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Glucose Utilization in Vertebrates as a Molecular Probe for the Study of Evolution

Utilización de glucosa en vertebrados como herramienta molecular para el estudio de la evolución

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Hexokinase isozymic profiles from the liver of 68 vertebrate species are presented. The comparison of the diverse patterns observed, as well as the kinetic and physicochemical properties of the isozymes, reveals that the hexokinases from mammals are very similar to those from turtles and amphibians. The hexokinases from birds, lizards and snakes on the other hand are similar within themselves and different from the enzymes from mammals and amphibians. Liver pyruvate kinases show about the same behavior. The hexokinase system from vertebrate muscle however is very uniform in all the species studied consisting mainly of hexokinase B.

ISOZYMES HEXOKINASES PYRUVATEKINASES EVOLUTION

The truly enormous variety of living organisms sharply contrasts with the (by comparison) monotonous uniformity of the biochemical machinery of the same organisms. Although a few reactions, *e.g.* pigment biosynthesis, building of cell walls, etc., may be considered typical for some taxa, some fundamental processes (best illustrated by the universality of the genetic code, the conservatism of protein synthesis and the ubiquity of glycolytic reactions) are found to be similar wherever investigated. Nevertheless, even within the uniformity of the metabolic blueprint some latitude *must* exist to account for such different results as *Escherichia coli* and *Elephas maximus*. It is important then to catalogue and understand molecular differences especially in the intermediary, exergonic reactions of the metabolism of organisms. In fact, these processes are the ones on which cells depend upon for energetic purposes and therefore presumably permit adaptation to diverse ecological niches. The great amount of scattered available information has not been yet comprehensively collated (see however Hochachka and Somero (1)).

The recognition of isozymes, *i.e.* multiple molecular forms of enzymes catalyzing the same reaction in a cell or organism, has permitted a profitable approach to the study of biochemical diversity since isozymes usually present qualitative and quantitative differences when studied in a variety of organisms. The elegant studies of Markert and his group (2, 3) and of several other investigators on the isozymes of lactate dehydrogenases and other systems (for a review see Masters and Holmes (4)) have illustrated the usefulness of this approach to the study of evolution and molecular taxonomy[1].

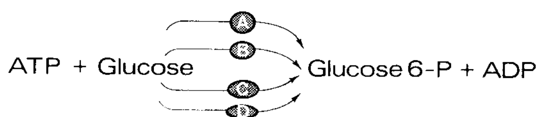
[1]The detection of isozymes has resulted in a useful device to study several problems of organismic biology. In fact, the use of isozymes (allozymes) as genetic markers has become a standard tool in taxonomy and population genetics. A proper utilization of the isozymic phenomenon, however, should be based on the understanding of

This essay will review data gathered in our laboratory on the isozymology of the hexokinases and pyruvate kinases from vertebrates in a attempt to offer a comparative view of glucose utilization which may be used as a probe to understand the mechanisms of enzyme evolution and its significance to the overall performance of organisms. Needless to say, no clear answer will be forthcoming but some proposals which can lead to experimental tests will be put forward.

The Hexokinases from the Liver of Vertebrates

Several reports from this laboratory (6-13) have dealth with the isozymic system catalyzing the phosphorylation of glucose in vertebrate liver.

Scheme 1



The four hexokinases (ATP:D-hexose 6-phosphotransferases, EC 2.7.1.1) were called A, B, C., and D, primarily according to their order of elution from diethylaminoethyl-(DEAE-) cellulose columns (6). Later on, their kinetic properties and substrate specificities (for reviews see 12, 14-17) were also taken into account.

A summary of the chromatographic profiles observed in the liver of 68 vertebrate species is depicted in Fig. 1. The animals studied have been grouped together within each Class of vertebrates according to the presence or absence of one or more isozymes, *i.e.* taking into account qualitative differences only. Quantitative variation (relative proportion of each isozyme) of the patterns has also been observed within the groups (18, 19) but because of assay restrictions, dietary conditions, etc., we think that quantitative enzyme profiles are a poor indication of the metabolic state of the tissues or of the underlying control mechanisms involved (see for instance, 20-22).

