Multiple Forms of Human Red Blood Cell Hexokinase

PREPARATION, CHARACTERIZATION, AND AGE DEPENDENCE*

Vilberto Stocchi, Mauro Magnani, Franco Canestrairi, Marina Dacha, and Giorgio Fornaini

From the Istituto di Chimica Biologica, Università degli Studi di Urbino, Via Saffi, 2, 61029 Urbino, Italy

Human red blood cell hexokinase (EC 2.7.1.1) has been shown to exist in multiple molecular forms which are separable by ion exchange chromatography. Of the major forms, designated hexokinase Ia, Ib, and Ic, only hexokinase Ia corresponds to hexokinase type I from human liver, while the others differ from every other previously reported hexokinase isozyme. Hexokinase Ib is the predominant form in the fetal erythrocytes, while it is present at lower levels in the red blood cells of adults. Analysis of the hexokinase isozymic pattern in red cells of different mean age shows that the level of hexokinase Ib is also dependent on the age of the cell. The three major forms of hexokinase have the same molecular weight of 100,000, by sedimentation velocity on sucrose density gradients, the same Michaelis constants, substrate and coenzyme specificity, pH-dependent activity, and the same thermal stability. The only significant differences were found in the isoelectric points which were 5.7 pH units for hexokinase Ia, 5.5 pH units for hexokinase Ib, and 5.35 pH units for hexokinase Ic. These data, together with that previously reported for rabbit erythrocytes (Stocchi, V., Magnani, M., Canestrairi, F., Dacha, M., and Fornaini, G. (1981) J. Biol. Chem. 256, 7856–7861) suggest that the presence of multiple forms of hexokinase is a common phenomenon in mammalian red blood cells.

Hexokinase in mammalian tissues exists as four isoenzymes with distinct kinetic properties and tissue distribution (1, 2). The proportion of these isozymic forms appears to be adaptive and can be altered by developmental, hormonal, and dietary changes (1, 2).

It is commonly accepted that red blood cell hexokinase is mainly of type I, but the presence of subtypes has also been described. Multiple forms of red blood cell hexokinase were first reported by Eaton et al. (3) because of the observation of several electrophoretic bands. More recently, many authors have confirmed the existence of two or more distinguishable forms of hexokinase in different mammalian erythrocytes (4–18), but usually, the reported results were considerably different. Probably, many discrepancies are due to the age dependence of the various forms of hexokinase (11–17), to the different distribution in the red cells of different mammals (6), and to the approach utilized for the evaluation of the isozymic pattern.

In previous papers, we have reported the presence of two distinct forms of hexokinase in rabbit erythrocytes and reticulocytes (18, 19) and their age dependence (17). Compared to the rabbit erythrocyte hexokinase, the isozymic pattern in human red blood cells is much more complex. In this paper, we report the presence of at least three major forms1 of hexokinase and of some minor components in adult and fetal erythrocytes. The cell age dependence of the major forms, the procedure for their preparation, and their biochemical properties are also described.

EXPERIMENTAL PROCEDURES

Materials—Coenzymes, enzymes, substrates, and dithiothreitol were obtained from Sigma. DEAE-Sephadex A-50 and Sephadex G-25 were purchased from Pharmacia, Inc., Piscataway, NJ. DE-52 from Whatman, and ampholines (pH range 4 to 6 and 5 to 8) from LKB Instruments, Inc., Rockville, MD. All other reagents were of an analytical grade.

Hexokinase Assay—Hexokinase activity was measured at 37 °C spectrophotometrically in a system coupled with glucose-6-phosphate dehydrogenase (EC 1.1.1.49) and 6-phosphogluconate dehydrogenase (EC 1.1.1.44), as previously described (18). For each molecule of glucose utilized, 2 molecules of NADP+ are reduced. One unit of hexokinase activity is defined as the amount of enzyme which catalyzes the formation of 1 μmol of glucose 6-phosphate/min at 37 °C.

In the sugar 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) as in Ref. 18. Hence, 1 unit of hexokinase activity is defined as the amount of enzyme which catalyzes the production of 1 μmol of ADP/min at 37 °C.

