The Acyl-enzyme Intermediate and the Kinetic Mechanism of the Glyceraldehyde 3-Phosphate Dehydrogenase Reaction

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The acyl-enzyme compound formed by d-glyceraldehyde 3-phosphate dehydrogenase has been studied by several workers in a variety of ways (1), primarily in experiments at high enzyme concentrations and in many instances with model substrates. In the present investigation an attempt is made to apply kinetic criteria to the mechanism of the reversible oxidative phosphorylation of the natural substrates. Although the oxidative and acyl transfer steps are clearly separable at substrate level enzyme concentrations, the initial velocity kinetics at low enzyme concentration is determined by a random order of substrate addition to enzyme with the formation of a kinetically important quaternary enzyme-substrate complex. One does not obtain, in initial velocity measurements, the same kinetic signs of group transfer through an enzyme-bound intermediate that are observed, for example, with the glutamate-oxaloacetate transaminase (2). It is found that the rate-limiting step in glyceraldehyde 3-phosphate oxidation at pH 7.4 is the acyl group transfer from a site on the enzyme to an external acceptor. The properties of the partial reactions are such that in the physiological pH region the kinetics is dominated by a high steady state concentration of the intermediate. There is a large effect of the intermediate on the typical interactions of pyridine nucleotide with enzyme, apparently mediated by protein isomerizations. Events which might not be recognized in a purely kinetic approach are clarified by an examination of isolated enzyme-substrate interactions and of the equilibria of the partial reactions in which the enzyme is a stoichiometric participant.

EXPERIMENTAL PROCEDURE

dl-Glyceraldehyde 3-Phosphate—This compound was obtained as the barium salt of the diethylacetel from Sigma Chemical Company and from California Corporation for Biochemical Research. It was converted to the free aldehyde by heating 20 mg for 3 minutes at 100° in 1.5 ml of an exhaustively washed and recycled suspension of Dowex 50, 20% by volume, in the acid form. Total concentration of the aldehyde in the chilled filtrate was determined as alkali-labile phosphate (3). The concentration of the d isomer was determined enzymatically in a solution which contained 0.1 mM sodium carbonate-bicarbonate, pH 8.6, 0.01 mM sodium arsenate, 1.5 mM DPN, and enzyme sufficient to catalyze complete reaction in less than 1 minute. Absorbance changes were read at 340 mp in a Beckman DU or Zeiss spectrophotometer.

3-Phosphoglyceroyl Phosphate—This derivative was prepared by the oxidative phosphorylation of the aldehyde. Conditions for the oxidation were those of Negelein and Bromel (4) except that 0.02 mM sodium pyruvate and lactic dehydrogenase, 0.06 mg per ml, were substituted for acetaldehyde and alcohol dehydrogenase to reoxidize the DPNH. The crude product, precipitated with acid-acetone as described by the above authors, was dissolved in a small volume of cold water and brought to pH 7 by the addition of solid imidazole. Diluted aliquots containing about 20 μmoles in 20 ml of 0.01 M imidazole chloride, pH 7.5, were placed on a column (0.9 x 10 cm) of DEAE-Sephadex, “fine” grade. The column was developed by a gradient in which 0.6 M sodium chloride in the imidazole buffer was fed into a mixer containing 200 ml of buffer. Fractions of approximately 4 ml were collected at a rate of 0.7 ml per minute. The product appeared in tubes 34 to 37 in about 65% yield and contained no detectable nucleotide or aldehyde. Aliquots from each tube were assayed spectrophotometrically in test solutions which contained 0.15 mM DPN and sufficient enzyme for rapid reaction at pH 7. The acyl phosphate can be stored frozen at pH 7.0 to 7.4 but must be used promptly after melting.

Pyridine Nucleotides—The presence of nucleotide impurities in DPN, up to 4% in the best samples examined (5), was confirmed. In the present case, however, there was no sign of inhibitory action of the impurities. DPNH (Sigma) before or after chromatography on DEAE-cellulose contained no detectable inhibitors and was used directly. Concentrations of the 3-acetylpyridine analogue of DPN were determined spectrophotometrically as the cyamide complex (6) and also by enzymatic reduction at pH 9.6 in 0.1 M glycine-10 mM arsenate-1 mM glyceraldehyde-3-P and 50 μg of enzyme per ml with measurement of absorbance changes at 363 mp. The 3-acetylpuridine analogue of DPNH was prepared with ethanol and yeast alcohol dehydrogenase (7) and purified on columns identical with those employed for 3-phosphoglyceroyl phosphate purification.

Enzyme—Enzyme was prepared from rabbit skeletal muscle (8) with 4 mM EDTA present in all solvents, and was recrystallized three or more times. Dilute stock solutions, when kept cold, lost approximately 5% of initial activity over the course of several hours, for which corrections were made. Published absorption coefficients (9) were used in determining protein concentrations spectrophotometrically. In experiments in-
volving AcPyDPN, the bound DPN of the enzyme was first removed by treatment with charcoal.

Commercial preparations (Sigma) of triosephosphate isomerase and \( \alpha \)-glycerophosphate dehydrogenase contained no interfering enzyme activities and could be used directly. However, crystalline preparations of phosphoglycerate kinase from yeast were heavily contaminated with the isomerase and also with the glyceraldehyde-3-P dehydrogenase. These impurities were removed by ion exchange chromatography. It may be noted that the dehydrogenase from rabbit muscle, after several recrystallizations, still contains traces of the isomerase. The contamination is not detectable at the low enzyme concentrations used in kinetic work, but may be sufficient to equilibrate the triose phosphates in experiments of long duration carried out at high enzyme concentration, for example, during ultracentrifugation or dialysis equilibrium.

**Fluorometric Method**—Rates of DPN reduction were measured in a Farrand model A fluorometer with a low pressure mercury source, primary filter Corning No. 5860 and secondary filters Corning Nos. 3385 and 4308. Reactions in a final volume of 1 ml were run in test tubes and were initiated by the addition of enzyme. The temperature of the tube holder was controlled by a water jacket and circulating water bath. An adjustable zero, adjustable range recorder (Leeds and Northrup) allowed, at highest sensitivity, a full scale deflection from a change in DPNH concentration of 0.5 \( \mu \)M. This sensitivity was frequently necessary to minimize product inhibition and to determine the smaller Michaelis constants. A fluorescent impurity in the imidazole buffer was reduced to a low level by filtration of stock imidazole solutions through Norit A. To minimize troublesome noise from dust particles at high instrumental sensitivities, the test solutions were also filtered through Whatman No. 50 filter paper. In order to obtain equivalent sensitivities in studying the reverse reaction fluorometrically, initial DPNH concentrations had to be kept below 5 \( \mu \)M to avoid phototube fatigue and drift.

