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SURFACE AFFINITY CHROMATOGRAPHIC SEPARATION OF BLOOD CELLS

II. INFLUENCE OF MOBILE PHASE COMPOSITION ON THE CHROMATOGRAPHIC BEHAVIOUR OF HUMAN PERIPHERAL BLOOD CELLS ON POLYETHYLENE GLYCOL-BONDED SEPHAROSE

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SUMMARY

The influence of mobile phase composition on the chromatographic behaviour of human platelets, granulocytes, lymphocytes and erythrocytes has been studied by using a bisoxirane-coupled polyethylene glycol 20M–Sepharose 6B column at pH 7.5. Lowering of the concentration of dextran T40 from 8 to 2% (w/w) produced the highest separation factor between platelets and granulocytes. Addition of 0.5% (w/w) of DEAE-dextran to a mobile phase containing 2% dextran T40 or T500 increased the retention of platelets, and discriminated the cells from erythrocytes. Addition of sodium chloride increased the retention volumes of lymphocytes, granulocytes and platelets. These blood cells were adsorbed to the column in isotonic phosphate-buffered eluent, whereas in imidazole-buffered eluent about 0.15–0.154 *M* sodium chloride improved their resolution.

INTRODUCTION

In the previous paper[1] we described the partition of blood cell particles between the bonded stationary phase and the mobile phase in a manner similar to aqueous polymeric two-phase (APTP) systems established by Albertsson[2] by means of binding of polyethylene glycol (PEG) to the support material in liquid chromatography. The chromatographic separation of an artificial mixture of erythrocytes, granulocytes and lymphocytes from human and rabbit peripheral blood was achieved by the use of bisoxirane-coupled PEG 20M–Sepharose 6B (PEG 20M–Sepharose) as the column packings and phosphate-buffered solution of 4.5 or 8.0% (w/w) dextran as the mobile phase. Bonded PEG stationary phases and mobile phases containing dextran systems offer an approach to the chromatographic separation of blood cell populations.

It is, therefore, necessary to determine the effects of variables on the behaviour of the blood cells in such chromatographic systems. The present paper describes the

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influence of the mobile phase composition on the elution behaviour of platelets, granulocytes and lymphocytes from human peripheral blood on a PEG 20M–Sepharose column. The variables studied include: concentrations of dextran T40 and T500; several anionic or cationic buffers; addition of a neutral salt, such as sodium chloride, and of a positively charged dextran, *e.g.*, DEAE-dextran.

EXPERIMENTAL

Materials

Epoxy-activated Sepharose 6B, dextran T40 (weight-average molecular weight $M_w = 40,000$), dextran T500 ($M_w = 500,000$) and diethylaminoethyldextran (DEAE-dextran, $M_w = 500,000$) were obtained from Pharmacia (Uppsala, Sweden). Polyethylene glycol, number-average molecular weight $M_n = 6000$–7500 and 15,000–20,000 was purchased as PEG 6000 and 20M, respectively (extra pure grade; Wako, Osaka, Japan). Other reagents were of analytical reagent grade.

Instruments

A Hitachi Model 034 liquid chromatograph, equipped with a Model 0037 multi-wavelength effluent monitor (Hitachi, Tokyo, Japan), was used. An LKB 2112 RediRac fraction collector (LKB, Bromma, Sweden) was employed for fractionation of eluates. A Coulter Model D counter (Coulter Electronics, Harpenden, Great Britain) was used for counting the number of blood cells.

Preparation of column packing

Bisoxirane-coupled PEG 20M–Sepharose 6B was prepared by coupling of epoxy-activated Sepharose 6B with PEG 20M for 16 h at 40°C in a solution of pH 12.0, as described previously[1]. The product was treated with 1 *M* 2-aminoethanol for the purpose of blocking residual free epoxy groups. The amount of PEG 20M coupled under the optimal conditions as the bonded stationary phase was determined by spectrophotometry[1] as 16 μmol per gram of dry powder.

Collection and isolation of blood cells

Human blood was drawn from normal male adult donors by venous puncture and heparin was added, 0.05 ml of a 1000 U/ml solution per 10 ml of the blood. Siliconized glassware was used in all procedures.

