Alterations in phosphofructokinase isoenzymes during early human development

Establishment of adult organ-specific patterns

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Human 6-phosphofructokinase (EC 2.7.1.11) exists in tetrameric isoenzymic forms composed of muscle (M), liver (L) and platelet (P) subunits, which are under separate genetic control. In the adult, the proportion of these subunits in different organs reflects the relative activity of glycolysis versus gluconeogenesis. To elucidate the developmental basis for the observed distribution, we investigated the isoenzymic transitions of phosphofructokinase in human fetuses (12–40 weeks' gestation) by using high-resolution chromatography and monoclonal antibodies. We studied skeletal muscle, heart, liver and brain because these organs show very different glycolytic fluxes and isoenzymic patterns in adult individuals. Our results demonstrate that there is no unique 'foetal' form of phosphofructokinase in humans, but all three loci are variably expressed in all foetal organs during early gestation. As development proceeds, muscle and liver isoenzyme patterns show dramatic changes, with disappearance of P and L subunits in muscle and transient reappearance of M and P subunits in liver; in contrast, phosphofructokinase isoenzymes change little in brain and heart. Most changes occur at mid-gestation and near term, and adult isoenzyme patterns are expressed at birth, indicating that organ differentiation is complete. These studies show that phosphofructokinase undergoes changes of isoenzyme patterns similar to, but not identical with, those of other multilocus isoenzyme systems of glycolysis. The observed changes probably reflect changing patterns of gene expression, with repression of some loci and activation of others.

Biochemical, immunochemical and genetic studies have shown that human PFK is under the control of three structural loci, which encode muscle-type (M), liver-type (L) and platelet-type (P; same as fibroblast-type, F) subunits (Kahn *et al.*, 1979; Meienhofer *et al.*, 1979; Vora *et al.*, 1980; Vora, 1981; for review see Vora, 1982). Variable expression of these loci in different human tissues leads to the formation of tissue-specific patterns of various homo- and hetero-tetrameric isoenzymes. Muscle and liver PFK enzymes are homotetramers, M₄ and L₄, whereas erythrocyte PFK consists of an entire five-

membered isoenzyme set composed of the M and L subunits, i.e. M₄, M₃L, M₂L₂, ML₃ and L₄ (Vora *et al.*, 1980). Platelet PFK consists of three isoenzymes composed of a distinct subunit 'P' in combination with L subunits, i.e. P₄, P₃L, P₂L₂. Cultured fibroblasts and lymphoblastoid cell lines express all three subunits in variable amounts (Vora, 1981). Recently we have found that the distribution of human PFK subunits in various adult organs accurately reflects the relative activity of glycolytic and gluconeogenic pathways (M. Davidson & S. Vora, unpublished work). The M-type subunit predominates in organs depending mostly on glycolysis, i.e. skeletal muscle, heart and brain, whereas the L-type predominates in organs with active gluconeogenesis, i.e. liver and kidney cortex. The P-type is found to predominate in rapidly replicating cultured cells largely using aerobic glycolysis (Vora, 1982, 1983).

Abbreviation used: PFK, phosphofructokinase.

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Previous developmental studies of human PFK have largely defined changes in total PFK content of brain (Bennett *et al.*, 1977), erythrocytes (Kahn *et al.*, 1977; Travis & Garvin, 1977; Gahr *et al.*, 1979), skeletal muscle (Colling-Saltin, 1978) and adipose tissue (Novak *et al.*, 1972) during early human development. Only few studies have attempted to characterize isoenzyme composition(s) of foetal brain (Bennett *et al.*, 1975), erythrocytes (Chen *et al.*, 1977; Kahn *et al.*, 1977) and other organs (Kahn *et al.*, 1980), and their changes, during ontogeny. Therefore in the present study we investigated the changes of PFK isoenzymes during early human development by using high-resolution chromatography and subunit-specific monoclonal antibodies. We studied skeletal muscle, heart, brain and liver, four tissues with markedly different needs for carbohydrate metabolism. Our results demonstrate that human PFK exhibits changes of isoenzyme profiles very similar to, although not identical with, those of other multigenic isoenzyme systems of glycolysis. In general, there is a tendency towards uniformity in early foetal life, i.e. simultaneous expression of all three PFK loci. However, as development proceeds, recognizable isoenzymic alterations occur and distinct tissue-specific patterns are established for all four organs at the end of gestation.

