Studies with Specific Enzyme Inhibitors[1]

X. Mechanism of Action of L-(+)-β-Monofluorolactate on Crystalline Heart Muscle Lactate Dehydrogenase[2]

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SUMMARY

The L-(+) stereoisomer of β-monofluorolactic acid is a specific inhibitor of lactic acid dehydrogenase. The inhibition is competitive with respect to L-(+)-lactate, uncompetitive with DPN and pyruvate, and noncompetitive with DPNH. Kinetic analyses of substrate pairs in the presence of fluorolactate revealed that K_i with respect to DPN is a linear function of the concentration of lactate, while K_i with respect to lactate is directly proportional to the reciprocal of the concentration of DPN. In the reverse direction K_i with respect to pyruvate is independent of the concentration of DPNH, and K_i with respect to DPNH is a linear function of the reciprocal of the concentration of pyruvate. Fluorolactate in the forward reaction can combine only with the enzyme-DPN complex, while in the reverse direction the prerequisite for inhibition is the formation of an enzyme–pyruvate–DPNH complex.

INTRODUCTION

Oxalate has been recognized as an inhibitor of LDH[4] by Quastel and Wooldridge (1), later by Neilands (2), and used extensively for the kinetic analysis of the catalytic mechanism of LDH by Novoa *et al.* (3), Anderson *et al.* (4), Nisselbaum *et al.* (5), and more recently by Zewe and Fromm (6). It is predictable from known properties of oxalate (e.g., complex formation with Ca^{++}) that it cannot be employed as a selective inhibitor of LDH in a complex enzyme system. Oxamate, which was found by Hakala *et al.* (7), Novoa *et al.* (3) to inhibit LDH, was recently used as a presumably specific inhibitor of this enzyme in tumor cells by Goldberg and Colowick (8) and Goldberg *et al.* (9). It was, however, shown by Kun and Achmatowicz (10) that oxamate also competitively inhibits the reductive amination of pyruvate catalyzed by crystalline glutamate dehydrogenase. It follows that interpretations of metabolic effects of oxamate on tumor cells (8, 9) which are based on the assumption that this substance acts only on LDH are oversimplified. Recent experiments indicate (11) that the enzymic interconversion of alanine, aspartate, and glutamate is a part

[1] The preceding paper in this series is listed as reference (10).

[2] A preliminary report containing part of this paper was presented at the annual meeting of the American Society of Biological Chemists, April 11, 1965; E. Kun, R. J. Dummel and B. Achmatowicz, *Fed. Proc.* **24**, (2), 2971, p. 667 (1965).

[3] Recipient of the Research Career Award of the USPHS.

[4] The abbreviation LDH will be used throughout this text for lactate dehydrogenase (E.C.1.1.1.27).

of an "energy-controlled"[5] system, connecting metabolic pathways coupled to intracellular transport of keto and amino acids (unpublished experiments).

The bisulfite adduct of acetaldehyde is also an inhibitor of LDH, as shown by Zelitch (12), but it is not certain whether or not the adduct itself or the dissociated bisulfite ion is the actual inhibitory species. Bisulfite is known to inhibit LDH (13). Busch and Nair (14) reported that β-monofluoropyruvate inhibited LDH. However, this substance is an alkylating agent, thus unstable in biological systems. Moreover, as found independently by Lee *et al.* (15) and in our laboratory, fluoropyruvate is a good substrate of LDH, similar to other α-oxo-acids (16). It seemed predictable that the product of reduction of fluoropyruvate, monofluorolactate, might be a more selective inhibitor of LDH. Racemic β-monofluorolactate was synthesized (17), and resolved into both enantiomorphs; the absolute configuration of these isomers was established (18). Only the dextrorotatory isomer corresponding to L configuration was found to be an enzyme inhibitor. It is of interest that trifluorolactate (cf. 4) had no inhibitory action on LDH. The present work is concerned with the kinetic interaction of L-(+)-β-monofluorolactate with crystalline LDH; fluorolactate as an enzyme reagent in complex systems will be dealt with in a future publication.

MATERIALS AND METHODS

Reagents. Solutions of sodium (+) lactate were prepared by ion exchange (Dowex 50) from analytical grade calcium salt. Crystalline potassium pyruvate was prepared from freshly distilled pyruvic acid. Pyridine nucleotides were obtained from Sigma or California Biochemicals. Calculations of concentrations of pyridine nucleotides were based on their known molar extinction coefficients, measured at 260 and 340 mμ. There was no indication of the presence of inhibitory impurities in freshly prepared coenzyme solutions.

Kinetic measurements were carried out either with the Gilford multichannel recorder attached to a Beckman monochromator, in cuvettes with a 1-cm light path, or with a Zeiss spectrophotometer; in the latter case absorbance changes in a cuvette with a 5-cm light path were recorded on a Honeywell chart recorder. Rates of the forward and reverse reaction catalyzed by lactic dehydrogenase were determined by recording the formation or disappearance of the DPNH band at 340 mμ at 26°. Both forward and reverse reactions were determined at pH 7.53, checked with a microelectrode attached to a Beckman research pH-meter in the cuvette containing the final reaction system.

