Kinetic and Isotope-Exchange Studies on Shikimate Dehydrogenase from *Pisum sativum**

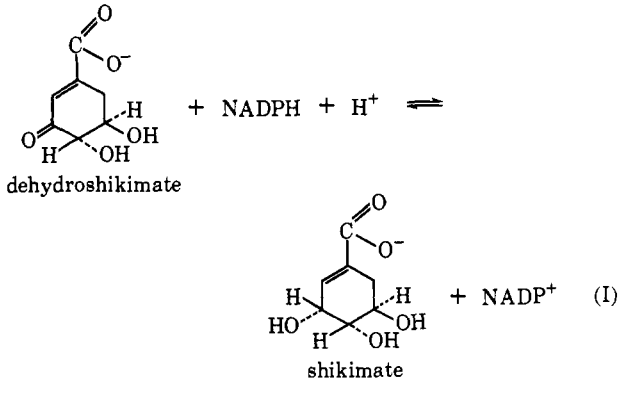
Doris Balinsky, A. W. Dennis,† and W. W. Cleland‡

ABSTRACT: Shikimate dehydrogenase, extracted from etiolated pea seedlings, has been purified 209-fold and stabilized by the addition of 1 mM cysteine to all buffers during the purification procedure. The kinetic parameters (Michaelis and dissociation constants) for the combination of the enzyme and its substrates in both the forward and reverse directions were determined at pH 7.4. The initial velocity patterns indicated a sequential mode of addition of substrates to the enzyme.

Product inhibition studies were consistent with an ordered mechanism and isotope-exchange studies at equilibrium confirmed that the mechanism is probably ordered.

Shikimate dehydrogenase (shikimate:NADP$^+$ oxidoreductase, EC 1.1.1.25) is on the pathway of biosynthesis of the aromatic amino acids (Davis, 1951; Sprinson, 1960) and of the biologically important quinones (Cox and Gibson, 1964) and lignin in higher plants (Brown, 1961). The enzyme occurs in bacteria and higher plants but not in animals (Yaniv and Gilvarg, 1955). It has been purified to some extent from *Escherichia coli* (Yaniv and Gilvarg, 1955) as well as from mung bean seedlings (Nandy and Ganguli, 1961), the epicotyls of etiolated pea seedlings (Balinsky and Davies, 1961a), and recently from the tea plant (Sanderson, 1966).

The enzyme catalyzes the NADPH-linked reduction of 5-dehyroshikimate to shikimate (see reaction I). A few



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kinetic properties of the enzyme have been reported (Yaniv and Gilvarg, 1955; Nandy and Ganguli, 1961; Balinsky and Davies, 1961a; Sanderson, 1966). Some light has been shed on the mode of attachment of shikimate and 5-dehydroshikimate to the enzyme by means of studies with structural analogs of these substrates (Balinsky and Davies, 1961b).

The present paper describes the purification of a stable enzyme preparation from whole pea seedlings, and kinetic studies aimed at elucidating further the mechanism of the reaction. Results of isotope-exchange studies are also reported.

Experimental Section

Chemicals. NADP and NADPH were obtained from Sigma while shikimic acid was obtained from Sigma, BDH, or Calbiochem. The Sigma preparation was recrystallized from ethanol before use in order to obtain a chromatographically pure sample, while the BDH and Calbiochem preparations were found to be pure. Dithiothreitol was a Calbiochem product. The [^{14}C]shikimic acid (10.15 μCi/μmole) and [^{14}C]NADP (18.5 μCi/μmole) were purchased from the New England Nuclear Corp.

Dehydroshikimic acid was synthesized by the platinum oxidation of shikimic acid using a modification of the method of Haslam *et al.* (1957). A 5% solution of shikimic acid was adjusted to pH 4.5 with solid $NaHCO_3$ and the oxidation carried out for 15 hr at 40° to obtain maximum yields of dehydroshikimate. The dehydroshikimate was fractionated on a Dowex 1 column, concentrated, and recrystallized from ethyl acetate as described by Haslam *et al.* (1957). Protocatechuic acid, a breakdown product of dehydroshikimate, remained in the mother liquor. Since protocatechuate is a competitive inhibitor *vs.* shikimate for the reverse reaction (Balinsky and Davies, 1961b), solutions of dehydroshikimate were checked for absence of protocatechuate by running thin-layer plates in benzyl alcohol–isopropyl alcohol–*sec*-butyl alcohol–water (3:1:1:1, v/v) containing 2% formic acid, and spraying with alkaline silver nitrate. In this solvent protocatechuate runs near the solvent front, while dehydroshikimate has a low R_F.

