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Submitochondrial Location and Electron Transport Characteristics of Enzymes Involved in Proline Oxidation

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ABSTRACT

Isolated corn mitochondria (*Zea mays* cv. B73 x Mo17) were fractionated and the fragments were separated on a 20–45% (weight/weight) continuous sucrose gradient. Soluble enzymes remained at the top of the gradient overlapping with the outer membranes, while inner membrane vesicles and intact inner membranes were distributed farther down the gradient. Proline oxidase and Δ^1-pyrroline-5-carboxylic acid dehydrogenase activities were associated only with the inner mitochondrial membrane. Glutamate dehydrogenase was confirmed as a matrix enzyme.

Both proline and Δ^1-pyrroline-5-carboxylic acid supported oxygen uptake in isolated mitochondria. Proline dependent oxygen uptake was relatively independent of pH with a maximum rate at pH 7.2. In contrast, Δ^1-pyrroline-5-carboxylic acid-dependent oxygen uptake was sensitive to pH with an optimum at pH 6.1. The oxidation of proline and Δ^1-pyrroline-5-carboxylic acid was inhibited by 10 micromolar rotenone. This indicates that electrons from these substrates enter the respiratory chain prior to at least one of the rotenone sensitive iron-sulfur proteins. Both substrates yielded ADP:O ratios of around 1.9 as compared to malate plus pyruvate (2.1), succinate (1.3), and exogenous NADH (1.2).

Proline oxidation is a mitochondrial process (2, 10) that can be inhibited by water stress (16). Although extensive research has been conducted on proline accumulation and its significance during water stress, little has been directed toward elucidating the inhibition of proline oxidation. Inhibition of proline oxidation contributes to proline accumulation. To determine the mechanism of this process, it is necessary to establish the intramitochondrial location and properties of the enzymes involved. The first enzyme involved in the oxidation of proline is proline oxidase, which catalyzes the conversion of proline to P5C[1]. P5C is then oxidized to glutamate by P5C dehydrogenase. Glutamate can be converted to the Krebs cycle intermediate α-KG by glutamate dehydrogenase, or it can be metabolized by other pathways.

The location of these enzymes in animal mitochondria is fairly well established. Proline oxidase is associated with the outer phase of the inner mitochondrial membrane, P5C dehydrogenase and glutamate dehydrogenase are considered matrix enzymes (1, 5). In plants, their submitochondrial location is not well defined. The location of proline oxidase and P5C dehydrogenase is not known, while glutamate dehydrogenase is considered a matrix enzyme (4). The purpose of this paper is to further characterize these enzymes in isolated corn mitochondria.

[1] Abbreviations: P5C, Δ^1-pyrroline-5-carboxylic acid; α-KG, α-ketoglutarate; SRM, standard reaction medium; RCR, respiratory control ratio; OAA, oxaloacetic acid; DCIP, 2,6-dichloroindophenol.

MATERIALS AND METHODS

Corn seedlings (*Zea mays* cv. B73 x Mo17) were grown in the dark at 30 ± 2 C in moist vermiculite. Mitochondria were isolated from the shoots of 3- to 4-day-old seedlings according to Day and Hanson (6). Protein was estimated by the method of Lowry *et al.* (12) using BSA (Fraction V) as the standard. Assays were conducted at 26 ± 2 C in 3.0 ml of SRM (6) unless otherwise indicated, and were initiated upon addition of substrate. SRM consisted of 0.25 M sucrose, 5 mM KH_2PO_4, 5 mM $MgCl_2$, and 1 mg/ml BSA buffered with either 10 mM Tes or 20 mM Mes (pH as indicated in legends).

Oxygen Uptake. Oxygen utilization was measured at 26 ± 1 C in 3.0 to 3.2 ml of SRM using a Clark O_2 electrode (Model 53, Yellow Springs Instrument Co., Yellow Springs, OH). ADP:O ratios, RCR, and O_2 content of air saturated water were determined according to Estabrook (9).

