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Resolution of serum aluminum-binding proteins by size-exclusion chromatography: identification of a new carrier of aluminum in human serum

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ABSTRACT

We have examined the use of TSK-GEL HW 55S for determining the distribution of aluminum in human serum by size-exclusion chromatography (SEC). In comparison to other SEC matrices, this material has less affinity for ionized aluminum and separates serum proteins and their aluminum complexes with greater resolution. This enabled the identification of a previously unknown protein carrier, provisionally called albindin, that binds aluminum with great stability. Albindin appears to be distinct from the previously described aluminum carriers albumin and transferrin and may be important in the pathogenesis of disease secondary to hyperaluminemia.

INTRODUCTION

Aluminum is known to participate in the pathogenesis of osteodystrophy and encephalopathy associated with chronic hemodialysis [1] and has been proposed to be involved in central nervous system degeneration in Alzheimer's disease (AD) [2–4] and the Parkinsonism dementia and amyotrophic lateral sclerosis syndromes of Guam [5]. Despite interest in the role of aluminum in these disorders, the processes determining the uptake, body distribution and excretion of aluminum are poorly understood.

Over 90% of the aluminum in normal and uremic serum is non-dialyzable, suggesting it is bound to serum proteins [6]. Previous workers have employed size-exclusion chromatography (SEC) on soft-gel media to identify serum carriers for aluminum. King *et al.* [7] employed atomic absorption spectroscopy (AAS) to monitor serum fractionation by Sephacryl S-200. Serum aluminum was found to exist primarily as protein–aluminum complexes and free aluminum was not detected in normal or uremic serum. One aluminum-containing component, which lacked absorbance at 280 nm, was suggested to consist of aluminum complexed with inorganic anions or small polypeptides. This component was proposed to play a role in the pathogenesis of dialysis encephalopathy. Subsequent studies identified proteins solely as carriers of aluminum although divergent conclusions were reached regarding their number, identities and the relative amounts of aluminum bound. Using Sephadex G-200 and G-50, Trapp [8] identified albumin and transferrin (Tf) as the major acceptors of aluminum added to serum *in vitro*. No binding by low-molecular-mass species was observed. Employing a competitive binding assay in conjunction with Sephacryl S-200 SEC, Bertholf *et al.* [9] concluded that albumin is the major serum acceptor of

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aluminum. They proposed that a shift in aluminum distribution towards low-molecular-mass, low-affinity binding species occurs in hemodialysis patients. Using Sephacryl S-300 to analyze pooled hemodialysis plasma and commercial preparations of albumin and Tf incubated with aluminum *in vitro*, Cochran *et al.* [10] concluded that Tf is the sole carrier of aluminum. However, using Sephadex G-75, Kahlil-Manesh *et al.* [11] concluded that high-molecular-mass fractions containing albumin and Tf were the major carriers of aluminum in plasma from normal and dialysis patients without aluminum intoxication whereas a low-molecular-mass (approximately 8000 daltons) protein was the major carrier of aluminum in plasma from aluminum-intoxicated hemodialysis patients. Presumably, the inability of these SEC methods to resolve albumin and Tf contributed to the lack of consensus on serum aluminum speciation. Recently, employing an ion-exchange high-performance liquid chromatographic (HPLC) procedure that resolved albumin and Tf, Garcia Alonso *et al.* [12] concluded that Tf is the unique aluminum-binding protein of serum. However, the evidence cited above, together with the established capacity of albumin for metal ions [13,14] and the relatively low stability of albumin–metal ion complexes [15], suggests that ion-exchange techniques may displace aluminum from albumin. At present it appears that the number and nature of aluminum-binding components in serum are not known with certainty. The disparate conclusions reached by various groups presumably reflect variation in the samples examined in addition to methodological differences.

Since nearly all serum aluminum is non-dialyzable, recent attempts to treat aluminum intoxication have relied on dialyzable chelators to facilitate renal clearance of aluminum or its removal during hemodialysis. Several studies have used SEC to examine serum aluminum speciation following chelation therapy with desferrioxamine (DFO). Leung *et al.* [16] used Bio Gel P-2 to demonstrate marked increases in the ultrafiltrable fraction of total serum aluminum which corresponded to reductions in protein-bound aluminum and the appearance of DFO–aluminum complexes following DFO treatment of dialysis patients. Similarly, Bertholf *et al.* [17] demonstrated redistribution of aluminum from large proteins to a smaller component, suggested to be aluminum-DFO, using Sephacryl S-200 and Bio Gel P-2 SEC of serum from a DFO-treated dialysis patient. In contrast, DFO treatment of aluminum-intoxicated dialysis patients was shown to increase the overall protein-bound portion of total plasma aluminum and cause significant increases in the content of both aluminum and protein in a low-molecular-mass fraction (approximately 8000 daltons) resolved by SEC on Sephadex G-75 [11].

To further investigate serum aluminum distribution we have examined the use of a mechanically rigid SEC packing, TSK-GEL HW-55S, which has several advantages over the soft gels employed previously. This material has a lower affinity for aluminum than other SEC media and the greater mechanical stability facilitates the analysis of large numbers of samples without column repacking. The small particle size and narrow particle size distribution compared to Bio Gel, Sephacryl and Sephadex packings, in conjunction with the pore characteristics of TSK-GEL HW-55S, facilitate higher-resolution fractionations of high-molecular-mass serum components. In order to maximize the precision and reproducibility of sample fractionations, a high-performance liquid chromatography (HPLC) pump was used to regulate elution. We have employed this SEC system to determine serum aluminum distribution in normal individuals and to study the effect of DFO treatment on serum aluminum distribution in hyperaluminemic patients with normal renal function. In sera containing elevated levels of aluminum, we found aluminum in an extremely stable complex with a protein fraction that has not been described previously.

EXPERIMENTAL

Sample handling

The brands of plastic tubes and pipet-tips used in this study were selected on the basis of possess-

ing low leachable aluminum contamination. Glassware was washed with 10 m*M* EDTA followed by 10% nitric acid and two water rinses. High-purity deionized water (specific resistivity 18 MΩ/cm at 25°C, Milli-Q+, Millipore, Bedford, MA, USA) was used throughout.

Serum preparation

Venous blood from healthy volunteers and patients with possible aluminum intoxication as a result of occupational exposure to aluminum dust was collected in vacutainer tubes (Becton Dickinson, Rutherford NJ, USA) and allowed to clot for 1 h at room temperature. Serum, prepared by centrifugation for 10 min at 1500 *g* in a clinical centrifuge, was transferred to polystyrene tubes (Falcon 2003, Becton Dickinson, Lincoln Park, NJ, USA) and stored at −80°C until required.

