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SURFACE AFFINITY CHROMATOGRAPHIC SEPARATION OF BLOOD CELLS

I. SEPARATION OF HUMAN AND RABBIT PERIPHERAL GRANULOCYTES, LYMPHOCYTES AND ERYTHROCYTES USING POLYETHYLENE GLYCOL-BONDED COLUMN PACKINGS

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SUMMARY

Two different types of column packings with bonded polyethylene glycol (PEG) as the stationary phase, PEG 20M-bonded Porasil AX and bisoxirane-coupled PEG 20M–Sepharose 6B, were prepared for chromatographing human and rabbit peripheral blood cells. The best separation, especially of granulocytes and lymphocytes (separation factor 2.65), was obtained with the latter packing and a phosphate-buffered solution (pH 7.5) of 8% (w/w) dextran as the mobile phase.

The selectivity of the column for blood cells depended on the concentration and molecular weight of dextran and on the ionic composition of the electrolytes in the mobile phase. The recoveries of human granulocytes, lymphocytes and erythrocytes by this chromatographic system were about 67, 82 and 50%, respectively.

INTRODUCTION

Improvements in fractionation methods used in the investigation of cells have long been required. In column chromatographic methods, which seem to have potential for cell fractionation, many practical difficulties are experienced, such as the adsorption of a large number of cells on the column. The subsequent separation of blood cells according to their adherence characteristics is exemplified by the technique of adherence chromatography, which uses a column of siliconized glass beads about 200 μm in diameter[1]. The glass bead column does not separate human lymphocytes from erythrocytes. The recovery from the column is about 60–70% for small lymphocytes and 35–45% for polymorphonuclear leukocytes[1]. Several reports have described the chromatographic behaviour of certain types of mammalian cells on columns filled with agarose beads[2], poly(methyl methacrylate) beads[3], cross-linked dextran beads[3,4], etc. However, only a few papers have described the adherence of cells to packing materials[3,5].

On the other hand, the use of immiscible aqueous two-phase systems for the fractionation of cell particles and biological macromolecules was established by Albertsson[6]. Of various aqueous polymeric two-phase (APTP) systems, partition in aqueous dextran–polyethylene glycol (PEG) two-phase systems is useful in many instances, these containing a PEG-rich top and a dextran-rich bottom phase.

The manual operation of a counter-current device is tedious; separation with immiscible aqueous systems is particularly slow as the solutions used are viscous. Such systems can be rendered isotonic by incorporation of inorganic salts and are suitable for partition and separation by a counter-current distribution apparatus of a special design for the partitioning of cell particles and cell organelles.

It is clear that partition in APTP systems could be carried out in a column if one of the polymer phases were held immobile. By binding one of the polymers to the support material in liquid chromatography, it should be possible to achieve partition of cell particles between the bonded stationary phase and the mobile phase in a manner similar to the APTP systems. In this work, we have attempted to prepare column packing materials with chemically bonded PEG as the stationary phase. By the use of such a packing, fractionation of peripheral blood cells from human and rabbit could be carried out by means of column chromatography, based on the partition between chemically bonded PEG and dextran constituting the aqueous mobile phase.

EXPERIMENTAL

Materials

Porasil AX (particle size 75–125 μm) and Corasil II (particle size 37–75 μm) (Waters Assoc., Milford, Mass., U.S.A.), epoxy-activated Sepharose 6B, dextran T40 (weight-average molecular weight $M_w = 40{,}000$) and dextran T500 ($M_w = 500{,}000$) (Pharmacia, Uppsala, Sweden) were commercial products. Polyethylene glycol, number-average molecular weight $M_n = 6000$-7500 and 15,000-20,000, was purchased as PEG 6000 and 20M, respectively. 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 for chromatography. An LKB 2112 RediRac fraction collector (LKB, Bromma, Sweden) was used 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 PEG-bonded column packings

