HISTOCHEMICAL ASPECTS OF STEROID METABOLISM.

BY

D. McK. Hart,

Department of Anatomy, The University, Glasgow.

ProQuest LLC.
789 East Eisenhower Parkway
P.O. Box 1346
Ann Arbor, MI 48106 – 1346

ACKNOWLEDGEMENTS.

I am deeply indebted to Professor G.M. Wyburn for research facilities and materials made available to me in the Department of Anatomy of the University of Glasgow and to Professor O. Eranko and Professor M. Niemi for research facilities and materials in the Department of Anatomy, the University of Helsinki, Finland.

A personal grant from the Medical Research Council is gratefully acknowledged.

The work performed in the University of Helsinki was supported in part by the Board of Management for Glasgow Northern Hospitals.

Organon Laboratories, Searle Laboratories and Boots Pure Drug Co. kindly donated a large range of steroid substrates.

I am grateful to Dr. A.H. Baillie for helpful discussion of many points in this work.

To Dr. W. Armstrong, Prof. I. Donald, Dr. McKay Hart, Mr. J. Hutchison, Mr. W.S. Mack and Sir Hector Maclennan of Glasgow and Prof. Goligher of Leeds I am grateful for the specimens of human tissues obtained from their units.

Miss J. Kerr kindly typed the manuscript.

HISTOCHEMICAL ASPECTS OF STEROID METABOLISM.

Contents

		Page
INTRODUCTION		1
	Section I.	
1.	HISTOCHEMICAL DEMONSTRATION OF 11β-HYDROXY-STEROID DEHYDROGENASE. A. H. Baillie, M.M. Ferguson, K.C. Calman and D. McK. Hart. J. Endocrin. (1965) 33: 119 - 125.	4
2.	HISTOCHEMICAL DEMONSTRATION OF 20β-HYDROXY-STEROID DEHYDROGENASE. A. H. Baillie, K.C. Calman, M. M. Ferguson and D. McK. Hart. J. Endocrin. (1965) 32: 337 - 339.	2
3.	HISTOCHEMICAL DEMONSTRATION OF 3α-, 6β-, 11α-, 12α-, 16α-, 16β-, 17α-, 21- AND 24-HYDROXY-STEROIDS. A. H. Baillie, K. C. Calman, M. M. Ferguson and D. McK. Hart. J. Endocrin (In Press).	39
4.	OBSERVATIONS ON THE HISTOCHEMICAL DISTRIBUTION OF ALCOHOL DEHYDROGENASE IN ENDOCRINE TISSUE. M. M. Ferguson, A. H. Baillie, K. C. Calman and D. McK. Hart. Nature (In Press).	78

Page

Section II

1. 3β-HYDROXYSTEROID DEHYDROGENASE IN THE ADRENAL GLAND AND PLACENTA. 87

A. H. Baillie, E. H. D. Cameron, K. Griffiths and D. McK. Hart. J. Endocrin. (1965) 31: 227 - 233.

2. 3β HYDROXYSTEROID DEHYDROGENASE DEVELOPMENT IN MOUSE ADRENAL AND GONADS. 113

D. McK. Hart, A. H. Baillie, K. C. Calman and M. M. Ferguson. J. Anat. (Lond) (In Press).

3. HYDROXYSTEROID DEHYDROGENASES IN THE HUMAN ADRENAL CORTEX. 145

K. C. Calman, A. H. Baillie, M. M. Ferguson and D. McK. Hart. J. Endocrin. (In Press).

4. HYDROXYSTEROID DEHYDROGENASE IN HUMAN FOETAL GONADS. 166

D. McK. Hart, K. C. Calman, M. M. Ferguson, M. Niemi and A. H. Baillie. Acta Endocrin. (In Press).

5. EVIDENCE OF STEROID METABOLISM AND POSSIBLE BIOSYNTHESIS IN THE HUMAN GENITAL RIDGE MESENCHYME. 179

A. H. Baillie, M. M. Ferguson and D. McK. Hart.

6. HISTOCHEMICAL DISTRIBUTION OF HYDROXYSTEROID DEHYDROGENASES IN KIDNEY AND LIVER. 191

A. H. Baillie, K. C. Calman, M. M. Ferguson and D. McK. Hart. Histochemie (In Press).

Page

7. ONTOGENETIC DISTRIBUTION OF HYDROXYSTEROID DEHYDROGENASE IN HUMAN PRO-, MESO- AND METANEPHRIC KIDNEYS: PHYLOGENETIC DISTRIBUTION OF HYDROXYSTEROID DEHYDROGENASES IN THE VERTEBRATE KIDNEY. 223

A. H. Baillie, M. M. Ferguson and D. McK. Hart.

— — — —

Section III.

1. HYDROXYSTEROID DEHYDROGENASE ACTIVITY IN NORMAL HUMAN PLACENTA FROM SIX WEEKS TO FORTY-TWO WEEKS OF GESTATION. 241

D. McK. Hart. J. Endocrin. (In Press).

2. THE HISTOCHEMICAL DISTRIBUTION OF HYDROXY-STEROID DEHYDROGENASES IN THE HUMAN FOETAL MEMBRANES AT TERM. 270

D. McK. Hart. Histochemie (In Press).

3. HISTOCHEMICAL DEMONSTRATION OF HYDROXY-STEROID DEHYDROGENASES IN HYDATIDIFORM MOLES. 288

D. McK. Hart. Obstet. Gynec. (In Press).

4. HISTOCHEMICAL INVESTIGATION OF THE LOCALIS-ATION OF PLACENTAL 17β-HYDROXYSTEROID DEHYDRO-GENASES. 304

D. McK. Hart.

5. EVIDENCE OF STEROID METABOLISM IN A HUMAN YOLK-SAC. 321

D. McK. Hart.

— — — —

INTRODUCTION.

In 1958 Wattenberg described a histochemical method for demonstration of 3β-hydroxysteroid dehydrogenase an important enzyme in steroid hormone biosynthesis, in tissue slices, using a tetrazolium dye and provided a means of cellular localisation of this enzyme. Methods for 3α- and 17β-hydroxysteroid dehydrogenases were described by Pearson and Grose in 1959 and for 20α-hydroxysteroid dehydrogenase by Balogh in 1964.

Other hydroxysteroid dehydrogenases have been described biochemically and little is known of the metabolism of the corresponding hydroxysteroids. It was therefore decided to undertake in the Department of Anatomy at Glasgow University a search for other hydroxysteroid dehydrogenases using histochemical techniques and to explore the substrate specificity and co-factor requirements of these enzymes.

Section I of this thesis deals with the description of techniques for ten hydroxysteroid dehydrogenases not previously described histochemically. Alcohol dehydrogenase, a source of control difficulties experienced/

experienced in localising hydroxysteroid dehydrogenases histochemically in endocrine tissues has been characterised histochemically, and is described in this section.

Section II deals with the localisation, and in some cases the age distribution both foetal and post-natal of the hydroxysteroid dehydrogenases described in Section I. Additional information about the histochemical distribution of 3β- and 17β-hydroxysteroid dehydrogenases is adduced.

Evidence of steroid synthesis and metabolism in the human and murine genital ridge is presented and some suggestions as to the possible mechanism of excretion of steroids in the kidneys is discussed.

Section III consists of observations on hydroxysteroid dehydrogenase activity in normal and abnormal (hydatidiform degeneration) human placenta and in the human term foetal membranes.

The previous literature on 3β- and 17β-hydroxy-steroid dehydrogenase activity is reviewed and knowledge of these enzymes is advanced by an extensive age series of normal human placentae; the cellular and age distribution of the hydroxysteroid dehydrogenases described in Section I are detailed and the biochemical significance of hydroxysteroid dehydrogenase activity in

placenta/

placenta is discussed.

Conflicting views on 17β-hydroxysteroid dehydrogenase in placenta are reviewed and some of the existing discrepancies are explained; moreover evidence for more than one placental 17β-hydroxysteroid dehydrogenase is adduced.

The final paper shows evidence of steroid metabolism in the yolk-sac of a 14 mm. human embryo; hydroxysteroid dehydrogenase activity has not previously been described in the human yolk sac.

Histochemical Demonstration of 11β-Hydroxysteroid Dehydrogenase.

BY

A. H. Baillie,
M. M. Ferguson
K. C. Calman
D. McK. Hart

Department of Anatomy, University of Glasgow, Glasgow.

SUMMARY.

11β-Hydroxysteroid dehydrogenase can be demonstrated histochemically by incubating tissues with nitro blue tetrazolium (2,2'-di-p-nitrophenyl-5,5'-diphenyl-3,3'-(3,3'-dimethoxy-4,4'diphenylene) ditetrazolium chloride), NAD or NADP and an appropriate steroid. Suitable steroid substrates are:- (1) 11β-hydroxyandrost-4-ene-3, 17-dione (11β-hydroxyandrostenedione), (2) 3,11β-dihydroxyoestra-1,3,5(10)-trien-17-one (11β-hydroxyoestrone), (3) 3α, 11β-dihydroxy-5α-androstan-17-one, (4) 3α,11β-dihydroxy-5β-androstan-17-one and (5) 11β-hydroxypregn-4-ene-3, 20-dione (11β-hydroxyprogesterone).

11β-Hydroxysteroid dehydrogenase activity was found in the Leydig cells of six human testes from subjects ranging in age from 12 to 57 yr. with all five substrates.

The Leydig cells of the mouse testis contain demonstrable 11β-hydroxysteroid dehydrogenase activity and the volume of reactive tissue increases regularly between birth and the end of the 10th week of postnatal life; this growth curve is sigmoid in form. An extremely weak histochemical reaction/

with human placenta obtained at term was observed, 11β-hydroxyandrostenedione being the only substrate utilized to any extent. A specimen of hydatid mole, however, showed intense 11β-hydroxysteroid dehydrogenase activity with all substrates surveyed. 11β-Hydroxysteroid dehydrogenase was also found in the ova, granulosa, theca interna, interstitial tissue and corpora lutea of the mouse ovary.

INTRODUCTION.

In recent years Nitro-BT has been used for the histochemical demonstration of steroid dehydrogenases and methods are now available for the localization of 3β-, 17β-, 20α-, and 20β-hydroxysteroid dehydrogenases (Wattenberg, 1958; Pearson & Grose, 1959; Balogh, 1964; Baillie, Calman, Ferguson & Hart, 1965). This paper deals with the histochemical localization of 11β-hydroxysteroid dehydrogenase and with its substrate specificity.

MATERIAL AND METHODS.

Testicular biopsies were performed on six healthy human males aged 12, 13, 28, 29, 30 and 57 yr. In addition, testes were obtained, with 1 min. of/

of death by decapitation, from 44 Swiss white mice, killed in groups of four at weekly intervals between birth and the end of the 10th week of postnatal life. Pieces of tissue of about 1 cm.^{3} were cut from a human placenta at Caesarean section performed at term for obstetric reasons not related to placental function and from one vaginally delivered hydatid mole in the 6th month of gestation. Mouse ovaries were also studied. Each tissue specimen was frozen in solid carbon dioxide within 3 min. after interruption of its blood supply and sectioned at 12 μ on a cryostat maintained at -20°. The sections were attached to clean dry glass slides by momentary thawing. They were incubated for 1 hour at 37° in phosphate buffer (0.05 M) containing 1% (w/v) magnesium chloride and the following reagents ; NAD or NADP (final conc. 0.0 - 0.4 mM), nicotinamide (final conc. 1.0 mM), nitro blue tetrazolium (2,2'-di-p-nitrophenyl-5,5'-diphenyl-3,3'-(3,3'-dimethoxy-4,4'-diphenylene) ditetrazolium chloride) (final conc. 0.5 mg./ml.) (Sigma) together with a steroid substrate dissolved in propylene glycol/

glycol (final conc. 0.0 - 1.0 mM). The steroids studied were:-

(1) Androst-4-ene-3, 17-dione (androstenedione) (Steraloids),

(2) 11β-Hydroxyandrost-4-ene-3, 17-dione (11β-hydroxyandrostenedione) (Medical Research Council),

(3) 3-Hydroxyoestra-1,3,5(10)-trien-17-one (oestrone) (Steraloids),

(4) 3,11β-Dihydroxyoestra-1,3,5(10)-trien-17-one (11β-hydroxyoestrone) (Dr. K. Griffiths),

(5) 3α,11β-Dihydroxy-5α-androstan-17-one (Medical Research Council),

(6) 3α,11β-Dihydroxy-5β-androstan-17-one (Steraloids)

(7) 11β-Hydroxypregn-4-ene-3, 20-dione (11β-hydroxyprogesterone) (Steraloids),

(8) 11α-Hydroxypregn-4-ene-3, 20-dione (11α-hydroxyprogesterone) (Steraloids),

(9) Progesterone (Steraloids).

In addition the effects of varying pH from 5.5 to 8.0 were observed. To compare results for individual substrates a quantitative analysis of one human testis was made using the point method of Glagolev/

Glagolev (1934) and Chayes (1949). Sections at a magnification of 90 diameters were scanned with a grid with intersections 1 cm. apart on two axes at right angles to one another. The volume of Leydig tissue exhibiting 11β-hydroxysteroid dehydrogenase activity when incubated with a given substrate was expressed as a percentage. In the same manner the volume of reactive Leydig tissue in the age series of mouse testes after incubation with 11β-hydroxyprogesterone was estimated as a percentage. The testicular volume at different ages is already known in this strain (Baillie, 1961) and from the percentage and the testicular volume the total volume of reactive Leydig tissue was calculated.

RESULTS.

Testis

All the human testes studied presented a closely similar picture when incubated with the various steroids. With androstenedione no colour was seen in any part of the testis. After incubation with 11β-hydroxyandrostenedione blue diformazan particles (Plate,/

(Plate, fig. 1) and pink monoformazan deposits were seen in many of the Leydig cells. No colour was seen in the seminiferous tubules. After incubation with oestrone traces of monoformazan were seen in one or two Leydig cells, but the majority were not reactive. With 11β-hydroxyoestrone mono- and diformazan deposits were seen in many Leydig cells but the testis was less reactive with this substrate than with 11β-hydroxyandrostenedione. Incubation with 3α, 11β-dihydroxy-5α-androstan-17-one produced mono- and diformazan deposition in some Leydig cells but this substrate was not as well used as 3α,11β-dihydroxy-5β-androstan-17-one. With this steroid pink monoformazan and blue diformazan deposits were seen in most of the Leydig cells and its reactivity approached that of 11 - hydroxyandrostenedione. The human testes failed to react with progesterone and 11α-hydroxyprogesterone but most of the Leydig cells reacted strongly with 11β-hydroxyprogesterone, blue diformazan predominantly being deposited.

The adult mouse testes showed a similar picture with each substrate but the dehydrogenase activity was/

was less than that observed in the human material. Little difference was noted between 3α,11β-dihydroxy-5α-androstan-17-one and 3α,11β-dihydroxy-5β-androstan-17-one. Using 11β-hydroxyprogesterone many of the Leydig cells in the neonatal mouse testis were reactive and contained mono- and diformazan. With advancing age the picture remained little changed (Plate, fig. 2).

The quantitative results obtained with different substrates in the human testis are summarized in Table 1, and show that 11β-hydroxyandrostenedione and 11β-hydroxyprogesterone are the best utilized of the substrates surveyed. The quantitative results of the studies on mouse testes (Table 2) show that the volume of Leydig tissue with 11β-hydroxysteroid dehydrogenase activity increases regularly with age; the growth curve is sigmoid in form (Text-fig. 1).

Placenta

In the placenta at term no colour was obtained after incubation with any of the steroid substrates surveyed except 11β-hydroxyandrostenedione. With this/

TABLE I.

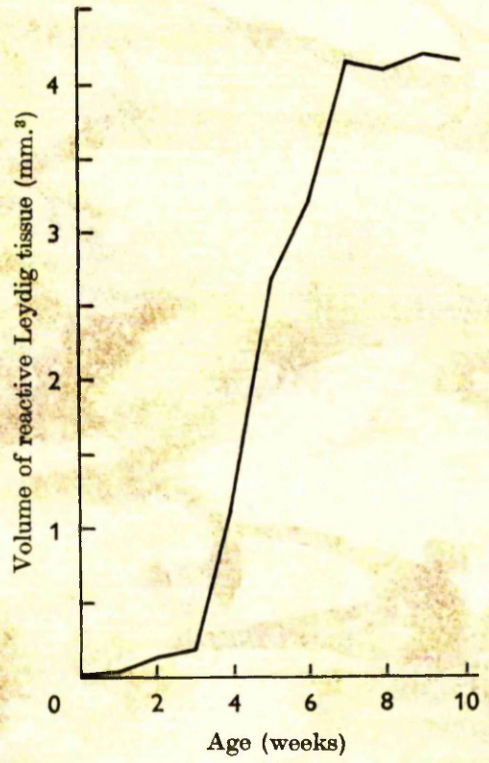
11β-Hydroxysteroid Dehydrogenase Activity of Human Leydig Tissue Incubated with Various Substrates.

Reactive Leydig Tissue (%)	Substrate
<0.1	Androstenedione
4.5	11β-Hydroxyandrostenedione
<0.1	Oestrone
1.8	11β-Hydroxyoestrone
2.5	3α, 11β-Dihydroxy-5α-androstan-17-one
4.0	3α, 11β-Dihydroxy-5β-androstan-17-one
<0.1	Progesterone
<0.1	11α-Hydroxyprogesterone
4.4	11β-Hydroxyprogesterone

TABLE 2.

11β-Hydroxysteroid Dehydrogenase Activity of Murine Leydig Tissue Incubated with 11β-Hydroxyprogesterone.

Age in Weeks	Testicular Volume ($mm.^3$)	Leydig Tissue Reacting with 11β-Hydroxyprogesterone as % Testis	Volume of Leydig Tissue Reacting with 11β-Hydroxyprogesterone ($mm.^3$)
0	0.8	5.0	0.04
1	6.1	1.0	0.06
2	18.3	1.3	0.2
3	31.7	1.2	0.3
4	88.9	1.5	1.3
5	169.2	1.7	2.7
6	195.4	1.7	3.4
7	196.1	2.2	4.3
8	196.1	2.1	4.2
9	196.0	2.2	4.3
10	196.1	2.2	4.3



Text-fig. 1. The volume of mouse Leydig tissue capable of metabolizing 11β-hydroxyprogesterone increases steadily with age and the growth curve is sigmoid in form.

this substrate very weak activity was seen in the cells of the syntrophoblast consisting almost entirely of faint pink monoformazan deposition.

With the hydatid mole a quite different picture was obtained. No colour developed after incubation with androstenedione, oestrone, progesterone or 11α-hydroxyprogesterone. Incubation with 11β-hydroxyandrostenedione, 11β-hydroxyoestrone and 11β-hydroxyprogesterone resulted in intense mono- and diformazan deposition in the chorionic epithelium (Plate, fig. 3). A similar result was obtained with 3α, 11β-dihydroxy-5β-androstan-17-one but 3α,11β-dihydroxy-5α-androstan-17-one was extremely poorly utilized, only traces of monoformazan being visible in the chorionic epithelium.

Mouse Ovary.

The ovarian sections incubated in the control media without steroids showed no formazan deposition. When the mouse ovary was incubated with androstenedione, no formazan was seen in the sections. However, with 11β-hydroxyandrostenedione as substrate both mono- and diformazan were observed in/

in the corpora lutea, interstitial tissue, theca interna and the granulosa of atretic and post-ovulatory follicles. Oestrone and 11β-hydroxyoestrone did not appear to be utilized by any of the ovarian tissues. Using 3α,11β-dihydroxy-5α-androstan-17-one, mono- and diformazan were deposited in the corpora lutea, interstitial tissue, theca interna and the granulosa of atretic and post-ovulatory follicles. Furthermore, activity was also apparent in the ova (Plate, fig. 4). A similar picture was obtained with 3α,11β-dihydroxy-5β-androstan-17-one. Progesterone produced no formazan deposition in the ovarian sections but with 11β-hydroxyprogesterone as substrate mono- and diformazan were deposited in the corpora lutea, interstitial tissue, theca interna and the granulosa of atretic post-ovulatory follicles. The mouse ovary was not capable of utilizing the 11α-hydroxyprogesterone as a substrate.

Experimental variables.

NAD and NADP gave equally good histochemical results with all tissues and no benefit was obtained from increasing the concentration of either above 1 mM/

1 mM; no reaction occurred if both were omitted. No improvement was seen after the substrate concentration rose above 0.5 mM and no colour developed if sections were incubated in medium containing no steroid. Nicotinamide is not an essential constituent of the reaction medium. The pH optimum lay between 7 and 7.2.

DISCUSSION.

11β-Hydroxysteroid dehydrogenase carried out the important cortisol-cortisone dehydrogenation but this reaction can unfortunately not be utilized histochemically because of the presence of two additional hydroxyl groups. However, the substrates used in this investigation give an adequate picture of the histochemical localization and activity of the enzyme.

Dehydrogenation of 11β-hydroxysteroids occurs much more readily in human than in rodent tissues and these results agree well with the corresponding enzymic reaction utilizing cortisol in human and rodent tissues (Koerner & Hellman, 1964). This may/

may be due to the fact that rodent tissues reduce the A ring at a faster rate (McGuire & Tomkins, 1960) with subsequent conjugation and elimination of cortisol and corticosterone and that 11β-hydroxy-steroid dehydrogenase activity is decreased to maintain a higher concentration of the physiologically active 11β-hydroxy derivative.

A number of biochemical studies now indicate that dehydrogenation of the 11β-hydroxyl function depends on the presence of a specific stereochemical 5αH configuration of the A ring, or the presence of a Δ^4-3 ketone grouping in both human and rodent tissues (Hubener, Fukushima & Gallagher, 1956; Bush & Mahesh, 1959; Mahesh, 1960; Koerner & Hellman, 1964). In human tissues, however, it has been demonstrated that steroids with the 5βH configuration may also be oxidized at the 11β-position (Meigs & Engel, 1961). Histochemically this problem is difficult to resolve because of the difficulty in obtaining suitable steroids. However, our results with human testis indicate that 3α,11β-dihydroxy-5β-androstan-17-one is used by testis more readily/

readily than the corresponding 5αH compound. The quantitative results indicate that the activity was due not solely to the 3α-hydroxyl group (Baillie, unpublished observations). Human placenta, however, in agreement with the biochemical results, reacted only with the 11β-hydroxy Δ^4-3 keto substrate, 11β-hydroxyandrostenedione. This finding is in surprising contrast to the results with a hydatid mole. With the latter tissue, as with human testis, the 5βH compound was better utilized than the 5αH. No obvious differences were found between these two substrates with mouse testis and ovary, and this may indicate a species difference in the enzyme specificity, but the present histochemical technique is not sufficient to resolve this problem.

The pH optimum of the reaction in this histochemical method was 7.0 - 7.2. This contrasts with biochemical studies in which the optimum was pH 9 - 10 (Meigs & Engel, 1961; Koerner & Hellman, 1964). The reverse reaction, that of reduction of the ketone group, takes place at pH 7.1 (Meigs & Engel, 1961) which may partly explain the difference in activity of this enzyme as compared with the 3β-hydroxysteroid dehydrogenase/

dehydrogenase which has been shown in this laboratory to be much more active. Histochemically, however, incubation at pH values greater than pH 8 are thought to result in complications because of the so-called "nothing dehydrogenase" (Pearse, 1960).

The 11β-hydroxysteroid dehydrogenase has been shown to utilize both NAD and NADP in biochemical systems (Hurlock & Talalay, 1959) and this has been confirmed in the present histochemical investigation.

The progressive increase in the volume of Leydig tissue in the growing mouse testis which reacts with 11β-hydroxysteroids resembles the growth curves described elsewhere for tissue with 3β-hydroxysteroid dehydrogenase activity.

ACKNOWLEDGEMENTS.

We are indebted to Professor Klyne, Westfield College, for samples of 11β-hydroxy-androstenedione and 3α, 11β-dihydroxy-5α-androstan-17-one and to Dr. K. Griffiths for samples of 11β-hydroxyoestrone. One of us (D. McK. H.) is indebted to the M.R.C. for a personal grant.

REFERENCES.

BAILLIE, A.H. (1961). Observations on the growth and histochemistry of the Leydig tissue in the postnatal prepubertal mouse testis. J. Anat. Lond., 95, 357 - 370.

BAILLIE, A.H., CAIMAN, K.C., FERGUSON, M.M. & HART, D. McK. (1965). Histochemical demonstration of 20β-hydroxysteroid dehydrogenase. J. Endocrin. 32, 337 - 339.

BALOGH, K. (1964). A histochemical method for the demonstration of 20α-hydroxysteroid dehydrogenase activity in rat ovaries. J. Histochem. Cytochem. 12, 670 - 673.

BUSH, I.E. & MAHESH, V.B. (1959). Metabolism of 11-oxygenated steroids. (1) Influence of the A/B ring junction on the reduction of the 11-oxogroup. Biochem. J. 71, 705 - 718.

CHAYES, F. (1949). The theory of thin section analysis. J. Geol. 62, 92 - 101.

GLAGOLEV, A.A. (1934). Quantitative analysis by the point method. Eng. & Mining J. 135, 399 - 400.

HUBENER, H.J., FUKUSHIMA, D.K. & GALLAGHER, T.F. (1956). Substrate specificity of enzymes reducing the 11- and 20-keto groups of steroids. J. biol. Chem. 220, 499 - 511.

HURLOCK, B. & TALALAY, P. (1959). Microsomal 3α- and 11β-hydroxysteroid dehydrogenases. Arch. Biochem. Biophys. 80, 468 - 469.

KOERNER, D.R. & HELLMAN, L. (1964). Effect of thyroxine administration on the 11β-hydroxy-steroid dehydrogenases in rat liver and kidney. Endocrinology. 75, 592 - 601.

McGUIRE, J.S. & TOMKINS, G.M. (1960). The heterogeneity of Δ^4-3 keto steroid reductase. J. biol. Chem. 235, 1634 - 1638.

MAHESH, V.R. (1960). In vitro metabolism of steroids in the rat kidney. Fed. Proc. 20, 174.

MEIGS, R.A. & ENGEL, L.L. (1961). The metabolism of adrenocortical steroids by human tissues. Endocrinology, 69, 152 - 162.

PEARSE, A.G.E. (1960). Histochemistry, theoretical and applied. London: Churchill.

PEARSON, B. & GROSE, F. (1959). Histochemical demonstration of 17β-hydroxysteroid dehydrogenase by use of tetrazolium salt. Proc. Soc. exp. Biol., N.Y. 100, 636 - 638.

WATTENBERG, L.W. (1958). Microscopic histochemical demonstration of steroid-3β-ol-dehydrogenase in tissue sections. J. Histochem. Cytochem. 6, 225 - 232.

DESCRIPTION OF PLATE.

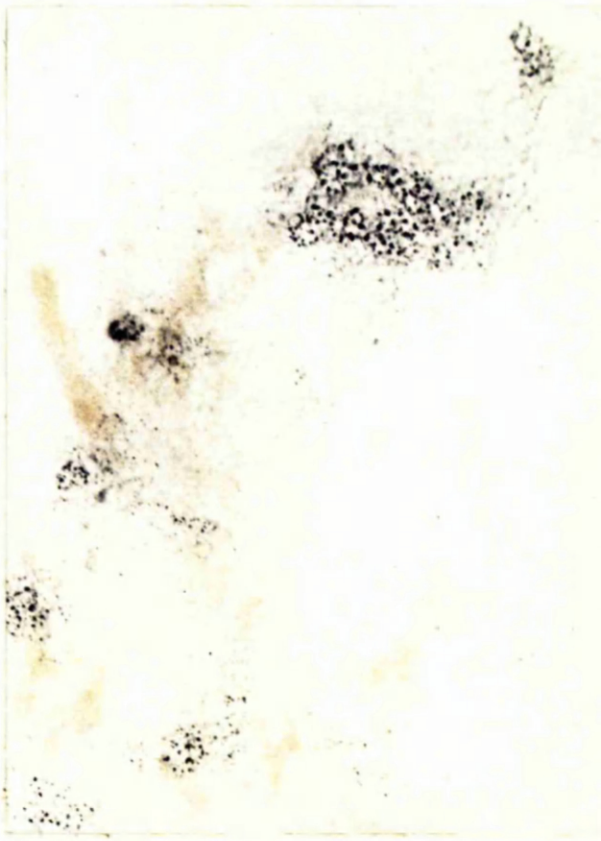
Fig. I. Adult human testis after incubation with 11β-hydroxyandrostenedione. Intense 11β-hydroxysteroid dehydrogenase activity is seen in the Leydig cells. (x 90).

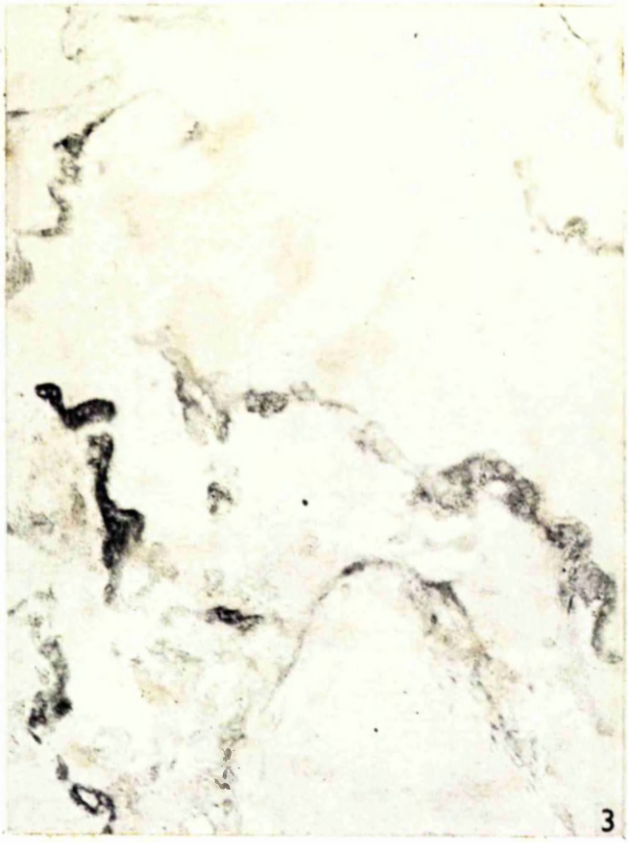
Fig. 2. Four-week-old mouse testis after incubation with 11β-hydroxyprogesterone. 11β-Hydroxysteroid dehydrogenase activity is seen in the interstitium. (x 200.)

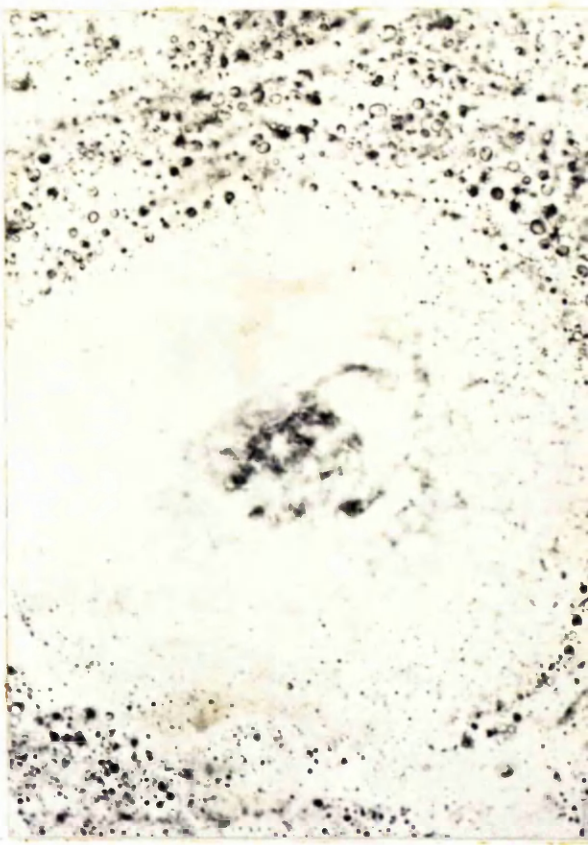
Fig. 3. Hydatid mole incubated with 3α,11β-dihydroxy-5β-androstan-17-one. Intense 11β-hydroxysteroid dehydrogenase activity is seen in the trophoblast. (x 90).

Fig. 4. Mouse ovum; intense 11β-hydroxysteroid dehydrogenase activity can be seen in the cytoplasm of the ovum and in the theca interna. A trace of activity is present in the granulosa. (x 300).

PLATE







Histochemical Demonstration of 20β-Hydroxysteroid Dehydrogenase.

BY

A. H. Baillie,
K. C. Calman,
H. M. Ferguson,
D. McK. Hart.

Department of Anatomy, University of Glasgow, Glasgow, W.2, Scotland.

SUMMARY.

NAD-dependent 20β-hydroxysteroid dehydrogenase activity can be demonstrated histochemically using Nitro-BT. 20β-Hydroxysteroid dehydrogenase activity was found in the Leydig cells of human and mouse testis, in the zona fasciculata of the mouse adrenal and in the theca interna of the mouse ovary.

INTRODUCTION.

The biosynthetic pathways of the steroid hormones have become increasingly well understood in the last few years and techniques employing the tetrazolium salt Nitro-BT have been developed for the histochemical demonstration of 3β-, 17β- and 20α-hydroxysteroid dehydrogenase (Wattenberg, 1958; Pearson & Grose, 1959; Balogh, 1964). This paper describes the histochemical localization of another such enzyme, 20β-hydroxysteroid dehydrogenase.

MATERIAL AND METHODS.

Specimens of testis, adrenal, ovary, lung, heart, liver, kidney and gut were obtained from adult Swiss white mice within 1 minute of death by decapitation. In addition, testicular biopsies were performed on two healthy boys aged 12 and 13 years. The tissue samples were frozen in solid carbon dioxide and sectioned at 15 μ. on a cryostat maintained at -20°. The sections were attached to clean dry glass slides by momentary thawing.

They were incubated for 1 hour at 37° in phosphate buffer (0.05 **M**) containing 1% (w/v) magnesium sulphate and the following reagents: NAD or NADP, final conc. 0.0 - 4.0 mM; 20β-hydroxy-pregn-4-en-3-one (Steraloids), final conc. in propylene glycol 0.0 - 1.0 mM; nicotinamide, final conc. 1.0 mM and Nitro-BT (Sigma: 2,2'-di-p-nitrophenyl-5,5'-diphenyl-3,3'-dimethoxy-4,4'-diphenylene ditetrazolium chloride).

In addition, the effects of varying the pH from 5.5 to 8.0 were observed.

RESULTS.

In the mouse testes and in both the immature human testes studied a positive reaction was noted after incubation with 20β-hydroxypregn-4-en-3-one and NAD. In the rodent testes the reaction was weak and pink monoformazan was seen in many Leydig cells. Occasionally interstitial cells contained blue diformazan. No colouring was seen in the seminiferous tubules. The human Leydig cells reacted much more intensely than the rodent Leydig cells (Plate, fig. I) and many cells contained abundant diformazan deposits. Again no staining of the tubules was seen.

In the adrenal glands moderate 20β-hydroxy-steroid dehydrogenase activity in the presence of NAD was seen in the outer half of the zona fasciculata. Many of the clear cells contained lipid droplets coloured pink with monoformazan (Plate, I, fig. 2) but some diformazan crystals were also seen. No reaction products could be detected in the zona glomerulosa or zona reticularis.

In/

In the ovary an extremely weak reaction was noted, mainly in the cells of the theca interna. The other tissues surveyed did not apparently react, but since no satisfactory controls for lung, liver and kidney could be obtained it is not possible to make a definite statement about these organs.

No histochemical reaction was observed in testes or ovaries incubated with 20β-hydroxy-pregn-4-en-3-one and N.A.D.P. The optimum concentration of N.A.D. lay between 0.5 and 2 mM; the reaction did not proceed in the absence of N.A.D. No benefit was derived from increasing the substrate concentration above 0.5 mM and sections of testis, ovary and adrenal incubated in the medium containing no steroid remain colourless. The reaction developed satisfactorily in a medium lacking nicotinamide. The pH optimum lay between 7 and 7.4; a weak histochemical reaction was obtained at pH 6, but not at pH 5.5. The reaction deteriorated as the pH rose above 7.4.

DISCUSSION.

While 20β-hydroxypregn-4-en-3-one has been isolated from bovine corpora lutea only the 20α-epimer appears to occur in rodent tissues (Engel & Langer, 1961) and this may explain the very poor histochemical reaction elicited by the 20β-epimer in rodent testis and ovary. In contrast, however, Balogh (1964) has shown that the 20α-epimer gives an intense histochemical reaction in the rat ovary. The occurrence of both isomers in human adrenal and ovarian tissue has been established (Zander, Forbes, von Munstermann & Neher, 1958) and both can be formed by incubation of human ovarian homogenates with progesterone (Sweat, Berliner, Bryson, Nabors, Haskell & Holmstrom, 1960). The strong histochemical reaction obtained with the 20β-epimer in human but not mouse testicular tissue points to a species difference and it is interesting to note that the presence of 20β-hydroxysteroid dehydrogenase has been established biochemically in foetal human testes (Acevedo, Axelrod, Ishikawa & Takaki, 1963).

The/

The organ and species differences between our findings with the 20β-epimer and those of Balogh (1964) with the 20α-epimer underline the substrate specificity of the 20α- and 20β-hydroxysteroid dehydrogenases. Further, the specificity of the two dehydrogenases for the reduced pyridine nucleotides is interesting since some steroid dehydrogenases, such as the 11β-hydroxysteroid dehydrogenase, can use both (Hurlock & Talalay, 1959; Osinski, 1960; Baillie, Calman, Ferguson & Hart, 1965). NAD diaphorase and NADP diaphorase are both histochemically demonstrable in the tissues exhibiting 20β-hydroxysteroid dehydrogenase activity (unpublished observations) and it therefore seems likely that the specificity for NAD resides with the steroid dehydrogenase and is not secondary to a lack of NADP diaphorase.

20β-Hydroxysteroid dehydrogenase was first isolated and crystallized from bacterial sources (Hubener, Sahrholtz, Schmidt-Thome, Nesemann & Junk, 1959; Hubener & Sahrholtz, 1960) and is known to be a/

a NAD-linked enzyme. Its pH optimum in this histochemical reaction seems to lie between 7.0 and 7.4.

The difficulty in controlling lung and liver sections has already been noted by Wattenberg (1958) in connexion with another steroid dehydrogenase and work in hand in this laboratory suggests that the falsely positive reactions are due to the presence in certain tissues of substrate-specific alcohol dehydrogenases. Different steroid vehicles have not so far proved satisfactory but suitable inhibitors hold more promise.

ACKNOWLEDGEMENT.

We are indebted to the Anatomy Department of the University of Glasgow for research facilities, to Mr. W. S. Mack (Western Infirmary) for human testicular material and Mr. J. McGadey for technical assistance.

REFERENCES.

ACEVEDO, H.F., AXELROD, L.R., ISHIKAWA, E. & TAKAKI, F. (1963). Studies in fetal metabolism. II. Metabolism of progesterone-4-C^{14} and pregnenolone-7α-H^{3} in human fetal testes. J. clin. Endocr. 23: 885 - 890.

BAILLIE, A.H., CALMAN, K.C., FERGUSON, M.M. & HART, D.McK. (1965). Histochemical demonstration of 11β-hydroxysteroid dehydrogenase. J. Endocrin. 33: 119 - 125.

BALOGH, K. (1964). A histochemical method for the demonstration of 20α-hydroxysteroid dehydrogenase activity in rat ovaries. J. Histochem. Cytochem. 12: 670 - 673.

ENGEL, L.L. & LANGER, L.J. (1961). Biochemistry of steroid hormones. Ann. Rev. Biochem. 30: 499 - 524.

HÜBENER, H.J. & SAHRHOLTZ, F.G. (1960). 20β-Hydroxysteroid dehydrogenase. Biochem. Z. 333: 95 - 105.

HUBENER, H.J., SAHRHOLTZ, F.G., SCHMIDT-THOME, J., NESEMANN, G. & JUNK, R. (1959). 20β-Hydroxysteroid-Dehydrogenase, ein neues kristallines Enzym. Biochim. biophys. Acta. 35, 270 - 272.

HURLOCK, B. & TALALAY, P. (1959). Microsomal 3α-, and 11β-hydroxysteroid dehydrogenases. Arch. Biochem. 80, 468 - 469.

OSINSKI, P.A. (1960). Steroid 11β-ol-dehydrogenase in the human placenta. Nature, Lond., 187, 777.

PEARSON, B. & GROSE, F. (1959). Histochemical demonstration of 17β-hydroxysteroid dehydrogenase by use of tetrazolium salt. Proc. Soc. exp. Biol.,N.Y., 100, 363 - 638.

SWEAT, M.L., BERLINER, D.L., BRYSON, M.J., NABORS, C., HASKELL, M. & HOLMSTROM, E.G. (1960). The synthesis and metabolism of progesterone in the human and bovine ovary. Biochim. biophys. Acta. 40, 289 - 296.

WATTENBERG, L.W. (1958). Microscopic histochemical demonstration of steroid-3β-ol dehydrogenase in tissue sections. J. Histochem. Cytochem. 6, 225 - 232.

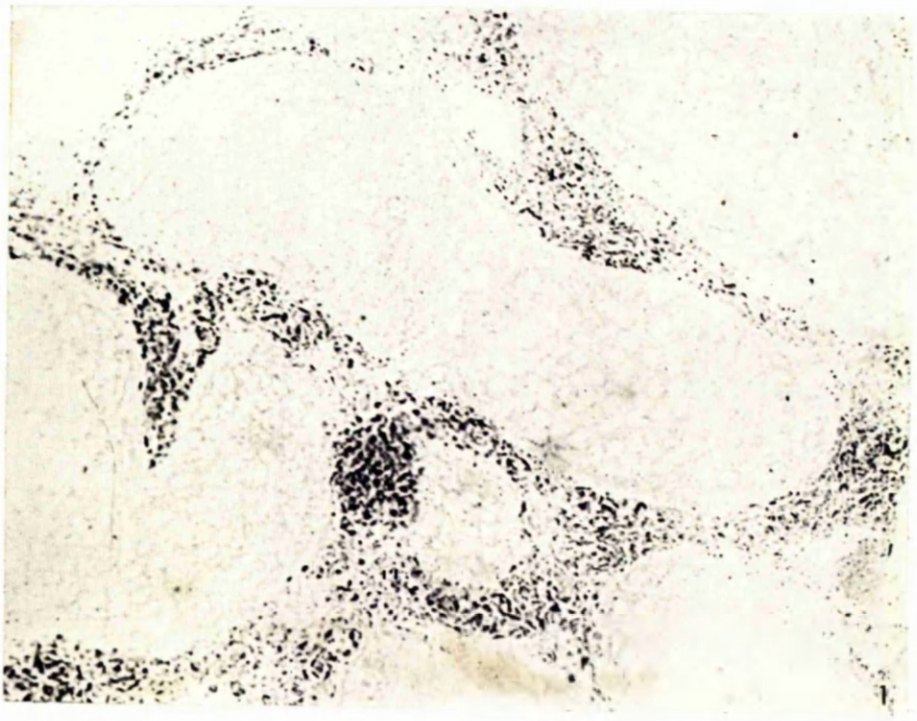
ZANDER, J., FORBES, T.R. VON MUNSTERMANN, A.M. & NEHER, R. (1958). Δ^4-3-Ketopregnene-20α-ol and Δ^4-3-ketopregnene-20β-ol, two naturally occurring metabolites of progesterone isolation, identification; biologic activity and concentration in human tissues. J. clin. Endocr. 18, 337 - 353.

DESCRIPTION OF PLATE.

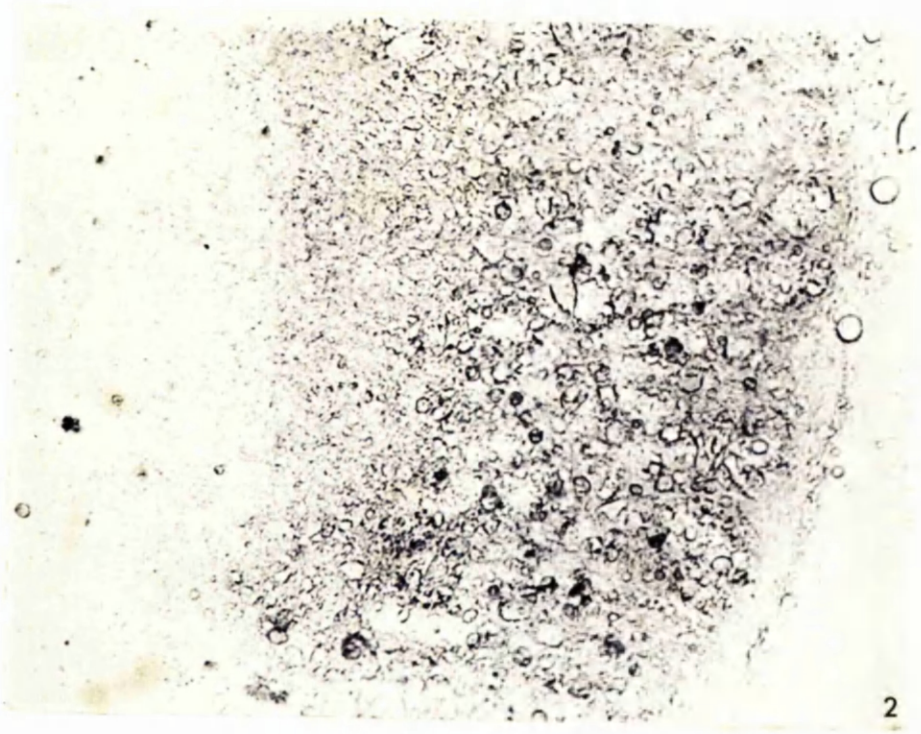
Fig. I. Testis of a boy aged 12 year. 20β-Hydroxy-steroid dehydrogenase activity is seen in the Leydig cells. (x 100).

Fig. 2. Mouse adrenal, adult. 20β-Hydroxysteroid dehydrogenase activity is seen in the zona fasciculata. Monoformazan mainly was deposited. (x 80).

PLATE.



1



2

Histochemical Utilization of 3α-, 6β-, 11α-, 12α-, 16α-, 16β-, 17α-, 21- and 24-Hydroxysteroids.

BY

A. H. Baillie
K. C. Calman
M. M. Ferguson
D. McK. Hart

Department of Anatomy, The University,
GLASGOW.

SUMMARY.

The histochemical utilization of 3α-, 6β-, 11α-, 12α-, 16α-, 16β-, 17α-, 21- and 24-hydroxysteroids in human and mouse testis, human placenta, mouse ovary and rat adrenal has been investigated using a coupling method and the tetrazolium salt, Nitro BT. 3α-Hydroxysteroid dehydrogenase was present in the human Leydig cells and placental syntrophoblast, but there was little in rat adrenal zona fasciculata and in mouse ovary; the enzyme is N.A.D. or N.A.D.P. dependent. 6β-Hydroxysteroid dehydrogenase was present in human Leydig cells, mouse Leydig cells, ova granulosa, theca interna, corpora lutea and interstitial tissue; it is N.A.D dependent. 11α-Hydroxysteroid dehydrogenase activity was very poorly developed, being N.A.D. dependent and demonstrable only in some human Leydig cells. 12α-Hydroxysteroid dehydrogenase could be demonstrated in some human Leydig cells and slight activity was noted in the ovary; it was both N.A.D. and N.A.D.P. dependent. 16α-Hydroxysteroids were very poorly used by all the tissues surveyed. 16β-Hydroxysteroids gave an intense histochemical reaction with N.A.D. in human Leydigand Sertoli cells, in placental trophoblast/

trophoblast, in adrenal zonae glomerulosa, fasciculata and reticularis and in all ovarian tissues. 17 -, 21- and 24-Hydroxysteroids were poorly utilized by human Leydig cells, but not by the other tissues. The first two were N.A.D. dependent; 24-hydroxysteroid utilization was both N.A.D. and N.A.D.P. dependent.

The techniques used are believed to demonstrate specific hydroxysteroid dehydrogenases because of variations in pyridine nucleotide requirement and in the location in the tissues of different hydroxysteroid dehydrogenases. Moreover, stereoisomers of the same hydroxysteroid behave differently in this system. The role of steroid 5 - and 5 -dehydrogenases is discussed in connexion with the histochemical results.

INTRODUCTION.

In the past few years the biosynthetic pathways of the steroid hormones have become better understood and techniques have been developed for the histochemical localization of 3 -, 11 -, 17 -, 20 - and 20--hydroxysteroid dehydrogenases, using the tetrazolium salt Nitro-BT (Wattenberg, 1958; Pearson & Grose, 1959; Balogh, 1964; Baillie, Calman, Ferguson & Hart, 1965; Baillie, Ferguson, Calman & Hart, 1965).

This paper describes the tissue utilization of a further nine hydroxysteroids.

MATERIAL AND METHODS.

Tissues: Testicular biopsies were performed on three healthy men aged 31, 73 and 82; a biopsy of a cryptorchid testis from a 49 year old man was also studied. Lastly, pieces of tissue about 1 $cm.^3$ were secured from two placentae at Caesarian section at term for obstetric reasons not related to placental function.

In addition, testes were secured from four adult Swiss white mice. Ovaries from four adult female Swiss white mice were obtained together with adrenal glands from three adult rats of the Royal Wistar albino strain. The mice and rats used were killed by decapitation and the tissues excised and frozen on solid carbon dioxide within 1 min. of death. The human tissues were similarly frozen within 2 min. of having had their blood supply interrupted.

Incubations: Each tissue was sectioned at 12μ in a cryostat maintained at -20°. The sections were attached to clean dry glass slides by momentary thawing. They were incubated for 30 min. at 37° in phosphate buffer (0.05 mM), pH 7.4, containing 1% (w/v) magnesium chloride and with the following reagents:

N.A.D./

N.A.D. or N.A.D.P. (final conc. 0.0 - 0.4 mM): nicotinamide (final conc. 1.0 mM), Nitro BT (= Sigma, 2,2'-di-p-nitrophenyl-5,5'-diphenyl-3,3'-(3,3'-dimethoxy-4,4'-diphenylene) ditetrazolium chloride) (final conc. 0.1 mg./ml.) together with a steroid substrate dissolved in propylene glycol or dimethyl formamide (final conc. 0.0 - 1.0 mM). The steroids studied were: (1) 3α-Hydroxysteroid dehydrogenase; (a) 3α-hydroxy-5α-androstan-17-one (Steraloids), (b) 3α-hydroxy-5β-androstan-17-one (Steraloids). (2) 6β-Hydroxysteroid dehydrogenase; (c) 6β-hydroxypregn-4-ene-3, 20-dione (M.R.C.) (6β-Hydroxyprogesterone). (3) 11α-Hydroxy-steroid dehydrogenase; (d) 11α-hydroxypregn-4-ene-3, 20-dione (Steraloids) (11α-hydroxyprogesterone). (4) 12α-Hydroxysteroid dehydrogenase; (e) 12α-hydroxy-pregn-4-ene-3, 20-dione (Steraloids) (12α-hydroxy-progesterone). (f) 12α-hydroxy-5α-pregnane-3, 20-dione (Steraloids). (5) 16α-Hydroxysteroid dehydrogenase; (g) 3-hydroxy-estra-1,3,5(10)trien-17-one (oestrone), (h) 3,16α-dihydroxy-estra-1,3,5(10)trien-17-one (16α-hydroxyoestrone), (i) 16α-hydroxypregn-4-ene-3, 20-dione (M.R.C.)(16α-hydroxyprogesterone). (6) 16β-Hydroxysteroid/

(6) 16β-Hydroxysteroid dehydrogenase; (j) 3β-hydroxyandrost-5-en-16-one 3-methyl ether (Steraloids), (l) 3β,16β-dihydroxyandrost-5-ene 3-methyl-ether (Steraloids). (7) 17α-Hydroxysteroid dehydrogenase; (l) 17α-hydroxyandrost-4-en-3-one (Steraloids), (m) 17α-hydroxypregn-4-ene-3, 20-dione (Steraloids) (17α-hydroxyprogesterone), (n) Progesterone. (8) 21-Hydroxysteroid dehydrogenase; (o) 21-hydroxypregn-4-ene-3, 20-dione (Steraloids) (21-hydroxyprogesterone). (9) 24-Hydroxysteroid dehydrogenase; (p) 24-hydroxy-5-cholane (Steraloids).

RESULTS.

Conditions of Incubation.

The pyridine nucleotide specificity of the steroid dehydrogenases is summarized in Table 1. No reaction developed with any steroid if both N.A.D. and N.A.D.P. were omitted. The development of the histochemical colour reaction for all the steroids surveyed was best within the pH range 7.0 - 7.6. At pH values outside this range the solubility of Nitro BT was altered, and the results were therefore difficult to evaluate/

evaluate. A substrate concentration of 0.5 mM appeared to give the strongest colour reaction; increasing the substrate concentration above this level did not enhance the reaction with any of the steroids. A colour reaction developed in the absence of nicotinamide or $MgCl_2$, but no colour was seen if steroid was omitted from the incubation medium.

Tissues: Human Testis.

The three healthy males had testes which were histologically normal, although the seminiferous tubules in the 82 year old testis showed a slight degree of atrophy. The cryptorchid testis of 49 years duration had a histologically typical appearance with atrophied seminiferous tubules comprising a Sertoli syncytium containing occasional spermatogonia and bounded by a slightly thickened basement membrane. The interstitium of this cryptorchid testis contained the prominent Leydig cells usually described as hyperplastic. From a histochemical standpoint all four human testes gave identical results which are summarized in Table 2.

TABLE 1. Pyridine nucleotide specificity of hydroxysteroid dehydrogenases

Position of hydroxyl group...	3α	6β	11α	12α	16α	16β	17α	21	24
N.A.D.	-	-	-	-	-	-	-	-	-
N.A.D.P.	-	-	-	-	-	-	-	-	-

TABLE 2. Histochemical activity of the testis

Position of hydroxyl group...	3α	6β	11α	12α	16α	16β	17α	21	24
Human testis									
Leydig tissue	M	MD	M	MD	M	MD	MD	MD	MD
Seminiferous epithelium	M	MD	-	M	-	D	M	M	M
Mouse testis									
Leydig tissue	M	MD	-	M	-	MD	M	-	-
Seminiferous epithelium	-	M	-	-	-	MD	-	M	-

TABLE 3. Histochemical activity of the mouse ovary

Position of hydroxyl group...	3α	6β	11α	12α	16α	16β	17α	21	24
Mouseovary									
Ovum	-	M	-	-	M	D	-	-	-
Granulosa	M	MD	-	M	-	D	-	-	-
Theca interna	M	MD	-	-	M	D	-	-	-
Corpora lutea	M	MD	-	M	M	D	-	-	-
Interstitial tissue	M	MD	-	M	M	D	-	-	-

M = Monoformazan D = Diformazan
MD = Intense reaction, Mono- and Diformazan
D = Intense reaction, Diformazan only.

Table 4. Histochemical activity of the rat adrenal gland

Position of hydroxyl group...	3α	6β	11α	12α	16α	16β	17α	21	24
Rat adrenal									
Zona glomerulosa	-	M	-	-	-	D	-	-	-
Zona fasciculata	M	D	-	-	M	D	-	M	-
Zona reticularis	-	D	-	-	-	D	-	-	-
Medullary rests	-	-	-	-	-	D	-	-	-

Table 5. Histochemical activity of the human placenta

Position of hydroxyl group...	3α	6β	11α	12α	16α	16β	17α	21	24
Human placenta	M	M	-	-	-	D	-	-	-

M = Monoformazan D = Diformazan

D = Intense deposition of Diformazan

Some variability was noted in the human testes in the intensity of reaction given by a particular hydroxysteroid. Pl. 1 and the first two figures in Pl. 2 illustrate typical reactions.

