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Nicotinamide Adenine Dinucleotide-specific Glyceraldehyde 3-Phosphate Dehydrogenase from *Pisum sativum*

ASSAY AND STEADY STATE KINETICS*

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SUMMARY

The assay for NAD^+-specific glyceraldehyde 3-phosphate dehydrogenase (EC 1.2.1.12) was critically examined in order to obtain reliable kinetic data. Product inhibition by 1,3-diphosphoglyceric acid was relieved by the inclusion of ADP, magnesium chloride, and 3-phosphoglyceric acid kinase in the assay. Reactions were initiated with glyceraldehyde 3-phosphate since this substrate was found to be unstable under assay conditions. Rate measurements were made within 20 s of the addition of this substrate.

Initial velocity and product inhibition studies indicated two possible Bi Uni Uni Uni Ping Pong kinetic mechanisms, and one of these (NAD^+ on, phosphate on, 1,3-diphosphoglyceric acid off, glyceraldehyde 3-phosphate on, NADH off) was chosen on the basis of published studies of the enzyme which have indicated that a stable acyl enzyme is an obligatory intermediate in the reaction. The proposed mechanism predicts the existence of a stable enzyme-NAD^+ complex, and this form is well known.

The mechanism agrees with many of the published observations on the enzyme but differs from those indicated by previous kinetic studies. It is suggested that these workers have not fully considered the problems associated with the assay, and that this can account for these discrepancies.

There have been several kinetic studies of glyceraldehyde 3-phosphate dehydrogenase from rabbit muscle (1–3), pig muscle (4–7) and human erythrocytes (8). On the basis of these studies, a variety of mechanisms have been proposed. There are several discrepancies between the data obtained by different workers. For instance, NADH inhibition has been variously reported as competitive (1) or noncompetitive (3), with respect to the aldehyde substrate, and as linear (1, 3) or parabolic (7). However, a common feature of these mechanisms is that they are all sequential (9). In some cases, this feature of the mechanism is an implicit (4, 5, 8) or explicit (3) assumption, and the data may be explained in other ways if this assumption is abandoned.

In contrast to these kinetic studies, studies of the partial (10–12) and minor (13, 14) activities of the enzyme have invariably suggested that NADH release occurs before the binding of one of the substrates, *i.e.* the mechanisms are ping-pong. Furthermore, if the mechanism is sequential, it would be predicted that the only stable enzyme form which could be isolated would be the free enzyme. Studies using stoichiometric quantities of the enzyme (10, 15–19) have unequivocally demonstrated the existence of a stable acyl enzyme and an enzyme-NAD^+ complex. Attempts have been made to reconcile these differences (1, 3), but the explanations offered are not entirely satisfactory. It was therefore decided to reexamine the kinetics of glyceraldehyde 3-phosphate dehydrogenase, using the recently purified enzyme from pea seeds (20). This enzyme appears to be essentially identical to glyceraldehyde 3-phosphate dehydrogenase from other sources. Particular attention was paid to the validity of the assay method in order to obtain reliable kinetic data.

MATERIALS AND METHODS

Materials—Glyceraldehyde 3-phosphate dehydrogenase was purified from pea seeds as previously described (20). Other enzymes, biochemicals, and buffers were purchased from Sigma Chemical Co.; other chemicals were of the highest grade commercially available.

Assay—The assay will be described in some detail, since we believe that it is only by the use of this assay that reliable kinetic data can be obtained. Glyceraldehyde 3-phosphate dehydrogenase was assayed at 25° by following NADH production at 340 nm, using a Gilford-modified Beckman DU spectrophotometer, and reaction rates are reported as the change in absorbance per min. The assay mixture contained NAD^+, glyceraldehyde 3-phosphate, potassium phosphate and NADH as indicated in the text and figure legends, 3.3 mM cysteine hydrochloride, 0.2 mM EDTA, 0.2 mM magnesium chloride, 0.5 mM ADP, 1.7 units[1] per ml of yeast 3-phosphoglyceric acid kinase (Sigma type I-C or type IV), and 0.02 to 0.1 units per ml of glyceraldehyde 3-phosphate dehydrogenase in 135 mM Tris-chloride buffer, pH 8.79. The final volume was 3 ml.

All components, except NAD^+, ADP, 3-phosphoglyceric acid

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[1] In all cases, the unit of enzyme activity is the amount which will catalyze the formation of 1 μmole of product per min.

kinase, glyceraldehyde 3-phosphate dehydrogenase, and 3-phosphoglyceric acid, were pipetted into a 10-mm light path cuvette, then the missing components were added in the order given, allowing 1 to 3 min between the addition of glyceraldehyde 3-phosphate dehydrogenase and glyceraldehyde 3-phosphate. The addition of glyceraldehyde 3-phosphate initiates the reaction, and rate determinations were made over the period 5 to 20 s after the addition of this substrate. Glyceraldehyde 3-phosphate dehydrogenase was diluted to 0.1 to 0.2 mg per ml with 1.5 mM EDTA in 10 mM potassium phosphate buffer, pH 7.2, on the day of use. Corrections were applied for the slow (approximately 2% per hour) loss of activity from this diluted enzyme.

Previously determined amounts of 40 mM hydrochloric acid were added to ensure that the pH of the reaction mixture was 8.79. This is particularly important when the concentration of glyceraldehyde 3-phosphate is varied since this substrate is added as the free acid, pH 2.5. The phosphate concentrations reported are corrected for phosphate contamination in glyceraldehyde 3-phosphate, ADP, 3-phosphoglyceric acid kinase, and glyceraldehyde 3-phosphate dehydrogenase, as determined by the method of Fiske and SubbaRow (21). It should be noted that there is sufficient ADP and EDTA in this assay to ensure that there is no free magnesium in the assay which might otherwise form a complex with, and thereby reduce the concentration of, phosphate. Using this assay, there is a linear relationship between the rate of reaction and glyceraldehyde 3-phosphate dehydrogenase over the range employed.

