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Spectral Properties, Respiratory Activity, and Enzyme Systems of Bovine Corpus Luteum Mitochondria*

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ABSTRACT: Mitochondria isolated from bovine corpora lutea were shown to oxidize a number of tricarboxylic acid cycle substrates coincident with the conversion of cholesterol into pregnenolone.

The oxidative and phosphorylative capacities of these mitochondria were similar to those observed with heart and liver. With malate as substrate, respiratory control ratios exceeded 4 and ADP/O ratios approached 3. By contrast, mitochondria isolated from ovarian interstitial tissue had very low respiratory activity which was not stimulated by electron-donor substrate, ADP, or uncouplers of oxidative phosphorylation. Difference spectra of corpus luteum mitochondria demonstrated the presence of cytochromes *b*, *c* + c_1, and *a* + a_3. The presence of a carbon monoxide binding pigment with absorption maximum at 448 nm (cytochrome P-450) was confirmed, and its molar ratio to cytochrome *a* (+a_3) was 0.8:1. Data on steroid biosynthesis by corpus luteum mitochondria support the concept of interaction of the conventional cytochrome-containing respiratory chain with the cytochrome P-450 containing cholesterol side-chain cleavage enzyme(s) (cholesterol oxygenase) system.

In the adrenal and corpus luteum, the conversion of cholesterol into pregnenolone is generally considered to be the slowest step in steroid synthesis and therefore an important site for expression or influence by control mechanisms. Both extra- and intramitochondrial controls are known. The former includes luteinizing hormone in the case of the corpus luteum and adrenocorticotropic hormone for the adrenal, both presumably acting through the intermediacy of 3′,5′-cAMP and perhaps the supply of cholesterol and/or its precursors. Intramitochondrial factors limiting the rate of steroid synthesis are the availability of oxygen, electrons for oxygen activation associated with steroid hydroxylation, suitable enzyme system(s) for supplying these electrons, and quite possibly the rate of efflux of pregnenolone (Koritz and Kumar, 1970). For the past few years we have studied several of the intramitochondrial factors controlling the conversion of cholesterol into pregnenolone and its inhibition. We have sought to understand the relationship between the mitochondrial respiratory chain enzymes and the steroid hydroxylation pathway as well as the influence of various inhibitors on the molecular mechanism of steroid hydroxylation. This is the first of a series of publications dealing with these topics.

In the corpus luteum, unlike the adrenal cortex, the only steroid mixed-function oxidase present in the mitochondrial fraction is cholesterol oxygenase which carries out side-chain oxygenation and cleavage (Ichii *et al.*, 1963). Although less well studied, the cholesterol oxygenase system in the corpus luteum appears to be identical with the corresponding enzyme complex in the adrenal (Sulimovici and Boyd, 1968a). Each requires a NADPH-specific flavoprotein dehydrogenase and oxygen, and is inhibited by carbon monoxide. Moreover, the components of this electron transport pathway are probably the same since, as Yohro and Horie (1967) have shown, bovine corpora lutea contain cytochrome P-450 localized to the mitochondria, and pig ovaries contain an adrenodoxin-like nonheme iron protein (Kimura and Ohno, 1968).

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Despite the availability of considerable information regarding the mitochondrial cholesterol oxygenase system, much less is known about the normal respiratory chain, the major electron and energy transport system in the corpus luteum. In the present communication we wish to present new information regarding the electron carriers of the bovine corpus luteum respiratory chain and to demonstrate the oxidative, phosphorylative, and respiratory control capacities of corpus luteum mitochondria. Certain of these observations have been presented elsewhere in preliminary form (McIntosh *et al.*, 1968).

Experimental Section

Preparation of Mitochondrial Fraction. Bovine ovaries or corpora lutea, shelled out of ovaries obtained within 1-hr slaughter, were collected on ice and transported to the laboratory. After removing the fibrous capsule, the tissue was minced with scissors, passed through a tissue press, and homogenized gently by hand in an all-glass Ten-Broeck homogenizer equipped with a loose-fitting pestle. All operations were performed at 0–4°. A 10% (w/v) suspension of the homogenate in isolation solution consisting of 300 mM sucrose, 1 mM EDTA (pH 7.45), and 0.5% bovine serum albumin (hereafter simply called albumin) was then centrifuged in a refrigerated International centrifuge at 750*g* for 10 min.[1] Next, the supernatant was centrifuged at 1000*g* for 10 min to remove any nuclear debris or unbroken cells unavoidably transferred in the initial step. The mitochondrial pellet was obtained following centrifugation at 8700*g* for 10 min in rotor 30 of the Spinco, Model L-2. After carefully removing the loosely adherent "fluffy" layer, the pellet was homogenized by hand with a Ten-Broeck homogenizer, diluted to one-half the original volume with fresh isolation solution, and centrifuged again at 8700*g* for 15 min. This final mitochondrial pellet was homogenized gently by hand in a small volume of 300 mM mannitol, adjusted to a protein concentration of 30–40 mg/ml, and kept at 4° until used.

Mitochondria prepared by this method were used in the respiratory and steroid biosynthesis studies, but in order to reduce the hemoglobin contamination, mitochondria for the spectrophotometric studies were isolated only from perfused corpora lutea. For these experiments the internal genitalia were removed intact and the ovary containing the corpus luteum was perfused through the uterine and ovarian arteries with 100–200 ml of solution consisting of isotonic saline and 1 mM EDTA (pH 7.45). Subsequent isolation of the mitochondrial fraction was as described above.

