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REGULATION OF GLYCOLYSIS BY ADENOSINE DIPHOSPHATE IN *PISUM SATIVUM*

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Key Word Index—*Pisum sativum*; Leguminosae; glycolysis; fermentation; metabolic regulation; adenine nucleotides; pyruvate kinase.

Abstract—The role of ADP in controlling glycolysis has been examined in a soluble extract of germinating pea seeds. A shortage of ADP appears to retard glycolysis principally by restricting the conversion of phosphopyruvate to pyruvate rather than by restricting formation of phosphoglycerate. Upon addition of ADP to the extract there is an immediate decrease in the concentration of phosphopyruvate accompanied by an increase in pyruvate. Apparently the pyruvate-kinase step shows the most marked response to fluctuations in ADP availability. The glycolytic response to ADP depends on the concentration of ATP and magnesium ions. The relation of magnesium-ion availability to adenine-nucleotide control of glycolysis is discussed.

INTRODUCTION*

HIGHER plant glycolysis may be regulated by the availability of ADP as well as by concentrations of other nucleotides and/or orthophosphate.[1-4] ADP might control glycolytic flux at either of two steps, since this nucleotide is required by both the PGK and PK reactions. Thus far ADP regulation of glycolysis by cell-free extracts of higher-plant tissues does not appear to have been explored in any detail, though Hatch and Turner[3] mentioned that under certain conditions ADP did stimulate glycolysis.

An earlier study of the glycolyzing pea-seed extract preparation employed in the present work confirmed the findings of Hatch and Turner[3] that ATP, P_i, Mg^{2+}, and NAD^+ were required for maximum rates of glycolysis. However, ATP was found to retard glycolysis at low levels of Mg^{2+} owing to inhibition at the PFK step.[2] In the present work ADP control of glycolysis has been studied in order to determine which glycolytic reaction is the most sensitive to a shortage of ADP. The results are discussed with reference to current ideas on short-term glycolytic regulation in higher plants.

* *Abbreviations used*: ADP, adenosine diphosphate; ATP, adenosine triphosphate; P_i, orthophosphate; NAD^+, nicotinamide adenine dinucleotide; $NADP^+$, nicotinamide adenine dinucleotide phosphate; PFK, phosphofructokinase; PK, pyruvate kinase; GAP, glyceraldehyde-P; DHAP, dihydroxyacetone-P; 3-PGA, 3-phosphoglycerate; 2-PGA, 2-phosphoglycerate; 1,3-di-PGA, 1,3-diphosphoglycerate; FDP, fructose diphosphate; G6P, glucose 6-P; F6P, fructose 6-P.

[1] BEEVERS, H. (1961) *Respiratory Metabolism in Plants*, Row, Peterson, Evanston.
[2] GIVAN, C. V. (1972) *Planta* **108**, 29.
[3] HATCH, M. D. and TURNER, J. F. (1958) *Biochem. J.* **69**, 495.
[4] THOMAS, M., RANSON, S. L. and RICHARDSON, J. A. (1973) *Plant Physiology*, Longman, London.

RESULTS AND DISCUSSION

In the absence of supplied ATP and ADP there was some detectable glycolytic activity by the pea-seed extract system (Table 1). The presence of low amounts of residual adenine nucleotides in the dialyzed extract may therefore be inferred. However, addition of ADP markedly accelerated glycolysis in the absence of supplied ATP. This was true irrespective of the magnesium-ion concentration. Where the Mg^{2+} level was high (expt. II) ADP at a high concentration produced a much greater stimulation than that given by the lower ADP concentration. At low Mg^{2+} (expt. I), high ADP gave no more stimulation than low ADP. The magnesium dependence of the ADP response is possibly related to the magnesium-binding properties of ADP.[5] Owing to chelation, a shortage of magnesium ions might occur anywhere in the glycolytic sequence if the ADP concentration were too high, and this shortage could tend to offset the stimulation of glycolysis. Also, there may be limiting amounts of Mg^{2+} for formation of the Mg–ADP used in the PK reaction or the PGK reaction.