The forward reaction (i.e., lactate→ pyruvate) was carried out in 0.5 M glycine containing 0.2 M hydrazine in order to trap pyruvate, thus off-setting the unfavorable equilibrium of the reaction at close to neutral pH. Since kinetic constants obtained were not different from those determined under different conditions, we had no reason to suspect that hydrazine itself had an effect on the catalytic activity of the enzyme. Reduction of pyruvate was measured in the same buffer containing no hydrazine. In all kinetic measurements, initial velocities were read directly from recorder tracings and extrapolated to 0 time. Reactions were started by addition of 10-μl of appropriately diluted LDH.

Enzyme preparation. In all kinetic experiments, a single crystalline LDH isozyme (isozyme V) was employed, prepared from hog heart[6] by the procedure of Reeves and Fimognari (19). A solution of the crystalline enzyme (5 mg protein per milliliter) was dialyzed against 0.1 M phosphate buffer (pH 7.4). The test system used for the oxidation of lactate contained 0.15 μg enzyme protein per 1 ml, and for the

[5] The term "energy-controlled" is intended to define control mechanisms which are responsible for the inhibitory or activating effects of ATP (or ADP) or its energy-rich precursors (including those containing no phosphate), generated during oxidative phosphorylation, on either electron or ion transfer reactions of mitochondria.

[6] We are grateful to Mr. M. Magar for this contribution.

reduction of pyruvate 0.01 μg protein per 1 ml.

RESULTS

The catalytic mechanism of LDH, in terms of conventional steady-state kinetics, is fairly well documented (3, 4, 6); it consists most probably of an ordered sequence of additions with formation of isomerizing ternary complexes. The effects of fluorolactate were therefore examined with respect to all four reactants (lactate, DPN, pyruvate, DPNH), following the steady-state model of Alberty (20, 21) and the graphical analysis of Florini and Vestling (22), using Lineweaver-Burk plots (23).

Effects of L-(+)-β-*Fluorolactate on the Oxidation of* L-(+)-*Lactate*

When DPN and lactate concentrations are varied in the presence or absence of a fixed concentration (3×10^{-3} M) of fluorolactate and the results are expressed in a reciprocal plot, a family of straight lines is obtained which converge at a point in either of the left quadrants of the coordinate system (Figs. 1 and 2). The projections of the crossing points on the abscissa correspond in the absence of fluorolactate to the value[7]

$$-\frac{K_L}{K_{OL}}$$

(Fig. 1), while in presence of the inhibitor this is displaced by the value

$$1 + \frac{[I]}{K_i}$$

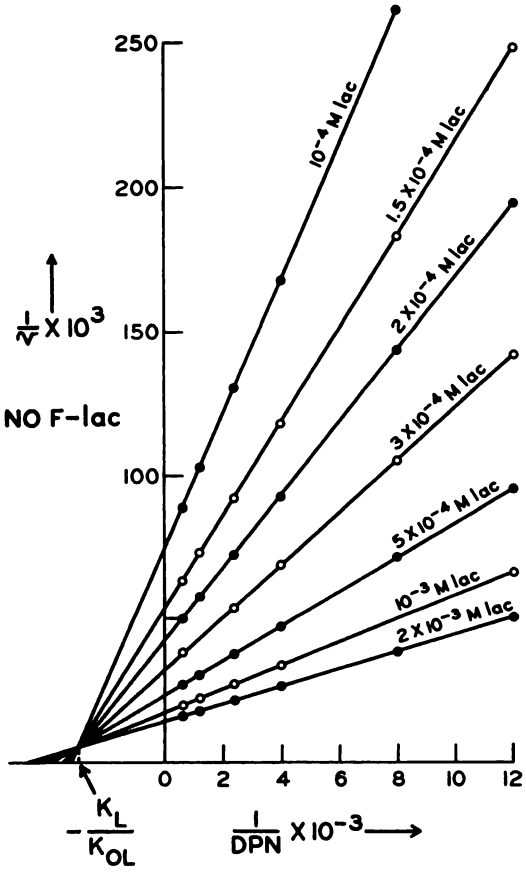

FIG. 1.

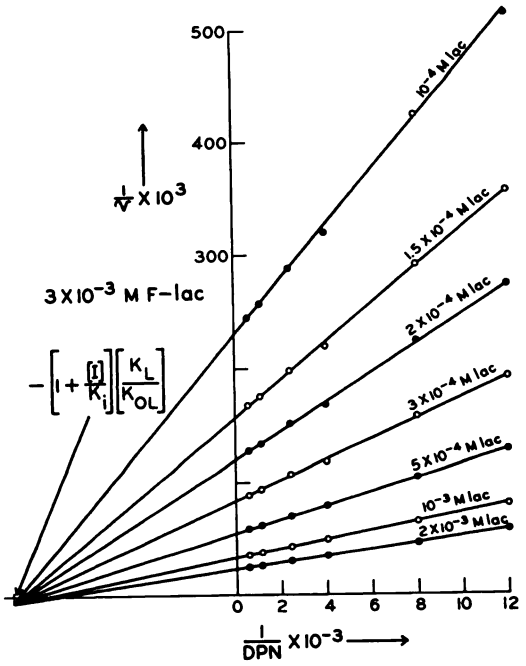

FIG. 2.

FIGS. 1 and 2. *The effect of fluorolactate on initial velocity (v) of the enzymic oxidation of lactate as a function of DPN concentration at varying levels of lactate*

Abbreviations: *lac* = lactate; *F-lac* = fluorolactate.

