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SURFACE AFFINITY CHROMATOGRAPHIC SEPARATION OF BLOOD CELLS

III*. EFFECT OF MOLECULAR WEIGHT OF POLYETHYLENE GLYCOL BONDED STATIONARY PHASES ON ELUTION BEHAVIOUR OF HUMAN BLOOD CELLS

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SUMMARY

The chromatographic behaviour of human peripheral blood cells on four kinds of oxirane-coupled polyethylene glycol (PEG)–Sepharose 6B columns was investigated by using an eluent containing 2% of dextran T40. The retention volumes of lymphocytes, granulocytes and platelets at pH 7.5 have a strong dependence on the average molecular weight of the bonded PEG in the range 400–20,000, increasing with increasing molecular weight. Further, the same tendency for the retention behaviour was also observed at the isoelectric points determined for the four kinds of blood cells by using the cross-partition method. For lymphocytes and granulocytes hydrophobic interactions with the bonded PEG phase were found to be predominant, whereas for erythrocytes and platelets electrostatic interactions were also taken into account.

INTRODUCTION

By using a column packing consisting of chemically bonded PEG 20M on Sepharose 6B (PEG 20M–Sepharose) and an isotonic buffered solution (pH 7.5) containing dextran T40 or T500 as the mobile phase, we previously effected the chromatographic separation of human blood cells and studied the influence of mobile phase composition on the retention behaviour[1,2]. Except in a few instances, the blood cells were eluted from the column in the order erythrocytes, platelets, granulocytes and lymphocytes. This elution order seems to be independent of the character of the cells, *e.g.*, size, adhesiveness and surface negative charge.

Addition of neutral salts, such as sodium chloride, to the mobile phase while maintaining the isonicity resulted in an increase in the retention volumes of erythrocytes, granulocytes and lymphocytes[2]. In the hydrophobic affinity chromatography

* For Part II, see ref. 2.

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of serum albumin and enzymes, etc., it is well known that the addition of neutral salts to the mobile phase increases the hydrophobic interaction between the stationary phase and the proteins[3]. It is presumed that hydrophobic interactions partly contribute to the retention of blood cells on a PEG 20M–Sepharose column.

In this work, to confirm the contribution of hydrophobic interactions to the retention of blood cells, we prepared column packings in which PEG of several molecular weights (400, 4000, 6000 and 20M) was bonded with epoxy-activated Sepharose 6B. The affinity of the blood cells to the bonded PEG phase was investigated by using these four kinds of column packings, which differ from one another in hydrophobicity. In practice, however, because of the negative charge of the surface of the blood cells based predominantly on sialic acid, there is a risk that electrostatic interactions may occur between these cells and the bonded PEG phase in which inorganic electrolytes are distributed unequally relative to the mobile phase. Therefore, the surface negative charge of the intact erythrocytes was decreased by means of either self-digestion[4,5] or neuraminidase treatment[6], and the chromatographic behaviour was investigated. The decrease in the sialic acid content of the cells led to a decreased retention on the all PEG–Sepharose columns used.

It has been reported that cross-partition in aqueous two-phase systems[7] can be applied to the determination of the isoelectric point not only of soluble proteins but also of subcellular organelles[8,9]. We showed that this method can also be applied to determine the isoelectric point of human erythrocytes, platelets, granulocytes and lymphocytes. Further, on the basis of the retention behaviour of these blood cells at their isoelectric point pH values, it may be possible to characterize the hydrophobic interactions between the cells and the bonded PEG stationary phase.

EXPERIMENTAL

Materials

Epoxy-activated Sepharose 6B and dextran T40 and T500 (weight-average molecular weight M_w = 40,000 and 500,000, respectively) were obtained from Pharmacia (Uppsala, Sweden). Polyethylene glycol 400, 4000, 6000 and 20M (number-average molecular weight M_n = 400, 3500–4000, 6000–7500 and 15,000–20,000, respectively) (extra-pure grade) were purchased from Wako (Osaka, Japan). Neuraminidase from *Clostridium perfringens* and N-acetylneuraminic acid were obtained from Sigma (St. Louis, MO, U.S.A.). Other reagents were of analytical-reagent grade.