Mammals have so far presented five diferent hexokinase patterns (Fig. 1, *top*). The pattern ABCD may be also present as ACBD in some rodents, *e.g. Mus, Cavia, Mesocricetus* (8). Hexokinase A is the only isozyme which is never absent in all the mammals so far studied. No simple taxonomic relationships can be derived from the profiles observed.

Kinetic and physicochemical characterization of the partially purified mammalian hexokinases have shown quite distinct properties for each member of the system (Table I). On the basis of several facts (for reviews see 12, 15, 16) it has been concluded that each mammalian hexokinase is the product of a separate gene[2].

On the other hand, almost no variation is observed in the case of the hexokinases from Aves and higher Reptiles (10, 11). In fact, the hepatic isozymic patterns from birds, lizards, serpentes and *Amphisbaena alba* are very similar (Fig. 1, *second and third rows).* None of these animals possess a substrate-inhibited isozyme (hexokinase C) or a high K_m isozyme (hexokinase D). Moreover, the properties of the isozymes from birds (Table II) and higher Reptiles (Table III) differ so markedly from those of mammals (Table I) that, for the time being we have chosen not to use the designation employed for mammals (ABCD system), and have used a provisory nomenclature (α β γ) instead).

The hexokinase pattern from turtles (Fig. 1, *third row, extreme left)* are qualitatively similar to the mammalian type ABCD. Also, the properties of turtle hexokinases (Table IV), albeit not so well studied, are very similar to those of mammals, except that hexokinase C has yet to be clearly identified in turtles (11).

The isozymic profile from the liver of an individual *Caiman latirostris* (Fig. 1, *third row, extreme right)* shows two hexokinases (T.

the biological role of the multiplicity of enzyme forms. The realization that research on the role of isozymes is just in its very beginnings (5) may become a surprise to many workers.

[2]The quaternary structure of vertebrate *non-mammalian* hexokinases has not been studied. It seems safe, however, to consider all vertebrate hexokinases as products of separate genes, *i, e.* not the result of hybrid combinations as in the lactate dehydrogenase system (24).