Enzyme Assays. Malate dehydrogenase was assayed by measuring the OAA dependent oxidation of NADH in SRM containing 50 μM NADH, 250 μM OAA, and 1 mM KCN. Glutamate dehydrogenase activity was determined by measuring the α-KG dependent oxidation of NADH in SRM containing 5 mM NH_4Cl, 50 μM NADH, 10 mM α-KG, and 1 mM KCN. P5C dehydrogenase was measured by the P5C dependent reduction of NAD^+ in SRM containing 0.05% Triton X-100, 2 mM NAD^+, and 0.19 mM DL-P5C. Changes in *A* at 340 nm were used to measure these reactions. P5C was synthesized according to Williams and Frank (18).

Proline oxidase, succinate dehydrogenase and NADH dehydrogenase were measured using Cyt *c* and DCIP as electron acceptors. Changes in *A* at 550 nm were used to measure Cyt *c* reduction in an assay medium containing substrate (10 mM proline, 10 mM succinate, or 0.5 mM NADH), 50 μM Cyt *c* and 1 mM KCN. DCIP reduction was measured by following changes in *A* at 600 nm in a reaction medium containing substrate, 60 μM DCIP and 1 mM KCN. Antimycin A (0.15 mM) and rotenone (3.0 mM) were solubilized in 80% ethanol. The following mM extinction coefficients were used: $\epsilon_{550} = 21.0\ mM^{-1}\ cm^{-1}$ for Cyt *c*; $\epsilon_{340} = 6.22\ mM^{-1}\ cm^{-1}$ for NADH; $\epsilon_{600} = 21.0\ mM^{-1}\ cm^{-1}$ for DCIP; and $\epsilon_{260} = 15.4\ mM^{-1}\ cm^{-1}$ for ADP (8, 11, 19).

Submitochondrial Fractionation. To determine the intramitochondrial localization of enzymes involved in proline oxidation, mitochondria were isolated from 120 g of tissue and suspended in 2.0 ml of 10 mM phosphate buffer (pH 7.4). Fractionation was achieved with a modification of the procedure of Sparace and Moore (15). The entire procedure was conducted at 4 C. The mitochondrial suspension was homogenized for 5 min (10 rotating strokes/per min) in a Duall 25 glass homogenizer, diluted to 120 ml with buffer and allowed to swell for 20 min.

The resulting suspension was centrifuged at 105,500*g* for 60 min in a Beckman Ti-50 rotor. The pellets were resuspended in a total of 2.0 ml of 10% sucrose (w/w) in 10 mM Tes (pH 7.4). The mitochondrial fragments were then layered on a 20–45% (w/w) continuous sucrose gradient containing 10 mM Tes (pH 7.4), and

