Biochimica et Biophysica Acta, 526 (1978) 116—128

BBA 68514

HYBRID ISOZYMES OF RAT PYRUVATE KINASE

THEIR SUBUNIT STRUCTURE AND DEVELOPMENTAL CHANGES IN THE LIVER

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(Received January 17th, 1978)

Summary

Thin-layer polyacrylamide gel electrophoresis of various rat tissues revealed three major isozymes (types L, M_1 and M_2) and various intermediate forms of pyruvate kinase (ATP: pyruvate 2-*O*-phosphotransferase, EC 2.7.1.40).

In vitro dissociation and reassociation of purified enzymes showed that the three major isozymes had homotetrameric structures. $L \cdot M_2$ hybrids and $M_1 \cdot M_2$ hybrids closely resembled some naturally occurring intermediates; the subunit structure of intermediates isolated from the small intestine (form 3 or form 4) were estimated to be $(L)_2(M_2)_2$ and $(L)(M_2)_3$, respectively.

Pyruvate kinase activity after electrophoresis could be estimated quantitatively from densitometric measurements of the electrophoretic pattern. Type L activity in fetal liver was separated from type R activity derived from intrahepatic erythropoietic cells. It changes in three distinct steps during development: it increased during the late fetal period, remained steady during the neonatal period and increased again after weaning. Some of the intermediates found in extracts of early fetal liver were shown to cross-react with both anti-L and anti-M_1 serum, suggesting that they might be $L \cdot M_2$ or $R \cdot M_2$ hybrids. These hybrid enzymes were shown to appear only during early fetal and neonatal periods.

Introduction

Electrophoretically, there are three major isozymes and several intermediate forms in mammalian pyruvate kinase (ATP: pyruvate 2-*O*-phosphotransferase,

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EC 2.7.1.40) [1—7]. The three major forms, i.e. types L, M_1 and M_2 have been well characterized in many species by studies on purified or partially purified enzymes [8—11]. They apparently consist of four identical or nearly identical subunits [12—16].

Susor and Rutter [17] produced L · M_2 hybrids in vitro for the first time and proposed their possible existence in rat tissue during ontogenesis. Very recently, Strandholm et al. [18] confirmed that the subunit compositions of two intermediate forms from bovine kidney were $(M_1)(M_2)_3$ and $(L)_2(M_2)_2$.

Erythrocytes have another intermediate form (type R), which is thought to be a hybrid of the L and M_2 types [19,20] or a modified L type [21]. Recent investigations with the enzyme from human erythrocytes [22—24] support the latter hypothesis, but the true subunit structure of the enzyme is not known for certain.

These studies, particularly with bovine enzymes, have increased our understanding of the structure of many intermediate forms, but those of rat tissues are still unknown, and thus interpretations of zymograms are often ambiguous.

The present study was done to obtain more definite information at the molecular level for interpreting zymograms of rat pyruvate kinase isozymes. Thin-layer polyacrylamide gel electrophoresis with immunological techniques was found to be useful for identification of the isozymes. L · M_2 (or R · M_2) hybrid enzymes in fetal and newborn rat liver, the existence of which has long been questionable [25,26], were demonstrated. We also report quantitative estimations of the activities of multiple form in developing rat liver by photometric measurements of zymograms. Activities of types L and R were determined separately for the first time.

Experimental Procedures

Materials. Dithiothreitol was obtained from Calbiochem and guanidine · HCl, extra pure, from Schwarz-Mann. Other chemicals were obtained as described in the previous paper [27]. Anti-M_1 and anti-L sera were prepared using type M_1 purified from rat muscle and type L from rat liver as antigens, as described previously [8]. 1 ml of samples of anti-M_1 serum and anti-L serum neutralized 30 units of type M_1 and 15 units of type L, respectively.

Animals. Sprague-Dawley albino rats were housed in an air-conditioned room (23°C) with alternating periods of light (9:00 a.m. to 9:00 p.m.) and dark (9:00 p.m. to 9:00 a.m.). The animals were given laboratory chow from Clea Inc., Japan and water ad libitum. Rats were weaned on the 22nd day of age. Both sexes were used until the 5th postnatal day of age, and thereafter only male rats were used. Fetal age was determined from the fetal body weight and crown-rump length [28]. The ages determined by these two criteria agreed very closely with each other. In the present paper, ages of fetal animals are measured backward from the day of birth, the 22nd day of gestation, with the prefix " — ", e.g. day —6. Ages of postnatal animals are expressed as days or weeks after birth, e.g. day 6. Rats were always killed between 12:00 noon and 5:00 p.m.

Enzyme assay and electrophoresis. The method of enzyme assay [29] and thin-layer polyacrylamide gel electrophoresis was described in the previous paper [27].

