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# Studies on the Interaction between Rabbit Liver Pyruvate Kinase and its Allosteric Effector Fructose 1,6-Diphosphate

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Preparation of the L form of rabbit liver pyruvate kinase (EC 2.7.1.40) in the presence of fructose 1,6-diphosphate yielded an enzyme which was kinetically identical with the M or muscle-type form of pyruvate kinase found in liver. Chromatographic and dialysis studies of this complex showed that most of the fructose 1,6-diphosphate molecules were loosely bound to the enzyme, but dilution–dissociation studies and binding experiments established that there was a high initial affinity between the enzyme and fructose 1,6-diphosphate ($K_{assoc.} = 2.3 \times 10^9$), and that binding of the loosely bound fructose 1,6-diphosphate was concentration-dependent and a necessary condition to overcome the co-operative interaction observed with the homotropic effector phosphoenolpyruvate. Preparation of the liver enzyme in the absence of EDTA did not yield a predominantly M form of the enzyme, and incubation of the M form in the presence of EDTA did not convert it into the L form, but resulted in inhibition of enzyme activity. Immunological studies confirmed that the L and M forms in liver were distinct, and that preparation of the L form in the presence of fructose 1,6-diphosphate did not produce an enzyme antigenically different from the L form prepared in the absence of this heterotropic effector.

Williams *et al.* (1969) reported that chicken liver pyruvate kinase prepared in the presence of fructose 1,6-diphosphate did not exhibit the pronounced allosteric behaviour of the enzyme prepared in the absence of this effector. This enzyme was kinetically indistinguishable from pyruvate kinase extracted from chicken muscle, even after passage through a Sephadex G-25 column to remove free fructose 1,6-diphosphate. In the light of this observation it appeared conceivable that the M or muscle-type form of the enzyme isolated by Tanaka *et al.* (1967) from rat liver may be stable L or liver-type form to which fructose 1,6-diphosphate was tightly bound.

Pogson (1968) reported that an L and an M form of pyruvate kinase could be separately isolated from rat epididymal adipose tissue, depending on whether or not EDTA was included in the extraction medium. It was also reported that the fructose 1,6-diphosphate-sensitive enzyme was converted into the M form when incubated with low concentrations of fructose 1,6-diphosphate and that this process was reversed on incubation with EDTA. It was possible that the EDTA in the extracting buffer had dissociated fructose 1,6-diphosphate from the enzyme (yielding a liver or L-form enzyme) whereas homogenization in its absence allowed isolation of a pyruvate kinase–fructose 1,6-diphosphate complex (which was kinetically identical with the M form of the liver enzyme), and thus Pogson's (1968) results were similar in many respects to those of Williams *et al.* (1969).

Sols (1968) showed that any significant pyruvate kinase activity during gluconeogenesis would create a wasteful adenosine triphosphatase cycle with interaction of glycolytic and gluconeogenic fluxes at the phosphoenolpyruvate level. This 'futile cycle' could be bypassed by conversion of the M form of pyruvate kinase, which had been shown to have a lower $K_m$ for phosphoenolpyruvate than the L form (Irving & Williams, 1973), into an L form, mediated by alterations in the steady-state concentration of fructose 1,6-diphosphate, and its subsequent removal from pyruvate kinase (reconverting it into the L form) by some other active metabolite in a manner analogous to the reported mode of action of EDTA *in vitro*. Knowledge of the nature of the binding between the enzyme and fructose 1,6-diphosphate would thus indicate whether the two forms of pyruvate kinase were interconvertible. The present paper reports an investigation of the nature of the binding of fructose 1,6-diphosphate to the rabbit liver L form of pyruvate kinase by using [U-$^{14}$C]fructose 1,6-diphosphate. Results are also reported on the action of EDTA on the L and M forms of rabbit liver pyruvate kinase, and the nature of the antigenic specificity of these enzyme forms.

## Experimental

### *Materials*

*Chemicals and enzymes.* Chemicals and enzymes used were as described by Irving & Williams (1973).

D-[U-$^{14}$C]Glucose 6-phosphate (100mCi/mmol) was obtained from The Radiochemical Centre, Amersham, Bucks., U.K.

*Experimental animals.* Animals used were as described by Irving & Williams (1973). Adult male albino Sprague–Dawley rats were obtained from the animal holding unit at Prince Henry Hospital, Sydney, N.S.W., Australia.

*Dialysis studies.* Dialysis tubing was prepared as described by Irving & Williams (1973).

*Preparation of [U-$^{14}$C]fructose 1,6-diphosphate*

The procedure detailed below was developed for the preparation and isolation of relatively large amounts (2$\mu$mol) of [U-$^{14}$C]fructose 1,6-diphosphate. D-[U-$^{14}$C]Glucose 6-phosphate (500$\mu$Ci, 2$\mu$mol) was dissolved in 5ml of water and added to 1ml of 0.4M-triethanolamine–HCl buffer, pH7.6, together with 10$\mu$mol of ATP, 35 units of glucose phosphate isomerase (EC 5.3.1.9), 15 units of phosphofructokinase (EC 2.7.1.11), 20$\mu$mol of $MgSO_4$ and 60$\mu$mol of KCl.

The reaction was allowed to proceed at 25°C for 50min. During this time 50$\mu$l samples were transferred to 0.5ml of water at regular intervals, deproteinized by heating at 100°C for 5min and, after centrifugation, the supernatant solution was assayed for fructose 1,6-diphosphate by the method of Bücher & Hohorst (1963). When the reaction had come to equilibrium (50min), 6ml of 0.6M-$HClO_4$ was added, the denatured protein was removed by centrifugation and the supernatant solution was adjusted to pH8.0 with 10M-KOH. After standing for 1h at 0°C, the precipitate of $KClO_4$ was removed by centrifugation.

Two columns (50cm×0.5cm) of Dowex AG 1 (X4) resin were prepared as described by Williams *et al.* (1971). The first column was used as a control to study the separation of glucose, glucose 6-phosphate, fructose 6-phosphate and fructose 1,6-diphosphate, and the hexose and hexose phosphates were isolated and identified by using the methods of Williams *et al.* (1971). The preparative column was loaded with the deproteinized solution of [U-$^{14}$C]-fructose 1,6-diphosphate contained in 5ml of water, adjusted to pH8.0 with 0.1M-KOH, and eluted with a linear gradient of 0.1–0.4M-ammonium tetraborate. Every fourth sample was analysed for hexose phosphate, and radioactivity was monitored by adding 10$\mu$l portions of the column eluate to 5ml of Diotol (Nuclear–Chicago Corp., 1967) and counting the radioactivity in a Packard Tri-Carb liquid-scintillation spectrometer model 2002. The fractions containing [U-$^{14}$C]fructose 1,6-diphosphate were pooled and evaporated to dryness *in vacuo* at 40°C, and the residue was suspended in a minimum volume of water. The ammonium tetraborate was removed by three successive evaporations, each with 300ml of redistilled A.R. methanol. [U-$^{14}$C]Fructose 1,6-diphosphate, free of borate, was dissolved in 5ml of water, pH6.5 (final sp. radioactivity 5$\mu$Ci/ml). A yield of 64% was obtained for the conversion of glucose 6-phosphate into fructose 1,6-diphosphate.

