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Membrane-associated pyruvate kinase in developing guinea-pig liver

Sheelagh M. FARROW* and Colin T. JONES†
Laboratory of Developmental Physiology, Nuffield Institute for Medical Research, University of Oxford, Headley Way, Oxford OX3 9DS, U.K.

During analysis of pyruvate kinase distribution in developing guinea-pig liver it was observed that a substantial proportion of the activity remained associated with the microsomal membrane fraction ('microsomes'). Although some of this could be removed by washing with sucrose, the majority required detergent treatment for liberation, and even then at least one-half remained attached to the microsomes. Estimates of the contribution of this fraction to total cell pyruvate kinase activity indicated that it was more than 50% of the total, and this is likely to be an underestimate because of the continued latency of the enzyme even in the presence of detergent. The susceptibility of the microsomal enzyme, whether released by detergent or sucrose washing, to inactivation by Triton X-100 suggested it to be different from the cytosolic enzyme, which was stable under such conditions. (The microsomal enzyme required the presence of additional protein, such as bovine serum albumin, to maintain stability.) This view was confirmed by DEAE-cellulose chromatography and particularly isoelectric focusing, where the microsomal enzyme was shown to consist of at least four forms, which were distinctly different from those in the cytosol. Those data and the kinetic properties of the four forms in the membrane fraction indicate that the microsomal pyruvate kinase could consist of four counterparts to the cytosolic isoenzyme forms. These results are discussed in relation to the two possible explanations for the phenomenon (not mutually exclusive): that the more hydrophobic membrane forms are precursors of the cytosolic enzyme and that they may be part of functional glycolytic pathway in the microsomes of developing liver.

INTRODUCTION

Pyruvate kinase exists as at least three non-interconvertible isoenzymes with different physical, chemical, immunological, kinetic and electrophoretic properties. Adult liver contains predominantly PK1 (variously known as type L or B), with some PK4 (M_2, K or C) (Tanaka *et al.*, 1967; Susor & Rutter, 1968; Imamura & Tanaka, 1972). PK4 is distributed widely among tissues, and its predominance in foetal tissues, cancers and regenerating livers has led to the suggestion that it is a 'prototype' isoenzyme (Imamura & Tanaka, 1972; Farina *et al.*, 1974; Dyson *et al.*, 1977; Saheki *et al.*, 1978). The third well-characterized isoenzyme, PK3 (M or A), is the major form in skeletal and cardiac muscle and in brain (Imamura & Tanaka, 1972; Whittell *et al.*, 1973). It is unclear whether pyruvate kinase activity in erythrocytes, PK2(R), is a fourth distinct isoenzyme or a modified form of PK1, to which it is related closely (Tanaka *et al.*, 1967; Marie *et al.*, 1977), both probably being products of the same gene (Marie *et al.*, 1981). PK2 exists in several forms which differ kinetically (Cartier *et al.*, 1968; Ibsen *et al.*, 1971; Kahn *et al.*, 1978; Lakomek *et al.*, 1983) and appear at separate times during red-cell aging (Kahn *et al.*, 1978). Heterogeneity of PK2 in erythrocytes may result also from coexistence of membrane-associated and cytosolic forms (Lakomek *et al.*, 1983). Membrane-bound pyruvate kinase has been found in other tissues, for example associated with F-actin in skeletal muscle (Clarke & Masters, 1975).

The phenomenon of membrane association has been reported for other glycolytic enzymes in skeletal muscle (Arnold & Pette, 1970; Clarke & Masters, 1975; Moreton *et al.*, 1977), erythrocytes (Green *et al.*, 1965; Tillman *et al.*, 1975), brain (Wilson, 1968; Knull *et al.*, 1974) and liver (Agostoni *et al.*, 1966; Foemmell *et al.*, 1975; Weiss *et al.*, 1981). Hence for glycolytic enzymes there are at least three sources of intracellular diversity; different isoenzyme forms, variation in intracellular processing and bimodal distribution between membrane and cytosolic fractions.

In view of this increasing evidence for glycolytic-enzyme heterogeneity, it was not surprising in preliminary experiments to find the foetal liver contained significant quantities of pyruvate kinase activity associated with the microsomal fraction. The objective of the present experiments was to establish the nature of the membrane-bound enzyme and quantify its contribution to total cell activity. The results show that more than 50% of pyruvate kinase in foetal guinea-pig liver is membrane-bound and chromatographically, electrophoretically and kinetically different from the classical cytosolic forms.

* Present address: Department of Medicine, Middlesex Hospital, Mortimer Street, London W1P 7PN, U.K.
† To whom communications and reprint requests should be addressed.

METHODS

Animals

All studies used guinea pigs of the Dunkin–Hartley strain maintained on Dixon's Diet 18 and hay *ad libitum*. Time of conception was assessed as described by Elvidge (1972). Gestational age of foetuses used ranged from 45 to 65 days.

