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MITOCHONDRIA FROM HUMAN TERM PLACENTA

II. CHARACTERIZATION OF RESPIRATORY PATHWAYS AND COUPLING MECHANISMS

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SUMMARY

Pathways of electron transport utilized for respiration in human term placental mitochondrial preparations were differentiated and characterized through the use of classical respiratory chain inhibitors and multiple sources of reducing equivalents. Mechanisms of associated energy conservation and utilization were examined in these preparations with uncouplers and inhibitors of phosphorylation.

Inhibition by rotenone, antimycin A and cyanide established the classical electron transport chain as the major pathway of respiration with glutamate and succinate as substrates. Approximately 20 % of glutamate-supported respiration was insensitive to inhibitors and may proceed by the cytochrome P-450 linked pathway of electron transport. Approximately 50 % of ascorbate-*N*,*N*,*N*′,*N*′-tetramethyl-*p*-phenylenediamine supported respiration was insensitive to 10^{-3} M cyanide and must utilize an undefined by-pass of cytochrome oxidase. A rotenone- and antimycin-insensitive, exterior pathway for NADH oxidation was demonstrated which could be artificially linked by exogenous cytochrome *c* to the cytochrome oxidase region of the classical electron transport system. Glycerol 3-phosphate also supported oxidative phosphorylation yielding ADP/O ratios of 2.

Respiration of placental mitochondria was stimulated by 2,4- dinitrophenol and gramicidin. With succinate, dinitrophenol-stimulated respiration exceeded that obtained in the presence of ADP. Oligomycin and atractyloside prevented the stimulation of respiration by ADP. Thus, respiration appeared coupled through normal mechanisms to ATP formation and ion transport. A preferential coupling of respiration to the energy-utilizing processes of steroid hormone biosynthesis may exist.

INTRODUCTION

Early studies of Villee and associates [1, 2] which first demonstrated the capacity for oxidative phosphorylation of mitochondria from the human term placenta yielded

Abbreviation: TMPD, *N*,*N*,*N*′,*N*′-tetramethyl-*p*-phenylenediamine.

variable and generally low levels of respiration and phosphorylation. Quantitative assessment of ATP formation, however, was uncertain due to the high ATPase activity in the mitochondrial preparations. More recently, Klimek et al. [3] found improved P/O ratios using bovine serum albumin throughout their isolation and assay procedures. In the preceeding paper [4], we have described procedures developed for the isolation and assay of mitochondria from human term placentas which have yielded preparations which consistently displayed good rates of respiration, respiratory control and near theoretical ADP/O ratios when glutamate, succinate or ascorbate-TMPD were used as substrates.

In addition to ATP formation, human placental mitochondria also utilize oxygen and reducing equivalents for steroid hormone biosynthesis [5, 6], by employing an alternative electron transport system linking NADPH to cytochrome P-450 as terminal mixed-function oxidase [7, 8]. A similar duality of oxidative functions and electron transport pathways characterizes mitochondria of other steroid-producing endocrine tissues, i.e. the adrenal cortex, ovary and testis.

Other differences in the properties of mitochondria from such steroid-producing endocrine tissues have been described. In bovine adrenal cortical mitochondria, a significant alternative pathway for NADH oxidation, not associated with hormone biosynthesis, has been differentiated [9]. A modification of the usual coupling mechanisms has also been proposed to exist in these mitochondria, such that energy derived from respiration may be preferentially utilized for hormone synthesis at the expense of ATP production [10]. In this current study, the pathways of electron transport utilized for respiration in human placental mitochondria were examined and defined through the use of classical inhibitors of mitochondrial respiration and the use of alternative sources of reducing equivalents. In addition, the processes of energy conservation and transferral associated with respiration were examined with uncouplers and inhibitors of phosphorylation.

MATERIALS AND METHODS

Mitochondria were isolated from human term placentas and were resolved by differential centrifugation into three fractions, heavy mitochondria (fraction I), light mitochondria (fraction II), and a less dense fraction III, as described in the previous paper [4]. Oxygen utilization and associated ATP production were measured polarographically with a Gilson KM Oxygraph using the assay conditions developed in the preceding study. Experimental details are given in the legends of tables and figures under Results.

