enzyme was achieved by the adsorption of impurities on the cation-exchange resin IRC-50 (NH_4^+ form) followed by column chromatography on cellulose. At this stage, the ratio of light absorption at 280 mμ to that at 260 mμ was 1.1 indicating the presence of other u.v.-absorbing material, but it is not known whether this constitutes a contaminant or forms part of the enzyme. The enzyme was relatively unstable after IRC-50 adsorption and gradually lost about 20 % of its activity on storage at 0° for 2 weeks.

Spectroscopic examination of the purified enzyme gave no indication of the presence of bacterial cytochrome[3]. The enzyme was completely inactive in the methylene blue assay system without added iron. The dependence of the reaction rate on iron concentration with different amounts of the enzyme is shown in Fig. 1. The concentration of iron required for half-maximum velocity calculated by the method of LINEWEAVER AND BURK[4] was found to be $4.6 \cdot 10^{-5}$ *M*. Essentially the same values were obtained when the amount of enzyme was varied from 0.45 to 1.8 μg.

The enzyme showed no activity when Fe was substituted by equivalent amounts of any of the following: Mn^{++}, Co^{++}, Ni^{++}, Zn^{++}, Pb^{++}, molybdenum (as molybdenum trioxide or ammonium molybdate), vanadate, tungstate, chromate or borate. None of these substances was found to enhance the activity of the enzyme in the presence of optimum amounts of iron salts. The addition of cytochrome c_3 isolated[3] from the bacteria was also without any effect.

These observations indicate that ionic iron is required for hydrogenase activity with methylene blue as hydrogen acceptor.

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Partial modification of baker's-yeast lactic dehydrogenase

The problem of the connection between the dehydrogenation of a substrate by a dehydrogenase such as succinic dehydrogenase or diaphorase and electron transport in the cytochrome system has been the subject of much conjecture. A definitive experimental approach to this study, however, had to await the purification of the enzymes and cytochromes related to these reactions. SINGER *et al.*[1] have succeeded in purifying succinic dehydrogenase to a homogeneous state, assaying its activity with phenazine methosulphate[2]. Their dehydrogenase, however, fails to reduce cytochrome *b* prepared with cholate[3], and diaphorase purified by the method of STRAUB[4] reduces neither cytochrome *b* nor c_1, though according to all of the schemes of "*a*, *b*, *c* and c_1" type electron-transferring systems variously proposed[5–7], these cytochromes should be the

first to receive electrons from succinate and reduced diphosphopyridine nucleotide. Accordingly some workers think that some factor other than the cytochromes, such as a lipid or vitamin may mediate between these enzymes and the cytochromes. Others favor the view that the primary dehydrogenase may be a hemoprotein, possibly identical with cytochrome b or c_1.

From baker's yeast, cytochrome c and b_2 have been crystallized in our laboratory[8,9], and lactic dehydrogenase originally containing cytochrome b_2 and flavine mononucleotide by APPLEBY AND MORTON[10]. Therefore, we[11,12] have studied some interrelationships between cytochrome c, cytochrome b_2 and baker's-yeast lactic dehydrogenase to clarify the connection between the dehydrogenation of lactate and cytochrome b_2. The results suggested the possibility that a certain linkage between the dehydrogenase and cytochrome b_2 moieties of the whole enzyme might be split. This led us to obtain a cytochrome-free lactic dehydrogenase moiety corresponding to the succinic dehydrogenase obtained by SINGER *et al.*

