Journal of Chromatography, 641 (1993) 279–289
Elsevier Science Publishers B.V., Amsterdam

CHROM. 25 098

Immobilized metal ion affinity partitioning of erythrocytes from different species in dextran–poly(ethylene glycol) aqueous phase systems

Harry Walter* and Kim E. Widen

Laboratory of Chemical Biology — 151, Veterans Affairs Medical Center, Long Beach, CA 90822-5201 (USA)

Gerd Birkenmeier

Institute of Biochemistry, University of Leipzig, D-04103 Leipzig (Germany)

(First received January 12th, 1993; revised manuscript received March 23rd, 1993)

ABSTRACT

Poly(ethylene glycol) (PEG)-bound ligands partition preferentially into the top, PEG-rich, phase of dextran (Dx)–PEG aqueous phase systems. The extraction of erythrocytes from beef, dog, horse, human, pig, rabbit, rat and sheep was examined in both non-charge-sensitive and charge-sensitive Dx–PEG phase systems containing PEG–iminodiacetate (IDA) which had been reacted with Cu(II) or Zn(II). PEG–IDA-Cu binds primarily to histidine (His) residues. Phase systems containing excess imidazole were used to obtain cell partition ratios not attributable to the metal chelate. In non-charge-sensitive phase systems having lower polymer concentrations a correlation has been reported between the partition, *P*, of erythrocytes and their membrane ratio of poly/monounsaturated fatty acids; while in charge-sensitive phases there is some correlation between the *P* values and the cells' relative electrophoretic mobilities. At higher polymer concentrations red blood cells accumulate at the interface and do not partition. Under such conditions addition of the PEG–IDA-Cu (Zn is less effective) causes erythrocytes to partition into the PEG-rich phase in a non-charge-related *or* charge-associated sequence reminiscent of that found in the absence of chelate in non-charge-sensitive *or* charge-sensitive phase systems, respectively, at lower polymer concentrations. PEG–IDA-Cu may thus be useful in extending the partitioning range of Dx–PEG systems to cells having such low *P* values in non-charge-sensitive and/or charge-sensitive phase systems as to preclude their partitioning even when phase systems are optimized by manipulation of their components.

From the cited experiments it would appear that either the His per unit surface area of erythrocytes from different species is about the same causing the non-charge-related or charge-associated surface properties (depending on the phase system used) to determine, to a large extent, the *P* even in the presence of chelate *or* that the non-charge-related or charge-associated surface properties outweigh the differences in His content and effect the observed correlations.

In contrast to these apparently "non-specific" extractions effected by PEG–IDA-Cu, there are cases in which PEG–IDA-Cu acts as a sensitive probe for recognizing differences in cell surface properties not detected by other means.

INTRODUCTION

Partitioning in two-polymer, most commonly dextran (Dx)–poly(ethylene glycol) (PEG), aqueous phase systems is an established method for the separation and fractionation of biomaterials including cells, membranes and organelles [1,2]. Depending on the Dx and PEG concentrations and the ionic composition and concentrations used, the phase system can either be charge-sensitive (*i.e.*, have a Donnan potential between the phases) or non-charge-sensitive [3,4]. In the case of erythrocytes from different

* Corresponding author.

0021-9673/93/$06.00 © 1993 Elsevier Science Publishers B.V. All rights reserved

species, which have been used as models, cell partitioning behavior in a charge-sensitive system with higher polymer concentrations is determined to a great extent by the cells' surface charge while in a non-charge-sensitive system with lower polymer concentrations it is lipid-related surface properties that effect the cells' partitioning behavior [3].

Affinity partitioning, utilizing the interaction between surface biomolecules and affinity ligands confined primarily to one of the phases by covalently linking them to one of the phase-forming polymers (usually PEG), has also been employed in cell separation technology. Initially, so called "general ligands", which were either charged (*e.g.*, DEAE-Dx, trimethylamino-PEG or PEG-sulfonate) or hydrophobic (*e.g.*, esters of PEG and fatty acids), were used in cell affinity partitioning [5,6]. Currently, immunoaffinity partitioning is being developed for the biospecific extraction of cell populations by use of polymer-linked antibodies [7–10].

Another approach to increase the selectivity of separation exploits the interaction of chelated transition metal ions with macromolecules [11]. This interaction depends on immobilized metal ions coordinating with electron-rich ligands on protein surfaces [12–14]. Accessible histidine residues localized in a favorable orientation on the protein surface have been reported to serve as predominant metal-binding sites [15–17]. Experiments with metal chelate-derivatized PEG in Dx–PEG and PEG–salt phase systems indicate that extraction of proteins by such ligands is histidine-mediated [18,19]. Immobilized metal ion affinity partitioning in Dx–PEG phase systems has recently been successfully applied to the extraction of cells [20].

Here we have examined the metal chelate affinity partitioning behavior of erythrocytes from different species in Dx–PEG aqueous phase systems. The influence of Cu(II) [and, in some experiments, of Zn(II)] chelated to PEG–iminodiacetate (IDA) on cell partitioning was studied in both charge-sensitive and non-charge-sensitive phase systems. Insights into factors governing the behavior of cells in affinity phase systems were obtained especially with regard to those aspects of partitioning that can be ascribed to "specific" *vs.* "non-specific" extractions by an affinity ligand.

