

Comparison of the Properties of Two Forms of Pyruvate Kinase in Rat Liver and Determination of their Separate Activities During Development

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1. Two forms of hepatic pyruvate kinase, designated type L and type M, were distinguished on the basis of kinetic, chromatographic, electrophoretic and immunological criteria. They were partially purified and their properties compared with each other and with the purified enzyme from skeletal muscle. 2. In contrast with type L, the type M enzyme showed no marked evidence of co-operative interactions with phosphoenolpyruvate and was not stimulated by fructose diphosphate. 3. The activity profiles of type L and type M enzymes were determined in developing rat liver by utilizing differences in the kinetic properties of the two forms. The high activity of type M enzyme in the early foetal rat decreased in late gestation and immediately after birth to reach a low value, which remained essentially constant for the remainder of the developmental period. The activity of type L enzyme, in contrast, was low in the early foetal and neonatal liver but increased markedly at the onset of weaning. 4. Possible roles of the two forms of hepatic pyruvate kinase in the control of glycolysis and gluconeogenesis are discussed.

The pyruvate kinase (EC 2.7.1.40) activity in adult rat liver and the rate of hepatic glucose utilization respond in a similar manner to changes in the diet (Krebs & Eggleston, 1965; Tanaka *et al.*, 1965; Yudkin & Krauss, 1967; Szepesi & Freedland, 1968; Bailey *et al.*, 1968*a*) and hormonal status (Weber *et al.*, 1965, 1966). Tanaka *et al.* (1965) demonstrated that hepatic pyruvate kinase consists of more than one form. The major component, designated type L, is subject to control by both positive (phosphoenolpyruvate and fructose 1,6-diphosphate) and negative (ATP and alanine) effectors (Taylor & Bailey, 1967; Tanaka *et al.*, 1967*a,b*; Carminatti *et al.*, 1968; Passeron *et al.*, 1967; Bailey *et al.*, 1968*b*; Carminatti *et al.*, 1969; Seubert *et al.*, 1968; Weber *et al.*, 1968). It is the activity of the type L hepatic enzyme that varies with dietary and hormonal conditions (Tanaka *et al.*, 1965, 1967*a*). Bailey *et al.* (1968*a*) distinguished two interconvertible forms of the type L enzyme; two forms, again distinguished by sensitivity towards fructose 1,6-diphosphate, were also noted by Susor & Rutter (1968). In view of these many reports, a summary of our own results on the type L enzyme will be given only as far as they are required for comparative purposes and estimation of the two forms.

The activity of the other main form of pyruvate kinase in liver, designated type M, whose properties are not well characterized, is relatively unchanged by dietary modifications (Tanaka *et al.*, 1965, 1967*a*)

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and appears to be related to the degree of differentiation of the organ; activity is increased in regenerating liver and certain hepatomas (Tanaka *et al.*, 1965; Suda *et al.*, 1966). The potential mechanisms for control of hepatic pyruvate kinase activity are thus both diverse and complex.

The increase in total hepatic pyruvate kinase activity (Walker & Vernon, 1967; Taylor *et al.*, 1967; Vernon & Walker, 1968) during the weaning period parallels a rise in hepatic glucose-phosphorylating capacity that is known to be due to the synthesis of a unique glucokinase (EC 2.7.1.2; Walker & Holland, 1965). This suggested that an examination of the nature and the activities of the various forms of pyruvate kinase present at several stages of development might provide a more detailed understanding of the events involved in hepatic maturation, and the results are now reported.

Materials and Methods

Materials

Phosphoenolpyruvate (potassium salt), fructose 1,6-diphosphate, ATP, NADH, tris and muscle lactate dehydrogenase (EC 1.1.1.27) were obtained from Boehringer Corp. (London) Ltd., London W.5, U.K.; ADP and *p*-chloromercuriphenylsulphonic acid were from Sigma (London) Ltd., London S.W.6, U.K.; bovine serum albumin was from Armour Pharmaceutical Co. Ltd., Eastbourne,

Sussex, U.K.; imidazole was from Eastman Kodak Co., Rochester, N.Y., U.S.A., and dithiothreitol was from Calbiochem, Los Angeles, Calif., U.S.A. All other chemicals were of AnalaR quality.

Animals

Rats were a Wistar albino strain fed *ad lib.* on a pellet diet. Litters were culled to a maximum of ten pups 2 days after birth and weanling rats were removed from their mothers when 30 days old. Neonatal animals were removed from such litters at random. Foetal age was assessed by the method of Stotsenburg (1915). All adult animals were males weighing 200–250 g.

Assay procedures

Homogenates (33%, w/v) were prepared in imidazole buffer (10 mM), pH 7.0, containing KCl (0.15 M) and EDTA (2 mM), and were centrifuged at 100000g for 50 min. The supernatant fraction was used for all experiments. Pyruvate kinase was assayed at 30°C by a coupled assay system similar to that described by Reynard *et al.* (1961). Except where modified for a particular purpose the final reaction mixture (1 ml) contained: imidazole buffer, pH 7.0, 100 mM; KCl, 100 mM; MgCl₂, 4.0 mM; phosphoenolpyruvate, 1.0 mM; ADP, 1.0 mM; NADH, 0.15 mM; bovine serum albumin, 0.5 mg/ml; lactate dehydrogenase, 10 units. One unit of pyruvate kinase activity was defined as that which catalysed the formation of 1 μmol of pyruvate/min at 30°C. Protein was determined in crude preparations by a biuret method (Gornall *et al.*, 1949) and in more purified preparations by the method of Warburg & Christian (1941). Protein in column eluates was monitored by absorption at 280 nm.

