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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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tion of citric acid cycle intermediates in conjunction with energy-dependent reversed electron transport coupled to $NAD(P)^+$ transhydrogenase activity (Harding *et al.*, 1965) or by (2) mitochondrial $NADP^+$-specific dehydrogenases (Purvis *et al.*, 1968; Grant, 1956) or through (3) the coupling of cytoplasmic and mitochondrial $NADP^+$-specific malic enzyme activities, the so-called malate shuttle (Simpson and Estabrook, 1969).

In corpus luteum homogenates several $NADP^+$-specific dehydrogenase activities have been demonstrated; however, the subcellular localization of these enzymes was not reported (Savard *et al.*, 1963). Therefore, the important question remains: Is mitochondrial cholesterol oxygenase dependent on a normally functioning respiratory chain as would be the case if reduction of $NADP^+$ depended solely on energy-linked transhydrogenase activity, or do the two electron transfer systems, *i.e.*, the cytochrome-containing respiratory chain and the P-450-containing cholesterol oxygenase system, diverge prior to the first site of energy conservation and consequently function more or less independently?

The report that rotenone does not block succinate- or malate-supported steroid 11β hydroxylation in bovine adrenocortical mitochondria (Harding *et al.*, 1968) raises one of the most critical objections to the interpretation that mitochondrial NADPH is generated by reversed electron transport coupled to an energy-dependent transhydrogenase. Furthermore, antimycin, at a concentration sufficient to inhibit respiration, does not inhibit steroid 11β hydroxylase activity supported by malate (Harding *et al.*, 1965). This finding has been taken as additional evidence against energy-linked reversed electron transport as a significant source of NADPH for mitochondrial steroid hydroxylations.

In contrast, the present studies support the idea of reversed electron transport by demonstrating that it is an important, if not the only, $NADP^+$-reducing pathway in corpus luteum mitochondria, since inhibition of reversed electron transport resulted in significantly decreased cholesterol oxygenase activity. In addition, it is suggested that amobarbital acts not only to inhibit the conventional respiratory chain enzymes, but also the cytochrome P-450 containing cholesterol oxygenase system. Certain of these observations have been presented in preliminary form (Užgiris *et al.*, 1969).

Experimental Section

Isolation of bovine corpus luteum mitochondria, analytical procedures, respiratory studies with the Clark oxygen electrode, and assay of cholesterol side-chain cleavage (cholesterol oxygenase) activity utilized methods previously described (McIntosh *et al.*, 1971). The incubation medium contained an electron donor (citric acid cycle intermediate or NADPH), one of the respiratory inhibitors, and cofactors as described in Table I. The mitochondrial respiratory chain inhibitors, amobarbital, antimycin, and rotenone, were dissolved in absolute ethanol and added in 10-μl aliquots to the incubation flasks. In all experiments the concentration of inhibitor was sufficient to block succinate- or NADH-linked substrate oxidation *via* the conventional respiratory chain (Slater, 1967). Amobarbital was used at a concentration of 4.6 mM, a concentration that allows a half-maximal rate of energy-controlled transhydrogenase (Harding *et al.*, 1968). Although the concentration of rotenone used (1 μg/mg of mitochondrial protein incubated) exceeded that needed to block respiratory chain mediated NADH-linked substrate oxidation, this concentration does not inhibit malate-supported adrenal mitochondrial 11β hydroxylation (Harding *et al.*, 1968). The concentration of antimycin was approximately 0.5 μg/mg of mitochondrial protein, *i.e.*, a concentration which is sufficient to provide maximal inhibition of the conventional respiratory chain enzymes. This concentration, however, does not inhibit malate-supported steroid 11β hydroxylation in adrenal mitochondria (Cammer and Estabrook, 1967).

TABLE I: Composition of Cholesterol Oxygenase Assay Mixture.

	Amount	Vol (ml)
Mitochondria	30 mg of protein/ml	1.00
Mannitol (300 mM)–Hepes[a] (5 mM)–EDTA (0.2 mM)–phosphate buffer (2.5 mM, pH 7.4)		1.40
Bovine serum albumin	75 mg/ml	0.01
Magnesium sulfate	5 μmoles	0.01
ADP	0.625 μmole	0.01
Citric acid cycle intermediate	25 μmoles	0.02
(or NADPH)	(19 μmoles)	0.02
[7α-^{3}H]Cholesterol (in propylene glycol)	10^6 dpm	0.05

[a] Hepes, *N*-2-hydroxyethylpiperazine-*N*-2-ethanesulfonic acid.

