α-AMINOACYL HYDROXAMATE ADSORBENTS—A NEW TYPE OF IMMOBILIZED CHELATOR

NABIL RAMADAN and JERKER PORATH*

*Institute of Biochemistry, Uppsala Biomedical Center, Box 576, S-751 23 Uppsala (Sweden)*

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SUMMARY

Several novel metal chelating ligands, monohydroxamic acid, bishydroxamic acid, trishydroxamic acid, and hexahydroxamic acid, with single hydroxyl functions have been synthesized and coupled to an epoxy-activated Sepharose 6B support. All of them have been found strongly to chelate several metal ions, particularly Fe^{3+}, for which they are superior to previously described ligands. The first member of the series, monohydroxamic acid–Sepharose 6B sorbent, charged with Fe^{3+}, was evaluated by chromatography of human serum. Several proteins were bound and could be displaced with satisfactory resolution. The resolved chromatographic fractions were further analysed by gel electrophoresis. The same sorbent was also tested for selective removal of heavy metal ions from aqueous solutions.

INTRODUCTION

Porath *et al.*[1] introduced chelones (carboxymethylated amines) immobilized on agarose as ligands for metals:
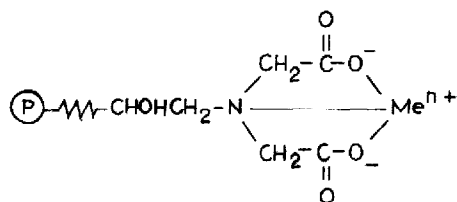
and demonstrated the separation of several serum proteins on chelated Zn^{2+} and Cu^{2+}. Metal chelate affinity chromatography is widely recognized as a popular technique for the purification of proteins[2].

Recently, we have expanded the use of the principle of immobilized metal affinity (IMA) adsorption along two lines: (a) exploration of new ligands for metal immobilization; (b) concurrent introduction of new types of metal. The synthesis of the tris(carboxymethyl)ethylenediamine ligand (TED) allowed the stable immobi-

lization of several additional metals: Fe^{3+}, Al^{3+}, Ga^{3+}, In^{3+}, and Tl^{3+} and their evaluation as sorbents for protein resolution utilizing serum as a model mixture[3,4].

In this report we introduce a series of potential new sorbents with a novel functional group together with amino or imino groups; the simplest sorbent has a single hydroxamate function, the most complex one has several. although these ligands can carry several types of metal, they are intended primarily for the immobilization of Fe^{3+} ions. The excellent stability of Fe^{3+} immobilization, in striking contrast to that on the IDA-function, make the new gels an attractive addition to the range of sorbents intended for immobilized metal affinity chromatography (IMAC).

MATERIALS AND METHODS

Hydroxylamine hydrochloride and glycine ethyl ester hydrochloride were purchased from EGA-Chemie (Steinheim/Albuch, F.R.G.). Ethylenediamine, tetraethylenepentamine, bromoacetylbromide, epichlorohydrin, sodium borohydride and formic acid were all obtained from Merck (Darmstadt, F.R.G.). Sephadex G-25 and Sepharose 6B were from Pharmacia (Uppsala, Sweden). All other chemicals were of reagent grade.

Glycyl hydroxamic acid was synthesized essentially according to a published procedure[5,6]. A 500-ml three-neck flask equipped with a mechanical overhead stirrer was placed in an ice-bath and used as a reaction vessel. A sample of hydroxylamine hydrochloride (7.9 g) was placed in the flask, and glycine ethyl ester (14 g) was added in 17 ml of water. The solution was stirred vigorously for 15–20 min. A solution of sodium hydroxide (13.2 g in 27 ml of water) was then added dropwise for *ca.* 40 min. The reaction was then allowed to proceed for an additional 15 min. The reaction mixture was then brought to neutral pH with formic acid and was used directly for coupling to an activated matrix.

The resulting glycylhydroxamic acid could be obtained, if desired, in crystalline form when the reaction mixture was acidified with 8.5 ml of concentrated hydrochloric acid (12 *M*) and left in the cold for 24 h. The crystals were filtered off and washed with a minimum amount of water. The yield was 5.9 g. The crystals can be recrystallized readily from ethanol. The values from elementary analysis agreed with the theoretical composition: calculated: C, 26.6; H, 6.7; N, 31.09; found: C, 26.13; H, 6.3; N, 31.07.

