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Pyruvate Kinase, a Possible Regulatory Enzyme in Higher Plants[1]

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ABSTRACT

A number of plant species were examined for the presence of pyruvate kinase (pyruvate-ATP phosphotransferase, EC 2.7.1.40), and of a phosphatase activity which hydrolyzes phosphoenolpyruvate. Of those examined, only cotton (*Gossypium* sp. L.) seeds were found to be sufficiently free of the phosphatase to permit a kinetic study of pyruvate kinase.

During germination of cotton seeds, pyruvate kinase activity rises for the first 3 days, after which it falls back to its original level. This developmental pattern is characteristic of enzymes involved in the conversion of fat into carbohydrate in fat-storing seeds. The phosphatase also rose rapidly during germination, which precluded the use of extracts from seedlings in the study of pyruvate kinase. No evidence was found for the presence of more than one pyruvate kinase in cotton seedlings.

In crude extracts from ungerminated seeds, the enzyme shows slight deviations from normal kinetics with respect to phosphoenolpyruvate, magnesium, and to a lesser extent, ADP. After partial purification of the enzyme by ion exchange chromatography, the enzyme shows normal kinetics. The enzyme is activated by AMP, and inhibited by both ATP and citrate, in both crude and partially purified preparations. It is suggested that cotton seed pyruvate kinase is a regulatory enzyme.

Pyruvate kinase (pyruvate-ATP phosphotransferase, EC 2.7.1.40) from a variety of tissues (2, 7, 18, 19, 34, 36) and organisms (10, 17, 21, 32, 36, 38, 41) appears to be a regulatory enzyme. It usually shows a sigmoid saturation curve towards PEP[3] and is activated by FDP in eucaryotes and by AMP in procaryotes. However, some workers have described pyruvate kinases which do not fit these generalizations (3, 6, 30, 42).

The kinetic properties of pyruvate kinase from higher plants have not been examined in detail. The activation of the enzyme by mono- and divalent cations in relation to plant nutrition has been studied (14, 26–28, 39), but the work to date (26, 28, 31) indicates that the higher plant enzyme does not show sigmoid kinetics towards PEP and is not activated by FDP. However, since neither of these properties is common to all pyruvate kinases, it was decided to re-examine the higher plant enzyme.

Most plant tissues contain phosphatases which interfere with the usual assay for pyruvate kinase. Therefore, a source of enzyme should be chosen which contains very little phosphatase. Also, since regulatory pyruvate kinases are usually associated with tissues capable of gluconeogenesis (16, 34), a fat-storing seed was chosen as the source of enzyme, since such seeds are gluconeogenic during the early stages of germination (8, 25). Bearing in mind these criteria, cotton seeds were found to be a satisfactory source of pyruvate kinase.

In a recent paper (13), we reported the kinetic properties of the enzyme from this source and suggested that the enzyme may be involved in regulation. In the present paper, we report some additional data on the higher plant enzyme.

MATERIALS AND METHODS

Materials. Cotton seeds (*Gossypium sp.* L)[4] were purchased from Carolina Biological Supply Co., and other seeds were obtained from local seed houses. Biochemicals, enzymes, and buffers were obtained from Sigma Chemical Co.; other chemicals were of the highest grade commercially available.

Enzyme Assays. Pyruvate kinase was assayed by coupling pyruvate production to NADH oxidation using lactic dehydrogenase and following the change in absorbance at 340 nm. The assay contained 0.1 mM NADH, 2.7 units[5] per ml of rabbit muscle lactic dehydrogenase (type II), ADP, PEP, magnesium chloride, potassium chloride, and other additions as indicated in the text and figure legends. The buffer used was either 100 mM tris, adjusted to pH 7.5 with hydrochloric acid; 50 mM TES, adjusted to pH 7.5 with tris; or appropriate mixtures of 100 mM tris and 100 mM MES to give the required pH. The assay was initiated by the addition of pyruvate kinase. Reaction rates (v) in Table IV and Figures 2, 4, 5, 6, and 7 are expressed as the change in absorbance at 340 nm/min and are corrected for the phosphatase activity (27, 28) under the same conditions, *i.e.* the same assay but omitting ADP. The rate of reactions was found to be proportional to the pyruvate kinase concentration over the range employed. Isocitrate lyase was assayed by the method of Dixon and Kornberg (12).

