SOME PROPERTIES OF CALCIUM-CONTAINING THERMOPRECIPITATED FRACTIONS OF HUMAN BLOOD SERUM

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ABSTRACT

SOME PROPERTIES OF CALCIUM-CONTAINING THERMOPRECIPITATED FRACTIONS OF HUMAN BLOOD SERUM

By

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Heat coagulation of human serum was employed to produce thermoprecipitable and nonprecipitable serum calcium fractions. Studies were undertaken to determine if the fractions produced had properties similar to those of the serum calcium fractions quantitated by other methods.

Changes in total protein, total calcium and hydrogen ion concentration as determined by the heat coagulation method employed were similar to those obtained by other methods.

The determination of serum calcium fractions by thermoprecipitation is rapid and requires no special reagents or equipment.

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Fred J. White

A THESIS

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To

Jo Ellen, Chris, and Gretchen

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INTRODUCTION

Calcium is one of the most precisely controlled constituents of serum; the normal diurnal fluctuations are within the range of 1.5 to 10%. Calcium plays an important role in formation of bones and teeth, regulation of membrane permeability, maintenance of cardiac rhythm (excess calcium increases contractility and prolongs systole of the cardiac muscle, while relative lack of calcium decreases contractility), regulation of neuromuscular excitability (decrease of calcium ions increases the excitability of both muscles and nerves and, if sufficiently marked, results in tetany), development of the fetus, lactation, and maintenance of enzyme activity (blood coagulation enzymes and muscular contraction).

Absorption and Distribution

Under ordinary conditions, daily absorption of calcium is matched by daily losses. Active transport out of the upper small intestine is aided by a high gastrointestinal calcium content, a low pH (to solubilize calcium complexes), vitamin D (which is essential) and, possibly, parathyroid hormone. Loss occurs through the gastrointestinal and urinary tracts.

Red blood cells contain almost no calcium, and therefore values presented refer to concentration in serum or plasma. Calcium takes 3 forms in the blood. The first is protein bound; roughly 40% of the total serum calcium is bound to serum proteins, 80% of this to albumin and 20%

to globulins. This fraction is not diffusible through the capillary membrane. The second form (5 to 15% of total serum calcium) is in complexes with anions such as phosphate, citrate and sulfate. These complexes are diffusible through the capillary membrane, although they are not ionized. The third form is free, ionized calcium; it represents approximately half of the calcium present in the blood[2] (see Figure 6).

The fine adjustments in serum calcium are made by the hormones, calcitonin and parathormone. Calcitonin is produced in the parafollicular "c" cells of the thyroid and is released in response to an increased serum calcium. It acts rapidly to lower serum calcium by promoting its deposition in bone and, perhaps, by inhibiting bone resorption. It may also diminish renal phosphate excretion.[5]

Parathormone, on the other hand, is secreted by the chief cells of the parathyroid glands. Stimulus to its production is hypocalcemia. Parathormone acts to raise serum calcium by mobilizing calcium from the bone mass (balances defective absorption or excess excretion), increases urinary excretion of phosphate by direct effect on the renal tubules, and increases absorption of calcium from the gut.[5]

These 2 hormones share with vitamin D in control of ionized serum calcium level. Vitamin D directly affects the ionized fraction by increasing intestinal absorption. Blood pH also influences the ionized portion of serum calcium. Changes in hydrogen ion concentration effect a change in the charge carried by serum proteins. As the charge is modified by hydrogen ions, albumin binds more of the free calcium ions or releases them to the free form.[2,5]

Need to Quantitate Serum Ionized Calcium

The reason for attempting to determine the ionized fraction is that it represents the portion of serum calcium that is responsible for the element's various physiologic functions. It is in a state of interaction with bone calcium and plasma proteins. Measurement of total serum calcium is of limited value in determining the most important aspect of serum calcium. Diseases that raise total protein concentration will cause a rise in total serum calcium, while a decreased total calcium is seen with a low total protein concentration. Total calcium is directly related to protein-bound calcium but not to ionized calcium. Also, in disturbances marked by changes in blood hydrogen ion concentration, total calcium will not change; the proportions of free and bound calcium will change, however.

Indications for the determination of free and bound calcium include: any clinical or radiographic evidence of bone disease, convulsions, investigation of renal calculi, and cardiac dysrhythmias.

Methods for Determining Ionized Calcium

Bioassay methods for determining ionized calcium were among the first. McLean and Hastings performed calcium ion determinations by observing the action of serum samples on the contractibility of the isolated frog heart.[7] The calcium ion concentration has also been determined by its *in vitro* effect on calcification of vitamin D-deficient rat cartilage.[6]

Since the calcium fractions in the blood are in equilibrium, attempts to separate one form for analysis tend to shift the concentrations to new equilibrium levels. Simple dialysis removes all of the serum calcium by upsetting the equilibrium. However, compensation dialysis may be employed in which serum is dialyzed against a number of solutions of different calcium content. The solution in which no change of calcium

concentration occurs contains calcium ions at the same level as the serum. With this method about 60% of the calcium of human serum was found to be diffusible. Calculations based on the composition of cerebrospinal fluid, edema fluid, or ascitic or pleural effusions indicate that the diffusible calcium of normal serum is about 1.25 mM/l.[7]

Ion-exchange methods have been developed that yield estimates of calcium-ion concentration comparable to those obtained in the compensation dialysis methods. One ion-exchange-resin system yields values of 63% ionized calcium.[4] By ion-exchange chromatography, values of 53.7% ionized calcium are reported.[12] These methods involve the determination of calcium before and after equilibration with either an ion-exchange strip or dry dextran gel.

Separation of the serum-calcium fractions has been attempted by ultracentrifugation, pressure ultrafiltration, and ultrafiltration by centrifugation. These various attempts have accounted for the wide range of values for ultrafiltrable or ionized calcium in the literature. Until recently there was no provision made for control of pH during the process of separating serum water from proteins. In 1968, G. A. Rose developed a method of pressure ultrafiltration calling for flooding the chambers and tubes of the apparatus with a 5% carbon dioxide and 95% oxygen gas mixture in order to maintain normal serum pH. This same gas mixture is needed for the ion-exchange methods as well. The Rose technique involves no less than 10 ml. of venous blood and a total of 4 to 5 hours to complete. This method yields an average of 56.5% ionized calcium.[11]

Ionized calcium may be calculated from the nomogram based on the work of McLean and Hastings. Total serum calcium and total protein are determined and the ionized value read from the chart.

The nomogram is based on the relationships between calcium and proteins at a pH of 7.35 at 25 C. Its use is questionable in cases where the serum pH differs from 7.35 or where the albumin:globulin ratio differs greatly from 1:8. The problem is further complicated by the observation that phospholipids bind calcium in a manner similar to proteins and may be responsible for 30 to 40% of the bound calcium in serum.[6,13]

The most accurate method available uses the calcium ion selective electrode.

