3-Phosphoglycerate Kinase from Rat Tissues. Further Characterization and Developmental Studies†

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ABSTRACT: Measurement of 3-phosphoglycerate kinase in soluble extracts of three rat tissues during postnatal development revealed that in cardiac muscle and liver the patterns were similar in that the activity decreased in the first two weeks of postnatal life followed by an increase after weaning. Rat skeletal muscle 3-phosphoglycerate kinase increased steadily from low levels at birth to a maximum value around 80 days after birth and then slowly declined to steady-state values. A peak of activity around 80 days was also observed in cardiac muscle and liver. Steady-state values were 300, 145, and 115 units per g of tissue for skeletal muscle, cardiac muscle, and liver, respectively. Using immunological techniques, it was demonstrated that the changes in activity during development were due to changes in enzyme protein. In the development studies, no differences were noted in the patterns of males and females. Throughout the life of the rat 3-phosphoglycerate kinase has been shown to be the same single molecular form in skeletal muscle, cardiac muscle, and liver. Evidence to support this conclusion was provided by electrophoretic, kinetic, and immunological studies. No significant changes in 3-phosphoglycerate kinase activities of adult rat skeletal muscle, cardiac muscle, or liver were observed for up to 3 days of fasting.

Developmental studies on glycolytic enzymes have usually dealt with the subject from the standpoint of whether the level of glycolytic enzymes is related to the glycolytic flux (Burch *et al.*, 1963; Schaub *et al.*, 1972; Middleton and Walker, 1972; Osterman *et al.*, 1973). Few (Ramponi *et al.*, 1968; Goetsch, 1966) developmental studies on the glycolytic enzyme 3-phosphoglycerate kinase (ATP:3-phospho-D-glycerate 1-phosphotransferase, EC 2.7.2.3) have appeared despite the fact that the other three kinases of the glycolytic pathway, glucokinase, phosphofructokinase, and pyruvate kinase, are generally conceded to be sites of metabolic control of glycolysis and gluconeogenesis (Scrutton and Utter, 1968). Phosphoglycerate kinase has also been identified as important in regulating the cellular ratio of $NAD^+/NADH$ through its effect on $\{ATP\}:\{ADP\}\{P_i\}$ (Veech *et al.*, 1970; Lagunas *et al.*, 1970; Greenbaum *et al.*, 1971). In addition, the enzyme catalyzes one of three glycolytic reactions that may compete directly for ADP and P_i with the mitochondrial respiratory systems (Weinhouse, 1972).

Aside from the possible role of phosphoglycerate kinase in regulating metabolic pathways, the enzyme is of interest because, along with phosphoglucomutase (Harshman and Six, 1969), it is one of only two glycolytic enzymes that does not occur as a subunit enzyme and thus may serve as a model for turnover studies of nonsubunit enzymes.

We have previously reported on the purification and characterization of rat skeletal muscle phosphoglycerate kinase (Hass *et al.*, 1974) and in the present communication present evidence that the same molecular form of the enzyme occurs in rat skeletal muscle, cardiac muscle, and liver. In addition, we report how the levels of phosphoglycerate kinase change in these three rat tissues during postnatal development.

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Experimental Section

Animals. Sprague-Dawley rats were housed in air-conditioned rooms (24 ± 1°) on a schedule of alternating periods of light (7:00 a.m. to 7:00 p.m.) and dark (7:00 p.m. to 7:00 a.m.). Except for the fasting experiments the animals had Purina Laboratory Chow and water *ad libitum*. In the developmental studies the number of animals in a litter was reduced at birth to 10 and the rats were weaned on the 22nd day of age. If not otherwise stated, rats were killed between 7:30 a.m. and 8:00 a.m. by decapitation.

Tissue Preparation. Tissues were removed and kept on ice. They were trimmed of fat, minced, and then weighed. Livers, hearts (ventricles), and skeletal muscle were homogenized in 9 (adults) or 19 (newborns) volumes of cold 0.02 M Tris-HCl–6 mM EDTA (pH 7.4) in a Potter-Elvehjem homogenizer. In older animals the quadriceps femoris was used for the skeletal muscle samples, whereas in newborns all muscle from both hind legs was used. The homogenates were centrifuged at 27,000*g* for 20 min and the supernatants were used for enzyme measurements.

