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Fe^{3+}–HYDROXAMATE AS IMMOBILIZED METAL AFFINITY-ADSORBENT FOR PROTEIN CHROMATOGRAPHY

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SUMMARY

The adsorbent glycinehydroxamate–Sepharose 6B, charged with Fe^{3+} under specified conditions, is reported. It was used at various pH values for chromatography of the following proteins: lysozyme, cytochrome *c*, avidin, bovine pancreatic RNase, myoglobin, ovalbumin and human serum albumin. The common naturally occurring amino acids were tested for their interactions with the new sorbent under neutral conditions.

INTRODUCTION

Porath *et al.* reported[1] that adsorption of proteins on immobilized metals is highly pH-dependent. Hard-metal Lewis acids such as Fe^{3+}, Ce^{3+} or Al^{3+} yield immobilized metal ions (IMA adsorbents) showing strong protein interaction at low ionic strength in slightly acidic media (pH 5). Such adsorbents have predominantly ion-exchange properties. Up to now, no detailed study of such specific ion exchangers has been made.

We have found that proteins can be adsorbed on immobilized Fe^{3+} over a wider range of pH. The adsorption affinities of proteins towards the IMA–Fe^{3+} sorbent are highly dependent on the nature of the buffer and the capacity of the adsorbent: α-aminohydroxamate adsorbent charged with Fe^{3+}–glycine shows selective ion-exchange properties, which have been studied in detail. The adsorption is different from that in ordinary ion-exchange processes and is interpreted on the basis of interactions that depend on the coordination characteristics of proteins with metal ions at various pHs.

In order to understand the results obtained by this approach, the retention of the common amino acids on a Fe^{3+}–hydroxamate gel was investigated. These studies allow estimation of relative contributions to the binding of glycoproteins to this kind of adsorbent. Although the atoms N, O and S are known to be responsible for the interaction with IMA–Me^{2+}, it was essential to study the individual amino acids in order to make some quantitive estimate of the relative contributions of the different functional groups present in proteins.

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MATERIALS AND METHODS

α-Aminohydroxamate–Sepharose 6B was prepared as previously reported[2]. Chicken lysozyme, horse heart cytochrome *c*, chicken avidin, bovine pancreatic RNase, sperm whale myoglobin, chicken ovalbumin and human serum albumin (HSA) were all purchased from Sigma (St. Louis, MO, U.S.A.). The amino acids L-phenylalanine, L-tyrosine, L-tryptophan, L-threonine, L-glutamic acid and L-valine were obtained from Fluka (Buchs, Switzerland). L-Alanine, L-arginine, L-asparagine, L-aspartic acid, L-cysteine, L-cystine, L-glutamine, L-histidine, L-isoleucine, L-leucine, L-lysine, L-serine, glycine, and ferric chloride were purchased from Merck (Darmstadt, F.R.G.). Tris(hydroxymethylaminoethane) was from Fluka. All other chemicals used were commercially available and of puriss grade.

The chromatographic columns and the peristaltic pump P3 used were from Pharmacia (Uppsala, Sweden). The gradient device used was constructed in the workshop of the Biomedical Center (Uppsala, Sweden). A continuously recording spectrophotometer (Uvicord from LKB, Bromma, Sweden) was used to monitor the protein concentration in the effluents, and the chromatographic fractions were measured manually using a Hitachi Model 101 spectrophotometer.

Charging of glycinehydroxamate adsorbent with metal ion

Glycinehydroxamate–Sepharose 6B (15 g) was prepared according to standard procedures[2] and washed thoroughly with 1 *M* glycine–sodium hydroxide buffer (pH 9.0). The excess solution was removed by gentle suction on a Büchner funnel. The moist gel was suspended in 30 ml of 1 *M* glycine–sodium hydroxide buffer (pH 9.0). Ferric chloride (150 ml of 0.2 *M*) was added dropwise and the solution was stirred for 16 h at room temperature. The Fe^{3+}–hydroxamate gel prepared was washed thoroughly with the glycine buffer then equilibrated with the buffer to be used in each of the separate experiments until no ferric ions could be detected in the eluate.