Purification of multiple forms of pyruvate kinase from rat small intestine. There are five electrophoretic forms of pyruvate kinase in rat intestine, named form 5 (type M_2) through form 1 in order of increasing mobility to the anode. Intestine was washed out with 0.9% NaCl and then homogenized in a Waring Blendor with two volumes of homogenization buffer containing 0.2 mM Fru-1,6-P_2 and 10 mM β-mercaptoethanol. The homogenization buffer contained 20 mM Tris · HCl buffer (pH 7.5), 100 mM KCl, 5 mM $MgSO_4$ and 1 mM EDTA. The 30—50% $(NH_4)_2SO_4$ fraction of the crude extract was collected. To obtain good separation of intermediate forms by phosphocellulose chromatography, the supernatant obtained with 50% $(NH_4)_2SO_4$ saturation was discarded, although it contained much form 5. The precipitated material was dialyzed and then chromatographed on a phosphocellulose column (Fig. 1). Further purifications of these enzymes were achieved by affinity elution chromatography on a phosphocellulose column under similar conditions to those used for types R, L, and M_2 [27]. The specific activities of purified form 3, form 4 and form 5 were 116, 206 and 236 units/mg protein and these fractions were obtained in 9, 11 and 27% yield, respectively, from the eluate of the first phosphocellulose column. On SDS polyacrylamide gel electrophoresis purified form 3, form 4 and form 5 gave single bands (data not shown).

Hybridization procedure. The purified enzyme [27] was dissociated in 4 M

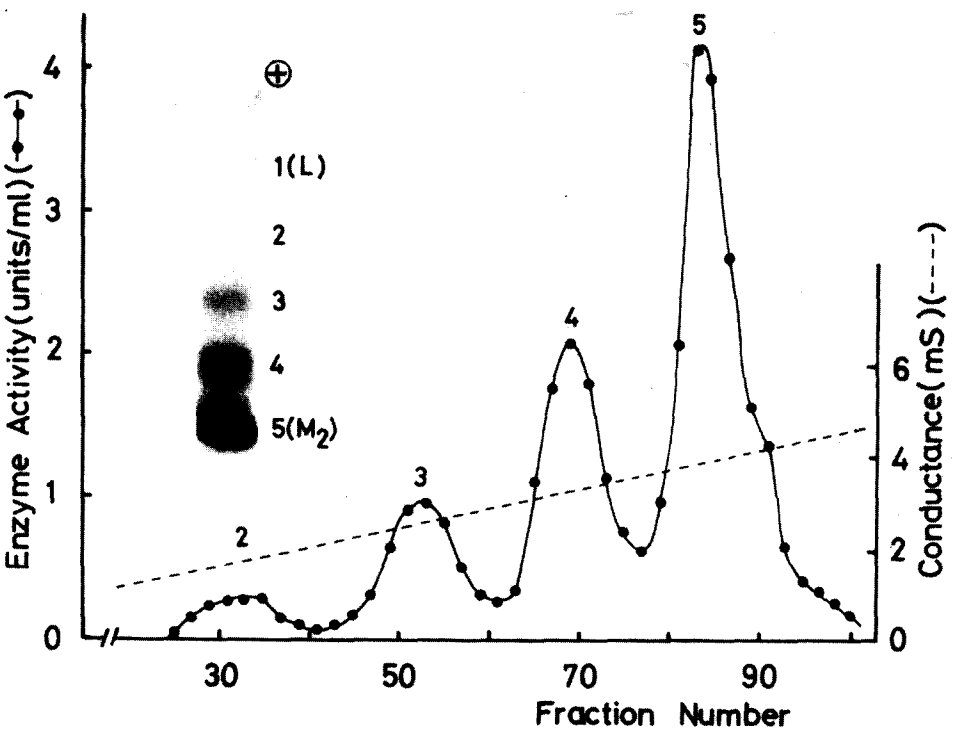

Fig. 1. Phosphocellulose chromatography of rat intestine pyruvate kinase. ●———●, pyruvate kinase activity; — — — —, electric conductance. The 30—50% $(NH_4)_2SO_4$ fraction of a crude extract was chromatographed on a phosphocellulose column. The enzyme was eluted with a linear concentration gradient of 5—140 mM phosphate buffer, pH 6, containing 2 mM $MgSO_4$, 10 mM β-mercaptoethanol and 0.2 mM Fru-1,6-P_2. Fractions in the individual peaks were combined, except for those of form 2 which showed very low total activity. Insert: Electrophoretic pattern of original crude extract.

guanidine · HCl and reassociated by dilution according in part to a procedure used in earlier studies on pyruvate kinase from rabbit muscle [30]. A mixture of type M_1 and type M_2 isozymes with equal activities, as a suspension in $(NH_4)_2SO_4$ solution, was centrifuged and the precipitate was dissolved in 50 mM Tris · HCl buffer (pH 7.5) containing 100 mM KCl, 5 mM $MgSO_4$ and 10 mM dithiothreitol to give a protein concentration of 8 mg/ml. The solution was mixed with an equal volume of 8 M guanidine · HCl at 0°C. The mixture was incubated at 0°C for 15 min and then diluted with renaturation buffer by gentle swirling to give a final protein concentration of about 0.04 mg/ml and incubation was continued for 12 h at 0°C. The renaturation buffer used was 50 mM Tris · HCl buffer (pH 7.5) containing 5 mM $MgSO_4$, 100 mM KCl, 5 mM phospho*enol*pyruvate and 10 mM dithiothreitol. Denaturation and reassociation of isozymes singly and as mixtures of types L and M_2, types L and M_1, and types R and M_2 were carried out in the same way. Purified form 3 and form 4 were each subjected to dissociation and reassociation by the same method.

