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NON-CLASSICAL ANTIMETABOLITES—VIII. THE BRIDGE PRINCIPLE OF SPECIFICITY WITH EXO-ALKYLATING IRREVERSIBLE INHIBITORS*

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Abstract—Five additional compounds related to the exo-alkylating irreversible inhibitor, 4-(iodoacetamido)salicylic acid (I), have been investigated as irreversible inhibitors of GDH and LDH.† All five compounds reversibly inhibited and three irreversibly inhibited both enzymes. N-methyl-4-(iodoacetamido)salicylic acid (II) inhibited LDH irreversibly, but not GDH. In contrast *m*-(iodoacetamido)-oxanilic acid irreversibly inhibited GDH ,but not LDH. These differences are attributed to the bridge principle of specificity.

WE RECENTLY presented[1–3] strong experimental evidence to support the concept[4] of a new class of irreversible inhibitors that operate by exo-alkylation. A properly designed compound such as 4-(iodoacetamido)salicylic acid (I) can complex reversibly with an enzyme such as GDH, then become irreversibly bound within the complex by formation of a covalent bond adjacent to the active site. In the detailed version of this experimental evidence,[3] the bridge hypothesis of specificity was proposed:

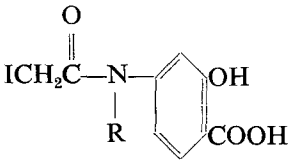
Compared with a reversible inhibitor, the exo-alkylating type of irreversible inhibitor can have an extra dimension of specificity; this extra specificity is dependent upon the ability of the reversibly-bound inhibitor to bridge to and alkylate a nucleophilic group on the enzyme surface and upon the nucleophilicity of the enzymic group being alkylated.

A series of compounds related to 4-ISA (I) that might be more selective in irreversible inhibition of GDH than LDH or *vice versa* by the bridge hypothesis was synthesized;[5] these compounds could be grouped as follows: (1) variation of the bridge length between the halogen and the reversible binding points of the inhibitor (compounds IV and VI); (2) bulk tolerance of the enzyme near the nucleophilic group (compound II); (3) restricted rotation of the iodoacetamido group of the inhibitor (compound V). (4) the effect of oxanilic acid as a carrier of the iodoacetamido group (compound III).

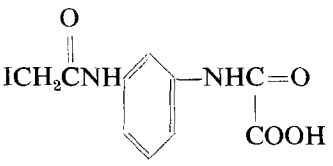
The evaluation of these six compounds presented in this paper gave experimental evidence for the bridge hypothesis that warrants raising its status from hypothesis to principle.

* This work was generously supported by Grant CY-5867 of the National Cancer Institute. A preliminary communication has appeared on this subject.[1]

† The following abbreviations are used: GDH, glutamic dehydrogenase; LDH, lactic dehydrogenase; DPNH, reduced diphosphopyridine nucleotide; Tris, tris(hydroxymethyl)aminomethane; ISA, iodoacetamidosalicylic acid.



I, R = H
II, R = CH_3



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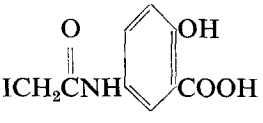
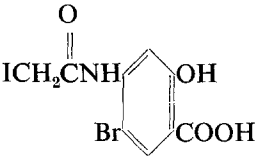
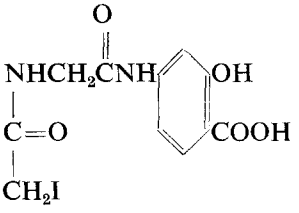


IV



V



VI

MATERIALS AND METHODS

Reagents. Sodium pyruvate, α-ketoglutaric acid, DPNH, LDH, GDH, and ammonium sulfate were the highest grade materials available by purchase from Nutritional Biochemicals Corp.; the LDH was crystalline enzyme isolated from rabbit muscle, and the GDH was crystalline enzyme isolated from mammalian liver.

Inhibitors. The synthesis of the 4-ISA (I) used in these studies has been described previously,[3] as have compounds II–VI.[5]

Reactivity of halogen. The procedure for determining the halogen reactivity of compounds II–VI was unchanged from that previously employed for 4-ISA (I).[3]

Reversible binding of inhibitors to enzymes (I_{50}). The I_{50} is defined as the millimolar concentration of inhibitor necessary to give 50% inhibition in the presence of 1 mM concentration of substrate.[6] The procedure employed for LDH was the same as described previously[6] except that the cuvet concentration of pyruvate was 1 mM and Tris buffer (pH 7·4) was employed. The I_{50} values for GDH were determined in the same manner as for LDH, with cuvet concentrations of 1 mM α-ketoglutarate and 75 mM ammonium sulfate in Tris buffer (pH 7·4).

