BBA 12285

POTASSIUM SORBATE INHIBITION OF YEAST ALCOHOL DEHYDROGENASE*

WINOTO MARTOADIPRAWITO** AND JOHN R. WHITAKER

Department of Food Science and Technology, University of California, Davis, Calif. (U.S.A.)

(Received March 19th, 1963)

SUMMARY

Potassium sorbate was found to inhibit yeast alcohol dehydrogenase (alcohol: NAD oxidoreductase, EC 1.1.1.1) maximally at the pH optimum of the enzyme; *viz.* pH 8.5. Potassium sorbate competed with NAD and ethanol for a site on the enzyme but in an irreversible manner. Sorbamide was found to be approx. 1000 times a better inhibitor of yeast alcohol dehydrogenase than was potassium sorbate. The evidence presented indicates that potassium sorbate inhibits yeast alcohol dehydrogenase irreversibly by either the formation of a covalent bond between the sulfur of the essential sulfhydryl group or the $ZnOH^-$ of the enzyme and the δ and/or β carbons of the sorbate ion.

INTRODUCTION

Previous work[1,2] has shown that yeast alcohol dehydrogenase is inhibited by sorbic acid. The present investigation was carried out to determine the mechanism of this inhibition with the hope this would aid in a further understanding of the action of yeast alcohol dehydrogenase and of the fungistatic properties of sorbic acid.

MATERIALS AND METHODS

Yeast alcohol dehydrogenase

This was twice crystallized material obtained from Worthington Biochemical Corporation, Freehold, New Jersey. A stock solution ($1 \cdot 10^{-3}$ g/ml) was made in 0.1% gelatin. Just prior to use an aliquot of this stock solution was diluted with cold, 0.01 M sodium pyrophosphate (pH 7.5) to contain 100 μg enzyme/ml and kept in an ice bath. Molar concentration was calculated using a molecular weight of 150 000 (see ref. 3). The enzyme preparation had an activity of 34 300 units per mg protein when assayed under the conditions described by RACKER[4] except the NAD concentration was $6.67 \cdot 10^{-5}$ M rather than $5.00 \cdot 10^{-5}$ M.

* Presented by W. MARTOADIPRAWITO in partial satisfaction of the requirements for the degree of Master of Science in Food Science, University of California, Davis, September 1962.

** Present address: Gadjah Mada University, Jogjakarta (Indonesia).

Horse-liver alcohol dehydrogenase

This was a suspension of one time crystallized material obtained from Nutritional Biochemicals Corporation, Cleveland, Ohio. Just prior to use an aliquot of the suspension was diluted with 0.01 M pyrophosphate buffer (pH 7.5) to 1.22 mg protein/ml as measured spectrophotometrically ($E^{1\%}_{1\,cm} = 4.55$ at 280 mμ (see ref. 5)). The molar concentration was calculated using a molecular weight of 73 000 (see ref. 6).

Nicotinamide adenine dinucleotide

NAD was obtained from Nutritional Biochemicals Corporation. Purity of the material was determined spectrophotometrically after its total reduction by yeast alcohol dehydrogenase[7] and by use of the molar extinction coefficient of $6.22 \cdot 10^6$ cm^2/mole at 340 mμ (see ref. 8). The NAD stock solution was $1.0 \cdot 10^{-3}$ M.

Potassium sorbate and sorbamide

Potassium sorbate was obtained from Union Carbide Chemicals Co., New York. Sorbamide was prepared in this laboratory. 2 g of sorbic acid were heated under reflux for 30 min with 10 ml thionyl chloride and then poured into 30 ml of cold, concentrated ammonium hydroxide. The precipitate was collected by filtration on a Buchner funnel and washed with a small amount of ice-cold water. It was recrystallized twice from ethyl acetate and twice from ethyl acetate–light petroleum (b.p., 30°–60°) and dried *in vacuo*. The yield was 0.10 g with a melting point of 170°–171.5°. Reported melting point[9] is 170°–171°.

All other reagents were reagent grade. Deionized water was used.