*Preparation of the L form of rabbit liver pyruvate kinase in the presence of [U-$^{14}$C]fructose 1,6-diphosphate*

A rabbit was killed by cervical dislocation and the chilled, chopped liver was divided into two equal portions (each 30g). One of these was homogenized with 300ml of 20mM-sodium phosphate, pH7.4, containing 1mM-EDTA and 1mM-fructose 1,6-diphosphate; 1.4$\mu$mol of [U-$^{14}$C]fructose 1,6-diphosphate ($2.2\times10^6$d.p.m.) was also added. The other portion of liver was homogenized in the same volume of buffer containing no fructose 1,6-diphosphate. The two different enzyme preparations were isolated as the $(NH_4)_2SO_4$ fractions by using methods detailed by Irving & Williams (1973), and the 0–45%-satd. $(NH_4)_2SO_4$ fraction, which contained the L form, was used for all studies.

*Preparation and isolation of rabbit liver pyruvate kinase in the presence and absence of EDTA*

A rabbit was killed by cervical dislocation and the chilled, chopped liver divided into two equal portions (each 25g). One of these was homogenized in 30mM-imidazole, 5mM-EDTA and 10mM-mercaptoethanol, adjusted to pH6.8 with 0.2M-acetic acid, and the enzyme isolated was defined as PK-A in the terminology of Pogson (1968). The other portion was homogenized in 20mM-imidazole, adjusted to pH6.8 with 0.2M-acetic acid, and this enzyme fraction was designated PK-B. The two enzyme preparations were isolated and stabilized by $(NH_4)_2SO_4$ fractionation as described by Irving & Williams (1973), and the supernatant solution as well as the 0–45%-, 45–55%- and 55–65%-satd. $(NH_4)_2SO_4$ fractions were used for enzyme studies.

*Assay of pyruvate kinase activity*

The procedure used was as described by Irving & Williams (1973) in assay medium containing 30mM-Tris, 40mM-KCl, 0.15mM-NADH, 4mM-$MgADP^-$ ($MgADP^-/ADP^{2-}$ = 50:1), adjusted to pH7.4 with 0.2M-HCl, and 10 units of lactate dehydrogenase, in a final volume of 1ml. Assay procedure for the intrinsic reaction involved incubation of all reactants except phosphoenolpyruvate, for 10min at 30°C, and the reaction was started by the addition of 0.25mM-phosphoenolpyruvate. For the fructose 1,6-diphosphate-stimulated reaction, the same procedure was used except that 0.1mM-fructose 1,6-

diphosphate and 0.25mM-phosphoenolpyruvate were added simultaneously to start the reaction.

*Chromatographic studies of the pyruvate kinase–[U-$^{14}$C]fructose 1,6-diphosphate complex*

Sephadex G-25 (coarse grade) was suspended in three changes of 20mM-sodium phosphate buffer, pH7.4, allowed to swell overnight, and the fine particles were decanted before the column was packed. A portion (2.5mg) of the pyruvate kinase–[U-$^{14}$C]-fructose 1,6-diphosphate complex ($2 \times 10^4$ d.p.m.) was washed on to the appropriate column and 0.5ml fractions were collected at the rate of one every 2min, the eluate being continuously monitored at 253.7nm with an Isco UA-2 analyser. All operations were carried out at 0–5°C.

*Protein determination*

All protein solutions were passed through a Sephadex G-25 column (10cm × 1.0cm) to remove $NH_4^+$ and EDTA, and protein was then measured as described by Irving & Williams (1973).

*Dilution and dissociation studies*

To monitor the proportional change in reaction velocity on serial dilution of the pyruvate kinase–fructose 1,6-diphosphate complex, the reaction was initiated by the simultaneous addition of sub-optimum concentrations of fructose 1,6-diphosphate and phosphoenolpyruvate. After the initial rate had been accurately recorded, one-third of the original reacting mixture was added to twice its volume of a standard assay mixture containing all reactants except pyruvate kinase and fructose 1,6-diphosphate. A further one-third was added to twice its volume of the assay mixture, which contained all reactants, including fructose 1,6-diphosphate at the original sub-optimum concentration, but no pyruvate kinase. The rate of reaction was followed spectrophotometrically as described by Irving & Williams (1973).

*Binding studies*

Amicon conical and flat XM-50 ultrafiltration membranes were obtained from Scientific and Research Equipment Co., Pennant Hills, N.S.W., Australia. Two ultrafiltration procedures were used to study the binding of fructose 1,6-diphosphate to the L form of the enzyme. The first procedure involved the use of flat membranes (XM-50) (molecular-weight 'cut off' = 50000), in an ultrafiltration cell as described by Paulus (1969), whereas the second approach used conical filters as described by Blatt *et al.* (1968). When the concentrations of free and bound [U-$^{14}$C]fructose 1,6-diphosphate had been determined by removal of 10$\mu$l portions into 5ml of Diotol and counting in a Packard Tri-Carb liquid-scintillation spectrometer, the number of mol of fructose 1,6-diphosphate bound/mol of pyruvate kinase was derived (Edsall & Wyman, 1958). A Scatchard (1949) plot was then constructed to determine the association constant between pyruvate kinase and fructose 1,6-diphosphate. The enzyme used for binding studies was the 0–45%-satd. $(NH_4)_2SO_4$ fraction, which had been suspended in 20mM-sodium phosphate, pH7.4, and subjected to heat treatment and chromatographic separation on DEAE-cellulose (Irving & Williams, 1973). The enzyme fractions studied had specific activities of 10–20 units/mg of protein and contained no detectable ketose 1-phosphate aldolase (EC 4.1.2.7), phosphofructokinase (EC 2.7.1.11), $\alpha$-glycerophosphate dehydrogenase (EC 1.1.1.8) or fructose 1,6-diphosphatase (EC 3.1.3.11) activity.

