Studies on Fluoropyruvate as a Substrate of Lactate Dehydrogenase*

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ABSTRACT: There are conflicting reports in the literature concerning the ability of fluoropyruvate to act as substrate or inhibitor of lactate dehydrogenase. In the studies reported here, a method for the purification of fluoropyruvate by preparative paper chromatography has been developed and a material has been separated from fluoropyruvate preparations which is a potent inhibitor of crystalline bovine heart lactate dehydrogenase.

Purified fluoropyruvate has been shown to be a good substrate for this enzyme, and fluorolactate has been shown to be the product. However, in concentrations as low as 200 μM at *p*H 7, fluoropyruvate shows substrate inhibition. The ternary complex of enzyme nicotinamide-adenine dinucleotide fluoropyruvate has been demonstrated and it is suggested that substrate inhibition by fluoropyruvate, as with pyruvate, is due to interference by this inactive intermediate.

Initial interest in the action of fluoropyruvate (FCH_2-COCOOH) in intermediary metabolism arose during studies on the repression of neoplastic growth (Busch, 1955; Traub and Ginzburg, 1959). Fluoropyruvate was shown to inhibit respiration in mitochondrial preparations as well as the oxidative and reductive metabolism of pyruvate in liver and kidney homogenates. The inhibition was thought to be due to the formation of fluorocitrate from fluoropyruvate. Insofar as glycolysis plays an important role in catabolism of glucose in tumors both *in vitro* and *in vivo*, it was suggested by Traub and Ginzburg (1959) that fluoropyruvate could serve as a specific inhibitor of the respiration of nonglycolyzing cells, while glycolyzing cells could possibly overcome the inhibition by reduction of fluoropyruvate to fluorolactate. In fact, these authors reported that fluoropyruvate is reduced to fluorolactate by crystalline rabbit muscle lactate dehydrogenase at about one-tenth the rate shown by pyruvate. However, it was not demonstrated that fluorolactate is the product of fluoropyruvate reduction nor were the inhibitory properties of fluoropyruvate at high concentrations reported.

Using this same enzyme, Busch and Nair (1957) reported that fluoropyruvate is a noncompetitive, partially reversible inhibitor and twice as effective as oxalic or oxamic acids, compounds which have previously been shown to be potent inhibitors of the bovine heart enzyme (Novoa *et al.*, 1959).

This communication reports on studies with purified fluoropyruvate and crystalline bovine heart lactate dehydrogenase, which indicate that fluoropyruvate is a good substrate for this enzyme and exhibits substrate inhibition, as does pyruvate (Kubowitz and Ott, 1943). The apparent Michaelis constants and maximal velocities in 0.2 ionic strength Tris-Tris·HCl–KCl buffer, *p*H 7.0, are reported and compared with corresponding values determined with pyruvate as substrate. It is suggested that substrate inhibition arises from the formation of an inactive ternary enzyme·NAD^+·fluoropyruvate intermediate.

Experimental

Isozyme "H_4" of bovine heart LDH[1,2] was prepared according to the method of Schwert *et al.* (1962) and isolated by column chromatography on DEAE-Sephadex. The chromatographed enzyme was recrystallized two to three times with ammonium sulfate. Diluted enzyme solutions, made from the crystals suspended in 0.6 M saturated ammonium sulfate, were prepared daily. The enzyme was assayed in the presence of L-lactate and NAD^+ at *p*H 10 in 0.1 M glycine-phosphate buffer as described previously (Schwert *et al.*, 1962). A value of 21.8×10^4 cm^{-1} M^{-1} was used as the molar extinction coefficient of crystalline "H_4" isozyme and

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[1] The following abbreviation has been used: LDH, lactate dehydrogenase.

[2] Isozyme "H_4" of bovine heart LDH, the fastest-moving isozyme of heart LDH on free-boundary electrophoresis. All kinetic experiments reported in this paper were performed on this isozyme since all previous rate data with pyruvate have been gathered using this isozyme. A number of preliminary experiments indicated that fluoropyruvate was an equally good substrate with both the rabbit muscle enzyme and the enzyme from bovine heart. However, all reported experiments were performed with the latter enzyme since it has been prepared in highly purified form in this laboratory.

the molecular weight was taken as 150,000 (Takenaka and Schwert, 1956).

