Changes in glycolytic enzyme activities in aging erythrocytes fractionated by counter-current distribution in aqueous polymer two-phase systems

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Human and rat erythrocytes were fractionated by counter-current distribution in charge-sensitive dextran/poly(ethylene glycol) two-phase systems. The specific activities of the key glycolytic enzymes (hexokinase, phosphofructokinase and pyruvate kinase) declined along the distribution profiles, although the relative positions of the activity profiles were reversed in the two species. These enzymes maintained their normal response to specific regulatory effectors in all cell fractions. No variations were observed for phosphoglycerate kinase and bisphosphoglycerate mutase activities. Some correlations between enzyme activities (pyruvate kinase/hexokinase, pyruvate kinase/phosphofructokinase, pyruvate kinase/pyruvate kinase plus phosphoglycerate kinase, pyruvate kinase/bisphosphoglycerate mutase and phosphoglycerate kinase/bisphosphoglycerate mutase ratios) were studied in whole erythrocyte populations as well as in cell fractions. These results strongly support the fractionation of human erythrocytes according to cell age, as occurs with rat erythrocytes.

INTRODUCTION

Fractionation according to cellular age of the whole erythrocyte population in blood allows the biochemical and functional changes associated with the cell aging process to be studied. Relationships between erythrocyte age and glycolytic enzyme activities may well provide a model for the basic understanding of the process of cell aging in other tissues and perhaps the entire organism [1,2]. The most common method for the separation of circulating aging erythrocytes is based on differences in cell density in a variety of buoyant centrifugation gradients [1,2]. Difficulties in distinguishing between reticulocyte maturation and erythrocyte aging with this procedure, and the confusion over the relationship between cell density and age-dependent properties [1–4], suggest a need for alternative fractionation methods.

Multiple partitions in dextran/poly(ethylene glycol) (Dx/PEG) two-phase systems with a thin-layer counter-current distribution (c.c.d.) procedure can fractionate erythrocytes from different species on the basis of subtle alterations in cell surface properties that occur as a function of normal or abnormal processes *in vivo* (or *in vitro*) [5–10]. This technique seems to be particularly useful for the fractionation by age of whole erythrocyte populations [8–15].

A decrease in the activity of pyruvate kinase (EC 2.7.1.40) (PK) and a parallel increase in bisphosphoglycerate mutase (EC 5.4.2.4) (BPGM), the enzyme responsible for 2,3-bisphosphoglycerate (BPG) synthesis in a glycolysis by-pass, were shown in anaemic rats, the blood reticulocyte level of which ranged from 95% down to 3% [15]. A gradual increase in the concentration of BPG, which is an allosteric haemoglobin (Hb) modulator that decreases oxygen affinity, was also shown from rat reticulocytes to erythrocytes [15,16]. A marked decrease in PK activity with a parallel increase in both BPG concentration

and BPGM activity was also observed during the switch from foetal to adult erythrocytes in blood at early stages of rabbit [17,18] and rat [19] development. All these findings mean that a clear inverse relationship exists between the PK/BPGM ratio and BPG concentrations in both the reticulocyte maturation and the erythrocyte switch processes. The enzyme ratio is then an adequate index for BPG formation in erythrocytes. Changes in PK and BPGM activities (i.e. the PK/BPGM ratio) have been used in our laboratory to illustrate the fractionation by c.c.d. of reticulocyte populations from anaemic rats (according to the degree of maturation) [15] and rat erythrocyte populations during development (according to the stage of cell switching) [19].

Changes in PK and BPGM activities [15,20] and the response of PK to allosteric effectors [20] have also been preliminarily studied in adult rat erythrocyte populations fractionated by c.c.d. Since PK decreased with cell age without BPG concentration and BPGM activity being significantly affected [15,20], the decrease in the PK/BPGM ratio with erythrocyte age is lower in the c.c.d. fractions than the decrease in the enzyme ratio during reticulocyte maturation [15] or erythrocyte switching [19].

The first step in this study was to look at changes in PK and BPGM activities and the PK/BPGM ratio in human erythrocyte populations and c.c.d. fractions. The fractionation was carried out under c.c.d. conditions basically similar to those previously used for rat erythrocytes (low-interfacial-tension charge-sensitive systems and short settling times) [11,15,19,20]. The results were then compared with those for rat erythrocyte populations and aging erythrocytes. Experimental conditions are discussed in the light of the differences in the top/bottom phase volume ratios used for rat and human erythrocyte fractionations.

