Biochemical Pharmacology, Vol. 23, pp. 2328–2331. Pergamon Press, 1974. Printed in Great Britain.

Allyl alcohol-induced irreversible inhibition of yeast alcohol dehydrogenase

(*Received* 13 *November* 1973; *accepted* 1 *February* 1974)

BOTH yeast and horse liver alcohol dehydrogenase oxidize allyl alcohol into the highly cytotoxic agent acrolein.[1] In the process of this conversion, the yeast enzyme is itself irreversibly inhibited, whereas the horse liver enzyme remains little affected. As we have been interested in the chemical and enzymological factors pertinent to the design of irreversible enzyme inhibitors which are self-generated by the target enzyme, we have further investigated the mechanism of inhibition in the yeast case. The results of this investigation are reported herein.

The oxidative conversion of allyl alcohol to acrolein by yeast alcohol dehydrogenase (ADH) represents an example of an enzyme catalyzing the formation of a chemically reactive species from its unreactive counterpart. Acrolein belongs to the class of acceptors in the Michael reaction,[2] and molecules of this sort are known to react in a quite facile manner with both proteins and nucleic acids.[3] When the yeast ADH is subjected to freshly distilled allyl alcohol, in the presence of oxidized nicotinamide adenine dinucleotide (NAD^+) as the hydride acceptor, the activity of the enzyme is irreversibly reduced (Fig. 1). Suitable controls show that it is, indeed, the product of the enzymatic conversion that is inhibitory (Fig. 1). Further evidence in support of this notion comes from independent experiments with added acrolein. Acrolein by itself will inactivate the enzyme, and this inactivation can be slowed down by the presence of NAD^+

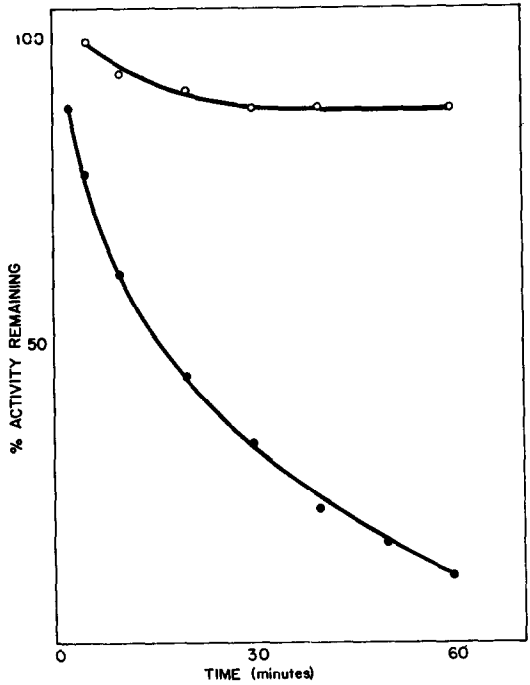

FIG. 1. Allyl alcohol-induced inactivation of yeast ADH. Two test tubes containing 0·6 ml of 0·032 M potassium pyrophosphate buffer at pH 8·8 were charged with 0·01 mg yeast ADH, sp. act. = 439 units/mg[4] (Sigma Chemical Co.), and 0·3 ml NAD^+ (167 mg NAD^+/10 ml) in pyrophosphate buffer. To the first tube was added 0·1 ml freshly prepared 2 M allyl alcohol and to the second 0·1 ml of 2 M ethanol. After various time periods, 10 μl of each of these solutions was assayed by the standard ethanol assay.[5] Acetaldehyde is produced in the tube containing ethanol. (○) and acrolein in the tube containing allyl alcohol (●). Thus, the graph shows the activity (NADH production with ethanol) of the incubation mixtures at various time points. The inhibited samples did not regain any activity after being dialyzed overnight against the pyrophosphate buffer containing 30 mg/l of reduced glutathione. In the absence of NAD^+, allyl alcohol has no effect on the activity of the enzyme which is consistent with the assumption that the product of the enzymatic reaction, acrolein, is the actual irreversible inhibitor.

TABLE 1. EFFECT OF ACROLEIN ON YEAST ADH*

Time (min)	Percentage activity remaining†		
	I ADH + ethanol (%)	II ADH + acrolein (%)	III ADH + acrolein + NAD^+ (%)
15	100	44	82
4040	100	18	46

* The yeast ADH (0·01 mg/ml) was incubated at room temp. in three test tubes containing 2 mM acrolein, 2 mM ethanol, and 2 mM acrolein + 7·5 mM NAD^+, all in 0·032 M sodium pyrophosphate buffer, pH = 8·8. Aliquots (100 μl) of each of these solutions were removed at various time points and assayed for activity by the standard ethanol assay.[4] The inhibition by acrolein proved to be irreversible, as expected, since continued dialysis of the inhibited samples led to no increase in activity.

† Activity of I taken as 100 per cent.

