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HYDROPHOBIC CHROMATOGRAPHY OF CELLS: ADSORPTION AND RESOLUTION ON HOMOLOGOUS SERIES OF ALKYLAGAROSES*

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SUMMARY

Implantation of hydrocarbon chains on beaded agarose results in column materials capable of adsorbing erythrocytes or lymphocytes from various sources. In a homologous series of such columns, identical in all structural respects (ligand density, charge density and ultrastructure) except for the length of their hydrocarbon chains, the capacity to adsorb these cells generally increases with increase in the number of carbon atoms per chain. The cells can be desorbed from the columns by repeated gentle pipetting in the presence of bovine serum albumin (for erythrocytes) or foetal calf serum (for lymphocytes). Under the conditions used for adsorption and desorption there is neither physical entrapment of the cells in the column nor apparent damage to their integrity, as indicated by the facts that (a) 95–98% of the cells applied on the column can be recovered; (b) erythrocytes eluted from the column and those yet to be applied are morphologically indistinguishable, exhibit an identical osmotic fragility profile and, (after desorption) retain the same adsorption profile for the columns; and (c) over 95% of the mouse spleen lymphocytes, eluted from the columns, continue to exclude the dye Trypan blue, suggesting that they preserve their viability. Cells from different sources exhibit different adsorption profiles on homologous series of alkylagaroses, allowing the detection of differences in the surface of these cells and their resolution by these columns. Exploratory experiments with artificial cell mixtures are described, illustrating the possibility of enriching the mixture with one of the cell types by preferential adsorption or exclusion on an appropriate alkylagarose. The best resolution was obtained with a mixture of erythrocytes and spleen lymphocytes (both from DBA/1 mice). After appropriate "tailoring" of the column material used (an ethylagarose) it was possible to apply a 1:1 mixture of these cells and to obtain (after preferential adsorption) essentially pure erythrocytes (98% of the excluded cells). Experiments aimed at assessing the type of interactions that mainly contribute to the adsorption and resolution of cells on to alkylagaroses show that: (a) columns with equal or even decreasing net positive charge exhibit an increased capacity to bind erythrocytes with increasing hydrocarbon chain length; (b) the binding of erythrocytes on to alkylagaroses, which decreases in the order mouse > rabbit > guinea pig > man, is distinctly different from their electrophoretic

* This paper is dedicated to Professor Esmond E. Snell on his 70th birthday.
** Incumbent of the Hella and Derrick Kleeman Chair in Biochemistry.

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mobility, which decreases in the order mouse > man > guinea pig > rabbit; (c) even after acetylation of the alkylagaroses (which abolishes their positive charge and somewhat reduces their adsorption capacity for the cells) they still exhibit a chain length-dependent gradation in the adsorption of erythrocytes; and (d) enzymatic removal of sialic acid residues from the surface of erythrocytes, which considerably decreases their negative surface charge, has essentially no effect on the adsorption profile of these cells on alkylagaroses. These findings indicate that hydrophobic, rather than ionic, interactions play a key role in the binding.

INTRODUCTION

On anchoring hydrocarbon chains on agarose, they acquire new adsorption properties[1–8]. Systematic studies with homologous series of alkylagaroses (Seph-C_n*) showed that the retention of proteins on these columns depends on the number of carbon atoms, *n*, in the hydrocarbon chains[1,2,9–13] and that their resolving power is derived mainly from differences in the size, number and lipophilicity of available hydrophobic patches or pockets in the various proteins[1,2,10–14]. These findings set the stage for the development of hydrophobic chromatography as a general, systematic approach for the purification of both water-soluble and lipophilic proteins. The major useful features of the homologous series approach lie in the fact that it is possible to adjust delicately the tightness of adsorption of the desired protein (by the very choice of the member in the series) and thus avoid an overly strong retention, which then requires drastic conditions for elution and, subsequently, might cause damage to the native (physiologically relevant) structure of the protein to be purified. At the same time, this approach provides an additional criterion for discriminating between proteins[1,2,9–14], and preliminary work suggests that it might also be useful in the adsorption, resolution and probing of the surface of cells[15,16].

This paper deals with the reversible adsorption of cells on alkylagaroses and provides evidence that this can be carried out under isotonic conditions and at physiological pH, without significant damage to the cells. Further, the columns are shown to discriminate between cells from various sources and can thus be used for the fractionation of cells. Mechanistic studies, aimed at the elucidation of the type of interactions which are involved in the function of these columns, show that hydrophobic (rather than ionic) interactions predominate, both in determining their binding capacity and their ability to discriminate between (and thus to resolve) cells from different sources.

* Abbreviations: Seph-C_n = Sepharose 4B activated with CNBr and allowed to react with an α-aminoalkane, *n* carbon atoms long; Seph-C_n-NH_2 = Sepharose 4B, activated with CNBr and allowed to react with α,ω-diaminoalkane, *n* carbon atoms long (for a schematic representation of column materials see ref. 11); BSA = bovine serum albumin; ChRC = chicken red blood cells; FCS = foetal calf serum; GPRC = guinea pig red blood cells; HRC = human red blood cells; MRC = mouse red blood cells; MSL = mouse spleen lymphocytes; PBSS = phosphate-buffered salt solution; PLL = poly-L-lysine; RbRC = rabbit red blood cells.

EXPERIMENTAL

Animals

Inbred guinea pigs (Heston), inbred mice (DBA/1) and random-bred albino rabbits were obtained from the Experimental Animal Unit of the Weizmann Institute of Science, Rehovot, Israel. Chicken (random-bred) were obtained from local sources.

Erythrocytes

Fresh heparinized blood was centrifuged (850 *g*, 5 min). The red cell pellet was washed three times and finally suspended in phosphate-buffered salt solution (PBSS, see *Buffers* section).