Plant Extracts. Plant extracts were prepared by homogenizing the tissue with 100 mM tris chloride buffer, pH 7.5, using a Servall Omnimixer. The homogenate was filtered through several layers of muslin, and centrifuged at 34,000*g* for 15 min. The supernatant was collected, taking care to avoid contamination from the upper lipid layer. All steps were performed at 0 to 4 C.

Cotton seed pyruvate kinase was routinely prepared by homogenizing finely ground kernels with 100 mM tris chloride

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[3] The following abbreviations are used: PEP: phosphoenolpyruvate; FDP: fructose 1,6-diphosphate; DEAE: diethylaminoethyl.

[4] The species used was not positively identified, but is probably *G. hirsutum* or *G. herbaceum*.

[5] In all cases, the unit of enzyme activity is defined as the amount which will catalyse the formation of 1 μmole of product per minute.

buffer, pH 7.5, containing an equal volume of glycerol. Ten milliliters of this buffer were used per g of tissue. A supernatant fraction was prepared as before.

In the growth experiment, cotton seeds were germinated in moist vermiculite at 30 C in the dark. At intervals, 20 seedlings were removed at random, washed, blotted dry, weighed and homogenized in 15 ml of 50 mM potassium TES buffer, pH 7.0, containing 50% (v/v) glycerol. The homogenate was filtered through several layers of muslin, and 1 ml of the filtrate was removed for lipid estimation. The remainder was used to prepare a supernatant fraction as described above. In calculations of the total lipid and enzyme content of seedlings, it was assumed that the dry weight remains constant over the experimental period, so that increases in wet weight of the tissue are equal to increases in tissue water content.

Partial Purification of Cotton Seed Pyruvate Kinase. The supernatant fraction of cotton seed extracts was applied to a 3.5 × 20 cm column of DEAE-cellulose which had been washed and equilibrated with 10 mM tris chloride buffer, pH 7.5, containing 50% (v/v) glycerol. The enzyme was washed on to the column with an equal volume of this buffer, then eluted with a 0.0 to 0.5 M potassium chloride gradient in this buffer. A flow rate of 0.5 to 1.0 ml/min was maintained, and 3- to 6-ml fractions were collected, all operations being performed at 0 to 4 C. The most active fractions were pooled and used without further treatment.

Analytical Methods. Protein was estimated by the method of Lowry *et al.* (23), after precipitating the protein with 5% trichloroacetic acid, or by the method of Murphy and Kies (29). Potassium was estimated by flame emission spectroscopy at 760 nm, using a Unicam SP 90A. Lipid in crude plant extracts was extracted and estimated gravimetrically, using the method of Bligh and Dyer (4).

Table I. *Pyruvate Kinase and Phosphatase Content of Various Seeds*

Extracts were prepared as described under "Materials and Methods," using 15 ml of tris chloride buffer and 3 g of seeds in all cases, except *Gossypium arboreum* (2 g) and *Gossypium barbadense* (2 g). The pyruvate kinase assay contained 0.5 mM PEP, 1.0 mM ADP, 10 mM magnesium chloride, and 100 mM potassium chloride in the tris chloride assay system. The phosphatase assay was the same as that for pyruvate kinase, except that ADP was omitted.

Species	Specific Activity		Pyruvate Kinase Phosphatase
	Pyruvate kinase	Phosphatse	
	units × 10³/ml extract		*ratio*
Pisum sativum	266	43	6.2
Helianthus annuus	128	90	1.4
Brassica rapa	139	23	6.0
Gossypium arboreum	169	4	42.3
Gossypium barbadense	161	7	23.0
Glycine max	428	40	10.7
Ricinus communis	71	44	1.6
Cucumis sativus var.			
Straight 8	71	18	3.9
Early Fortune	137	24	5.7
Ace	133	24	6.0
Chicago Pickling	212	32	6.6
Wisconsin SMR 12	172	27	6.4
Double Yield Pickling	216	28	7.7

Table II. *Pyruvate Kinase and Phosphatase Content of Plant Tissues*

Seeds were grown in moist perlite at 25 C in the dark for the indicated period. Extracts were prepared and assayed as in Table I, using 3 g wet weight of each tissue.