PEG-bonded silica beads. Porasil AX and Corasil II were used as supports for the stationary phases. Both types of silica bead were dried for 12 h at 120° before use. Replacement of the surface hydroxyl groups with chloro groups was carried out with titanium tetrachloride or silicon tetrachloride, according to the procedure of Locke *et al.*[7]. The chlorinated silica beads were dried over phosphorus pentoxide under reduced pressure at room temperature. A mixture of 15 g of melted PEG 6000 or 20M and 0.3 g of metallic sodium was heated for 2–3 h at 140° until the sodium had dissolved. To this melt, 2.5 g of chlorinated silica beads were added and the mixture was heated for about 15 h at 180° until the aqueous solution of an aliquot of this reaction mixture did not show a red colouration with phenolphthalein. The product

was washed repeatedly with tetrahydrofuran until the washings no longer had the blue colour due to peroxochromic acid [oxodiperoxochromium(VI)] and PEG[8] *. Either PEG 6000- or 20M-bonded Corasil II and/or Porasil AX was dried under vacuum at 130° for 48 h, and the carbon and hydrogen contents were determined by elemental analysis. The X-ray diffraction patterns showed characteristic diffractions due to PEG at $\theta = 25$, 37.7 and 47.8.

Bisoxirane-coupled PEG-Sepharose 6B. One gram of epoxy-activated Sepharose 6B was washed on a sintered-glass filter funnel (G3) with *ca.* 100 ml of water and then mixed with PEG solution. The solution was prepared by dissolving 10 g of either PEG 6000 or 20M in 20 ml of a solution adjusted to a pre-determined pH of 9.0, 10.0 or 11.0 with sodium hydrogen carbonate–sodium carbonate buffer, and pH 12.0 and 13.0 with sodium hydroxide solution. The reaction mixture was shaken for 16 h at 32.5°, 40° and 45° in a thermostat. The coupled product was washed on a sintered-glass filter funnel successively with water, 0.1 *M* sodium tetraborate–boric acid–0.5 *M* sodium chloride buffer (pH 8.0), and 0.1 *M* sodium acetate–acetic acid–0.5 *M* sodium chloride buffer (pH 4.0) until PEG was no longer detected in the filtrate[8]. The product, suspended in 25 ml of 1 *M* 2-aminoethanol, was shaken for 12 h at room temperature, thoroughly washed with water and dried under vacuum at 105° for 48 h. The amount of PEG bonded to epoxy-activated Sepharose 6B was determined by spectrophotometry[9] from the amount of unchanged PEG in the combined solution of the filtrate of the reaction mixture and the washings.

Collection of blood cells

Human blood was drawn from normal male adult donors by venepuncture and heparin (Shimizu Seiyaku, Shizuoka, Japan) added (0.05 ml of a 1000 U/ml solution per 10 ml of the blood). Rabbit defibrinated blood was purchased from Nippon Seibutsu Zairyo Centre, Tokyo, Japan.

Isolation of blood cells. Siliconized glassware was used in all procedures.

For 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.

For granulocytes, sodium metrizoate–dextran sedimentation technique of Bøyum[10] was used. A mixture of 10 ml of blood and 2 ml of 0.9% physiological saline was carefully layered on 10 ml of a mixed solution of 32.8% (w/w) sodium metrizoate (N-methyl-3,5-diacetamido-2,4,6-triiodobenzoate)–6% (w/w) dextran T500 (1:2). The system was allowed to stand for 1–2 h until the sedimentation of erythrocytes was complete. The supernatant plasma was pipetted off. The supernatant was centrifuged at 130 *g* for 10 min and the precipitated cells were then washed three times with 5-ml volumes of saline and centrifuged. The granulocyte preparation contained a variable amount of contaminating erythrocytes.

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* Polyethylene glycols are high-molecular-weight ethers and are able to stabilize CrO_5 [oxodiperoxochromium(VI)] through addition. This stabilization permits the detection of polyethylene glycols. *Procedure.* To one drop of polyethylene glycol solution, one drop of 5% K_2CrO_4, followed by a drop of 4% H_2SO_4 containing 3% H_2O_2 is added. A blank test without polyethylene glycol is also carried out. In the blank the blue colour initially obtained changes to green within a few minutes. The possitive response is indicated through the persistence of the blue colour during 15–20 min.

