(6) Schulert, A. R., and Weiner, M., *J. Pharmacol. Exptl. Therap.*, **110**, 451(1954).
(7) Frostad, S., *Acta Tuberc. Pneumol. Scand.*, **41**, 68(1961).
(8) Nelson, E., Cureton, G., Mirigian, R. A., and Campagna, F. A., THIS JOURNAL, **52**, 605(1963).
(9) Schroeter, L. C., Tingstad, J. E., Knoechel, E. L., and Wagner, J. G., THIS JOURNAL, **51**, 865(1962).
(10) Levy, G., *ibid.*, **52**, 1039(1963).
(11) Hogben, C. A. M., *Federation Proc.*, **19**, 864(1960).
(12) Nelson, E., THIS JOURNAL, **46**, 607(1957).
(13) Higuchi, W. I., Parrott, E. L., Wurster, D. E., and Higuchi, T., *ibid.*, **47**, 376(1958).
(14) Nelson, E., *ibid.*, **48**, 96(1959).
(15) Levy, G., and Gumtow, R. H., THIS JOURNAL, in press.
(16) Higuchi, T., Rao, A. N., Busse, L. W., and Swintosky, J. V., *ibid.*, **42**, 194(1953).
(17) Niebergall, P. J., and Goyan, J. E., *ibid.*, **52**, 29 (1963).
(18) Krebs, V. C., *Anales Fac. Quim. Farm. Univ. Chile*, **11**, 204(1959); through *Chem. Abstr.*, **54**, 25575f(1960).
(19) Higuchi, T., Elowe, L. N., and Busse, L. W., THIS JOURNAL, **43**, 685(1954).
(20) Nelson, E., personal communication.
(21) Levy, G., "Biopharmaceutical Considerations in Dosage Form Design and Development," J. B. Lippincott Co., Philadelphia, Pa., in press.

Nonclassical Antimetabolites XIV

Some Factors in Design of Irreversible Inhibitors Effective at Low Concentration

By B. R. BAKER, R. P. PATEL, and PRABODH I. ALMAULA

5-(Bromoacetyl)salicylic acid (XII) irreversibly inhibits glutamic dehydrogenase and lactic dehydrogenase about as well as the standard exoalkylating agent, 4-(iodoacetamido)salicylic acid, at one-tenth the concentration of the standard; most of this increased reactivity is because of the higher reactivity of the halogen in XII. Three of the four iodoacetamido compounds that reversibly bound to lactic dehydrogenase more tightly than the standard gave irreversible inhibition at 10–20 per cent the concentration necessary for the standard. Three carbophenoxyamino heterocyclic acids showed no irreversible inhibition of lactic dehydrogenase, but were effective irreversible inhibitors of glutamic dehydrogenase at one-tenth the concentration of the standard compound.

THE CONCEPT (2) that a properly designed inhibitor can reversibly complex with the active site of an enzyme, then become irreversibly bound within the complex by alkylation adjacent to the active site (exo-alkylation) has had strong experimental support (3, 4). Similar observations have been independently and subsequently made in the area of hapten immunochemistry (5) and with chymotrypsin (6).

The exo-alkylation phenomenon is illustrated (4) by Eq. 1. In the experimental evidence for this phenomenon, it was shown that the rate of irreversible reaction between the inhibitor AB and the enzyme, to form the irreversible complex, II, was dependent upon the concentration of reversible complex, I; that is, the rate of inactivation could not be increased once the enzyme was saturated with the inhibitor. The amount of reversible complex, I, can be calculated from the measurable dissociation constant, K_I, of the enzyme-inhibitor complex.

$$E\!\!<^{H} + AB \rightleftharpoons E\!\!<^{H} \cdots AB \rightarrow \underset{\text{I}}{} \quad E\!\!\overset{\frown}{\cdots A} + BH \quad \text{(Eq. 1)}$$
II

Of obvious importance to chemotherapy is that a sufficient intracellular concentration of an inhibitor must be obtained to effect the target enzyme site of an invading cell. In order to overcome an insufficient intracellular concentration of inhibitor, it would be necessary to find inhibitors that will operate at lower concentrations. There are two ways in which exo-alkylating irreversible inhibitors effective at lower concentrations could be obtained. First, a compound that can saturate the enzyme at a lower concentration (smaller K_I) will operate equally as effectively as an agent that requires a higher concentration to saturate the enzyme (larger K_I); second, if a more active alkylating group, B, in structure I is employed, the rate of inactivation of the enzyme should be the same at proportionally lower inhibitor concentrations. The design, synthesis, and evaluation of both types are the subject of this paper.

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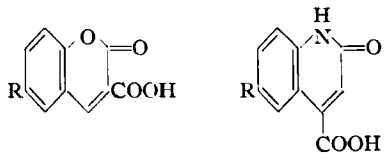
For previous paper in this series see Reference 1.