Enzyme Assay. Phosphoglycerate kinase activity was assayed by following the oxidation of NADH at 340 nm using a Gilford Model 240 recording spectrophotometer. The assay mixture contained (final concentrations): 6 mM EDTA, 3.6 units of glyceraldehyde-3-phosphate dehydrogenase (Boehringer), 0.071 mM NADH, 10 mM $MgSO_4$, 3.6 mM Na_2ATP, 11.2 mM 3-P-glycerate (dicyclohexylammonium salt), and 20 mM Tris-HCl (pH 7.4), in a final volume of 2 ml. The ATP was neutralized with 1 N NaOH before it was added to the assay mixture. Temperature was maintained at 25 ± 1°. All assays were performed in duplicate.

Electrophoresis was performed on cellulose polyacetate strips (Sepraphore III, 2.5 × 15 cm, Gelman Instrument Co., Ann Arbor, Mich.) and the phosphoglycerate kinase was detected by contact printing using the method described by Susor and Rutter (1971). Tissues were homogenized in 4 volumes of 0.02 M Tris-HCl–6 mM EDTA (pH 7.4). Electrophoresis was carried out at 0–4° for 2 hr at 17 V/cm. The staining mixture contained (final concentration): 6 mM EDTA,

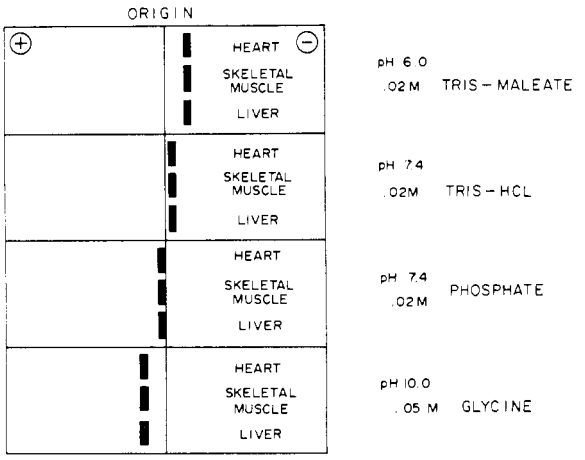

FIGURE 1: Electrophoresis of phosphoglycerate kinase from rat tissues in different buffers. Electrophoresis was performed as described in the Experimental Section.

1.8 mM Na_2ATP, 1.9 mM 3-P-glycerate (tricyclohexylammonium salt), 0.7 mM NADH, 1 mM $MgSO_4$, 6 units of glyceraldehyde-3-phosphate dehydrogenase, and 20 mM Tris-HCl (pH 7.4) in a final volume of 10 ml/strip.

Immunological Procedures. Phosphoglycerate kinase from rat skeletal muscle was purified as described by Hass *et al.* (1974). The final preparation showed a single band on disc gel electrophoresis when stained by Amido Black. The final specific activity of the phosphoglycerate kinase varied slightly with each purification but the final specific activity was always around 550 units/mg of protein. Antibody was prepared by injecting New Zealand white rabbits with the purified enzyme according to the following schedule. Protein (2 mg) was emulsified with an equal volume of complete Freund's adjuvant (Difco Laboratories, Detroit, Mich.) and injected subcutaneously in the back and hind legs. The procedure was repeated 1 week later. Two weeks after the first injection 3 mg of protein without adjuvant was injected into the back. Seven to ten days later the rabbits were bled by cardiac puncture. The antiserum was collected and the immunoglobin fraction was concentrated by the careful addition of an equal volume of cold, saturated ammonium sulfate (pH 7.0). The solution stood for 2 hr at 4° and was then centrifuged at 27,000*g* for 20 min. The antibody was dissolved in cold 0.15 M NaCl by standing overnight in enough saline to cover the precipitate. The solution was then centrifuged to remove insoluble protein and the supernatants were dialyzed against 0.15 M NaCl for 24 hr.

