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SEPARATION AND CHARACTERIZATION OF HEXOKINASE I SUBTYPES FROM HUMAN ERYTHROCYTES

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Summary

Hexokinase (ATP: D-hexose 6-phosphotransferase, EC 2.7.1.1) type I from human erythrocytes exists in four electrophoretical distinct forms, termed I_a, I_b, I_c and I_d in order of their increasing anodal electrophoretic mobility at pH 8.8. We were able to separate type I_a, I_b and I_{cd} on phosphocellulose by using a discontinuous gradient elution. The three chromatographically distinct forms do not differ in their affinity constants for the substrates glucose and $MgATP^{2-}$. In addition the inhibition by glucose 1,6-diphosphate does not differ significantly for all forms. However, the regulation of these inhibitions by inorganic phosphate is much less for type I_a compared to the other subtypes ($P = 0.001$)

Aging of the red cells is accompanied by a relative increase of the proportion of type I_c and I_a, which is the less regulated form of the enzyme. This shift in electrophoretic and regulatory properties is argued to be due to a post-translational modification of the primary enzyme.

Introduction

In mammalian tissues there are four isozymes of hexokinase (ADP: D-hexose 6-phosphotransferase, EC 2.7.1.1) designated as hexokinase type I, II, III and IV in order of their increasing electrophoretic mobility towards the anode [1].

Hexokinase type I, the most predominant isozyme of hexokinase in mammalian tissues, appears to be heterogeneous in nature. Easterby and O'Brien [2] separated two enzymatically active species from pig heart using chromatography on phosphocellulose. It was documented that these species represent a

reversible equilibrium between a dimer and monomer form of the enzyme [2,3], although in a later report the idea of a modification of one form into the other (e.g. proteolytic) was favoured, each form being subject to dimerization [4]. The same monomer-dimer equilibrium was described for bovine brain hexokinase by Chakrabarti and Kenkare [5]. Furthermore, in rat brain two different forms of hexokinase type I were found by Felgner and Wilson [6] using DEAE-chromatography, one form bindable to brain mitochondria, the other non-bindable.

Human erythrocytes have been reported to contain a 'complex' of electrophoretically distinguishable type I hexokinases [7—10]. Kinetic studies on purified hexokinase I from human red cells suggested the presence of at least two differently regulated forms of hexokinase type I [11,12]. The inhibition of the one form by hexosephosphates was regulated by inorganic phosphate (P_i), whereas the other one was insensitive to P_i. Polyacrylamide gel electrophoresis of partly purified hexokinase from erythrocyte populations fractionated according to cell age showed a three-banded pattern in the type I region, while the relative intensities of the bands were shifting with increasing cell age [7]. Comparison of the electrophoretic and kinetic properties of the normal enzyme from cell populations of different cell age with the properties of the enzyme from a patient with a partial hexokinase deficiency, suggested that the shift in electrophoretic mobility with increasing cell age might be accompanied by a loss of regulation by P_i [13].

The aim of this study was to isolate the hexokinase I subtypes from human erythrocytes and to characterize them separately in order to test the above mentioned hypotheses.

Materials and Methods

Materials. Glucose 1,6-diphosphate (Glc-1,6-*P*$_2$, tetracyclohexylammonium salt), ATP (disodium salt), NADP (disodium salt) and auxilliary enzymes for the determination of hexokinase were obtained from Boehringer (Mannheim, F.R.G.). P_{11}-phosphocellulose was from Whatman (Maidstone, U.K.). NaH_2PO_4 (P_i) and all other chemicals used were of analytical grade of purity.

Enzyme assays. Hexokinase activity was measured either by the glucose-6-phosphate dehydrogenase coupled assay or by the pyruvate kinase-lactate dehydrogenase coupled assay (in the inhibition studies with Glc-6-*P*) as described before [14]. 1 unit enzyme activity is defined as the amount of enzyme which catalyzes the formation of 1 μmol Glc-6-*P* or 1 μmol ADP, respectively, per min at 37°C. Glucose-6-phosphate dehydrogenase was determined according to Beutler [15].