Preparation and Assay of Glyceraldehyde 3-Phosphate—Glyceraldehyde 3-phosphate was obtained as barium diethylacetal-DL-glyceraldehyde 3-phosphate and converted to the free acid and assayed enzymically as described by the manufacturer (Sigma), except that 4 units of rabbit muscle glyceraldehyde 3-phosphate dehydrogenase were added to the assay without prior dilution. This enzyme assay method underestimates the concentration of the active (D) isomer by 9% (see "Results and Discussion"). The free acid could be stored as a frozen solution (pH 2.5) for several days without detectable decomposition. After thawing and use, solutions of glyceraldehyde 3-phosphate were discarded. The concentration of glyceraldehyde 3-phosphate is expressed as the concentration of the active isomer, as determined by the enzymic method.

The stability of glyceraldehyde 3-phosphate in 200 mM Tris-chloride buffer was determined by incubation under the conditions specified in the text at 25°. Samples were withdrawn at intervals, and the residual glyceraldehyde 3-phosphate was estimated as above. If glyceraldehyde 3-phosphate has undergone extensive decomposition, a plateau of absorbance is not reached in this assay. The change in absorbance after 5 min was routinely used as an estimate of glyceraldehyde 3-phosphate.

Data Analysis—The data of Figs. 3 to 9 were analyzed using a computer program for a nonlinear regression, assuming constant variance of velocities in each experiment and no error in concentrations of the substrates and NADH. The data of Figs. 3, 4, and 5 were fitted to the equations:

$$v = \frac{V_{max}}{1 + \frac{K_A}{[A]} + \frac{K_B}{[B]} + \frac{K_{AB}}{[A][B]}} \quad (1)$$

$$v = \frac{V_{max}}{1 + \frac{K_A}{[A]} + \frac{K_B}{[B]}} \quad (2)$$

where A and B are the varied substrates, K_A and K_B are their respective limiting Michaelis constants, V_{max} is the maximum velocity, and K_{AB} is an interaction term.

The data of Figs. 6 to 9 were fitted to the equations:

$$v = \frac{V'_{max}}{1 + \frac{K'_A}{[A]} + \frac{K'_A \cdot [I]}{[A] \cdot K_{IS}} + \frac{[I]}{K_{II}}} \quad (3)$$

$$v = \frac{V'_{max}}{1 + \frac{K'_A}{[A]} + \frac{K'_A \cdot [I]}{[A] \cdot K_{IS}}} \quad (4)$$

where A is the variable substrate, K'_A and V'_{max} are the apparent Michaelis constant and maximum velocity, respectively, I is NADH, and K_{IS} and K_{II} are the slope and intercept inhibition constants, respectively.

The significance of the K_{AB} term and the K_{II} term was evaluated using a variance ratio (F) test, using the equations:

$$F = \frac{RSS_r - RSS_c}{RSS_c(n - p)} \quad (5)$$

where RSS_r and RSS_c are the residual sums of squares (*i.e.* $\sum_{i=1}^{n} (\bar{v}_i - v_i)^2$, where $\bar{v}_i$ is the predicted velocity and v_i is the actual velocity of the ith assay) for the reduced (Equation 2 or 4) and the complete (Equation 1 or 3) models, respectively, n is the number of assays in a particular experiment, and p is 4, the number of parameters in the complete models. The reduced model was selected if the complete model did not give a significantly better fit to the data.

The lines in Figs. 3 to 9 were drawn according to the best estimates of the parameters in the model chosen. The line in Fig. 10 was drawn according to the parameters estimated from an unweighted least squares regression of the transformed data to the equation:

$$v = V_{max} - \frac{v \cdot [I]}{K_I} \quad (6)$$

in accordance with the recommendations of Dowd and Rigg (22). Other lines were fitted to the data by eye.

RESULTS AND DISCUSSION

Validity of the Assay—In any kinetic study, it is essential to establish a suitable assay procedure in order that the reaction rates measured may represent a good approximation to the true initial rate. Using a fairly simple assay mixture, the reaction rate of glyceraldehyde 3-phosphate dehydrogenase was found to decrease markedly with time (Fig. 1, *Curve A*). In part, this is due to product inhibition by 1,3-diphosphoglyceric acid since, in the presence of ADP, magnesium chloride and 3-phosphoglyceric acid kinase, a linear rate is maintained for a longer period of time (Fig. 1, *Curve B*). Replacing phosphate with arsenate, a procedure used by many workers on the assumption that the product (1-arseno-3-phosphoglyceric acid) is rapidly hydrolyzed, was found to aggravate the nonlinearity of the assay (Fig. 1, *Curve C*). Teipel and Koshland (23) have suggested that 1-arseno-3-phosphoglyceric acid is not rapidly hydrolyzed and is a potent inhibitor of the rabbit muscle enzyme. All kinetic experiments were therefore performed in the presence of the 3-phosphoglyceric acid kinase system so that the concentration of 1,3-diphosphoglyceric acid was reduced to very low levels. Other workers have not taken this precaution and this

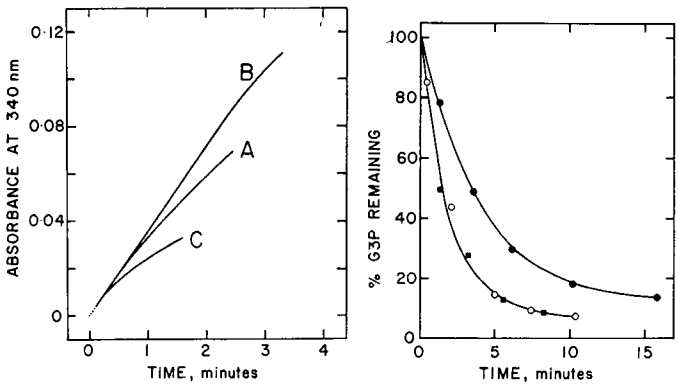


FIG. 1 (*left*). Time course of the assay for glyceraldehyde 3-phosphate dehydrogenase. Assays were performed as described by Duggleby and Dennis (20), using 0.017 unit of pea seed glyceraldehyde 3-phosphate dehydrogenase per assay. Modifications to this assay were: none (*Curve A*); addition of 1 mM ADP, 5 mM magnesium chloride, and 5 units of yeast 3-phosphoglyceric acid kinase (*Curve B*); or replacing phosphate with 17 mM sodium arsenate (*Curve C*). The curves are redrawn from recorder tracings.

