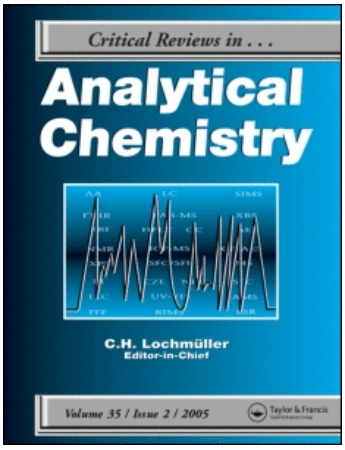

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DETERMINATION OF TOTAL AND IONIC SERUM CALCIUM

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SUMMARY OF SCOPE AND CONTENT

The analyst carrying out determinations of serum calcium should have some understanding of the physiology and metabolism of calcium. This review discusses this subject briefly and describes the important regulators of calcium metabolism that directly or indirectly affect the serum calcium level, such as parathyroid hormone, inorganic phosphate, thyrocalcitonin, vitamin D, glucocorticoids, and serum proteins. Since calcium levels are closely controlled by these several homeostatic mechanisms, only accurate and precise determinations of serum calcium will reveal a loss of steady state control and yield useful diagnostic information. The analyst familiar with calcium homeostasis can quickly alert the clinician to life-threatening states.

The newest technique of calcium determination, atomic absorption spectrophotometry, is discussed in some detail. Atomic absorption is a highly specific method for calcium, but the analysis of serum presents a number of chemical interferences, the most serious of which is due to serum proteins. Several solutions to the protein problem are presented, the choice among them being determined in part by the number of specimens that must be examined at one time. Recently published normal ranges are presented; they make it possible to appreciate why an error greater than 0.1 mmole/l. is intolerable for serum calcium.

Finally, the potentiometric method for determining the activity of ionized calcium with a liquid ion-exchanger electrode is discussed. The determination of ionized calcium is highly desirable since this is the physiologically active species. The potentiometric method is much simpler to carry out than the older chemical or ultrafiltration techniques. The collection and preservation of blood samples is discussed and the effect of pH on ionized calcium is presented in detail. The published normal values differ greatly, most likely because of the lack of agreement in the standardization of the electrode. This problem remains unsolved, and the method is, therefore, not quite ready for routine use in a clinical laboratory. The

electrode is much more difficult to use for determinations of ionized calcium than the glass electrode is for measurements of pH.

INTRODUCTION TO THE PHYSIOLOGY AND METABOLISM OF CALCIUM

The Normal State

Calcium is present in normal serum in three forms: 1. the largest fraction, roughly 47% of the total, is bound to protein, principally to albumin; 2. about 14% is complexed to ultrafiltrable ions such as phosphate and citrate; and 3. the balance is present as ionic or "free" calcium associated with monovalent ions, primarily chloride.[1] The physiologic response of the body's homeostatic mechanisms is believed to be controlled by the activity of the calcium ion in plasma. A rough estimate of the activity can be made by applying the Debye-Hückel equation to the known concentraton of free calcium and the ionic strength of the plasma. The activity can be estimated directly with a calcium-ion specific electrode. All of the ionic species dissolved in plasma contribute to the ionic strength and affect the activity of the calcium ion.

Before considering methods for the determination of total serum calcium and calcium-ion activity, it is desirable to have some understanding of the steady state mechanisms controlling calcium. It very quickly becomes apparent that only highly precise and accurate methods will yield useful diagnostic information. Second, the analyst must know when an abnormal concentration of serum calcium is potentially life-threatening. He can then provide an extra bit of service to the clinician by calling his attention to the abnormal level. After all, it is the analyst who sees the result first.

Normally, the activity of calcium ion in plasma is maintained within very narrow limits. The preservation of life depends upon a finely tuned control of the steady state. Even in the absence of an adequate calcium intake and a negative calcium balance, plasma calcium remains unchanged for a considerable length of time as the calcium stores in bone are called on.[2]

Calcium is required for the contraction of smooth, skeletal, and cardiac muscle; for the transmission of nerve impulses; for the functioning of lipase, succinic dehydrogenase, and certain proteolytic enzymes; and for blood clotting.

Regulators of Calcium Metabolism: PTH

The single most important regulator of the plasma calcium level is parathyroid hormone (PTH). PTH is elaborated by 4 small glands, weighing about 35 mg each, which are imbedded in the thyroid gland. PTH acts on bone, kidney, and intestine to raise the calcium activity of plasma. It stimulates the resorption of bone, causing calcium and phosphate to be released into the extracellular compartment, and it increases the renal clearance of phosphate by inhibiting its tubular reabsorption while at the same time enhancing the tubular reabsorption of calcium. Both of these effects on kidney tend to raise the plasma calcium. In conjunction with vitamin D, PTH increases the intestinal absorption of calcium.

PTH must always be present in the blood to counteract other mechanisms that operate to deposit calcium and phosphate in bone tissue. The release of PTH from the gland is, in turn, controlled by the plasma calcium activity. A reduced calcium level stimulates the release of PTH. Normally the balancing mechanism between calcium and PTH is very efficient, so that large swings in the plasma calcium are not observed. Large fluctuations of the calcium level have been attributed to a degree of loss of homeostatic control.[3]

Phosphate

The concentrations of plasma calcium and phosphate tend to change in opposite directions. Normally, the product of the total calcium and phosphorus concentrations, when both are expressed in mg/100 ml, is between 35 and 40. When phosphate is infused, plasma phosphate rises and calcium falls. Phosphate antagonizes the bone-mobilizing effect of PTH, the net effect being the deposition of calcium and phosphate in bone. In cases of phosphate depletion because of diet or other reasons, calcium leaves the skeleton to produce a moderate to slight hypercalcemia and a significant increase in urinary calcium. If the diet is adequate and the absorption of phosphate is normal, phosphate has only an indirect effect on maintaining calcium homeostasis.

Thyrocalcitonin (TCT)

The primary effect of TCT is the inhibition of bone resorption. TCT is secreted by the thyroid, and possibly by other glands,[4] and is normally present in plasma. Its concentration rises and falls

with the plasma calcium activity. In contrast to PTH, the older individual can maintain a normal plasma calcium after total thyroidectomy (provided that the parathyroid glands are left). TCT was once thought to counteract the effect of PTH and hence might be used to combat the hypercalcemia of hyperparathyroidism. Unfortunately TCT is ineffective, or if it is helpful its effect in reducing hypercalcemia may not be sustained.[5] TCT is believed to play a role in early childhood or adolescence in the prevention of hypercalcemic hyperparathyroidism during the period of rapid bone growth.[6]

Vitamin D

Vitamin D acts on the intestine to facilitate the absorption of calcium from the diet. PTH plays a similar role, but the absence of vitamin D may lead to rickets, osteomalacia, parathyroid hyperplasia, and secondary hyperparathyroidism. Vitamin D or, better, AT-10 (Hytakerol®) is an important therapeutic agent for the chronic hypocalcemia of hypoparathyroidism.

Glucocorticoids

Glucocorticoids oppose the action of vitamin D on the gut and hence tend to decrease plasma calcium. In cases of adrenal insufficiency, hypercalcemia may be observed. Glucocorticoids may also inhibit the action of PTH on bone resorption.

Proteins

Finally, plasma proteins indirectly influence the total serum calcium. Albumin binds calcium and so, to a lesser extent, do the globulins. PTH is released or is prevented from being released depending on the calcium activity, i.e., the parathyroids do not "see" the bound calcium. Total plasma calcium rises and falls with the albumin concentration while the calcium activity remains constant.[1] Serum calcium decreases by about 0.2 mmole/l. for each decrease of 1 g/100 ml in albumin.[5]

Abnormalities of Calcium Metabolism

Primary Hyperparathyroidism

Boonstra and Jackson found 55 cases of unsuspected primary hyperparathyroidism during the routine screening of 50,330 patients.[7] Consequently this is not a very common disease, but still it is a dangerous one. The single most important test for its diagnosis at present is an accurate determination of total serum calcium. Immunoassay for PTH and ionic calcium may become more important diagnostic tests for primary hyperparathyroidism when they become more readily available and the difficulties in performing them have been overcome.[5]

In primary hyperparathyroidism, the glands become autonomous and secretions of PTH are no longer controlled by the plasma calcium. The autonomous function, commonly due to adenomas and rarely to carcinomas of the parathyroids, is nearly always accompanied by a high total serum calcium and hypophosphatemia. When calcium is at the high-normal borderline level, ionized calcium has been said to be of more diagnostic significance.[8] Three quarters of the patients will show kidney and/or bladder stones and one quarter of them will have radiologically demonstrable (especially in the hands) bone disease. Serum alkaline phosphatase may be elevated, and peptic ulcer is seen in some patients.[9]

Acute hypercalcemia (i.e., total calcium above 3.5 to 4 m*M*) may produce cardiac arrest. Factors which make a high calcium level even more dangerous are alkalosis, which decreases the ionic calcium, and the use of digitalis.

Surgical removal of one or more of the parathyroid glands is one approach which has had some success. Postoperative hypocalcemia and tetany have been observed. Accommodation by a patient to a slow rise or fall in serum calcium to abnormal levels is well-known. It is the sudden change in calcium level that is extremely dangerous.

Other Diseases Exhibiting Hypercalcemia

Cancer is more important as a cause of hypercalcemia than hyperparathyroidism. Hypercalcemia has been observed in multiple myeloma, metastatic (to bone) carcinoma, and primary carcinoma not involving bone, particularly bronchocarcinoma. Other causes are vitamin D intoxication, milk-alkali syndrome, sarcoidosis, osteoporosis and disuse atrophy, and thyrotoxicosis.[9] The differential diagnostic tests that are used are beyond the scope of this discussion.

Hypocalcemia

Hypocalcemia has been observed in hypoparathyroidism, pseudohypoparathyroidism where a sufficient amount of an ineffective PTH is

secreted, and in malabsorption of calcium. It may also be a sequel to thyroidectomy if the parathyroids are removed. The danger is always hypocalcemic tetany leading to convulsions, laryngeal spasms, and death. Treatment in the acute phase is with intravenous calcium, while Hytakerol® and a high calcium-low phosphate diet are used for chronic hypocalcemia.

An excellent recent review by Kleeman et al.[6] on calcium metabolism should be consulted for a more complete discussion.

DETERMINATION OF TOTAL SERUM CALCIUM BY ATOMIC ABSORPTION SPECTROPHOTOMETRY (AAS)

Introduction

AAS is the most recently developed of the major analytical methods used for determining calcium. The possibility of applying AAS to problems of chemical analysis was discussed by Walsh in 1955.[10] In the short period that has elapsed since then, AAS has become a major analytical tool. Willis' paper in 1960 introduced the method to the clinical laboratory for the determination of serum calcium.[11] Since that time, AAS as applied to serum calcium has been examined in some detail in about 20 publications.

The fundamentals and theory of AAS are discussed by Elwell and Gidley,[12] Robinson,[13] and Slavin,[14] and will be discussed briefly here as they apply to the determination of calcium.

