Schuler, M. L., Tsuchiya, M. H., and Aris, R. (1973), *J. Theor. Biol. 41*, 347.

Von Korff, R. W. (1972), *in* Energy Metabolism and the Regulation of Metabolic Processes in Mitochondria, Mehlman, M. A., and Hanson, R. W., Ed., New York, N. Y., Academic Press, p 93.

Webb, J. L. (1963), Enzyme and Metabolic Inhibitors, New York, N. Y., Academic Press.

Irreversible Inhibition of Aspartate Aminotransferase by 2-Amino-3-butenoic Acid†

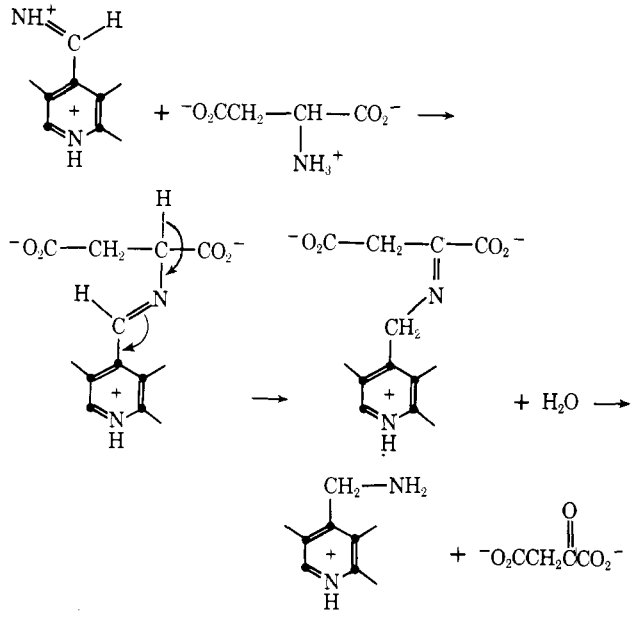
Robert R. Rando

ABSTRACT: Pyridoxal-linked aspartate aminotransferase is irreversibly inactivated by 2-amino-3-butenoic acid. The mode of inhibition of this inhibitor requires that it be chemically converted into its active form by the target enzyme. The inhibitor has no effect on either the apoenzyme or the holoenzyme in the pyridoxamine form. A profound ultraviolet spectral change accompanies the inactivation process and ^{14}C inhibitor is covalently incorporated into the inactivated enzyme.

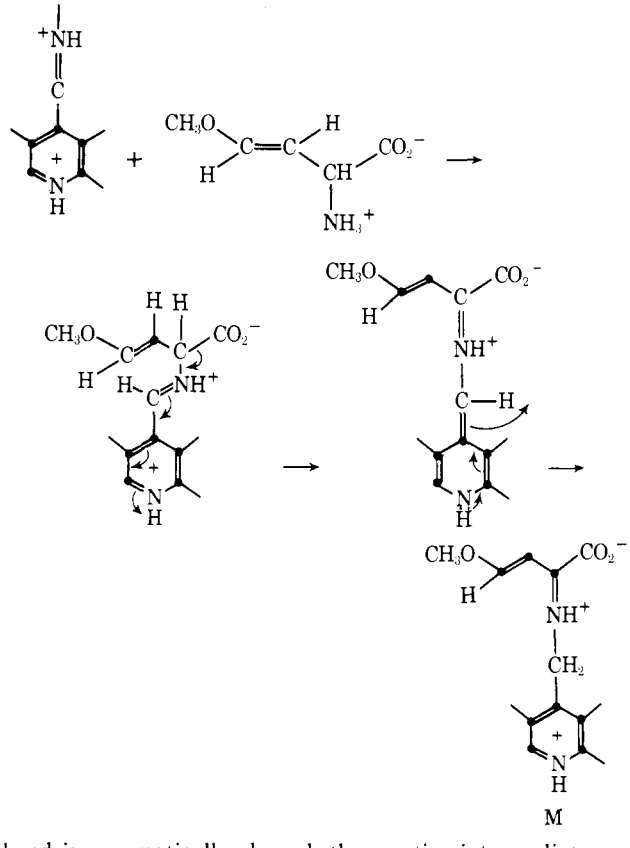
The microbial toxin *trans*-L-2-amino-4-methoxy-3-butenoic acid has been shown to be an irreversible inhibitor of soluble, pyridoxal linked, aspartate aminotransferase (Rando, 1974a). The inhibitor does not contain chemically reactive groups, as such, but is transformed into a reactive inhibitor by the target enzyme (Rando, 1974a). That is, aminomethoxybutenoic acid, a substrate for the enzyme, and one or several of the intermediates along the enzymatic pathway are sufficiently reactive to engage in a chemical reaction(s) with the enzyme. Thus, the enzyme is an agent of its own destruction.

