Subcellular Distribution of Δ^5 -3 β -Hydroxy Steroid Dehydrogenase in the Granulosa Cells of the Domestic Fowl (*Gallus domesticus*)

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1. The distribution of 3 β -hydroxy steroid dehydrogenase was examined in the subcellular fractions of granulosa cells collected from the ovary of the domestic fowl. 2. 3 β -Hydroxy steroid dehydrogenase activity was observed in the mitochondrial (4000g for 20 min) and microsomal (105000g for 120 min) fractions. 3. Approximately three times more 3 β -hydroxy steroid dehydrogenase activity was associated with the cytochrome oxidase activity (a mitochondrial marker enzyme) in anteovulatory-follicle granulosa cells than with that of the postovulatory follicle. 4. Comparison of the latent properties of mitochondrial 3 β -hydroxy steroid dehydrogenase with those of cytochrome oxidase and isocitrate dehydrogenase indicated that 3 β -hydroxy steroid dehydrogenase is located extramitochondrially. 5. This apparent distribution of 3 β -hydroxy steroid dehydrogenase is explained on the basis that the mitochondrial activity is either an artefact caused by a redistribution in the subcellular location of the enzyme, occurring during homogenization, or by the existence of a functionally heterogeneous endoplasmic reticulum that yields particles of widely differing sedimentation properties.

The oxidation of the Δ^5 -3 β -hydroxy group of both C₁₉ and C₂₁ steroids to form the Δ^4 -3-oxo group is an important step in the biosynthesis of biologically active steroids. The reaction was first demonstrated by Samuels *et al.* (1951) and requires the participation of two enzymes, namely 3 β -hydroxy steroid-NAD⁺ oxidoreductase (EC 1.1.1.51) and 3-oxo steroid Δ^4 - Δ^5 -isomerase (EC 5.3.3.1).

Recent papers have reported that 3 β -hydroxy steroid dehydrogenase is present in both the mitochondrial and microsomal fractions obtained from adrenal cortex tissue (Basch & Finegold, 1971; Kream & Sauer, 1976), ovarian tissue (Jackanicz & Armstrong, 1968; Dimino & Cambell, 1976), testicular tissue (Sulimovici *et al.*, 1973; Van der Molen *et al.*, 1975) and placental tissue (Koide & Torres, 1965; Ferre *et al.*, 1975).

Various investigators have attempted to explain this heterogeneous distribution by suggesting that the mitochondrial activity can arise from direct microsomal contamination (Beyer & Samuels, 1956), from a redistribution artefact (Cowan *et al.*, 1974) or from an actual mitochondrial location (Jackanicz & Armstrong, 1968; Sulimovici *et al.*, 1973).

In a previous report (Armstrong & Wells, 1976) of experiments with a whole ovarian homogenate obtained from mature laying birds, it was shown that all the 3 β -hydroxy steroid dehydrogenase activity was associated with the microsomal fraction. However, during the processing of this tissue the

large yolky follicles were split to expel the yolk, an operation that has since been shown to result in the removal of the granulosa cells from these follicles (Gilbert *et al.*, 1977). The present paper extends the previous work by examining the distribution of 3 β -hydroxy steroid dehydrogenase activity in the subcellular fractions obtained from anteovulatory-follicle and postovulatory-follicle granulosa cells.

Experimental

Animals

Sixteen mature light-hybrid laying birds (Shaver, type 288) were housed in individual cages with 14 h of light/day. Food and water were always available.

Chemicals

3 β -Hydroxypregn-5-en-20-one (pregnenolone), pregn-4-ene-3,20-dione (progesterone), 3 β -hydroxyandrost-5-en-17-one (dehydroepiandrosterone) androst-4-ene-3,17-dione (androstenedione) and all other fine chemicals were obtained from Sigma Chemical Co., Poole, Dorset, U.K. All radioactive steroids were obtained from The Radiochemical Centre, Amersham, Bucks., U.K., and were checked periodically for radiochemical purity by t.l.c.

Androst-5-ene-3,17-dione was prepared from dehydroepiandrosterone by the method of Nes *et al.* (1963). The product was purified by recrystallization from diethyl ether and had negligible

absorption at 241 nm in ethanol, indicating the absence of a conjugated ketone.

All other chemicals were obtained from BDH Chemicals, Poole, Dorset, U.K.