The second step was the comparative study of the changes in the activities of PK and other key glycolytic enzymes and their response to specific allosteric effectors, also examined in rat and human whole erythrocyte populations and c.c.d. fractions. The

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three enzymes additionally studied were: hexokinase (EC 2.7.1.1) (HK) and phosphofructokinase (EC 2.7.1.11) (PFK), both of which are involved in glycolytic regulation in the upper section (influx) of the glycolytic pathway, and phosphoglycerate kinase (EC 2.7.2.3) (PGK), the enzyme that is responsible for the degradation of 1,3-bisphosphoglycerate (1,3-BPG), the substrate for BPG formation by BPGM. A decrease in HK, PFK and PK activities, without the regulatory properties of these enzymes being affected, and constant levels of PGK and BPGM activity were observed during erythrocyte aging in both species.

Finally, variations in several enzyme ratios between the lower and upper section of glycolysis and the BPG cycle (PK/HK, PK/PFK, PK/PK+PGK, PK/BPGM and PGK/BPGM) were compared as indexes for BPG concentration in human and rat whole erythrocyte populations as well as in aging erythrocytes from both species.

MATERIALS AND METHODS

Chemicals

Reagents, substrates, cofactors and auxiliary enzymes were from Sigma or Boehringer. Dextran T-500 (Dx) was from Pharmacia and poly(ethylene glycol)-6000 (PEG) was from Serva. Heparin was from Leo (Madrid, Spain). Tris, EDTA and all other chemicals were also of analytical grade and were from Merck.

Preparation of cell suspensions and haemolysates

Human blood from healthy adults was collected in heparin (10 units/ml of blood). Wistar rats (150–180 g) were anaesthetized with diethyl ether, killed by exsanguination and blood was collected in 0.9 % (w/v) NaCl containing 1 unit of heparin/ml. After centrifugation (400 g, 10 min) of both blood samples, the supernatant and the top layer of packed cells (still containing leucocytes and platelets) were carefully removed. Pelleted erythrocytes were then washed three times with 0.9 % NaCl in the same way, so as to reduce progressively the presence of non-erythrocyte cells. All these steps were carried out at 4 °C. The resulting cell suspension was essentially free of leucocytes (less than 1 % of the original number) and platelets. Haemolysates were obtained by hypo-osmotic shock with 2 vol. of a stabilizing solution (2.7 mM-EDTA/0.7 mM-2-mercaptoethanol) followed by freezing–thawing. The erythrocyte suspension and haemolysate were prepared on the day of assay.

C.c.d. (multiple partitions) of cells

The following stock solutions (in water) were used to prepare phase systems: 20 % (w/w) Dx, standardized by polarimetry; 40 % (w/w) PEG; 1 M-NaCl; 0.2 M-sodium phosphate buffer (pH 6.8). Charge-sensitive systems (with an electrical potential difference between the phases) were formed by the asymmetric partitioning of phosphate towards the bottom phase and the symmetric distribution of chloride between the phases, so the top phase has a relatively higher positive charge than the bottom [5–10]. Enough 'charge-sensitive' two-phase system, containing 5 % (w/w) Dx, 4 % (w/w) PEG, 0.03 M-NaCl and 0.09 M-sodium phosphate buffer, was prepared by weight from the above stock solutions so that several experiments could be carried out with the same batches of polymers. The phase systems were allowed to equilibrate for 24 h in the cold (separatory funnel) before the top PEG-rich and bottom DX-rich phases were separated.

The theoretical basis for the fractionation of cells by this procedure has been described [5–10]. A c.c.d. apparatus [5] and two thin-layer units (60 concentric cavities) made at the Uni-

versity of Umeå (Umeå, Sweden) and the University of Sheffield, (Sheffield, U.K.) (BioShef TLCCD, MK 3), each one formed by two circular plates (bottom, or stator, plate and top, or rotor, plate), were used. The bottom capacity volume of the units were: 0.81 ml (unit 1) and 0.79 ml (unit 2). Experiments were carried out in both units after appropriate corrections were made to take into account differences in the bottom cavity capacities (see below).

For rat erythrocytes, each of three adjacent cavities (0–2) received a mixture of 0.62 ml (unit 1) or 0.6 ml (unit 2) of the Dx-rich bottom phase plus 0.1 ml of the erythrocyte suspension, whereas the remaining cavities (3–60) each received 0.72 ml (unit 1) or 0.70 ml (unit 2) of the same Dx-rich bottom phase. At this point the cavities on the bottom plates then contained about 89 % of their total capacity, thus ensuring that enough space (0.09 ml) had been left for cells from the top cavity to be adsorbed at the interface and remain with the bottom phase at each partition step. A 0.94 ml (unit 1) or 0.9 ml (unit 2) volume of the PEG-rich top phase was added to the 60 cavities. The volume ratio of the phases that were loaded into the cavities was similar in the two units [L = 0.94/0.72 (unit 1) or 0.9/0.7 (unit 2) = about 1.3]. Since 0.09 ml of the top phase was left in the bottom cavities, the effective top phases that were transferred were: 0.94 − 0.09 = 0.85 ml (unit 1) or 0.9 − 0.09 = 0.81 ml (unit 2). Therefore the 'effective volume ratios' (L') also had similar values in the two units: L' = 0.85/0.81 (unit 1) = 0.81/0.79 (unit 2) = 1.04.

