# Immobilized Metal Ion Affinity Adsorption and Immobilized Metal Ion Affinity Chromatography of Biomaterials. Serum Protein Affinities for Gel-Immobilized Iron and Nickel Ions†

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ABSTRACT: Immobilized metal ion affinity adsorption (IMA adsorption) is a collective term that is proposed to include all kinds of adsorptions whereby metal atoms or ions immobilized on a polymer cause or dominate the interaction at the sorption sites. IMA chromatography is one of the most powerful methods available to date for protein fractionation although this is not as yet widely recognized. This study deals with the theoretical aspects of IMA adsorption and its practical applications as exemplified by the various results reported here. The synthesis of iminodiacetate-substituted agarose (IDA–agarose) and tris(carboxymethyl)ethylenediamine–agarose (TED–agarose) is described. Many types of metal ions can easily be immobilized on these gel derivatives to form IMA adsorbents. We have not observed any damage to the proteins during the adsorption–desorption process. After performance of an experiment, the gels can easily be regenerated and can be loaded with the same or a different metal ion for an ensuing experiment. Specific adsorption is demonstrated for serum proteins on immobilized Ni(II) and Fe(III). Ligand-specific desorption (affinity elution) is also demonstrated by including in the buffer system certain solutes which are similar to or identical with some particular amino acids found in proteins. High concentrations of certain salts that affect the structure of water, such as $Na_2SO_4$, promote coordinate covalent bonding of proteins by a mechanism that is apparently similar to that found in hydrophobic interactions. Neutral detergents and aquoorganic solvents may be used. This opens up the possibility for the fractionation of membrane components. The IMA-adsorption method could also be expanded to other areas besides protein fractionation.

When metal chelate affinity chromatography was introduced (Porath et al., 1975), our purpose was to exploit the affinity between proteins, nucleic acids, and other biomaterials on the one hand, and heavy metals on the other, in order to develop a new separation principle. This idea is a logical extension of our earlier work on molecular sieving (Porath & Flodin, 1959) and biospecific adsorption–bioaffinity chromatography (Porath, 1968). Since then, some very important further contributions have corroborated the original suggestion that exposed imidazole and thiol groups on the protein molecules are the most important binding sites for the zinc- and copper-containing adsorbents (Sulkowski et al., 1982). Although its successful application to the purification of interferon should have stimulated increased interest in this new method, progress has been rather slow. We are convinced of the extraordinary merits of chromatography based on differential metal ion affinity and have therefore decided to develop it further ourselves. We feel that a new nomenclature is desirable.

About 20 years ago "ligand-exchange chromatography" (Helfferich, 1961) was introduced, a separation method based on selective adsorption and replacement of amines and other small molecular size solutes from heavy metal ions immobilized on resins. Since the term "ligand" is used differently in immunoaffinity chromatography and immunoassays, the Helfferich terminology may lead to confusion. Furthermore, as Helfferich pointed out, mechanism in addition to ligand exchange also contributes, a fact which is certainly true in metal chelate affinity chromatography as well. Also this latter term is not entirely satisfactory. It is too restricted, as it deals only with those adsorbents where the metal ions have been immobilized by chelate formation. We are of the opinion that the nomenclature should be elastic to include all kinds of chromatography dealing with interactions between solutes and immobilized heavy metals, regardless of whether the latter are in the form of ions or not. The metal may thus be fixed to the support by ionic or covalent bonds or even dispersed as a colloid in an insoluble matrix.

One reason for such a wide definition is the fact that metals frequently, but certainly not always, exhibit characteristic affinity behavior independent of their oxidation state. Mercury, for example, whether immobilized as $Hg^{2+}$ ions or in thiolated, organometallic or colloid metallic form always has an affinity for sulfur. Fe(III)- and Fe(II)-containing adsorbents will show big differences in adsorption properties, but also some similarities. We therefore propose the term immobilized metal affinity adsorption (abbreviated IMA adsorption) to cover all kinds of interactions between solutes and immobilized metals of whatever form. Ligand-exchange chromatography and metal chelate chromatography or IMIA chromatography ("immobilized metal ion affinity chromatography") may then, if necessary or desirable, be treated as subdivisions of the *IMA chromatography* or *IMAC*.

Although our project is intended to cover most of the relevant periodic table elements, our initial study is limited to two adsorbents, to a single model mixture, and to the use of extremely simple standardized procedures. We have synthesized two chelone-type agarose gels and loaded them with $Fe^{3+}$ and $Ni^{2+}$ (Figure 1). In view of the tremendous diversity among the IMA-desorption procedures (use of different kinds of chelators, competing ligand solutes, ion concentration gradients, mixed solvents, etc.), our present account can only give a glimpse of the potentialities and perhaps further stimulate the application of IMA adsorption in various fields.

In choosing human serum as our model mixture we are following the standard practice of earlier pioneering work on fractionation techniques for proteins and can easily demonstrate the power of IMA chromatography. We have determined the presence or absence of particular proteins in some

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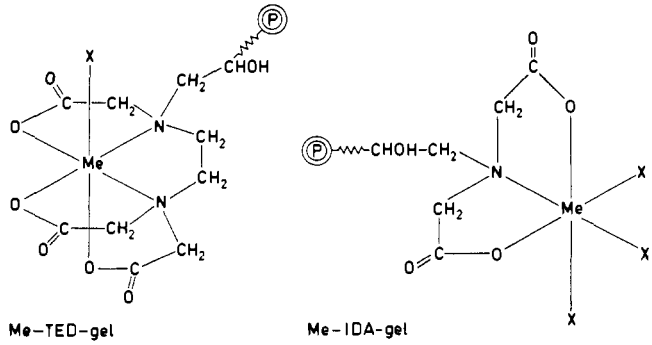



FIGURE 1: Schematic representation of metal chelone adsorbents for octahedral configuration about a hexacoordinate central metal ion; X may be $H_2O$, OH, buffer ion, protein ligand, etc. The stronger attachment of the metal to the TED adsorbent and the lesser sterical hindrance for a solute ligand to reach the metal bound to the IDA gel are clearly visualized.

of the fractions, but it is beyond the scope of this investigation to identify all of the components. At the present stage, protein yields and the protein patterns, as revealed by the analytical tools used, are sufficient to guide the development of the method and to demonstrate some of its inherent potentialities.

## Materials and Methods

All chemicals used are commercially available and are of pro analysi or puriss grade. Sepharose 6B or 4B (Pharmacia Fine Chemicals AB, Uppsala, Sweden) was used for the synthesis of all adsorbents.

The chromatography columns were constructed in the workshop of the Biomedical Center, Uppsala. A peristaltic pump P3 (Pharmacia Fine Chemicals AB) and a fraction collector (Stålprodukter AB, Uppsala) were used. The chromatographic separations were followed by a continuously recording spectrophotometer (UVICORD from LKB-Produkter, Bromma, Sweden) and manually by a Hitachi Model 101 spectrophotometer.

Electrophoretic analysis of the fractions was performed in slabs of a 4–30% polyacrylamide gradient gel (PAA 4/30 Pharmacia Fine Chemicals AB) according to the manufacturer's manual.

Fused rocket immunoelectrophoresis was performed in a "flat bed apparatus" (Pharmacia Fine Chemicals AB) as described in their manual.

Elementary analysis for nitrogen was done on dried gel samples by the micro-Kjeldahl method with Hg as catalyst (Gustafsson, 1960). The adsorption capacity of each column for the respective metal ions was determined by visual inspection and by measurement of the migration velocity of the moving boundary of the colored ion when a solution of metal ion was passed through the column.

