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Effect of the mobile phase on antibody-based enantiomer separations of amino acids in high-performance liquid chromatography

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Abstract

The effect of the mobile phase parameters flow rate, temperature, pH and ionic strength, as well as the addition of various organic modifiers on the enantiomer separation of various aromatic α-amino acids was investigated using two antibody-based chiral stationary phases that have opposing stereoselectivity. On both columns, a decrease in flow rate or temperature resulted in increased interaction with the retained enantiomer. It was found that the retention factor k_2 depends on the affinity between the analyte and the immobilized antibody and is not independent of the flow rate. Optimum separations of all amino acids investigated were obtained at pH 7.4 on both columns. While increased k_2 values were obtained at low ionic strength on the anti-D-amino acid antibody column, no such effect was observed on the anti-L-amino acid antibody column. The addition of organic modifiers did not improve separations. In all studies, the unretained enantiomer eluted with the void volume.

Keywords: Enantiomer separation; Antibodies; Chiral stationary phases, LC; Mobile phase composition; Amino acids; Proteins

1. Introduction

One of the most popular techniques for the analysis and direct separation of enantiomers is HPLC utilizing a chiral stationary phase (CSP). Chiral selectors commonly used for the preparation of CSPs include oligo- and polysaccharides, macrocyclic antibiotics, alkaloids, synthetic polymers, π-donor/π-acceptor systems, crown ethers, ligand exchange selectors, and various proteins [1,2]. Despite their success, these chiral selectors are generally not tailor-made for specific analytes, which can make the identification of a suitable CSP a time-consuming exercise. We have recently demonstrated that tailor-made CSPs for the direct separation of enantiomers in HPLC can be prepared by immobilizing suitably raised stereoselective antibodies onto a solid support material [3]. Using the stereoselective interaction between monoclonal anti-α-amino acid antibodies and D- and L-amino acids as a model system, we showed that such immunoaffinity stationary phases possess predicted selectivity and, if operated under mild isocratic conditions, are surprisingly stable. By employing phosphate buffered saline (PBS), pH 7.4, as the sole mobile phase, we were able to reuse the same antibody-columns for more than 2000 separations over a period of 3 years. This is in contrast to various other reports that demonstrated separation of stereoisomers in immunoaffinity systems [4–9]. In those studies, mixtures of enantiomers, dissolved in a neutral buffer, were passed through a column containing antibodies covalently linked to agarose beads [4–9] or silica [9]; after the unbound enantiomer was washed clear, elution of the bound enantiomer was achieved by altering to a mobile phase that disrupted its interaction with the antibody. As is typical for classical affinity and immunoaffinity chromatography systems [10–12], elution of bound material required harsh conditions, i.e., a drastic change in pH [9], or addition of organic solvents [4,6–8] or chaotropic salts [5]. Such severe elution conditions invariably cause protein denaturation and considerably shorten column lifetime, which rarely exceeded 100 separation cycles [9].

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Antibodies are glycoproteins, which are produced by the immune system of vertebrates in response to invading pathogenic microorganisms and "non-self" biological material, called antigens. Through their specific interaction with discrete structures on the antigen they mark it for elimination by other components of the immune system such as phagocytes and the complement system [13]. It is well known that antibodies can be raised against low-molecular weight compounds, haptens, if these are conjugated to suitable carriers, e.g., proteins; such antibodies may be stereoselective and may distinguish between the enantiomers of chiral haptens [14]. Despite the fact that antibodies are made from chiral building blocks, namely L-amino acids and sugars, and therefore possess an inherent chirality, their stereoselectivity appears to be primarily based on the specific interaction between amino acid residues in the binding site and complementary functional groups and moieties in the hapten structure [3]. The most abundant antibodies in blood are immunoglobulins of the IgG class, which possess two identical binding sites and have a molecular weight of approximately 150,000 Da [13]. As is the case for other types of protein–ligand interactions, such as enzyme-substrate, receptor-hormone, or lectin-sugar systems, the binding forces between an antibody and an antigen are of purely non-covalent nature and involve electrostatic interactions, electron acceptor–electron donor forces (hydrogen bonds), and non-polar interactions (Lifshitz-van der Waals forces) [15,16]. The strength of interaction, therefore, is affected by environmental parameters such as the temperature, and the pH, ionic strength and polarity of the solvent in which binding occurs. The effect of the mobile phase on protein–ligand interactions has successfully been utilized in a number of protein-based chiral separations to modulate the interaction between the immobilized chiral selector and the analyte, and to optimize separations [17,18].

Here, we describe how the mobile phase parameters flow rate, temperature, pH and ionic strength, as well as the addition of various organic modifiers influence enantiomer separation of several aromatic α-amino acids on two antibody-based CSPs that possess "opposing stereoselectivities" and bind to either D- or L-α-amino acids.

2. Experimental

2.1. Chemicals

POROS-OH was obtained from PerSeptive Biosystems (Cambridge, MA). *N*,*N*′-disuccinimidyl carbonate (DSC) and dimethylaminopyridine (DMAP) were from NovaBiochem (La Jolla, CA), HPLC-grade acetonitrile, ethanol, methanol, 1-propanol, and 2-propanol were purchased from Sigma (St. Louis, MO). Inorganic salts were from ACROS/Fisher (Fair Lawn, NJ); all other chemicals were from Sigma (St. Louis, MO).

Water was purified using a MilliQ water system (Millipore, Bedford, MA). Phosphate buffered saline (PBS) was prepared according to reference [19] and adjusted to pH 7.4 with 0.1 N HCl. All amino acids were of the highest purity available. D-Tryptophan, D- and L-phosphotyrosine, D- and L-*p*-aminophenylalanine, and D- and L-phenylalanine were purchased from Sigma (Deisenhofen, Germany). L-Tryptophan and D-tyrosine were from Aldrich (Munich, Germany), D- and L-DOPA were from Fluka (Neu-Ulm, Germany). L-Tyrosine was kindly provided by Degussa (Frankfurt, Germany).

