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An investigation of possible inhibitors for D-amino acid oxidase

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AN INVESTIGATION OF POSSIBLE INHIBITORS FOR D-AMINO ACID OXIDASE

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To My Family

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ABSTRACT

This thesis describes the preparation of several α-substituted butynoic acids and their inhibition of D-Amino Acid Oxidase. A strong time-dependent inhibitor was found. Three new compounds have been prepared as intermediates in these reactions: ethyl 2-keto-3-butynoate, butyl 2-tosyl-3-butynoate and butyl 2-phthalimido-3-butynoate. Inhibition by diethyl oxalate is also described.

AN INVESTIGATION OF POSSIBLE INHIBITORS FOR D-AMINO ACID OXIDASE

I. INTRODUCTION

A. Scope of the Project and Thesis

It has been the purpose of this project to identify likely suicide inhibitors for D-Amino Acid Oxidase (DAAO), to attempt to synthesize these substances and to test their inhibition qualities *in vitro*.

A thorough search of the literature provided information on the reaction of DAAO, and the nature of the likely enzyme-substrate complex. With this understanding, suicide inhibitors for other enzymes were studied and a comparable class of substrates for DAAO identified. 2-Amino-3-butynoate and other α-substituted β-acetylenic acids looked most promising as compounds most likely to be suicide inhibitors for DAAO. The inhibition of DAAO by several substances prepared during the course of this project has been studied and a very powerful, time-dependent inhibitor was found.

The first section of this thesis summarizes what is known about the DAAO reaction, the nature of the substrate-enzyme complex, and information on suicide inhibition used to decide upon an appropriate series of compounds to prepare. Section II describes the actual experiments performed. Section III discusses the results and implications of these experiments. Section IV reviews what has been accomplished and discusses possible future work.

B. The Chemistry of DAAO

1. Biological Role

D-Amino Acid Oxidase (DAAO) was first discovered in 1938 by Warburg and Christean[1]. Its presence in large quantities in pig liver and kidney lead to its easy isolation and widespread use as a model flavoenzyme. It is also used in synthetic work to purify L-amino acids by selectively destroying D-amino acids in a racemic mixture. However, the exact biological function of this enzyme remains elusive. It is thought that DAAO may have some role in the immune system since it could destroy the D-amino acids that comprise bacterial cell walls. A possible role for this enzyme in lipid metabolism has also been postulated[2].

In addition to its presence in liver and kidney, DAAO is found in the brain. Patek believes it may be used to remove glycine which is found in modest concentrations in the cerebellum[3]. However, glycine is not a particularly good substrate for DAAO.[4] This project was undertaken to provide one step on the road to determining the biological role of DAAO.

2. Inhibition

To determine the role of an enzyme <u>in vivo</u>, one usually either removes it or destroys its activity, then observes what reactions do not take place in its absence. To remove DAAO requires destruction of the <u>in vivo</u> system. Therefore, some method of destroying its activity <u>in vivo</u> without harming other components of the system is desired. To this end one requires an inhibitor that: (1) is specific for the enzyme in question, (2) has easy mobility from the point of

injection to the position of the enzyme, and, (3) does not react with other compounds before or after enzyme interaction. We shall concentrate on the design of a substrate that meets criteria (1) and (3).

Inhibition has been extensively exploited in the last two decades to study enzyme mechanisms and enzyme roles in vivo. Several types of inhibition have been characterized:

(1) Competitive inhibition: The inhibitor competes with normal substrate for reversible binding at the active site. This changes the apparent K_M (the dissociation constant of the enzyme-substrate complex) of the enzyme system, but V_{max} remains constant since the enzyme can be saturated with excess substrate, effectively excluding inhibitor from any binding sites.

(2) Noncompetitive inhibition: Reversible binding of the inhibitor does not interfere with substrate binding but alters the rate of the reaction so that V_{max} is never reached. The rate of substrate binding is unaffected.

(3) Uncompetitive inhibition. The inhibitor binds reversibly in such a way that both the ability of the substrate to bind and the ability of the enzyme to catalize reaction are altered.

(4) Irreversible inhibition: Inhibition in any of the above manners except forming a permanent covalent bond. This bond is judged to be permanent when it is stable to dialysis, chromatography and other nondegradative physical methods.[5-7]

It is this last form of inhibition, irreversible inhibition, that we are interested in. We wish to find a component that will irreversibly bind to DAAO, bind at the active site, and prevent any enzyme activity. Furthermore, we wish it to bind in such a way that

it has no chance to undergo spurious reactions before or after interaction with the DAAO. We also wish to have an inhibitor that does not react with another species before finding the target enzyme. Suicide Substrates are special substrates that require alteration by a target enzyme before they become inhibitors of that enzyme. Some experimental criteria necessary for suicide inhibition are[5]:

(1) Loss of activity must be (a) first order, and, (b) time-dependent. If inactivation is time-dependent the enzyme has probably been covalently modified. First order kinetics indicate that inactivation has occurred before the inactivator had a chance to be released from the enzyme active site.

(2) The rate of inactivation (K_{inact}) must be proportional to the concentration of the inhibitor at low concentrations of inhibitor and independent of the concentration of inhibitor at high concentrations. That is, the enzyme-inhibitor interaction must be competitive with the substrate and must display saturation kinetics, both necessary phenomena if inhibition is at the active site.

(3) K_{inact} must decrease as the concentration of normal substrate increases. This is also a necessary consequence of inhibitor binding at the enzyme active site.

(4) The binding must be irreversible.

3. The DAAO Reaction and Kinetics

What properties of DAAO can be exploited in designing a specific inactivator? Suicide Substrate criteria (2) and (3) require that binding be at the active site of the enzyme. Therefore we wish to understand as much as possible about the active site of this enzyme;

its structure, the reactions it catalyzes, and the changes occurring at the active site during catalysis. Since DAAO has been used as a model enzyme in much flavoenzyme research, a great deal has been learned about its active site.

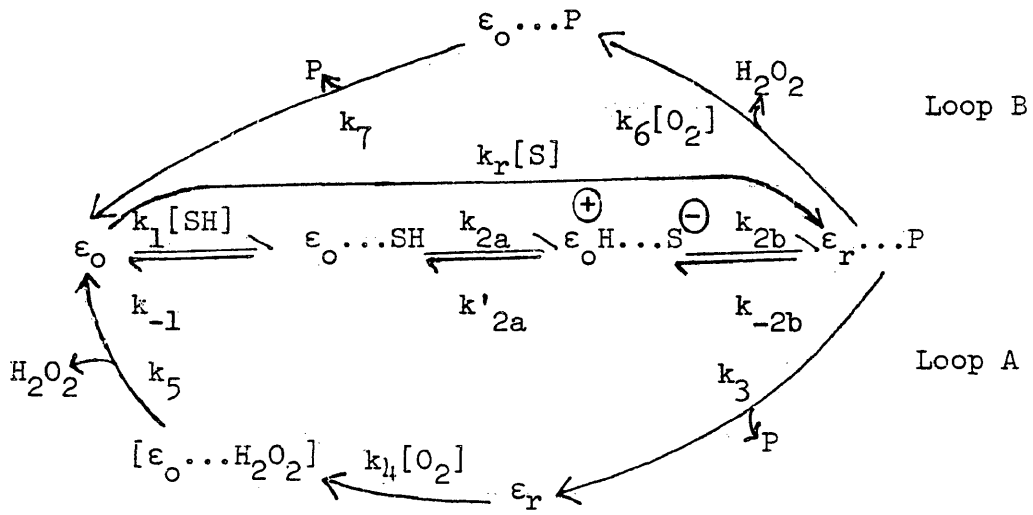
DAAO is a flavoenzyme. It contains flavin in the form of flavin adenine dinucleotide (FAD) (Fig. 1) as coenzyme. The proteinaceous apoenzyme has a high basic amino acid content[8]. The monomer has a molecular weight of about 38,000,[9-11] with 1 FAD for each monomer unit[12]. This flavin is intimately involved in enzyme catalysis. Spectral studies[13] show that it oscillates between its fully oxidized and fully reduced states during the catalytic reaction (Fig. 2). DAAO catalizes the oxidation of D-amino acids:

$$\begin{array}{l} \mathrm{NH_2} \\ \mathrm{R\text{-}C\text{-}COOH} \\ \mathrm{H\ [SH]} \end{array} + \mathrm{O_2} \xrightarrow{\mathrm{DAAO}} \begin{array}{l} \mathrm{NH} \\ \mathrm{RC\text{-}COOH} \\ \mathrm{[P]} \end{array} + \mathrm{H_2O_2} \qquad \text{(1a)}$$

$$\begin{array}{l} \mathrm{NH} \\ \mathrm{RCCOOH} \end{array} \xrightarrow{\mathrm{H_2O}} \begin{array}{l} \mathrm{O} \\ \mathrm{RCCOOH} \end{array} + \mathrm{NH_3} \qquad \text{(1b)}$$

where (1a) is the enzymatic oxidation of substrate and (1b) is the nonenzymatic hydrolysis to the final product. The reaction can also be run in reverse under anaerobic conditions[14].

Careful spectrophotometric and kinetic studies indicate that the specific stepwise reaction for flavoprotein oxidases can be represented as follows:[15]



(2)

That is, oxidized enzyme, E_o, combines with reduced substrate, [SH]. The enzyme oxidizes the substrate while itself being reduced, giving the complex $E_r...P$. At this point the enzyme has two pathways available to it. It can release oxidized substrate (P), then accept oxygen to return to its activated state (loop A) as one might intuitively expect. Or the enzyme-substrate complex can react with O_2 before the substrate is released (loop B). Figure 3 shows typical spectra for DAAO in its oxidized (E_o), reduced (E_r), and complexed ($E_r...P$) forms.

The complete steady-state rate equation for equation 2 is:

$$\frac{[E_T]}{v} = \frac{1}{k_2} + \frac{k_{-1}+k_2}{k_1k_2[S]} + \frac{k_{-1}k_{-2}}{k_1k_2(k_3+k_6[O_2][S])}$$

$$+ \frac{k_4k_5k_7(k_2+k_{-2})[O_2]+k_2(k_3k_7+k_5k_6[O_2])k_4[O_2]+k_2k_3k_5k_7}{k_2k_4k_5k_7(k_3+k_6[O_2])[O_2]} \qquad (3)$$

This rather unwieldy equation can be simplified by the method of Dalziel[17] into

$$\frac{[E_T]}{v} = \phi_o + \frac{\phi_1}{[S]} + \frac{\phi_2}{[O_2]} + \frac{\phi_{12}}{[S][O_2]} \qquad (4)$$

where the ϕ_i's are functions of the rate constants, and in particular, ϕ_o^{-1} is the maximum turnover number. As can be shown, ϕ_{12} usually = 0 because k_{-2} and/or $k_{-1} \simeq 0$[15,18] (see equation (3)) so that (4) becomes

$$\frac{[E_T]}{v} = \phi_o + \frac{\phi_1}{[S]} + \frac{\phi_2}{[O_2]} \qquad (5)$$

giving straight line kinetics.[15]

4. The Reaction Path

DAAO reacting with basic amino acids (especially aspartic and glutamic acids) follows loop A. $k_3 >> k_2$ so no $E_r...P$ can be detected.[16] However, when nonbasic amino acids are oxidized by DAAO, no correlation is observed between ϕ_o^{-1} and ϕ_2^{-1} and k_3 and k_4 of loop A; loop B must be operative instead. O_2 must react directly with $E_r...P$ and dissociation of oxidized substrate from reoxidized enzyme, k_7, must be the rate limiting step.

That loop B is indeed the proper path of equation (2) for most DAAO reactions was finally shown by Porter, Voet and Bright in a classic paper.[18] Using stopped flow kinetics and inhibitors of the oxidized enzyme, they were also able to measure k_6 and k_7.

Either loop in equation (2) can be divided into two ½-reactions. In the reductive ½-reaction (RHR), the substrate is oxidized and the flavin of DAAO is reduced. Under anaerobic conditions the reaction stops at this point. If O_2 is then added in a second step the oxidative ½-reaction (OHR) can take place. In this step the enzyme is reoxidized to its active state. In a stopped flow apparatus, two reaction mixtures (enzyme and substrate) simultaneously enter a

reaction chamber and the course of the reaction is monitored spectrophotometrically. In double stopped flow experiments this first reaction is allowed to reach equilibrium, then a third solution is injected (in this case O_2) and the next reaction, the re-oxidation of enzyme, is monitored. (Because E_r...P and E_o...P are only transient intermediates, they cannot be used as initiating reagents in the simpler single stopped flow experiments.)

To measure k_6, benzoate, a strong competitive inhibitor of E_o,[19] was added with the O_2 mix. Benzoate binds with the E_o formed in the OHR so that the reduction of enzyme is prevented and only the OHR takes place. The E_o-I complex is spectrally indistinguishable from E_o and E_o...P, and it is formed quickly compared to k_6 and k_7. Hence, by monitoring the increase of total E_o, E_o...P and E_o-I at 450 nm, or the decrease of E_r...P at 550 nm, a measurement of k_6 could be obtained. The rate equation for this reaction is:

$$k_{obs} = \frac{k_{-1}k_{-2}}{k_2+k_{-1}} + k_3 + k_6[O_2] \qquad (6)$$

The slope of a plot of k_{obs} vs $[O_2]$ gives $k_6 \approx \phi_2^{-1}$.

To measure k_7, anthranilate, an equally strong competitive inhibitor,[20] was added in the second mixture, again preventing the RHR from occurring. In this case, however, the E_o-I complex is spectrally distinct from E_o. Since again the complex forms very fast compared to k_6 and k_7, a measure of the decay of the 450 nm absorption peak should measure the rate of decay of E_o...P. The value thus obtained for k_7 agreed well with that measured for ϕ_o^{-1}, the maximum turnover number for the enzyme. From these experiments Porter, Voet and Bright concluded that:

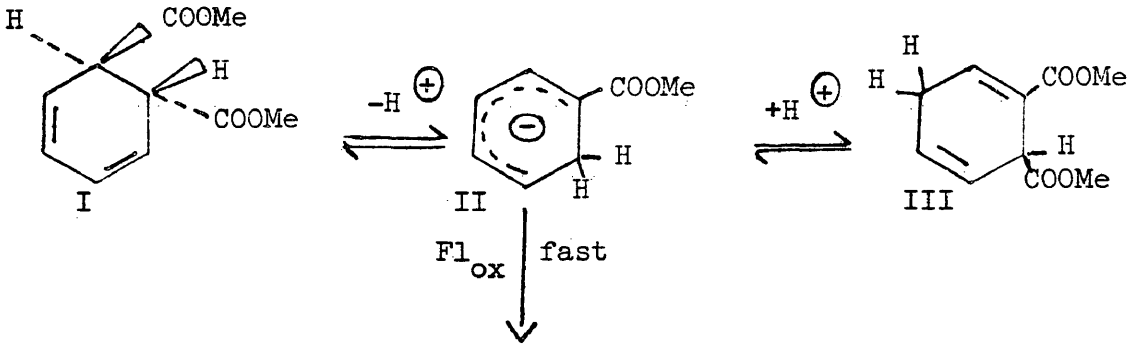
(1) Loop B is the operative reaction path for neutral amino acids; i.e., product is not released until after the flavin is reoxidized.

(2) Product release, step 7, is the rate limiting step in this loop. This means that the oxidized substrate does not leave the enzyme active site immediately upon oxidation, but stays around long enough to aid in the reoxidation of the enzyme. Therefore inhibition involving a compound that becomes destructive upon oxidation is a possibility.

5. The Nature of the Enzyme-Substrate Complex

Another long-standing problem which Porter, Voet and Bright addressed was the nature of the intermediate formed in the k_2 step. It was felt that this step involved H^+ abstraction with subsequent substrate carbanion formation (for a review of this literature see reference 15). Previous attempts at measuring linear free energy relationships led to confusing results because the differences between binding rates (k_1) and reaction rates (k_2) could not be sorted out for the various substrates used.[15] This difficulty arose because $k_{-2} = 0$ for most substrates so only the forward reaction could be monitored. With phenylalanine, however, $k_{-2} \neq 0$ and, again employing double stopped flow experiments, k_{-2} could be measured directly. Using a series of ring substituted phenylalanines to minimize the differences in hydrophobic, steric and hydrogen bonding interactions, Porter, Voet and Bright were able to measure the linear free energy relationships of $k_{rev} = k_{-2}$. They found that the magnitude of k_{-2} increased fairly consistently with the increasing electron withdrawing power of the

substrate, in accord with the concept of a developing negative charge in the transition state of step 2, as would be expected if the H is abstracted as a proton in the k_2 step. Furthermore, the kinetic isotope effect was the same in both the k_2 and the k_{-2} direction, indicating that the H^+ that has been removed from the substrate must remain bound at the enzyme active site during the two phases of step two. However, it is not clear whether it is removed by a basic residue of the apoprotein and later transferred to N(5) of the flavin, or is initially abstracted by the isoalloxazine ring. Abstraction by a basic residue has generally been assumed. This would leave an anionic species to interact with the isoalloxazine ring. Main, et. al.[21] have shown that an anion is the active species in at least one case. They studied the interaction of Fl_{ox} with dimethyldihydrophthalates. Compounds I and III, which have a common carbanion were used:



Fl-II complex

The rate of formation of II from III is 1/10 that of the formation of II from I. This matches the relative rates of flavin complex formation by each species at high flavin concentrations where the reaction is

zero order in flavin. The reaction has been found to be base-catalyzed indicating that it is indeed the carbanion species that is reacting and not some common neutral isomer.

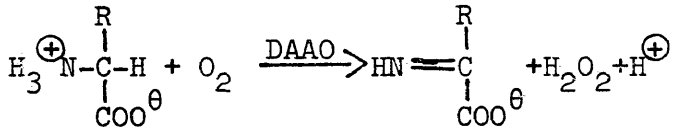
This concept of an anion as the active species is further supported by work on the unusual substrate NO_2Et where the preformed anion was found to be much more highly reactive than the neutral species.[22,23] NO_2Et has been trapped as a covalent adduct at N(5).[23]

Additional support comes from studies of the E_r...P complex which appears to be a charge-transfer complex between an anion and flavin. Massey and others have amassed considerable evidence that E_r...P is a charge-transfer complex where the substrate is the electron donor and the flavin the electron acceptor.[20, 24-33] This explanation of the long wavelength absorption of these species is supported by molecular orbital calculations.[34,35] Bruice and his colleagues, however, continue to try to prove the existence of free radicals as the intermediates in flavoenzyme oxidation reactions.[36-46] The major supporting evidence for these species being charge-transfer complexes rather than free radical systems is the lack of ESR spectra for these intermediates. However, the possibility of free radical transient intermediates cannot be ruled out. These various experiments all support the concept that (a) the α-H is abstracted as a proton by a basic residue at the active site and that (b) it is the anion that interacts with the isoalloxazine ring.

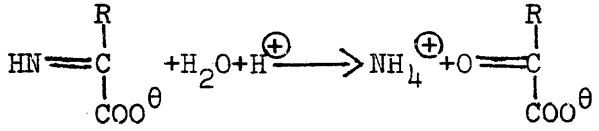
6. The Form of the Product Released

For a long time it was felt that the enzyme released the altered substrate as the oxidized product. Yagi, et. al.[47] were the

first to point out that an α-imino acid is released by the enzyme which is then immediately hydrolized by the aqueous medium. Yagi measured an initial drop, then subsequent increase in pH as the oxidation reaction proceeded. The rise in pH was not observed, however, when proline was oxidized. He interpreted this data to mean that an imino-acid was being initially released for linear amino acids:

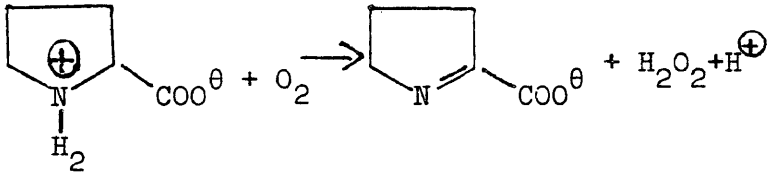


pk = 6-7 drop in pH (8)



pk = 9.3 rise in pH

But for proline:

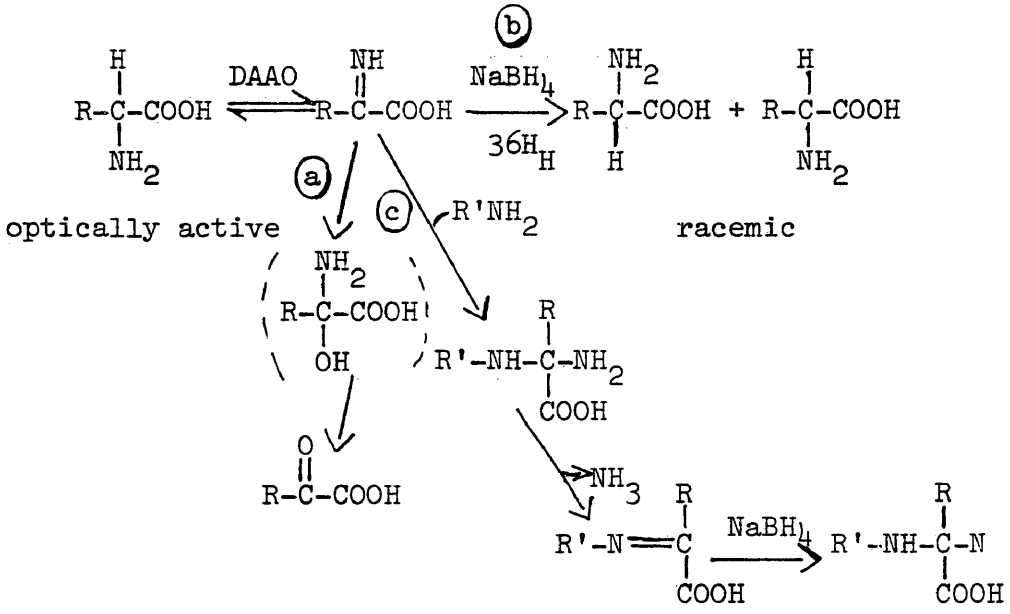


(9)

The imine is a stable species which is not hydrolyzed and therefore the secondary rise in pH was not observed.

Hafner and Wellner[48] succeeded in trapping the imine being released using sodium borohydride. If an imine is released from the enzyme before it is fully oxidized, a racemic reduction product should be obtained (10b). Because the rate of reduction was 36 times the rate of normal hydrolysis (10a) this reduction product could be and was observed. They also found that the released imines can form Schiff

bases with amino groups of proteins and free lysine (10c) which can be trapped by sodium borohydride reduction.



