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Kinetic Studies on the Regulation of Rabbit Liver Pyruvate Kinase

By M. G. IRVING and J. F. WILLIAMS
School of Biochemistry, University of New South Wales, Kensington, N.S.W. 2033, *Australia*

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Two kinetically distinct forms of pyruvate kinase (EC 2.7.1.40) were isolated from rabbit liver by using differential ammonium sulphate fractionation. The L or liver form, which is allosterically activated by fructose 1,6-diphosphate, was partially purified by DEAE-cellulose chromatography to give a maximum specific activity of 20 units/mg. The L form was allosterically activated by K^+ and optimum activity was recorded with 30mM-K^+, 4mM-$MgADP^-$, with a $MgADP^-/ADP^{2-}$ ratio of 50:1, but inhibition occurred with K^+ concentrations in excess of 60mM. No inhibition occurred with either ATP or GTP when excess of Mg^{2+} was added to counteract chelation by these ligands. Alanine (2.5mM) caused 50% inhibition at low concentrations of phosphoenolpyruvate (0.15mM). The homotropic effector, phosphoenolpyruvate, exhibited a complex allosteric pattern ($n_H = 2.5$), and negative co-operative interactions were observed in the presence of low concentrations of this substrate. The degree of this co-operative interaction was pH-dependent, with the Hill coefficient increasing from 1.1 to 3.2 as the pH was raised from 6.5 to 8.0. Fructose 1,6-diphosphate interfered with the activation by univalent ions, markedly decreased the apparent K_m for phosphoenolpyruvate from 1.2mM to 0.2mM, and transformed the phosphoenolpyruvate saturation curve into a hyperbola. Concentrations of fructose 1,6-diphosphate in excess of 0.5mM inhibited this stimulated reaction. The M or muscle-type form of the enzyme was not activated by fructose 1,6-diphosphate and gave a maximum specific activity of 0.3 unit/mg. A Michaelis–Menten response was obtained when phosphoenolpyruvate was the variable substrate ($K_m = 0.125$mM), and this form was inhibited by ATP, as well as alanine, even in the presence of excess of Mg^{2+}.

Pyruvate kinase catalyses the formation of pyruvate and ATP from phosphoenolpyruvate and ADP, and is a key enzyme involved in the co-ordination of flux of carbon atoms through both the glycolytic and gluconeogenic pathways. Control of the activity of this enzyme is of considerable interest, because any significant, simultaneous pyruvate kinase activity during gluconeogenesis would serve to create a 'futile cycle', recycling pyruvate at the expense of ATP (Sols, 1968), and it would appear that the cellular activity of this enzyme is subject to several types of control.

Hess *et al.* (1966) reported that yeast pyruvate kinase could be strongly and specifically activated by low concentrations of fructose 1,6-diphosphate, with kinetics characteristic of allosteric activation. This activation by fructose 1,6-diphosphate could be counteracted by ATP, and it was proposed that alternative operation of these components, together with the other phosphokinases of glycolysis, would produce a pulsed glycolytic turnover, whereby pyruvate kinase activity was increased during glycolysis and markedly inhibited during gluconeogenesis (Hess *et al.*, 1966). Pyruvate kinase extracted from rat liver (Tanaka *et al.*, 1967*a*), rat epididymal adipose tissue (Pogson, 1968) and *Escherichia coli* K_{12} (Malcovati & Kornberg, 1969) have also been shown to give kinetics with fructose 1,6-diphosphate characteristic of allosteric activation.

Several observations suggest, however, that pyruvate kinase activity may also be controlled through changes in the total enzyme concentration. Tanaka *et al.* (1967*b*) reported the existence of two kinetically and antigenically different species of pyruvate kinase in rat liver. The muscle-type (M) form gave Michaelis–Menten kinetics with phosphoenolpyruvate as the variable substrate, and was insensitive to fructose 1,6-diphosphate, whereas the liver (L) form gave a sigmoidal response to phosphoenolpyruvate and was allosterically activated by fructose 1,6-diphosphate. It was also shown that the concentration of this sensitive kinase was dependent on the hormonal and nutritional status, and a similar dependence has been observed with the enzyme from rat kidney cortex (Krebs & Eggleston, 1965) and yeasts (Hommes, 1964).

The primary objective of this present study was to investigate more fully the nature of the interaction between fructose 1,6-diphosphate and rabbit liver pyruvate kinase, as there was no published study of the factors modulating its activity. The purification of the enzyme was first attempted, and some of the

kinetic properties were systematically investigated. The results were used as a basis for an investigation of the nature of the binding of the allosteric effector, fructose 1,6-diphosphate, to the enzyme (Irving & Williams, 1973).

Experimental

Materials

Chemicals and enzymes. All enzymes, coenzymes and substrates were obtained from Boehringer Corp. (London) Ltd. (London W.5, U.K.) or Sigma Chemical Co. (St. Louis, Mo., U.S.A.). Substrates were in the form of their sodium salts, except fructose 1,6-diphosphate (tetracyclohexylammonium salt) and phosphoenolpyruvate (tricyclohexylammonium salt). Inorganic reagents and solvents were of A.R. grade (British Drug Houses Ltd., Poole, Dorset, U.K., or E. Merck A.G., Darmstadt, Germany). Sephadex resin and ion-exchange resins were obtained from Townson and Mercer (N.S.W.) Pty. Ltd., Sydney, Australia.

Rabbits. California–New Zealand white crossbred female rabbits aged 4–5 months (average body wt. 4kg) were obtained from the Tillside Stud, Bargo, N.S.W., Australia. Animals were not starved before being killed.

Buffer solutions. All buffer solutions were adjusted to the appropriate pH at 25°C (by using a Radiometer pH-meter model 22, fitted with a combined Radiometer electrode), with either 1M-HCl or aq. 1M-NH_3 soln.

Dialysis tubing for enzyme fractions. Dialysis tubing (6.35cm; Visking Co., Chicago, Ill., U.S.A.) was treated to remove plasticizing substances, as described by Garland *et al.* (1964), before dialysis of the enzyme fraction against the appropriate buffer.

Extraction of pyruvate kinase from rabbit liver

Animals were weighed, killed by cervical dislocation, and the chilled, chopped liver was homogenized in a Waring Blendor for 60s with 10vol. of extracting buffer. Two different buffer systems were used for the extraction of the enzyme from rabbit liver. Buffer A contained 20mM-sodium phosphate, 1mM-EDTA, 5mM-$MgSO_4$, 2mM-mercaptoethanol and 2.5mM-iodoacetate adjusted to pH7.4. Buffer B contained 45mM-imidazole, 0.15M-KCl, 5mM-$MgSO_4$, 2.5mM-EDTA and 1mM-dithiothreitol, and was adjusted to pH7.4 with 0.2M-acetic acid. The crude homogenate was centrifuged for 1h at 40000$g_{av.}$ at 4°C (Sorvall RC-2 centrifuge, SS-34 head, 18500rev./min), and the supernatant solution was filtered through glass wool to remove the fat pad.

The protein of the supernatant solution was fractionated with $(NH_4)_2SO_4$. The $(NH_4)_2SO_4$ concentration was successively raised to 45, 55 and 65% saturation, and after each $(NH_4)_2SO_4$ treatment the mixture was allowed to equilibrate at 4°C for 2h before it was centrifuged at 10000$g_{av.}$ for 1h.

The L form of pyruvate kinase sedimented in the 0–45%-satd. $(NH_4)_2SO_4$ fraction, and this fraction was suspended in 10vol. of 20mM-sodium phosphate buffer, pH7.4, containing 0.15M-KCl, and was then stirred at 4°C for 2h. The preparation was then heated at 55°C for 3min, quickly cooled and the denatured protein was removed by centrifuging at 40000$g_{av.}$ for 1h at 4°C. When the supernatant solution was adjusted back to 45% saturation with solid $(NH_4)_2SO_4$ it was found that the protein fraction which sedimented at this concentration of $(NH_4)_2SO_4$ did not contain ketose 1-phosphate aldolase (EC 4.1.2.7) or phosphofructokinase (EC 2.7.1.11).