Only three steroid products were found consistently after mitochondrial incubations: pregnenolone (3β-hydroxypregn-5-en-20-one), progesterone (pregn-4-ene-3,20-dione), and 20β-hydroxypregn-4-en-3-one. The latter was not treated as a product of *in vitro* incubation, since there was no significant increase in mass during incubation and only a trace of radioactivity was associated with the isolated steroid. Furthermore, in contrast to pregnenolone and progesterone, the mass and radioactivity of 20β-hydroxypregn-4-en-3-one did not change on incubation with the respiratory chain inhibitors or NADPH.

The cholesterol oxygenase activity was expressed as either net steroid synthesis in μg/mg of mitochondrial protein/30 min or % [^{3}H]cholesterol conversion/mg of mitochondrial protein per 30 min. Net synthesis was calculated by subtracting the total amount of endogenous C-21 metabolites (pregnenolone plus progesterone) at zero time from the content after 30-min incubation. Per cent [^{3}H]cholesterol conversion was calculated by summing disintegrations per minute of ^{3}H in both C-21 metabolites after 30-min incubation and dividing by disintegrations per minute of [^{3}H]cholesterol incubated. Per cent inhibition was calculated by dividing the net endogenous synthesis or per cent exogenous conversion in the presence of inhibitors by that measured in the control flasks and then subtracting the quotient expressed as per cent from 100%.

It will be noted that there are quantitative discrepancies between the mass of steroid produced and the amount of radioactive cholesterol converted into pregnenolone (Tables II–VI). A common explanation for such phenomena relate to the pool sizes and isotopic dilution factors for the individual preparations and the rates of equilibration of the added radioactive cholesterol with the endogenous pool(s). Furthermore, differential permeability of the mitochondrial membrane to the various inhibitors and the exogenous substrate

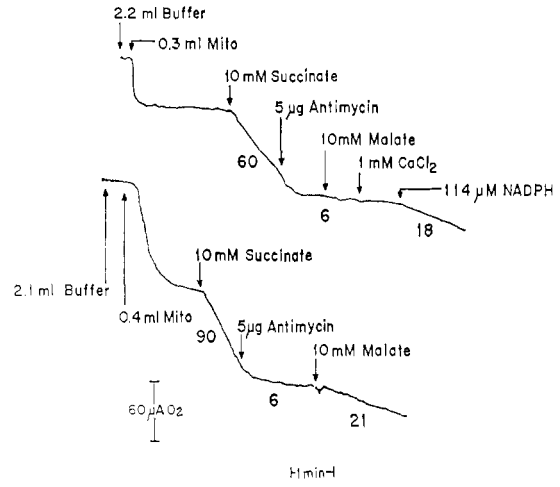

FIGURE 1: Effect of antimycin on the rate of oxygen utilization. Bovine corpus luteum (upper trace) and adrenal cortex mitochondria (lower trace), 4.3 and 3.2 mg of protein per ml, respectively, were diluted to a final volume of 2.5 ml with isotonic medium containing mannitol (300 mM), EDTA (0.1 mM), potassium phosphate (2.5 mM), and Hepes buffer (5 mM, pH 7.45). All subsequent additions were as noted. The numbers on the traces refer to the rate of oxygen utilization in microatoms of O_2 per minute. Room temperature, approximately 25°; expt BCL-666 and BA-6811.

would introduce a variable time factor in the inhibition of conversion of the exogenous substrate, especially as compared to endogenous substrate.

Results

Oxygen Utilization by Mitochondria Isolated from Bovine Corpus Luteum and Adrenal Cortex. Figure 1 is a typical polarographic trace demonstrating the different effect antimycin had on the rate of oxygen utilization in bovine corpus luteum and adrenocortical mitochondria. Although adrenocortical mitochondria had a higher respiratory rate than corpus luteum with succinate as electron donor substrate, both preparations were sensitive to added antimycin. In both cases, on adding antimycin O_2 consumption ceased, presumably as a result of electron transport inhibition in the cytochrome *b* region of the respiratory chain, resulting in decreased $NADP^+$ reduction through the energy-dependent transhydrogenase. Malate addition to adrenocortical mitochondria restored O_2 uptake *via* an antimycin-insensitive pathway, presumably involving $NADP^+$-linked malic dehydrogenase (Simpson and Estabrook, 1969). By contrast, mitochondria from the corpus luteum displayed no such resumption of respiration following the addition of malate to the antimycin-inhibited preparation. Respiration resumed only on addition of both $CaCl_2$ and NADPH. Presumably, this stimulation of O_2 utilization in the corpus luteum mitochondria is related to the conversion of endogenous cholesterol into pregnenolone. It is unlikely that the oxygen consumption noted after NADPH and $CaCl_2$ addition was related to oxidation of NADPH *via* the conventional respiratory chain since $NAD(P)^+$ transhydrogenase does not readily catalyze intramitochondrial NAD^+ reduction by NADPH (Krebs, 1967; Chance *et al.*, 1967). These observations indicate that mitochondria from bovine corpus luteum, unlike those from adrenal cortex, demonstrate antimycin-sensitive oxygen uptake when incubated with malate and presumably lack $NADP^+$-linked mitochondrial malate dehydrogenase activity.

