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Surface and metabolic properties of microcytic and macrocytic human anaemic red blood cells detected in polymer aqueous two-phase systems

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Abstract

Microcytic and macrocytic red blood cells from anaemic patients have been fractionated as a function of cell surface properties by the countercurrent distribution technique using charge-sensitive dextran/poly(ethylene glycol) aqueous two-phase systems. As deduced from the fractionation profiles, microcytic cells constitute a heterogeneous cell population with decreased surface charge properties while, macrocytic cells constitute a homogeneous cell population with behaviour similar to that of the control red blood cells. The specific activity of pyruvate kinase, an age-dependent enzyme, did not change along microcytic red blood cells fractionation profiles, suggesting that such cells have altered ageing properties. However, pyruvate kinase specific activity decreases from the left- to the right-hand side of the fractionation profile of macrocytic red blood cells, indicating that these cells follow the normal ageing process. Bisphosphoglycerate mutase specific activity did not change along the fractionation profile of any cell population under study, thus providing 2,3-bisphosphoglycerate during the life-span of the red blood cells from anaemic patients. © 1998 Elsevier Science B.V. All rights reserved.

Keywords: Aqueous two-phase systems; Red blood cells

1. Introduction

The β-thalassaemia, an inherited group of haemoglobinopathies that commonly affect Mediterranean populations, is often confused with iron deficiency anaemia, another leading cause of hypochromic microcytic anaemia. Several laboratory tests have been devised to spot the difference between these two pathologies [1–9]. Red blood cell (RBC) indices obtained from electronic Coulter counters, such as red cell distribution width (RDW), indicate a pronounced heterogeneity of these cells in patients with thalassaemia, which has been confirmed by fractionation in aqueous dextran/poly(ethylene glycol) (D/PEG) two-phase systems [10]. This technique was shown to be useful in distinguishing normal and β-thalassaemic (heterogeneous) RBCs [10]. In addition, it provided information about the cell surface charge properties and metabolism of RBCs. The purpose of this paper consisted of extending these studies to microcytic RBCs from iron deficiency anaemia and to macrocytic RBCs from anaemic patients. The results will be compared with normal and β-thalassaemic RBCs.

Consequently, RBCs from anaemic patients were fractionated by multiple partition in charge-sensitive D/PEG systems using a thin-layer countercurrent distribution (CCD) apparatus [11]. This is an estab-

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lished technique for the RBC fractionation on the basis of subtle differences inherent to cell surface charge properties that occur as a function of normal or abnormal in vivo or in vitro processes [12–17]. The specific activities of one of the main glycolytic regulatory kinase, pyruvate kinase (EC 2.7.1.40) (PK), as well as the specific activity of bisphosphoglycerate mutase (EC 5.4.2.4) (BPGM), the enzyme responsible for the synthesis of 2,3-bisphosphoglycerate (2,3-BPG) were measured in the separated cells. These studies permitted expanding the knowledge about the enzymatic changes that take place during the cellular ageing process of anaemic cells and, to obtain information about their metabolic function.

2. Experimental

2.1. Cell suspensions and haemolysates

Blood samples from eight normal adults, three microcytic (iron deficient, samples 1 to 3) and two macrocytic (pernicious anaemia, samples 4 and 5) patients donors from the University Hospital (Alcalá de Henares, Madrid, Spain) were collected in heparin (10 units/ml). Blood was centrifuged (400 *g* for 10 min) at 4°C and the supernatant and top layer containing white blood cells were removed. The packed RBCs were washed three times with cold 0.15 *M* NaCl. Haemolysates were obtained by hyposmotic shock with two volumes of stabilizing solution (2.7 m*M* EDTA-Na_2–0.7 m*M* mercaptoethanol), followed by freezing and thawing [10,17].

2.2. Haematologic parameters

Haematologic parameters, including RBC counts, haemoglobin content (HGB), haematocrit (HCT), mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH), mean corpuscular haemoglobin concentration (MCHC) and wide distribution haemoglobin (WDH) were monitored throughout the different processes in a Serono-Baker Diagnostic System 9000+ Coulter counter.

