CHROM. 17,380

SEPARATION OF SERUM PROTEINS ON A Fe^{3+}-MONOHYDROXAMATE ADSORBENT

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(Received November 9th, 1984)

SUMMARY

The Fe^{3+}-monohydroxamate adsorbent was tested by chromatography of increasing volumes of serum proteins. The specifically bound proteins were displaced in a stepwise manner by increasing the sodium chloride concentration. The resolved chromatographic fractions were further analysed by gel electrophoresis. A comparison between the properties of Fe^{3+}-monohydroxamate and commercially available adsorbents is presented.

INTRODUCTION

Several years ago, Porath *et al.*[1] described the use of metal chelate affinity chromatography involving Zn^{2+} and Cu^{2+} for protein separation and purification. Modification of this method allowed large-scale purification of α_2-macroglobulin[2,3]. In subsequent years, human serum has been used as a model to evaluate the usefulness of immobilized metals on gels[4], with several proteins being isolated using the new techndique, immobilized metal ion affinity chromatography (IMAC)[5]. Although the synthesis of tris-(carboxymethyl)ethylenediamine ligand (TED)[4] has increased the number of metals that can be immobilized on gels, further research is required to increase the scope, with a special emphasis on iron. The information reported so far is based on micro-scale fractionation, whereas the subject of this report is the fractionation, concentration and isolation of a specific class of proteins on a macro-scale.

The synthesis of α-aminohydroxamate-Sepharose 6B[6] permits new exploration of metal ions bound to gels. Although the α-aminohydroxamate sorbents can bind several types of metal, it is the excellent stability of the Fe^{3+} complex that makes it of special interest. Because the residence time of serum proteins on the Fe^{3+}–monohydroxamate sorbent was increased, the separation, concentration and fractionation of particular classes of proteins were achieved. The most interesting class of proteins that bind to the Fe^{3+}-monohydroxamate sorbent is the glycoproteins, particularly those having sialic acid as the terminal group in the carbohydrate moiety. A more detailed study of this class of proteins will be the subject of a separate report. The results show that hydroxamate gels have a large capacity for this specific class

of proteins and that the same chromatographic column can be used repeatedly without any significant decrease in efficiency. The capacity drops by only *ca.* 15% on passage of several hundred millilitres of human serum through a 5-ml column. The preliminary data on the resolution of serum proteins show that the isolated proteins are not identical with those resolved using TED–Fe^{3+} (ref. 4).

Human serum is a rich mixture of different kinds of protein, and is thus an excellent sample material for investigation of the selectivity and specificity of Fe^{3+}–monohydroxamate sorbent. The sorbent was prepared by chemical fixation of α-aminoacylhydroxamic acid to an inert permeable support in an alkaline medium[6]. Agarose was found to be a satisfactory matrix material for the fixation of the α-aminoacylhydroxamate ligand. Ferric chloride in water solution was used for saturating the support with Fe^{3+}.

Columns containing three different supports were prepared: (A) quaternary aminoethyl (QAE-Sephadex A-50); (B) sulphopropyl (SP-Sephadex C-50); (C) Fe^{3+}–monohydroxamate–Sepharose 6B. Three experiments were carried out using the three columns in series in the order ABC, CAB, and ABC. A fourth experiment using a column of monohydroxamate–Sepharose 6B free of metal ion was carried out as a control. All experiments were carried out using the same volume of dialysed serum (25 ml), the same flow-rate, and the same elution protocol. After introduction of the serum, the columns were washed with the respective equilibrating buffer to give a constant absorbance at 280 nm. The columns were then disconnected and the adsorbed proteins eluted separately in a stepwise manner by increasing the sodium chloride concentration.