Protein Estimation—In the hemolysate, hemoglobin concentration was determined spectrophotometrically, at 540 nm with Dabkin's solution as described by Beutler (20). During the purification procedure, protein was determined according to the method of Lowry et al. (21), with bovine serum albumin as a standard, or spectrophotometrically at 280 nm.

Molecular Weight Estimation—The molecular weights of hexokinase Ia, Ib, and Ic were determined by sedimentation velocity on sucrose density gradients. The procedure of Martin and Ames (22) was used at 4 °C. Linear 4.5-mI sucrose gradients from 5 to 25% sucrose (w/v) were prepared by automatic gradient former (Gilson) in 5 mM sodium potassium phosphate buffer, pH 7.5, containing 3 mM 2-mercaptoethanol, 3 mM KF, 10 mM glucose. Partially purified hexokinase isozymes (100 μI containing 1.5 units/ml, 10 μI of aldolase (EC 4.1.2.13) (rabbit muscle in (NH4)2SO4), 10 μI of lactate dehydrogenase (EC 1.1.1.27) (rabbit muscle, in (NH4)2SO4), 10 μI of phosphoglucomutase (EC 2.7.5.1) (rabbit muscle in (NH4)2SO4), and 10 μI of 3-phosphoglycerate kinase (EC 2.7.2.3) (yeast in (NH4)2SO4 and Na2HPO4) were dialyzed for 4 h against phosphate buffer and layered onto a gradient. The gradients were developed in the SW 65 rotor of the Beckman L5-65 ultracentrifuge at 4 °C for 16 h at 60,000 rpm and afterwards, 3-drop fractions were collected from the bottom of the tubes and assayed for enzyme activities.

Isoelectric Focusing—Isoelectric focusing of hexokinases was carried out on LKB 8100 electrophoresis equipment in a glycerol gradient solution and in a pH gradient of 4 to 8 at 1% ampholine concentrations according to the instructions of the manufacturer. The sample was layered after the pH gradient was formed. The voltage was 600 V and

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the focusing was completed after 16 h from the introduction of the sample. Cooling temperature was 4 °C, elution flow rate 30 ml/h, and fractions of 1 ml were collected and measured for the hexokinase activity and the pH value.

Preparation of Human Liver Hexokinase—The preparation of the soluble human liver hexokinase extract was performed using a procedure based in part on that described by Grossbard and Schimke (23). Fresh liver (1.5 g) obtained by surgical biopsy from adults, was minced and then homogenized with 3 volumes of 3 mM sodium potassium phosphate buffer, pH 7.5, containing 0.25 mM glucose and 5 mM dithiothreitol. The homogenate was centrifuged at 105,000 × g for 1 h at 4 °C. At this stage, the supernatant fluid contains 1.6 units/ml of solubilized hexokinase activity.

Red Cell Fractionation—Human blood was drawn from normal subjects ranging in age from 20 to 30 years and collected in heparin. After centrifugation at 1,200 × g for 10 min at 4 °C, the plasma anduffy coat were removed by suction and erythrocytes were washed twice with buffered isotonic NaCl (0.9% w/v of NaCl containing 1 mM sodium potassium phosphate, pH 7.4). The washed red cells were resuspended in an equal volume of cold isotonic saline solution and separated into fractions of different mean age by ultracentrifugation.
according to the method of Turner et al. (24), with some modifications (25). The gradients were spun for 1 h at 4°C and 40,500 × g in a SW 27 swinging bucket rotor in a Beckman L5-65 ultracentrifuge.