[7] The following symbols are used: L, lactate; O, oxidized diphosphopyridine nucleotide (DPN); P, pyruvate; R, reduced pyridine nucleotide (DPNH); I, inhibitor (i.e., fluorolactate).

(Fig. 2). It should be noted that the scales of ordinates in Fig. 1 and Fig. 2 differ; thus comparisons cannot be made by simple inspection.

Figure 3 illustrates the kinetic behavior of fluorolactate with respect to DPN at a

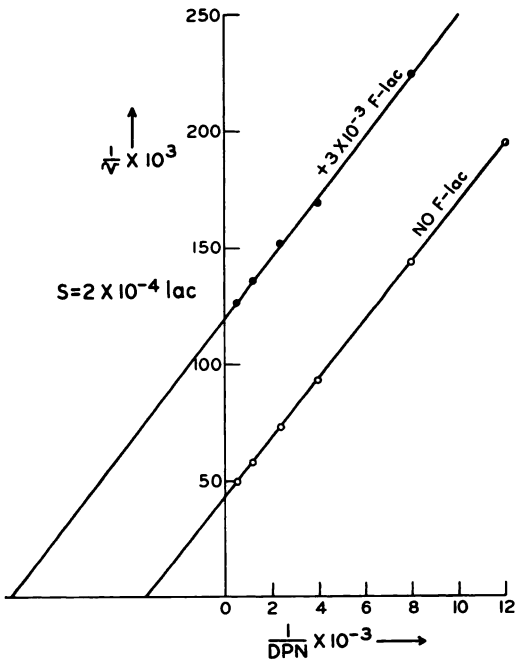

FIG. 3. *Uncompetitive relationship between DPN and fluorolactate, determined at one level of lactate*

S = substrate; substrate and inhibitor concentrations are expressed as moles/liter (as in previous and all subsequent figures).

single concentration of lactate. Typical uncompetitive behavior is observed, i.e., both K_m and velocity are altered by the same factor

$$1 + \frac{[I]}{K_i}$$

However, further comparisons made at various lactate concentrations reveal that the uncompetitive K_i for fluorolactate with respect to DPN (determined at various finite concentrations of lactate) is a linear function of the concentration of the second reactant (i.e., lactate) (Fig. 4).

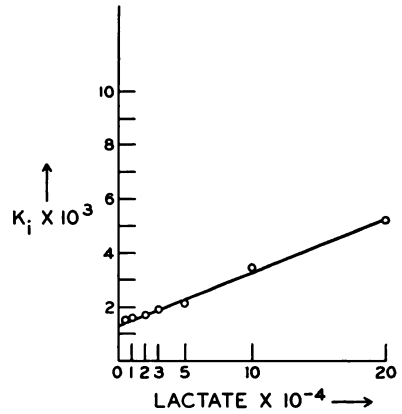

FIG. 4. *K_i with respect to DPN as a function of lactate concentration*

That this relationship is to be expected can be most easily shown by a graphical illustration. Taking the concentration of DPN as infinite, a double reciprocal plot ($1/V'_m$ vs. 1/lac, where V'_m = apparent maximal velocity) in presence and absence of fluorolactate results in a typical competitive type of graph (see below and Fig. 8). If a line is drawn parallel with the abscissa intersecting the ordinate at $1/V_{max}$, any chosen vertical linear distance between this line and the slope obtained in the presence of a competitive inhibitor will be defined as x, and any corresponding distance between the parallel line and the slope obtained in the absence of inhibitor will be defined as y; thus

$$\frac{1}{V'_m} = \left(\frac{1}{V_{max}}\right) + y$$

and

$$\frac{1}{V'_{m,(i)}} = \left(\frac{1}{V_{max}}\right) + x$$

(where $V'_{m,(i)}$ = apparent maximum velocity in presence of inhibitor). Returning to an uncompetitive kinetic relationship illustrated in Fig. 3, by definition

$$1 + \frac{[I]}{K_i} = \frac{1}{V'_{m,(i)}} \Big/ \frac{1}{V'_m}$$

which can be written (after substitution) as

$$\frac{K_i}{[I]}\left(\frac{x}{y} - 1\right) = \frac{V_{max}}{V_{max} - V'_m}$$

It follows from the Michaelis-Menten equation, where V'_m is analogous to v, that

$$\frac{K_i}{[I]}\left(\frac{x}{y} - 1\right) = \frac{s}{K_m} + 1$$

Since K_m, x/y, and [I] are constants, it follows that K_i (uncompetitive toward first substrate, see Fig. 3) must be a linear function of the concentration of the second substrate (see Fig. 8).

Fluorolactate is a competitive inhibitor with respect to lactate. Primary plots, demonstrating velocity changes as a function of lactate concentration at varying levels of DPN in absence and presence of 3×10^{-3} M L-(+)-β-fluorolactate, are shown in Figs. 5 and 6. In a Dixon type of plot (24), straight lines are obtained at varying concentrations of inhibitor, indicating that the kinetic nature of the competitive type of interactions between lactate and fluorolactate is independent of the concentration of the inhibitor. The purely competitive K_i of fluorolactate with respect to lactate shows a linear relationship to the reciprocal of the concentration of the second substrate, i.e., DPN (Fig. 7). It is also seen that at zero concentration of DPN, K_i is ∞, i.e., no reaction between fluorolactate and free enzyme occurs.