> "The calcium electrode functions similarly to the conventional pH electrode. The pH electrode employes a semipermeable glass membrane through which only hydrogen ions will diffuse and cause a change in voltage. Calcium electrodes also depend on a voltage potential change but accomplish this with a liquid ion exchanger (a calcium salt of an organophosphoric acid) which has a high specificity for ionized calcium. Another solution, calcium chloride, is used; this is separated from the liquid ion exchanger by a rigid porous membrane disk. Here the calcium creates a stable potential between it and the inner aspect of the membrane. Chloride stabilizes the potential between the calcium chloride and a silver-silver chloride reference electrode. In this situation, then, there is a stable potential, but when serum ionized calcium is added, a change in potential is recorded as the ionized calcium moves toward the liquid ion exchanger-impregnated disk. Bound calcium as well as complexing agents, such as citrates, and polyphosphates are not recorded. Specificity and sensitivity are excellent since the electrode responds only to the activity of ionized calcium."[1]

Values obtained by this instrument are much lower than others reported. Changed calcium normals as determined by W. E. Moore are 1.14 mM/l or 46% of total serum calcium[2] (Figure 6).

Although the calcium electrode is a rapid method for determining ionized calcium, there are added variables due to its basic working principle. The electrode is subject to change in its calibration curve. It requires frequent recalibration to insure accurate readings. The calibrating standards contain the electrolyte composition of normal human

serum in addition to different concentrations of calcium. In the case of a serum with abnormal electrolyte concentrations, a correction must be determined based on the change in calcium-ion activity coefficient.

Like the ultrafiltration technique it has the added variable of temperature dependency. No electrode available to date is thermostated. The electrode also is costly and requires skill to operate and maintain.

Because of the importance of the serum-calcium fractions in maintenance of normal functions, their accurate determination is desirable clinically. The current methods of quantitation are complex and expensive to the point of being limited, practically, for routine use.

Because of the ease with which a heat precipitate filtrate can be obtained, this study was undertaken to determine if the calcium fractions yielded were related to serum-calcium fractions.

MATERIALS AND METHODS

Distribution of serum calcium into various fractions has been shown to be dependent on changes in albumin, total serum calcium, and hydrogen-ion concentrations. In order to determine if the thermoprecipitation product under study is a useful estimate of important serum-calcium fractions, the responses of this fraction to pH and total calcium were compared to those obtained with the calcium electrode.

Both individual and pooled venous blood samples were obtained from hospitalized patients. For all studies hydrogen ion concentration determinations were made with the Radiometer pH meter at 37 C. Albumin determinations were made by the dye binding method of American Monitor (see Appendix). Total serum calcium and thermoprecipitated supernatant calcium determinations were performed by the automated procedure of Kessler and Wolfman (Technicon method N-3b).

In this method samples are mixed with 1 Normal hydrochloric acid to release the protein-bound calcium. These mixtures are dialyzed into a recipient stream of cresolphthalein complexone. Colored complexes between calcium and dye form upon addition of diethylamine. The solutions are warmed to 37 C. before entering the colorimeter. The developed color in the samples is measured at 580 nM (see Appendix).

Preparation of the coagulum's supernate consisted of heating a 3-ml. sample of serum in a 12 x 75-mm. test tube at 100 C. $\pm$ 5 C. for 3 minutes. Heating was carried out in a Tempblock Heater module. The serum became opaque as the proteins coagulated. The product was then mixed with a

stirring rod to break up the solid mass which formed. Then the tube was centrifuged at 3400 rpm for 10 minutes and a clear fluid was taken from the top of the samples for calcium studies.

The supernatant calcium value was considered nonprecipitable or free calcium. This value subtracted from the total serum calcium equalled the value of thermoprecipitable calcium.

Experiment I: Determination of Individual Levels of Total Serum Calcium and the Thermoprecipitation Products

Individual serum samples were employed for this study. All samples were adjusted to a pH range of 7.35 to 7.45. Coagulation supernatants were prepared as described above and the calcium content determined for comparison with the total serum calcium of the specimens.

Experiment II: Investigation of pH Dependency in Heat Precipitable and Nonprecipitable Calcium Fractions

A pooled serum sample was separated into 3-ml. aliquots for separate determinations. Changes in pH in successive tubes were made with a gas mixture of 11% carbion dioxide and 89% oxygen. The gas was bubbled through samples after it had been bubbled through physiological saline. To prevent foaming of serum samples a drop of caprylic alcohol was added before inserting the bubbling tube. The time of bubbling determined how low the pH could be forced. It was varied from 1 minute to 8 minutes for a broad pH range.

After the gas mixture had run, the pH was determined and the tube quickly sealed with parafilm and a stopper. The coagulum supernatant was prepared.

The calcium concentration was then determined in the whole serum sample and in each supernatant product.

Experiment III: Study of the Heat Coagulation Fractions as Related to Changes in Total Calcium Concentration

Aliquots of 15 ml. from a pooled serum sample were prepared. In successive tubes the total calcium of the sample was raised by adding .05 ml. of an 825 mg./dl. solution of calcium chloride. The addition of .05 ml. of the solution raised the total serum calcium by 1.0 mg./100 ml. Multiples of .05 ml. were added to successive tubes to raise their total calcium by multiples of 1.0 mg./dl. The concentrated calcium chloride solution was chosen so that quantities added to successive serum samples would raise total serum calcium without affecting a dilution of the samples, since protein concentration also alters calcium binding. After addition of the calcium chloride each 15-ml. aliquot of serum was divided into 2 3-ml. samples. The remaining serum was used for determination of total calcium and albumin.

The duplicate 3-ml. samples were adjusted with the gas mixture to a pH of 7.36. The tubes were sealed and the supernatant liquid prepared as before.

Samples from the pooled serum were also diluted with .85% sodium chloride solution. Dilutions from 5 parts serum and 1 part saline to 1 part serum and 5 parts saline were prepared. All dilutions were pH-adjusted and heat-precipitate prepared.

RESULTS

Experiment I

Twenty-six individual serum samples were used in the investigation of relation between the heat-precipitated fractions of calcium and total serum calcium. Results are tabulated in Table 1.

Plotting supernatant calcium values against total serum calcium (see Figure 1) revealed no apparent relationship between these values. However, plotting the value for heat-precipitated calcium against total calcium yielded a straight line with a slope of .863 ($r = .917$, see Figure 2). The values obtained with heat-precipitation of individual serum samples compared favorably with relative percentages obtained by other methods (see Table 4).

Experiment II

The investigation of pH dependency involved 30 serum samples and 3 different testing pools. The data are given in Table 2. The average change in supernatant calcium concentration in response to 1 pH unit was .57 mM/l.