NADH and NADPH were purchased from P-L Biochemicals, Milwaukee, Wisc., U.S.A. DL-α-Glycerophosphate, 2,4-dinitrophenol (grade II), cytochrome *c* (type VI), rotenone, antimycin A, gramicidin D, oligomycin and atractyloside were obtained from the Sigma Chemical Company, St. Louis, Mo., U.S.A.

RESULTS

1. Pathways of placental mitochondrial respiration

(*a*) *Studies with inhibitors.* The support of placental mitochondrial respiration by NAD-linked substrates, succinate and ascorbate-TMPD, and the nearly theoretical

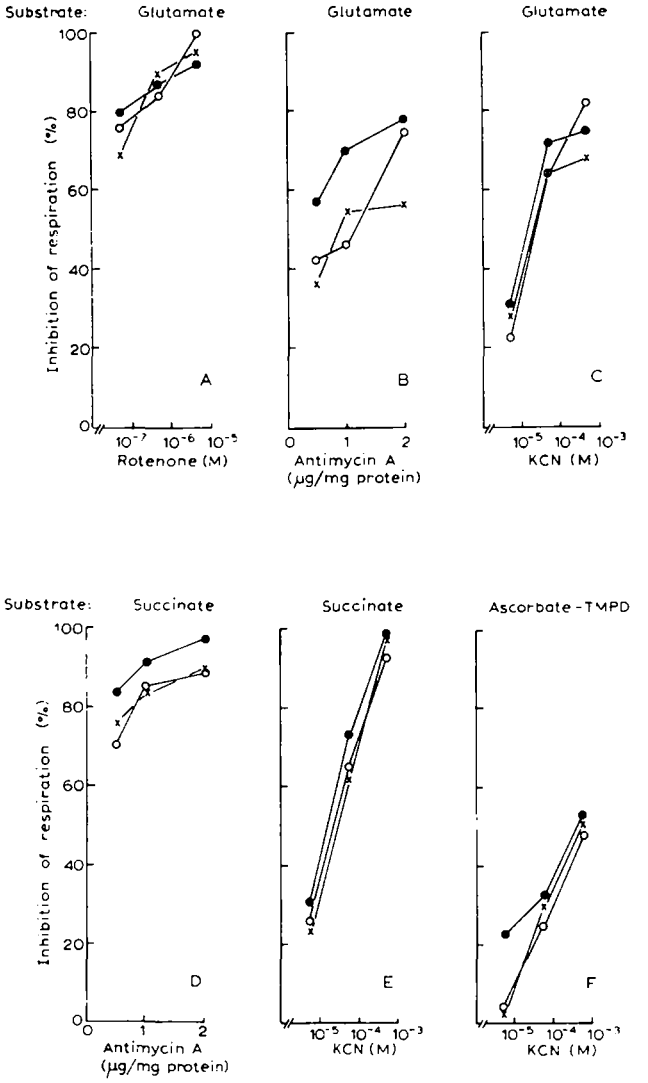

Fig. 1. The effect of rotenone, antimycin A and potassium cyanide on the respiration of human placental mitochondrial preparations. Incubation mixtures contained 1 mg of mitochondrial protein plus the indicated concentrations of inhibitors in a final volume of 2 ml of standard medium containing 15 mM Tris · HCl (pH 7.4), 10 mM potassium phosphate (pH 7.4), 1.5 mM EDTA, 0.13 M sucrose, 6 mM $MgCl_2$ and 4 mg bovine serum albumin (fatty acid free). Incubations, at 30 °C, were started by the addition of substrate: 10 mM glutamate for Figs 1A, 1B and 1C; 10 mM succinate for Figs 1D and 1E; and 10 mM ascorbate plus 0.12 mM TMPD for Fig. 1F. After 3–4 min, 0.25 mM ADP was added to incubations containing glutamate or succinate and 0.05 mM ADP to those containing ascorbate-TMPD. Oxygen uptake was measured polarographically with a Gilson KM Oxygraph and results compared to the maximum rates of respiration achieved in the absence of inhibitor but in the presence of ADP (absolute rates of respiration under these conditions are given in Table IV of ref. 4). Heavy mitochondria (fraction I) (● — ●), light mitochondria (fraction II) (○ — ○) and fraction III (× ··· ×) were tested. The results are the average of two experiments.