This fraction was dissolved in a minimal volume of 20mM-sodium phosphate buffer, pH7.4, and chromatographed on a column (40cm × 2cm) of Sephadex G-25, which was equilibrated with a solution containing 90mM-imidazole, 5mM-$MgSO_4$, 0.25M-sucrose and 0.5mM-dithiothreitol, adjusted to pH7.4 with 0.2M-acetic acid. The eluted enzyme was then dialysed at 4°C for 14h against 20vol. of the above buffer to remove residual Cl^-. The enzyme preparation was concentrated to a final volume of 15ml by immersing the dialysis tubing in polyvinylpyrrolidone powder (1.5g of powder/ml of enzyme solution) and was then applied to a column (20cm × 2.8cm) of DEAE-cellulose, which had been equilibrated with the dialysing buffer. After enzyme adsorption, the column was washed with 2 bed vol. of buffer, and the enzyme was eluted with a linear gradient of 0–0.3M-KCl (Fig. 1). The L form of rabbit liver pyruvate kinase was eluted at 0.12M-KCl and was stabilized by the addition of solid $(NH_4)_2SO_4$ to a final concentration of 45%. Further attempts to purify the enzyme by application to CM-cellulose or Sephadex G-200 columns resulted in further loss in enzyme activity, with little increase in the purity of the preparation.

Attempts to purify the M form of the enzyme, which sedimented in the 55–65%-satd. $(NH_4)_2SO_4$ fraction, by DEAE-cellulose chromatography were not successful, as the enzyme would not adsorb to the cellulose below pH8.5, and at this pH very poor recovery of the enzyme was obtained.

Assay of pyruvate kinase activity

Pyruvate kinase activity was assayed at 30°C by using a modification of the method of Bücher & Pfleiderer (1955). The reaction was coupled to lactate dehydrogenase, and the oxidation of NADH was followed continuously by measuring either the change in E_{340} with a Unicam SP. 800 multiple-recording spectrophotometer, coupled to a Honeywell–Brown Electronic recorder, or the E_{334} with an Eppen-

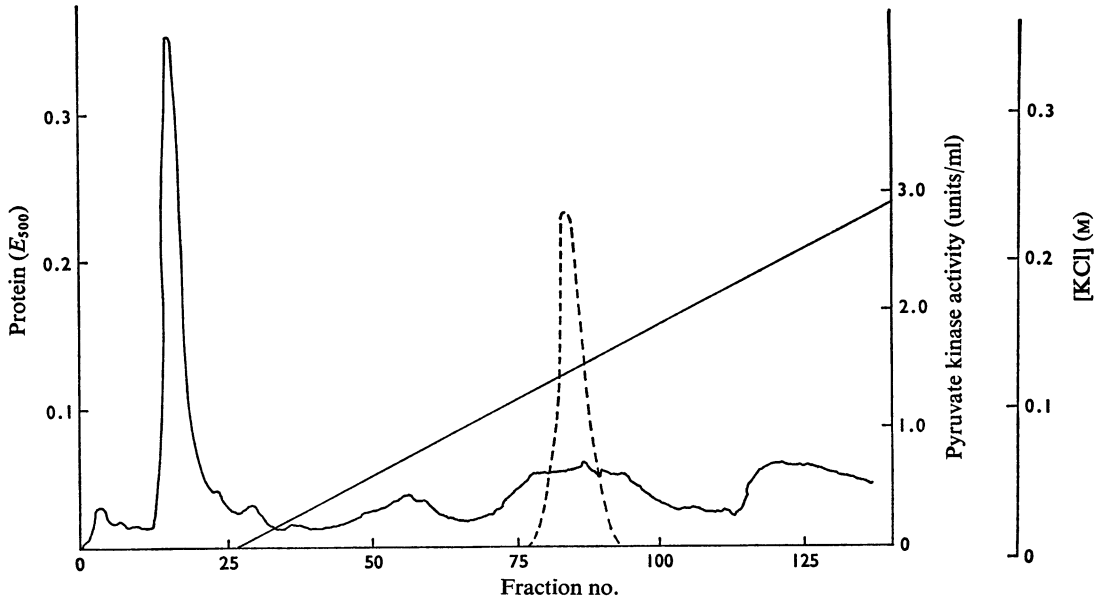

Fig. 1. *Elution profile of the L form of pyruvate kinase from DEAE-cellulose chromatography*

The preparation layered on the column contained 180mg of protein with a specific activity of 0.6 unit/mg. The column (40cm×2cm) had a flow rate of 90ml/h, and 6ml fractions were collected at 4°C. Enzyme activity and protein were measured as described in the Experimental section and the legend of Table 2. The straight line denotes [KCl]. ——, Protein; – – – –, pyruvate kinase activity.

dorf photometer with a log-linear converter and scale expander allowing a full-scale deflexion of 0–0.05 absorbance unit on a Ricken Denshi SP-J3 recorder. The assay medium contained: 30mM-Tris; 40mM-KCl; 0.15mM-NADH; 4.0mM-MgADP$^-$ ($MgADP^-/ADP^{2-} = 50:1$), adjusted to pH7.4 with 0.2M-HCl; lactate dehydrogenase, 10 units, in a final volume of 1ml. Assay procedure for the intrinsic pyruvate kinase reaction involved incubation of all reactants except phosphoenolpyruvate for 10min at 30°C and the reaction was started by the addition of phosphoenolpyruvate. For the fructose 1,6-diphosphate-stimulated reaction, the same procedure was used, with 0.1mM-fructose 1,6-diphosphate and phosphoenolpyruvate being added simultaneously to start the reaction. Pyruvate kinase activity was shown to be linear over a 10-fold range of enzyme concentration.

The following procedures were used to assay for contaminant enzymes in the pyruvate kinase preparation: ketose 1-phosphate aldolase (EC 4.1.2.7) (Bruns & Bergmeyer, 1963); fructose 1,6-diphosphatase (EC 3.1.3.11) (Pontremoli *et al.*, 1965); glycerol 3-phosphate dehydrogenase (EC 1.1.1.8) (Shonk & Boxer, 1964); hexokinase (EC 2.7.1.1) (Sharma *et al.*, 1963); lactate dehydrogenase (EC 1.1.1.27) (Bergmeyer *et al.*, 1963); malate dehydrogenase (EC 1.1.1.40) (Bergmeyer & Bernt, 1963); phosphofructokinase (EC 2.7.1.11) (Underwood & Newsholme, 1965).

Preparation of lactate dehydrogenase

Before most experiments, lactate dehydrogenase was passed through a column (10cm×1.0cm) of Sephadex G-25 (coarse grade) in 20mM-sodium phosphate buffer, pH7.4. In experiments designed to investigate the effect of univalent cations 1.0ml of rabbit muscle lactate dehydrogenase (5mg/ml, 360 units/mg) was dialysed against three changes of 250ml of 45mM-imidazole acetate, pH7.4, diluted to 400 units/ml and stored at −10°C in 1ml portions.

Reaction rates

These are expressed as initial velocities in μmol of product/min at 30°C (units) or specific activities (μmol/min per mg) under the assay conditions. Hill coefficients (n_H) were calculated from the equation $n_H = \log v/(V-v/\log[L])$, where [L] is the concentration of ligand, v is the initial velocity of the formation

of pyruvate at a given concentration of phosphoenolpyruvate and V is the velocity when the enzyme is saturated with phosphoenolpyruvate. For kinetic studies each experimental point is the mean of four separate determinations for that concentration of substrate. Each concentration of phosphoenolpyruvate was studied separately and was not due to an incremental addition of phosphoenolpyruvate to the assay mixture.

General methods

Protein. This was determined by the methods of either Lowry *et al.* (1951) or Warburg & Christian (1942). Bovine serum albumin (fraction V) was used as a protein standard.

Polyacrylamide gels. Polyacrylamide gels (6%) were prepared by using the procedures of Davis (1964). Gels were loaded with 100μg of protein and run in Tris–glycine buffer, pH8.9, at a current of 5mA per tube. After electrophoresis the gels were stained with Amido Schwarz fixative, destained and stored with 7% (v/v) acetic acid. Polyacrylamide-gel electrophoresis in a continuous molecular-sieve gradient was carried out by the methods of Margolis & Kenrick (1968). Gradipore electrophoresis apparatus supplied by Townson and Mercer (N.S.W.) Pty. Ltd. was used with a concave gel concentration of acrylamide of 4–27%.