(10)

Coffey and coworkers had trapped just such a Schiff base when adding sodium borohydride to DAAO/amino acid mixtures.[49,50] Their work generated great excitement initially when it was thought that the adduct might represent the active site of the enzyme. But tests showed that the presence of these adducts had no effect on the enzyme's activity and therefore they could not be at the active site.

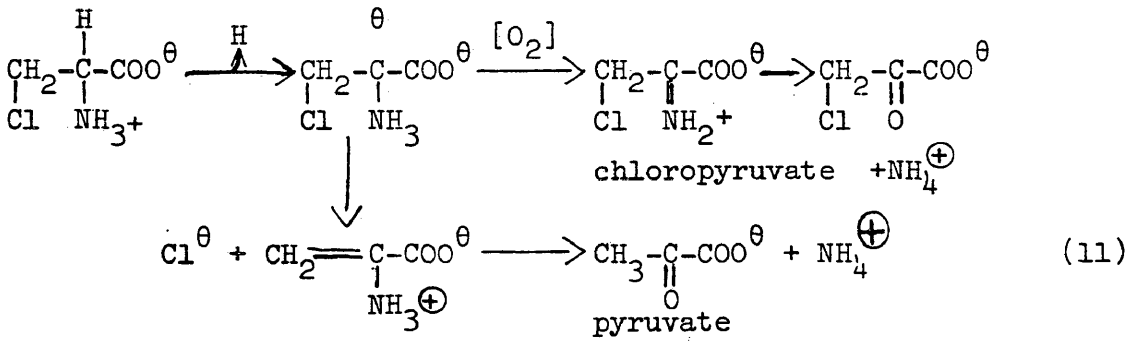
Walsh et al.[51] found a number of residues on DAAO capable of undergoing a similar reaction with D-propargylglycine oxidation products. In this case the enzyme's activity was altered but not stopped.

It now appears[52] that this transamination side reaction (10c) may be exploited by the oxidized substrate to aid hydrolysis. When imino acids react with the α-amino group of glycine or other

amino acids, they become more susceptible to hydrolysis. Thus the amino acid substrate acts as a posative effector in its own oxidation. However, if the imine product reacts with the ε-amino group of lysine or other primary amines as happened in Coffey's and Walsh's experiments, it becomes more stable to hydrolysis, slowing the reaction. This reaction has proven useful for trapping some DAAO oxidation products and showing that they are not suicide substrates since they leave the active site before undergoing this reduction.

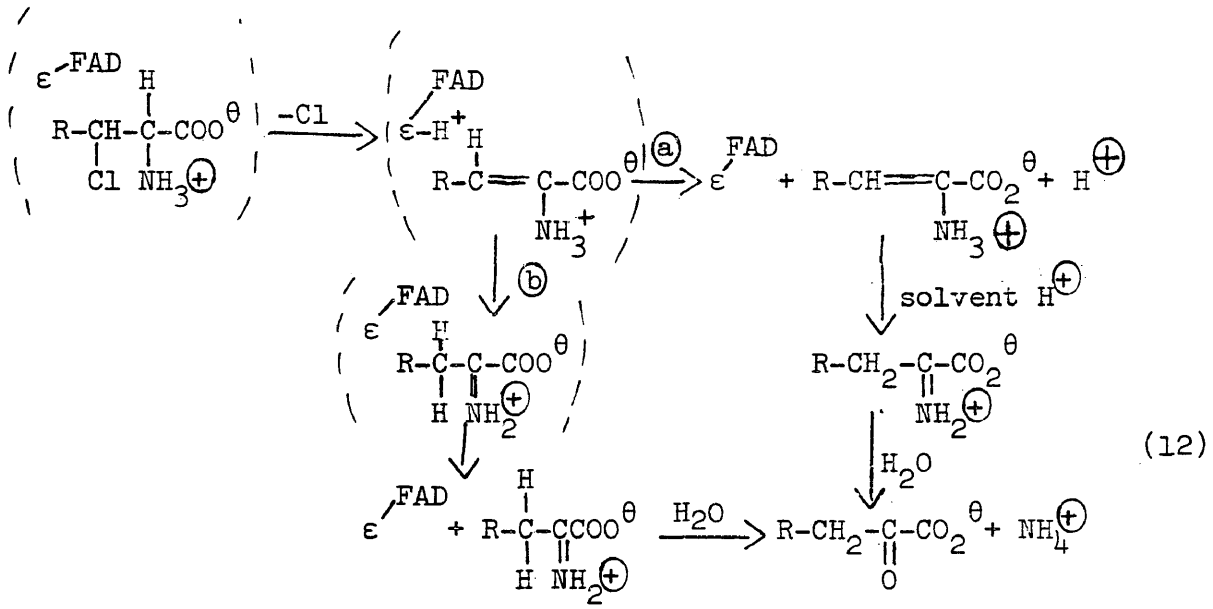
7. Oxidation vs Dehydrogenation

Flavoprotein oxidases catalyze both oxidation and dehydrogenation reactions. DAAO has been shown to be capable of catalyzing both types of reactions, depending upon the substrate used. For example, Walsh et al.[53] found that when β-chloroalanine was mixed with DAAO anaerobically (in 100% nitrogen) pyruvate was formed exclusively. In 100% oxygen only β-chloropyruvate was formed. At intermediate gas mixtures combinations of the two oxidation products were formed. This is illustrated in eq. 11.



(11)

With α-amino-β-chlorobutyrate, only α-ketobutyrate was formed, regardless of whether or not oxygen was available.[54] On the other hand, two competing elimination reactions were observed. From experiments with α-amino-β-chloro[α-^{2}H]-butyrate it was concluded that DAAO reacts with the substrate to form an enzyme-bound enamine. This enamine is either (a) realeased into the medium where it spontaneously ketonizes, or, (b) protonated at the β-position (with the former α-^{2}H) to form α-imino-butyric acid, as shown in eq. (12).



(12)

The relative rates of reactions (a) and (b) are temperature dependent, more elimination occurring at room temperature than at 4°C. It is possible that a suicide substrate could exploit this elimination reaction to become active.

8. Competitive Inhibition

Much work has been done on the inhibition of DAAO, but most substrates turn out to be competitive inhibitors.[19,20,55-57] However,

these studies provided some useful knowledge regarding substrate-enzyme interaction. In particular, it appears that good competitive inhibitors form charge-transfer complexes with DAAO in which the substrate acts as an electron donor and the oxidized flavin acts as an electron acceptor.[18,20,25,35]

This fact has been exploited in at least one case of irreversible inhibition, that of (-)$EtNO_2$ on DAAO as has already been mentioned.[23] Conversely, electron acceptors complex with the reduced enzyme[33] as expected from molecular orbital calculations.[34]

In most cases studied so far (all but those of the RNO_2 series) a -COOH group appears to be necessary for binding. The active site also has a hydrophobic portion which can accommodate a short aliphatic chain (C_4 molecules exhibit maximum binding) or a benzene ring.[56] Good competitive inhibitors usually have an unsaturated bond α,β to the negatively charged group. Compounds with two anionic groups do not inhibit the enzyme.[57]

9. Irreversible Inhibition

Two irreversible inhibitors of DAAO have recently been found: Propargylglycine[58] and $NO_2Et(-)$[23].

a. Propargylglycine

The reactions of propargylglycine with DAAO have now been studied in some detail. Horiike et al.[58] observed time-dependent inhibition of the enzyme, with covalent binding of an inhibitor species to the apoenzyme. This inactivation did not have first order kinetics, indicating that the altered propargylglycine must be released from

the enzyme before deactivating it in a second step. These observations were corroborated by Stock.[59] Extending Horiike's work one step further, she was unable to find irreversible inhibition using whole liver and kidney homogenates. This experiment supports the idea that the propargylglycine product is being released before it can inhibit the enzyme. If the product remained in the enzyme's vicinity until inactivation, then one would expect the rate of inactivation to be nearly independent of other species present. If, however, the product spent some time in solution before inactivation, then it might react with a number of components in the system besides DAAO.

Marcotte and Walsh have studied the reactions of propargylglycine in great detail.[60,51,61] Propargylglycine is an active substrate for DAAO with a K_M = 0.6 mM and V_{max} = 7 μmole/min·mg. Acetopyruvate was detected in large quantities and determined to be the final product of the oxidation process. No anaerobic turnover was observed with this substrate. (Anaerobic turnover is indicative of α,β-elimination, a process observed so far only with β-chloro species.) A slow time-dependent, irreversible inactivation was also observed. The number of turnovers was found to be a function of the inhibitor concentration implying that the inhibitor or its precursor competes with substrate for enzyme. Inactivation occurred approximately once for every 2000 turnovers. Acetopyruvate was shown not to be the inhibiting species, but the inhibitor was apparently released into solution before attacking the enzyme since (1) the enzyme could be protected by addition of butylamine·hydrochloride to the system, (2) increasing enzyme concentration increased the rate of inactivation, and (3) the inactivation rate was not first order. Where radioactive inhibitor was used, 1.7

labels per active site were added to the apoenzyme. Examination of the inhibited enzyme revealed not one, but a minimum of five distinct species of modified enzyme with changed activities and several totally inactive ones. Longer incubations with propargylglycine led to more different kinds of modified enzyme as determined by gel electrofocusing. Furthermore, modified DAAO showed a much decreased activity with D-alanine, but a greatly increased activity when the normally poor substrate, D-phenylalanine was used. D-Alanine is normally used to assay DAAO activity, and different laboratories have used different amounts of D-alanine in their assays. Because the measured activity of D-propargylglycine-modified-DAAO is a function of the concentration of D-alanine used, the measured inactivation of DAAO by D-propargylglycine was not consistent from one laboratory to another.[58,59,51] The rate determining step of the catalytic reaction was also different in the modified enzyme. In native enzyme, product release is the slow step (see earlier discussion); in propargylglycine-modified-enzyme, the slow step became H^+ cleavage from C_2. This and the changes in substrate specificity indicate that the enzyme has been modified in such a way that the environment at the active site is greatly changed, but the active site itself is not blocked. This modification was found to be stable under all conditions tested.

With the modified enzyme thus characterized, Marcotte and Walsh then undertook to analyze the sequence of reactions involved in enzymatic oxidation of propargylglycine.[61] In addition to the inactivating species, two species capable of forming intense charge-transfer complexes with DAAO were observed. The first complex formed very

quickly with an absorption maximum at 580 nm. It was shown to be in rapid noncovalent equilibrium by the fact that added sodium benzoate bound preferentially to the enzyme at a rate of 0.08/sec, the dissociation rate of the enzyme-inhibitor complex. The initial maximum turnover number was greater than this rate of charge-transfer complex dissociation, so the charge-transfer complex must be forming from the oxidized enzyme. This charge-transfer complex decayed over 60-90 minutes into a second charge-transfer complex which was less tightly bound. Native enzyme could not be recovered when protecting reagents are added to the first complex, but was recovered in preference to the second complex. This indicated that the first complex was formed while substrate was still at the active site, but the second was formed by reequilibration with a species from solution. Furthermore, this second complex could be formed using product generated in situ,which then had to migrate to the DAAO active site. (That is, previous reduction of DAAO itself was not required for formation of this charge-transfer complex.) This charge-transfer complex was not involved in covalent modification. No inactivation was observed during the lifetime of this charge-transfer complex. No inactivation was observed when this complexing species was generated in situ and allowed to migrate to DAAO to form the charge-transfer complex. Therefore, the inactivating species must lie on the pathway between the first and second complexes. Marcotte and Walsh have devised Scheme I to fit their observations.

The allenic intermediate (8 of Scheme I) would be subject to nucleophylic attack whether by a basic residue on the amino acid to form an alkylated enzyme species, or internal attack by the acid anion to form the lactone (6) in a 1,4 Michael-type addition. This

lactone was indeed observed and characterized by NMR. The availability of this ready internal attack is probably the reason why so many turnovers occur for each modified enzyme molecule formed. The fact that many different enzyme species were observed, shows that this alkylation is not specific for any one amino acid residue. Propargylglycine is not a satisfactory inhibitor for the study of DAAO.

b. Nitroethane Anion

Nitroethane anion, in the presence of cyanide, rapidly and irreversibly inhibits DAAO.[23] The absorption spectrum of the inhibited enzyme is characteristic of a flavin-substrate covalent adduct, with binding at N(5) of the isoalloxazine ring. When flavin is released from the apoenzyme by hot methanol, the adduct is found to remain with the flavin species. If water is added instead of cyanide, normal turnover is observed. Addition of any species with a free electron pair to the enzyme-substrate cpx. (see Scheme II) allows removal of the alkylated species and normal turnover. Cyanide does not have a free electron pair to donate to an elimination reaction and thus traps the species in an irreversible manner. Porter, Voet and Bright presented the equations of Scheme II to explain their results.

While $NO_2Et(-)$ has proven useful for the study of DAAO *in vitro*, this strong anion cannot be supposed to be specific for DAAO in an *in vivo* system. Furthermore, cyanide, a substance well known to be lethal to living organisms, is required for inhibition. Therefore, this system is not useful for studying DAAO *in vivo*.

C. The Chemistry of Flavin

Much fruitful knowledge has been gained from the studies of the inhibition of DAAO and other flavoenzymes. Another, equally fruitful area of investigation has been research into the nature of the flavin moiety of the enzyme system. It is known that the flavin portion of the enzyme is intimately involved in the catalytic process. Attempts have therefore been made to produce similar reactions in the simpler noncatalytic free flavin system, then deduce what must be happening at the enzyme active site.

The following questions can be asked about the flavin-substrate system:

(1) What geometric and electronic changes does the flavin undergo during its reduction and reoxidation phases?

(2) What is the nature of the flavin-substrate interaction?

(3) What portion of the flavin molecule is involved in substrate interaction and flavin reoxidation?

1. The Geometry of Flavin

Oxidized flavin (Fl_{ox}) is a planar structure with complete delocalization of its electrons (Fig. 4a). Fully reduced flavin (Fl_rH_2) has a butterfly configuration with each of the two side rings bending out of the plane (Fig. 4c). In a planar structure, the central ring of the fully reduced flavin would have 8π electrons, an unstable situation (Fig. 4b). In the enzyme environment, these changes in conformation may well be restricted. It is thought that such restructions may be responsible for the relative reactivities of different flavoenzymes

with O_2. (For example, dehydrogenases will not react with O_2 to reoxidize while oxidases react extremely quickly.)

Bruice has attempted to explain susceptibility toward O_2 attack in two contradictory ways.[36,38] First he notes the potential antiaromaticity of the central pyrazine ring of a planar reduced flavin (Fig. 4b). The two extra electrons in the planar form would occupy antibonding orbitals "to provide diradical character". This would allow direct covalent bonding by molecules having triplet states such as ground state O_2. Conversely, he considered the case of the fully bent system. Here the orbitals of the free electron pairs tend to interact (Fig. 4c). This interaction will be greater when both orbitals are equatorial as when both N(10) and N(5) are alkylated (presumably the case in the DAAO system where E_r...P remains intact until after O_2 attack). The result of this interaction is a splitting of the orbital energies so that one is higher than the other allowing for easy and stable radical formation. This radical species might exist as a transient intermediate activated toward attack by the triplet O_2 ground state.

These theories support exactly opposite conformational tendencies as the source of activation toward O_2 attack, leaving the student of these systems in a quandry. Presumably those enzymes not able to react with O_2 would have a geometry somewhere in the mid-range between these two extremes.

Tauscher et al.[62] synthesized a series of N(5) substituted reduced flavins and measured the angle between the two butterfly wing planes. They found a variation of from 9-35.5°. They measured the conformational energy of these structures and found it sufficiently

large to effectively "freeze" specific configurations providing an effectively chiral N for H transfer. Unfortunately they did not measure the susceptibility of any of these compounds for O_2 attack, so we are not any the wiser on this controversy. Flavin can also be found in the half-reduced free radical semiquinone state. The different forms flavin is able to take and the absorption maxima for each of these forms are shown in Fig. 2.[44]

2. The Flavin-Substrate Interaction

Because flavin is the major chromophore of a flavoenzyme system in the visible region, one can learn about the changes occurring in this coenzyme during catalytic turnover by spectrophotometric monitoring of the reaction (c.f. Fig. 3). The observed changes in the spectrum can then be compared with those occurring in well characterized simpler flavin systems. In this way, researchers have been able to learn about apoenzyme-coenzyme binding, when, furing turnover, the flavin is in its oxidized and reduced states, and something of the nature of the enzyme-substrate complex. Beinert[63,64] was the first to exploit the spectral properties of flavin in this way.

Spectral monitoring of the anaerobic catalytic reaction of DAAO with normal substrate shows an initial single exponential increase in A_{550} (k_{obs} for the RHR). This corresponds to the increase in $E_r...P$, an apparent charge-transfer complex between flavin in its reduced form and substrate[24,25,27] (see Fig. 3). This A_{550} peak then decays exponentially (k_3 of equation 2). k_3 is independent of [S].[15] Covalent intermediates have been postulated and, in some cases, trapped.[23,65-69] Thus, it appears that variation between a

charge-transfer complex transient intermediate and a transient covalent intermediate may be a function of the specific enzyme-substrate system.

All available sites of the flavin ring have at one time or another been postulated as the actual participating active site in substrate oxidation. Molecular orbital calculations[34,35] have shown that the N(5) and C(4a) positions of the oxidized flavin are most susceptible to nucleophulic attack. In the reduced flavin molecule, these are the sites most susceptible to electrophylic attack. The N(5) position should be the most prone to attack in either case.

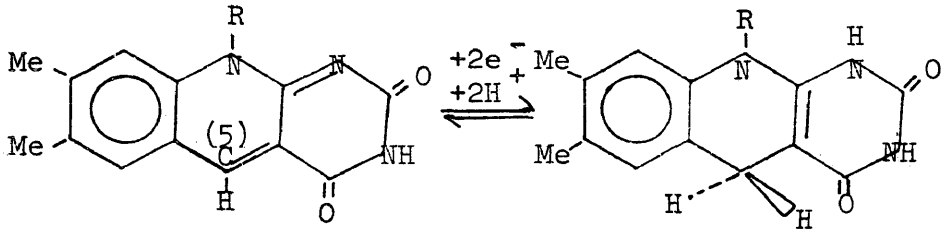
The molecular orbital calculations are supported by the experimental facts. Charge-transfer complexes seem to form most readily with electron donating species reacting with oxidized flavin or electron deficient species reacting with reduced flavin.[24,33] The only covalent species isolated to date from an enzyme system are bound either at N(5) or C(4a). Williams and Bruice[37] claim covalent bond formation is a spurious reaction and true oxidation of substrate occurs via radical intermediates. Nonetheless, it seems highly likely that these atoms are involved with the catalytic activity of the enzyme and design of inhibitors of flavin concentrate on exploiting reactions at these centers.

In the case of DAAO, it is known that the α-H is removed in conjunction with purple complex (E_r...P) formation. The resulting substrate carbanion is apparently being stabilized by interacting with the flavin ring at N(5). This could occur in either of two ways.[36] Planar overlap of the filled carbanion orbital lobe (sp^3) with the frontier orbital at N(5) should be prevented by the presence of a

non-bonding electron pair at N(5) (Fig. 5a). However, out-of-plane overlap between the filled carbanion orbital and the flavin anti-bonding molecular orbital can occur (Fig. 5b). Strong absorption is predicted in the long wavelength charge-transfer band for a planar complex, but only weak absorption is predicted for an out-of-plane complex. Weak absorptions are observed without exception for these complexes with DAAO.

3. Deazaflavin

Having decided that N(5) of the isoalloxazine ring was an important position for catalytic activity in flavoenzymes, the next step was to synthesize a flavin modified in this position so its importance could be tested. O'Brien, et al.[70] succeeded in synthesizing 7,8-dimethyl-5-deazaribo flavin (dFl) which can convert to the reduced 1,5-dihydrodeazaflavin (dFl_rH_2):



(13)

dFl dFl_rH_2

This modified flavin has been very valuable in clarifying some points of the normal flavin redox reaction.

Deazaflavin has so far been substituted into more than thirteen enzyme systems and has been shown to oxidize the same substrates

as normal enzymes, though at a somewhat slower rate. Deazaflavin has enhanced reactivity toward oxidized substrate (the reverse of k_2, equation 2), a reaction available to normal enzyme only under anaerobic conditions and with excess oxidized substrate and ammonia, or with certain unfavorable substrates (e.g., phenylalanine).[71,72,18]

Deazaflavin has about the same binding affinity to apoenzymes as flavin. Absorption, fluorescence and circular dichroism spectra are quite similar. Therefore, deazaflavin is probably binding at the same portion of the apoenzyme as normal flavin does.[71] Apoenzyme still provides its function of enhancing the catalytic reaction rate. The rate limiting step in oxidation (RHR) is the same for both types of flavin. These compounds are also complexed by electron donors.[72] Molecular orbital calculations show the C(5) position to be most prone to attack.[34,35] Free radical species are considerably more difficult to generate.[71] This is to be expected since the fully reduced state no longer has an unstable 8π electron central ring and is not capable of the lone pair--lone pair orbital interactions available to reduced flavin that enchance the probability of radical formation. (See above discussion of the properties of reduced flavin and Fig. 4.) This may account for the relative unreactivity of dFl_rH_2 towards O_2 (triplet O_2 would be more reactive toward a transient free radical or excited state species than toward the ground state reduced deazaflavin molecule).

Enzyme catalized transfer of substrate hydrogen to flavin has been difficult to demonstrate due to the rapid exchange rates of reduced flavins. Modifying the enzyme by replacing flavin with deazaflavin slows this reaction and prevents reoxidation via O_2 so that the

H transfer step (RHR) can be studied in detail. Furthermore, dFl_rH_2 now has a chiral center at the proposed active site so the stereochemistry of this H transfer can also be studied.

Using N-methylglutamine synthetase, Jorns and Hersch were able to show that the modified enzyme underwent the same 2-step catalytic reaction as native enzyme:[73,74]

$$\text{E-dfMN} + \text{L-glutamate} \longrightarrow \text{E}\begin{matrix}\diagup \text{dFMNH}_2 \\ \diagdown \text{glutaryl}\end{matrix} + \text{NH}_3 \qquad \text{(a)}$$

(14)

$$\text{E}\begin{matrix}\diagup \text{dFMNH}_2 \\ \diagdown \text{glutaryl}\end{matrix} + \text{methylamine} \rightarrow \text{E-dFMN} + \text{N-methyl-L-glutamate} \qquad \text{(b)}$$

at 1-5% the normal rate. Using [α-^{3}H] glutamate, they were able to show approximately a 1:1 relationship between ^{3}H incorporated into the enzyme and the amount of $\text{E}\begin{matrix}\diagup \text{dFMNH}_2 \\ \diagdown \text{glutaryl}\end{matrix}$ produced. When the enzyme was separated into the three components, the ^{3}H was found to be associated with the $dFMNH_2$ moiety. If the enzyme was treated with methylamine before analysis, the ^{3}H was then incorporated into N-methylglutamate. The fact that there were 2 protons, one labeled and one not, at C(5), yet only the labeled one was incorporated into N-methylglutamate indicated the stereoselectivity of the reaction. This ^{3}H was not exchangeable with solvent and therefore remained at a protected site on the enzyme during both steps of this reaction, probably at C(5) of the deazaflavin.