Ammonium sulfate saturations were calculated according to the nomogram of Dixon (1953).

Calcium phosphate gel was prepared by the method of Keilin and Hartree (1938), and DEAE-cellulose, purchased from Whatman, was freed from impurities as described by Peterson and Sober (1962). DEAE-cellulose paper (DE-81) was purchased from Sargent.

Buffers. Throughout the purification procedure sodium phosphate buffer (pH 7.4) containing 1 mM cysteine to protect the enzyme from oxidation was used unless otherwise

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stated. When the purified enzyme was diluted for kinetic studies, 0.1 mM dithiothreitol was used instead of cysteine.

Assay Procedure. During purification the enzyme was assayed at pH 9.0 using shikimate and NADP as substrates according to the method of Balinsky and Davies (1961a). A Beckman DB or a Unicam SP 800 recording spectrophotometer with scale expander attachments was used. The temperature was controlled by circulating water at 30° around the cuvet holder. Protein concentrations were measured by the method of Warburg and Christian (1941). A unit of activity is defined as the amount of enzyme catalyzing the production of 1 μmole of NADPH/min, assuming a molar extinction coefficient for NADPH at 340 mμ of 6.22×10^3 (Horecker and Kornberg, 1948). Specific activity is the activity per milligram of protein. For measurement of the kinetic parameters, a Gilford spectrophotometer was used, and water at 30° was circulated as above. Potassium phosphate buffer (0.1 M, pH 7.4) was used for the assays, as dehydroshikimate is unstable at basic pH values.

Data Processing. Reciprocal velocities were plotted against reciprocal substrate concentrations, and when the plots were linear, the data were fitted to eq 1 using a least-squares method and assuming equal variance for the velocities (Wilkinson, 1961). The computer programs of Cleland

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

(1963a) were used, which give values for the constants and their standard errors. Data conforming to a sequential initial velocity pattern were fitted to eq 2, and those conforming to competitive and noncompetitive inhibitions to eq 3 and 4, respectively. In eq 2, K_a and K_b are the Michaelis

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

$$v = \frac{VA}{K\left(1 + \frac{I}{K_{is}}\right) + A} \tag{3}$$

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

constants of the two substrates, and K_{ia} is the dissociation constant of the first substrate to attach if the mechanism is ordered. For a random mechanism, K_{ib}, the dissociation constant for B, is obtained from the relationship $K_{ia}K_b = K_aK_{ib}$. In eq 3 and 4, K_{is} and K_{ii} are the inhibitor constants affecting the slope and intercept, respectively.

Isotope Exchange. A mixture of all reactants was prepared in 0.1 M potassium phosphate buffer (pH 7.4) such that the ratio of products to reactants was equal to the equilibrium constant. When measuring incorporations of label from shikimate to dehydroshikimate, a trace of radioactive shikimate (not more than 5% of the concentration of unlabeled shikimate) was added to the reaction mixture, and the reaction started by addition of a suitable amount of enzyme. Alternatively, radioactive shikimate was added last after 10-min equilibration at 30°. The mixture was incubated at 30°. Aliquots (50 μl) were removed with a Hamilton syringe at suitable intervals, applied to DEAE-cellulose paper, and dried with a hair dryer. Shikimate and dehydroshikimate were separated by ascending chromatography in 2 N acetic acid (R_F (dehydroshikimate) 0.3, R_F (shikimate) 0.7) and located on marker strips with a periodate–aniline spray reagent (Yoshida and Hasegawa, 1957). The sections of paper containing each compound were counted in a liquid scintillation counter.