2.2. Monoclonal antibodies

Monoclonal antibodies were produced as previously described [20]. In brief, 8-week-old BALB/c mice were immunized with conjugates of keyhole limpet hemocyanin and either *p*-amino-D-phenylalanine or *p*-amino-L-phenylalanine, prepared by diazotization, in complete Freund's adjuvant following a standard immunization protocol. For the production of monoclonal antibodies splenocytes were fused with NS0 myeloma cells using polyethylene glycol and hybridomas were selected in hypoxanthine/aminopterin/thymidine medium [21]. Large quantities of the anti-D-amino acid antibody secreted by clone 67.36 and the anti-L-amino acid antibody produced by clone 29.2 were obtained by the preparation of ascites fluid. The antibodies were purified by ammonium sulfate precipitation followed by ion-exchange chromatography on DEAE-Sephacel (Amersham Biosciences, Piscataway, NJ). The antibodies did not contain any impurities as determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The binding characteristics of the anti-amino acid antibodies have been described in detail elsewhere [3,20,22]. Their affinities are highest for structures that resemble the hapten, i.e., bear aromatic side chains; however, also amino acids with different side chains, including aliphatic, charged and non-charged residues, are stereoselectively recognized by the respective antibodies. The exquisite stereoselectivity of the antibodies has previously been utilized in enzyme-linked immunosorbent assays [20,22,23], flow-injection immunoassays [24,25], immunosensors [26–28], and chromatography [3,20] for enantiomer detection and separation.

2.3. Chiral stationary phase

Following activation with DSC and DMAP, the POROS-OH stationary phase material was reacted with either the anti-D-amino acid antibody or the anti-L-amino acid antibody as described elsewhere [3]. In brief, 3.5 g POROS-OH (20 μm particles) were reacted with 350 mg DSC and 287 mg DMAP in 17.5 mL dry acetone for 1.5 h at 4 °C. One fraction of the support material (1 g) was reacted overnight with 30 mg of the anti-D-amino acid antibody in PBS at 4 °C, while the other fraction (1.5 g) was reacted with 50 mg of the anti-L-amino acid antibody overnight at 4 °C under salting-out conditions. Remaining active groups on the support were quenched by treatment with 0.2 M TRIS for 1 h, followed by extensive washing. The concentration of immobilized an-

tibody was determined using the dye binding method published by Bonde et al. [29], and was found to be as follows: anti-D-amino acid antibody column, 12 mg per gram of support material (wet); anti-L-amino acid antibody column, 15.5 mg/g. As previously reported, differences in column performance of the two antibody-columns are based on the fact that the anti-D-amino acid antibody 67.36 has a higher affinity towards D-amino acids than the anti-L-amino acid antibody 29.2 has towards the corresponding L-enantiomer [3]. In addition, it was found that the column capacities of the POROS-based antibody-columns are too low to enable enantiomer separation of aliphatic amino acids [3]. Genetic analysis of the antibody-binding sites has recently revealed that both antibodies possess considerable differences in their amino acid sequences and that they belong to different families [30]. Thus, it can be expected that the two antibodies interact with their corresponding binding partners via different amino acids, and that these interactions may show different susceptibilities to changes of environmental parameters.

2.4. Chromatography and instrumentation

The HPLC system consisted of a Hitachi L-7100 pump with a degasser, an L-7400 UV-detector equipped with an analytical flow cell, and a D-7000 interface with System Manager V 4.0 software. Injections were performed using a Rheodyne 7725i injection valve with a 20 μL loop (Hitachi, Naperville, IL). Columns were packed using an Alltech Slurry Packer Model 1666 (Alltech, Deerfield, IL).

Stainless steel columns (anti-D-amino acid antibody column: 2.3 mm × 200 mm; anti-L-amino acid antibody column: 4.6 mm × 250 mm) were slurry packed at a pressure of 160 bar in PBS. No leakage of antibody was detected during column equilibration. Unless stated otherwise, all chromatographic separations were performed at room temperature under isocratic conditions using PBS, pH 7.4, as mobile phase. Columns were stored under azide-containing PBS at 4 °C only when not used for an extended period of time. Flow rates used in this study varied from 0.5 to 4.5 ml/min. Mixtures of the pure enantiomers in PBS (10 μL) were injected. Tryptophan was detected at 280 nm, *p*-aminophenylalanine at 240 nm, phosphotyrosine at 254 nm, tyrosine and DOPA at 220 nm, and phenylalanine at 205 nm. The elution order was determined by injection of the pure enantiomers as well as by spiking. The void volume for the determination of chromatographic data was measured using water or buffer [31]. For the short retention times obtained in this study, errors in the determination of the void volume affect the calculation of chromatographic parameters. Slight variations may be explained by this fact. In addition, the results described in this paper were obtained over a period of approximately 2 years during which the antibody columns were subjected to various conditions, e.g., organic modifiers, which may have affected the structural integrity of the immobilized antibodies. To ensure the reproducibility of the results, all measurements were carried out at least in triplicate. Standard deviation of chromatographic parameters was typically less than 10%.

2.4.1. Influence of the flow rate

The effect of the flow rate on antibody-based chiral separations was investigated at flow rates between 0.5 ml/min and 4.5 ml/min. Due to the higher affinity of the anti-D-amino acid antibody towards D-amino acids, compared to the anti-L-amino acid antibody towards L-amino acids, more amino acids, namely DOPA, *p*-aminophenylalanine, phenylalanine, phosphotyrosine, and tryptophan could be baseline separated on the anti-D-amino acid column. In contrast, only *p*-aminophenylalanine, phenylalanine, and tyrosine yielded calculable results on the anti-L-amino acid column.

2.4.2. Effect of temperature

The temperature dependence of the enantiomer separation of D,L-phenylalanine was studied between 5 °C and 40 °C. Antibody columns and buffer reservoir were immersed in a water bath (Haake, Berlin, Germany), and the temperature was increased in increments from 5 °C to 40 °C. The antibody columns were equilibrated for at least 1.5 h before the corresponding series of injections were performed at the respective temperatures.

2.4.3. Effect of pH

The effect of the pH on enantiomer separations was studied using 10 mM phosphate buffer ranging from pH 4.5 to pH 10, to which sodium chloride and potassium chloride were added to yield final concentrations of 2.7 mM KCl and 137 mM NaCl, respectively, which corresponds to their molarity in PBS. Columns were equilibrated with each buffer for at least 1.5 h. Separations using the anti-D-amino acid antibody column were carried out at flow rates of 1 ml/min for *p*-aminophenylalanine, tryptophan, and DOPA and 2 ml/min for phenylalanine. Separations of tyrosine, phenylalanine and *p*-aminophenylalanine on the anti-L-amino acid antibody column were carried out at 1 ml/min.