The results obtained with the mouse testis are illustrated in Pl. 2, figs. 7 and 8, and listed in Table 2; much less variation was noted than in the human testis. The results in mouse ovaries are shown in Table 3 and Pl. 3, figs. 8 - 11. Results on rat adrenals are summarized in Table 4 and Pl. 3, fig. 12; Pl. 4, figs. 13 and 14 illustrate the intensity of reactions encountered.

The results obtained with human placentae are dealt with in Table 5 and Pl. 4, figs. 15 and 16.

DISCUSSION.

Histochemical Specificity.

The central difficulty in assessing and interpreting the above results is the problem of the histochemical specificity of the hydroxysteroid dehydrogenases. At present most workers accept that Wattenberg's (1958) histochemical reaction for the demonstration of 3β-hydroxysteroid dehydrogenase is specific, and it has even been claimed (Baillie & Griffiths, 1964) that in rodent testis this enzyme shows/

shows specific substrate preferences in histochemical systems and that these change with age. Nevertheless, Pearse (1960) has questioned the specificity of the histochemical 3β- and 17β-hydroxysteroid dehydrogenase techniques (Wattenberg, 1958; Pearson & Grose, 1959) and it has been suggested that, particularly in the adrenal cortex, the distribution of formazan seen after incubation of tissue sections with 3β-hydroxysteroids may be dependent on the solubility of the reaction products in tissue lipids. In this context it has been pointed out that lipids are far more abundant in the zona fasciculata (Currie, Symington & Grant, 1962) and mono- and di-formazan seem to be selectively deposited after 3β-hydroxysteroid dehydrogenase incubations at sites where large amounts of lipid are present. This criticism was foreseen by Wattenberg (1958) who suggested rinsing tissue sections in acetone to remove lipids, and this practice is now widespread.

The present findings indicate a high degree of specificity of the histochemical techniques for demonstrating hydroxysteroid dehydrogenases. Three principal features point to this conclusion.

First/

First, hydroxysteroid dehydrogenases in histochemical systems show marked preferences for the two different reduced pyridine nucleotides and on this basis three groups of hydroxysteroid dehydrogenases can be recognised: (a) N.A.D.-dependent dehydrogenases. This group includes the 6β-, 11α-, 16α-, 17α-, and 21-hydroxysteroid dehydrogenases described above, which together with the 3β-, and 20β-hydroxysteroid dehydrogenases (Wattenberg, 1958; Baillie, Calman et al., 1965) are specific for N.A.D.: These histochemical findings accord well with the known biochemical specificity of these hydroxysteroid dehydrogenases for N.A.D. (Breuer, Nocke & Knuppen, 1958: Breuer & Nocke, 1959: Hurlock & Talalay, 1959; Ryan, 1960). (b) N.A.D.- or N.A.D.P.-dependent dehydrogenases. This second group of hydroxysteroid dehydrogenase utilize either N.A.D. or N.A.D.P. histochemically and includes the 3α-, 12α-, 16β- and 24-hydroxysteroid dehydrogenases (see above) together with 11β-hydroxysteroid dehydrogenase (Baillie, Ferguson et al. 1965). Biochemically the 3α-, and 11β-hydroxysteroid dehydrogenases are also known to utilize both pyridine nucleotides (Meigs & Engel, 1961). (c) N.A.D.P.-dependent dehydrogenases. The third, smallest, group of hydroxysteroid dehydrogenases has so far only one representative, 20α-hydroxysteroid dehydrogenase/

dehydrogenase and this enzyme is known to be specific for N.A.D.P. both histochemically (Balogh, 1964) and biochemically (Wiest, 1959).

The second group of features pointing to specificity of hydroxysteroid dehydrogenases within this histochemical system is the striking difference in localization of different reactions in different tissues. Perhaps the three most obvious examples are: (A) The strong utilization of 16β-hydroxysteroids throughout the epithelium of the human seminiferous tubule while other hydroxysteroids so far examined only produce little or no formazan deposition in this tissue. (B) The deposition of formazan in the ovum after incubation with 11β-(Baillie, Ferguson et al., 1965), 3α-, 6β- and 16β-hydroxysteroids but with no other steroids yet reported and lastly: (C) The striking differences in human adrenal zonal distribution after incubation with 3β-hydroxysteroids (Wattenberg, 1958) and, for example, 16β-hydroxysteroids.

The last group of features pointing unmistakably to a high degree of enzyme substrate specificity is the

remarkable differences in reactivity of α-hydroxy and β-hydroxy derivatives of the same steroid. Some, such as 20α- and 20β-hydroxysteroids require different cofactors (Balogh, 1964; Baillie, Calman et al., 1965), while others, such as the 11α- and 11β-hydroxysteroids are very different in their histochemical activity. A further point of importance is that in many cases biochemical work has shown that these enzymes are specific.

The simplest explanation of these findings is that hydroxysteroid dehydrogenases are specific in these histochemical conditions and we prefer this view to other possible explanations, including that based on the influence of membrane permeability factors and on the lipid-solubility of reaction products. We can therefore see no advantage in continuing to wash sections in acetone before incubation. This practice has been shown in the ovary (Levy, Deane & Rubin, 1959) to remove 3β-hydroxysteroid dehydrogenase from the theca interna, and in the foetal human testis (Baillie & Niemi, unpublished observations) it considerably reduced 3β-hydroxysteroid dehydrogenase activity with/

with particular respect to the steroid sulphates. It may be argued that acetone-washing improves the cytological location of formazan produced in these reactions, but since the resolution of the light microscope does not permit precise cytological localization of the site of hydroxysteroid dehydrogenase activity it seems to us that the principal function of these techniques at present is to establish which cells in a given organ can carry out a given conversion. If this be so acetone-washing, which reduces and may, in certain sites, even abolish hydroxysteroid dehydrogenase activity, should be avoided.

Histochemical Considerations.

Recent work in this laboratory (Baillie, Calman, Ferguson & Hart, unpublished observations) have established that steroid 5α- and 5β-dehydrogenases can reduce Nitro BT in this histochemical system and it seems likely that some of the colour observed after the incubation of tissues with the saturated 3α-hydroxy compounds, 12α-hydroxy-5α-pregnane-3, 20-dione, and 24-hydroxy-5β-cholan-3-one is due to a steroid 5α- and 5β-dehydrogenase. This possibility must be born in mind when interpreting the histochemical findings.

3β-Hydroxysteroid dehydrogenase activity has previously been reported in the Leydig cell of the mouse (Hitzeman, 1962; Baillie, 1964) and of the rat (Niemi & Ikonen, 1962) and the cytological distribution of hydroxysteroid dehydrogenase activity in the rodent tissue used in the present investigation with 3α-, 6β-, 12α- and 16β-hydroxysteroids as substrates is identical with that described for the 3β-hydroxysteroid dehydrogenase. 16β-Hydroxysteroids gave a stronger histochemical reaction in the mouse testis than did any of the 3β-hydroxysteroids used in this laboratory (Baillie, 1965).

The histochemical localization of 3β-hydroxysteroid dehydrogenase activity in the Leydig cells of the human testis (Baillie, Niemi & Ikonen, 1965) is identical with the distribution of hydroxysteroid dehydrogenases revealed by incubation of human testis sections with 3α-, 11α-, 12α-, 16α-, 16β-, 17α-, and 21-hydroxysteroids and the wide range of hydroxysteroids dehydrogenated by the human Leydig cells is in marked contrast with the relatively limited range of dehydrogenase activity in the placenta and different zones of the adrenal. The removal of hydrogen from/

from the 24 position by the human Leydig cells was unexpected and the possibility that this reaction was catalysed by a non-specific alcohol dehydrogenase cannot be excluded. Alternatively the colour may be due to the removal of hydrogen from the 5β-position by a dehydrogenase. Nevertheless, using purified preparations of alcohol dehydrogenase it has been shown in vitro (Merritt & Tomkins, 1959) that steroids such as testosterone and cortisol are not attacked by this enzyme.

Goldberg, Jones & Borkowf (1964) have reported a histochemical colour reaction in human Leydig cells with 3β-, 17β-dihydroxyandrost-4-ene (androstenediol), 3α-hydroxy-5α-androstan-17-one and the dipropionate of androstenediol as substrates. While these workers attribute the diformazan deposition in their testicular sections to a reaction of 3β-hydroxysteroid dehydrogenase with these steroids, it seems likely that part at least of the formazan deposition they describe arises from 17β-hydroxysteroid dehydrogenase activity and part from a steroid 5α- or 5β-dehydrogenase. They failed to observe any colour with the 3α-, 17α- and 20β-hydroxysteroids in human testis while in our experience these steroids are all metabolised histochemically by human Leydig cells.

Incubation of 3α-, 12α-, 17α- and 21-hydroxy-steroids with human testicular sections results in the deposition of small amounts of formazan in the seminiferous tubules in relation to the developing spermatozoa. These reactions are difficult to control satisfactorily, due apparently to the presence in the seminiferous epithelium of an alcohol dehydrogenase acting on propylene glycol and very little formazan can be found in the tubules after incubation in media containing dimethyl formamide as solvent for 3α-, 12α-, 16α-, 17α- and 21-hydroxysteroids. The trace activity found with these steroids, distributed in droplets in relation to the maturing germ cells, contrasts with the generalized distribution of 16β-hydroxysteroid dehydrogenase activity throughout the seminiferous tubules. The 6β- and 16β-hydroxysteroid dehydrogenases are the first enzymes concerned in steroid metabolism to be definitely localized in the human seminiferous tubules and their biochemical significance has to be considered.

Ikonen, Niemi, Pesonen & Timonen (1961) first described 3β-hydroxysteroid dehydrogenase as being situated round the periphery of the cells in the granulosa, and this distribution was later confirmed in the atretic mouse granulosa by Ferguson (1965).

A/

A similar ring-like distribution in the periphery of granulosa cells was seen with both 6β-hydroxysteroid dehydrogenase and 16β-hydroxysteroid dehydrogenase: after incubation with the 16β-hydroxysteroid this pattern of diformazan deposition was sometimes also observed in corpora lutea, but tended to be obscured by monoformazan dispersed in cytoplasmic lipid. These ring patterns of distribution of the diformazan in the granulosa were only evident after incubation with substrates which were well utilized by the ovary. Other substrates may have been used at a similar cellular site, but this was not obvious owing to the sparse nature of the deposits. A further problem in the intracellular localization of the hydroxysteroid dehydrogenases is that some are soluble and tend to diffuse (Novikoff, 1959; Smith, 1964).

16β-Hydroxysteroid dehydrogenase was distributed in the ovary in a similar manner to that of 3β-hydroxysteroid, that is, in corpora lutea, interstitial tissue, theca interna, granulosa of atretic follicles, connective tissue stroma and theca externa. Of particular interest was the slight 16β-hydroxysteroid dehydrogenase activity/

activity noted in the connective tissue stroma and theca externa, indicated by scattered fine granules of diformazan. While other hydroxysteroid dehydrogenases may occur in these cells they could not be histochemically demonstrated.

In recent years the histochemical localization of 3β-hydroxysteroid dehydrogenase activity has been well studied in the adrenal cortex (Wattenberg, 1958; Levy et al. 1959; Cavallero & Chiappino, 1961; Dawson, Pryse-Davies & Snape, 1961) and it has been established that the zona fasciculata of various species can utilize the substrates 3β-hydroxyandrost-5-en-17-one (D.H.A.) and 3β-hydroxypregn-5-en-20-one (pregnenolone). A variety of other substrates have been shown to be satisfactory for the histochemical localization of this reaction (Baillie, Cameron, Griffiths & Hart, 1965) which, in normal human adrenal glands, appears to be confined to the zona fasciculata. The 3α-, 11α- and 21-hydroxysteroid dehydrogenases demonstrated in the rat adrenal have the same zonal location as the 3β-hydroxysteroid dehydrogenase; 6β-hydroxysteroid dehydrogenase activity differs from the foregoing enzymes in that it is found in the zona reticularis as well as in the zona fasciculata. 16β-Hydroxysteroid dehydrogenase/

dehydrogenase is peculiar in that it is particularly well developed in the zona glomerulosa in man, although present in both the other zones.

The distribution of hydroxysteroid dehydrogenase activity in the human placenta at term as shown by tetrazolium dye deposition after incubation with the appropriate substrates indicates that 3α- and 16β-hydroxysteroid dehydrogenases, in this respect, do not differ from 3β-hydroxysteroid dehydrogenase (Lobel, Deane & Romney, 1962; Baillie, Cameron et al. 1965) and NAD-linked 17β-hydroxysteroid dehydrogenase (Kellogg & Glenner, 1960).

Biochemical Considerations.

The majority of these enzymes are concerned in steroid metabolism in the widest sense and the histochemical findings discussed confirm and extend much biochemical work.

3α-Hydroxysteroid dehydrogenase is an important enzyme concerned in the detoxication and elimination of steroids from the organism. Thus cortisol with a Δ^4-3-keto grouping is reduced to tetrahydrocortisol, which can be conjugated with glucuronide and excreted, and it has been shown (Tomkins & Isselbacher, 1954) that the reduction of the keto group is secondary to the/

the reduction of the Δ^4 double bond. Theoretically, both 5α- and 5β-compounds could serve as substrates for this enzyme and our results with human testis agree with those of Tomkins (1956) in rat liver, kidney and testis, in that both 5α- and 5β-compounds were utilized, although the 5β-compound with cis A/B ring junction was better utilized. However, these results may in part have been due to a steroid 5α- or 5β-dehydrogenase. By contrast, in mouse testis, the reaction was weak and no difference in utilization was observed.

Biochemical investigations have indicated that the 3α-hydroxysteroid dehydrogenase may be both soluble (Tomkins, 1956) and microsomal (Hurlock & Talalay, 1959; Meigs & Engel, 1961), and our experience suggests that the testicular enzyme can easily be removed by pre-incubation of tissue sections for one minute in buffer. If the brief washing is omitted, occasionally diformazan particles can be seen in the incubation medium, indicating the possibility that the enzyme has been leached out of the tissue section, although other interpretations of this observation are possible. The pH optimum of this enzyme (7.2 - 7.4) is confirmed by/

by biochemical studies (Tomkins, 1956) and, again in accord with biochemical findings, both NAD and NADP can be used as cofactors for this enzyme in the histochemical system.

6β-Hydroxysteroids have been isolated from urine in two forms: (a) as a metabolite of cortisol by Burstein & Dorfman (1955) and Ulstrom, Colle, Burley & Gunville (1960), and these workers found no 6-keto form; (b) as 6β-hydroxy-oestradiol (Breuer et al. 1958). A 6β-hydroxysteroid dehydrogenase has been demonstrated histochemically in most tissues surveyed. The exact significance of this reaction is not understood, but the nature of the histochemical reaction may indicate an important metabolic role for the 6β-hydroxysteroid metabolites, or for the 6β-hydroxysteroid dehydrogenase.

The fact that a weak but definite histochemical reaction in some human testes with the 11α-hydroxy substrate has been demonstrated may indicate that a separate hydroxysteroid dehydrogenase is present. It is possible that the 11α-hydroxy group is oxidised by/

by the 11β-hydroxysteroid dehydrogenase, and that the reaction is weak because of the hindered position of the 11α-hydroxyl group. However, the difference between the coenzyme requirements of the two reactions is against this. 11α-Corticosteroids have been isolated from human adrenal glands (Neher & Wettstein, 1956).

Steroids with a 12α-grouping belong to the class of bile acids and a variety of 12-keto cholanic acids (Norman & Sjovall, 1958; Danielsson, Eneroth, Helstrom, Lindstedt & Sjovall, 1963) have been isolated from the faeces. These degradation products imply the existence of a 12α-hydroxysteroid dehydrogenase in the intestinal flora and our results suggest that this type of oxidation may also take place in human testis.

16α-Hydroxy progesterone has been isolated from adrenal tissue (Villee, Dimoline, Engel, Villee & Racker, 1962; Ward & Grant, 1963) and a number of oestrogens with either 16α- or 16β-hydroxy groups have been discovered (Breuer & Nocke, 1959; Levitz, Spitzer & Twombly, 1958). Moreover, it is known that/

that these 16-hydroxy compounds can be inter-converted via the 16-keto group (Levitz, Rosen & Twombly, 1960). Liver, placenta and hydatidiform mole have also been shown to metabolize the 16-hydroxy oestrogens (Breuer & Knuppen, 1958; Ryan, 1960; Klausner & Ryan, 1964; MacDonald & Siiteri, 1964) and recently a 16α-hydroxy oestriol conjugate with glucuronic acid has been isolated (Slaunwhite, Karsay & Sandberg, 1964; Slaunwhite, Lightman & Sandberg, 1964). The histochemicalresults obtained, namely high 16β-hydroxysteroid dehydrogenase activity and low 16α-hydroxysteroid dehydrogenase activity in endocrine tissues, when interpreted in the light of this work, would suggest that most of the 16β-hydroxy oestrogens are converted by way of the 16-keto form to the 16α-hydroxy derivatives and excreted in conjugated form as the 16α-hydroxy glucuronide.

Recently a 17β-hydroxysteroid dehydrogenase has been described in the spermatazoa of dogs and man (Hathaway & West, 1964); the substrate used by this enzyme appears to be 17β-oestradiol. Our results with the 6β- and the 16β-hydroxysteroid dehydrogenases and other/

other hydroxysteroids in the seminiferous tubules suggest that other hydroxysteroid dehydrogenases are present at these sites.

The 17α-hydroxy configuration is important since it is found in cortisol. However, dehydrogenation of the 17-position in the C-21 steroids is impossible, because of the absence of available hydrogen atoms. C-18 and C-19 17α-hydroxysteroids, however, can be dehydrogenated as shown by the histochemical reactions and 17α-hydroxyoestrogens have been isolated from bovine placenta (Gorski & Erb, 1959), liver (Breuer & Nocke, 1959) and blood (Axelrod & Werthessen, 1960). With blood and liver, interconversion with the keto-form has been demonstrated. It is possible then to visualize the interconversion of the 17α-hydroxyl and the 17β-hydroxyl group by way of the 17-ketone. Both 17α- and 17β-hydroxysteroid dehydrogenases would then presumably be involved.

NOTE INSERTED IN PROOF.

NADP-linked 16β-H.S.D. has now been detected in rat ovary and NADP-linked 3β-H.S.D. in certain tissues, in this laboratory.

REFERENCES.

AXELROD, I.R. & WERTHESSEN, N.T. (1960). The metabolism of estrone-16-C^{14} in bovine blood. Archs. Biochem. Biophys. 86: 53 - 55.

BAILLIE, A.H. (1964). Further observations on the growth and histochemistry of the Leydig tissue in the postnatal prepubertal mouse testis. J. Anat. Lond. 98: 403 - 420.

BAILLIE, A.H. (1965). 3β-Hydroxysteroid dehydrogenase activity in the foetal mouse testis. J. Anat. Lond. 99; 507 - 511.

BAILLIE, A.H., CALMAN, K.C., FERGUSON, M.M. & HART, D.McK. (1965). Histochemical demonstration of 20β-hydroxysteroid dehydrogenase. J. Endocrin. 32: 337 - 339.

BAILLIE, A.H., CAMERON, E.H.D., GRIFFITHS, K. & HART, D.McK. (1965). 3β-Hydroxysteroid dehydrogenase in the adrenal gland and placenta. J. Endocrin. 31: 227 - 233.

BAILLIE, A.H., FERGUSON, M.M., CALMAN, K.C. & HART, D.McK. (1965). Histochemical demonstration of 11β-hydroxysteroid dehydrogenase. J. Endocrin. 33: 119 - 125.

BAILLIE, A.H. & GRIFFITHS, K. (1964). 3β-Hydroxysteroid dehydrogenase activity in the mouse Leydig cell. J. Endocrin. 29, 9 - 17.

BAILLIE, A.H., NIEMI, M. & IKONEN, M. (1965). 3β-Hydroxysteroid dehydrogenase activity in the human foetal testis. Acta. Endocr., Copenh. (in the Press).

BALOGH, K. (1964). A histological method for the demonstration of 20α-hydroxysteroid dehydrogenase activity in rat ovaries. J. Histochem. Cytochem. 12, 670 - 673.

BREUER, H. & KNUPPEN, R. (1958). Metabolism of 16 oxoestrone by human liver slices in vitro. Nature, Lond. 182, 1512.

BREUER, H. & NOCKE, L. (1959). Formation of estriol-3,16β,17α by liver tissue in vitro. Biochim. biophys. Acta. 36: 271 - 272.

BREUER, H., NOCKE, L. & KNUPPEN, R. (1958). 6β-Hydroxylierung von Oestrogenen in der Rattenleber, Naturwissenschaften, 45, 397.

BURSTEIN, S. & DORFMAN, R.I. (1955). Steroid metabolism in guinea pigs. I. Metabolism of cortisol in vivo. J. biol. Chem. 213, 581.

CAVALLERO, C. & CHIAPPINO, G. (1961). Istologia ed istochimica della corteccia surrenale negli ipercorticalismi. IX Congresso Nazionale, Simposia su la m. di Cushing. Soc. ital. Endocrinal. Arti Graphiche Pacini Mariotti, Pisa, p.5.

CURRIE, A.R., SYMINGTON, T. & GRANT, J.K. (1962). The human adrenal cortex. Edinburgh: Livingston.

DANIELSSON, H., EMEROTH, D., HELLSTROM, K., LINDSTEDT, S. & SJOVALL, T. (1963). On the turnover and excretory products of cholic and chenodeoxycholic acid in man. J. biol. Chem. 238, 2299 - 2304.

DAWSON, I.M.P., PRYSE-DAVIES, J. & SNAPE, I.M. (1961). The histochemical distribution of steroid 3β-ol-dehydrogenase and diphosphopyridine nucleotide diaphorase in the adrenal cortex. Biochem. J. 78, 16P.

FERGUSON, M.M. (1965). 3β-Hydroxysteroid dehydrogenase activity in the mouse ovary. J. Endocrin. 32: 365 - 371.

GOLDBERG, B., JONES, G.E.S. & BORKOWF, H.I. (1964). A histochemical study of substrate specificity for the steroid-3β-ol dehydrogenase and isomerase systems in human ovary and testis. J. Histochem. Cytochem. 12, 880 - 889.

GORSKI, J. & ERB, R.E. (1959). Characterisation of estrogens in the bovine. Endocrinology, 64, 707 - 712.

HATHAWAY, R.R. & WEST, C.D. (1964). The conversion of 17β-estradiol to estrone by spermatozoa of dogs and man. Endocrinology, 75, 616 - 617.

HITZEMAN, Str. J.W. (1962). Development of enzyme activity in the Leydig cells of the mouse. Anat. Rec. 143, 351 - 362.

HURLOCK, B. & TALALAY, P. (1959). Microsomal 3α- and 11β-hydroxysteroid dehydrogenases. Archs. Biochem. Biophys. 80, 468 - 469.

IKONEN, M., NIEMI, M., PESONEN, S. & TIMONEN, S. (1961). Histochemical localisation of four dehydrogenase systems in the human ovary during the menstrual cycle. Acta endocr., Copenh., 38, 293 - 302.

KELLOGG, D.A. & GLENNER, G.G. (1960). Histochemical localization of human, term placental 17β-oestradiol dehydrogenases: implications for the transhydrogenase reaction. Nature, Lond. 187, 763 - 764.

KLAUSNER, D.A. & RYAN, K.J. (1964). Estriol secretion by human, term placenta. J. clin. Endocr. 24, 101 - 104.

LEVITZ, M., ROSEN, M.F. & TWOMBLY, G.H. (1960). Interconversions of 16-oxygenated estrogens. II. The metabolism of 16-ketoestradiol-17β-16-C^{14} in man. Archs. Biochem. Biophys. 88, 212 - 215.

LEVITZ, M., SPITZER, T.R. & TWOMBLY, G.H. (1958). Interconversions of 16-oxygenated estrogens. I. The synthesis of estriol-16-C^{14} and its metabolism in man. J. biol. Chem. 231, 787 - 797.

LEVY, H., DEANE, H.W. & RUBIN, B.L. (1959). Visualisation of steroid-3β-ol dehydrogenase activity in tissues of intact hypophysectomized rats. Endocrinology, 65, 932 - 943.

LOBEL, B.C., DEANE, H.W. & ROMNEY, S.L. (1962). Enzymic histochemistry of villous portion of human placenta from six weeks of gestation to term. Am. J. Obstet. Gynec. 83, 295 - 299.

MACDONALD, P.C. & SIITERI, P.K. (1964). Study of estrogen production in Women with hydatidiform mole. J. clin. Endocr. 24, 685 - 90.

MEIGS, R.A. & ENGEL, L.L. (1961). The metabolism of adrenocortical steroids by human tissues. Endocrinology. 69, 152 - 162.

MERRITT, D.A. & TOMKINS, G.M. (1959). Reversible oxidation of cyclic secondary alcohols by liver alcohol dehydrogenase. J. biol. Chem. 234,2778 - 2782

NEHER, R. & WETTSTEIN, A. (1956). Isolierung and Konstitutionsermittlung weiterer Pregnan Verbin dungen aus Nebennieren. Helv. chim. Acta, 39, 2062 - 2088.

NIEMI, M. & IKONEN, M. (1962). Cytochemistry of oxidative enzyme systems in the Leydig cells of the rat testis and their functional significance. Endocrinology, 70, 167 - 174.

NORMAN, A. & SJOVALL, J. (1958). On the transformation and enterohepatic circulation of cholic acid in the rat. J. biol. Chem. 233,872 - 885.

NOVIKOFF, A.B. (1959). Enzyme cytochemistry pitfalls in the current use of tetrazolium techniques. J. Histochem. Cytochem. 7, 301 - 302.

PEARSE, A.G.E. (1960). Histochemistry, theoretical and applied. London: Churchill.

PEARSON, B. & GROSE, F. (1959). Histochemical demonstration of 17β-hydroxysteroid dehydrogenase by use of tetrazolium salt. Proc. Soc. exp. Biol. Med. 100, 636 - 638.

RYAN, K.J. (1960). Metabolism of 16-keto-estrone by human placenta. Endocrinology, 66: 491 - 494.

SLAUNWHITE, W.R., KARSAY, M.A. & SANDBERG, A.A. (1964). Studies on phenolic steroids in human subjects. V. Excretion of urinary estriol glucosiduronate during pregnancy. J. clin. Endocr. 24, 263 - 266.

SLAUNWHITE, W.R., LICHTMAN, M.A. & SANDBERG, A.A. (1964). Studies on the phenolic steroids in human subjects. VI. Biosynthesis of estriol glycosiduronic acid by human liver. J. clin. Endocr. 24, 638 - 643.

SMITH, B. (1964). The localisation of enzymes within skeletal muscle fibres using the tetrazolium technique. J. Histochem. Cytochem. 12, 847 - 851.

TOMKINS, G.M. (1956). A mammalian 3α-hydroxysteroid dehydrogenase. J. biol. Chem. 218, 437 - 447.

TOMKINS, G.M. & ISSELBACHER, K.H. (1954). Enzymic reduction of cortisone. J. Amer. chem. Soc. 76, 3100 - 3101.

ULSTROM, R.A., COLLE, E., BURLEY, J. & GUNVILLE, R. (1960). Adrenocortical steroid metabolism in newborn infants. II. Urinary excretion of 6β-hydroxy cortisol and other polar metabolites. J. clin. Endocr. 20, 1080.

VILLEE, D.R., DIMOLINE, A., ENGEL, L.L., VILLEE, C.A., & RACKER, H. (1962). Formation of 16α-hydroxy-progesterone in hyperplastic adrenal tissue. J. clin. Endocr. 22, 726 - 734.

WARD, P.J. & GRANT, J.K. (1963). Metabolism of (4-^{14}C)-progesterone by adrenocortical tissue from patients with Cushing's syndrome. J. Endocrin. 26, 139 - 147.

WATTENBERG, L.W. (1958). Microscopic histochemical demonstration of steroid-3β-ol dehydrogenase in tissue sections. J. Histochem. Cytochem. 6, 225 - 232.

WIEST, W.G. (1959). Conversion of progesterone to 4-pregn-20α-ol-3-one by rat ovarian tissue in vitro. J. biol. Chem. 234, 3115 - 3121.

DESCRIPTION OF PLATES.

PLATE I.

Fig. I. Moderately strong 3α-hydroxysteroid dehydrogenase activity in the Leydig cells of human testis with traces of monoformazan in the seminiferous tubules. (x 90).

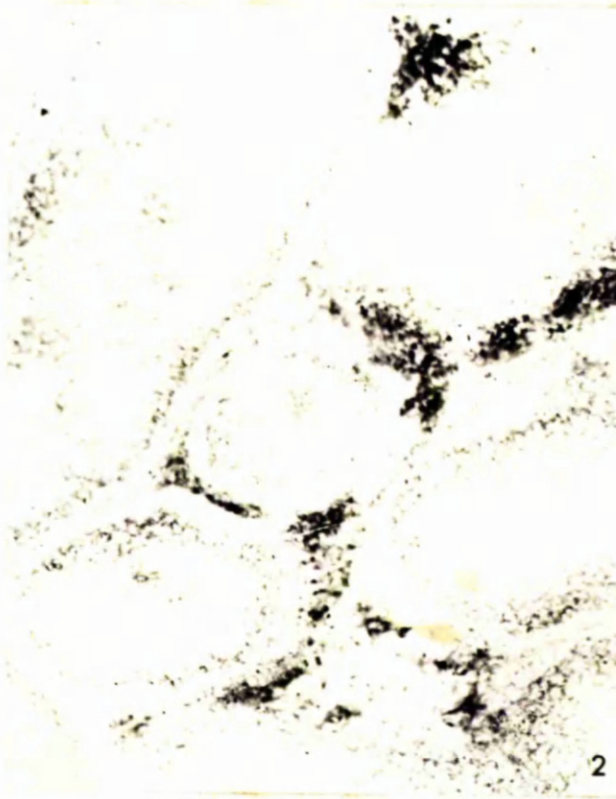
Fig. 2. Diformazan densely deposited in human Leydig cells and monoformazan in seminiferous tubules with 6β-hydroxyprogesterone as substrate. (x 90).

Fig. 3. Human Leydig cells showing diformazan deposition with a trace of colour in the seminiferous tubules using 12α-hydroxyprogesterone as a substrate. (x 90).

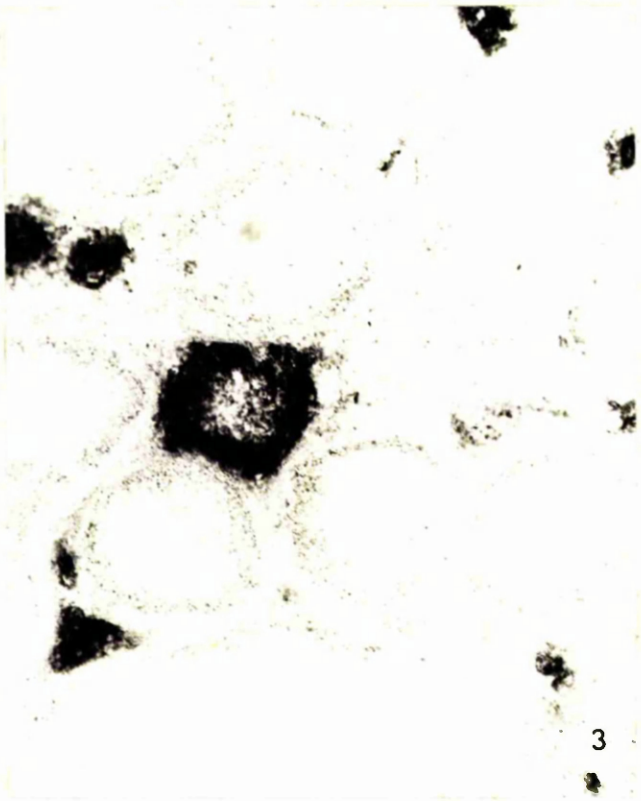
Fig. 4. Very weak 16α-hydroxysteroid dehydrogenase activity in human Leydig cells. (x 180).

PLATE I

1



2



3

4

PLATE 2.

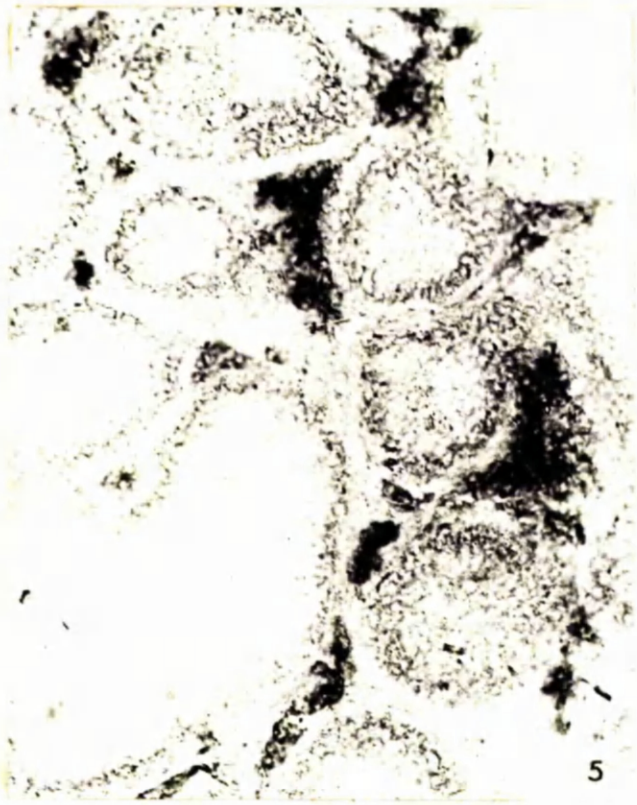
Fig. 5. Heavy deposit of diformazan in human Leydig cells and monoformazan in the seminiferous tubules indicating intense 16β-hydroxysteroid dehydrogenase activity. (x 90).

Fig. 6. Diformazan deposition in human Leydig cells with 5β-cholan-24-ol. (x 90).

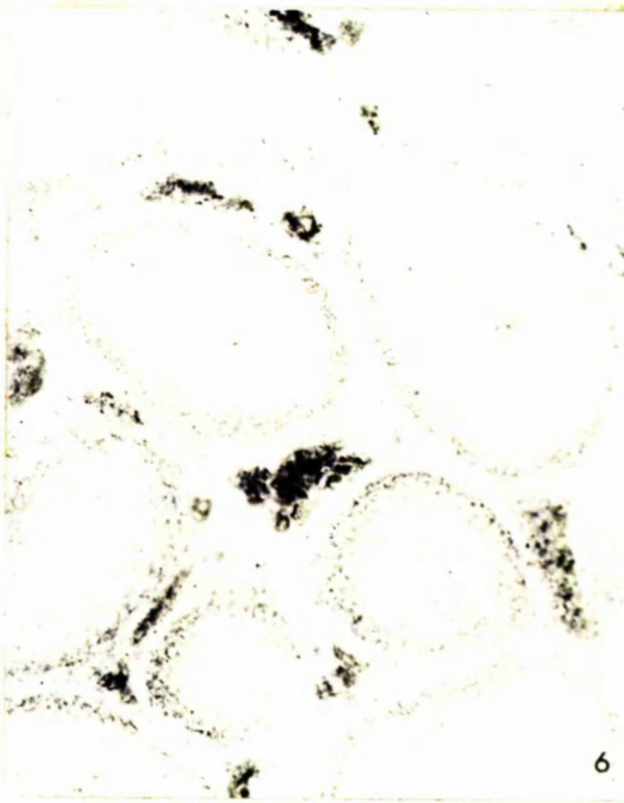
Fig. 7. 6β-Hydroxysteroid dehydrogenase activity in mouse testis; intense activity in the Leydig cells with some monoformazan in the seminiferous tubules. (x 120).

Fig. 8. Dense deposit of diformazan in mouse Leydig cells with no colour present in the seminiferous tubules when incubated with 3β, 16β-dihydroxyandrost-5-ene-3-methyl ether as substrate. (x 180).

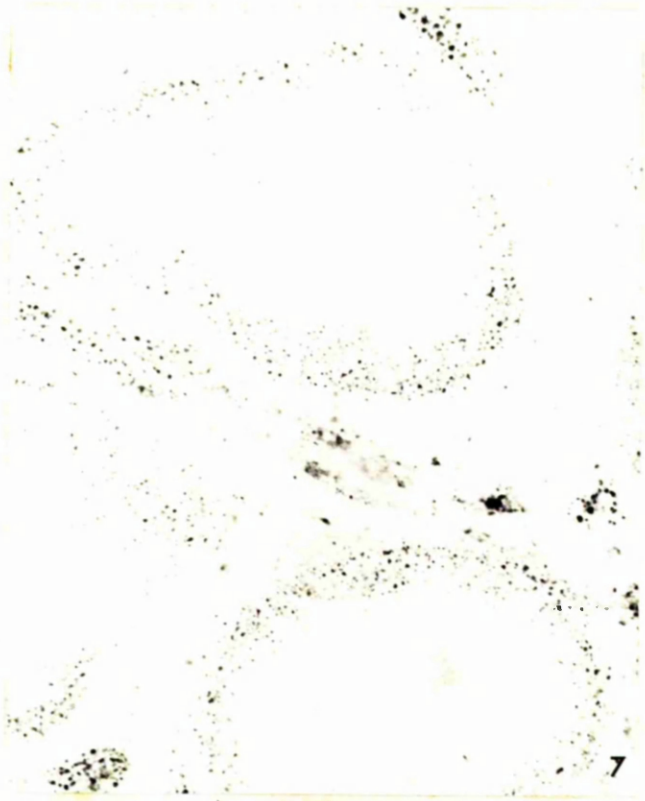
PLATE II



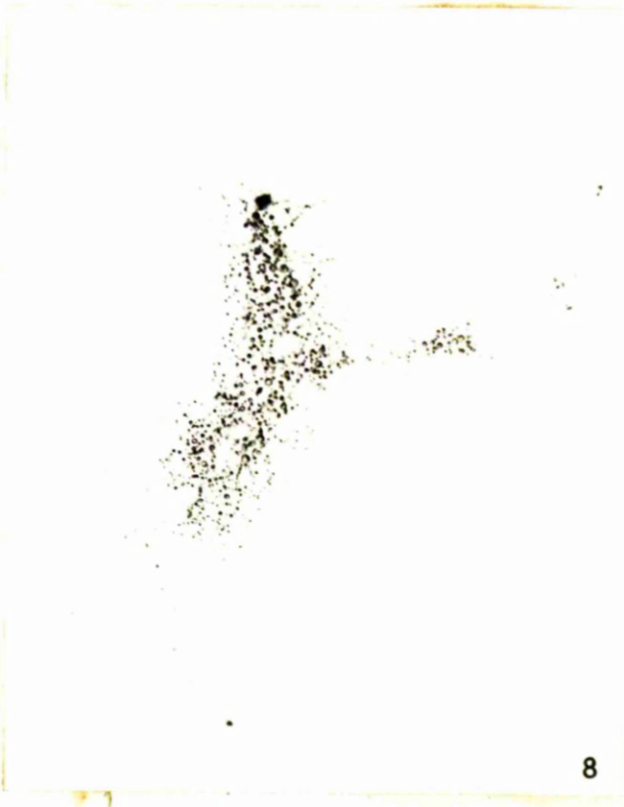
5



6



7



8

PLATE 3.

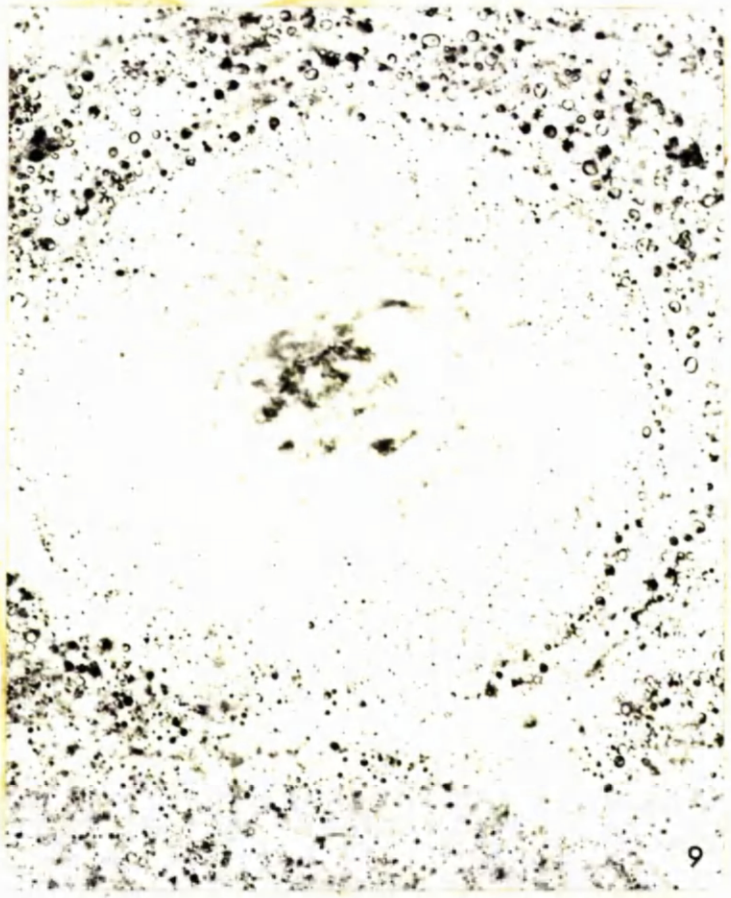
Fig. 9. Diformazan deposited in granulosa and ovum of mouse ovary with 6β-hydroxyprogesterone as substrate. (x 360).

Fig. 10. 6β-Hydroxysteroid dehydrogenase activity in mouse corpus luteum (CL), follicle (F), interstitial tissue (IT), granulosa and ovum (O). (x 60).

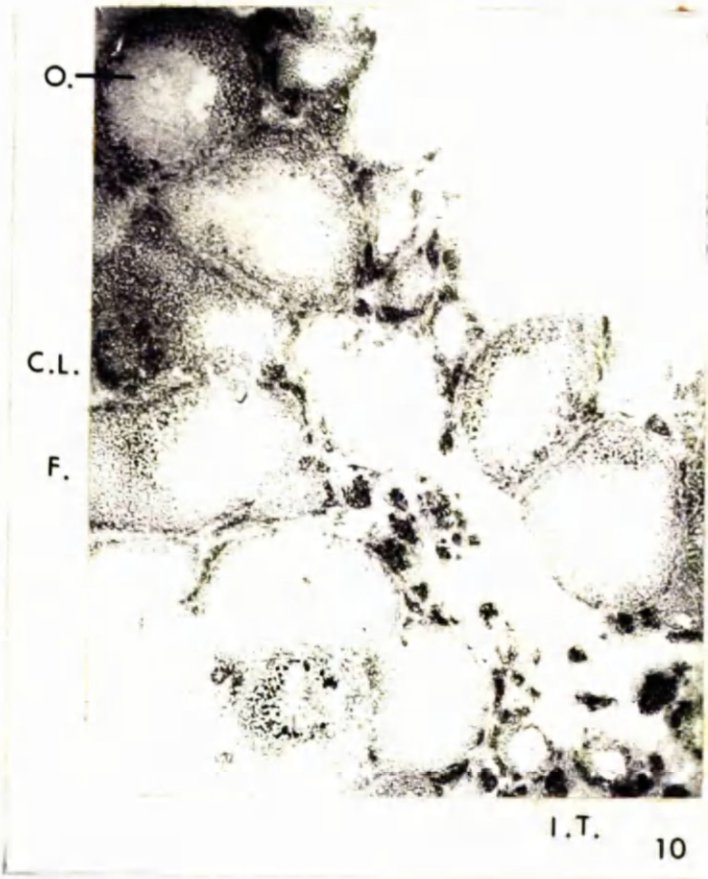
Fig. 11. Intense deposition of diformazan in the granulosa of mouse atretic follicles when incubated with 3β-, 16β-dihydroxyandrost-5-ene-3-methyl ether. Note the distribution of formazan granules in the periphery of the granulosa cells. (x 180).

Fig. 12. Mono- and di-formazan in the zona fasciculata and zona reticularis of the rat adrenal cortex demonstrating 6β-hydroxysteroid dehydrogenase activity. (x 90).

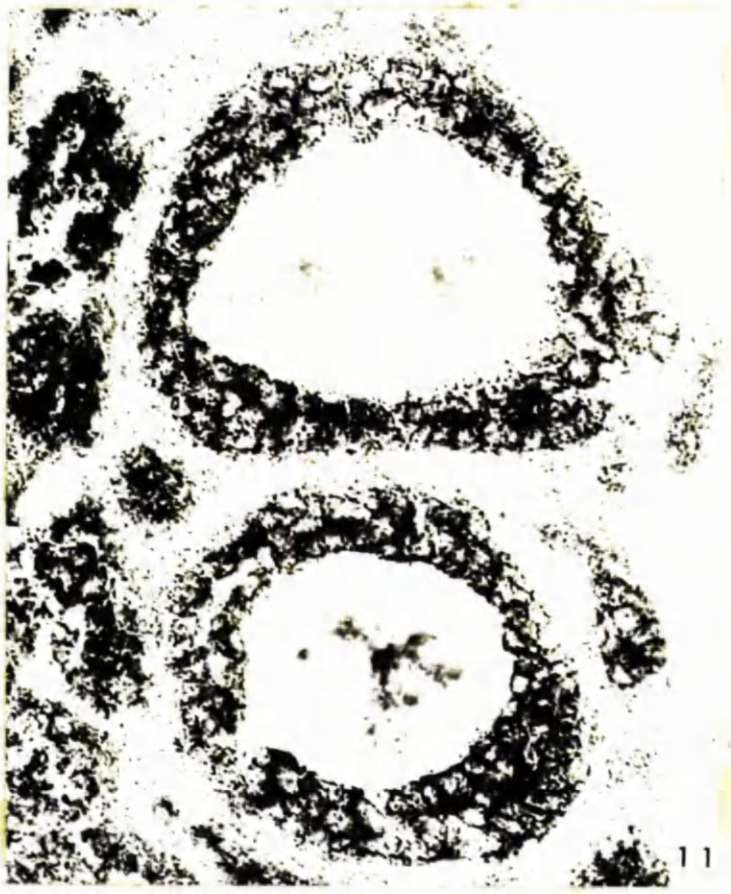
PLATE III



9



10



11

12

PLATE 4.

Fig. 13. Intense 16β-hydroxysteroid dehydrogenase activity in zona glomerulosa and zona reticularis with lesser activity in the zona fasciculata in the rat. (x 90).

Fig. 14. Diformazan heavily deposited in groups of fascicular cells in the rat adrenal medulla after incubation with 3β-,16β-dihydroxyandrost-5-ene-3-methyl ether. (x 120).

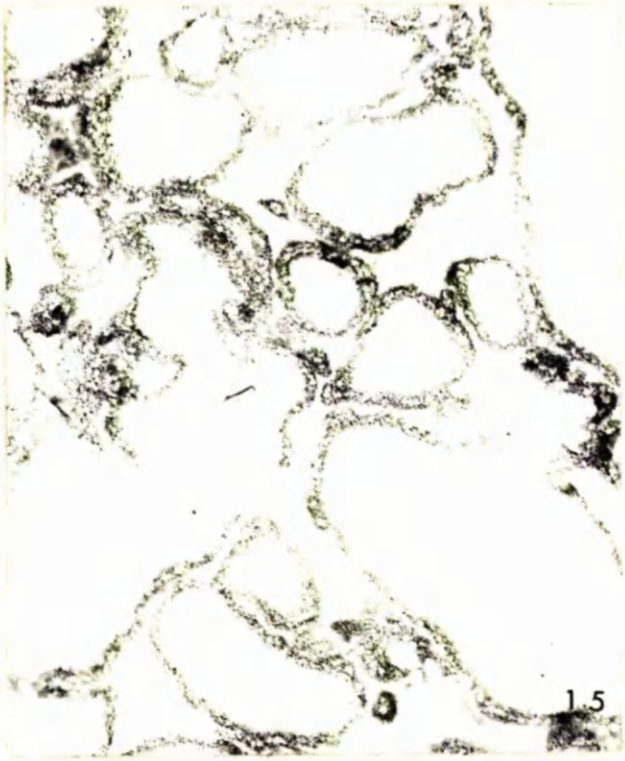
Fig. 15. 3α-Hydroxysteroid dehydrogenase activity in the syntrophoblast of human placenta. (x 90).

Fig. 16. Intense 16β-hydroxysteroid dehydrogenase activity in the syntrophoblast of human placenta. (x 90).

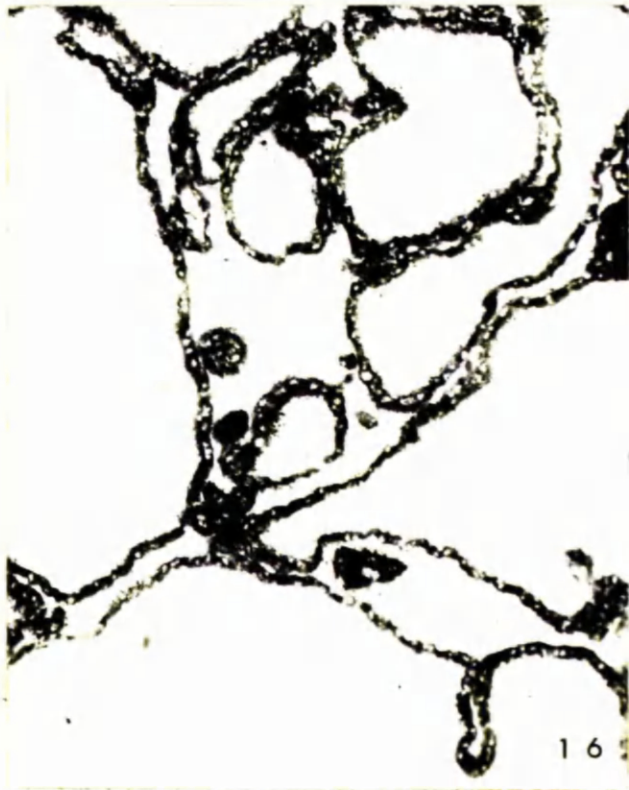
PLATE IV

13

14



15



16

Observations on the Histochemical Distribution of Alcohol Dehydrogenases in Endocrine Tissue

BY

M. M. Ferguson
A. H. Baillie
K. C. Calman
D. McK. Hart

Department of Anatomy, University of Glasgow.

Histochemical studies of alcohol dehydrogenases in rodents using various alcohols (Ferguson,1965) revealed no obvious differences in the sites of alcohol utilisation. Primary and secondary alcohols in general tissues were utilised at the same sites; the present investigation deals with the histochemical variations in sites of utilisation of different alcohols in steroid producing endocrine glands.

Human term placenta obtained at Caesarean section and rat (Royal Wistar) adrenal glands, testes and ovaries were frozen on solid carbon dioxide and the tissues were prepared and incubated as described by Ferguson (1965), using the following alcohols (final conc. 5%):- methanol, ethanol, propanol, butanol, pentanol, hexanol, heptanol, octanol, nonanol, decanol, undecanol, dodecanol, tridecanol, phenol, hydroquinone, 2-ethoxy-ethanol, allyl alcohol, 2-methyl-butan-1-ol, 3-methyl-butan-1-ol, nonyl alcohol, tertiary butanol, tertiary butyl carbonol, isopropanol, secondary butanol, pentan-2-ol, pentan-3-ol, octan-2-ol, cyclohexanol, ethylene glycol, propylene glycol, glycerol, butan-1: 3-diol, butan-1: 4-diol, pinacol and furfuryl alcohol.

Control/

TABLE I.

Alcohol Substrate.	Human Placenta	Rat Adrenal	Rat Ovary	Rat Testis
Methanol	-	-	-	-
Ethanol	-	F. R.	CL.	T.
Propanol	-	G. F. R.	O. G. TI. I. CL. GE	L. T.
Butanol	-	G. F. R.	O. G. TI. I. CL. GE.	L. T.
Pentanol	-	G. F. R.	O. G. TI. I. CL. GE.	L. T.
Hexanol	-	G. F. R.	O. G. TI. I. CL. GE.	L. T.
Heptanol	-	G. F. R.	O. G. TU. I. CL. GE.	L. T.
Octanol	-	F. R.	O. G. TI. I. CL. GE.	L. T.
Nonanol	-	-	O. G. TI. I. CL. GE.	-
Decanol	-	-	-	-
Undecanol	-	-	-	-
Dodecanol	-	-	-	-
Tridecanol	-	-	-	-
Phenol	-	-	-	-
Hydroquinone	-	-	-	-
2-Ethoxyethanol	-	G. F. R.	O. G. TI. I. CL. GE.	L. T.
Allyl alcohol	-	G. F. R.	O. G. TI. I. CL. GE.	L. T.
2-Methyl-butan-1-ol	-	-	-	T.
3-Methyl-butan-1-ol	-	G. F. R.	O. G. TI. I. CL. GE.	L. T.
Benzyl alcohol	-	-	O. G. TI. I. CL. GE.	L. T.
Tertiary butanol	-	-	-	-
Tertiary butyl carbinol	-	-	-	-
Isopropanol	-	G. F. R.	G. TI. I.	-
Secondary butanol	-	G. F. R.	CL.	-
Pentan-2-ol	T	-	O. G. TI. I. CL. GE.	T.
Pentan-3-ol	-	-	CL.	T.
Octan-2-ol	T	G. F. R.	O. G. TI. I. CL. GE.	L. T.
Cyclohexanol	-	-	-	-
Ethylene glycol	-	-	-	-
Propylene glycol	-	G. F. R.	O. G. TI. I. CL. GE.	L. T.
Glycerol	-	-	-	-
Butan-1:3-diol	-	-	-	-
Butan-1:4-diol	-	-	CL.	-
Pinacol	-	-	-	-
Furfuryl alcohol	++	G. F. R.	O. G. TI. I. GE. CL.	L. T.

T = Trophoblast (Placenta) G = Zona Glomerulosa L = Leydig Cells
T = Seminiferous Tubules (Testis) F = Zona Fasciculata O = Ova
I = Interstitial cells R = Zona Reticularis G = Granulosa
GE = Germinal Epithelium CL = Corpus Luteum TI = Theca Interna

Control sections were concurrently incubated in the buffered medium containing no alcohol.

The results are summarised in Table I.

DISCUSSION.

Since Bonnicksen (1951) isolated alcohol dehydrogenase from horse liver the biochemical properties and mechanism of this enzyme have been widely described (Thewell & Bonnicksen, 1951; Thewell & Chance, 1951; Thewell, Nygaard & Bonnicksen, 1955; Thewell, 1958; Winer & Thewell, 1960; Thewell, 1961; Thewell & McKinley -McKee, 1961; Dalziel, 1961; Plane & Thewell, 1961; Snyder, Vogel & Schulman, 1963; Plane & Long, 1963; Dalziel, 1963).

The reactions catalysed by alcohol dehydrogenase, namely -

I Primary alcohols

$$R.CHOH + NAD^+ \rightleftharpoons R.CHO + NADH + H^+$$

and II Secondary Alcohols

$$R.CHOH.R + NAD^+ \rightleftharpoons R.CO.R + NADH + H^+$$

tend to move farther to the right as the pH is increased and to the left when the p.H is decreased. p.H 7.4 was/

was chosen for the medium in this investigation due to maximal aqueous solubility of Nitro BT at this value. At higher values Nitro BT was preferentially soluble in the alcohol which was immiscible with the aqueous medium, so that the optimal p.H for the reaction could not be determined in this system.

Utilisation of alcohols by endocrine tissues can be classified according to the alcohol structure (Ferguson, 1965).

(1) Utilisation of straight-chain alcohols (methanol-tridecanol) improved with increasing chain length to an optimum of six carbon atoms and thereafter the longer chained alcohols were progressively less satisfactory substrates, perhaps because of their decreased solubility in the medium.

(2) Aromatic alcohols (phenol and hydroquinine): these two substrates are not usually dehydrogenated in tissues but are instead conjugated with glucuronic acid prior to excretion.

