Δ^5 - 3β -HYDROXYSTEROID DEHYDROGENASE ACTIVITY IN THE STEROID-HORMONE PRODUCING ORGANS OF THE FERRET (*MUSTELA PUTORIUS FURO*)

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Summary. A histochemical method was used for the detection of Δ^5 - 3β -hydroxysteroid dehydrogenase activity in the adrenal glands, testes, ovaries and placentae of ferrets. The enzyme was demonstrated in the adrenocortical tissue, in the testicular Leydig cells of the testes, and in the granulosa lutein cells, the theca lutein cells and the theca interna of healthy and atretic follicles of the ovary. There was no enzyme activity in any part of the placenta, even when incubation was prolonged. All the tissues of the organs examined were active for the NAD diaphorase reaction. Lipid staining did not parallel the activity of Δ^5 - 3β -hydroxysteroid dehydrogenase.

The pattern of distribution of Δ^5 - 3β -hydroxysteroid dehydrogenase activity resembles that seen in the guinea-pig, and differs from that seen in many other species—in particular the rabbit, another induced ovulator.

INTRODUCTION

The ferret (Carnivora) is a valuable species for the study of reproduction, because of its small size and marked sexual changes, and because the female ovulates only after coitus (Robinson, 1918; Hammond & Marshall, 1930; Rowlands, 1957). Both the male and female have a well-defined breeding season extending from about March to August. Oestrus in the absence of copulation persists till the end of the breeding season. The reproductive physiology of this animal has been a subject of special study by one of us (Galil, 1965).

A method is available for the detection and cellular localization of an enzyme, Δ^5 - 3β -hydroxysteroid dehydrogenase (Wattenberg, 1958; Levy, Deane & Rubin, 1959), known to hold a key position in the synthesis of the various steroid hormones (Samuels, 1960; Dorfman, 1962; Deane & Rubin, 1965). We thought it would be valuable to study the activity of this enzyme in the steroid-hormone producing organs of the ferret. The need for extending the studies of the activity of this enzyme to a larger variety of mammalian

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species has become evident from the results so far reported. For the most part the enzyme is demonstrable only in the steroid-hormone producing organs of vertebrates. The enzyme has been detected in various ovarian structures and the adrenal cortex of a number of mammals (Wattenberg, 1958; Rubin, Deane & Hamilton, 1963), in the interstitial cells of the testis in many species (Wattenberg, 1958; Baillie & Griffiths, 1964; Maeir, 1965), in the syncytiotrophoblast of the human placenta (Wattenberg, 1958; Lobel, Deane & Romney, 1962; Baillie, Cameron, Griffiths & Hart, 1965) and in the giant trophoblastic cells of the rat and mouse placentae (Deane, Rubin, Driks, Lobel & Leipsner, 1962). In a few instances, however, tissues expected to exhibit this reaction have failed to do so (Wattenberg, 1958; Rubin *et al.*, 1963; Maeir, 1965; Deane & Rubin, 1965).

MATERIALS AND METHODS

Eight pregnant female ferrets and one sexually active male were used. The animals were obtained from The Wellcome Veterinary Research Station, Frant, Tunbridge Wells. The pregnant animals were at different stages of gestation (7, 17, 20, 23, 29, 30, 37 and 41 days of pregnancy), full gestation in the ferret being 42 days (Hammond & Marshall, 1930). The adrenal glands, the testes, the ovaries and segments of placentae were removed under Nembutal anaesthesia (30 mg/kg). These were frozen directly on solid carbon dioxide. They were then sectioned at 16 μ in a Bright (Pearse) cryostat maintained at -20° C. The sections were mounted on clean coverslips, dried at room temperature for 30 min and stored in a refrigerator overnight.

Before incubation the sections were treated with ice-cold redistilled acetone for about 5 min to remove free lipid droplets. They were then passed through two changes of cold 0.1 M-phosphate buffer (pH 7.5).

The test medium, slightly modified from that used by Rubin *et al.* (1963), consisted of: 0.5 ml of acetone containing 0.1% dehydroepiandrosterone (DHA; Sigma); 4 ml of 0.1 M-phosphate buffer (pH 7.5); 2 ml of aqueous 0.1% Nitro-BT (Sigma), and 1 ml of aqueous 0.6% β -nicotinamide adenine dinucleotide [NAD (DPN), Sigma]. The control medium lacked DHA. Incubation was conducted in air at 37° C for $\frac{1}{2}$, 1 and 2 hr in cases of the adrenals, testes and ovaries, and for 3 and 5 hr for the placentae. Another medium containing β -NADH₂ (DNPH; Sigma) as substrate was used to demonstrate general diaphorase activity (Pearse, 1960; Rubin *et al.*, 1963). This incubation lasted 30 min.

