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Review Article

Factors in the Design of Active-Site-Directed Irreversible Inhibitors

By B. R. BAKER

CLASSICAL ANTIMETABOLITES—those having only a small change in structure compared to the substrate—have such a limitation in the structural changes that can made (1), that utility is considerably restricted. In contrast, nonclassical antimetabolites—those having large but appropriate structural changes compared to substrate—should have much wider application (1, 2). For example, the concept of irreversible inhibition by the exoalkylation mechanism with nonclassical antimetabolites was proposed in 1959 (2). A properly designed inhibitor such as 4-(iodoacetamido)salicylic acid can reversibly complex with an enzyme such as GDH,[1] then become irreversibly bound within the complex by alkylation of the enzyme adjacent to the binding site; an expression more general than exoalkylation is "active-site-directed irreversible inhibition." This review will attempt to show the evidence for the phenomenon of active-site-directed irreversible inhibition, how the design of the required inhibitors can be experimentally approached, and the possible utility of the phenomenon in chemotherapy and protein structure studies.

CHEMICALLY REACTIVE FUNCTIONAL GROUPS ON ENZYMES

Since proteins in general and enzymes in particular are highly organized but heterogeneous polymers linked through a polyamide backbone, it has long been obvious that along the polymer chain there will be reactive functional groups; these reactive functional groups result from the third group in trifunctional amino acids such as the ϵ-amino group of lysine, the γ-carboxyl of glutamic acid, the mercapto group of cysteine, the hydroxyl group of serine and threonine, the methylthio group of methionine, the imidazole of histidine, the phenolic hydroxyl of tyrosine, and others. Over the years, many studies have been made with relatively common reagents that might be specific for one of these functional groups (3)—for example, heavy metal ions are apparently specific for thiol groups. However, not all the available thiol groups will necessarily react since some may be buried in the hydrophobic center of the enzyme. Furthermore, all thiol groups may not be necessary for the functioning of the enzyme; for example, 10 thiol groups of aldolase can be destroyed without loss of activity (4). Thus, it is clear that chemical interaction of simple reagents with enzymes by S_N2 type reactions would not have the specificity necessary to have relatively specific effects on one enzyme, let alone the specificity with a multiplicity of enzymes required for chemotherapy.

MAJOR MILESTONES IN ACTIVE-SITE-DIRECTED IRREVERSIBLE INHIBITION

Diisopropyl Fluorophosphate.—A major milestone in specificity of chemical reaction on an enzyme occurred with the discovery (3,

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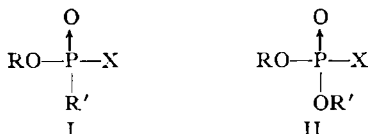
[1] The following abbreviations are used: GDH, glutamic dehydrogenase; LDH, lactic dehydrogenase; DFP, diisopropyl fluorophosphate; DPN, diphosphopyridine nucleotide.

5, 6) that DFP[1] was a specific reagent for the active site of esterases and proteolytic enzymes.



R and R′ = alkyl
X = —F, —CN, —$OC_6H_4NO_2$

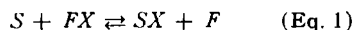
Many related compounds (I and II) have been found to have utility in clinical medicine and as insecticides (7). Although these compounds (I and II) will react with a number of esterases and proteolytic enzymes by formation of a phosphoryl serine in the active site, the action is limited only to this type of enzyme, and others are not inhibited (3). This specificity is clearly due to the unique arrangement in the active site and the mechanism of action of the enzymes that can be inhibited; in all cases an acylated enzyme is an intermediate in the enzymic hydrolysis of the substrate, and the acylated enzyme then undergoes further reaction to give the free enzyme and the acidic hydrolysis product. However, a properly substituted phosphate (II) can form a phosphorylated enzyme which may dephosphorylate to free the enzyme at such a slow rate that it results in selective inactivation at the active site; phosphonates can also interact in the same way. The interaction of DFP with cholinesterase (5, 6) can be considered to be the first example of *active-site-directed irreversible inhibition* that occurred by taking advantage of some unique property of an enzymic active site. It should be noted that of a total of 20 serine residues in chymotrypsin, only one reacts specifically with DFP (8), thus indicating the unique character of the single serine residue at the active site.

Azaserine.—The second milestone in specificity of chemical reaction with an enzyme occurred with the discovery that the L-glutamine (III) antagonists, L-azaserine (VII), and 6-diazo-5-oxo-L-norleucine (VIII) rapidly react chemically with the active site of the enzyme that converts formylglycinamide ribotide (V) to the corresponding amidine (VI) using L-glutamine (III) as the cofactor (9). Kinetic data indicated that L-azaserine (VII) was a competitive inhibitor for L-glutamine (III); rapid inactivation apparently occurred within the enzyme-inhibitor reversible complex by an internal alkylation reaction. Later work by Buchanan, *et al.* (10, 11), showed that a single cysteine residue had reacted specifically with azaserine, as determined by hydrolysis to S-carboxymethyl-L-cysteine. The internal alkylation reaction within the enzyme-azaserine complex can be considered the first example of *a properly constructed antimetabolite causing specific active-site-directed irreversible inhibition.* Azaserine chemically interacts with those enzymes that transfer the amide group of L-glutamine to substrate (12–14), but is not a general enzyme poison (2,10,11, 14). Thus, the unique specificity for this chemical interaction is dependent upon the nature of the active site.

ENDO AND EXOALKYLATION CONCEPTS

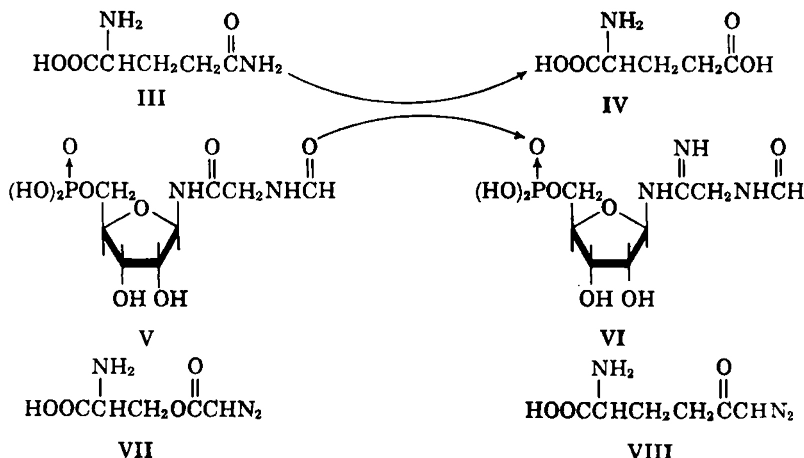
Endoalkylation.—The observations by Buchanan, *et al.* (9), on the selective irreversible inhibition by azaserine (VII) led Baker (2) to propose the concept of endoalkylation. When Buchanan's observations were combined with observations of over-sized inhibitors (15, 16), the concept of exoalkylation was also proposed (2).

Enzymatic reactions utilizing a cofactor can be written in the following general form



$$S + FX \rightleftarrows SX + F \qquad \text{(Eq. 1)}$$

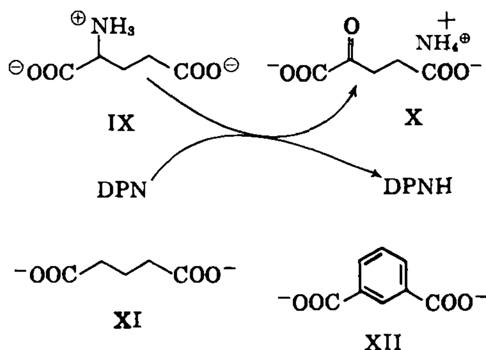
where S is the substrate and F is a cofactor bear-



ing a group X which is to be transferred to substrate. In order for the group X to be transferred to substrate, the group X must be held in juxtaposition between F and S by the active site of the enzyme as shown in Fig. 1; if more than a single interatomic distance exists between S and X, then new bond formation to form SX will be difficult. When the group, X, is replaced by B, as shown in Fig. 2, an inhibitor results if B is a group that cannot be transferred. If the group B is the diazomethyl group of azaserine (VII), then reaction with the SH group within the active site occurs as shown by the arrow; the formation of this new covalent bond stops the dissociation of the enzyme-inhibitor complex, and the active site becomes selectively denatured. Since the alkylating group, B, has replaced the transfer group, X, the alkylation most probably takes place *within the active site* (*endoalkylation*).

The active site may not be able to tolerate a group, B, that is bulkier than X since there may no longer be room for the juxtapositioning of S and FB; that such may be the case is indicated by the fact that the higher homolog of azaserine, α-diazopropionyl-L-serine shows no biological activity (17). This limitation on bulk greatly reduces the utility of the endoalkylation concept since only small changes in structure of the substrate or cofactor can be made. It was proposed (2) that another small B group that might be useful for endoalkylation would be the FCH_2CO- group; as will be described later, the $ClCH_2CO$- has been found useful for endoalkylation.

Exoalkylation.—To develop the concept of exoalkylation, the critical dimensions of an antimetabolite must be considered. Hellerman, *et al.* (16), observed that both glutarate (XI) and isophthalate (XII) effectively inhibited the GDH-catalyzed oxidation of L-glutamate (IX) to α-oxoglutarate (X)



by DPN. Since isophthalate (XII) can fit the active site of GDH as well as the substrate,

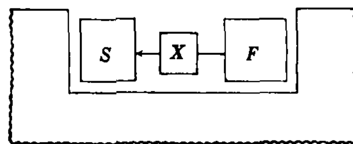


Fig. 1.—A simplified diagram of the enzyme-catalyzed transfer of the group X from a cofactor F to the substrate, S, both molecules being juxtapositioned at the active site of the enzyme.