Influence of inorganic phosphate. To evaluate the influence of inorganic phosphate (P_i) a function, $f(P_i)$, was derived describing the influence of P_i on the inhibition constant of hexokinase for any inhibitor as a function of P_i concentration [11]. A series of assays was performed with increasing concentrations of P_i (0.0—5.0 mM) in the presence and absence of 0.1 mM of the inhibitor Glc-1,6-*P*$_2$. The concentration of $MgATP^{2-}$ was 0.5 mM, free Mg^{2+} was 5.0 mM and the pH of the assay medium was 7.15. Higher pH values and variations in temperature and glucose concentration gave essentially the same effect

of P_i, although inhibition by Glc-1,6-P_2 at higher pH values was less extensive [12].

Electrophoresis. Electrophoresis on cellulose acetate gels and subsequent staining for enzyme activity was performed as described before [16], except for the time of the run which was extended to 3 h. After this period, hexokinase I subtypes are clearly separated, while hexokinase type III, which is present in minor amounts in human red blood cells [7], had moved out of the gel.

Disc electrophoresis on polyacrylamide gels was performed as described before [7]. The relative intensities of the bands showing hexokinase activity are estimated with a Helena Quickscan densitometer.

Fractionation of red cells according to cell age. Fresh heparinized blood from a healthy donor was fractionated into populations of different cell age by the method of Murphy [17]. The cells were centrifuged in their own plasma at 30°C using an angle rotor at 39 000 × *g*. The top fraction (about 10% of the cell volume, referred to as 'youngest' cells), the bottom fraction (about 10% of cell volume, referred to as 'oldest' cells) and the middle fraction were collected. Subsequently, leukocytes and platelets were removed by filtration through α-cellulose-crystalline cellulose mixture according to Beutler et al. [18]. The efficiency of the fractionation method was estimated by the determination of the cell age dependent enzymes glucose-6-phosphate dehydrogenase and hexokinase, and by determining the mean corpuscular haemoglobin concentration and mean corpuscular volume (Table I).

Hexokinase from these cell populations was partially purified by batchwise treatment with DEAE-Sephadex A-50 and by $(NH_4)_2SO_4$ precipitation as described before [14]. The precipitates were dissolved in 0.2 M Tris-HCl buffer (pH 8.0) to which 10 mM glucose and 3 mM β-mercaptoethanol were added. Prior to the electrophoresis experiments the samples were dialysed against the same buffer.

Partial purification and separation of subtypes. Human erythrocyte hexokinase was partly purified by batchwise treatment with DEAE-Sephadex A-50 (Pharmacia, Uppsala, Sweden) and concentrated by $(NH_4)_2SO_4$ precipitation as previously described [14]. The precipitate was dissolved in a phosphate buffer, pH 6.5/1.48 g/l K_2HPO_4/3.33 g/l KH_2PO_4/10 mM glucose/2 mM β-mercaptoethanol/1 mM Na_2EDTA. The sample was extensively dialysed against the same buffer and consecutively subjected to phosphocellulose chromatography as described by Easterby and O'Brien [2]. When the elution conditions as used in this report are modified by using a discontinuous KCl gradient, as shown in

TABLE I

FRACTIONATION OF HUMAN ERYTHROCYTES ACCORDING TO CELL AGE

	'top'	'middle'	'bottom'	ratio top/bottom
Glucose-6-phosphate dehydrogenase	12.0	11.5	10.1	1.20
Hexokinase	2.1	1.5	1.4	1.50
Mean corpuscular volume	117	103	99	1.18
Mean corpuscular hemoglobin concentration	17.6	20.8	21.5	0.82

