Europ. J. Cancer Vol. 15, pp. 1131–1137.

0014-2964/79/0901-1131 $02.00/0

Liver Fetal Isozymes in Hereditary Tyrosinemia

C. GUGUEN-GUILLOUZO,*† M. F. SZAJNERT,* F. SCHAPIRA,* L. BELANGER‡ and A. GRENIER‡

**Institut de Pathologie Moléculaire, Unité 129 de l'INSERM, 24, rue du Faubourg St Jacques, 75674 Paris Cedex 14, France*

‡Department of Biochemistry and Department of Human Genetics, Faculty of Medicine, Laval University, Quebec, Canada

Abstract—*The purpose of this work was to establish: (a) whether the persistence of liver alpha-fetoprotein production in hereditary tyrosinemia (tyrosinosis) is an isolated phenomenon or represents part of a generalized display of fetal liver functions and (b) whether this functional disturbance is correlated to the biochemical injury. To answer these questions, we have analyzed the pattern of aldolase and pyruvate kinase isozymes in five tyrosinemic liver extracts. Our data show, by kinetic, electrophoretic, isoelectrofocusing and immunological criteria, the presence of fetal-type isozymes, together with a decrease in normal adult-type liver isozymes. The fetal isozyme pattern was more pronounced for pyruvate kinase than for aldolase. These observations demonstrate that fetal isozyme production occurs in parallel with that of alpha-fetoprotein. The presence of a subnormal isozymic pattern in one case of early precirrhotic tyrosinosis, and the variations of this pattern according to the liver fragment tested in another case, suggest that the expression of fetal liver functions in tyrosinosis appears to depend on the existence of regeneration processes rather than on the biochemical alteration of the disease.*

INTRODUCTION

HEREDITARY tyrosinemia (tyrosinosis) is an autosomal recessive metabolic disorder with liver cirrhosis and renal tubular dysfunctions [1, 2]. The biochemical basis of the disease is uncertain, but blocks in the normal metabolism of tyrosine, and possibly methionine, appear to play a role in its development [1, 3–8]. One of the remarkable features of tyrosinosis is the persistence of active production of alpha-fetoprotein (AFP) by the liver [8–13]. This observation, with the notion that tyrosinemic livers are highly pre-malignant [14], raised considerable interest in regard to cellular and biochemical mechanisms involved in liver cell differentiation and malignant transformation.

Whether AFP expression in tyrosinosis is an isolated phenomenon or represents part of a generalized display of fetal liver functions and whether this functional disturbance is correlated to the biochemical injury, has not been established yet. To answer these questions, we have analyzed the pattern of aldolase and pyruvate kinase isozymes in tyrosinemic liver extracts.

Three types of aldolase are known to exist in higher animals: aldolase A (muscle type), B (liver type) and C (brain type). These isozymes differ in their electrical charge and immunological properties. Further, their relative activity towards the two substrates fructose-1,6-diphosphate (FDP) and fructose-1-phosphate (F1P) is different: the FDP/F1P activity ratios are 50, 1 and 7 for aldolase A, B and C, respectively. Aldolase B is almost the only form present in normal adult liver, whereas aldolase A and C are present in important amounts in fetal liver [15]. One of us has described the resurgence of the fetal liver forms of aldolase isozymes in rat and human hepatomas, along with a decrease of the normal adult liver form [16–17]. A moderate "fetal deviation" has also been observed during experimental liver regeneration and in some other hepatic diseases [18].

The exact distribution of pyruvate kinase isozymes is not clearly defined yet. Nevertheless, it is known that the liver-type

Accepted 20 February 1979.

†C. Guguen-Guillouzo's present address: INSERM, Unité de Recherche U 49 Hôpital Pontchaillou, 35011 Rennes Cédex, France.

pyruvate kinase (PK L) is predominant in normal adult liver, whereas the fetal-type (PK III, or M_2) is predominant in fetal liver and present in minor amounts in normal adult liver [19]. PK III is distinguished from PK L on the basis of their different isoelectric points. Recently, Marie *et al.* [20] reported the resolution, by isoelectric focalization, of several sub-fractions of PK III (M_2, M_3, M_4). In addition to fetal tissues, M_2 and M_3 are found in placenta, spleen and kidney. As for aldolase a shift of pyruvate kinase isozyme activities in hepatomas towards a fetal liver phenotype pattern has been documented [21].

In this study, electrophoretic, immunological and isoelectrofocusing analyses, and substrate relative activity measurements were all employed to assess the aldolase and pyruvate kinase isozyme distribution in tyrosinemic livers.