For human erythrocytes, three adjacent cavities (0–2) each received a mixture of 0.42 ml (unit 1) or 0.4 ml (unit 2) of the Dx-rich bottom phase plus 0.1 ml of the erythrocyte suspension while the remaining cavities (3–60) each received 0.52 ml (unit 1) or 0.5 ml (unit 2) of the above Dx-rich bottom phase. The cavities on the bottom plates then contained about 64 % of their total capacity. The space left for the cell adsorption at the interface was then 0.29 ml. A 0.73 ml (unit 1) or 0.7 ml (unit 2) volume of the PEG-rich top phase was added to the 60 cavities. The volume ratio used for human erythrocytes was similar to the one used for rat erythrocytes [L = 0.73/0.52 (unit 1) or 0.7/0.5 (unit 2) = about 1.4]. This top/bottom phase volume ratio was previously used by Walter & Krob [21]. Since 0.29 ml of the top phase was left in the bottom cavities of our units, the effective top phases were: 0.73 − 0.29 = 0.44 ml (unit 1) or 0.7 − 0.29 = 0.41 (unit 2). Thus the 'effective volume ratios' also had values that were similar in the two units [L' = 0.44/0.81 (unit 1) = 0.41/0.79 (unit 2) = about 0.53] but were lower than the 'effective volume ratio' used for rat erythrocytes (L' = 1.04).

A partition step was formed by a 20 s shaking time followed by 5 min settling time and a transfer of the top (rotor) plate. With each transfer the cells in the top phase were carried forward where they were re-extracted with fresh bottom phase, whereas the cells partitioned at the interface were left behind to be re-extracted with fresh top phase. Transfers, 57 in all, were repeated at 4 °C.

After a cycle of multiple partitions (known as a distribution run), 1 ml of 0.9 % NaCl was added to all cavities in order to transform the system into a single phase, and the content of each cavity was collected separately. The distribution profile for each erythrocyte population is given by the Hb absorbance values at 540 nm against the cavity number on the distribution unit. Cells with affinity for the top phase (high distribution coefficient, G) were distributed as fast-moving cells in the fractions of highest number, i.e. towards the right-hand side of the distribution profile. Cells with affinity for the interface (lower G values), as slow-moving cells, tended to remain in the fractions with the lowest number, i.e. towards the left-hand side of the distribution profile.

Enzyme assays

HK, PFK, PK, PGK and BPGM were measured in haemo-lysates of whole erythrocyte populations and pelleted cells from pooled adjacent c.c.d. fractions, as indicated in Figs. 1 and 2. HK was measured by the method described by Crane & Sols [22], PFK by the method of Layzer [23], PGK as described by Yoshida [24], PK as described by Valentine & Tanaka [25] and BPGM as described by Pineda & Luque [26].

Assays were carried out at 30 °C in a Kontron (Uvikon 810) double-beam recording spectrophotometer. Specific enzyme activities are expressed as units/g of Hb. The Hb content was determined as cyanohaemiglobin with a standard method [27].

RESULTS AND DISCUSSION

Enzyme activities in whole erythrocyte populations

The specific activities of the three key glycolytic kinases (HK, PFK and PK) and the two main enzymes in the BPG cycle (PGK and BPGM) were determined in whole erythrocyte populations.
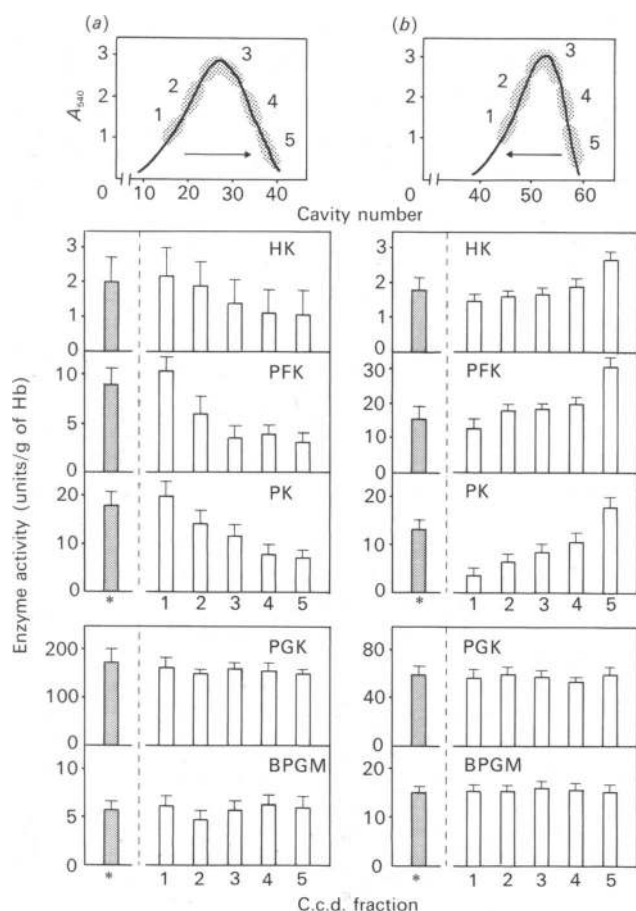