Species	Age	Tissue	Specific Activity		Pyruvate Kinase-Phosphatase
			Pyruvate kinase	Phosphatase	
	days		*units × 10³/ml extract*		*ratio*
Pisum sativum	0	seed	266	43	6.2
	2	embryo	252	29	8.7
	2	seed	316	42	7.5
	4	root	103	14	7.4
	4	shoot	115	15	7.7
	4	seed	248	21	11.8
Helianthus annuus	0	seed	128	90	1.4
	4	root	1.5	30	0.05
	4	seed	48	74	0.65
Cucumis sativus var.	0	seed	71	18	3.9
Straight 8	4	root	14	49	0.29
	4	seed	99	101	0.98
Spinacia oleracea		leaves	330	192	1.7

RESULTS

Source of Pyruvate Kinase. Extracts of a number of seeds were assayed for pyruvate kinase and phosphatase activity, and the results are shown in Table I. In all cases, except for cotton seeds, the extracts contain large amounts of phosphatase activity. Evans (14) reported that extracts of cucumber (*Cucumis sativus*) seeds are virtually free of phosphatase activity, but this observation was not confirmed.

Germination of pea (*Pisum sativum*), sunflower (*Helianthus annuus*), and cucumber seeds does not markedly alter the ratio of pyruvate kinase to phosphatase (Table II). Spinach (*Spinacia oleracea*) leaves also contain considerable phosphatase activity. Thus, cotton appears to be the best source of pyruvate kinase.

When cotton seeds are germinated for several days, the pyruvate kinase content more than trebles over the first 3 days, after which the activity falls back to its original level (Fig. 1). Isocitrate lyase activity also reaches a peak at about the same time as pyruvate kinase, and the peak coincides with the period of most rapid fat breakdown. Since it has been shown that this developmental pattern is typical of enzymes involved in the conversion of fat into carbohydrate (8, 25), pyruvate kinase may be implicated in gluconeogenesis. The phosphatase activity rises rapidly during germination, making a detailed study of the enzyme from germinated cotton seeds difficult. Chromatography of the enzyme from 3-day-old seedlings on DEAE-cellulose does not give a satisfactory separation of pyruvate kinase and phosphatase, but pyruvate kinase elutes as a single peak. This suggests that the increase of pyruvate kinase during germination does not represent the synthesis of a different enzyme. Thus, extracts from ungerminated cotton seeds were used as the source of pyruvate kinase for kinetic experiments. Extracts were prepared in the presence of 50% (v/v) glycerol, since the cotton seed enzyme was found to lose all activity after 8 hr at 0 C in the absence of glycerol. Miller and Evans (28) reported that cotton seed pyruvate kinase is stable for 20 hr in aqueous solution.

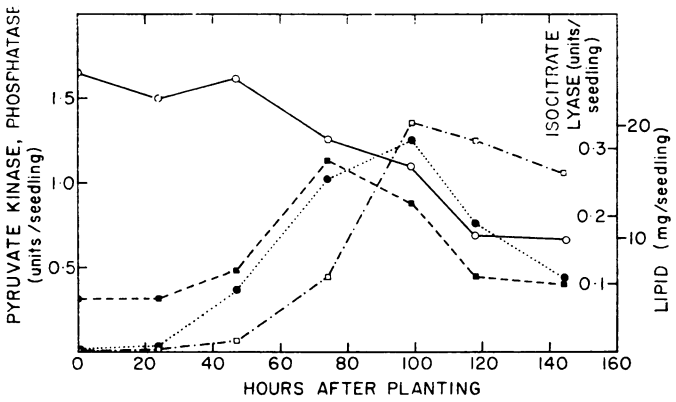

FIG. 1. Isocitrate lyase, pyruvate kinase, phosphatase, and lipid content of germinating cotton seeds. Seeds were germinated and extracted as described under "Materials and Methods." The extracts were assayed for pyruvate kinase (■) and phosphatase (□), as in Table I, and for isocitrate lyase (●) and lipid (○) as described under "Materials and Methods." The results are expressed as the amount of lipid per seedling.