For lymphocytes, a sodium metrizoate–Ficoll sedimentation technique based on that of Thorsby and Bratlie[11] was used. A 5-ml volume of blood was defibrinated by rotating the test-tube vertically for 10 min with five glass spheres of 5 mm diameter, and 5 ml of the defibrinated blood were mixed with an equal volume of saline in a centrifuge tube and carefully layered on 7.5 ml of Lymphoprep [a mixture of 9.6% (w/v) sodium metrizoate and 5.6% (w/v) Ficoll; Nyegaard, Oslo, Norway]. The tube was centrifuged at exactly 400 *g* for 30–40 min. The lymphocyte layer was separated and the cells were diluted with about 2 ml of saline and washed and centrifuged at 500 *g* for 10 min. The precipitated cells were washed and centrifuged three times with 5-ml volumes of saline. The contamination in the lymphocyte suspension of erythrocytes was usually between 1 and 5% of the total number of cells.

Chromatography

Four eluents were used, as follows: 0.03 *M* sodium dihydrogen orthophosphate–0.03 *M* disodium hydrogen orthophosphate buffer (pH 7.5) containing 4.5% (w/w) of dextran T500 and 0.1 *M* sodium chloride (eluent I); 0.045 *M* sodium dihydrogen orthophosphate–0.045 *M* disodium hydrogen orthophosphate buffer (pH 7.5) containing 4.5% (w/w) of either dextran T500 (eluent II) or T40 (eluent III); and 0.045 *M* sodium dihydrogen orthophosphate–0.045 *M* disodium hydrogen orthophosphate buffer (pH 7.5) containing 8.0% (w/w) of dextran T40 (eluent IV).

A jacketed glass column (10.5 × 0.9 cm I.D.), filled with PEG 20M-bonded Porasil AX, and a column (25 × 0.9 cm I.D.) filled with bisoxirane-coupled PEG 20M–Sepharose 6B were used. The packing materials were suspended in each of the above eluents, and the columns were filled with the slurried packings. The columns were thoroughly washed with the eluent to equilibrate the chemically bonded phases by the use of a reciprocating or a peristaltic pump.

The total amount of granulocytes, lymphocytes and erythrocytes prepared as above was suspended in 0.6 ml of the eluent used, 0.5 ml of the cell suspension containing $13.9 \cdot 10^4$–$19.8 \cdot 10^4$ of granulocytes, $9.9 \cdot 10^4$–$15.8 \cdot 10^4$ of lymphocytes or $2.4 \cdot 10^6$–$3.2 \cdot 10^6$ of erythrocytes was loaded in the column, and the column was eluted with each of the eluents. These operations were performed at 4° by circulation of cold water through the column jacket. A flow-rate of 1.5–12 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 test-tubes every 15 min (for PEG 20M-bonded Porasil AX) or 30 min (for bisoxirane-coupled PEG 20M-Sepharose 6B); in the former instance the volume was about 2 ml and in the latter about 1 ml. An aliquot of each fraction was diluted with 5 ml of Isoton (aqueous electrolyte diluent for blood cell counting; Coulter Diagnostics, Hialeah, Fla., U.S.A.) and the number of granulocytes, lymphocytes and erythrocytes was counted with a Coulter counter. The recovery of the eluted cells was calculated from the combined number in each fraction against the cells loaded on the column.

RESULTS

PEG bonded-phase column packings

Chlorination of the surface silanol hydroxyl groups of the silica support material Porasil C was investigated by Locke *et al.*[7]. In this study, chlorination of the surface silanol hydroxyl groups according to the method of Locke *et al.*[7] was compared

for the two types of spherical siliceous supports: deactivated Porasil AX, with totally porous silica particles, and Corasil II, with spherical glass particles that have a double layer of porous silica. Chlorination of silanol hydroxyl groups was carried out with both titanium tetrachloride and silicon tetrachloride in *n*-pentane as the solvent. After pyrolytic decomposition of the reaction products with sodamide, the chlorine content of the products was determined by argentimetric titration. As shown in Table I, the chlorine content of chlorinated Porasil AX was higher than that of Corasil II with both chlorination reagents. When these chlorinated siliceous supports were allowed to react with PEG 6000 at 180° for 15 h, the carbon content of the PEG-bonded Porasil AX was also higher than that of Corasil II.

