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PYRUVATE KINASE ISOZYMES IN CELLS ISOLATED FROM FETAL AND REGENERATING RAT LIVER

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Summary

There are at least three major mammalian isozymes of pyruvate kinase (ATP : pyruvate 2-*O*-phosphotransferase, EC 2.7.1.40), designated K_4, L_4, and M_4. Whereas parenchymal cells from adult rat liver contain only the type L isozyme, parenchymal cells isolated from fetal and regenerating liver were found to synthesize both the K_4 and L_4 isozymes. A small amount of K-M hybrid was seen in regenerating liver, but there were no detectable M-L or K-L hybrids. Thus, it appears that type L pyruvate kinase is not synthesized at the same time in the same liver cell with either of the other two isozymes. The intermediate electrophoretic bands seen with homogenates of whole fetal liver, and in some earlier work attributed to either hybrid isozymes or to the presence of M_4, are contributed by nonparenchymal cells which, in the fetus, are largely hemopoietic. These additional bands of pyruvate kinase are electrophoretically and immunologically similar to the pyruvate kinase isozymes found in adult erythrocytes.

The results reported here suggest a very rigorous control in the synthesis of K_4 and L_4 isozymes in parenchymal cells of both fetal and regenerating liver as opposed to developing neurons and glia, where the shift from synthesis of type K to type M subunits appears to occur gradually and results in the production of substantial amounts of hybrid isozymes.

Introduction

Mammals have three major noninterconvertible isozymes of pyruvate kinase (ATP : pyruvate 2-*O*-phosphotransferase EC 2.7.1.40), each of which is apparently composed of four identical or nearly identical subunits [1—8]. The early embryo contains only one of these isozymes, designated here as type K

for its apparent identity with the isozyme isolated from adult kidney. Although type K pyruvate kinase continues to be found at least in small quantities in probably all adult tissues, it is joined during fetal development by two more specialized isozymes: type L, an allosteric isozyme that is the dominant form in adult liver and a minor component in adult kidney and small intestine; and type M, a non-allosteric form that is dominant in adult striated muscle and nervous tissue. A fourth isozyme or isozyme hybrid has been postulated for rat erythrocytes [2—4,9], though other data suggest that erythrocyte pyruvate kinase may instead be a form of the type L isozyme [8,10—13].

Adult rat liver parenchymal cells normally synthesize only type L pyruvate kinase [14,15] but add the type K isozyme some time during the first few days following partial hepatectomy [16,17]. However, the mechanism of the shift from L to K is unclear since it is not known whether synthesis of both isozymes occurs simultaneously in the same cell, a situation that would be expected to result in formation of K-L hybrid isozymes. Previous studies did not reveal and K-L hybrids in regenerating liver [17], but the techniques used were not designed to look specifically for them, and the resolution attained in the earlier work was such that small quantities of hybrids could easily have been missed.

One or more intermediate electrophoretic forms have been observed in fetal liver [2,4,18—20] and have been variously identified as hybrids [1,4,18,21], a fourth isozyme [2], or type M [22]. Walker and Potter [23] first correlated the appearance of an intermediate electrophoretic form with the presence of hemopoietic cells in developing rat liver, while Schapira et al. [18] and Faulkner and Jones [20] determined that the intermediate bands of fetal rat and guinea pig have the same electrophoretic mobilities as those of erythrocytes.

The objectives of this paper are: (1) to examine the isozymes of fetal and regenerating liver, looking specifically for isozyme hybrids and (2) to determine the nature and cellular orgin of the intermediate electrophoretic bands seen with extracts from fetal liver. We have therefore performed cell separations, analyzed the isozyme patterns under electrophoretic conditions resulting in higher sensitivity and resolution than used previously, and combined electrophoresis with immunological techniques for isozyme identification. Although we found significant quantities of the K_4 isozyme in parenchymal cells of fetal liver and in parenchymal cells from regenerating liver only 24 h following partial hepatectomy, we could find no evidence for K-L hybrids in either case. These observations contrast sharply with the situation in developing brain, where K-M hybrids occur in abundance [24].

Materials and Methods

Chemicals

Special chemicals and enzymes, unless otherwise noted, were obtained from Worthington Biochemical Corporation or Sigma Chemical Co. Common chemicals were standard reagent grade, except for Schwarz-Mann's enzyme-grade sucrose. Distilled, deionized water was used throughout.