(3) Cellosolve (2-ethoxy-ethanol) was moderately used by the tissues. However, straight chain alcohols of a corresponding length appeared to be more suitable substrates. Thus the ethereal oxygen atom appears to exert some effect on the terminal hydroxyl group, impeding its dehydrogenation.

(4) The unsaturated alcohol (allyl alcohol) was fairly well utilized but the double bond seemed to impede dehydrogenation to a slight extent in comparison to straight chain alcohols.

(5) Branched alcohols: (iso butanol, 2-methyl butan-1-ol, 3 methyl butan-1-ol, nonyl alcohol, tertiary butanol, tertiary butyl-carbinol). The two tertiary alcohols were not used and the remaining branched alcohols were not as well utilised as were the corresponding straight chain alcohols. 3-Methyl-butan-1-ol tended to be a more suitable substrate than 2-methyl butan-1-ol, presumably due to the increased steric hindrance of the methyl group the nearer it is to the hydroxyl group.

Secondary alcohols (iso-propanol, secondary butanol, pentan-2-ol, pentan-3-ol, octan-2-ol). Alcohol dehydrogenase has been shown to utilise various alcohols biochemically but there do not as yet appear to be any references to different alcohol dehydrogenases. The present findings suggest that primary alcohol dehydrogenase and secondary alcohol dehydrogenase are two separate enzymes. With the primary alcohols, activity was demonstrable in all components of the ovary approximately to an equal extent but not at all in placenta. With secondary alcohols the corpora lutea/

lutea were extremely active whereas the rest of the ovarian tissues were only very slightly reactive. The placenta could also utilize some of the secondary alcohols although the primary alcohols were not suitable substrates.

(6) Cyclohexanol was not utilised to any significant extent by the endocrine tissues examined under the conditions of the experiment.

(7) Polyhydric alcohols (ethylene glycol, propylene glycol, glycerol, butan-1:3-diol, butan-1:4-diol, pinacol) did not appear to be utilised as avidly as were the monohydric alcohols.

(8) Furfuryl alcohol. Pentose metabolism involves the formation of furfuryl alcohol as a by-product and as the most notable metabolism of pentoses is connected with nucleic acids it is not surprising that furfuryl alcohol dehydrogenase was observed in all the tissues examined.

From this study it emerges that in any future histochemical investigation into alcohol dehydrogenases at least two substrates would be required; firstly a straight chain primary alcohol such as hexanol, in/

In preference to ethanol, which is the substrate quoted for the demonstration of this enzyme (Pearse, 1960). Secondly a secondary alcohol of a similar chain length to demonstrate secondary alcohol dehydrogenase.

REFERENCES.

BONNICKSEN, R.K. (1951). Acta chem. Scand. 4: 715.

DALZIEL, K. (1961). Biochem. J. 80: 440.

DALZIEL, K. (1963). J. biol. Chem. 238: 2850.

FERGUSON, M.M. (1965). Quart. J. micr. Sci. (In Press).

PLANE, R.A. & LANG, T.V. (1963). Acta chem. Scand. 17. Suppl. 91.

PLANE, R.A. & THEORELL, H. (1961). Acta chem. Scand. 15. 1797.

SNYDER, R., VOGEL, W.H. & SCHULMAN, P. (1963). Biochem. Biophys. Res. Comm. 13: 335.

THEORELL, H. (1958). Advance Enzymol. 20: 31.

THEORELL, H. (1961). Fed. Proc. 20: 967.

THEORELL, H. & BONNICKSEN, R.K. (1951). Acta chem. Scand. 5: 1105.

THEORELL, H. & CHANCE, B. (1951). Acta chem. Scand. 5: 1127.

THEORELL, H. & McKINLEY-McKEE, J.S.M. (1961). Nature. 192: 47.

THEORELL, H.M NYGAARD, A.P. & BONNICKSEN, R.K. (1955). Acta chem. Scand. 9: 1148.

WINER, A.D. & THEORELL, H. (1960). Acta chem. Scand. 14: 1729.

3β-Hydroxysteroid Dehydrogenase in the Adrenal Gland and Placenta.

BY

A. H. Baillie,*
E. H. D. Cameron, †
K. Griffiths, †
D. McK. Hart. ‡

*Department of Anatomy, † Department of Steroid Biochemistry; University of Glasgow; ‡ Department of Obstetrics and Gynaecology, Stobhill Hospital, Glasgow, Scotland.

SUMMARY.

3β-Hydroxysteroid dehydrogenase activity was studied histochemically in human, monkey, and rat adrenal glands and in human placentae. Tissue sections were incubated separately with each of the following substrates: (1) 3β-hydroxypregn-5-en-20-one (pregnenolone); (2) sodium 3β-sulphoxy-pregn-5-en-20-one (pregnenolonesulphate); (3) 3β-acetoxypregn-5-en-20-one (pregnenolone acetate); (4) 3β,16α-dihydroxypregn-5-en-20-one (16α-hydroxy-pregnenolone); (6) ammonium 3β-sulphoxy-17α-hydroxy-pregn-5-en-20-one (17α-hydroxypregnenolone ammonium sulphate); (7) 3β-hydroxyandrost-5-en-17-one (DHA); (8) 3β-sulphoxyandrost-5-en-17-one (DHA sulphate); (9) 3β-acetoxyandrost-5-en-17-one (DHA acetate); (10) androst-5-ene-3β,17β-diol (androstenediol).

The histochemical results obtained with pregnenolone and DHA as substrates resemble those described by other workers. Using pregnenolone sulphate and 17α-hydroxypregnenolone sulphate, a strong histochemical reaction with diformazan deposition/

deposition was found in the zona fasciculata of the adrenals of all species and in the placental syntrophoblast. With DHA sulphate an extremely weak histochemical reaction was obtained with the adrenal zona fasciculata, monoformazan only being deposited. The syntrophoblast, however, showed intense 3β-hydroxysteroid dehydrogenase activity when incubated with DHA sulphate. These results accord with recent findings regarding the secretion and metabolism of 3β-sulphoxysteroids.

A strong histochemical reaction was also obtained in both adrenal and placental tissues using 17α-hydroxypregnenolone, 16α-hydroxypregnenolone, androstenediol, pregnenolone acetate, and DHA acetate. These steroids have not previously been described as substrates for the histochemical demonstration of 3β-hydroxysteroid dehydrogenase in the adrenal or placenta.

INTRODUCTION.

In recent years the histochemical localization of 3β-hydroxysteroid dehydrogenase activity has been/

been well studied in the adrenal cortex (Wattenberg, 1958; Levy, Deane & Rubin, 1959; Cavallero & Chiappino, 1961; Dawson, Pryse-Davies & Snape, 1961) and placenta (Goldberg, Jones & Turner, 1963). This enzyme catalyses the conversion of a steroid Δ^5-3β-hydroxy group to a Δ^5-3-oxogroup and the reduced nicotinamide-adenine dinucleotide ($NADH_2$) formed in the reaction can be coupled to the reduction of a tetrazolium salt by $NADH_2$; lipoamide oxidoreductase. Pregnenolone and DHA have been used as substrates for this histochemical reaction by most workers but 17α-hydroxypregnenolone (Baillie & Griffiths, 1964a), 16α-hydroxypregnenolone, pregnenediol and androstenediol (Baillie & Griffiths, 1965) have been shown to be satisfactory as substrates and in the foetal mouse testis enzyme-substrate specificity has been sufficiently pronounced to suggest the existence of more than one 3β-hydroxysteroid dehydrogenase (Baillie & Griffiths, 1964b). The recent demonstration of sulphokinase activity in adrenal tissue (Cohn, Mulrow & Dunne, 1963; Sneddon & Marrian, 1963; Wallace & Lieberman, 1963) directed attention towards/

towards 3β-sulphoxysteroids, and it has been shown in the Leydig cells of the testis that pregnenolone sulphate and 17α-hydroxypregnenolone sulphate are better utilized in the histochemical demonstration of 3β-hydroxysteroid dehydrogenase activity than the free steroids. A poor reaction only was obtained with DHA sulphate (Baillie & Griffiths, 1965).

In the light of these observations, it was decided to investigate the histochemical reactivity of the adrenal cortex and placenta with particular respect to the sulphates of pregnenolone, 17α-hydroxypregnenolone and DHA.

MATERIAL AND METHODS.

Adrenal glands were obtained from two women operated on for mammary carcinoma, and from two rhesus monkeys and two rats; pieces of placental tissue of about 1 $cm.^3$ were secured from four placentae removed at Caesarean section at term for obstetric reasons not related to placental function. In addition, placental tissue from a normally delivered woman was studied.

Each/

Each tissue sample was immediately frozen on solid carbon dioxide, sectioned at 15 μ in a cryostat maintained at -20°, and the sections attached to clean dry glass slides by transient thawing. Before incubation, the slides were brought to room temperature and dried in air. Sections from each specimen were incubated separately at 37° with the steroids listed below for 30 min. in the medium described by Wattenberg (1958) buffered at pH 7 with either phosphate or phthallate. The final concentration of steroid was 0.1 mM. Nitro-BT (2,2'-di-p-nitrophenyl-5,5'-diphenyl-3,3'-(3,3'-dimethoxy-4,4'diphenylene)-ditetrazolium chloride) (L. Light and Co.) was employed as a final electron acceptor. The steroids, dissolved in propylene glycol, were: (1) pregnenolone, (2) pregnenolone sodium sulphate, (3) pregnenolone acetate, (4) 16α-hydroxypregnenolone, (5) 17α-hydroxypregnenolone, (6) 17α-hydroxypregnenolone ammonium sulphate; (7) DHA, (8) DHA sodium sulphate, (9) DHA acetate, and (10) androstenediol.

Preparation of 3β-sulphoxy-17α-hydroxypregn-5-en-20-one, ammonium salt (17α-hydroxypregnenolone ammonium sulphate).

17α-Hydroxypregnenolone ammonium sulphate was prepared by the method of Sobel & Spoerri (1941). Solvents were removed under reduced pressure and the solid residue was triturated with 15 ml. chloroform in which the sulphating reagent is insoluble. The resulting slurry was filtered on a sintered glass funnel and the residue washed repeatedly with warm chloroform. The chloroform solution (approx. 250 ml.) was then shaken vigorously with 1 vol. of 2N-NH_4OH and after two further extractions with 1 vol. of 2N-NH_4OH the combined ammoniacal layers were washed with 100 ml. of chloroform. The 17α-hydroxypregnenolone ammonium sulphate was extracted from the aqueous medium by the procedure of Edwards, Kellie & Wade, (1953). The ether-ethanol extract was evaporated to dryness in a rotary evaporator and inorganic salts were removed by treating the residue with warm ethanol and filtering. The ethanolic solution was concentrated in vacuo and the 17α-hydroxypregnenolone ammonium sulphate allowed to crystallize out at low temperature. A small amount (200μg.) of the crystalline/

crystalline material was chromatographed on 3 g. of alumina. No 17α-hydroxypregnenolone could be detected in the appropriate fraction with the sulphuric acid-ethanol reagent of Oertel & Eik-Nes (1959) in conditions which would normally detect 2μg. A further 100μ g. of the residue was chromatographed on Whatman No. 1 paper in the 'alkaline system' of Schneider & Lewbart (1956). A single rather streaked spot was detected by the methylene-blue test (Crepy & Judas, 1960) for sulphate esters.

Examination of the sulphate ester by the Oertel & Eik-Nes (1959) reaction gave an assay of 102.8% on the basis of the formula 17α-hydroxy-pregnenolone ammonium sulphate.

RESULTS.

(1) Adrenal Tissue.

Similar histochemical results were obtained with the adrenal glands from the three species studied. Using the conventional substrates pregnenolone and DHA, 3β-hydroxysteroid dehydroge-

nase/

dehydrogenase activity was localized by a heavy blue diformazan deposit in the zona fasciculata of the adrenal cortex (Pl. 1, fig. 1). Only a faintly positive reaction was observed in the zona glomerulosa and no significant formazan deposition was seen in the zona reticularis. A similar result was observed with 17α-hydroxy-pregnenolone.

The results obtained with the sulphates are of particular interest. The diformazan deposition after incubation with pregnenolone sulphate (Pl. 1, fig. 2) and with 17α-hydroxypregnenolone sulphate (Pl. 1, fig. 3) is very similar to that seen with the free steroids. In contrast however (Pl. 1, fig. 4) DHA sulphate was not utilized to any extent and only a slight pink monoformazan deposit could be detected in the zona fasciculata. Heavy diformazan deposition was also observed in the zona fasciculata after incubation with 16α-hydroxypregnenolone (Pl. 2, fig. 5), androst-enediol (Pl. 2, fig. 6) and the 3β-acetoxy derivatives of pregnenolone (Pl. 2, fig. 7) and DHA (Pl. 2, fig. 8).

(2) Placental tissue.

The chorionic villi of the human placenta at term are covered with a single layer of syntrophoblast although occasional cytotrophoblast cells can be recognised after a careful search, particularly on the placental septa.

Using pregnenolone, 17α-hydroxypregnenolone, and DHA as substrates, intense 3β-hydroxysteroid dehydrogenase activity was seen in the syntrophoblast. The cytoplasm was coloured deep pink due to monoformazan deposition and contained abundant dark blue minute diformazan deposits.

Pregnenolone sulphate (Pl. 3, fig. 9) 17α-hydroxypregnenolone sulphate (Pl. 3, fig. 10) and DHA sulphate (Pl. 3, fig. 11) were better utilized than the free steroids in that diformazan deposition occurred more rapidly in the syntrophoblast. With each steroid sulphate intense mono- and diformazan deposition was found in the trophoblast. Similar results were obtained with 16α-hydroxypregnenolone (Pl. 3, fig. 12). Incubation with androstenediol resulted in little diformazan deposition although monoformazan/

monoformazan could be detected in the syntrophoblast (Pl. 3, fig. 13). Use of the 3β-acetoxy derivatives of pregnenolone and DHA (Pl. 3, fig. 14) lead to heavy mono- and diformazan deposition in the syntrophoblast.

DISCUSSION.

Several groups of workers have demonstrated histochemically that 3β-hydroxysteroid dehydrogenase in the adrenal cortex of various animals can utilize the substrates pregnenolone and DHA (Wattenberg, 1958; Levy et al. 1959; Cavallero & Chiappino, 1961; Dawson et al. 1961) and the histochemical distribution in the adrenal cortices of rat, monkey and man, described in the present report is in accord with earlier descriptions. Histochemically the greatest activity of the 3β-hydroxysteroid dehydrogenase is found in the zona fasciculata, and this has been confirmed biochemically with DHA as substrate (Cameron, Magrini & Grant, 1964; Grant, 1964). Our observations indicate that the same histochemical distribution is obtained/

obtained with 17α-hydroxypregnenolone, androstenediol and 16α-hydroxypregnenolone as substrates.

Recently evidence has been obtained that DHA is secreted by the human adrenal cortex, mainly as the sulphate ester (Baulieu, 1960; Vande Wiele, MacDonald, Bolté & Lieberman, 1963) and sulphokinase activity has also been demonstrated in human adrenal tissue (Cohn et al. 1963; Wallace & Lieberman, 1963). DHA sulphate gave only a faintly positive histochemical reaction when used as substrate for the enzyme 3β-hydroxysteroid dehydrogenase in the adrenal cortices of rat, monkey and man. A strong reaction in the zona fasciculata was, however, obtained when the sulphates of pregnenolone and 17α-hydroxypregnenolone were used. This strong reaction was obtained even in a medium containing phosphate ions, known to inhibit sulphatase activity (Roy, 1957; Burstein & Dorfman, 1963). While the precise metabolic role of the steroid sulphates in steroid hormone biosynthesis remains unknown, several reports of the direct metabolism of steroid sulphates have recently/

recently appeared. Of especial interest are the conversions of (a) pregnenolone-^{3}H sulphate-^{35}S to 17α-hydroxypregnenolone-^{3}H sulphate-^{35}S in vitro using homogenates of hyperplastic adrenals (Calvin & Lieberman, 1964), (b) pregnenolone sulphate-^{35}S to DHA sulphate-^{35}S in vivo (Calvin, Vande Wiele & Lieberman, 1963) and (c) cholesterol-^{3}H sulphate-^{35}S to DHA-^{3}H sulphate-^{35}S (Roberts, Bandi, Calvin, Drucker & Lieberman, 1964). In each instance conversion occurred without cleavage of the sulphate ester group.

Whether the presence of a sulphate group influences the binding of a 3β-hydroxysteroid dehydrogenase with its substrate as suggested earlier (Baillie & Griffiths, 1965) remains to be investigated. Calvin & Lieberman (1964) in experiments with human adrenal tissue did not demonstrate ready transformation of pregnenolone sulphate to Δ^{4}-3 oxosteroids. The limited ability of the adrenal cortex to metabolize DHA sulphate, suggested from our observations, might account for the secretion of this conjugate by the adrenal gland.

An/

An active metabolic role for DHA sulphate was suggested by Roberts, Vande Wiele & Lieberman (1961) who observed that the administered conjugate can be cleaved and further metabolized in man. Baulieu, Wallace & Lieberman (1963) indicated that DHA sulphate may be utilized by the placenta for oestrogen formation, and Bolté, Mancuso, Eriksson, Wiqvist & Diczfalusy (1964a, b, c) have confirmed that this sulphate is an oestrogen precursor when perfused through human placenta in situ. Similarly, the utilization of circulating DHA sulphate for oestrogen synthesis during late pregnancy has been established (Baulieu & Dray, 1963; Siiteri & MacDonald, 1963). In these circumstances the fact that DHA sulphate is histochemically well utilized by the syntrophoblast of the placenta, in contrast to the poor reaction in the adrenal cortex, is of particular interest. It has also been observed (Baillie & Griffiths, 1965) that DHA sulphate gives a very weak reaction in the Leydig cells of mouse testis, whereas heavy formazan deposition occurs in the seminiferous epithelium of the mature gonad. It was therefore suggested that the seminiferous tubules may be a target organ for circulating/

circulating DHA sulphate. A similar role might be postulated in the female for the placenta.

The present findings accord with the distribution of 3β-hydroxysteroid dehydrogenase in human placental tissue at term described by Lobel, Deane & Romney (1962) who used DHA as substrate. No significant differences were observed in the distribution pattern obtained with the other free steroids. The biochemical significance of the results with the 3β-acetoxy steroids requires further investigation.

ACKNOWLEDGEMENTS.

The authors are indebted to Professor Goligher, University Department of Surgery, Leeds General Infirmary, for the adrenal glands; and to Dr. D. McKay Hart, Snr., Director of Obstetrics and Gynaecology, Stobhill Hospital, Glasgow for placentae; one of us (E.H.D.C.) is indebted to I.C.I. for a personal grant.

REFERENCES.

BAILLIE, A.H. & GRIFFITHS, K. (1964a). 3β-Hydroxysteroid dehydrogenase activity in the mouse Leydig cell. J. Endocrin. 29, 9 - 17.

BAILLIE, A.H. & GRIFFITHS, K. (1964b). 3β-Hydroxysteroid dehydrogenase in the foetal mouse Leydig cell. J. Endocrin. 31, 63 - 66.

BAILLIE, A.H. & GRIFFITHS, K. (1965). Further observations on 3β-hydroxysteroid dehydrogenase activity in the mouse Leydig cell. J. Endocrin. 31, 207 - 215.

BAULIEU, E.E. (1960). Ester-sulfates des stéroides hormonaux. Isolement de l'ester-sulfate de 5-androstène-3β-ol-17-one (déhydroépiandrosterone) dans une tumeur cortico-surrénalienne. Absence du stéroide libre. C.R. Acad. Sci., Paris, 251, 1421 - 1423.

BAULIEU, E.E. & DRAY, F. (1963). Conversion of ^{3}H-dehydroepiandrosterone (3β-hydroxy-Δ^{5}-androstene-17-one) sulfate to ^{3}H-estrogens in normal pregnant women. J. clin. Endocr. 23, 1298 - 1301.

BAULIEU, E.E., WALLACE, E. & LIEBERMAN, S. (1963). The conversion in vitro of Δ^5-androstene-3β, 17β-diol-17-H^3 to testosterone-17-H^3 by human adrenal and placental tissue. J. biol. Chem. 238, 1316 - 1319.

BOLTE, E., MANCUSO, S., ERIKSSON, G., WIQVIST, N. & DICZFALUSY, E. (1964a). Studies on the aromatization of neutral steroids in pregnant women. I. Aromatisation of C-19 steroids by placentas perfused in situ. Acta endocr., Kbh., 45, 535 - 559.

BOLTE, E., MANCUSO, S., ERIKSSON, G., WIQVIST, N. & DICZFALUSY, E. (1964b). Studies on the aromatization of neutral steroids in pregnant women. 2. Aromatization of dehydroepiandrosterone and of its sulphate administered simultaneously into a uterine artery. Acta endocr., Kbh., 45, 560-575.

BOLTE, E., MANCUSO, S., ERIKSSON, G., WIQVIST, N. & DICZFALUSY, E. (1964c). Studies on the aromatization of neutral steroids in pregnant women. 3. Over-all aromatization of dehydroepiandrosterone sulphate circulating in the foetal and maternal compartments. Acta endocr., Kbh., 45, 576 - 599.

BURSTEIN, S. & DORFMAN, R.I. (1963). Determination of mammalian steroid sulphatase with 7α-^{3}H-3β-hydroxyandrost-5-en-17-one sulfate. J. biol. Chem. 238, 1656 - 1660.

CALVIN, H.I. & LIEBERMAN, S. (1964). Evidence that steroid sulfates serve as biosynthetic intermediates. II. In vitro conversion of pregnenolone-^{3}H sulfate-^{35}S to 17α-hydroxy-pregnenolone-^{3}H sulfate-^{35}S. Biochemistry. 4, 259 - 264.

CALVIN, H. I., VANDE WIELE, R.L. & LIEBERMAN, S. (1963). Evidence that steroid sulfates serve as biosynthetic intermediates: In vitro conversion of pregnenolone-sulfate-^{35}S to dehydroisoandrosterone-sulfate-^{35}S. Biochemistry. 2, 648 - 653.

CAMERON, E.H.D., MAGRINI, U. & GRANT, J.K. (1964). Biochemistry of the histologically defined zones in the horse adrenal cortex: 3β-hydroxy-steroid dehydrogenase activity. F.E.B.S. 1st Meeting Abstracts, London, 1964, pp. 101 - 102.

CAVALLERO, C. & CHIAPPINO, G. (1961). Istologia ed istochimica della corteccia surrenale negli ipercorticalismi. IX Congresso Nationale. Simposia su la m. di Cushing, Soc. ital. Endocrinol. p.5. Pisa: Arti Graphiche Pacini Mariotti.

COHN, G.L., MULROW, P.J. & DUNNE, V.C. (1963). In vitro synthesis of dehydroepiandrosterone sulfate by an adrenal adenoma. J. clin. Endocr. 23, 671 - 676.

CREPY, D. & JUDAS, O. (1960). Determination qualitative et quantitative des sulfates des stéroides à l'aide du bleu methylene. II. Détection des sulfates des stéroides en chromatographie sur papier. Rev. franç. Étud. clin. biol. 5, 284 - 286.

DAWSON, I.M.P., PRYSE-DAVIES, J. & SNAPE, I.M.(1961). The histochemical demonstration of steroid 3β-ol-dehydrogenase and diphosphopyridine nucleotide diaphorase in the adrenal cortex. Biochem. J. 78, 16P.

EDWARDS, R.W.H., KELLIE, A.E. & WADE, A.P. (1953). The extraction and oxidationof urinary steroid conjugates. Mem. Soc. Endocrin. no. 2, 53 - 63.

GOLDBERG, B., JONES, G.E. & TURNER, D.A. (1963). Steroid 3β-ol dehydrogenase activity in human endocrine tissues. Amer. J. Obstet. Gynec. 86, 349 - 359.

GRANT, J.K. (1963). In The structure and function of corticosteroids, pp. 139 - 145. Eds. J. R. Pasqualini & M.F. Jayle. London: Academic Press.

LEVY, H., DEANE, H.W. & RUBIN, B.L. (1959). Visualisation of steroid-3β-ol-dehydrogenase activity in tissues of intact and hypophysectomised rats. Endocrinology. 65, 932 - 943.

LOBEL, B.L., DEANE, H.W. & ROMNEY, S.L. (1962). Enzymic histochemistry of villous portion of human placenta from six weeks of gestation to term. Amer. J. Obstet. Gynec. 83, 295 - 299.

OERTEL, G.W. & EIK-NES, K.B. (1959). Determination of Δ^5-3-beta steroids. Analyt. Chem. 31, 98.

ROBERTS, K.D., BANDI, L., CALVIN, H.I., DRUCKER, W.D. & LIEBERMAN, S. (1964). Evidence that cholesterol sulfate is a precursor of steroid hormones. J. Amer. chem. Soc. 86, 958 - 959.

ROBERTS, K.D., VANDE WIELE, R.L. & LIEBERMAN, S. (1961). The conversion in vivo of dehydroisoandrosterone sulfate to androsterone and etiocholanolone glucuronidates. J. biol. Chem. 236, 2213 - 2215.

ROY, A.B. (1957). The kinetics of sulphatase A. Experientia, Basel, 13, 32 - 34.

SCHNEIDER, J.J. & LEWBART, M.L. (1956). Enzymatic synthesis of steroid sulfates. J. biol. Chem. 222, 787 - 794.

SIITERI, P.K. & MACDONALD, P.C. (1963). The utilization of circulating dehydroepiandrosterone sulfate for oestrogen synthesis during human pregnancy. Steroids, 2, 713 - 730.

SNEDDON, A. & MARRIAN, G.F. (1963). Sulphation of oestrone in vitro by bovine adrenal tissue. Biochem. J. 86, 385 - 388.

SOBEL, A.E. & SPOERRI, P.E. (1941). Steryl sulfates. I. Preparation and properties. J. Amer. chem. Soc. 63, 1259 - 1261.

VANDE WIELE, R.L., MACDONALD, P.C., BOLTE, E. & LIEBERMAN, S. (1963). Precursors of the urinary 11-desoxy-17-ketosteroids; estimation of the secretory rate of dehydroisoandrosterone. J. clin. Endocr. 22, 1207 - 1221.

WALLACE, E.Z. & LIEBERMAN, S. (1963). Biosynthesis of dehydroisoandrosterone sulfate by human adrenocortical tissue. J. clin. Endocr. 23, 90 - 94.

WATTENBERG, L.W. (1958). Microscopic histochemical demonstration of steroid-3β-ol dehydrogenase in tissue sections. J. Histochem. Cytochem. 6, 225 - 232.

DESCRIPTION OF PLATE.

PLATE I.

Fig. 1. 3β-Hydroxysteroid dehydrogenase in the zona fasciculata of the human adrenal cortex using DHA as substrate. (x 130).

Fig. 2. Intense diformazan deposition with pregnenolone sulphate as substrate in the zona fasciculata of the monkey adrenal cortex. (x 90).

Fig. 3. Diformazan deposition in the zona fasciculata of the human adrenal cortex after incubation with 17β-hydroxypregnenolone sulphate. (x 180).

Fig. 4. Monoformazan in the zona fasciculata of the monkey adrenal cortex after incubation with DHA sulphate (x 90).

PLATE I


1


2


3


4

PLATE 2.

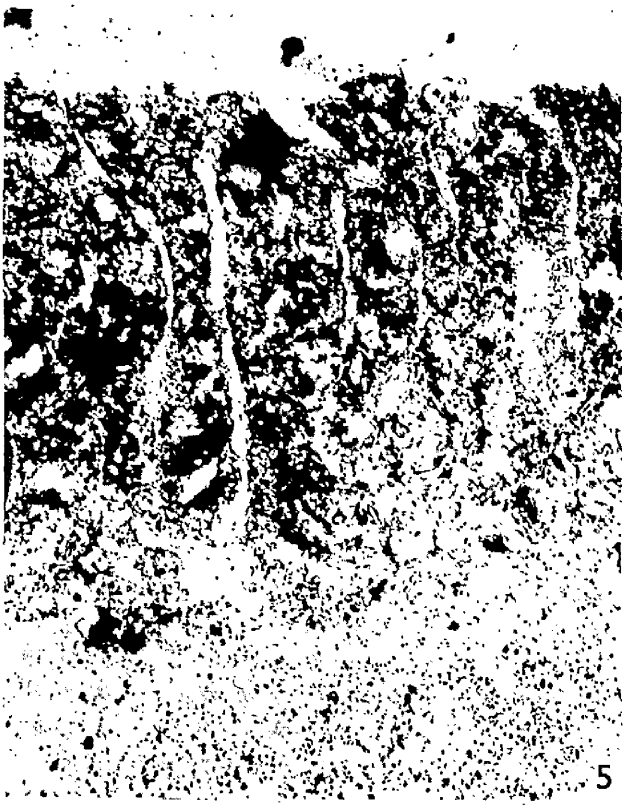
Fig. 5. Deposition of diformazan in the zona fasciculata of the human adrenal cortex after incubation with 16α-hydroxypregnenolone. (x 90).

Fig. 6. Deposition of diformazan in the zona fasciculata of the human adrenal cortex after incubation with androstenediol as substrate. (x 90).

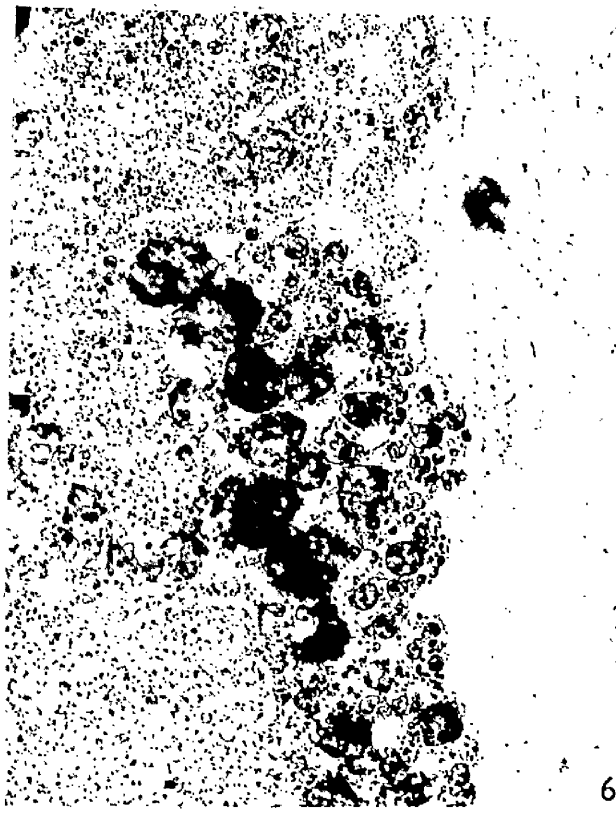
Fig. 7. Diformazan deposition in the monkey adrenal cortex after incubation with pregnenolone acetate. (x 90).

Fig. 8. Diformazan distribution in the human adrenal cortex after incubation with DHA acetate. (x 130).

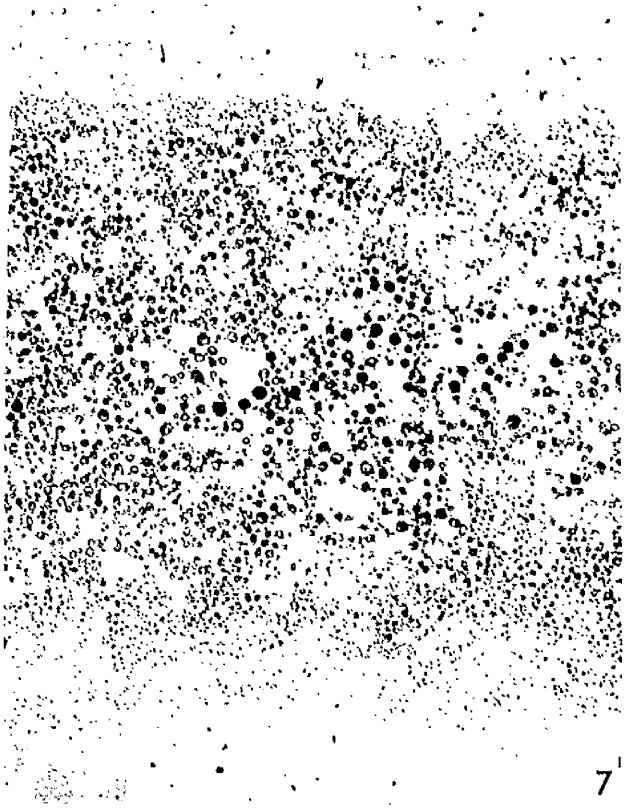
PLATE II



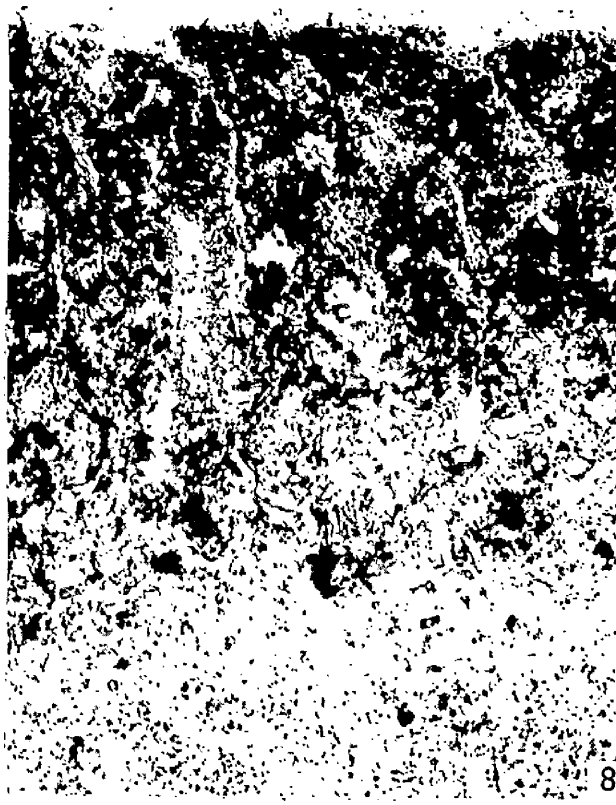
5



6



7



8

PLATE 3.

Fig. 9. Diformazan deposition in the syntrophoblast after incubation of human placental tissue with pregnenolone sulphate. (x 90).

Fig. 10. 3β-Hydroxysteroid dehydrogenase activity in the syntrophoblast of human placental tissue after incubation with 17α-hydroxypregnenolone sulphate. (x 90).

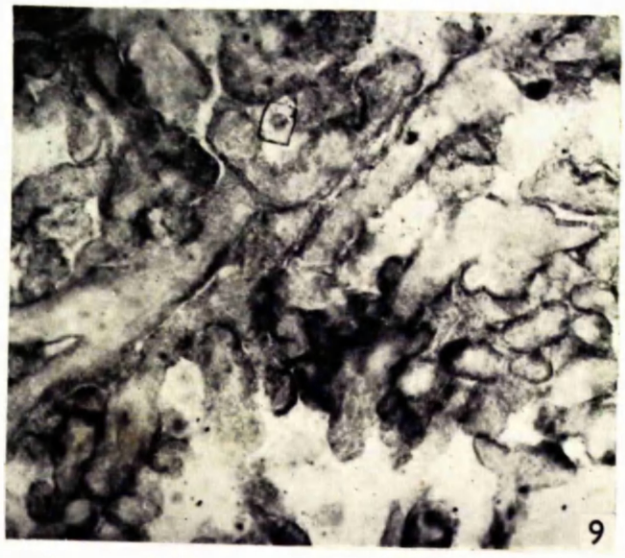
Fig. 11. 3β-Hydroxysteroid dehydrogenase activity in the syntrophoblast of human placental tissue after incubation with DHA sulphate. (x 90).

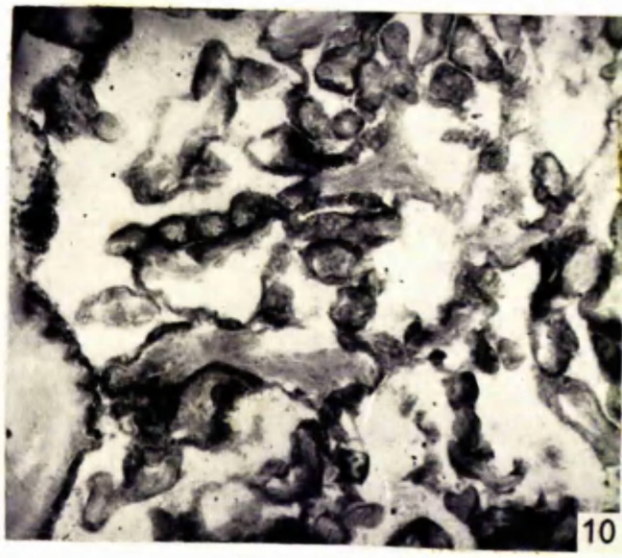
Fig. 12. Formazan deposition in the syntrophoblast of human placental tissue after incubation with 16α-hydroxypregnenolone. (x 90).

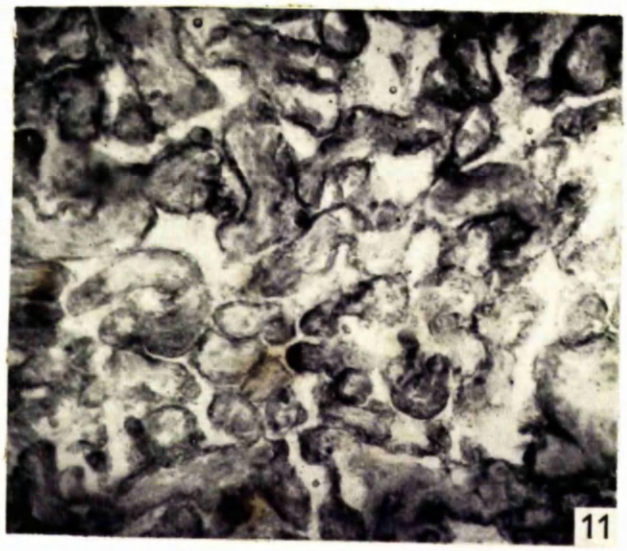
Fig. 13. A few diformazan particles in the syntrophoblast of human placental tissue after incubation with androstenediol. (x 280).

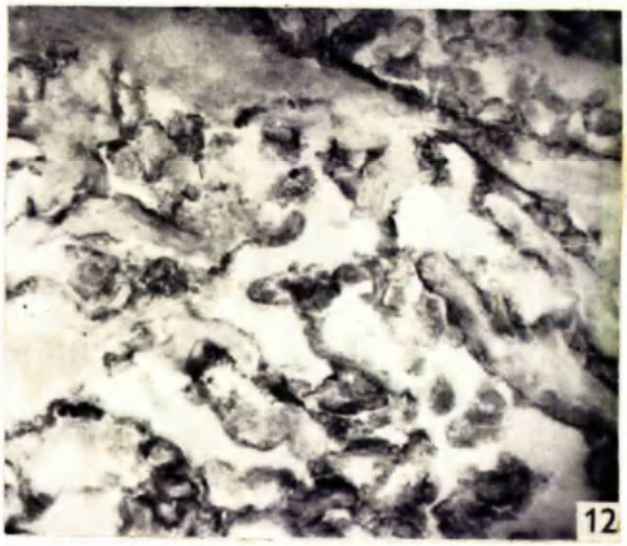
Fig. 14. Deposition of mono- and diformazan in the syntrophoblast of human placental tissue after incubation with DHA acetate. (x 90).

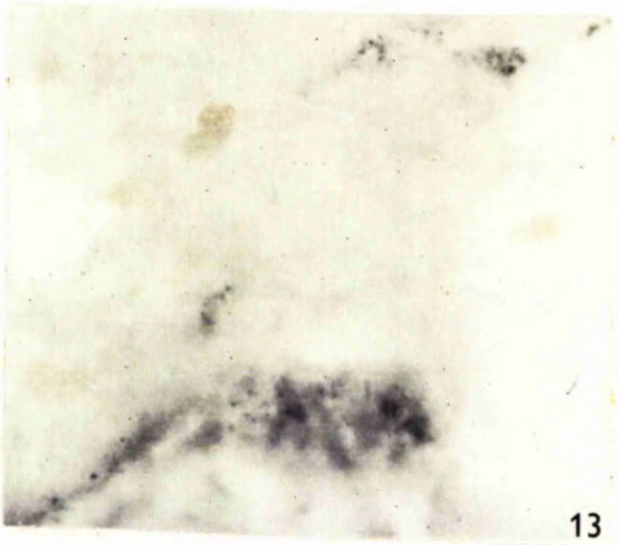
PLATE III

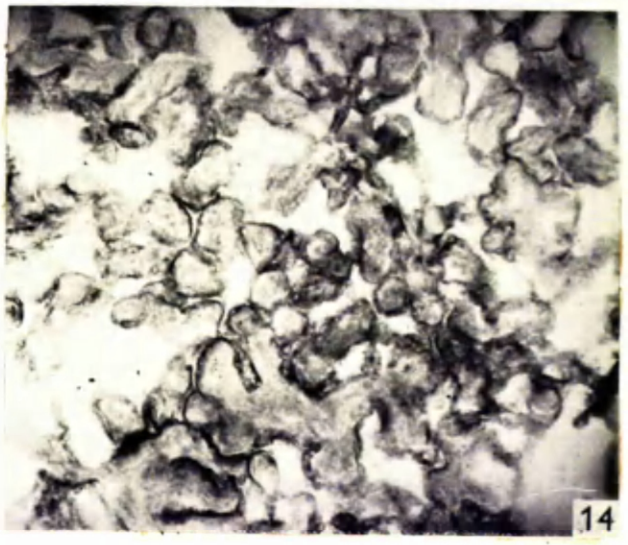

9


10


11


12


13


14

Hydroxysteroid Dehydrogenase Development in Mouse Adrenal and Gonads.

By

D. McK. Hart

A. H. Baillie

K. C. Calman

M. M. Ferguson.

Department of Anatomy, University of Glasgow.

SUMMARY.

The age distribution of hydroxysteroid dehydrogenases (H.S.D.s) in the mouse adrenal cortex and gonads, was studied histochemically between the 9th day of intrauterine life and the end of the 10th week of postnatal life using 3α-, 3β-, 6β-, 11α-, 11β-, 12α-, 16α-, 16β-, 17α-, 17β-, 20β-, 21 and 24-hydroxysteroids as substrates.

On the 12th day of embryonic life 16β-H.S.D. was demonstrable in the mouse adrenal cortex; 3β-H.S.D. became apparent on the 15th embryonic day. 3β- and 16β-H.S.D.s are by far the most active in this tissue; 11β-H.S.D. showed weak activity; 6β-, 11α-, 12α-, 17α-, 20β-, 21- and 24-hydroxysteroids were not utilised histochemically. Zone differences in H.S.D. activity are quantitative rather than qualitative.

16β-H.S.D. was active in the genital ridge from the 9th day and 3β-H.S.D. from the 11th day in the indifferent gonad. 11β-, 3α- and 17β-H.SD.s appeared in the testis on the 16th, 19th and 20th days of embryonic life respectively. After birth the volume of interstitial tissue undertaking hydroxysteroid dehydrogenation increased regularly with age. 6β-H.S.D. was first demonstrated 7 weeks post-partum.

3β- And 16β-H.S.D. activities were seen in the ovary at the 12th day in utero.

At birth, 16β-H.S.D. activity was seen in atretic ova and these showed 3α-, 11β- and 17β-H.S.D. activities by the third postnatal week.

Different patterns of granulosa cell H.S.D. activity were seen; some follicles contained no diformazan, others showed a deposit throughout the granulosa, while in a third group an outer, intensely reactive zone contrasted with the inner, weakly reactive region. Corpora lutea possessed 3α-, 3β-, 6β-, 11β-, 12α-, 16α-, 16β-, 17β- and 20β-H.S.D. activities.

The germinal epithelium showed only 16α-, 16β- and 17β-H.S.D. activities, first demonstrable in the 6th week of postnatal life.

Weak 3α- and 11β- and strong 3β- and 16β-H.S.D. activities were present in ovarian interstitial cells at birth and 17β-H.S.D. activity appeared during the 3rd week.

INTRODUCTION.

Techniques have been described for the histochemical demonstration of diverse hydroxysteroid dehydrogenases (Wattenberg, 1958; Pearson & Grose, 1959A, B; Balogh, 1964; Baillie, Calman, Ferguson & Hart, 1965A, B; Baillie, Ferguson, Calman & Hart, 1965). This investigation was undertaken to determine at what stage in the development of the mouse adrenal cortex and gonads these enzymes become demonstrable histochemically.

MATERIAL AND METHODS.

Two female Swiss white mice were killed by decapitation on each of the 9th to 21st days of pregnancy and the uteri removed immediately. The younger embryos were frozen on solid carbon dioxide in their containing segments of uterus, the larger ones after removal from the uteri; all were frozen within 3 minutes of interruption of their blood supply. The adrenal glands and gonads were located by serial sectioning of embryonic trunks at 12 in a cryostat (-25°C.) and staining every 10th section/

section with haematoxylin and eosin.

The requisite sections, thus defined, were retained for incubation.

Twenty-two male and twenty-two female Swiss white mice were killed by decapitation in groups of 2 of each sex at weekly intervals from birth until the end of the 10th week of post-natal life.

The adrenal glands and ovaries were excised from the female mice and the testes from the males within 1 minute of death and immediately frozen on solid carbon dioxide. These organs were sectioned at 10μ in a cryostat maintained at -25° and the sections attached to clean, dry glass coverslips by momentary thawing and incubated individually to demonstrate N.A.D.-dependent H.S.D. activity using techniques described previously (Baillie et al. 1965b). Dimethyl formamide was used as the vehicle for the following steroid substrates:-

(1) 3α-hydroxy-5α-androstan-17-one (Steraloids)

(2) 3α-hydroxy-5β-androstan-17-one (Steraloids)

(3) D.H.A.
(3β-hydroxyandrost-5-ene-17-one: Steraloids)

(4) Pregnenolone
(3β-hydroxypregn-5-ene-20-one: Steraloids)

(5) 6 /

(5) 6β-hydroxyprogesterone
(6β-hydroxypregn-4-ene-3, 20-dione: Steraloids)

(6) 11α-hydroxyprogesterone
(11α-hydroxypregn-4-ene-3, 20-dione: Steraloids)

(7) 11β-hydroxyprogesterone
(11β-hydroxypregn-4-ene-3, 20-dione: Steraloids)

(8) 12α-hydroxyprogesterone
(12α-hydroxypregn-4-ene-3, 20-dione: Steraloids)

(9) 16α-hydroxyoestrone
(1,3,5,(10)estratrien-3, 16α-diol-17-one:Steraloids)

(10) Oestrone (as control)
(1,3,5,(10)-estratrien-3-ol-17-one: Steraloids)

(11) 16α-hydroxyprogesterone
(16α-hydroxypregn-4-ene-3, 20-dione: M.R.C.)

(12) 3β-, 16β-dihydroxyandrost-5-ene,3-methyl ether
(Steraloids)

(13) 3β-hydroxyandrost-5-ene-16-one, 3-methyl ether
(as control) (Steraloids)

(14) 17α-hydroxyandrost-4-ene-3-one (Steraloids)

(15) Testosterone
(17β-hydroxyandrost-4-ene-3-one: Steraloids)

(16) 20β-hydroxyprogesterone
(20 -hydroxypregn-4-ene-3-one: Steraloids)

(17) 21-hydroxyprogesterone
(21-hydroxypregn-4-ene-3-one: Steraloids)

(18) 24-hydroxy-5β-cholan (Steraloids)

As an additional control, sections were incubated in the complete medium including steroid solvent (dimethyl formamide) but devoid of steroid.

Testicular/

Testicular volume for this closely inbred strain of mouse was established by Baillie (1961) and is summarised for convenience in Table IV as are the 11β-H.S.D. results which are described elsewhere (Baillie, Ferguson et al., 1965).

To enable direct comparison of utilisation of individual hydroxysteroids to be made in post-natal testis a quantitative analysis of the interstitial tissue of each age group was made. The reactive volume of Leydig tissue with a given H.S.D. was derived as a percentage, from which, together with the testicular volume, the total volume of Leydig tissue at each age with the ability to utilise a given steroid substrate was calculated.

RESULTS.

The results of this investigation are detailed in Tables I to V and Figures 1 to 11.

Little monoformazan was noted in foetal sections, the tetrazolium dye being deposited mainly in the form of blue diformazan particles. In general, much heavier dye deposition occurred in the adrenal cortex than in either the testis or the ovary.

Before/

TABLE I.

oetal ge in Days	TESTIS				OVARY				ADRENAL				GENITAL RIDGE			
	3α	3β	11β	16β	17β	3α	3β	11β	16β	17β	3α	3β	11β	16β	17β	
- 11											-	tr	-	+	-	
2 - 14	-	+	-	+	-	-	+	-	+	-	tr	-	+	-	-	
5 - 16	tr	+	+	+	-	-	+	-	+	-	-	+	-	+	-	
7 - 19	+	+	+	++	tr	tr	+	-	+	-	tr	+	+	+	-	
0 - 21	+	+	+	++	+	+	+	-	+	-	tr	+	+	+	-	

Hydroxysteroid dehydrogenase activity in foetal tissues.

tr = trace.

TABLE II

Position of Hydroxyl Group*

Age in Weeks From Birth	3α 5α config.			3α 5β config.			3β			11β			16α			16β			17β		
	G.	F.	X.	G.	F.	X.	G.	F.	X.	G.	F.	X.	G.	F.	X.	G.	F.	X.	G.	F.	X.
0 - 2	-	tr	-	tr	+	tr	+	++	+	tr	+	tr	tr	+	tr	++	++	++	-	-	-
3 - 4	-	tr	-	tr	+	tr	+	++	+	-	tr	-	tr	+	tr	+++	+++	++	-	tr	-
5 - 10	-	-	-	tr	tr	-	+	++	+	-	tr	-	tr	+	tr	+++	+++	++	-	tr	-

* 6β-, 11α-, 12α-, 17α-, 20β-, 21- and 24-hydroxysteroids did not give any colour in any zone.

The development of hydroxysteroid dehydrogenase activity in the post-natal female mouse adrenal.

G = Zona Glomerulosa

F = Zona Fasciculata

X = X Zone

TABLE III.

THECA INTERNA

Age in Weeks	3α *	3β	6β	11β	12α	16α	16β	17β	20β
0 - 4	NOT RECOGNISABLE								
4 - 6	tr	+	-	tr	-	tr	++	+	tr
7 - 10	tr	++	+	+	tr	tr	++	+	tr

CORPORA LUTEA

0 - 5	NOT RECOGNISABLE								
6 - 10	tr	++	+	+	tr	tr	++	+	tr

GRANULOSA

0 - 1	tr	++	-	-	-	-	++	-	-
2 - 6	tr	++	-	+	-	tr	++	+	-
7 - 10	tr	++	+	+	tr	tr	++	+	tr

INTERSTITIAL TISSUE

0 - 2	tr	++	-	tr	-	-	++	-	-
3 - 5	tr	++	-	+	-	tr	++	+	-
6 - 10	tr	++	+	+	tr	tr	++	+	tr

OVA

0 - 1	-	-	-	-	-	-	+	-	-
2 - 3	tr	tr	-	tr	-	-	+	+	-
4 - 6	tr	tr	-	+	-	tr	+	+	-
7 - 10	tr	tr	+	+	-	tr	+	+	-

GERMINAL EPITHELIUM

Age in Weeks	*3α	3β	6β	11β	12α	16α	16β	17β	20β
0 - 5	-	-	-	-	-	-	-	-	-
6 - 8	-	-	-	-	-	-	-	+	-
9 - 10	-	-	-	-	-	tr	+	+	-

The development of hydroxysteroid dehydrogenase activity in the post-natal mouse ovary.

* = 5β configuration

3α-hydroxy-5α-androstan-17-one and 11α-, 17α-, 21- and 24-hydroxysteroids did not give any colour in any component of post-natal mouse ovary.

TABLE IV.

osition of Hydroxyl Group		3α (5α-config.)	3α (5β-config.)	6β	16β	17β	Testicular Volume in mm³.
ge in Weeks	0	tr	7.6	-	18.7	tr	0.8
	1	tr	1.8	-	11.6	1.6	6.1
	2	tr	1.5	-	6.7	1.3	18.3
	3	tr	2.3	-	6.0	1.3	31.7
	4	tr	2.5	-	5.8	1.2	88.9
	5	tr	2.3	tr	5.8	1.4	169.2
	6	tr	2.0	tr	5.9	1.9	195.4
	7	tr	2.0	tr	5.0	1.9	196.1
	8	tr	2.1	0.9	6.0	1.8	196.1
	9	tr	2.2	1.1	5.2	1.7	196.1
	10	tr	2.1	1.2	5.0	2.0	196.1

The development of hydroxysteroid dehydrogenase activity in the testis, showing reactive Leydig tissue as a percentage of testicular volume.

Inconstant trace activity with 6α-, 11β-, 12α-, 16α-, 17α-, 20β-, 21-and 24-hydroxysteroids as substrates could not be quantitated.

TABLE V.

Age in Weeks:-	0	1	2	3	4	5
Position of Hydroxyl Group						
3α	0.06	6.11	0.27	0.72	2.22	3.9
6β	-	-	-	-	-	-
16β	0.14	0.71	1.2	1.9	5.16	9.81
17β	-	0.09	0.23	0.41	1.01	2.37

Age in Weeks:-	6	7	8	9	10
3α	3.9	3.92	4.01	4.31	4.01
6β	-	-	1.76	2.16	2.36
16β	11.52	11.76	11.76	12.15	11.76
17β	3.71	3.72	3.53	3.33	3.92

The absolute volume of Leydig tissue in $mm.^3$ reactive with 3α-(5β-configuration), 6β-, 16β-, and 17β-hydroxysteroids.

Before the 3rd week of postnatal life granulosa in most ovarian follicles contained an even diformazan deposit throughout the cells; thereafter, different patterns of activity in granulosa emerged. The granulosa of some follicles contained no diformazan, others showed a deposit of diformazan throughout, while the third consisted of two zones of granulosa of different activity; an outer peripheral layer of granulosa cells some four or five cells thick was intensively reactive, the individual tetrazolium particles being arranged in the periphery of the cytoplasm of the granulosa; the inner part of the granulosa was much more weakly reactive and pink monoformazan was deposited evenly throughout the cytoplasm of these granulosa cells.

DISCUSSION.

The adrenal cortex can first be recognised in the 12 day mouse embryo (Waring, 1935). Attempts have been made to establish the time of onset of cortical function in developing adrenal glands using histochemical tests for lipids in the chick (Dawson, 1953), rat (Van Dorp & Deane, 1950; Josimovich, Ladman & Deane, 1954; Lever, 1955) and mouse (Mooge, Bennett & Dean, 1954) but interpretation of functional status in terms of

of lipid content appears to be difficult.

3β-H.S.D. activity was investigated in the adrenal cortex of the mouse by Allen (1960) and in the adrenal glands of various mammals, including the mouse, by Rubin, Deane and Hamilton (1963). Both papers record that the zona glomerulosa and inner part of the zona fasciculata were active, while in the X zone activity was limited to the juxta-medullary portion in the female, appearing between the 13th and 16th days after birth and persisting into adult life, but being absent in the yound male. Allen considers that N.A.D.H. diaphorase activity is not a limiting factor in the study of 3β-H.S.D. in developing mouse adrenal gland (Allen, 1959) and our unpublished observations agree.

The findings in the present investigation of 3β- and 16β-H.S.D.s in the 12 day embryo suggests steroid biosynthesis in the adrenal cortical anlage as soon as it becomes recognisable. 17β-H.S.D. was not detected in foetal or neonatal mouse adrenal in agreement with Allen's (1960) results.

The significance of formazan deposition after incubation/

incubation with the 3α-hydroxysteroid substrates is not wholly clear; unpublished observations in this laboratory suggest that steroid 5α- or 5β-dehydrogenase may be in part responsible for the colour.