TABLE I

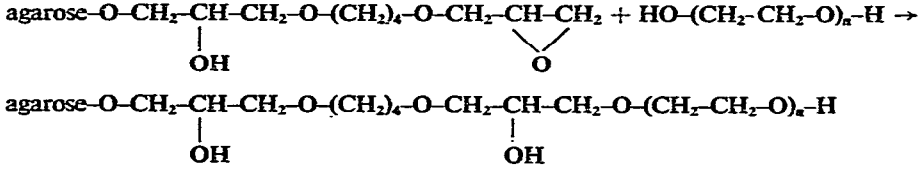
CHLORINE AND CARBON CONTENTS OF CHLORINATED AND PEG-BONDED SPHERICAL SILICA BEADS

Samples: silica beads chlorinated with (A) $TiCl_4$ or (B) $SiCl_4$; PEG-bonded silica beads with (C) PEG 6000 or (D) PEG 20M.

Starting material	*Chlorine content (mequiv. per 100 mg)*		*Carbon content (%)*	
	Sample A	*Sample B*	*Sample C*	*Sample D*
Porasil AX	26.89	12.86	3.17	3.59
Corasil II	0.91	0.61	0.61	

On the basis of this result, Porasil AX was condensed with PEG of two different molecular weights, PEG 6000 and 20M, at 180° for 15 h. The carbon contents are shown in Table I for the products from two types of silica beads: PEG 6000 or 20M bonded on porous beads (Porasil AX), and PEG 6000 bonded on solid core beads (Corasil II). These results also demonstrate the greater capacity of totally porous beads for binding with PEG. Because the carbon content of the former was five times greater than that of the latter, PEG 20M-bonded Porasil AX (PEG-bonded Porasil) was used as the column packing for subsequent liquid chromatography of blood cells.

As a soft gel type of packing material, PEG 20M was coupled to bead-shaped agarose through the mediation of bisoxirane [1,4-bis(2,3-epoxypropoxy)butane]. A terminal hydroxyl group in PEG 20M was directly coupled to an active oxirane group of epoxy-activated Sepharose 6B, and a stable hydrophilic uncharged ether linkage was formed between PEG and Sepharose 6B. The reaction is as follows:



As the coupling conditions depend greatly on the properties of the ligand and the functional group to be coupled[12], suitable conditions for the oxirane coupling of PEG 20M with epoxy-activated Sepharose 6B were investigated at 32.5°, 40.0° and 45.0° for 16-h periods in aqueous solution adjusted to pH 9.0, 10.0, 11.0, 12.0 or 13.0. The amount of PEG 20M coupled was determined from the recovered amount of unchanged PEG in the reaction mixture and washings of the products. As shown in

TABLE II

RETENTION VOLUMES AND SEPARATION FACTORS OF GRANULOCYTES, LYMPHOCYTES AND ERYTHROCYTES

Column: PEG 20M-bonded Porasil AX (10.5 × 0.9 cm I.D.)

Blood cells	*Mobile phase**							
	I		*II*		*III*		*IV*	
	V_R *(ml)*	*Separation factor*	V_R *(ml)*	*Separation factor*	V_R *(ml)*	*Separation factor*	V_R *(ml)*	*Separation factor*
Human:								
Erythrocytes	18.0	1.50	42.0	3.50	14.0	(1.56)**	14.0	1.33
Granulocytes	12.0	1.20	14.0	1.17	7.0	1.29	10.5	1.67
Lymphocytes	10.0		12.0		9.0		17.5	
Rabbit:								
Erythrocytes			44.0	(3.57)**	33.0	5.50	14.3	(1.14)**
Granulocytes	14.3	1.05	10.5	1.17	6.0	1.00	12.3	1.04
Lymphocytes	15.0		12.3		6.0		12.5	

* Mobile phase I contained 4.5% (w/w) of dextran T500, 0.03 *M* NaH_2PO_4, 0.03 *M* Na_2HPO_4 and 0.1 *M* NaCl (pH 7.5); mobile phase II contained 4.5% (w/w) of dextran T500, 0.045 *M* NaH_2PO_4 and 0.045 *M* Na_2HPO_4 (pH 7.5); mobile phase III contained 4.5% (w/w) of dextran T40, 0.045 *M* NaH_2PO_4 and 0.045 *M* Na_2HPO_4 (pH 7.5); mobile phase IV contained 8.0% (w/w) of dextran T40, 0.045 *M* NaH_2PO_4 and 0.045 *M* Na_2HPO_4 (pH 7.5).