When measuring incorporations of label from NADP to NADPH, a mixture of unlabeled and [^{14}C]NADP in a ratio of 6.5:1 was used in the equilibrium mixture, and the reaction started with enzyme. The nucleotides were separated by ascending chromatography on DEAE-cellulose paper in 0.15 M potassium phosphate buffer (pH 7.4) (R_F (NADPH) 0.3, R_F (NADP) 0.7) and visualized under ultraviolet light where the NADPH spot fluoresced and the NADP absorbed. The spots were cut out and counted in a liquid scintillation counter.

Purification Procedure. Etiolated pea seedlings (*Pisum sativum* var. Greenfeast) were harvested after 7-days growth, and washed in tap water. The seed coats together with all regions showing fungal infection were removed, and the seedlings were chilled to 4°. The succeeding steps took place in a refrigerated room.

Extraction and Ammonium Sulfate Fractionation. The chilled seedlings were homogenized in a Waring blender for 90 sec with 0.5 volume of 0.1 M buffer. The extract was squeezed through cheese cloth and solid ammonium sulfate added to 35% saturation. The precipitated protein was removed by centrifugation at 14,000g for 20 min in a Sorvall SS-3 high-speed centrifuge. The concentration of ammonium sulfate was raised to 52% saturation and the precipitated protein collected again by centrifugation at 14,000g for 20 min. The precipitate, redissolved in a minimal volume of 0.1 M buffer, was dialyzed for 4 hr against 12 volumes of the same buffer. The enzyme was then refractionated with ammonium sulfate using a 95% saturated solution at pH 7.4. The fraction precipitating between 28 and 44% saturation was collected as above, and redissolved in a minimal volume of 0.05 M buffer.

Calcium Phosphate Gel Fractionation. The protein solution was dialyzed overnight against 15 volumes of 5 mM buffer. It was adjusted to pH 6.3 by dialysis for a further 3 hr against 25 volumes of 5 mM buffer (pH 6.3). To the enzyme solution was added one-fifth of its volume of cold calcium phosphate gel. After 5 min the gel was centrifuged down at 800g for 5 min in an MSE Major refrigerated centrifuge at 0°. This process was repeated until all the activity had been adsorbed. The gel was then washed with cold water and the shikimate dehydrogenase eluted using 0.05 M buffer (pH 7.4).

Concentration of the Enzyme. The eluate was concentrated by overnight dialysis against a 95% saturated solution of ammonium sulfate (pH 7.4). The precipitate formed was collected at 0° by centrifugation for 10 min at 20,000g in an MSE refrigerated centrifuge, and redissolved in a minimal volume of 0.05 M buffer. This step, as well as concentrating the enzyme, brought about a further increase in specific activity. The enzyme was found to be stable for up to 6 weeks when stored at −15° at this stage.

DEAE-cellulose Column Fractionation. Six batches of enzyme were prepared to this stage; the fractions were then combined and dialyzed against 25 volumes of 5 mM buffer (pH 6.3) for 4 hr. The enzyme was adsorbed onto a DEAE-cellulose column previously equilibrated with the same buffer. The column was washed with 5 mM buffer to remove the unadsorbed protein. Shikimate dehydrogenase was eluted

TABLE I: Purification of Shikimate Dehydrogenase.[a]

Fraction	Total Act. (Units)	Total Protein (g)	Sp Act. (Units/mg of Protein)	Purificn Factor	Yield (%)
Crude homogenate	1030	770.1	0.0013	1.0	100
First ammonium sulfate fractionation (35–52% saturation)	1005	228.8	0.0044	3.3	98.6
Second ammonium sulfate fractionation, pH 7.4 (28–44% saturation)	806	87.6	0.0092	6.9	78.1
After dialysis	545	37.2	0.0147	10.9	52.8
Calcium phosphate gel eluate	288	4.1	0.0697	52.0	27.9
After concentration against saturated $(NH_4)_2SO_4$	281	2.6	0.109	81.5	27.2
DEAE-cellulose column eluate	112.5	0.42	0.268	200	10.9
After concentration against saturated $(NH_4)_2SO_4$	100.5	0.36	0.279	209	9.8

[a] The enzyme was assayed spectrophotometrically at 340 mμ in quartz cuvets of 1-cm path length containing 270 μmoles of glycine–NaOH buffer (pH 9.0), 3 μmoles of sodium shikimate, 0.5 μmole of NADP, and enzyme diluted to give an optical density change not greater than 0.03/min.

with 0.05 M buffer. The enzyme was concentrated by dialysis against 95% saturated ammonium sulfate and the precipitate redissolved in 0.05 M buffer and stored at −15° until required. The purified enzyme was found to be stable for at least 3 months.