TABLE I.—SYNTHESIS OF CARBOPHENOXYAMINO AND IODOACETAMIDO HETEROCYCLIC CARBOXYLIC ACIDS

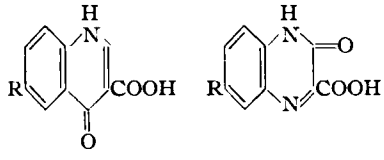
Compd. No.[a]	Method[b]	Yield, %	M.p., °C.	Anal. Calcd. C	H	N	Found C	H	N
III*d*	A	78	215–216[c]	38.6	2.16	3.75	38.8	2.30	3.84
III*e*	A	52	218–220[d]	62.9	3.42	4.32	62.9	3.51	4.32
IV*d*	C	33	above 300[e]	38.8	2.44	7.55	38.7	2.64	7.31
IV*e*	B	43	above 300[e]	63.1	3.73	8.65	62.9	3.90	8.71
V*d*	E	61	above 300[f]	38.8	2.44	7.55	39.0	2.61	7.48
V*e*	E	77	above 300[e]	63.1	3.73	8.65	62.8	3.82	8.42
VI*d*	D	43	above 300[g]	35.3	2.16	11.3	35.2	2.37	11.3
VI*e*	C[h]	31	above 300[e]	59.2	3.42	12.9	58.9	3.59	13.1

[a] Each compound had the expected infrared spectrum. [b] See *Experimental*. [c] Recrystallized from 95% ethanol. [d] Recrystallized from ethyl acetate. [e] Recrystallized from absolute ethanol-petroleum ether. [f] Recrystallized from 2-methoxyethanol-water. [g] Recrystallized from ethanol-water. [h] Although the sodium salt of VI*c* was insoluble, *Method C* worked better than *Method D*.

The prototype of exo-alkylating irreversible inhibitor was 4-(iodoacetamido)salicylic acid (3,



III IV



V VI

a series, R = H
b series, R = NO_2
c series, R = NH_2

d series, R = $ICH_2\overset{\overset{O}{\|}}{C}NH-$
e series, R = $C_6H_5O\overset{\overset{O}{\|}}{C}NH-$

4). A number of compounds have been observed (7) to give lower I_{50} values[1] for LDH and GDH[2] than the prototype, salicylic acid (8). For example, salicylate had I_{50} = 19 on LDH, coumarin-3-carboxylic acid (III*a*) had I_{50} = 1.7, 2-hydroxycinchoninic acid (IV*a*) had I_{50} = 0.18, and 1,6,7-trimethyl-2-quinolone-3-carboxylic acid had I_{50} = 0.20; thus these compounds had K_I values of 0.1 to 0.01 of the original salicylic acid. The structural feature necessary for inhibition of LDH and GDH that these compounds had in common was the 1,2- or 1,3- relationship of oxo (or hydroxyl) and carboxyl groups (7).

Two other compounds that should reversibly inhibit LDH and GDH are 4-hydroxyquinoline-3-carboxylic acid (V*a*) and 2-hydroxyquinoxaline-3-carboxylic acid (VI*a*). The latter was resynthesized (10) and showed reversible inhibition of LDH and GDH; however, the I_{50} concentration could not be reached because of the amount of absorption of light at 340 mμ by the compound. It was expected that the carbophenoxyamino (9, 11) and iodoacetamido derivatives of III–VI should show lower K_I values than the standard compound, 4-(iodoacetamido)salicylic acid (3, 4) and therefore could potentially operate as exo-alkylating agents at lower concentrations than that used for the standard compound.

EXPERIMENTAL

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

Determination of I_{50} Values.[1]—The procedure employed for LDH was the same as that previously described (7, 8), except that 0.05 *M* Tris buffer (pH 7.4) and 1 m*M* pyruvate were employed (12). 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 0.05 *M* Tris buffer (pH 7.4). In some cases with colored compounds (for example compound IV*d*), the I_{50} concentration could not be reached because of insufficient light transmission at 340 mμ for the kinetic study. In such cases, the highest concentration possible was used that allowed full light transmission at 340 mμ and the I_{21} or I_{30} was noted; these I_x values could be converted to the K_I values in Table II by the calculations shown in the section on *Results*.

Enzyme Inactivation Procedure.—The LDH inactivation procedure (4), as later modified (12), was employed. The GDH incubations were run as previously described (4). In all incubations for determination of irreversible inhibition, three incubation solutions were run simultaneously and were made from the same master enzyme-DPNH solution (4): (*a*) a solution of the test compound, (*b*) a standard of 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 elimi-

[1] The I_{50} is defined as the concentration of inhibitor (I) that will give 50% inhibition of enzymic reaction in the presence of 1 m*M* concentration of substrate *S* (8). The I_{50} and the enzyme-inhibitor (EI) dissociation constant K_I can be interrelated (9) by the dissociation constant, K_m, of the enzyme-substrate complex with use of the formula

$$K_I = K_m \times I/S = K_m \times I_{50}$$

[2] Abbreviations: LDH, lactic dehydrogenase; GDH, glutamic dehydrogenase; DPNH, reduced diphosphopyridine nucleotide; 4-ISA, 4-(iodoacetamido)salicylic acid.

TABLE II.—RELATIVE EFFECTS OF INHIBITORS ON LDH AND GDH

Compd. No.	Thiosulfate Reactivity	Enzyme	$K_I \times 10^4$	Inactivation, Rate	Incubation Concn.[a]
4-ISA	1.0[c]	LDH	17[b]	1.0[c]	2.0
		GDH	8.5[b]	1.0[c]	2.0
XII	19	LDH	13	1.2, 1.2	0.2
		GDH	11	0.65, 0.63	0.2
III*d*	1.13	LDH	3.1[d]	0.45, 0.59	0.4
IV*d*	0.63	LDH	3.2[e]	0.56, 0.46	0.2
V*d*	...[g]	LDH	0.87	0, 0	0.2
VI*d*	...[g]	LDH	1.4[d]	0.76, 0.83	0.2
III*e*	...	LDH	1.9	0, 0, 0	0.4
		GDH	2.0	0.62, 0.58	0.2
IV*e*	...	LDH	2.4	0, 0, 0	0.4
		GDH	2.4	1.0, 1.3[f]	0.2
V*e*	...	LDH	1.1[d]	0, 0	0.13[h]
		GDH	2.3[d]	0, 0	0.13[h]
VI*e*[i]	...	...	...	...	...

