

Ionized Calcium in Normal Serum, Ultrafiltrates, and Whole Blood Determined by Ion-Exchange Electrodes

EDWARD W. MOORE

From the Metabolic and Gastrointestinal Research Unit of the Medical Services, Lemuel Shattuck Hospital, Department of Public Health, Commonwealth of Massachusetts and the Departments of Medicine and Physiology, Tufts University Medical School, Boston, Massachusetts 02130

ABSTRACT Ion-exchange calcium electrodes represent the first practical method for the direct measurement of ionized calcium $[Ca^{++}]$ in biologic fluids. Using both "static" and "flow-through" electrodes, serum $[Ca^{++}]$ was within a rather narrow range: 0.94–1.33 mmoles/liter (mean, 1.14 mmoles/liter). Within a given individual, $[Ca^{++}]$ varied only about 6% over a several month period. Consistent pH effects on $[Ca^{++}]$ were observed in serum and whole blood, $[Ca^{++}]$ varying inversely with pH. Less consistent pH effects were also noted in ultrafiltrates, believed to largely represent precipitation of certain calcium complexes from a supersaturated solution. Heparinized whole blood $[Ca^{++}]$ was significantly less than in corresponding serum at normal blood pH, related to the formation of a calcium-heparin complex. $[Ca^{++}]$ in ultrafiltrates represented a variable fraction (66.7–90.2%) of total diffusible calcium. There was no apparent correlation between serum ionized and total calcium concentrations. Thus, neither serum total calcium nor total ultrafiltrable calcium provided a reliable index of serum $[Ca^{++}]$. Change in serum total calcium was almost totally accounted for by corresponding change in protein-bound calcium $[CaProt]$. About 81% of $[CaProt]$ was estimated to be bound to albumin and about 19% to globulins. From observed pH, serum protein, and $[CaProt]$ data, a nomogram was developed for estimating $[CaProt]$ without ultrafiltration. Data presented elsewhere indicate that calcium binding by serum proteins obeys the mass-law equation for a monoligand association. This was indicated in the present studies by a close correspondence of observed serum $[Ca^{++}]$ values with those predicted by the McLean-Hastings nomogram.

These studies were included as part of a symposium, January 1969 (1) and preliminary reports have appeared previously (2, 3).

Received for publication 25 March 1968 and in revised form 16 July 1969.

While these electrodes allow study of numerous problems not possible previously, they have not been perfected to the same degree of reliability obtainable with current pH electrodes. The commercial (Orion flow-through) electrode is: (a) expensive, (b) requires periodic replacement of membranes, and (c) has not yet been thermostated. As with blood pH measurements, (d) electrode response is logarithmic, i.e. small potential errors generate rather large $[Ca^{++}]$ errors, (e) loss of CO_2 should be prevented, and (f) errors due to other cations must be considered under certain conditions. Despite these limitations, we believe the electrode represents a major advance in calcium metabolism.

INTRODUCTION

The physiologic importance of calcium is broad and complex. Classical frog heart experiments of McLean and Hastings (4–6) clearly demonstrated that *ionized* calcium $[Ca^{++}]$ is the physiologically active species, and many important biologic processes are now known to be critically dependent on calcium ion activity (or concentration).

Serum ionized calcium is of particular interest since it plays a central role in over-all calcium metabolism. In 1911, Rona and Takahashi (7) found from dialysis experiments that the total calcium of serum is separable into diffusible and nondiffusible fractions. Numerous studies (4–6, 8–28) have subsequently shown that there are three distinct calcium fractions in normal serum: (a) nondiffusible (protein-bound) calcium which, depending on pH and temperature, represents about 30–55% of the total; (b) diffusible nonionized calcium (i.e. complexes and chelates) comprising about 5–15% of the total; and (c) ionized calcium.

One of the greatest difficulties in studies of calcium metabolism has been the lack of a practical method for

direct measurement of ionized calcium. At least three methods have been used: (a) frog-heart (4-6) (b) rachitic cartilage bioassay (29), and (c) metal ion indicators, particularly murexide (16, 18, 20, 23, 26-28, 30, 31). Each of these has had rather restricted usefulness; murexide, for example, requires separation of proteins and heavy metals before analysis and thus does not readily lend itself to direct study of serum or to calcium-protein interaction.

