*Biochimica et Biophysica Acta*, 972 (1988) 1–8
Elsevier



BBA 12332

# Improved metabolic properties of hexokinase-overloaded human erythrocytes

Mauro Magnani [a], Luigia Rossi [a], Marzia Bianchi [a], Giorgio Fornaini [a,†], Umberto Benatti [b], Lucrezia Guida [b], Elena Zocchi [b] and Antonio De Flora [b]

[a] *Institute of Biochemistry, University of Urbino, Urbino and* [b] *Institute of Biochemistry, University of Genoa, Genoa (Italy)*

(Received 6 April 1988)

Key words: Hexokinase; Encapsulation; Erythrocyte aging; (Human erythrocyte)

**Human erythrocytes were loaded with homogeneous hexokinase purified from human placenta (an enzyme species apparently identical to the erythrocyte enzyme), using a procedure of encapsulation based on hypotonic hemolysis, isotonic resealing and reannealing. The hexokinase-overloaded erythrocytes contained 4.77 ± 0.75 IU of hexokinase activity per ml of packed erythrocytes, a value 15-times higher than that of corresponding unloaded or native red cells. The hexokinase-loaded erythrocytes were found to metabolize twice the amount of glucose consumed by the unloaded cells through a nearly doubled glycolytic activity, while the activity of the hexose monophosphate shunt pathway was unmodified. Estimates of glycolytic intermediates showed increased levels of most metabolites with respect to the unloaded erythrocytes, while the intracellular concentrations of adenine nucleotides and 2,3-bisphosphoglycerate were unaffected by entrapment of hexokinase. The new steady-state condition characterized by improved glycolytic function was demonstrated to be directly related to enhanced levels of hexokinase activity and not to the use of a rejuvenation solution during the procedure of entrapment. These results are consistent with suggestions by several investigators that glucose metabolism in human erythrocytes is regulated by hexokinase, and they open new perspectives for manipulating erythrocytes with the ultimate aim of improving their survival under different storage conditions.**

## Introduction

In humans, all living functions of the mature erythrocyte are selectively and entirely dependent upon glucose utilization. A major part of glucose consumption (0–95%) occurs via the glycolytic pathway to yield lactate, while the remaining 5–10% is channeled through the hexose monophosphate shunt (HMS) pathway. These limited metabolic functions are compatible with a life-span of approx. 120 days, after which senescent erythrocytes are sequestered and phagocytosed in the reticuloendothelial system.

Hexokinase (EC 2.7.1.1.) catalyzes the first step of glucose metabolism in several cell types including erythrocytes, in which it is currently believed to be a crucial, rate-limiting enzyme of the glycolytic pathway [1–11]. Support of this view has recently been provided by studies of glucose metabolism in intact erythrocytes from patients who are heterozygous for hexokinase deficiency

† Deceased August 23, 1987; to the memory of whom the other authors wish to dedicate this paper.

Correspondence: M. Magnani, Istituto did Chimica Biologica, Università degli Studi, Via Saffi, 2, 61029 Urbino, Italy.

[12,12] or with increased hexokinase activity because of 'de novo' trisomy 10p [7,11].

Besides catalyzing the committed step of glycolysis, hexokinase seems to be of additional interest because of its half-life of only 33 days [13]. Such decay of hexokinase activity throughout the erythrocyte life-span has been related to the parallel decline in the glycolytic rate, on the basis of a progressively decreased ability of aging erythrocytes to produce ATP [13,14]. A convenient approach to studies on the role of hexokinase in both glucose metabolism and erythrocyte aging seems to be provided by use of procedures allowing purified hexokinase to be encapsulated in red cells [5]. Among such procedures, those based on hypotonic hemolysis and isotonic resealing are certainly the most conservative [15,16] and have been recognized to be useful for identifying sites of metabolic control in erythrocytes [5]. A previous study performed on these grounds on human erythrocytes has shown that entrapment of yeast hexokinase apparently enhances the metabolic flow through the glycolytic pathway, as measured from the amount of $^{14}$C-labeled anions formed from [$^{14}$C]glucose [5]. However, since yeast hexokinase is not regulated by product inhibition [17], the results obtained may not have physiological relevance in human erythrocytes and, indeed, use of autologous enzyme proteins [5] seems to be desiderable.