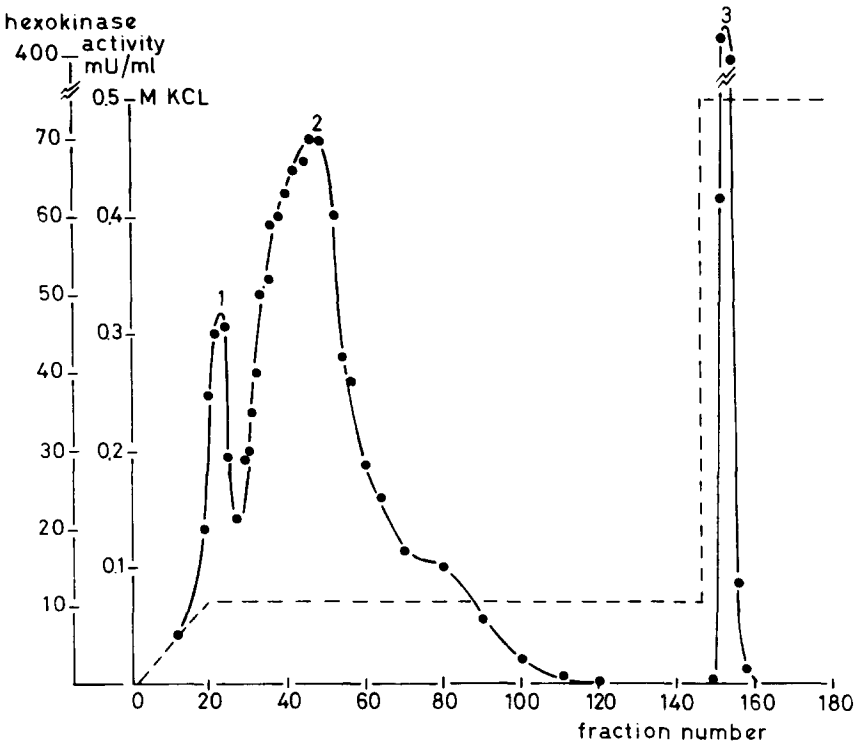


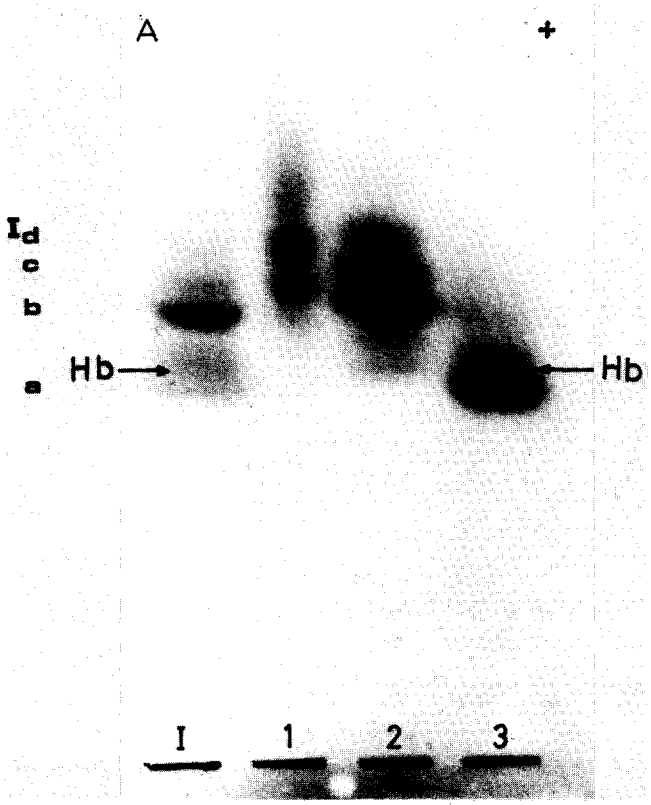
Fig. 1. Phosphocellulose chromatography of partially purified human red cell hexokinase. A 500 ml KCl gradient (0.0—0.3 M KCl) was applied until the first hexokinase activity was eluted from the column. 5 ml fractions were collected at a flow rate of about 40 ml/h. Column dismensions were 2.5 X 5 cm. Recovery of total activity was 60%.