Fig. 1. Fractionation according to cellular age of human (a) and rat (b) erythrocytes by c.c.d. in an aqueous two-phase system (top) and specific glycolytic enzymes activities in whole erythrocyte populations (stippled bars) and pooled c.c.d. fractions from human (a) and rat (b) erythrocytes (bottom)

Experimental conditions are described in the text. Top: distribution profiles are given by Hb absorbance values at 540 nm against the cavity number of the distribution rotor; arrows indicate the direction of cellular aging. Bottom: vertical bars represent the mean ± S.D. for six separate c.c.d. runs and quadrupled measurements.

The results are shown in Figs. 1(a) (humans) and 1(b) (rats) (stippled bars). A comparison between the two species highlights some interesting facts.

Non-significant differences between rat (1.7 units/g of Hb) and human (2.0 units/g of Hb) were found for HK, which has the lowest glycolytic activity. Some differences in activity between the two species can be noted for the four other enzymes: PFK, PK, PGK and BPGM (Fig. 1). With respect to PFK, which is the main allosteric regulatory step in the upper section of glycolysis, the difference between rats (14.3 units/g of Hb) and humans (9.2 units/g of Hb) is not significant. Many factors that affect the allosteric properties of PFK may influence PFK activity. The activity of the main regulatory enzyme in the lower section of glycolysis, PK, is slightly lower in rat (13.3 units/g of Hb) than in human (18.1 units/g of Hb) erythrocyte populations. The inverse relationship between BPG concentration and PK activity in mammalian erythrocytes with the capacity for BPG synthesis is well known [15,17–19,28]. In agreement with this, the relatively lower PK activity in rats should be related to the higher BPG concentration in rat (1.15 mol/mol of Hb) than in human (0.96 mol/mol of Hb) erythrocytes [16].

The enzyme BPGM is directly responsible for the synthesis of BPG from 1,3-BPG. The specific activity of BPGM is three times higher in rat (16.0 units/g of Hb) than in human (5.4 units/g of Hb) erythrocytes. Obviously, such a significantly higher BPGM activity in rats may by itself account for the higher concentration of BPG in rat erythrocytes than in human erythrocytes [16] rather than the relative lower PK activity.

The specific activity of PGK, the glycolytic kinase in the BPG cycle that degrades 1,3-BPG to 3-phosphoglycerate, is three times lower in erythrocyte populations in rats (59 units/g of Hb) than in humans (184 units/g of Hb). Since PGK uses the same phosphate (1,3-BPG) as BPGM as a substrate, the possibility exists that the lower PGK value in rat erythrocytes could favour higher rates of BPG formation from 1,3-BPG via BPGM, thus contributing to BPG accumulation in these cells. A similar suggestion was made on the basis of the changes in PGK and BPGM activities in different cell density fractions (Percoll) from rat bone marrow [29]. The simultaneous decrease in PGK and increase in BPGM from the basophilic erythroblast stage through to the reticulocyte stage in rats was parallel with the increase in BPG (and Hb) during the erythropoietic process [29].

Ratios of enzyme activities in whole cell populations

Several of the enzyme ratios that can be calculated from the above activities help to explain the specialization of the glycolytic pathway and differences in BPG formation between humans and rats in whole erythrocyte populations (this section) and in aging erythrocytes (see below).

These ratios are normally based on the activity of PK, the enzyme that is inversely related to the concentration of BPG in erythrocytes, and for this reason are used to explain differences between species and between different processes within the same species. The enzyme ratios calculated from the respective activities (Fig. 1) are shown in Table 1.

The PK/HK and PK/PFK ratios relate the activity of PK to the activities of the two main regulatory steps in glycolysis. They can be considered as indexes for BPG regulation in the upper section (influx) of glycolysis. An inverse relationship between BPG concentration and these enzyme ratios has been shown for embryonic, foetal and adult erythrocytes in rabbits and rats as well as other adult mammals and under pathological conditions in humans [17–19,28,30]. Because of the low HK activity in erythrocytes (Fig. 1), rather high values for the PK/HK ratio are observed, and the differences between human (9.05) and rat (7.82) erythrocytes (Table 1) are due more to differences in PK

Table 1. Ratios of enzyme activities in whole and fractionated erythrocyte populations

Arrows indicate the direction of cellular aging.