Previous attempts at Ca^{++} electrode measurements have been largely unsuccessful. Early studies with calcium amalgam electrodes by Fosbinder (32) showed that such electrodes were reliable only in aqueous solutions of calcium salts; the electrodes were poisoned by very small amounts of protein. In the 1940's, Sollner and co-workers (33-35) developed colloidion membrane electrodes for measurement of several ions, a technique later applied by Carr (36) to studies of calcium binding by fractionated proteins. The difficulty with these electrodes was that other ions also gave rise to potentials across the membrane. After Eisenman, Rudin, and Casbys' (37) development of sodium-selective glass electrodes, there was hope that a Ca^{++} glass electrode could be perfected (38). Again, some success was achieved in simple aqueous solutions, as noted by Truesdell and Christ (39), but little or no success was achieved in biologic fluids. The selectivity for Ca^{++} of existing glass electrodes is probably too low for accurate measurements in biologic systems. More recently, Gregor and Schonhorn (40-42) have described multilayer membrane electrodes with Ca^{++} sensitivity, but the selectivity of these electrodes is also probably too low for accurate measurements in biologic fluids.

In March 1965, we obtained from Orion Research, Inc., Cambridge, Mass. the first ion-exchange Ca^{++} electrode (43); these and subsequent "generation" electrodes have been applied in a variety of biologic fluids. The present paper describes results in normal subjects; these results were included in a recent symposium (1).

Although these electrodes allow study of numerous problems which would otherwise be quite difficult, it should be noted at the outset that they have not been perfected to the extent of current pH electrodes. The first commercial version (Orion Research, Inc., model 92-20) did not work satisfactorily in serum, a problem related to protein and to the specific membrane used in that electrode. While this problem was later resolved in the Orion flow-through system (model 98-20), the latter electrode requires skill and practice in fabrication.

METHODS

A total of 104 studies were made in 67 healthy volunteer hospital personnel, 46 females and 21 males whose age ranged from 16 to 61 yr (mean, 30.7 yr). Venous blood

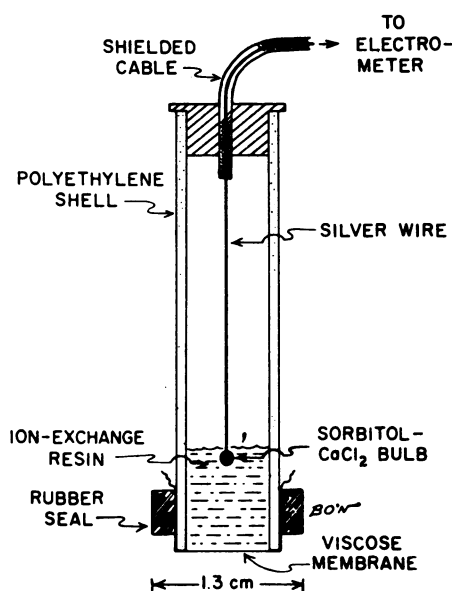


FIGURE 1 Static-type calcium electrode, prepared in the laboratory.

was obtained under oil, without anticoagulant, about 2 hr after breakfast. Care was taken to avoid prolonged venous stasis. Blood was centrifuged under oil, an aliquot of serum was removed for pH measurement, and the remaining serum separated and again placed under oil without exposure to air. A heparinized blood sample was also obtained for determination of whole blood pH. All pH measurements were made at 37°C with a Corning model 12 pH meter and a Corning blood pH electrode.

Calcium electrodes

Two types of electrodes have been employed: (a) "static" and (b) "flow-through."