The strong fascicular 3β-H.S.D. activity at birth, persisting unchanged into adult life, contrasts with the gradual increase from low activity at birth to the adult level at 21 days reported by Allen (1960). 16β-H.S.D. activity in the zona fasciculata, strong at birth, becomes intense during the third week of life and remained so. In this zone the enzyme appeared to be very soluble, with the reaction taking place mainly in the medium on the surface of the tissue section.

Allen (1959) and Moog et al. (1954), from diaphorase and lipid studies believed that the X zone played an important part in the physiology of the first two weeks of postnatal life, but the results of the present investigation indicate that functional activity is not limited to such a short period.

No obvious difference in the pattern of hydroxysteroid dehydrogenase is noted between the zona fasciculata and the X zone, differences in activity being quantitative/

quantitative rather than qualitative, and this accords well with the observation by Vinson and Chester Jones (1964) that the presence or absence of the X zone did not appear to influence the ratio of oestrogens to adrenocorticosteroids produced from progesterone by mouse adrenal glands in vivo. Chester Jones (1950) suggested that the X zone probably does not produce a steroid hormone, at least not in the same way as other tissues producing such hormones, but the presence of 3β- and other H.S.D.s in this zone does not support this view.

The mouse ovary can be recognised on the 12th day of foetal life by a process of exclusion, the testis being differentiated at this stage while the ovary remains indifferent (Brambell, 1927). The medulla becomes apparent on the 16th day and oöcytes surrounded by 3 or 4 flattened epithelial cells can be seen on the 18th day.

3β-H.S.D. has been demonstrated in 8 day post-natal rat ovaries (Presl, Jirasek, Horsky & Henzl, 1965) and in adult mouse ovaries (Ferguson, 1965A). The present investigation with mouse ovary reveals extremely/

extremely weak 3β-H.S.D. activity as early as the 12th day of foetal life, at which stage 16β-H.S.D. can also be detected.

The histochemical reactions observed in the foetal ovary are very much weaker than those seen in the foetal testis and this may reflect the widely known fact that androgens are required to secure differentiation towards the male phenotype, while oestrogens of placental or maternal origin suffice to produce differentiation towards the female phenotype (Price & Pannabecker, 1959). On examination of frozen sections it was not possible to be sure that those ova which contained formazan deposits after incubation with hydroxysteroids were not atretic although most appeared to be histologically normal; morphological alterations of ova are believed to be preceded by enzymic changes in the granulosa (Lobel, Rosenbaum & Deane, 1961) when the follicle is undergoing atresia.

Some ova at birth were seen to possess 16β-H.S.D. activity and by the third week of postnatal life the ova had further developed 3β-, 11β- and 17β-H.S.D.s.

Many/

Many morphologically normal follicles exhibited no activity in the ova whereas others showed some formazan deposited in the ova while the surrounding granulosa was devoid of formazan.

In follicles which showed evidence of atresia ova, when seen, were invariably possessed of H.S.D. activity.

From these findings it would appear that the presence of H.S.D.s in ova is indicative of atresia.

The utilization of such a narrow range of hydroxysteroids (17β-, 16β-, 16α-,) in the germinal epithelium points to limited metabolism of steroids such as 17β-oestradiol and oestriol, but definite proof is lacking. Despite the common origin of germinal epithelium and membrana granulosa, it is interesting to observe the divergence of H.S.D. pattern between these tissues.

The ovarian interstitial cells are said to have a dual origin (Rennels, 1951; Dawson & McCabe, 1951). The primary type is reputed to arise early in life, is of uncertain origin, and exhibits cytochemical changes suggestive of secretion (Dawson & McCabe, 1951). The secondary type is believed

to/

to be formed at a later stage from the theca interna of atretic follicles. Recent histochemical investigations for 3β-hydroxysteroid dehydrogenase confirm that this is probably an active site in the metabolism of steroids (Levy, Deane & Rubin, 1959; Deane, Lobel, Driks & Rubin, 1961; Ikonen, Niemi, Pesonen & Yimonen, 1961; Taylor, 1961; Goldberg, Jones & Turner, 1953; Rubin et al., 1963; Ferguson, 1965) and electron microscopic studies indicate a secretory function (de Groodt, Lagrasse & Sebruyns, 1957).

This rise and decline in enzymic activity seen in the formation and regression of the corpus luteum might account for the corresponding blood levels of hormones.

The mouse testis becomes recognisable on the 11th day of embryonic life (Brambell, 1927) and 3β-H.S.D. activity has been recorded by Hitzeman (1962) in the mouse testis on the 15th day of embryonic life.

Histochemically trace 3β-H.S.D. appears to be present in the mouse genital ridge and is certainly present as soon as the testis is recognisable/

recognisable as such. More striking is the unmistakable 16β-H.S.D. activity in the mesenchymal cells of the genital ridge and all stages of testis. These facts may reflect steroid metabolism in the genital ridge.

3β-H.S.D. has previously been described in the mouse (Hitzeman, 1962; Baillie, 1964A) interstitial cell and the cytological distribution of 3α-hydroxysteroid dehydrogenase in the mouse Leydig cell is identical with that described for the 3β-hydroxysteroid dehydrogenase. In a previous report (Baillie & Griffiths, 1964) the volume of Leydig tissue in this strain of mouse having a 3β-H.S.D. capable of acting on substrates such as D.H.A. (3β-hydroxyandrost-5-ene-17-one) has been established and the growth curves of reactive Leydig tissue with 3β-H.S.D. activity are very similar to the sigmoid growth curve presently observed with Leydig tissue having a histochemically demonstrable 3α-H.S.D. At any given age, however, the volume of Leydig tissues capable of dehydrogenating 3β-hydroxysteroids is considerably greater than the volume of interstitial tissue capable of dehydrogenating 3α-hydroxysteroids.

Notwithstanding/

Notwithstanding the uncertainty regarding the biochemical significance of the 16—hydroxysteroids, the present testicular results made it very clear that 16β-H.S.D. is an extremely active and therefore probably important, constituent of the testicular interstitium. Reference to Fig. 10 indicates that almost 20% of the testis can execute 16β-dehydrogenation at birth and about 6% - the entire interstitium (Baillie, 1961) - in the adult testis. The overall volume of interstitial tissue capable of carrying out this conversion (Fig. 11) increases steadily with age and is far greater at any given age than the volume of tissue capable of acting on any of the other hydroxysteroids surveyed in the present study.

Histochemical studies of 17β-H.S.D. appear not to have been undertaken following Pearson & Grose's (1959A) assertion that this enzyme was not histochemically demonstrable in testis. The present results indicate that it is not merely demonstrable in adult mouse testis but that it is present in the testicular interstitium from birth.

REFERENCES

ALLEN, J.M. (1959). A chemical and histochemical study of D.P.N.-diaphorase activity in the adrenal cortex of the developing mouse. Anat. Rec., 134, 385 - 395.

ALLEN, J.M. (1960). A histochemical study of 3 (β)-sterol dehydrogenase in the adrenal cortex of the developing mouse. Anat. Rec. 137: 57 - 63.

BAILLIE, A.H. (1961). Observations on the growth and histochemistry of the Leydig tissue in the postnatal prepubertal mouse testis. J. Anat. Lond., 95: 357 - 370.

BAILLIE, A.H. (1964). Further observations on the growth and histochemistry of the Leydig tissue in the postnatal prepubertal mouse testis. J. Anat. Lond., 98: 403 - 419.

BAILLIE, A.H., CALMAN, K.C., FERGUSON, M.M. & HART, D.McK. (1965A). Histochemical demonstration of 20β-hydroxysteroid dehydrogenase. J. Endocrin. 32: 337 - 339.

BAILLIE, A.H., CALMAN, K.C., FERGUSON, M.M. & HART, D.McK. (1965B). Histochemical utilisation of 3α-, 6β-, 11α-, 12α-, 16α-, 16β-, 17α-, 21- and 24-hydroxysteroids. J. Endocrin. (In Press).

BAILLIE, A.H., FERGUSON, M.M., CALMAN, K.C. & HART, D.McK. (1965). Histochemical demonstration of 11β-hydroxysteroid dehydrogenase. J. Endocrin. 33: 119 - 125.

BAILLIE, A.H. & GRIFFITHS, K. (1964). 3β-Hydroxysteroid dehydrogenase activity in the mouse Leydig cell. J. Endocrin. 29: 9 - 17.

BALOGH, K. (1964). A histochemical method for the demonstration of 20α-hydroxysteroid dehydrogenase activity in rat ovaries. J. Histochem. Cytochem. 12: 670 - 673.

BRAMBELL, F.W.R. (1927). The development and morphology of the gonads of the mouse Part I. The morphogenesis of the indifferent gonad and of the ovary. Proc. roy. Soc. B. 101: 391 - 409.

CHESTER JONES, I. (1950). The effect of hypophysectomy on the adrenal cortex of the immature mouse. Amer. J. Anat. 86: 371 - 403.

DAWSON, A. (1953). Histochemical evidence of early differentiation of the suprarenal gland of the chick. J. Morph. 92: 579 - 595.

DAWSON, A.V. & McCABE, M. (1951). The interstitial tissue of the ovary in infantile and juvenile rats. J. Morph. 88: 543 - 572.

DEANE, H.W., LOBEL, B.C., DRIKS, E.C. & RUBIN, B.L. (1961). Further observations on steroid 3β-ol-dehydrogenase activity in the reproductive system of the female rat. Ann. Histochem. 6: 283 - 291.

DE GROODT, M., LAGASSE,A., SEBRUYNS, M. (1957). L'espace perivasculaire dans le tissu interstitiel de l'ovaire, vu au microscope electronique. Bull. Micr. appl. 7: 101 - 103.

GOLDBERG, B., JONES, G.E.S. & TURNER, D.A. (1963). Steroid 3β-ol-dehydrogenase activity in human endocrine tissues. Am. J. Obstet. Gynec. 86: 349 - 359.

HITZEMAN, J.W. (1962). Development of enzyme activity in the Leydig cells of the mouse testis. Anat. Rec. 143: 351 - 362.

IKONEN, M., NIEMI, M., PESONEN, S. & TIMONEN, S. (1961). Histochemical localisation of four dehydrogenase systems in the human ovary during the menstrual cycle. Acta Endocr. (Kbh) 38: 293 - 302.

JOSIMOVICH, J.B., LADMAN, A.J. & DEANE, H.W. (1954). Histophysiologic study of developing adrenal cortex of the rat during foetal and early postnatal stages. Endocrinol. 54: 627 - 639.

LEVER, J. (1955). Adrenocortical histogenesis in the rat embryo with observations on lipids and ascorbic acid distribution. J. Anat. Lond. 89: 293 - 300.

LEVY, H., DEANE, H.W. & RUBIN, B.L. (1959). Visualisation of steroid-3β-ol-dehydrogenase activity in the tissues of intact and hypophysectomised rats. Endocrinol. 65: 932 - 943.

MOOG, F., BENNETT, D.J. & DEAN, C.M. (1954). Growth and cytochemistry of the adrenal gland of the mouse from birth to maturity. Anat. Rec. 120: 873 - 892.

PEARSON, B. & GROSE, F. (1959A). Histochemical demonstration of 17 -hydroxysteroid dehydrogenase by use of tetrazolium salt. Proc. Soc. exp. Biol. 100: 636 - 638.

PEARSON, B. & GROSE, F. (1959B). Histochemical study of some D.P.N.H. and D.P.N. dependent dehydrogenases. Fed. Proc. 18: 1,965.

PRESL, J., JIRASEK, J., HORSKY, J. & HENZL, M. (1965). Observations on steroid-3β-ol dehydrogenase activity in the ovary during early post-natal development in the rat. J. Endocrin. 31: 293 - 294.

PRICE, D. & PANNABECKER, R. (1959). Comparative responsiveness of homologous sex ducts and accessory glands of foetal rats in culture. Arch. Anat. microsc. et Morphol. exp. 48[2] (Suppl.) 223 - 243.

RENNELS, E.G. (1951). The influence of hormones on the histochemistry of ovarian interstitial tissue in the immature rat. Am. J. Anat. 88: 63 - 107.

RUBIN, B.L., DEANE, H.W. & HAMILTON, J.A. (1963). Biochemical and histochemical studies on Δ^5-3β-hydroxysteroid dehydrogenase activity in the adrenal glands and ovaries of diverse mammals. Endocrinol. 73: 748 - 763.

TAYLOR, F.B. (1961). Histochemical changes in the ovaries of normal and experimentally treated rats. Acta endocr. (Kbh) 36: 361 - 374.

VAN DROP, A. & DEANE, H.W. (1950). A morphological and cytochemical study of the postnatal development of the rat's adrenal cortex. Anat. Rec. 107: 265 - 281.

VINSON, G.P. & JONES, I., CHESTER (1964). The In Vitro production of oestrogens from progesterone by mouse adrenal glands. J. Endocrin. 29: 185 - 191.

WARING, H. (1935). Effect of hormones on degeneration of the X zone in the mouse adrenal. J. Endocrin. 3: 123 - 131.

WATTENBERG, L.W. (1958). Microscopic histochemical demonstration of steroid-3β-ol dehydrogenase in tissue sections. J. Histochem. Cytochem. 6: 225 - 232.

PLATE I.

Fig. 1. The genital ridge of a 10 day mouse embryo after incubation with a 16β-hydroxysteroid. x 90.

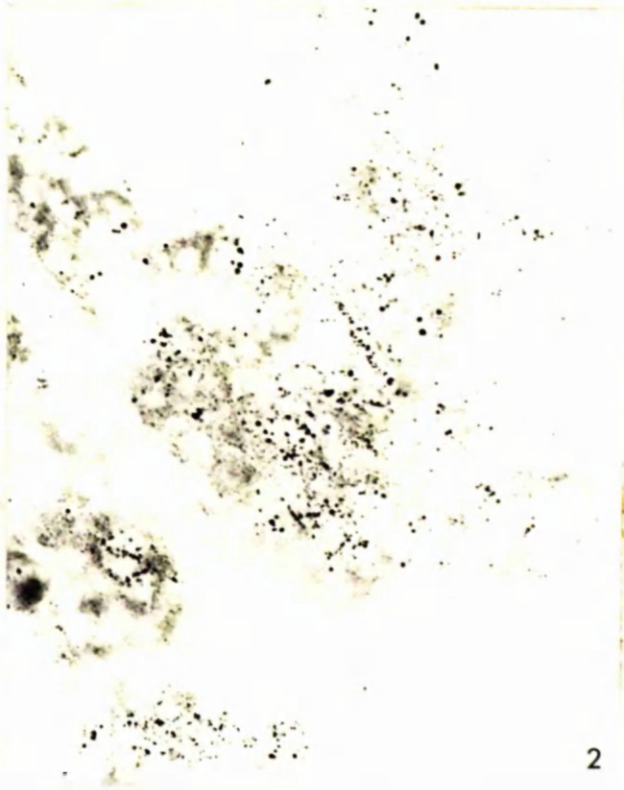
Fig. 2. Testis from a 19 day mouse embryo after incubation with a 16β-hydroxysteroid. Intense 16β-hydroxysteroid dehydrogenase activity is seen in the interstitial tissue. x 200.

Fig. 3. The adrenal gland of a 16 day embryo after incubation with a 16β-hydroxysteroid. Activity is present in all zones. The X zone at this stage is interlocked with the medulla. x 90.

Fig. 4. 3β-Hydroxysteroid dehydrogenase activity in the adrenal cortex of a 6 week old mouse after incubation with D.H.A. Activity is seen in all zones of the cortex, being strongest in the zona fasciculata; in the X zone activity is present near the medulla. The medulla (at foot of figure) shows no activity. x 150.

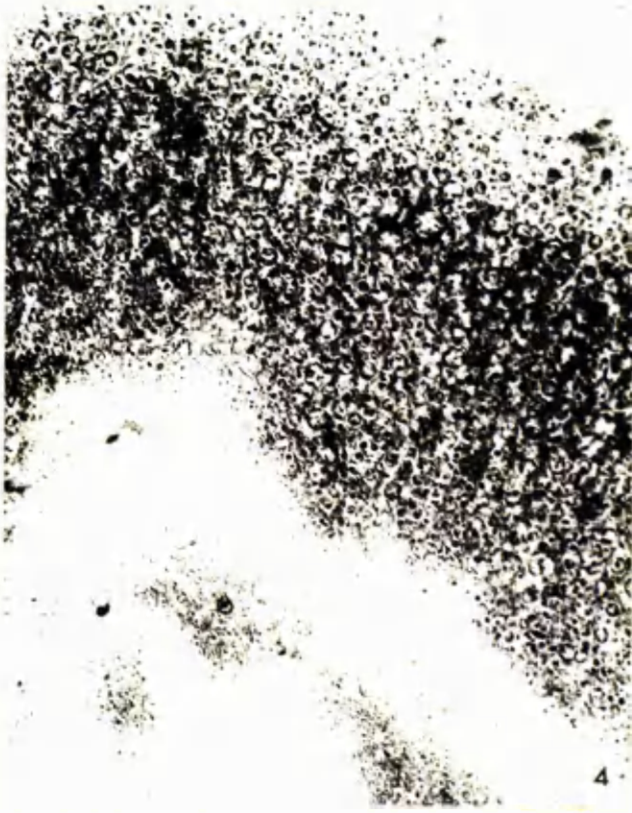
PLATE I

1



2

3



4

PLATE II.

Fig. 5. Diformazan in the neonatal interstitium and follicles with 16β-hydroxysteroid. x 200.

Fig. 6. Two week old ovary with 3β-16β-dihydroxy-androst-5-ene, 3-methyl ether showing activity in some granulosa, theca interna and in circumscribed groups of interstitium. x 90.

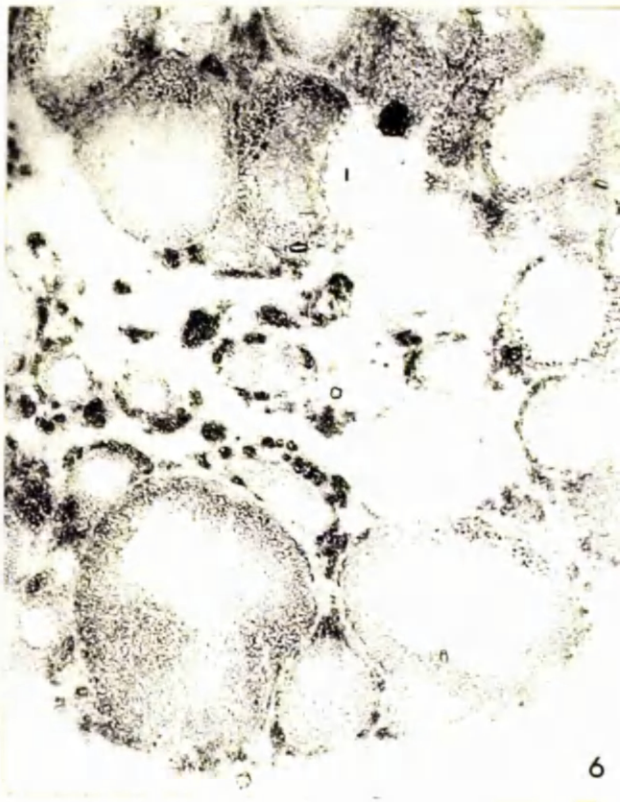
Fig. 7. Diformazan in the interstitium and granulosa indicating 3β-hydroxysteroid dehydrogenase in the neonatal ovary. x 50.

Fig. 8. Heavy deposition of diformazan in the germinal epithelium following incubation of a seven week ovary in 17β-hydroxyandrostene-3-one. x 90.

Fig. 9. 16β-Hydroxysteroid dehydrogenase activity in the 16 day old foetal ovary. x 80.

PLATE II

5



6

7

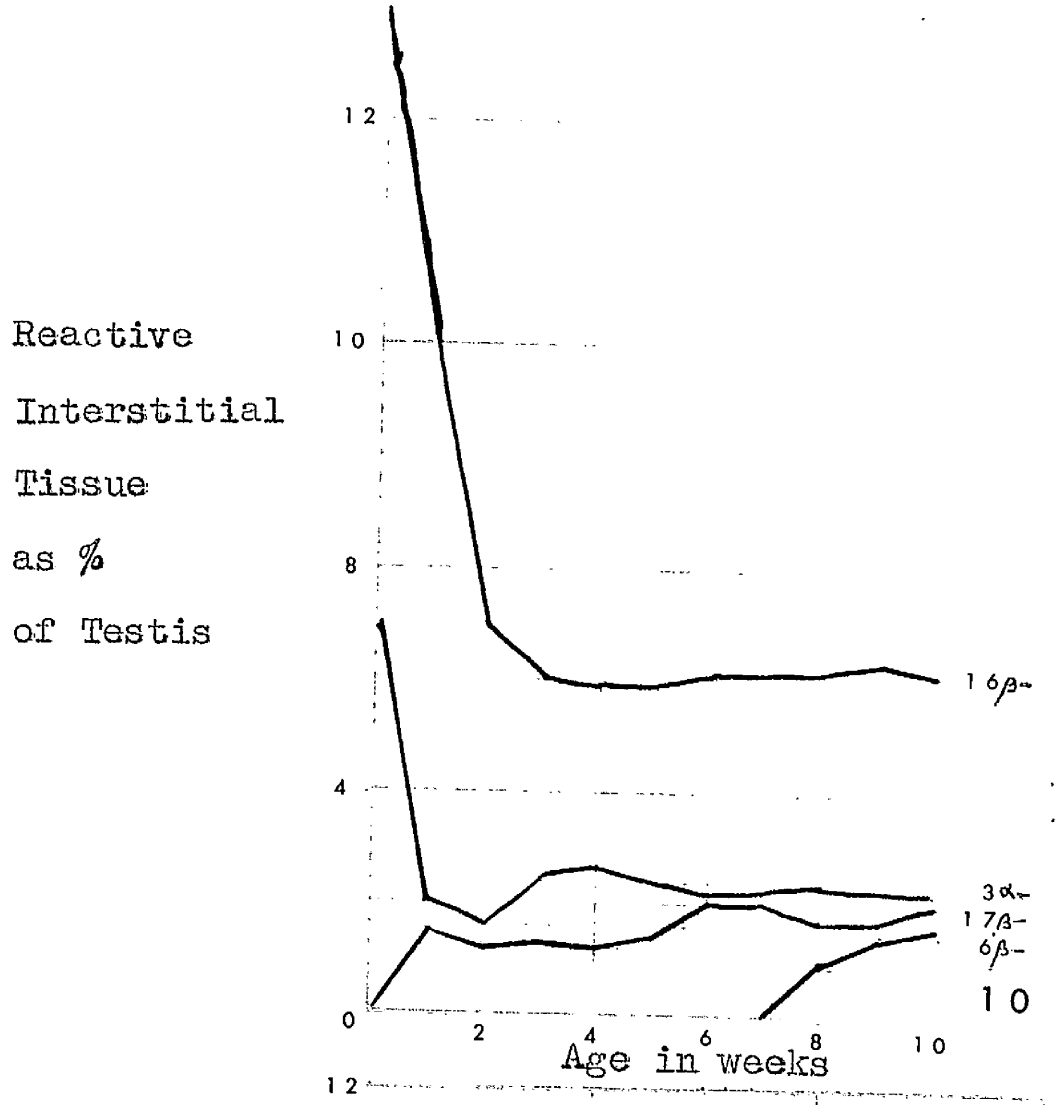
8

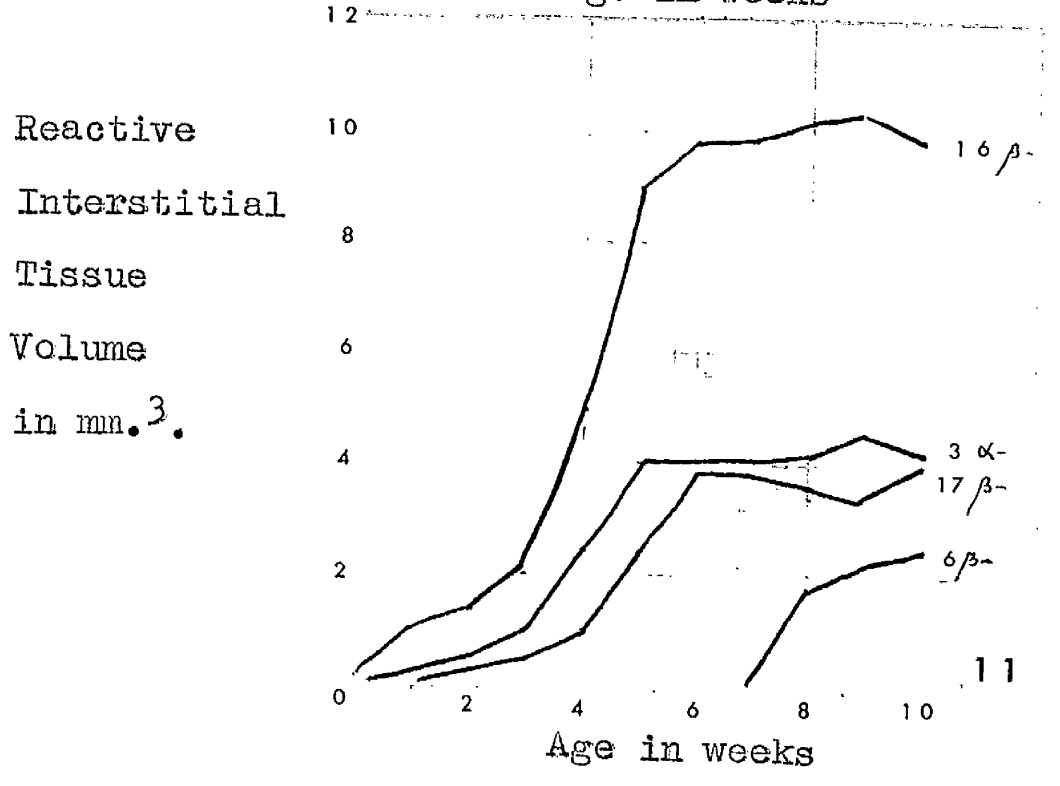
9

DESCRIPTION of FIGURES

Fig. 10. The graph shows the interstitial tissue reactive with 3α-, 6β-, 16β- and 17β- hydroxysteroids as a percentage of the testis during the first 10 weeks of post-natal life.

Fig. 11. This graph shows the hydroxysteroid dehydrogenase reactive interstitial tissue for 3α-, 6β-, 16β- and 17β- hydroxysteroid substrates as an absolute volume during the first 10 weeks of post-natal life.


Reactive
Interstitial
Tissue
as %
of Testis
12
10
8
4
0
2
4
6
8
10
16β-
3α-
17β-
6β-
10
Age in weeks


Reactive
Interstitial
Tissue
Volume
in mm.3.
12
10
8
6
4
2
0
2
4
6
8
10
16 β-
3 α-
17 β-
6β-
11
Age in weeks

Hydroxysteroid Dehydrogenases in the Human Adrenal Cortex.

BY

K. C. Calman,
A. H. Baillie,
M. M. Ferguson,
D. McK. Hart.

Department of Anatomy, The University, Glasgow.

INTRODUCTION.

In recent years the biosynthetic pathways of the steroid hormones have become progressively elucidated and since the development of a technique for the histochemical demonstration of 3β-hydroxysteroid dehydrogenase in 1958 (Wattenberg, 1958), similar techniques have been described for the utilisation in tissue sections of 3α- and 17β-, (Pearson & Grose, 1959A & B), 20α-, (Bajogh, 1964), 11β-, (Baillie, Ferguson, Calman & Hart, 1965), 20β-, (Baillie, Calman, Ferguson & Hart, 1965A) and 3α-, 6β-, 11α-, 12α-, 16α-, 16β-, 17α-, 21- and 24-hydroxysteroids (Baillie, Calman, Ferguson & Hart, 1965B).

This paper describes the histochemical utilisation of 3α, 3β-, 6β-, 11α-, 11β-, 12α-, 16α-, 16β-, 17α-, 17β-, 20β-, 21- and 24-hydroxysteroids in three normal adult human adrenal glands and in two human foetal adrenal glands. An adrenal adenoma and adrenal glands from three cases of Cushing's syndrome were also surveyed with these techniques.

MATERIAL AND METHODS.

Tissues: Three adrenal glands were obtained from women undergoing adrenalectomy for breast carcinoma and who had not previously received any systemic steroids. The adrenals were placed on solid carbon dioxide within two minutes of interruption of their blood supply. Adrenal glands were also secured from two foetuses of crown-rump lengths 12.4 cms. and 17.8 cms. respectively, at therapeutic termination of pregnancy performed for psychiatric reasons. These glands were similarly frozen on solid carbon dioxide immediately on excision. The 12.4 cms. foetus was male, the 17.8 cm. foetus female. Three adrenal glands were obtained from patients undergoing adrenalectomy for Cushing's syndrome; the first patient was a female aged 32 and only the 3β-hydroxysteroid dehydrogenase distribution was investigated in this case. The second was a male patient aged 47 and the third a female aged 22. These glands were treated as described above. The adrenal adenoma was from a 42 year old female.

Incubations: Each adrenal was sectioned at 12μ in a cryostat maintained at -20° and the sections attached to/

to clean dry glass coverslips by momentary thawing. They were then incubated with steroid substrates to demonstrate the following hydroxysteroid dehydrogenases:-

(1) 3α-hydroxysteroid dehydrogenase, using 3α-hydroxy-5α-androstan-17-one and 3α-hydroxy-5β-androstan-17-one (Steraloids).

(2) 3β-hydroxysteroid dehydrogenase, using 3β-hydroxypregn-5-en-20-one (pregnenolone; Steraloids);

sodium 3β-sulphoxypregn-5-en-20-one (pregnenolone-sulphate; Organon);

3β,17α-dihydroxypregn-5-en-20-one (17 -hydroxy-pregnenolone; Steraloids);

Ammonium 3β-sulphoxy-17α-hydroxypregn-5-en-20-one (17α-hydroxypregnenolone ammonium sulphate; Dr. E.H.D. Cameron):

3β-hydroxyandrost-5-en-17-one (D.H.A.; Steraloids);

3β-sulphoxyandrost-5-en-17-one (D.H.A. Sulphate; Organon);

(3) 6β-hydroxysteroid dehydrogenase, using 6β-hydroxyprogesterone (6 -hydroxypregn-4-ene-3, 20-dione; M.R.C.),

(4) 11 /

(4) 11α-hydroxysteroid dehydrogenase,
using 11α-hydroxyprogesterone (11α-hydroxypregn-4-ene-3, 20-dione; Steraloids),
(5) 11β-hydroxysteroid dehydrogenase,
using 11β-hydroxyprogesterone (11β-hydroxypregn-4-ene-3, 20-dione; Steraloids),
(6) 12α-hydroxysteroid dehydrogenase,
using 12α-hydroxyprogesterone (12α-hydroxypregn-4-ene-3, 20-dione; Steraloids),
(7) 16α-hydroxysteroid dehydrogenase,
using oestrone (1,3,5,(10) estratrien-3-ol-17-one; Steraloids)and 16α-hydroxyoestrone (1,3,5,(10)-estratrien-3,16α-diol-17-one; M.R.C.),
(8) 16β-hydroxysteroid dehydrogenase,
with 3β-hydroxyandrost-5-en-16-one-3-methyl ether (Steraloids) and 3β,16β-dihydroxyandrost-5-ene-3-methyl ether (Steraloids),
(9) 17α-hydroxysteroid dehydrogenase,
using epitestosterone (17α-hydroxyandrost-4-ene-3-one; Steraloids),
(10) 17β-hydroxysteroid dehydrogenase,
using testosterone (17β-hydroxyandrost-4-ene-3-one; M.R.C.) and oestradiol (1,3,5,(10)-estratrien-3, 17β-diol; Organon),
(11) 20 /

(11) 20β-hydroxysteroid dehydrogenase,
using 20β-hydroxyprogesterone (20 -hydroxypregn-4-ene-3, -dione; Steraloids),
(12) 21-hydroxysteroid dehydrogenase,
using 21-hydroxyprogesterone (21-hydroxypregn-4-ene-3, 20-dione; Steraloids),
(13) 24-hydroxysteroid dehydrogenase,
using 5β-cholan-24-ol (Steraloids).

The incubation medium consisted of 500μg. steroid substrate dissolved in 0.5 ml. dimethyl formamide, 3 mg. N.A.D., 2 mg. Nitro B.T. and 9.5 ml. O.I.M. Phosphate buffer (pH.7.4).

RESULTS.

The results are summarised in Table 1: the three adult glands behaved very similarly and their results are all grouped under one heading. The three Cushing's syndrome adrenals had three clearly recognisable zones. The adrenal adenoma specimen consisted mainly of normal adrenal tissue with occasional adenomatous nodules in the zona fasciculata. These nodules, 3 - 4 mm. in diameter, were of fascicular cell origin. Histochemically they could be divided into inner and outer zones.

Position of Hydroxyl Group	FOETAL ADRENAL C-R Length 12.4 cms.		FOETAL ADRENAL C-R Length 17.8 cms.		ADULT ADRENAL (3)				CUSHING'S ADRENAL ♦				ADRENAL ADENOMA			
	Foetal Cortex	Definitive Cortex	Foetal Cortex	Definitive Cortex	Zona Glomerulosa	Zona Fasciculata (Outer)	Zona Fasciculata (Inner)	Zona Reticularis	Zona Glomerulosa	Zona Fasciculata (Outer)	Zona Fasciculata (Inner)	Zona Reticularis	Zona Glomerulosa	Zona Fasciculata (Outer)	Zona Fasciculata (Inner)	Zona Reticularis
3α	$+_M$	tr	$+_{MD}$	tr	-	$+_M$	-	tr	-	-	-	-	-	tr_{MD}	-	tr
* 3β	$+_D$	$+_D$	$+_D$	$+_D$	-	$+_D$	-	$+_D$	$+_D$	$+_D$	-	$+_D$	-	$+_D$	$+_D$	tr
6β	$+_D$	tr	+	tr	-	tr_{MD}	-	-	-	-	-	-	-	tr_M	-	-
11β	$+_M$	tr	$+_D$	tr	tr	$+_D$	-	$+_M$	-	-	-	-	-	tr_M	-	-
12α	$+_M$	-	$+_M$	-	-	-	-	-	-	-	-	-	-	-	-	-
16α	$+_D$	-	$+_D$	-	-	tr	-	-	-	-	-	-	-	tr_{DM}	-	tr_M
16β	++	++	++	++	++	+++	tr	++	$++_D$	$+^+_D$	-	$++_D$	$++_D$	$+++_D$	tr_D	$++_D$
17β testosterone	$+_{MD}$	tr	$+_{MD}$	tr	tr	$+_M$	-	tr	-	-	-	-	-	tr_M	-	-
17β oestradiol	$+_M$	-	tr_M	-	-	tr	-	-	-	-	-	-	-	-	-	-
20β	$+_M$	-	$+_D$	-	-	-	-	-	-	-	-	-	-	tr	-	-
21	$+_D$	-	$+_D$	-	-	-	-	-	-	-	-	-	-	-	-	-

Hydroxysteroid Dehydrogenase Activity in Human Adrenal Cortex

M = Monoformazan, D = Diformazan, tr = Trace Activity.

* All 3β-OH Substrates except D.H.A. Sulphate

♦ One Cushing's Adrenal showed no 3β- or 16β-hydroxysteroid in the zona glomerulosa.

No colour was produced in any adranal when D.H.A. Sulphate, 11α-Hydroxyprogesterone, Epitestosterone, 5β-cholan-24-ol, Oestrone, Progesterone or 3β-hydroxyandrost-5-ene-16-one, 3 methyl ether were used as steroid substrate.

DISCUSSION.

The human adrenal cortex, is of the lipid rich type and the functional significance of the three morphologically distinguishable zones has been the subject of intensive investigations. The problem has been approached from a number of viewpoints; histologically, histochemically, biochemically and physiologically. The results which have been described here give support to the recent work of Griffiths, Grant and Symington (1963) concerning the significance of each zone. These authors, modifying their original view (Symington, 1962) suggest that the zona glomerulosa is the only site of aldosterone production, whereas the zona fasciculata and zona reticularis are a single unit, synthesising corticosteroids and sex hormones. The proportional contributions of each zone are not established.

Our results would seem to support the view that the zona fasciculata is the most active of the adrenal zones, but the activity of 16β-hydroxysteroid dehydrogenase in the zona glomerulosa is of interest (Fig. 1). Two additional points must be made; firstly, the more precise localisation possible with these/

these histochemical techniques indicates that the zona fasciculata itself can be divided into two parts, an inner and an outer. Only the latter is in fact active with the majority of the enzymes described. Under stress conditions, however, both parts of the zona fasciculata may become active. The original view of Symington (1962) that the zona fasciculata was principally a storage zone may thus in fact be true for the inner part of the zone. Secondly, of the enzymes described only the 3β-hydroxysteroid dehydrogenase which has been previously described in detail in the human adrenal (Baillie, Cameron, Griffiths, Hart, 1965), is in fact concerned in a biosynthetic pathway. The remaining enzymes have in many cases doubtful physiological significance, especially regarding the biosynthesis of steroid hormones. In the main they are concerned with the further metabolism of steroid in the widest sense. The important point is that whereas recent biochemical studies have been concerned with biosynthesis of steroids the present study concerns steroid metabolism as a whole, and although it would seem that our results confirm the biochemical ones, they are not strictly equivalent. Further work may distinguish differences between the two approaches.

With the Cushing's adrenal the most striking feature of our results is the change in zonation of the 3β-hydroxysteroid dehydrogenase. Previous investigation (Baillie et al., 1965), confirmed in this study, indicated that in normal adrenal, this enzyme could be detected histochemically only in the zona fasciculata. With the Cushing's adrenals, however, the enzyme is present in all zones, excepting the inner part of the zona fasciculata. Since this enzyme is intimately concerned with the biosynthesis of cortisol, it is not surprising that its activity is increased in this condition. Concomitant with this increase in 3β-hydroxysteroid dehydrogenase there is a decrease in the activity of all the other enzymes investigated. In fact only the 16β-hydroxysteroid dehydrogenase can be demonstrated, the remainder show no activity. It is possible then, that the activity of these subsidiary enzymes is curtailed due to the great increase in cortisol production.

In contrast to the decreased activity of the Cushing's adrenal, the adrenal adenoma shows an enzymic pattern similar to normal adrenal. This is not surprising in view of the histological appearance of the/

the adenoma. The main difference is the presence of an active 16α-hydroxysteroid dehydrogenase. The reason for the increase in activity of this enzyme is obscure but 16α-hydroxy compounds are important in oestrogen metabolism (Breuer & Nocke, 1959; Levitz, Rosen & Twombly, 1960) as metabolites of progesterone (Villee, Dimoline, Engel, Villee & Racker, 1962; Ward & Grant, 1963) and as a postulated salt losing hormone (Neher, Desaulles, Vischer, Wieland & Wettstein, 1958; Cope & Parry, 1959). Any or none of these factors may be involved, but histochemical techniques do not allow us to discriminate between these possibilities. 6β-Hydroxysteroid compounds have been isolated as urinary metabolites from an adrenal adenoma and also from adult adrenal cortex (Ulstrom, Colle, Burley & Gunville, 1960; Nowaczynski, Koiw & Genest, 1962) and as derivatives of oestradiol (Breuer, Nocke & Knuppen, 1958). Previous work with 3α-hydroxy compounds in human testis (Baillie et al. 1965B) showed that the 5β-isomer was better utilised than the 5α-compound. In the normal adrenal, no significant difference was observed, but with the adrenal adenoma, not only is the 5β-compound dehydrogenated to a greater extent in the zona fasciculata, but it is also metabolised in the zona reticularis.

reticularis. It is probable that some of the formazan deposited in this case could come from the 5α- or 5β-hydrogens, since the substrates used for the 3α-hydroxysteroid assay were saturated steroids.

The foetal zone of the human foetal adrenal can metabolise most of the steroids with which it is presented, with the exception of D.H.A. sulphate, 11α-hydroxyprogesterone, epitestosterone and 5β-cholan-24-ol. The definitive cortex on the other hand has an enzymic pattern much more like that of adult adrenal cortex. The high activity of 3α-hydroxysteroid dehydrogenase in the foetal part of the human prenatal cortex may indicate that it is actively concerned in the detoxication of steroids perhaps of placental or maternal origin. The definitive part is similar to the adult human adrenal cortex in its weak 3α-hydroxysteroid dehydrogenase activity. The histochemical distribution of 3α-hydroxysteroid dehydrogenase in the foetal human adrenal resembles the distribution of 3β-hydroxysteroid dehydrogenase (Bloch, Tissenbaum, Rubin & Deane, 1962; Niemi & Baillie, 1965) (Fig. 2). The relatively small amounts of 11-oxygenated steroids, in comparison to 17-oxosteroids (Solomon, Lanman, Lind & Lieberman, 1958; Bloch & Benirschke, 1959) present in/

in human foetal adrenal may be related to the low 11β-hydroxysteroid dehydrogenase activity noted histochemically.

The results with the 16β-hydroxysteroid dehydrogenase in the foetal definitive cortex, point to a functional and morphological zonation in embryos of crown-rump length 12.4 cm. and over. The activity is particularily intense in the outermost layer, suggesting that these cells may have undergone a considerable degree of differentiation towards the postnatal zona glomerulosa. Indeed Niemi & Baillie (1965) have shown that 3β-hydroxysteroid dehydrogenase is found mainly in the inner half of the definitive cortex and this suggests that the inner half has undergone similar histochemical and biochemical differentiation towards the zona fasciculata and zona reticularis. The view of Lanman (1962) that "the structure of the glomerular zone reflects a physiological inactivity in the foetal adrenal", seems to be at variance with our histochemical findings.

SUMMARY.

The histochemical utilisation of 3α-, 3β-, 6β-, 11α-, 11β-, 12α-, 16α-, 16β-, 17α-, 17β-, 20β-, 21- and 24-hydroxysteroids by three normal adult human adrenal/

adrenal glands, two human foetal adrenal glands, three Cushing's adrenals and one adrenal adenoma are described.

The normal adult human adrenal showed high 16β-hydroxysteroid dehydrogenase activity in the zona glomerulosa. Activity restricted to the outer part of the zona fasciculata was recorded with 3α-, 3β-, 6β-, 11β-, 16α-, 16β- and 17β-hydroxysteroids. The zona reticularis utilised 3α-, 3β-, 11β-, 16β- and 17β-hydroxysteroids less well than in the zona fasciculata.

The Cushing's adrenals showed activity only for 3β- and 16β-hydroxysteroid dehydrogenases; activity was noted in all three zones. The adrenal adenoma resembled the normal adult human adrenal except that greater activity for 16α-hydroxysteroid dehydrogenase was noted.

The foetal zone of the human foetal cortex was extremely active showing 3α-, 3β-, 6β-, 11β-, 12α-, 16α-, 16β-, 17β-, 20β- and 21-hydroxysteroid dehydrogenase activity. The definitive cortex was similar to that of the adult and possessed 3α-, 3β-, 6β-, 11β-, 16β- and 17β-hydroxysteroid dehydrogenase; some evidence of zoning of the definitive cortex was seen with the 16β-hydroxysteroids.

The relevance of these findings in the light of current knowledge of adrenal zonation is discussed.

ACKNOWLEDGEMENTS.

One of us (D.McK. H.) is grateful to M.R.C. for a personal grant. We would like to thank Mr. J. Hutchison, Royal Infirmary, Glasgow, for supply of tissues.

REFERENCES.

BAILLIE, A.H., FERGUSON, M.M., CALMAN, K.C. & HART, D.McK. (1965). Histochemical demonstration of 11β-hydroxysteroid dehydrogenase. J. Endocrin. 33: 119 - 125.

BAILLIE, A.H., CALMAN, K.C., FERGUSON, M.M. & HART, D.McK. (1965A). Histochemical demonstration of 20β-hydroxysteroid dehydrogenase. J. Endocrin. 32: 337 - 339.

BAILLIE, A.H., CALMAN, K.C., FERGUSON, M.M. & HART, D.McK. (1965B). Histochemical utilisation of 3α-, 6β-, 11α-, 12α-, 16α-, 16β-, 17α-, 21- and 24-hydroxysteroids. J. Endocrin. (In Press).

BAILLIE, A.H., CAMERON, E.H.D., GRIFFITHS, K. & HART, D.McK. (1965). 3β-Hydroxysteroid dehydrogenase in the adrenal gland and placenta. J. Endocrin. 31: 227 - 233.

BALOGH, K. (1964). A histochemical method for the demonstration of 20α-hydroxysteroid dehydrogenase activity in rat ovaries. J. Histochem. Cytochem. 12: 670 - 673.

BLOCH, E. & BENIRSCHKE, K. (1959). Synthesis in vitro of steroids by human fetal adrenal gland slices. J. biol. Chem. 234: 1085 - 1089.

BLOCH, E., TISSENBAUM, B., RUBIN, B.L. & DEANE, H.W. (1962). Δ^5-3β-hydroxysteroid dehydrogenase activity in human fetal adrenals. Endocrinology, 71: 629.

BREUER, H. & NOCKE, (1959). Formation of estriol 3-, 16β-, 17α- by liver tissue in vitro. Biochem. biophys. Acta. 36: 271 - 272.

BREUER, H., NOCKE, L. & KNUPPEN, R. (1958). 6β-Hydroxy lierung von oestrogenen in der rattenleber, Naturwissenschaften. 45: 397.

COPE, C.L., PARRY, E.D.O. (1959). A sodium excreting steroid. Brit. Med. J. 2: 1004 - 1005.

GRIFFITHS, K., GRANT, J.K. & SYMINGTON, T. (1963). A biochemical investigation of the functional zonation of the adrenal cortex in man. J. clin. Endocr. & Metab. 23: 776 - 785.

LANMAN, J.T. (1962). in "The human adrenal cortex", Ed. Currie, A.R., Symington, T., Grant, J.K. E. & S. Livingstone, Edinburgh.

LEVITZ, M., ROSEN, M.F. & TWOMBLY, G.H. (1960). Interconversions of 16-oxygenated oestrogens. II. Metabolism of 16-keto estradiol 17β 16-C^{14} in man. Arch. Biochem. Biophys. 88: 212 - 215.

NEHER, R., DESAULLES, P., VISCHER, E., WIELAND, P., WETTSTEIN, A. (1958). Isolierung, konstitution synthese eine neuen steroide aus nebennieren. Helv. Chim. Acta. 41: 1667 - 1692.

NIEMI, M. & BAILLIE, A.H. (1965). 3β-Hydroxysteroid dehydrogenase activity in the human foetal adrenal. Acta endocr. Kbh. 48: 423 - 428.

NOWACZYNSKI, W., KOIW, E. & GENEST, J. (1962). The isolation of 6β-hydroxycorticosterone, and its 11-dehydroderivative from the adrenal gland, adrenal adenoma and urine of a patient with primary aldosteronism. Canad. J. Bioch. & Phy. 4: 1779 - 1796.

PEARSON, B., & GROSE, F. (1959B). Histochemical demonstration of 17β-hydroxysteroid dehydrogenase by use of tetrazolium salt. Proc. Soc. exp. Biol. N.Y. 100: 636 - 638.

PEARSON, B. & GROSE, F. (1959A). Histochemical study of some D.P.N.H. and D.P.N. dependent dehydrogenases. Fed. Proc. 18: 1965.

SOLOMON, S., LANMAN, J.T., LIND. J. & LIEBERMAN, S. (1958). The biosynthesis of Δ^4-androstenedione and 17α-hydroxyprogesterone from progesterone by surviving human foetal adrenals. J. Biol. Chem. 233:1084 -

SYMINGTON, T. (1962). The Human Adrenal Cortex. Edited by Currie, Symington & Grant. Livingstone, Edinburgh.

ULSTROM, R.A., COLLE, E., BURLEY, J. & GUNVILLE, R. (1960). Adrenocortical steroid metabolism in newborn infants. II. Urinary excretion of 6β-hydroxy-cortisol and other polar metabolites. J. clin. Endocr. & Metabolism. 20: 1080 - 1095.

VILLEE, D.B., DIMOLINE, A., ENGEL, L.L. & VILLEE, C.A. & RACKER, H. (1962). Formation of 16α-hydroxyprogesterone in hyperplastic adrenal. J. clin. Endocr. & Metabolism. 22: 726 - 734.

WARD, P.J. & GRANT. J.K. (1963). Metabolism of (4-C^{14}) progesterone by adrenocortical tissue of patients with Cushing's syndrome. J. Endocrin. 26: 139 - 147.

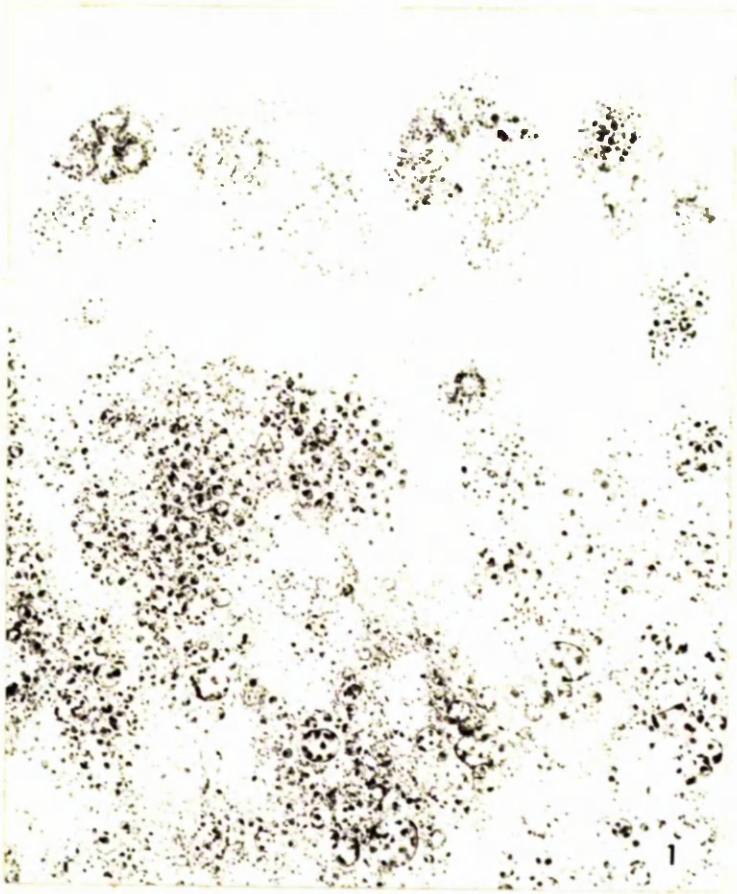
WATTENBERG, L.W. (1958). Microscopic histochemical demonstration of steroid 3β-ol-dehydrogenase in tissue sections. J. Histochem. Cytochem. 6: 225 - 232.

EXPLANATION OF PLATES.

Fig. 1. Adult adrenal showing 16β-hydroxysteroid dehydrogenase activity in the zona glomerulosa and zona fasciculata. x 180.

Fig. 2. Adrenal from 12.4 cm. male foetus showing prominent 3α-hydroxysteroid dehydrogenase activity in the foetal cortex. x 90.

PLATE



Hydroxysteroid Dehydrogenase in Hydroxysteroid Dehydrogenase in Human, Foetal Gonads.

BY

D. McK. Hart,
K. C. Calman,
M. M. Ferguson,
M. Niemi,
A. H. Baillie.

Department of Anatomy, The University of Helsinki, Finland,

and

Department of Anatomy, The University of Glasgow, Scotland.

SUMMARY.

This paper describes the histochemical utilization of 3α-, 3β-, 6β-, 11α-, 11β-, 16β-, 17α-, 17β- and 20β-hydroxysteroids in the testes of 11 human foetuses of crown-rump lengths 4.3 cm to 18.1 cm and in the ovaries of 10 foetuses of crown-rump lengths 4.5 cm. to 17.5 cm. The histochemical technique employed a Tetrazolium Salt (Nitro BT) as the final electron acceptor.

All testes showed activity with 3β- and 16β-hydroxysteroids as substrates throughout the age range studied. 3α-Hydroxysteroids produced a weak reaction in all testes. 11β- and 17β-hydroxysteroids were utilized only by the older testes. With 6β- and 20β-hydroxysteroids only weak and inconstant diformazan deposition occurred. 11α-Hydroxysteroid dehydrogenase activity was not demonstrated.

Hydroxysteroid dehydrogenase activity was not demonstrated in any human, foetal ovary between the 10th and 20th weeks of gestation despite the presence of abundant N.A.D.H. tetrazolium reductase activity.

INTRODUCTION

The development of hydroxysteroid dehydrogenase activity has been surveyed in mouse gonads (Hart, Baillie, Calman and Ferguson, 1966) using previously established techniques (Wattenberg, 1958; Pearson and Grose, 1959A, B: Baillie, Calman, Ferguson and Hart 1965A, B: Baillie, Ferguson, Calman and Hart, 1965). 3β-Hydroxysteroid dehydrogenase has been described in human, foetal testis recently, (Baillie, Niemi and Ikonen, 1965). This paper reports the distribution of 3α-, 3β-, 6β-, 11α-, 11β-, 16β-, 17α-, 17β- and 20β-hydroxysteroid dehydrogenases in foetal human ovaries and testes between the 10th and 20th weeks of gestation.

MATERIAL AND METHODS.

Ovaries were obtained from 10 female human foetuses of crown-rump lengths from 4.5 cm. to 17.5 cm. and testes from 11 male foetuses of crown-rump lengths from 4.3 cm. to 18.1 cm. at therapeutic termination of pregnancy. Each gonad was frozen on solid/

solid carbon dioxide within a few minutes of removal of the foetus from the uterus.

These tissues were sectioned at 12 μ in a cryostat maintained at -20°; the sections were attached to clean, dry glass slides by momentary thawing, then dried in air. Incubation was carried out at 37° for two hours in the following medium:-

0.1.M. Phosphate buffer (pH 7.4)	10 ml.
Steroid substrate (dissolved in 0.5 ml. D.M.F.)	0.5 mg.
Nitro B.T.	3 mg.
N.A.D.	3 mg.

The steroid substrates employed were:-

(1) 3α-hydroxy-5α-androstan-17-one (androsterone) Steraloids.

(2) 3α-hydroxy-5β-androstan-17-one (aetiocholanolone) Steraloids.

(3) 3β-hydroxyandrost-5-en-17-one (D.H.A.) Steraloids.

(4) 6β-hydroxypregn-4-en-3, 20-dione (6β-hydroxy-progesterone) Steraloids.

(5) 11α-hydroxypregn-4-en-3, 20-dione (11α-hydroxy-progesterone) Steraloids.

(6) 11β-/

(6) 11β-hydroxypregn-4-en-3, 20-dione (11β-hydroxy-progesterone) Steraloids.

(7) 3β, 16β-dihydroxyandrost-5-en-3-methyl ether Steraloids.

(8) 3β-hydroxyandrost-5-en-16-one-3 methyl ether Steraloids.

(9) 17α-hydroxyandrost-4-en-3-one (epitestosterone) Organon.

(10) 3, 17β-dihydroxy-oestra-1,3,5,(10)-trien (oestradiol) Organon.

(11) 20β-hydroxypregn-4-en-3-one (20β-hydroxyprogest-erone) Steraloids.

RESULTS.

The results with foetal human testes are summarised in Table I. It will be seen that the Leydig cells of all human testes studied exhibited 3β- and 16β-hydroxysteroid dehydrogenase activity. 3α-Hydroxysteroid dehydrogenase activity was also present in all testes studied but the activity was much/

TABLE I.

Foetal Crown-Rump Length in Cm.