[a] Millimolar concentration of inhibitor; 2 m*M* 4-ISA used for comparison (see *Methods*). [b] These values calculated from the I_{50} (7) (see *Results*) are slightly different from the K_I values obtained by Lineweaver-Burk plots (4) and agree within experimental error for both methods (9). [c] Arbitrary values for relative purposes; these are not the same absolute value for LDH and GDH. [d] Calculated from I_{50} value. [e] Calculated from I_{20} value. [f] This compound showed a rate saturation effect (4, 12) in two runs comparing 0.12 and 0.24 m*M* and comparing 0.15 and 0.30 m*M* (Fig. 3) with ratios of 1.4 and 1.1, respectively; calcd. ratios from Eq. 4: 1.5 and 1.4. [g] Sodium salt too insoluble to determine, but all N-aryl iodoacetamides previously measured have shown 1.0 ± 0.4. [h] Maximum concentration obtainable at 0° in Tris buffer (pH 7.4) was 0.20 m*M*. [i] Solubility less than 0.2 m*M* at 0° in Tris buffer (pH 7.4) and therefore K_I and irreversible inhibition not determined.

nated if the control or standard did not behave properly.

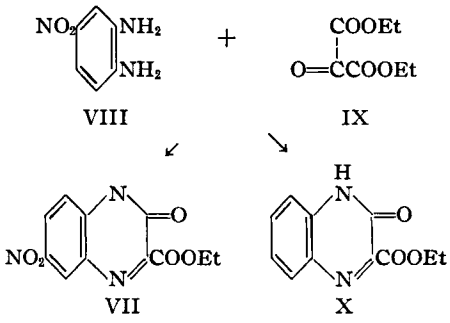
Reactivity of Halogen.—The method for determination of the relative halogen reactivity previously employed (4) was not satisfactory for the compounds listed in Table II. Whereas 4-ISA had a half reaction time with thiosulfate of about 60 minutes at 0°, 5-(bromoacetyl)salicylic acid (XII) had half reacted at this temperature and concentration in about 3 minutes. Satisfactory kinetics were obtained when the reaction and the blank were diluted tenfold; XII reacted 19 times faster than 4-ISA (Table II).

The other compounds in Table II (III*d*–VI*d*) formed insoluble sodium salts at 0° with the previous method (4). The method was modified by allowing the reaction to proceed at 24°, but the increased rate in reaction was compensated by running the kinetics at a fourfold dilution of that previously used (4). The standard compound, 4-ISA was rerun under these warmer, more dilute conditions; comparisons are listed in Table II where 4-ISA is given an arbitrary rate of 1.0.

CHEMISTRY

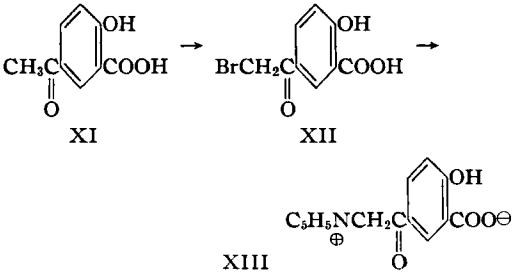
Methods.—All of the potential irreversible inhibitors were synthesized by reaction of III*c*–VI*c* with iodoacetyl chloride (7) or with phenyl chloroformate (9), as previously described or by suitable modification of the conditions as noted in Table I. The requisite 6-aminocoumarin-3-carboxylic acid (III*c*) was obtained by stannous chloride reduction of 6-nitrocoumarin-3-carboxylic acid (III*b*) (13); catalytic reduction gave deeply colored amorphous products that could not be purified. 6-Amino-2-hydroxycinchoninic acid (IV*c*) was best prepared by ferrous ammonium sulfate reduction of the nitro acid (IV*b*) (14); in this case catalytic reduction gave poor and unreproducible yields of IV*c*. Catalytic reduction of 4-hydroxy-6-nitroquinoline-3-carboxylic acid (V*b*) in the presence of two equivalents of hydrochloric acid afforded 6-amino-4-hydroxyquinoline-3-carboxylic acid (V*c*) in 37% yield.

Although condensation of 4-nitro-*o*-phenylenediamine (VIII) with ethyl oxomalonate might be expected to give two isomers, VII and X, only one isomer could be isolated. Since the reaction proceeds by initial condensation of the oxo-group of IX with the stronger *meta*-amino group of VIII, the product could be expected to be VII. Analogous reactions involving *o*-diamines of differing amine strengths



have been described (15, 16). Saponification of VII afforded VI*b* in 77% yield. Considerable difficulty was encountered in reducing VI*b* to the amine VI*c*. Catalytic reduction frequently led to over-reduction products. The best method found for this reduction was ferrous ammonium sulfate; the product was easily isolated in pure form, but the yields were only fair.

A candidate for an exo-alkylating inhibitor with a more reactive alkylating group was 5-(bromoacetyl)-salicylic acid (XII). This compound was prepared by bromination of 5-acetylsalicylic acid (XI) (17) in ether.



That the bromine had entered the methyl group and not the ring was shown by conversion of XII to the pyridinum betaine (XIII) and by the high reactivity of the halogen of XII towards thiosulfate (Table II). Compound XII has the same melting point described for XII prepared by another route (18) that appeared technically unattractive.