Enzyme inactivation procedure. The LDH inactivation procedure[3] was modified so that the LDH and the DPNH concentrations were 10-fold greater in the inactivation, but the inhibitor was still 2 mM; the amount of remaining enzyme was then determined by using a 0·1 aliquot in the cuvet. This procedure is favored with strong reversible inhibitors since the amount of reversible inhibition after 10-fold dilution becomes negligible. The GDH incubations were run as previously described.[3]

In all incubations for irreversible inhibition, three incubation solutions made up from the same master enzyme-DPNH solution were run simultaneously: (1) a control with no inhibitor, (2) a standard with 2 mM 4-ISA, and (3) a 2 mM solution of the test compound. All runs were duplicated at least twice, and an occasional bad run was readily eliminated if the control or standard did not behave properly.

RESULTS

Comparison of halogen reactivity. The rate data with sodium thiosulfate were plotted as second-order reactions, giving straight lines. The slope of a given line, compared with the slope obtained with 4-ISA, gave the relative halogen reactivity; this reactivity is recorded in Table 1 where the halogen reactivity[3] of 4-ISA has been given the arbitrary value of 1·0.

TABLE 1. RELATIVE EFFECTS OF INHIBITORS ON GDH AND LDH

Compound	Relative halogen reactivity	GDH I_{50}	LDH I_{50}	Rate of GDH inactivation	Rate of LDH inactivation
I	1·00	3·4	6·6	1·0	1·0
II	1·64	13	13	0	1·0
III	1·20	9·2	0·51	0·75	0
IV	0·70	11	2·0	2·0	0·78
V	0·88	0·23	2·9	1·3	1·6
VI	0·90	7·2	1·6	1·2	0·87

4-(β-Iodopropionamido)salicylic acid, 4-(α-iodopropionamido)salicylic acid, and 4-(α-chlorophenylacetamido) salicylic acid, the syntheses of which were described previously,[5] failed to react with thiosulfate at a reasonable rate and were therefore not investigated as irreversible inhibitors of GDH or LDH even though these three compounds were good reversible inhibitors.

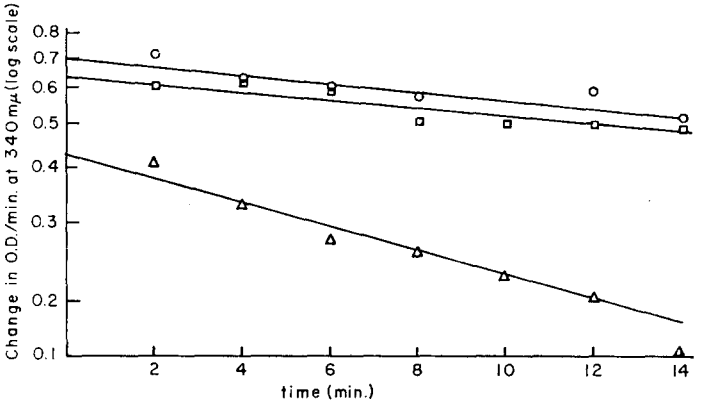


FIG. 1. Comparative irreversible inhibition of GDH·DPNH by 4-ISA (I) and N-methyl-4-ISA (II): ○, GDH·DPNH control; △, 2 mM 4-ISA; □, 2 mM N-methyl-4-ISA.

I_{50} values of inhibitors. The I_{50} values are recorded in Table 1. All these compounds (I–VI) complexed with and reversibly inhibited LDH (pyruvate → lactate)[7] and GDH (α-ketoglutarate → L-glutamate). Thus any compound (Table 1) that failed to inhibit GDH or LDH irreversibly did not fail for lack of reversible complex formation.