To confirm the stereospecificity of hydrogen transfer to deazaflavin, and to transfer to C(5), Spencer, et al.[75], compared the

kinetic isotope effect on oxidation of dFl_rH_2 reduced (a) by borodeuteride, and (b) by oxoreductase + 4-R-[^{2}H]NADH and monitored the changes by NMR. Borohydride presumably reduces randomly at either face of deazaflavin whereas the enzyme system should reduce stereospecifically on only one face of the deazaflavin molecule. Oxidation of system (a) gave complicated kinetics and NMR studies showed an absence of almost all of the C(5) proton absorption consistent with the expectation that most of the deazaflavin would still be deuterated at C(5). When the enzyme reduced species (system (b)) was reoxidized by riboflavin, the kinetic isotope effect was 3.0, reflecting chiral $^2H^+$ addition and subsequent abstraction. Further support for a chiral hydrogen transfer was obtained from studies with NAD:flavin oxidoreductase which reduces substrate 5-deazariboflavin. Preparation of [4-R-^{2}H]NADH and incubation with oxidoreductase and riboflavin indicated stereospecific transfer of this H by complete volatilization (>90%) of the counts with a kinetic isotope effect of 4.8. With deazariboflavin, a kinetic isotope effect of 3.3, for [4-R-^{2}H]NADH was observed. The 4S isomer showed no kinetic isotope effect. There was a large difference in the rate of H transfer between flavin and deazaflavin, but this step was rate determining in the RHR in each case. Jorns and Hersch[76,77], using glucose oxidase and DAAO + [α-^{3}H]-substrate also demonstrated stereospecific H transfer to and from deazaflavin.

Deazaflavin has indeed proved a useful model for clarifying the flavoenzyme oxidase system. It always binds at the active site. It is capable of oxidizing normal substrates, and undergoes the reverse of this reaction very readily. Its sluggish oxidation by O_2 indicates

that the N(5) unbonded electron pair is essential for rapid oxidation by O_2. It has helped confirm that α-H is transferred to C(5) (N(5) of the normal flavin system). This transfer may be either directly to C(5) or via an enzyme basic residue in a stereospecific manner. The fact that nonenzymatic transfer between substrate and deazaflavin has been observed argues for the direct transfer of this hydrogen to C(5), but the formation of charge transfer complexes during the hydrogen abstraction step of many flavoenzymes makes assistence from an enzyme basic residue more plausible. In any case, work on deazaflavin has conclusively shown that N(5) is intimately involved at the enzyme active site in flavoprotein oxidases.

D. Suicide Inhibition

How can we exploit the knowledge gained about the nature of the active site of DAAO and the reactions it catalizes, in order to design a suicide inhibitor of this enzyme? First, we need to find a compound sufficiently like normal substrate that it can bind at the active site. A modified D-amino acid seems most acceptable. Second, we want it to be unreactive until it has been modified by the enzyme. α-H abstraction appears to be the most likely step in catalysis to be exploitable for this purpose. Third, the modified compound must now be sufficiently reactive to bind at the active site before it dissociates from the enzyme. The generated active species might be an electrophile which could attack the proposed base present for initial abstraction of the α-H. However, if the species is not reactive until after the proton is removed, this base may no longer be available for attack. Alternatively, the active species could be a nucleophile, designed to

attack the electron deficient isoalloxazine ring of the flavin coenzyme. $NO_2Et(-)$ is an example of a compound sufficiently reactive to form a covalent bond and not just a reversible charge-transfer complex with flavin. However, as mentioned above, this compound lacks suicide substrate properties and is not suitable for _in vivo_ work.

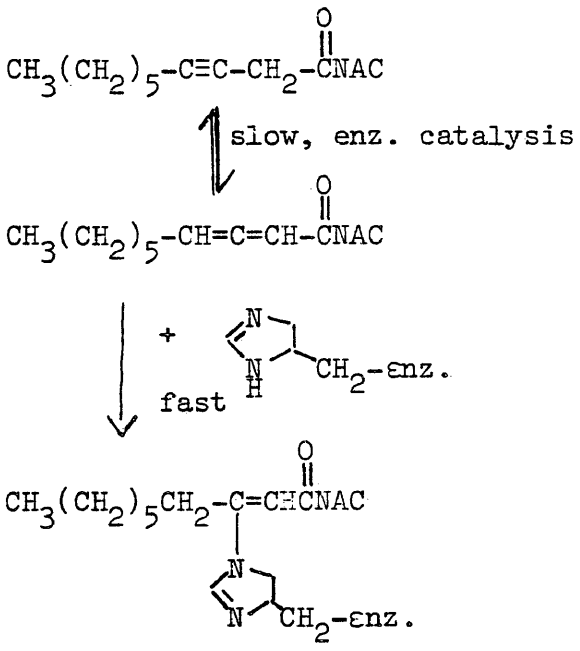
What kind of suicide substrates for enzymes have been prepared so far and how do they work?

1. 3-Decynoyl-N-Acetylcysteamine

Konrad Bloch was the first to prepare and characterize a suicide substrate.[78,79] He was studying the biosynthetic formation of fatty acids and found that 3-decynoyl-N-acetylcysteamine (3-decynoyl-NAC) an acetylenic analog of a normal β-hydroxy-decanoyl thioester dehydrase substrate was a potent and highly specific inhibitor of the enzyme. The inhibition was substrate protected, but completely irreversible. The optimal chain length for the inhibitor coincided with that for optimal substrate, indicating that binding was at the active site and that it occurred in the same manner as for normal substrate. Furthermore, only the N-acetyl cystamine form of the substrate was active, indicating high specificity of this substrate for the target enzyme.

Further studies[80] clarified the mode of inaction of this inhibitor. In these experiments both 3-decynoyl-NAC and 2,3-decadienoyl-NAC were tested. The allenic inhibitor was even more effective than the acetylenic one. Inhibition by it was not affected by [S]. The enzyme was known to catalize the transformation of _cis_-3-decenoyl-NAC to _trans_-2-decynoyl-NAC. An analogous enzymatic isomerization of the acetylenic thioester would produce the allenic NAC derivative. A

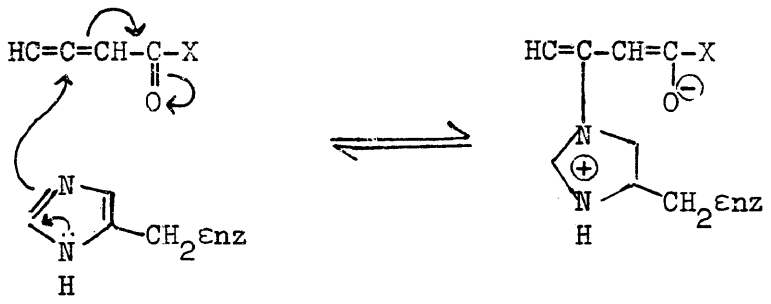
kinetic isotope effect was found with inhibition by the acetylenic substrate, but not with allene inhibition. Free 2,3-decadienoic acid also inhibited the enzyme. Thus, α-H must be removed from the acetylenic compound in a rate limiting step to generate the active allene species. This data supports equation 15 as being the mode of inhibition of β-hydroxydecanoyl thioester dehydrase.



(15)

pH studies[81] indicated that the active species was the neutral allene, not the anion.

Model inhibition studies on histidine interaction with allenes[82] indicated that one of the imidazole nitrogens of histidine can serve as nucleophile, attacking the central carbon of the allene in a 1,4-Michael type addition reaction:



(16)

The 3,4-dienoate has no inhibitory effect on the enzyme. Thus the reactivity of the allene must either be enhanced by the adjacent conjugated carbonyl or must be severely restricted by stereochemical conjunction of the allene and the histidine at the enzyme active site, or both.

This new tool in enzyme inhibition has been exploited by a number of workers, most notably in steroid biosynthesis.[83-86]

2. Other Acetylenic Inhibitors

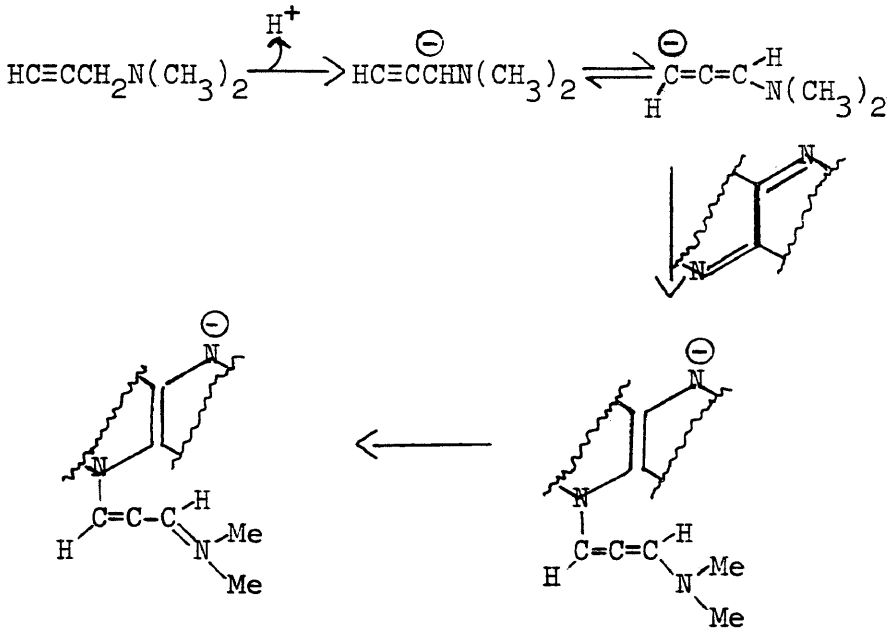
Similarly, propargylglycine was found to inhibit γ-cystathionase,[87] presumably by α-H abstraction, rearrangement to an allene and nucleophylic attack by a base at the active site. The reaction was never fully characterized.

Monoamine oxidase (MAO) has also been inhibited by an acetylenic substrate, but is inhibited 25 times more slowly with the comparable allenic substrate, in vitro.[88] In vivo these two species exhibited approximately equal potency. Perhaps the two substrates were converted in vivo into some common intermediate before reaching the enzyme's active site.

In a more detailed study Abeles and coworkers[89] followed the inactivation of MAO with 3-dimethylamino-1-propyne. Proteolytic

digestion of the inhibited enzyme yielded a flavin peptide containing one mole of inhibitor per mole of flavin. Where chemical and spectral properties were compared with model compounds, they were found to resemble those of an N(5) adduct containing the structure -N-CH=CH-CH=N+. This inhibition must therefore be different than that occurring with β-hydroxydecanoyl thioester. Using the knowledge compiled by Bruice on flavin reaction mechanism[44], they postulated three possible reaction mechanisms for adduct formation: carbanion formation, radical formation, and complete oxidation.

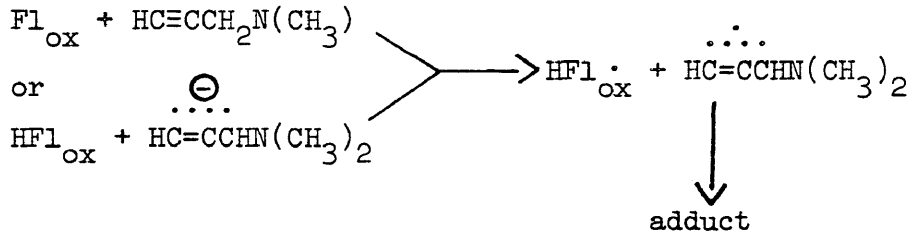
If the enzyme catalizes α-H abstraction, the intermediate carbanion could add to N(5) of oxidized flavin yielding the observed adduct after protonation:



(17)

That is, oxidized flavin reacts with reduced inactivator to form a stable covalent bond. This attack is in line with what we know about N(5) being electron deficient and susceptible to nucleophilic attack by substrate. But there is no activating group adjacent to the site of proton removal, so the likelihood of a carbanion intermediate is questionable.

Bruice[36] has suggested the possibility of the formation of a radical pair intermediate complex involving flavin and substrate. This complex could be formed either by H· transfer to flavin or from electron transfer from carbanion intermediate to flavin. Collapse of the complex plus adduct protonation would give the observed species.



(18)

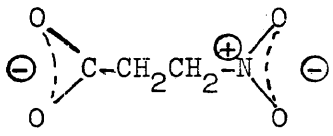
In this case partially reduced flavin and partially oxidized substrate interact. As noted above, such intermediates cannot be detected in many flavoenzyme systems.

Complete oxidation of inhibitor gives Fl_rH_2 + HC≡CCH=N(CH$_3$)$_2$ which is highly reactive and should be an excellent receptor in Michael addition reactions. Addition of N(5) across the triple bond would give the observed adduct. As mentioned in an earlier section, Fl_rH_2 is indeed nucleophylic.

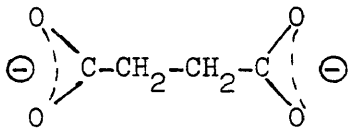
One should note that the usual oxidation catalized by MAO is at C(1) of the substrate amine. In the observed adduct, attachment is at C(3). Thus, either N(5) is not the immediate site of normal oxidation in this enzyme, or an abnormal oxidation is taking place with this inhibitor.

3. 3-Nitropropionate

Another interesting suicide inhibition is that of 3-nitropropionate on succinate dehydrogenase in rate liver mitochondria.[90] Succinate dehydrogenase catalizes the transformation of succinate into fumarate in the Krebs Cycle. Bright modeled this study on his successful inhibition of DAAO by nitroethane anion and looked at the isoelectronic nitro compound of succinate:

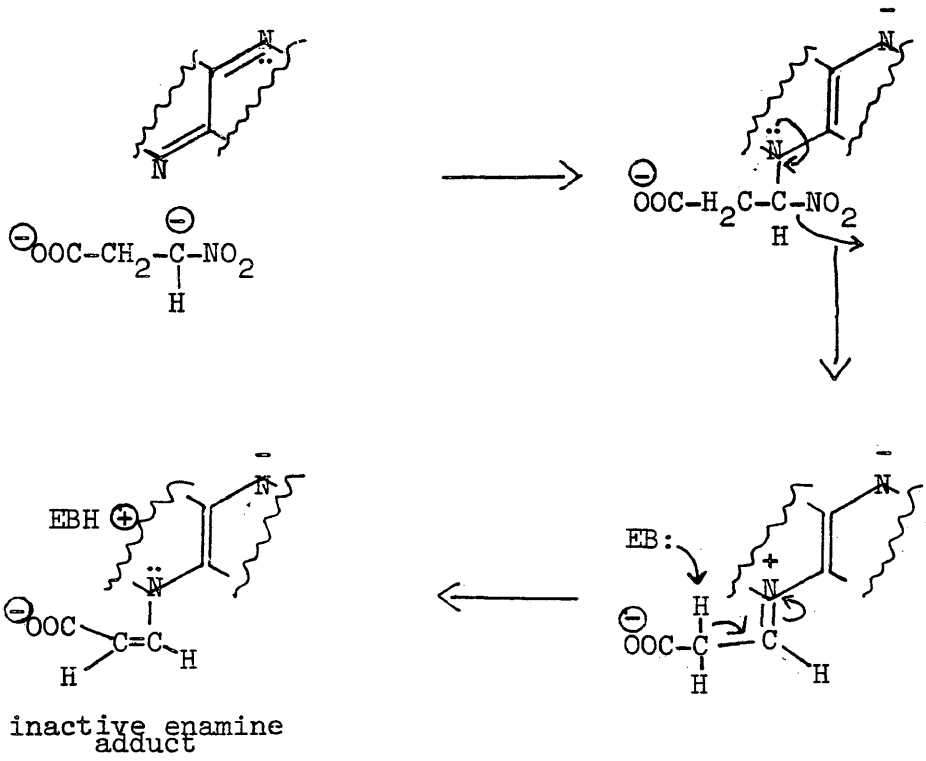


3-nitropropionate



succinate

Furthermore, he reasoned that since succinate dehydrogenase oxidizes the 2,3 position of the physiological substrate and not just one carbon as DAAO does, that the system could be inactivated by collapse of the N(5) adduct expected to be formed in analogy with the $NO_2Et^{\ominus}$/DAAO reaction (compare with Scheme II).



(19)

That is, there should be a second internal base available to catalize this secondary H^+ abstraction as it would in normal dehydrogenase activity.

When this compound was tested, a first order rate of inhibition was found, which was a function of [I] and [S]. The inactivated enzyme could not be revived by normal methods. No external nucleophile was required for inhibition. Respiratory rates, respiratory control rates and ADP/O ratios were unaffected by the concentration of the inhibitor, indicating that only the Krebs Cycle is stopped. All these data indicate that 3-nitropropionate is indeed a highly specific, time-dependent suicide substrate of succinate dehydrogenase.

4. 2-Hydroxy-3-Butynoate

Perhaps the work most relevant to the search for a suicide substrate for DAAO has been that done with the acetylenic substrate, 2-hydroxy-3-butynoic acid. It has been found to be a suicide substrate for lactate oxidase,[91-93] D-lactate dehydrogenase,[94] L-lactate dehydrogenase,[95] and glycollate oxidase.[96] In every case covalent adduct formation occurs between inhibitor and flavin. None of these enzymes form irreversible covalent adducts with 2-hydroxy-3-butenoate or 3-butynoic acid. That is, both the hydroxy and the acetylene functional groups appear to be necessary for inactivation.

a. Lactate Oxidase

The reaction with L-lactate oxidase (L-LO) from Mycobacterium smegmatis has been most thoroughly studied. 2-Hydroxy-3-butynoate reacts with the FMN coenzyme (see Fig. 1) of L-lactate oxidase to form a fluorescent species stable to autooxidation.[91] Using 2-hydroxy-3-[4-^{3}H]-butynoate and 2-hydroxy-3-[2-^{3}H]-butynoate, it was established that the α-H was lost, but the γ-H remained in the adduct although no longer attached to an acetylene group. Apoenzyme could be reactivated by exogenous FAD indicating that there were no detrimental reactions of inhibitor with the apoenzyme. The inhibitor was found to partition between catalytic oxidation and inactivation.[93] The amount of oxidation was a function of the oxygen cencentration, but independent of the concentration of the inhibitor. Under anaerobic conditions, enzyme and inhibitor interacted on a 1:1 basis (assuming only the L-hydroxy acid isomer reacts with L-lactate oxidase) to form the inactivated species. These data indicate that both reactions occur from a common intermediate.

This intermediate was considered to be the transient charge-transfer complex (λ_{max} = 600 nm) observed under rapid reaction techniques. The charge-transfer complex was assigned to an interaction between reduced enzyme and oxidized inhibitor: EFl_rH_2...2-keto-3-butynoate. This species did not fluoresce. Known complexes of α-ketoacids with reduced flavins exhibit similar spectral and fluorescent characteristics.[97] This complex was not stable and converted to an inactive species with λ_{max} = 318, 368 which was strongly fluorescent. This modified flavin was unstable and slowly (over 24 hours) converted into another, this time stable species. Absorption spectra and fluorescence characteristics of the initial adduct resembled those of C(4a)-N(5) bridged FMN derivatives.[97] In order to test the hypothesis that it is the α-ketoacid that is the actual inhibiting species, this highly unstable compound was generated <u>in situ</u> from 2-hydroxy-3-butynoate using lactate dehydrogenase and $NAD^{=}$.[93] Reduced enzyme was generated photochemically in a nitrogen atmosphere in order to avoid complications arising from slow formation of oxidized enzyme by reversal of the equation:

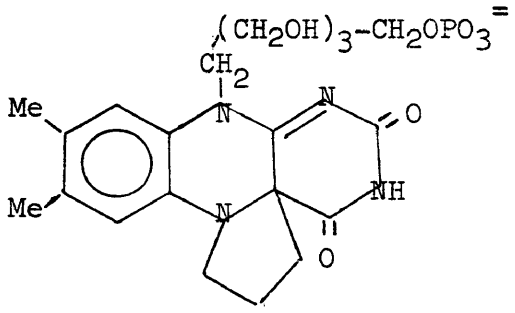
$$EFl_{ox} + \text{L-lactate} \rightleftharpoons EFl_{ox}\cdot\text{L-lactate} \rightleftharpoons$$

$$EFl_rH_2\cdot\text{pyruvate} \rightleftharpoons EFl_rH_2 + \text{pyruvate} \qquad (20)$$

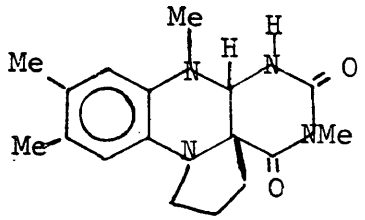
Other conventional methods of generating reduced enzyme could not be used as they would also reduce any ketoacid being formed. In this reaction a slow but measurable inhibition was indeed observed. The slowness of this reaction was probably due to the slow rate of association of α-ketoacid with reduced enzyme at such low oxidized substrate

levels. (The reverse RHR is normally run in a large excess of oxidized substrate and is still slow.) Thus, it was shown that the α-ketoacid can react with reduced enzyme and inactivate it.