Results

Table I shows the results of a typical purification procedure. An overall purification of 209-fold was obtained and the method was found to be reproducible. The presence of cysteine in the buffers was found to protect the enzyme from inactivation. However, it was only after the calcium phosphate gel step that the enzyme was sufficiently stable to be kept for weeks with little activity loss.

TABLE II: Kinetic Constants for Shikimate Dehydrogenase Determined from Initial Velocity and Product Inhibition Studies.[a]

Kinetic Constant	Initial Velocity	Product Inhibn
K_{NADPH}	4.3 ± 0.5 μM	
$K_{i\ NADPH}$	4.3 ± 0.7 μM	8.9 ± 0.4 μM
K_{DHS}[b]	0.34 ± 0.04 mM	
$K_{i\ DHS}$		9 ± 3 mM
K_{NADP}	10.3 ± 0.7 μM	
$K_{i\ NADP}$	21 ± 2 μM	
K_{SHIK}[b]	0.60 ± 0.04 mM	
$K_{i\ SHIK}$		34 ± 8 mM
V_1/V_2	1.57	

[a] All constants were measured in 0.1 M potassium phosphate buffer (pH 7.4) at 30°. [b] DHS = dehydroshikimate; SHIK = shikimate.

Initial Velocity Patterns. In order to determine the dissociation and Michaelis constants of the substrates dehydroshikimate and NADPH, reciprocal velocities were plotted against reciprocal NADPH concentration at several constant concentrations of dehydroshikimate. The family of lines obtained (Figure 1) intersected in a point, indicating a sequential mechanism, *i.e.*, both substrates must add to the enzyme before any product is released. The data were thus fitted to eq 2 and the resulting constants are shown in Table

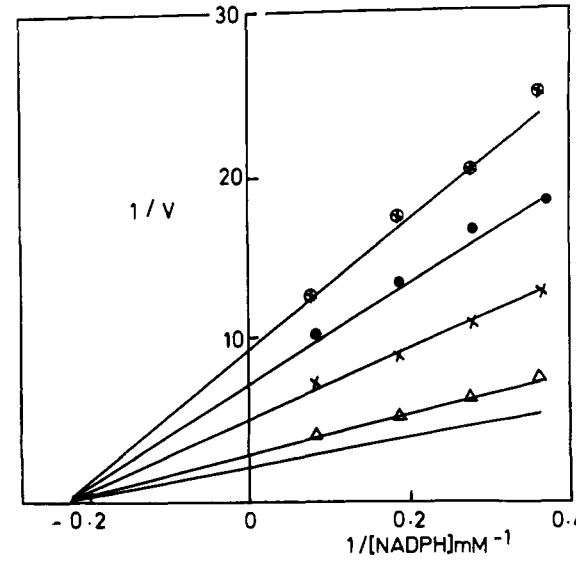


FIGURE 1: Reciprocal plots with NADPH as variable substrate. Dehydroshikimate concentrations: (⊗) 0.10 mM; (●) 0.143 mM; (×) 0.25 mM; and (△) 1.0 mM. The lowest line is extrapolated to infinite dehydroshikimate concentration. The enzyme was assayed at 30° in 0.1 M potassium phosphate buffer (pH 7.4) in silica cuvets of 5-cm path length. Velocity is expressed as the change in optical density at 340 mμ/min, as measured in a Gilford spectrophotometer.