Patients receiving DFO were given intramuscular injection (Desferal, Ciba-Geigy, Toronto, Canada) (7.0 mg/kg body weight) every 12 h for three days. Blood samples were taken prior to the first injection and at 0.5, 1.0, 1.5, 2.0, 3.0, 4.0, 6.0, 8.0 and 60 h after the first injection. During the course of therapy, subjects were housed in a clinical investigation unit in which aluminum intake was controlled. Informed consent was obtained from all subjects. Treatments were conducted in accordance with the protocol approved by the Medical Ethics Committee on Human Experimentation of the University of Toronto.

Size-exclusion chromatography

A 1-ml volume of serum was chromatographed at room temperature (20–22°C) on a 95 cm × 1.6 cm I.D. bed of TSK-GEL HW-55S (Supelco, Oakville, Canada) packed in a glass column equipped with an adjustable flow adapter (Model G16 × 1000, Amicon, Oakville, Canada). Samples were eluted at 12 ml/h with buffer containing 50 m*M* NaCl, 50 m*M* Tris–HCl pH 7.4 and 3.0 m*M* NaN_3 using a high-pressure chromatography pump (P-3500, Pharmacia, Uppsala, Sweden). Wetted surfaces of the SEC system consisted of glass, plastic and titanium. Fractions (2 ml) were collected in polypropylene tubes (Falcon 2053, Becton Dickinson) and protein concentrations determined by spectrophotometry at 280 nm. The elution volumes of human apotransferrin, bovine serum albumin, rabbit immunoglobulin G (IgG) (T2252, A7888, I5006, Sigma, St. Louis, MO, USA) and bovine α-2-macroglobulin (602-442, Boehringer Mannheim, Laval, Canada), applied separately and as a mixture, were used to calibrate the SEC system for molecular mass estimations.

Aluminum determination

The aluminum concentration of SEC fractions was determined directly by electrothermal atomic absorption spectrophotometry (EAAS) at 396.2 nm using either a Perking Elmer (Norwalk, CT, USA) 5100 PC or 305B instrument. Column buffer consistently contained less aluminum than could be detected by EAAS. Total serum aluminum was determined as described previously [18]. Calibration curves were obtained by dilution of an aluminum standard solution (SA 442-500, Fisher Scientific, Don Mills, Canada) with column buffer. The accuracy of total serum aluminum measurements was monitored with the aid of internal and external quality control programs. The external program was executed as part of an International Trace Element Quality Assessment Scheme organized by the University of Surrey.

Comparison of SEC media aluminum affinity

Aluminum-binding capacities of Sephadex G-200, Sephacryl S-500, Bio Gel P-2 and TSK-GEL HW-55S were compared by packing columns (16 cm × 1.5 cm I.D.) with each medium and determining the recovery of aluminum following application of equal aluminum loads. Before use, each column was washed overnight with column buffer containing 0.8 m*M* DFO followed by 8 h equilibration with column buffer to remove aluminum from the packings and chromatographic equipment. Subsequently, 125 ng of aluminum in 1 ml (aluminum standard diluted with column buffer) were applied and each column was eluted with 2.5 column volumes of buffer delivered by a peristaltic pump at 12 ml/h.

Fractions (2 ml) were collected and their aluminum content determined by EAAS. Aluminum recovery was calculated by summing the aluminum content of consecutive fractions in the eluted aluminum peak.

Aluminum addition to human serum in vitro

Aluminum concentrations, within the range determined commonly in this laboratory for hemodialysis patients (800–10 000 nmol/l), were attained in sera from normal individuals by adding freshly prepared aluminum lactate or aluminum acetylacetonate (17 000 nmol/l) dissolved in column buffer containing $NaHCO_3$ (40 mmol). The samples were then incubated at 37°C for 1 h, with intermittent mixing, prior to chromatography. Protein precipitation was not observed in any samples at the aluminum concentrations employed. For the experiment shown in Fig. 3b, 1 ml of sera previously incubated with aluminum acetylacetonate was dialyzed (Spectrapor 1 tubing, 6000–8000 daltons nominal exclusion limit, Fisher Scientific) against 1 l of buffer containing 10 m*M* Tris–HCl pH 6.0, 50 m*M* NaCl and 0.76 m*M* DFO for 36 h followed by dialysis against 1 l of column buffer for 4 h at 4°C, prior to SEC.

Protease treatment of sera

Protease digestion of serum samples was performed to determine the composition of serum aluminum-binding components. For the experiment shown in Fig. 5, 30 mg (approximately 1350 U) of Pronase (Calbiochem, La Jolla, CA, USA) was added to 1 ml of serum and incubated at 37°C for 20 h prior to SEC.

Protein electrophoresis

Sodium dodecylsulphate polyacrylamide gel electrophoresis (SDS-PAGE) was performed in slab gels prepared by modifications of the procedure described by Laemmli [19]. Acrylamide concentrations in the separating and stacking gels were 9 and 3% while bisacrylamide concentrations were 0.24 and 0.08%, respectively. The separating gel contained 370 m*M* Tris–HCl pH 8.8, 0.1% (w/v) SDS, 0.05% (w/v) ammonium persulfate and 0.00033% (v/v) N,N,N′,N′-tetramethylethylenediamine (TEMED). The stacking gel contained 125 m*M* Tris–HCl pH 6.8, 0.1% (w/v) SDS, 0.13% (w/v) ammonium persulfate and 0.001% (v/v) TEMED.

Aliquots of SEC fractions estimated by UV absorbance to contain approximately 60 μg of total protein were dried by vacuum centrifugation (Speed-Vac, Savant, Farmingdale, NY, USA) prior to analysis if 100 μl or less. More dilute samples were initially concentrated by ultrafiltration (UFC3-LGC-00, 10 000 daltons nominal exclusion limit, Millipore) and then dried. Protein molecular mass standards (Pharmacia, Dorval, Canada) were used to calibrate gels for molecular mass estimation.

Samples (20 μl), in buffer containing 10 m*M* Tris–HCl pH 7.5, 10 m*M* EDTA, 1.0% (w/v) SDS, 20% (v/v) glycerol and 0.00005% (w/v) bromophenol blue, were boiled for 5 min and then electrophoresed at 20 V during stacking (approximately 45 min) followed by 40 V for the remainder of the separation (approximately 2.75 h). Gels were stained overnight with 0.22% (w/v) Coomassie brilliant blue R-250 (CBR) in 50% (v/v) methanol and 10% (v/v) acetic acid and destained in the same solution without dye. Silver staining [20] was performed either directly after electrophoresis or after staining with CBR followed by extensive destaining.

