Blond, D. M., and Whittam, R. (1965), *Biochem. J. 92*, 158.
Chappell, J. B., and Crofts, A. (1966), *in* Regulation of Metabolic Processes in Mitochondria, Tager, J. M., Papa, S., Quagliariello, E., and Slater, E. C., Ed., New York, N. Y., American Elsevier Publishing Co., p 293.
Chappell, J. B., and Robinson, B. H. (1968), *in* Metabolic Roles of Citrate, Goodwin, T. W., Ed., New York, N. Y., Academic Press, p 123.
Christiansen, R. O., Loyter, A., and Racker, D. (1969), *Biochim. Biophys. Acta 180*, 207.
Gómez-Puyou, A., Sandoval, F., Chavez, E., and Tuena, M. (1970), *J. Biol. Chem. 245*, 5239.
Gómez-Puyou, A., Sandoval, F., Peña, A., Chávez, E., and Tuena, M. (1969b), *J. Biol. Chem. 244*, 5339.
Gómez-Puyou, A., Sandoval, F., Tuena, M., Peña, and Chávez, E. (1969a), *Biochem. Biophys. Res. Commun. 36*, 316.
Graven, S. N., Estrada-O., S., and Lardy, H. A. (1966), *Proc. Nat. Acad. Sci. U. S. 56*, 654.
Harris, E. J., Höfer, M. P., and Pressman, B. C. (1967), *Biochemistry 6*, 1348.
Kimmich, G. A., and Rasmussen, H. (1967), *Biochim. Biophys. Acta 131*, 413.
Krall, A. R., Wagner, M. C., and Gozansky, D. M. (1964), *Biochem. Biophys. Res. Commun. 16*, 77.
Lynn, W. S., and Brown, R. H. (1966), *Arch. Biochem. Biophys. 114*, 260.
Massari, S., and Azzone, G. F. (1970), *Eur. J. Biochem. 12*, 310.
Mitchell, P. (1968), Chemiosmotic Coupling and Energy Transduction, Bodmin, Cornwall, Glynn Research.
Moore, C., and Pressman, B. C. (1964), *Biochem. Biophys. Res. Commun. 15*, 562.
Opit, L. J., and Charnock, J. S. (1965), *Biochim. Biophys. Acta 110*, 9.
Papa, S., Tager, J. M., Guerrieri, F., and Quagliariello, E. (1969), *Biochim. Biophys. Acta 172*, 184.
Pinto, E., Gómez-Puyou, A., Sandoval, F., Chávez, E., and Tuena, M. (1970), *Biochim. Biophys. Acta 223*, 436.
Pressman, B. C. (1965), *Proc. Nat. Acad. Sci. U. S. 53*, 1076.
Pressman, B. C., and Lardy, H. A. (1952), *J. Biol. Chem. 197*, 547.
Pressman, B. C., and Lardy, H. A. (1955), *Biochim. Biophys. Acta 18*, 482.
Sandoval, F., Gómez-Puyou, A., Tuena, M., Chávez, E., and Peña, A. (1970), *Biochemistry 9*, 684.
Smith, E. H., and Beyer, R. E. (1967), *Arch. Biochem. Biophys. 122*, 614.
Sumner, J. B. (1944), *Science 100*, 413.

Inhibition and Kinetic Mechanism of Rabbit Muscle Glyceraldehyde-3-phosphate Dehydrogenase*

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ABSTRACT: The patterns of product inhibition by NADH, dead-end inhibition by nitrate, α-glycerophosphate, and 3-phosphoglycerate, and substrate inhibition by 3-hydroxypropionaldehyde-3-P permit deduction of the kinetic mechanism of rabbit muscle glyceraldehyde-3-phosphate dehydrogenase as ordered, with NAD, aldehyde, and phosphate or arsenate adding in that order, and acid release preceeding NADH release. The rate-limiting step with glyceraldehyde-3-P as substrate is NADH release. When 3-hydroxypropionaldehyde-3-P is used as substrate, with arsenate some previous step is equally rate limiting, while with phosphate a previous step is much slower, and the steady-state level of E·NADH is reduced sixfold from that present with arsenate. The kinetic constants with phosphate or arsenate are reported for eight aldehydes which act as substrates.

Glyceraldehyde-3-phosphate dehydrogenase (D-glyceraldehyde-3-phosphate:NAD oxidoreductase phosphorylating, EC 1.2.1.12) is capable of catalyzing a number of different reactions; however, the most important one from the physiological point of view is the oxidative phosphorylation of D-glyceraldehyde 3-phosphate (GAP).[1] The enzyme is not specific for GAP, but can catalyze the oxidative phosphorylation of other aldehydes such as D-glyceraldehyde, propionaldehyde, and acetaldehyde. While the enzyme is specific for the hydrogen acceptor, NAD, it is possible to replace the inorganic orthophosphate by arsenate and so convert the phosphorolysis to an irreversible arsenolysis. It has also been established that oxidation precedes phosphorolysis and that under certain conditions the two steps can be separated from one another (Velick, 1954; Boyer and Segal, 1954; Krimsky and Racker, 1963). For example, in the absence of phosphate the oxidation of GAP leads to an acyl-enzyme in which a 3-phosphoglyceryl thiol ester is found (Velick and Hayes, 1953; Segal and Boyer, 1953; Velick and Furfine, 1963).

The kinetic mechanism of this enzyme is of particular interest because (a) it has been investigated by three different groups of workers who came to different and apparently con-

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[1] Abbreviations used are: GAP, D-glyceraldehyde-3-phosphate; HPAP, 3-hydroxypropionaldehyde-3-phosphate; PGA, 3-phosphoglyceric acid.

flicting conclusions, and (b) it is a ter-bi reaction (Cleland, 1963a) involving three substrates in the forward direction and two substrates in the reverse direction.