Initial reaction rates were estimated from measurements of the rate of change of absorbancy at 340 mμ with a Cary Model 11 recording spectrophotometer equipped with a water-jacketed cell compartment maintained at a constant temperature by water circulated from a bath regulated to ±0.05°.[3] The cell compartment is capable of accommodating both 1- and 10-cm cuvets and, in most cases, cuvets with a 10-cm optical path were used in order to obtain sufficiently large changes in optical density to afford reasonable precision in estimating initial reaction rates. All rate determinations were made at 28° in Tris-Tris·HCl-KCl buffer at 0.2 ionic strength. Reported *p*H values were determined in a Beckman Model G *p*H meter at the completion of the measured reaction. Cuvets were charged with buffer, coenzyme, and pyruvate of fluoropyruvate, and the reaction was initiated by the addition of 10–20 μl of enzyme with an "adder-mixer" of the type described by Boyer and Segal (1954). Maximal initial velocities and Michaelis constants were calculated from Lineweaver-Burk plots of the rate data as described previously (Winer and Schwert, 1958).

Fluorescence measurements were preformed on a Farrand Model A recording fluorimeter equipped with a water-jacketed cell compartment. A Corning primary filter No. c.s. 7-60 was used at the excitation entrance and a secondary filter No. c.s. 3-74 at the fluorescence-emission exit.

β-NAD^+ (98% Sigma) was purified by chromatography on DEAE-cellulose (Winer, 1964) and assayed with yeast alcohol dehydrogenase. The β-NADH used was 100% pure, based on a comparison of total phosphorus content with the theoretical density change at 340 mμ, after complete enzymatic reduction of acetaldehyde with horse liver alcohol dehydrogenase at *p*H 7.0 directly before use. All buffers contained 0.001 M EDTA and were prepared in quartz-distilled water. Potassium pyruvate and L-lactate were prepared as described earlier (Takenaka and Schwert, 1956). Twice-recrystallized rabbit muscle lactate dehydrogenase was obtained from the Sigma Chemical Co. and was used without further purification.

Synthesis of fluoropyruvic acid was carried out by the method of Blank *et al.* (1955) as modified by Nair and Busch (1958). The product was purified by microsublimation at 75–80° at 1 mm mercury pressure. Purified fluoropyruvate has a mp of 88° after drying *in vacuo* over P_2O_5.

Anal. Calcd for $C_3H_3O_2F$: C, 33.9; H, 2.8; F, 17.9. Found:[4] C, 33.4; H, 2.9; F, 17.8.

The product is extremely hygroscopic and is unstable at room temperature. However, stored over calcium chloride at −15°, it has remained stable for a period of 1 year.

Preparative descending paper chromatography on Whatman No. 1 paper for 60 hours using the solvent system 1-butanol–acetic acid–pyridine (100:10:8, v/v), saturated with water, was used for further purification of the synthesized product. The spots were localized on a reference strip after spraying with saturated 2,4-dinitrophenylhydrazine in 95% ethanol. The strip was placed in an oven at 100° for 10 minutes and was subsequently sprayed with 5% potassium hydroxide in 95% ethanol. The main spot on the remaining paper (R_F 0.29) was eluted with water and assayed by the spectrophotometric method, based on its interaction with cysteine (Avi-Dor and Mager, 1956). The fluoropyruvate recovered was approximately 95% of the total material placed on the paper. A minor component (R_F 0.41), present in about 1–2%, was shown to undergo slow reduction by LDH but was otherwise unidentified. It is conceivable that fluoropyruvate is somewhat labile in 1-butanol and that the fluorine was replaced by the butanol to give the compound $C_4H_9OCH_2COCOOH$,[5] which would probably be a substrate for LDH. Pyruvate, which has an R_F value of 0.35 in this solvent system, could not be detected in the preparation. That inorganic fluoride was absent from the purified samples of fluoropyruvate was demonstrated by the paper chromatographic procedure of Peters and Hall (1957).

Commercial fluoropyruvate,[6] as well as purified material which had been standing at room temperature in solid form for 2–4 days, yielded a slow moving component (R_F 0.07) on paper chromatography. One commercial preparation yielded several other impurities in the solvent system used.