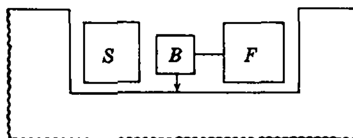
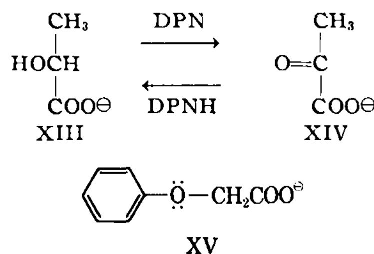


Fig. 2.—A simplified diagram of endoalkylation. A group, B, replaced the transfer group, X, in Fig. 1. B can then alkylate some nucleophilic group within (endo) the active site to form a covalent bond.

glutamate (IX), it is apparent that glutamate must assume the conformation equivalent to isophthalate when bound to the enzyme (16). It is also apparent that the remainder of the benzene ring of isophthalate (XII) constitutes a considerable change in gross measurement compared to the substrate, glutamate (IX) (2).

Inhibitors of the enzymatic oxidation of succinate to fumarate can vary considerably in gross measurements, providing the change is only at one α-position of succinate; thus α-alkylsuccinic acids, even with large alkyl groups, competitively inhibit succinic dehydrogenase (18).

Phenoxyacetic acid (XV) has been observed to be a competitive inhibitor of LDH;[1] it was proposed that phenoxyacetate (XV) binds to LDH[1] through its carboxylate group and an electron



pair of the ether oxygen the same as the substrate, lactate (XIII) (15). When bulky groups were placed on the benzene ring of phenoxyacetate (XV), little change in binding occurred (1), thus indicating that the benzene ring was not in contact with the enzyme surface (see Fig. 3).

If the alkylating group, B, (Fig. 3) is on the side of the inhibitor not in contact with the enzyme and is placed so that it can bridge to some nucleophilic group on the enzyme surface, then covalent bond formation might take place (shown

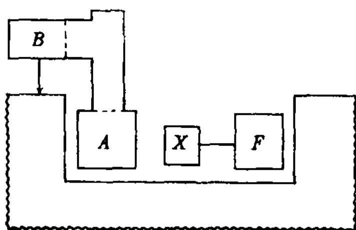


Fig. 3.—A simplified diagram of an oversize (nonclassical) inhibitor. Note that the excess size above the horizontal dotted line of substrate faces away from the enzyme surface. When the alkylating group *B* can bridge to a nucleophilic site on the enzyme surface adjacent to the active site, covalent bond formation (arrow) occurrs outside the active site (exoalkylation).

by arrow). The new bond formation outside the active site is termed exoalkylation; such bond formation can stop dissociation of the enzyme-inhibitor complex in the same fashion as the endoalkylation example with azaserine (VII).

EXAMPLES OF ENDO AND EXOALKYLATING IRREVERSIBLE INHIBITORS

After Buchanan's first announcement on the irreversible inhibition of an enzyme by azaserine (VII) (9), some 4 years elapsed before another example of an active-site-directed irreversible inhibitor appeared in the literature. In 1961, Baker, *et al.* (19), presented data that 4-(iodoacetamido)salicylic acid (XLVI) complexed with the active site of GDH, then caused irreversible inhibition by the exoalkylation mechanism. 4-(Iodoacetamido)salicylic acid is believed to be the first example of an active-site-directed irreversible inhibitor deliberately designed as such; the earlier examples, DFP and azaserine, were found to be active-site-directed irreversible inhibitors when their biological activity was investigated. In 1962, five other laboratories (20–24) announced their independently conceived work on active-site-directed irreversible inhibitors and in 1963 a seventh laboratory announced another example (25). Of these seven different examples, four were of the exoalkylation type and three were of the endoalkylation type. It is also of interest that five of the seven examples were irreversible inhibitors of chymotrypsin, one was an irreversible inhibitor of GDH, and one of a combining region of an antibody. Rather than discussing the seven examples in chronological order, the five examples with chymotrypsin will be discussed first.

Active-Site Labeling of Chymotrypsin.—The proteolytic enzyme, chymotrypsin (XVI), first complexes reversibly with the substrate (XVII, an ester or amide) to form XVIII; the reversible complex (XVIII) then undergoes an internal alcoholysis reaction with a serine residue to form the acyl chymotrypsin (XX) and an amine or alcohol (XVII*a*). Presumably through mediation of a nearby histidine residue, XX undergoes deacylation to give the acid (XIX) and regenerated enzyme (XVI). The enzyme is rather nonspecific and a variety of amides and esters, usually of L-configuration, can be hydrolyzed; the relative rates of acylation of XVI and deacylation can vary dependent upon the nature of XVII and the resultant seryl ester, XX. For example, pivalyl-chymotrypsin deacylates much slower than natural acyl chymotrypsins (26–28).

In an elegant study, Lawson and Schramm (22) selected *p*-nitrophenyl *N*-bromoacetyl-α-aminoisobutyrate (XXI) as a bifunctional reagent "so designed that it becomes covalently bound to an amino acid side chain at the active site, and then, fixed in position, reacts with another amino acid in the vicinity." The acyl group becomes bound to a serine residue at the active site (XX*a*), then attacks an adjacent amino acid (exoalkylation) which ultimately was shown to be a methionine residue (as in XXIII).

Perhaps fortuitously, but more likely by intent, Lawson and Schramm (22) selected an acylated chymotrypsin (XX*a*) that should deacylate slowly, analogous to pivalyl-chymotrypsin; such a slow deacylation gives a greater half-life for a molecule such as XX*a*, thus giving more time for a neighboring group reaction to take place with formation of XXIII. In 4 hours at ambient temperature, a 10:1 ratio of XXI to chymotrypsin (XVI) react to give a solution containing 20% of the original enzyme activity; this activity fails to decrease further with time. Evidence that the active site of chymotrypsin (XVI) is involved in this inactivation is as follows.

(*a*) The initial rapid "burst" of *p*-nitrophenol when XX*a* is formed is retarded by the presence of the reversible inhibitor, 3-indolepropionic acid, also retarding the rate of inactivation.

(*b*) *N*-Bromoacetyl-α-aminoisobutyric acid in a hundredfold molar excess causes no inactivation under the conditions used with XXI, thus giving evidence that an acyl-chymotrypsin (XX*a*) is an intermediate in the inactivation.

(*c*) When the serine residue of chymotrypsin is first blocked by conversion to the diisopropyl phosphoryl derivative with DFP (5, 6), no alkylation of methionine by XXI occurs (see Fig. 5), thus excluding a random alkylation reaction of the simple S_N2 type.

Lawson and Schramm (22) then showed by amino acid analysis that the 80% inactivated

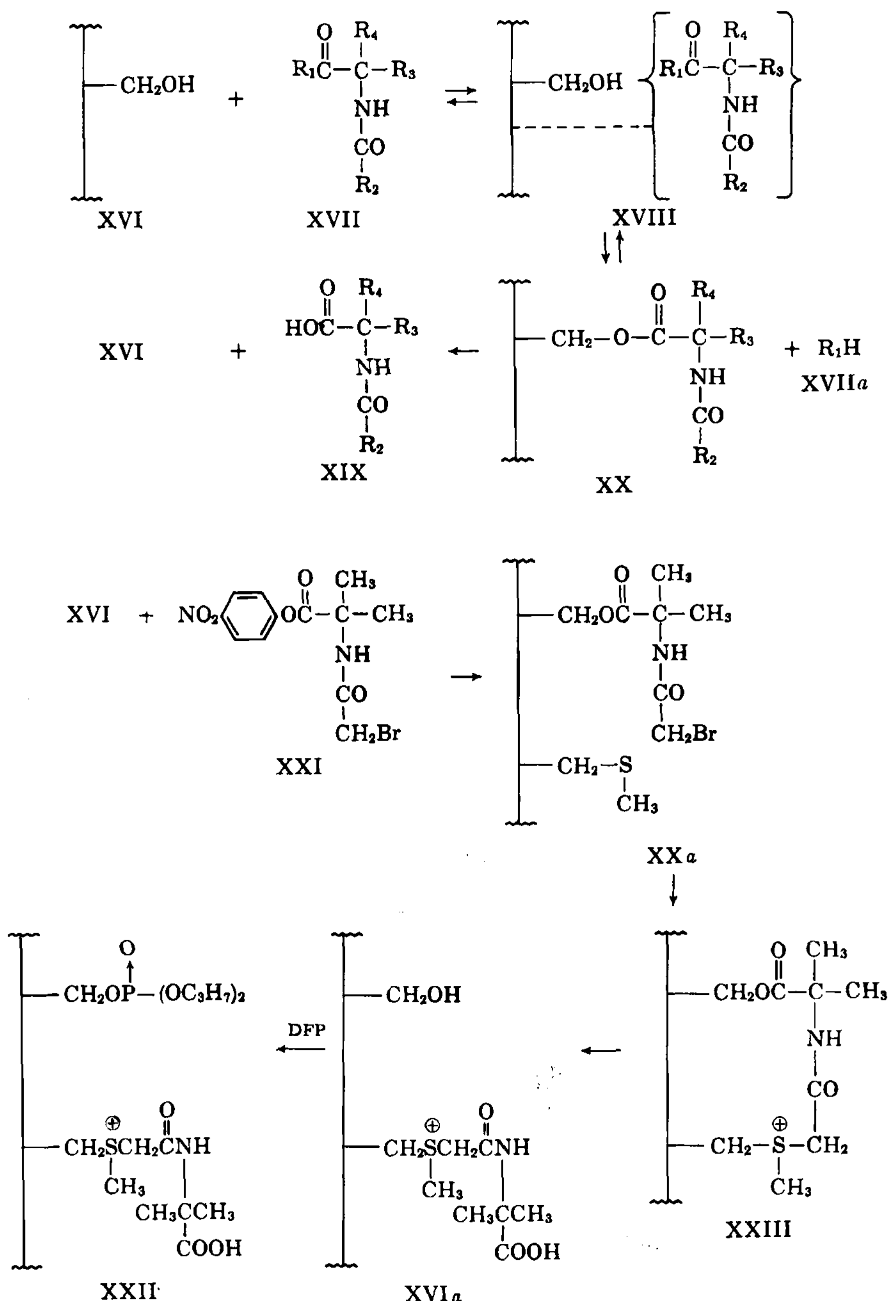


Figure 4.

chymotrypsin no longer contained one of its two methionine residues. The missing mole of methionine appeared as 0.2 mole of S-carboxymethyl-L-homocysteine (XXVI), 0.1 mole of homoserine (XXVII) and 0.3 mole of homoserine lactone (XXVIII); in addition, about 1 mole of α-aminoisobutyric acid (XXIV) was recovered that must have been bound (as in XVI*a*) to the 80% inactivated, but dialyzed, chymotrypsin. Such a splitting pattern of a carboxymethylated methionine residue in ribonuclease had been previously observed (28), and XVI*a* $\equiv$ XVI*b* could be expected to give a similar hydrolysis pattern.