High-performance liquid chromatography

The HPLC system consisted of two Waters 510 pumps, a Gilson 811 high-pressure dynamic mixer with a 1.5-ml chamber, a Rheodyne 7125 injection valve with a 2-ml sample loop, a Waters 440 absorbance detector with an extended wavelength module operating at 214 nm (Waters 440 and EWM) and a Gilson 201 fraction collector. Gradient elution and data collection were controlled by an Apple 2e computer (Cupertino, CA, USA) using Chromatochart software and Adalab interfaces (Interactive Microware, State College, PA, USA). All reagents and solvents employed were HPLC or analytical grade. HPLC was performed at room temperature.

After pooling SEC fractions of interest, trifluoroacetic acid (TFA) was added to 0.1% (v/v) pri-

or to filtration through a 0.22-μm filter (Lida, Bensenville, IL, USA). Samples were then analyzed by reversed-phase HPLC (RP-HPLC) on a Chromegabond octadecyl column (250 mm × 4.6 mm I.D., 5-μm particles with 30-nm pores, E.S. Industries, Marlton, NJ, USA) equilibrated with 0.1% TFA. Samples ranging from 2 to 10 ml were applied by syringe injections while larger samples were applied using the buffer A pump at 0.8 ml/min. When column effluent absorbance returned to baseline levels, retained components were eluted by a 60-min linear gradient from 100% buffer A to 100% buffer B at 0.8 ml/min. Buffer A was 0.1% TFA in water and buffer B was 0.1% TFA in 60% acetonitrile. Fractions (0.4 ml) were collected in 1.5-ml polypropylene tubes.

Amino acid analysis

Amino acid compositions were determined by RP-HPLC analysis of protein hydrolyzates derivatized with phenylisothiocyanate (PITC) [21]. Samples (1–5 μg) were hydrolyzed at 110°C under vacuum in 100 μl of constant-boiling hydrochloric acid for 20 h and then transferred to 1.5-ml polypropylene vials, diluted with 100 μl of water and dried by vacuum centrifugation. After resuspension and drying from 10 μl of ethanol–water–triethylamine (TEA) (40:40:20), samples were derivatized for 20 min at room temperature in 20 μl of ethanol–water–TEA–PITC (65:20:10:5). Excess reagent was then removed by vacuum centrifugation for 2 h and the tubes stored at –20°C prior to analysis.

Phenylthiocarbamyl (PTC)-amino acids were analyzed using the chromatograph described above except that detection was at 254 nm and a 100-μl sample loop was employed. Samples, dissolved in 100–150 μl of buffer A (20 m*M* ethylenediamine pH 7.0), were injected onto a Waters Resolve octadecyl column (150 mm × 3.9 mm I.D., 5-μm particles with 9-nm pores, Waters, Milford, MA, USA) and eluted at ambient temperature by a 30-min linear gradient from 100% A to 50% B (40 m*M* ethylenediamine pH 7.0, 20% methanol and 40% acetonitrile) at 1 ml/min. A 5-min wash with 100% B followed by a 10-min equilibration with 100% A was performed between analyses. The system was calibrated with aliquots of a commercial amino acid mixture (standard H, Pierce, Rockville, MD, USA) derivatized in parallel with experimental samples.

RESULTS

Comparison of SEC media aluminum affinity

Table I summarizes our findings concerning aluminum binding by chromatographic media that has been employed previously to determine serum aluminum distributions. Under the conditions employed here, only 24–36% of a 125-ng load of aluminum was eluted from Bio Gel P-2, Sephacryl S-500 and Sephadex G-200 in contrast to the 75% eluted from TSK-GEL HW-55S. Two

TABLE I

ALUMINUM-BINDING TO SIZE-EXCLUSION CHROMATOGRAPHY MEDIA

Columns (16.0 cm × 1.5 cm I.D.) were packed with the various SEC media and chelatable aluminum removed by washing with buffer containing DFO as described in the Experimental section. Identical loads of aluminum chloride were applied and aluminum recoveries determined by summing the aluminum content of consecutive fractions as determined by EAAS.

Medium	Aluminum loaded (ng)	Aluminum recovered (ng)	Recovery (%)
Bio Gel P-2	125	30	24
Sephacryl S-500	125	37	30
Sephadex G-200	125	45	36
Toyopearl HW 55 (S)	125	94	75

repetitions of this comparison yielded similar results.

Patient sera SEC

SEC on TSK-GEL HW-55S of serum form an aluminum worker before and after DFO treatment is shown in Fig. 1. This patient displayed cognitive and behavioral disturbances suggestive of aluminum intoxication. However, total serum aluminum (220 nmol/l) and the serum aluminum speciation prior to DFO treatment for this patient (Fig. 1a) were similar to those determined for healthy volunteers (Fig. 2a) [22,23]. Four major peaks with optical density at 280 nm, labelled 1–4 in Fig. 1a, were resolved. SDS-PAGE revealed that peak 1 contained a number of large proteins with α-2-macroglobulin constituting the major component. Peak 2 included immunoglo-

