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Research Articles

Nonclassical Antimetabolites XII

Bridge Principle of Specificity with Exo-Alkylating Irreversible Inhibitors V. Differences in Specificity of Enzymic Nucleophilic Sites as Detected by the Carbophenoxy Group

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

Investigation of four carbophenoxyamino derivatives of salicylic acid and oxanilic acid has shown that all four reversibly bind to lactic dehydrogenase (LDH) and glutamic dehydrogenase (GDH). Three of these compounds showed irreversible inhibition of GDH with no irreversible effect on LDH. Since 4-(iodoacetamido)-salicylic acid had previously been shown to inhibit both enzymes irreversibly, the specificity shown by the carbophenoxyamino group is attributed to its specificity for reaction only with a primary amino group in the nucleophilic site of an enzyme.

RELATIVELY LARGE molecules—compared to the substrate—have been found that can inhibit an enzyme and have accordingly been called nonclassical antimetabolites (3, 4), in contrast to classical antimetabolites that have only a small change in structure compared to the substrate. Based on the nonclassical antimetabolite theory, it was possible to propose the concept of irreversible inhibition by an exo-alkylation mechanism (3); a properly designed inhibitor such as 4-(iodoacetamido)salicylic acid can reversibly complex with glutamic dehydrogenase (GDH),[1] then become irreversibly bound within the complex by alkylation of the enzyme adjacent to the active site. Strong experimental evidence to support this exo-alkylation phenomenon has been presented (5, 6).

Experimental observations pertinent to the exo-alkylation phenomenon were subsequently

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A preliminary communication on part of this work has appeared (2).
For previous paper in this series see Reference 1.

[1] LDH, lactic dehydrogenase; GDH, glutamic dehydrogenase; DPNH, reduced diphosphopyridine nucleotide; Tris, tris-(hydroxymethyl)aminomethane hydrochloride buffer; 4-ISA, 4-(iodoacetamido)salicylic acid.

and independently made in two other laboratories. Lawson and Schramm (7) observed that the *p*-nitrophenyl ester of N-bromoacetyl-L-α-aminobutyric acid rapidly released *p*-nitrophenol when allowed to react with chymotrypsin; the resultant bromoacetyl-L-α-aminobutyryl derivative of chymotrypsin (presumably bound by an ester link to a serine residue) underwent intramolecular alkylation between the bromoacetyl group and one of the two methionine residues in chymotrypsin. Wofsy, Metzger, and Singer (8) have made related observations in the area of hapten immunochemistry.

In our detailed version of the experimental evidence for the exo-alkylation phenomenon (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."

Recently we presented experimental evidence (9, 10) for the first corollary of the bridge hypothesis of specificity; that is, the difference in ability of certain inhibitors to bridge to and alkylate an enzymic nucleophilic site which merited raising the status of the hypothesis to principle. Evidence has now been found to support the second corollary of the bridge hypothesis —namely, the difference in nucleophilicity of the enzymic group being alkylated; this evidence is the subject of this paper.

MATERIALS AND METHODS

Reagents.—Sodium pyruvate, α-ketoglutaric acid, DPNH, LDH, and GDH were purchased from Nutritional Biochemical Corporation and from Sigma Chemical Co.; LDH was the crystalline enzyme isolated from rabbit muscle and GDH the crystalline enzyme isolated from mammalian liver.

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 m*M* concentration of the substrate (4). The procedure employed for LDH was the same as previously described, except that Tris buffer (pH 7.4) and 1 m*M* pyruvate were employed; the I_{50} values for GDH were determined in the same fashion as for LDH using cell concentrations of 1 m*M* α-ketoglutarate and 75 m*M* ammonium sulfate in Tris buffer (pH 7.4).

