AFFINITY LABELING VIA DEAMINATION REACTIONS

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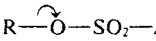
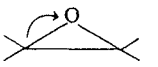
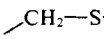
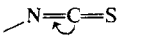
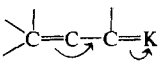
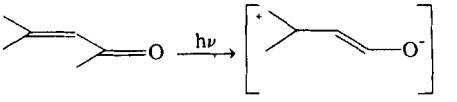
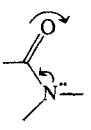
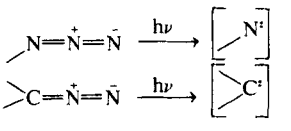
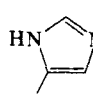
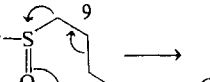
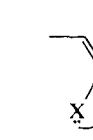
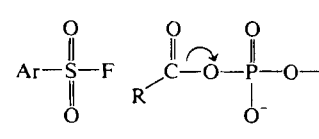
I. AFFINITY LABELING AND SUICIDE INACTIVATION

The specific covalent blocking of the active site of a protein which normally binds small molecules has many attractions as a biochemical tool. Information can be obtained about the location and function of the protein, and, in the special case of enzymes, degradation of the protein can indicate what functional groups are in, or at least near, the active site. Information can thereby be obtained about the catalytic mechanism. Very highly specific active-site-directed enzyme inactivators can have additional potential as therapeutic agents. The first requirement for such molecules is that they resemble the normal substrate of the target enzyme well enough to be bound noncovalently at the active site.[1,2] In fortunate cases, slight structural modification of a substrate will so slow down the decomposition of a covalent enzyme-substrate intermediate that the group covalently modified can be identified.[3,4] However, in the general case, once the requirement for active-site-binding is fulfilled, there are a number of ways in which the covalent attachment of the label (which is usually radioactive, so that the extent and site of attachment to the protein can readily be followed) can be brought about. Since proteins contain many nucleophilic sites capable of forming stable bonds with electrophiles, but no corresponding electrophilic sites (at least which can be manifested under conditions which proteins survive), bond formation usually involves reaction of a nucleophilic site on the protein with an electrophilic site on the reagent.

These electrophilic sites can be overtly reactive — the most common types of affinity labels are of this type (Table 1). The problem with reagents of this type — which have been termed *exo* affinity labels — is that their very reactivity creates difficulties. The N-bromoacetyl derivative of a substrate or coenzyme containing an amino group will still, in addition to any affinity labeling it does, react like bromoacetamide and to some extent selectively alkylate the more nucleophilic proteins (or more nucleophilic parts of the same protein).

The solution, in principle, to this problem of random labeling is to generate the electrophile in the active site directly. This can be done photochemically (see Table 1), but the technique of photoaffinity labeling often results in the labeling of several residues in the active site region,[5] a feature which can be turned to advantage and which can be naively rationalized from the high energy of the species formed photochemically (a photon of 350 nm corresponds to an energy of 82 kcal/mole) simply "bouncing around" in the active site. The technique cannot, however, be used in vivo. In addition to light, the catalytic action of the enzymes themselves can be used to generate electrophilic sites on the labels when bound to active sites.[6] The action of the enzyme required for successful labeling of the enzyme by such "k_{cat} inhibitors" or "suicide substrates", in addition to the provision of a suitable nucleophile, can be fairly minimal. Thus, in the case of the

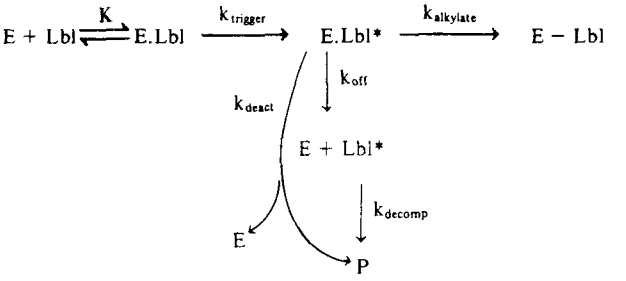
Table 1

Nucleophilic sites on proteins, protein nucleophiles	Electrophilic sites on affinity labels not arising from departure of nitrogen (Refs. 2 and 7, except where stated)
 (Cys SH)	 Activated aromatic halides and thio ethers
 (Ser, Thr)	
 (Tyr)	
 (Met)	
 (Lys)	 (Michael acceptor)
 (Glu, Asp)	
 (Glu, Asn, chain)	
 (His)	
 (O⁻ of Tyr or indole NH of Trp)	

cyclohexene epoxides studied as affinity labels for glucosidases,[2] proton donation by an active site group to the epoxide oxygen is apparently sufficient to augment the electrophilic properties of the epoxide sufficiently for it to open selectively in the active site. More often, however, the changes in molecular structure necessary to convert a precursor to an active electrophile are more deep-rooted than the simple formation of a hydrogen bond. The problem then becomes to stop the newly created electrophile from coming off the enzyme, accumulating in solution, and reacting indiscriminately. Formally, whereas for an *exo*-affinity label we have, where Lbl* is the electrophilic form of the label,

$$E + Lbl \overset{K}{\rightleftharpoons} E.Lbl \xrightarrow{k_{inact}} E - Lbl$$

but for a suicide substrate we have



Successful suicide substrates must have reasonably high ratios of $k_{alkylate}$ to k_{off}. Many successful suicide substrates have been designed for pyridoxal-dependent enzymes, the chemistry usually involving generation of Michael acceptors such as allenes or $\alpha\beta$ unsaturated imines via elimination and tautomerisation reactions. In these cases the pyridoxal moiety to which the alkylating agent is attached is tightly bound and ensures that k_{off} is small compared to $k_{alkylate}$. (The k_{deact} route, in which the alkylating agent reacts harmlessly with water while still in the active site, is not troublesome from the point of view of affinity labeling but does introduce complications into the kinetic analysis of the reaction. This is because measurements of how many moles of label are decomposed before a mole of active sites is inactivated necessarily give the ratio of $k_{alkylate}$ to $[k_{off} + k_{deact}]$).

Many enzymes, however, do not have cofactors which can be used as anchors to ensure that the newly created electrophile remains bound to the active site. With, for example, a simple hydrolase, K_i values for competitive inhibitors with structures resembling the substrate are commonly in the 10 μM to 10 m M range. If binding is diffusion-controlled (with a rate constant of 10^7-10^8 $M^{-1}s^{-1}$),[10] residence times are between 10 msec and 1 μsec. Therefore, if $k_{alkylate}$ is to be comparable with k_{off} for a label possessing only the same structural features as a competitive inhibitor, the triggered form of the label (Lbl*) must react with the protein at a rate of 10^2-10^6 s^{-1}. It must also not accumulate in free solution to any significant extent, otherwise one might as well deal with an *exo*-affinity label or even a non-specific agent.

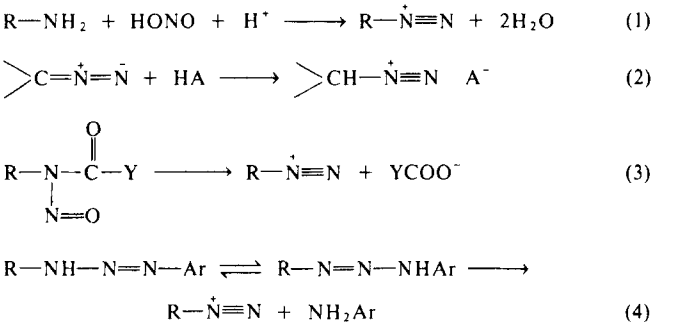
The functionality which stands the most chance of conforming to these criteria is the aliphatic diazonium ion; the leaving group is the highly stable molecular nitrogen molecule, a leaving group so good that, in some circumstances, its departure is faster than the diffusion apart from the components of an encounter-complex (*vide infra*). Moreover, it can be generated from nonelectrophilic (if unstable) precursors by either nucleophilic or acidic catalysis. In this review the methods of generation of aliphatic diazonium ions will first be described, followed by an account of the affinity labeling of pure enzymes by deamination precursors. Lastly, the in vivo activity of some such compounds will be described.

II. DEAMINATION REACTIONS

A traditional way of replacing $-NH_2$ by -OH in an aliphatic molecule is treatment with nitrous acid.[11] The method is preparatively unsatisfactory and attempts to rectify this have led to the discovery of a number of related reactions which, unlike the original reaction, conceivably can be used in biochemical systems.[12] These deamination reactions all involve the departure of molecular nitrogen from a saturated carbon center. The high proportion of products arising from carbonium-ion type rearrangements indicate that these reactions are qualitatively different from those involving the departure of more

nucleophilic leaving groups (such as *p*-toluenesulfonate anion). It was first thought that the carbonium ions formed in these reactions were not in vibrational equilibrium with their environment — hence, the adjective "hot". While this now seems unlikely,[12] it is useful to maintain the distinction between cationic species formed by departure of nitrogen (and some other molecules) and those formed directly from a stable precursor.

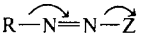
There are four currently known deamination routes: (1) the action of nitrous acid on amines; (2) the protonation of diazo compounds; (3) the decomposition of nitrosamides, nitroso ureas, and nitrosocarbamates; and (4) the decomposition of monoalkyl aryl triazenes.



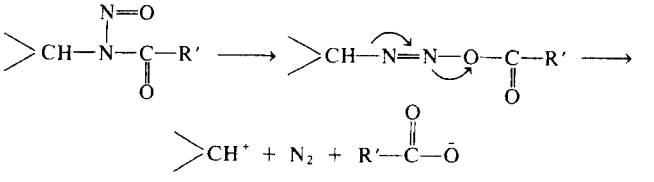
The chemistry of deamination reactions was reviewed in 1976 by Kirmse[13] and is thoroughly covered in the yearly reports on Organic Reaction Mechanisms from the point of view of the fate of the carbocationic species generated.

A. Carbonium Ions or Diazonium Ions?

One type of product from R, however, is relevant to the use of deamination precursors as affinity labels, since it bears on the question of whether alkane diazonium ions have a discrete existence or whether deaminations can be represented as synchronous fragmentations in general.

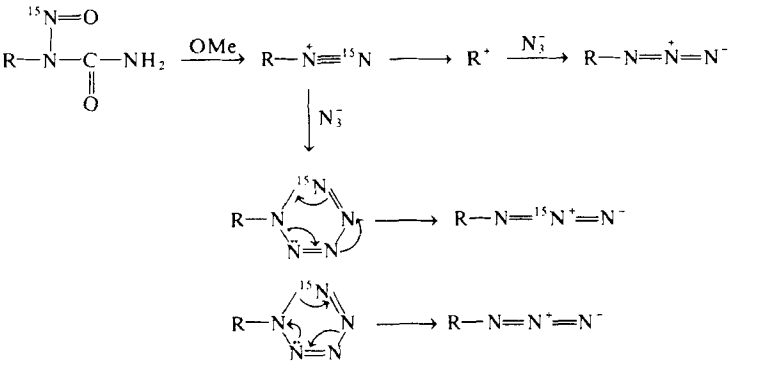


Decomposition of secondary alkyl aryl triazenes in glacial acetic acid gives substantial quantities of alkylated anilines, whereas primary alkyl aryl triazenes give only small amounts of such products.[14] It was concluded that, in the case of the secondary alkyl aryl triazenes, the derived secondary carbonium ion is sufficiently stable that the R-N bond breaks at the same time as the N-NH_2Ar bond, the "internal return" products R-NH-Ar arising from attack of the aniline on the carbocation within the same encounter complex in which it was formed. In the case of primary alkyl aryltriazenes, the lower stability of a primary carbocation ensures that the alkane diazonium ion has a real existence and can diffuse away from the aniline. Similar work with N-nitrosoamides in anhydrous acetic and butyric acids showed that attempts to generate secondary alkane diazonium ions only result in synchronous fragmentation of the neutral precursor, the diazonium carboxylate.[15] Acetolysis of N-nitrosobutyramides



gives butyrates; butyrolysis of N-nitrosoacetamides gives acetates. It is clear then that in acetic (and butyric) acids where the derived carbocation is of a stability comparable to, or greater than, a simple secondary alkyl cation, the diazonium ion does not exist and reaction can be regarded as proceeding via the carbocation.

In methanol, trapping experiments paint a similar picture. Decomposition of cyclopropyl[16] and 1-norbornyl[17] N-nitroso ureas with methanolic sodium methoxide gives diazonium ions long enough lived to be trapped by azide ion as a pentazole, pentazole formation being detected by isotopic labeling as shown:



Experiments with R=1-butyl failed to demonstrate any trapping. However, the 1-butanediazonium ion must be just on the borderline of a real existence, since it can be trapped by base, decomposition of 1-diazobutane in $NaOCH_3/CH_3OD$ giving largely $CH_3CH_2CH_2CD_2OCH_3$.[18]

(Decomposition of optically active N-nitroso-N-(1-phenylethyl)-2-naphthamide in dioxan at 25° C, which is considered to give the E stereoisomer of the covalent diazonium carboxylate, gives the same excess of retained 1-phenyl-ethyl-2-naphthoate as reaction of Z-1-phenylethyldiazotate ion with 2-*p*-naphthoyl chloride, which is considered to give the Z stereoisomer of the covalent diazonium carboxylate.[19] The interpretation of these results in terms of a general scheme involving ion pairs[20] seems unnecessarily complex, since performing the reaction such that nitrous oxide, rather than nitrogen, is interposed between electrophilic center and leaving group results in the same stereochemical result.[19])

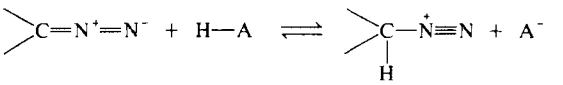
In water, the situation seems completely different. Alkane diazonium ions have a stability which is surprising at first sight. CH_3-$N^+{\equiv}N$ can be observed directly using stopped-flow techniques, and has a lifetime of 0.3 sec in water at 25° C.[21] Likewise, studies on the rates of H_3O^+-catalysed hydrolyses of various alkyl phenyl triazenes, R-N=N-NH-Ph, of widely varying structure indicated that the rate of decomposition is determined solely by the pK_a of RNH_3^+, even when R was *p*-methoxybenzyl.[22] It therefore appears that in order to get synchronous fragmentation in water, R^+ must be a very stable cation indeed.

This greater stability of alkane diazonium ions in water than in organic solvents is readily rationalized as an effect of the higher polarity of water. In the transition state for synchronous fragmentation, the incipient positive charge is delocalized over at least three atoms — both nitrogens and the carbocationic center. In the first transition state for stepwise fragmentation, it is delocalized only over the two nitrogens, and on going from the alkane diazonium ion to the transition state for the second step of stepwise fragmentation, charge is dispersed. Polar solvents will therefore hinder this step. In an enzyme active site, therefore, it will be very difficult to say whether any successful affinity

labeling arises from a carbonium ion or from a diazonium ion. In at least one case, however, the above ideas about the importance of solvent polarity allow sense to be made of very puzzling stereochemical observations.

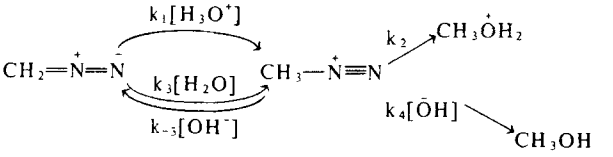
B. Protonation of Aliphatic Diazo Compounds

The simplest way of generating an alkane diazonium ion is protonation of a diazoalkane



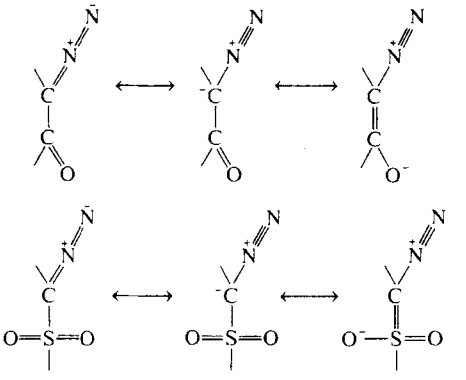
Depending on the structure of the diazo compound, protonation may be reversible or irreversible. In aqueous solution simple diazoalkanes protonate very rapidly; in the work mentioned above[21] the lifetime of the methane diazonium cation was measurable only because under conditions of excess diazomethane and 10 to 100 μM H_3O^+, protonation was so rapid that decomposition of the diazonium ion was rate-limiting.

A very careful study by stopped-flow techniques[23] of the pH-dependence of the decomposition of diazomethane in 60/40 v/v aqueous tetrahydrofuran has indicated that for the scheme



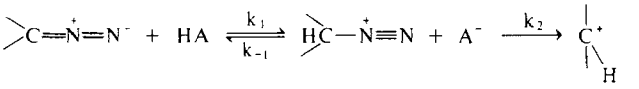
at 25° C, $k_2 = 2\,s^{-1}$, $k_4 = 10^4 M^{-1}s^{-1}$, $k_1 = 2 \times 10^8 M^{-1}s^{-1}$ and that the pKa of the methane diazonium ion is around 10 in this medium. Isotope exchange experiments indicate that protonation is irreversible at pH 3, partly reversible between pH 6 and 10, and reversible at pH 14. Conventional UV kinetic experiments gave $k_3k_2/k_{-3} = 3 \times 10^6 M^{-1}s^{-1}$. In this solvent which is less polar than water, reactions resulting in anihilation of charge are favoured — in water itself, measurements by conventional UV spectroscopy show that $k_4/k_2 = 0.6\ M^{-1}$. One would therefore expect that reversibility of the protonation would set in at higher pH than when an organic cosolvent was present.