From Figs. 1 and 2, a secondary plot (22) was obtained by relating velocities at

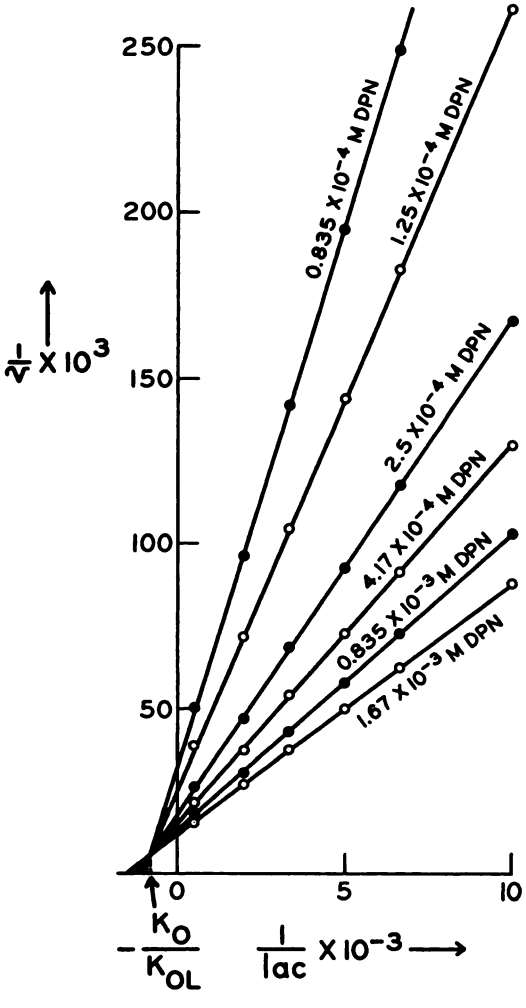

Fig. 5.

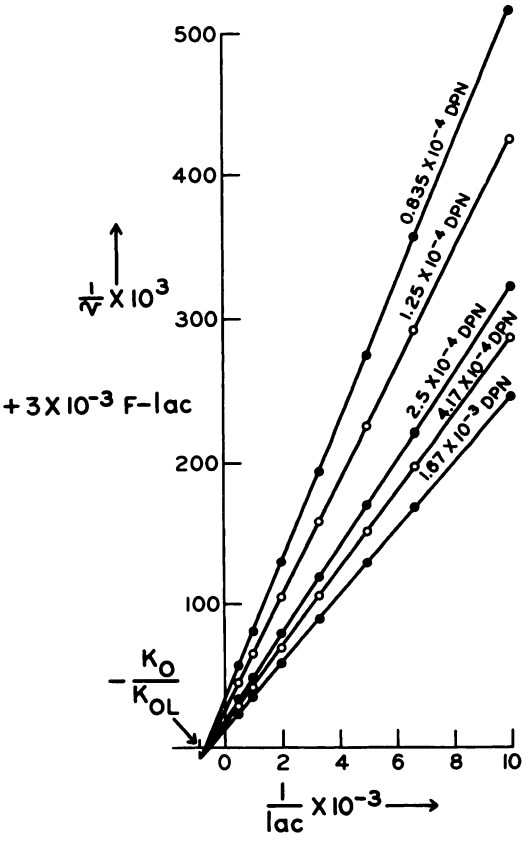

Fig. 6.

Figs. 5 and 6. *The effect of fluorolactate on initial velocity of the enzymic oxidation of lactate as a function of lactate concentration at varying levels of DPN*