Ammonium sulphate fractionations

These were performed at 0–4°C by additions of finely ground ammonium sulphate to produce, in successive steps, fractions precipitating in the ranges 0–25, 25–45, 45–52 and 52–70% saturation. After each addition stirring was continued for 30 min and the precipitate then collected by centrifugation. Precipitates containing activity were redissolved in and dialysed for 2 h against imidazole buffer (20 mM), pH 7.0, containing EDTA (2 mM) before use in further studies.

Kinetic results

The concentrations of the solutions of phosphoenolpyruvate and ADP used for the determination of K_m were checked enzymically. Inhibitors were preincubated with the enzyme for 3 min at 30°C in

the assay system minus phosphoenolpyruvate, which was then added to start the reaction. When experiments involved the inhibitory action of ATP, the concentration of Mg²⁺ was 12.5 mM. Fructose 1,6-diphosphate was added to the assay system just before the enzyme. All enzyme dilutions were made in imidazole buffer (20 mM), pH 7.0, containing bovine serum albumin (0.5 mg/ml), and were used within 30 min of dilution. Lactate dehydrogenase was in excess in all kinetic experiments.

Cellulose acetate electrophoresis

Cellulose acetate strips (17 cm × 2.5 cm) and the electrophoresis tank were obtained from Gelman Instrument Co. Ltd., Ann Arbor, Mich., U.S.A., and the procedure was essentially as described by Kohn (1960). Samples for electrophoresis were dialysed against the electrophoresis buffer (70 mM-sodium barbitone, pH 8.3, containing 1 mM-EDTA) for 3 h (two changes) before application. Constant portions (0.03 unit) of pyruvate kinase activity, usually contained in 2–4 μl, were applied to the strips 5.7 cm from the cathode end under constant current (0.4 mA/cm) and electrophoresis was continued for 2.5 h at 40°C. The strips were stained for pyruvate kinase activity by incubation with thin strips of gelled assay system by a procedure similar to that employed by Susor & Rutter (1968). The stain was examined under a Hanovia lamp (maximum emission at 366 nm), when regions of enzymic activity became visible as dark bands on a fluorescent background.

Preparation of anti-(muscle pyruvate kinase)

The purification of rat skeletal muscle pyruvate kinase followed the procedure described by Tietz & Ochoa (1962) as far as the second 55–70%-satd. ammonium sulphate fraction. The dissolved precipitate was passed through a column of Sephadex G-25 (Pharmacia, Uppsala, Sweden) equilibrated with acetate buffer (0.05 M), pH 5.0, and the protein eluate adsorbed on a column of CM-cellulose (Whatman CM-32) equilibrated with this buffer. The enzyme was eluted at 75 mM-KCl when a linear gradient (0–0.2 M) of this salt was applied in the acetate buffer. A 63-fold purification was achieved with 48% recovery of activity and the preparation had a final specific activity of 400 units/mg of protein.

The purified enzyme was dialysed for 12 h against 0.85% NaCl, adjusted to a protein concentration of 20 mg/ml and emulsified with an equal volume of Freund's Complete Adjuvant (Difco Laboratories, Detroit, Mich., U.S.A.). Emulsion (1 ml) was injected into each foot pad of a New Zealand white rabbit. This procedure was repeated after 2 weeks and followed by a booster dose (0.125 ml) 2 weeks later. Blood was collected and antisera were prepared 10 and 17

days after the last injection, as described by Leskowitz & Waksman (1960). The antisera were purified by the procedure of Marrack *et al.* (1951).

Liver supernatants containing 0.2 unit of pyruvate kinase activity were preincubated with the antiserum as described by Susor & Rutter (1968), except that preincubation with type M pyruvate kinase was for 5 min only. Excess of antiserum did not inhibit the lactate dehydrogenase employed in the coupled assay system. Qualitative antigen-antibody analyses were performed by double diffusion in agar gel (Ouchterlony, 1949).

Partial purification of type M pyruvate kinase from liver

The 100000g supernatant prepared from 30g of rat liver was fractionated by successive additions of ammonium sulphate and the fraction precipitated between 52 and 70% saturation was dissolved in imidazole buffer (5mM), pH7.0, containing EDTA (1mM), dithiothreitol (0.5mM) and MgSO₄ (2mM). The introduction of low concentrations of Mg²⁺ to this buffer (which was employed in all subsequent steps) enhanced the stability of the enzyme. The dissolved precipitate was desalted by passage through a column of Sephadex G-25 and the protein (210mg) then passed through a column of CM-cellulose. The protein eluate (180mg) from this column, containing about 80% of the calculated activity of type M enzyme, was adsorbed on a column of DEAE-

cellulose, and type M pyruvate kinase eluted at 17mM-KCl by applying a linear gradient (0–0.2M) of this salt in the above buffer (see Fig. 1). The final preparation, representing an eightfold purification and a 5.9% recovery of the activity in the 52–70%-satd. ammonium sulphate fraction, had a specific activity of 0.51 unit/mg of protein. The poor recovery, which was due mainly to the instability of the enzyme on DEAE-cellulose, was not improved by the addition of either substrate to the buffer.