Experimental

Chemicals and reagents

Adenine nucleotides, NADH, fructose 6-phosphate, fructose 1,6-bisphosphate and dithiothreitol were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Fructose bisphosphate aldolase, glycerol 3-phosphate dehydrogenase and triose phosphate isomerase were from Boehringer Mannheim (Indianapolis, IN, U.S.A.). DEAE-Sephadex A-25 was from Pharmacia Fine Chemicals (Piscataway, NJ, U.S.A.). Nonidet P-40 was purchased from Particle Data (Chicago, IL, U.S.A.), and staphylococci bearing protein A (IgGSorb) were from The Enzyme Center (Boston, MA, U.S.A.). All other chemicals were of reagent grade.

Human foetal tissues

Specimens (2–10 g) of foetal skeletal muscle, heart, liver and brain were obtained at various stages of development during the conduct of an epidemiological study of spontaneous abortions in women. Fresh placental tissue was obtained from full-term normal deliveries. The tissues were harvested within 6–8 h of delivery, rinsed with chilled phosphate-buffered saline (0.15 M-NaCl/10 mM-sodium phosphate buffer, pH 7.4) and stored at -20°C ; the

studies were performed within 1–7 days of tissue collection. Each organ was examined chromatographically from three or more donors at the following gestational ages: 10–12, 17–21, 24–28 and 36–40 weeks. However, a much larger number of foetal specimens from several other donors (six to eight) at various gestational ages were examined by immunoprecipitation with the use of three types of subunit-specific antibodies.

Assay of PFK activity

Assays of PFK activity were done with a Gilford model 2400 spectrophotometer at 26°C as described previously (Vora *et al.*, 1980). One unit of enzyme activity is defined as that amount of enzyme that converts $1\mu\text{mol}$ of fructose 6-phosphate into fructose 1,6-bisphosphate in 1 min.

Preparation of tissue extracts

A 2–5 g wet wt. portion of tissue was cut into small pieces and washed extensively with chilled phosphate-buffered saline to remove as much blood as possible. Minor degree of contamination with erythrocytes was unavoidable with liver and placental tissues. The tissue was subsequently homogenized with 2 vol. of ice-cold extraction buffer [50 mM-potassium phosphate buffer, pH 8.0, containing 10 mM- $(\text{NH}_4)_2\text{SO}_4$, 1 mM-EDTA, 0.5 mM-fructose 6-phosphate, 0.1 mM-ATP and 1.0 mM-dithiothreitol] in a VirTis 23 homogenizer. The extracts were centrifuged at $10000g$ for 20 min at 4°C , and the supernatants were used for chromatographic and immunochemical studies.

Chromatographic separation of PFK isoenzymes

PFK isoenzymes were resolved by using DEAE-Sephadex A-25 ion-exchange chromatography at 4°C as described previously (Vora *et al.*, 1980). Briefly, a $1.7\text{ cm} \times 30\text{ cm}$ column was equilibrated in 0.1 M-Tris/phosphate buffer, pH 8.0, containing 25 mM-NaCl, 0.2 mM-EDTA, 0.2 mM-AMP and 0.7 mM-dithiothreitol. The column was loaded with 0.1–0.12 unit of the enzyme preparation. A 300 ml concave elution gradient was prepared by using three chambers of a Varigrad nine-chambered gradient mixer, as described previously (Vora *et al.*, 1980). A total of 100 3 ml fractions were collected and assayed for PFK activity. The three homotetramers are distinguished by their relative positions of elution. M_4 tetramer is eluted first at 214 mosmol/kg (approx. 40th fraction), and L_4 tetramer is eluted last at 480 mosmol/kg (approx. 75th fraction); P_4 tetramer is eluted very close to M_4 tetramer, at 244 mosmol/kg (approx. 47th fractions) (Vora *et al.*, 1980; Vora, 1981).