**Absorption Spectrophotometry**—Kinetic measurements were also made in a Beckman DU spectrophotometer with cuvettes of 10-cm light path (6-ml volume) and with the same recorder that was employed with the fluorometer. At the maximal scale expansion a change of 1 \( \mu \)M in DPNH concentration gave a full scale recorder deflection. This instrument was used primarily with the AcPyDPN reactions because of the higher absorption coefficient and lower fluorescence quantum yield of AcPyDPNH. It was also useful in exploring higher concentration ranges of DPNH than were practical fluorometrically.

**Fluorometric Titration**—In these experiments the protein is excited by light of any wave length within the tryptophan absorption bands, and the right angle fluorescence is measured at 350 \( \mu \)M as a function of the concentration of added DPN (10). In order to minimize undesired absorption of the excitation beam in the tail of the 290 \( \mu \)M absorption band of DPN, the excitation was done at 305 \( \mu \)M in a quartz cuvette of 2-mm light path. Even under these conditions absorption corrections are required, based on measured absorbances at the wave lengths of interest, when the DPN concentration approaches or exceeds 100 \( \mu \)M. An Aminco spectrophotofluorometer was employed with an efficient alternating current voltage regulator on the photometer unit and the recently redesigned high pressure xenon arc (Hano-)

\[ \text{AcPyDPN} + \text{DPN}^+ + \text{HPO}_4^{2-} \rightarrow \text{AcPyDPNH} + \text{DPNH} + \text{H}^+ \]

**RESULTS**

**Limiting Conditions**—The equilibrium of the oxidative phosphorylation (Equation 1) favors aldehyde and DPN in the physiological pH region.

\[ \text{R} - \text{CHO} + \text{DPN}^+ + \text{HPO}_4^{2-} \rightarrow \text{R} - \text{CO} - \text{OPo}_4^{2-} + \text{DPNH} + \text{H}^+ \] (1)

It is therefore easy to measure initial velocities of acyl phosphate reduction by DPNH, but satisfactory initial velocity measurements of aldehyde oxidation require substrate concentrations that are too high to be of primary mechanistic interest. The equilibrium restriction at pH 7.4 can be avoided by using arsenate as external acyl acceptor in place of phosphate. This adds a hydrolytic step to the reaction and leads to the irreversible formation of 3-phosphoglycerate. The alternative way of circumventing the equilibrium restriction at this pH is to take advantage of the high oxidation-reduction potential of the 3-acetylpyridine analogues of DPN and DPNH (11). Both orthophosphate ion and the natural coenzyme may be used at pH 8.6. The equilibrium at the alkaline pH is more favorable for aldehyde oxidation, and secondary substrate and product effects are also greatly diminished. Each of these conditions has been examined.

**Graphical Analysis**—The general procedure in the three-substrate or forward reaction direction is to measure initial velocities as a function of the concentration of one substrate at a constant concentration of a second substrate and a series of fixed concentrations of the third. Reciprocal plots of velocity against concentration from the data of such experiments yield sets of lines which may be parallel or which may intersect at a common point in a left-hand quadrant. The former event in a three-substrate reaction provides evidence for a compulsory sequence of substrate addition to enzyme, but under all adequately defined conditions that have been examined the latter relation prevails.

**Aldehyde Oxidation at pH 8.6**—Reciprocal plots of the data obtained at pH 8.6 are collected in Fig. 1. The experiments illustrated all involve arsenate as the external acyl group acceptor. Results of the identical form are obtained when orthophosphate is employed. The Michaelis constants of DPN and glyceraldehyde-3-P are unaltered in phosphate, and essentially the same maximal velocity is observed. Within the limits of experimental error the families of lines all intersect on the negative abscissa. The negative reciprocal of the intersection point gives a Michaelis constant directly, as conventionally defined. Over a relatively wide concentration range the Michaelis constant of each substrate is seen to be independent of the concentration of the cosubstrates.

**Aldehyde Oxidation at pH 7.4**—The data obtained in the physiological pH region and plotted in Fig. 2 take a form identical with the results at the alkaline pH, but the concentration ranges are different and in certain instances are much more limited. In particular, the Michaelis constant of DPN is much higher than at pH 8.6 and that of aldehyde much lower. The range of aldehyde and arsenate concentrations employed in Fig. 2 is
FIG. 1 (left). Kinetics of DPN reduction by glyceraldehyde-3-P (GSP) at pH 8.6 in 0.1 M sodium carbonate bicarbonate buffer at 26°. A, reciprocal plots of velocity against DPN concentration at 420 μM arsenate and a series of fixed aldehyde concentrations; B, reciprocal plots of velocity against DPN concentration at 340 μM aldehyde and a series of fixed arsenate concentrations; C and D, reciprocal plots of velocity against substrate concentrations at 114 μM DPN and other variables as indicated. The enzyme concentration in A and B was 2.9 X 10⁻⁹ M, and in C and D 6.1 X 10⁻¹⁰ M. The ordinates in this and in the following reciprocal plots of kinetic data are in units of (millimicromoles per ml per second)⁻¹ unless otherwise indicated.

FIG. 2 (right). Kinetics of DPN reduction by glyceraldehyde-3-P (GSP) at pH 7.4 in 0.1 M imidazole (chloride) buffer at 26°. In A, the arsenate concentration was held constant at 15 μM and aldehyde was varied as indicated; in B, the aldehyde was held at 4.2 μM at a series of fixed arsenate concentrations. Enzyme concentration in A and B was 2.8 X 10⁻⁹ M. In C and D, the enzyme concentration was 1.3 X 10⁻⁹ M and the DPN concentration was constant at 260 μM.