Static-type electrode. The "first generation" static electrode (Fig. 1) was prepared in the laboratory as follows: Glass or plastic tubes were sealed at one end with viscose dialysis tubing, held in place by a silicone rubber sleeve, and filled with 2-3 ml of ion exchanger, the calcium salt of didecyl phosphoric acid dissolved in didecylphenyl phosphonate (Orion Research, Inc.). Electrical contact was made with a chloridized silver wire coated with 0.1 M KCl or CaCl_2 in fused sorbitol. The volume of sample required was about 3 ml and equilibration time was usually 1-3 min. Average life-span of these electrodes was about 3 wk. This electrode is not to be confused with the "second generation" static electrode marketed by Orion Research (model 92-20), which employed a Millipore membrane and did not work satisfactorily in serum.

The static electrode, while being rather crude, had the advantage of allowing direct study of pH effects on $[\text{Ca}^{++}]$. This required a suitable CO_2 chamber (Fig. 2) in which Pco_2 and sample pH could be carefully controlled. Both ionized calcium and pH were continuously monitored at 37°C through separate electrometers, using a calomel electrode as common reference.

Flow-through electrode. The "third generation" electrode, and the second type to be used in these studies, was a flow-through electrode marketed by Orion Research, Inc. (model

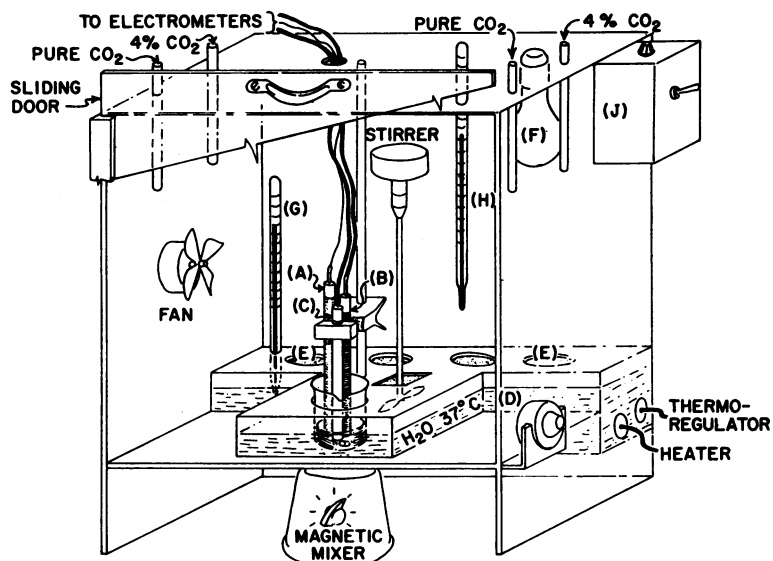


FIGURE 2 Plexiglas CO₂ chamber for Ca⁺⁺ measurements with static-type electrodes. Both [Ca⁺⁺] and pH are continuously monitored with separate electrometers. The calcium electrode (A) and pH electrode (B) are introduced into standard or test solution with calomel half-cell (C) as common reference electrode. Continuous stirring is achieved by a magnetic stirrer. Sample is maintained at 37°C by circulating water (D). Additional samples may be prewarmed (E). Air temperature is maintained at 37°C by a light bulb (F) connected to a rheostat. Water and air temperatures are monitored by thermometers (G) and (H), while (J) is the thermo-regulator control box. Sample pH is varied by alteration in chamber Pco₂.

98-20), shown diagrammatically in Fig. 3. All measurements with this electrode were made at 25°C.

The flow-through system has several advantages over static-type electrodes: (a) equilibration is more rapid, usually 30-90 sec; (b) it is more stable, with typical drift of 2-5 mv/day as compared with 3-10 mv/day with static electrodes; (c) less sample volume is required, several measurements being possible with a 1 ml sample; and (d) the measurement is anaerobic, analogous to a blood pH measurement.