*Preparation of antibodies against rabbit muscle and rabbit liver pyruvate kinase enzyme fractions*

Adult male albino Sprague–Dawley rats were used as host animals. Rabbit muscle pyruvate kinase and the L forms of rabbit liver pyruvate kinase prepared in the presence and absence of fructose 1,6-diphosphate were extensively dialysed against 0.89% NaCl (supplemented with 0.2mM-fructose 1,6-diphosphate for the L form prepared in the presence of this effector). Rabbit muscle pyruvate kinase (0.75mg) was homogenized with 2ml of Freund's adjuvant and injected intraperitoneally, and the L forms of pyruvate kinase were injected at a dosage of 1.15mg of protein in 2ml of adjuvant. Rats were injected every 28 days in the tail vein at a dosage of 0.5mg of protein, and five such treatments were required before a satisfactory antibody titre was obtained (Ballard & Hanson, 1969). Rats were then bled by heart puncture and sera were pooled for antigen–antibody studies. Thin-layer Ouchterlony double-diffusion precipitation analysis of pyruvate kinase antibodies was carried out in Petri dishes. A 1% agar solution containing arsenate was poured, and a centre well 1cm in diameter was filled with appropriate antigen and 0.2ml samples of antibody were pipetted into surrounding wells. The precipitation reaction was completed after 7 days at 30°C in a humidified chamber.

## Results

*Preparation of the [U-$^{14}$C]fructose 1,6-diphosphate–pyruvate kinase complex*

The enzyme–ligand complex in the fraction obtained at 45% saturation with $(NH_4)_2SO_4$ was isolated by passage through a Sephadex G-25 column

(40cm×0.5cm) and the kinetics of the homotropic effector, phosphoenolpyruvate, were ascertained in both the presence and absence of added fructose 1,6-diphosphate. Fig. 1 shows that the [U-$^{14}$C]-fructose 1,6-diphosphate–pyruvate kinase complex exhibited Michaelis–Menten kinetics when initial reaction velocity was plotted against increasing phosphoenolpyruvate concentration, and that there was only slight stimulation by the allosteric effector fructose 1,6-diphosphate.

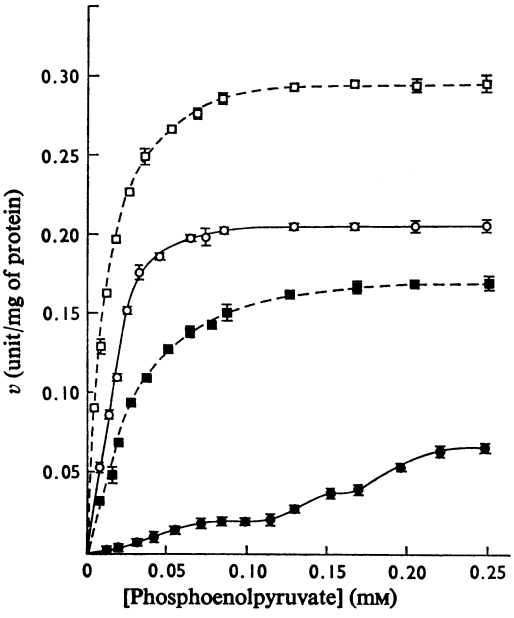


Fig. 1. *Influence of fructose* 1,6-*diphosphate on the relationship between the L form of pyruvate kinase activity prepared in the presence and absence of* [*U*-$^{14}$*C*]*fructose* 1,6-*diphosphate and phosphoenolpyruvate concentrations at a constant* $MgADP^-$ *concentration* (4mM-$MgADP^-$, $MgADP^-/ADP^{2-}=50:1$)

The enzyme preparations were chromatographed on a Sephadex G-25 column (40cm×0.5cm) and eluted with 20mM-sodium phosphate buffer, pH7.4. The reaction mixture including enzyme was incubated at 30°C for 10min, 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 of enzyme activity for each phosphoenolpyruvate concentration. ●, Pyruvate kinase (L form) prepared in the absence of fructose 1,6-diphosphate; ■, pyruvate kinase prepared in the absence of fructose 1,6-diphosphate, 0.1mM-fructose 1,6-diphosphate in the assay; ○, pyruvate kinase prepared in the presence of [U-$^{14}$C]-fructose 1,6-diphosphate; □, pyruvate kinase prepared in the presence of [U-$^{14}$C]fructose 1,6-diphosphate, 0.1mM-fructose 1,6-diphosphate in the assay. Standard errors for each experimental point are shown.

However, the enzyme prepared in the absence of fructose 1,6-diphosphate consistently and reproducibly gave a 'wavy' curve with phosphoenolpyruvate as the variable substrate (see also Irving & Williams, 1973), but there was a marked stimulation of activity and a change of the kinetic pattern to the Michaelis–Menten form in the presence of added fructose 1,6-diphosphate.

*Chromatographic profiles of the* [*U*-$^{14}$*C*]*fructose* 1,6-*diphosphate–pyruvate kinase complex*

The elution profile of the enzyme–ligand complex (which sedimented in the 0–45%-satd. $(NH_4)_2SO_4$ fraction) after chromatography on a Sephadex G-25 column (40cm×0.5cm) is shown in Fig. 2. The

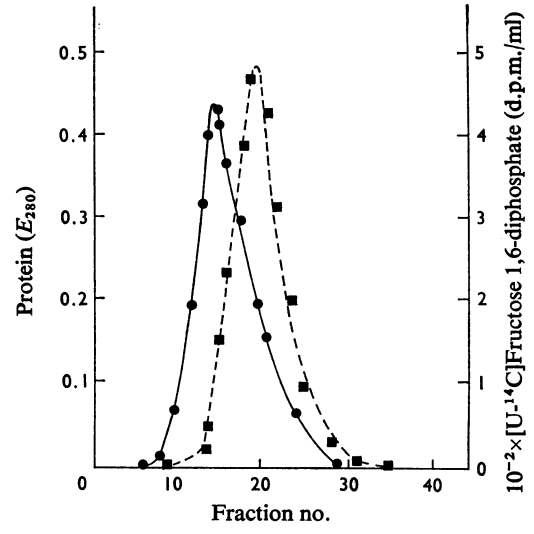


Fig. 2. *Chromatographic separation of* [*U*-$^{14}$*C*]*fructose* 1,6-*diphosphate from rabbit liver pyruvate kinase–* [*U*-$^{14}$*C*]*fructose* 1,6-*diphosphate complex*

The pyruvate kinase–[U-$^{14}$C]fructose 1,6-diphosphate complex (2.5mg) was applied to a Sephadex G-25 column (40cm×0.5cm; coarse grade; void volume 4.4ml; flow rate 15ml/h) and 20mM-sodium phosphate buffer, pH7.4, was used to elute the protein fractions. Each fraction was 0.5ml. Protein and [U-$^{14}$C]fructose 1,6-diphosphate were determined as described in the Experimental section. ●, Protein; ■, [U-$^{14}$C]fructose 1,6-diphosphate.