TABLE 1. EFFECT OF ADENINE NUCLEOTIDES ON GLYCOLYTIC EVOLUTION OF CARBON DIOXIDE BY PEA SEED EXTRACT

	Conditions	CO_2 evolved (μl/sample)
Experiment I (low Mg^{2+})	Minus ADP, minus ATP	78 ± 1
	Low ADP, minus ATP	192 ± 0
	High ADP, minus ATP	201 ± 4
	Minus ADP, plus ATP	116 ± 0
	Low ADP, plus ATP	82 ± 2
	High ADP, plus ATP	51 ± 6
Experiment II (high Mg^{2+})	Minus ADP, minus ATP	65 ± 3
	Low ADP, minus ATP	150 ± 4
	High ADP, minus ATP	277 ± 7
	Minus ADP, plus ATP	238 ± 4
	Low ADP, plus ATP	230 ± 6
	High ADP, plus ATP	241 ± 21

Reaction mixtures contained the following, in μmol: GlP-K_2, 27; K-PO_4, 50; NAD^+, 15; ATP (where indicated), 14; high ADP (where indicated), 14; low ADP (where indicated), 2·4; low Mg^{2+} (where indicated), 4; high Mg^{2+} (where indicated) 22. Dialyzed pea-seed extract in buffer, pH 7·0 (tricine, 50 mM, dithiothreitol, 0·5 mM, $MgSO_4$, 4 mM), 1 ml. Total vol. = 2·75 ml. Period of measurement, 60 min. Values are average ± range of duplicate samples.

Table 1 further shows that in the absence of supplied ADP, ATP stimulated glycolysis at both the high and low levels of magnesium ion, though the stimulation was much greater at high magnesium concentration. The previously reported inhibition by ATP at low Mg^{2+} (Ref. 2) thus appears to occur only in the presence of added ADP. Moreover, ADP accelerated glycolysis only in the absence of supplied ATP (Table 1). In the presence of ATP, ADP was markedly inhibitory at low magnesium, and had little effect either way at high magnesium.

Some of the above data are difficult to explain but they do indicate that the availability of magnesium ions is a critical factor in relation to adenine-nucleotide effects. Adenine nucleotides may thus exert regulatory effects by altering levels of free magnesium ions as well as by direct interactions with glycolytic enzymes. Although the critical role of Mg^{2+} was stressed earlier in connection with ATP control of glycolysis,[2] greater emphasis should

[5] O'SULLIVAN, W. J. and PERRIN, D. D. (1964) *Biochemistry* **3**, 18.

have been given to the possibility that inhibitory effects of adenine nucleotides could often result to a significant degree from removal of magnesium. Magnesium deficiency may specifically decrease the activity of a regulatory enzyme, such as PFK.[6] Therefore, much of the ATP inhibition reported previously[2] could have been due to reduction in availability of Mg^{2+} and not altogether to a direct inhibition of PFK by ATP. Magnesium deficiency alone may reduce the catalytic activity of PFK (cf. also the work of Dennis and Coultate[7]). Several authors[8–10] have recently discussed the effects of magnesium ions on enzymes mediating interconversions of sugar-phosphates in plants; these effects may possibly be of regulatory importance *in vivo*. Lack of ADP stimulation in the presence of added ATP suggests that there is sufficient hydrolysis of the latter compound to satisfy the glycolytic requirement for ADP.

The clearest and least complicated ADP effect was the one observed in the absence of supplied ATP. Here ADP gave a clear-cut acceleration of glycolysis, and no complication due to magnesium deficiency was apparent. Moreover, it is thought that glycolysis *in vivo* is normally accelerated during periods of high ADP availability, not retarded. I have therefore studied further the response of glycolysis under conditions where ADP was found to accelerate it. In these experiments P_i was supplied at a fairly high concentration so as to reduce the possibility of limitation of the glycolytic rate at the PFK or triose-*P* dehydrogenase steps and to make ADP the rate-limiting cofactor. Pool sizes of several glycolytic intermediates from hexosemonophosphate through to pyruvate were measured (GAP by itself, 2-PGA and 1,3-di-PGA were too low for accurate estimation with available instrumentation; the last of these compounds is unstable to the acid-deproteinisation procedure