2.4.4. Effect of ionic strength

To study the influence of the ionic strength on the separation of D,L-phenylalanine, the molarities of NaCl and KCl contained in the phosphate buffer mobile phase were changed by adding the chlorides to a solution of 8.5 mM Na_2HPO_4 and 1.5 mM KH_2PO_4 adjusted to pH 7.4. The antibody columns were equilibrated for at least 1.5 h before the corresponding series of injections were performed at the different ionic strengths. Separations were carried out at flow rates of 2 ml/min using the anti-D-amino acid antibody column and 1 ml/min on the anti-L-amino acid antibody column, respectively.

2.4.5. Effect of organic modifiers

To study the effect of organic modifiers on the separation of D,L-phenylalanine, the organic modifiers methanol, ethanol, 1-propanol, 2-propanol, and acetonitrile were added

to the PBS buffer. Using the anti-D-amino acid antibody column, the following maximum percentages of organic modifiers were employed: methanol and acetonitrile, 5% (v/v); ethanol, 9%; 1-propanol and 2-propanol, 8%. Using the anti-L-amino acid antibody column all organic modifiers were employed up to a maximum of 5%, except acetonitrile, which was added up to 3%. Flow rates of 1 ml/min and 2 ml/min were used with the anti-L-amino acid antibody column and the anti-D-amino acid antibody column, respectively.

3. Results and discussion

3.1. Influence of the flow rate

Both chiral stationary phases used in this study were prepared by immobilizing stereoselective monoclonal antibodies to either D- or L-α-amino acids onto the synthetic high flow-through type perfusion material POROS [32,33]. This support contains a network of large throughpores, which allow the liquid phase to flow through the sorbent and enable convective transport within the particle. Thus, diffusional mass transfer limitations are overcome, and, based on the reduced flow-resistance of the packing, analyses can be performed at higher flow rates compared to conventional silica supports [32,34]. In order to evaluate the effect of the flow rate on antibody-based chiral separations, several aromatic amino acids were investigated under isocratic conditions using PBS, pH 7.4, as mobile phase at flow rates of up to 4.5 ml/min. DOPA, *p*-aminophenylalanine, phenylalanine, phosphotyrosine, and tryptophan were studied using the anti-D-amino acid column, while *p*-aminophenylalanine, phenylalanine, and tyrosine were separated on the anti-L-amino acid column. As exemplified in Fig. 1, which shows the separation of D,L-phenylalanine on the anti-L-amino acid column at 1 ml/min and 4 ml/min, increasing the flow rate leads to an improvement in peak shape and enables rapid separations within a few minutes. Plots of R_s versus flow rate for the anti-L-amino acid column (Fig. 2) show a considerable decrease in resolution as the flow rate is increased. The same general trend was observed using the anti-D-amino acid column for all amino acids investigated. With the perfusion material, however, the loss in resolution is somewhat smaller than predicted by the theory for conventional supports. According to the extended plate height equation for large-pore supports developed by Rodrigues [35,36], the plate height becomes independent of the mobile phase velocity at high velocities, and the resolution R_s is expected to be independent of the flow rate. This, however, may not hold true for affinity-based separation systems, where the slow kinetics of non-covalent molecular interactions can contribute to an extra term in the plate height equation, which is proportional to the velocity. A decrease in column efficiency was also observed with both antibody columns studied here. The plate number *N* for the separation of phenylalanine on the anti-L-amino acid column, for example, dropped from 1080/m at 1 ml/min to 580/m at 4 ml/min. A similar loss in efficiency was observed using the anti-D-amino acid column, where plate numbers were lower by a factor of eight. The overall low efficiencies can be attributed to the slow dissociation kinetics, the large particle size of the POROS material as well as the high flow rates. In a previously published study on the separation of various amino acid derivatives and drugs using POROS-immobilized bovine serum albumin as a chiral stationary phase [37], we found that although the loss in resolution at increased flow rates is less significant than predicted by the theory for conventional supports, it is not independent of the flow rate as suggested by the Rodrigues equation. Based on chromatographic theory, the product of the resolution R_s and the square root of the flow rate $\sqrt{F}$ is expected to be constant. Therefore, the product of $\sqrt{F'}$, which represents the square root of the ratio of two different flow rates, and the ratio R'_s of the resolutions at these flow rates should have a value of one [37]. Table 1 summarizes the relationship between the change in flow rate and the change in actual resolution R'_s for the enantiomer sep-

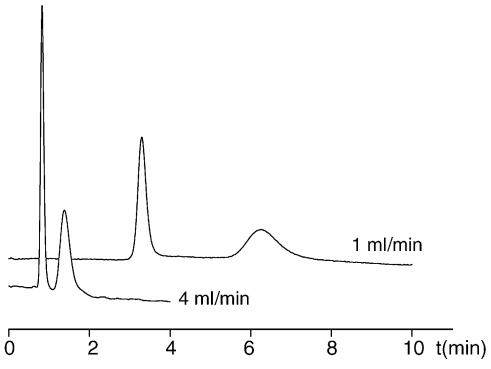

Fig. 1. Enantiomer separation of D,L-phenylalanine on the anti-L-amino acid column at 1 ml/min and 4 ml/min. The first peak corresponds to the D-enantiomer, the second to the L-enantiomer. Separations were carried out at RT using PBS as mobile phase. Other conditions are given in Section 2.

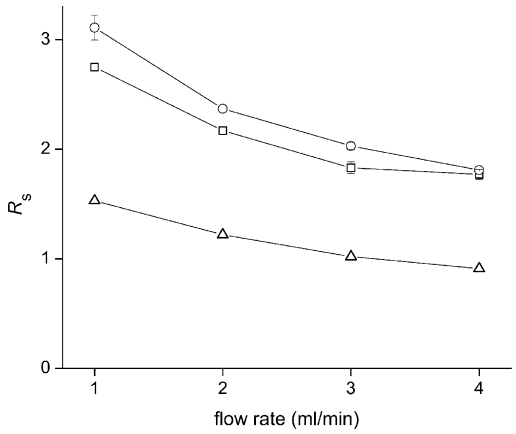

Fig. 2. Resolution values of the enantiomer separations of phenylalanine (○), tyrosine (□), and *p*-aminophenylalanine (△) on the anti-L-amino acid column in PBS at different flow rates. Separations were carried out at RT. Other conditions are given in Section 2. Values represent means of triplicate determinations. Missing error bars are obscured by the symbols.