Results

Evidence for two forms of hepatic pyruvate kinase

Tanaka *et al.* (1965) first demonstrated heterogeneity of the enzyme by electrophoresis in starch blocks. Evidence for the presence of a second form that accompanies the predominating type of enzyme is presented here, based on (i) kinetic properties of fractions prepared by ammonium sulphate fractionation, (ii) DEAE-cellulose chromatography, (iii) electrophoresis on cellulose acetate strips, and (iv) immunological properties.

Ammonium sulphate fractionation. Initial experiments showed that pyruvate kinase activity could be precipitated over a wide range of the salt concentrations without signs of separated peaks of activity. Attention was eventually focused on the protein fractions that precipitated between the limits of 25–45% and 52–70% saturation with ammonium sulphate. With increasing concentrations of phosphoenolpyruvate, sigmoidal substrate-saturation curves were observed for each dialysed fraction and the $K_{\frac{1}{2}}$ values for this substrate were similar. The values for n given by the slopes of Hill plots $\log[v/(V_{\max} - v)]$ against $\log[\text{phosphoenolpyruvate}]$, however, were about 3 for the 25–45%-satd. fraction and varied with different preparations between values of 1.3 and 1.8 for the 52–70%-satd. fraction. The value of n for the fraction precipitated between 45 and 70% saturation was higher (up to 2.5), suggesting that elimination of the activity precipitated between 45 and 52%-satd. ammonium sulphate from the 45–70%-satd. fraction resulted in the removal of activity possessing properties similar to that precipitated between 25 and 45%-satd. ammonium sulphate. The resultant fraction (52–70%-satd.) showed essentially normal Michaelis-Menten kinetics with increasing concentrations of phosphoenolpyruvate.

Other properties of the enzyme present in the two ammonium sulphate fractions are given in Table 1. The two fractions exhibited marked differences in sensitivity to inhibition by Cu²⁺. The results were in qualitative agreement with those of Passeron *et al.* (1967), who worked on mouse liver. At saturating substrate concentrations half-maximum inhibition of

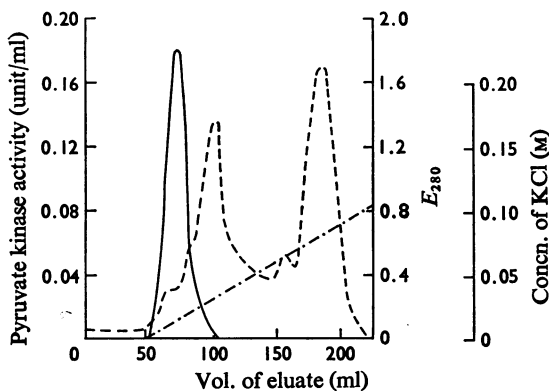


Fig. 1. Elution profile of the final stage in the partial purification of type M pyruvate kinase from rat liver by DEAE-cellulose chromatography

The column was 13.5cm × 2.2cm. The experimental procedure and the assay system are described in the text; 3–4ml fractions were collected. —, Enzyme activity (units/ml); ----, E_{280} ; - · - · - ·, concn. of KCl in eluate.

Table 1. *Summary of the properties of pyruvate kinase present in two ammonium sulphate fractions and two fractions prepared from rat liver supernatants by chromatography on DEAE-cellulose*

The various parameters were determined by the methods described in the text. n = Hill coefficient (see the text). $K_{\frac{1}{2}}$ is the concentration of phosphoenolpyruvate giving half-maximum velocity.

Parameter	Activity precipitated between 25 and 45% -satd. $(\text{NH}_4)_2\text{SO}_4$	Activity precipitated between 52 and 70% -satd. $(\text{NH}_4)_2\text{SO}_4$	Chromatography peak II	Chromatography peak I
K_m or $K_{\frac{1}{2}}$ (mM) for phosphoenolpyruvate	$K_{\frac{1}{2}}$ 5.5 ($n = 3.0-5.7$)	$K_{\frac{1}{2}}$ 4.4 ($n = 1.6$)	$K_{\frac{1}{2}}$ 5.8 ($n = 2.9$)	K_m 0.13 ($n = 1.0$)
Activation by fructose 1,6-diphosphate	+		+	-
K_m (mM) for phosphoenolpyruvate in the presence of fructose 1,6-diphosphate	0.065		0.08	0.13
K_m (mM) for ADP	0.28		0.8	1.1
Concn. of ATP giving 50% inhibition (mM)	0.14-0.38		0.75	1.0
Concn. of Cu^{2+} giving 50% inhibition (mM)	0.021	1.0	0.048	1.0
Concn. of KCl at elution of peak activity from DEAE-cellulose (M)	0.07	0.017	0.074	0.02
Percentage inhibition by excess of anti-(muscle pyruvate kinase)	—	46	0-6	46
Immunodiffusion (Ouchterlony) against anti-(muscle pyruvate kinase)	No reaction	Cross-reacts		
Relative mobility (R_m) during cellulose acetate electrophoresis			2.0	1.0
Estimated percentage of total activity present in the supernatant preparation	90-93	7-10	95	5

the activity in the 25-45%-satd. fraction occurred at 20-50 μM - Cu^{2+} . The activity of the 52-70%-satd. fraction was not inhibited at this concentration but 13% inhibition was observed at 0.25 mM.

The pyruvate kinase activity in dialysed preparations of the two fractions was inhibited to varying extents by very low (10^{-5} - 10^{-8} M) concentrations of *p*-chloromercuriphenylsulphonic acid. Although quantitative relationships could not be established owing to the lack of purity of the preparations, it was observed that addition of fructose 1,6-diphosphate

(final concn. 0.5 mM) to the 25-45%-satd. fraction resulted in complete reversal of previous inhibition by *p*-chloromercuriphenylsulphonate.