MATERIALS AND METHODS

Monohydroxamate–Sepharose 6B was prepared as reported previously[6]. Quaternary amino ethyl (QAE-Sephadex A-50) and sulphopropyl (SP-Sephadex C-A50) chromatographic columns and the peristaltic pump P3 were from Pharmacia (Uppsala, Sweden). Ferric chloride was obtained from Merck (Darmstadt, F.R.G.). All other chemicals were of reagent grade. The chelating, monohydroxamate–Sepharose 6B was saturated with an aqueous solution of ferric chloride and washed with water. The retained Fe^{3+} was measured as described in the preceding publication[6], and amounted to 1540 μmol/g dry gel. A continuously recording spectrophotometer (Uvicord from LKB, Bromma, Sweden) was used to monitor the protein concentration in effluents. The chromatographic fractions were measured manually using a Hitachi Model 101 spectrophotometer. Polyacrylamide gradient gels (4/30% PAA, Pharmacia) were used for the electrophoretic analysis of the fractions containing protein according to the manufacturer's instructions.

Chromatographic procedure

A column (7.4 × 1 cm I.D.) of monohydroxamate–Sepharose 6B was washed with water and charged with a 20 m*M* solution of ferric chloride until saturated. The column was then rinsed thoroughly with water and equilibrated with the buffer of choice. Human serum was dialysed for 24 h against the initial buffer to be used in the chromatographic experiments. Samples of human serum, 1.5, 5, 25, 125 and again 1.5 ml, were used in the first set of experiments (Fig. 1a and b). The unbound material
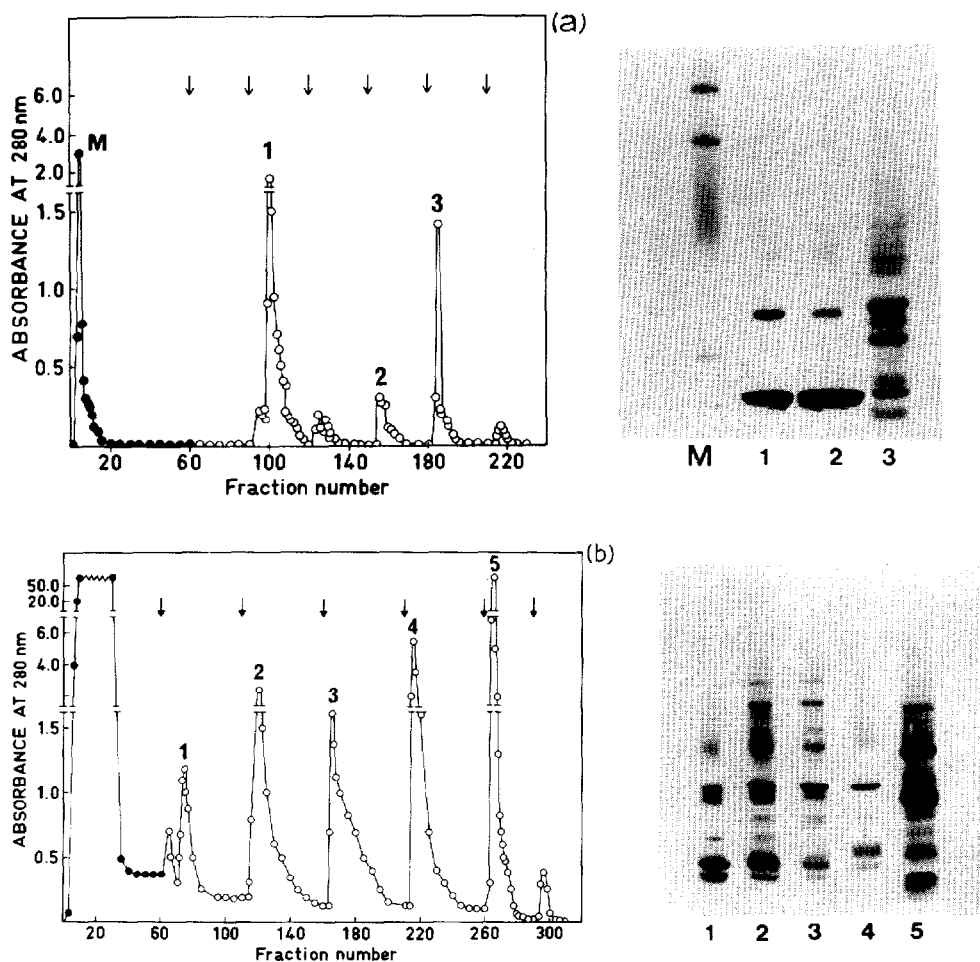
Fig. 1. Chromatography of (a) 1.5 and (b) 125 ml of serum dialyzed overnight against the starting buffer, respectively. As buffer, 50 mM sodium acetate (pH 5.5) was used throughout, with the following inclusions of sodium chloride: (1) nil; (2) 20 mM; (3) 60 mM; (4) 100 mM; (5) 200 mM; (6) 500 mM. The washing buffer (final buffer) was 0.2 M glycine–sodium hydroxide (pH 9.0), including 0.5 M sodium chloride. Flow-rates, 7.3 ml/h; total bed volume 6 ml (7.8 × 1 cm I.D.). The arrows indicate the introduction of the next buffer system. The fractionated proteins are represented from left to right in the corresponding electropherograms in the order of elution. The channel M shows the the unadsorbed proteins.

