THE D-α-HYDROXY ACID DEHYDROGENASE OF YEAST†

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The finding that anaerobically grown yeast, although devoid of cytochrome b_2, contrary to earlier impressions (Slonimski, 1953), rapidly oxidizes DL-lactate (Lindenmayer and Smith, 1957; Slonimski, 1958; Boeri et al., 1958; Singer and Kearney, 1958) assumed special interest when it was discovered (Labeyrie et al., 1959) that the enzyme concerned was active on D(-) lactate and could not oxidize the L(+) form of this substrate, while the enzyme associated with cytochrome b_2 was specific for L(+) lactate. A moderate degree of purification has been reported in preliminary communications from two laboratories but the details have not yet been published (Boeri et al., 1958; Slonimski and Tysarowski, 1958). Both groups considered the enzyme to be a flavoprotein but the nature of the prosthetic group was not unambiguously demonstrated.

In another paper (Singer et al., 1960),we have reported that the dehydrogenase is present in both anaerobic and aerobic yeasts, normal or "petite" mutants, but that the activity depends on the conditions of growth and is highest in anaerobically cultured cells, as also reported by Labeyrie et al. (1959). The purpose of this note is to show, (1) that the enzyme is not a specific D(-) lactic dehydrogenase but a D-α- hydroxy acid dehydrogenase, (2) that the catalyt:

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activity appears to depend on a divalent metal which may be reversibly removed from the holoenzyme, and (3) to describe some general properties of the dehydrogenase.

The dehydrogenase has been purified by a procedure to be published elsewhere from an autolysate of commercial distillation yeast. It was assayed by following the rate of reduction of ferricyanide spectrophotometrically at 420 mμ at pH 8 in phosphate or Tris buffer. In the best preparations so far obtained 32 μmoles of D-lactate were oxidized per min. per mg. protein (fixed lactate concentration, 22° C).

The purified enzyme oxidizes D(-) lactate, D-malate, DL-α-hydroxybutyrate, and DL-glycerate. Glycolate was essentially inert as a substrate and the following substances were not oxidized: L(+) lactate, L-malate, DL-β-hydroxybutyrate, DL-mandelate, D-alanine, D-leucine, DL-serine, L-leucine, and L-proline. At 30°, the relative rates of oxidation were as follows: D(-) lactate = 100; D-malate = 89; DL-α-hydroxybutyrate = 116, and DL-glycerate = 1.9. The latter two values are minimal, since the activities on the D-forms may be higher if the L-isomers should be competitive inhibitors (see below). The K_m values for D(-) lactate and D-malate, respectively, were 2 and 16 m$\underline{M}$ (Fig. 1).

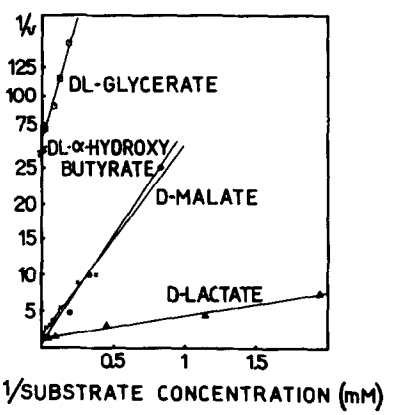

Fig. 1. Reactivity with various substrates.

At 20° the relative rates of oxidation were different: with the rate of oxidation of D(-) lactate taken as 100, those for D-malate, DL-α-hydroxybutyrate, and DL-glycerate were 43, 93 and 1.6. The K_m for D(-) lactate was 1.6 m$\underline{M}$. It

appears that the activation energies for the oxidation of the various substrates differ appreciably. Purification of the enzyme did not change the relative reaction rates with various substrates.

The following substances are efficient competitive inhibitors of the enzyme, in decreasing order of effectiveness, with the K_i values at 30°, determined with D(-) lactate as substrate, given in parentheses: oxalate (2.5×10^{-6} M); malonate (0.9×10^{-3} M); tartronate (0.8×10^{-3} M); α-ketoglutarate (1.4×10^{-3} M); L-malate (1.05×10^{-3} M), and L(+) lactate (6.2×10^{-2} M). Pyruvate and oxalacetate are less efficient inhibitors and the inhibition is of a mixed type (Dixon, 1958). The following substances were not inhibitory at the concentrations shown in parentheses: succinate (0.016 M), fumarate (0.016 M), citrate (0.032 M), glycolate (0.016 M), mandelate (0.016 M), D-alanine (0.008 M), and DL-serine (0.008 M). The remarkable inhibitory power of oxalate may explain the observations of Bernheim (1928) on the inhibition of the lactate-methylene blue reaction in yeast by oxalate, although it is not clear with which of the various lactic dehydrogenases of yeast this author was dealing.