That the inactivated chymotrypsin was a modified enzyme (XVI*a*) with only 20% of the activity of the original chymotrypsin (XVI) was also clearly demonstrated as follows.

(*a*) Further reaction of the modified chymotrypsin of 20% activity (XVI*a*) with DFP resulted in complete inactivation of the enzyme and 1 mole of phosphorus per mole of enzyme was introduced, as shown in structure XXII.

(*b*) The Michaelis constant (K_m) for tyrosine ethyl ester was increased by elevenfold; thus, the active site of XVI*a* is still capable of functioning, but offers some hindrance to reversible complexing with substrate in the normal fashion (XVIII).

At about the same time, Gundlach and Turba (23) reported a similar study with chymotrypsin (XVI*c*) and methyl iodoacetyl-phenylalaninate (XXIX). Since the conditions used by Gundlach and Turba for their inactivation studies are somewhat different than those reported by Lawson and Schramm (22), it is difficult to interpret the much slower rate of reaction of XXIX with chymotrypsin (XVI*c*) compared to XXI. In fact, the data of Gundlach and Turba could be interpreted to indicate that the two compounds inactivate by slightly different mechanisms, even though one methionine residue is attacked in both cases. Note the following information.

(*a*) The L-isomer of XXIX gives insignificant inhibition of chymotrypsin after 14 days at 0°, whereas the D-isomer of XXIX gives 35% inhibition under these conditions. Since *N*-acyl esters of D-phenylalanine are reversible inhibitors that do not form an acyl-chymotrypsin at a reasonable rate, it would appear that the inactivation by the D-isomer of XXIX might not occur through an acyl-chymotrypsin such as XX*a*, but might occur through the initial reversible noncovalent complex of type XXX with formation of XXXI without loss of the ester group. This point could be resolved by using a C^{14}-methyl ester group or a *p*-nitrophenyl ester (22).

(*b*) That inactivation may also occur through a reversible complex such as XXX is further supported by the fact that the rate of incorporation of nondialyzable C^{14} from C^{14} labeled XXIX parallels the rate of inactivation; if inactivation occurred *via* a covalent complex such as XX, labeling might be expected to proceed more rapidly than inactivation, particularly with a *p*-nitrophenyl ester.

(*c*) Iodoacetamide and iodoacetate gave no inactivation under the same conditions used for XXIX, excluding a random S_N2 inactivation mechanism.

Since the inactivation of chymotrypsin by XXI (Fig. 4) and by XXIX (Fig. 5) may proceed by slightly different mechanisms, it cannot be certain at this time that the same methionine is attacked in both cases. Needless to say, the experiments of Lawson and Schramm (22) are more clean-cut mechanistically than those of Gundlach and Turba (23); the latter workers could certainly clarify the situation by using some of the techniques of Lawson and Schramm.

Gundlach and Turba (23) consider the alkylation reaction that forms XXXI to be an introduction of "a reactive residue into the 'active center' of the enzyme." Since we consider the formation of XXXI (Fig. 6) and of XXIII (Fig. 4) to occur by the exoalkylation mechanism "adjacent to the active site" (1, 2, 19), some redefinition may be necessary, as will be discussed later.

The reports from the other three laboratories (20, 24, 25) on inactivation of chymotrypsin meet

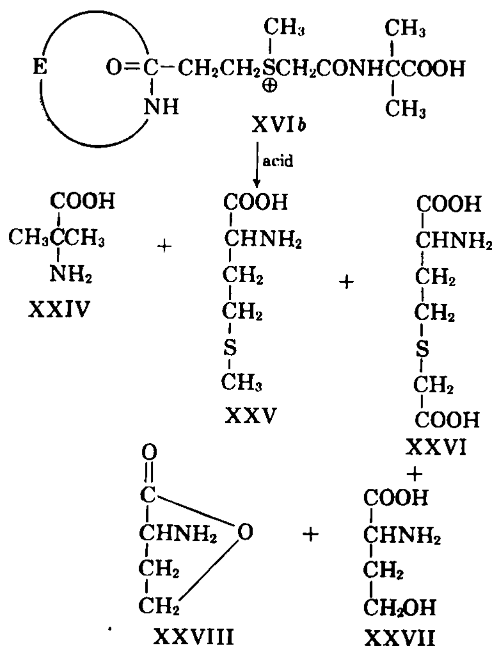


Figure 5.

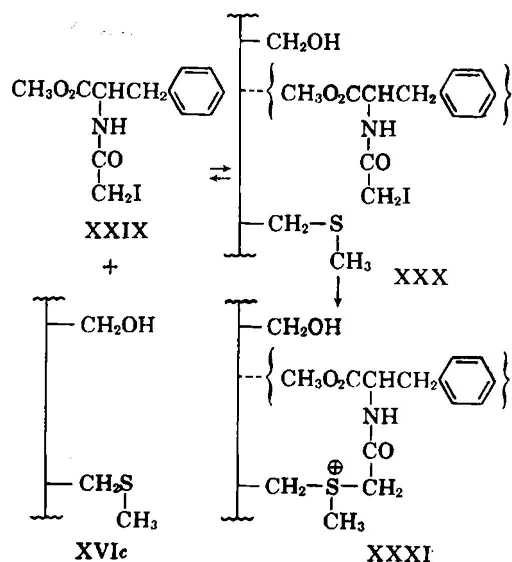


Figure 6.

our earlier criteria (2) for alkylation within the active site, so-called "endoalkylation."

Schoellmann and Shaw (20) wished "to develop new, specific reagents for locating the active centers of enzymes by designing molecules which combine two kinds of structural features—namely, those which provide affinity to the active center and, in addition, a chemically reactive grouping which may anchor irreversibly to the enzyme. . . ." Based on the observation that *N*-tosyl-L-phenylalanine ethyl ester was a substrate for chymotrypsin (29), they synthesized and evaluated the related chloromethyl ketone (XXXII) as an irreversible inhibitor (see Fig. 7) with the following results.

(*a*) The chloromethyl ketone (XXXII) gave 50% inactivation of chymotrypsin (XVI*d*) in 50 minutes at pH 6 and 37°. The rate of inactivation was slowed in the presence of the reversible inhibitor, β-phenylpropionic acid, indicating that the active site was involved.

(*b*) When C^{14}-chloromethyl ketone (XXXII) was employed, a 1:1 ratio of nondialyzable C^{14} to chymotrypsin was observed after inactivation was complete. In the presence of 8 *M* urea, less than 3% binding of C^{14}-XXXII took place under the same conditions, showing that the loss of the tertiary structure and active site caused by the urea also caused loss of chemical reaction with XXXII, implicating a complex with the active site (XVIII*a*) as being a necessary prerequisite for inactivation.

(*c*) *N*-Methylation has been shown to decrease greatly the ability of the modified derivatives to serve as substrates; the *N*-methyl derivative of XXXII gave no inactivation of chymotrypsin under the conditions that XXXII did, thus implicating a reversible complex with the active site (XVIII*a*) as being an intermediate to inactivation.

(*d*) The inactivation of chymotrypsin by XXXII showed a pH profile of dependence with a peak near pH 7.2, the same as the pH profile of the enzymatic reaction; these data also implicate participation of the active site in the inactivation.

(*e*) Amino acid analysis of chymotrypsin inactivated by XXXII showed the loss of one of the two histidine residues originally present in the enzyme; thus, the inactivated chymotrypsin probably has a partial structure of type XXXIII or the corresponding N_1-imidazolyl derivative.

(*f*) When DFP inactivated chymotrypsin was treated with XXXII, no loss of histidine was found, indicating that the reversible complex XVIII*a* is an intermediate to inactivation.

(*g*) That XXXII was not a general enzyme poison was shown by a comparable incubation with trypsin; no loss in activity occurred.