AAS is not the oldest "flame method" for calcium. Flame emission spectroscopy has been used for a long time; unfortunately, however, it is subject to interferences that are introduced by other constituents of serum, particularly sodium, and that can be overcome only with some difficulty.[15] By contrast, AAS methods for calcium are free from spectral interferences from other elements. In fact, there are no other elements which overlap or coincide with the absorption lines for calcium, and the method is, therefore, highly specific.

A diluted solution of the sample is introduced into a total consumption or a mist-chamber entrainment burner. A small fraction of the calcium ions in solution is reduced to the atomic state when they reach the flame (commonly air/acetylene). Of these, the overwhelming majority are in the ground electronic state.[12] The fraction in the ground state is quite insensitive to changes in the flame temperature; this is in contrast to flame emission methods, where the fraction of the atoms in the excited state is highly sensitive to fluctuations of the temperature of the flame.

The width of the absorption band of the calcium atoms in the flame is about 0.001 nm.[10] In theory, a continuous-spectrum light source could be used, but it would require a prism or grating of extraordinary dispersive ability, far beyond what is currently available, to produce an instrument with adequate sensitivity. Narrow slits and exceptionally stable and sensitive photodetectors would also be required. This problem has been solved by using a hollow-cathode lamp that contains the element to be determined. The element is excited electrically to emit its line spectrum, and one of the lines is selected as the source. The line corresponding to the transition from the ground state to the first excited state is the one generally chosen, since it will yield the highest sensitivity in the determination. Secondary lines have not been used in determinations of serum calcium.

With hollow-cathode lamps, the dispersion device need not have very high dispersive ability. It must only be able to resolve the desired line from any nearby lines and from any radiation due to the lamp filler gas (e.g., He, Ne). An obvious disadvantage is that a separate lamp is needed for each element to be determined. This is a minor problem in most clinical laboratories since besides calcium, only magnesium, lithium, iron, and, rarely, lead, mercury, chromium, and nickel are determined by atomic absorption.

Sensitivity

The 422.7-nm emission line of calcium has been used uniformly for determinations of calcium in serum. Zettner and Seligson[16] and Pybus[17] obtained the best sensitivity when the hollow-cathode lamp was operated at the lowest possible current. If the emission line from the lamp is broader than the absorption line of the calcium atoms in the flame, the sensitivity will be poorer than if the emission line is narrower than the absorption line. By keeping the lamp current at the usable minimum, the emission line can also be kept as narrow as possible. Increasing the path length in the flame will increase the sensitivity, as will increased efficiency of nebulization of the

liquid and increased reduction of the calcium to the atomic state. Nebulization and reduction in the flame are together very inefficient for producing atoms in the flame. Less than 1% of the calcium ions fed to the burner is responsible for the absorption signal that is recorded.[13] Technical improvements would be most welcome here. The sensitivities (defined as the ratios of change of absorbance to change in calcium concentration) for six recently published studies are given in Table 1. For the most part, the sensitivities were calculated from the published calibration curves. The Perkin-Elmer® Model 214 Atomic Absorption Spectrophotometer* gave somewhat lower sensitivity,[16] but there is remarkably good agreement among the rest. In some cases, where an arbitrary scale for absorbance or an unstated scale expansion was used, it was not possible to calculate the sensitivity obtained. The limited data in Table 1 indicate that the presence or absence of $HClO_4$ or the presence or absence of protein entering the burner does not affect the sensitivity for calcium determinations.

The hollow-cathode lamps have a limited life and must be operated at higher currents as they get older. The cathode element is vaporized slowly

TABLE 1

Sensitivity of Atomic Absorption Methods

Ref.	Instrument/method	Absorbance	Aspirate concentration, m*M*	Sensitivity, $\Delta A/\Delta C$, $(mM)^{-1}$
Zettner and Seligson[16]	Perkin-Elmer® 214, 100 mm, 1-slot burner, direct aspiration, 10x dilution	0.33 0.18	0.25 0.13	1.3
Trudeau and Freier[28]	Perkin-Elmer® 303, Boling burner, direct aspiration, 50x dilution	0.23 0.05	0.10 0.025	2.4
Pybus et al.[20]	I.L.,® Boling burner, direct aspiration, 50x dilution	0.15 0	0.05 0	3.0
Paschen and Fuchs[21]	Perkin-Elmer® 303, Boling burner, direct aspiration, 100x dilution	0.41 0.04	0.125 0.0125	3.3
Pybus[17]	Techtron AA3, $HClO_4$ filtrate, 25x dilution	0.28 0.13	0.10 0.05	3.0
Lott and Herman[27]	Perkin-Elmer® 303, Boling burner, aspiration of dialyzate, approx. 21x dilution	0.70 0.19	0.24 0.06	2.8

*The Perkin-Elmer Corp., Norwalk, Conn. 06852.

and deposits on the walls of the lamp. Calcium emission in the flame is nullified by either electrically or mechanically pulsing the hollow-cathode lamp output and then amplifying only the AC signal. The emission signal from the flame is DC or continuous and is thereby rejected. The increase or decrease in the AC signal is proportional to the concentration of calcium atoms in the flame.

Popularity of AAS

In a 1971 quality control survey of 1,576 laboratories by the College of American Pathologists (Comprehensive Set C-A, Chemical Procedures), only 105 or roughly 7% reported that they use either manual or automated AAS for determinations of serum calcium.[18] Assuming that AAS is the most specific method for calcium, why doesn't it have a larger following? Several answers are suggested: 1. The instrumentation is costly and complex, and skilled personnel are needed to run it. 2. Tanks of flammable gas (e.g., acetylene) must be kept on hand and constitute a hazard. 3. Laboratories are traditionally conservative and are reluctant to institute changes which may disrupt the routine. 4. The AutoAnalyzer® methods enjoy wide acceptance.* 5. The continuing appearance of papers on procedures for the determination of calcium by AAS indicates that there is some dissatisfaction with existing techniques and that the final word has not yet been said.

Chemical Interferences

Anions

Calcium forms complexes with proteins, phosphate, sulfate, citrate, and other constituents of serum. Some way must be found to release the calcium prior to analysis. Phosphate and (to a lesser extent) sulfate have been recognized as causes of chemical interference. Phosphate and sulfate depress the absorption of calcium in the flame. Willis[11] suggested the use of Sr^{2+}, which preferentially combines with phosphate or sulfate, or of EDTA, which forms a more stable complex with calcium than phosphate[19] but which will burn and release calcium in the flame. Zettner and Seligson found an 0.5% solution of EDTA to be unsatisfactory since their burner could not consume the organic solids fast enough, so that accumulation of matter and rapid clogging of the burner were observed.[16]

Strontium chloride has been used successfully by many authors to combat the depressive effects of phosphate and other anions on calcium.[11, 17,20-23] Strontium has one advantage over lanthanum, the other widely used antidepressant cation, and this is that clogging of an aspirator or burner will never be overlooked when strontium is used, due to its intense, scarlet-red emission spectrum.

The other antidepressant cation is lanthanum, usually added as $LaCl_3$. Solutions of lanthanum must be acidic, for otherwise lanthanum oxide or hydroxide tends to precipitate out. It has been said[16] that lanthanum salts substantially enhance the absorbance due to calcium. Monder and Sells indicated that the enhancement due to lanthanum was cancelled by the addition of lanthanum to the blank,[24] and concluded that there was calcium in the lanthanum. From their Figure 2, the divergence of the calibration curves obtained in the presence and in the absence of lanthanum can be attributed only to an enhancement effect by lanthanum on the calcium absorbance. The enhancement by lanthanum adds sensitivity and favors its use over strontium. Lanthanum salts commonly contain a trace of calcium which must be corrected for. A convenient method is to dilute the standards and unknowns with the same lanthanum solution.

The use of lanthanum has been recommended in most of the recent studies.[16,25-29]

Calcium is also complexed to protein at physiological pH, but in solutions containing 0.25 *M* HCl no binding of calcium to protein was observed.[27] Also, if the data in Figure 1 of Loken et al. can safely be extrapolated to pH 0-1, it appears that no significant calcium binding to protein occurs in the presence of strong acid.[32]

Cations

Magnesium does not absorb at the wavelength (422.7 nm) for calcium nor does it offer any chemical interferences.[11,16,27] There is some disagreement as to interference by sodium. Some authors have found that sodium enhances calcium absorbance,[11,22,28] while others report that it depresses calcium absorbance.[16] Another group found that sodium was without effect provided that strontium[17] or lanthanum salts were present.[20,25,27,28] Potassium has been found

*Technicon Corp., Tarrytown, N. Y. 10591

not to interfere[16,20,27] and to enhance calcium absorption.[11] The questions of what constitutes "interference" and what magnitude of enhancement or depression of calcium absorbance is considered to constitute interference must be decided individually.

Murdoch and Heaton[30] found that a hotter flame (acetylene/nitrous oxide) gave enhancement of calcium absorbance by sodium, whereas a "cooler" acetylene/air flame showed no enhancement. Others reported that the interference from sodium and potassium decreased as the flame was made leaner and hotter.[31]

The only conclusions which can be reached for this sometimes conflicting evidence is that when AAS is introduced into a laboratory, the peculiarities of the instrument, burner, aspirator, reagents, etc. must be evaluated for potential interferences by cations. This evaluation can be done by preparing calcium standards with and without sodium and potassium at normal and abnormal physiological levels. In the presence of 0.036 *M* $LaCl_3$ (0.5% La) and 0.25 *M* HCl, probably no cationic inteferences will be observed.

Protein

The high concentration of solids in serum, and in particular the presence of 6 to 8 g of protein/100 ml, greatly increases the difficulty of serum calcium determinations by AAS. Two problems related to the presence of protein must be considered. Near the isoelectric pH (pH 4 to 5) serum protein will begin to coalesce and precipitate. Precipitation can be avoided and the calcium released from the protein by acidifying with 0.1 to 0.25 *M* hydrochloric acid. Higher concentrations of acid are not recommended since precipitation of proteins may also occur as the concentration of hydrochloric acid approaches 1 *M*. Precipitation on dilution with 0.25 *M* HCl containing 0.036 *M* $LaCl_3$ has not been observed in this laboratory during the analysis of several thousand sera.[27] A recent exception was a myeloma patient whose serum contained 4.3 mmole/l. of calcium and 11 g/100 ml of total protein. The procedure of Savory et al.[25] (trichloracetic acid precipitation) was used in this one case for the unknown and standards.

The second problem due to proteins is that they are not volatile and do not burn well. Upon aspiration of dilute solutions of protein, precipitation or accumulation of protein in the aspirator or burner has often been observed. Protein accumulation may change the rate of sample consumption and affect the results. A number of authors have reported that protein and partly combusted material tend to contaminate or clog the burner.[16,27-29,33,34]

The burner supplied with the original Perkin-Elmer® 303 atomic absorption spectrophotometer was difficult to demount and clean, and was particularly annoying if it had to be cleaned in the middle of a group of samples. The newer stainless steel burner is a considerable improvement. Another consideration that is not always recognized with samples containing protein is that diluted sera have surface tensions different from those of aqueous calcium standards. The lower surface tensions of the diluted sera cause them to be aspirated somewhat more rapidly and may lead to results higher than those that would be obtained with aqueous solutions containing no protein.