The mechanism of action of this enzyme involves the initial sequence of steps shown in Scheme I (Hammes and Fasella, 1963). The transamination process is completed by the reversal of this sequence with α-ketoglutarate. The net reaction is: aspartate + α-ketoglutarate → oxaloacetate + glutamate. With 2-amino-4-methoxy-3-butenoic acid as the substrate, the following conversions can occur (Scheme II). Once the α C–H bond is enzymatically cleaved, the reactive intermediates can form. The highly reactive Michael acceptor (M) could be involved in the inactivation step by engaging in a reaction with an active-site Lewis base. However, the presence of the enol ether moiety renders the molecule potentially bifunctional in

SCHEME I



SCHEME II



† From the Department of Pharmacology, Harvard Medical School, Boston, Massachusetts 02115. *Received April 29, 1974.* This work was supported by the National Institutes of Health (1 R01 GM 20,366-01 and 1 R01 NS 11,550-01).

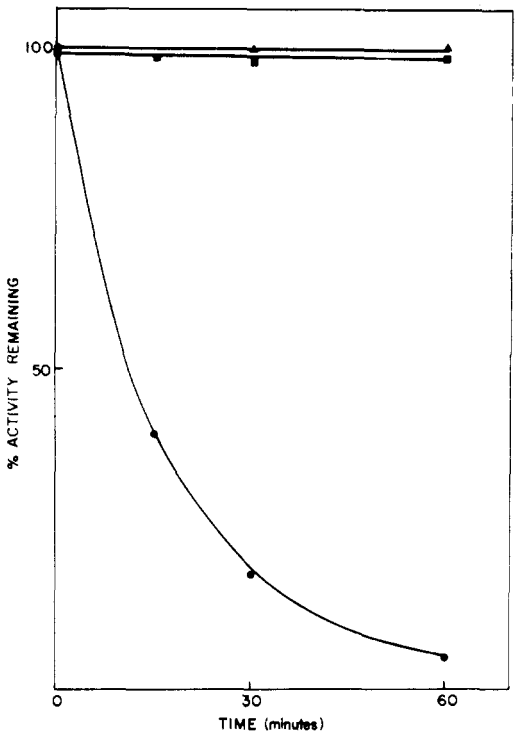


FIGURE 1: Irreversible inactivation of aspartate aminotransferase by 2-amino-3-butenoic acid.

its reactive characteristics. It was therefore of interest to test the parent β,γ-unsaturated amino acid, 2-amino-3-butenoic acid, to see if it is also an irreversible inhibitor of the enzyme. In this paper we confirm this notion and demonstrate the 2-amino-3-butenoic acid induced irreversible inactivation of aspartate aminotransferase.

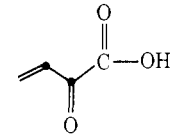
Results

Irreversible Inhibition of Aspartate Aminotransferase by 2-Amino-3-butenoic Acid. Incubation of aspartate aminotransferase with DL-2-amino-3-butenoic acid led to the irreversible inactivation of the enzyme as shown in Figure 1.[1] As expected, the enzyme cannot be in the least reactivated by continued dialysis. Furthermore, L-aspartic acid protects against inactivation (Figure 1). This observation is consistent with the notion that DL-2-amino-3-butenoic acid is active-site directed. Though unlikely here, one must consider the possibility that the enzyme is producing its own affinity labeling agent (Baker, 1967).[2] That is, the enzymatic product diffuses into solution only to later return to react with an active-site residue. This case has been shown to obtain in the allyl alcohol induced irreversible inhibition of yeast alcohol dehydrogenase (Rando, 1972, 1974b). This possibility can be ruled out here by the demonstration that 0.1 M mercaptoethanol has no effect on the rate of 2-amino-3-butenoic acid induced irreversible inhibition of aspartate aminotransferase at pH 8.0. Had the active inhibitor, in this case

TABLE I: Effect of 2-Amino-3-butenoic Acid on Apoaspartate Aminotransferase and Holoaspartate Aminotransferase in Pyridoxamine Form.