The supernatant obtained following centrifugation at 8700*g* was further centrifuged at 105,000*g* for 60 min and the pellet (microsomal fraction) saved. This pellet was homogenized by hand in isolation solution and centrifuged again at 105,000*g* for 60 min, and the washed microsomal pellet adjusted to a protein concentration of 10–20 mg/ml in 300 mM mannitol.

Analytical Procedures. Difference spectra were measured at room temperature (approximately 25°) in a Phoenix dual-wavelength scanning spectrophotometer according to the method of Chance and Williams (1955a). All spectral curves are presented as photographs of the original records. The concentration of cytochromes was determined using the reported molar extinction coefficients (Estabrook and Holowinsky, 1961; Omura and Sato, 1964).

Estimates of contamination of the mitochondrial fraction by elements of the endoplasmic reticulum were based on measurements of the cytochrome b_5 concentration determined from the NADH reduced minus oxidized difference spectrum of aerobic mitochondria. The spectrum was recorded immediately after adding 260 μM NADH (final concentration) to the experimental cuvet and an equal volume of buffer to the reference cuvet. Calculation of the concentration was made using an extinction coefficient of 163 cm^{-1} mM^{-1} for the absorbance increment $\Delta\epsilon$ 424–409 nm (Garfinkel, 1958). Although the mitochondrial pellets were rinsed carefully to remove as much of the fluffy layer as possible, and washed by recentrifuging, evidence of microsomal contamination was observed in every mitochondrial preparation examined. In some preparations the cytochrome b_5 concentration was as high as 10% of the cytochrome a ($+a_3$) concentration.

Hemoglobin contamination of the mitochondrial preparations was estimated from the carbon monoxide difference spectrum of aerobic mitochondria using an extinction coefficient increment ($\Delta\epsilon$ 421–407 nm) of 107 cm^{-1} mM^{-1} (Hosoya and Morrison, 1967). Only those mitochondrial preparations in which hemoglobin could not be detected spectroscopically were used for further spectroscopic studies.

Human hemoglobin solution was prepared by washing erythrocytes with isotonic saline, lysing in 20 mM Hepes[2] buffer at pH 7.45, and sedimenting the erythrocyte "ghosts" at 20,000*g*.

Mitochondrial protein was estimated by a biuret procedure (Gornall *et al.*, 1949) as modified by Szarkowska and Klingenberg (1963). Absorption at 578 nm due to mitochondrial lipids and other interfering substances was corrected for by subtracting the value obtained following addition of about 10 mg of KCN. Fatty acid and free and total cholesterol content of the mitochondrial fraction were measured as described in Levinson and MacFate (1956).

Oxygen Utilization. Oxygen uptake was measured with a Clark membrane-covered, oxygen electrode sealed in a fixed volume (2.5 ml), magnetically stirred Lucite cuvet. The polarographic measurements were performed at room temperature (approximately 25°) in air-saturated isotonic buffer solution which contained 300 mM mannitol, 0.1 mM EDTA, 2.5 mM potassium phosphate buffer (pH 7.45), and 5 mM Hepes buffer (pH 7.45). Additions were made in 5- to 10-μl aliquots. The final concentration of ethanol did not exceed 1% in any experiment.

Mitochondria Incubations and Steroid Extraction. Cholesterol side-chain cleavage (cholesterol oxygenase) activity was assayed *in vitro* by determining conversion rates of endogenous and added cholesterol to pregnenolone and progesterone. Bovine corpus luteum mitochondria, suspended in 2.5 ml of buffer solution, were incubated in 25-ml erlenmeyer flasks at 37° for 30 min with continuous shaking. Additions were made to the flasks in the following order: [7α-^{3}H]cholesterol (10^6 dpm/flask) in 50 μl of propylene glycol, albumin at a final concentration of 0.03% (w/v), $MgSO_4$ and ADP at final concentrations of 2 and 0.4 mM, respectively, about 30 mg of mitochondrial protein, and finally buffered

[1] All centrifugal forces reported are average values computed at the center of the sample tube.

[2] Abbreviation used is: Hepes, *N*-2-hydroxyethylpiperazine-*N*-2-ethanesulfonic acid.

mannitol (pH 7.4) to the final volume. Where indicated, electron donor substrates and gas phases were used as stated. The reaction was terminated by adding 0.2 ml of glacial acetic acid and freezing. [4-^{14}C]Pregnenolone and [4-^{14}C]progesterone were added in tracer amounts to the incubation solutions which were alkalinized to 0.2 N with 3 N NaOH before extracting three times with three volumes of diethyl ether. Extracts were washed twice with 2 ml of water and concentrated under N_2, and the residues suspended in 10 ml of 70% methanol overnight at −20°. Subsequently, the methanolic solutions were centrifuged at 3000 rpm for 30 min; the resulting supernatant was evaporated under N_2 and made up to 5 ml with water and the steroids were reextracted with diethyl ether. Final extracts were taken to dryness under N_2 and residues were chromatographed on 250-μ layer of silica gel GF in two dimensions as described by Armstrong *et al.* (1964). Preparatory thin-layer chromatography on 500-μ silica gel GF layer in *n*-hexane–ethyl acetate (5:2, v/v) system was used to remove contaminating [^{3}H]cholesterol from the pregnenolone and progesterone. Steroids were eluted from silica gel with absolute ethanol. Progesterone was estimated spectrophotometrically at 240 nm and pregnenolone was measured according to Oertel and Eik-Nes (1959). Radioactivity associated with these steroids was estimated by scintillation counting and the data were corrected for recovery losses. Average recoveries of [^{14}C]pregnenolone and [^{14}C]progesterone were 70% (n = 32) and 66% (n = 37), respectively.