Mouse spleen lymphocytes

Mouse spleens were ground on a stainless-steel mesh in a Petri dish containing PBSS, supplied with 10% (v/v) FCS. The resulting cell suspension was filtered through gauze mesh (four layers) to remove coarse particles, and was subsequently centrifuged on a Ficoll–Metrizoate gradient according to the procedure described by Davidson and Parish[17]. Lymphocytes collected from the interphase (by means of a Pasteur pipette) were immediately transferred to cold (4°C) PBSS, supplied with 5% (v/v) FCS. The lymphocyte suspension was centrifuged (300 *g*, 10 min) and the cells were then washed twice and finally suspended in PBSS. The viability of these cells was measured by Trypan blue staining. One volume of lymphocyte suspension was mixed with two volumes of Trypan blue solution (0.5% in saline). Cells were counted and the proportion of cells that excluded the dye (viable cells) was thus determined.

Cell counting

Cells were counted with a haemocytometer (in quadruplicate). With cell mixtures containing erythrocytes from various sources, differential cell counts were based on morphology or size differences. With cell suspensions containing both erythrocytes and lymphocytes, aliquots of the given suspension were diluted 1:10 in saline (for the total cell count) and in saline solution containing 1% acetic acid (to cause lysis of the red cells). The number of erythrocytes was determined by subtracting the lymphocyte count from the total cell count.

Buffers

Unless otherwise indicated, a PBSS buffer (pH 7.4) was used. This buffer was composed of NaCl (137 m*M*), KCl (2.7 m*M*), Na_2HPO_4 (6.5 m*M*) and KH_2PO_4 (1.46 m*M*). To decrease the ionic strength of PBSS while maintaining its isotonicity, decreasing volumes of this buffer were mixed with increasing volumes of isotonic PBS–sucrose. The latter buffer (pH 7.4) was composed of sucrose (274 m*M*), KCl (2.7 m*M*), Na_2HPO_4 (7.56 m*M*) and KH_2PO_4 (2.44 m*M*). A series of buffers (all at pH 7.4) was thus obtained, in which the NaCl constituent of the PBSS was gradually replaced with an iso-osmotic concentration of sucrose.

Alkylagaroses (Seph-C_n) and ω-aminoalkylagaroses (Seph-C_nNH_2)

A sample (10 g, wet gel*) of Sepharose 4B (Pharmacia, Uppsala, Sweden) was allowed to react with 10 mmole of CNBr and 40 mmole of either a normal α-aminoalkane or a normal α,ω-diaminoalkane, as described previously[10]. The resulting column materials were washed with five times their volume of a series of solvents[10], ending with an additional solvent composed of aqueous acetic acid (0.4 *M*) and N,N'-dimethylformamide (1:1, v/v). Finally, the column materials were washed with water (ten times their volume) and stored in 50 m*M* acetic acid (4°C).

Characterization of the column materials

The ligand and charge densities of the column materials were determined as described previously[9].

Acetylation of the alkylagarose columns

Acetylation was performed with acetic anhydride, according to the procedure described by Wilchek and Miron[18].

Determination of the adsorption–elution profile of cells on alkylagarose columns

The procedure described in detail in ref. 15 was used.

Osmotic fragility profile of erythrocytes

A series of buffers (pH 7.4), composed of Na^+/K^+ phosphate (25 m*M*) and different concentrations of NaCl (0–125 m*M*), was prepared. Samples of erythrocytes in PBSS ($4 \cdot 10^7$ cells/sample) were centrifuged (5 min at 850 *g*), and the cell pellet of each sample was resuspended in one of the above-mentioned buffers (1 ml). Lysis was allowed to proceed (in stoppered tubes) for 30 min at 37°C. The contents of each tube were then gently mixed with 1 ml of an appropriate buffer so as to bring the composition of the solution to 75 m*M* Na^+/K^+ phosphate, 75 m*M* NaCl (pH 7.4) and thus to stop the lysis of the cells. The tubes were centrifuged (5 min at 850 *g*) and the resulting pellets were lysed with 2 ml of water. From the $A_{543\ nm}$ values obtained for the supernatants and for the lysed pellets, the percentage of lysed cells at each NaCl concentration was determined.

Determination of sialic acid residues in cell membranes

Sialic acid residues were released from cell membrane by acid hydrolysis, according to the procedure described by Tischer and Peters[19], and the amount released was determined with resorcinol, as described by Saifer and Feldman[20]. All types of erythrocytes tested were simultaneously assayed, to ensure maximal comparative accuracy.

Enzymatic removal of sialic acid residues by neuraminidase

Washed erythrocytes (10^9) were suspended in 1 ml of saline solution (0.9% NaCl) containing 20 units of *Vibrio cholera* neuraminidase (Behringwerke, F.R.G.). The suspension was incubated for 1 h at 37°C (with occasional stirring) and the cells

* Before weighing, the agarose is placed on a Büchner funnel to drain off excess of water until the very first signs of dryness (cracks on the gel surface) appear.

were then washed twice and finally suspended in PBSS. The agglutinability of the cells by poly-L-lysine was tested on cell samples to monitor the removal of the sialic acid residues.

Agglutination of cells by poly-L-lysine

Agglutination of red cells by poly-L-lysine (MW 70,000, 95 Lys residues per molecule, purchased from Miles, Yeda, Israel) was monitored by means of a Fragilligraph Model D-2 (Elmedix, Tel-Aviv, Israel), equipped with an agglutination accessory and scaling unit, according to the procedure described elsewhere[21,22]. This instrument records (in arbitrary units) the change in light transmission of the cell suspension due to the agglutination of the cells and the subsequent decrease in the turbidity of the cell suspension.

RESULTS AND DISCUSSION

Gradation in the adsorption of cells on a homologous series of Seph-C_n or Seph-C_n-NH_2 columns

Alkylagaroses, as well as ω-aminoalkylagaroses, are capable of adsorbing cells from different sources. This adsorption, which occurs in an isotonic medium and under physiological conditions of pH and temperature, is readily observed under the microscope (Fig. 1). The number of cells adsorbed by a given amount of column