Introduction of substituents conjugated with the diazo function produces an increase in stability to acid, and diazoketones, diazoesters, and diazosulfones are all tractable enough to study by conventional methods.



The protonation of primary diazoesters (such as ethyl diazoacetate),[24] primary diazoketones,[25] and primary diazosulphones[26] is reversible, whereas that of secondary

diazoketones and diazoesters is irreversible.[27,28] Diphenyldiazomethane is irreversibly protonated.[29] If the kinetic scheme



is analyzed more closely, it is seen that the overall effects of diazocompound structure on the value of k_1/k_{-1}, which determines whether the protonation is reversible or not, will be difficult to predict since electron-withdrawing substituents increase to k_{-1}, but also increase k_2. What the scheme does predict is that as the buffer concentration is increased an apparently irreversible protonation (that of 3-diazobutanone and ethyl 2-diazopropionate) becomes partly reversible because of the increase in concentration of A^-; this has been observed.[30]

The decomposition of diazo compounds whose protonation is apparently irreversible has been studied extensively with a view to characterising proton transfer. Brønsted coefficients for the general-acid-catalyzed decomposition of 3-diazobutanone and ethyl diazopropionate are around 0.6.[31] Water can act as an acid, and there is evidence from isotope effects that the ^-OH so formed reacts with the diazonium ion before diffusing away.[32] An analogous process in the decomposition of 1-diazobutane in methanol[18] could explain the apparent trapping of the diazonium ion by methoxide but not by azide.

Brønsted plots for acid-catalyzed decomposition of diphenyldiazomethane are, however, curved, and the curvature has been interpreted in terms of the Marcus theory of proton-transfer, which treats solvent reorganization and chemical steps separately.[29]

C. Nitrosoamide Decompositions

The decomposition of N-nitroso-N-acyl alkylamines to diazonium ions can occur by three routes — a "no mechanism" reaction, an acid-catalyzed reaction, and a base-catalyzed acyl group transfer. Additionally, N-alkyl-N-nitrosoureas may decompose by a base-catalyzed elimination reaction.

N-Nitroso-N-acyl-arylamines[33] and -alkylamines[34,35] decompose in inert solvents to diazonium carboxylates and then to other products. The rearrangement step is rate-determining[36] and has the classic characteristics of an apolar, pericyclic reaction, insensitivity to electronic effects[33] and solvent polarity.[34] It is, however, strongly accelerated by steric crowding. If the rearrangement is written simply as an electrocyclic reaction, only four electrons are involved. The transition state is thus aromatic — and the reaction favored — only with Möbius geometry[37] in which the N=O and N—CO bonds are perpendicular. Such a geometry involves considerable strain and will be favored if the nitrosoamide is distorted from its preferred planar ground-state conformation by branching in R′. Conceivably also, binding of an N-nitroso-amide to a protein could have the same effect and, thus, a way could be found to affinity label binding proteins of no catalytic function.

In aqueous solution nitrosoamides decompose by pathways other than the simple electrocyclic reaction described above. They are rather unstable compounds, this instability being least around pH 5, in the case of simple aliphatic compounds. At more acid pH values, a representative example — *N-n*-butyl-*N*-nitrosoacetamide — undergoes both denitrosation and deamination, but the solvent deuterium isotope effects on the two processes is different ($k_{D_2O} > k_{H_2O}$ for deamination and $k_{H_2O} > k_{D_2O}$ for denitrozation).[38] The mechanisms of Figure 1 were proposed, the slow proton transfer to nitrogen in the denitrosation pathway being held to arise from the low basicity of the amide nitrogen. In less acid solution, as expected, denitrozation (of N-nitrosopyrrolidone) is subject to general acid catalysis.[39] Deamination was also shown to be subject to acid

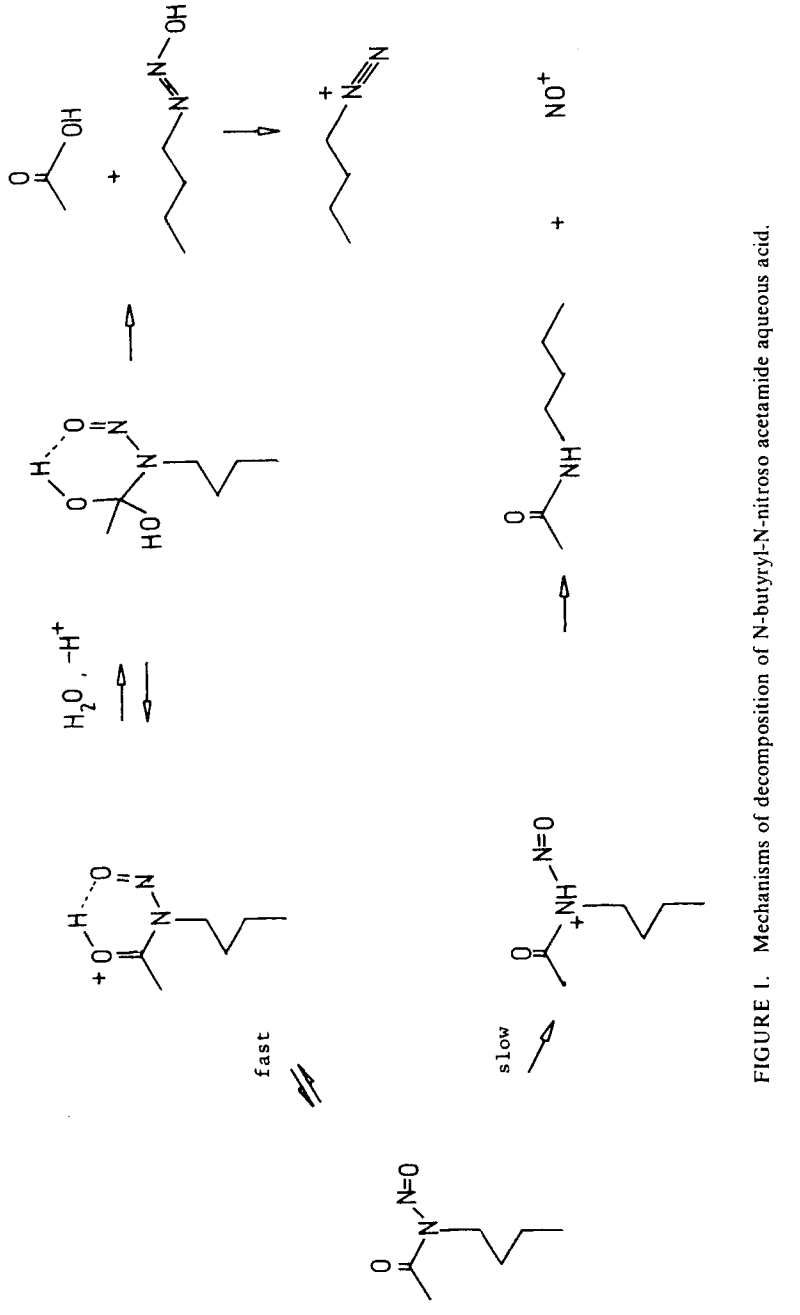
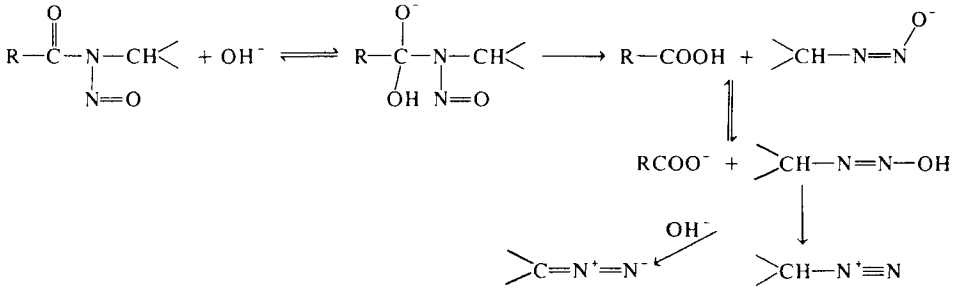


FIGURE 1. Mechanisms of decomposition of N-butyryl-N-nitroso acetamide aqueous acid.

catalysis, but by both acid and basic components of the buffer. Similar results have been obtained for N-alkyl nitrosoureas.[40]

It is clear from these results that attempts to use N-nitroso amides as suicide substrates for enzymes for which acid catalysis is of great importance could lead instead to nonspecific inactivation via the denitrozation pathway.

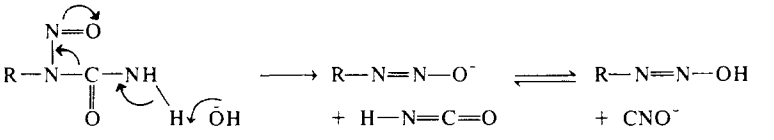
The base-catalyzed decomposition of nitrosoamides is a simple acyl transfer-reaction;[34] N-nitrosocarbamates (R = O alkyl) react similarly.[41] The alkane diazotate first formed can protonate, and then water can be lost to give a diazocompound or hydroxide ion to give a diazonium ion (which may itself deprotonate).



The base-catalyzed deamination of N-nitroso-2-pyrrolidone in water has been subjected to detailed kinetic analysis.[42] Buffer catalysis was observed, but the ineffectiveness of the sterically hindered 2,6-lutidine makes it likely that this is due to nucleophilic catalysis. Further, a second order term in imidazole is observed when this base is used as a buffer.[42]

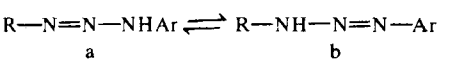
This nucleophilically-catalyzed pathway for nitrosoamide decomposition has been used in affinity labeling work (see Section III.F.).

A fourth pathway for nitrosoamide decomposition came to light when it was discovered that, although N-alkyl-N-nitrosoureas were labile to base, blocking the second nitrogen with methyl groups produced a compound which was practically inert.[43] Cyanate ion was the product of reaction with unblocked N-nitrosoureas.[44] This base catalyzed pathway is clearly only possible with nitrosoureas, and has been disputed even there.[40]



D. Decomposition of Monoalkyl Aryl Triazenes

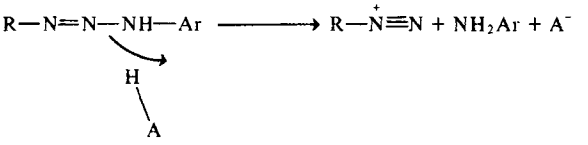
Deamination via monoalkyl aryl triazenes was introduced by White and Scherrer,[45] the triazenes being readily made by reaction of alkylamine with arene diazonium ion. The chemistry of alkyl aryl triazenes is complex,[46] the first complexity being the tautomeric equilibrium between the two forms a and b:



Generally, the equilibrium is in favor of tautomer b.[47-49] Its position is substituent-dependent[50] but substituent effects seem small — certainly nowhere near as large as the effects on rates of decomposition. The tautomerisation is fast, even in aprotic solvents, which has led to the suggestion that radical pathways are available.[51] Additionally, in

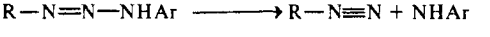
principle *cis-trans*-isomerism is possible about the double bond of both tautomers, although this has not been observed when it has been looked for.[48]

Under unambiguously heterolytic conditions, only products derived from ArNH-N and R-N cleavage are formed; there is no evidence that cleavage to arene diazonium ions and alkylamines, the reverse of the normal method of preparation, takes place in homogeneous solution in polar solvents to any significant extent.[14,15,22,46] The normal pathway for decomposition in protic media is protonation of the arene-bound nitrogen synchronous with cleavage of the N-N bond (and sometimes also the C-N bond (see Section II.A.).



As anticipated from this mechanism, in aqueous solution triazene decomposition is subject to buffer catalysis by the acid component of the buffer; this is associated with a rate-determining proton transfer as confirmed by a solvent isotope effect of $k_{H_2O}/k_{D_2O} = 1.7$ for the decomposition of 1-propyl *p*-nitrophenyl triazene.[52] The Brønsted coefficients for the general acid catalysed decomposition of a series of aryl propyl triazenes by oxygen acids show some sign of varying slightly with the aryl substituent (from 0.70 ± 0.02 for *p*-methoxyphenyl to 0.61 ± 0.04 for *p*-nitrophenyl) and, as one would expect if this were real, the β_{lg} values do vary with the strength of the catalysing acid (from 0.61 ± 0.05 for H_3O^+ to 0.44 ± 0.05 for $H_2PO_4^-$). Nitrogen acids do not seem to be effective catalysts. Decreasing the stability of the diazonium ion (by decreasing the pK_a of RNH_3^+) makes buffer catalysis less easy to observe.[52] These data can be rationalized in the now conventional way by considering the two-dimensional free energy profile of Figure 2. The substituent effects are characteristic of a transition state in the position shown with the reaction coordinate approximately in a "NNE-SSW" direction.[53]

However, with electron-withdrawing substituents in the arene moiety, a pH-independent triazene decomposition becomes apparent,[22] which is characterized by the absence of a solvent isotope effect and a *negative* value of β_{lg}. We proposed[22] (somewhat reluctantly, since anilines are far weaker acids than water) that the process being observed was a simple unimolecular heterolysis without proton-donation from solvent.



Some support for this proposal has since been obtained from the fact that the rates of pH-independent hydrolysis of *p*-nitro, *p*-cyano, 3,5-dichloro, and 3,4-dichlorophenyl-propyl triazenes correlate better with the pKa of $ArNH_2$ ($\beta_{lg} = -0.30$, $r = -0.99$) than with the pKa of $ArNH_3^+$ ($\beta_{lg} = -0.9$, $r = 0.97$).[54]

E. Related Reactions

There are a number of other reactions in which an electrophilic center can be generated by departure of a non-nucleophilic leaving group. Most promising from the point of view of potential affinity labels is deamination via nitroamides and nitrocarbamates.[55,56] The chemistry closely parallels that of nitrosamides but nitrous oxide, rather than nitrogen, is the inert leaving group. Only the no-mechanism pathway, in practice, leads readily to a deamination reaction since N-nitroamines are stable compounds and decompose to nitrous oxide only under vigorously acidic conditions.

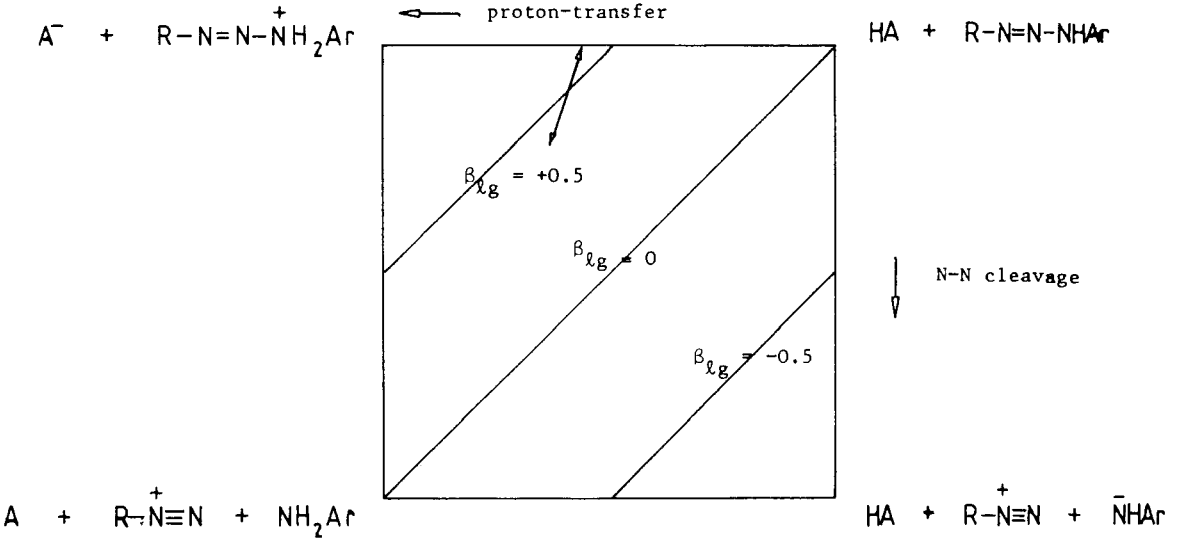
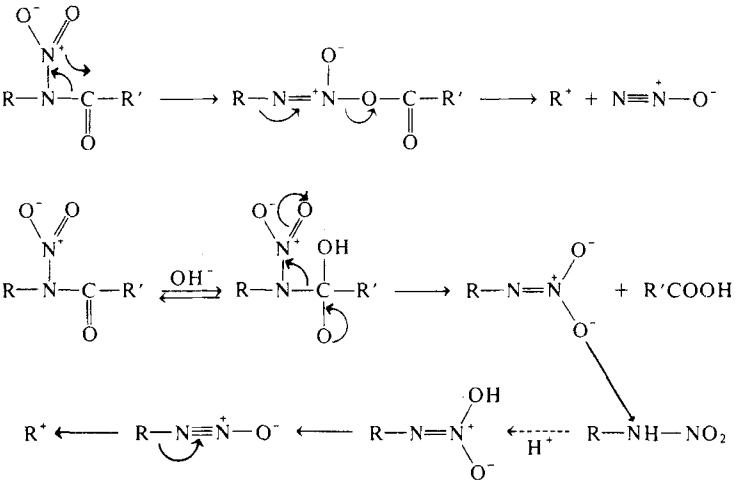
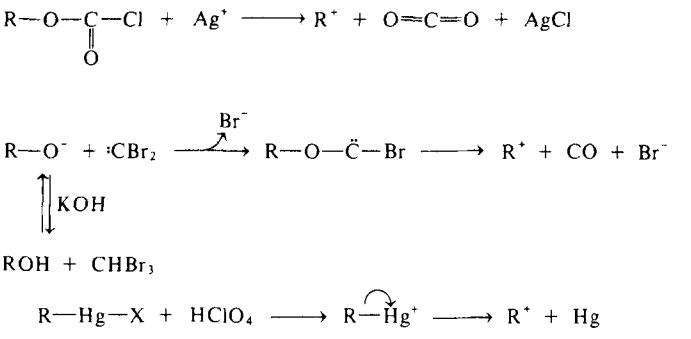


FIGURE 2. Jencks-More O'Ferrall diagram describing the position of the transition state in the general acid-catalyzed decomposition of alkyl aryl triazenes.