Chromatography on DEAE-cellulose. Preliminary experiments indicated that not all the pyruvate kinase activity in a liver supernatant was bound to DEAE-cellulose (Whatman DE-32) at pH 7.0. However, all the activity could be bound to this exchanger equilibrated with imidazole-chloride buffer (5 mM), pH 7.5, if the supernatant was first desalted (by a Sephadex G-25 column) and pretreated by passage

through a column of CM-cellulose equilibrated with the same buffer.

Two peaks of activity (see Fig. 2), designated peak I and peak II respectively, were eluted at 0.02 and 0.074M-KCl. A steep gradient of KCl was necessary to prevent undue dilution of peak I and hence to permit accurate assay. Under identical conditions, the activity in a 25–45%-satd. ammonium sulphate fraction was eluted in one symmetrical peak at 0.08M-KCl with 35% recovery of activity and one peak was eluted at 17mM-KCl from the 52–70%-satd. ammonium sulphate fraction with a 26% recovery. Assuming therefore the identity of the 25–45%-satd. fraction with the peak II enzyme from the DEAE-cellulose column and of the 52–70%-satd. fraction with peak I enzyme, and making further allowances for the relative stabilities of the two enzyme forms, it can be calculated that peak I represents about 5% of the total original pyruvate kinase activity and peak II approx. 95%.

The kinetic properties of the enzyme present in peaks I and II are summarized in Table 1. Although the K_m values for ADP were similar, the K_m or K_+ values for phosphoenolpyruvate were different. Fructose 1,6-diphosphate modified the effect of phosphoenolpyruvate only on the peak II enzyme from K_+ 5.8×10^{-3} M ($n = 1.8$) to K_m 8.0×10^{-5} M ($n = 1.0$). The inhibitory effects of ATP and Cu^{2+} were also quantitatively very different (Table 1). The slope of the Hill plot for the effect of ATP on the peak II enzyme gave $n = 2.5$, which suggests that co-operative interactions may occur as ATP molecules bind to this enzyme. There were no reasons to doubt the apparent

absence of sigmoidal kinetics with the peak I enzyme, but it should be noted that the peak II enzyme, prepared by column chromatography on DEAE-cellulose, was partially desensitized with respect to inhibition by ATP compared with the enzyme in the 25–45%-satd. ammonium sulphate fraction.

Electrophoresis on cellulose acetate strips. Two bands of enzymic activity were resolved from a rat liver supernatant with relative mobilities (muscle pyruvate kinase $M = 1.0$) of 1.3 and 0.7. The faster band stained more intensely than the slower. Neither presoaking the cellulose acetate strip with bovine serum albumin before electrophoresis (Rosalki & Montgomery, 1967) nor preincubation of the liver supernatant with fructose 1,6-diphosphate (2mM), EDTA (2mM) or dithiothreitol (1mM) altered the staining pattern. Two bands of hepatic pyruvate kinase activity have also been detected by Pogson (1968*a,b*) and Susor & Rutter (1968) after electrophoresis on cellulose acetate.

The pyruvate kinase activity in peaks I and II from chromatography on DEAE-cellulose, concentrated by dialysis against polyethylene glycol, had different mobilities after electrophoresis on cellulose acetate. The peak II enzyme was almost identical in m value with the principal band of a liver supernatant and could not be resolved from it. Peak I pyruvate kinase activity had $R_m = 0.9$, but it was not possible to show conclusively on the strips that it was identical with the minor component of a liver supernatant.

Immunological studies. Liver supernatant contained an antigen that cross-reacted with anti-(muscle pyruvate kinase); the addition of either EDTA or fructose 1,6-diphosphate did not alter this precipitin behaviour. The behaviour of a 52–70%-satd. ammonium sulphate fraction was essentially identical with that of the liver supernatant but no precipitin band could be detected between the 25–45%-satd. fraction and anti-(muscle pyruvate kinase) at concentrations of the former up to 4mg of protein/ml.

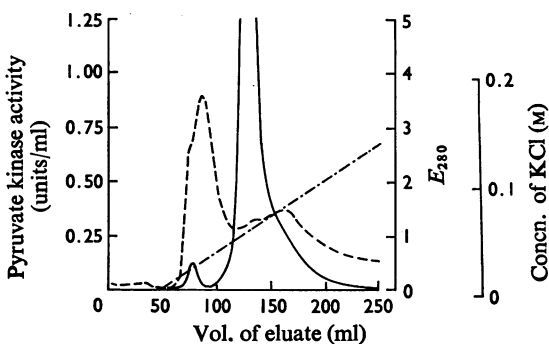


Fig. 2. Separation of two peaks of pyruvate kinase activity from a pretreated liver supernatant by DEAE-cellulose chromatography

The column was 13.0cm \times 1.8cm and was equilibrated with imidazole buffer (5mM), pH7.5. Fractions (3–4ml) were collected and assayed as described in the text. —, Enzyme activity (units/ml); ----, E_{280} ; - · - · - ·, concn. of KCl in eluate.