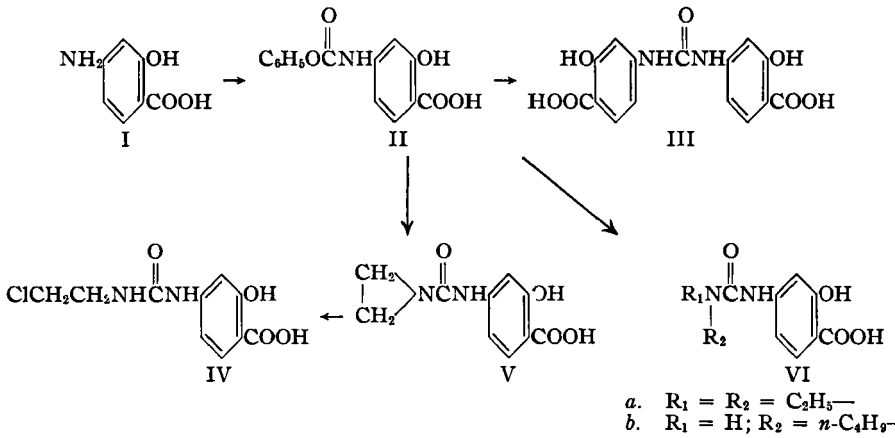
Enzyme Inactivation Procedure.—The LDH inactivation procedure (6), as later modified (10) was employed. The GDH incubations were run as previously described (6). In all incubations for determination of irreversible inhibition, three incubation solutions were run simultaneously and were made from the same master enzyme-DPNH solution (6): (*a*) a 2 m*M* concentration of test compound, (*b*) a standard with 2 m*M* 4-ISA, and (*c*) an enzyme control with no inhibitor. All runs were duplicated at least twice. An occasional bad run was readily eliminated if the control or standard did not behave properly.

The rate saturation experiments were run similarly, except that (*b*) was either a 1 m*M* solution or a 4 m*M* solution of the test compound depending upon the extent of reversible binding of inhibitor by the enzyme, that is, the I_{50}. Considerable care must be taken in making up solutions of the carbophenoxy compounds (Table II) so that the base labile phenyl ester group did not hydrolyze. Usually, 6 m*M* master solutions were made by stirring in Tris buffer at 0° with the gradual addition of 1 *N* sodium hydroxide to keep the pH at 7.4, but care was taken that the pH did not rise above 7.4; several hours were usually required to obtain complete solution. A 12.2 m*M* solution of the standard, 4-ISA, in pH 7.4 Tris buffer was stable for several weeks at 2–5°, as determined by the thiosulfate titration of covalently bound iodine (6). However, the carbophenoxy compounds as 6 m*M* solutions in Tris buffer (pH 7.4) were 25–30% hydrolyzed after 24 hours at 5° (determined spectrophotometrically) and thus were prepared fresh for each incubation.

CHEMISTRY

The synthesis of phenylurethanes and their further reaction with amines to give mixed ureas has been described by Crosby and Niemann (11). When 4-aminosalicylic acid (I) in aqueous sodium bicarbonate was treated under the usual conditions used for reaction with acid chlorides (12), 4-(carbophenoxyamino)salicylic acid (II) was obtained in low and variable yields and considerable amounts of the symmetrical urea (III) were formed.

The usual conditions (12) of dropwise addition of the acid chloride to a solution of I in aqueous sodium bicarbonate at near 0° were unsatisfactory because the initially formed product (II) in the alkaline solution could further react with as yet unreacted 4-aminosalicylic acid (I) to give the symmetrical urea (III). With amines soluble in an organic solvent, Crosby and Niemann (11) avoided this difficulty by reverse addition of the amine to the acid chloride; since the chloro group is more reactive than the phenoxy group, good yields of O-phenylurethanes could be obtained. Such a method would be incompatible with the aqueous alkaline method, since the acid chloride would merely undergo hydrolysis if insufficient amine were present. This enigma was resolved by adding the phenyl chloroformate all at once to the cold solution of I in aqueous sodium bicarbonate; this was possible since the chloroformate reacted slowly enough that the temperature was capable of being controlled. However, it is essential to have sufficiently vigorous stirring in this reaction to emulsify the acid chloride; magnetic stirring was insufficient and allowed the product (II) in solution to compete favorably for the 4-aminosalicylic acid (I), resulting in large amounts of the urea III being formed. The presence of the carbophenoxy group in II was readily determined by its distinctive "active ester" carbonyl at 1720 cm.$^{-1}$ in the in-



a. $R_1 = R_2 = C_2H_5$—
b. $R_1 = H$; $R_2 = n\text{-}C_4H_9$—

frared. The urea III, of course, did not show carbonyl absorption at higher frequency than that of the carboxyl at 1640 cm.$^{-1}$ Mixtures of II and III obtained in the earlier runs were readily separated by the high solubility of II, but relative insolubility of the urea III, in ethanol.

All other compounds could be prepared by this method, except 4-(carbophenoxyglycylamino)salicylic acid. In the latter case, sodium bicarbonate was not strong enough to remove the proton from the zwitterion structure of 4-(glycylamino)salicylic acid and it was necessary to use aqueous sodium hydroxide. If too much base was employed, the carbophenoxy group suffered cleavage, thus leading to unchanged amino acid; in addition, a shortened contact time with the aqueous base was advantageous.