*Iminodiacetate–Sepharose 6B ("IDA–Sepharose 6B").* Suction-dried Sepharose 6B (400 g) was mixed in a 3-L round-bottom flask provided with a stirrer with 200 mL of 2 M NaOH, 20 mL of epichlorohydrin, and 750 mg of $NaBH_4$. During 2 h of slow stirring at room temperature, an additional 200 mL of 2 M NaOH and 100 mL of epichlorohydrin were added in portions, and the suspension was allowed to react overnight. The gel was washed on a Büchner funnel, sucked dry, and returned to the reaction flask. A 500-mL sample of 2 M $Na_2CO_3$, 50 g of disodium iminodiacetate, and 0.6 g of $NaBH_4$ were added to the gel. The suspension was kept at 60 °C overnight with slow stirring. The gel was washed thoroughly on a Büchner funnel with water, with dilute acetic acid, and again with water until the washings were neutral.

The N content of the dried gel was 1.43%, and the gel adsorbed about 35 μg-atoms of $Ni^{2+}$/g of wet gel.

*Ethylenediamine–Sepharose 4B* ("ED–Sepharose 4B") was synthesized by mixing 100 g of Sepharose 4B (previously washed with water and suction dried on a Büchner funnel) with 50 mL of epichlorohydrin, 460 mL of 2 M NaOH, and 1.7 g of $NaBH_4$ in a 3-L round-bottom flask provided with a stirrer. To the suspension were added 460 mL of 2 M NaOH and 230 mL of epichlorohydrin in small portions over a period of 1.5 h. The suspension was stirred overnight, and the reagents were removed by filtration on a Büchner funnel. The gel was then washed with water, dilute acetic acid, water, and finally 0.2 M $NaHCO_3$. To the washed gel was added 300 mL 0.2 M $NaHCO_3$ and 200 mL of ethylenediamine (pH 12.5) and the suspension stirred for 20 h at 50 °C. The liquid was filtered off and the gel was washed with water, dilute acetic acid and again water to neutrality.

*Tris(carboxymethyl)ethylenediamine–Sepharose 4B* ("TED–Sepharose 4B") was synthesized as follows: Six hundred grams of sucton-dried ED–Sepharose was placed in the reaction vessel. To this gel was added 125 g of bromoacetic acid that had been neutralized with 400 mL of 2 M NaOH and 400 mL of 1 M $NaHCO_3$. After the suspension was stirred for 15 h at room temperature, the gel was washed with water, dilute acetic acid, and again water. This gel adsorbed 47 μg-atoms of Ni/mL of packed bed.

Nickel-loaded gel was washed with 1 M glycine followed by distilled water. Part of the nickel was removed by this treatment. Samples of metal-free gel, nickel-loaded gel, and glycine-washed nickel gel were dried and analyzed. The metal-free gel contained 2.93% N. The Ni-loaded gels were found to contain 7.09 and 4.59% Ni, respectively, before and after washing with glycine. All figures refer to dried gel material.

In the same fashion the gel was loaded wih $Fe^{3+}$ and washed. The gel contained 5.90% $Fe^{3+}$ before and 5.04% $Fe^{3+}$ after washing with glycine.

*Chromatography.* In all experiments, each chelator gel was packed in a separate column (1 × 13 cm, $V_t$ about 10 mL) in distilled water. Each column was then loaded with the metal ion in question by applying 2–4 mL of a 50 mM solution of the metal (usually in the form of the chloride or sulfate salt) followed by distilled water. During this process, the columns become colored as the metal ions attach to the gels. Usually the colored front extended to between half and two-thirds of the bed height, indicating that none of the columns used was fully loaded with metal ion. Each column was then washed with at least one total bed volume of each of the eluents that we expected to use in the adsorption–desorption process (see Results and legends to figures) in order to ensure that no leakage of the metal would occur during the actual experimental run later on. However, in the case of the nickel–IDA columns the nickel was completely displaced from the bed by passage of 1 M glycine, and washing with glycine was thus omitted.

A typical chromatographic experiment was performed as follows: Two or more columns packed with one type of chelator gel (e.g., TED–Sepharose) and loaded with different metal ions were connected in series to form "tandem columns". Usually a precolumn packed only with the chelator gel without metal was inserted in the system to ensure that the adsorption of solutes to the gels was due entirely to solute–metal ion interaction.

Unless otherwise stated in specific experimental sections, the following buffers were used throughout: buffer A, 0.05

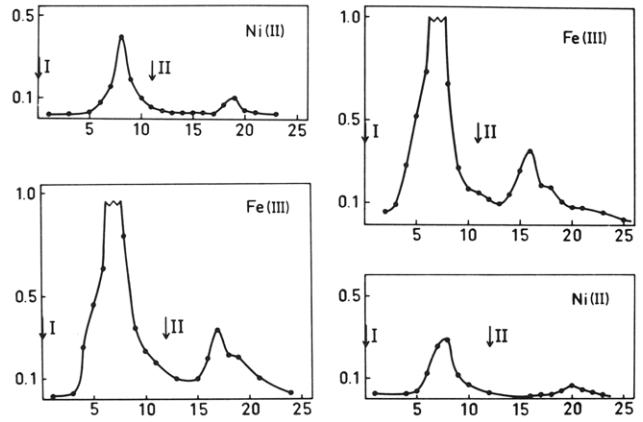



FIGURE 2: Ni → Fe and Fe → Ni tandem column chromatograms (above and below, respectively); equilibrium buffer, buffer A. The peaks corresponding to unadsorbed material have been omitted. First arrow indicates introduction of buffer C and the second arrow the introduction of buffer D. Abscissa, absorbance at 280 nm. Ordinate, fraction number.

M sodium acetate and 0.1 M NaCl, pH 5.5; buffer B, 0.1 M Tris-HCl, pH 8.1; buffer C, 0.5 M sodium acetate, pH 5.5; buffer D, 1 M glycine, pH 9.0.

In almost all cases investigated so far, very few proteins were adsorbed to the precolumns. The sample (normal human serum) which had been dialyzed against the initial eluent buffer (equilibrating buffer) was applied to the tandem column, and elution with the equilibrating buffer was continued until all nonadsorbed material was eluted. The tandem beds were then disconnected, and the adsorbed material from each bed was displaced by successive elution with different buffers, usually buffers C and D. The fractionated material was then pooled and further analyzed by electrophoresis in polyacrylamide gradient gels or by fused rocket immunoelectrophoresis for identification purposes.

## Results

*Effect of Metal Ion.* Four experiments, each with 4 mL of serum, were carried out in two-bed tandem columns loaded with 175 μg-atoms of $Ni^{2+}$ and 115 μg-atoms of $Fe^{3+}$, respectively. Two chromatographic runs were done with buffer, A and a further two runs were done with buffer B as initial buffers. In each case displacement of adsorbed proteins was made in two steps: (1) with buffer C and (2) with buffer D. Under these conditions the metal ions were not displaced, since the boundaries between colored and colorless parts of the bed remained stationary.

The order of the connected beds was then reversed such that the Fe(III)–TED bed preceded the Ni(II)–TED bed, and the above experiment was repeated. As seen in Figures 2 and 3, the chromatograms obtained were similar, in part almost identical, independent of the order in which the columns were connected. This strongly indicates the specific nature of the adsorption obtained.

At pH 5.5 the Fe(III) and Ni(II) columns adsorbed 10.2% and 1.7%, respectively, of the proteins applied to the tandem column, and at pH 8.1 the corresponding adsorption capacities were found to be 1.5% and 4%, respectively (based on absorbance values at 280 nm).