Identity and Radiochemical Purity of [^{3}H]Pregnenolone and [^{3}H]Progesterone. To identify the radioactive products of the cholesterol oxygenase reaction, steroid extracts from five replicate incubations were pooled, purified by a single two-dimensional thin-layer chromatography, diluted with 20 mg of the appropriate authentic steroid, and recrystallized four times from different solvent systems to constant specific activity. To ensure sufficient pregnenolone, 3′-5′-cAMP (1 mM), an inhibitor of pregnenolone conversion into progesterone (Koritz *et al.*, 1968), was included in the incubation solution. Since about half of the radioactivity appeared to be due to substrate and/or by-products, both preparatory and analytical chromatographies were used routinely in all other quantitative experiments.

Materials. [7α-^{3}H]Cholesterol (9.9 Ci/mmole), [4-^{14}C]-pregnenolone (52.4 mCi/mmole), and [4-^{14}C]progesterone (22 mCi/mmole) were purchased from New England Nuclear, Boston, Mass. Radioactive steroids were purified before use by thin-layer chromatography in benzene–ethyl acetate (4:1, v/v) system. Authentic pregnenolone, progesterone, and 20β-hydroxypregn-4-en-3-one were obtained from Steraloids, Inc., Pawling, N. Y. Antimycin (*Streptomyces kitazawanensis*), citric acid cycle intermediates, NADH, NADPH, 3′,5′-cAMP, and ADP were obtained from Sigma Chemical Corp. Redistilled solvents for chromatography were obtained from Curtis Co., Wilmington, Mass. Rotenone was purchased from Aldrich Chemical Co., recrystallized from ethanol–chloroform (4:1, v/v) in order to remove ultraviolet-absorbing contaminants, and stored in the dark prior to use.

Results

Lipid Content of Bovine Corpus Luteum Mitochondria. Samples of freshly prepared mitochondria were extracted and the isolated lipid compounds were measured. The sterol and fatty acid contents of mitochondria prepared from a pool of 12–14 corpora lutea were as follows: cholesterol (total and free), 75.1 and 37.1 μg per mg of protein, fatty acids, 342 ± 11 (standard error of the mean) μg/mg of protein.

TABLE I: Oxidative, Phosphorylative, and Respiratory Control Capacities of Bovine Corpus Luteum Mitochondria.[a]

Substrate	Oxygen Uptake[b] State 4 (−ADP)	State 3 (+ADP)	ADP/O	Respiratory Control[c]
Succinate (10 mM)	21	37	1.6	1.8
+ rotenone (10 μM)	20	45	1.8	2.3
L-Malate (10 mM)	3	13	2.7	4.3
	2	14	2.6	7.0
DL-Isocitrate	2	11	2.5	5.5
(10 mM)	3	10	2.4	3.3
NADH (100 μM)	<1			
NADPH (115 μM)	<1			

[a] In each experiment a 0.5-ml aliquot of mitochondria (11.2 mg of protein) was added to 2.0 ml of buffer solution. All reaction conditions were similar to those in Figure 1. The state 3 respiratory rate was measured following successive additions of 140 μM ADP. The state 4 respiratory rate was measured following consumption of the added ADP. [b] Rates of oxygen uptake in nanoatoms per minute per milligram of protein. [c] Respiratory control ratios were calculated as a ratio of the state 3 to state 4 respiratory rates as described by Chance and Hess (1959).

Pregnenolone (3β-hydroxypregn-5-en-3-one), progesterone (pregn-4-ene-3,20-dione), and 20β-hydroxypregn-4-en-3-one were present in the following concentrations: 0.05 ± 0.01, 0.37 ± 0.03, and 0.04 ± 0.1 (standard error of the mean) μg/mg of protein. These mitochondria contained large amounts of cholesterol. As will be demonstrated later, this endogenous cholesterol was readily converted to progesterone *in vitro*, successfully competing for the cholesterol oxygenase enzyme(s) with exogenous [^{3}H]cholesterol.

Respiratory and Phosphorylative Activities of Bovine Corpus Luteum and Ovarian Interstitial Tissue Mitochondria. As shown in Table I corpus luteum mitochondria were able to oxidize various tricarboxylic acid cycle intermediates, with succinate being oxidized at the highest rate. In agreement with the observations of Yago *et al.* (1967), NADPH was found not to be appreciably oxidized by fresh mitochondria, even in the presence of ADP. By contrast the respiratory rates with the other substrates were dependent on the presence (state 3) or absence (state 4) of added ADP. The state 3 respiratory rate of these mitochondria with succinate as substrate is comparable to that reported for bovine adrenocortical mitochondria (Cammer and Estabrook, 1967a). With the three Krebs' cycle intermediates tested, the state 4 oxygen uptake rate is considerably lower than that reported by Cammer and Estabrook (1967a) for adrenocortical mitochondria. These differences presumably reflect in part the biochemical intactness of the corpus luteum mitochondria used in this study and, in part, the lack of NADPH-dependent malate and isocitrate dehydrogenase activity in bovine corpus luteum mitochondria (see Užgiris *et al.*, 1971).