Fig. 1, three peaks of hexokinase activity could be eluted: as soon as the first fractions containing hexokinase activity were eluted, the gradient was flattened (concentration of KCl about 70 mM at this stage). In this way two peaks of hexokinase activity were eluted. The relative activity of these two peaks and the resolution of these peaks varied from experiment to experiment. The remaining hexokinase activity on the column was eluted with 0.5 M KCl in the buffer. When a continuous KCl gradient was used as in the method of Easterby and O'Brien [2] only a very poor resolution of the three peaks was obtained. The results of a representative experiment are shown in Fig. 1.

Results and Discussion

Cellulose acetate electrophoresis of partially purified red cell hexokinase results in a multibanded pattern consisting of four bands with hexokinase activity (Fig. 2). These four bands of hexokinase type I are referred to as type I_a, I_b, I_c and I_d in order of their increasing anodal electrophoretic mobility towards the anode.

Phosphocellulose chromatography of partially purified red cell hexokinase using a discontinuous KCl gradient to elute the enzyme results in the separation of three peaks of hexokinase activity. By comparison of the electrophoretic



pattern of the three peaks with the total hexokinase preparation, peak I can be identified as type $I_{c,d}$ (with some minor contamination of type I_b), peak 2 as type I_b (with some contamination of type I_c and peak 3 as type I_a (Fig. 2B). Band I_a is somewhat broadened at the anodal site by the interference of hemoglobin.

The nomenclature is in accordance with an earlier report, describing the electrophoretic behaviour of erythrocyte hexokinase on polyacrylamide gels [7]. The slowest moving band on cellulose acetate (peak 3 after phosphocellulose chromatography) also has the lowest mobility on polyacrylamide (Fig. 3). Type I_d is a minor component of the total type I complex and was not noticed originally on polyacrylamide gels.

Phosphocellulose chromatography of pig heart hexokinase using a continuous KCl gradient to elute the enzyme resulted in the separation of two peaks of hexokinase type I activity which were denominated as type I_A and I_B in order of their elution from the column [2]. We prefer to name the subisozymes in order of their increasing electrophoretic mobility in accordance with the nomenclature of type I, II, III and IV as designated by Katzen and Schimke [1]. The order of electrophoretic mobility (at pH 8.8) is reversed to the order of elution from a phosphocellulose column (at pH 6.5), which fact points at an isoelectric point of all subisozymes between 6.5 and 8.8. The chromatographically distinct peaks all belong to the hexokinase type I complex as is shown by the substrate