FIG. 3. Kinetics of reduction by glyceraldehyde-3-P (GSP) of the 3-acetylpyridine analogue of DPN (AcPyDPN) at pH 7.4 and 26° in 0.1 M imidazole buffer. In A, the phosphate concentration was 30 mM and the enzyme concentration 2 X 10⁻⁴ M; in B, the aldehyde concentration was 164 μM and the enzyme concentration 4.8 X 10⁻⁸ M.

FIG. 4. Kinetics of the reduction of 3-phosphoglyceroyl phosphate (PGP) by DPNH at pH 7.4 in 0.1 M imidazole at 26°, at an enzyme concentration of 4.6 X 10⁻¹ M. The Michaelis constant of each substrate is independent of the concentration of the other.

Reciprocal plots of kinetic data obtained at pH 7.4 with AcPy- DPN and phosphate, respectively, as the hydrogen and acyl acceptors in aldehyde oxidation are shown in Fig. 3. The Michaelis constant of AcPyDPN is independent of aldehyde and phosphate concentrations, and the kinetic parameters of the latter substrates are independent of the concentration of AcPy- DPN.

Restricted to avoid substrate activation and inhibition effects, which are described in a following section.

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Initial velocity kinetics of the reverse reaction, between the acyl phosphate and DPNH. The results take the same form as those of the forward reaction and are characterized by particularly small Michaelis constants for both substrates.

Secondary Substrate Effects

Enzyme Activation by Arsenate—The responses in initial velocity to changes in arsenate concentration at pH 7.4 differ in three separate arsenate concentration ranges and are illustrated in Fig. 5 by the effects of arsenate upon the kinetic parameters.
of glyceraldehyde-3-P. At arsenate concentrations below 20 
\( \mu \text{M} \), \( K_{\text{glyceraldehyde-3-P}} \) is independent of arsenate concentration. 
In the arsenate concentration range of 50 to 500 \( \mu \text{M} \), there is a 
pronounced secondary enzyme activation as shown in the lower 
pair of lines of Fig. 5A. \( K_{\text{glyceraldehyde-3-P}} \) in this concentration 
range is dependent on arsenate concentration. When arsenate 
is used in the millimolar concentration range (Fig. 5B), the high 
maximal velocity still prevails but arsenate is also seen to be an 
inhibitor, competitive with aldehyde. Reciprocal plots of re-
lated data in which arsenate is the independent variable are 
shown in Fig. 6. It is seen in Fig. 6A that in the concentration 
region in which arsenate is an activator \( K_{\text{arsenate}} \) is a function of 
aldehyde concentration. Fig. 6B shows a secondary plot for 
maximal velocity covering the normal and activating regions of 
arсенate concentration.

**FIG. 5.** The effects of three concentration ranges of arsenate 
on the kinetic behavior of glyceraldehyde-3-P (G3P) at pH 7.4 
in 0.1 M imidazole buffer at 26°C. In A, at low arsenate concen-
tration, the lines intersect on the abscissa, as they do in Fig. 2A, 
and \( K_{\text{glyceraldehyde-3-P}} \) is independent of arsenate concentration. 
At arsenate concentrations in the 50 to 100 \( \mu \text{M} \) range, the in-
tersections on the abscissa, and hence \( K_{\text{glyceraldehyde-3-P}} \), depend upon 
arseenate concentration and there is a secondary enzyme activation 
with an increase in maximal velocity. In B, at arsenate concen-
trations in the millimolar concentration range, there is secondary 
enzyme activation but arsenate is now an inhibitor, competitive 
with aldehyde. The DPN concentration in these experiments 
was 260 \( \mu \text{M} \) and the enzyme concentration 1.7 X 10^{-5} \text{M}.

**FIG. 6.** Reciprocal plots of velocity against arsenate concen-
tration in the concentration region in which enzyme is activated 
by arsenate. In A, the Michaelis constant of arsenate is seen 
to depend upon glyceraldehyde-3-P (G3P) concentration. Curve 
B is a reciprocal plot of velocities, which are maximal with respect 
to glyceraldehyde-3-P, against arsenate concentration. These 
experiments were done in 0.1 M imidazole, pH 7.4, at 26°C, at a 
DPN concentration of 260 \( \mu \text{M} \) and an enzyme concentration of 
1.7 X 10^{-5} \text{M}.

**FIG. 7.** Substrate inhibition by glyceraldehyde-3-P (G3P) at 
two levels of arsenate concentration in imidazole buffer, pH 7.4, 
at 26°C and an enzyme concentration of 1.7 X 10^{-5} \text{M} and a DPN 
concentration of 230 \( \mu \text{M} \). Initial velocities are plotted against 
log [glyceraldehyde-3-P].

**Rate Laws and Kinetic Parameters**

**Rate Laws**—The empirical rate laws for the forward and re-
verse reactions under conditions in which there are no kinetically 
detectable secondary substrate effects are given, respectively, in 
Equations 2 and 3.

\[
\begin{align*}
\frac{E_0}{v} &= \frac{1}{V_f} \left( 1 - \frac{K_A}{(A)} \right) \left( 1 + \frac{K_B}{(B)} \right) \left( 1 + \frac{K_C}{(C)} \right) \\
\frac{E_i}{v} &= \frac{1}{V_r} \left( 1 + \frac{K_D}{(D)} \right) \left( 1 + \frac{K_F}{(F)} \right)
\end{align*}
\]

where the \( K \) values are Michaelis constants and \( v \) and \( V \) are, 
respectively, initial and maximal velocities. The independence of 
the \( K \) values of the concentrations of the cosubstrates elimi-
nates the need for constants of the type \( K_{AB} \) or \( K_{ABC} \) dependent 
on two or more substrates, although constants of these types 
occur in the rate laws for reactions carried out at arsenate con-
centrations which produce secondary enzyme activation.

All of the pyridine nucleotide dehydrogenase reactions that 
have so far been examined kinetically (13–18) have been de-
scribed by steady state mechanisms that have been simplified 
by the assumption of an ordered sequence of substrate addition 
to enzyme. When this approach is attempted for the forward 
or three-substrate reaction in the present case, the resulting 
equations lack terms that are required in Equation 2 and hence 
are contrary to the experimental results. The desired equation 
can be derived in principle by the general steady state method 
by assuming a random order of substrate addition to enzyme, 
but the results are too complicated to be useful at the present
A + (EABC) are described by the identical dissociation constants measured by equilibrium binding methods, since the two types of constant are assumed to be formally analogous to that exerted by the aldehyde has been observed by Reynard, Hass, Jacobsen, and Boyer (21) in the pyruvate kinase reaction, which also exhibits rapid equilibrium kinetics.