Instruments

An ISCO Model UA-5 absorbance monitor (Instrumentation Specialties, Lincoln, NE, U.S.A.) was used for detecting the absorbance of the eluates at 254 and 580 nm simultaneously. An LKB 2112 Varioperpex II or 2132 Microperpex peristaltic pump and a RediRac fraction collector (LKB, Bromma, Sweden) were employed for chromatographing blood cells and for fractionation of the eluates. A Coulter Counter Model D (Counter Electronics, Harpenden, U.K.) was used for counting the number of blood cells.

Preparation of PEG–Sepharose 6B column packings

PEG 400, 4000, 6000 and 20M were coupled with epoxy-activated Sepharose 6B at 40°C for 16 h in a solution of pH 12.0, as described previously[1,2].

It was necessary to determine the optimal concentration of perchloric acid for hydrolysis of the epoxy groups in the epoxy-activated Sepharose 6B. To determine the epoxy groups, the method involving a reaction between the oxirane ring and sodium thiosulphate[10] was used. Epoxy-activated Sepharose 6B was treated with perchloric acid of various concentrations for 1 h at room temperature with shaking. As shown in Fig. 1, the epoxy groups of the epoxy-activated Sepharose 6B were thoroughly hydrolysed with perchloric acid of concentration higher than 0.06 *M*. Consequently, four kinds of PEG–Sepharose 6B, the products of oxirane coupling, were treated with 0.1 *M* perchloric acid for 1 h at room temperature with shaking to hydrolyse the residual free epoxy groups.

The contents of PEG 400, 4000, 6000 and 20M coupled under the above conditions in the bonded phase were determined by spectrophotometry according to the procedure reported previously[1], and were 1129, 80.4, 54.6 and 17.4 μmol per gram of dry powder, respectively. In order to make uniform the content of bonded PEG in PEG 400–, 4000– and 6000–Sepharose 6B with that of 17.4 μmol of PEG 20M per gram of PEG 20M–Sepharose 6B, the three column packings were diluted with epoxy-hydrolysed Sepharose 6B in which the epoxy groups of epoxy-activated Sepharose 6B had been hydrolysed with 0.1 *M* perchloric acid for 1 h at room temperature.

Collection and isolation of blood cells

Human blood was drawn from the veins of a donor with the use of heparin as anticoagulant. Siliconized glassware was used in all procedures.

Erythrocytes. Blood was centrifuged at 500 *g* for 10 min and plasma and the buffy coat layer* were removed. The erythrocytes were washed three times with saline.

Platelets. A centrifugation isolation technique based on that of Leeksma and Cohen[11] was employed.

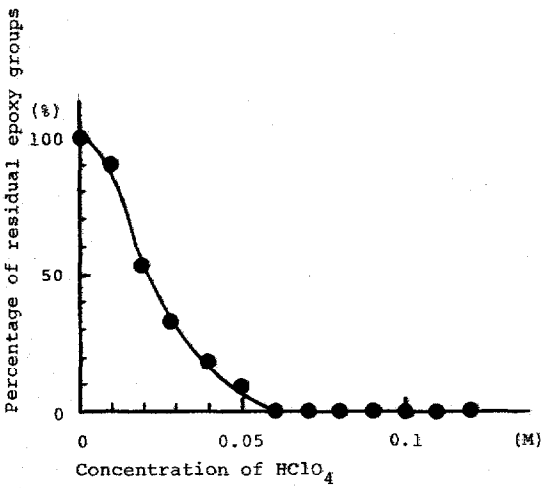

Fig. 1. Hydrolytic cleavage of epoxy groups of epoxy-activated Sepharose 6B as a function of concentration of perchloric acid.

* A yellowish white layer of leukocytes (granulocytes and lymphocytes) and platelets that, upon centrifugation of blood, covers the erythrocytes.

Granulocytes. The sodium metrizoate–dextran T500 sedimentation technique[12] was used.

Lymphocytes. The sodium metrizoate–Ficoll sedimentation technique[13] was used.

The above isolation procedures have been described in detail elsewhere[1,2].

Preparation of self-digested and neuraminidase-treated erythrocytes

Self-digestion of intact erythrocytes. A 4–6-ml volume of packed intact erythrocytes was suspended in 1 volume of Krebs–Ringer phosphate buffer (pH 7.4), placed in a dialysis bag and immersed in 200 volumes of the same solution. The suspension was incubated at 37°C for 25 h with shaking as described by Brovelli *et al.*[5]. After incubation the cell suspension was centrifuged at 1000 *g* for 5 min and the incubated erythrocytes were washed three times with saline. The sialic acid content in the incubation supernatant was determined by thiobarbituric acid assay[14].