Analysis by thin-layer chromatography (TLC) showed no traces of glycine or glycine ester. The substance also showed only a single peak when run on an automatic amino acid analyser without prior hydrolysis.

Iminodiacetodiethyl ester dihydrochloride
To a three-neck 1-l flask equipped with an overhead stirrer, a reflux condenser and a dropping funnel, 39.9 g (0.3 ml) of iminodiacetic acid was added in 350 ml (6 mol) of absolute ethanol. The mixture was gassed with dry hydrogen chloride under reflux with continuous stirring until the iminodiacetic acid was completely dissolved. The reaction was allowed to proceed under reflux for an additional 3–4 h, after which the reaction flask was allowed to cool slowly to room temperature. The reaction product was recovered from the flask after 2–3 days and was recrystallized from

absolute ethanol. TLC showed a single spot and no trace of iminodiacetic acid. Analysis on an amino acid analyser confirmed the purity of the product.

Iminodiacetodihydroxamate

This compound was prepared in a similar manner to glycine hydroxamic acid. Briefly, to an ice-cold solution of 13.8 g of iminodiacetodiethyl ester and 14 g of hydroxylamine hydrochloride in 20 ml of water, a cold solution of 26.4 g of sodium hydroxide in 25 ml of water was added dropwise. The reaction was allowed to proceed for an additional 20–30 min under stirring. A white precipitate which formed on acidification with 12 M hydrochloric acid was left to crystallize in the cold for 24 h. The crystals were filtered off rapidly in the cold, washed with a minimum amount of water and then recrystallized from ethanol. TLC and analysis on an amino acid analyser showed only a single product (m.p. $> 250°C$).

Monobromoacetylhydroxamic acid

To a 500-ml three-neck flask a solution of 13.8 g of hydroxylamine hydrochloride and 42 g of sodium carbonate in 400 ml of water was added. Bromoacetyl bromide (17.2 ml) was then added in small portions with vigorous stirring. The addition was complete within 2 h at $-10°C$. When the reaction mixture was stirred for an additional 30 min at room temperature a reddish-white precipitate formed, which dissolved on acidification with formic acid. The added formic acid also decomposed any remaining traces of hydroxylamine, so that this reaction mixture can be used, without further treatment, as a source of monobromoacetylhydroxamic acid for the coupling of the latter to produce di-, tri- and hexahydroxamate functions.

In order to obtain pure monobromoacetylhydroxamic acid a purification procedure was employed[7].

Epoxy-activated Sepharose 6B and Sephadex G-25

These compounds were prepared according to a published procedure[8].

Ethylenediamine–Sepharose 6B

This compound was prepared by coupling ethylenediamine to epoxy-activated Sepharose 6B as follows. Activated Sepharose 6B (60 g) was washed with 180 ml of 2 M sodium carborate–sodium bicarbonate (pH 10.0), and the excess liquid was removed on a filter under gentle suction. The moist gel was suspended in 90 ml of the same buffer and the suspension, in a round-bottomed flask, was treated with 120 ml of ethylenediamine, (pH 12.5), added over a period of 15–20 min. The pH of the suspension was adjusted to 10.5. The suspension was stirred at 45–50°C for 24 h. The gel was then filtered, washed with water, 10% acetic acid and water again until neutral.

Tetraethylenepentamine–Sepharose 6B

This compound was prepared as follows. Activated Sepharose 6B (60 g) was washed with 180 ml of 2 M sodium carbonate–sodium bicarbonate (pH 10), and the excess liquid was removed on a filter under gentle suction. The gel was suspended in 90 ml of the same buffer and treated with 120 ml of tetraethylenepentamine over a period of 20–30 min, after which the pH of the suspension was adjusted to 10.5 and

the reaction was continued at 45–50°C for 24 h. The gel was then filtered and washed with water, 10% acetic acid, and finally with water until neutral. The nitrogen content of the gel was 1290 μmol/g dry weight, as measured by the Kjeldahl method.

