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GLYCERALDEHYDE-3-PHOSPHATE DEHYDROGENASE (NADP) FROM *SINAPIS ALBA*: STEADY STATE KINETICS

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Key Word Index—*Sinapis alba* L.; chloroplast glyceraldehyde-3-phosphate dehydrogenase(NADP); kinetic mechanism.

Abstract—Substrate interaction and product inhibition kinetics of the forward reaction of glyceraldehyde-3-phosphate dehydrogenase (NADP) (EC 1.2.1.13) from *Sinapis alba* suggest an Uni Uni Uni Bi Ping Pong mechanism (NAD(P)H on, glyceraldehyde-3-phosphate off, 1,3-diphosphoglycerate on, phosphate off, $NAD(P)^+$ off) with an apparent Theorell Chance displacement between 1,3-diphosphoglycerate and phosphate. The proposed mechanism predicts the existence of stable enzyme-$NAD(P)^+$ and acyl-enzyme complexes as obligatory intermediates. A comparison of the present findings on the NADP-enzyme with an earlier kinetic analysis of the NAD-specific enzyme from plants (EC 1.2.1.12) by other authors shows that the kinetic mechanisms for the two enzymes, although similar in principle (both show Ping Pong kinetics), differ in some details.

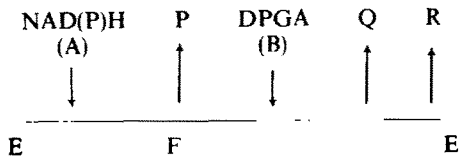
INTRODUCTION

In a previous publication [1] it was demonstrated that NADP-glyceraldehyde 3-phosphate dehydrogenase from *Sinapis alba* (EC 1.2.1.13) is structurally distinct from the NAD-specific enzyme (EC 1.2.1.12) and also differs from the latter in another important property: while NAD(H) transforms the NADP-dependent enzyme into an abnormal conformation (NAD-conformation), which aggregates (see also [2]) or interacts with Sepharose 6B, it has no such effect on the NAD-specific species. In addition two different kinetic mechanisms have been reported for the two enzymes. Whereas Duggleby and Dennis [3] determined a Ping Pong mechanism for the NAD-specific species from peas, the kinetic investigations of Pupillo *et al.* [4] seem to point to a sequential mechanism for the NADP enzyme from spinach. In view of the preliminary nature of the latter study, a more comprehensive examination of the NADP-enzyme from *Sinapis alba* was undertaken, including a complete set of substrate interaction and product inhibition kinetics for the forward enzyme reaction. The terminology and the diagnostic strategy of the present study follow essentially the suggestions of Cleland [5]. The results do not conform with a sequential mechanism, but suggest that the NADP enzyme also has a Ping Pong mechanism, which, however, differs from that of the NAD enzyme in important details.

RESULTS AND DISCUSSION

Substrate interaction kinetics

In experiment 1 (Fig. 1) kinetic patterns were determined by varying the coenzyme at several fixed levels of 3-phosphoglycerate. Since, as previously shown [1], the enzyme is active with both NADPH (K_m = 23 μM) and NADH (K_m = 300 μM), these patterns were determined for both coenzymes. In Fig. 1, A (NADPH oxidation activity) and B (NADH oxidation activity), the two coenzymes were varied in comparable concentration ranges, between 0.03 K_m (0.5 μM NADPH) and 0.08 K_m (40 μM NADH) up to the K_m concentration (16 μM NADPH and 320 μM NADH, respectively). Both patterns (Figs. 1 A and B) are parallel or very slightly intersecting. In the experiment of Fig. 1C, the concentration range of 3-phosphoglycerate was lowered to 5, 20, 40 and 500 μM (top to bottom) and that of the variable substrate NADH to 1 to 16 μM (0.003–0.05 K_m), using a fluorescence photometer (see Experimental). It can be seen that also under these conditions there are no significant slope effects, suggesting that the enzyme has the following general Ping Pong mechanism:



Mechanism 1. E and F symbolize two stable enzyme forms [5].

For Mechanism 1 the following rate equation is valid:

$$\frac{1}{v} = \frac{K_a}{VA} - \frac{1}{V}\left(1 + \frac{K_b}{B}\right) \quad (1)$$

where the slope term ($= K_a$) is constant and independent of B.

In the presence of product P equation (1) changes into (for derivation see Appendix):

$$\frac{1}{v} = \frac{1}{VA}\left(K_a + \frac{K_{ia}K_bP}{K_{ip}B}\right) + \frac{1}{V}\left(1 + \frac{K_b}{B} + \frac{K_bP}{K_{ip}B}\right) \quad (2)$$

Abbreviations: NADP-GPD: D-glyceraldehyde-3-phosphate dehydrogenase, NADP-linked, phosphorylating (EC 1.2.1.13); DPGA: 1,3-diphosphoglycerate; G3P: glyceraldehyde-3-phosphate; P_i: phosphate.