ADP/O ratios obtained in the presence of ADP, demonstrated in the previous study [4], gave suggestive evidence for the functioning of the classical mitochondrial electron transport chain. To obtain further evidence for the nature of the electron transport pathways present in placental mitochondria, the effects of several classical inhibitors of mitochondrial respiration were examined.

Varying concentrations of rotenone, antimycin A and potassium cyanide were tested for their effects on endogenous respiration, substrate-supported respiration (state 4) and ADP-stimulated respiration (state 3). Results are shown in Fig. 1. Endogenous rates of respiration of these preparations, which were 7–11 ngatoms of oxygen/min per mg of protein, were completely insensitive to high concentrations of inhibitors, (i.e. 10^{-4}M rotenone, 40 μg antimycin/mg protein, $5 \cdot 10^{-2}$ M cyanide).

Rotenone, at a concentration of $5 \cdot 10^{-8}$ M, inhibited glutamate-supported oxygen consumption by 70–80 % (Fig. 1A) preventing completely any response to ADP. With higher concentrations of rotenone, respiration with glutamate was suppressed by 90–100 %.

Antimycin A, present at 0.5 μg/mg protein, inhibited completely any respiratory response to ADP with glutamate as substrate. Inhibition of non-phosphorylating (state 4) respiration required higher concentrations of inhibitor but unlike the results with rotenone, only a maximum of 80 % inhibition was achieved (Fig. 1B). The effect of cyanide on glutamate-supported respiration was similar to that found with antimycin (Fig. 1C). A low concentration ($5 \cdot 10^{-6}$ M) blocked ADP stimulation of respiration, higher concentrations interfered with non-phosphorylating respiration but 20–30 % of total glutamate-supported respiration remained insensitive to cyanide at $5 \cdot 10^{-4}$ M.

These data (Figs 1A, 1B and 1C) suggest that at least two pathways of electron transport link glutamate to oxygen in these mitochondrial preparations. 70–80 % of glutamate-supported respiration was sensitive to rotenone, antimycin and cyanide and therefore appears to involve the classical pathway of electron transport (i.e. NADH dehydrogenase, cytochromes b, c_1, c, $a+a_3$. With all three inhibitors, coupled respiration (state 3) via this pathway was more sensitive to inhibition than was non-phosphorylating respiration (state 4). However, 20–30 % of glutamate-supported respiration was insensitive to high concentrations of antimycin or cyanide but was inhibited by very high concentrations of rotenone. Thus a second, inhibitor-insensitive pathway of electron transport must be available in these mitochondrial preparations. It appears unlikely that this alternative pathway corresponds to that reported for the oxidation of exogenous NADH in bovine adrenal cortical mitochondrial particles [9], which was insensitive to antimycin and rotenone but was inhibited by 10^{-3} M cyanide.

In contrast to the results with glutamate, respiration with succinate was essentially completely inhibited by antimycin or cyanide (Figs 1D and 1E). Thus, only the classical electron transport chain was utilized. As with glutamate, low concentrations of either inhibitor prevented the stimulation of oxygen uptake by ADP, while higher concentrations were required to inhibit non-phosphorylating respiration linked to succinate.

With ascorbate-TMPD as substrate, only 50 % of mitochondrial respiration could be inhibited by cyanide (Fig. 1F). Thus an alternative pathway of electron transport from ascorbate to oxygen, not involving cytochrome oxidase, must be present in these mitochondrial preparations.