VERTEBRATE HEXOKINASES

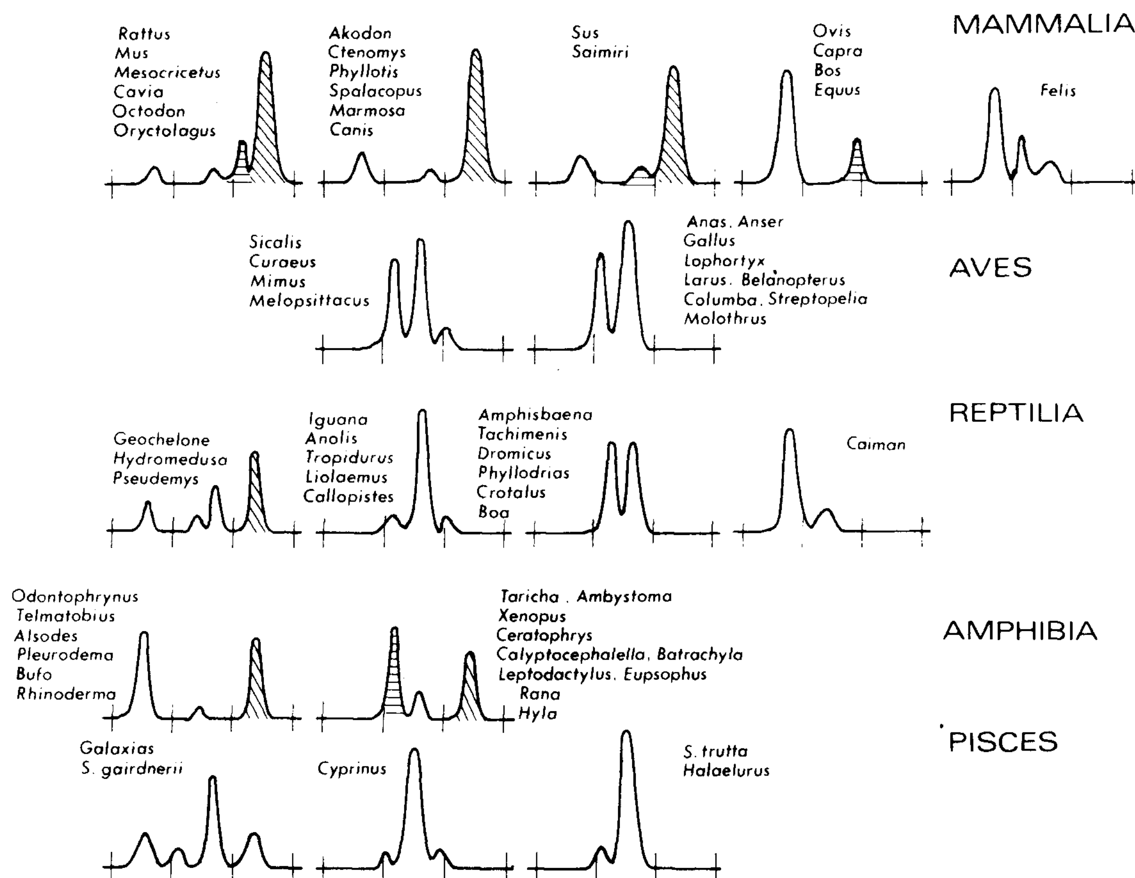


Fig. 1. The Hexokinases from Vertebrate Liver. A summary of chromatographic profiles of the hexokinases from 68 vertebrate species. In all cases DEAE-cellulose columns were used for the separation of isozymes. Small vertical lines indicate KCl concentrations (0-100-200-300 mM) at which the isozymes elute. Horizontal shades indicate that the enzyme peak is inhibited by high glucose concentrations. Inclined shades indicate that the enszyme peak has a K_m value for glucose > 1mM. The different categories of isozymic profiles are based on the absence of one or more isozymes within each Class of vertebrates. Because most species present a unique *quantitative* hexokinase pattern, the idealized profiles here represented constitute a rather average pattern within each category.

Ureta, unpublished). Because of the very low activity levels of the isozymes no kinetic characterization was possible. However, the chromatographic mobilities of both hexokinases are similar to those of the mammalian isozymes A and B. In other words, *Caiman* hexokinases are very different from those of birds and higher Reptiles[3]

[3]The Order Crocodilia is considered a derivation from an Archosaurian stock, *i.e.* the same ancestral stock that originated birds. In fact, several anatomical features of both groups have led to the hypothesis — with which many zoologists agree — that crocodiles are more closely related to Aves than to any other living reptiles (25). However, the common origin of birds and crocodiles is becoming a controversial area. Although space restrictions do not allow an extended discussion on this problem, we would like to point out the profound differences observed in karyotypes and DNA nuclear content between birds and crocodiles (26-28). Those differences, as well as our own observations on vertebrate hexokinases, suggest closer relations between birds and serpentes on the one hand, and crocodiles, turtles and mammals on the other.

Table I

Some properties of the glucoe phosphorylating isozymes from mammalian liver

Parameter	Hexokinase A	B	C[a]	D[b]
Substrate specificity[c]		relative V_{max}		
Glucose	1.0	1.0	1.0	1.0
Mannose	—	—	1.0	0.8
2-Deoxyglucose	—	—	1.0	0.4
Fructose	1.1	1.2	1.3	0.2
Michaelis constants		mM		
Glucose	0.039±0.004[d]	0.162±0.043[e]	0.035±0.008[f]	5.4±0.9[g]
Fructose[c]	3.1	3.0	1.2	12
ATP[c]	0.4	0.7	1.3	0.5
		mM KCl		
Chromatographic mobility[h]	70±10	152±10	153±19	224±9
Molecular weights[c]	100,000	100,000	100,000	55,000

[a]The substrate-inhibited isozyme; [b]The high $K_{m\ glucose}$ isozyme (also called glucokinase); [c]From Table I of Ureta (12); [d]Mean ± S.E. of measurements on hexokinase A from 15 species (T. Ureta, J. Radojković, S. Zepeda and M. Rozo, *manuscript in preparation); *[e]Mean ± S.E. of observations on hexokinase B from 7 species (Ureta *et al. in preparation;* [f]Mean ± S.E of measurements on hexokinase C from 5 species (Ureta *et al. in preparation);* [g]Mean ± S. E of values on hexokinase D reported by Niemeyer *et al.* (23); [h]Mean ± S. E of observations from 5 rodents reported by Ureta *et al.* (8).