Position of Hydroxyl Group	4.3		4.4		5.3		7.3		8.5		10.5		11.3		13.0		14.5		15.8		18.1	
	L	T	L	T	L	T	L	T	L	T	L	T	L	T	L	T	L	T	L	T	L	T
3 α	tr	-	tr	-	tr	-	tr	-	tr	-	tr	-	tr	-	tr	-	tr	-	tr	-	tr	-
3 β	+	-	+	-	+	-	+	-	+	-	+	-	+	-	+	-	+	-	+	-	+	-
11 α	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
11 β	-	-	-	-	-	-	-	-	tr	-	tr	-	tr	-	tr	-	tr	-	+	-	tr	-
16 β	++	tr	++	tr	++	tr	++	tr	++	tr	++	tr	++	tr	++	tr	++	tr	++	tr	++	tr
17 α	-	-	-	-	-	-	-	-	tr	-	-	-	-	-	-	-	tr	-	-	-	-	-
17 β	-	-	-	-	-	-	-	-	+	tr	+	tr	+	tr	+	tr	+	tr	+	tr	+	tr

Hydroxysteroid Dehydrogenase Activity in Human Foetal Testis.

T = Seminiferous Tubules
L = Leydig Cells
tr = Trace Activity

much weaker than that of 3β- or 16β-hydroxysteroid dehydrogenases. 11β- And 17β-hydroxysteroid dehydrogenases were present constantly in the older foetuses but not in the younger ones. 6β- and 20β-hydroxysteroid dehydrogenase activities were weak, inconstant and difficult to demonstrate. On two occasions when the 17α-hydroxysteroid was employed as substrate a weak reaction was obtained. After incubation with the 16β- or 17β-hydroxysteroids some diformazan was seen in the area of the seminiferous tubules.

Human foetal ovaries in the age series studied did not show any significant hydroxysteroid dehydrogenase activity. The human foetal ovaries of all ages studied showed strong N.A.D.H.$_2$ diaphorase activity.

DISCUSSION.

The results of the present investigation indicate the existence of 3α-, 11β-, 16β- and 17β-hydroxysteroid dehydrogenases in human foetal testis in addition to the 3β-hydroxysteroid dehydrogenase already described there (Baillie, Niemi et al., 1965).

The/

The possible significance of these hydroxysteroid dehydrogenases has already been discussed with regard to general biochemical considerations (Baillie et al., 1965B: Baillie, Ferguson, Calman & Hart, 1965) and with special reference to steroid metabolism in human testis (Baillie & Mack, 1966).

Comparison between the pattern of development of hydroxysteroid dehydrogenase activity in foetal mouse testis (Hart et al., 1966) and in human foetal testis shows considerable similarity. In contrast to this a marked discrepancy is noted between the foetal ovaries of these species. Whereas in the mouse 3β- and 16β-hydroxysteroid dehydrogenase activities are present in the ovary when first it becomes differentiated and 3α-hydroxysteroid dehydrogenase appears several days before birth, no hydroxysteroid dehydrogenase activity could be demonstrated in human, foetal ovaries between the 10th and 20th weeks of gestation. This failure to demonstrate hydroxysteroid dehydrogenase activity histochemically was not secondary to a lack of N.A.D. - tetrazolium reductase since this enzyme was present in all tissues of all ovaries studied. Hydroxysteroid dehydrogenase/

dehydrogenase activity may, of course, develop in the human ovary between the 20th week of gestation and term, but Diczfalusy, Cassmer, Alonso & DeMiguel (1961) found no evidence to suggest that human foetal or neonatal ovaries were capable of oestrogen metabolism. With human, foetal testis changes in the activity of the enzymes studied may occur outwith the age range investigated.

While the factors determining the differentiation and development of male and female reproductive tracts are imperfectly understood at present, it appears to be widely accepted (Price & Pannabecker, 1959) that in the absence of androgenic stimulation the embryo will differentiate into the female phenotype. Our results suggest that the testis may be able to fulfil a steroidogenic function at an early date in utero.

ACKNOWLEDGEMENT.

The authors are grateful to the Anatomy Department, University of Helsinki, for the research facilities and material provided. This work has been supported in part by grants from the Medical Research Council (U.K.), the Wellcome Foundation and the Board of Management for Glasgow Northern Hospitals.

REFERENCES.

BAILLIE, A.H., CALMAN, K.C., FERGUSON, M.M. & HART, D.McK. (1965A). Histochemical demonstration of 20β-hydroxysteroid dehydrogenase. J. Endocrin. 32: 337 - 339.

BAILLIE, A.H., CALMAN, K.C., FERGUSON, M.M. & HART, D.McK. (1965B). Histochemical utilisation of 3α-, 6β-, 11α-, 12α-, 16α-, 16β-, 17α-, 21- and 24-hydroxysteroids. J. Endocrin. (In Press).

BAILLIE, A.H., FERGUSON, M.M., CALMAN, K.C. & HART, D.McK. (1965). Histochemical demonstration of 11β-hydroxysteroid dehydrogenase. J. Endocrin. 33: 119 - 125.

BAILLIE, A.H. & MACK, W.S. (1966). Hydroxysteroid dehydrogenases in normal and abnormal human testes. J. Endocrin. (In Press).

BAILLIE, A.H., NIEMI, M. & IKONEN, M. (1965). 3β-Hydroxysteroid dehydrogenase activity in the human foetal testis. Acta Endocr. 48: 429 - 438.

DICZFALUSY, E., CASSMER, O., ALONSO, C. & de MIGUEL, M. (1961). Estrogen metabolism in the human fetus and newborn. Recent Progr. Hormone Res. 17: 147 - 161.

HART, D.McK., BAILLIE, A.H., CALMAN, K.C. & FERGUSON, M.M. (1966). Hydroxysteroid dehydrogenase development in mouse adrenal and gonads. J. Anat. (In Press).

PEARSON, B. & GROSE, F. (1959A). Histochemical demonstration of 17β-hydroxysteroid dehydrogenase by use of tetrazolium salt. Proc. Soc. exp. Biol. 100: 636 - 638.

PEARSON, B. & GROSE, F. (1959B). Histochemical study of some D.P.N.H. and D.P.N. dependent dehydrogenases. Fed. Proc. 18: 1,965.

PRICE, D. & PANNABECKER, R. (1959). Comparative responsiveness of homologous sex ducts and accessory glands of fetal rats in culture. Arch. Anat. microsc. et Morphol. exp. 48^2: 1959. Suppl. 223 - 243.

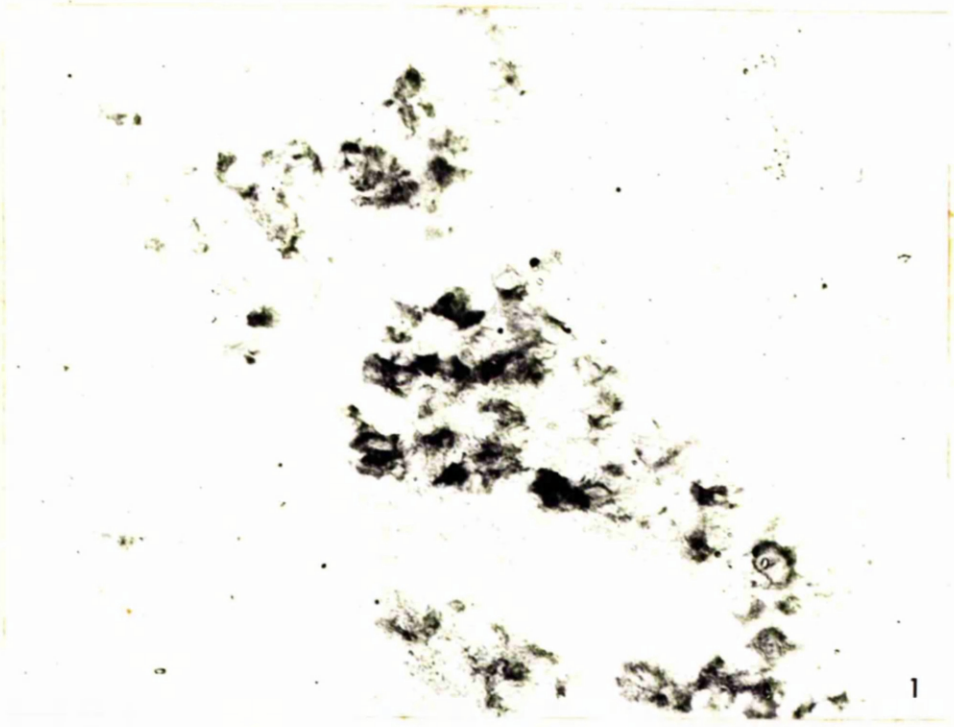
WATTENBERG, L.W. (1958). Microscopic histochemical demonstration of steroid-3β-ol dehydrogenase in tissue sections. J. Histochem. Cytochem. 6: 225 - 232.

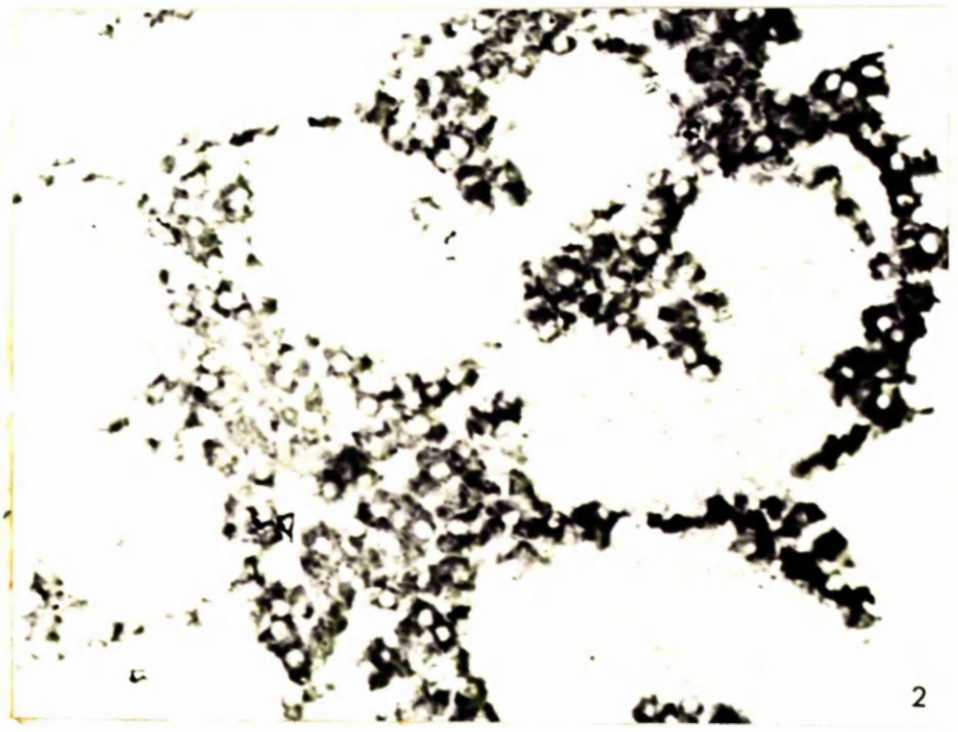
DESCRIPTION OF PLATE

Fig. 1. Leydig cells in the testis of a foetus of crown-rump length 7.3 cm. showing weak 3α-hydroxysteroid dehydrogenase activity.
(x 300)

Fig. 2. This section from the testis of a foetus of crown-rump length 14.5 cm. shows strong 16β-hydroxysteroid dehydrogenase activity in the Leydig cells and trace activity in the seminiferous epithelium.
(x 250)

PLATE





Evidence of Steroid Metabolism and Possible Biosynthesis in the Human Genital Ridge Mesenchyme.

BY

A. H. Baillie

M. M. Ferguson

D. McK. Hart.

Department of Anatomy,

University of Glasgow.

SUMMARY.

3β-, 16β- and 17β-hydroxysteroid dehydrogenases have been demonstrated in genital ridge mesenchyme in a 14 mm. human foetus. Trace 3α-hydroxysteroid dehydrogenase activity was also noted, but 11β-, 16α-, and 20β-hydroxysteroids gave no reaction. These findings point to possible steroid metabolism in the genital ridge mesenchyme. The relevance of this to the control of primordial germ cell migration to the genital ridge, and proliferation of the primordial germ cells and coelomic epithelium of the genital ridge, is discussed.

INTRODUCTION

There is increasing evidence to suggest an early assumption of hormonal activity by the testis (1,2 and 3). This paper adduces information pointing to steroid metabolism in the mesenchyme of the genital ridge in a 14 mm. human foetus.

MATERIAL AND METHODS.

A 14 mm. human embryo was secured in its membranes at therapeutic termination of pregnancy for psychiatric reasons/

reasons. The embryo was immediately frozen in pulverized solid carbon dioxide and 10 μ horizontal serial sections were cut in a cryostat maintained at -25°C. Sections at the level of genital ridge were attached to coverslips by momentary thawing and dried in air. Incubations were conducted separately at pH 7.4 in phosphate buffer to which N.A.D. and the following steroid substrates dissolved in dimethyl formamide were added:-

(1) 3α-hydroxy-5α-androstan-17-one

(2) 3α-hydroxy-5β-androstan-17-one

(3) 3β-hydroxy-pregn-5-ene-20-one (pregnenolone)

(4) 3β-hydroxy-androst-5-ene-17-one (D.H.A.)

(5) 3β, 17β-dihydroxy-androst-5-ene (androstenediol)

(6) 11β-hydroxy-androst-4-ene-3, 17-dione (11β-hydroxy-androstenedione)

(7) 16α-hydroxy-pregn-4-ene-3, 20-dione (16α-hydroxy-progesterone)

(8) 3β, 16β-dihydroxy-androst-4-ene 3-methylether

(9) 3β-hydroxy-androst-4-ene-16-one 3-methyl ether

(10) 1,3,5,(10)-estratriene-3 , 17β-diol (estradiol)

(11) 20β-hydroxy-pregn-4-ene-3-one (20 -hydroxyprogesterone).

The final concentration of steroid substrate in the incubation medium was 0.5 mM. Nitro BT (Sigma) was/

was employed as the final electron acceptor. Control incubations lacking steroid substrate were carried out. The presence of N.A.D.-diaphorase was routinely checked in the genital ridge by incubating with N.A.D.H. On completion of the 4 hr. incubation the sections were washed in buffer and mounted with an aqueous mountant.

In addition to the above reactions sections were incubated to demonstrate alkaline phosphatase in the germ cells (4). Haematoxylin and eosin stained sections were also prepared.

RESULTS

General Histology. The haematoxylin and eosin preparations disclosed that the coelomic epithelium covering the medial side of the genital ridge consisted in a mass of oval or rounded cells whose membranes were difficult to discern. In the subjacent mesenchyme large primordial sex cells could berecognised and these gave an intense alkaline phosphatase reaction (5). Reference to Figure I indicates that germ cell migration from the gut endoderm and mesentery into the genital ridge is incomplete in this embryo. The genital ridge/

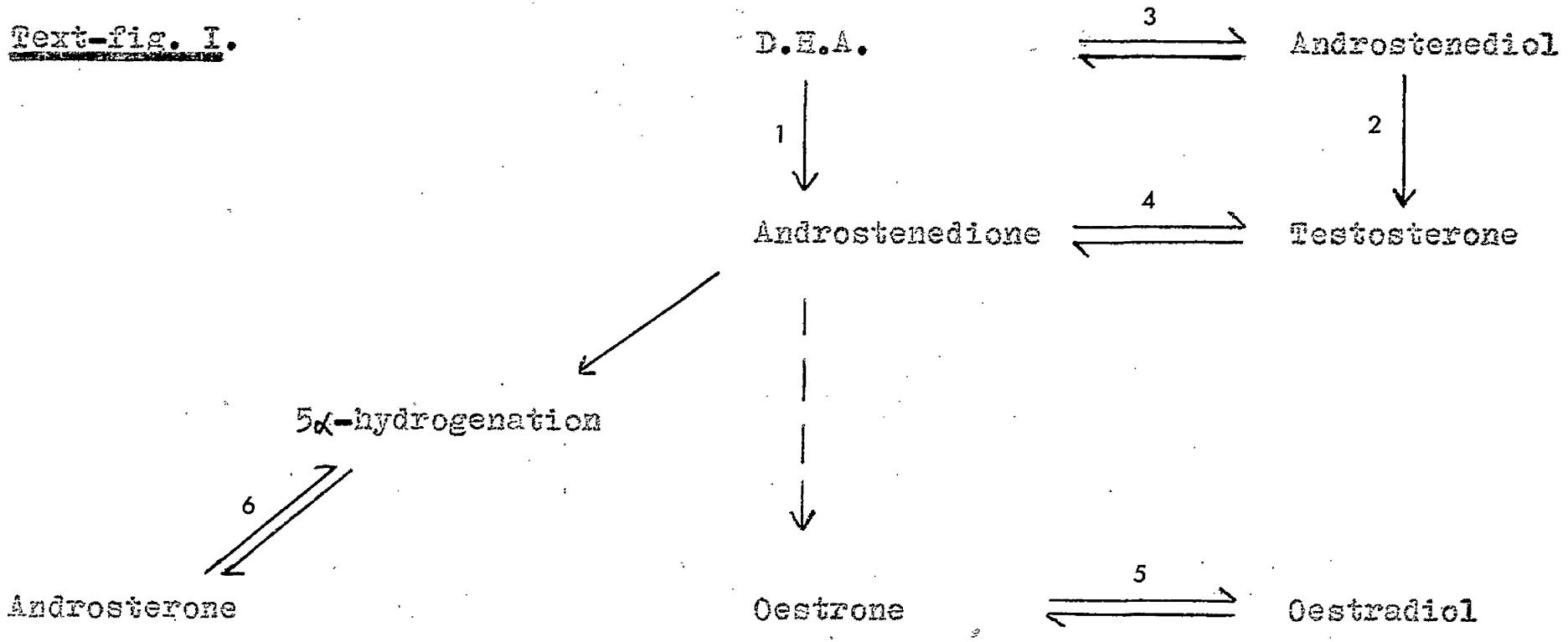
ridge is incomplete in this embryo. The genital ridge itself was not yet demarcated by folds and a mesogenitale from the mesonephros.

Hydroxysteroid dehydrogenases. Trace 3α-, moderate 3β- and 16β- and intense 17β-hydroxysteroid dehydrogenase activity was seen in the mesenchyme of the genital ridge (Figs. 2 - 4). The reaction product was deposited as minute blue diformazan granules. The diformazan was restricted to the genital ridge mesenchyme; no reaction was observed in mesonephric mesenchyme, the primordial germ cells or the outer part of the coelomic epithelium.

11β-, 16α- and 20β-hydroxysteroids were not utilized histochemically by any constituent of the genital ridge.

(The above noted reactions were carried out immediately on obtaining the foetus. It is of general histochemical interest to note that the N.A.D. and N.A.D.P. diaphorase in all the tissues of this foetus did not survive more than 6 days storage at -20°C. This is unique in our experience and places a time limit on storage of early foetal material for dehydrogenase histochemistry at -20°C).

Text-fig. I.


D.H.A.
3
Androstenediol
1
2
Androstenedione
4
Testosterone
5α-hydrogenation
6
Androsterone
Oestrone
5
Oestradiol

DISCUSSION.

These results indicate the existence of 3α-, 3β-, 16β- and 17β-hydroxysteroid dehydrogenases in a functional state in the mesenchyme of a very primitive human genital ridge and Conversions 1 - 6 in Text fig. 1 can clearly be undertaken, provided that the appropriate isomerase (6, 7) and other appropriate enzymes are present.

It will be seen from Text fig. 1 that the genital ridge mesenchyme apparently has the potential to produce a variety of androgens including probably androstenediol, androstenedione, testosterone and androsterone, but this would depend on the availability of D.H.A., perhaps from maternal or placental sources, or on the co-existence of pathways of cholesterol biosynthesis in the genital ridge together with a 17α-hydroxylase and a desmolase. In this connection it is interesting to note that in the youngest human foetal testes yet studied biochemically, pregnenolone-17α-H^3 can be converted to D.H.A., testosterone and androstenedione (8) and there is no evidence in that study to suggest that the 17α-hydroxylase or sidechain splitting enzymes/

enzymes lag behind 3β- and 17β-hydroxysteroid dehydrogenases in their activity. It seems reasonable to suggest that the human genital ridge mesenchyme can synthesise androgens, and possibly oestrogens, from placental or maternal D.H.A., or perhaps even from locally synthesised cholesterol. Such a state of affairs is clearly of profound importance in early gonadal embryogenesis.

In fishes (9 - 12), amphibia (13 - 18), reptiles, birds (19 - 21), mammals and man (22 - 24), the primordial germ cells are known to migrate from either intra- or extra-embryonic endoderm or its environs (Fig. 1) to the genital ridge. This migration is partly achieved by differential tissue growth, partly by active ameboid movements of the germ cells and partly by intravascular transport in some species, and is widely believed to be under humoral control. (For a review, see Baillie (25) 1966). Our results raise the possibility that the chemotactic agents are steroidal in nature. On arrival in the genital ridge the primordial germ cells undergo rapid and extensive multiplication (26) and it is tempting to speculate that this mitotic activity, together with that known to occur in the coelomic epithelium at this time, may be due to prevailing high local concentrations of steroid hormones in the genital ridge.

ACKNOWLEDGEMENT.

We are indebted to Dr. W. F. Harper, Stobhill General Hospital, Springburn, Glasgow, for the foetus. The work was financed by the M.R.C. whose support is gratefully acknowledged.

REFERENCES.

(1) BILLINGHAM, R.E. Heredity, 6: 201. 1952.

(2) LILLIE, F.R. J. Exp. Zool. 23: 371. 1917.

(3) OWEN, R.R., DAVIS, H.P., MORGAN, R.F. J. Heredity. 37: 291. 1946.

(4) MANHEIMER, L.H. & SELIGMAN, A.M. J. Nat. Cancer Inst. 9: 181. 1948.

(5) McKAY, D.G., HERTIG, A.T., ADAMS, E.C. & DANZIGER, S. Anat. Rec. 117: 201. 1953.

(6) EWALD, W., WERBIN, H. & CHAIKOFF, I.L. Biochim. Biophys. Acta 81: 199. 1964.

(7) KRUSKEMPER, H.L., FORCHIELLI, E. & RINGOLD, H.J. Steroids. 3: 295. 1964.

(8) ACEVEDO, H.F., AXELROD, L.R., ISHIKAWA, E. & TAKAKI, F. J. Clin. Endocrin. 21: 1611. 1961.

(9) ALLEN, B.M. Science N.Y. 33: 268. 1911.

(10) DODDS, G.S. J. Morph. 21: 563. 1910.

(11) MASCHKOWZIFF, A. Zool. Jahrb. Abt. Anat. u. Ont. 59: 1. 1934.

(12) JOHNSTON, P.M. J. Morph. 88: 471. 1951.

(13) ALLEN, B.M. Anat. Anz. 31: 339. 1907.

(14) BURNS, R.K. In "Analysis of Development" (B. H. Willier, P.A. Weiss & V. Hamburger. Eds.) pp. 462 - 491. 1955. W. B. Saunders & Co., Philadelphia & London.

(15) PERLE, G. C.R. Acad. Sci. Paris. 184: 303. 1927.

(16) CHENG, T. Zeit. f. Zellforsch. mikr. Anat. 16: 497. 1932.

(17) CHENG, T. Zeit. f. Zellforsch. mikr. Anat. 16: 542. 1932.

(18) JURAND, A. Daud. Folia Biol. 5: 123. 1957.

(19) OKKELBERG, P.O. J. Morph. 35: 1. 1921.

(20) EVERETT, N.B. J. Exp. Zool. 92: 49. 1943.

(21) GILLMAN, J. Carnegie Inst. Wash. Pub. 575. Contrib. to Embryol. 210: 83. 1948.

(22) FUSS, A. Anat. Anz. 39: 407. 1911.

(23) FELIX, W. In "Manual of Human Embryology" (Keibol & Mall) vol. 2: 752 - 979. J. B. Lippincott Co., Philadelphia & London.

(24) WITSCHI, E. Carnegie Inst. Wash. Pub. 575. Contrib. to Embryol. 32: 67. 1948.

(25) BAILLIE, A.H. "The Testis" Academic Press. N.Y. & London.

(26) MINTZ, B. J. Embryol. exp. Morph. 5: 396. 1957.

EXPLANATION OF PLATE.

Fig. 1. Alkaline phosphatase in germ cells in mesentery (M) and genital ridge (G).
(x 90).

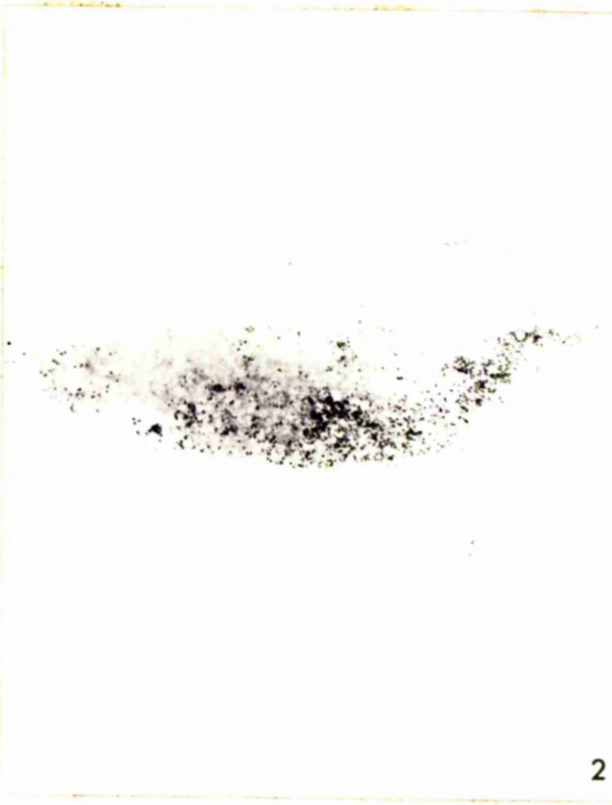
Fig. 2. 3β-Hydroxysteroid dehydrogenase in genital ridge mesenchyme.
(x 100).

Fig. 3. 16β-Hydroxysteroid dehydrogenase in mesenchymal cells of genital ridge.
(x 100).

Fig. 4. 17β-Hydroxysteroid dehydrogenase in genital ridge.
(x 100).

PLATE

1



2

3

4

Histochemical Distribution of Hydroxysteroid Dehydrogenases in Kidney and Liver.

BY

A.H. Baillie

K.C. Calman

M.M. Ferguson

D. McK. Hart

Department of Anatomy,

University of Glasgow.

SUMMARY.

Mouse, rat, hamster, guinea pig and sheep kidneys and foetal human, adult male and female human, mouse, rat, hamster and guinea pig livers were examined for hydroxysteroid dehydrogenase activity.

3α-Hydroxysteroids were utilised by all tissues, including neonatal mouse kidney, but the 5α-configuration was a more suitable substrate than the corresponding 5β-steroid. Both N.A.D. and N.A.D.P. were suitable co-factors.

Only trace 3β-hydroxysteroid dehydrogenase activity was demonstrable in renal tissue, but liver possessed a higher level of activity and lanosterol, a precursor of cholesterol, was an especially suitable substrate possibly indicating cholesterol synthesising ability in the liver.

6β-Hydroxyprogesterone was poorly utilised by renal and hepatic tissues and N.A.D. was found to be the only co-factor suitable for this reaction in our histochemical system.

All the tissues possessed 11β-hydroxysteroid dehydrogenase activity. In kidney, while activity of the other hydroxysteroid dehydrogenases was confined

to convoluted tubules and Henle's loop, the 11β- enzyme was only active in collecting tubules. It was further noted that in mouse kidney 11β-hydroxysteroid dehydrogenase was absent at birth but appeared within the first fourteen days. Activity with 11β-hydroxysteroids was observed to be more prominent in the liver of male species and this pattern was also found with 3α-, 3β-, 16α- and 16β-hydroxysteroids all of which are confirmed by previous biochemical findings.

Renal tissue was not capable of utilizing the 16 -hydroxysteroid in contrast to liver which could use this substrate fairly well. 16β- and 17β-hydroxysteroid dehydrogenases were demonstrable in the liver of all species and in all kidneys except those of mouse. The 20β-hydroxysteroid was only poorly utilized by hepatic tissue and not at all by renal tissue.

Slight activity was demonstrable with 5α- and 5β-androstans as substrates in liver and the diformazan deposition was presumably due to the action of a steroid reductase.

INTRODUCTION.

Methods have been described for the histochemical demonstration of 3β- (Wattenberg, 1958), 17β- (Pearson and Grose, 1959), 20α-, (Balogh, 1964), 11β- (Baillie, Ferguson/

11β- (Baillie, Ferguson, Calman and Hart, 1965), 20β- (Baillie, Calman, Ferguson and Hart, 1965a), 3α-, 6β-, 11α-, 16α-, 16β-, 17α-, 21- and 24-hydroxysteroid dehydrogenases (Baillie, Calman, Ferguson and Hart, 1966). The distribution of these enzymes has been extensively investigated in steroid-producing endocrine tissues and, more recently, in duodenal epithelium (Baillie, Calman and MacKay, 1966) sebaceous glands (Baillie, Calman and Milne, 1965) and extra-placental chorion (Hart, 1966).

The kidney and liver in man are widely accepted to be extensively involved in steroid synthesis, metabolism and excretion; this paper describes the histochemical utilisation of various hydroxysteroids by human (foetal and adult), mouse, rat, hamster, guinea pig, sheep and bullock kidney and liver.

Previous histochemical literature does not report the demonstration of hydroxysteroid dehydrogenase activity in the kidney but Wattenberg (1958) observed 3β-hydroxysteroid dehydrogenase in the livers of mouse, rat and rabbit and Pearson and Grose (1959) detected 17β-hydroxysteroid dehydrogenase in rat liver.

MATERIAL AND METHODS.

Kidneys were obtained from Swiss white mice, Royal Wistar rats, hamsters, guinea pigs, sheep and bullocks. An age series of mouse kidney was established by killing three animals at weekly intervals between birth and the end of the tenth week of post-natal life inclusive.

Biopsies of two adult male and female human livers and the livers of three human foetuses of crown-rump length 4.5 cm. to 19 cm. were studied. Livers of male and female Swiss white mice, Royal Wistar rats, hamsters and guinea pigs were also surveyed.

In each instance the tissue was frozen on solid carbon dioxide within two minutes of interruption of its blood supply, sectioned at 10 μ in a cryostat maintained at -25°C and the sections attached to clean, dry glass coverslips by momentary thawing. These sections were incubated at 37°C in phosphate buffer medium (pH 7.4) containing N.A.D. or N.A.D.P. and Nitro B.T. (Sigma: 2,2'-Di-p-nitrophenyl-5, 5'-diphenyl-3, 3'-(3,3'-dimethyl-4,4 diphenylene)-ditetrazolium Chloride) together with the following steroid substrates/

substrates dissolved in dimethyl formamide (final conc. 0.5 mM):

(1) 3α-Hydroxy-5α-androstan-17-one. Steraloids.

(2) 3α-Hydroxy-5β-androstan-17-one. Steraloids.

(3) 3β-Hydroxy-4,4,14α-trimethyl-8, 24, (5α)-Cholest adien (Lanosterol). Steraloids.

(4) 3β-Hydroxypregn-5-ene-20-one (pregnenolone). Boots.

(5) 3β, 17α-Dihydroxypregn-5-ene-20-one (17α-hydroxy pregnenolone). Boots.

(6) 3β-Hydroxyandrost-5-ene-17-one (D.H.A.). Steraloids.

(7) 3β-Sulphoxypregn-5-ene-20-one (pregnenolone sulphate). M.R.C.

(8) 3β-Sulphoxy-17α-hydroxypregn-5-ene-20-one (17α-hydroxypregnenolone sulphate). E. Cameron.

(9) 3β-Sulphoxyandrost-5-ene-17-one (D.H.A. sulphate). M.R.C.

(10) 6β-Hydroxypregn-4-ene-3, 20-dione (6β-hydroxy-progesterone). Steraloids.

(11) 11α-Hydroxypregn-4-ene-3, 20-dione (11α-hydroxy-progesterone). Steraloids.

(12) 11β-Hydroxyandrost-4-ene-3, 17-dione (11β-hydroxy-androsterone). Steraloids.

(13) 11β/

(13) 11β-Hydroxypregn-4-ene-3; 20-dione (11β-hydroxy-progesterone). Steraloids.

(14) 12α-Hydroxypregn-4-ene-3; 20-dione (12α-hydroxy-progesterone). Steraloids.

(15) 16α-Hydroxypregn-4-ene-3, 20-dione (16α-hydroxy-progesterone). Steraloids.

(16) 3β-Hydroxyandrost-5-ene-16-one 3-methyl ether. Steraloids.

(17) 3β, 16β-Dihydroxyandrost-5-ene-3-methyl ether. Steraloids.

(18) 17α-Hydroxyandrost-4-ene-3-one (epitestosterone). Steraloids.

(19) 3, 17α-Dihydroxyoest-1,3,5,(10)-trien (epioestradiol) Organon.

(20) 17β-Hydroxyandrost-4-ene-3-one (testosterone). Organon.

(21) 3, 17β-Dihydroxyoestra-1,3,5,(10)-trien (oestradiol) Organon.

(22) 3-Hydroxyestra-1,3,5,(10)-trien-17-one (oestrone). Organon.

(23) 17β-Hydroxy-5α-androstan-3-one. Organon.

(24) 17β-Hydroxy-5β-androstan-3-one. Organon.

(25) 17β-Sulphoxyandrost-4-ene-3-one (testosterone sulphate). Organon.

(26) 20β/

(26) 20β-Hydroxypreg-4-ene-3-one (20β-hydroxy-progesterone). Steraloids.

(27) 21-Hydroxypregn-4-ene-3-one (21-hydroxyprogest-erone). Steraloids.

(28) 24-Hydroxy-5β-cholan-3-one. Steraloids.

(29) 5α-Androstan-3, 17-dione. Koch Light.

(30) 5β-Androstan-3, 17-dione. Koch Light.

In order to ascertain the pyridine nucleotide specificity of the various hydroxysteroid dehydrogenases, sections of each tissue were incubated separately in N.A.D.- and N.A.D.P.- containing media with each steroid substrate. Control sections were incubated in media containing N.A.D. or N.A.D.P., but no steroid.

RESULTS.

Kidney: The results are summarised in Tables 1 and 2. Hydroxysteroid dehydrogenase activity was strongest in the collecting tubules and the proximal and distal convoluted tubules, being less marked in Henle's loop (Figs. 1 and 2). The 3α-Hydroxysteroid with the 5α-configurationwas better utilised histochemically than the corresponding 5β-steroid. 11β-Hydroxysteroid dehydrogenase was present in the collecting tubules of the mouse and to a lesser extent in rat (Fig. 3). The

5α-/

TABLE I.

SUBSTRATE:- / SPECIES	3α-Hydroxy-5α-androstan-17-one	3α-Hydroxy-5β-androstan-17-one	Lanosterol	Pregnenolone	17α-Hydroxyprogenen.	D.H.A.	11β-Hydroxyandrost.	11β-Hydroxyprogest.	3β,16β-dihydroxyandrost-5-ene-3 methyl ether	Testosterone	Oestradiol	17β-Hydroxy-5α-androstan-3-one	17β-Hydroxy-5β-androstan-3-one
Mouse	+	+	-	tr	tr	tr	+	+	tr	tr	tr	-	-
Rat	+	+	tr	tr	tr	tr	+	+	tr	+	+	-	-
Hamster	+	+	tr	tr	tr	tr	+	+	+	+	+	+	+
Guinea Pig	+	+	tr	tr	tr	tr	+	+	+	+	+	+	+
Sheep	+	+	tr	tr	tr	tr	+	+	+	+	+	+	+
Bullock	+	+	tr	tr	tr	tr	+	+	+	+	+	+	+
Co-factor													
N.A.D.	+	+	+	+	+	+	+	+	+	+	+	+	+
N.A.D.P.	+	+	-	-	-	-	+	+	+	+	+	+	+

Hydroxysteroid Dehydrogenase Activity in Kidney.

No colour was obtained in any kidney when Pregnenolone Sulphate, 17α-Hydroxypregnenolone Sulphate, D.H.A. Sulphate, 11α-Hydroxyprogesterone, 12α-Hydroxyprogesterone, 16α-Hydroxyprogesterone, 3β-Hydroxyandrost-5-ene-16-one-3 methyl ether, Epitestosterone, Epioestradiol, Oestrone, Testosterone Sulphate, 20β-Hydroxyprogesterone, 21-Hydroxyprogesterone, 24-Hydroxy-5β-cholan-3-one, 5α-Androstan-3,17-dione or 5β-Androstan-3, 17-dione was used as steroid substrate.

TABLE 2.

Mouse Kidney Age Series.

Age in Weeks	3 -Hydroxysteroid Dehydrogenase	11 -Hydroxysteroid Dehydrogenase
0	+	-
1	+	trace
2	+	+
3	+	+
4	+	+
5	+	+
6	+	+
7	+	+
8	+	+
9	+	+
10	+	+

TABLE 3.

SUBSTRATE:- / SPECIES	3α-Hydroxy-5α-androstan-17-one	3α-Hydroxy-5β-androstan-17-one	Lanosterol	Pregnenolone	17α-Hydroxyprogesn.	D.H.A.	6β-Hydroxyprogest.	11β-Hydroxyandrost.	11β-Hydroxyprogest.	16α-Hydroxyprogest.	3β,16β-dihydroxy-androst-5-ene 3-methyl ether	Testosterone	Oestradiol	17β-Hydroxy-5α-androstan-3-one	17β-Hydroxy-5β-androstan-3-one	20β-Hydroxyprogest.	5α-Androstan-3, 17-dione	5β-Androstan-3, 17-
uman Foetal	+	tr	+	tr	tr	tr	+	+	+	-	tr	+	+	+	+	tr	tr	tr
uman Adult (♂)	+	tr	+	tr	tr	tr	+	+	+	tr	+	+	+	+	+	tr	tr	tr
uman Adult (♀)	+	tr	+	tr	tr	tr	+	+	+	tr	+	+	+	+	+	tr	tr	tr
ouse (♂)	+	+	+	tr	tr	tr	tr	+	+	+	+	+	+	+	+	tr	-	-
ouse (♀)	+	+	+	tr	tr	tr	tr	+	+	tr	tr	+	+	+	+	tr	-	-
at (♂)	+	+	+	tr	tr	+	tr	+	+	+	+	+	+	+	+	tr	tr	tr
at (♀)	+	±	+	tr	tr	tr	tr	tr	tr	tr	tr	+	+	+	+	tr	tr	tr
amster (♂)	+	+	+	+	+	+	+	*	+	+	+	+	+	+	+	tr	-	tr
amster (♀)	+	+	+	+	+	+	tr	+	+	+	tr	+	+	+	+	tr	-	tr
uinea Pig (♂)	+	+	+	tr	tr	+	tr	+	-	+	+	+	+	+	+	tr	tr	tr
uinea Pig (♀)	+	+	+	tr	tr	+	tr	tr	tr	tr	+	+	+	+	+	tr	-	-
o-factor																		
.A.D.	+	+	+	+	+	+	+	+	-	+	+	+	+	+	+	+	+	+
.A.D.P.	+	+	-	-	-	-	-	+	+	-	-	+	+	+	+	-	+	+

Hydroxysteroid Dehydrogenase Activity in Liver

No colour was obtained in any liver when Pregnenolone Sulphate, 17α-hydroxypregnenolone ulphate, D.H.A. Sulphate, 11α-Hydroxyprogesterone, 12α-Hydroxyprogesterone, 3β-Hydroxyandrost -ene-16-one-3 methyl ether, Epitestosterone, Epiostradiol, Oestrone, Testosterone, Sulphate, l-Hydroxyprogesterone or 24-Hydroxy-5β-cholan-3-one was employed as steroid substrate.

5α- and 5β-androstans were not utilised to any significant extent.

Liver: The liver results are summarised in Table 3. As with kidney, the 3α-hydroxysteroid having the 5α-configuration was better utilised in this histochemical system than the corresponding 5β-steroid. Of particular interest was the development of a colour in liver cells incubated with the androstans. Incubating with the 3β-hydroxysteroid lanosterol gave a good histochemical reaction and pregnenolone, 17α-hydroxypregnenolone and D.H.A. were less well used. All the 17β-hydroxysteroids were well used; testosterone gave a slightly stronger reaction than oestradiol in one of the foetal livers, but in the remainder both substrates were equally well used. With 3α-, 3β-, 6β-, 16α- and 16β-hydroxysteroids there was a marked sex difference; the male livers utilised these hydroxysteroids better than female livers.

DISCUSSION.

3α-Hydroxysteroid dehydrogenase is thought to be concerned with the detoxication of steroids and their elimination from the circulation (Dorfman and Ungar, 1953); compounds such as cortisol, with a Δ^4-3-keto grouping, can be reduced prior to conjugation and subsequent excretion. A transhydrogenase function has also been ascribed to 3α-hydroxysteroid dehydrogenase

Hurlock and Talalay, 1958; Baron, Gore, Pietruszko and Williams, 1963) which with its dual nucleotide specificity, is capable of hydrogen transfer between di- and triphosphopyridine nucleotides. 3α-Hydroxy-steroid dehydrogenase has been isolated from foetal and adult human liver, as well as from mouse, rat, guinea pig, rabbit and dog livers and kidneys (Hurlock and Talalay, 1958; Koide, 1963; Baron, Gore, Pietruszko and Williams, 1963; Villee and Loring, 1963; Aoshima, Kochakian and Jadrijevic, 1964), and, as in our histo-chemical system, they are N.A.D. or N.A.D.P. linked. 3α-Hydroxysteroids are believed to be interconverted by this specific enzyme or enzymes with the corresponding 3-keto steroids and biochemical examples known to occur include the conversions of androstenedione and testo-sterone to androsterone and aetiocholanolone (Vande Wiele, MacDonald, Gurpide and Lieberman, 1963) respectively and it has been shown that this step is a prelude to glucuronide conjugation and excretion of the steroid concerned (Tomkins and Isselbacher, 1954).

Theoretically both 5α- and 5β-compounds can serve as substrates for this enzyme and our results agree with those of Tomkins (1956) in that both are used, but it is interesting to note that 3α-hydroxy-5α-androstan-17-one gives a good reaction in kidney and liver/

liver while the corresponding 5β-steroid is poorly used. Clinically, aetiocholanolone (3α-hydroxy-5β-androstan-17-one) gives rise to pyrexia on injection (Kappas, Hellman, Fukushima and Gallagher, 1956; Shulman, Herrman and Petersdorf, 1964) and this would appear to be function of the cis A/B ring junction; 3α-hydroxysteroids with a 5α-configuration, such as androsterone, do not appear to cause pyrexia. Kappas, Soybel, Fukushima and Gallagher,(1958) found that injected aetiocholanolone is excreted mostly unchanged in the urine and not converted to the diketone form. The inaptitude of the liver to metabolise 3α-hydroxy-5β-androstan-17-one to a considerable extent histochemically may rationalise the clinical effects of this steroid.

The poor 3β-hydroxysteroid dehydrogenase activity noted in kidney and liver compared with the stronger 3α-hydroxysteroid dehydrogenase forms a marked contrast with steroid synthesing endocrine tissues where 3β-hydroxy-steroids are the much better utilized and this observation may be added to the reasons (Baillie et al. 1965C) for supposing that the histochemical techniques are specific. The presence of 3β-hydroxysteroid dehydrogenase in renal (Aoshima et al., 1964) and hepatic tissue (Hamm,/

(Hamm, Kochakian and Carroll, 1956; Klempien, Voigt and Tamm, 1961; Rubin, Strecker and Koff, 1963) is well known and Rubin and Strecker (1961) and Rubin et al. (1963) reported a much higher level of activity in the male than in the female rate, apparently a function of circulating testosterone; our histochemical findings are in accord with the biochemical results of Rubin et al. (1961 and 1963) in that a sex difference was noted in the ability of male rat liver to utilise 3β-hydroxysteroids better than the female liver. Lanosterol was a more suitable substrate than pregnenolone, 17α-hydroxypregnenolone or D.H.A. for the demonstration of 3β-hydroxysteroid dehydrogenase in the liver and in conjunction with the finding of desmosterol, the immediate precursor of cholesterol (Blohm, Karuja and Laughlin, 1959; Stokes and Fish, 1960) and of cholesterol in the liver parenchyma (Clementi and Fumagalli, 1962), probably indicates cholesterol biosynthesis in the liver (Goodwin, 1963).

6α- and 6β-hydroxylation has been established in rat (Axelrod and Miller, 1954; Breuer and Knuppen, 1959; Breuer, Nocke and Pangels, 1960; Breuer Knuppen and Pangels, 1962) and human liver (Cohn, Upton and Bondy, 1961; Lipman, Katz and Jailer, 1962) and the further metabolism/

metabolism of such steroids may involve a 6β-hydroxy-steroid dehydrogenase (Breuer et al. 1962) so it is not surprising to find an N.A.D. dependent 6β-hydroxy-steroid dehydrogenase histochemically in liver. 6-Keto and 6β-hydroxyl groups are reversibly interconverted by 6β-hydroxysteroid dehydrogenase (Dorfman, 1957).

Koerner and Hellman (1964) noted the presence of an 11β-hydroxysteroid dehydrogenase in rat liver, more active in the male than in the female and both N.A.D.- and N.A.D.P.-linked. The present histochemical findings support these biochemical observations with sex differences especially noteable in rat and guinea pig livers. Renal 11β-hydroxysteroid dehydrogenase has been shown to convert cortisol to cortisone (Mahesh, 1960; Koerner and Hellman, 1964) and, as with liver, both N.A.D. and N.A.D.P. could be used as cofactors. The occurrence of strong 11β-hydroxysteroid dehydrogenase in the collecting tubules of the mouse kidney was unexpect These cells show no 11β-hydroxysteroid dehydrogenase activity at birth and the reactivity gradually becomes established over the next fourteen days.

The presence of a 16α-hydroxysteroid dehydrogenase has previously been recorded in rat liver (Corrcale and Balestreri/

(Correale and Balestreri, 1962) and the present results both support and extend this work to include several other species. 16α-Hydroxysteroid dehydrogenase could not be demonstrated histochemically in foetal human liver although it was apparent in adult human liver. Paradoxically, 16α-hydroxylase occurs in foetal human liver (Engel, Baggett and Halla, 1962) but not in adult human liver (Engel, Baggett and Halla, 1958). On the other hand, Breuer, Nocke and Pangels (1960) and Colas (1962) found 16α-hydroxylase in the liver of adult rats and similarly Axelrod, Miller and Herling (1956) noted activity in adult dog livers. Male rats have been shown to possess a much higher level of 16α-hydroxylase than do females (Rubin, 1957; Forchielli, Brown-Grant and Dorfman, 1958; Colas, 1962) and the present results indicate that the livers of male mice, rats and guinea pigs have a higher level of 16α-hydroxysteroid dehydrogenase than do those of females.

An enzyme system which readily converts oestriol to 16-Keto oestradiol has been described in the kidneys of mature female rats (Ryan, Meigs, Petro and Moorison, 1963); full enzymic activity also occurred in immature rats of either sex or in male rats after administration of oestradiol. 16α- And

and 16β-hydroxysteroids occur as progesterone (Villee, Dimoline, Engel, Villee and Racker, 1962) and oestrogen derivatives (Levitz, Spitzer and Twombly, 1958; Breuer and Nocke, 1959) and it would appear that these 16-hydroxy compounds can be interconverted via the 16-keto group (Levitz, Rosen and Twombly, (1960). A 16α-hydroxy oestriol glucuronide conjugate has recently been isolated (Slaunwhite, Karsay and Sandberg, 1964; Slaunwhite, Lichtman and Sandberg, 1964) and our finding 16β-hydroxysteroid dehydrogenase in the renal tubules might indicate that 16β-hydroxy oestrogens are converted via the 16-keto form to 16α-hydroxy derivatives, subsequently to be excreted conjugated with glucuronic acid.

Steroid 17α-hydroxylase activity has been detected biochemically in rat (Breuer and Knuppen, 1961) and rabbit liver (Schneider and Mason, 1948; Breuer and Pangels, 1960). Our failure to demonstrate 17α-hydroxy-steroid dehydrogenase activity may be related to Breuer and Pangels' (1960) observation that oestrone is preferentially metabolised to oestradiol-17β.

Soluble and insoluble N.A.D. and N.A.D.P. linked 17β-hydroxysteroid dehydrogenases have been described in/

in foetal and adult human liver (Stylianou, Forchielli, Tummillo and Dorfman, 1961; Nocke, Breuer and Knuppen, 1961; Engel, Baggett and Halla, 1962) as well as in rat, rabbit, guinea pig, mouse, hamster and dog livers (Endahl, Kochakian and Hamm, 1960; Villee and Spencer, 1960; Breuer and Pangels, 1960; Breuer, Knuppen and Pangels, 1962; Endahl and Kochakian, 1962; Aoshima and Kochakian, 1963; El Attar, Mosebach and Dirscherl, 1964) and the histochemical observations accord well with the biochemical findings.

17β-Hydroxysteroid dehydrogenase has been demonstrated biochemically in kidney (Velle and Erichsen, 1960; Aoshima and Kochakian, 1963) and may now be anatomically assigned mainly to the convoluted tubules. Oestradiol-17β and oestrone are interconvertible in bovine kidney cells (Ville and Erichsen, 1960) and 17α-oestradiol is the excretion product. Our demonstration of 17β-hydroxysteroid dehydrogenase and not of 17α-hydroxysteroid dehydrogenase in the tubules of kidneys examined, other than the mouse, supports the biochemical evidence that 17β-hydroxy oestrogens are converted via the 17-keto form to be excreted as the 17α-hydroxy form.

20β-Hydroxysteroid dehydrogenase has been demonstrated biochemically/

biochemically (Acevedo, Axelrod, Ishikawa and Takaki, 1963) and Baillie et al. (1965a) described its histochemical localization in endocrine tissues wherein 20β-hydroxysteroid dehydrogenase was found in human tissues but not to any significant extent in rodent tissue. The present results, however, showed 20β-hydroxysteroid dehydrogenase activity existed in both human and rodent liver.

21-Hydroxysteroid dehydrogenase (N.A.D. linked) occurs in ovine, bovine and porcine livers (Mander and White, 1963) and the equilibrium appears to very strongly favour reduction; this might account for the absence of 21-hydroxysteroid dehydrogenase in the tissue sections. Taylor (1959) using rabbit liver homogenate could not demonstrate any obvious 21-hydroxysteroid dehydrogenase either.

ACKNOWLEDGEMENTS.

The authors are grateful to the Department of Anatomy, Glasgow University, for the research facilities provided and are further indebted to M.R.C., Boots Pure Drug Co. Ltd., Searle Ltd., and Organon Laboratories for the generous supply of various steroids.

REFERENCES.

ACEVEDO, H.F., L.R. AXELROD, E. ISHIKAWA & F. TAKAKI. Studies in fetal metabolism. II. Metabolism of progesterone-4-C^{14} and pregnenolone-7α-H^3 in human fetal testis. J. clin. Endocr. 23: 885 - 890 (1963).

AOSHIMA, Y. & C.D. KOCHAKIAN. Activity, intra-cellular distribution and some properties of 17β-hydroxy-c-19-steroid dehydrogenases in liver and kidney. Endocrinology. 72: 106 - 114 (1963).

AOSHIMA, Y., C.D. KOCHAKIAN & D. JADRIJEVIC. T.P.N.- and D.P.N.-specific 3α-hydroxy and Δ^5-3β-hydroxy-steroid dehydrogenases of liver and kidney. Endocrinology. 74: 521 - 531 (1964).

AXELROD, L.R. & L.I. MILLER. The bio-oxidation of steroids at carbon 6 by isolated perfused rat livers. Arch. Biochem. 49: 248 - 249. (1954).

AXELROD, L.R., L.L. MILLER & F. HERLING. The metabolism of testosterone in the isolated perfused dog liver. J. biol. Chem. 219. 455 - 461 (1956).

BAILLIE, A.H., M.M. FERGUSON, K.C. CALMAN & D.McK. HART. Histochemical demonstration of 11β-hydroxy-steroid dehydrogenase. J. Endocr. 33: 119 - 125 (1965).

BAILLIE, A.H., K.C. CALMAN, M.M. FERGUSON & D.McK. HART. Histochemical demonstration of 20β-hydroxysteroid dehydrogenase. J. Endocr. 32: 337 - 339. (1965).

BAILLIE, A.H., K.C. CALMAN, M.M. FERGUSON & D.McK. HART. Histochemical utilisation of 3α-, 6β-, 11α-, 12α-, 16α-, 16β-, 17α-, 21- and 24-hydroxysteroids. J. Endocr. 34: 1[illegible]-12 (1966).

BAILLIE, A.H., K.C. CALMAN & C. MACKAY. Histochemical distribution of hydroxysteroid dehydrogenases in human alimentary tract. J. Roy. micr. Soc. (In Press). (1966).

BAILLIE, A.H., K.C. CALMAN & J.A. MILNE. Histochemical distribution of hydroxysteroid dehydrogenases in human skin. Brit. J. Derm. (in Press). (1965).

BALOGH, K. A histochemical method for the demonstration of 20α-hydroxysteroid dehydrogenase activity in rat ovaries. J. Histochem. Cytochem. 12: 670 - 673. (1964).

BARON, D.N., M.B.R. GORE, R. PIETRUSZKO & D.C. WILLIAMS. Purification and properties of the 3α-hydroxysteroid dependent nicotinamide-adenine dinucleotide transhydrogenase of rat liver. Biochem. J. 88: 19 - 25. (1963).

BLOHM, T.R., T. KARUJA & M.W. LAUGHLIN. Effects of MER-20, a cholesterol synthesis inhibitor, on mammalian tissue lipides. Arch. Biochem. 85: 250 - 263. (1959).

BREUER, H. & R. KNUPPEN. Isolation of 6-hydroxy-oestradiol-17β and oestriol after incubation of oestradiol-17β with rat liver slices. Biochim. biophys. Acta (Amst.) 39: 408 - 411 (1960).

BREUER, H. & R. KNUPPEN. The formation and hydrolysis of 16α, 17α-epoxy-oestratrien-3-ol by rat liver tissue. Biochim. biophys. Acta (Amst.) 49: 620-621. (1961).

BREUER, H., R. KNUPPEN u. G. PANGELS. Konfiguration, Biogenese und Stoffwechsel von 6α- und 6β-hydroxylierten phenolischen Steroiden. Biochim. biophys. Acta (Amst.) 65: 1 - 12. (1962).

BREUER, H. & L. NOCKE. Formation of estriol-3, 16β, 17α by liver tissue in vitro. Biochim. biophys. Acta (Amst.) 36: 271 - 272 (1959).

BREUER, H., L. NOCKE u. G. Pangels. Untersuchungen uber den zeitlichen Verlauf der 6-und 16α-Hydroxylierung von Oestrogen durch Rattenleberschnitte. Acta endocr. (Kbh.) 34: 359 - 365. (1960).

BREUER, H. u. G. Pangels. Stoffwechsel von Oestron Oestradiol-17α und Oestradiol-17β in der Kaninchenleber. Acta endocr. (Kbh.) 33: 532 - 538 (1960).

CLEMENTI, F. & R. FUMAGALLI. Intracellular localization in the liver of exogenous 3-H cholesterol: autoradiographic and radiochemical assay. Exp. Cell Res. 28: 604 - 608.(1962).

COHN, G.L., V. UPTON & P.K. BONDY. The in vivo conversion of cortisol-4-C^{14} to 6β-hydroxycortisol-4-C^{14} by the human cirrhotic liver. J. clin. Endocr. 21: 1328 - 1330. (1961).

COLAS, A. The 16α-hydroxylation of D.H.A. (3β-hydroxy-androst-5-en-17-one) by rat liver slices. Biochem. J. 82: 390 - 394 (1962).

CORREALE, L. & R. BALESTRERI. Metabolism of oestrogens oxygenated in position C-16. Conversion in vitro of estriol 16 keto 17β-Oestradiol by rat liver. Arch. Maragliano Pat. Clin. 18: 653 - 658 (1962).