Results

Extraction of the L and M forms of pyruvate kinase

The fraction which sedimented at 0–45%-satd. $(NH_4)_2SO_4$ was designated as the L form of the enzyme, since it showed a co-operative interaction with the homotropic effector, phosphoenolpyruvate, and was also allosterically activated by the heterotropic effector, fructose 1,6-diphosphate. Isolation of this fraction in imidazole buffer (buffer B) resulted in a preparation with a higher specific activity than when the enzyme was extracted in phosphate buffer (buffer A). It was also observed that the enzyme was more stable when extracted in buffer B, but that a five- to six-fold increase in activity was the maximum activation brought about by fructose 1,6-diphosphate. However, with pyruvate kinase extracted in buffer A it was observed that freshly prepared fractions exhibited only a two- to three-fold stimulation by fructose 1,6-diphosphate, but after aging for 3 weeks at 0–5°C, a 10–15-fold stimulation was consistently observed. Table 1 shows that for the L form of the enzyme isolated in buffer A the fructose 1,6-diphosphate-stimulated rate remained relatively constant regardless of the age of the preparation. However, the intrinsic rate decreased as the preparation aged, and the high stimulation by fructose 1,6-diphosphate could be attributed to this effect. The L form of the enzyme isolated in buffer B was not subject to this rapid aging effect.

Table 1. *Effect of buffer extraction on fructose* 1,6-*diphosphate stimulation of the L form of pyruvate kinase*

Chilled, chopped rabbit liver (10g) was homogenized in 10vol. of buffer A and the supernatant solution was adjusted to 45% saturation with $(NH_4)_2SO_4$ as described in the Experimental section. A further 10g of the same liver was homogenized in 10vol. of buffer B and processed identically to that in buffer A. Both fractions were assayed for pyruvate kinase activity at 7-day intervals by using the assay mixture described in the Experimental section. All assays were performed in triplicate, and the reaction was started by the addition of phosphoenolpyruvate, to a final concentration of 0.25mM, and fructose 1,6-diphosphate (0.1mM). v = Reaction velocity in the absence of allosteric modifiers. $v_{Fru1,6\text{-}diP}$ = reaction velocity in the presence of 0.1mM-fructose 1,6-diphosphate.

$$\text{Stimulation coefficient} = \frac{v_{Fru1,6\text{-}diP} - v}{v} \times 100.$$

Age of pyruvate kinase (days)	Buffer A			Buffer B		
	v (unit/g)	$v_{Fru1,6\text{-}diP}$ (unit/g)	Stimulation coefficient (%)	v (unit/g)	$v_{Fru1,6\text{-}diP}$ (unit/g)	Stimulation coefficient (%)
0	0.25	0.29	16	0.38	0.425	12
7	0.13	0.285	119	0.322	0.375	16
14	0.105	0.274	160	0.201	0.256	27
21	0.065	0.269	314	0.150	0.235	56
28	0.029	0.241	731	0.143	0.212	48
35	0.017	0.205	1105	0.135	0.202	49
42	0.004	0.175	4275	0.095	0.135	43

Table 2. *Purification of rabbit liver pyruvate kinase*

The extraction buffer had the following composition: 45mM-imidazole, 0.15M-KCl, 5mM-$MgSO_4$, 2.5mM-EDTA and 1mM-dithiothreitol, adjusted to pH7.4 with 0.2M-acetic acid. Extraction procedures were as described in the Experimental section. All assays were carried out at 30°C in an assay mixture of the following composition: 40mM-Hepes (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulphonic acid), 30mM-KCl, 4mM-$MgADP^-$ ($MgADP^-/ADP^{2-} = 50:1$), adjusted to pH6.5 with 0.1M-KOH, and 10units of lactate dehydrogenase, in a total volume of 1.0ml. The reaction was started by the addition of phosphoenolpyruvate to a final concentration of 6.0mM. Values are for an extract obtained from 50g of rabbit liver.

Step	Volume (ml)	Total activity (units)	Total protein (mg)	Specific activity (units/mg)	Yield (%)	Purification
Crude extract	500	468	17000	0.028	100	1
0–45%-satd. $(NH_4)_2SO_4$ fraction	15	500	3680	0.24	100	8.7
Heat-treatment and $(NH_4)_2SO_4$ fractionation	1.9	316	210	1.5	66	53.5
DEAE-cellulose	20	12.3	0.6	20.05	2.6	715
45–55%-satd. $(NH_4)_2SO_4$ fraction	3	17.0	618	0.028	3.4	1
55–65%-satd. $(NH_4)_2SO_4$ fraction	5	60.0	1100	0.052	8.0	2

The results in Table 2 show that there was a 700-fold increase in the specific activity of the L form after DEAE-cellulose chromatography. The L form was highly susceptible to inactivation on dilution or in the absence of univalent ions. Although the 0–45%-satd. $(NH_4)_2SO_4$ fraction of the homogenate supernatant was stable for 10–12 weeks at 4°C, it was found that, after heat treatment, dialysis for 14h of the L form resulted in a 60% loss of enzyme activity, and there was a further loss of activity if the fraction obtained after DEAE-cellulose chromatography was not fractionated with $(NH_4)_2SO_4$. This inactivation of the L form was not alleviated by the addition of ADP (0.1mM) and phosphoenolpyruvate (0.1mM) to the extraction buffer, and was the cause of the low yield of the highly purified L form of the enzyme. Polyacrylamide- and Gradipore-gel (concave 4–27%) electrophoresis revealed only two protein bands; pyruvate kinase was the major component with a molecular weight in excess of 175000, and there was a minor band with a molecular weight less than 60000. No ketose 1-phosphate aldolase, fructose 1,6-diphosphatase, phosphofructokinase, glycerol 3-phosphate dehydrogenase, lactate dehydrogenase, malate dehydrogenase or hexokinase activity was detected in the L form of pyruvate kinase.

The fraction that sedimented at 55–65%-satd. $(NH_4)_2SO_4$ was designated the M, or muscle-type, form. This enzyme differed from the L form in that it did not adsorb to DEAE-cellulose until the pH was raised to 8.5, and it exhibited Michaelis–Menten kinetics with phosphoenolpyruvate as the variable substrate (Fig. 2). This fraction contained a very active aldolase–glycerol 3-phosphate dehydrogenase couple, which gave high values for the rate of the reaction in the presence of fructose 1,6-diphosphate, but with the use of suitable controls to compensate for this contamination, fructose 1,6-diphosphate stimulation of the M form was not observed. Reduction with $NaBH_4$ (Grazi *et al.*, 1962) of the Schiff base between the contaminant aldolase and its substrate

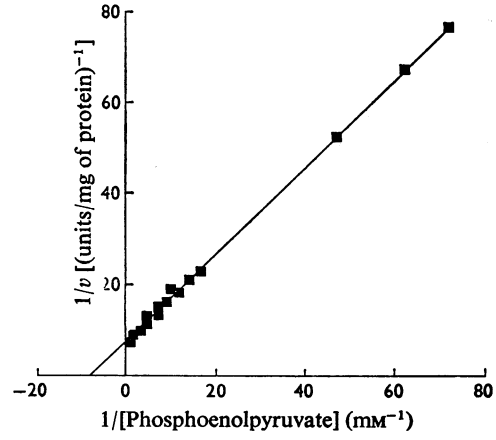

Fig. 2. *Double-reciprocal plot of the rate of reaction of the M form of rabbit liver pyruvate kinase with various concentrations of phosphoenolpyruvate*

A fixed concentration of $MgADP^-$ was used (4mM-$MgADP^-$, $MgADP^-/ADP^{2-} = 50:1$). The assay system was as described in the Experimental section, and assay mixture plus enzyme was incubated at 30°C for 10min before the reaction was started by the addition of phosphoenolpyruvate.

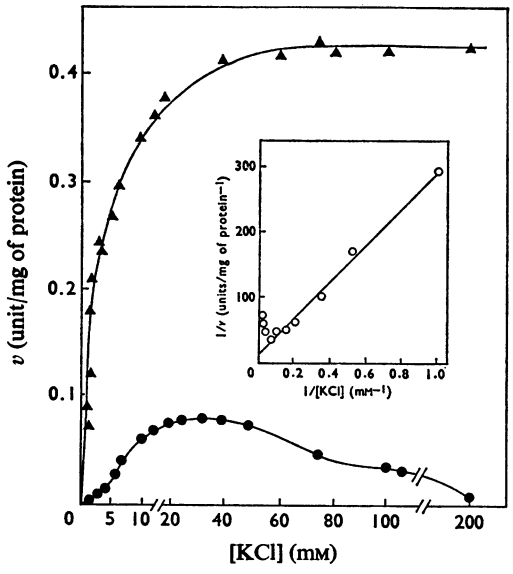

Fig. 3. *Influence of K^+ on the relationship between L form of pyruvate kinase activity and phosphoenolpyruvate concentration*

The reaction mixture, including enzyme, was preincubated at 30°C for 10min, and the reaction was started by the addition of phosphoenolpyruvate to give a final concentration of 0.25mM. ●, No fructose 1,6-diphosphate; ▲, fructose 1,6-diphosphate (0.2mM) added at the same time as phosphoenolpyruvate. The inset depicts the double-reciprocal plot of the reaction in the absence of fructose 1,6-diphosphate.

dihydroxyacetone phosphate was not successful in eliminating the influence that these contaminating enzymes had on the rate of the reaction in the presence of fructose 1,6-diphosphate.

