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# A PROLINE SHUTTLE IN INSECT FLIGHT MUSCLE

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## SUMMARY

A proline shuttle for oxidation of extramitochondrial NADH was reconstituted from soluble and mitochondrial fractions of blowfly (Phormia regina) flight muscle. The soluble fraction catalyzed reduction of Δ'-pyrroline-5-carboxylate to proline via the action of Δ'-pyrroline-5-carboxylate reductase (EC 1.5.1.2). The reaction required NADH as hydrogen donor, NAD(P)H being ineffective in this regard. Mitochondria catalyzed regeneration of Δ'-pyrroline-5-carboxylate from proline via action of proline oxidase. The capacity of the shuttle to operate under conditions of possible competition for Δ'-pyrroline-5-carboxylate between Δ'-pyrroline-5-carboxylate reductase and Δ'-pyrroline-5-carboxylate dehydrogenase (EC 1.5.1.12) was investigated. Results of these investigations indicate that dehydrogenase activity does not significantly interfere with shuttle activity.

Nicotinamide nucleotides do not readily cross the inner mitochondrial membrane (1). Consequently, various shuttle mechanisms have been proposed for the transport of hydrogen from cytoplasmically generated NADH to the respiratory chain localized inside the permeability barrier (2,3). In highly active insect flight muscle, hydrogen transfer across the permeability barrier has been classically associated with a glycerol-phosphate shuttle (4,5). In this report evidence is presented indicating the existence of another mechanism for

ABBREVIATIONS: P-5-CA= DL-Δ'-pyrroline-5-carboxylate; HEPES= N-2-Hydroxyethylpiperazine-N'-2-ethanesulfonic acid.

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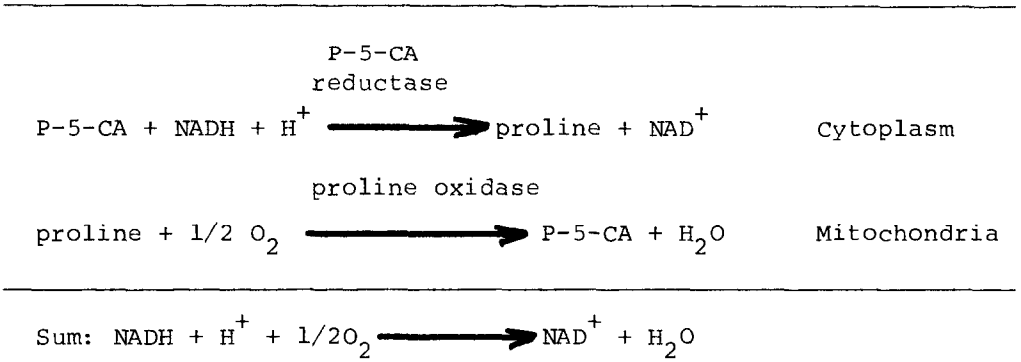
hydrogen transfer, a proline shuttle, which catalyzes oxidation of extramitochondrial NADH according to the following scheme: (2)




## METHODS

Blowflies (Phormia regina), 7-21 days old, were immobilized by chilling. The thoraces (90-100) were isolated and placed in an ice-cold mortar containing 10 ml isolation medium consisting of 0.25 M sucrose, 0.5 mM HEPES, 10 mM EDTA, 0.5% bovine serum albumin, pH 7.3. The thoraces were gently pounded and the resultant brei filtered through four layers of cheesecloth previously moistened with isolation medium. The filtrate was centrifuged at 120 x g for 3 minutes, the supernatant drawn off and the sediment discarded. The supernatant was centrifuged at 35,000 x g for 20 minutes. The reddish-brown mitochondrial pellet was resuspended in 2 ml isolation medium (minus EDTA). The supernatant fraction, designated below as soluble fraction, was clarified by filtering through four layers of cheesecloth. Unless otherwise noted, all operations were carried out at 0-4°C.

Nicotinamide nucleotide oxidation was followed fluorometrically. Excitation, 364 nm; emission, 425 nm. Oxygen consumption was measured with a Gilson Oxygraph with Clark-type electrode. DL-$\Delta$'-pyrroline -5-carboxylate synthesis and quantitative assay with O-aminobenzaldehyde was according to Williams and Frank(6). Solutions of pyrroline-5-carboxylate were stored in 1.0 M HCl at 4°C. and in the dark. Prior to use they were neutralized with 1.0 M NaOH. Protein was estimated employing the biuret reagent (7).

