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Regulation of Phosphoenolpyruvate Carboxylase of *Zea mays* by Metabolites

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Crude preparations of phosphoenolpyruvate carboxylase obtained from aetiolated seedlings of *Zea mays* are unstable but can be stabilized with glycerol. At the pH optimum of 8.3, the K_m value for phosphoenolpyruvate is 80μM. When assayed at 30°C, the enzyme shows normal Michaelis–Menten kinetics, but when assayed at 45°C sigmoid kinetics are exhibited. At pH7.0 the enzyme is inhibited by a number of dicarboxylic acids and by glutamate and aspartate. D and L forms of the hydroxy acids and amino acids are inhibitory and the kinetics approximate to simple non-competitive inhibition. The same compounds produce less inhibition at pH7.6 than at pH7.0 and the kinetics of inhibition are more complex. The enzyme is activated by P_i, by SO_4^{2-} and by a number of sugar phosphates. Maximum activation occurs at acid pH values, where enzyme activity is lowest. The enzyme is activated by AMP and inhibited by ADP and ATP so that the response to energy charge is of the R type and is thus at variance with Atkinson's (1968) concept of energy charge. The physiological significance of the response to metabolites is discussed.

The replenishment of the tricarboxylic acid cycle can be accomplished by pyruvate carboxylase or phosphoenolpyruvate carboxylase. Plants and many bacteria contain phosphoenolpyruvate carboxylase and, as may be expected from its anaplerotic role (Kornberg, 1965), the enzyme in micro-organisms is subject to allosteric control, being activated by acetyl-CoA (Canovas & Kornberg, 1965, 1966) and sometimes by fructose diphosphate (Sanwal & Maeba, 1966) and being inhibited by aspartate and sometimes by organic acids (Maeba & Sanwal, 1965; Corwin & Fanning, 1968; Liao & Atkinson, 1971).

The enzyme has been highly purified from peanut cotyledons (Maruyama *et al.*, 1966) and from potatoes (Smith, 1968) but no evidence of control mechanisms was obtained. Various attempts to demonstrate allosteric effectors have produced limited success. Ting (1968) found that the enzyme from maize roots was inhibited by malate, oxaloacetate and aspartate. C. R. Slack (unpublished work; cited by Hatch & Slack, 1970), however, reported that the enzyme from maize leaves is inhibited by oxaloacetate but not by malate and aspartate. Waygood (1971) has reported that of the many metabolites studied as possible effectors of phosphoenolpyruvate carboxylase only one, glucose 6-phosphate, behaved as an effector. Stimulation of CO_2 fixation by low concentrations of AMP in a particulate system has been observed by Graham & Young (1959) and stimulation by low concentrations of orthophosphate was noted by Tchen & Vennesland (1955).

The lack of metabolic regulation noted with purified preparations of phosphoenolpyruvate carboxylase from plants could represent a loss of allosteric properties, which frequently occurs during enzyme purification. In particular it is known that thiol compounds, which are often used in the preparation of enzymes from plants, frequently inhibit allosteric responses. Consequently we have used a plant low in polyphenol oxidase activity, to avoid the use of thiol compounds, and have used relatively crude preparations to retain maximum allosteric properties.

Experimental

Materials

Buffers. All buffers were prepared with glass-distilled water. Tris was obtained under the trade name Trizma from Sigma (London) Chemical Co., London S.W.6, U.K. It was dissolved in water at 25°C and adjusted to the required pH with HCl (A. R. grade).

Materials for scintillation counting. 2,5-Diphenyloxazole was obtained from British Drug Houses Ltd., Poole, Dorset, U.K., and 1,4-bis-(4-methyl-5-phenyloxazol-2-yl)benzene was obtained from Packard Instruments Co., Wembley, Middx., U.K.

Chemicals. Sodium salts of phosphoenolpyruvate, NADH, AMP, ADP, ATP and various sugar phosphates were obtained from Sigma (London) Chemical Co. $NaH^{14}CO_3$ (40mCi/mmol) was obtained from The Radiochemical Centre, Amersham, Bucks., U.K. Other chemicals used were obtained from Hopkin and Williams Ltd., Chadwell Heath, Essex, U.K.

