## The purification of hydrogenase of *Desulfovibrio desulfuricans*

The purification and properties of the hydrogenase of *D. desulfuricans* have been described in earlier communications[1,2]. The purified enzyme so obtained was strongly colored and showed absorption bands at 555 mμ, 525 mμ and 419 mμ in the reduced state. Further, the increase in activity of the enzyme by the addition of iron salts was only about 2- to 2.5-fold, so that an absolute requirement of iron for enzyme activity could not be established.

The present report describes a modified purification procedure by which the specific activity of the enzyme was increased 5-fold to a value of $11.4 \cdot 10^6$ μl $H_2$ absorbed/h/mg enzyme N. The purified enzyme was completely inactive without added iron. The absorption bands that were observed earlier have now been shown to be due to cytochrome impurities.

The purification of the enzyme was carried out as follows: Hydrogenase was extracted from the acetone-dried cells as described earlier[2]. The supernatant was left at —18° overnight, thawed and precipitated impurities removed by centrifugation. The enzyme was then precipitated by adjusting the pH to 3.9 to 4.0 with 0.2 *M* acetic acid and the pink precipitate collected by centrifugation. The precipitate was redissolved in 0.2 *M* phosphate, pH 6.8, heated at 60° for 10 min and centrifuged to remove denatured proteins. The supernatant was then adjusted to pH 5.0 with 0.2 *M* acetic acid, reheated to 50° for 1.5 min and centrifuged. Hydrogenase was then precipitated by the addition of 3 vol. acetone at —10° and dried over $P_2O_5$ at 0° *in vacuo*. All the following operations were carried out at 0°. The precipitate was re-dissolved in 0.1 *M* phosphate, pH 6.5, and protamine sulfate solution added to remove most of the nucleic acids. The precipitate was centrifuged off and discarded. The enzyme was then adsorbed on $Ca_3(PO_4)_2$ gel at pH 6.8, eluted by 0.2 *M* phosphate, pH 8.0, and evaporated to about one-sixth of its volume. Further purification of the

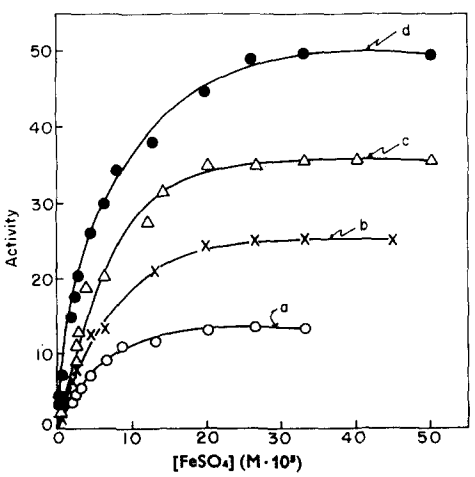


Fig. 1. Effect of iron concentration on hydrogenase activity. Activity is expressed as μl $H_2$ absorbed/min at 34°. The test system contained 200 μmoles tris(hydroxymethyl)aminomethane buffer, 2 mg crystalline bovine albumin, 20 μmoles cysteine, enzyme and $FeSO_4$ as indicated, in a final vol. of 1.5 ml; pH, 7.6. One side arm contained 10 μmoles methylene blue in 0.2 ml water. Alkaline pyrogallol was placed in the centre well and in the second side-arm: a – 0.45 μg enzyme; b – 0.9 μg enzyme; c – 1.35 μg enzyme; d – 1.8 μg enzyme.

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 m$\mu$ to that at 260 m$\mu$ 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 $\mu$g.

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