PERILLYL ALCOHOL DEHYDROGENASE FROM A SOIL PSEUDOMONAD*

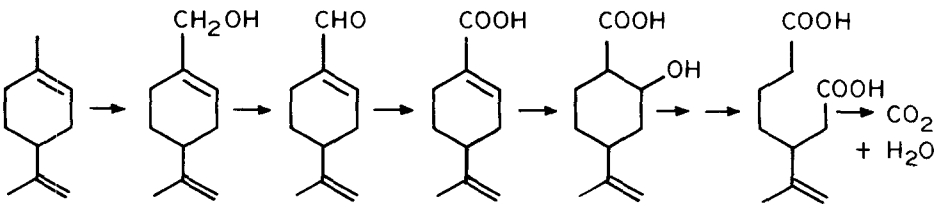
N. R. Ballal, P. K. Bhattacharyya and P. N. Rangachari

National Chemical Laboratory, Poona-8, India.

Received April 11, 1966

Extensive work has been done on the alcohol dehydrogenases from microorganisms. Yeast alcohol dehydrogenase has been crystallized and the kinetics and mechanism of its action have been very thoroughly investigated. These alcohol dehydrogenases which usually catalyze the oxido-reduction of primary alcohols have a fairly wide range of substrate specificity (1). NAD is the common cofactor in these alcohol dehydrogenases. A NADP-specific alcohol dehydrogenase has been partially purified from Leuconostoc mesenteroides (2). A dehydrogenase, active on a number of isomeric bornanols has been crystallized from a diphtheroid strain adapted to D(+) camphor (3).

During the course of the work pursued in this Laboratory on the microbiological transformations of simple terpenes, a NAD-linked alcohol dehydrogenase was isolated from a soil pseudomonad grown on limonene as the sole source of carbon. Dhavalikar and Bhattacharyya (4) have shown the probable pathway for the degradation of limonene to be as shown below:



* Communication number 920 from the National Chemical Laboratory

This postulated pathway is based on the isolation of the various intermediates and on the pattern of growth, adaptation and oxidation of these intermediates with intact cells. Further, the presence of some of the enzymes catalyzing the different steps has also been shown by Dhavalikar (5). The first enzyme in the sequence, a hydroxylating enzyme, which converts limonene to perillyl alcohol, is presented in the 100,000 x g sediment of sonicated cells, while most of the other activities tested are present in the soluble supernatant (5). With a view to get a better understanding of the pathway, a study of the soluble enzymes in the system was taken up. The first soluble enzyme in this sequence is the dehydrogenase carrying out the reversible NAD-dependent oxidoreduction of the alcohol, perillyl alcohol.

The enzyme has been purified 6-7 fold from the sonicates of limonene-grown cells in four steps consisting of (i) streptomycin sulfate treatment, (ii) ammonium sulfate fractionation, (iii) adsorption and elution on calcium phosphate gel (7), and (iv) a second fractionation with ammonium sulfate. Detailed purification procedure will be published elsewhere.

The enzyme activity is assayed by measuring the increase in optical density at 340 mμ in a Beckman DU spectrophotometer. The test system consists in a total volume of 3 ml: Tris-HCl buffer, 150 μmoles, pH 8.6; NAD, 10 μmoles; substrate, 200 μmoles and enzyme sufficient to give an O.D. change of 0.010 to 0.015 in 30 secs. The reaction is started by the addition of the enzyme and the average change in reading of the first two minutes is taken as the change in O.D. per minute. One unit of enzyme activity is one μmole of NADH formed per minute under the assay conditions, taking the molar extinction coefficient of NADH, ϵ_{340}^{1cm} as 6.22×10^3. The specific activity is the units of activity per mg protein. Protein is determined by the spectrophotometric method of Warburg and Christian (6).

The specific activity of the sonicate varied from 0.5 with freshly grown cells to about 0.17 in cells stored at -18°C for a period of 3

months. The activity of the purified enzyme preparation is about 3.0 to 3.5.

The enzyme at this stage of purity has been studied for its substrate specificity and cofactor requirements. It is found to be absolutely specific towards NAD, NADP being inactive. Substrate specificity studies employing different aliphatic, aromatic and hydroaromatic alcohols revealed that certain structural features are necessary in the substrate before it can be dehydrogenated. Tables I & II show the relative rates of dehydrogenation of the different alcohols with respect to that of perillyl alcohol; the structurally closely related terpenic alcohols such as phellandrol, cumyl alcohol and 1-hydroxymethyl-1-cyclohexene are dehydrogenated at comparable rates. The more soluble alcohol, 8-hydroxy phellandrol, is 3 times as active as perillyl alcohol. Benzyl