Furfine and Velick (1965) working with the enzyme from rabbit muscle concluded that the kinetic mechanism was best described by a random addition of substrates and release of products with the rate-limiting step occurring in the reaction of a kinetically important quaternary enzyme–substrates complex (rapid equilibrium random ter bi). In contrast to this Keleti and Batke (1965) using the enzyme from pig muscle found that the initial rate data could be explained by a random addition of NAD and GAP followed by a rate-limiting reaction with phosphate as the compulsory third substrate. However, in a similar study with the same enzyme but D-glyceraldehyde as the substrate Keleti (1965) found that the data supported a rapid equilibrium random ter-bi mechanism. To complicate the picture further Fahien (1966) also with D-glyceraldehyde but using rabbit muscle enzyme concluded that the kinetic mechanism was ordered with the compulsory order of addition of substrates being NAD, D-glyceraldehyde, arsenate, and a second molecule of NAD.

Initial rate equations for mechanisms involving three substrates have been considered by various authors (Frieden, 1959; Cleland, 1963a; Keleti and Batke, 1965; Keleti, 1965) and more recently Dalziel (1969) has carried out a systematic and comprehensive treatment of the analysis and interpretation of initial rate data for ter-reactant enzymes. However, due to the confusing picture that has emerged from the initial rate data for glyceraldehyde-3-phosphate dehydrogenase it was considered that an approach through inhibition studies might clarify the situation. Furfine and Velick (1965) used inhibition by products in their studies and Fromm (1967) considered, in detail, the use of dead-end inhibitors to distinguish possible ter-reactant systems. Most recently Cleland (1970) suggested that some of the problems of kinetic mechanisms might possibly be resolved by detailed analysis of substrate inhibition in multisubstrate systems. In this present study product, substrate and dead-end inhibition kinetics have been used in an attempt to establish a kinetic mechanism for glyceraldehyde-3-phosphate dehydrogenase.

Experimental Section

Materials. DL-Glyceraldehyde 3-phosphate (monobarium salt) was obtained as the diethyl acetal derivative from Sigma. It was converted into the sodium salt of the free aldehyde by heating a solution at 100° for 3 min in the presence of Dowex 50 (H^+). NAD, NADH, and DL-α-glycerophosphate (disodium salt) were also obtained from Sigma. Aldol, *n*-butyraldehyde, propionaldehyde, and acetaldehyde were obtained from Eastman Kodak and were freshly distilled before use. The tricyclohexylammonium salt of 3-phosphoglyceric acid was obtained from Boehringer.

3-Hydroxypropionaldehyde was prepared by heating a 20% aqueous solution of acrolein in a sealed flask at 100° for 24 hr (Neff, 1904). The yellow solution was filtered to remove a small amount of white solid and the filtrate concentrated to one-fifth the volume by vacuum distillation at 10 mm. The thick yellow liquid was then subjected to vacuum fractional distillation in a 10-ml short-path distillation apparatus (Bantamware, Kontes Glass Co.). At a pressure of 12 mm the fraction distilling between 74 and 77° was collected. The reported boiling point at this pressure is 76° (Heilbron and Bunbury, 1953). The semicarbazone and 2,4-dinitrophenylhydrazone derivatives showed sharp melting points at 113–114 and 150–152°, respectively.

The diethyl acetal of 3-hydroxypropionaldehyde was prepared by the ethyl orthoformate–NH_4NO_3 method described in Rabjohn (1963). The fraction boiling between 97 and 100° at 20 mm was collected (Heilbron and Bunbury, 1953). Phosphorylation of the diethyl acetal of 3-hydroxypropionaldehyde was achieved by the method of Tener (1961). The diethyl acetal (1.48 g; 10 mmoles) was dissolved in 5 ml of pyridine and to this was added 20 ml of an aqueous pyridine solution containing 20 mmoles of cyanoethyl phosphate (pyridinium salt). The solution was reduced to dryness by vacuum distillation at 30° and the residue was taken up in 25 ml of anhydrous pyridine and again taken to dryness at 30° *in vacuo*. This process was repeated twice more. Finally the residue was dissolved in 15 ml of anhydrous pyridine, and 25 ml of a pyridine solution containing 25 mmoles of dicyclohexylcarbodiimide was added. The flask was sealed and left at room temperature. After 2 days 30 ml of water was added and after a further hour at room temperature the mixture was taken to dryness at 30° *in vacuo*. The residue was taken up in 50 ml of water and filtered to remove the cyclohexylurea. Excess cyanoethyl phosphate was removed from the filtrate as the ethanol insoluble barium salt at pH 8. Excess ethanol was removed by vacuum distillation and the remaining aqueous solution made to 0.1 N NaOH and heated at 50° for 10 min. The pH was adjusted to 8, 10 mmoles of $BaCl_2$ was added, and the white precipitate formed was removed by filtration. The pH of the filtrate was readjusted to eight and two volumes of ethanol added. After standing in the cold overnight the barium salt of 3-hydroxypropionaldehyde 3-phosphate diethyl acetal crystallized as pale yellow plates. The crystals were filtered, washed with ethanol and dried. The yield of 275 mg was about 8%. *Anal.* Calcd for $C_7H_{15}BaO_6P$: P, 8.55. Found: P, 8.21. The infrared spectrum showed major peaks at 2970, 1122, and 1062 cm^{-1}; the latter two being consistent with an ether and alkyl phosphate ester, respectively. Minor peaks at 1640, 1395, 1005, 950, and 817 cm^{-1} were also present. A similar spectrum of the barium salt of DL-glyceraldehyde 3-phosphate diethyl acetal showed major peaks at 2970, 2530, 1136, and 1080 cm^{-1} and minor peaks at 1660, 1510, 1460, 1395, 1000, 945, and 772 cm^{-1}. Both these spectra are consistent with the postulated structures.

Glyceralde-3-phosphate dehydrogenase from rabbit muscle was purchased from Boehringer as a crystalline suspension in 2.5 M ammonium sulphate solution (10 mg of protein/ml). The enzyme was used without further treatment.

