STUDIES IN THE BIOGENESIS OF STEROID HORMONES

(Summary)

by

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Summary.

The purpose of the investigation was to determine whether the histochemically defined picture of 3β-hydroxy-steroid dehydrogenase distribution in the adrenal cortex of horse and man could be confirmed biochemically. Attempts were also made to investigate the significance of this distribution.

PART I.

Histochemistry has shown that the highest 3β-hydroxy-steroid dehydrogenase activity is in the outer zona fasciculata with little or no activity in the zona reticularis. This result is obtained with substrates dehydroepiandrosterone, pregnenolone and 17α-hydroxy-pregnenolone.

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Evidence is now presented that DHA- and pregnenolone--3β-hydroxy dehydrogenase activity (and possibly 17α--hydroxy-pregnenolone-3β-hydroxy dehydrogenase activity) is higher in the zona fasciculata of the adrenal cortices of horse and man. The results obtained, however, do not indicate the large difference in activity suggested by the histochemical evidence, and indeed it was found that reticular tissue contains substantial amounts of 3β-hydroxy-steroid dehydrogenase activity.

PART II.

The ability of fascicular and reticular cells of the horse adrenal cortex to transform [7α-^{3}H] pregnenolone, [4-^{14}C] progesterone, [7α-^{3}H] 17α-hydroxypregnenolone and [4-^{14}C] 17α-hydroxyprogesterone to cortisol was measured.

It was found that:-

1. All four steroids are transformed to cortisol by both types of cell.

2. The transformation of all four steroids to cortisol is higher in fascicular tissue.

3. The sequences pregnenolone ⟶ 17α-hydroxypregnenolone ⟶ 17α-hydroxyprogesterone and pregnenolone ⟶ progesterone ⟶ 17α-hydroxyprogesterone are both slower than the succeeding steps from 17α-hydroxyprogesterone ⟶ ⟶ cortisol.

4. The step 17α-hydroxypregnenolone ⟶ 17α-hydroxyprogesterone is rate-limiting in the transformation of 17α-hydroxypregnenolone ⟶ cortisol and there is approximately 2.4 times more 17α-hydroxypregnenolone--3β-hydroxy dehydrogenase activity in fascicular than in reticular tissue.

5. Progesterone-17α-hydroxylase activity is between 1.67 and 2.91 times higher in fascicular than in reticular tissue.

Following the experiments with horse adrenal cells, an attempt was made with human adrenal tissue to investigate the alternative metabolic pathways which convert pregnenolone to 17α-hydroxyprogesterone with a view to the elucidation of the role of 3β-hydroxysteroid dehydrogenase. [7α-^{3}H] pregnenolone and [4-^{14}C] progesterone were incubated simultaneously with fascicular and with reticular slices from a normal human adrenal cortex. Conversions of each substrate to 16α-hydroxyprogesterone, 11-deoxycorticosterone, 17α-hydroxyprogesterone and cortisol were measured.

Evidence was found suggesting that:-

1. Both fascicular and reticular tissue convert pregnenolone and progesterone to the four metabolites mentioned above.

2. Pregnenolone is converted to these metabolites in greater yield in fascicular tissue compared with reticular tissue.

3. The conversion of progesterone to these metabolites is only marginally greater in fasicular tissue.

4. Pregnenolone ⟶ progesterone ⟶ 11-deoxycorticosterone is the only major pathway for the formation of 11-deoxycorticosterone in both zones.

5. The main route from pregnenolone to 16α-hydroxyprogesterone is *via* 16α-hydroxypregnenolone in both zones.

6. The pathway pregnenolone ⟶ 17α-hydroxypregnenolone ⟶ 17α-hydroxyprogesterone ⟶ 11-deoxycortisol ⟶ cortisol is the major route to cortisol from pregnenolone *in vitro* in the adrenal cortex and the preference for this pathway is greater in fascicular tissue.

7. It is possible that a pathway exists from 17α-hydroxypregnenolone to cortisol in fasicular tissue independent of 17α-hydroxyprogesterone.

The theoretical factors involved in making an accurate determination of the magnitude of alternative pathways of steroid biosynthesis were discussed.

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A Thesis presented for the Degree of Doctor of Philosophy

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3β-HYDROXYSTEROID DEHYDROGENASE AND THE BIOSYNTHESIS OF CORTISOL

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ABBREVIATIONS.

Δ^5-3β-hydroxysteroids.

	Systematic Name.	Trivial Name in Text.
C_{27}	cholest-5-en-3β-ol	cholesterol
	sodium cholest-5-en-3β-yl sulphate	cholesterol sulphate
C_{21}	3β-hydroxypregn-5-en-20-one	pregnenolone
	3β-sulphoxypregn-5-en-20-one, ammonium salt	pregnenolone sulphate
	3β,16α-dihydroxypregn-5-en-20-one	16αOH-pregnenolone
	3β,17α-dihydroxypregn-5-en-20-one	17αOH-pregnenolone
	3β-sulphoxy-17α-hydroxypregn-5-en-20-one, ammonium salt	17αOH-pregnenolone sulphate
	3β,21-dihydroxypregn-5-en-20-one	21 OH-pregnenolone
	3β,17α,21-trihydroxypregn-5-en-20-one	17α,21 OH-pregnenolone
	3β,11β,17α,21-tetrahydroxypregn-5-en-20-one	11β,17α,21 OH-pregnenolone

Δ^5-3β-hydroxysteroids.

	Systematic Name.	Trivial Name in Text.
C_{19}	3β-hydroxyandrost-5--en-17-one	dehydroepiandrosterone (DHA)
	3β-acetoxyandrost-5--en-17-one	DHA acetate
	sodium 3β-sulphoxy-androst-5-en-17-one	DHA sulphate
	androst-5-ene-3β,17β--diol	androstenediol
	androst-5-ene-3β,17β--yl diacetate	androstenediol diacetate

Δ^4-3-oxosteroids.

C_{21}	pregn-4-one-3,20-dione	progesterone
	16α-hydroxypregn-4-ene-3, 20-dione	16αOH-progesterone
	16α-acetoxypregn-4-ene--3,20-dione	16αOH-progesterone acetate
	pregn-4-ene-3,16,20--trione	16-oxoprogesterone
	17α-hydroxypregn-4-ene--3,20-dione	17αOH-progesterone
	21-hydroxypregn-4-ene--3,20-dione	deoxycorticosterone (DOC)

Δ^4-3-oxosteroids.

	Systematic Name.	Trivial Name in Text.
	21-acetoxypregn-4-ene--3,20-dione	DOC acetate
	11β,21-dihydroxypregn--4-ene-3,20-dione	corticosterone
	17α,21-dihydroxypregn--4-ene-3,20-dione	11-deoxycortisol
	11β,17α,21-trihydroxy-pregn-4-ene-3,20-dione	cortisol
	21-acetoxy-11β,17α--dihydroxypregn-4--ene-3,20-dione	cortisol acetate
	17α,21-dihydroxypregn--4-ene-3,11,20-trione	cortisone
	21-acetoxy-17α-hydroxy-pregn-4-ene-3,11,20-trione	cortisone acetate
C_{19}	androst-4-ene-3,17-dione	Δ^4-androstenedione
	11β-hydroxyandrost-4--ene-3,17-dione	11βOH-androstenedione
	androst-4-ene-3,11,17--trione	adrenosterone
	17β-hydroxyandrost-4--en-3-one	testosterone
	17β-hydroxyandrost-4--ene-3,11-dione	11-oxotestosterone

Miscellaneous.

	Systematic Name.	Trivial Name in Text.
C_{19}	3α-hydroxy-5β-androstane--11,17-dione	11-oxoaetiocholanolone

GENERAL INTRODUCTION

Harley (1858) and Arnold (1866) first described the histological division of the human adrenal cortex into three concentric zones or layers of cells. Immediately below the capsule lies the zona glomerulosa, often only two or three cells thick. This zone is not continuous but occurs in islets of cells beneath the capsule of the gland, and in places the zona fasciculata is adjacent to the capsule. The zona fasciculata constitutes the bulk of the cortex, consisting of cords of large "clear cells". (Symington, 1960). These cells have a high lipid content, and appear vacuolated in paraffin embedded sections stained with haematoxylin and eosin. Between the zona fasciculata and the adrenal medulla lies the zona reticularis comprising small groups of eosinophilic "compact cells" (Symington, 1960). The last twenty-five years have seen many attempts to formulate a theory of cell function of the histologically defined zones of the adrenal cortex compatible with all the experimental evidence. An excellent and comprehensive review of the morphology and theories concerning function of zones with their supporting evidence, covering the earlier work until 1960, has been compiled by Deane (1962). A very brief outline of the most important work is given below.

In 1940, Bennett extended an earlier theory of

Gottshau (1883) claiming that cortical cells were formed in the subcapsular region and migrated centripetally towards the adrenal medulla. The zona reticularis was said to be a senescent zone where the cells degenerated and were removed by the vascular system. Salmon & Zwemer (1941), also claimed to be able to label subcapsular cells with azo-dyes and observe this migration. Later workers (Calna & Foster, 1943; Baxter, 1946) were unable to confirm this, and indeed Mitchell (1948) and Race (1955) showed the occurrence of mitoses in all zones of the cortex. Until this point, no real progress had been made in the understanding of the biochemistry of the adrenal cortex. Swann (1940) observed that adrenalectomy, but not hypophysectomy, causes electrolyte disturbance. Hypophysectomy causes atrophy of the fascicular and reticular zones, although no degeneration of the zona glomerulosa appears to occur. This indicates that there is a distinction in control and probably cell function of the zona glomerulosa on the one hand and the zona fasciculata and zona reticularis on the other. Chester-Jones (1948) incorporated these and other findings into a general "zonal theory", proposing that the adrenal zones are independent, with different secretory functions. From the histochemical work of Deane & Greep (1946, 1947,

1948, 1949) and Greep & Chester-Jones (1950), it appeared that the glomerular zone was the secretory zone of a hormone controlling electrolyte balance. Aldosterone has since been shown to be synthesised exclusively by this zone in ox (Ayres, Gould, Simpson & Tait, 1956) and rat (Biraud, Stachenko & Pilette, 1958) adrenals. The zona fasciculata was credited with the biosynthesis of adrenocorticol steroids, cortisol and cortisone; adrenal androgens (not clearly defined) and oestrogens were thought to be formed in the zona reticularis (Albright, 1943; Blackman, 1946). An elevated excretion of androgens in patients with adrenogenital syndrome is associated with hyperplasia of the zona reticularis (Blackman, 1946). Support for these ideas came from Seligman & Ashbel (1952), who reported a histochemical technique for visualising "oxo-steroid" in the reticular zone, but this was subsequently shown to be a non-specific reaction for unsaturated lipid (Wolman & Greco, 1952; Karnovsky & Deane, 1954). Interest in the possibility of the zona reticularis being an active secretory zone was stimulated by the histochemical observations of Koffey & Baxter (1949) and Koffey (1953, 1955) in the rat. They demonstrated that cholesterol was present in much higher quantities in the zone

fasciculata than in the zona reticularis, and that adrenal glands removed a few minutes after ACTH administration had apparently lost cholesterol from the zona reticularis. Yoffey's results seemed to suggest that the reticular zone might be the active secretory zone for adrenocortical steroids, and the fascicular zone merely a storage site for cholesterol, the steroid hormone precursor. It is worth noting that cholesterol and its esters seem to be the only steroids stored by the adrenal. Steroid hormones are not stored in the cells, but pass out into the bloodstream almost immediately. Throughout this Thesis "secretion" is intended to signify biosynthesis and release from cells. Symington, Duguid & Davidson (1956) and Symington et al. (1958) described the effect of administered ACTH on adrenal glands which were being removed in the course of treatment of women with breast cancer. A two-stage bilateral adrenalectomy procedure was used whereby the first gland was removed and used as a control. After an interval of about three weeks, 100 units of ACTH per day were administered intramuscularly for the four days immediately prior to removal of the second gland. The adrenals removed at the second stage showed marked histological and chemical changes. Initially, certain regions of the zona fasciculata next

to the fasciculer/reticular border became depleted of lipid, and if ACTH administration is prolonged, these cells are apparently transformed into "compact" cells with consequent increased acid and alkaline phosphatase and Krebs cycle dehydrogenase activities and with higher RNA content. The total effect is one of apparent movement of the fasciculer/reticular border toward the capsule. Grant, Symington & Duguid (1957) were further able to show that there is a correlation between degree of lipid depletion and increase of steroid 11β-hydroxylase activity. Thus the evidence at this point seemed to imply that the reticular zone is the actively secreting zone and the zona fasciculata a storage zone. It also suggested that the immediate effect of ACTH is to act on the cells of the zona fasciculata at the fasciculer/ /reticular border possibly to mobilise stored cholesterol or cholesterol ester for steroid hormone synthesis, and in the longer term to transform the zona fasciculata cells into zona reticularis-like cells with increased enzymic activity (Symington et al. 1958; Symington, 1960). Grant & Griffiths (1962) and Griffiths, Grant & Symington (1963) now adapted a sliding technique (Stadie & Riggs, 1944) to separate fasciculer from reticular tissue with a microtome specially designed for working with human

adrenal glands. They were able to show that, *in vitro*, slices of both zones are capable of synthesising cortisol and 11β-OH-androstenedione when incubated in Krebs-Ringer bicarbonate solution containing glucose. The 11β-hydroxylase activity of both zones is approximately equal using DOC as substrate. Perhaps the most important observation reported by these authors was that only slices of fascicular tissue show a significant response to stimulation by ACTH. The small response of the reticular tissue was attributed to contamination of slices from this zone with fascicular cells.

This work confirmed an earlier view expressed by Ofstad, Lamvik, Stoa & Emberland (1961) that a substantial proportion of the secreted cortisol might be produced in the zona fasciculata and that both zones might be capable of androgen biosynthesis (Deane, 1962). Ofstad *et al.* (1961) described histological and urinary studies on a patient having adrenal glands with a completely fibrotic zona reticularis but with an apparently intact zona fasciculata. The 17α-hydroxycorticosteroid levels in the urine were within the normal range, although the 17-oxosteroid levels were below normal.

Ward & Grant (1963) were subsequently able to show that both "clear" and "compact" cells were capable of synthesising testosterone *in vitro* from [4-^{14}C] progesterone.

From the investigations reported since 1961, it became apparent that the inter-relationship between zona fasciculata and zona reticularis was not simply that of storage and secretory zones, and the earlier theory was modified accordingly (Griffiths et al. 1963).

Their main points were -

(i) With respect to the biosynthesis of C_{19} and C_{21} steroids other than aldosterone, the fascicular and reticular zones should be regarded as a single functional unit (Symington, 1958).

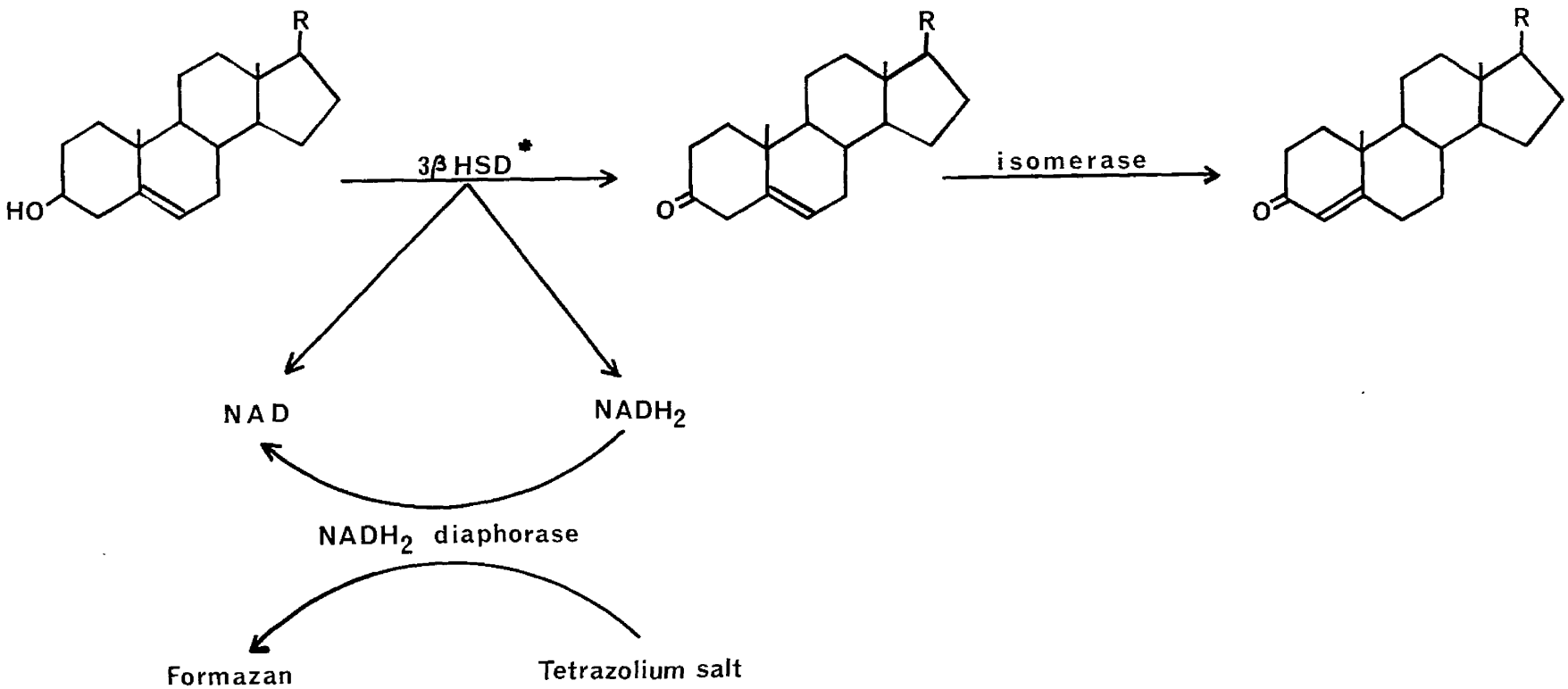
(ii) In vivo, ACTH is needed for cortisol secretion since this is abolished by hypophysectomy. At resting blood concentrations of ACTH, the cells of the zona reticularis and zona fasciculata both produce cortisol and androgens with the zona reticularis possibly maximally stimulated.

(iii) Under conditions of stress with raised concentrations of ACTH in the blood, the fascicular cells are stimulated to produce much greater amounts of cortisol, which probably originates from their stored cholesterol ester.

(iv) If stress is prolonged, the raised ACTH concentrations cause the "clear cells" to become almost completely

depleted of lipid, and assume the form of "compact cells" with the concurrent increase of RNA content and build up of enzymic activity described earlier. If this picture is correct, then both the zona fasciculata and zona reticularis must possess all the enzymes necessary for transforming cholesterol to cortisol, including 3β-hydroxysteroid dehydrogenase(s) and isomerase(s) (see fig. 1, p. 9) to transform pregnenolone to progesterone and 17αOH--pregnenolone to 17αOH-progesterone. The transformation DHA to Δ^4-androstenedione also requires such a system (see fig. 2, p.10). (Throughout the Thesis, 3β-hydroxysteroid dehydrogenase is intended to mean 3β-hydroxysteroid dehydrogenase + isomerase, unless otherwise stated.)

It might also be expected that the zona reticularis would possess reasonably high 3β-hydroxysteroid dehydrogenase activity if it is an actively secreting zone. However, histochemical evidence does not seem to support this. In 1958, Wattenberg devised a method purporting to demonstrate 3β-hydroxysteroid dehydrogenase activity in steroid hormone producing tissues. An unfixed tissue section is incubated in a medium containing the substrate, e.g. DHA, plus NAD, a tetrazolium salt and buffer. Hydrogen removed from the substrate by the enzyme is transferred to NAD, then from $NADH_2$ to the tetrazolium



* 3βHSD = 3β-hydroxysteroid dehydrogenase

fig. 1

METABOLIC PATHWAYS.

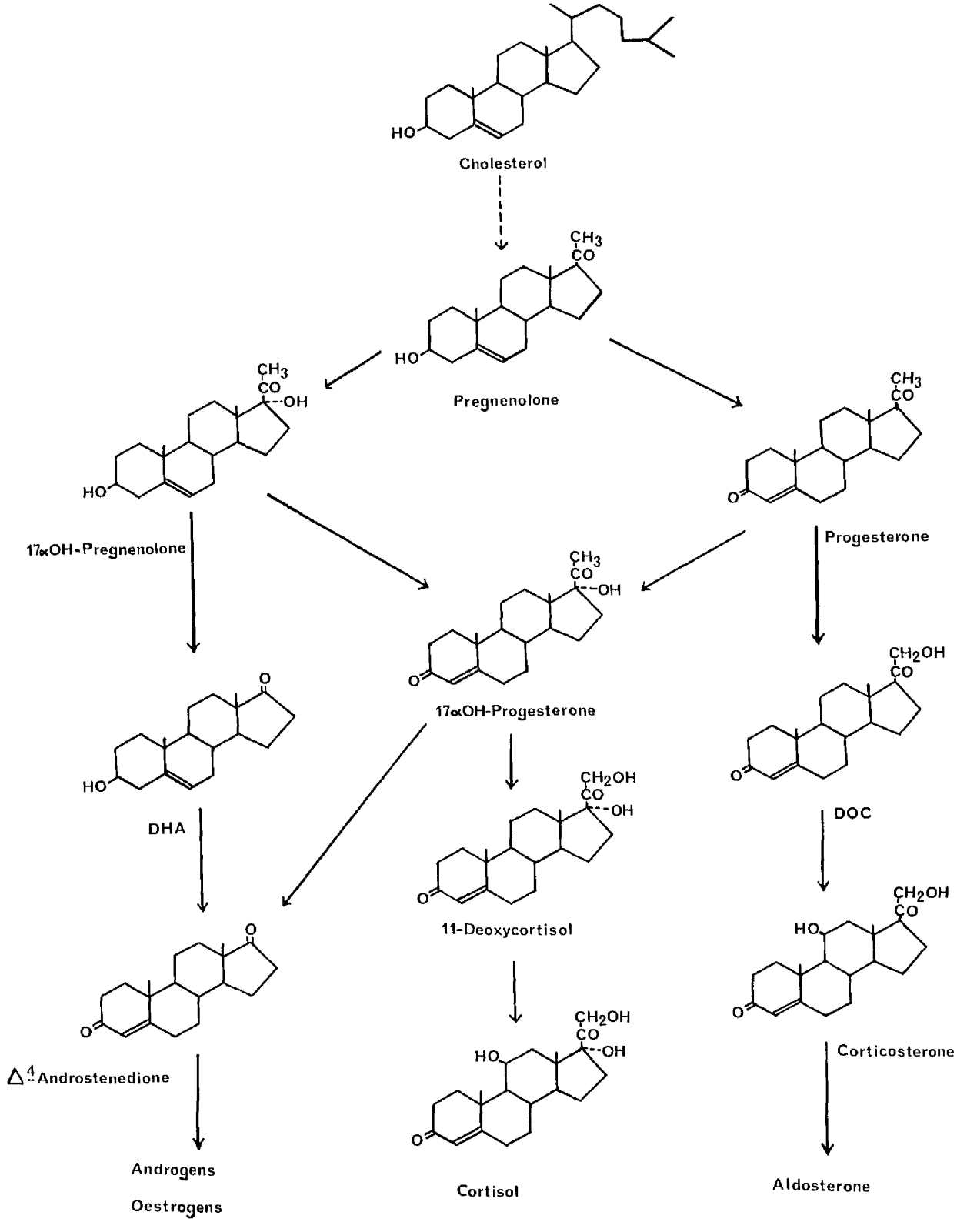


Fig. 2

salt by a portion of the electron transport system involving $NADH_2$:lipoamide oxidoreductase (fig. 1, p.9). The tetrazolium salt is thereby reduced to a coloured formazan dye which is deposited in the section. Since Wattenberg's initial observation, the histochemical distribution of 3β-hydroxysteroid dehydrogenase in the adrenals of several species including man, has been extensively studied (Levy, Deane & Rubin, 1959, 1959a; Dawson, Pryse-Davies & Snape, 1961; Cavallero & Chiappino, 1961, 1962, 1963). In the "fatty" (Cook, 1958; Symington, 1960) type gland, which microscopically shows a clear distinction between the fascicular and the reticular zones, e.g. in man, monkey and rat, there is a consistent pattern of 3β-hydroxysteroid dehydrogenase activity — formazan deposition in zona glomerulosa and outer zona fasciculata, and little or none in the inner zona fasciculata and the zona reticularis, suggesting greatest 3β-hydroxysteroid dehydrogenase activity in the zona glomerulosa and outer zona fasciculata, and least in the zona reticularis. Wattenberg (1958) and Pearson & Grose (1959) also reported that the same tissues which oxidised DHA, a steroid not involved in the biosynthesis of cortisol, also oxidised pregnenolone, although formazan deposition was always very much less with pregnenolone as

substrate. Perhaps it is not unreasonable to expect high 3β-hydroxysteroid dehydrogenase activity in the outer zona fasciculata since it might be described as an "emergency" zone and as such, ought to have the ability for rapid hormone biosynthesis. However, it is disturbing to find little or no histochemical 3β-hydroxysteroid dehydrogenase activity in the zona reticularis, in view of its proposed role. Thus it was considered that one of the most important steps in elucidating cell function and metabolic pathways for steroid hormone biosynthesis in the different zones of the adrenal cortex was to determine whether the histochemical distribution of 3β-hydroxysteroid dehydrogenase activity could be confirmed biochemically. Some preliminary biochemical experiments with homogenates of human adrenal slices indicate that with DHA as substrate, there is probably a higher 3β--hydroxysteroid dehydrogenase activity in the zona fasciculata (Grant, 1964). ACTH is known to stimulate the conversion of cholesterol to pregnenolone (Stone & Hechter, 1954), but it is readily seen from fig. 2 (p.10) that the 3β-hydroxysteroid dehydrogenases could control the ultimate biosynthetic fate of pregnenolone, e.g. pregnenolone-3β-hydroxydehydrogenase or 17αOH-pregnenolone--3β-hydroxydehydrogenase activities <u>could</u> dictate to a

large degree the ratio of cortisol to corticosterone produced (see fig. 2, p.10), and it is known that this ratio is altered in favour of cortisol on administration of ACTH (Grant, Forrest & Symington, 1957).

There is abundant evidence in the literature that the classical pathway for the biosynthesis of cortisol via pregnenolone ⟶ progesterone ⟶ 17αOH-progesterone ⟶ 11-deoxycortisol ⟶ cortisol (Hechter & Pincus; 1954; Samuels, 1960; Dorfman, 1961) is not unique (Eichhorn & Hechter, 1957, 1959; Berliner, Berliner & Dougherty, 1958; Schindler & Knigge, 1959: Cox, 1961; Mulrow, Cohn & Kuljian, 1962; Welikу & Engel, 1962, 1963). A second pathway via pregnenolone ⟶ 17αOH-pregnenolone ⟶ 17αOH-progesterone etc. was identified by Mulrow et al. (1962) in human adrenal slices. Welikу & Engel (1962) incubated a human adrenal tumour simultaneously with $[4\text{-}^{14}C]$ progesterone and $[7\alpha\text{-}^{3}H]$ 17αOH-pregnenolone, and found efficient conversion of the latter to cortisol. In a similar experiment, in which human hyperplastic adrenal tissue was incubated simultaneously with $[4\text{-}^{14}C]$ progesterone and $[7\alpha\text{-}^{3}H]$ pregnenolone, Welikу & Engel (1963) found by examination of the isotopic content of various metabolites that the pregnenolone ⟶ progesterone step had not occurred to any measurable degree

— the 17αOH-progesterone and cortisol isolated contained ^{3}H and ^{14}C but progesterone, DOC, corticosterone and 16αOH--progesterone contained only ^{14}C (see fig. 2). These results indicated clearly for the first time that there might be more than one 3β-hydroxysteroid dehydrogenase enzyme system or at least strong evidence for enzyme--substrate specificity. These authors suggested, on the basis of their own experiments and on the accumulated evidence in the literature, that "the activity measured or localised should be considered as a measure of the Δ^{5}-3β-hydroxysteroid dehydrogenase specific for the substrate used".

There is supporting evidence for further hydroxylation of pregnenolone before dehydrogenation at the C-3 position in association with the demonstration of a possible 3β-hydroxysteroid dehydrogenase deficiency in foetal adrenal tissue (Villee, Engel & Villee, 1959; Villee, Loring & Villee, 1962; Cathro, Birchall, Mitchell & Forsyth, 1963) and in the more serious congenital lack of a 3β-hydroxysteroid dehydrogenase system observed in the newborn (Bongiovanni, 1962). However, no parallel studies of the biochemistry and histochemistry of 3β-hydroxysteroid dehydrogenase activity of any one cell type have been performed.

All the accumulated evidence seemed to indicate that a biochemical study of the 3β-hydroxysteroid dehydrogenase activity of the adrenal cortex with particular reference to the different zones and their ability to metabolise various Δ^5-3β-hydroxysteroids should provide useful information as to the significance, if any, of the histochemical distribution of 3β-hydroxy-steroid dehydrogenase enzyme activity, and throw further light on alternative biosynthetic pathways to cortisol.

Normal human adrenal glands for biochemical study, once readily available from patients undergoing adrenalectomy for breast cancer, are now very rare. With this in mind, it was decided at the outset to try to develop analytical methods by working with the horse adrenal cortex, which shows histological and histochemical similarities to that of man, and to apply experience gained to the scarcer human material when available. Two general approaches to the problem seemed likely to be fruitful.

PART I.

Attempts were made to measure the transformation by fascicular and reticular tissue prepared according to the technique of Griffiths et al. (1963) of Δ^5-3β-hydroxy-

steroids DHA, pregnenolone and 17αOH-pregnenolone to the corresponding Δ^4-3-oxosteroids or their metabolites.

PART II.

Attempts were also made to measure the transformation of radioactive steroids by fascicular and reticular tissue to the end-product cortisol, in order to gain a picture of the total biosynthetic activity of the cell types found in these tissues. In addition, experiments were undertaken to determine the relative importance of alternative pathways.

PART I

MEASUREMENT OF 3β-HYDROXYSTEROID DEHYDROGENASE ACTIVITY

INTRODUCTION

The initial purpose of the present study was to determine whether the results of a biochemical assay of 3β-hydroxysteroid dehydrogenase enzyme activity in the zona fasciculata and zona reticularis of the human adrenal cortex would reflect the distribution of this activity observed histochemically with respect to substrates DHA, pregnenolone and 17αOH-pregnenolone. As mentioned in the General Introduction (p.15) the horse adrenal gland was used as a model system in order to assist in the development of analytical methods. Preliminary experiments were performed to investigate the metabolism of $[4\text{-}^{14}C]$ progesterone by homogenates of horse adrenal fascicular and reticular tissue. The metabolism of $[7\alpha\text{-}^{3}H]$ pregnenolone by homogenates of this type was also investigated, and the effects of the following were determined.

a) Versene (diamino-ethane-tetra-acetic acid) - this substance will chelate Cu^{++} ions which are necessary for the action of 11β-hydroxylase (Grant, 1956)

b) Anaerobic conditions - molecular oxygen is required for steroid hydroxylations (Sweat et al. 1956; Bloom, Hayano, Saito, Stone & Dorfman, 1956; Hayano, Saito,

Stone & Dorfman, 1956).

c) Medium Composition.

These experiments were performed in order to find conditions of maximal transformation of pregnenolone to progesterone with minimal transformation of either steroid to other substances by hydroxylation reactions. Such a system was required in order to simplify analysis. It had also been noted that in 1956, Beyer & Samuels showed that 3β-hydroxysteroid dehydrogenase activity appeared to be confined to the microsomal fraction of adrenal cells. (Activity was also found in relatively large amounts in the nuclear fraction of these cells. However, on repeated washing of the nuclei, the activity of the fraction declined in parallel with the RNA content, indicating that the activity was due to microsomal contamination.) Thus a higher 3β-hydroxysteroid dehydrogenase activity should be manifest in a microsomal preparation derived from cells of the zona fasciculata than in an equivalent fraction from the zona reticularis. Working with this cell fraction 11β--hydroxylation is eliminated.

With regard to methods of determination of 3β--hydroxysteroid dehydrogenase activity, one way, as in

all enzyme assays, measure the amount of product formed or of substrate remaining in an incubation at the end of an arbitrary time interval. Both approaches were extensively investigated by means of paper and absorption column chromatography in order to find a reliable system. Latterly a method was developed for the determination of pregnenolone by gas-liquid chromatography but since this procedure was not actually used in the estimation of the 3β-hydroxysteroid dehydrogenase of a tissue, these experiments are described in Appendix V (p.206).

EXPERIMENTAL.

A. Adrenal Tissue.

1. Adrenal Glands.

Horse adrenal glands were obtained from a local slaughterhouse, normally within 20 - 25 minutes of the death of the animal. Adrenal glands from human subjects were obtained at operation from a number of patients undergoing treatment for Cushing's syndrome or breast cancer. Those from patients with breast cancer were assumed to be normal (Grant et al. 1957). The time taken for human glands to reach the laboratory varied according to their source from 0.5 - 24 hours. The relevant information concerning each gland used is given with the appropriate section (p.48).

All tissue for biochemical study was transported to the laboratory in polythene bags on crushed ice.

2. Histology.

Pieces of every gland investigated, together with samples of tissue, presumed to be taken from the zona fasciculata or zona reticularis, were fixed separately in 10% neutral formalin, for subsequent preparation of paraffin embedded haemotoxylin and eosin sections.

3. Histochemistry.

As soon as possible after removal of a gland, a piece was cut from one end and dropped into solid carbon dioxide snow in a vacuum flask. 3β-hydroxysteroid dehydrogenase activity was localised in thin sections of this material by the method of Wattenberg (1958) as modified by Levy, Deane & Rubin (1959). DHA, pregnenolone and 17αOH-pregnenolone were used as substrates.

4. Slicing Technique.

Slices of fascicular and reticular tissue were prepared by the use of a modified Stadie-Riggs microtome (Stadie & Riggs, 1944) as described by Griffiths et al. (1963).

5. Preparation of Homogenates and Mitochondria-free Supernatant Fractions.

All homogenates were prepared by vertical strokes of a steel plunger in a uniform bore glass tube (Philpot & Stander, 1956).

Mitochondria-free supernatant fractions were obtained by centrifugation of 20% (w/v) homogenates at 5000 x g for 10 minutes in the SW39 rotor of a "Spinco" Preparative Ultracentrifuge, Model L (Beckman Instruments, Ltd., Palo Alto, California; now Glenrothes, Fife and Frankfurt,

Germany).

B. Analytical Procedures.

1. Chromatography of Steroids on Paper.

The solvent systems used for paper chromatography were the propylene glycol/toluene (PG/T) system of Burton, Zaffaroni & Keutmann (1951), the benzene/chloroform/formamide (Bz/$CHCl_3$/F) system of Zaffaroni & Burton (1951), and several systems of the type described by Bush (1952) see Table 1. (Griffiths et al. 1963) below. Whatman No. 42 paper was used for the PG/T and Bz/$CHCl_3$/F systems, and Whatman No. 1 for the Bush-type systems. The paper was washed, prior to use, with a mixture of chloroform and methanol for two days in a Soxhlet apparatus. All chromatograms were equilibrated overnight (16 hours) and developed at 22°.