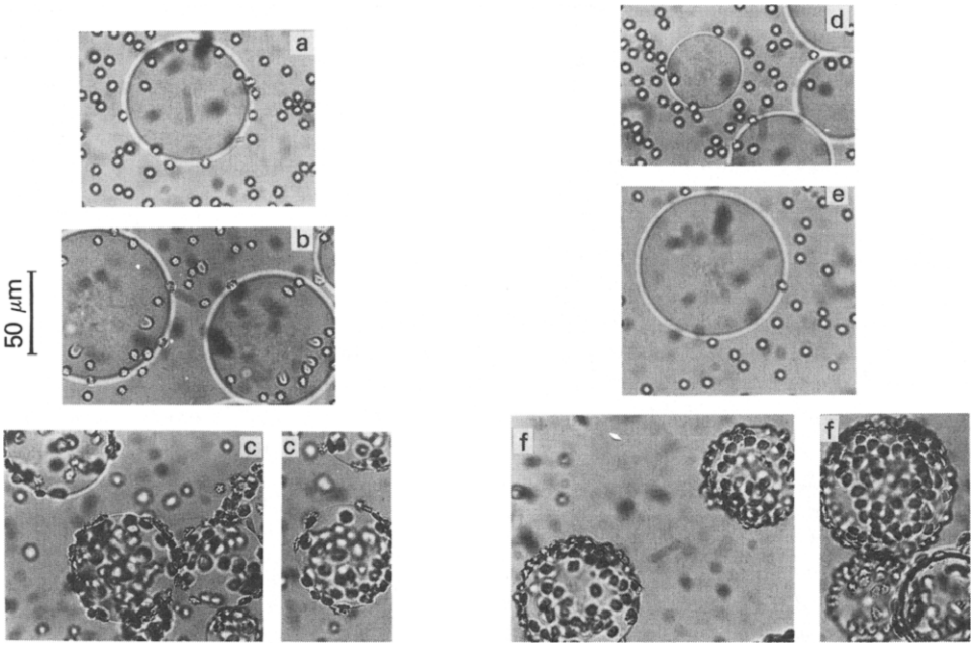


Fig. 1. Adsorption of GPRC on Seph-C_n and Seph-C_n-NH_2 columns, as viewed under the microscope. Samples (0.15 ml) of GPRC suspension (10^9 cells/ml in PBSS) were applied on each of the columns indicated (0.85 ml, settled volume). The cells were allowed to be adsorbed (15 min at 22°C) and the columns were then washed with 10 ml of PBSS. Aliquots of the column materials were then examined under the microscope. (a) Seph-C_0; (b) Seph-C_1; (c) Seph-C_9; (d) Seph-C_6^N (representing Sepharose 4B activated by CNBr, then allowed to react with ammonia); (e) Seph-C_2-NH_2; (f) Seph-C_6-NH_2.

material gradually increases with the elongation of the hydrocarbon chains for both the Seph-C_n and Seph-C_n-NH_2 series (Fig. 2). In these column series, this gradation is different for erythrocytes from different animals (*e.g.*, mouse, rabbit, guinea pig and man, Fig. 2A), suggesting the potential use of such columns for discriminating between these cell populations. Similarly, different types of cells from the same animal (*e.g.*, spleen lymphocytes and erythrocytes from a DBA/1 mouse) display a different adsorption profile on the Seph-C_n series (not illustrated), the most pronounced difference occuring with a Seph-C_2 column, which was found to have a *ca.* 25% higher capacity for the lymphocytes.

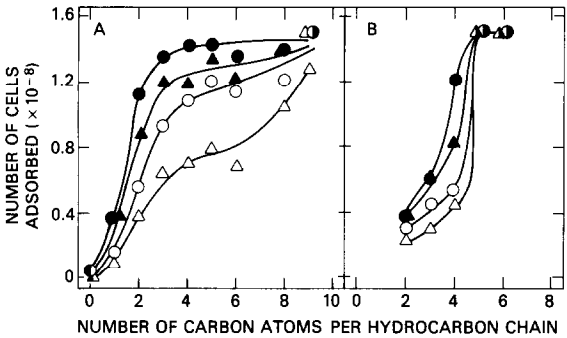


Fig. 2. Adsorption profiles of erythrocytes from various sources on (A) Seph-C_n and (B) Seph-C_n-NH_2 columns. Samples (0.15 ml) of the indicated cells ($1.5 \cdot 10^8$ per sample) were applied to each column (0.85 ml, settled volume). Adsorption was allowed to proceed for 15 min at 22°C and then the columns were washed, each with 10 ml of PBSS. The cells in the resulting effluents were centrifuged (5 min at 850 *g*) and each of the pellets was lysed with 2 ml of water. The absorbance of the resulting lysates ($A_{543\ nm}$) was determined and converted into cell numbers by comparison with a set of standards (erythrocyte pellets containing a known number of cells which were similarly lysed with 2 ml of water). The number of adsorbed cells was determined by subtracting the number of excluded cells from the number of applied cells. (●) MRC obtained from DBA/1 mice; (▲) RbRC obtained from random-bred albino strain of rabbits; (○) GPRC obtained from Heston guinea pigs; (△) HRC, blood type A^+.

It should be noted that adsorption of erythrocytes on the higher members of the Seph-C_n-NH_2 series ($n > 6$) is frequently accompanied by cell lysis. The reason for this damage to the cells is not yet understood. It could not be prevented by extensive washing with solvents that would have removed any excess of the diaminoalkane, if this had been the cause of cell lysis. However, such damage to the cells was never observed with any of the Seph-C_n columns used (n = 1–12). Therefore, our work was concentrated on this series.

Reversibility and mildness of the adsorption-desorption process

The binding of erythrocytes to Seph-C_n columns is reversible. Cells adsorbed on these columns can be desorbed in high yield under mild conditions, which cause no apparent damage to the cells. Of several methods tested, optimal desorption was achieved by soaking the loaded column (0.85 ml) with 1 ml of PBSS containing 20 mg of BSA (pH 7.4), followed by repeated gentle pipetting of the suspension with a Pasteur pipette (about ten times). BSA presumably prevents re-adsorption of the cells on the column material by competing for the unmasked alkyl chains. Subsequent

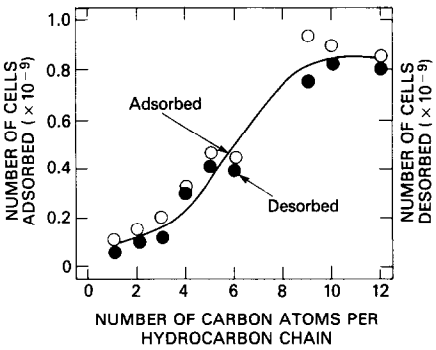


Fig. 3. Desorption of GPRC from Seph-C_n columns. Samples (0.15 ml) containing $1.1 \cdot 10^9$ GPRC (in PBSS) were applied to each of the columns (0.85 ml, settled gel). Adsorption was allowed to proceed for 15 min at 22°C. Non-adsorbed cells were excluded by washing the columns with 10 ml of PBSS, then 1.0 ml of PBSS containing 20 mg of BSA (pH 7.4) was allowed to soak into the gel. Detachment of adsorbed cells was achieved by repeated gentle pipetting (*ca.* 10 times) of the gel suspension into a Pasteur pipette, followed by washing the column with 10 ml of PBSS. The number of adsorbed and desorbed cells was determined according to the procedure described in Fig. 2. (○) Number of adsorbed GPRC; (●) number of desorbed GPRC.

washing of the column with an excess (10 ml) of PBSS leads to the recovery of >95% of the cells (Fig. 3).