Prompted by these considerations and by current availability of homogeneous hexokinase type I purified from human placenta [18], i.e., an enzyme species apparently identical to the human erythrocyte enzyme, we started an investigation on the metabolic properties of erythrocytes overloaded with human hexokinase. The purpose of this study was twofold: on one hand, manipulated erythrocytes containing higher than normal hexokinase activity should reproduce a metabolic condition found in the young cells, thus providing a useful model for the study of erythrocyte aging; on the other hand, such erythrocytes are expected to be metabolically more competent than the corresponding unloaded cell, this opening the way to possible improvement of storage techniques. The results reported in this paper tend to support both expectations as they showed that this specific type of manipulation results in a metabolic re-modelling of human erythrocytes, the extent of which is somewhat restricted by intracellular regulation of encapsulated human hexokinase.

## Materials and Methods

*Materials.* Commercial enzymes, coenzymes and substrates for the determinations described below were obtained from Sigma (St. Louis, MO, U.S.A.) or Boehringer Mannheim (F.R.G.). Solvents for HPLC were from Fluka (Buchs, Switzerland). All other chemicals were from standard supply houses, and were of the highest grade available. [1-$^{14}$C]Glucose was from the Amersham International, Amersham, U.K.

*Purification of hexokinase.* Hexokinase type I was purified from human placenta as described in Ref. 18. The specific activity was $190 \pm 5$ IU/mg protein in the four different purifications performed from the studies described below. The enzyme protein was found to be homogeneous as shown by sodium dodecyl sulfate gel electrophoresis.

*Enzymatic and metabolic assays.* Hexokinase activity was measured spectrophotometrically at 37 °C in a system coupled with glucose-6-phosphate dehydrogenase (EC 1.1.1.49) as described previously [19]. The activities of all other glycolytic enzymes as well as of glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase were estimated according to Beutler [20]. Intraerythrocytic levels of glycolytic intermediates were determined spectrofluorimetrically as described in Ref. 20. Concentrations of 2,3-bisphosphoglycerate were determined by an ultraviolet test (Boehringer Mannheim, F.R.G.), while levels of adenine and pyridine nucleotides were measured by reverse-phase HPLC as described previously [21]. Cell counts, mean cell volume, mean cell hemoglobin and mean cell hemoglobin concentration were determined by a Coulter Model S7.

Lactate production (taken as an estimate of the amount of glucose metabolized in the Embden-Mayerhof-Parnas pathway, assuming that the concentration of 2,3-bisphosphoglycerate remains unchanged) was measured on neutralized perchloric

acid extracts as reported in Ref. 20. Incubations of erythrocytes were performed at 37°C and at a 30% hematocrit for the times indicated in the Results and in the experimental conditions described in Ref. 7. The amount of glucose metabolized through the pentose phosphate pathway was evaluated from the measurement of $^{14}CO_2$ production derived from [1-$^{14}$C]glucose [5,7].