Quantitative measurements of zymograms. The absorbancy of each spot due to pyruvate kinase activity was measured by scanning at 600 nm using a reflex type of densitometer (TLC-scanner Model CS-900, Shimazu). The integrated absorbancy was found to be linearly proportional to the activity (the correlation is reported in Results and Discussion), and so the relative ratio of integrated absorbancy of each spot represented the relative activity. The activities of each form were calculated by multiplication of the original enzyme concentration in each tissue (units/g tissue) by the relative ratio.

Results and Discussion

Electrophoretic patterns of pyruvate kinase in rat tissues

Our improved electrophoretic method with high sensitivity and high resolving capacity enabled us to make more detailed observations on the multiple forms of pyruvate kinase. Fig. 2 shows the electrophoretic patterns of pyruvate kinase from fetal tissues of rats. Eight different samples were subjected to electrophoresis on a gel plate and activities were detected on photographic paper. All the bands were due to pyruvate kinase activity, because none of them were detected by staining a mixture without phospho*enol*pyruvate.

The figure shows three major isozymes (types L, M_1, M_2) and various intermediate forms. The three equally spaced intermediate forms between type M_1 and type M_2 (as seen in heart) must be $M_1 \cdot M_2$ hybrid enzymes produced from tetrameric parental isozymes. The three other equally spaced major intermediate forms between type L and type M_2 (as seen in small intestine) must be $L \cdot M_2$ hybrid enzymes.

Blood cells gave several unique bands as well as a faint band of type M_2. Type M_2 is derived from leucocytes and/or platelets [8] and the three bands seen further to the anode are derived from erythrocytes. Although human erythrocytes [21] give only two bands, rat erythrocytes give at least three. The structures of the components in these bands are unknown, so we named the bands the R complex, or R_1, R_2 and R_3, respectively, in order from the cathodic side.

Type L is present in a limited number of tissues: i.e. liver, kidney and

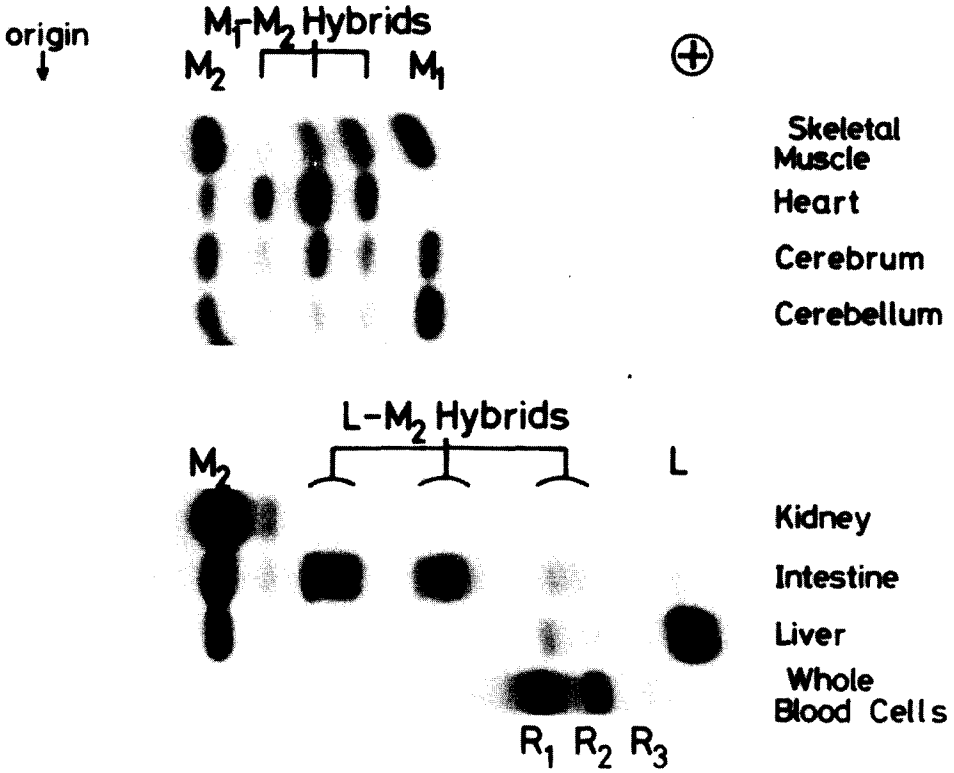

Fig. 2. Thin-layer polyacrylamide gel electrophoretic patterns of pyruvate kinase isozymes from fetal (day −0.5) rat tissues. Tentative designations are indicated. Fetal rats were removed from the uterus and placed in a dish of saline containing 10 mM EDTA (pH 7.4) for a few min at room temperature to remove the blood from their umbilical cord. Blood cells were disrupted by freezing and thawing, and the tissues were homogenized in 2—4 volumes of cold homogenization buffer containing 0.2 mM Fru-1,6-P_2 and 10 mM β-mercaptoethanol and centrifuged. For electrophoresis, tissue extracts were diluted with stabilizing buffer (homogenization buffer containing 0.2 mM Fru-1,6-P_2, 10 mM β-mercaptoethanol and 1% bovine serum albumin) to the following final concentrations: skeletal muscle, heart, cerebrum, cerebellum, 2 units/ml; liver, kidney, 4 units/ml; intestine, 6 units/ml; and whole blood cells, 3 units/ml.

intestine. Three intermediate forms between types L and M_2 were found in intestine and kidney, as described previously [5—7]. We examined the chorionic membrane of the fetus for the first time and found that it had the same isozyme pattern as intestine, but the placenta and the amnion (unpublished data) had type M_2 and $M_1 \cdot M_2$ hybrids.

