THE D-LACTIC CYTOCHROME REDUCTASE OF YEAST: ITS CHEMICAL NATURE, ORIGINS, AND RELATION TO THE RESPIRATORY CHAIN*

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In a previous publication (Singer et al., 1960), we have described the oxidation of D(-)lactate by cytochrome c in cell-free extracts of aerobic yeast. This reaction could not be ascribed to either of the known lactic dehydrogenases of yeast (the hemoflavoprotein cytochrome b_2 and the general D-α-hydroxy acid dehydrogenase) and thus indicated the existence of a hitherto unrecognized enzyme. The enzyme has now been isolated at approximately 500-times the specific activity previously reported for autolysates. The purpose of this communication is to report the general characteristics of the enzyme and its relation to the other lactic dehydrogenases and the electron transport system of yeast.

Nygaard, who has independently observed the D-lactate—cytochrome c reaction in yeast preparations (Nygaard, 1960, a,b), has reported that the majority of the activity resides in the "respiratory particles". We have found that about 90% of the D-lactic cytochrome reductase activity of baker's yeast (Red Star) is recovered in the particles isolated by breaking the cells with a Nossal shaker followed by differential ultracentrifugation, as previously described, except for the omission of versene (Hebb et al., 1959). Such particles have been shown to contain all the usual components of the respiratory chain, the enzymes of the Krebs cycle, and show good oxidative phosphorylation. They have been

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referred to as mitochondria, although in reality they are probably membrane fragments.

The particles or extracts thereof oxidize D(-)lactate vigorously with either cytochrome c or phenazine methosulfate as electron acceptor. In contrast to the D-α-hydroxy acid dehydrogenase of yeast (Boeri et al., 1960) which reduces ferricyanide, menadione, methylene blue, and 2,6-dichlorophenolindophenol (DCPIP) but not phenazine methosulfate or cytochrome c, the reductase fails to react with DCPIP, ferricyanide or menadione. While in the assay to be described the measured rate varies only slightly with cytochrome concentration, there is an appreciable dependence on dye concentration in the phenazine assay. At V_{max} with respect to the acceptor and 30° the rate with phenazine is 8.4 times that obtained with cytochrome c. In view of the inactivity of DCPIP, a convenient spectrophotometric method for the assay of the enzyme utilizes the latter dye as terminal acceptor and phenazine methosulfate as a catalyst. The rate of decolorization is measured at 600 mμ, varying the concentration of the phenazine dye. The usual assay is performed at 30°, pH 7.5, in 0.05 M imidazole buffer, with 0.01 M D(-)lactate as substrate and either cytochrome c (0.7 mg./ml.) or phenazine methosulfate (varied between 0.06 and 0.66 mg./ml.) as oxidant. The inclusion of versene (Nygaard, 1960 c) is ill-advised for it may dissociate the metal component of the enzyme. Interference in the cytochrome c assay by the cytochrome oxidase contained in the particles and in partially purified extracts may be overcome, without anaerobiosis, by the inclusion of 5×10^{-5} M HCN, which inhibits the oxidase almost completely with only a negligible effect on the D-lactic cytochrome reductase. A more convenient alternative is to adjust the extracts to pH 4 at 0° for a few minutes, followed by neutralization, which causes complete inactivation of the oxidase without harming the reductase.

Digestion of the yeast particles with a bacterial lipase of broad

specificity, or a concentrate of the autolyzing enzyme of yeast, failed to solubilize the enzyme. Cobra venom (a source of phospholipase A) caused extensive inactivation without major solubilization. The extraction of acetone powders of the particles with various buffers has shown that as the pH is raised both inactivation and solubilization increase. At pH values where the enzyme is stable (at and below 7.8) little or no activity was extracted. Butanol-ether powders (Singer et al., 1957) behaved similarly: at pH 8 only some 18% solubilization was obtained; at higher pH values the enzyme was inactivated. In contrast, some 60% of the activity is extracted with 4% (w/v) Triton X-100 at pH 6.5 without inactivation and the resulting extract may be 20-fold purified with calcium phosphate gel treatment, as compared with the particles. Although extraction with detergents, even of the non-ionic type, such as Triton, seldom, if ever, yields soluble enzymes in the strict sense, such preparations have been nonetheless useful in characterizing the reductase since they possessed high specific activity and were very stable.

As compared with the D-α-hydroxy acid dehydrogenase, this enzyme has a very restricted specificity. The substrates found to be active are oxidized at the following rates relative to D(-)lactate (V_{max} values at 30°, with K_M in parentheses): D(-)lactate, 1 (2.8×10^{-4} M); D-α-hydroxybutyrate, 0.41 (1.4×10^{-3} M, determined with DL substrate, uncorrected for possible inhibition by L component). D-malate, α-hydroxyisobutyrate, α-hydroxyisovalerate, α-hydroxycaproate, α-hydroxyisocaproate, glycollate, L-α-hydroxy acids, and β-hydroxybutyrate were not oxidized. Dicarboxylic acids, such as oxalate, L-malate, succinate, and malonate inhibit the enzyme, but its sensitivity to these agents is much less marked than that of the D-α-hydroxy acid dehydrogenase. The pH optimum is 7.5 (at 30° in imidazole or Tris buffer). The enzyme is very stable at neutral and mildly acid reactions and extremely unstable above pH 8. While in other respects the reductase differs sharply from the D-α-hydroxy acid

