THE EFFECTS OF CHANGES IN ACID CONCENTRATION ON FREE AND BOUND SERUM CALCIUM

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ABSTRACT

THE EFFECTS OF CHANGES IN ACID CONCENTRATION

ON FREE AND BOUND SERUM CALCIUM

By

William Lee Madden

A revised method of thermoprecipitation for the determination of free and protein-bound calcium concentrations in human serum has been developed. The revisions are (1) the addition of small amounts of a 2N hydrochloric acid solution to adjust the serum between 6.8 and 7.8, and (2) to allow the heated tubes to equilibrate to room temperature before centrifugation. The addition of small amounts of acid provides a stable pH and eliminates calcium phosphate precipitation in the serum.

The number of calcium binding sites on the albumin molecule, dissociation constant, pK value, and changes in the hydrogen ion concentration as determined by the revised thermoprecipitation method are similar to the results of more complicated and expensive methods.

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William Lee Madden

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For Joan,

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INTRODUCTION

Until McLean and Hastings showed in 1935 that the calcium of serum existed partially bound to protein, data obtained from clinical and laboratory investigations could not be satisfactorily explained. The total calcium appeared to be beyond those levels at which phosphates should precipitate, and hypotheses were offered suggesting that calcium phosphates were present in serum in a supersaturated state. Under these circumstances, no rational concepts could be developed to explain nutritional or physiological data.

McLean and Hastings used perfused frog hearts to detect and quantify the concentration of ionized calcium in the sera that they studied. Although this method was effective for the problems that they were investigating, this perfusion method is too difficult and cumbersome for application to the clinical problems involved in the diagnosis of parathyroid and nutritional disorders.

Various attempts to develop ultrafiltration or dialysis methods for the measurement of calcium ion concentrations were abandoned after Loeb demonstrated that the movement of calcium ions across a membrane was accompanied by a release of protein-bound calcium. More recently Moore[9] has developed an electrode for the direct measurement of calcium ions in biological solutions. The apparatus for this system is not yet commercially available, and it is clear from the published descriptions that when it does become available, the equipment will be expensive

and will probably require more than the usual skill and sophistication to operate and maintain.

Review of Calcium Metabolism

Dietary Source

The chief source of dietary calcium is milk and milk products with a small amount being contributed by vegetables.

Absorption and Excretion of Calcium

The intestinal absorption of dietary calcium is regulated by intestinal acidity, parathormone, and vitamin D. Since the upper duodenum is the most acid, thus favoring calcium salt dissolution, this is the major site of calcium absorption in man. There is some evidence that small amounts of calcium are absorbed in the lower ileum and even in the large intestines. The degree of absorption of calcium diminishes with age, but the average daily absorption is estimated to be 10 milligrams per kilogram of body weight.

Fatty acids prevent absorption of dietary calcium by forming insoluble calcium salts. If the amount of fat in the diet is increased, the amount of calcium bound to the fatty acids in the stools increases, producing a decrease in serum calcium concentration.

The excretion of calcium in humans is by the kidneys and the intestines. Except when active bone formation is taking place, the average daily urinary excretion of calcium approximates the net intestinal absorption of calcium. Renal excretion is a function of renal tubular reabsorption of filtered calcium. Reabsorption is regulated by parathormone which is liberated in response to a low ionized calcium concentration in the blood plasma. Intestinal excretion of

calcium consists of calcium salts in the bile, pancreatic secretions, and unabsorbed dietary calcium.

Calcium Metabolism in Pregnancy and Lactation

The requirements for calcium are considerably increased during the later months of pregnancy due to fetal bone formation. If supplies are not adequate for the needs of the fetus, its mineral requirements will be met by calcium mobilization from the maternal skeleton with resulting demineralization. Lactation results in a negative calcium balance due to the need for large quantities of calcium in the milk.

Calcium Metabolism in Pancreatic Disease and Other Malabsorption Syndromes

Acute pancreatitis often causes hypocalcemia. The lipase of the pancreatic juice escapes from the diseased pancreas and hydrolyzes the neutral fat in the omentum into fatty acids. These fatty acids then are changed into soaps by conjugation with calcium and magnesium. Large quantities of these soaps are formed, absorbing large quantities of calcium that would normally be used to meet the requirements of the body, thus producing hypocalcemia.

Hypercalcemia may also precipitate pancreatitis by the deposition of calcium salts within the lumen of the small ducts. The resulting obstruction of ducts brings about the pancreatitis.

Other malabsorption syndromes that may produce hypocalcemia are intestinal obstruction, duodenal ulcers, anorexia, persistent diarrhea, and constipation.

Effects of Vitamin D Deficiency and Excess

Vitamin D deficiency is characterized by rickets in children and osteomalacia in adults. This is brought about by a decreased intestinal absorption of calcium, which is vitamin D-dependent, producing hypocalcemia. This hypocalcemia stimulates the parathyroid glands to increased activity to maintain the plasma calcium level at the expense of the skeleton. As this deficiency in the absorption of dietary calcium progresses, the available mineral reserves of the skeleton may become depleted to the extent that the serum calcium level cannot be supported adequately despite the increased parathyroid activity. Hypocalcemia then develops and, if severe, is manifested by neuromuscular hyperexcitability and deformity and softening of the bones.

Hypervitaminosis D results in an increased intestinal absorption of calcium with resulting hypercalcemia. The excessive amount of calcium in the plasma may lead to metastatic calcification of the kidneys, arteries, bronchi, muscles, and gastric mucosa. Renal failure may develop leading to death.

Calcium Metabolism in Renal Disease and Parathyroid Adenoma

Renal disease, due to impaired or defective renal function, produces a retention of phosphorus and a decrease in plasma calcium resulting from increased excretion of urinary calcium.

Parathyroid adenoma invariably raises the level of ionized calcium in the plasma. This is the result of increased production of parathormone which stimulates bone mobilization of mineral and increases renal tubular reabsorption of calcium. The increased ionized fraction may or may not be reflected in an increase in serum total calcium. Renal failure may develop from nephrocalcinosis and/or nephrolithiasis

complicated by pyelonephritis. When the disease has progressed to this stage, the classical plasma calcium elevation and phosphorus depression may be obscured since the renal disease produces a retention of phosphorus and a decrease in plasma calcium.

LITERATURE REVIEW

Calcium Binding to Protein

That calcium is bound to serum proteins was recognized in 1913 when Rona and Takahashi[3] dialyzed serum against water showing that part of the calcium was not diffusible through the semipermeable membrane. McLean and Hastings[7] later showed that the diffusible portion of the calcium was partially ionized and partially complexed with other substances such as citrate. Most investigators have confirmed these findings but have disagreed as to what proportions are in each fraction.[4,6,15]

Scatchard[12] stated in 1949, in reference to protein-binding, "We want to know about the binding of the molecule or ion, how many, how tightly bound, where and why."