Gradient gel electrophoretic analysis revealed marked differences in the protein patterns (Figures 4 and 5). Here again the adsorption was shown to be independent of the order in which the tandem beds were connected. $\alpha_2$M-macroglobulin was identified immunoelectrophoretically in peaks displaced by buffer C from the pH 8.1 nickel columns and corresponds

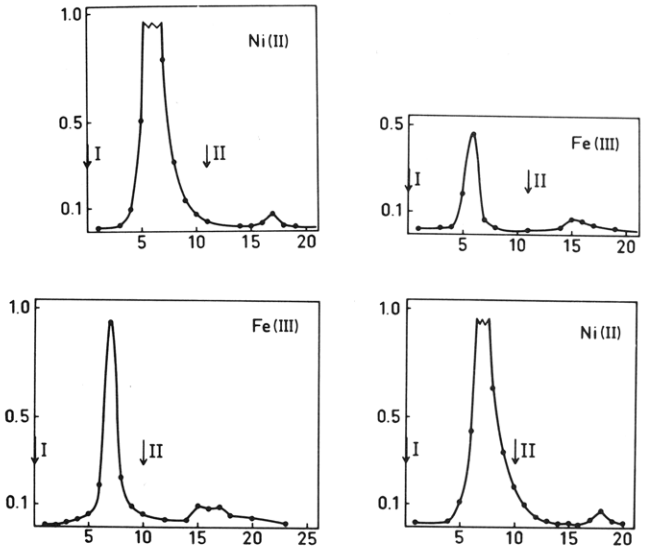



FIGURE 3: Ni–Fe and Fe–Ni tandem column chromatograms (above and below, respectively); equilibrium buffer, buffer B. Further explanations as in the legend to Figure 2.

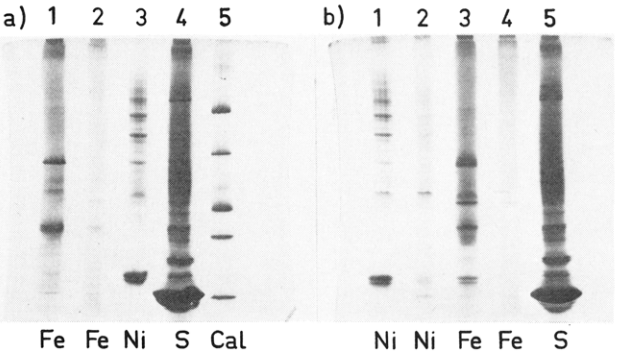



FIGURE 4: Gradient gel electrophoretograms. (a) (From Figure 2a) First diagram was obtained with buffer C displaced material from the Fe(III) column, the second was obtained with buffer D displaced proteins, and the third is the buffer C displaced substances from Ni column. S is the starting material (human serum), and Cal is the diagram for the calibration substances (from above to below in order): thyroglobulin (669 kdaltons), ferritin (440 kdaltons), catalase (232 kdaltons), LDH (140 kdaltons), and albumin (67 kdaltons). (b) (From Figure 2b) The first and second diagrams were obtained from the nickel column, first and second displaced peak, respectively, and the third and fourth diagrams were obtained from the Fe column, first and second displaced peak, respectively.

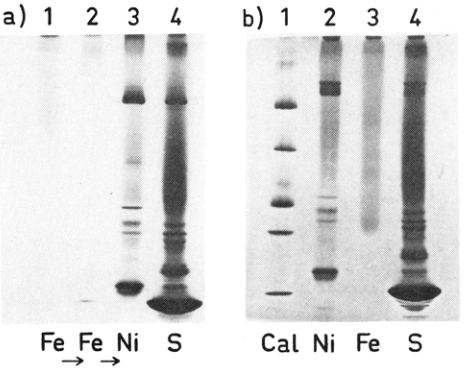



FIGURE 5: Gradient gel electrophoretograms of materials from the experiment referred to in Figure 3. (a) Fe–Ni–TED tandem column: (1) Fe(III) bed, buffer C peak; (2) buffer D peak; (3) Ni(II) bed, buffer C peak; (4) serum. (b) Ni–Fe–TED tandem column: (1) calibration mixture as in Figure 4; (2) Ni(II) bed, buffer C peak; (3) Fe(III) bed, buffer C peak; (4) serum.

to one or both of the strong bands in the gradient gel electrophoresis positioned above thyroglobulin. $\alpha_2$M-macro-

Table I: Identification of Proteins in Fractions from $Fe^{3+}$- and $Ni^{2+}$-TED Columns

| | initial buffer | | |
|---|---|---|---|
| | pH 5.5 | | pH 8.1 |
| | Fe | Ni | Ni |
| IgG | + | − | + |
| haptoglobin | + | + | (+) |
| hemopexin | − | + | + |
| $\alpha_2$-macroglobulin | − | − | + |
| transferrin | − | − | − |
| ceruloplasmin | − | − | − |
| orosomucoid | − | − | − |
| $\alpha_1$-antitrypsin | − | − | − |
| Gc-globulin | − | − | − |
| C1q | (+)? | − | + |
| C3 | + | + | − |
| C4 | + | − | − |
| albumin | − | − | − |

globulin had earlier been purified by Zn–IMA chromatography (Kurecki et al., 1979; Sottrum-Jensen et al., 1980). The strong bands above albumin (Figure 5a, column 3; Figure 5b, column 2) apparently contain hemopexin, plasminogen, and complement factors. Transferrin was found among the proteins that passed through the columns without adsorption. No trace of this protein was found in the desorbed materials. The proteins in many of the other bands have not yet been identified. It is particularly noteworthy that the Ni(II) column adsorbs the proteins more efficiently at pH 8.1 than at pH 5.5, whereas the opposite is true for the Fe(III) column. The results of the analysis are compiled in Table I.

Nearly identical results were obtained after repeated use of the same columns, and the metal ion remained strongly attached to the column.

In those cases where loss of proteins due to strong adsorption is suspected or observed, the columns should be washed with ethylenediaminetetracetic acid (EDTA) and the desorbed material dialyzed and desalted by chromatography on a molecular-sieving gel to remove the EDTA–metal complex. It should be noted though that such proteins may tenaciously bind EDTA. The adsorbent is then regenerated by loading it with metal ions as previously outlined.

*Effect of Chelator Structure.* TED- and IDA-agarose columns were charged with 175 $\mu$g-atoms of $Ni^{2+}$ each and connected to form a tandem column. The column was then equilibrated with buffer B containing 0.5 M NaCl, and 4 mL of dialyzed serum was chromatographed as described in the previous section. Upon elution with buffer D the nickel was eluted from the Ni(II)–IDA column in a well-defined peak just behind the protein. After the IDA–agarose was recharged with $Ni^{2+}$, a new chromatographic run was performed which was identical with the previous one with the exception that the IDA–agarose preceded the TED–agarose.

The amount of adsorbed proteins from the TED gel was found to be 4.2% of the serum proteins applied. The proteins contents of buffer C and buffer D eluted material from the IDA column were 8.3% and 2.8%, respectively. The gel electrophoretic patterns of fractionated materials are shown in Figure 6.

The Ni(II)–TED gel gives a clear-cut group separation as in the previous experiments. Some of the proteins interact with the Ni(II)–IDA adsorbent. This material contains entirely different proteins than those bound to the Ni(II)–TED bed. Only two electrophoretic bands coincide and may correspond to identical proteins. The main part of the immunoglobulins are adsorbed on the IDA column and only a minor portion on

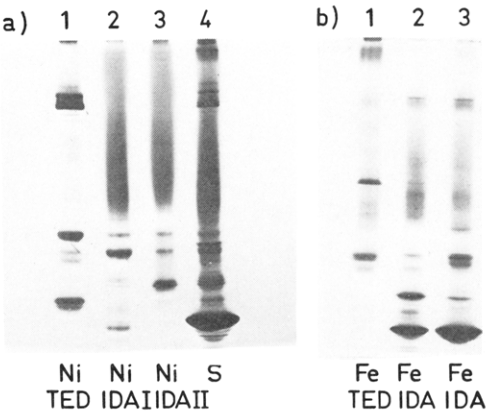



FIGURE 6: Gradient gel electrophoretograms of serum fractions from TED–IDA–Sepharose 6B tandem column experiments. (a) TED bed precedes the IDA-bed. (From left to right) Ni(II)–TED column, buffer C peak, Ni(II)–IDA column, buffer C peak followed by the buffer D peak, and human serum. (b) (From left to right) Fe(III)–TED column, buffer C peak, and IDA column, buffer C peak and buffer D peak.

the TED column. Serum albumin is totally absent and so is antitrypsin in all fractions except the one that passed through the tandem column without adsorption. Transferrin is present in small amounts in the buffer D eluted peak from the IDA column. Whereas hemopexin was adsorbed to the TED gel, not a trace of this protein could be detected in the material eluted from the IDA bed.

