Nonclassical Antimetabolites XV

Bridge Principle of Specificity with Active-Site-Directed Irreversible Inhibitors VI. Highly Selective Irreversible Inhibition of Lactic Dehydrogenase

By B. R. BAKER and R. P. PATEL

Eleven compounds, all of which were reversible inhibitors of both heart and skeletal muscle lactic dehydrogenase, were investigated as irreversible inhibitors of the two enzymes. 4- and 5-(Iodoacetylglycylamido)salicylic acids (V and VI) irreversibly inhibited both enzymes. However, specificity was shown with 4- and 5-(iodoacetamido)salicylic acids (I and II), which irreversibly inhibited skeletal muscle lactic dehydrogenase, but not heart lactic dehydrogenase. A crossover specificity was noted with 5-(carbophenoxyamino)salicylic acid (IV); this compound irreversibly inhibited heart lactic dehydrogenase, but not skeletal muscle lactic dehydrogenase. It is proposed that these specific irreversible inhibitions are due to exploitable differences in the secondary-tertiary structure of these two substrate-identical enzymes from different tissues.

Classical antimetabolites having only a small change in structure compared to the substrate have such a limitation in the structural changes that can be made (2) that utility is greatly limited. In contrast, nonclassical antimetabolites, defined as inhibitors that have large structural changes compared to the substrate (1), have much wider application. For example, the concept of irreversible inhibition by the exoalkylation mechanism with nonclassical antimetabolites was proposed (1). A properly designed inhibitor such as 4-(iodoacetamido)-salicylic acid (I) 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 active-site; an expression more general than exoalkylation is active-site-directed irreversible inhibition.[2] In support of this phenomenon, strong experimental evidence has been presented (3, 4). Further evidence for active-site-directed irreversible inhibition requiring initial reversible complexing with the enzyme has been presented more recently; compounds with a lower reversible dissociation constant (K_I) between enzyme and inhibitor give about the same speed or irreversible inhibition at a lower concentration dependent upon the relative amount of reversible binding of the compounds to the enzyme (10). Five other laboratories have subsequently and independently made observations pertinent to active-site-directed irreversible inhibition with chymotrypsin (16, 18, 22, 23) and in the field of hapten immunochemistry (25).

Our definition of exoalkylation (1) and its experimental support (3, 4, 10) led to the concept of (4) and experimental evidence (5–7, 11) for the bridge principle of specificity, which is now restated to replace the word "exoalkylation."

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.

With the mechanistically closely related enzymes, LDH[1] and GDH, experimental evidence in support of both facets of the bridge principle has been observed. Specificity of irreversible

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For previous paper in this series see *Reference 10*.

[1] The following abbreviations are used: LDH, lactic dehydrogenase; GDH, glutamic dehydrogenase; DPNH, reduced diphosphopyridine nucleotide; tris, tris-(hydroxymethyl)aminomethane hydrochloride buffer; 4-ISA, 4-(iodoacetamido)salicylic acid (I); 4-IGSA, 4-(iodoacetylglycylamido)salicylic acid (V).

[2] The expression "exoalkylation" is not strictly correct for a carbophenoxy compound such as IV; the covalent linkage between the reversible bound inhibitor and the enzyme occurs in this case by acylation, but the principle is the same. The more general expression "site-directed irreversible inhibition" is preferable.

inhibition of GDH, but not LDH, or *vice versa* by inhibitors related to 4-ISA[1] (I) was attributed to differences in the spatial relationship of the reversible binding sites and the nucleophilic sites of the two enzymes (5, 6). The differences in nucleophilicity of the enzymic groups being covalently linked in LDH and GDH was demonstrated with compounds related to 5-(carbophenoxyamino)salicylic acid (IV), whereby GDH was irreversibly inhibited, but not LDH (7, 11).[2]

Substrate-identical enzymes from different tissues frequently give little or no cross-reaction with specific antisera (14, 15, 17), thus showing differences in the secondary or tertiary structure of the protein *other than the active-site* (6); we suggested (6) that—*via* the bridge principle—it may be possible to obtain highly selective irreversible inhibitors of the substrate-identical enzyme from two different tissues. Such specific irreversible inhibition of LDH from heart and skeletal muscle has recently been reported (12) wherein 4-(iodoacetylglycylamido)salicylic acid (V) could irreversibly inhibit LDH from either heart or skeletal muscle, but 4-ISA (I) irreversibly inhibited only the skeletal muscle LDH and not heart LDH. Further observations on selective irreversible inhibition of these two enzymes is the subject of this paper.

