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Potential Anticancer Agents*—L. Non-Classical Antimetabolites—II. Some Factors in the Design of Exo-Alkylating Enzyme Inhibitors, particularly of Lactic Dehydrogenase

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Introduction

Among the earliest known biochemical differences between normal and neoplastic tissues were the lowered oxygen uptake and high lactate formation of some neoplastic tissues compared to most normal tissues.[1] The enzymatic shunt of pyruvate to lactate with resultant regeneration of DPN† from DPNH is probably necessary for oxidative anabolism in an oxygen-deficient cell. Since this function of LDH is probably unessential for most, if not all, normal tissues, then a strong selective blockade of this enzyme could result in selective inhibition of a tumour that is dependent on this reaction for regeneration of DPN.

The observation by Ottolenghi and Denstedt[2] that phenoxyacetic acid is an inhibitor of LDH, in either of the directions that the enzymatic reaction is run, suggested that this moiety, and related rather large molecules (compared to pyruvate), be investigated further in cancer chemotherapy. The fact that phenoxyacetic acid and a variety of its common derivatives failed to inhibit

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† The following abbreviations are used: DPN, diphosphopyridine nucleotide; DPNH, reduced diphosphopyridine nucleotide; LDH, lactic dehydrogenase; GDH, glutamic dehydrogenase.

the growth of Sarcoma 180, Adenocarcinoma 755 or Leukemia L-1210* might be explained by the high *in vivo* concentration of pyruvate with which these inhibitors must compete, and the relatively poor inhibitions[2] of LDH-catalyzed pyruvate reduction by phenoxyacetic acid.† An inhibitor that could combine irreversibly with LDH should be much more effective,[4] since one inhibitor molecule could combine with one active site on LDH and selectively denature the enzyme, rather than competing with substrate pyruvate.‡

There are two known types of irreversible inhibitors:[4] those that bind ionically to the enzyme much more strongly than to the

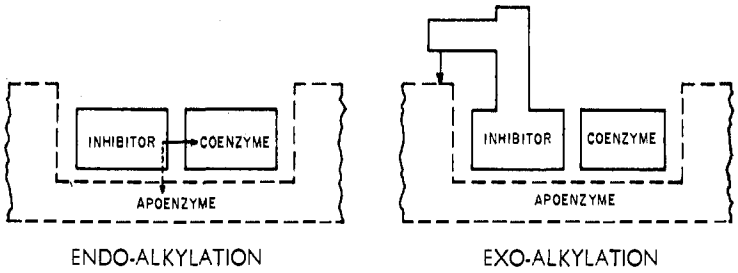


Fig. 1. Oversimplified diagram of endo- and exo-alkylation. The arrows represent the attack of an alkylating group on an active site. The two different routes for endo-alkylation are shown by the solid arrow and by the dotted arrow.

substrate (pseudo-irreversible[6, 7]) and those that combine chemically with the enzyme through covalent bond formation to stop desorption. The latter can be divided into two classes:[4] those that react by endo-alkylation such as azaserine, and those that could theoretically react by exo-alkylation (Fig. 1).

In order to design an effective exo-alkylating irreversible inhibitor of LDH, the following factors should be considered:

(1) Relatively large moieties (compared to pyruvate), other than phenoxyacetic acid, that could serve as a carrier for the alkylating group giving exo-alkylation of the enzyme;

* Personal communication from Dr. Howard W. Bond, Cancer Chemotherapy National Service Center; *cf.* also reference 3.

† These negative results could also be interpreted to mean that the phenoxyacetic acids are not selective enough in their inhibitor action, or even that the pyruvate to lactate shunt is of no value to a tumour cell.

‡ Busch *et al.*[5] have recently observed that dichloropyruvate strongly inhibited the growth of Walker rat Carcinosarcoma 256 and attributed the drug action to irreversible inhibition of LDH.

(2) The size, position, and inductive effect of substituents on the phenoxyacetic acid and other moieties that can be made without losing inhibitory action;

(3) Determination of the effect of the inhibitors in (1) and (2) on an enzyme with similar binding sites, such as GDH, in order to obtain an approximate estimate of the comparative specificity;

(4) The placing of a suitable alkylating group on an inhibitor, such as phenoxyacetic acid, that has sufficient chemical reactivity and the proper dimensions to be able to alkylate a nucleophilic group of the enzyme, such as NH, OH, or SH, near the active site (see Fig. 1).

Factors (1), (2) and (3) are the experimental subjects of this paper and factor (4) the future projection of this work.

Experimental

Enzyme Measurements

Reagents

DL-Lactate, L-glutamate, and DPN were commercial preparations. Crystalline LDH, isolated from rabbit muscle, and crystalline GDH, isolated from mammalian liver, were purchased from Nutritional Biochemicals Corporation. The sources of inhibitors are indicated in the Tables.

Methods of Assay

The enzymatic activities of LDH[2] and GDH were measured by the rate of change of concentration of DPNH. The concentration of DPNH was determined by the change in optical density of the medium at 340 mμ with either a Beckman DU spectrophotometer or a Cary 14 recording spectrophotometer.

In the reverse reaction (lactate → pyruvate), the reaction mixture contained 0·05 molar phosphate buffer (pH 8·4), DL-lactate at a final concentration of 8 mmolar, DPN at a final concentration of 2·4 mmolar, and, when used, an inhibitor. All the ingredients, except the enzyme, were placed in a cuvette with a total volume of 3·0 ml. The reaction was started by adding the proper quantity of LDH in 0·10 ml of the phosphate buffer. Sufficient enzyme was used to give a rate of formation of DPNH of about 0·4 optical density units per minute when the above

concentrations of DL-lactate and DPN were employed. It was observed that D- and L-lactate gave the same rate of reaction; therefore the convenient DL-lactate was employed.

The GDH assays were run similarly[8] in 0·05 molar phosphate buffer (pH 8·4) with a final concentration of 2 mmolar L-glutamate, 0·8 mmolar DPN, and sufficient enzyme to give a rate of formation of DPNH of about 0·25 optical density units per minute.

The forward reaction (pyruvate → lactate) was run similarly in 0·05 molar tris buffer (pH 7·4) with a final concentration of 2 mmolar sodium pyruvate, about 0·6 mmolar DPNH, and sufficient enzyme to give a rate of change of about 0·6 absorbence units per minute.

Chemistry

Commercial materials, if not pure, were recrystallized until their melting points agreed with accepted literature values.