2.3. RBCs fractionation

The RBC fractionation experiments were carried out as previously reported for normal and β-thalassaemic erythrocytes [10,17]. Briefly, two-phase systems containing 5% (w/w) D (Dextran T-500, Pharmacia Biotech, Uppsala, Sweden); 4% (w/w) PEG (PEG 8000, Serva, Heidelberg, Germany); 0.03 *M* NaCl; 0.09 *M* Na phosphate buffer, pH 6.8 were prepared. The affinity of phosphate for the D-rich bottom phase gives a positive PEG-rich top phase allowing cell partition on the basis of surface charge properties [11–13]. Multiple partitions were carried out in a CCD apparatus (Bioshef MK3; University of Sheffield, Sheffield, UK) with a circular thin-layer unit (60 concentric cavities) formed by a bottom or stator plate and a top or rotor plate. The experimental conditions were as detailed for human RBCs [10,17]. Cell distribution profiles are given by the haemoglobin absorbance at 540 nm against cavity number. In order to secure sufficient number of cells required for the enzyme activities measurements, some of those adjacent CCD cavities (with a similar partition ratio) were joined into five pools (5–6 cavities).

2.4. Enzyme assays

Pooled CCD fractions were washed twice with 0.15 *M* NaCl prior to haemolysis and PK [18] and BPGM [19] activities measured. Enzyme specific activity was expressed as U/cell. Reagents, substrates, cofactors and auxiliary enzymes were all from Sigma (St. Louis, MO, USA) and Boehringer (Mannheim, Germany).

3. Results and discussion

3.1. Haematologic parameters

The RBC haematologic parameters of studied samples are summarized in Table 1. As it can be observed, the RBC number, haemoglobin concentration and haematocrit decreased with respect to controls in all analyzed samples. Microcytic RBCs (samples 1 to 3) show lower MCV, MCH and MCHC values than the control cells while macrocytic RBCs

Table 1
Haematologic parameters of microcytic (samples 1 to 3) and macrocytic (samples 4 and 5) RBCs from anaemic patients

	Control	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5
WBC $10^3/\mu l$	4.8–10.8	5.17	8.25	5.94	5.83	11.20
RBC $10^6/\mu l$	M 4.7–6.1		M 4.41			
	F 4.2–5.4	F 2.57		F 4.22	F 3.38	F 4.01
HGB g/dl	M 14–18		M 10.1			
	F 12–16	F 5.6		F 10.9	F 10.3	F 12.8
HCT %	M 42–52		M 35.3			
	F 37–47	F 19.6		F 33.9	F 34.2	F 41.7
MCV fl	M 80–94		M 79.9			
	F 81–99	F 76		F 80.4	F 101.1	F 104
MCH pg	27–31	21.8	23	25.7	30.4	31.9
MCHC g/dl	33–37	28.6	28.8	32	30.1	30.6

Control values represent the range for four separate control samples. M=Male and F=female.

(samples 4 and 5) show higher MCV, practically normal MCH and slightly decreased values of MCHC. RBC abnormalities including anisocytosis (sample 3), which suggests some cellular heterogeneity, and anisochromia (samples 2 and 3) as well as hypochromia (samples 2, 3 and 4), were also observed by optical microscopy. An increase in the WDH index in sample 3 with respect to controls coincides with the existence of anisochromia observed in these cells.

3.2. CCD fractionation of RBCs

The CCD profiles in charge-sensitive two-phase systems for normal RBCs were reproducible with respect to those previously obtained in our laboratory under identical conditions [10,17]. In short, cells were distributed in a homogeneous curve, around cavities 20–50 (the highest cellularity around cavities 35–40). The partition ratio (affinity for the top phase) of RBCs increased from the left- to the right-hand side of the CCD curve parallel to the increase in cell surface charge properties. A representative profile is shown in Fig. 1 in which RBCs distribute among cavities 25–50 with the highest cellularity in cavity 37.