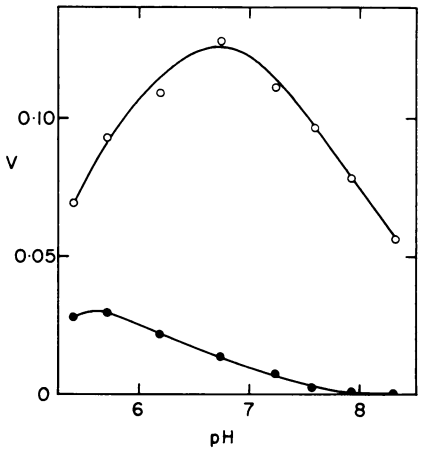

FIG. 2. Effect of pH on the activity of cotton seed pyruvate kinase. A crude extract of cotton seeds was prepared as described under "Materials and Methods," and assayed for pyruvate kinase (○) and phosphatase (●) as described in Table I, except that the buffer used was tris MES, and the pH was as indicated.

Kinetic Studies. The enzyme has a pH optimum of approximately 6.7 (Fig. 2). The activity of the phosphatase is very low above pH 7, but rises as the pH is lowered and is maximal around pH 5.7 under the conditions employed. Thus cotton seeds appear to contain an acid phosphatase which has a low activity at the pH of the pyruvate kinase assay (pH 7.5). Extracts from cucumber (var. Double Yield Pickling), on the other hand, showed the greatest phosphatase activity at alkaline pH values. Cucumber seed pyruvate kinase has a similar pH optimum to the cotton seed enzyme. In order to avoid interference from the phosphatase activity in cotton seed extracts, most of the subsequent work was performed at pH 7.5, although the activity of pyruvate kinase is 22% lower at this pH than at 6.7.

In studies of the kinetics of crude extracts from cotton seeds, the enzyme was found to display curved double reciprocal plots when the concentration of PEP, ADP, or magnesium chloride was varied (data not shown), but the purified enzyme does not show such effects (13). In crude extracts, the enzyme was shown to be markedly activated by the addition of potassium, but the presence of extraneous monovalent cations in the assay (4.7 mM sodium and 2.0 mM ammonium) made it impossible to determine if the monovalent cation requirement is absolute, as it is for other eucaryotic pyruvate kinases (17, 26, 28).

Pogson (33) has reported that pyruvate kinase from adipose tissue shows normal kinetics when extracted using imidazole buffer, but that pretreatment of the enzyme with various compounds will convert it into a form which shows sigmoid kinetics. This regulatory form of the enzyme is characterized by a high ratio between the activities observed at high and low PEP concentrations. This criterion was used to determine if the cotton seed enzyme could be converted from a nonregulatory to a regulatory form. However, no change was noted after pretreatment of the enzyme with EDTA, ATP, citrate, 2-mercaptoethanol, magnesium chloride, or alanine, or at pH 6.0 and 8.2

Pyruvate kinase from several sources (22, 35, 37, 43) only exhibits regulatory properties under certain conditions of pH or temperature. When the cotton seed enzyme was assayed over a range of pH values from 5.7 to 8.3, and a range of temperatures from 15 C to 40 C, no marked change in the kinetics towards PEP was found.

We have previously reported that the cotton seed enzyme is inhibited by trisodium citrate (13). The enzyme in extracts of cucumber (var. Double Yield Pickling) and soybean (*Glycine max*) also show this inhibition, whereas the commercially obtained rabbit muscle enzyme (Sigma type II) does not. Thus, citrate inhibition appears to be a property of the higher plant enzyme, rather than some artifact of the assay system. The cotton seed enzyme is also inhibited by ATP (20% at 2 mM) and activated by AMP (15% at 2 mM). However, no effect was noted on addition of up to 2 mM FDP, a characteristic activator of pyruvate kinase from a variety of sources (2, 5, 15, 17, 21, 24, 32, 38, 40).

Since these activations and inhibitions were first observed in crude extracts, the cotton seed enzyme was partially purified by chromatography on DEAE-cellulose. The enzyme eluted as a single peak between 0.10 and 0.15 M potassium chloride, and is not separated from the phosphatase activity (Fig. 3). The eluted enzyme is activated by AMP and inhibited by citrate, and both effects are constant across the peak of activity (Table III). The most active fractions were pooled.