Tissue collection and fractionation

Anteovulatory-follicle and postovulatory-follicle granulosa cells were collected from mature birds as described previously (Gilbert *et al.*, 1977; Armstrong *et al.*, 1977).

The cells were suspended in an ice-cold 25 mM-sodium phosphate buffer, pH 7.2, supplemented with sucrose (0.25 M), KCl (0.15 M) and EDTA (1 mM). Homogenization was carried out in a loose-fitting all-glass homogenizer (three passes). The resultant homogenate was centrifuged at 600g for 15 min at 2°C in an MSE Mistral 6L centrifuge to remove cell debris and the nuclear fraction. The volume of the supernatant was adjusted to give a protein concentration of approx. 0.5 mg/ml.

The mitochondrial pellet was obtained by centrifugation in a Beckman JA-20 angle-head rotor for 20 min at 2°C and 4000g (r_{av} 7.0 cm). The microsomal pellet was obtained by centrifugation of the post-mitochondrial fraction in an MSE 8×35 ml aluminium angle head rotor for 120 min at 2°C and 105 000g (r_{av} 6.5 cm).

The supernatant from this last spin was taken to be the soluble fraction. Each particulate fraction was washed twice in 10 ml of the homogenizing medium and finally resuspended in 5 ml of the same medium. The suspensions were stored in 1 ml portions at -20°C until required. All assays were completed within 3 days of the tissue fractionation and no loss in enzyme activity was observed over this period.

Mitochondrial preparations were lysed either by osmotic shock or by sonication.

Enzyme assays

3 β -Hydroxy steroid dehydrogenase, 3-oxo steroid Δ^4 - Δ^5 -isomerase, cytochrome oxidase (EC 1.9.3.1), carboxyl esterase (EC 3.1.1.1), lactate dehydrogenase (EC 1.1.1.27) and isocitrate dehydrogenase (EC 1.1.1.42) were assayed by the methods described by Armstrong *et al.* (1977), Neville & Engel (1968), Appelmans *et al.* (1955), Bier (1955) and Bergmeyer (1974a,b) respectively. Protein was measured by the method of Lowry *et al.* (1951) with bovine serum albumin as standard.

Results

The distribution of a number of enzymes in the various subcellular fractions of anteovulatory-follicle and postovulatory-follicle granulosa cells is given in Tables 1 and 2.

The major portion of the 3 β -hydroxy steroid dehydrogenase activity was located in the two particulate fractions and the recoveries with pregnenolone or dehydroepiandrosterone as substrate were in the order of 100%. Addition of a portion of boiled soluble fraction to either of the particulate fractions had no effect on the specific activity of the enzyme.

Centrifugation of the postmitochondrial fraction at 105 000g for 1 h resulted in appreciable 3 β -hydroxy steroid dehydrogenase activity remaining in the soluble fraction. However, recentrifugation for 2 h at the same speed resulted in the sedimentation of material that contained a large proportion of this activity. A similar observation was made by Cheatum

Table 1. *Enzymic activities in subcellular fractions isolated from ovarian follicular anteovulatory granulosa cells*
Enzymic activities are expressed as μ mol of product formed/min by the cell-free homogenate at 37°C. The reported activities are the mean values from two pools of 12 granulosa preparations having an average protein content of 40.8 mg. Assay techniques are as described in the text. The subcellular distribution is presented as a percentage of the cell-free homogenate activity. C₂₁ and C₁₉ indicate that the dehydrogenase is specific for pregnenolone and dehydroepiandrosterone as substrates respectively.

Enzymes	Enzyme activity (μ mol/min)	Distribution in subcellular fractions (%)			Recovery (%)
		M*	P*	S*	
3 β -Hydroxy steroid dehydrogenase (C ₂₁)	0.088	45.7	43.6	7.3	96.6
3 β -Hydroxy steroid dehydrogenase (C ₁₉)	0.075	45.9	45.9	11.2	103.0
Δ^4 - Δ^5 -Isomerase	0.998	43.7	45.7	7.4	96.8
Cytochrome oxidase	1.23	80.8	10.4	0	91.2
Carboxylesterase	0.452	28.6	61.2	4.4	94.2
Lactate dehydrogenase	70.1	2.0	1.7	90.7	94.4
Protein		23.2	21.8	52.0	97.0

* M, P and S represent the mitochondrial, microsomal and soluble fractions respectively.