Table 1
Relationship between the change in flow rate and the change in actual resolution R'_s

Chiral selector	Analyte	$F_2/F_1=F'$	$\sqrt{F'}$	$R_{s,2}/R_{s,1} = R'_s$	$R'_s\sqrt{F'}$
Anti-L-amino acid antibody	*p*-Aminophenylalanine	4/1 = 4	2	0.91/1.53=0.59	1.19
		3/1 = 3	1.73	1.02/1.53 = 0.67	1.16
	Tyrosine	4/1 = 4	2	1.71/2.75 = 0.62	1.24
		3/1 = 3	1.73	1.83/2.75 = 0.67	1.16
	Phenylalanine	4/1 = 4	2	1.81/3.11 = 0.58	1.16
		3/1 = 3	1.73	2.03/3.11 = 0.65	1.12
Anti-D-amino acid antibody	Phosphotyrosine	4/1 = 4	2	0.69/1.17=0.59	1.18
		3/1 = 3	1.73	0.8/1.17 = 0.68	1.18
	DOPA	4/0.5 = 8	2.83	0.78/1.73 = 0.45	1.27
		3/0.5 = 6	2.45	0.98/1.73 = 0.56	1.37
	Tryptophan	4/1 = 4	2	1.01/1.62 = 0.62	1.24
		3/1 = 3	1.73	1.07/1.62 = 0.66	1.14
	Phenylalanine	4/2 = 2	1.41	0.92/1.19 = 0.77	1.09
		4/3 = 1.33	1.15	0.92/1.03 = 0.89	1.02
	p-Aminophenylalanine	4.5/2 = 2.25	1.5	1.19/1.84 = 0.65	0.98
		4/2 = 2	1.41	1.20/1.84 = 0.66	0.93

Conditions: PBS, pH 7.4; room temperature.

aration of several amino acids on both antibody columns. In most cases, the values of $R'_s\sqrt{F'}$ are greater than one, which indicates that the perfusion-type support material has some advantage over conventional supports. However, a slightly higher loss in resolution at increased flow rates was observed for the enantiomer separations of *p*-aminophenylalanine on the anti-D-amino acid column, which might be based on the higher affinity of the D-enantiomer of this amino acid towards the immobilized antibody. The major advantage of the perfusive support appears to be the greater range of flow rates that can be applied; this is especially valuable in cases where the retained enantiomer possesses a higher affinity towards the immobilized antibody, which would lead to long retention times and extreme peak-broadening at lower flow rates [3]. The use of higher flow rates in such cases allows relatively rapid elution of the retained enantiomer under mild isocratic buffer conditions as a well-defined peak.

A plot of the retention factor *k* as a function of the flow rate (Fig. 3) suggests that, for the immunoaffinity-based enantiomer separations investigated here, *k* is not independent of the flow rate as would be expected according to chromatographic theory for isocratic separations. As exemplified in Fig. 3, the decrease in *k* with increasing flow rates appears to be more prominent for analytes that have a relatively higher affinity towards the immobilized antibody. Thus, the k_2 values for D-*p*-aminophenylalanine decrease from 7.33 at 2 ml/min to 5.60 at 4.5 ml/min on the anti-D-amino acid antibody column, while k_2 for D-phosphotyrosine remains almost constant between 1 ml/min and 4 ml/min (Fig. 3a). Similarly, the decrease in k_2 is more prominent for L-tyrosine than for L-*p*-aminophenylalanine on the anti-L-amino acid antibody column (Fig. 3b). This disparity may be based on different dissociation rate constants. While association rate constants of antibody–hapten interactions are typically high and may

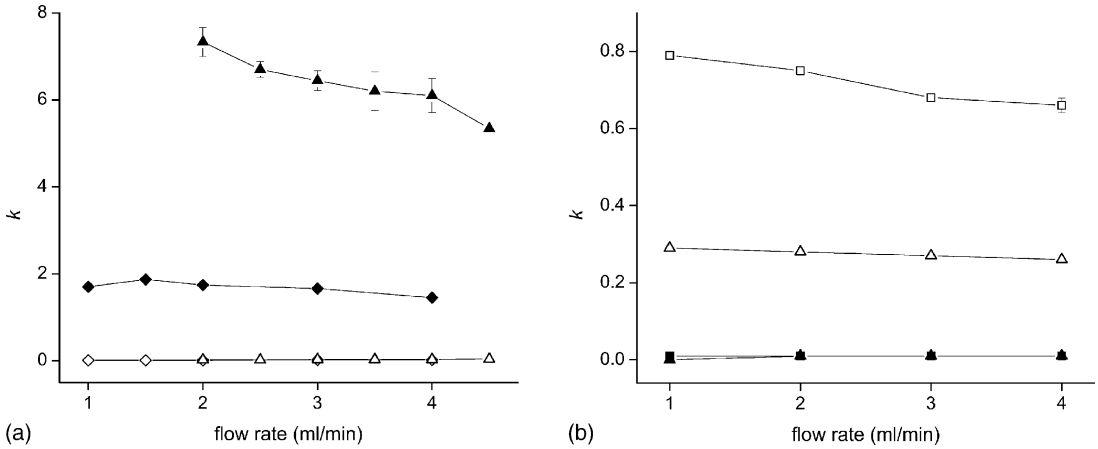

Fig. 3. Relationship between the retention factor *k* and the flow rate. Separation of (a) *p*-aminophenylalanine (triangles) and phosphotyrosine (diamonds) on the anti-D-amino acid column, and of (b) tyrosine (squares) and *p*-aminophenylalanine (triangles) on the anti-L-amino acid column. Filled symbols represent the D-enantiomer, open symbols the L-enantiomer. Separations were carried out at RT using PBS as mobile phase. Other conditions are given in Section 2. Values represent means of triplicate determinations. Missing error bars are obscured by the symbols.

approach the diffusion-controlled limit, the affinity of the interaction is primarily determined by the dissociation rate constant. Thus, analytes that are more strongly bound, that is, have a higher affinity towards the immobilized antibody, dissociate slower from the CSP. We believe that the local concentration ratio of free versus bound analyte decreases at high flow rates due to dilution effects, and that this reduces the amount of dissociating analyte that is rebound. If both the association and dissociation kinetics are fast enough, as is the case for more weakly bound analytes, such a dilution effect will not be discernible. However, if the dissociation step is slow and the flow rate high enough, such an effect could result in a relatively lower retention at higher flow rates, which would manifest itself in a decrease in k. It should be noted that on both columns the k_1 values for the first eluting enantiomer (that is, the L-enantiomer on the anti-D-amino acid column and the D-enantiomer on the L-amino acid column) were close to zero and independent of the flow rate; this indicates that no noticeable specific or non-specific interactions between the CSP and this enantiomer occur. This, furthermore, implies that the retention of the second eluting enantiomer is solely based on specific interactions with the CSP.