EXPERIMENTAL

Reagents

Dx T500 (lot No. 01 06905) was obtained from Pharmacia-LKB (Piscataway, NJ, USA). PEG 8000 ("Carbowax 8000") was a product of Union Carbide (Long Beach, CA, USA). Neuraminidase (*Vibrio cholerae*) was purchased from Calbiochem (San Diego, CA, USA). Monomethoxy-PEG 5000, trypsin, phenylhydrazine hydrochloride and imidazole were from Sigma (St. Louis, MO, USA). All salts used were of analytical-reagent grade.

Preparation of metal chelate–poly(ethylene glycol)

PEG–IDA was synthesized by reacting bromoacetic acid with aminomonomethoxy-poly(ethylene glycol) as previously described [18]. Charging of the chelated PEG with Cu(II) and Zn(II) was performed in 50 m*M* sodium acetate buffer (pH 4.0); and the product was extracted repeatedly with chloroform. The content of copper and zinc per mol of PEG–IDA was 0.64 to 0.83 and 0.80 mol, respectively.

Blood from animals and from human donors

Blood from eight different species was collected in acid–citrate–dextrose (ACD) anticoagulant solution. The ratio used was 10 ml of blood to 3 ml of ACD. Human blood was obtained by venipuncture from presumably hematologically normal individuals; dog blood was from the femoral vein; rabbit blood from the ear marginal vein; and rat blood by heart puncture. Beef blood came from Shamrock Meats (Vernon, CA, USA); while horse, pig and sheep blood were obtained from the Animal Resource Facility, University of California, Irvine. Erythrocytes were used within one week of collection in the experiments outlined below.

Neuraminidase- or trypsin-treatment of human erythrocytes

A 2-ml volume of packed human red blood cells was washed three times with 10 vols. of

phosphate-buffered saline (PBS, pH 7.0 in neuraminidase and pH 7.4 in trypsin experiments). A 0.5-ml aliquot of such washed red cells + 3.5 ml of PBS was put into a 40-ml glass centrifuge tube and incubated together with 200 μl (1 I.U./ml) of neuraminidase or with 200 μl (1 mg/ml) of trypsin at 37°C for 60 min. At the same time a similarly treated aliquot of the same cell population was incubated in the absence of enzyme. The treated cells were washed three times with PBS and used in the partition experiments described below.

Phenylhydrazine injection of rats

Some of the rats were injected subcutaneously, on each of 5 successive days, with a neutralized solution of phenylhydrazine (3 mg phenylhydrazine/100 gm rat body weight). On the eighth day these rats' red blood cell populations consisted of at least 90% reticulocytes. The cells were washed three times with PBS and used in the partition experiments described below.

Preparation of aqueous two-phase systems

A number of different aqueous two-phase systems, having different physical properties and prepared as described by Walter [3], were used. In short, stock solutions were prepared of each of the components needed to make the various phase systems [*i.e.*, a 20% (w/w) Dx T500 solution; 40% (w/w) PEG 8000 solution; 5% (w/w) PEG–IDA-Cu or PEG–IDA-Zn solution; 0.44 *M* sodium phosphate buffer, pH 6.8; and 0.60 *M* NaCl]. These solutions were weighed out in appropriate amounts to yield the required quantity of a desired phase system. The compositions of the phases used as well as their physical properties are indicated below. The shorthand employed for phase system composition is as follows: the first number given is the % Dx (w/w). This is followed by a colon and a number giving the % PEG (w/w); followed again by a colon and a number giving the % PEG–IDA-Cu (or Zn, if so indicated) (w/w). These numbers are followed by a # designating the salt composition. #1 is 0.11 *M* sodium phosphate buffer, pH 6.8; #2 is 0.09 *M* sodium phosphate buffer, pH 6.8 + 0.03 *M* NaCl; and #5 is 0.15 *M* NaCl + 0.01 *M* sodium phosphate buffer, pH 6.8. Thus, a phase system given as 5:3.74:0.66 #2 contains 5% (w/w) Dx, 3.74% (w/w) PEG 8000, 0.66% (w/w) PEG–IDA-Cu, 0.09 *M* sodium phosphate buffer, pH 6.8 + 0.03 *M* NaCl.

Excess imidazole interferes with binding of the copper chelate to cell surface histidine while it does not affect the partition of cells in charge-sensitive or non-charge-sensitive phase systems devoid of PEG–IDA-Cu [20]. To establish that the partition obtained in phases containing PEG–IDA-Cu were predominantly due to the copper chelate (and not to other physical properties of the phases), partitioning was also carried out in "control" phases with imidazole (1 m*M* unless otherwise indicated).

Partitioning of erythrocytes in aqueous two-phase systems

The phase system which was to be used for partitioning, at 21–24°C, was mixed and poured into 50-ml centrifuge tubes. The tubes were centrifuged to speed phase separation and the top and bottom phase volumes were adjusted to be equal. The phase system was then mixed and 3-ml aliquots were delivered into 12 × 75 mm tubes. A 50-μl volume of the washed, packed erythrocytes which were to be partitioned was added to 200 μl saline. A 50-μl volume of this suspension, corresponding to between $6 \cdot 10^7$ and $1.7 \cdot 10^8$ cells, was pipetted into a mixed 3-g phase system which was mixed again. The phase systems were allowed to settle 15 min with the tubes in the vertical position. An 0.8-ml aliquot was withdrawn from the middle of the top phase. The quantity of cells in each top phase was determined by lysing the cells and measuring hemoglobin absorbance at 540 nm [21]. The quantity of cells initially added to the partition tubes was similarly determined.