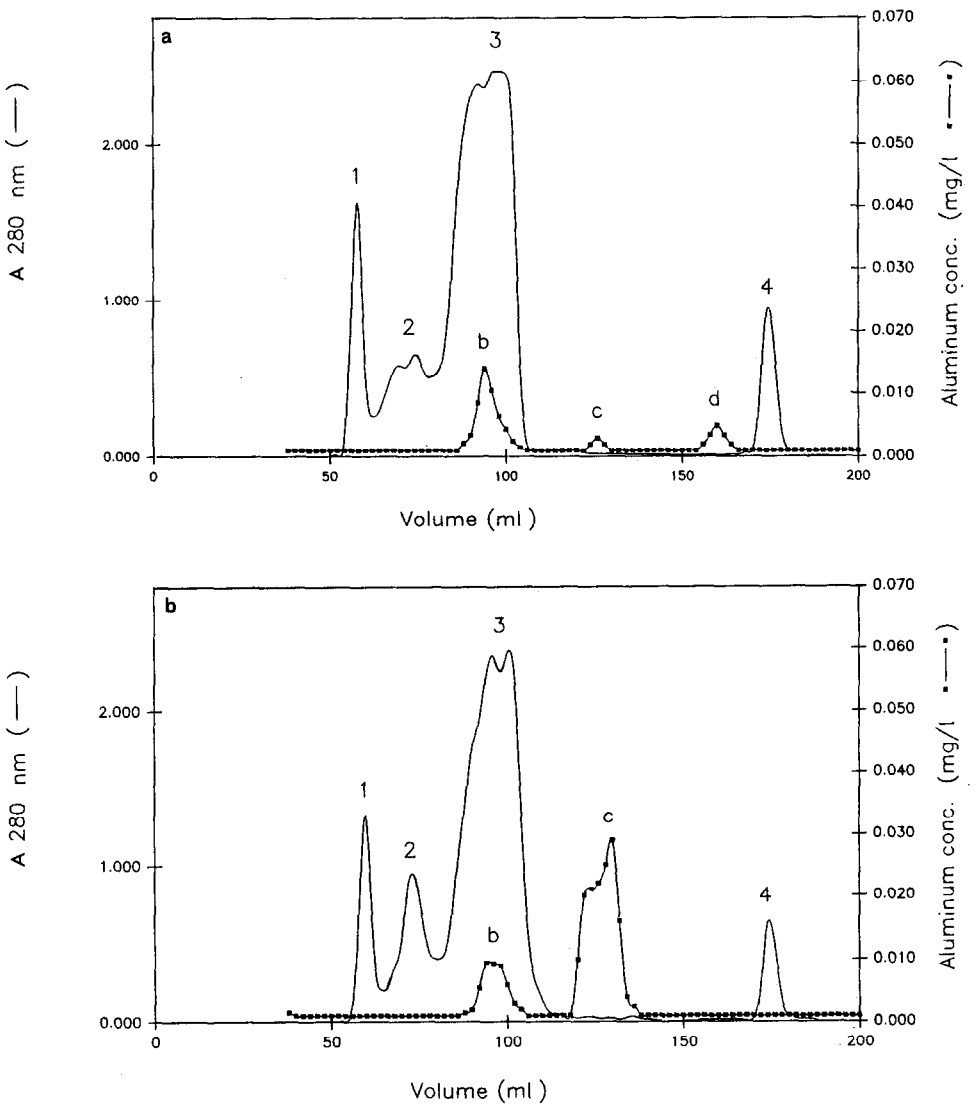

Fig. 1. Resolution of serum proteins and aluminum complexes by SEC. Effect of DFO on serum aluminum speciation. SEC analysis of serum from an aluminum worker before (a) and 3 h after (b) injection with DFO. Total serum aluminum levels were 220 and 1074 nmol/l, respectively. Solid line, absorbance at 280 nm; squares, aluminum concentration in mg/l. Numbered protein peaks and lettered aluminum peaks are discussed in the text.

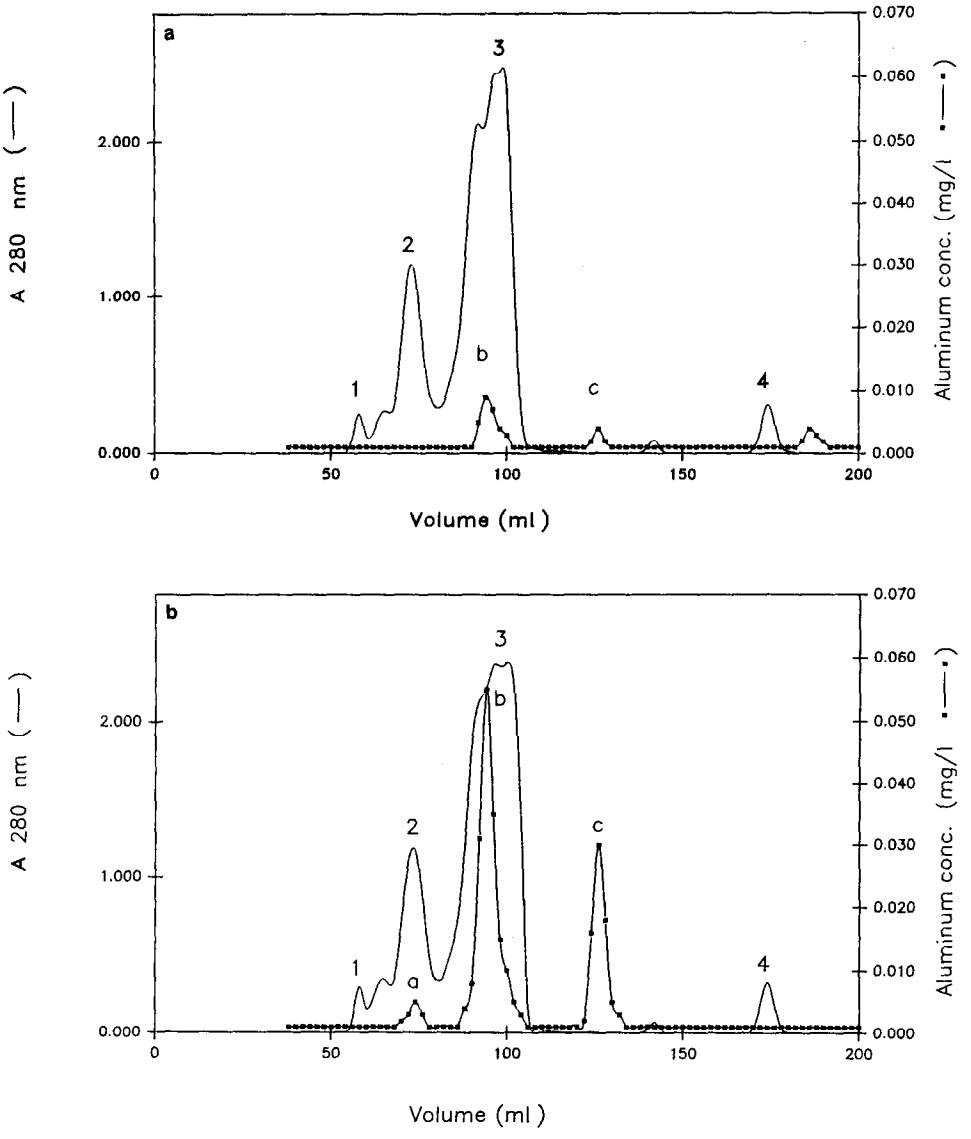

Fig. 2. Serum aluminum speciation following addition of aluminum to serum *in vitro*. SEC analysis of serum from a healthy volunteer before (a) and after (b) incubation with aluminum lactate. Total serum aluminum levels were 120 and 1700 nmol/l, respectively.

bulins and haptoglobin in addition to several unidentified proteins. Peak 3 was composed primarily of albumin and Tf. Peptides and low-molecular-mass compounds (<6000 daltons) eluted in peak 4. Similar protein elution profiles, with individual differences in peak shape and amplitude but with constant elution volumes, have been obtained with this same column for more than one hundred samples.

Direct EAAS of sera SEC fractions revealed that aluminum typically eluted from TSK-GEL HW-55S in three to five discrete peaks. Three peaks, labelled b–d in Fig. 1a, were resolved for this patient. The bulk of aluminum (peak b) co-eluted with albumin and Tf (peak 3). Somewhat less aluminum eluted in peak c, a portion of the chromatogram corresponding to a molecular mass of approximately 18 000 daltons. This re-

gion typically lacked absorbancy at 280 nm as did another region (peak d), corresponding to a molecular mass of approximately 8000 daltons, where a similar amount of aluminum eluted. Another aluminum peak, termed peak a, which co-eluted with immunoglobins (peak 2) in some patient samples, was absent in this sample.