Properties of type L pyruvate kinase

Type L hepatic pyruvate kinase was prepared essentially by the method of Susor & Rutter (1968). A 38-fold purification, giving a specific activity of 21 units/mg of protein, was achieved. The kinetic properties of this preparation (summarized in Table 2) are similar to those described by Taylor & Bailey (1967), Susor & Rutter (1968) and Carminatti *et al.* (1969). The principal properties for purposes of comparison with the type M form (see the next section) were: (a) a sigmoidal saturation curve for phosphoenolpyruvate at several concentrations of ADP, with $n = 3.5$; (b) Michaelis–Menten hyperbolic kinetics with a K_m for phosphoenolpyruvate (that was independent of the ADP concentration) of

$3.6 \times 10^{-5} \text{ M}$ for the enzyme activated by fructose 1,6-diphosphate; (c) no inhibition by or precipitin reactions with anti-(muscle pyruvate kinase); (d) inhibition by ATP with half-maximum activity at $8.5 \times 10^{-4} \text{ M}$ and $n = 1.35$, i.e. the slope of the Hill plot for the effect of ATP on the purified L-type enzyme appeared to be less than that for the peak II enzyme (see above). This desensitization towards ATP on purification has also been noted by Carminatti *et al.* (1969).

Properties of type M pyruvate kinase

About 25% of the enzyme activity was lost during storage for 12h at 0°C in the preparative buffer. The rate of reaction in control assays (i.e. in the absence of either phosphoenolpyruvate or ADP) was low (<2%) and was unaltered by fructose 1,6-diphosphate. In marked contrast, very high rates were obtained when fructose 1,6-diphosphate was added to control assays containing the unpurified enzyme. These high rates are almost certainly due to the presence of an enzyme system that utilizes fructose as a substrate and ultimately results in the oxidation of NADH; they were allowed for in calculating the pyruvate kinase activities.

There are marked differences in the kinetic, chromatographic and immunological properties of type M and type L hepatic pyruvate kinase (Table 2). This contrast was also noted by Tanaka *et al.* (1965, 1967a). The type M enzyme exhibited normal Michaelis-Menten kinetics ($n = 1.0$) towards phosphoenolpyruvate, and the K_m value for this substrate (Fig. 3) varied between 1.1×10^{-4} and $8.0 \times 10^{-4} \text{ M}$ when the pH of the preparative buffer was altered from 7.0 to 7.6. The K_m was unaltered by the addition of fructose 1,6-diphosphate or by the concentration of the other substrate (Mg-ADP⁻). Apparent differences between our results and those of Tanaka *et al.* (1970), who reported briefly some limited sigmoidal behaviour and partial activation of an unspecified preparation by fructose 1,6-diphosphate, may be related to the instability of the type M enzyme. The latter was not markedly inhibited by low concentrations of Cu^{2+} or *p*-chloromercuriphenylsulphonate. In the presence of 12.5 mM-Mg^{2+} , the activity of type M pyruvate kinase was not significantly inhibited by concentrations of ATP up to 5 mM. Inhibition at lower Mg^{2+} concentrations was attributed to complex-formation of the added Mg^{2+} with ATP, which thus decreased the concentration of Mg-ADP⁻ substrate (see, e.g., Wood, 1968).

Table 2. Summary of properties of types L and M pyruvate kinase prepared from rat liver and the enzyme purified from rat muscle

The various parameters were determined by the methods described in the text. n = Hill coefficient. $K_{\frac{1}{2}}$ is the concentration of phosphoenolpyruvate giving half-maximum velocity.

Parameter	Type L enzyme	Type M enzyme	Muscle enzyme
K_m or $K_{\frac{1}{2}}$ (mM) for phosphoenolpyruvate	$K_{\frac{1}{2}}$ 0.4 ($n = 3.5$)	K_m 0.11–0.8 ($n = 1.0$)	0.087 ($n = 1.0$)
Activation by fructose 1,6-diphosphate	+	–	–
K_m (mM) for phosphoenolpyruvate in the presence of fructose 1,6-diphosphate	0.036 ($n = 1.0$)	0.13–0.8	0.087
K_m (mM) for ADP	0.7	0.63–0.91	0.74
Concn. of ATP giving 50% inhibition (mM)	0.35–0.85	5.0	20.0
Concn. of Cu^{2+} giving 50% inhibition (mM)	0.022	1.0	10.0
Principal $(\text{NH}_4)_2\text{SO}_4$ fraction (% satn.)	25–45	52–70	54–70
Concn. of KCl at elution of peak activity from DEAE-cellulose (M)	0.07	0.017	0.02
Relative mobility (R_m) during cellulose acetate electrophoresis	2.0	1.0	1.0
Percentage inhibition by excess of anti-(muscle pyruvate kinase)	0	46	93
Immunodiffusion (Ouchterlony) against anti-(muscle pyruvate kinase)	No reaction	Cross-reacts	Reaction of identity

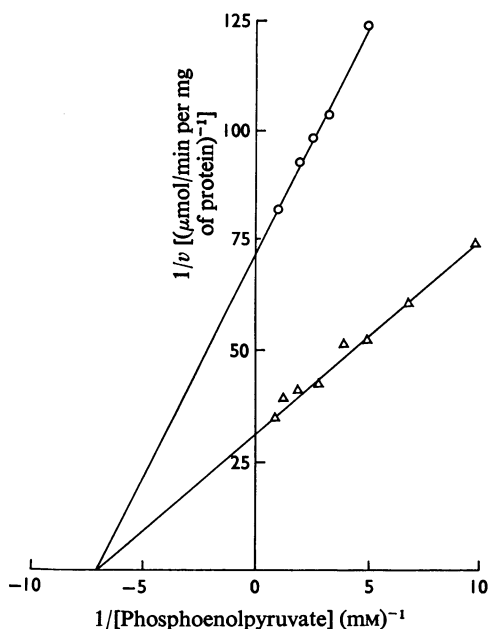


Fig. 3. Double-reciprocal plots for the effect of concentration of phosphoenolpyruvate on the velocity of type M hepatic pyruvate kinase at fixed concentrations of ADP

The enzyme preparation was the combined fractions containing peak specific activity from the DEAE-cellulose column illustrated in Fig. 1. The assay system was as described in the text. \circ , ADP (0.4 mM); Δ , ADP (1.0 mM). K_m for phosphoenolpyruvate is 1.3×10^{-4} M.