Results

Identity of 3-Phosphoglycerate Kinase from Rat Skeletal Muscle, Cardiac Muscle, and Liver. As recently as 15 years ago multiple forms of enzymes were virtually unknown and it was generally accepted that enzymes from different tissues of an animal were the same. Now, of course, isozymes seem to be the rule rather than the exception for soluble intracellular enzymes and it becomes necessary to demonstrate experimentally that a particular enzyme from different tissues is the same. For phosphoglycerate kinase we have used electrophoretic, kinetic and immunological studies to demonstrate that a single molecular form of the enzyme occurs in rat skeletal muscle, cardiac muscle, and liver.

(A) ELECTROPHORETIC STUDIES. As seen in Figure 1 phosphoglycerate kinase from rat skeletal muscle, cardiac muscle, and liver migrates electrophoretically as a single molecular form at three different pH values and in four different buffer systems. Also the enzyme from the three different tissues has the same electrophoretic mobility. That rat phosphoglycerate kinase binds P_i is evident from the fact that anodic migration is noted when the electrophoresis is performed in phosphate buffer but not when the experiment is done in Tris buffer at the same pH (Figure 1). Similar behavior has been reported for yeast and rabbit muscle phosphoglycerate kinase and indeed, the affinity of phosphoglycerate kinase for phosphate is the basis for employing phosphocellulose in the purification procedure (Hass *et al.*, 1974).

TABLE 1: Michaelis–Menten Constants (K_m) of Rat Phosphoglycerate Kinase.[a]

Tissue	Adenosine Triphosphate (mM)	3-Phosphoglycerate (mM)
Heart	0.151	1.397
Liver	0.238	1.654
Skeletal muscle	0.193	1.663
Skeletal muscle (purified)	0.127	1.097

[a] The concentration of ATP varied from 0.0454 to 2.27 mM and of 3-phosphoglycerate from 0.279 to 27.9 mM. Tissue homogenates were prepared in the manner described in Experimental Section. The purified rat skeletal muscle phosphoglycerate kinase was prepared as described by Haas *et al.* (1974) and had a specific activity of 600 units/mg of protein. The data were computer analyzed by obtaining a least-squares fit to a double-reciprocal plot according to the method of Lineweaver and Burk (1934).

(B) KINETIC STUDIES. (1) Michaelis–Menten Constants. Michaelis–Menten constants (Michaelis and Menten, 1913; Briggs and Haldane, 1925; Lineweaver and Burk, 1934) were obtained by running the enzyme assay at 25° over a 100-fold concentration range of ATP at a fixed 3-phosphoglycerate concentration of 11.2 mM and over a 100-fold range of 3-phosphoglycerate concentration at a fixed ATP concentration of 3.6 mM. The fixed concentrations of ATP and 3-phosphoglycerate were saturating concentrations of these substrates and the hyperbolic nature of the substrate concentration curves indicated that the enzyme followed Michaelis–Menten kinetics. Substrate saturation curves were run for the purified skeletal muscle enzyme and for the phosphoglycerate kinase in supernatants from homogenates of rat skeletal muscle, cardiac muscle, and liver. The results, shown in Table I, indicate that the constants obtained for the enzymes from the different rat tissues are not significantly different. It can also be seen from Table I that roughly eight times as much 3-phosphoglycerate as ATP is required to saturate the enzyme.

(2) Temperature Effect on Enzyme Activity. When the enzymes from the supernatant fraction of homogenates of rat skeletal muscle, cardiac muscle, and liver were assayed over a range of temperature from 15 to 40°, the results, shown in Figure 2, were similar for each preparation and revealed that in each case there was more than a 2-fold increase in activity from 25 to 40°. In contrast, the activity of the purified rat skeletal muscle enzyme increased only 1.6-fold from 25 to 40° (Figure 2). The reason for such differences between purified enzyme and the same enzyme in homogenates is not known with certainty.