ANALYTICAL PROCEDURES

All intermediary compounds, gels and final products (sorbents) were analysed for nitrogen content (Kjeldahl) and metal content (atomic absorption) as previously described[9]. The presence, if any, of amino acids was established on an amino acid analyser. The protein concentration in the chromatographic fractions was estimated spectrophotometrically at 280 nm. The analysis of the complexity of the chromatographic fractions was performed by electrophoresis in slabs of a 4–30% polyacrylamide gradient gel (PAA 4/30 Pharmacia) according to the manufacturer's manual. TLC of glycine, glycine ethyl ester, glycine hydroxamic acid, iminodiacetodiethyl ester and iminodiacetic acid was performed on precoated silica plates (Merck DC Fertig Platte, Kieselgel 60 F_{254}) in the solvent system ethyl acetate–acetone–acetic acid–water (5:3:1:1). The plates were developed with ninhydrin.
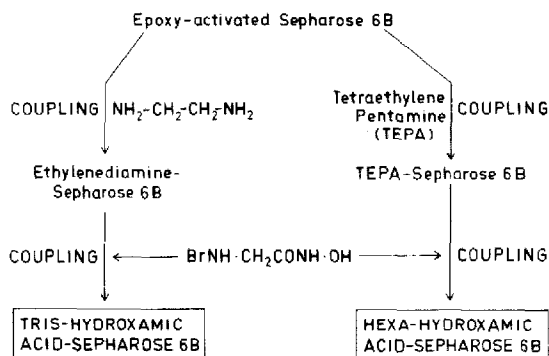
RESULTS

Synthesis of adsorbents

Four novel metal ligands containing the hydroxamic acid function were syn-
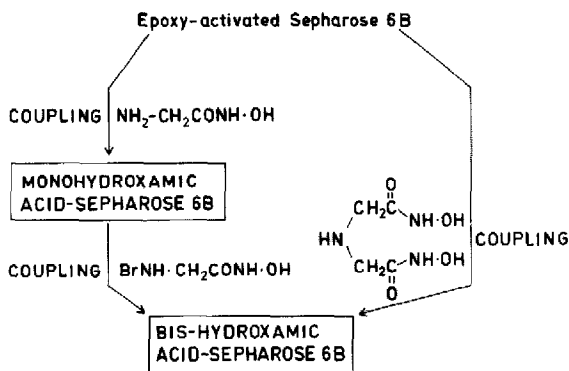
Scheme 1.

Scheme 2.

thesized (Schemes 1 and 2). These are monohydroxamic acid–Sepharose 6B and bis-hydroxamic acid–Sepharose 6B prepared by two synthetic routes (Scheme 1) and tris-hydroxamic acid–Sepharose 6B and hexahydroxamic acid–Sepharose 6B (Scheme 2).

Monohydroxamic acid–Sepharose 6B and monohydroxamic acid–Sephadex G-25

These two sorbents were prepared in an identical manner. Activated gel (epoxy gel, 30 g) was washed with 100 ml of 2 M sodium carbonate–sodium bicarbonate (pH 10.5), and the excess moisture was removed by gentle suction on a Büchner funnel. The moist gel was suspended in 45 ml of 4 M sodium carbonate buffer and transferred to a 250-ml round-bottomed flask. A solution of 4–6 g of glycine hydroxamic acid in 45 ml was added dropwise to the reaction mixture with slow stirring, during 2 h. The pH was adjusted continuously to 10.5 with 4 N sodium hydroxide for another 2–4 h. We observed that the maximum capacity of the gel, as determinated by both nitrogen content and binding of metal ion (Cu^{2+}), was reached after 12–16 h of coupling. No further increase in nitrogen content or capacity for metal occurred on standing an additional 24 h. Afterwards, the gel suspension was neutralized with formic acid and washed thoroughly on Büchner funnel with water, 10% acetic acid and with water until neutral. The gel so prepared showed the following characteristics: (a) the nitrogen content of the Sephadex G-25 gel was 665 μmol/g dry weight and that of the Sepharose 6B gel was 1535 μmol/g dry weight; (b) both gels (Sephadex G-25 and Sepharose 6B) were stable when kept at pH 10.5 at 4°C for 40 days, as judged by the constant nitrogen content and undiminished capacity for the retention of metal ions.

Iminodiacetodihydroxamic acid–Sepharose 6B

This gel was prepared by two synthetic routes.