The incomplete inhibition of the type M enzyme by anti-(muscle pyruvate kinase) may have been a consequence of the short incubation times (5 min) that were necessary to prevent loss of activity in control and experimental samples. The type L enzyme was found not to be inhibited by the antiserum during incubation periods up to 1.5 h.

There are marked similarities in the properties of type M hepatic pyruvate kinase and the enzyme from skeletal muscle (Table 2), but small significant immunological and kinetic differences (e.g. K_m value for phosphoenolpyruvate) were observed.

Activity of type L and type M pyruvate kinase during development

The equivocal nature of the inhibition of total and type M hepatic pyruvate kinase activity by the anti-(muscle pyruvate kinase) preparation made it necessary to utilize (a) the differential behaviour towards

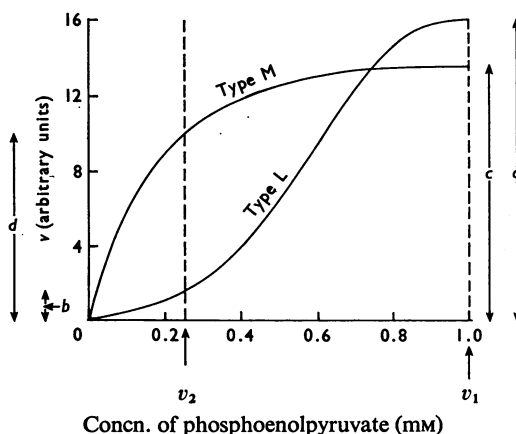


Fig. 4. Effect of concentration of phosphoenolpyruvate on the velocity of type L and type M hepatic pyruvate kinase

The curve for type L enzyme is derived from measurements on five adult liver supernatants treated under conditions identical with those used in Fig. 6; the ratio of the rates at 1.0 mM-phosphoenolpyruvate (v_1) to those at 0.25 mM-phosphoenolpyruvate (v_2) was then corrected for the calculated 5% of type M enzyme in the supernatants (see the text for fuller explanation). The curve for type M enzyme is obtained from measurement of the rates v_1 and v_2 of a partially purified preparation of type M pyruvate kinase. Parameters a , b , c and d are explained in the Results section. The assay system is described in the text.

phosphoenolpyruvate and (b) inhibition by Cu^{2+} of the two enzymes to estimate their respective contributions to the total activities as follows.

(a) From Fig. 4, which is constructed from the results obtained in Table 1, the measured rate at 1.0 mM-phosphoenolpyruvate is given by

$$v_1 = V_L + V_M \quad (1)$$

where V_L and V_M are the V_{\max} values of types L and M pyruvate kinase respectively.

The rate at 0.25 mM-phosphoenolpyruvate is given by

$$v_2 = (b/a) V_L + (d/c) V_M \quad (2)$$

so that

$$\frac{v_1}{v_2} = \frac{V_L + V_M}{(b/a) V_L + (d/c) V_M} \quad (3)$$

and where b/a and d/c are ratios of two activities as described below and shown on Fig. 4. The derivation of eqn. (3), which assumes that the two enzymes work independently, is probably valid so long as the

concentration of phosphoenolpyruvate is much greater than the concentration of each of the enzyme-substrate complexes. The K_m value of the type M enzyme (for phosphoenolpyruvate) in the supernatant was assumed to be most closely approximated by the lowest measured K_m value for this substrate. The constant value of b/a for the type L enzyme was dependent on the sigmoidal nature of the phosphoenolpyruvate-saturation curve, and as this alters during the purification procedure it was necessary to determine the constant by measurements on supernatants treated in a manner identical with those for the experimental samples. This included dialysis for 3 h to remove endogenous substrates. A value for v_1/v_2 of 8.0 ± 0.35 (mean \pm S.E.M. for five animals) was obtained for supernatants from adult livers. By substituting this value into eqn. (3) and putting d/c equal to 0.74 and assuming a value of 5% for the amount of type M enzyme in the adult liver supernatant (see Table 1), b/a was calculated to be 0.0925. Eqn. (3) therefore becomes:

$$\frac{v_1}{v_2} = \frac{V_L + V_M}{0.0925 V_L + 0.74 V_M} \quad (4)$$

By inserting a series of values for the relative activities of types L and M enzyme (where the total activities of type L and type M = 1) the corresponding values of v_1/v_2 can be calculated; the relationship obtained is illustrated in Fig. 5. Thus from experimental measurements of the ratio v_1/v_2 , the relative proportions of type L and M enzyme can be calculated.

(b) In the presence of concentrations of phosphoenolpyruvate and ADP that were optimum for both type L and type M enzyme, the rate $v_1 = V_L + V_M$ where V_L and V_M were as defined as above. In the presence of 1 mM- Cu^{2+} type L activity is inhibited completely (see Table 1) and type M by 30% (see Table 1), so that in the presence of this concentration of inhibitor $v_2 = 0.7 V_M$. Hence $v_2/v_1 = 0.7(V_M/V_L + V_M)$.