3.2. Effect of temperature

It is well known that temperature changes can have a dramatic effect on chiral separations using protein CSPs. An increase in temperature typically results in decreased analyte retention, although unusual effects have been reported [38]. Also the interaction between antibodies and antigens may diminish with increasing temperature [39], which is typically a result of a decrease in the negative value of ΔH, the enthalpic component of the free-energy change of binding. However, a loss in enthalpy is often compensated by a gain in entropy, which can be explained by increased dehydration of the protein at elevated temperatures, and the release of previously oriented water molecules into the solvent [16]. The overall effect of temperature changes, therefore, is difficult to predict and may actually be negligible [15,16].

In order to study the effect of temperature on antibody-based chiral separations and to compare the anti-L-amino acid antibody CSP with the anti-D-amino acid antibody CSP, D,L-phenylalanine was separated on both columns at temperatures between 5 °C and 40 °C. Higher temperatures were not investigated to avoid protein denaturation. As seen in Fig. 4, on both columns an increase in temperature leads to reduced retention times of the second peak, while the retention time of the unretained enantiomer does not change. Plots of ln k versus $1/T$ show a non-linear behavior of k_2 (Fig. 5). Non-linear van't Hoff plots have been reported for protein-based chiral separations [38,40] as well as for antibody-antigen interactions [41], and are usually attributed to temperature-induced changes of the protein conformation. A detailed study by Theriault et al. [42] on the interaction between several anti-DNP antibodies and DNP-derivatized dipeptides, for example, demonstrated that the anomalous temperature-dependence of the antibody AN01 is related to structural heterogeneity in the antibody binding site. While the antibody is structurally constrained and remains in a single conformation at 5 °C, it can assume multiple conformations at 45 °C.

The van't Hoff plot for L-phenylalanine on the anti-L-amino acid column (Fig. 5a) is linear at temperatures between 37.5 °C and 20 °C ($y = -27.88 + 8.09x$; $R = 0.999$) and between 20 °C and 5 °C ($y = -15.85 + 4.55x$; $R = 0.999$). This biphasic behavior suggests that the anti-L-amino acid antibody undergoes a conformational change around 20 °C. The temperature-dependence of the interaction of D-phenylalanine with the anti-D-amino acid antibody (Fig. 5b) appears to be more complex and the non-linear plot can be divided into at least three phases with transition temperatures around 15 °C and 37.5 °C. However, the positive slopes of the van't Hoff plots that are obtained for the retained enantiomer on both columns indicate that the interaction between the L-enantiomer and the anti-L-amino acid antibody as well

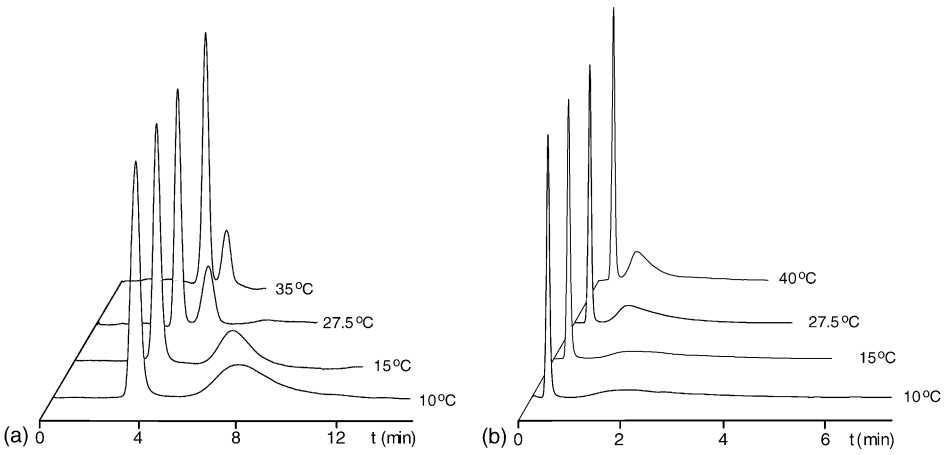

Fig. 4. Enantiomer separation of phenylalanine at different temperatures on the anti-L-amino acid column at 1 ml/min (a) and on the anti-D-amino acid column at 4 ml/min (b) using PBS as mobile phase. Other conditions are given in Section 2. The D-enantiomer elutes first in (a) and second in (b), while the elution of the L-enantiomer is vice versa.

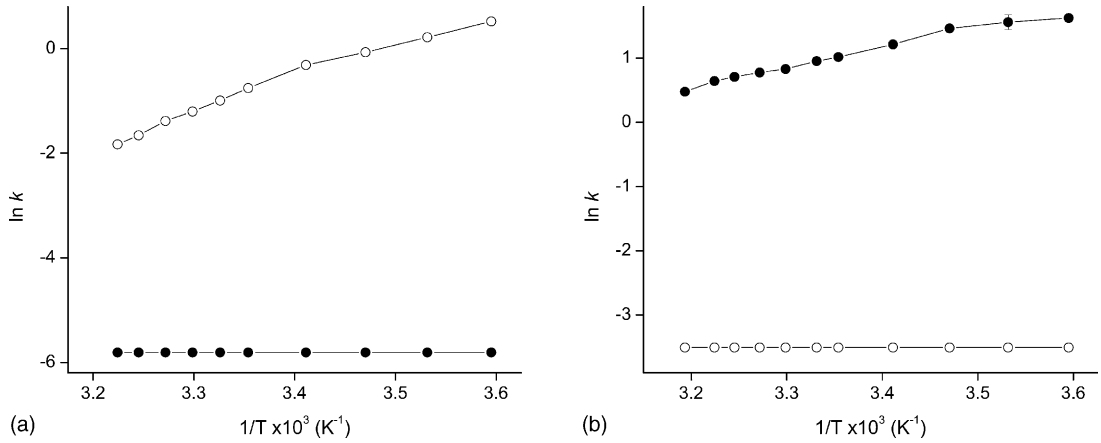

Fig. 5. Van't Hoff plots for the enantiomer separation of phenylalanine on the anti-L-amino acid column at 1 ml/min (a) and on the anti-D-amino acid column at 4 ml/min (b). Separations were carried out using PBS as mobile phase. Filled circles represent the D-enantiomer, open circles the L-enantiomer. Values represent means of triplicate determinations. Missing error bars are obscured by the symbols.

