Identification of the Serum Binding Proteins for Iron, Zinc, Cadmium, Nickel, and Calcium

Barbara J. Scott and Arthur R. Bradwell

The binding of five biologically important metals to serum proteins has been studied. After suitable radioactive isotopes were added to serum proteins separated and precipitated by two-dimensional immunoelectrophoresis, the sample plates were exposed to roentgenogram film. $^{59}$Fe bound to transferrin alone; $^{65}$Zn bound mostly to albumin, but also to another 12 proteins; $^{109}$Cd was mostly associated with $\alpha_2$-macroglobulin, but was also present on albumin, immunoglobulins G and A, and prealbumin; $^{63}$Ni, added in high concentration, was associated with an $\alpha_2$-mobility protein and albumin; and, finally, $^{46}$Ca was mostly bound to albumin, but seven other binding proteins were also identified, with transferrin predominant. The results are not quantitative, but the technique is simple and specific, and the information gained can direct further studies on isolated proteins.

Additional Keyphrases: radioassay • albumin • transferrin • two-dimensional immunoelectrophoresis • immunoglobulins • macroglobulin • prealbumin

Many metals play an essential role in biological pathways as enzyme regulators, catalysts, cell depolarizers, and in molecular structure. The correct functioning of these pathways is ready disrupted by a deficiency or excess of these metals or by the presence of other toxic metals. Therefore, an understanding of the biochemistry of metals and their measurement in biological fluids is important. The results of measurements in serum or plasma, however, may be of doubtful value for two reasons: they may bear little relationship to intracellular concentrations; and they may not relate to the biological availability if there is considerable protein binding. The latter problem is well recognized for various substances routinely measured in serum. For example, thyroxin (T4) is extensively bound by serum proteins, particularly thyroxin-binding globulin, so that the concentration of the latter must be assessed when interpreting total T4 concentrations. Similarly, total metal concentrations in serum will not necessarily relate to the "free" or available metal concentration if protein binding is extensive.

Chromatographic, equilibrium dialysis, and electrophoretic methods for identifying and quantifying binding proteins all have poor resolution, making results difficult to interpret. For example, approximately 45% of serum calcium is protein-bound, 80% of this being bound to albumin (1); despite considerable effort, however, the proteins binding the remaining 20% have not been identified (2, 3). The present knowledge of the serum binding of the five metals we have studied is summarized in Table 1.

In an attempt to improve on the resolution and sensitivity of available methods we have used an immunological technique, i.e., two-dimensional immunoelectrophoresis (2-D IEP) combined with autoradiography (4). Human serum proteins are separated and precipitated on a glass plate by 2-D IEP with use of an antisera raised against whole human serum. The plate is immersed in a buffer, at physiological pH, that contains a radioactive species of the ligand, then is dried and exposed to roentgenogram ("x-ray") film. Comparison of the autoradiograph with the protein-stained plate positively identifies the binding proteins. Where there is doubt about identity, immunoprecipitation and autoradiography by radial immunodiffusion (RID) is used with a panel of monospecific antisera.

We have used these techniques to identify the serum proteins binding five metals: iron, zinc, cadmium, nickel, and calcium.

Materials and Methods

Reagents

Radioisotopes: The radioisotopes were obtained from Amersham International, Amersham, Bucks., U.K. Specific activities and modes of decay are shown in Table 2.

Antisera: All antisera were produced in sheep and purified in our laboratory.

Procedures

Two-dimensional immunoelectrophoresis was performed on 80 × 80 mm glass plates as described previously (13), with 4 mL of pooled normal human serum (from 100 donors) and 2.0 mL of anti-whole human serum (Figure 1). After electrophoresis the gels were washed in phosphate-buffered saline (NaH2PO4, 2H2O 2.5 mmol/L, Na2HPO4 7.5 mmol/L, NaCl 0.15 mol/L, pH 7.2), to remove both the barbital buffer used for electrophoresis and the uncomplexed sheep proteins. The plates were then placed in Petri dishes containing sufficient phosphate-buffered saline to just cover the gel (about 6.0 mL). A suitable concentration of a radioactive species of each metal was then added to the 2-D IEP plates (Table 2). The Petri dishes were sealed and stored at 4 °C.