TABLE II

Some properties of the glucose phosphorylating isozymes from avian liver

Parameter	Hexokinase α	β
Substrate specificity[a]	relative V_{max}	
Glucose	1.00	1.00
Mannose	2.00	0.87
2-Deoxyglucose	2.04	1.12
Fructose	2.24	1.89
Michaelis constants	mM	
Glucose	0.113±0.026[b]	0.083±0.012[c]
Mannose[a]	0.079	0.099
Fructose[a]	4.32	2.62
ATP[a]	1.24	0.66
	mM KCl	
Chromatographic mobility[d]	119±15	174±12
Molecular weights[a]	98,000	95,000

[a]Measurements on the hexokinases from chicken liver (9); [b]Mean ± S.E of observations on hexokinase α from 6 avian species (10); [c]Mean ± S. E of measurements on hexokinase β from 7 avian species (10); [d]Mean ± S.E of observations on 13 avian species (10).

TABLE III

Some properties of the glucose phosphorylating isozymes from the liver of higher reptiles

Parameter	Hexokinase α	Hexokinase β	Hexokinase γ
Substrate specificity		relative V	
Glucose	1.00		1.00
Fructose	1.32[a]	—	1.49[b]
Michaelis constants		mM	
Glucose	0041±0.004[c]	0.079±0.028[d]	0.114[b]
Fructose	3.9[a]	10.8[b]	4.9[b]
		mM KCl	
Chromatographic mobility	134±13[e]	166±14[e]	201±21[f]

[a]A single measurement on the enzyme from *Tachimenis peruviana* (unpublished experiments of J. Radojković and T. Ureta); [b]A single measurement on the enzyme from *Liolaemus gravenhorstii* (unpublished); [c]Mean ± S. E of measurements on hexokinase α from the liver of *Liolaemus tennuis, Amphisbaena alba, T. peruviana* and *Dromicus chamissonis* (11); [d]Mean ± *S. E* of measurements on hexokinase β from *L. gravenhorstii, L. tennuis* and *A. alba* (11); [e]Mean ± S. E of measurements on the hexokinases from 10 species of reptiles excluding turtles (11); [f]Mean ± S. E of observations on 7 species of reptiles excluding turtles (11). Dashes indicate measurements not performed.

Four hexokinases can be demonstrated in amphibian liver and their properties (Table V) are almost identical to those of mammals. However, any given amphibian displays only three isozymes and the profiles so far observed are either of the type ABD or CBD (Fig. 1, *fourth row*). A detailed study of the amphibian hexokinases has recently been reported (13, 30) and

TABLE IV

Some properties of the glucose phosphorylating isozymes from turtle liver

Parameter	Hexokinase *A*	Hexokinase *B*	Hexokinase *C*[a]	Hexokinase *D*
Substrate specificity		relative V_{max}		
Glucose	—	1.00	1.00	1.00
Mannose	—	1.21[b]	—	0.89*
2-Deoxyglucose	—	1.11[b]	—	0.31*
Fructose	—	1.22[b]	1.22[b]	0.25[b]
Michaelis constants		mM		
Glucose	0.051±0.025[c]	0.119±0.017[d]	0.111; 0.211[b]	4.6[e]
Mannose	—	0.143[f]	—	11.7*
Fructose	—	24.3[b]	—	—
		mM KCl		
Chromatographic mobility[g]	48±5	146±17	173±6	237±7