A set of experiments similar to those above was made with Fe(III) instead of Ni(II). In this case the tandem column was equilibrated with buffer A. The gel electrophoretograms of the materials desorbed from the column by buffers C and D are shown in Figure 6b.

*Effect of High Concentration of Salts.* Already in our first publication (Porath et al., 1975) we demonstrated that IDA–agarose (with long spacer) adsorbed serum proteins effectively in the presence of up to 0.8 M NaCl. Evidently IMA chromatography is well suited to explore protein behavior and protein purification in the presence of very high concentrations of salts.

We have therefore carried out a number of experiments to investigate the effects of high concentrations of salts included in the buffer on the adsorption capacity for proteins. Nickel–TED–Sepharose 6B columns (175 $\mu$g-atoms of Ni) were thus equilibrated in buffer B containing NaCl of a specified molarity. In each experiment 4 mL of serum, previously dialyzed against the buffer system used, was introduced into the column. Adsorbed proteins were eluted as described earlier. Almost all adsorbed proteins were displaced by buffer C. The gradient gel electrophoretograms of the materials desorbed by buffer C from the 0.1, 1, and 5 M NaCl experiments are shown in Figure 7a. Apparently, NaCl increases the adsorption capacity without greatly affecting the number and types of protein components adsorbed.

Serum (3-mL samples) was also fractionated on Fe(III)–TED columns (1 × 15.2 cm) containing 200 $\mu$g-atoms of $Fe^{3+}$ equilibrated with buffer A. One experiment was carried out in 4 M NaCl and another without inclusion of salt in the equilibrating buffer. In the absence of NaCl the material desorbed by buffers C and D accounted for 26 and 11%, respectively, of the proteins applied. A considerable reduction of adsorption capacity was observed in the presence of 4 M NaCl. Buffers C and D desorption peaks in this case contained only 3 and 6%, respectively, of the serum proteins applied.

Two other chromatographic experiments with Ni(II)–TED gel were also performed as above with the exception that 0.5

Table II: Recovered Amount of Proteins from Ni(II)–TED–Agarose Experiments with Indicated Components Included in Buffer B

| | $[Na_2SO_4]$ | | % $NaDodSO_4$ | | % Tween 80 | | | | | % $Me_2SO$ | | 40% ethylene glycol | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 0.5 M | 1.0 M | 0.01 | 0.1 | 0.01 | 0.1 | 1 | 2 | 4 | 10 | 20 | buffer B | buffer B + 1.7 M NaCl |
| optical units over the peaks | 19.0 | 35.8 | 7.05 | 2.1 | 9.8 | 9.7 | 10.9 | 12.3 | 12.2 | 16.1 | 15.5 | 24.1 | 13 |
| % of material applied | 9.5 | 25.0 | 3.8 | 1.1 | 5.2 | 5.1 | 5.7 | 6.5 | 6.4 | 8.3 | 8.7 | 8.3 | 4.5 |

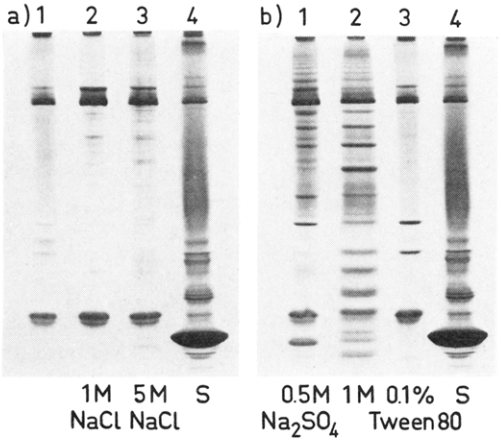



FIGURE 7: Gradient gel electrophoretograms of acetate-eluted peaks from Ni(II)–TED column chromatography performed with different equilibration buffers containing buffer B and various concentration of salts. The included characteristic solute species are indicated below the diagrams. (a) Effect of NaCl concentration. (b) Effect of $Na_2SO_4$ at different concentrations and 0.1% Tween 80.

or 1 M $Na_2SO_4$ was included in the equilibrating buffer A instead of NaCl. Some precipitate that formed during dialysis against 1 M $Na_2SO_4$ buffer was removed. The supernatant containing 71.5% of the 280-nm absorbing material was applied to the column. No precipitate was observed when serum was dialyzed against the 0.5 M $Na_2SO_4$ containing buffer. The adsorbed proteins were in each case displaced by elution with buffer C. About 9.5% of the applied proteins were adsorbed to the column equilibrated in the presence of 0.5 M $Na_2SO_4$, and about 19.9% to the column equilibrated with the buffer B containing 1 M $Na_2SO_4$. The proteins in these elution peaks gave the gel electrophoretic patterns shown in Figure 7b. Only trace amounts of proteins were found in the peak desorbed by buffer D.

Some preliminary experiments were made on columns of Ni–TED–Sepharose 6B equilibrated with buffer B containing 3 M LiCl or 2 M potassium phosphate adjusted to pH 8.1. The results show that the adsorption of the serum proteins (soluble in these systems) was considerably increased in the phosphate buffer but was decreased in the presence of the chaotropic salt LiCl.

*Effect of Urea and Detergents.* A set of three columns packed with Ni–TED–Sepharose was equilibrated with buffer B containing either 8 M urea or 0.01% or 0.1% sodium dodecyl sulfate ($NaDodSO_4$). As before, 4 mL of serum, previously dialyzed against the equilibrating buffer, was applied to each column and chromatographed as described above. These structure perturbants suppressed the adsorption considerably (Table II).

In a series of similar experiments, Tween 80 at different concentrations was included in buffer B. The Tween was slightly colored and showed considerable absorbance at 280 nm. After the adsorption step, therefore, the bed was washed free from Tween by buffer B. Desorption was then performed as above with buffer C and then buffer D. The yields are shown in Table II. Proteins that may have been eluted upon removal of the Tween are not accounted for. The protein patterns obtained by gradient gel electrophoresis of the peak desorbed by buffer C from the 0.1% Tween experiment are shown in Figure 7b.

Table III: Effect of Sample Load on a Ni(II)–IDA Column, Equilibrated with 0.1 M Tris-HCl, pH 8.1, and Buffer B in 5 M NaCl

| | volume of serum applied (mL) | | | |
|---|---|---|---|---|
| | 1 | 2 | 4 | 16 |
| material desorbed by buffer C (% of applied) | 14.6 | 10.2 | 10.6 | 8.0 |
| material desorbed by buffer D (% of applied) | 9.4 | 7.4 | 6.3 | 0.9 |
| yield in optical units ($A_{280}$) | 11.2 | 16.4 | 31.6 | 65.7 |

*Effect of Organic Solvents.* Ethylene glycol (40% v/v) mixed either with buffer B or with buffer B containing 1.7 M NaCl (close to saturation) was used to equilibrate two otherwise identical Ni(II)–TED–Sepharose 6B columns. The serum sample (4 mL) was dialyzed against each of the two solvents and applied to the corresponding column. Due to the high viscosity of the equilibrating buffers the flow rate was, of necessity, kept low (0.5–1 mL/h). After all nonadsorbed material had passed, the adsorbed material in each case was displaced by elution with buffer C and then buffer D (see Table II). The high viscosity of such solvents makes chromatographic runs on agarose gels somewhat cumbersome. Batch operations or high-pressure chromatography on more rigid supports might circumvent this problem.