*Encapsulation of hexokinase into erythrocytes.* Encapsulation of hexokinase in human erythrocytes was obtained according to Ropars et al. [22], as described [23,24]. This procedure involves three sequential steps, i.e., hypotonic hemolysis, isotonic resealing and reannealing of erythrocytes. Briefly, blood was collected in heparin immediately before use and centrifuged at 2500 rpm at 4°C to separate the plasma, which was then maintained at 0°C until use. Erythrocytes were washed twice in 5 mM sodium phosphate buffer (pH 7.4), containing 0.9% (w/v) NaCl and 5 mM glucose and finally resuspended in the same buffer containing hexokinase (10 IU/ml packed erythrocytes) at a hematocrit of 70% in a dialysis tube (Spectrapor, Spectrum Medical Industries, Los Angeles, CA, U.S.A. molecular size cut off, 12–14 kDa). Hypotonic lysis of erythrocytes was obtained by dialysis of 2 ml of cell suspensions in a Falcon 50 ml sterile tube containing 10 mM sodium phosphate/10 mM sodium bicarbonate/20 mM glucose (pH 7.4) and rotated at 15 rpm for 1 h at 4°C. The hemolysate was then collected and 1 vol. of resealing solution (5 mM adenine/100 mM inosine/100 mM sodium pyruvate/100 mM sodium phosphate/100 mM glucose/12% (w/v) NaCl pH 7.4) solution A was added to every 10 vols. of hemolysate. Reannealing of the cells was then performed by incubation at 39°C for 20 min. Three additional washes of lysed and resealed erythrocytes were performed at 4°C with a physiological saline solution. Finally, erthrocytes were resuspended in their native plasma and utilized for metabolic studies. For each experiment, two additional erythrocyte suspensions were used, the first one (referred to as 'native') corresponding to the native, untreated cells and the second one (designated 'unloaded') corresponding to erythrocytes which had been processed as for the entrapment technique, but without any hexokinase addition.

## Results

*Encapsulation of human hexokinase in erythrocytes*

Table I shows the yield of encapsulation and the percentage of cell recovery as well as some hematological parameters of the hexokinase-overloaded erythrocytes. Levels of hexokinase activity in the 'unloaded' erythrocytes were similar to those estimated in the native, i.e., untreated cells, thus ruling out any selective loss of hexokinase throughout the dialysis step. This was also confirmed by measuring specific activities of all glycolytic enzymes, of glucose-6-phosphate dehydrogenase and of 6-phosphogluconate dehydrogenase (not shown), whose ranges were 90–100% of the corresponding specific activities measured in the native erythrocytes. No appreciable absorption of the added hexokinase to the external surface of erythrocytes could be recorded following the procedure of hemolysis/resealing, suggesting that the

TABLE I

ENCAPSULATION OF HUMAN HEXOKINASE IN HUMAN ERYTHROCYTES

Values are mean ± S.D. and ranges obtained in ten different experiments performed as described under Materials and Methods, at a starting concentration of 10 IU hexokinase/ml packed erythrocytes. The percentage of incorporation was calculated as described previously [5], by considering the enzyme added outside the cells at the beginning of the hemolysis steps as 100%. No externally absorbed hexokinase activity was detected upon performing the enzyme assay with 'loaded' erythrocytes at 0.5% hematocrit in isotonic glycylglycine (pH 8.1), while the encapsulated hexokinase activity became apparent by addition of 0.1% Triton X-100.

| | Hexokinase activity (IU/ml erythrocytes) |
|---|---|
| Unloaded erythrocytes | 0.33 ± 0.1 (0.26–0.40) |
| Hexokinase-loaded erythrocytes | 4.77 ± 0.75 (3.72–5.90) |
| % entrapment | 19.7 ± 6.2 (11.7–30.0) |
| % cell recovery | 77.9 ± 8.1 (70.2–87.2) |
| Mean cell volume (fl) | 73.3 ± 1.15 (72–74) |
| Mean cell hemoglobin (g/dl) | 27.58 ± 2 (24.42–29.9) |

encapsulated enzyme was confined within erythrocytes.

The efficiency of the encapsulation procedure was found to be approx. 20%, a value not remarkably different from those of other entrapped proteins [5] when appropriate allowance is made for the large molecular mass of human hexokinase (110 kDa). Such efficiency was the result of several experiments aimed at optimizing the amount of enzyme protein to be used, the hematocrit of cells undergoing hemolysis/resealing/reannealing and the duration of hemolysis.

The opened/resealed erythrocytes had almost normal hematological parameters [22], with the exception of a reduced cellular volume, and they were slightly hypocromic (Table I). This is in agreement with results obtained by most investigators using the hemolysis/resealing technique [15,16].