MATERIALS AND METHODS

Reagents.—Sodium pyruvate and DPNH[1] were purchased from the Nutritional Biochemical Corp. Heart LDH, skeletal muscle LDH, tris,[1] and tris hydrochloride were purchased from the Sigma Chemical Co.; heart LDH was the crystalline enzyme isolated from beef heart, and skeletal muscle LDH was the crystalline enzyme isolated from rabbit skeletal muscle.

Reversible Binding of Inhibitors to LDH.—The concentration for 50% inhibition by compounds I–XI was determined for heart LDH in the presence of 0.3 m*M* pyruvate and 0.23 m*M* DPNH in 0.05 *M* tris buffer (pH 7.4) by observing the change in optical density per minute at 340 mμ; sufficient enzyme was employed to give a change of about one optical density unit per minute with no inhibitor. The 50% inhibition values for skeletal muscle LDH were done similarly in the presence of 1 m*M* pyruvate and 0.6 m*M* DPNH (2). These 50% inhibition values were then converted to the apparent K_I values in Table I described under *Results*.

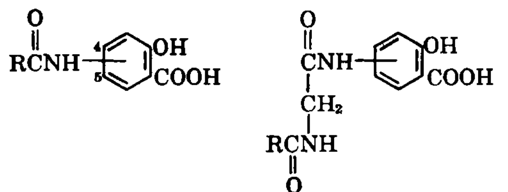
Enzyme Inactivation Procedure.—The inactivation procedure for skeletal muscle LDH has been previously described in detail (4, 6). The same procedure was used for heart LDH, except that the incubation time at 37° was increased from 60 minutes to 120 minutes. In all incubations for determination of irreversible inhibition of heart LDH, incubation solutions containing 2.3 m*M* DPNH and sufficient enzyme to give 9–11 optical density units change/minute/ml. of tris buffer were run simultaneously; the solutions were made from the same master enzyme-DPNH solution (4, 6): (*a*) the test compound in a concentration determined by the relative apparent K_I, (*b*) a standard of 4 m*M* 4-IGSA,[1] and (*c*) an enzyme control with no inhibitor. An 0.4-ml. aliquot was removed at the proper time interval, and the irreversible reaction was quenched by placement of the aliquot in an ice-bath. When time allowed, 0.30 ml. of the aliquot was added to 2.7 ml. of tris buffer (pH 7.4) in a cell. Then 0.10 ml. of 9.3 m*M* pyruvate was added, and the rate of oxidation of DPNH was followed at 340 mμ in a Cary 11 recording spectrophotometer. Under these conditions the change in optical density per minute was directly proportional to the remaining enzyme concentration in the aliquot. Since the concentration of inhibitor is in excess and does not change, the reaction is unimolecular with respect to enzyme concentration; therefore, the enzyme concentration (expressed in optical density units) is plotted on a log scale in Figs. 2–4. All runs were duplicated at least twice. An occasional bad run was readily eliminated if the control or standard did not behave properly.

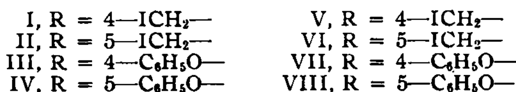
When two concentrations of the same inhibitor were compared, the experiments were run similarly, except that (*b*) was a solution of the test compound at one-half the concentration of (*a*).

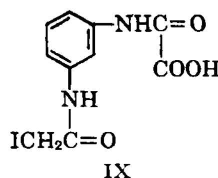
Considerable care, as previously described (11), must be taken in making up solutions of the carbophenoxy compounds listed in Table I so that the base-labile phenyl ester group does not hydrolyze.