[A]Inhibition by excess substrate has not been documented for turtle hexokinase C; [b] A single measurement on the enzyme from *Geochelone chilensis* (J. Radojković and T. Ureta, unpublished); [c]Mean ± S.E. of measurements on hexokinase A from three species of turtles (11); [d]Measurements on the enzyme from two species of turtles (11); [e]$K_{0.5}$ of the enzyme from *G. chilensis* (11, 23); [f]A simple measurement on the enzyme from *Hydromedusa tectifera* (J. Radojković and T. Ureta, unpublished); [g]Mean ± S.E. of observations on three epecies of turtles (11). Dashes indicate measurements not performed. *A single measurement on the enzyme from *G. chilensis* (M.L. Cárdenas, personal communication).

TABLE V

Some properties of the glucose phosphorylating isozymes from amphibian liver

Parameter	Hexokinase *A*	*B*	*C*	*D*
Substrate specificity		relative V_{max}		
Glucose	1.00[a]	1.00[a]	—	1.00[b]
Mannose	1.04[a]	1.74[a]	—	0.64[b]
2-Deoxyglucose	1.44[a]	1.84[a]	—	0.31[b]
Fructose	1.62[a]	1.14[a]	—	0.70[b]
Michaelis constants		mM		
Glucose	0.061±0.006[c]	0.094±0.015[d]	0.044±0.005[d]	2.5±0.4[e]
Mannose	0.54[a]	—	—	—
2-Deoxyglucose	0.28[a]	—	—	—
Fructose	9.8[a]	15.2[a]	1.35[f]	430[f]
ATP	—	—	1. 0[f]	2[f]
		mM KCl		
Chromatographic mobility[g]	42±7	147±11	114±32	258±20
Molecular weights	100,000[h]	—	—	55,000[i]

[a]Measurements on hexokinases A and B from *Bufo spinulosus;* [b]Observations on hexokinase D from four anuran species; [c]Measurements on hexokinase A from *B. spinulosus, Odontophrynus americanus* and *Telmatobius halli;* [d]Mean ± S. E of measurements on hexokinases from five anuran species; [e]Mean ± S. E of values obtained on hexokinase D from four amphibian species; [f]A single measurement on the enzyme from *Calyptocephalella caudiverbera* (29); [g]Mean ± S.E of observations performed on 28 amphibian species; [h]A single observation on hexokinase A from *B. spinulosus* using sucrose gradient centrifugation; [i]Mean of measurements on hexokinase D from *Ambystoma mexicanum, B. spinulosus* and *C. caudiverbera.* All results, except those in[f], from Ureta *et al.* (13). Dashes indicate measurements not performed.

the relevance of the isozymes for the taxonomy and phylogeny of chilean anuran species is discussed elsewhere in this Symposium (31).

A few fishes have been analyzed and their chromatographic patterns of hexokinases have been included in Fig. 1, *bottom.* The very low hexokinase levels of fish liver have so far precluded the study of their kinetic properties.

A comparison of the chromatographic and catalytic properties of the hexokinases from vertebrate liver shows that the isozymes from mammals, turtles and amphibians are closely similar within themselves, and at the same time markedly differ from the isozymes from birds and higher reptiles. The scheme of Fig. 2, which is still "rather an expression of feeling than a painting" simply postulates that a mutation occurred in the evolutionary line leading to Aves and higher Reptiles, markedly affecting their hexokinase genes (13). Note that crocodiles are thought to have escaped this putative mutational event[3].

We have asked ourselves whether this mutational event has affected in some way the utilization of glucose in the animals concerned. After all, glucose phosphorylation *do* occur in the liver of birds and reptiles, and the variation of Michaelis constants, although significant, is rather small. The disappearance of hexokinases C and D in avian and reptilian liver is not without parallel in mammals (see Fig. 1, *top*) However, the following facts show that the regulation of glucose utilization in birds and higher reptiles may be quite different to that of mammals: *a)* blood glucose levels in birds are twice those observed in mammals; *b)* the regulation of blood glucose levels is, by far, less strict in reptiles than in mammals; *c)* the injection of high doses of insulin to birds does not provoke hyperglycemic convulsions as it does in mammals; *d)* total pancreatectomy in birds, lizards and serpentes is followed by a prolonged period of hypoglycemia lasting several days instead of the marked permanent hyperglycemia which it produces in mammals, turtles and alligators (for reviews see 32-34). It is tempting to speculate that at least some of those differences are causally related to the diverse isozymic repertoire of glucose phosphotransferases.