Enzyme	Concn Inhibitor (mM)	Incubation Time (hr)	% Act. Remaining[a]
Holoenzyme (pyridoxal form)	20	3	0
Holoenzyme (pyridoxal form)	0	3	100
Holoenzyme (pyridoxamine form)	20	3	100
Apoenzyme	20	3	100[b]

[a] Experimental error is ±5%. [b] After reconstitution.



diffused into solution first it would have been trapped by the mercaptoethanol at pH 8. Thus, we can be confident that direct inactivation of the enzyme is occurring. The fact that the time course of inactivation does not exhibit a lag is also consistent with this view.

Lack of Effect of 2-Amino-3-butenoic Acid on Apoenzyme and Holoenzyme in Pyridoxamine Form. The experiments described above show that 2-amino-3-butenoic acid is an active-site directed, irreversible inhibitor of the enzyme. They in no way demonstrate that enzymatic conversion of 2-amino-3-butenoic acid to a reactive form must precede the inactivation step. If the hypothesis that catalytic conversion must precede inactivation is correct, then anything which blocks this conversion should also prevent inactivation from occurring. This can be accomplished here in two ways. The holoenzyme can be resolved into apoenzyme and cofactor (Bertland and Kaplan, 1968). After removal of the resolved pyridoxal phosphate cofactor the enzyme is, of course, catalytically inactive. The activity can be restored by readdition of the cofactor. Apoenzyme prepared in this manner and treated with aminobutenoic acid for long periods of time can be completely reactivated after removal of the aminobutenoic acid and readdition of fresh pyridoxal phosphate (Table I). This is precisely what is expected. A different experiment can also be conducted along these lines. The aldehyde form of the enzyme can be completely converted into the pyridoxamine form by treatment with L-cysteinesulfinate (Jenkins and D'Ari, 1966). Holoenzyme prepared in this manner should not be affected by aminobutenoic acid since Schiff base formation with the pyridoxal form of the enzyme is required prior to further enzymatic conversion. This cannot occur when the enzyme is in the pyridoxamine form. This expectation is also confirmed, as shown in Table I. These experiments, taken together, indirectly demonstrate that aminobutenoic acid is not a simple affinity labeling agent of the enzyme, but must first be converted into an active form prior to inactivating the enzyme.

Ultraviolet Spectrum of Inactivated Enzyme and Radioactive Labeling with [1-^{14}C]-2-Amino-3-butenoic Acid. More direct evidence for the requirement of enzymatic transformation prior to the inactivation comes from experiments on the ultra-

[1] The concentration of L-2-amino-3-butenoic acid used (10 mM) is well below that required for saturation. We estimate the binding constant for reversible inhibitor binding to be in the 0.1 M range. This is only an estimate because at the higher inhibitor concentrations the rate of enzyme inactivation is too rapid to make careful measurements.

[2] The term affinity labeling agent is used here to mean a chemically reactive molecule approximately isosteric with the substrate which can undergo covalent bond formation with a specified receptor. The specificity of a reagent of this type is solely determined by the magnitude of its binding constant to the receptor.