2. Elution of Steroids from Paper Chromatograms.

Steroids were eluted from paper chromatograms by cutting the area involved into small pieces and shaking with 5 ml. methanol:ethyl acetate (1:1, v/v) for 1 hour at 37° (see Appendix IV, p.196).

3. Chromatography of Steroids on Thin-Layer Plates.

Glass plates (20 cm. x 20 cm.) were coated by Desaga

Solvent System	Mobile Phase	Stationary Phase
P10	Light Petroleum (80-100°) (P.E.)	methanol:water (7:3)
PB91	P.E.:Benzene (9:1)	methanol:water (7:3)
PB21	P.E.:Benzene (2:1)	methanol:water (7:3)
PB11	P.E.:Benzene (1:1)	methanol:water (7:3)
B10	Benzene	methanol:water (7:3)
ET19	Ethyl Acetate:Toluene (1:9)	methanol:water (7:3)
PG/T	Toluene	propylene glycol
Bz/$CHCl_3$/F	Benzene:$CHCl_3$ (1:1)	formamide

Table 1. (solvent proportions are shown by volume).

applicator (Camlab Glass Co., Cambridge) with a slurry of Merck Kieselgel G (25 gm. in 65 ml. water) containing approximately 0.4% of an inorganic phosphor (H 913, Levy West Laboratories, Ltd., Harlow). After 10 minutes drying at room temperature, the thin layers were activated for 60 minutes at 110°. Steroids were applied in methylene chloride and the chromatograms developed in the solvent systems shown in Table 2 , below.

4. Elution of Steroids from Thin-Layer Chromatograms.

All steroids were eluted by scraping the silica gel containing the steroid on to black glazed paper, transferring it to a tube containing 5 ml. of benzene or ether (cortisol elutions) and mixing vigorously with a motor-rotated stainless steel wire bent at the tip into a figure eight. Water (1 ml.) was added and the tube shaken for 1 minute. After centrifugation, the upper layer was removed and the aqueous layer re-extracted with 5 ml. solvent. This procedure was found to give residues virtually free from non-steroidal impurities. Extracts were combined and dried in a stream of air at 50°. Recoveries normally lie between 90 - 100% with this method (see Appendix IV, p.197), which is a modification of that described by Griffiths, Grant, Browning, Whyte &

Thin-Layer Chromatography Systems (System No.)	Solvent Proportions by Volume
I	Chloroform:methanol:water (187:12:1)
II	Benzene:methanol (170:30)
III	Benzene:hexane:ethanol (140:50:10)
IV	Cyclohexane:ethyl acetate (90:110)

Table 2.

Sharp (1965).

5. Detection of Steroids on Paper and Thin-Layers.

UV-absorbing steroids were located on paper and thin-layer chromatograms by viewing with a "Chromato--lite" lamp (Hanovia, Ltd., Slough, Bucks.).

Non-UV-absorbing steroids were detected by means of a 15% ethanolic solution of phosphomolybdic acid. Paper chromatograms were dipped and thin-layer chromatograms sprayed with the reagent and then warmed in an oven at 60° for 5 minutes. The steroids were located as blue spots against a yellow background. Most steroids seem to react well with this reagent, with a sensitivity of about 2 $\mu g/cm^2$, but Δ^5-3β-hydroxysteroids, particularly on thin-layer chromatograms, show up more quickly.

6. Column Chromatography on Alumina.

Residues from the aqueous methanolic fraction from extracts of incubations described in experiment 7 (p.47) were dissolved in 8 ml. benzene and applied to 0.8 cm. internal diameter glass columns containing 3 g. alumina (Savory & Moore) deactivated with 11% (w/v) of water. The columns were developed with a mixture of 0.1% ethanol in benzene and the first 25 ml. eluate was discarded.

The next 40 ml., containing DHA and pregnenolone, was evaporated to dryness and assayed for Δ^5-3β-hydroxysteroid and radioactivity content. The columns were further developed with 30 ml. of 1% ethanol in benzene and this fraction, containing 17αOH-pregnenolone was again assayed for Δ^5-3β-hydroxysteroid and radioactivity content (see also Appendix IV, p.198).

7. Detection and Measurement of Radioactivity on Paper Chromatograms.

Radioactive steroids were located on chromatograms with an automatic recording gas-flow strip counter (Scanogram II, Chromatogram Scanner, Atomic Accessories Inc., New York). The amount of radioactivity associated with each steroid was proportional to the area of its tracing on the recording chart, which was determined by planimetry.

8. Measurement of Radioactivity in Extracts, Etc.

Portions of steroid residues, etc., to be counted were placed in glass vials of low potassium content (Wheaton Glass Co., Millville, N.J.) and dissolved in 10 ml. of toluene containing 3 g./l. of 2,5-diphenyl-oxazole (PPO) and 0.1 g./l. of 1,4-bis-2(4-methyl-5-

-phenyloxazolyl)-benzene (dimethyl-POPOP). Radioactivity was determined by Packard Tri-Carb Liquid Scintillation Spectrometer, Model 314EX (Packard Instrument Co., Inc., La Grange, Ill.). At voltage tap 6.2 (1130 volts), Channel I was set with a voltage discriminator gate of 100 - 1000 and amplifier gain of 100% giving an efficiency of counting of approximately 64% for ^{14}C (see Appendix III, p.192). Channel II was set with a voltage discriminator gate of 100 - 1000 and amplifier gain of 20% giving an efficiency of counting of approximately 26% for ^{3}H. No quenching (loss of counts due to interference of scintillation system at the molecular level by sample) was observed under these conditions.

Aqueous samples were counted in a system containing 0.5 ml. of the aqueous material mixed in a vial with 5 ml. scintillator solution and 4.5 ml. of ethanol to make the mixture homogeneous. Severe quenching is observed with this type of system; counting efficiencies were determined by use of internal standards. Steroid sulphates which are insoluble in toluene were dissolved in 1 ml. of ethanol before addition of 9 ml. scintillator solution to the vial. Again counting efficiencies were determined by means of the internal standard technique.

9. Preparation of Derivatives.

a) Oxidation.

A chromic acid oxidising reagent was prepared by the method of Kiliani & Merck (1901) as modified by Griffiths, Grant & Whyte (1963). Concentrated H_2SO_4 (23 ml.) was added to 92 ml. of water. The mixture was cooled, stirred into a solution of 32.2 g. $Na_2Cr_2O_7.2H_2O$ in 70 ml. of water, and washed in with a further 10 ml. of water. The reagent (100 ml.) was stirred into 10 ml. of acetone. 500 µl of the resulting mixture was then added to the dry residue and allowed to react for 20 minutes at room temperature. The reaction was stopped by the addition of 2 ml. of water and the steroids extracted with ethyl acetate.

b) Acetylation.

Steroids were acetylated by the procedure of Zaffaroni & Burton (1951). Two drops each of freshly distilled acetic anhydride and pyridine were added to the dried steroid residue and the reaction allowed to proceed at room temperature overnight in a tightly stoppered test-tube. The reaction mixture was then diluted with 1 ml. of methanol and the solvents evaporated completely to dryness in a stream of air.

c) Saponification.

Steroid acetates were saponified by a modification of the method of Neher, Desaulles, Vischer, Wieland & Wettstein (1958) as described by Ward & Grant (1963). A solution (250 µl) of 2% aqueous K_2CO_3 (w/v) was added to the steroid residue dissolved in 1 ml. methanol, and the mixture kept overnight at room temperature. The steroids were recovered by extraction with ethyl acetate after addition of 2.5 ml. water.

d) Reduction.

Dried steroids were reduced by 100 ml. of an ice--cold 0.05% methanolic solution of $NaBH_4$ at 0^o for 45 minutes. This is a modification of the method of Southcott, Bandy, Newson & Darrach (1956) as described by Griffiths et al. (1963). The reaction was stopped by the addition of one drop of glacial acetic acid. Steroids were extracted with ethyl acetate after addition of 2.5 ml. of water.

10. Determination of Steroids.

a) Δ^4-3-oxosteroids.

Following purification by chromatography, Δ^4-3-oxo-steroids were dissolved in 5 ml. or 2 ml. of ethanol, and

their optical densities measured at 240 mμ against ethanol in 1 cm. cells of a Unicam SP500 spectrophotometer. Steroid present was found by reference to a calibration curve (see Appendix IV, p.202). Blanks from the chromatographic material were taken through the elution procedure.

b) Δ^5-3β-hydroxysteroids.

After column chromatography on alumina (p.26), Δ^5-3β-hydroxysteroids were subjected to treatment with a sulphuric acid-ethanol reagent (Oertel & Elk-Nes, 1959). The reagent is prepared by adding 2 volumes of concentrated H_2SO_4 slowly to 1 volume of ethanol with cooling and stirring. 5 ml. of the reagent was added to dry steroid residues and the tubes shaken vigorously for 1 minute. Mixtures were then left to stand for 5 minutes to allow small air bubbles to come to the surface, and their optical densities were measured at 380, 405 and 430 mμ in 1 cm. cells of a Unicam SP600 spectrophotometer against a reagent blank. Steroid present was determined by means of standards, using

a) Allen corrected optical densities (Allen, 1950). With the above three wavelengths:-

Allen corrected optical density (O.D.) at 405 mμ

$$= \text{O.D. at 405 m}\mu - \frac{(\text{O.D. at 380 m}\mu + \text{O.D. at 430 m}\mu)}{2}$$

or b) a simple corrected optical density (Saffran & Schally, 1955) where

O.D. = O.D. at 405 mμ - O.D. at 430 mμ.

Tissue blanks were carried through the entire procedure.

11. Determination of Protein Nitrogen.

The protein nitrogen content of mitochondria-free supernatant fractions of horse adrenal tissue was measured by a modification of the method described by Nayyar & Glick (1954).

Reagents:-

(i) 1.0 N NaOH.

(ii) 0.1 N NaOH.

(iii) Bromsulphalein Reagent:-

To 1 ml. of 5% Bromsulphalein solution (Hynson, Westcott & Dunning, Inc., Baltimore, Md.) 100 ml. 1.0 N HCl, 50 ml. of 1.0 M citric acid (pH 1.8) and distilled water were added to a final volume

of 250 ml. (final concentration 0.02% Bromsulphalein).

A portion (100 µl) of the supernatant fraction was diluted to 2.5 ml. with distilled water. Portions (25 µl, i.e. 1/100th) of the diluted solution were pipetted into small test tubes and dried *in vacuo*. 40 µl 1.0 N NaOH was then added and the mixtures agitated by vibrating the tubes against a rapidly rotating bent pin. After 1 hour at room temperature, 100 µl of the 0.02% Bromsulphalein reagent was added and, after mixing, the tubes were centrifuged in a miniature centrifuge (Misco, Microchemical Specialities Co., Berkeley, California) for 5 minutes at 1500 x g. 60 µl of each supernatant was diluted with 1.0 ml. of 0.1 N NaOH and, after shaking, the optical densities of the mixtures were observed in 1 cm. glass cells of a Unicam SP600 spectrophotometer at 580 mµ against a water blank. Triplicate reagent blanks were taken through the whole procedure.

The optical densities observed for the tissue extracts are subtracted from those of the reagent blanks giving a $\triangle$O.D. figure.

($\triangle$O.D. = optical density of "blank" at 580 mμ - optical density of extract at 580 mμ)

$\triangle$O.D. x 5.84 x 2 = μg protein nitrogen.

5.84 is the factor for adrenal tissue relating protein content to the dye-bound determined by Nayyar & Glick (1954) by comparison of results obtained by the above method with those obtained using a micro-Kjeldahl procedure .

C. Studies with Horse Adrenals.

1. Incubations with [4-^{14}C] Progesterone.

[4-^{14}C] progesterone, stored in benzene:methanol (9:1, v/v), was added to incubation vessels in this solution, together with 100 µl of propylene glycol. The benzene:methanol was evaporated at 50° in a stream of air, leaving the steroid dissolved in a film of propylene glycol. Tissue slices, 500 mg. from each zone, were homogenised in 2 ml. 0.25M sucrose containing 0.12M nicotinamide (Handler & Klein, 1942) medium and transferred to the incubation vessels with a further 2 ml. of the medium. To each vessel was added a solution (4.8 ml.) containing 360µmoles KCl, 365.0µmoles TRIS buffer (pH 7.4), 26.5µmoles $MgSO_4.7H_2O$, 72 µmoles potassium fumarate, 7.92µmoles ATP (dipotassium salt), 120µmoles glucose-6-phosphate (dipotassium salt), 1.57µmoles NADP and 180 µg (8.4 Kornberg Units) glucose-6-phosphate dehydrogenase (Umbreit, Burris & Stauffer, 1957). The mixtures were incubated at 37° for 2 hours with shaking in air. Products of incubation were extracted three times with 10 ml. benzene:chloroform (6:1, v/v) and twice with 10 ml. ethyl acetate. The combined extracts were evaporated to dryness at 50°

under a stream of air, and the residues partitioned between 30 ml. 75% aqueous methanol and 30 ml. light petroleum (80-100°). The petroleum was extracted twice more with 30 ml. volumes of aqueous methanol and the combined methanolic layers reduced to approximately 20 ml. in a rotary evaporator. Water (20 ml.) was then added and the aqueous mixture extracted four times with 40 ml. chloroform. The combined chloroform layers were evaporated to dryness at 50° under a stream of air and aliquots of the residues were taken for radioactivity counting and investigation by paper chromatography.

Radioactive steroids in samples from the extracts were tentatively identified by comparison of their chromatographic behaviour in a variety of solvent systems. Conversions were determined by planimetry of radioactivity peaks from the chromatogram scans (see p.27), and the area of each peak as a percentage of the total was calculated. Cortisol was further identified by the carrier technique of Berliner & Salhanick (1956) which involves the addition of the non-radioactive steroid considered to be present in the eluate of the radioactive zone and determination of specific activities before and after the formation of

derivatives.

2. Effect of Versene and Anaerobic Conditions on the Metabolism of [7α-^{3}H] Pregnenolone.

Four homogenates were prepared, two of 250 mg. of fascicular tissue and two of 250 mg. of reticular tissue, in 2.2 ml. of 0.1M phosphate buffer (pH 7.4) containing 0.154M NaCl. To one of each pair was added 50 µl water, and to the other, 50 µl of 0.45M Versene (diamino-ethanetetra-acetic acid), pH 7.4, to give a final concentration of 10mM. 100 µl aliquots of each homogenate were placed in incubation vessels together with 20 µg of [7α-^{3}H] pregnenolone (1.026 µC) dissolved in 20 µl propylene glycol, and 1 mg. NAD. Incubations were performed either under air or nitrogen. Those performed under nitrogen were done in Thunberg tubes, the tubes being evacuated several times with an oil pump and the atmosphere replaced by nitrogen. All incubations were carried out at 37° for 30 minutes. Details of the experiment are set out in the following table. At the conclusion of the incubation, each mixture was diluted with 4 ml. water and extracted four times with 4 ml. ethyl acetate:diethyl ether (1:1,v/v).

Incub.	Zone *(F or R)	Versene 10 mM	H_2O	Atmosphere air	Atmosphere N_2	Code
1.	F	-	+	+	-	F/W/A
2.	R	-	+	+	-	R/W/A
3.	F	+	-	+	-	F/V/A
4.	R	+	-	+	-	R/V/A
5.	F	-	+	-	+	F/W/N
6.	R	-	+	-	+	R/W/N
7.	F	+	-	-	+	F/V/N
8.	R	+	-	-	+	R/V/N

Table 3.

*F = zona fasciculata

R = zona reticularis

Solvents were removed from the combined extracts at 50° under a stream of air, and the residues partitioned between 75% aqueous methanol and light petroleum (80--100°) as in the previous experiment.

Part of the aqueous methanolic fraction was taken for radioactivity counting, and the remainder chromatographed on paper in the P10 system (p.23). Radioactive steroids were located by automatic strip counter (p.27).

3. Effect of Medium Composition on the Metabolism of [7α-^{3}H] Pregnenolone.

of [7α-^{3}H] Pregnenolone, senate (25%, w/v) were prepared with a mixture of fascicular and reticular tissue in different media:-

(i) 0.1M phosphate buffer (pH 7.4) containing 0.154M NaCl (PO_4 x 1)

(ii) 0.05M phosphate buffer (pH 7.4) containing 0.077M NaCl (PO_4 x ½)

(iii) 0.25M sucrose containing 0.12M nicotinamide (S/N)

(iv) 0.075M TRIS buffer (pH 7.4) (TRIS)

1.25 ml. of each homogenate was diluted with 1.25 ml.

Incub.	Homog. Medium	Medium Added	Versene (50µl of 0.5M)	Water (50µl)	Code
1.	PO_4 x 1	PO_4 x 1	-	+	PO_4 x 1/PO_4 x 1/W
2.	PO_4 x 1	PO_4 x 1	+	-	PO_4 x 1/PO_4 x 1/V
3.	PO_4 x $\frac{1}{2}$	PO_4 x $\frac{1}{2}$	-	+	PO_4 x $\frac{1}{2}$/PO_4 x $\frac{1}{2}$/W
4.	PO_4 x $\frac{1}{2}$	PO_4 x $\frac{1}{2}$	+	-	PO_4 x $\frac{1}{2}$/PO_4 x $\frac{1}{2}$/V
5.	S/N	PO_4 x 2	-	+	SN/PO_4 x 2/W
6.	S/N	PO_4 x 2	+	-	SN/PO_4 x 2/V
7.	S/N	PO_4 x 1	-	+	SN/PO_4 x 1/W
8.	S/N	PO_4 x 1	+	-	SN/PO_4 x 1/V
9.	S/N	TRIS x 2	-	+	SN/TRIS x 2/W
10.	S/N	TRIS x 2	+	-	SN/TRIS x 2/V
11.	TRIS	TRIS	-	+	TRIS/TRIS/W
12.	TRIS	TRIS	+	-	TRIS/TRIS/V

Table 4.

of the medium in which it was prepared, or with

a) 0.2M phosphate buffer (pH 7.4) containing 0.308M NaCl (PO_4 x 2)

or

b) 0.15M TRIS buffer (pH 7.4) (TRIS x 2)

as indicated in the table below.

Each diluted homogenate was incubated with 20 µg [7α-^{3}H] pregnenolone (1 µC) dissolved in 100 µl propylene glycol, 1 mg. NAD and 50 µl 0.5M versene, pH 7.4 (final concentration 10mM) or water. Incubations were performed at 37° for 30 minutes. Incubation media were extracted, partitioned and the aqueous methanolic fractions investigated as in experiment 2.

4. Rate of Metabolism of [7α-^{3}H] Pregnenolone.

a) A mixture (1 gm.) of fasciculer and reticular tissue was homogenised in 4 ml. 0.25M sucrose containing 0.12M nicotinamide. A series of incubation mixtures was prepared, each mixture consisting of 1.25 ml. homogenate, 1.25 ml. 0.2M phosphate buffer (pH 7.4) containing 0.308M NaCl, 1.026 µC of [7α-^{3}H] pregnenolone (20 µg) dissolved in 100 µl propylene glycol, and

1 mg. NAD. Incubations were performed for several different time periods at 37° with shaking.

At the conclusion of each incubation, 5 ml. of water was added and the mixtures were extracted four times with 5 ml. ethyl acetate:diethyl ether (1:1, v/v). Combined extracts were taken to dryness at 50° in a stream of air (fraction 1). The aqueous phase was treated in one of two ways:-

(i) In order to ensure complete extraction of free steroid from the aqueous phase, 5 volumes of hot acetone were added and the mixture centrifuged. The aqueous acetone supernatant was removed and the tissue residue extracted twice more with 5 volumes of hot acetone. The combined acetone extracts (fraction 2a) were taken to dryness in a rotary evaporator and an aliquot taken for radioactivity counting.

(ii) To determine if appreciable steroid sulphate formation had occurred during incubation, the extraction and solvolysis procedure of Burstein & Lieberman (1958) was carried out. Ammonium sulphate (2 gm.) was added to the aqueous phase, the mixture brought to pH 1 with H_2SO_4 and extracted three times with equal volumes of ethyl acetate. The combined extracts (fraction 2b)

were left overnight at room temperature.

After removal of the solvent in a rotary evaporator, an aliquot of the residue was taken for radioactivity counting.

Fraction 1 was partitioned between 75% aqueous methanol and light petroleum (80-100°) as in experiment 2. Portions of the light petroleum phase (fraction 3) and the aqueous methanolic fraction (fraction 4) were taken for radioactivity counting. The remainder of fraction 4 was subjected to paper chromatography in the P10 system (p.23). Chromatograms were then examined.

b) A second series of incubation mixtures was prepared similar to that described above, containing 50, 100 or 150 µg of [7α-^{3}H] pregnenolone per incubation. All incubations were performed at 37° for 5 minutes.

"Recovery" mixtures were prepared and recoveries estimated at the 50 µg pregnenolone level with 1 µC [7α-^{3}H] pregnenolone added, either immediately before adding, or after shaking with, the first 5 ml. volume of ethyl acetate:diethyl ether.

The aqueous methanol fractions were investigated by paper chromatography and automatic strip scanning as before.

5. Incubations with [4-^{14}C] DHA.

Mitochondria-free supernatant fractions were obtained from 20% (w/v) homogenates of fascicular and reticular tissue prepared in 0.25M sucrose containing 0.12M nicotinamide. Each supernatant (1.25 ml.) was incubated with 1.25 ml. 0.2M phosphate buffer (pH 7.4) containing 0.308M NaCl, 6 mg. NAD and 150 µg [4-^{14}C] DHA (591 mµC) for 15 minutes at 37°. Extraction and partition were as described in experiment 1 (p.35). After paper chromatography, the relative proportions of [^{14}C] DHA and [^{14}C] Δ^4-androstenedione were measured by planimetry of their peaks on automatic strip scanner traces.

6. Incubations with [7α-^{3}H] Pregnenolone, [7α-^{3}H] 17αOH-Pregnenolone and [4-^{14}C] DHA.

In this series of experiments, incubations with substrates [7α-^{3}H] pregnenolone, [7α-^{3}H] 17αOH-pregnenolone or [4-^{14}C] DHA (500 µg of each) were performed in a similar fashion to those already described in experiment 5. Modifications, however, were introduced in extraction, and recovery of the aqueous methanol fraction, in order to remove "blank" material.

(i) At the conclusion of the incubation period,

20 ml. 2N NaOH was added to make the medium strongly alkaline. Extraction of the lipid fraction was effected with ethyl acetate:diethyl ether (1:1, v/v) - - four times with equal volumes.

(ii) The pooled extract was then washed with 5 ml. of 10% HCl and twice with 5 ml. of water. It was then evaporated to dryness under a stream of air.

(iii) After partition between 75% aqueous methanol and light petroleum (80-100°), the combined methanolic extracts were taken completely to dryness under reduced pressure in a rotary evaporator.

Attempts to measure the conversion of the substrates to Δ^4-3-oxosteroids were made by three different methods.

a) after paper chromatography by the planimetric method described in experiment 5 (p.44).

b) by measurement of the absorption of the extract at 240 mμ due to the presence of Δ^4-3-oxosteroids (p. 30).

c) by measurement of Δ^5-3β-hydroxysteroids remaining in the extract by a specific colour reaction (Oertel & Eik-Nes, 1959) involving the use of the sulphuric acid-ethanol reagent described on page 31. Details of incubations performed are set out in Table 5

Expt.	Zone (F or R)	Time of Incub. (min.)	Substrate Incubated (µg)	Code
1.	F + R	0	[4-^{14}C] DHA (462.5)	D recov.
2.	F + R	0	[7α-^{3}H] Preg.[1] (512.5)	P recov.
3.	F	15	[4-^{14}C] DHA (462.5)	DF15
	R	15	" (")	DR15
4.	F	30	" (")	DF30
	R	30	" (")	DR30
5.	F	60	" (")	DF60
	R	60	" (")	DR60
6.	F	15	[7α-^{3}H] Preg. (512.5)	PF15
	R	15	" (")	PR15
7.	F	30	" (")	PF30
	R	30	" (")	PR30
8.	F	60	" (")	PF60
	R	60	" (")	PR60

Table 5.

[1] [7α-^{3}H] pregnenolone.

7. Incubations with Pregnenolone, 17αOH-Pregnenolone and DHA.

For experiments involving smaller amounts of tissue, e.g. from atrophic human adrenals, the above experiments 5 (p.44) and 6 (p.44) are too wasteful of rare material. Experiment 7 involved the reduction of all quantities except substrate by a factor of five. The steroid substrate was fixed at 100 µg/incubation, i.e. 250 µl mitochondria-free supernatant fraction (equivalent to 100 mg. of tissue) was incubated with 250 µl 0.2M phosphate buffer (pH 7.4) containing 0.308M NaCl, 1.2 mg. NAD and 100 µg pregnenolone, 17αOH-pregnenolone or DHA for 2 hours at 37°. To estimate recovery of substrate in the case of pregnenolone, a tracer amount of [7α-^{3}H] pregnenolone was added at the conclusion of the incubation period. Extraction and partition, on the reduced scale, were as in experiment 6 (p.44). Aliquots of the dried residues from the aqueous methanolic fractions were taken for radioactivity counting and column chromatography on alumina (p.26).

D. Studies with Human Adrenals.

1. Data on Patients.

Patient	Sex	Age (yr.)	Left or Right Gland (g.)	Diagnosis[1]	Source & Time Lapse[3] (hr.)
A.W.	F	45	L (4.2)	Ca. Br. (P.I.)	Cardiff (24)
M.S.	F	39	L (2.8)	Ca. Br. (P.I.)[2]	Cardiff (24)
C.W.	F	63	L (2.7)	Ca. Br. (P.I.)	Cardiff (24)
H.R.	F		L (5.8)	Hyp.	Glasgow (0.5)
M.B.	F	29	L	Hyp.	Glasgow (0.5)
J.M.	M	33	L (8.9)	Hyp.	Dundee (5.0)
B.H.	M	22	R (10.9)	Hyp.	Glasgow (0.5)
R.V.	F		L (5.35)	Hyp.	Glasgow (0.5)

Table 6.

1. Ca. Br. = Breast Cancer.

   Hyp. = Cushing's syndrome due to bilateral adrenal hyperplasia.

   Ad. = Adenoma.

2. P.I. = Patient received Pituitary Implant Therapy prior to Adrenalectomy.

3. Source = Cardiff = Cardiff Royal Infirmary.
   Glasgow = Glasgow Royal Infirmary.
   Dundee = Maryfield Hospital, Dundee.

Time Lapse:- Approximate time taken for gland to reach laboratory after removal from patient.

2. Effect of Versene and Medium Composition on Metabolism of [7α-^{3}H] Pregnenolone.

The cortex of an adrenal gland (A.W.) was removed by scraping with a scalpel. Two 20% homogenates were prepared:-

(i) in 0.25M sucrose containing 0.12M nicotinamide (S/N) or

(ii) in 0.1M phosphate buffer (pH 7.4) containing 0.154M NaCl (PO_4 x 1). Aliquots of each homogenate were diluted with an equal volume of the medium in which they were prepared, or with

a) 0.2M phosphate buffer (pH 7.4) containing 0.308M NaCl (PO_4 x 2)

or

b) 0.15M TRIS buffer at pH 7.4 (TRIS x 2) as set out in the table below. 2.5 ml. of each diluted homogenate was incubated with 20 µg [7α-^{3}H] pregnenolone (1 µC) dissolved in 100 µl propylene glycol, 50 µl 0.5M versene (final concentration 10mM), and 1 mg. NAD at 37° for 30 minutes.

Incub.	Homog. Medium	Medium Added	Versene. (50 µl of 0.5M)	Water (50 µl)	Code
1.	PO_4 x 1	PO_4 x 1	-	+	PO_4 x 1/PO_4 x 1/W
2.	PO_4 x 1	PO_4 x 1	+	-	PO_4 x 1/PO_4 x 1/V
3.	S/N	PO_4 x 2	-	+	SN/PO_4 x 2/W
4.	S/N	PO_4 x 2	+	-	SN/PO_4 x 2/V
5.	S/N	TRIS x 2	-	+	SN/TRIS x 2/W
6.	S/N	TRIS x 2	+	-	SN/TRIS x 2/V

Table 7.

3. Incubations with Pregnenolone, 17αOH-Pregnenolone and DHA.

All experiments in this section involved the incubation of a mitochondria-free supernatant fraction (0.25 ml.) and 0.2M phosphate buffer (pH 7.4) containing 0.308M NaCl (0.25 ml.) with pregnenolone, 17αOH--pregnenolone or DHA (100 µg) dissolved in 100 µl of propylene glycol, and 1 mg. NAD for 30 minutes at 37°.

Extraction, partition, column chromatography on alumina and measurement of Δ^5-3β-hydroxysteroids by the Oertel & Eik-Nes (1959) reagent were performed exactly as described in experiment 7 of Section C (p.47).

RESULTS

A. Adrenal Tissue - Histology and Histochemistry.

With all adrenal tissue investigated, haematoxylin and eosin stained sections showed that material taken to represent the zona fasciculata contained 93% ± 5 "clear" cells and that taken to represent the zona reticularis contained 85% ± 5 of "compact" cells.

The distribution of histochemically demonstrable 3β-hydroxysteroid dehydrogenase activity in both horse and human adrenal gland sections gave the classical picture of highest activity in the outer zona fasciculata with lowest activity in the zona glomerulosa and inner zona reticularis (see p.11). This pattern of activity was obtained with DHA, pregnenolone and with 17αOH--pregnenolone as substrates (see Plate I).

B. Studies with Horse Adrenals.

1. Incubations with [4-^{14}C] Progesterone.

Table 8 (p.55) shows that the recovery of radioactivity was uniformly high, and Table 9 (p.56) gives the percentage conversions of [4-^{14}C] progesterone to radioactive products having the chromatographic mobilities

PLATE 1.

of the steroids named. These products had the same mobilities as the reference steroids in several systems. Since the percentage conversions were derived from planimetric measurements, the figures quoted are approximate (error $\pm$ 15%). They do, however, give the correct order of magnitude. It should be noted (Table10 p.62) that in every case, more progesterone was metabolised by reticular tissue than by fascicular tissue and in two experiments out of three, a greater percentage of the progesterone metabolites was represented by 17αOH--progesterone, 11-deoxycortisol, cortisol and cortisone in fascicular tissue (Table12 , p.64). Tables 11 & 12 (pages 63 & 64) indicate a similar situation with regard to the transformation of progesterone to DOC and corticosterone.

Table12 (p.64) shows yet another facet of the results of this preliminary experiment, viz. the sum total of the percentage transformation of the substrate [4-^{14}C] progesterone to 17αOH-progesterone, 11-deoxycortisol, cortisol, cortisone, DOC and corticosterone, is remarkably constant.

Proof of the identity of the peak ascribed to cortisol is given in Table 13 (p.65).

Expt.	Zone (F or R)	[4-^{14}C] progesterone incubated (mμmole)	(μC)	^{14}C recovered in MeOH aq. fraction (μC)	(%)
1.	F	191.0	5.0	4.58	91.6
	R	191.0	5.0	4.65	92.9
2.	F	76.4	2.0	1.96	98.0
	R	76.4	2.0	1.74	87.0
3.	F	38.2	1.0	0.96	96.1
	R	38.2	1.0	0.93	93.4

Table 8.

Expt.	Zone (F or R)	Steroid Associated with Radioactive Peak	Peak Area (arbitrary units)	% of total radioactivity
1.	F	progesterone	265	68.5
		20α(β)-hydroxypregn--4-en-3-one	-	-
		DOC	15	3.9
		17αOH-progesterone	15	3.9
		Unknown	15	3.9
		16αOH-progesterone	10	2.6
		corticosterone	26.5	6.8
		11-deoxycortisol	23.5	6.8
		cortisol	7.5	1.9
		cortisone	5	1.0
		polar unknown	8.5	2.2

Expt.	Zone (F or R)	Steroid Associated with Radioactive Peak	Peak Area (arbitrary units)	% of total radioactivity
1.	R	progesterone	140	36.7
		20α(β)-hydroxypregn--4-en-3-one	-	-
		DOC	52.5	13.8
		17αOH-progesterone	25	6.6
		Unknown	10	2.6
		16αOH-progesterone	15	3.9
		corticosterone	52	13.6
		11-deoxycortisol	38	10.0
		cortisol	15	3.9
		cortisone	5	1.3
		polar unknown	28	7.3

Expt.	Zone (F or R)	Steroid Associated with Radioactive Peak	Peak Area (arbitrary units)	% of total radioactivity
2.	F	progesterone	60	38.5
		20α(β)-hydroxypregn--4-en-3-one	4	2.6
		DOC	4	2.6
		17αOH-progesterone	10	6.4
		Unknown	6	3.8
		16αOH-progesterone	6	3.8
		corticosterone	6	3.8
		11-deoxycortisol	22	14.1
		cortisol	26	16.7
		cortisone	6	3.8
		polar unknown	6	3.8

Expt.	Zone (F or R)	Steroid Associated with Radioactive Peak	Peak Area (arbitrary units)	% of total radioactivity
2.	R	progesterone	38	21.3
		20α(β)-hydroxypregn--4-en-3-one	4	2.2
		DOC	6	3.4
		17αOH-progesterone	12	6.7
		Unknown	20	11.2
		16αOH-progesterone	6	3.4
		corticosterone	6	3.4
		11-deoxycortisol	24	13.5
		cortisol	34	19.1
		cortisone	18	10.1
		polar unknown	10	5.6

Expt.	Zone (F or R)	Steroid Associated with Radioactive Peak	Peak Area (arbitrary units)	% of total radioactivity
3.	F	progesterone	46.6	43.1
		20α(β)-hydroxypregn--4-en-3-one	-	-
		DOC	5.5	5.1
		17αOH-progesterone	15.0	13.9
		Unknown	4.2	3.9
		16αOH-progesterone	6.3	5.8
		corticosterone	9.4	8.7
		11-deoxycortisol	14.8	13.7
		cortisol	6.0	5.6
		cortisone	-	-
		polar unknown	-	-

Expt.	Zone (F or R)	Steroid Associated with Radioactive Peak	Peak Area (arbitrary units)	% of total radioactivity
3.	R	progesterone	13.7	14.4
		20α(β)-hydroxypregn-4-en-3-one	4.4	4.6
		DOC	4.4	4.6
		17αOH-progesterone	12.0	12.6
		Unknown	5.5	5.8
		16αOH-progesterone	7.4	7.8
		corticosterone	6.5	6.8
		11-deoxycortisol	21.7	22.8
		cortisol	14.5	5.4
		cortisone	5.1	15.3
		polar unknown	-	-

Table 9.

Expt.	Zone (F or R)	% Prog. Metab.	% of Radioactivity Incub. Rep. by 17αOH-P, S, F & E†	Ratio of Metabs. 17αOH-P, S, F & E (F/R)
1.	F	31:5	12:6	0:58
	R	63:3	21:8	
2.	F	61:5	41:0	0:83
	R	78:7	49:4	
3.	F	56:9	33:2	0:59
	R	85:6	56:1	

Table 10.

†% of Radioactivity Incubated Represented by:-

17αOH-progesterone (17αOH-P)

11-deoxycortisol (S)

cortisol (F)

cortisone (E)

Expt.	Zone (F or R)	% of Radioactivity Incub. Rep. by DOC + B†	Ratio of Metabs. DOC & B (F/R)
1.	F	10.7	0.39
	R	27.4	
2.	F	6.4	0.94
	R	6.8	
3.	F	13.8	1.21
	R	11.4	

Table 11.