MATERIALS AND METHODS

Liver specimens from 5 patients with tyrosinosis were studied: one (T_1), obtained by surgical biopsy from a 4 month-old infant, had a limited AFP production (1 μg/ml) and presented a subnormal histology. Three liver specimens (T_2, T_3, T_4) were obtained at autopsy from 2-yr old patients; T_2 and T_3 had an actively regenerating, high AFP-producing [8] cirrhosis (serum AFP levels 60 and 180 μg/ml, respectively). T_4 presented a subnormal liver histology with minimal liver regeneration and limited AFP production (5 μg/ml of serum). Another specimen (T_5) from a 15-yr old patient, obtained by surgical biopsy, had an active AFP production (120 μg/ml) and presented cirrhotic nodules. Two different fragments from this specimen were studied.

Normal liver specimens were taken from infants and children of different ages by autopsy or during surgery for non-hepatic diseases. All specimens were kept at −70°C until the time of analysis. The tissues were homogenized in Tris 10 mM, EDTA 1 mM, pH 7.5 buffer for aldolase assays, and in Tris–HCl 50 mM, pH 8.0, 2 mM dithiothreitol for pyruvate kinase. The homogenates were centrifuged for 10 min at 15,000 *g* and the supernatants submitted to kinetic and electrophoretic experiments. Total proteins were measured by the Lowry's procedure [22].

Aldolase and pyruvate kinase specific activities were determined, respectively, by Sibley and Lehninger's method [23], with adaptations for the FIP substrate and by Bücher and Pfleiderer's method [24].

Aldolase isozymes were separated by polyacrylamide gel electrophoresis and stained according to Penhoet *et al.* [25]. The presence of aldolase A and C was also demonstrated by double diffusion reactions against anti-aldolase A and anti-aldolase C antisera. These antisera were obtained by immunization of chickens with purified rabbit isozymes isolated from muscle (aldolase A), liver (aldolase B) and brain (aldolase C), as previously described [15].

Pyruvate kinase isozymes were resolved by isoelectrofocusing in polyacrylamide gels, according to Vesterberg [26], with modifications detailed elsewhere [27]. Immunoinactivation experiments were performed with anti PK L antiserum, obtained by immunization of rabbits with pure human liver PK L prepared according to Kahn *et al.* [28]; the immunoinactivation assays were conducted in a "protective solution", as described by Marie *et al.* [20].

RESULTS

Aldolase

In one tyrosinemic liver (T_1), electrophoretic analyses showed a subnormal isozymic pattern similar to those from normal infants. In the other tyrosinemic specimens studied, electrophoretic patterns showed, in addition to the normal adult liver aldolase isozyme (aldolase B), the presence of the fetal aldolase isozyme A together with a hybrid A–C, especially in T_3 and in one fragment of T_5 (T_5b). By contrast the other fragment of T_5 (T_5a) showed a subnormal pattern (Fig. 1). These electrophoretic differences in T_5 were associated with histological differences. Cirrhotic nodules and fibrosis were seen in T_5b but not in T_5a (not shown). The presence of aldolase A and C was also demonstrated by double immuno-diffusion experiments with the corresponding specific antisera (Fig. 2). In immunoinactivation assays, the inhibition of aldolase activity by anti-aldolase A antiserum was greater in three tyrosinemic extracts (T_2, T_3, T_5b) than in the controls (up to 35%, compared to 7.5%) (Table 1) and in T_1 and T_5a. The FDP/F1P relative activity ratios were also higher in tyrosinemic livers except in T_1 and T_5b which had a normal ratio (Table 2).

Thus, all these data indicate that in tyrosinemic liver, except at the early stage of the