A graph of the pH-dependent change (see Figure 3) illustrates the linear or slightly curved decrease in free calcium as serum pH rises.

Experiment III

The data for this experiment appear in Table 3. Figure 4 represents the relationship between the heat-precipitable fraction of calcium and total serum calcium.

Table 1. Concentration of calcium in the heat fractions of individual serum samples

Total Serum Calcium (mM/1)	Super-natant Calcium (mM/1)	Precipi-table Calcium (mM/1)	Albumin (gm./dl.)	pH	% Free
2.5	1.5	1.0	4.0	7.38	60.0
2.45	1.55	.9	3.9	7.36	63.3
2.65	1.45	1.2	3.75	7.44	54.7
2.35	1.5	.85	3.9	7.31	63.8
2.7	1.5	1.2	4.75	7.36	55.5
2.55	1.45	1.1	4.3	7.45	56.9
2.59	1.5	1.09	4.8	7.30	57.9
2.9	1.5	1.4	4.5	7.35	51.7
2.8	1.55	1.25	5.0	7.37	55.4
2.75	1.55	1.2	3.2	7.30	56.4
2.55	1.5	1.05	4.0	7.45	58.8
2.31	1.51	.8	3.5	7.445	65.4
2.29	1.47	.82	3.2	7.310	64.2
2.4	1.43	.97	4.1	7.45	59.6
2.29	1.36	.93	3.9	7.38	59.4
2.48	1.48	1.00	4.1	7.45	59.7
2.29	1.33	.96	3.8	7.36	58.1
2.34	1.5	.84	3.6	7.445	64.1
2.5	1.56	.94	4.2	7.45	62.4
2.4	1.53	.87	3.8	7.40	63.8
2.4	1.62	.78	3.4	7.38	67.5
2.60	1.57	1.03	3.4	7.45	60.4
2.55	1.61	.94	4.0	7.42	63.1
2.55	1.57	.98	4.0	7.41	61.6
2.35	1.5	.85	3.9	7.31	63.8
2.55	1.5	1.05	4.0	7.45	58.8
$\bar{x}$=2.51	$\bar{x}$=1.50	$\bar{x}$=1.00	$\bar{x}$=3.96	$\bar{x}$=7.39	$\bar{x}$=60.2%
SD=.165	SD=.065	SD=.15	SD=.45	SD=.05	SD=3.8%

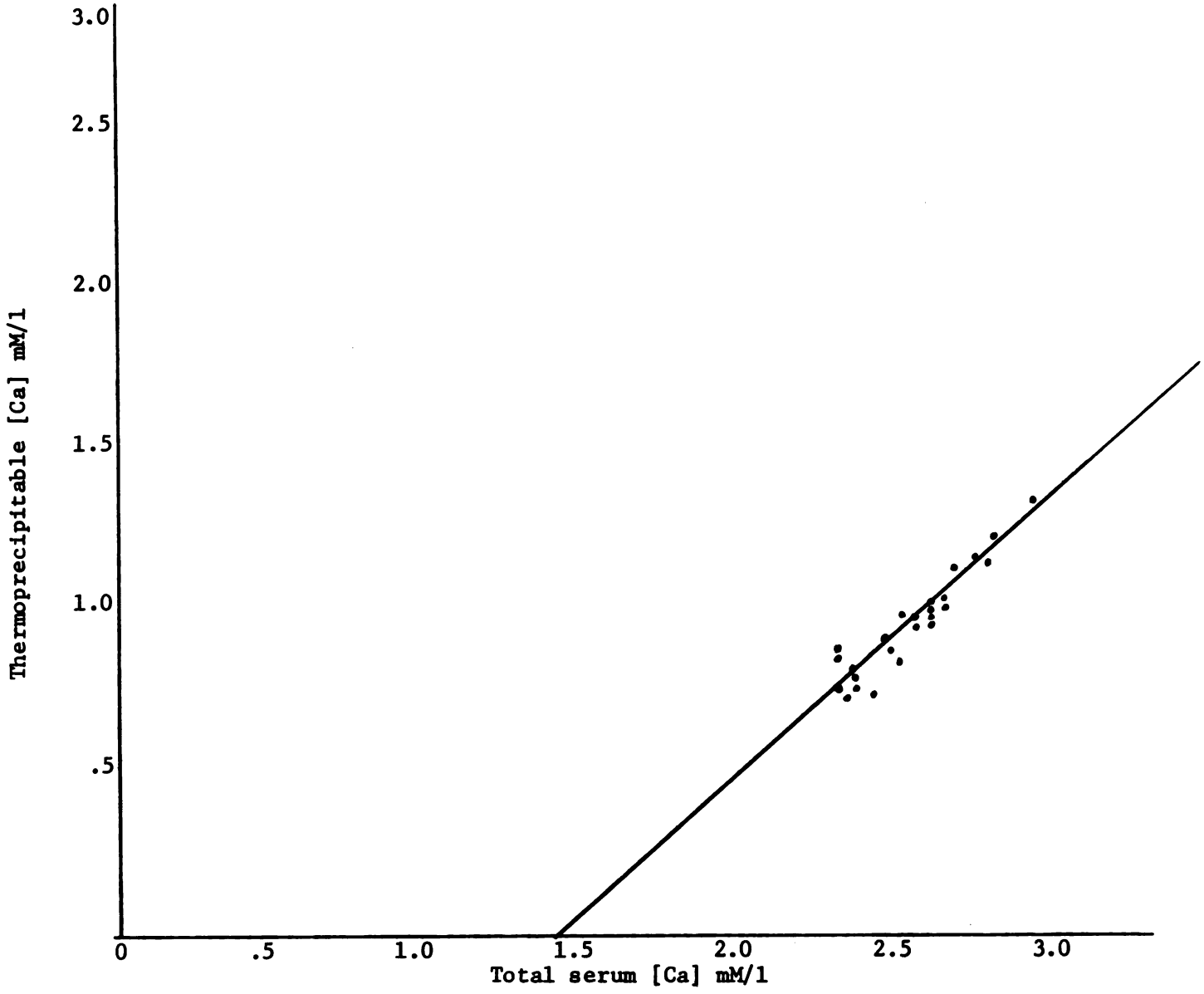

Figure 2. Relation of precipitable calcium to total serum calcium in individual sera.