Synthesis[3]

6-Nitrocoumarin-3-carboxylic Acid (*IIIb*).—Condensation of 10 Gm. of 5-nitrosalicylaldehyde with 10 Gm. of ethyl malonate in 200 ml. of boiling ethanol containing 1 ml. of piperidine gave a 98% yield of ethyl 6-nitrocoumarin-3-carboxylate, m. p. 194–196° [lit. m.p. 198° (13)]. Considerable difficulty was experienced in finding proper conditions for saponification of the ester. Precipitation by ordinary acidification gave a product still containing potassium and apparently containing an opened lactone ring. The following procedure gave a good product each time.

A mixture of 1.00 Gm. of ester and 10.6 ml. of 5% alcoholic potassium hydroxide was stirred for 90 minutes, during which time the orange potassium salt of the product separated. Sufficient water was added to just dissolve the potassium salt; the solution was added in a thin stream with good mechanical stirring to 60 ml. of 3 *N* hydrochloric acid. The product, which gradually separated, was collected on a filter, washed with water, and recrystallized from ethyl acetate; yield, 0.50 Gm. (56%), m.p. 227–229° [lit. m.p. 234–235° (13)].

6-Aminocoumarin-3-carboxylic Acid (*IIIc*).—A 2.00-Gm. (9.5 mmole) quantity of *IIIb* was added to 6 Gm. of stannous chloride dihydrate in 6 ml. of 12 *N* hydrochloric acid. The mixture was warmed gently on the steam bath until the exothermic reaction had started, then was controlled by cooling in an ice bath. The solution was then heated on the steam bath for 2 hours. Spin-evaporation *in vacuo* gave a thick syrup to which 100 ml. of water was added. The yellow product gradually crystallized; yield, 1.8 Gm. (95%), m.p. 224–226°, that was suitable for further transformations. Recrystallization from dilute alcohol gave crystals, m.p. 234°; $\nu_{max.}^{KBr}$ 3400 (OH, NH); 1725 (lactone and carboxyl C=O); no nitro bands near 1520 and 1340 cm.$^{-1}$.

Anal.—Calcd. for $C_{10}H_7NO_4 \cdot H_2O$: C, 53.8; H, 4.07; N, 6.27. Found: C, 53.6; H, 4.01; N, 6.26.

6-Amino-2-hydroxycinchoninic Acid (*IVc*).—A solution of 2.0 Gm. (8.5 mmoles) of *IVb* (14) in 6 ml. of water and 6 ml. of concentrated ammonium hydroxide was poured with stirring into a boiling solution of 23 Gm. of ferrous ammonium sulfate hexahydrate in 50 ml. of water. Then 20 ml. of concentrated ammonium hydroxide was added in small portions over a period of 20 minutes with stirring, but without additional heating. The mixture, after being heated an additional 30 minutes on the steam bath, was filtered, and the cooled filtrate acidified to about pH 4 with acetic acid. The product was collected on a filter and washed with cold water; yield 1.0 Gm. (57%), m.p. above 300°; this material is suitable for further transformations. Recrystallization from water gave pale yellow needles, m.p. above 300°, whose infrared spectrum indicated a zwitterion structure; $\nu_{max.}^{KBr}$ 3450 (NH); 1670 (amide C=O); 1600 cm.$^{-1}$ (carboxylate). Catalytic reduction was not as satisfactory since the product could not always be isolated and at best gave a 46% yield.

Anal.—Calcd. for $C_{10}H_8N_2O_3$: C, 58.8; H, 3.93; N, 13.7. Found: C, 58.5; H, 4.24; N, 13.5.

6-Amino-4-hydroxyquinoline-3-carboxylic Acid (*Vc*).—A mixture of 1.00 Gm. (4.2 mmoles) of *Vb*, 200 ml. of 70% ethanol, 0.083 ml. of 12 *N* hydrochloric acid, and 100 mg. of platinum oxide was shaken with hydrogen at 2–3 Atm. until reduction was complete (about 24 hours). The filtered solution was spin-evaporated to dryness *in vacuo* leaving 0.20 Gm. of product. An additional 0.10 Gm. was obtained by extracting the catalyst precipitate with 5 ml. of 1 *N* NaOH, then acidification of the filtrate; total yield, 0.30 Gm. (37%), m.p. above 300°, that was suitable for the next step. Recrystallization from ethanol-water gave the analytical sample, m.p. above 300°; $\nu_{max.}^{KBr}$ 3500, 3400 (NH); 1675 (carboxyl C=O), 1620 cm.$^{-1}$ (C=N or amide C=O).

Anal.—Calcd. for $C_{10}H_8N_2O_3$: C, 58.8; H, 3.93; N, 13.7. Found: C, 58.7; H, 3.80; N, 13.6.

Ethyl 3-Hydroxy-7-nitroquinoxaline-2-carboxylate (VII).—A 6.96-Gm. (0.04 mole) quantity of ethyl ketomalonate was added to a mixture of 6.12 Gm. (0.04 mole) of 4-nitro-*o*-phenylenediamine and 125 ml. of absolute ethanol. The mixture was refluxed with gentle stirring for 7 hours. The hot solution was filtered, the filtrate was diluted with 100 ml. of water, treated with 1 Gm. of charcoal at the boiling point, then filtered. On being cooled, the filtrate deposited 5 Gm. (48%) of product, m.p. 225–227°. By recrystallization from absolute ethanol an analytical sample was obtained, m.p. 227°; $\nu_{max.}^{KBr}$ 3500 (NH); 1750 (ester C=O), 1652 (amide C=O); 1525 cm.$^{-1}$ (NO_2).

Anal.—Calcd. for $C_{11}H_9N_3O_5$: C, 50.2; H, 3.42; N, 15.9. Found: C, 50.2; H, 3.54; N, 15.7.