The quantitative recovery of the cells clearly indicates that during the adsorption–desorption procedure there is very little lysis and there is essentially no entrapment of cells in the column. Moreover, the cells desorbed from the columns are morphologically indistinguishable from those yet to be applied (Fig. 4A and B) and they have an identical osmotic fragility profile (Fig. 4C). In addition, it was found that the desorbed cells retain their affinity for Seph-C_n columns and exhibit re-adsorption similar to their original adsorption profile.

The mildness of the adsorption–desorption procedure was also demonstrated with spleen lymphocytes from DBA/1 mice. In a typical experiment, $1.5 \cdot 10^7$ lymphocytes, suspended in 0.15 ml of PBSS, were applied to a Seph-C_{10} column (0.85 ml). The cells were incubated (15 min at 22°C) with the column material, at which time it was determined that essentially all of the cells (99%) had been adsorbed. Elution was carried out according to the procedure described above, with two modifications: the column material was soaked with PBSS containing 10% FCS (instead of BSA), and the same FCS-containing medium (10 ml) was used for the subsequent washing (FCS contributes significantly to the preservation of lymphocyte viability). As with erythrocytes, quantitative (98%) elution was achieved, and over 95% of the cells in the effluent excluded the Trypan blue dye (*i.e.*, were viable), compared with 98% of the cells in the control lymphocyte population not applied on the column.

Characterization of the column materials with respect to ligand density and charge density

Activation of agarose with CNBr and coupling with alkylamines results in several types of ligand-to-matrix bridges. These include (*cf.*, Fig. 2 in ref. 9) substituted carbamates, unsubstituted carbamates, substituted imidocarbamates and substituted

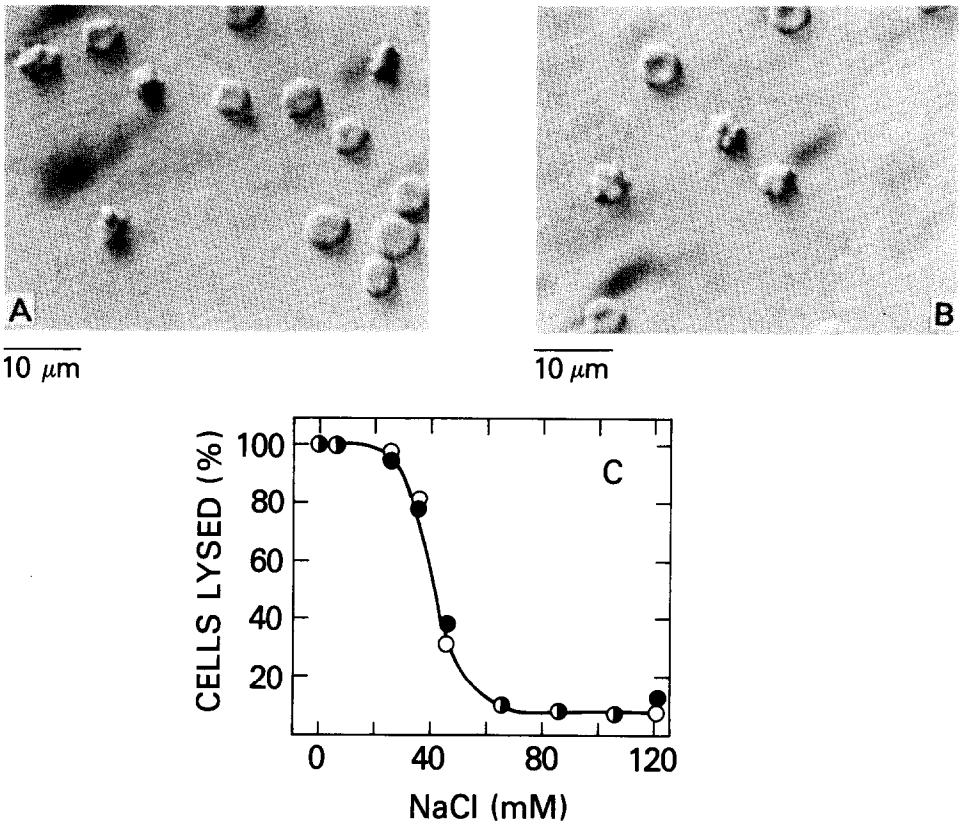


Fig. 4. The adsorption–desorption procedure does not affect the morphology and the osmotic fragility of GPRC. (A) Microscopic examination of GPRC after detachment from a Seph-C_{10} column. Adsorption of GPRC on the column and their subsequent desorption was performed according to the procedure described in Fig. 3. Samples of the effluent containing the desorbed cells were examined under the microscope. (B) Microscopic examination of GPRC which were not applied to a column. GPRC were suspended in PBSS, containing BSA (20 mg/ml), pH 7.4. The resulting suspension was mixed with 10 ml of PBSS and the cells were examined under the microscope. These cells served as a control. (C) Osmotic fragility profile of GPRC before and after application to a Seph-C_{10} column: (●) GPRC obtained as described in (A); (○) control cells prepared as described in (B). The cells were centrifuged (5 min at 850 *g*) and washed twice with PBSS. Aliquots, each containing $4 \cdot 10^7$ cells, were then suspended in 1 ml of 25 m*M* phosphate buffer, containing the indicated concentration of NaCl. The results represent the percentage of lysed cells in each of these aliquots.