Phases to be used in the neuraminidase or trypsin partition experiments, at 21–24°C, were mixed and about 12 ml was poured into partition tubes (*i.e.*, calibrated tubes, 125 mm × 16 mm). The tubes were centrifuged to speed phase separation and the top and bottom phase volumes were adjusted to be equal at 5 ml. A 30-μl aliquot of washed, packed erythrocytes was added to the mixed phases. The tubes were capped (with Parafilm), inverted a number of

times and the phases were then permitted to settle in horizontal position for 7 min. The tubes were gently raised to the vertical position (without agitating the contents) and the phases permitted to settle for one additional minute. A 1-ml aliquot of top phase was withdrawn. The quantity of cells in each top phase was determined as above.

The partition of cells is given by a P value, defined as the quantity of cells in the top phase, at the time of sampling, as a percentage of the total quantity of cells added. The distribution ratio is the quantity of cells in the top phase divided by quantity of cells at the interface plus bottom phase. In the case of phases containing PEG–IDA-Cu the P values for cells obtained both without and with imidazole are given, for purposes of discussion, in the tables; while the distribution ratios shown in the figures represent partitions of cells in phases at polymer concentrations which were so selected that the cells' partitions in the presence of imidazole are very small. Graphs were plotted using Sigma-Plot 3.0 (Jandel Scientific, Corte Madera, CA, USA). Regression lines are of single order.

RESULTS AND DISCUSSION

Basic phenomena relating to affinity partitioning using a general ligand

Partitioning of erythrocytes in Dx T500:PEG 8000 systems takes place between the top phase and the interface [3]. In non-charge-sensitive phase systems (see below), at adequately low polymer concentrations (*e.g.*, 5% Dx:3.5% PEG), the P value of red cells is species-specific and correlates extremely well with the erythrocytes' membrane ratio of poly/monounsaturated fatty acids [22]. Red cells with high P values (*e.g.*, those from dog) have been shown to be held more weakly at the interface than are erythrocytes with low P values (sheep, beef) [23,24]. For cell affinity partitioning with a ligand which potentially interacts with all cells albeit to a differing extent (hereafter called "general" affinity ligand, *e.g.*, PEG–IDA-Cu), the non-charge-sensitive phase system selected should be one which has the lowest polymer concentrations at which virtually all cells are at the interface [3]. Even under these conditions it is clear that some species' red cells (*e.g.*, dog) would, if all else were equal, be more easily pulled into the upper phase by an affinity ligand than some other species' erythrocytes (*e.g.*, those from sheep, beef).

An analogous argument can be made with respect to charge-sensitive phase systems (see below) at higher polymer concentrations (*e.g.*, 5:4) in which erythrocytes from different species yield P values which have some correlation with the cells' relative electrophoretic mobilities [3].

Relationship of the distribution ratios of red blood cells from different species to their surface properties in non-charge-sensitive and charge-sensitive Dx–PEG aqueous phase systems with and without PEG–IDA-Cu

Non-charge-sensitive Dx–PEG aqueous phase systems are those in which the predominant salt has ions with essentially equal affinities for the two phases (*e.g.*, NaCl) and there is, hence, no Donnan potential between the phases [3,22]. With increasing polymer concentrations cells added to the phases tend to be increasingly adsorbed at the interface. 5:3.8 is close to the

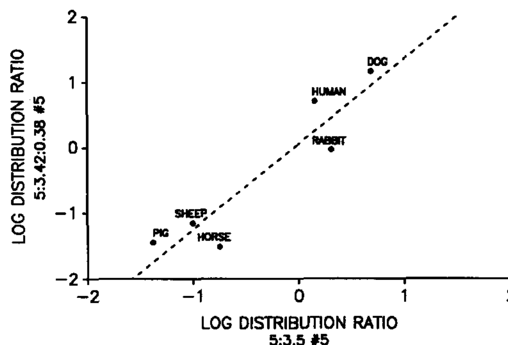

Fig. 1. The log distribution ratios of RBC from different species in a non-charge-sensitive phase system containing PEG–IDA-Cu (5% Dx:3.42% PEG:0.38% PEG–IDA-Cu, 0.15 M NaCl and 0.01 M sodium phosphate buffer, pH 6.8) plotted against those obtained in a non-charge-sensitive system with lower polymer concentration and without chelate (5% Dx:3.5% PEG, 0.15 M NaCl and 0.01 M sodium phosphate buffer, pH 6.8 [22]). A general similarity is in evidence between the groupings of extractions of RBC from different species in the two systems. Note that because of its higher polymer concentration cells do not partition in the phase system used with the PEG–IDA-Cu in its absence but remain at the interface [3]. See text for discussion.

lowest polymer concentrations at which the red blood cells of all species examined (except for rat) are almost (but not) completely adsorbed at the interface.

In Fig. 1 we depict distribution ratios of erythrocytes from a number of different species obtained in a non-charge-sensitive system with higher PEG concentrations (*i.e.*, 5:3.8 #5) in which 10% of the PEG has been replaced by PEG–IDA-Cu (ordinate). The fact that erythrocytes partition in this phase system at all reflects that the PEG–IDA-Cu, which itself partitions predominantly into the top phase [18], interacts with the cells and "pulls" cells out of the interface and into the top phase. The erythrocyte distribution ratios appear to be species-specific. For comparison we also show in this figure the previously reported partition results in a non-charge-sensitive phase system at lower polymer concentrations (5:3.5 #5) devoid of PEG–IDA-Cu (abscissa) [22]. Note a general similarity in the grouping of distribution ratios of red cells from different species in the phase systems with and without PEG–IDA-Cu (*i.e.*, dog, human, rabbit have high and horse, sheep, pig low ratios).