Similar experiments with 10 and 20% dimethyl sulfoxide ($Me_2SO$) and buffer B were also performed, with the results shown in Table II.

*Effect of Sample Load.* Chromatographic runs with the Ni(II)–TED column equilibrated with buffer B containing 5 M NaCl were made with varying volumes of the dialyzed serum. The results (see Table III) show that the total amount of proteins adsorbed to the column increases with increase in the amount of sample applied. In contrast, the amount of material adsorbed relative to that applied to the column (expressed as percent) decreases as the sample load is increased. This indicates that the adsorbent has varying affinities for different components present in serum. With increase in sample load, the components having higher affinity for the adsorbent apparently displace those with lower affinity. An efficient and sharp displacement of weakly adsorbed materials is effected by proteins having higher affinity for the Ni adsorbent. However, the major bands, from $\alpha_2$M-macroglobulin and hemopexin, are present in all peaks desorbed by buffer C.

*pH Dependence.* Coordinative binding of serum proteins is expected to be more extensive as the pH of the medium increases. It is therefore of considerable interest to learn about

Table IV: Effect of pH[a]

| | pH | | |
|---|---|---|---|
| | 7.6 | 8.1 | 8.6 |
| material desorbed by buffer C (% of applied) | 11.5 | 10.2 | 4.2 |
| material desorbed by buffer D (% of applied) | 3.3 | 6.2 | 3.0 |

[a] Ni-IDA column; 0.1 M Tris-HCl and 5 M in NaCl.

the magnitude of such a pH effect. Chromatograms were obtained from experiments with Ni(II)–TED–Sepharose 6B at pH 7.6 and 8.6 to supplement the previous findings at pH 8.1. Sodium chloride was included (5 M). Otherwise, the experiments were made as described in earlier sections. The results of the experiments are summarized in Table IV.

*Affinity Elution.* In a number of preliminary experiments with buffer B and buffer A, with and without 1 M $Na_2SO_4$, we noticed that the proteins adsorbed could be displaced by $NH_4^+/NH_3$, imidazole, or histidine at pH 8.1 and, at pH 4.5, by cysteine. We also tried two other routes of desorption at pH 8.1. The first was lowering the $Na_2SO_4$ concentration to 0.2 M which resulted in the displacement of more than 50% of the adsorbed materials. The major portion of proteins remaining on the column could then be displaced by stepwise elution with imidazole. In the second alternative, imidazole was added to the eluent buffer containing $Na_2SO_4$. In this case, practically all of the adsorbed material was displaced from the Ni(II)–TED column and a subsequent decrease in the $Na_2SO_4$ concentration did not release much more material. The chromatograms from the TED and IDA columns showed similarities as well as differences.

In a typical experiment a Ni(II)–TED–IDA tandem column, with adsorption capacity similar to that described earlier, was equilibrated with buffer B containing 1 M $Na_2SO_4$. A sample was prepared by mixing 4 mL of serum with 16 mL of 1.2 M $Na_2SO_4$. A small precipitate was removed and the supernatant applied to the column. The elution steps are indicated in the chromatogram (Figure 8). Adsorption due to "hydrophobic" forces seems to be more pronounced on the IDA gel than on the TED gel and thus appears to be dependent on the number of water molecules ligated to the immobilized metal ion.

The chromatogram indicated that a similar type of group separation occurs in the two beds. The fact that only a small amount of material is eluted by $(NH_4)_2SO_4$ is somewhat surprising. The peak desorbed by cysteine seems to contain small molecular size substances and a minute amount of proteins. The protein distribution in some of the fractions has been analyzed by gel electrophoresis (Figure 9).

A separate run on Ni-free gels was made in buffer B containing 1 M $Na_2SO_4$ to check the hydrophobic interaction between the serum proteins and the gel matrix. Less than 0.01% of the proteins was adsorbed which could be desorbed by buffer B alone. This control experiment indicates the minor role played by hydrophobic forces in the total adsorption seen in IMA chromatography.

## Discussion

*Choice of Metal.* Nature serves as a rough guide for the selection of metal ions suitable for use in IMA chromatography. Those metals which are essential for life and those that are poisonous seem to be useful candidates for such applications. For our purpose the list can be somewhat extended to include other metals as well. It appears, nevertheless, that those metal ions known for their great tendency for complexation offer the best prospects for successful application in IMA chromatography. Although some metals, e.g., mercury, can exist as ions strongly fixed to a matrix or as covalently bound complexes to a carrier group, our study has been limited entirely to the ionic form of the metal under investigation.

A screening of selected elements of the periodic table has proceeded this study. Earlier work published by us and others (Porath et al., 1975; Conlon & Murphy, 1976; Lönnerdal et al., 1977) has been limited to the first series of transition

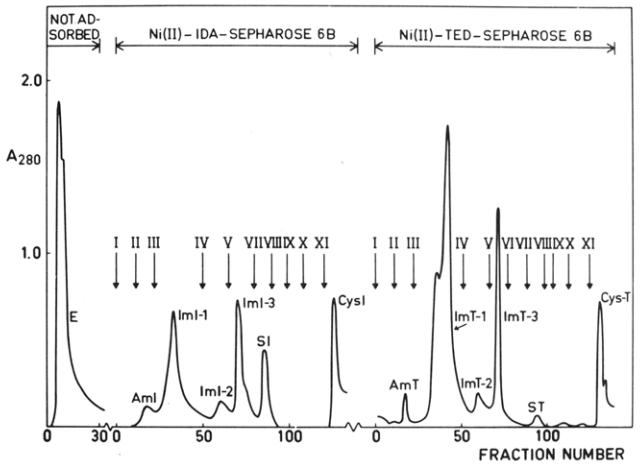



FIGURE 8: Chromatogram of 4 mL of serum supernatant from precipitation with 1 M $Na_2SO_4$ in buffer B. Column, Ni–TED → Ni–IDA tandem column. Equilibrium buffer, buffer B + 1 M $Na_2SO_4$. Eluents: I, buffer B + 0.5 M $(NH_4)_2SO_4$ + 1 M $Na_2SO_4$; II, buffer B + 1 M $Na_2SO_4$; III, buffer B + 0.006 M imidazole + 1 M $Na_2SO_4$; IV, buffer B + 0.01 M imidazole + 1 M $Na_2SO_4$; V, buffer B + 0.04 M imidazole + 1 M $Na_2SO_4$; VI, buffer B + 0.04 M histidine + 1 M $Na_2SO_4$; VII, buffer B + 0.5 M $Na_2SO_4$; VIII, buffer B + 0.2 M $Na_2SO_4$; IX, buffer B; X, buffer A; XI, buffer A + 0.03 M cysteine. Am, Im, S, and Cys denote peaks obtained by elution with $NH_4^+/NH_3$, elution with imidazole, withdrawel of salt, and elution with cysteine, respectively. Flow rate, 10 mL $h^{-1}$. Fraction volume, 2.7 mL.

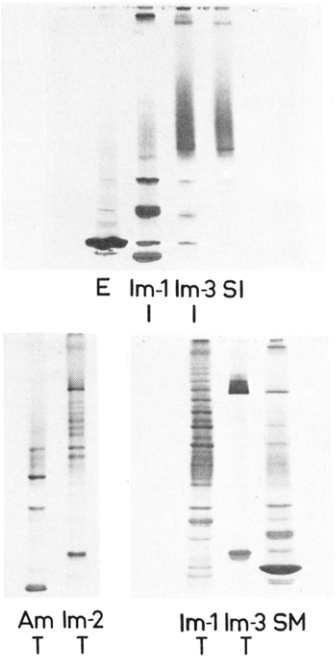



FIGURE 9: Gel electrophoretic analysis of fractions from the experiment referred to in Figure 8. SM denotes starting material. I and T refer to IDA and TED columns, respectively.

elements with calcium as the only exception (Borrebaeck et al., 1981), and it was deemed desirable to continue with these elements in the developmental phase of IMA-adsorption studies.