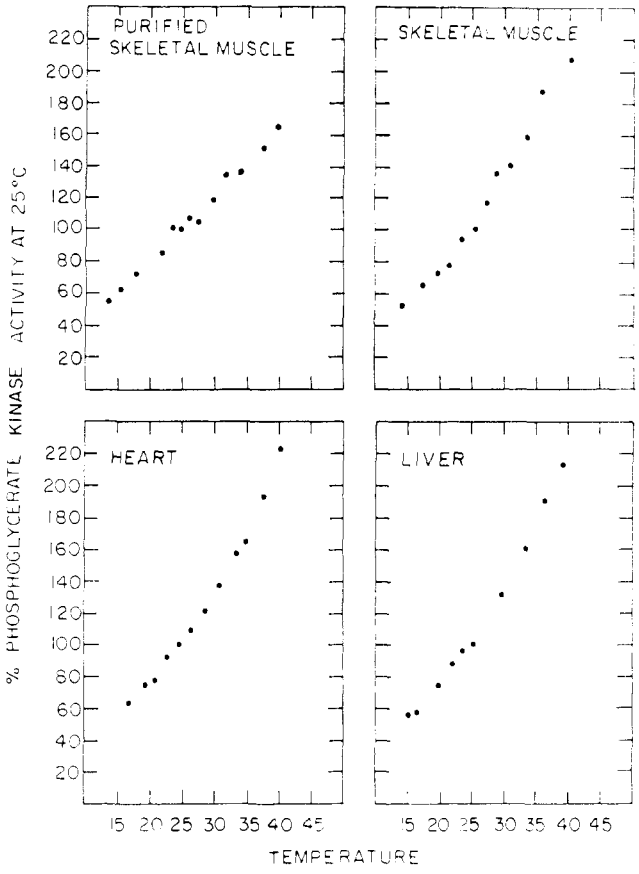

FIGURE 2: Temperature coefficient of phosphoglycerate kinase. The temperature was determined in the cuvette after the assay. Tissue homogenates were prepared in the manner described in the Experimental Section. The purified skeletal muscle PGK had a specific activity of 600 units/mg of protein.

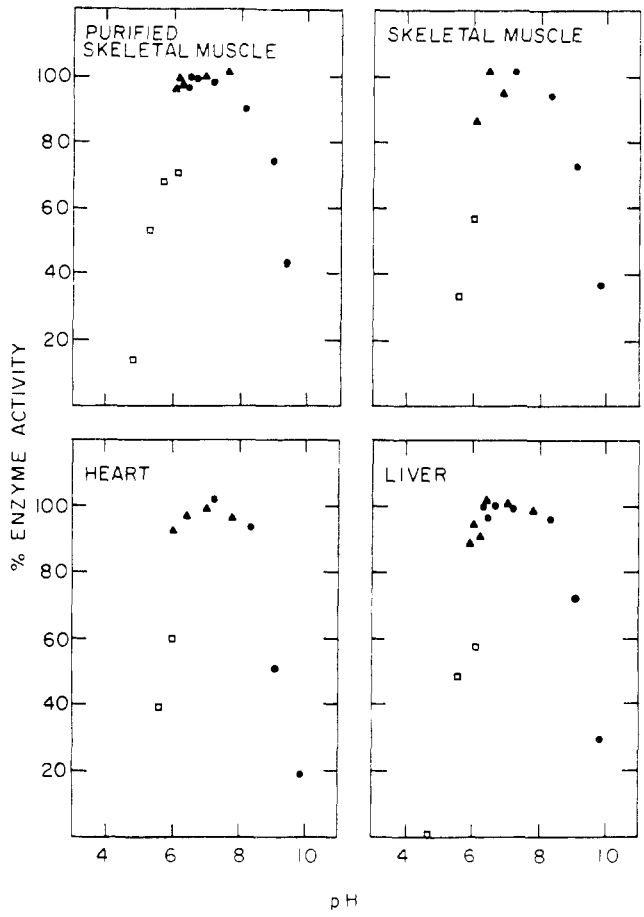

FIGURE 3: pH optimum of phosphoglycerate kinase. pH optimum was determined in the following buffers: (▲) 0.05 M Tris-HCl, (□) 0.05 M citrate, and (●) 0.05 M glycine. The pH was measured in the cuvette after the assay. The purified skeletal muscle phosphoglycerate kinase was prepared as described by Hass *et al.* (1974) and had a specific activity of 600 units/mg of protein. Tissue homogenates were prepared as described in the Experimental Section. The enzyme activity is expressed as the per cent of the average phosphoglycerate kinase activity for pH 7.0.

(3) pH Effect on Enzyme Activity. 3-phosphoglycerate kinase from homogenates of the three rat tissues, as well as the purified rat skeletal muscle enzyme, exhibited a broad pH optimum extending from about pH 6.0 to 8.0. On either side of these pH values, the activity dropped sharply (Figure 3).