There is also some tendency for the distribution ratios of erythrocytes from different species, in a system containing high polymer concentrations and PEG–IDA-Cu, to increase with a larger membrane ratio of their poly/monounsaturated fatty acids (data not shown). Thus, the sequence of extraction of cells in this phase system appears to reflect a non-charge-dependence attributable to membrane lipid parameters but not to a specificity of the chelate.

Charge-sensitive Dx–PEG aqueous phase systems are those in which the predominant salt has ions which have unequal affinities for the two phases (*e.g.*, sodium phosphate) giving rise to a Donnan potential between the phases, top phase positive [3]. With increasing polymer concentrations cells added to the phases tend to be increasingly adsorbed at the interface. 5:4.4 is close to the lowest polymer concentrations at which the red blood cells of all species examined (except for rat) are almost (but not) completely adsorbed at the interface.

Fig. 2 shows the distribution ratios of erythrocytes from a number of different species obtained in a charge-sensitive system with higher PEG concentrations (*i.e.*, 5:4.4 #2, ordinate) in which 15% of the PEG has been replaced by PEG–IDA-Cu. Again, PEG–IDA-Cu causes the red cells to partition into the top phase at polymer concentrations at which they would not partition in the ligand's absence. For comparison we also show in this figure the previously reported partition results in a charge-sensitive phase system at lower polymer concentrations (5:4 #1) devoid of PEG–IDA-Cu (abscissa) [3]. Note the rather good correlation between distribution ratios of red cells from different species in the phase systems with and without PEG–IDA-Cu.

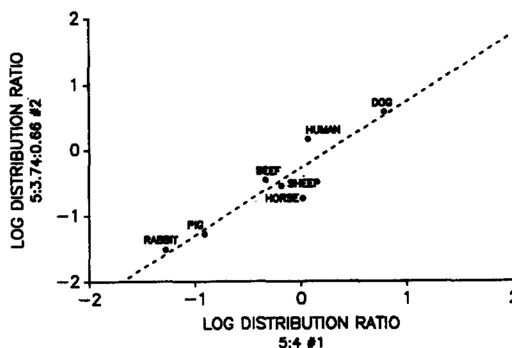

Fig. 2. The log distribution ratios of RBC from different species in a charge-sensitive phase system containing PEG–IDA-Cu (5% Dx:3.74% PEG:0.66% PEG–IDA-Cu, 0.09 *M* sodium phosphate buffer, pH 6.8, and 0.03 *M* NaCl) plotted against those obtained in a charge-sensitive phase system with lower polymer concentration and without chelate (5% Dx:4% PEG and 0.11 *M* sodium phosphate buffer, pH 6.8 [22]). A similarity is in evidence between the sequence of extractions of RBC from different species in the two phase systems. Beef RBC belonged to partition class I (see ref. 22 and below). Note that because of its higher polymer concentration cells do not partition in the phase system used with the PEG–IDA-Cu in its absence but remain at the interface [3]. See text for discussion.

The distribution ratios of erythrocytes from different species, in a system containing a high polymer concentration and PEG–IDA-Cu were also found to correlate well with the cells' relative electrophoretic mobilities (data not shown). Thus, the sequence of extraction of cells in this phase system appears to reflect a charge-dependence attributable to the Donnan potential but not to a specificity of the chelate.

The relationship between the partition ratios of red cells from different species in non-charge-sensitive and charge-sensitive phase systems with and without PEG–IDA-Cu suggests that a major effect of the PEG–IDA-Cu is the facilitation of partitioning of the cells at polymer concentrations higher than those at which they could partition without the chelate. The sequence of extraction of the cells is reminiscent of, though not identical with (see below), that observed in phases at lower polymer concentrations devoid of chelate. This implies that the histidine per unit surface area of red blood cells from different species is about the same causing the non-charge-related *or* charge-associated surface properties to determine the distribution ratios even in the presence of chelate *or* that these properties outweigh the differences in histidine content and effect the observed correlations.

This general effect of PEG–IDA-Cu on partitioning of red blood cells (RBC) has also been corroborated by counter-current distribution studies on rat red blood cell populations of different ages (which are known, based on a combination of isotopic and partitioning experiments, to have distinct partition ratios in non-charge-sensitive phase systems without chelate [3,25]). The results indicate (data not shown) that the reticulocytes, young mature erythrocytes, middle-aged erythrocytes and old erythrocytes have relative partition ratios with respect to the total cell population that mimic those

TABLE I

THE PARTITION, *P*, OF RAT RED BLOOD CELLS FROM NORMAL AND FROM PHENYLHYDRAZINE-INJECTED RATS IN NON-CHARGE-SENSITIVE PHASE SYSTEMS WITH AND WITHOUT PEG–IDA-Cu

P, the partition, is defined as the quantity of cells in top phase as a percentage of total cells added. Values given are means ±S.D. RBC (10-μl packed cells) were partitioned in a 3-g phase system having equal top and bottom phases. Settling time was 15 min. Rats were injected with a solution containing phenylhydrazine (3 mg/100 g of body mass) on 5 successive days and were bled on the eighth day. Reticulocytes then constituted more than 90% of the total red cell population.