Irreversible inhibition by compounds I–VI. The rate of irreversible inhibition by a given compound was compared with the rate of irreversible inhibition by 4-ISA in a simultaneous experiment; thus the inactivation rate by 4-ISA is assigned an arbitrary value of 1·0 for each enzyme, and the other compounds gave the relative rates listed in Table 1. Compounds IV, V, and VI irreversibly inhibited both LDH and GDH at 75 to 200% with respect to the inhibition produced by 4-ISA. *In contrast, N-methyl-4-ISA (II) failed to inhibit GDH irreversibly (Fig. 1) but did irreversibly inhibit LDH (Fig. 2). A crossover specificity was noted with m-(iodoacetamido)oxanilic acid (III); i.e., GDH was irreversibly inhibited (Fig. 3) but LDH was not (Fig. 4).* Although the

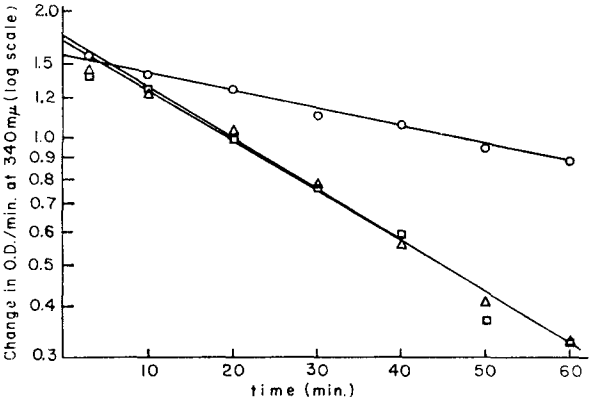


FIG. 2. Comparative irreversible inhibition of LDN·DPNH by 4-ISA (I) and N-methyl-4-ISA (II): ○, LDH·DPNH control; △, 2 mM 4-ISA; □, 2 mM N-methyl-4-ISA.

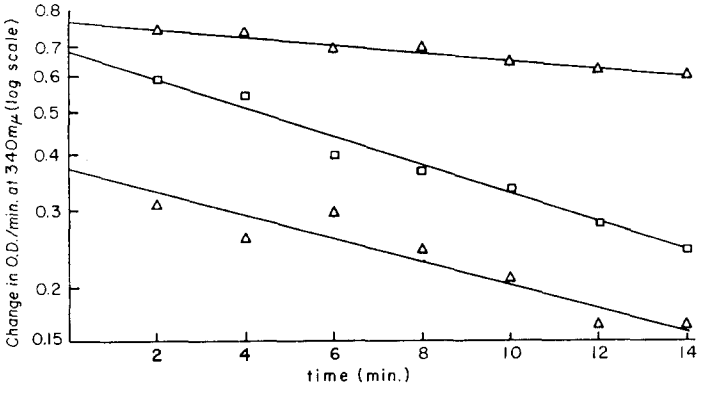


FIG. 3. Comparative irreversible inhibition of GDH·DPNH by 4-ISA (I) and *m*-(iodoacetamido) oxanilic acid: ○, LDH·DPNH control; △, 2 mM 4-ISA; □, 2 mM *m*-(iodoacetamido)oxanilic acid.

lack of irreversible inhibition is recorded in Table 1 as zero, the rate is actually between one-tenth the rate of 4-ISA and zero. The experimental design for testing the original bridge hypothesis[3] called for a minimum of a 10-fold rate difference; a rate more accurate than one-tenth to zero cannot be measured within the experimental error of the current experimental design.

DISCUSSION

In N-methyl-4-ISA (II), the N-methyl and the carbonyl carbon of the iodoacetyl group necessarily have a fixed distance between them since they are bound to the same nitrogen. Thus when the iodoacetyl group of II approaches the enzymic nucleophilic group within the reversible enzyme complex (Fig. 5), there must be space available in the LDH-II complex for the counterbalancing N-methyl group, but *there is no space for this N-methyl group in the GDH-II complex as the iodoacetyl group approaches the nucleophilic site for bridging.*

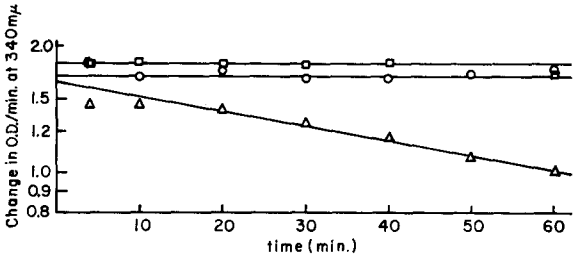


Fig. 4. Comparative irreversible inhibition of LDH·DPNH by 4-ISA (I) and *m*-(iodoacetamido) oxanilic acid: ○, LDH·DPNH control; △, 2 mM 4-ISA; □, 2 mM *m*-(iodoacetamido)oxanilic acid.