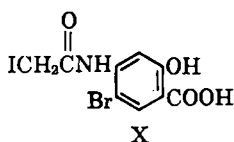
CHEMISTRY

Of the 11 compounds in Table I studied as irreversible inhibitors of heart LDH, nine have previously been studied as inhibitors of skeletal muscle LDH. The synthesis of compounds I, II, V, IX, and X have been previously described (8), as have their effects on skeletal muscle LDH (6). The synthesis and effects of compounds III, IV, and VII on skeletal muscle LDH have also been described (11), as have similar data on compound XI (10). The synthesis of the remaining two compounds (VI, VIII) were carried out as follows:

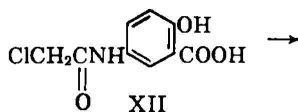








5-(Chloroacetamido)salicylic acid (XII) (20) was prepared in 92% yield from 5-aminosalicylic acid and chloroacetyl chloride in aqueous sodium bicarbonate as previously described for related compounds (8). When XII was allowed to react with concentrated ammonium hydroxide, the amino acid (XIII) was obtained in 54% yield; the infrared spectrum of XIII indicated that it was not a zwitterion, in contrast to 4-(glycylamido)salicylic acid (8). Reaction of XIII with iodoacetyl chloride or phenyl chloroformate in cold aqueous sodium bicarbonate afforded VI and VIII, respectively, in good yield.



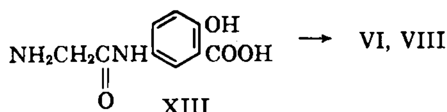


TABLE I.—REVERSIBLE AND IRREVERSIBLE INHIBITION OF LDH FROM HEART AND SKELETAL MUSCLE

	Skeletal Muscle LDH[b]			Heart LDH		
		Rate of Inactivation			Rate of Inactivation	
Compd.[a]	Apparent[a] $K_I \times 10^4$	mM Concn.	Relative Rate	Apparent[a] $K_I \times 10^4$	mM Concn.	Relative Rate
4-$ICH_2C(=O)$—ASA (I)	17	2	1.0[c]	45	2	0
					4	0.2[d]
4-$ICH_2C(=O)$—GSA (V)	4.0	2	0.87	25	4	1.0[c,e,f]
5-$ICH_2C(=O)$—ASA (II)	5.0	2	0.78	19	2	0[f]
4-$C_6H_5OC(=O)$—ASA (III)	11	2	0.98[g]	19	4	0
5-$C_6H_5OC(=O)$—ASA (IV)	15	2	0	14	3	0.58[h]
5-$ICH_2C(=O)$—GSA (VI)	23	2	1.9	34	4	1.0
4-$C_6H_5OC(=O)$—GSA (VII)	16	2	0	17	4	0
5-$C_6H_5OC(=O)$—GSA (VIII)	4.6	2	0	18	2	0
$ICH_2C(=O)$—AOA (IX)	1.3	2	0	1.2	2	0[f]
5-Br-4-$ICH_2C(=O)$—ASA (X)	7.3	2	1.6	28	2	0[f]
$ICH_2C(=O)$—ACC (XI)	3.1	0.4	0.52	9.6[i]	1	0.67

[a] Abbreviations used: ASA, aminosalicylic acid; AOA, *m*-aminoxanilic acid; GSA, glycylaminosalicylic acid; ACC, 6-aminocoumarin-3-carboxylic acid. The units of the apparent K_I values are moles/liter. [b] Data taken from *References 6, 10,* and *11*, except for new data on compounds VI and VIII. [c] Arbitrary assignment of rate for relative purposes; the absolute values for the two LDH's are not the same. [d] Since no irreversible inhibition was detectable at 2 mM and slight irreversible inhibition was detectable at 4 mM, it is probable that the slight amount of irreversible inhibition at 4 mM is due to a small amount of bimolecular inactivation (tail-alkylation) of the type previously observed with iodoacetamide on skeletal muscle LDH (4). Even if this small amount of inactivation could be experimentally established as occurring in the same manner as inactivation by 4-IGSA, this amount of inactivation is on the border of the experimental error; any value in the table listed as 0 is actually less than 0.1, the limits of detection in this experimental design. Therefore, this compound (I) is considered to give negligible irreversible inhibition under these conditions. [e] The relative rates of inactivation in two runs when 4 mM V was compared to 2 mM V were 1.5 and 1.6; Calcd.: 1.4. (See Fig. 2.) [f] Data taken from *Reference 12.* [g] Comparison of the relative rates of inactivation of 4 mM *versus* 2 mM of compound III did not give a ratio less than 2, in disagreement with the ratio calculated from the ratio of the amounts of enzyme-inhibitor complex; see text of *Results* and *Reference 11.* [h] Showed relative rates of inactivation of 1.4 and 1.4 in two runs when 4 mM IV was compared with 2 mM IV; Calcd.: 1.3. [i] Estimated from 20% inhibition obtained from a saturated solution and may have a larger error than other apparent K_I's; see *Reference 10.*

EXPERIMENTAL

Melting points were taken in capillary tubes with a Mel-temp block and are uncorrected. Infrared spectra were determined with a Perkin-Elmer model 137B spectrophotometer.