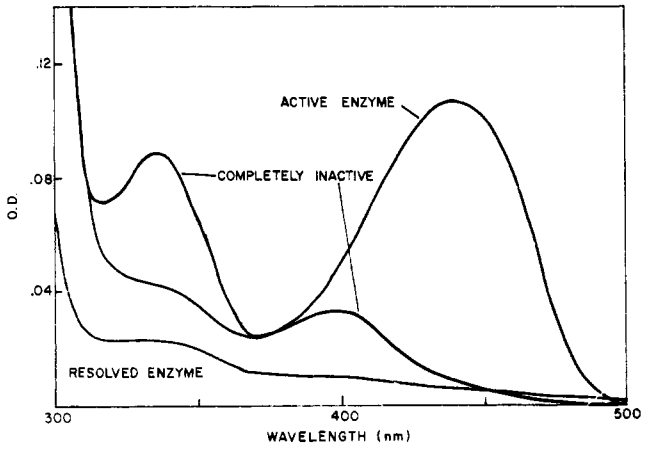


FIGURE 2: Ultrviolet spectra and inactivation of aspartate aminotransferase.

violet spectra of the holoenzyme as a function of the inactivation process. At pH 6.2 the active enzyme possesses a λ_{max} at 435 nm (Martinez-Carrion *et al.*, 1965). In the presence of aminobutenoic acid the spectrum drastically changes as can be seen in Figure 2. The completely inactive enzyme has a λ_{max} centered at 335 nm. The shape and position of this peak are affected neither by pH nor sodium borohydride. These observations taken together strongly suggest the presence of a pyridoxamine derivative (Khomutov *et al.*, 1963). Now, importantly, this holoenzyme can be resolved under the usual conditions (Figure 2). However, upon the readdition of pyridoxal phosphate to the resolved apoenzyme, no significant activation occurs even though the pyridoxal phosphate binds to the enzyme as judged from the ultraviolet spectrum of the reconstituted holoenzyme. This experiment requires that the primary action of aminobutenoic acid is to react with an active-site residue and not with the cofactor. Had the reaction occurred with the cofactor, readdition of fresh pyridoxal phosphate should have reactivated the enzyme.

The hypothesis that aminobutenoic acid directly attaches itself to the enzyme can be directly confirmed by demonstrating covalent attachment of radioactive aminobutenoic acid to the enzyme as a function of the time course of inhibition. As a control we use holoenzyme in the pyridoxamine form. As can be seen in Figure 3 counts are increasingly incorporated into the holoenzyme in the aldehyde form until full inactivation occurs. In a striking way, no counts above background are shown to be incorporated into the holoenzyme in the pyridoxamine form. Thus, we have shown that incorporation of aminobutenoic acid is synonymous with inactivation and conversely that lack of incorporation of aminobutenoic acid is synonymous with lack of inactivation. In addition, these experiments also confirm the notion that the mechanism of action of aminobutenoic acid involves a chemical reaction with an active-site residue and not with the cofactor. We say this because the inactivated enzyme is denatured with Cl_3CCOOH, spun down, and washed five times with 10% Cl_3CCOOH. Under these conditions, the B_6 cofactor is removed with the Cl_3CCOOH washes.