THE EVOLUTION OF HEXOKINASES

Still "Mehr Ausdruck der Empfindung als Mahlerei"

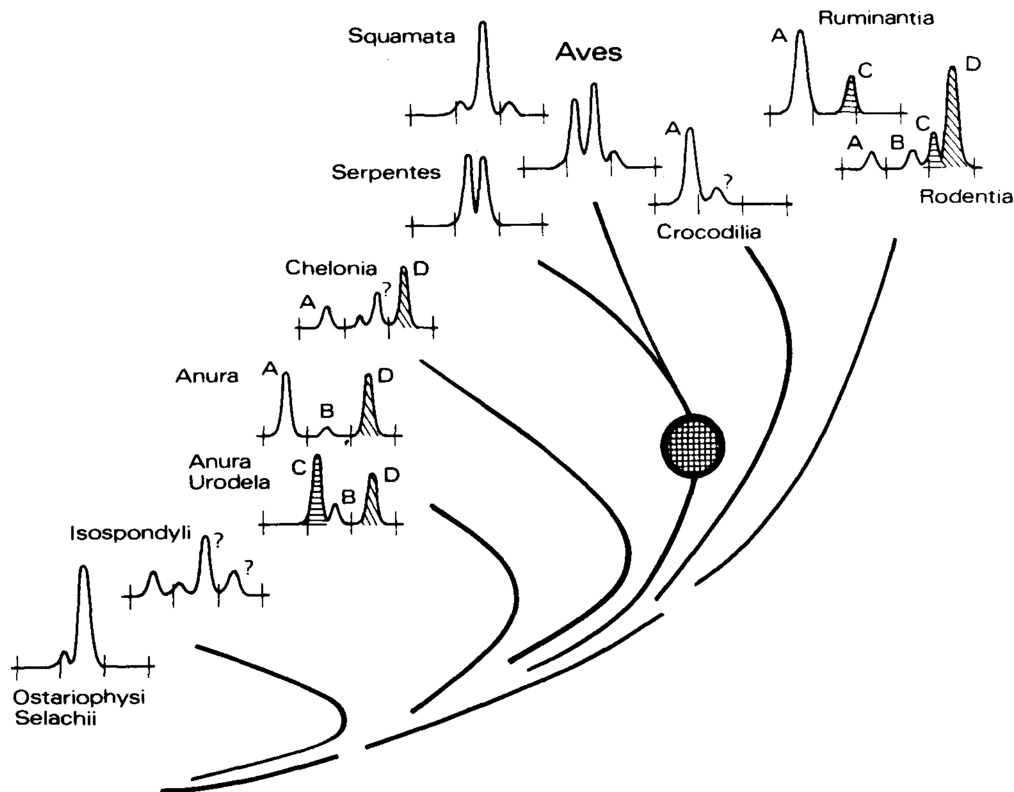


Fig. 2. The Evolution of Hexokinases. A second edition of a simplified scheme of hexokinase evolution (12). The idealized patterns at top are intended to illustrate examples of chromatographic profiles of *some* species, but within each taxa the relative proportion of the isozymes may vary considerably. See Fig. 1 for identification of the examples shown in this scheme. The circle on the line leading to Aves and Squamata marks the postulated mutational event referred to in the text. It must be stressed that this is NOT a phylogenetic tree of Vertebrates but a proposed phylogeny of a particular enzyme system in the realm of vertebrate species. Needless to say, no temporal scale is intended.

The hexokinases from vertebrate muscle

The variability of the hexokinase isozymic patterns observed in the liver of vertebrates sharply contrasts with the uniformity of isozymic profiles of the same system in skeletal muscle. Fig. 3 shows the hexokinase patterns of hind-leg muscle from some vertebrates. In some species a small amount of hexokinase A may be observed but most of the activity is accounted for by hexokinase B. The chromatographic mobility, as well as the kinetic properties (not shown) of the major muscle hexokinase are almost constant in all the species so far studied.