Animals

Rats were obtained from a local colony of Wistar origin. In determining

gestational age, the day of mating was designated day zero. Sprague-Dawley rats inoculated with Novikoff hepatoma were a generous gift of Dr. Sidney Weinhouse.

Cell isolation

Separation of parenchymal and non-parenchymal cells was performed using procedures described elsewhere [17,25] except for perfusion. Perfusion fluid contained 7 mM glucose, 103 mM NaCl, 3 mM KCl, 0.5 mM $MgCl_2$, 0.4 mM KH_2PO_4, 24 mM $NaHCO_3$, 0.6 mM Na_2HPO_4, and 0.1% collagenase, adjusted to pH 7.4 by bubbling 95% O_2: 5% CO_2 through the solution. Perfusion was accomplished in situ by cutting the portal vein, clamping the inferior vena cava between the liver and hepatic veins, then passing a catheter through the right atrium and into the inferior vena cava toward the liver. A ligature was placed around the catheter and a syringe used to force 20—30 ml of perfusing fluid slowly through the hepatic sinusoids and out of the portal vein into the peritoneal cavity. The success of the procedure can be judged by the degree of blanching of the liver as blood is flushed from it. The perfused liver was removed from the animal, minced, placed in a 125 ml erlenmeyer flask containing another 30—50 ml of the perfusion buffer and subjected to gentle shaking until dispersed (30 to 90 min) at 37° C in an atmosphere of 95% air, 5% CO_2. The cells were separated by low-speed differential centrifugation as described by Berry and Friend [25] and disrupted by 5—10 0.5-s bursts from a Kontes sonicator.

Fetal livers were not perfused but were rinsed with saline, minced, and placed directly in the shaker bath. Differential sedimentation with gravity alone was usually sufficient to separate the parenchymal cells from the slower-sedimenting hemopoietic and reticuloendothelial cells. However, in some cases the viscosity was great enough to require gentle centrifugation as described for cells from adult livers.

Electrophoresis

Cellulose acetate electrophoresis was carried out at 4° C and 200 V (14 V/cm) in a buffer containing 0.02 M Tris · HCl (pH 7.5 at room temperature), 1 mM fructose 1, 6-diphosphate, 1 mM EDTA, 0.5 M sucrose, and 10 mM β-mercaptoethanol. Sonicated extracts were cleared by centrifugation at 10 000 × *g* for 25 min and dialyzed in 1/4-inch casing against the electrophoresis buffer for at least 2 h. Detection of pyruvate kinase activity was performed by pressing the electrophoresis strip against an agar film containing the components of the assay medium as described elsewhere [2,26]. Before application of the sample to the cellulose acetate, solutions were diluted to approximately 9 units (μmol per min) of pyruvate kinase activity per ml if more concentrated, unless otherwise noted. More dilute solutions were spotted repeatedly to keep the total amount of enzyme per strip for a given experiment as constant as possible. Variations in intensities from strip to strip result from differences in incubation times and temperatures and in photographic exposure times. However, for a single electrophoretic strip, the intensities of the bands are approximately proportional to the relative amounts of enzyme activities.

In order to maximize our ability to detect hybrid isozymes, electrophoresis

of all samples was carried out for at least 19 h, which was several times as long as the separation time used in most of the earlier work and which produced much higher resolution. In many experiments, the longer electrophoresis time was coupled with multiple applications of sample to the electrophoresis strip, thereby producing the equivalent of using samples containing up to 60 units of pyruvate kinase activity per ml. By combining long electrophoresis time, heavy sample applications, and long incubations of the electrophoresis strips with the activity detection medium, we could detect individual electrophoretic forms representing only about 0.2 percent of the total activity.

Heart and whole liver extracts (homogenized, centrifuged, and dialyzed as before) were used as electrophoretic standards, since small variations in mobility occurred from experiment to experiment. Electrophoretic patterns were recorded on Kodabromide F-5 paper. All recorded bands were shown to be pyruvate kinase by demonstrating a requirement for both phospho*enol*-pyruvate and ADP in the detection medium.

Immunological techniques

Antibodies were produced in adult chickens by intramuscular injections of bovine skeletal muscle pyruvate kinase purified by the method of Cardenas et al. [27] or rat type L pyruvate kinase partially purified as described in reference 28. Two injections of 2—5 mg pyruvate kinase each in Freund's complete adjuvant were given at one month intervals. Chickens were bled via wing veins approximately one week after the second injection.