Methods. The reaction was followed by measuring the formation of NADH at 340 nm using a Beckman DU monochromator with a deuterium lamp coupled to a Gilford Model 200 optical density converter and a 10-mV recorder equipped with an adjustable zero and multispeed chart drive. Full-scale sensitivity of 0.1–0.2 OD unit and chart speeds from 0.2–10 in./min were used.

Kinetic experiments were carried out using a 0.4-ml reaction mixture in 10-mm silica microcuvets. All components, except the enzyme, were prewarmed in the cuvet to 30° in a water bath and the reaction compartment was also maintained at 30° with thermospacers. The reaction was started by the addition of 5 μl of enzyme solution. Initial velocities were determined by measuring the tangent to the recorded curve extrapolated to the time of addition of the enzyme. The basic incubation mixture contained 0.02 M *N,N*-bis(2-hydroxyethyl)glycine (Bicine)–acetate buffer (pH 8.6), 0.5 mM EDTA, and 1 mM dithiothreitol; to this were added substrates and

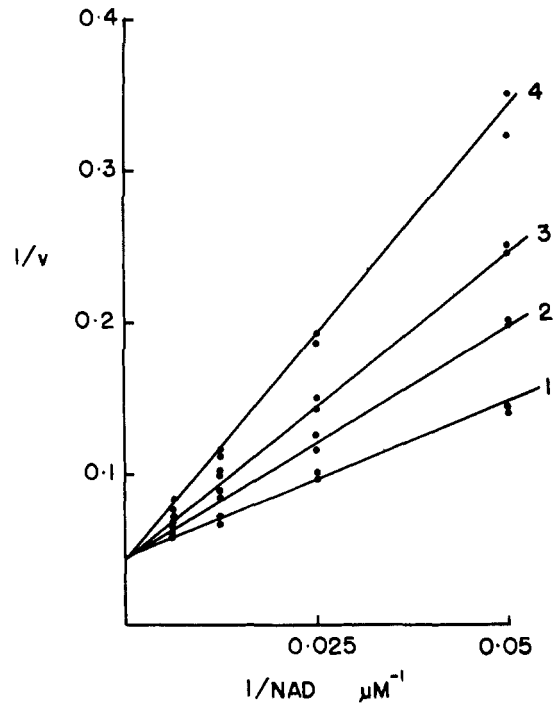


FIGURE 1: Reciprocal plots with NAD as the variable substrate and NADH as the inhibitor. HPAP concentration: 1 mM; arsenate concentration: 62 μM. NADH concentrations: (1) 0, (2) 15.5 μM, (3) 31 μM, (4) 62 μM. Enzyme concentration: 80 μg/ml. Data fitted to eq 4.

inhibitors at the appropriate concentrations. A standard assay was established containing 0.16 mM NAD, 0.8 mM GAP, and 20 mM sodium arsenate. The stock enzyme suspension was freshly diluted with basic incubation medium immediately prior to use; this diluted enzyme showed little loss of activity over 24 hr. The enzyme was assayed by the standard procedure at the beginning and end of each kinetic run; in no instance was there a significant loss of activity. For comparison between different kinetic runs a unit of enzyme activity was conveniently defined as that amount of enzyme that causes the reduction of 1 μmole of NAD/min. It was found that 18 μg of protein was equivalent to 1 unit of activity and that depending on the reaction 1–40 μg of protein was necessary to produce reasonable rates.

Data Processing. Reciprocal velocities were plotted graphically against reciprocals of the substrate concentrations. In all cases but three (see below) a reasonably linear relationship was obtained. The data were then fitted to eq 1 using a

$$v = \frac{VA}{K + A} \tag{1}$$

least-squares method and assuming equal variance for the velocities (Wilkinson, 1961). All calculations were performed on an IBM 360/44 digital computer using a FORTRAN IV program (Cleland, 1967) which provides values of K, V, K/V, $1/V$, and the standard errors of their estimates. Slopes (K/V) and intercepts ($1/V$) were then plotted graphically against the inhibitor concentration or the reciprocal of the second substrate concentration. All replots except one were linear. On the basis of the replots the proper pattern was chosen for each experiment and all the data points fitted to the appropriate equation (Cleland, 1963b). In the three cases where the reciprocal plots indicated substrate inhibition, the data were fitted to eq 2. Data fitting a sequential initial velocity

$$v = \frac{VA}{K + A + A^2/K_I} \tag{2}$$

pattern were fitted to eq 3, for linear competitive inhibition to eq 4, for linear uncompetitive inhibition to eq 5, for linear noncompetitive inhibition to eq 6, and for uncompetitive substrate inhibition to eq 7.

$$v = \frac{VAB}{K_{ia}K_b + K_bA + K_aB + AB} \tag{3}$$

$$v = \frac{VA}{K(1 + I/K_{is}) + A} \tag{4}$$

$$v = \frac{VA}{K + A(1 + I/K_{ii})} \tag{5}$$

$$v = \frac{VA}{K(1 + I/K_{is}) + A(1 + I/K_{ii})} \tag{6}$$

$$v = \frac{VAB}{K_{ia}K_b + K_bA + K_aB + AB + AB^2/K_I} \tag{7}$$

The lines in the figures are the calculated lines after fitting the data to the appropriate equation; the points represent the actual experimental values. Velocities in all the figures correspond to $\Delta OD(\times 10^2)$/min.

TABLE I: Kinetic Constants of Some Aldehydes.