The partially purified enzyme shows normal kinetics towards PEP and ADP (Fig. 4), and the presence of ATP, citrate, or AMP does not make the kinetics sigmoidal. In the absence of citrate, the enzyme shows normal kinetics towards magnesium (Fig. 5, A and B), but the presence of 2 mM citrate causes some

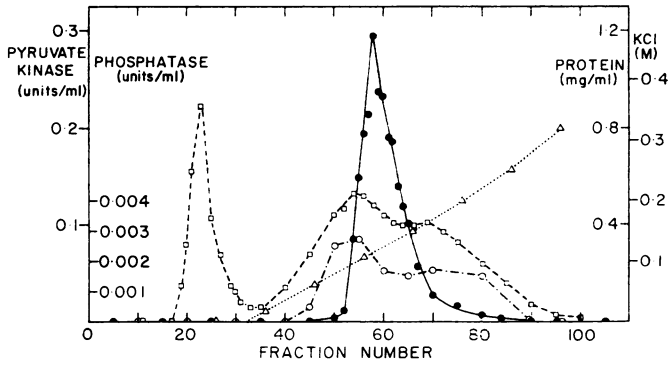

FIG. 3. Purification of cotton seed pyruvate kinase on DEAE-cellulose. Cotton seed pyruvate kinase was purified as described under "Materials and Methods," using 17 ml of cotton seed extract. The flow rate was maintained at 1.0 ml/min, and 5.3-ml fractions were collected. Pyruvate kinase (●) and phosphatase (○) were assayed as in Table I, and potassium (△) and protein (□) were estimated as described under "Materials and Methods."

sigmoidicity (Fig. 5C). This effect is undoubtedly due to magnesium chelation, since the addition of EDTA has a similar effect (Fig. 5D). The amount of activation by AMP is greatly affected by the ionic composition of the assay medium (Table IV); the chloride ion reduces AMP activation to a considerable extent. It is for this reason that the tris chloride buffer system was replaced by the tris TES system.

The partially purified enzyme, like the enzyme from other sources, is inhibited by ATP (Fig. 4). This inhibition, coupled with the activation of the enzyme by AMP, should cause the enzyme to respond to energy charge (1), as has been reported for the enzyme from *Azotobacter vinelandii* (21). The effect of

Table III. *Citrate Inhibition and AMP Activation of the Peak of Pyruvate Kinase Eluted from DEAE-cellulose*

The enzyme was purified as in Figure 3, and the active fractions were tested for citrate inhibition and AMP activation. The assay contained 0.05 mM PEP, 0.1 mM ADP, 10 mM magnesium chloride, 100 mM potassium chloride, and additions as indicated, in the tris chloride assay buffer.

Fraction	Inhibition by 3 mM Citrate	Activation by 1 mM AMP
No.	%	%
54	32	12
56	30	14
58	29	12
60	30	13
62	32	10
64	34	12
66	31	11

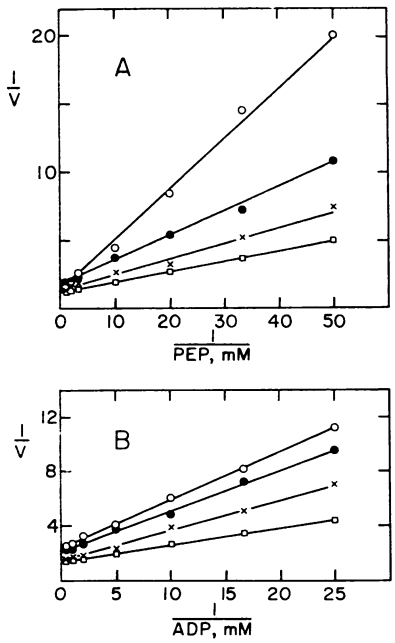

FIG. 4. Effect of AMP, citrate, and ATP on the kinetics of purified cotton seed pyruvate kinase. The enzyme was purified and assayed as described under "Materials and Methods," using an assay containing 10 mM magnesium chloride, 45 mM potassium chloride, and tris TES buffer. Additions to this assay were: none (×), 1 mM AMP (□), 2 mM citrate (●), or 3 mM ATP (○). A: assays contained 0.1 mM ADP and PEP as indicated; B: assays contained 0.05 mM PEP and ADP as indicated. The data are plotted in double reciprocal form.