A typical polarographic trace is shown in Figure 1. With malate as substrate, addition of ADP caused a fourfold

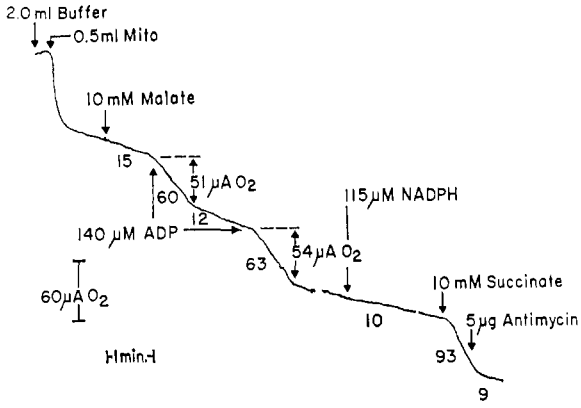

FIGURE 1: Illustration of respiratory control in bovine corpus luteum mitochondria. The buffer solution consisted of 300 mM mannitol, 0.1 mM EDTA, 2.5 mM phosphate buffer (pH 7.45), and 5 mM Hepes buffer (pH 7.45). Mitochondrial protein was 4.6 mg/ml; all subsequent additions are noted on the record. The numbers below the trace refer to the rate of oxygen utilization in microatoms of O_2 per minute. The ADP/O ratios were determined as described by Chance and Williams (1955b). Final volume, 2.5 ml. Room temperature, approximately 25°, expt BCL-671.

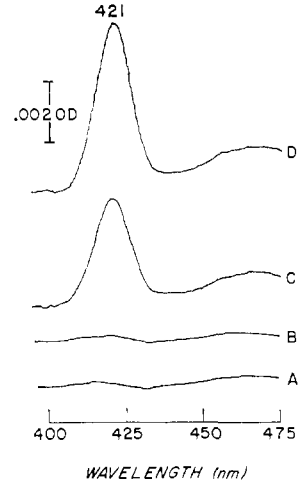

FIGURE 2: Detection of hemoglobin contamination in corpus luteum mitochondria. Both the experimental and reference cuvets contained mitochondria at a concentration of 2.7 mg of protein/ml of the same buffer solution described in Figure 1. Curve A is an aerobic minus aerobic base line. Curve B is the carbon monoxide aerobic minus aerobic difference spectrum produced by saturating the contents of the experimental cuvet with CO just prior to recording the spectrum. Curves C and D are the difference spectra resulting from the successive addition of 10-μl aliquots of a human hemoglobin solution (4.2 μM) to both the experimental and reference cuvet of curve B. Slit width, 0.2 mm; expt BCL-6717.

increase in the rate of respiration (state 3). After conversion of the added ADP into ATP, as indicated by the sharp cutoff in the polarographic trace, the respiratory rate returned to the low level indicative of the ADP-deficient state (state 4). These cyclic bursts of rapid respiration on adding ADP demonstrate the high respiratory control capacity of these mitochondria and are characteristic of intact, well-preserved mitochondria (Chance, 1956). As expected, the addition of NADPH produced no change in the oxidation rate. Had these mitochondria been permeable to exogenous NADPH, the rate of oxygen uptake would have increased. For this experiment, the calculated ADP/O ratios with malate were 2.9 and 2.6 and the respiratory control ratios, 5.0 and 6.3, respectively. As summarized in Table I a similar effect of ADP was observed with both succinate and isocitrate but not with NADPH and NADH.

For comparison, we attempted to isolate a mitochondrial fraction from bovine ovarian interstitial tissue which has only limited steroid synthesis capability. Exactly the same isolation procedure was used. Unlike the corpus luteum which breaks up very easily, interstitial tissue, consisting mostly of connective tissue elements, does not, and it is extremely difficult to homogenize even after passing through a tissue press. Centrifugation of the postnuclear supernatant at 8700*g* for 10 min yielded only a dull white pellet. Typically, the mitochondrial pellet isolated from heart or liver tissue is tan colored. After suspending the pellet in a small volume of 300 mM mannitol, aliquots were taken to measure the respiratory activity of these particles. Even at a concentration of 10 mg of protein/ml, more than twice the concentration of corpus luteum mitochondria used in Figure 1, the rate of oxygen uptake in the presence of succinate and ADP never exceeded 1–2 natoms of oxygen/min per mg of protein in any experiment. Moreover 50 μM dinitrophenol (DNP) did not stimulate this respiration nor was it inhibited by fresh 1 mM KCN.

When aliquots of the nuclear pellet, the light mitochondrial pellet (13,500*g* for 15 min), and the microsomal pellet (105,000*g* for 60 min) were examined polarographically, similar low, cyanide-insensitive rates of oxygen utilization were observed with each fraction. In view of these low rates, the respiratory activity of crude ovarian interstitial tissue homogenate was measured to ensure that the mitochondria were not being lost or damaged during the isolation procedure. Using 50- to 100-mg samples (wet weight) the average rate of oxygen uptake for six experiments was 8 natoms/hr per mg wet weight. Addition of either succinate or succinate and DNP to the interstitial tissue homogenate did not change the endogenous respiratory rate. In marked contrast the endogenous rate for corpus luteum homogenate was 27 while in the presence of both succinate and DNP the rate increased, on the average, to 40 natoms/hr per mg wet weight. These studies suggest that the cells of bovine ovarian interstitial tissue, unlike those of the corpus luteum, contain relatively few mitochondria. In further support of this conclusion these fractions were found to be spectroscopically devoid of mitochondrial cytochrome $a + a_3$ but contained varying levels of cytochrome b_5, presumably of microsomal origin.