The actual site on the protein molecule for the binding of calcium and other metals was discussed by Vallee.[16] The binding sites on the protein molecule have been determined through the study of the interactions between metals and amino acids, peptides, and their derivatives. In general the amino acid side chains of proteins, having dissociable hydrogen ions, serve as the ligands for metal interactions, although peptide nitrogens can also participate. Practially all of the reactive groups of amino acids have been posulated to bind metals. Steinhart and Reynolds[14] stated that calcium is bound on the C-terminal end and the epsilon amino groups of the protein molecule; however, the most probable binding sites are single amino acid residues. Other binding

sites could be formed through the displacement of the hydrogen ion on the thiol group between dimer complexes, and the formation of 5 membered rings with the calcium ion.

Since cations other than calcium bind to proteins, competition for the calcium binding sites would be expected. However, human serum proteins have a greater specificity for calcium than for sodium, potassium, or magnesium ions.[8] The absolute error in calcium-binding studies due to the binding of these cations, is probably well below 5 percent. Hopkins, Howard and Eisenberg,[4] using ultrafiltration techniques, found no interference from these cations.

The studies of the effects of temperature on calcium-binding to proteins indicated that there is an increased dissociation of calcium from serum proteins at low temperatures.[6] At a pH of 7.35, there was a decrease in the percentage of free calcium at 37 C. from that found at 12 C. Toribara, Terepka and Dewey[15] reported the change in free calcium per degree Centigrade was 0.5 percent. Hopkins *et al.*,[4] however, reported no difference in free calcium between 26 C. and 38 C. with their anaerobic ultrafiltration technique.

The effect of hydrogen ion concentration on calcium-protein binding is just the opposite of temperature. With an increase in pH there is a decrease in free calcium with a corresponding increase in the calcium-protein fraction.[8] Moore[8] described this phenomenon as a competition between hydrogen ions and calcium ions for the binding sites on the protein molecule. With an increase in pH there is an increase in calcium binding to proteins. Between pH 6.8 and 7.8, Moore[8] reported a 16.8 percent (0.42 mM/l) decrease in the ionized calcium fraction of serum. The absolute change in the calcium-protein fraction produced by a pH change would be expected to be in proportion to the

to the level of the existing protein, but the percentage change would be expected to be constant. The decrease in the percentage of free calcium in serum, corresponding to an increase of 1 pH unit, varies with the method used to determine the free calcium. Methods such as the use of the isolated frog heart technique,[7] the calcium electrode,[8] or thermoprecipitation[18] showed decreases of 14.4, 16.8, and 22.8 percent, respectively, per pH unit. Ultrafiltration techniques, however, showed decreases varying from 1.5 to 3.5 percent per pH unit.[15]

At a pH above the physiologic level, calcium salts begin to precipitate out of solution.[6] Normally calcium salts are present in the serum as mono- and di-hydrogen phosphates. Between pH 7.8 and 8.0, calcium phosphates begin to precipitate, and above pH 8.0, other salts of calcium begin to precipitate. The precipitated calcium alters the percentage of free and protein-bound calcium from that which would be found at normal physiological pH levels.

At a pH of 5.1, Hopkins *et al.*[4] using ultrafiltration techniques, found the free calcium to be 97 percent of the total calcium concentration.

Most investigators of calcium binding to proteins have considered only binding to total protein. One of the first reports of calcium binding to the various protein fractions was by McLean and Hastings.[7] Using purified albumin and globulins, they reported the binding of 0.716 milligrams of calcium per gram of globulin and 0.716 milligrams of calcium per gram of albumin. These values are similar to those reported later by Rawson and Sunderman[10] of 0.84 milligrams of calcium per gram of albumin and 0.83 milligrams of calcium per gram of globulin. With the advent of improved methods of separating the globulins, Prasad and Shiraz[9] found that normally 1 mole of albumin bound to 1.23 moles

of calcium, alpha and gamma globulins did not bind to calcium, but some calcium was bound to the beta fraction. Gamma globulin in patients with multiple myeloma binds some calcium.[9] More recently Moore,[8] using the calcium electrode, reported that in normal individuals 81.1 percent of the calcium was bound to albumin and 18.9 percent to the globulins. In cancer patients with hypercalcemia, 66.5 percent of the calcium was bound to albumin and 33.5 percent was bound to the globulins. Moore stated that the change in the binding of calcium in cancer patients might have been the result of excessive parathormone in some of the patients. It is possible that in some diseases there may be molecular change in the protein molecules which alters the protein-binding capacity. This aspect of calcium binding needs further clarification.

Scatchard's question of "how many and how tightly bound" is best answered by applying the law of mass action to the results of calcium-binding studies. Using their classical frog heart technique, McLean and Hastings[7] showed that the binding of calcium to serum proteins obeys the mass action law which they expressed as follows:

$$(1) \quad \frac{[Ca^{++}] \times [Prot^{=}]}{[CaProt]} = K_{CaProt}$$

If we assume that calcium is quantitatively bound to albumin, equation (1) becomes:[7]

$$(2) \quad K_{CaAlb} = \frac{[Ca^{++}] \times [Alb^{-}]}{[CaAlb]}$$

where $[Alb^{-}]$ is the total negative-site concentration available for calcium binding on the albumin molecule, and is equal to:

$$(3) \quad [Alb^{-}] = (n[Alb[- [CaAlb])$$

where [Alb] is the molar albumin concentration and n is the maximum number of calcium binding sites per albumin molecule. Substituting equation (3) into equation (2) we have:

$$(4) \quad K_{CaAlb} = \frac{[Ca^{++}](n[Alb] - [CaAlb])}{[CaAlb]}$$

This equation gives us 2 unknowns, K and n. The serum concentrations of free calcium, albumin, and calcium-albumin can be determined by suitable chemical methods. If the range of the free calcium is sufficiently large, values for K and n can be obtained by rearrangement as follows:

$$(5) \quad \frac{[Alb]}{[CaAlb]} = \frac{1}{n} + \frac{K}{n} \times \frac{1}{[Ca^{++}]}$$

From formula (5) a plot of $\frac{[Alb]}{[CaAlb]}$ against $\frac{1}{[Ca^{++}]}$ should yield a straight line with intercepts on the ordinate of 1/n and a slope of K/n from which both K and n are readily calculated. Formula (5) assumes that successive dissociation sites on the albumin molecule are identical and independent. The protein-binding studies of Scatchard[12] indicated that this assumption is probably not exact, but differences are not detectable at the pH of 7.35 and with a sufficiently large free calcium range. Loken *et al.*[6] by ultracentrifugation have observed that a plot of [Prot]/[CaProt] against $1/[Ca^{++}]$ was linear at a pH of 7.35. Formula (5) is further complicated by the fact that other serum proteins bind calcium.[6,11,12]

Methods of Estimating Free and Bound Calcium in Serum

A variety of methods for the determination of calcium fractions in serum has been developed. The normal values for most of these methods are in disagreement with each other due principally to the various

techniques of controlling the pH, temperature, and ionic strength of the serum during the procedure.