(Table 1). This latter observation strongly suggests that the acrolein is active-site directed. Indeed, studies have already demonstrated that yeast ADH possesses active-site sulfhydryl groups, the chemical modification of which inactivates the enzyme, and that these modifications can be blocked with NAD^+.[6] Acrolein, of course, reacts exceedingly rapidly with sulfhydryl groups in a Michael addition reaction. Thus, as a plausible working hypothesis, we suggest that the acrolein inactivates the enzyme by reacting with its active-site sulfhydryl group.[2] Further studies will determine the validity of this suggestion.

A priori there are two limiting modes by which the generated acrolein can inactivate the enzyme. In the first instance, the acrolein could immediately react with an active-site group without first diffusing into solution. Ample precedent for this kind of inhibitor exists.[7–9] On the other hand, the acrolein might first diffuse into solution and later inactivate the enzyme by an affinity-labeling mechanism.[10] Since these two modes are asymmetric in time, they can be differentiated by carrying out an experiment which measures the rate of acrolein production as a function of the rate of inactivation of the enzyme. The direct inactivation mode requires parallelism between these rates, whereas the affinity-labeling mode does not. This experiment is shown in Fig. 2. Since most of the total acrolein that can be formed in the presence of limiting NAD^+ is formed before measurable inactivation occurs, we conclude that an affinity-labeling

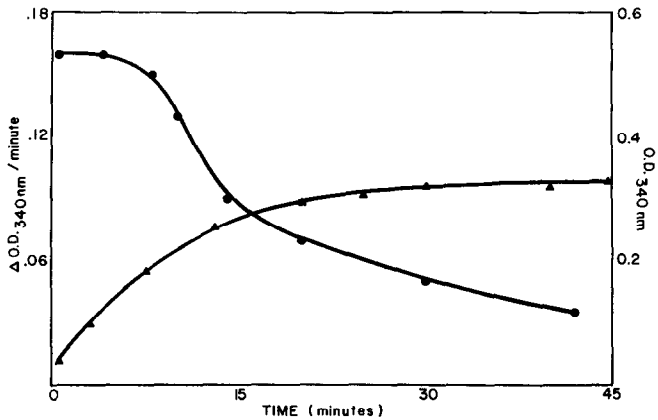

FIG. 2. Yeast ADH-catalyzed generation of acrolein and its inactivation as a function of time. Yeast ADH (0·439 units) was incubated in 1 ml pyrophosphate buffer containing allyl alcohol and NAD^+. The amounts of allyl alcohol and NAD^+ used were the same as in Fig. 1. At various times, 50 μl of the solution was removed, and the activity of the enzyme was assayed by the standard ethanol assay. At the same time, 50-μl samples were removed, added to 1 ml distilled water and the O.D. was read at 340 nm to determine the amount of NADH (acrolein) that had formed. Thus, the left ordinate (●) gives a measure of the ADH activity, and the right ordinate (▲) gives the amount of acrolein formed, both as function of time.

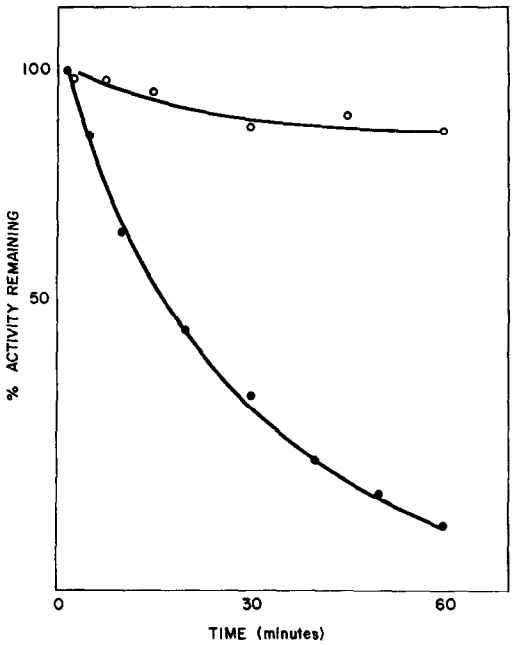

FIG. 3. Protection against allyl alcohol-induced inhibition of yeast ADH by DTT. Experiments identical to the allyl alcohol experiment in Fig. 1 were run except that one tube contained 1 mM DTT. At various times aliquots were removed from the DTT-protected tube (○) and the control (●) and their activities assayed by the standard assay.[5]

mechanism is operative here with little or no direct inactivation. Further evidence for this viewpoint comes from experiments carried out in the presence of excess of a reagent that can irreversibly trap free acrolein. When reduced dithiothreitol (DTT) is present in a reaction mixture that contains allyl alcohol NAD^+ and ADH, the enzyme is protected against inactivation (Fig. 3). This experiment is consistent only with the affinity-labeling mode. If the acrolein had reacted with an active-site residue prior to diffusing from the surface of the enzyme, the DTT would have had very little, if any, effect in the rate of inactivation. This argument is based on the assumption that a high affinity binding site for DTT does not exist at the enzyme's active site. The DTT would have to be bound in such a way that an immediate reaction could occur between it and the acrolein. By the rule of William of Ockham, we prefer the alternative mechanism, where the acrolein diffuses into solution prior to inactivating the enzyme. This latter mechanism is also the only one consistent with the time course of enzymatic acrolein production vs inactivation (Fig. 2).