TABLE I

SUBSTRATE SPECIFICITY

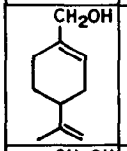
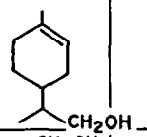
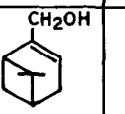
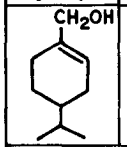
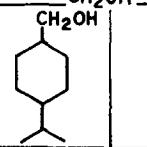
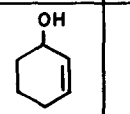
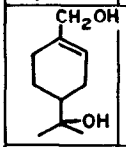
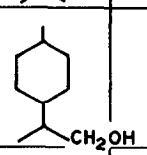
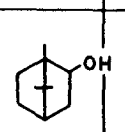
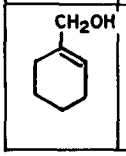
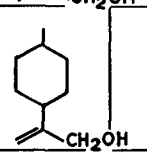
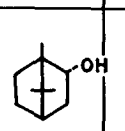
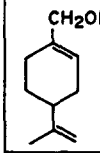
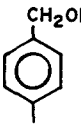
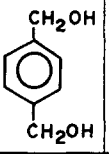
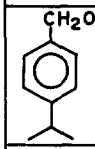
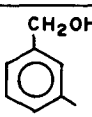
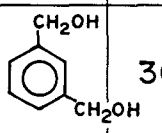
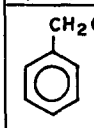
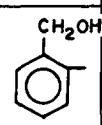
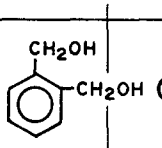
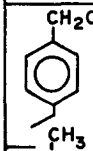
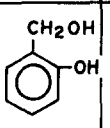
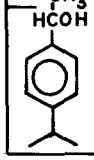
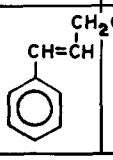
SUBSTRATE	RELATIVE ACTIVITY	SUBSTRATE	RELATIVE ACTIVITY	SUBSTRATE	RELATIVE ACTIVITY
	100		0		0
	70		0		0
	280		0		0
	150		0		0

TABLE II

SUBSTRATE SPECIFICITY

SUBSTRATE	RELATIVE ACTIVITY	SUBSTRATE	RELATIVE ACTIVITY	SUBSTRATE	RELATIVE ACTIVITY
	100		200		170
	170		140		30
	65		0		0
	140		0	$CH_2=CHCH_2OH$	5
	0		170	CH_3CH_2OH	0

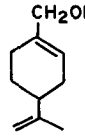
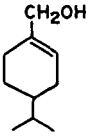
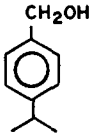
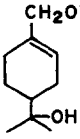
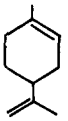
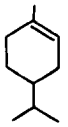
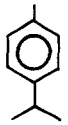
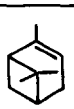
alcohol showed about 60% and allyl alcohol 3-5% activity. Menthan-7-ol, p-menthan-10-ol and 8-p-menthen-10-ol do not serve as substrates. Neither the bicyclic terpenic alcohol, myrtenol nor the secondary alcohols such as 7-methyl cumic alcohol, borneol, isoborneol and cyclohexene-1-ol are dehydrogenated. Substitution in the 4-position of the ring favours dehydrogenation as shown by p-tolyl carbinol, p-ethyl benzyl alcohol and terephthalyl alcohol. Substitution in the meta-position to the primary alcoholic group decreases the activity to a certain extent but does not inhibit it totally as is evident from the behaviour of m-tolyl carbinol and isophthalyl alcohol. But ortho-substituted alcohols such as salicyl and phthalyl alcohols, are not dehydrogenated. Furthermore, simple alcohols like methanol and ethanol do not serve as substrates.

These facts lead one to the conclusion that the following structural features may be necessary in the molecule to be dehydrogenated by this enzyme: (1) a primary alcoholic group, preferably allylic to an endocyclic double bond, (2) a six-membered ring, either aromatic or hydroaromatic, (3) an alkyl substituent in the para position tends to increase the enzyme activity.

It is shown that a similar alcohol dehydrogenase active on perillyl alcohol is present in the pseudomonad cells adapted to α-pinene, p-cymene and Δ^1-p-menthene as sole source of carbon. Table III shows the relative rates of alcohol dehydrogenase activity in the cells grown on the four different types of substrates, with regard to four different alcohols, the

TABLE III

RELATIVE ACTIVITY OF ADH
$[NH_4]_2SO_4$ FRACTION (30-65%)

SUBSTRATE / INDUCER				
	100	55	105	280
	100	63	95	200
	100	63	120	160
	100	82	130	204

activity with perillyl alcohol being taken as 100 in each case. Taking into consideration the wide substrate specificity of the alcohol dehydrogenase and yet certain rigid structural requirement in the substrate molecule it appears likely that the same alcohol dehydrogenase is induced in the cells during growth on the different terpenic hydrocarbons investigated. This constitutes another example of cell economy in microbial systems where a number of substrates can be degraded by the same enzyme systems (8).

ACKNOWLEDGEMENT: One of the authors (N.R.B.) wishes to thank the Council of Scientific & Industrial Research (India) for the award of a Junior Research Fellowship.

REFERENCES

1. Sund, H. and Theorell, H., The Enzymes, Vol. 7 (Boyer,Lardy & Myrback, Eds) 1963, p. 26.
2. DeMoss, R.D., J.Bact. 68, 252 (1954).
3. Kuo, J.F., Ph.D. Thesis, University of Illinois (1964).
4. Dhavalikar, R.S. and Bhattacharyya, P.K. (in press).
5. Dhavalikar, R.S. Ph.D. Thesis, University of Poona (1964).
6. Warburg, O., and Christian, W., Biochem. Z., 310, 384 (1942).
7. Keilin, D. and Hartree, E.F., Proc.Roy.Soc. London, B 124, 397 (1938).
8. Gunsalus, I.C., Chapman, P.J. and Kuo, J.F. Biochem.Biophys.Res. Comm. 18, 924 (1965).