The chief advantage of this deamination route is then the stability of nitroamides; its disadvantage is that this stability is also manifest in the nitroamine.

Carbon dioxide,[57] carbon monoxide,[58] and atomic mercury[59] can also be used as non-nucleophilic leaving groups as shown, although without extensive modification of the precursors these routes have little prospect of being used in biological chemistry.



III. AFFINITY LABELING OF PURIFIED ENZYMES WITH DEAMINATION PRECURSORS

Inactivation and modification of enzymes is easy to bring about. Confidence that any observed enzyme inactivation and/or labeling has come about because of a modification of the active site should only come from conformity of the reaction to the following criteria:

1. Accurately exponential decay of the enzymic activity of (noncooperative) enzymes to zero in the presence of a large, constant excess of active site reagent, i.e.,

$$(\text{activity}) = (\text{starting activity})\, e^{-kt}$$

2. The first-order rate constants for loss of enzymic activity should show a saturation dependence on the concentration of the labeling agent: for noncooperative enzymes the following relationship should hold:

$$k = \frac{k_{max}[Lbl]}{K + [Lbl]}$$

If indeed the labeling takes place at the active site, once all the active sites are occupied by noncovalently-bound label, the enzyme cannot be inactivated any faster. With a really good affinity label, however, k_{max} can be so high that measurable values of k are obtained only at concentrations of label will below K (the dissociation constant of the E.Lbl complex), so that it is possible only to get a second-order rate constant (k_{max}/K) for the inactivation.

3. Quantitative protection by a reversible inhibitor of catalysis must be observed. This is the most important kinetic criterion for true affinity labeling. If, for a Michaelian enzyme, K_i is the dissociation constant of the complex between a competitive inhibitor I and enzyme (measured by inhibition of catalysis), then the following relationship must hold:

$$K = \frac{k_{max}[Lbl]}{[Lbl] + K\left(1 + \frac{[I]}{K_i}\right)}$$

4. The stoichiometry of the inactivation must be such that 1 mole of label is incorporated into the protein for every mole of active sites destroyed. Ratios of greater than 1 mole of inhibitor incorporated per mole of active sites destroyed are a sure sign that the label is not completely active-site directed. Stoichiometries of less than unity are less worrying, and are usually a sign of partially denatured enzyme. However, in the case of enzymes exhibiting "half-of-the-sites" reactivity, it is reasonable that ratios of 0.5 should be observed since, with a true affinity label, one would not expect other sites in the enzyme molecule to distinguish between a site temporarily occupied by substrate and a site permanently occupied by a close structural analog. There is an additional criterion for suicide inactivation — the obvious one that $k_{trigger}$ must be greater than the rate of spontaneous decomposition of the label.

It is unfortunately rare to find affinity labeling studies in which the inactivation has been this rigorously examined. Criteria (3) and (4) are the most important, and work in which either of these has been met in at least a representative case will be discussed. Such work using deamination precursors falls into five broad classes: (1) labeling of enzymes which at some point in their action cleave R—CO—X bonds (to RCOOH and HX) with substrate-derived diazoketones R—CO—CH=$\overset{+}{N}$=$\bar{N}$ and related compounds; (2) labeling of pepsin-type proteases with N-diazoacetyl amino acid esters. There is reason for doubting that a deamination reaction occurs in the active site, since the reagent is commonly incubated with Cu^{++} before addition to enzyme (*vide infra*); (3) use of a simple unconjugated diazocompound to affinity label a glycosidase; (4) use of a aryl carbohydrate triazenes to affinity label glycosidases; and (5) use of N-nitroso amino acid amide derivatives to affinity label chymotrypsin.

A. Substrate-Derived Diazoketones and Related Compounds

The field of "deamination-biochemistry" can be dated from the isolation, from culture filtrates of a *Streptomyces* species found in Peruvian soil, of two compounds detected by their tumor inhibitory properties. They were characterised, in what was at the time a *tour de force* of natural products chemistry, as O-diazoacetyl-L-serine[60] (azaserine) and

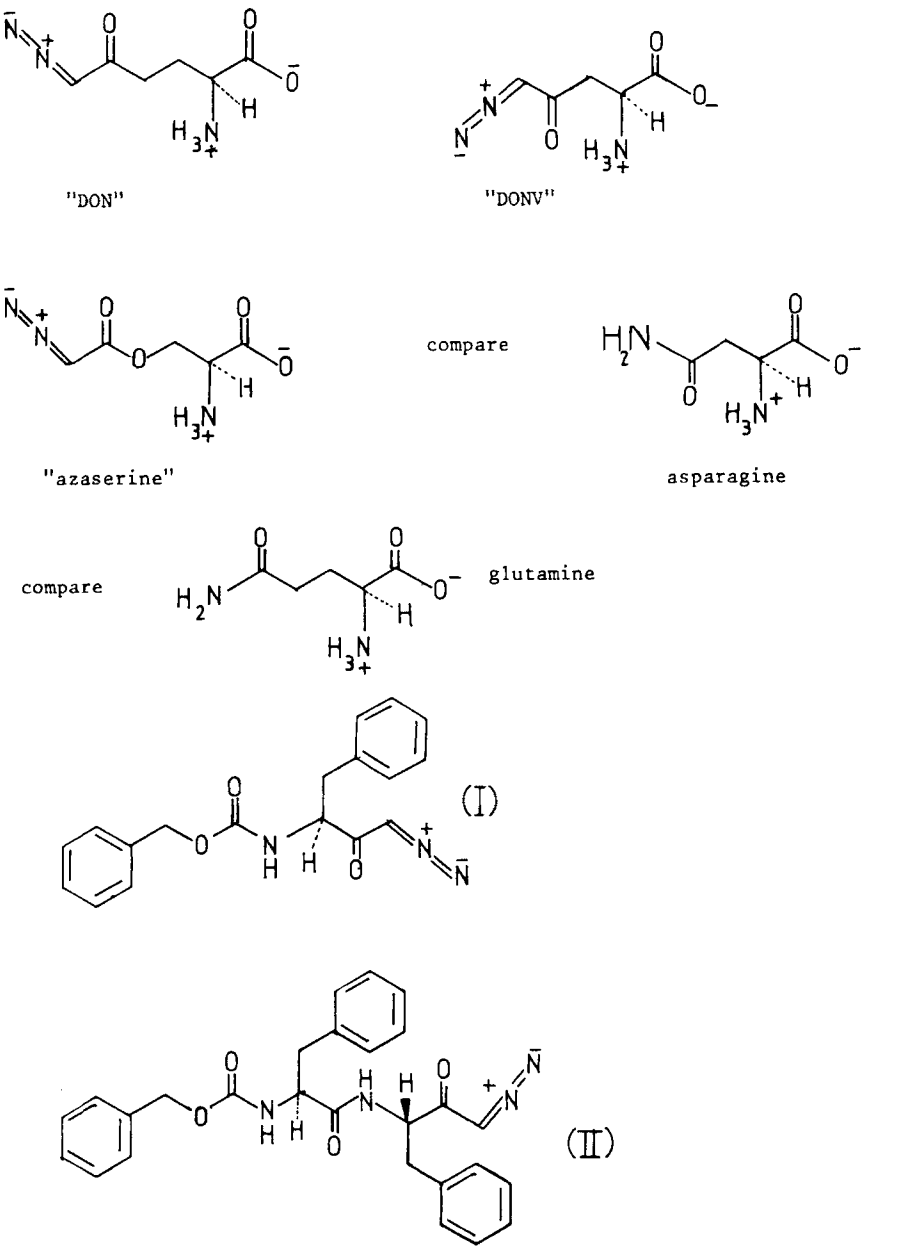


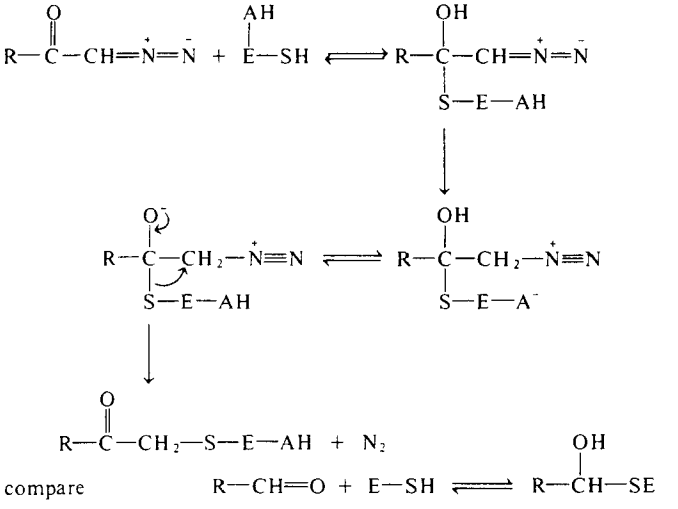
FIGURE 3. Substrate derived diazoketones.

6-diazo-5-oxo-L-norleucine (DON).[61] The two compounds are isosteric, and DON can be regarded as glutamine in which the amide nitrogen has been replaced by a diazomethane residue (Figure 3: the diazoketone grouping being shown in the preferred conformation, with the diazo group *cis* to the carbonyl[62]). DON and azaserine inactivate enzymes which cleave the γ-carboxamide of glutamine to glutamic acid. Since NH_2^- is a very poor leaving group, some proton donation to the nitrogen in all probability takes place during this process. If DON or azaserine is bound in the same orientation, proton transfer will take place to the carbon of the diazo group, thus generating a diazonium ion which

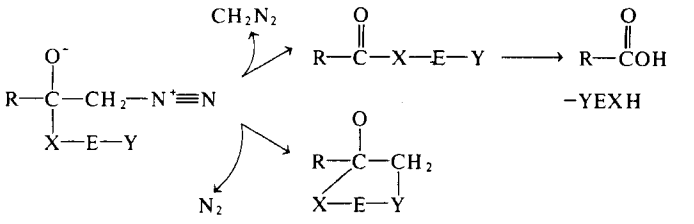
subsequently alkylates the protein. Similar considerations apply to the sythetic compounds 5-diazo-4-oxo-L-norvaline (DONV) which is an affinity label of the L-asparaginase of *E. coli,*[63] and diazoketones I and II which inactivate the -SH proteases papain[64] and Cathepsin B_1.[65]

As yet, it is not clear which diazonium ion is the species which alkylates the protein. The catalytic pathway of at least some of the enzymes against which these compounds are active involves an acyl-enzyme intermediate. It is possible, therefore, that the nucleophile to which the acyl group is normally attached adds to the diazoketone before the carbon of the diazo-group is protonated (a process which is much readier once conjugation with the carbonyl is destroyed). The inertness of compounds I and II to thiols in free solution (unsurprising since protonation of primary diazoketones is reversible[25]), and certain perceived anomalies in the pH-dependence of the inactivation reaction, led to the suggestion that a thiohemiketal was an intermediate in the alkylation of the active site thiol of papain.[66] This suggestion receives support from the recent discovery that only thiol proteases, but not serine, carboxyl, or metallo proteases, are inactivated by substrate-derived diazoketones.[67,68]

The reversible formation of a thiohemiacetal with a substrate-derived aldehyde, in an analogous fashion, has been demonstrated,[69] so the scheme below seems reasonable:



There is evidence that tetrahedral intermediates similar to the above — but not necessarily derived from thiol nucleophiles — may decompose to diazomethane and (eventually) acid. The glutaminase A of *E. coli* reacts with DON in a catalytic fashion, decomposing 70 molecules to glutamic acid and diazomethane for every active site alkylated.[70] The factor of 70 is independent of pH, suggesting that alkylation of the protein and generation of diazomethane are both only one step from a common intermediate. A tetrahedral intermediate as above could be such an intermediate:



One of the attractions of DON, DONV, and azaserine as affinity labels is that synthesis of radiolabeled material is fairly simple (Figure 4).

1. Thiol Proteases

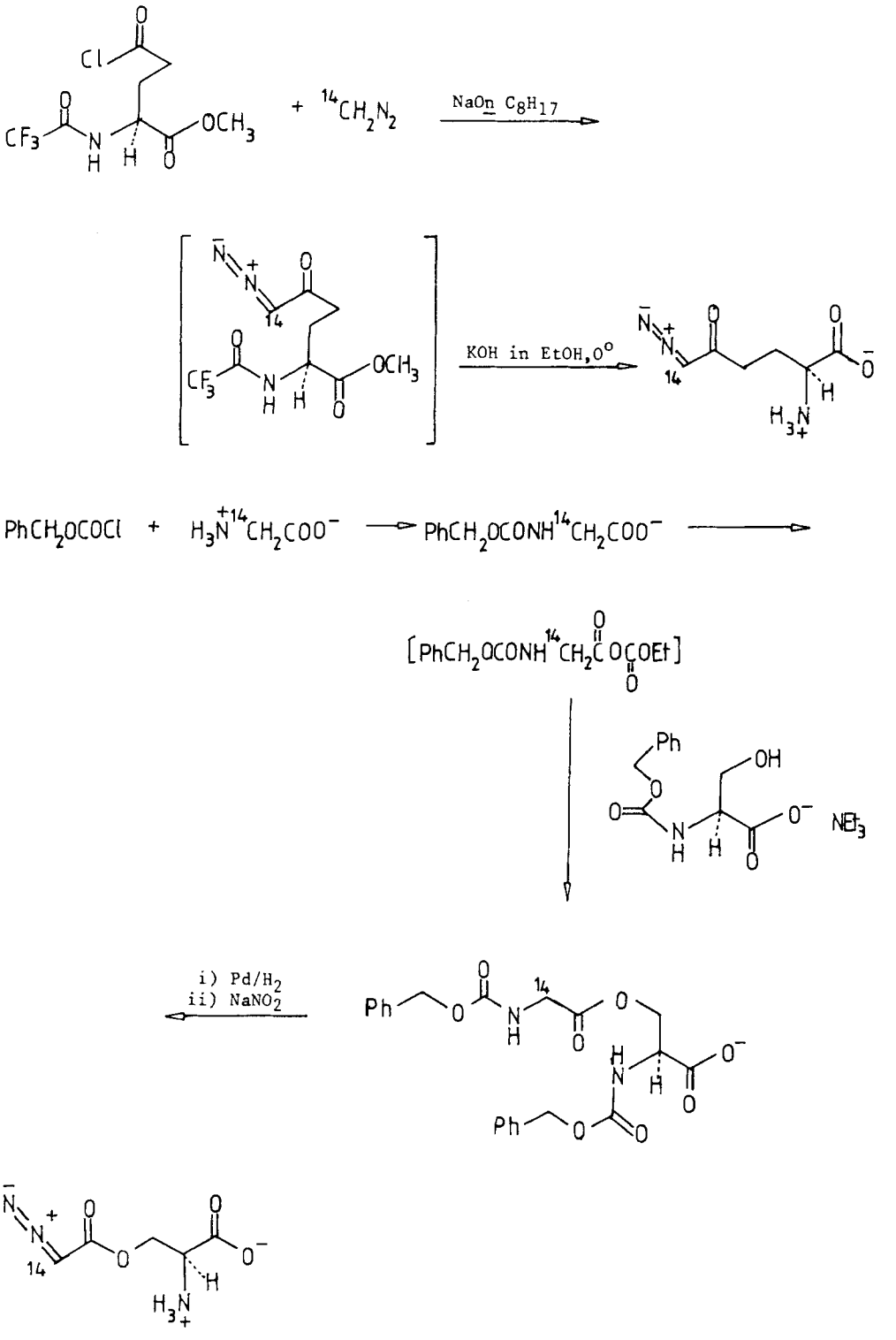
Diazoketones (I) and (II) (Figure 3) are active-site-directed irreversible inhibitors of papain, the site of alkylation being identified as cysteine from the difference in amino-acid composition of active and inactivated protein. Kinetic data are given in Table 2 — the inactivation being so efficient that saturation of active sites by the reagents was not observed.[64] This discovery prompted a search for related specific inactivators of thiol proteases which could be used in vivo.[65,67,68] The results of this search are shown in Table 2; cathepsin B, streptococcal proteinase, and clostripain are all endopeptidases, and cathepsin C is an aminodipeptidase. The second-order rate constant for inactivation by a diazoketone parallels the ability of the enzyme in question to cleave that peptide bond in the parent peptide which has been modified to form the diazoketone. Thus clostripain, which cleaves a peptide chain after basic residues, exhibits a selectivity of 4½ orders of magnitude between Z-Lys CHN_2 and Z-Phe-AlaCHN_2. Cathepsin B, which cleaves a peptide chain after a residue which is itself after a hydrophobic residue, is rapidly inactivated by the latter compound. Z-Phe-Gly-PheCHN_2, as would be anticipated from this specificity, is hydrolyzed to Z-Phe-Gly and PheCHN_2, without alkylating the enzyme.