Route 1. A sample (30 g) of monohydroxamic acid–Sepharose 6B, prepared as described above, was washed with 100 ml of 2 M sodium carbonate–sodium bicarbonate buffer (pH 10.5), sucked free of excess liquid on a Büchner funnel, and resuspended in 50 ml of 4 M sodium carbonate buffer. A 250-ml volume of the monobromohydroxamic acid (see Materials and methods) was added dropwise while the pH was maintained at 10.5 with 4 N sodium hydroxide. The suspension was stirred at room temperature for 24 h. The suspension was neutralized with formic acid, and the gel, collected on Büchner funnel, was washed with water, 10% acetic acid and again with water until neutral. The nitrogen content of the gel was 960 μmol/g dry weight. After treatment of the gel with 1 N sodium hydroxide at 60°C for 24 h the nitrogen content decreased to 890 μmol/g dry weight.

Route 2. Activated Sepharose 6B (30 g) was washed with 100 ml of M sodium carbonate–sodium bicarbonate (pH 10.5), and excess moisture was removed by suction on a Büchner funnel. To this gel, suspended in 45 ml of 4 M sodium carbonate buffer, a reaction mixture containing *ca.* 6–8 g of iminodiacetodihydroxamic acid, prepared as described in Materials and methods, was added dropwise. After complete addition, the pH of the reaction mixture was adjusted to 10.5 with 4 N sodium hydroxide and the reaction was allowed to continue for an additional 24 h. The gel was then washed as described above. The nitrogen content and the metal binding capacity were found to be identical with those of the gel prepared by the first synthetic route.

Trishydroxamic acid–Sepharose 6B

Ethylenediamine–Sepharose 6B (30 g), prepared as described in Materials and methods, was washed with 100 ml of 2 M sodium carbonate–sodium bicarbonate (pH 10.5), and gently dried under suction. The gel was suspended in 45 ml of 4 M sodium carbonate buffer, and monobromohydroxamic acid (12–14 g), contained in 400 ml of the reaction mixture described in Materials and Methods, was added dropwise. The pH was adjusted to 10.5 with 4 N sodium hydroxide, and the suspension was stirred at room temperature for 24 h with occasional adjustment of the pH. The suspension was then neutralized with formic acid, and the gel was washed with water, 10% acetic acid and finally with water until neutral. The nitrogen content of the gel was 2070 μmol/g dry weight. When the gel was treated with 1 N sodium hydroxide at 60°C for 24 h the nitrogen content was reduced to 1950 μmol/g dry weight.

Hexahydroxamic acid–Sepharose 6B

This gel was prepared by coupling monobromohydroxamic acid, prepared as described in Materials and Methods, to tetraethylenepentamine–Sepharose 6B, also prepared as described earlier. The coupling conditions were those used for the preparation of trishydroxamic acid–Sepharose 6B, except for the use of approximately double the amount (20–30 g) of monobromohydroxamic acid. All other conditions were changed appropriately. The nitrogen content of hexahydroxamic acid–Sepharose 6B was 1290 μmol/g dry weight, although the coupling conditions have not yet been optimized.

The nitrogen content of all hydroxamate gels and the amount of metal ions, Cu^{2+} and Fe^{3+}, retained by the gels after extensive washing with water, are given in Table I. In the case of monohydroxamic and trishydroxamic gels, the retention of Fe^{3+} is somewhat higher than that of Cu^{2+}, and for the dihydroxamic and hexahydroxamic gels the retentions of the two metals are about the same. This is a very favourable experimental outcome compared with the retention of Cu^{2+} and Fe^{3+} on IDA-type gels. The stability of the retention of several metal ions toward various washing solutions is given in Table II for monohydroxamic acid–Sephadex G-25.

Of particular interest is the fact that the retention of Fe^{3+} ions by the monohydroxamic gel remains high after washing with ordinary buffers, but falls off as expected on treatment with EDTA. The small amount of Fe^{3+} that remains after

TABLE I

METAL BINDING CAPACITY IN μmol/g DRY WEIGHT AND LIGAND CONCENTRATION FOR DIFFERENT HYDROXAMATE SEPHAROSE 6B ADSORBENTS

A sample of 1 g of moist gel was saturated with metal ion solution in water (0.02 M) for 24 h. The excess of metal ion was washed with 200-fold water, and the remaining metal values are given here.