Antisera were incubated with tissue extracts in 4% NaCl, 3 mM Tris · HCl, pH 7.5, 0.5 mM β-mercaptoethanol, and 0.05 mM EDTA for 90 min at 37°C and then at least 4 h at 0—4°C before centrifuging 10 min at 10 000 × *g* to remove the precipitated antigen-antibody complex. The supernatants were then dialyzed 2—4 hours against the electrophoresis buffer described above.

Results

Fig. 1 shows the zymograms obtained with homogenates of whole liver at various times after hepatectomy. As early as 24 h after surgery, an increase in the ratio of type K to type L was already visible, with the amount of type K pyruvate kinase reaching a maximum by about six days after partial hepatectomy. By 17 days, the pattern was reverting to that seen in normal animals but still had not reached the normal ratio. Throughout liver regeneration, the total amount of pyruvate kinase extracted per g wet weight remained fairly constant at 26.4 ± 5.4 units per g.

Fig. 2 shows the electrophoretic patterns obtained from normal and regenerating liver, and from parenchymal cells (PC) and nonparenchymal cells (NPC) of regenerating liver. Very light bands are visible between K_4 and L_4 in the zymograms of regenerating liver. If types K and L subunits are synthesized simultaneously in the same cells, then one would expect K-L hybrids to occur. However, the electrophoretic mobilities of the intermediate bands, shown in Fig. 2a, cannot be K-L hybrids, as the three hybrids should be evenly spaced between K_4 and L_4, a prediction that has been verified by Osterman and Fritz [29] and by Strandholm et al. [13]. Rather, the intermediate electrophoretic