From Figs. 1 and 2, a secondary plot (22) was obtained by relating velocities at

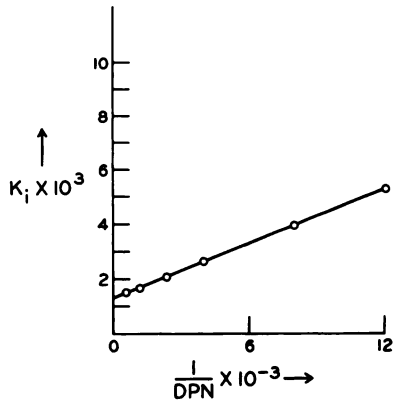

FIG. 7. *K_i with respect to lactate as a function of [DPN]*

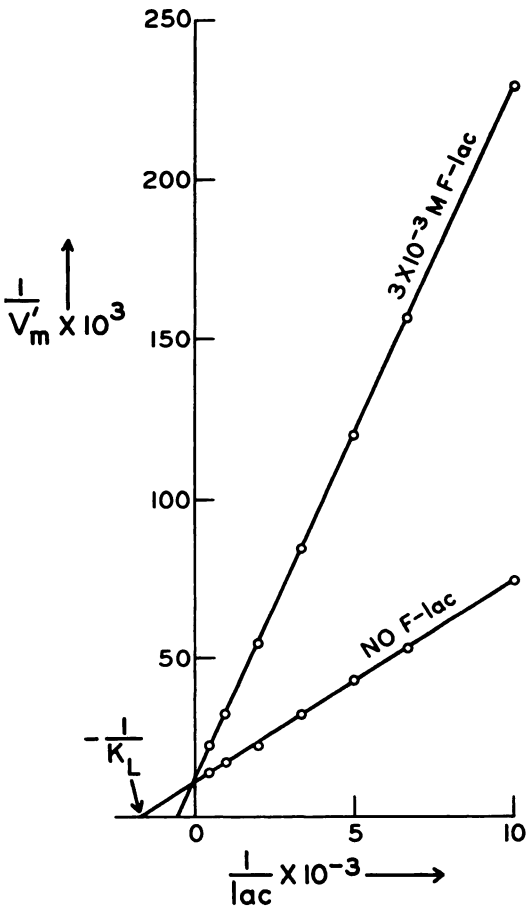

FIG. 8. *Competitive relationship between lactate and fluorolactate*

Correlation between apparent maximal velocity and lactate concentration in absence and presence of fluorolactate

infinite DPN concentrations against 1/lac. The true K_i

$$\frac{[EO][I]}{[EOI]}$$

determined from this plot (Fig. 8) is 1.3×10^{-3} and is in agreement with the K_i at infinite concentration of DPN, read off from Fig. 7.

Another secondary plot (Fig. 9) was constructed (from Figs. 5 and 6) by relating

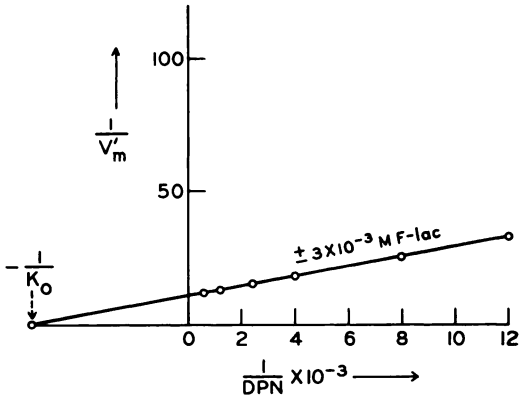

FIG. 9. *Correlation between apparent maximal velocity and concentration of DPN at infinite concentration of lactate in presence and absence of fluorolactate*

apparent maximal velocities with DPN concentrations. These values were obtained at infinite concentrations of lactate; therefore the presence of fluorolactate has no effect on this relationship.

Effect of (+)-β-Fluorolactate on the Reduction of Pyruvate

Primary plots (Figs. 10 and 11) show the relationship between initial velocities as a function of DPNH concentration, at various levels of pyruvate, in absence and presence of fluorolactate (3×10^{-3} M). A secondary plot (22) obtained from Figs. 10 and 11 correlates apparent maximal velocities with 1/DPNH in absence and presence of fluorolactate. Since only V_{max} changes, this relationship defines noncompetitive interaction between DPNH and fluorolactate (Fig. 12).

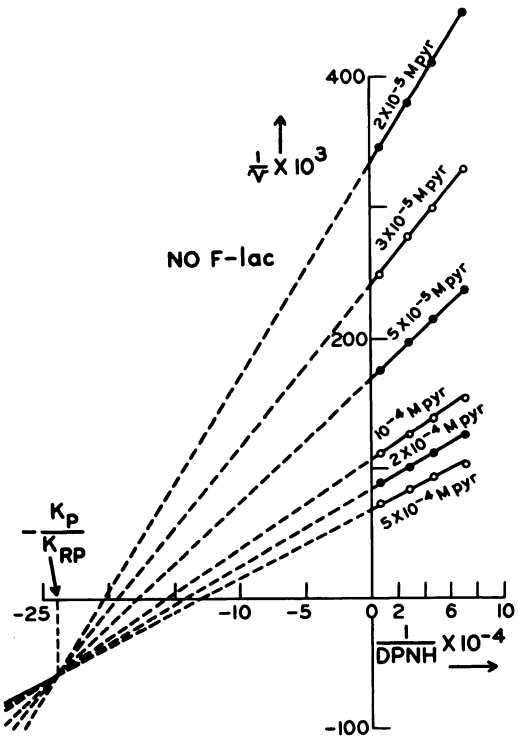

FIG. 10.

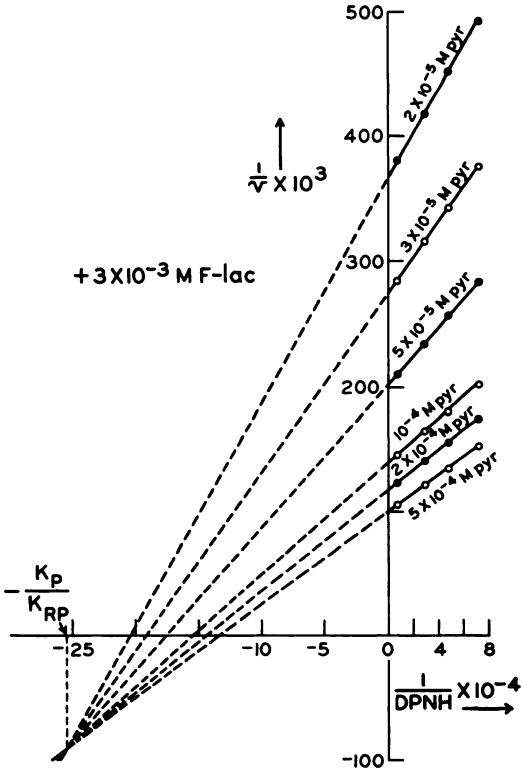

FIG. 11.