## RESULTS AND DISCUSSION

In Fig. 1 is a fluorometric recording of NADH oxidation (solid line) catalyzed by soluble P-5-CA reductase of the proposed proline shuttle. The oxidation was rotenone-

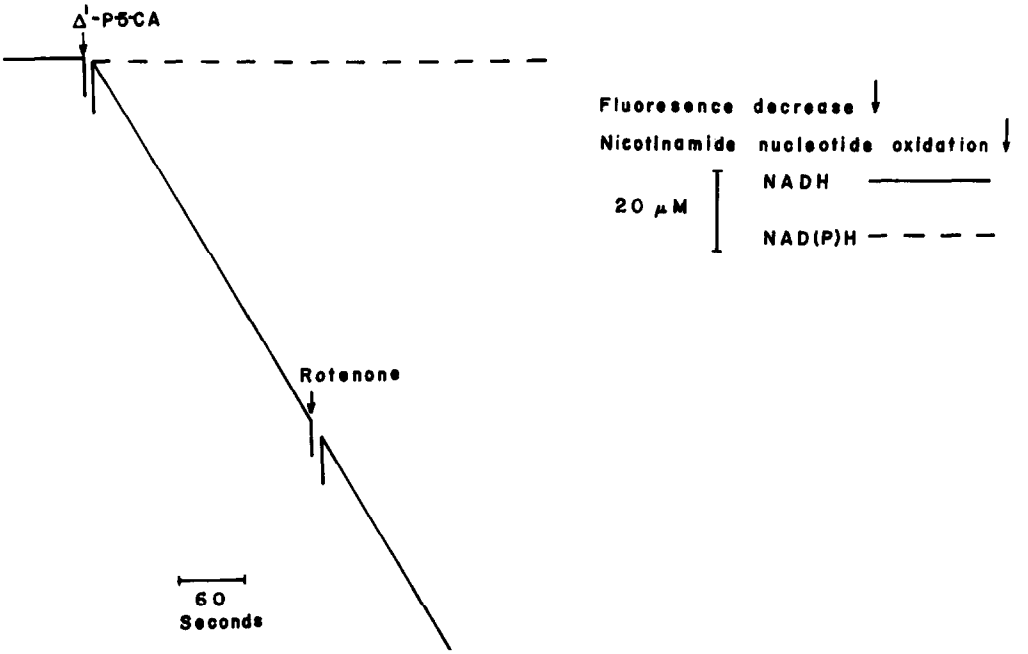



Fig. 1. Soluble NADH-linked Δ'-P-5-CA reductase activity of blowfly flight muscle. The reaction mixture contained 0.25 M sucrose, 5 mM HEPES, 0.5% bovine serum albumin, 20 mM sodium phosphate, 1.2 mM $MgCl_2$ and 0.62 mM NADH (solid line) or NAD(P)H (dashed line). At the points indicated, 0.78 mM Δ'-P-5-CA and 5.4 μM rotenone were added. Soluble protein, 6.8 mg. pH 7.3. Volume, 1.3 ml. Temperature, 25°C.

insensitive. NAD(P)H used in place of NADH supported no significant reductase activity (dashed line). This specificity of the reductase for NADH is an important consideration (2) in assigning to the proline shuttle a role in the transfer of hydrogen from extramitochondrial NADH to the respiratory chain. Other reductases show no similar nicotinamide nucleotide specificity. Apparent $K_m$ values for P-5-CA and NADH were 190 μM and 57 μM respectively. Irreversibility of the reductase reaction (8,9) was confirmed for the flight muscle enzyme under a variety of conditions. In homogenates of rat liver, kidney and