Rat	Phase system[a]		*P*			
			Percentage of PEG substituted with PEG–IDA-Cu[a]			
			0	3	5	10
Normal	5:3.9 #5		14 ± 1	32 ± 4	61 ± 0	92 ± 1
		with imidazole		26 ± 1	53 ± 1	75 ± 1
	ΔP			6	8	17
Injected	5:3.9 #5		5 ± 0	15 ± 2	20 ± 2	63 ± 1
		with imidazole		7 ± 2	7 ± 1	18 ± 3
	ΔP			8	13	45
Normal	5:4.0 #5		9 ± 0	25 ± 1	30 ± 1	92 ± 2
		with imidazole		9 ± 1	10 ± 1	20 ± 1
	ΔP			16	20	72
Injected	5:4.0 #5		4 ± 0	12 ± 0	18 ± 2	42 ± 1
		with imidazole		4 ± 0	7 ± 1	8 ± 1
	ΔP			8	11	34
Normal	5:4.2 #5		7 ± 0	14 ± 1	21 ± 1	60 ± 4
		with imidazole		9 ± 1	12 ± 1	9 ± 0
	ΔP			5	9	51
Injected			n.d.	n.d.	n.d.	n.d.[b]

[a] The various Dx, PEG phase systems contained 0.15 *M* NaCl and 0.01 *M* sodium phosphate buffer, pH 6.8, with the indicated percentages of PEG replaced by PEG–IDA-Cu. Control systems also contained 1 m*M* imidazole (see text for discussion).
[b] n.d. = Not determined.

obtained in non-charge-sensitive phase systems containing lower polymer concentrations and no chelate. Again, PEG–IDA-Cu facilitates the extraction of cells into the top phase in a sequence reflecting the relative avidity of rat red cells of different ages for the interface [23,24].

Partitions of rat red blood cells from normal and from phenylhydrazine-injected rats in non-charge-sensitive phase systems containing PEG–IDA-Cu

The problems encountered in selecting an appropriate phase system for affinity partitioning using a "general" affinity ligand with cells is illustrated with the example shown in Table I. Repeated injection of rats with phenylhydrazine yields red blood cell populations composed almost entirely of reticulocytes [3]. Such cells have previously been found to have *P* values lower than those of erythrocytes from untreated (normal) animals [25]. In Table I we present the *P* values of red blood cells from normal and from injected rats in a series of non-charge-sensitive phase systems having different polymer concentrations and containing increasing quantities of PEG–IDA-Cu (*i.e.*, the latter replacing from 0 to 10% of the PEG present in the phase systems). Partitions were also carried out, as control, in phase systems containing 1 m*M* imidazole.

Rat red cells have a very high membrane ratio of poly/monounsaturated fatty acids which correlates with their higher *P* value in non-charge-sensitive phase systems, higher than that of red cells from other species [22]. Thus, the lowest polymer concentrations at which virtually all rat red cells will be at the interface has to be higher than for any other species' erythrocytes. In a 5:3.9 #5 system (Table I) the *P* value of rat normal RBC, in the absence of PEG–IDA-Cu, is still about 14% in the top phase. With increasing quantities of PEG–IDA-Cu this value increases to 92%. Phase systems having the same phase compositions but also containing imidazole (which interferes with the Cu chelate-cell interaction) also give an increase in the RBC *P* value. The latter increase illustrates the rule that substitution of a polymer of lower molecular weight (as in the case of the PEG 5000-ligand) for one of higher molecular mass (the PEG 8000 which constitutes the bulk of the phase system) causes biomaterials (including cells) to favor the phase which contains the polymer with reduced average molecular mass [4]. High control *P* values obtained in the presence of imidazole should not be subtracted from *P* values obtained in its absence because misleading, low "net" values can result. That is because cells partitioning into the top phase in the presence of imidazole under these conditions may form part of the population that can also be extracted with PEG–IDA-Cu if a phase system with adequately high polymer concentrations were used in which the cells are retained at the interface initially (see results in 5:4 #5 and 5:4.2 #5, Table I). It follows that the *P* values obtained for normal rat RBC in all 5:3.9 #5 systems containing PEG–IDA-Cu do not give quantitative information on the extractability of these cells with the chelate. The same argument applies to previously published data obtained in phase systems which were close to the critical point [20].

Rat reticulocytes have lower *P* values than do normal mature rat RBC in non-charge-sensitive phase systems. Hence reticulocytes tend to adhere to the phase interface more strongly [23]. In a 5:3.9 #5 system with increasing quantities of PEG–IDA-Cu reticulocyte *P* value increases while the *P* values obtained in the systems containing 3 or 5% PEG–IDA-Cu and imidazole are very low. In the system containing 10% PEG–IDA-Cu and imidazole the PEG–ligand molecular mass effect on the *P* value (see above) is again in evidence (although less so than in the case of normal mature rat RBC).

To gauge the relative affinity of rat RBC from normal (mature RBC) from phenylhydrazine-injected (reticulocytes) animals for the copper chelate, polymer concentrations of the phase systems are increased (Table I) to the point at which inclusion of PEG–IDA-Cu (at a useful concentration) yields low *P* values in the presence of imidazole. Increasing the concentrations of polymers beyond this will cause cells to be held more and more firmly at the interface and result in ever-decreasing cell affinity extractions. Comparison of *P* values for normal RBC and for reticulocytes in phase systems having the same composition and in which both of these cell populations have low *P* values (*i.e.*, 10% or less)

in the presence of imidazole (*e.g.*, 5:4 #5 with 3 or 5% PEG–IDA-Cu), indicates that normal RBC are extracted by PEG–IDA-Cu to a greater extent than reticulocytes.

