Clinical Studies of Protein-Bound Calcium in Various Diseases

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A recently developed gel-filtration technique allows protein-bound calcium fractions to be separated and quantitated; the protein is separated under physiological conditions of pH, temperature, and concentrations of Na, Mg, and Ca to assure that the calcium–proteinate equilibrium is not disturbed. We used this gel-filtration technique to study the protein-bound calcium fractions in 18 patients with hyperparathyroidism, multiple myeloma, diabetes, osteoporosis, or liver cirrhosis. We calculated the amount of calcium bound per gram of protein for each of the three protein peaks and the intrinsic association constant ($K_a$) for calcium/albumin. Results with the multiple myeloma patients (three IgG, one IgA) indicated that IgG did not bind calcium appreciably, that IgA had about the same affinity as albumin for Ca, and that $K_a$ was slightly low for one patient of the IgG type (79 L/mol) and normal for the other three myeloma patients (106, 90, and 91 L/mol). Results for patients with the other diseases were also essentially normal, except for the osteoporosis patients (two men, one woman), whose $K_a$ values (69, 75, and 73 L/mol) were lower than normal.

Additional Keyphrases: gel filtration · immunoglobulins · albumin · hyperparathyroidism · multiple myeloma · diabetes · osteoporosis · cirrhosis · reference interval

Ionized calcium, the physiologically active species of blood calcium, ordinarily is maintained within very narrow limits by a rigidly controlled homeostatic mechanism. Strict control is of utmost importance because such life-sustaining processes as nerve conduction and muscle contraction are regulated by the concentration of this calcium species in serum. If the concentration of ionized calcium in serum drops below or increases above the tolerable limit, the ensuing changes in these life-sustaining processes can be fatal. For this reason, there is considerable interest in the calcium fractions found in serum specimens of persons who have diseases that are usually characterized by either hypocalcemia or hypercalcemia. Most investigators of the calcium fractions in patients with hyperparathyroidism, multiple myeloma, diabetes, osteoporosis, or liver cirrhosis report total ionized, ultrafilterable, and protein-bound calcium ($1–6$). Because suitable methodology has been lacking, few attempts have been made to examine calcium binding to the different protein fractions. Some investigators have conducted calcium-binding studies on purified protein (7) or on protein fractions separated by precipitation (8) or electrophoresis (9); however, a gel-filtration technique (10) separates serum protein fractions under physiological conditions of pH, temperature, and concentrations of sodium, magnesium, and calcium. Under these conditions, the calcium–protein equilibrium is not disturbed, and the calcium bound to the separated proteins can be quantitated.

We have used this gel-filtration technique to examine the calcium-binding affinity of serum proteins in cases of hyperparathyroidism, multiple myeloma, diabetes, osteoporosis, and liver cirrhosis. We report the micromoles of calcium per gram of protein (Ca, μmol/g of protein) for each protein fraction and the intrinsic association constants ($K_a$) for calcium/albumin in these disease states.

Materials and Methods

Apparatus. For the gel filtration we used jacketed columns, 0.9 × 100 cm (Glenco 3400; Glenco Scientific, Inc., Houston, TX 77007), fitted with a reagent reservoir (Pharmacia R25; Pharmacia Fine Chemicals, Piscataway, NJ 08854), and maintained at 37 °C by a circulating water bath (Lauda K-2/R; Brinkmann Instruments, Westbury, NY 11590). The eluant was collected with an automated fraction collector (ISCO Model 328; Instrumentation Specialties Co., Lincoln, NB 68504). For automated analyses we used a microcentrifugal analyzer (Instrumentation Laboratory, Lexington, MA 02173) and a fluoronephelometer, AutoAnalyzer II proportioning pumps, samplers, and dialysis unit (all from Technicon Instruments Corp., Tarrytown, NY 10591). All pH values were measured with a research pH meter (Digital 112; Corning Glass Works, Corning, NY 14830). Ionized calcium was analyzed with an Ionized Calcium Analyzer (Orion Biomedical, Cambridge, MA 02139).

Methods. Serum samples were fractionated by gel filtration (10). Automated procedures for total calcium (11), total protein (12), albumin (12), and dialyzable calcium (13) were used as reported or with minor modifications as appropriate. The precision (mean ± 1 SD; n = 20) obtained with these four methods was: total calcium 2.5 ± 0.33 mmol/L, total protein 15 ± 0.4 g/L, albumin 10 ± 0.03 g/L, and dialyzable calcium 1.0 ± 0.02 mmol/L. The total protein and albumin concentrations were similar to those found in the column fractions. The calcium methods were calibrated with National Bureau of Standards (NBS) calcium carbonate. The total protein and albumin methods were standardized with NBS protein standard. We used least-squares linear regression (14) to calculate concentrations.