Preparation of tissue extracts

Animals were killed by cervical dislocation and bled from the neck. The digestive process was a modified method of Leffert & Paul (1972). Liver was chopped, washed in Krebs bicarbonate buffer, placed in a 100 ml Erlenmeyer flask with Ca^{2+}-free Krebs bicarbonate buffer (2 ml/g wet wt. of liver) containing 3 mg of collagenase (Sigma, type I)/ml and 20 mM-Hepes, pH 7.4, and incubated at 37 °C in a shaking water bath for 8 min. The supernatant was collected and kept on ice. The process was repeated five times and pooled supernatants were centrifuged at 50 *g* for 1 min, and the pellets washed in Krebs bicarbonate buffer and reprecipitated. Sedimented cells were resuspended in 9 vol. of 0.2 M-sucrose/50 mM-Tris/HCl, pH 7.5, containing 1 mM-EDTA, 0.1 mM-dithiothreitol and 5 mM-$MgCl_2$, and sonicated for 2 × 5 s at 150 W (instrument from Ultrasonics Ltd., Shipley, W. Yorks., U.K.). When hepatocyte preparation was attempted by perfusion of the liver, as described below, with Krebs buffer containing collagenase, rapid breakdown of liver vasculature blocked perfusion.

Tissues were homogenized with a motor-driven Teflon pestle (clearance 0.07 mm) in 9 vol. of 0.2 M-sucrose/50 mM-Tris/HCl (pH 7.5) containing 1 mM-EDTA, 0.1 mM-dithiothreitol, 5 mM-$MgCl_2$ and 1 mM-phenylmethanesulphonyl fluoride.

Sonicated preparations and homogenates were separated by centrifugation as described by de Duve *et al.* (1955) into a nuclear and an unbroken-cell fraction (600 *g* for 10 min), a mitochondrial (10000 *g* for 30 min), a microsomal and a cytosolic fraction (74000 *g* for 60 min), by using a 16 × 15 ml angle rotor in a MSE High-Speed 25 centrifuge at 2 °C. Each pellet was washed once with 2 ml of the sucrose/Tris buffer and re-centrifuged. For determination of enzyme activities, the pellets were resuspended in 0.2 M-sucrose/Tris buffer, pH 7.5, in the presence and absence of 0.25% Triton X-100. To obtain a sample of microsomal pyruvate kinase, the washed pellet was resuspended in buffer containing 0.25% Triton X-100 and 1 mg of bovine serum albumin (Sigma, fraction V)/ml, which was necessary to maintain enzyme activity in the presence of detergent. The suspension was sonicated, left for 15 min at 0 °C, centrifuged at 74000 *g* for 60 min, and the supernatant, containing solubilized enzyme activity, was collected.

PK2 activity was prepared from adult guinea-pig erythrocytes; blood (15 ml) was centrifuged at 1500 *g* at 4 °C for 20 min and the cells were diluted (25%, v/v) in sucrose/Tris/HCl buffer, pH 7.5, containing 0.25% Triton X-100. The lysed cell suspension was centrifuged for 60 min at 74000 *g* at 2 °C, and the PK2 activity in the supernatant was precipitated by the addition of $(NH_4)_2SO_4$ to 40% (w/v) saturation at 0 °C. The precipitate was redissolved in sucrose/Tris/HCl buffer.

Preparation of extracts of perfused liver

Adult guinea pigs were anaesthetized with sodium pentobarbitone (30 mg/kg) and the liver was exposed. The inferior vena cava was cut and the liver perfused through the hepatic portal vein with Krebs bicarbonate buffer. The tissue was excised and treated as described above.

Foetuses from anaesthetized pregnant guinea pigs (47–67 days gestational age) were exteriorized, catheters inserted into the umbilical vein and inferior vena cava, and the liver was perfused at 37 °C at a flow rate of approx. 12 ml/min with Krebs bicarbonate buffer containing 1 mM-glucose and gassed with CO_2/O_2 (1:19). Once the effluent appeared free of blood (5–10 min), the liver was removed and homogenized as described above.

Assays

Pyruvate kinase was assayed as described by Bucher & Pfleiderer (1955); the final reaction mixture (1 ml) contained 0.1 M-Tris/HCl, pH 7.5, 50 mM-KCl, 25 mM-$MgCl_2$, 0.15 mM-NADH, 0.5 mM-phosphoenolpyruvate, 0.1 mM-fructose 1,6-bisphosphate, 1 mM-ADP, 5 units of lactate dehydrogenase and extract. After preincubation for 10 min at 25 °C, the reaction was started by the addition of ADP and the decrease in A_{340} followed continuously on a Pye–Unicam SP.1800 spectrophotometer.

K_m values for phosphoenolpyruvate of pyruvate kinase isoenzymes were calculated by the Lineweaver–Burk method with V_4 weighting as described by Wilkinson (1961).