Nature of the Electron Carriers of Bovine Corpus Luteum Mitochondria. Because the absorption bands of hemoglobin and the respiratory chain cytochromes tend to overlap, hemoglobin contamination of the mitochondrial fraction, even in small amounts, distorts the appearance of the absorption spectra and makes quantitation of the individual cytochromes nearly impossible. To avoid this problem, only perfused corpora lutea (see Experimental Section), grossly free of red blood cells, were used. This treatment was usually sufficient to reduce hemoglobin contamination in the isolated mitochondrial fraction to spectrophotometrically undetectable levels. But, as a final check the carbon monoxide aerobic minus aerobic difference spectrum of each mitochondrial preparation was examined. Figure 2 demonstrates the sensitivity of this procedure to detect and measure hemoglobin contamination. After dividing the dilute suspension of aerobic mitochondria equally between the two cuvets, the base line of equal light absorption (curve A) was recorded by

scanning the Soret region (500–400 nm).[3] Following this, the experimental cuvet was saturated with carbon monoxide and the wavelength interval rescanned (curve B). If, as in this case, carbon monoxide treatment produced no change in the base line, the mitochondrial preparation was considered to be spectroscopically free of hemoglobin. Curves C and D were recorded following successive 10-μl additions of fresh human hemoglobin solution (4.2 μM) to each of the cuvets (final concentration 17 and 33 nM) used to obtain curve B. In our hands the lower limit of hemoglobin measurable in turbid solutions by this method is 5 nM; however, evidence of hemoglobin contamination is detectable at about one-half this concentration. Since the concentration of any one of the cytochromes in liver mitochondria is at least 200 times greater than this (Estabrook and Holowinsky, 1961), spectral studies should not be appreciably affected by hemoglobin contamination of this degree.

The succinate anaerobic minus aerobic difference spectrum of bovine corpus luteum mitochondria is shown in Figure 3 (solid-line curve). To obtain this spectrum, first, the base line of equal light absorption was recorded. Next, succinate was added to the experimental cuvet and following utilization of the dissolved oxygen the difference spectrum was recorded. This spectrum exhibits the cytochrome absorption bands characteristic of mitochondria isolated from other tissue. The α-absorption band of cytochrome a ($+a_3$) is at 604 nm, that of cytochrome $c + c_1$ at 551 nm, and the shoulder at 562 nm represents the α band of cytochrome b. In the Soret region the γ-band of cytochrome a_3 ($+a$) is located at 444 nm with the shoulders at about 430 and 420 nm representing the γ bands of cytochromes b and $c + c_1$, respectively. The symmetry of the α band of cytochrome a ($+a_3$) at 604 nm together with the presence of a single Soret peak at 444 nm provide added proof that hemoglobin contamination has been reduced to spectroscopically undetectable levels.

The dashed-line curve (Figure 3) was observed after bubbling the experimental cuvet, containing the reduced cytochromes, with carbon monoxide for 2 min. Notable features of this carbon monoxide anaerobic minus aerobic difference spectrum are the presence of a small shoulder at about 590 nm, due to the absorption band of the newly formed cytochrome $a_3 \cdot CO$ complex (Yonetani, 1960), and the splitting of the single Soret peak into two peaks with absorption maxima at 431 and 448 nm. The peak at 431 nm represents the combined absorbance of the γ band of cytochrome b and that due to the $a_3 \cdot CO$ complex which has an absorption maximum at about 430 nm (Yonetani, 1960). The peak at 448 nm, on the other hand, has been observed only in those mitochondrial and microsomal fractions prepared from animal tissues which contain the carbon monoxide binding pigment, cytochrome P-450 (Harding and Nelson, 1966; Klingenberg, 1958; Cooper *et al.*, 1965). Demonstration of cytochrome P-450 in these mitochondria confirms the findings of Yohro and Horie (1967). In the same communication these authors also reported that the carbon monoxide anaerobic minus anaerobic difference spectrum, obtained by adding dithionite to both cuvets, had two Soret peaks, one at 450 and the other at 420 nm. By contrast in the present study the carbon monoxide anaerobic minus anaerobic difference spectrum (not shown) exhibited only a single Soret peak at about 450 nm. As suggested by them (Yohro and Horie, 1967) the peak at 420 nm was probably due to hemoglobin contamination and not to the presence of P-420, the enzymatically inactive form of P-450 (Ichikawa and Yamano, 1967).