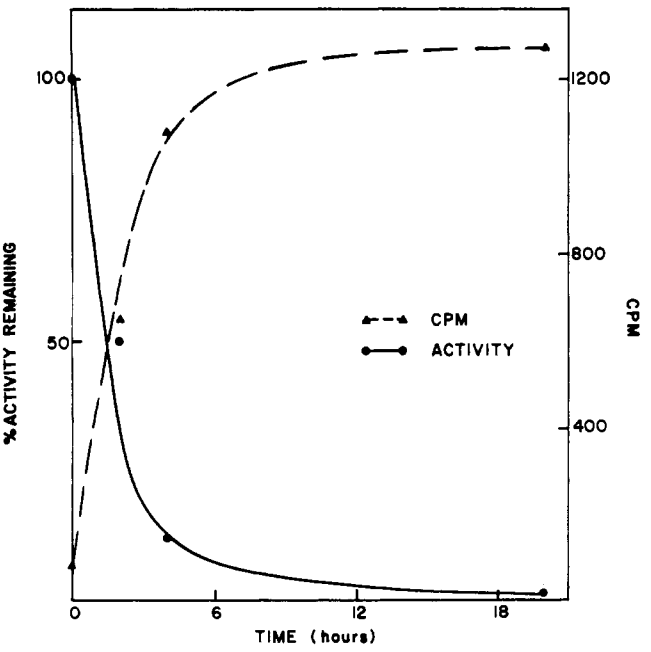
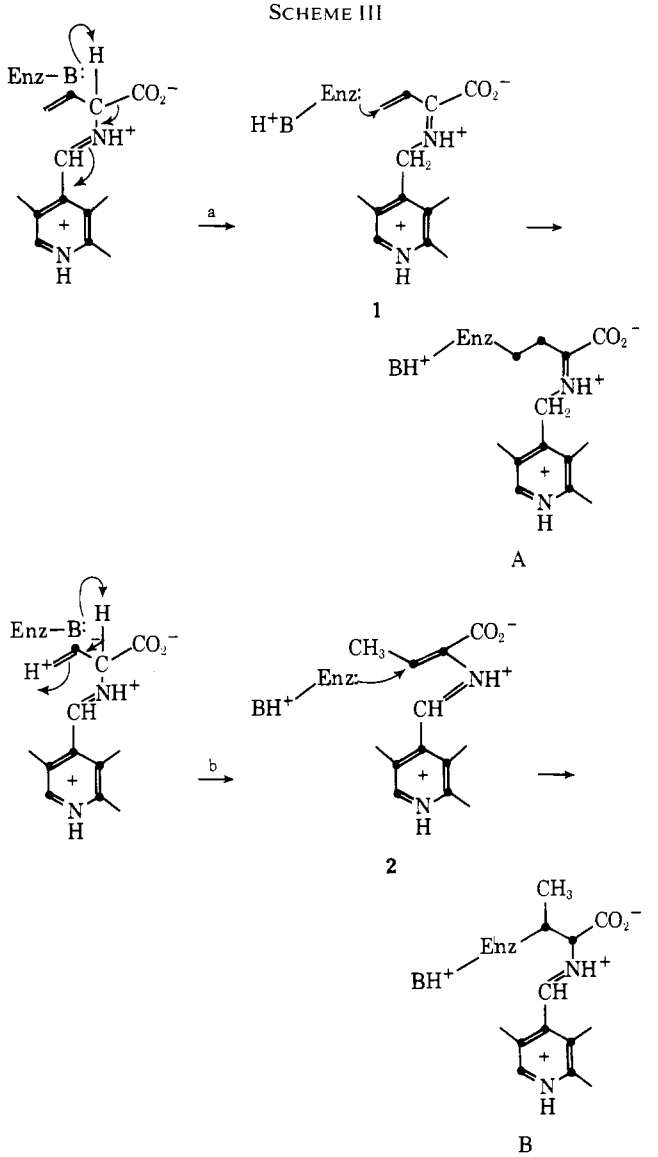


FIGURE 3: Labeling of aspartate aminotransferase by [1-^{14}C]-2-amino-3-butenoic acid.

Discussion

The results reported here demonstrate that DL-2-amino-3-butenoic acid irreversibly inactivates aspartate aminotransferase by a mechanism that requires enzymatic conversion prior to inactivation. Two possible mechanisms for this conversion are shown in Scheme III. Pathway 2 can be confidently ruled

SCHEME III



out as a possibility on the following grounds. First and foremost, structure B is inconsistent with the ultraviolet spectral change which accompanies the inactivation. The λ_{max} for B should be about 435 nm at pH 6.2 since it is a pyridoxal derivative. Secondly, the ultraviolet spectrum of the inactivated enzyme is neither perturbed by pH nor by sodium borohydride. Both pH and borohydride would drastically change the spectrum of B. Finally, the conversion labeled b would be unnatural for the transaminase inasmuch as once the α C–H bond is cleaved the electrons should flow toward the strongly electropositive pyridoxal ring. On the other hand, the pathway labeled a leading to A is completely consistent with all of the data available. Its spectrum is consistent with the one observed, being a pyridoxamine derivative. In addition, neither pH nor borohydride should perturb it. And, finally, the mechanism of its formation would not require the enzyme to follow an unnatural pathway. Thus, we argue that it is the oxidative conversion of aminobutenoic acid to the powerful Michael acceptor **1** that is crucial to the inactivation process. Furthermore, the fact that the chemically activated aminobutenoic acid is held at the active site by its covalent linkage to the cofactor increases the probability of a reaction with an active-site residue prior to diffusion into solution. This latter point is an important, though not completely obvious one. The mere formation of a chemically reactive molecule at the active site of an enzyme does not ensure that a chemical reaction with a nearby residue will occur (Rando, 1974c).