Since this result is also obtained in non-charge-sensitive phase systems at lower polymer concentrations without PEG–IDA-Cu there is, again, no indication of an extraction specificity attributable to PEG–IDA-Cu.

Partitions in charge-sensitive and non-charge-sensitive phase systems with and without PEG–IDA-Cu of human normal erythrocytes (RBC) and RBC treated with neuraminidase or trypsin

It has previously been reported that when human RBC are treated with neuraminidase (thereby removing virtually all of the main charge-bearing groups on the surface of these cells) or trypsin their *P* value is markedly reduced in charge-sensitive phases (*e.g.*, 5:3.9 #1) and appreciably increased in non-charge-sensitive systems (*e.g.*, 5:3.4 #5), see ref. 22 and Table II. The increase in partition of neuraminidase- or trypsin-treated cells in non-charge-sensitive phases may be a consequence of the closer interaction between the top, PEG-rich, phase and the non-charge-related surface properties possible after sialic acid or peptide and peptide-bound sialic acid removal [3,22]. In a non-charge-sensitive phase system having higher polymer concentrations and containing PEG–IDA-Cu (5:3.78:0.42 #5), selected in a manner as outlined in the previous section, the *P* value of neuraminidase-treated cells, but not of trypsin-treated cells, is also found to increase (Table II). In a charge-sensitive phase system having higher polymer concentrations and containing PEG–IDA-Cu (5:3.74:0.66 #2) the *P* value of neuraminidase-treated red cells is not only not reduced but is actually increased. This is an example of specific extraction of the treated

TABLE II

THE PARTITION, *P*, OF HUMAN NORMAL ERYTHROCYTES (RBC) AND ERYTHROCYTES TREATED WITH NEURAMINIDASE OR TRYPSIN IN CHARGE-SENSITIVE OR NON-CHARGE-SENSITIVE PHASE SYSTEMS WITH AND WITHOUT PEG–IDA-Cu

P, the partition, is defined as the quantity of cells in top phase as a percentage of total cells added. Values given are means ±S.D. with the number of experiments in parentheses. RBC (30-μl packed cells) were partitioned in a 10-g phase system having equal volumes of top and bottom phase. Settling time was 7 min in the horizontal position + 1 min in the vertical position.

Phase system[a]		*P*		
		Normal RBC	Neuraminidase-treated RBC	Trypsin-treated RBC
5:3.9 #1		63 ± 3 (12)	7 ± 3 (7)	23 ± 1 (5)
5:3.74:0.66 #2		61 ± 2 (12)	80 ± 6 (8)	20 ± 2 (5)
	with imidazole	9 ± 2 (7)	10 ± 0 (3)	10 ± 1 (5)
5:3.4 #5		44 ± 3 (12)	89 ± 3 (7)	82 ± 2 (5)
5:3.78:0.42 #5		21 ± 2 (5)	81 ± 1 (3)	13 ± 1 (3)
	with imidazole	3 ± 0 (5)	8 ± 1 (3)	7 ± 1 (3)

[a] 5:3.9 #1 contained 5% Dx, 3.9% PEG, and 0.11 *M* sodium phosphate buffer, pH 6.8 (NaPB). It has a Donnan potential between the phases and is charge-sensitive. 5:3.74:0.66 #2 contained 5% Dx, 3.74% PEG, 0.66% PEG–IDA-Cu, 0.09 *M* NaPB and 0.03 *M* NaCl. The control system also contained 1 m*M* imidazole. These systems have a higher interfacial tension than 5:3.9 #1 due to the higher polymer concentrations and have a Donnan potential between the phases. They are thus charge-sensitive and contain PEG–IDA-Cu. 5:3.4 #5 contained 5% Dx, 3.4% PEG, 0.15 *M* NaCl and 0.01 *M* NaPB. It has virtually no Donnan potential between the phases and is non-charge-sensitive. 5:3.78:0.42 #5 contained 5% Dx, 3.78% PEG, 0.42% PEG–IDA-Cu, 0.15 *M* NaCl and 0.01 *M* NaPB. The control system also contained 1 m*M* imidazole. These systems have a higher interfacial tension than 5:3.4 #5 due to the higher polymer concentrations and have virtually no Donnan potential between the phases. They are thus non-charge-sensitive and contain PEG–IDA-Cu.

cells by the copper chelate [*i.e.*, an extraction that cannot be attributed to the physical (*i.e.*, charge-sensitive) properties of the phases devoid of chelate]. The removal of sialic acid may permit increased binding of PEG–IDA-Cu to the cell surface thereby raising the treated cells' *P* value. The removal of peptides and peptide-bound sialic with trypsin apparently results in diminished binding sites for the chelate as reflected by the reduced *P* value.

In other experiments (not shown) we have found that identical partitioning results are obtained over a five-fold increase in cell concentration over that used here.