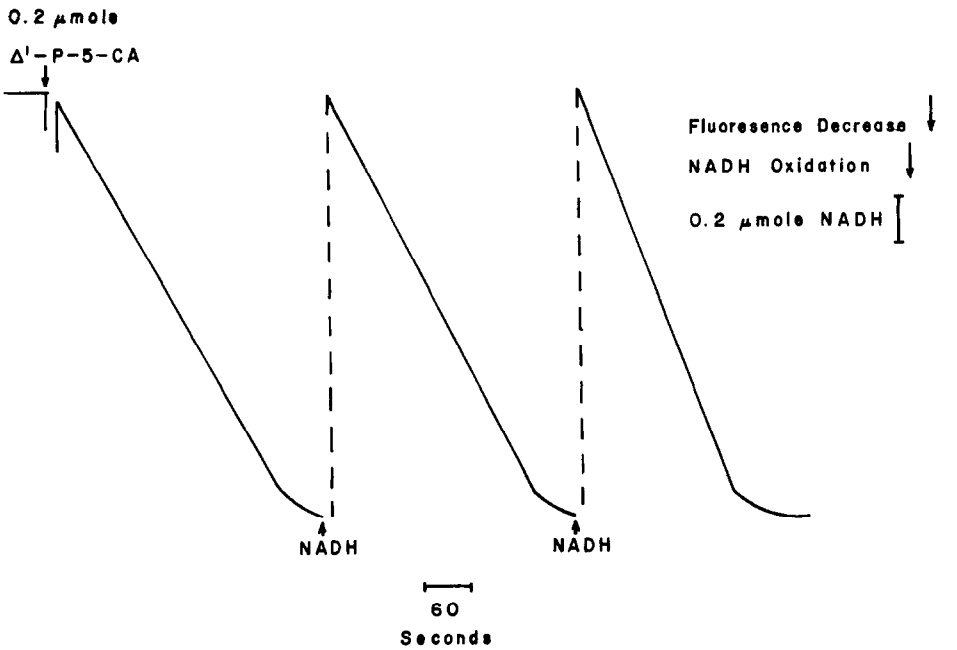



Fig. 2. Oxidation of NADH by reconstituted proline shuttle. The reaction mixture contained 0.25 M sucrose, 5 mM HEPES, 0.5% bovine serum albumin, 20 mM sodium phosphate, 1.2 mM $MgCl_2$, 16 mM ADP, 6.2 μM rotenone and 2.0 μmoles NADH. At the points indicated, 0.2 μmole Δ'-P-5-CA and 2.0 μmoles NADH were added. Soluble protein, 0.7 mg. Mitochondrial protein, 0.65 mg. pH 7.3. Volume, 1.13 ml. Temperature, 25°C.

small intestine the reductase is inactivated by cold treatment (10). The flight muscle reductase exhibited no similar cold inactivation.

Addition of catalytic amounts of P-5-CA to a reaction system consisting of soluble and mitochondrial components of the shuttle plus NADH should foster shuttle activity which in turn should persist until all available NADH is oxidized. In Fig. 2, it can be seen that addition of 0.2 micromole P-5-CA to spark shuttle activity resulted in the oxidation of all available NADH, initially present in excess (2.0 micromoles) to

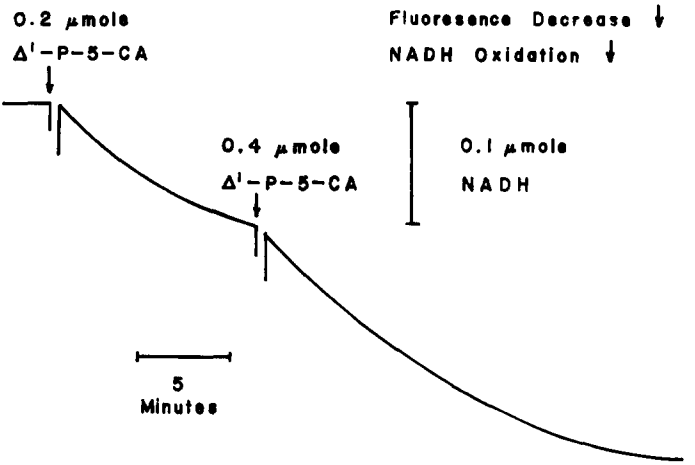



Fig. 3. Dependence on mitochondrial fraction for shuttle activity. The reaction system was similar to that employed in the Fig. 2 studies with the exception that the mitochondrial fraction was omitted.

P-5-CA. Addition of another aliquot of excess NADH fostered a resumption of shuttle activity until NADH supply was exhausted again.

No reductase activity was associated with mitochondrial fractions. Requirement of the mitochondrial fraction for shuttle activity is demonstrated in the studies reported in Fig. 3. Here, it can be seen that as a result of the omission of the mitochondrial fraction, NADH supply was not limiting. Rather, P-5-CA became limiting. Also, from the data in Fig. 3 it can be seen that P-5-CA and NADH utilization was in accordance with the established 1:1 stoichiometry for the reductase reaction, assuming, that only the L-isomer of P-5-CA is utilized (6,11).

In the case of flight muscle, P-5-CA is also oxidized to glutamate via the action of intramitochondrial P-5-CA

dehydrogenase, EC 1.5.1.12 (12). The question arose as to whether a proline shuttle is feasible given the possibility of competition between the reductase and the dehydrogenase for P-5-CA. In the shuttle studies of Fig. 2, such competition was ruled out because the shuttle was run in the presence of rotenone which would inhibit $NAD^+$-linked dehydrogenase activity. When shuttle activity was assayed in the absence of rotenone, activity was about the same as it was in the presence of rotenone. A lack of interference on the part of the dehydrogenase reaction may be due to the fact that the dehydrogenase exhibits a low affinity for P-5-CA. Brosemer and Veerabhadrappa (13) reported that in the case of homogenates of Locusta migratoria flight muscle, affinity of the dehydrogenase was so low that $K_m$ could not be reliably determined with their assay procedure. We have found that blowfly mitochondria oxidized P-5-CA (50 mM) very slowly (25 nano-gm-atoms oxygen/min/mg protein).

Whether both a glycerol-phosphate and a proline shuttle are operational in the flight muscle of blowflies awaits further investigation. At this point, however, the following observation may be relevant. In fly homogenates, ring-substituted cinnamic acids are inhibitory to the glycerol-phosphate shuttle. Yet, when injected directly into the thorax of the intact fly, they are non-toxic (14). Several explanations might account for this lack of lethal effects in vivo. One is, that mechanisms other than the glycerol-phosphate shuttle exist for the oxidation of extra-mitochondrial NADH. The work presented here suggests that a proline shuttle could be such a mechanism.

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