A purified sample of fluoropyruvate donated by Professor E. D. Bergmann, as judged by fluorine content, melting point, and R_F value, was identical with the purified material used in the present studies. However, traces of an inhibitory material (R_F 0.07) were detected. Presumably this arose from exposure to room temperature during shipping.

Fluorolactate was prepared from fluoropyruvate by reduction with sodium borohydride in aqueous solution at *p*H 9.5 with subsequent purification on Dowex 1 (formate).[7] Descending chromatography on Whatman No. 1 paper for 12 hours using the solvent system 3-butanol–formic acid–water (80:10:10, v/v) and sprayed with the β-naphthol-sulfanilamide reagent (Schmidt *et al.*, 1963)[8] showed the following R_F values: fluorolactate, 0.62; lactate, 0.69; and fluoropyruvate, 0.45 (see Figure 4). Lactate, prepared from pyruvate by reduction with borohydride, was identical with

[3] This jacketed compartment was designed and built by Mr. Thomas B. Orr of the Research Machine Shop of this institution.

[4] Microanalyses by Galbraith Laboratories, Inc., Knoxville, Tenn.

[5] Suggested by Professor E. D. Bergmann, Hebrew University, Jerusalem, Israel.

[6] K and K Laboratories, New York City.

[7] The method for the preparation of fluorolactate is in preparation and will appear elsewhere.

[8] This chromatographic procedure was brought to our attention by Dr. Robert L. Lester.

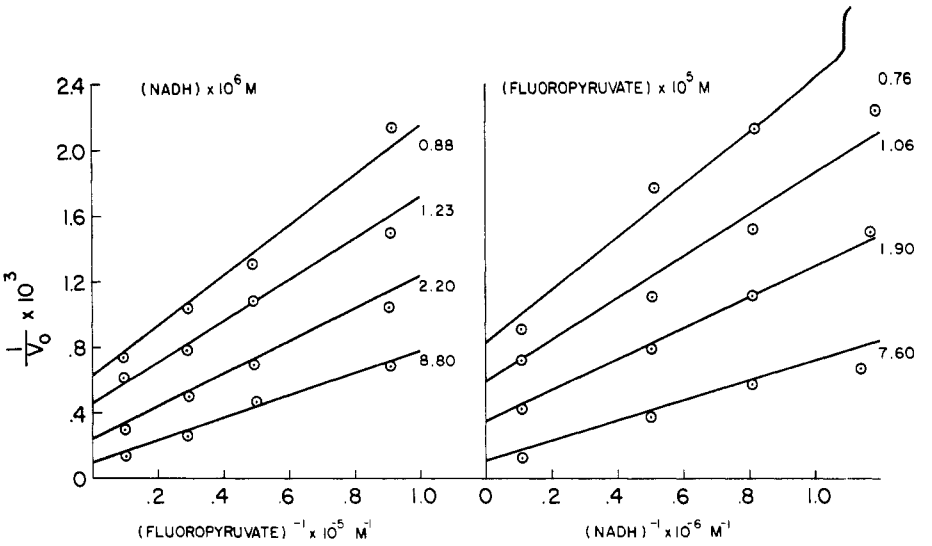

FIGURE 1: Lineweaver-Burk plots of initial rate data at *p*H 7.0. The concentrations of fixed substrates are shown under each line. The points are experimental points for measurements made in 0.2 ionic strength Tris-Tris·HCl-KCl buffer at 28°. The lines are calculated using equations previously derived (Hakala *et al.*, 1956). All concentrations are in moles per liter and reaction velocities in moles per liter per minute per mole of enzyme.