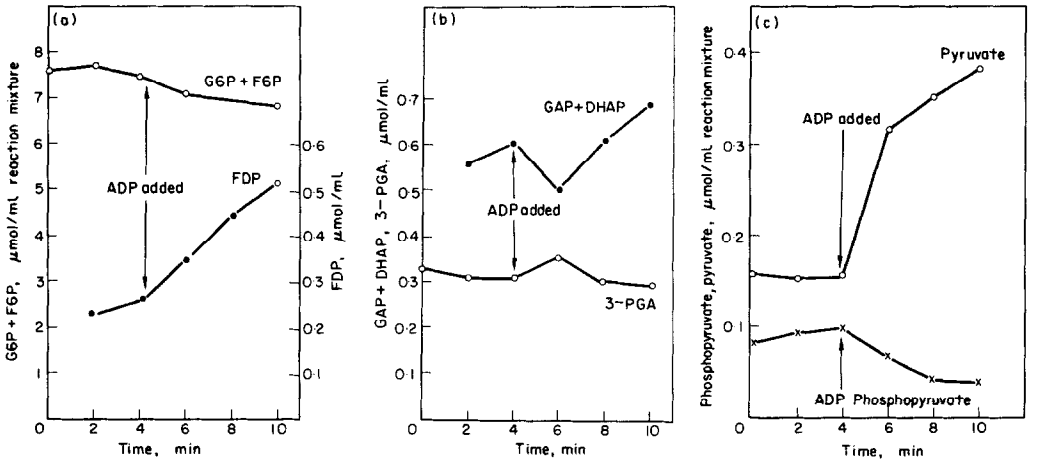

FIG. 1. EFFECT OF ADP ON POOL SIZES OF GLYCOLYTIC INTERMEDIATES.
Reaction mixture contained 15 ml of dialyzed pea extract (in tricine, 50 mM; $MgSO_4$, 4 mM, dithiothreitol, 0·5 mM), plus the following, in μmol: GlP-K_2, 400, NAD^+, 225; K-PO_4, 750. pH 7·0. At time indicated by arrow, ADP (24 μmol) added. Preincubation 15 min at 30° before time zero on abscissa. Total vol. = 40 ml.

[6] KELLY, G. J. and TURNER, J. F. (1969) *Biochem. J.* **115,** 481.
[7] DENNIS, D. T. and COULTATE, T. P. (1966) *Biochem. Biophys. Res. Commun.* **25,** 187.
[8] JENSEN, R. G. (1971) *Biochim. Biophys Acta* **234,** 360.
[9] PREISS, I. and KOSUGE, T. (1970) *Ann. Rev. Plant Physiol.* **21,** 433.
[10] WALKER, D. A. (1973) *New Phytol.* **72,** 209.

used). Measurements were first made for a period prior to addition of ADP; then similarly for a period following ADP addition, with readjustments in the sizes of these pools being noted. Comparison of the pool sizes before and after addition of ADP allowed identification of the reactions showing inhibition prior to ADP addition which were specifically accelerated upon presentation of additional ADP to the system.

Figures 1a, 1b and 1c show plots of glycolytic intermediate concentrations for a period starting 4 min before addition of ADP and ending 6 min after ADP addition. Before addition of ADP, there were considerable amounts of all glycolytic intermediates present, including those occurring both before and after the PGK step in the glycolytic pathway. There was therefore no obvious block in the vicinity of the PGK step, given the presence of the residual amount of ADP present in the dialyzed extract. It is noteworthy that the concentration of phosphopyruvate was nearly as high as the concentration of pyruvate (Fig. 1c). Since the equilibrium position of the PK reaction, which catalyzes conversion of phosphopyruvate to pyruvate, lies very heavily in favour of pyruvate,[4] it is clear that reduced availability of ADP has caused a considerable accumulation of phosphopyruvate. The primary effect of a shortage of ADP therefore appears to be a restriction of the flow of substrate through the PK step.