†% of Radioactivity Incubated Represented by:-

11-deoxycorticosterone (DOC) and

corticosterone (B)

Expt.	Zone (F or R)	% of Metabs. Represented by 16αOH-P, S, F & E[1]	% of Metabs. Represented by DOC & E[1]	% of Metabolites Represented by 17αOH-P, S, F, E, DOC & E.
1.	F	40.0	34.0	74.0
	R	34.4	43.3	77.7
2.	F	66.7	10.4	77.1
	R	62.8	8.6	71.4
3.	F	58.3	24.3	82.6
	R	65.5	13.3	78.8

Table 12.

[1] see Tables 10 and 11) (pages 62 and 63).

Reaction	Product	System (Rf)	Specific Activity (mμC/μmole)
-	cortisol	B10 (0.23)	22.66
acetylation	cortisol acetate	PB55 (0.26)	21.92
reduction	11β,17α,20β,21-tetra-hydroxypregn-4-en--3-one	ET19 (0.10)	22.45

Table 13.

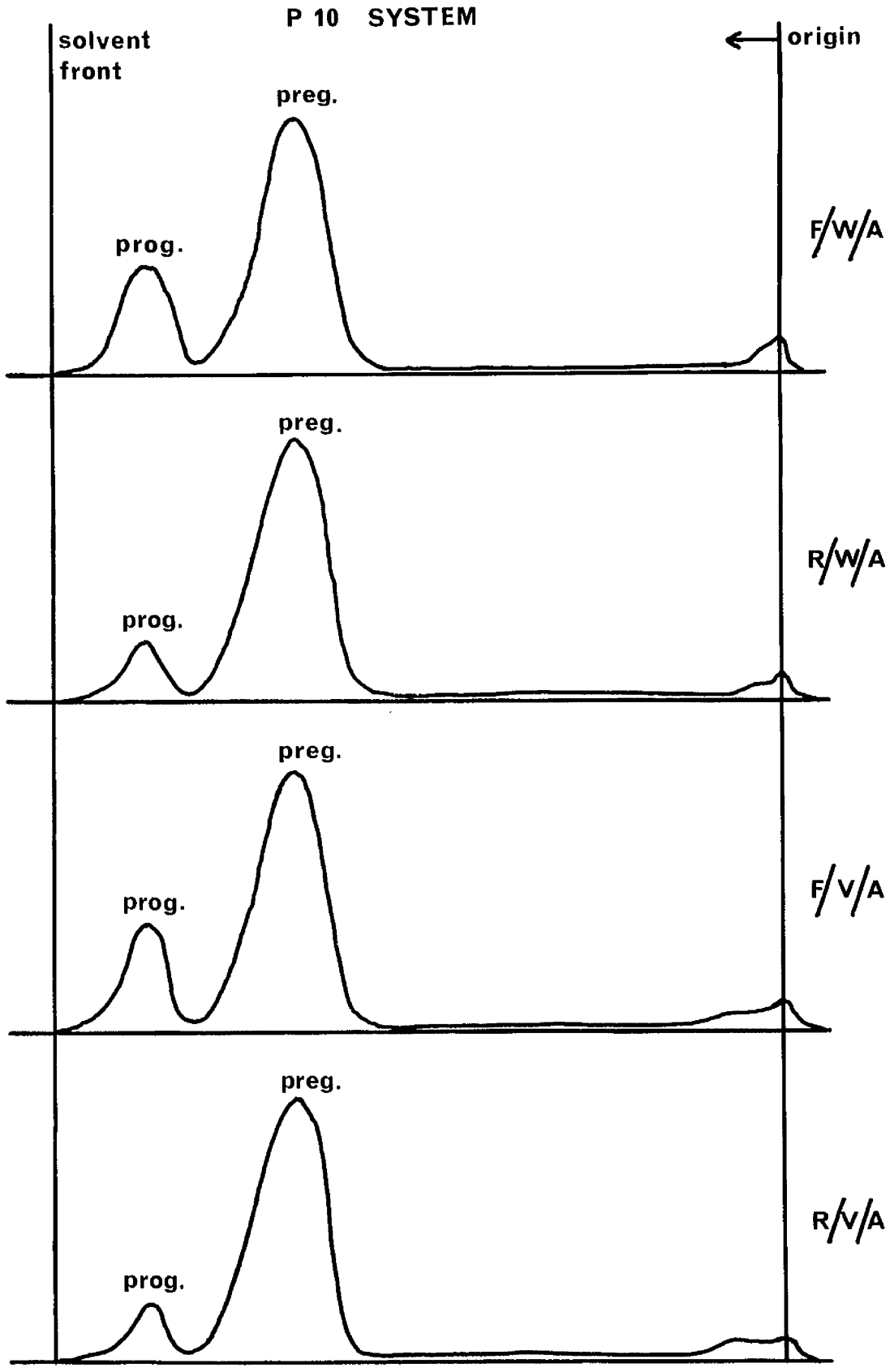
2. Effect of Versene and Anaerobic Conditions on the Metabolism of [7α-^{3}H] Pregnenolone.

The figures obtained for the recovery of ^{3}H are uniformly high (Table 14, p.67) indicating that the radioactivity scans (Figs. 3 & 4) are comparable. Of the incubation conditions investigated, it can be seen that there is little to chose between incubations 1 & 2 and incubations 3 & 4 i.e. Versene has no appreciable effect on the metabolism of the [7α-^{3}H] pregnenolone under aerobic conditions. From the scans from incubations 5 & 6 and 7 & 8, it is apparent that the use of anaerobic conditions alone simply depresses the total metabolism of the substrate although the addition of Versene causes a slight improvement.

Incub.	Code†	^{3}H Recovered (mμC)	(%)
1.	F/W/A	831.1	81.0
2.	R/W/A	847.5	82.6
3.	F/V/A	821.8	80.1
4.	R/V/A	837.2	81.6
5.	F/W/N	846.5	82.5
6.	R/W/N	887.5	86.5
7.	F/V/N	835.2	81.4
8.	R/V/N	835.2	81.4

Table 14.

†see p.38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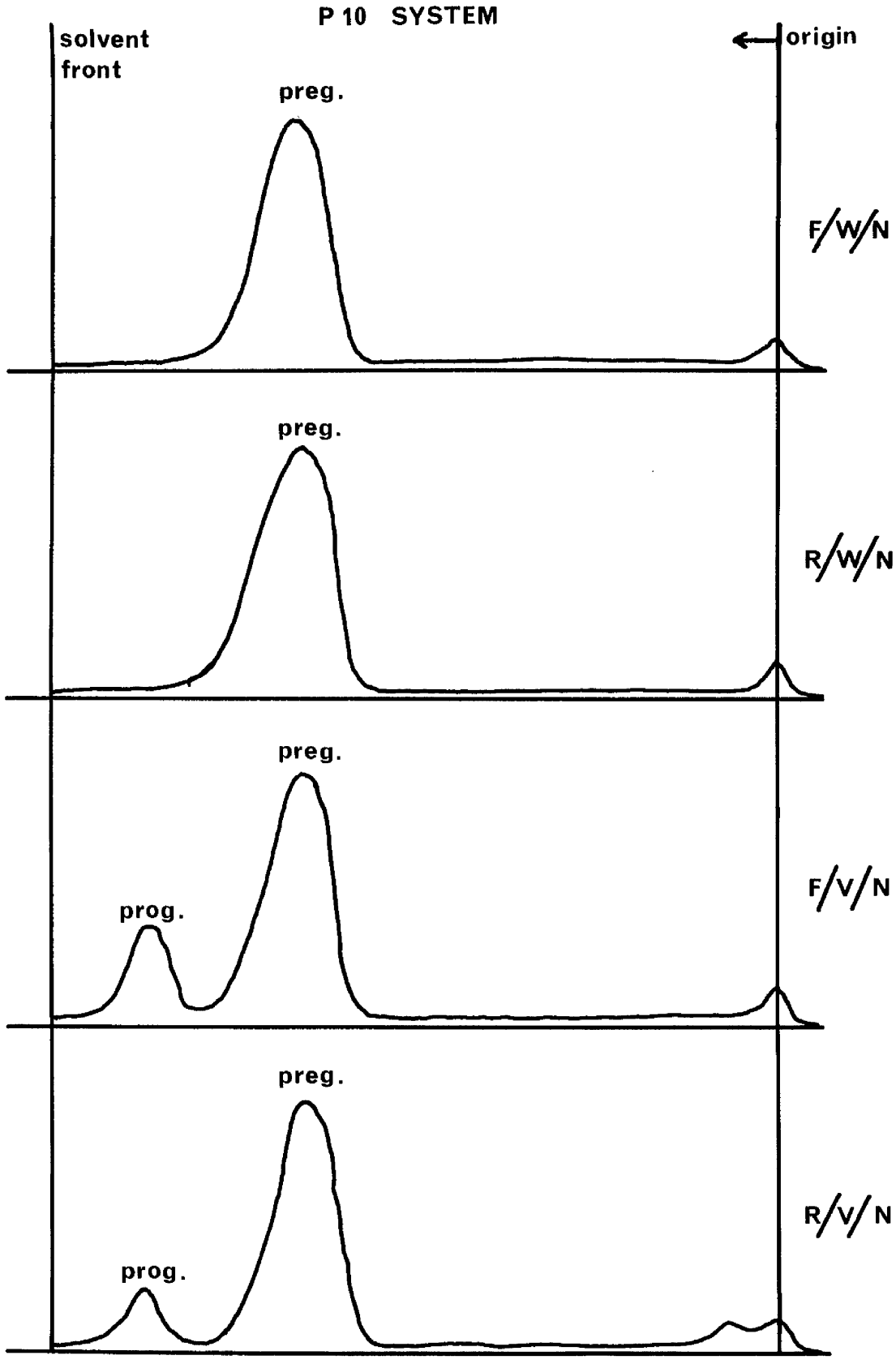
prog.
R/V/A

fig . 3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F/W/N
preg.
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preg.
prog.
F/V/N
preg.
prog.
R/V/N

fig. 4

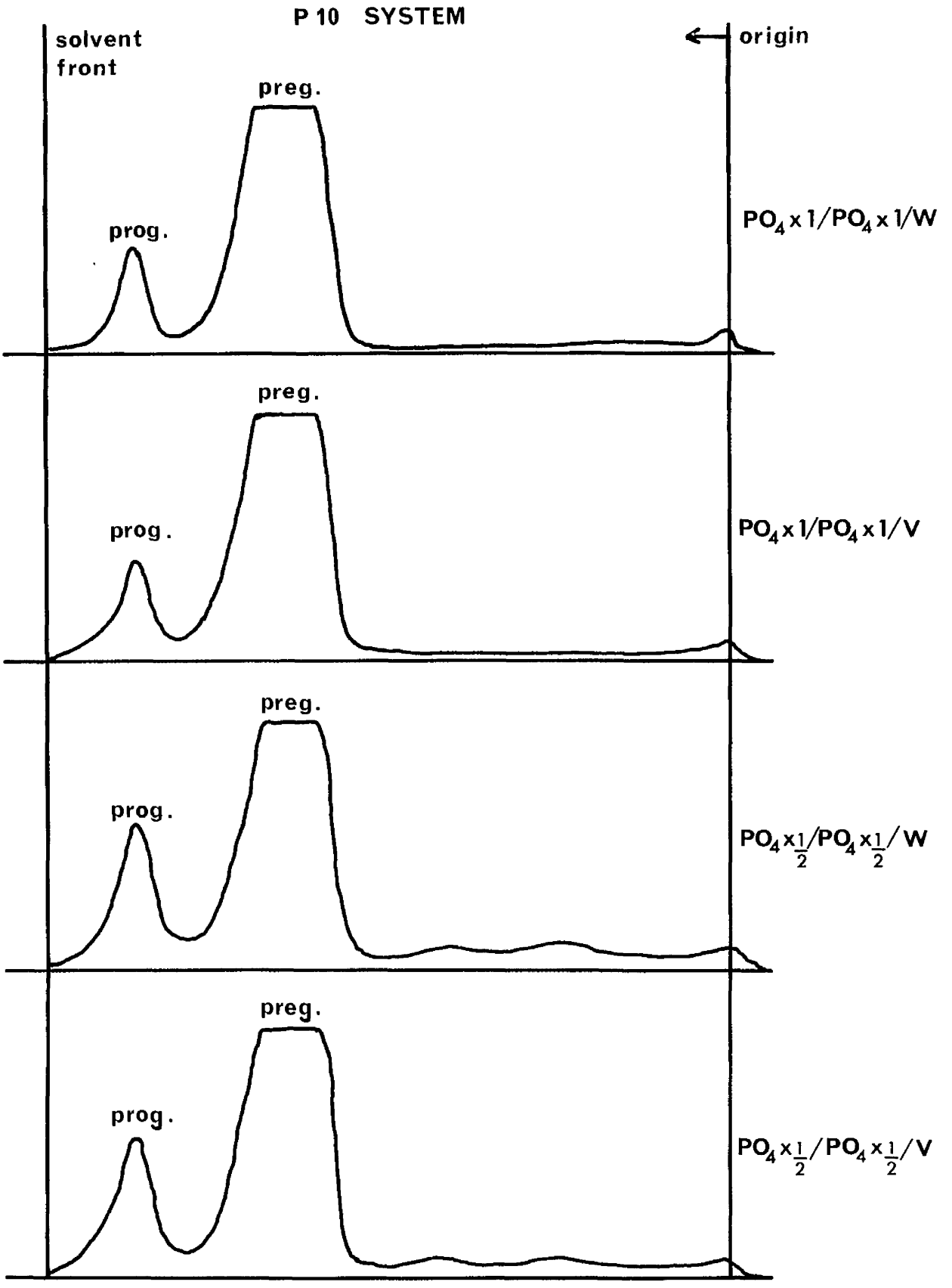
3. Effect of Medium Composition on the Metabolism of [7α-^{3}H] Pregnenolone.

Recovery of radioactivity was again high in this series of incubations with the exception of those in which sucrose/nicotinamide medium was used for the preparation of the homogenate (Table 15, p.69). The radioactivity scan traces (figs. 5, 6 & 7) show as in the previous experiment, that the addition of versene has little effect on the course of metabolism under the conditions studied. The metabolism of the substrate, [7α-^{3}H] pregnenolone, to products other than progesterone can be seen to be very low in all cases. It is also apparent that the optimal conditions of conversion of pregnenolone to progesterone are to be found in incubation 5, viz. the case in which tissue is homogenised in sucrose/nicotinamide medium and the homogenate diluted with 0.2M phosphate buffer (pH 7.4) containing 0.308M NaCl.

Incub.	Code†	^3H Recovered in Aqueous Methanol Fraction (mμC)	% Recovery of ^3H in Aqueous Methanol Fraction
1.	PO_4 x 1/PO_4 x 1/W	821.8	80.1
2.	PO_4 x 1/PO_4 x 1/V	898.8	87.6
3.	PO_4 x ½/PO_4 x ½/W	792.1	77.2
4.	PO_4 x ½/PO_4 x ½/V	840.3	81.9
5.	SN/PO_4 x 2/W	564.3	55.0
6.	SN/PO_4 x 2/V	656.6	64.0
7.	SN/PO_4 x 1/W	506.8	49.4
8.	SN/PO_4 x 1/V	517.1	50.4
9.	SN/TRIS x 2/W	709.0	69.1
10.	SN/TRIS x 2/V	660.7	64.4
11.	TRIS/TRIS/W	893.6	87.1
12.	TRIS/TRIS/V	873.1	85.1

Table 15.

†see p. 40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prog.
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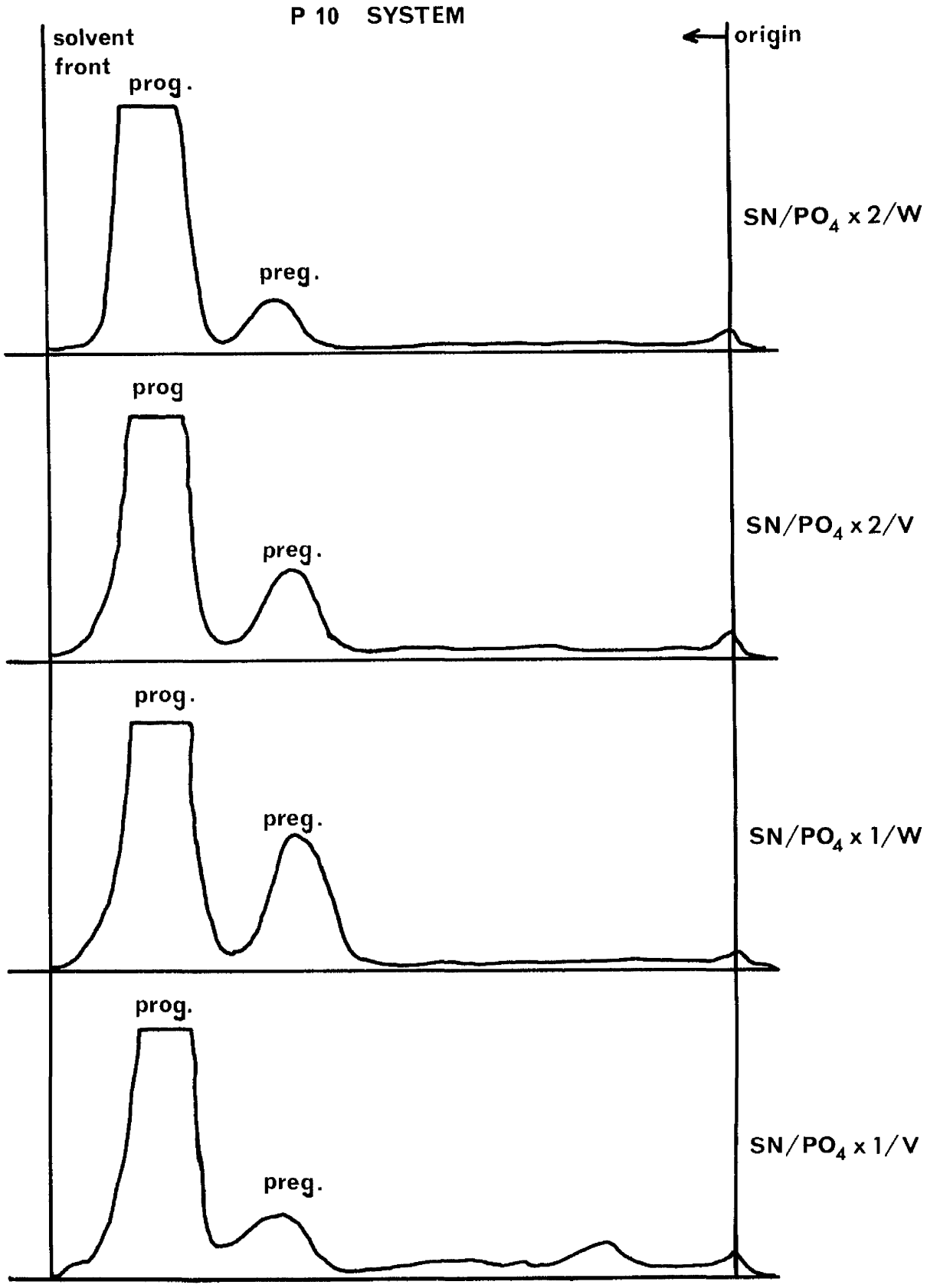
preg.
prog.
PO4 x 1/2/PO4 x 1/2/V

fig. 5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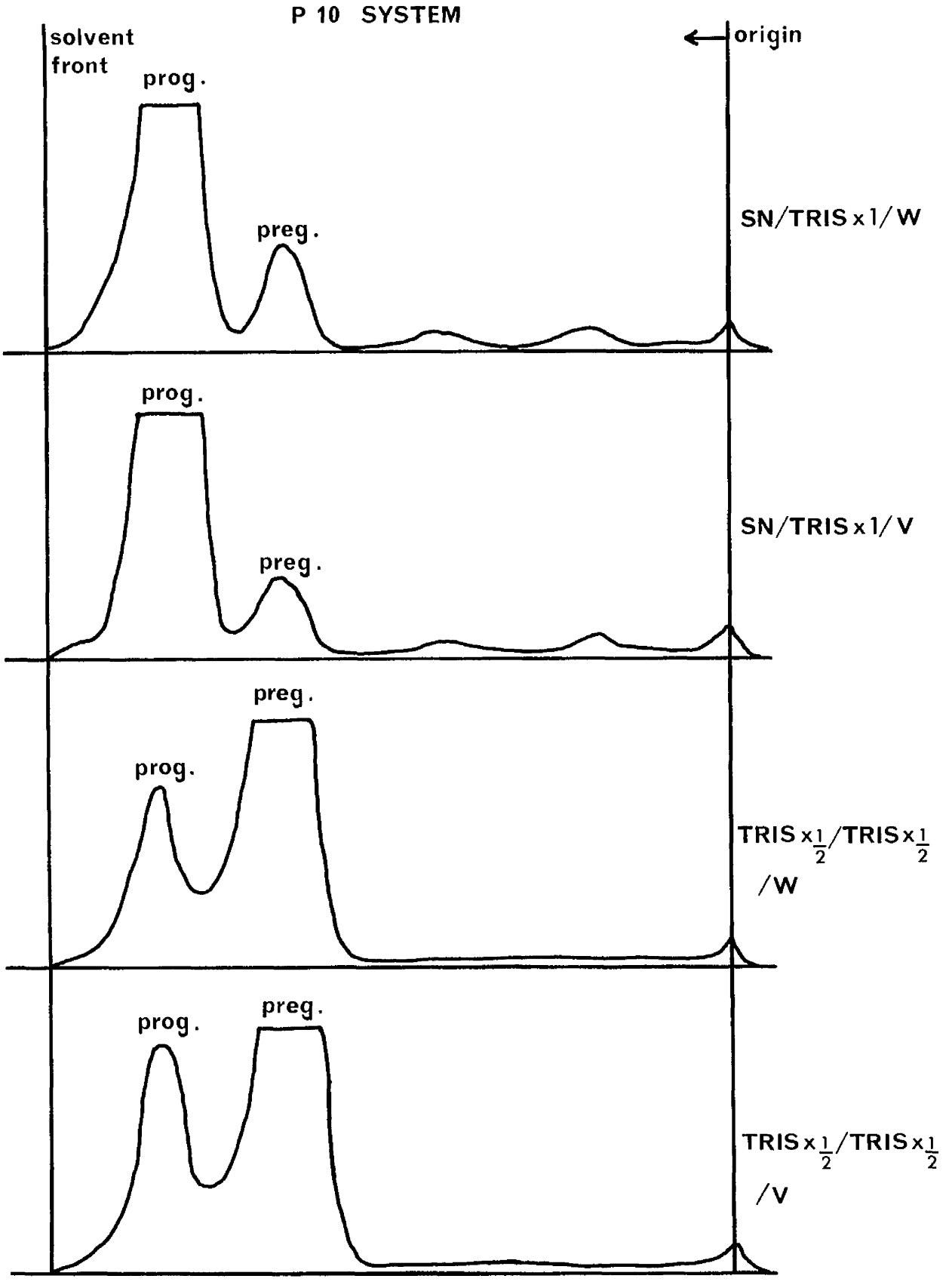
prog.
preg.
SN/PO4 x 1/V

fig. 6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solvent front
origin
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preg.
SN/TRIS x 1/W
prog.
preg.
SN/TRIS x 1/V
preg.
prog.
TRIS x 1/2 /TRIS x 1/2 /W
prog.
preg.
TRIS x 1/2 /TRIS x 1/2 /V

fig. 7

4. Rate of Metabolism of [7α-^{3}H] Pregnenolone.

a) From Table 16, (p.72), it is very noticeable that as the time of incubation is increased from 5 to 30 minutes (incubations 1 - 5), the recovery of ^{3}H in the aqueous methanol fractions (Fraction 4, see p.43) steadily falls. The missing radioactivity is not found in any of the other fractions. (When a large quantity of substrate (200 μg) is employed, a much higher percentage recovery of radioactivity is observed - - 80.0% ± 1.1 as a mean of four determinations of the radioactivity content of the aqueous methanol fraction, Fraction 4.)

From fig. 8 it can be seen that 20 μg. of pregnenolone is rapidly metabolised by the quantity of homogenate used and indeed after 5 minutes little more transformation of pregnenolone to progesterone appeared to occur although there was a small increase in the peak having the chromatographic mobility of DOC.

b) In this section, recoveries of radioactivity are set out in Table 17 (p.73) and, as in previous experiments, the bulk of the incubated label is found in the aqueous methanol fraction. In experiments 1 - 6 the recovery

tends to increase as larger quantities of pregnenolone are used.

As might be expected, a gradual decrease in the degree of metabolism is seen as the mass of steroid incubated increases (fig. 9), from ~ 50% metabolism at the 50 µg level to ~ 25% metabolism at the 150 µg level. Appreciable quantities of products more polar than progesterone were not observed.

Incub.	Time (mins.)	Fraction 2a† % of ^3H incub.	Fraction 2b % of ^3H incub.	Fraction 3 % of ^3H incub.	Fraction 4 % of ^3H incub.	Total % recov. of ^3H
1.	5	-	9.6	4.5	72.9	87.0
2.	10	-	9.2	4.4	64.5	76.1
3.	15	-	8.6	3.9	63.6	76.1
4.	20	-	9.7	4.2	55.7	69.6
5.*	30	5.0	-	4.0	51.7	60.7

Table 16.

*mean of four determinations.

†see page 42

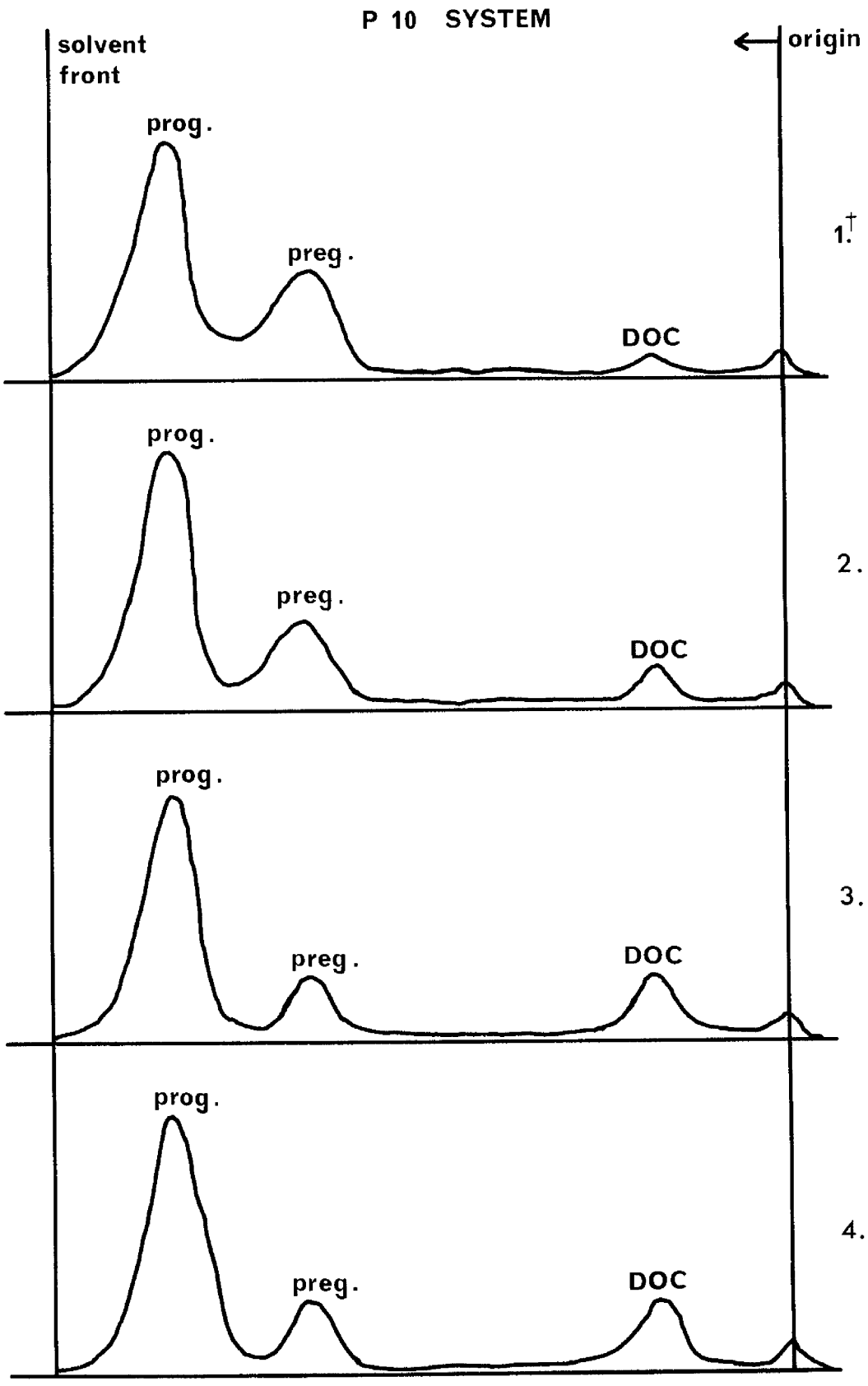

fig. 8 († see Table 16.)

Incub.	Pregnenolone Incubated (µg.)	(mµC)	Time (mins.)	Recovery of ^{3}H in MW Fraction.[3] (mµC)	(%)
1.	50	2052	5	1729	84.2
2.	50	2052	5	1477	72.0
3.	100	4104	5	3476	84.7
4.	100	4104	5	3443	83.9
5.	150	6156	5	5325	86.5
6.	100	6156	5	5491	89.2
7.[1]	50	1026	-	999	97.4
8.[1]	50	1026	-	989	96.4
9.[2]	50	1026	-	1014	98.8
10.[2]	50	1026	-	1090	106.2

Table 17.

[1] [7α-^{3}H] pregnenolone added to incubation mixture + pregnenolone before addition of extracting solvents.

[2] [7α-^{3}H] pregnenolone added to incubation mixture + pregnenolone after addition of extracting solvents.

[3] aqueous methanol fraction.

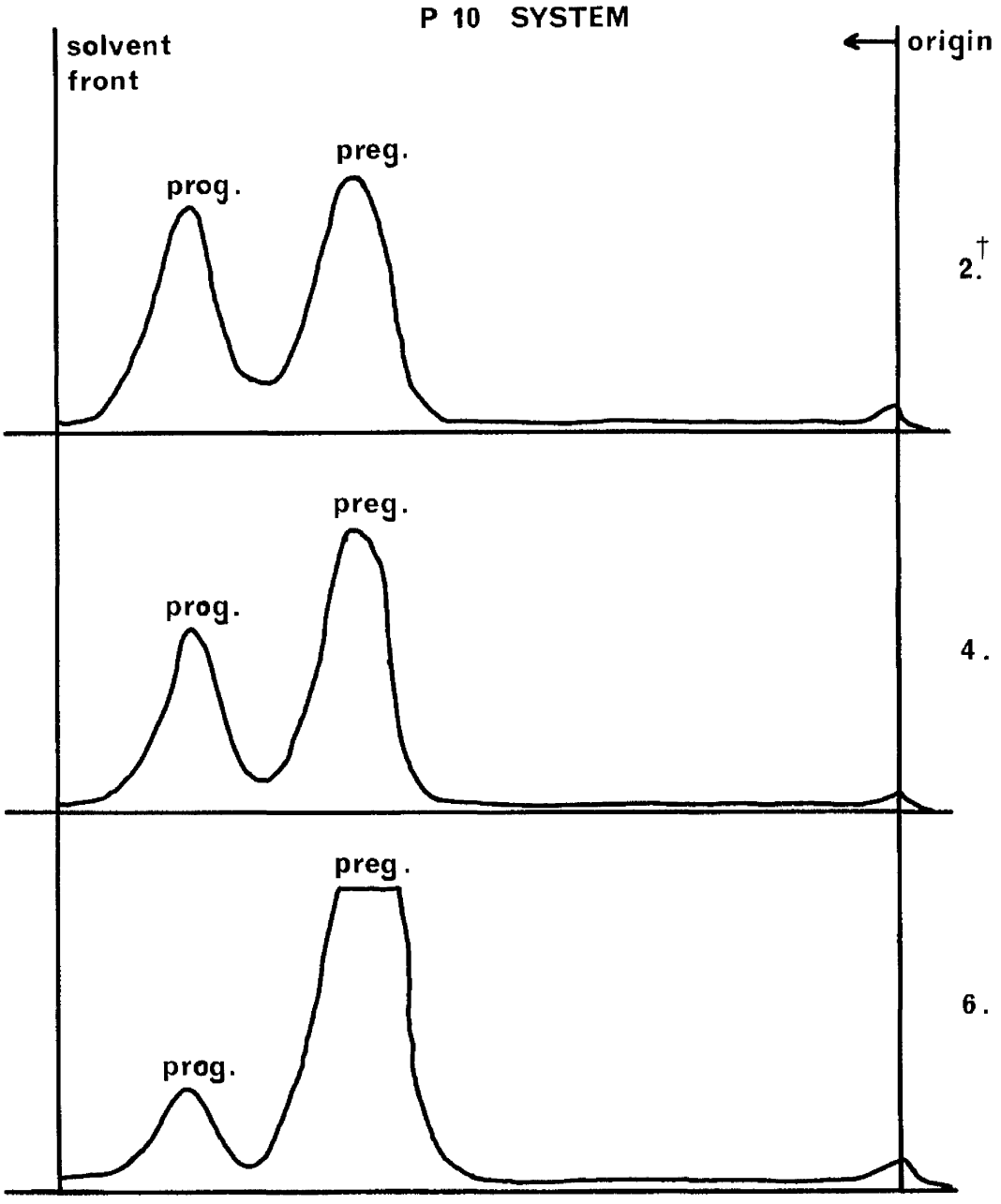

fig. 9

(†see Table 17.)

Expt. 2 :– $\frac{1}{2}$ aqueous methanol fraction.

" 4 :– $\frac{1}{4}$ " " " .

" 6 :– $\frac{1}{6}$ " " " .

5. Incubations with [4-^{14}C] DHA.

Five determinations of 3β-hydroxysteroid dehydrogenase activity in fasicular and reticular tissue from the horse adrenal cortex were made with [4-^{14}C] DHA as substrate. The recoveries of radioactivity in the aqueous methanol fraction (Table 18, p.76) are almost invariably greater than 90%. After paper chromatography in the P10 system (p.23) of the dried residue from this fraction, only peaks of radioactivity corresponding in mobility to Δ^4-androstenedione (Δ^4-A) and DHA standards were observed (fig. 10).

The areas of the paper chromatograms corresponding to Δ^4-androstenedione and DHA respectively were eluted and the eluates divided into two parts. The first part of each eluate was acetylated and the second part reduced (pages 29 & 30). On re-chromatography, it was found that a) the radioactive substance associated with Δ^4-androstenedione did not acetylate, and on reduction

had the mobility of testosterone, and b) the radioactive substance associated with DHA ran with DHA acetate upon acetylation and with androstenediol on reduction. Finally, the radioactive reduction products with the mobilities of testosterone and androstenediol were acetylated and subsequently found to have the chromatographic mobilities of testosterone acetate and androstenediol diacetate respectively.

Table 19 (p. 77) shows that 25 - 30% greater 3β-hydroxysteroid dehydrogenase activity is associated with the 5000 x g supernatant fraction prepared from homogenates of fascicular tissue compared with the same fraction prepared from reticular tissue.

