mM hydrogen peroxide. This is in accordance with results obtained by other "rapid" methods. With "slow" methods a gradual leveling off and eventual decline in the initial velocity is observed[2], because the increase in substrate concentration accelerates the transition to the less active form of the enzyme. Variations in k values with pH in the range 6 to 8 were only about 10%, which again demonstrated the absence of interference from the transition of the enzyme, since the rate of the transition has been shown to increase with acidity[2]. The k values were also independent of buffer concentration in the range 5 to 100 mM and identical results were obtained in Tris–HCl and in phosphate buffers.

Fig. 3 illustrates the usefulness of the method for the assay of catalase activity in samples of widely varying purity. Linearity between k values and protein concentration has been obtained with crystalline catalase, a partly purified tissue catalase preparation and erythrocyte suspensions with lysed and intact cells. In each case the linearity has been established over at least a 10-fold variation in protein concentration.

Division of Endocrinology,
Institute for Experimental Medicine,
University of Copenhagen,
Copenhagen (Denmark)

MIKAEL RØRTH
P. K. JENSEN

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Benzylalcohol dehydrogenase, a new alcohol dehydrogenase from Pseudomonas sp.

We have isolated and partially purified from a toluene-cultured pseudomonad an NAD-linked dehydrogenase which catalyzes the interconversion of benzyl alcohol and benzaldehyde. The enzyme, which will be referred to as "benzylalcohol dehydrogenase" (alcohol:NAD^+ oxidoreductase, EC 1.1.1.1), is unstable, has a half-life of a few hours under conventional conditions and differs from any known alcohol dehydrogenases with respect to substrate specificity and the effectiveness of inhibitors. This report describes briefly the procedure for the purification of benzylalcohol dehydrogenase and some properties of the enzyme.

An aerobic, Gram-negative rod organism used throughout this work was isolated from soil by enrichment culture with toluene as a sole carbon source and was kindly identified as a pseudomonad by Professor T. FUJINO and Dr. Y. TAKEDA of the Research Institute for Microbial Diseases, Osaka University. The cells were grown with

reciprocal shaking at 30° for about 40 h in 5-l erlenmeyer flasks, each containing 1.5 l of the following medium: 0.5% NH_4NO_3, 0.15% K_2HPO_4, 0.05% KH_2PO_4, 0.02% $MgSO_4 \cdot 7\,H_2O$, 0.0002% $FeSO_4 \cdot 7\,H_2O$, 0.01% yeast extracts and 25 ml of toluene which was enclosed in a Visking cellulose tubing (2.1 cm diameter and 15 cm long) and floated on the medium. Cell-free extracts were prepared by grinding the harvested wet cells mechanically in a chilled mortar with twice the weight of levigated alumina (Wako W. 800). The mixture was then slurried with the same volume of cold 0.05 M potassium phosphate buffer (pH 7.5) containing 10% acetone (subsequently referred to as "acetone buffer") and centrifuged at 13 000 × *g* for 20 min to yield active supernatant.

For the assay, each cuvette (1-cm light path) contained 20 μmoles of benzyl alcohol, 0.5 μmole of NAD^+, 50 μmoles of sodium pyrophosphate (pH 8.5) and enzyme in a total volume of 3.0 ml, and the increase in absorbance at 340 mμ was followed with a manual spectrophotometer at 20° between 15 and 45 sec after the reaction had been started by addition of the enzyme preparation.

Purification of the enzyme was carried out in five steps. The crude extract was treated with protamine sulfate (final concentration, 0.25%) and fractionated by the addition of cold acetone (between 55 and 70%). The active fraction was dissolved in the acetone buffer and further fractionated with $(NH_4)_2SO_4$ (between 0 and 25 g per 100 ml). The preparation, after dialysis against the acetone buffer, was purified by chromatography on DEAE-cellulose and then on DEAE-Sephadex, both of which were equilibrated with the acetone buffer. The active fractions from the column were concentrated by $(NH_4)_2SO_4$ precipitation, dialyzed against the acetone buffer and stored at 0° without appreciable inactivation. Further details of the purification method will be published elsewhere. The enzyme preparation from the DEAE-Sephadex column was purified 10-fold with respect to the crude extract and with 34% yield. The purified enzyme was free of benzaldehyde dehydrogenase[1] (EC 1.2.1.3), but still contained minor impurities after final chromatography. The molecular weight of the enzyme was determined using a Sephadex G-200 column essentially according to the method of ANDREWS[2]. By reading off the elution volume of the enzyme activity from the calibration curve for the column, the value was tentatively estimated as 116 000.

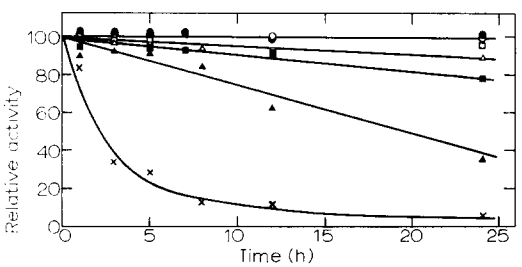

Fig. 1. Stability of benzylalcohol dehydrogenase in organic solvents. The purified fraction (46 μg protein) was stored at 5° in 2 ml of 0.05 M phosphate buffer (pH 7.5) containing different concentrations of organic solvents. After the indicated period, an aliquot was taken and the activity was measured by the standard method. Without organic solvents: ×—×; with acetone: 2% △—△; 5% □—□; 10% ○—○; and with ethanol: 2% ▲—▲; 5% ■—■; 10% ●—●.