Three approaches to the protein problem have been used. The first is to precipitate the protein with trichloroacetic acid (TCA)[11,22,23,25] or perchloric acid,[17] centrifuge, and aspirate the supernatant into the flame. Standards must be treated in the same way to correct for dilution. There is some loss of sensitivity[25] when trichloroacetic acid is used, possibly because of an increase in viscosity of the solutions. Coprecipitation of calcium with the protein has been cited as a possible source of error,[16] but the experience of several groups using a protein-free filtrate obtained by treatment with trichloroacetic acid seems to indicate that coprecipitation is not a problem.[11,22,23,25]

An obvious disadvantage of methods that involve the precipitation of protein is the extra handling and pipetting steps that are required and the resultant greater possibilities of error and contamination. The antidepressant and precipitating agent can be added in one step, as Thin and Thomas[23] (TCA plus strontium) and Savory et al.[25] (TCA plus lanthanum) have shown. A number of reports indicate that better precision is obtained when protein is excluded from the burner than when diluted serum was aspirated directly.[25,27,29]

The second approach to the protein problem is to aspirate sera directly after dilution with lan-

thanum,[16,26,28,34] strontium,[21] lanthanum plus strontium,[20] or EDTA.[11,33] Tenfold,[16,33] 50-fold,[20,28] and 100-fold[21] dilutions have been used. No protein separation step is employed. The standards are treated the same way. This approach is very convenient and requires only minimal sample handling, particularly if an automated dilutor[16] is available. Clogging of the burner has been reported to be considerably reduced if the diluent contains 6% *n*-butanol and 0.02% octanol[16] (as an antifoaming agent) or 4% *n*-butanol.[34] Willis[11] considered that dilution (with water or EDTA) and direct aspiration of sera are adequate for "rapid routine purposes" but that "removal of protein by coagulation is desirable for the most accurate work." Zettner and Seligson[16] made a detailed study of protein interference in their dilution and direct aspiration technique. The interference was largely overcome by $LaCl_3$. When the calcium level was above about 2.5 mmole/l., the protein content of the samples affected the calcium absorbance. The absorbance was found to go down as the protein went up at a constant calcium concentration. The effect is small and probably would have been ignored by most analysts, but these authors chose to include 6.5% calcium-free protein in their standards as an additional precaution. In their method, the difference between the protein contents of the standards and specimens would have to exceed 4 g/100 ml for the relative error to be greater than 2%.

Pybus et al. used a dual channel instrument (I.L.®-153*) with strontium serving as the reference or internal standard channel.[20] During the measurement of the absorption of light by strontium in the flame, the addition of calcium and protein did not change the strontium absorbance. It was deduced from this evidence that protein also does not interfere in the measurement of calcium. This is a reasonable assumption in view of the similarities between the characteristics and behaviors of calcium and strontium in the flame. Paschen and Fuchs found that different dilution ratios of serum and diluent gave the same results (the standards were diluted the same way) for calcium concentrations and concluded that there was no protein interference in their method.[21]

Are dilution and direct aspiration methods capable of sufficient accuracy and precision? The answer is a qualified yes, as shown by Pybus et al.,[20] provided that certain conditions and precautions are fulfilled. Regular or daily cleaning of the burner has been recommended and is probably required in all direct methods.[28,33] The rate of burner/aspirator clogging is best determined on an individual basis. The newer, easily demounted burners facilitate cleaning. Butanol is a useful aid to reduce protein build-up. The appearance of glowing specks on top of the burner always necessitates stopping the analysis and cleaning. Frequent recalibration (e.g., with every other sample[20]) is essential. Highly accurate and precise results can be obtained with the direct method of Pybus et al.,[20] and in fact this may be the long-sought-for reference method for total serum calcium.

Two of the dilution and direct aspiration methods were intended for the often forgotten pediatric patient. Rodgerson and Moran[26] used the method of Zettner and Seligson[16] but made a 200-fold dilution of 20 µl of serum. Gochman and Givelber were able to measure both calcium and magnesium on 30 µl of sample with a combined AutoAnalyzer® – IL®-153 system.[34]

Interference by protein in direct aspiration methods may or may not be observed, depending on the instrument and burner/aspirator that are used, on the antidepressant and its concentration, on the type and concentration of acid used, and on the extent to which the serum is diluted. Since the technique is complex, a dilution and direct aspiration technique should not be introduced into a laboratory before the effect of protein is checked. This can be accomplished by examining calcium standards at low, normal, and high concentrations in replicate with and without decalcified protein. It is also desirable to examine solutions having the same concentration of calcium but having protein concentrations that vary from 3 to 11 g/100 ml. If these last specimens give a constant calcium absorbance, protein probably does not interfere. The rate of clogging should be checked to assess how often complete cleaning is necessary. This can be done by repeatedly analyzing a serum pool and noting any change in the aspiration rate and drift in the absorbance. Recovery studies of samples prepared from decalcifed, pooled serum to which known amounts of calcium have been added should be carried out. Decalcification is readily carried out with a

*Instrumentation Laboratories, 113 Hartwell Avenue, Lexington, Mass. 02173.

strong-acid cation-exchange resin (e.g., Dowex® 50 x 8, 100 mesh) in the sodium form.[27,35]

The third approach to the protein problem is separation from calcium by dialysis and use of the AutoAnalyzer®. The protein-free dialyzate is led to the aspirator of the burner in a continuous stream. It is advisable to debubble the stream before it enters the burner to prevent surging in the aspirator. Klein et al. proposed such a system and found that the peaks were smoother and showed less noise when the dialyzate was aspirated than when diluted serum was aspirated.[29]

Protein does not reach the flame and no interference from protein would be expected. Aqueous calcium standards were used by Klein et al., who stated that "the recommended addition of physiologic concentrations of protein to the standard solutions did not apply in the present case and standard solutions were, therefore, prepared in dilute acid."[29] Lott and Herman[27] examined this method and found higher-than-labeled amounts of calcium in control sera, particularly those with normal total protein contents. Further studies revealed that addition of decalcified albumin to the aqueous standards increased the slope of the calibration curve (Figure 1). Surprisingly, protein does have an effect even when dialysis is used. The protonated and hence positively charged protein enhanced the dialysis of calcium (and magnesium) ions, possibly by a Donnan membrane effect phenomenon. The use of aqueous calcium standards therefore leads to falsely elevated results with sera. The magnitude of this error can be estimated from Figure 2. The most accurate results are obtained when the standards and unknowns have exactly the same concentrations of both calcium and protein. The magnitude of the error depends on the difference between the concentrations of protein in the standards and the unknown, and it also depends on the calcium concentration. With a given difference in protein concentration, the absolute error increases as the calcium concentration increases. At a calcium concentration of 3.75 mmole/l. and with 7 g/100 ml protein in the specimen, aqueous standards will give results that are 0.1 mmole/l. too high. With 7 g/100 ml albumin in the standards, the error in calcium at the upper normal limit of about 2.6 mmole/l. would be less than 0.05 mmole/l. if the concentration of protein in the serum is between 3 and 11 g/100 ml. At the lower normal limit of about 2.1 mmole/l., the error is less than 0.05 mmole/l. if the concentration of protein in the serum falls between 2 and 12 g/100 ml.

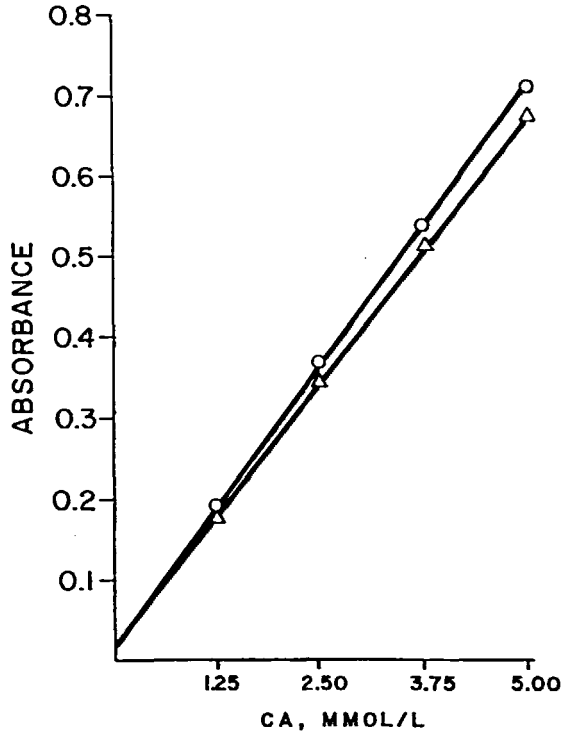

FIGURE 1. Calcium calibration curves of standards with (o) and without (Δ) 7 g of albumin per 100 ml. (From Lott, J. A. and Herman, T. S., *Clin. Chem.*, 17, 614 (1971). With permission.)

Preparation of decalcified albumin[27] for the standards is easy but time consuming. It is advisable to add an antibacterial agent such as Zephiran® to the albumin. The absence of calcium from the treated albumin must be confirmed before the standards are prepared.

Amador and Neeley confirmed these reports that the dialysis of calcium is enhanced by protein.[36] The enhancement they observed was even greater when a miniature (12-in.) dialyzer was used. They found that the serum calcium can be falsely elevated by as much as 0.28 mmole/l., which exceeds half the width of the normal range. Instead of decalcified albumin, the addition of 8% polyvinylpyrrolidone to the standards was recommended. They also suggested that other phenomena beside the Donnan effect may be operating, such as the osmotic pressure due to the serum proteins. Amador and Neeley measured a difference in potential across the dialysis membrane which may contribute to, or be symptomatic of the effect of proteins. The enhancing

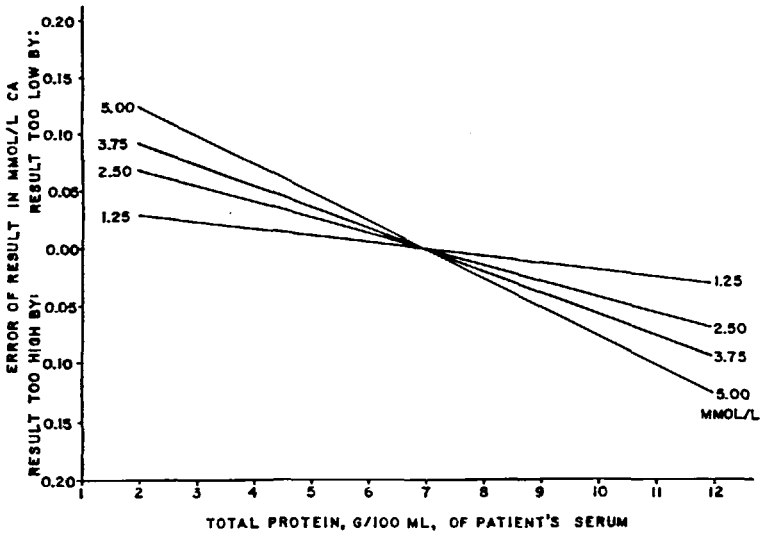

FIGURE 2. Estimate of error in calcium result when protein concentration of serum and standards differ, for various calcium concentrations. Slopes in order of increasing concentration of calcium are −0.005, −0.014, −0.018, and −0.025 mmole of calcium/l./g of protein (with permission).

effect of proteins was decreased by the presence of 1 *M* hydrochloric acid, but then occasional serum coagulation was observed.[36]

Increasing the concentration of hydrochloric acid in the recipient stream of the dialyzer has been shown to decrease the enhancing effect of protein, but does not eliminate it.[27] There is a limit to the concentration of hydrochloric acid that can be aspirated. Even 0.25 *M* hydrochloric acid has a corrosive effect on the stainless steel nebulizer offered by Perkin-Elmer for the Model 303. The Teflon® and polyethylene aspirator was found to be difficult to adjust and had several positions of the capillary where maximum sensitivity was observed in the author's laboratory.