Table 2. Calcium concentrations in heat fractions of pooled sera resulting from pH variation

pH	Total Serum Calcium (mM/l)	Supernatant Calcium (mM/l)	Precipitable Calcium (mM/l)	% Free
7.945	2.55	1.2	1.35	47
7.865	2.55	1.25	1.3	49
7.83	2.55	1.25	1.3	49
7.65	2.55	1.525	1.025	60
7.535	2.55	1.56	.99	61
7.42	2.55	1.6	.95	63
7.41	2.55	1.575	.975	62
7.38	2.55	1.56	.99	61
7.315	2.55	1.675	.875	66
7.185	2.55	1.56	.99	61
7.13	2.55	1.67	.88	65
8.01	2.4	1.0	1.4	42
7.96	2.4	1.07	1.33	44.5
7.885	2.4	1.25	1.15	52
7.835	2.4	1.07	1.33	44.5
7.745	2.4	1.145	1.255	48
7.66	2.4	1.2	1.2	50
7.52	2.4	1.08	1.32	45
7.42	2.4	1.375	1.025	57
7.37	2.4	1.46	.94	61
7.13	2.4	1.56	.84	65
7.615	2.75	1.525	1.225	55
7.48	2.75	1.72	1.03	63
7.41	2.75	1.56	1.19	57
7.365	2.75	1.77	.98	64
7.265	2.75	1.77	.98	64
7.250	2.75	1.72	1.03	63
7.210	2.75	1.72	1.03	63
7.175	2.75	1.775	.975	65
7.11	2.75	1.775	.975	65

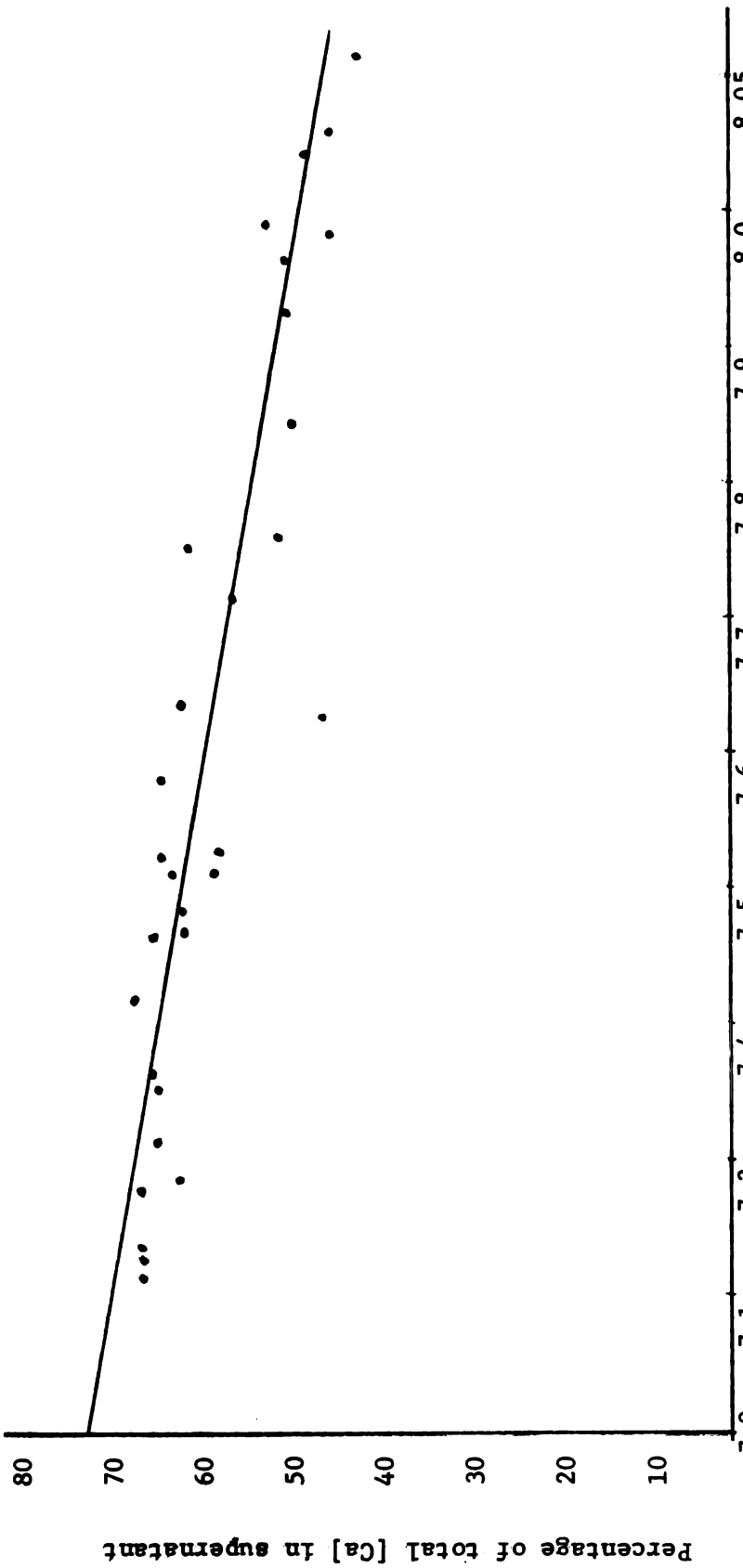

Figure 3. pH dependency of free calcium in pooled sera.

Table 3. Calcium levels in heat fractions after the addition of calcium chloride

Total Serum Calcium (mM/1)	Super-natant Calcium (mM/1)	Precipi-table Calcium (mM/1)	Albumin (gm./dl.)	pH
2.45	1.55	.9	3.9	7.36
3.0	1.95	1.05	3.9	7.36
3.5	1.8	1.7	3.9	7.36
4.0	2.2	1.8	3.9	7.36
4.5	2.35	2.15	3.9	7.36
5.0	2.6	2.38	3.9	7.36
5.5	2.8	2.7	3.9	7.36
6.0	3.35	2.65	3.9	7.36
2.5	1.5	1.0	4.0	7.38
2.06	1.45	.62	3.4	7.38
1.69	1.19	.5	2.8	7.38
1.25	.94	.31	1.9	7.38
.9	.75	.155	1.5	7.38
.49	.45	.046	.7	7.38
2.75	1.625	1.13	4.0	7.38
3.0	1.75	1.25	4.0	7.38
3.25	1.875	1.375	4.0	7.38
3.5	2.1	1.44	4.0	7.38
3.75	2.125	1.6	4.0	7.38
4.0	2.25	1.75	4.0	7.38
4.44	2.52	1.91	4.0	7.38
5.13	2.71	2.42	4.0	7.38
5.37	2.89	2.48	4.0	7.38
6.0	3.28	2.73	4.0	7.38
6.38	3.625	2.75	4.0	7.38
7.13	3.78	3.35	4.0	7.38
7.31	4.23	3.1	4.0	7.38