as between the D-enantiomer and the anti-D-amino acid antibody is enthalpy-driven. Since temperature changes do not affect the interaction of the unretained enantiomer with the CSPs, a higher selectivity for the enantiomer separation can be achieved on both columns by lowering the temperature. The resolution on the anti-L-amino acid column at 1 ml/min shows a maximum at 20 °C (R_s = 2.34), which shifts to 15 °C at higher flow rates. The resolution on the anti-D-amino acid column at 2 ml/min, on the other hand, drops between 10 °C (R_s = 1.77) and 20 °C (R_s = 1.19) and remains constant at higher temperatures, but is constant (R_s = 1.20) at all temperatures at higher flow rates.

As described above, our studies on the influence of the flow rate on the retention factor *k* demonstrated that, depending on the affinity between the analyte and the CSP, *k* may decrease with an increase in flow rate (Fig. 3). Our observation that the strength of interaction between the retained enantiomer and the immobilized antibody can be modulated by changing the temperature, therefore, prompted us to investigate the influence of the flow rate on the retention factor at different temperatures. For that, the enantiomers of phenylalanine were separated on both columns at various flow rates and at the highest and lowest temperature that yielded calculable results. As seen in Fig. 6, the retention factor for the retained enantiomer (k_2) decreases significantly at 5 °C, where the affinity to the immobilized antibody is the highest. In contrast, k_2 remains constant at the investigated flow rates at 30 °C on the anti-L-amino acid column and at 40 °C on the anti-D-amino acid column, respectively. This result supports our theory that the effect of the flow rate on the retention factor depends on the affinity of the studied system.

3.3. Effect of pH

The pH of the aqueous mobile phase can have a significant influence on chiral separations using protein CSPs. In general, changes in pH appear to have a stronger effect on charged analytes than on uncharged ones [17]. A convenient

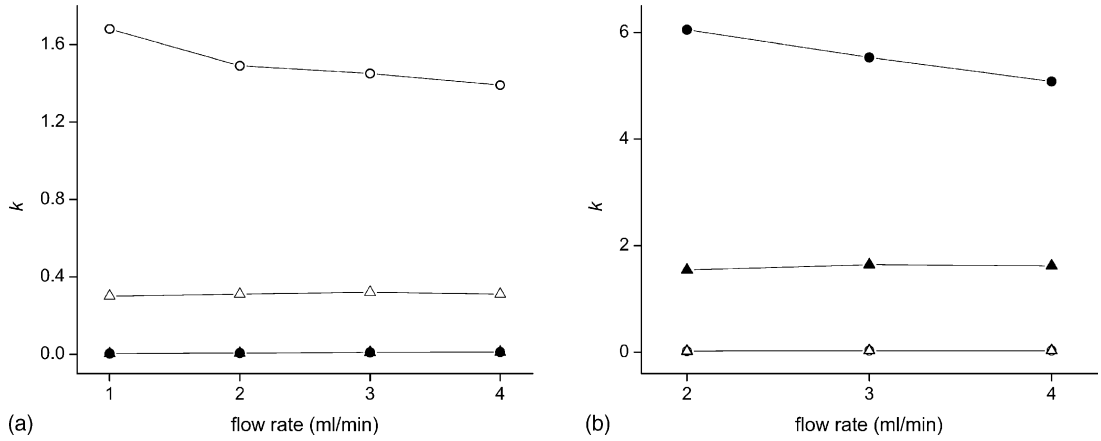

Fig. 6. Relationship between the retention factor *k* and the flow rate at different temperatures. Enantiomer separation of phenylalanine (a) at 5 °C (circles) and 30 °C (triangles) on the anti-L-amino acid column, and (b) at 5 °C (circles) and 40 °C (triangles) on the anti-D-amino acid column. Filled symbols represent the D-enantiomer, open symbols the L-enantiomer. Separations were carried out using PBS as mobile phase. Other conditions are given in Section 2. Values represent means of triplicate determinations; error bars are obscured by the symbols.

explanation is that an increase in pH reduces the number of positively charged amino acid residues on the protein, which results in a decrease of electrostatic interactions with negatively charged analytes, e.g., carboxylic acids. A decrease in pH, on the other hand, reduces the number of negatively charged residues on the protein, thus reducing the interaction with cationic analytes [17]. While this behavior is reminiscent of the binding of charged analytes to ion-exchange materials, the pH-dependence of specific protein–ligand interactions within distinct binding sites often appears to be more complex. The binding site may contain buried or partly buried ionizable groups with perturbed pK_a values that are stabilized by charges on the protein surface [43]. Electrostatic interactions between proteins and ligands may also be mediated through metal counter-ions complexed within the binding site by appropriate pH-sensitive amino acid residues [16,44]. Changes in pH may furthermore induce conformational changes in the protein that interfere with the correct positioning of amino acid residues participating in ligand binding [45,46]. Such conformational changes may be restricted to certain loops or domains, or, especially at extremely high or low pH, may affect the overall structure and lead to protein denaturation [47]. In order to minimize the risk of denaturation of the antibody CSPs, we limited our studies of the effect of the mobile phase pH to the range between pH 4.5 and pH 10.