3-Ethoxyphenoxyacetic acid (Method A). To a refluxing solution of *m*-ethoxyphenol (1·38 g, 10 mmoles) in 4 per cent aqueous sodium hydroxide (10 ml, 10 mmoles) was added dropwise over 30 min a solution of chloroacetic acid (0·95 g, 10 mmoles) in 2 per cent aqueous sodium hydroxide (20 ml, 10 mmoles). The solution, after being refluxed for 1–2 h, was acidified to pH 1 and cooled in an ice-bath. The product was collected on a filter and washed with water; yield, 1·01 g (52 per cent), m.p. 89–90°.

Recrystallization from benzene–petroleum ether (b.p. 30–60°) gave colourless crystals, m.p. 89–89·5°.

Anal. Calcd. for $C_{10}H_{12}O_4$: C, 61·2; H, 6·12. Found: C, 61·3; H, 6·33.

In cases where the phenol was not sufficiently soluble and precipitated with the substituted phenoxyacetic acid, the crude product was dissolved in ethyl acetate and the phenoxyacetic acid extracted out with 5 per cent aqueous sodium bicarbonate. Acidification gave the phenoxyacetic acid, usually in pure form. Recrystallization was employed when necessary.

The 3-phenoxypropionic acids were prepared in the same manner using 3-bromopropionic acid.

3-Aminophenoxyacetic acid hydrochloride (Method B). A mixture of *m*-acetamidophenoxyacetic acid (1·0 g, 4·8 mmoles) (Table I) and 12 N hydrochloric acid (15 ml) was refluxed for 2 h,

then chilled in an ice-bath. The product was collected on a filter and washed with ice-cold 6 N hydrochloric acid; yield, 0·70 g (72 per cent), m.p. >300°. The compound gave a single spot (R_f 0·75) when chromatographed on acetylated paper in benzene–methanol–water (2 : 6 : 1).

Anal. Calcd. for $C_8H_9NO_3.HCl$: C, 47·2; H, 4·90; Cl, 17·4. Found: C, 46·9; H, 4·91; Cl, 17·4.

3-(2-Aminophenoxy)propionic acid (Method C). A mixture of 3-(2-nitrophenoxy)propionic acid[9] (1·00 g, 4·72 mmoles), absolute ethanol (50 ml), and 5 per cent palladium–charcoal (75 mg) was shaken with hydrogen for 15 min, when reduction was complete. The filtered solution was evaporated to dryness *in vacuo*, leaving 0·70 g (82 per cent) of pure product, m.p. 106–107°.

Anal. Calcd. for $C_9H_{11}NO_3$: C, 59·6; H, 6·07. Found: C, 59·3; H, 5·97.

Results

A plot of V_0/V against I for two or more concentrations of the inhibitor was used to determine the concentration of I necessary to give 50 per cent inhibition ($V_0/V = 2$) in the presence of 8 mmolar lactate, where V_0 = velocity of reaction with no inhibitor, V = velocity with inhibitor, and I = concentration of inhibitor.[10] Only those concentrations of inhibitor giving 30–70 per cent inhibition were employed for the plots in order to avoid the high errors in higher or lower inhibitions. With most of the inhibitors, a straight line was obtained and the point of 50 per cent inhibition was readily estimated using a few points only. Some obviously non-competitive* inhibitors gave a bowed curve. In these cases more points were obtained, with one near 50 per cent inhibition, so that the 50 per cent inhibition point could be determined with reasonable accuracy; the obvious non-competitive inhibitors are marked in the Tables. The 50 per cent inhibition concentrations were then divided by the concentration of substrate to give the I_{50} values, which are defined as the millimolar concentration of inhibitor required to give 50 per cent inhibition in the presence of 1 millimolar concentration of substrate. The I_{50} values for GDH

* Throughout this paper, non-competitive inhibition is defined as any type of inhibition that is not competitive, rather than the strict kinetic definition of non-competitive,[10] unless otherwise indicated.

and the LDH forward reactions were determined similarly; the I_{50} values are recorded in the Tables. This definition of I_{50} allows a more direct comparison of the effect of the same inhibitor on two different enzymes such as GDH and LDH, although kinetically speaking one cannot reduce the 50 per cent inhibition values at a given concentration of substrate to the I_{50} value at 1 millimolar substrate concentration unless the inhibitor is competitive. Nevertheless, if one compares the experimentally observed 50 per cent inhibition values for oxidation by GDH and LDH, one comes to the same general conclusions set forth in the Discussion.

Discussion

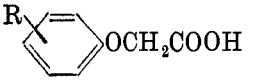
Rationale

The purpose of the present investigation was to find relatively large moieties that could inhibit LDH and would be suitable for later conversion to exo-alkylating inhibitors.[4] With this objective in mind, we felt that the examination of a wide variety of compounds for inhibition was more important than the achievement of maximal quantitative accuracy for any particular inhibitor.*

Attempts to compare the I_{50} values of competitive *vs.* non-competitive inhibitors may be grossly inaccurate and unsatisfying from the enzymologist's strictly kinetic standpoint. Nevertheless, the chemotherapist should not ignore attempts to make these comparisons, since an invading intact cell may be killed as selectively by blockade of an enzyme essential to this cell by a non-competitive antagonist with poor kinetics as by a competitive antagonist with good kinetics. The major objection to non-competitive antagonists—the commonly believed statement[23] that 'it may be anticipated that competitive antagonists will exhibit greater specificity than non-competitive'—is subject to severe criticism in the light of some of the results given in Tables III and IV. For example, Hellerman *et al.*[8] have done careful kinetic studies showing that isophthalate and glutarate are equally effective ($I_{50} = 1$) competitive antagonists of GDH.

