Molecular forms of red blood cell hexokinase

Giorgio Fornaini, Marina Dachà, Mauro Magnani, Vilberto Stocchi
Istituto di Chimica Biologica, Università degli Studi di Urbino, Via Saffi, 2, 61029 Urbino, Italy

Summary

Mammalian red blood cell hexokinase has been shown to exist in two or more distinct molecular forms, which are separable by ion-exchange chromatography. Of these forms just one corresponds to hexokinase type I from other tissues, while the others differ from any previously reported hexokinase isozyme. Analysis of several molecular properties of the three major forms (Ia, Ib and Ic in the order of their elution from DE-52 columns) of hexokinase prepared from human red cells and of the two forms purified from rabbit reticulocytes, shows significant differences in the isoelectric point. The kinetic and regulatory characteristics, the molecular weight, the temperature and pH-dependence of the various isozymes were similar.

The hexokinase isozymic pattern is largely dependent upon red blood cell age. Among all, hexokinase Ib is the predominant form in rabbit reticulocytes and becomes the minor component in the older cells; a similar situation has also been found in the human erythrocyte. At present the molecular basis of hexokinase heterogeneity remains unknown, however preliminary experimental findings indicate a post-translational modification as a possible mechanism.

Introduction

It has become clear from the literature data of the last years that the occurrence of multiple forms of enzyme appears to be a general phenomenon. Mammalian hexokinase (EC 2.7.1.1), as many others, exists in multiple forms (1–3). Four isozymes have been separated by electrophoresis or ion-exchange chromatography and designated I, II, III and IV according to their order of elution from DEAE-columns (1–3). All the four isoenzymes were found present in liver (1). Brain and kidney were shown to contain mainly type I hexokinase, skeletal muscle mainly type II and fat pad, heart, and intestine approxiamately similar amount of types I and II, while low amounts of type III were detected in kidney and intestine (4).

These isozymes were also readily distinguished by their Km values for glucose (3) which are 10^{-5} M for hexokinase I, 10^{-4} M for hexokinase II, 10^{-6} M for type III and 10^{-2} M for glucokinase or type IV, while, with the exception of the latter, they show the same molecular weight. A more general discussion of mammalian hexokinase is beyond the scope of this paper, but many excellent reviews are presently available (5–10).

In the red blood cells the situation is quite different. The presence of multiple forms of hexokinase in the human erythrocyte was first described, fifteen years ago, by Eaton et al. (11) because of the observation of several electrophoretic bands. Subsequently, many authors have confirmed the existence of two or more distinguishable forms of hexokinase in different mammalian erythrocytes (12–25), but usually the reported results have been considerably different. Many discrepancies are probably due to the fact that the erythrocyte hexokinase multiplicity cannot be simply explained on

Molecular and Cellular Biochemistry 49, 129–142 (1982). 0300-8177/82/0493-0129/$02.80.

the basis of the four hexokinase isozymes found in other tissues, but, as documented in this paper, are due to the presence of sub-types, or multiple forms of hexokinase type I. Other causes of discrepancies are the different distribution in the red cells of different mammals (13), the age dependence of the various forms of hexokinase (18–21, 23, 24) and the approach utilized in the evaluation of the isozymic pattern.

Only recently these questions have been solved by the isolation identification, and characterization of the major forms of hexokinase from human and rabbit red blood cells. The approach followed, and the main results, are summarized in this article.

Material and methods

Some of the methods used in these studies have been published and references are given in the corresponding figures.

Preparation of tissue extracts

Human, rabbit, rat, cow, horse, mouse, pig and dog blood was collected using EDTA or heparin as anticoagulants. Rabbit reticulocytes were induced by phenylhydrazine treatment as in (26). Cell washing and haemolysis in 0.4% (w/v) saponin solution were performed as described (27).

Soluble human liver hexokinase extract and rabbit liver, kidney, spleen, heart and skeletal muscle were obtained using a procedure based, in part, on that described by Grossbard and Schimke (28). Fresh tissues were minced and homogenate at 25% (w/v) in 3 mM sodium potassium phosphate buffer, pH 7.5, containing 5 mM glucose, 3 mM KF, 3 mM 2-mercaptoethanol, 1 mM dithiothreitol and 9% (v/v) glycerol and were centrifuged at 105 000 **g** for 1 h at 4 °C. Rabbit brain mitochondrial bound hexokinase was prepared as described in (29).

Rabbit bone marrow cells were obtained from normal rabbits and phenylhydrazine treated animals (26). In the latter case the rabbit bone marrow contained about 90% of erythroid cells precursors (mainly basophilic and polychromatic erythroblasts). The bone marrow was washed twice with a Krebs-Ringer solution in order to remove the red blood cells as contaminant. Single cell suspensions were prepared by collagenase (type IV from Sigma) digestion (0.025% w/v) for 30 min at 37 °C. Residual aggregates and fat cells were removed by filtration through two layers of gauze. The cells were washed twice by centrifugation in cold Krebs-Ringer solution.

The bone marrow erythroyd cells were lysed by adding 2.5 mM sodium potassium phosphate buffer, pH 7.5, containing 3 mM KF, 2.5 mM glucose and 0.4% (w/v) saponin. The nuclei mitochondria and membranes were discarded after centrifugation at 15 000 **g** for 30 min.

Leucocytes were obtained from heparinized rabbit blood samples by repeated centrifugation at 2 000 rpm at 4 °C followed by remotion of the upper layer of packed cells enriched in leucocytes. Contaminant red cells were finally removed by ipotonic hemolysis in cold 5 mM sodium potassium phosphate buffer, pH 7.4, followed by centrifugation at 3 000 rpm with a further washing in isotonic NaCl solutions. If necessary this last procedure was repeated twice until the cell suspension was colourless. Leucocyte lysate was obtained by repeated cycles of freeze-thawing treatment in 5 mM sodium potassium phosphate buffer, pH 7.4, containing 3 mM KF, 3 mM 2-mercaptoethanol, 2.5 mM glucose and 0.4% (w/v) saponin then centrifuged at 15 000 **g** for 30 min.