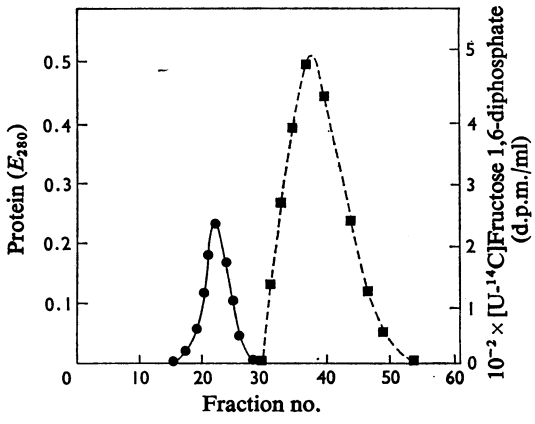


Fig. 3. *Chromatographic separation of [U-$^{14}$C]fructose 1,6-diphosphate from rabbit muscle pyruvate kinase*

Rabbit muscle pyruvate kinase (2.5mg) and 0.1 $\mu$mol of [U-$^{14}$C]fructose 1,6-diphosphate made 0.1mM with non-radioactive fructose 1,6-diphosphate were chromatographed on a Sephadex G-25 column (40cm×0.5cm; coarse grade; void volume 4.4ml; flow rate 15ml/h) and 20mM-sodium phosphate buffer, pH7.4, was used to elute the protein fraction. Each fraction was 0.5ml. Protein and [U-$^{14}$C]fructose 1,6-diphosphate were determined as described in the Experimental section. ●, Protein; ■, [U-$^{14}$C]fructose 1,6-diphosphate.

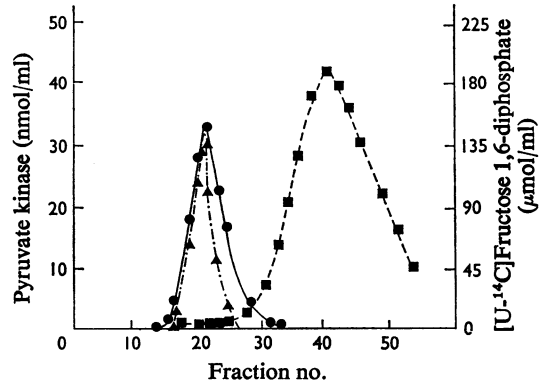


Fig. 4. *Chromatographic separation of [U-$^{14}$C]fructose 1,6-diphosphate from rabbit liver pyruvate kinase–[U-$^{14}$C]fructose 1,6-diphosphate complex*

The pyruvate kinase–[U-$^{14}$C]fructose 1,6-diphosphate complex (2.5mg) was chromatographed on a Sephadex G-25 column (35cm×2.2cm; coarse grade; void volume 15.0ml; flow rate 15ml/h) and 20mM-sodium phosphate buffer, pH7.4, was used to elute the protein fractions. Each fraction was 0.5ml. Protein and [U-$^{14}$C]fructose 1,6-diphosphate were determined as described in the Experimental section. ●, Protein; ■, [U-$^{14}$C]fructose 1,6-diphosphate; ▲, enzyme activity (units/ml), 0.25mM-phosphoenolpyruvate used to start the enzyme assay.

resultant preparation still exhibited Michaelis–Menten kinetics with phosphoenolpyruvate as the variable substrate. Enzyme–ligand association was judged to have occurred because [U-$^{14}$C]fructose 1,6-diphosphate could not be chromatographically separated from the enzyme. On the basis of molecular size, fructose 1,6-diphosphate should travel far behind the protein peak, and this was shown to be the case with the enzyme prepared from rabbit muscle (Fig. 3), and a similar pattern was also observed when haemoglobin was used as a marker protein. In both of these latter examples, fructose 1,6-diphosphate does not bind or interact.

It was considered that passage of [U-$^{14}$C]fructose 1,6-diphosphate–pyruvate kinase through a large Sephadex G-25 column might completely dissociate the complex, and the kinetics would then exhibit the unstimulated sigmoidal behaviour with phosphoenolpyruvate as the variable substrate. A Sephadex G-25 column (35cm×2.2cm) was prepared, and the elution profiles of protein, enzyme activity and [U-$^{14}$C]fructose 1,6-diphosphate were determined. Pyruvate kinase activity and the protein were coincident (Fig. 4), and although the [U-$^{14}$C]fructose 1,6-diphosphate peak had been displaced further behind the protein peak, there was always a significant residual amount of radioactivity associated with the pyruvate kinase fraction. The kinetic pattern of this preparation (Fig. 5) was complex, and the rectangular hyperbolic Michaelis–Menten kinetics were no longer observed with phosphoenolpyruvate as the variable substrate. However, this preparation exhibited a fivefold-stimulated rate when compared with the activity of the enzyme prepared in the absence of [U-$^{14}$C]fructose 1,6-diphosphate.

*Dilution and dissociation studies*

It was shown (Irving & Williams, 1973) that the rate of the pyruvate kinase reaction depended on the concentrations of enzyme, $K^+$, fructose 1,6-diphosphate, $MgADP^-$ and phosphoenolpyruvate. If rabbit liver pyruvate kinase binds to fructose 1,6-diphosphate to form a complex, and the concentration of $K^+$, $MgADP^-$ and phosphoenolpyruvate were maintained constant throughout all dilution steps, then the velocity of the reaction may be expressed as:

$$v_0 = [\text{pyruvate kinase} - \text{fructose 1,6-diphosphate}] \times k \quad (1)$$

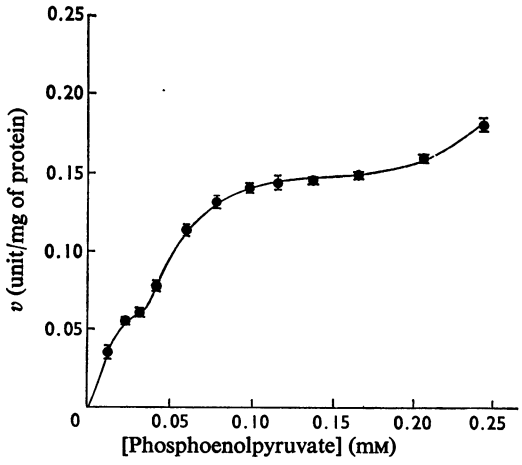


Fig. 5. *Effect of phosphoenolpyruvate concentration on the rate of reaction of the L form of rabbit liver pyruvate kinase–[U-$^{14}$C]fructose 1,6-diphosphate complex after chromatography on a Sephadex G-25 column (35 cm × 2.2 cm; coarse grade)*