(C) Immunological studies. Purity of the rabbit anti-rat skeletal muscle phosphoglycerate kinase was verified by double diffusion in agar (Figure 4). Figure 4 also shows that the antibody to the rat skeletal muscle phosphoglycerate kinase gives a line of identity whether the antigen is the purified rat skeletal muscle enzyme or is derived from homogenates of rat liver, cardiac muscle, or skeletal muscle. Further evidence that phosphoglycerate kinase from these three rat tissues is immunologically the same is presented in Figure 5 where it is seen that when increasing amounts of antiserum are added to a constant amount of phosphoglycerate kinase contained in homogenates of liver, heart, or skeletal muscle, the curves of decreasing enzyme activity as well as the amount of protein precipitated at the equivalence point are virtually the same.

Fasting Studies. When food was withheld from the rats, there were no significant changes in phosphoglycerate kinase activities of heart, skeletal muscle, or liver for up to 3 days after beginning the fast (Table II). Thus, phosphoglycerate kinase differs from the other three kinases of the glycolytic pathway. Rat liver pyruvate kinase decreases almost 50% after a 48-hr fast while the same enzyme from skeletal muscle is virtually unaffected (Tanaka *et al.*, 1965). Rat liver glucokinase decreases more than 60% after a 48-hr fast (Sharma *et al.*, 1964) while rat liver phosphofructokinase decreases at least 80% when the animals are fasted for 48 hr (Weber *et al.*, 1966). In contrast to phosphoglycerate kinase, pyruvate kinase, glucokinase, and phosphofructokinase are all subunit enzymes present in the various rat tissues in the form of isozymes but whether this is related to dietary and hormonal control of the enzyme levels is not presently known.

Developmental Studies. Changes in 3-phosphoglycerate kinase activity in the supernatant fraction of rat skeletal muscle, heart, and liver homogenates at various times during postnatal development are shown in Figure 6. The data shown are for male and female rats. There was no difference in the phosphoglycerate kinase activities between the sexes during this period. As seen in Figure 6 each of the three tissues had its own characteristic pattern although there were similarities among them. The developmental patterns in liver and heart were virtually the same in both the changes with time and the absolute amounts. The primary difference between heart and liver was that in liver there was a sharp rise between birth and 5 days followed by a decline until 15–20 days whereas in heart the activity simply declined from birth until 15–20 days. In skeletal muscle the activity was low at birth and increased steadily until reaching a peak around 80 postnatal days. This peak in activity was seen in all three tissues at about the

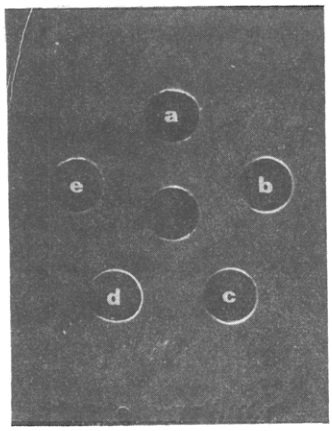

FIGURE 4: Double diffusion in agar of rat phosphoglycerate kinase. Wells a, b, and c contain rat tissue homogenates from heart, skeletal muscle, and liver, respectively. Well d contains purified rat skeletal muscle phosphoglycerate kinase and well e contains buffer. The center well contains serum from a rabbit injected with the antigen that is in well d. The plate was kept at room temperature for 48 hr.

same time; thereafter the activity declined to steady-state values of 300, 145, and 115 units per g for skeletal muscle, heart, and liver, respectively. The changes in activity were due to changes in enzyme protein for as noted in Figure 5 when constant amounts of enzyme activity from extracts of the three tissues at different stages in development were mixed with antiserum, the amount of protein precipitated was not significantly different.

Discussion

These studies clearly establish that 3-phosphoglycerate kinase exists throughout the life of the animal as a single form, identical in rat liver, heart, and skeletal muscle. This knowledge is consistent with the fact that the enzyme is a single polypeptide chain (Hass *et al.*, 1974), and implies that a single genetic locus is responsible for the presence of the enzyme in these three rat tissues at all times during the life of the animal. Such findings simplify consideration of factors involved in regulating the intracellular levels of the enzyme because it no longer is necessary to take into account the possible role of subunit assembly or exchange.