To study carbophenoxy compounds such as II as potential irreversible inhibitors of LDH or GDH it was necessary to determine the stability of II in Tris buffer at pH 7.4 and 37°. Two possible reactions could take place, leading to one of three products. First, the phenoxy group might be displaced by the tris-(hydroxymethyl)aminomethane to give a mixed urea of type VI. Second, hydrolysis to *p*-aminosalicylic acid (I) might occur; the latter then might react with unchanged II to give the symmetrical urea III. To follow the course of this decomposition reaction, a model compound (VI*b*) was prepared in excellent yield by reaction of the carbophenoxy salicylic acid (II) with excess *n*-butylamine in dimethylformamide by short heating on a steam bath.

Table I lists the ultraviolet absorption spectra of the compounds involved in the decomposition of the carbophenoxy group of II. Although there was little difference in the position of the two peaks, the ϵ ratio of these two peaks was considerably higher in compound II than in the others; thus by following the change in the ϵ ratios it was possible to check the stability of II.

TABLE I.—RELATIVE ULTRAVIOLET PEAK RATIOS OF SUBSTITUTED 4-AMINOSALICYLIC ACIDS IN TRIS BUFFER (pH 7.4)

Compd.	Peak I, mμ (ϵ)	Peak II, mμ (ϵ)	ϵ Ratio of Peak I to Peak II
I	267 (13,000)	300 (8600)	1.51
II	264 (20,000)	300 (7750)	2.60
VI*b*	268 (20,800)	300 (10,000)	2.08

In 1% aqueous sodium bicarbonate (pH 8.4) at 37°, the ϵ ratio rapidly dropped from 2.60 to 1.75 in 15 minutes and 1.57 in 45 minutes; after 24 hours at 37°, the ϵ ratio was 1.50, the same as *p*-aminosalicylic acid (I). It is highly probable that at the high dilution this experiment is run (0.55 m*M*), hydrolysis to I occurs rather than urea III formation.

In Tris buffer (pH 7.4) at 37°, II was considerably more stable. In 1 hour, the ϵ ratio dropped from 2.60 to 2.42; after 24 hours at 37°, the ϵ ratio was 2.01. The latter number is probably within experimental error for formation of a mixed urea such as VI with the Tris; it is unlikely that hydrolysis to I (ratio of 1.51) took place since a greater change in ϵ ratio from 1 hour to 24 hours should have taken place. In either case, it is clear that the maximum decomposition that could occur in 1 hour in the Tris buffer (the length of the enzyme incubation period) was about 34% if the mixed urea VI with Tris was formed and less if I was formed.

The ease with which the phenoxy (II) could be converted to a mixed N-*n*-butyl-urea (VI*b*) with *n*-butylamine suggested that the synthesis of the mixed urea V with aziridine be attempted, since V could be a potentially useful irreversible inhibitor. When II was reacted with 3–4 equivalents of aziridine in dimethylformamide at about 90°, these conditions employed for preparation of VI*b* led to polymeric materials. That diethylamine could react smoothly with II to give a mixed urea (VI*a*) showed that a secondary amine could be used for this reaction. When a solution of II in dimethylformamide containing two equivalents of triethylamine was allowed to react with one equivalent of aziridine at room temperature, then the reaction mixture poured into dilute hydrochloric acid, slow separation of the chloroethyl urea IV took place over several days. That IV could be isolated certainly indicated the aziridinyl urea V had been formed. However, attempts to isolate V directly when the reaction was run or worked up under a variety of conditions proved to be fruitless, even though mixed ureas of aziridine have been previously prepared by the carbophenoxy route (13).

4-(Carbophenoxyamino)salicylic Acid (II).—A solution of 5.0 Gm. (32.6 mmoles) of 4-aminosalicylic acid in 130 ml. of water containing 11 Gm. of sodium bicarbonate was cooled to 3° in an ice bath. Then 7.55 Gm. (48 mmoles) of phenyl chloroformate

was added in one portion; the mixture was vigorously stirred with a mechanical paddle stirrer for 3 hours. During this time, the temperature stayed between 3–8°; some of the sodium salt of the product separated from solution. The solid sodium salt was collected on a filter and the filtrate was acidified to about pH 2 with 3 *N* hydrochloric acid. The product was collected on a filter, washed with water, and dried.