** Separation factor against lymphocytes.

Fig. 1, the optimum coupling condition for PEG 20M was a 16-h period at 40.0° in solution of pH 12.0. The content of PEG coupled under these optimal condition as the bonded stationary phase was 15.7 μmol per gram of dry powder (314 mg per gram of dry powder). In addition, the amount of PEG 20M bound was also expressed by means of the carbon and nitrogen content of the products, the latter obtained from the hydroxyethylamino substituent which was introduced by treatment with 1 *M* 2-aminoethanol after the coupling, for the purpose of blocking residual free epoxy groups. The analysis of the dried product prepared under optimal conditions was C 47.09, H 6.64 and N 0.30%. The carbon content was the highest and the nitrogen content the lowest compared with the contents in the products prepared according to the bisoxirane coupling conditions shown in Fig. 1. On the other hand, epoxy-activated Sepharose 6B hydrolysed under optimal coupling conditions gave values of C 45.08, H 6.69 and N 0.46% as a control.

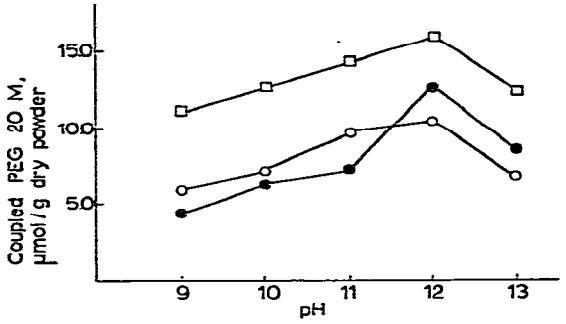


Fig. 1. Dependence of coupling of PEG 20M to epoxy-activated Sepharose 6B on pH and temperature. Carbonate–hydrogen carbonate buffers were used in the pH range 9–11 and sodium hydroxide solution in the range pH 12–13. PEG 20M concentration, 500 mg/ml. Temperature: ○, 45°; □, 40°; ●, 32.5°.

Chromatography of peripheral blood cells

Each suspension of granulocytes, lymphocytes and erythrocytes from human and rabbit peripheral blood was chromatographed on chemically bonded PEG stationary phases. Four kinds of mobile phases containing either dextran T40 orT500 were used, with isotonic phosphate buffers containing either sodium chloride or no salt except as a buffer. One of the principal advantages of these mobile phases was that the fundamental behaviour of some of the blood cells in APTP systems is known[13,14].

Table II shows retention volumes and separation factors for human and rabbit granulocytes, lymphocytes and erythrocytes eluted independently from PEG-bonded Porasil column. It can be readily seen that the order of elution of human granulocytes and lymphocytes was reversed by the use of dextran T500 and T40 as the mobile-phase. Decreasing the molecular weight of dextran would reverse the elution order with both kinds of human cells, whereas it gave similar retention volumes for rabbit cells.

The increased retention volume of human lymphocytes was observed from the increase in dextran concentration in the mobile phase from 4.5 to 8.0% (w/w). The delayed elution of human lymphocytes resulted in an improved separation from granulocytes. Of the four mobile phases used, eluent IV, containing 8% (w/w) of dextran T40, produced the highest separation factor for human granulocytes and

TABLE III

RETENTION VOLUMES AND SEPARATION FACTORS OF GRANULOCYTES, LYMPHOCYTES AND ERYTHROCYTES

Column: bisoxirane-coupled PEG 20M-Sepharose 6B (25 × 0.9 cm I.D.)