Partitions of human and rabbit erythrocytes (RBC) in a non-charge-sensitive phase system with PEG–IDA-Cu or PEG–IDA-Zn

PEG–IDA-Zn is less efficient than PEG–IDA-Cu in extracting RBC in non-charge-sensitive aqueous phases. This becomes evident when PEG–IDA-Zn is substituted for PEG–IDA-Cu in a phase system of otherwise identical composition. In Table III we present, for comparison, the *P* values of human and rabbit RBC in a non-charge-sensitive phase system close to the critical point, 5:3.5 #5 [22], and in a phase system with higher polymer concentrations in which part of the PEG has been replaced by either PEG–IDA-Cu or PEG–IDA-Zn. It is clear that the *P* value of rabbit erythrocytes is higher than that of human RBC in the non-charge-sensitive phase devoid of metal chelate, a result which fits into the general pattern correlating *P* values of erythrocytes from different species with their membrane ratio of poly/mono-unsaturated fatty acids [22]. In the presence of PEG–IDA-Cu the indicated sequence is reversed with human red cells having the higher *P* value (Table III and Fig. 1). Thus, the Cu chelate specifically extracts human erythrocytes to a greater extent than rabbit red blood cells. Furthermore, since PEG–IDA-Zn causes no reversal of the partitioning sequence of human and rabbit RBC, it appears that the interactions of these chelates with these cells' surfaces is metal-specific.

Partitioning beef red blood cells belonging to different partition classes in charge-sensitive and non-charge-sensitive phase systems containing PEG–IDA-Cu

Beef red blood cells collected from different animals have previously been found to fall into three partition classes with low (class I), intermediate (II) and high (III) *P* values in charge-sensitive phase systems [26]. These classes ap-

TABLE III

THE PARTITION, *P*, OF HUMAN AND RABBIT ERYTHROCYTES (RBC) IN A NON-CHARGE-SENSITIVE PHASE SYSTEM WITH AND WITHOUT PEG–IDA-Zn OR PEG–IDA-Cu

P, the partition, is defined as the quantity of cells in the top phase as a percentage of total cells added. Values given are means ±S.D. with the number of experiments given in parentheses. RBC (10-μl packed cells) were partitioned in a 3-g system having equal volumes of top and bottom phase. Settling time was 15 min.

Phase system[a]		*P*	
		Human RBC	Rabbit RBC
5:3.5 #5[b]		59 ± 6	67 ± 11
5:3.42:0.38 (Cu) #5		84 ± 6 (5)	49 ± 6 (5)
	with imidazole	4 ± 1 (2)	4 ± 1 (2)
5:3.42:0.38 (Zn) #5		8 ± 2 (4)	22 ± 3 (4)

[a] 5:3.5 #5 contained 5% Dx, 3.5% PEG, 0.15 *M* NaCl and 0.01 *M* sodium phosphate buffer, pH 6.8 (NaPB). 5:3.42:0.38 (Cu) #5 contained 5% Dx, 3.42% PEG, 0.38% PEG–IDA-Cu, 0.15 *M* NaCl and 0.01 *M* NaPB. The control system also contained 1 m*M* imidazole. 5:3.42:0.38 (Zn) #5 contained 5% Dx, 3.42% PEG, 0.38% PEG–IDA-Zn, 0.15 *M* NaCl and 0.01 *M* NaPB.
[b] Data from ref. 22.

pear to have different quantities of charge-bearing surface components (*e.g.*, class I has far less sialic acid on its surface than does class III). Each of these classes breaks into additional and sometimes overlapping classes when partitioned in non-charge-sensitive phase systems [27].

When beef erythrocytes are partitioned in a charge-sensitive phase system with higher polymer concentrations and containing PEG–IDA-Cu no difference in partitioning behavior based on class (determined in a 5:3.9 #1 system) is in evidence (note relatively small S.D. and range of *P* values, Table IV). On the other hand, beef red blood cells from different animals yield a variety of *P* values in a non-charge-sensitive phase system at higher polymer concentrations and containing PEG–IDA-Cu (note large S.D. and range of *P* values, Table IV). The latter do not correlate with the class determined in the charge-sensitive phase system devoid of PEG–IDA-Cu.

Beef red blood cells belonging to classes II and III have *P* values that are intermediate and high compared to other species' red cells in charge-sensitive phases while all beef erythrocytes have low *P* values in non-charge-sensitive phases. The uniform extraction of beef red blood cells, irrespective of class, in charge-sensitive phases containing PEG–IDA-Cu and their varied extraction in non-charge-sensitive phases containing PEG–IDA-Cu thus appears to reflect a specific extraction by the chelate.

TABLE IV

THE PARTITION, *P*, OF BEEF ERYTHROCYTES (RBC) IN CHARGE-SENSITIVE AND NON-CHARGE-SENSITIVE PHASE SYSTEMS WITH PEG–IDA-Cu

P, the partition, is defined as the quantity of cells in the top phase as a percentage of total cells added. Values given are means ±S.D. with the number of experiments given in parentheses. RBC (10-μl packed cells) were partitioned in a 3-g system having equal volumes of top and bottom phase. Settling time was 15 min.

Phase system[a]		*P*
5:3.52:0.88 #2		26 ± 4 [b] (15)
	with imidazole	2 ± 1 (4)
5:3.04:0.76 #5		14 ± 13 [c] (24)
	with imidazole	5 ± 1 (5)

[a] 5:3.52:0.88 #2 contained 5% Dx, 3.52% PEG, 0.88% PEG–IDA-Cu, 0.09 *M* sodium phosphate buffer, pH 6.8 (NaPB) and 0.03 *M* NaCl. The control system also contained 1 m*M* imidazole. 5:3.04:0.76 #5 contained 5% Dx, 3.04% PEG, 0.76% PEG–IDA-Cu, 0.15 *M* NaCl and 0.01 *M* NaPB. The control system also contained 1 m*M* imidazole.