Removal of metal ion (Fe^{3+}) and recharge of the adsorbent

The column was washed with 5–6 volumes of 2 *M* sodium hydrogen sulphite at a rate of 15 ml/h. Alternatively, washing of the column with one volume of 0.5 *M* oxalic acid was also sufficient to remove all the ferric ions. In both cases, the column could, on requilibration, be recharged without any noticable decrease in metal binding capacity.

Chromatography of amino acids and proteins

For each set of experiments the same Fe^{3+}–glycinehydroxamate column (6.4 × 1 cm I.D., V_t *ca.* 5 ml) was used. The column was equilibrated with the starting buffer and one total volume (5 ml) of sample solution (1–2 mg protein/ml of starting buffer) was applied. In the case of avidin the sample concentration was 0.4 mg/ml. The sample was followed by four total volumes of starting buffer. The proteins were then displaced as described in the individual experiments.

The amino acids were dissolved separately in the equilibration buffer to a final concentration of 3–5 m*M*, except for L-tyrosine, which was dissolved in the equilibration buffer to saturation by gentle heating.

The amino acid samples (500 μl) were applied to the column and elution was

performed at 15 ml/h. Fractions of 1 ml were collected and the amino acids were detected with trinitrobenzene sulphonic acid[3], ninhydrin[4,5] or by continuous recording of the absorption at 280 nm. The retention of amino acids is expressed as the reduced elution volume V_e/V_t, where V_e is the measured volume at the peak maximum and V_t is the total volume of the gel bed. The experiments were carried out at room temperature.

RESULTS

Chromatography of amino acids

The retention data for amino acids are presented in Tables I and II. The amino acid retention was determined in 0.1 *M* Tris–hydrochloric acid (pH 7.0) (Table I), and in the same buffer at different pH values (Table II). Cysteine was markedly retained, with a much longer retention time than any other amino acid. No numerical value is presented for cysteine.

Table II illustrates the pH-dependence of the retention of aromatic amino acids and cystine. The retention of cystine compared with the retention of aromatic amino acids is remarkable. The retention of aromatic amino acids is insensitive to pH in the range 5–9.

TABLE I

THE RETENTION OF AMINO ACIDS ON Fe^{3+}–HYDROXAMATE ADSORBENT

All amino acid samples were applied on a column (6.4 × 1 cm I.D., V_t *ca.* 5 ml) equilibrated with 100 m*M* Tris–hydrochloric acid (pH 7.0). All other details are given in Materials and methods. V_e denotes the effluent volume.

Amino acid	V_e/V_t
Alanine	1.2
Arginine	1.4
Asparagine	1.3
Aspartic acid	2.0
Cysteine	>20
Cystine	3.8
Glutamine	1.3
Glutamic acid	1.7
Glycine	1.2
Histidine	1.3
Isoleucine	1.2
Leucine	1.2
Lysine	1.4
Methionine	1.3
Phenylalanine	1.1
Serine	1.2
Threonine	1.2
Tryptophan	1.3
Tyrosine	1.3
Valine	1.2

TABLE II

INFLUENCE OF pH ON THE RETENTION OF AROMATIC AMINO ACIDS AND CYSTINE ON Fe^{3+}-HYDROXAMATE ADSORBENT

The same column as described in Table I was used. The buffers used were 0.1 *M* Tris–acetate at pH 5 and 6 and 0.1 *M* Tris–hydrochloric acid at pH 7, 8 and 9. V_e indicates the elution volume of the amino acid and V_t the total column volume.