Blood cells	*Mobile phase**							
	I		*II*		*III*		*IV*	
	V_R *(ml)*	*Separation factor*	V_R *(ml)*	*Separation factor*	V_R *(ml)*	*Separation factor*	V_R *(ml)*	*Separation factor*
Human:								
Erythrocytes					5.4		3.8	
Granulocytes	12.0	1.25	12.1	1.49	9.0	1.66	8.3	2.18
Lymphocytes	15.0		18.0		21.0	2.33	22.0	2.65
Rabbit:								
Erythrocytes	22.0		17.0		5.0		4.5	
Granulocytes	31.0	1.24	21.0	1.24	21.0	4.20	22.0	(2.33)**
Lymphocytes	14.3	2.17	5.6	3.56	47.0	2.24	10.5	2.10

* Mobile phases I–IV as in Table II.

** Separation factor against lymphocytes.

lymphocytes. The separation factor for both kinds of rabbit blood cells was lower than that for human cells, and the highest value was obtained by the use of eluent II. The separation factor for human erythrocytes and lymphocytes was 1.56 with eluent III and 3.57 for those of the rabbit with eluent II. Because of irreversible adsorption on the column, the best recoveries of human granulocytes and lymphocytes were not more than 24% and 16%, respectively, of the number of these cells applied, when using eluent III. Similarly, 23% of rabbit granulocytes and 14% of lymphocytes were recovered by using eluent IV.

Table III shows the retention and separation of human and rabbit granulocytes, lymphocytes and erythorcytes chromatographed independently on bisoxirane-coupled PEG–Sepharose 6B (PEG-bonded Sepharose) column using the same eluents. In eluent I the affinity of lymphocytes for the mobile phase increased and the separation from granulocytes was not good. Eluent II, with a similar composition to eluent I but without sodium chloride, gave a better separation. Human granulocytes and lymphocytes were eluted from the column with increasing retention volumes with every eluent. A decrease in the molecular weight of dextran in the mobile phase from T500 to T40 appreciably increased the retention of human lymphocytes. The separation factor against granulocytes was improved by the delayed elution of lymphocytes. When the concentration of dextran T40 was increased from 4.5 to 8.0% (w/w), the elution of lymphocytes was retarded and the separation factor against granulocytes became 2.65. The separation factor of human erythrocytes against granulocytes was 1.66 with eluent III and 2.18 against granulocytes with eluent IV. With eluents I and II the erythrocytes aggregated and were adsorbed on the column.

On the other hand, eluents I, II and IV reversed the order of elution of rabbit granulocytes and lymphocytes in contrast to that of human cells. A reversal of values of the retention volume, similar to human cells in Table II, was also obtained when dextran T500 was replaced with 4.5% (w/w) of T40 (eluent III). An increase in the concentration of dextran T40 resulted in lower separation factors for both cells, similar to that observed in Table II. The highest separation factor for rabbit granulocytes and lymphocytes was obtained with eluent II, and this result was comparable to that obtained on a PEG-bonded Porasil column. By taking advantage of the anomalous behaviour of rabbit lymphocytes in eluent II, an almost complete separation from granulocytes was accomplished by the use of either eluent II or III. Rabbit erythrocytes gave a separation factor of 4.20 against granulocytes with eluent III and 2.33 against lymphocytes with eluent IV. It was considered that the anomalous behaviour of rabbit lymphocytes may be species-dependent owing to the difference in the surface properties compared with human cells.

A good selectivity of the PEG-bonded Sepharose column for human and rabbit blood cells was shown. High separation factors for these cells were observed compared with the PEG-bonded Porasil. The selectivity of the column depended greatly on the molecular weight and concentration of dextran in the mobile phases used.

In order to demonstrate the capability of the PEG-bonded Sepharose column, a mixture of human granulocytes and lymphocytes was chromatographed, using eluent IV on the basis of the results described above. As shown in Fig. 2, the two kinds of cells were completely separated and eluted within 12 h. The recoveries of granulocytes and lymphocytes from the column were 67 and 82%, respectively, of the number of cells applied.