Bioassay, by the use of the isolated frog heart, was one of the first methods used for the determination of calcium ion.[7] In this method the difference in calcium ion concentration in serum samples produced proportional variations in the contractions of isolated frog hearts. From these experiments, McLean and Hastings[7] developed a nomogram for estimating ionized calcium. Serum total calcium and total protein concentrations were determined and with these values the ionized calcium was read from the chart. Their reported normal values for ionized calcium are 1.05 to 1.44 mM/1. The clinical use of this nomogram is not entirely satisfactory. The nomogram was developed with the assumption that the pH of the unknown serum was at 7.35 at 25 C., and had an albumin:globulin ratio of 1:8. Any deviations of the serum from these set values therefore produced erroneous results.

A physicochemical method of determining ionized calcium was developed by Weir and Hastings.[17] They estimated the calcium ion in serum by bringing it into equilibrium with solid calcium carbonate for 5 to 6 hours at a definite carbon dioxide tension, and at 38 C. The calcium ion was calculated from the following formula:

$$[Ca^{++}] = \frac{K_{\text{solubility product}}}{[CO_3]}$$

The $[CO_3]$ concentration was determined from the total carbon dioxide and pH. The solubility product of calcium carbonate was known. Calcium-protein concentrations were calculated by subtracting the calculated calcium ion concentration from the total serum calcium concentration. With this method the authors were able to approximate closely the dissociation constant of serum calcium-albumin obtained by McLean and Hastings.[7]

With the development of the ultracentrifuge, attempts were made to separate free and protein-bound calcium. One of the first investigators used an artificial protein, casein, for the determination of these fractions. With this method estimates of the concentration of free calcium in human serum ranged from 51 to 65 percent of the total calcium concentration.[1]

With the further development of temperature control of the ultracentrifuge, Loken *et al.*[6] were able to control some of the variables affecting calcium-protein relationships. With the control of temperature and the addition of small amounts of a hydrochloric acid solution to control the pH of the serum, they reported values for free and bound calcium which were in close agreement with those of McLean and Hastings.[7]

In using this method, sera are added to special cups and placed into the ultracentrifuge. The samples are centrifuged at a force of approximately 115,000 to 200,000 times gravity for 8 hours. The proteins are thus concentrated in the bottom half of the sample cup with a clear supernate above. Each fraction can then be sampled for calcium content. Ultracentrifugation is usually unavailable in the routine laboratory due to the expense of the equipment, the time required, and the large sample volume required.

Several methods of ultrafiltration have been developed. Many of the early investigators determined the ultrafiltrable calcium of serum using techniques in which there was no control of the carbon dioxide tension. This was considered unimportant at the time, but more recently it has been shown that ultrafiltrable calcium changes with the carbon dioxide tension.[5] Rose[11] was one of the first to attempt to control the carbon dioxide tension, and his method has been used by many other investigators.

In principle, all ultrafiltration procedures utilize some type of membrane of small pore size to prohibit the passage of proteins and some means of supplying filtration pressure to force fluid and smaller molecules through the membrane. Toribara *et al.*[15] used centrifugal force as a means of supplying filtration pressure, while others used direct pressure by a column of mercury.[5,10] A determination of the calcium content of the ultrafiltrate, being protein free, was equivalent to the free calcium concentration in the serum.

Equilibration of free calcium in serum with dry dextran gel has also been used to estimate the concentration of free calcium. Dry dextran gel added to serum will take up water and solutes while excluding proteins and polypeptides of certain size. The intra-gel solution may be regarded as an ultrafiltrate or dialysate of serum. The concentration of unbound calcium in the gel, multiplied by an appropriate Donnan correction factor, equals the concentration of the unbound or free calcium in the original serum. The normal range of free calcium by this technique is 49.7 to 57.8 percent of the total calcium.[13]

The most accurate method to date of determining ionized calcium is with the calcium-ion selective electrode. This instrument operates on the same principle as the pH electrode. In place of the glass electrode, there is a liquid ion exchanger which has a high specificity for calcium ions. A stable potential is obtained between the ion exchanger and a silver-silver chloride reference electrode. When serum is added to the electrode, a change in potential is recorded as the ionized calcium moves toward the liquid ion exchanger. Bound calcium and complexed calcium salts are not recorded. The ionized calcium fraction in normal individuals as reported by Moore[8] is 1.14 mM/l., or 46 percent of the total serum calcium. This value is much

lower than other methods give as the instrument does not record the complexed calcium fraction, which is about 0.35 mM/l. If this value were subtracted from the free calcium concentration obtained by some of the other methods, the two would be in close approximation.

White[18] has recently described a method for the determination of serum protein-bound and free calcium using thermoprecipitation. The method consists of adjusting the pH of the serum sample to 7.35 to 7.45 by passing a mixture of carbon dioxide and oxygen through it. Upon reaching the desired pH, the serum is added to a test tube, sealed with a stopper, and incubated at 100 C. for 3 to 4 minutes. The heat denatures the proteins and a solid mass of coagulum is formed in the tube. Immediately after the incubation period the tube is removed from the heat source and the coagulum agitated with a stirring rod. While still hot, the tube is centrifuged at 3400 rpm for 10 minutes, forming a clear supernate on top of the coagulum. The supernate is virtually protein-free and the calcium content is very nearly equivalent to the free calcium of the serum. The mean free calcium value by this method was found to be 60 percent of the total calcium with a standard deviation of 3.8 percent. This value closely parallels the results of ultrafiltration techniques.

Critique of the Thermal Precipitation Method of White

The measurements obtained by White showed good general agreement with the results obtained electrometrically by Moore.[8] Furthermore, a series of measurements performed by Smith (1971) with this method compared closely with similar published data using more complex methods. It was surprising, therefore, that analysis of the effect of increasing concentrations of calcium to obtain an estimate of the number of binding

sites always yielded a negative value. At the beginning of the series of investigations reported here, several alternative explanations of this phenomenon were considered. Since the chemical method of estimating serum calcium may possibly include variable amounts of magnesium, this was considered as a possible source of error. A second possible error was considered as arising from the failure of these simple methods to distinguish between ionized calcium and un-ionized protein-free calcium bound to various organic anions. However, both of these potential explanations of our apparently anomalous results may be ruled out by detailed mathematical analysis of combined mass-action effects.

Examination of the supernatant fluid obtained after agitating and centrifugation revealed that the pH had risen above 8.0 (the upper limit of the expanded scale pH meter used in this study). Thus even though White's procedure involves restoration of carbon dioxide content and stoppering the tubes while heating, enough carbon dioxide must be lost to allow the pH to rise excessively. That the anomalous results were caused by precipitation of calcium phosphate during heating was confirmed by analysis of acid-treated coagulum. Loken *et al.*[6] have noted that calcium phosphate begins to precipitate out of the serum between pH 7.8 and 8.0 and, above pH 8.0, other calcium salts also precipitate.