In fact this possibility is mitigated against by the much higher rate of enzyme–substrate dissociation *vs.* covalent bond formation. For example, NAD^+-linked alcohol dehydrogenase will catalyze the formation of acrolein (a powerful Michael acceptor) from allyl alcohol without simultaneous inactivation of the enzyme (Rando, 1972, 1974b). This occurs because the acrolein, once generated, is not held by covalent linkage to the active-site region and simply diffuses rapidly into solution without reacting with an active-site residue.

As mentioned in the introductory statement, the naturally occurring toxin, 2-amino-4-methoxy-3-butenoic acid, is also an irreversible inhibitor of aspartate aminotransferase (Rando, 1974a). It is of interest to compare the modes of the irreversible inhibition by these two molecules as it relates to their structure. Both inhibitors must inactivate the enzyme by similar mechanisms since the kinetics of inactivation as well as the effects of the inhibitors on apoenzyme and the pyridoxamine form of the enzyme are identical. This is expected since the reactive intermediates in both cases will be similar. This is because the salient feature of the two inhibitors is the presence of the β,γ double bond which allows for the formation of the reactive Michael acceptor intermediates. The mechanism of action of the two inhibitors differs in one important respect, though. The ultraviolet spectral changes accompanying the inactivations are qualitatively different. The spectral changes with 2-amino-4-methoxy-3-butenoic acid are more complicated than with 2-amino-3-butenoic acid. In the former case the spectrum of the inhibited enzyme is a triplet with a λ_{max} of approximately 350 nm (Rando, 1974a). The identification of the molecular species giving rise to this complicated spectrum is the subject of current consideration. This difference is probably due to the potential bifunctionality of the enol ether moiety in the molecule once enzymatic inactivation to the Michael acceptor has occurred.

In summary, DL-2-amino-3-butenoic acid has been demonstrated to be an irreversible enzyme inhibitor which requires chemical activation by the target enzyme. The molecule is oxidatively activated by the transaminase to generate a highly reactive Michael acceptor which engages in a chemical reaction with an active-site residue.

Materials and Methods

Cytoplasmic, pig heart, aspartate aminotransferase (predominantly the α subform) (Martinez-Carrion *et al.*, 1965), α-ketoglutaric acid, L-aspartic acid, NADH, and L-cysteinesulfinic acid were all products of the Sigma Chemical Co. The specific activity of the enzyme is 85 units/mg, where one unit of enzyme will convert 1 μmol of aspartic acid to oxaloacetate under standard conditions (Lis, 1958). DL-2-Amino-3-butenoic acid was prepared from 2-hydroxy-3-butenoic acid by standard synthetic routes (Glattfield and Hoen, 1935). The yield of 2-amino-3-butenoic acid was consistently in the 50% range starting from 2-hydroxy-3-butenoic acid after purification by ion exchange on Amberlite IR-120 HCP resin (H^+ form) and crystallization from ethanol–water mixtures. The amino acid is a white crystalline solid of mp 215° dec with an r_f value (descending chromatography) of 0.21 on Whatman No. 1 paper with butanol–water–acetic acid (18:5:2 mixture). The amino acid shows color changes of yellow to grey to purple with ninhydrin. This color change is characteristic of β,γ-unsaturated amino acids (Levenberg, 1968). *Anal.* Calcd of the amino acid: C, 47.52; H, 6.93; N, 13.86. Found: C, 47.22; H, 6.70; N, 13.57.

The nmr (D_2O) and infrared spectra of the 2-amino-3-butenoic acid are totally in accord with the proposed structure and the molecule can be hydrogenated in 10% H_2O–MeOH with 5% palladium on carbon to afford DL-2-aminobutanoic acid which is identical in every way with authentic material (Eastman Organic Chemical). The amino acid is highly soluble in water, sparingly soluble in ethanol, and insoluble in benzene. [1-^{14}C]-2-Amino-3-butenoicacid of specific activity 0.016 mCi/mmol was prepared by the usual method from [1-^{14}C]-2-hydroxy-3-butenoic acid.