Lactate dehydrogenase was determined as described by Kornberg (1955), glutamate dehydrogenase as described by Hogeboom & Schneider (1953), citrate synthase as described by Srere (1969), 5′-nucleotidase as described by Michell & Hawthorne (1965), acid phosphatase as described by Alvarez (1962), NADPH–cytochrome *c* reductase as described by Sottocasa *et al.* (1967), cytochrome oxidase as described by Wharton & Tzagoloff (1967), and nucleoside diphosphatase as described by Plaut (1963). Glucose-6-phosphatase was assayed in 50 mM-sodium cacodylate (pH 6.5) containing 50 mM-sucrose, 0.2 mM-EDTA, 0.25% Triton X-100 and 25 mM-glucose 6-phosphate at 37 °C. Liberated P_i was measured as described by Fiske & Subba Row (1925); P_i liberation was linear with time for at least 30 min. Phospholipids in the microsomal fraction were extracted with chloroform/methanol (2:1, v/v). The organic phase after evaporation to dryness and resuspension was separated (400–800 μg of lipid) on 20 cm × 20 cm silica-gel t.l.c. plates developed with chloroform/methanol/acetic acid/water (25:15:4:2, by vol.). Phosphorus in the phospholipid fraction was determined as described by Bartlett (1959). Recovery of phosphatidyl-choline and -ethanolamine was 86.7 ± 3.4 (6)% and 83.1 ± 4.6 (6)% (S.D.) respectively.

DEAE-cellulose chromatography

Pyruvate kinase isoenzymes were separated on DEAE-cellulose (Whatman DE52). Tissue extracts were applied to 8 cm × 1 cm columns pre-equilibrated with 20 mM-Tris/HCl, pH 7.5, containing 1 mM-KCl, 1 mM-$MgCl_2$, 0.1 mM-EDTA and 0.1 mM-dithiothreitol. A continuous linear concentration gradient of KCl (0–0.5 M) was used to elute pyruvate kinase isoenzymes, at 0.3–0.5 ml/min.

Isoelectrofocusing

Isoelectrofocusing columns (110 ml; LKB 8100-1) containing linear (5–50% (w/v) sucrose gradients were prepared with the anode at the top. The required range of Ampholines (LKB) was added to a final concentration of 1% (w/v), the column equilibrated at 5 °C and 150 V, 0.5 mA, for 24 h, then the sample was introduced into the middle of the column with a peristaltic pump. Proteins were separated at 320 V, 1.0 mA and 5 °C for 26 h, then eluted by pumping distilled water into the top of the column at 60–72 ml/h. Fractions (2.0–2.5 ml) were collected, assayed for pyruvate kinase activity and their pH was measured.

RESULTS

In the foetal guinea-pig liver at around 50 days, the haematopoietic-cell fraction is a small proportion of that of the hepatocytes (Faulkner & Jones, 1979). Moreover, the hepatocyte isolation left most of the haematopoietic cells in the supernatant fraction, and electron micrographs of the hepatocyte pellet indicated there was little contamination by erythroblastic cells.

After sonication of foetal hepatocytes, nearly 90% of lactate dehydrogenase and 10% of glutamate dehydrogenase were recovered in the supernatant fraction, indicating that cell disruption was virtually complete (Table 1). However, less than three-quarters of the apparent pyruvate kinase activity was liberated, 25% remaining associated with the microsomal fraction, compared with 5% and 13% of lactate dehydrogenase and glutamate dehydrogenase respectively. Doubling sonication time to 4 × 5 s did not increase recovery of pyruvate kinase in the supernatant, although approx. 30% of total cell glutamate dehydrogenase activity was recovered in the cytosolic fraction, suggesting mitochondrial damage. The proportion of the liver cell activity for other enzymes recovered in the microsomal fraction was: 5′-nucleotidase, 94±18% (S.D.); glucose-6-phosphatase, 76±24%; NADPH–cytochrome *c* reductase, 63±21%; nucleoside diphosphatase, 72±23%; acid phosphatase, 4±1%; citrate synthase 6±2%, cytochrome oxidase, 4±3%. The microsomal protein recovered was 30 mg/g of liver, and 15.6±5.1 g of microsomal phospholipid was recovered/g of liver.

Fractionation of homogenized whole foetal liver showed a similar relatively high recovery of pyruvate kinase activity in the microsomal fraction. It represented 17.7±6.5 (7)% of total when prepared in sucrose buffer, 14.1±5.5 (6)% of total when 0.5 M-KCl was added to the homogenizing buffer, and 35±10 (7)% of total in the presence of 0.25% Triton X-100.

Initially the pyruvate kinase activity recovered in the microsomal fraction appeared to be loosely bound, as washing the pellet in sucrose/Tris buffer (pH 7.5) removed 90%, or with 0.5 M-KCl 80%, of that detected. However, when Triton X-100 was added to the initial homogenizing buffer or to the microsomal fraction after isolation, both total and microsomal pyruvate kinase activity increased 2–3-fold (Table 2). This appeared to be release of latent activity rather than enzyme activation, since addition of detergent to the cytosolic fraction had no effect on pyruvate kinase activity, whereas in the absence of bovine serum albumin it completely inactivated solubilized microsomal activity. In the presence of 0.25% Triton X-100, the proportion of pyruvate kinase associated with the microsomal fraction increased to at least 50% of total cell activity; less than half of this could be removed by washing with sucrose/Tris buffer or with 0.5 M-KCl. Even the value of 50% associated with the microsomal fraction may be an underestimate, as after detergent treatment not all latent activity was released. A higher concentration of Triton X-100 (0.5%) inactivated the enzyme, an effect which was not observed with cytosolic pyruvate kinase. The addition of albumin was essential to maintain activity in the presence of detergent.