A single injection of DFO resulted in an approximately five-fold increase in total aluminum, from 220 to 1074 nmol/l, in serum taken from this individual 3 h after injection. While the protein profile is similar to that of the pre-injection sample, monitoring aluminum elution revealed a dramatic increase in the quantity of aluminum eluting in peak c (Fig. 1b). The quantity of aluminum in peak b was similar to pre-injection levels while that in peak d decreased to baseline level. This altered aluminum distribution was also seen in a sample taken 8 h after injection and a sample taken three days later following a total of six DFO injections (see Fig. 5a).

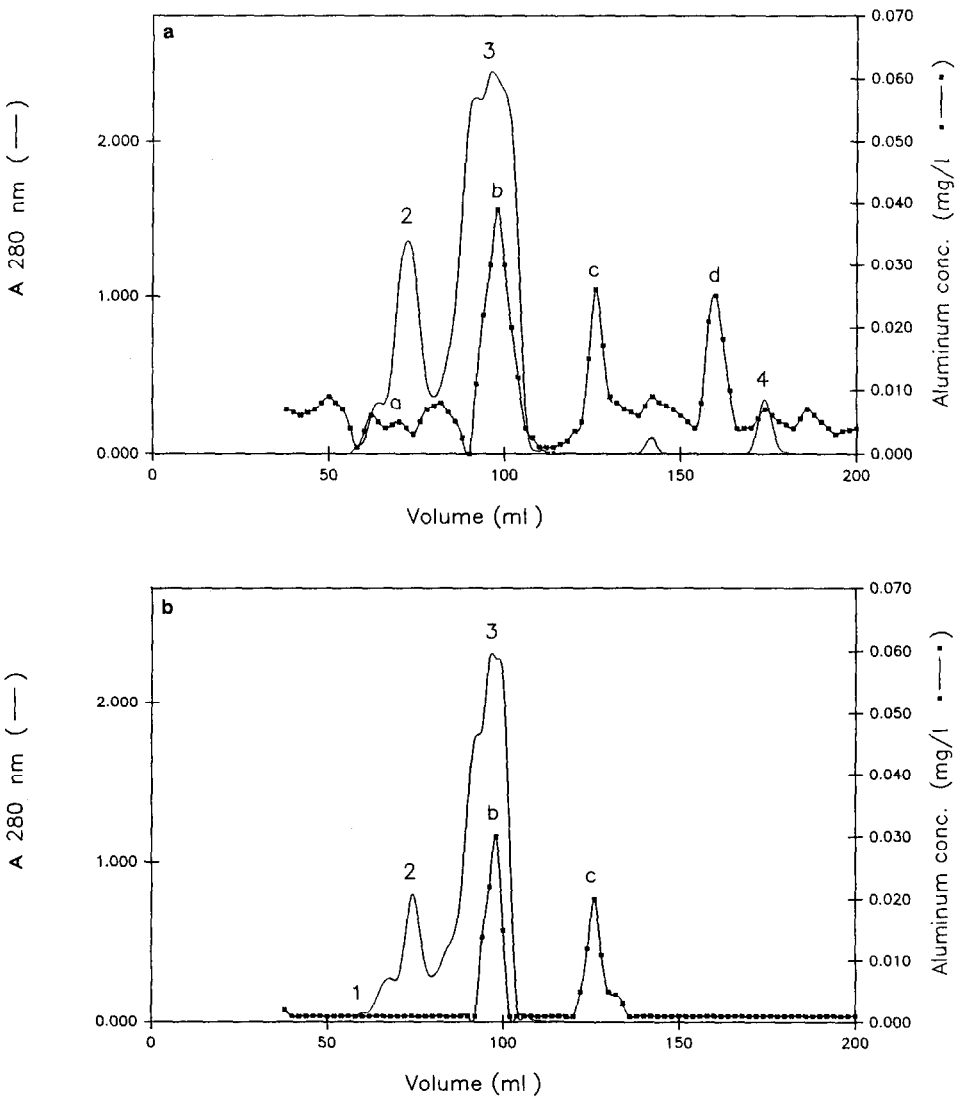

Fig. 3. Stability of serum aluminum complexes in the presence of DFO *in vitro*. SEC analysis of serum from a healthy volunteer following incubation with aluminum acetylacetonate (a) and after subsequent dialysis against DFO (b). Total serum aluminum in (a) was 1700 nmol/l.

Aluminum distribution in serum incubated with aluminum in vitro

The altered serum aluminum distribution resulting from DFO injection can be mimicked, in part, by addition of aluminum to serum *in vitro*. Fig. 2a shows the profile obtained upon SEC of serum from a healthy volunteer (total Al = 120 nmol/l). Aluminum peaks a and d were not detected in this serum sample. Incubation of serum with aluminum lactate (final Al = 1700 nmol/l) prior to SEC resulted in the appearance of aluminum peak a and significant increases in the elution of aluminum in peaks b and c (Fig. 2b).

Stability of aluminum complexes in serum

Fig. 3a shows the aluminum distribution in the same serum as that shown in Fig. 2a, following incubation with aluminum acetylacetonate *in vitro* (final Al = 1700 nmol/l). As with the addition of aluminum lactate, incubation of serum with aluminum acetylacetonate resulted in a marked increase in the quantity of aluminum eluting in peaks b and c and the appearance of peak a. In addition, the complexes represented by peak d and several components of higher and lower molecular mass were formed. Peaks b and c apparently contain the most stable complexes since only these complexes remained following exhaustive dialysis of serum against a high concentration of DFO (Fig. 3b). It should be noted that the disappearance of peak d and other small complexes may be due to loss during dialysis.

Characterization of aluminum-binding serum components

The aluminum-binding components resolved by SEC could be inorganic or organic species, with the latter possibly proteinaceous and non-proteinaceous molecules. SDS-PAGE analyses of peaks a–d showed that fractions of peak a contained chiefly immunoglobulins while those of peak b contained both albumin and Tf, as expected on the basis of the co-elution of standard proteins. The elution of aluminum peaks c and d was usually not accompanied by elution of material with significant absorbance at 280 nm. Only trace amounts of protein were detected when 1.0-ml aliquots of peak c fractions were analyzed by SDS-PAGE and stained with CBR. However, as shown in Fig. 4, silver-staining of gels revealed the presence of numerous polypeptide species ranging in molecular mass from 16 000 to 150 000 daltons in pooled fractions (58–70) comprising peak c. The most abundant polypeptide, present in similar amounts in each of the four samples analyzed in Fig. 4, had an approximate molecular mass of 67 000 daltons and was appar-