The protein nitrogen content of each supernatant fraction was measured by the method of Nayyar & Glick (1954) and the results are given in Table 20 (p. 78).

Incub.	Zone (F or R)	^{14}C Recovered in Aqueous Methanol Fraction (mμC)	(%)
1.	F	577.4	97.7
	R	552.6	93.5
2.	F	582.1	98.5
	R	594.5	100.6
3.	F	487.6	82.5
	R	604.0	102.2
4.	F	525.4	88.9
	R	545.5	92.3
5.	F	523.7	90.3
	R	533.7	90.3

Table 18.

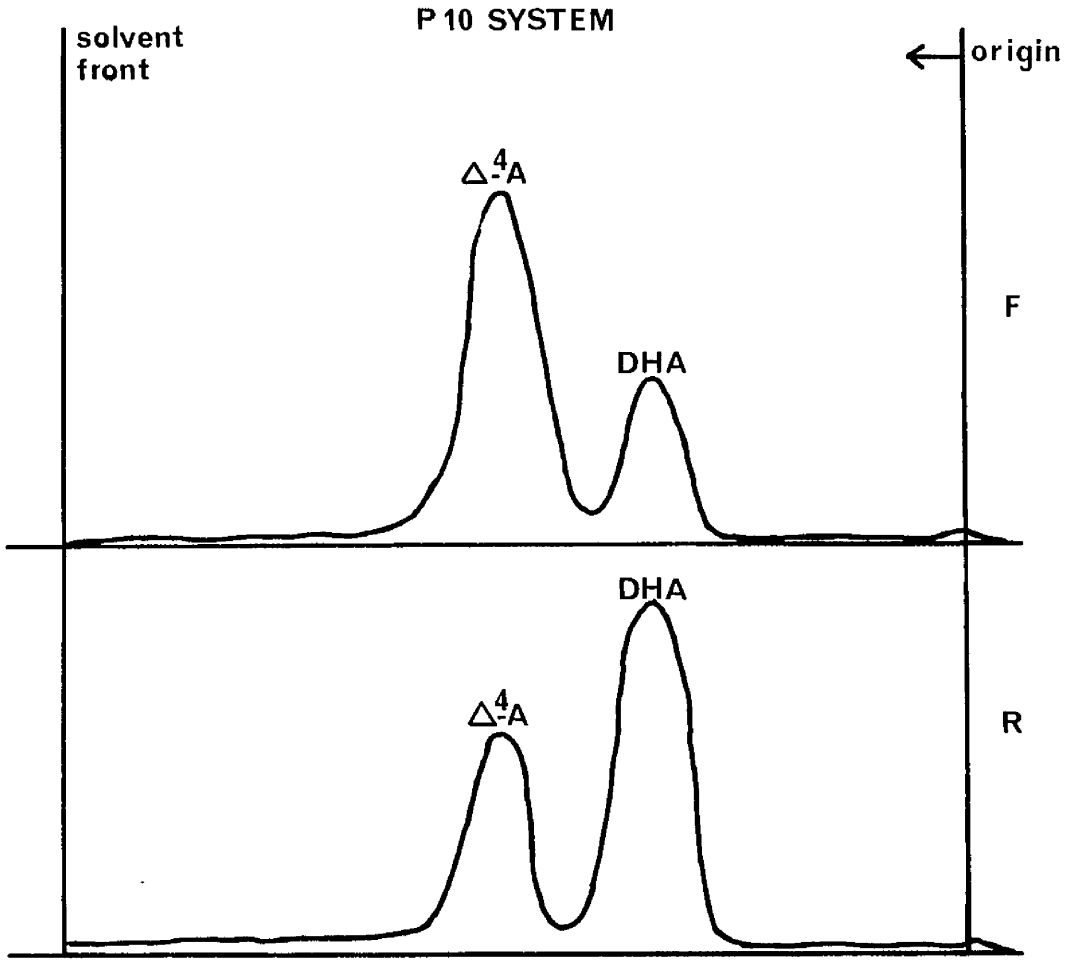

fig. 10

Incub.	Zone (F or R)	Area of DHA Peak (arbitrary units)	Area of Δ^4-A Peak (arbitrary units)	Δ^4-A formed (%)	Ratio of Δ^4-A formed (F/R)
1.	F	56	534	90.5	1.20
	R	165	508	75.5	
2.	F	389	579	60.4	1.32
	R	677	570	45.7	
3.	F	357	576	61.7	1.27
	R	539	512	48.7	
4.	F	288	572	66.5	1.29
	R	450	478	51.5	
5.	F	336	523	60.9	1.26
	R	518	484	48.3	

Table 19.

Incub.	Zone (F or R)	Protein N per Incub. (mg.)	Ratio of protein N (F/R)
1.	F	3.21	1.15
	R	2.80	
2.	F	3.05	1.12
	R	2.73	
3.	F	2.98	1.06
	R	2.81	
4.	F	2.90	1.04
	R	2.79	
5.	F	3.13	1.12
	R	2.78	

Table 20.

6. Incubations with [7α-^{3}H] Pregnenolone, [7α-^{3}H] 17αOH-Pregnenolone and [4-^{14}C] DHA.

a) Measurements based on the planimetric method.

With the larger quantity (500 μg) of [4-^{14}C] DHA, the radioactivity scan traces were similar to those described previously (see fig. 10) and require no further comment.

[7α-^{3}H] pregnenolone did not give such a simple result. Peaks due to other products were observed in the case of incubations of the mitochondria-free supernatant fractions from both zones (see fig. 11). A fifth (approx. 1 μC) of the extract in each case was chromatographed in the P10 system. Although the incubation of the [7α-^{3}H] pregnenolone appeared to give more [^{3}H] progesterone in the extract from the fascicular tissue preparation, substantial quantities of radioactivity with the mobility of DOC and some which did not move from the origin were observed in both cases. The radioactivity associated with DOC moved with the standard in the P10 and PB21 systems and, on acetylation, with DOC acetate in the P10 system. On elution of the origin material and re-chromatography in the PB55 system,

several small peaks were detected, the major one corresponding in mobility to corticosterone. After acetylation of the eluate from the area of the chromatogram corresponding to corticosterone, the radioactivity ran with corticosterone acetate in the PB55 system. No evidence was obtained to suggest that appreciable quantities of 17aOH-pregnenolone were formed but traces of radioactivity appeared to be associated with DHA. In view of the multiplicity of the products formed, the planimetric method could not be justified.

Extracts from a similar series of incubations with [7a-^{3}H] 17aOH-pregnenolone were chromatographed in various systems (P10; PB21; PB55; B10; PG/T; and Bz/$CHCl_3$/F). In no case was a satisfactory chromatogram radioactivity scan trace obtained. Streaking invariably occurred and ruined any possibility of planimetry.

b) Measurement of product formed by UV-absorption.

Preliminary experiments with extracts not treated by alkali and acid washing were found to have a very high "background" absorption which was non-linear between 225 and 255 mμ in ethanolic solution. Table 21 (p. 81) shows some examples of how this absorption was drastically reduced by these washing processes, yet the final duplicate

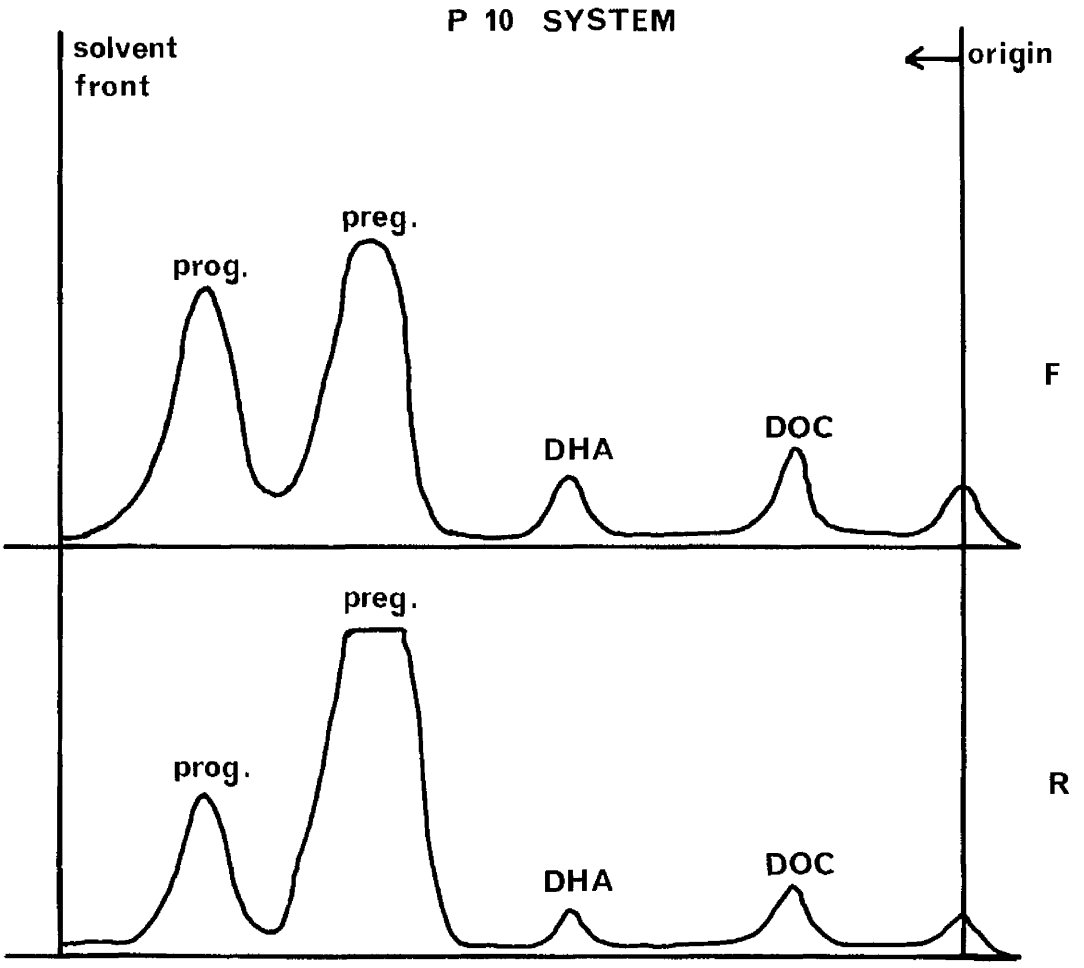
P 10 SYSTEM
solvent front
origin
prog.
preg.
DHA
DOC
F
prog.
preg.
DHA
DOC
R

fig .11

Experiment	Treatment of Extract	Optical Densities in 5 ml. EtOH (1/5th of extract)		
		E_{225}	E_{240}	E_{255}
1.	none	0.838	0.345	0.345
2a.	NaOH wash	0.712	0.288	0.288
2b.	" "	0.528	0.220	0.216
3a.	NaOH and HCl washes	0.214	0.108	0.088
3b.	" " " "	0.136	0.072	0.048

Table 21.

values obtained differ so widely as to make the method valueless. Measurement of known quantities of steroid agains "backgrounds" of the magnitude and variability of those described in Table 21 was found to introduce unacceptable errors in the determination of 3β-hydroxy-steroid dehydrogenase activity.

c) Measurement of residual substrate by H_2SO_4/ /ethanol reagent.

Table 22 shows how the extracts described in Table 21 gave rather different results when treated with the sulphuric acid/ethanol reagent of Oertel & Eik-Nes (1959). The situation was not improved by the washes and if anything was made slightly worse. It did seem, however, that the "background" was approaching linearity more quickly between 405 and 430 mμ than between 380 and 405 mμ and so a simplified corrected optical density value (Saffran & Schally, 1956) was employed from this point forward for the quantitative estimation of Δ^5-3β-hydroxysteroids where

$$\Delta O.D. = E_{405} - E_{430} \qquad \text{(cf. p. 32).}$$

Tables 23 and 24 give the results of experiments

designed to show the degree of oxidation of DHA and pregnenolone over a period of time from 15 - 60 minutes (see p.46).

Experiment	Treatment of Extract	Optical Densities in 5 ml. Reagent (1/5th of Extract)			Allen†
		E_{380}	E_{405}	E_{430}	
1.	none	0.168	0.114	0.084	-0.006
2a.	NaOH wash	0.168	0.100	0.062	-0.007
2b.	" "	0.170	0.108	0.070	-0.006
3a.	NaOH and HCl washes	0.222	0.150	0.104	-0.007
3b.	" " " "	0.194	0.126	0.086	-0.007

Table 22.

†Allen corrected optical density (see p. 32).

Expt.	Code[1]	Substrate recov. (µg.)	% recov. of ^{3}H or ^{14}C	µg. recov. (corrected for loss of ^{3}H or ^{14}C	µg. recov. (corrected for "quenching"[2])
1.	D recov.	238	89.6	266 (57.5%)	
2.	P recov.	250	91.9	262 (51.0%)	
3.	DF15	138	90.1	153	266
	DR15	200	88.9	225	391
4.	DF30	84	89.8	94	164
	DR30	142	92.6	153	266
5.	DF60	70	83.5	84	146
	DR60	115	83.5	138	240
6.	PF15	215	92.5	232	455
	PR15	245	92.5	265	520
7.	PF30	198	89.7	221	433
	PR30	153	90.8	169	331
8.	PF60	132	87.1	152	299
	PR60	132	83.1	159	311

Table 23.

Expt.	Code	Substrate recov. (see Table 23) (µg.)	Product formed Δ^4-A or Prog.† (µg.)	Ratio of product formed (F/R)
3.	DF15	266	197	2.73
	DR15	391	72	
4.	DF30	164	299	1.52
	DR30	266	197	
5.	DF60	146	317	1.42
	DR60	240	223	
6.	PF15	455	71	11.80
	PR15	520	6	
7.	PF30	433	93	0.48
	PR30	331	195	
8.	PF60	299	227	1.06
	PF60	311	215	

Table 24.

[1] see page 46

[2] The figures in this column are derived from those in the preceding one on the assumption that the loss of chromogenicity (quenching) due to interference from the tissue extract is constant for any given steroid in any given experiment. The justification for this is given in Tables 25 and 26.

† Δ^4-androstenedione (Δ^4-A)
progesterone (prog.)

Table 24 shows the values for the quantities of Δ^4-androstenedione and progesterone calculated to be formed in the respective incubations. The figures were not corrected for the loss of hydrogen from the substrate since this error was considered to be negligible. Table 25a shows the results of recovery experiments for DHA at three levels (133, 266 and 443 µg.) from a horse adrenal mitochondria-free supernatant + buffer + NAD mixture (p. 44) which had been incubated for 30 minutes at 37° prior to addition of the steroid ([4-^{14}C] DHA, 120,000 dpm, was also added to enable the determination of true losses).

The % recoveries are reasonably constant at all three levels.

Table 25b shows a similar set of results obtained under similar conditions to those described for Table 25a except that the enzyme + buffer + NAD mixture was not incubated before addition of the steroid.

Table 26 demonstrates how the "blank" residue from a tissue extract lowers the observed value for a known quantity of a Δ^5-3β-hydroxysteroid (DHA) as measured by the sulphuric acid/ethanol reagent. The "% recoveries" are relatively constant.

Expt.	DHA added to incubation mixture (µg.)[1]	% recovery of ^{14}C in DN fraction	µg. of DHA observed	µg. of DHA corr. for loss of ^{14}C	"% recovery" of DHA
1a	133	90.7	76	84	63.2
b	"	93.1	78	84	63.2
2a	266	91.9	157	171	64.3
b	"	90.1	159	176	66.2
3a	443	91.1	271	297	67.0
b	"	91.7	279	304	68.6

Table 25a.

[1] enzyme + buffer + NAD mixture incubated for 30 min. at 37° before addition of steroid.

Expt.	DHA added to incubation mixture (µg.)[1]	% recovery of ^{14}C in MW fraction	µg. of DHA observed	µg. of DHA corr. for loss of ^{14}C	"% recovery" of DHA
1a	133	89.7	76	85	63.9
b	"	85.1	70	82	61.7
2a	266	90.6	128	141	53.0
b	"	91.8	139	151	56.8
3a	443	91.2	248	272	61.4
b	"	89.0	259	291	65.7

Table 25b.

[1] enzyme + buffer + NAD not incubated before addition of steroid.

Tube	DHA added to "blank" residue (µg.)	DHA measured (µg.)	"% recovery" of DHA
1.	8.9	5.4	60.7
2.	17.7	9.7	54.8
3.	35.4	22.0	62.1

Table 26.

7. Incubations with Pregnenolone, 17αOH-Pregnenolone and DHA.

In Table 27, the substrate recovered unchanged from each incubation after column chromatography on alumina as measured by the sulphuric acid/ethanol reagent is recorded in column 4. (The statistics of recovery of the three Δ^5-3β-hydroxysteroids is discussed more fully in Appendix IV, p.198). "Blank" preparations were also chromatographed on alumina and the appropriate fraction evaporated to dryness. In most cases, the residue did not appear to quench the sulphuric acid/ethanol chromogen peak (see Appendix IV, p.198). The percentage recovery of each substrate is also given as determined

a) in the case of pregnenolone by the addition of a trace amount of [7α-^{3}H] pregnenolone at the conclusion of the incubation and measurement of the recovery of ^{3}H and

b) in the cases of 17αOH-pregnenolone and DHA by measurement of the quantities of these steroids which could be isolated from non-incubated steroid + tissue mixtures.

The amounts of product formed were calculated by

difference, the effect of the loss of the two hydrogen atoms being neglected.

Expt.	Substrate	Zone (F or R)	Substrate recov. (µg.)	% recov. of substrate (see a & b p. 92)	Substrate recov. corrected for losses (µg.)	Product formed (µg.)	Ratio of products formed (F/R)
1.	preg.	F	62.5	81.4	76.8	23.2	1.38
	"	R	70.1	84.3	83.2	16.8	
2.	"	F	39.2	88.0	44.6	55.4	1.36
	"	R	53.3	89.8	59.4	40.6	
3.	"	F	59.5	83.1	71.6	28.4	1.92
	"	R	67.3	79.0	85.2	14.8	
4.	"	F	42.3	97.1	43.6	56.4	1.07
	"	R	45.8	97.1	47.2	52.8	
5.	DHA	F	57.5	75.5	76.2	23.8	2.70
	"	R	68.9		91.2	8.8	

Expt.	Substrate	Zone (F or R)	Substrate recov. (µg.)	% recov. of substrate (see a & b p. 92)	Substrate recov. corrected for losses (µg.)	Product formed (µg.)	Ratio of products formed (F/R)
6.	DHA	F	59.5	75.5	78.8	21.2	1.71
	"	R	66.1		87.6	12.4	
7.	17aOH-Preg.	F	48.4	80.6	60.1	39.9	1.26
	"	R	55.1		68.4	31.6	
8.	"	F	52.4	79.1	66.3	33.7	0.96
	"	R	51.3		64.8	35.2	
9.	"	F	53.2	82.1	64.8	35.2	1.02
	"	R	53.7		65.4	34.6	

Table 27.

* Recovery of substrate corrected for extraction and partition losses (µg.).

D. Studies with Human Adrenals.

1. Effect of Versene and Medium Composition on the Metabolism of [7α-^{3}H] Pregnenolone.

As in similar experiments with horse adrenal tissue preparations (p. 66), the recovery of ^{3}H from all incubations is high (Table 28). This again enabled a comparison to be made of the radioactivity scan traces of paper chromatograms and consequently of the various incubation conditions.

It should be noted that the use of sucrose/nicotinamide medium for the preparation of the homogenate did not appear adversely to affect the recovery of ^{3}H in contrast to the results obtained with horse adrenal preparations. In figs. 12 and 13 it can be seen, again in contrast to the horse tissue experiment, that almost complete metabolism of the [7α-^{3}H] pregnenolone was observed in all cases. Versene had no discernible effect on the course of metabolism under the conditions used. With homogenates prepared in and diluted with phosphate buffer, considerable quantities of metabolites with the chromatographic mobilities of DOC and corticosterone were observed. Such a marked conversion to these

Incubation	Code†	^3H recovered in MW fraction	
		(mμC)	(%)
1.	PO_4 x 1/PO_4 x 1/W	930	84.3
2.	PO_4 x 1/PO_4 x 1/V	926	83.8
3.	SN/PO_4 x 2/W	901	81.5
4.	SN/PO_4 x 2/V	935	84.6
5.	SN/TRIS x 2/W	1,010	91.4
6.	SN/TRIS x 2/V	1,007	91.1

Table 28.

†for code, see p. 51

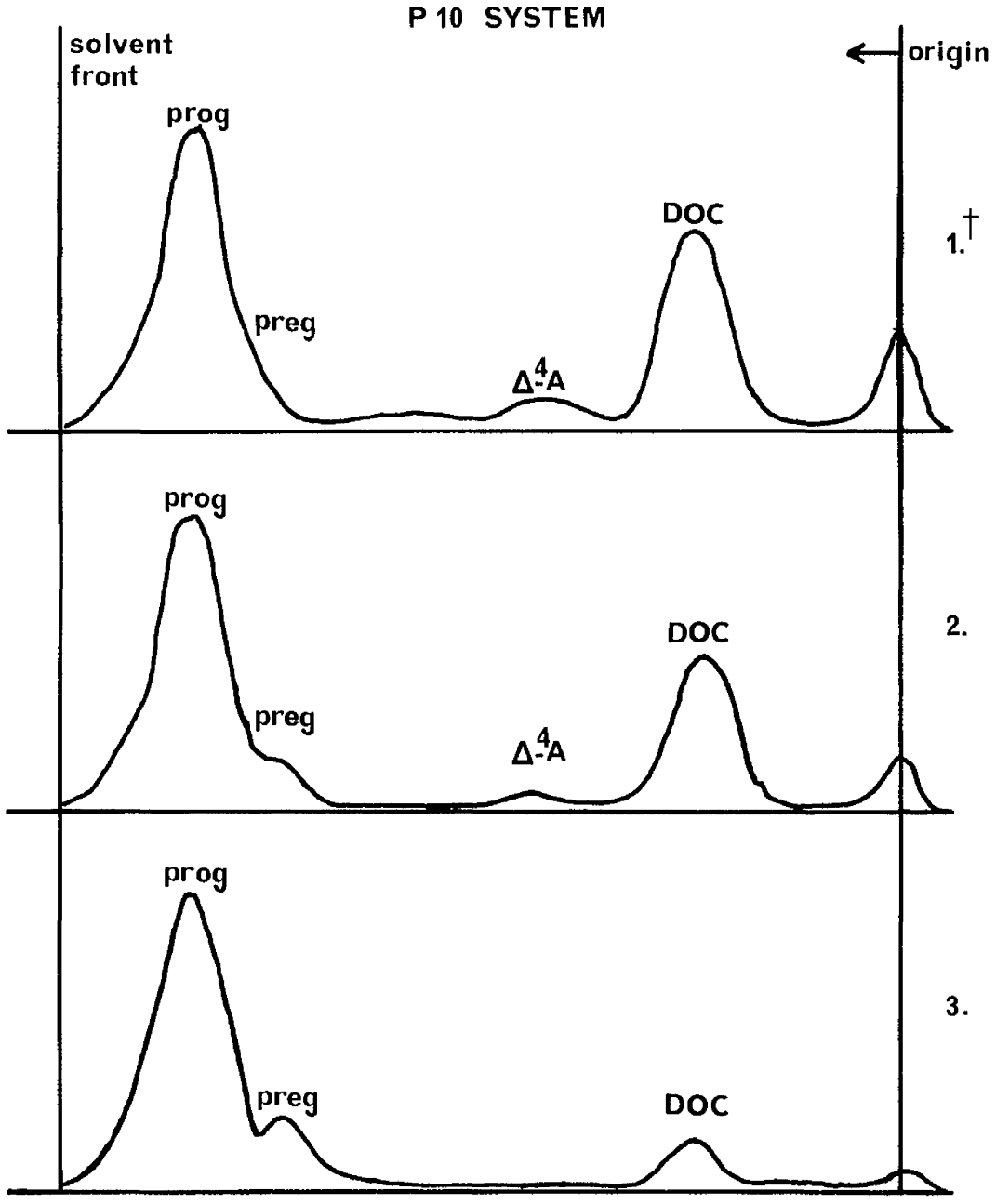

fig. 12

†1. :– $PO_4 \times 1/PO_4 \times 1/W$

2. :– $PO_4 \times 1/PO_4 \times 1/V$

3. :– $SN/PO_4 \times 2/W$ (see Table 28.)

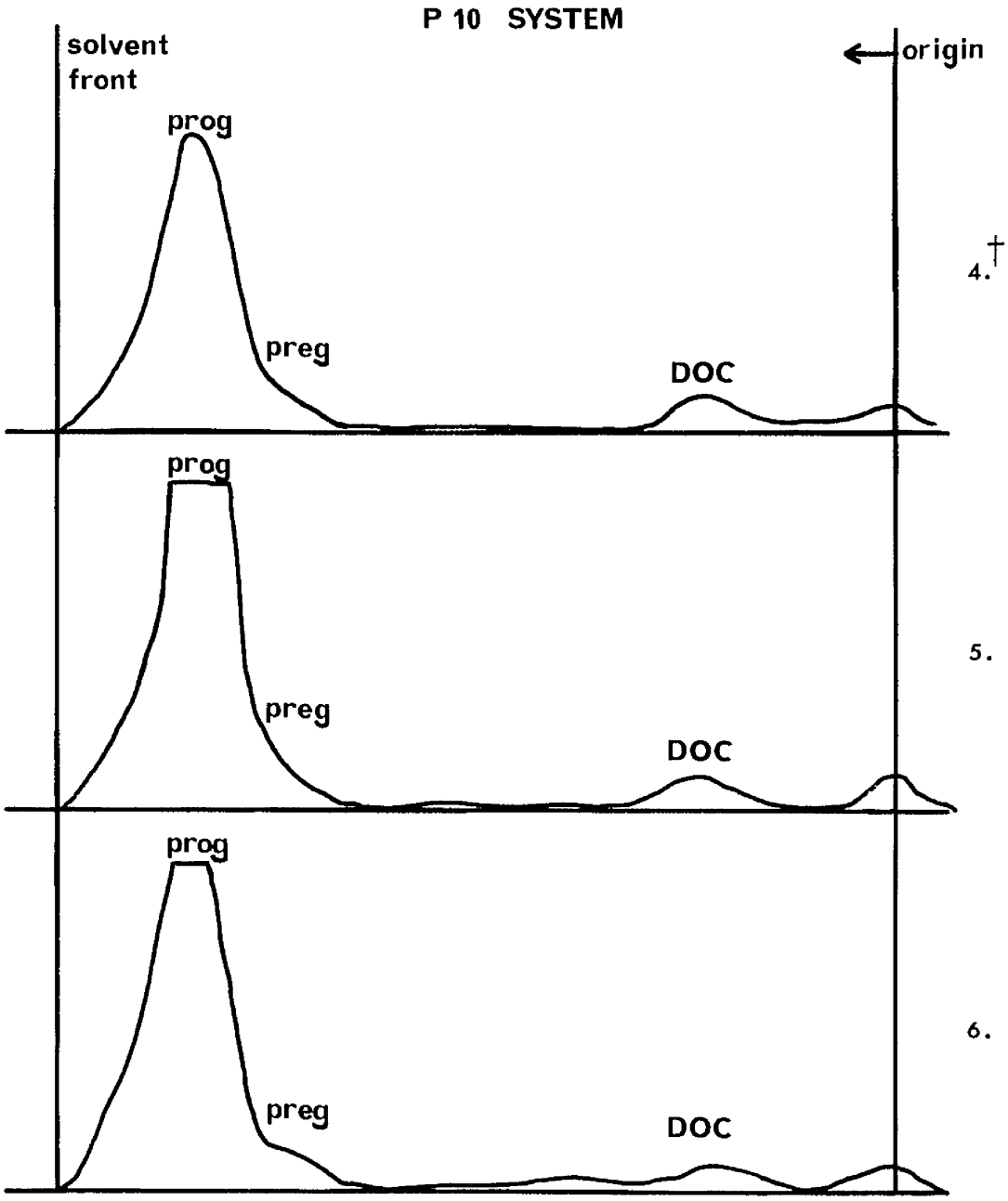

fig. 13

† 4. :– SN/PO_4 x 2 / V

5. :– SN/TRIS x 2 / W

6. :– SN/TRIS x 2 / V (see Table 28.)

metabolites was not observed under the other conditions described.

2. Incubations with Pregnenolone, 17αOH-Pregnenolone and DHA.

Table 29 summarises the results obtained. The tissue used in these experiments came from a variety of sources (see Table 6, p.48). Glands which arrived at the laboratory more than 4 hours after surgical removal from the patient were not satisfactory. Despite maintenance at about 0°, they had usually deteriorated to such an extent that the tissue was too soft for slicing with the Stadie-Riggs hand microtome and homogenates were prepared with a mixture of cell types. Adrenals obtained without delay from the Glasgow Royal Infirmary, on the other hand, were normally in excellent condition for slicing and some (B.H. and R.V.) actively metabolised the substrates incubated.

In column 5 of Table 29, the figures marked with an asterisk were determined by estimation of the recovery of a trace of labelled substrate added at the end of the incubation. The remainder were found by measurement of 100 µg. quantities of substrate from non-incubated

tissue + buffer mixtures as determined by the sulphuric acid/ethanol reagent following column chromatography on alumina. Recoveries of all three substrates, estimated by either technique, appeared to have a wide variation. Nevertheless, in the instances when good separation of fascicular and reticular tissue was possible, it is evident that DHA and pregnenolone are metabolised to a greater extent in fascicular tissue. With 17αOH-pregnenolone, however, the situation is equivocal.

Patient	Substrate	Zone (F or R)	Substrate recov. (µg.)	% recov. of substrate	Substrate recov. corrected for losses (µg.)	Product formed (µg.)†	Ratio of products formed (F/R)
M.S.	DHA	F + R	75.6	91.6	82.5	17.5	-
C.W.	"	F + R	65.1	97.0	67.1	32.9	-
N.R.	DHA	F	50.2	68.0	73.8	26.2	1.72
	"	R	57.2		84.8	15.2	
	Preg.[a]	F	37.6	74.4	50.6	49.4	1.92
	"	R	55.4		74.4	25.6	
	17αOH-Preg.[b]	F	47.5	56.1	84.7	15.3	0.60
	"	R	41.8		74.5	25.5	

Patient	Substrate	Zone (F or R)	Substrate recov. (µg.)	% recov. of substrate	Substrate recov. corrected for losses (µg.)	Product formed (µg.)+	Ratio of products formed (F/R)
M.E.	DHA	F	36.2	64.4*	56.2	43.8	1.30
	"	R	38.7	58.6*	66.0	34.0	
	Preg.	F	52.0	81.2*	64.1	35.9	1.23
	"	R	59.1	83.4*	70.9	29.1	
	17αOH--Preg.	F	50.5	87.6*	57.7	42.3	3.33
	"	R	76.8	88.0*	87.3	12.7	
J.M.	DHA	F + R	44.4	69.0	64.4	36.6	-
	Preg.	F + R	49.6	81.8	60.6	39.4	-
	17αOH--Preg.	F + R	67.6	82.8	81.6	18.4	-

Patient	Substrate	Zone (F or R)	Substrate recov. (µg.)	% recov. of substrate	Substrate recov. corrected for losses (µg.)	Product formed (µg.)†	Ratio of products formed (F/R)
B.H.	DHA	F	3.6	72.5*	5.0	95.0	1.12
	"	R	11.8	79.8*	14.8	85.2	
	Preg.	F	6.1	71.6*	8.4	91.6	1.16
	"	R	15.6	74.9*	20.8	79.2	
R.V.	17αOH-Preg.	F	13.9	62.6*	22.2	77.8	1.13
	"	R	24.6	78.7*	31.3	68.7	

Table 29.

[a]Pregnenolone

[b]17αOH-pregnenolone

*recovery of substrate estimated by recovery of radioactivity - trace of labelled substrate added to incubation mixture at the end of the incubation period.

†DHA ⟶ Δ^4-androstenedione; pregnenolone ⟶ progesterone; 17αOH-pregnenolone ⟶ 17αOH-progesterone.

DISCUSSION

As stated in the Results section (p.53), the tissue taken to represent the zona fasciculata and zona reticularis contained a high percentage of "clear" and "compact" cells, respectively. It is obviously important to achieve an efficient separation of cell types in order that subsequent observations of biological activity may be related to one particular cell type. The low degree of contamination observed in the present experiments should not seriously prejudice an assessment of biochemical differences between the two zones.

The fact that 17αOH-pregnenolone gives a histochemically determined picture of 3β-hydroxysteroid dehydrogenase activity similar to that found by other workers with DHA and pregnenolone (e.g. Wattenberg, 1958 and Pearson & Grose, 1959) is of particular interest.

The use of the horse adrenal gland for a study of the zonation of adrenal cells has not been described before and the incubation of homogenates of fascicular and reticular tissue from this source with [4-^{14}C] progesterone permitted a rapid preliminary determination of the biosynthetic activity of these tissues. It is apparent from the results that the tissue preparations

actively metabolise progesterone, and that the reticular cell preparations are invariably the more active of the two. The fascicular cell preparations, however, appear to convert a higher proportion of the substrate metabolised to 17αOH-progesterone, 11-deoxycortisol, cortisol and cortisone. In other words, these results indicate that the fascicular zone is better adapted to the production of cortisol than the reticular zone. This agrees with the evidence of Griffiths et al. (1963) that ACTH acts on fascicular rather than on reticular cells in vitro causing a rapid outpouring of cortisol.

Rigorous proof of the biosynthesis of [^{14}C] cortisol from [4-^{14}C] progesterone by homogenates of horse adrenal tissue was considered to be important in view of the failure of Ward & Grant (1963) to find this steroid in similar incubations with adrenal tissue from human subjects.

Interpretation of the figures obtained for the amounts of DOC and corticosterone isolated from incubations of each zone is difficult. It is possible, however, that there may be some relationship between the amount of progesterone incubated and the figures in column 4 of Table 11 (p. 63) i.e. the ratio of the relative proportions of DOC and corticosterone formed in each zone. The

different concentrations of progesterone may have affected the kinetics of a particular transformation in the sequence progesterone ⟶ DOC ⟶ corticosterone more in one zone than in the other.

No comment can be offered at this time on the apparent constancy of the total percentage transformations to the steroids on the pathways to cortisone and corticosterone (Table 12, p. 64).

Experiments 2, 3 and 4 were designed to find the optimum conditions for the conversion of pregnenolone to progesterone with minimum subsequent transformation of the progesterone formed. (The conditions described by Rubin, Leipsner & Deane (1961) for 3β-hydroxysteroid dehydrogenase assay were reproduced in Experiment 3, incubation No. 3.) Initially the use of sucrose/ /nicotinamide solution for the preparation of homogenates seemed to coincide with low recoveries of radioactive steroids (Table 15, p. 69). The chromatogram radioactivity scans, however, were encouraging in that a high percentage conversion of pregnenolone to progesterone appeared to have occurred with little further metabolism of the progesterone and no appreciable metabolism of pregnenolone to compounds other than progesterone. Eventually with increased quantities of substrate,

conditions which might be satisfactory for 3β-hydroxy-steroid dehydrogenase assay by planimetric measurement were found (Table 17, p. 73 and fig. 9).