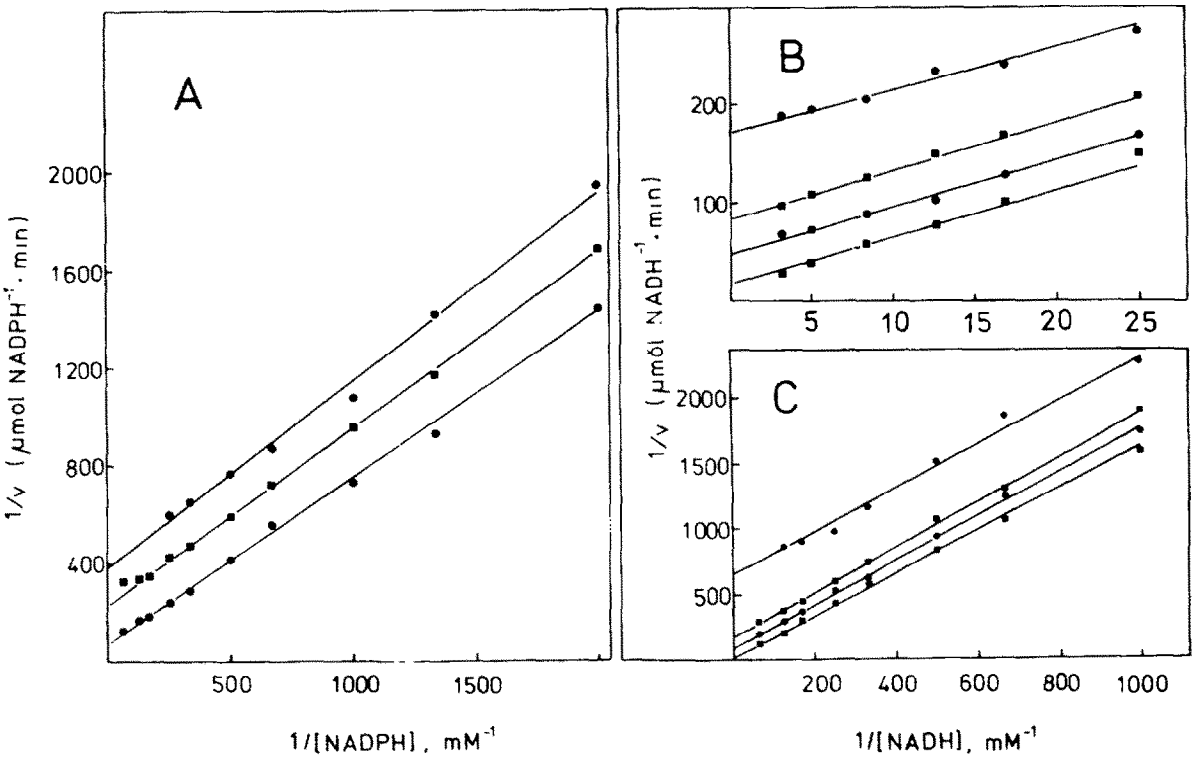


Fig. 1. Kinetics of *Sinapis alba* NADP-glyceraldehyde 3-phosphate dehydrogenase with NADPH (A) and NADH (B and C) as the variable substrate and at several fixed levels of 3-phosphoglycerate. The curves are Lineweaver Burk plots. Concentrations of 3-phosphoglycerate (from top to bottom); A: 50, 100, 500 μM; B: 50, 100, 200, 4500 μM; C: 5, 20, 40, 500 μM. Enzyme concentrations; A: 0.10 μg/ml; B: 0.70 μg/ml; C: 0.80 μg/ml. In B, initial rates were measured as decrease in absorbance (total assay volume 1 ml), in A and C as decrease in fluorescence (total assay volume 3 ml). Assays were performed as described under Experimental.

where K_{ia} and K_{ip} are the dissociation constants for the EA and EP complexes. The interesting feature of equation (2) is that the slope term is not only dependent on P, but also on B, predicting intersecting kinetics when B is varied and P is kept constant. Therefore, as suggested by Cleland [5], the transformation of parallel substrate interaction kinetics into an intersecting pattern in the presence of a finite amount of P can be used as an additional diagnostic for a Ping Pong mechanism. This approach has been applied in the experiment of Fig. 2, where substrate interaction kinetics of the kind shown in Fig. 1 B, were determined in the presence of 1 mM glyceraldehyde 3-phosphate. Fig. 2 clearly demonstrates that the parallel pattern of Fig. 1 B transforms into an intersecting one, suggesting that glyceraldehyde 3-phosphate is the first product released after binding of NAD(P)H, as depicted in the following scheme:

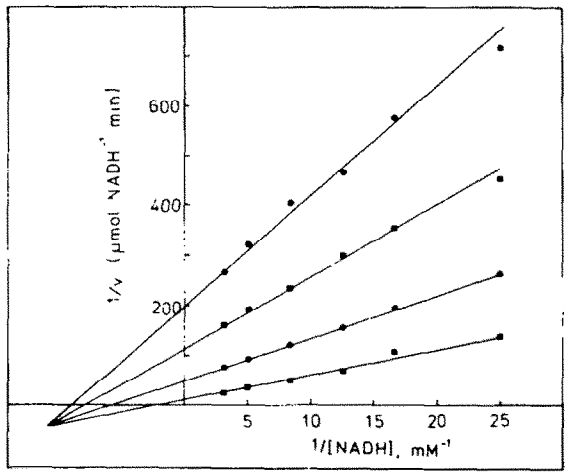
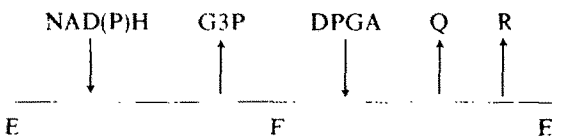


Fig. 2. Kinetics of *Sinapis alba* NADP-glyceraldehyde 3-phosphate dehydrogenase in the presence of 1 mM glyceraldehyde 3-phosphate. NADH was varied at several fixed levels of 3-phosphoglycerate (from top to bottom: 100, 200, 500, and 4500 μM). Enzyme concentration: 0.70 μg/ml. Initial rates were measured as decrease in absorbance. Assays were performed as described under Experimental.



Mechanism 2.

Product inhibition kinetics

Given Mechanism 2, product inhibition analysis can be used to test the validity of the conclusions drawn from substrate interaction kinetics and also to determine the order of release of the products phosphate and NAD-

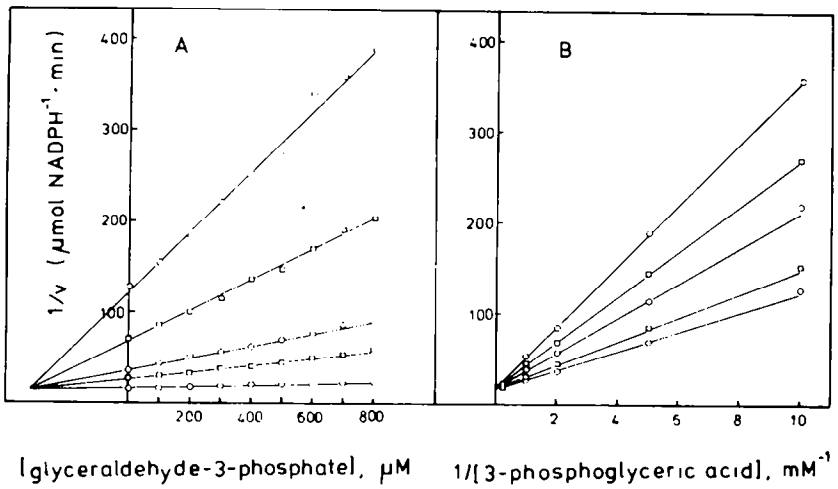


Fig. 3. Competitive inhibition by glyceraldehyde 3-phosphate vs 3-phosphoglycerate as the variable substrate and at 80 μM NADPH. A: Dixon plots for 100, 200, 500, 1000, 4500 μM 3-phosphoglycerate (from top to bottom). B: Lineweaver–Burk plots of the same data for 0, 100, 300, 500, and 700 μM glyceraldehyde 3-phosphate (from bottom to top). Enzyme concentration: 0.64 μg/ml. Initial rates were measured as decrease in absorbance. Assays were performed as described under Experimental.

$(P)^+$. For Mechanism 2 Cleland's rules of enzyme inhibition [5] predict three different inhibition patterns for the following 3 products vs 1,3-diphosphoglycerate (DPGA) as the variable substrate: I. Glyceraldehyde 3-phosphate (G-3-P): competitive (slope effects only), because substrate and product bind to the same enzyme form (F).

II. Product Q: noncompetitive (slope and intercept effects), because substrate and product bind to different enzyme forms, which are reversibly connected.

III. Product R: uncompetitive (intercept effects only), because substrate and product bind to two different enzyme forms, which are not reversibly connected (DPGA is surrounded by two product release steps).