[b] Range: 20–31.

[c] Range: 1–47.

CONCLUSIONS

PEG–IDA-Cu binds to red cells from different species and causes them to be extracted in a sequence reminiscent of *either* that obtained in charge-sensitive *or* non-charge-sensitive phase systems at lower polymer concentrations without the PEG–IDA-Cu. While the above-indicated basis for the extraction involves interaction of the affinity ligand with common binding sites on the cell surfaces and can be deemed "non-specific", PEG–IDA-Cu may prove useful in extending the partitioning range of Dx–PEG systems to cells having such low partitions (*P* values) in non-charge-sensitive and/or charge-sensitive phase systems as to preclude their partitioning even when phase systems are optimized by manipulation of their components.

Instances of specific extractions by PEG–IDA-Cu also occur. Examples are the increase in the *P* value of human neuraminidase-treated red blood cells in charge-sensitive phases containing PEG–IDA-Cu; the higher *P* value of human compared to rabbit erythrocytes in non-charge-sensitive phases containing the copper chelate; and the uniform extraction of beef red blood cells, belonging to different partition classes in charge-sensitive phases, in charge-sensitive phases containing PEG–IDA-Cu.

ACKNOWLEDGEMENTS

This work was supported by the Medical Research Service of the United States Department of Veterans Affairs and by the German Ministry of Science and Technology (0319792A).

REFERENCES

1 H. Walter, D.E. Brooks and D. Fisher (Editors), *Partitioning in Aqueous Two-Phase Systems—Theory, Methods, Uses, and Applications to Biotechnology*, Academic Press, Orlando, FL, 1985.
2 H. Walter, G. Johansson and D.E. Brooks, *Anal. Biochem.*, 197 (1991) 1.
3 H. Walter, in H. Walter, D.E. Brooks and D. Fisher (Editors), *Partitioning in Aqueous Two-Phase Systems—Theory, Methods, Uses, and Applications to Biotechnology*, Academic Press, Orlando, FL, 1985, pp. 327–376.
4 D.E. Brooks, K.A. Sharp and D. Fisher, in H. Walter, D.E. Brooks and D. Fisher (Editors), *Partitioning in Aqueous Two-Phase Systems—Theory, Methods, Uses, and Applications to Biotechnology*, Academic Press, Orlando, FL, 1985, pp. 11–84.
5 H. Walter and F.W. Selby, *Biochim. Biophys. Acta*, 148 (1967) 517.
6 E. Eriksson, P.-Å. Albertsson and G. Johansson, *Mol. Cell. Biochem.*, 10 (1976) 123.
7 K.A. Sharp, M. Yalpani, S.J. Howard and D.E. Brooks, *Anal. Biochem.*, 154 (1986) 110.
8 L.J. Karr, J.M. Van Alstine, R.S. Snyder, S.G. Shafer and J.M. Harris, *J. Chromatogr.*, 442 (1988) 219.
9 S.J. Stocks and D.E. Brooks, *Anal. Biochem.*, 173 (1988) 86.
10 C. Delgado, R.J. Anderson, G.E. Francis and D. Fisher, *Anal. Biochem.*, 192 (1991) 322.
11 J. Porath, J. Carlsson, I. Olsson and G. Belfrage, *Nature*, 258 (1975) 598.
12 E. Sulkowski, *BioEssays*, 10 (1989) 169.
13 R.J. Sundberg and R.B. Martin, *Chem. Rev.*, 74 (1974) 471.
14 E. Sulkowski, *TIBTECH*, 3 (1985) 1.
15 E.S. Hemdan, Y.-J. Zhao, E. Sulkowski and J. Porath, *Proc. Natl. Acad. Sci. U.S.A.*, 86 (1983) 1811.
16 F.H. Arnold, *BioTechnology*, 9 (1991) 151.
17 S.-S. Suh, B.L. Haymore and F.H. Arnold, *Protein Engin.*, 4 (1991) 301.
18 G. Birkenmeier, M.A. Vijayalakshmi, T. Stigbrand and G. Kopperschläger, *J. Chromatogr.*, 539 (1991) 267.
19 S.-S. Suh and F.H. Arnold, *Biotechnol. Bioeng.*, 35 (1990) 682.
20 H. Goubran-Botros, G. Birkenmeier, A. Otto, G. Kopperschläger and M.A. Vijayalakshmi, *Biochim. Biophys. Acta*, 1074 (1991) 69.
21 H. Walter, A. Miller, E.J. Krob and G.S. Ascher, *Exp. Cell Res.*, 69 (1971) 416.
22 H. Walter, E.J. Krob and D.E. Brooks, *Biochemistry*, 15 (1976) 2959.
23 D. Fisher, F.D. Raymond and H. Walter, in D.S. Kompala and P. Todd (Editors), *Cell Separation Science and Technology*, (*ACS Symposium Series*, No. 464), American Chemical Society, Washington, DC, 1991, pp. 175–188.
24 H. Walter, F.D. Raymond and D. Fisher, *J. Chromatogr.*, 609 (1992) 219.
25 H. Walter and E.J. Krob, *Br. J. Haematol.*, 38 (1978) 43.
26 H. Walter, R. Tung, L.J. Jackson and G.V.F. Seaman, *Biochem. Biophys. Res. Commun.*, 48 (1972) 565.
27 H. Walter and E.J. Krob, *FEBS Lett.*, 78 (1977) 105.