The activity of the enzyme was routinely assayed by the published method on a Guilford No. 240 recording spectrophotometer (Amador and Wacker, 1962). Ultraviolet spectra were determined with a Cary 118 spectrophotometer. Measurements of radioactivity were made on a Packard Model 3375 liquid scintillation counter. Protosol (New England Nuclear) was used to dissolve precipitated and denatured enzyme prior to counting. Counting was then conducted in toluene with 4 g of 2,5-diphenyloxazole plus 0.05 g of 1,4-bis[2-(5-phenyloxazolyl)]benzene per liter. Efficiencies were determined with standard amounts of [^{14}C]valine.

The enzyme was resolved into apoenzyme and cofactor by the method of Bertland and Kaplan (1968). The pyridoxamine form of the enzyme was prepared with L-cysteinesulfinic acid according to the procedure of Jenkins and D'Ari (1966).

Irreversible Inactivation of Aspartate Aminotransferase by 2-Amino-3-butenoic Acid (*See Figure 1*). Aspartate aminotransferase (0.08 mg/ml) was incubated at 37° in 0.1 M potassium phosphate (pH 7.6) and 20 mM racemic 2-amino-3-butenoic acid. At the indicated times aliquots were removed and the remaining activity was determined by the standard assay (Rando, 1972, 1974b) (●). The control consisted of incubating the enzyme as above minus 2-amino-3-butenoic acid (▲). Protection against inactivation by substrate (aspartic acid) was demonstrated by incubating the enzyme in buffer and 0.2 M aspartate in addition to 20 mM 2-amino-3-butenoic acid (■).

The activity of the inactivated enzyme was not restored by dialysis against the phosphate buffer with several changes over a 36-hr period. Furthermore, the activity of the inactivated en-

zyme could also not be restored by incubation with 1 mM pyridoxal phosphate for several hours.

In a separate experiment the enzyme was incubated with and without 20 mM 2-amino-3-butenoic acid at pH 8.0 in the presence of 0.1 M mercaptoethanol. The thiol had no effect on the rate of inactivation of the enzyme nor did it have any effect on the stability of the enzyme.

Effect of 2-Amino-3-butenoic Acid on Apoaspartate Aminotransferase and Holoaspartate Aminotransferase in Pyridoxamine Form (*See Table I*). RESOLUTION EXPERIMENT. Aspartate aminotransferase (4 mg/ml) was resolved into catalytically inactive apoenzyme and pyridoxal phosphate by the published method (Rando, 1974a). Complete dialysis of the enzyme against 0.1 M potassium phosphate (pH 7.4) led to the catalytically inactive apoenzyme whose ultraviolet spectrum indicated a virtually complete absence of cofactor. The apoenzyme was split into two samples; one sample was incubated with 20 mM 2-amino-3-butenoic acid for 3 hr and the second served as a control. The two samples were then thoroughly dialyzed times 1000 vol of phosphate buffer and then activated by adding fresh 1 mM pyridoxal phosphate (Rando, 1974a). The two reactivated samples had exactly the same activity by the usual assay.

PYRIDOXAMINE EXPERIMENT. Two samples of enzyme (2 mg/ml) were prepared. The first was treated with L-cysteinesulfinate by the published method and the second served as a control (Jenkins and D'Ari, 1966). The ultraviolet spectrum of the treated sample showed that it was completely in the pyridoxamine form. Both samples were thoroughly dialyzed against 0.1 M potassium buffer at pH 7.5. The cysteinesulfinate treated and dialyzed enzyme was then incubated with 20 mM 2-amino-3-butenoic acid at room temperature for 3 hr. Both samples were then thoroughly dialyzed against the buffer. The activities of aliquots of these samples were shown to be identical by the standard assay.