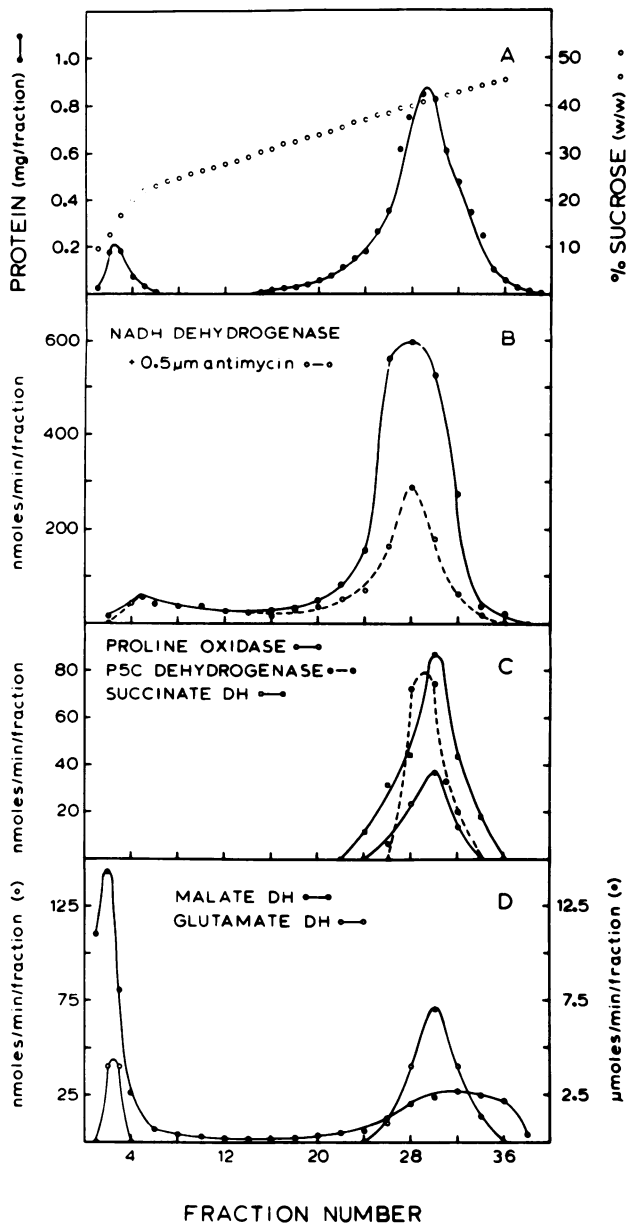

FIG. 1. Submitochondrial fractionation. Percentage of sucrose was determined using a Bausch and Lomb Abbe-3L refractometer. Protein was assayed using 0.10 ml samples. Activities of NADH dehydrogenase (0.05-ml sample), succinate dehydrogenase (0.10 ml-sample), and proline oxidase (0.10-ml sample) were measured using Cyt c as the electron acceptor. P5C dehydrogenase was assayed by following the reduction of NAD^+ in 0.05% Triton X-100 solubilized samples (0.10 ml). Malate dehydrogenase activity was determined with 0.01-ml samples and glutamate dehydrogenase with 0.10-ml samples. Assays were conducted on all fractions in SRM buffered with 10 mM Tes (pH 7.2). Fractions with no activity were not plotted. Other fractions were not plotted for clarity.

centrifuged at 82,500g for 120 min in a Beckman SW 27 rotor. The gradient was fractionated into 1-ml fractions using an ISCO Model D density gradient fractionator, and the fractions assayed for various enzyme activities.

For the isolation of outer membranes, fractions 1 to 10 were collected, diluted to 20 ml with 10 mM Tes (pH 7.4) and centrifuged at 105,500g for 60 min in a Beckman Ti-50 rotor. The pellets were resuspended in a total of 1.0 ml of 10 mM Tes (pH 7.2) and assayed for various enzyme activities.

Table I. *Enzyme Activities Associated with the Outer Membrane Fraction*

NADH dehydrogenase and proline oxidase activities were measured using Cyt c as the electron acceptor. P5C dehydrogenase was determined by following NAD^+ reduction in 0.05% Triton X-100 solubilized samples. The reaction medium was buffered with 10 mM Tes (pH 7.2).

Assay	Protein	Oxidation Rate
	μg/assay	*nmol/min·mg protein*
NADH Dehydrogenase +0.5 μM antimycin	10	657
		573
Proline Oxidase	83	0
P5C Dehydrogenase	83	0