Methods

Preparation of enzyme. Seeds of *Zea mays* L. var. Horsetooth were soaked overnight in water and then germinated in the dark for 6 days at 25°C. Aetiolated leaves and coleoptiles (158g) were ground with 7ml of Tris–HCl buffer (0.2M, pH7.6) and sand (approx. 5g) in a mortar and pestle. After passage through four layers of muslin the homogenate was centrifuged at 40000*g* for 20min. The supernatant was then desalted on a column (2.5cm×30cm) of Sephadex G-25 previously equilibrated with Tris–HCl buffer (10mM, pH7.6). The resulting crude extract was immediately used for the assay of phosphoenolpyruvate carboxylase activity. All procedures were carried out at 4°C.

Enzyme assay. Phosphoenolpyruvate carboxylase activity was assayed at 30°C by coupling the reaction with malate dehydrogenase. The oxidation of NADH was measured at 340nm in a Unicam SP. 500 spectrophotometer equipped with a Vitatron recorder. The standard assay system contained Tris–HCl buffer (33.3mM; pH varied as indicated), sodium phosphoenolpyruvate (0.67mM), NADH (0.13mM), $NaHCO_3$ (10mM), $MgCl_2$ (16.7mM), maize extract and water in a total volume of 3ml. Alternatively, enzyme activity was assayed by measuring the amount of ^{14}C incorporated into malate from $NaH^{14}CO_3$. The assay system was the same as for the spectrophotometric system except that 2μCi of $NaH^{14}CO_3$ was also included. The reaction was started by the addition of the enzyme and after 10min was stopped by the addition of $HClO_4$ (final concentration 5%). After aeration and neutralization with Tris base, 0.1ml of each sample was counted for ^{14}C radioactivity in a Packard liquid-scintillation spectrometer. A stock scintillation fluid was prepared by dissolving 2,5-diphenyloxazole (7g) and 1,4-bis-(4-methyl-5-phenyloxazol-2-yl)benzene (600mg) in 400ml of Cellosolve (2-ethoxyethanol) and 600ml of toluene. Samples of radioactive solutions were mixed with 15ml of scintillation fluid and their radioactivity was counted for 5min.

The enzyme was also assayed by following the formation of oxaloacetate at 270nm by using a double-beam Unicam SP.800 spectrophotometer. The assay medium used was the same as for the coupled-enzyme assay except that NADH was omitted. The reference cuvette contained all the components except phosphoenolpyruvate. This assay could not be used when nucleotides were being examined as allosteric effectors.

Results

Stability

The enzyme was very unstable, losing as much as 40% of its original activity in 8h. Compounds such as 2-mercaptoethanol and cysteine were inhibitory, but when glycerol was added to the extract to give a final concentration of 25% (v/v) the enzyme lost very little activity after 50h at 4°C.

pH optimum

Studies on the effect of pH on enzyme activity are complicated by the fact that the concentration of H^+ ion affects the equilibrium between CO_2 and bicarbonate. Maruyama *et al.* (1966) produced evidence that bicarbonate was the reactive substrate, but Waygood *et al.* (1969) have reported that CO_2 is the substrate for the carboxylase of maize. In the present study, with saturating concentrations of bicarbonate (10mM), the pH optimum was found to be near 8.3.

Effect of concentration of phosphoenolpyruvate

When bicarbonate and $MgCl_2$ were kept at saturating concentrations ($NaHCO_3$, 10mM; $MgCl_2$, 16.7mM) and the enzyme activity was assayed at 30°C, plots of rate versus phosphoenolpyruvate concentrations gave normal Michaelis–Menten curves at pH7.0, 7.5 and 8.3. Saturation occurred at approx. 800μM-phosphoenolpyruvate, and concentrations above this value were inhibitory. The K_m values for phosphoenolpyruvate were 180, 160 and 80μM at pH7.0, 7.5 and 8.3 respectively. The effects of metabolites on the activity of the enzyme were studied at 30°C. However, when the enzyme was assayed at 45°C, in the presence of glycerol (2%, v/v) to stabilize the enzyme, the plot of rate versus phosphoenolpyruvate concentration gave a sigmoid curve (Fig. 1). The use of glycerol completely stabilized the enzyme

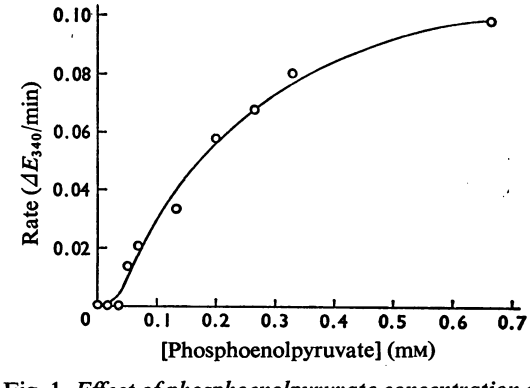


Fig. 1. *Effect of phosphoenolpyruvate concentration on the activity of phosphoenolpyruvate carboxylase at 45°C*

Enzyme activity was assayed at 340nm at pH7.6. Experimental details are given in the text.

for the duration of the assay. When the results were treated according to the Hill equation the value of $n_H = 1.6$ was obtained.