The relative proportions of type L and M enzyme can thus be calculated from measurements of the ratio v_1/v_2 .

Liver supernatants were not dialysed before determination of v_1 and v_2 in this method because dialysis alters the susceptibility of purified skeletal muscle enzyme to inhibition by Cu^{2+} , and it is possible that preferential loss of one form of hepatic enzyme could occur during dialysis. No account was taken of endogenous effectors that could alter the degree of inhibition.

The above two methods were used to examine the effect of starvation of adult rats on the two forms and the results, which showed that it is only type L enzyme whose activity is decreased, were in agreement with those of Tanaka *et al.* (1965, 1967a).

The separate activities of type L and type M

enzyme were determined during the foetal and neonatal periods by the two methods. The development profiles (Fig. 6) obtained by both methods were qualitatively very similar, although there were small quantitative differences. The specific activity of the type M enzyme as determined by the phosphoenolpyruvate method (see Fig. 6) was highest in the early foetal liver (27 units/100 mg of supernatant protein in the 16-17-day foetus) and decreased to reach the low values typical of the adult liver (2 units/100 mg of supernatant protein) immediately after birth. The activity of type M enzyme remained essentially constant during the suckling and weaning periods. Type L pyruvate kinase, on the other hand, had a relatively low specific activity in the foetal

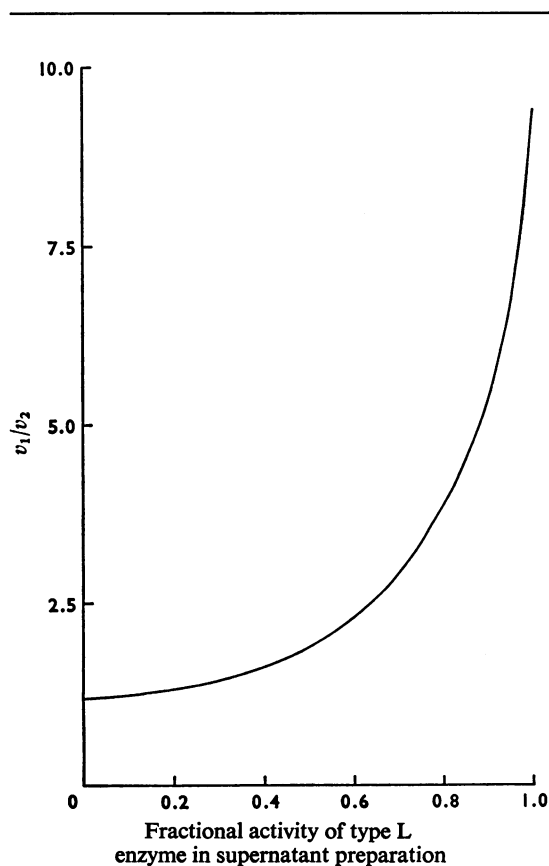


Fig. 5. Relationship between the ratio of the velocities of pyruvate kinase at 1.0 mM- (v_1) and 0.25 mM-phosphoenolpyruvate (v_2) and the proportion of type L enzyme activity in the supernatant

The results were obtained by inserting a series of values for the relative activities of type L and M enzyme (where activity of type L + activity of type M = 1) into eqn. (4), which is derived in the text.

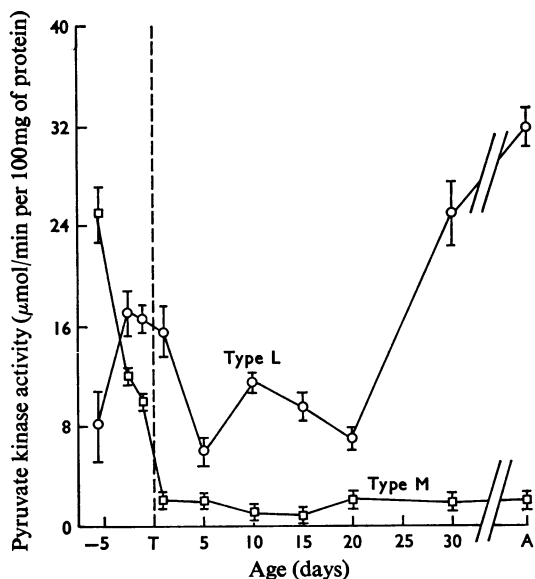


Fig. 6. Activity of type L and type M pyruvate kinase in liver supernatant determined by the phosphoenolpyruvate method as a function of age

The individual activities of types L (○) and M (□) enzyme for each age group were calculated from the respective total activities and the relative proportions of types L and M enzyme determined as described in the text. Activities are expressed as $\mu\text{mol}/\text{min}$ per 100mg of supernatant protein and given as means \pm S.E.M. (four or five determinations). Each individual determination of foetal liver enzymic activity represents the average value for the pooled livers from one litter. T, Term; A, adult.

liver (8 units/100mg of supernatant protein in the 16–17-day foetus). There was a slight increase around birth and then a decrease during the suckling period. At the onset of weaning (around 20 days *post partum*) there was a rapid and large increase in the activity of the type L enzyme up to the thirtieth day, followed by a more gradual increase to the adult values of 30–33 units/100mg of supernatant protein (Fig. 6).

The development profile was not significantly altered when the results were expressed per 100g body wt., except that the increase in activity of type L enzyme before birth was a little less than that depicted in Fig. 6.