What AAS Method Should Be Used?

The AAS method of choice for a clinical laboratory is determined in part by the number of samples that must be examined in one run. When a limited number of specimens (say 10 to 20) must be analyzed, a direct dilution and aspiration technique could be used advantageously.[16,20,26,34] An easily demounted burner is recommended in any case and is a definite advantage with direct dilution methods. In a large laboratory where many samples are examined daily, the method of Klein et al.[29] as modified by Lott and Herman[27] is worth considering. Aspiration of the dialyzate absolutely minimizes clogging of the burner by incombustible residue and gives much more trouble-free performance. The AutoAnalyzer® components in the author's laboratory have proven to be highly reliable and are rarely the cause of problems.

Normal Range and Diagnostic Significance of Total Serum Calcium

A total serum calcium value is actually a composite of several variables. As discussed earlier, PTH is the most important regulator of ionic calcium. The total calcium is in turn a reflection of the levels of PTH and the total protein, especially albumin, in serum.[1] Moore found no correlation between the total and the ionic serum calcium. However, he did find a correlation between the serum albumin and total calcium in the normal state, and in this sense Moore concluded that total calcium is an indirect measure of the albumin level.[37] Total protein and albumin are believed to change independently of the serum calcium.

Keating et al., in a study of a large group of normal men and women over 20 years of age, observed a decrease in calcium with increasing age in men which paralleled a decrease in both serum proteins and albumin.[38] They found in women that the serum calcium did not change significantly with increasing age, but a decrease in total protein and albumin was observed which was smaller than the same decrease in men.

Diet has[39] and has not[40] been found to affect serum calcium. In both these studies, the population was small and this question needs further investigation. There is little information of the effect of method of collection and recumbency of the patient on the serum calcium but venous stasis would be expected to concentrate serum proteins and raise calcium.

Some qualitative data are available on the effect of drugs on serum calcium.[41] Colorimetric methods are affected more by interferents while AAS seems to be less prone to drug interference. Singh et al. found that of a large number of drugs tested, only *p*-aminophenol, a metabolite of *n*-acetyl-*p*-aminophenol, at the 0.001 *M* level interfered in the colorimetric method (SMA®-12/60) for calcium and gave a "calcium" value of 0.85 mmole/l.[42] Christian presented qualitative data on drugs which induce physiological changes or give chemical interferences in the determination of calcium.[43] Drug-induced physiological changes will affect all calcium methods. Finally, the method, its error, and method of standardization will affect the calcium results. An accurate determination of serum calcium is the best currently available test for hyperparathyroidism. Given all the variables that can affect the result, some of which cannot be or are not controlled, it is amazing that meaningful values are produced at all!

Gowenlock discussed the effect of method error on the normal range.[44] He gives the equation

$$S_0 = \sqrt{S_b^2 + S_m^2}$$

where S_0 is the observed standard deviation for a test of a group of normals and S_b and S_m are the standard deviations of the biological variation and methodological variation, respectively. The normal range for calcium ($4S_b$) is quite narrow, probably below 0.4 m*M*. If we consider acceptable results to be those for which biological variation is responsible for 90% of S_0, then the methodological error must not exceed S_b or one fourth of the normal range. For calcium, then, S_m must not exceed 0.1 m*M*. The value of S_m can be estimated by examining a single serum pool entering the laboratory as fictitious patient samples or blinds over a period of time. Allen et al.[45] and Gowenlock and Broughton[46] have shown that blind samples (controls unknown to the analyst) give a more reasonsable measure of S_m. Replicate analyses of a serum pool or an aqueous standard run on a single day will yield an underestimate of S_m.

In Table 2, 19 of the recently published normal ranges for total serum calcium for adults are given. The first eight used AAS, which is presumably more specific than the chemical methods. The differences in the observed means are possibly due to variations in the populations examined, but more likely the methods were standardized differently. The mean value, 2.65 m*M*, obtained by Oreskes et al., lies distinctly above the mean for all the rest.[47] If it is valid to consider the eight AAS methods together, then the mean total calcium lies between 2.3 and 2.4 m*M* and 4 S.D. lies between 0.3 and 0.4 m*M*. The remaining 11 methods had a slightly higher mean of 2.4 to 2.5 m*M* and 4 S.D. of 0.3 to 0.4 m*M*.

Admittedly, the combining of a number of normal ranges from the literature is open to question. Elveback et al. questioned the validity of assuming that the normal patient data distribution is Gaussian.[48] There is really no reason why it should be. For calcium, they found the distribution of results for 576 healthy persons to be slightly skewed to the low side, but there was good agreement between the mode and the mean. The 95% range excludes an approximately equal number of healthy persons at each end of the curve (see Reference 48, Figure 3).

In the face of the inbred tradition that the 95% range is "normal," the only recourse is to discourage the overinterpretation of borderline low and high values. These "normal" ranges will be used rather arbitrarily until we reach the ideal situation where each individual has his own normal range that has been developed over a period of years with unchanging, highly precise, and accurate methods.

A viable alternative proposed by Elveback is that each laboratory develop distribution of results for a healthy population by age and sex for each test.[49] A large number of individuals will have to be examined from each sex and age group to make the distributions valid. Results for a patient are then reported with the percentile corresponding to this value for healthy persons of the patient's age group and sex, e.g., calcium 2.6 mmole/l., 97th percentile. This is certainly a major improvement over the use of one normal range for all patients regardless of age and sex.

TABLE 2

Normal Values for Total Serum Calcium in Adults

Ref.	Method	Mean, mmole/l.	95% range (mean ± 2 S.D.)	n
Thin and Thomas, 1967[23]	AAS[a]	2.30	2.09 - 2.51	137
Pybus, 1968[17]	AAS	2.45	2.32 - 2.58	60
Oreskes et al., 1968[47]	AAS	2.65	2.24 - 3.06	50
Johnson and Riechmann, 1968[76]	AAS	2.39	2.20 - 2.58	304
Pybus et al., 1970[20]	AAS	2.38	2.20 - 2.55	NS[b]
Hattner et al., 1970[63]	AAS	2.45	2.31 - 2.59	23
Lott and Herman, 1971[27]	AAS	2.34	2.18 - 2.50	67
Li and Piechocki, 1971[64]	AAS	2.29	2.05 - 2.53	231
Yendt and Gagne, 1968[78]	EDTA titration	2.41	2.26 - 2.56	113
Sachs et al., 1969[68]	EDTA titration	2.41	2.21 - 2.61	22
Moore, 1970[37]	EDTA titration	2.48	2.19 - 2.77	70
Arnold et al., 1968[65]	AutoAnalyzer®	2.44	2.18 - 2.70	18
Robertson and Peacock, 1968[69]	AutoAnalyzer®	2.40	2.23 - 2.55	47
Seamonds et al., 1972[39]	AutoAnalyzer®	2.50	2.34 - 2.66	84
Radde et al., 1971[66]	Flame photometry	2.49	2.13 - 2.85	13
Harris and DeMets, 1971[79]	Flame photometry	2.55	2.41 - 2.69	68
Farese et al., 1970[80]	Glyoxal	2.58	2.40 - 2.76	22
Raman et al., 1970[77]	Glyoxal	2.41	1.85 - 2.96	17
Elvebach et al., 1970[48]	NS	2.37	2.22 - 2.52	576

[a]Atomic Absorption Spectrophotometry
[b]Not Specified

Quality Assurance and Standardization

The concept of quality control or quality assurance is well accepted in most clinical laboratories. The goal of a quality assurance program should be to upgrade the system and increase its reliability. The methods by which quality assurance is carried out differ widely. Many laboratories run control sera with their unknowns. However, long-term data-keeping of the control results is often not done, so that drift or shifts of means are overlooked. If blind controls are used, the feedback of out-of-control values to the laboratory should be very rapid to prevent, if possible, incorrect patient results from leaving the laboratory. It does little good to use sophisticated computer routines in a quality assurance scheme if the out-of-control results do not reach the laboratory for several days or weeks. The ultimate goal is an on-line quality assurance program which provides instantaneous comments when blind results are out of control.

A simple goal-oriented scheme for quality assurance was described recently by Grannis et al.[50] An "operational line" is obtained by analyzing mixtures of high- and low-value controls prepared in simple ratios. The determined values are plotted against the calculated values for the controls and from this, the precision and the presence of standardization errors can be estimated. An estimate of the accuracy can also be made if the commercially available serum controls are assumed to contain the labeled values. No elaborate knowledge of statistics is needed and the program can be implemented in small or large laboratories.

Any doubts about the need for a quality assurance program for calcium determinations by AAS can be quickly dispelled by reading the paper of Sideman et al.[51] Their interlaboratory survey revealed that even the most specific method for calcium can give quite unsatisfactory results. Although, as was concluded above, the error

should not exceed one fourth of the normal range or 0.1 mmole/l., approximately 22% of the results in his survey were outside these limits.[51]

Gross errors in calcium determinations have been reported in cases where calcium was leached out of cork stoppers into serum.[52] Rubber stoppers did not cause contamination. Foster et al.[53] and Pragay et al.[54] found calcium contamination in red-top Vacutainers.* The first group found up to 14 μg of calcium in a 15-ml tube, which would add as much as 0.025 mmole/l. to the measured calcium value in a full tube of blood. Pragay et al. found between 0.9 and 3.1 μg of calcium in a 10-ml Vacutainer.[54] Acid washing reduced the calcium contamination to a negligible amount.

Highly purified (99.9%) and certified calcium carbonate is available from the National Bureau of Standards (NBS) as Standard Reference Material (SRM) number 915.[55] Calcium carbonate is a highly stable reagent. It can be freed of superficial moisture at 210° without decomposition; it decomposes only at temperatures in excess of 625°, and it has a high equivalent weight. NBS samples equilibrated with air at 90% relative humidity picked up only 0.02% water by weight at the most.[55] SRM calcium carbonate is expensive but it can serve as the primary standard to which other sources of calcium carbonate can be compared (see, e.g., Reference 56). It is the reference standard of choice for calcium determinations in the clinical chemistry laboratory.