Activators of phosphoenolpyruvate carboxylase

Phosphate and phosphate esters. In the coupled enzyme assay of phosphoenolpyruvate carboxylase activity it is necessary to establish that the reaction is not limited by malate dehydrogenase. To check this point commercial malate dehydrogenase was added and was found to increase the rate of the coupled reaction. However, commercial malate dehydrogenase is stabilized with $(NH_4)_2SO_4$ (3M) and subsequent investigation has shown that the rate of the coupled reaction is not limited by malate dehydrogenase but rather that the phosphoenolpyruvate carboxylase is activated by $(NH_4)_2SO_4$ and specifically by SO_4^{2-} ions. This stimulation has been confirmed by means of the ^{14}C assay and by the direct spectrophotometric method. The similarity between SO_4^{2-} and HPO_4^{2-} suggests the possibility that the enzyme responds to two closely positioned negative charges, and P_i has been shown to be an activator of the enzyme.

A number of phosphate esters were examined as possible allosteric effectors and were shown to be activators (Fig. 2). In all cases, with the exception of 3-phosphoglycerate, control experiments established that effectors did not interfere with any of the assay systems.

However, 3-phosphoglycerate reacts in all the assay systems and investigation has shown that it is converted into phosphoenolpyruvate. Nevertheless, the rate of reaction with 3-phosphoglycerate exceeds the rate with phosphoenolpyruvate and it is therefore probable that 3-phosphoglycerate is also an activator of the enzyme.

Effect of pH on the activation produced by phosphate esters. The stimulation produced by P_i and all phosphate esters examined is a function of pH, maximum activation occurring at low pH values (Fig. 3).

Mode of activation. The enzyme preparation is too crude to permit detailed experiments to test the

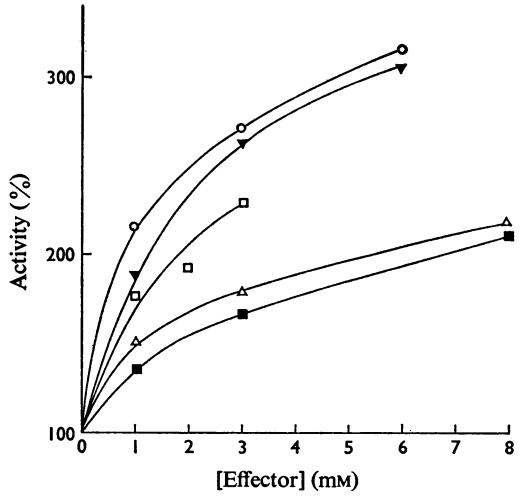


Fig. 2. *Effect of various compounds on the activity of phosphoenolpyruvate carboxylase*

Activities are expressed as a percentage of the control rate measured in the absence of an effector. Enzyme activity was assayed at 340nm at pH7.0. Experimental details are given in the text. o, Glucose 6-phosphate; △, P_i; ▼, α-glycerophosphate; ■, sulphate; □, 3-phosphoglycerate.

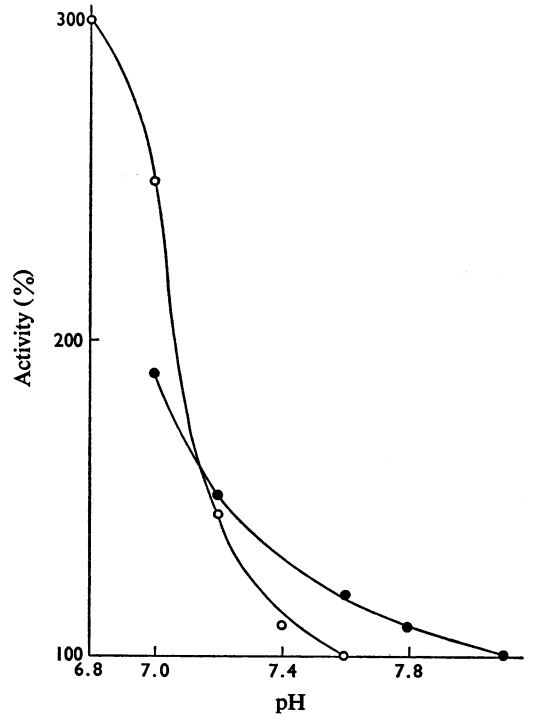


Fig. 3. *Effect of pH on the activation of phosphoenolpyruvate carboxylase by P_i and fructose* 1,6-*diphosphate*