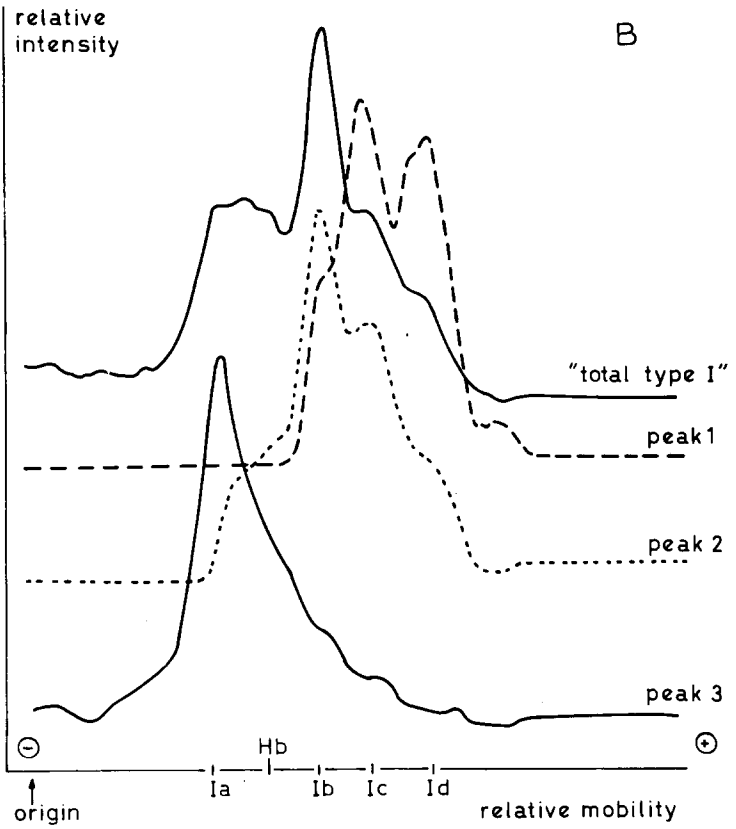


Fig. 2A. Cellulose acetate electrophoresis of hexokinase from the first (1), second (2) and third (3) peak eluted from a phosphocellulose column (see Fig. 1) compared to the total hexokinase type I preparation before phosphocellulose chromatography (I). The total activity in each peak was combined and 2 munits of hexokinase activity per sample were applied to the gel. B. Graphic representation of the electrophoretic patterns as shown in Fig. 2A.

affinities (Table III). The Michaelis-Menten constants for the substrates glucose and $MgATP^{2-}$ are the same and are in accordance with previous reported values for hexokinase type I [7,14]. In addition the inhibition by Glc-1,6-P_2 of the various forms is not significantly different ($P > 0.5$), neither does the inhibition by Glc-6-P seems to be much different. However, there is a difference in the regulation by inorganic phosphate of these inhibitions. As shown by the $f(P_i)$, describing the influence of P_i on the inhibition constant for Glc-1,6-P_2 (Fig. 4) type I_a is much less regulated by P_i than the other subtypes. The difference was found to be highly significant by analysis of variance ($P = 0.001$). It was hypothesized before [11,12] that there were two types of hexokinase I in erythrocytes: one form regulated by P_i and another form insensitive to regulation by P_i. This hypothesis appears to be partially valid in the respect that there is one subtype of hexokinase type I, which is less sensitive to phosphate regulation than the other subtypes.

Fractionation of a normal red cell population into fractions of different cell age produces a shift in the electrophoretic pattern. The isozymic patterns of

TABLE II

KINETIC PARAMETERS

Hexokinase type	I	I_a	I_b	I_{cd}
$K_{m,\,app}$ glucose * (mM)	0.069 S.D. = 0.014 (n = 5)	0.062 S.D. = 0.011 (n = 5)	0.078 S.D. = 0.018 (n = 4)	0.074 S.D. = 0.020 (n = 4)
$K_{m,\,app}$ $MgATP^{2-}$ ** (mM)	0.78 S.D. = 0.26 (n = 5)	0.69 S.D. = 0.19 (n = 6)	0.71 S.D. = 0.21 (n = 6)	0.78 S.D. = 0.19 (n = 6)
Inhibition by Glc-1,6-P_2 *** (%)	64 S.D. = 8 (n = 5)	58 S.D. = 12 (n = 6)	67 S.D. = 15 (n = 6)	62 S.D. = 15 (n = 6)
K_i Glc-1,6-P_2 (mM)	0.040 ; 0.043	0.057 ; 0.070	0.038 ; 0.070	0.054 ; 0.070
K_i Glc-6-P (mM)	0.011	0.009	0.017	0.020

* Determined at 5.0 mM $MgATP^{2-}$.