* This philosophy of approach for a broad survey of an enzymatic phenomenon has been previously expressed by Levy and Vennesland.[22]

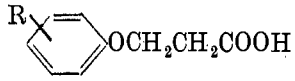
Table I. Inhibition of LDH (reverse reaction) and GDH by substituted phenoxyacetic acids



R	LDH[a] I_{50}	GDH[a] I_{50}	GDH I_{50} / LDH I_{50}	m.p., °C	Literature m.p., °C	Method or source[b]
H	3·0	41	13			D
2-CH_3	6·3	28	4·4	155–156	151–152[11]	A
3-CH_3	6·6	28	4·3	104–105	102[11]	A
4-CH_3	14	28	2·0	140–141	135[11]	A
3,5-$(CH_3)_2$	4·1	109	27	110–111	110–111[12]	A
2-Cl	2·4	11	4·6			E
3-Cl	3·2	13	4·1	109–109·5	108–110[13]	A
4-Cl	3·4	11	3·2			F
2,4-Cl_2	1·6	3·5	2·2			E
2,4,5-Cl_3	0·67	2·5	3·7			D
2,4,5-Cl_3-α-CH_3	1·0	2·0	2·0			D
2,4,6-Cl_3	1·5	10	6·7			G
4-HO	22	45	2·3			E
3-CH_3O	4·5	34	7·6	117·5–118	118[14]	A
4-CH_3O	6·7	35	5·1			E
3-C_2H_5O	6·1	17	2·8	89–90[c]		A[c]
4-C_2H_5O	4·2	26	6·1			E
3-n-C_4H_9O	2·4	3·5	1·5	98–98·5[d]		A[d]
4-NH_2	12	39	3·2	200–210(d.)	200–210(d.)[15]	C[e]
3-NH_2[f]	4·0	89	22	>300		B[e]
2,4-Cl_2-5-NH_2	1·4	6·5	4·7			H
3-AcNH	8·3	80	9·6	170–171	171–172[16]	A
2-AcNH	5·5	50	9·1	149–149·5	153–154[17]	A
3,4-Benzo	0·93	2·0	2·1			J

[a] mmolar concentration of inhibitor required to give 50 per cent inhibition in the presence of 1 mmolar concentration of substrate. [b] For Methods A, B, and C, *cf.* Experimental; D, Matheson, Coleman and Bell; E, Eastman Kodak Co.; F, California Foundation for Biochemical Research; G, K. and K. Laboratories; H, Dr. Howard W. Bond, Cancer Chemotherapy National Service Center; J, Berkeley Chemical Corporation. [c] See Experimental. [d] *Anal.* Calcd. for $C_{12}H_{16}O_4$: C, 64·3; H, 7·14. Found: C, 64·4; H, 7·18. [e] Product precipitated during hydrogenation. The mixture was filtered through a Celite pad. The latter was continuously extracted in a Soxhlet apparatus with ethanol to recover the product. [f] As the hydrochloride; the free base had been prepared previously.[17]

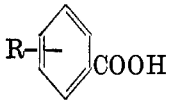
Table II. Inhibition of LDH (reverse reaction) and GDH by substituted 3-phenoxypropionic acids



R	LDH[a] I_{50}	GDH[a] I_{50}	GDH I_{50} / LDH I_{50}	m.p., °C	Method or source[b]
H	3·9	15	3·8	94–95[c]	A
3-AcNH	4·0	37	9·2	132–134[d]	A
2-NH_2	6·3	72	11	106–107[e]	C

[a] mmolar concentration of inhibitor required to give 50 per cent inhibition in the presence of 1 mmolar concentration of substrate. [b] For Methods A and C, *cf.* Experimental. [c] Lit. m.p. 96–97°.[18] [d] *Anal.* Calcd. for $C_{11}H_{13}NO_5$: C, 54·7; H, 6·23. Found: C, 54·4; H, 6·48. [e] See Experimental.

Table III. Inhibition of LDH (reverse reaction) and GDH by substituted benzoic acids



R	LDH[a] I_{50}	GDH[a] I_{50}	GDH I_{50} / LDH I_{50}	Source[b]
H	3·4	50[f]	15	A
2-HO	0·75	20	27	B
3-HO	0·87	14[f]	16	A
2-COOH	34	126[f]	3·7	B
3-COOH	6·1	1·0[f]	0·16	C
2,4-$(COOH)_2$	35	30	0·86	D[g]
3,5-$(COOH)_2$	34	3·0[f,h]	0·088	A
3-COOH-5-CH_3	7·4	40	5·4	D[c]
3-COOH-5-AcNH	2·3	30	13	D[d]
3-COOH-4-AcNH	1·9	30	16	D[e]
3-COOH-5-OH	1·9[h]	2·5	2·3	A
3,4-Benzo	1·8	4·0	2·2	A
2,3-Benzo	2·4[i]	10	4·2	A
3,4-Benzo-2-HO	0·062	0·10[h]	1·6	A
3,4-Benzo-6-HO	0·19	0·70[h]	3·7	A

[a] mmolar concentration of inhibitor required to give 50 per cent inhibition in the presence of 1 mmolar concentration of substrate. [b] A, Eastman Kodak Co.; B, J. T. Baker Co.; C, Matheson, Coleman and Bell; D, synthetic. [c] Uvetic acid, m.p. 270–280°; synthesized in two steps from pyruvic acid.[19] [d] Prepared from 5-aminoisophthalic acid by acetylation.[20] [e] Prepared from 4-aminoisophthalic acid by acetylation.[21] [f] Previously studied by Hellerman *et al.*[8] but data determined in this laboratory. [g] m.p. 220–224°(d.); prepared by hydrolysis of anhydride obtained from Amoco Chemical Corporation. [h] Strongly non-competitive. [i] Moderately non-competitive.

Table IV. Inhibition of LDH (reverse reaction) and GDH by miscellaneous compounds

Compound	LDH[a] I_{50}	GDH[a] I_{50}	GDH I_{50} / LDH I_{50}	Source[b]
Oxalate	0·21	100	480	A
Glutarate	82	0·60[c]	0·0073	A
Methoxyacetate	>100	192	<1·9	A
Ethoxyacetate	23	238	10	A
Phenylmercaptoacetate	4·7	25	5·3	E
DL-Mandelate	8·3	80	9·6	A
Phenylpyruvate	2·7	36	13	B
3-Benzoylpropionate	4·6	48	10	C
Oxanilate	2·2	19	8·6	A
Cinnamate	3·1	33[c]	11	A
Hydrocinnamate	>100	45	<0·45	A
Furoate	6·4	18[c]	2·8	A
5-Bromofuroate	3·3	0·050[c]	0·015	D

[a] mmolar concentration of inhibitor required to give 50 per cent inhibition in the presence of 1 mmolar concentration of substrate. [b] A, Eastman Kodak Co.; B, Nutritional Biochemical Corporation; C, K. and K. Laboratories; D, Dr. Howard W. Bond, Cancer Chemotherapy National Service Center; E, Evans Chemetics, Inc. [c] Previously studied by Hellerman *et al.*[8]

Table V. Inhibition of LDH forward reaction (pyruvate → lactate)