Baker's-yeast lactic dehydrogenase was purified to a specific activity (Q_{MB}[13] at 30°) of about 4,000 by a slight modification of the method of BACH, DIXON AND ZERFAS[13]. The purified enzyme was not contaminated with other enzymes which could metabolize lactate such as a DPN-dependent lactic dehydrogenase, while it rapidly reduced cytochrome c and methylene blue in the presence of lactate. The enzyme was stored in a precipitated state at neutral pH in ammonium sulphate solution (about 60 % saturation) without a notable loss of its activity. Before use it was dissolved in distilled water and dialysed against several volumes of distilled water for 3 h in a refrigerator. The enzymic activity was assayed both by methylene blue reduction and oxygen uptake with phenazine methosulphate. The reduction of methylene blue was measured anaerobically in Thunberg tubes at 35° with 3.0 ml of a solution containing 2.5 μmoles methylene blue, 0.01 M sodium lactate and 0.1 M sodium acetate, pH 6.0 in the main chamber and 0.5 ml of the enzyme solution in the side arm. After 5-min preincubation, the components were mixed and the time required completely to bleach the methylene blue was measured. The relative activity was expressed as the reciprocal of this time in sec. The measurements with phenazine methosulphate were carried out aerobically in a Warburg manometer at 38° with 0.5 ml of the enzyme solution, 0.5 ml 0.1 M sodium phosphate, pH 6.0, 0.2 ml sodium lactate and 0.8 ml distilled water in the main chamber, and 0.2 ml 1 % phenazine methosulphate in the side bulb. After 5-min preincubation, the components were mixed and O_2 uptake measured for the initial 5 min. Over a certain range, the O_2 uptake was proportional to the enzyme concentration. In these experiments, 1 unit enzyme is defined as the amount which consumes 20 μl O_2/5 min.

Immediately after dialysis, the enzyme consumed O_2 and reduced methylene blue as shown by Fig. 1, curves A_{PM} and A_{MB}. If the dialysed enzyme was kept in a refrigerator, the methylene blue-reducing activity was lost more rapidly than the oxygen-consuming activity. After 3 days storage, the methylene blue-reducing activity was greatly depressed (curve B_{MB}) compared with its activity with phenazine methosulphate (curve B_{PM}).

This may fit in with the assumption mentioned above that during storage baker's-yeast lactic dehydrogenase is partially modified and there is a break-down of the electron-transferring pathway of the whole enzyme from its dehydrogenase moiety to the cytochrome b_2 moiety, but that there is no denaturation of the dehydrogenase

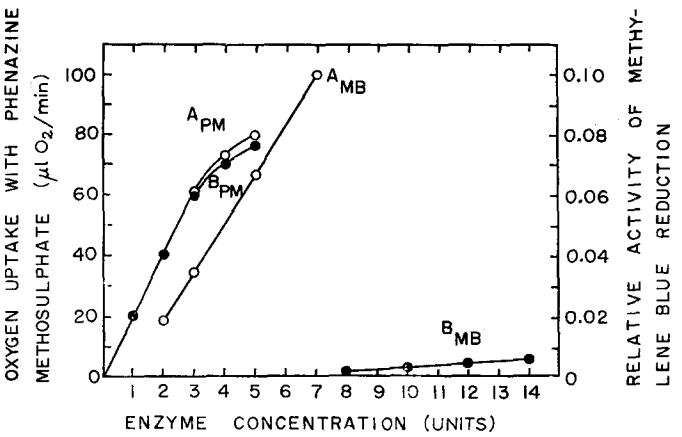

Fig. 1. Methylene blue-reducing and oxygen-consuming activities of baker's-yeast lactic dehydrogenase before and after modification. Curves A, the enzyme prior to storage; curves B, the enzyme after storage. A_{PM} and B_{PM}, oxygen-consuming activity assayed by the phenazine methosulphate method; A_{MB} and B_{MB}, methylene blue-reducing activity.

moiety itself. This modification was completely prevented by addition of lactate to the enzyme. It seems likely that this partial modification of baker's-yeast lactic dehydrogenase corresponds to the phenomenon observed by SINGER *et al.* with succinic dehydrogenase in which their dehydrogenase lost methylene blue-reducing activity prior to loss of phenazine methosulphate-reducing activity.

A similar modification of the lactic dehydrogenase was demonstrated within minutes at pH's 5 or 9 at 35–40°. These results are being further studied, as is the purification of the partially modified baker's-yeast lactic dehydrogenase.

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