Aldehyde	With Arsenate[a] K (mM)	With Arsenate[a] V[b]	With Phosphate[a] K (mM)	With Phosphate[a] V[b]
GAP	0.155	100	0.137	95
HPAP	5.6	47	6.9	7.3
DL-Glyceraldehyde	73	7	76	5.2
3-Hydroxypropionaldehyde	136	0.9	106	1.2
Aldol	193	1.1	314	1.0
n-Butyraldehyde	347	1.3	266	1.1
Propionaldehyde	400	0.8	378	0.6
Acetaldehyde	667	1.0	1030	0.7

[a] The assays were carried out with the aldehyde as the variable substrate. The NAD concentration was 0.16 mM and assays contained either arsenate at 20 mM or phosphate at 84 mM. These concentrations of arsenate or phosphate are over 500 × K_m; the K_m for NAD varies with aldehyde substrate, but is always less than 0.1 mM. [b] The values for V are all relative to the V value for GAP with arsenate.

Results

Specificity. A number of aldehydes were found to be substrates, confirming previous reports (Harting and Velick, 1954). Table I shows K and V for a series of aliphatic aldehydes in both the phosphate and arsenate systems. Although there is a considerable change in K and V within the series, for any

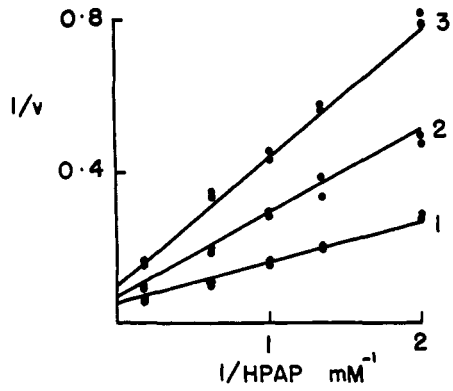


FIGURE 2: Reciprocal plots with HPAP as the variable substrate and NADH as the inhibitor. NAD concentration: 40 μM; arsenate concentration: 62 μM. NADH concentrations: (1) 0, (2) 62 μM, (3) 124 μM. Enzyme concentration: 68 μg/ml. Data fitted to eq 6.

given aldehyde there is little difference between the K in arsenate and phosphate and, except for HPAP, between the V in the two systems. In the case of HPAP the $V_{arsenate}$ is about sixfold greater than the $V_{phosphate}$, suggesting a change in the rate-limiting reaction as the acyl acceptor changes. The results with GAP are in good agreement with previous workers (Furfine and Velick, 1965) in that K and V are essentially the same in both systems. However, the values of K for GAP are somewhat higher than the value of 0.09 mM obtained by these authors. The results with DL-glyceraldehyde confirmed the value of K as 80 mM obtained by Fahien (1966). The kinetic constants for the other aldehydes have not been reported before; in addition values for 3-hydroxypropionaldehyde and HPAP were obtained, neither of which had been previously used.

Product Inhibition. Attempts to make the acyl phosphate product of the oxidation of HPAP (1,3-diphospho-3-hydroxypropionic acid) were unsuccessful. Consequently only one of the reaction products, NADH, was tested as a product inhibitor. NADH was found to be a linear competitive inhibitor of NAD (Figure 1) with a K_{is} of 33.5 ($\pm$3.1) μM. In contrast NADH was a linear noncompetitive inhibitor against HPAP

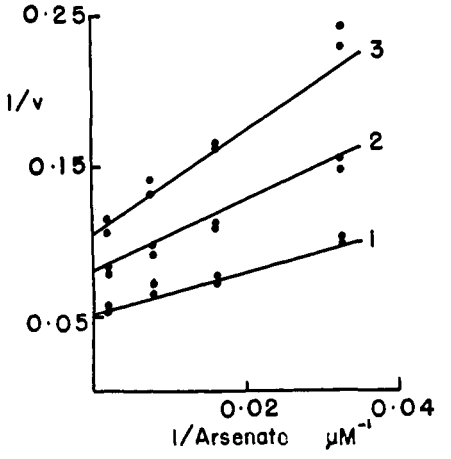


FIGURE 3: Reciprocal plots with arsenate as the variable substrate and NADH as the inhibitor. NAD concentration: 80 μM; HPAP concentration: 1 mM. NADH concentrations: (1) 0, (2) 62 μM, (3) 124 μM. Enzyme concentration: 79 μg/ml. Data fitted to eq 6.

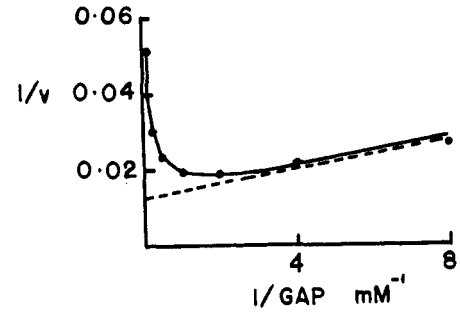


FIGURE 4: Reciprocal plot with GAP as the variable substrate. NAD concentration: 160 μM; arsenate concentration: 120 μM. Enzyme concentration: 6 μg/ml. Data fitted to eq 2.

(Figure 2) and arsenate (Figure 3). With HPAP as the variable substrate, a K_{is} of 65 ($\pm$9) μM and a K_{ii} of 75 ($\pm$16) μM were obtained; with arsenate as the variable substrate, the K_{is} and K_{ii} values were 76 ($\pm$18) μM and 140 ($\pm$15) μM, respectively. The competitive inhibition between NAD and NADH confirms the earlier observation of Furfine and Velick (1965); however, these workers also observed a competitive effect between NADH and GAP.

Substrate Inhibition. In 1963 Velick and Furfine observed that GAP showed substrate inhibition. This was confirmed (Figure 4) and it was established that the inhibition was essentially the same (K_I = 2.7 $\pm$ 0.5 mM) in both the arsenate and phosphate systems. HPAP was also found to give substrate inhibition (Figure 5); however, this only occurred with arsenate (K_I = 2.4 $\pm$ 0.4 mM) and was completely absent with inorganic phosphate over the same concentration range of HPAP.