Zymograms of rat liver at various times after partial hepatectomy

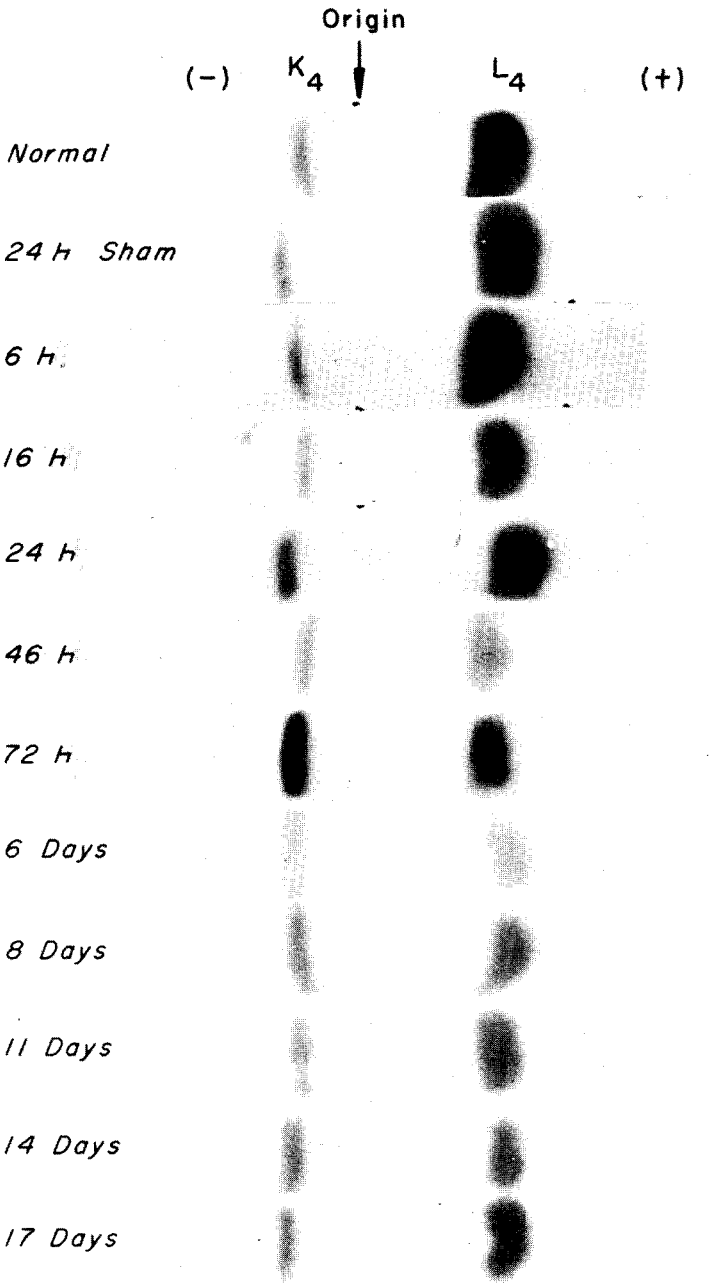

Fig. 1. Pyruvate kinase isozymes in liver extracts after partial hepatectomy. Origins are marked by the black triangles between the K_4 and L_4 positions. Electrophoresis time was 21 h at 4°C. Note the changing ratios of K_4 and L_4. Absolute intensities cannot be compared from strip to strip, as they are affected by photographic conditions.

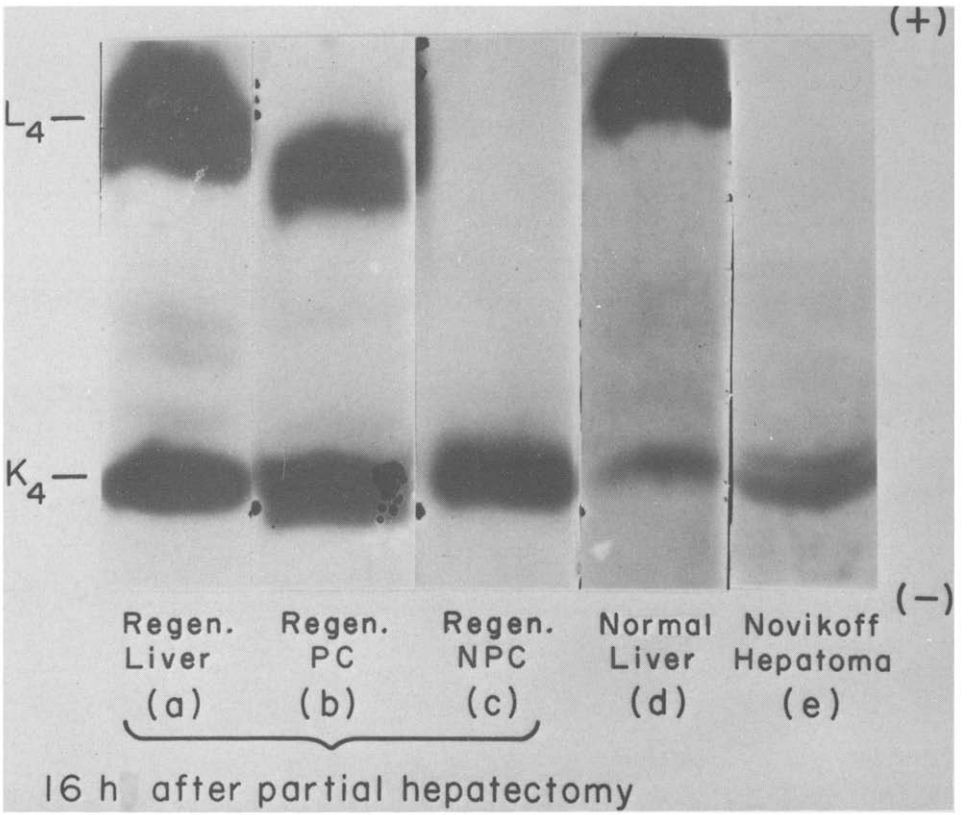

Fig. 2. Pyruvate kinase isozymes in parenchymal cells (PC) and nonparenchymal cells (NPC) of regenerating rat liver. Electrophoretic origins are marked by black triangles near the K_4 position. Electrophoresis time was 21 h. (a) Regenerating rat liver taken the day following partial (70%) hepatectomy. (b) Parenchymal cells isoalted from a similar regenerating rat liver. Note the substantial amount of K isozyme and the absence of K-L hybrid isozymes. (c) Nonparenchymal (endothelial and Kupffer) cells from a regenerating rat liver. (d) Normal, non-resected control. (e) Novikoff hepatoma cells, a rapidly growing and poorly differentiated line, showing K_4 and some K_3M but no L_4.

bands in Fig. 2a migrate as K-M hybrids, a finding that is consistent with other work from this laboratory [13] showing K-M hybrids in all bovine tissues examined. The intensity of the K-M hybrids seems to be somewhat less in parenchymal cells of regenerating liver than from whole regenerating liver extracts (see Fig. 2 parts a and b). Though it appears that these hybrids occur in regenerating parenchymal cells themselves, we cannot exclude the possibility that they originate from some other cell type that is present at low levels in our parencymal cell preparations. In any case, there is no evidence for the occurrence of K-L hybrids in regenerating liver or parenchymal cells isolated therefrom.