Even though the type of inactivation ("endoalkylation") given by the chloromethyl ketone (XXXII) has precedent in the azaserine (VII) inactivation discussed earlier, the beautiful work of Schoellmann and Shaw (20) stands as a milestone in the progress toward design of active-site-directed irreversible inhibitors. Kallos (25) has independently found a closely related "endoalkylation" of chymotrypsin with "phenylalaninol ditosylate" (XXXIV). The data are meager, and the

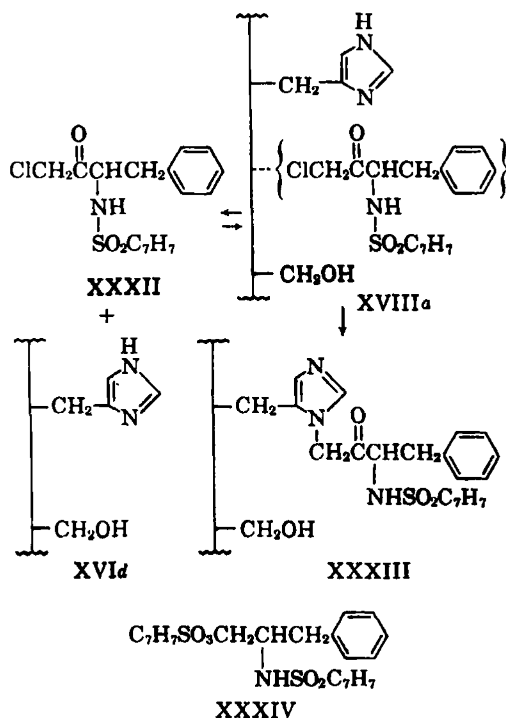


Figure 7.

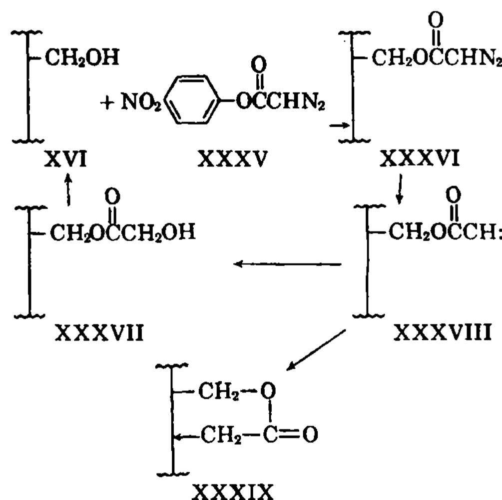


Figure 8.

only evidence that a simple S_N2 inactivation was not the mode of inactivation was that the D-isomer of XXXIV leveled off at 40% inactivation, whereas the L-isomer leveled off at 80% inactivation. Without the thorough work of Schoellmann and Shaw (20) preceding Kallo's work, XXXIV could not even be implicated as an active-site-directed irreversible inhibitor; certainly further criteria such as those used by Schoellmann and Shaw (20) will have to be applied by Kallos before XXXIV can be reasonably accepted as an active-site-directed irreversible inhibitor.

Westheimer, *et al.* (24), have used a photolytic method for introducing a C^{14}-carbene into chymotrypsin (XVI). When the enzyme was reacted with *p*-nitrophenyldiazoacetate (XXXV), (Fig. 8), an acyl-chymotrypsin (XXXVI) was obtained that was stable to deacylation, as previously observed with pivalyl-chymotrypsin and XX*a* (22); however, chymotrypsin (XVI) could be regenerated by treatment of XXXVI with hydroxylamine, then assayed. Since the diazoacetyl chymotrypsin (XXXVI) had a weak absorption band at 370 mμ, this wavelength was used in the photolytic decomposition to minimize photolysis of the parent enzyme. Unfortunately, the resultant carbene (XXXVIII) underwent solvolysis to glycolyl-chymotrypsin (XXXVII) in 80% yield and only 20% inactivation by an insertion reaction (symbolized by XXXIX) took place; the 20% loss of enzyme by formation of XXXIX paralleled a 20% uptake of C^{14} from C^{14}-labeled XXXVI. An investigation of the position of attack by the carbene is being actively pursued, and results will be awaited with interest.

Affinity Labeling of Antibodies.—Wofsy, Metzger, and Singer (21) have used an approach similar to "exoalkylation" for attaching covalently bound groups to antibody molecules, which they termed "affinity labeling." It had been known for a number of years that when a protein is coupled with a diazonium salt such as diazotized arsanilate (XL*a*), then injected into a rabbit, antibodies (XLI*a*) were produced that had specific combining regions dependent upon the character of the attached azo grouping, called a hapten (XL). The use of other compounds attached to the same protein (XL) gave antibody molecules (XLI) that were specific for the particular haptenic determinate in forming insoluble complexes (XLII). It was also known that the hapten (XL*a*)-antibody (XLI*a*) complexing to give the insoluble XLII*a*, could be decreased if the combining region of XLI*a* was complexed with a small molecule such as *p*-nitrobenzenearsonic acid. Wofsy, *et al.* (21), chose this system for studying the nature of the combining regions by covalently attaching specific reagents that were related in structure to the hapten; this system has the advantage that haptenic combining regions can be designed more or less to order.

They reasoned that the diazonium salt (XLIII), from which the original modified protein (XL) was prepared, could combine with the specific antibody (XLI) to form a reversible complex (XLIV), then undergo irreversible reaction by formation of a covalent linkage as depicted in XLV. That affinity labeling (XLV) of the combining region (XLIV) of an antibody could take place is demonstrated in Figs. 9 and 10.

(*a*) The antibody for azobenzenearsonic acid (XLI*a*) reacted rapidly at 0° with the arsonic diazonium salt (XLIII*a*) to form a colored azo derivative (XLV*a*), the rate of formation being followed spectrophotometrically. Of the three types of azo compounds that can be formed in a protein—namely, azohistidine, azolysine, and azotyrosine—only the latter was formed with a peak at about 475 mμ. No azohistidine peak was formed.

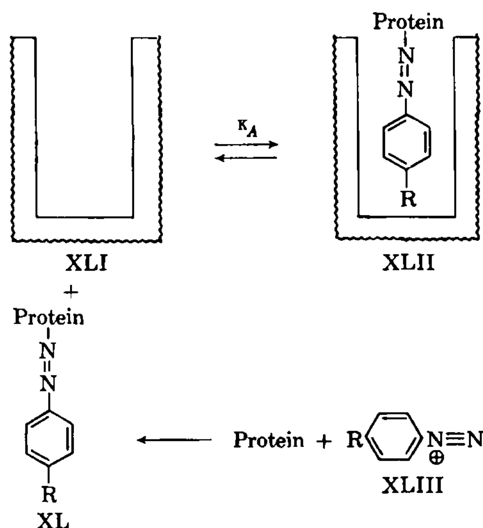
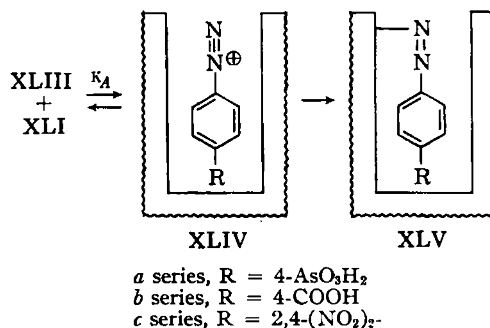


Figure 9.



a series, R = 4-AsO_3H_2
b series, R = 4-COOH
c series, R = 2,4-$(NO_2)_2$-

Figure 10.

(*b*) Normal γ-globulin reacted with the arsonic diazonium salt (XLIII*a*) at one-four-hundredth the rate of XLI*a*. With the assumption that there was only one tyrosine in each of two active sites on the antibody molecule (XLI*a*), but that there were 60 tyrosine residues in a normal γ-globulin molecule, the enhancement of the rate of reaction with XLI*a* was about 10,000; "such a large enhancement is explicable only by the mechanism of initial reversible complex formation [XLIV*a*], according to the theory proposed."

(*c*) The heterologous reagent, *p*-carboxybenzene diazonium salt (XLIII*b*), reacted with the arsonic antibody (XLI*a*) at the same slow rate it reacted with normal γ-globulin, thus showing that there is not a uniquely reactive tyrosine in the arsonic antibody (XLI*a*); these results were consistent with the concept of affinity labeling; that is, when an initial reversible complex (XLIV) is formed, the rate of azo reaction is greatly accelerated by an anchimeric reaction.

(*d*) When the combining region (XLI*a*) of the arsonic antibody was protected by initial complexing with *p*-nitrobenzenearsonic acid, then XLI*a* reacted at the same slow rate with either the arsonic diazonium salt (XLIII*a*) or the carboxybenzene diazonium salt (XLIII*b*) at other regions than the haptenic site.

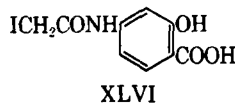
(*e*) An antibody (XLI*c*) specific for the 2,4-dinitrophenyl hapten reacted with a 5-mole ratio of 2,4-dinitrobenzenesulfonyl chloride to give 50% loss of antibody activity in 2 hours at 25°. In the presence of a 50-mole ratio of ε-(2,4-dinitrophenyl)lysine, no loss of antibody activity occurred due to the protective effect of the latter by complex formation.

(*f*) Treatment of the antiarsonic antibody (XLI*a*) with a 10-mole ratio of 2,4-dinitrobenzenesulfonyl chloride under the conditions of (*e*) gave no loss of antibody activity.

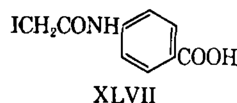
(*g*) Kinetic studies showed that increasing the concentration of diazonium reagent (XLIII) increased the rate of reaction with antibody (XLI) clearly less markedly than would be expected for a simple second-order reaction for formation of the labeled antibody (XLV). They demonstrated "that the concentration dependence is entirely accounted for by the unimolecular mechanism" *via* the obligatory formation of a reversible complex (XLIV); that is, the rate of reaction was dependent on the concentration of XLIV.

Wofsy, *et al.* (21), consider that the combining region has been labeled in XLV, again differing, as have Gundlach and Turba (23), with our definition "adjacent to the active site;" some redefinition of "exoalkylation," as discussed later, could clarify these differences in semantics.

Inactivation of Lactic and Glutamic Dehydrogenases.—Evidence for the inactivation of LDH and GDH by 4-(iodoacetamido)salicylic acid (XLVI) by active-site-directed irreversible inhibition has been presented by Baker, *et al.* (19).