Activation of the L form of pyruvate kinase by K^+

Pyruvate kinase isolated from yeast and animal tissues has an absolute requirement for univalent ions (Kachmar & Boyer, 1953; Suelter *et al.*, 1966), and the response of the L form of rabbit liver pyruvate kinase to increasing concentrations of K^+ at a constant concentration of phosphoenolpyruvate (0.25mM) is shown in Fig. 3. A sigmoidal response was recorded for the intrinsic reaction, with optimum activity at 50mM-K^+ and an apparent K_m of 9.3mM for K^+. Inhibition of the intrinsic reaction occurred when the K^+ concentration exceeded 60mM, and it would appear that the rabbit liver enzyme was more sensitive to increasing concentrations of K^+ than the enzyme isolated from other sources (Melchior, 1965; Williams *et al.*, 1969). The optimum K^+ concentration for the intrinsic reaction was close to the reported concentration of K^+ (47mM) in the cytosol (Murdoch & Heaton, 1968), but the fructose 1,6-diphosphate-stimulated reaction was unaffected by these elevated K^+ concentrations.

Activation of the L form of pyruvate kinase by Mg^{2+} and ADP^{2-}

The bivalent cations Mg^{2+}, Co^{2+} and Mn^{2+} are known to be activators of the pyruvate kinase reaction, and considerable evidence (Reynard *et al.*, 1961; Melchior, 1965) indicates that the adenosine nucleotides participate in this reaction as their magnesium salts, with the bivalent cations forming a bridge between the enzyme and ADP (Mildvan & Cohn, 1965). By using a value of 4×10^3 for the stability constant of $MgADP^-$ (O'Sullivan & Perrin, 1964), different concentrations of $MgADP^-$ were prepared with known $MgADP^-/ADP^{2-}$ ratios.

Table 3 shows that a $MgADP^-/ADP^{2-}$ ratio of 50:1, equivalent to a total Mg^{2+} concentration of 13.5mM, gave optimum activity for both the intrinsic and fructose 1,6-diphosphate-stimulated reaction. Fig. 4 shows the effect of increasing the concentration of $MgADP^-$, at a fixed ratio of $MgADP^-/ADP^{2-}$ of 50:1, for both the intrinsic and fructose 1,6-diphosphate-stimulated reactions. Saturation curves were obtained for both expressions of activity, with K_m values of 0.5 and 0.455mM respectively, which were in close agreement with other reported values (Boyer, 1962).

Activation of L form of pyruvate kinase by fructose 1,6-diphosphate

The effect of increasing concentrations of fructose 1,6-diphosphate on the velocity of the L form of pyruvate kinase at a possible physiological concentration of phosphoenolpyruvate (0.25mM) is shown in Fig. 5. The kinetics followed a Michaelis–Menten relationship, and a modified Lineweaver–Burk plot of these data (Fig. 5 inset) gave a K_a value for fructose 1,6-diphosphate of 0.3μM, which was similar to values reported for pyruvate kinase extracted from other sources (Taylor & Bailey, 1967; Susor & Rutter, 1968).

Inhibition of the stimulated reaction occurred with fructose 1,6-diphosphate concentrations in excess of 0.5mM, and it would appear that the rabbit liver enzyme exhibited maximum sensitivity at lower fructose 1,6-diphosphate concentrations than did the enzyme extracted from yeast. The structural specificity of fructose 1,6-diphosphate activation was tested with a number of glycolytic intermediates, but only fructose 1,6-diphosphate and fructose 6-phosphate (0.1mM) stimulated the enzyme. The activation by

Table 3. *Effect of different $MgADP^-/ADP^{2-}$ ratios at constant concentration of $MgADP^-$ (1 mM) on the reaction velocity of the liver (L) form of rabbit liver pyruvate kinase in the presence and absence of added fructose* 1,6 *diphosphate*

The enzyme was incubated for 10min at 30°C with the assay mixture described in the Experimental section, and the reaction was started by the addition of phosphoenolpyruvate to a final concentration of 0.25mM. The fructose 1,6-diphosphate-stimulated rate was studied by using identical conditions in the presence of 0.2mM-fructose 1,6-diphosphate.

$MgADP^-/ADP^{2-}$	Total [Mg] (mM)	Free Mg^{2+} (mM)	Total [ADP] (mM)	Free $[ADP^{2-}]$ (mM)	Reaction velocity (unit/mg) v	$v_{Fru\,1,6-diP}$
2:1	1.5	0.5	1.764	0.764	0.0087	0.013
4:1	2.0	1.0	1.47	0.47	0.0172	0.024
5:1	2.25	1.25	1.412	0.412	0.0183	0.025
10:1	3.5	2.5	1.294	0.294	0.0252	0.042
15:1	4.75	3.75	1.254	0.254	0.0263	0.056
20:1	6.0	5.0	1.253	0.253	0.0274	0.064
50:1	13.5	12.5	1.200	0.200	0.0280	0.064
100:1	26.0	25.0	1.188	0.188	0.0278	0.061

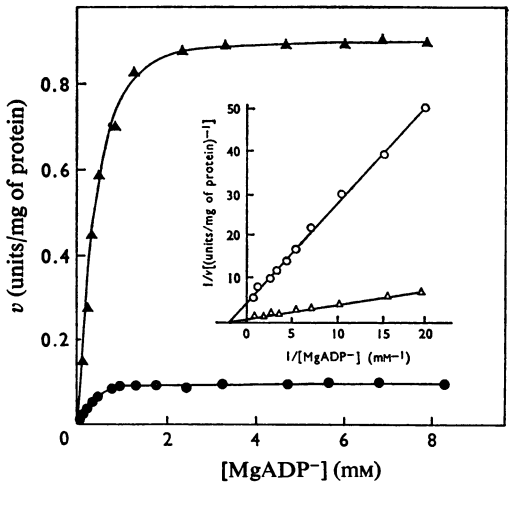

Fig. 4. *Influence of $MgADP^-$ concentration on the relationship between the L form of pyruvate kinase activity and phosphoenolpyruvate concentration at a constant $MgADP^-/ADP^{2-}$ ratio of* 50:1

The reaction mixture, including enzyme, was pre-incubated at 30°C for 10min, and the reaction was started by the addition of phosphoenolpyruvate to give a final concentration of 0.25M. ●, No fructose 1,6-diphosphate; ▲, fructose 1,6-diphosphate (0.1mM) added at the same time as phosphoenolpyruvate. The inset depicts the double-reciprocal plot of the reaction in the presence (△) and in the absence (○) of fructose 1,6-diphosphate.

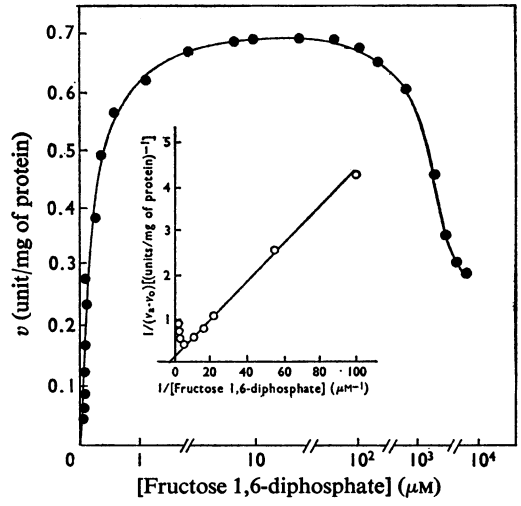

Fig. 5. *Effect of fructose* 1,6*-diphosphate on the rate of reaction of the L form of pyruvate kinase at constant phosphoenolpyruvate concentration*

The reaction mixture, including enzyme, was pre-incubated at 30°C for 10min, and the reaction was started by the addition of phosphoenolpyruvate to give a final concentration of 0.25mM, together with the stated concentrations of fructose 1,6-diphosphate. The inset depicts the double-reciprocal plot of this reaction.

fructose 6-phosphate was not reproducible and was attributed to trace impurities of fructose 1,6-diphosphate.