Enzyme activity was assayed at 340nm as described in the text, except that 2-(*N*-morpholino)ethanesulphonic acid buffer (33.3mM) was used to obtain pH6.8, and is expressed as a percentage of the control rate. ●, Fructose diphosphate (2mM); o, P_i (30mM).

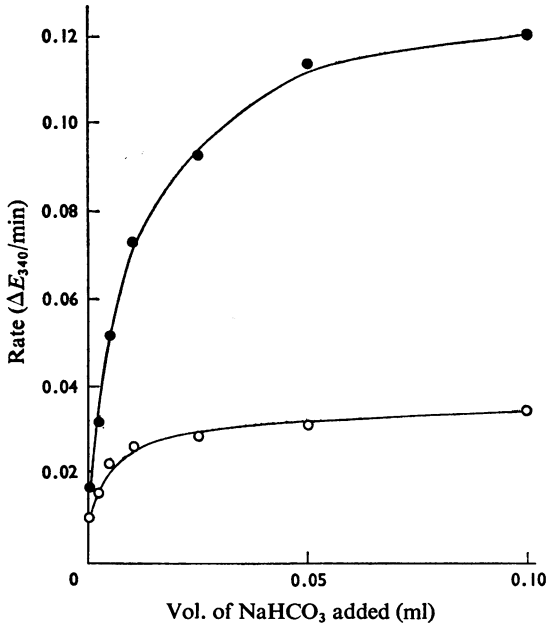


Fig. 4. *Effect of CO_2 and $NaHCO_3$ on the activity of phosphoenolpyruvate carboxylase in the presence and in the absence of the activator glucose 6-phosphate*

Enzyme activity was assayed at 340nm at pH7.35 as described in the text, except that $NaHCO_3$ and CO_2 concentrations were varied. $NaHCO_3$ was added as a 0.35M solution in equilibrium with 100% CO_2 (pH7.35). o, Control; •, glucose 6-phosphate (3.3mM).

mechanism of activation. However, enzyme activity in the presence of high concentrations of a phosphate ester is not increased by adding a second phosphate ester that is itself an activator, suggesting a common allosteric site for the sugar phosphates.

The possibility that activation involves an effect on the affinity of the enzyme for CO_2 (or HCO_3^-) was examined. The results presented in Fig. 4 suggest that glucose 6-phosphate does not increase the affinity of the enzyme for CO_2 (or HCO_3^-). On the other hand, as illustrated in Fig. 5, fructose diphosphate (0.67mM) decreased the K_m value for phosphopyruvate from 230μM to 80μM, whereas $V_{max.}$ was not significantly changed.

Nucleotides. The effect of a number of nucleotides on the activity of phosphoenolpyruvate carboxylase was examined and the results are shown in Table 1. The stimulation by AMP is pH-sensitive, maximum stimulation occurring at low pH values. The most effective concentration for activation by AMP was 0.7–1.0mM, higher concentrations proving inhibitory.

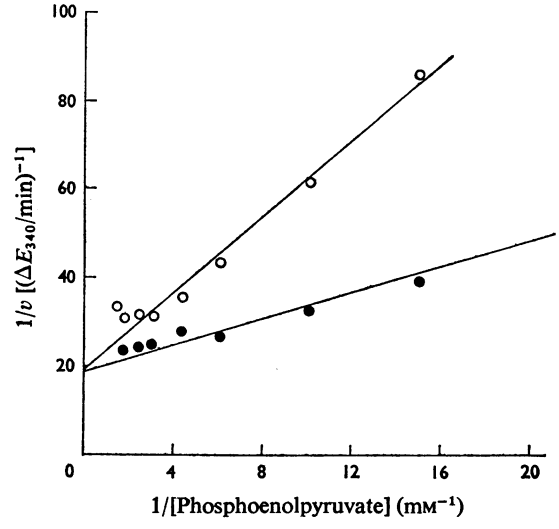


Fig. 5. *Double-reciprocal plot of rate against phosphoenolpyruvate concentrations in the presence* (•) *and in the absence* (o) *of fructose 1,6-diphosphate*

Enzyme activity was assayed at 340nm at pH7.3 as described in the text, except that the phosphoenol pyruvate concentration was varied. Fructose diphosphate when present was 0.67mM (•).