Metal ion(s)	Monohydroxamate–Sepharose 6B	Dihydroxamate–Sepharose 6B	Trishydroxamate–Sepharose 6B	Hexahydroxamate–Sepharose 6B
Fe^{3+}	428	321	446	587
Cu^{2+}	376	308	358	582
Ligand concentration	1540	957	2070	1290

TABLE II

METAL BINDING CAPACITY TO GLYCINE HYDROXAMIC ACID–SEPHADEX G-25

A sample, 0.5 g, of moist gel was saturated with metal ion solution in water. The excess of metal ion was washed with water and the remaining metal(s) is given in column 2. Another sample, 0.5 g, was saturated and washed with water and then with glycine buffer and water again, the results are shown in column 3. The same treatment was extended to washes with sodium acetate (column 4), and EDTA (column 5).

Metal ion	Washing step			
	100 ml H_2O	100 ml 1 M Gly–NaOH, pH 9.0, and 100 ml H_2O	100 ml 1 M Sodium acetate, pH 5.5 and 100 ml H_2O	100 ml 0.1 M Na_2EDTA and 100 ml H_2O
Fe^{3+}	192	160	138	5
Co^{2+}	166	61	45	49
Ni^{2+}	204	58	138	47
Cu^{2+}	290	129	280	47
Zn^{2+}	171	15	19	3
Ag^+	240	27	9	38

extensive washing with EDTA was found to be of no importance as regards the binding of proteins. The sorbent, after all washings including that with EDTA, can be recharged to the original capacity with all the metal ions under study. The capacity, selectivity, and ultimately the usefulness, of all hydroxamate gels remain largely unexplored and are at present undergoing thorough scrutiny in this laboratory. However, it may be of interest to report at this time some preliminary data on the resolving power of these novel sorbents.

Adsorption of metal ions

In many cases it is desirable to remove certain chemical species selectively from a multicomponent mixture, *e.g.* when highly toxic or valuable constituents, such as certain heavy metal ions, are to be removed from industrial waste water. Ultrafiltration and reverse osmosis have become standard procedures for the separation of molecular solutions[10–13]. Table III illustrates the effective removal of heavy metal ions from Uppsala tap water by one of our gels.

TABLE III

REMOVAL OF METAL IONS FROM 8 l OF DEGASSED TAP WATER BY MEANS OF GLYCINE HYDROXAMIC ACID–SEPHADEX G-25

Ligand concentration 664 μmol/g dry weight.

Metal ion	μmol/g dry weight
Mg^{2+}	7
Ca^{2+}	93
Fe^{3+}	6
Co^{2+}	0
Ni^{2+}	<0.5
Cu^{2+}	132
Zn^{2+}	2
Ag^+	0

There are many different molecular complexing agents available today, *e.g.* iminoacetic acid, methylthiourea, quaternized amine, 8-hydroxyquinoline, LIX 64 N (trademark of General Mills), LIX 64 N 336 (trademark of Ashland Chemicals), etc. These agents require relatively long residence times and intensive mixing, which makes their use time-consuming and uneconomical in comparison with the hydroxamate sorbent.

The chelator gel without metal was packed into a column (1×0.6 cm I.D., V_t *ca.* 0.5 ml) in distilled water. Degassed tap water, 8 l, was then pumped through at a rate of 27–30 ml/h using a peristaltic pump. The column became coloured as the metal ion(s) attached to the gel. The coloured front extended halfway down the column, indicating that the bed was not fully loaded with metal ion(s) after passage of 8 l. The sorbent was analysed for nitrogen content (Kjeldahl) and metal content (atomic absorption) as previously described[9] (Table III). The hydroxamate adsorbent showed high selectivity, and high binding capacity at low cost. For valuable metals such as gold, silver, etc., the cost of separation by the hydroxamate method would be generally low compared with the value of the metals.