TABLE II: Quantitation of Rat Phosphoglycerate Kinase During Fasting.[a]

Days of Fasting	Tissue		
	Heart	Skeletal Muscle	Liver
0 (control)	187.4 ± 6.13	386.4 ± 18.81	169.8 ± 2.96
1	175.2 ± 12.73	422.2 ± 6.22	173.6 ± 4.44
2	197.2 ± 5.94	440.8 ± 24.9	167.4 ± 9.17
3	185.0 ± 5.82	422.0 ± 14.3	189.8 ± 10.4

[a] The values are expressed in units per gram of tissue and are the means ± SE of 5 animals. The values were determined at 24, 48, and 72 hr after withdrawing food. The animals had water *ad libitum*.

The rate-limiting factors in glycolysis have been the subject of numerous investigations and the conclusions of such studies have been varied enough to indicate that no single factor is rate limiting under all conditions. The subject has been extensively reviewed (Scrutton and Utter, 1968; Villar-Palasi and Larner, 1970) and is much too complex to discuss here in detail. It is, however, a widely held view that the glycolytic pathway is extensively regulated by feedback mechanisms which affect the activity of certain enzymes catalyzing particular steps in the pathway. In assessing such theories it should be kept in mind that they are based on *in vitro* enzyme assays and of necessity are carried out using enzyme concentrations 1000 times or more below those found in the cell. It seems clear that if an enzyme is not saturated with substrate *in vivo*, then changes in the substrate concentration could alter the rate of metabolic flux. The concentration of ATP in rat liver is 2.74 μmol/g (Hems and Brosnan, 1970) which is ten times the intracellular 3-phosphoglycerate concentration of 0.28 μmol/g (Hems and Brosnan, 1970). Assuming that each

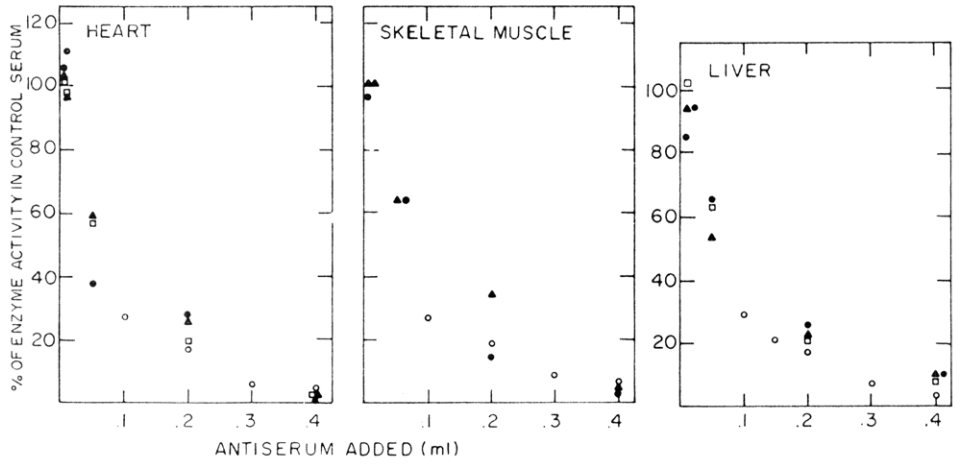

FIGURE 5: Immunoprecipitation of rat liver, heart, and skeletal muscle phosphoglycerate kinase. Tissue extracts from 1-day-old (●), 42-day-old (▲), 207-day-old (□), and 334-day-old (○) rats were prepared as described in the Experimental Section. A constant amount of enzyme activity (approximately 0.7 unit) was mixed with increasing volumes of antiserum or control serum. The samples were incubated overnight at 4° and then centrifuged for 20 min at 27,000*g*. The supernatant was assayed for enzyme activity and the precipitate analyzed for protein. The micrograms of protein precipitated at the equivalence point plus and/or minus the standard error of three determinations for rat liver at the different ages was as follows: 1 day old, 59.2 ± 1.1; 42 days old, 50.9 ± 4.7; 207 days, 52.9 ± 7.7; 334 days, 48.1 ± 1.9; for rat heart: 1 day, 35.2 ± 4.0; 42 days, 37.8 ± 2.2; 207 days, 46.9 ± 5.3; 334 days, 49.2 ± 4.8; for rat skeletal muscle: 1 day, 58.7 ± 3.4; 42 days, 43.1 ± 2.7; 334 days, 39.3 ± 1.5. There were no statistically significant differences between any of these values.