The inactivation has been established as unique to thiol proteases. Serine proteases are not inactivated even by those reagents which closely resemble preferred substrates — chymotrypsin is inert to Z-Phe-CHN_2, trypsin to Z-Lys CHN_2, and elastase to Z-Phe-Ala CHN_2. Thermolysin, a metallo-protease, was likewise inert to Z-PheCHN_2 and cathepsin D, a carboxyl protease to Z-Phe-Phe-CHN_2. Chymotrypsin however catalyses the decomposition of Z-PheCHN_2[67] — to what is not known.

These data show that, although proton-donation to the substrate-leaving group may be a necessary condition for the derived diazoketone to be an affinity label, it is not a sufficient condition. Since the catalytic mechanism of thiol and serine proteases is grossly similar in all except the nucleophilic atom, reaction via a thiohemiketal provides the most convincing rationalization of the susceptibility of thiol proteases, and inertness of serine proteases, to substrate-derived diazomethyl ketones.

2. Glutaminase

As mentioned above, DON is a good suicide substrate for the glutaminase A of *E. coli*. Protection by substrate was shown in the original experiments,[74] and use of ^{14}C-labeled reagent eventually enabled the enzyme to be shown to be a tetramer of 28,000 mol. wt. subunits.[75] The enzyme is a cooperative one, sigmoid dependence of rate on substrate concentration being observed and, as expected for a true suicide substrate, alkylation of some of the sites with DON increases the affinity of the remaining ones, both for DON and substrate. In the absence of detailed kinetics, a qualitative impression of the effectiveness of the reagent can be had from the completeness of inactivation after 30 min in the presence of an excess of it.[74] The site of attachment of the label to the *E. coli* glutaminase is apparently not known, but the sequence of peptides containing the labeled residue has been determined for the closely-related enzymes, the glutaminase-asparaginases of *Pseudomonas 7A* and *Acinetobacter glutaminasificans*.[76] The *Pseudomonas* enzyme has comparable glutaminase and asparaginase activity,[77] but the *Acinetobacter* enzyme is really a glutaminase (V_{max} / Km values for the two substrates differ by a factor of $10^{4.5}$).[76] Both enzymes were however inert to DONV, the active site reagent for asparaginase and, in accord with the idea of their fundamental similarity, in both cases the label is attached to the underlined threonine in the sequence

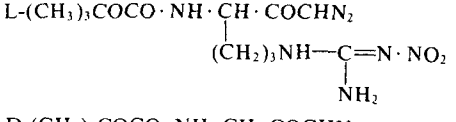
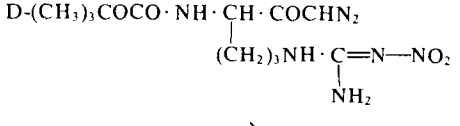


DONV is made similarly to DON.[67]

FIGURE 4. Syntheses of ^{14}C DON,[71] DONV,[72] and azaserine.[73]

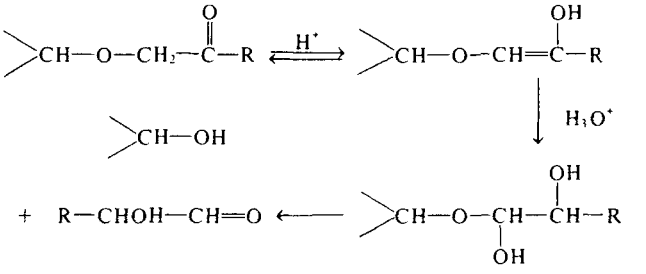
Table 2
SECOND-ORDER RATE CONSTANTS FOR INACTIVATION OF PROTEASES BY SUBSTRATE-DERIVED DIAZOMETHYL KETONES AT 25° C EXCEPT WHERE STATED

Reagent	Enzyme	pH	$\log_{10}$ $\lvert k_{max}/K)/s^{-1}M^{-1}\rvert$	Ref.
(I) ≡ ZPheCHN$_2$	Papain	6.5 (at room temperature)	1.01	64
(I)	Papain	5.4 (at room temperature)	0.91	64
(II) ≡ ZPhePheCHN$_2$	Papain	6.5 (at room temperature)	3.31	64
(I)	Cathepsin B	5.4 (at room temperature)	−0.57	65
(II)	Cathepsin B	5.4	2.29	67
Z-D-Phe-L-PheCHN$_2$	Cathepsin B	5.4	0.40	67
Z-D-Phe-D-PheCHN$_2$	Cathepsin B	5.4	0.39	67
Z-Phe-GlyCHN$_2$	Cathepsin B	5.4	2.84	67
Z-Phe-AlaCHN$_2$	Cathepsin B	5.4	3.10 (K_i = 1.7 μM)	67
Z-Ala-Phe-AlaCHN$_2$	Cathepsin B	5.4	3.07	67
Z-Pro-Gly-CHN$_2$	Cathepsin B	5.4	0.48	67
Z-LysCHN$_2$	Cathepsin B	5.4	2.48	68
Z-Ala-Ala-CHN$_2$	Cathepsin B	5.4	2.15	68
Z-Gly-Gly-MetCHN$_2$	Cathepsin B	5.4	0.76	68
Z-Gly-Gly-PheCHN$_2$	Cathepsin B	5.4	0.66	68
Z-Gly-Gly-ValCHN$_2$	Cathepsin B	5.4	−0.30	68
Z-Gly-Gly-ProCHN$_2$	Cathepsin B	5.4	−0.88	68
Z-Phe-Gly-PheCHN$_2$	Cathepsin B	5.4	(inhibitor destroyed)	68
Z-Ala-Ala-ProCHN$_2$	Cathepsin B	5.4	~0 (inhibitor destroyed)	68
Z-Gly-Gly-LeuCHN$_2$	Cathepsin B	5.4	0.47	68
Gly-PheCHN$_2$	Cathepsin B	5.4	Not detected	68
Gly-PheCHN$_2$	Cathepsin C	6.0	4.24	68
Z-Phe-AlaCHN$_2$	Cathepsin C	6.0	1.26	68
Z-Phe-Gly-PheCHN$_2$	Cathepsin C	6.0	1.34	68

Z-Ala-Phe-AlaCHN$_2$	Streptococcal protease	5.4	4.47	68
Z-Phe-AlaCHN$_2$	Streptococcal protease	5.4	3.88	68
Z-Phe-GlyCHN$_2$	Streptococcal protease	5.4	2.53	68
Z-Ala-AlaCHN$_2$	Streptococcal protease	5.4	2.13	68
Z-Ala-Ala-ProCHN$_2$	Streptococcal protease	5.4	0.78	68
Z-Phe-Gly-PheCHN$_2$	Streptococcal protease	5.4	0.00	68
Z-Gly-Gly-ProCHN$_2$ Z-Pro-GlyCHN$_2$ Gly-PheCHN$_2$	Streptococcal protease	5.4	Not detected	68
Z-Lys-CH$_2$N$_2$	Clostripain	7.8	2.67	68
(I)	Clostripain	7.8	1.30	68
(II)	Clostripain	7.8	1.18	68
	Clostripain	7.8	0.04	68
	Clostripain	7.8	−0.13	68
Z-Phe-AlaCHN$_2$ Z-Pro-GlyCHN$_2$ Z-Gly-Gly-MetCHN$_2$	Clostripain	7.8	$<\sim -2$	68

Ala—Thr—Gly—Gly—Thr—Ile—Ala—Gly—Ala—Gly—Ala

Before this sequence could be determined,[76] it was found necessary to stabilize the protein-label linkage to acid pH by sodium borohydride reduction.[76] It is possible that the acid lability arose via enolization of the alkoxyketone, and acid-catalyzed hydrolysis of the resulting enol ether.



3. Asparaginase

The naive expectation that this enzyme would behave in an identical fashion with DONV as glutaminase does with DON is not fulfilled. The *E. coli* enzyme does indeed catalyse the decomposition of DONV[63] — only one decomposition in 400 results in inactivation of an active site — but the product of this decomposition is the normal product of hydrolysis of a diazoketone, 5-hydroxy-4-oxo-norvaline, not aspartic acid and diazomethane. Further, at 20° C and pH 7 the K value for inactivation is 73 μM but the K_m for DONV decomposition is 9.5 μM, and the decomposition (but not the inactivation of the enzyme) can be suppressed by the addition of organic solvents, most effectively dimethyl sulfoxide.[63] The different K_m values would seem to indicate that DONV hydrolysis and enzyme inactivation do not proceed through any common E. DONV intermediate. However, β-cyanoalanine (asparto-β-nitrile) is hydrolysed (to ammonia) by the enzyme, and the K_m for this reaction, and the K_i for the inhibition of DONV hydrolysis by β-cyanoalanine are the same, i.e., these two processes proceed at the same site.[63] In view of the now-recognized very different steric requirements for attack on double and triple bonds,[78] it seems likely that, in addition to an asparaginase site, the enzyme used had a nitrile-hydrolyzing site and that inactivation by DONV took place at the amidase site and decomposition to hydroxyketone at the nitrilase site. Studies with crystalline enzyme,[79] although considered to support the case for hydrolysis of asparagine and β-cyanoalanine at the same site, in fact weaken it since the pH-dependences of the two processes are different, and the extent of inhibition of asparaginase activity of β-cyanoalanine is less than expected from the K_m for its hydrolysis.

The amino-acid sequence containing the site of attachment of the label is

Val—Gly—Ala—Met—Arg—Pro—Ser—Thr—Ser—Met

the label being attached to one of the adjacent triplet of hydroxyamino acids,[80] probably to the serine distal to the N-terminus. This sequence is very different from that obtained with glutaminase, but the full amino acid sequence does contain, near the N-terminus, an eight-residue sequence (Ala-Thr-Gly-Gly-Thr-Ile-Ala-Gly) which also occurs in the active site of glutaminase-asparaginase.[76] DON does not inactivate the enzyme.[73]

4. Mammalian Kidney γ-Glutamyl Transpeptidase

The reaction catalyzed by this enzyme is given in Figure 5. DON is an excellent affinity label for the enzyme from rat kidney.[81-83] At the pH optimum for inactivation (7.4) and

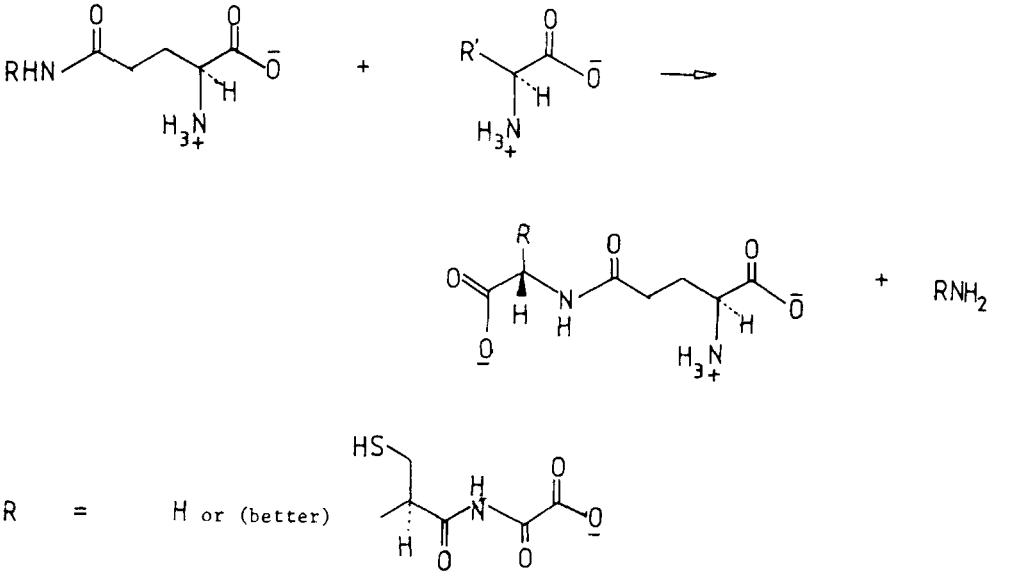


FIGURE 5. Reaction catalyzed by γ-glutamyl transpeptidase.

25° C, DON inactivates with K = 6 m*M*, $k_{max} = 4 \times 10^{-3} s^{-1}$:[81] protection by reduced glutathione is observed, but maleate accelerates the inactivation. With pure enzyme, one mole of label is incorporated into 102 Kg detergent-solubilized enzyme and 64 Kg papain-solubilized enzyme.[82] Radioactivity from ^{14}C-DON is incorporated only into the lighter of the two types of subunit.[82,83] Azaserine is also an active-site-directed irreversible inhibitor, but only about 1/3 as good as DON. Essentially similar results were obtained with human enzyme.[84]

In the following sections the inhibition of enzymes which transfer the amido group of glutamine to other molecules will be considered. DON and azaserine are very effective active site reagents for all these enzymes.[85] The amide group of glutamine is relatively non-nucleophilic, but on the other hand it is difficult to see a simple mechanism whereby an enzyme could hold a small molecule such as ammonia tightly enough to stop it coming off the enzyme. The amino group in a tetrahedral intermediate will however be much more nucleophilic than the parent amide. If this tetrahedral intermediate were the true reactive species, then this conceptual problem is solved since, once the lone pair of electrons on the nitrogen ceases to be conjugated with the carbonyl group, it is available to make the NH_2-group nucleophilic. Were the enzyme constructed so as to stabilize the tetrahedral intermediate from glutamine, moreover, an analogous process with DON would account for its effectiveness as an active site reagent: proton donation to the diazomethyl carbon from water would in all probability be sufficiently fast to make acid catalysis by the enzyme redundant (cf. Ref. 23).

5. Glucosamine-6-Phosphate Synthetase

The reaction catalyzed, together with a plausible mechanism for the amination, is shown in Figure 6. Azaserine does not inactivate the rat liver enzyme,[86] but DON is a good inactivator of both this enzyme and the one from *E. coli*.[87] The rat liver enzyme is subject to allosteric feedback inhibition by UDP-*N*-acetyl glucosamine and, in accord with the status of DON as a suicide substrate, both this compound and glutamine inhibit the inactivation. Moreover, the individual inactivation-retarding effects of the two ligands individually is enhanced when they are both present.[88]

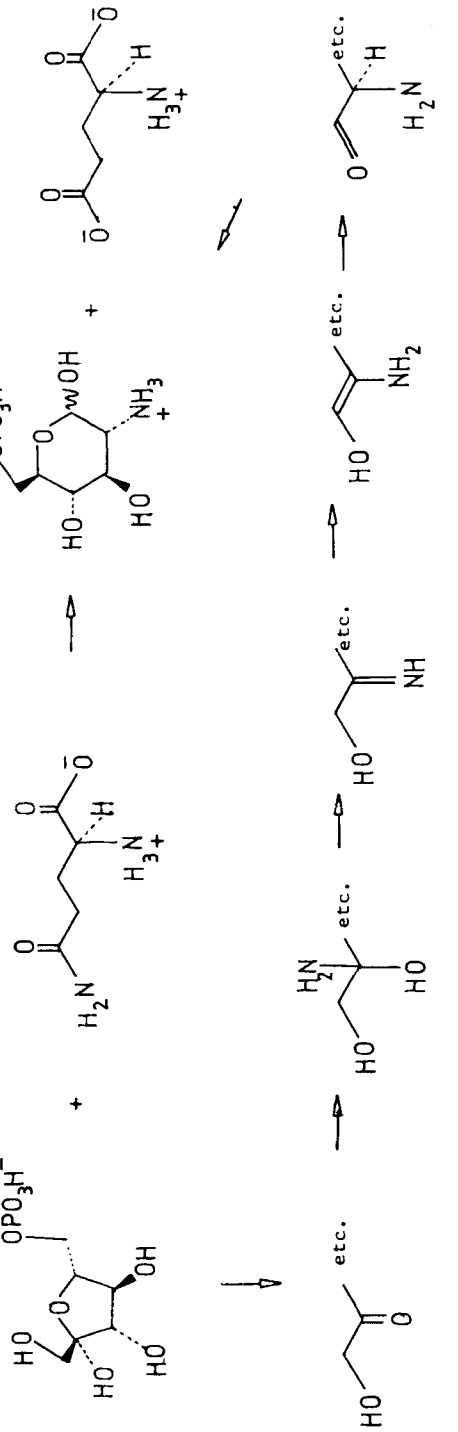


FIGURE 6. Reaction catalyzed by glucosamine 6-phosphate synthetase.