Fig. 3 demonstrates that the inhibition by glyceraldehyde 3-phosphate with 1,3-diphosphoglycerate is linear competitive, confirming prediction I. From the linearity of the double reciprocal plots (Fig. 3 B, see also Figs. 4 and 5) it can also be inferred that the concentration of 1,3-diphosphoglycerate is proportional to the concentration of 3-phosphoglycerate varied in the assay mixture. Assuming that the dehydrogenase reaction and the spontaneous hydrolysis of 1,3-diphosphoglycerate are slow compared to the kinase reaction (the assay mixture contains 3-phosphoglycerate kinase in excess), the relationship between the two substances is expressed by the equilibrium equation:

$$[1,3\text{-diphosphoglycerate}] = K_{eq} \cdot ([ATP]/[ADP]) \times [3\text{-phosphoglycerate}]$$

with K_{eq} (the equilibrium constant) = 2.9×10^{-4} (at pH 6.9, 25°, in phosphate buffer). Proportionality implies that the ratio ([ATP]/[ADP]) remains essentially constant when 3-phosphoglycerate is varied, which would be expected if the reciprocal changes in [ATP] and [ADP] are small compared to the absolute concentrations initially present. The initial ATP concentration is 2 mM. Given a 2% contamination with ADP* the initial ADP concentration would be 40 μM and the relationship between the two substances approximates to:

$$[1,3\text{-diphosphoglycerate}] \approx 1.5 \times 10^{-2} [3\text{-phosphoglycerate}]$$

From intercept replots of Figs. 1 A and B (not shown) and from Fig. 3 B (intercept of the bottom curve with the horizontal axis) the 'K_m' for 3-phosphoglycerate would be at the most 300 to 600 μM†, which yields a 1,3-diphosphoglycerate concentration of 4.5–9 μM. If the equilibrium assumption holds, the true K_m for 1,3-diphosphoglycerate cannot be much larger than these values but may well be considerably smaller, in which case the initial ADP-concentration (which is unknown) must be higher than 40 μM. It follows from this rough estimate that the K_m for 1,3-diphosphoglycerate may be at least 30–60 times lower than the dissociation constant (K_i) for glyceraldehyde 3-phosphate, which is *ca* 300 μM as determined in Fig. 3 A (see also Table 1).

As shown in Fig. 5, $NADP^+$ is uncompetitive with 1,3-diphosphoglycerate corresponding to prediction III. It is suggested therefore that $NADP^+$ and phosphate represent products R and Q, respectively. However, as opposed to prediction II, phosphate is *competitive* with 1,3-diphosphoglycerate (Fig. 4) indicating an apparent Theorell-Chance relationship between these two reactants. Since slight intercept effects cannot be excluded (compare bottom curve with the three upper curves in Fig. 4), the mechanism should not be taken as an actual displacement but rather as an indication that

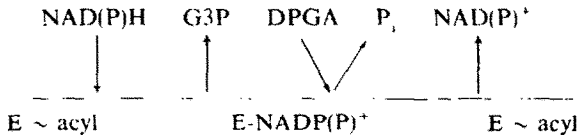
* According to the manufacturer (Boehringer Co) the average contamination of ATP with ADP and AMP is 1%, but ADP also accumulates spontaneously because 1,3-diphosphoglycerate is unstable.

† The 'K_m' for 3-phosphoglycerate, extrapolated from substrate interaction kinetics, appears to be considerably smaller at low enzyme concentrations; e.g. an intercept replot from Fig. 1 C (not shown) gives a 'K_m' of about 50 μM.

Table 1. Constants of product inhibition (K_i) of glyceraldehyde-3-phosphate dehydrogenase(NADP) from *Sinapis alba* L. (Figs. 3, 4, 8)

Product inhibitor	Varied substrate	K_i (μM)	Origin
Glyceraldehyde-3-phosphate	1,3-diphosphoglycerate	290	Fig. 3 A
Phosphate	1,3-diphosphoglycerate	5500	Slope replot of Fig. 4
$NADP^+$	NADPH	48	Fig. 8 A
NAD^+	NADPH	1200	Fig. 8 B

the concentrations of the intervening complexes are relatively low. The kinetic mechanism may now be further defined as follows:



Mechanism 3. E ~ acyl and E-NAD(P)$^+$ symbolize two separate stable enzyme forms (see text).

Mechanism 3 suggests that the enzyme oscillates between the two stable forms E ~ acyl and E-NAD(P)$^+$ containing firmly bound 3-phosphoglycerate and NAD(P)$^+$, respectively. Probably 3-phosphoglycerate is linked to the enzyme by a high energy bond (E ~ acyl), possibly a thioester, which preserves the C-1 phosphate bond energy after dissociation of P_i, as postulated for the NAD-specific glyceraldehyde-3-phosphate dehydrogenase (EC 1.2.1.12). There is no indication from the present study that free 3-phosphoglycerate (present in large excess in the assay mixture) interacts with the enzyme.