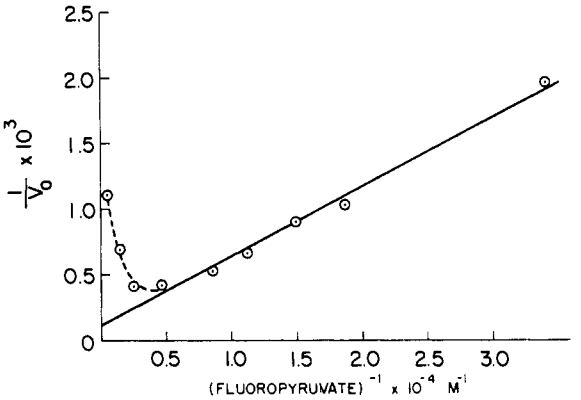

FIGURE 2: Variation of the rate of the LDH-catalyzed reduction of fluoropyruvate concentration at *p*H 7.0. The solid curve was calculated from the equation of Lineweaver and Burk (1934) for substrate inhibition by using the Michaelis constant shown in Table I and 8.4×10^{-3} M for the dissociation constant for the second bound molecule of pyruvate. The points are experimental points. Velocities are in moles per liter per minute per mole of enzyme. The NADH concentration was constant at 2.2 μM and all velocity determinations were made in Tris buffer, $\mu = 0.2$, at 28°.

lactate prepared enzymatically from pyruvate as identified by chromatography in the foregoing solvent system. Fluorolactate was prepared enzymatically from fluoropyruvate as indicated in the legend of Figure 4. The reaction mixture, at the end of 1 hour, was evaporated to dryness at 20° *in vacuo* and the residue was dissolved in 0.2 ml of water and chromatographed using the foregoing solvent system.

Results and Discussion

A number of β-substituted halogen derivatives of lactic acid have been reported to be oxidized by crystalline rabbit muscle LDH but at a rate about one-hundredth the rate at which lactate is oxidized (Franks and Holz, 1959). Busch and Nair (1957) have reported that the β-chloro and bromo derivatives of pyruvic acid show no substrate properties with the rabbit muscle enzyme and, in fact, bromopyruvate was found to be slightly inhibitory in the concentration range used.

That fluoropyruvate is a substrate for bovine heart lactate dehydrogenase is illustrated in Figure 1. The range of concentrations over which fluoropyruvate can be varied is about ten. At concentrations as low as 2×10^{-4} M, at *p*H 7.0, there is already observable substrate inhibition. Incubation of LDH with purified fluoropyruvate in the concentrations used for the kinetic studies for 30 minutes in 0.1 M K_2HPO_4 buffer, *p*H 7.0, produced no inorganic fluoride as indicated by the paper chromatographic method of Peters and Hall (1957). Figure 2 shows the variation of the rate of the LDH-catalyzed reduction of fluoropyruvate with fluoropyruvate concentration at *p*H 7.0. Increasing the concentration of fluoropyruvate to 2×10^{-3} M decreased the initial velocity about 50% of that observed at noninhibitory concentrations. The concentration of pyruvate necessary to show similar inhibition under identical experimental conditions is about 5×10^{-4} M (Winer and Schwert, 1958). An experiment in which pyruvate at a final concentration of 1×10^{-4} M was added to fluoropyruvate at a concentration of 0.5×10^{-4} M indicated a strictly additive substrate effect, and this occurred regardless of the addition sequence of enzyme, substrate, or coenzyme. The slow-moving component with R_F 0.07, when eluted with water and tested in an assay system containing 0.3 μmole of pyruvate, 0.06