Erythrocytes. Blood was centrifuged at 500 *g* for 10 min, and the supernatant and buffy coat layer were removed. The cells were washed three times with saline and packed by centrifugation.

Granulocytes. The sodium metrizoate–dextran T500 sedimentation technique[3] was used. The granulocyte preparation contained a variable amount of contaminating erythrocytes.

Lymphocytes. The sodium metrizoate–Ficoll sedimentation technique[4] was used. The erythrocyte contamination of the lymphocyte preparation was usually between 1 and 5% of the total number of cells. The isolation procedures for both the above kinds of blood cells have been described in detail[1].

Platelets. A centrifugal isolation technique based on that of Leeksma and Cohen[5] was employed. A 10-ml volume of heparinized whole blood was mixed with

1 ml of 1% (w/v) disodium ethylenediaminetetraacetate in 0.9% (w/v) saline, and was centrifuged at 65 *g* for 20 min after filtration through an absorbent gauze. The supernatant (platelet-rich plasma, PRP) was removed with a siliconized pipette. Generally, not all of the PRP could be removed without disturbing the buffy coat. The PRP was centrifuged at 250 *g* for 20 min. Most of the leukocytes and erythrocytes (if present) were then collected on the bottom of the tube. On the top of these the platelets were already partly sedimented. These were resuspended, precaution being taken to prevent stirring of the bottom layer. The PRP was pipetted into another tube, centrifuged at 250 *g* for 20 min and the sedimented platelets were then washed thrice with 0.9% (w/v) saline to remove other contaminating cells.

Chromatography

Nineteen kinds of eluents were used, the compositions of which are shown in Tables I and II.

A jacketed glass column (25 × 0.9 cm I.D.) filled with bisoxirane-coupled PEG 20M–Sepharose 6B was used. The packing material was suspended in each of the eluents, and the column was filled with the slurried packing. The column was thoroughly washed with the eluent to equilibrate the chemically bonded phase, using a reciprocating or peristaltic pump.

The total amount of platelets, granulocytes, lymphocytes and erythrocytes prepared as above was suspended in 0.6 ml of the eluent used. A 0.5-ml volume of the cell suspension containing $6.6 \cdot 10^5$–$24.4 \cdot 10^5$ platelets, $13.7 \cdot 10^4$–$20 \cdot 10^4$ granulocytes, $9.5 \cdot 10^4$–$14.5 \cdot 10^4$ lymphocytes or $2.5 \cdot 10^4$–$3.4 \cdot 10^4$ erythrocytes was loaded in the column, and the column was eluted with each of the eluents. These operations were performed at 4°C, the temperature being maintained by circulation of cold water through the column jacket. A flow-rate of 3–12 ml/h was maintained by the use of a pump. The absorbance of the eluate at 230, 260 and 570 nm was monitored continuously with a multi-wavelength effluent monitor. The fractions were collected in glass vials every 10 min or 15 min, the volume of each fraction being about 0.75–2.0 ml. An aliquot of each fraction was diluted with 5 ml of Isoton (aqueous electrolyte diluent for blood cell counting; Coulter Diagnostics, Hialeah, FL, U.S.A.) and the number of blood cells was counted with a Coulter counter. The recovery of the eluted cells was calculated from the combined number in each fraction compared with the cells loaded on the column.

RESULTS

Influence of concentration and molecular weight of dextran in the mobile phase

Each suspension of erythrocytes, platelets, granulocytes and lymphocytes from human peripheral blood was chromatographed by the use of a chemically bonded PEG 20M–Sepharose column. Seven kinds of mobile phases (I–VII) containing either dextran T40 or T500 (except for eluent V) were used. These mobile phases were kept essentially isotonic with 0.09 *M* sodium phosphate buffer, pH 7.5.