Compound	LDH[a] I_{50}	GDH[a, b] I_{50}	GDH I_{50} / LDH I_{50}
Oxalate	0·50	100	200
Oxamate[c]	0·30	9·0	30
Oxanilate[d]	14	19	1·4
Phenoxyacetate	70	41	0·58
	35[f]	41	1·2
Benzoate	240	50	0·21
m-Hydroxybenzoate	48	14	0·29
	22[f]	14	0·63
Salicylate	19	20	1·1
	17[f]	20	1·2
5-Bromosalicylate[e]	5·5[e]	1·8	0·33
5-Chlorosalicylate[e]	5·5[e]	2·5	0·45
Phenylpyruvate	21	36	1·7
Levulinate[d]	>500[g]		
Cinnamate	33	33	1·0
Isophthalate	71	1·0	0·014
5-Bromofuroate	104	0·050	0·00048

If sources are not indicated, see previous Tables. [a] mmolar concentration of inhibitor required to give 50 per cent inhibition in the presence of 1 mmolar concentration of substrate. [b] Glutamate → α-oxoglutarate. [c] Matheson, Coleman and Bell Co. [d] Eastman Kodak Co. [e] Non-competitive. [f] pH 8·4, 0·05 M tris buffer. [g] This result does not agree with that recorded by Busch and Nair.[26]

However, glutarate (Table IV) has little effect on the LDH reverse reaction except at high concentrations ($I_{50} = 82$), whereas isophthalate (Table III) is a reasonably good inhibitor ($I_{50} = 6 \cdot 1$) of the LDH reverse reaction. Thus two equally effective competitive inhibitors for GDH vary by a factor of 22 on specificity of inhibition for LDH; therefore, the fact that an inhibitor is competitive for a given enzyme affords no assurance that the inhibitor is selective.

On the other hand, trimesic acid (5-carboxyisophthalate, Table III) is a good non-competitive inhibitor of GDH ($I_{50} = 3 \cdot 0$) but is still more effective on GDH than on LDH ($I_{50} = 34$); thus, a non-competitive inhibitor may be more selective than a competitive inhibitor. Admittedly, any one of the compounds in the tables may be a more effective inhibitor for some other enzyme in an intact cell; nevertheless, conclusions on specificity drawn from comparison of two enzyme systems have infinitely more validity than any invalid conclusions on specificity drawn from measurements on only one enzyme.

Ideally, one should measure the effect of an inhibitor on all the enzymes present in a cell to understand the specificity. Since this is obviously impractical, chemotherapy has developed mainly on the basis of the effect of an inhibitor on an intact organism, the selectivity of the effect being measured as the chemotherapeutic index. In the final analysis, the drug is useful only if it has an acceptable chemotherapeutic index in the intact organism. Many thousands of references can be quoted, including some from this laboratory,[24, 25] where structure–activity relations have been attacked by a purely empirical approach, albeit successfully. However, the empirical approach to chemotherapy and the design approach attempted in this manuscript are not mutually exclusive; in fact, there is considerable crossover between the two approaches—intentional or not. Thus the possible validity or non-validity of certain concepts in chemotherapy can be supported at the enzyme level by comparison of the effects of inhibitors on two enzyme systems such as GDH and LDH, as discussed in the following sections.

Chemotherapeutic Implications

The efficacy of a drug is best measured by its chemotherapeutic index, that is, the ratio of the maximum tolerated dose to the

minimum effective dose; these are usually expressed as 50 per cent values for obvious experimental reasons. Then the ED_{50} is the dosage regimen that will be effective in 50 per cent of the host organisms, LD_{50} is the dose given with the same regimen that causes 50 per cent lethality, and the chemotherapeutic index, C.I., is LD_{50}/ED_{50}. Thus, the ED_{50} is a measure of effect of the drug on the desired enzyme (or receptor site), whereas the LD_{50} is a measure of the effect on the most critical, but undesired, enzyme or receptor site of the host. If one reduces this to its simplest terms and ignores the less lethal side-effects of the drug for the moment, then one can make an analogy with any two enzyme systems.

For the sake of discussion, let us assume that the LDH reverse reaction is the target enzyme that must be inhibited for the desired physiological effect on a tumour; then the I_{50} of an inhibitor of this reaction can be likened to the ED_{50}. Similarly, if GDH is assumed to be a critical enzyme for the host, inhibition of which will lead to lethality, then the I_{50} of the inhibitor on GDH can be likened to the LD_{50}.

In structure–activity studies, one varies the active structure empirically, then measures the chemotherapeutic index in the given host system.[24, 25] The most effective change—that is, the change giving the best chemotherapeutic index—makes the compound more effective against the target receptor site than the lethal receptor site.

Among the substituted phenoxyacetic acids in Table I, phenoxyacetic acid has $I_{50} = 3 \cdot 0$ against LDH and $I_{50} = 41$ against GDH. Thus the ratio of the I_{50}'s, namely 13, would be the chemotherapeutic index for this system. In chemotherapy, the compound active at the lowest dose in a series is not necessarily the best compound. For example, (2,4,5-trichlorophenoxy)acetic acid is the most active compound in Table I with an I_{50} of $0 \cdot 67$, but its selectivity (chemotherapeutic index) is decreased to $3 \cdot 7$. In contrast, (3-aminophenoxy)acetic acid is less active ($I_{50} = 4 \cdot 0$) than the parent phenoxyacetic acid, but has greater selectivity with an inhibition ratio of 22.

In Table III, benzoic acid ($I_{50} = 3 \cdot 4$) is almost as good an inhibitor of the LDH reverse reaction as phenoxyacetic acid. This activity is greatly enhanced by substitution of a hydroxyl

group on position 2 ($I_{50} = 0 \cdot 75$) or 3 ($I_{50} = 0 \cdot 87$) of the ring. Of these two compounds, salicylic acid has the better selective effect with a ratio of 27. The corresponding naphthalene analogue, 1-hydroxy-2-naphthoic acid, is twelve times more effective ($I_{50} = 0 \cdot 062$) than salicylic acid in inhibiting the LDH reverse reaction; in fact, it is the most active compound that we have observed in this system. However, it is far from the most selective compound, since its inhibition ratio compared to GDH is only $1 \cdot 6$.