** Determined at 10 mM glucose.

*** Percentage of inhibition by 100 μM Glc-1,6-P_2 at 0.5 mM $MgATP^{2-}$ at pH 7.15.

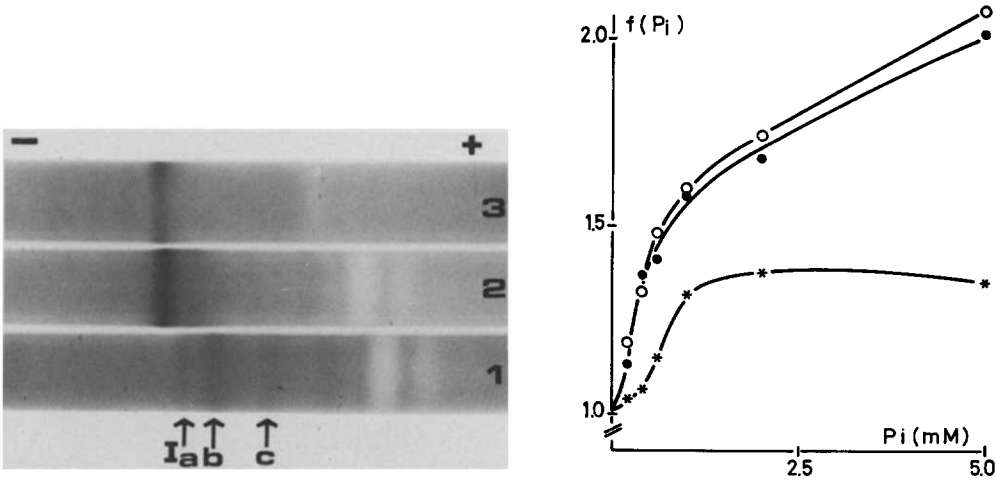


Fig. 3. Polyacrylamide gel electrophoresis of partially purified hexokinase from human erythrocytes (total type I complex) (lane 1) and of peak 3 after phosphocellulose chromatography (lane 3) (type I_a). Lane 2 is a mixture of lane 1 and 3.

Fig. 4. Influence of inorganic phosphate on the inhibition by Glc-1,6-P_2 of hexokinase type I_a (*———*), I_b (○———○) and I_{cd} (●———●). $f(P_i)$ is determined as defined in the Materials and Methods section. The figure is the mean of five separate experiments.

the partially purified preparations are shown in Fig. 5. Although the fractionation into cell populations of different ages is of course rather imperfect (see also Table I) and although the exact estimation of the extent of the electrophoretic shift is troubled by the interference of hemoglobin at the cathodal site of band I_a, it must be concluded that the proportion of hexokinase type I_b is decreasing in favour of the other subtypes with increasing cell age.

In an earlier report it was stated that the electrophoretic shift was entirely in favour of type I_c [7]. However, closer examination of the results reveals that it is more correct to mention only a relative decrease of type I_b. The result of this decrease is an increase of the proportion of type I_a and I_c during the lifespan of the erythrocyte. This means that there is an increasing proportion of less regulated enzyme (type I_a). However, the overall loss of regulation by P_i appears to be only marginal and it is doubtful whether this alteration in regulatory properties is of physiological importance.

The observed shift is due to an in vivo process and is not a result of artifacts of the fractionation and purification procedure, as it became more pronounced with increasing cell age, all enzymes being prepared under the same conditions. As the mature erythrocyte lacks the ability to synthesize protein, the observed changes in electrophoretic and regulatory properties have to be the result of a post-translational phenomenon.