3-Hydroxy-7-nitroquinoxaline-2-carboxylic Acid (*VIb*).—A mixture of 3.0 Gm. (11.4 mmoles) of ethyl ester (VII) and 60 ml. of 3 *N* sodium hydroxide was stirred for 4 hours. Then 500 ml. of water was added and the nearly clear solution was filtered. The filtrate was added to 60 ml. of 3 *N* hydrochloric acid with stirring. The yellow product was collected on a filter and washed with water; yield, 1.8 Gm. (67%), m.p. 265° dec. suitable for the next step. For analysis a sample was recrystallized from 2-methoxyethanol to give yellow crystals, m.p. 267° dec.; $\nu_{max.}^{KBr}$ 3500 (NH); 1750 (carboxyl C=O); 1640 (amide C=O); 1525 cm.$^{-1}$ (NO_2).

Anal.—Calcd. for $C_9H_5N_3O_5$: C, 45.9; H, 2.14; N, 17.9. Found: C, 46.1; H, 2.31; N, 17.9.

3-Hydroxy-7-aminoquinoxaline-2-carboxylic Acid (*VIc*).—This was prepared by ferrous reduction of *VIb* as described for the *preparation of* *IVc* in 40% yield, m.p. above 300°. A sample was dissolved in 10 ml. of 1 *N* sodium hydroxide for analysis. The solution was filtered, then acidified with 3 *N* hydrochloric acid with ice cooling; the product was collected and washed with water. This reprecipitation was repeated once more to give yellow crystals, m.p. above 300°; $\nu_{max.}^{KBr}$ 3400, 3350, 3200 (NH); 1690 (carboxyl C=O); 1630, 1580 cm.$^{-1}$ (C=N or amide C=O).

[3] Melting points were taken in capillary tubes in a Meltemp block and are uncorrected. Infrared spectra were determined in KBr pellets with a Perkin-Elmer 137B recording spectrophotometer. Enzyme kinetics and ultraviolet spectra were determined with a Cary 11 recording spectrophotometer.

Anal.—Calcd. for $C_9H_7N_3O_3$: C, 52.7; H, 3.44; N, 20.5. Found: C, 52.6; H, 3.58; N, 20.2.

6-(Carbophenoxyamino)coumarin-3-carboxylic Acid (IIIe).—A 1.04-Gm. (6.72 mmoles) quantity of phenyl chloroformate in one portion was added to a rapidly stirred solution of 1.00 Gm. (4.4 mmoles) of 6-aminocoumarin 3-carboxylic acid (III*c*) in 50 ml. of water containing 1.12 Gm. of sodium bicarbonate and cooled to 3° in an ice bath. After being stirred for 3 hours in an ice bath, during which time part of the yellow product separated as the sodium salt, the mixture was filtered. The filtrate was acidified to pH 3 with 3 *N* hydrochloric acid; the product was collected on a filter and washed with water.

The insoluble sodium salt was added to 50 ml. of water preheated to 70°. After being stirred for a few minutes, the solution was cooled, acidified, and the product collected. This process should be repeated until no more product is obtained on acidification of an extract. Extended heating of the sodium salt in water causes hydrolysis of the carbophenoxy group.

The dried, combined precipitates were recrystallized from ethyl acetate to give yellow crystals; yield, 0.75 Gm. (52%), m.p. 218–220°; $\nu_{max.}^{KBr}$ 3300 (NH); 1730 (lactone C=O); 1705 (urethane C=O); 1690 (carboxyl C=O); 737 and 685 cm.$^{-1}$ (monosubstituted phenyl). (See Table I for analytical data.)

All compounds prepared by this method are listed in Table I under *Method A*. In some cases, the sodium salt was soluble and all of the product was obtained by acidification of the filtered alkaline solution (*Method B*); in some cases all of the product separated as sodium salt and none was obtained by acidification of the filtered alkaline solution (*Method C*). In one case, the sodium salt of the starting material was insoluble at 0° and it was necessary to run the reaction at 40° (*Method D*).

4-Hydroxy-6-(iodoacetamido)quinoline-3-carboxylic Acid (V*d*).—To a stirred solution of 250 mg. (1.2 mmoles) of V*c* in 5 ml. of 2% sodium hydroxide (2.4 mmoles) cooled in an ice bath was added 0.45 Gm. (2.4 mmoles) of iodoacetyl chloride in one portion. The mixture was stirred for 30 minutes, then the product (free acid) was collected on a filter and washed with water; yield, 270 mg. (61%), m.p. above 300°. Recrystallization from 2-methoxyethanol by addition of water gave the pure product, m.p. above 300°; $\nu_{max.}^{KBr}$ 3400 (NH); 1660 (amide and carboxyl C=O); 1600 cm.$^{-1}$ (C=C). (See Table I for analytical data.)

Compound V*c* could not be acylated by *Method A* in sodium bicarbonate, since the proton was not removed from the zwitterion with aqueous sodium bicarbonate. Compounds prepared by this route are listed in Table I under *Method E*.

5-(Bromoacetyl)salicylic Acid (XII).—A solution of 0.28 ml. of bromine in 50 ml. of ether over a period of 2 hours was added to a magnetically stirred solution of 0.92 Gm. (5.11 mmoles) of 5-acetylsalicylic acid (XI) (17) in 100 ml. of reagent ether protected from moisture. The filtered solution was spin-evaporated to dryness *in vacuo;* yield, 1.3 Gm. (98%), m.p. 132°. Recrystallization from chloroform gave 0.90 Gm. (68%) of white crystals, m.p. 152°; $\nu_{max.}^{KBr}$ 3100 (OH); 1670 cm.$^{-1}$ (C=O).