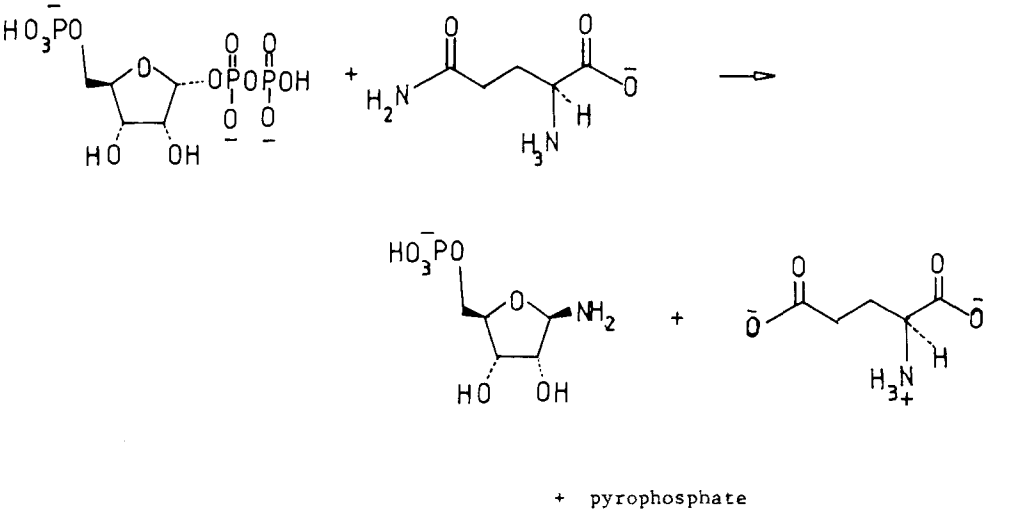


FIGURE 7. Reaction catalyzed by phosphoribosyl pyrophosphate transamidase.

6. Phosphoribosyl Pyrophosphate Amido Transferase

The reaction catalyzed by this enzyme involves transfer of the amide group of glutamine to C-1 of ribose, with pyrophosphate as a leaving group (Figure 7). Detailed work on the chicken liver enzyme by Hartman[71] established that inactivation by DON and azaserine is active-site-directed and irreversible. The rate of inactivation varies with the concentration of phosphoribosyl pyrophosphate and Mg^{2+}; at saturating concentrations of these species at pH 8 and 25°, K for DON is 19 μM and for azaserine is 4.2 mM; k_{max} for DON is $4.9 \times 10^{-4} s^{-1}$. The molecular weight of the holoenzyme is 2×10^5, and one molecule of ^{14}C DON is attached for every molecule of the molecular weight inactivated.

As is the case with many glutamine-dependent transamidases, the glutamine-transforming activity and the amination reaction can be uncoupled: the enzyme catalyses the reaction of methanol and sugar amines with the phosphoaldosyl pyrophosphate, and formation of the γ-hydroxamic acid of glutamic acid in the presence of hydroxylamine.[89] The transglycosylation reactions are still catalyzed by DON-inactivated enzyme.[71] The trans-γ-glutamylation activity implies the existence of a covalent γ-glutamyl enzyme and hence, again, a tetrahedral intermediate at some stage of the reaction. If inactivation by DON and azaserine proceeded through an analogous adduct, then the much higher Km for azaserine than for DON would be explained since nucleophilic additions to the carbonyl of a ketone are readier than such additions to the carbonyl of an ester.

7. Cytidine Triphosphate Synthetase

The value of effective suicide substrates in probing the subunit interactions of an allosteric enzyme are well illustrated by the work of Koshland and his co-workers on cytidine triphosphate synthetase.[90] The enzyme consists of identical subunits which associate as dimers in the absence of ligands and tetramers in the presence of ATP, UTP, and Mg^{2+}.[91] The covalency changes involved in catalysis are plausibly represented by the scheme of Figure 8, since it was shown that a covalent γ-glutamyl enzyme intermediate could be isolated, and that incorporation of ^{18}O into inorganic phosphate product occurs if 4′-^{18}O-UTP is used as substrate.[92] The glutamyl enzyme is drawn as a thiol ester since DON became attached to a thiol group.[91]

The kinetics of inactivation of the tetramer with DON in the presence of UTP and ATP are biphasic, the second site being inactivated ten times slower than the first. Only two of

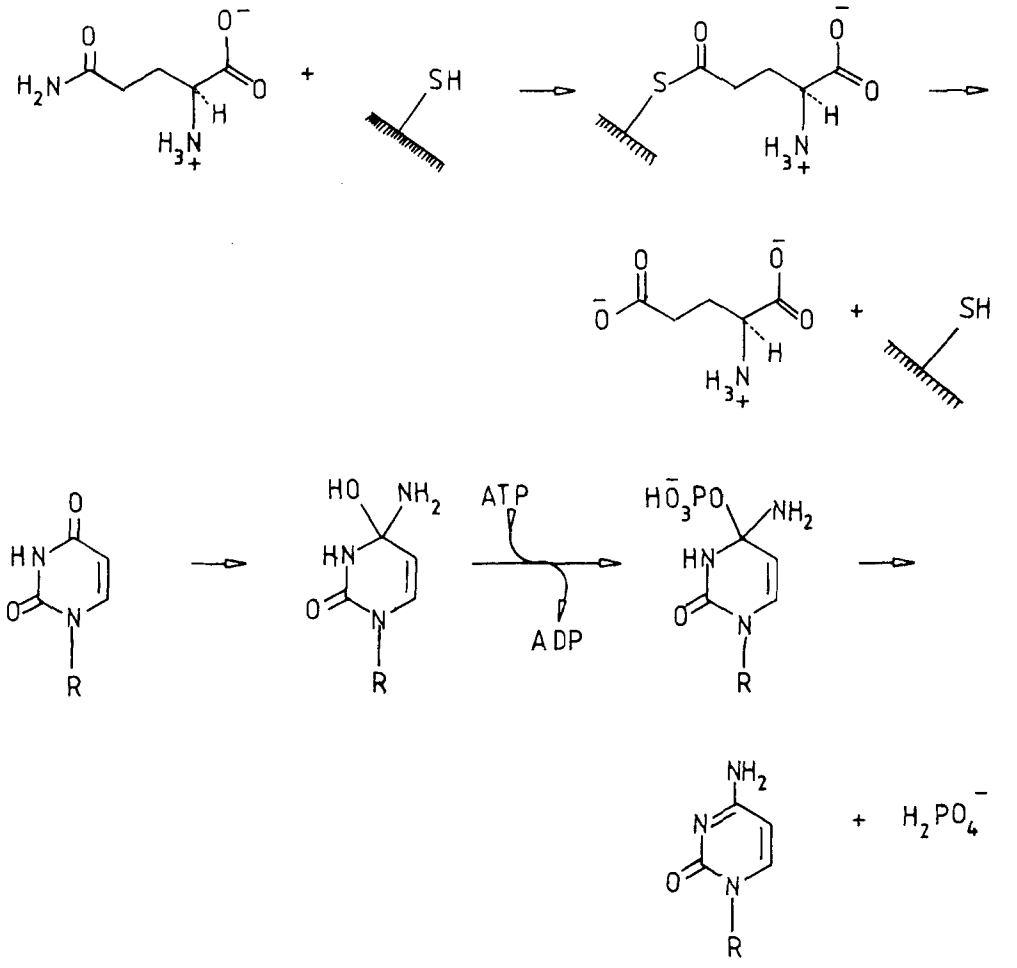


FIGURE 8. Reaction catalyzed by cytidine triphosphate synthetase.

the four sites of the tetramer are ever alkylated, but these two sites can be made to react at the same rate by the addition of GTP, an allosteric effector.[90] Inactivation of the dimeric form of the enzyme by DON was simply first order, but only one molecule of DON per dimer was incorporated; it was accelerated by the presence of GTP. These inactivations of both the dimeric and tetrameric forms of the enzyme refer only to the ability to use glutamine as an amino-group donor — the ability of the enzyme to use ammonia to synthesize CTP is unimpaired by reaction with DON.

8. 2-Formamido-N-Ribosylacetamide 5'-Phosphate: L-Glutamine Amido-Ligase

The DON and azaserine inactivation of this enzyme, which catalyses the reaction

$$6\text{—phospho—}\beta\text{—D—Rib—}f\text{—NH—}\overset{\overset{O}{\|}}{C}\text{—CH}_2\text{—NH—CHO} + \text{H}_2\text{NCO(CH}_2)_2\text{—CH(NH}_3^+\text{)COO}^- + \text{ATP} \longrightarrow 6\text{—phospho—}\beta\text{—D—Rib—}f\text{—NH—}\overset{\overset{NH}{\|}}{C}\text{—CH}_2\text{—NH—CHO} + {}^-\text{OOC(CH}_2)_2\text{.CH(NH}_3^+\text{)COO}^- + \text{ADP} + \text{H}_2\text{PO}_4^-$$

, has been studied with

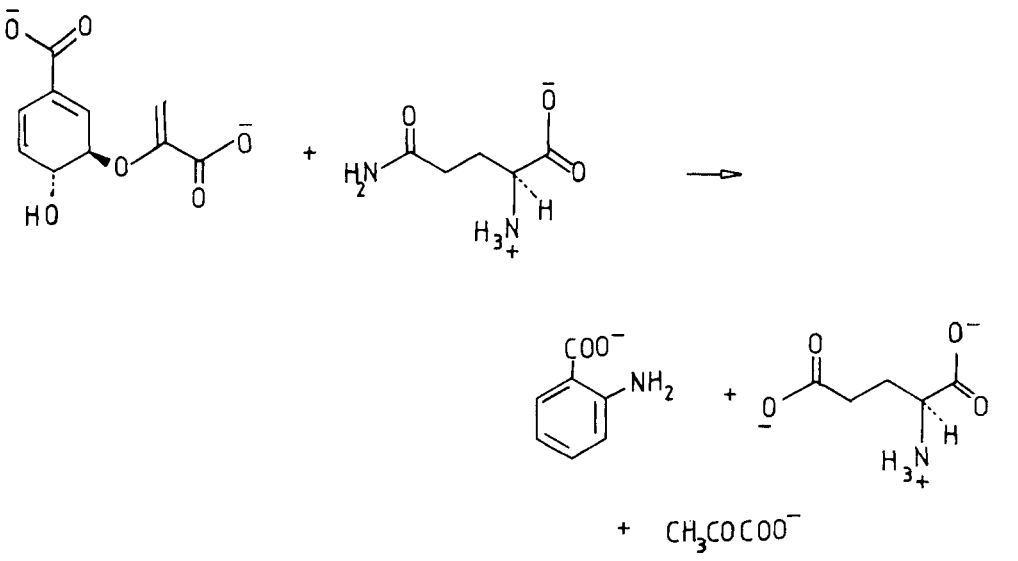
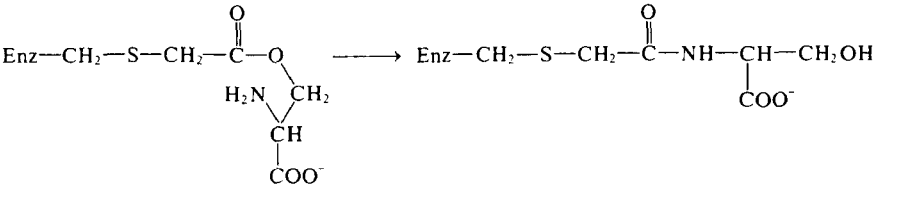


FIGURE 9. Reaction catalyzed by anthranilate synthetase.

enzymes from pigeon liver[93] and *Salmonella typhimurium*.[72] The relative effectiveness of DON and azaserine against the enzyme from these two sources differs. DON is the much more effective at inactivating the pigeon liver enzyme, but the bacterial enzyme is comparably sensitive to the two reagents.

The site of attachment of azaserine was shown to be cysteine, ^{14}C-*S*-carboxymethyl-cysteine being identified in the complete acid hydrolysate of labeled enzyme.[94] Digestion with pronase or papain led to small peptides; further degradation showed the sequence around the alkylated cysteine to be Ala-Leu-Gly-Val-*Cys*.

The first formed α-thioalkoxy ester undergoes a rearrangement of the azaserine moiety of an α-thioalkoxy amide;[95] once conjugation with the diazo grouping is removed the ester function becomes more reactive:



9. Anthranilate Synthetase

The DON inactivation of anthranilate synthetase from three sources — *Serratia marcescens*,[96] *Salmonella typhimurium*,[97] and *Pseudomonas putida*[98,99] — has been examined in detail. The enzyme catalyses the reaction shown in Figure 9.

The *S. marcescens* enzyme has an $\alpha_2\beta_2$ quaternary structure, the molecular weights of the two subunits being 60,000 and 21,000; DON alkylation, a process which leaves ammonia-dependent anthranilate synthetase activity intact but abolishes the glutamine-dependent activity, occurs on an -SH group in the light subunit. The number of active sites could be correctly estimated by reaction with less than two equivalents of DON — this would imply the enzyme is not catalyzing DON decomposition. From the experimentation reported, it is possible to extract an approximate value of $10^{3.2}M^{-1}s^{-1}$ for k_{max}/K for DON inactivation of this enzyme at room temperature.

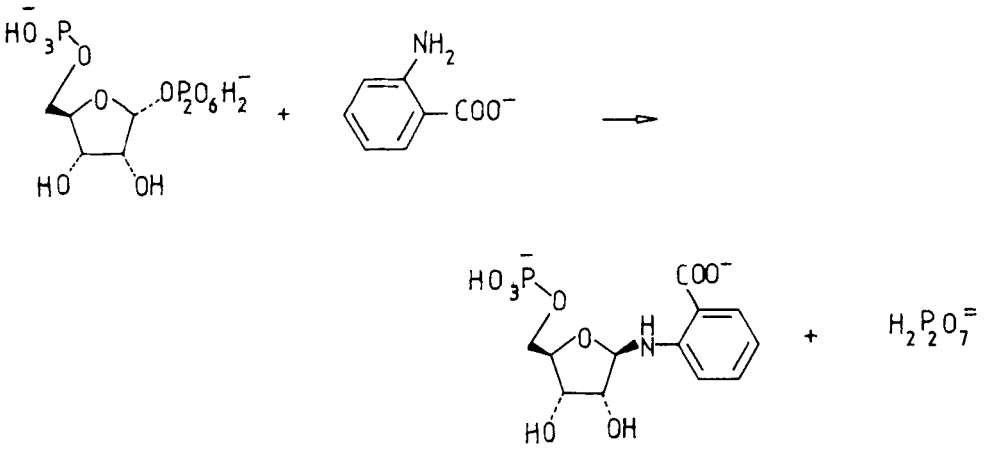
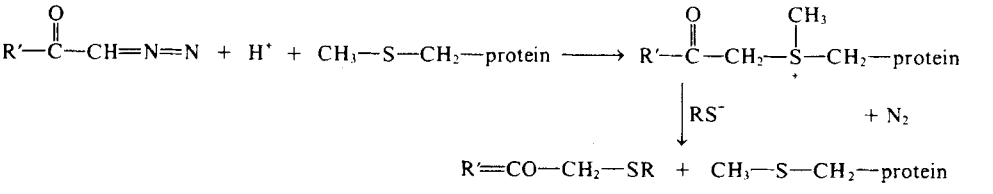


FIGURE 10. Step in tryptophan biosynthesis subsequent to the synthesis of anthranilate.

The enzyme from *S. typhimurium* again has an $\alpha_2\beta_2$ quaternary structure; again one subunit is responsible for the aromatization reaction and another for cleavage of glutamine; again DON alkylates a sulfhydryl group on the glutamine-cleaving subunit which blocks anthranilate synthesis from glutamine but not from ammonia. In this case, however, the glutamine-cleaving subunit has another catalytic function, the promotion of the reaction in Figure 10 which is the next step in the biosynthesis of tryptophan. Moreover, DON alkylation only occurs in the presence of chorismate. One can conclude that DON decomposition is not catalyzed for the same reasons as for the *S. marcescens* enzyme.

The enzyme from *P. putida* has an $\alpha_n\beta_n$ quaternary structure but association and dissociation of subunits is fast on the time scale of protein purification. Again there is an aromatization subunit and a glutamine-cleaving subunit, and DON inactivates only the glutamine-cleaving subunit, but the presence of the second subunit and Mg^{2+} and chorismate speed up the inactivation. Azaserine as well as DON inactivates; K for DON is 0.3 μM.[99]

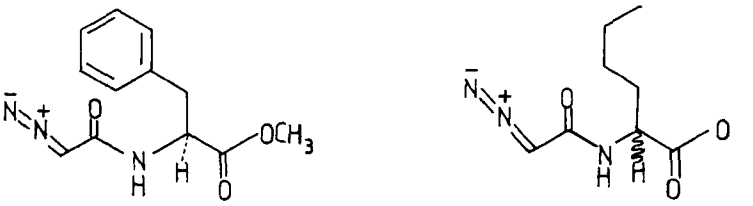
The restoration of DON-inactivated enzyme by thiols[99] — although claimed to be an experimental artefact[98] — would, if real, imply that the label has attacked methionine rather than cysteine. Alkylation of methionine sulfur would given an α-carbonyl sulfonium salt; such species are known[100] to be subject to attack by thiols with regeneration of methionine.