These conditions appear to be:

a) the use of 0.25M sucrose containing 0.12M nicotinamide for the preparation of homogenates,

b) dilution of homogenates prior to incubation with an equal volume of 0.2M phosphate buffer (pH 7.4) containing 0.308M NaCl.

c) incubation of 2.5 ml. of the diluted homogenate with 100 µg. (in the case of DHA, a further increase to 150 µg. was found desirable) of steroid substrate dissolved in 100 µl of propylene glycol at 37° for 5 minutes.

In experiment 5 (p. 74), planimetric measurement of peak areas from paper chromatogram radioactivity scan traces provided a simple means of determining the amounts of unchanged DHA and Δ^4-androstenedione present in incubation extracts. Since equivalent amounts of tissue preparations from each zone were incubated with

the same weight of DHA for the same period of time, the quantities of Δ^4-androstenedione formed may be used to measure DHA-3β-hydroxy dehydrogenase activity. DHA--3β-hydroxy dehydrogenase activity proved to be greater in fascicular tissue than in reticular tissue preparations by a factor of 20 - 30% (Table 19, p. 77). One might have expected a much greater difference, however, in view of the histochemical evidence (p. 53). The peaks of radioactivity observed by Grant (1964) at the solvent front and origin in radioactivity scan traces from chromatograms of extracts from similar incubations of adrenal tissue from human subjects were not seen.

The protein nitrogen content of each tissue preparation was determined in order to see if there was an obvious relationship between the protein nitrogen content of each zone and the corresponding DHA-3β--hydroxy dehydrogenase activity. No such relationship could be found. It can also be seen from the protein nitrogen values in Table 20 (p. 78) that if 3β-hydroxy-steroid dehydrogenase activity were determined on a protein nitrogen basis, the results would still show higher 3β-hydroxysteroid dehydrogenase activity in fascicular tissue.

It has been noted above that results of incubation

of mitochondria-free supernatant fractions from fascicular and reticular tissue with 150 µg of DHA did not show dramatic differences in their DHA-3β-hydroxy dehydrogenase activities. Perhaps these differences might have been more apparent during the early stages of incubation while the reaction kinetics approximated more closely to first order. Since the quantities of product formed at this stage are very small, however, the planimetric method of measurement would be of no value.

With regard to Experiment 6, attempts were made to measure 3β-hydroxysteroid dehydrogenase activity by three different methods involving a) planimetry, b) UV absorption and c) Oertel reagent (see pages 44 and 79). Preliminary experiments had indicated that larger quantities of 3β-hydroxysteroids would require to be used if the residual substrate at the end of an incubation was to be measured accurately by the Oertel & Eik-Nes (1959) reagent. This was due to the fact that the "blank" values were high and the steroids themselves did not form very strongly absorbing chromogens. Consequently 500 µg quantities of the substrates DHA, pregnenolone and 17αOH-pregnenolone were used. As stated in the Results section (p. 79), the results of the incubation of the larger quantity of DHA were not significantly

different from those of the first determination of DHA-
-3β-hydroxy dehydrogenase activity.

A curious feature of the experiments involving incubation of the larger quantity (500 μg.) of pregnenolone was the appearance of small but discernable quantities of steroids with the chromatographic mobilities of DOC and corticosterone. This observation had not been made in the earlier set of experiments with whole homogenates. In view of the fact that the amounts of these more polar metabolites were small, and that comparative experiments with fascicular and reticular tissue preparations showed that the fascicular tissue preparation had the higher pregnenolone-3β-
-hydroxy dehydrogenase activity (fig. 11), it was decided to pursue this approach and to try to measure a) the total Δ^4-3-oxosteroids present by their collective absorption at 240 mμ. or b) the residual substrate by a specific chemical reaction for Δ^5-3β-hydroxysteroids (Oertel & Eik-Nes, 1959). The measurement of the 240 mμ absorbing steroids formed was used by Rubin et al. (1961) for the determination of the 3β-hydroxysteroid dehydrogenase activity of adrenal tissue from human subjects by a simple subtraction of a "tissue blank"

from the absorption at 240 mμ of incubation extracts. As the results of the present investigation show (Table 21, p. 81), this method proved completely useless. Webb & Munro (1965) have attempted to measure 3β-hydroxysteroid dehydrogenase activity by this procedure, also without success. In the opinion of these workers, small amounts of the large quantity of NAD (6 mg.) incubated may be extracted with the lipid fraction and interfere with the absorption spectrum of the Δ^4-3-oxosteroids. Washing the "blank" extracts with NaOH and HCl lowered the observed optical density at 240 mμ but the final "blank" values obtained were still equivalent to between 7 and 10 μg of progesterone. This meant that there could be a substantial error in the determination of small quantities of steroid.

The "tissue blank" observed using the sulphuric acid/ /ethanol reagent of Oertel & Elk-Nes (1959) appeared to be approximately linear between 405 mμ and 430 mμ (Table 22, p. 84) which indicated that the simplified optical density correction (p. 82) described by Saffran & Schally (1956) might be used. It was found, however, that recoveries of Δ^5-3β-hydroxysteroids as measured by

(i) determination of radioactivity and (ii) Oertel reaction, did not agree (Tables 25a and 25b, pages 89 and 90). The values obtained by the Oertel reaction were consistently low i.e. "quenching" was taking place due to interference by some constituent of either the tissue lipid fraction and for solvent residues with the sulphuric acid chromogen. This effect was found to be relatively constant over a considerable concentration range of steroid and after thorough investigation, it was found to be due to impurities of both tissue and solvent origin.

The results shown in Table 24 (p. 86) obtained by measurement of residual substrate indicate once again that there is almost invariably a higher DHA- and pregnenolone-3β-hydroxy dehydrogenase activity in fascicular tissue. In view of the observed "quenching effect", however, in the determination of 3β-hydroxy-steroids by sulphuric acid/ethanol reagent, it was felt that estimates of enzyme activity could only be approximate, especially if the amount of substrate metabolised was low (see e.g. Incubation 6, Table 24, p. 86). It was obviously desirable, therefore, to eliminate this "quenching effect" and to determine the

recovery of substrate by more precise means.

Appendix IV (p.198) shows that very consistent recoveries of DHA, pregnenolone and 17αOH-pregnenolone at different levels are obtainable after column chromatography on alumina. From Table 27 (p. 94) it is also apparent that the wide variation in the ratios of 3β--hydroxysteroid dehydrogenase activity (F/R) using DHA and pregnenolone as substrates was eliminated through the use of column chromatography. The third substrate used, 17αOH-pregnenolone, gave equivocal results. Although it is possible that the results obtained did indeed indicate a greater 17αOH-pregnenolone-3β-hydroxy dehydrogenase activity in fascicular tissue, the difference in the activities of the two zones is not large.

The foregoing discussion has dealt with experiments on horse tissue. In the experiments with adrenal tissue from human subjects, the best conditions for 3β-hydroxysteroid dehydrogenase assay were again found to involve the use of homogenates prepared in sucrose/nicotinamide and diluted with phosphate buffer, although TRIS buffer could have been substituted for the phosphate buffer without deleterious effect. As with the horse tissue experiments, DHA- and pregnenolone-3β-hydroxy dehydrogenase activities were shown to be greater in fascicular

tissue but again the results obtained using 17αOH-pregnenolone were equivocal. It is interesting to note, however, that even with widely differing degrees of metabolism, the ratios of the amounts of Δ^4-androstenedione or progesterone formed (F/R) are remarkably constant.

The experiments in Part I have established that there is a greater 3β-hydroxysteroid dehydrogenase activity in fasicular tissue from horse and human adrenals using substrates DHA and pregnenolone. In view of the analytical problems described, more sophisticated methods for 3β-hydroxysteroid dehydrogenase assay may be developed in the future. Nevertheless, the present results show that reticular tissue is capable of dehydrogenating Δ^5-3β-hydroxysteroids and, in fact, the difference in activity between the two types of tissue is not nearly so large as the histochemical observations might suggest (e.g. Plate I). One possible explanation for this might be that tissues used in the histochemical and biochemical assays are treated in quite different ways. The tissue section for histochemical assay is deep frozen, dried, acetone washed and then incubated with an enormous excess of substrate. All of these factors, especially the last, could greatly influence the

final result.

A biochemical assay of 3β-hydroxysteroid dehydrogenase, however accurately it may be in terms of Δ^4-3-oxosteroid formed or 3β-hydroxysteroid unchanged remaining per unit time, can tell us nothing about other reactions which involve Δ^5-3β-hydroxysteroids which, although they were not detectable under the conditions of assay used in Part I, may still play an important role in the metabolism of these steroids in vivo. These are now discussed.

1. 3-oxosteroid Δ^5- Δ^4-isomerase.

Throughout the experimental work of the Thesis, it has been assumed that the transformation of a Δ^5-3β--hydroxysteroid to a Δ^4-3-oxosteroid is a one-step process. As stated in the General Introduction (p. 8), however, there are really two steps involved (see fig. 1, p.9) viz. a Δ^5-3β-hydroxysteroid dehydrogenase and a 3-oxosteroid Δ^5- Δ^4-isomerase. Kawahara (1960) isolated a crystallisable bacterial isomerase which will convert Δ^5-3-oxosteroids to Δ^4-3-oxosteroids with the intramolecular transfer of hydrogen from C-4 to C-6 (Talalay & Wang, 1955) but without exchange of hydrogen with the isomerase (Kawahara & Talalay, 1960). Similar

studies in mammalian systems indicate that rat liver and human serum (Talalay & Wang, 1955) also contain isomerase activity and that all tissues capable of steroid hormone biosynthesis, contain isomerase activity (Kawahara, 1962). Recent work (Ewald, Werbin & Chaikoff, 1964; Kriskemper, Forchielli & Ringold, 1964) indicates that ox and rat adrenal preparations contain at least two isomerases - - possibly a C_{19} and a C_{21} isomerase. It would seem to be quite possible that similar isomerases exist in human and horse adrenal tissue. It is equally possible that the type of adrenogenital syndrome ascribed to a 3β-hydroxysteroid dehydrogenase deficiency (Bongiovanni, 1962) could be due to an isomerase deficiency.

2. Sulphates (3β-sulphoxysteroid esters).

In 1957, Baulieu isolated DHA sulphate from the urine and plasma of a patient with an adrenal tumour and later (Baulieu, 1960) he was able to show that this substance is an adrenal secretory product. The *in vivo* conversion of cholesterol sulphate to DHA sulphate (Roberts, Bandi, Calvin, Drucker & Lieberman, 1964), pregnenolone sulphate to DHA sulphate (Calvin, Vande Wiele & Lieberman, 1963) and the *in vitro* conversion of pregnenolone sulphate to

17αOH-pregnenolone sulphate (Calvin & Lieberman, 1963) have all been demonstrated. There is no evidence, however, that the interconversion of free steroid and sulphate ester occurs very easily. 3β-hydroxysteroid dehydrogenase (Beyer & Samuels, 1956) and steroid sulphatase are both stated to be firmly bound to the microsomal fraction. Indeed, Roberts et al. (1964) showed that cholesterol sulphate labelled with ^{3}H and ^{35}S was metabolised to DHA sulphate in vivo with a very similar $^{3}H/^{35}S$ ratio. This must mean that the peripheral transformation of steroid sulphates to the corresponding free steroids must be very slow. No evidence in the present series of experiments indicated that significant quantities of the radioactive substrates [4-^{14}C] DHA, [7α-^{3}H] pregnenolone or [7α-^{3}H] 17αOH-pregnenolone had been transformed to the corresponding sulphate esters.

3. Alternative Pathways.

Although considerable efforts were made to determine optimal incubation conditions for the transformation of Δ^{5}-3β-hydroxysteroids to Δ^{4}-3-oxosteroids (e.g. of pregnenolone to progesterone), it is entirely probable that this situation is not representative of the

physiological state. As already pointed out (p. 13), we know that pregnenolone is transformed to cortisol *via* progesterone but that an alternative pathway exists *via* 17αOH-pregnenolone. Indeed, the latter route might even be the major one. There is also the possibility that pregnenolone may be transformed by various hydroxylation reactions to e.g. 21 OH-pregnenolone, 17α,21 OH--pregnenolone or even 11β,17α,21 OH-pregnenolone before dehydrogenation to DOC, 11-deoxycortisol or cortisol respectively. Such transformations from 21 OH-pregnenolone and 17α,21 OH-pregnenolone have been shown to occur in ox adrenal tissue (Berliner, Cazes & Nabors, 1962). No information was obtainable on this type of metabolic pathway with the techniques used in Part I of the Thesis.

In summary, Part I has presented evidence that DHA- and pregnenolone-3β-hydroxy dehydrogenase activity (and possibly 17αOH-pregnenolone-3β-hydroxy dehydrogenase activity) is higher in the zona fasciculata of the adrenal cortices of horse and man. The results obtained, however, do not indicate the large difference in activity suggested by the histochemical evidence and indeed it was found that reticular tissue contains substantial amounts of 3β--hydroxysteroid dehydrogenase activity.

At this stage in the investigations, it seemed possible that an interpretation of the results in terms of cell function might include a difference in major routes from pregnenolone to cortisol in the two cell types. Perhaps pregnenolone ⟶ progesterone ⟶ ⟶ 17αOH-progesterone ⟶ 11-deoxycortisol ⟶ ⟶ cortisol is the main route in the fascicular zone, in view of its higher pregnenolone-3β-hydroxy dehydrogenase activity, whereas the pregnenolone ⟶ ⟶ 17αOH-pregnenolone ⟶ 17αOH-progesterone ⟶ ⟶ 11-deoxycortisol ⟶ cortisol is the main route in the reticular zone. These questions were further investigated by the more sophisticated techniques described in Part II.

PART II

3β-HYDROXYSTEROID DEHYDROGENASE AND THE BIOSYNTHESIS OF CORTISOL

INTRODUCTION

In the experiments already described in Part I, attempts were made to compare the conversion of Δ^5-3β--hydroxysteroids to their corresponding Δ^4-3-oxosteroid derivatives by fascicular and reticular tissue in cell--free systems designed to minimise subsequent transformations. It should be remembered, however, that this situation is not entirely relevant to the conditions which obtain *in vivo* or even in intact cell preparations *in vitro*. All experiments in Part II were performed with intact cell preparations.

The main concern of the investigations described throughout the Thesis is to examine the relationship between the histochemically observed distribution of 3β--hydroxysteroid dehydrogenase and the activity of this enzyme in the different zones observed biochemically. It is also important, however, to try to explain the *significance* of the distribution of the enzyme in the cell types found in the two zones —— to determine how the activity of this enzyme or group of enzymes influences the biosynthetic patterns of the zones.

The first section of Part II deals with experiments

designed to measure the relative abilities of fascicular and reticular tissue from the horse adrenal cortex to transform [7α-^{3}H] pregnenolone, [4-^{14}C] progesterone, [7α-^{3}H] 17αOH-pregnenolone and [4-^{14}C] 17αOH-progesterone to cortisol. If the only pathway from 17αOH-pregnenolone to cortisol is *via* 17αOH-progesterone and 11-deoxycortisol (fig. 14), and if the step 17αOH-pregnenolone ⟶ 17αOH--progesterone is rate-limiting in the transformation of 17αOH-pregnenolone to cortisol in both zones, then comparison of the ability of the two tissues to transform 17αOH-pregnenolone and 17αOH-progesterone to cortisol will indicate if either tissue has a higher 17αOH--pregnenolone-3β-hydroxy dehydrogenase activity. Having obtained such information, one may then attempt to compare, in a similar fashion, the pregnenolone-3β--hydroxy dehydrogenase activities although the situation is necessarily more complicated, since pregnenolone is a branching point for different biosynthetic pathways (fig. 14).

As already stated in the General Introduction (p.13), Weliky & Engel (1962) investigated the metabolism of [4-^{14}C] progesterone and [7α-^{3}H] 17αOH-pregnenolone by an adrenal tumour from a human subject. In a later

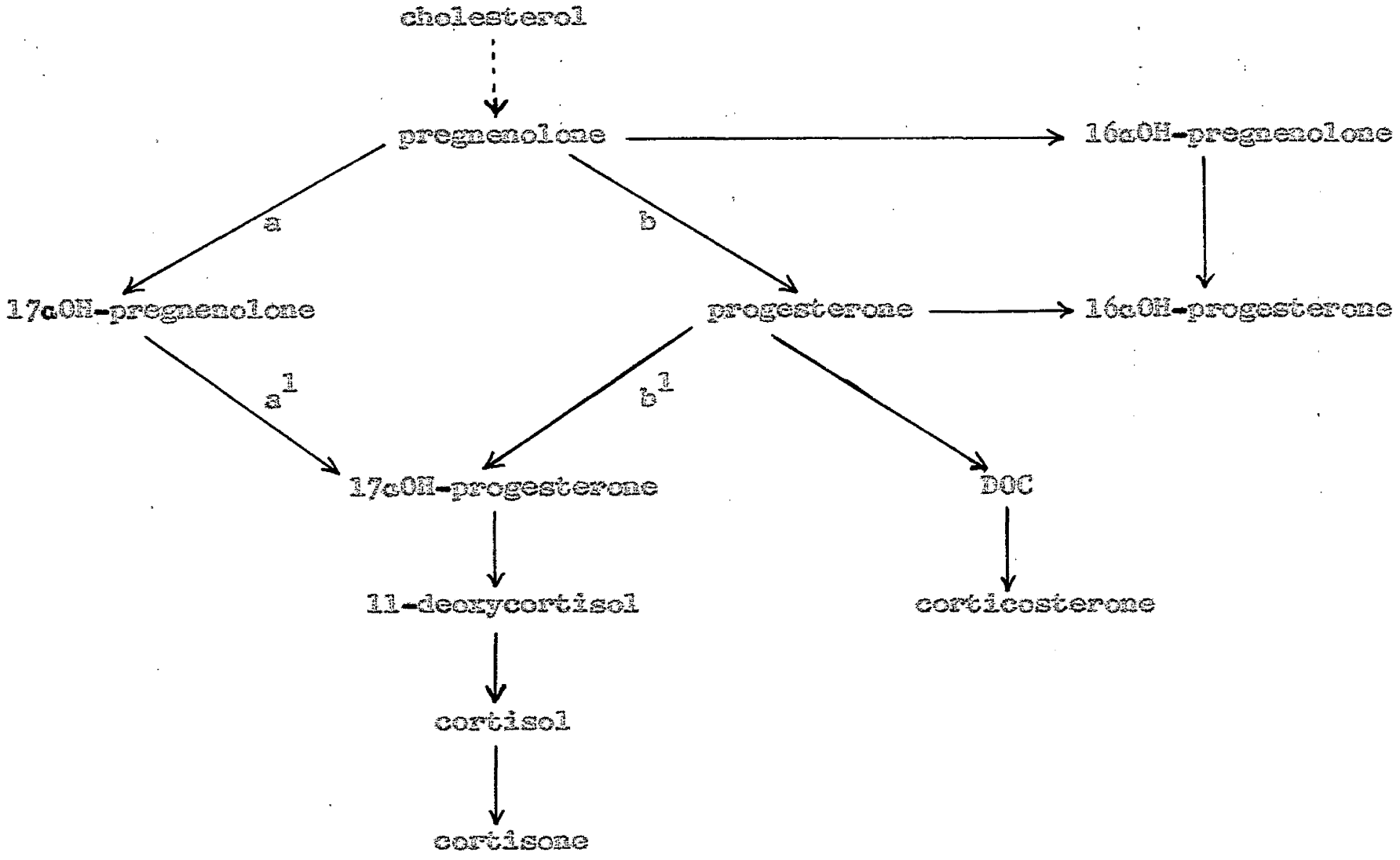
cholesterol
pregnenolone
16αOH-pregnenolone
a
b
17αOH-pregnenolone
progesterone
16αOH-progesterone
a1
b1
17αOH-progesterone
DOC
11-deoxycortisol
corticosterone
cortisol
cortisone

fig. 14.

examined the metabolism of [7α-^{3}H] pregnenolone by huma

experiment, the same authors (Wellky & Engel, 1963) examined the metabolism of [4-^{14}C] progesterone and [7α-^{3}H] pregnenolone by human hyperplastic adrenal tissue. In the case of the tumour, no details of the cell type or types present were given, neither was it stated whether the tumour was an adenoma or a carcinoma. No attempt was made in the case of the hyperplastic adrenal to separate fascicular from reticular tissue (the results which these workers obtained will be discussed more fully at a later stage, in conjunction with those of the present findings).

In view of the lack of any correlation between cell type and biosynthetic activity in the experiments of Wellky & Engel (1962, 1963) just described, it seemed essential to extend their work. The second section of Part II describes this extention viz. the simultaneous incubation of [4-^{14}C] progesterone and [7α-^{3}H] pregnenolone with slices of fascicular and with slices of reticular tissue from a normal adrenal gland surgically removed from a patient with breast cancer. The purpose of the experiment was to investigate the possible routes which may be followed in the biosynthesis of cortisol from pregnenolone by comparison of the ^{3}H/^{14}C ratios of certain metabolites of [4-^{14}C] progesterone and [7α-^{3}H] pregnenolone

[7α-^{3}H]

after incubation with tissue from each zone. Is the biosynthetic pathway *via* 17αOH-pregnenolone or *via* progesterone or both since pregnenolone may be metabolised to cortisol by route $\underline{aa}^1$ or by route $\underline{bb}^1$?

For each incubation, the radioactive [7α-^{3}H] pregnenolone was diluted to provide a pool of substrate. This was done in order that the effect of the introduction of a trace amount of ^{14}C labelled intermediate which could be either [4-^{14}C] progesterone or [4-^{14}C] 17αOH-pregnenolone should be minimised. These ^{14}C-labelled compounds are obtainable only in states of low specific activity compared with the ^{3}H-labelled substrate pregnenolone. Only ^{14}C--labelled progesterone was, in fact, available for the experiment, and this was used. Moreover, it was necessary to determine the ^{3}H/^{14}C ratios in metabolites of the intermediate and since, for example, 16αOH-progesterone and DOC (metabolites of progesterone) are easier to measure than the Δ^5-3β-hydroxysteroid metabolites of 17αOH-pregnenolone, progesterone was the obvious choice of intermediate. A quantity of unlabelled 17αOH-pregnenolone equivalent to the [4-^{14}C] progesterone was also added, in order that the route $\underline{aa}^1$ should not be favoured.

EXPERIMENTAL

A. Studies with Horse Adrenal Tissue.

Incubations with [4-^{14}C] Progesterone, [7α-^{3}H] Pregnenolone, [4-^{14}C] 17αOH-Progesterone and [7α-^{3}H] 17αOH-Pregnenolone.

Slices of fascicular and reticular tissue were prepared and batches of 300 mg. chopped with a safety razor blade until the tissue appeared to contain no lumps. These minces were then incubated with [4-^{14}C] progesterone, [7α-^{3}H] pregnenolone, [4-^{14}C] 17αOH-progesterone or [7α-^{3}H] 17αOH-pregnenolone, as shown in Table 30 (p.125) in 4 ml. of Krebs-Ringer bicarbonate + glucose medium (see Appendix II, p.191) at 37° for 2 hours in an atmosphere of 95% O_2:5% CO_2.

After incubation, 20 ml. of acetone + 1 ml. of ethanol containing 250 µg each of cortisol and cortisone were added. The mixture was homogenised with a Silverson (Silverson Ltd., 55 Tower Bridge Road, London, S.E.1.) homogeniser, and after centrifugation, the supernatant was decanted and the tissue residue washed twice more with 20 ml. of acetone. The combined acetone extracts were evaporated almost to dryness in a rotary evaporator, and partitioned between 10 ml.

Incubation	Zone (F or R)[a]	Steroid[b]	µg.	mµmoles	µC.
1.	F	[4-^{14}C] progesterone	29.00	92.35	2
2.	R	[4-^{14}C] progesterone	29.00	92.35	2
3.	F	[7α-^{3}H] pregnenolone	29.22	92.35	10
4.	R	[7α-^{3}H] pregnenolone	29.22	92.35	10
5.	F	[4-^{14}C] 17α0H-progesterone	22.80	69.09	1
6.	R	[4-^{14}C] 17α0H-progesterone	22.80	69.09	1
7.	F	[7α-^{3}H] 17α0H-pregnenolone	22.90	69.09	10
8.	R	[7α-^{3}H] 17α0H-pregnenolone	22.90	69.09	10

Table 30.

[a] F = zona fasciculata.
R = zona reticularis.

[b] Steroid dissolved in 100 µl propylene glycol.

light petroleum (80-100°) and 10 ml. 80% aqueous methanol in a stoppered test-tube. The petroleum layer was extracted twice more with equal volumes of aqueous methanol. Combined extracts were again taken almost to dryness in a rotary evaporator and 10 ml. of water was added. The aqueous mixture was then extracted three times with equal volumes of chloroform and the combined chloroform extracts blown to dryness in a stream of air (Fig. 14).

The residue from the chloroform extract was chromatographed on thin-layer System I to separate cortisol from cortisone. These steroids were then eluted and their specific activities (S.A.) measured before and after the formation of derivatives (p. 29). When the specific activities of a steroid and its derivatives varied by less than 10%, the mean specific activity was used to calculate the percentage conversion from the original radioactive steroid. A flow-sheet, using cortisol as the example, is shown in Fig. 15 .

Calculation

Using the conversion of $[4\text{-}^{14}C]$ progesterone to ^{14}C cortisol as an example,

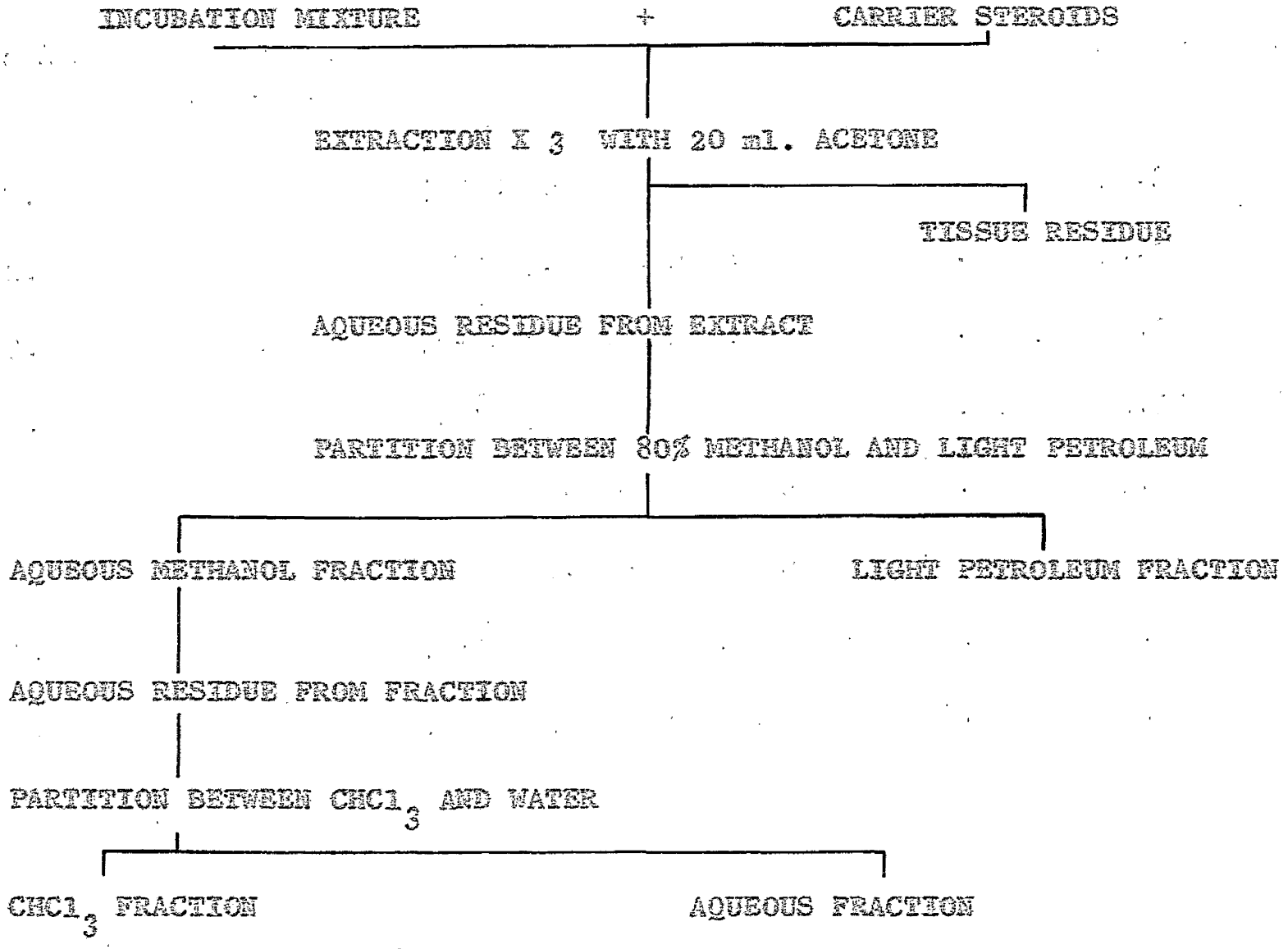
INCUBATION MIXTURE
+
CARRIER STEROIDS
EXTRACTION X 3 WITH 20 ml. ACETONE
TISSUE RESIDUE
AQUEOUS RESIDUE FROM EXTRACT
PARTITION BETWEEN 80% METHANOL AND LIGHT PETROLEUM
AQUEOUS METHANOL FRACTION
LIGHT PETROLEUM FRACTION
AQUEOUS RESIDUE FROM FRACTION
PARTITION BETWEEN $CHCl_3$ AND WATER
$CHCl_3$ FRACTION
AQUEOUS FRACTION

Fig. 14.

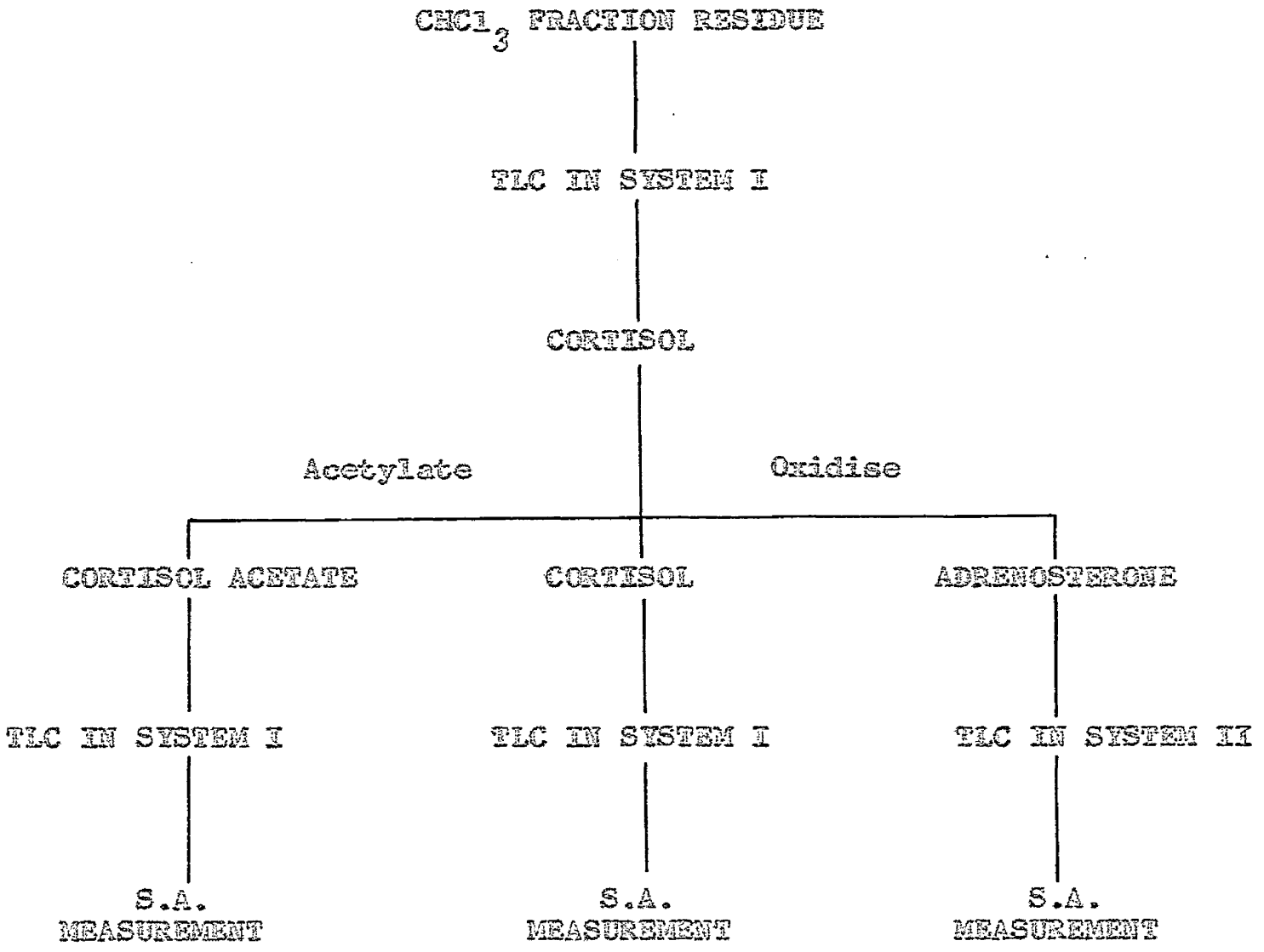
$CHCl_3$ FRACTION RESIDUE
TLC IN SYSTEM I
CORTISOL
Acetylate
Oxidise
CORTISOL ACETATE
CORTISOL
ADRENOSTERONE
TLC IN SYSTEM I
TLC IN SYSTEM I
TLC IN SYSTEM II
S.A. MEASUREMENT
S.A. MEASUREMENT
S.A. MEASUREMENT

Fig.15.

$$\% \text{ conversion of } [4\text{-}^{14}C] \text{ progesterone to } {}^{14}C \text{ cortisol} = \frac{\text{m}\mu C/\text{m}\mu\text{mole of cortisol isolated} \times \text{m}\mu\text{mole cortisol added}}{\text{m}\mu C \text{ of } [4\text{-}^{14}C] \text{ progesterone incubated}} \times 100$$

(the mass of the radioactive steroid actually transformed to cortisol during the incubation is neglected).

B. Simultaneous Counting of ^{3}H and ^{14}C (Double Isotope Counting).

Samples for counting were prepared in glass vials containing 10 ml. scintillator medium, as described in Part I (p. 27). Radioactivity was determined by Packard Tri-Carb Liquid Scintillation Spectrometer, Model 3214 (Packard Instrument Co., Inc., La Grange, Illinois). With a voltage of 3.797, the Red Channel was set with a voltage discriminator gate of 50-210 and amplifier gain of 25% giving efficiencies of counting of approximately 3.8% for ^{14}C and 18% for ^{3}H. The Green Channel was set with a voltage discriminator gate of 190-1000 and amplifier gain of 5% giving efficiencies of counting of approximately 48% for ^{14}C and 0% for ^{3}H. The disintegrations/min. of ^{3}H and ^{14}C in each vial were determined by application of the data obtained to the equations

below (Packard, 1962), which are essentially those described by Okita, Kabara, Richardson and Le Roy (1957).

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

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

where A_1 = activity of isotope 1 (^3H)

A_2 = " " " 2 (^{14}C)

R_R = counts/min. in the Red Channel.

R_G = counts/min. in the Green Channel.

E_{1R} = counting efficiency of isotope 1 in the Red Channel.

E_{1G} = counting efficiency of isotope 1 in the Green Channel.