FIG. 2 (*right*). Stability of glyceraldehyde 3-phosphate (*G3P*) in solution. Incubations and assays were performed as described under "Materials and Methods." The conditions of the incubations were: pH 8.90 (○), pH 8.20 (●), or pH 8.90 in the presence of 3.3 mM cysteine (■). The data are expressed as a per cent of the glyceraldehyde 3-phosphate concentration at zero time (1.22 mM).

may have introduced artefacts into the kinetic patterns that they observe.

The nonlinearity is more pronounced when low concentrations of glyceraldehyde 3-phosphate are used. We believe that the reason for this is that glyceraldehyde 3-phosphate is unstable in solution, for the following reasons.

1. When glyceraldehyde 3-phosphate is preincubated in the assay and the reaction initiated with enzyme, the initial rate is dependent on the preincubation time; thus, preincubation of assay mixtures containing 1 mM glyceraldehyde 3-phosphate for 0.0, 1.1, 3.0, and 5.1 min gave initial rates of 0.0974, 0.0934, 0.0596, and 0.0309 absorbance unit per min, respectively, when the enzyme was added. These rates are similar to the rates observed in a single assay where there has been no preincubation, and tangents are drawn to the curve over the same time period. In this case the rates are 0.0974, 0.0865, 0.0562, and 0.0276 absorbance unit per min.

2. If a reaction is run for 15 min, the rate approaches zero, although the change in absorbance indicates that only a small percentage of the substrates could have reacted. Addition of glyceraldehyde 3-phosphate dehydrogenase, NAD^+, or phosphate has no effect, but addition of glyceraldehyde 3-phosphate restarts the reaction.

3. Assays are more linear when high enzyme concentrations are employed, indicating that in these faster reactions, glyceraldehyde 3-phosphate has less time in which to break down.

When the degradation of glyceraldehyde 3-phosphate is examined directly, a rapid breakdown of glyceraldehyde 3-phosphate is observed (Fig. 2), the rate of which is pH-dependent. The rate of breakdown is unaffected by the presence of cysteine, so the formation of a hemimercaptal (24) would not appear to be involved. There appears to be no hydrolysis of the phosphate moiety of glyceraldehyde 3-phosphate, and no nonenzymic oxidation to 3-phosphoglyceric acid.

Trentham *et al.* (25), and later workers (26, 27) have described

TABLE I

Effect of glyceraldehyde 3-phosphate dehydrogenase concentration on the estimation of glyceraldehyde 3-phosphate

Estimations were performed as described under "Materials and Methods," except for the concentration of glyceraldehyde 3-phosphate dehydrogenase, which was as indicated. The routinely used amount is 4 units, which gives an estimate of glyceraldehyde 3-phosphate which is 9% lower than that obtained by extrapolation to infinite enzyme concentration. The same solution contained 23.48 mM alkali-labile phosphate. Assuming that this is all derived from glyceraldehyde 3-phosphate, and that the glyceraldehyde 3-phosphate is 50% D isomer, the solution contains 11.74 mM D-glyceraldehyde 3-phosphate.

Enzyme added	Estimated D-glyceraldehyde 3-phosphate concentration
units	*mM*
1	9.39
4	10.75
10	11.31
15	11.42
20	11.51
Infinite	11.70–11.80[a]

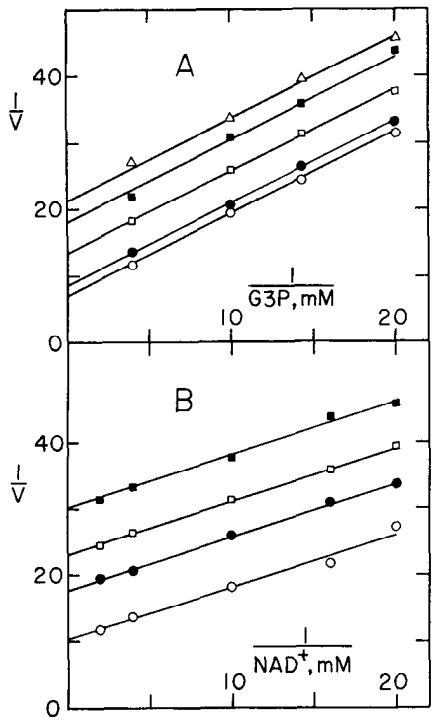
[a] Extrapolated from a plot of [glyceraldehyde 3-phosphate] against 1/[enzyme].

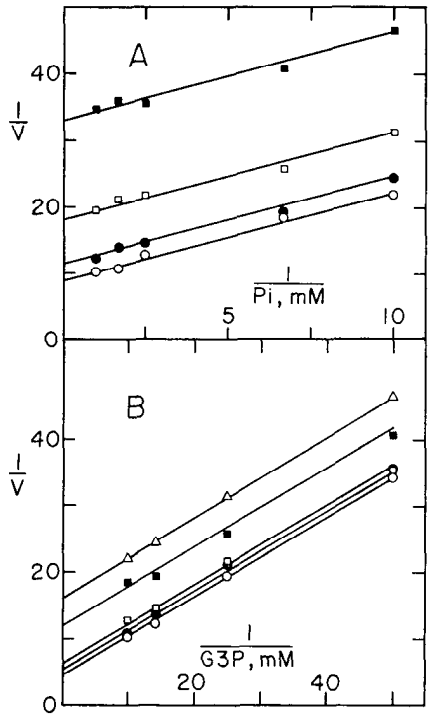
the hydration of the active aldehyde to the inactive *gem*-diol, but the rate of this reaction is considerably greater than the phenomenon described above. Assays for glyceraldehyde 3-phosphate do not reach a plateau of absorbance if glyceraldehyde 3-phosphate has broken down extensively, indicating a reconversion of the breakdown product to glyceraldehyde 3-phosphate. This reaction is much slower than the diol to aldehyde conversion (25) and is unaffected by the addition of beef erythrocyte carbonic anhydrase, which will catalyze the diol to aldehyde interconversion (28, 29).