Summary of AAS

Atomic absorption spectrophotometry can be a highly specific, accurate, and sensitive technique for the measurement of total serum calcium. It does not enjoy a wide following in clinical laboratories, probably because it is a complex method and requires costly instrumentation. Chemical interferences due to sodium, potassium, phosphate, and other anions have been overcome by diluting sera with acidic solutions of lanthanum or strontium salts. The more serious interference due to serum proteins has been dealt with by making a protein-free filtrate, by dilution and direct aspiration of the sample, and by aspiration of a dialyzate from the AutoAnalyzer.® All of these approaches have disadvantages such as many pipetting steps, clogging of the burner/aspirator, and preparation of complex standard solutions. The method of choice depends largely on how many samples must be examined at one time.

ESTIMATION OF IONIZED CALCIUM ACTIVITY

Introduction

More than 35 years ago, McLean and Hastings showed rather clearly that it is the ionized form of calcium that is the physiologically active form.[57-59] Ionized calcium means that part of the total serum calcium that is not associated with protein or with complexing anions, such as phosphate or sulfate, and a host of other anions present in low concentration which form molecular aggregates with calcium. The ionized calcium concentration can be estimated quite well from the McLean-Hastings nomogram if the total protein and serum calcium are known.[58] The assumptions that are made in the nomogram are a physiological pH, a constant binding affinity of the serum proteins, and a constant albumin/globulin ratio. A refinement would be to introduce the effect of a pH other than 7.4 and the effect of varying albumin/globulin ratios. This would make the estimate of ionic calcium better but would also make it much more cumbersome to calculate. The ionic calcium activity can be measured directly with an electrode. The equipment for doing this is expensive and requires special attention. It is safe to predict that the McLean-Hastings nomogram will be in use for a long time to come and will continue to provide useful information as long as its limitations are kept in mind.

The many methods that have been used to estimate ionized calcium are listed by Moore.[37] The bioassay methods with the frog heart were difficult to perform. The chemical methods using murexide required separation of proteins and heavy metals before analysis.

Calcium-specific Electrodes

Potentiometric methods for ionized calcium in serum were unsuccessful until Ross introduced his liquid ion-exchanger electrode in 1967.[60] Measurement with the calcium-specific electrode yields values of the ionized calcium *activity* rather than the ionized calcium *concentration.* If the standards that are used to calibrate the electrode have the same ionic strength as serum, then the measured

*Becton-Dickinson and Co., Rutherford, N. J. 07070.

activities can be expressed as concentrations. Expressing ionized calcium in concentration units assumes that the standards and unknowns are in fact identical in every way and behave in the same way toward the electrode.

The first generation calcium electrode as described by Ross and marketed by Orion* contained the calcium salt of didecylphenylphosphoric acid.[60] The salt was dissolved in a water-immiscible solvent, diphenylphosphonate. The solution was used to impregnate an inert matrix such as a filter membrane and in this way the membrane became the ion-selective surface, analogous to the sensitive glass in a pH-responsive electrode. The inert matrix membrane saturated with exchanger separated the sample to be measured from the electrode's internal filling solution and the internal reference electrode (silver/silver chloride). The potential developed across the membrane was related to the calcium-ion activity of the sample in contact with the membrane. As with any potentiometric measurement, a reference electrode was required which also had to contact the sample.

The exchanger in the original, and also in the current, calcium electrode is slowly leached from the membrane due to its finite water solubility, and this in part limits the useful life of the electrode. The electrode can be "rebuilt" by renewing the membrane, exchanger solution, and the internal filling solution.

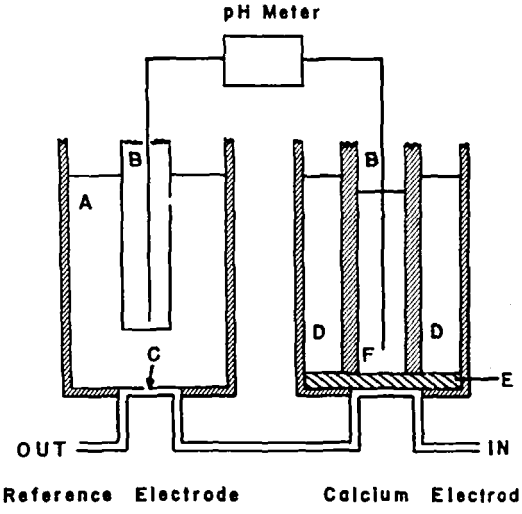

FIGURE 3. Diagrammatic representation of cross section of calcium and reference electrodes. A, 1 *M* KCl; B, Ag/AgCl reference electrodes; C, pinhole junction; D, calcium organophosphate salt dispersed in organic solvent; E, membrane saturated with liquid exchanger; F, 0.02 *M* Ca^{++} plus chloride internal filling solution.

Selectivity of the Calcium Electrode (Orion®)

The calcium liquid ion-exchanger electrode has a high specificity for calcium over sodium, potassium, and magnesium. The selectivity constant, K_{Ca}^{Na}, of sodium over calcium is 10^{-4}, i.e., calcium is much preferred over sodium. The constants for potassium, K_{Ca}^{K}, and magnesium, K_{Ca}^{Mg}, are 10^{-4} and 10^{-2}, respectively.[37] Ross found similar results for the selectivity constants, K_{Ca}^{Na} and K_{Ca}^{Mg} in serum, namely 10^{-4} and 1.4×10^{-2}.[60] Above pH 6 and at 10^{-4} *M* Ca^{++} and above, the electrode does not respond to hydrogen ions. Below about pH 6, strong interference from hydrogen ions occurs at 10^{-4} *M* Ca^{++} and below. Below pH 5, the electrode acts as a pH-sensitive electrode and cannot be used for calcium.

Assuming Nernstian behavior, a 10-fold change in the activity of calcium ion produces a 29.5-mV change in response at 25° and 30.8 mV at 37°. A 10-fold change in the sodium activity would produce a 59.1×10^{-4} mV change in response. The equation proposed by Ross[60] for the measured response is

$$E_{measured} = \text{Constant} + \frac{0.0591}{2} \log_{10} \left[(Ca^{++}) + \sum_i K_i (A_i) \right]^{2/z_i}$$

The "constant" in the equation includes the potential of the reference electrode, the potential of the calcium electrode at unit activity of calcium and unit activity of the interfering cations, and the liquid-junction potential. A_i is the activity of the interfering ion of charge z_i, and K_i is the selectivity constant described above.

Although the change in response of the electrode to a change in sodium activity is small, sodium at physiological levels affects the measured potential. In serum, the presence of 150 m*M* sodium and 0.5 m*M* magnesium enhances the response to calcium by about 2%.[1] Inclusion of 150 m*M* sodium chloride in the standards is common practice and corrects for part of the enhancing effect. Ladenson and Bowers also added magnesium to the standards and reported greater stability in its presence.[61] Moore found a linear

*Orion Research Inc., 11 Blackstone Street, Cambridge, Mass. 02139.

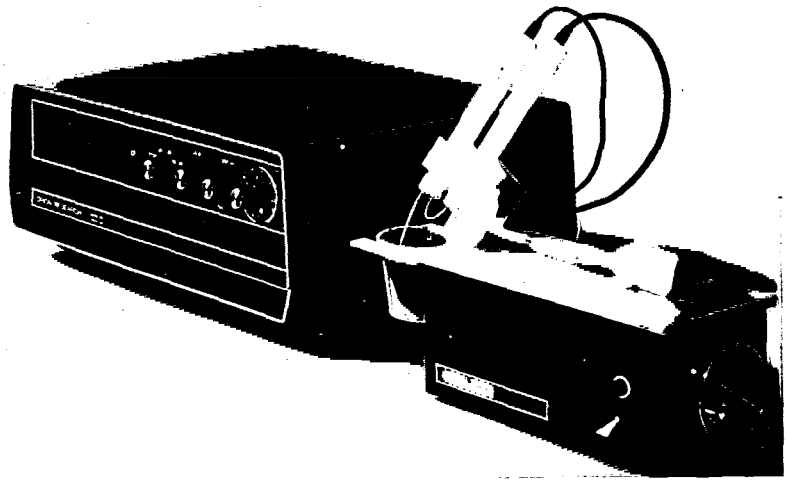

FIGURE 4. Exterior view of Orion® calcium and reference electrodes. (Courtesy Orion Research, Inc.)

response of the electrode between 0.5 and 10 m*M* $CaCl_2$ when all of the solutions contained 150 m*M* NaCl.[1] He estimated the activity coefficient of calcium ion in these standards to be about 0.54. Since the calibration curve was linear, it is reasonable to assume that the activity coefficient for calcium was constant over the range of calcium concentrations used. The ionic strength of serum is about the same as that of the standards, hence the assumption that the *change in response* of the electrode in serum is also proportional to the *change in concentration* or ionized calcium is reasonable. But since the uncertainty in the activity coefficient of calcium in serum is large, the uncertainty in the expressed concentration of serum ionized calcium in a sample is also large. It is possible to talk of ionized calcium "concentrations" when measured by electrode provided the conditions of standardization are clearly defined and that the liquid-junction potentials for standards and unknowns are not very different in the measuring system.[62]

Responsivity of the Orion® Electrode

Hattner et al. reported a 7.5-8-mV change in potential when the calcium concentration was changed from 0.5 to 1 m*M* or from 1 to 2 m*M* at 23° with the Orion® 99-20 calcium electrode.[63] Li and Piechocki found an 8 to 8.8-mV change (temperature not given) for the same conditions and electrode.[64] The Nernst equation requires a 8.9-mV change at 25° for the above changes in concentration, i.e., when the calcium concentration is doubled. The change in potential over the normal range of ionized calcium of, e.g., 1 to 1.3 m*M*, is only 3.9 mV, so accurate and drift-free measurements of potential are required. Given an 8-mV span between the 1 and 2 m*M* calcium standards, an error of only 1 mV introduces an 8.5% error, which corresponds to one fourth of the width of the normal range. An arbitrary span of 7.5 mV between the above standards has been given by Orion® as the point below which the calcium and/or the reference electrode should be rebuilt. The error introduced by a 1-mV drift becomes even larger as the span gets smaller.

Dip-type vs. Flow-through Electrodes

The first type of calcium electrode was of the probe or dip type (Orion® 92-20). This electrode and a reference electrode were immersed in the standard or serum, usually in a micro dish. This technique was used by Arnold et al.[65] and Oreskes et al.[47] The serum sample was exposed to air and the measurement had to be made quickly to minimize the error caused by a change in pH due to a loss of CO_2. The ionized calcium concentration is very sensitive to pH changes, as will be discussed shortly. Moore enclosed the electrodes in a chamber where both the temperature (37°) and the CO_2 tension could be controlled.[1]

The currently available flow-through electrode assembly (Orion® 99-20) greatly simplifies the determination of ionized calcium. The system is wholly enclosed so that determinations are possible without exposing the sample to air. Greater precision is possible with this electrode and the

measurements can be made in a more leisurely manner.