Fig. 3 shows the isozyme distribution in fetal rat liver. The homogenate of whole fetal liver shows substantial quantities of K_4 and L_4, as expected, plus two major intermediate bands. However, these extra bands are neither K-M nor K-L hybrids, for they do not have the predicted electrophoretic mobility. To verify that conclusion, we separated parenchymal cells from nonparenchymal

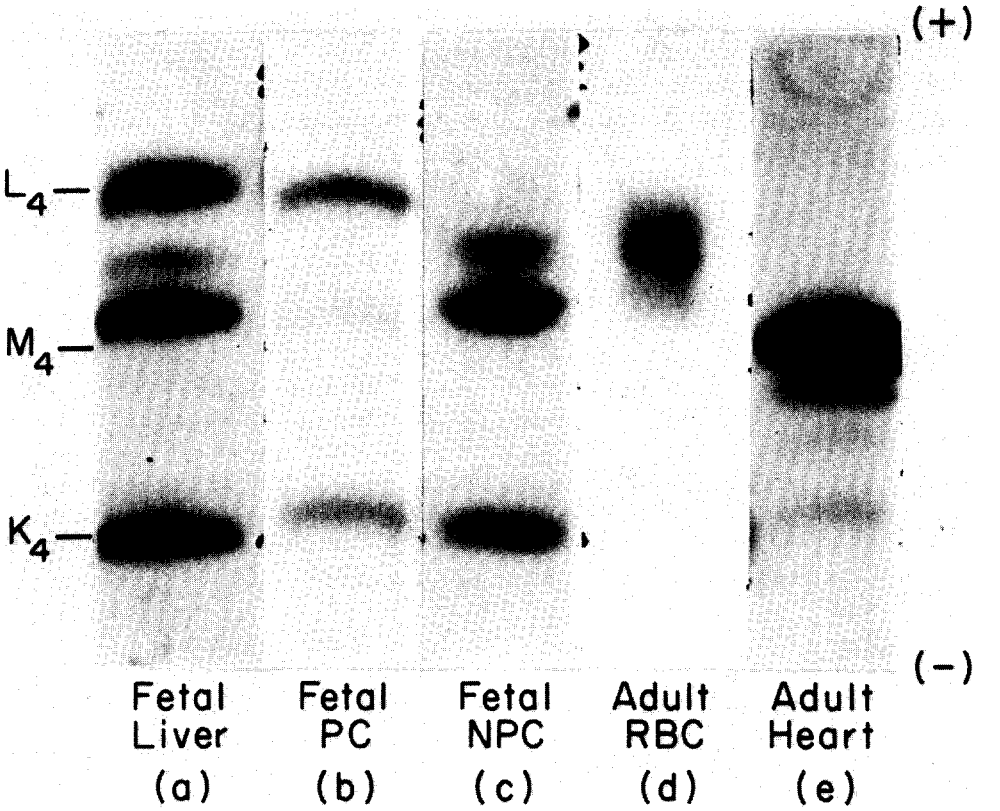

Fig. 3. Pyruvate kinase isozymes in parenchymal cells (PC) and nonparenchymal cells (NPC) of fetal rat liver. Electrophoresis time was 19 h. Origins are marked by black triangles near the K_4 position. (a) Fetal rat liver, 18 days gestation. (b) Parenchymal cells from a similar fetal rat liver. The light bands between the K_4 and L_4 position correspond in intensity to the level of NPC contamination. Note the absence of K-L hybrids. (c) Nonparenchymal (almost exclusively hemopoietic) cells from fetal rat liver. Note absence of the L_4 isozyme. (d) Comparison zymogram of adult erythrocyte pyruvate kinase. (e) Comparison zymogram of adult rat heart, establishing the electrophoretic position of the M_4 isozyme.