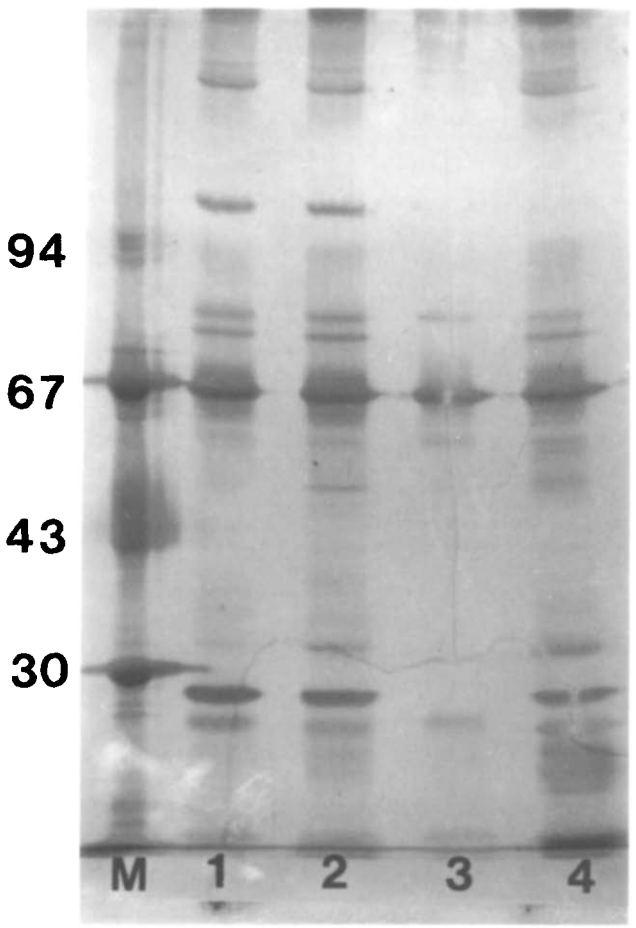

Fig. 4. SDS-PAGE analysis of albindin (peak c) isolated by SEC. Fractions 58–70 from SEC of sera from a normal volunteer (1), an Alzheimer's disease patient (2) and from a hemodialysis patient (4) were pooled and aliquots concentrated by ultrafiltration prior to electrophoresis. Total serum aluminum levels were 120, 269 and 3220 nmol/l, respectively. An aliquot of peak c from normal serum dialyzed after incubation with aluminum acetylacetonate (shown in Fig. 3b) was also analyzed (3). Proteins were visualized by silver staining. M = protein markers, molecular mass in thousands of daltons.

ently albumin since it co-migrated with the albumin protein standard.

To test whether the aluminum-binding activity of peak c was due to protein, serum was incubated overnight with a mixture of non-specific proteases prior to SEC. Fig. 5a shows the aluminum distribution in an aluminum worker 60 h after the start of DFO therapy. At that time, this individual had received a total of 2.5 g of DFO and the total serum aluminum was 770 nmol/l. The major fraction of aluminum was found in peak c with a lesser amount (peak b) associated with Tf and albumin. Aluminum eluting at 50 ml was apparently associated with a component with a molecular mass in excess of 725 000 daltons since it eluted prior to α-2-macroglobulin (peak 1). Protease digestion resulted in major alterations in both the protein and aluminum elution profiles (Fig. 5b). The magnitude of peaks 1 and 2 were much diminished and the aluminum peak eluting

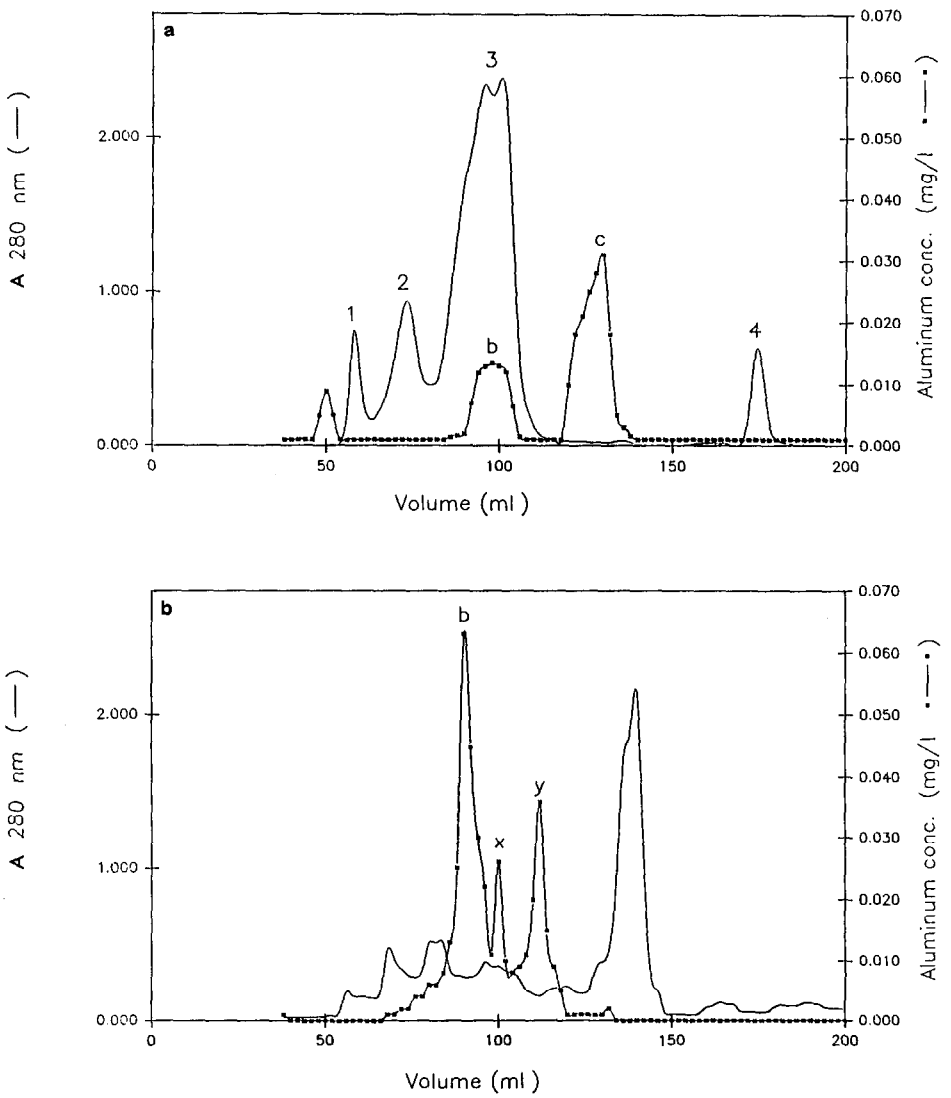

Fig. 5. Protease digestion of serum demonstrates that albindin is proteinaceous. Serum of an aluminum worker taken three days after starting DFO therapy (total serum aluminum level = 770 nmol/l) was analyzed by SEC before (a) and after (b) incubation with Pronase.

at 50 ml disappeared. The amount of protein eluting in peak 3 was also greatly diminished, yet the aluminum signal (peak b) increased markedly. Virtually no aluminum eluted in the position of peak c. Two novel aluminum components (peaks x and y) eluted immediately after peak b. As discussed below, peaks x and y presumably represent aluminum bound by partially degraded forms of Tf or possibly aluminum-binding proteins present in the protease reagent employed.