Table I shows retention volumes and separation factors for erythrocytes, platelets, granulocytes and lymphocytes eluted independently from the PEG 20M–Sepharose column. All reported retention volumes of the blood cells are the means of triplicate or further determinations. It can readily be seen that erythrocytes, plate-

TABLE I

RETENTION VOLUMES AND SEPARATION FACTORS OF HUMAN ERYTHROCYTES (e), PLATELETS (p), GRANULOCYTES (g) AND LYMPHOCYTES (l)

Column: bisoxirane-coupled PEG 20M–Sepharose 6B (25 × 0.9 cm I.D.). Mobile phases I–X contained 0.045 *M* NaH_2PO_4 and 0.045 *M* Na_2HPO_4 (pH 7.5). All retention volumes are the means of triplicate or further determinations.

Mobile phase	*Concentration (% w/w)*			*Retention volume (ml)*				*Separation factor*		
	Dextran T40	*Dextran T500*	*DEAE-dextran*	*e*	*p*	*g*	*l*	*p/e*	*g/p*	*l/g*
I	8.0	—	—	3.8*	7.6	8.3*	22.0*	2.00	1.09	2.65*
II	4.5	—	—	5.4*	8.9	9.0*	21.0*	1.64	1.01	2.33*
III	2.0	—	—	5.8	7.4	13.2	16.2	1.28	1.78	1.23
IV	1.0	—	—	5.6	5.7	6.1	14.6	1.02	1.07	2.39
V	—	—	—	5.1	5.7	6.2	4.9	1.12	1.15	1.04(e/l)
VI	—	4.5	—	ads.*	11.6	12.1*	18.0*	—	1.04	1.49*
VII	—	2.0	—	ads.	8.3	10.6	12.5	—	1.28	1.18
VIII	4.5	—	0.5	8.7**	8.6	14.0	15.4	0.99	1.63	1.10
IX	2.0	—	0.5	5.9**	11.0	15.4	18.8	1.86	1.40	1.22
X	—	2.0	0.5	10.3**	10.3	12.6	14.8	1.00	1.22	1.17

* Data from ref. 1. ads.: adsorbed on the column.

** A proportion of the erythrocytes was adsorbed at the top end of the column.

lets, granulocytes and lymphocytes were eluted from the column in the order of their increasing retention volumes with every mobile phase used, except for eluent V in which dextran was absent. Of the various factors investigated, the concentration and the molecular weight of dextran in the eluent had a significant effect on the retention volume of these blood cells. As shown previously[1], the retention volumes of erythrocytes and granulocytes increased with decreasing concentration of dextran T40 [from 8.0 to 4.5% (w/w)]. In the present work, however, when the concentration of dextran T40 and T500 was decreased from 4.5 to 2.0% (w/w), the retention volumes of platelets, lymphocytes and granulocytes were decreased, the only exception being those of granulocytes with eluent III. Retention volumes of erythrocytes, platelets and granulocytes became very similar because of the reduced retention of the last two groups of blood cells with eluent IV containing 1.0% (w/w) of dextran T40, whereas lymphocytes were still appreciably retained with this eluent. Evidently, in the absence of dextran in the mobile phase (V), all these kinds of blood cells were only weakly retained on the stationary phase.

Of the seven kinds of mobile phases used, eluent III, containing 2.0% (w/w) of dextran T40, produced the best separation between platelets and granulocytes, whereas that of erythrocytes and platelets was not good because of the reduced retention of the latter on the stationary phase. On the other hand, the best resolution of granulocytes and lymphocytes was achieved by the use of eluent I. Increasing the concentration of dextran T40 or T500 from 2.0 to 4.5% (w/w) retarded the elution of these blood cells, and the separation factor between platelets and granulocytes was reduced to 1.01–1.04 because of the delayed elution of the former cells.

An increase in the molecular weight of dextran from T40 to T500 appreciably increased the retention of platelets and decreased those of granulocytes and lympho-

cytes. With eluents VI and VII erythrocytes aggregated and were adsorbed on the column. In general, of the seven mobile phases used, eluent II, containing 4.5% (w/w) of dextran T40, produced the best resolution for all these kinds of blood cells.

Effect of addition of DEAE-dextran to the mobile phase

In order to examine the influence of a polycationic polymer in the eluents, three kinds of mobile phases containing 0.5% (w/w) of DEAE-dextran in addition to either dextran T40 or T500 were also used. It has been pointed out in APTP systems that dextran–PEG systems can be modified by incorporation of DEAE-dextran to enhance separations based on the surface charge of mammalian erythrocytes of a number of species[6,7].