E_{2R} = counting efficiency of isotope 2 in the Red Channel.

E_{2G} = counting efficiency of isotope 2 in the Green Channel.

No quenching was observed under the conditions described above. Determination of the optimal counting conditions for the separation of the β-particle spectra of ^3H and ^{14}C was performed during installation of the instrument.

C. Studies with Human Adrenal Tissue.

1. Adrenal Tissue.

The patient (E.R.), 56 years old, was suffering from breast cancer. Prior to operation, she had no steroid, ACTH nor pituitary implant therapy. The gland received at the laboratory, approximately 8 hours after removal, was of normal appearance and weighed 5.5 g. (sub-total adrenalectomy, left side).

2. Simultaneous Incubation of [7α-^{3}H] Pregnenolone and [4-^{14}C] Progesterone.

Two incubation vessels were prepared each containing

100 µl propylene glycol

10 µC [7α-^{3}H] pregnenolone (2.12 µg)
27.10 µg pregnenolone — total 92.35 mµmoles

½µC [4-^{14}C] progesterone (7.25 µg) — total 23.09 mµmoles

17αOH-pregnenolone — total 23.09 mµmoles

Chopped slices (150 mg.) of fascicular or reticular tissue were incubated with the above mixture at 37° for 2 hours in 4 ml. of Krebs-Ringer bicarbonate + glucose medium in an atmosphere of 95% O_2 + 5% CO_2.

Incubations were stopped by the addition of 20 ml. of

acetone, and 300 µg. each of cortisol, 17αOH-progesterone, 16αOH-progesterone and DOC were added in 1 ml. of ethanol. Mixtures were thereupon homogenised, extracted and partitioned, as described on page 124.

Following preliminary separation of steroids in the $CHCl_3$ extract on a thin-layer (System IV, page 25), specific activities of the four steroids added were determined in a similar fashion to that described on page 126. Details of derivatives formed and solvent systems used, are given below:

Thin-layer solvent systems I, II, III and IV are described on page 25 .

acetylation)
oxidation) refer to the reactions described on pages 29 and 30 .
reduction)

Steroids shown in parenthesis are the end products used in the determination of the specific activities (S.A.)

a) 17αOH-progesterone.

17αOH-progesterone was first subjected to the acetylation reaction to convert any acylable impurities to their corresponding acetates. Subsequently, 17αOH-progesterone, which does not acetylate under these conditions, may be readily purified by thin-layer chromatography.

17α0H-progesterone

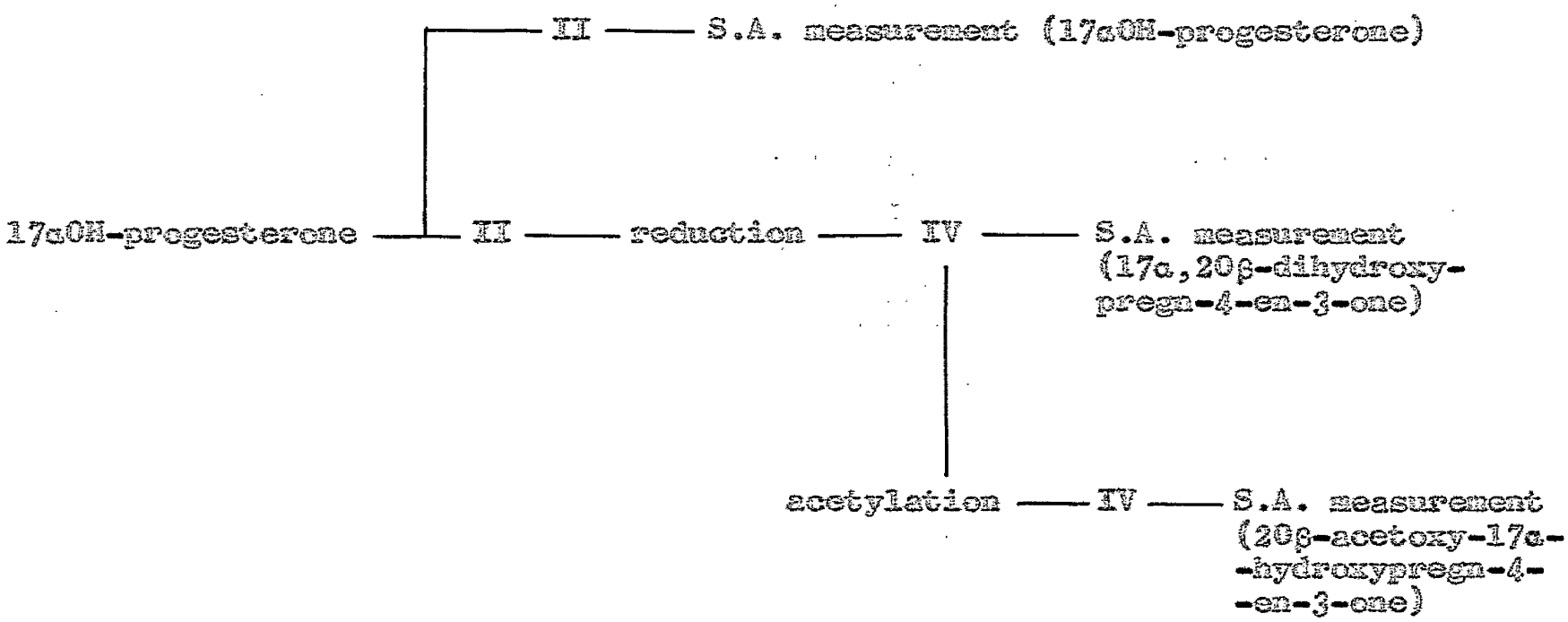

Fig. 16.

b) DOC.

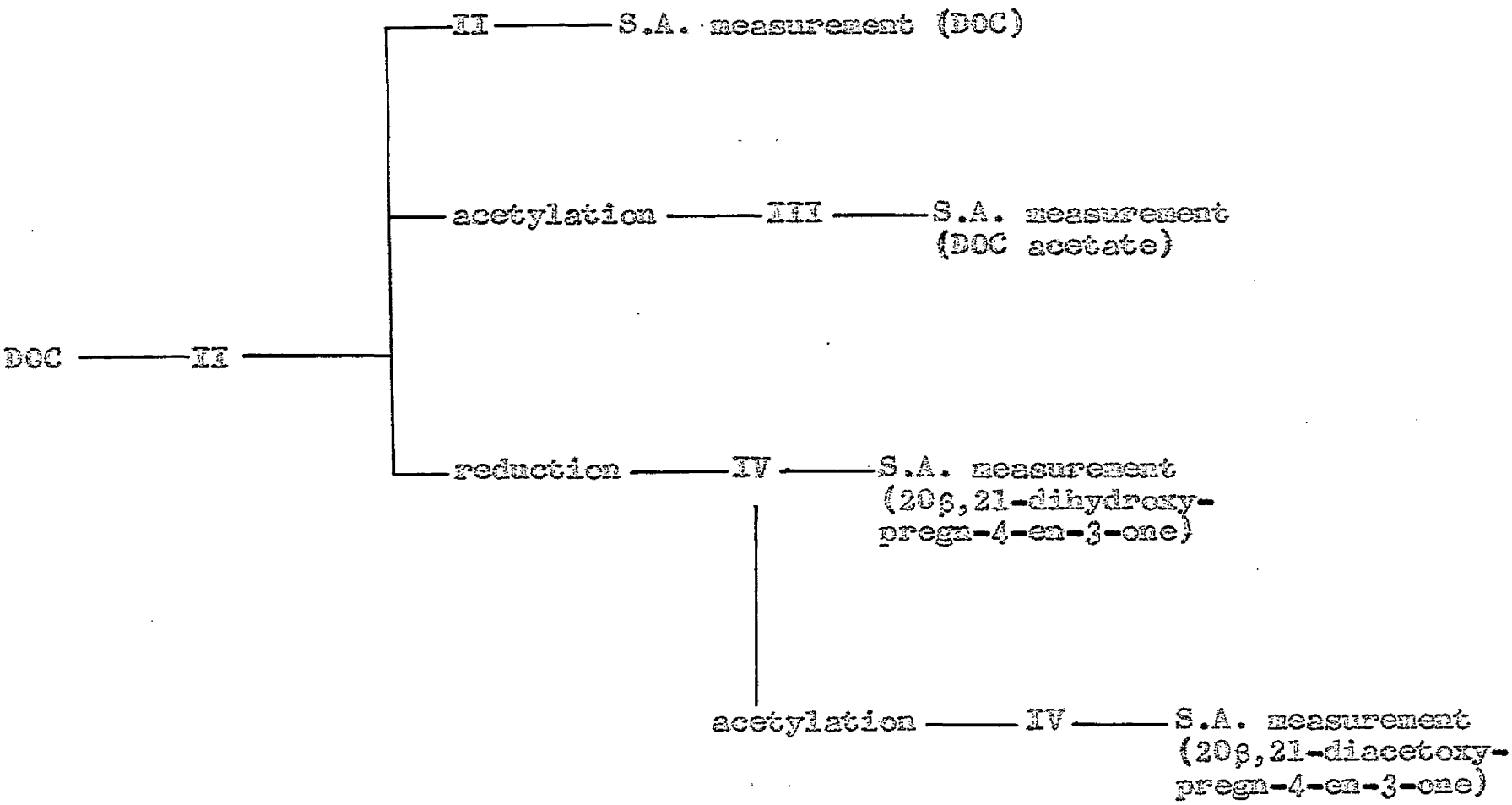
DOC
II
II
S.A. measurement (DOC)
acetylation
III
S.A. measurement (DOC acetate)
reduction
IV
S.A. measurement (20β,21-dihydroxy-pregn-4-en-3-one)
acetylation
IV
S.A. measurement (20β,21-diacetoxy-pregn-4-en-3-one)

Fig. 17.

c) 16α0H-progesterone

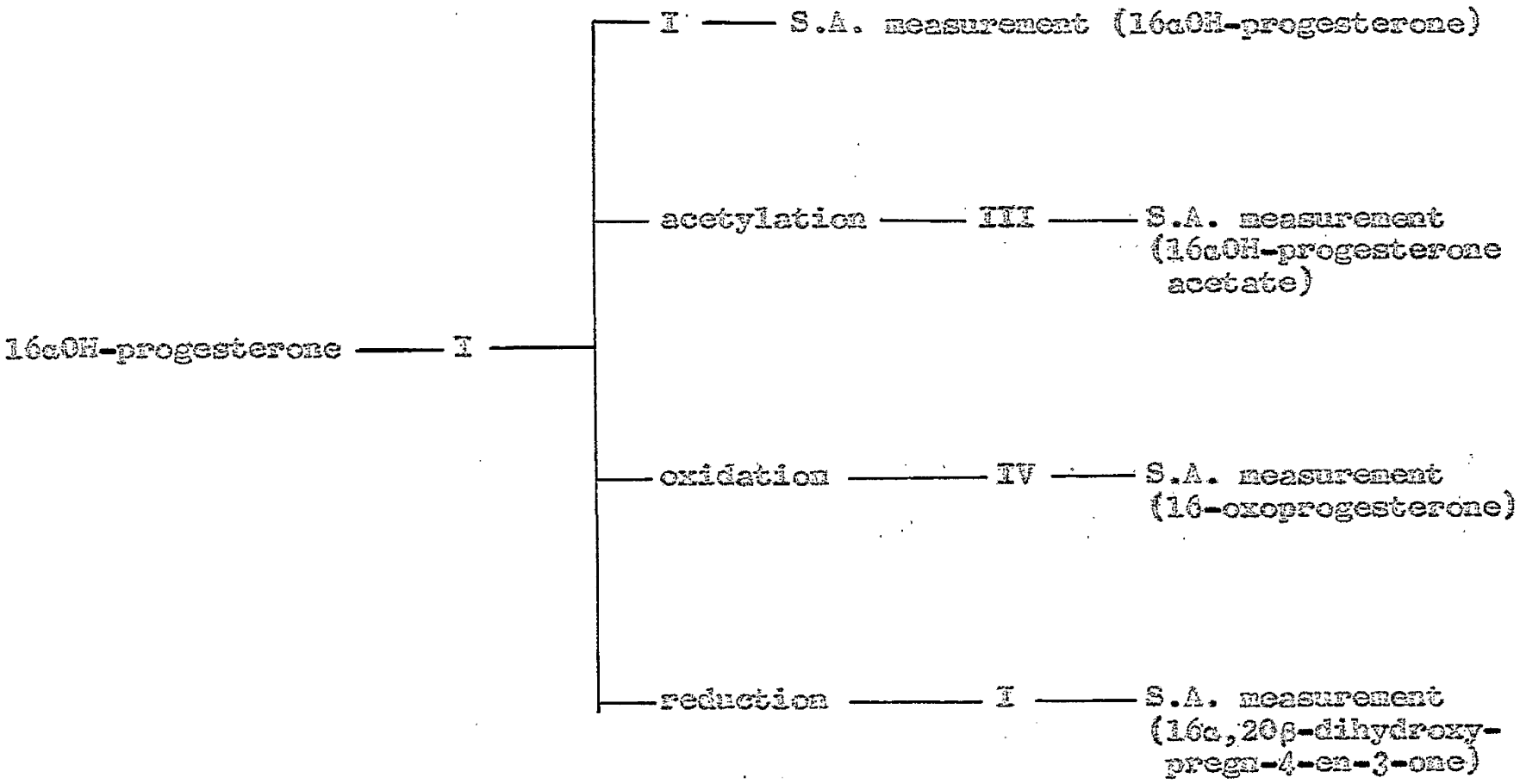

Fig.18.

d) cortisol

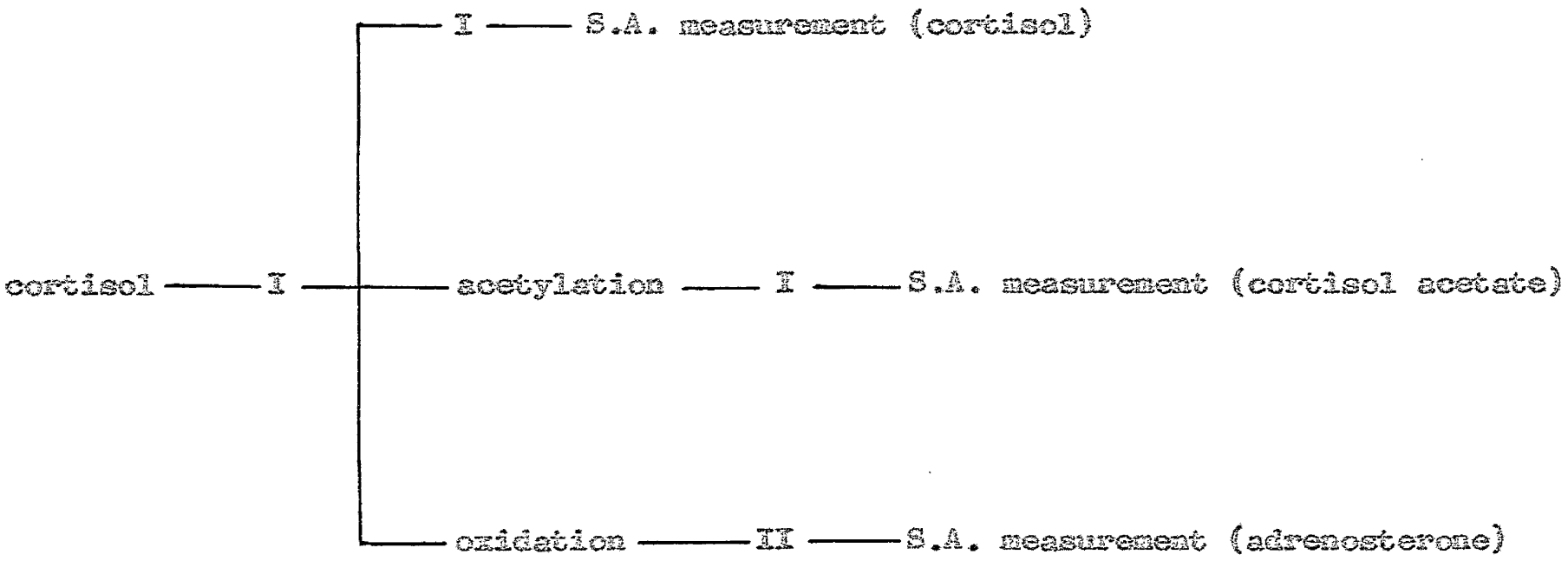

Fig.19.

The calculated percentage conversion to each of these steroids from the radioactive substrates then allowed certain conclusions to be drawn as to the relative magnitude of alternative pathways to cortisol and 16αOH-progesterone in fasicular and reticular tissue from the human adrenal gland *in vitro*.

RESULTS

Studies with Horse Adrenal Tissue

The radioactivity recovered in the aqueous methanol fraction from each incubation extract is set out in Table 31. In most cases, the recovery is over 80%. Low recoveries (60 - 65%) were observed when [7α-^{3}H] pregnenolone was incubated with fascicular tissue. The fact that recoveries of radioactivity in experiments involving the incubation of [7α-^{3}H] 17αOH-pregnenolone and [4-^{14}C] 17αOH-progesterone were uniformly high, would seem to indicate that in experiments 3 and 4, the low recoveries were specifically due to loss of substrates [7α-^{3}H] pregnenolone or [4-^{14}C] progesterone.

Table 32 shows the specific activities of the diluted metabolite cortisol isolated from each incubation, and its derivatives. In a few instances, cortisone was also isolated. Repeated thin-layer chromatography was often necessary to obtain three specific activities within the 10% maximum limit of error. Indeed, in several cases of very low ^{3}H activity (cortisone), this was impossible and only two figures can be quoted. From the mean specific activities of the cortisol and its derivatives, it was

possible to calculate the percentage conversion from the original radioactive steroid incubated (see p.126). Table 33 shows the figures obtained. It should be noted that:-

a) all steroids incubated are more readily converted to cortisol in the fascicular zone than in the reticular zone.

b) the 3β-hydroxysteroids are less effective precursors of cortisol in either zone than their corresponding A-ring oxidation derivatives progesterone and 17αOH--progesterone.

c) 17αOH-progesterone is converted to cortisol in much higher yields in both zones than the other precursors investigated.

Expt. No. & Tissue Incubated (F or R)*	Steroid Incubated			^{3}H Recovered (MeOH aq. fraction)		^{14}C Recovered (MeOH aq. fraction)	
		mμC	mμmole	mμC	%	mμC	%
1a (F)	[4-^{14}C] 17α0H-progesterone	922	69.09	-	-	755.0	81.9
" (R)	"	"	"	-	-	868.4	94.2
1b (F)	[7α-^{3}H] 17α0H-pregnenolone	9,077	69.09	8,768	96.6	-	-
" (R)	"	"	"	9,204	101.4	-	-
2a (F)	[4-^{14}C] 17α0H-progesterone	922	69.09	-	-	808.5	87.7
" (R)	"	"	"	-	-	830.6	90.1
2b (F)	[7α-^{3}H] 17α0H-pregnenolone	9,077	69.09	8,614	94.9	-	-
" (R)	"	"	"	8,614	94.9	-	-
3a (F)	[4-^{14}C] progesterone	2,104	92.35	-	-	1,549	73.6
" (R)	"	"	"	-	-	1,812	86.1

Expt. No. & Tissue Incubated (F or R)*	Steroid Incubated			^{3}H Recovered (MeOH aq. fraction)		^{14}C Recovered (MeOH aq. fraction)	
		mμC	mμmole	mμC	%	mμC	%
3b (F)	[7α-^{3}H] pregnenolone	10,269	92.35	6,716	65.4	-	-
" (R)	"	"	"	8,780	85.5	-	-
4a (F)	[4-^{14}C] progesterone	2,104	92.35	-	-	1,503	71.4
" (R)	"	"	"	-	-	1,448	68.8
4b (F)	[7α-^{3}H] pregnenolone	10,269	92.35	6,172	61.0	-	-
" (R)	"	"	"	8,975	87.4	-	-

*F = fascicular

R = reticular

Table 31.

Expt. No. & Tissue Incubated (F or R)*	Steroid Incubated	Steroid Isolated	Chemical Reaction	Product	Solvent System	† Sp. Act.
1a (F)	[4-^{14}C] 17αOH-prog.[1]	cortisol	-	cortisol	I	398
			acetylation	cortisol acetate	I	382
			oxidation	adrenosterone	II	412
1a (R)	"	"	-	cortisol	I	153
			acetylation	cortisol acetate	I	141
			oxidation	adrenosterone	II	141
1b (F)	[7α-^{3}H] 17αOH-preg.[2]	"	-	cortisol	I	545
			acetylation	cortisol acetate	I	569
			oxidation	adrenosterone	II	578
1b (R)	"	"	-	cortisol	I	209
			acetylation	cortisol acetate	I	232
			oxidation	adrenosterone	II	229

Expt. No. & Tissue Incubated (F or R)*	Steroid Incubated	Steroid Isolated	Chemical Reaction	Product	Solvent System	Sp. Act.†
2a (F)	[4-^{14}C] 17αOH-prog.	cortisol	-	cortisol	I	574
			acetylation	cortisol acetate	I	548
			oxidation	adrenosterone	II	604
2a (R)	"	"	-	cortisol	I	280
			acetylation	cortisol acetate	I	270
			oxidation	adrenosterone	II	259
2b (F)	[7α-^{3}H] 17αOH-preg.	"	-	cortisol	I	1,489
			acetylation	cortisol acetate	I	1,418
			oxidation	adrenosterone	II	1,516
2b (R)	"	"	-	cortisol	I	624
			acetylation	cortisol acetate	I	615
			oxidation	adrenosterone	II	627

Expt. No. & Tissue Incubated (F or R)*	Steroid Incubated	Steroid Isolated	Chemical Reaction	Product	Solvent System	Sp. Act.†
3a (F)	[4-^{14}C] prog.[3]	cortisol	-	cortisol	I	783
			acetylation	cortisol acetate	I	819
			oxidation	adrenosterone	II	799
3a (R)	"	"	-	cortisol	I	272
			acetylation	cortisol acetate	I	290
			oxidation	adrenosterone	II	262
3b (F)	[7α-^{3}H] preg.[4]	"	-	cortisol	I	2,021
			acetylation	cortisol acetate	I	1,923
			oxidation	adrenosterone	II	2,037
3b (R)	"	"	-	cortisol	I	414
			acetylation	cortisol acetate	I	405
			oxidation	adrenosterone	II	447

Expt. No. & Tissue Incubated (F or R)[=]	Steroid Incubated	Steroid Isolated	Chemical Reaction	Product	Solvent System	Sp. Act.[+]
3a (F)	[4-^{14}C] prog.	cortisone	-	cortisone	I	-
			acetylation	cortisone acetate	I	45
			oxidation	adrenosterone	II	42
			reduction	11-oxotestosterone	II	43
3a (R)	"	"	-	cortisone	I	-
			acetylation	cortisone acetate	I	23
			oxidation	adrenosterone	II	23
			reduction	11-oxotestosterone	II	25
3b (F)	[7α-^{3}H] preg.	cortisone	-	cortisone	I	-
			acetylation	cortisone acetate	I	98
			oxidation	adrenosterone	II	95
			reduction	11-oxotestosterone	II	-
3b (R)	"	"	-	cortisone	I	-
			acetylation	cortisone acetate	I	39
			oxidation	adrenosterone	II	39
			reduction	11-oxotestosterone	II	-

Expt. No. & Tissue Incubated (F or R)*	Steroid Incubated	Steroid Isolated	Chemical Reaction	Product	Solvent System	Sp. Act.†
4a (F)	[4-^{14}C] prog.	cortisol	-	cortisol	I	131
			acetylation	cortisol acetate	I	136
			oxidation	adrenosterone	II	144
4a (R)	"	"	-	cortisol	I	81
			acetylation	cortisol acetate	I	81
			oxidation	adrenosterone	II	87
4b (F)	[7α-^{3}H] preg.	"	-	cortisol	I	607
			acetylation	cortisol acetate	I	562
			oxidation	adrenosterone	II	558
4b (R)	"	"	-	cortisol	I	128
			acetylation	cortisol acetate	I	133
			oxidation	adrenosterone	II	123

Expt. No. & Tissue Incubated (F or R)*	Steroid Incubated	Steroid Isolated	Chemical Reaction	Product	Solvent System	Sp. Act.†
4a (F)	[4-^{14}C] prog.	cortisone	-	cortisone	I	-
			acetylation	cortisone acetate	I	7.2
			oxidation	adrenosterone	II	7.6
			reduction	11-oxotestosterone	II	7.0
4a (R)	"	"	-	cortisone	I	-
			acetylation	cortisone acetate	I	8.1
			oxidation	adrenosterone	II	7.8
			reduction	11-oxotestosterone	II	7.3
4b (F)	[7α-^{3}H] preg.	cortisone	-	cortisone	I	-
			acetylation	cortisone acetate	I	27.0
			oxidation	adrenosterone	II	29.5
			reduction	11-oxotestosterone	II	-

Expt. No. & Tissue Incubated (F or R)*	Steroid Incubated	Steroid Isolated	Chemical Reaction	Product	Solvent System	Sp. Act.†
4b (R)	[7α-^{3}H] preg.	cortisone	-	cortisone	I	-
			acetylation	cortisone acetate	I	20.2
			oxidation	adrenosterone	II	18.7
			reduction	11-oxotestosterone	II	18.9

Table 32.

*F = fascicular

R = reticular

†Specific Activities in mμC/μmole

1 [4-^{14}C] 17αOH-progesterone

2 [7α-^{3}H] 17αOH-pregnenolone

3 [4-^{14}C] progesterone

4 [7α-^{3}H] pregnenolone

Expt. No. & Tissue Incubated (F or R)*	Steroid Incubated	Steroid Isolated	Mean Specific Activity (mμC/μmole)	Total Conversion mμC	Total Conversion %	F/R†
1a (F)	[4-^{14}C] 17αOH-progesterone	cortisol	397	277.0	30.05	2.73
" (R)	"	"	145	101.4	11.00	
1b (F)	[7α-^{3}H] 17αOH-pregnenolone	"	564	392.4	4.32	2.53
" (R)	"	"	223	155.6	1.71	
2a (F)	[4-^{14}C] 17αOH-progesterone	"	575	400.4	43.44	2.13
" (R)	"	"	270	187.7	20.36	
2b (F)	[7α-^{3}H] 17αOH-pregnenolone	"	1,474	1,026.7	11.31	2.35
" (R)	"	"	622	436.6	4.81	
3a (F)	[4-^{14}C] prog.	"	800	557.0	26.47	2.91
" (R)	"	"	275	191.7	9.11	

Expt. No. & Tissue Incubated (F or R)*	Steroid Incubated	Steroid Isolated	Mean Specific Activity (mμC/μmole)	Total Conversion mμC	Total Conversion %	F/R†
3b (F)	[7α-^{3}H] preg.	cortisol	1,994	1,388.0	13.52	4.72
" (R)	"	"	422	294.1	2.86	
3a (F)	[4-^{14}C] prog.	cortisone	43	30.3	1.44	1.88
" (R)	"	"	23	16.1	0.77	
3b (F)	[7α-^{3}H] preg.	"	97	67.6	0.66	
" (R)	"	"	39	27.2	0.27	
4a (F)	[4-^{14}C] prog.	cortisol	137	95.3	4.53	1.67
" (R)	"	"	82	57.2	2.72	
4b (F)	[7α-^{3}H] preg.	"	575	400.4	3.90	4.48
" (R)	"	"	128	89.3	0.87	

Expt. No. & Tissue Incubated (F or R)*	Steroid Incubated	Steroid Isolated	Mean Specific Activity (mμC/μmole)	Total Conversion mμC	Total Conversion %	F/R†
4a (F)	[4-^{14}C] prog.	cortisone	7.3	5.0	0.024	0.93
" (R)	"	"	7.7	5.4	0.026	
4b (F)	[7α-^{3}H] preg.	"	28.3	19.6	0.002	1.46
" (R)	"	"	19.3	13.4	0.001	

Table 33.

*F = fascicular
R = reticular

$$\dagger_{F/R} = \frac{\text{\% conversion in fascicular zone}}{\text{\% conversion in reticular zone}}$$

[4-^{14}C] prog. = [4-^{14}C] progesterone

[7α-^{3}H] preg. = [7α-^{3}H] pregnenolone

Studies with Human Adrenal Tissue

Good recoveries (>88%) were obtained for both ^{3}H and ^{14}C from both incubations (Table 34). The specific activities of the steroids isolated and of their derivatives are given in Table 35. In the case of DOC, it proved impossible to obtain three ^{3}H specific activities within the 10% maximum limit of error and two only are given. Table 36 shows the total conversions of the radioactive substrates to the steroids listed. Table also shows the $^{3}H/^{14}C$ ratios in these steroids and the ratios of conversion in fasicular conversion in reticular tissue to each steroid from [7α-^{3}H] pregnenolone and [4-^{14}C] progesterone.

It should be noted that:-

a) throughout, the conversion of pregnenolone to the metabolites isolated was greater in fascicular than in reticular tissue and in the case of pregnenolone

cortisol, it was very much greater.

b) the conversion of progesterone to cortisol was greater in fascicular tissue, but the conversion of progesterone $\longrightarrow$ 17αOH-progesterone was greater in reticular tissue. Marginally greater conversions in fascicular tissue were

Tissue Incubated (F or R)	[7α-^{3}H] Preg. Incubated		^{3}H Recovered (MeOH aq. fraction)		[4-^{14}C] Prog. Incubated		^{14}C Recovered (MeOH aq. fraction)	
	mμC	mμmole	mμC	%	mμC	mμmole	mμC	%
F	11,131	92.35	10,067	88.84	603.8	23.09	593.9	98.36
R	11,131	92.35	10,174	89.79	603.8	23.09	579.8	96.03

Table 34.

Tissue Incubated (F or R)	Steroid Isolated	Chemical Reaction	Product	Solvent System	Specific Activity ($m\mu C/\mu mole \times 10^2$) ^3H	^{14}C
F	cortisol	-	cortisol	I	15,163	1,586
		acetylation	cortisol acetate	I	13,689	1,512
		oxidation	adrenosterone	II	14,609	1,663
R		-	cortisol	I	1,707	849
		acetylation	cortisol acetate	I	1,597	839
		oxidation	adrenosterone	II	1,697	878

Tissue Incubated (F or R)	Steroid Isolated	Chemical Reaction	Product	Solvent System	Specific Activity ($m\mu C/\mu mole \times 10^2$) ^3H	^{14}C
F	17αOH-prog.	-	17αOH-prog.	II	9,933	1,296
		reduction	17α,20β-di-hydroxypregn-4-en-3-one	IV	10,608	1,405
		acetylation	20β-acetoxy-17α-hydroxypregn-4-en-3-one	IV	9,787	1,280
R		-	17αOH-prog.	II	4,758	1,479
		reduction	17α,20β-di-hydroxypregn-4-en-3-one	IV	4,908	1,440
		acetylation	20β-acetoxy-17α-hydroxypregn-4-en-3-one	IV	4,474	1,466

Tissue Incubated (F or R)	Steroid Isolated	Chemical Reaction	Product	Solvent System	Specific Activity ($m\mu C/\mu mole \times 10^2$)	
					^3H	^{14}C
F	16α-OH-prog.	-	16α-OH-prog.	I	2,730	1,330
		acetylation	16α-OH-prog. acetate	III	2,553	1,280
		oxidation	16-oxoprog.	IV	-	1,325
		reduction	16α,20β-dihydroxypregn-4-en-3-one	I	2,488	-
R		-	16α-OH-prog.	I	1,490	1,204
		acetylation	16α-OH-prog. acetate	III	1,518	1,136
		oxidation	16-oxoprog.	IV	-	1,101
		reduction	16α,20β-dihydroxypregn-4-en-3-one	I	1,488	-

Tissue Incubated (F or R)	Steroid Isolated	Chemical Reaction	Product	Solvent System	Specific Activity ($m\mu C/\mu mole \times 10^2$)	
					^3H	^{14}C
F	DOC	-	DOC	II	-	6,577
		acetylation	DOC acetate	III	2,205	-
		reduction	20β,21-dihydroxypregn-4-en-3-one	IV	2,400	6,952
		acetylation	20β,21-diacetoxypregn-4-en-3-one	IV	-	7,294
R		-	DOC	II	499	6,168
		acetylation	DOC acetate	III	-	6,015
		reduction	20β,21-dihydroxypregn-4-en-3-one	IV	-	5,561
		acetylation	20β,21-diacetoxypregn-4-en-3-one	IV	546	-

Table 35.

Tissue Incubated (F or R)	Steroid Isolated	Mean Specific Activity (mμC/μmole x 10^2)		Total Conversion (mμC)		$^3H/^{14}C$	F/R	
		^3H	^{14}C	^3H	^{14}C		^3H	^{14}C
F	cortisol	14,487	1,587	117.25	12.85	9.12	8.69	1.86
R	"	1,668	855	13.49	6.92	1.95		
F	17α-OH-prog.	10,109	1,327	89.75	11.79	7.61	2.14	0.91
R	"	4,713	1,462	41.85	12.98	3.22		
F	16α-OH-prog.	2,590	1,312	23.00	11.65	1.97	1.74	1.14
R	"	1,488	1,147	13.21	10.19	1.30		
F	DOC	2,303	6,941	20.45	61.62	0.33	4.40	1.17
R	"	523	5,915	4.65	52.51	0.09		

Table 36.

observed in the cases of progesterone ⟶ DOC and progesterone ⟶ 17αOH-progesterone.

c) the results also show that in

(i) fascicular tissue

$^{3}H/^{14}C$ ratio of cortisol > $^{3}H/^{14}C$ ratio of 17αOH-progesterone ≫ $^{3}H/^{14}C$ ratio of 16αOH-progesterone ≫ $^{3}H/^{14}C$ ratio of DOC.

(ii) reticular tissue

$^{3}H/^{14}C$ ratio of 17αOH-progesterone > $^{3}H/^{14}C$ ratio of cortisol > $^{3}H/^{14}C$ ratio of 16αOH-progesterone ≫ $^{3}H/^{14}C$ ratio of DOC.

DISCUSSION

It is probably a reasonable assumption that the only major pathway to cortisol from 17αOH-pregnenolone is via 17αOH-progesterone and 11-deoxycortisol (Mulrow et al., 1962). The results of the incubation of horse adrenal gland fascicular and reticular tissue with [4-^{14}C] progesterone, [7α-^{3}H] pregnenolone, [4-^{14}C] 17αOH-progesterone and [7α-^{3}H] 17αOH-pregnenolone illustrate the following points:-

1. The sequences pregnenolone ⟶ 17αOH-pregnenolone ⟶ ⟶ 17αOH-progesterone and pregnenolone ⟶ progesterone ⟶ 17αOH-progesterone are both slower than the succeeding steps from 17αOH-progesterone to cortisol.