Collection of Blood for Determinations of Ionized Calcium

A variety of techniques have been used to collect blood and to protect it from interferences. Venous stasis must be avoided, since this tends to concentrate plasma proteins and decreases the ionized calcium fraction. Heparinized blood has been used and was found to give lower values for ionized calcium than serum.[37,47,64] Radde et al. added 2.4 to 2.5 units of heparin per ml of blood and found heparin to be without effect.[66] Moore collected blood with oil in the syringe, carried out the centrifugation under oil, and removed a sample for the estimation of ionized calcium without allowing the sample to be exposed to air.[37] Hattner et al. transferred the blood from a syringe directly into a test tube containing oil, allowed it to clot, and then centrifuged.[63] Li and Piechocki used siliconized Vacutainers for collection and found that incompletely filled tubes gave lower results for ionized calcium than completely filled ones did, presumably because of the loss of carbon dioxide and increase in the serum pH.[64] They found no difference between samples collected in syringes, filled into Vacutainers and capped, and samples collected directly into Vacutainers. Oreskes el al. also recommended the use of siliconized Vacutainers.[47] Schwartz et al. found that serum in a well-filled Vacutainer and in contact with the clot maintained its pH for at least eight hours at an unstated, but probably ambient, temperature.[67]

Siliconized Vacutainers have a disadvantage in that clotting is often delayed and it is common to have a clot that is hard to spin down. Plain Vacutainers are superior here, provided that they are completely filled and the possibility of calcium contamination is checked. Handling specimens under oil is a nuisance and should be avoided if possible. Oil has a way of getting over everything to the detriment of the measurement, particularly if any oil reaches the calcium-ion-selective membrane.[65]

Preservation and Storage of Samples

The stability of the blood specimens after collection is an important consideration if the analysis cannot be carried out at once. Serum has extremely little buffering ability. Loss of carbon dioxide after exposure to air is rapid and is accompanied by undesirable changes in pH. The control of pH is vital and is discussed in the next section. The several published studies invariably recommended or performed the determination of ionized calcium shortly after collection. Moore found that serum specimens could be frozen under oil for an unstated period of time and the original pH restored with 5% CO_2 prior to measurement.[37] Freezing apparently did not have an effect. Schwartz et al. recommended storage at refrigerator temperature if necessary, and were able to reproduce the fresh-serum ionized calcium value to within 0.8% of the original value provided the pH was adjusted to the original value by bubbling a 10% CO_2/air mixture.[67] The original (fresh) ionized calcium value could not be restored within a change of less than 3.1% from the original when samples were stored at room temperature or frozen and treated with 10% CO_2/air to reconstitute the pH. They recommended against storage at room temperature or freezing.[67]

Hattner et al. found that ionized calcium values were reproduced within ± 2.5% during a 12-hr period after collection, presumably at room temperature, for samples stored under oil.[63] They also found that freezing and subsequent reconstitution of pH with 5% CO_2 − 95% O_2 gave results that were not statistically different from the fresh-serum values. They correctly questioned the validity of an approach where all sera are reequilibrated with 5% CO_2 − 95% O_2, particularly those sera with an abnormal original pH or partial pressure of carbon dioxide.[63] Li and Piechocki found the ionized calcium of serum in contact with the clot in an unopened (presumed) container to be stable for two days at 4°, but to decrease 3% after 7 days at 4°.[64] Oreskes et al. recommended storage in ice water if prompt analysis is not possible.[47] Radde et al., who used heparin, found that exposure of whole rat blood to air for 5 minutes was without effect on the ionized calcium value, but this was not true for rat plasma.[66] Whole rat blood could be stored for up to two hours in a syringe at refrigerator temperatures without changing the ionized calcium. Oreskes et al. found that plasma (heparin) could be stored in the refrigerator for up to six hours without a significant change in the ionized calcium.[47] But after 24 hours a significant drop occurred.

The simplest storage technique appears to be successful. If the analysis cannot be done at once, the blood in the full, unopened plain Vacutainer is

allowed to clot, the clot spun down with the top on, and the tube stored unopened at 4°. The data of Li and Piechocki indicate that the value decreases only 0.04 mmole/l. after 7 days of storage under these conditions.[64] The container is brought to room temperature and opened only immediately before analysis. It is recommended to check the pH after storage and to be alert to possible contamination of the glassware by calcium.

Effects of pH *in vivo* and *in vitro*

Hypocalcemic tetany, commonly associated with convulsions, is a well-known clinical manifestation. Hypocalcemia is particularly dangerous in the alkalotic patient since he may have an uncompensated decrease in ionized calcium due to increased binding of calcium by proteins. A simplistic view is that hydrogen and calcium ions compete for binding sites on the plasma proteins. Hydrogen ions can displace calcium from protein to raise the ionized calcium; conversely, more calcium is taken up by proteins as the hydrogen-ion activity drops or the pH increases. This phenomenon has now been amply demonstrated both in vitro and in vivo.

In the first 3 studies listed in Table 3, serum ultrafiltrates were examined for pH effects. The pH was varied using HCl and NaOH;[68] CO_2, NaOH, and lactic acid;[69] and HCl and CO_2.[70] The concentration of calcium in the ultrafiltrates was measured by chemical methods. Moore[37] and Schwartz et al.[67] varied the pH with CO_2 mixed with air and used the electrode to measure ionized calcium. The in vivo variation of pH in the study of Seamonds et al. was produced by hyperventilation.[39]

The ultrafiltrable calcium measured chemically and the ionized calcium activity as determined potentiometrically are not the same, as discussed earlier, but nevertheless, the change in calcium concentration (ultrafiltrable or ionized) with changing pH is remarkably similar in the six studies. In all cases, the slope of the line was measured as close to pH 7.4 as possible (Table 3, column 2). In some cases the slope had to be estimated from figures available in the papers, so that there is at least a 5% uncertainty in the stated values.

The effect of pH on ionized calcium activity would be expected to be greater than the effect of pH on the ultrafiltrable calcium. The change in

TABLE 3

Influence of pH on Ionized and Ultrafiltrable Serum Calcium

Ref.	Slope of line, $\Delta [Ca^{++}]/\Delta$ pH, mmole/pH unit	Slope determined at pH	T	Samples	pH range studied (shape of curve $[Ca^{++}]$ vs. pH)
Loken et al., 1960[32]	−0.39 (ultrafiltrable calcium)	7.4	37	15 sera	4.65 - 9.7 (sigmoid)
		7.4	37	30 sera	6.5 - 8.5 (sigmoid)
Robertson and Peacock, 1968[69]	−0.41 (ultrafiltrable calcium)	7.4	37	ca.10 sera	6.5 - 7.7 (curved)
Pederson, 1971[70]	−0.47 (ultrafiltrable calcium)	7.4 7.4	37	12 pooled sera	7 - 8 (sigmoid)
Moore, 1970[37]	−0.42 (ionized calcium)	7.4	37	52 sera	6.8 - 7.8 (sigmoid)
Schwartz et al., 1971[67]	−0.56 (ionized calcium)	7.46	27	4 pooled sera	7 - 8 (linear)
Seamonds, 1972[39]	−0.50 (ionized calcium, in vivo)	7.4	37	3 subjects	7.38 - 7.61 (linear)

ultrafiltrable calcium concentration is due to the change in protein binding with pH. The change in ionized calcium with pH reflects both the change in protein binding and the increase or decrease in complexation with ultrafiltrable ions such as phosphate, sulfate, citrate, etc. Moore observed a change in the potentiometrically determined calcium-ion activity of an ultrafiltrate when the pH of the ultrafiltrate was changed.[37] This was attributed primarily to a change in solubility of the ultrafiltrable calcium complexes. The slopes in Table 3 largely agree with this view. Schwartz et al. observed the largest change in ionized calcium with pH.[67] Their data imply that a change of 0.1 unit in the pH will change the concentration of ionized calcium by 0.06 mmole/l., which is about 20% of the normal ionized calcium span.

Jacobs et al. surprisingly did not observe a regular decrease in calcium binding of bovine serum albumin with decreasing pH.[71] They varied the pH from 7.2 to 7.6 and state that "only at a high total calcium concentration is there any substantial pH influence in calcium binding. In the physiologic range of calcium concentration (i.e., 2.5 mequiv [sic]) there is virtually no difference between bound calcium over the hydrogen ion concentrations studied."[71]

Effects of Temperature on Ionized Calcium

The effect of temperature on the binding of calcium by protein is complex. When the temperature is raised above 25°, the value of pK_w for water decreases, so that the extent of dissociation into hydrogen and hydroxyl ions increases and the pH-value coresponding to neutrality decreases below 7. Accordingly, the hydrogen-ion activity increases, and this would be expected to decrease the fraction of the calcium that is bound. In addition, carbonic acid becomes a stronger acid at higher temperatures. Gupta found that increasing the temperature increases the binding of calcium by protein.[72] The net effect of increasing the temperature on the ionized calcium was found by Moore[37] to be very small. He found the mean of two groups of normals for values obtained at 25° and 37° to be 1.161 and 1.169 mmole/l. for ionized calcium. By contrast, Hansen and Theodorson found values at 37° to be, on average, 0.02 mmole/l. *lower* than at 24°.[73]

There is no particular reason why ionized calcium should be determined at "ambient" temperatures rather than at 37°. The sensitivity is slightly better at 37°, where a 10-fold increase of the calcium-ion activity causes the Nernstian potential to increase 30.8 mV rather than 29.6 mV as it does at 25°, but this small increase of sensitivity entails careful thermostating of the system, which is not easy. As 37°, the in vivo state is mimicked more closely, which has some advantage. What would be desirable is to select a uniform temperature which everyone would use and to thermostat the system at that temperature. A good choice would be 25°, which could be maintained with a suitable enclosure for the electrodes, a thermostat having a small heater and a circulating fan. Physiological temperatures (37° to 38°) would be harder to maintain since the system would have to reestablish temperature after every disturbance in which a new sample is put in place, the pump is started, etc. A water-jacked system for the Orion® 99-20 electrode would certainly facilitate thermostatting.

Standardization

The standards used to calibrate the calcium ion-specific-electrode should resemble the unknowns as closely as possible. Bates and Alfenar have examined the effect of different liquid-junction potentials between unknowns and standards and stated that "the uncompensated liquid-junction potential may cause some of the most serious errors encountered in the experimental pA measurements".[62] The liquid-junction potential cannot be eliminated but its effect on the accuracy of the measurement can be minimized by having standards with the same ionic strength and compositions as the unknowns.

Ideally, the standards should be prepared from freshly pooled sera containing known amounts of protein, calcium, sodium, potassium, and magnesium, as well as of chloride, phosphate, and other anions, and have a pH of 7.45 ± 0.05. These standards would then be appropriate for all sera except those with a marked electrolyte or protein imbalance.

The standards in current use all contain 0.150 *M* sodium chloride and 0.5 to 2 m*M* calcium chloride. The sodium chloride brings the ionic strength of the standards close to that of serum and compensates for the sodium response of the electrodes. Magnesium has been omitted from the standards in most cases, but there has been one exception.[61] Trypsin (for its proteolytic effect?) and triethanolamine (TEA) (to bring the pH above

6?) have been added by some authors to their standards (see Table 4). Hattner et al.[63] found equilibration to be more rapid in the presence of trypsin and TEA and Lindgaerde and Zettervall[74] reported that the reproducibility was better when trypsin and TEA were present.