5-(Glycylamido)salicylic Acid (XIII).—Reaction of 5-aminosalicylic acid with a 50% excess of chloroacetyl chloride in aqueous sodium bicarbonate by the previously described general method (8), gave a 92% yield crude product (XII), m.p. 236–238° (Raiziss and Clemence (20) recorded 242–244°), that was suitable for the next step.

A solution of 5.0 Gm. (0.0218 mole) of XII in 50 ml. of concentrated ammonia water was allowed to stand overnight. The solution was spin evaporated to dryness *in vacuo* and the residue triturated with 10 ml. of cold water; yield, 2.7 Gm. (54%), m.p. 240°. Recrystallization from water gave buff-colored crystals of unchanged melting point; $\nu_{max.}^{KBr}$ 3625, 3140 (NH, OH); 1687, 1650 cm.$^{-1}$ (C=O).

Anal.—Calcd. from $C_9H_{10}N_2O_4 \cdot H_2O$: C, 47.4; H, 5.26; N, 12.3. Found: C, 47.6; H, 5.35; N, 12.6.

5-(Iodoacetylglycylamido)salicylic Acid (VI).—To a stirred solution of 2.28 Gm. (10 mmoles) of XIII in 40 ml. of water containing 3.4 Gm. (40 mmoles) of sodium bicarbonate and cooled in an ice-bath, was added 4.08 Gm. (20 mmoles) of iodoacetyl chloride. After being stirred for 75 minutes, the solution was clarified by filtration, and the filtrate was acidified to about pH 3; yield, 3.0 Gm. (79%), m.p. 185–190°. Several recrystallizations from ethanol-ethyl acetate gave the pure material, m.p. 214°; $\nu_{max.}^{KBr}$ 3290, 3100 (NH, OH); 1675, 1638 cm.$^{-1}$ (C=O).

Anal.—Calcd. for $C_{11}H_{11}N_2O_5$: C, 34.9; H, 2.91; N, 7.40. Found: C, 35.0; H, 3.03; N, 7.22.

5 - (Carbophenoxyglycylamido)salicylic Acid (VIII). This was prepared similarly from 2.28 Gm. of XIII and 2.34 Gm. of phenyl chloroformate; yield, 2.7 Gm. (82%), m.p. 207–209°. Three recrystallizations from ethyl acetate gave the pure compound, m.p. 217–219°; $\nu_{max.}^{KBr}$ 3300 (NH, OH); 1735, 1710, 1670 cm.$^{-1}$ (C=O).

Anal.—Calcd. for $C_{16}H_{14}N_2O_6$: C, 58.2; H, 4.24; N, 8.48. Found: C, 58.4; H, 4.13; N, 8.30.

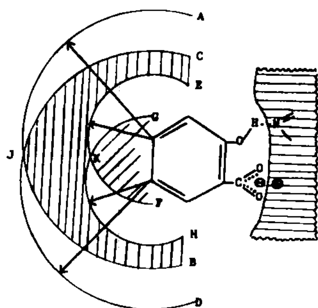


Fig. 1.—Scale model cross section of a possible relationship of the active site to the enzymic nucleophilic site. The horizontal cross-hatch represents the part of the enzyme with the active-site. See *Discussion* for the significance of the vertical and angular cross-hatch. The short arrow represents the most extended conformation of the —CH$_2$-CONH— group and is 3.7Å. long from the center of the methylene carbon to the *N*-phenyl bond; the long arrow represents the —CH$_2$CONHCH$_2$-CONH— group which is 7.7Å. long when fully extended. The measurements were made with Leybold models.

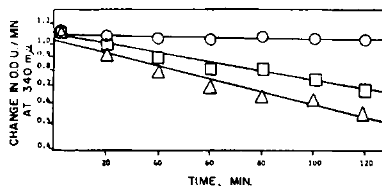


Fig. 2.—Effect of inhibitor concentration on the rate of inactivation of heart LDH·DPNH by 4-IGSA (V). Key: O, heart LDH·DPNH control; □, 2 m*M* 4-IGSA; Δ, 4 m*M* 4-IGSA. The change in O.D. units/minute is proportional to the remaining enzyme concentration. (See *Materials and Methods*.)