		PK/HK	PK/PFK	PK/BPGM	PK/PK+PGK	PGK/BPGM
Humans						
Whole populations		9.05	1.96	3.35	0.08	34.07
	1	9.09	1.88	3.22	0.11	26.10
	2	7.36	2.18	2.91	0.08	32.20
C.c.d. fractions	3	8.50	3.15	2.26	0.07	31.50
	4	7.27	1.81	1.25	0.05	24.30
	5 ▼	7.09	2.69	1.30	0.05	25.00
Rats						
Whole populations		7.82	0.93	0.83	0.18	3.70
	1 ▲	2.06	0.24	0.19	0.05	3.70
C.c.d. fractions	2	4.06	0.36	0.40	0.09	4.04
	3	5.00	0.44	0.48	0.11	3.77
	4	5.31	0.51	0.61	0.15	3.35
	5	7.22	0.61	1.21	0.24	3.85

activity than to differences in HK activity. PK activity is slightly lower in rat than in human erythrocytes, whereas differences in HK activity between the two populations are not significant (Fig. 1). So the PK/HK ratio apparently does not have any special significance that, by itself, would help to explain the differences in BPG concentrations between the two species. With respect to the PK/PFK ratio, differences between human (1.96) and rat (0.93) erythrocytes (Table 1) are due to variations in activity in both enzymes (Fig. 1). Since the values for the PK/PFK ratio (twice as high in humans as in rats) are inversely related to the concentration of BPG, this ratio could be used as an index of BPG regulation [30].

More appropriate indexes of BPG regulation, also based on PK activity, have been proposed [15,17–20,28,30] and are confirmed and extended here. The PK/BPGM and PK/PK+PGK ratios relate the activity of PK to the activity of the two enzymes of the BPG cycle that are either directly responsible (BPGM) or indirectly involved (PGK) in BPG synthesis.

As discussed above, a higher BPG concentration is related to a decrease in PK activity and an increase in BPGM activity. In consequence, the relationship between the concentration of BPG and the PK/BPGM ratio is inverse. This inverse relationship has been demonstrated during the erythrocyte switch in animal development, reticulocyte maturation and the erythrocyte aging processes [15,17–20,28,30]. In fact, the values for the PK/BPGM ratio in the two erythrocyte populations (Table 1) are four times lower in rats (0.83) than in humans (3.35). This again agrees with the BPG concentration being slightly higher in rat than in human erythrocytes [16].

In contrast with the parallelism between BPG concentration and BPGM activity, a higher BPG concentration in rats seems to be related to a slightly lower PK activity and a significantly lower PGK activity (see above). As a consequence, a direct relationship must exist in rats between BPG concentration and the enzyme ratio that relates PK and PGK activities (the PK/PK+PGK ratio). As shown in Table 1, the PK/PK+PGK ratio is twice as high in rats (0.18) as in humans (0.08). This significant difference between the erythrocyte populations may also serve to explain the higher BPG concentration in rats.

Finally, the PGK/BPGM ratio, which relates the activity of two enzymes in the BPG cycle, is probably a more appropriate index than the PK/BPGM ratio. The lower PGK (and higher BPGM) activity in rat than in human erythrocytes makes the PGK/BPGM ratio in rat (3.7) a tenth of the value in human (34.0) erythrocytes, clearly confirming the higher BPG con-

centration in rats. Therefore a higher PGK activity with respect to BPGM (i.e. an increase in the PGK/BPGM ratio) must be inversely related to the concentration of BPG.

Fractionation of erythrocytes according to cellular age

A representative distribution profile (c.c.d. curve) for whole erythrocyte populations, as given by the Hb absorbance values against cavity number, is shown for humans (Fig. 1a, top) and rats (Fig. 1b, top). Each of the five fractions in Fig. 1 is formed by a pool of erythrocytes from adjacent cavities. Reproducibility of the c.c.d. curves was observed in experiments with the blood from at least six different healthy adults, human or rat. Differences in the volume ratio of phases added to the c.c.d. units are taken into account to explain the location of rat and human erythrocyte profiles [31,32]. The volume ratio of loaded phases used is similar to the one employed by Walter and co-workers ($L = 1.4$) [8–10,21,32]. However, the effective volume ratios are higher for rats ($L' = 1.04$) and lower for humans ($L' = 0.53$) than the one used by Walter et al. [8–10,21,32] for both species ($L' = 0.7$).