Anal.—Calcd. for $C_9H_7BrO_4$: C, 41.7; H, 2.70; Br, 30.7. Found: C, 41.9; H, 2.89; Br, 30.6.

The halogen was lost if the compound was recrystallized from protic solvents; the compound should be stored in a desiccator. A melting point of 152° has been reported for XII prepared by a different route (18).

5-(ω-Pyridiniumacetyl)salicylate Betaine (XIII).—A solution of 1.0 Gm. (3.8 mmoles) of XII in 10 ml. of reagent pyridine was heated on a steam bath for 30 minutes. On cooling, 1.0 Gm. of a crystalline precipitate was obtained that was a mixture of XIII and its hydrobromide salt, m.p. 180–200° dec. A hot solution of the salt in 20 ml. of water was neutralized with a solution of 0.24 Gm. of anhydrous sodium acetate dissolved in 0.5 ml. of water. After standing overnight at 3°, the mixture was filtered and the product washed with ice water; yield, 0.42 Gm. (42%), m.p. 262–264°. Recrystallization from water gave white crystals, m.p. 271°.

Anal.—Calcd. for $C_{14}H_{11}NO_4$: C, 65.4; H, 4.30; N, 5.44. Found: C, 65.1; H, 4.46; N, 5.37.

RESULTS[4]

Comparison of Halogen Reactivities.—When the rate data with sodium thiosulfate and the iodo compounds were plotted as second-order reactions, straight lines were obtained. The slope of a given line gave the relative halogen reactivity compared to 4-ISA (4, 12). The relative reactivities are recorded in Table II where 4-ISA is given an arbitrary value of 1.0. The bromoketone, XII, was 19 times as reactive as 4-ISA. The iodoacetamido derivatives (III*d*–VI*d*) had the same order of halogen activity as 4-ISA, as expected from previous comparisons (12, 19).

K_I Values of Inhibitors.—The I_{50} value[1] could be determined from a plot of Vo/V against I where Vo = velocity of the enzyme reaction without inhibitor, and V = velocity in the presence of I concentration of inhibitor. Where $Vo/V = 2$, $I = I_{50}$; the K_I could be calculated (9) from

$$K_I = K_m \times I_{50} \qquad \text{(Eq. 2)}$$

Both α-ketoglutarate and pyruvate had $K_m = 2.5 \times 10^{-4}$ in our system. These K_I values are listed in Table II.

In some cases noted in Table II, the I_{50} concentration could not be reached because of insufficient transmittance of light at 340 mμ; absorption by the compound did not allow observation of the decrease in 340 mμ absorption necessary for following the enzyme rate. In such cases, the near maximum Vo/V obtainable with the slits not quite wide open in the spectrophotometer could be used for calculating the K_I; obviously these results will have about twice the error in the absolute value of K_I at I_{20} or I_{30}. These I_{20} or I_{30} could be converted to K_I values by use of the corresponding I_{20} or I_{30} values of 4-ISA and the $K_I = 17 \times 10^{-4}$ for the latter compound. Since I_{50} measures the amount of EI complex at 50% inhibition and I_{20} measures the amount of EI complex at 20% inhibition, the ratio of Eq. 3 is derivable. Thus from the I_{20} and I_{50} values of 4-ISA and

$$I'_{20}/I_{20} = I'_{50}/I_{50} = K_I'/K_I \qquad \text{(Eq. 3)}$$

[4] The capable technical assistance of Maureen Vince and Dorothy Ackerman is gratefully acknowledged.

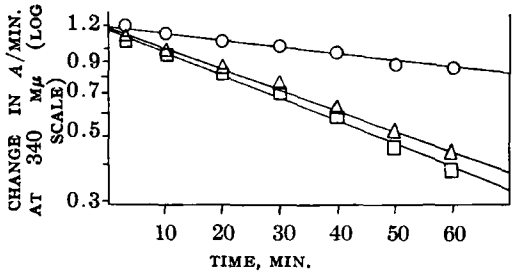


Fig. 1.—Comparative irreversible inhibition of LDH·DPNH by 4-ISA and 5-(bromoacetyl)salicylic acid (XII). Key: ○, LDH·DPNH control; △, 2 m*M* 4-ISA; □, 0.2 m*M* XII.

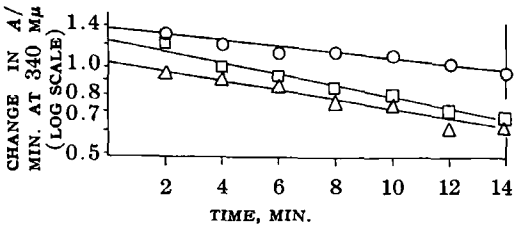


Fig. 2.—Comparative irreversible inhibition of GDH·DPNH by 4-ISA and 6-carbophenoxyamino-2-hydroxycinchoninic acid (IV*e*). Key: ○, GDH·DPNH control; △, 2 m*M* 4-ISA; □, 0.2 m*M* IV*e*

the I_{20} of an unknown compound, the K_I of an unknown compound can be estimated. The K_I values calculated from I_n values less than I_{50} are noted in Table II.

5-(Bromoacetyl)salicylic acid (XII) was reversibly bound to LDH and GDH slightly stronger than 4-ISA. The carbophenoxyamino (III*e*–V*e*) and iodoacetamido (III*d*–VI*d*) heterocyclic carboxylic acids were bound to LDH and GDH 3.5 to 15 times stronger than 4-ISA.