RESULTS

A plot of V_o/V against I for several concentrations of inhibitor was used (2) to determine the concentration of I necessary for 50% inhibition ($V_o/V = 2$); where V_o = velocity of the enzymic reaction with no inhibitor, V = velocity with inhibitor, and I = concentration of inhibitor. If possible, several of the points should be in the 30–70% inhibition range to avoid the higher errors in higher or lower inhibitions. A reasonable approximation of the reversible dissociation complex (apparent K_I) between enzyme and inhibitor could be calculated from the previously derived (11) expression:

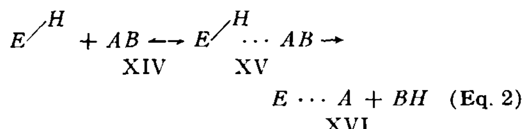
$$K_I = Km \times I/S \qquad \text{(Eq. 1)}$$

where Km is the enzyme-substrate dissociation constant and I is the concentration of inhibitor necessary to give 50% inhibition in the presence of a concentration of substrate S three to four times Km (see *Methods*); K_I and Km are expressed in moles/liter. This derivation contains the assumption that a complex between enzyme and inhibitor or enzyme and substrate are mutually exclusive, and does not distinguish "competitive" from "noncompetitive" reversible inhibition. Reiner (21) has pointed out that "competitive" kinetics determined by the reciprocal plot method do not prove that the inhibitor combines at the actual site, a common misinterpretation, but that a complex between enzyme and inhibitor or enzyme and substrate are mutually exclusive, regardless of where the inhibitor complexes with the enzyme. Since neither the reciprocal plot method nor the I_{50} method can prove unequivocally that complexing occurs at the active site, Eq. 1 is therefore sufficiently accurate to indicate the relative magnitudes of enzyme-inhibitor (*EI*) complex formation. In our system, pyruvate had $K_m = 0.95 \times 10^{-4}$ for heart LDH and $K_m = 2.5 \times 10^{-4}$ for skeletal muscle LDH, determined by a plot of $1/V$ *versus* $1/S$.

The apparent K_I values are listed in Table I, as are the effects of the compounds as irreversible inhibitors on both types of LDH. It should be noted that there is no difficulty in separating reversible from irreversible inhibition; the reversible inhibition is determined in 1 minute at 25°, an interval in which no measurable irreversible inhibition occurs (see Figs. 2–4). All 11 compounds

in Table I reversibly inhibited both types of LDH. Of these 11 compounds in Table I, skeletal muscle LDH was not irreversibly inhibited by four, but was irreversibly inhibited by seven compounds (I, II, III, V, VI, X, XI). With heart LDH, seven of the 11 compounds gave no irreversible inhibition and four showed irreversible inhibition (IV, V, VI, XI).

By definition of site-directed irreversible inhibition (1, 4), a reversible complex (XV) between the inhibitor (XIV) and the active-site of the enzyme is an obligatory intermediate for the formation of a covalent bond (XVI) between the inhibitor and the enzyme resulting in inactivation as shown in



$$E\diagup^{H} + \underset{\text{XIV}}{AB} \rightleftharpoons \underset{\text{XV}}{E\diagup^{H} \cdots AB} \rightarrow \underset{\text{XVI}}{E \cdots A} + BH \quad \text{(Eq. 2)}$$

The rate of inactivation is therefore dependent upon the concentration of the enzyme-inhibitor (EI) complex (XV), and the concentration of EI is expressed (4)

$$[EI] = \frac{E_t}{(K_I/I) + 1} \quad \text{(Eq. 3)}$$

where E_t is the total enzyme concentration excluding that portion of the enzyme that has become inactivated.

If a compound such as 5-(carbophenoxyamino)-salicylic acid (IV) inactivates heart LDH by active-site-directed irreversible inhibition, then doubling the concentration of inhibitor should give less than a doubling of the inactivation rate, according to Eqs. 2 and 3. For example, IV has an apparent $K_I = 1.4 \times 10^{-3}$ for heart LDH; then when $[I] = 4$ mM, $[EI] = 0.74\ E_t$ and when $I = 2$ mM, $[EI] = 0.59\ E_t$. Therefore, increasing $[I]$ from 2 mM to 4 mM increases $[EI]$ by 0.74/0.59 or 1.3. The actual observed values in two runs (Table I) were 1.4 and 1.4 (Table I), the so-called "rate-saturation effect" on rate of inactivation (4).