Table I shows that addition of 0.5% (w/w) of DEAE-dextran to a mobile phase containing 4.5% (w/w) dextran T40 (eluent VIII) decreased the retention volumes of all these kinds of blood cells and the separation factors were reduced as compared with those obtained with eluent II. On the other hand, the cationic polymer increased the retention of these cells in mobile phases containing 2.0% (w/w) of either dextran T40 (eluent IX) or T500 (eluent X) in comparison with those values obtained with eluents III or VII. In every case the separation factor between platelets and granulocytes was appreciably reduced because of the increased retention volume of the platelets. The usefulness of mobile phase systems containing DEAE-dextran was demonstrated by the fact that one can discriminate between erythrocytes and platelets due to the retarded elution of the latter when using the eluent IX.

Effect of addition of neutral salt to the mobile phase

The effect of neutral salts, such as sodium chloride, on the retention of blood cells in mobile phases buffered with either phosphate or organic amines was investigated. Table II summarizes the results of increasing the concentration of sodium chloride on the retention volumes of platelets, granulocytes and lymphocytes. An

TABLE II

RETENTION VOLUMES AND SEPARATION FACTORS OF HUMAN PLATELETS (p), GRANULOCYTES (g) AND LYMPHOCYTES (l) USING MOBILE PHASES CONTAINING PHOSPHATE OR CATIONIC BUFFER (pH 7.5) IN THE PRESENCE OF SODIUM CHLORIDE

Column as in Table I. Mobile phases XI–XIX contained 2.0% (w/w) of dextran T40.

Mobile phase	*Concentration (M)*					*Retention volume (ml)*			*Separation factor*	
	NaCl	*NaH_2PO_4*	*Na_2HPO_4*	*Imidazole*	*Tris*	*p*	*g*	*l*	*g/p*	*l/g*
XI	0.07	0.03	0.03	—	—	5.8	8.6	12.6(ads.)	1.48	1.47
XII	0.11	0.02	0.02	—	—	5.5	10.4(31.4)	12.1(ads.)	1.89	1.16
XIII	0.13	0.01	0.01	—	—	5.3	17.2(ads.)	21.9(ads.)	3.25	1.27
XIV	0.12	—	—	0.465	—	3.6	3.6	3.6	—	—
XV	0.13	—	—	0.465	—	4.2	5.3	5.3	1.26	—
XVI	0.14	—	—	0.465	—	4.2	5.3	12.0	1.26	2.26
XVII	0.15	—	—	0.465	—	4.2	8.7	12.4	2.07	1.43
XVIII	0.154	—	—	0.465	—	5.3	9.8	13.1	1.85	1.34
XIX	0.03	—	—	—	0.07	4.5	9.5	15.8	2.11	1.66

increase in sodium chloride concentration with a concomitant decrease in phosphate concentration to keep the overall salt concentration essentially isotonic caused a slight decrease in the retention volume of platelets. On the other hand, the retention volumes of granulocytes and lymphocytes decreased and a proportion of these cells were adsorbed on the column when eluent XI containing 0.07 *M* of sodium chloride was used. In particular, the affinity of granulocytes for the stationary phase was significantly increased by the use of eluents XII and XIII, and lymphocytes were also adsorbed with eluent XIII. Erythrocytes were entirely adsorbed on the column with eluents XII and XIII.