DORFMAN, R.I. Comments on the metabolism of steroid hormones. Cancer Res. 17: 535 - 536 (1957).

DORFMAN, R.I. & F. UNGAR. Metabolism of steroid hormones. Minneapolis: Burgess Publ. Co. 1953.

EL ATTAR, T., K.O. MOSEBACH, & W. DIRSCHERL. The initial metabolism of 4-^{14C}-testosterone in the rat. II. In liver, blood plasma, seminal vesicles and ventral prostate. Acta endocr. (Kbh.) 45: 437 - 446 (1963).

ENDAHL, G.L. & C.D. KOCHAKIAN. Partial purification and further characterization of the triphospho-pyridine nucleotide specific C19-17β-hydroxy-steroid dehydrogenase of guinea pig liver. Biochim. biophys. Acta (Amst.) 62: 245 - 250 (1962).

ENDAHL, G.L., C.D. KOCHAKIAN & D. HAMM. Separation of a TPN-specific from a DPN-specific 17β-hydroxy (testosterone) dehydrogenase of guinea pig liver. J. biol. Chem. 235: 2792 - 2796 (1960).

ENGEL, L.L., B. BAGGETT & M. HALLA. The formation of ^{14}C-labelled estriol from 16-^{14}C-estradiol-17β by human fetal liver slices. Biochim. biophys. Acta (Amst.) 30: 435 - 436 (1958).

ENGEL, L.L., B. BAGGETT & M. HALLA. In vitro metabolism of estradiol-17β by human fetal liver: formation of estriol, 16-epiestriol estrone and an estriol glucosiduronic acid. Endocrinology 70: 907 - 914 (1962)

FORCHIELLI, E., K. BROWN-GRANT & R.I. DORFMAN. Steroid Δ^4 hydrogenases of rat liver. Proc. Soc. exp. Biol. (N.Y.) 99: 594 - 596. (1958).

GOODWIN, T.W. The biosynthesis of vitamins and related compounds. London: Academic Press 1963.

HAMM, D.I., C.D. KOCHAKIAN & B.R. CARROLL. 3β-hydroxysteroid dehydrogenase in both supernatant and particulate fractions from livers of male guinea pigs. Proc. Soc. exp. Biol. (N.Y.) 93: 493 (1956).

HART, D.McK. The histochemical distribution of hydroxysteroid dehydrogenases in the human foetal membranes at term. Histochemie (In Press) (1966).

HURLOCK, B. & P. TALALAY. 3α-hydroxysteroids as co-enzymes of hydrogen transfer between di- and triphosphopyridine nucleotides. J. biol. Chem 233: 886 - 897 (1958).

KAPPAS, A.L. HELLMAN, D.K. FUKUSHIMA & T.F. GALLAGHER. The pyrogenic effect of etiocholanolene (3α-hydroxyetiocholan-17-one). J. clin. Endocr. 16: 948 (1956).

KAPPAS, A., W. SOYBEL, D.I.K. FUKUSHIMA & T.F. GALLAGHER. The thermogenic effect and metabolic fate of etiocholanolene in man. J. clin. Endocr. 18: 1043 - 1055 (1958).

KLEMPIEN, E.J., K.D. VOIGT u.J. TAMM. Der Umsatz von Dehydroisoandrosteron in der Hundelber. Acta endocr. (Kbh.) 36: 498 - 510 (1961).

KOERNER, D.R. & L. HELLMAN. Effect of thyroxine administration of the 11β-hydroxysteroid dehydrogenase in rat liver and kidney. Endocrinology. 75: 592 - 601 (1964).

KOIDE, S.S. Purification of 3α-hydroxysteroid dehydrogenase obtained from the soluble fraction of rat liver. Arch. Biochem. 101: 278 - 285. (1963).

LEVITZ, M., M.F. ROSEN & G.H. TWOMBLY. Interconversions of 16-oxygenated estrogens. II.Metabolism of 16 keto estradiol 17β 16-C-^{14} in man. Arch. Biochem. 88: 212 - 215 (1960).

LEVITZ, M., J.R. SPITZER & G.H. TWOMBLY. Interconversions of 16-xoygenated steroids. I.The synthesis of estriol-16-C^{14} and its metabolism in man. J. biol. Chem. 231: 787 - 797 (1958).

MAHESH, V.B. In vitro metabolism of steroids in the rat kidney. Fed. Proc. 29: 174 - (1960).

MANDER, C. & A. WHITE. Purification and properties of a sheep liver 21-hydroxysteroid N.A.D.-oxidoreductase J. biol. Chem. 238: 767 - 774 (1963).

NOCKE, W., H. BREUER u. R. KNUPPEN. Isolierung und quantitative Bestimmung der 4 epimer Ostriole nach Injektion von 16α- und 16β-Hydroxyoestron beim Menschen. Acta endocr. (Kbh.) 36: 393 - 403 (1961).

PEARSON, B. & F. GROSE. Histochemical demonstration of 17β-hydroxysteroid dehydrogenase by use of tetrazolium salt. Proc. Soc. exp. Biol. (N.Y.) 100: 636 - 638 (1959).

PEDERSON, K. J. & O.Z. DALGAARD. Microscopical histochemical studies on fifty human percutaneous renal biopsies. Acta path. Microbiol. scand. 50: 237 - 256 (1960).

RUBIN, B.L. Sex differences in orientation of reduction products of 3-keto, C^{19} steroids by rat liver homogenates. J. biol. Chem. 227: 917 - 927 (1957).

RUBIN, B.L. & H.J. STRECKER. Further studies on the sex difference in 3β-hydroxysteroid dehydrogenase activity of rat livers. Endocrinology 69: 257 - 267 (1961).

RUBIN, B.L., H.J. STRECKER & E.B. KOPF. Further observations on sex-influenced activity of 3β-hydroxysteroid dehydrogenase of rat liver. Endocrinology 72: 764 - 770 (1963).

RYAN, K.J., R.A. MEIGS, Z. PETRO & G. MOORISON. Estrogen-induced 16-hydroxysteroid dehydrogenase activity in rat kidney. Science 142: 243 (1963).

SCHNEIDER, J.J. & H.L. MASON. Studies on intermediary steroid metabolism. I. Isolation of Δ^5-androstene-3β, 17α-diol and Δ^5-androstene-3β,16β,17α-triol following the incubation of dehydroisoandrosterone with surviving rabbit liver slices. J. biol. Chem. 172: 771 - 782 (1948).

SHUIMAN, J.A., W.L. HERRMAN & R.G. PETERSDORF. Experimental etiocholanolone fever. J. clin. Endocr. 24: 1136 - 1142 (1964).

SLAUNWHITE, W.R., M.A. KARSAY & A.A. SANDBERG. Studies on phenolic steroids in human subjects. V. Excretion of urinary estriol glucosiduronate during pregnancy. J. clin. Endocr. 24: 263 - 266 (1964).

SLAUNWHITE, W.R., M.A. LIGHTMAN & A.A. SANDBERG. Studies on the phenolic steroids in human subjects. VI. Biosynthesis of estriol glucosiduronic acid by human liver. J. clin. Endocr. 24: 638 - 643 (1964).

STOKES, W.M. & W.A. FISH. Sterol metabolism. II. The occurrence of dermosterol (24 dehydrocholesterol) in rat liver. J. biol. Chem. 235: 2604 - 2607 (1960).

STYLIANOU, M., E. FORCHIELLI, M. TUMMILLO & R.I. DORFMAN. Metabolism in vitro of 4-C^{14}-testosterone by a human liver homogenate. J. biol. Chem. 236: 629 - 694 (1961

TAYLOR, W. The metabolism of 21-hydroxy-5β-pregnane-3, 20-dione by rabbit liver homogenate. Acta endocr. (Kbh.) 32: 187 - 194 (1959).

TOMKINS, G.M. A mammalian 3α-hydroxysteroid dehydrogenase. J. biol. Chem. 218: 437 - 447 (1956).

TOMKINS, G.M. & K.J. ISSELBACHER. Enzymic reduction of cortisone. J. Amer. chem. Soc. 76: 3100 - 3101 (1954).

VANDE WIELE, R.L., P.C. MACDONALD, E. GURPIDE & S. LIEBERMAN. Studies on the secretion and interconversion of the androgens. Rec.Progr.Hormone Res. 18: 275 - 310.

VELLE, W. & S. ERICHSEN. Studies on oestrogens in cattle. Acta endocr. (Kbh.) 33: 277 - 286 (1960).

VILLEE, C.A. & J.M. LORING. 20α-hydroxysteroid dehydrogenase and 3α-hydroxysteroid dehydrogenase in human fetal liver. Endocrinology 72: 824 - 828 (1963).

VILLEE, C.A. & J.M. SPENCER. Some properties of the pyridine nucleotide specific 17β-hydroxysteroid dehydrogenase in guinea pig liver. J. biol. Chem. 235: 3615 - 3619 (1960).

VILLEE, D.B., A. DIMOLINE, L.L. ENGEL, C.A. VILLEE & E. RACKER. Formation of 16α-hydroxyprogesterone in hyperplastic adrenal tissue. J. clin. Endocr. 22: 726 - 734.

WATTENBERG, L.W. Microscopic histochemical demonstration of steroid-3β-ol dehydrogenase in tissue sections. J. Histochem. Cytochem. 6: 225 - 232 (1958).

DESCRIPTION OF PLATE.

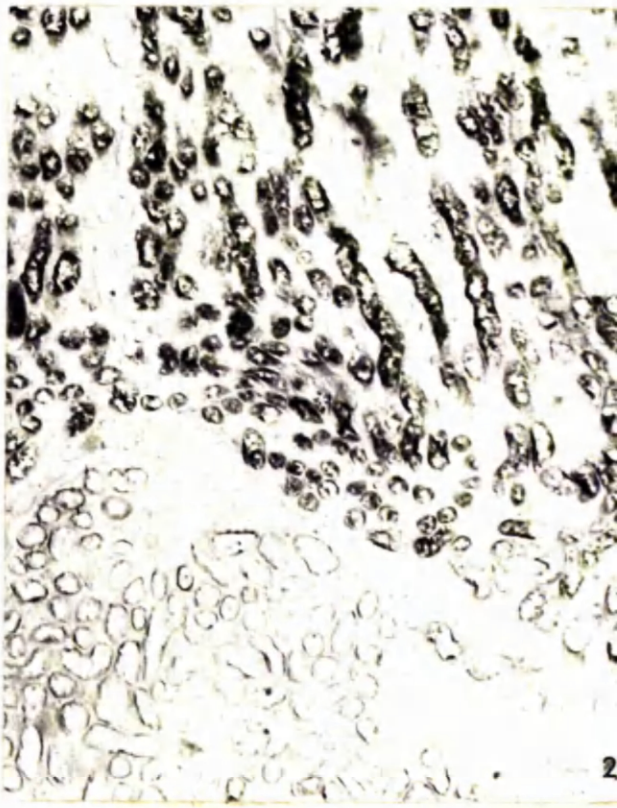
Fig. 1. 3α-Hydroxysteroid dehydrogenase activity in mouse kidney, showing a fairly intense deposition of diformazan in the renal tubules. x 200.

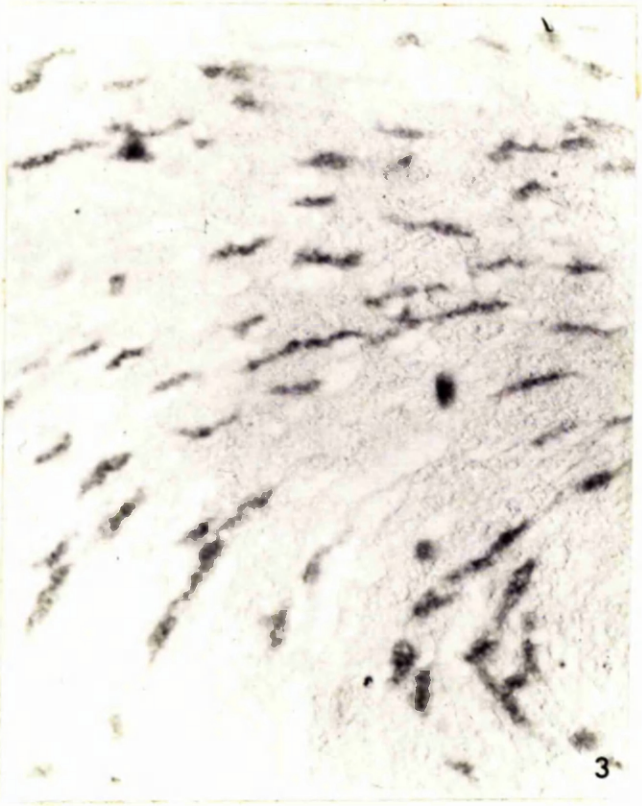
Fig. 2. Bullock kidney incubated in 3α-hydroxy-5α-androstan-17-one demonstrating enzymic activity in the tubules and loop of Henle but not in the collecting tubules. x 100.

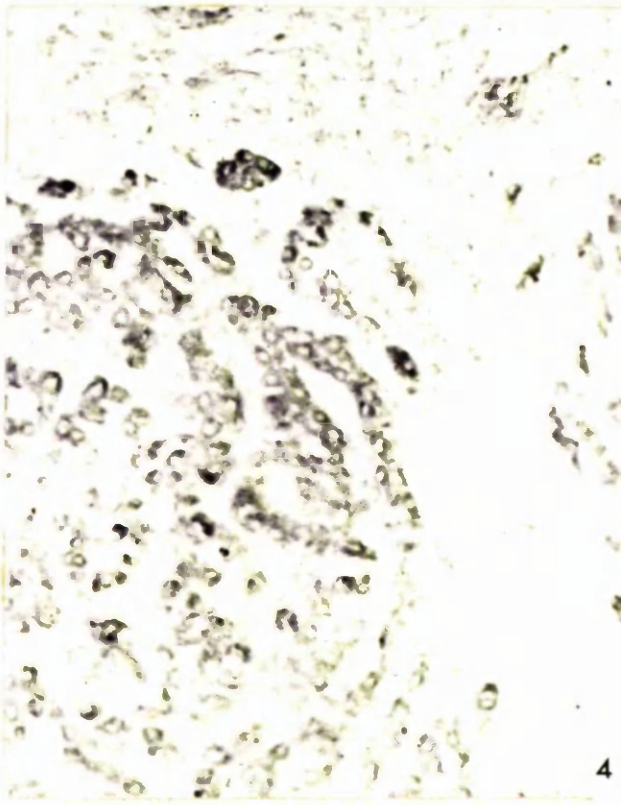
Fig. 3. Diformazan deposited in the collecting tubules of mouse kidney when incubated with 11β-hydroxyprogesterone x 100.

Fig. 4. 17β-Hydroxysteroid dehydrogenase in cirrhotic human liver with intense activity in the parenchymal cells and no significant activity in the fibrous tissue. x 200.

PLATE







Ontogenetic Distribution of Hydroxysteroid Dehydrogenase in Human Pro-, Meso- and Metanephric Kidneys; Phylogenetic Distribution of Hydroxysteroid Dehydrogenases in the Vertebrate Kidney.

BY

A. H. Baillie,

M. M. Ferguson,

D. McK. Hart.

Department of Anatomy, The University, Glasgow, W.2.

SUMMARY.

The human pronephros exhibited no hydroxysteroid dehydrogenase activity. The human mesonephros, like the amphibian and piscine mesonephros exhibits 16β- and 17β-hydroxysteroid dehydrogenase activity and on this basis possible human mesonephric function is suggested. Metanephric kidneys exhibit 3α-, 3β-, 6β-, 16α-, 16β- and 17β-hydroxysteroid dehydrogenase and 11β-hydroxysteroid dehydrogenase is present in all adult mammalian metanephric kidneys. 3α-Hydroxysteroid dehydrogenase occurs selectively and is very active in the proximal and distal convoluted tubules particularly of the juxta-medullary glomeruli, and this is thought to be related to the excretion of 3-hydroxy and 3-ketoxy steroids. 11β-Hydroxysteroid dehydrogenase is confined to a predominant cell type in the collecting tubules and its possible involvement with cortisol or androgen metabolism is noted. 17β-Hydroxysteroid dehydrogenase may be concerned in the excretion of the sex steroids and it occurs throughout the nephron, excluding the collecting tubules. 3β-, 6β-, 16α- and 16β-hydroxysteroid dehydrogenases are not as active histochemically in the kidney as the 3α-, 11β-/

11β- and 17β-hydroxysteroid dehydrogenases.

INTRODUCTION.

In a preliminary investigation of mouse, rat, hamster, guinea pig, sheep and bullock kidney Baillie et al. (1966) noted strong 3α-, 11β- and 17β-hydroxysteroid dehydrogenase activity and weak or moderate 3β-, 6β-, 16α- and 16β-hydroxysteroid dehydrogenase activity. The 3α- and 17β-hydroxysteroid dehydrogenases were mainly located in the proximal and distal convoluted tubules of the kidney while the 11β-hydroxysteroid dehydrogenase was confined to the collecting tubules. In view of the relevance of these observations to the metabolism and excretion of steroids by the kidney, it was decided to examine the three generations of human kidney histochemically for hydroxysteroid dehydrogenases; in addition it was felt that a phylogenetic study of the vertebrate kidney might prove rewarding.

MATERIAL AND METHODS.

The pro- and mesonephroi of a 14 mm. human foetus together with the metanephric kidneys of four foetuses ranging in length from 45 mm. to 190 mm. were studied. Four/

Four adult human kidneys from individuals ranging in age from 45 to 70 were obtained at nephrectomy. Two of the adult human kidneys had renal carcinomata, one was pyelonephrotic and the fourth was severely hydronephrotic. This constituted the material studied in the human ontogenetic survey.

In the phylogenetic survey representative examples of fish, amphibian, reptilian, avian and mammalian kidneys were chosen and the species details are summarised in Table 1.

In each case the kidney or part thereof was frozen on carbon dioxide within four minutes of interruption of blood supply and sectioned at 10μ, in a cryostat maintained at -20°C. The 14 mm. human foetus was frozen intact on removal and serially sectioned. The pro- and mesonephric areas were identified by histological examination and the appropriate sections selected for incubation. Incubations were conducted for four hours at 35°C in media described elsewhere (Baillie, Calman et al., 1965) to demonstrate hydroxysteroid dehydrogenase activity. The following steroid substrates, dissolved in dimethyl formamide, were used:- (1) 3α-Hydroxy-5α-androstan-17-one, (2) 3α-/

(2) 3α-Hydroxy-5β-androstan-17-one, (3) Pregnenolone, (4) D.H.A., (5) 6β-Hydroxyprogesterone, (6) 11α-Hydroxyprogesterone, (7) 11β-Hydroxyandrostenedione, (8) Progesterone, (9) 12α-Hydroxyprogesterone, (10) 16α-Hydroxyprogesterone, (11) 3β, 16β-Dihydroxyandrost-5-ene-3-methyl ether, (12) 3β-Hydroxyandrost-5-ene-16-one-3-methyl ether, (13) Epitestosterone, (14) Testosterone, (15) Oestradiol-17β, (15) 20β-Hydroxyprogesterone.

Sections of every kidney were routinely examined histologically and the presence of N.A.D. and N.A.D.P. diaphorase in all parts of the nephron and collecting duct system confirmed histochemically. Inhibition of 11β-hydroxysteroid dehydrogenase with equimolar spironolactone (Searle) was attempted, without success.

RESULTS.

The results are summarised in Tables 1 and 2, and in Plates 1 and 2. One or two additional remarks are necessary.

With regard to the adult human kidneys, no significant hydroxysteroid dehydrogenase activity was noted in the specimen from the grossly hydronephrotic kidney.

The two kidneys from individuals with clear cell carcinoma, or hypernephroma, were in a dubious functional state on account of encroachment of the tumour on the renal pelvis although the tissue blocks surveyed

TABLE I.

Human Kidney - Ontogenetic Study.

		3α-H.S.D.	3β-H.S.D.	6β-H.S.D.	11β-H.S.D.	16α-H.S.D.	16β-H.S.D.	17β-H.S.D.	20β-H.S.D.
Pronephros,	14 mm. foetus	-	-	-	-	-	-	-	-
Mesonephros,	14 mm. foetus	-	trace	-	-	-	+(1)	+(1)	-
Metanephros,	45 mm. 75 mm. 110 mm. 190 mm. foetuses	-	-	-	-	-	-	-	-
Metanephros,	Adult	+ poor (2)	trace (1)	-	+ (3)	-	+ poor (1)	+ poor (1)	trace

(1) Hydroxysteroid dehydrogenase activity noted in the entire nephron.

(2) Hydroxysteroid dehydrogenase activity mainly located in the proximal and distal convoluted tubules.

(3) Hydroxysteroid dehydrogenase activity confined to the collecting tubules.

TABLE 2.

Vertebrate Kidney - Phylogenetic Study.

Animal	Type of Kidney	3α-hydroxysteroid dehydrogenase	3β-hydroxysteroid dehydrogenase	6β-hydroxysteroid dehydrogenase	11β-hydroxysteroid dehydrogenase	16α-hydroxysteroid dehydrogenase	16β-hydroxysteroid dehydrogenase	17β-hydroxysteroid dehydrogenase	20β-hydroxysteroid dehydrogenase
DOGFISH - Scyliorhinus Canicula	Mesonephros	-	-	-	-	-	-	+	-
SKATE - Raja Batis	Mesonephros	+	-	-	-	-	-	-	-
SEA SCORPION - Cottus Bubalis	Mesonephros	trace	-	-	-	-	-	-	-
COD - Gadus Morhua	Mesonephros	trace	-	-	-	-	-	+	-
WHITING - Gadus Merlangus	Mesonephros	-	-	-	-	-	-	+	-
PLAICE - Platessa Platessa	Mesonephros	-	-	-	-	-	+	+	-
FROG - Rana temporaria	Mesonephros	-	-	-	-	-	trace	+(1)	-
TORTOISE - Testudo Graeca	Metanephros	+(1)	-	-	-	+(1)	+(1)	+(1)	-
CHICKEN - Gallus Domesticus	Metanephros	+(1)	-	-	+(3)	-	trace	+(1)	-

(1) Hydroxysteroid dehydrogenase activity noted in entire nephron.

(2) Hydroxysteroid dehydrogenase activity located mainly in the proximal and distal convoluted tubules.

(3) Hydroxysteroid dehydrogenase activity confined to the collecting tubules.

TABLE 2 (Cont'd).

Vertebrate Kidney - Phylogenetic Study.

Animal	Type of Kidney	3α-hydroxysteroid dehydrogenase	3β-hydroxysteroid dehydrogenase	6β-hydroxysteroid dehydrogenase	11β-hydroxysteroid dehydrogenase	16α-hydroxysteroid dehydrogenase	16β-hydroxysteroid dehydrogenase	17β-hydroxysteroid dehydrogenase	20β-hydroxysteroid dehydrogenase
RAT - Rattus Rattus	Metanephros	+(2)	trace	-	+(3)	-	trace	+(1)	-
MOUSE - Mus Muris	Metanephros	+(2)	trace	trace	+(3)	-	trace	+(1)	-
CAT - Felis Catus	Metanephros	+(2)	trace	trace	+(3)	trace	+(1)	+(1)	-
DOG - Canis Familiaris	Metanephros	+(2)	trace	trace	+(3)	+(1)	+(1)	+(1)	-
GUINEA PIG - Cavia Porcellus	Metanephros	+(2)	trace	trace	+(3)	-	+(1)	+(1)	-
COW - Bos Taurus	Metanephros	+(2)	trace	trace	+(3)	-	trace	+(1)	-
PIG - Sus Scrofa	Metanephros	trace	-	-	+(3)	-	-	+(1)	-
HAMSTER - Mesocricetus Auratus	Metanephros	+(2)	trace	trace	+(3)	-	+(1)	+(1)	-
MONKEY - Rhesus Macacus	Metanephros	trace	-	-	+(3)	trace	trace	trace(1)	-

(1) Hydroxysteroid dehydrogenase activity noted in entire nephron.

(2) Hydroxysteroid dehydrogenase activity located mainly in the proximal and distal convoluted tubules.

(3) Hydroxysteroid dehydrogenase activity confined to the collecting tubules.

surveyed histochemically were histologically normal. The pyelonephrotic kidney was severely damaged, from a histological point of view. These last three kidneys behaved similarly histochemically (Table I) but in our opinion the material was far from optimal and it seems likely that the reactions obtained with the pathological adult human kidneys surveyed may not represent the full range of hydroxysteroid dehydrogenase activity demonstrable in normal functional human kidney.

The results stated in Table 2 from amphibian kidney specifically exclude the 3α-, 3β-, 16β- and 17β-hydroxysteroid dehydrogenases demonstrable in the adrenal tissue which is closely associated with the amphibian kidney.

While 3α-hydroxysteroid dehydrogenase activity was demonstrable with both substrates used in the present investigation, the 5α-configuration was invariably much better utilised than the corresponding 5β-steroid in the metanephric kidney.

In collecting tubules of mammalian kidneys incubated with 11β-hydroxysteroids, while the majority of cells exhibit/

exhibit 11β-hydroxysteroid dehydrogenase activity, a second rarer cell type exists (Pl. 2, fig. 7) which has no demonstrable 11β-hydroxysteroid dehydrogenase. Sections incubated in control solutions devoid of steroid or containing progesterone or 3β-hydroxyandrost-5-ene-16-one-3-methyl ether remained colourless.

DISCUSSION.

The failure to demonstrate any hydroxysteroid dehydrogenase activity in the extremely rudimentary human pronephros is not surprising.

The human mesonephros and the fish and frog mesonephros are very similar in that they possess a moderate 17β- and 16β-hydroxysteroid dehydrogenase in their nephrons. To our knowledge this is the first histochemical evidence of ontogeny repeating phylogeny. The functional status of the mammalian mesonephros varies enormously; the rat mesonephros (Jovrey, 1961) is rudimentary while the pig mesonephros exhibits both glomerular and tubular activity. Histologically the human embryonic mesonephros falls between these two extremes, but our histochemical observations raise the possibility that the human mesonephros, from the stand-point of steroid excretion at least, is functional.

The/

The most primitive (tortoise) metanephric kidneys studied possess 3α-, 16α-, 16β- and 17β-hydroxysteroid dehydrogenases while all the adult mammalian metanephric kidneys have, in addition, a strong 11β-hydroxysteroid dehydrogenase in their collecting tubules. In the neonatal mouse kidney (Baillie et al., 1966) 3α- and 17β-hydroxysteroid dehydrogenases are histochemically demonstrable; the 11β-hydroxysteroid dehydrogenase activity does not develop until the animal is 14 or 21 days old. It is thus clear that, in the metanephric kidney, 11β-hydroxysteroid dehydrogenase is the last steroid dehydrogenase to appear, both phylogenetically and ontogenetically. This appears to be another example of ontogeny repeating phylogeny histochemically.

3α-Hydroxysteroid dehydrogenase has been isolated from mouse, rat, guinea pig, rabbit and dog livers and kidneys (Hurlock & Talalay, 1958; Koide, 1963; Baron et al., 1963; Villee & Loring, 1963; Aoshima et al. 1964) and with its dual nucleotide specificity both biochemically (Hurlock & Talalay, 1958; Baron et al., 1963) and histochemically (Baillie et al., 1966) has been ascribed a transhydrogenase function. 3α-Hydroxy steroids/

3α-Hydroxysteroids and the corresponding 3-keto steroids are interconverted by this enzyme and it therefore seems reasonable to suggest that such steroids are metabolised and excreted mainly by the proximal and distal convoluted tubules.

The particular strength of the 3α-hydroxysteroid dehydrogenase reaction in the convoluted tubules of the juxta-medullary glomeruli suggests the possibility that the inner and outer cortical nephrons behave rather differently with respect to steroid excretion, but further work is required to elucidate this point.

The massive N.A.D. and N.A.D.P.-linked 11β-hydroxysteroid dehydrogenase in the collecting tubules of all mammalian and primate kidneys studied is the most striking feature of the present results. Although the physiological significance is not clear, biologically active 11β-hydroxysteroids include aldosterone, cortisol and the 11β-hydroxy androgens. At the present time it is not possible to differentiate histochemically between these possibilities, but further experimental work is in hand.

17β-Hydroxysteroid dehydrogenase, known biochemically to exist in the kidney (Velle & Erichsen, 1960; Aoshima & Kochakian, 1963) can now be assigned to the convoluted/

convoluted tubules, and, to a lesser extent, to the thick loops of Henle. It seems reasonable, in the light of these observations, to assume the inter-conversion of oestradiol and oestrone, testosterone and androstenedione and androstenediol and D.H.A. in these sites in the kidney and it would thus seem that the excretion of 17-steroids including the sex steroids, can occur throughout the nephron, although predominantly in the convoluted tubules.

The 3β-, 6β-, 16α- and 16β-hydroxysteroid dehydrogenases are present, but as a rule poorly active in the kidneys surveyed histochemically. They are known to exhibit sex differences histochemically in the kidney (Baillie et al., 1966) and the present survey has disclosed species variations as well. Biochemical precedents for their existence in renal tissues exist (Aoshima et al., 1964; Breuer et al., 1962; Ryan et al., 1963). Their physiological role in the kidney with respect to steroid excretion has not been clarified.

ACKNOWLEDGEMENT.

This work was supported by an M.R.C. Grant, which is gladly acknowledged.

REFERENCES.

AOSHIMA, Y. & KOCHAKIAN, C.D. (1963). Activity, intracellular distribution and some properties of 17β-hydroxy-C-19-steroid dehydrogenase in liver and kidney. Endocr. 72: 106 - 114.

AOSHIMA, Y., KOCHAKIAN, C.D. & JADRIJEVIC, T.P.N. and D.P.N.-specific 3α-hydroxy and Δ^5-3β-hydroxysteroid dehydrogenases of liver and kidney. Endocr. 74: 521 - 531.

BAILLIE, A.H., CALMAN, K.C., FERGUSON, M.M. & HART, D.McK. (1966). Histochemical Distribution of Hydroxysteroid Dehydrogenases in Kidney and Liver. Histochemie (In Press).

BARON, D.N., GORE, M.B.R., PIETRUSZKO, R. & WILLIAMS, D.C. (1963). Purification and properties of the 3α-hydroxysteroid-dependent nicotinamide-adenine dinucleotide transhydrogenase of rat liver. Biochem. J. 88: 19 - 25.

BREUER, H., KNUPPEN, R. & PANGELS, G. (1962). Konfiguration Biogenese und Stoffwechsel von 6α- und 6β-hydroxylierten phenolischen Steroiden. Biochim. Biophys. Acta. 36: 271 - 272.

HURLOCK, B. & TALALAY, P. (1958). 3α-Hydroxysteroids as co-enzymes of hydrogen transfer between di- and tri-phosphopyridine nucleotides. J. biol. Chem. 233: 886 - 897.

KOIDE, S.S. (1963). Purification of 3α-hydroxysteroid dehydrogenase obtained from the soluble fraction of rat liver. Arch. Biochem. 101: 278 - 285.

RYAN, K.J., MEIGS, R.A., PETRO, Z. & MOORISON, G. (1963). Estrogen-induced 16α-hydroxysteroid dehydrogenase activity in rat kidney. Science. 142: 243

TORREY, T.W. (1961). Morphogenesis of the vertebrates. John Wiley & Sons Inc. New York & London. 362 - 406.

VELLE, W. & ERICHSEN, S. (1960). Studies on oestrogens in cattle. Acta Endocr. (Kbh) 33: 277 - 286.

VILLEE, C.A. & LORING, J.M. (1963). 20α-Hydroxysteroid dehydrogenase and 3α-hydroxysteroid dehydrogenase in human foetal liver. Endocrinol. 72: 824 - 828.

DESCRIPTION OF PLATES.

PLATE I.

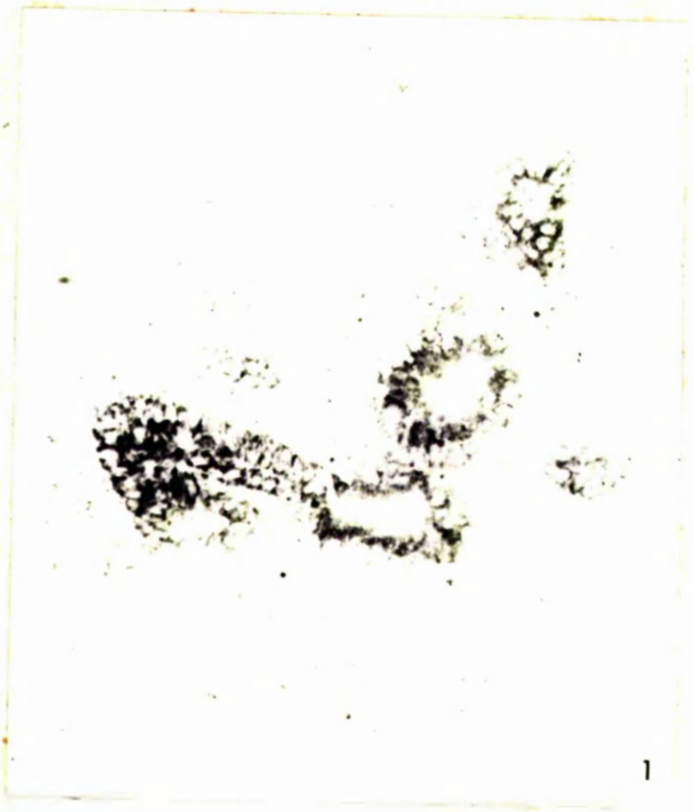
Fig. 1. 17β-Hydroxysteroid dehydrogenase activity in the mesonephros of a 1¼ mm. human foetus.
(x 300)

Fig. 2. Mesonephros of the frog, showing 17β-hydroxysteroid dehydrogenase activity in the tubules.
(x 180)

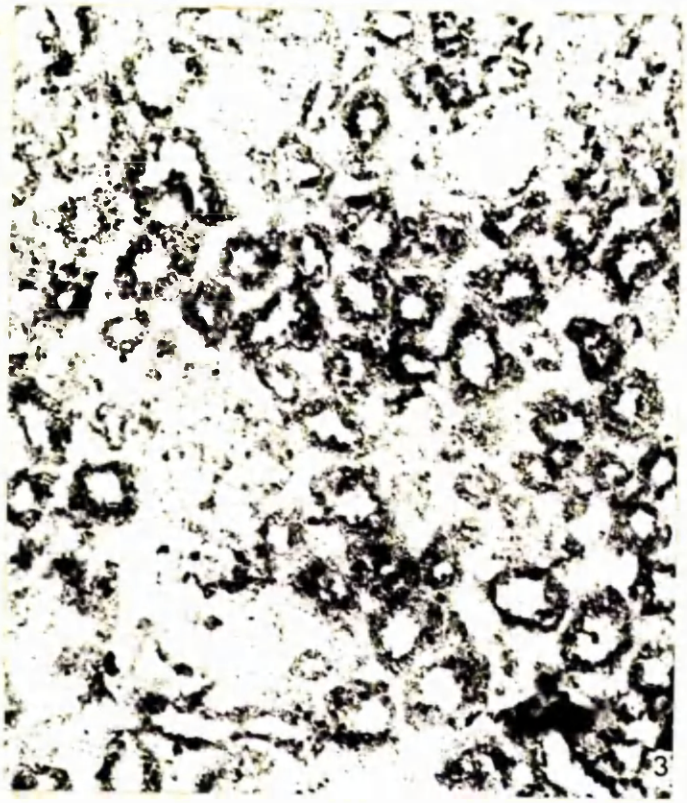
Fig. 3. Tortoise metanephros, exhibiting strong 16β-hydroxysteroid dehydrogenase activity in the tubules.
(x 130)

Fig. 4. 3α-Hydroxysteroid dehydrogenase activity in the nephron of the dog.
(x 180)

PLATE I


1


2


3

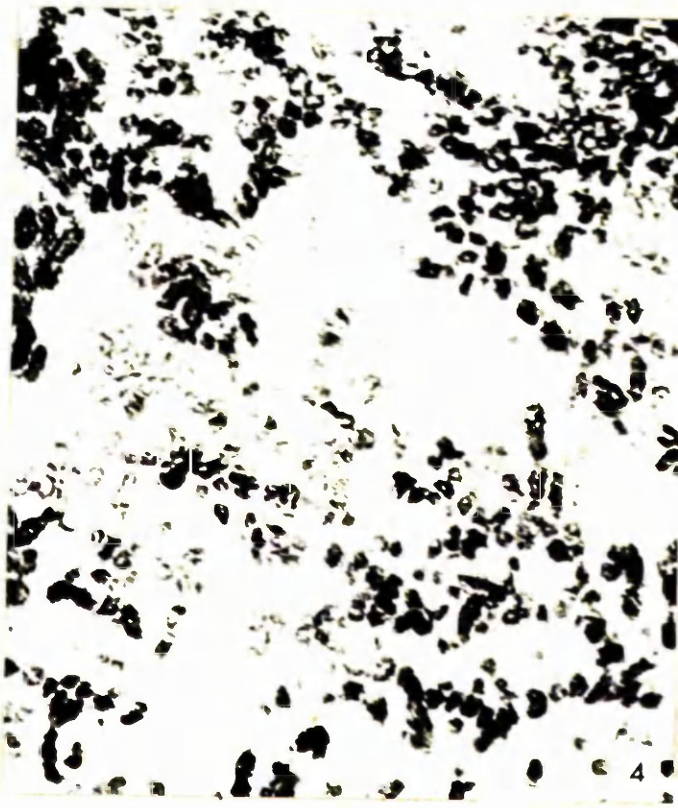

4

PLATE II.

Fig. 5. Rat kidney with 3α-H.S.D. activity in the convoluted tubules of juxta-medullary glomeruli (x 350).

Fig. 6. Collecting tubules in mouse kidney showing 11β-H.S.D. activity. (x 80).

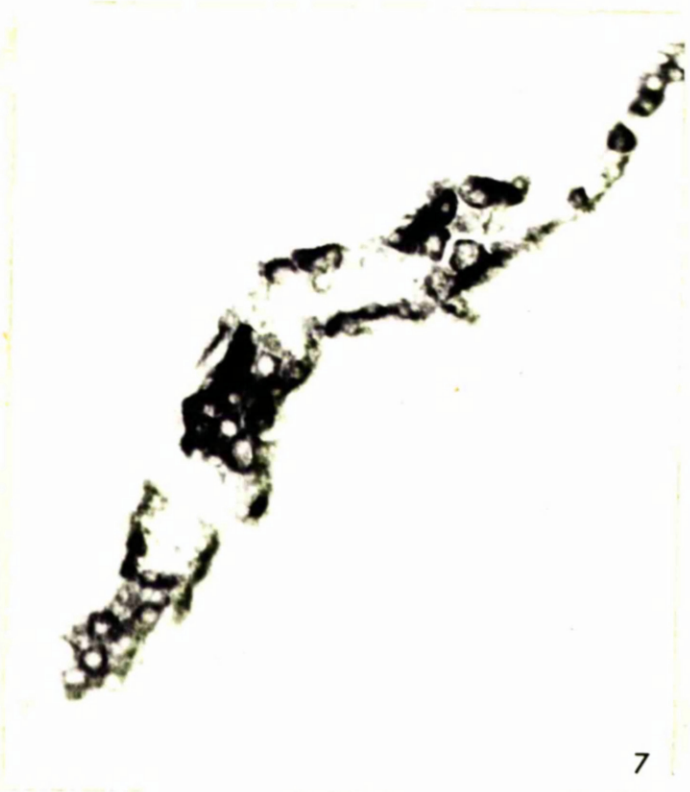
Fig. 7. Part of a collecting tubule in a mouse kidney, showing 11β-H.S.D. activity. Note the inactive cells (x 400).

Fig. 8. Mouse kidney. 17β-H.S.D. activity is seen in the nephron. (x 80).

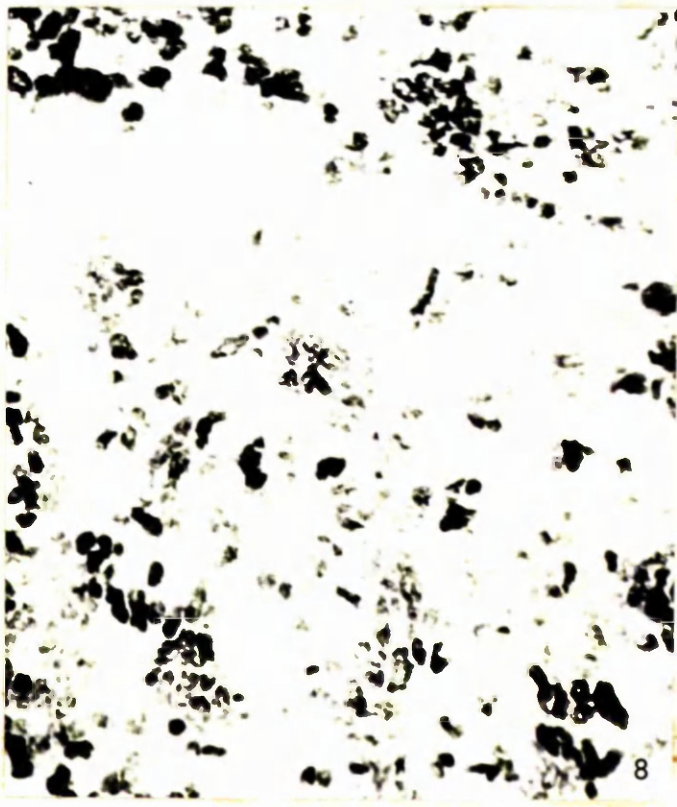
PLATE II

5

6



7



8

Hydroxysteroid Dehydrogenase Activity in Normal Human Placenta from Six Weeks to Forty-Two Weeks of Gestation.

BY

D. McKay Hart

Department of Anatomy, The University, Glasgow, W.2.

SUMMARY.

Fifty-one normal human placentae from the 6th to the 42nd week of pregnancy were surveyed for 3α-, 3β-, 6β-, 11α-, 11β-, 12α-, 16α-, 16β-, 17α-, 17β-, 20β-, 21- and 24-hydroxysteroid dehydrogenase (H.S.D.) activity.

Strong N.A.D.-linked 3β-, 16β-, and 17β-H.S.D. activity and moderate N.A.D.P.-linked 3β-, 16β- and 17β-H.S.D. activity were found in the villous trophoblast at all ages. Weak 3α- and weak or trace 6β- and 11β-H.S.D. activity was noted in the trophoblast of some placentae using N.A.D. as cofactor, with no obvious age distribution. Moderate 17β-H.S.D. activity, N.A.D.-linked only, was found in the villous stroma and vasculature.

INTRODUCTION.

Interconversions of hydroxy- and ketosteroids are catalysed by a class of widely distributed, pyridine nucleotide dependent hydroxysteroid dehydrogenases (H.S.D.s), which are specific with respect to the position and steric configuration of the groups undergoing reactions (Talalay, 1957).

Histochemical/

Histochemical methods for the demonstration of 3α-, 3β-, 6β-, 11α-, 11β-, 12α-, 16α-, 16β-, 17α-, 17β-, 20α-, 20β-, 21- and 24-H.S.D.s in tissue sections have been described (Wattenberg, 1958: Pearson & Grose, 1959A & B: Balogh, 1964: Baillie, Calman, Ferguson & Hart, 1965A & B: Baillie, Ferguson, Calman & Hart, 1965), using a tetrazolium salt as final hydrogen acceptor.

The histochemical distribution of 3β-H.S.D. has been described in term, human placenta and membranes and in hydatidiform mole (Wattenberg, 1958: Fuhrman, 1961: Baillie, Cameron, Griffiths & Hart, 1965: Koide & Mitsudo, 1965: Hart, 1966A & B) and in a series of 9 human placentae from the 6th week of gestation to term (Lobel, Deane & Romney, 1962). 3α-, 6β-, 11β-, 16β- (Baillie et al., 1965B: Baillie, Ferguson, Calman & Hart, 1965) and 17β-H.S.D.s (Kellogg & Glenner, 1960: Koide & Mitsudo, 1965) have also been noted in term, human placenta, but at the present time there is no information regarding the age distribution of these enzymes in placenta.

This paper describes the histochemical utilisation of 3α-, 3β-, 6β-, 11α-, 11β-, 12α-, 16α-, 16β-, 17α-, 17β-, 20β-, 21- and 24-hydroxysteroids in 51 normal/

normal, human placentae from the 6th to the 42nd weeks of pregnancy and discusses the significance of the enzymes demonstrated in placenta.

MATERIAL AND METHODS.

Portions of placental material measuring approximately 1 $cm.^3$ were obtained at legal therapeutic termination of pregnancy from 31 patients at 6 - 25 weeks of gestation and similar specimens were obtained from 20 patients undergoing Caesarean section for reasons not connected with placental malfunction at maturities of from 34 weeks to 42 weeks.

All specimens were frozen on solid carbon dioxide within a few minutes of removal from the uterus and sectioned at 15 μ in a cryostat maintained at $-25^{\circ}C$. The sections were attached to clean, dry glass coverslips and incubated at $37^{\circ}C$. in the following medium:-

O.I.M. Tris or phosphate buffer pH. 7.4	10 ml.
N.A.D. or N.A.D.P.	3 mg.
Nitro Blue Tetrazolium	3 mg.

Steroid substrate, 50 μ g. to 5 mg. dissolved in 0.5 ml. dimethyl formamide.

As a control, sections were incubated in medium containing/

TABLE I.

3α-Hydroxy-5α-androstan-17-one (androsterone)	-	Steraloids
5α-Androstan-3, 17-dione	-	Koch Light
3α-Hydroxy-5β-androstan-17-one (aetiocholanolone)	-	Steraloids
5β-Androstan-3, 17-dione	-	Koch Light
3β-Hydroxypregn-5-ene-20-one (pregnenolone)	-	Boots
3β-Hydroxyandrost-5-ene-17-one (D.H.A.)	-	Steraloids
3β-Sulphoxyandrost-5-ene-17-one (D.H.A. sulphate)	-	Organon
6β-Hydroxypregn-4-ene-3, 20-dione (6β-hydroxyprogesterone)	-	Steraloids
11α-Hydroxypregn-4-ene-3, 20-dione (11α-Hydroxyprogesterone)	-	Steraloids
11β-Hydroxyandrost-4-ene-3, 17-dione (11β-Hydroxyandrosterone)	-	Koch Light
11β-Hydroxypregn-4-ene-3, 20-dione (11β-Hydroxyprogesterone)	-	Steraloids
12α-Hydroxypregn-4-ene-3, 20-dione (12α-Hydroxyprogesterone)	-	Steraloids
3, 16α-Dihydroxyoestra-1,3,5,(10)-trien (16α-hydroxyoestrone)	-	Steraloids

TABLE I (continued).

3,16β-Dihydroxyoestra-1,3,5,(10)-trien (16β-hydroxyoestrone)	-	Steraloids
3β,16β-Dihydroxyandrost-5-ene-3-methyl ether	-	Steraloids
3β-Hydroxyandrost-5-ene-16-one-3-methyl ether	-	Steraloids
3, 17α-Dihydroxyoestra-1,3,5,(10)-trien (epioestradiol)	-	Organon
17β-Hydroxyandrost-4-ene-3-one (testosterone)	-	Organon
3,17β-Dihydroxyoestra-1,3,5,(10)-trien (oestradiol)	-	Organon
3-Hydroxyoestra-1,3,5,(10)-trien (oestrone)	-	Organon
20β-Hydroxypregn-4-ene-3-one (20β-Hydroxyprogesterone)	-	Steraloids
21-Hydroxypregn-4-ene-3-one (21-hydroxyprogesterone)	-	Steraloids
24-Hydroxy-5β-cholan-3-one	-	Steraloids

Steroid Substrates employed for the demonstration of hydroxysteroid dehydrogenase activity.

containing all the reagents other than steroid substrate and in addition oestrone, 5α-androstan-3, 17-dione, 5β-androstan-3, 17-dione and 3β-hydroxy-androsten-17-one-3-methyl ether were employed as controls for 16α-hydroxyoestrone, androsterone, etiocholanolone and 3β, 16β-dihydroxyandrost-5-en-17-one 3-methyl ether.

The steroid substrates employed are listed in Table I.

RESULTS.

No significant evidence of 11α-, 12α-, 16α-, 17α-, 20β-, 21- or 24-H.S.D. activity was detected in any component of human placenta at any age studied. Sections incubated in control solutions did not show activity.

Table II and figures 1 to 8 detail the positive results.

Strong 3β-, 16β- and oestradiol-17β-H.S.D. activities, N.A.D.-linked and moderate N.A.D.P.-linked 3β-, 16β- and oestradiol-17β-dehydrogenase activity were found in the trophoblastic epithelium in all placentae examined: the activity of 3β-, and 16β-H.S.D.s declined slightly towards term. In immature placentae where cytotrophoblast and syncytiotrophoblast were distinguishable both types of trophoblastic epithelium

LEGEND FOR TABLE II.

H.S.D. activity after 2 hrs. incubation, using N.A.D. as co-factor except where indicated ("Oe_2 17β N.A.D.P.").

A blank space indicates that the corresponding substrate was not employed.

Oe_2	=	Oestradiol
Tr	=	Trace Activity
+	=	Weak Activity
++	=	Moderate Activity
+++	=	Strong Activity

TABLE II

Foetal Crown Rump Length or Maturity	3α (5α epimer)	3α (5β epimer)	3β D.H.A.	3β Preg.	3β D.H.A. SO_4	6β	11β	16β	17β Oe_2 NAD	17β Oe_2 NADP	17β testosterone
1.4 cm.	+	+	++	++		-	-	+++	+	+	tr
1.6 cm.	+	+	++			+	+	+++	+	+	tr
3.8 cm.	-	tr		++	+	-	tr	+++	+	tr	+
4.4 cm.	+	+	++			-	-	++	+	tr	*
4.5 cm.	+	-	++		-	-	-	+++	+	+	+
4.7 cm.	+	+	++			+	+	++	+	+	+
5.2 cm.	-	-		+		tr	-	+++	+	tr	+
5.3 cm.	+	+	++			-	tr	+++	++	+	+
6.5 cm.	+	tr	++			-	+	+++	++	+	+
7.5 cm.	+	+	++			+	tr	++	+	tr	+
7.7 cm.	-	-	++			-	-	+++	++	+	+
7.8 cm.	+	+	++			+	+	++	++	+	+
8.0 cm.			+			tr	tr	++	++	+	+
8.4 cm.	-	-	++			-	-	++	+	tr	+
8.5 cm.	-	-	++			-	tr	+++	++	+	+
10.5 cm.	+	+	++			+	+	++	++	+	+
10.5 cm.	-	tr	++			-	tr	+++	+	+	+
11.0 cm.	+	+	++			tr	tr	++	+	tr	+
11.3 cm.	+	tr	++			-	-	+++	++	+	tr
12.5 cm.	-	tr	++	++	+	tr	-	+++	++	+	+
13.0 cm.	+	+	++			-	-	++	+	+	+
14.5 cm.	tr	tr	+			-	-	++	+	tr	+
15.0 cm.	+	tr	++			-	tr	+++	++	+	+
15.0 cm.	-	+	+			tr	-	++	+	tr	+
15.5 cm.	-	tr	++			-	tr	+++	++	+	+
15.7 cm.	+	+	++			+	+	++	++	+	+
17.5 cm.	+	tr	++			-	-	+++	++	+	+
18.1 cm.	-	-	+			-	-	++	+	tr	+
19.5 cm.	-	tr	++			tr	tr	++	+++	++	+
22.0 cm.	+	+	++	++	+	+	tr	+++	++	+	+
34 weeks (2 cases)	-	tr	++			-	tr	+++	++	+	+
36 weeks (2 cases)	tr	+	++			tr	tr	+++	++	+	+
Term (Average of 15)	tr	+	++	++	+	tr	tr	++	+++	+	+
42 weeks (1 case)	-	tr	++			-	tr	++	++	+	+

were seen to be active (Fig. 2). Oestradiol-17β-dehydrogenase activity was evident in stroma and blood vessels with N.A.D. but not with N.A.D.P. as cofactor and 17β-H.S.D. activity of moderate intensity was observed in the small foetal blood vessels in the chorionic villi of mature placentae (Fig. 4) using testosterone as substrate with N.A.D., but not N.A.D.P. as cofactor; weaker activity was present in the blood vessels of immature placentae. Weak 3α-H.S.D. activity was noted in the trophoblast and to a lesser extent in the villous stroma of 60% of placentae and weak or trace 6β- and 11β-H.S.D. activities in 50% of placentae with no obvious age distribution, N.A.D.-linked only.

DISCUSSION.

The reduction of α/β -unsaturated 3-ketosteroids to their corresponding saturated 3-alcohols comprises a major pathway of metabolism of C.19 and C.21 steroids (Dorfman & Ungar, 1953) and a cell-free preparation of rat liver capable of catalysing the two-step reduction of cortisone to tetrahydro-cortisone with reduced pyridine nucleotides as hydrogen donors has been described (Tomkins & Isselbacher, 1954). The significance of the weak 3α-H.S.D. activity demonstrated in/

in human placenta is uncertain. The present findings agree with those of Baillie et al. (1965B) in term placenta and Hart (1966A, B) in human extra-placental chorion at term and hydatidiform moles. Koide & Mitsudo (1965) did not obtain a positive reaction in term placenta using 5α-androstan-3α-ol-17-one in a similar system, but their sections were washed in 80% alcohol for 10 mins. after incubation and pink monoformazan ignored, conditions which would abolish evidence of trace activity.

D.H.A. has been shown to be an oestrogen precursor in the human placenta in vivo (Bolte, Mancuso, Eriksson, Wiqvist and Diczfalusy (1964A, B) and Bolte et al. (1964A) have suggested that the biosynthetic sequence D.H.A. sulphate ⟶ D.H.A. ⟶ androstenedione or testosterone ⟶ oestradiol or oestrone is responsible for placental formation of oestradiol and oestrone. 3β-H.S.D. is involved in the step D.H.A. ⟶ androstenedione or testosterone.

Placental 3β-H.S.D. activity was first described histochemically by Wattenberg, (1958) in the trophoblastic cytoplasm of human, term placenta using D.H.A. as the steroid substrate. Fuhrman (1961) obtained similar

similar results using pregnenolone and D.H.A. Löbel, Deane & Romney (1961) examined a series of 9 normal placentae from 6 weeks of gestation to term using D.H.A. and found 3β-H.S.D. activity in both cyto- and syncytiotrophoblast; this activity declined slightly towards term. The results of the present age series are in agreement.

In an investigation of substrate specificity of 3β-H.S.D. Baillie, Cameron, Griffiths & Hart (1965) demonstrated activity in human, term placental trophoblast using pregnenolone, pregnenolone sulphate, pregnenolone acetate, D.H.A., D.H.A. sulphate, D.H.A. acetate, 16α-hydroxypregnenolone, 17α-hydroxypregnenolone and 17α-hydroxypregnenolone sulphate. Pulkkinen (1961) described steroid sulphatase in human placenta and this has been confirmed by Warren & Timberlake (1962), who showed that a 22 week placenta was as active as term placenta, and Warren & French (1965). Baulieu & Dray (1963), Siiteri & Macdonald (1963) and Bolte, Mancuso, Eriksson, Wiqvist & Diczfalusy (1964A, B, C) have shown that D.H.A. sulphate is an oestrogen precursor in the human placenta. The demonstration of 3β-H.S.D. activity at 10, 14, 17, 25 and 40 weeks in human placental trophoblast using D.H.A. sulphate as substrate implying the removal

of/

of the sulphate group is in agreement with these reports.

All the foregoing reports are of N.A.D.-linked 3β-H.S.D. activity, but Koide & Mitsudo (1965) described the histochemical demonstration of 3β-H.S.D activity both N.A.D.- and N.A.D.P.-linked in human, term placenta using D.H.A., 5β-androstan-3β-ol-17-one, 5α-androstan-3β-ol-17-one, androst-4-en-3β-ol-17-one, pregn-5-en-3β-ol-17-one and 5α-pregnan-3β-ol-20-one, formazan being deposited only in the trophoblast in all cases. It has now proved possible to repeat the demonstration of 3β-H.S.D. using N.A.D.P. as co-factor in this laboratory.