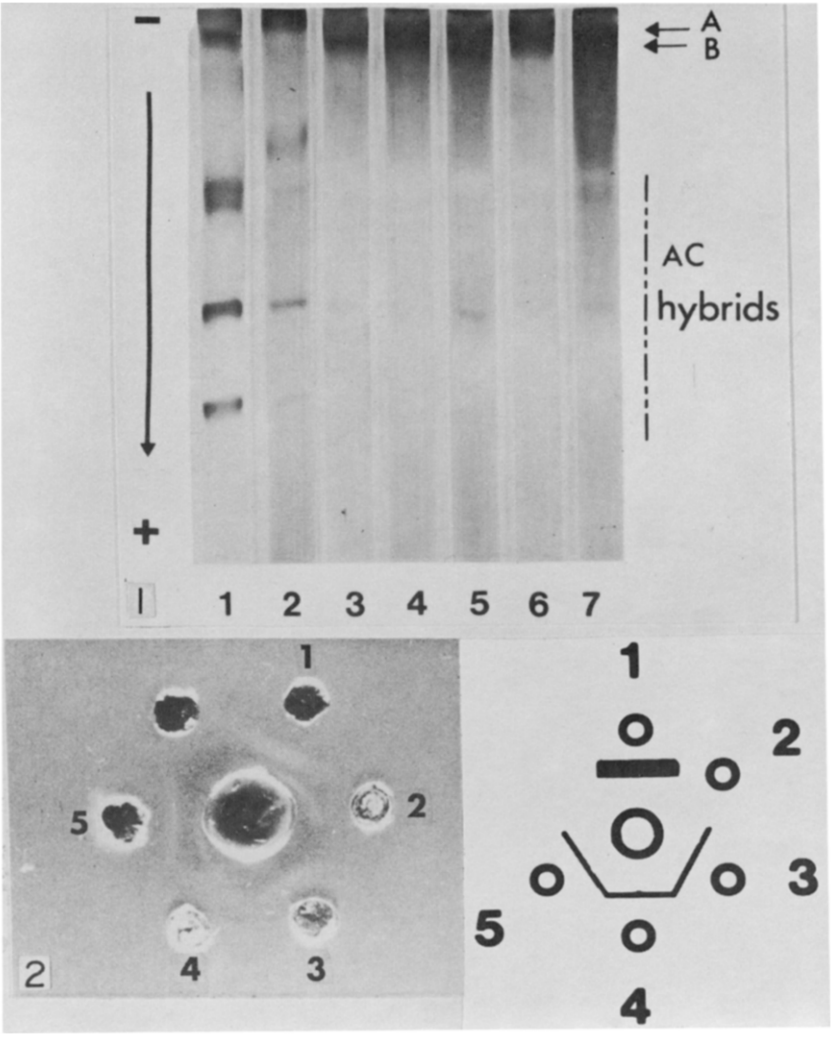

Fig. 1. *Polyacrylamide gel electrophoresis pattern of aldolase isozymes in normal and tyrosinemic human tissue extracts:* 1, *normal adult brain;* 2, *tyrosinemic liver* T_3*;* 3, 10-yr *old normal liver;* 4, *tyrosinemic liver* T_5b; 5, *tyrosinemic liver* T_5a; 6, *newborn normal liver;* 7, *tyrosinemic liver* T_1.

Fig. 2. *Double diffusion reaction of human tissue extracts against anti-aldolase C antiserum:* 1, *normal adult brain;* 2, *normal adult liver;* 3, 4, 5, *tyrosinemic livers* T_2, T_3, T_4, *respectively. Central well, anti-aldolase C antiserum.*

The extracts were assayed at uniform protein concentration. All three tyrosinemic liver extracts show a precipitin line, indicative of the presence of fetal liver-type aldolase C.