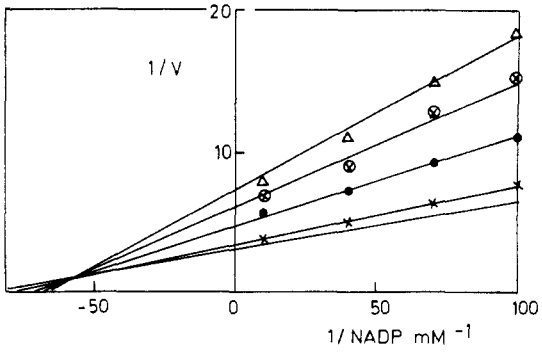


FIGURE 2: Reciprocal plots with NADP as the variable substrate. Shikimate concentrations: (△) 0.47 mM; (⊗) 0.67 mM; (●) 1.17 mM, and (×) 4.69 mM. The lowest line is extrapolated to infinite shikimate concentration. Other experimental details were as described for Figure 1, except that silica cells of 1-cm path length were used.

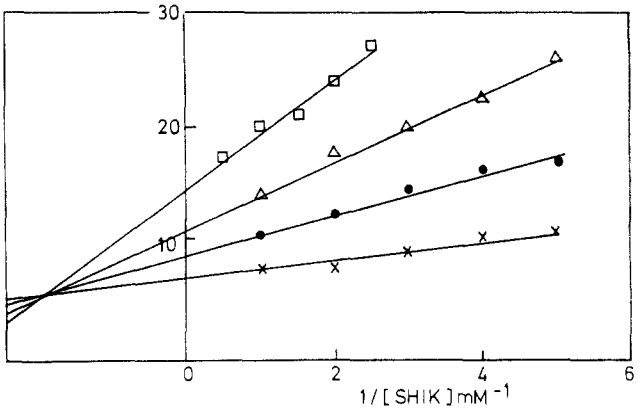


FIGURE 4: NADPH as product inhibitor *vs.* shikimate. NADPH concentrations: (×) 0, (●) 19 μM, (▲) 35 μM, and (□) 72 μM. NADP concentration: 52 μM. Other experimental details were as in Figure 2.

II. The constants for NADP and shikimate were obtained similarly (Figure 2).

Product Inhibition Studies. Several enzyme mechanisms can be distinguished by studies of the effects of reaction products on the initial velocity of the reaction. NADPH was found to give linear competitive inhibition with NADP as variable substrate both at unsaturating (Figure 3) and saturating levels of shikimate. NADPH was a noncompetitive inhibitor *vs.* shikimate at low NADP concentrations (Figure 4), but the inhibition was eliminated on raising the NADP concentration to 400 times its Michaelis constant. Figures 5 and 6 show the effects of dehydroshikimate on the back-reaction and shikimate on the forward reaction, respectively. When data were fitted both to eq 3 and 4 statistical analysis indicated a smaller residual least square for the noncompetitive than for the competitive fit, though K_{ii} values were large and had high standard errors. The true inhibition constants for shikimate and dehydroshikimate obtained by comparing the equations for product inhibition in an ordered bi-bi mechanism (Cleland, 1963b,c) with eq 3 and 4 are shown in Table II.

Equilibrium Constant. Yaniv and Gilvarg (1955) had obtained an apparent equilibrium constant for the reaction at pH 7.0 of 27.7 and at pH 7.8 of 5.7 by allowing the reaction to reach completion and measuring the concentrations of dehydroshikimate and NADPH present at equilibrium. We measured the equilibrium constant by mixing 85.7 μM NADP, 21.4 μM NADPH, 28.6 μM dehydroshikimate, and varying amounts of shikimate between 470 and 860 μM until a mixture was found which showed no change in optical density on incubation at 30° at pH 7.4. The apparent equilibrium constant, K_{eq}' = [shikimate][NADP]/[dehydroshikimate][NADPH], was found to be 10.3 and 8.2 in two separate experiments.