Rabbit platelets were prepared essentially as described in (32). Blood was collected in a plastic container by using EDTA (final concn. 5.4 mM) as anticoagulant. Platelet-rich plasma was prepared by centrifugation of whole blood at 370 **g** for 40 min at 12 °C and the platelets were then sedimented at 1 000 **g** for 50 min at 12 °C and resuspended in 0.02 M-Tris/HCl buffer (pH 7.4 at 4 °C), containing 0.15 M-NaCl, 5 mM glucose and 6 mM-EDTA. The platelet preparation was freed of residual erythrocytes by repeated centrifugation at 1 000 **g** for 1 min at 4 °C. The platelets were collected by centrifugation at 1 800 **g** for 15 min at 4 °C and washed twice in the above buffer. The pellet obtained in the final wash was resuspended in 4 vol. of 0.02 M-Tris/HCl buffer (pH 7.4 at 4 °C) lysed by three cycles of freeze-thawing at -20 °C followed by centrifugation at 15 000 **g** for 30 min.

Hexokinase assay

Hexokinase activity was measured at 37 °C spectrophotometrically in a system coupled with glu-

cose-6-phosphate dehydrogenase (EC 1.1.1.49) and 6-phosphogluconate dehydrogenase (EC 1.1.1.44). Initial rate measurements were performed by following the reduction of $NADP^+$ at 340 nm. For each molecule of glucose utilized two molecules of $NADP^+$ are reduced (28). In substrates specificity studies the initial rates of ADP production were measured in a coupled enzyme system with pyruvate kinase (EC 2.7.1.40) and lactate dehydrogenase (EC 1.1.1.28) according to Sapico and Anderson (31).

Chromatographic fractionation of multiple forms of hexokinase

Multiple forms of mammals' red blood cell hexokinase were separated by chromatography of 1 ml hemolyzates applied to DE-52 cellulose columns (0.35 × 24 cm) equilibrated in 5 mM sodium potassium phosphate buffer, pH 7.5, containing 0.25 mM glucose, 3 mM KF, 3 mM 2-mercaptoethanol and 1 mM dithiothreitol and operated at 5 ml/h. The columns were developed with 280 ml linear gradients of KCl from 0–0.4 M in the equilibrating buffer. Fractions of 0.7 ml were collected and assayed for hexokinase activity in the presence of 1 mM glucose. Hexokinase IV, or glucokinase was assayed at 100 mM glucose. Alternatively, tissue distribution of hexokinase isozymes in rabbits was examined by using 0.7 × 20 cm DE-52 columns. In this case a column flow rate of 15–20 ml/h was used and 1.5 ml fractions were collected.

Results

Evidence for the presence of multiple forms of red blood cell hexokinase

For many years it was suspected that a set of hexokinase isozymes might be present in the red blood cell.

The first evidence was produced by Eaton, Brewer and Tashian (10) following the observation of several electrophoretic bands. Soon after this report it was hypothesized that the presence of the multiple forms of hexokinase reported by Eaton et al. (11) could be attributed to an artefact (12, 15, 16). Subsequently, from many others laboratories, conflicting results have been reported about the hexokinase pattern in the erythrocyte (13, 14, 17–25). The discrepancies were mainly due to the difficulties related to their separation and to the evaluation of each form. Like many others, we have used electrophoresis on starch gel, agarose gel and cellulose acetate membrane as hexokinase separation techniques but without obtaining good results in resolution. Polyacrylamide disc gel electrophoresis or electrofocusing on polyacrylamide gel, on the other hand, inactivate selectively some isozymes (30). The question has been recently solved by introducing the use of small DE-52 ion-exchange columns (33). A further advantage of this approach is that it permits the separation and recovery of hexokinase isozymes starting from haemolysates. In fact, other systems (23–25) that employ the removal of the bulk of haemoglobin before the study of the red cell hexokinase pattern, inevitably cause a modification of the isozymic pattern leading to erroneous results as clearly shown in a previous paper (34). However, the successful application of this method requires consideration of several parameters such as column dimension, gradient, enzyme stability, etc. and depends on the optimization of all the experimental conditions. Since the method has been recently published (33) we will not report here any detailed information on its use but we would like to underline that it allowed us to give evidence for the presence of multiple forms of red cell hexokinase and to identify them. Some results obtained by this method are reported in Fig. 1 where the hexokinase isozymic pattern of human red cells is shown as compared with the pattern found in human liver. While all the four classical hexokinase isozymes (2) have been found in liver extracts, three major forms (that we labelled hexokinase Ia, Ib and Ic on the basis of their elution from the DE-52 column) and some other minor components, have been constantly observed in the red cell haemolysate of 30 normal adults. Co-chromatography experiments on human liver and red cell extracts have given further evidence that only hexokinase Ia corresponds to hexokinase type I, and that a low amount of hexokinase type III was also present in the human erythrocyte. All the other molecular forms were eluted at an intermediate position between hexokinase type I and type II (34).

A similar situation has been found in rabbit reticulocytes (24). Two hexokinase forms were separated from rabbit reticulocyte hemolysates but,