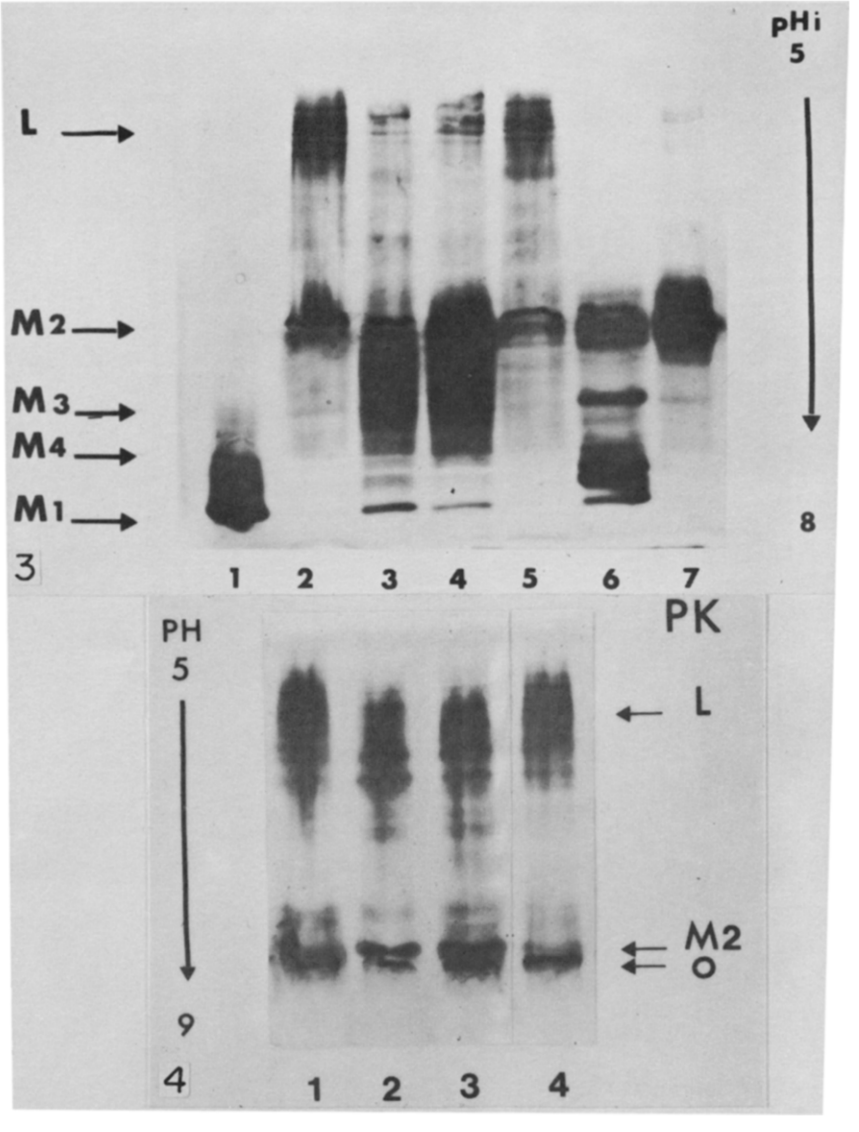

Fig. 3. Isoelectric focalization in polyacrylamide gel of pyruvate kinase isozymes from normal human tissue and tyrosinemic liver extracts: 1, adult muscle; 2, adult liver; 3, 4, 5, tyrosinemic livers T_2, T_3, T_4, respectively; 6, fetal brain; 7, placenta. Two tyrosinemic extracts T_2 and T_3 show a heavy dominance of the fetal-type M_2 and M_3 isozymes, together with a marked decrease of the normal adult liver-type isozyme PK L.

Fig. 4. Isoelectric focalization in polyacrylamide gel of pyruvate kinase isozymes from normal and tyrosinemic liver: 1, adult liver; 2, newborn liver; 3, 10-yr old liver; 4, tyrosinemic liver T_1.

Table 1. Percentage of inhibition of FDP activity by antisera anti-aldolase A and B in normal infant liver and in tyrosinemic liver

	Antialdolase A	Antialdolase B
Normal liver	7.5±2.1*	95.0±2.5*
Tyrosinemic liver	T_1 :10	91
	T_2 :20	94
	T_3 :35	85
	T_4 :12	96
	T_5a:10	97
	T_5b:18	95

*Standard error.

Table 2. Ratio of aldolase activities towards fructose-1-6-diphosphate and fructose-1-phosphate (FDP/F1P ratio) in normal and tyrosinemic human liver

Specimens	FDP/F1P ratio
Normal liver	
Adult and ≧10-yr old	1.15±0.1*
One month–10-yr old	1.25±0.1*
Tyrosinemic liver	
T_1	1.29
T_2	1.40
T_3	2.00
T_4	1.25
T_5a	1.35
T_5b	1.32

*Standard error.

disease, the aldolase isozyme distribution was slightly shifted towards a fetal pattern.

Pyruvate kinase

The gel electrofocusing analyses disclosed profound modifications in the pyruvate kinase isozyme pattern of T_2 and T_3 liver extracts, with a heavy predominance of the M_2 and M_3 isozymes (fetal types), and a remarkable relative decrease in the L form (adult liver type) (Fig. 3). The isozyme distribution in T_4 liver appeared relatively normal, with a dominant L isozyme and a minor M_2 component. The T_1 specimen showed the same isozyme distribution as that of the normal liver from a 2-month old infant and a 10-yr old child, which was characterized by the presence of small amounts of M_2 form (Fig. 4). Moreover, one of the two fragments of T_5 (T_5b) was almost normal whereas the other (T_5a) showed an important shift towards the fetal M_2 and M_3 forms.

The PK L immunoinactivation experiments confirmed electrofocusing analyses. By adding anti PK L antiserum to liver extracts, about 30% of pyruvate kinase activities were inhibited in T_2 and 10% were inhibited in T_3 and in T_5a. A nearly normal value was obtained for T_1, T_4 and T_5b (Table 3). Thus, these data demonstrate a shift of the pyruvate kinase isozyme profile towards a fetal pattern even more marked than for aldolase.