The CCD profiles of microcytic RBCs were clearly more heterogeneous, containing several cell subpopulations, than the control ones. CCD profiles were characteristic for each patient. In general, cells were distributed around cavities 14–45 the highest cellularity being around cavities 20–35. To illustrate the variability for RBCs from different patients, three representative CCD fractionation profiles of different microcytic RBCs (samples 1 to 3) are shown in Fig. 2. As can be seen, CCD fractionation curves appeared more irregular and skewed with the highest cellularity toward the left against the control CCD curves. It means that microcytic RBCs constitute a heterogeneous cell population with decreased surface charge properties (lower partition ratio) when comparing to control RBCs. Such results were similar to those previously obtained with microcytic RBCs from β-thalassaemia. These samples showed cellular heterogeneity and RBCs with slightly lower surface charge than the control ones [10].

Two representative CCD fractionation profiles of macrocytic RBCs (samples 4 and 5) are shown in Fig. 3. In general, cells were distributed around cavities 20–47 in CCD curves as homogeneous as the controls but slightly shifted toward the left (the highest cellularity among cavities 30–37). It infers that these homogeneous cell populations contain cells whose surface charge properties decreased slightly with respect to the controls.

The differences above described for CCD profiles

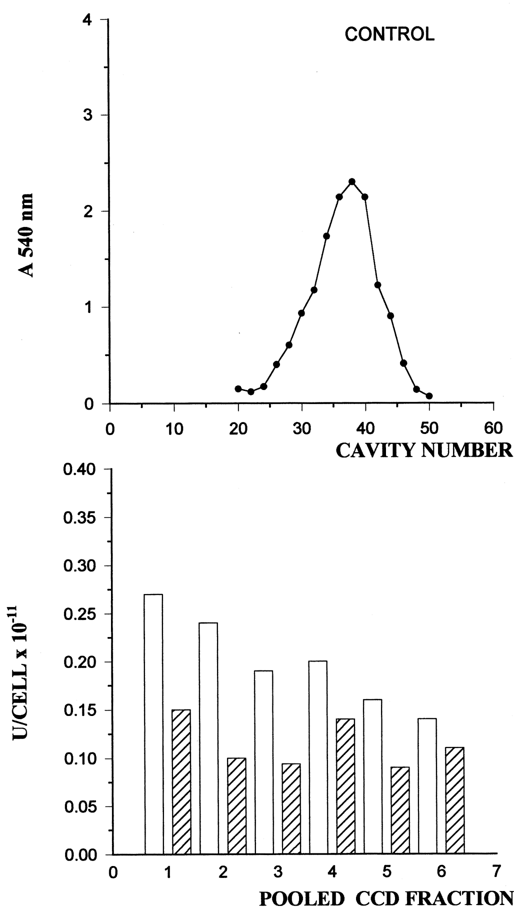

Fig. 1. Top: CCD fractionation profile of normal human RBCs in a 5% D/4% PEG charge sensitive two-phase system. Cellularity expressed as haemoglobin absorbance at 540 nm is plotted against the CCD cavity number. Bottom: PK (hollow bars) and BPGM (stripe bars) specific enzyme activities ($U/cell \cdot 10^{-11}$) in the five pooled CCD fractions (1 to 5). Data are the mean of three separate determinations.

of microcytic and macrocytic RBCs with respect to the controls, together with our previous results concerning the β-thalassaemic RBCs [10], allow us to suggest that the cell size was not affecting the obtained CCD fractionations. Thus, the biggest cells, the macrocytic ones, showed quite similar fractionation profiles to the controls. Instead, alterations in cell surface charge properties of these pathologic samples seem to account for these differences.

3.3. PK and BPGM enzyme activities in CCD fractionated RBCs

The progressive decrease in activity of the three key glycolytic enzymes (hexokinase, phosphofructokinase and PK) from young to old RBCs [20,21], has been used as an age-marker parameter to characterize the CCD fractionation. The decline of enzyme specific activities for these three key glycolytic kinases, from the left- to the right-hand side of the CCD profile, has been shown repeatedly in control human erythrocytes [10,17]. Thus, a relationship between partition ratio and RBCs ageing has been established; an increase in partition ratio indicates ageing of human erythrocytes [10,17]. This relationship was also reaffirmed by in vitro human ^{51}Cr-labelled RBCs experiments. Young human erythrocytes have a slightly lower partition value while older RBCs have higher partition values compared to the whole populations of RBCs [12]. The presence of reticulocytes in the RBC samples (which show higher PK and BPGM specific activity than mature erythrocytes) has also been taken into account. These cells are distributed at cell fractions located farther to the left [12,15]. The low PK and BPGM specific activities in cell fractions located to the left-hand side of the CCD profile, suggest the absence of reticulocytes.