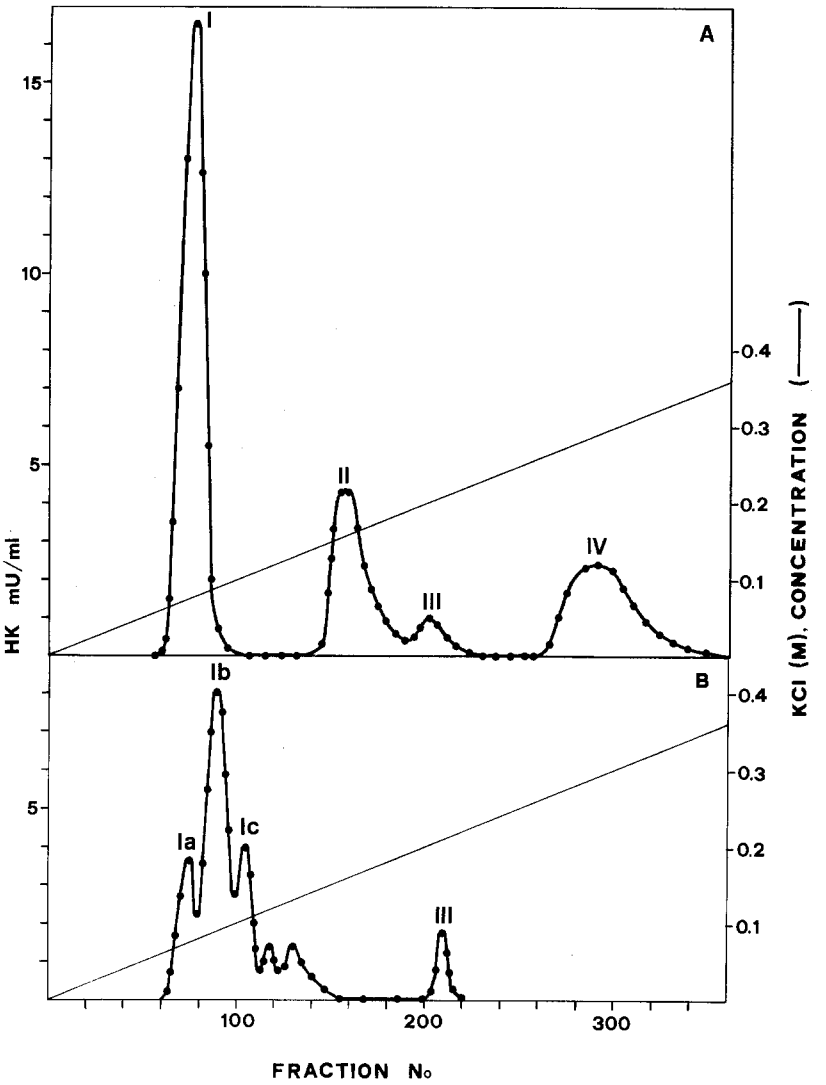


Fig. 1. DEAE-Cellulose chromatography of hexokinase activity from human liver (A), and adult erythrocytes (B). 1 ml of human liver homogenate and 1 ml of hemolysate from adult erythrocyte, were chromatographed on DE-52 columns (0.35 × 24 cm) equilibrated in 5 mM sodium potassium phosphate buffer, pH 7.5, containing 0.25 mM glucose and 5 mM dithiothreitol. The columns were developed with 280 ml of a linear gradient of KCl from 0 to 0.4 M in the same sodium potassium phosphate buffer, and operated at 5 ml/h. Fraction of 0.7 ml were collected and assayed for hexokinase activity. (Adapted from ref. 34.)

when the hemolysate from total red cells was chromatographed under the same conditions, the hexokinase form, which is eluted as the second peak, represents only a small fraction of the total activity. In Fig. 2 the chromatographic pattern of these two forms has been compared with the soluble hexokinase of many rabbit tissues and with the brain mitochondrial bound enzyme. Again, the first peak of hexokinase activity from rabbit reticulocyte extracts corresponds to hexokinase type I while the second peak lies at an intermediate position between hexokinase isozymes I and II. Interestingly this hexokinase type I multiplicity was not found in any other rabbit tissue examined (Fig. 2) and represents the first unequivocal evidence of the presence of sub-types of hexokinase in the erythrocyte.

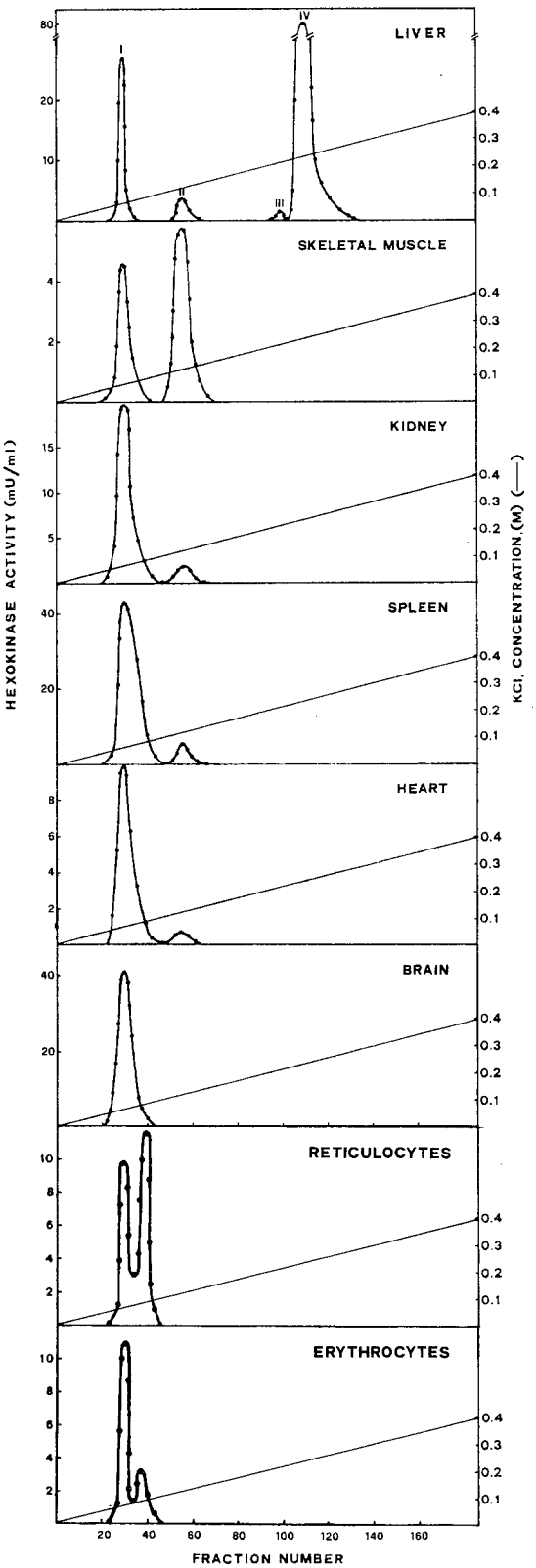


Fig. 2. DEAE-cellulose chromatography of hexokinase activity from rabbit liver, skeletal muscle, kidney, spleen, heart, brain, reticulocytes and adult red cell extracts. 1 ml of hemolysates or of tissue extracts were chromatographed on DE-52 columns (0.7 × 20 cm) equilibrated in a 5 mM sodium potassium potassium phosphate bufer (pH 7.5) containing 3 mM KF, 3 mM 2-mercaptoethanol and 5 mM glucose and operated at 15–20 ml/h. The columns were developed with 280 ml of a linear gradient of KCl from 0–0.4 M in the same buffer. Fractions of 1.5 ml were collected and assayed for hexokinase activity or glucokinase activity. (Adapted from ref. 30.)