MATERIALS AND METHODS

All pooled serum samples were obtained from hospital patients who had fasted overnight. Samples were centrifuged twice to obtain clear serum, and frozen until used. Freezing does not alter the calcium fractions in human serum.[8]

Calcium Addition Studies

Into a series of test tubes, increasing amounts of a calcium chloride solution (300 mg. Ca./dl.) were added (see Appendix for preparation). The addition of 0.02 ml. of this solution raised the concentration of calcium in 6 ml. of serum to 1.0 mg./dl. The volume of the calcium chloride solution was not sufficiently large to alter the concentration of the proteins to any great extent, nor to change the effect of the protein concentration on the binding of calcium.

An amount of 2N hydrochloric acid, sufficient to regulate the supernate pH between 6.8 and 7.8 (see Table 1) was added to 100 ml. of pooled serum and thoroughly mixed. Six milliliters of the adjusted pooled serum were added to each of the previously prepared calcium chloride tubes and mixed. Approximately 2.5 ml. of this serum mixture were separated into another test tube and later used for the determination of albumin and total calcium concentrations. The samples were then stoppered and placed into a Tempblock Heater at 100 C. for 6 minutes. This temperature was adequate to denature the proteins and form a solid coagulum in the test tube. After the incubation period,

the tubes were removed and allowed to reach room temperature. The tubes were then agitated thoroughly with a stirring rod, stoppered, and centrifuged at 3000 rpm for 15 minutes. This produced a clear, protein-free, supernate fluid on top of the packed coagulum; the calcium content of the supernate is regarded here as the concentration of calcium bound to protein.

Since approximately 80 percent of the calcium-protein fraction is bound to albumin,[8] the calcium-protein value multiplied by 0.80 gives the value [CaAlb] used in Formula 5 in the introduction. Albumin values were converted to moles per liter by assuming that the molecular weight of albumin is 69,000.

Albumin Determination

The concentrations of albumin in supernate fluid and in serum were measured with AlbuStrate reagent.* This method makes use of the ability of albumin to bind bromcresol green specifically. Since globulins do not react with this dye, albumin can be measured directly in the serum without prior salt fractionation or electrophoretic separation.

Method

Into 12 x 75 mm. cuvettes, 1.0 ml. of the dye solution and 4.0 ml. of distilled water were added. Using disposable micro-pipettes, 0.01 ml. of serum was added to each tube, except the reagent blank, and mixed well. Monitrol I, a commercial standard, was used as a control. After 2 minutes, the transmittance of each tube was read against the reagent blank at 630 nm. The concentration in each sample was obtained

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*General Diagnostics, Division Warner-Lampert, Morris Plains, N.J.

from a calibration curve previously prepared using Monitrol I and Monitrol II. All reagents are listed in the Appendix.

Calcium Determination

Using an Oxford Titrator,* the concentrations of calcium in serum and in supernate fractions were determined by ethylenediaminetetraacetate (EDTA) titration using trishydroxybisazo dye as the indicator. The end point was indicated by a sharp color change from red to blue at a pH of 12. The dye forms a red chelate with calcium. Magnesium does not interfere with the end point determination since magnesium ions are precipitated at this pH.

Method

The titrant reservoir was filled with the EDTA solution. Three drops of calcium diluent were placed into a disposable titration cup, followed by 0.05 ml. of serum. A 10 mg./dl. standard and Monitrol I were titrated with each run. After adding 1 drop of the calcium indicator, the cups were placed on the titrator turntable to agitate briefly. One drop of potassium hydroxide was added to the cup, the titrant reservoir lowered just below the surface of the solution in the cup, and the turntable and lamp were turned on. The calibrated dial was turned slowly, discharging the EDTA, until the red color in the cup changed to blue. The blue color was the end point of the titration and the dial reading was recorded. The concentration of calcium was calculated as follows:

$$\frac{\text{Dial units to titrate unknown}}{\text{Dial units to titrate standard}} \times \text{value of standard}$$

*Oxford Reagents, 107 North Bayshore Blvd., San Mateo, Calif.

The reagents are described in the Appendix.

For all studies the hydrogen ion concentrations were determined with a blood gas pH meter.*

Experiment I: Determination of the Amount of 2N HCl Required to Obtain a Supernate pH of 6.8 to 7.8

From a pooled serum sample, 3.5 ml. samples were added to separate test tubes. Various amounts of 2N HCl (from 0.01 to 0.05 ml.) were added to each serum. The tubes were stoppered and a supernate fluid obtained from each as described above. Serum calcium, supernate calcium, and pH were determined on each tube. This experiment also served to investigate the pH dependency of the calcium fractions.

Experiment II: Determination of the Maximum Number of Binding Sites for Calcium on the Albumin Molecule, Dissociation Constant, and pK Value

Pooled serum was used in this experiment. The procedure was the same as described for calcium addition studies above. Serum dilutions with sodium chloride were not recorded in the experiment, since the diluted coagula were not firm and the supernates were cloudy.

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*Instrumentation Laboratory, Inc., 9 Galen St., Watertown, Mass.

RESULTS

The results are presented in tabular and graphic form. Table 1 and Figures 1 and 2 relate to Experiment I. Table 2 and Figures 3, 4 and 5 relate to Experiment II.

Table 1. Effects of acid addition to 3.5 ml. pooled serum on free calcium and supernate pH (Experiment I)

ml. of 2N HCl	Free Calcium mM/l.	Supernate pH
0.010	1.26	8.00
0.015	1.46	8.00
0.020	1.48	7.83
0.025	1.56	7.75
0.030	1.61	7.62
0.035	1.69	7.36
0.040	1.85	7.15
0.050	1.89	6.82

Pooled serum concentrations: total protein 6.9 gm./dl.; total calcium 2.47 mM/l.