Amino acid	V_e/V_t				
	pH 5	*pH 6*	*pH 7*	*pH 8*	*pH 9*
Phenylalanine	1.0	1.0	1.1	1.1	1.2
Tyrosine	1.3	1.3	1.3	1.3	1.1
Tryptophan	1.3	1.3	1.3	1.3	1.1
Cystine	5	4.0	3.8	2.4	2.4

Chromatography of proteins of pI 4.8–11.2

The chromatography of selected proteins with isoelectric points in the range 4.8–11.2 was examined at two pH values (5.8 and 9.0). A salt gradient from 0 to 0.5 *M* sodium chloride was used for the elution in both cases. At higher pH values all proteins were eluted according to their p*I* values (Fig. 1a and Table III). The four proteins that had a net negative charge (p*I* values 4.8–7.8) were all retained slightly (the breakthrough volume of the columns was 5.0 ml) whereas the basic proteins eluted with the salt gradient. The adsorbent behaved as a cation exchanger.

At pH 5.8 in 0.04 *M* sodium phosphate buffer, the elution behaviour was more complex (Fig. 1b). The two proteins with a net negative charge (serum albumin and ovalbumin) were only slightly retarded. At the beginning of the salt gradient myoglobin and lysozyme, despite their very different p*I* values (6.8 and 11.2), eluted together. They were followed by RNase and cytochrome *c* at the same elution volume despite their different p*I* values (7.8 and 10.6). Avidin was strongly adsorbed and eluted as a trailing peak in 0.5 *M* sodium chloride. Although the adsorbent did behave as a cation exchanger, it is clear that additional factors contribute to the adsorption of proteins.

Chromatography of basic proteins

The effect of pH and ionic strength on the behaviour of the lysozyme and cytochrome *c* was investigated. At low ionic strength both proteins were adsorbed on the column at pH 5.8 and did not elute when the pH was increased to 7.8 (Fig. 2a and b): they also remained on the column at pH 9.0 (Fig. 1a). Owing to the risk of formation of an insoluble precipitate (iron hydroxide) in the gel bed, further increases in pH were not attempted.

The elution of proteins was achieved by increasing the ionic strength, either by increasing the buffer concentration (Fig. 2a) or by adding sodium chloride (Fig. 2b). The sodium chloride gradient used at pH values 5.8 and 9.0 shows that the two proteins elute at 0.15 *M* and 0.13 *M* at pH 9.0 and 0.14 *M* and 0.33 *M* sodium chloride, respectively, at pH 5.8. At pH 7.8, 0.5 *M* sodium chloride was used to achieve a stepwise elution of cytochrome *c* (Fig. 2b).