Mechanism 3 predicts three different inhibition patterns for the following three products versus NAD(P)H as the variable substrate:

IV. Glyceraldehyde-3-phosphate: noncompetitive, because substrate and product bind to two different enzyme forms (E ~ acyl and E-NAD(P)$^+$) which are reversibly connected.

V. Phosphate (P_i): uncompetitive, because substrate and product bind to two different enzyme forms which are not reversibly connected.

VI. NAD(P)$^+$: competitive, because substrate and product bind to the same enzyme form (E ~ acyl).

Fig. 6 shows that inhibition by glyceraldehyde 3-phosphate with NADH is noncompetitive as predicted (IV). Since the kinetics have a common crossover point on the horizontal axis, the question arises as to how this *pure* noncompetitivity can be rationalized in terms of the rate equation given by equation (2). Assuming that $K_a = K_{ia}$, equation (2) transforms and may be rearranged into:

$$\frac{1}{v} = \frac{K_{ia}K_b}{VBA}\left(\frac{B}{K_b} + \frac{P}{K_{ip}}\right) + \frac{K_b}{VB}\left(1 + \frac{B}{K_b} + \frac{P}{K_{ip}}\right) \quad (3)$$

which, if $\left(\frac{B}{K_b} + \frac{P}{K_{ip}}\right) > 1$, may account for the observed *pure* noncompetitivity. The assumption $K_a = K_{ia}$ implies that the transformation of the acyl-enzyme into E-NAD(P)$^+$ is slow compared to the binding of NAD(P)H.

Prediction V can also be confirmed as shown in Fig. 7. Inhibition by phosphate versus NADH (Fig. 7 A) and NADPH (Fig. 7 B) is clearly uncompetitive. At high phosphate concentrations (Fig. 7 B) the inhibition becomes nonlinear and intercept replots (not shown) are parabolic.

Fig. 8 demonstrates that inhibition by NAD(P)$^+$ vs NADPH is competitive (prediction VI). The inhibition constants are 48 and 1200 μM for $NADP^+$ and NAD^+, respectively (see also Table 1).

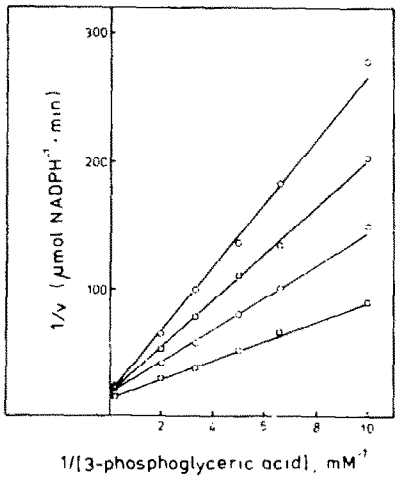


Fig. 4. Competitive inhibition by phosphate vs 3-phosphoglycerate and at 80 μM NADPH. The curves are Lineweaver-Burk plots. Phosphate concentrations (from bottom to top): 0, 4, 8, 12 mM. Enzyme concentration: 0.64 μg/ml. Initial rates were measured as decrease in absorbance. Assays were performed as described under Experimental.

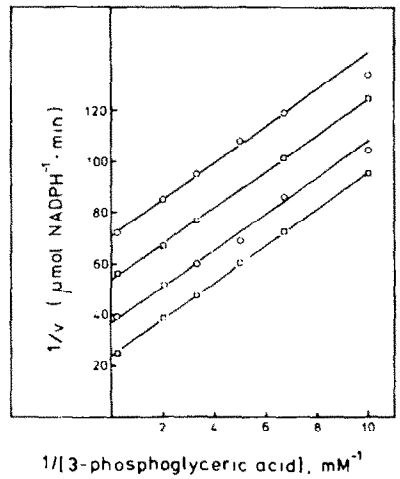


Fig. 5. Uncompetitive inhibition by $NADP^+$ vs 3-phosphoglycerate and at 80 μM NADPH. The curves are Lineweaver-Burk plots. $NADP^+$ concentrations (from bottom to top): 0, 160, 320, 480 μM. Enzyme concentration: 0.40 μg/ml. Initial rates were measured as decrease in absorbance. Assays were performed as described under Experimental.