isourea linkages[23–25]. Around pH 7, the column materials of the Seph-C_n series have a net positive charge owing to protonation of the substituted isourea linkages at the "root" of some of the alkyl chains. These cationic groups were shown to have an identical pK_a of 9.7 in all the members of the Seph-C_n series tested (n = 1–12)[11–15]. In a large number of Seph-C_n columns characterized in our laboratory with respect to their charge density and hydrocarbon chain density, it has been consistently observed[9,26,27] that when the columns are synthesized with an alkylamine to CNBr ratio of 2–6 mole/mole, the charge to ligand density ratio is 0.6 ± 0.1 equiv./mole. However, as can be seen in Table I, this charge to ligand density ratio can increase

TABLE I

EFFECT OF THE ALKYLAMINE TO CNBr RATIO USED DURING COUPLING ON THE CHARGE TO ALKYL CHAIN RATIO IN THE RESULTING Seph-C_n COLUMN

Aliquots of Sepharose 4B (10 g, wet gel) were activated each with the indicated amounts of CNBr and [^{14}C]butylamine (0.1 μCi/mmole). The alkyl-chain density of the final product was determined from its specific radioactivity and the charge density by potentiometric titrations, as described under Experimental.

Reactants used during coupling			*Properties of the resulting Seph-C_4*		
CNBr (mmole)	*Butylamine (mmole)*	*Butylamine/CNBr (mole/mole)*	*Alkyl-chain density (μmole/ml settled gel)*	*Charge density (μequiv./ml settled gel)*	*Charge/alkyl chain (equiv./mole)*
10	0.2	0.02	4.2	4.2	1
10	20	2	13.5	9.2	0.68
10	60	6	15	7.5	0.50

up to 1.0 equiv./mole when the column is prepared with an alkylamine to CNBr ratio of 0.02 mole/mole.

Assessing the relative importance of hydrophobic vs. ionic interactions

The relative importance of hydrophobic *vs.* ionic interactions in the binding of cells to Seph-C_n columns was studied by several approaches. As shown in Fig. 5, the number of guinea pig erythrocytes adsorbed on a given amount of Seph-C_n column materials clearly increases with increasing ligand density. However, as the charge density of the two columns tested increases with increasing ligand density (see the

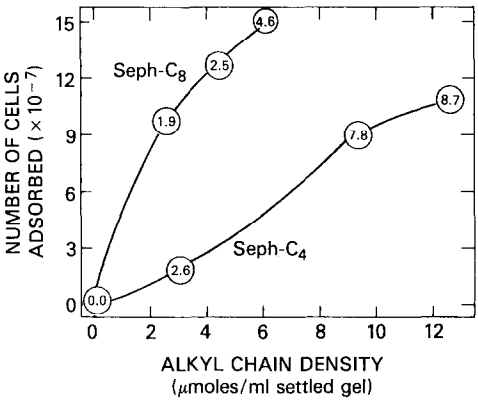


Fig. 5. Effect of alkyl chain density on the capacity of Seph-C_4 and Seph-C_8 to bind erythrocytes. The synthesis of Seph-C_4 and Seph-C_8 columns, varying in their chain density, was performed by activating 10 g (wet gel) aliquots of Sepharose 4B with varying amounts of CNBr (5–60 mmole) and then allowing the activated Sepharose to react with [^{14}C]butylamine or [^{14}C]octylamine (0.1 μCi/mmole). The molar ratio of the alkylamine to CNBr was 6 mole/mole in all instances. The alkyl chain density (μmole/ml settled gel) of the final product was determined from the specific radioactivity of the column material, and the net positive charge (μequiv./ml settled gel at pH 7.0) was determined by potentiometric titrations (see Experimental) and is represented by the numbers within the circles. The number of guinea pig erythrocytes adsorbed was determined according to the procedure described in Fig. 2.

values of charge density given along each curve), it is not possible from this experiment to deduce the relative contribution of these charges to the interaction between the cells and the column material.

It should be noted that the experiment depicted in Fig. 5 clearly illustrates the prominent effect of the length of the alkyl chains on the capacity of the columns to bind GPRC. Thus, the Seph-C_4 column with a ligand density of 9.5 μmole/ml and a charge density of 7.8 μequiv./ml adsorbed roughly the same number of cells as a Seph-C_8 column with *ca.* 3 times less ligand density and *ca.* 4 times less charge density. Similarly, the Seph-C_8 column with a charge density of 1.9 μequiv./ml and a ligand density of *ca.* 3 μmole/ml adsorbs about 7 times the number of cells adsorbed by a Seph-C_4 column with a similar ligand density and a slightly higher charge density (Fig. 5). In fact, the experiment depicted in Fig. 6 further stresses the importance of the hydrocarbon chain length, and points to a lack of correlation between the charge density of the Seph-C_n columns, which in this case *decreases* with increasing n, and their capacity to bind GPRC, which clearly *increases* with increasing n.

In spite of the above, it should be realized that, while hydrophobic interactions seem to play a major role in the adsorption of erythrocytes on Seph-C_n columns[14–16,28], this does not rule out the possibility that ionic interactions contribute to this adsorption. This is plausible in view of the fact that the Seph-C_n columns have a positive net charge[9,11–15], that the surface of erythrocytes has a net negative charge[29–31] and that the contact area between an erythrocyte and a Seph-C_n bead (compared with the density of alkyl chains and of positive charges on the beads) allows for several points of interaction. Further, there is also a possibility that the existence of a hydrophilic, positively charged functional group at the "root" of the alkyl chain may affect the conformation of the column material by repelling the hydrocarbon chain and preventing it from clinging to the matrix backbone, thus favourably presenting it for interaction with a hydrophobic locus on a protein or a cell.