Neuraminidase-treated erythrocytes. Intact erythrocytes were treated with *Cl. perfringens* neuraminidase as described Cook *et al.*[6]. One volume of packed erythrocytes (about 1 ml) was incubated at 37°C for 1 h with 1 volume of the neuraminidase aqueous solution (activity: 1500 units/ml in 0.145 *M* sodiumchloride–0.005 *M* calciumchloride buffered to pH 7.0 with 0.5 *M* sodium hydrogen carbonate.

Determination of isoelectric point of human peripheral blood cells

The partition phase systems used were as follows.

System A_1. 7.5% (w/w) of dextran T500, 7.5% (w/w) of PEG 4000, 90 mmol/kg of sodium phosphate buffer and 30 mmol/kg of sodium chloride.

System A_2. The same composition as in system A_1 but without the sodium chloride.

System B_1. 7.5% (w/w) of dextran T500, 7.5% (w/w) of PEG 4000, 10 mmol/kg of litium phosphate buffer and 120 mmol/kg of lithium chloride[8].

System B_2. The same composition as in system B_1 but with 60 mmol/kg of lithium sulphate instead of the lithium chloride[8].

System C_1. 6% (w/w) of dextran T500, 6% (w/w) of PEG 4000, 320 mmol/kg of sucrose, 5 mmol/kg of sodium citrate–phosphate buffer and 100 mmol/kg of sodium chloride[9].

System C_2. The same composition as in system C_1 but with 50 mmol/kg of sodium sulphate instead of the sodium chloride[9].

The cross-partition pH of the buffer for system A ranged from 5.0 to 7.5, and for systems B and C from 4.0 to 7.0. Each system was thoroughly mixed and portions were pipetted into each tube.

For erythrocytes, $1.3 \cdot 10^6$–$1.7 \cdot 10^6$ cells were added to systems A_1 and A_2. For platelets and granulocytes, $1.2 \cdot 10^5$–$3.3 \cdot 10^5$ platelets or $2.7 \cdot 10^5$–$6.5 \cdot 10^5$ granulocytes were added to systems B_1 and B_2. For lymphocytes, $1.2 \cdot 10^5$–$2.3 \cdot 10^5$ cells were added to systems C_1 and C_2.

The phase systems were thoroughly mixed by inverting the test-tubes, which were allowed to stand for 20 min. After phase separation, 0.1 ml of the upper phase was carefully pipetted from the systems in each tube and diluted with 10 ml of Isoton (aqueous electrolyte diluent for blood cells counting; Coulter Diagnostics, Hialeath, FL, U.S.A.). The number of cells in the upper phase was counted with a Coulter

Counter. The distribution of blood cells in the two-phase systems was expressed as the percentage of the number in the upper phase with respect to the total added the systems.

Chromatography of blood cells

Sodium phosphate buffer solutions (0.09 *M*) (pH 7.5 and $pH_{c.p.}$*) which contained 2% (w/w) of dextran T40 were used as the mobile phase. The column packings were suspended in the mobile phase and packed into the columns (25 × 0.9 cm I.D.) by the slurry-packing method. The columns were thoroughly washed with the mobile phase to equilibrate the bonded PEG phase by the use of a peristaltic pump. The total amount of erythrocytes, platelets, granulocytes and lymphocytes prepared as above was suspended in 0.3 ml of the mobile phase. A 0.2-ml volume of the cell suspension containing $2.5 \cdot 10^4$–$3.5 \cdot 10^4$ erythrocytes, $6.5 \cdot 10^5$–$20 \cdot 10^5$ platelets, $1.3 \cdot 10^5$–$2.0 \cdot 10^5$ granulocytes or $9.0 \cdot 10^4$–$14.5 \cdot 10^4$ lymphocytes was loaded on to the column, which was then eluted at a flow-rate of 6–12 ml/h, maintained by use of a peristaltic pump. The absorbance of the eluate at 254 and 580 nm was monitored continuously with a multi-wavelength effluent monitor. The fractions were collected in glass vials in volumes of 1.0–2.0 ml. An aliquot of each fraction was compared with the cells loaded onto the column.