All complexes of metal ions with electrons in $e_g$ orbitals are labile. Tripositive iron with its spherically symmetrical $d^5$ configuration $(t_{2g})^3(e_g)^2$ with five spin-unpaired electrons forms labile outer orbital complexes. In contrast metal ions with electrons in the $d^8$ configuraiton offer large field stabilization when subjected to strongly splitting ligands such as those occurring in some proteins. Dipositive nickel has two unpaired electrons and the configuration $(t_{2g})^6(e_g)^2$. With eight d electrons, Ni(II) is likely to give complexes that are more inert than those of Fe(III) with no ligand field stabilization.

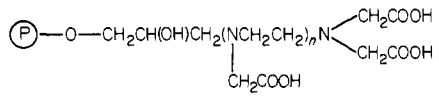
Concerning the kinetics of ligand replacement from octahedral Ni(II) complexes some guidance may be obtained from known water-exchange rates for the $Ni[(H_2O)_6]^{2+}$ ion. The half-life for such an exchange reaction is $<10^{-4}$ s (Eigen & Wilkins, 1965). Since half-lives of the order of 1 s may still be acceptable for the form of chromatography we are dealing with here, no difficulties should be caused by the possible relative inertness of the Ni(II)–protein complexes. This assumption seems to be verified by our experiments.

We may also compare iron and nickel ions from another aspect. According to the "hardness–softness" concept based on the polarizability of the interacting ions (Pearson, 1963), we may consider $Fe^{3+}$ as a "hard" Lewis acid or electron acceptor. $Ni^{2+}$ is on the borderline to "soft" acids.

The concept of hardness–softness may serve as a guide for understanding the principles and mechanisms underlying IMA chromatography. In general, it can be said that hard metal ions tend to coordinate with hard ligands and that soft ligands coordinate preferably with soft metal ions. According to this, therefore, the Fe(III) adsorbent will mainly be charge controlled while the frontal orbital energy term is important in the case of Ni(II)–protein complexes. It is thus apparent that the former depicts electrovalent and the latter covalent behavior. Fe(III) associates more strongly with oxygen as ligand atom while Ni(II) has a preference for nitrogen and sulfur.

*Choice of Matrix and Chelator.* The solid support for metal immobilization, the polymer matrix, should have the same properties as for bioaffinity adsorbents (Porath & Kristiansen, 1975) to be suitable for IMA chromatography. Agarose, which we have chosen as our matrix, fulfills these requirements, but cross-linked polyacrylamide, poly(vinyl alcohol), cellulose, nylon, etc., as well as granular hydrophobic polymers that are effectively coated with a hydrophilic layer may also be suitable matrices for IMA adsorbents.

The choice of chelator is of crucial importance for the proper function of the immobilized metal ion. Bidentate chelators afford too weak binding of the metals. Unwanted leakage of metal ion is high. Terdentate chelators are on the borderline as suitable metal binding groups. Due to this observation, our present investigation is limited entirely to ligand groups of chelones (i.e., carboxymethylated amines) of the general formula




Only experiments with adsorbents based on iminodiacetic acid (IDA), with $n = 0$, and tris(carboxymethyl)ethylenediamine (TED), with $n = 1$, are reported here. The IDA and TED groups tend to form a maximum number of five-membered fused ring systems when in contact with di- and tripositive ions such as $Ni^{2+}$ and $Fe^{3+}$ (Figure 1).

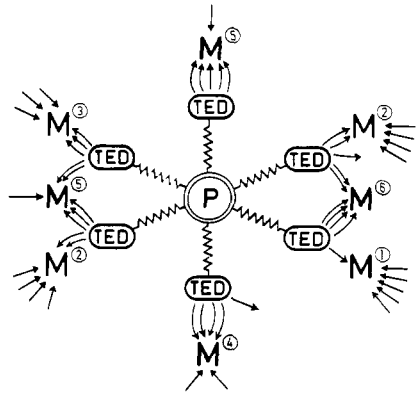



FIGURE 10: Schematic representation of some possible polymer-bound metal chelate structures that may account for the fact that some metal ions are weakly adsorbed while others are strongly bound to the gel.

In neutral and weakly basic solution the metal ions on Ni(II) and Fe(III) gels are displaced from the gels in the presence of buffers containing EDTA. Desorption with iminodiacetate is complete from IDA gel but incomplete from the TED gel. We can therefore conclude that the stability order of the metal complexes is as follows: IDA gel < iminodiacetate ≪ TED gel < EDTA. The trivalent iron chelates are usually more stronger than those of divalent nickel.

Besides the ability to form strong metal coordination complexes, the chelone groups possess the desirable property of being completely transparent to visible light. Adsorption and desorption of colored metal ions such as $Ni^{2+}$ and $Fe^{3+}$ can thus be followed easily by visual inspection.

In all of the chelone gels we have investigated so far the metal ions are very strongly bound. Figure 10 illustrates some possible structures for metal–TED adsorbents. A metal ion may be bound to two adjacent TED substituents. This would explain the fact that the metal concentration only reaches about 70% of the theoretically expected yield corresponding to a 1:1 stoichiometric-type metal–chelone complex (for a hexacoordinated metal ion) calculated from the nitrogen content.

It is important to eliminate too weakly bonded metal ions from the gel (such as $M^{(1)}$–$M^{(4)}$ in Figure 10) to avoid metal leakage and to make the gel more homogeneous with respect to the adsorption centers. EDTA removes all metals from the TED gel although long-time treatment might be required. In addition the use of TED as chelator solves the delicate problem how to properly balance the forces between the ligands in the solid phase and the solution. The TED group, therefore, seems to fulfill many of the requirements for an optimal chelator for use in IMA chromatography. Other small molecular size chelators are useful to complement that of the TED gels, as is exemplified by the IDA–agarose.

While only a single "free" site is likely to be available on a hexacoordinate metal ion bonded to the TED gel, there are at least two, ususally three, free coordination sites on an IDA gel (Figure 1). In the latter case chelate structures involving more than two ligands on the protein may be formed. Furthermore, the IDA–chelone offers less steric hindrance to proximal contact and interactions than does the TED group. These circumstances could explain the higher selectivity in some cases for the TED gel and in other cases for the IDA gel and, of course, the stronger metal binding to the TED gel.

*Choice of Medium and Elution Conditions.* The coordination selectivity in the binding of proteins and other solutes is strongly pH dependent. A negatively charged basic ligand,

$L_B^-$, $RCOO^-$, for example, will be involved in two equilibria:

$$HL_B \rightleftarrows L_B^- + H^+ \quad (1)$$

with the dissociation constant $K_a$ and

$$M^{n+} + L_B^- \rightleftarrows ML_B^{(n-1)+} \quad (2)$$

with formation constant $K$.

For the overall reaction

$$HL_B + M^{n+} \rightleftarrows ML_B^{(n-1)+} + H^+ \quad (3)$$

the equilibrium constant is

$$K_{eq} = K_a K \quad (4)$$

Even if the formation constant is unknown, we can easily see that adsorption will be strongly influenced by $K_a$. Since carboxyls will be deprotonated already on the acid side of neutrality, phenolic hydroxyls dissociate in basic solutions, and imidazole has a $pK_a$ in the pH region 6–7, it is clear that adsorption can be controlled by pH. The binding of competing buffer ion species also depends on the pH of the medium. Deprotonation of the aquoligands occurs with concomitant lowering of the charge.