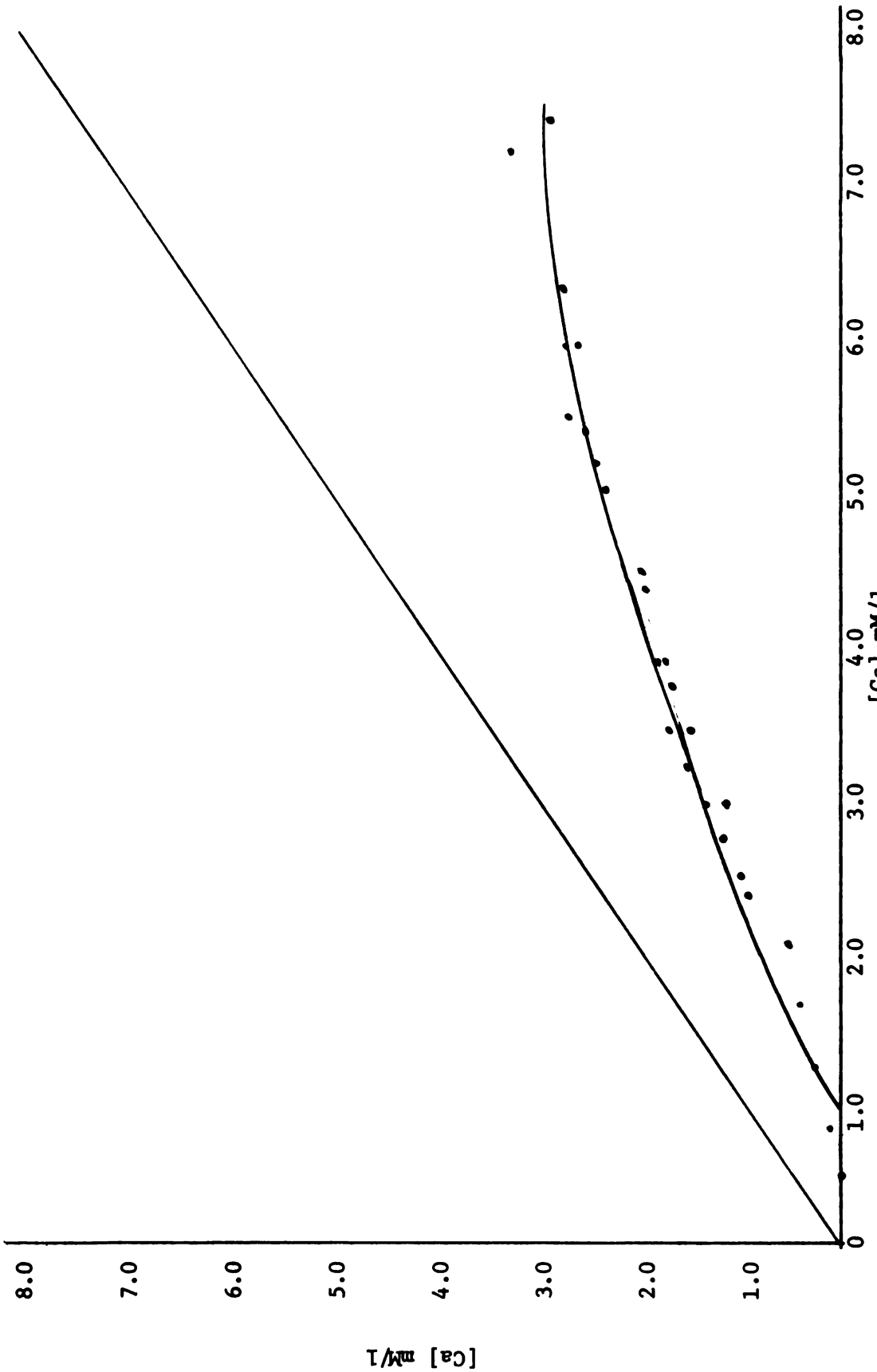

Figure 4. Relationship between total and heat-precipitated calcium with increasing calcium concentrations.

Table 4. Reported average ionized calcium percentages

Ref.	Method	Mean Percentage Ionized
6	McLean and Hastings Formula	53.0
11	Ultrafiltration and Murexide	56.5
9	Calcium Electrode	43.9
10	Ultrafiltration and Calcium Electrode	50.8
3	Ultrafiltration by Centrifugation	57.1
2	Calcium Electrode	46.0
4	Ion-Exchange Resin	63.0
12	Ion-Exchange Chromatography	53.7
3	Other Reported Ultrafiltration Values	51.7
		52.0
		54.5
		58.5
		59.1
		62.1
	Present Study	60.2