Production and characterization of subunit-specific antibodies

Subunit-specific monoclonal antibodies against the M and L subunits were produced by the secretory lymphocyte hybridoma technique. The details of the immunization protocol, technique of hybridization, screening of the secretory hybrids, production of antibody-rich mouse ascites fluids and characterization of the secreted antibodies are described elsewhere (Vora *et al.*, 1981). The production and characterization of a P-subunit-specific BALB/c antiserum has been described in detail elsewhere (Vora *et al.*, 1983). The five monoclonal antibodies (four anti-L and one anti-M) and P-subunit-specific antiserum react strictly with the respective subunit only (Vora *et al.*, 1981, 1983). At the dilutions used, these antibodies retained full precipitating efficacy and were always in excess of the amount of PFK used for immunoprecipitation (see below). An excess of each of these antibodies precipitates completely not only the respective homotetramer, but also heterotetramers containing one or more of the specific subunit/molecule. For instance, anti-M antibody precipitates completely not only M₄ homotetramer, but also the M₃L, M₂L₂ and ML₃ heterotetramers (Vora, 1981).

Enzyme-immunoprecipitation assay

Freshly prepared tissue extracts were diluted to a final concentration of 0.06 unit/ml with the extraction buffer described above. Samples (50 µl) of a given extract were mixed in duplicate with 50 µl of diluted mouse ascites fluid (1:200 in phosphate-buffered saline) containing either the anti-M (V96-26) or anti-L (V65-06) monoclonal antibody or with 50 µl of diluted anti-P serum (1:64). The mixtures were incubated for 30 min at 37°C, and then 100 µl of freshly washed (Kessler, 1976) 10% (w/v) suspension of staphylococci bearing protein A (IgGSorb) was added. The mixtures were incubated at 4°C for 30 min with continuous shaking, centrifuged at 8000g for 10 min and assayed for residual enzyme activity. The precipitated enzyme activities were expressed as percentages of the concurrent controls; only the precipitation values more than 7% were considered to be significant (Vora *et al.*, 1982). Each extract was tested in duplicate with duplicate controls.

Histopathological studies

In order to investigate whether the observed changes in hepatic isoenzyme patterns correlated with the hepatic haematopoiesis, the livers of 21 foetuses and infants were examined. As previously reported (Stowens, 1966; Kelemen *et al.*, 1979), granulopoietic elements showed marked increase in the 20–30-weeks-gestational-age group, a period during which hepatic isoenzyme pattern showed

dramatic changes. In contrast, erythropoietic elements, which were present from 10–35 weeks' gestation, showed minimal alterations during the same period.

Results and discussion

Since the establishment of diverse and unique metabolic pathways reflects the process of differentiation, alterations in isoenzyme complement(s) may be used as markers of organ differentiation. Among the multilocus glycolytic enzymes, the isoenzymes of pyruvate kinase, lactate dehydrogenase, fructose biphosphate aldolase and enolase have been studied in detail (Edwards & Hopkinson, 1977; Schapira, 1981). In contrast, very few studies have investigated the developmental changes of PFK isoenzymes in man (Bennett *et al.*, 1975; Chen *et al.*, 1977; Kahn *et al.*, 1980) and rodents (De Faria *et al.*, 1978; Thrasher *et al.*, 1981). The paucity of studies on PFK may be due to the relatively recent recognition of PFK isoenzymes, and to the fact that these extremely similar molecules are difficult to resolve and identify. For instance, on the basis of electrophoretic studies, a single isoenzyme was reported in foetal brain and two isoenzymes in adult brain (Bennett *et al.*, 1975), whereas both foetal and adult erythrocytes had two isoenzymes (Chen *et al.*, 1977). Since electrophoretic separation of PFK isoenzymes is at best inadequate, these results are inaccurate. On the basis of immunochemical studies with absorbed rabbit antisera, Kahn *et al.* (1980) have suggested that between 8 and 16 weeks of foetal development all human tissues variably express all three PFK loci; however, the time course of isoenzymic transitions that result in the establishment of the adult patterns were not determined. Slight differences in charge properties and relative molecular masses of the M and L subunits in foetal and adult tissues were attributed either to the presence of unique 'foetal' subunits coded by foetal genes or to post-translational modification of these subunits.

Our results demonstrate that, except for the liver, the other three organs express all three PFK loci in early gestation (before 17–21 weeks), as previously reported (Kahn *et al.*, 1980). However, as development proceeds, very orderly changes take place in all four organs at definite points in time, which culminate in the establishment of adult organ-specific patterns at term.