Although the crossed competitive product inhibitions provide formal support for the rapid equilibrium kinetic mechanism, the inhibition constants calculated according to Equations 4 and 5 are extremely small and are not identical with the corresponding Michaelis constants. If the glyceraldehyde-3-P generated in the reverse reaction were as inhibitory as that added prior to initiation of the reaction, the earliest measurable initial velocities would have been subject to product inhibition. An attempt was made, therefore, to relieve any product inhibition during initial velocity measurements in the reverse reaction by adding a large excess of triosephosphate isomerase, which converts most of the aldehyde to dihydroxyacetone phosphate. No acceleration was observed. When a-glycerophosphate dehydrogenase was added together with the isomerase, the rate of DPNH oxidation was doubled and this was accounted for entirely by the presence of the aldehyde.

Product Inhibition

Inhibition by Glyceraldehyde-3-P and DPNH—Both aldehyde and DPNH are potent inhibitors of the reactions in which they are produced. These inhibitions are competitive not only with the homologous precursor substrate but also with the opposing cosubstrate. Thus, as shown in Fig. 9, the aldehyde is competitive both with acyl phosphate and with DPNH. Conversely, DPNH, in Fig. 10, is seen to be competitive with DPN and aldehyde.

The rate laws which describe these inhibitions are given in Equations 4 and 5 for the forward and reverse reactions, respectively,

\[
\frac{E_t}{v} = \frac{1}{V_r} \left[ \left( 1 + \frac{K_a}{A} \right) \left( 1 + \frac{K_b}{B} \right) \left( 1 + \frac{K_c}{C} \right) \right]
\]

\[
\frac{E_t}{v} = \frac{1}{V_r} \left[ \left( 1 + \frac{K_b}{D} \right) \left( 1 + \frac{K_c}{F} \right) \left( 1 + \frac{I}{K_i} \right) \right]
\]

where \( I \) is the inhibitor and \( K_i \) the inhibition constant. Inhibitions of this type are not compatible with a simple compulsory order steady state mechanism but are readily derived for a rapid equilibrium mechanism by considering the complexes in Fig. 8 plus the inhibited complexes EI and EIC. A product inhibition analogous to that exerted by the aldehyde has been observed by Reynard, Hass, Jacobsen, and Boyer (21) in the pyruvate kinase reaction, which also exhibits rapid equilibrium kinetics.

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consumption of the extra mole of DPNH in the reduction of the ketone.

An independent test for product inhibition by DPNH in the forward reaction was provided by allowing the reaction to occur in the presence of sufficient beef heart lactic dehydrogenase to bind all DPNH formed up to concentrations of 28 \mu M. The lactic and glyceraldehyde-3-P dehydrogenases have approximately equal affinities for DPNH, but the 10,000-fold excess of lactic dehydrogenase allows it to compete successfully for the DPNH in a rapid binding reaction (10). Product inhibition by the DPNH generated in the reversible glyceraldehyde-3-P-arsenate reaction is seen in Fig. 11 to be very strong at low DPN concentrations. The same reaction run in the presence of lactic dehydrogenase shows the same initial velocity but proceeds nearly to completion. Competitive binding of the inhibitory DPNH by the lactic dehydrogenase thus relieves the inhibition in extended reactions at low DPN concentration, but does not accelerate initial rate or appreciably affect $K_{DPN}$.

**Fig. 11.** Removal of product inhibition by competitive binding of product (DPNH) on substrate level concentrations of a second protein. The initial concentrations in both reactions were: DPN, 9.5 \mu M; glyceraldehyde-3-P, 15 \mu M; arsenate, 1 \mu M; and enzyme, 7.9 \times 10^{-6} \mu M, at pH 7.4 and 20\degree C. The reaction described by the *upper curve* was performed in the presence of 1.4 mg of beef heart lactic dehydrogenase (LDH) per ml as a preferential DPN binder. Reaction was initiated at the arrow by addition of glyceraldehyde-3-P dehydrogenase. The *curves* are redrawn from recorder tracings, and the *upper curve* has been reduced by a factor of 1/2.1 to correct for the fluorescence enhancement of DPNH bound to lactic dehydrogenase observed with the particular filter combination that was used.

**Product Inhibition by DPN**—The action of DPN as an inhibitory product of the reaction between DPNH and 3-P-glyceroyl-P is illustrated in Fig. 12, where $1/v$ is plotted against DPNH concentration. In this type of plot for a two-substrate reaction, the inhibition constant is given by the projection on the *abscissa* of the intersection point at the left of the *ordinate*. DPN is found to be competitive with DPNH but is not competitive with the acyl-P in the concentration range that could be tested. The rate law for the single competitive inhibition is given in Equation 6.

$$\frac{E_0}{v} = \frac{1}{V_r} \left[ \frac{1 + K_D^{(D)}}{(1 + K_F^{(D)}) + (1 + K_D^{(F)}) + K_F^{(D)}} \right]$$

Although the inhibition constants of aldehyde and DPNH are both much smaller than the corresponding Michaelis constants, that of DPN is, within limits of error, equal to the Michaelis constant for DPN.

**Product Inhibition by 3-P-Glyceroyl-P**—The acyl phosphate is a competitive inhibitor of the oxidative phosphorylation at pH 8.6 with an inhibition constant in the range of 2 to 4 \mu M. At pH 7.4, the unfavorable equilibrium in phosphate and the occurrence of arsenolysis of the acyl phosphate in arsenate hinder a kinetic study of the product inhibition. A strong product inhibition by the acyl phosphate in the physiological pH region would be anticipated in view of its very low Michaelis constant. It has so far been possible to approach this inhibition only indirectly by examining the effects of differential substrate and product binding at equilibrium.

When the simpler pyridine nucleotide linked dehydrogenase reactions are run to equilibrium at low and at substrate levels of enzyme, it is found that at high enzyme concentration the equilibria are shifted toward increased DPNH formation (22-24). The preferential binding of DPNH over DPN becomes thermodynamically significant when the enzyme concentration is comparable to that of substrate or product. The product inhibition by the acyl phosphate in the physiological pH region would be anticipated in view of its very low Michaelis constant. It has so far been possible to approach this inhibition only indirectly by examining the effects of differential substrate and product binding at equilibrium.