The fact that almost all the compounds in Tables I–V do inhibit both enzymes at some level might be interpreted to mean that these compounds are general enzyme poisons and would affect many other enzymes. Actually, as will be discussed later, there is a fair degree of similarity between the binding sites on LDH and GDH and this was one of the reasons GDH was selected for comparison purposes. To show that even a highly effective but less selective inhibitor, such as (2,4,5-trichlorophenoxy)acetic acid, is not a general enzyme poison, a dehydrogenase enzyme system requiring a phosphate instead of a carboxylate for enzyme binding of the substrate was investigated. Thus, (2,4,5-trichlorophenoxy)acetic acid was totally ineffective as an inhibitor of glucose 6-phosphate dehydrogenase.*

It can be anticipated—or at least hoped—that basic knowledge in enzymology and chemotherapy will eventually reach the point where the chemotherapist will be able to compare the isolated target enzyme with the isolated enzyme in which the undesirable side-effect can occur. That such an approach could be useful in the future can be seen from the data on substituted isophthalates (Table III). If the target enzyme were lactic dehydrogenase, it is clear that isophthalate would not be detected as a possible inhibitor when tested in the intact organism, since its fair ($I_{50} = 6 \cdot 1$) inhibition of LDH would be masked by its stronger ($I_{50} = 1 \cdot 0$) inhibition of GDH. In contrast, with the isolated enzyme systems, it is possible to use isophthalate as a screening lead for structure–activity studies. Note that the bulk of a 5-methyl group on isophthalate makes little change ($I_{50} = 7 \cdot 4$) on the inhibition of LDH, but causes a 40-fold decrease ($I_{50} = 40$)

* We wish to thank Drs. W. Kilgore and J. Greenberg of these laboratories for this determination.

in inhibition of GDH, which is very sensitive to third-dimensional bulk.[8] Thus an unfavourable inhibition ratio of 0·16 that could not be detected in the intact organism has been changed to an inhibition ratio of 5·4, which could be a sufficiently favourable chemotherapeutic index in the intact organism to allow detection of its activity. Furthermore, the 4-acetamido group on isophthalate gives a compound more active ($I_{50} = 1 \cdot 9$) than phenoxyacetate ($I_{50} = 3 \cdot 0$) and with the good inhibition index of 16.

The fate of pyruvate in such a comparative study would be of obvious use in the design of LDH inhibitors for cancer chemotherapy since: (1) pyruvate reduction by LDH is an enzymic reaction probably essential for some types of cancer cells, but of no use for most normal cells, and (2) oxidation of pyruvate to acetyl coenzyme A with pyruvic dehydrogenase is a reaction essential for energy generation in all cells. Such a study should be made, since little is known about inhibition of pyruvic dehydrogenase; any inhibitor which would be as effective on pyruvic dehydrogenase as on LDH would be of no utility, as discussed below.

Busch and Nair[26] have stated the case for inhibitors of LDH as potential anticancer agents. They re-emphasized that inhibitors of the LDH oxidation of lactate do not necessarily inhibit the LDH reduction of pyruvate. In addition, they succinctly stated that 'Inasmuch as inhibition of glycolysis in tumours would require inhibition of the forward reaction, all the experiments in the present studies were carried out with DPNH and pyruvate as substrates. It was found that fluoropyruvate exhibited a more marked suppression of the reduction of pyruvate to lactate than the other available inhibitors.' Unfortunately, fluoropyruvate was 'too perfect' as a classical type antimetabolite. It not only inhibited the reduction of pyruvate* but also inhibited the oxidation of pyruvate to acetate by pyruvic dehydrogenase,[26] a reaction essential for normal cells; as a result, the compound was highly toxic and showed no inhibition of the mouse tumours Sarcoma 180, Adenocarcinoma 755, or Leukemia L-1210,† or

* Of more than passing interest is the fact that fluoropyruvate was observed to be an irreversible inhibitor of LDH,[26] most probably of the 'endo-alkylating' type.

† Private communication from Dr. Howard W. Bond, Cancer Chemotherapy National Service Center.

of the Walker rat Carcinosarcoma 256[5] at the maximum tolerated dose.

Classical antimetabolite theory, as expounded by Woolley[27] and Roblin,[28] greatly limits the change in the structure of a metabolite that should be made to convert it to an antimetabolite; that is, the antimetabolite should be as close in structure to the corresponding metabolite as possible. In a small molecule such as pyruvate, about the only small change that classical antimetabolite theory allows to be made in the structure is substitution of a fluorine for a hydrogen, since the remainder of the molecule is necessary for binding to the enzyme.[29] As already pointed out, Busch and Nair[26] have made this change, but the resultant fluoropyruvate suffered from lack of specificity. Thus, classical antimetabolite theory rules out any further chance of designing a selective inhibitor of LDH reduction of pyruvate.

In contrast, non-classical antimetabolite theory[4] states that 'an antimetabolite should be as close as possible in structure to that part of the molecule where the stereospecific requirements of the enzyme surface must be met.'

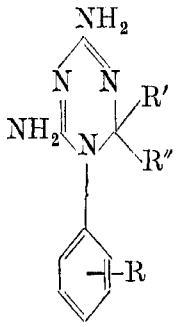
Furthermore, the conclusions reached earlier in this paper with non-classical inhibitors such as 4-acetamidoisophthalate and phenoxyacetate make it possible to propose a second corollary of non-classical antimetabolite theory. This corollary, a direct antithesis of classical theory, can be stated as follows:

In order for an antimetabolite to have maximum enzyme specificity, the greatest possible changes in the bulk of the antimetabolite should be made that still allow the stereospecific and binding requirements of the target enzyme to be met.

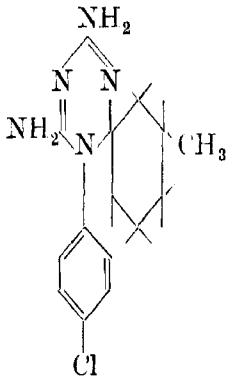
Available evidence for this corollary has been known for twenty years; in fact, this evidence appeared soon after the original Woods–Fildes antimetabolite theory[30,31] was proposed. The discovery of the first useful antibacterial agent, sulphanilamide, led directly to the antimetabolite theory when a few years later *p*-aminobenzoic acid was predicted and found[30] to be the competitive substrate. As a result of classical structure–activity relationship studies,[32] sulphanilamide was soon replaced by other sulphonamides, such as sulphathiazole, sulphadiazine, etc. in clinical practice since they exhibited fewer side reactions. Note that the heterocyclic rings of these drugs represent a bulky

change in structure compared to sulphanilamide, the classical antimetabolite of *p*-aminobenzoic acid. Thus, they represent an early example of non-classical antimetabolites that have been modified with greater bulk to give more specificity to the inhibitor.