Plots of the retention factor k versus pH show optima of k_2 at the physiological pH of 7.4 for all amino acids investigated here (Fig. 7). Similarly, the resolution values on both columns are highest at this pH. A higher or lower pH of the mobile phase results in reduced interaction between the retained enantiomer and the immobilized antibody. Increasing or decreasing the pH, however, does not affect the interaction with the opposite enantiomer, which at all pH values elutes with the void volume. While a change in pH will eventually also affect the degree of protonation of ionizable groups in the analyte, here aromatic amino acids, the observed pH effect is likely to be primarily based on changes within the antibody binding site. While the carboxyl groups of the aromatic amino acids investigated here have pK_a values ranging between ca. 1.8 (Phe) and 2.4 (Trp), the α-amino groups have pK_a values between 9.1 (Phe) and 9.4 (Trp). Thus, the analytes will predominantly be in the zwitterionic form at pH values between the lowest pH investigated here, pH 4.5, and approximately pH 8.1. This means that the decrease in interaction between analyte and antibody at pH values below pH 8 is not due to changes in the analyte structure. However, the aromatic amino group of *p*-aminophenylalanine, which has a pK_a at around pH 4.6, will become protonated at pH values lower than 5.6; this additional positive charge may be the reason for the drastic drop in k_2 observed with this amino acid on the anti-D-amino acid column. The decrease in interaction between analyte and antibody at pH values above pH 8 may be based on both the deprotonation of the α-amino groups of the analyte as well as changes in the antibody binding site.

3.4. Effect of ionic strength and organic modifiers

The variation of the ionic strength of the mobile phase buffer and the addition of organic modifiers are among the most widely used means to influence retention and resolution in protein-based chiral separations [17,18]. A decrease in ionic strength favors electrostatic interactions between charged analytes and opposite charges on the protein; consequently, these interactions are weakened at high ionic strength. However, an increase in the salt concentration may also cause dehydration, which, in turn, can increase hydrophobic interactions [16]. Organic modifiers, on the other hand, effectively reduce hydrophobic interactions, but are known to bear a considerable risk of denaturing proteins. In this study, the effect of ionic strength was investigated on both antibody columns by separating the enantiomers of the model analyte phenylalanine at ionic strengths ranging from 60 mM to 200 mM. As seen in Fig. 8a, the retention

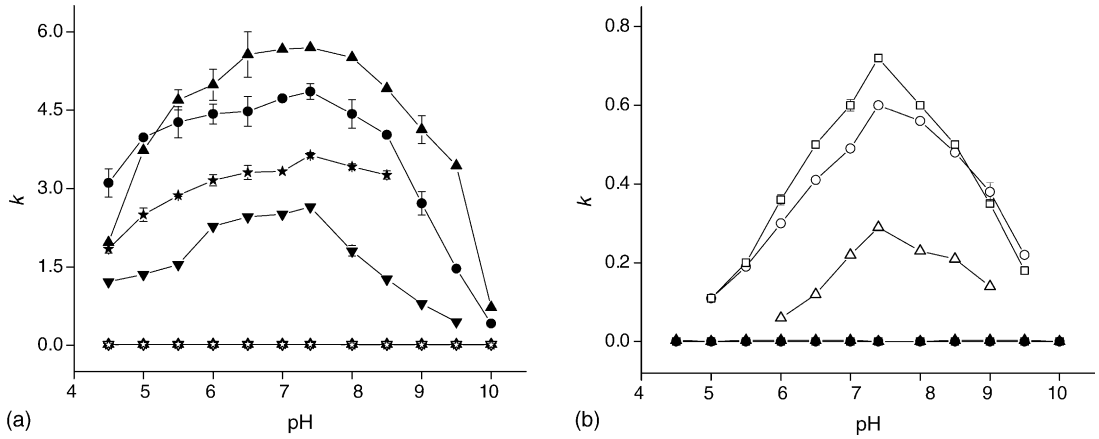

Fig. 7. Relationship between the retention factor k and the pH. Separation of (a) *p*-aminophenylalanine (triangles), phenylalanine (circles), tryptophan (stars), and DOPA (upside-down triangles) on the anti-D-amino acid column, and of (b) tyrosine (squares), phenylalanine (circles) and *p*-aminophenylalanine (triangles) on the anti-L-amino acid column. Filled symbols represent the D-enantiomer, open symbols the L-enantiomer. Chromatographic conditions are given in Section 2. Values represent means of triplicate determinations. Missing error bars are obscured by the symbols.

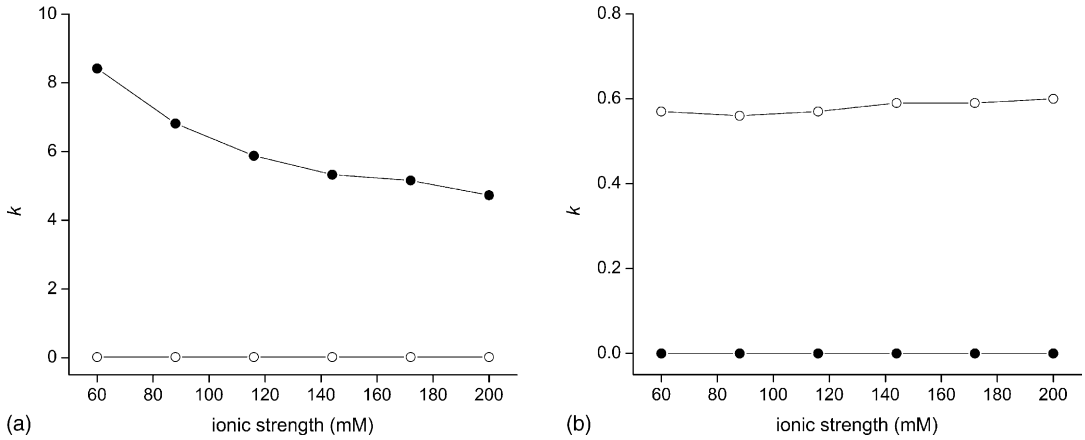

Fig. 8. Influence of the ionic strength on the retention factor k of the enantiomer separation of phenylalanine on the anti-D-amino acid column at 2 ml/min (a) and on an anti-L-amino acid column at 1 ml/min (b). Filled symbols represent the D-enantiomer, open symbols the L-enantiomer. Separations were carried out at RT using phosphate buffers of different ionic strength. Other conditions are given in Section 2. Values represent means of triplicate determinations. Missing error bars are obscured by the symbols.

of the D-enantiomer on the anti-D-amino acid column was significantly influenced by the ionic strength of the buffer, being highest at low ionic strength. Similarly, the resolution decreased from 60 mM to 200 mM (from R_s = 2.45 to R_s = 1.88). Interestingly, no such effect was observed for the retention of the L-enantiomer on the anti-L-amino acid column, where k_2 (Fig. 8b) as well as the resolution (R_s = 1.93) remained constant. This difference in susceptibility to changes in the ionic strength may be based on dissimilarities in the mode of interaction between the two antibodies and their binding partners. Although preliminary structural studies indicate considerable differences in the amino acid sequences within the binding sites of the two antibodies [30], only X-ray crystallographic analyses, which are currently under way, will allow elucidation of the molecular basis of interaction between the anti-D-amino acid antibody and D-amino acids, and between the anti-L-amino acid antibody and L-amino acids. On neither column did a change in the ionic strength of the mobile phase buffer affect the retention of the unretained enantiomer, which eluted with the void volume (Fig. 8).