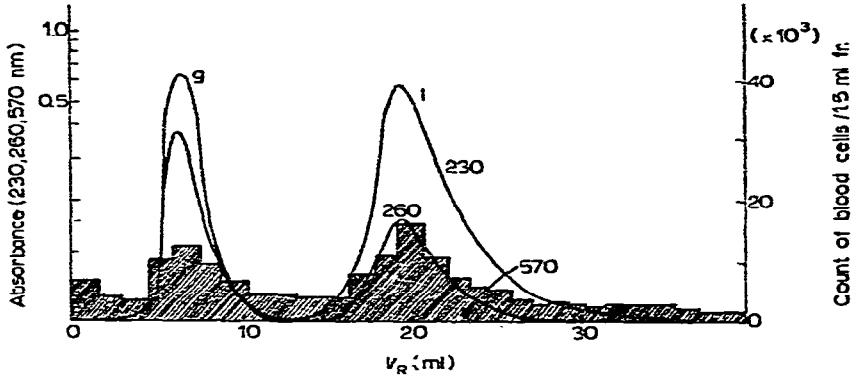


Fig. 2. Elution profile of human granulocytes (g) and lymphocytes (l). Column: 25 × 0.9 cm I.D., bisoxirane-coupled PEG 20M–Sepharose 6B. Eluent: IV, 8.0% (w/w) of dextran T40–0.045 *M* NaH_2PO_4–0.045 *M* Na_2HPO_4, pH 7.5; flow-rate, 3.0 ml/h. A mixture of $2.4 \cdot 10^4$ cells (g) and $3.9 \cdot 10^4$ cells (l) in 0.5 ml of the eluent was loaded on the column.

Fig. 3 shows a chromatographic pattern for a synthetic mixture of human erythrocytes and lymphocytes obtained by using the same column and eluent. The two kinds of cells were fairly well separated and eluted within about 5.8 h. The recovery of the erythrocytes from the column was 50%.

The selectivity of the chemically bonded PEG stationary phase for chromatographic separations is further illustrated in Fig. 4, which shows an elution profile for a synthetic mixture of human erythrocytes, granulocytes and lymphocytes with eluent IV. Three kinds of blood cells were well separated and eluted from the column within 15 h. A similar chromatogram was obtained at a higher flow-rate of 3.0 ml/h. The separation was attained without a serious decrease in column efficiency.

The viability of the blood cells recovered by these chromatographic separations was evaluated by means of phase-contrast microscopy and by respiratory and glycolytic activity.

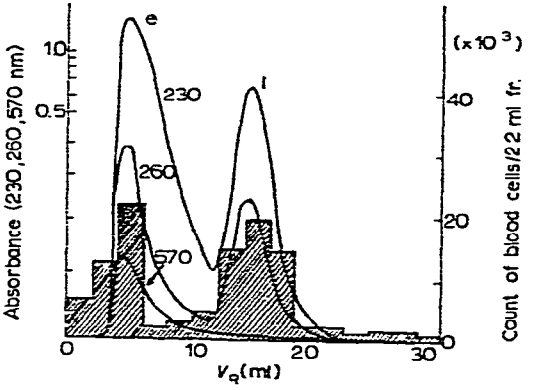


Fig. 3. Elution profile of human erythrocytes (e) and lymphocytes (l). Column and conditions as in Fig. 2, except the flow-rate was 4.4 ml/h. A mixture of $2.3 \cdot 10^6$ cells (e) and $5.8 \cdot 10^4$ cells (l) in 0.5 ml of the eluent was loaded on the column.

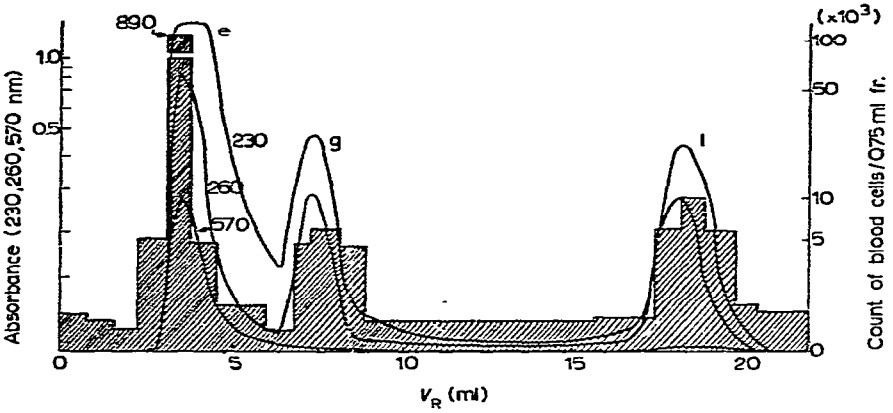


Fig. 4. Elution profile of human erythrocytes (e), granulocytes (g) and lymphocytes (l). Column and conditions as in Fig. 2, except the flow-rate was 1.5 ml/h. A mixture of $1.9 \cdot 10^6$ cells (e), 2.6×10^4 cells (g) and $2.7 \cdot 10^4$ cells (l) in 0.5 ml of the eluent was loaded on the column.