TABLE II: Inhibition of NADPH-Supported Cholesterol Oxygenase Activity of Bovine Corpus Luteum Mitochondria.[a]

Additions		Pregnenolone		Progesterone		Steroid Synthesis (μg/mg of Protein)	% Conversion of ^3H	% Inhibition	
Electron Donor	Inhibitor	μg/mg of Protein ± SEM	dpm/mg of Protein ± SEM	μg/mg of Protein ± SEM	dpm/mg of Protein ± SEM			Of Mass	Of ^3H
None[c]	None	0.08 ± 0.01		0.42 ± 0.06					
Ca^{2+} only	None	nm[b]	148 ± 4	0.68 ± 0.01	130 ± 11	0.18	0.45		
Ca^{2+} and NADPH	None	nm	205 ± 16	5.58 ± 0.33	14,800 ± 1,080	5.08	24.1		
Ca^{2+} and NADPH	Amobarbital (4.6 mM)	0.09 ± 0.04	458 ± 33	2.10 ± 0.15	3,940 ± 548	1.69	6.87	66.7	71.5
Ca^{2+} and NADPH	Rotenone (25 μM)	0.26 ± 0.03	4200 ± 161	4.29 ± 0.21	10,300 ± 733	4.05	21.5	20.3	10.8
Ca^{2+} and NADPH	Antimycin (11 μM)	0.14 ± 0.01	2700 ± 161	5.17 ± 0.39	12,400 ± 571	4.81	24.4	5.31	0

[a] Mitochondria (approximately 12 mg of protein/ml) were incubated with [7α-^{3}H]cholesterol (10^6 dpm/flask), NADPH (7.5 mM), $CaCl_2$ (11 mM), and the indicated inhibitors at 37° for 30 min, under 95% O_2 and 5% CO_2. Steroids were extracted, separated, and measured as described in Experimental Section. Values were corrected for recovery losses and are presented as the means of three separate incubations. [b] nm, nonmeasurable, *i.e.*, concentration was less than 0.02 μg/mg of protein incubated. [c] Zero time control.

TABLE III: Citric Acid Cycle Intermediate Supported Cholesterol Oxygenase Activity of Bovine Corpus Luteum Mitochondria.[a]

Additions	Pregnenolone		Progesterone		20β-Hydroxypregn-4-en-3-one		%
	μg/mg of Protein ± SEM	dpm/mg of Protein ± SEM	μg/mg of Protein ± SEM	dpm/mg of Protein ± SEM	μg/mg of Protein ± SEM	dpm/mg of Protein ± SEM	Conversion of ^3H
Control (zero time)	0.05 ± 0.02		0.23 ± 0.00		0.05 ± 0.01		
None	nm[b]	41 ± 6	0.47 ± 0.01	291 ± 22	0.05 ± 0.01	16 ± 6	0.98
DL-Isocitrate (10 mM)	0.28 ± 0.03	1160 ± 57	2.29 ± 0.06	2040 ± 111	0.05 ± 0.01	9 ± 1	9.05
L-Malate (10 mM)	0.27 ± 0.12	998 ± 226	2.73 ± 0.20	2840 ± 327	0.05 ± 0.01	35 ± 7	10.9
Succinate (10 mM)	0.76 ± 0.02	1850 ± 112	2.54 ± 0.05	2040 ± 145	0.08 ± 0.02	46 ± 1	11.1

[a] Mitochondria from bovine corpus luteum were incubated with one of the citric acid cycle intermediates as indicated. Other conditions were as described in the legend of Table II. [b] nm, nonmeasurable, *i.e.*, concentration was less than 0.02 μg/mg of protein incubated.