Purification of multiple forms of red cell hexokinase

The first problem in the attempt to investigate the molecular basis of hexokinase multiplicity is the availability of pure isozymes. Several methods have been published on the purification of animal hexokinases (35–41) but these methods cannot be used in the case of the red blood cell enzyme, which represents only approximately 0.0003% (w/v) of the total protein. The question has been solved by the application of affinity chromatography on Sepharose-N-aminohexanoyl glucosamine as a key step in red cell hexokinase purification (42). Rabbit red blood cell and reticulocyte hexokinase have been purified to homogeneity by a combination of ion-exchange chromatography, affinity chromatography, and preparative electrophoresis (30, 43). The highest specific activity obtained was 145 units/mg of protein for the red cell enzyme and 155 and 144 units/mg of protein for reticulocyte hexokinase Ia and Ib, respectively. Human red cell hexokinases have been prepared partially purified in order to study some of their molecular properties (34). In this last case the availability of homogeneous forms was limited by the amount of blood.

Molecular properties of multiple forms of red cell hexokinase

When homogeneous or partially purified hexokinase sub-types were available several molecular properties were determined and compared.
Isoelectric point: isoelectric focusing of purified hexokinase from rabbit red cells, rabbit reticulocytes, and human red cells, under native conditions, provides single peaks with the pI listed in Table 1.
Molecular weight: the molecular weights were determined under both native and denaturating con-

Table 1. Isoelectric points of red cell hexokinases.

Source of enzyme	Isoelectric points (pH units)		
	hexokinase Ia	hexokinase Ib	hexokinase Ic
Rabbit red cells	6.3	–	–
Rabbit reticulocytes	6.3	5.7	–
Human red cells	5.7	5.5	5.35

The isoelectric point of purified hexokinase isozymes, was determined by isoelectric focusing on LKB 8 100 equipment in a glycerol gradient solution and in pH gradients 3.5 to 10 and 4 to 8 at 1% ampholine concentration. Hexokinase Ia, Ib and Ic refer to their order of elution from DE-52 columns. The results have been confirmed by at least three experiments.

ditions, indicating that all hexokinases are monomers. A summary of the results obtained is given in Table 2. Interestingly, aggregation or polymerization can be excluded as causes of hexokinase multiplicity.

Substrate specificity: the apparent K_m values of the multiple forms of erythrocyte hexokinase were estimated in 0.1 M glycylglycine, pH 8.1 and 37 °C. The results obtained are reported in Table 3. Significant differences were found in the Michaelis constants of rabbit hexokinase Ia and Ib towards hexoses, while no differences were observed between the isozymic forms of human red cells.

Kinetic properties: rabbit erythrocyte hexokinase is inhibited competitively with respect to Mg · ATP^{2-} by uncomplexed ATP^{4-} which is, on the other hand, a non-competitive inhibitor for glucose; while N-acetyl glucosamine, a competitive inhibitor of glucose, is a non competitive inhibitor for Mg · ATP^{2-} (44). These findings are in agreement with previous data for hexokinase type I from other sources, including human erythrocytes, (45–50) and are consistent with a rapid random mechanism for mammalian red blood cell hexokinase (44). By the use of ATP^{4-} as substrate analog, we have seen that the inhibition pattern is competitive only at glucose concentrations above the K_m values. This allows us to assume that when both substrates are present at K_m values, the pathway via the E · glucose complex is highly preferred, as it is for yeast hexokinase (51).

Regulatory properties: the red blood cell hexokinase reaction is known to be a rate-limiting step of the erythrocyte glycolysis (52–54). This enzyme is markedly modulated by many compounds such as glucose 6-phosphate (47, 54–58), 2,3-diphosphoglycerate (59, 60), glucose 1,6-diphosphate (57, 61, 62) and others (57, 58) so that the main regulatory properties have been examined in detail.

Human erythrocyte hexokinase Ia, Ib and Ic are inhibited by glucose 6-phosphate and glucose 1,6-diphosphate competitively with respect to Mg · ATP. The secondary plot of slope of the Lineweaver-Burk plots, against the inhibitor, is linear over the range of concentrations used (0–77 μM for glucose 6-phosphate and 0–100 μM for glucose 1,6-diphosphate) (34) with K_i values of 15 μM for glucose 6-phosphate and 22 μM for glucose 1,6-diphosphate without differences among the three hexokinases. Furthermore, these dissociation constants are both in the range of the intracellular concentrations of these two phosphorylated compounds (43, 47, 61, 62).

Table 2. Molecular weight of red cell hexokinase.

Sample		Molecular weight (Daltons)		
		by gel filtration	by sedimentation velocity	by SDS-gel electrophoresis
Rabbit red cells	hexokinase Ia	110 000	112 000	110 000
Rabbit reticulocytes	hexokinase Ia	105 000	105 000	104 000
	hexokinase Ib	105 000	105 000	104 000
Human red cells	hexokinase Ia	–	100 000	–
	hexokinase Ib	–	100 000	–
	hexokinase Ic	–	100 000	–

Estimations of the molecular weight of red cell hexokinase were performed under native conditions by gel filtrations on Ultrogel AcA44 column and on sucrose density gradient sedimentation velocity, and under denaturing conditions on sodium dodecyl sulfate-gel electrophoresis. Detailed experimental conditions and the molecular weight standard used are reported in (30, 34, 43). The standard deviations calcualted by five experiments were ±5 000 for each method.

Table 3. Specificity of red cell hexokinases.

Compound	Rabbit red cell Hexokinase Ia		Rabbit reticulocyte				Human red cell					
			Hexokinase Ia		Hexokinase Ib		Hexokinase Ia		Hexokinase Ib		Hexokinase Ic	
	Km[a]	Relative velocity[b]	Km[a]	Relative velocity[b]	Km[a]	Relative velocity[b]	Km[a]	Relative velocity[b]	Km[a]	Relative velocity[b]	Km[a]	Relative velocity[b]
	(mM)	(%)	(mM)	(%)	(mM)	(%)	(mM)	(%)	(mM)	(%)	(mM)	(%)
Mg ATP	0.6	100	0.5	100	0.5	100	0.60	100	0.57	100	0.62	100
Mg GPT	–	–	–	–	–	–	–	–	–	–	–	–
Mg ITP	1.9	36	12.0	30	4.5	30	5	14	8	13.5	10	13.5
Mg UTP	–	–	–	–	–	–		–	–	–	–	–
Mg CTP	–	–	–	–	–	–	–	–	–	–	–	–
D(+)-Glucose	0.06	100	0.04	100	0.125	100	0.046	100	0.048	100	0.047	100
D(+)-Mannose	0.71	109	0.10	105	0.15	104	0.07	78	0.1	83	0.07	85
D(–)-Fructose	17.8	70	4	69	25	42	10	100	11.7	120	13	90
2-Deoxy-D-glucose	1.33	65.7	0.9	65	2	70	0.77	95	0.56	99	1.16	74
D(+)-Glucosamine	2.0	43.4	0.85	35	1.3	70	1.5	47	0.5	47	0.5	21
N-Acetyl-D-glucosamine	41.6	32.6	–	–	–	–	–	–	–	–	–	–
(D+)-Galactose		–	–	–	–	–	–	–	–	–	–	–

[a] Km values were determined using the Lineweaver-Burk Plots.