Exploratory study of hydroxamate adsorbents for IMAC on serum protein

To this end we have explored the resolution of serum on the monohydroxamic acid gel. The chromatogram obtained with 5 ml of human serum on a 6-ml bed of a monohydroxamic acid–Fe^{3+} gel is shown in Fig. 1. The electrophoretic analysis of the individual chromatographic fractions is illustrated in Fig. 2. Only *ca.* 25% of the serum protein content was retained under the solvent conditions employed, suggesting a measure of selectivity. Moreover, a larger amount of protein, representing a smaller proportion of the total, was retained when 125 ml of the serum was applied. Both the extent of retention and the capacity of the sorbent for retainable proteins indicate the potential usefulness of the simplest gel of the series for the resolution of the protein components of serum.
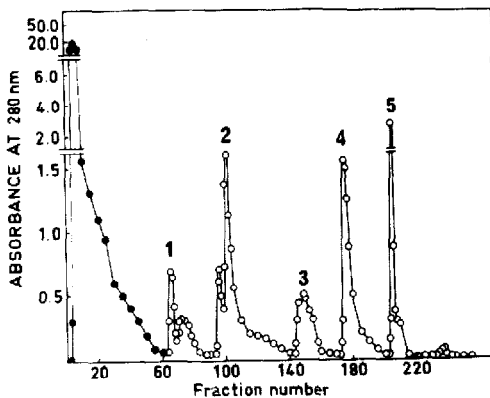

Fig. 1. Chromatography of human serum on monohydroxamic acid–Fe^{3+}–Sepharose 6B. Dialysed human serum (5 ml) was applied to the column (8×1 cm I.D.) equilibrated with 50 mM sodium acetate (pH 5.5) at *ca.* 7 ml/h, and the column was washed with *ca.* 10 total volumes of the equilibrating buffer. The column was developed stepwise with increasing concentrations of sodium chloride in the same buffer up to a final value of 0.5 M. Fractions of 3.6 ml were collected. The column was regenerated with 0.2 M glycine–sodium hydroxide buffer containing 0.5 M sodium chloride (pH 9.0), which removed traces of proteins still on the column (less than 0.5%).
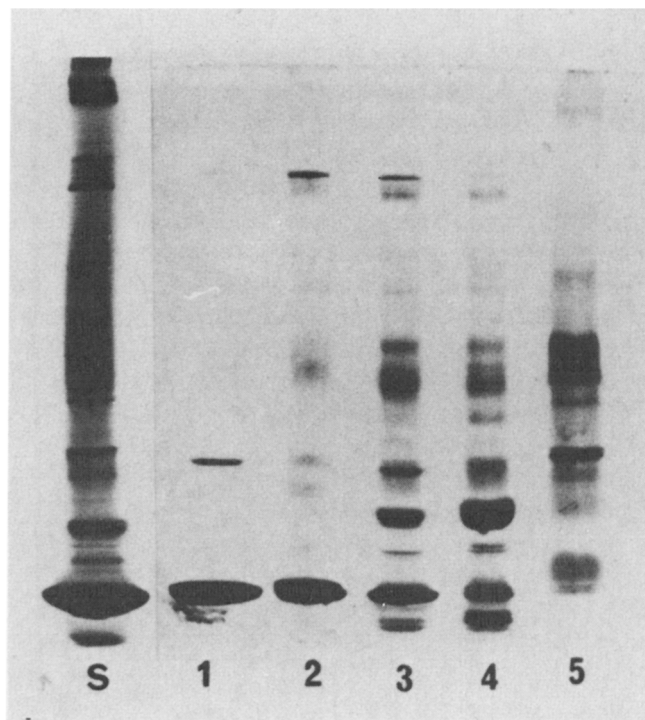
Fig. 2. Gradient gel electropherogram. Individual fractions taken from the experiment are in channels 1–5, analysed by electrophoresis as described in Analytical procedures. Left-hand channel: whole human serum.

DISCUSSION

A series of novel metal chelating ligands, mono-, bis-, tris- and hexahydroxamic acids, has been prepared. Although a hydroxamate function can firmly retain several metal ions (Tables II and III), it is the particular affinity for Fe^{3+} ions that is of special interest. Fig. 3 illustrates the coordination of Fe^{3+} ion to the hydroxamic acid sorbents. It is clear that the accessibility of the coordinated Fe^{3+} ion and its potential for interacting with electron donor groups on a protein surface may vary substantially among the four sorbents represented in Fig. 3. Therefore, each of these sorbents has to be evaluated individually as a potential tool for protein purification. However, our preliminary data for the separation of serum proteins on the simplest of the family of sorbents, i.e., Fe^{3+}–monohydroxamic acid–Sepharose 6B, are interesting. Figs. 1 and 2 show that the serum proteins can be resolved into several fractions. Furthermore, the contents of these fractions are not identical with those obtained earlier on TED–Fe^{3+} (ref. 3). These two sorbents, which retain Fe^{3+} better than the IDA ligand, may thus complement each other in a purification scheme. It is evident from Fig. 1 that monohydroxamic acid–Sepharose 6B has a large capacity for some serum proteins. In fact, the same column was used to adsorb selectively the same proteins from as much as 125 ml of human serum. Furthermore, the same column can be used repeatedly, after regeneration with a glycine buffer (Fig. 1), with
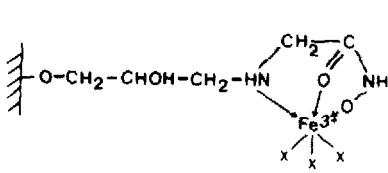
(1) Fe³⁺-Monohydroxamate adsorbent
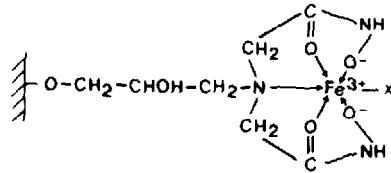
(2) Fe³⁺-Di-hydroxamate adsorbent