The PK and BPGM specific activities have been studied in the whole RBC population (Table 2) and in the five pooled CCD fractions after the fractionation of normal (Fig. 1, bottom), microcytic (Fig. 2, bottom) or macrocytic (Fig. 3, bottom) RBCs. Specific activity values are expressed as U/cell to eliminate the influence of low haemoglobin concentration in the anaemic cells. As a reference, the PK/BPGM ratio for control cells is 1.91, which falls within the range of 1.44–2.57 previously described [22].

PK and BPGM activities in the whole cell population of microcytic RBCs (Table 2) were lower than or similar to (except sample 1), respectively, those of controls, which is in agreement with our previous results concerning β-thalassaemic RBCs [10]. After the CCD fractionation, both PK and BPGM activities remained constant in the five pooled CCD fractions (Fig. 2, bottom). PK is a key, regulatory kinase of

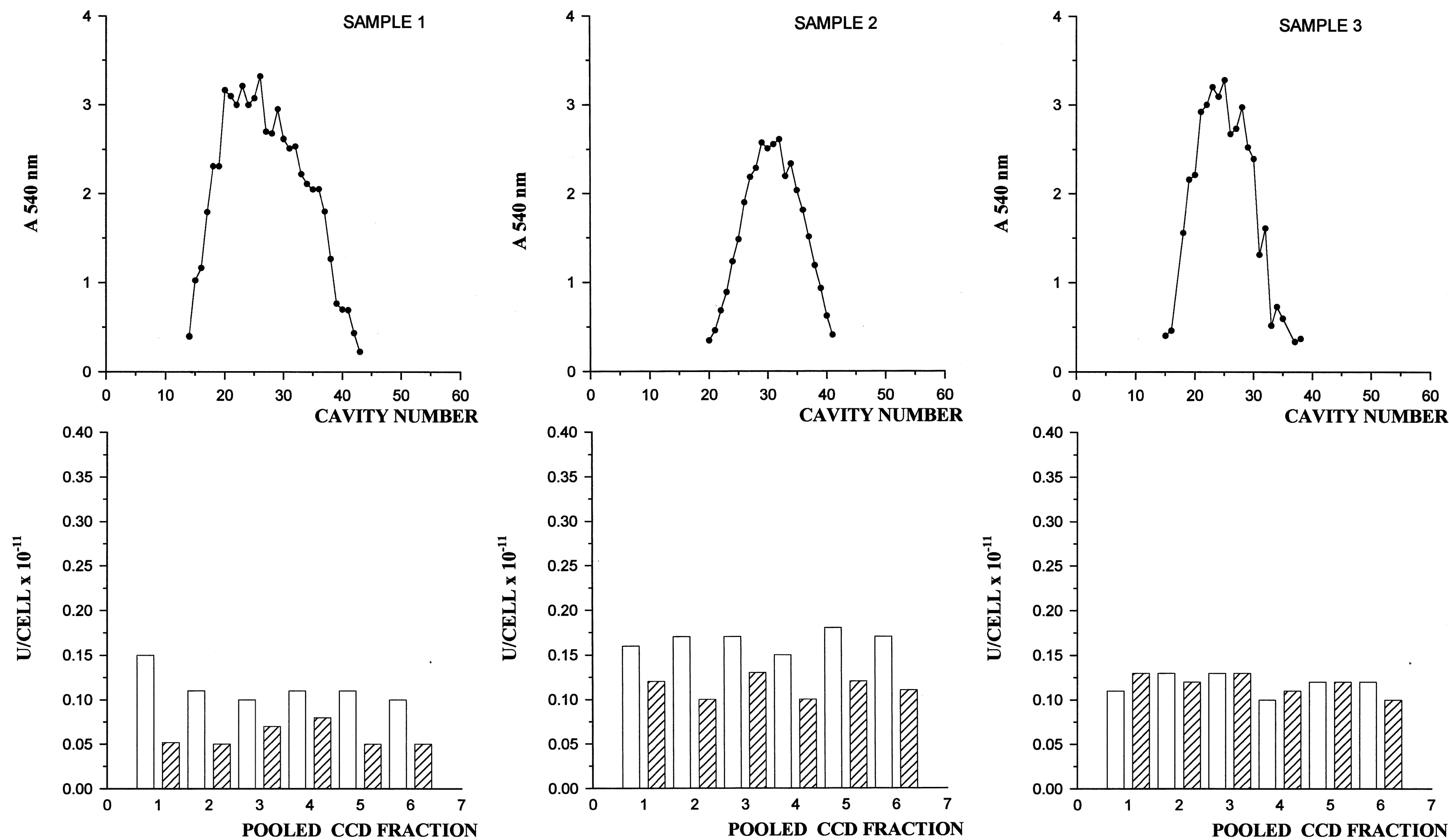

Fig. 2. Top: CCD fractionation profile for samples 1 (left), 2 (middle) and 3 (right) of microcytic human RBCs in a 5% D/4% PEG charge sensitive two-phase system. Cellularity expressed as haemoglobin absorbance at 540 nm is plotted against the CCD cavity number. Bottom: PK (hollow bars) and BPGM (stripe bars) specific enzyme activities (U/cell·10^{-11}) in the five pooled CCD fractions (1 to 5). Data are the mean of two separate determinations.