Aldehydes are known to undergo various oligomer- and polymerizations, and it has been shown that glyceraldehyde and related compounds can undergo these reactions under quite mild conditions (30). The pH dependence of the phenomenon which we have described suggests that glyceraldehyde 3-phosphate may undergo these reactions under assay conditions. The problem may be circumvented by assaying at a pH value near neutrality, but this is far from the pH optimum of the enzyme (20). Therefore, assays were initiated with glyceraldehyde 3-phosphate, and reaction rates were measured between 5 and 20 s, before glyceraldehyde 3-phosphate has the opportunity to undergo significant breakdown. This procedure, when used in conjunction with the 3-phosphoglyceric acid kinase system gives a good estimate of the true initial rate.

One consequence of the instability of glyceraldehyde 3-phosphate is that the enzymic estimation method tends to underestimate the concentration of this substrate. If the glyceraldehyde 3-phosphate dehydrogenase concentration in this assay is varied, the estimate of glyceraldehyde 3-phosphate also varies and extrapolates to a value 9% higher than the estimate at the recommended concentration of this enzyme (Table I). This extrapolated value agrees well with the glyceraldehyde 3-phosphate concentration estimated as alkali-labile phosphate.

Initial Velocity Studies—Kinetic patterns were determined, varying each substrate (the variable substrate) at several fixed concentrations of a second substrate (the changing fixed substrate), and at a constant concentration of the third substrate (the constant substrate). The nomenclature used here is that





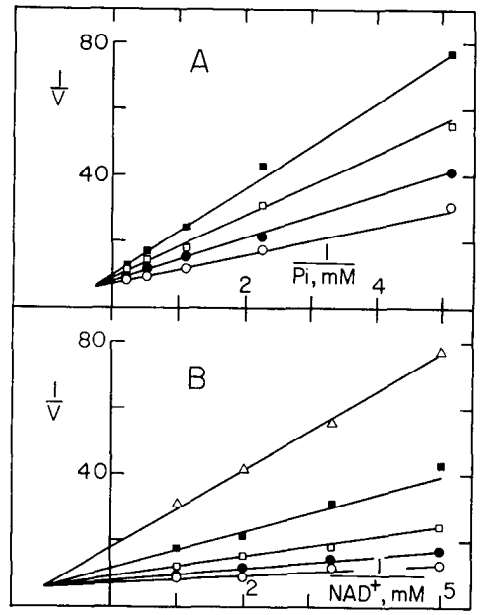


FIG. 3 (*left*). Kinetics of pea seed glyceraldehyde 3-phosphate dehydrogenase with respect to glyceraldehyde 3-phosphate (*G3P*) and NAD^+. Assays were performed as described under "Materials and Methods," using 10 mM phosphate and 0.7 μg of purified enzyme per assay. In *A*, glyceraldehyde 3-phosphate was varied as indicated, at fixed NAD^+ concentrations of 0.5 mM (○), 0.25 mM (●), 0.1 mM (□), 0.0625 mM (■), and 0.05 mM (△). In *B*, NAD^+ was varied as indicated, at fixed glyceraldehyde 3-phosphate concentrations of 0.25 mM (○), 0.1 mM (●), 0.07 mM (□), and 0.05 mM (■). The data are plotted in double reciprocal form.

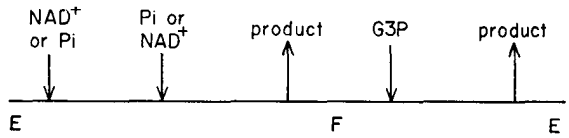
FIG. 4 (*center*). Kinetics of pea seed glyceraldehyde 3-phosphate with respect to phosphate and glyceraldehyde 3-phosphate. Assays were performed as described under "Materials and Methods," using 2.0 mM NAD^+ and 0.8 μg of purified enzyme per assay. In *A*, phosphate was varied as indicated, at fixed glyceraldehyde 3-phosphate concentrations of 0.1 mM (○), 0.07 mM (●), 0.04 mM (□) and 0.02 mM (■). In *B*, glyceraldehyde 3-phosphate was varied as indicated, at fixed phosphate concentrations of 1.0 mM (○), 0.6 mM (●), 0.4 mM (□), 0.15 mM (■), and 0.1 mM (△). The data are plotted in double reciprocal form.

FIG. 5 (*right*). Kinetics of pea seed glyceraldehyde 3-phosphate dehydrogenase with respect to phosphate and NAD^+. Assays were performed as described under "Materials and Methods," using 0.5 mM glyceraldehyde 3-phosphate and 0.7 μg of purified enzyme per assay. In *A*, phosphate was varied as indicated, at fixed NAD^+ concentrations of 1.0 mM (○), 0.5 mM (●), 0.3 mM (□), and 0.2 mM (■). In *B*, NAD^+ was varied as indicated, at fixed phosphate concentrations of 4.78 mM (○), 1.91 mM (●), 0.90 mM (□), 0.443 mM (■), and 0.195 mM (△). The data are plotted in double reciprocal form.

of Cleland (9). The range of concentrations over which the variable and changing fixed substrates can be varied is somewhat limited, as each substrate is inhibitory at high concentrations (20). This also limits the extent to which the constant substrate can be raised to a saturating concentration, and the kinetic constants determined in these experiments only approach the true limiting kinetic constants.