*Distinct effects of the 'rejuvenation solution' and of encapsulated hexokinase*

The procedure of encapsulation used in this study involves incubation of erythrocytes, during the resealing and reannealing steps, with a 'rejuvenation solution' (solution A). Use of this solution, containing metabolizable substrates, is justified by the need to re-equilibrate the hypotonically shocked erythrocytes, thereby obviating possible loss of metabolites. This may, however, result in a considerable perturbation of glycolytic metabolism of erythrocytes.

In order to discriminate between the 'solution A effect' and the 'hexokinase' effect on the metabolic properties of hexokinase-loaded erythrocytes, the intracellular concentrations of several glycolytic intermediates were measured at different times. The two erythrocyte populations submitted to such comparative analysis were the hexokinase-loaded and the unloaded cells, both of which had been treated with solution A during isotonic resealing and reannealing before being washed and incubated in their native plasma (see "Materials and Methods"). At the start of incubation in plasma, the concentration of many phosphorylated compounds was higher than in native cells in both erythrocyte populations, reaching steady-state levels after 1 h at 37°C (Fig. 1). It is important to note that after 1 h the concentrations of all glycolytic intermediates shown in Fig. 1 were higher in the hexokinase-loaded than in the corresponding unloaded cells, which had levels strictly comparable to those measured in the native erythrocytes (not shown).

Additional evidence for the 'solution A effect' came from estimates of phosphorylated metabolites in human erythrocytes that had been first hemolyzed and then resealed and reannealed both in the presence and in the absence of solution A (without any hexokinase addition). Thus, increased concentrations of glycolytic intermediates were observed only when PIGPA-C was present (not shown).

Therefore, these metabolic analyses indicated a background of enhanced glycolytic potential as induced by the 'rejuvenation solution', while additional improvement of glycolytic function over that afforded by solution A per se was the result of selective overloading with hexokinase.

*Metabolic properties of hexokinase-loaded erythrocytes*

The glycolytic rates of human erythrocytes overloaded with human hexokinase and, comparatively, those of control cells are reported in Table II. Estimates of lactate production from glucose were taken as more reliable determinations of the glycolytic pathway than were the measures of the bulk of $^{14}$C-labeled anions arising from [$^{14}$C]glucose [5]. The latter analysis, although indicative of the amount of glucose entering the Embden-Mayerhof-Parnas pathway, might, in fact, be influenced by accumulation of intermediate metabolites such as glucose 6-phosphate, as in the case of encapsulation of yeast hexokinase in human erythrocytes. Indeed, this treatment, when properly tuned to reproduce the same levels of hexokinase activity as those reached with encapsulation of human placenta hexokinase (i.e., 10- to 18-fold the activity of the untreated cells), resulted in a rapid loss of intracellular ATP levels, without any appreciable increase in the rate of lactate production (not shown).

Although the estimates of lactate output reported in Table II were carried out between 1 and 2 h of incubation at 37°C, comparable results were obtained between 2 and 3 h. The data shown in Table II indicate that the amount of lactate