In order to determine the type of substrate inhibition initial velocity studies were run with NAD as the variable substrate, HPAP as the changing fixed substrate and either arsenate or phosphate as the fixed third substrate. Figure 6 shows that with phosphate a normal intersecting initial velocity pattern was obtained with both intercepts and slopes being linear functions of the reciprocal of the HPAP concentration. However with arsenate (Figure 7) the pattern showed that while the slopes varied in a linear manner the intercepts showed substrate inhibition (Figure 8). This result clearly indicates that with arsenate HPAP was giving rise to uncompetitive substrate inhibition (Cleland, 1970). The K_I from a fit of the data to eq 7 was 3.7 $\pm$ 1.0 mM.

Dead-End Inhibition. A number of compounds related to the substrates were found to act as dead-end inhibitors of

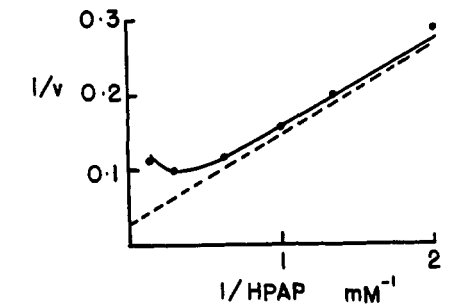


FIGURE 5: Reciprocal plot with HPAP as the variable substrate. NAD concentration: 160 μM; arsenate concentration: 500 μM. Enzyme concentration: 27 μg/ml. Data fitted to eq 2.

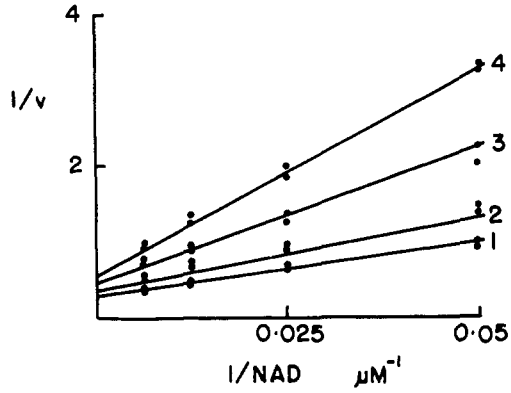


FIGURE 6: Reciprocal plots with NAD as the variable substrate and HPAP as the changing fixed substrate. HPAP concentrations: (1) 5 mM, (2) 3.31 mM, (3) 1.6 mM, (4) 1 mM. Phosphate concentration: 4.2 mM. Enzyme concentration: 38 μg/ml. Data fitted to eq 3.

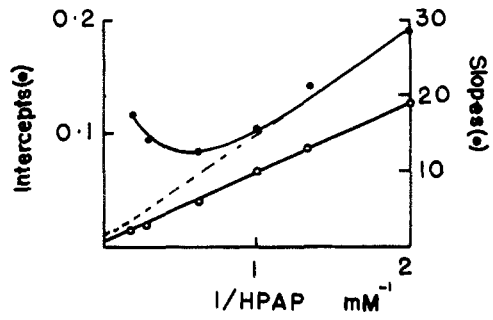


FIGURE 8: Replot of intercepts and slopes from Figure 7 against the reciprocal of the HPAP concentration.

glyceraldehyde-3-phosphate dehydrogenase. These included nitrate, DL-α-glycerophosphate, and 3-phosphoglyceric acid, none of which have previously been reported to be inhibitors. The inhibition patterns of these three inhibitors were determined with NAD, GAP, HPAP, arsenate, or phosphate as the variable substrate and the results are summarized in Table II.

An important point to be noted in these results is the large number of uncompetitive inhibitions. In particular 3-phosphoglyceric acid gave uncompetitive inhibition no matter which substrate was varied and, except for the phosphate system, the K_{ii}'s were all much the same. Although the inhibition was still uncompetitive (Figure 9) when phosphate was the variable substrate the K_{ii} was some sixfold greater when compared to the K_{ii} when arsenate was the variable substrate (Figure 10). Two other important uncompetitive inhibitions are those shown by α-glycerophosphate against NAD (Figure 11) and by nitrate against HPAP (Figure 12). In contrast when arsenate was the variable substrate nitrate was purely competitive (Figure 13). One other pattern of interest is that of α-glycerophosphate against GAP. Due to the close structural similarity one might have expected competitive inhibition, however, both slopes and intercepts varied with the inhibitor concentration, clearly indicating a noncompetitive type of inhibition (Figure 14).

Discussion

Initial rate data obtained by other workers (Furfine and Velick, 1965; Keleti, 1965; Keleti and Batke, 1965; Fahien, 1966) showed that all reciprocal patterns between any given pair of reactants were intersecting, thus effectively ruling out a Ping-Pong or substitution-type mechanism. Consequently this discussion will deal only with possible mechanisms of the sequential type.

One of the important results obtained from this work was the large number of uncompetitive inhibitions produced by dead-end inhibitors. Of particular interest is 3-phosphoglyceric acid which was uncompetitive no matter what the nature of the variable substrate. Uncompetitive dead-end inhibition is characteristically produced by combination of the inhibitor with an enzyme form that occurs after combination of the variable substrate and before release of all the products (Cleland, 1970). The results with 3-phosphoglyceric acid suggest that it is combining after the release of the first product and that the products are probably released in an ordered manner. The first product is most likely to be RCOX (Scheme I) as 3-phosphoglyceric acid is a structural analog of this product rather than NADH and consequently would fit into the slot in the active site previously occupied by this compound. That NADH is the last product released is further indicated by the