One of the most recent examples of proper bulk for enzyme specificity has been observed in the folic reductase area.[33] The 4,6-diamino-1-aryl-1,2-dihydro-*sym*-triazines (I) have been known to be folic reductase inhibitors for some years,[34] are useful for the treatment of malaria,[34,35] and show some anticancer activity,[34] particularly when R′ and R″ are small groups such as methyl



(I)



(II)

and R is a *meta* or *para* group such as halogen. The anticancer and antimalarial activity, as well as toxicity, were lost when R was an *ortho* group or R′ and R″ became bulky. In contrast, pinworms such as *Syphacia obvelata* in mice were very susceptible to the diaminotriazines (I), if R was a bulky group in the *o*-position or R′ and R″ were bulky groups. The compound of choice,[33] 1-(*p*-chlorophenyl)-4,6-diamino-1,2-dihydro-2,2-(3′-methylpentamethylene)-*sym*-triazine (II) had high anthelmintic activity in mice and exceedingly low toxicity. There is little doubt that the phenyl group of (II) is restricted in its rotation and cannot assume a planar conformation with the triazine ring. Even though Roth *et al.*[33] state that compounds such as (II) no longer have any significant antifolic activity—presumably when assayed by the usual microbiological techniques such as *Lactobacillus casei*, *L. arabinosus*, or *Streptococcus faecalis*—it is logical to assume that

the folic reductase enzyme of *Syphacia obvelata* can still be blocked by (II) in its nonplanar conformation, whereas the folic reductase of mammals, *L. arabinosus*, *L. casei*, or *S. faecalis*, can only accept a relatively planar biaryl conformation. The reality of this proposition could best be verified by inhibitor studies on folic reductase prepared from cell extracts of *Syphacia obvelata*. That the same enzyme can differ in inhibitor specificity from species to species,[36] from 'wild type' cells to resistant cells,[37] or even from tissue to tissue of the same animal,[38] has been observed previously.

Thus, in order to design an effective inhibitor, it would be most useful if data were available on the requirements and limitations of the active site on the enzyme of the species and tissue in question.* Ideally, this study should employ LDH obtained from the tumour tissue for which inhibition is desired, preferably a human tumour. Since this ideal cannot be met at this time for obvious technical reasons, this pilot study was initiated with commercial LDH isolated from rabbit muscle so that a suitable experimental approach could be found for determining the nature of the enzyme site.

Nature of the Active Site on LDH

The need for and use of knowledge of the stereospecificity, binding, and allowance for bulk at the active site on LDH were discussed in the previous section. As Busch and Nair[26] point out, it is necessary to block the reduction of pyruvate catalyzed by LDH and not the oxidation of lactate in order for an LDH inhibitor to be an effective anticancer agent. Nevertheless, considerable useful information about the enzyme site can be obtained by study of the inhibition of lactate oxidation that cannot be as readily obtained by inhibition of the forward reaction. The fact that a variety of inhibitors that compete with the reverse reaction are not inhibitors of the forward reaction[26] can be, and has been,[39]

* The proposition that the same enzyme from different tissues of the mouse differs in its ability to be inhibited by the 6-aminonicotinamide analogue of DPN[38] and that the triazine (II) presumably also differs in its ability to inhibit the same enzyme from different organisms, suggests that additional studies should be made on key anabolic enzymes to see if there are exploitable differences in enzyme specificity between a variety of normal tissues and different types of tumour tissues.

explained by invoking different sites for pyruvate and lactate on LDH. This concept has been refuted by the elegant work of Schwert *et al.*[40] on LDH. They state:

'In our model this hypothesis is not necessary. The location of the binding site for lactate and for pyruvate is presumably the same as regards the geometry of the enzyme surface and in part in the bound coenzyme. Since DPN and DPNH differ in both steric and chemical properties, the configuration of the binding site for substrate or inhibitors on the enzyme–DPN complex is quite different from that on the enzyme–DPNH complex.'

Further studies by Winer and Schwert[29] on the quenching of

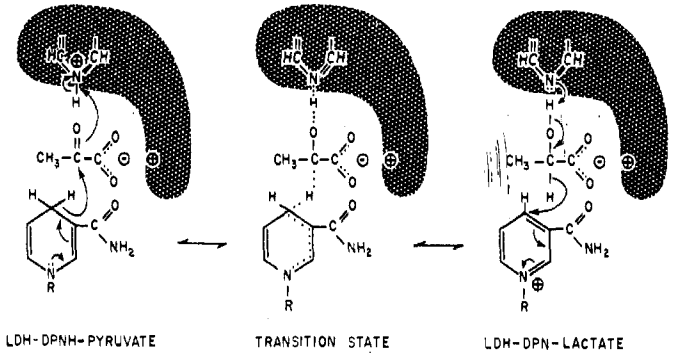


Fig. 2. Modified Winer–Schwert two-dimensional model for LDH. The shaded area represents protein surface. Note that the activated complex at the transition state is identical.

the fluorescence spectra of the LDH–DPNH complex by the classical-type antimetabolite, oxamate, caused them to propose a two-dimensional working model of the LDH–DPNH–pyruvate and the LDH–DPN–lactate complexes, a slight modification of which is presented in Fig. 2. Our present studies with non-classical inhibitors of LDH agree with the type of transition state proposed by Winer and Schwert. A three-dimensional model would be more desirable, since it more closely approximates the true nature of the enzyme site; our experiments were designed for this purpose.

The Dennis–Kaplan[41] three-dimensional model for oxidation of lactate, when applied to non-classical inhibitors (Fig. 3), fails to show why phenoxyacetate with an α-CH_2 group is a good inhibitor of the oxidation of lactate (Table I) but a poor inhibitor of the reduction of pyruvate (Table V). In Fig. 3, where the DPN is

in the imidazole plane, the equatorial hydrogen of DPNH[22] is in about the same position as the 4-hydrogen of DPN. Thus, the DPN and DPNH, when placed in this position, do not modify the geometry of the enzyme appreciably. Molecular models* show that phenoxyacetate can fit either the LDH–DPNH complex or the LDH–DPN complex equally well. Experimentally, this is not the case with phenoxyacetate. The model in Fig. 3 also does not account for the logical assumption of Levy and Vennesland[22] that the attack of pyruvate by the hydride of DPNH axial to the plane of the DPNH would involve the smallest shift of atoms from the positions which they occupy in the planar DPN form.