dehydrogenase, the two show remarkable similarity in that a divalent metal appears to be required for the activity of both, functioning as the substrate binding site in both enzymes. Boeri et al. (1960) have shown that the latter enzyme, a metalloflavoprotein, is resolved by anaerobic dialysis against a chelator, followed by a second dialysis to remove the chelator. The resulting inactive enzyme is reactivated by various divalent metals. In unpublished studies with Dr. A. Ghiretti of this laboratory we have shown that anaerobic dialysis of the reductase for 12 hours against 3.5 mM o-phenanthroline (or 4 mM versene) in 0.01 M phosphate, pH 6.5, followed by 3 hours dialysis against 0.01 M phosphate, and finally passage through a column of Sephadex G 25 results in complete inactivation of the reductase. Reactivation has been observed at very low concentrations of Zn^{++}, Co^{++}, Mn^{++}, Mg^{++}, and Fe^{++}. The V_{max} and the K_M varies not only with the metal but also with the substrate employed, even at infinite concentration of the latter. It may be mentioned that on the basis of inhibition studies Tubbs (1960) has very recently suggested that the D-α-hydroxy acid dehydrogenase of kidney mitochondria may also employ a metal component as the substrate binding site.

While it would be of interest to compare the results reported here with the properties of Nygaard's preparations, it is difficult to do so in view of the conflicting and contradictory claims in his publications (e.g., the presence vs. the absence of heme in the enzyme, absolute vs. partial stereospecificity, reactivity with ferricyanide and DCPIP in some preparations but not in others (Nygaard, 1960, a,b,c,d)). One point raised by Nygaard may be examined, however, in the light of the information presented. On the basis of the sequence of formation of L-lactic dehydrogenase (cyt. b_2) and D-lactic cytochrome reductase activities during O_2 —adaptation of anaerobic yeast, Nygaard (1960 b,c) revived an earlier hypothesis suggesting that the D-α-hydroxy acid

dehydrogenase of anaerobic cells is a precursor of L-lactic dehydrogenase and added that the D-lactic cytochrome reductase may be an intermediate in this transformation. Elsewhere (Singer et al., 1960; Gregolin et al., 1961) we have shown that at present there remain no grounds for assuming that the D-α-hydroxy acid dehydrogenase is a precursor of cyt. b_2. The same reasoning may be extended to D-lactic cytochrome reductase. Under suitable conditions of adaptation this enzyme is synthesized without disappearance of D-α-hydroxy acid dehydrogenase and under other conditions the latter disappears without formation of the former. Further, the synthesis of the reductase does not necessarily precede that of cyt. b_2. Further, the substrate specificity of the reductase is so different from that of either the D-α-hydroxy acid dehydrogenase or of cyt. b_2 as to render any suggestion of a precursor-product relation very tenuous.

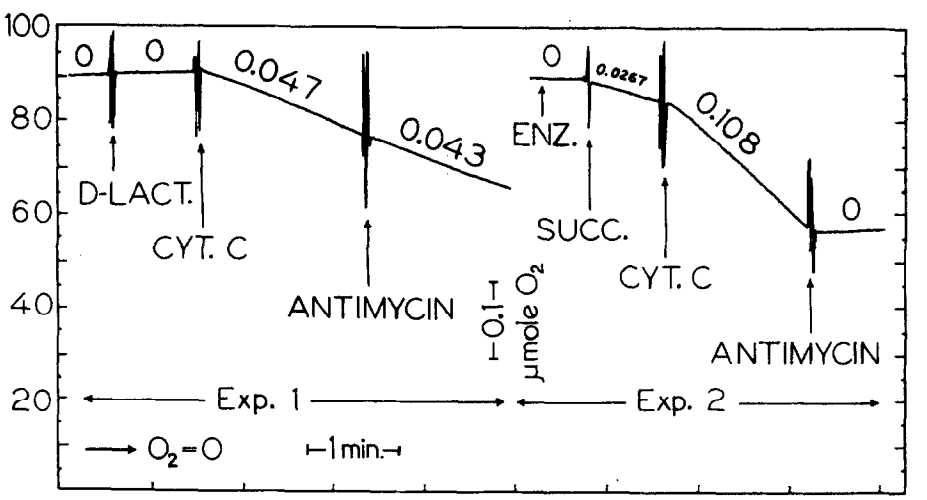

Fig. 1. The oxidation of D(-) lactate and of succinate by a respiratory chain preparation from baker's yeast. The experiments were performed with a rotating Pt microelectrode at room temperature (20°). In a total volume of 2 ml. the following components were present: 0.05 M imidazole buffer, pH 7.5; 0.01 M NaCl, and 6.6 mg. particles (biuret protein). Exp. 1, 20 μmoles D-lactate, 1 mg. cyt. c, and 6 mμmoles of antimycin A added at points indicated. Exp. 2, same as 1 except that 40 μmoles succinate were added instead of lactate. The numbers above the tracings are μmoles O_2 uptake per min.

It should be emphasized that the functioning of cytochrome c as an acceptor for the reductase need not indicate that cytochrome c is the normal reaction partner of the enzyme in the cell. In fact, the experiment reproduced in Fig. 1 strongly argues against the possibility that the reductase is functionally linked to the respiratory chain. As shown in the Figure, D-lactate cannot pass electrons to the respiratory chain under conditions that lead to vigorous oxidation of a genuine respiratory chain-linked substrate, succinate. When external cytochrome c is added oxidation of D-lactate is initiated, since reduced, soluble cytochrome c is readily reoxidized by the cytochrome oxidase present, but the ensuing O_2 uptake is antimycin-insensitive. In contrast, the oxidation of succinate, whether or not stimulated by addition of cytochrome c, is completely abolished by antimycin.

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