Assay of activity

Enzymatic activity was determined by the spectrophotometric method of RACKER[4] at 27° and in 0.06 M pyrophosphate buffer. All reagents except ethanol were mixed in the cuvette and incubated for 5 min at 27° in the thermostated compartment of a DU spectrophotometer. Ethanol was then added and the first absorbancy reading taken 15 sec later. Readings were taken at 15-sec intervals for 3 min. The increment in absorbancy between the 15- and 45-sec readings multiplied by two was taken as enzyme activity per minute. All reactions were carried out in triplicate. The inhibition was the difference between the activity of a control system and the inhibited system.

RESULTS

Effect of incubation time and order of addition of ethanol and NAD

WHITAKER[1] reported that incubation of yeast alcohol dehydrogenase with sorbic acid and NAD before addition of ethanol increased the degree of inhibition. This was confirmed by AZUKAS *et al.*[2]. KAPLAN AND CIOTTI[10] found that incubating hydroxylamine with enzyme and NAD enhanced the inhibition. The order of addition of ethanol and NAD did not influence the degree of inhibition of yeast alcohol dehydrogenase by hydroxylamine. In contrast, incubation of liver alcohol dehydrogenase with

TABLE I

EFFECT OF INCUBATION TIME AND ORDER OF ADDITION OF ETHANOL AND NAD ON POTASSIUM SORBATE INHIBITION OF YEAST ALCOHOL DEHYDROGENASE

Concentration of reagents were: 0.10 M ethanol, $6.67 \cdot 10^{-5}$ M NAD, $1.0 \cdot 10^{-2}$ M potassium sorbate, and 3.33 μg/ml ($2.22 \cdot 10^{-8}$ M) yeast alcohol dehydrogenase. Reaction carried out at 27° in 0.06 M pyrophosphate buffer (pH 8.5).

Incubation time (min)	*Percent inhibition Reaction started with*	
	ethanol	*NAD*
1	16	21
5	39	23
10	41	26
15	42	32
30	43	36
60	45	43

hydroxylamine and NAD gave more inhibition than incubation of the enzyme with hydroxylamine and ethanol.

The results of this investigation are shown in Table I. While prolonged incubation of yeast alcohol dehydrogenase with potassium sorbate in the presence of either ethanol or NAD gave the same degree of inhibition, incubation for short intervals gave more inhibition in the presence of ethanol than in the presence of NAD. This would be expected from the K_m values for ethanol ($1.75 \cdot 10^{-2}$ M) and NAD ($3.12 \cdot 10^{-5}$ M) determined during this investigation, provided that the sorbate ion did not react with NAD as was found for the reaction of hydroxylamine in the inhibition of horse-liver alcohol dehydrogenase[11]. Since the degree of inhibition was found to be essentially constant after 5 min incubation in the presence of ethanol, these conditions were used for most of the remainder of this work.

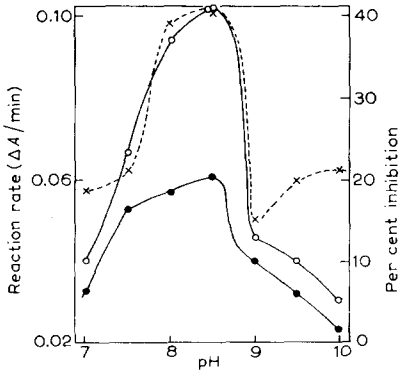

Fig. 1. pH-dependence of the inhibition of yeast alcohol dehydrogenase by potassium sorbate. Reactant concentrations were: $6.67 \cdot 10^{-5}$ M NAD, 0.10 M ethanol, $1.0 \cdot 10^{-2}$ M potassium sorbate, and 3.33 μg/ml ($2.22 \cdot 10^{-8}$ M) yeast alcohol dehydrogenase. Buffer was 0.06 M pyrophosphate. Reaction was started after 5 min incubation at 27° by the addition of ethanol. ○—○, control; ●—●, with inhibitor; × — — ×, per cent inhibition.

pH dependence

The effect of pH on the potassium sorbate inhibition of yeast alcohol dehydrogenase is shown in Fig. 1. Maximum inhibition was observed at pH 8.5, which was also found to be the pH optimum of the enzyme. 50% of maximum inhibition was found at pH 7.7–7.8. The degree of inhibition then decreased on the alkaline side of pH 8.5.