After three days the surface of the gels was briefly washed with distilled water to remove nonspecific radioactivity, then dried under a sheet of filter paper, in air. Autoradiography was performed at room temperature with the films listed in Table 2. The choice of films was based on previous work (14).

The plates were finally protein-stained in Kenacid Blue R (Figure 1), for comparison with the autoradiographs.

Radial immunodiffusion: Serum samples from pooled normal human serum were incubated with the appropriate concentration of radioactive metal ions for three days at 4 °C (Table 2). RID was performed as described previously (15), with agarose in phosphate-buffered saline and the monospecific antisera listed in Table 2. Concentrations of the antisera were adjusted to give ring diameters of between 8 and 10 mm.

Samples of serum and radioactive metal, equivalent to 10 μL of serum, were added to the plates and allowed to diffuse in a moist atmosphere for as long as three days. The plates

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Table 1. Protein Binding of the Metals Studied

<table>
<thead>
<tr>
<th>Protein Binding of the Metals Studied</th>
<th>Iron</th>
<th>Zinc</th>
<th>Cadmium</th>
<th>Nickel</th>
<th>Calcium</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal serum concn</td>
<td>1.13 - 1.27 mg/L (5)*</td>
<td>0.9 - 1.2 mg/L (6)</td>
<td>1.1 - 3.3 μg/L (6)</td>
<td>2.6 μg/L (10)</td>
<td>2.25 - 2.75 mmol/L (90 - 110 mg/L) (1)</td>
</tr>
<tr>
<td>% protein-bound in human serum</td>
<td>100 (5)</td>
<td>90 (6)</td>
<td>Approx. 90</td>
<td>60 (10)</td>
<td>40 (1)</td>
</tr>
<tr>
<td>Known serum binding proteins*</td>
<td>Transferrin</td>
<td>60% albumin, 30% α2-macroglobulin, haptoglobin, ceruloplasmin, transferrin, immunoglobulin (7)</td>
<td>Albumin, α2-macroglobulin (9)</td>
<td>96% albumin (11)</td>
<td></td>
</tr>
</tbody>
</table>

*Nos. in parentheses refer to references.

% of total bound is given when known.

*In rabbit serum 44% is bound to nickeloplasmin, 40% to albumin (12).

Table 2. Materials Used in the Assays

<table>
<thead>
<tr>
<th>Isotope</th>
<th>Iron (59Fe)</th>
<th>Zinc (65Zn)</th>
<th>Cadmium (110Cd)</th>
<th>Nickel (65Ni)</th>
<th>Calcium (40Ca)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Specific acty, Ci/g</td>
<td>13.9</td>
<td>357</td>
<td>684</td>
<td>11.28</td>
<td>17.8</td>
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<tr>
<td>2-D IEP:</td>
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<tr>
<td>Radioactive</td>
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<td></td>
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<tr>
<td>ligand concn*</td>
<td></td>
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<tr>
<td>RID:</td>
<td></td>
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<td></td>
<td></td>
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<tr>
<td>Ligand concn</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Antisera*b</td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Autoradiography</td>
<td></td>
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<tr>
<td>film</td>
<td></td>
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<tr>
<td>Cronex 4 (Du Pont)</td>
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<tr>
<td>2-D IEP:</td>
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<td></td>
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<tr>
<td>Exposed:</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>70 h</td>
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<td></td>
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</tbody>
</table>

*P = physiological concentration of analyte.

Common to zinc, cadmium, nickel, and calcium methods were antisera to the following: albumin, transferrin, α2-macroglobulin, orosomucoid, ceruloplasmin, α1-antitrypsin, haptoglobin, prealbumin, α1-9.5S glycoprotein, and IgG. α2-HS, M, 49 000 glycoprotein of unknown function (usual concentration, 400-850 mg/L in adults).

were dried under a sheet of filter paper in air and autoradiographed.