Table 1. *Effect of nucleotides on phosphoenolpyruvate carboxylase activity*

Enzyme activity was assayed by the spectrophotometric method at 340nm, at a phosphoenolpyruvate concentration of 0.17mM and pH7.0. All nucleotides tested were at 0.67mM.

Addition	Activity (% of control)
ATP	55
ADP	75
Adenosine	100
AMP	145
CMP	130
GMP	124

A kinetic analysis of activation by AMP shows that AMP increases the apparent affinity of the enzyme for phosphoenolpyruvate; thus AMP (670μM) decreased the K_m value for phosphoenolpyruvate from 170 to 35μM at pH7 (Fig. 6). When the results are plotted in the form of $1/(V_a - V_0)$ (where V_a is the rate in the presence of effector and V_0 is the rate in the absence of effector) against 1/[AMP] a straight line is obtained, giving a K_a value of 1.55mM. The linearity

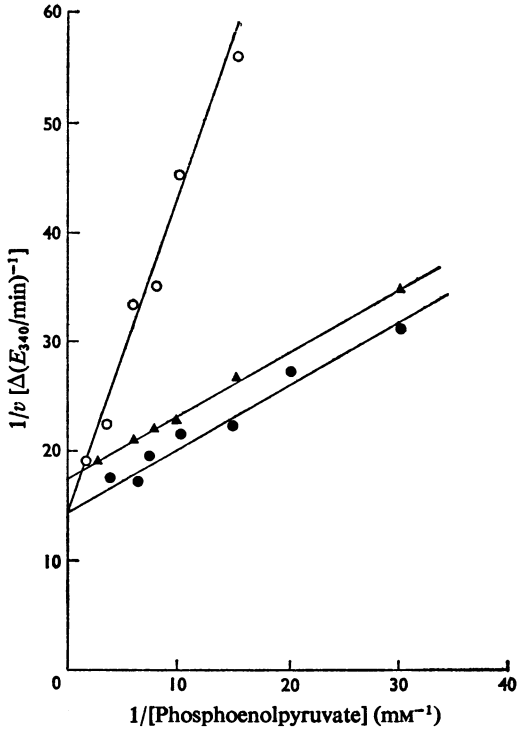


Fig. 6. *Double-reciprocal plot of rate against phosphoenolpyruvate concentration in the presence and in the absence of AMP*

Enzyme activity was assayed at 340nm and pH7.0 as described in the text, except that phosphoenolpyruvate concentration was varied. o, Control, no AMP; ●, AMP (0.67mM); ▲, AMP (2mM).

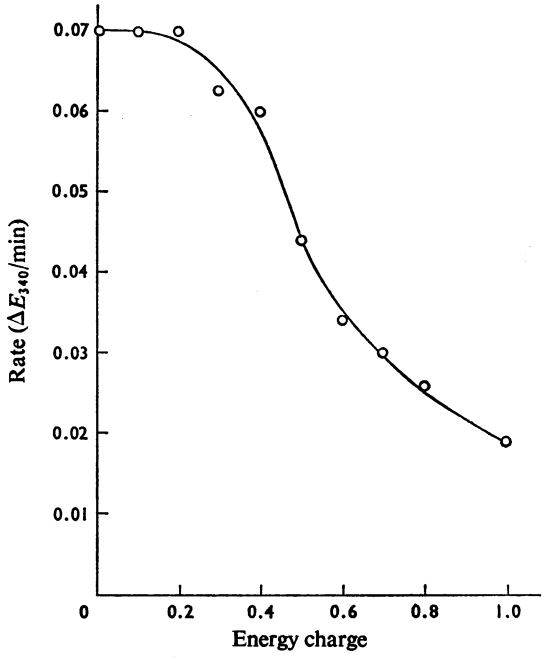


Fig. 7. *Effect of energy charge on the activity of phosphoenolpyruvate carboxylase*

Enzyme activity was assayed at 340nm at pH7.1 as described in the text. The equilibrium constant for the adenylate kinase was taken as 0.8 and the energy charge was calculated as described by Atkinson (1968).

suggests that activation occurs when 1 molecule of AMP is bound on the enzyme surface.