In general, the inhibitor sensitivity of the three mitochondrial preparations

was the same, indicating that similar patterns of utilization of reducing equivalents prevailed.

(*b*) *Studies with alternative electron donors.* To further define the pathways of electron transport available for respiration in placental mitochondria, alternative source of reducing equivalents were examined.

NADH: Low concentrations of exogenous NADH produced little or no stimulation of respiration. But with higher concentrations of NADH (2.5–5.0 mM) somewhat higher respiratory rates were obtained. Table I gives results for heavy mitochondria, however, similar rates of NADH oxidation were observed with all three mitochondrial preparations. Addition of cytochrome *c* (50 mM) in the presence of NADH stimulated respiration 2.3-fold with heavy mitochondria, 2.6-fold with light mitochondria and 3.1-fold with fraction III. With heavy mitochondria, respiration in the presence of NADH and cytochrome *c* was further stimulated by ADP, with ADP/O ratios of 1 being observed (Table I). This indicated an entry of reducing equivalents into the classical phosphorylating pathway between the second and third sites of energy conservation. Both lighter mitochondrial preparations, previously shown to be uncoupled at site III [4], showed no increase in respiration with NADH plus cytochrome *c* upon the addition of ADP.

The effects of rotenone, antimycin A, and potassium cyanide, upon the oxidation of exogenous NADH in the presence of cytochrome *c* were examined. With all three mitochondrial preparations, only 20 % of the NADH-supported respiration was sensitive to antimycin (or to low concentrations of rotenone). However, 90 % inhibition could be achieved with high concentrations of cyanide, suggesting that the major route for the oxidation of exogenous NADH was via an antimycin-insensitive, external pathway which could be artificially linked by exogenous cytochrome *c* to the cytochrome oxidase region of the internal electron transport chain.

TABLE I

RESPIRATION OF HEAVY MITOCHONDRIAL PREPARATIONS FROM HUMAN PLACENTA

Incubation conditions were similar to those of Fig. 1. State 4 respiration rates were measured in the presence of 2.5 mM reduced pyridine nucleotides or 10 mM DL-glycerol-3-phosphate. State 3 respiration rates were measured after the further addition of 0.05 mM ADP. Where indicated, cytochrome *c* was present at a concentration of 0.05 mM. The results are the average of two experiments. Respiratory control ratios and ADP/O ratios were calculated from polarographic measurements of respiration as previously described [4].

Substrate	Oxygen uptake (ngatoms/min per mg protein)		Respiratory control ratio	ADP/O
	State 4	State 3		
NADH	17			
NADH + cytochrome *c*	28	43	1.5	1.0
NADPH	12			
NADPH + cytochrome *c*	21	20	0.95	0
Glycerol 3-phosphate	20	40	2.0	1.8

NADPH: NADPH also stimulated the respiration of all three mitochondrial fractions, although to a lesser degree than did NADH. Exogenous cytochrome *c* stimulated NADPH-supported respiration. As with NADH, this stimulation was more pronounced with the lighter mitochondrial fractions. However, unlike the situation with NADH, ADP did not stimulate respiration with NADPH in the presence of cytochrome *c*. Thus a functional distinction exists between cytochrome *c* reduced by NADH and that reduced by NADPH in regard to its accessibility to cytochrome oxidase of the internal respiratory chain.

Glycerol 3-phosphate: An additional pathway for the entry of reducing equivalents into the electron transport chain of placental mitochondria was demonstrated using glycerol 3-phosphate as substrate (Table I). To our knowledge, this is the first demonstration of glycerol 3-phosphate dehydrogenase activity (EC 1.1.99.5) in the human placenta. ADP/O ratios approaching 2 were obtained with heavy mitochondria, indicating an entry of electrons at the same general level utilized by succinate dehydrogenase (EC 1.3.99.1). As with succinate, ADP/O ratios of 1 were obtained with both lighter mitochondrial preparations uncoupled at site III. Respiratory control ratios observed with glycerol 3-phosphate were also similar to those previously obtained with succinate.