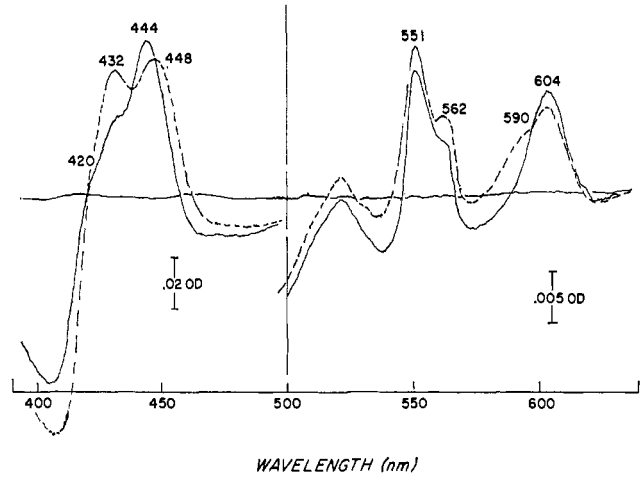

FIGURE 3: Corpus luteum mitochondrial anaerobic minus aerobic and carbon monoxide anaerobic minus aerobic difference spectra. Experimental conditions were as described in Figure 2. The solid-line curve is the anaerobic minus aerobic difference spectrum. Succinate (10 mM) was added to the experimental cuvet, an equal volume of solvent, water, to the reference cuvet, and the spectrum recorded after the experimental cuvet had become anaerobic. The dashed-line curve is the carbon monoxide anaerobic minus aerobic difference spectrum and was produced by saturating the experimental cuvet which contained the reduced respiratory chain cytochromes and P-450 with carbon monoxide; expt BCL-6717.

As shown previously in Figure 2 (curves A and B), saturating the experimental cuvet, containing aerobic mitochondria, with carbon monoxide induced no spectral changes. Since only reduced cytochrome P-450 can complex with carbon monoxide, in the absence of exogenous electron donor apparently little, if any, of the P-450 present in these mitochondria exists in a form capable of interaction with carbon monoxide.

From additional spectral studies of this nature the concentration of the individual respiratory chain cytochromes and P-450 was determined. These results are summarized in Table II. For each respiratory chain cytochrome the concentration was calculated by determining the absorbance difference between the measure (cytochrome peak) and reference wavelength and applying the appropriate difference extinction coefficient. Cytochrome P-450 concentration in both the mitochondrial and microsomal fractions was determined from carbon monoxide anaerobic–anaerobic difference spectra using dithionite as the reducing agent. The concentration of the respiratory chain cytochromes in bovine corpus luteum mitochondria compares favorably with those listed in Table II for adrenocortical mitochondria (Cammer and Estabrook, 1967b), as well as being in close agreement with the values reported by Estabrook and Holowinsky (1961) for rat liver mitochondria. Our value of 0.18 nmole of P-450/mg of mitochondrial protein compares well with the value of 0.17 nmole of P-450/mg of protein calculated from the spectral data of Yohro and Horie (1967). By contrast the concentration of cytochrome P-450 in bovine adrenocortical mitochondria is seven to eight times greater (Cammer and Estabrook, 1967; Harding and Nelson, 1966). The major reason for this difference is probably due to the absence

[3] The most intense absorption bands for oxy- and carboxyhemoglobin, the γ bands, are located at 407 and 421 nm in the carbon monoxide difference spectrum (Hosoya and Morrison, 1967).

TABLE II: Concentration of Mitochondrial Respiratory Chain Cytochromes and P-450 and of Microsomal P-450 in Bovine Corpus Luteum.[a]

Component	Wavelength Pairs (nm) ($\lambda_{measure} - \lambda_{ref}$)	$\Delta\epsilon$ (mM^{-1} cm^{-1})	Content (nmoles/mg of Protein)		
			Corpus Luteum[b] Mean ± Std Dev, $n = 7$	Corpus Luteum	Adrenal
Mitochondrial fraction					
Cytochrome *a* ($+a_3$)	605–630	16	0.23 ± 0.03		0.23[d]
b	562–575	20	0.15 ± 0.03		0.17[d]
$c + c_1$	551–540	19	0.35 ± 0.02		0.29[d]
P-450·CO	450–490	91	0.18 ± 0.08	0.17[c]	1.5[d]
Microsomal fraction					
Cytochrome P-450·CO	450–490	91	0.07 ± 0.02	0.03[c]	

[a] The concentration of the mitochondrial respiratory chain cytochromes was determined from succinate anaerobic minus aerobic difference spectra using the indicated molar extinction coefficients (Estabrook and Holowinsky, 1961). Mitochondrial and microsomal cytochrome P-450 was determined from carbon monoxide anaerobic minus anaerobic difference spectra. In calculating the concentration of cytochrome P-450 the molar extinction coefficient determined by Omura and Sato (1964) was used. [b] These values are the means of seven experiments with one standard deviation. [c] Values calculated from the data of Yohro and Horie (1967). [d] Data of Cammer and Estabrook (1967b).