The uniformity of hexokinase expression in muscle may be accounted for by the marked functional specialization of the tissue which forbids almost any variation of isozymic profiles to occur since those variations may be detrimental to its contractile task. The liver tissue, with its variety of functions, may be considered as an organ which has to deal with the changes in amount and quality of nutrients. Thus, a larger number of isozymes may be necessary for a

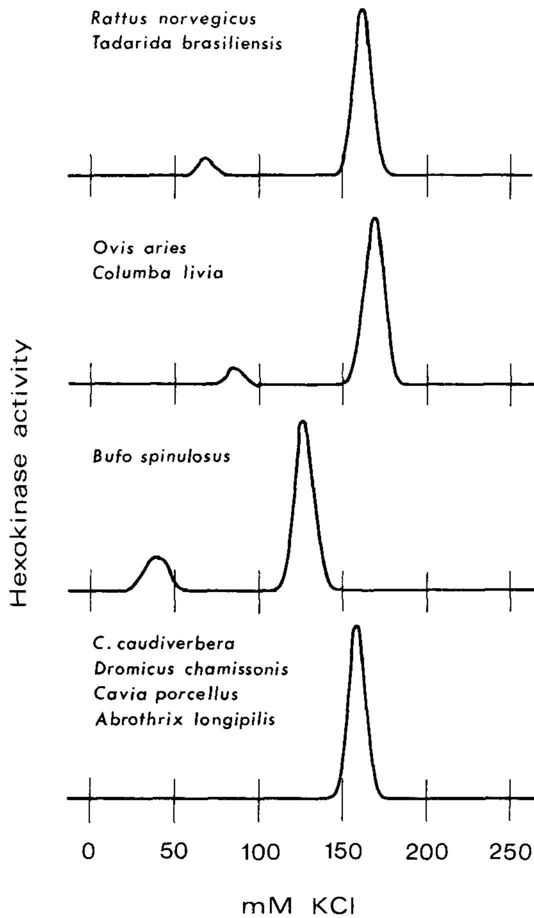


Fig. 3. The Hexokinases from Vertebrate Muscle. The isozymes from hindleg muscle were separated by DEAE-cellulose column chromatography. The horizontal scale represents the KCl concentration at which the isozymes elute from the column. The major activity peak corresponds to hexokinase B.

proper overall hepatic function and the differences in the patterns displayed may be related to the type of environment in which the animals live.

The Pyruvate Kinases from Vertebrate Liver

The differences in the regulation of carbohydrate metabolism among aves and reptiles on the one hand and mammals on the other, are not directly explained by the diverse hexokinase isozymic patterns of those animals. Nonetheless, these different isozymic profiles give us a lead to further explore the molecular basis of such metabolic differences. Thus, one may ask whether or not hexokinases are the only glycolytic enzyme system which differs in the animals studied. Fig 4 shows isozymic profiles of the