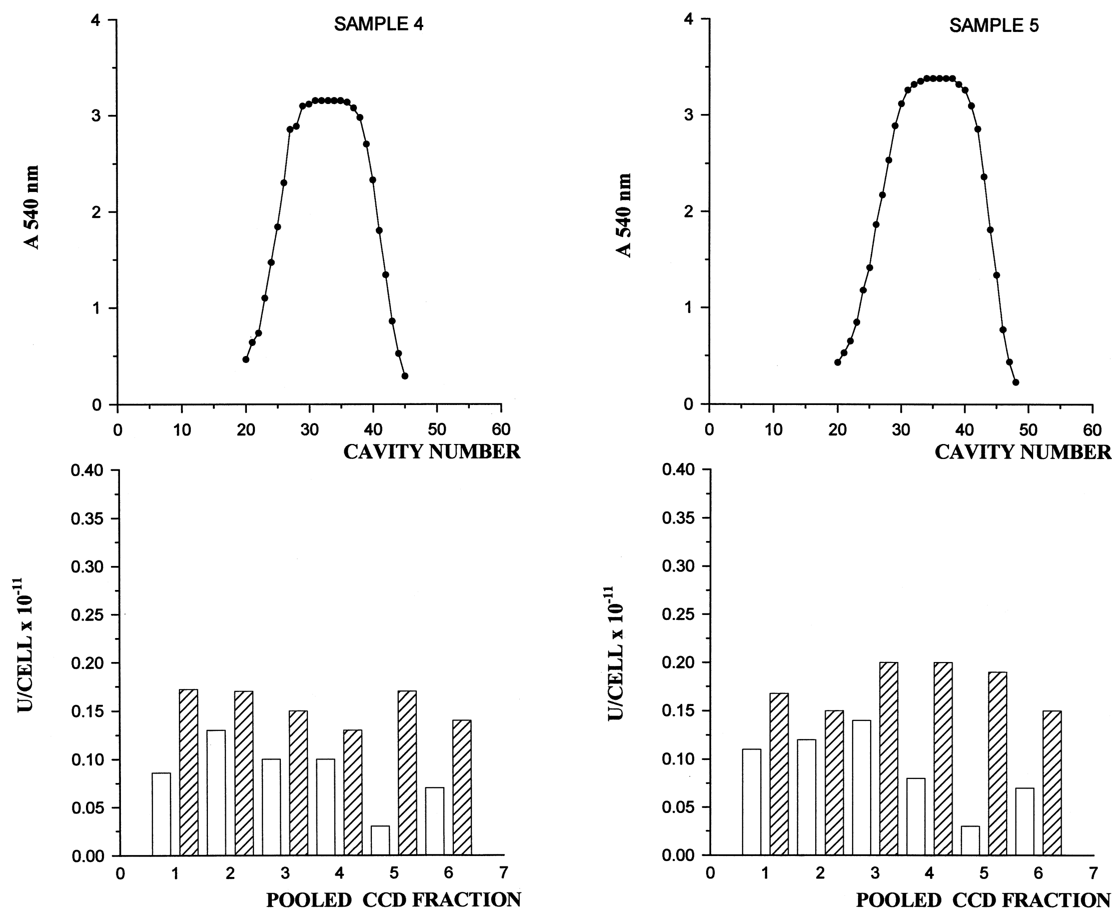

Fig. 3. Top: CCD fractionation profile for samples 4 (left) and 5 (right) of macrocytic human RBCs in a 5% D/4% PEG charge sensitive two-phase system. Cellularity expressed as hemoglobin absorbance at 540 nm is plotted against the CCD cavity number. Bottom: PK (hollow bars) and BPGM (stripe bars) specific enzyme activities (U/cell$\cdot 10^{-11}$) in the five pooled CCD fractions (1 to 5). Data are the mean of two separate determinations.

Table 2
Enzyme specific activities (U/cell$\cdot 10^{-11}$) in the microcytic (samples 1 to 3) and macrocytic (samples 4 and 5) of whole RBC populations

	PK	BPGM
Control	0.21±0.05	0.11±0.03
Sample 1	0.15	0.05
Sample 2	0.16	0.12
Sample 3	0.11	0.13
Sample 4	0.10	0.17
Sample 5	0.11	0.17

Control values represent the mean±S.E. for four separate measurements. Sample (1 to 5) values are the mean of three separate measurements.

the glucolytic pathway, validated as an age-marker enzyme in erythrocytes since PK activity decreases as the cell gets older. The constant PK activity along CCD profile, suggests that microcytic RBCs do not yield to the normal ageing process (Fig. 1, bottom). These cells behave as a young erythrocyte population.

The whole macrocytic RBC populations also showed a decreased PK activity (Table 2) with respect to the controls as also was observed for the microcytic cells. The BPGM activity appeared slightly higher than the observed in control RBCs. A decrease of the PK activity from pool 1 to pool 5 (Fig. 3, bottom) was observed after the CCD frac-