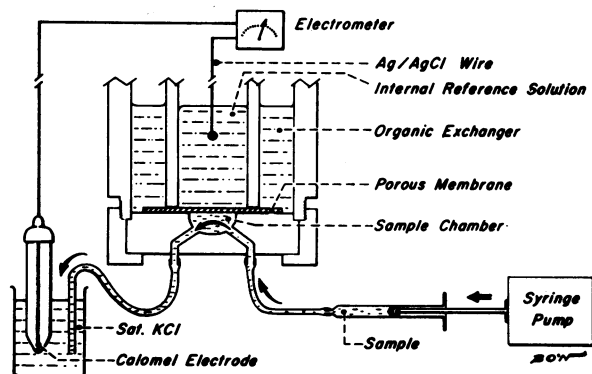


FIGURE 3 Diagram of Orion flow-through electrode. The sample flows through the electrode at about 0.05 ml/min.

Although we now use only this type of electrode in our laboratory, it has two disadvantages: (a) pH effects on ionized calcium cannot be directly monitored, and (b) it has not yet been thermostated, so that measurements are most conveniently made at room temperature. Since the binding of calcium to serum proteins is temperature as well as pH dependent, some difference between serum [Ca⁺⁺] at 37°C (static electrode) and 25°C (flow-through electrode) would be expected and will be noted later.

Electrode selectivity

No glass or ion-exchange electrode behaves perfectly, inasmuch as all electrodes will respond to more than one species (so-called "sodium error" for glass pH electrodes at high pH). Thus, one is necessarily concerned with the "selectivity" of a given electrode, i.e., the potential which will be generated by one ion species in preference to other ion species. Eisenman et al. (37) developed the following empirical equation to describe glass electrode potentials in mixtures of two univalent cations:

$$E = E_0 + \frac{2.3 RT}{zF} \times \log [(A^+)^{1/n_{AB}} + k_{AB}^{1/n_{AB}} (B^+)^{1/n_{AB}}]^{n_{AB}} \quad (1)$$

where (A⁺) and (B⁺) are the activities of ions A and B respectively, and k_{AB} and n_{AB} are adjustable parameters, constant for a given electrode and cation pair. The selectivity constant (k_{AB}) denotes how well the electrode "sees" one cation (A⁺) in preference to the other cation (B⁺).

TABLE I

Selectivity Constants (k_i) of Ion-Exchange Calcium Electrodes with Respect to Principal Cations in Extracellular (ECF) and Intracellular (ICF) Fluids

Ion	k_{CaA_i} (approx)	Ratio $[A_i]:[Ca^{++}]$	
		Serum and ECF	ICF
Na ⁺	10,000:1	150	10-15
K ⁺	10,000:1	4-5	150
Mg ⁺⁺	100:1	0.5-1	1.5-20
H ⁺	$\infty > \text{pH } 6$	10^{-4}	10^{-4}

$$E = \text{constant} + \frac{2.3 RT}{zF} \log_{10} [(Ca^{++}) + \sum_i k_i (A_i)^{z_i/z_1}]$$

Ross (43) has found that the potential of ion-exchange calcium electrodes is similarly given by the following empirical equation:

$$E = \text{constant} + \frac{2.3 RT}{zF} \log_{10} [(Ca^{++}) + \sum_i k_i (A_i)^{z_i/z_1}] \quad (2)$$

where A_i and z_i are the activity and charge of interfering ions i . The selectivity (Table I) for Ca^{++} over Na^+ (and also K^+) is so high that precise selectivity values are rather difficult to obtain. Ross (43) has found k_{CaNa} and k_{CaMg} values of 10^{-4} and 0.014, respectively, at normal serum concentrations of these ions. Similar results have been obtained in our laboratory (3). Thus in serum, the presence of 150 mM Na^+ and 0.5 mM Mg^{++} would result in about 2-3% enhancement of apparent Ca^{++} concentration. Hydrogen ions and K^+ errors must be considered in gastric juice and intracellular fluids respectively, but are of no consequence in extracellular fluids. The electrodes appear to be unresponsive to anions or to chelates of calcium. Below pH about 5.5, the electrodes no longer show Ca^{++} selectivity but behave as pH electrodes (43). We have obtained reproducible $[Ca^{++}]$ values in gastric juice, however, after alkalization to pH 6-7 with NaOH (44).