Skeletal-muscle isoenzyme(s)

In early gestation, skeletal muscle consistently shows the presence of multiple isoenzymes: the hybrids of the M and P subunits predominate, but L₄ tetramer and the hybrids of the L subunit with the M and P subunits are also seen at this stage (Fig. 1a).

Fig. 1. Representative PFK isoenzyme profiles of foetal skeletal muscle (a, b and c) and cardiac muscle (d, e and f) at about 17 weeks (a and d), at about 28 weeks (b and e) and at about 40 weeks (c and f) of gestation

For experimental details see the text. —, PFK activity; ----, elution gradient. The L- subunit- and P-subunit-containing isoenzymes disappear in skeletal muscle at mid-term and term respectively. In contrast, only the L-subunit-containing isoenzyme(s) either decline or disappear in cardiac muscle.

By mid-gestation, L_4 tetramer and hybrids of L subunits have declined and disappeared, but the broad chromatogram indicates the presence of P-subunit-containing isoenzymes at this stage (Fig. 1b). Only in late gestation and near term is the adult pattern, characterized by the exclusive presence of M_4 tetramer, clearly evident (Fig. 1c). These chromatographic findings were confirmed by immunochemical studies (Table 1). Colling-Saltin (1978) reported a sharp rise in skeletal-muscle PFK activity at about 25 weeks of gestation, a point in

time when the muscle-specific isoenzyme M_4 is predominantly expressed. In their study, the increase in the PFK activity also correlated with morphological and ultrastructural findings suggesting concordant enhancement of the metabolic capacity and contractile elements of the foetal muscle. Thus the changes in PFK isoenzyme pattern appear to reflect the process of cellular differentiation and to herald the maturation of metabolic pathways (glycolysis and glycogenolysis) in developing skeletal muscle. Like early foetal muscle, muscle

Table 1. *Immunoprecipitation values of PFK from various human foetal organs during intrauterine development*
For experimental details see the text. Results are given as means $\pm$ s.d. Abbreviation: N.D., not done.

Tissue	Gestational age (weeks)	No. of specimens	Precipitation (%)		
			Anti-M antibody	Anti-P antibody	Anti-L antibody
Muscle	16–20	7	86.0 $\pm$ 8.5	38.8 $\pm$ 3.9	30.9 $\pm$ 9.3
	21–26	4	87.3 $\pm$ 11.1	16.5 $\pm$ 2.5	3.7 $\pm$ 4.9
	36–40	7	93.0 $\pm$ 5.7	3.6 $\pm$ 4.7	3.0 $\pm$ 3.6
Heart	10–12	3	78.0 $\pm$ 15.3	45.0 $\pm$ 5.7	N.D.
	17–21	5	62.5 $\pm$ 12.0	64.5 $\pm$ 2.1	10.7 $\pm$ 3.2
	24–28	3	63.0 $\pm$ 17.0	60.0 $\pm$ 5.7	6.7 $\pm$ 6.0
	36–40	4	71.0 $\pm$ 15.7	58.4 $\pm$ 7.9	16.0 $\pm$ 5.8
Brain	10–12	3	52.9 $\pm$ 13.7	26.2 $\pm$ 10.5	69.5 $\pm$ 15.2
	17–21	5	73.2 $\pm$ 16.8	31.0 $\pm$ 12.7	60.7 $\pm$ 12.0
	24–28	4	74.8 $\pm$ 9.9	33.0 $\pm$ 8.7	31.7 $\pm$ 12.1
	36–40	4	75.2 $\pm$ 16.4	34.2 $\pm$ 1.6	19.7 $\pm$ 9.3
Liver	10–12	3	14.2 $\pm$ 2.6	10.7 $\pm$ 3.8	83.8 $\pm$ 1.8
	17–21	6	7.8 $\pm$ 5.2	15.5 $\pm$ 4.1	82.6 $\pm$ 7.6
	24–28	3	6.1 $\pm$ 8.7	26.0 $\pm$ 2.8	60.1 $\pm$ 15.7
	36–40	3	21.3 $\pm$ 13.4	38.0 $\pm$ 4.2	53.0 $\pm$ 15.2
Placenta	36–40	5	14.6 $\pm$ 1.0	34.5 $\pm$ 11.8	87.1 $\pm$ 6.0

cultures from adults also express all three PFK loci. However, unlike foetal muscle, where the M subunit predominates at all stages of development, in muscle cultures the P and L subunits predominate (Davidson *et al.*, 1983). These findings indicate that the expression of muscle-specific PFK *in vivo* may be controlled by a number of other factors, such as innervation and/or hormonal influences.