Effect of potassium sorbate concentration

The percentage inhibition by potassium sorbate was found to be a linear function of the logarithm of the inhibitor concentration for both yeast and horse-liver alcohol dehydrogenase (Fig. 2). While the I_{50} values are similar ($1.3 \cdot 10^{-2}$ M and $4.6 \cdot 10^{-2}$ M

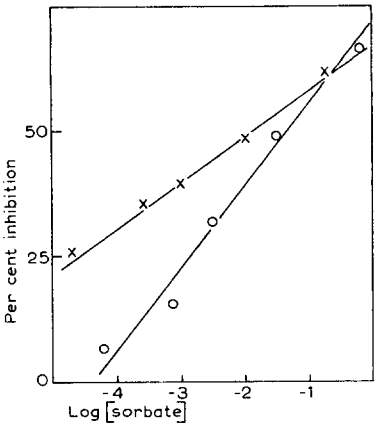

Fig. 2. Effect of potassium sorbate concentration on the inhibition of yeast alcohol dehydrogenase (×—×) and horse-liver alcohol dehydrogenase (○—○). Reactant concentrations were: $6.67 \cdot 10^{-5}$ M NAD, 0.10 M ethanol, 3.33 μg/ml ($2.22 \cdot 10^{-8}$ M) yeast alcohol dehydrogenase and 40.7 μg/ml ($5.58 \cdot 10^{-7}$ M) horse-liver alcohol dehydrogenase. Buffer was 0.06 M pyrophosphate (pH 8.5). Reaction was started after 5 min incubation at 27° by addition of ethanol.

respectively for yeast and horse-liver enzymes in the presence of 0.1 M ethanol and $6.67 \cdot 10^{-5}$ M NAD) yeast alcohol dehydrogenase was inhibited to a much greater extent by low concentrations of potassium sorbate than was horse-liver alcohol dehydrogenase. It should be noted that in this experiment 25 times more horse-liver enzyme was used than yeast enzyme. It may be of significance to compare the K_m values of these enzymes. For yeast alcohol dehydrogenase, K_m (NAD) and K_m (ethanol) were reported[3] to be $1.7 \cdot 10^{-4}$ M and $1.8 \cdot 10^{-2}$ M, respectively at pH 7.9 and for horse-liver alcohol dehydrogenase, K_m (NAD) and K_m (ethanol) were reported to be $1.2 \cdot 10^{-5}$ M (pH 7.0) and $5.4 \cdot 10^{-4}$ M (pH 8.2), respectively[6].

Analysis of the data of KAPLAN AND CIOTTI[10] gave an estimated I_{50} of $1 \cdot 10^{-3}$ M for hydroxylamine inhibition of yeast alcohol dehydrogenase in the presence of 0.1 M ethanol and $3.3 \cdot 10^{-4}$ M NAD. This would indicate hydroxylamine is a slightly better inhibitor of yeast alcohol dehydrogenase than is potassium sorbate.

Effect of NAD concentration

Effect of NAD concentration on the potassium sorbate inhibition of yeast alcohol dehydrogenase is shown in Fig. 3 where the data is plotted according to the modified LINEWEAVER–BURK equation[12]. In the absence of potassium sorbate, V_{max} and the apparent K_m were found to be $2.78 \cdot 10^{-5}$ moles/l/min and $3.12 \cdot 10^{-5}$ moles/l, respectively. In the presence of $1.0 \cdot 10^{-2}$ M potassium sorbate V_{max} was found to be $3.03 \cdot 10^{-5}$

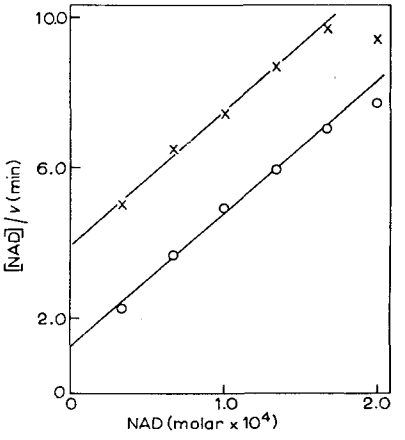

Fig. 3. Effect of NAD concentration on the potassium sorbate inhibition of yeast alcohol dehydrogenase. Reactant concentrations were: 0.10 M ethanol, $1.0 \cdot 10^{-2}$ M potassium sorbate and 3.33 μg/ml ($2.22 \cdot 10^{-8}$ M) yeast alcohol dehydrogenase. Buffer was 0.06 M pyrophosphate (pH 8.5). Reaction was started after 5 min incubation at 27° by addition of ethanol. O—O, control; ×—×, with inhibitor.

moles/l/min and the apparent K_i was $3.76 \cdot 10^{-3}$ moles/l. These data, calculated by the method of least squares, indicate that NAD and sorbate ion compete for the same site on the enzyme.