Some workers found that TEA, and possibly also trypsin, decreased the mV response of the

TABLE 4

Normal Values for Serum Ionic Calcium in Adults: Orion® Flow-Through Electrode

Ref.	Composition of standards	Temp.	Mean, m*M*	95% range (mean ± 2 S.D.)	n
Sachs et al., 1969[68]	$CaCl_2$, 0.25-2 m*M* NaCl, 150 m*M*	Ambient	1.10	0.90 - 1.30	22
Raman, 1970[77]	$CaCl_2$, 0.1 - 2 m*M* NaCl, 150 m*M*	Ambient	1.18	0.83 - 1.53	17
Hattner et al., 1970[63]	$CaCl_2$, 0.5 - 2 m*M* NaCl, 150 m*M* trypsin, 60 mg TEA[a], 3 drops/100 ml[b]	23°	0.96	0.88 - 1.04	23
Moore, 1970[37]	$CaCl_2$, 0.5 - 10 m*M* NaCl, 150 m*M*	37°. 25°	1.14	1.01 - 1.27	70
Schwartz et al., 1971[67]	$CaCl_2$, 0.5 - 1 m*M* NaCl, 150 m*M* trypsin, 60 mg TEA[a], 0.15 ml/100 ml[b]	27°	0.97	0.88 - 1.06	30
Lingärde and Zetterval, 1971[74]	$CaCl_2$ 1 - 2 m*M* NaCl, 150 m*M* trypsin, 60 mg TEA[a], 0.15 ml/100 ml	Ambient	1.23	1.12 - 1.34	297
Li and Piechochi, 1971[64]	$CaCl_2$ 0.5 - 2 m*M*, NaCl 150 m*M* trypsin TEA[c]	25°	1.22	1.13 - 1.21	397
Radde et al., 1971[66]	$CaCl_2$, 0.1 - 4 m*M* NaCl, 150 m*M* tryspin, 60 mg TEA[a], 0.015 ml (sic)/100 ml	Ambient	1.11	1.00 - 1.22	13
Hansen and Theodorson, 1971[73]	N.S.[c]	24°	1.10	1.02 - 1.18	35
Seamonds et al., 1972[39]	$CaCl_2$, 0.5 - 2 m*M* NaCl, 150 m*M* trypsin, 60 mg TEA[a], 0.060 ml/100 ml	37°	1.08	1.02 - 1.14	84
Ladenson and Bowers, 1972[61]	$CaCl_2$ plus Na, K, and Mg in physiological amounts	Ambient	1.29	1.20 - 1.38	N.S.

[a] 1 *M* triethanolamine solution
[b] Value of standards adjusted for depressing effect on ionic calcium of trypsin and TEA
[c] N.S.: Not Specified

calcium electrode below that obtained with the same standards without trypsin and TEA.[39, 63,67] Seamonds et al. reported that TEA had an effect on the standards: as the concentration of TEA was increased, higher ionized calcium values were found for unknowns.[39] If the standards turn out to have lower values than the assumed values, then the unknowns read from the calibration curve will of course yield falsely high results. This may explain why some normal values are generally higher than the rest.[74] It is unlikely that these substantially higher values for ionized calcium represent a genuine difference in the populations being examined. Rather, it is due to the difference in standardization.

Others recognized the depressing effect of TEA and trypsin and adjusted the values of their standards accordingly.[63,67] Schwartz et al. found the calcium activity of their standards was reduced by 12.5, 11.8, and 8.5% for their 0.5, 1, and 2 m*M* calcium standards, respectively, when TEA and trypsin were present.[67] But are the normal values of Schwartz et al.[67] and Hattner et al.[63] too low? Is the correction they applied to their standards possibly an overcorrection? The answer to both questions is, possibly, yes, in view of Moore's findings in which no TEA or trypsin were used,[37] and the studies by Seamonds et al., in which about 60% less TEA was used.[39] Some careful experimentation with TEA and trypsin can lay these questions to rest. This is not to imply that the stated normals in Table 4 are invalid. They have validity, provided the specified standards continue to be prepared and used the same way.

Li and Piechocki reported a marked change (0.22 mmole/l.) in the measured serum ionized calcium when 4.2 *M* KCl was substituted for 1 *M* KCl in the reference electrode filling solution.[64] The mV span observed for the standards shifted in position on the scale but the span value remained unchanged. The KCl must of course contact the serum sample being measured. Since 4.2 *M* KCl causes a slow denaturation and coagulation of serum proteins, this may be the cause of their observed effect on serum. One molar KCl usually does not precipitate proteins and should be retained.

Table 4 includes studies made with the Orion® flow-through calcium electrode only, with the exception of some data from Moore obtained with the dip-type electrode in a thermostatted chamber of controlled CO_2-content.[37] Other results with the older dip-type electrode have not been included since the sample was exposed to air and it was very difficult to control the pH of the serum.

Normal Range of Ionized Calcium

Judging from Table 4, the normal range for ionized calcium is a composite of the population variation, the analytical error, and the method of standardization. The last factor appears to play a major role in determining the midpoint of the normal range for some of the studies. It is very hazardous to estimate the "true" normal range from the available data.

Summary of Ionized Calcium Determinations

The editor of *Laboratory Medicine,* Dr. John B. Henry, introduced an article on ionized calcium measurements by M.J. Arras (1969) with the following comments:[75]

> "Measurements of serum ionized calcium appear to offer greater diagnostic sensitivity as well as pathophysiologic reflections of disturbances in calcium homeostasis. Indeed, an ionized calcium determination may supplement if not supplant serum total calcium measurements in clinical laboratories in the near future due to availability of an improved specific calcium electrode described in this report."

Unfortunately, the technical developments of the calcium-specific electrode have not kept pace and even at this writing, the calcium electrode is not ready to replace total serum calcium determinations. The calcium electrode is considerably inferior for ionized calcium measurements to the glass electrode for pH measurements. Determinations of ionized calcium are time consuming and require an operator with skill and judgment. Small changes in pH and drift of the potential lead to large errors in the determined values. The equipment is costly, and the electrode requires frequent maintenance and tends to be temperamental.

There is disagreement in the published reports as to which standards are suitable and this is obvious in the wide variation of reported normal values. Thermostatting at 25° is recommended and will require some modification of the Orion® flow-through system. To date, the only calcium electrode to be investigated in some detail for use in the clinical laboratory is the Orion® electrode. Its preeminence in this area is yet to be challenged. At present, the calcium electrode is primarily a research tool and has some way to go before it will be found in most large routine clinical chemistry laboratories.

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REFERENCES

1. Moore, E. W., Studies with ion-exchange calcium electrodes in biological fluids: some applications in biomedical research and clinical medicine, in *Ion-Selective Electrodes,* Durst, R. A., Ed., NBS Special Pub. No. 314, Washington, D. C., 1969, chap. 7.
2. West, E. S., Todd, W. R., Mason, H. S., and van Bruggen, J. T., *Textbook of Biochemistry,* 4th ed., Macmillan, New York, 1966.
3. Drach, G. W. and King, J. S., Jr., Estimating aberrant homeostasis: variance in serum calcium concentrations as an aid in diagnosis of hyperparathyroidism, *Clin. Chem.,* 16, 792 (1970).
4. Galante, L., Gudmundsson, T. V., Mathews, E. W., Tse, A., Williams, E. D., Woodhouse, N. J. Y., and MacIntire, I., Thymic and parathyroid origin of calcitonin in man, *Lancet,* 2, 537 (1968).
5. Raisz, L. G., The diagnosis of hyperparathyroidism (or what to do until the immunoassay comes), *N. Engl. J. Med.,* 285, 1006 (1971).
6. Kleeman, C. R., Massry, S. B., and Coburn, J. W., The clinical physiology of calcium homeostasis, parathyroid hormone, and calcitonin, *Calif. Med.,* 114, 16 (1971).
7. Boonstra, C. E. and Jackson, C. E., Serum calcium survey for hyperparathyroidism: results in 50,000 clinic patients, *Am. J. Clin. Pathol.,* 55, 523 (1971).
8. Transbol, I., Hahnemann, S., and Hornum, I., Ionized, ultrafiltrable and total calcium in serum in hyperparathyroidism, *Acta Endocrinol.,* 65, 385 (1970).
9. Netter, F. H., Parathyroid disorders and metabolic bone disease, in *The Ciba Collection of Medical Illustrations,* Vol. 4, *The Endocrine System,* Forsham, P. H., Ed., Ciba Pharmaceutical Prod., Summit, N. J., 1965.
10. Walsh, A., The application of atomic absorption spectra to chemical analysis, *Spectrochim. Acta,* 7, 108 (1955).
11. Willis, J. B., The determination of metals in blood serum by atomic absorption spectroscopy. I. Calcium, *Spectrochim. Acta,* 16, 259 (1969).
12. Elwell, W. T. and Gidley, J. A. F., *Atomic Absorption Spectrophotometry,* Macmillan, New York, 1962.
13. Robinson, J. W., *Atomic Absorption Spectroscopy,* Marcel Dekker, New York, 1966.
14. Slavin, W., *Atomic Absorption Spectroscopy,* Interscience, New York, 1968.
15. Thiers, R. E. and Hviid, K., Interference-free flame photometry of calcium in serum and urine, *Clin. Chem.,* 8, 35 (1962).
16. Zettner, A. and Seligson, D., Application of atomic absorption spectrophotometry in the determination of calcium in serum, *Clin. Chem.,* 10, 869 (1964).
17. Pybus, J., Determination of calcium and magnesium in serum and urine by atomic absorption spectrophotometry, *Clin. Chim. Acta,* 23, 309 (1968).