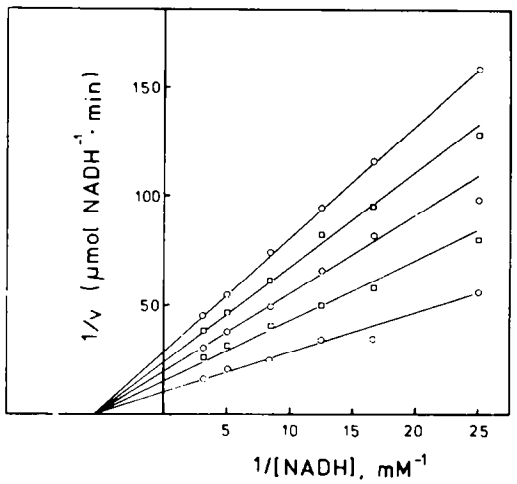


Fig. 6. Pure noncompetitive inhibition by glyceraldehyde 3-phosphate vs NADH as the variable substrate and at 500 μM 3-phosphoglycerate. The curves are Lineweaver–Burk plots. Glyceraldehyde 3-phosphate concentrations (from bottom to top): 0, 200, 400, 600, and 800 μM. Enzyme concentration: 1.3 μg/ml. Initial rates were measured as decrease in absorbance. Assays were performed as described under Experimental.

Several lines of evidence presented above indicate that the kinetic mechanism of glyceraldehyde-3-phosphate dehydrogenase(NADP) is Ping Pong and not sequential as suggested by Pupillo *et al.* [4] from studies on the reverse reaction. The main argument for a sequential mechanism in the analysis by Pupillo *et al.* are intersecting substrate interaction kinetics with $NAD(P)^+$ as the variable substrate, glyceraldehyde-3-phosphate as the changing fixed substrate and arsenate as the fixed substrate. However, as pointed out above, for a Ping Pong mechanism, parallel substrate interaction kinetics can only be expected in the *absence* of the product which separates the variable substrate from the changing fixed substrate (compare equations (1) and (2)). A K_m for 1,3-diphosphoglycerate of 10 μM or lower (see above) would probably not permit initial velocity studies of the reverse reaction in the *absence* of effective concentrations of this product, unless fluorescence techniques are used or 3-phosphoglycerate kinase is included in the assay mixture to remove the accumulating 1,3-diphosphoglycerate [3]. This argument also applies when arsenate is used as fixed substrate, since the results of Teipel and Koshland [6] indicate that the spontaneous hydrolysis of the product 1-arseno-3-phosphoglycerate is probably not fast enough to make its concentration essentially zero under steady state conditions. In addition no cooperative effects could be demonstrated for the pure *Sinapis alba* enzyme under the present conditions (all Lineweaver–Burk plots are linear). This contrasts with a previous publication by Ziegler *et al.* [7] reporting nonlinear kinetics for the partially purified enzyme from spinach chloroplasts. It remains to be established how far these 'biphasic' kinetics found by Ziegler *et al.* are characteristic for the *in vivo* situation of the enzyme.

A comparison of the present results with those of Duggleby and Dennis [3] for the NAD-specific enzyme from peas shows that the kinetic mechanisms for the two enzymes, although similar in principle (both show Ping Pong kinetics), differ in some details. For the NAD-specific enzyme, NADH was shown to be competitive with glyceraldehyde-3-phosphate, suggesting a Theorell-Chance relationship between these two reactants. This could not be confirmed for the mechanism of the present enzyme where inhibition by glyceraldehyde-3-phosphate vs NADH is purely noncompetitive (Fig. 6). Instead, for the NADP-enzyme an apparent Theorell-Chance displacement is observed between phosphate and 1,3-diphosphoglycerate (Fig. 4). Finally it should be noted that the dissociation constants of the NADP-enzyme for NAD^+ (1200 μM) and phosphate (5500 μM, see Table 1) are at least 10 times higher than the Michaelis constants for NAD^+ (97–152 μM) and phosphate (322–470 μM) reported for the NAD-specific enzyme [3]). Therefore, it seems unlikely that NAD^+ and phosphate are physiological substrates of the NADP-enzyme and that the latter plays a role as glycolytic enzyme in addition to its function in photosynthesis [4].

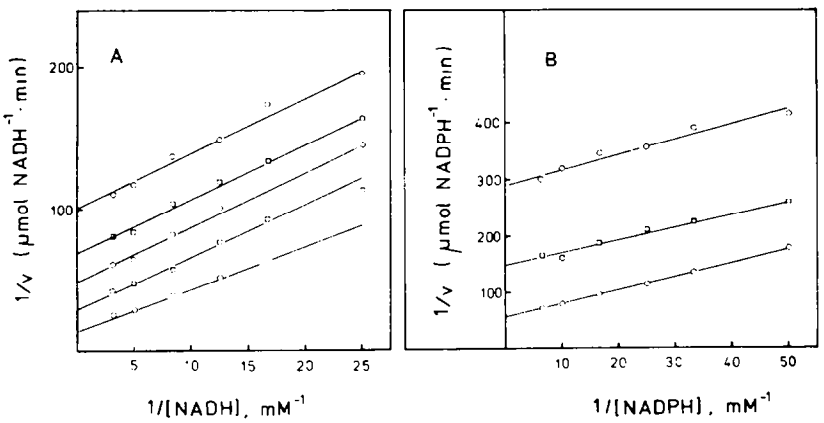


Fig. 7. Uncompetitive inhibition by phosphate vs NADH (A) and NADPH (B) as the variable substrate and at 0.5 (A) and 4.5 mM (B) 3-phosphoglycerate, respectively. The curves are Lineweaver–Burk plots. Phosphate concentrations (from bottom to top); A: 0, 4, 8, 12, and 16 mM; B: 0, 20, and 40 mM. Enzyme concentrations: 0.80 and 0.14 μg/ml for A and B, respectively. Initial rates were measured as decrease in absorbance. Assays were performed as described under Experimental.