As was also observed by Curdel et al. (1959), in the presence of ethylene diamine tetraacetate (EDTA), the dehydrogenase is gradually inactivated. In order to see whether this effect was due to the presence of a metal essential for the activity of the enzyme, the dehydrogenase was dialyzed anaerobically against 4 mM EDTA, 7 mM K_2HPO_4, and 3 mM Na thioglycolate at pH 7.4, 0°, for 12 hours, followed by 3 hours dialysis in the same medium except for the absence of EDTA. The second dialysis served to remove residual EDTA. The dialyzed enzyme was completely inactive without added metal ions, but upon treatment with low concentrations of Zn^{++} enzymic activity reappeared (Fig. 2). In practice a concentrated solution of the enzyme was preincubated for 10 minutes at 0° with the metal and a small aliquot was then diluted with the assay medium; the concentrations shown in Fig. 2 are those in the final assay medium. Half-maximal activity was reached at circa 2.8×10^{-6} M Zn^{++}.

Mg^{++}, Mn^{++}, and Co^{++}, in decreasing order of effectiveness, were less

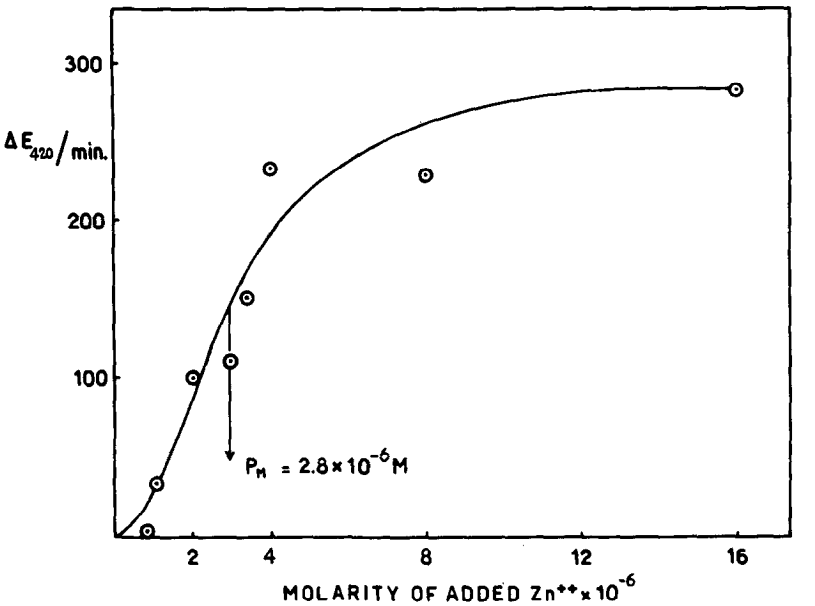

Fig. 2. Reactivation of resolved enzyme by Zn^{++}

active than Zn^{++} both with regard to the extent of activation reached and apparent affinity for the enzyme, and Ni^{++} was inactive. While these results strongly suggest that a divalent metal is required for the activity of the dehydrogenase, the fact that Zn^{++} is the best reactivator of the dialyzed enzyme cannot be taken as proof that it is the metal ion normally present in the enzyme. Identification of the latter will have to await the isolation of highly purified or homogeneous preparations. During the preparation of this paper a preliminary note by Curdel et al. (1959) appeared, in which they concluded that Zn^{++} is a component of this enzyme. This conclusion is based on the relief of the EDTA-inhibition of the dehydrogenase by an amount of Zn^{++} slightly in excess of the chelator present. As detailed elsewhere (Singer and Massey, 1957), inhibition by chelators and its reversal does not constitute unambiguous proof of the role of a metal in the action of an enzyme. In this instance, the metals which reversed the EDTA inhibition (Zn^{++} and Co^{++}) may well have acted by removing the inhibitor, owing to their high affinity for EDTA, whereas those which they found to be inactive in this test but which we have found capable of reactivating the resolved enzyme (Mg^{++}, Mn^{++}) form less

stable complexes with EDTA. Failure to remove the inhibitor prior to addition of the metal may explain also the high concentration of Zn^{++} required for reactivation in the experiments of Curdel et al. (1959).

Some other properties of the dehydrogenase are as follows. The pH optimum at 20° is 8.0. The measured activity depends significantly on the concentration of ferricyanide. The enzyme is relatively labile but may be preserved for several days at -20° under anaerobic conditions. Thioglycolate stabilizes the enzyme. Its flavoprotein nature has now been demonstrated by the reduction of purified preparations with D(-) lactate; the resulting difference spectrum shows typical flavoprotein peaks at 450 and 380 mμ.

With $FMNH_2$ as electron donor the enzyme catalyzes the reduction of pyruvate and thus it may be responsible for the considerable amounts of D(-) lactate present in yeast (Singer et al., 1960).

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