Overall similar results were obtained with both columns upon addition of the organic modifiers methanol, ethanol, 1-propanol, 2-propanol, and acetonitrile (Fig. 9). An increase in the concentration of ethanol, 1-propanol, 2-propanol, or acetonitrile resulted in a significant reduction in interaction between the immobilized antibody and the retained enantiomer on both columns. An analogous behavior was observed for the resolution, which decreased upon addition of these modifiers. Methanol, in contrast, had no discernible effect on k_2 or the resolution. It should be noted that the addition of organic modifiers had a considerable deteriorating effect on column efficiency, which ultimately led to reduced retention even in the absence of modifier. Especially acetonitrile was found to negatively affect the anti-D-amino acid antibody, which

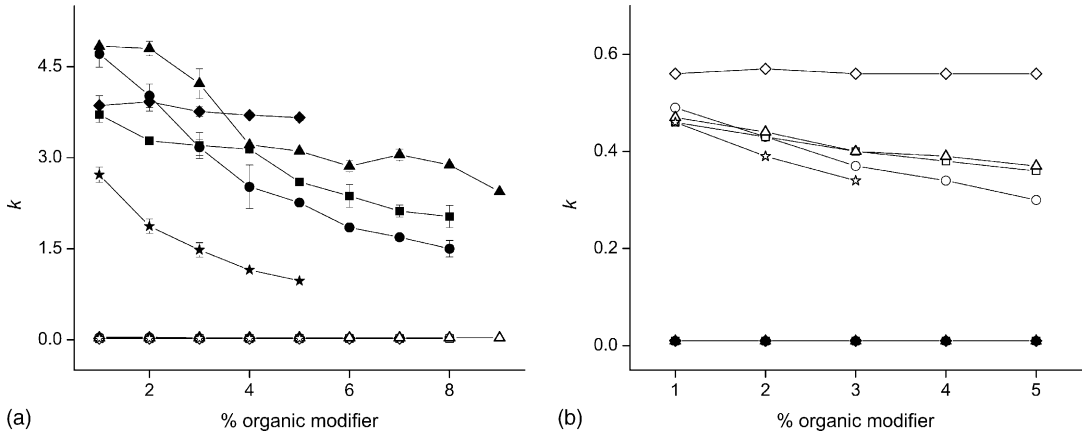

Fig. 9. Influence of the organic modifiers methanol (diamonds), ethanol (triangles), 1-propanol (circles), 2-propanol (squares), and acetonitrile (stars) on the retention factor k of the enantiomer separation of phenylalanine on the anti-D-amino acid column at 2 ml/min (a) and on the anti-L-amino acid column at 1 ml/min (b). Filled symbols represent the D-enantiomer, open symbols the L-enantiomer. Separations were carried out at RT using PBS containing modifier at the given percentages as additives. Other conditions are detailed in Section 2. Values represent means of triplicate determinations. Missing error bars are obscured by the symbols.

probably undergoes partial denaturation in the presence of this solvent. The reduced column capacity is obvious in the overall lower values of k_2 obtained on the anti-D-amino acid column in the presence of methanol, which was the last modifier whose influence was investigated.

4. Conclusions

In this study, the effect of the mobile phase parameters flow rate, temperature, pH, ionic strength, and organic modifiers on the enantiomer separation of several aromatic amino acids on two antibody-based CSPs was investigated. All results indicate that the retention of the second eluting enantiomer, i.e., the D-enantiomer on the anti-D-amino acid column and the L-enantiomer on the anti-L-amino acid column, is solely based on specific interactions between the analyte and the CSP, while the opposite enantiomer is not retained and elutes with the void volume. The use of a high flow-through type support material offers the possibility of increasing the flow rate without a significant increase in back-pressure, which leads to an improvement in peak shape and enables rapid separations. The observed loss in resolution at higher flow rates is generally lower than predicted by chromatographic theory for conventional silica supports. It was found that, depending on the affinity of the retained enantiomer toward the immobilized antibody, the retention factor k_2 is not independent of the flow rate as would be expected for separations under isocratic elution conditions. On both columns, a decrease in temperature resulted in increased values of k_2, which suggests that the binding is enthalpy-driven. Van't Hoff plots, however, are not linear, which may be due to temperature-induced conformational changes in the antibody-structure. The enantiomer separations of all amino acids investigated showed pH optima at pH 7.4 on both columns; while a decrease in k_2 and R_s at lower pH values is likely to result from changes in the antibody binding sites, a deterioration of the separation at higher pH values may also be caused by deprotonation of the analytes. Changes in the ionic strength of the mobile phase had different effects on the two antibody columns. While a decrease in ionic strength resulted in increased k_2 values and resolutions of D,L-phenylalanine on the anti-D-amino acid column, no effect was observed with the anti-L-amino acid column. The addition of the organic modifiers ethanol, 1-propanol, 2-propanol, and acetonitrile significantly reduced the interaction between the immobilized antibody and the retained enantiomer of phenylalanine on both columns; methanol, however, had no effect.

Our studies demonstrate that a variety of mobile phase parameters can be modulated to influence enantiomer separations on immunoaffinity columns. Although the two antibody-based CSPs show considerable similarities, the differences observed, particularly with regard to the susceptibility towards ionic strength, show that it is difficult to predict the influence of changes in the mobile phase. While optimum conditions for enantiomer separations on both columns combine a low flow-rate with a neutral buffer of pH 7.4 at a reduced temperature, only separations on the anti-D-amino acid column can be further improved by reducing the ionic strength of the mobile phase. Despite the fact that the two antibodies employed in this study bind to analytes having the same chemical structure (they just have opposite configuration), it is reasonable to assume that the physical-chemical forces involved in these interactions differ, and that changes in chromatographic conditions affect the antibody-analyte interactions in different ways.

Acknowledgements

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