The insoluble sodium salt was quickly dissolved in 200 ml. of water preheated to about 75°, then filtered. The filtrate was immediately acidified with 3 *N* hydrochloric acid. The separated product was combined with the first fraction and recrystallized from aqueous ethanol; yield, 7.9 Gm. (89%) of pure material, m.p. 215–217°; $\nu_{max.}^{KBr}$ 3340 (NH); 1720 (urethane C=O); 1637 cm.$^{-1}$ (carboxyl C=O); $\lambda_{max.}^{EtOH}$ 264 (ϵ 25,000), 302 mμ (ϵ 9,800).

Anal.—Calcd. for $C_{14}H_{11}NO_5$: C, 61.5; H, 4.02; N, 5.12. Found: C, 61.3; H, 4.06; N, 5.26.

5-(Carbophenoxyamino)salicylic Acid.—This compound was prepared from 5-aminosalicylic acid hydrochloride in the same manner as described for II. Recrystallization from alcohol-water gave 7.0 Gm. (97%) of product as nearly white crystals, m.p. 264–275° dec.; $\lambda_{max.}^{EtOH}$ 221 (ϵ 41,200), 246 (ϵ 16,900), 325 mμ (ϵ 3,800); $\nu_{max.}^{KBr}$ 3310(NH); 1705 (urethane C=O); 1620 cm.$^{-1}$ (carboxyl C=O).

Anal.—Calcd. for $C_{14}H_{11}NO_5$: C, 61.5; H, 4.02; N, 5.12. Found: C, 61.3; H, 3.78; N, 5.31.

m-(Carbophenoxyamino)oxanilic Acid.—From 5 Gm. of *m*-aminooxanilic acid (14) and 5.9 Gm. of phenyl chloroformate, as described for the preparation of II, a 4.5-Gm. (59%) quantity of product was obtained. Recrystallization from chloroform afforded white crystals, m.p. 182–183°; $\lambda_{max.}^{EtOH}$ 237 mμ (ϵ 35,900); $\nu_{max.}^{KBr}$ 3310 (NH); 1760 (urethane C=O); 1710 (carboxyl C=O); 1680 cm.$^{-1}$ (amide C=O).

Anal.—Calcd. for $C_{15}H_{12}N_2O_5$: C, 60.0; H, 4.00; N, 9.33. Found: C, 59.9; H, 4.16; N, 9.29.

4-(Carbophenoxyglycylamino)salicylic Acid.—A solution of 1.00 Gm. (4.76 mmoles) of 4-(glycylamino)salicylic acid (12, 15) in 40 ml. of 0.25 *N* sodium hydroxide was cooled in an ice bath. When the temperature reached 3°, 1.14 Gm. (7.14 mmoles) of phenyl chloroformate was added in one portion. The mixture was rapidly mixed in with a mechanical paddle stirrer with continued ice bath cooling for 90 minutes. The cold solution was filtered through a Celite pad and the filtrate immediately acidified with 3 *N* hydrochloric acid. The product was collected on a filter and washed with water; yield, 0.70 Gm. (45%), m.p. 236–238°. Recrystallization from ethyl acetate gave white crystals, m.p. 242–243°; $\nu_{max.}^{KBr}$ 3400 (NH); 1720 (sh.) (urethane C=O); 1640 (carboxyl C=O); 1680 (amide C=O); 732, 690 cm.$^{-1}$ (monosubstituted phenyl). Longer reaction times or more base gave lower yields.

Anal.—Calcd. for $C_{16}H_{14}N_2O_6$: C, 58.2; H, 4.24; N, 8.48. Found: C, 58.1; H, 4.44; N, 8.29.

N - n - Butyl - N′ - (3 - hydroxy - 4 - carboxyphenyl)urea (VI*b*).—To a solution of 1.00 Gm. (3.66 mmoles) of 4-(carbophenoxyamino)salicylic acid (II) in 10 ml. of dimethylformamide was added 0.80 Gm. (11 mmoles) of *n*-butylamine. After being heated on a steam bath for 1 hour protected from moisture, the solution was diluted with 40 ml. of water and acidified to about pH 3 with 3 *N* hydrochloric acid. The product was collected on a filter and washed with water. Recrystallization from dilute alcohol gave 0.80 Gm. (95%) of white crystals, m.p. 205–206°; $\nu_{max.}^{KBr}$ 3325 (NH), 1650 (urea and carboxyl C=O); and no urethane C=O near 1720 cm.$^{-1}$; $\lambda_{max.}^{EtOH}$ 274 (19,800), 302 mμ (ϵ 12,300).

Anal.—Calcd. for $C_{12}H_{16}N_2O_4$: C, 57.1; H, 6.34; N, 11.1. Found: C, 57.5; H, 6.57; N, 11.3.