After incubation the sections were fixed in alcoholic formalin, washed and mounted with Gurr's Water Mounting Medium.

Parallel sections were stained with Sudan black for lipids.

RESULTS

Adrenal gland

The adrenal gland exhibited strong Δ^5 -3 β -hydroxysteroid dehydrogenase activity in the inner zones of the cortex and somewhat less intense activity in the zona glomerulosa (Pl. 1, Fig. 1). Enzymic activity in the capsular and

medullary regions was restricted to the few scattered cortical cells that occurred in these areas. There was no non-specific activity evident in the control sections. The cortical tissue showed increased activity with advancing pregnancy.

NAD diaphorase activity was demonstrable in both cortical and medullary tissues at almost uniform intensity (Pl. 1, Fig. 2). There was a strong staining in the zona glomerulosa but only a weak one in the capsular region. There was no detectable increase in the amount of staining with advancing pregnancy.

Sections stained for lipids showed strong sudanophilia in the cortex and the ectopic cortical tissue in the capsule and medulla. The zona glomerulosa stained more strongly than the rest of the cortex (Pl. 1, Fig. 3).

Testis

Sections of the testes treated for the visualization of Δ^5 -3 β -hydroxysteroid dehydrogenase were characterized by an intense reaction in the Leydig cells, whereas the seminiferous tubules were lacking in specific enzymic activity (Pl. 2, Fig. 10). There was a slight coloration of the outer border of the seminiferous tubules, which also occurred in the control sections (cf. Pl. 2, Figs. 10 and 11), suggesting the utilization of endogenous substrates.

Both Leydig cells and the seminiferous tubules showed NAD diaphorase activity, but the Leydig cell reaction was far more intense than that of the tubules (Pl. 2, Fig. 12).

When sections were stained for lipids, the Leydig cells were the only part of the testis that contained stainable droplets (Pl. 2, Fig. 13).

Ovary

The ovary of the pregnant ferret showed strong Δ^5 -3 β -hydroxysteroid dehydrogenase activity in the granulosa lutein cells (Pl. 1, Fig. 4). There was far less enzymic activity in what are judged to be the theca lutein cells of corpora lutea and in the interstitial tissue. In both healthy and atretic follicles, in the pregnant animal, only the cells of the theca interna seemed to be reactive. There seemed to be a slight increase in the activity of the enzyme in lutein tissue in late gestation.

All ovarian tissues displayed a strong NAD diaphorase reaction, with the greatest activity being shown by the granulosa lutein cells (Pl. 1, Fig. 5).

In the sections stained with Sudan black, the granulosa lutein cells exhibited greater sudanophilia than did the theca lutein cells, which were almost devoid of stainable lipids (Pl. 1, Fig. 6). There was no conspicuous increase in the amount of luteal lipids with advancing pregnancy.

Placenta

Even with as much as 3 to 5 hr of incubation, Δ^5 -3 β -hydroxysteroid dehydrogenase activity could not be detected at any stage of pregnancy in the placental labyrinth or in the underlying uterine segment (Pl. 1, Fig. 7).

The NAD diaphorase reaction revealed a strong staining of all elements in the placental labyrinth and underlying decidua, but relatively weak staining of the myometrium (Pl. 1, Fig. 8).

Lipids could only be detected in the symplasma of the junctional zone and

in the maternal endothelium in the labyrinth (Pl. 1, Fig. 9). The lipids in the maternal endothelium were in the form of very fine droplets and were demonstrable only during the last stages of pregnancy (37 and 41 days).

DISCUSSION

The control sections were generally free of non-specific activity, except in the case of the seminiferous tubules, thus corroborating the findings of others (Wattenberg, 1958; Levy *et al.*, 1959; Rubin *et al.*, 1963; Baillie & Griffiths, 1964). This result dispels Pearse's misgivings about the technique (Pearse, 1960).

What raises more serious concern about this technique is the occasional failure to detect the enzyme in some organs or tissues expected to be active in steroid-hormone production. For example, in a canine adrenal, Rubin *et al.* (1963) reported the inactivity of the inner half of the cortex. These authors also showed that part of the adrenocortical tissue in both adult and foetal armadillos is lacking in enzymic activity. An even more striking example has been the common failure to detect this reaction in the Leydig cells of the normal primate testis (Goldberg, Jones & Turner, 1963; Maeir, 1965; Deane & Rubin, 1965). However, Goldberg, Jones & Borkowf (1964) have shown that hydroxysteroid dehydrogenase activity in the human testis can be demonstrated when substrates other than DHA are employed, notably Δ^4 - 3β -hydroxysteroids.