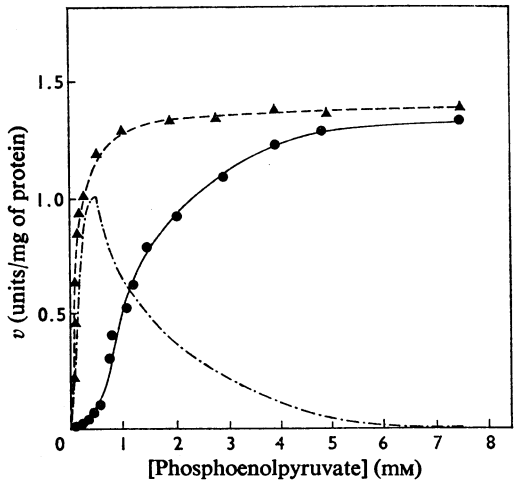

Fig. 6. *Influence of fructose* 1,6-*diphosphate on the relationship between the L form of pyruvate kinase activity and phosphoenolpyruvate concentrations at a constant* $MgADP^-$ *concentration* (4 mM-$MgADP^-$, $MgADP^-/ADP^{2-} = 50:1$)

The reaction mixture, including enzyme, was incubated at 30°C for 10 min, and the reaction was started by the addition of phosphoenolpyruvate and fructose 1,6-diphosphate. All concentrations of phosphoenolpyruvate were studied separately and were not due to incremental additions of phosphoenolpyruvate to the assay mixture. Each experimental point represents quadruplicate determinations for each phosphoenolpyruvate concentration. ●, No fructose 1,6-diphosphate; ▲, 0.1 mM-fructose 1,6-diphosphate; -·-·-·-, $v_{\text{Fru 1,6-diP}} - v$.

Activation of the L and M forms of pyruvate kinase by phosphoenolpyruvate

The results in Fig. 6 revealed that there was a positive co-operative interaction between the L form of pyruvate kinase and phosphoenolpyruvate, and a Hill coefficient of 2.5 was measured for the data in the range of phosphoenolpyruvate concentrations 0–5.0 mM. Loss of this homotropic interaction occurred in the presence of 0.1 mM-fructose 1,6-diphosphate, and the double-reciprocal plot of the data gave a K_m value of 0.2 mM for the fructose 1,6-diphosphate-stimulated reaction, compared with the 'apparent K_m' of 1.2 mM for the non-stimulated reaction. It was noteworthy that the difference between the stimulated and non-stimulated reactions was decreased by phosphoenolpyruvate concentrations above 0.5 mM, indicating that the fine control exerted by the heterotropic effector fructose 1,6-diphosphate could be replaced by the homotropic effect when phosphoenolpyruvate concentrations were high.

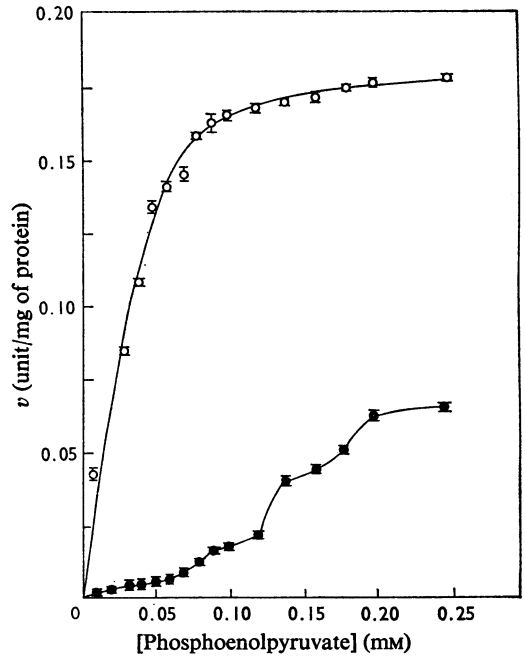

Fig. 7. *Influence of fructose* 1,6-*diphosphate on the relationship between the L form of pyruvate kinase activity and phosphoenolpyruvate concentrations within the normal physiological range of phosphoenolpyruvate concentrations* (0.05–0.25 mM)

Each experimental point represents quadruplicate determinations for each phosphoenolpyruvate concentration. All concentrations of phosphoenolpyruvate were studied separately and were not due to incremental additions of phosphoenolpyruvate to the assay mixture. Reaction conditions were identical with those detailed in Fig. 6. ●, No fructose 1,6-diphosphate; ○, 0.1 mM-fructose 1,6-diphosphate. Standard errors for each experimental point are shown.

The physiological concentration of phosphoenolpyruvate in liver cytosol is 50 μM (Greenbaum *et al.*, 1971), and a detailed analysis of the homotropic interaction with the L form within a possible physiological range of phosphoenolpyruvate concentrations of 0–0.25 mM is shown in Fig. 7. For every preparation of the L form studied (10), a wavy, non-linear curve was consistently and reproducibly observed for the intrinsic reaction. This kinetic pattern indicated that a combination of both positive and negative co-operative interactions occurred with the L form of pyruvate kinase within the physiological range of phosphoenolpyruvate concentrations but this interaction could be overcome by the addition of fructose 1,6-diphosphate (0.1 mM) to the enzyme assay.

It was observed that the M form of the enzyme did not exhibit any obvious co-operative interaction with the homotropic effector phosphoenolpyruvate (Fig. 2), a Hill coefficient of 1.3 being found. The M form of the enzyme had a K_m value for phosphoenolpyruvate of 0.125mM, which was some ten times lower than the K_m observed for the non-stimulated reaction for the L form of the enzyme. Under no circumstances was any activation of this fraction by fructose 1,6-diphosphate observed.

Interactions of the L and M forms of pyruvate kinase with ATP, GTP and alanine

Hess *et al.* (1966) reported that ATP was able to inhibit fructose 1,6-diphosphate activation of yeast pyruvate kinase, and proposed that inhibition of pyruvate kinase by elevated concentrations of ATP would provide an inhibitor for glycolysis, particularly when the main flux of carbon atoms was through the gluconeogenic sequence. GTP, a substrate for the phosphoenolpyruvate carboxylase (EC 4.1.1.32) reaction, and the glycogenic amino acid alanine, have both been proposed as inhibitors of chicken liver pyruvate kinase (Williams *et al.*, 1969), and the effect of these ligands on the L and M forms of rabbit liver pyruvate kinase was investigated.

Wood (1968) reported that inhibition of pyruvate kinase by ATP may be attributed to chelation of Mg^{2+} by ATP, together with an inadequate control of pH. As it had been determined that excess of Mg^{2+} did not inhibit the L form of rabbit liver pyruvate kinase (Table 2), an equimolar amount of Mg^{2+} was added to prevent inhibition by ATP or GTP caused solely by chelation of Mg^{2+}, and solutions of all phosphorylated effectors were adjusted to the experimental pH before assay.

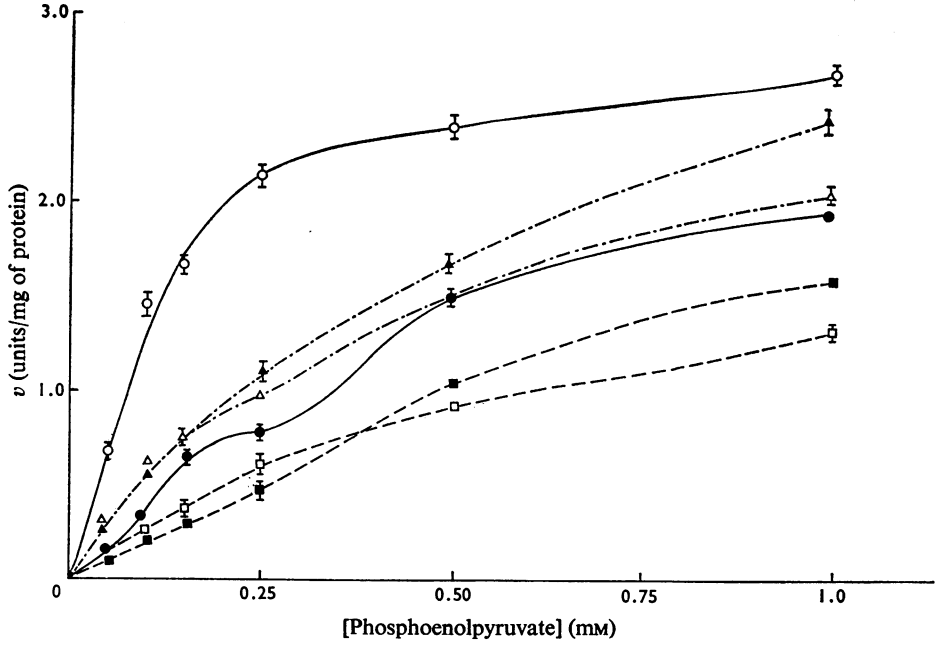

Fig. 8. *Effect of ATP, GTP and alanine on the relationship between the L form of pyruvate kinase activity and phosphoenolpyruvate concentration*