FIGS. 10 and 11. *The effect of fluorolactate on the initial velocity of enzymic reduction of pyruvate as a function of DPNH concentration at varying levels of pyruvate*

A fourth series of primary plots (Figs. 13 and 14) summarizes the relationship between initial velocity and pyruvate concentration, at various levels of DPNH, in the absence (Fig. 13) and presence (Fig. 14) of fluorolactate. Interpretation of these results is the same as in Figs. 1 and 2, since $-(K_R/K_{RP})$ is displaced in the presence of fluorolactate by

$$\left(1 + \frac{[I]}{K_i}\right).$$

Uncompetitive inhibition is also illustrated by the secondary plot, constructed from Figs. 13 and 14 (Fig. 15), in which both V_{max} and K_m are displaced to the same extent by fluorolactate.

K_i with respect to DPNH is a linear function of the reciprocal of pyruvate concentration (Fig. 16b). This is similar to the relationship between K_i (with respect to lactate) and DPN concentration (Fig. 7), as would be expected since fluorolactate is an uncompetitive inhibitor of both pyruvate and DPN. By similar reasoning to that used in connection with Fig. 4, it can be readily demonstrated that

$$\frac{1}{V'_m} = \frac{K_i(x)}{[I]}$$

(in this case, x is the vertical distance between slopes $\pm$ fluorolactate). Since $1/V'_m$ is a linear function of 1/pyr and x and [I] are constants, K_i has to be a linear function of 1/pyr. As shown in Fig. 16b, at zero concentration of pyruvate, noncompetitive K_i with respect to DPNH is infinite; therefore in the reverse reaction pyruvate must be bound to the enzyme before fluorolactate can inhibit. An extension of the above reasoning leads to the prediction that the concentration of the second substrate should have no influence on K_i (with re-

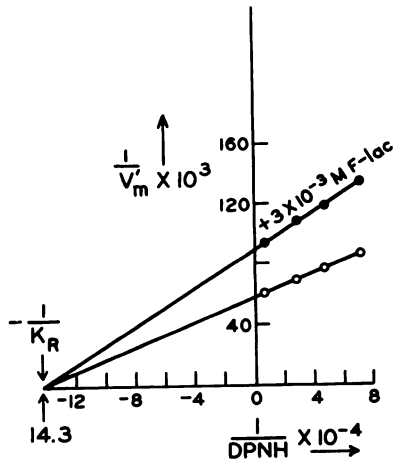

FIG. 12. *Noncompetitive relationship between DPNH and fluorolactate*

Correlation between apparent maximum velocity and DPNH concentration in presence and absence of fluorolactate at infinite concentration of pyruvate.

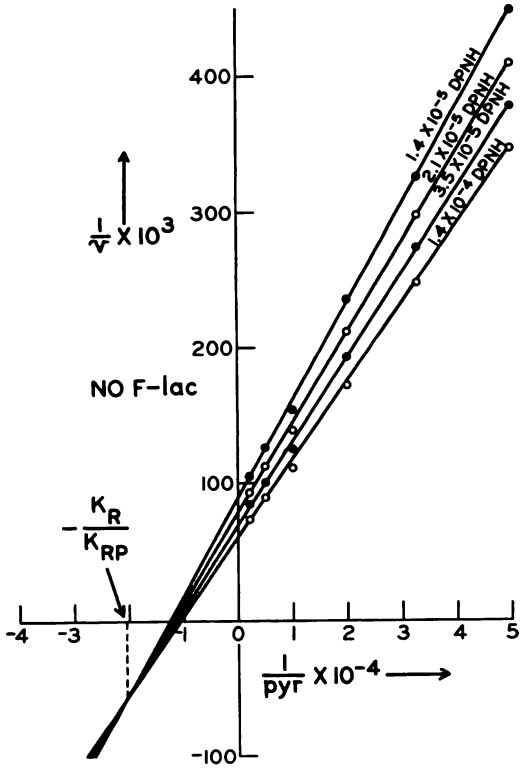

FIG. 13.

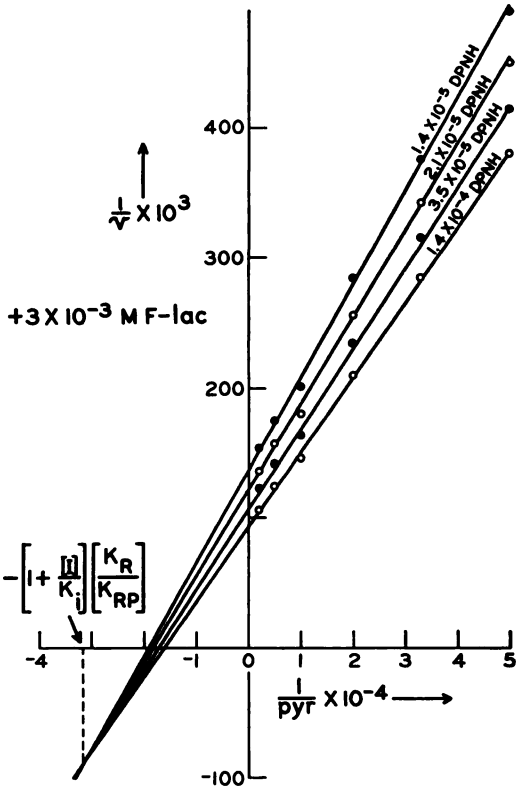

FIG. 14.