Effect of ethanol concentration

The results of this study are shown in Fig. 4. In the absence of potassium sorbate V_{max} and K_m were found to be $1.68 \cdot 10^{-5}$ moles/l/min and $1.75 \cdot 10^{-2}$ moles/l respectively. HAYES AND VELICK[3] reported K_m for ethanol to be $1.8 \cdot 10^{-2}$ M. In the presence of $1.0 \cdot 10^{-2}$ M potassium sorbate V_{max} was found to be $1.23 \cdot 10^{-5}$ moles/l/min and the apparent K_i was $4.30 \cdot 10^{-3}$ moles/l. The data, calculated by the method of least squares, indicate that ethanol and potassium sorbate compete for the same site on the enzyme, although there is some indication of a mixed type inhibition.

Effect of incubation with inhibitor in absence of NAD and ethanol

If K_i is real, that is, if it does represent a true dissociation constant of the enzyme–sorbate ion complex as analysis of the data by the modified Lineweaver–Burk plot indicates, one should be able to partially reverse the inhibition by the addition of NAD

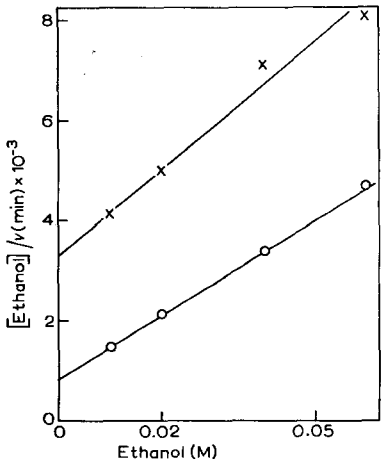

Fig. 4. Effect of ethanol concentration on the potassium sorbate inhibition of yeast alcohol dehydrogenase. Reactant concentrations were: $6.67 \cdot 10^{-5}$ M NAD, $1.0 \cdot 10^{-2}$ M potassium sorbate and 3.33 μg/ml ($2.22 \cdot 10^{-8}$ M) yeast alcohol dehydrogenase. Buffer was 0.06 M pyrophosphate (pH 8.5). Reaction was started after 5 min incubation at 27° by addition of NAD. ○—○, control; ×—×, with inhibitor.

and/or ethanol at any stage of the inhibition. That this was not found to be the case is shown by the data of Table II. This data indicate that the enzyme–sorbate ion complex is irreversible and that K_i therefore cannot represent the dissociation constant of this complex. The inhibition also cannot be reversed by the addition of cysteine. The value found for K_i reflects the effect of NAD and ethanol on the rate of formation of the irreversible enzyme–inhibitor complex.

TABLE II

EFFECT OF INCUBATION WITHOUT NAD AND ETHANOL ON POTASSIUM SORBATE INHIBITION OF YEAST ALCOHOL DEHYDROGENASE

Concentration of reagents: $1.0 \cdot 10^{-2}$ M potassium sorbate and 3.33 μg/ml ($2.22 \cdot 10^{-8}$ M) yeast alcohol dehydrogenase. Buffer was 0.06 M pyrophosphate (pH 8.5). Reaction was started after 5 min incubation at 27° by simultaneous addition of NAD and ethanol.