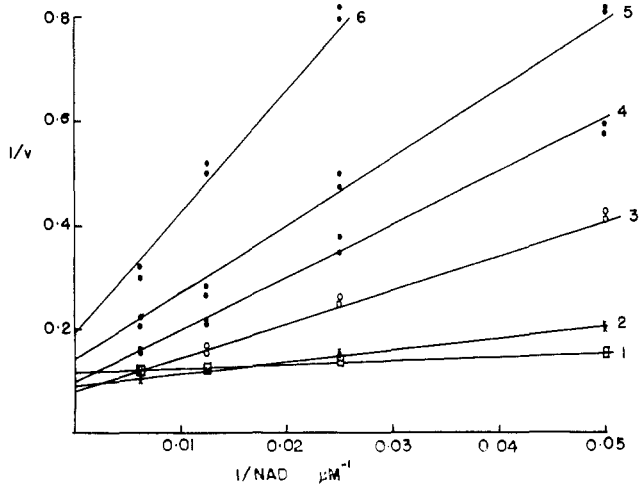


FIGURE 7: Reciprocal plots with NAD as the variable substrate and HPAP as the changing fixed substrate. HPAP concentrations: (1) 5 mM, (2) 3.31 mM, (3) 1.6 mM, (4) 1 mM, (5) 0.74 mM, (6) 0.5 mM. Arsenate concentration: 500 μM. Enzyme concentration: 27 μg/ml. Data fitted to eq 7.

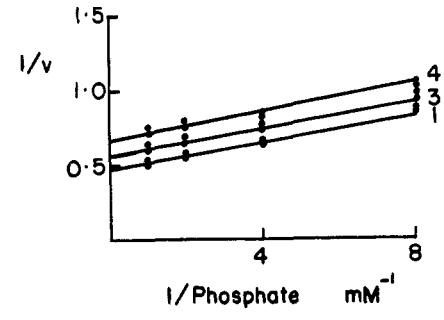


FIGURE 9: Reciprocal plots with phosphate as the variable substrate and 3-phosphoglyceric acid as the inhibitor. NAD concentration: 80 μM; HPAP concentration: 1 mM. 3-Phosphoglyceric acid concentrations: (1) 0, (3) 25 mM, (4) 50 mM. Enzyme concentration: 45 μg/ml. Data fitted to eq 5.

TABLE II: Inhibition Patterns and Constants.

Inhibitor	Substrates (mM)					Inhibn	Inhibn Constants (mM)	
	NAD	GAP	HPAP	Arsenate	Phosphate		K_{is}	K_{ii}
Nitrate	Var[a]		1.0	0.12		UC[b]		35.7 ± 1.5
Nitrate	0.08		Var	0.062		UC		8.7 ± 1.7
Nitrate	0.08		1.0	Var		C	8.1 ± 1.4	
α-Glycerophosphate	Var	0.5		0.062		UC		26.0 ± 1.2
α-Glycerophosphate	0.08	Var		0.062		NC	64 ± 14	82 ± 12
α-Glycerophosphate	0.08	0.5		Var		NC	42 ± 7	27.5 ± 2.2
3-Phosphoglycerate	Var		1.0	0.062		UC		21.5 ± 0.8
3-Phosphoglycerate	0.08		Var	0.062		UC		15.9 ± 1.9
3-Phosphoglycerate	0.08		1.0	Var		UC		20.0 ± 1.0
3-Phosphoglycerate	0.08		1.0		Var	UC		119 ± 8

[a] This indicates the variable substrate. [b] UC, uncompetitive inhibition and data fitted to eq 5; C, competitive inhibition and data fitted to eq 4; NC, noncompetitive inhibition and data fitted to eq 6.

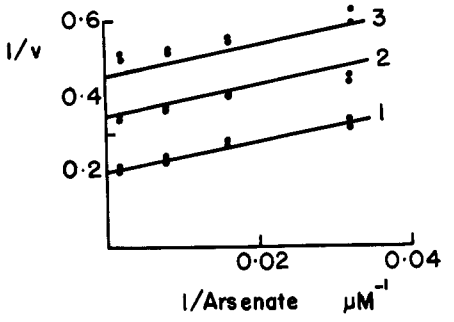
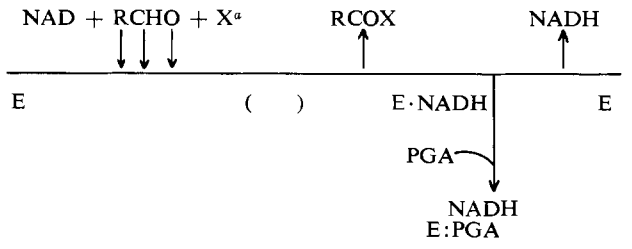


FIGURE 10: Reciprocal plots with arsenate as the variable substrate and 3-phosphoglyceric acid as the inhibitor. NAD concentration: 80 μM; HPAP concentration: 1 mM. 3-Phosphoglyceric acid concentrations: (1) 0, (2) 25 mM, (3) 50 mM. Enzyme concentration: 28 μg/ml. Data fitted to eq 5.

SCHEME I



[a] X = arsenate or phosphate.