XLVI



XLVII

(*a*) GDH was about 50% inactivated in 10 minutes at 37° by a 2 m*M* solution of XLVI; iodoacetamide showed no inactivation under the same conditions, thus indicating that a complex between enzyme and inhibitor was necessary for inactivation.

(*b*) GDH was inactivated eight times more rapidly by 2 m*M* 4-(iodoacetamido)salicylic acid (XLVI), then by the same concentration of 4-(iodoacetamido)benzoic acid (XLVII).

This difference in rate was attributed to the difference in enzyme-inhibitor dissociation constants; the K_i for XLVI was smaller, thus giving (at the same inhibitor concentration) a larger amount of reversible enzyme-inhibitor (XLVI) complex; the rate of inactivation is presumably dependent on the concentration of reversible complex.

(*c*) Increasing the concentration of XLVI from 1 m*M* to 2 m*M* increased the rate of inactivation of GDH by 1.23-fold, rather than twofold; this is considered evidence that the active-site is involved in the irreversible inhibition since the ratio of the rates calculated from K_i is 1.23.

(*d*) Although 4-acetamidosalicylic acid was a reversible inhibitor of GDH as good as XLVI, the former showed no irreversible inhibition of GDH; this experiment was considered evidence that the inactivation by XLVI was not due to a chelation effect of the salicylate structure.

(*e*) The inactivation of GDH by traces of common metallic ions such as silver, iron, copper, chromium, nickel, cobalt, or zinc was considered unlikely since there was no correlation between the trace metal content of XLVI, XLVII, and 4-acetamidosalicylic and their respective rates of inactivation of GDH.

(*f*) That the active site was presumably involved in the inactivation of GDH by XLVI was further indicated by the slower rate of inactivation in the presence of the reversible inhibitor, isophthalate (16)—a protective effect.

The criteria for the inactivation of LDH by XLVI by the exoalkylation mechanism were the same as used for GDH; the same results and con-

clusions were obtained as in the GDH case with two exceptions. The first exception was that the reversible inhibitor, oxamate (30), was used in the protection experiment (*f*) and slowed the rate of inactivation. The second exception was that iodoacetamide as well as XLVI showed irreversible inhibition of LDH, However, 2 m*M* XLVI inactivated LDH at about twice the rate of 4.48 m*M* iodoacetamide. As will be discussed later under the bridge principle, several *N*-substituted iodoacetamides, including 3-(iodoacetamido)oxanilic acid (XLIX), failed to give any measurable irreversible inhibition of LDH, indicating that a properly oriented reversible complex with XLVI is obligatory to inactivation.

ELUSIVE DEFINITION OF AN ACTIVE-SITE

Some difference of opinion exists regarding whether the active-site has been labeled with the specific reagents discussed in the previous section; at this early stage in the science of "mapping the active site," such *a priori* arguments can at best be only differences in philosophy. We have defined alkylation within the active-site stringently as "endoalkylation" (2). Of the work described in the previous section, only that on azaserine (VII), DFP, Shaw's chloromethyl ketone (XXXII), phenylalaninol ditosylate (XXXIV), and diazoacetyl-chymotrypsin (XXXVI) clearly meet these criteria. Those compounds that presumably operate by our definition of "exoalkylation" include Lawson's *N*-bromoacetyl derivative (XXI), Gundlach's *N*-iodoacetyl derivative (XXIX), Singer's hapten-type diazonium salts (XLIII), and Baker's iodoacetamido salicylic acid (XLVI). The argument is dependent upon how large to define the active-site, which obviously cannot yet be done. Two points emerge on which the various involved investigators will most probably agree. (*a*) The "exoalkylation" type definitely forms a covalent bond *adjacent to the reversible combining region of the inhibitor* (region *A* in Fig. 3). The combining region for a substrate or inhibitor is obviously smaller than the total active-site since other groups on the enzyme must be present in the active-site to catalyze the enzymatic reaction. (*b*) Initial complexing with the active-site is an obligatory intermediate to the formation of an irreversible covalent bond; that is, the reaction is controlled by the active-site with its inherent specificity.

The term "exoalkylation" also breaks down in describing adequately the mode of irreversible inhibition caused by 5-(carbophenoxyamino)-salicylic acid (LI), to be discussed in a following section; LI is clearly an acylating agent. Other irreversible inhibitors are neither an alkylating nor an acylating agent, but operate by Michael addition, such as LVI.

At the current level of knowledge concerning the active site, an expression is needed which will cover all of the cases so far discussed. Two expressions emerge which appear to describe the phenomenon adequately without defining the size of the active-site: (*a*) "active-site-directed irreversible inhibition" (31), and (*b*) "affinity-labeling," (21) providing "the remaining question whether the bond is formed with a group actually in the active site or one very close to, but not part of, the site" is left open for future definition when more data become available from mapping studies.

As the title of this review indicates, we plan to use expression (*a*).

BRIDGE PRINCIPLE OF SPECIFICITY

As pointed out in an earlier section, the first two milestones in active-site-directed irreversible inhibition were found by studying the biological mechanism of action of DFP and azaserine (VII). Except for the work in our laboratory, the main concern of subsequent investigators in the period of 1962–1963 was to use the phenomenon for the labeling of active sites of pure enzymes or of specific, though heterogeneous (21*c*), antibodies. Depending upon the goal of the particular investigator, two different criteria for specificity can be envisioned. If the investigator wishes to label the active-site of a pure protein, then he is concerned with finding a reagent that will react specifically with the active-site and have a near zero effect on other parts of the pure protein. In contrast, if the investigator wishes to use "active-site-directed irreversible inhibitors" in chemotherapy, the problem is less concerned with exactly how a given enzyme becomes inactivated and more concerned with the specific inactivation of a single enzyme in an intracellular mixture of enzymes in a functioning cell.

That these two apparently different goals initially converge into one approach becomes apparent when one realizes that the specificity required for inactivation of one enzyme in an intracellular mixture of enzymes must logically reside in the more or less subtle differences in the active-site of enzymes that perform different—but at times mechanistically closely related—enzymic reactions. Once the labeling reagent for an active-site has been found, the experimental approaches to the two goals then diverge dramatically in methodology. The purpose of studying the labeling of the active-site of a pure enzyme is to determine the amino acid sequence surrounding the

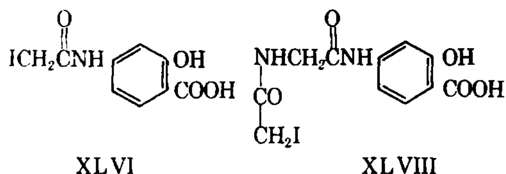
label in order to learn what part of the sequence is at (or close) to the active-site (mapping the active-site). In chemotherapy, the problem is different since the labeling reagent must now be studied to determine how specific it may be in reacting with only one of a number of mechanistically closely related enzymes; it is the latter problem to which we have been devoting our attention, particularly how an active-site-directed irreversible inhibitor may be modified to build in further specificity. This problem has been approached in the following way.

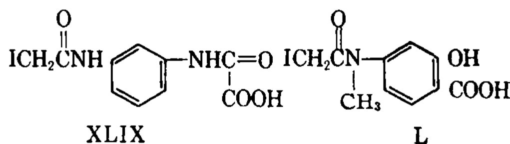
The earlier definition of "exoalkylation" (2) and the experimental support for this phenomenon (19, 21–23, 32) led to the concept (19*b*) of and experimental evidence (31–36) for the bridge principle of specificity; this principle has been restated (31), replacing the word "exoalkylation" by "active-site-directed irreversible inhibition:" "Compared to a reversible inhibitor, the active-site-directed type of irreversible inhibitor can have an extra dimension of specificity; this extra specificity is dependent upon the ability of the reversibly bound inhibitor to bridge to and form a covalent bond with a nucleophilic group on the enzyme surface and upon the nucleophilicity of the enzymic group being covalently linked."

Nucleophilic Sites.—The bridge principle can be envisioned by inspection of Fig. 3. The group, *B*, of an active-site-directed irreversible inhibitor can form a covalent bond with a nucleophilic group adjacent to the reversible binding region where group *A* is reversibly complexed. In order for this covalent bond to form (shown by the arrow), there must be a proper distance between the *A* and *B* regions of the inhibitor so that bridging can take place; in addition, the electrophilic group, *B*, and the enzymic nucleophilic group must have the ability to interact, since obviously not all electrophilic groups react with all nucleophilic groups. Furthermore, the approach of the electrophilic group to the enzymic nucleophilic group must meet the combined steric and conformational requirements of the active-site, the nucleophilic site, and the environment in between these two sites. These dual site requirements are obviously far more restrictive than the mere additive requirements of each site alone (34). Therefore, the use of the bridge principle should make it possible to obtain highly selective irreversible inhibitors within any group of enzymes that are closely related by the nature of their substrates. Furthermore, it should be noted that an irreversible inhibitor will have a much greater effect on the operation of an enzyme than a reversible inhibitor. The enzyme molecule is still more or less operational in the presence of a reversible inhibitor and substrate, but an irreversibly inhibited enzyme molecule can be completely inoperative. Since theoretically only one irreversible inhibitor molecule is necessary to inactivate one enzyme molecule, and since one active enzyme molecule can convert many substrate molecules to product, an irreversible inhibitor will be far more effective than a reversible inhibitor. Thus, an inhibitor will be more effective on an enzyme that can be first reversibly then irreversibly inhibited than a closely related enzyme that is only reversibly inhibited since enzymes have an estimated concentration of 10^{-10} to 10^{-12} M, while substrates have a concentration in the range of 10^{-3} to 10^{-6} M.