[b] Maximum velocities are expressed relative to the Vmax for glucose (100%) or Mg ATP (100%) when the enzyme is saturated by the considered substrate.

The inhibition of red cell hexokinase by phosphorylated compounds is known to be partly relieved by orthophosphate (47, 54–58). As we have shown (34), no differences can be found among the three human red cell hexokinases; however, a paper from Rijksen et al. (25) reports that type Ia hexokinase is less regulated by orthophosphate than are the other subtypes. A more extensive investigation on the effect of orthophosphate on hexokinase has been carried out for the rabbit erythrocyte enzyme (hexokinase Ia) (63).

We have also found that reduced and oxidized glutathione at their intracellular concentrations are physiological modulators of red cell hexokinase (64). Reduced glutathione at 1 mM concentration is able to mantain rabbit red blood cell hexokinase in the reduced state with full catalytic activity. At higher concentrations a marked inhibition is observed. In contrast, oxidized glutathione is a strong inhibitor of reduced erythrocyte hexokinase at all the concentrations studied.

Unfortunately comparative data among the multiple forms of red cell hexokinase are not presently available.

Other properties: the different forms of red blood cell hexokinase exhibited quite similar pH dependence of enzyme activity with the optimum at pH 8.0–8.2. All the molecular forms can be stabilized by hexoses, glycerol and sulphydryl protecting agents. The only differences in enzyme stability were observed between rabbit hexokinase Ia and Ib. Hexokinase Ia appears to be more stable than hexokinase Ib when stored for several days at 4 °C. Contrariwise, hexokinase Ib is more stable than hexokinase Ia when stored at 37 °C in the same conditions.

All the molecular forms of hexokinase show a break in the Arrehenius plot at 32 °C when studied in the presence of 5 mM glucose, 5 mM Mg · ATP and 5 mM excess Mg in 0.135 M glycylglycine pH 8.1. The calculated activation energy was 11 K cal/mol at temperatures lower than 32 °C and 5 Kcal/mol at higher temperatures.

Age-dependence of red cell hexokinase

Many authors have reported a marked decrease in the hexokinase level during cell ageing (18–21, 65–67). This age-related hexokinase decline is a complex phenomenon, the enzymic activity appearing to have a biphasic decay pattern (18, 21, 66), followed by changes in the kinetic (20, 21, 68) and electrophoretic (12, 17–20, 69) properties of the enzyme. The exact behaviour of the hexokinase decay during cell ageing has been clarified recently in our laboratory.

Figure 3 shows that the decay of the rabbit enzyme is a process that involves a decay of hexokinase Ia and Ib at different rates. Hexokinase Ib is the predominant form of the soluble glucose phosphorylating activity in the reticulocyte but becomes the minor component in the mature cell, and is also present in the older red cells (Fig. 4) (27). The changes in kinetic properties of the glucose phosphorylating activities observed during cell ageing (21, 68) can be explained by the fact that the affinity constant for glucose of hexokinase Ib is one order higher than hexokinase Ia, and young red cells contain mainly the former.

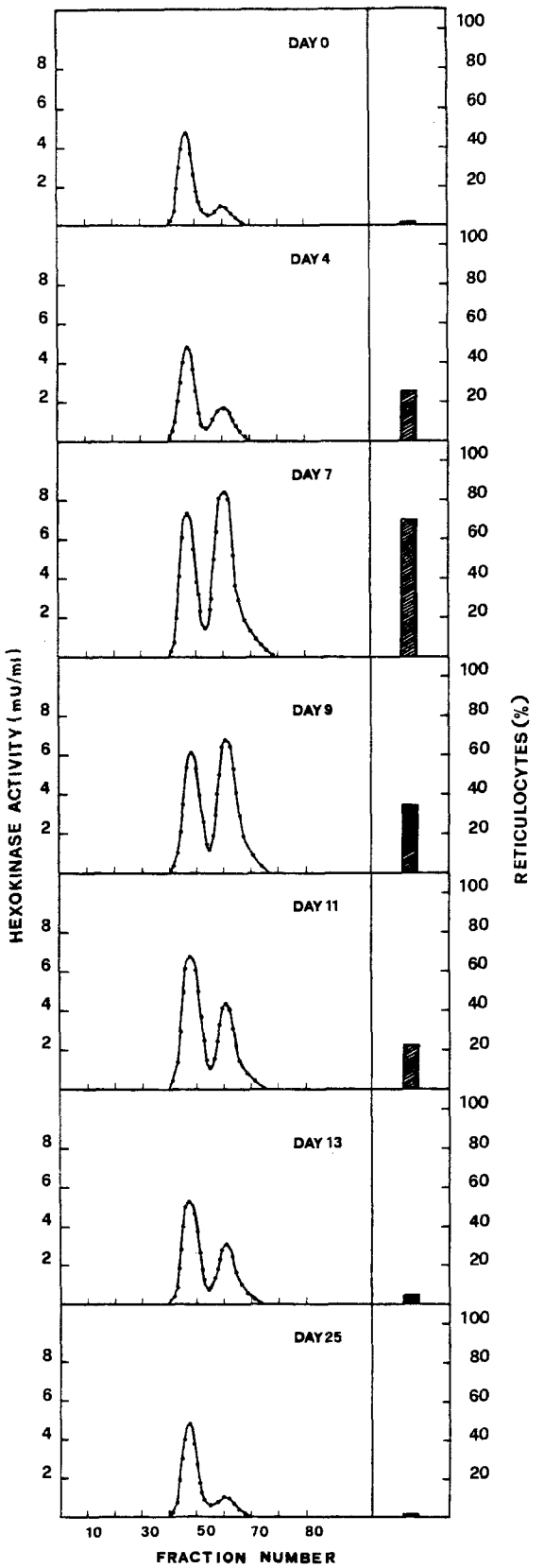
Hexokinase Ib is also the predominant form in young human red cells. As for the rabbit enzyme, it shows a faster decay during cell ageing among the various forms and is also present in the older cells (34).