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MINUTES

FIG. 13. Product inhibition by 3-P-glyceroyl-P. Concentrations in all of the reactions illustrated were: DPN, 250 \( \mu \)M; glyceraldehyde-3-P, 100 \( \mu \)M; and potassium phosphate, 4 mM, in 0.05 M imidazole buffer, pH 7.5, at 25\(^\circ\). The reaction illustrated by Curve A had come to a stop within 10 seconds and remained constant. Curve B represents an identical mixture which also contained 1.4 mg of lactic dehydrogenase per ml. Curve C describes a reaction similar to A but containing 5 \( \mu \)g of phosphoglycerate kinase per ml and 0.5 mM MgCl\(_2\). Extended reaction was initiated at the arrow by addition of ADP at an initial concentration of 250 \( \mu \)M. The reaction of Curve D is identical with C except that the initial mixture also contained 1.4 mg of lactic dehydrogenase per ml.

That DPNH binding by lactic dehydrogenase is operative under the above conditions is shown in Fig. 13, Curves C and D. In Curve C, which is identical with Curve A but contains an incomplete 3-phosphoglycerate kinase system, the kinase reaction is initiated at the arrow by addition of any missing component: magnesium, ADP, or kinase. Additional DPNH production occurs when the acyl-P is converted to 3-phosphoglycerate. When the same experiment is performed in the presence of high lactic dehydrogenase, the rate and extent of the subsequent glyceraldehyde-3-P oxidation are greatly increased. Thus the weaker DPNH inhibition is detected and relieved by binding to lactic dehydrogenase when the acyl-P restriction is removed.

Product inhibition by the acyl-P in initial velocity measurements involving the coenzyme analogue AcPyDPN in phosphate at pH 7.4 are not obvious in the results shown in Fig. 3. It is likely that the inhibition is weaker in the AcPyDPN than in the DPN reaction. There are several possible reasons for the difference, but further investigation is required.

Table I contains a summary of the kinetic parameters that have so far been obtained. The low Michaelis constant of 3-P-glyceroyl-P and the relatively high \( V_\text{max} / K_m \) value it necessary, for rapid equilibrium conditions to be maintained, that the bimolecular complex formation between 3-P-glyceroyl-P and enzyme approach a diffusion-controlled rate. There is now precedent for rate constants of this magnitude in the combination of proteins with small ligands (2, 29, 30).

**Kinetic Parameters and Dissociation Constants**

Contrary to the simple kinetic theory that describes the form of the results, several discrepancies have been noted between dissociation constants of enzyme-substrate complexes determined kinetically in different ways or kinetically and by equilibrium titration. The latter difference, in the case of DPN, is three orders of magnitude and raises the question whether the catalytic sites are distinct from the strong DPN-binding sites or whether the multiple binding sites are all equivalent but become modified under reaction conditions. The second explanation is the correct one. It is directly demonstrable and may be reconciled with the known properties of the enzyme and with the proposed kinetic mechanism.

**Partial Reactions under Initial Velocity Conditions**—At the limits of instrumental sensitivity, initial velocities of aldehyde oxidation can be measured during the formation of 0.3 \( \mu \)M product. Under such conditions with arsenate as external acyl acceptor and DPN in the range of 100 \( \mu \)M, there should be no appreciable direct product inhibition. It is pertinent to inquire, however, what the steady state concentration of acyl-enzyme intermediate might be in the initial steady state. The equilibrium constant of the oxidative step with enzyme as a stoichiometric participant in the absence of external acceptor was determined a number of years ago (19) and expressed as in Equation 8.

\[
K_1 = \frac{(acyl-enzyme)(DPNH)/(H^+) \times 10^{-4}}{(free enzyme)(DPN)(aldehyde)} \tag{8}
\]

Contributions from differential substrate and product binding

\[
K_1 = \frac{(acyl-enzyme)(DPNH)/(H^+) \times 10^{-4}}{(free enzyme)(DPN)(aldehyde)} \tag{8}
\]

**Table I**

**Kinetic parameters of glyceraldehyde 3-phosphate dehydrogenase reactions**

<table>
<thead>
<tr>
<th>Reaction</th>
<th>Substrate</th>
<th>Michaelis constant</th>
<th>Inhibitory product</th>
<th>Inhibition constant</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glyceraldehyde-3-P + DPN + arsenate; 0.1 M imidazole; pH 7.4; 26(^\circ); ( V_f = 1,250 ) min(^{-1} )</td>
<td>DPN</td>
<td>90</td>
<td>DPNH</td>
<td>0.3</td>
</tr>
<tr>
<td></td>
<td>Glyceraldehyde-3-P</td>
<td>2.5</td>
<td>3-P-Glyceroyl-P</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>Arsenate</td>
<td>28</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3-P-Glyceroyl-P + DPNH; pH 7.4; 26(^\circ); ( V_f = 14,900 ) min(^{-1} )</td>
<td>DPNH</td>
<td>3.3</td>
<td>DPN</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>3-P-Glyceroyl-P</td>
<td>0.8</td>
<td>Glyceraldehyde-3-P</td>
<td>0.06</td>
</tr>
<tr>
<td>Glyceraldehyde-3-P + DPN + arsenate or phosphate; pH 8.6; 26(^\circ); ( V_f = 22,000 ) min(^{-1} )</td>
<td>DPN</td>
<td>13</td>
<td>DPNH</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>Glyceraldehyde-3-P</td>
<td>90</td>
<td>3-P-Glyceroyl-P</td>
<td>2-4</td>
</tr>
<tr>
<td></td>
<td>Arsenate</td>
<td>69</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Phosphate</td>
<td>290</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glyceraldehyde-3-P + AcPyDPN + phosphate; pH 7.4; 26(^\circ)</td>
<td>AcPyDPN</td>
<td>28</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Glyceraldehyde-3-P</td>
<td>37</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Phosphate</td>
<td>6,800</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
are not separated but are included in $K_d$. This equation would also apply to the initial steady state of irreversible glyceraldehyde-3-P oxidation in arsenate if the acyl transfer step were rate-limiting. In such a case one may calculate the fractional acylation of enzyme sites at a series of substrate concentrations when the DPNH concentration has reached 0.3 $\mu$M in an initial velocity measurement. Thus, if (acyl-enzyme)/(free enzyme), calculated from Equation 8, equals $a$, then the fractional acylation equals $a/(a + 1)$. In Table II, calculations are presented in which (DPN) is varied and (aldehyde) is fixed at 5 $K_{d,aldehyde}$, which is just below the onset of substrate inhibition. Fractional acylation of the enzyme is seen to parallel fractional apparent maximal velocity, $v/v'$, which is in accord with the assumption that acyl transfer is rate-limiting. Similar results are obtained when (DPN) is fixed at 5 $K_{DPN}$ and (aldehyde) is varied. The calculations indicate, moreover, that in all of the concentrations ranges that have been experimentally accessible, an average of one or more of the three or four acyl acceptor sites of the enzyme have been acylated during initial velocity measurements. The kinetic $K_{DNP}$ therefore applies to acylated protein, and it is thus necessary that it be identical with the $K_{DPN}$ determined fluorometrically in the absence of cosubstrate.