DISCUSSION

This study establishes, by kinetic, electrophoretic, isoelectrofocusing and immunological criteria, the presence of fetal pyruvate kinase isozymes in the liver of 4 patients with hereditary tyrosinemia and the presence of fetal aldolase in 2 out of 5 patients. The fetal isozymic pattern was less pronounced for aldolase than for pyruvate kinase, except in case T_3. Thus, the expression of AFP in this disease coincides with that of other fetal proteins: this points to a general pattern of abnormal liver cell differentiation.

Early observations had shown a direct relation between tyrosinemic liver regenerative activity and serum AFP levels [13]; direct immunolocalization studies recently confirmed that AFP-containing cells are mainly confined to the regenerative areas of the cirrhotic livers [29]. The present data demonstrating a close quantitative relationship between the expression of fetal isozymes, that of AFP, and the intensity of liver regeneration processes, provides strong indirect evidence that the expression of fetal isozymes is also mainly connected with regenerative cell activity. This suggests some link between expression of immature liver functions and cell multiplication and growth: in this respect, we recall that the fetal isozyme pattern occurring with a decrease of adult types has been observed principally in hepatomas and to a more moderate extent in liver regeneration [18]. The

Table 3. Immuno-inactivation of liver pyruvate kinase activities by anti PK L antiserum in normal and tyrosinemic human liver

Specimens	Percentage of inhibition
Normal liver	
adult*	80
newborn	63
10-yr old	65
Tyrosinemic liver	
T_1	55
T_2	30
T_3	10
T_4	60
T_5a	63
T_5b	10

*Mean of 5 samples.
Inhibition tests were performed by incubating different extracts (at final comparable concentration) with 0.3 μl of antiserum at 37°C for 1 hr and at 4°C for 1 hr. After centrifugation supernatants were estimated for PK activity by comparison with extracts treated with normal rabbit serum.

presence of a normal pattern in the tyrosinemic liver T_1, an early precirrhotic stage as well as the wide differences in the isozyme pattern of the 2 areas of T_5 demonstrate that the production of fetal proteins is not linked to the genetic disease itself. A likely explanation for the resurgence of fetal liver functions in tyrosinosis appears to depend on this existence of regenerating cells which keep their fetal protein complement. It is tempting to speculate that such regenerating cells may be highly pre-malignant and form the basis for the strong incidence of neoplastic transformation of tyrosinemic livers [14]. Hereditary tyrosinemia may provide a powerful system to study the mechanisms of liver differentiation and malignant transformation in man.

Acknowledgements—We thank Dr. A. Kahn for kind gift of anti-pyruvate kinase antiserum; and Dr. J. Larochelle, Dr. M. Belanger, Dr. L. Prive, and Dr. C. Laberge for their clinical collaboration. L.B. is supported by a post-doctoral Fellowship from the Medical Research Council of Canada.

REFERENCES

1. J. Larochelle, A. Mortezai, M. Belanger, M. Tremblay, J. C. Claveau and G. Aubin, Experience with 37 infants with tyrosinemia. *Can. med. Ass. J.* **97,** 1051 (1967).
2. P. Bergeron, C. Laberge and A. Grenier, Hereditary tyrosinemia in the province of Quebec; prevalence at birth and geographic distribution. *Clin. Genet.* **5,** 157 (1974).
3. B. N. La Du, The enzymatic deficiency in tyrosinemia. *Am. J. Dis. Child.* **113,** 54 (1967).
4. J. Gentz, R. Jagenburg and R. Zotterstrom, Tyrosinemia. *J. Pediat.* **66,** 670 (1965).
5. C. R. Scriver, The phenotypic manifestations for hereditary tyrosinemia and tyrosyluria: a hypothesis. *Can. med. Ass. J.* **97,** 1073 (1967).
6. G. E. Gaul, D. K. Rassin and J. A. Sturman, Significance of hypermethioninemia in acute tyrosinosis. *Lancet* **i,** 1318 (1968).
7. T. L. Perry, Tyrosinemia associated with hypermethioninemia and islet cell hyperplasia. *Can. med. Ass. J.* **97,** 1067 (1967).
8. L. Belanger, J. Larochelle, M. Belanger and L. Prive, Tyrosinosis: hereditary persistence of alpha-1-fetoprotein. In *Onco-Developmental Gene Expression.* (Edited by W. H. Fishman and S. Sell) p. 155. Academic Press, New York (1976).
9. L. Belanger, M. Belanger and J. Larochelle, Presence of circulating alpha-1-fetoprotein in 8 patients with hereditary tyrosinemia. *Un. méd. Can.* **101,** 877 (1972).