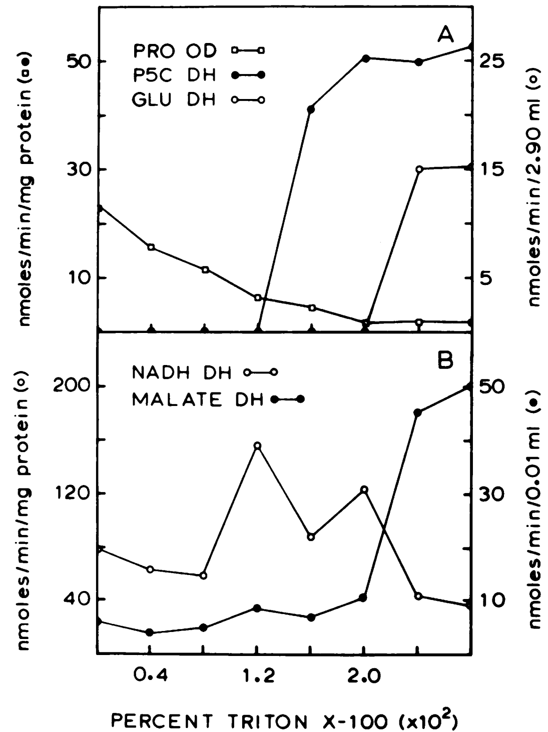

FIG. 2. Triton X-100 disruption of mitochondria. Mitochondria (about 500 μg/assay) were incubated at 26 ± 2 C for 5 min prior to assay. The incubation medium consisted of 3.0 ml SRM (10 mM Tes, pH 7.2) containing the appropriate percentage of Triton X-100 (v/v). Proline oxidase (DCIP reduction), P5C dehydrogenase (NAD^+ reduction), and NADH dehydrogenase (Cyt c reduction) activities were measured using 3.0, 3.0, and 0.4 ml of the incubated mitochondria, respectively. Other incubations were centrifuged at 30,000g for 10 min and the supernatant used for assay of glutamate and malate dehydrogenase activities. The NADH dehydrogenase activity shown is that which is antimycin sensitive (total activity minus antimycin insensitive activity).

RESULTS AND DISCUSSION

Submitochondrial Location. Figure 1 represents an experiment used to establish the intramitochondrial location of proline oxidase, P5C dehydrogenase, and glutamate dehydrogenase. As seen in Figure 1A, submitochondrial fragments were separated into two distinct regions on the sucrose gradient. Soluble mitochondrial enzymes remained at the top of the gradient overlapping with the outer membranes, while inner membrane vesicles and intact inner membranes were distributed further down the gradient. Inner membrane vesicles were not separated from intact inner membranes using this technique. The distribution of NADH dehydrogenase activity in the gradient is given in Figure 1B. Antimycin A

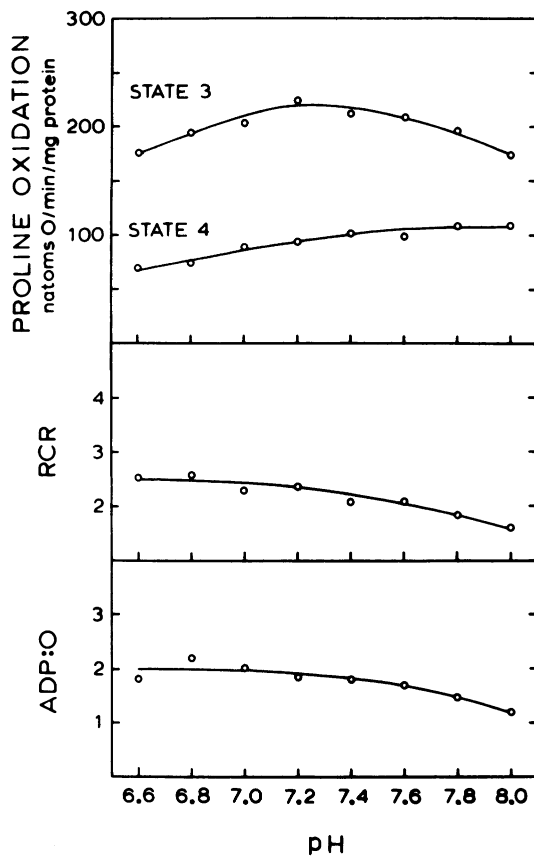

FIG. 3. The effect of pH on proline oxidation. Rates and RC ratios represent the mean of three pH curves normalized to the curve with the highest ADP:O ratios. Activity was measured in 3.2 ml SRM containing 10 mM Tes with an average of 1.15 mg mitochondrial protein/assay. The reaction was initiated with 10 mM L-proline. Rates are for the second addition of ADP (200 nmol).