N,N - Diethyl - N′ - (3 - hydroxy - 4 - carboxyphenyl)urea (VI*a*).—This was prepared in the same manner as described for VI*b* using diethylamine; yield, 0.90 Gm. (98%). Recrystallization from dilute alcohol gave white crystals, m.p. 161–162°; $\nu_{max.}^{KBr}$ 3450 (NH); 1640 (urea and carboxyl C=O); and no urethane C=O near 1720 cm.$^{-1}$; $\lambda_{max.}^{EtOH}$ 273 (ϵ 19,400), 304 mμ (ϵ 23,500).

Anal.—Calcd. for $C_{12}H_{16}N_2O_4$: C, 57.1; H, 6.34; N, 11.1. Found: C, 57.1; H, 6.35; N, 10.8.

N - (2 - Chloroethyl) - N′ - (3 - hydroxy - 4 - carboxyphenyl)urea (IV).—To a solution of 1.00 Gm. (3.66 mmoles) of 4-(carbophenoxyamino)salicylic acid (II) and 1.02 ml. (7.32 mmoles) of triethylamine in 10 ml. of dimethylformamide a 0.188-ml.

TABLE II.—IRREVERSIBLE INHIBITION OF LDH AND GDH BY CARBOPHENOXY COMPOUNDS

	GDH						LDH					
		Inactivation, Rate		Saturation Rate				Inactivation, Rate		Saturation Rate		
Compd.	I_{50}	mM Concn.	Rate[a]	mM Concn.	Ratio[b] Calcd.	Found	I_{50}	mM Concn.	Rate[a]	mM Concn.	Ratio[b] Calcd.	Found
4-(Iodoacetamido)-salicylic acid[c]	3.4	2	1.0	2/1	1.26	1.23	6.6	2	1.0	3/1	1.93	2.0
4-(Carbophenoxy-amino)salicylic acid (II)	3.4	1.5	0, 0				4.4	2	0.98	2/1	1.34	2.6, 3.7
		3.0	0, 0	—	—	—		2	0.85	4/2	1.23	2.3
		2	0, 0, 0							4/2		3.4[d]
5-(Carbophenoxy-amino)salicylic acid (VII)	1.6	2	2.0	2/1	1.17	1.1	5.9	2	0, 0			
		2	2.0	2/1		1.2, 1.0		2	0, 0	—	—	—
								2	0.1, 0.1			
4-(Carbophenoxy-glycylamino)sali-cylic acid (VIII)	6.7	2	1.3	4/2	1.20	1.0	6.3	2	0[e]			
		2	1.6	4/2		1.2		2	0	—	—	—
m-(Carbophenoxy-amino)oxanilic acid (IX)	4.0	2	1.85	2/1	1.21	1.3	0.87	2	0[e]			
		2	1.87	2/1		1.3		2	0[e]	—	—	—
								2	0[e]			

[a] Rate compared to 4-(iodoacetamido)salicylic acid, the standard that is assigned an arbitrary value of 1.0 and is run simultaneously. [b] Ratio of rates of inactivation when run simultaneously. [c] The figures on 4-(iodoacetamido)salicylic acid were previously reported in Reference 6. [d] Different preparation of inhibitor than those preceding. [e] This value was less than zero; that is, the compound protected the enzyme against the small amount of thermal inactivation observed in the control.

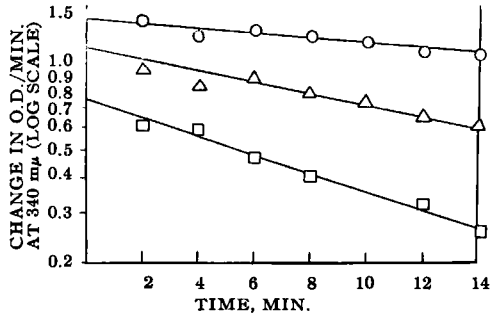


Fig. 1.—Comparative irreversible inhibition of GDH·DPNH by 4-ISA and 5-(carbophenoxyamino) salicylic acid (VII). Key: O, GDH·DPNH control; □, 2 m*M* 5-(carbophenoxyamino)salicylic acid; △, 2 m*M* 4-ISA.