Pyruvate kinase in the cytosol isolated from maternal liver, lung or muscle showed no tendency to bind to the microsomal fraction prepared from foetal liver. The adult liver fractionated as described in the Methods section, < 1% of total pyruvate kinase activity was recovered in the microsomal fraction.

Thus foetal liver contains pyruvate kinase in the microsomal fraction which appears to differ from cytosolic enzyme in respect to sensitivity to detergent and solubility. To investigate this further, the chromatographic and kinetic properties of pyruvate kinase from both sources were studied.

Cytosolic pyruvate kinase isoenzymes

Separation of cytosolic pyruvate kinase from adult and foetal liver (Figs. 1*a* and 1*b*) yielded one peak of activity, which did not bind to DEAE-cellulose, and another, which was eluted at approx. 150 mM-KCl, assumed to be PK4 and PK1 respectively (Jimenez de Asua *et al.*, 1971; Middleton & Walker, 1972; Osterman *et al.*, 1973; Faulkner & Jones, 1975). A separate peak representing PK2 was not recovered, although this isoenzyme is

Table 1. Subcellular distribution of pyruvate kinase, lactate dehydrogenase and glutamate dehydrogenase in fractionated foetal liver cells

Results are given as means ±S.D. Foetal liver cells were obtained by digestion with collagenase (see the Methods section) and sonicated (150 W for 2 × 5 s). Fractionation was by differential centrifugation as described in the Methods section. Total enzyme activities (units/g of protein) in homogenates: pyruvate kinase 42.4±8.9, lactate dehydrogenase 222.3±40.2, glutamate dehydrogenase 462±198.

Enzyme	No. of experiments	Percentage of total activity			
		Nuclear	Mitochondrial	Microsomal	Supernatant
Pyruvate kinase	11	< 0.1	2.3±5.8	24.4±11.5	73.2±12.5
Lactate dehydrogenase	6	2.7±3.0	5.3±4.0	4.9± 3.8	87.3±9.4
Glutamate dehydrogenase	11	27.5±7.6	48.8±9.3	13.0±3.6	8.7±4.7

Table 2. Liberation of pyruvate kinase activity from microsomal fractions of foetal guinea-pig liver

Results are expressed as means ±S.D. for six to seven experiments. Microsomal fractions were prepared from foetuses of 47–53 days gestational age and treated as described in the Methods section.

Treatment of microsomal fraction	Microsomal pyruvate kinase activity		
	Enzyme activity (units/g of microsomal protein)	Percentage of total cell activity	Percentage of apparent total cell activity remaining microsomally associated after treatment
None	11.5±1.1	34.9±16.4	34.9±16.4
Sucrose buffer	11.5±1.1	34.9±16.4	4.5±2.1
0.5 M-KCl	8.7±1.7	29.5±15.2	6.3±2.9
0.05% Triton X-100	21.2±2.7	47.8±17.8	22.0±12.5
0.2–0.25% Triton X-100	32.4±10.5	55.7±17.4	26.8±12.5