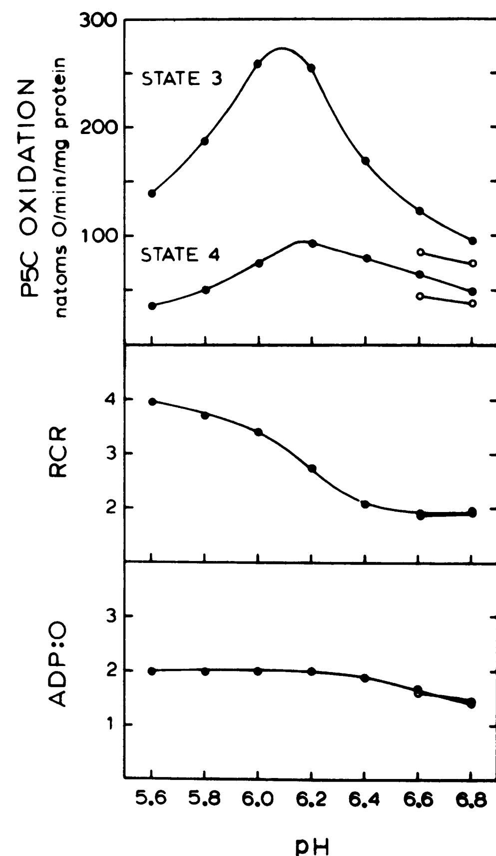

FIG. 4. The effect of pH on P5C oxidation. Activity was measured in 3.2 ml SRM containing 20 mM Mes (●) or 10 mM Tes (○) with 1.03 mg mitochondrial protein/assay. The reaction was initiated with 10 mM DL-P5C. Rates are for the second addition of ADP (200 nmol).

insensitive NADH oxidation is a marker for the outer mitochondrial membrane, since only the inner membrane dehydrogenases are inhibited by antimycin (8). It can be seen that the outer membrane peak (fraction 5) is well separated from that of the inner membranes, and is relatively uncontaminated with inner membrane fragments.

Figure 1C gives the distribution of succinate dehydrogenase (a marker for the inner membrane), proline oxidase, and P5C dehydrogenase. There was no measurable activity of these enzymes in the outer membrane region, suggesting that they occur only on the inner membrane. Because of the low protein content of the outer membrane peak, it could be argued that insufficient recovery of outer membranes was responsible for the lack of activity. To differentiate between these possibilities, outer membranes were isolated and assayed at protein concentrations known to yield activities of these enzymes (Table I). The results indicate that proline oxidase and P5C dehydrogenase are associated only with the inner mitochondrial membrane.

Glutamate dehydrogenase and malate dehydrogenase (a marker for the mitochondrial matrix) yielded two peaks of activity on the gradient (Fig. 1D). The peak at the bottom of the gradient corresponds to activity retained within intact inner membranes and that at the top to enzyme released by inner membrane breakage during resuspension of the 105,500g pellet. Thus, glutamate and malate dehydrogenases are soluble enzymes; however, this experiment cannot differentiate between matrix enzymes and intermembrane enzymes. Fumarase, which is considered a better matrix marker than malate dehydrogenase, was not used since measurable activity was lost during the fractionation process.

Table II. *Effects of Respiratory Chain Inhibitors on Substrate Oxidation*

Assays were conducted in 3.1 ml SRM (20 mM Mes, pH 6.6) with an average of 1.20 mg mitochondrial protein/assay. State 3 rates were maintained with inclusion of 0.6 mg hexokinase (Sigma Type III) and 50 mM glucose. Data are compiled from five individual experiments.