Isotope Exchange. When the concentration of the NADP–NADPH pair was raised in constant ratio, keeping the mixture under equilibrium conditions, the rate of exchange between shikimate and dehydroshikimate increased, and plots of reciprocal exchange rate *vs.* reciprocal substrate concentration showed no evidence of substrate inhibition (Figure 7). A similar plot was obtained when exchange between NADP and NADPH was measured under these conditions (Figure 7). When the NADP and NADPH concentrations were held constant and the concentration of shikimate and dehydroshikimate were varied in constant ratio, NADP–NADPH exchange was eliminated as the concentration of the substrate pair was raised (Figure 8), but exchange between shikimate

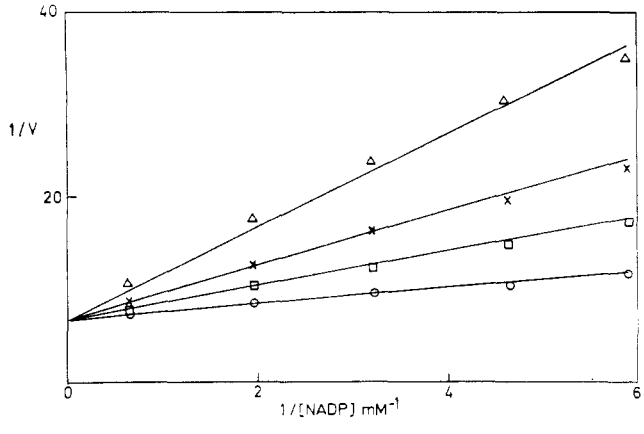


FIGURE 3: NADPH as product inhibitor *vs.* NADP. NADPH concentrations: (○) 0, (□) 10 μM, (×) 20 μM, and (△) 40 μM. Shikimate concentration: 12 mM. Other experimental details were as in Figure 2.

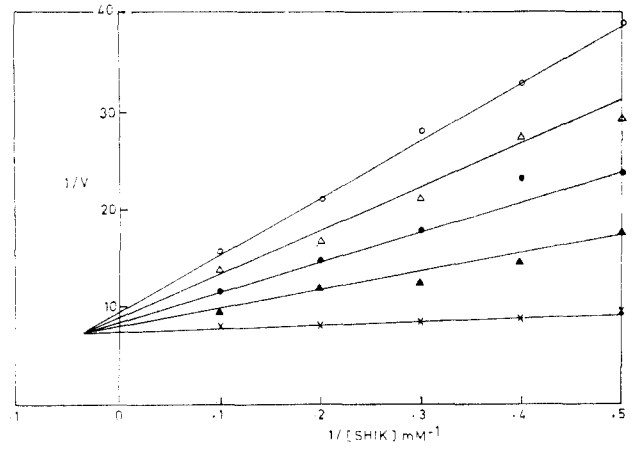


FIGURE 5: Dehydroshikimate as product inhibitor *vs.* shikimate. Dehydroshikimate concentrations: (×) 0, (▲) 0.63 mM, (●) 1.25 mM, (△) 1.88 mM, and (○) 2.5 mM. NADP concentration: 527 μM. Other experimental details were as described for Figure 2.

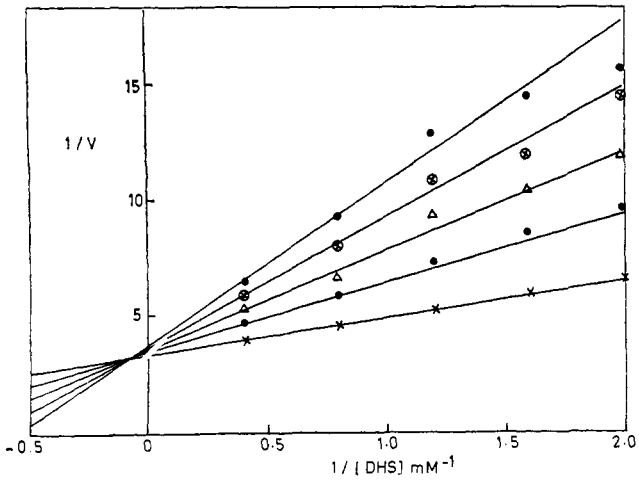


FIGURE 6: Shikimate as product inhibitor *vs.* dehydroshikimate. Shikimate concentrations: (×) 0, (●) 1.5 mM, (△) 3.0 mM, (⊗) 4.5 mM and (●) 6 mM. NADPH concentration: 50 μM. Other experimental details were as described for Figure 2.