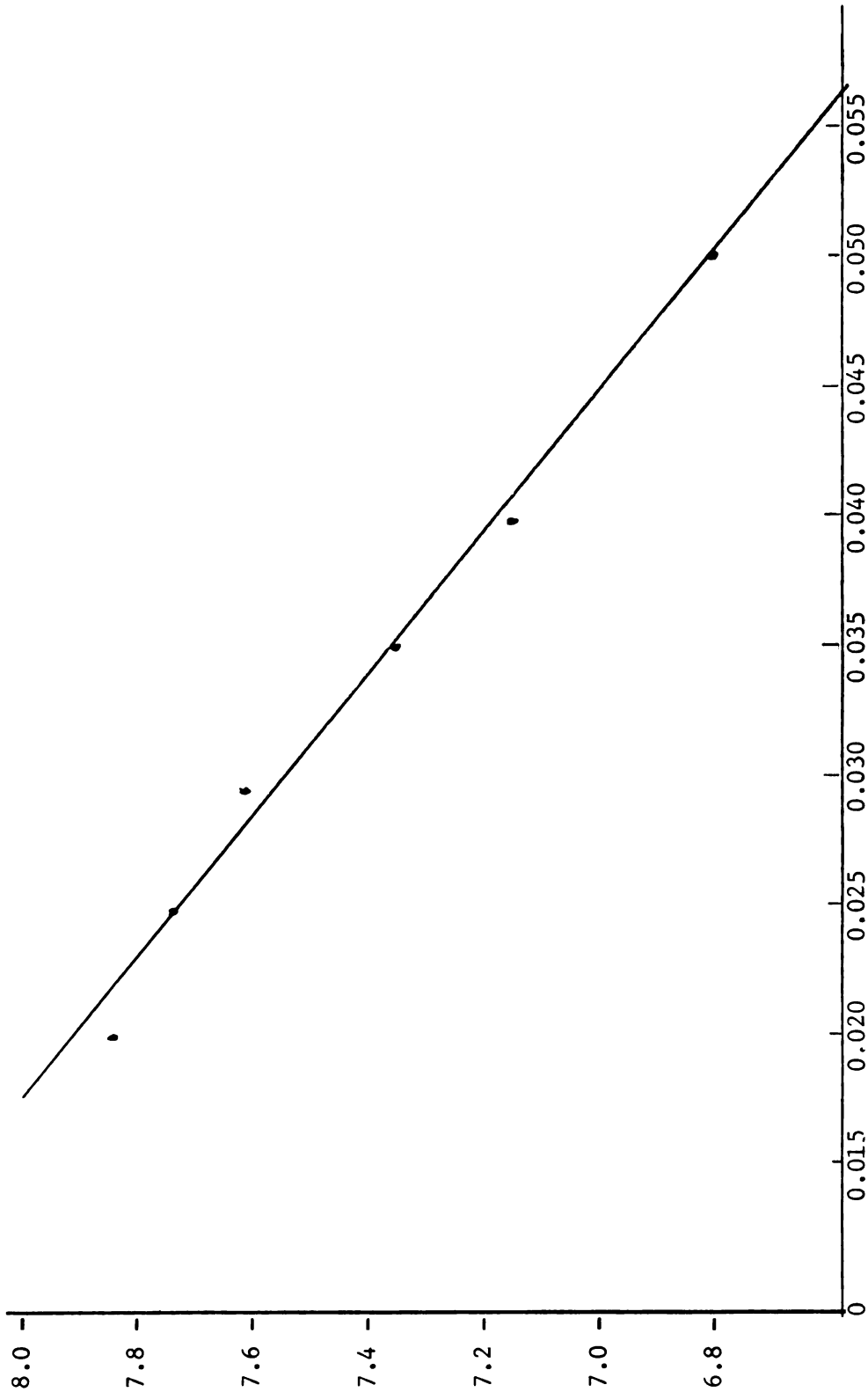


Figure 1. Effect of acid addition on the supernate pH of pooled sera (Experiment I).