Upon addition of ADP there was an immediate and sharp rise in the level of pyruvate, with a concomitant fall in the size of the phosphopyruvate pool. Six min after adding ADP the phosphopyruvate:pyruvate ratio had fallen from a pre-ADP value of about 0·7 to a new value of about 0·12 (Fig. 1c). Changes in levels of other glycolytic intermediate pools were much less striking. There was not any continuing change in the size of the triosephosphate or 3-PGA pools. This would suggest no major control response at the PGK step, though there was indeed a slight transitory decrease in triosephosphates and increase in 3-PGA (Fig. 1b).

The data suggest that lack of ADP primarily limits the passage of substrate through the PK step. Ricardo and ap Rees[11] have correctly pointed out that it is difficult to imagine glycolysis being controlled only at the PK step and not at the PFK step, since in such circumstances a glycolytic steady state might not be obtained. The properties of pea-seed PFK may resolve this problem; this enzyme is inhibited by phosphopyruvate.[6] Accumulation of phosphopyruvate during a period of ADP shortage could produce a feed-back inhibition of glycolysis at the PFK step. The substrate would then be held as hexosemonophosphate so that the pools of glycolytic intermediates subsequent to the PFK step would not increase indefinitely. In Fig. 1a it can be seen that the FDP level continued to rise after ADP addition, suggesting the occurrence of faster flux through the PFK step which more than compensated for the faster utilisation of FDP as glycolysis accelerated.

As regards the *in vivo* situation, it is difficult to know how often a situation might arise where lack of ADP would be the sole rate-limiting factor. One might expect that usually ADP would be low only when P_i was also low, and ATP high, so that direct control of the PFK step might override control of PK. On the other hand, if P_i were present in excess, availability of ADP could become the main limiting factor, leading to a primary constraint at the PK step. Adams[12,13] suggested that in freshly-cut carrot slices the glycolytic rate

[11] RICARDO, C. P. P. and REES, T. (1972) *Phytochemistry* **11**, 623.
[12] ADAMS, P. B. (1970) *Plant Physiol.* **45**, 495.
[13] ADAMS, P. B. (1970) *Plant Physiol.* **45**, 500.

was limited by ADP availability and he presented data implicating PK as an important rate-limiting step in this tissue. (Cf. also studies on *Ricinus*-endosperm glycolysis[14]).

Why does a shortage of ADP appear to affect the PK step more seriously than the PGK step? The K_m for ADP of plant PKs is said to be less than 100 μm.[15] A published K_m for ADP of plant PGK has not come to my attention, though the enzyme does respond to changes in "energy charge".[16] Perhaps the PGK has an even lower K_m for ADP than does PK. A further possibility would be that conversion of GAP to 3-PGA proceeds by an alternative reaction which does not require ADP, e.g. catalyzed by the enzyme discussed by Kelly and Gibbs[17] which directly converts GAP to 3-PGA, bypassing 1,3-di-PGA and thus eliminating the ADP requirement. This enzyme is, however, NADP-specific, whereas the glycolytic activity of the dialyzed extract used in the present work was found to be negligible in the absence of NAD^+. (Of course, some conversion of NAD^+ to $NADP^+$ cannot be ruled out.) The clear requirement for P_i for passage of substrate as far as 3-PGA[2] would suggest that the conventional P_i-dependent GAP dehydrogenase and PGK enzymes are probably operative and responsible for most of the oxidation of GAP to 3-PGA by the pea seed extract.

EXPERIMENTAL

Techniques employed in preparing the soluble extracts of germinating *Pisum sativum* seeds, measuring fermentative CO_2 evolution, and assaying glycolytic-intermediate pools have been described in a previous report.[2] Specific details of reaction mixtures are given in figure and table legends.

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[15] TOMLINSON, J. D. and TURNER, J. F. (1973) *Biochim. Biophys. Acta* **329**, 128.
[16] PACOLD, I. and ANDERSON, L. E. (1973) *Biochem. Biophys. Res. Commun.* **51**, 139.
[17] KELLY, G. J. and GIBBS, M. (1973) *Plant Physiol.* **52**, 111.