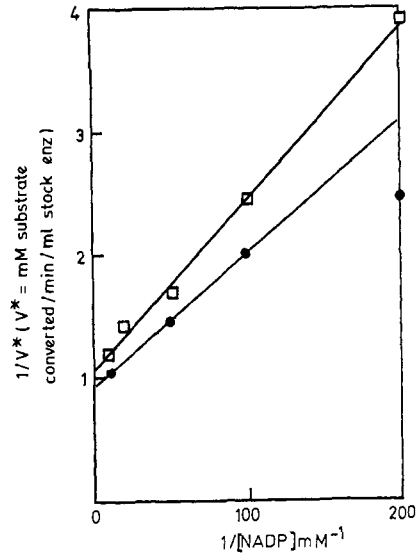


FIGURE 7: Isotope exchange varying the NADP–NADPH pair. (□) NADP* → NADPH exchange and (●) shikimate* → dehydroshikimate exchange. The reaction mixture contained 0.1 M potassium phosphate buffer (pH 7.4), 1.0 mM shikimate, 0.33 mM dehydroshikimate, and NADP and NADPH in a ratio of 2.7:1.

and dehydroshikimate under these conditions showed no evidence of substrate inhibition (Figure 8).

Discussion

A stable preparation of shikimate dehydrogenase from pea seedlings, purified 209 times, has been obtained. The stabilizing effects of cysteine and dithiothreitol are presumably due to protection by these compounds of enzyme sulfhydryl groups from oxidation since the enzyme is susceptible to inactivation by the sulfhydryl group reagent *p*-hydroxymercuribenzoate (Balinsky and Davies, 1961a). The increased stability of the enzyme following the calcium phosphate gel step may be due to separation from proteolytic enzymes at this stage. A similar finding has been reported for the preparation of quinate dehydrogenase from mung bean seedlings (Gamborg, 1966) where it was found that the enzyme was stabilized after hydroxyapatite treatment.

The limiting Michaelis constants obtained for shikimate and NADP are very similar to the apparent Michaelis constants of 0.19–0.28 mM and 7 μM, respectively, found at pH 9.0 by Balinsky and Davies (1961a) for the enzyme from pea epicotyls. The Michaelis constants are affected by pH, and values found at pH 9.0 correspond even more closely to the above (A. W. Dennis and D. Balinsky, unpublished results). The K_m values for shikimate are of the same order of magnitude as the apparent Michaelis constants of 0.09 mM for mung bean seedlings at pH 8 (Nandy and Ganguli, 1961) and 0.43 mM for tea shoots at pH 9 (Sanderson, 1966). The values for NADP are, however, much lower than the value of 0.117 mM found for mung bean seedlings (Nandy and Ganguli, 1961), but comparable to the value of 32 μM found for tea shoots (Sanderson, 1966). Some caution should be exercised with these comparisons, since the K_m value appears to be affected by the buffer used; *e.g.*, at pH 8.0, $K_{m,app}$ values for shikimate in 0.1 M potassium phosphate and Tris buffers were 0.2 and 0.5 mM, respectively (Galvin, Sutherland, and Balinsky, unpublished results).

The findings that the plots of reciprocal velocity *vs.* reciprocal substrate concentration at various fixed concentrations of the other substrate are linear and meet in a point (Figures 1 and 2) indicate that the mechanism is sequential, *i.e.*, both substrates must attach to the enzyme before any product is released. From these results it is not possible, however, to determine whether reactants can add in a random order, or must follow an obligatory order of addition. Recent computer simulation experiments (W. W. Cleland, unpublished) have indicated that random mechanisms, even those in which interconversion of the central complex is not the rate-limiting step, usually give linear reciprocal plots.