Electrode calibration

Electrode potentials are a function of calcium ion activity (Ca^{++}). The absolute value of (Ca^{++}) in any given solution is uncertain, however, since individual ion activity coefficients cannot be experimentally determined. Using a Na-electrode, Ag/AgCl electrode system, Moore and Ross (45) have estimated γ_{CaCl_2} in mixed $CaCl_2$ -NaCl solutions to be about 0.54 when NaCl = 0.15 mole/liter. From Debye-Hückel theory, this suggests that $\gamma_{Ca^{++}}$ is about 0.3 in biological fluids.

All Ca^{++} values will therefore be given in concentration $[Ca^{++}]$ terms, relative to mixed $CaCl_2$ -NaCl standard solutions with total ionic strength (μ) near that of serum ($\mu \approx 0.16$ mole/liter). It is assumed that $\gamma_{Ca^{++}}$ is similar in standard and unknown solutions; this seems reasonable, since activity coefficients are primarily related to total ionic strength (38, 46, 47). Electrodes were calibrated with solutions containing 150 mM NaCl and 0.5-10.0 mM $CaCl_2$ ($\mu = 151.5$ -180 mmoles/liter). Electrode response was linear over the 1.0-10.0 mM range, suggesting that $\gamma_{Ca^{++}}$ was similar in the different solutions, in accordance with Harned's rule (38, 48). The pH of these standards varied from about pH

6.0 to 7.0; no buffers were employed because: (a) the buffer would have uncertain effects on (Ca^{++}), and (b) there is, as noted above, no detectable H^+ error with these electrodes above about pH 5.5.

Neglecting the effects of other cations, electrode potentials in unknown and standard solutions are given by:

$$E_{\text{unk}} = E_0 + S \log ([Ca^{++}] \cdot \gamma_{Ca^{++}})_{\text{unk}} \quad (3a)$$

$$E_{\text{std}} = E_0 + S \log ([Ca^{++}] \cdot \gamma_{Ca^{++}})_{\text{std}} \quad (3b)$$

where S is the Nernst slope factor: $(2.3 RT)/(zF)$. Assuming $\gamma_{Ca^{++}}^{\text{unk}} = \gamma_{Ca^{++}}^{\text{std}}$, the potential difference (ΔE) between unknown and standard is given by:

$$[Ca^{++}]_{\text{unk}} = [Ca^{++}]_{\text{std}} \cdot 10^{\frac{\Delta E}{S}} \quad (4)$$

While equation 4 is easily solved graphically, we have found it convenient to record ΔE , using a suitable interfacing device between electrometer (Orion 801 digital pH meter) and typewriter. $[Ca^{++}]_{\text{unk}}$ is then obtained by manually feeding ΔE into an Olivetti-Underwood Programma 101 computer, with printout of $[Ca^{++}]_{\text{unk}}$.

It should be emphasized that small errors in potential measurements may yield rather large errors in estimated $[Ca^{++}]$ in unknown solutions. Thus, with an electrode slope of 28 mv, an error of 1 mv in measured potential would yield about an 8% error in apparent $[Ca^{++}]$.

Determination of ionized calcium

All measurements with static-type electrodes, in both standards and unknowns, were made at 37°C in a Plexiglas chamber (Fig. 2), in which sample pH was varied by alteration in P_{CO_2} . Electrode potentials were monitored with either a Corning model 12 pH meter or Orion model 801 digital pH meter. $[Ca^{++}]$ measurements in each serum, whole blood, and ultrafiltrate were made in duplicate over the pH range 6.8-7.8. Resulting curves were bracketed by measurements in standard $CaCl_2$ -NaCl solutions. Because of pH dependency of ionized calcium in sera and ultrafiltrates, all $[Ca^{++}]$ values below, for the static electrode, are those at the original whole blood pH. In the flow-through electrode studies, $[Ca^{++}]$ was determined in duplicate at $25^\circ \pm 2^\circ C$ either immediately (anaerobically) or, in a few instances, in samples frozen under oil, reequilibrated with 5% CO_2 before analysis.

It should be emphasized that no pH effect on $[Ca^{++}]$ was observed in standard solutions.