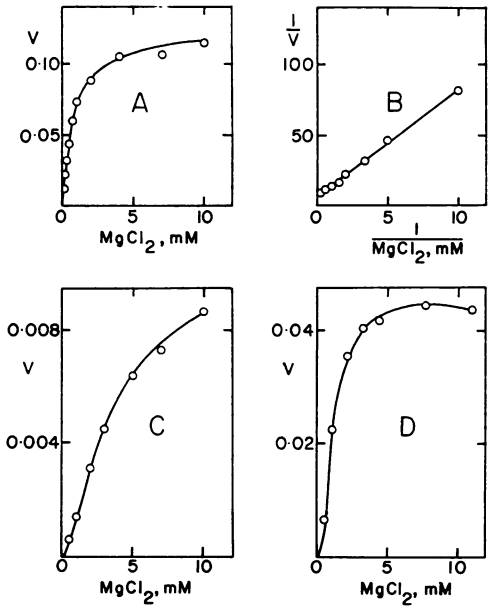

FIG. 5. Kinetics of purified cotton seed pyruvate kinase with respect to magnesium. The enzyme was purified and assayed as described under "Materials and Methods." A: Assays contained 0.05 mM PEP, 0.1 mM ADP, magnesium chloride as indicated and 45 mM potassium chloride, in tris TES buffer; B: the same data plotted in double reciprocal form; C: assays were as in A, except that assays contained 2 mM citrate and 100 mM potassium chloride, in tris chloride buffer; D: assays contained 0.055 mM PEP, 1.1 mM ADP, magnesium chloride as indicated, 110 mM potassium chloride, and 3.3 mM EDTA, in tris chloride buffer.

Table IV. *Effect of Salts on the Activation of Cotton Seed Pyruvate Kinase by AMP*

The enzyme was purified and assayed as described under "Materials and Methods," using the assay system described in Figure 4, with additions as indicated, 0.05 mM PEP, 0.1 mM ADP, and 0.2 ml of partially purified enzyme.

Addition	Rate		Activation by 1 mM AMP
	No AMP	1 mM AMP	
	10^4 × *value*		%
None	242	325	34
10 mM NaCl	248	314	28
30 mM NaCl	237	283	20
50 mM NaCl	217	234	18
10 mM tris Cl	262	326	24
30 mM tris Cl	225	269	15
50 mM tris Cl	196	215	10
10 mM Na TES	226	321	42
30 mM Na TES	180	269	49
50 mM Na TES	144	214	42

energy charge on the cotton seed enzyme is shown in Figure 6, at three total adenylate concentrations. The concentration of ADP appears to be the main determining factor in the shape of these curves, but ATP inhibition and AMP activation skews the curves somewhat. In order to determine the effect of the individual nucleotides of the energy charge mixture on the curves, the composition of energy charge mixtures was calculated, and assays were performed using the calculated concentration of ADP, in the presence and absence of each of the other two adenine nucleotides (Fig. 7). Clearly, the ADP con-

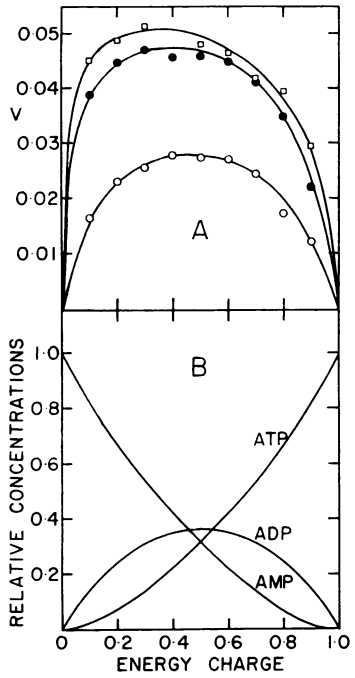

FIG. 6. Effect of energy charge on purified cotton seed pyruvate kinase. A: the enzyme was purified and assayed as described under "Materials and Methods," using 0.05 mM PEP, 10 mM magnesium chloride and 45 mM potassium chloride in tris TES buffer. Mixtures of AMP and ATP, with the appropriate energy charge and a total adenylate concentration of 6 mM, were incubated with 20 mM magnesium chloride and 20 units of rabbit muscle adenylate kinase per ml, in tris TES buffer. Appropriate amounts of these mixtures were added to the assays to give a final adenylate concentration of 0.2 mM (○), 1.0 mM (●), or 2.0 mM (□). B: The calculated relative concentrations of AMP, ADP, and ATP are plotted against energy charge. Calculations were performed as described by Atkinson (1), assuming an equilibrium constant ([ATP][AMP]/[ADP]^2) of 0.8.