RESULTS

Evidence for the Existence in Human Erythrocytes of Multiple Forms of Hexokinase—Hexokinase in mammalian liver exists as four isoenzymes which can be separated by electrophoresis or ion exchange chromatography (23, 26–28). Accordingly, when the soluble hexokinase prepared from human liver was chromatographed on DE-52 column we obtained the pattern shown in Fig. 1A. Compared to the isozyme profile reported for rat liver, we found a much lower level of hexokinase IV or glucokinase (in the nomenclature of Katzen and Schimke (26)). However, this difference can be explained by the fact that glucokinase in under dietary control and we usually obtained our liver samples after 12 to 20 h of fasting. When human red blood cells hemolysate, prepared as in Ref. 19, was chromatographed under the same conditions a very complex isozymic pattern appears (Fig. 1C). Three major forms (that we labeled hexokinase la, Ib, and IC on the basis of their elution from the DE-52 column) and some minor components have been constantly observed in the red cell hemolysate of 15 normal adults. By co-chromatography of hexokinase from human liver and human red cells, we have been able to demonstrate that only hexokinase Ia corresponds to hexokinase type I from liver and that a low amount of hexokinase type III was also present. All the other molecular forms can not be identified with any other known hexokinase isozyme and were eluted at intermediate positions between hexokinase type I and type II.

Red Blood Cell Age Dependence of the Hexokinase Isozymic Pattern—Fetal erythrocytes are characterized by a higher level of red blood cell hexokinase when compared to the red cells of adults. Many authors have proposed that this hexokinase increase can be due to a different regulation of synthesis of one type of hexokinase with respect to the others (4–6, 8, 13, 14). In Fig. 1B, we show that the chromatographic profile of the hexokinases from cord red blood cells and adult erythrocytes are significantly different. No hexokinase type III was present in fetal red cells and a higher level of type Ib was also found.

Studies performed on the modification of the hexokinase isozymic pattern during red cell ageing provide an explanation for the differences found between fetal and adult erythrocytes. Fig. 2 shows that in young red blood cells, obtained by density gradient ultracentrifugation, hexokinase Ib is the predominant form and that the amount decreases as the cell age increases. Recalling that blood samples obtained from umbilical cord contains a higher proportion of young cells compared to sample obtained from adult (the life span of fetal red blood cells is shorter than that of the adults (29)), we propose that the cell age is responsible of the differences found between the hexokinase isozymic pattern of fetal and adult erythrocytes. Furthermore, the pattern of Fig. 2 also provides evidence that the decrease in hexokinase Ib is responsible for the lower hexokinase activity found during cell ageing (15, 30, 31).

Preparation and Molecular Properties of Hexokinase Ia,
TABLE I

<table>
<thead>
<tr>
<th>Compounds</th>
<th>Hex Ia</th>
<th>Hex Ib</th>
<th>Hex Ic</th>
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<tr>
<td></td>
<td>$K_a$</td>
<td>$K_a$</td>
<td>$K_a$</td>
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<tr>
<td>$mM$</td>
<td>%</td>
<td>$mM$</td>
<td>%</td>
</tr>
<tr>
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</tr>
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<td>5</td>
<td>14</td>
<td>8</td>
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<tr>
<td>MgUTP</td>
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<tr>
<td>MgCTP</td>
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<tr>
<td>D(+)-Glucose</td>
<td>0.046</td>
<td>100</td>
<td>0.048</td>
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<td>D(+)-Mannose</td>
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<td>78</td>
<td>0.1</td>
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<td>100</td>
<td>11.7</td>
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<tr>
<td>2-Deoxy-D-glucose</td>
<td>0.77</td>
<td>95</td>
<td>0.56</td>
</tr>
<tr>
<td>D(+)-Glucosamine</td>
<td>1.5</td>
<td>47</td>
<td>0.5</td>
</tr>
<tr>
<td>N-Acetyl-D-glucosamine</td>
<td>1.9</td>
<td>100</td>
<td>1.16</td>
</tr>
</tbody>
</table>

$^a$ $K_a$ values were determined using the Lineweaver-Burk plots.

$^b$ Maximum velocities are expressed relative to the $V_{max}$ for glucose (100%) when the enzyme is saturated by the considered substrate.

**FIG. 5.** Heat stability of human hexokinase Ia (○, ◆), Ib (○, ▲, △), and Ic (□, ■, □). Experiments have been performed at 44 °C in 5 mM Tris-HCl, pH 7.2, containing 9% (v/v) glycerol and 3 mM 2-mercaptoethanol (○, ▲, △), or in the same buffer plus 5 mM glucose (○, △, □); or 5 mM glucose and 5 mM sodium potassium phosphate, pH 7.2, (○, ▲, ■).