Product inhibition experiments can be used to distinguish between various sequential mechanisms. The present results indicate competitive inhibition by NADPH *vs.* NADP but noncompetitive inhibition *vs.* shikimate, which is con-

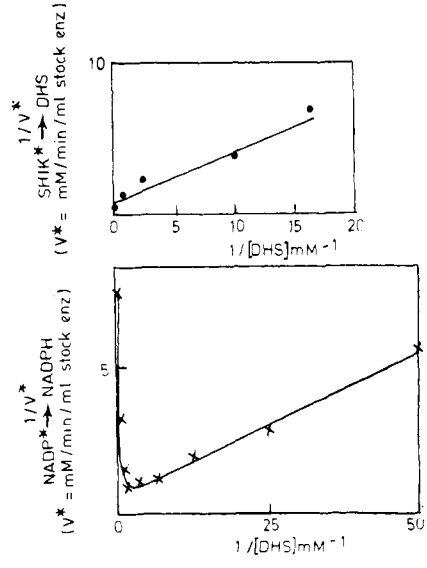
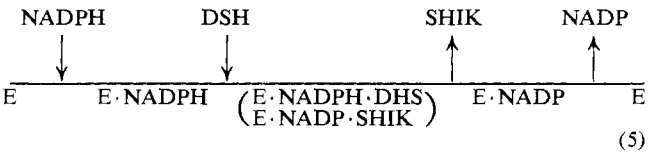


FIGURE 8: Isotope exchange varying the shikimate–dehydroshikimate pair. (●) Shikimate* → deydroshikimate exchange; (×) NADP* → NADPH exchange. The reaction mixture contained 0.1 M potassium phosphate buffer (pH 7.4), 82 μM NADP, 30 μM NADPH, and shikimate and dehydroshikimate in a 3:1 ratio.

sistent with either ordered or random addition of substrates. Results for shikimate (SHIK) inhibition *vs.* dehydroshikimate (DHS) and *vice versa* indicate slightly noncompetitive inhibition, though the effect on the intercepts in Figures 5 and 6 is very small and very high K_{ii} values are obtained compared to the K_{is} values. These inhibitions would be competitive in a random mechanism, so these data suggest the mechanism is ordered.

Further evidence that this mechanism is an ordered one comes from an examination of the Haldane relationships. For an ordered bi-bi mechanism as shown in eq 5, the Haldane



(5)

relationship is given by

$$K_{eq}' = \frac{V_1}{V_2}\frac{K_pK_{iq}}{K_{ia}K_b} = \left(\frac{V_1}{V_2}\right)^2\frac{K_{ip}K_q}{K_aK_{ib}}$$

Substituting the values shown in Table II into these equations gives $K_{eq}' = 13.5$ and 22.6. These results agree reasonably well with the equilibrium constant of 8–10 determined directly. Since the values for K_{ip} and K_{ib} are large and not very reliable, the first Haldane relationship above will give a more accurate result and is, in fact, closer to the measured equilibrium constant.

The most convincing evidence for an ordered mechanism is given by the results of the isotope-exchange studies. The rate equation for isotope-exchange at equilibrium from A to Q for an ordered bi-bi mechanism is

$$v^* = \frac{V_1}{1 + \frac{V_1}{V_2} + \frac{K_a}{A} + \frac{K_b}{B} + \frac{K_{ia}K_b}{AB} + \frac{K_qK_{ia}K_bP}{K_{iq}K_pAB} + \frac{V_1K_p}{V_2P} + \frac{K_qK_bP}{K_{iq}K_pB} + \frac{V_1K_aK_pB}{V_2K_{ia}K_bP} + \frac{P}{K_{ip}} + \frac{V_1P}{V_2K_{ib}}}$$

where A, B, P, and Q represent NADP, shikimate, dehydroshikimate, and NADPH, respectively. The numerator is multiplied by the factor $(1 + K_qP/K_qK_{iq} + K_aB/K_{ia}K_b)$ when measuring the velocity of exchange between shikimate and dehydroshikimate (B and P). It can be seen that A → Q exchange will be eliminated on raising the B–P pair to very high levels, but not B → P exchange. This is in fact found. Figure 8 shows that NADP–NADPH exchange is eliminated as the shikimate–dehydroshikimate pair is raised, but that shikimate–dehydroshikimate exchange is not. In a mechanism in which there are alternate pathways, a "substrate inhibition" pattern might occur, but the line would cross the ordinate at a finite value of $1/V$. The apparently total substrate inhibition seen here strongly argues for an ordered mechanism as shown in eq 5.

Acknowledgments

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