NAD (moles/l × 10^5)	*Ethanol (moles/l)*	*Reaction rate*		*Inhibition (%)*
		Control (moles/l/min × 10^5)	*Inhibited (moles/l/min × 10^5)*	
6.67	0.20	2.66	1.57	41.0
6.67	0.10	2.58	1.54	40.3
5.00	0.10	2.51	1.45	42.1
3.33	0.10	2.45	1.35	44.9
1.66	0.10	0.64	0.35	45.3

Effect of zinc acetate

Table III shows that Zn^{2+} was inhibitory for yeast alcohol dehydrogenase which has been reported previously[13] and did not protect the enzyme against potassium

TABLE III

EFFECT OF ZINC ACETATE ON INHIBITION OF YEAST ALCOHOL DEHYDROGENASE BY POTASSIUM SORBATE

Reactant concentrations were: $6.67 \cdot 10^{-5}$ M NAD, 0.10 M ethanol, $1.0 \cdot 10^{-2}$ M potassium sorbate and 3.33 μg/ml ($2.22 \cdot 10^{-3}$ M) yeast alcohol dehydrogenase. Buffer was 0.06 M pyrophosphate (pH 8.5). Reaction was started after 5 min incubation at 27° by addition of ethanol.

Zinc acetate (moles/l)	*Reaction rate*		*Inhibition (%)*
	Control (moles/l/min × 10^5)	*Inhibited (moles/l/min × 10^5)*	
0	2.06	1.29	37.4
$1.0 \cdot 10^{-5}$	1.48	0.87	41.2
$1.0 \cdot 10^{-4}$	1.32	0.80	39.4
$1.0 \cdot 10^{-3}$	1.00	0.58	42.0
$1.0 \cdot 10^{-2}$	0.87	0.50	42.5
$2.0 \cdot 10^{-2}$	0.87	0.49	43.6

sorbate inhibition. The data strongly indicate that Zn^{2+} and sorbate ion react with different sites on the enzyme.

Effect of sorbamide

Sorbamide is a much better inhibitor of yeast alcohol dehydrogenase than is potassium sorbate (Table IV). Under the same conditions the I_{50} values were found to be approx. 10^{-2} M for potassium sorbate and 10^{-5} M for sorbamide.

TABLE IV

INHIBITION OF YEAST ALCOHOL DEHYDROGENASE BY SORBAMIDE

Concentrations of reactants were: 0.10 M ethanol, $6.67 \cdot 10^{-5}$ M NAD and 3.33 μg/ml ($2.22 \cdot 10^{-3}$ M) yeast alcohol dehydrogenase. Buffer was 0.06 M pyrophosphate (pH 8.5). Reaction was started after 5 min incubation at 27° by addition of ethanol.

Sorbamide (moles/l)	*Inhibition (%)*
$2.0 \cdot 10^{-5}$	52
$2.5 \cdot 10^{-4}$	63
$1.0 \cdot 10^{-3}$	93

DISCUSSION

There are a number of possible explanations for sorbate ion inhibition of alcohol dehydrogenase. Additions of nucleophilic reagents to the 4-position of NAD have been described[14]. There is no evidence that sorbate ion inhibits in this fashion as it does not appear to react with NAD alone, it inhibits equally well when incubated with enzyme alone (Table II) and sorbamide, not as nucleophilic as sorbate ion, is much more inhibitory (Table IV).

One might speculate that the inhibition is due to non-specific binding of sorbate ion to the protein. However, sorbate ion is competitive with NAD and ethanol and sorbamide is approx. 1000 times more inhibitory than sorbate ion.

With liver alcohol dehydrogenase, THEORELL and coworkers[15–17] found that fatty acids are competitive inhibitors of ethanol but not acetaldehyde, while fatty acid amides are competitive inhibitors of acetaldehyde but not ethanol. As sorbamide is a potent inhibitor of the oxidation of ethanol by yeast alcohol dehydrogenase (Table IV) it would appear that sorbamide inhibits yeast alcohol dehydrogenase in a different manner than isobutyramide inhibits liver alcohol dehydrogenase.

Synthetic dihydropyridines can reduce quinoid dyes[18]. We have not found any spectral evidence that sorbate ion (or sorbamide) oxidizes NADH in the absence or presence of enzyme. Inhibition by sorbate ion, which is competitive with both NAD and ethanol, is a time-dependent reaction which takes place in the absence of NAD and ethanol.

THEORELL AND MCKINLEY-MCKEE[17] postulated that one of the reactive groups in liver alcohol dehydrogenase is $EnZnOH^-$ with a pKa of 8.6. It would appear that the nucleophilic species, $EnZnOH^-$, might be quite reactive with one or both of the double bonds of sorbate ion and that sorbamide would be an even better electrophilic reagent. One would expect that reaction of sorbate ion at zinc, either as a unidentate or bidentate ligand inhibitor, would appear to be competitive with both NAD and ethanol. This would also explain the effect of pH on the inhibition and the apparently non-competitive action of Zn^{2+} and sorbate ion (Table III).