(3.66 mmoles) quantity of aziridine was added. After standing about 18 hours in a stoppered flask, the solution was diluted with 40 ml. of water and strongly acidified with 3 *N* hydrochloric acid. During a period of 1 week at about 3°, white crystals gradually separated; yield, 0.3 Gm. (37%), m.p. 177–179°. Recrystallization from absolute alcohol–petroleum ether gave white crystals, m.p. 185–187°; $\nu_{max.}^{KBr}$ 3375 (NH); 1650 (urea and carboxyl C=O); and no urethane near 1720 cm.$^{-1}$ The compound gave a positive test for halogen.

Anal.—Calcd. for $C_{10}H_{11}ClN_2O_4$: C, 46.5; H, 4.26; N, 10.9. Found: C, 46.5; H, 4.46; N, 10.9.

N - (2 - Chloroethyl) - N′ - (3 - carboxy - 4 - hydroxyphenyl)urea.—This was prepared from 5-(carbophenoxyamino)salicylic acid in 50% yield, m.p.

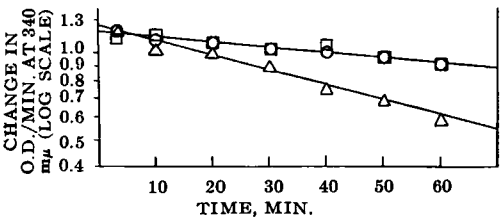


Fig. 2.—Comparative irreversible inhibition of LDH·DPNH by 4-ISA and 5-(carbophenoxyamino)-salicylic acid (VII). Key: O, LDH·DPNH control; □, 2 m*M* 5-(carbophenoxyamino)salicylic acid; △, 2 m*M* 4-ISA.

180–183°. Recrystallization from absolute alcohol–petroleum ether gave white crystals, m.p. 188–189°.

Anal.—Calcd. for $C_{10}H_{11}ClN_2O_4$: C, 46.5; H, 4.26; N, 10.9. Found: C, 46.9; H, 4.13; N, 11.1.

RESULTS

Comparisons of the reversible and irreversible inhibition of LDH and GDH by the four carbophenoxy derivatives are listed in Table II. All four compounds were good to excellent reversible inhibitors of both enzymes. Three of the four compounds, 5-(carbophenoxyamino)salicylic acid (VII), 4-(carbophenoxyglycylamino)salicylic acid (VIII), and *m*-(carbophenoxyamino)oxanilic acid (IX) inactivated GDH even more rapidly than the standard compound, 4-(iodoacetamido)salicylic acid; in contrast, none of these three compounds showed inactivation of LDH. These three compounds also showed a rate saturation in inactivation of GDH.

The theoretical rate saturations values can be calculated in the following manner if the rate of inactivation is dependent upon the concentration of reversible complex, *EI*, formed in Eq. 1.

$$E + I \longleftrightarrow EI \qquad \text{(Eq. 1)}$$

where E is the free enzyme concentration, I is the inhibitor concentration and EI is the concentration of enzyme-inhibitor reversible complex.

$$K_I = \frac{[E]\,[I]}{[EI]} \qquad \text{(Eq. 2)}$$

$$E_t = [E] + [EI] \qquad \text{(Eq. 3)}$$

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

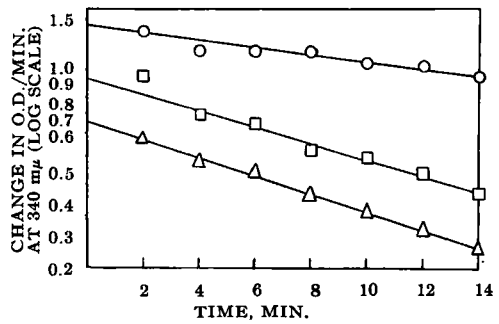


Fig. 3.—Rate saturation effect during inactivation of GDH·DPNH by 5-(carbophenoxyamino)salicylic acid (VII). Key: O, GDH·DPNH control; □, 1 m*M* VII; △, 2 m*M* VII.

Substituting Eq. 3 in Eq. 2 gives Eq. 4

$$[EI] = \frac{[E_t]}{\frac{K_I}{[I]} + 1} \qquad \text{(Eq. 4)}$$

Similarly, Eq. 5 can be derived (16) where S = the substrate concentration and ES = the concentration of enzyme-substrate complex and Km = the substrate-enzyme dissociation constant

$$[ES] = \frac{E_t}{\frac{Km}{[S]} + 1} \qquad \text{(Eq. 5)}$$

When $[ES] = [EI]$, 50% inhibition will be obtained, the I_{50}. Thus, equating Eqs. 4 and 5 gives Eq. 6