The reaction mixture, including enzyme, was preincubated at 30°C for 5min, and then ATP, GTP or alanine was added to the cuvette and allowed to incubate at 30°C for a further 5min before the reaction was started by the addition of phosphoenolpyruvate or phosphoenolpyruvate and fructose 1,6-diphosphate. All concentrations of phosphoenolpyruvate were studied separately and were not due to incremental additions of phosphoenolpyruvate to the assay mixture. Each experimental point represents quadruplicate determinations for each phosphoenolpyruvate concentration. ●, Phosphoenolpyruvate with no fructose 1,6-diphosphate; ○, phosphoenolpyruvate plus 0.2mM-fructose 1,6-diphosphate; ▲, either 5mM-ATP or 2.5mM-GTP, no fructose 1,6-diphosphate; △, 10mM-ATP, no fructose 1,6-diphosphate; ■, 2.5mM-alanine, no fructose 1,6-diphosphate; □, 5mM-alanine, no fructose 1,6-diphosphate. Reaction conditions were as described in the Experimental section except that the Mg^{2+} concentration was doubled to give a total value of 33mM. Standard errors for each experimental point are shown.

The results in Fig. 8 showed that ATP in no way inhibited either the intrinsic or fructose 1,6-diphosphate-stimulated reactions for the L form of pyruvate kinase, and in fact some activation occurred at high phosphoenolpyruvate concentrations. To test further whether any inhibition by ATP could be observed, pyruvate kinase was assayed at normal concentrations of Mg^{2+} in both the presence and absence of ATP, and also by using the assay procedure of Boyer (1969) with glycerol 1-phosphate to buffer the free Mg^{2+} concentration. Table 4 shows that under normal assay conditions (4mM-$MgADP^-$, $MgADP^-/ADP^{2-}$ = 50:1, total $[Mg^{2+}]$ = 16.5mM) some inhibition did occur, which was not observed when the Mg^{2+} concentration was doubled or when the Mg^{2+} concentrations were buffered by using the system of Boyer (1969). It would thus appear that inhibition by ATP or GTP may be attributed to chelation of Mg^{2+} in the assay system.

Alanine was the most effective inhibitor of the L form of the enzyme, causing a 50% inhibition at low phosphoenolpyruvate concentrations (0.5mM), and this inhibition was not relieved by elevated phosphoenolpyruvate concentrations. The inhibition pattern for 5mM-alanine when analysed as a Hill plot gave a n_H value of 2.7 compared with the Hill coefficient of 2.1 for the intrinsic reaction. However, the inhibitory effect of alanine was completely counteracted by low concentrations of fructose 1,6-diphosphate, the sigmoidal response being converted into a hyperbolic curve when phosphoenolpyruvate was the variable substrate.

The kinetic patterns caused by interactions of ATP and alanine with the M form of pyruvate kinase are shown in Fig. 9, where additional Mg^{2+} had been added to the assay medium to counteract any inhibition caused by chelation of Mg^{2+}. Unlike the L form of pyruvate kinase, the M form was sensitive to ATP inhibition, particularly at high concentrations of ATP (5mM). Alanine was again the best inhibitor, but unlike its behaviour with the L form of the enzyme, did not impart a sigmoidal response with increasing phosphoenolpyruvate concentrations, but paralleled the Michaelis–Menten pattern of the intrinsic reaction.

Allosteric interactions of the L form of pyruvate kinase as a function of pH

Rozengurt *et al.* (1969) reported that the allosteric properties of rat liver pyruvate kinase (type L) were strongly influenced by changes in H^+ concentration in the assay medium. Since phosphoenolpyruvate and fructose 1,6-diphosphate allosterically activate the enzyme in strikingly different ways, the effect of pH on these two modes of allosteric effect was studied (Fig. 10) at a constant phosphoenolpyruvate concentration of 0.25mM. Maximum activity was recorded at pH6.5, and there was a 30% decrease in activity when the pH was raised to 7.4. The pH effect found was independent of the buffer employed in the assay medium but the greatest activity was exhibited with Hepes. The pH optimum for the M form of the enzyme was 6.0 (Fig. 11).

The effect of pH on the shape of the curves of both the intrinsic and fructose 1,6-diphosphate-stimulated reactions for the L form of pyruvate kinase when phosphoenolpyruvate was the variable substrate is

Table 4. *Effect of Mg^{2+} concentration on the inhibition of the L form of pyruvate kinase by ATP*

All assays were carried out at 30°C in a reaction mixture of the following composition: 50mM-Tris, 30mM-KCl, 5.0mM-ADP, adjusted to pH7.4 with 0.2M-HCl, and 5 units of lactate dehydrogenase in a total volume of 1.0ml. The use of 50mM sodium glycerol 1-phosphate, as described by Boyer (1969), in the above reaction mixture resulted in a buffered free Mg^{2+} concentration of 8.0mM when the total Mg^{2+} concentration was 30mM. In the presence of 3.0mM-ATP, 99% of the ADP was present as $MgADP^-$, and 99% of the ATP as $MgATP^{2-}$. In all assays the enzyme was preincubated in the reaction mixture at 30°C for 5min before the addition of ATP, which was incubated for a further 5min before the reaction was started by the addition of phosphoenolpyruvate to a final concentration of 0.25mM, and fructose 1,6-diphosphate to a total concentration of 0.2mM. Results are expressed as the means of four measurements.

	Pyruvate kinase activity (unit/ml)					
[ATP] (mM) ...	0		2.5		5.0	
Total $[Mg^{2+}]$ in assay (mM)	v	$v_{Fru\ 1,6\text{-}diP}$	v	$v_{Fru\ 1,6\text{-}diP}$	v	$v_{Fru\ 1,6\text{-}diP}$
16.5	0.29	0.48	0.25	0.48	0.21	0.50
33.0	0.30	0.50	0.31	0.51	0.34	0.53
30 (in the presence of 50mM-glycerol 1-phosphate)	0.28	0.47	0.275	0.46	0.28	0.49

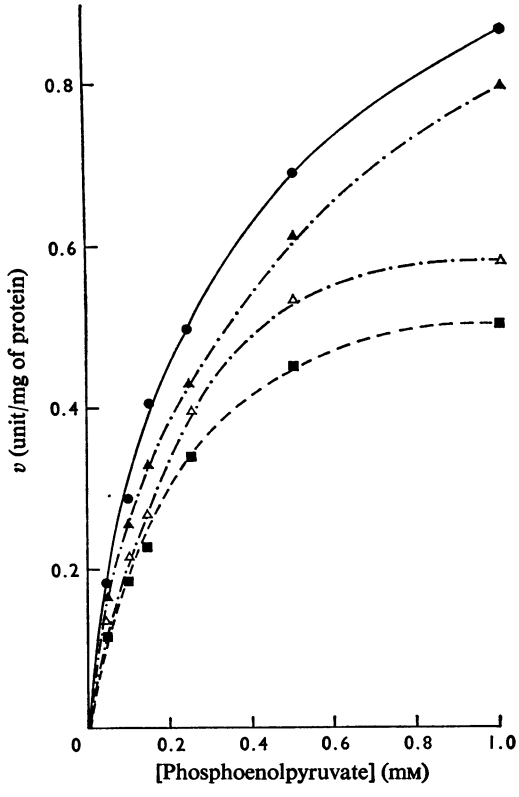

Fig. 9. *Effect of ATP and alanine on the relationship between the M form of pyruvate kinase activity and phosphoenolpyruvate concentration*