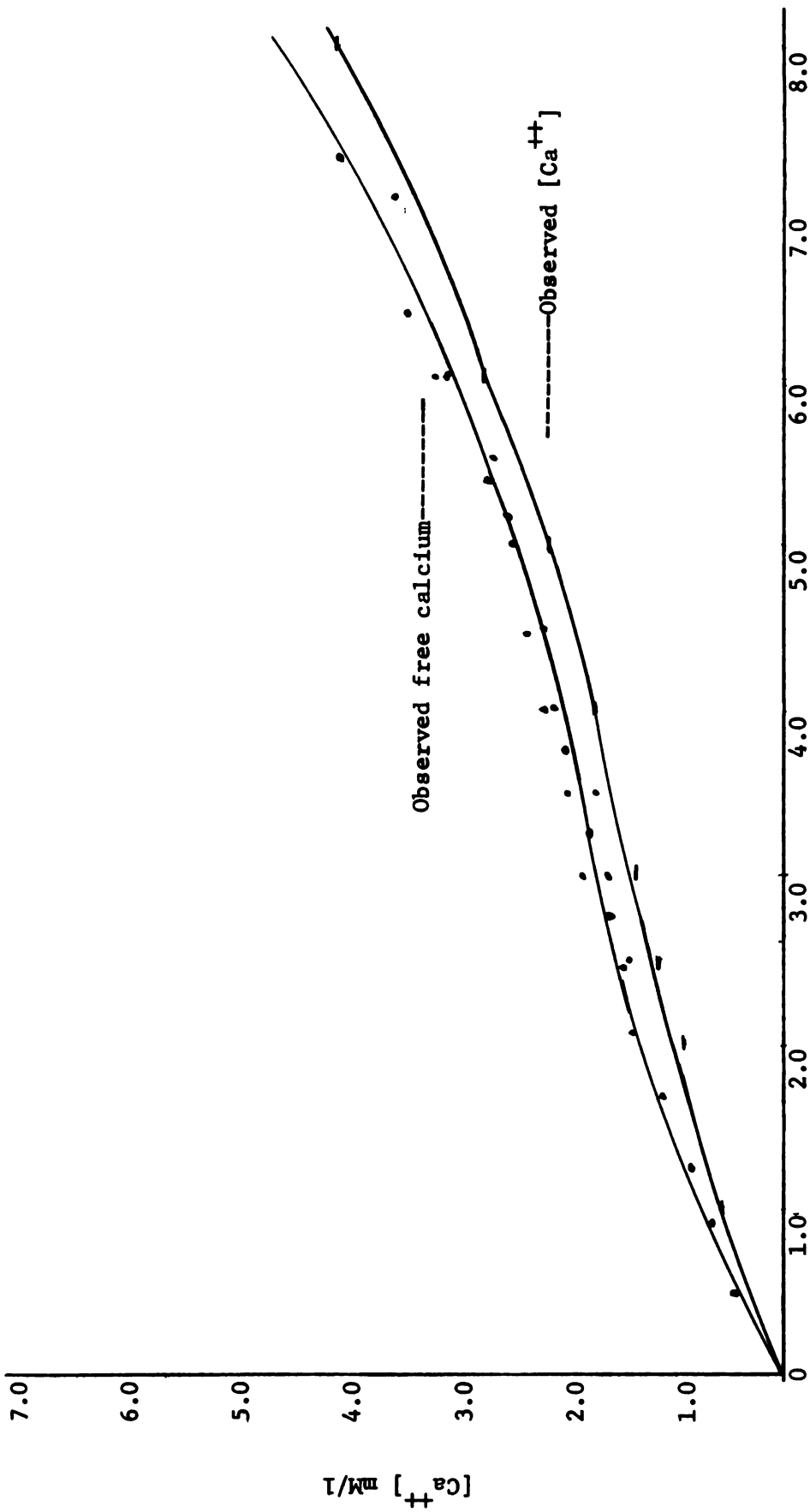

Figure 5. Relationship between free calcium and ionized calcium.

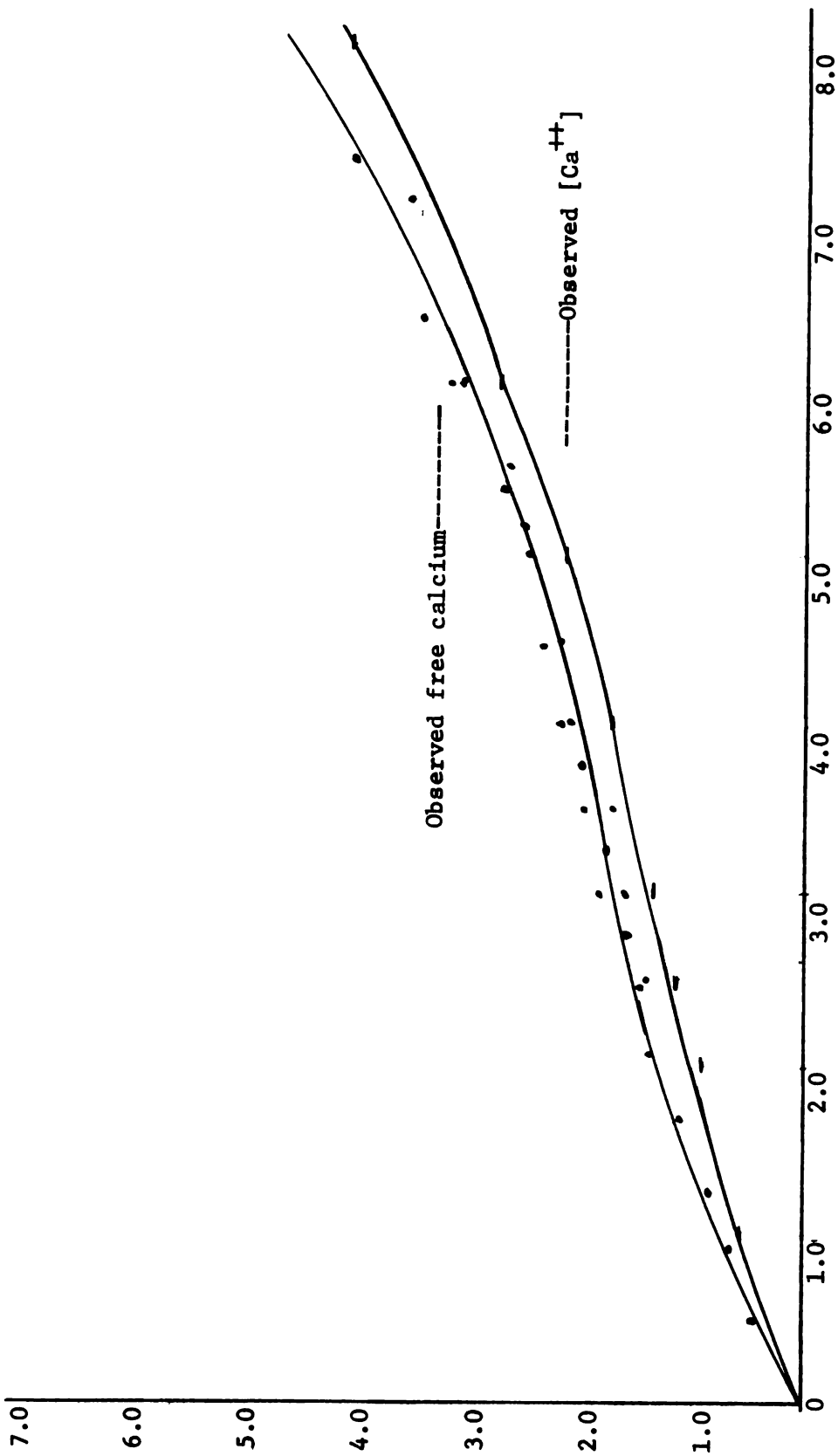

Figure 5. Relationship between free calcium and ionized calcium.