From the results reported it is possible to propose hexokinase Ib as a fine marker of cell age in human and rabbit erythrocytes.

Studies performed on the modification of the hexokinase isozymic pattern during red cell ageing (Fig. 5) also provide an explanation for the differences found between fetal and adult erythrocytes. Recalling that blood samples obtained from umbelical cords (as a source of fetal erythrocytes) contain a higher proportion of young cells as compared with samples obtained from adults (the life span of the fetal red blood cell is shorter than that of adults (70) we propose that the cell age is responsible for the differences found between the hexokinase activity and the isozymic pattern of fetal erythrocytes and that of adults.

Distribution of hexokinase in mammalian erythrocytes and in other blood cells

The presence of multiple forms of hexokinase is not only related to rabbit and human erythrocytes. Cow, dog, horse, monkey and mouse possess a set of isozymes in the hexokinase type I area. Contrariwise, rat erythrocytes contain only hexokinase type I. The pig red cell does not shows any hexoki-



nase in the type I area but possesses mainly hexokinase type III (71) and a glucose phosphorylating activity with a low affinity for glucose (72, 73).

In order to gain more information on the distribution of multiple forms of hexokinase in blood cells we have investigated the isozymic pattern in the white blood cells and in platelets. Figure 6 shows the presence of hexokinase type I in platelets and type I and II in white cells.

The results reported above indicate that the presence of hexokinase sub-types is related only to the erythrocytes. On the other hand it is well known that the mature red blood cell is not able to synthesize proteins and so a post-translational modification can be considered as a probable mechanism in the formation of multiple forms of hexokinase. In an attempt to identify a probable native enzyme we have studied the hexokinase chromatographic profile of bone marrow cells.

Figure 7 shows that bone marrow cells from normal rabbits contain both hexokinase type I and II, while, during induction of severe anemia, when the erythroid precursor cells become predominant over the nonerythroid cells, a great increase of hexokinase type I can be observed. This increase in hexokinase type I associated with the increase in the proportion of erythroid cells, is also concomitant with the appearence of hexokinase Ib, and the decrease of hexokinase type II.

In conclusion, hexokinase Ia seems to be the native enzyme at least in the erythroid precursor cells.

Discussion

Red blood cell hexokinase is of central importance for erythrocyte metabolism and, as many other red cell enzymes, (65) is present in multiple molecular forms which have not been identified in other tissues.

Fig. 3. In-vivo dependence of rabbit red blood cell hexokinase isozymic pattern. Reticulocytosis was induced in a group of 5 rabbits by 3 days phenylhydrazine administration and blood was collected in EDTA at day 0, 4, 7, 9, 11, 13 and 25 after treatment. A sample of 1 ml was applied to a DE-52 cellulose column (0.7 × 20 cm) equilibrated in 5 mM sodium potassium phosphate buffer (pH 7.5) containing 3 mM KF, 3 mM 2-mercaptoethanol and 5 mM glucose and operated at 15–20 ml/h. The columns were developed with 280 ml of a linear gradient of KCl from 0–0.4 M in the equilibrating buffer. Fractions of 1.0 ml were collected and assayed for hexokinase activity. (Reproduced from Ref. 27.)

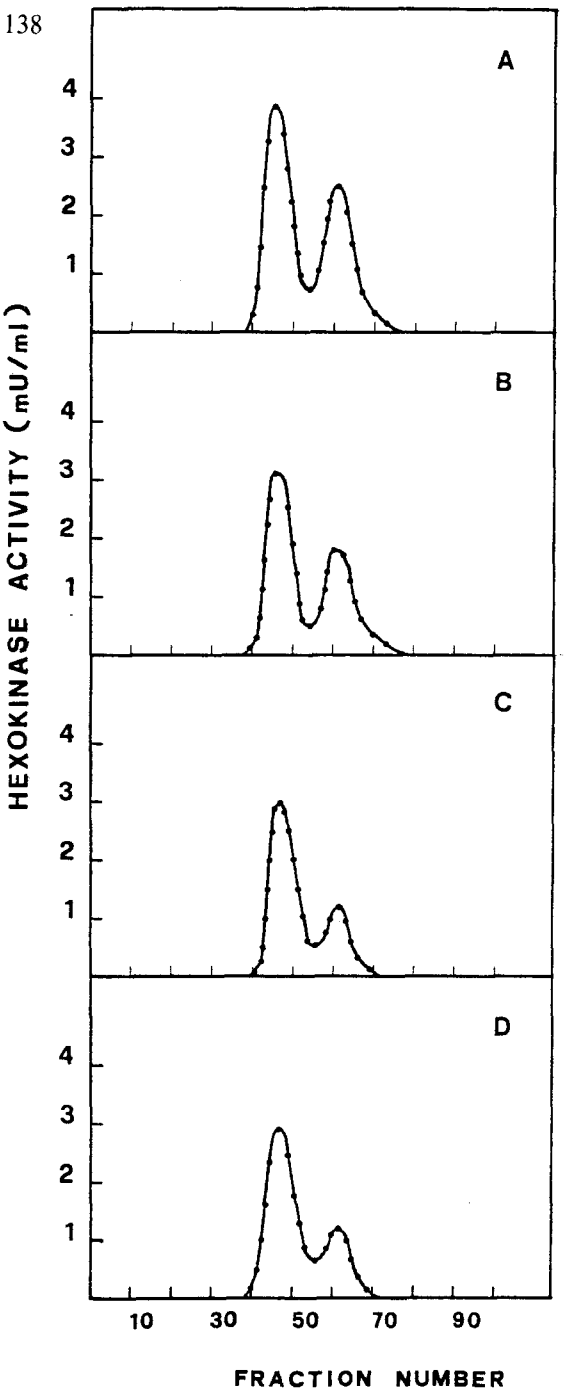
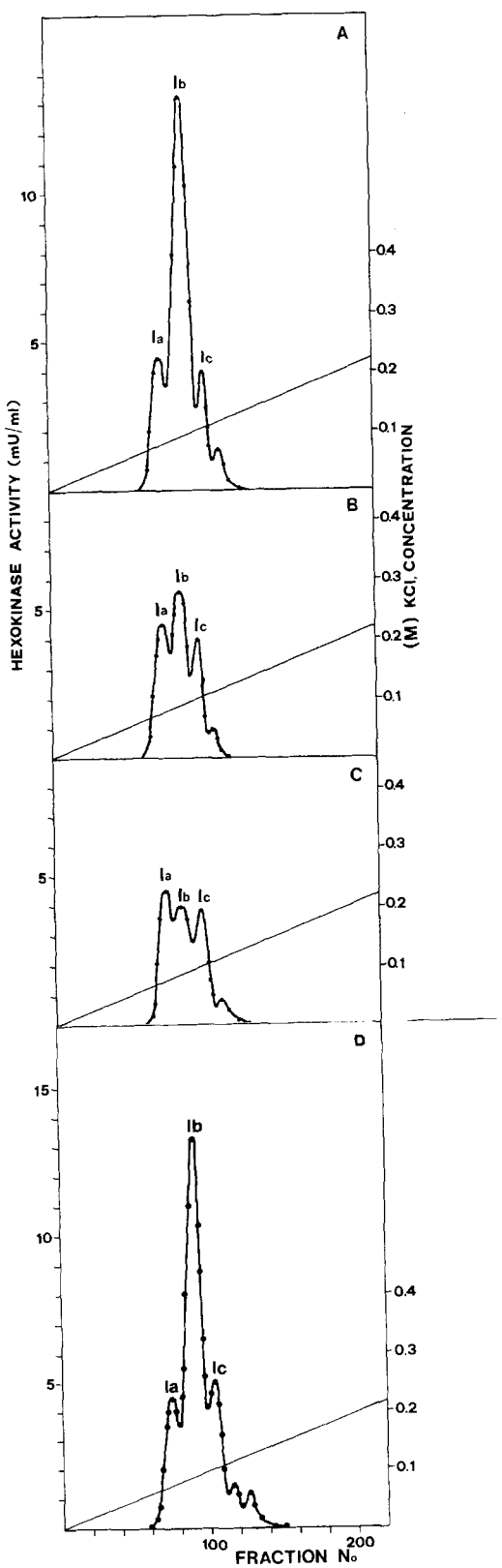