If inactivation occurred by bimolecular collision (tail-alkylation) between enzyme and inhibitor as shown in Eq. 4, it was previously assumed (4, 7, 10–12) that doubling of the concentration should

$$E\diagup^{H} + AB \rightarrow \widehat{EA} + BH \quad \text{(Eq. 4)}$$

double the rate of inactivation. This assumption is only true if the compound shows no reversible inhibition. If a reversible inhibitor operates by bimolecular collision with the active-site, then the compound can only inactivate the enzyme when the active-site is unoccupied by inhibitor, that is, in addition to Eq. 4, the equilibrium part of Eq. 2 is operating. Stated in another way, the rate of bimolecular inactivation (Eq. 7) is dependent upon the concentration of free enzyme $[E]$ in Eq. 5

$$[E_t] = [E] + [EI] \quad \text{(Eq. 5)}$$

$$K_I = \frac{[E]\,[I]}{[EI]} \quad \text{(Eq. 6)}$$

$$\text{rate} = k'[E]\,[I] = \frac{k'}{K_I}\,[EI] = k''[EI] \quad \text{(Eq. 7)}$$

Equation 7 for bimolecular inactivation with self-protection is kinetically indistinguishable from Eq. 8, the rate for site-directed irreversible inhibition. When two concentrations of inhibitors are compared, the ratio of the rates cancels k or k'' from

$$\text{rate} = k\,(EI) \quad \text{(Eq. 8)}$$

the equation. Thus, the "rate-saturation" effect does not distinguish between the two mechanisms, but at best can only be taken as evidence that the active-site is involved in the inactivation.[3] However, consideration of structure-activity relationships make the bimolecular inactivation mechanism practically untenable (see *Discussion*).

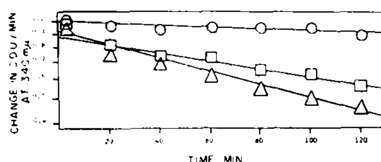


Fig. 3.—Comparative rate of irreversible inhibition of heart LDH·DPNH by 4-IGSA (V) and 5-(carbophenoxyamino)salicylic acid (IV). Key: ○, heart LDH·DPNH control; □, 3 mM 5-(carbophenoxyamino)salicylic acid (IV); △, 4 mM 4-IGSA. The change in O.D. units/minute is proportional to the remaining enzyme concentration. (See *Materials and Methods*.)

DISCUSSION

Our earlier observation (12) that 4-(iodoacetylglycylamido)salicylic acid (V) could irreversibly inhibit LDH from either heart or skeletal muscle, but 4-(iodoacetamido)salicylic acid (I) showed irreversible inhibition of only the skeletal muscle LDH, has been further extended with six additional compounds. Of these six compounds, three (IV, VI, XI) showed irreversible inhibition of heart LDH.

Similar to the 4-series, 5-(iodoacetylglycylamido)-salicylic acid (VI) irreversibly inhibited both heart and skeletal muscle LDH (Table I), but in contrast, 5-(iodoacetamido)salicylic acid (II) showed irreversible inhibition only of the skeletal muscle LDH. The facts that (*a*) the two iodoacetyl compounds with a longer side chain (V, VI) showed irreversible inhibition of heart LDH, (*b*) the shorter side chain compounds (I, II) did not irreversibly inhibit heart LDH, but (*c*) all four compounds showed irreversible inhibition of skeletal muscle LDH, indicates that the position of the enzymatic nucleophilic group (that has been bridged to and alkylated) with respect to the binding points for the hydroxyl and carboxyl of the salicylate moiety is different in these two substrate-identical enzymes. Thus the 4- and 5-(iodoacetamido)-salicylic acids (I and II) show a specificity for irreversible inhibition of skeletal muscle LDH since the nucleophilic site is apparently closer to the binding site, but the nucleophilic site apparently cannot be bridged in heart LDH.