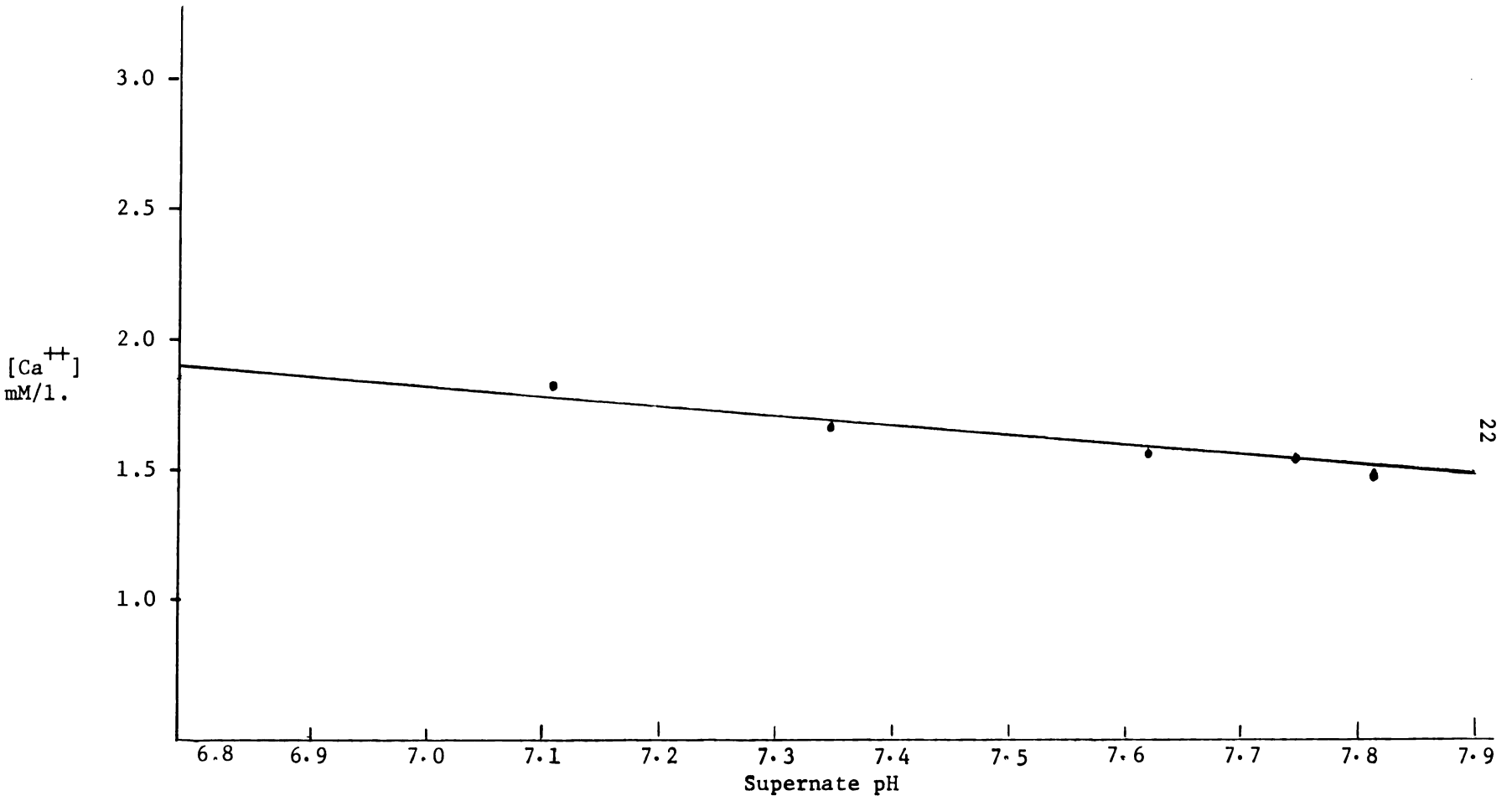


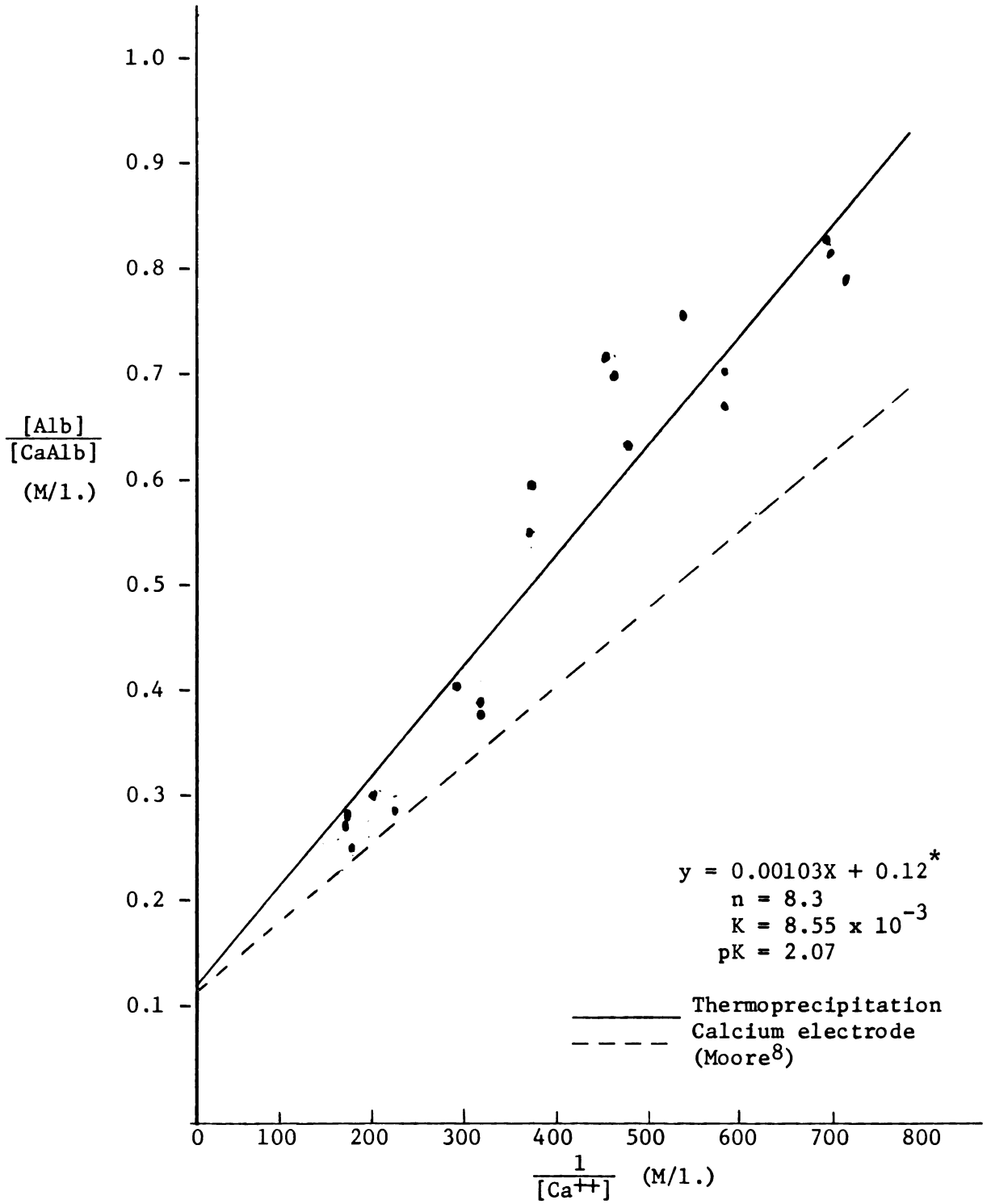
Figure 2. Effects of supernate pH on serum free calcium concentration (Experiment I).

Table 2. Results of calcium additions to 3 pooled sera (Experiment II)

Total Calcium mM/l.	Free Calcium mM/l.	[CaProt] mM/l.	[CaAlb] M/l. x 10^{-4}	$\frac{1}{[Ca^{++}]}$ M/l.	$\frac{[Alb]}{[CaAlb]}$ M/l.
2.43	1.53	0.90	7.2	653	0.75
2.68	1.82	0.86	6.9	549	0.78
3.05	2.12	0.93	7.4	472	0.73
3.68	2.56	1.12	8.9	391	0.60
4.93	3.10	1.83	14.6	322	0.37
6.18	3.84	2.34	18.7	260	0.29
7.43	5.03	2.40	19.2	199	0.28
2.47	1.48	0.99	7.9	676	0.68
2.72	1.71	1.01	8.1	585	0.67
3.10	2.06	1.04	8.4	488	0.64
3.72	2.56	1.16	9.4	392	0.57
4.97	3.33	1.64	13.1	300	0.41
6.22	4.65	1.57	12.6	215	0.43
7.47	5.20	2.27	18.2	192	0.30
2.43	1.55	0.88	7.0	645	0.77
2.68	1.71	0.97	7.8	585	0.70
3.05	2.15	0.90	7.2	465	0.75
3.68	2.56	1.12	9.0	391	0.60
4.93	3.09	1.84	14.7	324	0.37
6.18	4.16	2.02	16.2	240	0.33
7.43	5.16	2.27	18.2	194	0.30

Albumin concentration for all sera was 3.75 gm./dl.

Supernatant pH of all samples was between 7.63 and 7.72.



* = formula for the line
n = number of binding sites per mole of albumin
k = dissociation constant
pK = negative logarithm of dissociation constant

Figure 3. Results of calcium addition studies on normal sera (3 studies, Experiment II, with Moore's data superimposed).