When phosphate is the constant substrate, NAD^+ is the changing fixed substrate and glyceraldehyde 3-phosphate is the variable substrate, a parallel pattern is obtained (Fig. 3*A*). Parallel patterns are also obtained when glyceraldehyde 3-phosphate is the changing fixed substrate and NAD^+ is the variable substrate (Fig. 3*B*); when glyceraldehyde 3-phosphate is the changing fixed substrate and phosphate is the variable substrate (Fig. 4*A*); and when phosphate is the changing fixed substrate and glyceraldehyde 3-phosphate is the variable substrate (Fig. 4*B*). However, when glyceraldehyde 3-phosphate is the constant substrate, NAD^+ and phosphate give intersecting patterns (Fig. 5, *A* and *B*). On the basis of these studies, a general mechanism may be proposed (see Mechanism 1).

No sequential ordered, or random mechanism will account both for the patterns observed and for the linear nature of the data.[2]



MECHANISM 1. *G3P*, glyceraldehyde 3-phosphate.

Product Inhibition Studies—Given the above tentative mechanism, product inhibition studies can be used to determine the order of addition of NAD^+ and phosphate and the order of release of products. Since 1,3-diphosphoglyceric acid is unstable, product inhibition studies were only performed with NADH.

When the concentrations of glyceraldehyde 3-phosphate and phosphate are moderately high, NADH inhibition is competitive with NAD^+ (Fig. 6). However, this could be due to the formation of a dead-end complex, with NADH combining with the NAD^+ site on the enzyme. This experiment was, therefore, repeated at low concentrations of glyceraldehyde 3-phosphate and phosphate in order that true product effects might be fully expressed. Again, the inhibition is found to be competitive with NAD^+ (Fig. 7). NADH inhibition is also competitive with

[2] A random BC mechanism will explain these data if it is assumed that the curvature of the data is undetectable. However, this mechanism will not explain the product inhibition studies to be described and will not be considered further.

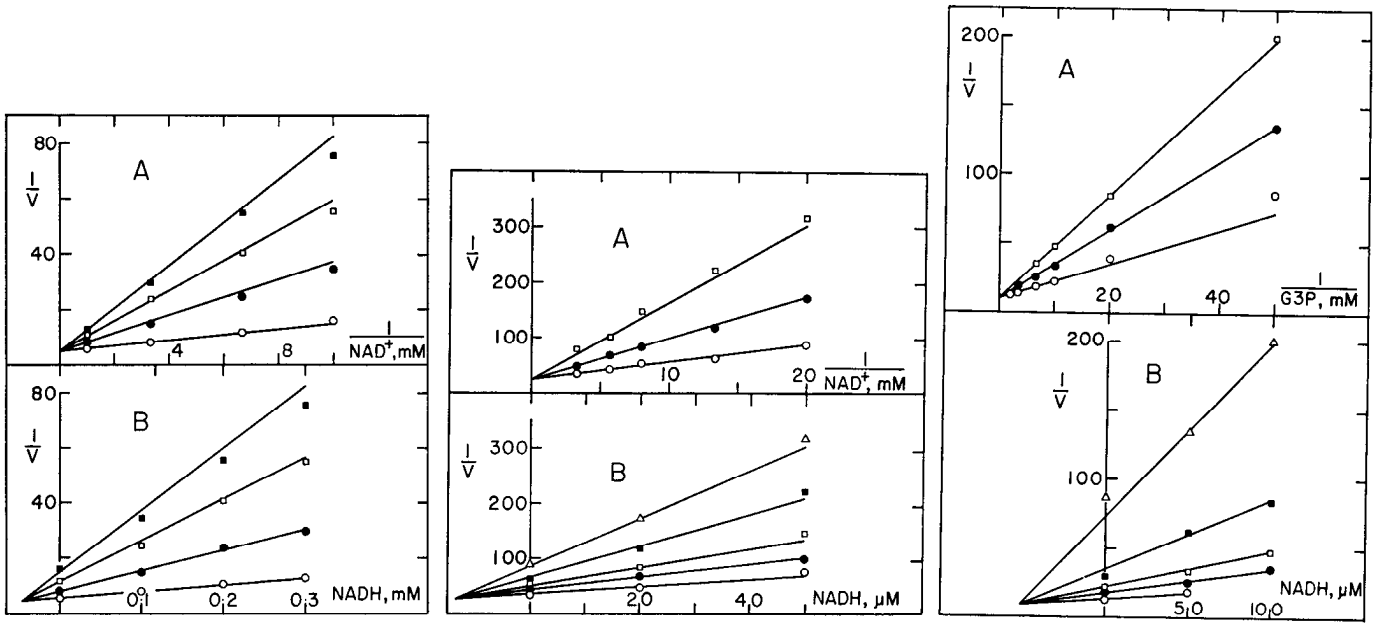


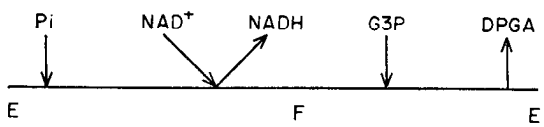
FIG. 6 (*left*). NADH inhibition of pea seed glyceraldehyde 3-phosphate dehydrogenase, with NAD^+ as the variable substrate, and at high concentrations of glyceraldehyde 3-phosphate and phosphate. Assays were performed as described under "Materials and Methods," using 1.0 mM glyceraldehyde 3-phosphate, 10 mM phosphate, and 0.75 μg of enzyme per assay. In *A*, NAD^+ was varied as indicated, at fixed NADH concentrations of 0.0 mM (○), 0.1 mM (●), 0.2 mM (□), and 0.3 mM (■). In *B*, NADH was varied as indicated, at fixed NAD^+ concentrations of 1.0 mM (○), 0.3 mM (●), 0.15 mM (□), and 0.1 mM (■). The data are plotted as $1/v$ against $1/[NAD^+]$ (*A*), and as $1/v$ against [NADH] (*B*).