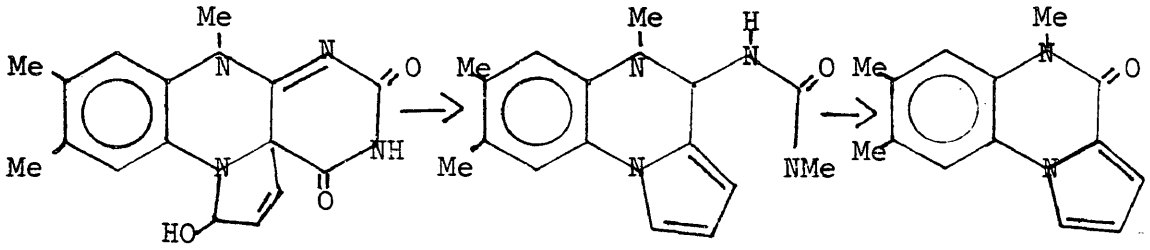
The next step was to determine the structure of the initial and final covalent adducts. Model flavins were synthesized and their characteristics compared to those of the modified L-LO species. When C(4a),N(5)-propanodihydro-FMN:



was prepared and its absorption spectra and fluorescence measured, they were found to be very similar to those of the initial adduct. When this initial adduct was reacted with sodium borohydride, it exhibited new spectral and fluorescence characteristics. These changes could only be matched when model flavin-N(5)-C(4a) adducts were reduced with borohydride. The structure of the product obtained from 4a,5-dihydro-4a,5-propano-3-methyl lumiflavin reduction was found by NMR and elemental analysis to be:

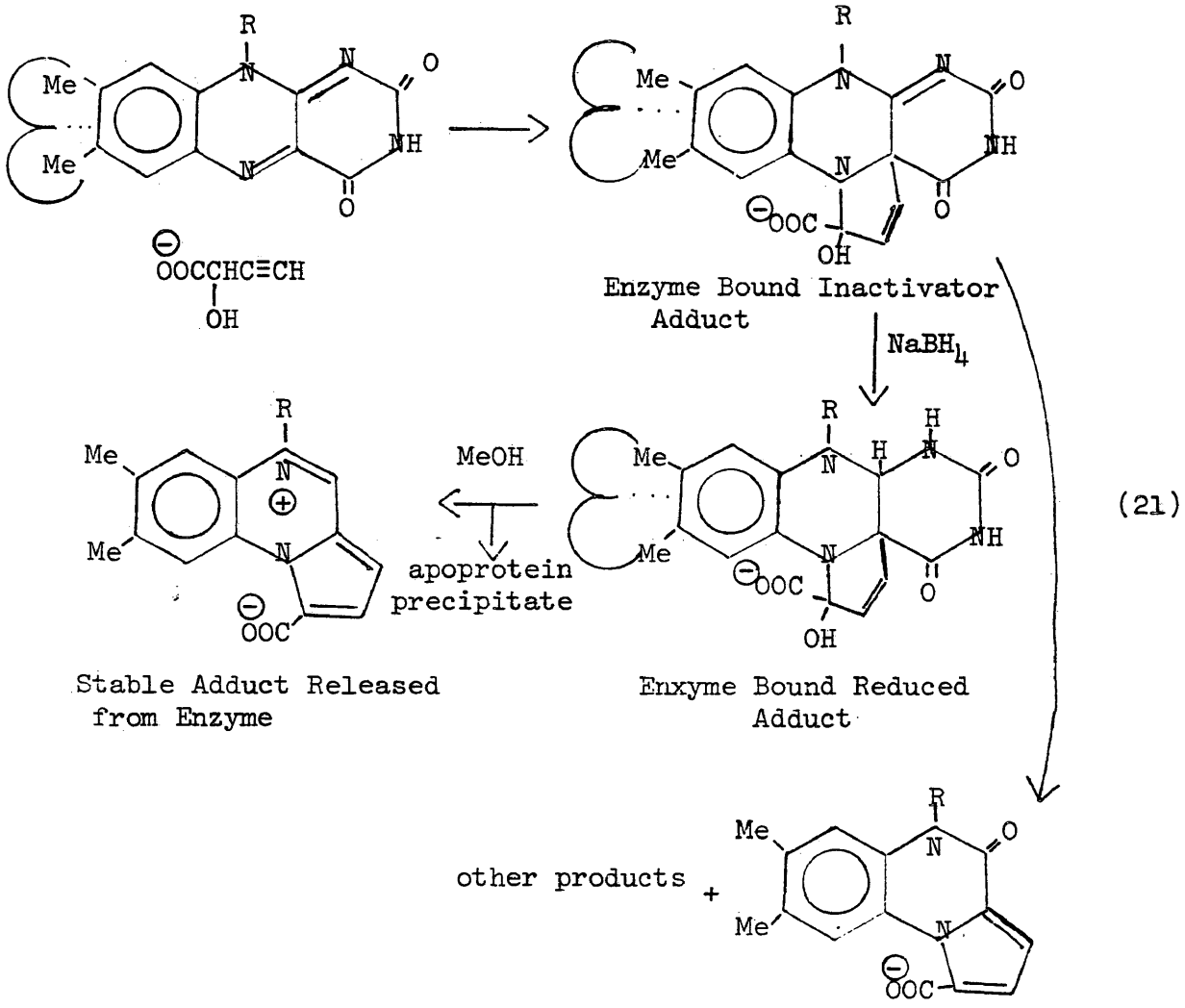


Hemmerich and coworkers[98] have synthesized compound A and found its spectral properties to be similar to those of the initial adduct. It is unstable in water and undergoes the following decomposition:



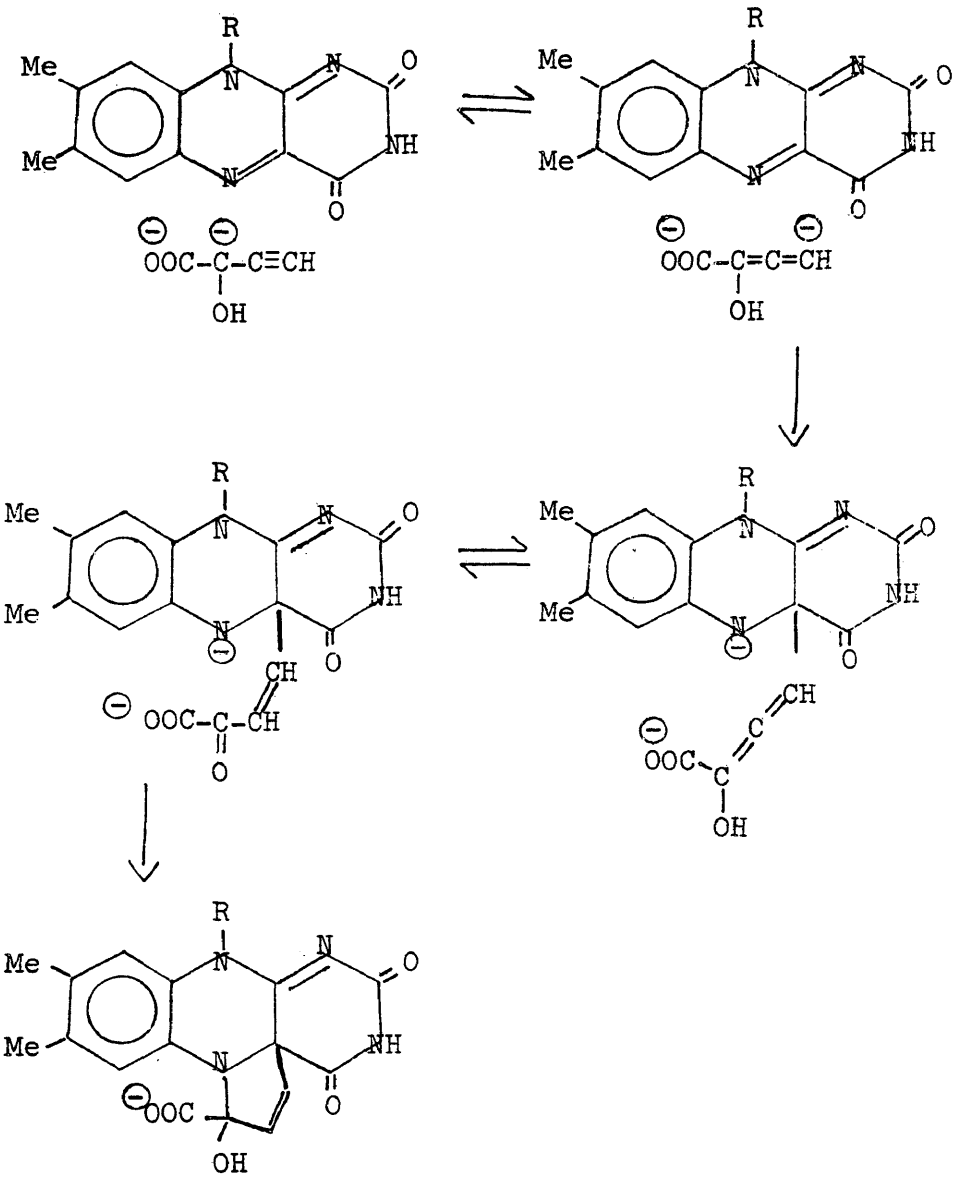
A B C

These reactions are consistent with the observed instability of the initial adduct formed on inactivation of L-lactate oxidase by 2-hydroxy-3-butynoic acid. Furthermore, the final adduct, when released from the holoenzyme with hot methanol and purified, exhibited an absorption spectrum very similar to that of compound C above. Studies of ^{14}C labeled adduct established that the carboxyl group of the hydroxy acid and C_2 of the flavin remained bound in the initial adduct, but that the flavin C_2 was lost on formation of the final adduct. Equation 21 was proposed for the sequence of reactions occurring during isolation of the borohydride stabilized adduct.



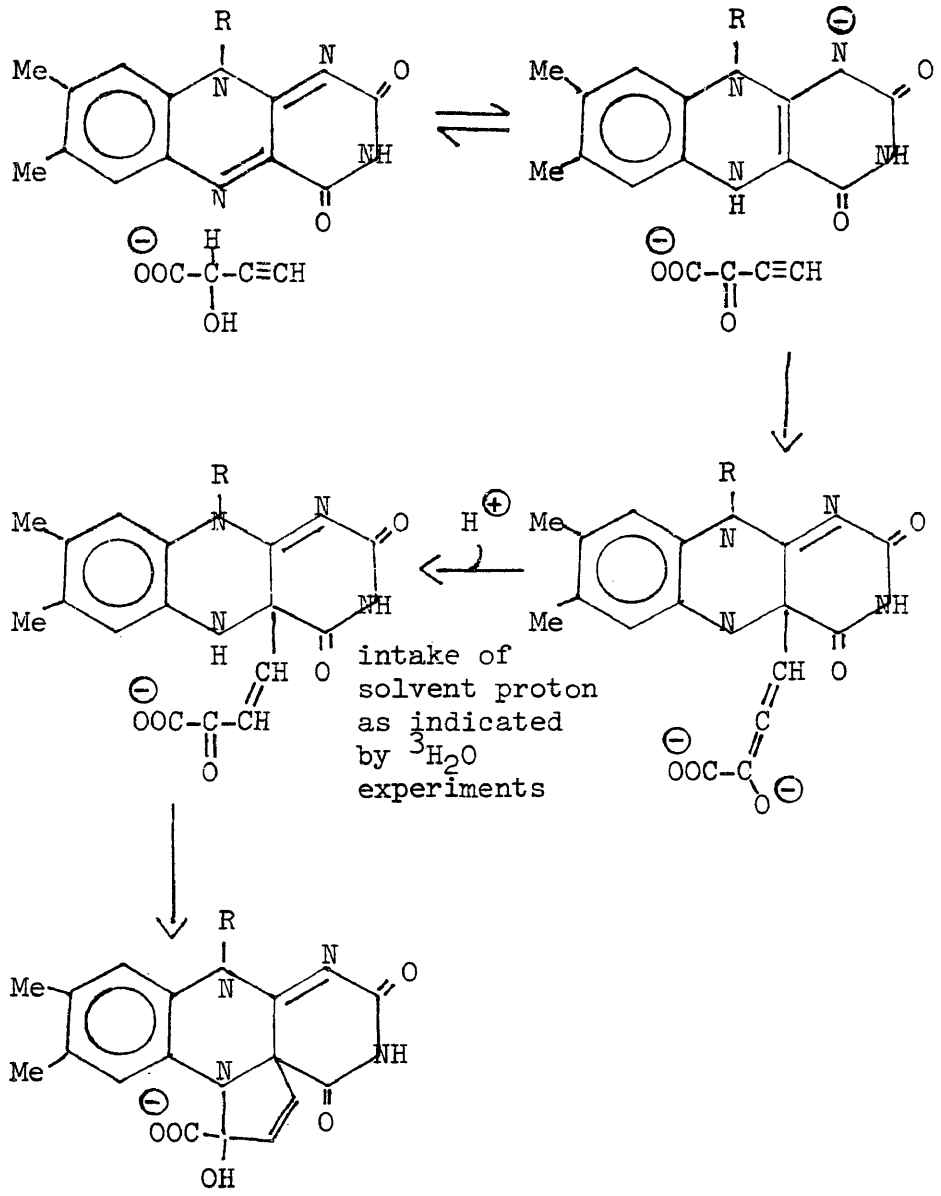
(21)

Several mechanisms can be envisioned for the formation of the flavin-inhibitor adduct. It is unlikely that, as in the case of β-hydroxy-decanoyl thioester[79] attack is from an allenic intermediate. An allene would be expected to undergo nucleophylic attack at the central allene carbon, not at C_2 and C_4, the observed attachment sites. However, an allene anion could initiate attack at C_{4a} of flavin, leading to the observed adduct:



Alternatively, attack could be by the keto acid on reduced flavin.

A possible mechanism for this reaction might be:



The two mechanisms are difficult to distinguish kinetically since E_{ox}...S and E_r...P are in equilibrium. But the analogous vinylglocollate, which should be equally prone to the Michael type addition of equation (23) does not inhibit any of the enzymes inhibited by α-hydroxybutynoic acid.

The significant result of these experiments is that the α-C binds to N(5) and therefore is presumably located next to it during normal oxidation. This adds to the growing evidence that N(5) of the isoalloxazine ring is the center on the flavin coenzyme involved in substrate oxidation.

Related experiments done in Massy's laboratory on D-lactate oxidase[99] indicate that inhibition by 2-hydroxy-3-butynoate results in an N(5)-C(6) bridged adduct with the α-C again attached to N(5). If this is indeed the case, then this enzyme must be holding the D-α-hydroxy acid in the same stereospecific orientation vis a vis the flavin N(5) as the L-α-hydroxy acid is held by L-lactate oxidase.

Regardless of the mechanism of inactivation, these studies provide valuable insight into the position and orientation of the substrate at the active site. Both D- and L-lactate oxidase form adducts with α-hydroxybutynoic acid which have the α-C attached to N(5). This confirms the theory that N(5) of the isoalloxazine ring is intimately involved at the active site in substrate oxidation in at least some of the flavooxidases. Furthermore the fact that the L-2-hydroxybutynoic acid is bound to the flavin L-lactate oxidase at C(4a) whereas D-2-hydroxybutynoic acid is bound to the flavin of D-lactate oxidase at C(6) implies that the flavin may require a specific orientation of the α-C of the substrate for oxidation. Thus it appears that N(5) is indeed the crucial center on the flavin nucleus for oxidation and that the enzyme orients the substrate so that the stereochemistry of this active site interaction ($\alpha\text{-C}\overset{\ominus}{\ldots}\text{N(5)}$) is fixed.

b. Other Enzymes

Glycollate oxidase from pea leaves has also been inhibited by 2-OH-3-butynoate in its sodium salt and methyl ester forms.[96] This enzyme normally oxidizes glycollic acid, but any straight chain L-2-hydroxy acid up to and including L-2-hydroxy caproate is easily oxidized by the enzyme. In in vitro studies, a substrate dependent inactivation occurred, but it was not first order. As with DAAO inhibition by propargylglycine which was also not first order in vitro, no time-dependent irreversible inhibition was observed in vivo. However, competitive inhibition was observed and the methyl ester was a more effective competitive inhibitor than the sodium salt.

A series of acetylenic substrates, RC≡CCH(OH)COOH, where R=H-, Me-, Et-, Pr- and Bu- were tested with LAAO and found to be active substrates.[100] Where R=H, inactivation occurred after about 25 turnovers, only the flavin and no amino acid residues were modified. All other substrates exhibited much poorer inhibition qualities. Perhaps the C_4-H is involved somehow in the inactivation process. More likely, groups larger than -H provide steric hindrance to the proper orientation for inhibition (both equations 22 and 23 postulate initial attack on the flavin by C_4 of the inhibitor which may be prevented where a bulky side group is attached to this carbon).

D- and L- lactate dehydrogenase (LDH) have also been inhibited by 2-hydroxy-butynoic acid.[94,95] These enzymes catalize the removal of 2- and 3- hydrogens rather than oxidation of C_2.

D-lactate dehydrogenase and D-lactate dehydrogenase-coupled transport in E. coli membrane vessicles were both specifically inhibited

by the acetylenic substrate.[94] D-lactose protected the enzyme against inhibition. Neither 3-butynoate nor 2-hydroxy-3-butenoate had any inhibitory affect of enzyme activity. The enzyme underwent an average of 35 turnovers with inhibitor before being rendered inactive. All these facts imply that the inhibitor must have substrate properties and must be altered by the enzyme's natural reactions into an active inhibitor species. This is supported by inhibition specificity studies. In vitro, both D-lactose and succinate-NADH oxidation were monitored. Only the D-lactose oxidation was affected. In vivo, D-lactate dehydrogenase-coupled lactose, prolene and valinomycin-induced rhubidium transport were compared with ascorbate-phenzaine methosulfate-dependent transport and α-glycerol-P-dependent threonine transport. Only the D-lactate dehydrogenase dependent transport was inhibited. Thus, the inhibitor appears to be quite specific for the target enzyme.

As in the lactate oxidase case, adduct was found between the inhibitor and the flavin coenzyme. Addition of fresh FMN to apoenzyme separated from the inhibited holoenzyme reconstituted active enzyme. In an interesting aside, the authors noted that the lactate dehydrogenase from rabbit heart muscle, which uses pyridine nucleotide rather than FMN as a coenzyme, was not affected by 2-hydroxy-3-butynoic acid. This inhibitor appears to be specific for the flavin nucleus.

Using L-lactate dehydrogenase (cytochrome b_2) from Baker's Yeast, Lederer[95] also observed inhibition by 2-hydroxy-3-butynoate in a first order process. Spectra of the inhibited species are similar to those for D-lactate dehydrogenase and lactate oxidase reported by Walsh.[91,94]

In this series of studies, both oxidases and dehydrogenases which use flavin as coenzyme were inhibited in a suicidal manner and always only on the flavin portion of the holoenzyme. By comparison of spectra, the adduct species appears to be the same in each case. Thus this inhibitor appears to be general for a broad class of flavoproteins.

2-Hydroxy-3-butynoate was also tested as a inhibitor for DAAO[99] but the kinetics were complicated and the reaction was never characterized.

5. Justification of the Choice of Inhibitors for DAAO

When choosing the most likely set of suicide inhibitors for DAAO, a major consideration was the similarity between lactate oxidase (which catalizes the transformation of α-hydroxy acids to α-keto acids) and DAAO (which catalizes the transformation of α-amino acids to α-imino acids). The catalized reactions are very similar as is the substrate size. Both abstract a proton so that the substrate anion can interact with flavin. Both appear to orient the substrate so that the α-C anion is adjacent to N(5) of the flavin.

The inhibitor species for DAAO most analogous to 2-hydroxy-3-butynoate for lactate oxidase is 2-amino-3-butynoate. The C_4 compound should be more reactive than any longer chain, as indicated by the studies of Dixon and Kleppe on maximum chain length,[56] and those of Cromartie and Walsh using LAAO[100] showing protection from inhibition when groups larger than -H were attached to the far side of the acetylene. If the acetylene is removed farther from the α-C, other reactions than the 2-hydroxy-3-butynoic acid inhibition appear to take place as indicated

by the complicated propargylglycine story. Other α-substituted butynoic acids whose α-H can be abstracted by the enzyme should also exhibit inhibitory properties. Therefore, we proposed to synthesize some 2-substituted-3-butynoic acids to test for suicide inhibition properties on DAAO.

II. MATERIALS AND METHODS

All reagents were analytical grade unless otherwise stated. Solvents were evaporated on a Buchi-Rotavapor-R. Infrared spectra were recorded on either a Bausch and Lomb Spectronic 250 or a Perkin-Elmer 337 Spectrophotometer. Gas Chromatographs were run on a Hewlett-Packard Research Chromatograph, Model 5750 using a 6 foot column of 15% carbowax, 20M on chromosorb AW-DCMS. High pressure liquid chromatography (HPLC) was performed on a Waters Associates High Pressure Liquid Chromatograph, Model 244 using either an Analytical μBondapak C_{18} column with a 10μ silica particle size or a Preparative Porasil C_{18} column with silica particle size 37-75. NMR spectra were obtained on a Hitachi Perkin-Elmer R-20B High Resolution NMR Spectrometer. pH adjustments were made using a Fisher Accumet pH Meter, Model 230A, equipped with a glass body combination ε-5A electrode. A Precision Scientific Company Model 25 Shaker Bath was used to incubate all enzyme solutions. The absorbance at 525 nm was measured on a Beckman Acta MCI Spectrophotometer.

A. Synthesis of n-Dibutyltartrate

1.75 g 4-methyl-benzene-sulfonic acid monohydrate were rinsed into a 1 liter 3-necked flask with 600 ml benzene. The flask was equipped with a Dean-Stark trap and a reflux condenser. The mixture was dried by refluxing 1-1/2 hours. Then 150 g tartaric acid and

276 ml n-butanol were added and the mixture was allowed to reflux 24 hours. Water was removed from the trap periodically.

The reaction mixture was transferred to a 2 liter separatory funnel and washed with 3x200 ml portions of water, then 3x165 ml portions of 2.5% sodium hydroxide, dried over magnesium sulfate, and filtered. The solvent was removed by rotary evaporation and the residue distilled. The fraction boiling at 156-158° C/2.5 mm was collected. The NMR was identical with that of Sadtler file number 6795 for n-dibutyl tartrate. The yield on separate runs varied from 63-71%.

B. Synthesis of n-Butyl Glyoxalate

1. Periodic Acid Method.[102]

360 ml dry ether and 48 ml (0.2 mole) di-n-butyl tartrate were placed in a 3-necked round bottom flask in a nitrogen atmosphere. The mixture was cooled in a water bath and stirred continuously. 44.8 g (0.2 mole) periodic acid were added in portions over one hour. The solution was stirred a few minutes more. The ether phase was decanted, dried over magnesium sulfate, filtered and evaporated. The residue was distilled under vacuum and the portion boiling at 53-69°C/7 mm collected

2. Lead Tetraacetate Method.[103]

In a 250 ml, 3-necked round bottom flask, 100 ml dry benzene and 31 g di-n-butyl tartrate were placed under nitrogen and stirred rapidly. 44.5 g lead tetraacetate were slowly added over 25 minutes, keeping the temperature below 30°C with a cold water bath. The mixture was stirred one hour after addition was complete, during which time the

gummy salts turned crystalline. The salts were removed by suction filtration and washed with 40 ml benzene. The solvent was removed by distillation at 65°C/50 mm, and the residue distilled at 65-79°C/20 mm under nitrogen.

3. Sodium Periodate Method.[101]

72 ml di-n-butyl tartrate and 800 ml 0.402 M sodium periodate were mixed in a 1 liter flask under nitrogen and stirred vigorously for one hour. The solution was extracted with ether, dried over magnesium sulfate, filtered, the solvent evaporated, and the residue distilled under vacuum. The portion boiling at 48-58°C/7 mm was collected. Yields ranged from 74-83%. A strong NMR peak was observed at δ9.0.