Determination of total calcium and proteins

Total calcium $[Ca]$ was determined in duplicate by ethylenediaminetetraacetate (EDTA) titration in 1 M KOH using Cal-Red (K. & K. Chemicals, Plainview, N. Y.) as indicator (31, 49). 25 samples (20 sera, 5 ultrafiltrates) were also analyzed by atomic absorption spectroscopy (AAS) (model 290, Perkin-Elmer Corp., Norwalk, Conn.). A close correlation ($r = 0.96$) between ethylenediaminetetraacetate (EDTA) (y) and AAS (x) values was observed in both sera and ultrafiltrates: $y = 0.16 + 0.96 x$; AAS values thus averaged about 0.1 mmole/liter lower than EDTA values in serum and about 0.06 mmole/liter lower in ultrafiltrates. All subsequent $[Ca]$ values are those obtained by EDTA titration. Serum total protein was determined by the biuret method (50); albumin and globulin were determined by electrophoresis.

Ultrafiltration

Ultrafiltrates of sera (29 studies) were obtained at $25 \pm 2^\circ\text{C}$ with a dialysis cell obtained from National Instrument Laboratories, Inc., Rockville, Md. Viscose dialysis tubing was soaked in running water and then in distilled water for about 20 min before use. Serum (10 ml), which had been separated under oil and its pH determined, was introduced anaerobically into the cell (preaerated with 5% CO_2) and pressure applied with pure nitrogen gas at 4–45 psi. Ultrafiltrates were removed anaerobically and immediately introduced into the Plexiglas chamber (also preaerated with 5% CO_2) for electrode analysis. All ultrafiltrates were clear; occasional cloudy samples were discarded and the experiment terminated.

In early studies, ultrafiltrate pH was somewhat higher (0.1–0.2 pH U) than original serum; the chamber was therefore modified to allow continuous gassing with moisturized 5% CO_2 during ultrafiltration. pH regulation was then quite close, average ΔpH between ultrafiltrate and original serum in 9 normal and 10 pathologic sera was 0.039 ± 0.013 (SE) U.

Since binding of calcium to serum proteins is temperature- as well as pH dependent, it is important to note that ultrafiltrates were obtained at 25°C . From ultracentrifugation data of Loken, Havel, Gordan, and Whittington (22), protein-bound calcium, $[\text{CaProt}]$, is about 5% lower at 25°C than at 37°C .

RESULTS

Do Ca^{++} electrodes work in biologic fluids? There are several ways in which it can be shown that the electrodes respond to changes in ionized calcium. In protein-free solutions such as ultrafiltrates, this is indicated by linear "recovery" upon addition of CaCl_2 . A representative study is shown in Fig. 4; a quantitative linear in-

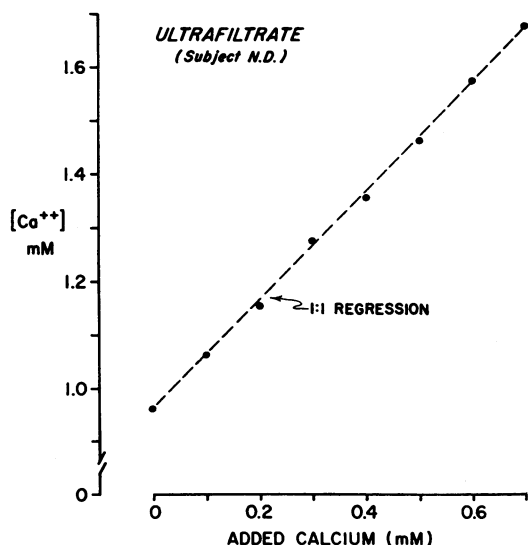


FIGURE 4 Representative recovery study in an ultrafiltrate of serum from a normal subject. Pure CaCl_2 (10.0 mmoles/liter) was added serially (30 μl) to a 3.0 ml ultrafiltrate sample at constant pH (pH 7.37). Resulting abscissa values are expressed as mM concentration.