Energy charge. The effect of nucleotides on the activation of the enzyme have been examined in relation to Atkinson's (1968) concept of energy charge. The energy charge is defined as

$$\frac{[ATP]+0.5[ADP]}{[ATP]+[ADP]+[AMP]}$$

being fully charged (1.0) when only ATP is present and being completely discharged (0) when only AMP is present. The results are shown in Fig. 7.

Inhibitors of phosphoenolpyruvate carboxylase

Organic acids. Using the ^{14}C-assay method at pH8.0 we have confirmed the inhibition of the enzyme by L-malate and oxaloacetate reported by other workers. The effect of various organic acids on the activity of the enzyme is shown in Table 2. The plots of percentage inhibition against concentrations of inhibitor are hyperbolic (Fig. 8). However, at

Table 2. *Effect of organic acids on phosphoenolpyruvate carboxylase activity*

Enzyme activity was assayed by the spectrophotometric method at 340nm. Phosphoenolpyruvate concentration was 0.67mM. Each inhibitor was tested at a concentration of 2.67mM.

Compounds	Inhibition (%) pH7.0	pH7.5
Citrate	0	11.5
DL-Isocitrate	0	20
α-Oxoglutarate	36	4
Succinate	88	0
Malonate	0	0
L-Tartrate	100	50
meso-Tartrate	85	5
D-Tartrate	77	66

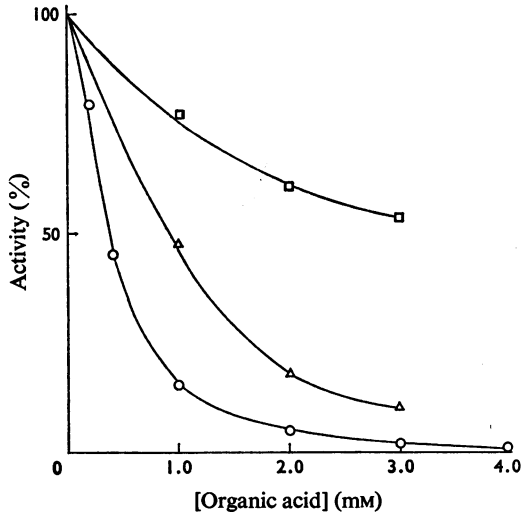


Fig. 8. *Effect of organic acids on the activity of phosphoenolpyruvate carboxylase*

Enzyme activity was assayed at 340nm at pH7.0 as described in the text and is expressed as a percentage of the control activity in the absence of an organic acid. □, α-Oxoglutarate; △, succinate; ○, L-tartrate.

Table 3. *Effect of amino acids on phosphoenolpyruvate carboxylase activity*

Enzyme activity was assayed by the spectrophotometric method at 340nm. Phosphoenolpyruvate concentration was 0.67mM, and that of amino acids was 13.3mM.

	Inhibition (%)	
Amino acid	pH7.0	pH7.6
L-Alanine	0	0
L-Asparagine	12	0
D-Aspartate	36	0
L-Aspartate	100	47
D-Glutamate	61	27
L-Glutamate	100	50
Glutamine	0	0
DL-Homoserine	6	6
Phenylalanine	0	0
Tryptophan	0	0

pH7.8 a plot of percentage inhibition versus isocitrate concentration gave a sigmoid inhibition curve, suggesting co-operative effects.

Amino acids. By using the coupled enzyme assay L-glutamate and L-aspartate were found to be inhibitors of the enzyme, and the D forms were also inhibitors (Table 3). Plots of percentage inhibition against the concentration of glutamate and aspartate gave hyperbolic inhibition curves at pH7.0. At pH7.4 the kinetics of inhibition appear to be classical non-competitive with respect to phosphoenolpyruvate (Fig. 9*a*). At pH7.6, higher concentrations of amino acids were required for inhibition and complex kinetics were observed (Fig. 9*b*).

The sigmoid inhibition curve for aspartate at pH7.6, when replotted in the form of the Hill equation, gave a Hill coefficient (n_H) of 1.4. The sigmoid inhibition response to aspartate was altered by heat treatment; heating at 50°C for 1min completely abolished the sigmoid response (Fig. 10).