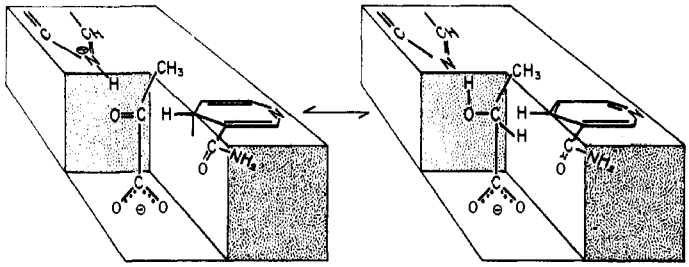


Fig. 3. Modified Dennis–Kaplan three-dimensional model for LDH.

The Levy–Vennesland hypothesis is satisfied if the DPN and DPNH are in a plane perpendicular to the plane of the imidazole ring on the protein surface and parallel to the line between the carboxyl carbon and the α-carbon of the substrate (Fig.4). In this configuration, the equatorial hydrogen of the bound DPNH (axial with respect to DPNH) in its LDH complex constitutes a change in the enzyme geometry compared to the DPN–LDH complex. Construction of molecular models clearly showed that the phenoxyacetate would readily fit the enzyme site for LDH–DPN; in contrast, phenoxyacetate could bind only poorly to the LDH–DPNH site *because of the interaction of an α-hydrogen of phenoxyacetate with the equatorial hydrogen of the DPNH.* This equatorial hydrogen plus the slight extension of the 4-carbon of the pyridine ring in DPNH from the vertical plane in the model (Fig. 4), due to the boat form[22] of the pyridine ring, most prob-

* Catalin Molecular Models manufactured by Catalin Products, Ltd., Waltham Abbey, Essex, England.

ably account for the difference in the specificity between LDH–DPN and LDH–DPNH.

Non-classical antimetabolites did fit the LDH–DPNH complex provided the carbon next to the carboxyl binding had a double-bonded oxygen rather than a $—CH_2—$ group. Thus, oxanilate was five times as good an inhibitor ($I_{50} = 14$) (Table V) and phenylpyruvate ($I_{50} = 21$) more than three times as good an inhibitor as phenoxyacetate ($I_{50} = 70$). However, in the model in Fig. 4, there was considerable restricted rotation of the benzene ring in both oxanilate and phenylpyruvate, which can account for part of the poorer I_{50} demonstrated by these compounds on reduc-

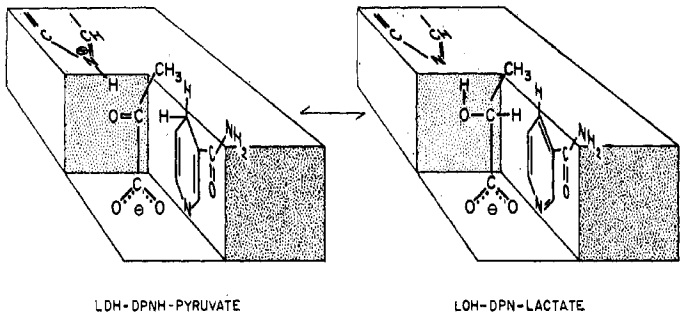


Fig. 4. Three-dimensional model of LDH satisfying the current inhibition studies.

tion of pyruvate compared to oxidation of lactate; an important factor that makes the LDH–DPNH complex more difficult to inhibit than the LDH–DPN complex is that pyruvate binds about 10^4 times more strongly to the LDH–DPNH complex than lactate binds to the LDH–DPN complex.[42] It should be possible to overcome this difficulty with an exo-alkylating irreversible inhibitor.[4] The fact that oxanilate and phenylpyruvate are still reasonably good inhibitors shows that outside the range of interference by the equatorial hydrogen of DPNH at the α-carbon of the inhibitor, gross changes in structure can be made.

A less obvious molecule, salicylate (Table V) is also a better non-classical inhibitor ($I_{50} = 19$) of the LDH–DPNH complex than phenoxyacetate. The salicylate inhibition of LDH–DPNH can be increased by halogen substitution on the 5-position of salicylate ($I_{50} = 5 \cdot 5$); although specificity is decreased, it could most likely be increased by proper positioning of bulk, as proposed earlier.

Salicylate ($I_{50} = 0 \cdot 75$) and its isomer, 3-hydroxybenzoate ($I_{50} = 0 \cdot 87$), gave useful information on the height of the plane of the imidazole ring, since both compounds are excellent and almost equally effective inhibitors of the LDH–DPN complex.* The height of the imidazole plane must be just above the hydroxyl of salicylate when its carboxyl is bound to the enzyme, but just below the hydroxyl of 3-hydroxybenzoate when its carboxyl is bound to the enzyme. This height is still satisfactory for the LDH–DPNH–pyruvate and LDH–DPN–lactate complexes, as shown in Fig. 4.

The substituted phenoxyacetic acids, when measured as inhibitors of the oxidation of lactate by DPN (Table I), gave considerable further information on the nature of the active site of LDH. Phenoxyacetic acid should have its primary binding to the LDH–DPN complex (Fig. 4) through the carboxylate group as usual. Secondary binding of a hydrogen-bond type can then occur between the ether oxygen of phenoxyacetate[2] and the NH of the imidazole; note that it must be assumed that the imidazole ring is protonated for this binding to the LDH–DPN complex. The ether oxygen fits just below the imidazole NH in a fashion similar to the LDH–DPNH–pyruvate complex (Fig. 4). Molecular models then show that there is still room for the phenyl group, but the adjacent 2- and 6-hydrogens cause some restricted rotation of the phenyl ring; this restricted rotation is increased only slightly by 2,6-dichloro-substituents, but the fit is still good and the inductive effect of the halogens (discussed later) more than overcomes the increased restriction of rotation.

* Among the assumptions made in the classical derivation of the mathematical expressions for enzyme inhibition[10] are: (1) only competitive inhibitors can fit the active site of an enzyme, (2) non-competitive inhibitors combine with some site other than the active enzyme site (ESI complex), and (3) the rate-limiting step in an enzymic reaction is the decomposition of the enzyme–substrate complex to renewed enzyme and products. Some workers, including ourselves, disagree that these assumptions are invariably valid.[44-49] In fact, it is possible to make other assumptions, not invoking an ESI complex, to explain the kinetics of non-competitive inhibition;[49] thus, non-competitive inhibitors can, and probably do, involve the active site. In order to avoid obscuring the more pertinent issues, we determined that salicylate and 3-hydroxybenzoate gave strictly competitive inhibition of LDH–DPN–lactate when plotted by the Lineweaver–Burk method;[10] phenoxyacetate has previously been shown to be competitive.[2] Thus, in the discussion that follows, the active site of LDH must account for the fit of these inhibitors.