**FIG. 6.** Reciprocal plots of hexokinase Ia (a), hexokinase Ib (b), and Ic (c), with MgATP as the varied substrate in the absence and presence of glucose 6-phosphate and glucose 1,6-diphosphate. These experiments have been performed in 80 mM Tris-HCl, pH 7.2, at 37 °C. Glucose concentration was 2.5 mM and free Mg$^{2+}$ was 5 mM. Orthophosphate from the enzyme solution was removed by passage through Sephadex G-25 columns (1.5 × 15 cm) equilibrated in 5 mM Tris-HCl, pH 7.2, containing 5 mM glucose, 5 mM dithiothreitol, and 9% (v/v) of glycerol. Upper panel, hexokinase inhibition by (○), 0 μM; (▲) 19 μM; (△) 38.5 μM, and (□) 77 μM of glucose 6-phosphate. Lower panel, effect of (○) 0 μM; (▲), 25 μM; (△), 50 μM, and (□), 100 μM glucose 1,6-diphosphate on hexokinase activity.
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values of \( f(P_i) \) were determined according to the method of Kosow et al. (35) from the equation \( f(P_i) = \frac{V_{\text{ADP}}(P_i) - V_{\text{ADP}}}{V_{\text{ADP}}(P_i)} \). where \( V_{\text{ADP}} \) is the reaction velocity in the presence of the inhibitor and orthophosphate, \( V_{\text{ADP}}(P_i) \) equals the reaction velocity in the presence of inhibitor and absence of \( P_i \), and \( V_{\text{ADP}}(P_i) \) equals the reaction velocity in the absence of inhibitor and \( P_i \).

**Molecular Weight**—The molecular weight of the partially purified hexokinases has been found to be about 100,000 by sucrose density gradient sedimentation velocity with four other standard enzymes. Co-sedimentation of the three hexokinases gives only symmetrical peak of activity indicating that the three forms have the same molecular weight (Fig. 3).

**Isoelectric Focusing**—Isoelectric focusing of the multiple forms of hexokinase, prepared as above, under native conditions, in glycerol gradient solutions provide single peaks with a pI of 5.7 for the hexokinase Ia, a pI of 5.5 for hexokinase Ib, and a pI of 5.35 for the hexokinase Ic. The same isoelectric points were also found if all the different forms of hexokinase were co-electrofocused under the same conditions (Fig. 4). The fetal and adult hexokinase isozymes show the same corresponding isoelectric points.

**pH Dependence of Enzyme Activity**—The different forms of human fetal and adult hexokinase exhibited quite similar pH dependence of enzyme activity. The enzyme activity increases more than 40% over the range of pH 7.0 to 8.0 after which the activity remains constant until pH 9.0 (data not shown).

**Thermal Stability**—All three different forms of human erythrocyte hexokinase were inactivated as a function of time when incubated at 44 °C in 5 mM Tris-HCl, pH 7.2, and 5 mM sodium potassium phosphate buffer, pH 7.2. The results of these experiments are presented in Fig. 5. Glucose was found to protect human erythrocyte hexokinases from being inactivated at high temperatures.

**Kinetic Properties and Specificity**—The apparent \( K_m \) values of the multiple forms of erythrocyte hexokinase for hexoses and nucleotides triphosphate were estimated in 0.1 mM glycylglycine, pH 8.1, at 37 °C. The double reciprocal plot, \( 1/v \) against \( 1/S \), shows similar \( K_m \) values for the hexoses and for the nucleotides triphosphate utilized (Table I). N-Acetyl-d-glucosamine and D(+)-galactose were not phosphorylated. MgGTP, MgUTP, and MgCTP did not serve as phosphate donors at 20 mM concentration. Hexokinase Ic is less efficient than hexokinase Ia and Ib in utilizing 2-deoxy-d-glucose.