2. Energy coupling in placental mitochondria

To analyze the nature of the coupling of respiration to energy production in these preparations, the effects of reagents known to differentially influence the processes of energy conservation, transferral and dispersal in other mitochondria were studied. Results are given in Table II. 2,4-Dinitrophenol, an uncoupler believed to act at the earliest stages of energy conservation to alleviate the dependence of electron flow upon energy acceptors, stimulated the respiration of heavy mitochondria with all three substrates,

TABLE II

THE EFFECT OF REAGENTS WHICH INFLUENCE ATP FORMATION UPON THE RESPIRATION OF HEAVY MITOCHONDRIAL PREPARATIONS FROM HUMAN PLACENTA

Incubation conditions were the same as those of Fig. 1. Glutamate, succinate or ascorbate were present at 10 mM, TMPD at 0.12 mM for the determination of state 4 respiration rates. The effects upon these respiration rates of 0.05 mM ADP, 0.1 mM dinitrophenol (DNP), 5 μM gramicidin and 0.75 μM oligomycin were determined except that 0.025 mM ADP and 3.8 μM oligomycin were used when ascorbate-TMPD served as substrate.

Addition	Oxygen uptake in presence of indicated substrate (% of state 4 respiration rates)		
	Glutamate	Succinate	Ascorbate-TMPD
None	100	100	100
ADP	400	209	135
DNP	375	282	141
DNP+ADP	363	270	131
Gramicidin	288	143	130
Gramicidin+ADP	263	143	123
Oligomycin	100	76	100
Oligomycin+ADP	100	76	100

and prevented any additional response upon the addition of ADP. With succinate as substrate, dinitrophenol-induced respiration exceeded that obtained in the presence of ADP alone. This augmentation of state 3 respiration rates was observed with all three mitochondrial preparations.

Gramicidin gave results qualitatively similar to those obtained with dinitrophenol, stimulating respiration but not to the extent achieved with ADP or with dinitrophenol (Table II). Like dinitrophenol, it prevented any additional stimulation of respiration by ADP. With other mitochondria, this reagent is believed to achieve its uncoupling effect through a facilitation of energy utilization in ion transport. Its effectiveness upon placental mitochondria is therefore indicative of the existence of such mechanisms in placental mitochondria for the coupling of respiration to such other mitochondrial activities.

Oligomycin, which inhibits mitochondrial ATPase and prevents the utilization of respiratory energy for ATP synthesis, blocked the response of placental mitochondria to ADP but by itself did not alter state 4 respiration (Table II).

Similar patterns of response to dinitrophenol, gramicidin and oligomycin were found with the lighter mitochondrial fractions when glutamate or succinate were utilized as substrates. However, with ascorbate-TMPD as substrate, oxygen consumption was neither stimulated nor inhibited by addition of these reagents. These findings are additional evidence for the uncoupled state of site III in these two mitochondrial preparations.

Further evidence for the coupling of placental mitochondrial respiration to ATP generation was obtained in studies of the effect of atractyloside, which interferes with transport mechanisms allowing exogenous ADP access to the inner mitochondrial compartment. Table III shows the effects of this reagent upon succinate-supported respiration in the three placental mitochondrial preparations. Atractyloside when added in the presence of ADP, reduced state 3 respiration rates without decreasing ADP/O ratios. When added prior to ADP, atractyloside blocked completely any response to ADP without impairing state 4 respiration. Similar effects of this reagent were obtained with all three mitochondrial fractions indicating the intactness of the inner membrane barrier to ADP in all three placental mitochondrial preparations.

TABLE III

THE EFFECT OF ATRACTYLOSIDE ON RESPIRATION AND PHOSPHORYLATION OF HUMAN PLACENTAL MITOCHONDRIA

Incubation conditions were similar to those of Fig. 1. 10 mM succinate was present in all incubations. ADP and atractyloside were added in the indicated sequences 1.5–2 min apart to yield final concentrations of 1.0 mM and 0.05 mM, respectively. Results are the average of two experiments. MF, mitochondrial fraction.