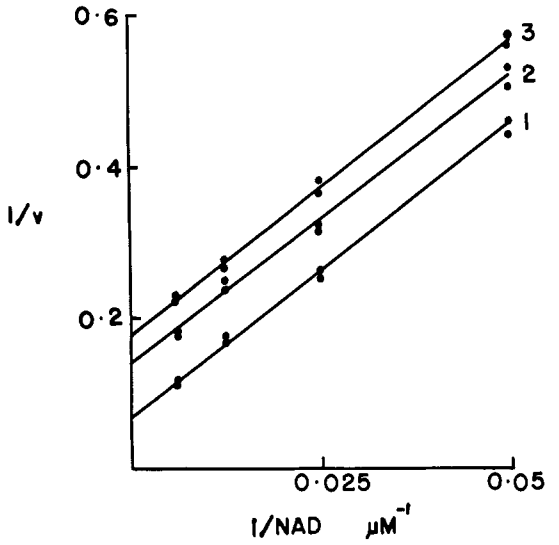


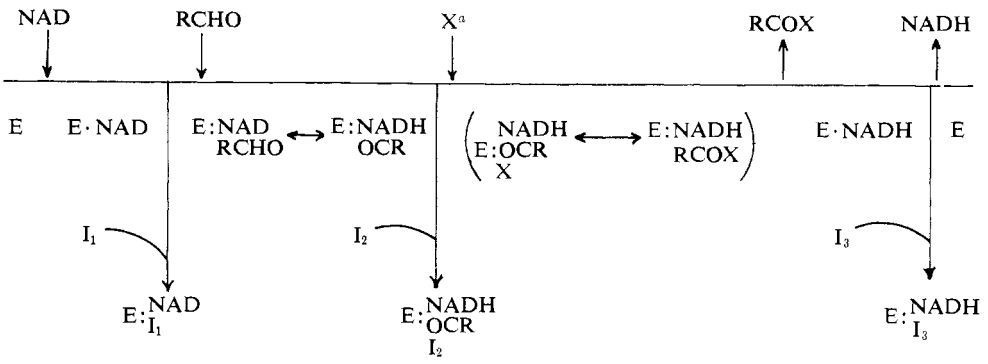
FIGURE 11: Reciprocal plots with NAD as the variable substrate and α-glycerophosphate as the inhibitor. GAP concentration: 0.5 mM; arsenate concentration: 62 μM. α-Glycerophosphate concentrations: (1) 0, (2) 25 mM, (3) 40 mM. Enzyme concentration: 15 μg/ml. Data fitted to eq 5.

strictly competitive effect between NADH as a product inhibitor and NAD as the substrate.

One further important observation was the increase in apparent K_{ii} for 3-phosphoglyceric acid when phosphate was substituted for arsenate as the variable substrate. This six-fold increase (Table II) matches closely the decrease in V_{max} on going from arsenate to phosphate with HPAP as the variable substrate (Table I). This is indicative that with GAP and either arsenate or phosphate, or with HPAP and arsenate, the rate-limiting step is largely the release of the last product, NADH. With HPAP and arsenate another step is equally rate limiting, since V is half that with GAP, but the steady-state level of E·NADH is still large. With HPAP and phosphate, or with other aldehydes in either system the rate-limiting step now becomes either the release of RCOX or some reaction involved in the intermolecular conversions of the central complexes, resulting in a low steady-state concentration of the E·NADH complex. This interpretation is strongly supported by the observation that substrate inhibition occurs with HPAP only in the arsenate system, although inhibition is the same in either phosphate or arsenate with GAP as the variable substrate. These results indicate that GAP or HPAP, when acting as a substrate inhibitor, combine with E·NADH in a similar manner to 3-phosphoglyceric acid. If this is the case and the release of products is ordered, then the substrate inhibition should be of the uncompetitive type, which is what was observed with HPAP in arsenate.

Assuming that the release of products is ordered and that

SCHEME II



[a] X = arsenate or phosphate; I_1 = α-glycerophosphate; I_2 = nitrate; I_3 = 3-phosphoglyceric acid, α-glycerophosphate, GAP, HPAP.

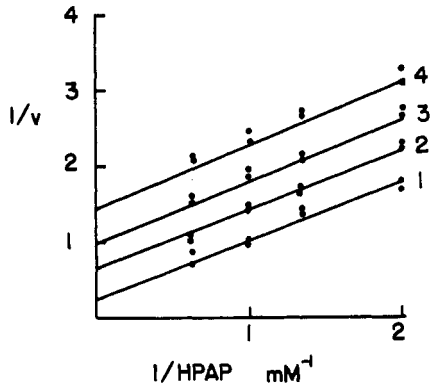


FIGURE 12: Reciprocal plots with HPAP as the variable substrate and nitrate as the inhibitor. NAD concentration: 80 μM; arsenate concentration: 62 μM. Nitrate concentrations: (1) 0, (2) 12.95 mM, (3) 25.9 mM, (4) 41 mM. Enzyme concentration: 54 μg/ml. Data fitted to eq 5.

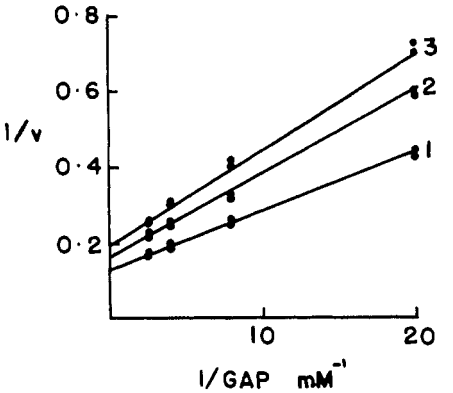


FIGURE 14: Reciprocal plots with GAP as the variable substrate and α-glycerophosphate as the inhibitor. NAD concentration: 80 μM; arsenate concentration: 62 μM. α-Glycerophosphate concentrations: (1) 0, (2) 25 mM, (3) 40 mM. Enzyme concentration: 18 μg/ml. Data fitted to eq 6.

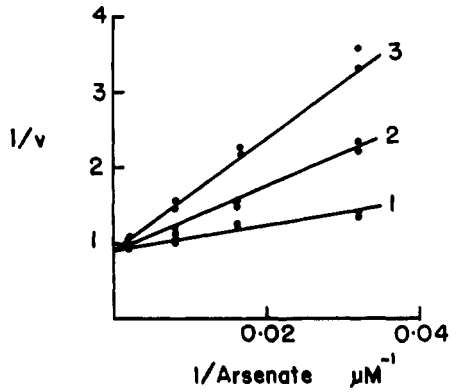


FIGURE 13: Reciprocal plots with arsenate as the variable substrate and nitrate as the inhibitor. NAD concentration: 80 μM; HPAP concentration: 1 mM. Nitrate concentrations: (1) 0, (2) 12.95 mM, (3) 25.9 mM. Enzyme concentration: 7 μg/ml. Data fitted to eq 4.