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**Table II**

<table>
<thead>
<tr>
<th>Substrate or effector</th>
<th>Hex Ia</th>
<th>Hex Ib</th>
<th>Hex Ic</th>
</tr>
</thead>
<tbody>
<tr>
<td>MgATP, ( K_m ) (mM)</td>
<td>0.60 ± 0.04</td>
<td>0.58 ± 0.04</td>
<td>0.62 ± 0.03</td>
</tr>
<tr>
<td>Glucose, ( K_m ) (mM)</td>
<td>46 ± 3</td>
<td>48 ± 4</td>
<td>47 ± 3</td>
</tr>
<tr>
<td>Glucose-6-phosphate, ( K_i ) (mM)</td>
<td>15 ± 2</td>
<td>13 ± 2</td>
<td>15 ± 2.5</td>
</tr>
<tr>
<td>Glucose-1,6-diphosphate, ( K_i ) (mM)</td>
<td>22 ± 2</td>
<td>22 ± 2.5</td>
<td>21 ± 2</td>
</tr>
</tbody>
</table>

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2 A portion of this paper (including the preparation of Hex Ia, Ib, and Ic, Fig. 1, and Tables I and II) is presented in detail in miniprint at the end of this paper. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopics are available from the Journal of Biological Chemistry, 9650 Rockville Pike, Bethesda, MD 20814. Request Document No. 81M-1755, cite authors, and include a check for $1.20 per set of photocopies. Full size photocopies are also included in the microfilm edition of the Journal that is available from Waverly Press.
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Regulatory Properties—Red blood cell hexokinase is markedly suppressed by its product glucose 6-phosphate (32-38) and by glucose 1,6-diphosphate (36, 39-41); however, this inhibition can be partially relieved by orthophosphate (P<sub>i</sub>) (32-35, 40, 41). Furthermore, the dissociation constants of glucose 6-phosphate and glucose 1,6-diphosphate for human red blood cell hexokinase are both in the range of the intracellular concentrations of these two phosphorylated compounds (37, 40). In order to have more information about the age dependence of the in vivo regulation of hexokinase, it seems of interest to study the effects of glucose 6-phosphate and glucose 1,6-diphosphate on the three major hexokinase isozymes. As shown in Fig. 6, human erythrocyte hexokinase Ia, Ib, and Ic are inhibited by glucose 6-phosphate and glucose 1,6-diphosphate competitively with respect to MgATP. The secondary plot of slope of the Lineweaver-Burk plots, against inhibitor is linear over the range of concentrations used and the determined K<sub>i</sub> values are reported in Table II. These experiments have been performed in 80 mM Tris-HCl at the physiological pH of 7.2 at 37°C. From the data reported in Tables I and II, it can also be observed that K<sub>i</sub> value for glucose and MgATP do not change over the pH range of 7.2 to 8.1 (data not shown). Contrary to previous published results (42), identical kinetic constants were obtained for hexokinase Ia, Ib, and Ic. Furthermore, no differences have been found among the three hexokinases in orthophosphate regulation. In fact, as shown in Fig. 7, the inhibition of hexokinase Ia, Ib, and Ic by glucose 6-phosphate or glucose 1,6-diphosphate is relieved to the same extent by addition of orthophosphate.

**DISCUSSION**

The presence of multiple forms of red blood cell hexokinase appears to be a common phenomenon in mammals. The hexokinase isozymic pattern has been examined by several authors and with different methods (3-18). The results reported, also by using the same technique, were considerably different, and this is probably due to the difficulties in investigating the true isozymic pattern in the hemolysate. All the methods used were based on the different net surface charge of the multiple forms of hexokinase. After some preliminary experiments with polyacrylamide disc gel electrophoresis, isoelectric focusing and agarose gel electrophoresis, we have decided to approach the study of the hexokinase isozymic pattern by utilizing the ion exchange column chromatographic method. Using the conditions employed in this paper, DE-52 column chromatography provided a powerful tool for the comparative analysis of multiple forms of hexokinase. The method is simple, quick, and capable of being done with a relatively small number of erythrocytes. Furthermore, recovery of the hexokinase activity applied to these columns was always not lower than 98 to 100%. Another factor affecting the human hexokinase isozymic pattern is the age of the red blood cell. In a population rich of young erythrocytes, obtained by density gradient ultracentrifugation, it can be observed that hexokinase Ib is the predominant form which contributes more than the others to the decay of the total glucose-phosphorylating activity found in cell ageing (15, 16, 24, 30). Comparison of the hexokinase isozymic pattern of the fetal and adult erythrocytes gives evidence of further differences. The adult erythrocyte contain hexokinase type III which was not found in the fetal cell, and lower levels of hexokinase Ib. Blood samples from umbilical cord contain a higher proportion of young cells compared to samples obtained from adults. Recalling that hexokinase Ib is the more age-dependent of the three major hexokinase forms, we propose that the presence of higher hexokinase Ib in fetal red cells may be interpreted as a red cell age-dependent phenomenon and not as the presence of a special fetal isozyme as suggested by some authors (13).