FIG. 7 (*center*). NADH inhibition of pea seed glyceraldehyde 3-phosphate dehydrogenase, with NAD^+ as the variable substrate, and at low concentrations of glyceraldehyde 3-phosphate and phosphate. Assays were performed as described under "Materials and Methods," using 0.05 mM glyceraldehyde 3-phosphate, 0.24 mM phosphate, and 1.3 μg of purified enzyme per assay. In *A*, NAD^+ was varied as indicated, at fixed NADH concentrations of 0.00 mM (○), 0.02 mM (●), and 0.05 mM (□). In *B*, NADH was varied as indicated, at fixed NAD^+ concentrations of 0.3 mM (○), 0.175 mM (●), 0.125 mM (□), 0.075 mM (■), and 0.05 mM (△). The data are plotted as $1/v$ against $1/[NAD^+]$ (*A*), and as $1/v$ against [NADH] (*B*).

FIG. 8 (*right*). NADH inhibition of pea seed glyceraldehyde 3-phosphate (*G3P*) dehydrogenase, with glyceraldehyde 3-phosphate as the variable substrate. Assays were performed as described under "Materials and Methods," using 0.2 mM NAD^+, 10 mM phosphate, and 0.6 μg of purified enzyme per assay. In *A*, glyceraldehyde 3-phosphate was varied as indicated, at fixed NADH concentrations of 0.00 mM (○), 0.05 mM (●), and 0.10 mM (□). In *B*, NADH was varied as indicated, at fixed glyceraldehyde 3-phosphate concentrations of 0.3 mM (○), 0.15 mM (●), 0.10 mM (□), 0.05 mM (■), and 0.02 mM (△). The data are plotted as $1/v$ against 1/[glyceraldehyde 3-phosphate] (*A*), and as $1/v$ against [NADH] (*B*).

glyceraldehyde 3-phosphate (Fig. 8), and on the basis of these results, two mechanisms may be proposed[3]:

MECHANISM 2. *DPGA*, 1,3-diphosphoglyceric acid; *G3P*, glyceraldehyde 3-phosphate.



MECHANISM 3. *G3P*, glyceraldehyde 3-phosphate; *DPGA*, 1,3-diphosphoglyceric acid.

Mechanisms involving two Theorell-Chance displacements were not considered since derivation of the rate equation shows that such mechanisms predict that the rate of reaction is infinite at saturating concentrations of substrates and in the absence of products.

[3] The Theorell-Chance mechanism should not be taken as an actual displacement but it indicates that the intervening complexes are not detectable in the assay conditions employed.

Both Mechanisms 2 and 3 predict NADH inhibition to be noncompetitive with phosphate, and this was confirmed (Fig. 9). In all NADH inhibition experiments, plots of $1/v$ against [NADH] were straight lines (Figs. 6 to 9). No departure from linearity was observed over a wide range of NADH concentrations (Fig. 10). The kinetic constants for the models chosen in the initial velocity and product inhibition studies are given in Table II. The *F* values, which were used to select the appropriate model are given in Table III.

Several lines of evidence suggest that Mechanism 2 is correct, and these can be best understood when this mechanism is rewritten in cyclical form (Fig. 11). Both an enzyme-NAD^+ complex (IV) and an acyl enzyme (I) have been isolated as stable forms of the enzyme which is consistent with the proposed mechanism (Fig. 11). Mechanism 3, on the other hand, would require the existence of a reduced (*E*) and an oxidized (*F*) form, with the enzyme mediating in the hydrogen transfer from glyceraldehyde 3-phosphate to NADH. The evidence for direct hydride transfer is substantial (31–33) and this argues against Mechanism 3.

Trentham (34) has proposed a mechanism for glyceraldehyde 3-phosphate dehydrogenase from sturgeon and lobster which resembles Mechanism 2. However, he postulates a Theorell-Chance displacement between phosphate and 1,3-diphosphoglyceric acid, and intervening complexes between complexes IV and I. His mechanism, and Mechanism 2, are thus limiting

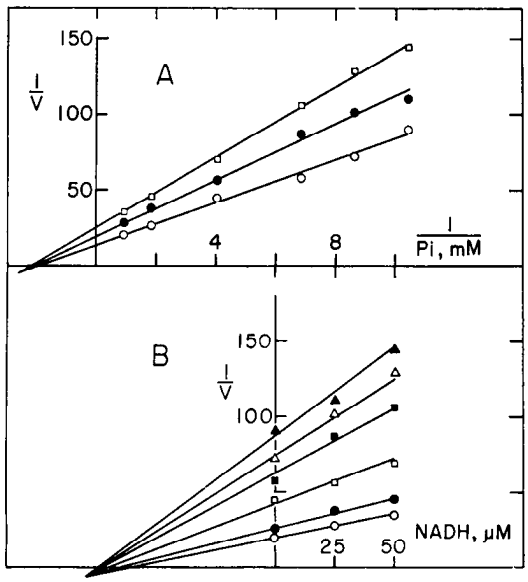


FIG. 9. NADH inhibition of pea seed glyceraldehyde 3-phosphate dehydrogenase, with phosphate as the variable substrate. Assays were performed as described under "Materials and Methods," using 0.2 mM NAD^+, 0.2 mM glyceraldehyde 3-phosphate, and 0.9 μg of purified enzyme per assay. In *A*, phosphate was varied as indicated, at fixed NADH concentrations of 0.00 mM (○), 0.025 mM (●), and 0.05 mM (□). In *B*, NADH was varied as indicated, at fixed phosphate concentrations of 1.05 mM (○), 0.546 mM (●), 0.246 mM (□), 0.146 mM (■), 0.116 mM (△), and 0.096 mM (▲). The data are plotted as $1/v$ against 1/[phosphate] (*A*), and as $1/v$ against [NADH] (*B*).