RESULTS

Chromatography on PEG–Sepharose columns at pH 7.5

Effect of molecular weight of PEG as bonded phases on the retention. Each suspension of erythrocytes, platelets, granulocytes and lymphocytes from human peripheral blood was independently chromatographed on the four PEG–Sepharose columns. In our previous conventional method[1,2] for the preparation of oxirane-coupled PEG 20M–Sepharose 6B, the reaction of PEG with epoxy-activated Sepharose 6B was followed by treatment with 1 *M* 2-aminoethanol to block the residual free epoxy groups. The latter reaction resulted in hydroxyethylamino substituents on the PEG 20M–Sepharose 6B. As such charged secondary amino groups may cause considerable electrostatic interaction with the charged blood cell surface, in this work we used 0.1 *M* perchloric acid to cleave hydrolytically the residual epoxy groups of PEG–Sepharose, as described under Experimental. As mentioned under Experimental, the amount of PEG 20M bonded to Sepharose 6B (17.4 μmol of PEG 20M per gram of dry powder) was minimal among the four different molecular weights of PEG used. In order to equalize the total content in terms of molarity of PEG for all the PEG–Sepharose packings, the PEG–Sepharose obtained in each preparation was adjusted so as to contain 17.4 μmol of PEG per gram of dry powder by dilution with the epoxy-hydrolysed Sepharose 6B that contained no PEG.

Sodium phosphate buffer solution (0.09 *M*) (pH 7.5) containing 2% (w/w) of dextran T40 was used as the mobile phase. As we have shown previously[1,2], this mobile phase was the best for the separation of these blood cells and for its low viscosity. Table I shows the effects of the molecular weight of bonded PEG on the retention volume and the separation factor of human blood cells. All the retention

* $pH_{c.p.}$ = pH at the cross-point; see *Determination of pH at the cross-point of human bloodcells.*

TABLE I

RETENTION VOLUMES AND SEPARATION FACTORS OF HUMAN ERYTHROCYTES (e), PLATELETS (p), GRANULOCYTES (g) AND LYMPHOCYTES (l) AT pH 7.5 USING BONDED PEG OF FOUR MOLECULAR WEIGHTS

Bonded phase	*Retention volume (ml)**				*Separation factor*		
	e	*p*	*g*	*l*	*e/p*	*p/g*	*g/l*
PEG 400	4.7	5.6	9.4	10.3	1.19	1.68	1.10
PEG 4000	5.3	5.5	11.1	11.4	1.04	2.02	1.03
PEG 6000	5.3	6.4	11.3	12.7	1.20	1.77	1.12
PEG 20M	5.8	7.2	13.2	15.9	1.24	1.83	1.20

* Column: bisoxirane-coupled PEG–Sepharose 6B (25 × 0.9 cm I.D.). Mobile phase: 2% (w/w) dextran T40 containing 0.045 *M* NaH_2PO_4 and 0.045 *M* Na_2HPO_4 (pH 7.5).

volumes reported are averages of several measurements. It can be seen from Table I that the order of elution from the columns is erythrocytes, platelets, granulocytes and lymphocytes, and that for all the different cells the retention volume increases with increasing molecular weight of bonded PEG, within experimental error. Good separation factors between erythrocytes and platelets and between granulocytes and lymphocytes were obtained with the use of the PEG 20M–Sepharose column. The bonded PEG phase of highest molecular weight seems to give a better separation than the others.

Chromatography of self-digested erythrocytes. Electrostatic interactions may occur between the charged surface of blood cells and unequally distributed inorganic electrolytes on the bonded PEG phase relative to the mobile phase. In order to evaluate the electrostatic contribution to the retention in the chromatographic systems, erythrocytes for which 50% of the surface sialic acid had been released by self-digestion[5] were eluted. As shown in Table II, the retention volume of self-digested erythrocytes is small compared with those of intact cells on all four columns.

The chromatograms of intact and self-digested erythrocytes with the PEG 20M–Sepharose column are shown in Fig. 2. It is clear that the elution of self-digested

TABLE II

COMPARISON OF RETENTION VOLUMES OF HUMAN INTACT ERYTHROCYTES WITH THOSE OF SELF-DIGESTED ERYTHROCYTES AT pH 7.5 USING OF BONDED PEG OF FOUR MOLECULAR WEIGHTS

Bonded phase	*Retention volume (ml)**	
	Intact	*Self-digested*
PEG 400	4.7	3.5
PEG 4000	5.3	4.4
PEG 6000	5.3	4.5
PEG 20M	5.8	4.5

* Column and mobile phase as in Table I.