Details of the 2-D IEP and RID techniques have been published elsewhere (4).

Results and Discussion

The autoradiographs of the 2-D IEP plates are shown in Figures 2-6; Figure 7 shows an example of a positive and negative RID result. The serum binding proteins identified by these two techniques are listed in Table 3.

Fig. 1. An example of two-dimensional immunoelectrophoresis of serum and anti-whole human serum, stained for protein

Fig. 2. Autoradiograph of 59Fe (1.0 mg/L) bound to transferrin in 2-D IEP

Exposure: 70 h
Iron

To illustrate the specificity of the technique, we measured the serum binding of iron (Figure 2); the results were in agreement with previous reports (5) that show iron binding only to transferrin. Lactoferrin concentrations being very low in normal serum (0.1–0.15 mg/L) (16), binding of iron to this protein was not detected.

Zinc

In contrast to iron, the binding of zinc was complex (Figure 3). Albumin was confirmed as the major binding protein, but 12 other proteins also bound zinc. Changes in the concentrations of these proteins will clearly contribute to changes in total serum zinc, and must be considered when evaluating zinc status. Although Okunewick et al. (17) reported that $^{65}$Zn added to serum in vitro did not equilibrate with native zinc atoms but bound nonspecifically to all serum proteins in a ratio closely paralleling their concentrations in serum, our results disagree with this report. When the radioactive label was added in physiological concentrations, several proteins of relatively high concentration did not bind zinc. For example, prealbumin bound zinc, whereas complement component $C_3$, which is present in approximately threefold greater concentration, did not.

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Lower concentrations of $^{63}$Ni failed to produce an autoradiograph because of the low energy of the $\beta^-$ particle. RID identified one of these proteins as albumin; the other, an $\alpha_2$-protein, could not be identified from the panel of monospecific antisera available, but is probably $\alpha_2$-nucleoplasmin, first described by Himmelhoch et al. in 1966 (18). From the 2-D IEP results, the $\alpha_2$-protein appeared to have an in vitro binding capacity for nickel approximately equal to that of albumin. This finding contradicts previous reports that the specific nickel-binding protein of human serum binds only small amounts of nickel in vitro (10, 11). Our result could reflect the high concentrations of $^{63}$Ni used, but the fact that the $\alpha_2$-protein has the capacity to bind as much nickel as does albumin in vitro suggests it may bind more free nickel in vivo than is currently believed. The production of a monospecific antiserum would help to answer this question.

**Calcium**

Calcium bound to eight proteins when $^{46}$Ca (8.4 mg/L as Ca$^{2+}$) was added to the serum (Figure 6). As expected, albumin was the major serum binding protein; of the seven other serum proteins, transferrin was the most important. Alterations in the concentrations of these binding proteins may contribute to the discrepancy between observed calcium/albumin corrections and free Ca measurements.

**Method Evaluation**

This study demonstrates that the identification of metal-binding proteins in serum by immunoprecipitation techniques is relatively sensitive, simple, and apparently accurate when compared with other techniques. However, the limitations of this approach need to be outlined.

The presence of antibody requires the following assumptions to be considered: (a) that the anti-whole human serum contains all the relevant antibodies; (b) that the presence of sheep antibody does not sterically inhibit normal protein binding (in the case of small ligands and metals this is a reasonable premise (19)); and (c) that the sheep immunoglobulins do not bind the ligand. This last assumption, though true for many ligands, was not the case for zinc, which was found to bind to both human IgG and sheep IgG. Positive or negative binding to human proteins was consequently assessed by "relative" binding capacity, because each precipitate would demonstrate binding if autoradiography was continued for long enough. This procedure ap-

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**Table 3. Serum Proteins Found to Bind the Five Metals**