Effect of Respiratory Chain Inhibitors on NADPH-Supported Cholesterol Oxidation. Harding and Nelson (1966) and Cammer and Estabrook (1967) demonstrated the presence in adrenocortical mitochondria of two electron transport systems—a conventional respiratory chain and a steroid hydroxylating pathway—which are probably interlinked. By analogy the two electron transport systems in corpus luteum mitochondria might be expected to function in a similar manner.

In the absence of electron donor substrate corpus luteum mitochondria converted little, if any, cholesterol to pregnenolone and progesterone (Table II). However, when incubated with various Krebs cycle intermediates or NADPH in the presence of $CaCl_2$, a swelling agent (Tables II and III), endogenous, as well as [^{3}H]cholesterol, was readily oxidized. As shown in Figure 2, on increasing the concentration of NADPH to 10 mM, cholesterol oxygenase activity increased more than 10-fold. With freshly prepared mitochondria, this response was observed only in the presence of 11 mM $CaCl_2$ which, by causing maximal mitochondrial swelling, destroyed the permeability barrier to exogenous NADPH.

The data demonstrating the effect of the three respiratory chain inhibitors on NADPH-supported cholesterol side-chain cleavage are presented in Table II. When antimycin was added at a concentration of 0.34 μg/mg of protein, a concentration which causes maximal inhibition of the conventional respiratory chain enzymes, cholesterol conversion was not significantly inhibited. Similarly, rotenone, an inhibitor of oxidative phosphorylation at site I in avian and mammalian mitochondria (Chance *et al.*, 1967), had little effect on NADPH-supported cholesterol oxidation by corpus luteum mitochondria. These results were anticipated on the assumption that NADPH-supported cholesterol oxidation involved only the direct transfer of reducing equivalents from NADPH to the P-450-containing electron transport system. In contrast, amobarbital inhibited cholesterol oxygenase activity significantly. This observation is in agreement with previous studies on bovine adrenocortical mitochondrial 11β hydroxylation (Harding *et al.*, 1968), but are at variance with those by Péron and McCarthy (1968), who were unable to demonstrate any effect of amobarbital on rat adrenal mitochondrial 11β hydroxylation of DOC in the presence of high levels of Ca^{2+} and NADPH.

Effect of Respiratory Chain Inhibitors on Cholesterol Oxygenase Activity Supported by Citric Acid Cycle Intermediates. The data presented in Table IV show that succinate-supported cholesterol side-chain cleavage was significantly inhibited ($p < 0.01$) on addition of any one of the respiratory chain inhibitors to the assay system. Although amobarbital was the most effective inhibitor of the three compounds tested, the degree of inhibition was no greater than that observed with NADPH as the electron donor substrate. Since amobarbital is known to inhibit a number of flavoprotein dehydrogenases (Ernster *et al.*, 1955), presumably including the $NADP^+$-specific flavoprotein dehydrogenase of the cholesterol side-chain cleavage system, in addition to the flavoprotein of the respiratory chain, one would have expected a greater degree of inhibition with succinate as the electron donor substrate. Because the degree of inhibition was no greater with succinate as electron donor, or, for that matter, with malate or isocitrate

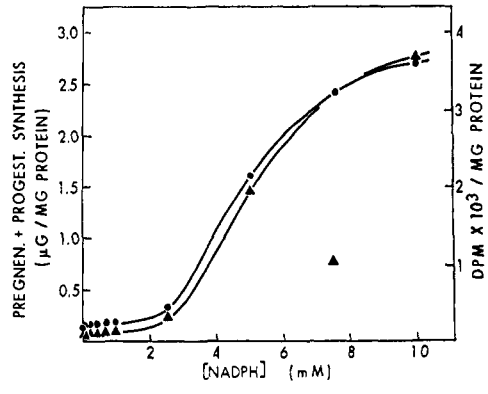

FIGURE 2: Effect of increasing concentration of NADPH on cholesterol oxygenase activity in mitochondria from bovine corpus luteum. Mitochondria (12 mg of protein/ml) were incubated with [7α-^{3}H]-cholesterol at 37° for 30 min, under 95% O_2 and 5% CO_2. Other additions were as shown in Table I. Pregnenolone and progesterone were measured as described under Experimental Section. Values were corrected for recovery losses and are presented as the results of individual incubations with the concentration of NADPH as noted. (▲) ^{3}H-labeled products; (●) unlabeled products.