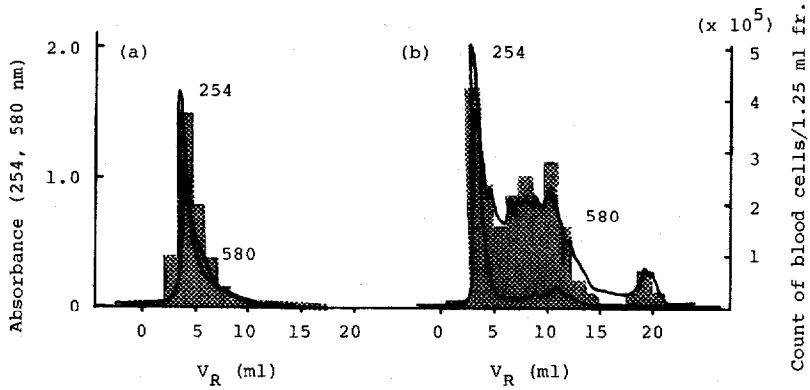

Fig. 2. Chromatograms of (a) intact and (b) self-digested erythrocytes. Column: bisoxirane-coupled PEG 20M–Sepharose 6B (25 × 0.9 cm I.D.). Mobile phase: 2% (w/w) dextran T40 containing 0.045 *M* NaH_2PO_4–0.045 *M* Na_2HPO_4 (pH 7.5). V_R = Retention volume.

erythrocytes is better than that of intact cells even though the chromatographic peak exhibits significant tailing, probably because of the heterogeneous distribution of negative charges on the surface of the cells. It appears that electrostatic interactions make a minor contribution to the retention of the blood cells.

As shown in Table II, relatively large changes in the retention of self-digested erythrocytes occur between phases of PEG of molecular weight 400 and 4000, but that the retention volume is nearly constant for PEG of molecular weight above 4000 or depends only slightly on the molecular weight of PEG. Using *Cl. perfringens* neuraminidase[6], we prepared an erythrocyte sample that further released sialic acid up to 70%. However, the retention volume of the neuraminidase-treated erythrocytes was not reproducible because of time-dependent aggregation after enzyme treatment.

Determination of pH at the cross-point of human blood cells

The partition of blood cells in two-phase systems with a given polymer composition [7.5% (w/w) dextran T500–7.5% (w/w) PEG 4000] is plotted against pH in Fig. 3. The two sets of curves in each instance correspond to two different alkali salt solutions of the two-phase systems.

Human platelets and granulocytes were only slightly partitioned in the PEG-rich upper phase of the dextran T500–PEG 4000 phase systems containing sodium phosphate (A_1 and A_2) because of their low surface charge compated with that of lymphocytes and erythrocytes. To obtain reasonable partition into two-phases, we used two sets of lithium chloride and lithium sulphate systems (B_1 and B_2, respectively) for platelets and granulocytes. For the same reason, systems containing chloride and sulphate buffered with sodium citrate–phosphate were chosen as appropriate two-phase systems (C_1 and C_2) for lymphocytes.

Sulphate, phosphate and citrate are known to increase the partition coefficient of negatively charged proteins in PEG phases[15], as a consequence of the interaction between dextran and these anions[16]. It has been also shown that the value of the cross-point determined from the intersection of two curves varies only slightly with different salts in the partition systems[17].