10. M. BELANGER, B. ST.-HILAIRE and L. BELANGER, Screening for inborn metabolic diseases. *Un. méd. Can.* **102,** 294 (1973).
11. D. BUFFE and C. RIMBAUT, L'alpha-1-foetoprotéine dans les atteintes hépatiques et les maladies métaboliques de l'enfant. *Biomedicine* **19,** 172 (1973).
12. A. GRENIER, L. BELANGER and C. LABERGE, α_1-Fetoprotein measurement in blood spotted on paper: discriminating test for hereditary tyrosinemia in neonatal mass screening. *Clin. Chem.* **22,** 1001 (1976).
13. L. BELANGER, M. BELANGER, L. PRIVE, J. LAROCHELLE, M. TREMBLAY and G. AUBIN, α_1-Fetoprotein in hereditary tyrosinemia. I. Clinical use. *Path. Biol. (Paris)* **21,** 449 (1973).
14. A. G. WEINBERG, C. E. MIZE and H. G. WORTHEN, The occurrence of hepatoma in the chronic form of hereditary tyrosinemia. *J. Pediat.* **88,** 434 (1976).
15. A. HATZFELD and F. SCHAPIRA, The ontogeny of aldolase in rat liver and brain. *Biochimie* **55,** 153 (1973).
16. F. SCHAPIRA, J. C. DREYFUS and G. SCHAPIRA, Anomaly of aldolase in primary liver cancer. *Nature (Lond.)* **200,** 995 (1963).
17. F. SCHAPIRA, Isozymes and cancer. *Adv. Cancer Res.* **18,** 77 (1973).
18. A. WEBER and F. SCHAPIRA, Les isozymes du foie en régénération. *C. R. Soc. Biol. (Paris)* **166,** 312 (1972).
19. C. GUGUEN, C. GREGORI and F. SCHAPIRA, Modification of pyruvate kinase isozymes in prolonged primary cultures of adult rat hepatocytes. *Biochimie* **57,** 1065 (1975).
20. J. MARIE, A. KAHN and P. BOIVIN, Pyruvate kinase isozymes in man. I. M-type isozymes in adult and fetal tissues, electrofocusing and immunological studies. *Human Genet.* **31,** 35 (1976).
21. F. SCHAPIRA and C. GREGORI, Pyruvate kinase de l'hépatome, du placenta et du foie foetal de rat. *C. R. Acad. Sci.* (Série D). **272,** 1169 (1971).
22. O. H. LOWRY, N. J. ROSENBROUCH, A. L. FARR and R. J. RANDALL, Protein measurement with the Folin phenol ragent. *J. biol. Chem.* **193,** 265 (1951).
23. J. A. SIBLEY and A. D. LEHNINGER, Determination of aldolase in animal tissues. *J. biol. Chem.* **177,** 859 (1949).
24. T. BUCHER and G. PFLEIDERER, Pyruvate kinase from muscle. In *Methods in Enzymology.* (Edited by S. P. Colowick and N. O. Kaplan) Vol. 1, p. 435. Academic Press, New York (1955).
25. E. E. PENHOET, T. RAJKUMAR and W. J. RUTTER, Multiple forms of fructose diphosphate aldolase in mammalian tissues. *Proc. nat. Acad. Sci. (Wash.)* **56,** 1275 (1966).
26. O. VESTERBERG, Isolectric focusing of proteins in polyacrylamide gels. *Biochim. biophys. Acta (Amst.)* **257,** 11 (1972).
27. C. GUGUEN-GUILLOUZO, M. F. SZAJNERT, J. MARIE, D. DELAIN and F. SCHAPIRA, Differentiation *in vivo* and *in vitro* of pyruvate kinase isozymes in rat muscle. *Biochime* **59,** 65 (1977).
28. A. KAHN, J. MARIE, C. GALAND and P. BOIVIN, Molecular mechanisms of erythrocyte pyruvate kinase deficiency. *Humangenetik* **29,** 271 (1975).
29. A. GUILLOUZO, G. FELDMANN and L. BELANGER, Localization of alpha-fetoprotein-containing liver cells in tyrosinosis and in newborn rat. In *Onco Developmental Gene Expression.* (Edited by W. H. Fishman and S. Sell) p. 647. Academic Press, New York (1976).