TABLE IV: Succinate-Supported Cholesterol Oxygenase Activity of Bovine Corpus Luteum Mitochondria.[a]

Inhibitor	Pregnenolone		Progesterone		Steroid Synthesis (μg/mg of Protein)	% Conversion of ^{3}H	% Inhibition	
	μg/mg of Protein ± SEM	dpm/mg of Protein ± SEM	μg/mg of Protein ± SEM	dpm/mg of Protein ± SEM			Of Mass	Of ^{3}H
Expt 9 control (zero time)	0.15 ± 0.00		0.20 ± 0.00					
None	nm[b]	400 ± 38	1.13 ± 0.01	387 ± 27	0.78	3.11		
Amobarbital (4.5 mM)	nm	89 ± 9	0.69 ± 0.05	63 ± 13	0.34	0.63	56.4	79.7
Rotenone (25 μM)	nm	58 ± 6	0.92 ± 0.02	150 ± 17	0.57	0.82	26.9	73.6
Expt 10 control (zero time)	0.04 ± 0.01		0.60 ± 0.00					
None	1.12 ± 0.22	3540 ± 153	3.67 ± 0.10	3170 ± 130	4.15	13.2		
Antimycin (11 μM)	nm	228 ± 25	1.58 ± 0.03	538 ± 33	0.94	1.51	77.4	88.6

[a] Mitochondria from bovine corpus luteum were incubated with succinate (10 mM) and one of the inhibitors as marked. Values are presented as the means of four separate incubations. Other conditions were as described in the legend of Table II. [b] nm, nonmeasurable, *i.e.*, concentration less than 0.02 μg/mg of protein incubated.

TABLE V: L-Malate-Supported Cholesterol Oxygenase Activity of Bovine Corpus Luteum Mitonchondria.[a]

Inhibitor	Pregnenolone		Progesterone		Steroid Synthesis (μg/mg of Protein)	% Conversion of ^{3}H	% Inhibition	
	μg/mg of Protein ± SEM	dpm/mg of Protein ± SEM	μg/mg of Protein ± SEM	dpm/mg of Protein ± SEM			Of Mass	Of ^{3}H
Expt 5 control (zero time)	nm[b]		0.37 ± 0.01					
None	0.03 ± 0.01	516 ± 43	1.88 ± 0.03	1890 ± 135	5.25	1.54		
Amobarbital (4.6 mM)	nm	100 ± 20	0.87 ± 0.02	375 ± 54	1.10	0.50	67.5	79.1
Rotenone (25 μM)	nm	129 ± 30	0.55 ± 0.01	303 ± 37	0.94	0.18	88.3	82.1
Expt 7 control (zero time)	0.08 ± 0.03		0.31 ± 0.01					
None	0.53 ± 0.08	1380 ± 146	2.24 ± 0.06	2010 ± 132	9.92	2.38		
Antimycin (11 μM)	0.02 ± 0.00	329 ± 23	0.86 ± 0.02	409 ± 32	2.30	0.49	79.4	76.8

[a] Mitochondria from bovine corpus luteum were incubated with L-malate (10 mM) and one of the inhibitors as marked. Values are presented as the means of four separate incubations. Other conditions were as described in legend of Table II. [b] nm, nonmeasurable, *i.e.*, concentration less than 0.02 μg/mg of protein incubated.

(Tables V and VI, respectively), this suggests that the inhibitory action of amobarbital may not be related solely to its action on flavoprotein dehydrogenases.

The finding that succinate-supported cholesterol oxygenase activity was blocked by antimycin is expected. Several authors (Harding *et al.*, 1968; Cammer and Estabrook, 1967) have observed that in the adrenal, mitochondrial steroid 11β hydroxylation supported by succinate is extremely sensitive to antimycin. By contrast the observation that rotenone-blocked succinate-supported cholesterol oxygenase activity is distinctly different from that found in the adrenal. In the adrenal, rotenone at a concentration achieving almost complete inhibition of the respiratory chain causes little or no inhibition of steroid 11β hydroxylation (Harding, 1968). Rotenone also significantly blocked malate- and isocitrate-supported cholesterol side-chain cleavage (Tables V and VI). Taken together, these observations strengthen the view that reduction of intramitochondrial $NADP^+$ in corpus luteum mitochondria occurs

TABLE VI: DL-Isocitrate-Supported Cholesterol Oxygenase Activity of Bovine Corpus Luteum Mitochondria.[a]