From the effect of DEAE-dextran on retention of blood cells described above, it is considered that positively charged organic amines are distributed in the bonded PEG stationary phase rather than in the mobile phase, in contrast to the anionic phosphate. In order to examine the influence of buffer ions and of the concentration of sodium chloride, three kinds of blood cells were chromatographed by using mobile phases containing 2.0% (w/w) of dextran T40 and several organic amines instead of phosphate. Table II shows the retention volumes of these blood cells with eluents XIV–XVIII which comprised 0.465 *M* imidazole–hydrochloric acid buffer solution, pH 7.5, and 0.12–0.145 *M* sodium chloride. In these eluents, from hypotonic to hypertonic concentrations there was very little lysis of human erythrocytes. As shown in Table II, a marked change of the retention was observed for granulocytes and lymphocytes. Retention volumes of the three kinds of cells became the same with eluent XIV containing 0.12 *M* of sodium chloride. These values were still approximately the same for platelets and granulocytes in 0.13 and 0.14 *M* sodium chloride. The retention of lymphocytes was enhanced in 0.15 *M* sodium chloride, and the most remarkable distinction of these cells was attained with 0.154 *M* sodium chloride. Lysis of erythrocytes due to the hypertonicity of the eluent was observed above this concentration.

By use of isotonic 0.07 *M* Tris–hydrochloric acid–0.03 *M* sodium chloride buffer, pH 7.5, containing 2% (w/w) dextran T40 (eluent XIX), the retention volumes of these cells were differentiated; however, their recoveries from the column were very poor. Four kinds of Good's buffer materials, N,N-bis(2-hydroxyethyl)glycine (Bicine), N-2-hydroxyethylpiperazine-N'-2-ethanesulphonic acid (HEPES), N-tris-(hydroxymethyl)methyl-2-aminoethanesulphonic acid (TES) and 2-(N-morpholino)-ethanesulphonic acid (MES), in concentrations of 0.01–0.1 *M* resulted in irreversible adsorption on the column and/or poor recoveries of these cells in the presence of isotonic 0.03–0.09 *M* sodium chloride.

DISCUSSION

In the previous paper[1], mobile phases containing either 8.0 or 4.5% (w/w) of dextran T40 or T500 and 0.09 *M* sodium phosphate buffer, pH 7.5, were used for the sub-fractionation of human and rabbit peripheral blood cells, except platelets. These concentrations of dextran were chosen according to the concentrations used in the APTP systems. It is convenient to imagine that charge-associated properties of the membrane surface are a major determinant of the partition of blood cells between the bonded PEG stationary phase and these mobile phases, since there is an electrostatic potential difference between the two phases.

It was shown in the present work that erythrocytes were eluted first, followed by platelets, granulocytes and lymphocytes, with every eluent containing dextran. It was also found the retention of lymphocytes and platelets to the stationary phase requires the presence of dextran in the mobile phase. As is seen from Table I, it was very difficult to resolve platelets and erythrocytes by means of this chromatographic system using eluents containing $<2.0\%$ (w/w) dextran T40. In APTP systems, Walter[8,9] partitioned platelets obtained from a number of different species in a system containing 5.5% (w/w) dextran–4% (w/w) PEG and 0.11 *M* sodium phosphate buffer, pH 6.8, and found that the partitions of platelets and erythrocytes from the same species are remarkably similar. This indicates that platelets and erythrocytes in a given species have similar surface properties, at least with respect to those characteristics which determine partition.

Lowering of the concentration of dextran T40 in the eluents from the 8% (w/w) used in the previous work to 2% (w/w) results in several advantages such as an improvement of the separation factor between platelets and granulocytes and an increase in flow-rate based on the decrease in the viscosity of the eluent.

In APTP systems, it has long been known[10], but not further explored, that the partition coefficient of cells increases when the polymer concentration is reduced in the presence of a constant salt composition and concentration. Walter *et al.*[11] pointed out that since the electrostatic potential difference between the phases diminishes with reduction of polymer concentration, the species-specific increase in partition coefficients of mammalian erythrocytes must be due to membrane surface properties other than charge. Furthermore, since the potential difference between the two aqueous polymeric phases caused by unequal partition of salts, notably phosphate, diminishes as the critical point is approached, it follows that the increase in partition of sells in phases close to the critical point must be related to something other than membrane surface charge. It is most likely that the reduction in interfacial tension that accompanies reduction in polymer concentration[12] results in less cell adsorption at the interface. The extent of the interaction of the cell surface with polymer determines the cell partition.