<table>
<thead>
<tr>
<th>Method of Protein Identification</th>
<th>Iron</th>
<th>Zinc</th>
<th>Cadmium</th>
<th>Nickel</th>
<th>Calcium</th>
</tr>
</thead>
<tbody>
<tr>
<td>2-D IEP Transferrin</td>
<td>At least nine binding proteins visible</td>
<td>$\alpha_2$-Macroglobulin</td>
<td>Albumin</td>
<td>$\alpha_2$-Antitrypsin</td>
<td></td>
</tr>
<tr>
<td>RID</td>
<td>Albumin</td>
<td>IgG</td>
<td>Albumin</td>
<td>Albumin</td>
<td></td>
</tr>
<tr>
<td></td>
<td>$\alpha_2$-Macroglobulin</td>
<td>IgA</td>
<td>Albumin</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Transferrin</td>
<td>IgM</td>
<td>Albumin</td>
<td></td>
<td></td>
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<tr>
<td></td>
<td>Ceruloplasmin</td>
<td>Prealbumin</td>
<td></td>
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<tr>
<td></td>
<td>IgG</td>
<td>Prealbumin</td>
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<td></td>
</tr>
<tr>
<td></td>
<td>IgA</td>
<td>Prealbumin</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>IgM</td>
<td>Prealbumin</td>
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<tr>
<td></td>
<td>Complement C3</td>
<td>Prealbumin</td>
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<td></td>
<td>Haptoglobin</td>
<td>Prealbumin</td>
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<td></td>
<td>Prealbumin</td>
<td>Prealbumin</td>
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<tr>
<td></td>
<td>C-reactive protein</td>
<td>Prealbumin</td>
<td></td>
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<tr>
<td></td>
<td>$\alpha_1$-Antitrypsin</td>
<td>Prealbumin</td>
<td></td>
<td></td>
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<tr>
<td></td>
<td>$\alpha_1$-9.5S</td>
<td>Prealbumin</td>
<td></td>
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<tr>
<td></td>
<td>Glycoprotein</td>
<td>Prealbumin</td>
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</tr>
<tr>
<td></td>
<td>Albumin + seven other proteins</td>
<td></td>
<td></td>
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<td></td>
</tr>
</tbody>
</table>

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peared to be valid in view of the selective binding of zinc to human proteins (Figure 7; see above).

The immunoprecipitation techniques cannot be quantit-

tative for several reasons: (a) the presence of antibody; (b) the
dilution of the metal in phosphate-buffered saline in 2-D IEP
(the concentrations quoted do not take this into account); (c)
the restrictions of phosphate-buffered saline as a general-
purpose physiological buffer; (d) the inability of these tech-
niques to detect binding to low-molecular-mass compounds
or to proteins present in such low concentrations that no
immune complex is formed; and (e) the nonlinear response of
photographic film. Despite these limitations, however, rela-
tive image density can give an approximation of binding
capacities, as is illustrated by the good agreement, when
comparisons can be made, between our results and others in
the literature involving different techniques.

Detection of radioisotopes by autoradiography is inherent-
ly less efficient than direct counting. However, the resolution
required with 2-D IEP makes counting procedures too
tedious. Moreover, the speed of autoradiography is variable,
being determined by the nature of the isotope. For isotopes
emitting strong β-particles and gamma rays, detection is
reasonably efficient; but for 65Ni, which emits a weak β−
particle, high concentrations of isotope had to be added to
the plates to produce an autoradiograph. The detection of 3H
is even slower, but it is a commonly used radiolabel for
biological compounds. To overcome the restriction this
places on the broader usefulness of these techniques, we are
developing an electronic detection system (a multiwire
proportional counter), which increases the speed of detection
of weak β− particles several thousand-fold (20).

The main advantages of the immunoprecipitation tech-
niques are their simplicity and specificity, together with the
facility to study ligand binding within a complex mixture of
proteins. Quantitative data and information on binding
affinities are not available, but once a binding protein is
identified, such data can be produced with isolated proteins.

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from the Medical Research Council.

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