Conformational and Steric Aspects.—For the initial studies on the bridge principle, Baker (33, 34) selected the mechanistically closely related enzymes, GDH and LDH. Earlier, a study of a variety of reversible inhibitors showed that almost all of the compounds that inhibited GDH also inhibited LDH (19*b*, 37); since these two enzymes perform similar reactions, *i.e.*, dehydrogenation at the α-position of an anionic substrate, and are therefore by mechanistic necessity closely related, it is not surprising that the two enzymes are inhibited by similar compounds. In contrast, *the adjacent nucleophilic sites would most likely be dissimilar*, since these nucleophilic sites probably have no function in the mechanistic operation of the enzymes, but are probably part of the protein molecule necessary for the integrity of the protein's secondary-tertiary structure. Thus, it was anticipated that relatively minor changes in inhibitor structure could greatly influence the bridging ability of the inhibitor.

Previously 4-(iodoacetamido) salicylic acid (XLVI) had been shown (19) to be an irreversible inhibitor of both LDH and GDH. A number of closely related compounds were then investigated as irreversible inhibitors of the two enzymes in a search for irreversible specificity (33, 34). When the length of the bridge was increased as in XLVIII, both GDH and skeletal muscle LDH were inhibited both reversibly and irreversibly, thus showing no irreversible specificity (Table I). However, two other compounds showed a crossover in selectivity of irreversible inhibition. Although XLIX was a better reversible inhibitor of skeletal muscle LDH than XLVI, the former showed no irreversible inhibition of skeletal muscle LDH; in contrast, XLIX was an irreversible inhibitor of GDH almost as good as XLVI. A direct antithesis of irreversible inhibition pattern was shown with L; although skeletal muscle





LDH was irreversibly inhibited by L, the latter showed no irreversible inhibition of GDH (Table I).

It was previously noted (1) in a crude model of the active-site of LDH that the oxanilic-LDH complex does not appear to have sufficient space for the complete free rotation of the phenyl group; in addition, it can be demonstrated with molecular models that the iodomethylene group of the oxanilate (XLIX) can approach any point in space that the iodomethylene group of XLVI can approach when the respective enzyme-binding points of the inhibitors are anchored. Therefore, the conformation necessary for the iodomethylene group of XLIX to bridge to the nucleophilic site for reaction within the enzyme-inhibitor complex may not be tolerated due to a restriction of rotation.

The carbonyl carbon of the iodoacetyl group and the *N*-methyl groups of L have a fixed relationship between them; when the iodomethylene group of L attempts to bridge to the nucleophilic site within the GDH-inhibitor reversible complex, there is apparently insufficient space for the counter-balancing *N*-methyl group and bridging for inactivation cannot be completed.

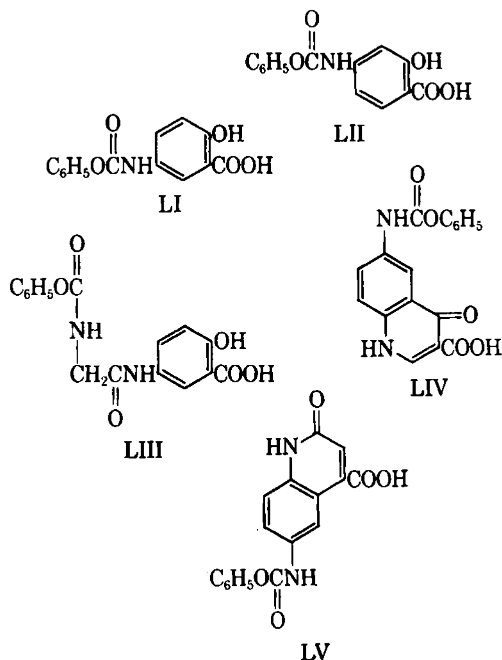
Inactivation via the Carbophenoxy Group.—The second corollary of the bridge principle of specificity is based on the difference in nucleophilicity of the enzymic group being alkylated. It is at this point that the approach for labeling the active-site of a pure enzyme and the approach to chemotherapy become divergent. The α-halogen carbonyl group of an iodoacetamide such as XLVI or a chloroketone such as XXXII have little functional specificity. Of about 15 amino acids in proteins containing a third functional group, a majority of them—such as methionine, histidine, arginine, lysine, cysteine, cystine, tyrosine, aspartic acid, and glutamic acid—have the nucleophilic ability to become alkylated. The α-halogen carbonyl compounds, therefore, have the broadest utility in labeling the active site of a pure enzyme, the selectivity of reaction being controlled by the initial complexing between the inhibitor and the active-site; this very broadness of utility for a pure enzyme is undesirable for the mixture of enzymes in a cell involved in chemotherapy. Ideally, for chemotherapy the greatest selectivity should be obtained by using an attacking group for the enzymic nucleophilic group that is specific for a single amino acid of the group of 15, thus affording a further dimension of specificity. The phenyl ester group such as that of 5-(carbophenoxyamino)salicylic acid (LI) apparently can

TABLE I.—IRREVERSIBLE INHIBITION OF LDH AND GDH BY SELECTED COMPOUNDS

No.	Compd.[a]	Liver GDH $K_i \times 10^4$	Liver GDH Rate of Inactivation[b]	Skeletal Muscle LDH $K_i \times 10^4$	Skeletal Muscle LDH Rate of Inactivation[b]	Heart Muscle LDH $K_i \times 10^4$	Heart Muscle LDH Rate of Inactivation[b]
XLVI	4-ICH_2CONH—SA	8.5	1.0[c]	17	1.0[c]	45	0
XLVIII	4-ICH_2CONH—GSA	18	1.2	4.0	0.87	25	1.0[c,g]
XLIX	3-ICH_2CONH—OA	23	0.75	1.3	0	1.2	0
L	4-$ICH_2CON(CH_3)$—SA	33	0	33	1.0	...	...
LI	5-C_6H_5OCONH—SA	4.0	2.0	15	0	14	0.58[h]
LII	4-C_6H_5OCONH—SA	8.5	0	11	0.98	19	0[g]
LIII	5-C_6H_5OCONH—GSA	1.6	2.0	5.9	0	18	0
LIV	6-C_6H_5OCONH—QA	2.3	0[d]	1.1	0[d]	...	...
LV	6-C_6H_5OCONH—CA	2.4	1.3[e]	2.4	0[f]	...	...
LVI	4-cis-HOOCCH=CH—CO—NH—SA (SA—NHCO)	43	0	18	0.9	30	0[g,i]
LVIII	Maleanilic acid	...	...	83	0	114	0

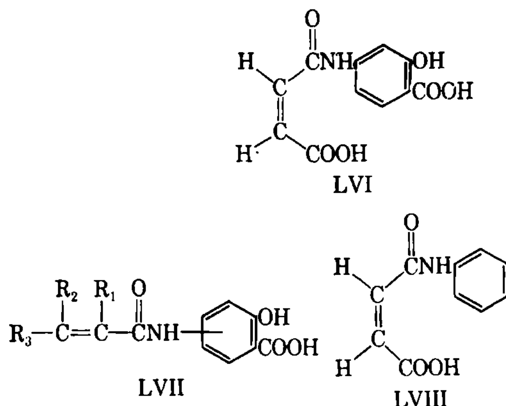
[a] Abbreviations used: SA = salicylic acid; OA = oxanilic acid; GSA = glycylamidosalicylic acid; QA = 4-hydroxyquinoline-3-carboxylic acid; CA = 2-hydroxycinchoninic acid; data taken from References 31–36, 38. [b] Concentration of inhibitor was 2 m*M* unless otherwise indicated. [c] Arbitrary standard rates that are not the same for the three enzymes. [d] Concentration of inhibitor was 0.13m*M*, the maximum solubility at pH 7.4 that could be attained in the inactivation procedure [e] Concentration of inhibitor was 0.2 m*M*. [f] Concentration of inhibitor was 0.4m*M*. [g] Concentration of inhibitor was 4m*M*. [h] Concentration of inhibitor was 3m*M*. [i] Preliminary unpublished data.

only react with a primary amino group, thus having complete functional specificity (35, 36).



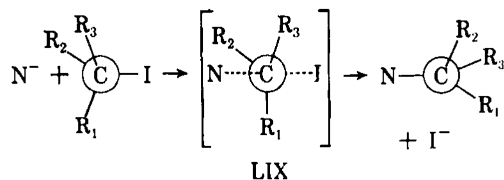
5-(Carbophenoxyamino) salicylic acid (LI) inactivated GDH at twice the rate of the standard, 4-(iodoacetamido) salicylic acid (XLVI); in contrast, LI showed no irreversible inhibition of skeletal muscle LDH (Table I). A cross-over specificity was noted with LII, which inactivated skeletal muscle LDH about the same rate as the standard (XLVI), but showed no inactivation of GDH. The peptide, LIII, with a longer side-chain still inactivated GDH, presumably by folding the chain back to the point in space equivalent to the position of the carbophenoxy group of LI. Although LIII can also fold back to the point in space equivalent to the position of the carbophenoxy group of LII, LIII failed to show the inactivation of LDH that LII showed; this difference is attributed to hindrance at the transition state which is discussed in a later section. In contrast, LIV failed to inactivate either LDH or GDH, showing that the fixed position of the carbophenoxy group in LIV cannot bridge to the available enzymic amino group (32). That a bicyclic compound could inactivate GDH at low concentration was shown with LV; again LDH was not inactivated by this compound (32).