Several underlying mechanisms can be mentioned. Hexokinase from many tissues is partly bound to mitochondria. The site of the hexokinase binding appears to be a specific hexokinase binding protein on the outer membrane [19]. The equilibrium between 'soluble' and 'particulate' hexokinase is shifted to the soluble form by e.g., Glc-6-*P*, ATP and ADP. This effect is counteracted by P_i. In reticulocytes part of the hexokinase is particulate, too [20]. During

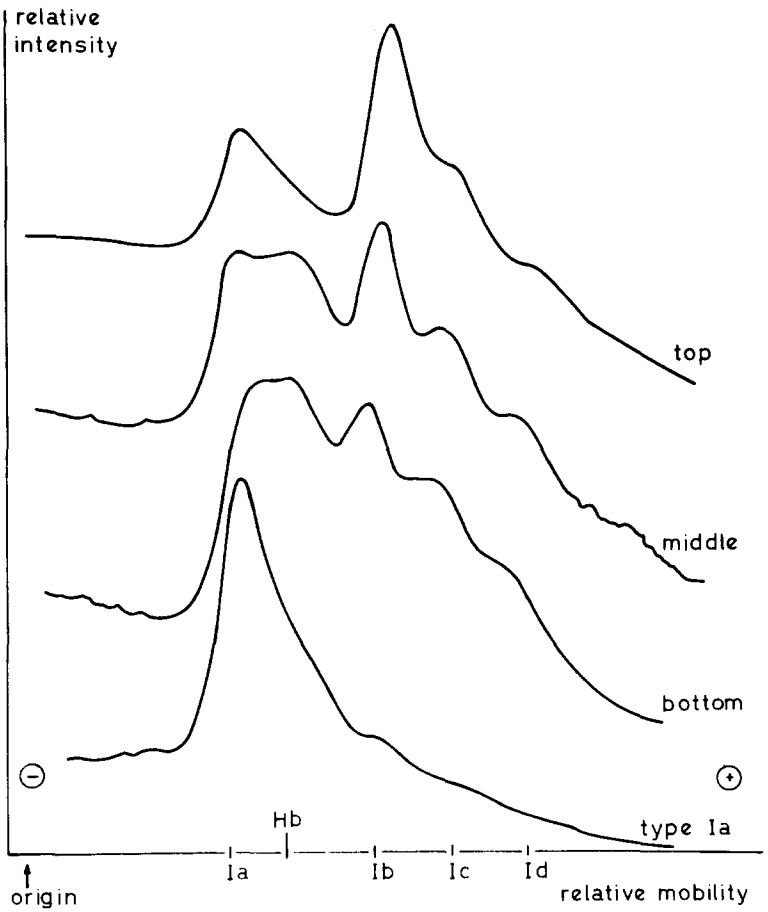


Fig. 5. Graphic representation of the electrophoretic pattern on cellulose acetate of partly purified hecokinase from cell populations of different age. Type I_a hexokinase obtained after phosphocellulose chromatography (peak 3) served as a reference.

the transition from reticulocyte to mature erythrocyte the mitochondria are destroyed, but the possibility that part of the hexokinase remains attached to membrane fragments must be considered. If this is so, one can assume that the equilibrium between soluble and particulate hexokinase persists in the mature red cell, although the particulate hexokinase would be better termed 'lipid-bound' hexokinase. In favour of this assumption is the finding that purified red cell hexokinase contains a considerable amount of lipid [14]. In this concept the shift in electrophoretic and regulatory properties could correspond to a changing position of the equilibrium with increasing cell age.

Another explanation for the presence of different subtypes of hexokinase I is the existence of a slow reversible equilibrium between a monomer and a dimer form of the enzyme as described in pig heart [3] and bovine brain [5]. In addition a limited in vitro tendency to form polymers has been described [3].

Glc-6-*P* is known to be a crucial agent in both the solubilization of particu-

late hexokinase [20] as well as in the dimerization of hexokinase [3,5]. However, preincubation of the samples with 1 mM Glc-6-*P* for 24—48 h at 4°C prior to electrophoresis, did not produce any change in the isozymic patterns (data not shown). Therefore, it seems unlikely that one of these mechanisms is responsible for the heterogeneity of erythrocyte hexokinase. In this view the concept of a post-translational modification of the primary enzyme is favoured, which may include a chemical modification of reactive residues or a limited proteolysis as described for erythrocyte pyruvate kinase [21].

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