Irreversible Inhibition by Compounds in Table II.—The inactivation rates of LDH and GDH by 4-ISA are assigned arbitrary values of 1.0; it should be noted that the absolute rates of inactivation of the two enzymes by 4-ISA are not identical. The relative rate of inactivation by a given compound is listed in Table II. In each experiment, 4-ISA was compared simultaneously with the new compound, since for reasons still unknown (4) the absolute rate of inactivation of the enzyme can vary twofold from experiment to experiment, but the relative rates usually duplicate with 30%.

The halogen of 5-(bromoacetyl)salicylic acid (XII) was 19 times as reactive as that of 4-ISA, and XII irreversibly inhibited LDH at 120% rate of 4-ISA with concentrations of 0.2 m*M* and 2 m*M*, respectively (Fig. 1); 0.2 m*M* of XII also irreversibly inhibited GDH at 65% of rate of 4-ISA at 2 m*M*.

None of the carbophenoxyamino compounds (III*e*–V*e*) irreversibly inhibited LDH, but two irreversibly inhibited GDH at one-tenth the concentration needed for 4-ISA. For example, 6-carbophenoxyamino-2-hydroxycinchoninic acid (IV*e*) at 0.2 m*M* give about the same rate of inactivation of GDH as 2 m*M* 4-ISA (Fig. 2); IV*e* also showed a rate saturation effect (Fig. 3).

Three of the four iodoacetamido compounds (III*d*, IV*d*, VI*d*) at one-tenth to one-fifth the concentration of 4-ISA showed irreversible inhibition of LDH at 45–83% the rate shown by 4-ISA. An example is 7 - (iodoacetamido) - 3 - hydroxyquinoxaline - 2-carboxylic acid (VI*d*) shown in Fig. 4. The fourth compound (V*d*) was a strong reversible inhibitor of LDH but showed no irreversible inhibition.

DISCUSSION

As shown in Fig. 1 and Table II, 0.2 m*M* of 5-(bromoacetyl)salicylic acid (XII) irreversibly inhibited LDH at 1.2 times the rate of 2 m*M* 4-ISA. There are four factors that can influence the rate of irreversible inhibition by exo-alkylation: (*a*) the extent of *EI* complex formation which can be calculated (4) from the K_I by Eq. 4, (*b*) the ability of the reversibly bound inhibitor to bridge to a nucleophilic group on the enzyme surface adjacent to the active site (12, 19), (*c*) the nucleophilic character of the

$$EI = \frac{E_t}{\frac{K_I}{I} + 1}$$

where E_t = total enzyme concentration (Eq. 4)

enzyme being alkylated (9, 11), and (*d*) the electrophilic character of the alkylating group. If factors (*b*), (*c*), and (*d*) were identical for 4-ISA and XII, then XII should have irreversibly inhibited LDH at about 3.8 times the rate of 4-ISA, which can be calculated from the ratio = 19 × 0.13/0.70, where 19 is the increased thiosulfate reactivity, 0.70 is the fractional amount of enzyme in the *EI* complex with 2 m*M* 4-ISA, and 0.13 is the fractional amount of enzyme in *EI* complex with 0.2 m*M* of XII. Therefore, factors (*b*), (*c*), or (*d*) or any combination of the three are contributing to the ratio of irreversible inhibition. In addition, the enzymic nucleophilic group, if not SH, probably has a different ratio of rate of reaction between XII and 4-ISA than the 19 observed with thiosulfate.

It can be calculated from Eq. 4 that at a concentration of 0.2 m*M* only 13% of total LDH is reversibly complexed by XII. This 13% appears to be close to the point of bimolecular alkylation of other enzymes where no complexing is required, which could be quite disadvantageous in the intact cell. In fact, however, the practically nonexistent energy barrier for complexing contrasts sharply with the energy barrier involved in a bimolecular reaction; this differential has been given an elegant mathematical treatment by Singer, *et al.* (5).

Although the carbophenoxy compounds in Table II were strongly bound to LDH reversibly, these showed no irreversible inhibition; the lack of irreversible inhibition of LDH by other carbophenoxy compounds has been previously noted (9, 11). Since all but one of these earlier carbophenoxy compounds could irreversibly inhibit GDH (11), it was not surprising that the carbophenoxy compounds, III*e*–IV*e*, irreversibly inhibited GDH. Of importance to this discussion is the low concentrations necessary for irreversible inhibition, as expected from the low K_I values; only a concentration one-

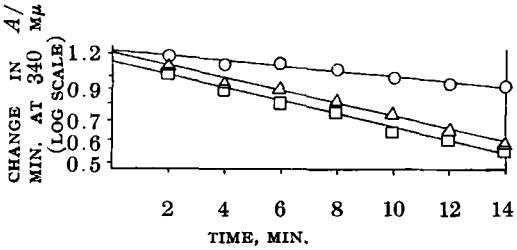


Fig. 3.—Rate saturation of irreversible inhibition of GDH·DPNH by 6-carbophenoxyamino-2-hydroxycinchoninic acid (IV*e*). Key: ○, LDH·DPNH control; △, 0.15 m*M* of IV*e*; □, 0.30 m*M* of IV*e*.