Baillie et al. (1965B) described weak 6β-H.S.D. activity in the trophoblast of two term, human placentae using 6β-hydroxyprogesterone as substrate. In the present investigation only trace evidence of activity of this enzyme could be detected.

Term extraplacental chorion did not display evidence of 6β-H.S.D. (Hart, 1966A) and activity was seen in only 1 of 5 hydatidiform moles (Hart, 1066B).

Hurlock & Talalay (1959) described a rat liver microsomal/

microsomal 11β-H.S.D. which catalyses a freely reversible interconversion of cortisol and cortisone. Following the observation that term, human, placental homogenates transform 11β-hydroxysteroids into 11-keto compounds, Osinski (1960) investigated the action and properties of the corresponding enzyme and found that placental 11β-H.S.D. was both N.A.D.P.- and N.A.D.-linked and had low substrate specificity, transforming both C_{19} and C_{21} steroids with or without a 17-hydroxy group in the side-chain. The enzyme was sensitive to freezing and this may be in part responsible for the poor histochemical demonstration in placenta. Hart (1966A) likewise found poor evidence of 11β-H.S.D. activity in term extra-placental chorion. The physiological role of the placental enzyme is uncertain, but its presence may explain the origin of the cortisone found in normal human liquor amnii (Baird & Bush, 1960).

An intensely strong histochemical reaction for 16β-H.S.D. reported by Baillie et al. (1965B) in human, term placenta has been confirmed by this study and extended to immature placentae where the activity is as strong, or stronger.

The/

The biochemical significance of this enzyme in placenta is uncertain, though a number of oestrogens with either 16α- or 16β-hydroxyl groups have been isolated (Breuer & Nocke, 1959; Levitz, Spitzer & Twombly, 1958) and it is known that these 16-hydroxy compounds can be interconverted via the 16-keto group (Levitz, Rosen & Twombly, 1960). Liver, placenta and hydatidiform mole have been shown to metabolise the 16-hydroxy oestrogens (Breuer & Knuppen, 1958; Ryan, 1960; Klausner & Ryan, 1964; Macdonald & Siiteri, 1964).

The failure to demonstrate 17α-H.S.D. in this series of placentae is in keeping with the findings of Kellogg & Glenner (1960) and Baillie et al. (1965B) in term placenta and Hart (1966A, B) in the extra-placental chorion at term and in hydatidiform moles. Ryan & Engel (1953), Levitz, Condon & Dancis (1956) and Troen (1961) have described the interconversion of oestrone and oestradiol by human tissue slices and in the perfused human placenta, and the existence of a 17β-H.S.D. is now well established.

Biochemically and histochemically placental 17β-H.S.D. has been the subject of dispute and diversity of opinion. Langer & Engel (1958) partially purified/

purified (50 fold) and characterised a 17β-H.S.D. both N.A.D.- and N.A.D.P.-linked with a K_M value compatible with transhydrogenase function under physiological conditions and possessing high specificity for aromatic 17β-hydroxysteroids; Hagerman & Villee (1959), using continuous flow curtain electrophoresis on paper, described three discrete fractions with N.A.D.-linked oestradiol-17β-dehydrogenase, N.A.D.P.-linked oestradiol dehydrogenase and oestrogen-sensitive pyridine nucleotide transhydrogenase functions; Jarabak, Adams, Williams-Ashman & Talalay (1961) purified (2,500 fold) a soluble 17β-H.S.D. from human placenta and found that this enzyme reacted with both N.A.D. and N.A.D.P. and appeared to be responsible for most, if not all, of the 17β-oestradiol mediated hydrogen transfer between pyridine nucleotides. Kellogg & Glenner (1960) investigated the histochemical localisation of human, term, placental 17β-oestradiol dehydrogenase in an attempt to determine whether one or several enzymes existed. They described N.A.D.-linked 17β-oestradiol dehydrogenase in the trophoblast and decidua and to a lesser extent in the villous stroma, while N.A.D.P.-linked 17β-oestradiol dehydrogenase was found only in the perivascular stroma. Preferential binding of/

of the tetrazolium salt and differences in N.A.D.H.$_2$ and N.A.D.P.H.$_2$ diaphorases were declared to have been excluded and it was concluded that at least two distinct 17β-oestradiol-sensitive enzymes were being demonstrated.

Koide & Mitsudo (1965) examined 17β-H.S.D. in human term, placenta by the method of Kellogg & Glenner (1960) using 17β-oestradiol, oestriol, testosterone, oestr-4-ene-17β-ol-3-one, 5α-androstan-17β-ol-3-one and 5α-androstan-3α,17β-diol. With 17β-oestradiol and N.A.D. these workers found moderate staining of trophoblast, vessels and stroma, while with 17β-oestradiol and N.A.D.P. a positive reaction was limited to the vessel walls (endothelial cells and perivascular stroma); activity with testosterone, oestr-4-ene-17β-ol-3-one, 5α-androstan-17β-ol-3-one and 5α-androstan-3α,17β-diol with both N.A.D. and N.A.D.P. was limited to the vessel walls.

The findings in the present series of 51 placentae are in agreement with previous workers with regard to N.A.D.-linked oestradiol-17β-dehydrogenase and N.A.D.-linked 17β-H.S.D. using testosterone. Using N.A.D.P. as co-factor, however, a quite different distribution of 17β-oestradiol dehydrogenase was observed. Moderate diformazan/

diformazan deposition occurred in the trophoblastic epithelium and no activity whatsoever was seen in vessel walls or perivascular stroma, even after sixteen hours incubation (Figs. 4 & 5).

Testosterone did not cause formazan deposition with N.A.D.P. as co-factor.

The difference between the present findings and those of Kellogg & Glenner (1960) and Koide & Mitsudo (1965) does not appear to be due to their use of bovine serum albumen in the medium or the use of propylene glycol as steroid solvent.

Lauritzen (1963) has shown that 20β-hydroxy-progesterone is a true progestogen in women, though only half as effective as progesterone itself, with respect to its effects on uterus and vagina. Using this steroid as substrate it was not possible to produce convincing evidence of 20β-H.S.D. activity in human placenta from 6 to 24 weeks of gestation. Similar findings have been reported elsewhere in term, extra-placental chorion and vesicular mole (Hart, 1966A, B).

The endocrine functions of the human placenta have been reviewed by Diczfalusy (1960); there is adequate evidence for the production of chorionic gonadotrophin/

gonadotrophin and oestrogens and strong evidence for progesterone secretion, though no progesterone production has so far been demonstrated in implantation or tissue culture studies.

The results of this investigation indicate that steroid synthesis and metabolism in human placenta, in as far as these activities are demonstrable by hydroxysteroid dehydrogenase activity, are well advanced by the sixth week of gestation and confirms that the trophoblast is the principal active placental component.

The problem of 17β-H.S.D. nucleotide specificity, localisation and transhydrogenase function requires further study.

ACKNOWLEDGEMENTS.

The author is grateful to the Department of Anatomy, the University of Glasgow, Scotland and the Department of Anatomy, the University of Helsinki, Finland, for research facilities and materials. This work was supported by the Medical Research Council and the Board of Management for Glasgow Northern Hospitals.

REFERENCES.

BAILLIE, A.H., CALMAN, K.C., FERGUSON, M.M. & HART, D.McK. (1965A). Histochemical demonstration of 20β-hydroxysteroid dehydrogenase. J. Endocrin. 32: 337 - 339.

BAILLIE, A.H., CALMAN, K.C., FERGUSON, M.M. & HART, D.McK. (1965B). Histochemical utilisation of 3α-, 6β-, 11α-, 12α-, 16α-, 16β-, 17α-, 21- and 24-hydroxysteroids. J. Endocrin. (In Press).

BAILLIE, A.H., CAMERON, E.H.D., GRIFFITHS, K. & HART, D.McK. (1965). 3β-Hydroxysteroid dehydrogenase in the adrenal gland and placenta. J. Endocrin. 31: 227 - 233.

BAILLIE, A.H., FERGUSON, M.M., CALMAN, K.C. & HART, D.McK. (1965). Histochemical demonstration of 11β-hydroxysteroid dehydrogenase. J. Endocrin. 33: 119 - 125.

BAIRD, G.W. & BUSH, I.E. (1960). Cortisol and cortisone content of amniotic fluid from diabetic and non-diabetic women. Acta Endoc. 34: 97 - 104.

BALOGH, K. (1964). A histochemical method for the demonstration of 20α-hydroxysteroid dehydrogenase activity in rat ovaries. J. Histochem. Cytochem. 12: 670 - 673.

BAULIEU, E.E. & DRAY, F. (1963). Conversion of ^{3}H-dehydroepiandrosterone (3β-hydroxy-Δ^{5}-androsten-17-one) sulphate to ^{3}H-estrogens in normal pregnant women. J. clin. Endocr. 23: 1298 - 1301.

BOLTE, E., MANCUSO, S., ERIKSSON, G., WIQVIST, N. & DICZFALUSY, E. (1964A). Studies on the aromatisation of neutral steroids in pregnant women. I. Aromatisation of C-19 steroids by placentas perfused in situ. Acta endocr., Kbh., 45: 535 - 559.

BOLTE, E., MANCUSO, S., ERIKSSON, G., WIQVIST, N. & DICZFALUSY, E. (1964B). Studies on the aromatisation of neutral steroids in pregnant women. 2. Aromatisation of dehydroepiandrosterone and of its sulphate administered simultaneously into a uterine artery. Acta endocr., Kbh., 45: 560 - 575.

BOLTE, E., MANCUSO, S., ERIKSSON, G., WIQVIST, N. & DICZFALUSY, E. (1964C). Studies on the aromatisation of neutral steroids in pregnant women. 3. Overall aromatisation of dehydroepiandrosterone sulphate circulating in the foetal and maternal compartments. Acta endocr., Kbh., 45: 576 - 599.

BREUER, H. & KNUPPEN, R. (1958). Metabolism of 16-oxo oestrone by human liver slices in vitro. Nature (Lond.) 182: 1512.

BREUER, H. & NOCKE, L. (1959). Formation of oestriol-3, 16β, 17α by liver tissue in vitro. Biochem. Biophys. Acta. 36: 271 - 272.

DICZFALUSY, E. (1960). Endocrine functions of the human placenta. Acta endocr., Kbh. Suppl. 50: 129 - 134.

DORFMAN, R.I. & UNGAR, F. (1953). Metabolism of steroid hormones. Minneapolis. Burgess. 1953.

FUHRMAN, K. (1961). Uber der histochemischen Nachweis der 3β-ol-steroiddehydrogenase-activitat in geweben endokriner Organen. Zbl. Gynak. 1961 (i): 565 - 572.

HAGERMAN, D.D. & VILLEE, C.A. (1959). Separation of human placental estrogen-sensitive transhydrogenase from estradiol-17β-dehydrogenase. J. biol. Chem. 234: 2031 - 2036.

HART, D.McK. (1966A). The histochemical distribution of hydroxysteroid dehydrogenases in the human foetal membranes at term. Histochemie. (In Press).

HART, D.McK. (1966B). Histochemical demonstration of hydroxysteroid dehydrogenases in hydatidiform moles. Obstet. Gynec. (In Preparation).

HURLOCK, B. & TALALAY, P. (1959). Microsomal 3α- and 11β-hydroxysteroid dehydrogenases. Arch. Biochem. 80: 468 - 470.

JARABAK, J., ADAMS, J.A., WILLIAMS-ASHMAN, H.G. & TALALAY, P. (1962). Purification of a 17β-hydroxysteroid dehydrogenase of human placenta and studies on its transhydrogenase function. J. biol. Chem. 237: 345 - 357.

KELLOGG, D.A. & GLENNER, G.G. (1960). Histochemical localisation of human, term placental 17β-oestradiol dehydrogenases: implications for the transhydrogenase reaction. Nature (Lond.) 187: 763 - 764.

KLAUSNER, D.A. & RYAN, K.J. (1964). Estriol secretion by human, term placenta. J. clin. Endocr. 24: 101 - 104.

KOIDE, S.S. & MITSUDO, S.M. (1965). Histochemical study of 3β- and 17β-hydroxysteroid dehydrogenases in human term placenta. Endocr. 76: 403 - 407.

LANGER, L.J. & ENGEL, L.L. (1958). Human placental estradiol-17β-dehydrogenase. I. Concentration, characterisation and assay. J. biol. Chem. 233: 583 - 588.

LAURITZEN, C. (1963). Biologische Wirkungen des 20β-hydroxypregn-4-en-3-one. Acta endocr., Kbh. 44: 225 - 236.

LEVITZ, M., CONDON, G.P. & DANCIS, J. (1956). The interconversion of estrone and estradiol in the perfused human placenta. Endocrinol. 58: 376 - 380.

LEVITZ, M., ROSEN, M.F. & TWOMBLY, G.H. (1960). Interconversions of 16-oxygenated oestrogens. II. Metabolism of 16-keto estradiol 17β 16-C^{14} in man. Arch. Biochem. Biophys. 88: 212 - 215.

LEVITZ, M., SPITZER, T.R. & TWOMBLY, G.H. (1958). Interconversions of 16-oxygenated estrogens. I. The synthesis of estriol 16-C^{14} and its metabolism in man. J. biol. Chem. 231: 787 - 797.

LOBEL, B.L., DEANE, H.W. & ROMNEY, S.L. (1962). Enzymic histochemistry of the villous portion of the human placenta from six weeks of gestation to term. Amer. J. Obstet. Gynec. 83: 295 - 299.

MACDONALD, P.C. & SITTERI, P.K. (1964). Study of estrogen production in women with hydatidiform mole. J. clin. Endocr. 24: 685 - 690.

OSINSKI, P.A. (1960). Steroid 11β-ol dehydrogenase in human placenta. Nature (Lond.) 187: 777.

PEARSON, B. & GROSE, F. (1959A). Histochemical demonstration of 17β-hydroxysteroid dehydrogenase by the use of tetrazolium salt. Proc. Soc. Exper. Biol. Med. 100: 636 - 638.

PEARSON, B. & GROSE, F. (1959B). Histochemical study of some D.P.N.H. and D.P.N. dependent dehydrogenases. Fed. Proc. 18: 1,965.

PULKKINEN, M.O. (1961). Arylsulphatase and the hydrolysis of some steroid sulphates in developing organism and placenta. Acta physiol. Scand. 52: Suppl. 180: 7 - 92.

RYAN, K.J. (1960). Metabolism of 16-keto-estrone by human placenta. Endocr. 66: 491 - 494.

RYAN, K.J. & ENGEL, L.L. (1953). The interconversion of estrone and estradiol by human tissue slices. Endocr. 52: 287 - 291.

SITTERI, P.K. & MACDONALD, P.K. (1963). The utilisation of circulating dehydroepiandrosterone sulphate for estrogen synthesis during human pregnancy. Steroids. 2: 713 - 730.

TALALAY, P. (1957). Enzymatic mechanisms in Steroid Metabolism. Physiol. Rev. 37: 362 - 389.

TOMKINS, G.M. & ISSELBACHER, K.H. (1954). Enzymic reduction of cortisone. J. Amer. chem. Soc. 76: 3,100 - 3,101.

TROEN, P. (1961). Perfusion studies of the human placenta. II. Metabolism of C^{14}-17β-estradiol with and without added human chorionic gonadotropin. J. clin. Endocr. 21: 895 - 908.

WARREN, J.C. & FRENCH, A.P. (1965). Distribution of steroid sulphatase in human tissues. J. clin. Endocr. 25: 278 - 285.

WARREN, J.C. & TIMBERLAKE, C.E. (1962). Steroid sulphatase in the human placenta. J. clin. Endocr. 22: 1,148 - 1,151.

WATTENBERG, L.W. (1958). Microscopic histochemical demonstration of steroid-3β-ol dehydrogenase in tissue sections. J. Histochem. Cytochem. 6: 225 - 232.

DESCRIPTION OF PLATES.

Plate I.

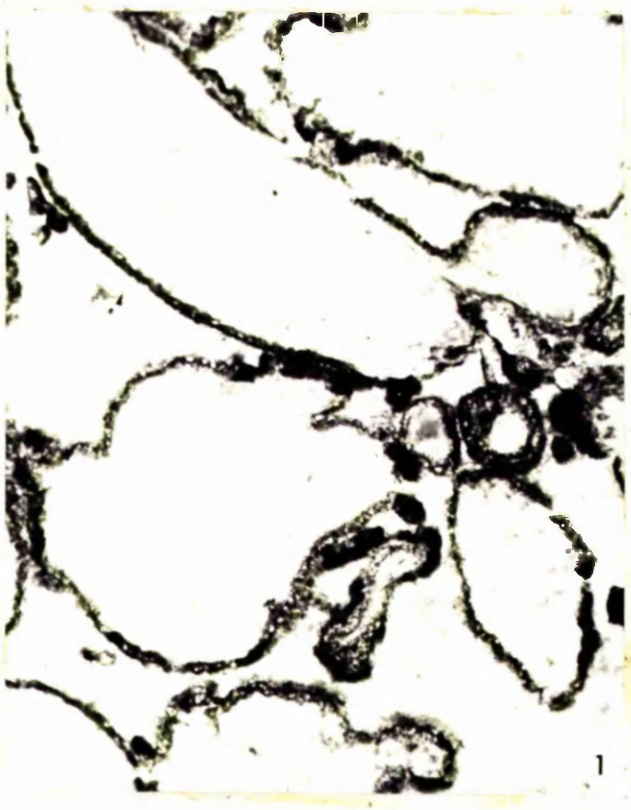
Fig. 1. N.A.D.-linked 16β-H.S.D. activity in the trophoblast of immature placenta (Foetal Crown-Rump Length 4.5 cm.).
(x 140)

Fig. 2. N.A.D.-linked 16β-H.S.D. activity is present in both cyto- and syncytio-trophoblast. (Foetal C.R. Length 4.5 cm.).
(x 140)

Fig. 3. N.A.D.-linked 16β-H.S.D. activity in the trophoblast of term placenta.
(x 200)

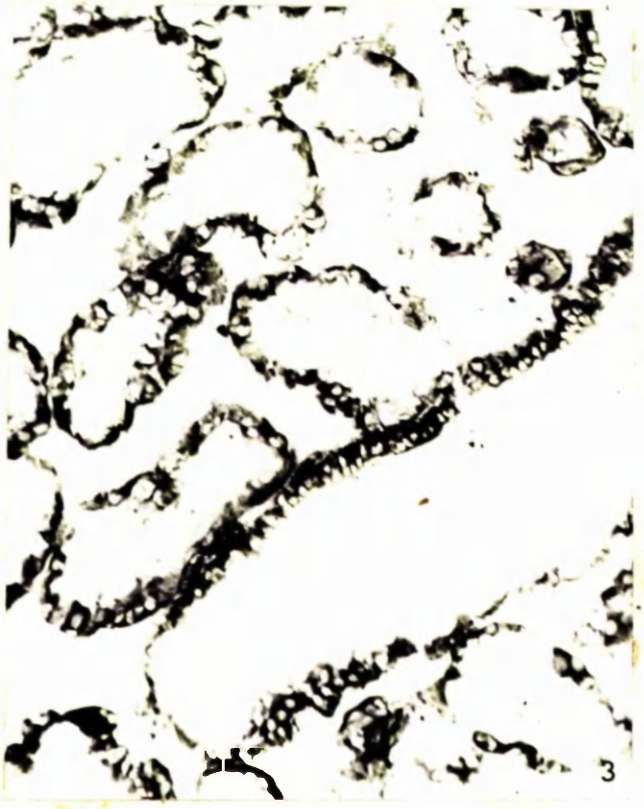
Fig. 4. N.A.D.-linked oestradiol-17β-dehydrogenase activity in term placenta. Activity is seen in the walls of several larger blood vessels and in the perivascular stroma.
(x 90)

PLATE I

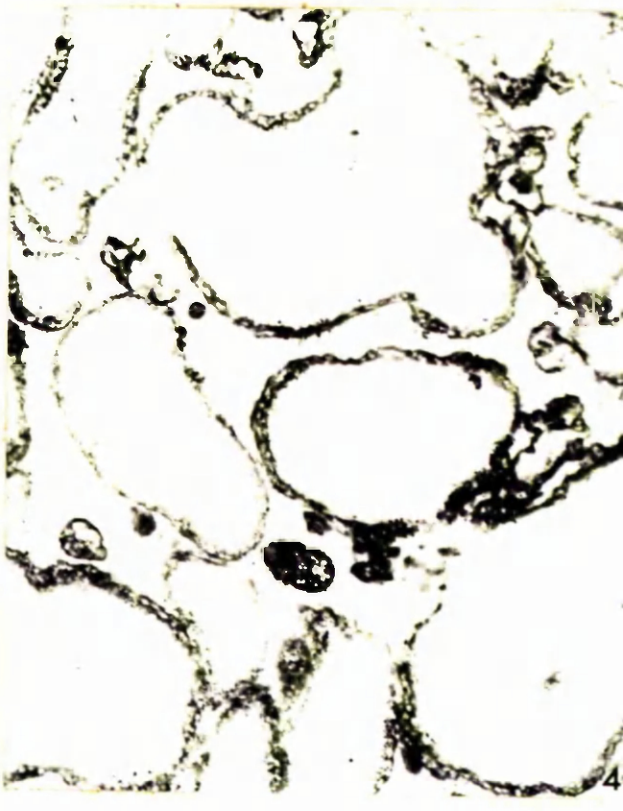


1

2



3



4

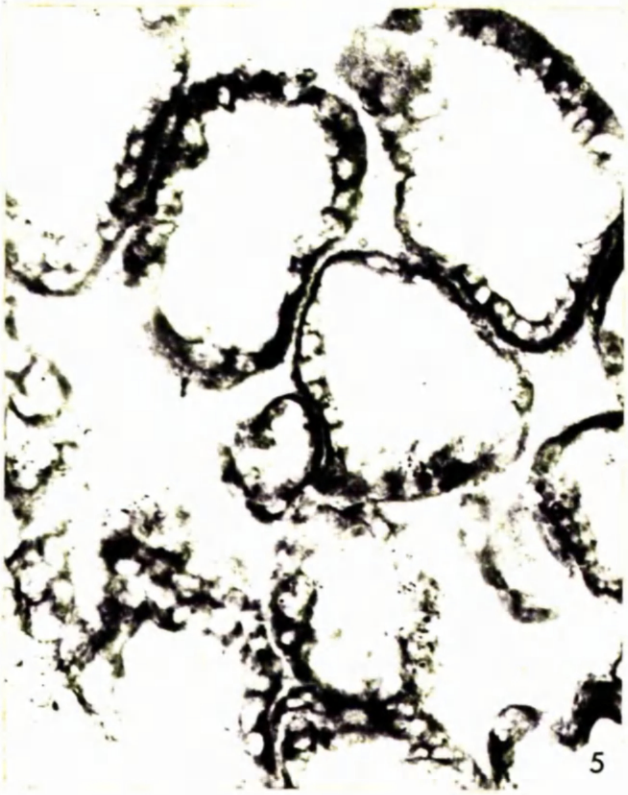
Plate II.

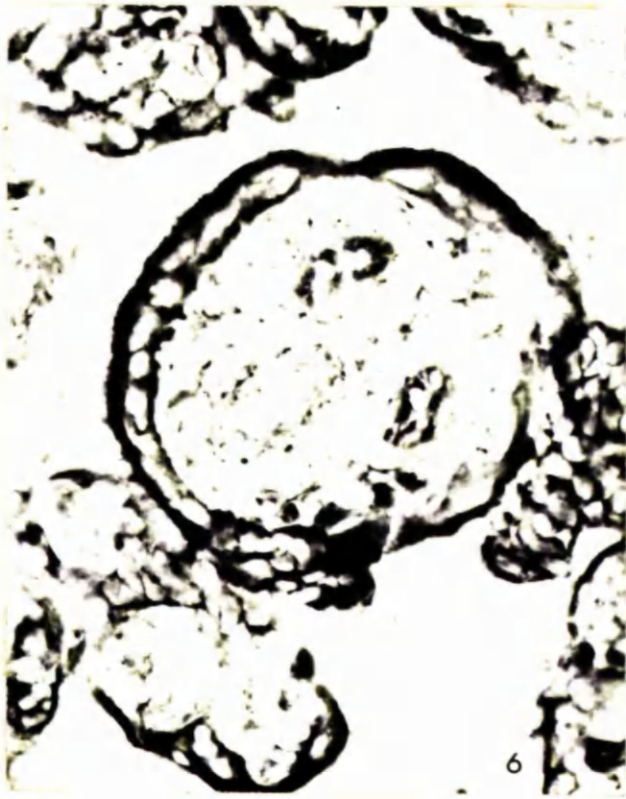
Fig. 5. N.A.D.P.-linked oestradiol-17β-dehydrogenase in term placenta. The activity is limited to the trophoblast.
(x 300)

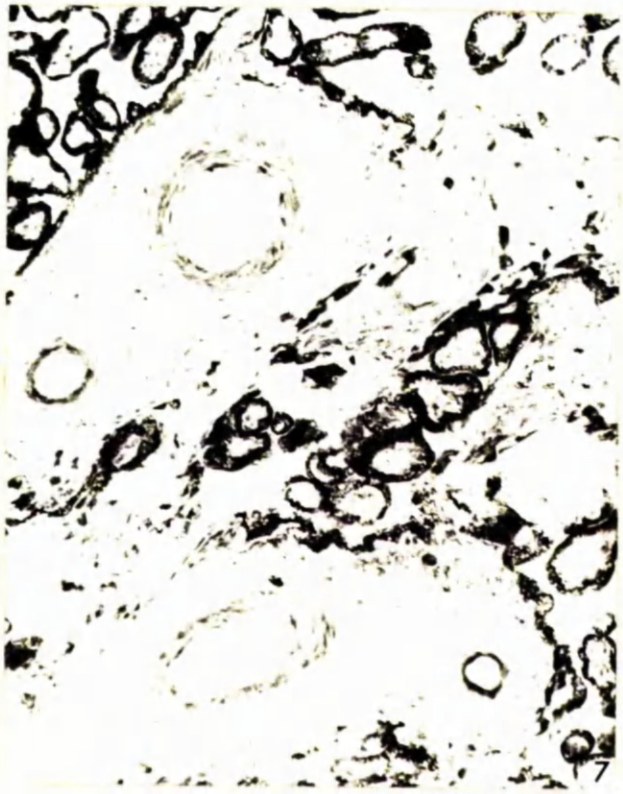
Fig. 6. N.A.D.-linked oestradiol-17β-dehydrogenase in term placenta. Activity is present in villous stroma and blood vessels as well as in the trophoblast.
(x 350)

Fig. 7. N.A.D.P.-linked oestradiol-17β-dehydrogenase activity in the trophoblastic epithelium of immature placenta (Foetal C.R. Length 8.5 cm.).
(x 120)

Fig. 8. Using testosterone as steroid substrate and N.A.D. as co-factor, 17β-H.S.D. activity is demonstrable in foetal blood vessels in term placenta.
(x 400)


5


6


7


8

The Histochemical Distribution of Hydroxysteroid Dehydrogenases in the Human Foetal Membranes at Term.

By

D. McKay Hart,
F.R.C.S. (Glasg.), M.R.C.O.G.

Department of Anatomy, The University,
Glasgow, W.2.

INTRODUCTION

Since the development, in 1958, of a technique for the histochemical demonstration of 3β-hydroxysteroid dehydrogenase using a post-coupling method with a tetrazolium dye as final electron acceptor (Wattenberg, 1958) similar techniques have been described for the demonstration in living tissue sections of 3α-, 6β-, 11α-, 11β-, 12α-, 16α-, 16β-, 17α-, 17β-, 20β-, 20α-, 21- and 24-hydroxysteroid dehydrogenases (Pearson & Grose, 1959 A & B; Balogh, 1964; Baillie, Calman, Ferguson & Hart, 1965A & B; Baillie, Ferguson, Calman & Hart, 1965).

Osinski (1960) found that, biochemically, the human nonplacental chorion was a rich source of 11 - hydroxysteroid dehydrogenase. Foetal membranes were therefore surveyed using the above techniques and this paper describes the histochemical utilisation of 3α-, 3β-, 6β-, 11α-, 11β-, 12α-, 16α-, 16β-, 17α-, 17β-, 20β-, 21- and 24-hydroxysteroids in the human foetal membranes at term.

MATERIAL AND METHODS.

Portions of the conception sac, measuring 6 cm. by 6 cm. were obtained at 12 elective Caesarean sections, including one case of dichorial twin pregnancy, performed at term for reasons not connected with placental malfunction. Folded several times to protect the amniotic epithelium and produce a more easily handled specimen, these portions were frozen on solid carbon dioxide within 2 minutes of removal from the uterus. Each specimen was sectioned at 12μ in a cryostat maintained at -25^{o}C and the sections attached to clean, dry coverslips by momentary thawing then dried in air at room temperature.

Incubation was carried out for 30 to 60 minutes at 37^{o}C in a medium consisting of 10 ml. Tris or phosphate buffer (pH. 7.4) containing 3 mg. N.A.D. or N.A.D.P., 3 mg. Nitro Blue Tetrazolium and 5 mg. steroid substrate dissolved in 0.5 ml. propylene glycol or dimethyl formamide. The steroid substrates employed were:-

(androsterone) 3α-hydroxy-5α-androstan-17-one.

Steraloids.

(Aetiocholanolone) 3α-hydroxy-5β-androstan-17-one. Steraloids.

(D.H.A.) 3β-hydroxyandrost-5-ene-17-one. Organon.

(Pregnenolone) 3β-hydroxypregn-5-ene-20-one. Steraloids.

(6β-Hydroxyprogesterone) 6β-Hydroxypregn-4-ene-3, 20-dione. Steraloids.

(11α-Hydroxyprogesterone) 11α-hydroxypregn-4-ene-3, 20-dione. Steraloids.

(11β-Hydroxyandrostenedione) 11β-hydroxyandrost-4-ene-3, 17-dione. Koch Light.

(12α-Hydroxyprogesterone) 12α-hydroxypregn-4-ene-3, 20-dione. Steraloids.

(16α-Hydroxyoestrone) 3, 16α-dihydroxyoestra-1,3,5, (10)-trien-17-one. Steraloids.

3β,16β-dihydroxyandrost-5-ene-3-methyl ether. Steraloids

(17α-Hydroxyprogesterone) 17α-hydroxypregn-4-ene-3, 20-dione. Steraloids.

(Epioestradiol) 3, 17α-dihydroxyoestra-1,3,5,(10)-trien. Organon.

(Epitestosterone) 17α-hydroxyandrost-4-ene-3-one. Organon.

(Oestradiol-/

(Oestradiol-17β) 3, 17β-dihydroxyoestra-1,3,5,(10)-trien. Organon.

(Testosterone) 17β-hydroxyandrost-4-ene-3-one. Organon.

(20β-hydroxyprogesterone) 20β-hydroxypregn-4-ene-3-one. Steraloids.

(21-Hydroxyprogesterone) 21-hydroxypregn-4-ene-3, 20-dione.

5β-Cholan-24-ol. Steraloids.

As a control, sections were incubated in medium containing all reagents, including steroid solvents, except steroid substrate. In addition, 5α-androstan-3, 17-dione, 5β-androstan-3, 17-dione, (Koch Light), 3β-hydroxyandrost-5-ene-16-one-3-methyl ether, pregn-4-ene-3, 20-dione and 3-hydroxyoestra-1,3,5, (10)-trien-17-one, Steraloids, were used as controls for related hydroxysteroids.

Sections of each specimen were stained with haematoxylin and eosin.

RESULTS.

The structure of the human, foetal membranes has been fully described by Bourne (1962). In the histochemical/

histochemical preparations described in this paper, the amniotic epithelium fibroblast and spongy layers of amniotic connective tissue, chorionic connective tissue and trophoblastic layer could be defined and Table I and Figures 1 to 4 show the distribution of hydroxysteroid dehydrogenase activity in these components of human, term membranes.

All hydroxysteroid dehydrogenase activity demonstrated was N.A.D.-linked.

No formazan deposition occurred in sections incubated in steroid-free medium or with control steroids.

D.H.A. was a better substrate than pregnenolone for the demonstration of 3β-hydroxysteroid dehydrogenase activity.

Of the 17β-hydroxysteroids employed, only oestradiol-17β was well used, with moderate diformazan deposition in the amniotic epithelium and trophoblastic layer.

Significant evidence of 6β-, 11α-, 12α-, 16α-, 20β-, 21- and 24-hydroxysteroid dehydrogenase activity was not obtained in any component of term foetal membranes.

The/

TABLE I.

Position of OH- Group	Amniotic Epithelium	Amniotic Connective Tissue	Chorionic Connective Tissue	Trophoblast Layer	Decidua
3α	D weak	D weak	D weak	D weak	M trace
3β	D weak	D weak	D weak	D ++	-
6β	-	-	-	-	-
11α	-	-	-	-	-
11β	-	-	-	D weak	-
12α	-	-	-	-	-
16α	-	-	-	-	-
16β	-	-	-	D ++	-
17α	-	-	-	-	-
17β	D+	-	-	D ++	-
20β	-	-	-	-	-
21	-	-	-	-	-
24	-	-	-	-	-

Distribution of Hydroxysteroid Dehydrogenase Activity.

M = Monoformazan D = Diformazan

The dividing membrane of the dichorionic twin pregnancy showed the same pattern of activity as the other specimens but was quantitatively much less reactive.

Dimethyl formamide was a more satisfactory steroid solvent than propylene glycol.

DISCUSSION.

Interconversions of hydroxy- and ketosteroids are catalysed by a class of widely distributed, pyridine nucleotide dependent hydroxysteroid dehydrogenases which are specific with respect to the position and steric configuration of the groups undergoing reactions. (Talalay, 1957).

The human placental trophoblast from 6 weeks of gestation to term has been shown histochemically to be the site of 3α-, 3β-, 6β-, 11β-, 16β- and 17β-hydroxysteroid dehydrogenases and the literature on this subject has been reviewed by Hart (1966). In the present investigation 3α-, 3β-, 11β-, 16β- and 17β-oestradiol hydroxysteroid dehydrogenases have been demonstrated in human foetal membranes at term, principally in the extra-placental trophoblast; this pattern of activity is similar to, but weaker than, that in the placental trophoblast.

The/

The reduction of $\alpha\beta$ -unsaturated 3-ketosteroids to their corresponding saturated 3-alcohols comprises a major pathway of metabolism of C_{19} and C_{21} steroids (Dorfman & Ungar, 1953); 3α-hydroxysteroid dehydrogenase is an important enzyme in the detoxication of steroids and their elimination from the organism.

3β-Hydroxysteroid dehydrogenase is directly involved in steroid synthesis, by the conversion of the Δ^5-3β-hydroxyl group to the Δ^5-3-oxo group.

11β-Hydroxysteroid dehydrogenase carried out the important cortisol -cortisone dehydrogenation; it has been shown biochemically to be present in human placenta and membranes (Osinski, 1960) and may be part of the foeto-maternal endocrine regulatory mechanism. Its presence in placenta and membranes may explain the origin of the cortisone found in normal human liquor amnii (Baird & Bush, 1960).

Placenta and hydatidiform mole have been shown to metabolise 16-hydroxy oestrogens (Ryan, 1960; Klausner & Ryan, 1964; MacDonald & Siiteri, 1964) but the role of 16β-hydroxysteroid dehydrogenase is uncertain in placenta and membranes.

The absence of 17α-hydroxysteroid dehydrogenase activity from the membranes is in agreement with the findings of Baillie et al. (1965B), Hart (1966) and Kellogg & Glenner (1960) in term placenta.

Levitz, Condon & Dancis (1956) and Troen (1961) have described the interconversion of oestrone and oestradiol in the perfused human placenta and there has been some dispute as to whether one or several oestradiol-sensitive enzyme systems exist in placenta (Hagerman & Villee, 1959; Jarabak, Adams, Williams-Ashman & Talalay, 1962). The absence from the membranes of N.A.D.P.-linked oestradiol dehydrogenase, which is present in the placenta, would tend to support the existence of more than one enzyme though the alternative possibility that the histochemical technique was insufficiently sensitive to detect weak N.A.D.P.-linked activity cannot be completely excluded. When testosterone is employed as steroid substrate 17β-hydroxysteroid dehydrogenase activity in term placenta is demonstrable apparently only in blood vessel walls; blood vessels are not present in the membranes and the absence of activity with testosterone as substrate in the membranes is confirmatory evidence for this vascular distribution (Koide & Mitsudo, 1965; Hart, 1966), of testosterone 17β-dehydrogenase in term placenta.

The/

The foetal membranes have been shown to be metabolically active and there is adequate evidence that the amnion can transport both fluid and electrolytes (Bourne & Lacy, 1960; Garby, 1957). The results of the present investigation indicate that the non-placental trophoblast is potentially capable of steroid hormone synthesis and metabolism, possessing a pattern of hydroxysteroid dehydrogenase activity closely resembling that of the villous trophoblast. The significance of the weaker reactions in amniotic epithelium and in connective tissue layers is less clear.

Dimethyl formamide was a more satisfactory steroid solvent than propylene glycol; some mono- and diformazan deposition in the amniotic epithelium and the connective tissue layers after prolonged incubation using the latter solvent was probably attributable to alcohol dehydrogenase activity (Ferguson, 1965).

SUMMARY.

The histochemical distribution of various hydroxysteroid dehydrogenases in human, term, foetal membranes has been investigated using the tetrazolium dye, Nitro-B.T.

The/

The trophoblastic layer was the most active, showing 3α-, 3β-, 11β-, 16β- and 17β-hydroxysteroid dehydrogenase activities, a pattern of activity similar to that of the placental villous trophoblast.

The amniotic epithelium showed weak 3α-, 3β-, 16β- and 17β-hydroxysteroid dehydrogenase activity; weak 3α- and 3β-hydroxysteroid dehydrogenase activity was noted in the connective tissue layers.

All activity demonstrated was N.A.D.-linked.

ACKNOWLEDGEMENTS.

The author is deeply grateful to the Medical Research Council, by whom this work was supported and to Organon Laboratories for donating steroids.

REFERENCES.

BAILLIE, A.H., K.C. CALMAN, M.M. FERGUSON & D. McK. HART. Histochemical demonstration of 20β-hydroxysteroid dehydrogenase. J. Endocrin. 33: 337 - 339, (1965A).

BAILLIE, A.H., K.C. CALMAN, M.M. FERGUSON & D. McK. HART. Histochemical utilisation of 3α-, 6β-, 11α-, 12α-, 16α-, 16β-, 17α-, 21- and 24-hydroxysteroids. J. Endocrin. (In Press) (1965B).

BAILLIE, A.H., M.M. FERGUSON, K.C. CALMAN & D. McK. HART. Histochemical demonstration of 11β-hydroxysteroid dehydrogenase. J. Endocrin. 33: 119 - 125 (1965).

BAIRD, C.W. & I.E. BUSH. Cortisol and cortisone content of amniotic fluid from diabetic and non-diabetic women. Acta Endocr. (Kbh.) 34: 97 - 104 (1960).

BALOGH, K. A histochemical method for the demonstration of 20α-hydroxysteroid dehydrogenase activity in rat ovaries. J. Histochem. Cytochem. 12: 670 - 673 (1964).

BOURNE, G. The human amnion and chorion. Lloyd-Luke, London. (1962).

BOURNE, G.L. & D. LACY. Ultrastructure of human amnion and its possible relation to the circulation of amniotic fluid. Nature (Lond.) 185: 952 - 954 (1960).

DICZFALUSY, E. Endocrine functions of the human placenta. Acta Endocr. (Kbh.) Suppl. 50, 129 - 134 (1960).

FERGUSON, M.M. Observations on the histochemical distribution of alcohol dehydrogenase. Quart. J. Micr. Sci. (In Press) (1965).

GARBY, L. Studies on transfer of matter across membranes with special reference to the isolated human amniotic membrane and the exchange of amniotic fluid. Acta Physiol. Scand. 40 Suppl. 137 (1957).

HAGERMAN, D.D. & C.A. VILLEE. Separation of human placental estrogen-sensitive transhydrogenase from estradiol-17β dehydrogenase. J. biol. Chem. 234: 2031 - 2036 (1959).

HART, D.McK. Hydroxysteroid dehydrogenase activity in normal, human placenta from 6 weeks of gestation to 42 weeks. J. Endocrin. (In Press).

JARABAK, J., J.A. ADAMS, H.G. WILLIAMS-ASHMAN & P. TALALAY. Purification of a 17β-hydroxysteroid dehydrogenase of human placenta and studies on its transhydrogenase function. J. biol. Chem. 237: 345 - 357 (1962).

KELLOGG, D.A. & G.G. GLENNER. Histochemical localisation of human, term placental 17β-oestradiol dehydrogenases; implications for the transhydrogenase reaction. Nature (Lond.) 187: 763 - 764 (1960).

KLAUSNER, D.A. & K.J. RYAN. Estriol secretion by human, term placenta. J. clin. Endocr. 24: 101 - 104 (1964).

KOIDE, S.S. & S.M. MITSUDO. Histochemical study of 3β- and 17β-hydroxysteroid dehydrogenases in human term placenta. Endocrinol. 76: 403 - 407 (1965).

MACDONALD, P.C. & P.K. SITTERI. Study of estrogen production in women with hydatidiform mole. J. Clin. Endocr. 24: 685 - 690 (1964).

OSINSKI, P.A. Steroid 11β-ol dehydrogenase in human placenta. Nature (Lond.) 187: 777 (1960).

PEARSON, B. & GROSE, F. Histochemical demonstration of 17β-hydroxysteroid dehydrogenase by use of tetrazolium salt. Proc. Soc. exp. Biol. 100: 636 - 638 (1959).

PEARSON, B. & F. GROSE. Histochemical study of some D.P.N.H. and D.P.N. dependent dehydrogenases. Fed. Proc. 18: 1,965 (1959B).

RYAN, K.J. Metabolism of 16-keto-estrone by human placenta. Endocrinol. 66: 491 - 494 (1960).

TALALAY, P. Enzymic mechanisms in steroid metabolism. Physiol. Rev. 37: 362 - 389 (1957).

TROEN, P. Perfusion studies of the human placenta. II. Metabolism of C^{14}-17β-estradiol with and without added human chorionic gonadotropin. J. clin. Endocr. 21: 895 - 908 (1961).

WATTENBERG, L.M. Microscopic histochemical demonstration of steroid-3β-ol dehydrogenase in tissue sections. J. Histochem. Cytochem. 6: 225 - 232 (1958).

DESCRIPTION OF PLATE.

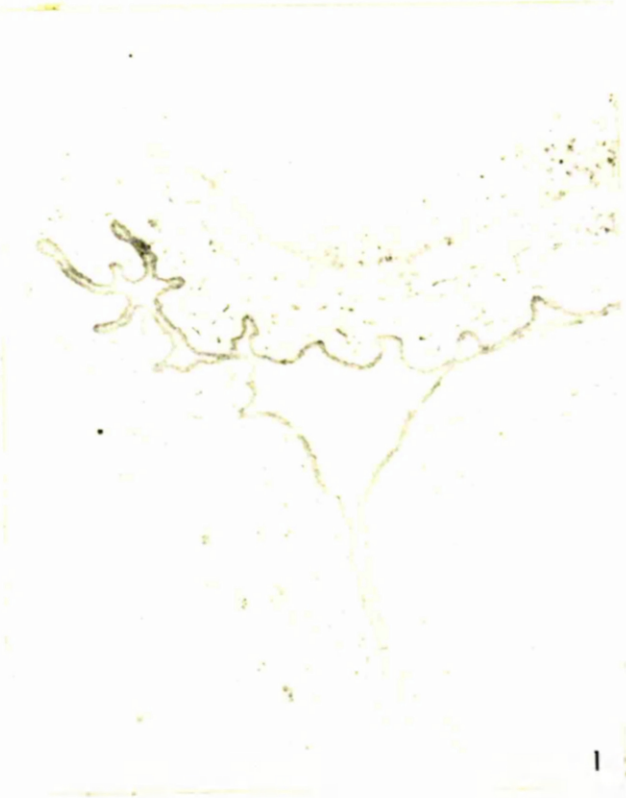
Fig. 1. Weak diformazan deposition in amniotic epithelium, in connective tissue and in trophoblast after incubation with a 3α-hydroxysteroid.
(x 70)

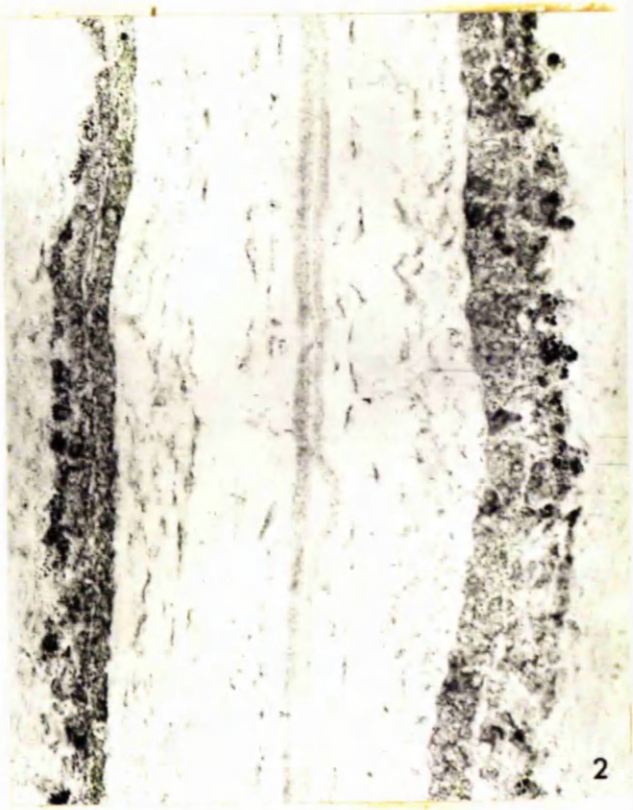
Fig. 2. Two apposed layers of membranes showing 3β-hydroxysteroid dehydrogenase activity in amniotic epithelium, in connective tissue and in trophoblast.
(x 180)

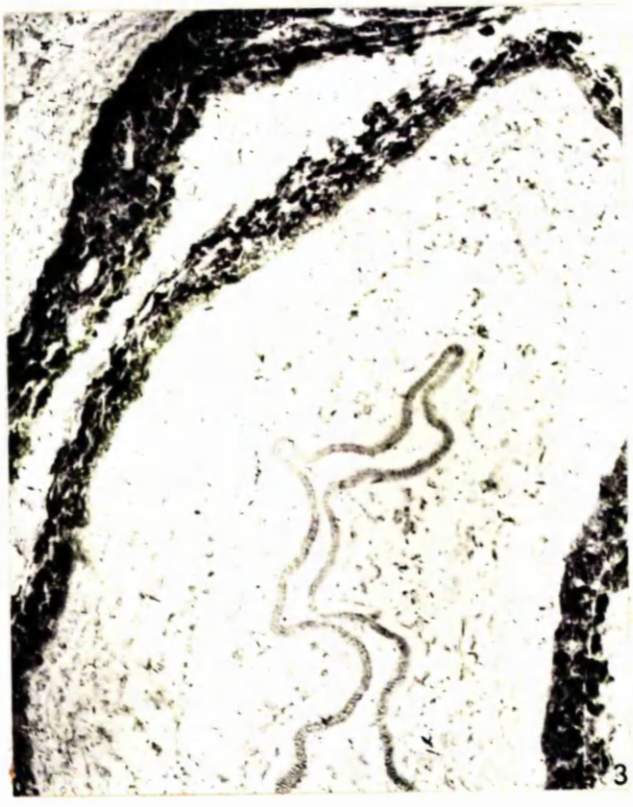
Fig. 3. 11β-Hydroxysteroid dehydrogenase activity is limited to the trophoblastic layer of chorion.
(x 360)

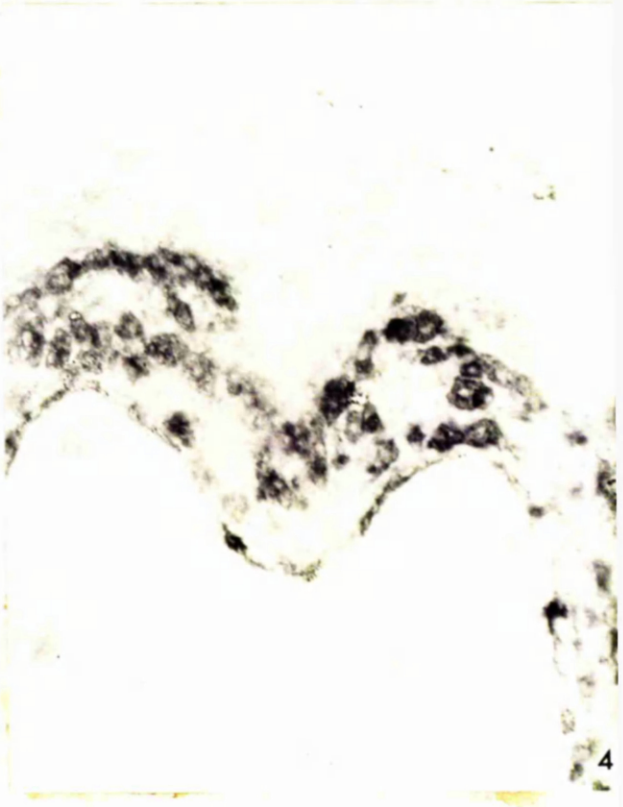
Fig. 4. Strong 16β-hydroxysteroid dehydrogenase activity is seen in the trophoblastic layer of chorion.
(x 150)

PLATE


1


2


3


4

Histochemical Demonstration of Hydroxysteroid Dehydrogenases in Hydatidiform Moles.

BY

D. McKay Hart, F.R.C.S. (Glasg.), M.R.C.O.G.

Department of Anatomy,
University of Glasgow.

INTRODUCTION.

The histochemical distribution of 3α-, 3β-, 6β-, 11β-, 16β-, and 17β-hydroxysteroid dehydrogenase (H.S.D.) activities has been described in immature and adult human placenta [2,3, 4, 10, 11, 14, 15, 16, 22] and in the extra-placental chorion at term [12].

This paper describes the histochemical utilization of 3α-, 3β-, 6β-, 11α-, 11β-, 12α-, 16α-, 16β-, 17α-, 17β-, 20β-, [1]21- and 24-hydroxy-steroids in five hydatidiform moles and 3β-hydroxy-steroids in one further mole.

MATERIAL AND METHODS.

Portions of tissue from six hydatidiform moles expelled spontaneously at 12, 15, 16, 19, 20 and 26 weeks of gestation respectively were frozen on solid carbon dioxide within a few minutes of expulsion from the uterus. Portions of the 15 week and 20 week moles were also obtained at subsequent curettage, performed to complete the evacuation of the uterus, and were similarly treated.

The/

The specimens were sectioned at 10μ. in a cryostat maintained at -25° and the sections attached to clean, dry glass coverslips by momentary thawing. Incubation was then carried out at 37° for two hours in 10 ml. 0.1.M. Tris or phosphate buffer (pH 7.4) containing the following reagents:-

N.A.D. or N.A.D.P.	3 mg.
Nitro Blue Tetrazolium	3 mg.
Steroid Substrate (dissolved in 0.5 ml. propylene glycol or dimethyl formamide)	5 mg.

The steroid substrates employed are listed in Table I.

At the time of examination of the 20 week mole only the method for 3β-H.S.D. was available.

As a control, sections were incubated in medium containing all reagents, including steroid solvent, except the steroid substrate; in addition, estrone, 5α-androstan-3, 17-dione, 5β-androstan-3, 17-dione and 3β-hydroxy-androsten-17-one-3-methyl ether were used as controls for 16α-hydroxyestrone, androsterone, etiocholanolone and 3β, 16β-dihydroxy-androst-5-en-17-one, 3-methyl ether.

Histologically/

TABLE I.

3α-Hydroxy-5α-androstan-17-one (androsterone)	Steraloids
5α-Androstan-3, 17-dione	Koch Light
3α-Hydroxy-5β-androstan-17-one (aetiocholanolone)	Steraloids
5β-Androstan-3,17-dione	Koch Light
3β-Hydroxypregn-5-ene-20-one (pregnenolone)	Boots
3β-Hydroxyandrost-5-ene-17-one (D.H.A.)	Steraloids
3β-Sulphoxyandrost-5-ene-17-one (D.H.A. sulphate)	Organon
6β-Hydroxypregn-4-ene-3, 20-dione (6β-hydroxyprogesterone)	Steraloids
11α-Hydroxypregn-4-ene-3, 20-dione (11α-hydroxyprogesterone)	Steraloids
11β-Hydroxyandrost-4-ene-3, 17-dione (11β-hydroxyandrosterone)	Koch Light
11β-Hydroxypregn-4-ene-3, 20-dione (11β-hydroxyprogesterone)	Steraloids
12α-Hydroxypregn-4-ene-3, 20-dione (12α-hydroxyprogesterone)	Steraloids

3,16α-Dihydroxyoestra-1,3,5,(10)-trien (16α-hydroxyoestrone)	Steraloids
3,16β-Dihydroxyoestra-1,3,5,(10)-trien (16β-hydroxyoestrone)	Steraloids
3β,16β-Dihydroxyandrost-5-ene-3-methyl ether	Steraloids
3β-Hydroxyandrost-5-ene-16-one-3-methyl ether	Steraloids
3,17α-Dihydroxyoestra-1,3,5,(10)-trien (epioestradiol)	Organon
17β-Hydroxyandrost-4-ene-3-one (testosterone)	Organon
3,17β-Dihydroxyoestra-1,3,5,(10)-trien (oestradiol)	Organon
3-Hydroxyoestra-1,3,5,(10)-trien (oestrone)	Organon
20β-Hydroxypregn-4-ene-3-one (20β-Hydroxyprogesterone)	Steraloids
21-Hydroxypregn-4-ene-3-one (21-hydroxyprogesterone)	Steraloids
24-Hydroxy-5β-cholan-3-one	Steraloids

List of Steroid Substrates Employed.

Histologically and clinically all six moles appear to be benign.

RESULTS.

No colour was obtained in control sections.

No significant evidence of 11α-, 12α-, 16α-, 17α-, 20β-, 21- or 24-H.S.D. activity was detected in any component of any of the five moles examined for the full range of enzymes.

Table 2 and figures 1 - 4 detail the positive results.

It will be seen that D.H.A. sulphate was much less well used than free D.H.A. and that 16β-hydroxy-estrone was a less suitable substrate than 3β, 16β-dihydroxyandrost-5-en-3-methyl ether for 16β-H.S.D.

Testosterone was very poorly used as compared with estradiol-17β.

Histological demonstration of 3α-, 6β- and 11β-H.S.D. activities was only possible using N.A.D. as pyridine nucleotide co-factor, whereas 3β-, 16β- and oestradiol-17β-H.S.D. activities were demonstrable using either N.A.D. or N.A.D.P; in all cases the activity was present in the trophoblastic epithelium and was greatest in areas of cellular proliferation.

There/

TABLE II.

Substrate	H.S.D.	Co-factor	12 Wks. Mole	15 Wks. Mole	16 Wks. Mole	19 Wks. Mole	20 Wks. Mole	26 Wks. Mole
Androsterone	3∝	N.A.D.	Tr	-	+	-		-
Aetiocholanolone	3∝	N.A.D.	+	+	+	+		-
Pregnenolone	3β	N.A.D. or N.A.D.P.	++	++	++	++	++	++
D.H.A.	3β	N.A.D. or N.A.D.P.	++	++	++	++	++	++
D.H.A. Sulphate	3β	N.A.D.	Tr	Tr		Tr	+	Tr
6β-Hydroxyprogest.	6β	N.A.D.	+	-	-	-		-
11β-Hydroxyandrost.	11β	N.A.D.	+	Tr	+	-		Tr
11β-Hydroxyprogest.	11β	N.A.D.	Tr	Tr		-		Tr
16β-Hydroxyoestrone	16β	N.A.D. or N.A.D.P.	+	+	+	+		+
3β,16β-Dihydroxy-androst-5-ene-3-methyl ether	16β	N.A.D. or N.A.D.P.	+++	+++	+++	+++		+++
Testosterone	17β	N.A.D.	Tr	+	-	-		-
Oestradiol-17β	17β	N.A.D.	++	++	++	++		++
Oestradiol 17β (N.A.D.P.)	17β	N.A.D.P.	+	+	+	+		+

Distribution of H.S.D. activity in hydatidiform mole trophoblast.