Protons can affect the immobilized metal ion as well as the surface properties of the proteins in the sample. Thus, when the pH is varied, the adsorption and/or desorption process could easily be assessed. On the other hand, an increase in the efficiency of the separation could be obtained by varying the concentrations of the eluents gradually. Gradients of pH have been used most frequently (Lönnerdal et al., 1977; Edy et al., 1977; Lebreton, 1977; Torres et al., 1979; Bollin & Sulkowski, 1981).

As in other chromatographic methods, water is the most important solvent for the fractionation of biopolymers by the IMA method. But it is also possible to include salts, detergents, urea, or organic solvents in the aqueous eluent when the IMA method is used. The results in Figure 7 and Table II show that varying the eluent composition affects the adsorption capacity and/or selectivity of the adsorbent. These preliminary results indicate the versatility of the IMA method and give impetus for further developments along similar lines aiming at optimizing the conditions necessary for solving a particular separation problem. Unlike ion-exchange chromatography, adsorption can take place at high concentrations of NaCl (see Figure 7). In fact, the adsorption capacity and the degree of selectivity under such circumstances seem to increase as the salt concentration in the equilibrating buffer is increased. This dependence on the salt concentration indicates the concomitant operation of at least three different kinds of adsorption factors. Charge-controlled attraction forces (ion exchange or ionic adsorption) predominate at low ionic strength, whereas the adsorption at very high salt concentration may be due to a frontier orbital controlled electron transfer which, together with the desolvation energy, results in the formation of the coordinate bonded adsorption complex. The Fe(III)-adsorption complexes in acidic medium are largely of the electrovalent type, and the adsorption strength therefore decreases with increasing concentration.

Our results corroborate the hypothesis that adsorption should be increasingly selective with increasing ionic strength. Since the adsorption capacity for serum proteins on the Ni–TED–agarose passes a minimum at about 1 M NaCl, it should be possible to obtain partial elution of proteins adsorbed at a higher salt concentration by decreasing the salt concentrations in the eluent to the "minimum concentration". However, the similarities in the gradient gel elctrophoretograms (Figure 7a) indicate that such an elution procedure is not very efficient. However, in the case of water-structure forming salts such as $Na_2SO_4$ or $KH_2PO_4$, the feasibility of such an elution procedure is indicated (Figures 7b, 8, and 9).

Another way of achieving selective desorption, apart from changing the pH, is to incorporate in the eluent certain solutes which have a higher affinity for the adsorption sites (coordination sites) on the proteins than does the immobilized metal or, alternatively, by using solutes that effectively compete with the proteins for adsorption. In the former case other kinds of metal ions can be used whereas in the latter instance specific displacers which contain groups such as $-NH_2$, –COOH, –SH, or imidazole may be effective.

The use of more selective gradient substances has been reported: imidazole (Rijken et al., 1979), histidine (Ohkubo et al., 1980), and histamine (Kikuchi & Watanabe, 1981).

The possibility of using such elution techniques is shown in Figure 8. It should be pointed out, however, that no attempt was made to optimize the elution parameters. Other elution programs such as those involving concentration gradients may turn out to be much more effective. The potentiality of the IMA method is further demonstrated by analysis of the fractions from this multiparameter based elution chromatogram (Figure 9).

The kinetics of the adsorption and desorption of proteins to gel-bound metals have so far not been studied in detail. Our results show that, although the Ni(II) and Fe(III) gels give reproducible results, the desorption process is time dependent. Double peaks are obtained if elution is stopped shortly after the emergence of a peak and then continued some hours later. This indicates that a sufficiently long time should be allowed for the adsorption and desorption steps to obtain maximum capacity. If, on the other hand, association and dissociation take place almost instantaneously, i.e., equilibrium is reached within a fraction of a second, then isocratic elution may be possible. Although we have, as yet, no experimental results to clearly demonstrate the existence of very fast equilibria, isocratic behavior has occasionally been observed.

Various effects can also be obtained by inclusion of denaturants in the eluent. Little or no adsorption takes place in 8 M urea. Sodium dodecyl sulfate at concentrations above 0.1% virtually abolishes the adsorption of all serum proteins on Ni(II) columns. On the other hand, an uncharged detergent does not seem to influence the adsorption at low concentrations while Tween 80 at concentrations above 1% in fact somewhat increases the adsorption capacity. These observatons may be used to advantage for the fractionation of solubilized membrane proteins.

Ethylene glycol, being a vicinal diol, is able to form chelates. Although they are weak, a concentration as high as 40% is likely to affect the adsorption due to chelation as well as by its other perturbant actions.

*Some Notes on the Adsorption Mechanism.* No results so far contradict the assumption that adsorption is stereoheterotopically determined; i.e., it is selective according to the spatial distribution of various ligands on the protein surface. For nickel, in neutral and basic solutions, the preferential ligand atoms may be sulfur and nitrogen in cysteine and histidine residues and to a minor extent amino and tryptophan indole groups. Octahedral nickel thus prefers uncharged ligands. Iron, in acid solution, is likely to have a preference for sulfur atoms and for carboxylic oxygen and to a minor extent also for phenolic hydroxyls.

While the complexation of many ligands with Ni(II) and Fe(III) ions cannot be studied in free alkaline solution due to

the precipitation of the metal hydroxides, this restriction does not hold for gel immobilized ions. Moreover, practially all adsorption sites seem to react independently of each other provided that the concentration of immobilized metal ion is not excessive, i.e., the degree of substitution of IDA or TED groups is not excessively high. These facts make solid phase complexation chemistry very different from that in solution.

From the experimental point of view a closer examination of the affinity-determining factors are also likely to give important clues as to how further exploitation of the IMA methods might be accomplished. Such studies can be carried out along three different lines: (1) by attempts, with model proteins of known three-dimensional structure, to correlate adsorption with stereochemistry (Sulkowski et al., 1982); (2) by collection of adsorption data for amino acids, peptides, and other small-size model substances; (3) by use of affinity elution. Our orienting experiments according to the second alternative (J. Porath and G. Jacobson, unpublished results) have revealed, for nickel gels, the following order of decreasing affinity at pH 7: His > Trp > Tyr > Phe > Arg ~ Met ~ Gly.

Affinity elution adequately performed should give an unequivocal answer to the question what kind of groups in the proteins are involved in the complexation. In the chromatographic experiment referred to in Figures 8 and 9 ammonium ions at a high concentration were first included in the buffer system. It is demonstrated beyond doubt that only a very small amount of proteins is adsorbed by unidental interaction of amino groups. The elution with imidazole, on the other hand, proves the specificity in the interaction between the immobilized nickel ions and exposed histidine residues. In addition, the experiment proves that this kind of interaction in the Ni(II)–TED gel bed is unidental since the major portion of imidazole-eluted material is eluted without admixture of ammonia or ammonium ions in the buffer system. The proven specificity is not surprising. Imidazole is an efficient electron donor capable of forming strong coordinate covalent bonds with nickel ions. Another reason for the effectiveness of histidine side chains as metal ion binding sites is the flexibility of the metal–imidazole bond.

Due to charge–dipole interactions water molecules surrounding the metal ions must be highly organized (also outside the coordination sphere). Upon contact with hydrophobic groups a similar situation prevails as between two hydrophobic surfaces. The entropic gain accompanying the expulsion of strongly organized water into the bulk phase is conducive for adsorption. The desorption of proteins in the absence of $Na_2SO_4$ from the buffer system is most likely due to the release of structural water. Hydrophobic interaction with the metal-free gel cannot be the main cause for water structure-dependent adsorption as is demonstrated by the control experiment.