Inhibitor	Pregnenolone		Progesterone		Steroid Synthesis (μg/mg of Protein)	% Conversion of ^{3}H	% Inhibition	
	μg/mg of Protein ± SEM	dmp/mg of Protein ± SEM	μg/mg of Protein ± SEM	dpm/mg of Protein ± SEM			Of Mass	Of ^{3}H
Control (zero time)	0.04 ± 0.03		0.28 ± 0.00					
None	0.38 ± 0.07	2078 ± 7	1.25 ± 0.05	1150 ± 137	1.31	12.5		
Amobarbital (4.6 mM)	0.02 ± 0.01	472 ± 75	0.68 ± 0.03	490 ± 48	0.38	3.97	71.0	68.2
Rotenone (25 μM)	0.20 ± 0.03	1600 ± 93	0.80 ± 0.03	684 ± 43	0.68	9.42	48.1	24.6
Antimycin (11 μM)	0.16 ± 0.06	910 ± 70	0.79 ± 0.03	499 ± 54	0.63	5.82	51.9	53.4

[a] Mitochondria from bovine corpus luteum were incubated with DL-isocitrate (10 mM) and one of the inhibitors as marked. Other conditions were as described in the legend of Table II.

almost exclusively by way of reversed electron transport coupled with energy-dependent transhydrogenase activity. On the basis of these findings, it is unlikely that succinate provides reducing equivalents for steroid synthesis through the formation of malate and its subsequent oxidation by the $NADP^+$-dependent malate dehydrogenase as is the presumed mechanism in the adrenal.

Previous studies by a number of investigators (Cammer and Estabrook, 1967; Fonzo *et al.*, 1967) have shown that malate-supported 11β hydroxylation in adrenal cortex is insensitive to inhibitors of the respiratory chain. This suggests that electron transport for 11β hydroxylation does not involve the respiratory chain enzymes or energy-requiring steps. In the present study, when mitochondria from the corpus luteum were incubated with malate, all three respiratory inhibitors significantly reduced cholesterol oxidation (Table V). Most significantly, a concentration of antimycin (0.35 μg/mg of mitochondrial protein) just sufficient to block malate oxidation *via* the respiratory chain caused nearly 80% inhibition of steroid synthesis.

With isocitrate as electron donor (Table VI), the extent of inhibition with amobarbital and antimycin was similar to that obtained when the mitochondria were incubated with either malate or succinate. A lower and more variable degree of inhibition was demonstrated with rotenone. This may indicate that an energy-dependent reaction is only incompletely involved in the isocitrate-supported conversion of cholesterol into pregnenolone and progesterone. In addition, these observations indicate that in bovine corpus luteum mitochondria isocitrate dehydrogenase is mostly NAD^+-linked rather than $NADP^+$, contrary to the observations on rat adrenocortical mitochondria (Purvis *et al.*, 1968).

On the basis of the present studies on cholesterol oxygenase activity and mitochondrial respiration, it is concluded that in bovine corpus luteum, mitochondrial steroid synthesis supported by citric acid cycle intermediates is sensitive to respiratory inhibitors. This implies that electron transport from these substrates requires the respiratory chain enzymes or energy-requiring steps. By contrast corpus luteum mitochondrial steroid synthesis supported by NADPH is insensitive to the respiratory chain inhibitors, antimycin and rotenone.

Discussion

One of the most critical tests for the evaluation of the mitochondrial $NADP^+$-reduction mechanisms is the inhibitory effect of respiratory chain inhibitors and uncouplers on succinate-supported mitochondrial hydroxylations. The reports that antimycin and cyanide, at concentrations sufficient to inhibit respiration, interfere with succinate-supported steroid 11β hydroxylation (Péron *et al.*, 1966; Harding *et al.*, 1966) were interpreted to mean that mitochondrial $NADP^+$ is reduced by NADH by way of an energy-linked transhydrogenase coupled with ATP-dependent succinate-supported reversed electron transport. Simpson and Estabrook (1969) have questioned the significance of the energy-linked transhydrogenase in bovine adrenal cortex when malate and succinate are used as substrates, since (1) rotenone did not influence the experimental pattern during succinate- or malate-supported hydroxylation; (2) pyruvate accumulated suggesting the presence of malic enzyme; and (3) mitochondrial oxidative phosphorylation could not compete with 11β hydroxylation for reducing equivalents (Cammer *et al.*, 1968). In contrast, the experiments described here can only be interpreted to mean that reducing equivalents for cholesterol side-chain cleavage in corpus luteum mitochondria are derived exclusively from energy-dependent reversed electron transport, since malate-, succinate-, and isocitrate-supported steroid synthesis was significantly depressed by antimycin (Figure 3). Furthermore, the fact that rotenone also depressed steroid synthesis in the presence of malate and succinate argues strongly against any involvement of malic enzyme in mitochondrial $NADP^+$ reduction. But, it does not necessarily exclude the presence of a "malate shuttle" as a source of intramitochondrial malate (Simpson and Estabrook, 1969).