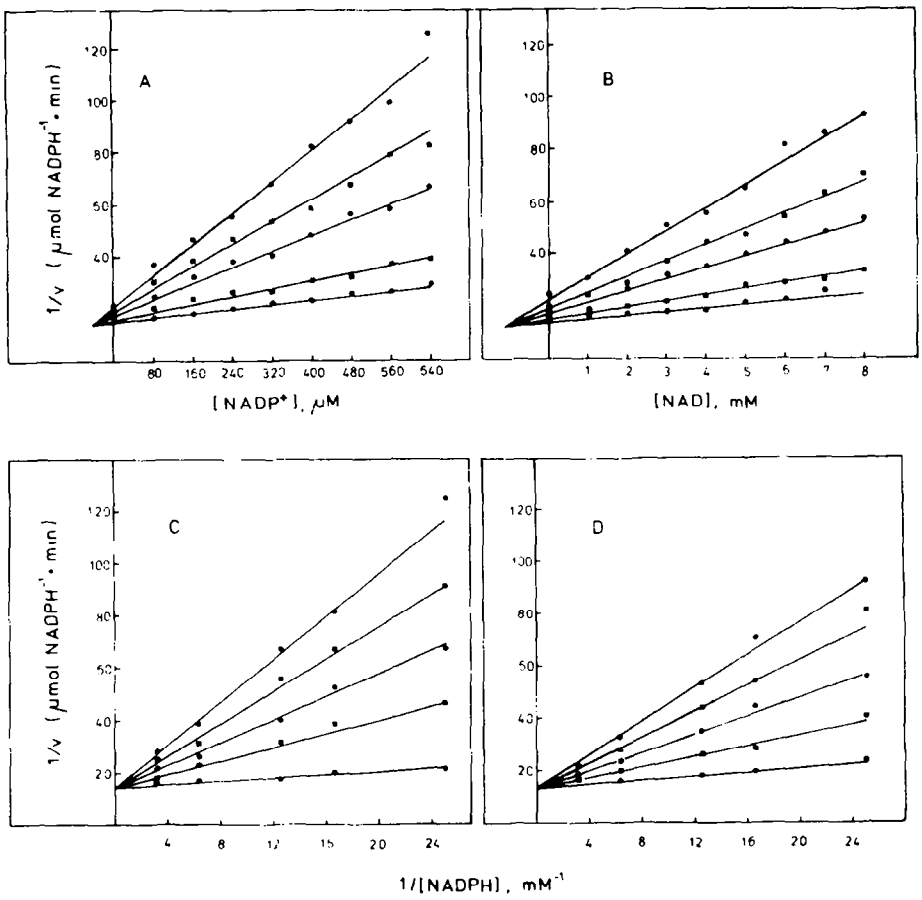


Fig. 8. Competitive inhibition by $NADP^+$ (A and C) and NAD^+ (B and D) vs various NADPH concentrations and at 4.5 mM 3-phosphoglycerate. A and B: Dixon plots for 40, 60, 80, 160, and 320 μM NADPH (from top to bottom). C and D: Lineweaver–Burk plots of the data from A and B, respectively. Inhibitor concentrations (bottom to top); C: 0, 160, 320, 480, 640 μM $NADP^+$; D: 0, 2, 4, 6, 8 mM NAD^+. Enzyme concentration: 0.50 μg/ml. Initial rates were measured as decrease in absorbance. Assays were performed as described under Experimental.

EXPERIMENTAL

Materials. Chloroplast glyceraldehyde-3-P dehydrogenase-(NAPD) from the white light grown *Sinapis alba* seedlings was purified as described previously [2]. The pure enzyme was frozen in liquid air, lyophilized and stored in aliquots at −20°. Prior to each expt an enzyme aliquot was redissolved and diluted to the appropriate concn. The enzyme activity in these solns (containing 0.1 M Tris Cl buffer, pH 7.8, 0.2 mM NAD^+, 2 mM dithioerythritol, and 1 mM EDTA) was completely stable for the duration of the expt.