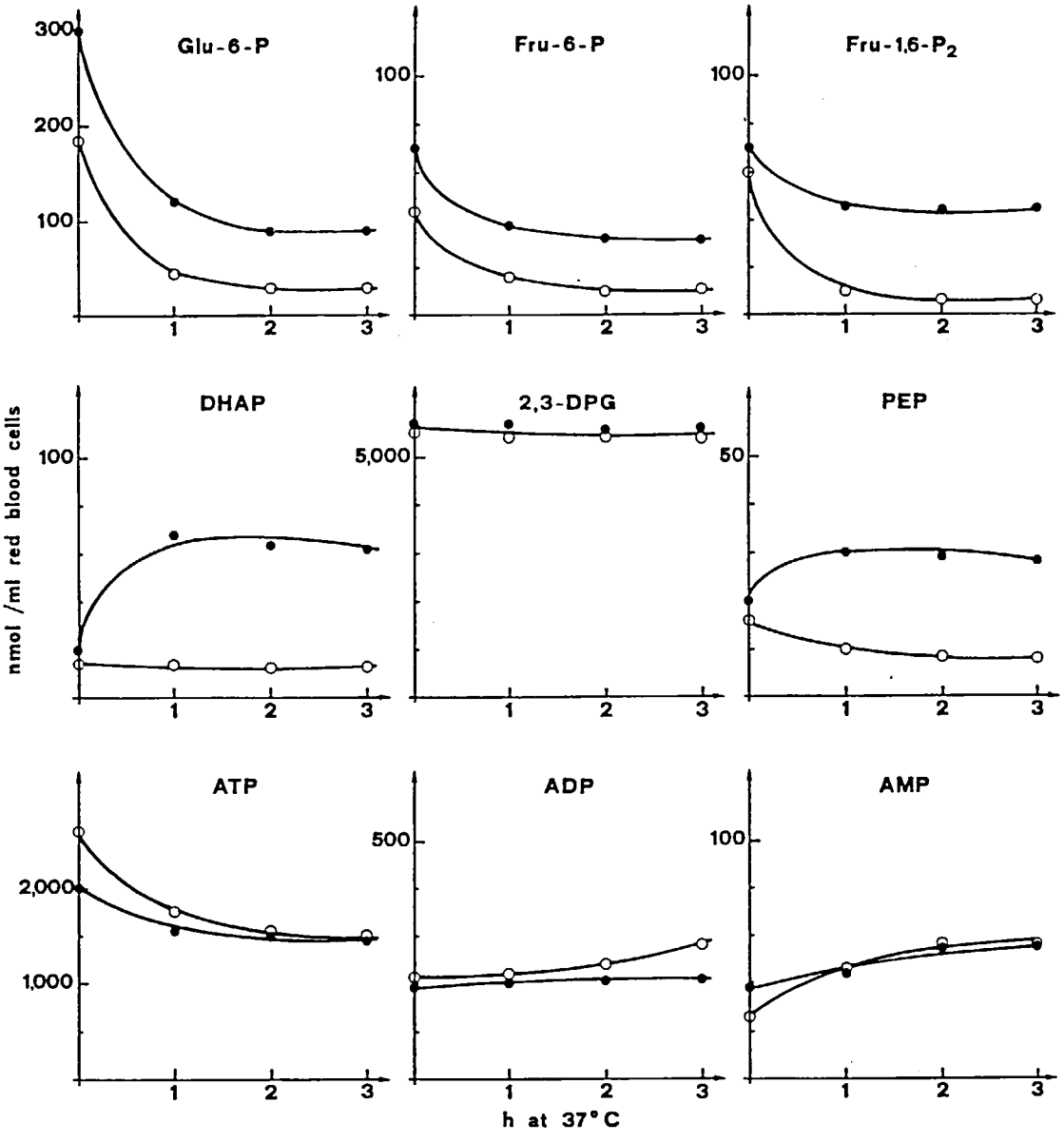


Fig. 1. Time-dependent changes of some glycolytic intermediates in unloaded (○) and hexokinase loaded (●) human erythrocytes. Human erythrocytes were processed following a procedure based on hypotonic lysis, isotonic resealing and reannealing both in the absence (unloaded erythrocytes) or in the presence of human hexokinase type I (hexokinase-loaded erythrocytes). Further details are reported in the Materials and Methods. Incubations were performed in autologous plasma at an hematocrit of 30% at 37 °C for the times indicated. The results are the means of three different experiments that agreed within 5%. Glu-6-P, glucose 6-phosphate; Fru-6-P, fructose-6-phosphate; Fru-1,6-$P_2$, fructose 1,6-bisphosphate; DHAP, dihydroxyacetone phosphate; DPG, diphosphoglycerate; PEP, phospho*enol*pyruvate.

formed from glucose almost doubled in the hexokinase-loaded erythrocytes with respect to both control cells (native and unloaded). Encapsulation of lower amounts of hexokinase than those reported in Table I resulted in some variability of results, although the trend of enhanced lactate formation was clearly maintained. Assays of intraerythrocytic glycolytic intermediates and