Addition	Oxygen uptake (ngatoms/min per mg protein)			ADP/O		
	MF I	MF II	MF III	MF I	MF II	MF III
None	15	15	15			
ADP	44	26	28	2.0	1.1	1.3
ADP; atractyloside	32	24	23	2.0	1.1	1.3
Atractyloside; ADP	15	15	15	0	0	0

DISCUSSION

The pathways for electron transport in placental mitochondria demonstrated or suggested by this study are outlined in Fig. 2, together with the previously established system for steroid mixed-function oxidation. The principal oxidative pathway is the classical respiratory chain which links reducing equivalents derived from NAD-linked substrates to oxygen. It is characterized by susceptibility to inhibition by rotenone, antimycin and cyanide and includes three coupled sites of energy conservation and ATP formation. Segments of this pathway are utilized in the oxidation of flavoprotein-linked substrates such as succinate and glycerol 3-phosphate, as well as by cytochrome-linked artificial electron donors such as ascorbate-TMPD and exogenously reduced cytochrome *c*.

The approximately 20% of glutamate-supported respiration which was resistant to rotenone and insensitive to antimycin and cyanide may utilize the cytochrome P-450-linked pathway. This might proceed either via NAD, since previous studies from this laboratory have shown that exogenous NADH could reduce placental mitochondrial cytochrome P-450 [7] or directly via NADP, since solubilized placental glutamate dehydrogenase (EC 1.4.1.2) has been shown to utilize this cofactor as well [11].

A rotenone- and antimycin-insensitive, external pathway for the oxidation of exogenous NADH is also present in these preparations. While this could result from microsomal contaminants, a similar electron transport system involving NADH-cytochrome b_5 reductase (EC 1.6.2.2.) and cytochrome b_5 has been demonstrated in the outer membrane fraction of liver mitochondria [12, 13] and similar components have also been described in mitochondrial preparations from adrenal cortex and corpus luteum [14, 15].

Upon the addition of exogenous cytochrome *c* to placental mitochondria, a shunt is established allowing electrons from the external pathway access to the interior,

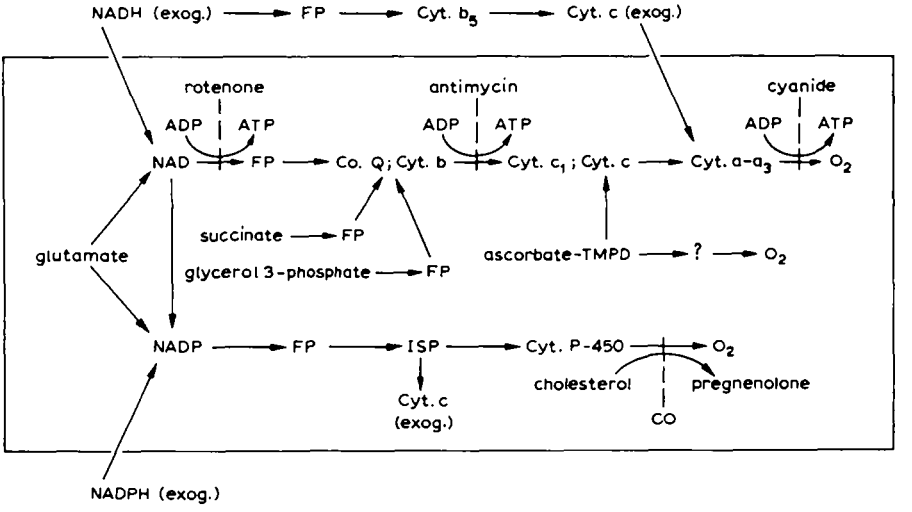

Fig. 2. Proposed pathways of respiration and electron transport in mitochondria from human term placenta. Inhibitory effects are indicated by broken lines. The following abbreviations are used: FP, flavoprotein; ISP, iron-sulfur protein; Co. Q, ubiquinone; TMPD, *N,N,N',N'*-tetramethyl-*p*-phenylenediamine; Cyt., cytochrome; exog., exogenously added.