Fetal and newborn livers gave several intermediate forms. The two major forms may be type R due to intra-hepatic erythropoietic tissues, as described by several workers [25,31]. $M_1 \cdot M_2$ hybrids were also present. In addition, small amounts of other intermediate forms, which seemed to be $L \cdot M_2$ or $R \cdot M_2$ hybrids, were detected though their existence in rat liver [17] has long been questionable [25,26].

Each of the intermediate forms which were considered to be $L \cdot M_2$ hybrids characteristically gave a broad band, but when higher resolution was attained, these broad bands were resolved into two or more sub-bands. The significance of this microheterogeneity is unknown.

The molecular structures of intermediate forms have been studied recently

by several groups [17,18] but the structures of the intermediates in rat tissues have not yet been determined, The following chapter describes some of the properties of these intermediate forms observed during their purification and hybridization and in immunological studies.

Hybridization studies

In denaturation and renaturation experiments, the recovery of enzyme activity during renaturation was affected by various factors, such as the pH of the solvents, the enzyme concentration and the temperature [30]. Using 50 mM Tris · HCl buffer, pH 7.5, containing 5 mM $MgSO_4$ and 100 mM KCl, optimal reversal were obtained with type M_2 at 16°C and with type L at 0°C. Primers, such as valine which is essential for the yeast enzyme [32], did not seem to be required in the renaturation process, but phospho*enol*pyruvate in Tris · HCl buffer appeared to increase the recovery by about 3—7%. The recovery was better, with the more purified enzyme preparations and so in the following experiments highly purified enzymes were used.

(a) Type L, type M_1 and type M_2. On denaturation and renaturation of any of the three isozymes individually a single electrophoretic band was obtained with the same mobility as the native isozyme (Fig. 3a). At least half the activity of type M_1 or M_2, but only 24% of that of type L was recovered on renaturation for 10—12 h at 0°C.

When the three pairs of isozymes (i.e. types M_1 and M_2, types L and M_1, and types L and M_2) were denaturated and renatured, they produced three intermediate bands in addition to the parental bands. Recoveries of parental type L and type M_2 were frequently poor, although that of parental type M_1 was always good.

These results indicate that types L, M_1 and M_2 consist of four electrophoretically identical subunits and that their hybrid enzymes are produced by random hybridization of the dissociated subunits.

(b) Intestinal intermediate (form 3 and form 4). Purified form 3 and form 4 each gave a single electrophoretic band with nearly the same mobility as the original crude enzyme (Fig. 3b).

Denaturation and renaturation of purified preparations of either form 3 or form 4 alone produced a five-membered set which closely resembled the five-membered set found in intestine and also the $L \cdot M_2$ hybrid set produced in vitro. The products with form 3 were slightly skewed towards type M_2, and the products with form 4 were highly skewed towards type M_2, probably because of an imbalance in the subunit composition, or because of the low recovery of type L. The results confirm that form 3 and form 4 are tetrameric hybrids of type L and type M_2, i.e. $(L)_2(M_2)_2$ and $(L)(M_2)_3$, respectively.

(c) Type R. As described in previous paper [27], purified type R showed a single band on electrophoresis and migrated faster than crude type R.

The product obtained by denaturation and renaturation of type R gave a single band with the same electrophoretic mobility as the native purified enzyme. Only 9% of the original activity was recovered on renaturation for 17 h. There might be a possibility that type R cannot be dissociated under these conditions. To examine this type R and type M_2 were denatured and renatured together and then the products were subjected to electrophoresis.