Ultraviolet Spectra and Inactivation of Aspartate Aminotransferase (*See Figure 2*). The ultraviolet spectra of 1.7 mg/ml of aspartate aminotransferase at pH 6.2 in 0.1 M potassium phosphate were recorded. One sample at this concentration was treated with 20 mM 2-amino-3-butenoic acid and a second served as a control. After 12 hr the ultraviolet spectra of the two samples were determined as well as their activities. The untreated sample lost little activity (10%) and had the same ultraviolet spectrum as the starting material, whereas the totally inactivated had a completely different spectrum as shown. The inactivated enzyme could be resolved by the usual method to give the resolved spectrum shown (Rando, 1974a). Fresh pyridoxal phosphate could be added back to this resolved enzyme and the ultraviolet spectrum of the starting holoenzyme could be largely regenerated. However, the catalytic activity of the enzyme was not regained as determined by the standard assay system (Lis, 1958). Finally, the resolved cofactor from the inactivated enzyme could activate fresh apoenzyme. This is taken to mean that the pyridoxamine has not been chemically modified by the inhibitor.

Labeling of Aspartate Aminotransferase by [1-^{14}C]-2-Amino-3-butenoicAcid (*See Figure 3*). Aspartate aminotransferase (4 mg/ml) at pH 8.0 in 0.1 M potassium phosphate was incubated with 10 mM[1-^{14}C]-2-amino-3-butenoic acid (specific activity, 0.016 mCi/mmol). As a separate control, the same amount of enzyme pretreated with cysteinesulfinate and dialyzed to get rid of the excess was also treated with the radioactive amino acid. At the various times indicated 1-ml aliquots of the samples were removed and added to 10 ml of 10% trichloroacetic acid. The precipitation was allowed to proceed for 5 min. In the meantime the activity of separate aliquots of the enzyme was determined using the standard assay (Lis, 1958). After the precipitation was completed the sample was spun down using a clinical centifuge. The supernatant was poured off and the pellet was resuspended in 10 ml of 10% Cl_3CCOOH and again respun. The supernatant was again poured off. This process was repeated five more times. At the last step the pellet was carefully drained and transferred to a counting vial in 0.25 ml of H_2O. Protosol (2 ml) was added and the mixture was heated on a water bath (50–60°) until dissolved. At this point 10 ml of scintillation fluid was added and the vials counted in the usual manner. The cysteinesulfinate treated enzyme contained between 100 and 110 cpm at all time points. The zero time inhibitor control was about 100 cpm. Therefore, inhibitor was not incorporated into holoenzyme in the pyridoxamine form.

Acknowledgment

The excellent technical assistance of Edie Miller is gratefully acknowledged.

References

Amador, E., and Wacker, W. E. C. (1962), *Clin. Chem. 8,* 343.
Baker, B. R. (1967), Design of Active-Site-Directed Irreversible Enzyme Inhibitors, New York, N. Y., Wiley.
Bertland, L. H., and Kaplan, N. U. (1968), *Biochemistry 7,* 134.
Glattfield, J. W. E., and Hoen, R. E. (1935), *J. Amer. Chem. Soc. 57,* 1405.
Hammes, G. G., and Fasella, P. (1963), *in* Chemical and Biological Aspects of Pyridoxal Catalysis, Snell, E. E., *et al.,* Ed., New York, N. Y., Macmillan, p 185.
Jenkins, W. T., and D'Ari, L. (1966), *Biochem. Biophys. Res. Commun. 22,* 376.
Khomutov, R. M., Karpeisky, M. Ya., and Severin, E. S. (1963), *in* Chemical and Biological Aspects of Pyridoxal Catalysis, Snell, E. E., *et al.,* Ed., New York, N. Y., Macmillan, p 313.
Levenberg, B. (1968), *J. Biol. Chem. 243,* 6009.
Lis, H. (1958), *Biochim. Biophys. Acta 28,* 191.
Martinez-Carrion, M., Riva, F., Turano, C., and Fasella, P. (1965), *Biochem. Biophys. Res. Commun. 20,* 206.
Rando, R. R. (1972), *Nature (London), New Biol. 237,* 53.
Rando, R. R. (1974a), *Nature (London)* (in press).
Rando, R. R. (1974b), *Biochem. Pharm.* (in press).
Rando, R. R. (1974c), *Science 185,* 320 (1974).