Discussion

Although the molecular nature of types L and M hepatic pyruvate kinase is not known, sufficient kinetic, chromatographic and electrophoretic differ-

ences have been observed to suggest that the two forms are different. The limited immunological information suggests that they may be different proteins. If so, types L and M pyruvate kinase may be termed isoenzymes within the context of the definition of Latner & Skillen (1968). Rutter (1969) has described a form of the enzyme in the late foetal liver that has electrophoretic behaviour between that of type L and type M enzyme. The possibility of molecular interconversions between type L and M enzyme cannot therefore be excluded. There are many similarities (Table 2) between the properties of the type M liver enzyme and the enzyme from skeletal muscle, but there do appear to be some immunological differences between them. Jiménez de Asúa *et al.* (1971) have reported that they also differ with respect to their inhibition by phenylalanine and other amino acids.

The activity of type M pyruvate kinase in rat liver is unlikely to be caused by leucocyte contamination because, although leucocyte pyruvate kinase has properties similar to those of the type M hepatic enzyme (Campos *et al.*, 1965) it has a low specific activity in the leucocyte, and perfusion of the liver to remove most of the blood does not alter the amount of type M enzyme detected. The kinetic properties of type M pyruvate kinase in the present study are in general agreement with those summarized by Tanaka *et al.* (1970). The absence of marked co-operative interactions with phosphoenolpyruvate or stimulation by fructose 1,6-diphosphate in our preparation (*n* values a little above 1 for the 52–70% satd. ammonium sulphate fraction were, however, noted; see above and Table 1) could be a result of the purification procedure desensitizing the enzyme, but no stimulation by fructose 1,6-diphosphate was ever observed at any stage of the preparation. The small stimulation reported by Tanaka *et al.* (1970) might conceivably be due to contamination of their type M enzyme with type L; this cannot be decided in the absence of information on the purity of their enzyme.

The proportion of the total activity due to the activities of type L and M hepatic pyruvate kinase reported by Tanaka *et al.* (1965, 1967a) in adult Sprague-Dawley rats (67 and 33% respectively) differ from the values obtained in our Wistar strain (95 and 5% respectively). This may well reflect genuine differences between the strains, since Bartley *et al.* (1967) and Eggleston & Krebs (1969) have reported different total pyruvate kinase activities in various strains of rats.

The intracellular concentration of phosphoenolpyruvate (0.13 mM; Burch, 1965; Williamson, 1966) is such that the activity of the type L pyruvate kinase will be very susceptible to changes in the concentration of fructose 1,6-diphosphate. It seems likely (see also Schoner *et al.*, 1970; Llorente *et al.*, 1970)

that the activity of this enzyme *in vivo* will be almost completely inhibited under conditions favouring gluconeogenesis by cellular concentrations of ATP and alanine. Under such conditions the activity of the type M enzyme, which is relatively unaffected by fructose 1,6-diphosphate or ATP, would probably remain at around 1–2 $\mu\text{mol}/\text{min}$ per g wet wt. of liver (depending on the actual K_m value of the enzyme *in vivo*). Thus the residual available hepatic pyruvate kinase activity during gluconeogenesis, even assuming that type L enzyme is not active, could be significant compared with the overall rate of gluconeogenesis (2 $\mu\text{mol}/\text{min}$ per g wet wt. of liver; Exton & Park, 1967). If the pathways of glycolysis and gluconeogenesis are not compartmented at this level then, in the absence of any metabolic inhibitors of the type M enzyme, some recycling of phosphoenolpyruvate will occur. This may increase the sensitivity of the control system, as suggested by Newsholme & Gevers (1967). Although this argument is of necessity based on several assumptions about the intracellular substrate concentrations and enzyme activities, it is apparent that the contribution of minor isoenzymes to the overall activity of rate-limiting enzymes cannot be omitted from theories of metabolic control.

The activity of the type M enzyme is unaffected by the increase in carbohydrate content of the diet at weaning (Fig. 6). The evidence for a high activity of type M pyruvate kinase in the early foetal liver is in accord with the qualitative observations of Rutter (1969), who used cellulose acetate electrophoresis. The changes in activity of type M enzyme are similar to those of the hepatic hexokinases (Burch *et al.*, 1963) except that the activity of type M pyruvate kinase decreases rapidly just before and after birth whereas the decrease in hexokinase activity is more gradual. As the decrease in activity of type M enzyme is approximately paralleled by the decrease in the proportion of haemopoietic cells during this period (Oliver *et al.*, 1963), the possibility arises that type M pyruvate kinase is localized in these cells. The results of Linder-Horowitz (1969) and Oliver *et al.* (1963) were used to calculate the number of haemopoietic cells in the developing rat liver. The correlation between the activity of type M enzyme and the number of haemopoietic cells was found to be very poor. It is therefore unlikely that type M enzyme is localized solely in these cells. Tanaka *et al.* (1967a) demonstrated that both type L and M hepatic pyruvate kinases were present in the parenchymal cells by means of a fluorescent-antibody technique.

The increase in total hepatic pyruvate kinase activity that occurs as the supply of carbohydrate increases at weaning (Walker & Vernon, 1967; Taylor *et al.*, 1967; Vernon & Walker, 1968) is due to a different type of enzyme adaptation from that effecting the increase in hepatic glucose-phosphorylating activity during this period. The latter is caused

by the synthesis at weaning of a unique glucokinase (EC 2.7.1.2). For pyruvate kinase both isoenzymes are already present in the early foetal liver and it is the type L form that increases in parallel with glucokinase.

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