Fig. 4. Hexokinase isozymic pattern in rabbit red cells of different ages. Rabbit red cells were separated into 5 fractions of increasing mean age by ultracentrifugation through gradients of Ficoll-Triosil. Fractions 1 and 2, which contain the less dense cells, have been pooled. These cells and those of fractions 3, 4 and 5 were collected in separate tubes, washed and hemolized in an equal volume of 0.4% (w/w) saponin solution. Samples of 1 ml were applied to DE-52 columns and chromatographed as reported in Fig. 1. Chromatographic profile of hexokinase: A) from the pooled fractions 1 and 2 (young cells); B) from fractions 3; C) from fraction 4; D) from fraction 5 (old cells). (Reproduced from Ref. 27.)

The results reported above show that on the basis of kinetic properties and chromatographic behaviour, only hexokinase Ia, of all the multiple forms present in the erythrocyte, corresponds to the classical hexokinase type I (in the nomenclature of Katzen and Schimke (2). All the other isozymes differ from every other previously reported hexokinase and are eluted from DE-52 columns intermediate between hexokinase type I and type II. Of course, this chromatographic profile reflects different isoelectric points of these molecular forms (Table 1). Molecular weight, temperature and pH dependence, kinetic and regulatory properties were all similar. In other words, at least for the human erythrocyte this hexokinase multiplicity does not seem to be able to affect the metabolic regulation of red cell metabolism. In fact, the action of the main metabolic effectors of the hexokinase reaction (glucose 6-phosphate, glucose 1,6-diphosphate and ortophosphate) is the same for each molecular form.

Any research into a posible physiological meaning of the isozymic pattern of hexokinase must be related to its age dependence. The mature erythrocyte lacks the ability to synthesize new protein and in view of the central role of hexokinase in red cell ageing the biological significance of the hexokinase multiplicity could be of importance in the establishing of a metabolic cause for red cell senescence (54). Among the various forms, (Ia, Ib, Ic) hexokinase Ib shows a faster decrease during cell ageing. The mechanisms responsible for this hexokinase decay (probably a proteolytic system) are at present unknown, however hexokinase Ia and Ic seem to be more resistent to this action so that the described multiplicity of hexokinase could represent a protection mechanism against its age-dependent decay.

Several mechanisms can be hypothesized as responsible for the hexokinase multiplicity. However, the low amount of hexokinase Ib present in rabbit erythroid precursor cells and its absence from many other rabbit tissues taken, together with the lack of protein synthesis in the erythrocytes, suggests a post-translational modification as a probable mechanism. Of course, other hypotheses cannot at present be discarded. Among all the several possible mechanisms involved in post-translational modifications of proteins (74), we have tested and discarded several possibilities. Aggregation or polymerization can be excluded, in fact hexokinase Ia, Ib and Ic show the same molecular weight under native and denaturing conditions. Furthermore,