Effect of acetyl-CoA on phosphoenolpyruvate carboxylase

Bacterial phosphoenolpyruvate carboxylases are activated by acetyl-CoA (Canovas & Kornberg, 1966). The enzyme from maize used in this study was neither activated nor inhibited by acetyl-CoA when tested over the concentration range 0–0.2mM.

Discussion

In the nomenclature proposed by Monod *et al.* (1965), the phosphoenolpyruvate carboxylase of maize behaves as a 'V' system. However, on heating to 45°C the enzyme behaves as a 'K' system. Similar observations have been made with glyceraldehyde 3-phosphate dehydrogenase (Kirschner *et al.*, 1966). The kinetics of the enzyme appear to be complex, and in view of the impure preparation used in these studies it seems unwise to discuss possible models that might explain the observed kinetics.

The use of crude preparations to study allosteric properties is necessary when purification eliminates the allosteric properties. However, observations made with crude preparations must be particularly carefully checked for artifacts. For example, in the present study when the concentration of phosphoenolpyruvate is not saturating, part of the activation produced by 3-phosphoglycerate is due to its conversion into phosphoenolpyruvate. Another example is the inhibition produced by glutamate. When studied by the direct spectrophotometric assay at 270nm, glutamate appears to be a much more potent inhibitor than when assayed by the coupled-enzyme system at 340nm. The explanation is that in the direct assay oxaloacetate accumulates and is measured at 270nm, but when glutamate is added the oxaloacetate is rapidly removed in a transamination reaction to form aspartate, which does not absorb at 270nm.

Phosphoenolpyruvate carboxylase can be regarded as the first enzyme in a pathway leading to the tricarboxylic acid cycle. Thus the intermediates of the

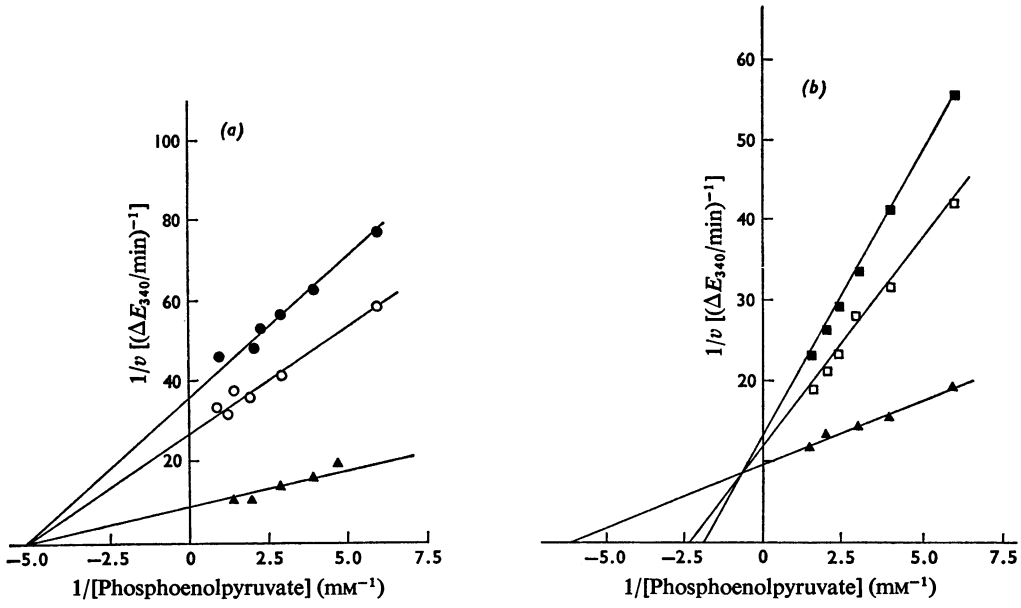
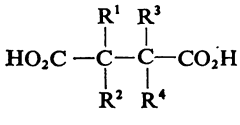


Fig. 9. *Double-reciprocal plots of rate against phosphoenolpyruvate concentration in the presence and in the absence of aspartate (a) at pH 7.4 and (b) at pH 7.6*

For experimental details see the text. ▲, Control (no aspartate); o, aspartate (0.67 mM); ●, aspartate (1.33 mM); □, aspartate (6.7 mM); ■, aspartate (11.5 mM).

cycle and the amino acids derived from them can be regarded as end products, which inhibit the first reaction involved in their biosynthesis. The results of the inhibitor studies suggest that compounds of the general formula:



(where R^1 and R^2 can be H or OH, R^3 can be H, OH or NH_2, and R^4 can be H, OH, NH_2, CH_2CO_2H or $CHOHCO_2H$) are inhibitors. This implies a relatively unspecific allosteric site, but it should be noted that the results in the present paper do not establish the existence of a single allosteric site for amino and organic acids.