The reaction mixture, including enzyme, was pre-incubated at 30°C for 5min, and then ATP or alanine was added to the cuvette and allowed to incubate at 30°C for a further 5min before the reaction was started by the addition of phosphoenolpyruvate. Reaction conditions were as described in the Experimental section except that the Mg^{2+} concentration was doubled to give a total value of 33mM. Each experimental point represents quadruplicate determinations for each phosphoenolpyruvate concentration. ●, Phosphoenolpyruvate with no effectors; ▲, 2.5mM-ATP; △, 5mM-ATP; ■, 2.5mM-alanine.

shown in Fig. 12. As the pH was raised the shapes of the curves became increasingly sigmoidal, and the enzyme became more susceptible to fructose 1,6-diphosphate activation at low phosphoenolpyruvate concentrations. The value of the Hill coefficient increased from 1.1 at pH6.5 to 3.2 at pH8.0. For the four pH values studied, the value of the Hill coefficient for the fructose 1,6-diphosphate-stimulated reaction

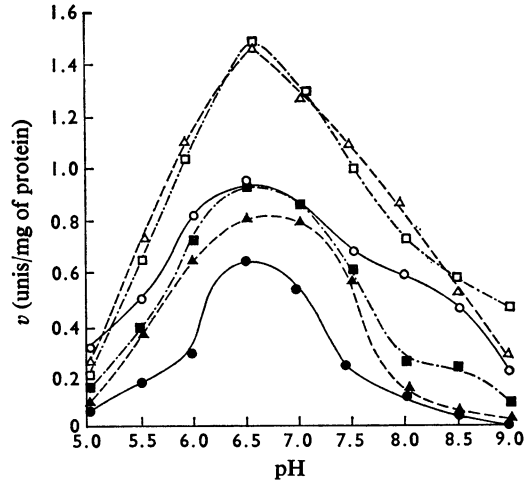

Fig. 10. *Effect of pH on the activities of the fructose 1,6-diphosphate-stimulated and non-stimulated activities of the L form of pyruvate kinase at a constant phosphoenolpyruvate concentration*

The reaction mixture, including enzyme, was pre-incubated at 30°C for 10min, and then the reaction was started by the addition of phosphoenolpyruvate to a final concentration of 0.25mM. ●, 50mM-Tris, no fructose 1,6-diphosphate; ○, 50mM-Tris, 0.2mM-fructose 1,6-diphosphate; ▲, 50mM-glycylglycine, no fructose 1,6-diphosphate; △, 50mM-glycylglycine, 0.2mM-fructose 1,6-diphosphate; ■, 50mM-Hepes, no fructose 1,6-diphosphate; □, 50mM-Hepes, 0.2mM-fructose 1,6-diphosphate.

was 1.0±0.2, indicating that increasing pH values did not affect the heterotropic interaction between the enzyme and fructose 1,6-diphosphate.

The results in Fig. 12, when plotted (Fig. 13) as a Dixon (1953) plot, indicated that an ionizing group having a pK value between 6.9 and 7.2 may be associated with the co-operative interaction between the L form of the enzyme and phosphoenolpyruvate.

Discussion

The specific activity of pyruvate kinase in rabbit liver is low, being 8–10 units/g wet wt. of tissue, but this low activity is sufficient to divert the flux of gluconeogenesis by wasteful cycling (Exton & Park, 1967). As in rat liver, two distinct forms of pyruvate kinase exist in rabbit liver, and the measured activity of each preparation depends on the extracting buffer used. The L form of the enzyme was preferentially extracted and stabilized by imidazole buffer, pH7.4, whereas the M form was the more active species

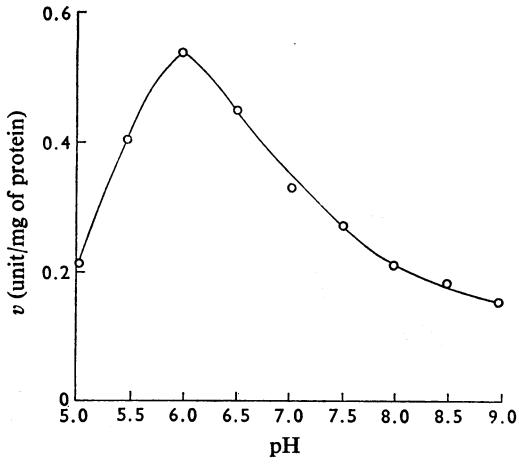

Fig. 11. *Effect of pH on the activity of the M form of pyruvate kinase*

The reaction mixture, including enzyme, was pre-incubated at 30°C for 10min and the reaction was started by the addition of phosphoenolpyruvate to a final concentration of 0.25mM. The reaction buffer was 50mM-Hepes.

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when extracted with phosphate buffer, pH7.4. Enzyme activity showed linear kinetics over a 10-fold range of enzyme concentrations (Irving, 1969), suggesting that the activity of the enzyme may not depend on the concentration of associated subunits.

The procedure used to assay enzyme activities gave much higher activity than the original Bücher & Pfleiderer (1955) procedure. Several workers, including Williams *et al.* (1969) with chicken liver pyruvate kinase and Haeckel *et al.* (1968) with yeast pyruvate kinase, have compared this coupled-assay procedure with others involving the chemical determination of pyruvate or monitoring of the disappearance of the enol band at 230nm, and have shown that the coupled-assay system did not interfere with the rate of the pyruvate kinase reaction. Williams *et al.* (1969) have also shown that a 10min preincubation at 30°C resulted in linear reaction kinetics, in contrast to the curvilinear kinetics obtained in the non-incubated experiments for both the fructose 1,6-diphosphate-stimulated and non-stimulated rates of reaction, and this procedure was incorporated into all assays.

The steady-state kinetics of the L form of rabbit liver pyruvate kinase demonstrated a co-operativity between the enzyme activity and concentrations of phosphoenolpyruvate and K^+, indicating allosteric properties of the enzyme. The enzyme was inhibited at lower concentrations of K^+ and fructose 1,6-diphosphate than had been reported for pyruvate kinase extracted from other sources (Haeckel *et al.*, 1968; Williams *et al.*, 1969), indicating that further fine controls could be exerted by the prevailing concentrations of these effectors.

The homotropic interaction with phosphoenolpyruvate yielded a sigmoidal curve (Fig. 6), and the Hill coefficient of 2.5 indicated a positive co-operative interaction between this ligand and the L form of pyruvate kinase. The physiological range of concentration of phosphoenolpyruvate in rat liver cytosol (Greenbaum *et al.*, 1971) and rabbit liver cytosol (M. G. Irving, unpublished work) is between 0.05mM and 0.25mM, and a detailed investigation of the homotropic interaction within this range (Fig. 7) consistently revealed 'wavy', non-linear kinetics. Levitzki & Koshland (1969), using a sequential model for subunit interactions, have described this kinetic pattern as indicating that both positive and negative co-operativity could occur for the same ligand. The molecular events which may explain this combination of positive and negative homotropic kinetics are the following.

(1) The binding of the first phosphoenolpyruvate molecule makes it easier for the second to bind, which in turn makes it more difficult for the third molecule of substrate to bind to the enzyme, and this pattern is extended to the binding of subsequent molecules of this ligand. (2) The binding of the homotropic effector may initially strengthen subunit interactions and thus distort the active site on each subunit and decrease substrate affinity, particularly if the dissociated form of the enzyme is the active species, as is seen with aspartate carbamoyltransferase (EC 2.1.3.2) (Gerhart & Pardee, 1962). (3) The homotropic kinetic pattern reflects subunit interactions with intermediary dissociation steps, as have been postulated by Benesch *et al.* (1966) to account for the allosteric nature of the oxygen–haemoglobin system. (4) A continuation of negative and positive co-operativity in $k_{cat.}$, but a negative co-operativity and Michaelis–Menten pattern of substrate binding. (5) A combination of two or more of the alternatives.