When separated by ion exchange chromatography, all multiple hexokinase forms present in human erythrocytes except type III lie in the type I hexokinase area. A similar phenomenon has been previously observed for the rabbit red cell enzyme (17-19). Thus, it appeared of interest to study some molecular properties of these hexokinases in order to have more information concerning the possible mechanism of their appearance. In human red cells, as in the rabbit erythrocytes (18), the multiple forms of hexokinase show no differences in molecular weight and possess similar biochemical properties. The only significant differences occur in the isoelectric points. So, a possible mechanism of the formation of multiple forms of hexokinase in mammalian erythrocytes is a post-translational modification of the native enzyme, that on the basis of the biochemical properties can be tentatively identified in hexokinase Ia. In fact, this last form has the same molecular properties of hexokinase type I from other tissues and is the only hexokinase isozyme in the bone marrow erythroid precursor cells.

In the well known case of rabbit muscle aldolase, the microheterogeneity has been shown to be due to deamination of a single asparagine residue (43). Robinson and Rudd (44) have proposed that deamination is a common phenomenon and is specifically designed to regulate the turnover of aldolase. A similar mechanism could also explain the heterogeneity of human red blood cell hexokinase which, as we show in this paper, is characterized by isozymes with similar molecular weight but different net electric charges. At present, it seems quite difficult to explain the molecular basis of the presence of multiple forms of hexokinase and the exact relationship between the different isozymes as found in human erythrocytes.

The availability of pure isozymes and the possibility of having some information on the primary structure of these multiple forms of hexokinase could definitively solve the question of their origin. Unfortunately, it seems quite difficult to obtain a sufficient amount of pure protein since hexokinase is present in the erythrocyte to the extent of only 0.0004% (w/w) of the total proteins.

**Addendum—**After this manuscript was submitted, Rijken et al. (45), using phosphocellulose chromatography, reported the separation of human red cell hexokinase I subtypes into three distinct forms. Their results do overlap considerably with those reported in this paper; however, some differences appear. Contrary to Rijken et al. (45), we have shown that the inhibition of hexokinases Ia, Ib, and Ic by glucose 6-phosphate or glucose 1,6-diphosphate was relieved to the same extent by addition of orthophosphate. Furthermore, some differences in the age dependence of the hexokinase pattern are probably due to the approach utilized. Cellulose acetate electrophoresis performed according to the method of Rijken et al. (45) has not given the same isozymic pattern obtained by loading the hemolysate directly onto DE-52 columns. These discrepancies are due to the fact that the enzyme purification step, unavoidable before hexokinase electrophoresis, produces significant differences as can be observed by comparing Fig. 1, B, and C, of this paper, with Fig. 1, A and B, presented in the miniprint section. In conclusion, our results agree with the data of Rijken et al. (45) on the presence of multiple forms of hexokinase in human red cells and on the similarities among their kinetic properties, but differ mainly in the approach. In the further study of intracellular metabolism and their regulation in human red cells, we believe that the present work will be useful.

REFERENCES

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Age-dependence

by

Vittorio Giordano, Massimo Magnani, Franco Terruzzi, Martin Dacki and Giampaolo Rijksen

all samples were performed at 37°C. All buffers contained 3 M-mercaptoethanol and 2 M-NAG.