It was observed in previous studies of the effect of $p$-mercuribenzoate on DPN binding that 4 mole equivalents of the mercureial promote the release of all of the bound DPN from the enzyme (9) and that 1 mole equivalent of mercureial per mole of protein weakens the affinity of the protein for DPN at all sites (10, 31). This effect was attributed to a conformational perturbation of the protein induced by the large anionic ligand in mercaptide linkage with a reactive thiol group. A similar effect may occur as a result of acylation of an acceptor site with the 3-phosphoglyceroyl group. In such a case the equilibria and relative rates of the partial reactions are such that all enzyme species are modified or isomerized during initial velocity measurements.

**Correlation of Active Sites with DPN-binding Sites**—If the above interpretation is correct, it should be possible to observe kinetically the strong DPN binding that is characteristic of the nonacylated enzyme-DPN when a substrate is employed which does not give a high initial steady state concentration of the acyl intermediate or which forms an intermediate that does not perturb the DPN-binding site. This condition may be realized by the use of the nonphosphorylated $a$-glyceraldehyde, which has an extremely high Michaelis constant and which at substantial concentrations reacts at less than 0.001 the rate of glyceraldehyde-3-P. Such an experiment was described several years ago (9) and is repeated here with a much lower concentration of enzyme, more sensitive recording, and a wider range of DPN concentrations. With this substrate the enzyme is used in the micromolar rather than millimicromolar concentration range, and one may therefore measure directly the initial rates as a function of the concentration of bound DPN. The results are shown in Fig. 14.

Starting with charcoal-treated, DPN-free enzyme at a concentration of 3.2 $\mu$M (assumed molecular weight of 136,000), the initial rates increase linearly with added DPN, level off abruptly at 9.4 $\mu$M DPN, and remain constant up to DPN concentrations as high as 1,400 $\mu$M. If the enzyme is only 95% saturated with DPN at the apparent equivalence point, which corresponds to 2.9 binding sites per molecule, the dissociation constant of the active enzyme-DPN complex could be no larger than $10^{-7}$ M. This agrees with the equilibrium binding of DPN by the nonacylated enzyme.

One can also approach the above condition kinetically with glyceraldehyde-3-P by working at pH 8.6. At this pH there is a large increase in maximal velocity associated with an acceleration of the acyl transfer step. The initial steady state concentration of the acyl-enzyme intermediate should therefore be below the maximum permitted by $K_0$ of Equation 8, and the kinetic $K_{DPN}$ should become more nearly equal to the equilibrium binding value. The number observed kinetically is 13 $\mu$M, about one-seventh the value at pH 7.4. The number obtained by fluorimetric quenching titration at this pH is approximately 3 $\mu$M. A similar value is obtained as described below by measuring the rates of spontaneous inactivation of the enzyme at 39° as a function of DPN concentration.

The inactivation of the apoenzyme follows first order kinetics, and the rate constants decrease as an inverse function of the DPN concentration as a result of the increased stability of the enzyme DPN complex. The first order rate constant so obtained are plotted against log [DPN] in Fig. 15. A sigmoid curve is obtained with a value of 6 $\mu$M, within a factor of 2 of the kinetic $K_{DPN}$ at the same pH.

**Effect of S-P-Glyceroyl-P on Dissociation of Enzyme-DPN**
in 0.1 M sodium carbonate-bicarbonate buffer at 39.5°, contained charcoal-treated apo-enzyme at a concentration of 8.2 X 10^-5 M in increasing concentrations of added DPN. At time intervals, aliquots of each mixture were assayed for enzyme activity in the same buffer at 25° with DPN (300 μM), glyceraldehyde-3-P (800 μM), and orthophosphate (500 μM). Total enzyme concentration in the activity tests was 6.8 X 10^-10 M. Loss of activity in the incubation mixtures was found in each case to follow first order kinetics. The first order rate constants are plotted against the log of the DPN concentration in the incubation mixture.

The equilibrium of the acyl transfer reaction, with enzyme as a stoichiometric participant, has been formulated as in Equation 9, and has been estimated to have an equilibrium constant, K2, of 10^-2 (19). In a phosphate-free solvent, the enzyme should therefore be nearly quantitatively acylated by very low concentrations of 3-P-glyceroyl-P. In the studies of DPN inhibition of the acyl-P-DPNH reaction (Fig. 12), the initial acyl-P concentrations were 4.4 μM, a level which approaches kinetic saturation. This condition can be duplicated in an equilibrium binding experiment in which apo-enzyme is titrated fluorometrically with DPN in the presence and absence of 3 μM 3-P-glyceroyl-P. In these titrations, illustrated in Fig. 16, complex formation is measured by the quenching within the complex of the 350 nm emission of the tryptophan residues of the protein as originally described for pyridine nucleotide complexes of the glyceraldehyde-3-P and lactic dehydrogenases (10) and for the combination of dinitrophenyl hapten with their specific purified antibodies (32). In the absence of 3-P-glyceroyl-P or in the presence of an incomplete 3-P-glyceroyl-P-generating system lacking 3-phosphoglycerate or ATP, the fluorescence quenching approaches a limit of about 12 μM DPN. In the presence of the complete kinase system, calculated to yield an equilibrium 3-P-glyceroyl-P concentration of 3 μM, the fluorescence quenching approaches approximately the same limit at 800 μM DPN. From the magnitudes of the quenching effects, the DPN binding stoichiometries in the two cases are very nearly the same. In both cases the original fluorescence is restored by the addition of 100 μM p-mercuribenzoate. The apparent dissociation constants in the absence and presence of 3-P-glyceroyl-P are, respectively, 10^-7 and 10^-4 M.