was washed out using the equilibration buffer until a constant absorbance at 280 nm was achieved. The adsorbed protein was then displaced by increasing the sodium chloride concentration to 0.5 M in a stepwise manner in the same buffer. The regeneration of the column was performed with 0.2 M glycine–sodium hydroxide buffer (pH 9.0) including 0.5 M sodium chloride. The column was developed at a flow-rate of 7.3 ml/h.

In the second set of experiments (Fig. 2a–d) columns containing (A) quaternary aminoethyl (QAE-Sephadex A-50), (B) sulphopropyl (SP-Sephadex C-50), and (C) Fe^{3+}-monohydroxamate–Sepharose 6B (loaded as described shortly) were connected

in series in the order ABC, CAB, and ABC, as shown in Fig. 2a–c, respectively. A fourth experiment using a column of monohydroxamate–Sepharose 6B free of metal ion was carried out as control (Fig. 2d). The bed volume of each of these columns was the same, 6.4×1 cm I.D. All experiments were carried out using the same volume of dialysed serum (25 ml), the same flow-rate (15 ml/h) and the same elution protocol as described above. After introduction of the serum the columns were washed with the respective equilibrating buffer to give a constant absorbance at 280 nm. The columns were then disconnected and the adsorbed proteins were eluted separately.

In Fig. 2a, b and d the equilibrating buffer was 0.05 M sodium acetate (pH 5.5) and for Fig. 2c, 0.03 M Tris–hydrochloric acid. (pH 8.1) was used. The protein elution was carried out by addition of 0.02, 0.06, 0.1, 0.2, and 0.5 M sodium chloride to the respective equilibrating buffers, followed by elution with 0.2 M glycine–sodium hydroxide (pH 9) containing 0.5 M sodium chloride. Fractions of 1 ml were collected for all experiments and protein elution was measured spectrophotometrically at 280 nm (Fig. 2a–d).
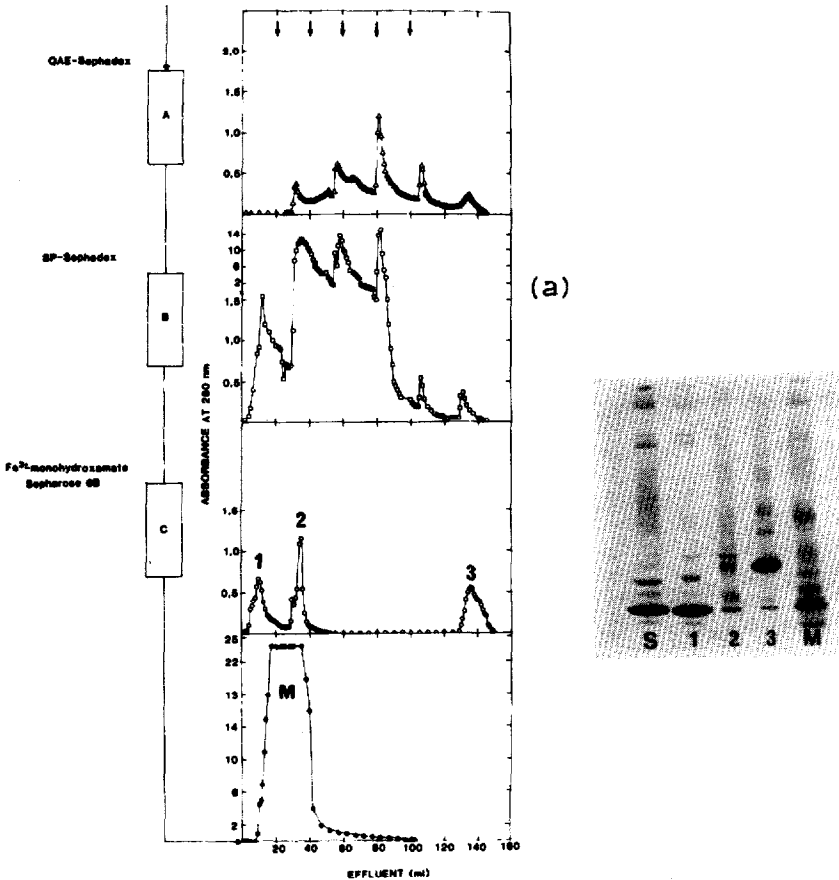

Fig. 2.

RESULTS

The chromatography of a series of increasing volumes (1.5, 5, 25, 125, and again 1.5 ml) of serum (Table I) on a Fe^{3+}-monohydroxamate–Sepharose 6B column at pH 5.5 in 50 mM sodium acetate buffer was carried out. The chromatograms for the 1.5 and 125 ml samples are shown in Fig. 1: 60% and 2.8% of the applied sample was adsorbed, respectively, in the starting buffer. The resolved fractions were analysed by gel electrophoresis and the proteins were identified by their positions in the electropherograms. In doubtful cases this was confirmed by immunoelectrophoresis (Table II).