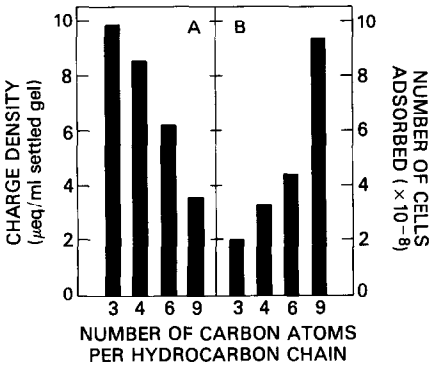


Fig. 6. Lack of correlation between the charge density of Seph-C_n columns and their capacity to bind GPRC. Samples (0.15 ml) containing 10^9 GPRC were applied to Seph-C_n columns (0.85 ml, settled gel), and the number of adsorbed cells was determined by subtracting the number of excluded cells from the number of cell applied. The charge density (μequiv./ml settled gel at pH 7.0) of the column materials was determined by potentiometric titrations, as described under Experimental. (A) Charge density of the columns; (B) number of cells adsorbed.

In an attempt to gain further insight into the mechanism operating in the binding of erythrocytes on Seph-C_n columns, the net charge of the columns was removed by acetylation[18] and the surface net negative charge of erythrocytes was considerably reduced by treatment with neuraminidase (from *Vibrio cholera*)[21,32,33]. We then studied the effect of each of these modifications on the interaction between the cells and the Seph-C_n columns.

Abolishing the positive charge of the columns by acetylation

Wilchek and Miron[18] have shown that acetylation of Seph-C_n columns removes their positive charge at neutral pH by modifying their N-substituted isourea linkages (*cf.*, Fig. 2 in ref. 9). Fig. 7 compares the capacity of acetylated and non-acetylated Seph-C_n columns to bind GPRC (A) and MRC (B). It is evident that with both types of columns and both types of erythrocytes, the adsorption capacity clearly increases with elongation of the alkyl chains. Nevertheless, acetylation does reduce to some extent the binding capacity of the column materials. As expected, this effect is more pronounced with columns having short hydrocarbon chains ($n = 2$). It gradually decreases with the higher members in the series and finally vanishes with Seph-C_9. These results suggest that while ionic interactions may assist the binding of erythrocytes to Seph-C_n columns, these interactions do not constitute a prerequisite for cell adsorption.

The capacity of Seph-C_n columns to bind erythrocytes is not necessarily related to the surface negative charge of the cells

In certain animal species, most of the surface negative charge of erythrocytes is due to sialic acid residues[29,34]. For example, HRC that have been treated with neuraminidase (which splits off the sialic acid residues) are no longer agglutinated by PLL and have a considerably reduced electrophoretic mobility[21,32–34]. As the capacity of Seph-C_n columns to adsorb HRC is lower than their capacity for GPRC (Fig. 2) (at least for $n = 4$–6), an attempt was made to find out whether this difference could be attributed to the differences in the surface negative charge of these two types of erythrocytes. A comparative quantitative analysis, carried out according to the procedures of Tischer and Peters[19] and Saifer and Feldman[20], revealed that the content of the sialic acid residues in the membranes of the HRC was 1.4 times higher than

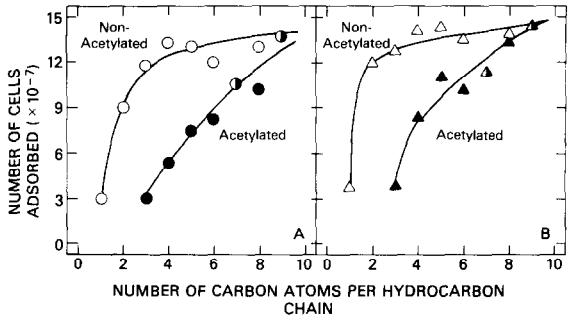
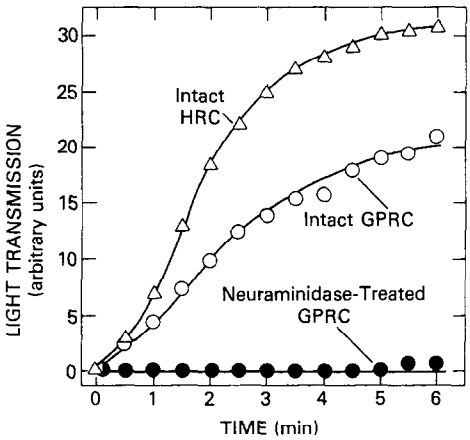


Fig. 7. Acetylation of Seph-C_n columns and its effect on the adsorption profile of (A) GPRC and (B) MRC. Adsorption profiles were determined as described in Fig. 2 for the unmodified (○, △) and the acetylated (●, ▲) Seph-C_n columns.

that of GPRC, showing that at least in this instance there is an inverse correlation between sialic acid content and binding to the Seph-C_n columns. This is further supported by the experiment depicted in Fig. 8, which shows that HRC are agglutinated by PLL at a higher rate and to a greater extent than GPRC. These findings regarding the sialic acid content and the agglutination by PLL are compatible not only with each other but also with the data presented by Bier[35], showing that the electrophoretic mobility of HRC is higher than that of GPRC. In fact, similar evidence is obtained when the adsorption of various types of erythrocytes on Seph-C_n columns is compared with data on their electrophoretic mobility. Fig. 9 depicts the adsorption of MRC, RbRC, GPRC and HRC on Sep-C_3, Seph-C_5 and Seph-C_8 columns, as related to electrophoretic mobility reported for these erythrocytes[35]. It is evident that the binding of these erythrocytes to the columns, which decreases in the order MRC, RbRC, GPRC and HRC, does not follow their electrophoretic mobility, which decreases in the order MRC (1.40), HRC (1.31), GPRC (1.11) and RbRC (0.55 μm/sec).

Reducing the available net negative charge of the cell surface by treatment with neuraminidase

It has previously been reported that sialic acid residues contribute essentially all of the surface negative charge of HRC, and that pre-treatment of these erythrocytes with neuraminidase prevents their agglutination by PLL[21,32–34]. As the same is true also for GPRC (Fig. 8), it is possible to compare, for two different types of erythrocytes, their adsorption profiles before and after treatment with neuraminidase. As can be seen in Fig. 10, the adsorption profiles of intact and neuraminidase-treated HRC are identical, and this is also true for the intact and neuraminidase-treated GPRC. Interestingly, the difference in the adsorption profiles of HRC and GPRC



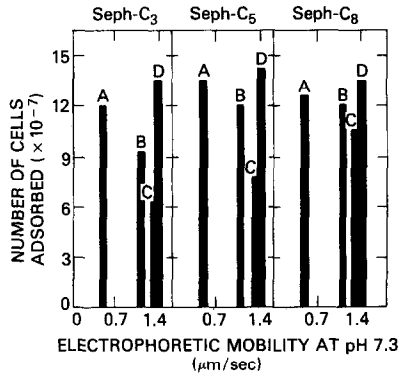


Fig. 8. Rate of agglutination of HRC, GPRC and neuraminidase-treated GPRC by PLL. Samples (1.2 ml) of cell suspensions, containing 10^8 erythrocytes, and 20 μg of PLL were incubated with continuous stirring in the agglutination accessory of the Fragilligraph, and the rate of agglutination was monitored, as described under Experimental. (△) HRC; (○) GPRC; (●) neuraminidase-treated GPRC.