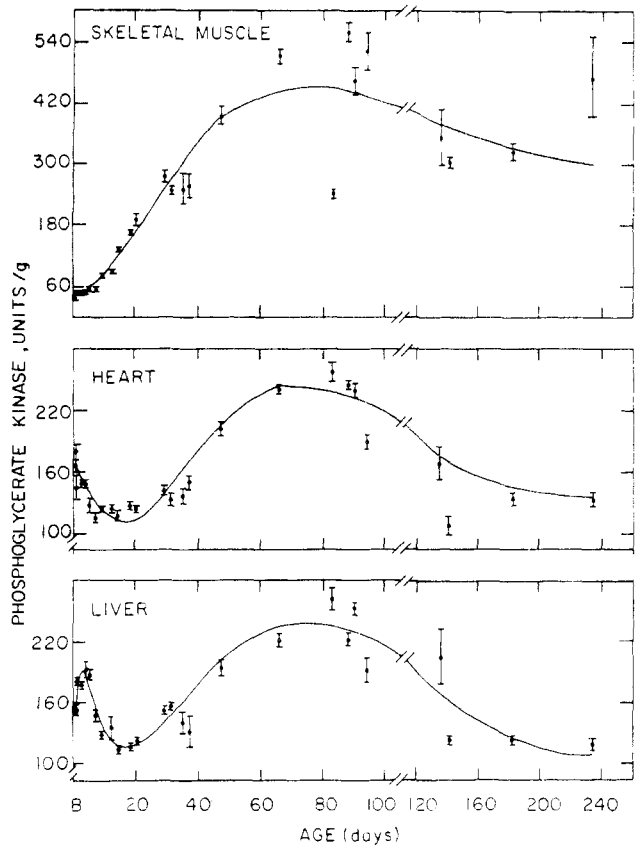

FIGURE 6: Developmental changes in phosphoglycerate kinase activity. The results are means ±SE of 4–11 animals. The line represents the best fit to the data as determined by computer analysis using a nonlinear least-squares program designated BMDX 85 and developed at the Health Science Computing Facility of the University of California at Los Angeles.

gram of tissue represents approximately 1.0 ml, it can be estimated that the intracellular ATP concentration is about 2.74 mM and of 3-phosphoglycerate is about 0.28 mM. Our data (Table I) indicate that the 3-phosphoglycerate concentration required for half-saturation (K_m) of rat liver 3-phosphoglycerate kinase is about seven times greater than the corresponding concentration of ATP (1.6 mM *vs.* 0.24 mM). Thus it seems that, *in vivo*, if the substrates are distributed uniformly throughout the cell, the enzyme is saturated with ATP but not with 3-phosphoglycerate. If 3-phosphoglycerate kinase is saturated *in vivo* with its substrate ATP then increasing the level of the enzyme might result in increased breakdown of ATP and qualify the enzyme as having a regulatory role in the metabolic pathway.

Looking at the reverse reaction which is actually the direction of glycolytic flux, the situation seems similar with respect to ADP. Krietsch and Bücher (1970) have estimated for the rabbit skeletal muscle enzyme that the K_m for ADP is 0.35 mM and we calculate from the data of Hems and Brosnan (1970) that the intracellular liver ADP concentration is about 1.3 mM. Consideration of the other substrate, 1,3-phosphoglycerate, is difficult because the intracellular concentrations are so low as to be hardly measurable. It is interesting to note, however, that the K_m for 1,3-diphosphoglycerate for rabbit muscle 3-phosphoglycerate kinase is more than 500 times lower than that for 3-phosphoglycerate, 0.0022 mM *vs.* 1.22 mM (Krietsch and Bücher, 1970). Thus, much lower concentrations of the substrate 1,3-diphosphoglycerate are required

TABLE III: 3-Phosphoglycerate Kinase (3-PGK) Levels in Developing Rat Liver.