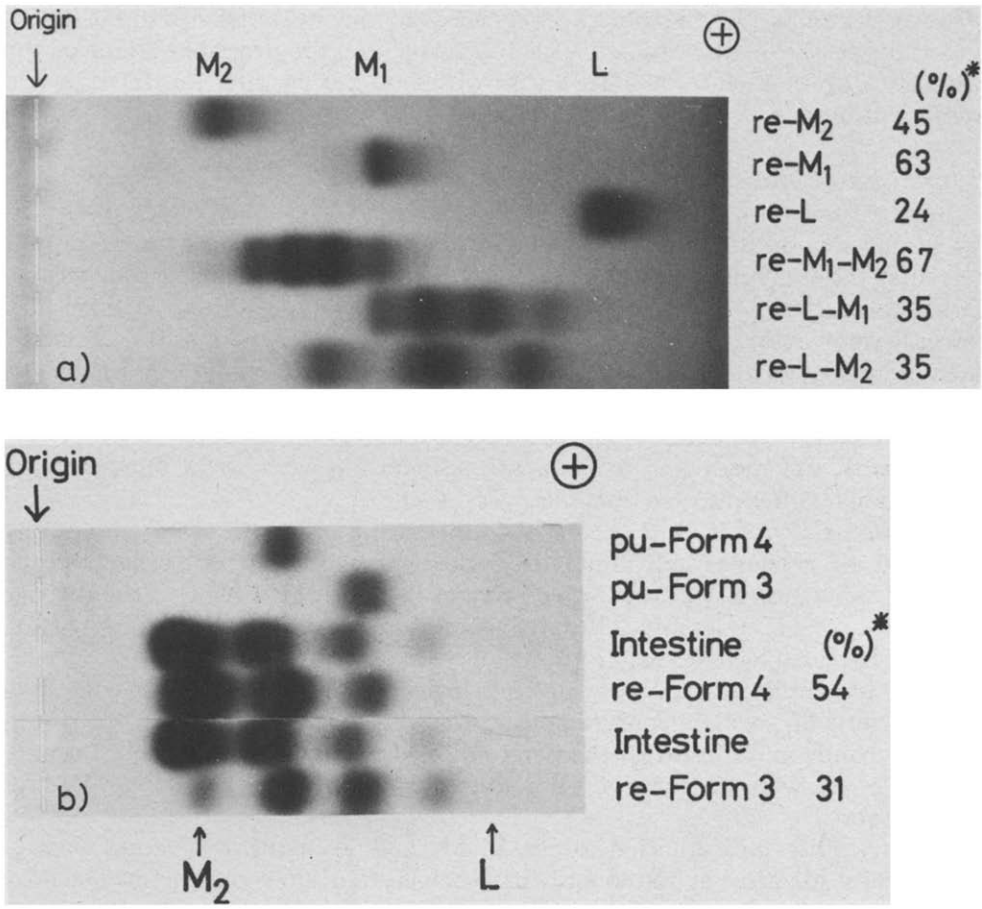

Fig. 3. Thin-layer polyacrylamide gel electrophoresis of purified rat pyruvate kinase isozymes and products of their dissociation and reassociation reactions. Types M_1, M_2 and L individually or in pairs (a), intestinal intermediate forms (form 3 and form 4) (b) were denatured in 4 M guanidine · HCl and renatured by dilution. Pu-, purified native enzyme; re-, denatured and renatured enzyme. *Recoveries of activities after denaturation are shown as percentages of the control activity after similar treatment but without 4 M guanidine · HCl.

Three equally spaced hybrid bands were found between those of the parental forms. An R · M_2 hybrid set was quite similar to an L · M_2 hybrid set (data not shown).

These results suggest that purified type R consists of four nearly identical subunits which are very similar to type L.

Immunological studies

As described previously [8], types L and R were neutralized with anti-L serum, but not with anti-M_1 serum. On the contrary, types M_1 and M_2 were neutralized with anti-M_1 serum, but not with anti-L serum.

Fig. 4 shows the electrophoretic patterns of crude extracts of rat tissues after incubation with anti-L serum or anti-M_1 serum. Type M_1, type M_2 and M_1 · M_2 hybrids from heart were neutralized with anti-M_1 serum but not with anti-L

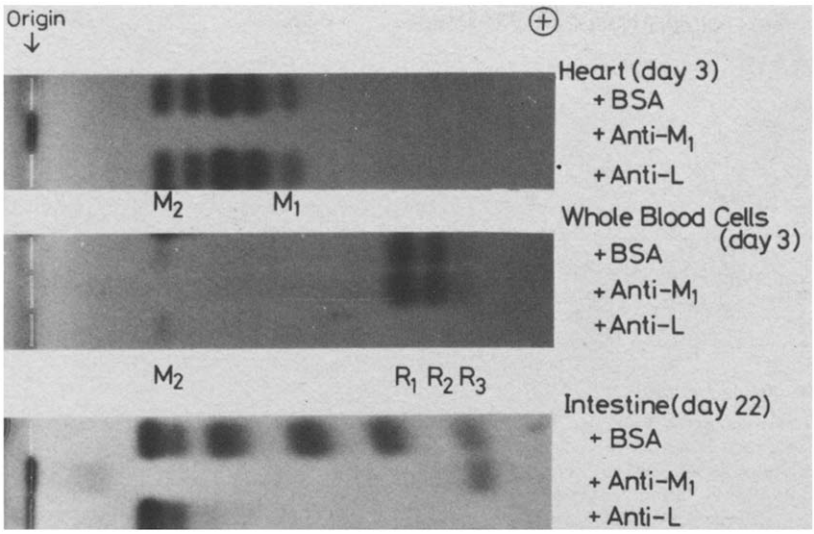

Fig. 4. Electrophoretic patterns of the multiple forms of pyruvate kinase in various rat tissues after incubation with anti-M_1 serum and anti-L serum. The supernatants from homogenates and hemolyzates with cold homogenization buffer containing 0.2 mM Fru-1,6-P_2 and 10 mM β-mercaptoethanol were diluted with stabilizing buffer to the following units/ml: whole blood cells, 8; intestine, 15; heart, 8. Before electrophoresis portions of the preparation were incubated for 10 min at room temperature with an equal volume of anti-M_1 serum, anti-L serum or 1% bovine serum albumin solution in homogenization medium as a control.

serum. All three bands of type R in erythrocytes were neutralized with anti-L serum, but not with anti-M_1 serum. The three equally spaced L · M_2 hybrids between type L and type M_2 from intestine was neutralized with both kinds of anti-serum. Type M_2 and several M_1 · M_2 hybrids were not neutralized with anti-L serum.