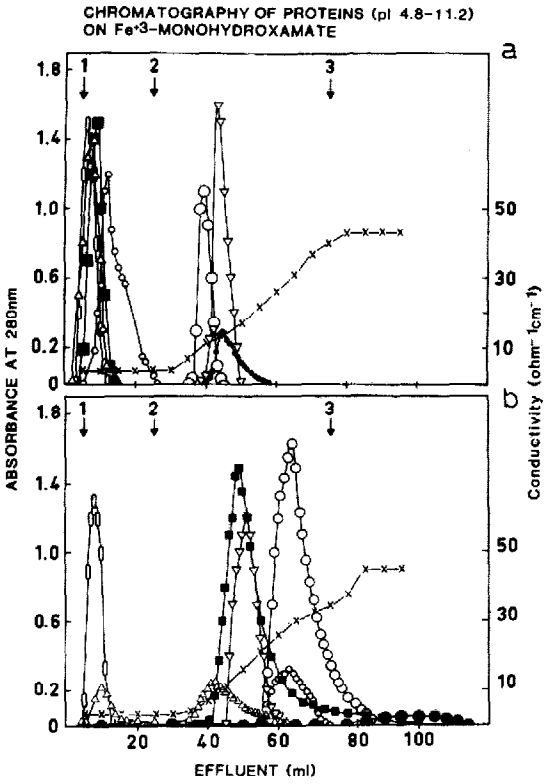


Fig. 1. Chromatography of proteins with different p*I* values (4.8–11.2) on Fe^{3+}–monohydroxamate adsorbent. A column (6.4 × 1 cm I.D.) was equilibrated with (a) 0.1 *M* Tris–hydrochloric acid (pH 9.0) and (b) 0.04 *M* sodium phosphate (pH 5.8). The column was then eluted with a linear gradient (0–0.5 *M*) of sodium chloride. Fractions of 1 ml were collected and the absorbance at 280 nm was measured. The arrows indicate application of sample, equilibrating buffer (1), gradient (2) and final buffer including 0.5 *M* sodium chloride (3). ▽–▽ = Lysozyme; ○–○ = cytochrome *c*; ●–● = avidin; ○–○ = RNase-A; ■–■ = myoglobin; □–□ = ovalbumin; △–△ = HSA.

TABLE III

DESORPTION OF PROTEINS OF DIFFERENT p*I* VALUES FROM Fe^{3+}–GLYCINE HYDROXAMATE GEL

Protein	*pI*	*Elution volume (ml) (0.1 M Tris–HCl, pH 9.0)*	*mM NaCl added to the buffer**	*Elution volume (ml) (0.04 M phosphate, pH 5.8)*	*mM NaCl added to the buffer**
HSA	4.8	8	–	10 and 42 (2 peaks)	–
Ovalbumin	5.0	6	–	8	–
Myoglobin	6.8	9	–	49	100
RNase-A	7.8	12	–	58	317
Avidin	10	44	159	95	500
Cytochrome *c*	10.6	38	129	59	328
Lysozyme	11.2	43	147	51	138

* Required to effect elution.

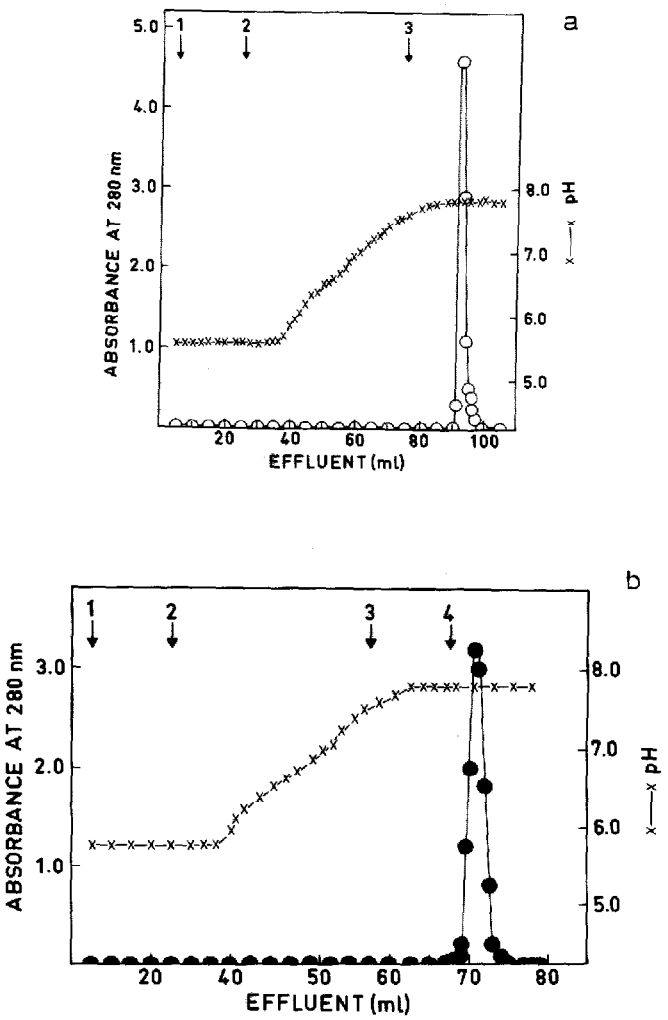


Fig. 2. Chromatography of (a) lysozyme and (b) cytochrome *c*. The first step is 0.02 *M* sodium phosphate (pH 5.8); the second step is a linear pH gradient (5.8–7.8); the third step is (a) 0.05 *M* sodium phosphate (pH 7.8) or (b) 0.02 *M* sodium phosphate (pH 7.8) followed by a fourth step where sodium chloride is added to this buffer at a concentration of 0.5 *M*.