10. Nicotinamide Adenine Dinucleotide Synthetase

The enzyme from bakers yeast has been purified and shown to be inactivated by DON and azaserine.[101] As with other reactions in which glutamine is the better, but not the only, amido-donor the glutamine-dependent (but not the ammonia-dependent) amidation reaction is stopped. The reaction catalyzed by the enzyme

$$R{-}COO^- + NH_2CO{-}R' + ATP \longrightarrow RCONH_2 + COO\,R' + AMP + H_2P_2O_7^{2-}$$



(III) (IV)

FIGURE 11. Diazoamides used against pepsin-type proteases.

is unusual in that ATP is cleaved to AMP and pyrophosphate. Similar results had been obtained previously with azaserine and the less extensively purified enzymes from yeast and rat liver.[102] A report of the inactivation of xanthosine-5′-phosphate amido transferase by DON has appeared.[103]

B. Diazoacetyl Amino Acid Esters and Pepsin-Type Proteases

Whereas the relationship of inactivation by azaserine and substrate-derived diazomethyl ketones to reasonable mechanisms for catalysis by the target enzymes described above is clear, this is not the case for the N-diazoacetyl amino acid derivatives used against pepsin and related proteases. The first difficulty is that pepsin reacts in an apparently active-site-directed way with aliphatic diazocompounds bearing little or no structural resemblance to the substrate.[104] The second difficulty is that inactivation of pepsin with compounds (III)[105] and (IV)[106] (Figure 11) requires Cu^{2+} ions. Ag^{+} is also effective with compound (IV).[107] Lundblad and Stein[107] showed that prior mixing of Cu^{2+} and compound (IV) altered the apparent pH-rate profile for inactivation of pepsin and suggested that the true affinity label was a Cu^{2+}-carbene complex. In the light of the well-known carbenoid behavior of diazocarbonyl compounds in the presence of copper,[108] this seems very reasonable, although involvement of Cu^{I} or Cu^{0} formed by reduction of added Cu^{2+} would accord better with subsequent experience of transition-metal carbene complexes.[109] This type of affinity labeling is thus not, strictly, via a deamination reaction. It has been used to detect aspartate residues at the active site of pig pepsin,[105,106] bovine pepsin,[110] and penicillopepsin.[111] Other acid proteases from various sources[112-122] have been studied by the technique, aspartate residues being identified in those cases where investigations have been taken beyond kinetics and stoichiometry.

C. Affinity Labeling with a Simple Diazocompound

Brockhaus and Lehmann[123-124] prepared a methanolic solution containing β-D-galactopyranosyl diazomethane by the route shown in Figure 12. Addition of this solution portionwise to aqueous solutions of the *lacZ* β-galactosidase of *E. coli* resulted in stepwise inactivation of the enzyme decomposition of the simple, unstabilized diazocompound in aqueous solution was too fast for the kinetics of inactivation to be followed, but protection by the competitive inhibitor isopropyl-1-thio-β-D-galacto-pyranoside was demonstrated. A second control was the absence of any effect of the reagent on β-glucosidase.

About 50% of the label made radioactive as shown in Figure 12 was removed as alcohol V with hydroxylamine; the remainder as the sulphide VI by heating in neutral buffer.[124] The proposal that the diazocompound — isosteric and isoelectronic with the substrate, β-D-galactopyranosyl azide[125] — was protonated in the active site and that the resulting

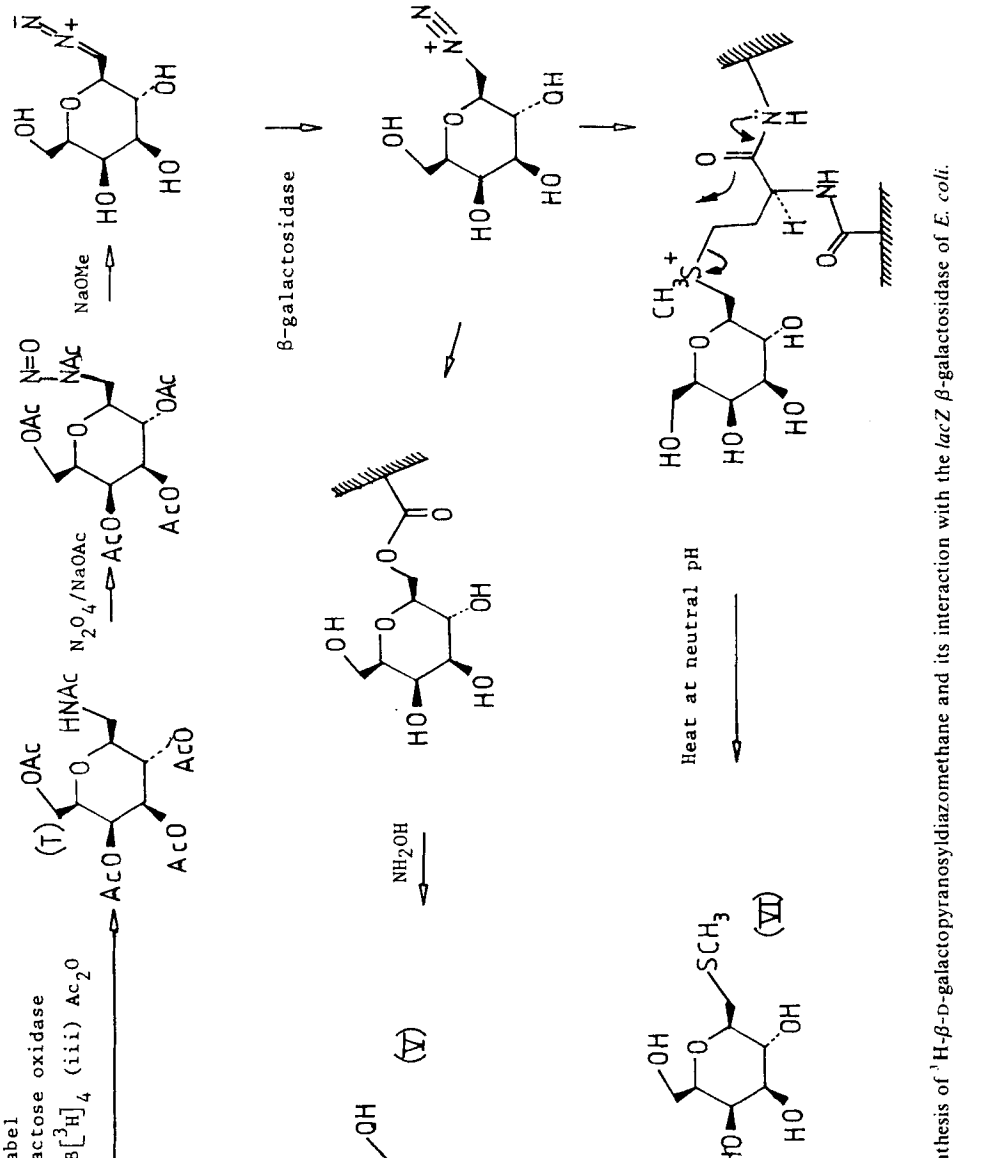


FIGURE 12. Synthesis of ^{3}H-β-D-galactopyranosyldiazomethane and its interaction with the *lacZ* β-galactosidase of *E. coli*.

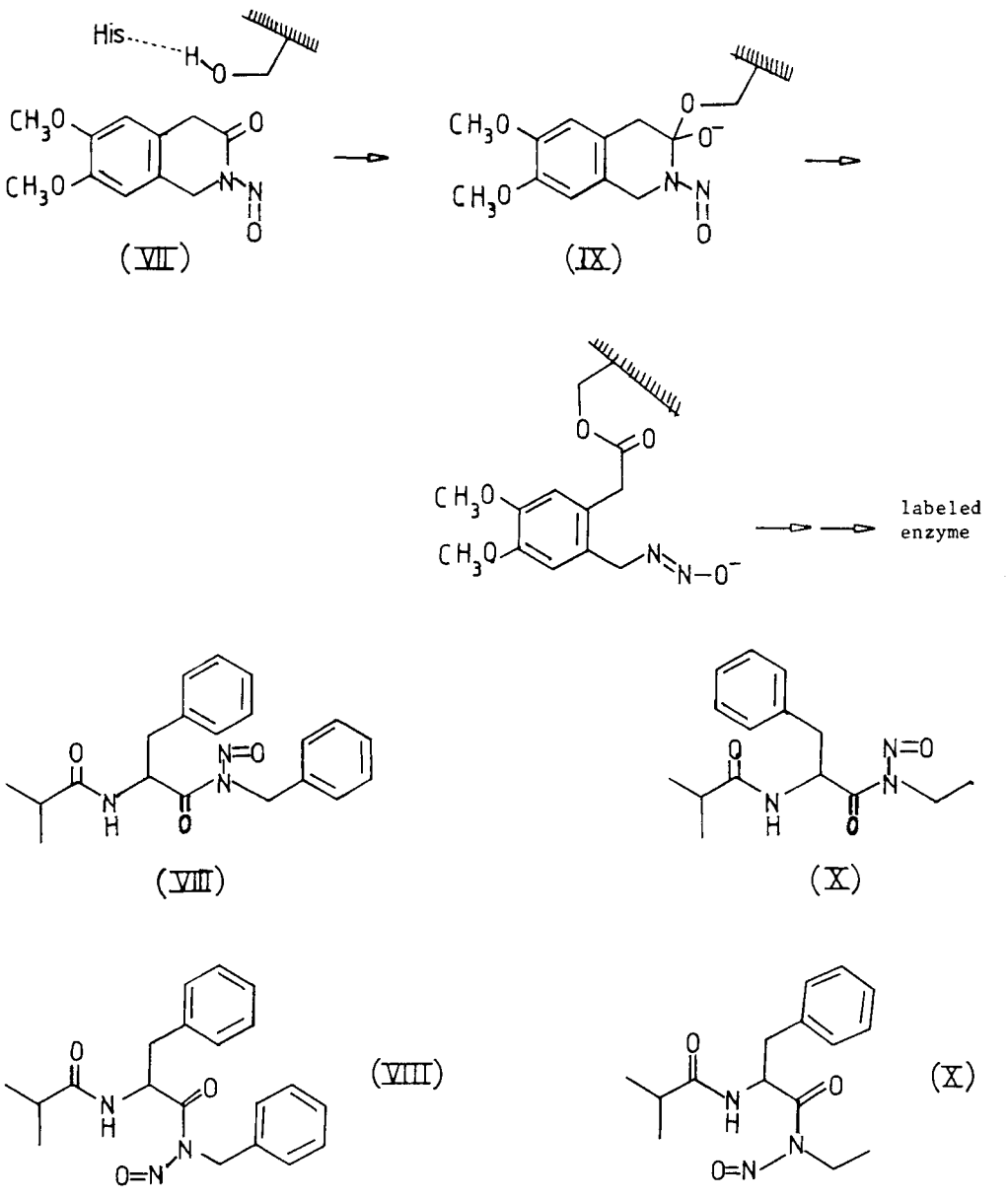


FIGURE 13. Interaction of substrate-derived *N*-nitrosoamides with chymotrypsin.

diazonium reacted either with a carboxylate or a methionine in the active site seemed very reasonable at the time.

D. Affinity Labeling of Chymotrypsin with N-Nitrosoamides

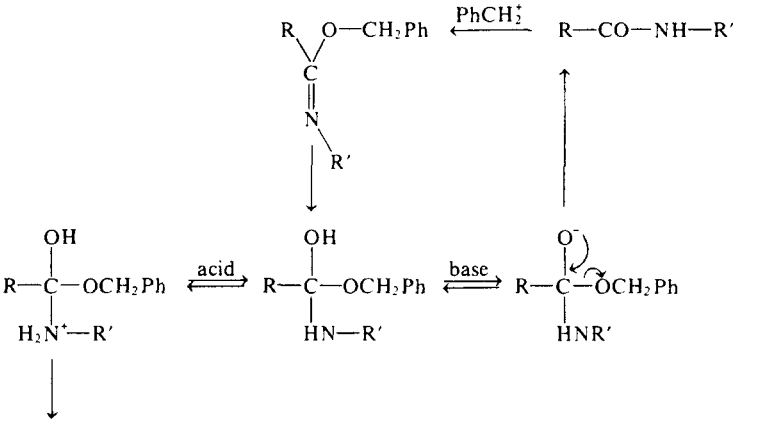
The first systematically planned approach to affinity labeling via deamination reactions was the work of White et al. on chymotrypsin.[126-128] It was found that nitrosolactam (VII) and nitrosoamide (VIII) (Figure 13) were suicide substrates for α-chymotrypsin,[126] inactivation by (VII) resulting in the decomposition of five molecules of the label for every active site alkylated. Complete protection by the parent lactam against nitrosolactam (VII) was observed, and 1.6 moles of label were attached per mole

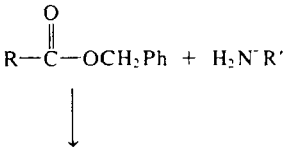
of active sites alkylated. Almost certainly the generation of the alkylating species results from a slight diversion of the normal catalytic mechanism: attack by active-site Ser 195 to give the diazolate shown, in a pathway very similar to the normal one for base-catalyzed hydrolysis of nitrosoamides. A high residence time of the actual alkylating species is ensured by the aromatic ring and by the formation of a transient covalent link to the catalytic Ser 195. This high residence time ensures that the diazotate, diazonium hydroxide or diazonium ion has time to alkylate the protein. Acid catalysis of the departure of the diazotate from the tetrahedral intermediate IX is unlikely to be of importance, since such processes also lead to denitrosation (see Section II.C.).

Completely different results were obtained with the nitrosoamide VIII.[127] Both enantiomers were hydrolysed by the enzyme, the L-isomer some six times faster than the D, to N-isobutyryl phenylalanine and benzyl alcohol. However only the enantiomer of the unnatural, D, configuration inactivated the enzyme by benzylation. Examination of a molecular model of chymotrypsin revealed that the D isomer could be fitted into the active site of chymotrypsin, with the catalytic Ser 195 poised to attack the Phe carbonyl, if the benzylamine moiety occupies the hydrophobic site which normally binds the hydrophobic amino-acid side chains of substrates of natural (L) configuration.

In aqueous solution, $PhCH_2N_2^+$ is a free species — synchronous fragmentation of deamination precursors to carbonium ions is not readily observed. Neither $Ph-CH_2-N=N-O^-$, $Ph-CH_2-N=N-OH$, or $PhCH_2N_2^+$ have any structural features which will be recognized by the enzyme. Therefore, it is likely that they will simply come harmlessly off the enzyme if they have a discrete existence. In a hydrophobic (i.e., nonpolar) environment, however, synchronous fragmentation of the diazonium-ion precursor to the resonance-stabilized benzyl cation will be favored. Hence, affinity labeling by the benzyl cation is observed only when the immediate precursor is in a hydrophobic environment. In accord with this explanation, which the reviewer advances, neither of the isomers of the ethyl compound (X) (Figure 13) alkylated the enzyme, although both isomers were substrates — synchronous fragmentation to $C_2H_5^+$ would not be expected to be observed.[14] (White and co-workers accounted for this observation by invoking the preferential formation of diazoethane from the ethanediazonium ion, compared with the formation of phenyldiazomethane from the phenylmethane diazonium ion).

A useful feature of benzyl cations is that they O-alkylate secondary amides in preference to N-alkylating them.[128] The resulting imidate esters can hydrolyse with either C-O or C-N cleavage, the former pathway being favored in alkali, the latter in acid — in this case the ester may hydrolyse further.





Therefore, if a peptide chain is labeled, treatment of the labeled protein with mild acid should lead to cleavage of the chain, possibly with loss of label as the carboxylic acid ester hydrolyses further. This is what happens with chymotrypsin labeled with compound VIII: at pH 5 the chain is cleaved between residues Ser-214 and Trp-215.[129]

E. Affinity Labeling of Glycosidases with Carbohydrate Triazenes

The triazene deamination route has been used successfully to affinity label glycosidases. Detailed studies have been done with the β-galactosidases of *E. coli* with β-D-galactopyranosylmethyl *p*-nitrophenyl triazene.[130-133] Introduction of radioactivity into almost all carbohydrate-based affinity labels has been greatly simplified by the discovery of Koch and Stuart[134] that simply boiling a nonreducing carbohydrate with Raney Ni and D_2O results in exchange of all the carbon-bound hydrogens next to OH groups: with β-D-galactopyranosylmethylamine, the precursor of the triazene active-site reagent, the amino-bound methylene exchanges as well.[135] (Figure 14). With ^3H_2O, highly tritiated precursor has been obtained.[136]

Triazenes derived from the five carbohydrate primary amines XI-XV shown in Figures 14 and 15 have been tested as active-site-directed irreversible inhibitors towards various glycosidases: the results are summarized in Table 3. The triazenes are made by reacting 2 mol of amine with 1 mol of diazonium tetrafluoroborate in water, and are extracted away from ionic material by 1-butanol.

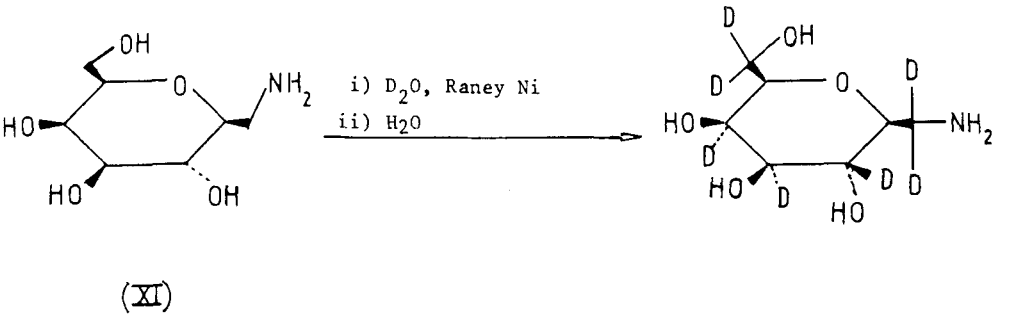
$$2\,RNH_2 + ArN_2^+ \longrightarrow R{-}NH{-}N{=}N{-}Ar + RNH_3^+$$

Where rates of inactivation are low, the data in Table 3 are approximate since not only does the inactivator concentration change over the time course of the experiment, but the decomposition products (e.g., (V), Figure 12) are often competitive inhibitors and hence protecting agents.