TABLE II

LACTATE PRODUCTION BY HEXOKINASE-LOADED AND BY CONTROL ERYTHROCYTES

All determinations were performed as described under Materials and Methods between 1 and 2 h incubation at 37°C of erythrocytes overloaded with hexokinase, as in Table I. Values are means ± S.D. of lactate production and ranges obtained in different experiments (10 for normal cells and 5 for both the unloaded and the hexokinase-loaded erythrocytes).

| | μmol/h per ml erythrocytes |
|---|---|
| Normal erythrocytes | 3.69 ± 0.75 (2.85–4.82) |
| Unloaded erythrocytes | 3.77 ± 0.33 (3.50–4.14) |
| Hexokinase-loaded erythrocytes | 6.68 ± 1.31 (5.33–7.89) |

adenine nucleotides in hexokinase-loaded and in unloaded erythrocytes were also performed in the experiments aimed at measuring the lactate output. The relevant data are reported in Table III, showing a substantial stability of ATP in spite of a 2-fold enhanced glycolytic activity. Accordingly, encapsulation of human hexokinase to reach an activity 15-fold that of the untreated erythrocytes resulted in a new steady-state in the red cells manipulated, thus with increased levels of glycolytic intermediates. Exceptions to this pattern were the concentrations of 2,3-bisphosphoglycerate and of adenine nucleotides whose concentrations were unmodified by overloading of hexokinase. This unmodified adenylate charge was also predicted by theoretical studies, because of the several mechanisms operating in the erythrocyte to keep ATP constant [4].

TABLE III

INTRACELLULAR GLYCOLYTIC INTERMEDIATES IN UNLOADED AND HEXOKINASE-LOADED ERYTHROCYTES

All values were determined after 2 h incubation at 37°C and are the mean ± S.D. of five experiments. All values are nmol/ml erythrocytes.

| | Unloaded erythrocytes | Hexokinase-loaded erythrocytes |
|---|---|---|
| Glucose 6-phosphate | 19.5 ± 8.6 | 84.9 ± 5.2 |
| Fructose 6-phosphate | 9.6 ± 3.4 | 30.4 ± 6.5 |
| Fructose 1,6-bisphosphate | 7.4 ± 3.0 | 46.4 ± 9.3 |
| Glyceraldehyde 3-phosphate | 0–0.3 | 1.8 ± 0.1 |
| Dihydroxyacetone phosphate | 14.4 ± 2.8 | 61.7 ± 10 |
| 2,3-Bisphosphoglycerate | 5370 ± 430 | 5.630 ± 570 |
| 3-Phosphoglyceric acid | 63 ± 8 | 223 ± 21 |
| 2-Phosphoglyceric acid | 5.1 ± 1.5 | 9.0 ± 2.0 |
| Phospho*enol*pyruvate | 8.3 ± 1.2 | 29.6 ± 4.8 |
| ATP | 1568 ± 86 | 1529 ± 168 |
| ADP | 254 ± 28 | 209 ± 43 |
| AMP | 57 ± 8.1 | 56 ± 14 |
| Glucose 1,6-bisphosphate | 135 ± 15 | 150 ± 16 |

The amount of glucose flowing through the hexose monophosphate pathway of hexokinase-loaded erythrocytes proved be strictly comparable to the values observed in both native and unloaded cells, i.e., 50 ± 5 (mean ± S.D., $n = 5$) nmol glucose/h per ml erythrocytes at 37°C. Also, when performing these experiments, the amount of $^{14}CO_2$ produced from [1-$^{14}$C]glucose was estimated between 1 and 2 h of incubation, in order to make the 'solution A effect' negligible (see above) and also to permit complete equilibration of the specific activity of [$^{14}$C]glucose 6-phosphate with that of [$^{14}$C]glucose [5].