Fig. 9. Lack of correlation between the electrophoretic mobility of erythrocytes and their binding to Seph-C_n columns (n = 3, 5, 8). RbRC (A), GPRC (B), HRC (C) and MRC (D) were allowed to be adsorbed on the indicated column materials as described in Fig. 2, and the number of cells adsorbed was plotted against the electrophoretic mobility of these cells, obtained from ref. 35.

before and after treatment with neuraminidase is preserved. It can therefore be concluded that not only the adsorption capacity but also the discrimination power of the Seph-C_n columns is independent of the surface charge of the cells. This observation seems important, because it indicates that the resolution of these erythrocytes by Seph-C_n columns is based mainly on a criterion that does not depend on charge, so that these columns can be used to purify cell populations that are not resolved by charge-dependent separation methods.

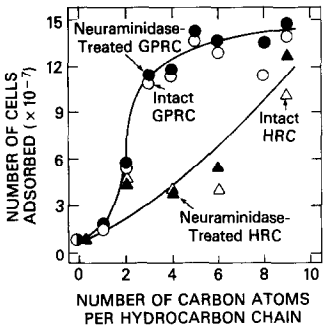
It might be argued that the enzymatic removal of the sialic acid residues makes the cell surface more hydrophobic (*e.g.*, by exposing previously buried hydrophobic residues, or by triggering a structural reorganization in the cell membrane). Thus, the impairment of the ionic interactions might be compensated for by the enhanced hydrophobicity of the modified cells. If this had been the case, then one would expect an increased affinity between the modified cells and the non-charged (acetylated) columns. When intact and neuraminidase-treated GPRC were applied to a non-acetylated and an acetylated Seph-C_5 column (by the procedure described in the legend to Fig. 10), it was found that acetylation of the Seph-C_5 column reduced its capacity to adsorb both intact and neuraminidase-treated GPRC, but the extent of this reduction was similar for both forms of GPRC.

When the effect of the ionic strength on the interaction between GPRC and Seph-C_n columns was studied (replacing the NaCl constituent in PBSS by iso-osmotic concentrations of sucrose) it was found that, although the ionic strength of the medium (0–140 m*M* NaCl) did affect the extent of cell adsorption, this effect was identical for both intact and neuraminidase-treated cells (Fig. 11). It seems, therefore, that in the adsorption of HRC and GPRC on Seph-C_n columns the contribution of the sialic acid residues (and thus of the cell surface negative charge) is negligible.

Resolution of mixed cells

Exploratory experiments published previously[15] showed that erythrocytes from different sources exhibit different adsorption profiles on Seph-C_n columns. These experiments were extended in the work presented here (*cf.*, Fig. 2). On the basis of these results it can be deduced, for example, that the separation of HRC and MRC should be optimal with $n = 4$–6. Indeed, when an artificial mixture (1:1) of HRC and MRC is applied on a Seph-C_6 column, 85% of the applied HRC were excluded, and the proportion of these cells in the effluent rose to 80%. The HRC/MRC ratio thus rose from 1:1 in the applied mixture to 4:1 in the effluent (Fig. 12A). Similar resolutions were achieved with a mixture of MRC and GPRC (Fig. 12B) and a mixture of HRC and ChRC (Fig. 12C), both on Seph-C_2 columns.

When the adsorption profile of MSL on Seph-C_n was compared with that of MRC (not illustrated), it was found that optimal separation of these two types of cells would be obtained on Seph-C_2. However, a high proportion of both types of cells (80% of the applied number of MRC and 100% of the applied MSL) was not excluded by this column. As a decrease in the amounts of both CNBr and alkylamine during the coupling step of the column synthesis leads to a decrease in chain density and in the adsorption capacity of the column (*cf.*, Fig. 6), we screened several Seph-C_2 columns (with different chain densities) for optimal resolution of these two cell types. When a Seph-C_2 column was synthesized with half the usual amount of CNBr and ethylamine (5 mmole of CNBr and 20 mmole of ethylamine, instead of



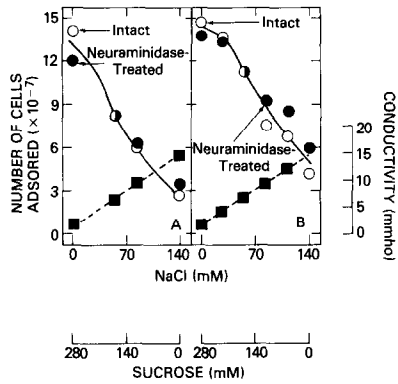


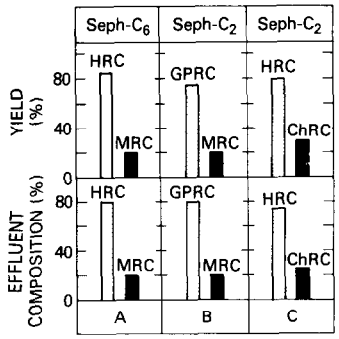
Fig. 10. Comparison of the adsorption profiles of intact and neuraminidase-treated erythrocytes on Seph-C_n columns. Samples (1 ml) of 10^9 cells in 0.9% NaCl were incubated with *Vibrio cholera* neuraminidase (20 units) for 60 min at 37°C. The cells of each sample were then washed twice and finally suspended in PBSS to a final concentration of 10^9 cells/ml. Aliquots (0.15 ml) of these suspensions were applied to each of the indicated Seph-C_n columns by the procedure described in Fig. 2. Erythrocytes that were similarly incubated without neuraminidase served as control (intact) cells: (○) intact GPRC, (●) neuraminidase-treated GPRC, (△) intact HRC and (▲) neuraminidase-treated HRC.