Determination of the capacity of the adsorbent

Fig. 3a shows a frontal analysis determination of the capacity of the adsorbent for cytochrome *c* in 0.1 *M* Tris–hydrochloric acid (pH 9.0). A solution of 1 mg of protein per millilitre of buffer (A_{280} = 1.4) was used. A plateau of A_{280} = 1.4 was reached after 120 ml of effluent. The calculated capacity was thus 21 mg of cytochrome *c* per millilitre of bed volume.

A similar capacity determination was carried out at pH 5.8 in 0.02 *M* sodium phosphate buffer (Fig. 3b). Here, a lower concentration (0.1 mg of protein per millilitre of buffer, A_{280} = 0.22) had to be used for quantitative estimation. The absor-

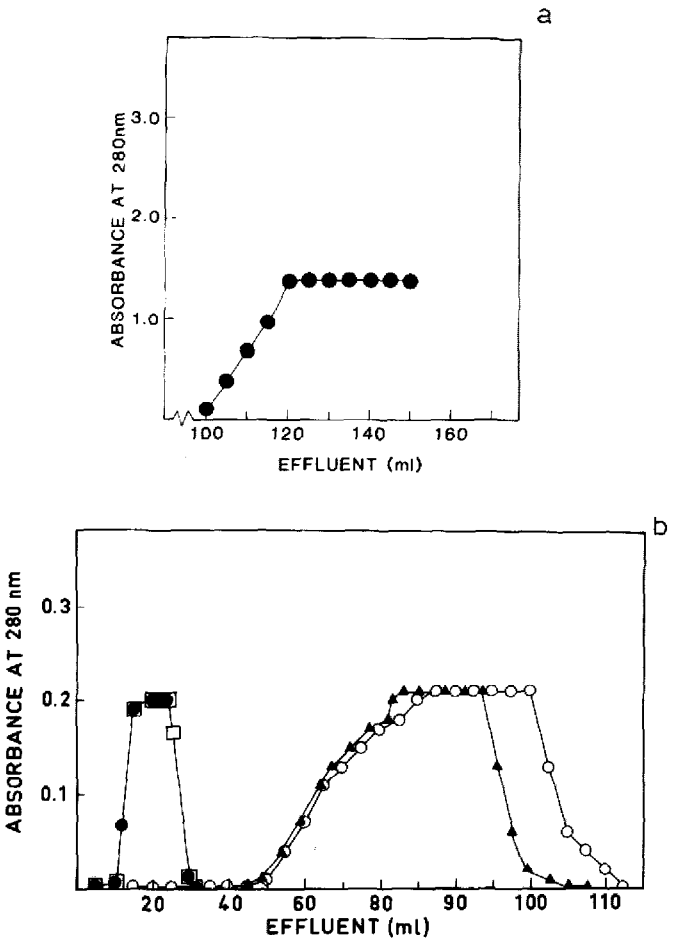


Fig. 3. Frontal analysis in (a) 0.1 *M* Tris–hydrochloric acid (1 mg cyt *c*/ml) and (b) 0.02 *M* sodium phosphate (pH 5.8). ○–○ = Fe^{3+}-monohydroxamate; ●–● = monohydroxamate after removal of metal ion; ▲–▲ = monohydroxamate recharged with Fe^{3+}; □–□ = activated/deactivated Sepharose 6B (blank).

bance of the plateau was equal to that of the sample solution after passage of 75 ml. The capacity thus calculated was 1.5 mg of cytochrome *c* per millilitre of bed volume.

Non-specific adsorption

The same experiments were also run after the iron had been removed from the adsorbent by treatment with 0.1 *M* EDTA at pH 4.5. We also examined a gel prepared by deactivation of an epoxy-activated Sepharose 6B followed by treatment with iron. Both of these blank gels had a capacity for cytochrome *c* of 0.2 mg per millilitre of bed volume (Fig. 3b). Finally, when the gel from which the iron had been removed with EDTA treatment was recharged with iron, the capacity was determined again and found to be identical with the initial value (Fig. 3b). The results are summarized in Table IV.