It would be difficult to reconcile our present findings with the previous demonstration of $NADP^+$-linked malate dehydrogenase activity in bovine corpus luteum homogenate (Savard *et al.*, 1963) unless it is assumed that malic enzyme is restricted entirely to the cytosol. Although no direct demonstration of malic enzyme activity was attempted in the present study, the fact that oxygen utilization and cholesterol oxygenase activity in the presence of malate were extremely sensitive

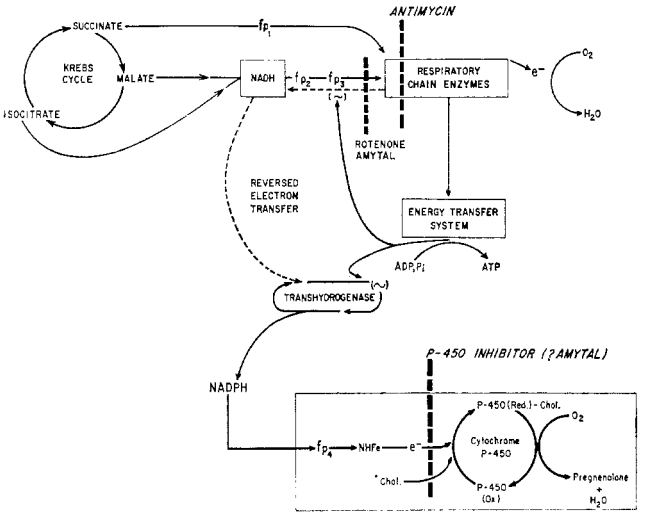

FIGURE 3: The proposed pathway in bovine corpus luteum mitochondria for supplying reducing equivalents to support cholesterol conversion to pregnenolone. The succinate, malate, isocitrate, and NADPH-specific flavoprotein (fp) dehydrogenases are indicated by fp_1, fp_2, fp_3, and fp_4, respectively; the nonheme iron containing protein by NHFe. The proposed sites of energy utilization (~) and the sites of inhibition by the various inhibitors (heavy dashed lines) are also shown.

to antimycin is consistent with the assumption that no malic enzyme was present in mitochondria isolated from bovine corpus luteum. The relative importance of CO_2 fixation by cytosol malic enzyme (Péron, 1968) in generating malate for the shuttle was not investigated, again because our data would suggest the presence of only NADH-linked dehydrogenase in corpus luteum mitochondria. Thus, any actual steroidogenic response would be directly related only to the function of the conventional respiratory chain.

Our observations on amobarbital inhibition of di- and tricarboxylic acid supported cholesterol oxygenase activity in corpus luteum mitochondria are in agreement with similar observations on cholesterol cleavage in adrenocortical mitochondria (Hall, 1967). The unexpected inhibition of NADPH-supported steroid synthesis by amobarbital implies the presence of an amobarbital-sensitive site in the cytochrome P-450 containing cholesterol oxygenase pathway and raises a question regarding the suitability of this respiratory chain inhibitor in studies of reversed electron transport and steroid mixed-function oxidases. The induction of an inhibitor-type difference spectrum (type II) with amobarbital in bovine corpus luteum mitochondria implies barbiturate binding to oxidized cytochrome P-450 and, as will be shown in a subsequent publication, suggests the basis for the inhibitory effect of amobarbital (Figure 3).

One final possibility must be taken into consideration. Current evidence in support of the participation of malic enzyme in adrenal steroid hydroxylation is based on studies of a single mitochondrial enzyme system, steroid 11β-hydroxylase. The corpus luteum was selected for study because of the absence of this enzyme system which might have complicated the interpretations (Hayano *et al.*, 1954; Savard *et al.*, 1965). Although there is evidence that different cytochrome P-450's participate in 11β hydroxylation and cholesterol side-chain cleavage (Jefcoate *et al.*, 1970), it might be postulated, in addition, that these enzyme systems are compartmentalized separately. Thus, the very active 11β-hydroxylase may have access to NADPH generated by both malic enzyme and reversed electron transport coupled to energy-linked transhydrogenase whereas the cholesterol oxygenase may be compartmentalized to receive reducing equivalents solely from the latter. Appropriate experiments designed to answer this question are currently under way.