We observed a rather selective retention of most of the glycoproteins. Ceruloplasmin, transferrin, α_2-macroglobulin, γ-globulin and IgG were found to be retained in an increasing proportion as the sample volume increased. The adsorbent shows a varying affinity for the different components present in serum. Proteins with a higher affinity thus displace those with a lower affinity. The results were reproducible even when the same column was used for all sets of experiments. Most of the
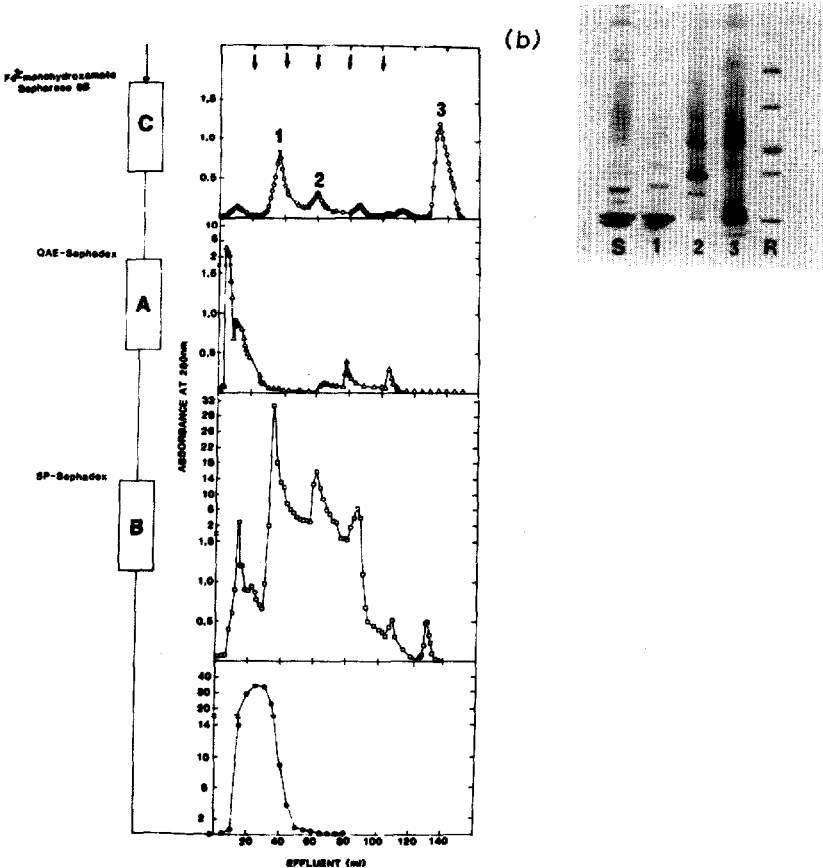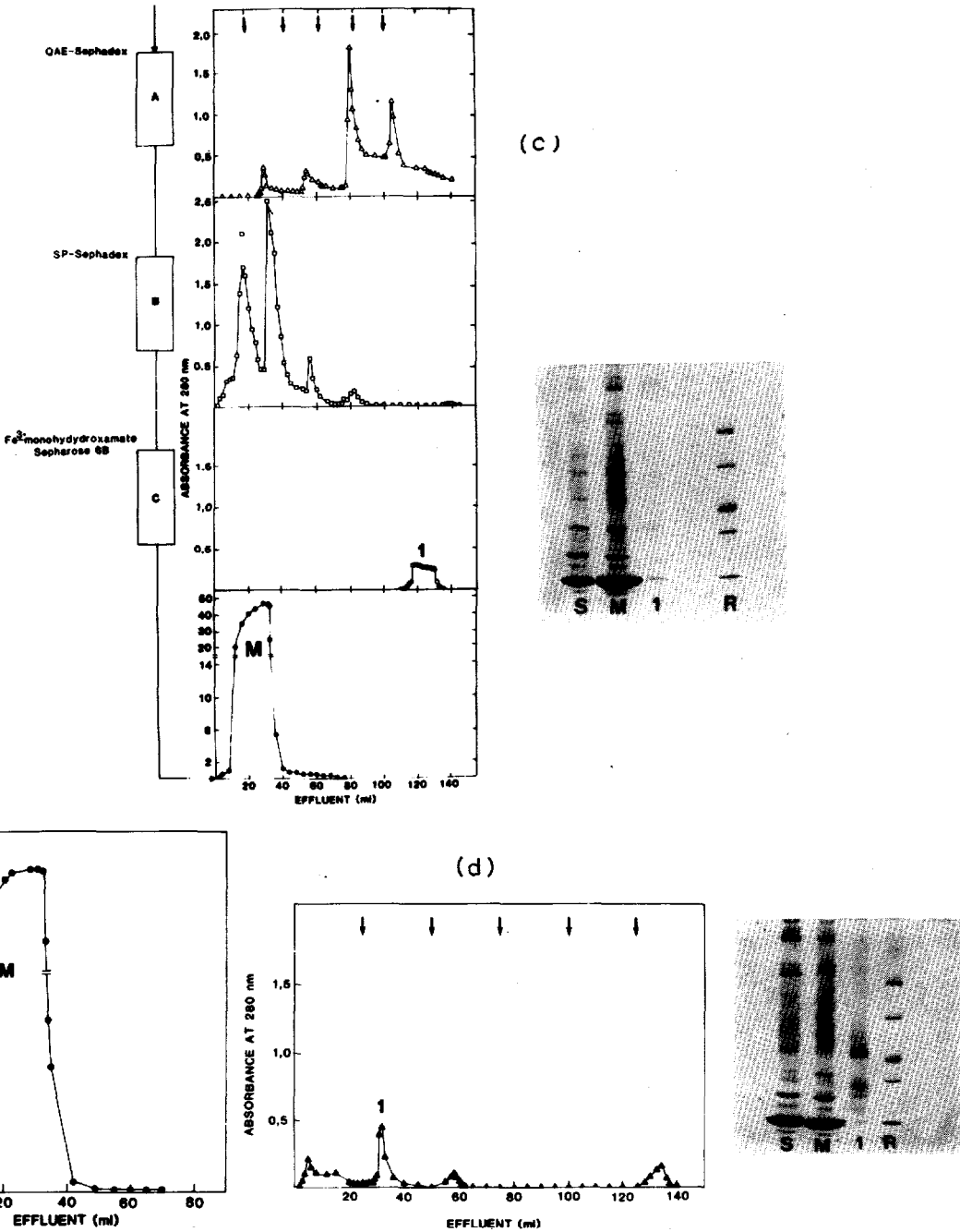