Although it is not presently possible experimentally to verify any of these proposals for pyruvate kinase, this combination of co-operative effects would have several advantages. Positive co-operativity in $k_{cat.}$ per site would result in increased sensitivity of the enzyme to changes in the ligand concentration, and the increased sensitivity at this key branch point would be advantageous, particularly for a system in glycolytic flux. Negative co-operativity at very low concentrations of phosphoenolpyruvate would tend to insulate the enzyme from extreme fluctuations in phosphoenolpyruvate concentrations (Conway & Koshland, 1968). The M form of the enzyme did not exhibit any co-operative interaction with phosphoenolpyruvate and the K_m of 0.125mM was much

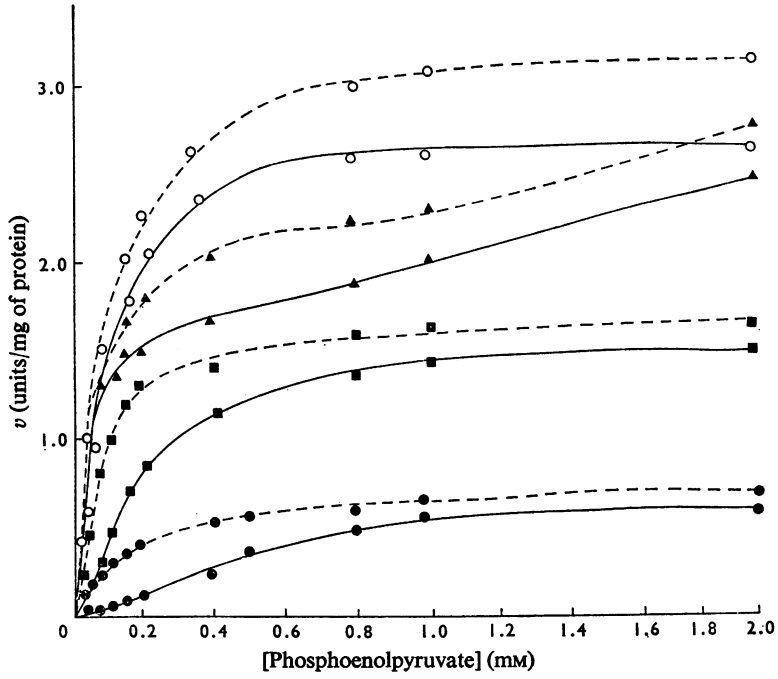

Fig. 12. *Influence of pH on the relationship between the L form of pyruvate kinase and phosphoenolpyruvate concentrations in the presence and absence of fructose* 1,6-*diphosphate*

The reaction mixtures, including enzyme, were preincubated at 30°C for 10min, and the reaction was started by the addition of phosphoenolpyruvate. Each experimental point represents quadruplicate determinations for each phosphoenolpyruvate concentration. o—o, 50mM-Hepes, pH6.5, no fructose 1,6-diphosphate; o---o, 50mM-Hepes, pH6.5, 0.2mM-fructose 1,6-diphosphate; ▲—▲, 50mM-Hepes, pH7.0, no fructose 1,6-diphosphate; ▲---▲, 50mM-Hepes, pH7.0, 0.2mM-fructose 1,6-diphosphate; ■—■, 50mM-Hepes, pH7.5, no fructose 1,6-diphosphate; ■---■, 50mM-Hepes, pH7.5, 0.2mM-fructose 1,6-diphosphate; ●—●, 50mM-Hepes, pH8.0, no fructose 1,6-diphosphate; ●---●, 50mM-Hepes, pH8.0, 0.2mM-fructose 1,6-diphosphate.

lower than the values recorded for either the intrinsic or the fructose 1,6-diphosphate-stimulated reactions of the L form, but was similar to the values obtained by Reynard *et al.* (1961) for the rabbit muscle enzyme and by Tanaka *et al.* (1967*a*) for the M form of rat liver pyruvate kinase.

Fructose 1,6-diphosphate was shown to activate the enzyme five- to six-fold. As the preparation aged, much greater activation was recorded, but this was accompanied by a decrease in the intrinsic activity. Unlike the yeast enzyme, the heterotropic effector did not exhibit any co-operative interaction with the L form of rabbit liver pyruvate kinase and the K_a (activator constant) value determined (0.3μM) was close to the reported concentration of fructose 1,6-diphosphate in liver (Burch, 1965). Because the dissociation constant of the Mg^{2+}–fructose 1,6-diphosphate and Mn^{2+}–fructose 1,6-diphosphate complexes are large enough to preclude the chelation of these bivalent ions by fructose 1,6-diphosphate (Haeckel *et al.*, 1968), and because the fructose 1,6-diphosphate-stimulated reaction is not dependent on the concentrations of bivalent or univalent cations (Figs. 3 and 4), it may be inferred that the binding site for fructose 1,6-diphosphate is different from the catalytic site. No fructose 1,6-diphosphate activation of the M form could be detected.

The allosteric properties of the L form of pyruvate kinase were strongly affected by changes in pH. Below pH7.0 the enzyme obeyed Michaelis–Menten kinetics with respect to phosphoenolpyruvate and there was little activation by the heterotropic effector, fructose 1,6-diphosphate. As the pH was raised, the kinetic response to increasing phosphoenolpyruvate concentrations became increasingly sigmoidal and fructose 1,6-diphosphate activation was more pronounced. This altered kinetic profile pointed to activation of an ionizing group having a pK value

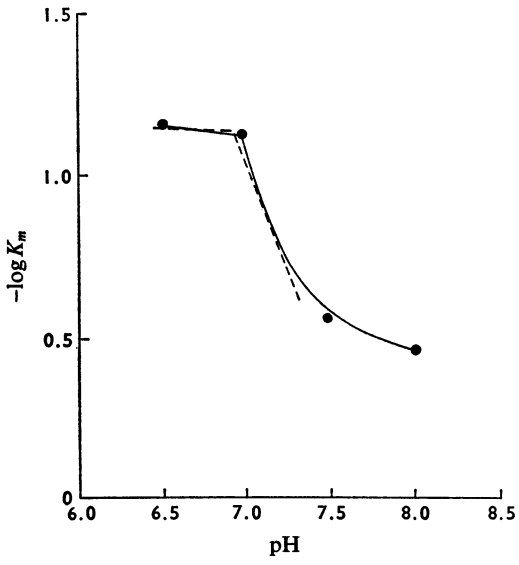

Fig. 13. *Influence of pH on the K_m of the L form of pyruvate kinase for various concentrations of phosphoenolpyruvate*

K_m values were determined from double-reciprocal plots of the data in Fig. 12. Experimental procedures were as described in the legend of Fig. 12.

around 7.0. Rozengurt *et al.* (1969), using the L form of rat liver pyruvate kinase, and Wieker & Hess (1971), with yeast pyruvate kinase, have noted the importance of a group, possibly imidazole, having this pK value in the regulation of the degree of co-operativity between the enzyme and phosphoenolpyruvate. The pH profiles revealed that the optimum pH was 6.5 for the L form and 6.0 for the M form in the presence and the absence of fructose 1,6-diphosphate. This pH-dependency could have considerable significance in pathological conditions involving intracellular acidosis, e.g. hepatocarcinoma, since not only would pyruvate kinase be more activated in this condition but the change to Michaelis–Menten kinetics would mean that the enzyme would be saturated at much lower phosphoenolpyruvate concentrations, thus promoting a rapid glycolytic flux. The decreased activity of pyruvate kinase when Tris–HCl was used as the assay buffer probably reflects the perturbation of tryptophan residues in this enzyme, as noted by Wilson *et al.* (1967), resulting in decreased enzyme stability.

Inhibition of the L form of pyruvate kinase by ATP and GTP was shown to be due solely to the removal of the Mg^{2+} from the assay medium and it would appear most unlikely that these nucleotide triphosphates could act as the physiological inhibitors of the enzyme. Gevers & Krebs (1966) suggested that the extrusion of Ca^{2+} out of, and the influx of Mg^{2+} into, mitochondria during gluconeogenesis could be an effective method of controlling pyruvate kinase activity, and Meli & Bygrave (1972), by altering the $[Mg^{2+}]/[Ca^{2+}]$ ratio in rat liver mitochondria were able to switch on and off pyruvate kinase activity alternately and rapidly. It would appear from our studies that the nature of the ionic environment could be the major mode for the control of the activity of the L form of pyruvate kinase. Alanine was the most effective inhibitor, particularly at low phosphoenolpyruvate concentrations, and elevated concentrations of this glycogenic amino acid could provide a further mode of control of pyruvate kinase during gluconeogenesis. Fructose 1,6-diphosphate was at all times able to overcome any inhibition by alanine. The M form of the enzyme was, however, subject to inhibition by ATP and alanine, and this effect could not be attributed to chelation of Mg^{2+}.

In summary, the present paper describes the isolation of two distinct forms of pyruvate kinase from rabbit liver, and details of experiments showing that these forms differ from each other in respect of (1) solubility in $(NH_4)_2SO_4$, (2) adsorption to DEAE-cellulose, (3) interaction with phosphoenolpyruvate and fructose 1,6-diphosphate, (4) pH optima and (5) inhibition by ATP and GTP.

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