TABLE III: Cholesterol Side-Chain Cleavage in Bovine Corpus Luteum Mitochondria.[a]

Additions	Pregnenolone		Progesterone		% Conversion of ^3H
	μg/mg of Protein ± SEM	dpm/mg of Protein ± SEM	μg/mg of Protein ± SEM	dpm/mg of Protein ± SEM	
Control (zero time)	0.09 ± 0.05		0.09 ± 0.01		
None	nm	41 ± 6	0.47 ± 0.01	291 ± 22	0.01
Succinate (10 mM)	0.32 ± 0.04	1,440 ± 22	1.19 ± 0.10	4,230 ± 103	14.8
L-Malate (10 mM)	0.14 ± 0.07	1,120 ± 92	1.22 ± 0.04	3,970 ± 217	13.2
DL-Isocitrate (10 mM)	0.20 ± 0.01	1,160 ± 87	1.12 ± 0.05	3,310 ± 591	11.6
NADPH (1 mM)	nm	19 ± 3	0.66 ± 0.02	830 ± 64	3.06
Ca^{2+} (11 mM)	nm	125 ± 5	0.77 ± 0.30	270 ± 86	0.55
Ca^{2+} (11 mM) + NADPH (11.5 mM)	nm	158 ± 25	4.55 ± 0.15	10,350 ± 42	12.0

[a] Mitochondria were incubated with [7α-^{3}H]cholesterol, 10^6 dpm (17.8 ng) for 30 min, under 95% O_2 + 5% CO_2. Steroids were extracted, separated, and measured as described in text. Values were corrected for recovery losses and are presented as means of three separate incubations. [b] nm, nonmeasurable, *i.e.*, concentration less than 0.02 μg/mg of protein incubated.

of the very active 11β- and 18-hydroxylases in corpus luteum mitochondria.

In the present investigation in contrast to that of Yohro and Horie (1967), no attempt was made to separate the so-called light mitochondrial fraction from the microsomal fraction. Presumably, this difference in technique accounts for the higher concentration of cytochrome P-450 in the microsomal fraction reported here.

Steroid Synthesis in Bovine Corpus Luteum Mitochondria. Previous studies have amply demonstrated that in both the adrenal and corpus luteum the cholesterol oxygenase enzymes are localized in the mitochondrial fraction. In the present study the enzymatic assay of this mixed-function oxidase system is based not only on the conversion of [^{3}H]cholesterol to pregnenolone and progesterone but also on the accumulation of these steroids derived from endogenous sources.

As mentioned previously, freshly isolated corpus luteum mitochondria contained large amounts of cholesterol. Progesterone and pregnenolone levels, on the other hand, were much lower, while only trace amounts of 20β-hydroxypregn-4-en-3-one were measured. After a 30-min incubation the main radioactive product identified was [^{3}H]progesterone along with smaller amounts of [^{3}H]pregnenolone (Table III). During the same interval progesterone mass increased proportionally indicating a rapid synthesis from endogenous sources as well. Because the level of 20β-hydroxypregn-4 en-3-one did not change, even after incubation, only data for progesterone and pregnenolone are presented.

The data in Table III demonstrate that mitochondrial steroid synthesis was largely dependent on the availability of reducing equivalents derived from oxidation of the citric acid intermediates. As expected from reports in the literature

(*e.g.*, Yago *et al.*, 1967), steroid synthesis with NADPH alone was minimal because of the relative impermeability of these intact mitochondria to external NADPH. With the addition of Ca^{2+}, which induces maximal swelling of the mitochondria, this permeability barrier was destroyed. Under these conditions NADPH now readily supported cholesterol conversion to progesterone.

Since progesterone was the main product recovered in these incubations, it must be assumed that the mitochondrial fraction contained sufficient Δ^5-3β-hydroxysteroid dehydrogenase–isomerase to convert most of the pregnenolone into progesterone. However, we were unsuccessful in our attempts to determine whether the hydroxysteroid dehydrogenase–isomerase activity resulted from microsomal contamination or actually represented localization of this pair of enzymes to the mitochondrial fraction as suggested by Sulimovici and Boyd (1968b).

Discussion

In the present study, bovine corpus luteum mitochondria were shown to contain two types of electron transfer systems—a conventional, cytochrome-containing respiratory chain, and a second system containing cytochrome P-450. The conventional system consists of cytochromes *b*, $c + c_1$, and $a + a_3$ in a ratio similar to that present in mitochondria from other tissues (Cammer and Estabrook, 1967b; Estabrook and Holowinsky, 1961). This system is supported by reducing equivalents derived from the oxidation of NADH- and succinate-linked substrates. The high respiratory control ratios (Table I) obtained with the various electron donor substrates, and most critically, the inability of NADH and NADPH to stimulate oxygen utilization attest to the biochemical "intactness" of these mitochondria. Furthermore, since the ADP/O ratios with malate and succinate approach the theoretical maxima of 3 and 2, respectively, for these substrates, corpus luteum mitochondria, like heart and liver mitochondria, probably contain the normal complement of phosphorylation sites.

The second electron transfer system present in these mitochondria supplies reducing equivalents to drive the cholesterol oxygenase reaction. In this system reducing equivalents derived from intramitochondrial NADPH are transferred to cytochrome P-450, the terminal enzyme in this system, and by a still incompletely understood mechanism are utilized in the side-chain cleavage of cholesterol. The nature of the components of this system is not known in the same detail as is the adrenocortical mitochondrial steroid 11β-hydroxylase system. However, each has the characteristics of a mixed-function oxidase requiring a NADPH-specific flavoprotein dehydrogenase and oxygen and being inhibited by carbon monoxide. Therefore, it is probable that the bovine corpora luteal system is similar to that described for the adrenal (Sulimovici and Boyd, 1968a). This conclusion is further strengthened by the recent observation by Kimura and Ohno (1968) that pig ovaries contain an adrenodoxin-like nonheme iron protein.