Tr = Trace Activity.

There was no difference in the range of steroids utilised by the spontaneously expelled and the curetted specimens of the 15 and 20 week moles, nor any obvious difference between moles of different ages.

DISCUSSION.

Hydatidiform mole is characterised by trophoblastic proliferation, hydropic degeneration of the villous stroma and scantiness of blood vessels[18] and there is more epithelial proliferation in that part of the mole which is attached to the uterine wall than in expelled vesicles. The Langhan's cells are cuboidal or polyhedral and well differentiated, with large, round, heavily stained nuclei while the syncytium consists of fenestrated sheets of amorphous cytoplasm which take a reddish-pink colour with H & E; the two layers are thus almost identical histologically with those in normal placenta.

Bur, Hertig, McKay and Adams[9] made a histochemical study of ribonucleoprotein, glycogen, glycoprotein, inorganic iron, alkaline phosphatase, 5-nucleotidase and non-specific esterase in fifteen cases of non-invasive mole, two invasive moles and three chorio-carcinomas/

choriocarcinomas. None of their findings pointed obviously to disordered steroid metabolism.

Hertig and Mansell[13] considered hydatidiform mole to be a degenerative process, resulting from agenesis of foetal vasculature in conjunction with functioning maternal vascular apparatus and many expelled vesicles show atrophic rather than hypertrophic trophoblast; MacDonald and Siiteri[17] have shown that estriol secretion by three patients with hydatidiform moles was much less than that in normal pregnant women. These factors might lead one to expect decreased evidence of steroid synthesis and metabolism in mole trophoblast but the H.S.D. activity of the moles in this series closely resembles that in fifty-one normal human placentae from 6 weeks to 42 weeks of gestation and in the extra-placental chorion at term as described by Hart[11, 12] both with regard to the range of hydroxy-steroids utilised and the relative intensities of the reactions. In particular, the utilisation of estradiol-17β both with N.A.D. and with N.A.D.P. as co-factors mirrors that in normal placenta.

D.H.A./

D.H.A. is aromatised by normal placenta in situ[6]. Siiteri and MacDonald[20], Baulieu and Dray[5], and Bolte et al.[7, 8], showed that circulating D.H.A. sulphate is also an estrogen precursor in human placenta while Pulkkinen[19] described steroid sulphatase in normal, human placenta. Warren and French[21] demonstrated that hydatidiform moles also possess steroid sulphatase activity and the uitlisation of D.H.A. sulphate by mole trophoblast in the histochemical system described in this report accords well with the above data.

The possible biochemical significance of H.S.D. activity in human trophoblast has been discussed elsewhere[II] and as far as the histochemical distribution of such activity is concerned hydatidiform mole formation does not involve a major upset in trophoblastic steroid biosynthesis or metabolism.

SUMMARY.

Five hydatidiform moles were surveyed for 3α-, 3β-, 6β-, 11α-, 11β-, 12α-, 16α-, 16β-, 17α-, 17β-, 20β-, 21- and 24-hydroxysteroid dehydrogenase activity and/

3β-hydroxysteroid and one hydatidiform mole for 3β-hydroxysteroid dehydrogenase activity alone.

Strong 3β-, 16β-, and 17β-H.S.D. activity, N.A.D.-linked, was found in the trophoblast when the appropriate steroid substrates were employed.

Poor 3α- and 11β-H.S.D. activity, N.A.D.-linked, was detected in three out of four moles examined and poor 6β-H.S.D. activity in only one out of four.

N.A.D.P.-linked 3β-, 16β- and estradiol-17β-dehydrogenases were constantly present in the trophoblast.

Other hydroxysteroids were not utilised by mole trophoblast.

H.S.D. activity in hydatidiform moles closely resembles that in normal, human placenta.

ACKNOWLEDGEMENTS.

This work was supported by the Medical Research Council of Great Britain.

REFERENCES.

BAILLIE, A.H., CALMAN, K.C., FERGUSON, M.M. & HART, D.McK. Histochemical demonstration of 20β-hydroxysteroid dehydrogenase. J. Endocrin. 32: 337. 1965.

BAILLIE, A.H., CALMAN, K.C., FERGUSON, M.M. & HART, D.McK. Histochemical utilisation of 3α-, 6β-, 11α-, 12α-, 16α-, 16β-, 17α-, 21- and 24-hydroxysteroids. J. Endocrin. (In Press).

BAILLIE, A.H., CAMERON, E.H.D., GRIFFITHS, K. & HART, D.McK. 3β-Hydroxysteroid dehydrogenase in the adrenal gland and placenta. J. Endocrin. 31: 227. 1965.

BAILLIE, A.H., FERGUSON, M.M., CALMAN, K.C. & HART, D.McK. Histochemical demonstration of 11β-hydroxysteroid dehydrogenase. J. Endocrin. 33: 119.1965.

BAULIEU, E.E. & DRAY, F. Conversion of ^{3}H-dehydroepiandrosterone (3β-hydroxy-Δ^{5}-androsten-17-one) sulphate to ^{3}H-estrogens in normal pregnant women. J. Clin. Endocr. 23: 1298. 1963.

BOLTE, E., MANCUSO, S., ERIKSSON, G., WIQVIST, H. & DICZFALUSY, E. Studies on the aromatisation of neutral steroids in pregnant women. I. Aromatisation of C-19 steroids by placentas perfused in situ. Acta Endocr., Kbh. 45: 535. 1964.

BOLTE, E., MANCUSO, S., ERIKSSON, G., WIQVIST, N. & DICZFALUSY, E. Studies on the aromatisation of neutral steroids in pregnant women. 2. Aromatisation of dehydroepiandrosterone and of its sulphate administered simultaneously into a uterine artery. Acta Endocr., Kbh., 45:560. 1964.

BOLTE, E., MANCUSO, S., ERIKSSON, G., WIQVIST, N. & DICZFALUSY, E. Studies on the aromatisation of neutral steroids in pregnant women. 3. Over-all aromatisation of dehydroepiandrosterone sulphate circulating in the foetal and maternal compartments. Acta Endocr., Kbh., 45: 576. 1964.

BUR, G.E., HERTIG, A.T., McKAY, D.G. & ADAMS, E.C. Histochemical aspects of hydatidiform mole and choriocarcinoma. Obstet. Gynec. 19: 156. 1962.

FUHRMAN, K. Uber der histochemischen nachweis der 3β-ol-Steroiddehydrogenase-Activitat in geweben endocriner Organen. Zbl. Gynak. 1961 (i): 565:1961.

HART, D.McK. Hydroxysteroid dehydrogenase activity in normal, human placenta from 6 weeks to 42 weeks of gestation. J. Endocrin. (In Preparation).

HART, D.McK. The histochemical distribution of hydroxysteroid dehydrogenases in the human foetal membranes at term. Histochemie. (In Press).

HERTIG, A.T. & MANSELL, H. Atlas of Tumor Pathology, Section IX, Fascicle 33. Armed Forces Institute of Pathology, Washington, D.C., 1956.

KELLOGG, D.A. & GLENNER, G.G. Histochemical localisation of human, term, placental 17β-oestradiol dehydrogenases; implications for the transhydrogenase reaction. Nature (Lond.) 187: 763. 1960.

KOIDE, S.S. & MITSUDO, S.M. Histochemical study of 3β-and 17β-hydroxysteroid dehydrogenases in human term placenta. Endocrinology, 76: 403. 1965.

LOBEL, B.L., DEANE, H.W. & ROMNEY, S.L. Enzymic histochemistry of the villous portion of the human placenta from six weeks of gestation to term. Am. J. Obst. & Gynec., 83: 295. 1962.

MACDONALD, P.C. & SITTERI, P.K. Study of estrogen production in women with hydatidiform mole. J. Clin. Endocr., 24: 685. 1964.

NOVAK, E.R. & WOODRUFF, J.D. Obstetric and Gynecologic Pathology. W.B. Saunders Co. 5th Ed. Philadelphia & London, 1962.

PULKKINEN, M.O. Arylsulphatase and the hydrolysis of some steroid sulphates in developing organism and placenta. Acta Physiol. Scand. 52: Suppl. 180, 1961.

SIITERI, P.K. & MACDONALD, P.C. The utilisation of circulating dehydroepiandrosterone sulphate for estrogen synthesis during human pregnancy. Steroids 2: 713. 1963.

WARREN, J.C. & FRENCH, A.P. Distribution of steroid sulphatase in human tissues. J. Clin. Endocr. 25: 278. 1965.

WATTENBERG, L.W. Microscopic histochemical demonstration of steroid-3β-ol dehydrogenase in tissue sections. J. Histochem. Cytochem. 6: 225. 1958.

DESCRIPTION OF PLATE.

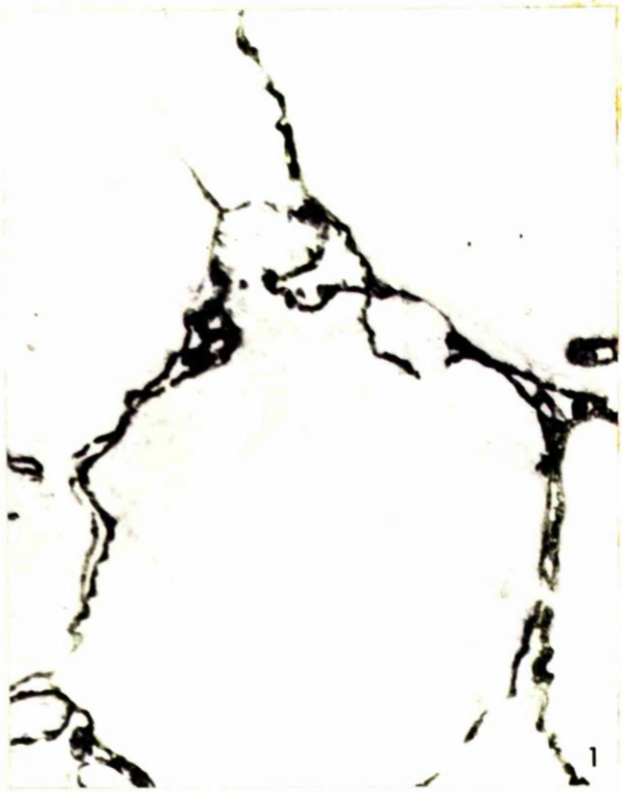
Fig. 1. 3β-Hydroxysteroid dehydrogenase activity in the trophoblast of a 20 weeks hydatidiform mole. x 90.

Fig. 2. 16β-Hydroxysteroid dehydrogenase activity in a proliferative area of the trophoblast of a 15 weeks mole. x 350.

Fig. 3. N.A.D.P.-linked 17β-oestradiol dehydrogenase activity in the trophoblast of a 19 weeks hydatidiform mole. x 300.

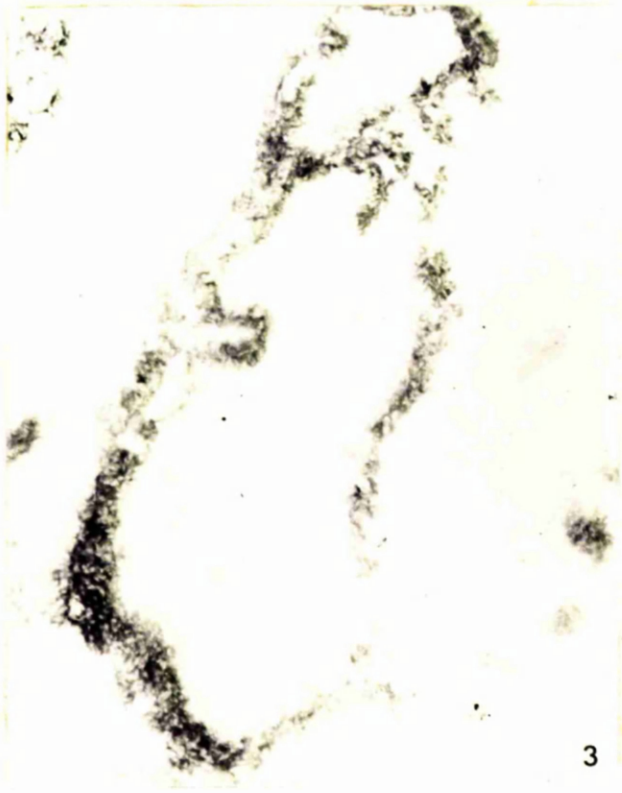
Fig. 4. N.A.D.-linked 17β-oestradiol dehydrogenase activity in a 19 weeks hydatidiform mole. x 90.

PLATE

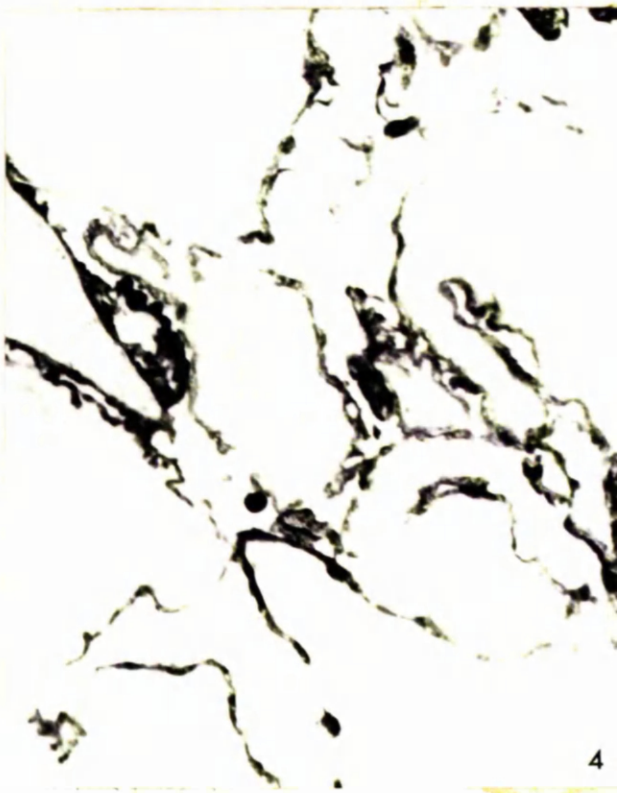


1

2



3



4

Histochemical Investigation of the Localisation of Placental 17β- Hydroxysteroid Dehydrogenases.

BY

D. McKay Hart

Department of Anatomy,
University of Glasgow.

SUMMARY.

The distribution of N.A.D. and N.A.D.P. diaphorases in immature and term human placenta and the localisation of placental 17β-hydroxysteroid dehydrogenases were investigated by histochemical techniques in an attempt to explain conflicting histochemical reports on the latter enzymes.

N.A.D. diaphorase and N.A.D. dependent oestradiol-17β dehydrogenase were present in the trophoblast, vessels and villous stroma; N.A.D.P. diaphorase was mainly evident in the trophoblast and N.A.D.P. dependent oestradiol-17β dehydrogenase was only demonstrated in the trophoblast; 17β-hydroxysteroid dehydrogenase activity using testosterone as substrate was N.A.D.-linked only and was restricted to the vessels and peri-vascular stroma.

It is concluded that the distribution of N.A.D. and N.A.D.P. dependent dehydrogenation of oestradiol-17β is compatible with both functions being performed by one enzyme with dual nucleotide specificity, while testosterone is dehydrogenated by a separate enzyme.

INTRODUCTION

Statements regarding the pyridine nucleotide specificity of placental oestradiol-17β dehydrogenase and its transhydrogenase function have been numerous and conflicting.

Talalay and his co-workers (Talalay & Williams-Ashman, 1958; Talalay, Hurlock & Williams-Ashman, 1958) showed that soluble enzyme preparations from human placenta promoted reversible transhydrogenation between pyridine nucleotides in the presence of low concentrations of 17β-hydroxysteroids or ketosteroids, especially 17β-oestradiol and oestrone. These preparations contained a 17β-hydroxysteroid dehydrogenase which interconverted 17β-oestradiol and oestrone using N.A.D. or N.A.D.P. as co-factor (Langer & Engel, 1958; Langer, Alexander & Engel, 1959) and there was close correlation between steroids that undergo dehydrogenation and those that mediate the transhydrogenation function.

Jarabak, Adams, Williams-Ashman & Talalay (1961) purified a soluble 17β-hydroxysteroid dehydrogenase from human placenta by an eight step procedure resulting in an approximately 2,500-fold purification with an over-all yield of 29% and were unable to separate the dehydrogenase from the transhydrogenase functions. They concluded

that/

that a soluble 17β-hydroxysteroid dehydrogenase in human placenta reacted with both N.A.D. and N.A.D.P. and that this enzyme was responsible for most, if not all, of the 17β-oestradiol mediated transfer of hydrogen between pyridine nucleotides.

On the other hand, Villee and his co-workers (Hagerman, Villee & Wellington, 1959; Hagerman & Villee, 1959; Villee, 1961) have claimed to have separated N.A.D.- and N.A.D.P.-linked oestradiol dehydrogenases and an oestradiol-17β-sensitive transhydrogenase.

From biochemical reports (Langer & Engel, 1958; Adams et al. 1962) it seems probable that oxidation of oestradiol-17β and of testosterone may be mediated by different 17β-hydroxysteroid dehydrogenases.

Histochemical reports of 17β-hydroxysteroid dehydrogenase in human placenta have likewise been conflicting.

Kellogg and Glenner (1960) described different cellular localization for N.A.D.- and N.A.D.P.-linked 17β-oestradiol dehydrogenase reactions in ten human, term placentae; N.A.D.-linked activity was most prominent in the cells of the trophoblast and decidua and less intense in the stroma, while N.A.D.P-linked activity was intense in the perivascular stroma and only minimal in the trophoblast after three hours incubation.

These/

These workers found no difference in the distribution or intensity of N.A.D. and N.A.D.P. diaphorases as demonstrated with the reduced nucleotides and no preferential binding of tetrazolium salt (Nitro B.T.) in decidua or trophoblast. They concluded that their observations strongly suggested that at least two different 17β-oestradiol-sensitive enzymes were being demonstrated. Dehydrogenation of testosterone was not investigated.

Koide and Mitsudo (1965), in a histochemical study of 3β- and 17β-hydroxysteroid dehydrogenases in human, term placenta found moderate N.A.D.-linked oestradiol-17β-dehydrogenase activity in both trophoblasts and vasculature and stroma, while N.A.D.P.-linked oestradiol-17β-dehydrogenase was demonstrated only in the vessel walls. With testosterone, estr-4-en-17β-ol-3-one and 5α-androstan-17β-ol-3-one hydroxysteroid dehydrogenase activity, both N.A.D.- and N.A.D.P.-linked was limited to the vessel walls. It was concluded that separate N.A.D.- and N.A.D.P.-linked oestradiol-17β-dehydrogenases had been demonstrated and that N.A.D.-linked testosterone dehydrogenase was a separate entity from the N.A.D.-linked oestradiol dehydrogenase, while the N.A.D.P.-linked dehydrogenases for oestradiol and testosterone were not differentiated by their results.

A large number of normal human placentae obtained at Caesarean section or therapeutic termination of pregnancy have been examined in this laboratory, including an age series of fifty-two placentae (Hart, 1966). While the distribution of N.A.D.-linked Oestradiol 17β dehydrogenase and 17β-hydroxysteroid dehydrogenase using testosterone reported by these previous authors were confirmed N.A.D.P.-linked oestradiol dehydrogenase was found in the trophoblast but not in the stroma or vasculature and no N.A.D.P.-linked dehydrogenation of testosterone was observed.

In an attempt to explain the discrepancies referred to above, the histochemical distributions of N.A.D. and N.A.D.P. diaphorases and of dehydrogenation of oestradiol-17β and testosterone with N.A.D. and N.A.D.P. as co-factor were examined.

MATERIAL AND METHODS.

Portions of placenta measuring approximately 1 $cm.^3$ were obtained at therapeutic termination of two pregnancies (foetal crown-rump lengths 1.4 cm. and 7.5 cm.) and at five Caesarean sections performed at term for reasons not connected with placental malfunction. These portions were frozen on solid carbon dioxide within two minutes of removal from the uterus, sectioned at 12μ in a cryostat

maintained/

maintained at -25° and attached to clean, dry coverslips by momentary thawing then dried in air.

To exclude preferential tissue binding of Nitro B.T. sections were incubated in O.I.M. Tris or phosphate buffer (pH 7.4) containing 0.3 mg. Nitro B.T. per ml. for 1 hour, then washed in buffer and treated with 0.1% ammonium sulphide or Ascorbic acid (Pearsön & Hess, 1961).

To demonstrate N.A.D. and N.A.D.H. diaphorase activities, sections were incubated in O.I.M. Tris or phosphate buffer pH 7.4, 10 ml. containing 3 mg. Nitro B.T. and 3 mg. reduced N.A.D. or N.A.D.P. (Sigma) per ml. Incubation was carried out for lengths of time varying from 5 minutes to 24 hours; the sections were then washed in buffer and mounted with an aqueous mountant.

For the demonstration of hydroxysteroid dehydrogenase activity sections were incubated at 37° in O.I.M. Tris or phosphate buffer (pH 7.4) 10 ml. containing N.A.D. or N.A.D.P. 3 mg., Nitro B.T., 3 mg. and with and without 25 mg. bovine Serum Albumen and oestradiol-17β (Organon) or testosterone (Organon) 5 mg. dissolved in 0.5 ml. dimethyl formamide. Incubations were carried out for two hours and for 24 hours.

As

As a control, sections were incubated in media containing all reagents other than steroid substrate. On removal from the media, sections were washed in buffer and mounted with an aqueous mountant (Hydramount) (Gurr).

RESULTS

Preferential binding of Nitro B.T. was not demonstrated; all components of immature and mature placentae were heavily stained with diformazan after treatment with Nitro B.T. and the reducing agents.

Differences in the intensity and distribution of N.A.D. and N.A.D.P. diaphorases were infact demonstrated in human placenta. After a short incubation in the media for the demonstration of diaphorase activity (5 - 15 mins.) heavy diformazan deposition was seen in the trophoblast with both reduced nucleotides, but evidence of activity in the stroma and vasculature was seen with N.A.D.H. only (Fig. 1 & 2). After incubation for 24 hours to ensure the development of maximum formazan deposition, intense diformazan deposition was seen in the trophoblast with both reduced nucleotides though less intense with N.A.D.P.H. than with $N.A.D.H._2$; in the stroma and vasculature, there was/

TABLE I.

SUBSTRATE	NUCLEOTIDE					
	N.A.D.			N.A.D.P.		
	Trophoblast	Vessels	Stroma	Trophoblast	Vessels	Stroma
Oestradiol-17β	++	+	+	+	-	-
Testosterone	-	+	tr	-	-	-

Hydroxysteroid Dehydrogenase Activity

was evidence of intense $N.A.D.H._2$ diaphorase activity, but only weak $N.A.D.P.H._2$ activity (Fig. 3). The findings with respect to oestradiol and testosterone dehydrogenase activities are summarised in Table 1.

There was no fundamental difference between the immature and term placentae, though the former possessed much less stromal connective tissue and vasculature.

The presence or absence of bovine serum albumen in the medium did not affect the results.

After incubation for 24 hours in media containing testosterone and N.A.D. sections of term placenta showed a trace of monoformazan in the trophoblast.

DISCUSSION.

It has been shown that selective binding of Nitro B.T. is not a problem in human placenta. Contrary to the findings of Kellogg and Glenner (1960) differences have been found between the activities of $N.A.D.H._2$ and $N.A.D.P.H._2$ diaphorases in human placenta; particularly after a short incubation (producing an intensity of formazan deposition similar to that produced in the demonstration of oestradiol-17β dehydrogenase activity) but even after 24 hours incubation, $N.A.D.P.H._2$ activity is weaker in the trophoblast and much weaker in the vessels and stroma than is $N.A.D.H._2$ activity (Fig. 3).

These/

These differences in diaphorase activity rationalise the different patterns of oestradiol-17β dehydrogenase activity reported by Hart (1966) with N.A.D. and N.A.D.P. as co-factors. Ubiquitous N.A.D.-linked oestradiol-17β dehydrogenase activity and N.A.D.P.-linked activity limited to the trophoblast are compatible with demonstration of one enzyme with dual nucleotide specificity, formazan deposition being similar to, but weaker than that observed on demonstration of the respective diaphorases.

It has been suggested biochemically that testosterone may be dehydrogenated by a separate enzyme from oestradiol-17β dehydrogenase (Langer & Engel, 1958; Adams et al. 1962). Histochemically, the distribution of dehydrogenase activity is different when oestradiol-17β and testosterone are employed as substrates, testosterone dehydrogenase activity being N.A.D.-linked and limited to the vessels and stroma, only a fraction of the distribution of Oe_2 17β-dehydrogenase.

Trace activity seen in the trophoblast after 24 hours incubation with testosterone as substrate and N.A.D. as co-factor is explicable as dehydrogenation of testosterone by oestradiol-17β dehydrogenase in the trophoblast as this enzyme has been shown to dehydrogenate testosterone at/

at less than 5% of the extent of oestradiol (Langer & Engel, 1958).

It is thus concluded that the histochemical distribution of N.A.D. and N.A.D.P.-linked dehydrogenation of oestradiol-17β is compatible with both these functions being performed by the same enzyme, while testosterone is dehydrogenated by a separate enzyme.

REFERENCES.

ADAMS, J.A., JARABAK, J. & TALALAY, P. (1962). The steroid specificity of the 17β-hydroxysteroid dehydrogenase of human placenta. J. biol. Chem. 237: 3069 - 3073.

JARABAK, J., ADAMS, J.A., WILLIAMS-ASHMAN, H.G. & TALALAY, P. (1962). Purification of a 17β-hydroxysteroid dehydrogenase of human placenta and studies on its transhydrogenase function. J. biol. Chem. 237: 345 - 357.

HAGERMAN, D.D., VILLEE, C.A. & WELLINGTON, F.M. (1959). Separation of human placental estrogen-sensitive transhydrogenase from estradiol-17β dehydrogenase. Fed. Proc. 18: 240.

HART, D.McK. (1966). Hydroxysteroid dehydrogenase activity in the normal human placenta from six weeks to forty-two weeks of gestation. J. Endocrin. (In Press).

KELLOGG, D.A. & GLENNER, G.G. (1960). Histochemical localisation of human, term placental 17β-oestradiol dehydrogenases: implications for the transhydrogenase reaction. Nature (Lond.). 187: 763 - 764.

KOIDE, S.S. & MITSUDO, S.M. (1965). Histochemical study of 3β- and 17β-hydroxysteroid dehydrogenases in human, term placenta. Endocrinology. 76: 403 - 407.

LANGER, L.J., ALEXANDER, J.A. & ENGEL, L.L. (1958). Human placental estradiol-17β-dehydrogenase. II Kinetics and substrate specificities. J. biol. Chem. 234: 2,609 - 2,614.

LANGER, L.J. & ENGEL, L.L. (1958). Human placental estradiol-17β dehydrogenase. I Concentration, characterisation and assay. J.biol.Chem. 233: 583 - 588.

PEARSE, A.G.E. & HESS, R. (1961). Substantivity and other factors responsible for formazan patterns in dehydrogenase histochemistry. Experientia, 17: 136 - 141.

TALALAY, P., HURLOCK, B. & WILLIAMS-ASHMAN, H.G. (1958). On a coenzymatic function of estradiol-17β. Proc. Natl. Acad. Sci. U.S. 44: 862-884.

TALALAY, P. & WILLIAMS-ASHMAN, H.G. (1958). Activation of hydrogen transfer between pyridine nucleotides by steroid hormones. Proc. Natl. Acad. Sci. U.S. 44: 15 - 26.

VILLEE, C.A. (1961). Die Beeinflussung von Enzymen in Uterus und Placenta durch Oestrogene. Klin. Wochschr. 39: 173-17

EXPLANATION OF PLATES.

PLATE I.

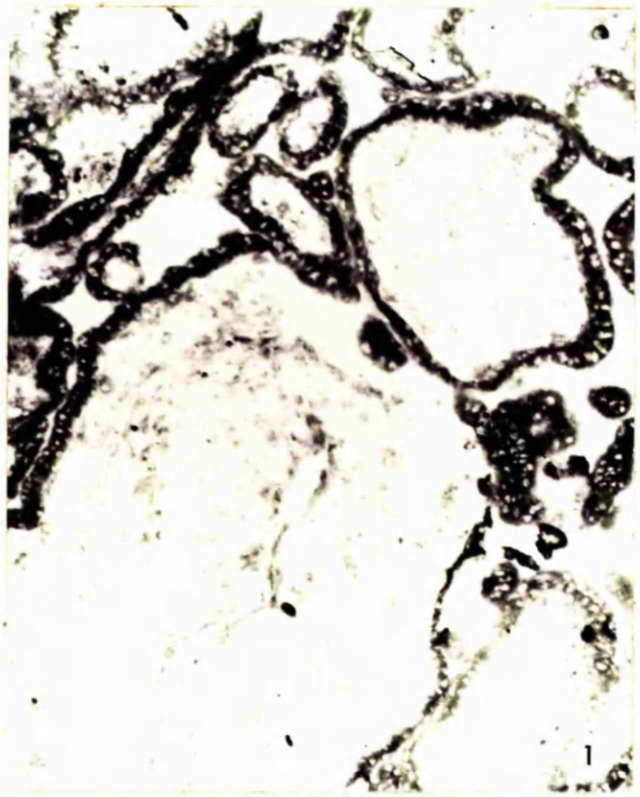
Fig. 1. N.A.D. diaphorase activity in trophoblast and stroma of a six weeks placenta. (x 300).

Fig. 2. N.A.D.P. diaphorase activity is seen only in the trophoblast of the six weeks placenta. (x 300).

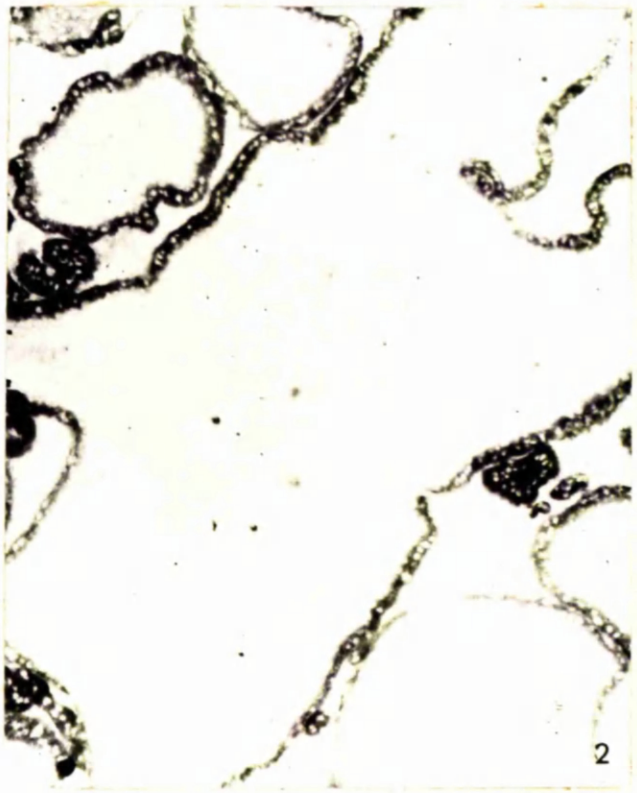
Fig. 3. Two serial sections of term placenta after twenty-four hours incubation to demonstrate diaphorase activity. N.A.D. diaphorase activity (above) is strong in trophoblast, vessels and villous stroma, while N.A.D.P. (below diaphorase is very weak in the vessels and stroma (x 80).

Fig. 4. Testosterone is dehydrogenated in the vessels only (x 200).

PLATE I

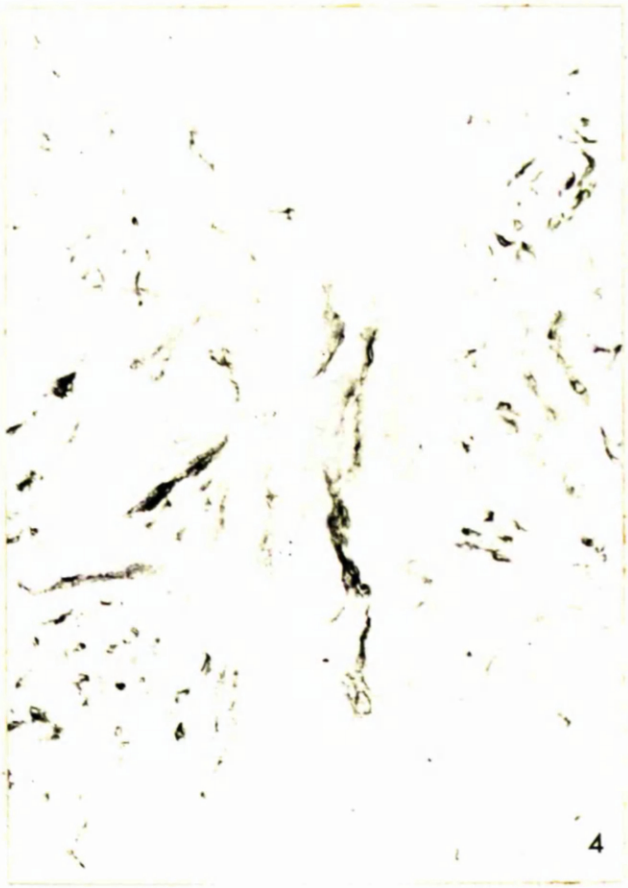


1



2

3



4

PLATE II.

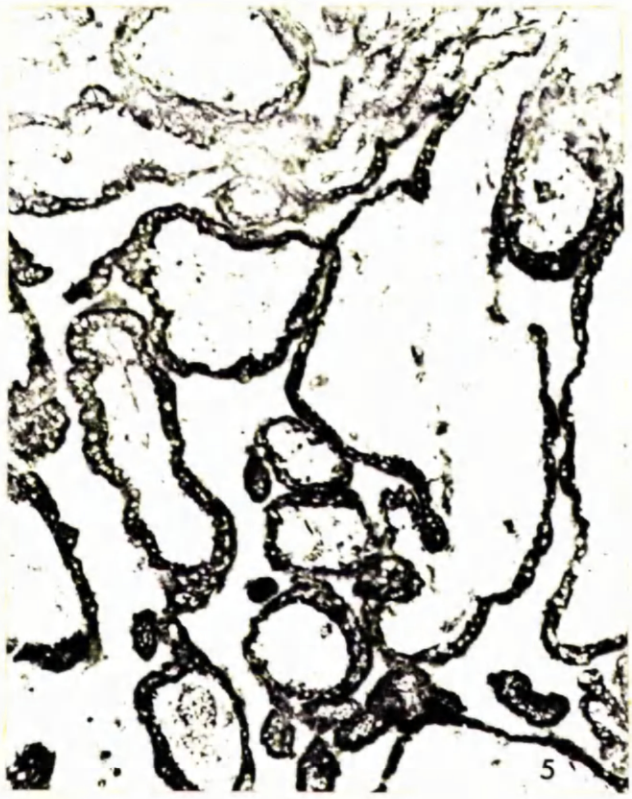
Fig. 5. N.A.D.-linked 17β-oestradiol dehydrogenase activity in trophoblast and the scanty stroma of a six weeks placenta (x 180).

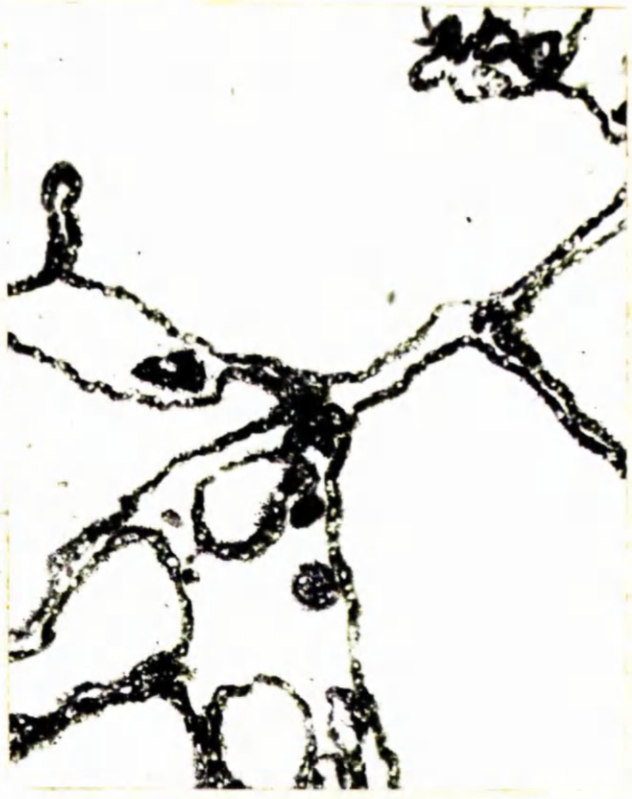
Fig. 6. N.A.D.P.-linked 17β-oestradiol dehydrogenase activity in the same six weeks placenta in the trophoblast only (x 200).

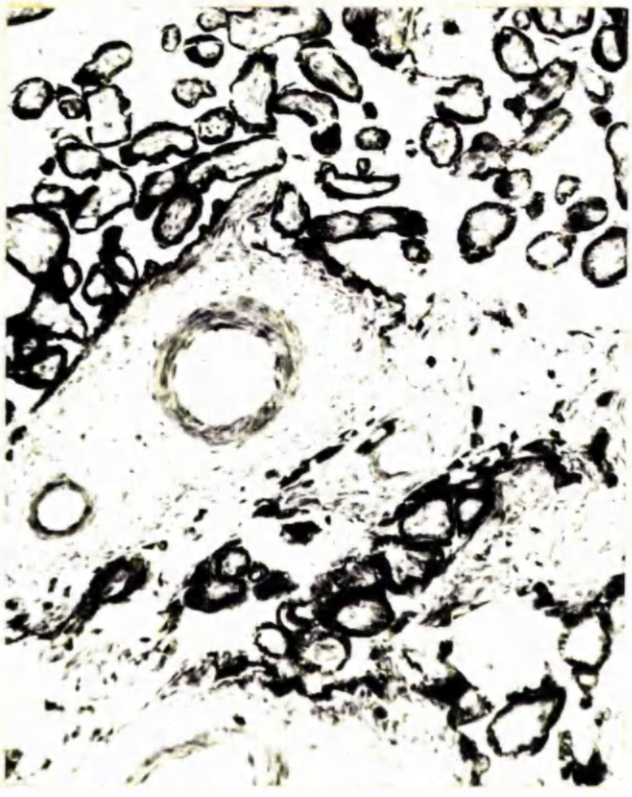
Fig. 7. N.A.D.-linked oestradiol-17β dehydrogenase activity in trophoblast, vessels and stroma of a term placenta. (x 100).

Fig. 8. N.A.D.P.-linked oestradiol-17β dehydrogenase in term placenta. Activity is seen in the trophoblast only. (x 150).

PLATE II







Evidence of Steroid Metabolism in a Human Yolk Sac.

BY

D. McKay Hart

Department of Anatomy,
University of Glasgow.

SUMMARY.

The yolk sac of a 14 mm. crown-rump length human foetus was surveyed for N.A.D. and N.A.D.P. diaphorase activities using the reduced nucleotides and Nitro-B.T. and for 3α-, 3β-, 6β-, 11β-, 16β- and 17β-hydroxysteroid dehydrogenases using the modification of Wattenberg's (1958) technique employed by Hart (1966).

N.A.D. and N.A.D.P. diaphorases were detected in the mesenchymal and endodermal cells of the yolk sac; N.A.D.- and N.A.D.P.-linked oestradiol-17β dehydrogenase activity was found also in both mesenchymal and endodermal cells.

INTRODUCTION.

Botte, Chieffi and Materazzi (1966) described 17β-oestradiol dehydrogenase and testosterone dehydrogenase in the yolk sac endodermal cells and trophoblastic giant cells of the rat placenta, both N.A.D. and N.A.D.P. dependent.

It has been suggested that migration of the germ cells into the genital ridge may be due to the chemotropic influence of local concentrations of steroids there (Baillie, Ferguson & Hart, 1966), on the basis of

of histochemical evidence of steroid hormone metabolism and possible biosynthesis in the human genital ridge. Human germ cells have been noted as far afield as the yolk sac epithelium (Florian, 1931; Debeyre, 1933; Polizer, 1933) and as the yolk sac of a 14 mm. human embryo was available for study it was decided that investigation of the steroid metabolic potentialities of this tissue should be carried out using histochemical techniques for the demonstration of hydroxysteroid dehydrogenase activity (Wattenberg, 1958; Pearson & Grose, 1959 A, B; Baillie, Calman, Ferguson & Hart, 1965, 1966; Baillie, Ferguson, Calman & Hart, 1965).

MATERIAL AND METHODS.

The yolk sac of a human foetus of crown-rump length 14 mm. was obtained at hysterotomy performed to terminate the pregnancy for psychiatric reasons, and was frozen on pulverised solid carbon dioxide within 10 minutes of removal from the uterus.

Using a cryostat maintained at -25°, 10μ sections were cut and were attached to clean, dry, glass coverslips by momentary thawing then dried in air.

To demonstrate diaphorase activity, sections were

were incubated at 37° in 0.1 M. phosphate buffer (p.H. 7.4) 10 ml. containing 3 mg. Nitro-B.T. and 3 mg. N.A.D.H_2 or N.A.D.P.H_2 (Sigma Chemical Co.) for 5 to 15 minutes, then washed in buffer and mounted with an aqueous mountant.

Hydroxysteroid dehydrogenase activity was demonstrated by incubation at 37° in 0.1 M. phosphate buffer (p.H. 7.4) 10 ml. containing Nitro-B.T. 3mg., N.A.D. or N.A.D.P. 3mg. and steroid substrate 5 mg. dissolved in 0.5 ml. dimethyl formamide. The following steroid substrates were employed :-

1 3α-hydroxy-5α-androstan-17-one (Steraloids),
2 3α-hydroxy-5β-androstan-17-one (Steraloids),
3 3β-hydroxyandrost-5-en-17-one (Steraloids),
4 3β-hydroxyprogn-5-en-20-one (Steraloids),
5 6β-hydroxyprogn-4-en-3,20-dione (Steraloids),
6 11β-hydroxyandrost-4-en-3,17-dione (Schering),
7 3β,16β-dihydroxyandrost-5-en-3-methyl ether (Steraloids)
8 3β-hydroxyandrost-5-en-16-one-3-methyl ether (Steraloids)
(as control for 8)
9 3,17β-dihydroxyoestra-1,3,5,(10)-trien (Organon)
10 17β-hydroxyandrost-4-en-3-one (Organon).

As a control, sections were incubated in medium containing all reagents, including steroid solvent, other than steroid substrate.

In order to confirm the presence of germ cells in the yolk sac epithelium, sections were incubated by the method of McKay, Hertig, Adams and Danziger (1953) for the demonstration of alkaline phosphatase.

RESULTS.

The presence of germ cells was confirmed.

N.A.D. and N.A.D.P. diaphorase activities were present in the mesenchymal and endodermal cells of the yolk sac, both being more pronounced in the endodermal cells. As depicted in figures 1,2 and 3, N.A.D. diaphorase was the more active.

Moderate N.A.D.-linked oestradiol-17β dehydrogenase and weak N.A.D.P.-linked oestradiol-17β dehydrogenase activities were noted in both mesenchymal and endodermal cells, the latter exhibiting the stronger activity (fig.4). Weak 17β-hydroxysteroid dehydrogenase, N.A.D. dependent only, was apparent when testosterone was the steroid substrate.

Significant evidence of other hydroxysteroid dehydrogenases was not obtained.

Sections incubated in the control media remained colourless.

DISCUSSION.

By the 14 mm. stage in the human the placental trophoblast has already assumed an active steroid biosynthetic and metabolic role (Hart, 1966). In comparison, the yolk sac of a 14 mm. human embryo has been shown to possess little hydroxysteroid dehydrogenase activity. It has moreover been demonstrated that this poor exhibition of hydroxysteroid dehydrogenase activity is not due to a lack of N.A.D. or N.A.D.P. diaphorase activity.

Oestradiol-17β dehydrogenase, which was present, interconverts oestrone and oestradiol and has a trans-hydrogenase function (Jarabak, Adams, Williams-Ashman & Talalay, 1962) and the finding of this enzyme together with dehydrogenation of testosterone accords well with the findings of Botte et al. in the yolk sac endodermal cells of the rat. The absence of 3β-hydroxysteroid dehydrogenase is also in agreement with the situation reported in the rat by Deane, Rubin, Driks, Lobel and Leipsner (1962) and Botte et al. (1966).

In comparison with the genital ridge of the 14 mm. human embryo (Baillie, Ferguson & Hart, 1966) the yolk sac shows little evidence of steroid biosynthesis; if, in fact

germ cell migration to the genital ridge is controlled by steroid hormone produced in that structure it is clear that the yolk sac is not a serious competitor as an alternative attractor of germ cells at the stage of development studied, if at all.

ACKNOWLEDGEMENTS.

Research facilities and materials in the Department of Anatomy, the University of Glasgow, are gratefully acknowledged.

I am grateful to the Medical Research Council, by whom this work was supported; Organon Laboratories kindly donated oestradiol and testosterone.

REFERENCES.

BAILLIE, A.H., CALMAN, K.C., FERGUSON, M.M. & HART, D.McK. (1965). Histochemical demonstration of 20β-hydroxysteroid dehydrogenase. J. Endocrin. 32: 337-339.

BAILLIE, A.H., CALMAN, K.C., FERGUSON, M.M. & HART, D.McK. (1966). Histochemical utilisation of 3α-, 6β-, 11α-, 12α-, 16α-, 16β-, 17α-, 21- & 24-hydroxysteroids. J. Endocrin. 34: 1-12.

BAILLIE, A.H., FERGUSON, M.M., CALMAN, K.C. & HART, D.McK. (1965). Histochemical demonstration of 11β-hydroxysteroid dehydrogenase. J. Endocrin. 33: 119-125.

BAILLIE, A.H., FERGUSON, M.M. & HART, D.McK. (1966). Evidence of steroid metabolism and possible biosynthesis in the human genital ridge mesenchyme. In Hart, D.McK., Thesis submitted to the University of Glasgow for the degree of M.D.

BOTTE, V., CHIEFFI, G. & MATERAZZI, G. (1966) Histochemical distribution of Δ^5-3β-hydroxysteroid dehydrogenase and 17β-hydroxysteroid dehydrogenase in the placenta and foetal membranes of rat. J. Endocrin. (In Press).

DEANE, H.W., RUBIN, B.L., DRIKS, E.C., LOBEL, B.L. & LEIPSNER, G. (1962). Trophoblastic giant cells in placentas of rats and mice and their probable role in steroid-hormone production. Endocrinology 70: 407-419.

DEBEYRE, A. (1933). Sur la presence de gonocytes chez un embryon humain au stade de la ligne primitive. Comp. rend. Ass. Anat. 28: 240-250.

FLORIAN, J. (1931). 'Urkeimzellen' bei einem 625μ langen menschlichen Embryo. Anat. Anz. 72: 286.

HART, D.McK. (1966). Hydroxysteroid dehydrogenase activity in normal human placenta from six weeks to forty-two weeks of gestation. J. Endocrin. (In Press).

JARABAK, J., ADAMS, J.A., WILLIAMS-ASHMAN, H.G. & TALALAY, P. (1962). Purification of a 17β-hydroxysteroid dehydrogenase of human placenta and studies on its transhydrogenase function. J. biol. Chem. 237: 345-357.

McKAY, D.G., HERTIG, A.T., ADAMS, E.C. & DANZIGER, S. (1953). Histochemical observations on the germ cells of human embryos. Anat. Rec. 117: 201-219.

PEARSON, B. & GROSE, F. (1959 A). Histochemical demonstration of 17β-hydroxysteroid dehydrogenase by use of tetrazolium salt. Proc. Soc. exp. Biol. 100: 636-638.

PEARSON, B., & GROSE, F. (1959 B). Histochemical study of some D.P.N.H. and D.P.N. dependent dehydrogenases. Fed. Proc. 18: 1,965.

POLITZER, T. (1933). Die Keimbahn des Menschen. Zeit. Ges. Anat. abt. I. Zeit. Anat. u. Entwicklungsges. 100: 331-361.

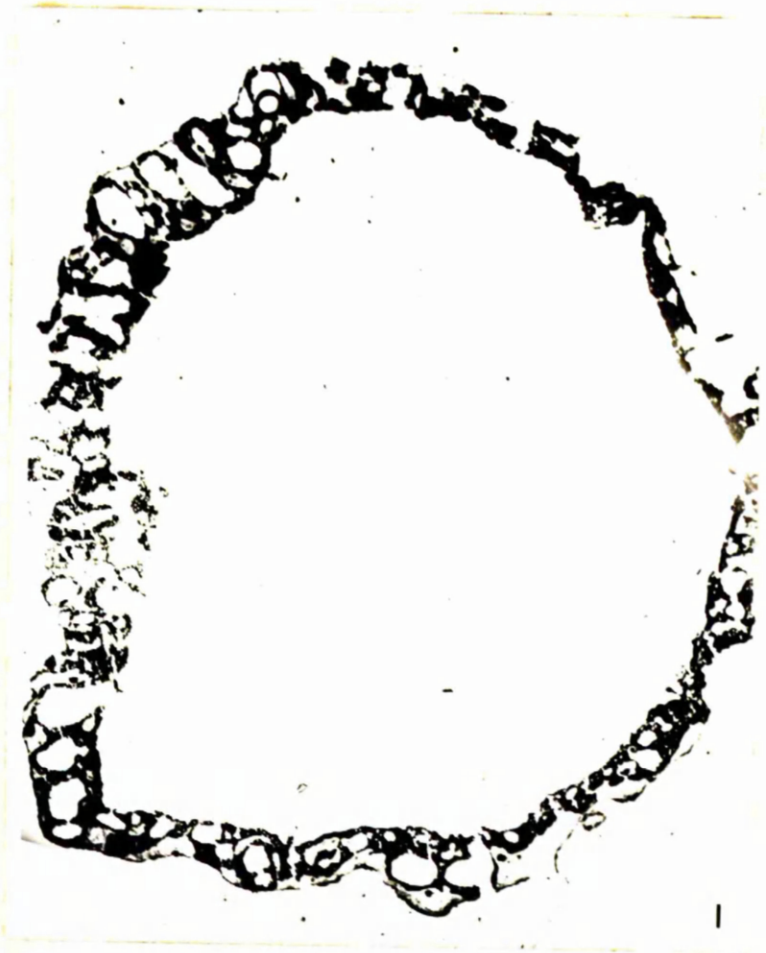
WATTENBERG, L.W. (1958). Microscopic histochemical demonstration of steroid-3β-ol dehydrogenase in tissue sections. J. Histochem. Cytochem. 6: 225-232.

EXPLANATION OF PLATES.

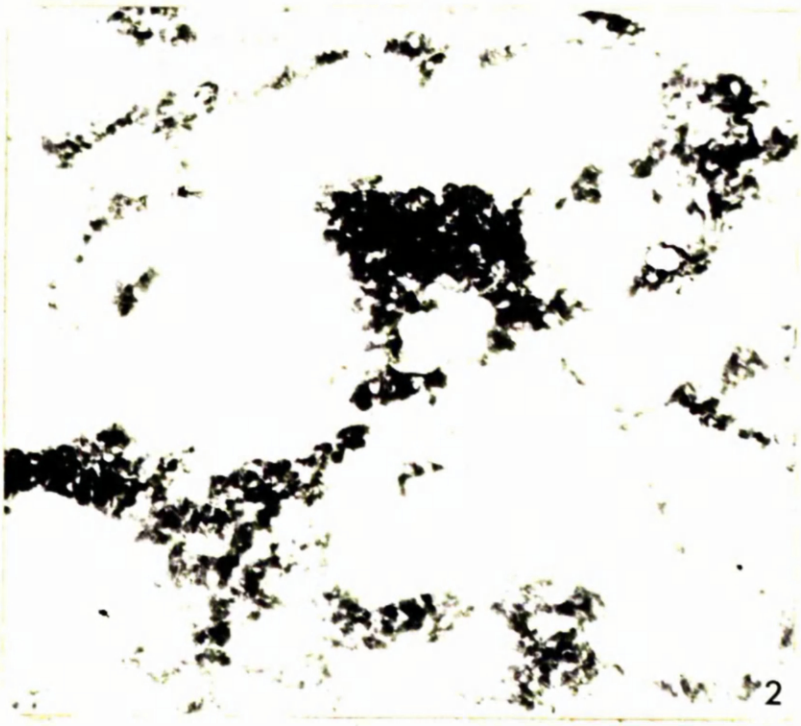
Fig. 1. Section of entire yolk sac, showing intense N.A.D.-diaphorase activity in the yolk-sac endodermal cells. The weaker mesenchymal reaction is not readily visible. (x 50).

Fig. 2. N.A.D.P. diaphorase in the wall of the yolk sac, more intense in the endodermal cells. (x 400).

PLATE I



1



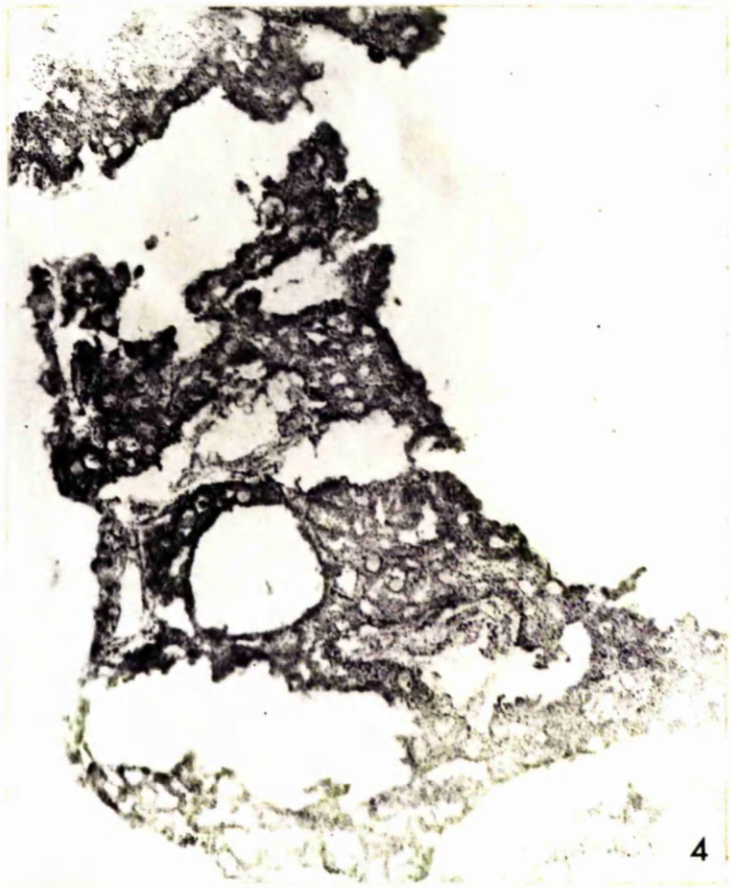
2

Fig. 3. N.A.D. diaphorase activity is intense in the endodermal cells. (x 300).

Fig. 4. N.A.D.-linked 17β-oestradiol dehydrogenase activity is much less intense than N.A.D. diaphorase activity. The endodermal cells show the stronger reaction. (x 300).

PLATE II

3



4