$$K_I = \frac{Km[I]}{[S]} = Km \times I_{50} \qquad \text{(Eq. 6)}$$

Since $Km = 2.5 \times 10^{-4}$ for α-oxoglutarate on GDH has previously been determined (6), the K_I for any of the compounds in Table II can be calculated. Thus, from Eq. 6 and an I_{50} of 1.6, 5-(carbophenoxyamino)salicylic acid (VII) has $K_I = 4 \times 10^{-4}$. Then when $[I] = 2$, Eq. 4 gives $[EI] = 0.83\,[E_t]$ and when $[I] = 1$, $EI = 0.71\,[E_t]$. An increase in $[I]$ from 1 m*M* to 2 m*M* should increase $[EI]$ by 0.83/0.71 or 1.17-fold. Therefore, the ratio of rates of inactivation should be 1.17 if the inactivation is

dependent upon initial reversible complex at the active site (exo-alkylation) and should be 2.0 if the inactivation were due to a random bimolecular collision between inhibitor and enzyme (tail-alkylation).

Other rate saturation ratios calculated similarly are listed in Table II. In the case of compound VIII, where the I_{50} value is greater than five it is better to use a 4/2 ratio to show rate saturation, rather than the standard 2/1 ratio. It is clear that compounds VII, VIII, and IX (Table II) inactivate GDH with rate saturation in agreement with the prediction for the exo-alkylation mechanism.

Not all carbophenoxy compounds showed inactivation of GDH, as the bridge principle would predict. Thus, 4-(carbophenoxyamino)salicylic acid (II) showed no irreversible inhibition of GDH in four runs. Irreversible inhibition of LDH at a slightly slower rate than the standard 4-ISA was shown by II; since II did not show a rate saturation effect, this irreversible inactivation of LDH by II is by some mechanism different than the exo-alkylation mechanism. (See *Discussion*.)

To conserve space, only three of the many inactivation runs listed in Table II are shown. Figure 1 shows the inactivation of GDH by 5-(carbophenoxyamino)salicylic acid (VII) and Fig. 2 shows the lack of inactivation of LDH by VII. A rate saturation effect in the inactivation of GDH by VII is shown in Fig. 3.

DISCUSSION

In seeking evidence for the exo-alkylation phenomenon (3), we deliberately chose the experimental design of an alkylating group with low specificity; in this way, the chances were increased that the alkylating group could covalently link with some enzymic nucleophilic site adjacent to the active site after a reversible complex had been formed at the active site. A group with the desired low specificity was the iodoacetyl group; strong experimental evidence was obtained (5, 6) that 4-(iodoacetamido)-salicylic acid inactivated both LDH and GDH by the exo-alkylation mechanism. It was then proposed (6) that the ability of the alkylating group to bind covalently with an enzymic nucleophilic site was dependent both on the ability of the bound inhibitor to bridge to the nucleophilic group and upon the nucleophilicity of the enzymic site being alkylated. Such an hypothesis, if true, could give an extra magnitude of specificity not possible with reversible inhibitors.

Specificity with the iodoacetyl group was obtained by influencing the bridging (9, 10), even though the iodoacetyl group has little functional specificity. Of some 15 amino acids in proteins containing a third functional group, about one-half have the nucleophilic capacity to react with the iodoacetyl group to form a covalent bond. In contrast, the carbophenoxy group of a molecule such as 5-(carbophenoxyamino)salicylic acid (VII), has complete functional specificity; that is, it will react only with a primary amino group on a protein; neither the indole-NH of tryptophane or the imidazole-NH of histidine will react with phenyl esters (17). Although no information concerning the reaction of phenyl esters with the guanidino group of arginine could be found, the guanidino group is not a probable site since it would be fully protonated at pH 7.4. Thus, the most likely nucleophilic site of a protein that could bind covalently with the carbophenoxy group by exo-alkylation would be the ϵ-amino group of a lysine and, less likely, a terminal α-amino group.[2]

All four carbophenoxy compounds listed in Table II were good to excellent reversible inhibitors of LDH and GDH. Of these, three (VII–IX) irreversibly inactivated GDH but had no inactivation effect on LDH; it is highly probable that these three compounds inactivated GDH by the exo-alkylation mechanism since all three showed the rate saturation effect indicating that a reversible complex between GDH and the inhibitor was an obligatory intermediate to inactivation. Although it would appear that the inactivity of II is because of the inability of the urethane group of 4-(carbophenoxyamino)salicylic acid (II) to reach the enzymic amino group for covalent bond formation, it is also possible that the enzyme hinders the relatively large phenyl group from assuming the transition state necessary for covalent bond formation.