FIGS. 13 and 14. *The effect of fluorolactate on initial velocity of enzymic reduction of pyruvate as a function of pyruvate concentration at varying levels of DPNH*

spect to the first substrate) when the inhibitor interacts with the second substrate in a purely noncompetitive manner. This is actually found to be the case, as shown in Fig. 16a.

A summary of kinetic constants is given in Table 1.

Substrate Properties and Specificity of (+)-β-Fluorolactate

Fluorolactate has also been tested as a substrate for lactate dehydrogenase. No detectable oxidation of (+)-fluorolactate was observed under conditions in which (+)-lactate was rapidly oxidized. When the enzyme concentration was raised several thousandfold over that normally used in enzymic assays, a very slow oxidation was

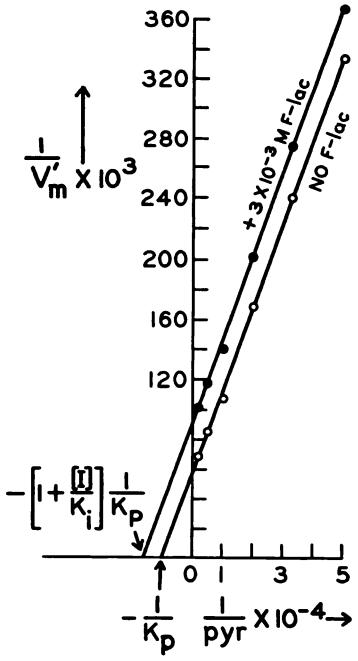

FIG. 15. *Uncompetitive relationship between pyruvate and fluorolactate*

Correlation between apparent maximum velocity and pyruvate concentration in absence and presence of fluorolactate.

detected. Calculated in terms of specific activity, this oxidation is less than 0.00002% of activity as compared to L-(+)-lactate.

The effect of L-(+)-β-fluorolactate was tested on the oxidation of isocitrate (TPN-linked), α-glycerophosphate (DPN-linked), glyceraldehyde-3-phosphate (DPN-linked), and glutamate, by crystalline enzymes. No effect was observed at concentrations of up to 10^{-2} M (+)-β-fluorolactate. The rate of reductive amination of α-ketoglutarate by TPNH + NH_4^+ (see conditions in reference 10) catalyzed by a soluble extract of sonically disrupted liver mitochondria was increased 30% by 2×10^{-2} M racemic fluorolactate. This acceleration had a maximum at $2 \times 10^{-2} M$ fluorolactate. The mechanism of this effect of fluorolactate on crude glutamate dehydrogenase is at present unknown. Since crystalline glutamate dehydrogenase did not exhibit this anomaly, it is probable that in crude form this enzyme may respond to catalytic modifiers differently. Oxidation of malate by mitochondrial extracts (by DPN) was slightly inhibited (about 10%) by 10^{-2} M fluorolactate, while the malic enzyme reaction (TPN-linked) of cytoplasmic extracts of liver was unaffected by the same concentration of this inhibitor. A detailed account of these effects of fluorolactate extended also to various multienzyme systems will be described elsewhere.

DISCUSSION

It is of interest to compare the kinetic mechanism of inhibition of LDH by fluorolactate with the known actions of oxamate and oxalate (cf. 3). This is shown in Table 2. The most probable interpretation of the effect of oxalate and oxamate is that both form an inactive ternary complex with the enzyme–coenzyme complex. This interaction, however, is not exclusive, since a direct reaction of these inhibitors with the enzyme itself also occurs (3). This complicated kinetic picture is probably a consequence of the nonspecific nature of these inhibitors. In contrast to oxamate or oxalate, fluorolactate in the forward reaction (lactate → pyruvate) appears to behave in a manner kinetically identical with lactate, since it can combine only with the enzyme–DPN complex. However, in the reverse reaction the mechanism of inhibitory action of fluorolactate involves further kinetic limitations. Since K_i decreases at increasing concentrations of pyruvate, the binding of fluorolactate (I) must require the presence of pyruvate (P), thus:

$$EP + I \rightleftharpoons EPI$$

while $$ER + I \not\rightleftharpoons$$

and $$E + I \not\rightleftharpoons$$

It was previously determined that pyridine nucleotides bind to the enzyme prior to carboxylic acid substrates (25, 26); thus K_i in the reverse direction must be

$$\frac{[ERP][I]}{[ERPI]}$$

i.e., fluorolactate can bind only to the enzyme–pyruvate–DPNH complex. A further consequence of this mechanism is that