Substrate	Average Control Rate	10 μM Rotenone	0.5 μM Antimycin	1 mM KCN	1 mM NaN_3
	natoms O/min· mg protein	*% inhibition*			
State 3					
10 mM Malate + Pyruvate	136	61	88	53	79
10 mM Succinate	346	+1	95	84	84
0.5 mM NADH	566	1	96	90	94
10 mM L-Proline	193	55	90	75	85
10 mM DL-P5C	135	54	87	10	74
State 4					
10 mM Malate + Pyruvate	81.5	27	68	67	69
10 mM Succinate	173	+24	82	80	79
0.5 mM NADH	206	0	88	86	79
10 mM L-Proline	93.5	13	79	90	71
10 mM DL-P5C	88.1	33	66	24	38

To obtain further evidence on the location of glutamate dehydrogenase, the sequential solubilization of mitochondrial mem-

Table III. *P5C Interference with Cyanide Inhibition of Oxygen Uptake*

Assays were conducted in 3.1 ml SRM (20 mM Mes, pH 6.6) containing 1.08 mg mitochondrial protein/assay. State 3 rates were maintained with inclusion of 0.6 mg hexokinase (Sigma Type III) and 50 mM glucose.

Substrate	State 3 Rate	+1 mM KCN	Inhibition
	natoms/min·mg protein		%
10 mM Succinate	349	78.5	78
5 mM DL-P5C	47.2	43.2	8
10 mM Succinate + 5 mM DL-P5C	387	274	29

Table IV. *Comparative ADP:O Ratios of Respiratory Substrates*

Values represent the mean (and corresponding average deviation) of four separate experiments. Experiments were run in 3.2 ml of SRM buffered in one experiment with 20 mM Mes (pH 6.1), in two with 20 mM Mes (pH 6.6), and in one with 10 mM Tes (pH 7.2). Mitochondrial protein averaged 1.23 mg/assay.

Substrate	ADP:O	RCR
10 mM Malate + Pyruvate	2.06 ± 0.17	2.24 ± 0.23
10 mM Succinate	1.30 ± 0.11	2.08 ± 0.28
0.5 mM NADH	1.17 ± 0.14	2.43 ± 0.18
10 mM L-Proline	1.87 ± 0.17	2.31 ± 0.08
10 mM DL-P5C	1.83 ± 0.16	1.97 ± 0.13

branes with Triton X-100 was investigated using a modification of the procedure of Woo (21). Figure 2B shows the activity of marker enzymes used to follow this solubilization. Antimycin-sensitive NADH oxidation was used to determine the rupture of the outer membrane, while malate dehydrogenase was used to determine inner membrane solubilization.

Since both mitochondrial membranes are essentially impermeable to Cyt *c* (7), it was used as the electron acceptor for the NADH dehydrogenase assay. In mitochondria with intact outer membranes, Cyt *c* can be reduced only by electrons from the outer membrane NADH dehydrogenase which is insensitive to antimycin. However, upon rupture of the outer membrane, Cyt *c* can accept electrons from endogenous Cyt *c* which is associated with the outer surface of the inner mitochondrial membrane. NADH oxidation by inner membrane dehydrogenases is sensitive to antimycin, therefore, a large increase in antimycin sensitive NADH oxidation was observed (Fig. 2B) upon rupture of the outer membrane at 0.012% Triton (v/v). Solubilization of the inner membrane occurred around 0.024% as shown by the increase in malate dehydrogenase activity in the 30,000*g* supernatant. Thus it can be seen that glutamate dehydrogenase (Fig. 2A) is only located within the mitochondrial matrix.

The second peak of antimycin sensitive NADH dehydrogenase activity at 0.020% Triton (Fig. 2B) occurred because the inner mitochondrial membrane became leaky to NADH since the inner membrane was not ruptured at this Triton concentration. This was observed as an increase in total NADH dehydrogenase activity and was confirmed by a comparison of the total malate dehydrogenase activity with that of the 30,000*g* supernatant (data not shown).