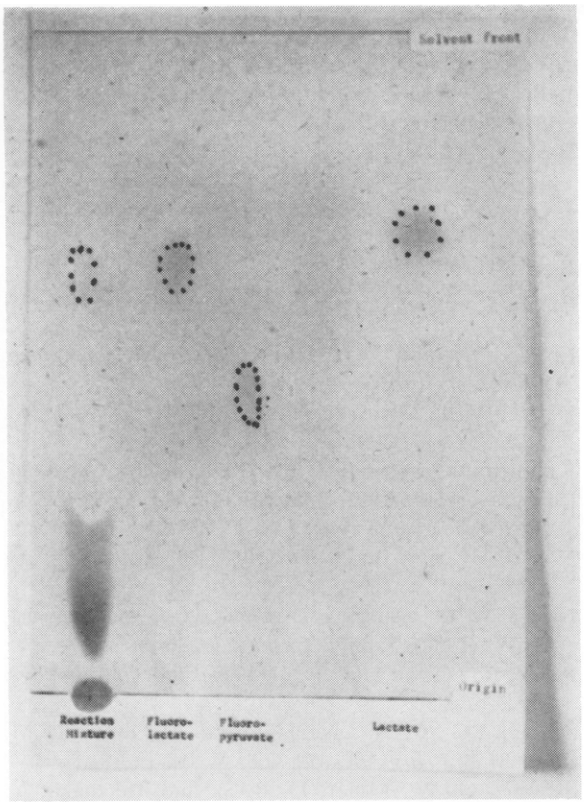

FIGURE 3: Chromatography (10 μg of each component) on Whatman No. 1 paper of fluorolactate, lactate, fluoropyruvate, and a reaction mixture at *p*H 7.0 and 23°, consisting of the following components in final concentrations: fluoropyruvate, 6×10^{-4} M; β-NADH, 5.4×10^{-4} M; K_3PO_4, 2×10^{-3} M. Reaction initiated with 5×10^{-9} M LDH. The fluoropyruvate was added in six installments of 1×10^{-4} M each, at intervals of 10 minutes, so as not to accumulate inhibitor products. (See text for further details.)

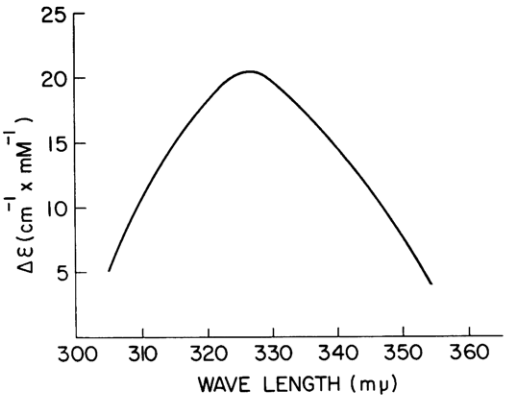

FIGURE 4: Difference spectra of the LDH·NAD$^+$·fluoropyruvate compound in Tris buffer, ionic strength 0.2, *p*H 8.15. Final concentrations in the sample cuvet as follows: LDH, 2×10^{-6} M; β-NAD$^+$, 5×10^{-4} M; pyruvate, 1×10^{-3} M. Two reference cuvets were used with the following reactants: pyruvate and LDH in one cuvet; β-NAD$^+$ in the other. Spectra were taken 30 minutes after addition of reactants.

TABLE I: Comparison of Kinetic Constants Using Pyruvate and Fluoropyruvate as Substrates for LDH in Tris Buffer, $\mu = 0.2$, *p*H 7.0, at 28°.

	Pyruvate	Fluoro-pyruvate
V_m[a] (moles/liter/min^{-1} per mole site)	15,000	5,000
K_P or K_{FP} (M $\times 10^4$)	1.1	1.2
K_R (M $\times 10^5$)	1.6	1.0
K_{RP} or $K_{R(FP)}$ (M$^2 \times 10^{10}$)	2.1	1.7

[a] Based on a molecular weight of 150,000 and four active centers per mole of enzyme. See text for definitions of other constants.

μmole NaDH, and 3×10^{-3} μmole of LDH in Tris buffer, *p*H 7.0, showed 65% inhibition in a dilution of 1:10 and 25% inhibition in a dilution of 1:25. The apparent Michaelis constant of NADH (K_R) for both pyruvate and fluoropyruvate is about the same as are the complex constants K_{RP} and $K_{R(FP)}$, as shown in Table I. These constants have the same operational relationship to the products of concentrations RP and R(FP) as do Michaelis constants to single substrate concentrations. The maximum velocity with fluoropyruvate as a substrate is one-fifth that with pyruvate as substrate.

That the product of fluoropyruvate reduction is fluorolactate is demonstrated chromatographically as shown in Figure 3. The fluorolactate formed enzymatically from fluoropyruvate has the same R_F value as the fluorolactate prepared chemically from fluoropyruvate by reduction with borohydride. That no fluoropyruvate remained in the reaction mixture is demonstrated by the lack of a spot corresponding to fluoropyruvate. The other spots in the complete reaction mixture as shown on the chromatogram are due to phosphoric acid and β-NAD$^+$. That β-hydroxypyruvate is not formed from fluoropyruvate to produce glyceric acid is indicated by the lack of a spot corresponding to glyceric acid, i.e., R_F 0.43. An 8:1:1 3-butanol–formic acid–water system has been used to develop all chromatograms. It produces excellent separations of fluorolactate and fluoropyruvate, causes little or no tailing, and gives compact spots.