Fig. 2.

Fig. 2. Chromatography on tandem columns. (a) Sequence ABC; (b) sequence CAB; (c) sequence ABC; (d) metal-free monohydroxamate–Sepharose 6B alone (control). The fractionated proteins on Fe^{3+}–monohydroxamate are represented from left to right in the attached electropherograms. S = The starting protein material; M = unadsorbed material; R = protein reference (marker). See Chromatographic procedure for details of buffers, etc.

TABLE I

EFFECT OF SAMPLE LOAD ON Fe^{3+}-MONOHYDROXAMATE-SEPHAROSE 6B EQUILIBRATED WITH SODIUM ACETATE EXPRESSED IN TERMS OF PROTEIN RECOVERY

NaCl concentration added to the buffer (M)	Fe^{3+}-monohydroxamate								Sample 1.5 ml*		Metal-free monohydroxamate-Sepharose 6B (Sample 25 ml)	
	Sample 1.5 ml		Sample 5 ml		Sample 25 ml		Sample 125 ml					
	A_{280} units	% of applied	A_{280} units	% of applied	A_{280} units	% of applied	A_{280} units	% of applied	A_{280} units	% applied	A_{280} units	% of applied
0.00 (in 0.05 M Na acetate, pH 5.5)		60		18		7		3		44		ca. 1
0.02 (in 0.05 M Na acetate, pH 5.5)	0	0	23	3	6	0.2	72	0.4	12	13	0	0
0.06 (in 0.05 M Na acetate, pH 5.5)	42	44	46	6	90	3	91	0.5	18	19	12	0.2
0.10 (in 0.05 M Na acetate, pH 5.5)	5	5	15	2	30	1	109	0.6	0	0	0	0
0.20 (in 0.05 M Na acetate, pH 5.5)	5	5	39	5	45	1.5	127	0.7	0	0	0	0
0.50 (in 0.05 M Na acetate, pH 5.5)	4	4	15	2	30	1	91	0.5	11	12	0	0
0.50 (in 0.2 M Gly–NaOH, pH 9.0)	1	1	2	0.3	9	0.3	18	0.1	0.8	0.8	6	0.1
Sum of adsorbed protein	57	59	140	18.3	210	7.0	508	2.8	42	45	18	0.3
Total recovery		92		90		90		89		90		92

* Control of column condition after the four runs amounting to 156.6 ml of serum. The efficiency was 75% of the initial run.