phosphorylating pathway as evidenced by the effects of cyanide and ADP. Yielding et al. [16] also found a stimulation of NADH oxidase of placental 'particulate preparations' by cytochrome *c* via a steroid-insensitive pathway which they believed to be the same as the antimycin-insensitive pathway of liver and kidney mitochondria. A similar antimycin-insensitive, cyanide-sensitive NADH oxidase system activated by the presence of exogenous cytochrome *c* has been studied in bovine and rat adrenal cortical mitochondrial preparations [17, 18]. The antimycin-insensitive, cyanide-sensitive pathway for NADH oxidation described by Simpson and Estabrook [9] in sonicated bovine adrenal cortical mitochondrial preparations was detected in the absence of exogenous cytochrome *c*. However, it seems probable that in these preparations of Simpson and Estabrook, an endogenous component served to artificially link external and internal pathways, as suggested by Sauer [18], or, alternatively, direct interaction may have become possible upon the disruption of structural barriers.

An external to internal pathway of NADPH oxidation similar to that observed with NADH in the presence of cytochrome *c* was not evident from studies with ADP, suggesting that contamination with placental microsomes (which contain both NADH- and NADPH-cytochrome *c* reductase activities) is not likely to be the principal cause for external nucleotide oxidations. Exogenously generated NADPH has previously been shown to support the mixed-function steroid oxidase of placental mitochondria in hypotonic medium [6]. The solubilized NADPH diaphorase plus iron sulfur protein of bovine adrenal mitochondria have been shown to constitute a NADPH-cytochrome *c* reductase system [19, 20] and this most probably applies to the placental components as well. Any reduced cytochrome *c* formed by such a mechanism must, however, be inaccessible to the cytochrome oxidase of the respiratory chain to account for our experimental observations.

Finally, a significant portion of respiration with ascorbate was insensitive to cyanide and thus by-passed the cytochrome oxidase of the respiratory chain. No information on the components responsible for this alternative pathway was obtained.

Mitochondria from steroid hormone-producing endocrine tissues appear to possess the same general pathways of electron transport for respiration and for steroid biosynthesis but differ markedly in the nature of the substrates which can be utilized as sources of reducing equivalents. Thus both glutamate and glycerol 3-phosphate were oxidized by placental mitochondria but cannot support respiration in bovine adrenal cortical mitochondria [10].

The coupling of respiration to processes of energy conservation and transferral at three sites of the respiratory chain in placental heavy mitochondria was previously indicated by the effects of ADP on respiration supported by glutamate, succinate and ascorbate-TMPD [4]. The inhibition of this response to ADP by oligomycin, shown in this study, indicates that placental mitochondria possess mechanisms for ATP generation qualitatively similar to those of other mitochondria. The effects of gramicidin on placental mitochondrial respiration suggest that respiration is also coupled to other energy-requiring mitochondrial processes such as ion transport.

In mitochondria from other steroid-producing endocrine tissues, a third aspect of respiratory control, i.e. the utilization of energy derived from respiration for the delivery of reducing equivalents for steroid hormone biosynthesis, has been shown to be important [21–25]. Cammer and Estabrook [10] have suggested that in bovine adrenal cortical mitochondria energy coupling and transferral mechanisms may be adapted or

modified such that this process takes precedence over the customary mitochondrial function of ATP synthesis. Experimental data leading to this suggestion were, in part, their findings of a much greater increase in respiration in response to dinitrophenol than was observed when ADP was added. While with placental mitochondria the difference in response to dinitrophenol and ADP was not as striking as that obtained by Cammer and Estabrook [10] with bovine adrenal cortical mitochondria, it was still significantly greater than that for liver mitochondria observed by these workers and supports their theory of a specialized coupling arrangement in mitochondria capable of steroid oxidations. Similar studies of dinitrophenol with mitochondria from other tissues and species would be of interest in evaluating this proposition.

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