Since pyruvate has been shown to form a ternary complex with enzyme and NAD$^+$ with high concentrations of pyruvic acid by absorbancy (Zewe and Fromm, 1962) and fluorescence measurements (Winer, 1963), it was of interest to determine whether fluoropyruvate could form a similar complex. Figure 4 shows the ter-

nary complex formed with fluoropyruvate, enzyme, and NAD^+. The millimolar absorbancy of the ternary compound is about 25 cm^{-1} mM^{-1}, which is about the same absorbancy reported for the pyruvate ternary intermediate. The wavelength maximum is at 325 mμ. Addition of fluoropyruvate to the fluorescent enzyme·NAD^+ binary complex (Winer, 1963) results in a quenching of the fluorescence when the solution is activated by light at 335 mμ and the fluorescence emission is measured at 445 mμ. From fluorescence-quenching titration experiments, performed as described recently (Winer, 1963), it can be estimated that the intrinsic dissociation constant of fluoropyruvate from the ternary intermediate is of the order of 10^{-5} M at *p*H 7.0 in Tris buffer. The equilibrium constant for the dissociation of the enzyme·NAD^+ pyruvate complex to give pyruvate and enzyme·NAD^+ is also of the order of 10^{-5} M under identical experimental conditions of buffer and *p*H (Winer, 1963).

Thus the formation of the inactive complex of enzyme·NAD^+·fluoropyruvate could account for substrate inhibition as seen with this substrate. Inhibitory properties of fluoropyruvate reported by Busch and Nair (1957) with the rabbit muscle enzyme are probably due in part to the formation of this inactive ternary intermediate and in part to the presence of the slow-moving impurity (R_F 0.07) present in even purified samples of fluoropyruvate which have remained at room temperature for short periods of time.

Acknowledgment

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References

Avi-Dor, Y., and Mager, J. (1956), *Biochem. J. 63*, 613.
Blank, I., Mager, J., and Bergmann, E. D. (1955), *J. Chem. Soc.*, 2190.
Boyer, P. D., and Segal, H. L. (1954), *in* The Mechanism of Enzyme Action, McElroy, W. D., and Glass, B., eds., Baltimore, Williams & Wilkins, p. 520.
Busch, H. (1955), *Cancer Res. 15*, 365.
Busch, H., and Nair, P. V. (1957), *J. Biol. Chem. 229*, 377.
Franks, W., and Holz, E. (1959), *Z. Physiol. Chem. 314*, 22.
Hakala, M. T., Glaid, A. J., and Schwert, G. W. (1956), *J. Biol. Chem. 221*, 191.
Kubowitz, F., and Ott, P. (1943), *Biochem. Z. 314*, 94.
Lineweaver, H. and Burk, D. (1934), *J. Am. Chem. Soc. 56*, 658.
Nair, P. V., and Busch, H. (1958), *J. Org. Chem. 23*, 137.
Novoa, W. B., Winer, A. D., Glaid, A. J., and Schwert, G. W. (1959), *J. Biol. Chem. 234*, 1143.
Peters, R. A., and Hall, R. J. (1957), *Biochim. Biophys. Acta 26*, 433.
Schmidt, G. C., Fischer, C., and McOwen, J. M. (1963), *J. Pharm. Sci. 52*, 468.
Schwert, G. W., Millar, D. B. S., and Takenaka, Y. (1962), *J. Biol. Chem. 237*, 2131.
Takenaka, Y., and Schwert, G. W. (1956), *J. Biol. Chem. 223*, 157.
Traub, A., and Ginzburg, Y. (1959), *Exptl. Cell Res. 17*, 246.
Winer, A. D. (1963), *Acta Chem. Scand. 17*, S203.
Winer, A. D. (1964), *J. Biol. Chem. 239*, PC 3598.
Winer, A. D., and Schwert, G. W. (1958), *J. Biol. Chem. 231*, 1065.
Zewe, V., and Fromm, H. J., (1962), *J. Biol. Chem. 237* 1668.