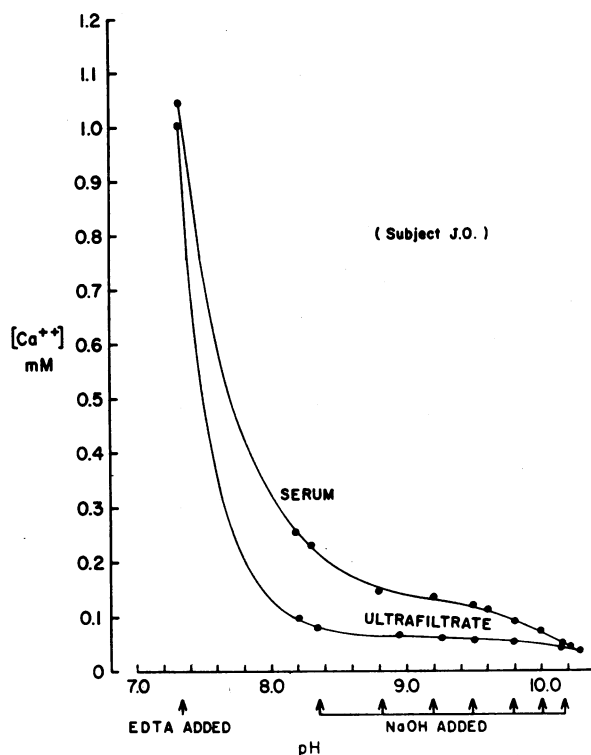


FIGURE 5 Effect of ethylenediaminetetraacetate (EDTA) on ionized calcium concentrations in serum and ultrafiltrate as monitored with the static-type calcium electrode. 10 μmoles (in 30 μl) of di-sodium EDTA was added to 3.0 ml samples, yielding final EDTA concentrations of about 3.3 mmoles/liter. Sample pH was then increased by addition of 1 M NaOH in 10–20 μl increments.

crease in $[\text{Ca}^{++}]$ was also observed in six additional studies.

Recovery of added calcium should be linear in ultrafiltrates but should not be quantitative, owing to the formation of complexes with various diffusible anions. From observed dissociation constants of the inorganic ligands present (see below), calculations indicate that observed Ca^{++} values (Fig. 4) were about 4–5% too high at a Ca^{++} level of 1.6 mmoles/liter. While this may reflect electrode error, linearity of response suggests a constant fractional error, as might occur in serial pipetting. In serum, the recovery of added calcium is not quantitative because of partial binding to serum proteins. This is noted elsewhere (1) and will be developed more fully in a subsequent paper.

The electrodes have also been used as monitors for chelating titrations. As shown in Fig. 5, upon addition of an excess of EDTA to serum or ultrafiltrate, there was a rapid decrease in $[\text{Ca}^{++}]$, followed by a further decrease upon addition of NaOH. Virtually identical results were obtained in four additional studies. Note

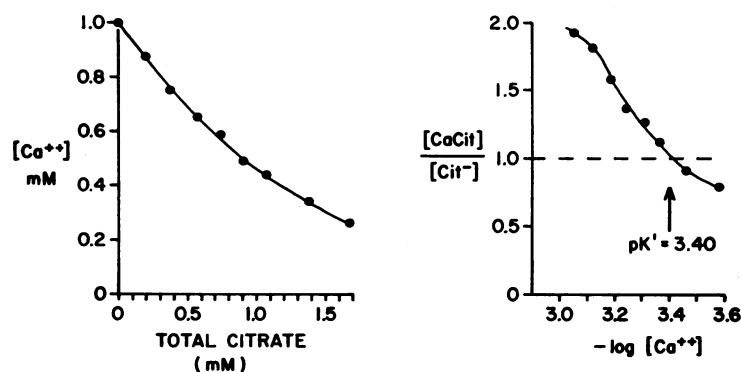


FIGURE 6 Determination of the dissociation constant of calcium citrate by serial addition of citrate to a 1 mM CaCl_2 solution (25°C , pH 7.0, $\mu \approx 0.15$ mole/liter).