DISCUSSION

The usefulness of APTP systems for fractionation by partition and counter-current distribution of blood cells is illustrated by the successful resolution of erythrocytes of different ages[15,16], the separation of erythrocytes from leukocytes[14], etc. With blood cells, partition in APTP systems depends considerably on their surface properties, on the polymer composition and concentration, on the ionic composition and concentration (including the ratio between the ions present, pH), etc. In general, it has been found that a phase system composed of 5% (w/w) of dextran T500 and 4% (w/w) of PEG 6000 is suitable for the partition of most blood cells. Inorganic salts show characteristic partition behaviour in aqueous two-polymer phases[17]. Sodium and phosphate ions distribute unequally between the phases, giving rise to an electrostatic interfacial potential difference between them[6,18]. This phenomenon explains why the surface charge of suspended cells is involved in determining their partition and why the partition of such materials is sensitive to the ionic composition of the phases.

In order to carry out the separation of peripheral blood cells by means of column chromatography, we prepared two types of packing materials with chemically bonded PEG as the stationary phase. Dextran was chosen as the polymer constituent of the mobile phase because its viscosity must be decreased by the use of a branched polymer. The PEG-bonded Sepharose packing performs excellent separations of human erythrocytes, granulocytes and lymphocytes, as described above. This chromatographic method eliminates many of the problems usually associated with the operation of counter-current distribution in APTP systems. It was also found that the capability of the PEG-bonded Sepharose column depended greatly on the concentration and molecular size of dextran and the ionic composition of coexisting inorganic electrolytes in the mobile phase. The results indicate that the selectivity of the column decreased on the addition of sodium chloride. As the salt partitions equally between the phases and there is virtually no electrical interfacial potential[17], the negatively charged blood cells should have a preferential affinity for the mobile phase and the cells should be eluted more rapidly from the column.

PEG-bonded Sepharose shows a much lower adsorption for blood cells than

PEG-bonded Porasil. It should be pointed out that Sepharose, the support material for the stationary phase, has a hydrophilic surface, and the soft and bulky bead-shaped agarose gel particles do not substantially adsorb proteins. In contrast, Porasil forms fine and rigid particles with a large surface area, and it is known to adsorb proteins spontaneously so that irreversible adsorption can take place between such a surface and proteins of the cell membrane. PEG-bonded Sepharose is easily prepared from epoxy-activated Sepharose 6B and PEG 20M, and the column can be used repeatedly. Scaling up of this technique can readily be achieved by use of a larger bore and/or a longer column.

It is known that lamb leukocytes show heterogeneity of population in their distribution curve by means of an APTP system composed of 5% (w/w) of dextran T500, 4% (w/w) of PEG 6000 and phosphate buffer, but lymphocytes and granulocytes were not separated sufficiently well[14]. Preliminary studies of the counter-current distribution of human leukocytes showed only a single peak[14] or partial resolution[19]. Similarly, separation of rabbit erythrocytes and leukocytes is feasible in similar phase systems[20].

The versatility and usefulness of PEG-bonded Sepharose for the separation and subfractionation of blood cells and cell populations have been demonstrated. The proposed method makes it possible to separate leukocyte populations without difficulty, but the chromatographic separation of lymphocyte populations has not been accomplished.

Mobile phase systems in this method can often be modified so as to solve a particular problem. Once a suitable mobile phase has been found, this technique should prove useful for a wide variety of fractionations and characterizations of blood cells that differ in surface properties, size, specific gravity of the cells and hydrophobicity of the cell surface. This aspect and some applications to the chromatographic separation of platelets from other blood cells will be reported in a subsequent paper.

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