A small amount of UV-absorbing material elutes at pH 5.5 with cysteine, indicating that the Ni(II) gel exhibits specific affinity for thiol groups in the peptides and proteins. In serum, however, such a mechanism seems to play a minor role in the adsorption process.

The experimental results obtained so far do not permit a detailed analysis of the mechanism underlying adsorption of proteins to the Fe(III)–agarose besides the general statements already made. However, it is appropriate to mention that even if the adsorption is largely charge controlled, the effect on the serum proteins is widely different from that exerted by ordinary ion exchangers. No doubt this difference reflects a steric specificity in the protein interacton also in the case of immobilized tripositive iron.

The fact that transferrin and ceruloplasmin were not adsorbed on TED–Sepharose loaded with Ni(II) or Fe(III) ions suggests that the strong metal combining sites in these proteins are located internally and are therefore not accessible for complex formation with the metal ion fixed to the gel. These proteins are adsorbed to Zn and Cu gels (Porath et al., 1975). We therefore believe that this fact illustrates the specificity in terms of the metal ion as well as differences in the chelator (IDA) and its surroundings.

The absence of albumin in the Ni–TED columns is remarkable and demonstrates the profound metal ion specificity in the adsorption of proteins. Serum albumin is found in the eluates from the Fe(III)–IDA gel. For purification of albumin, copper–IDA gels are particularly effective (Hanson & Kågedal, 1981).

Whereas urea and $NaDodSO_4$ prevent adsorption as a consequence of a drastic change in the surface properties of the proteins, uncharged detergents such as Tween 80 seem to have no significant influence on the protein adsorption behavior of the Ni columns at low concentrations (<1%). The outcome of the preliminary experiments we have made so far with mixed solvents (ethylene glycol and $Me_2SO$) also suggests that IMA chromatography may be a useful tool for the systematic study of processes involved in the unfolding of proteins and may yield information regarding the changes in molecular surface topography of the proteins occurring under such conditions.

We still do not know how a number of factors such as temperature or chelator and matrix densities affect protein adsorption and chromatographic behavior.

## Acknowledgments

We express our appreciation to Drs. D. Eaker and M. Belew for helpful comments and linguistic suggestions. We are indebted to Dr. B. Granstrand, Kabi-Vitrum, for help with protein identification. We also thank B. Ahlström for skillful technical assistance.

**Registry No.** IDA–Sepharose 6B, 76632-76-3; agarose, 9012-36-6; iminodiacetate, 142-73-4; ED–Sepharose 4B, 81859-05-4; ethylenediamine, 107-15-3; TED–Sepharose 4B, 84680-76-2; Ni, 7440-02-0; Fe, 7439-89-6; NaCl, 7647-14-5; disodium sulfate, 7757-82-6; urea, 57-13-6; $NaDodSO_4$, 151-21-3; Tween 80, 9005-65-6; ethylene glycol, 107-21-1; $Me_2SO$, 67-68-5; $NH_4^+$, 14798-03-9; cysteine, 52-90-4; imidazole, 288-32-4; histidine, 71-00-1; ceruloplasmin, 9031-37-2; $\alpha_1$-antitrypsin, 9041-92-3; C1q, 80295-33-6; C3, 80295-41-6; C4, 80295-48-3; catalase, 9001-05-2; LDH, 9001-60-9.

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# Effect of Metal Substitution on the Chromophore of Bovine Serum Amine Oxidase†

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ABSTRACT: The spectral and chemical properties of the putative organic chromophore in bovine serum amine oxidase (BSAO) have been characterized by metal ion substitutions of the enzyme. Native BSAO is yellowish pink on account of a broad electronic absorption band at 476 nm. Copper(II)-depleted BSAO (Cu-depBSAO) was prepared by dialyzing pale yellow BSAO, which had been obtained by a reduction of native BSAO with sodium dithionite, against KCN-containing buffer solution. The Cu-depBSAO exhibited no electronic absorption extremum in the region 350–650 nm. Dialysis of Cu-depBSAO against pH 7.2 buffer containing Ni(II) ion gave pink Ni(II)-substituted BSAO (Ni(II)BSAO), which displayed an absorption extremum at 484 nm. Upon removal of Ni(II) from Ni(II)BSAO by dialyzing Ni(II)-BSAO against dimethylglyoxime and KCN-containing buffer solution, pink (instead of pale yellow) Ni-depBSAO was obtained, which showed an electronic absorption peak at 470 nm. The absorption maxima of Ni(II)BSAO and Ni-depBSAO immediately disappeared on addition of a substrate, benzylamine, and did not reappear even under aerobic conditions. This indicates that the chromophore in Ni(II)BSAO or Ni-depBSAO was reduced by benzylamine. It was suggested that yellowish pink or pink BSAO which exhibits an absorption maximum at 470–484 nm possesses the chromophore in oxidized form (resting state) whereas pale yellow or colorless BSAO which exhibits no absorption peak in the region 350–650 nm possesses the chromophore in reduced form. A comparative inspection of electronic absorption and CD spectra of native BSAO, Ni(II)BSAO, and Ni-depBSAO treated with phenylhydrazine revealed that the chromophore is located near the metal ion at the active site of BSAO. The close location of the chromophore to Cu(II) ion was also supported by ESR data. The role of the chromophore is considered to bind a substrate and to catalyze the subsequent deamination while the Cu(II) ion operates the transfer of electrons from the reduced chromophore to oxygen molecule. The cooperative catalysis of the chromophore and Cu(II) ion in BSAO may correspond to the function of flavin–cofactor of mitochondrial amine oxidases.

Amine oxidases [amine:oxygen oxidoreductase (deaminating), EC 1.4.3] are known to catalyze the oxidative deamination of amines by accepting two electrons from amines and transferring them to molecular oxygen. The reactions are expressed by the equation

$$RCH_2NH_2 + O_2 + H_2O \rightarrow RCHO + NH_3 + H_2O_2$$

They may be conveniently divided into two classes: copper-containing amine oxidases (Malmström et al., 1975) and FAD-containing ones. The copper enzymes are widely distributed in plasma of bovine (Yamada & Yasunobu, 1962a,b, 1963; Yamada et al., 1963; Inamasu & Yasunobu, 1974; Ishizaki & Yasunobu, 1976; Suva & Abeles, 1978; Berg & Abeles, 1980; Zeidan et al., 1980) and pig (Buffoni & Blaschko, 1964; Buffoni et al., 1968; Taylor et al., 1972; Lindström et al., 1973, 1974; Lindström & Pettersson, 1974; Yadav & Knowles, 1981), kidney tissue of pig (Mondovi et al., 1967, 1968; Yamada et al., 1967; Finazzi-Agrò et al., 1977), pea seedling (Hill & Mann, 1964; Hill, 1967; Kluetz et al., 1980), and fungus (Yamada et al., 1965, 1969; Adachi & Yamada, 1969). The copper(II) ion in these proteins is known to be type 2 copper [nonblue and electron spin resonance (ESR)[1] detectable] (Malkin & Malmström, 1970; Peisach & Blumberg, 1974) and to restore the activity of copper-depleted oxidases (Yamada & Yasunobu, 1962b; Hill

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[1] Abbreviations: BSAO, bovine serum amine oxidase; Ni(II)BSAO, Ni(II)-substituted BSAO; Co(II)BSAO, Co(II)-substituted BSAO; Zn(II)BSAO, Zn(II)-substituted BSAO; Cu-depBSAO, Cu(II)-depleted BSAO; Ni-depBSAO, Ni(II)-depleted BSAO; CHR, organic chromophore; CT, charge transfer; ESR, electron spin resonance; CD, circular dichroism; MCD, magnetic circular dichroism; Tris, tris(hydroxymethyl)aminomethane; TCNQ, tetracyanoquinodimethane.

0006-2960/83/0422-1630$01.50/0 