Serum collection. Blood was collected without stasis into evacuated blood-collection tubes (with no preservatives or anticoagulants added) from fasting patients who had hyperparathyroidism, diabetes, osteoporosis, multiple myeloma, or liver cirrhosis. After the blood was allowed to clot, we separated the serum by centrifugation at 4 °C in the stopped

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3 Use of trade names is for identification only and does not constitute endorsement by the Public Health Service or the U.S. Department of Health and Human Services.
blood-collection tube, to minimize the loss of CO₂. We exposed
the sample to air as little as possible by immediately trans-
fering the serum to a syringe, expelling all air, and capping
the syringe tip. The serum was then stored at 4 °C.

Sample preparation. To prepare samples for gel filtration,
add 20 µL of 2-[tris(hydroxymethyl)methyl]aminoethane-
sulfonic acid, 1 mol/L, pH 7.35, to a 2.0-mL aliquot of the
serum. Then adjust the pH to 7.43 ± 0.01 at 37 °C with 5–10
µL of 1 mol/L HCl or 0.1 mol/L NaOH, to eliminate variations
in the calcium fractions related to differences in pH among
individual specimens.

Analyses. For gel filtration, we applied 1.5 mL of serum (as
prepared above) to the top of the column (flow rate 16–17
mL/h), and collected 110 fractions, about 1 mL each. The
fractions were frozen until they could be analyzed. Each
fraction was analyzed for total calcium, and appropriate
fractions were analyzed for total protein and albumin. We also
analyzed the serum from each patient (original, not gel-fil-
tered) for total calcium, dialyzable calcium, ionized calcium,
total protein, and albumin.

Results

Results obtained with the 18 original serum specimens in
this study are tabulated in Table 1, with abnormal results
indicated. Reference intervals for healthy adults are as follows:
total calcium, 2.12–2.60 mmol/L (16); ionized calcium, 1.00–1.12 mmol/L (16); protein-bound calcium, 0.86–1.12 mmol/L (16);
total protein, 66–83 g/L (17); and albumin, 35–50 g/L (17).

We also fractionated sera from 18 patients by gel filtration.
The elution pattern for the fractionation technique has been
previously described (10). Figure 1 represents a typical gel-
filtration elution pattern for total protein, albumin, and
calcium fractions. Total protein is separated into three distinct
peaks (1, 2, and 3 from left to right), and total calcium is sepa-
rated into protein-bound (shown directly above protein
peaks), complexed, and ionized fractions (at the extreme right)
(10).

Relative calcium binding by each protein peak. We cal-
culated the amount of calcium bound per gram of protein in

![Fig. 1. Typical gel-filtration elution pattern](image-url)

The composition of the protein peaks (left to right) are as follows (10): peak 1, IgM; peak 2, IgG; peak 3, primarily albumin with transferrin and α₁-antitrypsin. Directly above each protein peak is the concentration of calcium bound to that protein fraction. Complexed and ionized calcium are to the far right of the protein-bound calcium (10).
each of the three protein peaks for each of the 18 patients (Table 1). The total calcium associated with a protein peak was divided by the total protein in that peak. Toffaletti et al. (10) reported calcium ranges of 14–22, 6–14, and 15–19 μmol/g of protein (mean ± 2 SD) for peaks 1, 2, and 3, respectively, for 12 healthy individuals. We found calcium ranges of 17–19, 7–14, and 12–16 μmol/g of protein, respectively, for peaks 1, 2, and 3 for four normal individuals. Our calcium ranges (μmol/g of protein) for peaks 1, 2, and 3, respectively, for the different disease types are as follows: hyperparathyroidism, 13–22, 6–12, and 12–13; osteoporosis, 17–21, 7–10, and 10–13; diabetes, 17–19, 4–13, and 11–16; multiple myeloma, 17–24, 5–20, and 10–20; and liver cirrhosis, 16–20, 4–10, and 10–18.

**Albinin** $K_a$. For each patient’s specimen, we calculated $K_a$ for calcium/albinin according to the following equation (10):

$$\frac{1}{K_a} = \frac{[\text{Calcium}^{2+}]}{[\text{Albumin}] - [\text{protein-bound calcium}]}$$

These calculations were based on concentrations of ionized calcium, albumin, and protein-bound calcium in individual column fractions where the total protein was at least 90% albumin and the calcium concentration was at least 0.1 mmol/L. The $K_a$’s for our patients’ specimens (Table 1) showed the following ranges (L/mol) for the different diseases: osteoporosis, 69–75; diabetes, 84–108; hyperparathyroidism, 94–102; liver cirrhosis, 86–113; and multiple myeloma, 79–106. Toffaletti et al. (10) reported 91–111 L/mol (mean ± 2 SD) for 12 normal individuals; we found a range of 91–98 L/mol for four normal individuals.

**Discussion**

Our purpose in examining the calcium–protein fractions in different diseases was to present data that might be useful in explaining calcium abnormalities associated with these diseases. The diseases our patients had were diagnosed as follows: multiple myeloma, by presence of atypical plasma cells and soft tumors in the bone marrow; hyperparathyroidism, by surgical removal of the parathyroid gland; diabetes, by glucose-tolerance testing; osteoporosis, by radiological examination of bone; and liver cirrhosis, by biopsy.