A working hypothesis of the differences in position of the nucleophilic site attacked by an iodoacetyl group can be gleaned by considering Fig. 1. The salicylate is probably ionically bound to the enzyme (horizontal crosshatch) by a cationic point and an imidazole NH on the enzyme surface (2). Within

[3] The authors thank Dr. David Pressman, Roswell Park Memorial Institute, for bringing this oversight to our attention.

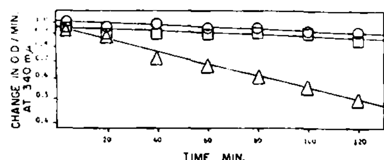


Fig. 4.—Comparative rate of irreversible inhibition of heart LDH · DPNH by 4-IGSA (V) and 4-(carbophenoxyglycylamido)salicylic acid (VII). Key: O, heart LDH · DPNH control; □, 4 m*M* 4-(carbophenoxyglycylamido)salicylic acid (VII); Δ, 4 m*M* 4-IGSA. The change in O.D. units/minute is proportional to the remaining enzyme concentration. (See *Materials and Methods.*)

the limits tolerated by the enzyme surface, the salicylate should be able to rotate in a cylinder on an axis which has as its radius a line segment connecting the two enzymic binding points. Thus, the enzyme and salicylic acid moiety in Fig. 1 would be a cross section of this cylinder. Attached to the salicylate at the 4- or 5-position can be a short or long side chain indicated by the short and long arrows. In its most extended conformation, the long side chain can rotate in a spherical segment with its center at the 4-position. Figure 1 then has one cross section of this spherical segment, *AJB*, which is in the plane of the benzene ring. *CJD* is then the cross-section circle segment for the 5-acetylglycylamido group of V, *EKF* the circle segment for the 4-acetamido group of I, and *GKH* the circle segment for the 5-acetamido group. The side chains can also fold to give a locus of spheres with the same center but shorter radii.

Since of the four compounds, only compounds V, VI, and XI can irreversibly inhibit heart LDH, the nucleophilic group most probably is in a three-dimensional figure with the cross section shown by the vertical hatch in Fig. 1 limited by the lines *EKH* and *CJB*. In contrast, the nucleophilic group of skeletal muscle LDH, which is attacked by both the long and short side chains of I, II, V, and VI most probably is inside a three-dimensional figure with a cross section limited to GDH on the outside and the benzene ring on the inside (shown as an angular cross-hatch); the longer side chains can fold back to the proper locus.

Another hypothesis is equally compatible with the current data. The relative position of the nucleophilic site could be identical in both enzymes, but a carbon chain may protrude from the heart LDH not present in skeletal muscle LDH that may require the side chain of the inhibitor to be sufficiently long to loop over the protruding group in heart LDH. Studies are being pursued to gain further evidence to differentiate between the two explanations or even give evidence for some yet unconceived explanation.

Although inactivation by bimolecular collision (tail-alkylation) cannot be eliminated unequivocally, considerations of structure-activity relationships make the bimolecular mechanism highly improbable. For example, *m*-(iodoacetamido)oxanilic acid (IX), an excellent reversible inhibitor of skeletal muscle LDH (Table I), shows no irreversible inhibition. Due to the increase in degrees of bond rotation in IX, compared to 4-ISA (I), there is no apparent reason why IX should not inactivate skeletal muscle LDH if I operates by bimolecular inactivation. A similar argument can be advanced against the bimolecular mechanism for inactivation of heart LDH. For example, 6-(iodoacetamido)-coumarin-3-carboxylic acid (XI) irreversibly inhibits heart LDH. Since XI is bulkier than I, there is no apparent reason why I should not inactivate heart LDH if XI had operated by the bimolecular mechanism.

A cross-over specificity was noted with 5-(carbophenoxyamino)salicylic acid (IV) (Fig. 3). *This compound irreversibly inhibited heart LDH, but showed no irreversible inhibition of skeletal muscle LDH* (Table I). That the active-site was most probably involved in this inactivation was shown by a "rate saturation effect"; doubling of the concentration from 2 m*M* to 4 m*M* increased the rate of inactivation by only 1.4-fold, compared to a "rate saturation" of 1.3 calculated from the apparent K_I for IV. In contrast (4-carbophenoxyamino)salicylic acid (III) irreversibly inhibited skeletal muscle LDH, but not heart LDH, even though a "rate saturation effect" was not observed.