tionation while, the BPGM activity was kept constant. Such behaviour is quite similar to that observed in control RBCs (Fig. 1, bottom). The decrease in PK activity from the left- to the right-hand side of the CCD profile suggests that macrocytic RBCs could be fractionated according to age. This is possible, in spite of their lower surface charge properties as deduced from their fractionation profile.

With respect to the BPGM activity, the enzyme directly related to 2,3-BPG synthesis, a constant value has been observed along the CCD profiles in both, microcytic and macrocytic RBCs (Figs. 2 and 3, bottom). BPGM activity behaviour results are similar to those previously observed in both, normal (Fig. 1, bottom) and β-thalassaemic RBCs [10,17]. In fact, non-significant variations in the 2,3-BPG level were observed in the pooled CCD fractions of normal and β-thalassaemic RBCs [10]. The maintenance of 2,3- BPG during the RBC ageing is crucial for the oxygenation throughout the erythrocytic lifespan. It has been explained on the basis of a fairly constant BPGM and PGK activities [17]. The stability of both enzymes may be the mechanism by which the RBCs maintain an appropriate level of 2,3-BPG. Therefore, these enzymes have to be far away of the normal age-related decay suffered by most enzymes.

In conclusion, the results shown here as well as those from previous studies illustrate that CCD in charge-sensitive two-phase systems is a technique highly sensitive for distinguishing between normal and altered cell surface properties of RBCs. Therefore, it could become useful in haematology mainly for a better understanding of enzymatic changes that take place in the course of some RBC diseases.

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