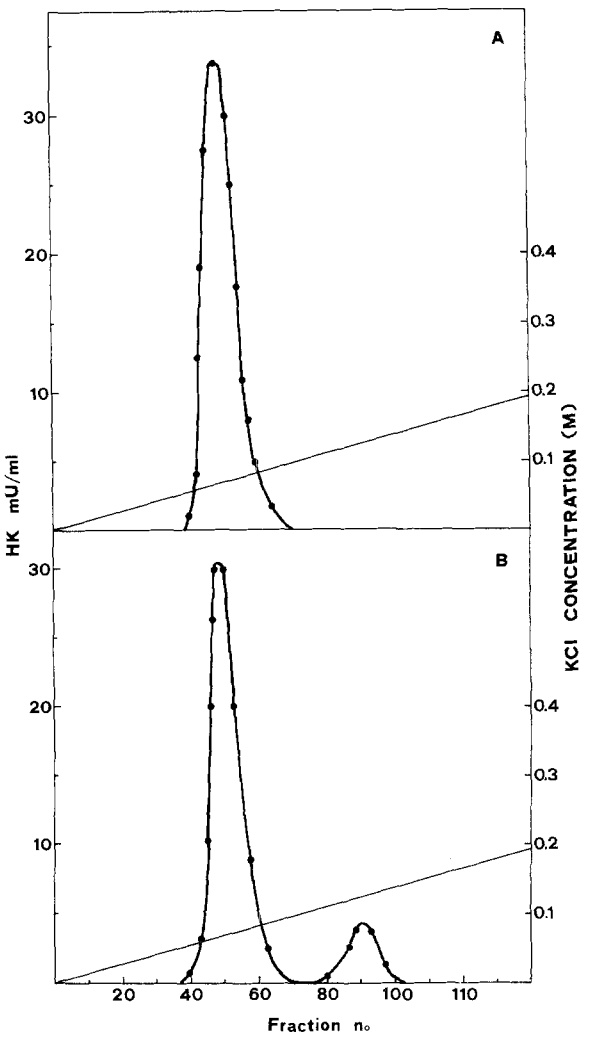


Fig. 6. Hexokinase isozymic pattern in rabbit platelets (A) and white blood cells (B). 1 ml of white blood cells and platelet extracts, prepared as described under methods, were chromatographed on DE-52 columns as in Fig. 2. Fractions of 1.0 ml were collected and assayed for hexokinase activity.

Fig. 5. Hexokinase isozymic pattern of human red cells of different ages. Human erythrocytes were separated into 6 fraction of increasing mean age (density) by ultracentrifugation through gradients of Ficoll-Triosil. Fraction 1 and 2, which contain the less dense cells (the younger), have been pooled. These cells and those of fractions 3 and 4 (the middle aged), and cells of fractions 5 and 6 (the older) were collected in separated tubes, washed and hemolyzed in an equal volume of 0.4% (w/v) saponin solution. Samples of 1 ml were applied to DE-52 columns and chromatographed as reported in Fig. 1. A) chromatographic profile of hexokinase from young red cells; B) from the middle aged cells; C) from old erythrocytes; D) fetal red blood cells. ●—●, hexokinase activities; ——, KCl gradient. (Adapted from ref. 34.)

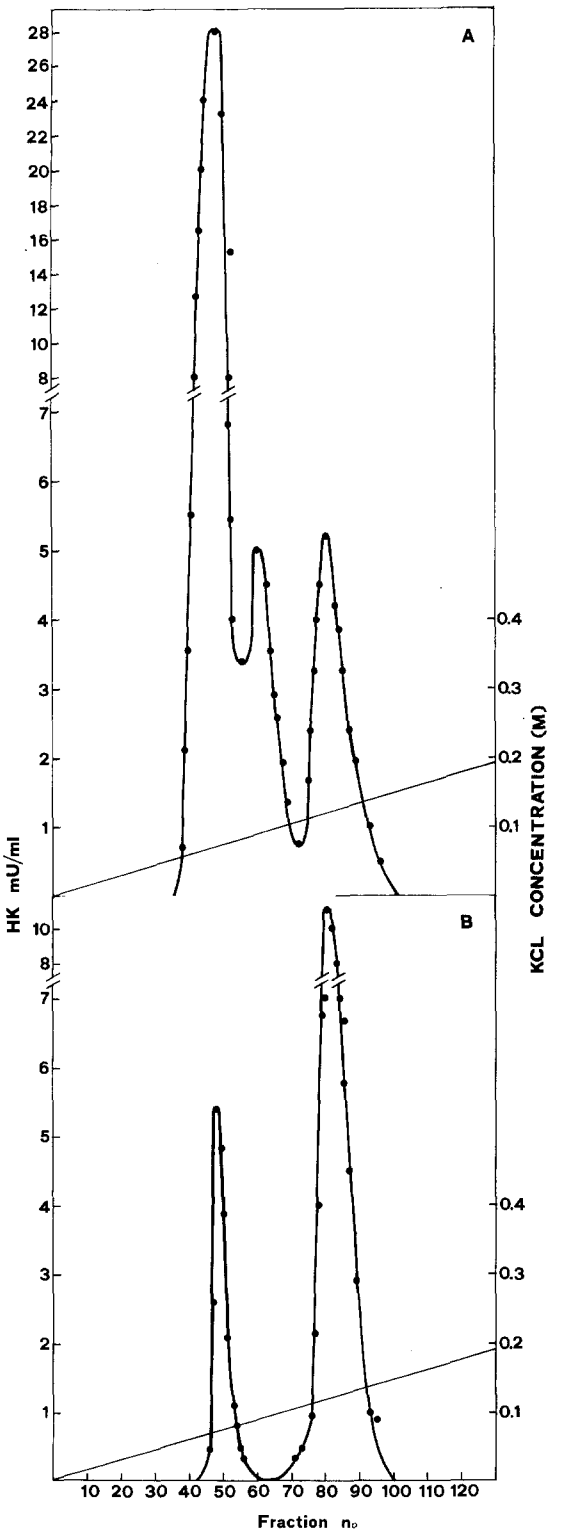


Fig. 7. Hexokinase isozymic pattern in rabbit bone marrow cells. Bone marrow cells from severely anemic animals (A) (with 70–80% of circulating reticulocytes) or normal rabbits (B) were prepared as in methods. 1 ml extracts were chromatographed on DE-52 columns as described in Fig. 2 and 1.0 ml fractions collected and assayed for hexokinase activity.

mitochondrially bound hexokinase from rabbit reticulocytes, and plasma membrane bound hexokinase from reticulocytes and erythrocytes have been solubilized and found to show the same chromatographic pattern as the isozymes designated Ia. The result excludes the possibility that the multiple forms of hexokinase could be of cytoplasmatic and (or) mitochondrial origin. Oxidation of SH groups by oxidized glutathione or oxidizing agents involves inactivation of the enzyme (64) so that it seems unlikely to be a cause of hexokinase multiplicity. Finally, any conversion between the various forms of hexokinase has been observed during prolonged storage of reticulocyte hemolyzates for three months at –20 °C or of single hexokinase Ia or Ib. Other possibilities (limited proteolysis, deamination, etc.) are currently under investigation in our laboratory.

In conclusion, the occurrence of multiple forms of hexokinase, as for many other enzymes, appears to be a very interesting phenomenon but, at the present time, of unknown biological significance. From all these studies, it is clear that the knowledge of the mechanism that leads to the formation of multiple forms of hexokinase is fundamental in the comprehension of hexokinase activity decrease during cell ageing. This fact, associated with the crucial role of hexokinase in the erythrocyte metabolism could explain the cell age-dependent metabolic decline which in turn determines the life span of the red cell.

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Received 1 May 1982.