Experimental details are the same as those described in the legends of Figs. 1 and 4. Each experimental point represents quadruplicate determinations for each phosphoenolpyruvate concentration from three preparations of pyruvate kinase–[U-$^{14}$C]fructose 1,6-diphosphate complex. Enzyme and assay mixture were incubated at 30°C for 10min before the reaction was started by the addition of phosphoenolpyruvate. Standard errors for each experimental point are shown. All concentrations of phosphoenolpyruvate were studied separately and were not due to incremental additions of phosphoenolpyruvate to the assay mixture.

where $k$=[K$^+$]×[phosphoenolpyruvate]×[MgADP$^-$]. If the enzyme remained as the intact undissociated enzyme–ligand complex under assay conditions, then on dilution of the complex by one-third, the rate of reaction should decrease to one-third of the original stimulated rate ($V_0$).

$$\therefore v' = [\text{pyruvate kinase–fructose 1,6-diphosphate}]/3 \times k = \frac{v_0}{3} \quad (2)$$

If, however, the enzyme–ligand complex dissociated under assay conditions, then the rate of reaction should fall to a value approaching one-ninth of the original stimulated rate if non-saturating concentrations of fructose 1,6-diphosphate and phosphoenolpyruvate were employed.

$$v'' = [\text{pyruvate kinase}]/3 \times [\text{fructose 1,6-diphosphate}]/3 \times k = \frac{v_0}{9} \quad (3)$$

If dissociation was to occur then addition of fructose 1,6-diphosphate to the original fructose 1,6-diphosphate concentration should result in the reaction rate increasing to one-third of the original stimulated rate, as in eqn. (2).

It was found that three sequential dilution steps could be accurately assayed (Table 1). For each dilution step, in both the presence and absence of added fructose 1,6-diphosphate, the reaction rate approximated to one-third of the previous stimulated rate. The 27-fold dilution of the enzyme did not produce any apparent increase in the dissociation of the enzyme–ligand complex, which was consistent with the view that there was a tight association between the enzyme and fructose 1,6-diphosphate.

*Studies of the relative strength of binding between [U-$^{14}$C]fructose 1,6-diphosphate and pyruvate kinase before and after dialysis*

To investigate further the nature of the relative strength of binding between [U-$^{14}$C]fructose 1,6-diphosphate and pyruvate kinase, a series of dialysis experiments was performed as follows. The enzyme–ligand complex was studied in five ways.

(1) A sample (1ml) of complex, containing 5mg of protein, was dialysed against 200ml of 20mM-sodium phosphate buffer, pH7.4, at 4°C with no buffer change for 24h, and samples were taken from the dialysis sac for the determination of the amount of [U-$^{14}$C]fructose 1,6-diphosphate associated with the protein.

(2) A sample (1ml) of the complex was dialysed against 200ml of 20mM-sodium phosphate buffer, pH7.4, at 4°C, which was renewed after 1, 3, 5, 8 and 24h. Samples were taken from the dialysis sac at each interval for determination of the amount of [U-$^{14}$C]fructose 1,6-diphosphate associated with the protein.

(3) A sample (1ml) of the complex was dialysed against 200ml of 20mM-sodium phosphate buffer, pH7.4, at 4°C, which was 0.1mM with respect to non-radioactive fructose 1,6-diphosphate. Samples were taken from the dialysis sac after 1, 3, 8 and 24h, but the dialysing buffer was not renewed at any stage.

(4) A sample (1ml) of pyruvate kinase, prepared in the absence of fructose 1,6-diphosphate [5mg of protein, 0–45%-satd. $(NH_4)_2SO_4$ fraction], was dialysed at 4°C against 100ml of 20mM-sodium

Table 1. *Effect of sequential dilution of the pyruvate kinase–fructose 1,6-diphosphate enzyme complex on the velocity of the pyruvate kinase-catalysed reaction in the presence and absence of added fructose 1,6-diphosphate*

Assay mixture contained 30mM-Tris, 30mM-KCl, 4mM-$MgADP^-$ ($MgADP^-/ADP^{2-}$ = 50:1) adjusted to pH 7.4, lactate dehydrogenase (20 units) and 1mg of pyruvate kinase (L form), and the reaction was started by simultaneous addition of phosphoenolpyruvate (0.1mM) and fructose 1,6-diphosphate. For subsequent dilution steps all reactants except pyruvate kinase and fructose 1,6-diphosphate were maintained at the original concentrations, and reaction velocities were determined as described in the Experimental section. Velocities are expressed in μmol of product/min per mg of enzyme protein. Numbers in parentheses indicate the numbers of measurements.

| | | Reaction velocity after sequential dilution into reaction mixture | | | | | |
|---|---|---|---|---|---|---|---|
| | | Without fructose 1,6-diphosphate | | | With fructose 1,6-diphosphate at initial concentration | | |
| [Fructose 1,6-diphosphate] (μM) | Initial velocity | Dilution factor | Reaction velocity | % of initial velocity | Dilution factor | Reaction velocity | % of initial velocity |
| 10.0 | 0.420 | 1/3 (5) | 0.125 | 30.0 | 1/3 (5) | 0.13 | 30.0 |
| 10.0 | 0.420 | 1/9 (5) | 0.04 | 9.3 | 1/9 (5) | 0.045 | 10.4 |
| 10.0 | 0.420 | 1/27 (5) | 0.015 | 3.6 | 1/27 (5) | 0.016 | 3.8 |
| 0.1 | 0.201 | 1/3 (4) | 0.064 | 32 | 1/3 (4) | 0.069 | 33.3 |
| 0.1 | 0.201 | 1/9 (4) | 0.020 | 9.9 | 1/9 (4) | 0.022 | 10.9 |
| 0.1 | 0.201 | 1/27 (4) | 0.008 | 4.0 | 1/27 (4) | 0.006 | 3.0 |

phosphate buffer, pH 7.4, which contained 0.14μmol of [U-$^{14}$C]fructose 1,6-diphosphate, and samples of the enzyme and the diffusate were taken after 24h.

(5) A sample (1ml) of pyruvate kinase, prepared in the absence of fructose 1,6-diphosphate [5mg of protein, 0–45%-satd. $(NH_4)_2SO_4$ fraction], was made 0.1mM with respect to non-radioactive fructose 1,6-diphosphate, and was dialysed against 100ml of 20mM-sodium phosphate buffer, pH 7.4, which contained 0.14μmol of [U-$^{14}$C]fructose 1,6-diphosphate, and samples of the enzyme and the diffusate were taken after 24h.