Assay. Initial velocities of glyceraldehyde-3-P dehydrogenase-(NADP) were measured at 25° and pH 7.8 by following NAD(P)-H oxidation photometrically. For NAD(P)H concns ⩾ 20 μM an Eppendorf Photometer (model 1101 M with recorder) was used (with 334 or 366 nm filter). Measurements at NAD(P)H concns below 20 μM were taken with a fluorescence photometer (Perkin-Elmer, model MPT-2A) with wavelengths of 340 nm and 460 nm for excitation and emission, respectively. Corrections were made for the non-proportionality between fluorescence yield and NAD(P)H concn above 4 μM NAD(P)H.

The assay mixture contained NAD(P)H, 3-phosphoglycerate, and reaction products as indicated in the text and figure legends. 2 mM ATP, 2 mM dithioerythritol, 8 mM $MgSO_4$, 1 mM EDTA, and per ml of assay mixture 1.8 units (4 μg) 3-phosphoglycerate kinase and 0.01–0.2 units of NADP-linked glyceraldehyde-3-phosphate dehydrogenase in 0.1 M Tris-Cl buffer, pH 7.8. This mixture was incubated for 1–2 min at 25° before 3-phosphoglycerate kinase was added to initiate the reaction. Readings were taken over the period 5–30 sec after the initiation of the reaction. The measurable maximum velocity of the enzyme is reached at 4.5 mM 3-phosphoglycerate, 2 mM ATP and 0.32 mM NADPH and 1 unit of enzyme activity is defined as the amount which will catalyse 1 μmol of product per min under these conditions. A further increase in the concn of 3-phosphoglycerate and ATP has no significant influence on the initial velocity, whereas a further increase in the NADPH concn causes substrate inhibition [1].

In kinetic expts with glyceraldehyde-3-phosphate this substance was added at the very end, immediately after the addition of 3-phosphoglycerate kinase, to minimize errors resulting from the spontaneous decomposition of glyceraldehyde-3-phosphate at pH 7.8 [3]. Glyceraldehyde 3-phosphate was added as free acid (pH 5), which is stable in soln. To compensate for the variation in the amount of acid added, equivalent amounts of a buffered H_2SO_4 soln (pH 5) were used to ensure a constant pH of 7.8 in the assay mixture. Phosphate was added as $Na_2HPO_4 \cdot 2H_2O$ and NaCl was used to keep the Na^+ concn in the assay mixture constant.

Preparation and assay of glyceraldehyde 3-phosphate. Before each expt glyceraldehyde 3-phosphate was freshly prepared from D,L-glyceraldehyde 3-phosphate-diethylacetal, dicyclo-

hexylammonium salt, by acid hydrolysis (100 mM H_2SO_4) as described by the manufacturer (Boehringer). After 2 min incubation at 80° the soln was adjusted to pH 5 by addition of $NaHCO_3$. The concn of the active isomer was determined enzymatically as decrease in NADH concn in the presence of 4 μg of a 10:1 mixture (w/w) of glycerol 3-phosphate dehydrogenase and triosephosphate isomerase in Tris–Cl buffer (0.1 M, pH 7.2). Because of the instability of glyceraldehyde 3-phosphate at pH 7.2, this enzyme assay method may underestimate the concn of the active isomer by *ca* 10% [3].

APPENDIX

Derivation of Rate Equation (2)

Rate equation (2) was derived from the general rate equation for a Bi Uni Uni Uni Ping Pong mechanism in the presence of all products

$$v = \frac{V_1V_2(ABC - PQ/K_{eq})}{K_{ia}K_bV_2C + K_cV_2AB + K_bV_2AC + K_aV_2BC + V_2ABC + \frac{K_pV_1Q}{K_{eq}} + \frac{K_qV_1P}{K_{eq}} + \frac{V_1PQ}{K_{eq}} + \frac{K_qV_1AP}{K_{ia}K_{eq}} + \frac{K_aK_{ic}V_2BQ}{K_{iq}} + \frac{K_{ia}K_bV_2CQ}{K_{iq}} + \frac{K_qV_1ABP}{K_{ia}K_{ib}K_{eq}} + \frac{K_aV_2BCQ}{K_{iq}} + \frac{K_aK_{ic}V_2BPQ}{K_{ip}K_{iq}}}$$

and the corresponding Haldane equation

$$K_{eq} = \frac{V_1K_pK_{iq}}{V_2K_{ia}K_bK_{ic}}$$

published by Cleland [8]. By transformation into a Uni Uni Bi Ping Pong mechanism substrates A,B,C, become products R,Q,P, and products P,Q become substrates B,A, and initials and indices have to be exchanged accordingly. Derivation of equation (2) from this general equation was accomplished by eliminating all those terms which contain initials for products absent in the assay mixture. The above Haldane relationship was then used (after exchange of initials and indices) to substitute K_{eq} in this equation.

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