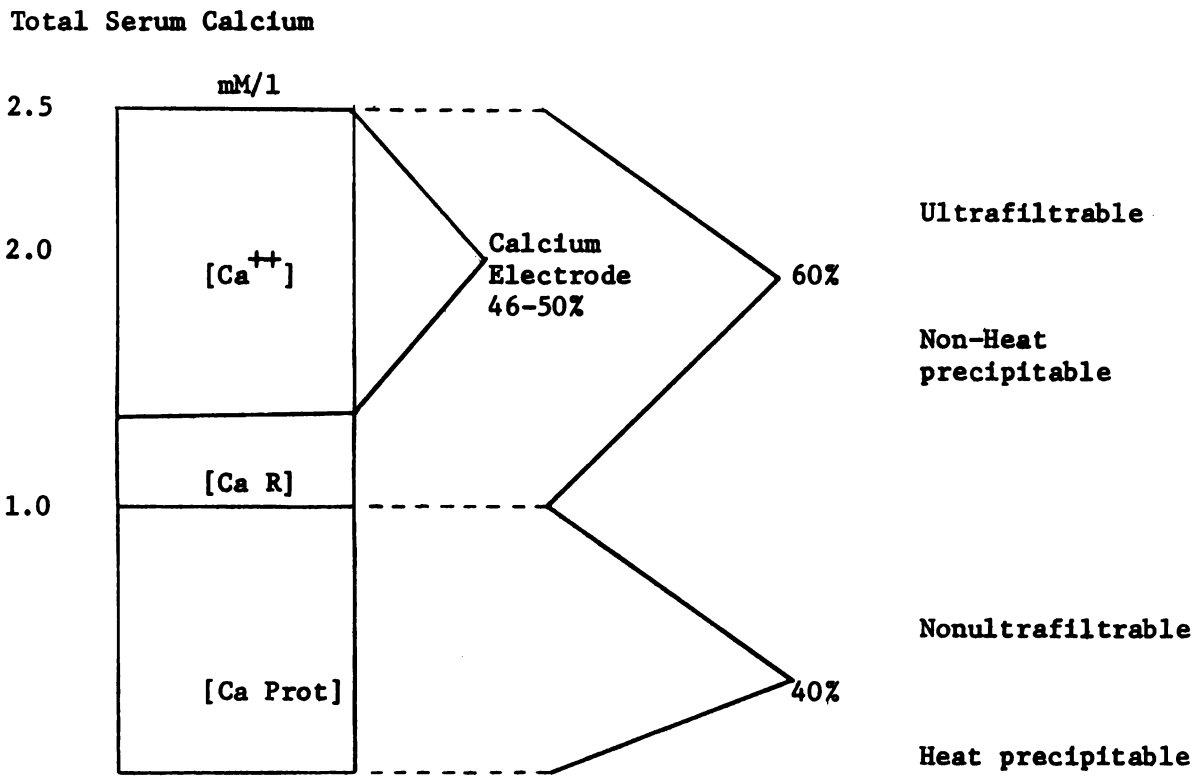

Figure 6. Serum calcium fraction as revealed by ultrafiltration, calcium electrode, heat precipitation.

It will be noted that the precipitable form is nearly linearly related to total calcium up to a total calcium of 7.5 mM/l. At this point the quantity precipitable is approximately 2.8 mM/l. The precipitated form then levels off indicating saturation in the coagulated protein fraction.

DISCUSSION

Figures published for various ultrafiltration techniques are within .01 mM/l from mean values for the heat precipitation products--both supernatant and thermoprecipitable--in this study. The values not in close agreement are those obtained with the use of the calcium electrode. The mean value for ionized calcium is much lower than measurements of free calcium by other methods, because the electrode does not respond to complexes of unbound calcium. These complexes represent a relatively stable 5 to 15% of total serum calcium and are included in measurements by other methods, including the one under study here, with the possible exception of the murexide method[2] (Figure 6).

The relationships between supernatant calcium, precipitable calcium and total serum calcium obtained in this study of individuals agree closely with the relationships reported by Moore with the calcium electrode. He found that ionized calcium remains within rather close limits despite wide variation in total serum calcium in individuals, and that there is no apparent relationship between the 2 values.[2] These same conclusions were drawn from the relation of thermoprecipitation supernatant calcium values to total serum calcium values.

The thermoprecipitated fraction, however, was found to be directly related to total serum calcium (see Figure 2). The slope of the line for this relationship was found to be .863, while Moore obtained a value of .92 for this slope, with protein-bound calcium plotted against total serum calcium.

The change in total serum calcium is almost quantitatively accounted for by a change in the heat-precipitable fraction. This relationship was reported by Moore in connection with the protein-bound fraction of serum calcium. The mean thermoprecipitable calcium value obtained in this study is nearly identical to the mean values for protein-bound calcium by various other methods. It is reasonable to conclude from these similarities that the serum calcium precipitated along with heat-coagulated protein represents the protein-bound fraction in normal individuals.

The degree of change in serum calcium fractions dependent on hydrogen-ion concentration has been determined employing the calcium electrode (Moore). The present study reveals similar changes. He observed that the soluble calcium complexes were not changed over the pH ranges studied. It can be concluded that the change observed is due to a shift from free ionized calcium to protein-bound calcium. The mechanism presumably involves a competition between calcium ions and hydrogen ions for negative sites on the plasma proteins. As the hydrogen-ion concentration goes up (decreasing pH) protein-bound calcium is displaced from the negative sites by hydrogen ions resulting in higher free ionized values. The reverse occurs with increasing pH. This is consistent with the explanation offered for loss of consciousness resulting from hyperventilation; increasing blood pH results in a shift of calcium from the free to bound form.[5]

The results of the present study indicate a mean change of .57 mM/l in supernatant calcium in response to a change of 1 pH unit. Moore reported a change of .42 mM/l in ionized calcium in response to a change of 1 pH unit. The difference in observations of .15 mM/l or approximately 6% of total serum calcium could be accounted for by the range of pH studied. Moore studied the change in ionized calcium over a pH range

of 6.8 to 7.8. In this area the change is almost linear, while the change becomes more abrupt at both higher and lower pH values. In the present pH dependency study the pH range was extended to over 8.0 where change is greater in ionized values. One additional difference in the pH dependency studies is that of protein concentration of serum samples. The various serum proteins bind calcium to differing degrees under normal conditions and would likely change in binding to differing degrees with pH change. It was not determined in this study whether the various serum proteins were equal in concentration to those in the study using the calcium electrode.

The response of thermoprecipitation fractions to change in total calcium and total protein was reflected in the degree of protein binding in the third experiment. The shift in proportions of free and bound calcium were noted by McLean and Hastings[8] and Moore.[2] Both reports related the loss of bound calcium in dilutions of serum to a shift in equilibrium between the original serum-calcium fractions.

At the lowest values of total serum calcium studied (1 mM/l) there was no appreciable amount of bound calcium detected (see Figure 4). The same observation was reported by Moore.

The many points of similarity exist in the calcium addition and saline dilution studies. The bound form showed slightly curved line with progressive additions of calcium. Saturation of serum proteins occurred at 2.8 mM/l at a total serum calcium of approximately 7.5 mM/l. The protein-bound fraction dropped to zero at a total serum calcium of 1.0 mM/l. These points indicate that the thermoprecipitation products represent a very close approximation of the serum calcium fractions studied by Moore.[2]

Values of free calcium (from Experiment III) are plotted against their respective total serum calcium values in Figure 5. Ionized calcium data taken from a graphic representation of results of a similar study with the calcium electrode are also shown.[2] It will be noted that free calcium concentrations by heat coagulation are higher throughout the calcium range studied. The important observation is the relatively constant relationship between the 2 measurements at all total serum calcium values except those below 1.0 mM/l. The uniform difference between these values appears to be no greater than .4 mM/l.