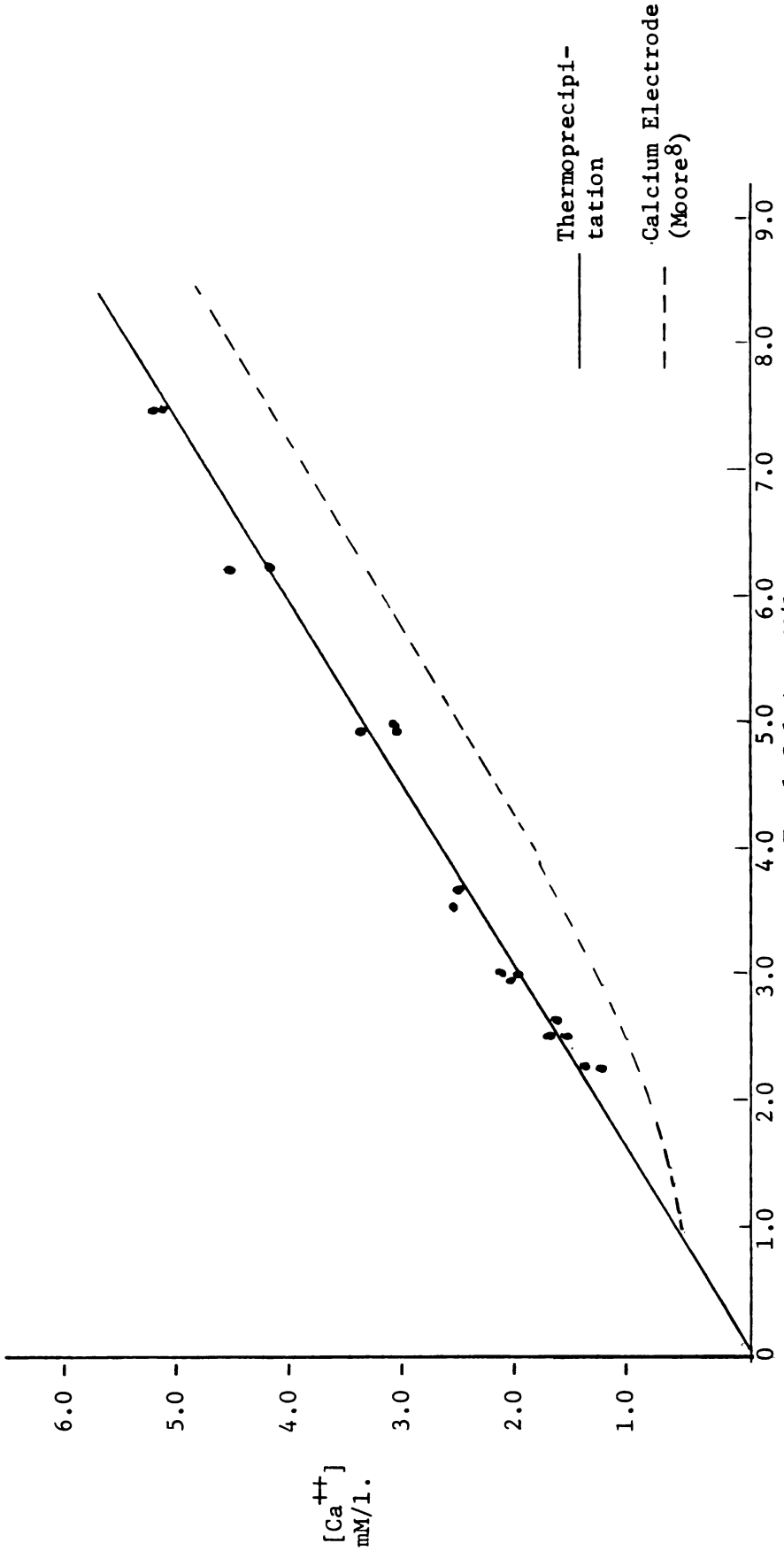


Figure 4. Relationship between free and total calcium in pooled sera (Experiment II, with Moore's data superimposed).

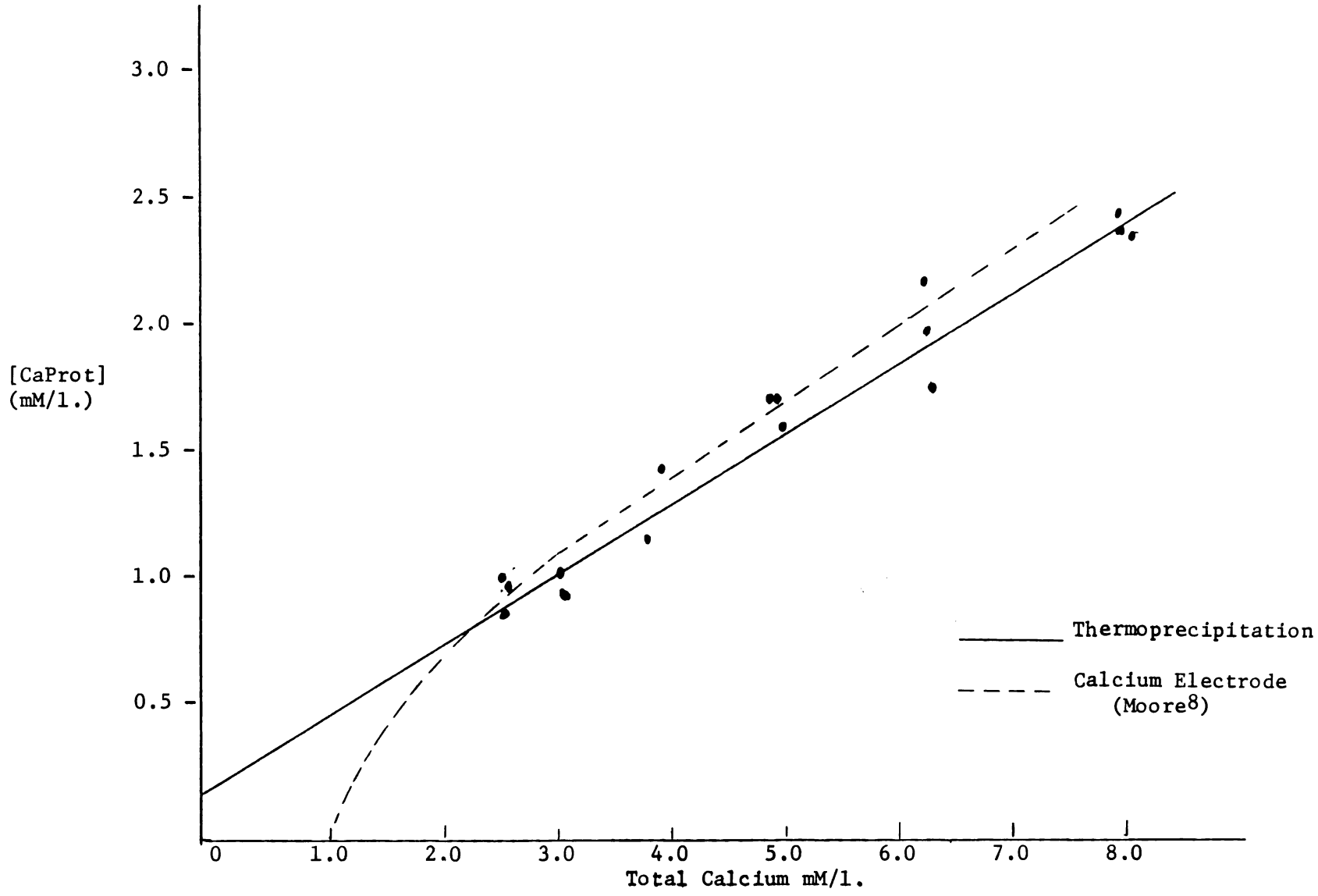


Figure 5. Relationship between calcium-protein and total calcium concentrations in pooled sera (Experiment II, with Moore's data superimposed).

DISCUSSION

One of the most important factors in quantitating calcium fractions in serum is the control of the pH during their determination. Loken[6] found that at a pH above 7.8, calcium phosphates began to precipitate. Stored serum loses its carbon dioxide content and thus becomes alkaline. The method of thermoprecipitation, introduced by White,[18] attempted to correct for this variable by restoration of the lost carbon dioxide content. As it was found that this method produced unstable pH values, especially when the specimen was heated, an attempt was made to stabilize the pH of the serum between 6.8 and 7.8, where a straight line relationship exists between pH and free calcium.[6]

From Figure 1 it was found that between 0.025 and 0.050 ml. of 2N hydrochloric acid was sufficient to regulate the supernate pH between 6.8 and 7.8. Above and below this pH range, the straight line relationship between free calcium and pH does not exist.