As shown in Fig. 1, the presence of an organic solvent protected the enzyme almost completely from inactivation. However, otherwise no significant protection of

the enzyme from inactivation was observed even when kept frozen at $-20°$ or in the presence of a thiol such as glutathione (1 mM). Quite a similar solvent effect for catechol 2,3-oxygenase (EC 1.13.1.2) from *Pseudomonas arvilla* has been reported by NOZAKI, KAGAMIYAMA AND HAYAISHI[3] showing that the enzyme is extremely sensitive to oxygen but is protected by an organic solvent almost completely from inactivation by air. However, benzylalcohol dehydrogenase is not stabilized in an atmosphere of nitrogen gas with a Thunberg tube unless an organic solvent is present. This observation suggests that the inactivation of the dehydrogenase is not due to the destruction of any oxygen-labile groups in the protein molecule.

Recently we have also found marked dependence on the presence of an organic solvent of the stability of beef liver apo-homogentisate oxygenase[4] (EC 1.13.1.5) and yeast alcohol dehydrogenase (EC 1.1.1.1) (S. TAKEMORI, E. FURUYA, H. SUZUKI AND M. KATAGIRI, unpublished work). These observations lead one to suggest that the solvent effect is not restricted to oxygenases, bacterial enzymes, enzymes specific for aromatic substrates or oxygen-sensitive enzymes. Although the mechanism and biological

TABLE I

SUBSTRATE SPECIFICITY OF BENZYLALCOHOL DEHYDROGENASE

Assayed in the standard system where benzyl alcohol was replaced by one of the compounds listed.

Compound	*Relative activity (%)**
Salicyl alcohol	100
Phenylethyl alcohol	25
3-Phenyl-1-propanol	13
Furfuryl alcohol	59
n-Hexanol	19
n-Heptanol	6
n-Octanol	2

* Activity with benzyl alcohol was taken as 100.

significance of the stabilization are still unknown, organic solvents may perhaps be useful stabilizers during the preparation, storage and transportation of many other enzymes regardless of the origin, type of enzyme reaction, nature of bound cofactor and so on.

The purified preparation of benzylalcohol dehydrogenase has been studied for its substrate specificity. Of a number of alcohols tested, an appreciable increase in absorbance at 340 mμ was observed with those listed in Table I, whereas the following compounds did not act as substrates of the enzyme: methanol, ethanol, *n*-propanol, isopropanol, *n*-butanol, isobutanol and cyclohexanol. Judging from these results, benzylalcohol dehydrogenase appears to be specific for primary alcohols, preferably those with an aromatic ring, although it also catalyzed the dehydrogenation of some non-aromatic primary alcohols having about 6 carbon atoms. NAD^+ was required as an electron acceptor for the enzyme and $NADP^+$ did not replace NAD^+. The reverse reaction was demonstrated by NADH oxidation in the presence of benzaldehyde (20 μmoles/3 ml) in 0.05 M potassium phosphate buffer (pH 6.5). NADPH did not replace NADH under the same conditions.

Inhibition experiments were carried out under standard assay conditions by preincubation of 10 μg of enzyme protein with the inhibitor at 20° for 20 min and the reaction was then started by adding NAD^+. Chelating reagents such as EDTA, *o*-phenanthroline or α,α'-dipyridyl at 1 mM did not show appreciable inhibition. Monoiodoacetate and hydroxylamine are known to be potent inhibitors of yeast[5] and liver[6] alcohol dehydrogenases, respectively. However, neither of these inhibitors at 1 mM affected the activity of benzylalcohol dehydrogenase. *N*-Ethylmaleimide at 1 mM showed 90% inhibition and *p*-chloromercuribenzoate at 1 μM showed 100% inhibition.

The substrate specificity studies and inhibition experiments indicate that this enzyme belongs to the EC 1.1.1.1 category, but that it differs from any known alcohol dehydrogenases[7,8]. We have shown in other experiments (unpublished) that benzylalcohol dehydrogenase is produced exclusively by toluene- or benzyl alcohol-cultured cells. These facts may suggest that the significance of the enzyme in the cell is limited to an involvement in the degradation pathway of toluene.

Further purification and additional investigations on the nature of the enzyme are in progress.

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Department of Chemistry,
Faculty of Science,
Kanazawa University,
Kanazawa (Japan)

MASAYUKI KATAGIRI
SHIGEKI TAKEMORI
KIYOSHI NAKAZAWA
HIROYUKI SUZUKI

Department of Biochemistry,
Osaka University Medical School,
Osaka (Japan)

KATSUKO AKAGI*

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* Present address: Cancer Laboratory, Osaka University Medical School, Osaka, Japan.