Inactivation via the Maleamyl Group.—The α,β-unsaturated system of LVII should react in general most rapidly with SH groups, much slower with amino groups, and extremely slowly with hydroxyl groups by a Michael addition reaction. For initial studies, 4-(maleamyl)salicylic acid (LVI) was selected for

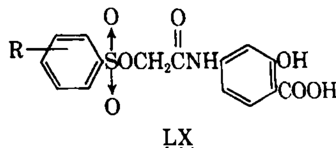


study (38). 4-(Maleamyl)salicylic acid (LVI) inactivated skeletal muscle LDH at about the same rate as the standard compound, XLVI; however, LVI failed to inactivate GDH, thus showing the selectivity of the maleamyl group. That a reversible complex between LVI and skeletal muscle LDH was an obligatory intermediate to inactivation was strongly indicated by the failure of maleanilic acid (LVIII)—which does not have the necessary groups for reversible binding—to inactivate skeletal muscle LDH. Preliminary studies have shown that 4-(acrylamido)salicylic acid (LVII, R = H) irreversibly inhibited both GDH and skeletal muscle LDH (39); further studies on the selectivity that might be obtained by varying the R groups LVII are in process (39).

Hindrance to the Transition State.—In a displacement reaction of the S_N2 type, whether bimolecular or intramolecular, the transition state appears to be planar (LIX) (40). There-

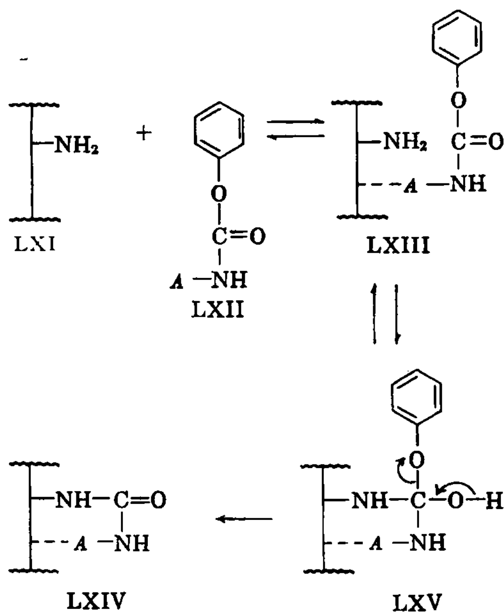


fore the attacking group should not be hindered by a bulky leaving group, since the two groups are on opposite sides of the carbon atom undergoing reaction; that such is the case has been indicated experimentally (41). When such an intramolecular reaction takes place in an enzyme-inhibitor reversible complex, the enzymic nucleophilic group which is attacking in



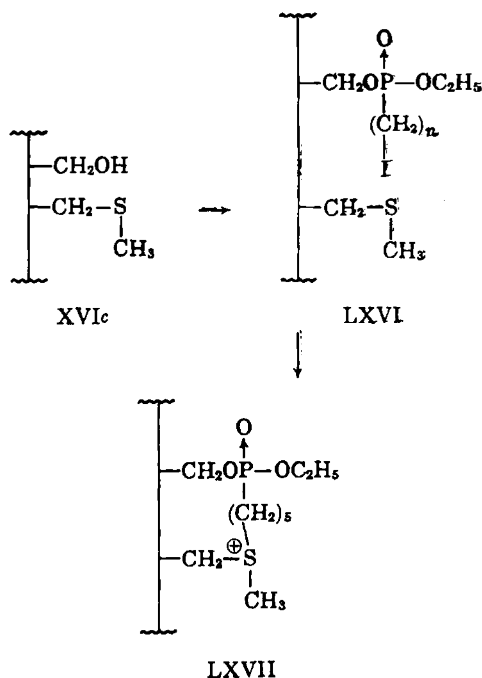
the displacement reaction is part of the extremely large (compared to inhibitor) enzyme molecule; as a result, there may be hindrance to the formation of the transition state by steric interaction of the enzyme and a bulky leaving group. Such a study is underway with the sulfonate leaving group (LX); when R = Me, LX is an irreversible inhibitor of GDH and skeletal muscle LDH (42) and studies with bulky R groups are in process.

Another type of reaction which may be subject to specificity dependent upon the transition state is the inactivation mechanism by phenyl esters such as LI and LIII. The inactivation is



The enzyme (LXI) initially forms a reversible complex with the inhibitor (LXII) through the binding area, *A* (see Fig. 3). In order for an amine on the enzyme surface in the complex (LXIII) to form a covalent bond, it is necessary that the transition state, LXV, be formed; LXV can then lose the phenoxy group resulting in the mixed urea, LXIV, the inactivated enzyme. For this reaction to proceed, there can be no steric interaction between the phenoxy group and the enzyme surface. Stated in another way, in order for the transition state to form, the leaving phenoxy group in the transition state must not be in contact with the enzyme—else the transition state could not form.

5-(Carbophenoxyglycylamido)salicylic acid (LIII) fails to inactivate skeletal muscle LDH, whereas LII can inactivate this enzyme. Since the carbonyl in the carbophenoxy group of LIII can reach any point in space that the same carbonyl group of LII can reach when the reversible binding groups of the salicylic acid structure are



anchored, the carbophenoxy group of LIII most probably cannot approach this critical point for formation of the transition due to a steric interaction between the phenoxy group and the enzyme (31, 36). A useful system for study of possible specificity by the steric interaction of the phenoxy leaving group and the enzyme could be the inactivation of both GDH and heart LDH by LI (Table I) (31). A number of related compounds with substituents on the phenoxy group have been synthesized and are currently being evaluated to determine if such a specificity of the irreversible reaction between these two enzymes can be found (43).

In another approach to find selectivity, some compounds related to 4-(iodoacetylglycylamido)-salicylic acid (XLVIII) having optically active D and L forms of alanine or valine instead of the glycine residue have been synthesized (42); there is a possibility for asymmetric tolerance of the bulky group of the substituted glycine residue in the enzyme-inhibitor reversible complex that may influence whether the bridge can be completed as in Fig. 3. These compounds are being evaluated as irreversible inhibitors, the hope being that only a D-isomer may be an irreversible inhibitor of one enzyme, such as LDH, and only an L-isomer an irreversible inhibitor of a closely related enzyme, such as GDH.

Bridging Phenomenon with Chymotrypsin.—In the earlier discussion on chymotrypsin, it was pointed out that α-haloacetamidoacylchymotrypsins (XX*a*) (Fig. 4) could undergo

intramolecular alkylation by attack of a methionine sulfur atom to form XXIII (22). That the proper bridge length is necessary for the reaction to occur is shown by the fact that iodoacetyl-chymotrypsin does not undergo such an intramolecular reaction with methionine or any other amino acid of chymotrypsin (44). In preliminary studies with ethyl 5-iodopentylphosphonofluoridate, Gold (45) has shown the phosphonyl-chymotrypsin (LXVI, $n = 5$) undergoes loss of iodide presumably by an internal alkylation reaction; the ethyl 5-iodopentylphosphonofluoridate does not release bound iodine under the same conditions without enzyme. Furthermore, XLVI with shorter or longer alkyl groups gave zero release of iodide in the presence of enzyme. It is probable that the same methionine attacked intramolecularly in the acylated chymotrypsin of type XX*a* is also attacked in this case to give XLVII, since the bridging distance is nearly the same.

That azaserine (VII) inactivates the appropriate enzyme while diazoacetyl-chymotrypsin (XXXVI) is stable, unless photolyzed, is an interesting comparison. The diazo group of XXXVI certainly has the capacity to react with a carboxyl, phenolic, hydroxyl, or mercapto group; apparently such a group is not within bridgeable distance to the diazomethyl group of XXXVI (Fig. 8).

SELECTIVE INHIBITION OF LACTIC DEHYDROGENASE FROM TWO DIFFERENT TISSUES

The enzymic nucleophilic site that covalently binds an active-site-directed irreversible inhibitor can be, but is not necessarily, adjacent to the active-site in the secondary-tertiary structure of the protein (see Fig. 3). The substrate-identical enzyme from different tissues frequently gives no cross-reaction with specific antisera (46, 47), indicating differences in tertiary structure. Therefore, Baker (34) suggested that it may be possible to obtain highly selective irreversible inhibition of the substrate-identical enzyme from different tissues by use of the bridge principle.

By antisera cross-reaction studies (47, 48) and by amino acid analysis (48), it has been shown that LDHs from heart and skeletal muscle in the same animal are quite different, but that heart LDHs from different species are more similar; also skeletal muscle LDHs from different species are more similar. As a first approximation, a study on selective irreversible inhibition of LDHs from rabbit skeletal muscle and beef heart was undertaken (31, 49).

The standard compound for irreversible inhibition of skeletal muscle LDH, 4-(iodoacetamido)salicylic acid (XLVI), failed to inhibit heart LDH irreversibly (Table I). When the bridge distance was lengthened (as in the peptide, XLVIII), both LDHs were irreversibly inhibited, but at different absolute rates. For further work, XLVIII was used as a standard for heart LDH. Selectivity of action was shown with 5-(carbophenoxyamino)salicylic acid (LI) which could inactivate heart LDH, but failed to inactivate skeletal muscle LDH. A cross-over in specificity was noted with the 4-isomer (LII), which inactivated skeletal muscle LDH, but not neart LDH. It was previously noted that 4-(maleamyl)salicylic acid (LVI) could inactivate skeletal muscle LDH; in contrast, preliminary studies indicate that heart LDH is not inactivated by LVI.

If this irreversible specificity for substrate-identical enzymes from different tissues can be transposed to such critical areas for cell division as (*a*) purine or pyrimidine biosynthesis or (*b*) the folic cofactor area, the benefits that could accrue to chemotherapy would be obvious. Such studies in area (*a*) (50) and area (*b*) (51–56) are continuing in our laboratory.

EXPERIMENTAL DESIGN FOR UNEXPLORED ENZYMES

Lest the medicinal chemist who has had the patience to read this far be tempted to dash into the laboratory and attach diazoketone, fluorophosphate, halomethyl carbonyl, diazonium, maleamyl, carbophenoxy, or other covalent forming groups to the nearest available substrate or inhibitor, he would be wise to be cautious with the suggestions that follow, else he is apt to be disappointed in his laboratory labors.