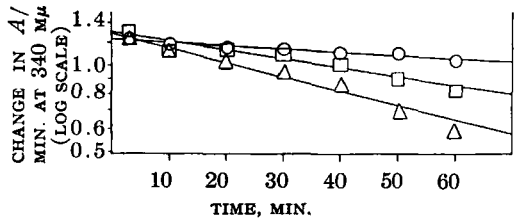


Fig. 4.—Comparative irreversible inhibition of LDH·DPNH by 4-ISA and 7-(iodoacetamido)-3-hydroxyquinoxaline-2-carboxylic acid (VI*d*). Key: ○, LDH·DPNH control; △, 2 m*M* 4-ISA; □, 0.2 m*M* VI*d*.

tenth that of 4-ISA (Table II) or one-twentieth of other carbophenoxyamino derivatives of salicylic acid (11) was required. As yet no carbophenoxy compounds have been found that give irreversible inhibition of LDH by the exo-alkylation mechanism, but the search for such a compound is continuing.

One of the carbophenoxy compounds was investigated further to demonstrate that reversible complexing at the active site was a necessary prerequisite for irreversible inhibition. 6-Carbophenoxyamino-2-hydroxycinchoninic acid (IV*e*) showed a rate saturation effect (Fig. 3); doubling the concentration of inhibitor from 0.15 m*M* to 0.30 m*M* gave an increase in inactivation rate of 1.1, somewhat less than the calculated ratio of 1.4 from Eq. 4 and definitely less than 2.0 if the inactivation occurred by bimolecular reaction (tail-alkylation). In a check run comparing 0.12 m*M* and 0.24 m*M*, the ratio of inactivation rates was 1.4 compared to a calculated value of 1.5.

All of the iodoacetamido compounds (III*e*–VI*d*) showed reversible inhibition of LDH with K_I's one-fifth to one-twelfth of 4-ISA. In addition, three of the four showed at least one-half the rate of irreversible inhibition of 4-ISA at one-fifth to one-tenth the concentration required for 4-ISA. The fourth compound (V*d*) showed no irreversible inhibition. This difference is most probably because of the bridge principle of specificity (12, 19), since in V*d* the distance of the iodomethylene group from the 1,2-oxo-carboxylic acid groups involved in binding is quite different than the distance between the corresponding groups of III*d*, IV*d*, and VI*d*.

The fact that a number of these compounds with lower K_I could inhibit LDH and GDH at correspondingly lower concentrations than used for 4-ISA gives another line of evidence supporting the exo-alkylation phenomenon as an explanation for the mode of selective denaturation of LDH and GDH by these alkylating inhibitors.

It can be concluded that in the design of an exo-alkylating irreversible inhibitor for a given enzyme careful attention should be given to use compounds that give the most tight-reversible binding possible. The more reactive bromoacetyl group of XII gives faster inactivation and allows design of a potential *in vivo* inhibitor that can vary in alkylating ability from the slow-reacting chloroacetamido group to the fast-reacting iodomethyl ketone group; in this way the proper balance between the highest reactivity with the least destruction of inhibitor by random reactions prior to the attack of the target site by the irreversible inhibitor can be maintained.

By placing the bromoacetyl group on III–VI, compounds should be obtained that would give irreversible inhibition of LDH *in vitro* at concentrations approaching 10^{-5} *M*, within the utilizable range for *in vivo* activity. As pointed out earlier (1, 4), our *in vitro* approach must by necessity ignore for the time the important problems of transport and membrane permeability present in an *in vivo* system. However, if necessary the carboxyl group of inhibitors such as II–VI could be masked by ester or amide functions to give a latent activity that could be regenerated by intracellular nonspecific amidases or esterases. It is our opinion that such an approach to LDH inhibitors is worthy of further pursuit.

REFERENCES

(1) Baker, B. R., and Sachdev, H. S., This Journal, **52**, 933(1963).
(2) Baker, B. R., *Cancer Chemotherapy Rep.* (No. 4), **1** (1959).
(3) Baker, B. R., Lee, W. W., Tong, E., and Ross, L. O., *J. Am. Chem. Soc.*, **83**, 3713(1961).
(4) Baker, B. R., Lee, W. W., and Tong, E., *J. Theoret. Biol.*, **3**, 459(1962).
(5) Wofsy, L., Metzger, H., and Singer, S. J., *Biochemistry*, **1**, 1031(1962).
(6) Lawson, W. B., and Schramm, H. J., *J. Am. Chem. Soc.*, **84**, 2017(1962).
(7) Baker, B. R., Lee, W. W., Tong, E., Ross, L. O., and Martinez, A. P., *J. Theoret. Biol.*, **3**, 446(1962).
(8) Baker, B. R., Lee, W. W., Skinner, W. A., Martinez, A. P., and Tong, E., *J. Med. Pharm. Chem.*, **2**, 633(1960).
(9) Baker, B. R., and Patel, R. P., This Journal, **52**, 927(1963).
(10) Gowenlock, A. H., Newbold, G. T., and Spring F. S., *J. Chem. Soc.*, **1945**, 622.
(11) Baker, B. R., and Patel, R. P., *Biochem. Biophys. Res. Commun.*, **9**, 199(1962).
(12) Baker, B. R., *Biochem. Pharmacol.*, **12**, 293(1963).
(13) Ichibagase, H., *J. Pharm. Soc. Japan*, **75**, 1480 (1955).
(14) Borsche, W., and Wenkmann, H., *Ber.*, **57**, 1150 (1924).
(15) Purrmann, R., *Ann.*, **548**, 284(1941).
(16) Leese, C. L., and Rydon, H. N., *J. Chem. Soc.*, **1955**, 303.
(17) Amin, J. H., and Desai, R. D., *J. Sci. Ind. Res. India*, **13B**, 178(1954).
(18) Granger, R., Corbier, M., and Vinas, J., *Compt. Rend.*, **234**, 1058(1952).
(19) Baker, B. R., *J. Med. Pharm. Chem.*, **5**, 654(1962).