Heart isoenzymes

In early gestation, heart predominantly expresses the P and M subunits, and the L subunit comprises a minor proportion of the total PFK (Fig. 1d). At mid-gestation, L₄ tetramer and L-subunit-containing hybrid isoenzymes disappear, but the presence of the L subunit in small amounts is demonstrable immunologically (Table 1). Near term, the adult pattern is established, generally consisting of three isoenzymes M₄, P₄ and a peak eluted in the position between the M₄ and P₄ isoenzymes. The latter peak probably results from the co-elution of M₃P, M₂P₂ and MP₃ isoenzymes. However, few hybrid isoenzymes containing the L subunit are present in some specimens (Fig. 1e). In contrast with skeletal muscle, in cardiac muscle the L subunit does not disappear at term but persists in minor amounts throughout life (Table 1).

Brain isoenzymes

Brain shows early maturation of the isoenzyme pattern, and there are minimal changes during gestation; all three subunits are expressed at all stages of development (Figs. 2a, 2b and 2c), although in variable amounts. The M subunit

predominates throughout life, whereas the relative amount of the L subunit declines around mid-gestation (Fig. 2b and Table 1), so that L₄ tetramer is lost at this time. Near term, brain shows the adult profile, where the M-subunit- and P-subunit-containing isoenzymes predominate, although the late-eluted hybrids also indicate the presence of L subunit in minor amounts (Fig. 2c). The lack of dramatic alterations in isoenzymic profile of brain appears to be accompanied by a similar lack of quantitative changes in total PFK content during development. Unlike pyruvate kinase and hexokinase, which show sharp rises in activity near term, PFK shows relatively low but stable activity throughout gestation (Bennett *et al.*, 1977).

Liver isoenzymes

Liver shows an interesting isoenzymic transition, probably related to its haematopoietic function during gestation. Except at about 20–30 weeks of gestation, L₄ isoenzyme predominates at all stages of development, indicating an early establishment of the liver-specific isoenzyme complement (Figs. 2d and 2f). By analogy with rat PFK isoenzymes (Thrasher *et al.*, 1981), the major isoenzyme L₄ is probably derived exclusively from hepatocytes, whereas the minor P₄ homotetramer (seen distinctly around term) is probably derived from Kupffer cells (Fig. 2f). The absence of P₄ isoenzyme in early gestation suggests the absence of Kupffer cells (Fig. 2d). During 20–30 weeks' gestation, liver consistently exhibits multiple isoenzymic species (Fig. 2e) composed of the P and L subunits (Table 1). These isoenzymes appear to originate from foetal

Fig. 2. Representative PFK isoenzyme profiles of foetal brain (a, b and c) and liver (d, e and f) at about 17 weeks (a and d), at about 28 weeks (b and e) and at about 40 weeks (c and f) of gestation

For experimental details see the text. —, PFK activity; ----, elution gradient. Hybrid isoenzymes appear transiently in liver at about 24–28 weeks of gestation, whereas only the L_4 isoenzyme is present in early gestation and the L_4 and P_4 isoenzymes are present at term. In contrast, brain shows simultaneous expression of all three subunits throughout gestation and relatively few changes.

haematopoietic cells, since the quantitative change in hepatic granulopoiesis shows good temporal correlation with the isoenzymic changes. In agreement with this interpretation, foetal liver near term shows the adult pattern of two homotetramers, which is coincident with the regression of visceral haematopoiesis. However, it is conceivable that foetal erythroid precursors may also show an isoenzyme transition from L subunits only to P + L subunits to M + L subunits at early, mid- and late gestation respectively. A similar shift in foetal hepatic isoenzymes coincident with the height of

hepatic haematopoiesis is also observed for fructose biphosphate aldolase (Schapira, 1981).