cells and examined the isozymic pattern of each. Nonparenchymal cells were isolated without contamination by parenchymal cells and were found to have K_4 and the major intermediate bands without detectable amounts of L_4 itself (Fig. 3c). Inspection of the parenchymal cell zymograms reveals K_4 and L_4 but relatively lesser amounts of the intermediate bands, the intensities of which correspond to the low level of nonparenchymal cell contamination in these preparations (Fig. 3b). We conclude that all detectable intermediate bands in fetal liver are associated entirely with nonparenchymal cells, which in the fetus are almost exclusively hemopoietic [30]. Thus, fetal liver parenchymal cells resemble their counterparts in adult regenerating liver in that synthesis of the K and L subunits of pyruvate kinase appears to be mutually exclusive for a given cell at any one time.

A question remained regarding the nature of the intermediate bands of fetal liver extracts that, as discussed above, apparently originate from the nonparenchymal cells. The suggestion has been made previously that fetal liver con-

tains substantial quantities of the type M isozyme [22]. To test this hypothesis, we performed electrophoresis with mixtures of fetal liver and adult skeletal muscle in order to compare the electrophoretic mobilities of the intermediate bands with that of type M pyruvate kinase. As seen in Fig. 4b, the electro-

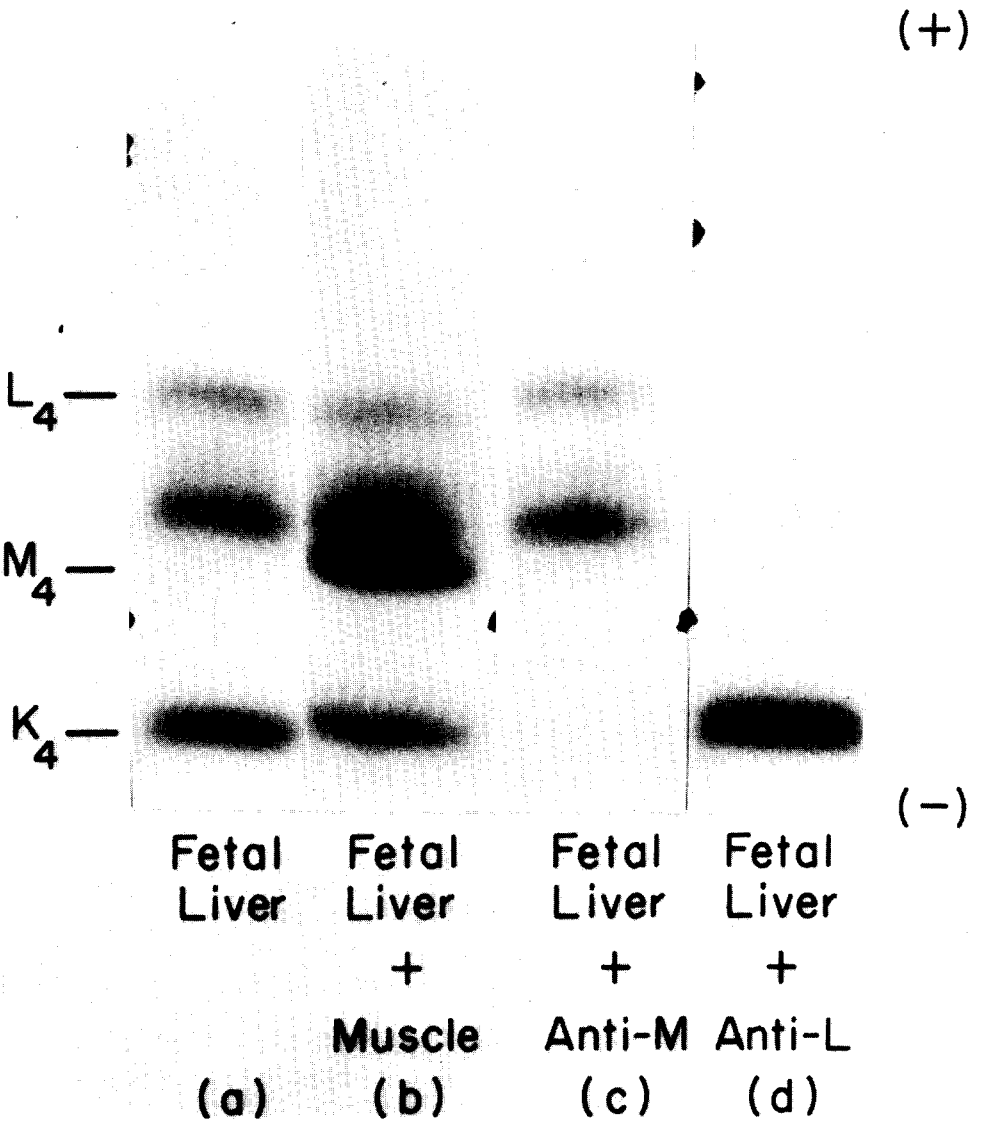

Fig. 4. Pyruvate kinase isozymes in fetal rat liver at 2.7 units per ml. Electrophoresis time was 21 h. Origins are marked by black triangles between the K_4 and M_4 positions. (a) Fetal liver control, 18 days gestation. (b) Fetal liver plus adult skeletal muscle. Note the electrophoretic separation between the muscle isozyme (M_4) and the hemopoietic bands of fetal liver. (c) Fetal liver extract containing 0.27 units of pyruvate kinase activity incubated with 10 μl of anti type M antiserum in a total volume of 100 μl. Note the loss of the K_4 band without loss of the type L or hemopoietic bands. The latter, therefore, do not contain type K or type M subunits. (d) Fetal liver extract containing 0.27 units of pyruvate kinase incubated with 4 μl of anti type L antiserum in a total volume of 100 μl. Note removal of the hemopoietic and type L pyruvate kinases without loss of K_4.

phoretic mobility of pyruvate kinase from muscle extracts (type M) is clearly distinguishable from any of the bands of fetal liver. Comparison of electrophoretic patterns reveal that at least the lighter of the two intermediate bands of fetal liver has the same electrophoretic mobility as the heavier band of adult erythrocytes. Although difficult to determine from the zymogram shown, the heavier intermediate band of fetal liver extracts appears to have the same electrophoretic mobility as a lighter, slower moving band of adult erythrocytes. Resolution of the two bands of adult