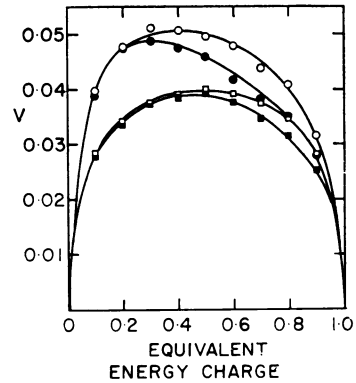

FIG. 7. Factors affecting the energy charge response of purified cotton seed pyruvate kinase. Methods were as in Figure 6A, using AMP, ADP, and ATP concentrations calculated as in Figure 6B, assuming a total adenylate concentration of 1 mM. The components added to the assays were: ADP only (□), AMP and ADP (○), ADP and ATP (■), or AMP, ADP, and ATP (●).

centration determines the general appearance of the curves. AMP activation is evident over most of the curve, although the effect is most pronounced at low energy charge, where the concentration of AMP is greatest, and ATP inhibition is appreciable above an energy charge of 0.3. In the range of 0.7 to 1.0, where most cells appear to operate (9), the two effects virtually compensate for one another.

DISCUSSION

A major problem in the study of pyruvate kinase from higher plants is the presence of a phosphatase activity in plant extracts which interferes with the assay. Of the tissues examined, only ungerminated cotton seeds were found to exhibit a low enough phosphatase activity under the assay conditions. These extracts do contain an acid phosphatase but the pH optimum of this activity is sufficiently far removed from the pH of the pyruvate kinase assay for it to cause little interference.

Pyruvate kinase in crude extracts showed nonhyperbolic kinetics towards PEP, ADP, and magnesium, the saturation curves being sigmoidal. However, a variety of pretreatments or assaying the enzyme under various conditions of pH and temperature failed to accentuate this effect. It is probable that the deviation from normal kinetics is an artifact of the assay of the crude preparation. It is possible that the pyruvate kinase is a regulatory enzyme *in vivo* which has been modified on extraction. The failure to separate the preparation into two isoenzymes indicates that two forms of the enzyme are not present. The crude enzyme is inhibited by citrate and ATP and is activated by AMP, indicating that it may possess regulatory properties.

After purification of the enzyme by chromatography on DEAE-cellulose, the AMP, ATP, and citrate effects are still seen, but the enzyme displays normal kinetics towards PEP and ADP. The addition of ATP, citrate or AMP is unable to induce a change to sigmoid kinetics. The kinetics of the purified enzyme towards magnesium are hyperbolic, but the addition of citrate gave a sigmoid magnesium saturation curve. However, a similar effect was noted on addition of EDTA, so the sigmoidicity is probably an artifact resulting from magnesium chelation. The shape of the energy charge response curve is typical of enzymes involved in the regulation energy-generating pathways (1); this is mainly an effect of ADP concentration.

In the conversion of fat into carbohydrate, PEP is a key branchpoint intermediate which can be utilized for energy generation or for gluconeogenesis. Pyruvate kinase would therefore be expected to be subject to regulation. The regulation might be expected to be somewhat similar to that exhibited by phosphofructokinase, which is inhibited by ATP, citrate, and PEP and is activated by phosphate (11, 20). A similar regulatory pattern was, in fact, found for pyruvate kinase. The effects of ATP and AMP are less marked than have been reported for phosphofructokinase, but this is offset by the fact that ADP is the substrate of pyruvate kinase, whereas ADP is a product of phosphofructokinase. The data presented, in conjunction with that presented elsewhere (13), lead us to conclude that pyruvate kinase from cotton seeds is a regulatory enzyme.

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