THE COUPLED OXIDATION OF SUCCINATE AND L-CYSTEINESULFINATE BY SOLUBLE ENZYMES*

by

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The authors have previously reported[1,2] that mitochondrial acetone powders prepared from a variety of mammalian and avian tissues and high-speed centrifuged extracts of *Proteus vulgaris* OX-19, grown under somewhat different conditions than used in the authors' earlier work[3] catalyze the rapid oxidation of L-cysteinesulfinate (L-CSA), without the formation of cysteate, in the presence of a previously described pyridine nucleotide preparation[4] from yeast and a suitable dye. When this coenzyme preparation was passed through a column of Dowex-1-Cl, neither the effluent (which contains all the pyridine nucleotide initially present) nor any of the eluates could induce the oxidation of L-CSA, but the effluent and a narrow fraction of 0.01 *N* HCl eluate together reestablished maximal rate of L-CSA oxidation. The pyridine nucleotide component in the effluent was quantitatively replaced by DPN but not by TPN. Under anaerobic conditions no reduction at 340 mμ was observed in the system.

The factor in the eluate ("eluate factor", E.F.) was assayed by the initial rate of O_2 uptake in the presence of excess L-CSA, DPN, phenazine methosulfate, and enzyme (Fig. 1). In routine work beef heart or rat liver mitochondrial acetone powders or *Proteus* extract served as a source of the enzyme.

Considerable purification of the factor was achieved by chromatography of the pyridine nucleotide preparation[4] on Dowex-1-Cl, using increasing strength of HCl (gradient elution technique[5])**. The preparation thus obtained contained 5-adenylic acid (5-AMP) as the only recognizable impurity; no P, S, N, carbohydrate, or ultraviolet absorbing material was present beyond that attributable to 5-AMP. The latter compound is not concerned with the reaction under study.

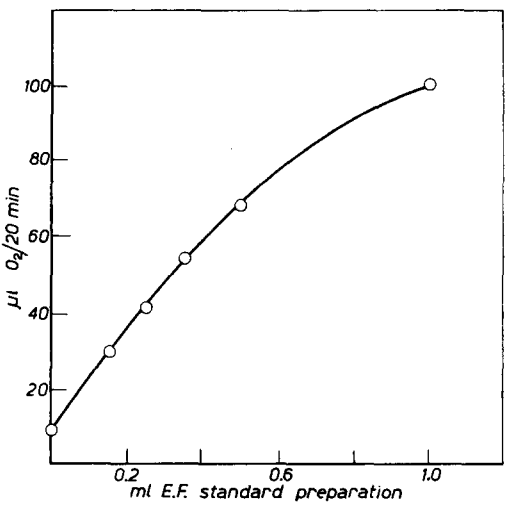

Fig. 1. Manometric assay of "eluate factor". Each Warburg vessel contained 6 mg of a homogenate of rat liver mitochondrial acetone powder, 0.05 *M* phosphate, pH 7.6, 2.4 μM DPN, 90 μM L-CSA, 2 mg phenazine methosulfate, and varying quantities of the factor. Temp., 38°. A soluble extract of rat liver, beef heart, or *P. vulgaris* serves equally well in the assay.

Without L-CSA, the purified factor was very slowly oxidized by the enzyme preparations in the presence of DPN and faster in the absence of DPN. With excess L-CSA and DPN, the factor was rapidly and stoichiometrically oxidized in a coupled reaction: one mole each of CSA and of the factor reacted to give one mole of pyruvate and one of aspartate and sulfate. Pyruvate was identified by paper chromatography, crystalline lactic dehydrogenase, and by means of its 2,4-dinitrophenylhydrazone. Aspartate was identified by chromatography on Dowex-50 (H^+), paper chromatography (4 solvents), and by isolation, followed by decarboxylation with chloramine T, and by conversion to oxaloacetate with purified α-ketoglutaric-aspartic transaminase. The products shown could have arisen from CSA + malate (or fumarate) in view of the coupled reaction demonstrated in the preceding note[6], but the factor, although a dicarboxylic acid, contained no fumarate or malate.