erythrocytes becomes clearer if hemoglobin is removed from the sample prior to electrophoresis. However, we felt it better to include here a zymogram of the whole hemolysate, rather than the pattern of a sample that had been further manipulated through partial purification. The finding of two intermediate bands, with the cathodic band dominant in hemopoietic cells and the anodic band dominant in adult erythrocytes, agrees with the work of Nakashima [11], who reported a similar shift in young versus old erythrocytes from human blood. Furthermore, incubation of fetal liver extracts with antibodies against type M prior to electrophoresis removes only the K_4 band of fetal liver (Fig. 4c), an isozyme with which M_4 is known to cross-react [3]. Antibodies against type L, on the other hand, do not remove the K_4 band but do eliminate type L and the intermediate bands, as seen in Fig. 4d. In additional experiments, not shown, we find that antibodies produced against type L remove all the pyruvate kinase activity from hemolysates of adult erythrocytes, an observation that is consistent with reports that type L and erythrocyte pyruvate kinases are immunologically very similar if not identical to each other but immunologically distinct from either K_4 or M_4 [3,10,11,31].

If these intermediate bands in fetal liver are to be identified with erythrocyte pyruvate kinase, then the presence of blood in the fetal livers must, of course, contribute to their intensity. However, the specific activity of pyruvate kinase in fetal erythrocytes is only about half that of the intact fetal liver, so that the relatively small amount of blood in the tissue could not appreciably alter the liver zymograms.

Discussion

The three major pyruvate kinases can be distinguished from one another on the basis of their immunological, kinetic, and electrophoretic behavior, and they are most likely the products of three separate genes [32]. However, they are clearly closely related proteins, for isozyme hybrids seem to occur in vivo when two subunit types are synthesized simultaneously in the same cell [2,8,13] and can be produced in vitro by denaturing and renaturing together any combination of two of the three isozymes [2,8,13,26]. Natural hybrids between K and L are seen in kidney [3,8,13,19] and intestine [29], but K-L hybrids are not normally seen in adult liver [19]. The latter result is consistent with the observation that the two isozymes in adult liver are distributed to different cell types: the L isozyme to parenchymal cells and the K isozyme to non-parenchymal cells [14—17]. However, parenchymal cells from regenerating liver produce both the K and L isozymes [16,17], a situation that we show here to be true also of parenchymal cells from fetal liver. Since no K-L hybrids

are seen in parenchymal cells of either fetal or regenerating liver, it appears that the K and L isozymes are not produced simultaneously by the same cell.