a

b
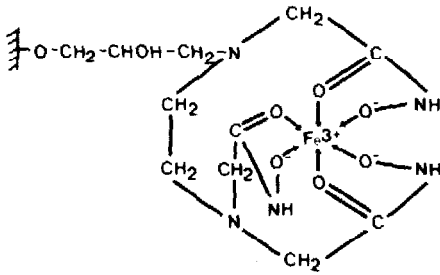
(3) Fe³⁺-Tris-hydroxamate adsorbent
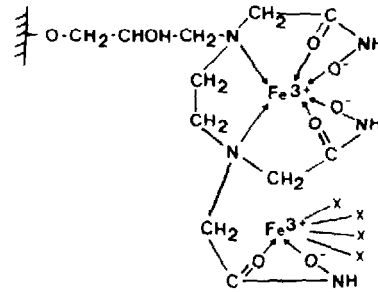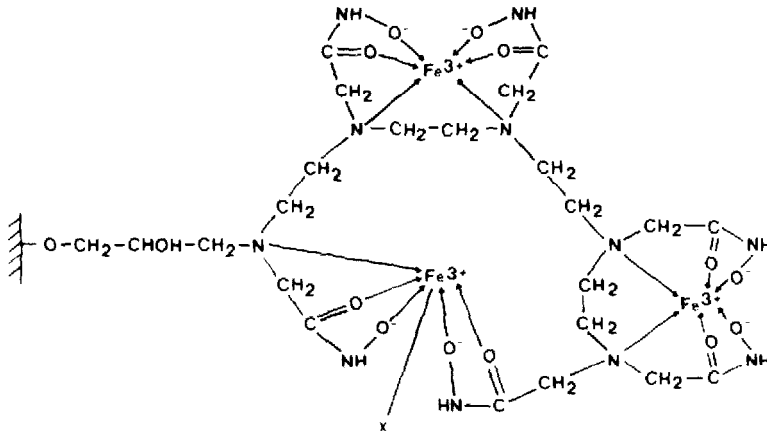
(4) Fe³⁺-Hexa-hydroxamate adsorbent
x=Solution counter ion

Fig. 3. The proposed coordination of Fe³⁺ ions to the hydroxamic acid sorbents.

no loss of capacity. In the elution protocol used for the fractionation of serum, the proteins are displaced by a stepwise increase in the ionic strength of the solvent. It remains to be seen whether the modification of other experimental parameters (pH, type of buffer, temperature, etc.) might also be exploited to alter the selectivity of elution. We shall also support the observations made with serum proteins by a study of somewhat simpler protein mixtures or individual proteins.

Further studies were carried out to evaluate the bis-, tris- and hexahydroxamate gels. The binding strengths for metal ions increased in the expected order. The adsorption of Fe^{3+} ions on the hexahydroxamate gels was so strong that most of the metal could not be eluted with solutions of any of the available chelators (iminodiacetic acid, EDTA, etc.) at acidic, or neutral or basic pH. Indeed, only by hydrolysing the gel could the metal ion be brought into solution, which means that the hydroxamate gels are extremely efficient for removal of transition metal ions, such as Fe^{3+}, from aqueous solutions. The heterogeneous binding of metal ions to hydroxamate adsorbents (Tables II and III) can be suppressed by washing the gel with an appropriate buffer system. This is necessary to obtain uniformity in the adsorption sites essential for IMAC.

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