The method simplified the distinction of L · M_2 hybrid set from the M_1 · M_2 hybrid set, which was sometimes difficult by electrophoresis alone.

Quantitative measurements from zymograms

The method used for quantitative determination of multiple forms of pyruvate kinase in a tissue extract is described above. This method is based on the fact that the relations between the amounts of activity of each multiple form and the integrated absorbancy were linear and had the same slope. The relation was demonstrated with the three main isozymes, i.e. type L, M_1 and M_2. Fig. 5 shows that the relation between the amount of activity applied and the integrated absorbancy of types L and M_1 was linear with up to 3 units/ml of activity on incubation for 10—29 min and that the two lines had the same slope. Similar results were obtained using mixtures of type M_2 (from a crude extract of AH-130 Yoshida ascites hepatoma cells) and crude type L. The good reproducibility of these curves was confirmed in at least nine separate measurements. The results indicate that quantitative estimation of the relative activity ratios of types L, M_1 and M_2 is possible and accurate on incubation for up to 30 min, when the concentrations of applied activities are each less than 3 units/ml. It seems probable that under these conditions the relative ratios of other

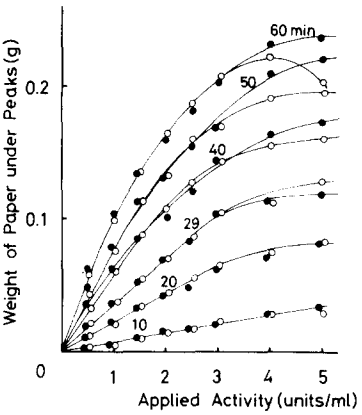

Fig. 5. Correlations between activity applied and integrated absorbances after various incubations times. Mixtures of equal amounts of activities of type L (from a crude extract of liver) and type M_1 (from a crude extract of skeletal muscle) were diluted stepwise with stabilizing buffer. The diluted samples were subjected to electrophoresis on a polyacrylamide gel plate. After electrophoresis, the activities of types L and M_1 on the plates were recorded on photographic enlarging paper. Integrated absorbancy is expressed as the weight of paper under the peaks recorded on chart paper by densitometry of the electrophoretic patterns. ●———●, type M_1; ○———○, type L.

multiple forms, such as L · M_2 hybrids and type R can also be estimated. Since extracts of liver always contain multiple forms, measurements could be made on them even at higher concentrations than 3 units/ml.

To estimate the low activity of L · M_2 hybrid in the liver of fetal and newborn rats, a wider range of activity must be applied to the gel. Accordingly an excess, but known amount, of activity was applied to one slot and a sample diluted to 2 units/ml was applied to the adjacent slot as an internal standard. After electrophoresis, the activity of L · M_2 hybrid was calculated by comparing its integrated absorbancy to that of the internal standard and the relative activity ratio were estimated.

Changes in isozymes of pyruvate kinase during development in the liver

Fig. 6 shows the changes in concentrations of multiple forms of pyruvate kinase. Type L activity showed a stepwise change during development. For convenience the changes were divided into three stages; the first stage was in the late fetal period, the second in the first 2 weeks after birth, and the third in the weaning period. In the first stage, type L appeared on day −7 to −6 and its activity rose to the prenatal level of about 10 units/g of tissue during the last 3 fetal days. In the second stage, type L activity increased slightly at the time of birth and remained steady until weaning. In the third stage, it increased steadily and reached the adult level between the 4th and 5th weeks. Type M_2 was the predominant form in the liver of day −7 fetuses. Its activity decreased during the late fetal period, while type L activity was increasing. At the time of birth and during the early second stage, it rose about 2-fold and then gradually decreased to the adult level. Type R was predominant during the early fetal period and its activity was higher than that of type L. During the late fetal period its activity decreased rapidly and in the first week after birth it disappeared almost completely.