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Structural Studies on *Dendrostomum pyroides* Hemerythrin*

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ABSTRACT: The N-terminal sequence has been determined for the first 34 residues of the nonheme iron respiratory protein hemerythrin from the sipunculid worm *Dendrostomum pyroides* using an automated sequencer. Additionally, the composition and sequence of the tryptic peptides of *D. pyroides* hemerythrin have been compared to the previously determined sequence of *Golfingia gouldii* hemerythrin. The data indicate that there are four amino acid sequence differences between these two proteins and suggest that *Dendrostomum* and *Golfingia* separated from a common ancestor fairly recently in terms of the evolutionary history of the sipunculids.

In a previous study we reported the physicochemical properties of the hemerythrin from the sipunculid worm *Dendrostomum pyroides*. The pigment of this species was shown to have a molecular weight of approximately 100,000 and to consist of eight subunits. Amino acid analysis, peptide mapping, and immunological studies indicated that *D. pyroides* hemerythrin was very similar in structure to the hemerythrin from another sipunculid worm, *Golfingia gouldii*. The amino acid sequence of the *Golfingia* pigment has been reported by Klotz and his coworkers (Groskopf *et al.*, 1966a,b; Subramanian *et al.*, 1968; Klippenstein *et al.*, 1968).

This paper is concerned with the N-terminal sequence and with the composition and sequence of typtic peptides of *D. pyroides* hemerythrin and compares the structure of this protein to that from *G. gouldii*.

Materials and Methods

D. pyroides were obtained from Pacific Bio-Marine Supply Co., Venice, Calif. *G. gouldii* were from the Marine Biological Laboratory, Woods Hole, Mass. Hemerythrin was prepared as described previously (Ferrell and Kitto, 1970) and converted into the apoprotein by the procedure of Groskopf *et al.* (1966a). Chemicals for the automated sequence analysis were purchased as a kit from Beckman Instruments, Palo Alto, Calif.

Amino Acid Analysis. Amino acid analyses were performed according to the standard methods of Moore *et al.* (1958), using a Beckman–Spinco automatic amino acid analyzer. Iron-free hemerythrin, prepared according to Groskopf *et al.* (1966a), was hydrolyzed at 110° in 6 N HCl in sealed, evacuated tubes for 24, 48, and 72 hr. Serine and threonine were extrapolated to zero time of hydrolysis to account for their destruction. The highest values of valine and isoleucine were taken to represent complete liberation of these residues. Peptides were hydrolyzed under the same conditions for 24 hr. A mixture of β-2-thienylalanine and L-α-amino-β-guanidinopropionic acid hydrochloride at a concentration of 1 μmole/ml of each was used as an internal standard (Siegel and Roach, 1961; Walsh and Brown, 1962).

Enzymatic Digestion. Tryptic digests of iron-free hemerythrin were prepared by dissolving the apoprotein in deionized water at a concentration of 1 mg/ml and heated on a boiling-water bath for 10 min to denature the protein. This solution was allowed to cool to room temperature and made 0.5% in ammonium bicarbonate. TPCK[1]-treated trypsin (Worthington Biochemical Corp.) was added to give an enzyme to substrate ratio of about 5:100, and digestion allowed to proceed for 24 hr at 37°. The reaction was terminated by adjusting the pH of the reaction mixture to 2.0 by the dropwise addition of 2 N HCl. The resulting solution was reduced to a convenient volume on a rotary evaporator under reduced pressure and stored at −10° until used. Tryptic digestion was also carried out, in essentially the same manner, on hemerythrin in which the amino groups of lysine residues were trifluoroacetylated by the method of Goldberger and Anfinsen (1962).

High-Voltage Electrophoresis. All electrophoretic separations employed a modified Michl apparatus (Michl, 1951). Electrophoresis was carried out under three conditions of pH: pH 1.9 (glacial acetic acid–formic acid–water at 8:2:90, v/v), pH 3.5 (pyridine–glacial acetic acid–water at 1:10:89, v/v), and pH 6.0 (glacial acetic acid–pyridine–water at 1:10:90, v/v). The pH 1.9 and 3.5 electrophoresis tanks contained Savant mineral spirits (paraffin oil) as a coolant and the pH 6.0 tank contained xylene (Humble Oil and Refining Co).

Paper electrophoresis was first carried out at pH 6.0 following the procedures of Brown and Hartley (1966). Analytical separations utilized Whatman No. 1 paper, and preparative runs utilized Whatman No. 3MM paper. Samples were applied to the middle of the paper, in a narrow band, and the

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[1] Abbreviations used are: TPCK, L-(1-tosylamido-2-phenyl)ethyl chloromethyl ketone; PTH, phenylthiohydantoin.