NADH is the last product to leave then a reasonable first assumption about the addition of substrates, in analogy with other NAD hydrogenases (Theorell and Chance, 1951; Raval and Wolfe, 1962; Dalziel, 1963; Dalziel and Dickinson, 1965; Wratten and Cleland, 1965; Hsu *et al.*, 1967) is that these are also ordered with NAD as the first substrate. The product inhibition patterns given by NADH are of no help as the observed patterns can be given not only by the ordered mechanism but also by the rapid equilibrium random mechanism.

In addition to the rapid equilibrium random and ordered mechanisms ter-reactant enzymes may show a number of other possible mechanisms all of the partial random type. These have been considered in detail by Fromm (1967) and Dalziel (1969). Very briefly these may be described as (a) the random addition of the first two substrates with the ordered addition of the third substrate (partial random AB), (b) the compulsory addition of the first substrate followed by the random addition of the second two substrates (partial random BC), and (c) the random addition of the first and third substrates but with the compulsory addition of the second substrate (partial random AC). If equilibrium conditions are assumed then it is possible to distinguish all these five mechanisms by initial rate studies (Fromm, 1967). However Dalziel (1969) in his excellent theoretical treatment of ter-reactant enzymes gives sound kinetic reasons for considering partial random mechanisms AB and AC very unlikely. He states that only partial random BC is reasonable and the equation given is based on the assumption that only the quaternary complex (EABC) is in equilibrium (this equation contains an extra BC term in the denominator when compared to the equation derived by Fromm (1967) which is based on equilibrium conditions for EA as well as EABC). The three most likely sequential mechanisms—ordered, rapid equilibrium random, and partial

random BC—may still be distinguished by initial rate studies and dead-end inhibitors.

Consider first the inhibition patterns produced by inorganic nitrate. Its purely competitive effect with arsenate and the uncompetitive inhibition *vs.* either NAD or HPAP indicates that arsenate adds as the compulsory third substrate. This inhibition pattern effectively rules out the rapid equilibrium random and partial random AC, both of which would give one competitive and two noncompetitive patterns. Also the partial random BC is ruled out, as the inhibition patterns here would be one competitive, one noncompetitive, and one uncompetitive pattern. This leaves only the ordered mechanism and, as a very unlikely possibility, partial random AB. The uncompetitive inhibition against NAD and the noncompetitive inhibitions against the other two substrates by α-glycerophosphate indicate that NAD is the compulsory first substrate. If it is assumed that α-glycerophosphate is competing with the aldehyde substrate for the active center of the enzyme, then the absence of a competitive inhibition pattern may be explained by considering that α-glycerophosphate not only binds to E·NAD but also to E·NADH, thus producing a noncompetitive pattern. Such an explanation is reasonable since the aldehyde substrates can bind to E·NADH. This interpretation effectively rules out a partial random AB mechanism leaving only an ordered mechanism for serious consideration.

Scheme II shows a proposed kinetic mechanism for glyceraldehyde-3-phosphate dehydrogenase based on the results reported in this paper. Included in this scheme are the partial reactions involving acyl-enzyme formation which appear to be important in the molecular mechanism.

References

Boyer, P. D., and Segal, H. L. (1954), *in* Mechanism of Enzyme Action, McElroy, W. D., and Glass, B., Ed., Baltimore, Md., John Hopkins Press, p 250.

Cleland, W. W. (1963a), *Biochim. Biophys. Acta 67*, 104.

Cleland, W. W. (1963b), *Nature (London) 198*, 463.

Cleland, W. W. (1967), *Advan. Enzymol. 29*, 1.

Cleland, W. W. (1970), *Enzymes 2*, 1.

Dalziel, K. (1963), *J. Biol. Chem. 238*, 2850.

Dalziel, K. (1969), *Biochem. J. 114*, 547.

Dalziel, K., and Dickinson, F. M. (1965), *Nature (London) 206*, 255.

Fahien, L. A. (1966), *J. Biol. Chem. 241*, 4115.

Frieden, C. (1959), *J. Biol. Chem. 234*, 2891.

Fromm, H. J. (1967), *Biochim. Biophys. Acta 139*, 221.

Furfine, C. S., and Velick, S. F. (1965), *J. Biol. Chem. 240*, 844.

Harting, J., and Velick, S. F. (1954), *J. Biol. Chem. 207*, 867.

Heilbron, I., and Bunbury, H. M. (1953), Dictionary of Organic Compounds, Vol. II, Oxford, University Press, p 816.

Hsu, R. Y., Lardy, H. A., and Cleland, W. W. (1967), *J. Biol. Chem. 242*, 5313.

Keleti, T. (1965), *Acta Physiol. Hung. 48*, 19.

Keleti, T., and Batke, J. (1965), *Acta Physiol. Hung. 48*, 195.

Krimsky, I., and Racker, E. (1963), *Biochemistry 2*, 512.

Neff, U. (1904), *Ann. 335*, 219.

Rabjohn, N. (1963), Organic Syntheses, Collect. Vol. IV, New York, N. Y., Wiley, p 21.

Raval, D. N., and Wolfe, R. G. (1962), *Biochemistry 1*, 263.

Segal, H. L., and Boyer, P. D. (1953), *J. Biol. Chem. 204*, 265.

Tener, G. M. (1961), *J. Amer. Chem. Soc. 83*, 159.

Theorell, H., and Chance, B. (1951), *Acta Chem. Scand. 5*, 1127.

Velick, S. F. (1954), *in* Mechanism of Enzyme Action, McElroy, W. D., and Glass, B., Ed., Baltimore, Md., John Hopkins Press, p 491.

Velick, S. F., and Furfine, C. (1963), *Enzymes 7*, 243.

Velick, S. F., and Hayes, J. E., Jr. (1953), *J. Biol. Chem. 203*, 545.

Wilkinson, G. N. (1961), *Biochem. J. 80*, 324.

Wratten, C. C., and Cleland, W. W. (1965), *Biochemistry 4*, 2442.