2. The step 17αOH-pregnenolone ⟶ 17αOH-progesterone is thus rate-limiting in the transformation of 17αOH-pregnenolone to cortisol, and so

$$\frac{\text{cortisol from 17αOH-pregnenolone in fascicular tissue}}{\text{cortisol from 17αOH-pregnenolone in reticular tissue}}$$

$$=\frac{\text{17αOH-pregnenolone-3β-hydroxy dehydrogenase activity in fascicular tissue}}{\text{17αOH-pregnenolone-3β-hydroxy dehydrogenase activity in reticular tissue}}$$

i.e. the percentage conversions of 17αOH-pregnenolone to cortisol in fascicular and reticular tissue must also reflect the conversions of 17αOH-pregnenolone ⟶ 17αOH-progesterone in the two zones. This means that, in the horse adrenal, there is approximately 2.4 times more 17αOH-pregnenolone-3β-hydroxy dehydrogenase activity in fascicular than in reticular tissue. It should also be noted, however, that dehydrogenation of 17αOH-pregnenolone takes place in reticular tissue (see also p.112) which appears to be contrary to the histochemical picture (Plate I), found by Baillie et al. (1965). (The histochemically defined distribution of 17αOH-pregnenolone-3β-hydroxy dehydrogenase activity is the classical one of virtually all activity in the outer zona fasciculata and none in the inner zona reticularis.)

3. We know from Table 33 (p.149) that progesterone is transformed to cortisol at a faster rate than pregnenolone in both zones. This means that if pregnenolone were converted to cortisol only via progesterone, then pregnenolone ⟶ progesterone would be the rate-limiting step in the sequence and,

$$\frac{\text{cortisol from pregnenolone in fascicular tissue}}{\text{cortisol from pregnenolone in reticular tissue}} \simeq 4.6$$

would represent

$$\frac{\text{pregnenolone-3}\beta\text{-hydroxy dehydrogenase activity in fascicular tissue}}{\text{pregnenolone-3}\beta\text{-hydroxy dehydrogenase activity in reticular tissue}}$$

i.e. the pregnenolone $\longrightarrow$ progesterone step would be carried out more efficiently in fascicular tissue and there would thus be a higher pregnenolone-3β-hydroxy dehydrogenase activity in this tissue. We know, however, that a second pathway from pregnenolone to cortisol exists *via* 17αOH-pregnenolone (Wolfky & Engel, 1961, 1962, 1963; Mulrow & Cohn, 1961; Mulrow *et al.* 1962; Lipsett & Hökfelt, 1961) and hence there are obvious alternative explanations. In the transformation of pregnenolone to cortisol, the reactions which convert pregnenolone to 17αOH-progesterone (fig. 14) are slower than the succeeding sequence from 17αOH-progesterone to cortisol (section 1, above) in both fascicular and reticular tissue. It has also been shown (section 2, above) that the transformation of 17αOH-pregnenolone

17αOH-progesterone is carried out at a faster rate in fascicular tissue. Thus it is quite possible that the figures obtained in Table 33 (p.149) for the conversion of [7α-^{3}H] pregnenolone and [4-^{14}C] progesterone to cortisol could be accounted for by

(i) a higher pregnenolone-17α-hydroxylase activity

(ii) a higher 17αOH-pregnenolone-3β-hydroxy dehydrogenase activity

(iii) a higher pregnenolone-3β-hydroxy dehydrogenase activity

in fascicular tissue or a combination of any or all of these three possibilities.

4. In comparing the conversions of progesterone and 17αOH-progesterone to cortisol in fascicular and reticular tissue, one may use similar criteria to those described above. If it is a reasonable assumption that progesterone ⟶ 17αOH-progesterone ⟶ 11-deoxy-cortisol ⟶ cortisol is the only major pathway for the transformation of progesterone to cortisol, then the figures in Table 33 (p.149) show that the progesterone ⟶ 17αOH-progesterone step is rate-limiting in this

sequence. Therefore, by similar reasoning to that described in section 2 (p.160), progesterone-17α--hydroxylase activity is between 1.67 and 2.91 times higher in fascicular than in reticular tissue from the horse adrenal cortex.

As already seen in Part I (p.113) and above (p.161), the histochemical picture of little or no DHA-, pregnenolone- or 17αOH-pregnenolone-3β-hydroxy dehydrogenase activities in the reticular zone is false or at least misleading, since DHA, pregnenolone and 17αOH--pregnenolone are all dehydrogenated by reticular tissue. In the results discussed above, again the histochemical picture does not seem to fit the biochemical one with respect to the metabolism of 17αOH-pregnenolone. As far as pregnenolone is concerned, it was only possible at this stage to say that pregnenolone was probably metabolised to progesterone at a higher rate in the fascicular zone.

The situation is thus more complicated than it appears at first sight. Following the experiments with horse adrenal slices, the attempt was made with <u>human</u> adrenal tissue, to investigate the alternative metabolic pathways which convert pregnenolone to 17αOH-progesterone

with a view to the elucidation of the role of 3β-hydroxy-steroid dehydrogenase. If a parallel situation exists in human and horse adrenal tissue, the pattern of 3β--hydroxysteroid dehydrogenase activity may dictate the ultimate fate of the steroid nucleus (p.12). As might be expected, however, in a gland having an "emergency" zone capable not only of normal activity but of meeting sudden demands for a particular hormone, control is probably exerted by means of a complex enzyme system of dehydrogenase, isomerase and hydroxylase activities and enzyme-substrate affinities. Therefore, it is really necessary to compare the metabolic pathways which convert pregnenolone to cortisol as a whole in both zones.

It was pointed out in section a and b (p.152) that pregnenolone is converted to all metabolites isolated in greater yield in fascicular tissue compared with reticular tissue. As shown in Table 36 (p.158), however, the conversion of progesterone to the various steroids isolated is only marginally higher in fascicular tissue incubations.

The extremely low $^{3}H/^{14}C$ ratios found in the DOC isolated strongly suggest that pregnenolone ⟶ progesterone ⟶ DOC is the only major pathway for the

formation of DOC in both zones. Furthermore, there is no evidence in the literature to indicate the existance of another major pathway. There is also no evidence in the literature to indicate that 16αOH-progesterone is readily metabolised by adrenal tissue in vitro. Indeed, this steroid is something of an enigma. We know, too, that the major metabolite of DOC in human adrenal tissue is corticosterone (Grant et al. 1957). There are thus a number of possible metabolic situations:-

(a) progesterone is the sole precursor of 16αOH-progesterone.

(b) progesterone is not the sole precursor of 16αOH--progesterone.

(c) DOC is metabolised rapidly to corticosterone.

(d) DOC is not metabolised rapidly to corticosterone.

These situations may be combined in four ways and compared with the results obtained, viz. $^{3}H/^{14}C$ ratios found in the DOC and 16αOH-progesterone isolated.

1. Progesterone is the sole precursor of 16αOH-progesterone and DOC is not rapidly metabolised.

The $^{3}H/^{14}C$ ratios in 16αOH-progesterone and DOC would then be similar. They are not and, therefore,

this combination is false.

2. Progesterone is the sole precursor of 16αOH-progesterone and DOC is rapidly metabolised.

In this case, the $^{3}H/^{14}C$ ratio in DOC would exceed that of 16αOH-progesterone, and since this is not so, this combination is also false.

3. Progesterone is not the sole precursor of 16αOH--progesterone and DOC is rapidly metabolised.

The figures obtained do not preclude this situation but the extremely low $^{3}H/^{14}C$ ratios in DOC make it unlikely.

4. Progesterone is not the sole precursor of 16αOH--progesterone and DOC is not rapidly metabolised.

This picture seems to fit the results best and, therefore, makes it the most likely explanation.

Welikky & Engel (1963) found no ^{3}H in 16αOH-progesterone isolated from their incubation of hyperplastic human adrenal tissue with $[7\alpha\text{-}^{3}H]$ pregnenolone and $[4\text{-}^{14}C]$ progesterone, i.e. the reactions, pregnenolone $\longrightarrow$ progesterone and 16αOH-pregnenolone $\longrightarrow$ 16αOH--progesterone did not occur. This may be compared with the present results, where the evidence points to the

fact that, in both zones of a normal human adrenal cortex, these transformations took place. Indeed, the figures suggest that the main route to 16αOH--progesterone may be via 16αOH-pregnenolone in normal tissue.

The examination of the relative magnitude of the alternate pathways of pregnenolone to cortisol in the two zones involves a comparison of the $^{3}H/^{14}C$ ratios in the cortisol and 17αOH-progesterone isolated with those of the 16αOH-progesterone and DOC. If the results obtained with horse tissue are any guide to what may occur in human tissue, the transformation of 17αOH-progesterone to cortisol is probably rapid in comparison with the preceding reactions (p.160), especially in the zona fasciculata. Since progesterone requires fewer steps than pregnenolone for transformation to cortisol, one might expect the $^{3}H/^{14}C$ ratio in 17αOH-progesterone to be similar to that found in DOC or 16αOH-progesterone if the pathway pregnenolone $\longrightarrow$ $\longrightarrow$ 17αOH-pregnenolone $\longrightarrow$ 17αOH-progesterone is a minor one. However, the $^{3}H/^{14}C$ ratios in 17αOH--progesterone and cortisol, particularly the material isolated from the fasciculer tissue incubation, are

very much higher than those of the DOC or 16αOH-progesterone. This shows quite clearly that the pathway independent of progesterone, far from being a minor one, is probably the major route to cortisol from pregnenolone *in vitro* and that the preference for this pathway is greater in fascicular tissue.

An interesting point of comparison of the two zones is that, whereas the $^{3}H/^{14}C$ ratio in the 17αOH-progesterone is greater than that in the cortisol from the reticular tissue incubation, the reverse is true of the steroids isolated from the fascicular tissue incubation. A possible explanation for this may be that yet another pathway independent of 17αOH-progesterone exists in fascicular tissue for the transformation of pregnenolone to cortisol. This might involve further hydroxylation of the Δ^{5}-pregnene nucleus before dehydrogenation at C-3.

It is an extraordinary fact that, although in the case of the testis and ovary, much is made of the distinction in histological, histochemical and biochemical properties of the different cell types present in these glands (e.g. Short, 1962), little attention has been focussed on the metabolism of isotopically labelled steroids by the separated fascicular and reticular cells of the adrenal cortex. In most instances, normal and

pathological adrenal cortices and adrenal tumours are treated as if they contained only one cell type. This must surely present a false picture of the nature of adrenal steroid biogenesis and provides no information on cell function. Great care should be taken with the interpretation of results obtained from isotopic tracer experiments, yet a direct comparison of the ability of hyperplastic adrenal tissue to synthesise various steroids including cortisol from 2.2 µg. of [7α-^{3}H] pregnenolone and 37 µg. of [4-^{14}C] progesterone (Weliky & Engel, 1963) has been made. From the results quoted by the authors, it can be calculated that the $^{3}H/^{14}C$ ratios of the 17αOH--progesterone, 11-deoxycortisol and cortisol were 0.88, 0.94 and 1.50, respectively. These figures could be explained by a rapid metabolism of a substantial proportion of the small quantity of [7α-^{3}H] pregnenolone to 17αOH-pregnenolone and 17αOH-progesterone in the very early stages of the incubation followed by a steady build--up of ^{14}C from the much larger quantity of [4-^{14}C] progesterone. This means that the correct conditions for the use of radioisotopes were not applied since the introduction of the "tracers" to be compared interfered disproportionately with the metabolic pathways under examination. In an earlier publication (Weliky & Engel,

1962), the same authors compared the efficiency of conversion of 5.46 µg. of [7α-^{3}H] 17αOH-pregnenolone and 35.6 µg. of [4-^{14}C] progesterone by an adrenal tumour. Such an experiment can tell little about the route of formation of cortisol in view of the difference in quantity of the substrates and also in view of the fact that there is no information available in these experiments on the transformation of pregnenolone to either 17αOH-pregnenolone or progesterone. The impression is given that 17αOH-pregnenolone is efficiently converted to cortisol, whereas progesterone is a poor precursor of this hormone. However, on the basis of the figures given in the paper, the ratio of conversion of progesterone and 17αOH-pregnenolone to cortisol is really 1.76:1.

Throughout the investigations described in Part II, only one cell type was present in each incubation (as far as the techniques available would allow). Any two substrates under direct comparison were made equimolar prior to the start of the incubation, and the tissue:total steroid ratio was ~10,000:1. As stated in the introduction to Part II (p.123), in the final experiment with human adrenal slices, it was reasoned that pregnenolone was the steroid under investigation, and so the initial

ratio of pregnenolone to progesterone was made as high as practicable, consistent with a measurable conversion of pregnenolone to the metabolites of interest. Furthermore, in order not to favour one pathway, a quantity of non-labelled 17αOH-pregnenolone equimolar with the [4-^{14}C] progesterone was also added.

The future development of the investigation of cell function in the adrenal cortex must now be considered. Interesting points have been raised concerning the effect of one steroid on the biosynthesis of another, e.g. 11β-hydroxylation of DOC (Sharma, Forchielli & Dorfman, 1963) and C-21 hydroxylation of pregnenolone and 17αOH-pregnenolone (Sharma & Dorfman, 1964) appear to be inhibited by certain androgens. Others have investigated the role of various pyridine nucleotides on steroid biosynthesis (Brownie & Grant, 1956; Grant, 1956; Tautsui, Marks & Reich, 1961; Koritz, 1963 & 1964; Villee, 1964) and the mechanism of action of ACTH (Griffiths et al. 1963; Farese, 1964) or of adenosine-3',5'-cyclic monophosphate (Studzinski & Grant, 1962; Creange & Roberts, 1965). Yet the fundamental problem of estimating the relative magnitude of the various metabolic pathways within the different cell types of the adrenal cortex has still to be solved.

In 1963, Kopin reviewed general methods of estimating the magnitude of alternative metabolic pathways *in vivo*. One of the model systems (see fig. 20 below) is described as "a convergent metabolic pathway where all of the products are derived from a single precursor. One of the products, C_2, is common to both intermediates B_1 and B_2".

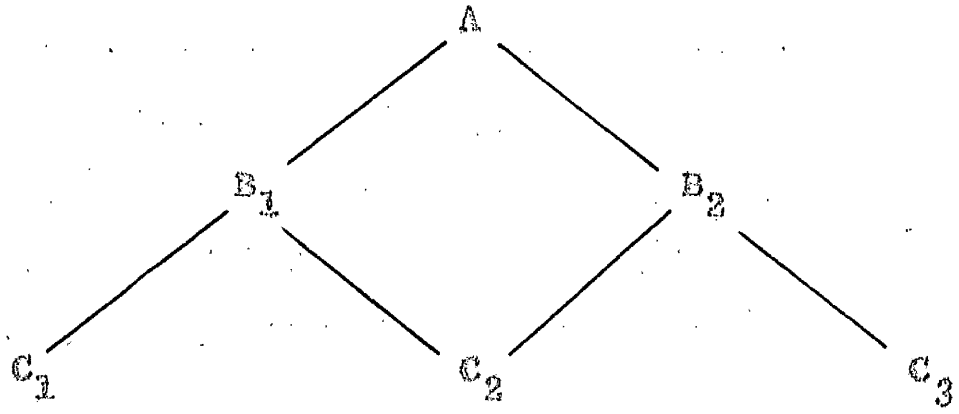

fig. 20.

On examination of the kinetics involved, it was concluded that the fraction of A converted to B_2 is

$$f_{AB_2} = \left[\frac{R}{S}\right]_{C_3} \Big/ \frac{R_o}{S_o}$$

and the proportion of C_2 derived from B_2 is

$$C_2 = \left[\frac{R}{S}\right]_{C_3} \Big/ \left[\frac{R}{S}\right]_{C_2}$$

where a) the amount of an isotope, R, administered as A is R_o

b) the amount of an isotope, S, administered as intermediate B_2 is S_o

c) $\left[\frac{R}{S}\right]_{C_2}$ and $\left[\frac{R}{S}\right]_{C_3}$ are the ratios of isotope R to isotope S in C_2 and C_3 respectively.

It is readily seen that the system of steroid metabolic pathways in fig. 21 is very similar to that of the model in fig. 20 .

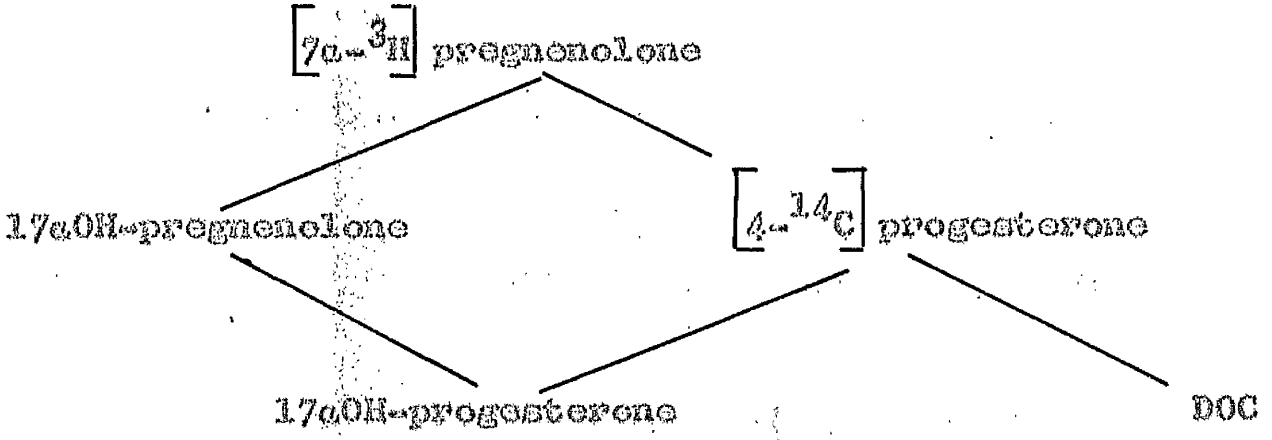

fig. 21.

However, we are immediately faced with the problem that,

in vitro, neither 17αOH-progesterone nor DOC is an end product, but each may be further metabolised quite readily by adrenal tissue. Thus examination of the isotopic content of these substances can give no *quantitative* information on the route of formation of 17αOH-progesterone. It is necessary to determine the total quantity of ^{3}H and ^{14}C labelled steroid which is at, or has passed through the 17αOH-progesterone and DOC "gateways".

In Appendix I, a further examination of the factors involved shows how future estimations of the relative magnitude of such alternative pathways might be achieved which should give a reasonably close approximation to the true picture. The reasoning is freely adapted from the article by Kopin (1963).

SUMMARY.

Summary.

The purpose of the investigation was to determine whether the histochemically defined picture of 3β--hydroxysteroid dehydrogenase distribution in the adrenal cortex of horse and man could be confirmed biochemically. Attempts were also made to investigate the significance of this distribution.

Part I.

Histochemistry has shown that the highest 3β-hydroxy-steroid dehydrogenase activity is in the outer zona fasciculata with little or no activity in the zona reticularis. This result is obtained with substrates dehydroepiandrosterone, pregnenolone and 17α-hydroxy--pregnenolone.

Evidence is now presented that DHA- and pregnenolone--3β-hydroxy dehydrogenase activity (and possibly 17α--hydroxy-pregnenolone-3β-hydroxy dehydrogenase activity) is higher in the zona fasciculata of the adrenal cortices of horse and man. The results obtained, however, do not indicate the large difference in activity suggested by the histochemical evidence, and indeed it was found that reticular tissue contains substantial amounts of 3β-hydroxy-steroid dehydrogenase activity.

Part II.

The ability of fascicular and reticular cells of the horse adrenal cortex to transform [7α-^{3}H] pregnenolone, [4-^{14}C] progesterone, [7α-^{3}H] 17α-hydroxypregnenolone and [4-^{14}C] 17α-hydroxyprogesterone to cortisol was measured.

It was found that:-

1. All four steroids are transformed to cortisol by both types of cell.

2. The transformation of all four steroids to cortisol is higher in fascicular tissue.

3. The sequences pregnenolone ⟶ 17α-hydroxypregnenolone ⟶ 17α-hydroxyprogesterone and pregnenolone ⟶ progesterone ⟶ 17α-hydroxyprogesterone are both slower than the succeeding steps from 17α-hydroxyprogesterone ⟶ ⟶ cortisol.

4. The step 17α-hydroxypregnenolone ⟶ 17α-hydroxyprogesterone is rate-limiting in the transformation of 17α-hydroxypregnenolone ⟶ cortisol and there is approximately 2.4 times more 17α-hydroxypregnenolone-3β-hydroxy dehydrogenase activity in fascicular than in reticular tissue.

5. Progesterone-17α-hydroxylase activity is between 1.67 and 2.91 times higher in fascicular than in reticular tissue.

Following the experiments with horse adrenal cells, an attempt was made with human adrenal tissue to investigate the alternative metabolic pathways which convert pregnenolone to 17α-hydroxyprogesterone with a view to the elucidation of the role of 3β-hydroxysteroid dehydrogenase. [7α-^{3}H] pregnenolone and [4-^{14}C] progesterone were incubated simultaneously with fascicular and with reticular slices from a normal human adrenal cortex. Conversions of each substrate to 16α-hydroxyprogesterone, 11-deoxycorticosterone, 17α-hydroxyprogesterone and cortisol were measured.

Evidence was found suggesting that:-

1. Both fascicular and reticular tissue convert pregnenolone and progesterone to the four metabolites mentioned above.

2. Pregnenolone is converted to these metabolites in greater yield in fascicular tissue compared with reticular tissue.

3. The conversion of progesterone to these metabolites is only marginally greater in fascicular tissue.

4. Pregnenolone ⟶ progesterone ⟶ 11-deoxycorticosterone is the only major pathway for the formation of 11-deoxycorticosterone in both zones.

5. The main route from pregnenolone to 16α-hydroxyprogesterone is *via* 16α-hydroxypregnenolone in both zones.

6. The pathway pregnenolone ⟶ 17α-hydroxypregnenolone ⟶ 17α-hydroxyprogesterone ⟶ 11-deoxycortisol ⟶ cortisol is the major route to cortisol from pregnenolone *in vitro* in the adrenal cortex and the preference for this pathway is greater in fascicular tissue.

7. It is possible that a pathway exists from 17α-hydroxypregnenolone to cortisol in fascicular tissue independent of 17α-hydroxyprogesterone.

The theoretical factors involved in making an accurate determination of the magnitude of alternative pathways of steroid biosynthesis were discussed.

APPENDICES

APPENDIX I.

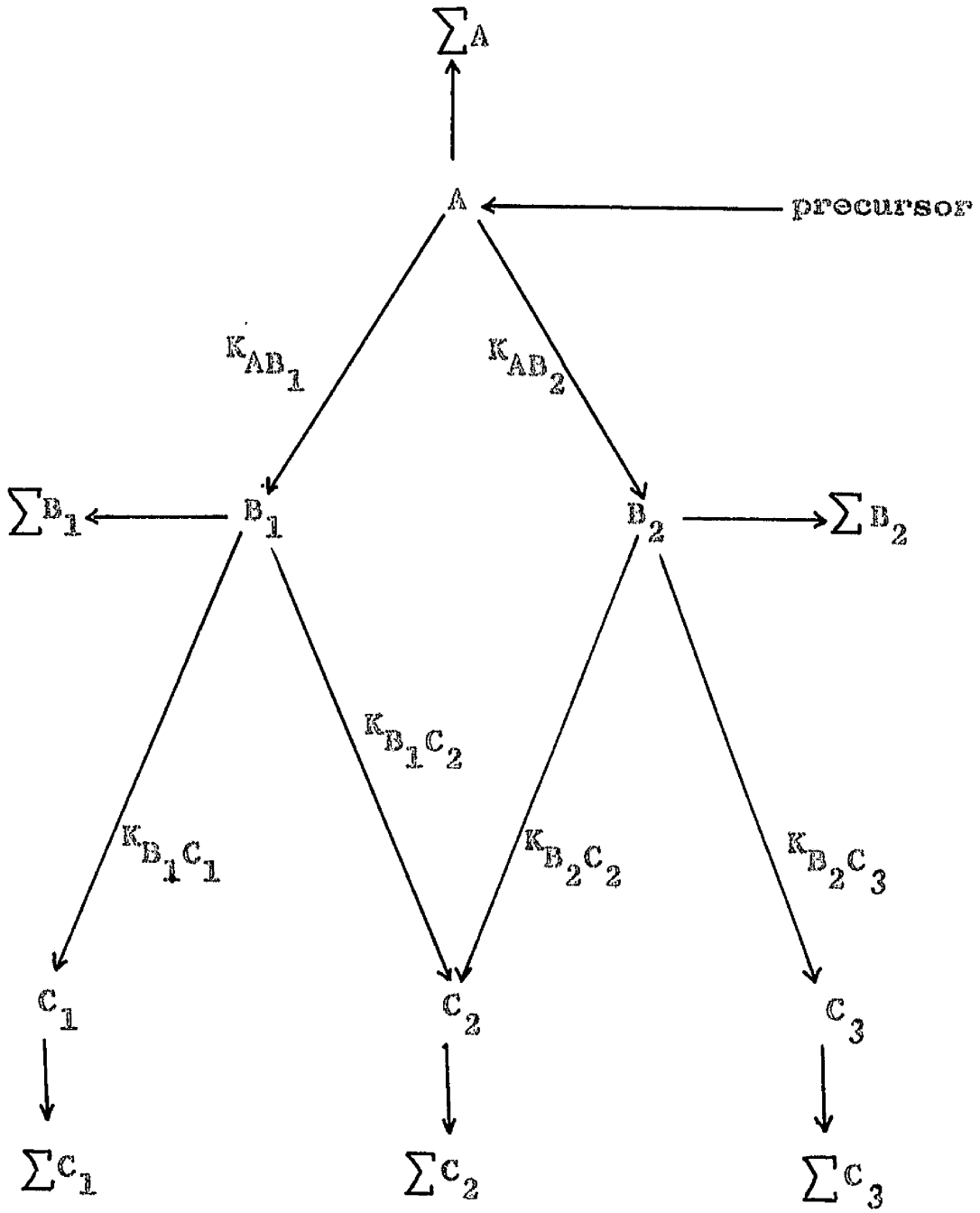

fig. 22

A represents all metabolites of A excluding B_1, B_2 and their products.

$\sum B_1$ and $\sum B_2$ represent all metabolites of B_1 and B_2 respectively excluding C_1, C_2 and C_3 and their products.

$\sum C_1$, $\sum C_2$ and $\sum C_3$ represent all metabolites of C_1, C_2 and C_3 respectively and their products.

Let A be the unique precursor of all intermediates and products in fig. above, and assume that the concentration of A ([A]) does not alter throughout the experiment.

If the rate of reaction is assumed to be proportional to a rate constant K_{XY}, where X is the precursor and Y the product, then

the rate of conversion $A \longrightarrow B_1 = [A]K_{AB_1}$

Precursor $A \longrightarrow B_1$ at a rate $[A]K_{AB_1}$ and to B_2 at a rate $[A]K_{AB_2}$ etc.

The amount of B_1 formed during time $T = [A]K_{AB_1}.\ T$

(A is assumed to be formed at the same rate as it disappears).

Rate of removal of A = $[A]K_{AB_1} + [A]K_{AB_2} + \ldots [A]K_{AB_X}$

(X = total number of metabolites of A)

Amount of A destroyed during the interval, T, is

$$A = [A](K_{AB_1} + K_{AB_2} + \ldots\ldots K_{AB_X}) \cdot T$$

The proportion of A which has been converted to B_1 during this interval is, therefore,

$$f_{AB_1} = \frac{[A]K_{AB_1} \cdot T}{[A](K_{AB_1} + K_{AB_2} + \ldots\ldots K_{AB_X}) T.}$$

$$= K_{AB_1} \Big/ \sum_{B_1}^{B_X} K_{AB}$$

$$\left(\sum_{B_1}^{B_X} K_{AB} = K_{AB_1} + K_{AB_2} + \ldots\ldots K_{AB_X} \right)$$

thus:-

$$f_{AB_1} = \frac{K_{AB_1}}{\sum_{B_1}^{B_X} K_{AB}} \; ; \quad f_{AB_2} = \frac{K_{AB_2}}{\sum_{B_1}^{B_X} K_{AB}}$$

(x is total number of metabolites of A.)

$$f_{B_1C_1} = \frac{K_{B_1C_1}}{\sum_{C_1}^{C_X} K_{B_1C}} \; ; \quad f_{B_1C_2} = \frac{K_{B_1C_2}}{\sum_{C_1}^{C_X} K_{B_1C}}$$

(x is total number of metabolites of B_1.)

$$f_{B_2C_2} = \frac{K_{B_2C_2}}{\sum_{C_1}^{C_X} K_{B_2C}} \; ; \quad f_{B_2C_3} = \frac{K_{B_2C_3}}{\sum_{C_1}^{C_X} K_{B_2C}}$$

(x is total number of metabolites of B_2.)

If a) all (or almost all, for practical purposes) of the labelled molecules of A, B_1 and B_2 are completely converted to C_1, C_2, C_3 and their products and b) the amount of isotope ^3H incubated as A is ^3H_o, then the total amounts of ^3H transformed to metabolites C_1, C_2 and C_3 are

$$C_1^{3H} = f_{AB_1} \cdot f_{B_1C_1} \cdot {}^3H_o.$$

$$C_2^{3H} = (f_{AB_1} \cdot f_{B_1C_2} + f_{A\,B_2} f_{B_2C_2}) \cdot {}^3H_o.$$

$$C_3^{3H} = f_{AB_2} f_{B_2C_3} \cdot {}^3H_o.$$

Similarly the amounts of isotope, ^{14}C, transformed to

C_2 and C_3 following incubation of ^{14}C labelled B_2 are:

$$C_2^{14}C = f_{B_2C_3} \cdot {}^{14}C_o.$$

$$C_3^{14}C = f_{B_2C_3} \cdot {}^{14}C_o.$$

(It is essential to remember that, in practice, $C_1^{3}H$ will not be formed by determination of the ^3H content of C_1, but by summation of the ^3H content of C_1, and of all its metabolites).

The ratio of ^3H to ^{14}C in C_3 + all its metabolites is

$$\left[\frac{^3H}{^{14}C}\right]_{C_3 + \sum C_3} = \frac{C_3^{3}H + {}^3H \sum C_3}{C_3^{14}C + {}^{14}C \sum C_3}$$

$$= \frac{f_{AB_2} \cdot f_{B_2C_3} \cdot {}^3H_o}{f_{B_2C_3} \cdot {}^{14}C_o}$$

$$= f_{AB_2} \ (^3H_o/^{14}C_o)$$

$$f_{AB_2} = \frac{{}^{3}H}{{}^{14}C}\ C_3 + \sum C_3 \Big/ {}^{3}H_o/{}^{14}C_o$$

or

$$= \frac{C_3^{3}H + {}^{3}H \sum C_3 \Big/ {}^{3}H_o.}{C_3^{14}C + {}^{14}C \sum C_3 \Big/ {}^{14}C_o} \qquad (1)$$

"The portion of a product (C_2) common to both routes formed through the pathway of the labelled intermediate (B_2) may be estimated from the ratio of the isotopes in this product and one uniquely derived from the intermediate (B_2)."

$$\frac{{}^{3}H}{{}^{14}C}\ C_2 + \sum C_2 = \frac{(f_{AB_2}.\ f_{B_2C_2} + f_{AB_1}.\ f_{B_1C_2})\ {}^{3}H_o}{f_{B_2C_2}.\ {}^{14}C_o.}$$

$$\frac{{}^{3}H}{{}^{14}C}\ C_3 + \sum C_3 = f_{AB_2}.\ \frac{{}^{3}H_o}{{}^{14}C_o.}$$

The proportion of $C_2 + \sum C_2$ derived from B_2 is

$$C_2 + \sum C_2 = \frac{f_{AB_2}.\ f_{B_2C_2}}{f_{AB_1}.\ f_{B_1C_2} + f_{AB_2}.\ f_{B_2C_2}}$$

This fraction may be calculated by dividing the ratio of $^{3}H/^{14}C$ in $C_3 + \sum C_3$ by the ratio of $^{3}H/^{14}C$ in $C_2 + \sum C_2$

$$C_2 + \sum C_2 = \left[\frac{^{3}H}{^{14}C}\right] C_3 + \sum C_3 \Big/ \frac{^{3}H}{^{14}C}\ C_2 + \sum C_2 \qquad (2)$$

Particularly in the steroid field, even a system as complicated as the one in fig. is an oversimplification. It is quite possible, for example, for C_1 and C_2 to be transformed by different processes into the same metabolite. Since both C_1 and C_2 are derived from B_1, they would both contain ^{3}H from A. The common metabolite of C_1 and C_2 would then receive ^{3}H from two sources. Thus to apply equations (1) and (2) correctly, it would be necessary to distinguish ^{3}H from C_1 and ^{3}H from C_2 which is clearly impossible. The problem, however, should not be insoluble given a number of conditions, the most important of which are that all the major steroid metabolic pathways in the tissue under examination are known. It might then be possible to perform two incubations simultaneously, one with $[^{3}H]A$ and $[^{14}C]B_2$, and the other say with $[^{3}H]B_1$ and $[^{14}C]C_1$. The second experiment would then provide information which could be applied in an assessment of the

relative contributions of C_1 and C_2 to their common metabolite. Thus the data required for the solution of equations (1) and (2) would become available.

From the statements already made, it would seem that in order to satisfy the criteria for correct application of the equations, we would need to know the origin and quantity of both isotopes in every metabolite of C_2 and C_3 formed. However, let us examine the situation in fig. a little further with respect to simultaneous incubation of $[7\alpha\text{-}^3H]$pregnenolone (A) and $[4\text{-}^{14}C]$ progesterone (D_2). The major metabolites of 17αOH-progesterone (C_2) in adrenal tissue incubations in vitro are 11-deoxycortisol, cortisol and cortisone, and the major metabolite of DOC (C_3) is corticosterone. Thus, if we know the ^3H and ^{14}C content of 17αOH-progesterone, DOC and their major metabolites, we have a reasonable approximation for the ^3H and ^{14}C values for "$C_2 + \sum C_2$" and "$C_3 + \sum C_3$" given the conditions of complete metabolism of the labelled steroids 17αOH-progesterone, DOC and their metabolites. If the reasoning set out above is valid, this may, therefore, be a way in which any future development of the in vitro investigation of the biochemistry of the adrenal cortex may proceed. The reasoning might also be applied to the investigation

of alternative metabolic pathways in other tissues.