TABLE IV

CAPACITY MEASUREMENTS OF Fe^{3+}–HYDROXAMATE ADSORBENT USING CYTOCHROME *c*

Adsorbent	*Iron content (μmol/g dry gel)*	*Capacity for cytochrome, pH 5.8 (mg/ml bed volume)*	*Capacity for cytochrome, pH 9.0 (mg/ml bed volume)*
Fe^{3+}–hydroxamate–Sepharose 6B	428	1.5	21
EDTA-treated Fe^{3+}–hydroxamate–Sepharose 6B	3	0.2	–
Reloaded EDTA-treated Fe^{3+}–hydroxamate–Sepharose 6B	401	1.5	–
Activated/deactivated Sepharose 6B	3	0.2	–

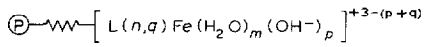
DISCUSSION

The basic principle of IMA chromatography is that a metal ion is fixed on a solid support via a chelating ligand. The interaction between transition metal and the solute (protein) occurs with the formation of coordination bonds inside the coordination sphere of the complex-forming ion, if the coordination sphere of this ion is unsaturated (the coordination sites are temporarily occupied by weakly bound solvent molecules). Various mechanisms could be responsible for the interaction between protein and IMA adsorbents. An obvious possibility is ion exchange, because both adsorbent and solutes are charged molecules. Hydrophobic interactions might also contribute, because both the ligands on the gel and the solute have some hydrophobicity. Finally, the specific coordination properties of the adsorbents are expected to play an important role. In the case of the IDA–Cu^{2+} and IDA–Ni^{2+} gels, the ionic forces evidently play a minor role, because the interaction increases on addition of salt to the solvent.

In contrast to IDA–Cu^{2+} and IDA–Ni^{2+} adsorbents, the Fe^{3+}–hydroxamate gel appears to bind proteins at low ionic strength. The interaction seems to be due mainly to coulombic forces: at pH 9.0, a set of proteins eluted in the order of increasing net positive charge. The conclusion is that Fe^{3+}–hydroxamate gel charged with ferric chloride in 1 *M* glycine–sodium hydroxide (pH 9.0) functions as a cation exchanger. This fact was substantiated by a more detailed study of the behaviour of the basic proteins cytochrome *c* and lysozyme. These proteins were eluted by increasing the ionic strength (Table II).

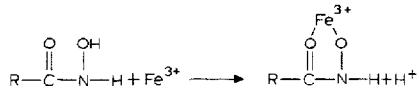
At pH 5.8, the situation was very different. The gel at pH 9 had a 14-fold higher capacity for basic proteins (Table IV). Some of the proteins with net negative charge were slightly retarded, whereas all proteins with net positive charge remained on the column but could be eluted at much lower ionic strength than at pH 9. It appears that the adsorbent still behaves as a cation exchanger but with a weaker effect. The elution order of the proteins indicates that mechanisms other than ion exchange also contribute to adsorption.

Iron has strong tendency to form complexes of different types[2,6,7]. Trivalent iron is very weakly coordinated to amino groups compared with metals of the first transition series[8]. It exists in solution in a hexacoordinated form with water, hydroxyl groups and other ions if present. In an immobilized form some of the coordination sites are occupied by ligands fixed to a polymer matrix. The expected formula of the metal ion–ligand complex can be represented schematically as follows:



Ⓟ signifies the polymer matrix; L is a ligand with (n-dentation) and negative charge q, because $m + n + p = 6$; $|p + q| < 3$ indicates a positively charged adsorbent (anion exchanger); $|p + q| = 3$ indicates an adsorbent with zero net charge (neutral); $3 < |p + q| < 6$ negatively charged adsorbent (cation exchanger). The number n can vary from 1 to 5. For $n < 3$, the iron ion is not strongly bound to the gel. If $n = 5$ the adsorption capacity is generally low.