The acid-adjusted serum remained relatively stable over a period of 4 hours, with a pH increase of less than 0.2. The acid addition proved to be an improvement over pH adjustment with carbon dioxide in which the pH increased 0.2 units within a period of 2 to 3 minutes. Heating the acid-adjusted sera did produce small increases in the supernate pH, which was probably due to small amounts of residual carbon dioxide being expelled from the serum. The slight increase in pH was considered insignificant so long as the supernate pH did not rise above 7.8.

Dependency of the supernate pH on free calcium concentration was established. A change in 1 pH unit (6.8 to 7.8) resulted in a decrease of 0.35 mM/l. of free calcium. This value is almost identical with that found with the isolated frog-heart technique[7] and only 0.07 mM/l. less than the value reported using electrometric techniques.[8] The percentage of free calcium in this study was found to be within 4 percent of the reported values obtained with other techniques (see Table 3).

In the first experiment, the free calcium ranged from 76.9 to 62.3 percent of the total calcium in the pH range of 6.8 to 7.8. The slope of the distribution curve was found to be practically linear. Therefore sera for free calcium determinations need only be adjusted to obtain a supernate pH between 6.8 and 7.8. The percentage of free calcium at pH 7.35 can be calculated by adding 14.6 times the difference in observed pH and 7.35.

To determine if this revised thermoprecipitation method produced calcium-protein relationships similar to those reported by other techniques, calcium addition studies were performed. The results of this experiment are expressed graphically in Figure 3, and comparisons with other methods can be made in Table 3.

Figure 3 compares data of calcium addition studies using the calcium electrode and the thermoprecipitation methods. There is an increasing spread between the two values as the reciprocal of the free calcium increases. The increasing difference can be explained from the observation by Moore[8] that, as the amount of calcium chloride increases in each tube, the fraction of the complexed form also increases. As free calcium is a combination of ionized and complexed calcium, the difference in the two curves could be explained by the increase in the

Table 3. Comparison of calcium-protein relationships as determined by various methods

Author	Method	$K_{CaProt.} \times 10^{-3}$	pK	Maximum Binding Sites	Change in Ca per pH Unit (mM/l.)
McLean and Hastings[7]	Isolated frog heart	9.47	2.03	N.D.*	0.36 (14.4%)
Moore[8]	Calcium electrode	6.55	2.18	8.4	0.42 (16.8%)
Loken[6]	Ultra-filtrate	6.55	2.18	8.0	0.46 (18.5%)
Greenberg and Larson[3]	Ultra-filtrate	3.63	2.44	N.D.*	N.D.*
Weir and Hastings[17]	Calcium carbonate solubility	7.75	2.11	N.D.*	N.D.*
White[18]	Thermo-precipitation	N.D.*	N.D.*	N.D.*	0.57 (22.8%)
Present Study	Thermo-precipitation	8.55	2.07	8.3	0.35 (14.6%)

*N.D. = not determined.

complexed calcium. The calcium electrode eliminates this difficulty by measuring only the ionized form.

A comparison of free calcium and ionized calcium can also be seen in Figure 4. Values of free calcium were plotted against their respective total serum calcium values. Ionized calcium values, taken from a graph of the results with the calcium electrode, are also shown.[8] The calcium electrode recorded no ionized calcium at a total calcium of 1.0 mM/l. This was explained by the author that at this low concentration of total calcium and albumin, accurate measurements of the calcium and protein fractions were not possible. In the present study, serum dilutions were not analyzed due to these difficulties and to the presence of turbidity in the supernate and incomplete separation of the calcium fractions. Although differences did occur between the two methods, it is important to note the constant relationship of the two values at most total calcium concentrations, except where dilutions of the serum affect the accuracy of the measurements as determined with the calcium electrode.

A similar relationship exists in Figure 5, representing a plot of total calcium against its corresponding calcium-protein concentration. In this instance, the results of the thermoprecipitation study were somewhat lower than those values obtained with the calcium electrode. The difference could be explained by the fact that the protein values of the two separate studies were not identical. Again, there was a constant relationship between the two values except where dilution of the serum introduced error in the electrode method. A study needs yet to be done to determine the normal values of the calcium fractions with this method. When this is done, it should

be a simple task to correlate the free calcium values with ionized calcium concentration.

SUMMARY AND CONCLUSIONS

A revised method has been presented for the separation of free calcium and calcium-protein concentrations in human sera. The addition of small amounts of 2N hydrochloric acid corrects for the pH changes found in the original procedure introduced by White.[18] The stabilized pH prevents the precipitation of calcium phosphates which produced the anomalous results found in the original procedure.

From the calcium addition studies, the derived 8.3 binding sites on the albumin molecule, dissociation constant of 8.55×10^{-3}, and pK value of 2.07 were in close agreement with the results obtained using other methods. Thermoprecipitation might therefore prove to be a useful technique in studying the effects of calcium binding *in vitro*.

Although the values for the free calcium in the supernate did not exactly match the values of ionized calcium obtained with the calcium electrode, parallel relationships were found to exist, which indicates that a conversion factor could be obtained to convert the free calcium values to ionized calcium concentration.

The thermoprecipitation technique offers the advantage of requiring inexpensive equipment, short completion time, and calcium-protein relationships which closely approximate values obtained with other more expensive and cumbersome methods.

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APPENDIX

APPENDIX

Reagents for Calcium Addition Studies:

1. Hydrochloric acid, 2N. Dilute 16.8 ml. concentrated hydrochloric acid to 100 ml. with distilled water.

2. Calcium solution, 300 mg./dl. Dilute 1.1005 gm. calcium chloride dihydrate to 100 ml. with distilled water.

Reagents for Serum Albumin:

1. AlbuStrate reagents:* Contains a dye (bromcresol green), buffer, surface active agent, and preservative.

Reagents for Serum and Supernate Calcium:**

1. Calcium titrant. Disodium EDTA, approximately 25 x 10^{-5} molar.

2. Calcium diluent. Deionized water.

3. Calcium indicator. Calchrome 10-B stable aqueous solution.

4. Potassium hydroxide. Aqueous solution approximately 6.25 N.

5. Calcium standard. Synthetic serum standard, 10 mg./dl.

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**Oxford Reagents, 107 North Bayshore Blvd., San Mateo, California.

VITA

The author was born in Grayville, Illinois, on December 27, 1929. He graduated from Grayville High School in 1947. After attending four quarters at Southern Illinois University, he enlisted in the U.S. Navy in 1950. After being discharged in 1954, he attended the University of Evansville and received his B.S. in Medical Technology in June, 1957. He passed his qualifying examination and was certified as a medical technologist by the American Society of Clinical Pathologists in 1957. After working approximately 12 years in various clinical laboratories, he enrolled in a program of Graduate School in Clinical Laboratory Science in the Department of Pathology, Michigan State University, in June, 1970. He is married and has three children.