C. Grignard and Related Reagents: Preparation and Use.

Ethyl magnesium bromide and ethyl magnesium chloride were purchased from Ventron Chemical Company, Alfa Division, Cleveland, Ohio 44128. Methyl magnesium iodide was prepared by the method described below. Ethynyl magnesium halide was prepared from each of these reagents by the method of Jones, et al.[104]

1. Lithium Acetylide.[105]

220 mM industrial grade acetylene were bubbled into 200 ml of tetrahydrofuran in a 500 ml 3-necked round bottom flask equipped with a sealed dropping funnel and a gas inlet and outlet. The system was kept in a nitrogen atmosphere at -78° throughout the reaction. 50 ml n-butyllithium were transferred to the dropping funnel and added to the reaction mixture over 20 minutes, then stirred 10 more minutes.

2. Methyl Magnesium Iodide

Methyl magnesium iodide was prepared by the method described by Jones, et al., for the preparation of ethyl magnesium bromide.[104] 9 g magnesium turnings (Fisher Scientific Company) and about 30 ml tetrahydrofuran were placed in a dry 500 ml 3-necked round bottom flask equipped with a mechanical stirrer, reflux condenser and a pressure-equalizing dropping funnel. The entire apparatus was kept under nitrogen during the reaction. About 1 ml methyl iodide was added from the dropping funnel to initiate the reaction. When the reaction had begun, the rest of the tetrahydrofuran (270 ml) was added. 24 ml methyl iodide were added dropwise and rinsed in with a few ml of tetrahydrofuran. The reaction was allowed to continue for two hours. A thick grey syrup formed.

3. Ethynyl Magnesium Halide.[104]

A 1 liter 3-necked round bottom flask was equipped with a sealed mechanical stirrer, a frittered glass disk gas inlet, a pressure equalizing dropping funnel, and a gas outlet. The entire system was flamed out under nitrogen. 500 ml of dry tetrahydrofuran were placed in the flask. (Use of less tetrahydrofuran resulted in some precipitation of the product Grignard reagent; use of more tetrahydrofuran made the subsequent reaction easier.) Industrial grade acetylene was passed through a -80° trap, concentrated sulfuric acid, and an ascarite drying tube for cleaning, then into the tetrahydrofuran in the reaction vessel. When the solution was saturated with acetylene, the alkyl magnesium halide (130 ml of 2.94 M ethyl magnesium bromide in ether for this amount of tetrahydrofuran) was slowly added in portions from

the dropping funnel. This addition took 2-3 hours. During the last part of the reaction, the mixture was warmed slightly (to about 30-40°C) to insure complete dissolution of the Grignard Reagent for easy transfer to a 500 ml dropping funnel for the subsequent reaction.

4. Butyl 2-Hydroxy-3-Butynoate.

a. From Ethynyl Magnesium Halide.[106]

A 2 liter 3-necked round bottom flask was equipped with a magnetic stirring bar, a gas inlet, a 500 ml pressure-equilibrating dropping funnel and a gas outlet, all flamed out under nitrogen. The following reaction was kept under nitrogen until addition of the Grignard was completed. 47.6 g of n-butyl glyoxalate was added to the flask and rinsed in with 50-100 ml tetrahydrofuran. The flask was cooled to $\leq$-20°C in a carbon tetrachloride/dry ice bath. Warm Grignard Reagent/tetrahydrofuran solution (from part 3 above) was transferred to the dropping funnel. This mixture was added slowly to the cold solution with stirring. (Directions in the literature called for addition over 1/2 hour; we were never able to add the Grignard this fast while maintaining an internal temperature $\leq$-20°C. A typical addition time was 1.5-2 hours. Best results were obtained on one run when the addition time was extended to more than 3 hours and the internal temperature remained $\leq$-30°C.) After addition was completed, the reaction mixture was allowed to warm to room temperature.

A mixture of 1 liter crushed ice, 30 g ammonium chloride and 50 ml concentrated hydrochloric acid were added to hydrolize the reaction mixture. The organic layer was decanted and the aqueous layer

extracted with ether. The organic portions were combined, dried over magnesium sulfate and filtered. The solvents were removed by rotary evaporator and the residue distilled. The portion boiling at 93-105° C/10 mm was collected.

IR: 3500, 2900 and 1150 cm^{-1}(OH), 2100 (C≡C)

NMR: δ.9, 1.5, 4.0 (Bu ester); δ2.4(HC≡C), δ4.6(α-H); OH shift varies.

Yield averaged 30%. The product remains stable for at least a month when stored at 5°C under nitrogen.

b. From Lithium Acetylide

11.4 g (0.088 moles) n-butyl glyoxalate were transferred to the dropping funnel and added over 5 minutes. The reaction mixture was allowed to reach room temperature, then hydrolized with 40 ml of water. Anhydrous potassium carbonate was added until the aqueous layer became pasty and the organic layer was then washed twice with 30 ml portions of ether. The organic layers were combined, dried over magnesium sulfate, filtered and the solvents removed. The residue was distilled at 9.5 mm pressure.

5. Ethyl 2-Keto-3-Butynoate.

Diethyl oxalate (Fisher Chemical Corporation) was distilled at 79-80°C/10 mm and used in place of n-butyl glyoxalate in the above reaction with ethynyl magnesium bromide (III-C-4). A two molar excess of diethyl oxalate over Grignard was used to decrease the likelihood of double attack on any one molecule. The fraction boiling at 76-80°C/ 9.5 mm upon distillation was collected. A strong IR acetylene peak was observed at 2100 cm^{-1}. The acetylenic proton had shifted to δ3.85 in

the NMR. HPLC analysis showed two components (starting diethyl oxalate and 2-keto-3-butynoate, ethyl ester). Comparison of the acetylenic peak weight to that for the $-CH_2-$ of the ethyl group gave a ratio of 1:6.05 by weight corresponding to a yield of 28% of the keto ester.

D. Synthesis of Butyl 2-Tosyl-3-Butynoate.[108]

6.7g of recrystallized tosyl chloride[107] were dissolved in 3.7 ml pyridine and cooled quickly to obtain short crystals. 5g of butyl 2-hydroxy-3-butynoate were added to the tosyl chloride/pyridine mixture which was kept on ice. The mixture was stirred by hand, then transferred to a 10-20° water bath for one hour. This mixture turned a glassy wine color within 10 minutes, becoming very viscous and usually solidifying. The reaction vessel was then transferred back to the ice bath and 25 ml of water were slowly added. The aqueous solution was extracted with ether to a total volume of 125 ml. The ether extract was then back-washed with 150 ml 0.01 N sulfuric acid and 100 ml 2.5% sodium bicarbonate. The ether solution was dried over magnesium sulfate, filtered, and the solvent evaporated. The tosylate crystallized on one attempt, but usually remained as an oily residue.

IR: 2100 cm^{-1} (C≡C) + varying amounts of 1950 cm^{-1} (C=C=C); 1160, 1170 cm^{-1} (tosyl group doublet).

NMR: δ7.1-7.5 (phenyl H's); δ2.35 (φMe-masks acetylenic H); δ4.5 (C≡C<u>C</u>H).

E. Preparation of Butyl 2-chloro-3-butynoate.[106]

1. In Diethyl Ether with Pyridine.

Ten ml butyl 2-hydroxy-3-butynoate were stirred in 50 ml anhydrous ether in a 250 ml 3-necked round bottom flask in an ice bath, equipped with two pressure-equalizing dropping funnels. 6 ml thionyl chloride were placed in one dropping funnel and 6.35 ml pyridine in the other. These solutions were added simultaneously over 1/2 hour. Then 50 ml ice water were added, the organic layer decanted and the aqueous layer extracted with ether. The organic portions were combined, dried over magnesium sulfate, filtered, and the solvent evaporated. The residue was distilled and a fraction boiling between 89-101°C/9.5 mm was collected.

IR: decrease in 3600 cm^{-1} and 1400 cm^{-1} OH bands; sharp C≡C stretch at 2100 cm^{-1}.

NMR: δ4.7 (≡C-C<u>H</u>(Cl)), δ4.5 (≡C-C<u>H</u>(OH)), δ2.6 (<u>H</u>C≡C[CCl]), δ2.3 (<u>H</u>C≡C[COH]).

NMR shows a mixture of starting alcohol and desired chloride in about a 60:40 ratio, with respect to the relative heights of the above cited NMR peaks. Verny and Vessière claim a yield of 95% for this reaction; in our hands the yield never exceeded 50%, and the two products could not be separated by distillation with the equipment in our possession.

2. In Isopropyl Ether.

A mixture of 3 g (0.0192 moles) butyl 2-hydroxy-3-butynoate, 20 ml isopropyl ether and 0.02 ml thionyl chloride were refluxed for

two days under nitrogen, cooled and the solvent evaporated. There was insufficient material to distill the chloride away from polymerized by-products.

IR: 2100 cm^{-1} (C≡C) and expected decrease in -OH bands.

NMR: δ4.7 and δ2.7 and absence of other acetylenic peaks indicated the chloro rather than the hydroxy species is present.

HPLC: 3 peaks in the cleanest preparation. These peaks correspond to three of the peaks in the distilled preparation of the mixture of the α-chloro and α-hydroxy esters (part (1) above).

F. Alumina Column Chromatography

Aluminum oxide (alumina) (Chemie-Ergeugnisse and Absorption-technik AG, Hombergerstrasse 24, 4132 Muttenz, Schweitz) was activated by drying for two days at 210°, then stored in a dessicator. The dry alumina was poured into ether in a 2 cm column to a height of 16 inches. The column was washed with about 200 ml ether to insure complete settling. 4 ml of sample were layered on the column and washed in with ether. 50 ml fractions were collected to a total of 1200 ml. Methanol was then rinsed through the column and 50 ml fractions again collected to a total of 1200 ml.

To restore the alumina, it was stirred in ethyl acetate and allowed to sit for two days, dried at 80°, then at 210° for two days.

Deactivated alumina was prepared by adding 10% water to activated alumina.[109]

G. Attempts to Prepare Butyl 2-Amino-3-Butynoate

1. With Ammonia[110]

a. Preparation of Cuperous Chloride[111]

8.5 g cuprous chloride dihydrate were dissolved in 25 ml of water and filtered into a 125 ml erlenmeyer flask. 10 g copper wire were added and the mixture heated under nitrogen almost to boiling for 12 minutes. In the meantime, 10 ml ether and 300 ml water were mixed in a 1 liter erlenmeyer flask, covered, shaken, and allowed to stand. The copper solution and white precipitate was then poured into the ether/water solution. The copper wire was rinsed with 10 ml of 3N hydrochloric acid which was also added to the ether/water solution. This was allowed to settle for 10 minutes under nitrogen in the hood. The white crystals were collected on a buchner funnel. A thin film of liquid was kept over the crystals at all times. The crystals were rinsed with 2x10 ml ethyl alcohol, then 2x10 ml ether and spread on a watch glass to dry. When dry, the crystals were stored under nitrogen in a vial in a dissicator.

b. Preparation of Amine

1 ml concentrated ammonium hydroxide and a few grains of cuprous chloride were added to a flask containing ~1 ml butyl 2-tosyl-3-butynoate. The mixture was stirred at room temperature four hours. Then 5 ml ether plus 5 ml water were added to the reaction vessel and the entire contents transferred to a separatory funnel. The aqueous layer was extracted with ether, these extractions combined, dried over magnesium sulfate, filtered and the solvent evaporated. A white, flaky residue was left. m.p. = 123-4.

IR: 1900 cm^{-1} (C=C=C); only minor 2100 cm^{-1} (C≡C) absorption; 36-3200 cm^{-1} triplet, 1600, 1450, 1300 cm^{-1} ($-NH_2$).

IR is consistent with the formation of the allenic amine, butyl 2-amino-2,3-butadienoate.

2. Cyanoborohydride on Ethyl 2-Keto-3-Butynoate.

In a modification of a method described by Borch, et al., for the preparation of α-amino acids,[112] 20.0 g diethyl oxalate/2-keto-3-butynoate were added to a mixture of 16 g ammonium bromide and 3.2 g sodium cyanoborohydride in dry methanol. The mixture was stirred for 48 hours under nitrogen. (The yellow ketone solution turned orange within minutes after addition; the solution was light yellow at the end of incubation.) Concentrated hydrochloric acid was added to pH<2 and the methanol removed under vacuum. A white residue was obtained which was dissolved in about 100 ml of water and extracted with 4.70 ml ether. The aqueous layer was made basic (pH>10) with potassium hydroxide and saturated with sodium chloride. This basic aqueous layer was extracted with 5x100 ml ether, dried over magnesium sulfate, filtered and evaporated. The white residue collected from this fraction did not melt below 300°C and was probably salt.

If the amine ester had been hydrolized to the zwitterionic acid, it would not dissolve in ether at this high pH but rather at its isoelectric point. Therefore the basic aqueous layer was neutralized (most simple neutral amino acids have their isoelectric points at pH 6-7), and extracted with ether. The ether extracts were combined, dried over magnesium sulfate, filtered and the ether evaporated.

NMR: δ2.1 ($-CH_3$), δ3.4 ($-CH_2-$), δ4.6 broad (?$-NH_2$)

IR: 3300-3400 cm^{-1} broad, 2980, 2900 cm^{-1}

The acid extracted ether fraction was also dried and analyzed.

IR: broad absorbance with little detail, 3600-2300 cm^{-1}, peaks at 1750, 1450, 1330, and a doublet at 1220 cm^{-1}.

NMR: a complicated spectrum that could not be analyzed.

3. Grabriel Synthesis

a. Preparation of the phthalimide[113]

4.0 g of 2-tosyl-3-butynoate were mixed with 5.75 g potassium-phthalimide salt (Eastman Chemical Company, Rochester, N.Y. 14650), 16 ml dimethyl formamide and 1 crystal potassium iodide and refluxed 1.5 hours. (The mixture quickly thickened and turned black.) Where refluxing was finished, 25 g ice were added and the solid material (brown-black crystals) was collected by suction filtration. These crystals were dissolved in 225 ml chloroform, washed with 3x33 ml 1N potassium hydroxide, 3x33 ml 0.5N hydrochloric acid and 3x33 ml water. The chloroform was then evaporated. 1.6 g of a black oil remained.

IR: 2100 cm^{-1} (C≡C); 2900, 1700-1750 cm^{-1} (C=O); 1450, 1370, 1270, 1200, 1060 cm^{-1} (phthalimide).

NMR: δ8.4 broad, 8.15 sharp (Ar<u>H</u>); δ2.2 (<u>H</u>C≡C); δ4.55 (?CNH).

b. Hydrazinolysis of the phthalimide[144]

Crude phthalimide from part a above was rinsed into a 50 ml round bottom flask with 20 ml 95% ethanol. 2.2 g hydrazine hydrate were added and the mixture was refluxed one hour. After it had cooled, 3.7 ml 10N hydrochloric acid were added to the solution which was kept cool on ice. The resulting precipitate was collected by suction

filtration and triturated with 20 ml water, filtered again and the combined filtrates evaporated to dryness. The residue was treated with a solution of 4.4 g potassium hydroxide in 10 ml water and extracted with 5x6 ml ether. The ether extract was dried over potassium hydroxide and filtered. The ether was removed by distillation through a column of glass beads. The residue was insoluble in carbon tetrachloride.

IR: broad 3500 and 1600 cm^{-1} peaks indicative of either acid or amine; no 1200 or 800 cm^{-1} peak as expected for a secondary amine.

H. Hydrolysis

1. Alkaline.[115]

1.8 g (0.032 moles) potassium hydroxide were dissolved in 80 ml water and mixed with 5 g (0.032 moles) butyl 2-hydroxy-3-butynoate. This solution was stirred overnight at room temperature. Concentrated hydrochloric acid was then added until a test drop of the solution turned Congo Red paper blue. This addition was carried out in an ice bath. The solution was then extracted with ether and the ether extract dried over magnesium sulfate, filtered and the solvent distilled. NMR analysis showed that hydrolysis did not take place.

2. Acidic[116]

2.7 g butyl 2-hydroxy-3-butynoate and 50 ml 10% hydrochloric acid were mixed and refluxed four hours. The solution was concentrated under vacuum and put on ice but crystallization could not be induced. The solution was therefore extracted with ether and the ether extract dried over magnesium sulfate, filtered and the solvent removed under

vacuum. An impure oily acid remained. HPLC analysis showed two major components with small quantities of a number of other components also present. This material could not be recrystallized from hexane.

3. Transesterification[117]

A mixture of 3 g (0.0192 moles) butyl 2-hydroxy-3-butynoate, 8 ml acetic acid and 4 ml concentrated hydrochloric acid were refluxed 1.5 hours in the hood, and allowed to cool. The solution was concentrated in an oil bath at 100°/50 mm and cooled. 33 ml of saturated sodium bicarbonate solution were added to the residue and the mixture stored overnight at 5°C.

When warmed to room temperature the solution was brought to neutral pH with additional solid sodium bicarbonate and extracted with 2x11 ml ether. The aqueous solution was then acidified with 12N sulfuric acid. (This addition was done slowly in an ice bath as a great deal of heat and frothing was produced.) The oily suspension was extracted with 3x12 ml ether. The ether extracts were combined and washed with water, dried over magnesium sulfate, filtered and the ether evaporated. The oily residue was insoluble in carbon tetrachloride and had the broad 3600-3000 cm^{-1} IR absorption expected for acids. The 2100 cm^{-1} C≡C stretch was also observed.

Where the above hydrolysis was performed on fractions containing butyl 2-chloro-3-butynoate the residue from the acidic ether extract was dissolved in 18.5 ml warm (50-60°) concentrated hydrochloric acid and the solution allowed to cool with occasional swirling. The hydrochloric acid was then removed under vacuum. The resulting residue was subjected to HPLC analysis and used in enzyme inhibition studies.

I. Enzyme Assays-Irreversible Inhibition[3]

1. Inhibition

a. Solutions

i. 0.02M pyrophosphate buffer, pH 8.3.

4.465g sodium pyrophosphate decahydrate were diluted to 10 ml with water and adjusted to pH 8.3 with 6N hydrochloric acid.

ii. Catalase, 4 mg/ml.

0.0040g catalase (ICN Pharmaceuticals, Inc. Life Sciences Group, Cleveland, Ohio 44128) were weighed out and diluted to 10 ml with water the day of use.

iii. FAD, 2.8×10^{-4}M.

0.0022g FAD (Flavin Adenine Dinucleotide) (ICN Pharmaceuticals, Inc. Life Sciences Group, Cleveland, Ohio 44128) were weighed out and diluted to 10 ml with buffer. This solution was kept in the dark at all times.

iv. DAAO, 6.25 µg/ml

0.0005g DAAO (U.S. Biochem Corp., Cleveland, Ohio 44128) were weighed out and dissolved in 0.4 ml FAD solution plus 7.6 ml buffer. This solution was prepared just before use and kept in the dark.

v. Inhibitor

Amounts as described in Results and Discussion were weighed out and diluted with buffer.

vi. TCA, 30% w/v

7g TCA (trichloracetic acid) were diluted to 25 ml with water.

b. Procedure

Solution A was prepared by mixing 2 ml FAD solution, 2 ml catalase and 8 ml buffer. 0.4 ml of this solution A was added to each of 15 test tubes. 0.5 ml DAAO was added to each of the first 14 test tubes and 0.5 ml buffer to the last (the blank). The test tubes were allowed to equilibrate in a constant temperature bath of 37°C. 1 ml of inhibitor was added to each odd numbered tube and 1 ml of buffer to each even numbered tube. One tube each of inhibitor and buffer solutions was incubated for exactly 1, 5, 10, 20, 50, 100 and 120 minutes. At the end of the incubation period, 0.3 ml D-alanine was added to the tube and the solution incubated for another 20 minutes. At the end of this period 0.5 ml TCA was added to stop all further enzyme activity.

2. Pyruvate Assay

a. Solutions

i. 1 M hydrochloric acid saturated with 2,4-dinitrophenyl hydrazine (DNPH)

46 ml DNPH were ground with an increasing volume of 1 M HCL to a total volume of 50 ml.

ii. 2 M Sodium Hydroxide

40g sodium hydroxide were dissolved in water and diluted to 500 ml. The solution was allowed to cool before being used.

b. Procedure

To each of 15 test tubes 0.9 ml water and 0.2 ml DNPH solution were added, then 0.1 ml of killed enzyme solution from part 1 above. Exactly 15 minutes after addition of the enzyme solution the incubation

was stopped by adding 3.0 ml of 2.0M sodium hydroxide. The absorbance at 525 nm was measured within 1/2-hour.

III. RESULTS AND DISCUSSION

A. Preparation of Butyl-2-Hydroxy-3-Butynoate

α-hydroxy butynoic acid has been found to be an inhibitor of DAAO.[99] Its ester is a useful intermediate whose hydroxy group can be replaced by a number of other functional groups. Therefore it was prepared as the starting material for a number of projects. Because the reported yield in the standard synthetic procedure[106] was only 30%, considerable initial effort was spent searching for a method that would give a higher yield.

1. Preparation of n-Butyl Glyoxalate

Initial work was done on redistilled n-dibutyl tartrate found in the stockroom. Little or no n-butyl glyoxalate could be generated via any of the procedures described. Careful re-examination of the NMR data for this starting material showed an extra peak at δ2.3. Although other physical properties of this material appeared to match those for n-dibutyl tartrate, every other possible error in the synthesis was systematically eliminated. When starting with dibutyl tartrate we had synthesized ourselves, good yields of the n-butyl glyoxalate were obtained.

The best yields of n-butyl glyoxalate were obtained from the reaction with sodium periodate (Section III-B-3). This reaction was

also the easiest to perform. Therefore, this procedure was followed preferentially whenever the reagents were available.

There was some discrepancy in the literature[101-103] as to the actual boiling point of the aldehyde. We found that the best fraction, by NMR analysis, was the cut boiling between 48 and 58°C/7 mm.

2. Preparation of Butyl 2-Hydroxy-3-Butynoate

Because the reported yield for butyl 2-hydroxy-3-butynoate, via the Grignard reaction was only 30%, initial attempts were made to find a substitute means of adding the acetylenic functional group to the aldehyde end of n-butyl glyoxalate. Lithium acetylide-ethylene diamine complex has been used in the laboratory to add acetylene to other compounds in good yield.[118] Therefore addition with this reagent was tried first. A method similar to that described for the ethynylmagnesium halide addition (Section II-C-4) was used. The only product isolated from the ether extraction was n-butanol. Subsequent extraction of the aqueous layer with tetrahydrofuran yielded a carbon tetrachloride insoluble component. Therefore, it appears that this reagent hydrolizes the ester rather than adding acetylene to the aldehyde end of the molecule.