The hydroxamate group acts as a bidentate chelate that forms a very stable five-membered ring with iron:

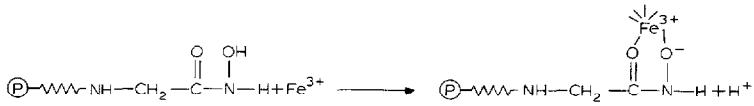


(1)

Hydroxamic acids are weak acids by virtue of the ionization of the –N–OH hydrogen (pK_a *ca.* 9)[9]. It is this hydrogen that is displaced by the metal on chelation, so efficiently that it will also occur at acidic pH values.

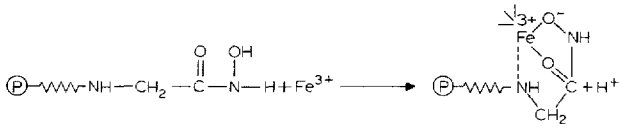
In α-aminohydroxamate the α-amino and keto groups coordinate with the metal ion in an uncharged form. The hydroxylamino group coordinates, as explained, with negative charge.

However, the Fe^{3+}/NH_2 coordination has weak binding properties[8]. Either a bi- or tridentate complex will thus be formed (eqns. 2 and 3).



(2)

where $n = 2$; $q = -1$, and $m + p = 4$;



(3)

where $n = 3$; $q = -1$ and $m + p = 3$.

In pure water the residual ligand positions will be occupied by water molecules or hydroxyl ions. The number of hydroxyl ions will depend on the pH (eqns. 4–6).

$$[L(3,-1)\ Fe^{3+}\ (H_2O)_3]^{2+} \rightarrow [L(3,-1)\ Fe^{3+}\ (H_2O)_2(OH^-)]^{+} + H^{+} \quad (4)$$

$$[L(3,-1)\ Fe^{3+}\ (H_2O)_2(OH^-)]^+ \rightarrow [L(3,-1)\ Fe^{3+}\ (H_2O)(OH^-)_2] + H^+ \quad (5)$$

$$[L(3,-1)\ Fe^{3+}\ (H_2O)(OH^-)_2] \rightarrow [L(3,-1)\ Fe^{3+}\ (OH^-)_3]^- + H^+ \quad (6)$$

In the case of the bidentate form, an additional equilibrium can be formulated (eqn. 7, Fig. 4)

$$[L(2,-1)\ Fe^{3+}\ (H_2O)_4]^{2+} \rightarrow [L(2,-1)\ Fe^{3+}\ (H_2O)_3(OH^-)]^+ + H^+, \text{ etc.} \quad (7)$$

In water, Fe^{3+} is known to exist as a $Fe^{3+}(OH^-)_3(H_2O)_3$ complex[10], even under acidic conditions (pH <3). The equilibria shown here are thus expected to apply even at acidic pH. A titration curve (mmol $[H^+]$/mmol ligand *vs.* pH, Fig. 4) did not indicate several distinct deprotonation steps in the range around neutrality. We therefore believe that the gel ligand structures are negative at all pH values of interest for chromatography. This is confirmed by the observation that the gel behaves mainly as a cation exchanger and must therefore be a polyprotic acid.

If counter-ions other than hydroxyl are present, such as phosphate or chloride, these can also be part of the structure[10]. Some preliminary experiments showed that such binding occurred with phosphate and acetate (to be reported elsewhere). The structure of the ligand in these cases may be more complex, but the net charge is still expected to be negative at all pH values of interest for chromatography. The hydroxamate gel would still be expected to behave as a cation exchanger at both weakly basic and acidic pH values (pH 9.0 and 5.8).

Our conclusion is that the immobilized iron ion acts as an ion exchanger with specific properties that differ from transition metal ions normally used for IMAC.