The uniformity shown between free calcium by coagulation methods and ionized calcium measured by the electrode throughout the total serum calcium range likely to be encountered clinically could lead to the employment of a correction factor to reduce nonprecipitable or free calcium to ionized calcium concentrations.

SUMMARY AND CONCLUSIONS

Heat coagulation of 83 samples of human serum was employed to produce thermoprecipitable and nonprecipitable serum calcium fractions. Studies were undertaken to determine if the fractions produced had properties similar to those of the serum calcium fractions quantitated by other methods.

Although the values for supernatant calcium did not match exactly the values obtained by the calcium electrode (because of the inclusion of the complexed form of calcium) the heat fractions were virtually identical in properties, and their concentrations paralleled those obtained with the calcium electrode.

The ultrafiltration methods employed by Rose, Moore, and Loken, while giving the same figures as the thermoprecipitation studied here, require special equipment, special pH control, large quantities of blood (10 to 30 ml.) and in excess of 4 hours to complete.

The thermoprecipitation method requires only 4 ml. of blood or less (depending on the method of calcium quantitation), a heat source such as boiling-water bath, and any calcium quantitation method currently being employed.

Due to the fact that this process can be completed with inexpensive equipment, small samples, and little time, it promises to be a valuable clinical laboratory procedure.

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APPENDIX

APPENDIX

Technicon Auto Analyzer Methodology (N-3b)-- Technicon Corporation, Tarrytown, N.Y.

This procedure is a modification of the method of G. Kessler and M. Wolfman, Clin. Chem. 10: 686-703 (1964). The method, as reported by H. J. Gitelman (Anal. Biochem. 18: 521 (1967), incorporates the use of 8-hydroxyquinoline to virtually eliminate the interference of magnesium.

0.25 Normal Hydrochloric Acid

Chemical composition:

1.	Hydrochloric acid, conc.	21 ml.
2.	Distilled water, q.s.	1000 ml.

Preparation:

1. Add concentrated hydrochloric acid to approximately 500 ml. water in a one-liter volumetric flask.
2. Dilute to volume and mix.

Cresolphthalein Complexone (0.010%) plus 8-Hydroxyquinoline (T01-0216)

Chemical composition:

1.	Cresolphthalein Complexone	0.100 gm.
2.	8-Hydroxyquinoline	2.5 gm.
3.	0.25 N Hydrochloric acid, q.s.	1000 ml.

Preparation:

1. Place approximately 500 ml. of 0.25 N hydrochloric acid in a one-liter volumetric flask.
2. Add the cresolphthalein complexone and mix until completely dissolved.
3. Add 8-hydroxyquinoline and mix until dissolved.
4. Dilute to volume with 0.25 N hydrochloric acid and mix.
5. *Do not* add any wetting agents to this reagent.

Calcium Base--3.75% Diethylamine

Chemical composition:

1.	Diethylamine	37.5 ml.
2.	Potassium cyanide	0.125 gm.
3.	Distilled water, q.s.	1000 ml.

Preparation:

1. Place approximately 500 ml. of distilled water in a one-liter volumetric flask.
2. Add the potassium cyanide and mix until dissolved.
3. Add the diethylamine, dilute to volume and mix.

Standards

Stock Calcium Standard (50 mg. Ca/100 ml.)

Chemical composition:

1. Calcium carbonate	1.250 gm.
2. Hydrochloric acid, conc., approx.	7 ml.
3. Distilled water, q.s.	1000 ml.

Preparation:

1. Weigh the calcium carbonate and transfer to a one-liter volumetric flask.
2. Add approximately 100 ml. of distilled water.
3. Carefully add the hydrochloric acid and swirl flask until all of the calcium carbonate has been dissolved.
4. Dilute to volume and mix.

Stock Magnesium Standard (100 mg. Mg./100 ml.)

Chemical composition:

1. Magnesium chloride ($MgCl_2 \cdot 6H_2O$)	8.36 gm.
2. Distilled water, q.s.	1000 ml.

Preparation:

1. Place approximately 500 ml. of distilled water in a one-liter volumetric flask.
2. Add the magnesium chloride and mix until dissolved.
3. Dilute to volume and mix.

Working Calcium Standards (Containing 2 mg. Mg./100 ml.)

Dilute the stock standards with distilled water. Add 1 drop conc. HCl per 100 ml.

ml. Stock Calcium	+ ml. Stock Magnesium	Dilute to	mg./100 ml. Magnesium	mg./100 ml. Magnesium
10	2	100 ml.	5.0	2
15	2	100 ml.	7.5	2
20	2	100 ml.	10.0	2
25	2	100 ml.	12.5	2
30	2	100 ml.	15.0	2

Operating Procedure Notes

1. The calcium procedure can be run at 60 determinations per hour.
2. Calcium can be determined in serum or heparinized plasma. Do not use EDTA, citrate, or oxalate as anticoagulant.
3. Magnesium (2 mg./100 ml.) equivalent to the level normally present in serum is added to the working calcium standards. This compensates for the slight color reaction of the dye with magnesium.
4. The reagent baseline must be set with water being aspirated through the sample line. With air through the sample line a slightly higher %T is recorded.
5. Be sure to use 0.5 ml. of Brij-35 per liter of hydrochloric acid to obtain good bubble patterns and low noise.

Albumin--American Monitor Corp., Indianapolis, Ind.

Principle:

Serum albumin binds to the dye 3, 3', 5, 5' tetrabromo-m-cresol-sulfonphthalein quantitatively and specifically. The albumin-dye combination produces an intense blue color with maximum absorption at 600 nM.

Procedure:

1. Rinse contents of dye vial and q.s. to 50 ml. with buffered detergent.
2. Pipette 3.0 ml. detergent dye reagent for each patient and a reagent blank.
3. Add 0.02 ml. of serum to each patient tube.
4. Mix well by inversion.
5. Set instrument to 100%T with the reagent blank and read transmittance of patient's test at 600.

Standards:

1. Add 0.01 ml. of prepared standard to 3.0 ml. of dye-detergent for the 2.5 gm./dl. standard.
2. Add 0.02 ml. of prepared standard to 3.0 ml. of dye-detergent for the 5 gm./dl. standard.
3. Treat as patient serum. Read and record %T values of the 2 standards as patients are read.
4. Plot the %T values of the standards against their concentrations of albumin--passing through 100%T for 0 concentration of albumin.

Color development:

The final color is stable for 1 hour. Since the reagent has a yellow color and the albumin-dye combination is blue, these colors blend to appear green. The blue color is the only one read at 600 nM.

VITA

The author was born in Ionia, Michigan, on June 2, 1946. He was graduated from Ionia High School in 1964.

During the next 4 years he attended Lansing Community College and Michigan State University, where he received a Bachelor of Science degree in Medical Technology in 1968.

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