The results in Fig. 16 establish by a nonkinetic method that a cosubstrate interaction on the acyl-enzyme is responsible for the high inhibition constant of DPN in the reduction of the acyl phosphate by DPNH. From the value of K2 in Equation 9, the predominant enzyme species in which the perturbation of DPN binding occurs should be the acyl-enzyme intermediate. This is in accord with the preceding calculations concerning initial steady state concentrations of the intermediate in the forward reaction. Acyl-enzyme is the only enzyme species common to the forward reaction between aldehyde and arsenate, the reverse reaction between acyl-P and DPNH, and the equilibrium titration with DPN in the presence of acyl-P. The results of Fig. 15 are in accord with the recent qualitative observation of Krimsky and Racker (33) that 3-P-glyceroyl-P promotes the release of bound DPN.

**Dissociation of Enzyme-AcPyDPN Complex**—For comparison with the kinetic determination of KAcPyDPN in Fig. 3, the binding of AcPyDPN by apo-enzyme was studied at pH 7.4 and 25° by spectrophotometric titration and at 39° by protection of the protein against thermal inactivation. The spectrophotometric method is similar in principle to one originally employed in measuring the binding of DPN (34) and is based upon the absorption band in the 360 nm region characteristic of the enzyme complex with the oxidized form of the dinucleotide. The present results are analyzed by the method of Stockell (35). In this method the mass law is expressed as in Equation 10.

\[
d/p = K/(e - p) + n
\]

where K is the apparent dissociation constant, n is the number of sites per molecule, d is the total concentration of AcPyDPN added, e is total enzyme concentration, and p is the concentration of occupied sites, i.e. the fraction of total enzyme bound times e. The plot of d/p against 1/(e - p) is linear with a slope of K and an intercept of n. K in Fig. 17 is found to be 28 μM, identical with the kinetic KAcPyDPN. The intercept is somewhere between 2 and 4 but cannot be accurately determined. A determination of K by protection against thermal inactivation at 39° yields a sigmoid plot analogous to that in Fig. 14 and an apparent KAcPyDPN of 31 μM. The coenzyme analogue appears to be bound similarly to DPN in that it gives the same type of 360 nm absorption band in the complex, but, unlike the natural coenzyme, the dissociation constants of the complex in...
the presence and absence of substrate are identical. It is possible that the oxidative step in the reaction with AcPyDPN is the slow one and that the initial steady state concentration of the intermediate is too low to produce a detectable effect. More detailed investigation is required.

**DISCUSSION**

The results that have been described are unusual in the sense that an enzyme that is particularly susceptible to modification by substrate catalyzes a reaction described by a random order, mutually independent substrate binding. The apparent paradox is a consequence of the fact that the major cosubstrate effect is produced by a nondissociable intermediate and is fully expressed under initial velocity conditions at the lowest substrate concentrations that can be handled experimentally. For this reason all enzyme species that are dealt with kinetically in reactions with the natural substrates are modified or isomerized and are hence designated as $E'$ rather than $E$ in the diagram of Fig. 8.

**Isomerization**—Use of the term isomerization to explain the observed effects may be questioned. For example, the interference by acyl-1 in DPN binding and the crossed competitive product inhibitions exerted by aldehyde and DPNH could be the result of direct steric interactions between the nonhomologous substrate-product pairs. Such direct steric interactions, in contrast with the behavior of the normal cosubstrate pairs, could arise from conformational differences in the oxidized and reduced bound forms both of the nucleotides and of the 3 carbon substrates. However, this interpretation does not account for events in the aldehyde-DPN-arsenate reaction at pH 7.4. If the acyl-enzyme intermediate interacted exclusively with the DPN at the same catalytic site, then the $1/e$ against $1/(DPN)$ plots would be linear only if all sites were fully acylated during the initial steady state. This possibility is not excluded by the accuracy of $K_1$ (Equation 8), but it would require that all measurable initial velocities be zero order with respect to aldehyde, and this is not the case. $K_{alddehyde}$ is quite measurable kinetically.

The acyl-enzyme-DPN interaction is therefore viewed as a special example of enzyme modifier action in which the modifier is a normal reaction intermediate. Formation of the intermediate at one site causes a protein isomerization which affects the properties of the unoccupied but otherwise equivalent catalytic sites. On a multisite enzyme of this type the cosubstrate inter-

**Quaternary Complex**—The isomerization effect is closely associated with the occurrence of a kinetically important quaternary enzyme-substrate complex. Prior indication of the need for such a complex was provided by the observation that the isolated acyl transfer step, studied by phosphate exchange or arsena
tysis on acyl phosphates (37), requires or is strongly promoted by the presence of bound pyridine nucleotide. This function can be provided by bound DPN and hence is a reverse manifestation of the effect of acylation upon DPN binding. The two types of ligand are presumed to produce antagonistic or counteracting changes in the secondary structure of the protein, and each therefore promotes the release or increases the reactivity of the other. If bound DPN promotes acyl transfer to phosphate, it should also promote the acylation of the protein by acyl phosphates in the reverse reaction. Hilvers and Weenen (38) have in fact reported that under rather special conditions the presence of DPN is necessary for the catalysis of acetyl phosphate reduction by DPNH. There is no sign of such a requirement in the present studies of 3-P-glyceroyl-P reduction. It is likely that if there is a secondary nucleotide requirement it can be provided by DPNH itself. Such a function is not readily tested in the partial reaction because DPNH rapidly reduces P-glyceroyl-P.