The results in Table 2 show that most of the radioactive fructose 1,6-diphosphate was dissociated from pyruvate kinase by dialysis, and that this separation was independent of the concentration of fructose 1,6-diphosphate in the dialysing buffer. It was found that 1–2% of the [U-$^{14}$C]fructose 1,6-diphosphate was consistently associated with pyruvate kinase after dialysis, and it would seem that the process of dissociation observed by using either Sephadex chromatography or dialysis was essentially the same. In methods (4) and (5) the dialysis procedure was reversed to ascertain whether the [U-$^{14}$C]fructose 1,6-diphosphate would bind to pyruvate kinase against a concentration gradient of fructose 1,6-diphosphate. Table 2 shows that the concentration of [U-$^{14}$C]fructose 1,6-diphosphate associated with pyruvate kinase doubled when the enzyme was associated with a higher concentration of fructose 1,6-diphosphate (0.1mM), indicating that this relatively high concentration of fructose 1,6-diphosphate could change the enzyme to a state that was characterized by maximum binding of fructose 1,6-diphosphate.

*Binding studies*

The high-specific-activity enzyme preparation obtained after chromatography of the L form of pyruvate kinase on DEAE-cellulose (Irving & Williams, 1973) was used for the binding studies. This fraction contained no ketose 1-phosphate aldolase, α-glycerophosphate dehydrogenase, fructose 1,6-diphosphatase or phosphofructokinase activity, and polyacrylamide-gel electrophoresis (Irving & Williams, 1973) revealed only two protein bands. It was reasoned that the magnitude of the fructose 1,6-diphosphate-binding constant obtained with this enzyme fraction would provide a quantitative insight into the interaction between fructose 1,6-diphosphate and the L form of pyruvate kinase, which resulted in the large heterotropic activation that was observed in the reaction rates.

By using the ultrafiltration procedure of Paulus (1969), the binding of [U-$^{14}$C]fructose 1,6-diphosphate to pyruvate kinase was interpreted as a Scatchard (1949) plot (Fig. 6) and gave a value of $2.3 \times 10^9$ for the association constant, and a binding ratio of approx. 1mol of fructose 1,6-diphosphate

Table 2. *Relative strength of binding between [U-$^{14}$C]fructose 1,6-diphosphate and the L form of rabbit liver pyruvate kinase before and after dialysis*

Expt. (1): a sample (5mg) of pyruvate kinase containing 0.1mmol of [U-$^{14}$C]fructose 1,6-diphosphate and made 0.1mM with non-radioactive fructose 1,6-diphosphate was dialysed against 200ml of 20mM-sodium phosphate buffer, pH7.4, for 24h at 4°C. Expt. (2): as for Expt. (1), except that dialysis buffer was renewed after 1, 3, 5, 8 and 24h. Expt. (3): as for Expt. (1) except that dialysis buffer was made 0.1mM with respect to fructose 1,6-diphosphate and not renewed at the sampling intervals. Expt. (4): a sample (5mg) of pyruvate kinase was dialysed for 24h against 100ml of 20mM-sodium phosphate buffer, pH7.4, containing 0.14$\mu$mol of [U-$^{14}$C]fructose 1,6-diphosphate. Expt. (5): as for Expt. (4) except that pyruvate kinase was made 0.1mM with non-radioactive fructose 1,6-diphosphate. Radioactivity measurements are corrected for background. Numbers in parentheses indicate the numbers of measurements.

| Expt. no. | Radioactivity of pyruvate kinase–fructose 1,6-diphosphate complex | | Radioactivity (d.p.m./mg of protein) |
|---|---|---|---|
| (1) | Before dialysis | (3) | 31000 |
| | After 24h dialysis | (3) | 300 |
| (2) | Before dialysis | (3) | 31000 |
| | After 1h dialysis | (3) | 13200 |
| | After 3h dialysis | (3) | 6800 |
| | After 5h dialysis | (3) | 2800 |
| | After 8h dialysis | (3) | 1300 |
| | After 24h dialysis | (3) | 100 |
| (3) | Before dialysis | (3) | 31000 |
| | After 1h dialysis | (3) | 31000 |
| | After 3h dialysis | (3) | 14800 |
| | After 8h dialysis | (3) | 2600 |
| | After 24h dialysis | (3) | 700 |
| (4) | After 24h dialysis | (5) | 26300* |
| | Radioactivity of diffusate after 24h dialysis | (5) | 49100 |
| (5) | Radioactivity of pyruvate kinase after 24h dialysis | (5) | 45300 |
| | Radioactivity of diffusate after 24h dialysis | (5) | 49600 |

* Radioactivity expressed as d.p.m./ml of enzyme (5mg) or ml of diffusate, both corrected for background.

bound/mol of pyruvate kinase, taking a value of $2\times10^5$ for the molecular weight of pyruvate kinase (Haeckel *et al.*, 1968). By using the ultrafiltration procedure of Blatt *et al.* (1968), with conical ultrafiltration cones, a value of $2.5\times10^8$ was obtained for the association constant and the binding ratio again approximated to 1mol of fructose 1,6-diphosphate bound/mol of pyruvate kinase. These results confirmed that there was a strong initial affinity between rabbit liver pyruvate kinase and its allosteric effector, fructose 1,6-diphosphate. It was observed, by using both methods to study ligand binding, that there was a critical upper value (1.2$\mu$mol of fructose 1,6-diphosphate/$\mu$mol of pyruvate kinase) beyond which the binding of fructose 1,6-diphosphate was no longer proportional to the increasing concentrations of [U-$^{14}$C]fructose 1,6-diphosphate. This suggested that, after the initial binding of ligand to the enzyme, factors other than the availability of fructose 1,6-diphosphate may influence the binding of this ligand to the enzyme.

*Interaction with EDTA*

Rabbit liver pyruvate kinase was extracted by using the buffer systems described by Pogson (1968). Extraction of the enzyme with buffer which contained no EDTA (PK-B), consistently produced a species of pyruvate kinase in the supernatant fraction that was stimulated by fructose 1,6-diphosphate (Table 3, Expt. 1), and it also exhibited sigmoidal kinetics with respect to phosphoenolpyruvate as the variable substrate. By using identical conditions with pyruvate kinase isolated from rat epididymal adipose tissue, Pogson (1968) isolated an M (muscle-type) form which exhibited Michaelis–Menten kinetics with phosphoenolpyruvate and was unaffected by fructose 1,6-diphosphate.