Table 2. Enzymic activities in subcellular fractions isolated from ovarian follicular postovulatory granulosa cells

Enzymic activities and assays are the same as those described in Table 1. The measurements were carried out on two pools of ten granulosa-cell preparations having an average protein content of 28.0mg and the activities represent means for two determinations. C₂₁ and C₁₉ indicate that the dehydrogenase is specific for pregnenolone and dehydroepiandrosterone as substrates respectively.

Enzymes	Enzyme activity ($\mu\text{mol}/\text{min}$)	Distribution in subcellular fractions (%)			Recovery (%)
		M*	P*	S*	
3 β -Hydroxy steroid dehydrogenase (C ₂₁)	0.039	35.8	49.2	3.4	88.4
3 β -Hydroxy steroid dehydrogenase (C ₁₉)	0.027	31.2	54.0	11.1	96.3
Cytochrome oxidase	0.932	76.3	8.4	6.1	90.8
Carboxylesterase	0.605	31.8	66.6	2.6	101.0
Protein		18.2	15.7	70.0	103.9

* See footnote to Table 1.

Table 3. Latency of mitochondrial enzymes isolated from ovarian follicular anteovulatory granulosa cells

Enzymic activities are expressed as nmol of product/min in the mitochondrial fraction at 37°C. In Expts. 1 and 2, mitochondria were lysed by sonication at 21 kHz (5 $\times$ 1 s bursts) with intermittent cooling in an ice-bath. In Expt. 3, lysis was achieved by suspending the mitochondria in water for 15 min at 0°C before returning to a medium containing 0.25M-sucrose. Mitochondria were prepared from pools of three, six and five granulosa-cell preparations for Expts. 1, 2 and 3 respectively. Abbreviation: n.d., not determined. The steroid dehydrogenase activity was measured with pregnenolone as substrate in the presence and absence of 1 mM-NAD⁺ at pH7.2.

Enzymes	Mitochondria	...	Expt. 1		Expt. 2		Expt. 3	
			Whole	Lysed	Whole	Lysed	Whole	Lysed
3 β -Hydroxy steroid dehydrogenase								
+NAD ⁺			7.13	6.63	14.78	13.77	4.73	5.03
-NAD ⁺			0.30	0.23	0.10	0.08	0.27	0.30
Cytochrome oxidase			9.4	94.8	42.7	502.4	66.4	455.0
Isocitrate dehydrogenase			n.d.	n.d.	n.d.	n.d.	24.5	241.3

et al. (1967) during subcellular fractionation of the bovine adrenal cortex.

The distribution of the 3 β -hydroxy steroid dehydrogenases specific for pregnenolone and dehydroepiandrosterone as substrates and of 3-oxo steroid Δ^4 - Δ^5 -isomerase were similar in the subcellular fractions of granulosa cells.

Contamination of the mitochondrial fraction with endoplasmic reticulum was assessed biochemically from the distribution of carboxylesterase activity (Tables 1 and 2). In both granulosa-cell preparations, the esterase activity that was found in the mitochondrial fraction amounted to approx. 30% of the total activity and was decreased only slightly on further washing/centrifugation cycles. Carboxylesterase was chosen as a microsomal marker enzyme rather than glucose 6-phosphatase since the activity of this latter enzyme was too low to measure accurately by using spectrophotometry with the avian granulosa homogenates.

The analysis of the distribution of the 3 β -hydroxy

steroid dehydrogenases specific for pregnenolone and dehydroepiandrosterone as substrates throughout the subcellular fractions shows that in the anteovulatory-follicle granulosa cells approximately 30% of the dehydrogenase activity follows the cytochrome oxidase activity (a mitochondrial marker enzyme). In postovulatory-follicle granulosa cells the proportion following cytochrome oxidase was about 10%.

The activities of three enzymes in whole and lysed mitochondrial preparations are compared in Table 3.

Cytochrome oxidase and isocitrate dehydrogenase display activities that are typical of latent enzymes. No difference was observed in the activities of 3 β -hydroxy steroid dehydrogenase in whole or lysed mitochondria incubated with or without NAD⁺. Low NAD⁺ blanks in the whole mitochondrial preparations indicate that the 3 β -hydroxy steroid dehydrogenase is unable to utilize endogenous pools of mitochondrial NAD⁺.