The location of the profiles for rat erythrocytes to the right of the c.c.d. train (Fig. 1b) was as previously observed under similar Dx/4% (w/w) PEG charge-sensitive systems, short (5 min) settling time, and high ($L' = 1.04$) effective volume ratio] [11,15,19,20]. This positioning agrees with the high partition ratio of the whole rat erythrocyte population in charge-sensitive systems [31].

As shown by ^{59}Fe-labelling studies in vivo, subtle differences in the surface properties of aging rat erythrocytes (i.e. slightly different partitioning behaviour) give rise to age-related fractionation. This fractionation by age of rat erythrocytes was first shown by Walter et al. [10,33], and confirmed by our group [11], by ^{59}Fe-labelling studies. These were supported by enzymological studies [15,19,20]. According to our results [11], fraction 5 was enriched in younger erythrocytes (mean age approx. 3 days), fraction 4 had cells of approx. 26 days of age and fraction 1 contained the oldest cells (approximate age 55 days). From these [10,11] and previous [15,19,20] results, fractions 3 and 2 should contain erythrocytes of increasing intermediate age (between 26 and 55 days). This means that, in spite of profile positioning towards the high-numbered cavities, rat erythrocytes are fractionated by age.

These c.c.d. conditions ($L' = 1.04$) also allow the fractionation of different cell populations. Because of differences in the partition ratio for each cell population, a displacement of c.c.d.

profiles was shown during rat development from the late-foetal stage (where profiles are at low-numbered cavities) to 0-, 6-, 8-, 12- and 24-day-old and adult animals (where profiles are at high-numbered cavities). The values for the PK/BPGM ratio in the c.c.d. fractions suggest that the fractionation of these cell populations takes place according to the type of cells or cell age in each population [19].

Human erythrocytes distributed themselves between cavities 10 and 40 (Fig. 1a). This agrees with the lower (with respect to rats) partition ratio in adult human erythrocyte populations in charge-sensitive systems [10], and the low effective volume ratio used here ($L' = 0.53$). Although initial studies indicated that human erythrocytes of different ages were not subfractionated by c.c.d., ^{51}Cr-labelling studies in vitro have shown that fractionation of human erythrocytes also reflects surface changes during aging [10,21]. However, the relative positioning of the fractionated erythrocytes are reversed in rats and humans. Young human erythrocytes have a slightly lower G value than the population as a whole and are distributed towards the left of the c.c.d. curve [10,21]. Younger erythrocytes would then be collected to the left of the c.c.d. profile (Fig. 1a). Old human erythrocytes have a somewhat higher G value than the population as a whole [10,21] and would probably be progressively distributed towards the right of the c.c.d. profile. Distributions like the one in human erythrocytes were first shown in dogs by Walter et al. [33] and in chickens by our group [11–13]. Alterations in erythrocyte surface properties as a function of cell age are species-specific [10]. Therefore c.c.d. fractionation is a useful technique for studying erythrocyte distribution according to age in the peripheral blood of different species.

In general, the number of reticulocytes present in the erythro-cyte preparations is almost too small to be detected in the c.c.d. fractions. If present, rat reticulocytes (which have a low G value, and are positioned very close to older erythrocytes) or human reticulocytes (which also have a low G value, and are positioned close to younger erythrocytes) would fractionate towards the left of the c.c.d. curves [10,20,21]. For this reason, the pooled cells in fraction number 1 in Figs. 1(a) and 1(b) do not include those erythrocytes that are fractionated to the left of cavities 13–15 (Fig. 1a, humans) or cavities 43–45 (Fig. 1b, rats). This means that the profiles shown here are exclusively due to the frac-tionation of aging erythrocytes.

Enzyme activities in the c.c.d. fractions

Specific activities have been measured in the five pooled cell fractions, which contain cells of increasing age (see above). The results, in terms of Hb concentration, are shown in Fig. 1(a) (humans) and Fig. 1(b) (rats) as the mean of six separate c.c.d. runs.

The activities of the three key regulatory kinases (HK, PFK and PK) decrease in pooled human erythrocyte fractions from 1 to 5 (Fig. 1a), and in pooled rat erythrocyte fractions from 5 to 1 (Fig. 1b). Thus the slopes for the decreasing kinase activities in the two species are opposite. Changes in glycolytic enzyme activities (mainly HK and PK) have often been used as age-dependent markers since they decrease progressively from young to older erythrocytes [1,2,34]. A general observation on the basis of these results is that the catalytic efficiency of regulatory kinases decreases with age but a significant proportion of activity is still present just before the cell disappears from the circulation [4,35].