The rabbit's corpus luteum has provided another exception, where a tissue, presumably active in steroidogenesis, showed no detectable Δ^5 - 3β -hydroxysteroid dehydrogenase activity (Wattenberg, 1958; Rubin *et al.*, 1963; Deane & Rubin, 1965). Recently, however, Deane (unpublished), using the conditions and medium employed in this study, has been able to detect moderate enzymic

EXPLANATION OF PLATE 1

FIGS. 1-3. Adjacent sections of the adrenal gland of a pregnant ferret, 23 days of gestation. $\times 20$. g = Glomerulosa, c = inner cortex (fasciculata and reticularis), ec = ectopic cortical cells, m = medulla.

FIG. 1. Δ^5 - 3β -Hydroxysteroid dehydrogenase, 1 hr inc.; enzymic activity is localized in the cortical tissue only.

FIG. 2. NAD diaphorase, 30 min inc.; all tissues are reactive.

FIG. 3. Sudan black, 30 min staining.

FIGS. 4-6. Adjacent sections of the ovary, 23 days of gestation. $\times 20$. cl = Corpus luteum, te = thecal tissue, hf = healthy follicle, af = atretic follicle.

FIG. 4. Δ^5 - 3β -Hydroxysteroid dehydrogenase, 1 hr inc.; strong activity in granulosa lutein cells; weak activity in theca of corpus luteum and theca interna of healthy and atretic follicles, no activity in granulosa cells.

FIG. 5. NAD diaphorase, 30 min inc.; all ovarian tissues show enzymic activity.

FIG. 6. Sudan black, 30 min staining; lipids are restricted almost entirely to the luteal tissue.

FIGS. 7-9. Adjacent sections of the placenta, 30 days of gestation. $\times 20$. l = Labyrinth, e = endometrium, s = symplasma, m = myometrium.

FIG. 7. Δ^5 - 3β -Hydroxysteroid dehydrogenase, 5 hr inc.; no enzymic activity in either the labyrinth or the underlying uterine segment.

FIG. 8. NAD diaphorase, 30 min inc.; all components show enzymic activity, the myometrium being somewhat less reactive than the placenta.

FIG. 9. Sudan black, 30 min staining; lipids are demonstrable only in the symplasma.

PLATE I

(Facing p. 336)

Adjacent sections of the testis of an adult male ferret. $\times 20$. Lc = Leydig cells; arrow, outer cell layer of seminiferous tubules.

FIG. 10. Δ^5 -3 β -Hydroxysteroid dehydrogenase, 1 hr inc.; activity is limited to the Leydig cells; the reaction of the outer cell layer of seminiferous tubules is non-specific—compare with Fig. 11.

FIG. 11. Control (NAD without DHA), 1 hr inc.; staining is absent from Leydig cells but occurs in the outer layer of seminiferous tubules.

FIG. 12. NAD diaphorase, 30 min inc.; both Leydig cells and seminiferous tubules show activity, although staining is stronger in the Leydig cells.

FIG. 13. Sudan black, 30 min staining; lipids are limited to the Leydig cells.

activity in the rabbit's corpus luteum. Possibly this success is attributable to the use of redistilled acetone for the washes and media.

The degree of enzymic activity in the various tissues of the ferret parallels quite closely the condition in the guinea-pig (Deane & Rubin, 1965). Activity is strong in the adrenal cortex, in the Leydig tissue of the testis and in the luteal tissue of the ovary. Relatively little activity is seen in the ovarian interstitial tissue in comparison with the strong activity exhibited by the corresponding tissue in the ovaries of rabbits (Wattenberg, 1958; Rubin *et al.*, 1963). In the past, comparison has been made between the ovarian interstitial tissue in these two species, implying close functional similarity (Hammond & Marshall, 1930).

The failure of the placenta to show enzymic activity raises especial problems, as discussed above, since progesterone has been detected in the uterine-vein blood of the pregnant ovariectomized ferret (Galil, 1965). Again this absence of enzymic activity is reminiscent of the extensive experience of one of us with the guinea-pig placenta (Deane & Rubin, 1965; Heap & Deane, unpublished). The failure of the ferret placenta to show any Δ^5 - 3β -hydroxysteroid dehydrogenase activity occurs in the presence of an active NAD diaphorase system. Therefore the primary dehydrogenase itself must in some way be implicated in this failure of activity. The situation may arise as a result of: (a) the enzyme being exceptionally labile, (b) another factor, necessary for the activation of the enzyme, not being supplied in the medium, (c) steroid-hormone biosynthesis in the placenta following another pathway that does not require this enzyme, or, finally (d) the progesterone in the uterine-vein blood emanating not from the placenta but from foetal organs, possibly the adrenals. We have no basis for selecting among these alternatives at present.

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