Table 3 shows the effect of the interactions of EDTA and fructose 1,6-diphosphate on the 0–45%-satd. $(NH_4)_2SO_4$ fraction (L form) and the 55–65%-satd. $(NH_4)_2SO_4$ fraction (M form) of rabbit liver pyruvate kinase prepared in both the presence and absence of EDTA; 5mM-EDTA appeared to inhibit the L form of the enzyme, and this inhibition was

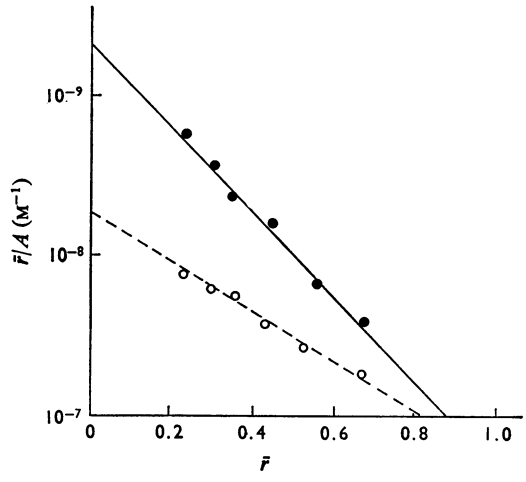


Fig. 6. *Binding of fructose* 1,6-*diphosphate by the L form of rabbit liver pyruvate kinase*

$\bar{r}$, the number of mol of fructose 1,6-diphosphate bound/mol of pyruvate kinase, was plotted against $\bar{r}/A$, where $A$ is the concentration of free fructose 1,6-diphosphate, by the method of Scatchard (1949). Methods for the determination of free and bound fructose 1,6-diphosphate are described in the Experimental section. ●, Binding measured with ultrafiltration disks; ○, binding measured with ultrafiltration cones.

partly removed by the subsequent addition of fructose 1,6-diphosphate (Table 3, Expt. 4). Stimulation was at a maximum with 0.2mM-fructose 1,6-diphosphate (Table 3, Expt. 2), in agreement with previous observations by Irving & Williams (1973), but elevated fructose 1,6-diphosphate concentrations (2.0mM) were more successful in relieving EDTA inhibition (Table 3, Expts. 3 and 4).

Preincubation of the L form with 2.5mM-EDTA for 20min resulted in increased rates of both the intrinsic and fructose 1,6-diphosphate-stimulated reactions for pyruvate kinase prepared in both the presence and absence of EDTA. However, by using Pogson's (1968) criteria to obtain 100% L form of the enzyme (preincubation for 20min at 34°C with 33.3mM-EDTA), complete inhibition of enzyme activity occurred, which could not be reversed or relieved by fructose 1,6-diphosphate concentrations as high as 20mM (Table 3, Expt. 5). This effect occurred for both the enzymes of the supernatant fraction and the $(NH_4)_2SO_4$ fractions of the enzyme prepared in the presence and absence of EDTA, and was always accompanied by an increased turbidity in the enzyme preparation. Preincubation of the L form of pyruvate kinase prepared in 20mM-sodium phosphate buffer with 33.3mM-EDTA showed a similar effect, indicating that the site of interaction of EDTA is with the enzyme itself and that the effect is not due solely to chelation of bivalent ions in the assay medium.

There was no evidence to suggest that the rabbit liver pyruvate kinase preparations of either the supernatant enzyme or the $(NH_4)_2SO_4$ fractions in the absence of EDTA gave an M (muscle-type) form of enzyme, which could be converted into an L form by incubation with EDTA.

### *Antigen–antibody interactions*

Tanaka *et al.* (1967) reported that the L and M forms of pyruvate kinase isolated from rat liver were antigenically distinct. However, Sorger *et al.* (1965) showed that the presence of univalent cations altered the complex immunoelectrophoretic patterns observed for the pyruvate kinase–antibody complex, and it was considered feasible that a similar effect owing to the binding of fructose 1,6-diphosphate might explain the different immunoelectrophoretic patterns observed by Tanaka *et al.* (1967).

No cross-reaction occurred between the antibody to pyruvate kinase extracted from rabbit muscle and any of the liver enzyme species. Identical antigen–antibody patterns were obtained with the L form prepared in both the presence and absence of fructose 1,6-diphosphate, showing that the binding of this ligand to the L form of pyruvate kinase did not produce an antigenically different species. No cross-reaction occurred between the M form of the liver enzyme and any of the L-form antigens or the muscle enzyme antigen, indicating that it was an antigenically distinct species.

## Discussion

Preparation of rabbit liver pyruvate kinase in the presence of [U-$^{14}$C]fructose 1,6-diphosphate produced an enzyme that was kinetically identical with the M (muscle-type) form of the enzyme after passage through a Sephadex G-25 column, which supported the observation by Williams *et al.* (1969) with the chicken liver enzyme. Chromatographic profiles of the [U-$^{14}$C]fructose 1,6-diphosphate–enzyme complex after passage through Sephadex G-25 columns (Fig. 2) showed that most of the [U-$^{14}$C]fructose 1,6-diphosphate molecules were loosely associated with pyruvate kinase and were separated from the enzyme by chromatography. The dialysis experiments further confirmed this observation, but the consistent residue of radioactivity that was always associated with the enzyme peak in the chromatographic studies, and with the enzyme in dialysis studies, was of the order of 1 mol of fructose

Table 3. *Effect of EDTA on the interaction of the L and M forms of rabbit liver pyruvate with fructose* 1,6-*diphosphate*

The enzyme was prepared by using the stated buffer systems. 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, together with the stated concentrations of fructose 1,6-diphosphate and EDTA. Expt. (1) was carried out on the initial supernatant solution. Expts. (2), (3), (4) and (5) were carried out on the 0–45%-satd. $(NH_4)_2SO_4$ fraction (L form) and Expt. no. (6) involved the 55–65%-satd. $(NH_4)_2SO_4$ fraction (M form). Reaction conditions were as described in the Experimental section. PK-A, Pyruvate kinase extracted in the buffer containing 20mM-imidazole acetate, 5mM-EDTA and 10mM-mercaptoethanol, pH6.8; PK-B, pyruvate kinase extracted in buffer containing 20mM-imidazole acetate, pH6.8.