The glycolytic enzyme with the lowest activity is HK, which is difficult to determine accurately in c.c.d. fractions. However, a slight decline in HK activity is found in human (fraction 1 to 5; Fig. 1a) and rat (fraction 5 to 1; Fig. 1b) erythrocytes. The

biphasic decay behaviour proposed for HK in several species [4,35] is not observed here, probably because we are dealing with cell fractions that only contain aging erythrocytes and are not contaminated with reticulocytes. The biphasic decrease in HK activity in human erythrocytes, seen after cell separation by density ultracentrifugation or a discontinuous arabinogalactane gradient, served to indicate that HK-R (the predominant iso-enzyme in reticulocytes but also present in the human erythro-cytes along with HK1) is the isoenzyme that decreases the most during aging [35]. The biphasic behaviour may therefore be due to the presence of reticulocytes in the original erythrocyte sample and not to the aging process [4,35].

A decrease in PFK activity is also shown in Figs. 1(a) and 1(b). Differences in the profile between human and rat erythrocytes are probably a consequence of the influence of numerous effectors on enzyme activity or of the presence in the samples of different subunits associated in various hybrid forms (isoenzyme com-plexity). The decrease in enzyme activity is considered to be monophasic during the aging process.

A monophasic decrease in PK activity is clearly observed in c.c.d. fractions from both species (Figs. 1a and 1b); this indicates that only one PK isoenzyme (PK-R2 [15]) is present in these erythrocytes. A similar monophasic decay in PK activity has also been shown by Murakami et al. [35].

The decrease with age in HK, PFK and PK activity throughout the profiles of human (Fig. 1a) and rat (Fig. 1b) erythrocytes confirms the validity of c.c.d. for cell fractionation according to age. As in rats, the human erythrocyte characteristics reported in this paper imply age-related surface differences that can be revealed by studying the variations in the activities of the 'age-dependent' enzymes throughout the profile. These results are consistent with preliminary studies reported by Walter et al. [10,33] on the activity of aspartate aminotransferase (as an enzyme marker for young cells). These authors found a decrease in activity from left (young) to right (older cells) in human c.c.d. fractions [10,33].

The specific activities of PGK and BPGM, both of which are involved in the glycolytic by-pass for BPG formation, do not exhibit significant differences in the c.c.d. fractions of human (Fig. 1a) or rat (Fig. 1b) erythrocytes. The stability of these enzymes is a significant finding, since enzymic stability would maintain a steady BPG concentration during the entire erythro-cyte life span, in keeping with the importance of this metabolite in Hb oxygenation at any stage of erythrocyte aging [15].

Variations in the ratios of enzyme activities in the c.c.d. fractions

As described above, the enzyme ratios were calculated from the information in Figs. 1(a) and 1(b) and are given in Table 1.

The PK/HK ratio in the c.c.d. fractions seems to decrease with the increase in age in humans (from fraction 1 to 5) and in rats (from fraction 5 to 1) (Table 1). As for erythrocyte populations (see above), the decrease is mainly due to the decrease in PK activity with age (Fig. 1) and has no special significance in itself.

The variation with age of the PK/PFK ratio, which relates two regulatory enzymes in the lower (PK) and upper (PFK) section of glycolysis, seems to be clearer in rats (a decrease appears from fraction 5 to 1) than in humans (from fraction 1 to 5). This is probably due to different rates of decreasing PFK activity in human and rat c.c.d. fractions (see above). In any case, the decrease in the PK/HK ratio (in both species) and the decrease in the PK/PFK ratio (in rats) may contribute to maintaining BPG concentrations during the erythrocyte aging process.

The decrease with age of the PK/PK + PGK ratio is clear in humans (from fraction 1 to 5) and rats (from fraction 5 to 1) (Table 1). Again, this decrease would also contribute to main-
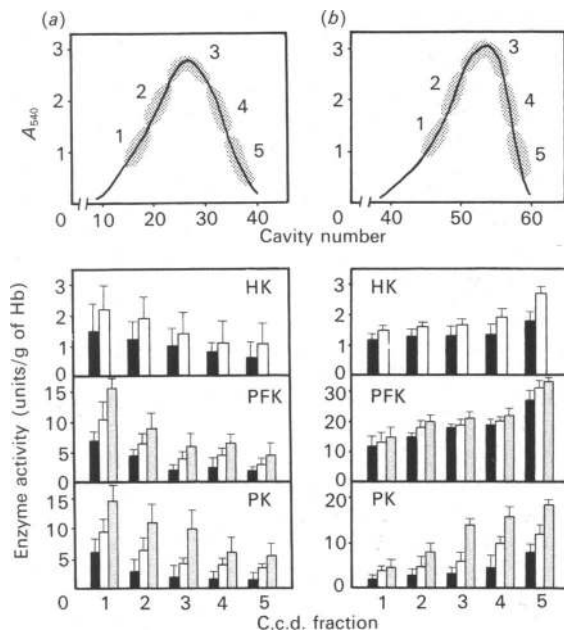
Fig. 2. Response of regulatory glycolytic kinases to regulator effectors in human (a) and rat (b) pooled erythrocyte c.c.d. fractions