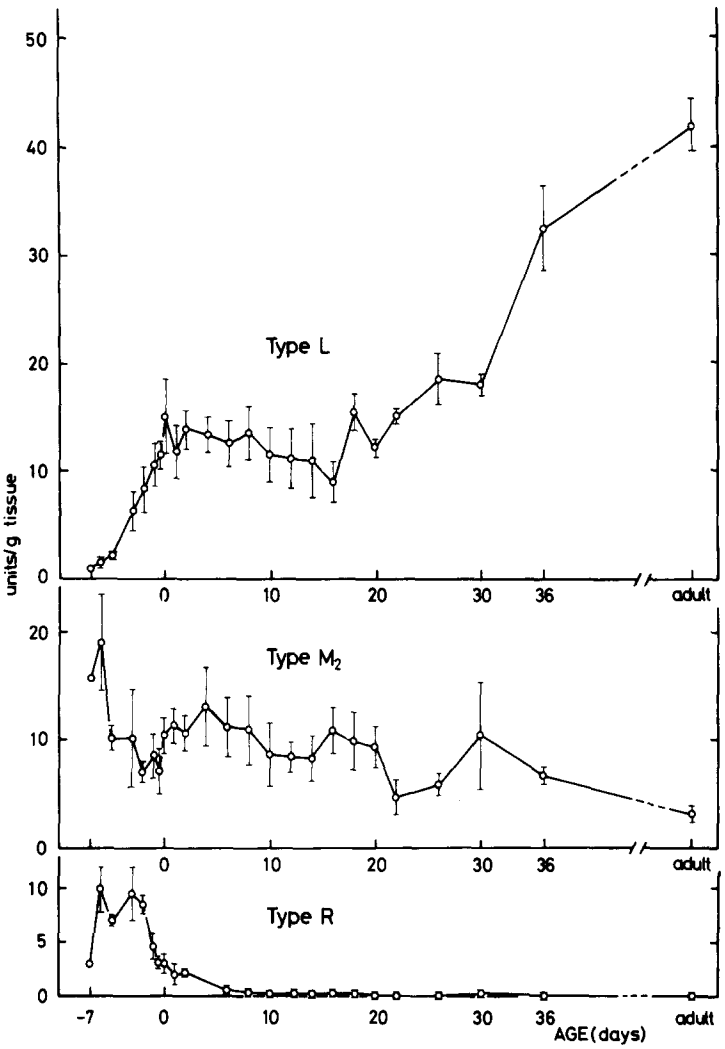

Fig. 6. Developmental changes in the activities of each of the multiple forms of rat liver. Rats after birth were decapitated and exsanguinated. The liver from fetal or postnatal rat was kept on ice after removal from the body and homogenized in four volumes of cold homogenization buffer containing 0.2 mM Fru-1,6-P_2 and 10 mM β-mercaptoethanol. The homogenates were centrifuged at $1 \cdot 10^5 \times g$ for 30 min and the supernatants were used for assays of enzymatic activities. All these procedures were accomplished within 3 h. For experiments 6—10 fetal rats or one postnatal rat was used. The results are means ± S.D. of 3—10 measurements. Where standard deviations are not shown, the results are means of two measurements.

New intermediate forms were found in fetal and newborn liver by applying an excess amount of activity to the gel plate.

Fig. 7 shows the typical new intermediate forms (indicated as a, b, c and d) in the liver of the early fetus (day —7). These bands apparently consist of unknown forms. However, if they were due to L · M_2 hybrids, they would be neutralized with both kinds of anti-serum. Anti-M_1 serum neutralized these activities as well as those of type M_2. Anti-L serum also neutralized these activities as well as those of type L and R. These results strongly suggests that bands a, b, c, and d are those of hybrids between type L and type M_2, although they

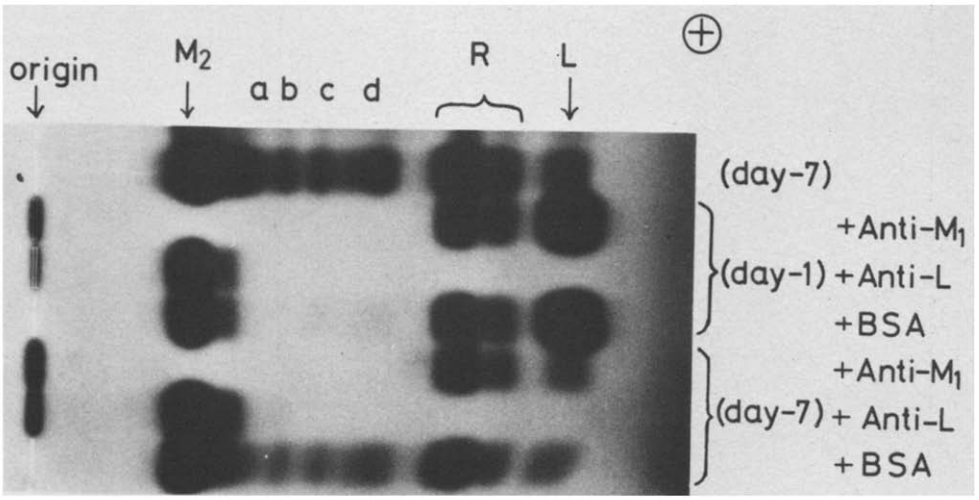

Fig. 7. Electrophoretic patterns of the multiple forms of pyruvate kinase in fetal rat liver. Livers from several littermates were combined and homogenized. Multiple intermediate forms, indicated by a, b, c, and d, were detected as well as types L, R and M_2 by applying an excess amount of activity to the gel. The procedure with antisera was the same as that described in the legend of Fig. 4. Multiple forms, indicated by a, b, c, and d, were neutralized with both kinds of anti-serum. BSA, bovine serum albumin.

may be those of hybrids between type R and type M_2. The changes in the concentrations of these hybrids during development are shown in Fig. 8. It shows that the activities of the hybrids are much less than those of types L, M_2 and R, but that they change significantly during development. The activity was remarkably high during the early fetal period; it decreased rapidly as term approached; it rose abruptly at the time of birth, and then rapidly disappeared after a few days.