The presence of protein in peak c was further indicated by RP-HPLC as presented in Fig. 6. An aliquot (1 ml) of the center tube of peak c prepared from a hyperaluminemic patient was made 0.1% (v/v) in TFA, filtered and injected onto an octadecyl column. Following the elution of unretained SEC buffer components (Tris, NaN_3) as a large peak immediately following the void volume, gradient elution resolved numerous retained components of which albumin, labelled HSA in Fig. 6, gave the largest peak area in agreement with SDS-PAGE analyses. A small amount of Tf was also present as indicated in Fig. 6. The elution positions of albumin and Tf were confirmed using appropriate standards. When only the center tube of peak c was analyzed by RP-HPLC, as in Fig. 6, the component labelled A was more abundant than any other component except albumin. However, in analyses of pooled fractions encompassing the total of peak c, numerous components were found in approximately equivalent abundance. The amounts of albumin and Tf detected in analyses of pooled fractions varied essentially linearly with the number of peak c fractions pooled. The apparent coincidence of the elution of component A from the SEC column with the apex of aluminum peak c suggested that component A was an aluminum-binding component. As shown in Table II, the amino acid composition of component A differs from that of albumin and transferrin. The levels of proline and serine are significantly greater than those in albumin and transferrin. Also, the level of glycine is several times that of albumin. The proportions of tyrosine, lysine and histidine are lower than in the other two proteins. Conversion of asparagine and glutamine during acid hydrolysis precludes comparison of the contents of these residues and also those of aspartic and glutamic acids.

DISCUSSION

The resolution of serum proteins by TSK-GEL HW-55S is comparable to, or better than, that achieved previously employing Sephacryl S-200

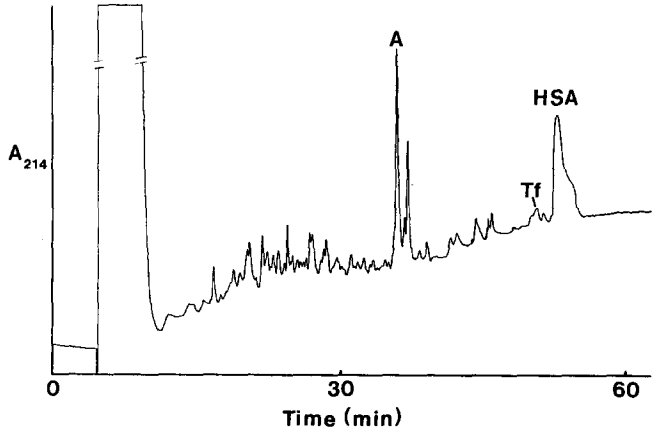

Fig. 6. RP-HPLC of the central portion of the albindin peak yielding a principal component. A 1-ml volume from the center fraction of the albindin peak from a SEC analysis was chromatographed on an octadecyl column using a 1-h gradient from 0 to 60% acetonitrile in 0.1% TFA. The peak marked A was collected for amino acid analysis (Table II). The elution positions of human apotransferrin and serum albumin are denoted by Tf and HSA, respectively.

TABLE II

AMINO ACID COMPOSITION OF COMPONENT A OF THE ALBINDIN FRACTION

The center tube of the albindin peak (peak c) from one SEC analysis was fractionated by RP-HPLC. The RP-HPLC component labelled A in Fig. 6 was prepared by acid hydrolysis and PTC-amino acids quantitated by RP-HPLC as described in the Experimental section. Literature values for human serum albumin and transferrin are presented for comparison.

Amino acid	Albindin (mol%)	Albumin (mol%)	Transferrin (mol%)
Non-polar			
Ala	10.45	10.34	8.50
Val	6.56	7.06	5.96
Leu	11.38	10.51	8.66
Ile	1.09	1.48	2.12
Pro	10.33	3.94	5.15
Met	0.65	1.15	1.42
Phe	6.02	5.75	4.11
Trp	N.D.[a]	0.33	1.13
Polar			
Gly	8.21	2.13	7.33
Ser	9.62	4.60	5.63
Thr	5.04	4.76	4.35
Cys	N.D.[a]	5.75	5.76
Tyr	1.11	3.12	3.75
Asn	N.D.[a]	2.79	N.D.[a]
Gln	N.D.[a]	3.28	N.D.[a]
Acidic			
Asp	7.36	5.91	11.80
Glu	11.93	10.02	8.89
Basic			
Lys	4.22	10.02	8.66
Arg	5.45	4.43	4.00
His	0.56	2.63	2.78
Basics/acidics[b]	0.53	0.78	0.75

[a] N.D. = not determined.

[b] Using the sum of Asp, Glu, Asn and Gln for acidic residues.

[7,9,17], Sephacryl S-300 [10] or Sephadex G-200 [8] and is distinctly higher than that achieved on Bio Gel P-2 [16,17].

Since potential effects on protein structure, charge and ligand binding resulting from adsorption to matrices or solvent composition are minimal in SEC compared to other modes of liquid chromatography, SEC appears to be the best method currently available to study aluminum distribution among serum proteins. Unfortunately, resolution in SEC is limited by both the lack of selectivity of available matrices and the possibility that proteins with different molecular mass can fold to assume structures of similar dimensions. Thus, we are unable to distinguish whether the major protein components alone of peaks a–d, identified by SDS-PAGE or RP-HPLC, are the aluminum-binding species in these fractions or whether other components participate.

We have demonstrated that TSK-GEL HW-55S has a lower affinity for aluminum than SEC matrices employed previously to study serum aluminum speciation. The aluminum load (125 ng) we used to compare matrices is two to ten times greater than the total aluminum contained in 1–2 ml of sera from normal and hyperaluminemic individuals and the column dimensions (16 cm × 1.5 cm I.D.) used in our comparison were considerably smaller than those normally employed in SEC of sera. Thus, our finding that up to 76% of the aluminum applied to small columns was taken up by polysaccharide and polyacrylamide-based supports suggests that protein–aluminum stoichiometries are more likely to be altered during SEC employing supports other than TSK-GEL HW-55S. This could preclude the detection of aluminum complexes of low stability. Redistribution of aluminum from low-stability complexes with proteins to sites on SEC media may result in diminuition of both the number and stoichiometry of aluminum–protein complexes detected. Furthermore, subsequent redistribution of aluminum from sites on SEC media to high-affinity ligands such as Tf ($K_1 = 10^{12.9}$, $K_2 = 10^{12.3}$) [24] or DFO ($K = 10^{22}$) [25] in later samples may also produce artifacts.