Midland[105] has described a procedure for the preparation of ethynyl carbinols and terminal acetylenes from aldehydes and ketones using monolithium acetylide at low temperatures. In our hands, using n-butylglyoxalate, none of the desired acetylene could be isolated. It should be noted, however, that this reaction was never repeated after the aldehyde preparation was satisfactorily worked out and may yet provide a means of significantly improving the yield at this step.

The addition of a terminal acetylene to n-butyl glyoxalate by the method of Verny and Vessière[106] using ethynyl magnesium bromide was repeated consistently in this laboratory in the yields reported (30% average) and became the accepted route to the 2-hydroxy-3-butynoate, butyl ester.

When ethyl magnesium chloride was substituted for ethyl magnesium bromide in the systhesis, a much lower yield was obtained. Both ethyl magnesium chloride and ethynyl magnesium chloride were significantly less soluble in tetrahydrofuran than the corresponding bromo compounds and were therefore much more difficult to work with. It was uncertain whether the decrease in the amount of product formed was due to a decreased reactivity of the ethynyl magnesium chloride or simply to its being less soluble.

It was felt that since the chloride was less soluble than the bromide in tetrahydrofuran, the corresponding iodides ought to be more soluble and more reactive, providing a higher yield of the acetylide hydroxy ester. Ethynyl magnesium iodide was therefore prepared and used in this reaction. It formed a thick grey slush rather than the expected solution. When distillation of the residue from ether extraction of the reaction mixture was attempted, a dark brown-black material filled the distillation apparatus at a temperature of about 40°C/9.5 mm. None of the desired product could be collected.

Varying amounts of polymerized by-product were obtained in all Grignard reactions depending upon the type of Grignard used, the temperature during addition, and the pressure during distillation. Once the hydroxy ester was distilled away from this mixture, however, it could be redistilled without appreciable polymerization.

B. Preparation of Butyl 2-Tosyl-3-Butynoate

A series of 3-butynoic acids, substituted in the α-position are desired for inhibition studies on DAAO. Therefore, one would like to find an intermediate which can be the common precursor to all these products. Because the tosyl group is such a good leaving group, it was hoped that the tosylate would become such an intermediate. Preparation of the tosylate by the method of Eglinton and Whiting,[108] developed especially for use on acetylenic compounds, was very straightforward. However, the tosylate was unstable, rearranging first to the allene, then loosing its unsaturation with time. (Within 16 hours under refrigeration, almost all the acetylene had disappeared; within 3 days no unsaturation could be detected.) In one preparation only the acetylene peak was observed by IR immediately after preparation; usually both the acetylene and allene peaks were present. The tosylate was therefore always prepared immediately prior to its use in subsequent reactions.

When it was determined that unsaturation was being lost in tosylate prepared by the above method, an attempt was made to use the method for tosylate synthesis described by Fieser and Fieser.[107] No acetylenic tosylate could be isolated. A search of the literature indicated that the presence of pyridine (of which a much larger concentration is used in the latter procedure) increases the likelihood of rearrangement of the acetylenic moiety to the allene which is susceptible to attack by nucleophiles and that this might be the cause of unsaturation loss.[106,119] Eglinton and Whiting[108] found a marked decrease in tosylate production when the amount of pyridine was increased,

supporting this theory. In light of these problems, an attempt was made to synthesize the tosylate in a non-pyridine medium. When the method of Prib and Malinovskii[120] for propargyl esters, using ether as the solvent, was employed, most of the acetylene was again lost with some allene and significant double bond formation as judged by both NMR and IR analysis. Since no better method could be found, that of Eglinton and Whiting[108] for the preparation of 2-tosyl-3-butynoate was used for all synthetic work.

C. Preparation of Butyl 2-Chloro-3-Butynoate

1. Synthesis

Because of the instability of the 2-tosyl-3-butynoate, a great deal of time was spent trying to prepare a pure sample of 2-chloro-3-butynoate as an alternative intermediate. This compound is also interesting in its own right as a potential inhibitor of DAAO.

Verny and Vessière[106] prescribed two methods for the preparation of this chloride, neither of which proved very satisfactory in our hands. They reported a 67% recovery of esters consisting of 95% of the α-chloro ester and 5% of the α-hydroxy ester when using method II-E-1; we were never able to achieve better than 50% conversion. Using method II-E-2, they obtained a 55% yield consisting solely of the α-chloro ester; we obtained a small amount of the α-chloro ester (as determined by NMR) plus mostly polymerization by-products.

2. Separation of Butyl 2-Chloro-3-Butynoate from Butyl 2-hydroxy-3-Butynoate

Since the overall yield from method II-E-1 was much better than that for method II-E-2, we concentrated on trying to separate the

α-chloro and α-hydroxy esters using column chromatography on alumina. We chose alumina column chromatography for separation of the two ester species because other chloro and hydroxy compounds had previously been successfully separated by alumina column chromatography in this laboratory.[118]

The esters boiled within 4° of each other and hence could not be separated by distillation. The acetylenic moiety partially rearranges to an allene during gas chromatography. The HPLC preparative column available to us at the time could not separate large quantities of material. Therefore, we felt the alumina column offered the best potential for separation of these two species.

We were hoping to be able to separate the two esters and thereby obtain the α-chloro ester in pure form for subsequent preparative work. The chloroester should go right through an alumina column when applied and eluted with ether. Then changing the solvent to methanol should elute the hydroxy ester. Preliminary work with a mixture of n-butyl chloride and n-butyl hydroxide verified these column characteristics. 50 ml fractions were collected during elution with first 1200 ml of ether, then 1200 ml of methanol and the contents of these fractions were monitored by gas chromatography (GC). The GC spectra indicated that a much more complicated process than the expected one was taking place. When ether fractions 2 and 3 were combined and evaporated, an IR spectrum indicated that partial hydrolysis and partial to full rearrangement to the allene had taken place. The multiple peaks in the GC spectra were apparently from the sum of these rearrangement products and the products of rearrangements known to take place

during GC analysis[106]. Methanol fractions from these columns showed equally complicated spectra. These fractions were never completely analyzed.

Farrar, et al.[109] have described the hydrolysis of esters on activated alumina columns and recommended deactivating the columns by adding 10% water to the alumina. They claimed this eliminated all hydrolysis by alumina of the esters they were working with. When we prepared a column in this manner, the flow rate was extremely slow and IR analysis indicated the same partial hydrolysis and partial rearrrangement to the allene as before. Perhaps the added length of time the ester was exposed to the column counteracted the decreased activity of the alumina.

Due to the above described complications in separation of the α-chloro ester by distillation and chromatography a pure sample of α-chloro butynoate was never obtained for further preparative work.

Future separations of butyl 2-hydroxy-3-butynoate and butyl 2-chloro-3-butynoate should by done on the preparative HPLC now available in this laboratory since analytical HPLC analysis of this mixture resulted in easy separation of the esters in methanol:water solutions with no apparent rearrangement taking place.

D. Attempts to Make 2-Amino-3-Butynoate

1. Using Ammonia

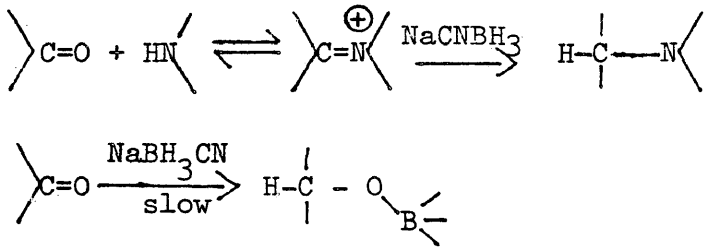
Monroe et al.[110] claim to have prepared 2-amino-3-butynoic acid for use as a corrosion retardant. They obtained the amine from the corresponding α-chloro-acid. We were never able to prepare the α-chloro-acid in sufficient purity to use as a preparative reagent.

However, the tosyl ester proved to be quite easy to make although it also easily rearranged to the allene. We attempted to use the tosylate in the prescribed procedure, to prepare butyl 2-amino-3-butynoate, but were only able to isolate what IR analysis indicated was the allenic amine, butyl 2-amino-2,3-butadienoate. At the time these reactions were run, we were not interested in the allenic amine and did nothing with those products. In light of subsequent inhibition studies (discussed elsewhere in this work), however, purification of this allenic amine and inhibition studies with it might prove very interesting.

2. The Cyanoborohyde Reaction

When the only preparation described in the literature for the synthesis of α-aminobutynoate failed in our hands, a complete search of the literature was made for other possible pathways. Two distinctly different possibilities presented themselves: Cyanoborohydride attack on an α-keto acid and the well-known Gabriel synthesis.

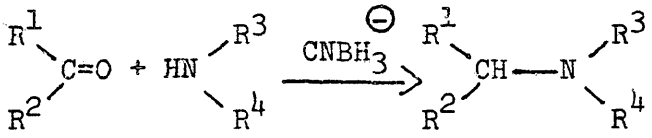
The cyanoborohydride reagent seemed like an attractive reagent for synthesis of the amine from the ketoester. Cyanoborohydride reduces an iminium ion much faster than it reduces a carbonyl group:[112,121]



(24)

Reduction of the carbonyl group is negligible under neutral conditions and must be run in a system buffered at pH 3-4. These reactive properties of cyanoborohydride allow one to aminate an aldehyde or ketone

by simply reacting the carbonyl compound with amine at pH 6-8 in the presence of sodium cyanoborohydride:

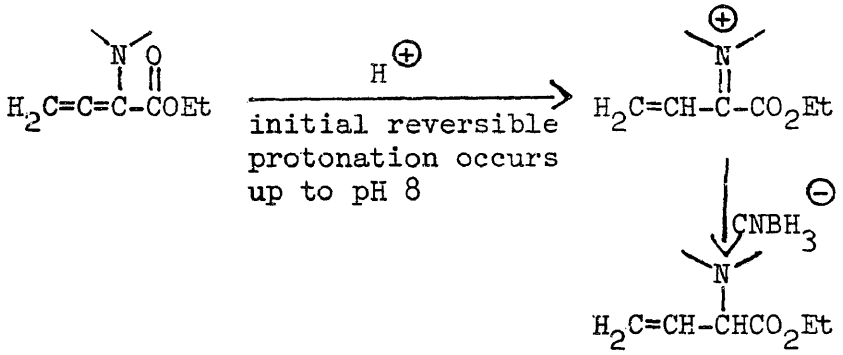


(25)

This reaction is general for aldehydes, and unhindered ketones with ammonia, primary and secondary amines.[121]

A further attraction of this reagent is that it does not react with ester or acid groups.[112, 121, 123] Broch, et al. have used this method to synthesize six of the common amino acids.[112] It is a particularly convenient method for preparing ^{14}N labeled amino acids.

Although some α,β-unsaturated compounds show a tendency toward 1,4 attack and bond migration,[124-126] and the allenic derivative of α-aminobutynoate should be readily reduced via its enamine form,[121]



(26)

Examination of the literature indicated that the acetylenic function might be left intact.[122,127]

Therefore, we prepared ethyl-2-keto-3-butynoate in order to attempt to repalce the keto group with an amine.

a. Ethyl 2-Keto-3-Butynoate

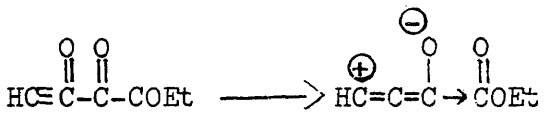
The idea for the actual reaction used in this synthesis came from the work of I. I. Lapkin and Y. S. Andreichikov[128] who first succeeded in preparing a series of α-keto acetylenic esters. Ethyl 2-keto-4-phenyl-3-butynoate was reported in a 20% yield from ethyl magnesium bromide, phenyl acetylene and diethyl oxalate using different conditions from ours. We felt that the method we had for the preparation of an α-hydroxy acetylenic ester was at least as good as the method reported for this synthesis and ought to give the desired α-oxo ester if diethyl oxalate were substituted for n-butyl glyoxalate in reaction II-C-4a. In order to avoid attack by a second Grignard on the keto ester, leading to the diacetylenic alcohol, a 2:1 ratio of diethyl oxalate: Grignard was used.

From planimeter measurements of the relative peak heights for the acetylenic H and the methylenyl H, a yield of 50% of the keto ester was calculated. When the sample was subjected to HPLC (high pressure liquid chromatography) analysis, only two components were observed, the excess starting material, diethyl oxalate, and ethyl 2-keto-3-butynoate. From the relative weights of these two peaks a yield of 28% was calculated. This is a much more accurate measurement than the planimeter measurement of the NMR peaks as the NMR peaks were small and sharp and difficult to measure accurately. Even this lower yield is considerably better than the 20% yield reported by Lapkin and Andreichikov for a related compound.

The acetylenic proton was shifted downfield in the NMR by the α-keto ester. We observed it at δ3.9 instead of near δ2.35 as observed

for the α-hydroxy ester. This large shift downfield is apparently due to the intense electron withdrawing nature of the α-keto ester. Jouve and Lecomte[129] have studied the δ shift for the acetylenic H in a series of α-substituted species. These studies showed that the more the electron withdrawing character adjacent to the acetylenic moiety, the stronger the δ shift downfield. Their values ranged from δ1.60 for MeC≡C<u>H</u> to δ3.0 for MeCOC≡C<u>H</u>. The addition of the ester group β to the acetylene apparently increases this shift significantly. Jouve and Lecomte also correlated the increase in the shift toward higher δ in the NMR with an increase in the amplitude of the IR peak. We did indeed observe a much stronger acetylenic peak in the IR for this compound.

One can also explain this shift in terms of resonance structures. If one considers the possible resonance structures that can be written for this species:



a b (27)

In accordance with this decrease in triple bond character both the HC≡C δ in NMR and the C≡C stretch in the IR are shifted toward that expected for a double bond. The shift for a vinylic proton in NMR is δ4.6-5.9; this proton has been shifted from 2.35 to 3.9. The allenic stretch in the IR is at 1950 cm^{-1}; the stretch in this molecule has moved from the expected 2100 cm^{-1} to 2060 cm^{-1}.

This compound appears to be quite stable. HPLC work was not done until two months after the synthesis was complete, yet showed only

the two expected components. Repeat NMR and IR analysis five months after synthesis gave the same spectra except that the HC≡C NMR peak had shifted upfield to δ3.6.

b. The Cyanoborohydride Reaction

No product could be isolated from the ether extract of the basic aqueous solution of the cyanoborohydride reaction. Thus the amine ester, butyl 2-amino-3-butynoate must not have been formed. If the ester had been hydrolized during this reaction however, it would no longer be insoluble in base and could only be extracted near its isoelectric point. Since most neutral amino acids have their isoelectric points between pH 6 and 7, the aqueous layer was neutralized and reextracted with ether. The NMR spectra of the residue left when the ether was evaporated was not inconsistent with the 2-amino-3-butynoate structure still in the ester form, but IR shows no carbonyl stretch at 1700-1800 cm^{-1}. It is unlikely that an acid was formed since the material was soluble in carbon tetrachloride.

When the ether extract of the material originally dissolved in water before being made basic was examined by NMR and IR, it appeared to be composed of a number of products and could never be analyzed.

3. The Gabriel Synthesis

The Gabriel Synthesis has been used to prepare amines from a variety of halo compounds.[130] In particular, amines have been successfully prepared from both α-halo esters[131] and propargylic halides[113] in good yields. Therefore we were hopeful that 2-amino-3-butynoic acid, which contained both of these functional groups, could be synthesized by this method.

The Gabriel Synthesis is a 2-step preparation consisting of initial reaction with potassium phthalimide to form the addition phthalimide plus water, and subsequent acid hydrolysis or hydrazinolysis to the amine.

a. Preparation of Butyl 2-Phthalimide-3-Butynoate

Assuming the acetylinic group to be the one most prone to give us difficulties, we followed the reaction procedure described for 2-butynylchloride.[113] It is interesting to note that although the tosyl starting material contained both allenic and acetylenic absorption peaks in the IR, only a clean acetylenic peak was observed for the phthalimide.

Although a large amount of brown-black crystals were obtained from the water solution after reflux, only 1.6g (a 33% yield) of a black oil were left after the described work-up procedure had been followed. If this reaction is to become a significant step on the pathway to the α-amino butynoic acid, then a better work-up procedure needs to be found. When preparing 1,4-diphthalimide but-2-yne, Fraser and Raphael[132] simply washed the precipitated solid obtained when water is added to the refluxed mixture with cold water and used it in the next step. They were also able to recrystallize this material from acetic acid. These modifications may be sufficient in our case. Certainly a good yield of precipitate was obtained from water in our case and if it could be used without further purification, a much higher yield of the product amine should be obtained.

Examination of the IR and NMR data indicated that the phthalimide had indeed been prepared.

b. Hydrazinolysis of Butyl 2-Phthalimido-3-Butynoate

The hydrazinolysis was performed by a method used to produce N-crotyl amine from the phthalimide, again under the assumption that a method successful for an unsaturated system was most likely to give us our desired unsaturated amine. However examination of the IR of the product of this reaction showed only a faint trace of the 2100 cm^{-1} acetylene peak. No allene absorption was observed. The spectrum showed the broad absorbance at 3500^{-1} and 1600^{-1} indicative of an amine, but the expected 1200 and 800 cm^{-1} peaks were missing. The product was insoluble in carbon tetrachloride and the NMR spectrum in heavy water indicated that the butyl group had been lost. This was not a problem for our purposes as we wished to produce the acid ultimately. However, no acetylenic peak could be observed in the NMR. The NMR spectrum was not inconsistent with the product's being 2-aminobutyric acid.

Although this hydrozinolysis step was unsuccessful in this initial trial, the Grabriel Synthesis appears to hold promise as a route to α-aminobutynoate. The phthalimide reaction was successful and there are a variety of ways to remove this functional group. All early work with phthalimides involved hydrolyzing the phthalimide in refluxing acid.[130] We have shown the acetylenic group to be stable to refluxing acid.[116] However, it may be that the α,β-unsaturated bond will be susceptible to saturation under any of the required preparative conditions.

E. Hydrolysis

Several different hydrolysis techniques were tried on butyl 2-hydroxy-3-butynoate. Mild basic hydrolysis yielded only starting

material. Acid hydrolysis gave the acid in a mixture of products (as determined by IR and HPLC). Transesterification gave the best results although HPLC data still showed a number of components in the residue. This method was used to generate all acids for use in enzyme studies. Both butyl 2-hydroxy-3-butynoate and butyl 2-chloro-3-butynoate were oils under these conditions and no means was ever found of inducing them to crystallize.

F. Enzyme Inhibition

1. Chloro Compound

Although a pure 2-chloro-3-butynoic acid fraction could never be prepared, it was decided to run preliminary inhibition studies on the mixture containing this species (Inhibitor, path 1, scheme III). Then if no inhibition were found, we would not waste valuable time trying to purify it. However, when this mixture was tested it exhibited powerful time-dependent inhibition essentially stopping all enzyme activity within 20 minutes. A repeat run one month later gave similar results (Fig. 6). When a second preparation containing purer 2-chloro-3-butynoic acid according to IR analysis (Inhibitor, path 2, scheme III) was tested, no time-dependence could be observed and inhibition was not complete.

In order to determine where the inhibitory species first appeared in the preparative procedure, a third sample was prepared, testing for inhibition at each step (path 3, scheme III). That is, tests were performed on the mixture of 2-hydroxy-3-butynoate and 2-chloro-3-butynoate,butyl esters before column chromatography, the first ether fractions from the column and the product of hydrolysis. The end

product displayed the same qualities as the product of the second preparation. Only the product directly off the column, which has been shown to be a combination of partially hydrolized chloro compounds, primarily the α-chloro allene, shows time-dependent inhibition. However the inhibition only decreased to 37%. Presumably this was because the inhibitor was present only in small quantities, insufficient to react with the full amount of enzyme present. In this test the inhibition had nearly reached its maximum after only 5 minutes.

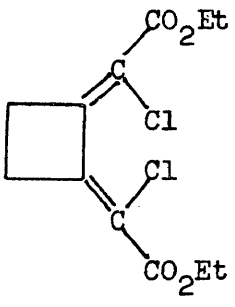
To determine which component in the original (path 1) inhibitor fraction was the powerful inhibitor of DAAO, we separated this fraction into 3 components by preparative HPLC using 20:80 methanol: water as the solvent and a 2ml/min. flow rate to separate a 1.5 ml injection sample. Three components were isolated and tested for inhibition. All 3 components were inhibitors of DAAO; the first and third displaying non-time dependent inhibition, and the second one showing time-dependent, but less strong inhibition. None of these species alone displayed the powerful inhibition properties of the mother liquor. Perhaps the powerful time-dependent inhibition is due to some type of symbiotic relationship in the interaction of this combination of inhibitors with DAAO.

Scheme III is a flow chart showing how each inactivating species was prepared and the results it gave. In interpreting the data given in scheme III it is important to recognize that the reported inhibitor concentrations are unreliable because we had some difficulty making sure the solvent was completely removed in every case and because the inhibitor fractions were, in most cases, not a pure

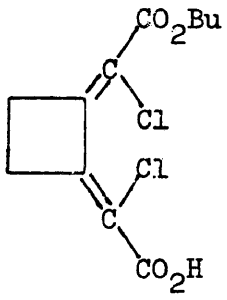
substance. Therefore, absolute numbers for the degree of inhibition are not reliable; only the general inhibition characteristics can be considered significant. Furthermore, one must be aware that the phrase "non time-dependent inhibition" means that inhibition had reached its maximum before the first measurement, after one minute of incubation, was made. Thus "non time-dependent inhibition" could be either rapid (<1 min.) time-dependent inhibition or true non time-dependent inhibition. These reactions are run at large inhibitor concentrations, and it is possible, that time dependence might be observed as inhibitor concentration decreased.

Since the acetylenic peak was present to a greater degree in samples that did not inhibit DAAO in an observably time-dependent manner, this species (2-chloro-3-butynoic acid) was ruled out as the "time-dependent" inhibitor.

Vessière and Verny have shown that esters of 2-chloro-3-butynoate easily isomerize to their allenic isomers, 2-chloro-2,3-butadienoate.[106] Where the crude allenic material was left several days at room temperature, crystals separated out which, upon analysis, were found to be:[133]



It is possible that we observed a similar dimerization and that the inhibiting species is the mono-acid of the butyl ester dimer:



However, if this were the case, one would expect more inhibition from the hydrolysate of path 2 (see scheme III) where the allene is known to have been present in large quantity. Confirmation of the structures of the observed inhibitors awaits Fourier Transform NMR analysis.