Step 1: Preparation of Hemolysates. Human red blood cells from normal adults and fetal cow blood were collected using EDTA as an anticoagulant. The red cells were washed twice with isotonic saline chloride solution. The red cells were diluted with an equal volume of 0.5% BSA in phosphate-buffered saline. The red cells were then washed three times with 0.2 M-NaCl, followed by centrifugation at 10,000 x g for 30 min.

Step 2: Assay of Hexokinase Activity. Hemolysates were incubated in buffer (0.1 M-NaCl, 50 mM-KH2PO4, 10 mM-MgCl2, 10 mM-ATP, 20 mM-2-mercaptoethanol, pH 7.4) containing 5 mM-glucose and 0.1 mM-3-[(N4-ethyl-N4-isopropylamino)-2-oxopropionylamino]-2-deoxy-D-glucose. After 10 min, the reaction was stopped by 5 mM-2-deoxy-D-glucose. The assay was performed at 37°C with the reaction mixture incubated for 10 min.

Step 3: Determination of Hexokinase Activity. The reaction mixture for the assay was incubated at 37°C for 30 min. The reaction was stopped by 5 mM-2-deoxy-D-glucose. The hexokinase activity was determined by measuring the formation of glucose-6-phosphate using a spectrophotometer.

Step 4: Determination of Glucose-6-Phosphate. The glucose-6-phosphate was measured using a glucose-6-phosphate dehydrogenase assay. The reaction mixture was incubated at 37°C for 10 min. The reaction was stopped by 5 mM-2-deoxy-D-glucose. The glucose-6-phosphate was measured using a spectrophotometer.

Step 5: Determination of Glucose-6-Phosphate Dehydrogenase. The enzyme activity was determined by measuring the formation of NADPH using a spectrophotometer.

Flow Cytometry

Flow cytometry was performed using a FACS Calibur (BD Biosciences) flow cytometer. The samples were stained with propidium iodide (PI) and analyzed using the WinMDI software. The fluorescence was measured at 633 nm excitation and 640 nm emission.

Fig. 1. Flow cytometric analysis of cells stained with PI. The x-axis represents the fluorescence intensity, and the y-axis represents the number of cells. The histogram shows the distribution of fluorescence intensity for the cells.

Fig. 2. Analysis of the cell cycle distribution using flow cytometry. The x-axis represents the percentage of cells in each phase of the cell cycle, and the y-axis represents the number of cells. The histogram shows the distribution of cell cycle phases for the cells.
### Table 1

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<th>Volume</th>
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*The volume and activity are the sum of two individual experiments and the specific activity of hemolysate (4.91) is the average from these experiments.*

### Table 2

<table>
<thead>
<tr>
<th>Step</th>
<th>Protein</th>
<th>Volume</th>
<th>Protein</th>
<th>Activity</th>
<th>Specific Activity</th>
<th>Yield</th>
<th>Purity</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Hemolysate</td>
<td>180</td>
<td>185</td>
<td>0.112</td>
<td>0.298 d</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>DEAE-Sephadex A-50</td>
<td>16.0</td>
<td>18.6</td>
<td>0.035</td>
<td>0.0156</td>
<td>90</td>
<td>35</td>
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<td>3</td>
<td>Acetone pellet precipitation 25 to 75%</td>
<td>9.5</td>
<td>10.2</td>
<td>0.322</td>
<td>95</td>
<td>14</td>
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<tr>
<td>4</td>
<td>DEAE-Sephadex A-50</td>
<td>1.6</td>
<td>2.39</td>
<td>0.159</td>
<td>1.52</td>
<td>15</td>
<td>15</td>
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<tr>
<td></td>
<td>Acetone pellet precipitation 25 to 75%</td>
<td>1.9</td>
<td>2.48</td>
<td>0.112</td>
<td>1.23</td>
<td>15</td>
<td>15</td>
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<tr>
<td></td>
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Multiple forms of human red blood cell hexokinase. Preparation, characterization, and age dependence.
V Stocchi, M Magnani, F Canestrari, M Dachà and G Fornaini


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