Age (Days after Birth)	3-PGK (Units/g)	DNA (mg/g)	3-PGK (Units/mg of DNA)
10	130	6.0	21.6
20	120	6.0	20.0
30	140	3.3	42.3
40	160	2.9	55.1
50	210	2.4	87.5
60	220	2.3	95.6
100	220	2.5	88.0
200	120	2.5	48.0

to saturate the enzyme than any of its other substrates. Whether the enzyme is saturated *in vivo* with 1,3-diphosphoglycerate cannot be determined until more sensitive methods are developed for measuring the substrate.

It is not yet known whether the fluctuations in 3-phosphoglycerate kinase activity during development (Figure 6) have physiological significance with respect to regulating the rate of glycolysis. It is interesting that the minima in the activity *vs.* time curves in heart and liver occur at precisely the same time during development as minima in the corresponding pyruvate kinase curves (Osterman *et al.*, 1973). The decrease in liver pyruvate kinase at this time was thought to be at least partially responsible for the increased rate of gluconeogenesis during this suckling period (Osterman *et al.*, 1973; Middleton and Walker, 1972).

Interpretation of changing enzyme levels during development is clouded by consideration of factors relating to changes in type, number, and size of cells. In other words, it is important to know, for example, if an observed increase in the amount of an enzyme per gram of tissue over a period of time is due to an actual accumulation of the enzyme within a particular type of cell or whether the manner of expressing the data is masking the fact that the rate of synthesis equals the rate of degradation within the cell. The recent work of Greengard *et al.* (1972) on cellular changes in developing rat liver has been helpful in dealing with these problems. With regard to cell type they found that at birth there were very few hematopoietic cells and that these diminished even more thereafter. They found that the absolute number of Kupffer cells in a given cell volume increased only 1.3-fold from fetus to adult while the relative number of Kupffer cells during this time increased from 4 to 37%. These differences were accounted for by a 3-fold increase in parenchymal cell volume during this time. They reported that the fraction of liver volume occupied by Kupffer cells was never more than 0.02 from fetal life to 100 days after birth. Thus it seems that during the developmental period we have been concerned with (Figure 6) there is some assurance that the measured rat liver 3-phosphoglycerate kinase levels are derived primarily from parenchymal hepatocytes.

Enesco and Leblond (1962), using weight gain as an index of growth of rat organs and tissues during development, measured DNA levels to estimate cell number and used histological examination to estimate cell size. They concluded in general that the rapid growth of rat organs and tissues from birth to about 17 days of age was due primarily to increases in cell number with little change in cell size while between 17

and about 48 days of age the rate of cell division was reduced whereas cell size rapidly increased. After about 48 days of age the rate of cell division continuously slowed as did the rate of increase in cell size. Winick and Noble (1965) attributed the slowing of growth with approaching maturity to an attainment of a steady state between protein synthesis and protein destruction.

Greengard *et al.* (1972), in considering various possible ways of expressing enzyme concentrations, point out that the vast majority of publications express enzyme amounts per unit weight of tissue even though cells or milligrams of DNA are frequently advocated as more "physiologically meaningful" bases for comparison. They suggest that the only reasonable procedure is to record the concentrations of both constituents as per gram of tissue and then comparisons can be made for interpretive purposes if desired. We have used the data of Enesco and Leblond (1962) to estimate the amount of rat liver 3-phosphoglycerate kinase per milligram of DNA during development (Table III). It is noted that although this procedure reveals quantitative differences in the 3-phosphoglycerate kinase levels, qualitatively the shape of the developmental curve is not altered appreciably. Thus it appears that there are indeed fluctuations in the level of rat liver 3-phosphoglycerate kinase during development and eventually an accumulation up to about 100 days after birth.

When the level of the enzyme increases with time, it means that the rate of synthesis exceeds the rate of degradation whereas when the enzyme level decreases with time, the rate of degradation is greater than the rate of synthesis. Furthermore, when the enzyme level is constant with time, the two rates are equal. Thus, it seems that if the enzyme levels change during development, the rates of synthesis and degradation most likely change also. It is not known what factors might be responsible for changing the rates of synthesis and degradation.

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