It is clearly established that glyceraldehyde-3-P reduction leading to acetyl-enzyme formation can occur in the absence of external acceptor (19, 20). Such a step is included in the rapid equilibrium reaction scheme of Fig. 7 but is designated by broken lines since it need not be considered in the derivation of the rate law of Equation 2. Although oxidation of aldehyde can occur under such conditions, it occurs much more rapidly when arsenate or phosphate is already present in the quaternary enzyme-substrate complex. In view of the general anion binding tendencies of glyceraldehyde-3-P dehydrogenase and the strong secondary enzyme activation produced by intermediate ranges of arsenate concentration (Fig. 4), this behavior is not surprising. Some years ago Koeppe, Boyer, and Stulberg (39) compared initial rates of glyceraldehyde oxidation at high enzyme concentration in the presence and absence of phosphate and observed no stimulation by phosphate. However, the conditions of the experiments and the kinetic behavior of glyceraldehyde and glyceraldehyde-3-P are sufficiently different that the two sets of results are not necessarily comparable.

In the absence of other information, the kinetic evidence for a quaternary complex would be interpreted mechanistically as a sign that under conditions of optimal catalysis the oxidative phosphorylation occurs by a modified nucleophilic displacement or $S_N2$ mechanism, in which the oxygen of a phosphate ion attacks the carbonyl carbon of the aldehyde at the same time that the hydrogen atom is transferred to the pyridine C-4 of bound DPN. Such a mechanism is in fact a limiting case of the two-step oxidation and acyl transfer mechanism as the time...
interval between the two steps approaches zero. The rate-limiting step at pH 7.4 would then not be acyl transfer but dissociation of acyl phosphate (or arsenate) from the protein. In terms of all of the evidence at present available, however, the acyl acceptor function at the catalytic site still appears to be real and not virtual even though it occurs within a quaternary enzyme-substrate complex.

Other Approaches—The present results describe only the gross features of the glyceraldehyde-3-P dehydrogenase reaction kinetics but provide the basis for a more extended attack on the mechanism both by standard and by newer methods. In several laboratories, applications are being made of fast reaction techniques which may clarify the events occurring in the ternary and quaternary complexes. From tryptic digests of glyceraldehyde-3-P dehydrogenase, Harris, Meriweather, and Park (40) and Perham and Harris (41) have isolated and determined the structure of an octadecapeptide which in the native protein carries the thiol groups that act as acyl acceptors with model substrates. By limited proteolysis with chymotrypsin, Krimsky and Racker (32) have destroyed phosphorylating activity without a commensurate loss in the activity of the oxidizing step and are exploring the possibility of intramolecular acyl migrations which on other grounds have so far been contraindicated.

Conditions in Muscle—In considering the performance of glyceraldehyde-3-P dehydrogenase in skeletal muscle, a chief point of interest is the fact that the enzyme concentration, expressed as active sites without correction for excluded volumes, is of the order of 70 μM or higher, whereas the steady state concentrations of the aldehyde and acyl phosphate have been reported to be in the range of 5 and less than 1 μM, respectively (42). The enzyme concentration thus normally exceeds those of its 3 carbon substrates but is considerably less than the concentrations of DPN and orthophosphate, which are in the millimolar range. Any restriction in the activity of the 3-phosphoglycerate kinase, such as might occur during rest by a limitation of the magnesium ion or ADP supply, would lead to a nearly total inhibition of glyceraldehyde-3-P dehydrogenase in the acylated form. Free 3-P-glyceroyl-P as such would not accumulate to any appreciable extent under any circumstances. Some of the DPNH produced can be removed by pyruvate reduction. To the extent that pyruvate is oxidized in mitochondria, there is an excess of free DPNH which must be removed by another pathway. DPNH production is self-limiting in the sense that it can take over product inhibition of the dehydrogenase when the restraint by acyl-P is removed. To what extent this may occur under physiological conditions is unknown.

SUMMARY

1. The oxidative and acyl group transfer steps previously shown to be catalyzed by glyceraldehyde 3-phosphate dehydrogenase under a variety of restricted conditions have been considered under conditions of rapid enzyme turnover in the reversible oxidative phosphorylation of the natural substrates.

2. Initial velocity kinetics has been measured in both reaction directions. The kinetic mechanism at pH 8.6 is described by a random order of substrate addition to enzyme and rate-limiting steps that occur in the breakdown of a kinetically important quaternary enzyme-substrate complex.

3. In the physiological pH region, similar kinetics is observed at low substrate concentrations, but at higher substrate concentrations a variety of secondary activations and inhibitions occur.

4. By kinetic criteria, the dissociation constant of the enzyme-diphosphopyridine nucleotide complex at pH 7.4, tested with diphosphopyridine nucleotide both as a substrate and as an inhibitory product, is several orders of magnitude larger than the value obtained by equilibrium titration in the absence of the phosphorylated 3 carbon substrates. It is identical, however, with the KDpN determined by fluorescence quenching titration in the presence of very low concentrations of 3-phosphoglyceroyl phosphate.

5. This result is accounted for by the equilibrium of the partial reactions and the fact that in the physiological pH region the acyl transfer to external acceptor is rate-limiting. The kinetic KDpN under such conditions describes the dissociation of DPN from acyl-enzyme rather than from free enzyme. At pH 8.6 the acyl group transfer is greatly accelerated and the kinetic KDpN approaches the equilibrium value determined in the absence of substrate. Perturbation of DPN binding at pH 7.4 disappears when the nonphosphorylated glyceraldehyde is employed as substrate. There is also no perturbation of pyridine nucleotide binding by the acyl intermediate at pH 7.4 when phosphorylated substrate when the 3-acetylpyridine analogue is used as hydrogen acceptor in place of DPN.

6. The interaction between DPN and the acyl intermediate is reciprocal. Thus the binding of DPN promotes acyl group transfer to external acceptor. A reciprocal interaction also occurs between glyceraldehyde 3-phosphate and DPNH, each of which, as an inhibitory product, competes with the other as well as with its homologous precursor.

7. The effect of acyl intermediate on KDpN is analogous to the weakening of DPN binding at three sites, promoted by 1 equivalent of p-mercuribenzoate per mole of enzyme. It is therefore believed that a protein isomerization occurs. The presumed isomerization does not involve the dissociation of the protein subunits but may involve their mutual reorientation.

REFERENCES

16. RAYAL, D. N., and WOLFE, R. C., Biochemistry, 1, 64 (1962).
The Acyl-enzyme Intermediate and the Kinetic Mechanism of the Glyceraldehyde 3-Phosphate Dehydrogenase Reaction
Charles S. Furfine and Sidney F. Velick