The inhibition of the enzyme by organic acids is of particular importance for plants, which respond to excessive cation uptake by increasing the production of malate (Hiatt & Hendricks, 1967). Excessive production of malate could be controlled by the allosteric inhibition noted in the present study. These allosteric properties reinforce the homeostatic function of the enzyme, which is a consequence of its steep pH–activity curve, and enables it to function as part of a metabolic pH-stat (Davies, 1972).

The response of the enzyme to nucleotides is somewhat unexpected. The anaplerotic role of phosphoenolpyruvate carboxylase makes it difficult to predict the response to energy charge. However, it is clear that in terms of Atkinson's (1968) proposals the enzyme could give either a flat response or a U-type curve, which is typical of regulatory enzymes in ATP-utilizing sequences. The phosphoenolpyruvate carboxylase of maize is activated by AMP rather than by ATP and thus the response to energy charge is of the R type, characteristic of ATP-generating sequences, and this response seems to be at variance with Atkinson's (1968) concept.

The activation of phosphoenolpyruvate carboxylase by AMP, P_i and sugar phosphates is pH-dependent, activation being greatest at low pH values. Low pH decreases the activity of the carboxylase and thus the allosteric effectors over-ride the pH-control. Excessive uptake of anions could lead to a fall in pH, and plant cells respond by lowering the concentration of organic acids (Jakoby & Laties, 1971). If the decrease in concentration of organic acids reaches a point where the operation of the tricarboxylic acid cycle is decreased, elevated concentrations of AMP and P_i would result and these metabolites could override the effect of pH and stimulate the production of oxaloacetate.

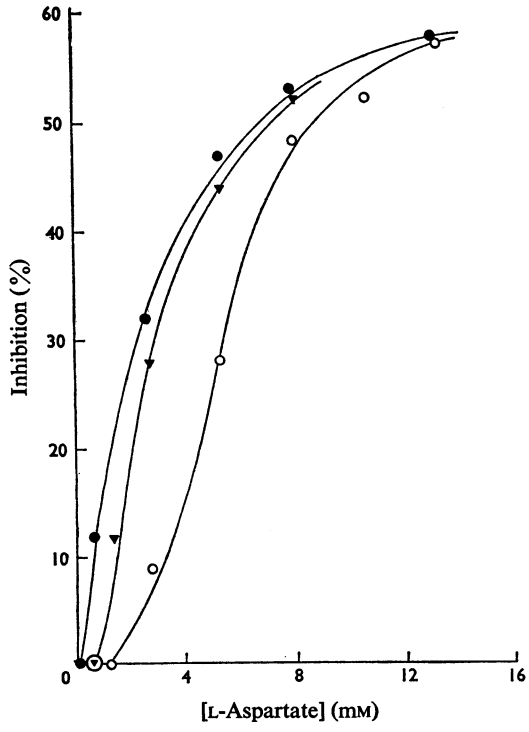


Fig. 10. *Effect of* L-*aspartate on the activity of a heat-treated preparation of phosphoenolpyruvate carboxylase*

Enzyme activity was assayed at 340nm at pH7.6 as described in the text. o, Control (not heated); ▼, heated for 1 min at 40°C; ●, heated for 1 min at 50°C.

Activation of the carboxylase by sugar phosphates could be considered as an example of metabolic interlock. Phosphofructokinase is inhibited by citrate (Dennis & Coultate, 1967) and by phosphoenolpyruvate (Kelly & Turner, 1969). If the organic acids are removed in response to excessive anion uptake the constraint on phosphofructokinase will be decreased, leading to an increased production of fructose diphosphate, which could activate phosphoenolpyruvate carboxylase and so replenish the tricarboxylic acid cycle.

The activation of phosphoenolpyruvate carboxylase by sugar phosphates is of particular significance in maize, which is a 'Hatch–Slack' plant (Hatch & Slack, 1970). This activation could be considered as positive feed-back, in which products of the photosynthesis cycle activate the first step in the fixation of CO_2. Additionally it should be noted that phosphate is both product and activator of phosphoenolpyruvate carboxylase.

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