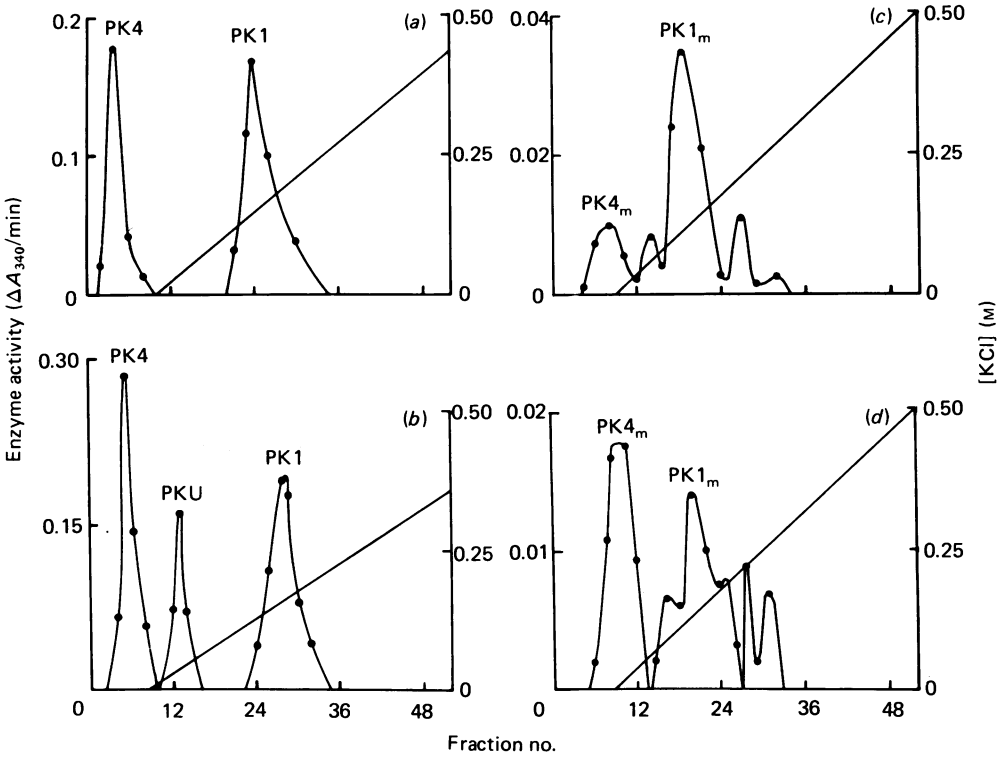

Fig. 1. DEAE-cellulose chromatography of pyruvate kinase activity

Enzyme activity prepared from adult liver cytosol (*a*); cytosol from foetal liver of 50 days gestational age (*b*); two separate examples microsomal fractions of foetal liver of 50–52 days gestational age are given in (*c*) and (*d*). ●, Enzyme activity; ——, [KCl].

present in foetal liver extracts (Faulkner & Jones, 1975). However, when erythrocyte PK2 was applied to the column, no activity was recovered, either. On occasions there was an unidentified peak, PKU, eluting at 50 mM-KCl (Fig. 1*b*).

Isoelectrofocusing gave better resolution of pyruvate kinase isoenzymes (Fig. 2). PK4 separated from lung extracts and adult and foetal liver had pI 6.22±0.03 ($n = 22$). PK1 from adult and foetal liver had pI 5.62±0.02 ($n = 22$). The distribution profile of PK2 obtained from adult blood cells showed two peaks of activity (Fig. 2*a*), one with pI 5.20±0.06 ($n = 6$) and another in the same region as PK1. Unlike adult liver (Fig. 2*b*), foetal liver contains a significant proportion of PK2 (Fig. 2*c*), which could be decreased by perfusion of the liver before homogenization (Fig. 2*d*). Thus the activity from DEAE-cellulose ascribed to PK1 in this study and elsewhere (Osterman *et al.*, 1973; Faulkner & Jones, 1975) is likely to be contaminated significantly by PK2. Perfusion of foetal liver and separation by isoelectrofocusing decreases the contribution of PK2 and shows, in contrast with previous reports (Faulkner & Jones, 1975), that foetal guinea-pig liver at about 50 days gestation contains more PK4 than PK1 activity (Fig. 2*d*).

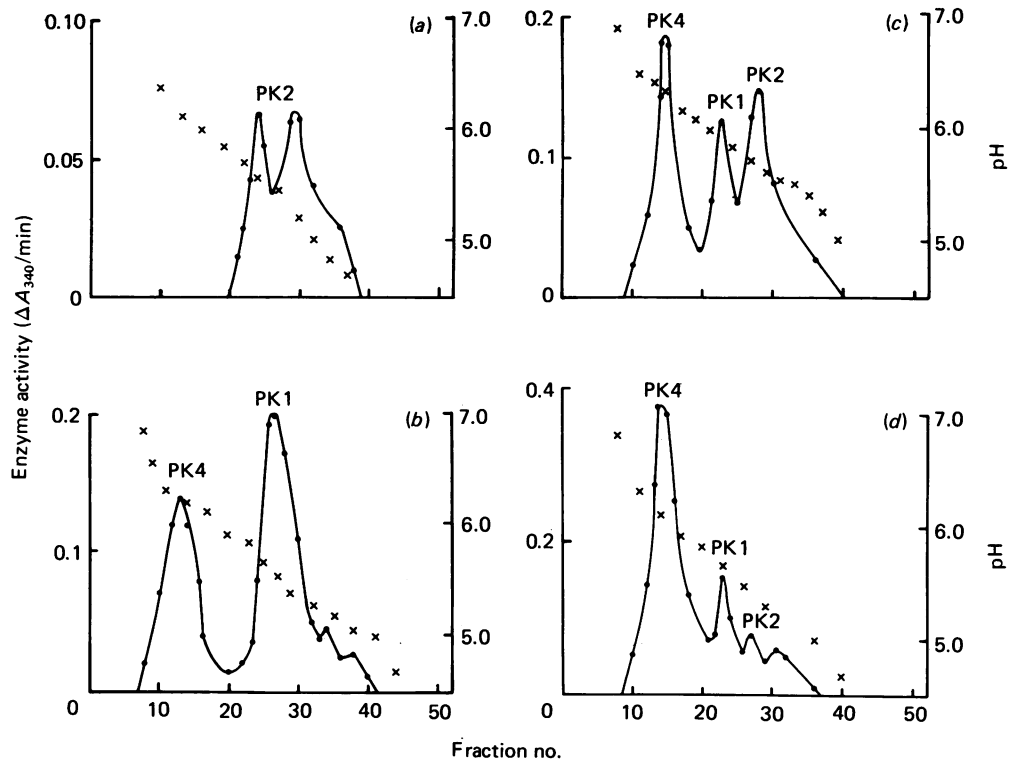

Fig. 2. Elution profiles of cytosolic pyruvate kinase isoenzymes separated by isoelectrofocusing on a gradient of pH 5–7

PK2 from adult blood cells (*a*); PK4 and PK1 from adult liver (*b*); PK4, PK1 and PK2 from foetal liver of 50 days gestational age (*c*); and from perfused foetal liver of the same age (*d*). ●, Enzyme activity; X, pH gradient.