The work presented here also demonstrates directly that the major intermediate electrophoretic bands of fetal liver, earlier thought to be possibly K-L hybrids [2,4,18], are in fact derived from hemopoietic cells. This proposal was first advanced by Walker and Potter [23] and later several others [18,20,22], based on correlations of time of disappearance of hemopoietic cells and the extra bands from the liver during development. Due to technical limitations, the hemopoietic isozyme was designated as type II, apparently equivalent in our nomenclature to type M. In addition, these earlier studies were carried out on homogenates of whole fetal liver only, so that it was not possible to separate the relative contribution of parenchymal vs. nonparenchymal cells. From the present work, we can say that hemopoietic cells contain little or no type L or type M, while parenchymal cells probably contain type K and type L only. Our electrophoretic and immunological results exclude the presence of significant amounts of M_4 in fetal rat liver and demonstrate instead isozymes comparable to those found in adult erythrocytes.

The electrophoretic patterns shown in the zymograms of this paper represent those actually occurring in vivo and are not artefactually produced in vitro. This was demonstrated directly in an earlier paper [24], where two or more tissues were homogenized, centrifuged, dialyzed, and electrophoresed together under conditions similar to those used in this paper without interconversion of electrophoretic bands or production of new ones.

As fetal liver matures, less K_4 and more L_4 is produced [23], and the opposite shift occurs in livers of adult rats during the early stages of regeneration following partial hepatectomy [16,17]. We have looked for, but failed to find, K-L hybrid isozymes in either fetal or regenerating liver.

When two homotetrameric isozymes known to be hybridizable are found in a tissue homogenate without hybrids, as is the case here, there is no way a priori of knowing whether they were synthesized in different cell types, in different compartments of the same cells, or by the same cells but at different times. However, since liver pyruvate kinase has been demonstrated to be a cytosolic enzyme [33], the presence of the K_4 and L_4 isozymes without hybrids in a purified preparation of liver parenchymal cells (Figs. 2 and 3) becomes strong, albeit indirect, evidence that the isozymes are synthesized at different times within these cells, just as the finding of hybrids would indicate an overlap in the expression of the K and L isozymes. Thus, the results reported here indicate that the switch from synthesis of one subunit type to the other probably occurs rapidly and completely in both fetal and regenerating liver parenchymal cells, avoiding hybrid isozyme formation, and that assembly of pyruvate kinase tetramers must be rapid and the assembled subunits stable so that K_4 and L_4 can coexist in the same cells, at least transiently.

This sharp separation of the synthesis of K and L type subunits of pyruvate kinase in parenchymal cells of fetal and regenerating liver has prompted us to consider the pyruvate kinase isozyme content of hepatoma cells. Shown in Fig. 2e is the pattern obtained with the rapidly growing, poorly differentiated Novikoff hepatoma. As shown, these cells produce mainly K_4 pyruvate kinase. A second, very light band can be seen near the heavy K_4 band, and its electro-

phoretic mobility suggests that it is K_3M; no evidence for L_4 or for hybrids containing type L subunits could be obtained for this type of hepatoma.

The studies reported here reveal shifts in the ratio of type K to L pyruvate kinase by 24 h after partial hepatectomy, but the K to L ratio does not reach a maximum until the third to sixth day, in agreement with previous studies [17]. By comparison, mitotic figures are already evident in regenerating liver by 24 h; they reach a maximum level quickly and then decline to very low levels by 72 h [34—36]. Thus, mitosis appears to precede the time of appearance of the maximum amount of type K pyruvate kinase; however, further analysis of these relationships must await measurements of rates of synthesis and of degradation of the pyruvate kinase isozymes.

The replacement of type K by type L pyruvate kinase in developing liver is analogous to the replacement of type K by type M in developing brain. However, studies on isolated neurons and glia from fetal rat brain clearly show a gradual replacement, with K and M subunit types being produced in the same cells at the same time, leading to substantial amounts of K-M hybrid [24]. In developing liver, on the other hand, the transition for a given parenchymal cell is apparently rapid and complete, implying a very different genetic mechanism for coupling isozyme expression to cellular differentiation.

Acknowledgements

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