The composition of SEC mobile phases also may influence analytical accuracy. The omission of bicarbonate or carbonate salts, necessary for aluminum binding to Tf [26], should prevent possible uptake of aluminum from the SEC matrix or other ligands by apotransferrin during chromatography. The inclusion of bicarbonate by previous workers [10,12,16] may have facilitated redistribution of aluminum from lower-affinity ligands to apotransferrin during chromatography,

resulting in a reduction in the number of aluminum complexes detected. Analyses of samples with both high and normal total serum aluminum concentrations in our system showed no variation in either the number or stoichiometry of aluminum complexes between repeated fractionations, demonstrating that significant redistribution of aluminum did not occur.

Due to the general lack of information regarding the nature of the protein–aluminum complexes and differences in experimental conditions, it is difficult to assess the extent to which systematic error has affected our results and those of previous workers. The number of high-molecular-mass aluminum complexes resolved here is similar to that achieved by groups employing Sephacryl S-200 [7,9,17]. Using different matrices, others have found only one high-molecular-mass aluminum complex suggested to contain albumin or transferrin or both. These discrepancies could be due to factors limiting the sensitivity of aluminum detection such as high background levels, incomplete resolution of serum components or loss of aluminum from complexes with low stability during chromatography.

Using our SEC system, we have resolved up to five high-molecular-mass aluminum complexes in sera of hyperaluminemic individuals. In contrast, between one and three aluminum complexes were resolved in sera from individuals with normal serum aluminum concentrations. This suggests that at least five serum proteins can bind aluminum. We infer that the number of aluminum complexes resolved and the quantity of aluminum contained within each complex reflects the total serum aluminum concentration. In sera with normal concentrations of aluminum, we suggest that Tf, and possibly albumin, are the major carriers of aluminum (peak b). With higher concentrations of aluminum, other proteins, tentatively identified here as immunoglobulin and albindin (peaks a and c), also bind aluminum. An additional aluminum complex formed at high serum aluminum concentrations, peak d, may be identical to the 8000-dalton polypeptide described previously [11].

The abundance of oxygen-rich ligands (*e.g.* organic acids, proteins and carbohydrates) that can complex with aluminum makes the existence of ionized aluminum in serum virtually impossible. However, the existence of aluminum complexes with low or moderate stability in serum could be biologically significant. We have demonstrated that the complexes in peaks a and d are less stable than those of peaks b and c due to their disappearance upon exposure to DFO either *in vivo* (Fig. 1) or *in vitro* (Fig. 3). Since these complexes are non-dialyzable, they could participate in aluminum intoxication by distributing aluminum from body regions with high local aluminum concentrations to regions of lower aluminum abundance.

The complexes eluting as peaks b and c are more stable since they retain the major fraction of bound aluminum during extensive dialysis against DFO (Fig. 3). Given the low stability determined for Gd–albumin complexes [15], it seems likely that the decrement in peak b following exposure to DFO is due to dissociation of aluminum–albumin complexes while aluminum–Tf complexes remain intact. Even though the stabilities of aluminum–Tf (peak b) and aluminum–albindin (peak c) appear to be similar, the affinity of albindin for aluminum may be less than that of Tf since little or no aluminum elutes in peak c unless total serum aluminum levels exceed 200 ng/l whereas some aluminum complexed with Tf or albumin in peak b has been detected in every sample analyzed to date [22,23].

Two previous reports have described metal ion-binding serum components that behave similarly to albindin in SEC. King *et al.* [7] suggested that an aluminum-containing component, which lacked significant absorbance at 280 nm and eluted after the Tf and albumin peak from Sephacryl S-200, consisted of aluminum complexed with either small inorganic species or small polypeptides. Assuming this component (also peak c in their figures) is synonymous with the albindin fraction described here, we have demonstrated that the ligand is proteinaceous since its elution was altered by protease digestion. Similarly, we have shown elsewhere that peak c is not due to the elution of aluminum–DFO [23]. The lack of

absorbance of peak c at 280 nm suggests albumin or Tf are not responsible for its aluminum-binding capacity. The similar amount of albumin in the four peak c samples from sera with different aluminum concentrations and peak c intensities analyzed by SDS-PAGE (Fig. 4) suggests that the albumin detected in peak c is merely the tail of the major albumin peak eluting just prior to peak c. This is further supported by the RP-HPLC analyses in which a maximal percentage of total peak area for component A of peak c was determined at the apex of the aluminum peak while similar percentages of total peak area were determined for albumin and Tf in analyses of single fractions or pooled fractions of peak c. While the RP-HPLC and amino acid analyses presented here demonstrate that the principal component of the albindin fraction is not albumin or Tf, they do not address the possibility that albindin is derived from proteolysis of these or other proteins. Using Sephacryl S-200 or TSK-GEL HW55, Borguet *et al.* [27] resolved a chromium-binding component eluting in SEC after Tf and albumin. They suggested that this component arose through proteolysis of components of the Tf and albumin peak since it was detected upon rechromatography of the Tf and albumin peak. However, in our experience, rechromatography in our SEC system yields only intact Tf and albumin eluting in the same volume with the same aluminum–protein stoichiometry as detected initially. No protein or aluminum has been detected in the elution position of peak c upon rechromatography of peak b, indicating that albindin is not the result of proteolysis during serum preparation or SEC [28]. We suggest that the phenomena described by Borguet *et al.* [27] is due to processes other than enzymatic degradation given the known resistance of native Tf to proteolysis [29]. We suggest that the increase in aluminum elution in peak b following protease treatment of serum (Fig. 5b) is due to the binding of aluminum present in the protease reagent by apotransferrin which had survived digestion. The Pronase reagent was found to contain a significant quantity of aluminum (approximately 27 ng aluminum per mg protein) and we estimate that 800 ng of aluminum was contained in the protease solution employed in Fig. 5b. Peaks x and y may represent partially degraded forms of Tf which bound some of the added aluminum. Alternatively, peaks x and y may represent the elution of aluminum complexes originating from the protease reagent. The disappearance of peak c following protease treatment of serum strongly suggests that the aluminum-binding activity of the albindin fraction is due to protein.

The potential for facilitated uptake of protein–aluminum complexes through receptor–mediated endocytosis, as occurs for Tf [26], or interference by bound aluminum with recognition or signalling events mediated by proteins, may be significant in mechanisms of aluminum toxicity. Only methods capable of preserving protein–aluminum stoichiometries during their resolution will allow appropriate correlations with physiological states to be made.

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