1. lacZ β-Galactosidase of E. coli

This enzyme is a tetramer and with Mg^{2+}-free enzyme and β-D-galactopyranosylmethyl *p*-nitrophenyl triazene it was shown that clean first order inactivation was observed to 95% inactivation; inactivation resulted in the incorporation of one mole of label per enzyme protomer.[130] Therefore no "half-of-the sites" reactivity was being observed. The Mg^{2+}-free enzyme is inactivated faster than the Mg^{2+}-enzyme, even though K values are lower for the metalloenzyme (Table 3). These higher K values probably account for the lower suicidal efficiency of the Mg^{2+}-free enzyme because of the consequent higher k_{off} values. For every four molecules of triazene decomposed by the enzyme, three active sites of Mg^{2+}-enzyme, but only one active site of Mg^{2+}-free enzyme, is alkylated.

The site of alkylation in both Mg^{2+}- and Mg^{2+}-free enzyme is methionine 500.[132] This was shown by sequencing of an active site peptide, the complete amino acid sequence being known. A dipeptide derivative containing the label was isolated by conventional methods[131] and shown to possess structure XVI (Figure 16) by hydrolysis to proline and by field desorption mass spectrometry. This technique for generating the molecular ion of an involatile compound would seem to be the method of choice for identifying labeled



(XI)

FIGURE 14. Sites of hydrogen isotope exchange during introduction of tritium into β-D-galactopyranosylmethylamine.

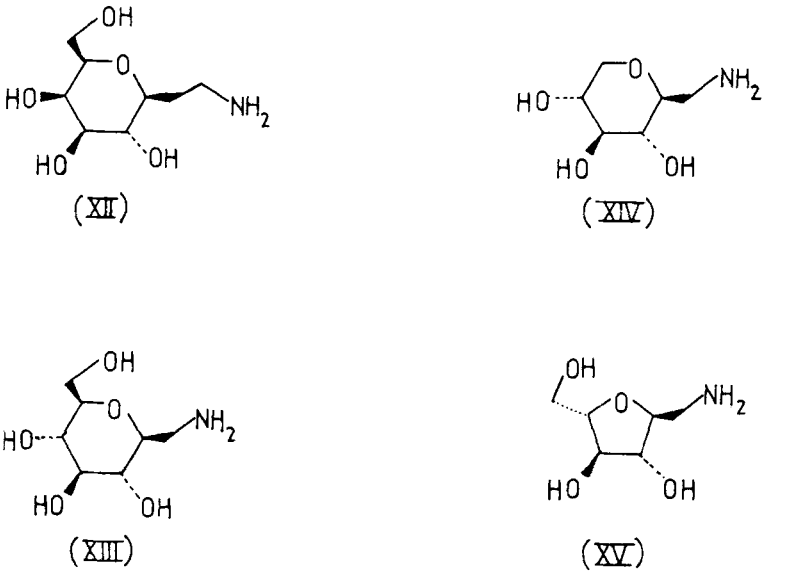


FIGURE 15. Structures of precursors of carbohydrate triazenes.

residues in proteins since, given the residue attached, the molecular weight (with only one or two ambiguities) immediately identifies the residue. Plausible pathways for formation of the derivative — and for the removal of label by cyanogen bromide — are given in Figure 16.

Lehmann's work[123-124] showed that β-D-galactopyranosyldiazomethane labeled both a methionine sulfur and a carboxylate. If both labels involve active site generation of a common diazonium ion this seems very puzzling. However, the stepwise addition technique made necessary by the extreme lability of the diazocompound in water may be the reason for the discrepancy, since it is possible that local high concentrations of the diazonium ion in free solution are the true labeling species.

The role of the methionine in the catalytic action of the enzyme is not clear. If it is the same methionine as labeled by N-bromoacetyl β-D-galactopyranosylamine,[137] then it is catalytically inessential. There is circumstantial evidence, however, that the adjacent residue (Tyr 501) may be catalytically important.[138] It is certainly strange that an electrophilic center generated exactly at the site to which, in a substrate, any acid catalysis would be applied, does not become attached to that catalytic group. If acid catalysis were

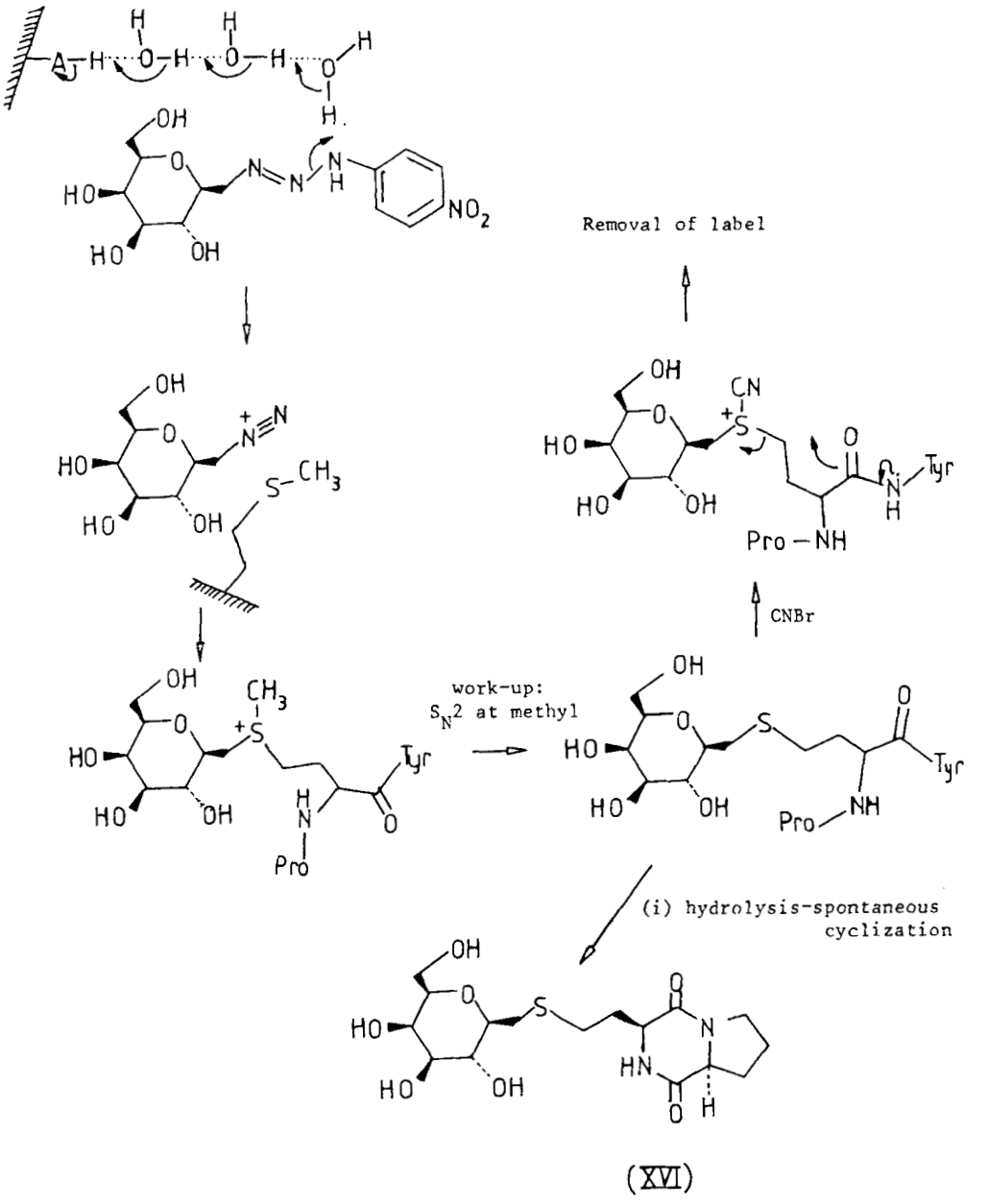


FIGURE 16. Affinity labeling of *lacZ* β-galactosidase of *E. coli* by β-D-galactopyranosyl methyl *p*-nitrophenyltriazene.

applied by the phenolic hydroxyl of Tyr 501, the rigidity and size of the residue make it plausible that it swings into action in a discrete kinetic step (for which there is some additional evidence[139]). If the enzyme has a conformational state in which the acid catalyst is not in place, this could explain why this group is not alkylated.

The *p*-nitrophenyl triazene is a "suicide substrate" — the rate of its decomposition in the E.Lbl complex with Mg^{2+}-free enzyme is 800 times that in free solution; but, the source of such a (by enzymic standards) modest rate increase is difficult to pin down.[131] Both k_{max} and k_{max}/Km vary with pH in a simple manner, a system of pK_a 7.5 in the E.Lbl complex and 6.5 in the free enzyme having to be in the protonated form for inactivation

to take place.[133] But the acid catalysis implied by this is in conflict with the β_{lg} value for inactivation of the enzyme by β-D-galactopyranosylmethyl aryl triazenes calculated from the data in Table 3. The value (−0.1 to −0.2, depending on pH and whether log k_{max} or log k_{max}/K is correlated with the pK_a of $ArNH_2$), indicating a transition state much more like that for unimolecular processes in triazene decomposition than those for bimolecular processes with appreciable proton transfer.

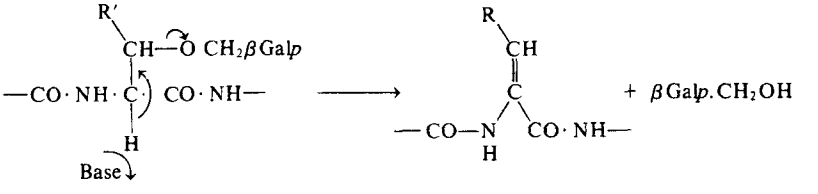
The β_{lg} values for enzyme inactivation are nonetheless significantly higher than those for the pH-independent triazene decomposition. Since the rate-enhancement is only 800 and acid catalysis has to be applied to the nitrogen atom of the triazene remote from the glycon, slight proton-transfer from an acid group via a water chain (or similar), as shown in Figure 16, is the likely mechanism for this inactivation.

Support for ideas of this type comes from the fact that aryl 2-(1-β-D-galactopyranosyl) ethyl triazenes are less effective than their lower homologs (Table 3), even though the extra methylene group insulates the reaction center from the electron-withdrawing effect of the carbohydrate moiety and the spontaneous triazene decompositions are, in consequence, faster.[54] A yet more remote site in the E.Lb1 complex is protonated even more ineffectively.

2. ebg β-Galactosidases of E. coli

E. coli possesses two β-galactosidases, the less effective *ebg* enzyme coming to light when it was found that after a time *lacZ* mutants of the bacterium could grow on lactose.[140] The hexameric enzyme[141] has been the subject of elegant studies on experimental evolution by Hall.[141,142] Enzymes of increased catalytic competence compared to the wild-type (*ebg*°) enzyme can be produced as a consequence of mutations in the genome of two general types.[143] *ebg*[a] Enzyme is a representative of Type I, and *ebg*[b] of Type II.

Like the *lacZ* enzyme the *ebg*° enzyme shows maximum activity towards O glycosides in the presence of Mg^{2+},[144] but maximal susceptibility to β-D-galactopyranosylmethyl *p*-nitrophenyl triazene inactivation in its absence (Table 3). The site of attachment of the label is a hydroxyamino acid, since the linkage between label and peptide is stable to the violent acidic conditions used for complete peptide hydrolysis, but is removed (as compound V) by comparatively mild base (pH 11 at 60° C).[135] This base lability is reminiscent of the base lability of glycopeptides glycosylated on serine or threonine, which, as peptides, can undergo $E1_{CB}$-like elimination reactions.[145]



Complete inactivation of *ebg*° enzyme of literature[141] specific activity results in the incorporation of 1 mole of label per 259 kg of protein.[135] The subunit molecular weight is 120,000.[141] As yet it is unclear whether the essentially similar enzyme isolation procedure used in both laboratories results in a preparation of this labile enzyme which is half denatured (although still hexameric), or whether half of the sites reactivity is being observed.

3. The Generality of Active-Site-Directed Irreversible Inhibition of Glycosidases by Glycosylmethyl p-Nitrophenyl Triazenes

The data in Table 3 allow the following conclusions to be drawn as to the likelihood of a glycosylmethyl *p*-nitrophenyl triazene inactivating a given glycosidase:

Table 3
ACTIVE-SITE-DIRECTED IRREVERSIBLE INHIBITION OF GLYCOSIDASE BY CARBOHYDRATE TRIAZENES

Parent amine	Parent diazonium ion	Enzyme	Source	pH	Temperature	10^3 k_{max}/s^{-1}	K/m*M*	$\log_{10}$ k_{max}/K	Ref.
XI	pCl $C_6H_4N_2^+$	Mg^{2+}-free β-galactosidase	*E. coli, lacZ*	8.0	25	0.34	1.6	−.679	54
XI	3,4 $Cl_2C_6H_3N_2^+$	Mg^{2+}-free β-galactosidase	*E. coli, lacZ*	8.0	25	0.45	0.9	−.302	54
XI	3,5 $Cl_2C_6H_3N_2^+$	Mg^{2+}-free β-galactosidase	*E. coli, lacZ*	8.0	25	0.43	0.55	−.112	54
XI	pCN $C_6H_4N_2^+$	Mg^{2+}-free β-galactosidase	*E. coli, lacZ*	8.0	25	0.55	1.6	−.468	54
XI	p$NO_2C_6H_4N_2^+$	Mg^{2+}-free β-galactosidase	*E. coli, lacZ*	8.0	25	1.3	.3	.637	54, 133
XI	p$NO_2C_6H_4N_2^+$	Mg^{2+}-free β-galactosidase	*E. coli, lacZ*	7.0	25	9.8	0.48	1.31	131
XI	p$NO_2C_6H_4N_2^+$	Mg^{2+}-β-galactosidase	*E. coli, lacZ*	7.0	25	0.4	0.07	0.74	131
XI	p$NO_2C_6H_4N_2^+$	Mg^{2+}-β-galactosidase	*E. coli, ebg*^{a}	7.5	25	0.023	0.2	−1.0	133
XI	p$NO_2C_6H_4N_2^+$	Mg^{2+}-free β-galactosidase	*E. coli, ebg*^{a}	7.5	25	0.14	1.8	−1.1	133
XI	p$NO_2C_6H_4N_2^+$	Mg^{2+}-free β-galactosidase	*E. coli, ebg*^{a}	7.5	4	0.01	3	−2.5	133
XI	p$NO_2C_6H_4N_2^+$	Mg^{2+}-free β-galactosidase	*E. coli, ebg*^{a}	7.5	25	0.2	0.6	−0.5	135
XI	p$NO_2C_6H_4N_2^+$	Mg^{2+}-free β-galactosidase	*E. coli, ebg*^{b}	7.5	25	0.7	~20	−1.5	135
XI	p$NO_2C_6H_4N_2^+$	β-glucosidase B	Sweet almonds		25			−3 to −4	133
XI	p$NO_2C_6H_4N_2^+$	(Lac repressor)	*E. coli, laci*	8.0	4	<0.01 at [triazene] = 1 m*M*			133
XI	p$NO_2C_6H_4N_2^+$	(Ricin RCA)	*Ricinus communis*		25	$<3\times10^{-3}$			54
XII	p$NO_2C_6H_4N_2^+$	Mg^{2+}-free β-galactosidase	*E. coli, lacZ*		25	0.5			54
XIII	p$NO_2C_6H_4N_2^+$	Mg^{2+}-free β-galactosidase	*E. coli, lacZ*	7.0	25			<−2.5	133
XIII	p$NO_2C_6H_4N_2^+$	β-glucosidase B	Sweet almonds	6.0	25			−1.2	133
XI	p$NO_2C_6H_4N_2^+$	β-galactosidase	Human liver lysosomes	6.0	25	6.8	0.02	2.6	133
XIV	p$NO_2C_6H_4N_2^+$	β-xylosidase	*Bacillus pumilus*	7.2	25			<−2	133
XIV	p$NO_2C_6H_4N_2^+$	β-xylosidase	*Penicillium wortmanni*	7.0	25			1.5_8	133
XV	p$NO_2C_6H_4N_2^+$	α-L-arabinofuranosidase	*Monilinia fructigena*, AFI	7.0	25			<−2.8	133
XV	p$NO_2C_6H_4N_2^+$	α-L-arabinofuranosidase	*Monilinia fructigena*, AFIII	7.0	25			−2	133
XI	p$NO_2C_6H_4N_2^+$	α-galactosidase	Coffee bean	6.0	25			−2	133
XIII	p$NO_2C_6H_4N_2^+$	α-glucosidase	Yeast	7.0	25			<−3	133
XIII	p$NO_2C_6H_4N_2^+$	Gluco amylase	Yeast	6.0, 7.0	25			<−3	133

1. Where both the aglycon of the substrate and the hydroxyl group of the aldose product occupy the same position with respect to the sugar ring as does the $-CH_2NH_2$ of the amine from which the triazene is derived, active-site-directed irreversible inhibition is observed. This is the case for the *lacZ* and *ebg* β-galactosidases of *E. coli,* human liver lysosomal β-galactosidase, sweet almond β-glucosidase B, the β-xylosidase from *Pencillium wortmanni,* and the α-L-arabinofuranosidase of *Monilinia fructigena.*[133] The only exception to this rule is another α-L-arabinofuranosidase from *Monilinia fructigena,* AF I. This enzyme however has a very acidic pH-optimum, and is strongly inhibited by triazene decomposition-products, so its apparent inertness could well have arisen from a combination of these two unfavorable effects.
2. Glycosidases are only inactivated by triazenes derived from the "wrong" sugar if they are truly nonspecific with regard to glycon, e.g., sweet almond β-glucosidase B, which has β-galactosidase activity and is inactivated by galacto triazene to some extent.
3. Some feeble inactivation can be observed with retaining α-glycosidases — e.g., coffee bean α-galactosidase[133] and human lysosomal α-glucosidase.[146] The phenomenon is not general, however.
4. Inverting glycosidases are not inactivated — the β-xylosidase of *Bacillus pumilus* and glucoamylase from *Aspergillus niger* being the cases in point. Work on this β-xylosidase (a thiol enzyme normally handled in the presence of protecting dithiothreitol or 2-mercaptoethanol) however revealed the *p*-nitrophenyltriazenes catalyze the air oxidation of such thiols[133] — possibly by acting as radical initiators (cf. Ref. 51).
5. Two proteins which bind but do not transform galactosides — *lac* repressor[133] and the castor oil bean lectin, RCA ricin,[54] are inert to the galacto triazene. This is in accord with the status of these triazenes as suicide substrates (rather than exo affinity labels) and is a *sine qua non* of their effective use in vivo.