Effect of 6 mM-2,3-BPG (■) on HK activity (□) for a 2 mM-D-glucose concentration. Effect of 0.3 mM-AMP (▨) and 1 mM-ATP (■) on PFK activity (□) for a 1.5 mM-fructose 6-phosphate concentration. Effect of 0.3 mM-FBP (▨) and 1 mM-ATP (■) on PK activity (□) for a 0.30 mM-phosphoenolpyruvate concentration. Vertical bars represent the mean ± S.D. for six separate runs and quadrupled measurements.

taining stable BPG concentrations during the erythrocyte life span.

On calculation of the PK/BPGM ratio in the c.c.d. fractions, a decrease from young to old erythrocytes in humans (from fraction 1 to 5) and rats (from fraction 5 to 1) was found. The decrease in the PK/BPGM ratio with age is due to a decrease in PK activity but not in BPGM activity (Fig. 1). Therefore the concentration of BPG tends to remain constant during the aging process. Only a marked decrease in the PK/BPGM ratio produced by both a decrease in PK activity and a parallel increase in BPGM activity, for instance during reticulocyte maturation or erythrocyte switching [15,19,20], is accompanied by a marked increase in BPG concentrations.

Finally, because PGK and BPGM activities do not undergo significant changes during the aging process, no variations in PGK/BPGM ratios are observed during cellular aging in either species.

Influence of enzyme effectors on the regulatory properties of glycolytic kinases in aging erythrocyte fractions

The influence of specific effectors on the activities of regulatory glycolytic kinases was studied in c.c.d. fractions from human and rat erythrocyte populations. The results are shown in Figs. 2(a) and 2(b). Enzyme activities at the several substrate concentrations needed for a kinetic study cannot be measured with the amount of cells present in the pooled cell fractions. Kinetic studies of HK, PFK and PK of whole erythrocyte populations are well established [36,37,39,41]. From these studies, an appropriate substrate concentration can be chosen to measure the influence of allosteric effectors in the c.c.d. fractions. In the case of HK, the

substrate concentration (D-glucose) used was 2 mM (i.e. adequate for maximum velocity) [36,37]. In the case of PFK and PK, the substrate concentrations used to study the influence of their allosteric effectors were taken from allosteric kinetic studies in cell haemolysates [39,41]. PFK and PK showed positive co-operativity ($n = 3.7$ and $n = 2.7$ respectively). The $S_{0.5}$ value of PFK for fructose 6-phosphate was 1.5 mM [39]. The $S_{0.5}$ value of PK for phosphoenolpyruvate was 0.3 mM [41]. The following effectors were studied, at fixed concentrations close to those found in erythrocytes: BPG (6 mM), as an inhibitor of HK; AMP (0.3 mM), as an activator of PFK; fructose 1,6-bisphosphate (FBP) (0.3 mM), as an activator of PK; and ATP (1 mM), as an inhibitor of PFK and PK.

Inhibition of HK activity by 6 mM-BPG in aging erythrocytes from human and rats was observed along the c.c.d. profiles. BPG has been shown to be a competitive inhibitor of HK activity in haemolysates of human and rabbit erythrocyte populations [36,37]. The inhibitory effect of BPG on HK activity suggests that the enzyme maintains its capacity of response to specific effectors during the aging process.

Activation of the PFK activity by 0.3 mM-AMP in aging erythrocytes from humans and rats was also observed along the c.c.d. profiles (Figs. 2a and 2b). It has been shown that the AMP activator physiologically acts on the PFK in several mammalian cells [38,39]. The inhibition of PFK activity by 1 mM-ATP was also observed in the c.c.d. fractions from humans and rats. This well-known inhibitory effect is especially important in the regulation of glycolysis [39]. PFK maintains its capacity to respond to its effectors in aging erythrocytes in both species; therefore the regulatory properties of the major rate-controlling enzyme are preserved during this process.

Finally, the response of PK to its allosteric effectors (FBP and ATP) was studied in c.c.d. fractions from human and rat erythrocytes. Activation by 0.3 mM-FBP and inhibition by 1 mM-ATP were observed along the c.c.d. profiles in both species (Figs. 2a and 2b). These results agree with the kinetic behaviour of the enzyme in whole erythrocyte populations (a typical sigmoidal curve with FBP activation and ATP inhibition [40,41]) and equally suggest that the enzyme is allosterically regulated during the whole life span of the erythrocytes.

These results demonstrate that the decrease in activity with age of HK, PFK and PK does not seem to affect the regulatory properties of these enzymes.

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