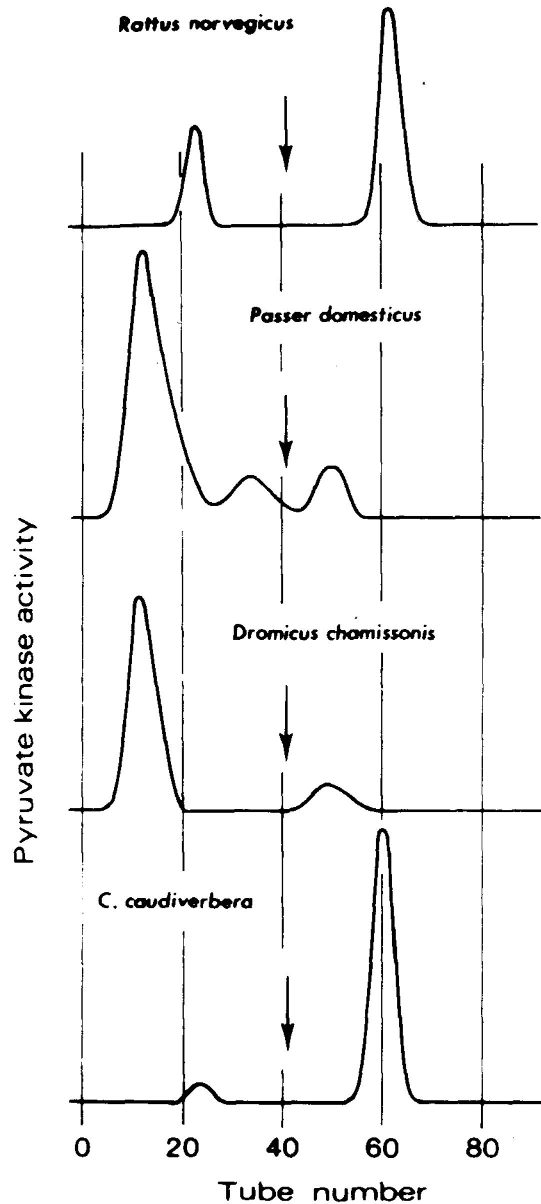
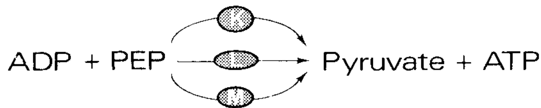


Fig. 4. Pyruvate Kinases in Vertebrate Liver. The isozymes were separated by chromatography on DEAE-cellulose columns according to Garnett *et al.* (35) and the enzyme activity assayed with the hexokinase-coupled radioassay described by Preller and Ureta (36). The same column was employed in the four experiments here depicted. From *top* to *bottom:* albino rat, common sparrow, long-tailed snake and chilean frog. The arrows (↓) mark the addition of KCl to the eluting buffer.

system catalysing the formation of pyruvate in the liver of a few vertebrates (ATP: pyruvate phosphotransferases, EC 2.7.1.40, trivial name: pyruvate kinases).

Scheme 2



Although a very small number and range of species have been so far studied with respect to pyruvate kinases, it is clear that mammals and amphibians present an almost identical pattern of pyruvate kinases which is different from the patterns present in avian and reptilian liver. We have not studied the kinetic properties of the pyruvate kinases in the various taxa to ascertain whether the diverse patterns are also accompanied by altered properties of the isozymes. Ochs and Harris (37) in a comparative study of the regulation of pyruvate kinases from rat and chicken liver reported marked differences within the two enzymes in their response to the administration of glucagon and cyclic AMP.

Propositions

Future work of our research group will be aimed to document further differences in isozymic patterns of vertebrates including other enzyme systems of the glucose utilization pathways. All of this work is leading towards the ultimate goal of detailed knowledge of the role of enzymes and isozymes in metabolism which is necessary for definitive understanding of their role in evolution. We will proceed with the following working hypotheses: *a)* A species may be considered as a unique molecular solution to the thermodynamic problem of environmental adjustment; *b)* the history of this unique solution has occurred through changes at the genetic material affecting both the primary structure of proteins and the regulatory mechanisms of gene expression; *c)* natural selection acts only sorting out mutations affecting the genetic material resposible for the regulation of protein activity; *d)* enzymes (isozymes) of a metabolic pathway are coordinately affected during evolution.

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RESUMEN

Se presentan los perfiles isoenzimáticos de hexoquinasa de hígado de 68 especies de vertebrados. La comparación de los diversos perfiles observados, así como de las propiedades cinéticas y fisicoquímicas de las isoenzimas, revela que las hexoquinasas de mamíferos son muy similares a las de tortugas y anfibios. Las hexoquinasas de aves, lagartijas y serpientes, por otra parte, son similares entre sí y diferentes a las enzimas de mamíferos y de anfibios. Las piruvatoquinasas de hígado muestran aproximadamente la misma conducta. El sistema hexoquinasa de músculo de vertebrados es muy uniforme en las especies estudiadas y consiste principalmente de hexoquinasa B.

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