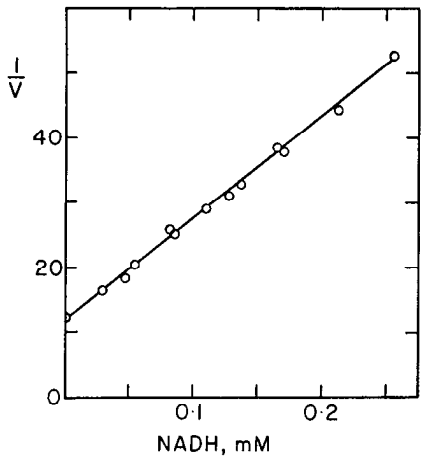


FIG. 10. NADH inhibition of pea seed glyceraldehyde 3-phosphate dehydrogenase, at low concentrations of all substrates. Assays were performed as described under "Materials and Methods," using 0.5 mM NAD^+, 0.1 mM glyceraldehyde 3-phosphate, 0.14 mM phosphate, and 1.3 μg of purified enzyme per assay. NADH was varied as indicated and is plotted against $1/v$.

cases of a general mechanism with no Theorell-Chance displacements. Fig. 11 shows the general mechanism with the glyceraldehyde 3-phosphate·*E*·NAD^+ (VI) and acyl·*E*·NADH (VII) complexes indicated in brackets. It is assumed that the general mechanism is correct but under the conditions of the assay this may degenerate into either Mechanism 2 or the mechanism proposed by Trentham.

The general mechanism would require that NADH inhibition was competitive with NAD^+ but noncompetitive with glyceraldehyde 3-phosphate. The results show that NADH is competitive with both. It can be shown that if the rates of conversion of

TABLE II

Kinetic constants of pea seed glyceraldehyde 3-phosphate dehydrogenase

The data of the experiments shown in Figs. 3 to 9 were fitted to the appropriate models as described under "Materials and Methods." The calculated values of the kinetics constants are shown, together with their standard deviations.

Substrates		Kinetic constants (standard deviation)			
A	B	$10^4 V_{max}$	K_A	K_B	$10^3 K_{AB}$
			μM	μM	mM^2
Glyceraldehyde 3-phosphate	NAD^+	1912 (61.2)	239 (11.1)	152 (7.15)	
Glyceraldehyde 3-phosphate	P_i	3518 (329)	212 (25.7)	470 (53.0)	
NAD^+	P_i	1471 (58.6)	97 (21.8)	322 (73.4)	318 (29.8)
Substrate	Remarks	$10^4 V_{max}'$	K'_A	K_{IS}	K_{II}
			μM	μM	μM
NAD^+	High substrates	2063 (42.6)	209 (11.7)	45.2 (2.24)	
NAD^+	Low substrates	409 (23.1)	130 (16.3)	14.7 (1.41)	
Glyceraldehyde 3-phosphate		1015 (41.7)	128 (13.9)	50.2 (6.63)	
P_i		740 (22.2)	527 (31.6)	77.5 (12.2)	60.9 (7.26)

Complex VI to Complex IV and of Complex VII to Complex I are rapid in comparison with the rates of combination of Complexes IV and I with glyceraldehyde 3-phosphate and NADH, respectively, and furthermore, if the interconversion of Complexes VI and VII is rapid and the rate of conversion of Complex VI to Complex VII is not large in comparison to the rate of conversion of Complex VII to Complex VI, then NADH inhibition will be competitive with glyceraldehyde 3-phosphate.

Trentham (34) detected these intermediate complexes using a stopped flow technique. He also discusses the problem of competitive inhibition between glyceraldehyde 3-phosphate and NADH and suggests that if glyceraldehyde 3-phosphate is competing with NADH by way of rapidly equilibrating intermediates, then there will be competitive inhibition between this substrate and product. The fact that the techniques of stopped flow analysis and enzyme kinetics have indicated a similar general mechanism would suggest that this mechanism may be operating and that the Theorell-Chance displacements in both the mechanism proposed here and in Trentham's mechanism are a function of the assay conditions.

Several studies using stoichiometric quantities of glyceraldehyde 3-phosphate dehydrogenase have demonstrated reactions which may be interpreted in terms of Mechanism 2. Thus, the enzyme may be acylated with glyceraldehyde 3-phosphate (11, 35, 36):

$$IV \rightarrow VI \rightarrow VII \rightarrow I$$

or with 1,3-diphosphoglyceric acid (19):

$$IV \rightarrow III \rightarrow II \rightarrow I$$

TABLE III

Variance ratio test of the kinetic models for pea seed glyceraldehyde 3-phosphate dehydrogenase

The residual sum of squares for each model was calculated as described under "Materials and Methods," and an F value was calculated. The appropriate model was then chosen from a comparison of this value with F from statistical tables.

Experiment	$10^8\ RSS_c$	$10^8\ RSS_r$	F			Model chosen
			Actual	(Degrees of freedom)	From tables $\alpha = 0.99$	
NAD^+/glyceraldehyde 3-phosphate	1412.4	1425.1	0.144	(1, 16)	8.53	Reduced
NAD^+/P_i	12145	94319	108	(1, 16)	8.53	Complete
Glyceraldehyde 3-phosphate/P_i	4817.4	5050.8	0.775	(1, 16)	8.53	Reduced
NAD^+/NADH (low substrate)	802.51	802.55	0.000548	(1, 11)	9.65	Reduced
NAD^+/NADH (high substrate)	4571.9	8609.6	10.6	(1, 12)	9.33	Reduced[a]
Glyceraldehyde 3-phosphate/NADH	6065.2	6692.7	1.14	(1, 11)	9.65	Reduced
P_i/NADH	710.36	5553.7	95.5	(1, 14)	8.86	Complete

[a] In this case, the complete model is marginally better than the reduced model. The reduced model was chosen, since the pattern of residuals obtained when the data are fitted to Equation 3 did not appear to be normally distributed and did not conform to the expected pattern when noncompetitive data is fitted to a competitive model. The intercept effect is only 4.7% of the slope effect and is probably not significant. At low concentrations of glyceraldehyde 3-phosphate and phosphate, there is no intercept effect. In all previous studies of NADH inhibition where NAD^+ is the varied substrate (1–3, 7), inhibition is invariably reported to be competitive.