The fact that GDH is attacked by either the carbophenoxy group of VII–IX or by the iodoacetyl group of 4- or 5-(iodoacetamido)salicylic acid (6) 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 VII–IX is the same group that is covalently bound by the (iodoacetamido)salicylic acids, even though it is possible for these two nucleophilic groups to be one and the same.

Conversely, it can be concluded that VII, VIII, and IX cannot bridge to an available amino group on LDH. It might have been expected that 4-(carbophenoxyglycylamino)salicylic acid (VIII), with its relatively long bridging ability, should have been able to form a covalent bond with an amino group on LDH; however, it is also possible that hindrance interaction between the enzyme and the phenyl group prevented the formation of the proper transition state. If a carbophenoxy compound is ultimately found that can irreversibly inhibit two enzymes, it would be of interest to see if specificity could be achieved by placing bulky groups on the phenoxy radical for selective hindrance of the transitional state.

Although 4-(carbophenoxyamino)salicylic acid (II) inactivated LDH almost as rapidly as the standard, 4-ISA, it is clear that this inactivation did not proceed by the exo-alkylation mechanism since no rate saturation effect was obtained. Several obvious explanations are readily eliminated: (*a*) the inactivation is not by the tail-alkylation mechanism, or VII, VIII, and IX should also have inactivated LDH by this bimolecular process; (*b*) it is highly unlikely that some extraneous impurity caused this effect, since two different samples prepared at 6-month intervals showed the same effect.

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[2] As pointed out by a referee, the carbophenoxy group is an acylating group and not an alkylating group. At the time that the first evidence for the exo-alkylation phenomenon was presented (5), an acylation reaction of this type was unforeseen. We have used "exo-alkylation" as equivalent to "covalent bond formation—adjacent to the binding site—within the enzyme-inhibitor reversible complex." It is apparent that the latter general expression was intended and that perhaps the word "exo-alkylation" should be literally limited to covalent bond formation by alkylation.

A possible explanation is that 4-(carbophenoxyamino)salicylic acid (II) can bind weakly to some site other than the active site, then form a covalent link that makes the active site inaccessible to the substrate; such reversible binding to an extraneous site would have to have a dissociation constant larger than 10^{-2} to show the lack of a rate saturation effect within experimental error. The fact that one compound does not show a rate saturation effect actually strengthens the interpretation that those compounds showing a rate saturation effect might operate by the exo-alkylation mechanism.

Although no compound in Table II inactivated LDH selectively by the exo-alkylation mechanism, it is probable that such a compound could be found by further investigation.

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

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Nonclassical Antimetabolites XIII

Simulation of the 5′-Phosphoribosyl Moiety of 5′-Adenylic Acid at the Enzyme Level by ω-Carboxyalkyl and Aralkyl Groups Attached to Adenine

By B. R. BAKER and H. S. SACHDEV

5′-Adenylic acid can inhibit both lactic dehydrogenase and glutamic dehydrogenase; since both the phosphate and adenine moieties are necessary for good inhibition, the 5′-adenylic acid presumably competes with DPN. This inhibition by 5′-adenylic acid can be duplicated by 9-(4′-carboxybutyl)-adenine (XVIII) on both enzymes, indicating that the ω-valeric acid side chain can simulate the binding of the 5-phosphoribosyl group when the latter is attached to adenine. 9-(*para*-Carboxybenzyl)-adenine (XVI), a compound with a conformation-fixed side chain, can simulate the binding of 5′-adenylic acid to glutamic dehydrogenase but not lactic dehydrogenase; this difference has been attributed to the difference in conformation of 5′-adenylic acid (and DPN) when bound to the two enzymes.

MANY IMPORTANT ENZYMES have substrates bearing the 5-phosphoribosyl moiety such as the ribotides involved in nucleic acid biosynthesis. A number of purines and pyrimidines useful in cancer chemotherapy operate by enzymic blockade at the nucleotide level (2). For example, 5-fluorouracil is converted intracellularly to 5-fluorouracil deoxyribotide; the latter inhibits cell growth by blockade of thymidylate synthetase (3, 4). Similarly, in the cell 6-mercaptopurine (I) is converted by inosinic pyrophosphorylase to 6-mercaptopurine ribotide (II) (5, 6). The latter compound is a potent inhibitor for inosinic dehydrogenase, the enzyme converting inosinic acid (IV) to xanthylic acid (V); at a somewhat higher concentration, II also inhibits adenylosuccinate synthetase, the enzyme forming adenylosuccinate (III) from inosinic acid (IV) (7, 8).

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