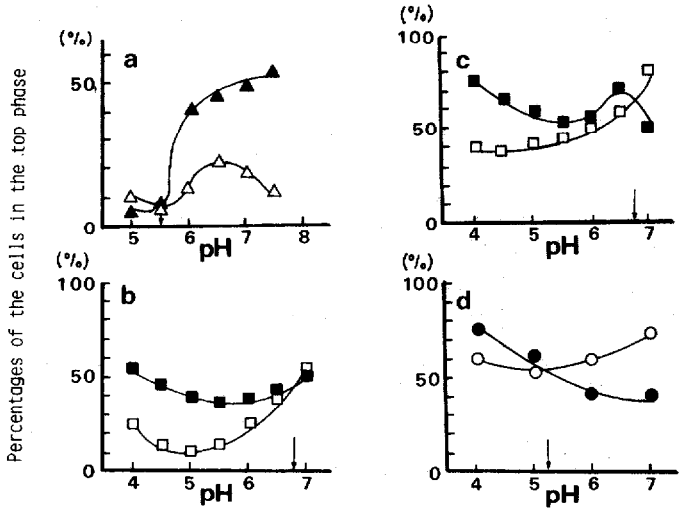

Fig. 3. Cross-partition curves of (a) human erythrocytes, (b) platelets, (c) granulocytes and (d) lymphocytes. Relative ratio as a percentage of the cells in the upper phase is plotted as a function of the pH of two phase systems. System A_1 and A_2 for erythrocytes containing NaCl (△) or nothing (▲). Systems B_1 and B_2 for platelets and granulocytes containing LiCl (□) or Li_2SO_4 (■). Systems C_1 and C_2 for lymphocytes containing NaCl (○) or Na_2SO_4 (●).

The partition curves of erythrocytes in the PEG-rich upper phase in systems A_1 and A_2 cross with each other at pH 5.5 (Fig. 3). In what follows, the symbol $pH_{c.p.}$ is used to express the pH value at this cross-point, *e.g.*, $pH_{c.p.} = 5.5$ for erythrocytes. In a similar manner, $pH_{c.p.} = 6.8$ for platelets and granulocytes and 5.2 for lymphocytes. It should be mentioned that the release of sialic acid from erythrocytes causes their $pH_{c.p.}$ to increase from 5.5 for the intact cells to 6.0 and 6.9 for self-digested and neuraminidase-treated erythrocytes, respectively.

Chromatography at $pH_{c.p.}$ for the blood cells

The retention volumes of the four different blood cells obtained using mobile

TABLE III

RETENTION VOLUMES OF HUMAN ERYTHROCYTES (e), PLATELETS (p), GRANULOCYTES (g) AND LYMPHOCYTES (l) AT $pH_{c.p.}$ USING BONDED PEG OF FOUR MOLECULAR WEIGHTS

$pH_{c.p.}$ values are as follows: 5.5 for erythrocytes, 6.8 for platelets and granulocytes and 5.2 for lymphocytes.

Bonded phase	*Retention volume (ml)**			
	e	*p*	*g*	*l*
PEG 400	4.5	5.5	8.4	9.6
PEG 4000	4.6	5.7	10.5	11.3
PEG 6000	4.6	6.0	11.1	11.4
PEG 20M	5.6	6.5	11.4	16.0

* Column and mobile phase as in Tables I and II.

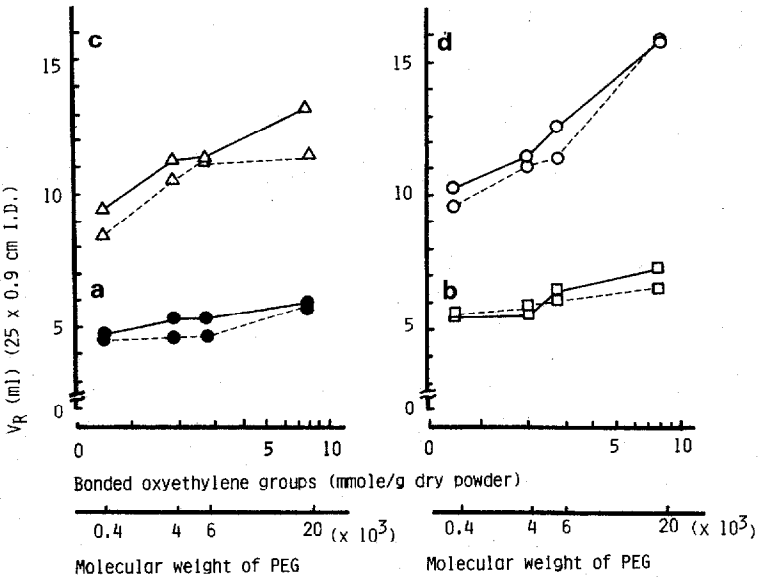

Fig. 4. Influence of pH of the mobile phase on the retention volumes of (a) human erythrocytes, (b) platelets, (c) granulocytes and (d) lymphocytes. pH of mobile phase: pH = 7.5 (●—●, □—□, △—△, ○—○); $pH_{c.p.}$ = 5.5 (●---●); $pH_{c.p.}$ = 6.8 (□---□, △---△); and $pH_{c.p.}$ = 5.2 (○---○).

phases buffered at each $pH_{c.p.}$ are shown in Table III. As the molecular weight of bonded PEG increases, the retention volumes of the blood cells increased, especially those of granulocytes and lymphocytes. Similar changes in retention volume were observed by using the mobile phase of pH 7.5 (see Table I).