Microsomal activity

For these studies, the activity released into the supernatant after homogenization of washed microsomal fractions with 0.25% Triton X-100 was used. DEAE-cellulose chromatography of this activity yielded consistently two major peaks in the same regions as cytosolic PK1 and PK4 (Figs. 1*c* and 1*d*). In addition, there were several smaller peaks at 35–1000 mM-, 227 ± 8.0 mM- and 278 ± 39 mM-KCl. These results imply that the majority of microsomal pyruvate kinase consists of isoenzymes comparable with cytosolic forms. However, this interpretation was not supported by isoelectrofocusing studies. These were optimal when a gradient of pH 4–6 was used, rather than that of pH 5–7 used for separation of cytosolic isoenzymes. There were three or four peaks of activity, with pI values ranging from 4.15 to 4.7 (Fig. 3), which bear little resemblance to values for cytosolic forms, suggesting that the microsomal fraction contains a different collection of isoenzymes. Microsomal activity was recovered consistently in the region of precipitation of bovine serum albumin. Bearing in mind that albumin was required to stabilize activity solubilized by Triton X-100, it probably combined with the enzyme. Hence the pI values may reflect the properties of an albumin/PK complex rather than of individual isoenzymes. Albumin had no effect on the pI values of cytosolic isoenzymes.

Isoelectrofocusing of microsomal extracts did not exhibit forms with pI values comparable with those of the classical isoenzymes. This implies that both loosely and firmly bound enzyme, which would be released together in the detergent solubilization, have no significant

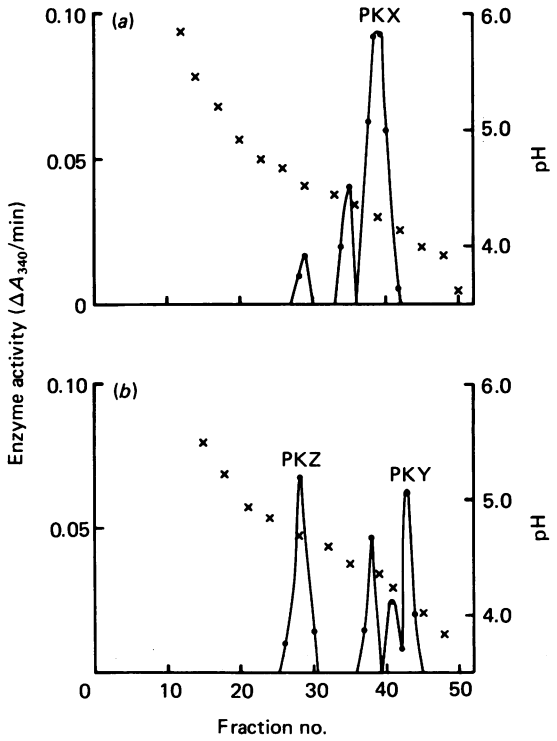

Fig. 3. Microsomal pyruvate kinase activity

Enzyme activity was prepared from foetal liver as described in the Methods section and separated by isoelectrofocusing in a gradient of pH 4–6. ●, Enzyme activity; X, pH gradient.

Table 3. Some kinetic properties of guinea-pig pyruvate kinase isoenzymes separated by DEAE-cellulose chromatography or isoelectrofocusing

Results are means ±S.D. for the numbers of experiments in parentheses. Pyrvuate kinase isoenzymes were separated from cytosolic and microsomal fractions prepared as described in the Methods section. PK1 and PK4 were obtained from perfused and non-perfused liver, PK2 from adult blood, PK3 from skeletal and cardiac muscle. Abbreviation: FBP, fructose 1,5-bisphosphate.

	K_m for phosphoenolpyruvate (mM)			
	DEAE-cellulose chromatography		Isoelectrofocusing	
Isoenzyme	0.1 mM-FBP	No FBP	0.1 mM-FBP	No FBP
Cytosolic				
PK1 (non-perfused liver) (10)	0.08±0.03	0.22±0.11	0.05±0.02	0.59±0.31
(perfused liver) (7)	—	—	0.26±0.12	1.18±0.9
PK2 (6)	—	—	0.09±0.06	0.21±0.19
PK3 (6)	0.05±0.02	0.03±0.01	0.04±0.01	0.05±0.01
PK4 (10)	0.30±0.09	0.35±0.1	0.20±0.05	0.26±0.10
Microsomal				
$PK1_m$ (3)	0.04±0.02	0.37±0.12	—	—
$PK4_m$ (3)	0.15±0.13	0.25±0.10	—	—
PKX	—	—	0.04	0.25
PKY	—	—	0.06	0.19
PKZ	—	—	0.08	0.19

cytosolic isoenzyme forms. Furthermore, cytosolic extracts prepared in the absence of detergent displayed no small peaks comparable with those of the microsomal forms.