Discussion

A characteristic of steroidogenic tissue is that it contains a large amount of smooth endoplasmic reticulum that forms a vast network of interconnected tubules throughout the cell (Christensen & Gillim, 1969). Some of this smooth endoplasmic reticulum is found in close association with the outer membrane of the mitochondria and the high activity of carboxyl-esterase found in the mitochondrial fraction may be a biochemical consequence of this special morphology of the steroid-producing cells.

The granulosa tissue used in the present study represented a very pure endocrine cell preparation. The only contaminating materials in the anteovulatory-follicle granulosa cell preparation were the basal lamina and the perivitelline layer (Gilbert *et al.*, 1977). The subcellular distributions of the marker enzymes were therefore not complicated by the presence of organelles from non-steroidogenic tissue that had the same marker enzymes, but could have exhibited slightly different sedimentation properties from the corresponding organelle in the endocrine cells.

The distribution of 3β -hydroxy steroid dehydrogenase activity throughout the fowl ovarian granulosa cells (Tables 1 and 2) showed the typical pattern found in other steroid-producing tissues (e.g. Jackanicz & Armstrong, 1968). However, the amount of 3β -hydroxy steroid dehydrogenase activity sedimenting in the mitochondrial fraction was dependent on whether the granulosa cells were collected from anteovulatory or postovulatory follicles.

Three times as much 3β -hydroxy steroid dehydrogenase activity was associated with the cytochrome oxidase activity of anteovulatory-follicle granulosa cells as with that of the postovulatory-follicle granulosa cells. This variation in the subcellular distribution of 3β -hydroxy steroid dehydrogenase activity may have important implications concerning the control of steroidogenesis in this tissue.

The results of the experiments testing the latent properties of 3β -hydroxy steroid dehydrogenase in anteovulatory-follicle granulosa cells (Table 3) suggested that the enzyme was located extramitochondrially. This is in contrast with the results obtained by Dimino (1977) and Kream & Sauer (1976), both of whom showed that 3β -hydroxy steroid dehydrogenase can utilize endogenous mitochondrial pools of NAD^+ , thus indicating an intramitochondrial location.

The properties of the fowl ovarian granulosa 3β -hydroxy steroid dehydrogenase, which sedimented in the mitochondrial fraction, appeared therefore to support the redistribution artefact as proposed by Cowan *et al.* (1974). However, the extent of this redistribution was dependent on whether or not the granulosa cells were collected before or after ovulation.

The 3β -hydroxy steroid dehydrogenase subcellular distribution is, however, open to another interpretation; Basch & Finegold (1971) and Moustafa & Koritz (1975) suggested that the smooth endoplasmic reticulum in steroidogenic tissue may be a functionally heterogeneous organelle similar to that found in the liver (Dallner & Ernster, 1968; Lewis & Tata, 1973). The disparity between the subcellular distribution of the esterase and the 3β -hydroxy steroid dehydrogenase activities in the fowl granulosa cells then becomes explicable on the assumption that a proportion of the 3β -hydroxy steroid dehydrogenase is located in a region of endoplasmic reticulum more likely to sediment with the mitochondria than with the microsomal fraction.

A lateral change in the location of 3β -hydroxy steroid dehydrogenase within the reticulum possibly occurs after ovulation so that the association of esterase and 3β -hydroxy steroid dehydrogenase activities increases in postovulatory-follicle granulosa cells.

This dual location of 3β -hydroxy steroid dehydrogenase within the endoplasmic reticulum may indicate the existence of two or more subcellular pools of progesterone. Dimino (1977) suggested that the progesterone produced by mitochondria containing an active 3β -hydroxy steroid dehydrogenase would be more likely to be secreted from the cell, whereas if progesterone was formed on the endoplasmic reticulum, the close proximity of other steroidogenic enzymes would tend to favour its further metabolism. In the case of the fowl ovarian granulosa cells, although there is no intramitochondrial 3β -hydroxy steroid dehydrogenase, perhaps the dehydrogenase that sediments with the mitochondria behaves similarly *in vivo* to the mitochondrial 3β -hydroxy steroid dehydrogenase discussed by Dimino (1977). Progesterone produced by this enzyme would then be available for secretion.

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