The good inhibition of LDH by (3,5-dimethylphenoxy)acetate ($I_{50} = 4 \cdot 1$) gives an important clue to the position of the carboxamide group of the DPN bound to LDH. If the relatively planar carboxamide group of DPN is in front as pictured in Fig. 4 (or at the rear) and held by the enzyme on a plane perpendicular to the DPN plane, then molecular models show that there is considerable interaction between a methyl group of (3,5-dimethylphenoxy)-acetate and the carboxamide group of the DPN. This can only be demonstrated by the presence of two *m*-methyl groups, since with only one *m*-methyl group, the phenyl moiety can be rotated 180° to remove the methyl group from the immediate vicinity of the carboxamide group, thus allowing much more freedom of rotation of the phenyl moiety. Since (3,5-dimethylphenoxy)-acetic acid is nearly as good an inhibitor of LDH as phenoxy-acetic acid, it is clear that the plane of the carboxamide group of the DPN cannot be perpendicular to the plane of the DPN, but that the carboxamide group must approach coplanarity with the DPN. However, no evidence appears to be available to indicate whether the carboxamide group is at the front (as indicated in Fig. 4) of the DPN or at the rear of the DPN, provided the carboxamide group is coplanar.

That there is open space to the left of the α-methylene group of lactate in the LDH–DPN–lactate binding, as shown in Fig. 4, is clearly indicated by the fact that substitution of the bulky methyl group on the α-position of (2,3,5-trichlorophenoxy)acetate ($I_{50} = 0 \cdot 67$) causes little change in binding ($I_{50} = 1 \cdot 0$).

The inductive effects of substituents on the binding of phenoxy-acetate (Table I) to the LDH–DPN complex deserves notice. In a general way, halogens in the *o*- or *p*-position tend to increase binding (lower I_{50}), in contrast to amino or hydroxyl groups in these positions, which decrease binding. However, a rigid correlation with the *sigma* constant or ionization constant of either the phenoxy acid or its corresponding phenol could not be demonstrated.

Active Site of Glutamic Dehydrogenase

The inhibition of GDH by 5-methyl-, 5-acetamido-, 5-carboxy-, and 5-H-isophthalate (Table III), with I_{50} values of 40, 30, $3 \cdot 0$, and $1 \cdot 0$, respectively, lends further credence to the conclusion

of Hellerman *et al.*[8] that the inhibitors of GDH must be planar in their conformation; that is, the active site can tolerate little third-dimensional bulk since the decrease in I_{50} values in this series of substituted isophthalates corresponds to the decrease in third-dimensional bulk as shown by molecular models. Further inhibitor studies would be necessary to establish whether or not third-dimensional bulk could be accepted by the enzyme if this

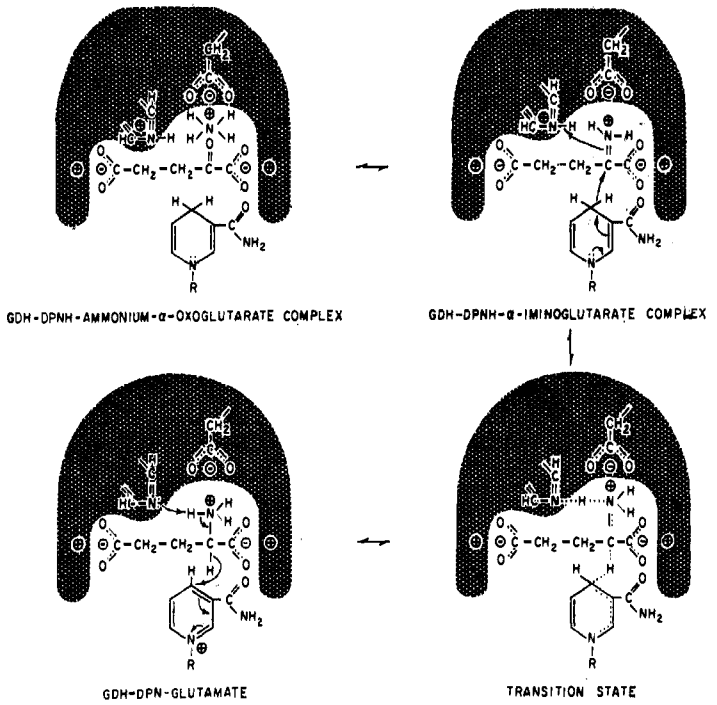


Fig. 5. Two dimensional model for GDH. The shaded area represents protein surface.

bulk were further removed from the binding groups of the inhibitor.

Sufficient data are available to propose a two-dimensional model of the GDH enzyme site (Fig. 5) that is similar to the two-dimensional model of LDH (Fig. 2). In addition to the imidazole ring on the protein surface for proton transfer, it would also be convenient to invoke the presence of a carboxylate group on the enzyme site with the proper conformation to bind the various forms of ammonia involved, that is, ammonium ion, the imino-glutarate zwitterion, and the glutamate zwitterion. Comparison of the two-dimensional model of LDH (Fig. 2) with that of GDH

(Fig. 5) shows some obvious similarities. Thus, one could expect some inhibitors to inhibit both enzymes. As discussed earlier, a particular inhibitor can be made to be more effective for one enzyme than the other, or *vice versa*, by judicious placing of other substituents.

Attempts to draw a three-dimensional model of GDH are probably not warranted with the data available, since the inhibitors studied were primarily chosen for the information they could give on the LDH active site (Fig. 4).

Conclusion

Further experimental work is warranted on compounds related to oxanilate, phenylpyruvate, and salicylate with proper substituents to give irreversible inhibition of LDH, since the current study has shown that space is available on the LDH site for inhibitors to have the large groups necessary for construction of the theoretically possible exo-alkylating type irreversible inhibitor.[4] Detailed information on inhibition of pyruvate dehydrogenase would be of considerable practical importance, although Bosund[43] has observed that salicylate does not inhibit pyruvic dehydrogenase in resting cells of *Proteus vulgaris*.

Summary. A second corollary of non-classical antimetabolite theory has been proposed, based on comparative inhibition of lactic dehydrogenase and glutamic dehydrogenase by several series of inhibitors. In addition, considerable insight has been gained on the three-dimensional aspects of the active site of lactic dehydrogenase, which could be of considerable use for the design of potential anticancer agents of the irreversible type.

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