As previously mentioned, the horse liver ADH is not susceptible to inactivation by acrolein. That allyl alcohol has no effect on this enzyme in the presence of oxidized NAD^+ is shown in Table 2. We take this to mean that the horse enzyme does not possess crucial amino acid residues, active-site or otherwise, susceptible to engaging in a rapid Michael reaction with acrolein.

The examples cited here are part of a more general phenomenon relating to the consequences of an enzyme generating a highly reactive molecule from an unreactive one. When this occurs, the following mutually exclusive events are possible: (1) the molecule might simply diffuse away and react with a biomolecule other than the enzyme itself; (2) the molecule may diffuse from the active site, but later irreversibly inhibit the enzyme by affinity labeling; and (3) the molecule may immediately react with an active site moiety without diffusing into solution. The horse liver and yeast ADH experiments discussed here belong to categories 1 and 2 respectively. It is important to understand those chemical factors which are important in determining which of the three cases will obtain when an enzyme generates a highly reactive molecule. For reasons already discussed, we believe that case 3 will only be found when the rate of enzyme–substrate dissociation is unusually slow.* In many cases this occurs with enzymes that utilize cofactors which require covalent interaction with the substrate (i.e. pyridoxal phosphate and flavin adenine dinucleotide). Consistent with this viewpoint are the examples reported here. It is well known that direct hydride transfer occurs between the substrate and nicotinamide portion of NAD^+ during the oxidation step. Thus, the

* R. R. Rando, *Science* (in press).

TABLE 2. EFFECTS OF ALLYL ALCOHOL AND ACROLEIN ON HORSE LIVER ADH*

	Percentage activity remaining†		
Time (min)	I ADH + ethanol + NAD^+ (ox) (%)	II ADH + 0·1 M allyl alcohol + NAD^+ (ox) (%)	III ADH + 10 mM acrolein (%)
5	100	100	100
20	100	100	100
45	100	100	100
90	100	100	100
150	100	100	100

* Horse liver ADH generates acrolein from allyl alcohol at a rate of 150 per cent of ethanol reaction. Acrolein is demonstrated to be the product of the allyl alcohol reaction by isolation and characterization of the 2,4-dinitrophenol, derivative. In the experiments reported here, the enzyme (0·01 mg/ml) (purchased from Boehringer-Mannheim Inc.) was incubated at room temp. with either 0·1 M allyl alcohol and 7·5 mM oxidized NAD^+ in 0·032 M sodium pyrophosphate, pH = 8·8, or in 10 mM acrolein. At various times, 100-μl aliquots were removed and assayed for activity by the standard ethanol assay.[4] In this assay, ethanol is oxidized to acetaldehyde and NAD is reduced to NADH. The rate of the reaction is followed by measuring the increase in O.D. at 340 nm (NADH absorption). These activities were compared to a control in which the enzyme was incubated with 0·1 M ethanol and 7·5 mM NAD^+ in the pyrophosphate buffer.

† Activity of I taken as 100 per cent.

acrolein which is formed is free to diffuse off the surface of the enzyme, and will do so much more rapidly than the time required for a direct chemical reaction with the enzyme.

The consequences of cases 1 and 2 are of pharmacological interest, since a rationale for drug design can be based on these phenomena—for example, if substrates could be found which are enzymatically converted into highly toxic Michael acceptors by an enzyme of the parasite, but not by the host, this would result in the selective destruction of the parasite. That this is, at least in theory, possible can be demonstrated by the alcohol dehydrogenase enzymes. The horse liver enzyme will convert cyclohex-2-en-1-ol into the highly reactive 2-cyclohexenone, whereas the yeast enzyme cannot effect this conversion.*

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REFERENCES

1. R. R. RANDO, *Nature New Biol.* **237,** 53 (1972).
2. E. D. BERGMANN, D. GINSBURY and R. RAPPO, in *Organic Reactions* (Eds. R. ADAMS *et al.*), Vol. 10, chap. 3. Wiley, New York (1959).
3. T. SABLES and L. WEIL, in *Methods in Enzymology* (Ed. C. H. W. HIRS), Vol. 11, p. 204. Academic Press, New York (1967).
4. H. SUND and H. THEORELL, in *The Enzymes* (Eds. P. D. BOYER, H. LARDY and K. MYRBACK), Vol. 1, p. 39. Academic Press, New York (1971).
5. B. L. VALLEE and F. L. HOCH, *Proc. natn. Acad. Sci. U.S.A.* **41,** 327 (1955).
6. J. TWU, C. C. Q. CHIN and F. WOLD, *Biochemistry, New York* **12,** 2856 (1973).
7. K BLOCH, *Accts chem. Res.* **2,** 193 (1969).
8. C. T. WALSH, A. SCHONBRUNN, O. LOCKRIDGE, V. MASSEY and R. H. ABELES, *J. biol. Chem.* **247,** 6004 (1972).
9. R. R. RANDO, *J. Am. chem. Soc.* **95,** 4438 (1973).
10. B. R. BAKER, *Design of Active-Site-Directed Irreversible Enzyme Inhibitors.* Wiley, New York (1967).

* R. R. Rando, unpublished experiments.