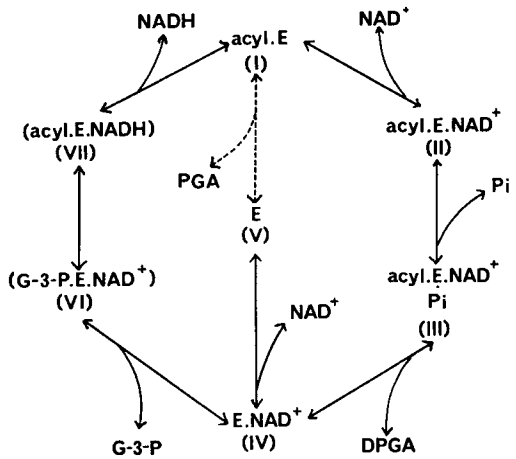


FIG. 11. Mechanism 2 in cyclical form. The catalytic cycle is represented by the reactions on the perimeter of the cycle, reading clockwise for the oxidative phosphorylation of glyceraldehyde 3-phosphate (*G-3-P*), and anticlockwise for the reductive dephosphorylation of 1,3-diphosphoglyceric acid (*DPGA*). The free enzyme (*V*) is also shown, although it is not catalytically active in these reactions. *PGA*, 3-phosphoglyceric acid.

Similarly, the acyl enzyme may be deacylated by incubation with NADH (10):

$$I \rightarrow VII \rightarrow VI \rightarrow IV$$

or with phosphate (or arsenate) plus NAD^+ (10, 12, 16, 19):

$$I \rightarrow II \rightarrow III \rightarrow IV$$

Mechanism 2 also explains the observation of Furfine and Velick (1) that the dissociation constant of NAD^+ from the enzyme differs by several orders of magnitude depending on whether it is determined in the presence ($I \leftrightarrow II$) or absence ($V \leftrightarrow IV$) of 1,3-diphosphoglyceric acid.

While the mechanism bears marked similarities to the mechanisms which have been proposed for the enzyme from studies of partial (10–12) or minor (13, 14) activities of the enzyme, all of which are ping-pong, previous steady state kinetic studies have invariably suggested sequential mechanisms. The most complete example of these studies is that of Furfine and Velick (1). All of the product inhibition data they present is in accord with Mechanism 2, including their observation of noncompetitive inhibition by NAD^+ with respect to 1,3-diphosphoglyceric acid, which these workers were unable to explain. The discrepancies between their data and Mechanism 2 are the observations of intersecting patterns between NAD^+ and glyceraldehyde 3-phosphate, between phosphate and glyceraldehyde 3-phosphate, and between NADH and 1,3-diphosphoglyceric acid. If we assume that in their studies of the forward reaction there was a small but finite concentration of 1,3-diphosphoglyceric acid, then Mechanism 2 predicts the observed intersecting patterns. In the present study, the inclusion of the 3-phosphoglyceric acid kinase system prevents the distortion of the expected parallel pattern in to an intersecting one. It is worth noting that other kinetic studies (1, 3, 5, 8) have indicated intersecting patterns between these two pairs of substrates, although the intersection point may vary considerably. If Mechanism 2 is correct, the intersection point will depend on the 1,3-diphosphoglyceric acid concentration, which in turn will depend on the experimental conditions employed. The intersecting pattern between NADH and 1,3-diphosphoglyceric acid observed by Furfine and Velick is less easy to explain, but Smith and Velick (37) have recently repeated this experiment under slightly different conditions and have obtained data which can be interpreted as indicating a parallel pattern with strong interference from substrate inhibition. Mechanism 2 predicts a parallel pattern.

Orsi and Cleland (3) have recently proposed an ordered sequential mechanism for glyceraldehyde 3-phosphate dehydrogenase, but it should be noted that they initiate the reaction by the addition of enzyme. Since glyceraldehyde 3-phosphate is unstable under their assay conditions (pH 8.6), this procedure could lead to erroneous conclusions. Some of their studies were performed using the glyceraldehyde 3-phosphate analog, 3-hydroxypropionaldehyde 3-phosphate, and it is not known if this compound is as unstable as glyceraldehyde 3-phosphate. They assume a sequential mechanism on the basis of earlier studies (1, 2, 4, 5), but the mechanism they propose is inconsistent with nearly half of the data of Furfine and Velick (1). They also assume that NADH is the last product to leave the enzyme, as it is for many dehydrogenases. We know of two dehydrogenases

(38, 39) where this does not appear to be the case, so this assumption may also be invalid. They rely heavily on the use of various dead-end inhibitors and observe a great many uncompetitive inhibitions. As Cleland himself has pointed out (40), dead-end inhibitors characteristically produce large numbers of uncompetitive inhibitions irrespective of mechanism, so the diagnostic power of these experiments is small.

It is concluded that either Mechanism 2 or 3 will explain the kinetic data presented in this report, but Mechanism 2 predicts the existence of enzyme-NAD^+ and acyl enzyme complexes as obligatory intermediates, and both of these complexes have been extensively described by many workers. It has not previously been realized that the former is a stable, rather than a transitory, complex. Mechanism 3, or any of the kinetic mechanisms proposed by other workers, does not predict that such forms could be isolated.

Certain difficulties in the assay procedure, namely product inhibition by 1,3-diphosphoglyceric acid and the instability of glyceraldehyde 3-phosphate, were examined, and these difficulties may have led other workers to draw erroneous conclusions. It is suggested that Mechanism 2 operates in the pea seed enzyme, as well as that from other sources. In the following paper (41) we will describe studies on a partial reaction catalyzed by glyceraldehyde 3-phosphate dehydrogenase, *viz.* the arsenolysis of 1,3-diphosphoglyceric acid, which provide further evidence for Mechanism 2.

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