Another possible explanation would be inhibition due to reaction of sorbate ion with the enzyme sulfhydryl group essential for combination with NAD and ethanol[4, 13,19–22]. We have found that sorbic acid reacts rapidly with cysteine and mercaptoethanol with a decrease in absorption at 256 mμ. Ficin, a sulfhydryl proteolytic enzyme, is markedly inhibited by sorbate ion[1]. The data implicates the involvement of a group with a pKa of 7.8 in both the activity of the enzyme and in the reaction with sorbate ion. Decreased inhibition by sorbate ion above pH 8.5 could be due to enzyme conformational changes at high pH; the enzyme is also less reactive in oxidizing alcohol at high pH. Sorbamide, because of more polarization of double bonds and elimination of negative charge, would be more reactive than sorbate ion with a sulfhydryl anion. On the other hand, the non-competitive action of Zn^{2+} and sorbate ion (Table III) is not explained by this hypothesis.

REFERENCES

1 J. R. WHITAKER, *Food Res.*, 24 (1959) 37.
2 J. J. AZUKAS, R. N. COSTILOW AND H. L. SADOFF, *J. Bacteriol.*, 81 (1961) 189.
3 J. E. HAYES, JR. AND S. F. VELICK, *J. Biol. Chem.*, 207 (1954) 225.
4 E. RACKER, in S. P. COLOWICK AND N. O. KAPLAN, *Methods in Enzymol.*, Vol. I, Academic Press, New York, 1955, p. 500.
5 R. K. BONNICHSEN AND N. G. BRINK, in S. P. COLOWICK AND N. O. KAPLAN, *Methods in Enzymol.*, Vol. I, Academic Press, New York, 1955, p. 495.
6 H. THEORELL AND R. K. BONNICHSEN, *Acta Chem. Scand.*, 5 (1951) 1105.
7 A. KORNBERG, *J. Biol. Chem.*, 182 (1950) 779.
8 B. L. HORECKER AND A. KORNBERG, *J. Biol. Chem.*, 175 (1948) 385.
9 A. BRUYLANTS AND J. ROWIES, *Bull. Soc. Chim. Belges*, 59 (1950) 244.
10 N. O. KAPLAN AND M. M. CIOTTI, *J. Biol. Chem.*, 201 (1953) 785.
11 N. O. KAPLAN AND M. M. CIOTTI, *J. Biol. Chem.*, 211 (1954) 431.
12 H. LINEWEAVER AND D. BURK, *J. Am. Chem. Soc.*, 56 (1934) 658.

[13] B. L. VALLEE, in P. D. BOYER, H. LARDY AND K. MYRBÄCK, *The Enzymes*, Vol. 3, Academic Press, New York, 1960, p. 225.
[14] N. O. KAPLAN, in P. D. BOYER, H. LARDY AND K. MYRBÄCK, *The Enzymes*, Vol. 3, Academic Press, New York, 1960, p. 105.
[15] A. D. WINER AND H. THEORELL, *Acta Chem. Scand.*, 14 (1960) 1729.
[16] H. THEORELL AND J. S. MCKINLEY-MCKEE, *Acta Chem. Scand.*, 15 (1961) 1811.
[17] H. THEORELL AND J. S. MCKINLEY-MCKEE, *Acta Chem. Scand.*, 15 (1961) 1834.
[18] K. WALLENFELS, in *Steric Course of Microbiological Reactions*, Ciba Foundation Study Group No. 2, J. and A. Churchill, London, 1959, p. 10.
[19] E. S. G. BARRON AND S. LEVINE, *Arch. Biochem. Biophys.*, 41 (1952) 175.
[20] M. DIXON, *Nature*, 140 (1937) 806.
[21] T. WAGNER-JAUREGG AND E. F. MÖLLER, *Z. Physiol. Chem.*, 236 (1935) 222.
[22] N. O. KAPLAN, in *Steric Course of Microbiological Reactions*, Ciba Foundation Study Group No. 2, J. and A. Churchill, London, 1959, p. 36.