Kinetic studies

Table 3 summarizes some of the kinetic properties of isoenzymes separated by DEAE-cellulose and isoelectrofocusing. The values for PK3 and PK4 from both techniques are similar to published results (Ibsen & Trippet, 1978; Middleton & Walker, 1972). In contrast, the properties of PK1 obtained from perfused liver were unlike those reported in guinea pig (Faulkner & Jones, 1975; Eigenbrodt & Schoner, 1977; Wu *et al.*, 1981) or those of PK1 from non-perfused liver or DEAE-cellulose. These differences could be explained by PK2 contamination of PK1.

The kinetic characteristics of the major peaks separated from microsomal activity on DEAE-cellulose, that is, $PK1_m$ and $PK4_m$ were not significantly different from their cytosolic counterparts (Table 3), except that inhibition of $PK4_m$ by alanine and phenylalanine (5 mM) was reversed by 0.1 mM-fructose 1,6-bisphosphate.

Three peaks of microsomal pyruvate kinase with pI values of 4.25, 4.15 and 4.7 (PKX, PKY and PKZ respectively) were studied. None of these behaved completely like any cytosolic isoenzyme, but there were similarities to PK1. However, the microsomal activity was unaffected by alanine or phenylalanine both of which inhibited PK1 in the absence of fructose 1,6-bisphosphate. Unlike the results from DEAE-cellulose, there was no evidence of any PK4-like activity.

DISCUSSION

Pyruvate kinase isoenzymes are tetramers, of total M_r 190000–250000 (Hall & Cottam, 1978). PK1, PK3 and PK4 differ sufficiently in their subunit structure and immunological properties to be products of three distinct genes. However, PK2 and PK1 appear to be interrelated; for example, Kahn *et al.* (1978) showed that PK1 was composed of four identical subunits, designated L_4, whereas erythrocyte PK2 was a heterotetramer of L_2L_2' where L' has a higher M_r. The enzyme in erythroblasts was composed of L_4', a subunit that could be transformed into the L form by mild tryptic attack, consistent with the report that both L and L' subunits were encoded by the same gene but with different mRNAs (Marie *et al.*, 1981). Electrophoresis of pyruvate kinase activity from cultured foetal hepatocytes (Guguen-Guillouzo *et al.*, 1980) revealed two forms of enzyme (R_1 and R_2), in the same region as erythrocyte PK2, which cross-reacted with PK1. These forms could be converted into PK1 by proteolysis. From these observations it was proposed that the R-types of pyruvate kinase were precursors of PK1 and their presence in foetal tissue was an indication that the proteolytic modification was less well developed than in the adult.

Further additions to the classical pyruvate kinase isoenzymes have been observed in developing and regenerating liver. Hybrid forms have been reported *in vitro* (Dyson & Cardenas, 1973; Saheki *et al.*, 1978) and occur naturally in bovine (Strandholm *et al.*, 1976), chicken (Ibsen *et al.*, 1976; Cardenas *et al.*, 1978) and guinea-pig tissues (Whittell *et al.*, 1973). Although it has been suggested that PK1 is synthesized in the parenchymal cells, whereas PK4 is produced in the Kupffer cells (van Berkel *et al.*, 1972), cultured hepatocytes from regenerating liver have been shown to shift from synthesizing PK1 to PK4 (Garnett *et al.*, 1974), indicating the potential of cells to produce both isoenzymes, and this is supported by the existence of hybrid forms.

Greater isoenzyme heterogeneity may exist if there are membrane-associated forms. Before the present study, although there was no direct evidence for membrane-bound pyruvate kinase and particularly for microsomally

associated enzyme, its potential existence could be inferred from observations in various tissues of glycolytic enzymes associated with intracellular structures. These include binding of aldolase, lactate dehydrogenase, glyceraldehyde-3-phosphate dehydrogenase and phosphofructokinase to F-actin and sarcoplasmic reticulum in skeletal muscle (Fahimi & Karnovsky, 1966; Arnold & Pette, 1970; Clarke & Masters, 1975; Moreton *et al.*, 1977), hexokinase and aldolase in brain (Clarke *et al.*, 1970; Knull *et al.*, 1974), aldolase and lactate dehydrogenase in liver microsomal fractions (Agostoni *et al.*, 1966; Foemmell *et al.*, 1975; Weiss *et al.*, 1981), phosphoglycerokinase and lactate dehydrogenase in kidney microsomal fractions (Jones *et al.*, 1963; Domenech & Domenech, 1970) and a collection of enzymes in erythrocyte membranes (Schrier, 1963; Green *et al.*, 1965; Tillman *et al.*, 1975). Wilson (1978) used the term 'ambiquitous' to describe enzymes which may exist in both bound and soluble states in an equilibrium which changes according to the environment, for example, concentration of metabolites. Mature erythrocytes have been reported to contain pyruvate kinase firmly associated with the cell membrane, which differs kinetically from the cytosolic enzyme (Lakomek *et al.*, 1983). This may explain the well-documented evidence of erythrocyte PK2 existing in several forms (Cartier *et al.*, 1968; Ibsen *et al.*, 1971; Kahn *et al.*, 1978). Thus the enzyme heterogeneity that exists for glycolytic enzymes and potentially for pyruvate kinase may be explained in part by membrane-associated forms.