Triton has also been shown to affect the activities of proline oxidase and P5C dehydrogenase. Proline oxidase is severely inhibited (2, 10) while P5C dehydrogenase activity (as measured by NAD^+ reduction) is dependent upon detergent activation in corn mitochondria as in those of barley (3). As shown in Figure 2A, proline oxidase exhibited a gradual inhibition with increasing detergent and had negligible activity at concentrations above 0.020%. In contrast, P5C dehydrogenase was inactive up to 0.016% Triton where it essentially became fully activated. Therefore, P5C dehydrogenase activity can be measured with a Triton concentration that is not sufficient to solubilize the inner mitochondrial membrane, and furthermore, at a concentration lower than is necessary for significant NADH permeability. This suggests that the detergent activation of P5C dehydrogenase may function by inhibition of exogenous NADH dehydrogenase activity which interferes with the measurement of NAD^+ reduction.

pH Responses. Since proline oxidation had been found to support O_2 uptake (2), an experiment was performed to see if P5C oxidation would also result in O_2 utilization. The addition of either proline or P5C was found to support O_2 uptake. However, when O_2 utilization was followed at or near the previously reported pH optima for enzyme activity low ADP:O and RC ratios were obtained. Higher ADP:O and RC ratios were obtained at lower pHs, therefore the pH dependence of O_2 uptake, ADP:O ratios, and RCR was determined using proline and P5C as substrates. These pH responses might better reflect *in vivo* oxidation.

At mitochondrial concentrations required to obtain reasonable ADP:O ratios (about 1 mg/assay), the effect of pH on proline oxidation was minimal (Fig. 3). State 3 rates were affected the most and consistently showed an optimum at pH 7.2. This optimum is different from the 8.0 to 8.5 observed by Huang and Cavalieri (10) using a DCIP assay. ADP:O ratios and RC ratios are comparable with those of Boggess *et al.* (2).

In contrast, P5C dependent O_2 uptake was sensitive to pH with an optimum at or near 6.1 (Fig. 4). This optimum is different from the 7.6 observed by Stewart and Lai (17) and the 8.0 observed by Boggess *et al.* (3). Both authors used NAD^+ as the electron acceptor. State 3 rates were affected the most and the ADP:O ratios were around 2. The RCR for P5C oxidation consistently showed this pattern of response (Fig. 4). The high RCR values at the lower pHs appear to be caused primarily by elevated state 3 rates.

Effects of Respiratory Chain Inhibitors. In further characterizing the oxidation of proline and P5C, it was of interest to determine where on the respiratory chain the electrons from these compounds entered. This was investigated with inhibitors using both state 3 and state 4 rates (Table II). Malate plus pyruvate represent substrates donating electrons to the respiratory chain prior to the rotenone sensitive iron-sulfur proteins, therefore showing sensitivity to rotenone. Electrons from succinate and exogenous NADH enter after the rotenone sensitive iron-sulfur proteins and are insensitive to rotenone (14). Proline and P5C oxidation were sensitive to rotenone similar to malate plus pyruvate. Therefore, electrons from proline and P5C enter the primary respiratory chain prior to at least one of the rotenone sensitive iron-sulfur proteins.

All substrates showed sensitivity to antimycin, KCN, and NaN_3 with the notable exception of P5C oxidation which showed cyanide insensitivity. It is possible that P5C interferes with cyanide inhibition. To test this possibility, the effect of P5C on cyanide inhibition of succinate oxidation was investigated. As presented in Table III, addition of P5C resulted in succinate oxidation becoming comparatively insensitive to cyanide inhibition, illustrating the proposed interference.

Relative Comparisons of ADP:O Ratios. Since electrons from P5C and proline enter the respiratory chain prior to at least one rotenone sensitive iron-sulfur protein, they could be involved in the initial proton translocating site as proposed by Mitchell (13). Depending upon the nature of the substrate and the submitochondrial position of the enzyme involved, the theoretical ADP:O ratio can be decreased due to proton dependent membrane transport systems (20). Therefore, only relative comparisons of ADP:O ratios are valid. As seen in Table IV, the oxidation of proline and P5C yielded similar ADP:O ratios. These ratios are slightly less than that obtained for malate plus pyruvate but considerably more than that for succinate or exogenous NADH. It is not possible to definitely say if proline and P5C have theoretical ADP:O ratios of 2 or 3 since the submitochondrial position of the enzymes involved

and the nature of the transport of these substrates have not been fully characterized. If a proton motive transport system is necessary for proline and P5C oxidation the theoretical ADP:O ratios would be 3, and if not necessary, they would be 2.

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