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In situ Generation of Irreversible Enzyme Inhibitors

MOLECULES which act as Lewis acids in the Michael reaction[1] profoundly inhibit the activities of biological molecules, no doubt because they can alkylate sulphydryl groups and certain activated amines. Acrylonitrile rapidly and irreversibly alkylates proteins in mild conditions[2] and recent findings strongly suggest[3] that the cytotoxic compounds produced by irradiating carbohydrates may also act as acceptors in the Michael reactions. A variety of α,β-unsaturated carbonyl-containing compounds possess anti-bacterial, anti-cancer, and anti-viral activities[3], and *in vivo* conversion of the potent anti-tumour agent cyclophosphamide into acrolein[4] suggests that acrolein may be the active cytotoxic agent. Furthermore, it is known that acrolein inhibits transcription *in vitro* by inhibiting RNA polymerase[5].

With these experiments in mind we set out to find an efficient enzymatic means of generating *in vivo* Michael acceptors from non-toxic reactants. We used alcohol dehydrogenase to generate the acrolein which subsequently irreversibly inhibited certain enzymes *in situ*.

Allyl alcohol is smoothly dehydrogenated to acrolein by dehydrogenases of both yeast and horse liver in the presence of oxidized nicotinamide adenine dinucleotide (NAD) as the hydride acceptor, and the incubation of either fumarase or β-galactosidase with these ingredients causes profound, irreversible inhibition of the enzymes (Table 1). In separate experiments it was shown that both enzymes are irreversibly inhibited by the addition of acrolein and Table 1 shows that this is the agent inhibiting the enzymatic system. Neither allyl alcohol nor aliphatic aldehydes (acetaldehyde) inhibit the enzymes.

In the experiments the enzymes to be inhibited were added to three vials containing 1.0 ml. 0.1 M KPO_4, *p*H 7.5. To vial I was added 30 μg yeast alcohol dehydrogenase (ADH), 5 μg malate dehydrogenase (MDH) and oxaloacetic acid (OAA) to 10^{-2} M (the last two were added to increase the acrolein produced), 1.3 mg NAD (2×10^{-3} M), and 35 μl. purified allyl alcohol. Vial II contained everything in vial I except for yeast ADH and vial III was the same as I except for the substitution of ethanol for allyl alcohol. Vials II and III were controls since no acrolein was produced in vial II and acetaldehyde was produced in III. The solutions were incubated for the requisite times and then dialysed separately against 1 l. 0.1 M KPO_4, *p*H 7.5, containing 0.01 M 2-mercaptoethanol with three buffer changes during 24 h. Aliquots of the solutions were then assayed, β-galactosidase activity by the method of Yariv *et al.*[6] and fumarase by the standard method[7].

Table 1 Irreversible Inhibition of Fumarase and β-Galactosidase

Enzyme	Incubation time (h)	Activity remaining (%)* I Enz, ADH, MDH, OAA; NAD and allyl alcohol	II Enz, MDH, OAA, NAD and allyl alcohol	III Enz, ADH, MDH, OAA, NAD and ethyl alcohol
Fumarase	5	0	100	100
β-Galactosidase	20	15	100	100

* Activity of III taken as 100%.
Enz refers to either pig heart fumarase or *E. coli* β-galactosidase, and MDH to pig heart malate dehydrogenase.
All enzymes were purchased from Boehringer Mannheim Corp.

Table 2 Irreversible Inhibition of Alcohol Dehydrogenase

Enzyme	Incubation time (h)	Remaining activity % I ADH, MDH, OAA, NAD and allyl alcohol	II ADH, MDH, OAA and allyl alcohol	III ADH, MDH, OAA, NAD and ethyl alcohol
Yeast alcohol dehydrogenase	3	0	25*	100
Horse liver alcohol dehydrogenase	24	75	100	100

* The activity of yeast ADH control II is lower than III because commercial yeast ADH carries adsorbed NAD. Acrolein was generated in II, albeit in smaller quantities than in I.

As well as inhibiting the added enzyme, the *in situ* generated acrolein irreversibly inhibited its maker, yeast alcohol dehydrogenase. The results of inhibition studies on both yeast and horse liver alcohol dehydrogenases are indicated in Table 2. The method of these experiments was similar to that outlined in Table 1 except that the inhibited samples were dialysed against 0.1 M sodium pyrophosphate buffer, *p*H 9.0, containing 30 mg/l. reduced glutathione. The ADH activity was assayed as before. The induced "suicide" by the substrate of yeast alcohol dehydrogenase is reminiscent of the case described by Bloch and coworkers[9] where β-hydroxydecanoyl dehydrase catalyses its own inhibition in the presence of $\Delta^{3,4}$-decynoyl-N-acetylcysteamine.

In vivo enzymatically generated acrolein ought to alkylate nucleic acids, sulphydryl-containing compounds such as glutathione and other nucleophilic biomolecules beside proteins.

The first part of our objective has been achieved by finding that alcohol dehydrogenase can produce sufficient quantities of acrolein to inhibit enzymes irreversibly. In this example a chemically unreactive species, allyl alcohol, was converted enzymatically into the highly reactive Michael acceptor, acrolein. Further work will test various allyl alcohol derivatives for pharmacological activity.

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ROBERT R. RANDO
Department of Chemistry, New York University, New York NY 10003

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