Susor and Rutter [17] first reported the existence of $L \cdot M_2$ hybrids in newborn rat liver; they separated an equally spaced five-membered set by electrophoresis and showed that it was consistent with $L \cdot M_2$ hybrids produced in vitro. Later workers, however, failed to confirm their existence [25,26,31, 33]. The present study unequivocally demonstrates the existence of hybrids, although these hybrids are quite unlike those reported by Susor and Rutter [17]; the hybrids did not form equally spaced bands, but much more complex bands. They were clearly seen during the early fetal period, but became much weaker during the late fetal and neonatal periods. Thus hybrids found in the present study may be different from those reported by Susor and Rutter [17].

The changes in pyruvate kinase activity in rat liver during perinatal period have been explained in relation to the mechanisms of control of glycolysis and gluconeogenesis during this period [34—36]. During the suckling period, the activities of the rate-limiting gluconeogenic enzymes are high, while those of enzymes metabolizing glucose, including pyruvate kinase, are low. But the changes in activities of the three isozymes type L, M_2 and R complex are actually more complicated. The time course of changes in types L and M_2 in developing liver were first investigated by Middleton and Walker [34], later by Osterman et al. [31] and Faulkner and Jones [33]. But these workers measured the activity of type L plus the R complex, because these activities could not be separated by kinetical, chromatographic or immunological techniques. In the

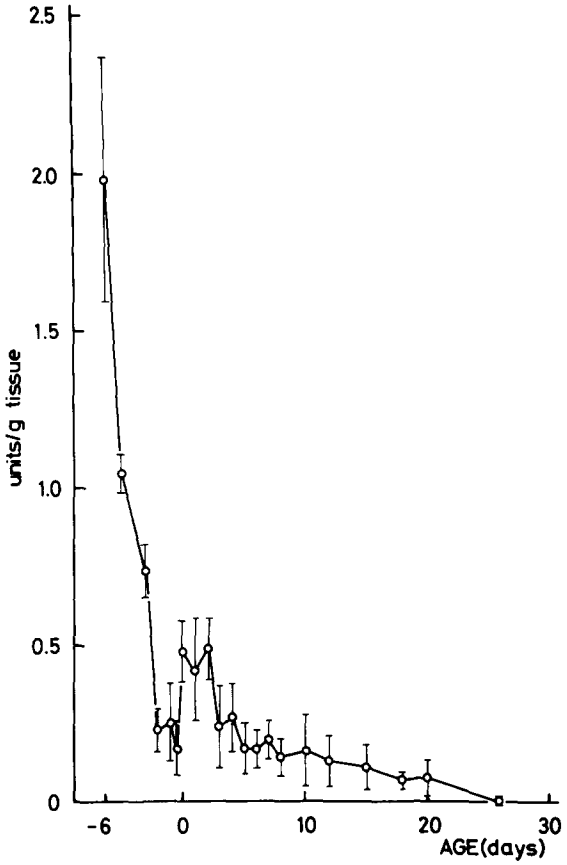

Fig. 8. Changes in activity of "L · M_2 of R · M_2 hybrid" in rat liver during development. The activity of this form was estimated quantitatively as described in the Experimental Procedures. The values represent the activities of hybrid bands c and d. Hybrid bands a and b were disregarded, because they had less activities than the formers and were a little contaminated with M_1 · M_2 hybrids. Note the extended scale of the ordinate.

present study type L activity and the activity of the R complex were determined separately for the first time. The R complex found in fetal liver [31] had the same unique electrophoretic pattern as that of erythrocytes and its appearance during development coincided with hepatic erythropoiesis [37, 38]. Therefore, the R complex seem to be due to erythropoietic tissues in fetal and neonatal liver.

Type L in the liver might be due to parenchymal cells and type M_2 to non-parenchymal cells [26,39–41]. Type M_2 in regenerating [26] and fetal liver [25], however, was recently reported to be partly due to parenchymal cells, suggesting that immature parenchymal cells have type M_2. From the various points of view, type M_2 in parenchymal cells would be a good marker for the immaturity of the cells and type L for the maturity. If the intermediate forms in the liver represent L · M_2 hybrids but not R · M_2 hybrids, they would be a marker for actively differentiating parenchymal cells. To understand the perinatal changes of type L, the kinetics of these cell populations must be investigated.

Acknowledgments

The authors wish to thank Dr. Zen-ichi Ogita of the Department of Biochemical Pathology, Research Institute for Oriental Medicines, Toyama University, and Dr. Yasuhiko Sakoyama of the Department of Genetics, Osaka University School of Medicine, for advice on thin layer polyacrylamide gel electrophoresis. This study was supported by a Grant-in-Aid for Cancer Research from Ministry of Education, Science and Culture, Japan.

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