The retention volumes of these four blood cells at pH 7.5 and $pH_{c.p.}$ are compared in Fig. 4. It can be seen that the retention volumes of erythrocytes, granulocytes and lymphocytes obtained at pH 7.5 are greater than those at $pH_{c.p.}$. There are a few exceptions for platelets with the PEG 4000–Sepharose and for lymphocytes with the PEG 20M–Sepharose column. When using PEG of higher molecular weight, such as PEG 6000 and 20M, as the bonded stationary phase, the retention volume of platelets obtained at pH 7.5 is higher than that obtained at $pH_{c.p.}$ 6.8. It seems that the retention volume of granulocytes shows a similar tendency when the molecular weight of bonded PEG is increased from 6000 to 20M. The retention volume of granulocytes increases with increasing molecular weight of bonded PEG from 400 to 6000 at $pH_{c.p.}$ 6.8, and that of lymphocytes also increases more markedly at $pH_{c.p.}$ 5.2. The above results suggest that the affinity between blood cells and bonded PEG phase at $pH_{c.p.}$ can be interpreted in terms of the hydrophobic interactions between them.

DISCUSSION

In previous work[2], the chromatographic behaviour of human blood cells was investigated by using PEG 20M–Sepharose 6B column and nineteen different mobile phases. It was shown that an increase in sodium chloride concentration in the mobile phase resulted in an increase in the retention volumes of granulocytes and lympho-

cytes. In the hydrophobic affinity chromatography of plasma proteins, viruses and even whole cells (baker's yeast)[18], these materials are eluted from column by decreasing the ionic strength of the mobile phase[18]. On the basis of these facts, it was assumed that the major affinity between blood cells and the bonded PEG 20M phase is hydrophobic interactions[2].

In this study, four column packings of PEG of different average molecular weight (400, 4000, 6000 and 20M) covalently bonded to Sepharose 6B were prepared and the effect of the molecular weight of PEG on the retention of human blood cells was investigated. In our conventional method[1,2], residual free epoxy groups of oxirane-coupled PEG 20M–Sepharose 6B were eliminated with the use of 2-aminoethanol after the coupling reaction. As a result, secondary amino groups were introduced into the PEG 20M–Sepharose 6B. In order to obviate the influence of this charged group on the retention, it is necessary to prepare non-charged column packings. Therefore, the non-reacted free epoxy groups in the PEG–Sepharose packings were hydrolytically cleaved. The content of PEG 400, 4000 and 6000 in the packings was also adjusted so as to be equal to that of PEG 20M in PEG 20M–Sepharose (17.4 μmol per gram of dry powder).

The retention volumes of the blood cells, except for erythrocytes, increase in order of increasing molecular weight of the bonded PEG phase. This implies that the enhancement of the hydrophobicity of these column packings based on an increase in the molecular weight of bonded PEG causes longer retentions for platelets, granulocytes and lymphocytes, especially the last two. Such an effect of molecular weight on retention is remarkable for lymphocytes with PEG of molecular weight between 400 to 4000, and also between 6000 and 20M (see Table I). Separation of granulocytes and lymphocytes is only possible by using a PEG 20M–Sepharose column. In general, the higher molecular weights of the bonded PEG phase tend to give a better separation than the use of lower molecular weights.

On the other hand, electrostatic interactions are also significant between the negative charge on the cells and the bonded PEG phase, because sodium and phosphate ions may be unequally distributed on the bonded PEG phase relative to the mobile phase. The surface negative charges on human erythrocytes are mainly due to the presence of an average of $2.4 \cdot 10^7$ molecules of sialic acid per cell such as sialoglycoprotein[19]. Human erythrocyte membranes are digested by their own sialoglycoproteins and release a glycopeptide containing sialic acid[5]. About 50% of sialic acid contained in the cell surface can be released by the self-digestion of intact cells[5]. The self-digested erythrocytes show a significant band broadening and tailing because of heterogeneity of the surface negative charges and of the dispersed cells, but the chromatographic peak was found to be somewhat faster than the intact erythrocytes from any of the columns used (Table II and Fig. 2). This result indicates that electrostatic interactions contribute to some extent to the retention of the cells in spite of the use of epoxy-hydrolysed column packings without charges. It is known that in an aqueous polymeric two-phase (APTP) system, some electrolytes, such as sodium phosphate, are distributed unequally and hence give rise to an electrical interfacial potential between the two aqueous polymeric phases[16,20,21]. Such an interaction in our case may be due to an electrical potential difference induced by unequal distribution of sodium and phosphate ions between the mobile and the bonded PEG phases[2]. In order to reduce electrostatic effects of the cells and to reveal hydrophobic