Fig. 11. Effect of ionic strength on the adsorption of intact *vs.* neuraminidase-treated GPRC on (A) Seph-C_2 and (B) Seph-C_4. A series of isotonic phosphate-buffered solutions (pH 7.4), decreasing in ionic strength, were obtained by gradually replacing the NaCl constituent of the PBSS with iso-osmotic concentrations of sucrose. A sample (0.15 ml) of GPRC, suspended in the buffer to be used (10^9 cells/ml), was applied to the indicated column (0.85 ml, settled gel), which was pre-equilibrated with the same buffer, also used to exclude the non-adsorbed cells. The number of adsorbed cells was determined as described in Fig. 2. The ligand density of the columns used was adjusted, as described in Fig. 5, so that under the usual standard conditions ($1.5 \cdot 10^8$ cells applied in PBSS) the columns adsorbed *ca.* 30% of the cells: (○) intact GPRC; (●) neuraminidase-treated GPRC; (■) conductivity.

the usual 10 and 40 mmole, respectively, for every 10 g of agarose) the yield of MRC obtained in the effluent of the resulting Seph-C_2 column was increased to 50%. Moreover, the proportion of these cells in the effluent reached 98% (Fig. 13), so that the MRC/MSL ratio rose from 1:1 in the applied mixture to 50:1 in the effluent, now containing essentially pure MRC.

CONCLUSION

Hydrophobic chromatography of cells on homologous series of alkylagaroses is not intended to replace the other methods for the resolution and purification of cells or for probing their surface. Rather, it is hoped that this approach will provide an additional (different) criterion for achieving these purposes. One of the important features of hydrophobic chromatography as presented above lies in the optimization of resolution and in the delicate adjustment of the adsorption forces (column series, member within the series, ligand density and even charge to ligand ratios), so as to avoid overly strong (often harmful) adsorption. This delicate adjustment is important if cells are to retain viability and to preserve, as much as possible, the native properties and structure of the cell surface. The effectiveness of the columns under physiological conditions of osmotic pressure, pH and temperature is certainly useful in that respect.



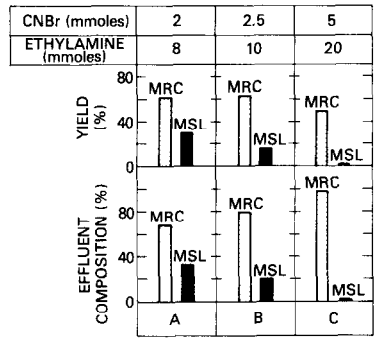


Fig. 12. Resolution of erythrocytes from various sources by Seph-C_n columns. A cell sample (0.15 ml), containing equal numbers of erythrocytes from two different sources, was applied to a Seph-C_n column that exhibited optimal discrimination between these two types of cells (*cf.*, Fig. 2). Adsorption was allowed to proceed for 15 min at 22°C, then the column was washed with 10 ml of PBSS. The cells in the effluent were counted (differential counts based on differences in cell size or morphology). The upper panels show the yield of each type of cell in the effluent (taking as 100% the number of cells of this type applied to the column). Lower panels show the composition of the cells in the effluent (taking as 100% the total number of cells in the effluent). (A) Resolution of HRC (blood type A^+) and MRC (DBA/1) on Seph-C_6; the applied sample contained $7.5 \cdot 10^7$ erythrocytes from each source. (B) Resolution of GPRC (Heston) and MRC (DBA/1) on Seph-C_2; the applied sample contained $7.5 \cdot 10^7$ cells from each source. (C) Resolution of HRC (blood type A^+) and ChRC (random bred) on Seph-C_2; the applied sample contained $1.2 \cdot 10^7$ cells from each source.

Fig. 13. Resolution of MRC (DBA/1) and MSL (DBA/1) on Seph-C_2. A series of three Seph-C_2 columns was prepared by allowing 10 g (wet gel) Sepharose 4B to react with (A) 2 mmole of CNBr and then 8 mmole of ethylamine, (B) 2.5 mmole of CNBr and then 10 mmole of ethylamine or (C) 5 mmole of CNBr and then 20 mmole of ethylamine. The resulting Seph-C_2 columns are denoted A, B and C, respectively. A sample (0.15 ml) of cell mixture in PBSS (containing $5 \cdot 10^7$ cells of each type) was applied to each of the three columns (0.85 ml settled gel). Fractionation was performed according to the procedure described in Fig. 12. Aliquots (0.1 ml) of each of the 10-ml effluents (from columns A, B and C) were diluted with either 0.9 ml of PBSS (for total cell counts) or 0.9 ml of a solution composed of 1% acetic acid in 0.9% saline (for lymphocyte count). Yields (upper panels) and composition (lower panel) as defined in Fig. 12. Shaded columns, MRC; filled columns, MSL.

The results presented above indicate that hydrophobic (rather than ionic) interactions play a key role in the binding of cells to Seph-C_n columns, because (a) columns with equal or even a decreasing net positive charge exhibit an increased capacity to bind erythrocytes with increasing n; (b) the binding of erythrocytes to Seph-C_n columns follows an order that is distinctly different from their electrophoretic mobility; (c) even after acetylation (which removes their positive charge[18]) the columns bind the cells with a chain length-dependent gradation; and (d) enzymatic removal of the sialic acid (negatively charged) residues from the cell surface does not affect their adsorption profile on Seph-C_n.

Alkylagaroses can be regarded as a mosaic of randomly distributed hydrophobic chains in a relatively hydrophilic inert matrix, and it is these chains that are mainly responsible for interaction with the surface of the cells and for distinguishing between these surfaces. The fact that erythrocytes from different species exhibit different adsorption profiles on Seph-C_n, the fact that small but reproducible differences can be detected, even with erythrocytes from two inbred strains of mice[15], and finally the effective separation of erythrocytes, from lymphocytes reported above, seem to

indicate that hydrophobic chromatography may find uses in the adsorption, resolution, probing and maybe immobilization of cells.

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