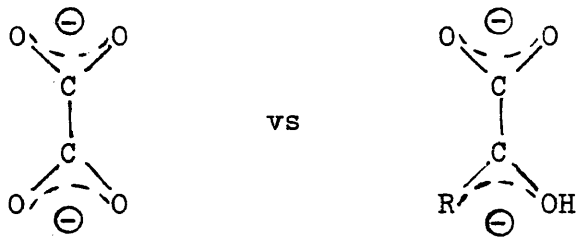
2. Oxalate Inhibition

Because 2-keto-3-butynoate was interesting as the ester form of a potential inhibitor of the reduced enzyme, 2-keto-3-butynoic acid, we wished to run a preliminary test on its inhibitory qualities before hydrolysis. However, it was still in solution with the starting material, diethyl oxalate. Therefore, a test was first run on diethyl oxalate to make sure any inhibitory qualities we saw were not due to this compound. Much to our surprise, we found strong inhibition by this species. A 10^4 molar excess of this inhibitor over enzyme reduced the observed enzyme activity to 15%. No time-dependence was observed.

Freisell et al.[57] tested a large number of compounds for DAAO inhibition and concluded that the essential features for inhibition were $C{=}C{-}X^{\ominus}$ where $X^{\ominus}$ was a carboxylate or phenolic hydroxyl

group and C=C could be part of an aliphatic, aromatic or heterocyclic structure. The saturated analogues of these species did not inhibit DAAO. The compounds they tested were competitive rather than irreversible inhibitors. Dianionic species such as fumarate and malonate exhibited no inhibition. However Dixon and Kleppe[56] observed inhibition by straight chain fatty acids. They also tested α-hydroxy and α-keto acids and found them to exhibit inhibition properties. From their work it was concluded that (1) the R group provides most of the affinity for the enzyme with a C_4 chain length having optimum binding ability and (2) the α-amino group is not essential for binding.

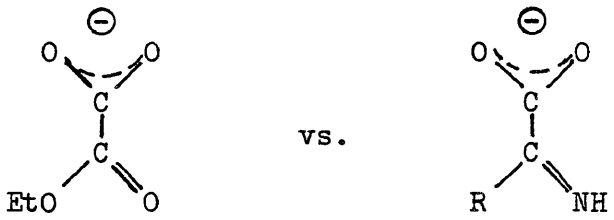
Oxalic acid has been found to be an inhibitor of lactate oxidase.[134-136] Oxalic acid is isoelectronic with the normal substrate anion for lactate oxidase:



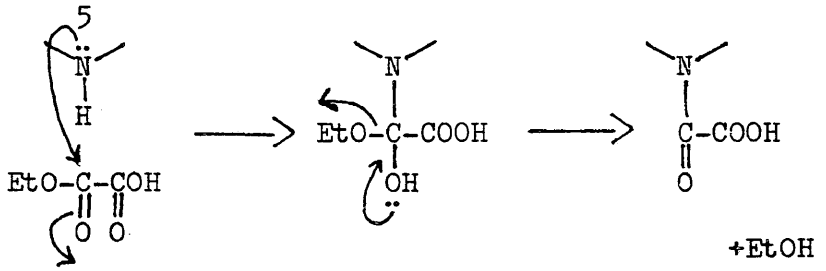
This similarity allows for initial binding of the pseudo-substrate which then inhibits the enzyme by decarboxylation and alkylation of the flavin N(5).

Diethyloxalate should not be an inhibitor for DAAO since it contains no acid group for binding at the active site. But esters have never been tested as possible inhibitors for DAAO since an anionic group (the carboxylic acid) has always been presumed to be necessary for initial substrate binding.

The monoacid, ethyloxate would be a much more likely inhibitor of DAAO. It is analogous to normal oxidized substrate:



and as such might form a very strong complex with the reduced enzyme. Some inhibitors (e.g., the propargylglycine derivative (8) of scheme I) have been shown to inhibit after reduction of the enzyme. $EtOCCOH$ would be most likely to react in this manner. The following acyl substitution can be envisioned to take place with reduced enzyme.



(28)

The presence of the leaving group, -OEt, provides a means of obtaining a stable adduct. To test this hypothesis the mono-acid would have to be specifically prepared and tested for inhibition. If irreversible inhibition were found, then the inhibited portion of the enzyme would have to be isolated and identified to see if it were indeed the proposed species.

IV. CONCLUSIONS AND PROPOSALS FOR FURTHER STUDY

This series of studies on the preparation of derivatives of α-substituted-3-butynoates and preliminary tests for inhibition by them have shown that these species are indeed inhibitors of DAAO and warrant further study.

Three new compounds have been made during the course of this study: ethyl 2-keto-3-butynoate, butyl 2-tosyl-3-butynoate and butyl 2-phthalimido-3-butynoate. Each of these species was prepared as an intermediate for further synthetic work and was never fully characterized in its own right. The structure of each species was consistent with IR and NMR analysis.

Ethyl 2-keto-3-butynoate appears to be a very stable species (no degeneration was observed during five months of storage). Mass spectrophotometry and elemental analysis should be done to confirm the assignment of this species. The acid analogue of 2-keto-3-butynoate has been reported to be a highly unstable species[93] which had to be generated *in situ* enzymatically for enzyme studies. This instability could be tested by hydrolysis of ethyl 2-keto-3-butynoate. If the acid does not decarboxylate, enzyme inhibition studies should be performed with this acid since it is an oxidized substrate analogue.

Butyl 2-tosyl-3-butynoate is an unstable species which rapidly rearranges into its allenic analogue, butyl 2-tosyl-2,3-butadienoate, and, more slowly, into a saturated compound. Conditions for

optimizing the production and lifetime of this compound need to be found to improve its use as an intermediate in a variety of substitution reactions.

Butyl 2-phthalimido-3-butynoate has been prepared, but the work-up procedure that was followed resulted in the loss of most of the product. Some possible improvements in the work-up procedure were suggested. These should be tried along with any other alterations in the procedure that might improve the yield.

The hydrazinolysis of this compound described in this thesis did not yield the expected 2-amino-3-butynoate, but the procedure up to this step is sufficiently promising, that some effort should be spent trying to find proper hydrazinolysis conditions. Some possible changes were discussed in Section III of this text.

2-Hydroxy-3-butynoate is the starting intermediate for all the synthetic work described in this thesis. This compound could only be prepared in a Grignard Reaction with a 30% yield. A possible alternative method was described. The yields reported in the literature for addition of lithium acetylide to aldehydes is sufficiently high (~90%)[105] to justify some effort to get this reaction to work with butyl glyoxalate.

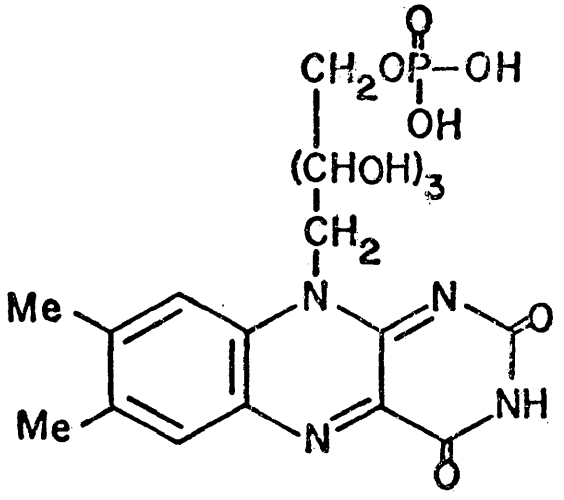
A mixture of 2-hydroxy-3-butynoate and 2-chloro-3-butynoate was separated, hydrolysed and tested for inhibition. A powerful inhibitor of DAAO was found which has been separated into three different fractions, each displaying unique inhibition properties. Possible identities of these species have been presented; definitive assignments of structures for them will require Fourier Transform NMR analysis because they are available in only minute quantities. It is hoped that

assignment of structure to inhibitor species will lead to a better understanding of the effects of an acetylenic group vs. an allenic group in DAAO inhibition. A method was described which led to preparation of butyl-2-amino-2,3-dienoate. This compound should again be prepared, and hydrolyzed and tested for inhibition to give additional information on inhibition by an allenic species.

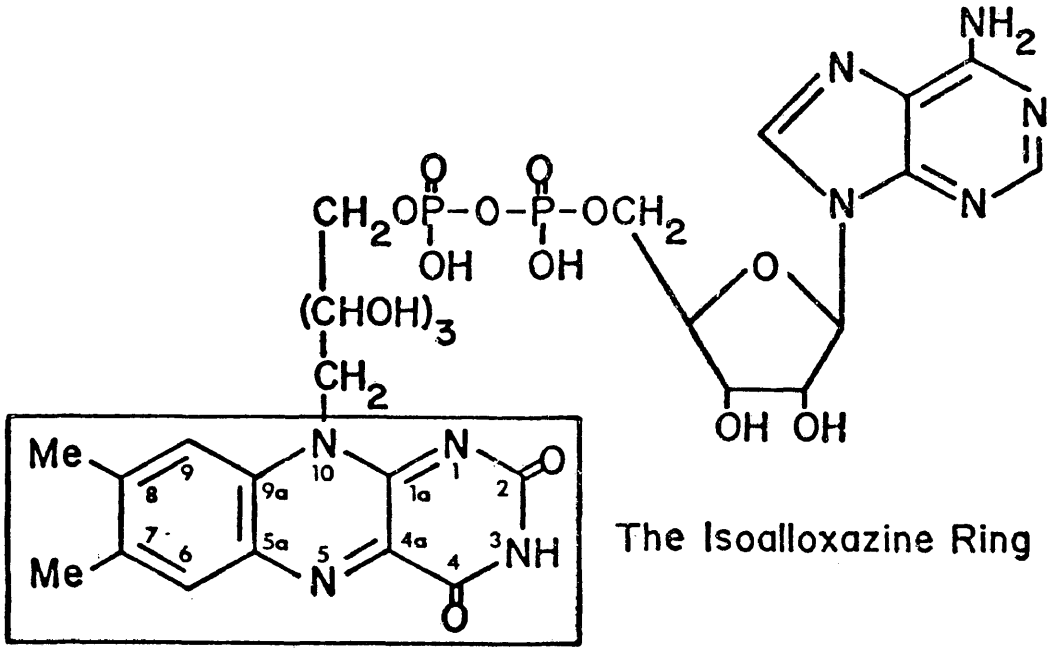
The inhibition studies described in this thesis are only preliminary. For those species displaying "non time-dependent" inhibition, competitive inhibition studies should be performed. All species should be tested at several lower concentrations to determine the amount of inhibitor necessary for inactivation and to determine the inhibition kinetics. Substrate protection should also be tested. The inhibited enzyme should then be tested for irreversibility of the inhibition--are there conditions under which the activity can be revived? The position of the irreversible bond between the enzyme and inhibitor should then be determined. This is usually done by reacting radioactive inhibitor with the enzyme, then isolating the radioactive fraction from degradation experiments. Identification of the nature of the enzyme--inhibitor bond can then be used to determine the mode of inactivation involved and thus a likely intermediate in the enzyme reaction. If this bond has been shown to be at the active site, then isolation of this portion of the enzyme can elucidate at least part of the enzyme active site.

Diethyl oxalate was also shown to be an inhibitor of DAAO at sufficiently large concentrations (85% inhibition at 10^4:1 I:E; no inhibition at 1:1 I:E). The inhibiting species was postulated to be

the mono-acid, ethyloxalate. This compound should be prepared to test for enzyme inhibition to confirm this theory. Since inhibition by this compound was "non time-dependent", competitive inhibition studies should also be performed. If irreversible inhibition is found, then characterization of the inhibited species should be undertaken as described above. Further work on these inhibitors and related compounds can be very fruitful. Characterization of the different inhibitor species and the types of inhibition they cause can lead to a greater understanding of intermediate steps in enzyme catalysis and the nature of the enzyme active site. In particular, it appears that significant differences between inhibition by acetylenic vs. allenic substrate analogues can be shown.



FMN



FAD

Fig. 1. The two biological forms of the flavin coenzyme. FMN = flavin mononucleotide. FAD = flavin adenine dinucleotide. The isoalloxazine ring portion of the coenzyme is at the enzyme active site.

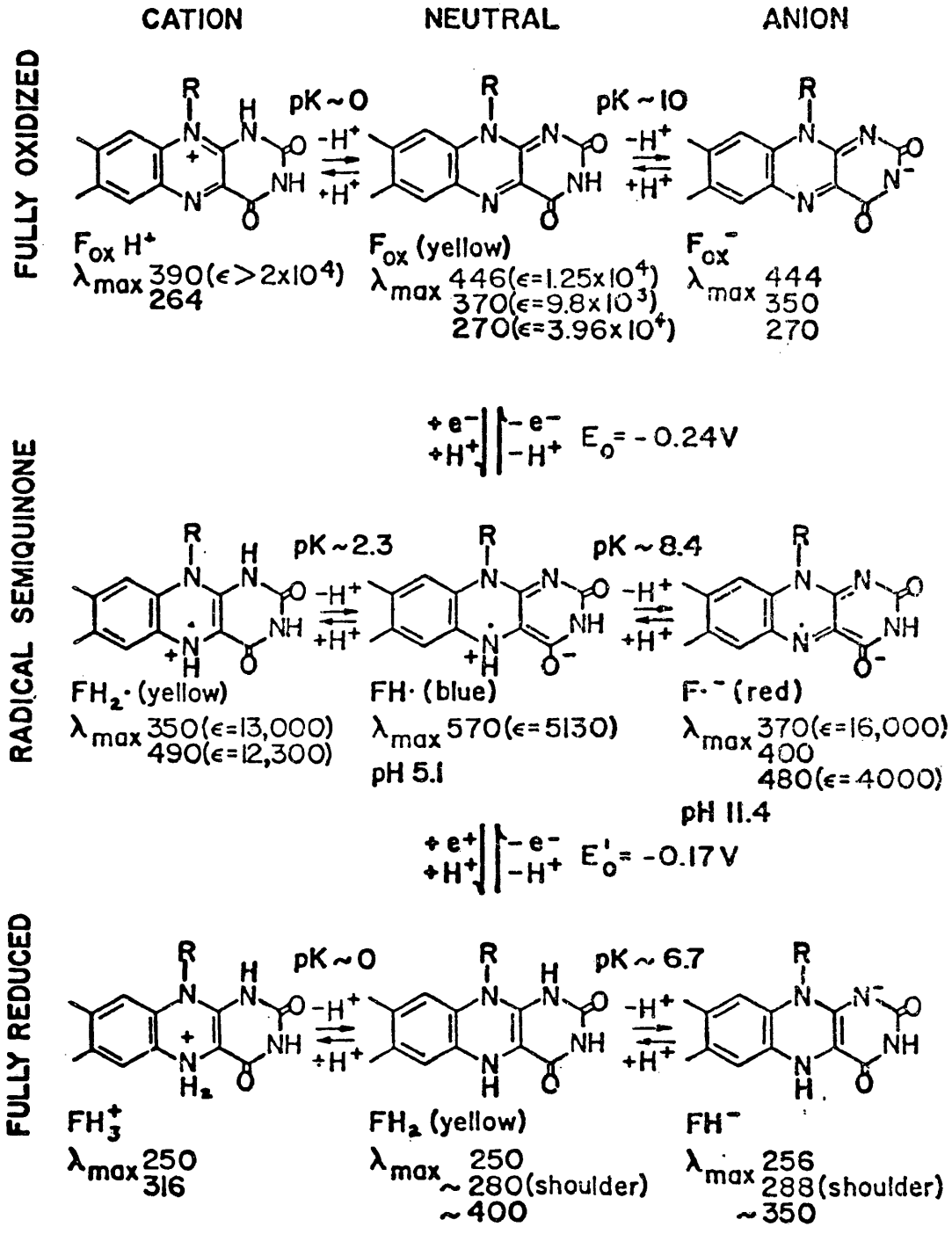


Fig. 2.[44] The oxidized, semiquinone and reduced states of flavin and their absorption maxima.

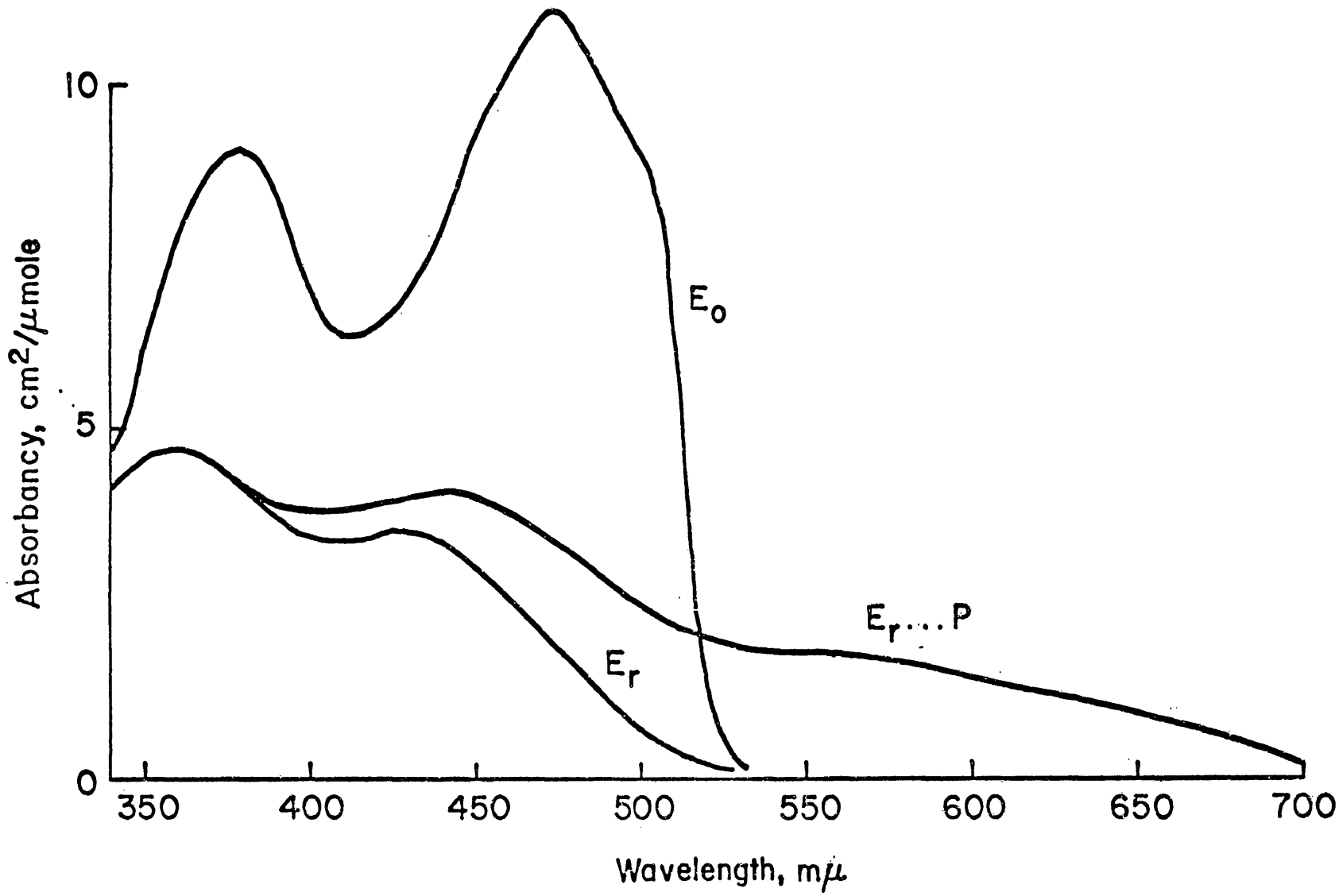
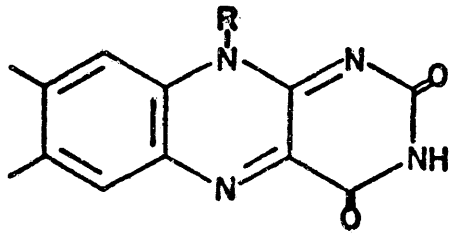
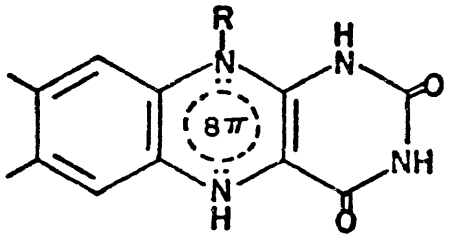


Fig. 3.[27] Changes in the DAAO spectrum during catalysis. E_0 = fully oxidized enzyme. $E_r...P$ = long wavelength intermediate complex between reduced enzyme and oxidized D-alanine as substrate.

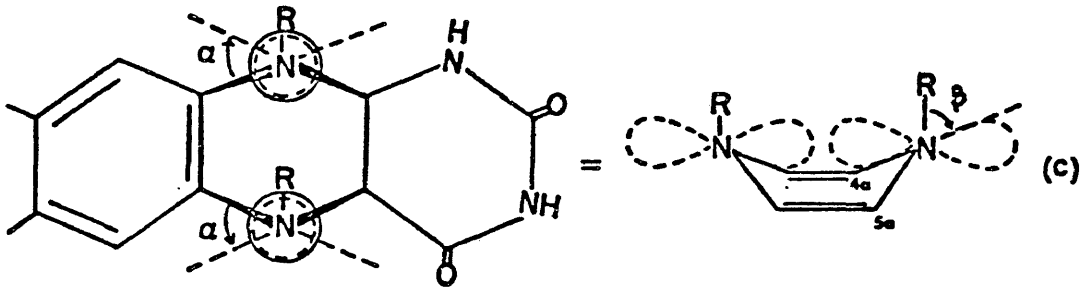


(a) Fl_{ox}



(b)

Fl_rH_2



(c)

Fig. 4. The geometry of oxidized and reduced flavin.

(a) Planar Oxidized Flavin.

(b) Planar Reduced Flavin, with an 8π antiaromatic central ring.

(c) Reduced Flavin in the normal butterfly configuration.