Placental isoenzymes

Because placenta is considered a 'foetal' tissue on the basis of its early gestational origin, we investigated the placental isoenzymic profile. Chromatographically, most placentas showed a five-membered set of P and L subunits, with the L_4 isoenzyme predominating (Fig. 3). However, immunologically some M-subunit-containing isoenzymes were always found (Table 1). Since

Fig. 3. (a) Representative PFK isoenzyme profile of placental tissue showing the presence of a five-membered set of P and L subunits (P_4 , P_3L , P_2L_2 , PL_3 and L_4) and (b) isoenzyme profile of platelet PFK (P_4 , P_3L and P_2L_2) shown for comparison

For experimental details see the text.

contamination with blood was unavoidable in placenta, the origin of the M subunit appears to be from erythrocytes.

General discussion

These ontogenetic alterations in human PFK are comparable with those exhibited by PFK from non-human mammalian species. During development, species-specific alterations in activity and isoenzymes occur in rat, rabbit and guinea pig (Baldwin *et al.*, 1977; De Faria *et al.*, 1978; Beitner *et al.*, 1979; Thrasher *et al.*, 1981). Like human PFK, the rabbit enzyme shows striking changes in the neonatal period in skeletal muscle and heart, but

not in brain and liver (De Faria *et al.*, 1978). Rabbit skeletal muscle and heart show the entire five-membered isoenzyme set composed of the A and C subunits (analogous to the human M and P subunits respectively), i.e. A_4 , A_3C , A_2C_2 , AC_3 and C_4 . Gradually, C_4 tetramer and C-subunit-containing hybrids disappear, so that by 2 and 8 postnatal weeks only the A_4 homotetramer is present in skeletal muscle and heart respectively. In contrast, rabbit brain and liver show the five-membered set of the A and C subunits and the B_4 (analogous to the human L_4) isoenzyme respectively at all stages of development. Since subunit structure(s) and genetic control of PFK in rat and guinea pig have not yet been elucidated, the precise nature of isoenzymic transitions in these species remains to be established. Available data are suggestive of species-specific alterations of PFK isoenzymes in various organs (De Faria *et al.*, 1978; Thrasher *et al.*, 1981).

As in the case of human skeletal muscle, isoenzymic alterations in the rodent skeletal muscle are generally accompanied by alterations in total enzymic activity. For instance, rabbit and rat skeletal muscle shows a 15–20-fold and 5-fold increases respectively in PFK activity during the first 4–6 weeks of life (Baldwin *et al.*, 1977; Beitner *et al.*, 1979; Thrasher *et al.*, 1981), when the muscle-specific isoenzyme pattern is established (De Faria *et al.*, 1978; Thrasher *et al.*, 1981). In contrast, like human organs, rabbit brain and liver exhibit neither quantitative changes nor isoenzymic alterations. Despite striking isoenzymic transitions (De Faria *et al.*, 1978), neonatal rabbit heart does not show quantitative increases in total PFK activity, whereas cardiac muscle from foetal and neonatal rat shows mild increases (1.3–1.8-fold) in PFK activity (Baldwin *et al.*, 1977; Thrasher *et al.*, 1981) accompanied by mild isoenzymic transitions (Thrasher *et al.*, 1981).

These studies indicate that the human PFK system shows developmental alterations very similar to, but not identical with, those of other multigenic isoenzyme systems involved in glycolysis, i.e. those of pyruvate kinase and fructose biphosphate aldolase (Edwards & Hopkinson, 1977; Schapira, 1981). In most such systems, there is a tendency towards uniformity in early foetal life, so that the isoenzyme patterns are similar in all organs. As development proceeds, distinct tissue-specific isozyme patterns are established; most changes occur during the later half of gestation and in the neonatal period. In general, skeletal muscle and cardiac muscle show loss of liver-type (acidic) isoenzyme(s) and acquisition of muscle-type (basic) isoenzyme(s) during the second half of gestation, when contractility is acquired. In contrast, brain and liver exhibit early maturation of isoenzyme profiles, which include mainly liver-type isoenzyme(s) and show relatively

few changes during the second half of gestation and early infancy (Edwards & Hopkinson, 1977).

The observed changes in PFK probably reflect changing patterns of gene expression resulting from the relative activation of one locus and occasional repression of another, e.g. *PFKL* and *PFKP* loci are distinctly repressed in human skeletal muscle at term. Although transcriptional control of PFK isoenzymes appears likely, it is noteworthy that the final isoenzyme patterns are not only determined by the relative activity of the genes, i.e. rates of enzyme synthesis, but also by the relative stabilities *in vivo* and rates of degradation of the synthesized enzyme molecules (Vora, 1982).

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