It was previously noted[6] that the oxanilic acid-LDH complex does not have sufficient space for complete free rotation of the phenyl group. Also, it can be clearly demonstrated with molecular models that the iodomethylene group of *m*-(iodoacetamido)-oxanilic acid (III) can approach any point in space that the iodomethylene group of 4-ISA (I) can approach when the respective binding points (carboxylate and adjacent oxygen) of both compounds are kept fixed; however, the total three-dimensional space required for rotation of the bonds of III to duplicate any conformation of I is considerably greater for III than I. Therefore, it follows that the LDH-III complex cannot tolerate the rotation of the benzene ring which will allow the iodomethylene group of III to bridge to the nucleophilic site.

Although compounds I, IV, V, and VI were not selective and showed irreversible inhibition of both enzymes, some inferences as to the nature of the nucleophilic site can be made. For example, the fact that compounds I, IV, and V can irreversibly inhibit eliminates the possibility that the acetamido group is in the plane of the benzene ring after covalent linkage to the enzyme is established, since the acetamido group of V cannot be planar in the direction of the 5-position owing to steric hindrance and since the acetamido group of 5-ISA (IV) cannot reach the point of acetamido group of V if the latter group is planar toward the 3-position. Since VI—with its longer bridge—irreversibly inhibits as well as I, it follows that the peptide sidechain folds back toward a position that the acetamido group of I must occupy after a covalent linkage to the enzyme has been established; therefore there must be space for this folded group in both enzymes. Whether compounds with additional optically active bulk, prepared by replacing the glycine of VI with optically active amino acids, can still inhibit both enzymes or only one enzyme (thus being stereospecific) remains to be determined.

The fact that two of the first five compounds related to 4-ISA have shown selectivity of irreversible inhibition (Table 1) seems remarkable at first sight, particularly since a crossover of specificity was noted. On further reflection it is not surprising that enzymes performing similar reactions—such as the LDH- and GDH-catalyzed hydrogenation of anionic substrates—would be reversibly inhibited by similar compounds since these sites are by mechanistic necessity closely related[7]; in contrast, there is reason to expect that the nucleophilic sites involved in exo-alkylation (Fig. 5)

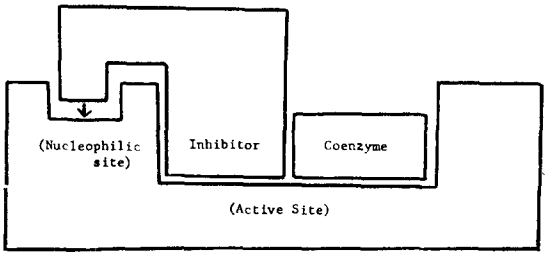


FIG. 5. Oversimplified diagram of exo-alkylation. The arrow represents the attack of the inhibitor on the nucleophilic site of the enzyme.

should be dissimilar, since these nucleophilic sites probably have no function in the mechanistic operation of the enzyme but are merely part of the secondary and tertiary structure of the protein that at best can have only limited effects on reversible specificity. Thus it could be anticipated that relatively minor changes in the structure of an exo-alkylating irreversible inhibitor could greatly influence its irreversible specificity but have much less influence on its reversible specificity, even though it has not been proven that the covalent bond formed in exo-alkylation is indeed on the secondary or tertiary structure of the protein; however, that enzymes of close mechanistic similarity can have different secondary-tertiary structures is clearly shown by cases of no cross reaction with specific antisera.[8] In fact, the mechanistically identical enzyme from different species,[8] or even from different tissues of the same animal, can frequently give different antisera reactions,[9] thus showing differences in secondary-tertiary structure.

In the inhibitor-enzyme complex, the approach of the alkylating group to the enzymic nucleophilic site (presumably in the secondary or tertiary structure) is dependent upon the combined environments of both the active site and nucleophilic site as well as the conformational and steric requirements between the two sites (Fig. 5). It should be possible to bring into play other factors in order to exploit the bridge principle, such as the space and conformational requirements for the transition state during nucleophilic attack of the inhibitor by the enzyme within the inhibitor-enzyme complex or the tolerance for optically active bulk in an inhibitor such as VI. These dual site requirements are obviously far more restrictive than the mere additive requirements of each site alone. 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; such a study has been started in the folic cofactor area.[10] A similar study on a mechanistically identical enzyme from different species or different tissues is warranted.[11] These types

of studies could have considerable utility in chemotherapy and in the determination of certain structural facets* of mechanistically related proteins.†

* A general method for labeling active regions of antibody molecules by a procedure related to exo-alkylation has been independently developed by Wofsky *et al.*[12] In addition, Lawson and Schramm[13] have recently demonstrated the exo-alkylation phenomenon with chymotrypsin where initial complex formation with the enzyme is through a labile ester bond.

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