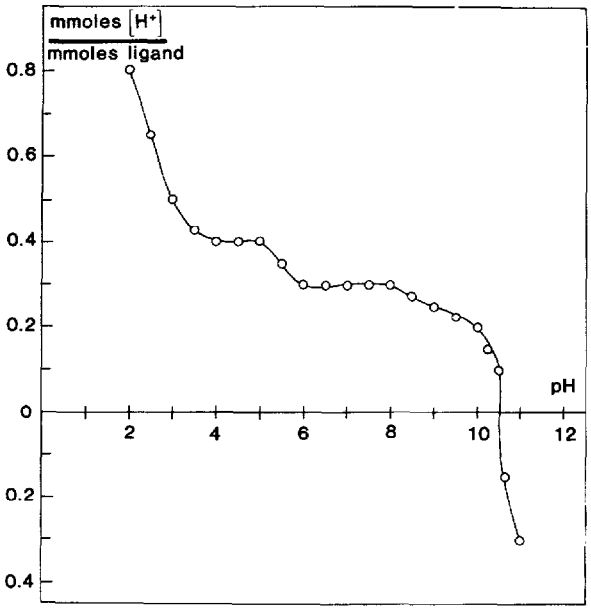


Fig. 4. Titration curve of Fe^{3+}–monohydroxamate adsorbent (5 g of sucked dry gel).

The ion-exchange properties of the gel do not exclude the possibility that other effects may come in to play in some cases. If structures on the solute can displace the negative counter-ions of the Fe^{3+} complex, the spatial binding properties of the IMA adsorbents become important. This seems to be an explanation for the high retention values of some amino acids, in particular, cysteine, cystine and the dicarboxylic acids aspartate and glutamate.

Amino acids are ampholytes, and their state of ionization depends on the environmental conditions. The α-amino and α-carboxyl and the third ionizable side-chain group are some of the factors influencing the interaction with IMA-metal. The amino acid at pH 7.0 (Tables I and II) exists predominantly as a neutral zwitterion. This explains the behaviour of most amino acids on the Fe^{3+}–monohydroxamate gel at pH 7.0. The pK_a values of the β- and γ-carboxyl groups of aspartic and glutamic acids, respectively, cause greater interactions than other amino acids (Table I). Even the imidazole group of histidine does not give rise to any significant interaction with the metal ion. The sulphydryl group of cysteine has a very high affinity for the Fe^{3+}–hydroxamate, showing that some other forces must be involved. According to Pearson's classification[8] Fe^{3+} is a hard metal acid and prefers a hard base ligand atom such as oxygen. Normally, valine, leucine and isoleucine are expected to interact with IDA-metal and TED-metal owing to the hydrophobic interaction between their side-chains and both ligands. This has not been observed in the case of Fe^{3+}–monohydroxamate gel. This is due to the fact that the ligand is very small and has no hydrophobic effect. The results obtained with serine and threonine provide further evidence that the ligand is not hydrophobic. Apparently, the nucleophilic character of the hydroxylic oxygen atom, resulting in the tridentate coordination, has no effect on the retention of these amino acids at pH 7.0. Aspartic and glutamic acids display retention values higher than all other amino acids except cysteine and cystine. This is explained in terms of coulombic interactions. Presumably other forces must also be involved, such as metal/oxygen affinity. On the other hand, the amides asparagine and glutamine show no significant interaction. The most important finding of this study is that the high affinity for cysteine and cystine, compared with other amino acids, especially histidine and tryptophan, differs from the other results reported so far in the IMA field. This observation can be of great value in the separation of proteins possessing these amino acid groups on their surface. It is clear, however, that the amino acid side-chains, which are most important for adsorption to other IMA adsorbents, show no significant interaction in case of IMA–Fe^{3+}. The retention behaviour of other amino acids may be enhanced by optimizing the experimental conditions such as pH, ionic strength, temperature, nature of cation and anion, etc. The exploitation of these properties for the chromatography of proteins will by reported elsewhere.

ACKNOWLEDGEMENT

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