interactions, it is necessary to investigate the chromatographic behaviour at the isoelectric pH valves for the cells.

Albertsson *et al.*[7,22] investigated the correlation between partition coefficients in APTP systems and the isoelectric points of proteins. In general, the partition behaviour of proteins in APTP systems depends to a great extent on the ionic composition of the systems. The partition in an APTP system with a given composition varies with the salt dissolved and the pH of the phase. Albertsson[23] found a series of salts that raise (or lower) the partition coefficients, K, of negatively charged proteins. For example, the partition between two phases is greatly affected by replacing K^+ by Na^+ or Li^+. This phenomenon is due to unequal distribution of the salt ions between two liquid phases[16]. Once a potential difference between those phases has been produced, the partition coefficient has a strong dependence on the charge of the proteins[23].

From a set of the pH-dependent partition curves in two-phase systems containing two different salts, a defined cross-point can be obtained at which the partition coefficients of the material are equal for both systems[15]. It has been shown for proteins that the so-called cross-point has values close to their isoelectric points. Such a cross-partition has been used to determine the isoelectric point of rat hepatic mitochondria[9], of submitochondrial particles[8], etc., by the use of dextran–PEG systems buffered with sodium citrate–phosphate.

We applied such a cross-partition method to determine the cross-point values of human blood cells. With the use of a suitable combination of a pair of salts, the pH values of the cross-points were found to be 5.5, 6.8, 6.8 and 5.2 for erythrocytes, platelets, granulocytes and lymphocytes, respectively (see Fig. 3). When the pH of the mobile phase in chromatography is adjusted to one of the $pH_{c.p.}$ values for the four kinds of blood cells, the effect of the surface negative charge of the cells may be quenched. Thus, the contribution of the hydrophobic interactions between bonded PEG phase and the cell surface to the retention of the cells may be manifested, and the electrostatic interaction can be minimized. The retention volumes of lymphocytes and granulocytes increase with increasing molecular weight of bonded PEG (Fig. 4). In general, as the hydrophobicity of the column packings must be enhanced increase in the molecular weight of bonded PEG, the retention of lymphocytes and granulocytes on PEG–Sepharose columns is mainly governed by the hydrophobic interactions at their $pH_{c.p.}$ values.

As described in the previous section, the retention volumes of the four kinds of blood cells at pH 7.5 were, in general, slightly greater than those at respective $pH_{c.p.}$ values (Fig. 4). This implies that the electrostatic interaction makes a relatively small contribution to the retention of the cells, compared with hydrophobic interaction. In conclusion, it has been shown that a higher hydrophobicity of the column packings derived from bonded PEG of higher molecular weight gives longer retentions for human blood cells. From a practical point of view, the best separation of the four different blood cells was obtained with a PEG 20M–Sepharose column at pH 7.5 in our chromatographic system. The separation of erythrocytes and platelets, however, remained inadequate even with this bonded phase.

In the surface affinity chromatography studied here, the factors affecting the retention of blood cells are as follows. First, the hydrophobic interactions between the hydrophobic regions on the cell surface membrane and the bonded PEG phase

are probably the main factor affecting the retention of lymphocytes and granulocytes on these columns. Second, both the electrostratic and hydrophobic interactions are important factors at pH values other than $pH_{c.p.}$. The latter must be the case for erythrocytes, as self-digested erythrocytes facilitate the elution compared with the intact erythrocycles. The contribution of the electrostatic interactions to the retention of blood cells seems to be a further reason for the longer retentions on these columns at pH 7.5 than at $pH_{c.p.}$, with the exception of a few cases.

A study of the relationship between the relative hydrophobicity of the blood cell surface and retention volumes will be reported in a subsequent paper.

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