18. College of American Pathologists Survey Program for 1971, listing of Laboratories and Methodologies; see Skendzel, L. P., Guidelines for the design of laboratory surveys, *Am. J. Clin. Pathol.*, 54, 437 (1970).
19. Laitinen, H. A., *Chemical Analysis*, McGraw-Hill, New York, 1960.
20. Pybus, J., Feldman, F. J., and Bowers, G. N., Jr., Measurement of total calcium in serum by atomic absorption spectrophotometry, with use of a strontium internal reference, *Clin. Chem.*, 16, 998 (1970).
21. Paschen, K. and Fuchs, C., A new micro-method for Na, K, Ca, and Mg determinations in a single serum dilution by atomic absorption spectrophotometry, *Clin. Chim. Acta*, 35, 401 (1971).
22. Sunderman, F. W., Jr. and Carroll, J. E., Measurements of serum calcium and magnesium by atomic absorption spectrophotometry, *Am. J. Clin. Pathol.*, 43, 302 (1965).
23. Thin, C. G. and Thomas, P. A., Estimation of calcium and magnesium in serum and urine by atomic absorption spectrophotometry, *J. Clin. Pathol.*, 20, 280 (1967).
24. Monder, C. and Sells, N., Influence of acids on the determination of calcium and magnesium by atomic absorption spectrophotometry, *Anal. Biochem.*, 20, 215 (1967).
25. Savory, J., Wiggins, J. W., and Heintges, M. G., Measurements of calcium and magnesium in serum and urine by atomic absorption spectrometry, *Am. J. Clin. Pathol.*, 51, 720 (1969).
26. Rodgerson, D. O. and Moran, I. K., Comparison of atomic absorption spectrophotometry and fluorometry for the measurement of serum calcium, *Clin. Chem.*, 14, 1206 (1968).
27. Lott, J. A. and Herman, T. S., Increased AutoAnalyzer dialysis of calcium and magnesium in presence of protein, *Clin. Chem.*, 17, 614 (1971).
28. Trudeau, D. L. and Freier, E. F., Determination of calcium in urine and serum by atomic absorption spectrophotometry (AAS), *Clin. Chem.*, 13, 101 (1967).
29. Klein, B., Kaufman, J. H., and Morgenstern, S., Determination of serum calcium by automated atomic absorption spectroscopy, *Clin. Chem.*, 13, 388 (1967).
30. Murdoch, J. A. and Heaton, F. W., Enhancement of calcium absorption in the nitrous oxide-acetylene flame by sodium and potassium, *Clin. Chim. Acta*, 28, 505 (1970).
31. Bowers, G. W., Jr., Smith, S. B., and Feldman, F. J., A study of factors affecting the determination of calcium in serum by atomic absorption spectroscopy, *Clin. Chem.*, 14, 846 (1968) (abst.).
32. Loken, H. E., Havel, R. J., Gordan, G. S., and Whittington, S. L., Ultracentrifugal analysis of protein-bound and free calcium in human serum, *J. Biol. Chem.*, 235, 3654 (1960).
33. Gimblet, E. G., Marney, A. F., and Bonsnes, R. W., Determination of calcium and magnesium in serum, urine, diet, and stool by atomic absorption spectrophotometry, *Clin. Chem.*, 13, 204 (1967).
34. Gochman, N. and Givelber, H., Automated simultaneous microdetermination of calcium and magnesium by atomic absorption, *Clin. Chem.*, 16, 229 (1970).
35. Rieman, W., III and Breyer, A. C., Chromatography: columnar liquid-solid ion-exchange processes, in *Treatise on Analytical Chemistry*, part I, Vol. 3, Kolthoff, I. M., Elving, P. J., and Sandell, E. B., Eds., Interscience, New York, 1961.
36. Amador, E. and Neeley, W. E., Automated serum calcium analysis corrected for dialysis effects, *Am. J. Clin. Pathol.*, 58, in press.
37. Moore, E. W., Ionized calcium in normal serum, ultrafiltrates, and whole blood determined by ion-exchange electrodes, *J. Clin. Invest.*, 49, 318 (1970).
38. Keating, F. R., Jr., Jones, J. D., Elveback, L. R., and Randall, R. V., The relation of age and sex to distribution of values in healthy adults of serum calcium, inorganic phosphorous, magnesium, alkaline phosphatase, total proteins, albumin, and blood urea, *J. Lab. Clin. Med.*, 73, 825 (1969).
39. Seamonds, B., Towfighi, J., and Arvan, D. A., Determination of ionized calcium in serum by use of an ion-selective electrode, *Clin. Chem.*, 18, 155 (1972).
40. Annino, J. S. and Relman, A. S., The effect of eating on some clinically important chemical constituents of the blood, *Am. J. Clin. Pathol.*, 31, 155 (1959).
41. Elking, M. P. and Kabat, H. F., Drug induced modifications of laboratory test values, *Am. J. Hosp. Pharm.*, 25, 485 (1968).
42. Singh, H. P., Hebert, M. A., and Gault, M. H., Effect of some drugs on clinical laboratory values as determined by the Technicon SMA 12/60, *Clin. Chem.*, 18, 137 (1972).
43. Christian, D. G., Drug interference with laboratory blood chemistry determinations, *Am. J. Clin. Pathol.*, 54, 118 (1970).
44. Gowenlock, A. H., The influence of accuracy and precision on the normal range, *Ann. Clin. Biochem.*, 6, 3 (1969).
45. Allen, J. R., Earp, R., Farrell, E. C., Jr., and Gruemer, H.-D., Analytical bias in a quality control scheme, *Clin. Chem.*, 15, 1039 (1969).
46. Gowenlock, A. H. and Broughton, P. M. G., The influence of accuracy and precision on the normal range and on acceptable limits for an analytical result, *Z. Anal. Chem.*, 243, 774 (1968).
47. Oreskes, I., Hirsch, C., Douglas, K. S., and Kupfer, S., Measurement of ionized calcium in human plasma with a calcium selective electrode, *Clin. Chim. Acta*, 21, 303 (1968).
48. Elveback, L. R., Guiller, C. L., and Keating, F. R., Health, normality, and the ghost of Gauss, *J. A. M. A.*, 211, 69 (1970).

49. Elveback, L. R., How high is high?, *Mayo Clin. Proc.*, 47, 93 (1972).
50. Grannis, G. F., Gruemer, H.-D., Lott, J. A., Edison, J. A., and McCabe, W. C., Proficiency evaluation of clinical chemistry laboratories, *Clin. Chem.*, 18, 222 (1972).
51. Sideman, L., Murphy, J. J., Jr., and Wilson, D. T., A collaborative study of the serum calcium determination by atomic absorption spectroscopy, *Clin. Chem.*, 16, 597 (1970).
52. Smith, F. E., Reinstein, H., and Braverman, L. E., Cork stoppers and hypercalcemia, *N. Engl. J. Med.*, 272, 787 (1965).
53. Foster, L. B., Frings, C. S., Dunn, T. T., Bowers, G. N., Jr., Pybus, J., and Doumas, B., Presence of calcium contamination in vacuum tubes for blood collection, *Clin. Chem.*, 16, 546 (1970).
54. Pragay, D. A., Howard, S. F., and Chilcote, M. F., Inorganic ion contamination in Vacutainer tubes and micropipettes used for blood collection, *Clin. Chem.*, 17, 350 (1971).
55. Meinke, W. W., Standard reference materials for clinical measurements, *Anal. Chem.*, 43, 28A (1971).
56. Barnard, A. J., Jr., Foy, E. F., and Michelotti, F. W., The practical analysis of high purity chemicals. IV. Characterization of high-purity chemicals for use as clinical laboratory standards, *Clin. Chem.*, 16, 527 (1970) (abs.).
57. McLean, F. C. and Hastings, A. B., A biological method for the estimation of calcium ion concentration, *J. Biol. Chem.*, 107, 337 (1934).
58. McLean, F. C. and Hastings, A. B., The state of calcium in the fluids of the body. I. The conditions affecting the ionization of calcium, *J. Biol. Chem.*, 108, 285 (1935).
59. McLean, F. C. and Hastings, A. B., Clinical estimation and significance of calcium-ion concentrations in the blood, *Am. J. Med. Sci.*, 189, 601 (1935).
60. Ross, J. W., Calcium-selective electrode with liquid ion exchanger, *Science*, 156, 1378 (1967).
61. Ladenson, J. and Bowers, G. N., Jr., Ionic calcium as a diagnostic service test in the hospital clinical chemistry laboratory, *Clin. Chem.*, 18 (1972) (Abstract).
62. Bates, R. G. and Alfenar, M., Activity standards for ion-selective electrodes, in *Ion-Selective Electrodes*, Durst, R. A. Ed., NBS Special Pub. No. 314, 1969.
63. Hattner, R. S., Johnson, J. W., Bernstein, D. S., Wachman, A., and Brackman, J., Electrochemical determination of apparent ionized serum calcium using a calcium-selective electrode: the method and values in normal humans and a comparison to total serum calcium, *Clin. Chim. Acta*, 28, 67 (1970).
64. Li, T.-K. and Piechocki, J. T., Determination of serum ionic calcium with an ion-selective electrode: evaluation of methodology and normal values, *Clin. Chem.*, 17, 411 (1971).
65. Arnold, D. E., Stansell, M. J., and Malvin, H. H., Measurement of serum ionic calcium using a specific-ion electrode, *Am. J. Clin. Pathol.*, 49, 627 (1968).
66. Radde, I. C., Hoeffken, B., Parkinson, D. K., Sheepers, J., and Luckham, H., Practical aspects of a measurement technique for calcium ion activity in plasma, *Clin. Chem.*, 17, 1002 (1971).
67. Schwartz, H. D., McConville, B. C., and Christopherson, E. F., Serum ionized calcium by specific-ion electrode, *Clin. Chim. Acta*, 31, 97 (1971).
68. Sachs, P. C., Bourdeau, A.-M., Balsan, S., and Presle, V., Detérmination du calcium ionisé dans le sérum avec une electrode spécifique à membrane, *Ann. Biol. Clin.*, 27, 487 (1969).
69. Robertson, W. G. and Peacock, M., New techniques for the separation and measurement of the calcium fractions of normal human serum, *Clin. Chim. Acta*, 20, 315 (1968).
70. Pederson, K. O., The effect of bicarbonate, PCO_2, and pH on serum calcium fractions, *Scand. J. Clin. Lab. Invest.*, 27, 145 (1971).
71. Jacobs, J. S., Hattner, R. S., and Bernstein, D. S., A physicochemical study of calcium-albumin aggregation employing a calcium-specific electrode, *Clin. Chim. Acta*, 31, 467 (1971).
72. Gupta, G. S., Effect of temperature on calcium interactions in normal human plasma, *Indian J. Biochem.*, 4, 58 (1967); *C.A.*, 67, 50971W (1967).
73. Hansen, S. O. and Theodorsen, L., The usefulness of an improved calcium electrode in the measurement of ionized calcium in serum, *Clin. Chim. Acta*, 31, 119 (1971).
74. Lindgaerde, F. and Zettervall, O., Serum ionized calcium in a normal population studied with a calcium ion-sensitive electrode, *Isr. J. Med. Sci.*, 7, 510 (1971).
75. Arras, M. H., Measuring ionized calcium, *Postgrad. Med.*, 45, 57 (1969).
76. Johnson, J. R. K. and Riechmann, G. C., Normal serum calcium levels by atomic absorption spectroscopy, *Clin. Chem.*, 14, 1218 (1968).
77. Raman, A., Determination of ionized calcium in serum with a calcium electrode, *Biochem. Med.*, 3, 369 (1970).
78. Yendt, E. R. and Gagne, R. J. A., Detection of primary hyperparathyroidism, with special reference to its occurrence in hypercalcemic females with "normal" or borderline serum calcium, *Can. Med. Assoc. J.*, 98, 331 (1968).
79. Harris, E. K. and DeMets, D. L., Biological and analytical components of variation in long-term studies of serum constituents in normal subjects, *Clin. Chem.*, 17, 983 (1971).
80. Farese, G., Mager, M., and Blatt, W. F., A membrane ultrafiltration procedure for determining diffusible calcium in serum, *Clin. Chem.*, 16, 226 (1970).