NON-CLASSICAL ANTIMETABOLITES. IX.[1] THE BRIDGE PRINCIPLE OF SPECIFICITY WITH EXO-ALKYLATING IRREVERSIBLE INHIBITORS. III. DETECTION OF DIFFERENCES IN SPECIFICITY OF ENZYMIC NUCLEOPHILIC SITES BY THE CARBOPHENOXY GROUP

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In addition to our original presentation (2) of strong experimental evidence to support the concept (3) of a new class of irreversible inhibitors that operate by exo-alkylation, two other laboratories have subsequently and independently made related observations in the areas of proteolytic enzymes (4) and hapten immunochemistry (5). A properly designed compound, such as I, can complex reversibly with an enzyme such as GDH[2], then become irreversibly bound within the complex adjacent to the active site. In the detailed version of this experimental evidence (6), the bridge hypothesis of specificity was proposed:

Compared to a reversible inhibitor, the exo-alkylating 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 alkylate a nucleophilic group on the enzyme surface and upon the nucleophilicity of the enzymic group being alkylated.

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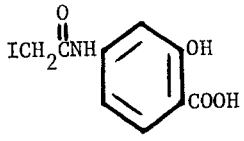
[2] LDH, lactic dehydrogenase; GDH, glutamic dehydrogenase.

Experimental evidence for the first corallary of the bridge hypothesis of specificity, namely, the difference in ability of certain inhibitors to bridge to and alkylate an enzymic nucleophilic site, has been more recently presented (1, 7), raising its status from hypothesis to principle. In this paper is presented experimental evidence for specificity based on the second corallary of the bridge principle, namely, the difference in nucleophilicity of the enzymic group being alkylated.

The iodoacetyl group of I has little functional specificity, that is, of about fifteen amino acids in proteins containing a third functional group, a considerable number of them such as cysteine, cystine, methionine, lysine, arginine, histidine, tyrosine, glutamic acid, and aspartic acid have the nucleophilic ability to become alkylated. In contrast, a phenyl ester such as that of 5-(carbophenoxyamino)salicylic acid (III) has greater functional specificity, that is, it reacts most rapidly with a primary amino group on a primary carbon, less rapidly with a primary group on a secondary carbon (α-amino group), and slowly with a primary amino group on a tertiary carbon; neither the imidazole-NH of histidine nor the indole-NH of tryptophane react with phenyl esters (8). Although no information concerning the reaction of phenyl esters with the guanidino group of arginine could be found, this group is a minor possibility since it would be fully protonated at pH 7.4. Thus, the most likely group of an enzyme to be attacked by a phenyl ester, via the exo-alkylation mechanism, would be the ϵ-amino group of a lysine and less likely a terminal α-amino group.

In Table I are listed a comparison of reversible and irreversible inhibition of LDH and GDH[2] by compounds I-III. In all incubations, an enzyme control and a 4-(iodoacetamido)salicylic acid (I) standard (2, 6) were run simultaneously with the test compound; all incubations were duplicated at least twice and the extent of irreversible inhibition is presented relative to I. Although the two phenyl esters (II,

III) could reversibly bind to both LDH and GDH, only GDH was inhibited irreversibly.



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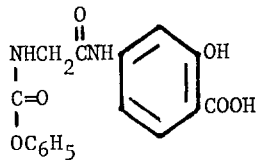

II

III

TABLE I

IRREVERSIBLE INHIBITION OF LDH AND GDH BY CARBOPHENOXYAMINO SALICYLIC ACIDS

Compound[a]	GDH[d] I_{50}	LDH[d] I_{50}	Ratio of rate of inactivation: Compound/I[e]	
			GDH	LDH
I	3.4	6.6		
II[b]	6.7	6.3	1.4	0
III[c]	1.6	5.9	2.0	0

[a] Compounds II and III were prepared from the corresponding amino salicylic acid by treatment with carbophenoxy chloride in alkaline solution. [b] M.p. 242-243°, from ethyl acetate; Anal. Calcd. for $C_{16}H_{14}O_6N_2$: C, 58.2; H, 4.24; N, 8.48. Found: C, 58.1; H, 4.44; N, 8.29. [c] M.p. 264-275° dec., from EtOH-H_2O; Anal. Calcd. for $C_{14}H_{11}O_5N$: C, 61.5; H, 4.02; N, 5.12. Found: C, 61.3; H, 3.78; N, 5.31. [d] An I_{50} is defined as the millimolar concentration of inhibitor necessary to reduce the rate of reaction to one-half in the presence of 1 millimolar substrate. The I_{50} values were determined for α-oxoglutarate ⟶ L-glutamate and for pyruvate ⟶ lactate as previously described (9). [e] The inactivation rates were determined as previously described (2, 6) and are presented relative to the standard compound I, 4-(iodoacetamido)salicylic acid.

That a reversible complex between GDH and III was an obligatory intermediate to inactivation of the enzyme was shown by a comparison of the rates of inactivation of GDH by 1 mM and 2 mM concentrations of III run simultaneously; the observed ratio of rates was near 1, definitively less than a ratio of 2 that would be expected if the inactivation occurred by a random bimolecular collision between GDH and III (2, 6).

The fact that the nucleophilic group on LDH covalently bound by the iodoacetyl group of I, is not covalently bound by the phenyl esters II-III indicates that this nucleophilic group is not an amino group and in addition shows that there is no other amino group that can be bridged by the carbophenoxy group within the reversible complexes formed by LDH and compounds II-III. The fact that GDH is attacked by either the carbophenoxy group or the iodoacetyl group indicates that within the reversible enzyme-inhibitor complex an amino group on the enzyme can be bridged for covalent bond formation; however, it cannot necessarily be concluded that the amino group on GDH covalently bound by II-III is the same group that is covalently bound by I, even though it is possible for these two nucleophilic groups to be one and the same.

Of importance to chemotherapy is the irreversible specificity noted with the phenyl esters (II-III), this specificity being due to the difference in the nucleophilic character of the enzymic groups being covalently bound on LDH and GDH. Groups on a reversible inhibitor that can specifically bridge to and bind covalently to other enzymic functions would be of use in both chemotherapy and protein structure studies, and such a study is continuing in these laboratories.

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