It has been also pointed out[6,7] in APTP systems that the partition of rabbit erythrocytes of low surface charge in a dextran–PEG phase system which also contains DEAE-dextran is dependent on the partition of DEAE-dextran itself. DEAE-dextran is distributed unevenly between the two aqueous polymeric phases[13]. In 5% (w/w) dextran T500 and 4% (w/w) PEG 6000 containing 0.11 *M* sodium phosphate, pH 6.8, *ca.* 90% of DEAE-dextran was found in the dextran-rich lower phase, which is negatively charged relative to the the upper phase[13]. The binding of DEAE-dextran to the anionic surface of blood cells causes the latter to behave as more positively charged particles.

Based on these results, in the phosphate-buffered mobile phase, it might be expected that the binding of DEAE-dextran would lead to a shift of the blood cells from the bonded PEG stationary phase to the mobile phase. By the use of a phosphate-buffered dextran mobile phase containing 0.5% (w/w) of DEAE-dextran (eluents VIII, IX and X), erythrocytes, and in particular platelets, were retained to the stationary phase with eluents VIII and X (Table I). On the other hand, erythrocytes were pulled into the mobile phase with eluent IX. The retention volumes of platelets, granulocytes and lymphocytes were slightly increased, however, with eluent IX compared to the

values obtained with eluent III. It is considered that this increase in retention may be due to an affinity of discharged cells binding with the cationic polymer for the stationary phase. The affinity is enhanced by increasing the concentration of dextran T40 from 2.0 to 4.5% (w/w). It must be noted that mobile phases containing DEAE-dextran are not ideal. As has been reported by Marikovsky *et al.*[14], the presence of charged polyelectrolytes has a tendency to cause agglutination of erythrocytes. In some of the mobile phases containing DEAE-dextran, after standing for a long time, the erythrocytes were aggregated.

It is known in APTP systems that some salts, such as phosphate, sulphate and citrate, are distributed unevenly, giving rise to an electrical and a zeta potential between the two aqueous polymeric phases[13,15,16]. This phase charge interacts with membrane surface-charge associated properties of cells added to the phases[17,18]. Thus the partition of suspended materials is dependent, although not exclusively, on charge. In addition, it has been pointed out that if sodium chloride is substituted for the phase close to the critical point, there is no potential difference between the phases[13] since this salt partitions almost equally[15]. Erythrocytes from most sources collect at the interface in such a system. Cells that do partition in such a phase system must do so through surface properties not related to their membrane charge.

In the present work, the interaction of blood cells with chemically bonded PEG stationary phase is apparently selective, as lymphocytes and granulocytes are more strongly retained on the column than platelets. The extent of retention of these cells is significantly affected by incorporation of sodium chloride in the eluent buffer. As shown in Table II, the retention behaviour of the blood cells gives an insight into the rôle played by the surface properties of the cells in this chromatographic system. Elution with mobile phases which are assumed to have zero potential difference with the bonded PEG stationary phase results in increased retention volume and adsorption of granulocytes. The retention of lymphocytes is increased in the presence of 0.13 *M* sodium chloride and these cells are adsorbed on the column with eluents XI–XIII. The chromatographic behaviour of platelets is little affected by the addition of sodium chloride. It is therefore considered that the retention of these blood cells when using a mobile phase containing sodium chloride and phosphate must depend on properties other than the membrane surface charge, since the mobile phase and the stationary phase provide no remarkable electrostatic potential difference with which the membrane charge could interact. The retention volumes of lymphocytes and granulocytes are also increased in the presence of 0.14–0.154 *M* sodium chloride in the mobile phase buffered with imidazole (eluents XVI–XVIII). It is assumed that these cells may be retained on the stationary phase by an interaction which is enhanced in sodium chloride concentrations above 0.14 *M*.

The effect of addition of sodium chloride to the mobile phase shows that the charge on the cell surface is one determinant of the retention of blood cells on the column. Ionic interactions may play a rôle in the retention of erythrocytes on the bonded PEG stationary phase; however, it is possible that the affinity of lymphocytes and granulocytes is considerably dependent on the interaction of the cell surface with the stationary phase. The contribution of hydrophobic interactions should also be taken into consideration. A study of the surface interaction of blood cells will be presented in a subsequent paper.

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