Recent evidence suggests that a decrease in renal function and unbalanced or excessive action of the parathyroid hormone may be among the causative factors of senile osteoporosis (18). Loss of estrogen secretion has been implicated as a contributing factor in postmenopausal women (19–21). To our knowledge, the different calcium fractions and the binding of calcium to individual serum proteins have not been previously reported for osteoporosis patients. In the three osteoporotic patients we studied, one patient (a man) had increased ionized and dialyzable calcium fractions; another (also a man) had increased total serum calcium and total protein-bound calcium. All other calcium fraction results were within the normal range (Table 1). The protein-bound calcium for peaks 1 and 2 were within normal ranges, but for peak 3 it was low normal for two patients and below normal for the third patient. The $K_a$ values for all three patients were lower than normal. The decreased calcium-binding affinity in these patients may be a contributing factor to the negative calcium balance seen in osteoporosis.

For our results for three diabetic patients indicated that protein-bound calcium was normal, total calcium was normal, dialyzable (ultrafilterable) calcium was increased in two patients and normal in one patient, and ionized calcium was increased in all three patients. We found that the micromoles of calcium per gram of protein for each of the three protein peaks was normal, except in one instance when one patient’s value for this in peak 3 was borderline low. The $K_a$’s listed in Table 1 were normal for two of the patients and slightly low for the third patient. Our results indicate that the calcium-binding affinity of proteins in diabetics is low to normal rather than increased.

Multiple myeloma is frequently complicated by hypercalcemia, but the patient often does not exhibit the clinical symptoms of hypercalcemia—polyuria, polydipsia, constipation, and confusion. Some investigators have indicated that this lack of symptoms may be explained by an increase in the protein-bound calcium fraction while the ionized fraction remains normal (3, 7, 22–24). Our results for five myeloma patients (Table 1) show no definite pattern for the calcium-binding affinity of serum proteins associated with this disease. The bound calcium (μmol/g of protein) for peak 1 was normal for three of the patients (two IgG, one IgA) and slightly increased for the other two (both IgG). In peak 2, the bound calcium content was normal for the three IgG myeloma patients and increased for the IgA patient (no separation for the fifth patient). Because peak 2 should represent mostly IgA or IgG in these myeloma patients, these data indicate that IgG paraprotein binds about the same amount of calcium as does normal IgG and that IgA binds about the same amount of calcium as albumin. The bound calcium content in peak 3 (albumin) was normal for two IgG patients, slightly low for one IgG patient, and slightly increased for the IgA patient (no separation on the fifth patient). The $K_a$ values were normal for two IgG patients and the IgA patient; it was decreased for one IgG patient ($K_a$ was not calculated for the fifth patient). Our group of myeloma patients appears to be unique regarding the total protein-bound calcium fraction. Four of the patients had abnormally low protein-bound calcium (total calcium minus dialyzable calcium) associated with abnormally low serum albumin—a finding not previously reported for myeloma patients.

Hyperparathyroidism is characterized by above-normal total and ionized calcium, which results in severe (sometimes fatal) mental and abdominal symptoms. Various investigators have attempted to evaluate the calcium-binding affinity of serum proteins in this disease, to identify factors responsible for producing the hypercalcemia. We examined the calcium-binding ability of serum proteins for three patients with primary hyperparathyroidism, and for all three we found the protein-bound fractions to be within the normal range (Table 1).

Patients having cirrhosis of the liver are of particular interest for the study of calcium binding to serum proteins because (a) they often have hypocalcemia, and (b) they frequently have low concentrations of serum albumin (the major calcium-binding protein in normal individuals) with a concurrent increase of serum globulins. We examined the calcium-binding to serum proteins in four cirrhotic patients, and our results indicated that the globulin fractions bind measurable amounts of calcium (Table 1). The amount of calcium bound by peak 1 was normal for all four patients. In peak 2, one patient had a low amount of calcium per gram of protein, and in peak 3, two patients had a low amount. $K_a$ values were normal for two patients, slightly lower than normal for one patient, and slightly higher than normal for another. The total protein-bound fraction was normal for three of our patients and lower than normal for one.

This study appears to be the first attempt to quantitate the calcium bound to the different protein fractions in instances of diabetes, osteoporosis, primary hyperparathyroidism, multiple myeloma, and liver cirrhosis. Our study is unique in that the binding of calcium to the serum proteins was studied.

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4 Five myeloma patients were included in the study; protein-bound calcium fractions were not separated in one instance (Table 1).
under physiological conditions of pH, temperature, and ionic strength. Our results indicate some slight deviations from normal, but because of large interindividual variations we found no definite trend in any of the diseases studied except in osteoporosis. Results for the osteoporotic patients showed $K_a$ values to be lower than normal.

References