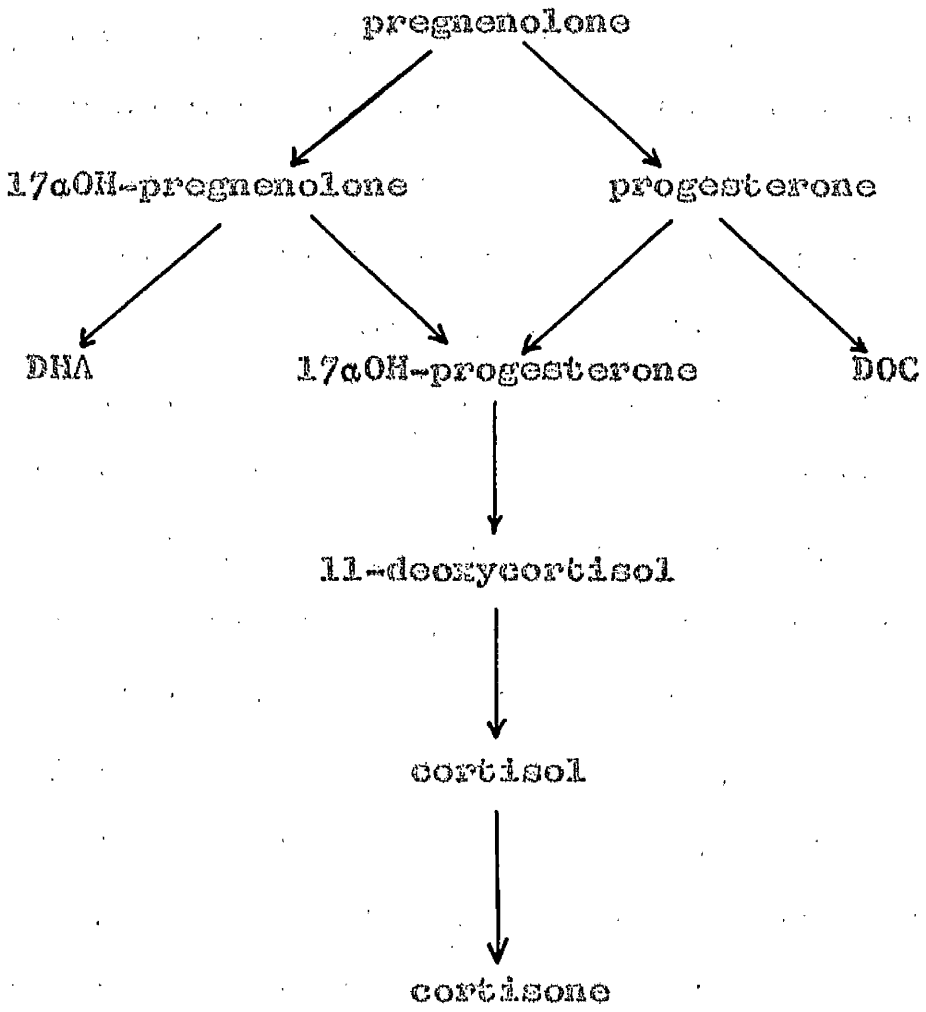

fig.23

APPENDIX II.

A. Reagents, Materials & Solvents (Sources etc.)

Chemicals & Reagents.

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° at an approximate concentration of 1 μC/ml. (^{14}C--steroids) or 10 μC/ml. (^{3}H-steroids) in a mixture of methanol/benzene (9:1, v/v).

Steroids.

Steroids were purchased from Koch-Light Laboratories, Ltd., Colnbrook, Bucks. or Steraloids, Ltd., Croydon, Surrey. Generous gifts of 16αOH-progesterone were

received from the Upjohn Co., Kalamazoo, Mich., U.S.A. and from Professor W. Klyne, Westfield College, London (M.R.C. Reference Collection).

Solvents.

Ethanol and methanol (Burroughs, A.R. grade), and diethyl ether (B.D.H., ANALar grade) were used without further purification. It was found necessary to purify methylene chloride, light petroleum and benzene by shaking with concentrated sulphuric acid; traces of acid were then removed by washing the solvents with water and after drying over anhydrous calcium chloride they were distilled. In particular, it was found to be essential to re-distil the purified benzene immediately before use for column chromatography on alumina. This procedure appeared to minimise interference by solvent residues with sulphuric acid-ethanol reagent chromogens.

All other solvents used were washed with water and re-distilled before use.

B. Krebs-Ringer Bicarbonate-Glucose Medium

The method of preparation of this medium is based on that described in "Manometric Methods" by Umbreit, Burris & Stauffer (1957).

	Vol. (ml.)	Conc. of Sol. added (M)	Conc. of Sol. added (g./100 ml.)	Final Conc. (M)
NaCl	10	1.16	6.78	0.116
KCl	10	0.465	3.47	0.0465
$NaHCO_3$ [a]	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

(final volume 100 ml.)

[a] previously gassed for 1 hr. with CO_2.

The final mixture was gassed for 10 minutes with 5% CO_2 in O_2, when the pH was found to be 7.4.

Appendix III.

A. Determination of Optimal Radioactivity Counting Conditions.

As stated in the Experimental section (Part I, p.28), the radioactivity content of extracts etc. was determined in earlier experiments by Packard Tri-Carb Liquid Scintillation Spectrometer, Model 314EX. These determinations involved only the measurement of ^{14}C or ^{3}H in any one sample. To avoid the necessity for changing counting conditions to give optimal efficiency for either ^{3}H or ^{14}C, a "compromise" set of conditions had to be found. This was achieved by plotting amplifier gain against counting efficiency for each isotope at the various E.H.T. settings available on the instrument. An "open window" voltage discriminator setting (gate width) of 100 - 1000 was used at all times.

The results obtained were used to plot the curves shown in fig. 24. From these curves the best "compromise" conditions for counting a set of samples containing ^{3}H or ^{14}C were found to be:-

^{14}C	Channel I	Gate Width	100 - 1000
		E.H.T.	6.2
		Amplifier Gain	20%
giving a Counting Efficiency of			64%
^{3}H	Channel II	Gate Width	100 - 1000
		E.H.T.	6.2
		Amplifier Gain	100%
giving a Counting Efficiency of			26%

From fig.24 it can be seen that a higher counting efficiency for ^{14}C could be obtained by using a lower E.H.T. The lower the E.H.T., however, the narrower is the ^{14}C peak, i.e. small fluctuations in line voltage might have a greater effect on counting efficiency. An E.H.T. of 6.2 was chosen, giving both high efficiency and stability.

B. Quenching.

Portions of extracts containing ^{3}H or ^{14}C were dissolved in scintillator medium (see p. 27) and counted. Scintillator medium (1 ml.) containing a known quantity of ^{14}C- or ^{3}H- labelled toluene was then added to each

vial and the samples recounted. The counts/min. due to the labelled toluene were then obtained by difference and compared with the counts/min. found for the same quantity of isotope under non-quenched conditions. The comparison enabled the degree of quenching due to the sample to be determined.

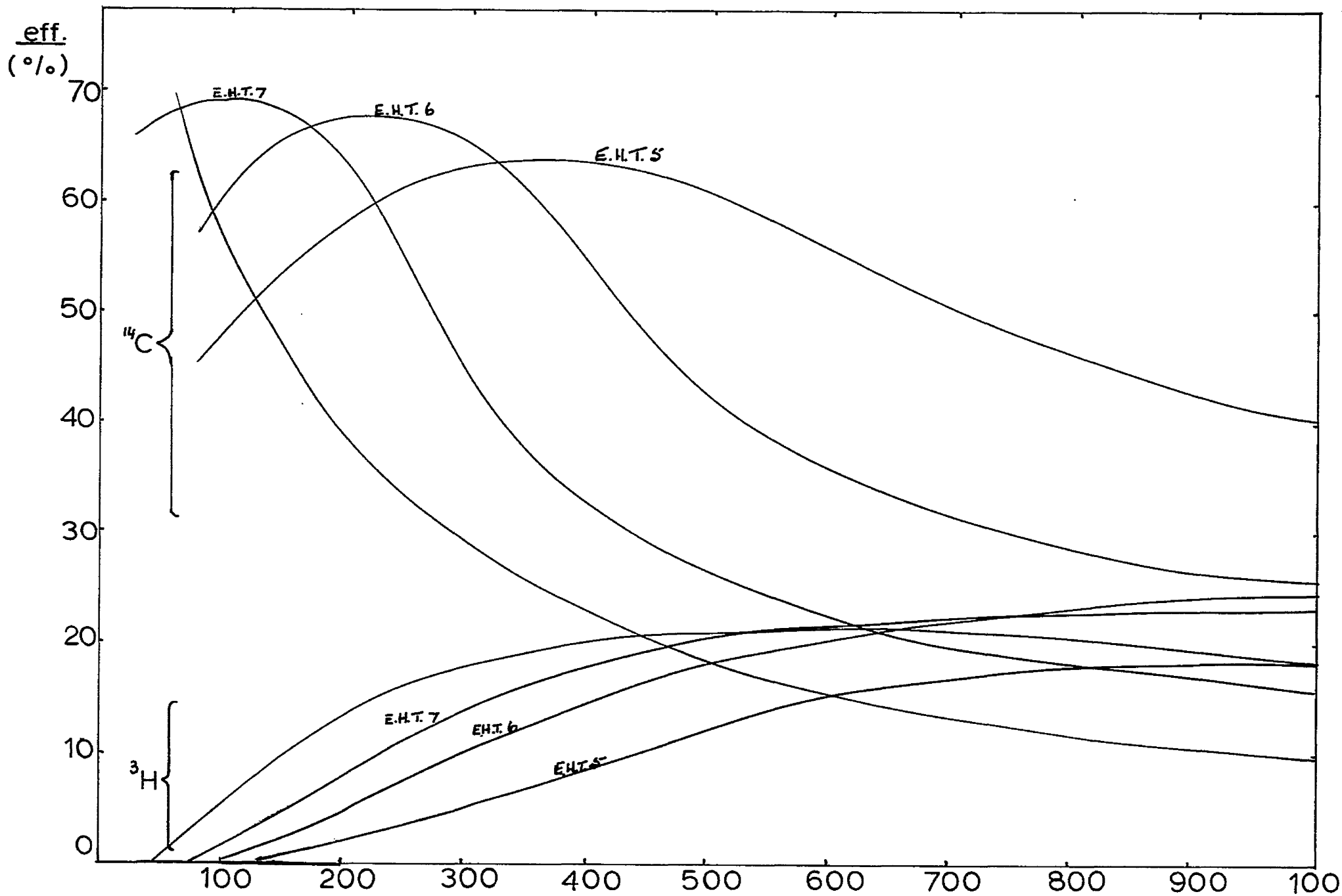
eff.
(%)
70
60
50
40
30
20
10
0
E.H.T. 7
E.H.T. 6
E.H.T. 5
^{14}C
^{3}H
E.H.T. 7
E.H.T. 6
E.H.T. 5
100
200
300
400
500
600
700
800
900
1000
amplifier gain.

fig. 24

Appendix IV.

A. Elution of Steroids from Paper Chromatograms.

Progesterone and cortisol, non-polar and polar steroids respectively, were chosen to test the elution procedure.

Solutions of [4-^{14}C] progesterone and [4-^{14}C] cortisol were prepared in ethanol and 100 µl quantities (containing 10 µg) spotted on 1" lanes of Whatman No. 1 paper. The [4-^{14}C] progesterone was chromatographed in the P10 solvent system (p. 23) and the [4-^{14}C] cortisol in the B10 solvent system. Steroids were located by UV lamp (p. 26) and the areas of paper involved cut into small pieces. The chopped paper was then shaken with 5 ml. of methanol:ethyl acetate (1:1, v/v) at 37° for 1 hr. Eluates were placed in counting vials and the solvents removed under a stream of air at 50°. Two further elutions were carried out on the paper residue.

[4-^{14}C] Progesterone.

100 µl of the stock solution was found to contain 1764 counts/min. of ^{14}C.

From a series of six lanes, the recoveries of radioactivity were 1636, 1658, 1676, 1653, 1611 and 1633 counts/

/min. giving a mean recovery of 94.2%. Less than 1% of the added radioactivity was recovered in the second and third elutions.

[4-^{14}C] Cortisol.

100 µl of the stock solution was found to contain 1630 counts/min. of ^{14}C.

From a series of four lanes, the recoveries of radioactivity were 1493, 1515, 1489 and 1491 counts/min. giving a mean recovery of 93.7%. Less than 0.5% of the added radioactivity was recovered in the second and third elutions.

B. Elution of Steroids from Thin-Layer Chromatograms.

A solution of [4-^{14}C] cortisol (1 mg./ml.) was prepared in ethanol. A series of 10 µl spots were placed on a thin-layer plate. The silica gel containing each spot was scraped off the plate and mixed vigorously with 5 ml. of ether by means of a rapidly rotating wire. Water (1 ml.) was then added and the tube shaken for 1 minute. After centrifugation, the upper layer was removed and the aqueous layer re-extracted with 5 ml. of ether. The ether extracts from each spot were combined

and evaporated to dryness at 50° under a stream of air.

The stock solution was found to contain 22,600 counts/min./10 µl.

In a series of nine spots, the recoveries of radioactivity were found to be 20,319; 20,179; 21,563; 20,964; 21,928; 20,521; 22,152; 21,597 and 22,020 counts/min. giving a mean recovery of 93.5%

When ether was used, the dried residue from "blank" silica gel extracts were found to give an optical density of 0.070 ± 0.005 with a 1 cm. light path when dissolved in 5 ml. of ethanol. Similar residues from benzene extracted silica gel gave optical densities of 0.023 ± 0.007 and this solvent was therefore used to extract steroids which were less polar than cortisol.

C. Recovery of DHA, Pregnenolone and 17αOH-Preg-nenolone.

a) Recovery of Pure Steroids from Alumina Columns.

Standard solutions of DHA, pregnenolone and 17αOH-pregnenolone were prepared in benzene. Percentage recoveries of steroid from alumina columns were then determined at the 10 µg and 50 µg levels using the elution procedure described on p. 26. Steroids were measured by

Oertel reaction.

DHA. 10 µg level:- 75.0, 78.6, 82.7, 92.0, 83.0, 82.1, 79.5, 83.0, 87.3, 83.9, 82.1, 79.5, 80.3, 84.8, 80.3, 80.3, 80.3

mean 82.8 ± 1.0% recovery.

50 µg level:- 92.3, 93.1, 94.1, 96.5, 103.9, 94.5, 91.6, 98.7, 81.5, 100.3, 92.6

mean 94.4 ± 1.8% recovery.

Pregnenolone

10 µg level:- 91.4, 92.6, 106.2, 95.1, 87.7, 81.5, 90.1, 87.6, 85.1, 91.1, 76.5

mean 91.4 ± 2.5% recovery.

50 µg level:- 93.3, 91.1, 94.0, 93.3, 92.6, 91.8, 100.0, 94.0, 97.0, 95.0, 98.0, 95.5.

mean 94.6 ± 0.8% recovery.

17αOH-Pregnenolone

10 µg level:- 98.9, 101.1, 105.3, 97.9, 91.6, 91.6, 84.4, 85.1, 90.5, 91.6, 92.6

mean 93.7 ± 2.4% recovery.

50 µg level:- 102.7, 100.8, 95.6, 102.9, 98.7,
86.4, 85.1, 97.3, 97.3, 97.3,
100.0, 94.2, 73.3
mean 94.7 ± 2.6% recovery.

b) Recovery of Steroids from Tissue Preparations.

(i) Steroid (100 µg) was dissolved in 100 µl propylene glycol and 450 µl horse tissue preparation (mitochondria free supernatant + buffer) together with 2.5N.NaOH (4 ml.) were then added.

(ii) Steroid extraction:- 3 x 5 ml. ethyl acetate/ /ether (1:1, v/v). Extract washing:- 1 x 1 ml. 10% HCl aq.; 2 x 1 ml. H_2O.

(iii) Defatting of extract residue:- 10 ml. 80-100° light petroleum/10 ml.; 75% methanol aq.; light petroleum re-extracted x 2 with 10 ml. portions of 75% methanol aq.

(iv) Aqueous methanol fraction chromatographed on column of 3 gm. alumina (see p. 26).

DHA.

Tissue Prep.	% Recov. of Steroid
1.	79.5, 79.5, 81.0, 84.3, 83.8, 85.2
	mean 82.2%
2.	82.1, 83.5, 74.1, 84.8, 74.4, 74.4
	mean 78.9%
3.	82.2, 90.4, 87.7, 91.5
	mean 88.0%

Pregnenolone.

1.	73.8, 74.9, 70.4, 73.4, 73.0, 73.8
	mean 73.2%
2.	76.9, 78.6, 78.6, 78.6
	mean 78.2%
3.	81.6, 84.2, 79.1, 76.1
	mean 80.3%

17αOH-Pregnenolone.

Tissue Prep.	% Recov. of Steroid
1.	65.9, 68.9, 71.1, 68.2
	mean 68.5%
2.	71.6, 71.6, 68.7, 61.2
	mean 68.3%
3.	76.9, 76.1, 69.4, 67.9
	mean 72.6%

D. Calibration Curves for Δ^4-3-oxosteroids and Δ^5-3β-hydroxysteroids.

a) Δ^4-3-oxosteroids.

Standard solutions of testosterone, cortisol, 11-deoxycortisol, 20β-hydroxypregn-4-en-3-one and Δ^4-androstenedione were prepared. Graphs of optical density at 240 mμ vs. concentration (mμmoles/5 ml. ethanol) were then plotted for each steroid (see fig. 25). It was found that all steroids examined had a molar extinction coefficient very close to 16,000 and this figure was used throughout the investigation.

b) Δ^5-3β-hydroxysteroids.

Standard solutions of DHA, pregnenolone and 17αOH-pregnenolone were prepared. The Oertel reaction was found to obey Beer's Law for concentrations of all three steroids up to 60 μg/5 ml. of reagent (see fig. 26). Standards were prepared, however, for every determination of Δ^5-3β-hydroxysteroids.

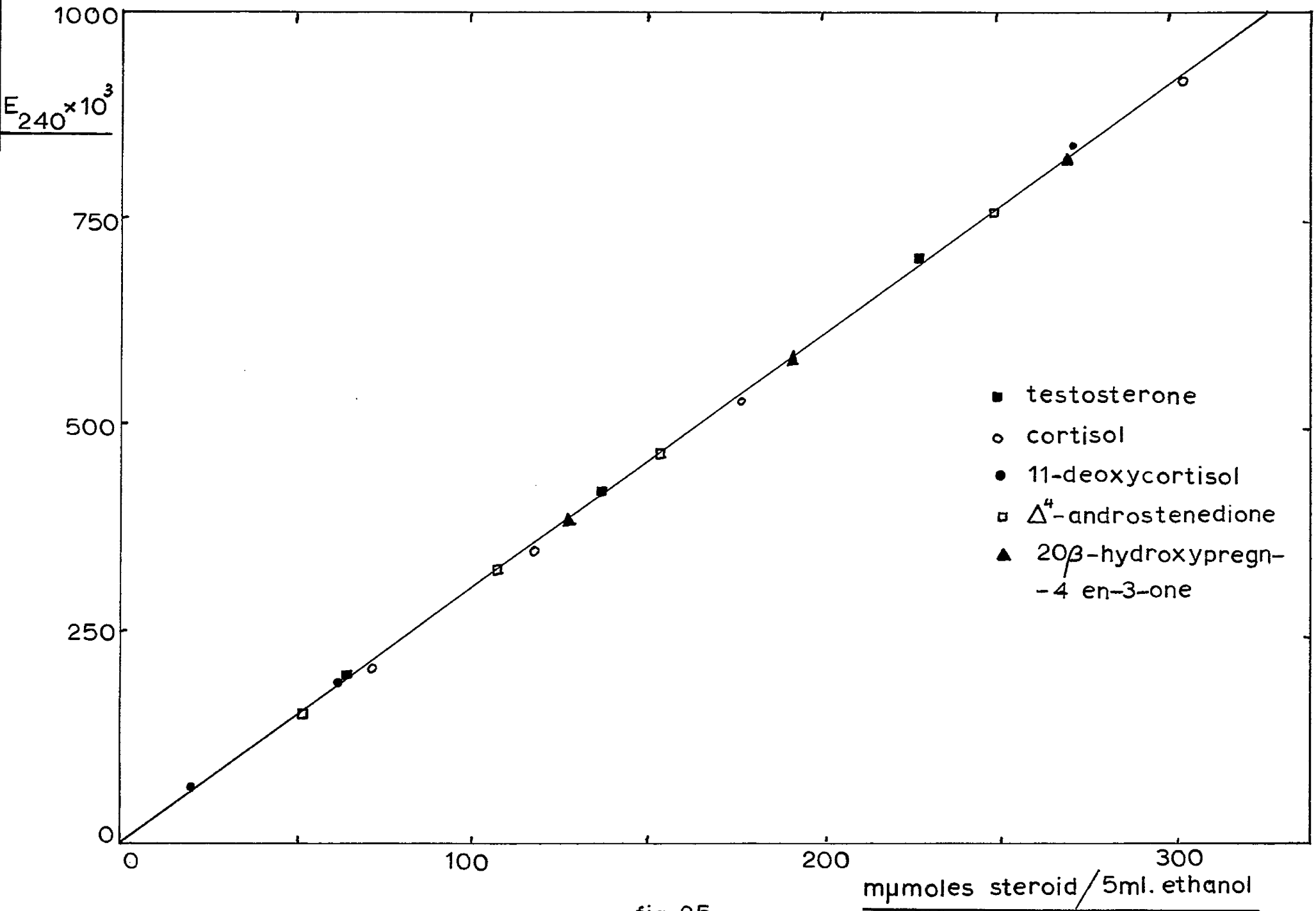
1000
750
500
250
0
$E_{240} \times 10^3$
0
100
200
300
mμmoles steroid/5ml. ethanol
testosterone
cortisol
11-deoxycortisol
Δ^4-androstenedione
20β-hydroxypregn-4 en-3-one

fig. 25

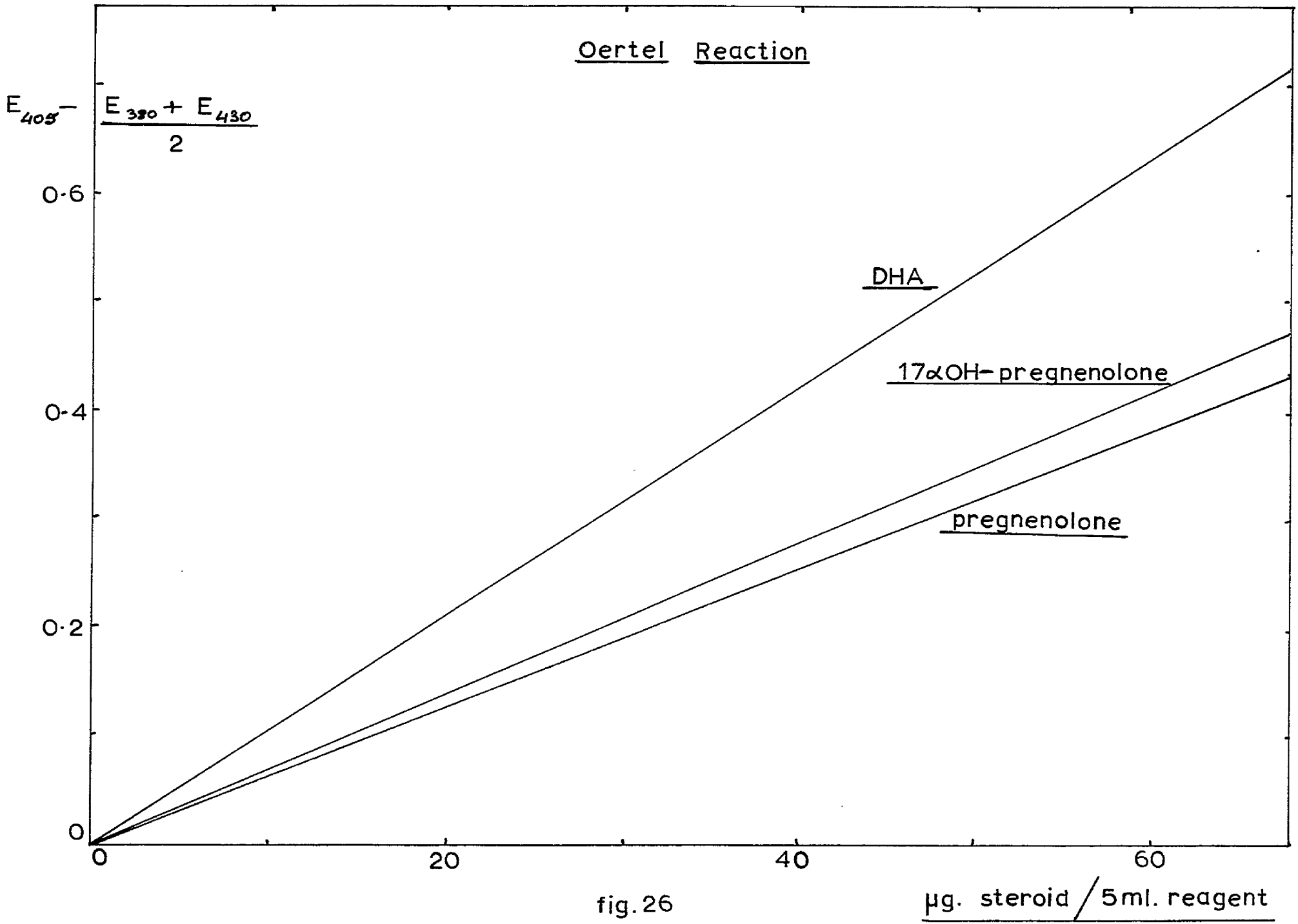
Oertel Reaction
$E_{405} - \frac{E_{380} + E_{430}}{2}$
0·6
0·4
0·2
0
0
20
40
60
DHA
17αOH-pregnenolone
pregnenolone
µg. steroid / 5ml. reagent

fig. 26

Appendix V.

Gas-Liquid Chromatographic Determination of Pregnenolone.

Instrument	:- Perkin-Elmer, Model 800 Gas Chromatograph.
Columns	:- 6 ft. long, made of stainless steel; internal diameter $\frac{1}{2}$".
Support	:- acid-washed, silanized Gas Chrom P, Mesh 100 - 120.
Stationary Phase	:- 1% neopentylglycol succinate (NGS).
Temp. of Column	:- 230°.
" " Injection Port	:- 280°.
" " Detector	:- 210°.

Pregnenolone and all other steroids used were chromatographed as the tri-methylsilyl ethers (TMSE). These were formed at room temperature by the action of hexamethyldisilazane and trichloromethylsilane in chloroform solution. Various urinary 17-oxosteroids were tested to find one suitable for use as an internal standard. Eventually 11-oxoaetiocholanolone (11-OA)-TMSE with a retention time of 14.2 minutes (retention time of pregnenolone-TMSE = 10.0 minutes)

under the conditions described above was chosen.

Peaks were measured in terms of relative peak area (RPA) where the

RPA = Peak Height (mm.) x Retention Time (mins.)

When the RPA was plotted against µg. of pregnenolone--TMSE or 11-OA-TMSE, straight lines were obtained in both cases (see fig. 27).

A series of samples containing various amounts of pregnenolone-TMSE together with a fixed amount (20 µg.) of 11-OA-TMSE were prepared. A graph was then plotted of µg. pregnenolone present in the original sample against

$$\frac{\text{RPA of Pregnenolone-TMSE}}{\text{RPA of 20 µg. 11-OA-TMSE}} \times 10^3$$

(see fig. 28).

i.e. after adding 20 µg. of 11-OA to a sample of pregnenolone and chromatographing the mixture as TMSE's, this graph could be used to determine the quantity of pregnenolone present in the original sample.

A series of samples containing [7α-^{3}H] pregnenolone (25 or 75 µg.) dissolved in propylene glycol, horse tissue preparation (mitochondria-free supernatant + buffer) and 2N.NaOH were prepared as described in Appendix IV (p.200). The aqueous methanol fraction was then obtained and this was then chromatographed on thin-layer

of silica gel together with standards Δ^4-androstene-dione and 17αOH-progesterone in solvent system III (p. 25). The standards were detected by UV light (p. 26), and the area of silica gel between them which contained the [7α-^{3}H] pregnenolone was eluted. The standard 11-OA (20 μg.) was then added to the dried residue and an aliquot of the mixture taken for counting. The steroids were then transformed to the TMSE's and subjected to gas chromatographic analysis as described above. The results below show that there is good agreement between recovery of ^{3}H and recovery of pregnenolone as measured by gas-liquid chromatography (GLC).

Sample	Preg. (μg.)	% recov. of ^{3}H	% recov. of preg. (GLC)
1.	25	61.9	63.2
2.	"	64.8	64.4
3.	"	60.9	60.4
4.	"	63.4	63.2
5.	"	56.7	56.7
6.	"	55.3	55.3
7.	"	52.4	52.1
8.	"	61.0	62.7

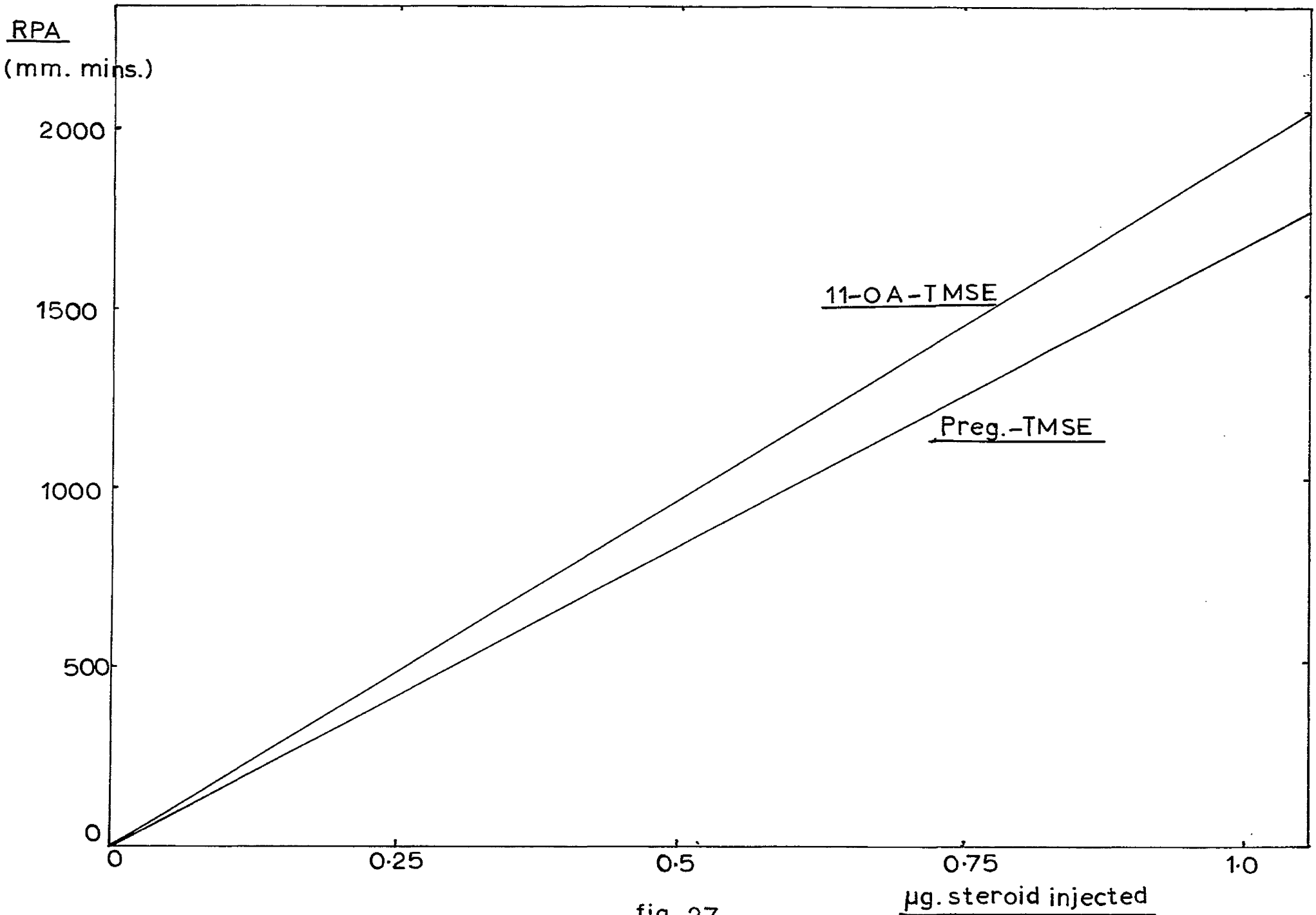
RPA
(mm. mins.)
2000
1500
1000
500
0
11-OA-TMSE
Preg.-TMSE
0
0·25
0·5
0·75
1·0
µg. steroid injected

fig. 27

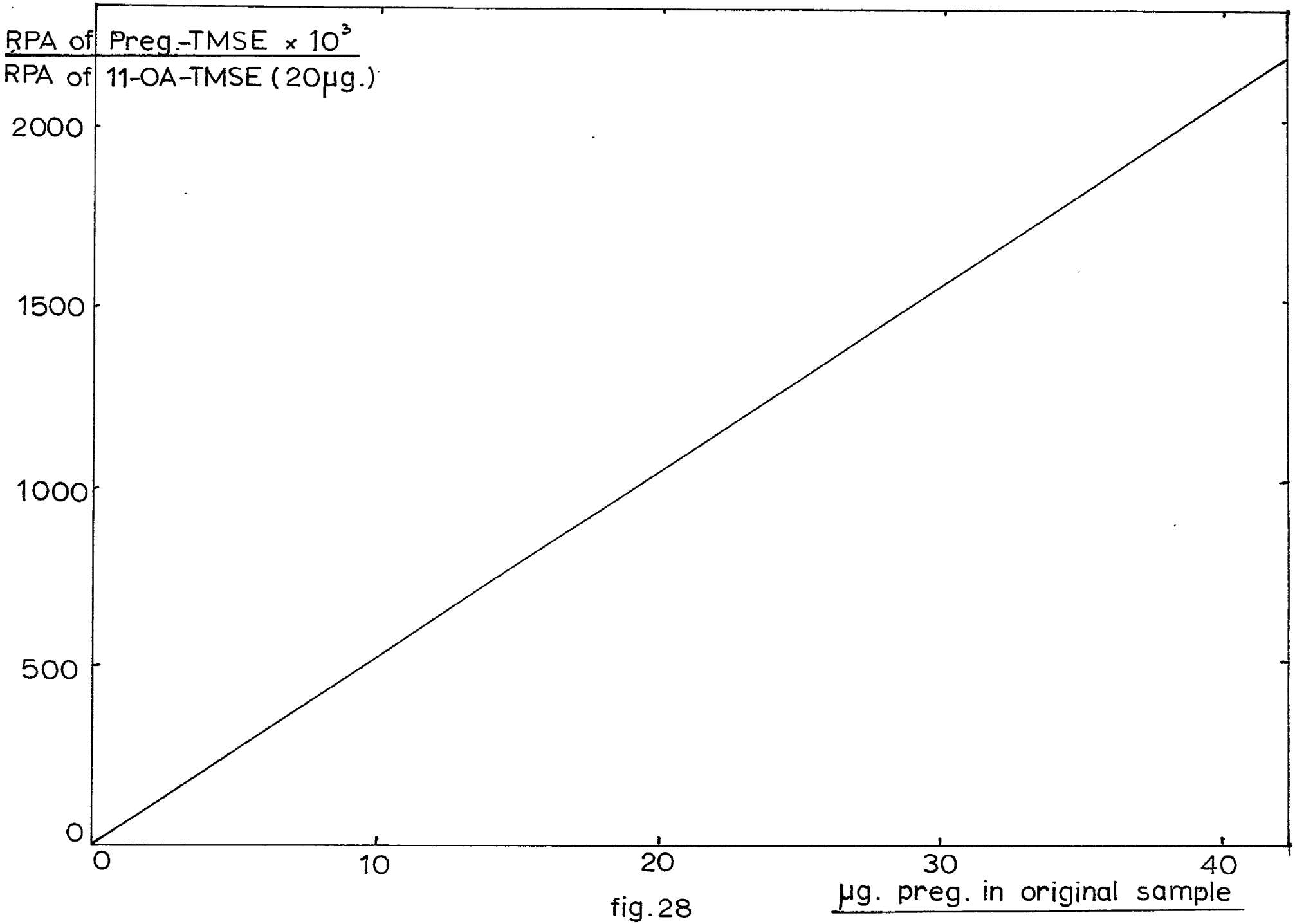
RPA of Preg.-TMSE × 10³
RPA of 11-OA-TMSE (20µg.)
2000
1500
1000
500
0
0
10
20
30
40
µg. preg. in original sample

fig.28

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