It is clear that the enzymic nucleophilic group of heart LDH attacked by IV is most probably not the same as that attacked by V and VI. From Fig. 1, the carbophenoxy group has a locus of possible conformations limited to a sphere less than the cross-section line *GKH*, since only a two atom bridge is formed by IV and a three atom bridge by II. Since the carbophenoxy group can react only with an amino group of the enzyme surface, it is probable that this amino group reacts too slowly with I and II to be detected by our experimental design.

The carbophenoxy compounds with the longer side chains, VII and VIII, could theoretically assume conformations that place their urethane carbonyls in position to react with the same enzymic group of heart LDH attacked by the carbophenoxy group of IV. A possible explanation for the fact that VII (Fig. 4) and VIII do not irreversibly inhibit heart LDH is that the proper transition state cannot be assumed by the carbophenoxy groups of VII and VIII; the folding back of the side chain is apt to put the phenyl group in a position toward the nucleophilic site causing a hindrance by the phenyl group of the approach of the carbonyl to the enzymic nucleophilic site. Since 5-(carbophenoxyamino)salicylic acid (IV) irreversibly inhibits both heart LDH and GDH, it may be possible to place groups on the phenyl group which may sterically hinder the approach of the carbophenoxy carbonyl to the nucleophilic site of only one of these two enzymes; such a specificity for the transition state at the nucleophilic site is actively being sought in our laboratory.

Again the possibility that the carbophenoxyamino compounds operate by the bimolecular mechanism is remote. Since there would be less hindrance to the approach of the 4- and 5-(carbophenoxyglycylamino)salicylic acids (VI and VII) to a nucleophilic site for bimolecular reaction than IV or III, then VI and VII would be expected to inactivate heart or skeletal muscle LDH even more rapidly than IV or III; as stated before, VI and VII do not inactivate these two substrate-identical enzymes.

In conclusion, by use of both corollaries of the bridge principle of specificity, cross-over specificity

for selective irreversible inhibition of heart LDH, but not skeletal muscle LDH, and vice versa, has been found, which is attributed to exploitable differences in the secondary-tertiary structure of these two substrate-identical enzymes. Differences in secondary-tertiary structure of these two substrate-identical enzymes have previously been shown by amino acid analysis (24) and by antisera cross reactions (14, 24). The LDH from skeletal muscle and heart in the same animal are distinctly different, but the skeletal muscle LDH from different species are more similar; also, heart LDH from different species are more similar. Even though the studies in this paper were carried out with the LDH's from two distinct tissues of two species, these experiments give a first approximation of the selective inhibition that may be obtained in tissues of the same animal.

If this irreversible specificity for substrate-identical enzymes, which is presumably due to exploitable differences in the secondary-tertiary structure of the enzymes, can be carried over to such critical areas for cell division as (*a*) purine (19) or pyrimidine biosynthesis of (*b*) the folic cofactor area, the potential utility in chemotherapy would be obvious. Such studies in area (*a*) (13) and area (*b*) (9) are continuing in these laboratories.

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Interaction of Weak Organic Acids with Insoluble Polyamides I

Sorption of Salicylic Acid by Nylon 66

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Insoluble polyamides have been shown to bind various chemical agents having acidic hydrogens. To explore in more detail the interaction of an acidic drug, salicylic acid, with a specific polyamide (nylon 66), a study was undertaken to ascertain the influence of concentration, temperature, pH, and solvent composition on the sorption phenomenon. From the sorption studies, a number of physical constants were evaluated: (*a*) saturation value, (*b*) standard affinities, (*c*) heat of sorption, (*d*) apparent diffusion coefficients, and (*e*) activation energy of diffusion.

Even though plastics have made a great impact in the various facets of pharmacy and medicine, some problems have occurred which should indicate that perhaps there should be a slower pace to the introduction of these items to the health professions. This laboratory in the past has reported on some of these problems (1, 2). To the industrial and hospital pharmacist an appreciation and understanding of drug-plastic interactions would help minimize costly errors and at the same time provide the many advantages to be gained by the use of plastics.

The insoluble polyamides (various types of nylon) have a number of applications as parts or component parts in various apparatus where strength and resistance to thermal changes are needed. In pharmaceutical and medical applications these nylon parts[1] may come in contact with drug or biological products for varying periods of time. Depending upon a number of factors, selective drugs and chemical

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[1] For example, component parts of heart lung machines, containers, tubings, syringes, valve parts for aerosol bottles, etc.