The studies described to this point have dealt only with irreversible inhibition of isolated enzymes—a far cry from chemotherapy in a host system. It is obvious that there are a variety of other factors—other than selective irreversible inhibition of isolated enzyme systems—that play important roles in whether the inhibitor would be effective in host system. Among these factors are (*a*) transport to and into the desired target cell from the site of administration of the inhibitor must be achieved; (*b*) the inhibitor must be reactive enough to give a reasonable rate of inactivation of the target enzyme, but not so reactive that insufficient inhibitor reaches the desired cell containing the target enzyme; and (*c*) selectivity of inhibition of an enzyme in the target cell such as a cancer cell or an invading organism. This review has been devoted only to one of these important

facets, namely (*c*); the other facets will have to be overcome simultaneously for the inhibitor to be a useful drug. If the candidate inhibitor is only studied for effectiveness in a host system, the jack-pot of (*a*), (*b*), and (*c*) working at the same time will have to be achieved, a remote possibility predicated mostly on extremely good luck.

The design of an effective agent in a host system is still so nebulous that successful design is more likely to be achieved by individual study of the three facets, followed by collective use in order to find eventually chemotherapeutic agents on a less hit or miss basis; that is not to say that synthesis and screening should be discontinued, but the screening approach is a calculated risk of hit or miss that has hit in infectious chemotherapy, but has been so much less successful in cancer chemotherapy to be termed a near miss. In fact, the medicinal chemist will even find it difficult to make a successful guess concerning where to put a covalent-forming group on a substrate or inhibitor to give an active-site-directed irreversible inhibitor for an unexplored enzyme. At this early stage of research in this area, it would appear wise to proceed systematically to determine the binding points of the inhibitor (area *A*, Fig. 3) and the noncontact area of the inhibitor and the enzyme. If the covalent-forming group (*B*) is unfortuitously placed in area *A* (Fig. 3), then an active-site-directed inhibitor will not be obtained since the reversible complex cannot form.

The logical development of where to place the covalent-forming group *B* (Fig. 3) to make an irreversible inhibitor for an unexplored enzyme can be gleaned by considering the previous development of irreversible inhibitors of LDH by reading in order the theoretical background (2), types of inhibitors and points of binding (1, 37), the noncontact areas (1, 37), and finally the bridge principle (31–36, 49); most of this information has been covered in this review, but not necessarily in the same order.

For two enzymes in the folic cofactor area, this approach has been systematically developed to the point where positionings for covalent-forming groups can now be done logically (51–56). An interesting sideline developed by serendipity may well solve the cell wall transport problem for active-site-directed irreversible inhibitors of folic reductase (56).

Having heeded my precautions, and introduced some new ones of his own on how to construct an active-site-directed irreversible inhibitor, and having been successful in synthesizing and enzymically evaluating the inhibitor, the researcher now must face the wall of prejudiced conservatism put up by his peers—the so-called "experts." Most of our work—and perhaps some of yours—may be based on kinetic evaluation of the inhibitors, giving results which may approach being, but do not become, unequivocal. We have criticized our own attempt to demonstrate that a reversible complex between inhibitor and enzyme is an obligatory intermediate to inactivation:

> "If it were possible to obtain ***unequivocal*** proof for the initial complex formation—and no such experiment for yielding unequivocal proof is apparent to us—solution of this problem would be relatively straight forward. Thus, one is left with the unsatisfying approach of eliminating all other possibilities than the one under consideration; such an approach has the obvious difficulty, as is the case for all kinetic experiments, that a yet unthought of mechanism may be the true one."

Thus, the "expert" may well sum up your work (and mine) by saying you (I) have not yet proved unequivocally your (my) thesis; therefore, the thesis must be wrong. At this point I like to chide the "expert" a little along the following vein, in an attempt to maintain my own sense of humor.

Too many of these "experts" treat the enzyme as a worshipper treats Mecca; they bow to the East and are dazzled by thoughts of the great enzyme temple that there resides. The more adventuresome will travel to Mecca, go inside the enzyme temple, and behold the glory of the active-site. Even the adventuresome worshipper is dazzled by this magnificient workmanship of nature. As past history has shown, some heretic worshipper or some worshipper of a different temple will—beneath his robes—put on his polarized sunglasses, then arise from his kneeling position. With the dazzle now eliminated, he can observe that the temple has been made from bricks, mortar, metal and paint, but of exquisite design; similarly, he may observe that the bricks of the enzyme temple are constructed from the long known elements of carbon, hydrogen, nitrogen, oxygen, and sulfur, then mortared together with peptide and hydrogen bonds and finally decorated with the metal and paint of functional groups—an exquisite and bedazzling design when the enzyme becomes functional for a substrate. Even with this dazzlement, three facts have emerged over the past 50 years that are the basis for active-site-directed irreversible inhibitors and their specificity: (*a*) The macromolecular enzyme has reactive functional groups on its surface; (*b*) this macromolecule can form complexes with simpler molecules such as substrates and inhibitors; (*c*) neighboring group reactions can be accelerated 10,000-fold or more

compared to the same chemical reaction occurring by a bimolecular reaction.

Some "experts" have accepted (*a*) and (*b*), and other "experts" have accepted (*c*). Is it not time for them to accept that the three tenets might be combined into one situation?

The macromolecular enzyme has functional groups on its surface which logically could be attacked selectively in the tremendously accelerated neighboring group reactions capable of taking place within the reversible complex formed between the enzyme and an inhibitor substituted with a properly placed neighboring group.

My own frustrations of researching in this area can be summed up in the following letter which this author sent to Dr. Samuel W. Goldstein, Associate Editor of THIS JOURNAL, in the process of considering another paper in this series for publication.

Thank you for your letter of September 9, 1963, with the referee's comments on our paper "Nonclassical Antimetabolites. XV." I share the quandary expressed by the referee; I too wonder how best to approach experimentally the "receptors of unknown structure." In fact, at times I wonder why I ever got started in this area of research which is so equivocal in its interpretation of data; at other times, I am sufficiently depressed about the reactions of reviewers to this work that I am tempted to drop this line of research.

However, everyone wants to know all about the active-site, but few are ever willing to try experiments to solve this tremendously important problem. Some not willing to experiment in such a difficult area are so conservative they are even unwilling to put their official seal of approval as a referee to such experiments. Although I feel our data are as solid as one can get at this early stage, I certainly agree that the interpretation of the data is equivocal and may even be wrong totally or in part. Nevertheless, I feel the main responsibility for statements in a publication that may ultimately prove to be wrong rests with the authors and not with the referees or editors, although the latter can certainly exercise their rights and reject any manuscript they wish. Actually, if anything turns out to be incorrect it will be my personal reputation that will be on the chopping block; it is this last reason that often tempts me to drop this area of research and stick to straight synthetic chemistry. However, the useful benefits that can potentially accrue to the field of chemotherapy keep me working in this difficult area.

The experimental approach that we have been using would appear to draw on knowledge available from the areas of organic chemistry, physical chemistry, biochemistry, and chemotherapy; I certainly feel my own shortcomings in some of these areas, and I imagine a referee may also feel shortcomings in one or more of these areas. Hence, it is difficult to set up an experimental design, carry out the research, and referee the results.

In conclusion, it is admittedly difficult to get started with a machete through the jungles of the so-called active-site. Until more sophisticated tools can be developed, some paths, including that which I have taken, may lead to naught; nevertheless, this or other paths may ultimately lead to the smooth four-lane highways for the band-wagon boys to jump aboard for the easy ride. Until my approach leads to a blind alley or a four-lane highway, I hope that the referees and editors will take into consideration that the experimental and interpretative difficulties are equivocal, but are being carried on with the highest scientific ideals that I am capable of reaching.

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Research Articles

Phytochemical Investigation of *Carya illinoensis*

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The leaves and petioles of *Carya illinoensis* and a lyophilized aqueous extract of this plant were investigated. Examination of the petroleum ether, ether, and chloroform extracts revealed the presence of unidentified phytosterols and a squalene-like substance in the unsaponified portions, and the presence of capric, lauric, myristic, palmitic, stearic, arachidic, oleic, linoleic, and linolenic acids in the saponified portions. Nonhydrolyzable tannins containing a phloroglucinol and a catechol nucleus were found in the ethanol and methanol extracts. Investigation of the plant extracts revealed the presence of carbohydrates and the absence of discernible amounts of glycosides and alkaloids. A crystalline neutral substance obtained from a neutral lead acetate treated aqueous extract was identified as the *m*-inositol. A crystalline acidic substance isolated from an aqueous extract of the crude drug was identified as 3,4-dihydroxybenzoic acid. Further pharmacologic studies of various extracts are presently in progress.

THIS PHYTOCHEMICAL STUDY was undertaken primarily on the basis of our preliminary screening tests which indicated that certain extracts of the leaves and petioles of *Carya illinoensis* (Wangh) K. Koch (Fam. *Juglandaceae*), common name—pecan, possessed the property of temporarily lowering the blood pressure of test animals on intravenous administration. A search of the available literature revealed little (1, 2) or no scientific information concerning the evaluation of this plant for medicinal properties. The fact that this species has been assigned eight official names (3) throughout its relatively short history may account for the lack of investigation in the *Carya* genus. In 1951, the plant was reclassified under the international code as *Carya illinoensis*.

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EXPERIMENTAL

Source and Preparation of Material.—The plant parts used in this investigation consisted of the air dried leaves and petioles collected in late summer from pecan trees growing near Covington, La. This material, sampled and authenticated,[1] was reduced to a moderately coarse powder with the aid of a W. J. Fitzpatrick, model D, comminuting machine.

Moisture Content and Ash Determination.—The moisture content of the powdered plant material, as determined by the toluene method (4) of the U.S.P.

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