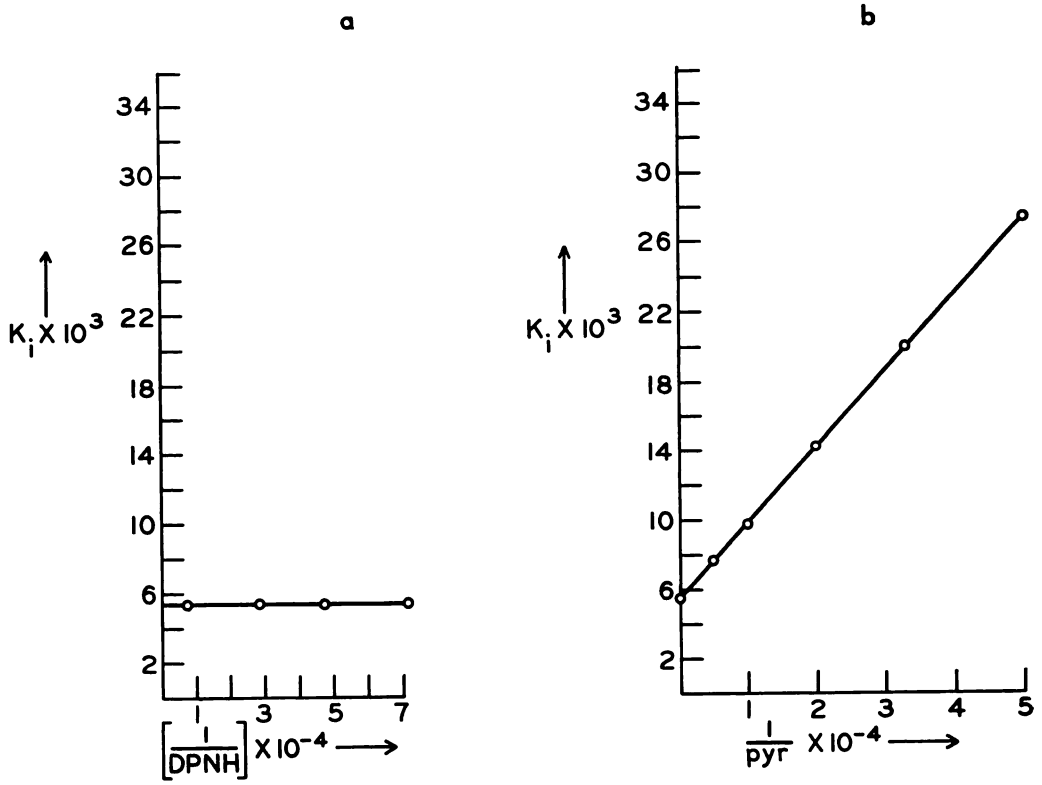

FIG. 16. (a) *K_i with respect to pyruvate as a function of DPNH concentration.* (b) *K_i with respect to DPNH as a function of pyruvate concentration*

TABLE 1

Kinetic constants[a] for the reaction $L + O \rightleftharpoons P + R$ at pH 7.53, 26°

Forward reaction	Reverse reaction
$K_i = \frac{[EO][I]}{[EOI]} = 1.3 \times 10^{-3}$ M	$K_i = \frac{[ERP][I]}{[ERPI]} = 5.4 \times 10^{-3}$ M
$K_O = 1.72 \times 10^{-4}$ M	$K_R = 7.0 \times 10^{-6}$ M
$K_L = 6.3 \times 10^{-4}$ M	$K_P = 1 \times 10^{-4}$ M
$K_{OL} = 2.1 \times 10^{-7}$ M	$K_{RP} = 4 \times 10^{-10}$ M
$V_f = 9.7 \times 10^4$ moles/min/mg enzyme	$V_r = 2.73 \times 10^5$ moles/min/mg enzyme
app. $K_{eq} = 6.65 \times 10^{-4}$ (calculated from Table 1 by the Haldane equation)	

[a] V_f = maximum velocity in the forward reaction
V_r = maximum velocity in the reverse reaction
app. K_{eq} = apparent equilibrium constant

uncompetitive K_i with respect to pyruvate is unaffected by DPNH concentration.

The inhibitory mechanism of fluorolactate and its chemical stability suggest that fluorolactate may be used as an enzyme reagent for the detection of the rate limiting role of LDH in complex multienzyme systems. In complex systems, metabolic flow can be measured by a variety of means (O_2 uptake, analyses of steady-state concentrations of intermediates, etc.), but distinction between network systems, mono-

TABLE 2

Summary of types of inhibition of LDH by fluorolactate, oxamate, and oxalate

The type of inhibition is correlated with each reactant (carboxylic acid substrate or coenzyme) by the first horizontal column of the table.

Inhibitor	Type of inhibition				References
	Lac	DPN	Pyr	DPNH	
F-lac	Comp.	Uncomp.	Uncomp.	Noncomp.	—
Oxamate	Noncomp.	Noncomp.	Comp.	Uncomp.	(3, 4, 6)
Oxalate	Comp.	Uncomp.	Noncomp.	Mixed	(3, 4, 6)

or polylinear or distributive or other pathways (cf. 27) is often not possible since the same steady-state concentrations of metabolites or overall metabolic rate can be reached by several different types of pathways. In such instances, the use of specific enzyme inhibitors as probes to detect the rate limiting role of selected enzymes in systems is a powerful experimental device, already employed in the study of control of metabolic pathways of glutamate (11). Theoretical considerations which call attention to the potential incorrectness of metabolic mechanisms constructed solely on the basis of quantitative analyses of intermediates were recently pointed out by Waley (28), with special reference to the lactate/pyruvate, glycerol-1-phosphate/dihydroxyacetone phosphate and malate/oxaloacetate systems.

ACKNOWLEDGMENTS

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