Isolation of the "eluate factor" was achieved by treatment of the purified preparation with 5-nucleotidase (to remove 5-AMP) and repetition of the ion-exchange chromatography on Dowex-1-Cl. The fractions active in the enzyme assay (Fig. 1) were combined, taken to dryness, and twice re-

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crystallized from water. The material was identified as succinic acid by its melting point (compound, 190°; succinic acid, 190.5°; mixed m.p., 190.5°), by paper chromatography (2 solvent systems), by its pK' values, and by its neutral equivalent (compound, 58.6; theory 59). Succinic acid quantitatively replaced the factor in the coupled oxidation in all of the enzyme preparations studied (Table I).

TABLE I

Substrate	*O_2 uptake* μ atom*	*CSA removed μM*	*Aspartate formed μM*	*Pyruvate formed μM*	*$SO_4^=$ formed μM*
10 μM succinate + 15 μM CSA	27.8	9.8	10.1	9.7	0.7–0.8
10 μM fumarate + 15 μM CSA	16.0	10.2	9.1	9.4	0.6

* At cessation of O_2 uptake. Note that in the presence of 1 mg methylene blue the oxidation of sulfite to sulfate is incomplete (0.6–0.8 atoms O_2 per mole). L-CSA and aspartate were determined, following chromatography on Dowex-50 (H^+ cycle), by means of ninhydrin and chloramine T, respectively, and pyruvate by the oxidation of DPNH with crystalline lactic dehydrogenase. Conditions, 12 mg rat liver mitochondrial acetone powder suspension, 0.05 M phosphate buffer, pH 7.6, 1.2 μM DPN, and 1 mg methylene blue per vessel; temp., 38°. All values are corrected for the slight blank given by CSA in the absence of dicarboxylic acids.

The stoichiometry of the reaction may then be written as:

$$\text{L-CSA} + \text{Succinate} + 1.5\ O_2 \xrightarrow{\text{DPN}} \text{Aspartate} + \text{Pyruvate} + SO_4^= + H^+ \qquad (1)$$

The following observations indicate that the reaction sequence is succinate $\longrightarrow$ fumarate $\longrightarrow$ malate $\xrightarrow{\text{DPN}}$ oxaloacetate; oxaloacetate + CSA $\longrightarrow$ aspartate + β-sulfinylpyruvate $\longrightarrow$ pyruvate + $SO_3^= \longrightarrow SO_4^=$. First, the coupled oxidation is competitively inhibited by malonate. Second, succinate is oxidized by the system in the absence of CSA only if DPN is omitted; this is in accord with the known great sensitivity of succinic dehydrogenase to oxaloacetate[7]. Third, CSA may be replaced by glutamic acid (plus internally contained transaminase) or by TPN plus "malic enzyme", either of which serve to remove oxaloacetate and thereby permit succinate oxidation to proceed. Neither system, however, is as efficient as the CSA transaminase, in accord with the fact that the fastest and most complete removal of oxaloacetate is provided by its transamination with CSA[6]. Fourth, the dependence of the entire reaction sequence on the initial dehydrogenation of succinate is shown by the following experiment. Homogenates of rat liver mitochondrial acetone powder catalyze both reaction (1) and the one-step oxidation of succinate to fumarate with either methylene blue or phenazine methosulfate as carrier. Soluble extracts prepared from this source catalyze reaction (1) and the succinate to fumarate step when phenazine methosulfate is the carrier, but neither reaction proceeds in the presence of methylene blue[8]. Fifth, the fact that all the enzymes in the postulated reaction sequence are present is shown by the observation that in soluble extracts, with methylene blue as carrier, fumarate plus CSA are smoothly oxidized to the products shown in reaction (1) but 1 atom less O_2 is consumed than in the presence of succinate.

In the coupled oxidation of CSA + succinate, in the presence of low succinate concentrations (as in Fig. 1), the rate of O_2 uptake is 5 to 6 times higher than with succinate alone, even when DPN is omitted and no oxaloacetate accumulates, and at high succinate concentrations the initial rate is maintained much longer in the coupled system. These findings may be explained by the competitive inhibition of succinic dehydrogenase by fumarate (which is particularly pronounced with the bacterial enzyme) and the efficient removal of the latter by conversion to aspartate in the coupled reaction[8].

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