F. Ouabain Receptor

An account has appeared of the attachment of a material produced from ouabain by the route shown in Figure 17 to the Na^+/K^+ ATPase of the electric organ of the electric eel. Three t.l.c. spots were produced, all of which were active against the digitalis binding sites to some extent. To the reviewer it seems extraordinary that the triazene function should have survived the treatment meted out to it (methanol at an "effective pH" of 5-6 at room temperature, followed by sodium cyanoborohydride) and, while an affinity label of sorts has unquestionably been produced, its molecular nature is not known.[147]

IV. IN VIVO ACTIVITY OF AFFINITY LABELS WHICH ARE ALSO DEAMINATION PRECURSORS

A. General Considerations

The selective inactivation of enzymes in vivo is realistically only attainable with enzyme inhibitors of the suicide type, since overtly reactive materials such as halocarbonyl compounds will alkylate transport proteins and (to some extent) most things which are remotely nucleophilic. A further complication of affinity labeling with these *exo* compounds in complex mixtures is made clear by data from Yon's group[148] on inactivation of *lacZ* β-galactosidase by N-bromoacetyl β-D-glucopyranosylamine and β-D-galactopyranosylamine. The second order rate constants, k_{max}/K, for inactivation of both compounds is very similar, with k_{max} and K being individually smaller for the *galacto* compound even though the enzyme has no mechanistically significant catalytic activity towards β-glucosides. It is also inert to β-glucopyranosylmethyl *p*-nitrophenyl

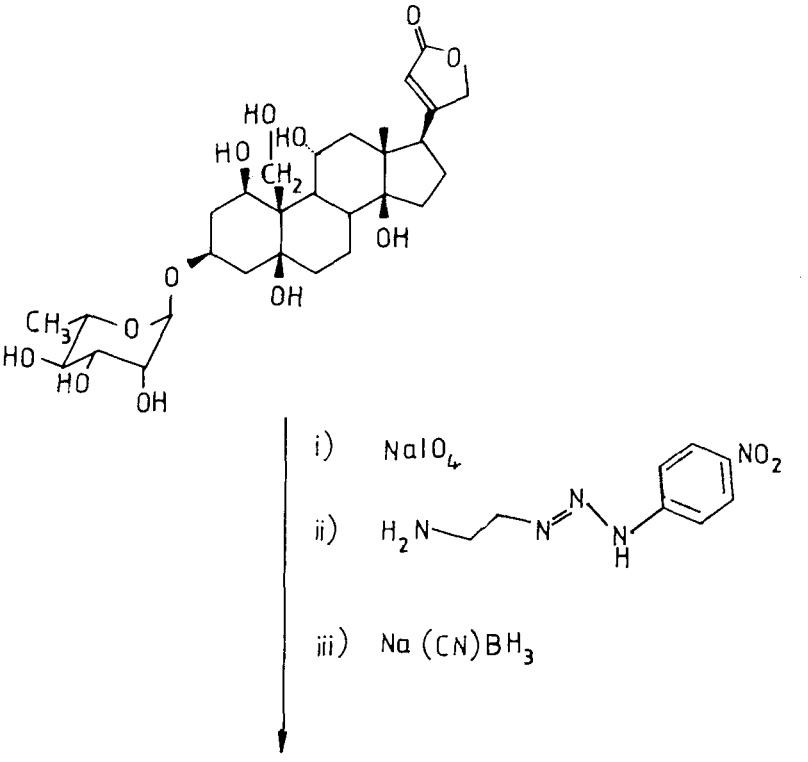


FIGURE 17. Production of inhibitory material from ouabain.

triazene; therefore, there is not a separate "glucose" binding site. Noncovalent binding of the N-bromoacetyl glucosylamine is disfavored but the unfavorable interactions resulting in this disfavored binding are presumably relieved when a covalent link is formed. Precisely the converse will happen when the alkylating agent fits snugly into the active site — the alterations in geometry consequent upon formation of a covalent link to the enzyme will disturb the snug fit in the noncovalent complex. The situation is entirely analogous to enzyme catalysis, in which low K_s values are associated with feeble catalysis, and a really good enzyme binds substrate weakly, if at all.[149]

Therefore, if an overtly electrophilic reagent is added to a complex mixture of proteins, these proteins will be labeled, not which best recognize the label noncovalently, but which best accomplish their own inactivation. Thus, addition of N-bromoacetyl glucosamine to a mixture of β-glucosidase and *lacZ* β-galactosidase would result in the alkylation of both enzymes.

If affinity labeling is performed with deamination precursors, however, the alkylating step is separate from and after the rate-limiting step, and there is thus a second chance at the diazonium ion stage of the noncovalent specificity of the enzyme being expressed. These ideas are considered in terms of free energy profiles for enzyme inactivation in Figure 18. The top profile refers to the data of Yon's group on *lacZ* β-galactosidase and N-bromoacetyl glycosylamines; the bottom to a hypothetical β-galactosidase which by some similar mischance of active-site chemistry decomposes the *gluco*-triazene faster than the *galacto*-triazene. Since the enzyme is a galactosidase, it will bind the *galacto*-diazonium ion more tightly than the *gluco*-diazonium ion — i.e., k_{off} for the "wrong" diazonium ion will be bigger with the consequently greater chance that it will come harmlessly off the protein before alkylating it.

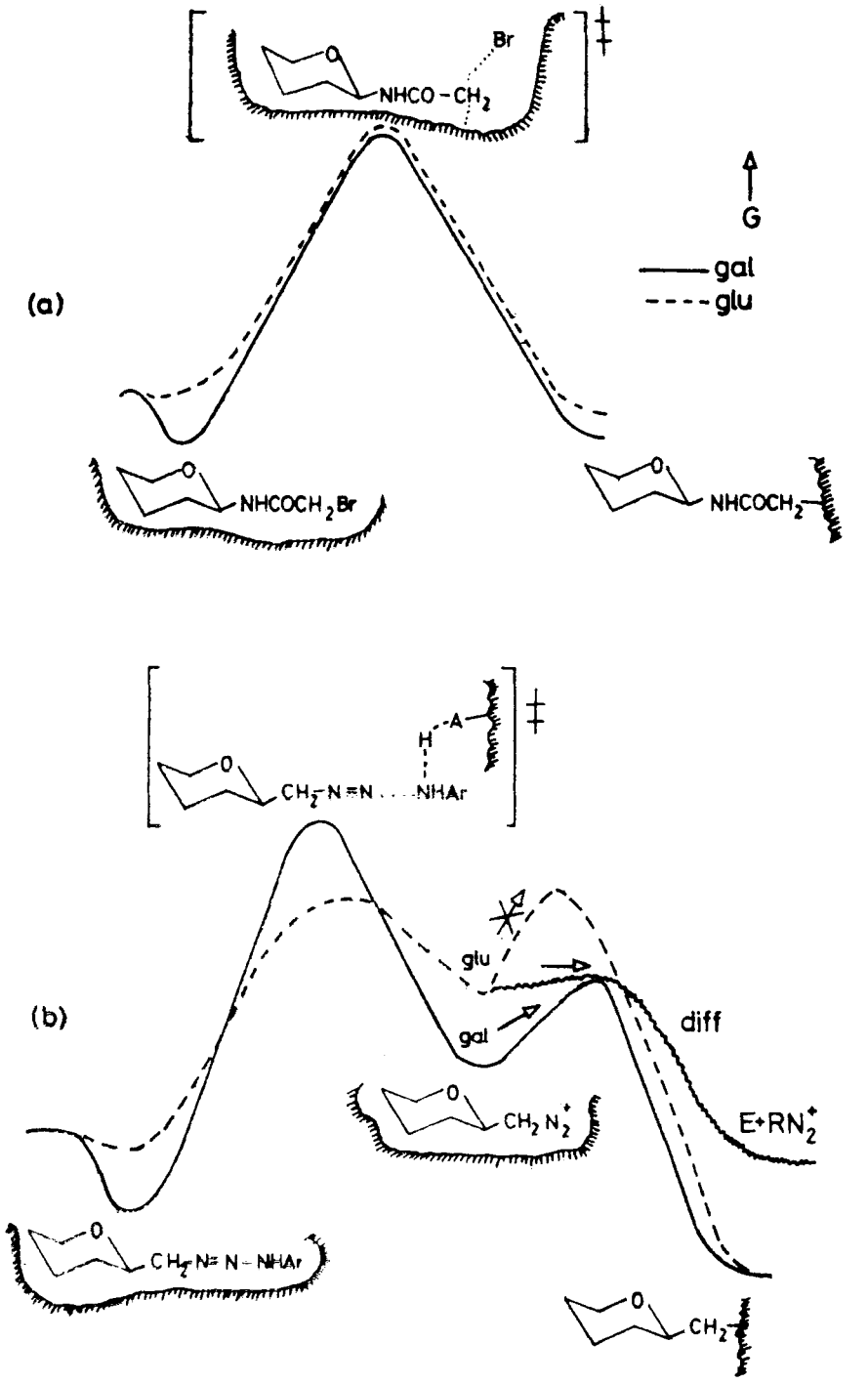


FIGURE 18. (A) Gibbs free energy profile for the alkylation of *lacZ* β-galactosidase by *N*-bromoacetyl-β-D-glucopyranosylamine (- - - -) and *N*-bromoacetyl-β-D-galactopyranosylamine (——). (B) Hypothetical free energy profile for reaction of, e.g., β-D-glucopyranosylmethyl- (- - - -) and β-D-galactopyranosyl- (——) (*p*-nitrophenyl)triazenes with a β-galactosidase where for some reason the calalyois of triazene decomposition is faster in the noncovalent EI complex with *gluco*- triazene rather than with *galacto*-triazene. The wavy line represents the common diffusional pathway of glucosylmethane- and galactosylmethane-diazonium ions to the activity site. (With permission from the Chemical Society.)

B. Diazoketones and Diazoesters

DON and azaserine were isolated and identified because of their action in vivo,[60,61] and with these compounds research was first of all concerned with identifying the enzymes which were inhibited as glutamine-dependent transamidases.[93,102] Interpreting results of in vivo experiments with these compounds becomes very complicated for two reasons. Firstly, there are many glutamine-dependent transamidases, so the potential sites of action are many and various, and without careful studies on purified enzymes it is difficult to know how much of what is inactivated. Secondly, since bacterial glutaminase on the average decomposes 70 molecules of DON to diazomethane before becoming deactivated itself,[70] there is a possibility of effects due to diazomethane rather than DON.

The use of DON and azaserine has however enabled the question of whether mammalian kidney γ-glutamyl transpeptidase is responsible for reabsorption of glutamine in the kidney to be settled in the negative. Use of ^{14}C DON enabled the membrane-bound enzyme to be shown to be facing the lumen.[82] Despite administration of DON having been shown to abolish the utilization of glutamine by human lymphoid cells,[150] in cultured human tumor cells it was shown that whereas γ-glutamyl transpeptidase activity was indeed abolished, the cells still retained the ability to transport amino acids.[151] Similar results were obtained with a line of rat tumor cells.[152]

Involvement of the enzyme in amino acid transport is made even more implausible by the discovery[153] that azaserine competitively and reversibly inhibits glutamine uptake by isolated rat renal brush-border membrane vesicles, but irreversibly inhibits γ-glutamyl transpeptidase activity.

DONV has been used to inactivate selectively the asparaginase of *E. coli* in vivo.[154] An auxotroph which required asparagine as a source of nitrogen treatment with DONV resulted in slower growth in a medium containing only asparagine, whereas normal growth was observed if the medium contained ammonia. On the growth of a different strain which required asparagine only for protein synthesis, DONV treatment had little effect. In addition to its irreversible action on asparaginase, DONV competitively inhibited asparagine uptake,[155] except for $\sim$20% of the activity which was irreversibly destroyed, most probably because of the inhibitory effects of DONV decomposition products. Interesting results have been obtained with the thiol protease inhibitor Z-Phe-AlaCHN$_2$.[156] This compound is taken up into cultured mouse peritoneal macrophages (apparently by pinocytosis) and there inactivates $\sim$40% of the protein-degrading activity and all the cathepsin B. Removal of the inhibitor results in a slow recovery of protease activity, presumably by *de novo* synthesis.

C. Triazenes

Little ambiguity attaches to the site of action of β-glucosylmethyl- and β-galactosyl-methyl *p*-nitrophenyl triazenes on cultured human fibroblasts. The *galacto*-triazene at 0.2 m*M* concentration in the culture medium inactivates the lysosomal β-galactosidase; the *gluco*-triazene inactivates the (membrane-bound) lysosomal β-glucosidase and also some of the α-glucosidase. No other effect is discernible; no other hydrolases are inactivated and the subsequent viability of the cells is not affected. Levels of β-glucosidase and β-galactosidase activity in triazene — treated cells recover exponentially to the original value — if zero-order-synthesis and first-order degradation of glycosidases is assumed, turnover times of 5 days for β-glucosidase and 10 days for β-galactosidase can be estimated.[146]

These times refer to cultured normal human fibroblasts. There are lysosomal storage diseases which are a consequence of a hereditary deficiency of lysosomal β-galactosidase. For the present purposes, three forms of this deficiency can be discerned: (1) classical GM_1-gangliosidosis, where β-galactosidase activity is completely absent; (2) variant GM_1-gangliosidosis, where it is present but at lower levels than normal; and (3) where it is

present at lower levels than normal and neuraminidase is also reduced, which we call β-gal$^-$/neur$^-$. By following the reappearance, after triazene treatment, of β-galactosidase activity in cultured cells of patients with variant GM_1-gangliosidosis, it was shown[136] that the turnover time was normal, whereas β-gal$^-$/neur$^-$ cells had a rapid turnover time ($\sim$1 day). Titration of the number of active sites in partially purified β-galactosidases from normal and defective cultured fibroblasts, using highly tritiated *galacto*-triazene, showed that the β-galactosidase associated with variant GM_1-gangliosidosis had a lower catalytic power per active site than normal, whereas that associated with β-gal$^-$/neur$^-$ galactosidase had the same activity as normal. It was thus possible to conclude that variant GM_1-gangliosidosis is caused by the synthesis of normal quantities of catalytically less effective β-galactosidase, whereas β-gal$^-$/neur$^-$ is caused by an accelerated degradation of unknown origin.

The *galacto* triazene has also been shown to selectively inactivate β-galactosidase in the liver and kidney of live mice, an injection of 3 μmol mouse^{-1} causing loss of approximately 80% of the β-galactosidase in these organs; however, brain β-galactosidase is unaffected.[157] The label clearly does not cross the blood-brain barrier.[157]

V. PROSPECTS

In the absence of coenzymes such as pyridoxamines to act as "anchors", deamination-chemistry offers the best hope of devizing active site reagents that will act in vivo. Diazoketones and diazoesters have the advantage of comparative stability, but the disadvantage that their reactivity is not properly understood.

Nitrosoamides are very unstable for general use and are impressively carcinogenic. Triazenes have considerably better stability if the parent alkylamine has a low pK_a, as with the glycosylmethyl derivatives, and they are less toxic, and triazenes could be designed to inactivate most enzymes which have nonacidic substrates — the triazene functionality is of course totally incompatible with any acidic function in the same molecule. If the "no-mechanism" rearrangement of N-nitrosoamides to diazonium carboxylates *can* be provoked by simple binding to proteins, then the more stable nitroamides would have advantages.

One intriguing possibility which arises from the unsuspected stability of diazonium ions in water is affinity labeling with actual diazonium ions in a quenched-flow apparatus.

Thus, there are prospects of designing affinity labels which incorporate deamination chemistry for the majority of enzymes. In view of the considerable superiority of these agents in point of specificity, the investment of somewhat greater effort in organic synthesis than is required for conventional reagents could be well rewarded.

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