## Discussion

Genetic disorders responsible for quantitatively abnormal amounts of specific enzyme proteins are unique 'natural' models for investigating sites of metabolic regulation. However, alternative strategies can be followed to this purpose, such as cloning and differential expression of the corresponding structural genes in several cell types. Human and animal erythrocytes are remarkable exceptions to this approach, since they can be directly 'loaded' with enzyme proteins (either purified from many sources or obtained by genetic engineering) through procedures easy to perform, reproducible and highly conservative [15,16,25,26]. Use of these procedures, notably those based on hypotonic hemolysis, isotonic resealing and eventual reannealing leads to intact and fully viable erythrocytes that have pre-defined levels of specific enzyme activities. Evaluation of metabolic functions and especially of metabolite levels and of rates of multi-step sequences in the erythrocytes thus engineered provides useful information on intracellular regulation of these enzyme activities.

When control mechanisms of erythrocyte-encapsulated enzyme proteins are under investigation, the ideal source of these proteins should be the erythrocyte from the same species. Use of heterologous proteins may prove misleading, because of unphysiological kinetic properties [5,27], of incorrect subcellular compartmentalization [5] or of protein instability in the intraerythrocytic environment [28]. The same consideration applies to encapsulated hexokinase, the most obvious example being the failure of the yeast enzyme to be product-inhibited [17], as indicated by rapid fall of ATP levels preventing proper overfunction of the glycolytic pathway (not shown). However, the established identity of the erythrocyte enzyme with the species present in human placenta and recently purified to homogeneity [18] allowed encapsulation of homologous hexokinase to be made, and analysis of metabolic properties of hexokinase-overloaded erythrocytes to be carried out.

The results reported in this paper, and especially the concentrations of glycolytic intermediates in the hexokinase-loaded erythrocytes, suggest some considerations on the manner which glycolytic pathway is regulated in human erythrocytes. It has been reported that, under steady-state conditions, glycolysis is mainly regulated by the combined hexokinase-phosphofructokinase system, the ATP-consuming processes of the erythrocyte and by 2,3-bisphosphoglycerate phosphatase [4]. Our finding that enhancing the levels of hexokinase activity several fold will produce a new steady-state characteristic of an increased glycolytic flux, seems to support the central role of hexokinase as a pace-maker of erythrocyte glycolysis. This conclusion is in agreement with data obtained with erythrocytes from patients affected by genetic disorders resulting in increased hexokinase activity (trisomy 10p, Ref. 7) or in increased phosphofructokinase activity (trisomy 21, Ref. 29): thus, the glycolytic function of erythrocytes was higher than normal in the former abnormality, while being unmodified in the latter. By adding purified enzymes to a hemolysate which displayed stable concentrations of ATP and other metabolities for at least 1 h, Reiman et al. [30] and Rapoport et al. [31] were able to draw similar conclusions, and these are fully consistent with theoretical predictions as detailed in refs. 2, 4, 30 and 31.

The present findings show that an enhanced activity of human hexokinase does not cause any increase in the amount of glucose metabolized in the hexose monophosphate shunt pathway under resting conditions. These data are in agreement with previous reports [32], but at variance with earlier findings obtained by overloading human erythrocytes with yeast hexokinase [5]. Therefore, the requirement of homologous enzyme proteins for biochemical studies on enzyme-loaded erythrocytes is clearly re-established.

The hexokinase-overloaded erythrocytes we have obtained and investigated seem to provide a workable model for studies on the mechanisms of aging of erythrocytes as they show metabolic properties that are comparable to those of young red cells [13]. Moreover, these manipulated erythrocytes seem promising as far as their use as potential bio-reactors (e.g., cells characterized by glucose over-consumption and by possibly extended life-span) is concerned. Studies are in progress in order to establish whether improved metabolic properties determined by entrapment of hexokinase are paralleled by prolonged survival under several conditions of storage and in different perturbing situations (e.g, osmotic, mechanic and oxidative stress).

## Acknowledgements

This work was supported in part by grants from the Biotechnology Action Programme of the E.E.C., Bruxelles (contracts No. BAP-0056 to ADF and BAP-0055 to M.M.) and from the Ministry of Education, Rome.

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