TABLE II

IDENTIFICATION AND QUANTIFICATION OF PROTEINS OBTAINED IN PURE FORM BY CHROMATOGRAPHIC SEPARATION USING Fe^{3+}-MONOHYDROXAMATE-SEPHAROSE 6B (TABLE I)

	Initial buffer, pH 5.5*
IgG	+ +
Haptoglobin	+ +
Hemopexin	+
α_2-Macroglobulin	+ + +
Transferrin	+
Ceruloplasmin	+ + + + +
α_1-Antitrypsin	(+)
GC-globulin	−
CIq	(+)
C_3	+
C_4	+
Albumin	(+)

* The symbols indicate the relative amount of proteins in the isolated fractions.

human serum albumin does not show specific affinity and passes through with the unadsorbed material. Some albumin was present in all eluents, as shown by the common bands. A protein band in the higher-molecular-weight region was identified as immunoglobulin. Hemopexin was also identified among the immunoglobulin proteins.

To determine the loss of efficiency of the column during the experiments, the elution using 1.5 ml of serum was repeated at the end of the series: the capacity was 75% of that observed in the initial run (Table I).

As described in the preceding publication[6], Fe^{3+}-monohydroxamate exhibits ion-exchange properties. To demonstrate the selectivity and specificity of the adsorbent in sorting out a protein mixture, a comparison between (A) quaternary aminoethyl (QAE-Sephadex A-50), (B) sulphopropyl (SP-Sephadex C-50) and (C) Fe^{3+}-monohydroxamate-Sepharose 6B, was carried out. The three columns were connected in series in the order ABC (Fig. 2a) and CAB (Fig. 2b). The proteins resolved at pH 5.5 on Fe^{3+}-monohydroxamate were in both cases completely different from those resolved using the other ion exchangers. The electropherograms of the resolved proteins on Fe^{3+}-monohydroxamate are shown in Fig. 2a and b. When the pH of the equilibrating buffer was changed to 8.1, the pattern obtained with the column order ABC was as shown in Fig. 2c.

DISCUSSION

The binding of a protein molecule to an immobilized metal ion occurs via the amino acid residues exposed on its surface, which are able to participate in a coordination bond as described previously[6]. The innate potential for interaction is modulated, to a significant extent, by the microenvironment, i.e. vicinal interacting groups, the solvent in which the protein molecule is bathed, and the time for which the protein will be in contact with the immobilized metal ion. In order to evaluate

the relative contributions of the contact time and the modulatory influence, it is necessary to isolate them experimentally. The simplest approach is to study the interaction of a protein mixture (human serum) with Fe^{3+}-monohydroxamate, and to vary the sample volume. The information derived can then be correlated with the behaviour of the individual proteins present in the mixture.

It was found that the fraction of the total applied protein that adsorbed on Fe^{3+}-monohydroxamate decreased as the sample volume increased. At the same time, the absolute amount of adsorbed protein, calculated in terms of absorbance units (A_{280}), increased in proportion to the increase in sample volume. The adsorbent thus shows different affinities for different components present in serum. The strongly adsorbed proteins, e.g. the glycoproteins, displaced other weakly adsorbed proteins. Further studies are required to increase our understanding of these interactions. The study of simpler protein "models" should serve to confirm or dispel the conclusions arrived at from the investigation of human serum. Both of these approaches are being implemented in this laboratory at present.

The Fe^{3+}-monohydroxamate adsorbent shows a selectivity and specificity that differ from both anion exchangers and cation-exchangers. Ferric ion adsorbents can therefore be expected to have properties that are complementary to commercial protein (ion-exchanger) adsorbents. The resolved protein mixtures are completely different from those isolated by using TED–Fe^{3+} as adsorbent[4]. These two sorbents together with the ion-exchanger adsorbents, which retain Fe^{3+} better than does the IDA ligand, may not show an excessive overlap in their chromatographic bias and could complement each other in a purification sequence. This observation clearly points to the potential usefulness of Fe^{3+}-hydroxamate gels as adsorbents for IMA chromatography. It remains to be seen whether the modification of other experimental parameters (pH, type of buffer, temperature, etc.) can be exploited to alter the selectivity of elution.

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