The adult liver pyruvate kinase (PK1) is clearly a soluble protein that showed little tendency to associate with microsomal fractions from adult or foetal liver. Hence the behaviour of the pyruvate kinase activity in foetal liver appears distinctly different. There are a number of potential explanations for this. The foetal liver could contain a relatively high proportion of enzyme monomers rather than tetramers, and these monomers could have greater hydrophobicity than the adult tissue enzyme forms. This seems unlikely, as pyruvate kinase tetramers can be dissociated, with attendant loss of enzyme activity, by urea or guanidine hydrochloride, for instance. However, on removal of the denaturing agent spontaneous re-association occurs, an event necessary for the return of activity (Cardenas & Dyson, 1973; Cardenas *et al.*, 1977; Porter & Cardenas, 1980). The microsomally associated activity could be caused by entrapment of soluble enzyme in membrane vesicle. However, on release it would be expected to show the properties of the cytosolic forms, which, on the basis of pI, sensitivity to detergent and kinetic properties, it does not. Moreover, calculations based on microsomal volume (Brattin *et al.*, 1982) suggest that, before washing, cytosolic entrapment should account for not more than about 6–7% of recovered activity. Finally, the microsomally associated pyruvate kinase activity could be an enzyme from with a greater than normal affinity for lipid. Association between phospholipid and a number of soluble proteins has been ascribed to predominantly electrostatic interaction (Papahadjopoulos *et al.*, 1975). The fact that the effects of washing with buffers containing high and low salt concentrations were equally ineffective at exposing latent pyruvate kinase activity, while removing with equal efficacy some that was loosely associated, indicates that much of the binding of latent activity was probably not electrostatic. Moreover, even that loosely associated did not inhibit the properties of soluble enzymes, but was sensitive to detergent and required albumin for stabilization. It seems probable that latent microsomal pyruvate kinase activity is firmly associated with membrane lipid, and even that which was released more rapidly could have been attached by hydrophobic bonds, whose strength is unlikely to be substantially increased by 0.5 M-KCl (Tanford, 1980). However, it is not possible at present to indicate whether the latent activity is that of permanent membrane protein or that of a protein in transit through, or in temporary binding with, cell membrane fractions. The high proportion of hepatocyte pyruvate kinase associated with the microsomal fractions makes the latter possibilities less likely.

In summary, the balance of evidence suggests that a substantial amount of pyruvate kinase in foetal liver is associated with the microsomal fractions and differs from cytosolic enzyme. Whether this membrane-bound activity is a product of the same gene as cytosolic pyruvate kinase is unclear, however, in view of the relationship between PK1 and PK2 (Kahn *et al.*, 1978); a potential explanation is that there are differences in processing of gene products. Hence, microsomal pyruvate kinase could be an intermediate isoenzyme (formed during production of mature enzyme), which is emphasized in foetal liver owing to low activity of a membrane proteinase (Guguen-Guillouzo *et al.*, 1980). In this respect, pyruvate kinase may be similar to mitochondrial enzymes such as glutamate dehydrogenase and aspartate aminotransferase, which are synthesized on the rough endoplasmic reticulum as a large precursor before processing and transportation into mitochondria (Kawajiri *et al.*, 1977; Jaussi *et al.*, 1982). In general, there is little evidence for precursor forms of glycolytic enzymes, although erythrocyte pyruvate kinase does appear to undergo proteolytic modification (Kahn *et al.*, 1978). One consequence of an accumulation of pyruvate kinase on the microsomal fractions in foetal liver should be that an increase in cytosolic activity would not necessarily be associated with a simultaneous increase in synthesis. The increase in cytosolic pyruvate kinase activity seen during development involves synthesis *de novo* (Hopkirk & Bloxham, 1980; Wu *et al.*, 1981). However, this does not exclude a microsomal storage form.

A potential function for microsomal pyruvate kinase is as part of a membrane-associated glycolytic pathway distinct from that in the cytosol. This would be consistent with such organization in bovine erythrocyte membranes, in which the bound enzymes catalysed the complete glycolytic pathway (Green *et al.*, 1965). Similarly, the glycolytic enzymes associated with plasma membranes of squid retinal axons produced ATP and GTP (Cecchi *et al.*, 1971). These authors suggested that the role of membrane-bound enzyme was to provide a local source of energy for membrane synthesis or a transport mechanism. Glycolysis in erythrocyte membranes may provide ATP for consumption by the Na^+/K^+-ATPase (Parker & Hoffman, 1967; Schrier *et al.*, 1975). A complete membrane-bound pathway in foetal hepatocytes is supported by the presence of other microsomally associated glycolytic enzymes, i.e. lactate dehydrogenase, aldolase, enolase, triose phosphate isomerase, phosphofructokinase, glyceraldehyde-3-phosphate dehydrogenase, phosphoglyceromutase and phosphoglucoisomerase (S. M. Farrow & C. T. Jones, unpublished work).

Although it is possible that these enzymes are storage forms, it is more likely that they are involved in a functional glycolytic pathway. The existence of such a pathway could be an important mechanism to explain the well-characterized resistance of foetal tissues to hypoxia. A microsomal pathway could maintain a glycolytic flux producing lactate, which may be removed from the cell into the lumen of the endoplasmic reticulum, while minimizing changes in cytosolic pH.

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