Previous authors (Yago *et al.*, 1967) have stressed the importance of the failure of exogenous NADH or NADPH to serve as a suitable electron donor for either oxidative phosphorylation or cholesterol oxygenase activity in intact mitochondria. As these authors demonstrated, there is little evidence to support the concept of a "shuttle" mechanism such as 3-hydroxybutyrate dehydrogenase to make the extramitochondrially generated NADPH available to the mitochondrial respiratory enzymes. Thus, for enhanced extramitochondrial NADPH production to be an important source of reducing equivalents for mitochondrial respiration or cholesterol oxygenase, there must remain some unknown, intervening process which controls the penetration of NADPH into intact mitochondria. By contrast the evidence that various Krebs cycle intermediates (Table III) can support cholesterol oxygenase activity suggests that in corpus luteum mitochondria these substrates are not only supplying electrons for oxidative phosphorylation *via* the conventional respiratory chain, but also being utilized by the cytochrome P-450 containing cholesterol oxygenase system.

Savard and Telegdy (1965) reported that progesterone and 20β-hydroxypregn-4-en-3-one were the end products in steroid synthesis by homogenates of bovine corpora lutea. This implies that bovine corpora lutea lack steroid 17α-hydroxylase and 17,20 lyase activity. Since these two enzymes are the most important ovarian microsomal steroid mixed-function oxidases, the presence of small but significant levels of cytochrome P-450 in the microsomal fraction requires explanation. It seems to us that some, if not all, of this P-450 represents contamination by lighter sedimenting fragments of broken mitochondria. Certainly, the slightly higher microsomal P-450 levels found by us in contrast to Yohro and Horie (1967) were due to differences in isolation techniques, *i.e.*, in the present study no attempt was made to separate off the so-called light mitochondrial fraction from the microsomal fraction.

Contrary to our expectation the main product formed from mitochondrial incubations with [^{3}H]cholesterol was progesterone although small amounts of pregnenolone and 20β-hydroxypregn-4-en-3-one were present. Cheatum *et al.* (1966) reported that in bovine corpora lutea the Δ^5-3β-hydroxysteroid dehydrogenase and isomerase activity which convert pregnenolone to progesterone was localized to the microsomal fraction. On the other hand, Sulimovici and Boyd (1968b) were unable to prepare mitochondria devoid of this activity from luteinized rat ovaries despite washing the mitochondrial pellets several times in different ways and preparing the mitochondria in two different ways, and, on the basis of electron microscopic examination of the mitochondrial pellet, which showed it to be essentially mitochondria, they concluded that mitochondria from luteinized rat ovaries contain Δ^5-3β-hydroxysteroid dehydrogenase and isomerase activity. Since we were never able to completely rid the mitochondrial pellet of cytochrome b_5, despite careful rinsing and repeated washing of the pellets in fresh isolation solution (see Experimental Section), the mitochondrial preparations used in our study obviously were contaminated by elements of the endoplasmic reticulum. Thus from our studies no conclusion can be drawn as to whether or not this enzyme system is localized to the microsomal fraction or both fractions. However, because of the inherent difficulties in correctly identifying and accounting for all of the elements in an electron micrograph of a mitochondrial pellet, it is doubtful that this technique can be considered a quantitative measure of purity.

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Role of Reversed Electron Transport in Bovine Corpus Luteum Mitochondrial Steroid Synthesis*

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ABSTRACT: Evidence for reversed electron transport in bovine corpus luteum mitochondria involving cholesterol side-chain cleavage (cholesterol oxygenase) was obtained from polarographic and steroid biosynthesis studies. Unlike mitochondria from bovine adrenal cortex, mitochondria from the corpus luteum displayed antimycin-sensitive, malate-supported oxygen consumption. Furthermore, cholesterol oxygenase activity supported by succinate, L-malate, and DL-isocitrate was significantly blocked by the respiratory chain inhibitors, rotenone, antimycin, and amobarbital, but activity supported by NADPH was inhibited only by amobarbital. The effect of these inhibitors indicates that the normal cytochrome-containing respiratory chain must transmit reducing equivalents directly to the cytochrome P-450 containing cholesterol oxygenase electron transport chain. Accordingly, it is proposed that reduction of intramitochondrial $NADP^+$ in bovine corpus luteum occurs almost exclusively *via* reversed electron transport coupled to an energy-dependent $NAD(P)^+$ transhydrogenase. In addition, it is unlikely that mitochondria $NADP^+$-specific malate and isocitrate dehydrogenases supply reducing equivalents for cholesterol oxidation in corpus luteum mitochondria.

Bovine corpus luteum mitochondria offer a unique system for the study of endogenous control of the side chain cleavage of cholesterol (cholesterol oxygenase; McIntosh *et al.*, 1971). Unlike the adrenal cortex, which contains at least three mitochondrial steroid mixed-function oxidases utilizing cytochrome P-450 in addition to the conventional respiratory chain enzymes (Simpson and Estabrook, 1969), corpus luteum mitochondria contain only cholesterol oxygenase (Sulimovici and Boyd, 1968). Thus, the corpus luteum provides the opportunity to study the interrelationships of these two electron transport systems, the cholesterol oxygenase and respiratory chain enzymes, without interference in the key steroidogenic reaction(s) leading to pregnenolone synthesis.

The source of intramitochondrial reduced nicotinamide adenine dinucleotide necessary to support steroid hydroxylation reactions has been extensively studied in adrenocortical mitochondria and several possible alternatives have been proposed. Intramitochondrial NADPH could be generated by: (1) oxida-

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