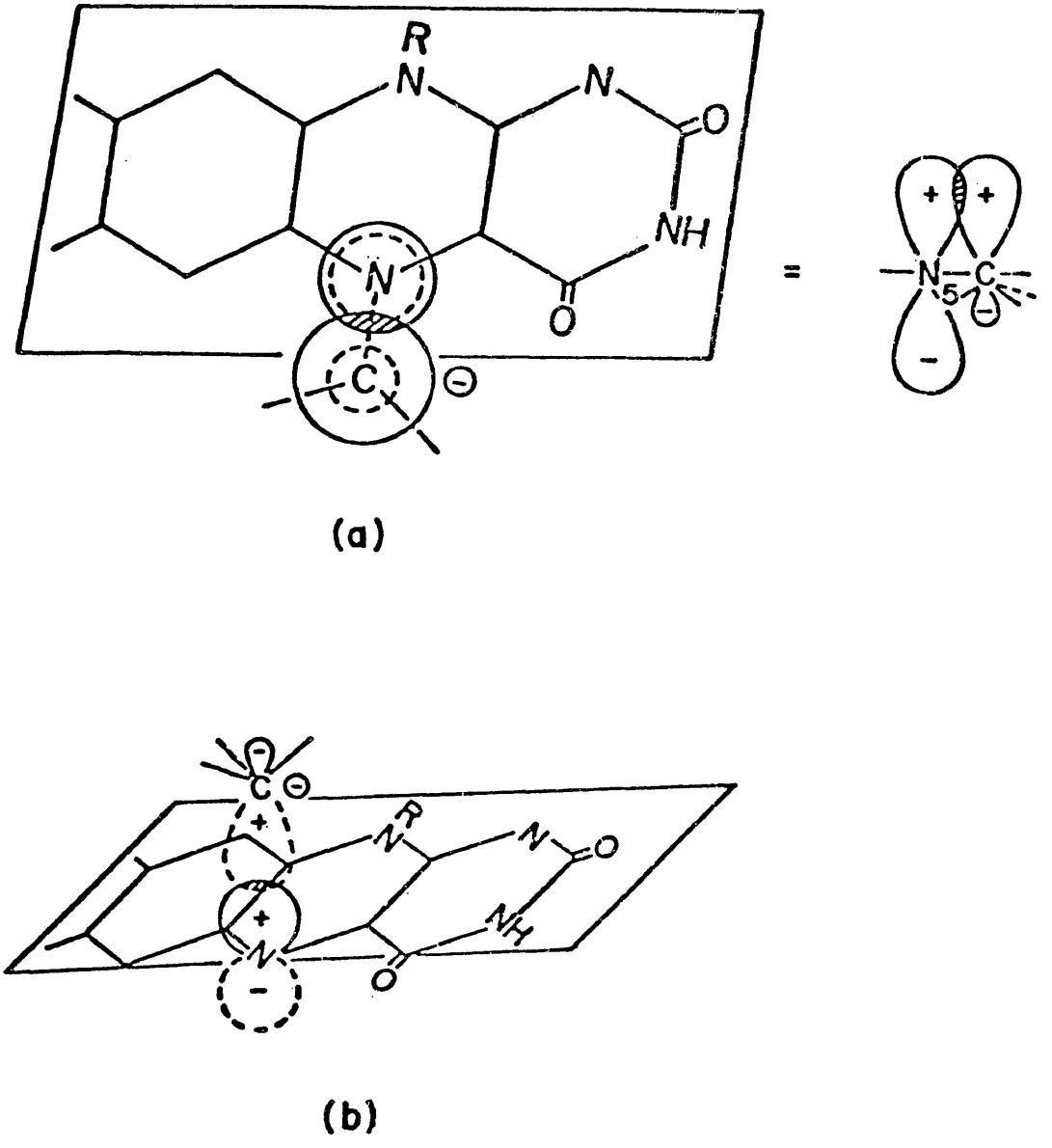


Fig. 5.[35] Orbital overlap for a carbanion-flavin adduct.
(a) in-plane overlap (2Pπ).
(b) out-of-plane overlap (2Pσ).

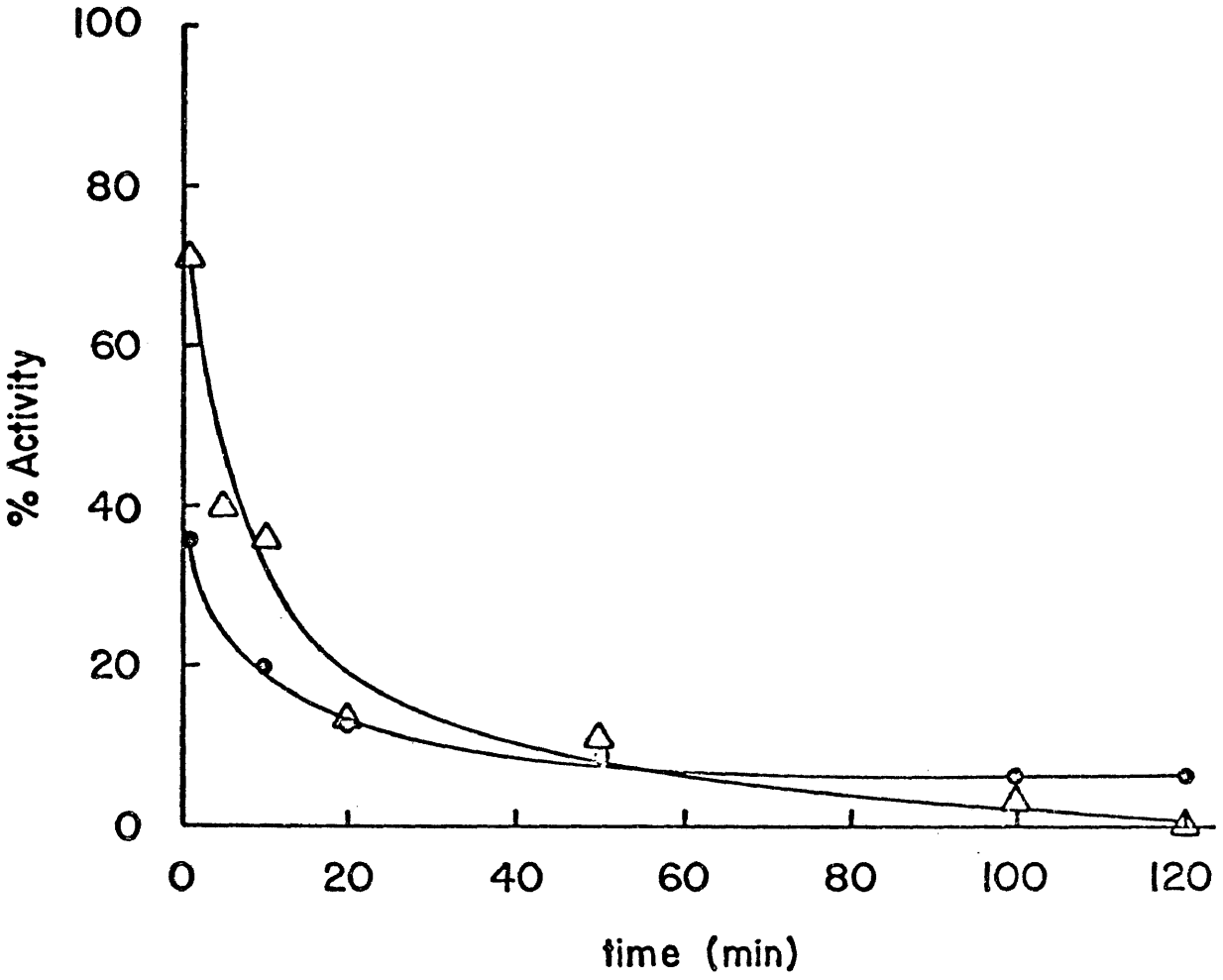
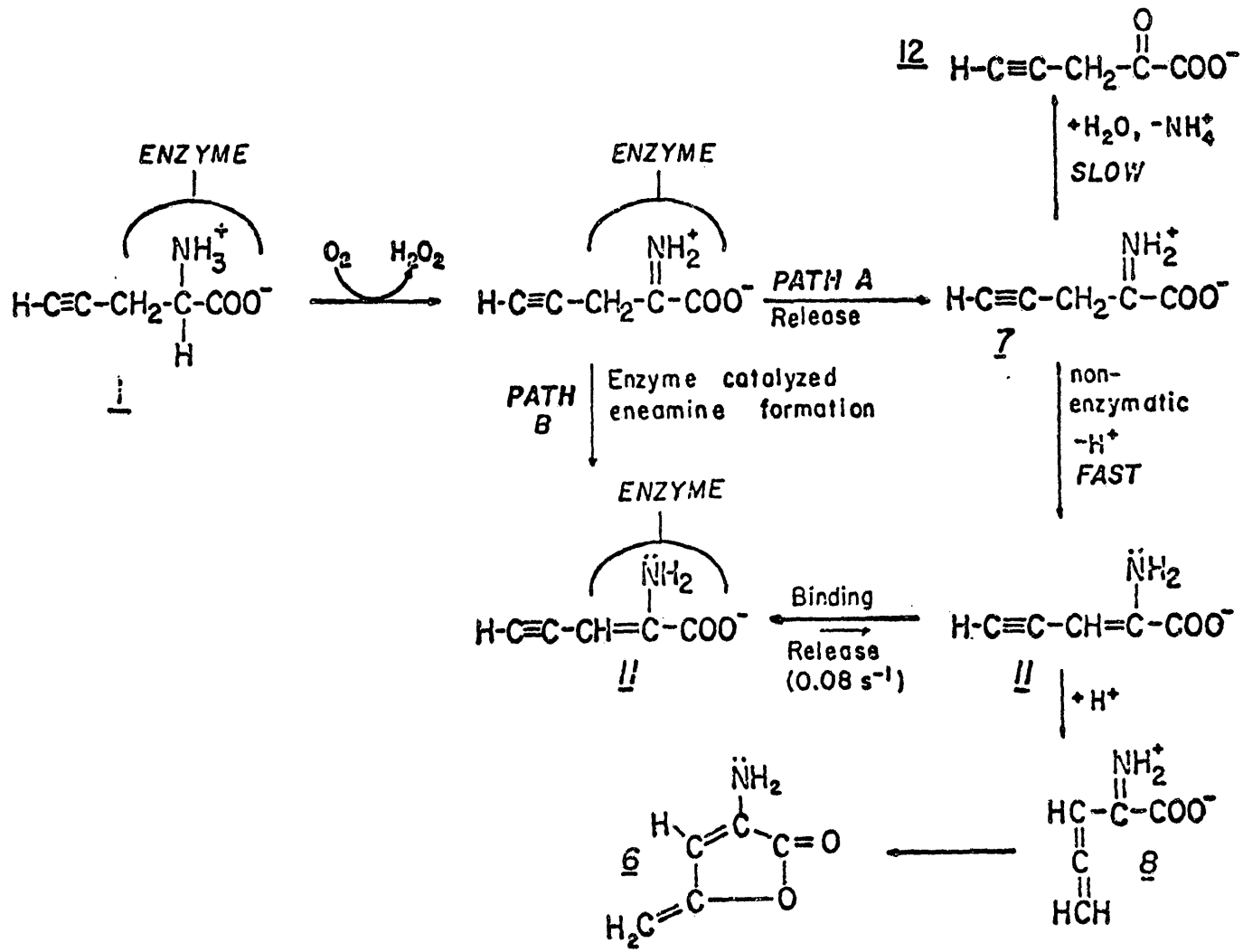
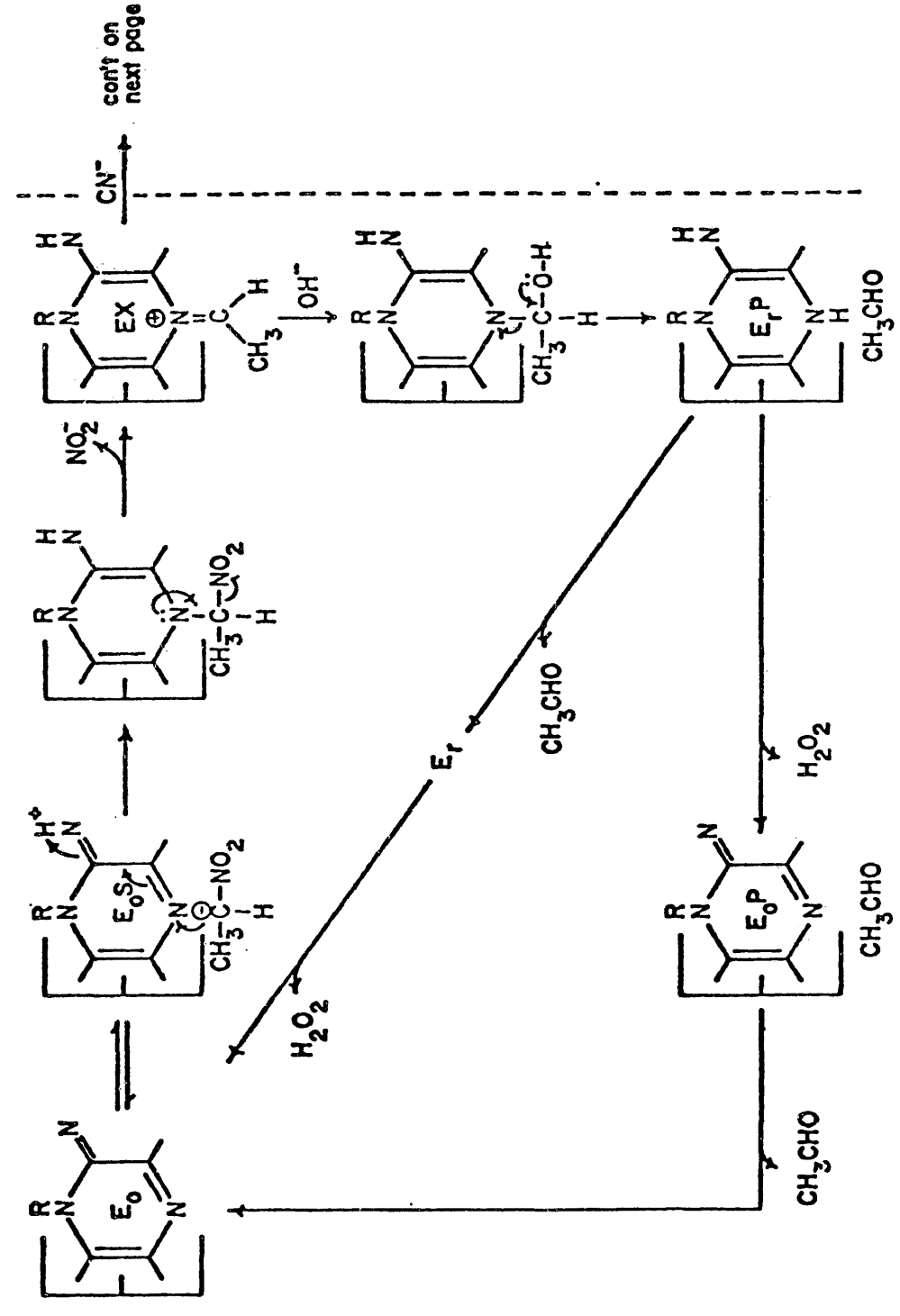


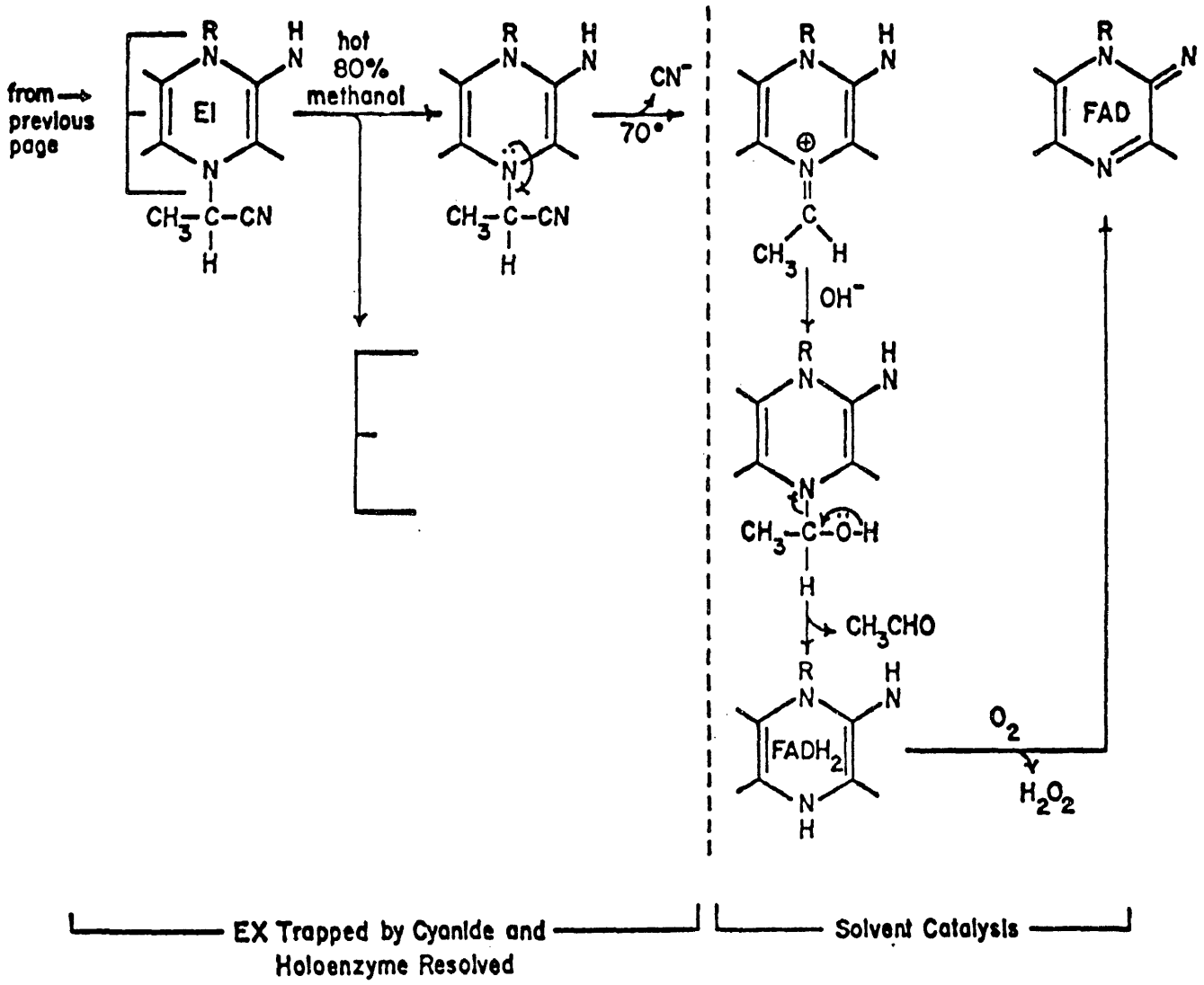
Fig. 6. Inhibition of DAAO by hydrolysate from Path I, Scheme III.
Δ = freshly prepared inhibitor. · = after 1 month's storage.



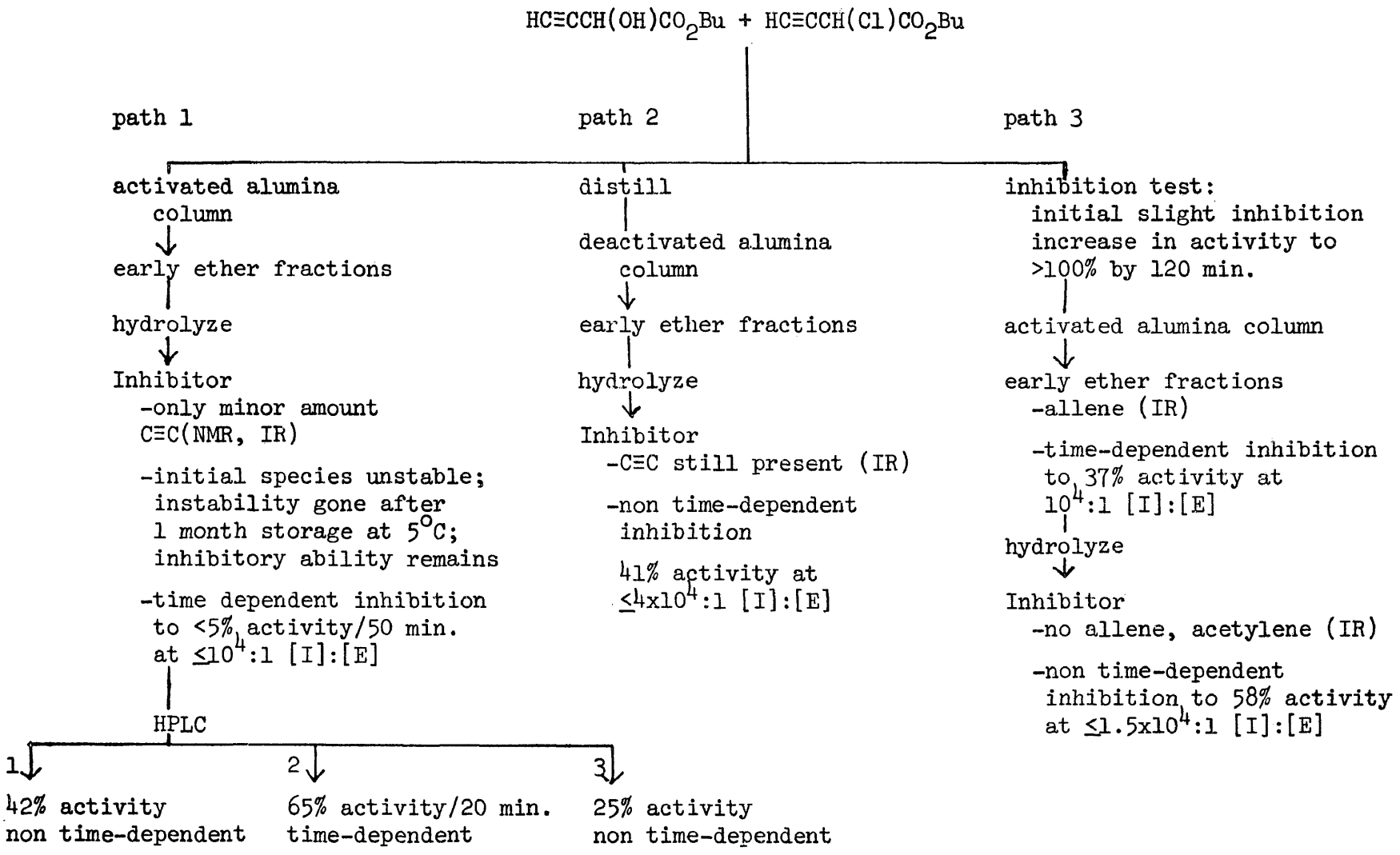
Scheme I.[61] The fate of propargylglycine during oxidation by DAAO. Compound 8 was found to be an irreversible inhibitor of DAAO. Compound 11 formed a tight charge-transfer complex with DAAO.



Scheme II, part 1. Mechanism proposed for flavin-catalyzed oxidation of nitro-ethane anion.[23]



Scheme II, part 2.



[I] = inhibitor concentration, [E] = enzyme concentration

Scheme III: Isolation of inhibitor species from a butyl 2-chloro-3-butynoate and butyl 2-hydroxy-3-butynoate mixture. See section III-F of text for discussion.

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