| Expt. no. | Additions | Reaction velocity (unit/mg of protein) PK-A | PK-B | Activation (%) PK-A | PK-B |
|---|---|---|---|---|---|
| (1) | Water | 0.36 | 0.37 | | |
| | Fructose 1,6-diphosphate (2.0mM) | 0.52 | 0.75 | +44.5 | +11.9 |
| | Fructose 1,6-diphosphate (2.0mM), EDTA (5mM) | 0.18 | 0.25 | −50.0 | −62.7 |
| (2) | Water | 0.79 | 0.48 | | |
| | Fructose 1,6-diphosphate (2.0mM) | 1.05 | 0.56 | +25.3 | +16.6 |
| | Fructose 1,6-diphosphate (0.2mM) | 1.19 | 0.67 | +50.5 | +40.5 |
| (3) | Fructose 1,6-diphosphate (2.0mM), EDTA (5mM) | 0.14 | 0.28 | −82.3 | −58.4 |
| | Fructose 1,6-diphosphate (0.2mM), EDTA (5mM) | 0.12 | 0.07 | −85.0 | −85.5 |
| (4) | EDTA (5.0mM) | 0.00 | 0.00 | −100 | −100 |
| | EDTA (5.0mM), fructose 1,6-diphosphate (2.0mM) | 0.68 | 0.35 | −13.9 | −28.1 |
| | EDTA (5.0mM), fructose 1,6-diphosphate (0.2mM) | 0.21 | 0.14 | −73.5 | −70.5 |
| (5) | After 20min incubation with 30mM-EDTA, phosphoenolpyruvate (0.25mM) added | 0.00 | 0.00 | −100 | −100 |
| | Fructose 1,6-diphosphate (20.0mM) | 0.00 | 0.00 | −100 | −100 |
| (6) | Water | 0.04 | 0.04 | | |
| | Fructose 1,6-diphosphate (2.0mM), EDTA (5mM) | 0.002 | 0.002 | −95 | −95 |

1,6-diphosphate bound/mol of pyruvate kinase, assuming that all the protein was pyruvate kinase of molecular weight $2\times10^5$ (Haeckel *et al.*, 1968).

After passage of the [U-$^{14}$C]fructose 1,6-diphosphate–pyruvate kinase complex through a Sephadex G-25 column (35cm×2.2cm), a stimulated kinetic response to the homotropic effector phosphoenolpyruvate was still observed (Fig. 5), but the kinetic pattern observed for all preparations chromatographed (three) was no longer a rectangular hyperbola. It is proposed that the maintenance of binding, or the presence of weakly bound molecules of [U-$^{14}$C]fructose 1,6-diphosphate that were easily separated by passage of the ligand–enzyme complex through the Sephadex column, is essential to overcome completely the negative co-operative interaction between phosphoenolpyruvate and pyruvate kinase (Irving & Williams, 1973). This proposal has been further supported by dialysis studies, where it was observed that the maximum association of [U-$^{14}$C]fructose 1,6-diphosphate with pyruvate kinase occurred when the enzyme was incubated with a high concentration of fructose 1,6-diphosphate (Table 2, Expt. 5). It would thus appear that after the initial 'tight' binding of [U-$^{14}$C]fructose 1,6-diphosphate to the enzyme, concentrations of fructose 1,6-diphosphate in excess of the $K_a$ value would be needed to stabilize the binding of the 'weakly' bound molecules of [U-$^{14}$C]fructose 1,6-diphosphate, which were easily removed by dialysis and Sephadex chromatography. This proposal may explain the non-proportional binding of the ligand to pyruvate kinase in the binding studies for fructose 1,6-diphosphate amounts in excess of 1mol of fructose 1,6-diphosphate/mol of pyruvate kinase.

Dilution–dissociation studies established that the pyruvate kinase–fructose 1,6-diphosphate complex did not undergo any dissociation or decomposition during the 27-fold dilution, consistent with the proposal that there was a 'tight' association between the enzyme and fructose 1,6-diphosphate. The binding studies supported this proposition, showing that there was an initial high affinity between the enzyme and fructose 1,6-diphosphate, probably reflecting the nature of the binding of the first molecule of ligand to the enzyme. Hess & Kutzbach (1971), using isoelectrofocusing techniques with L form of rat liver pyruvate kinase, obtained a binding ratio of 2mol of

fructose 1,6-diphosphate/mol of pyruvate kinase, but the number bound depended on the ionic strength of the medium. Thus with both rabbit and rat liver pyruvate kinase, once the first molecule of fructose 1,6-diphosphate has bound to the enzyme, the binding of subsequent molecules is random, being dependent on such factors as the ionic strength or the concentration of fructose 1,6-diphosphate in the medium or both.

There was no evidence to suggest that EDTA could in any way act as a modifier *in vitro* as reported by Pogson (1968) for the rat epididymal adipose tissue enzyme. The slight stimulation of intrinsic and fructose 1,6-diphosphate-modified enzyme that occurred with 1–5mM-EDTA has also been observed with the L form of rat liver pyruvate kinase (Bailey *et al.*, 1968). However, under the conditions used by Pogson (1968) (20min preincubation at 34°C with 33.3mM-EDTA) complete irreversible inhibition occurred with the rabbit liver enzyme. Pogson (1968) also reported that preparation of the rat epididymal adipose tissue pyruvate kinase with buffer containing no EDTA gave a mixture of both the L and M forms of the enzyme, the L form of which was converted into the M form on incubation with 25mM-fructose 1,6-diphosphate. It is possible, however, that incubation of rat epididymal pyruvate kinase, which had been prepared in the absence of EDTA, with 25mM-fructose 1,6-diphosphate may not have converted the enzyme into the M (muscle-type) form, but rather to have converted it into an enzyme–ligand complex with similar kinetic properties to the L form of rabbit liver pyruvate kinase that had been prepared in the presence of [U-$^{14}$C]-fructose 1,6-diphosphate. It would, however, appear that the rat epididymal adipose tissue enzyme was a unique pyruvate kinase species, differing from other tissue enzymes in its high $K_a$ value (29$\mu$M) for fructose 1,6-diphosphate, in its interaction with EDTA and fructose 1,6-diphosphate and in that fractionation with $(NH_4)_2SO_4$ did not resolve the supernatant enzyme into the L and M forms as had been noted in rat (Bailey *et al.*, 1968) and rabbit (Irving & Williams, 1973) liver enzyme preparations.

Immunological studies confirmed that rabbit muscle pyruvate kinase was antigenically different from any of the liver enzyme species. The M form of the enzyme in liver was antigenically distinct from the L form, and preparation of the L form in the presence of fructose 1,6-diphosphate did not produce a species that was antigenically different from the L form prepared in the absence of fructose 1,6-diphosphate.

In conclusion, it would appear that like rat liver, rabbit liver has two kinetically and antigenically distinct species of pyruvate kinase. EDTA was not able to mediate a conversion of the M form into the L form, and although fructose 1,6-diphosphate was shown to bind to the L form, the resultant ligand-induced conformation was not the same as the M (muscle-type) form of the enzyme in rabbit liver.

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