that the residual apparent value for $[\text{Ca}^{++}]$ at pH 10.3 was about 0.04 mmole/liter. This probably reflects sodium error in electrode potential. Thus at this point, sample sodium concentration was about 200 mmoles/liter. With a selectivity constant (Table I) of 10,000:1 the sodium potential would yield an apparent Ca^{++} value of about 0.02 mmole/liter (i.e. $[200]:[10,000]$). This selectivity "constant" is not a constant however, but is dependent on both Ca^{++} and Na^+ concentrations. Thus, the value 10,000:1 was obtained at 1 mM CaCl_2 -150 mM NaCl; at Ca^{++} and Na^+ concentrations of 0.1 mmole/liter and 200 mmoles/liter respectively, Ross (51) has found that $k_{\text{Ca:Na}}$ is reduced to about half, i.e., $\approx 5000:1$. This is consistent with the residual value of 0.04 mmole/liter in Fig. 5. These considerations emphasize that, in order to minimize Na^+ errors in biologic fluids with very low Ca^{++} concentrations, variation in selectivity constant must be considered and should be experimentally determined in the concentration range under study.

Another way of showing that the electrode responds to Ca^{++} is to measure the dissociation constant (K_d') of an important complex, such as calcium citrate. A single

experiment (flow-through electrode at 25°C) was therefore performed as follows: To an aqueous solution containing 1.0 mM CaCl_2 and 120 mM NaCl, 0.1 ml

TABLE II
Effect of Freezing on Serum Ionized Calcium Concentration

Subject	Ionized calcium concentration		
	Before	After	Δ
	mM		
1 R. C.	1.15	1.16	+0.01
2 C. G.	1.15	1.17	+0.02
3 N. G.	1.06	1.06	0
4 S. K.	1.12	1.16	+0.04
5 N. L.	1.26	1.26	0
6 L. M.	1.16	1.20	+0.04
7 S. S.	1.19	1.19	0
Mean	1.155	1.171	+0.016

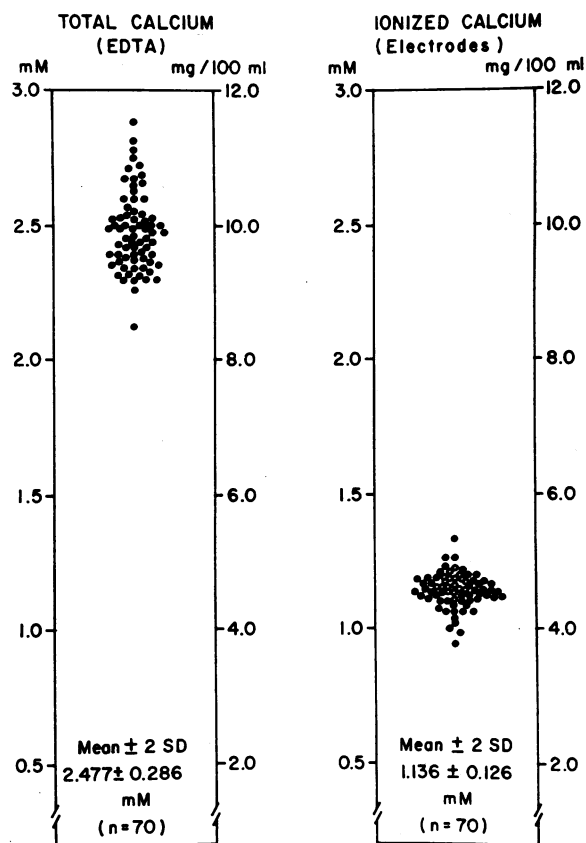


FIGURE 7 Serum ionized and total calcium concentrations in 52 subjects studied with static-type electrodes (\bullet) and 18 subjects studied with flow-through electrodes (\circ). Notice the narrow range of ionized calcium values. The 95% confidence limits for the population are indicated by respective means ± 2 SD: 2.19-2.76 mmoles/liter for total calcium and 1.01-1.26 mmoles/liter for ionized calcium.

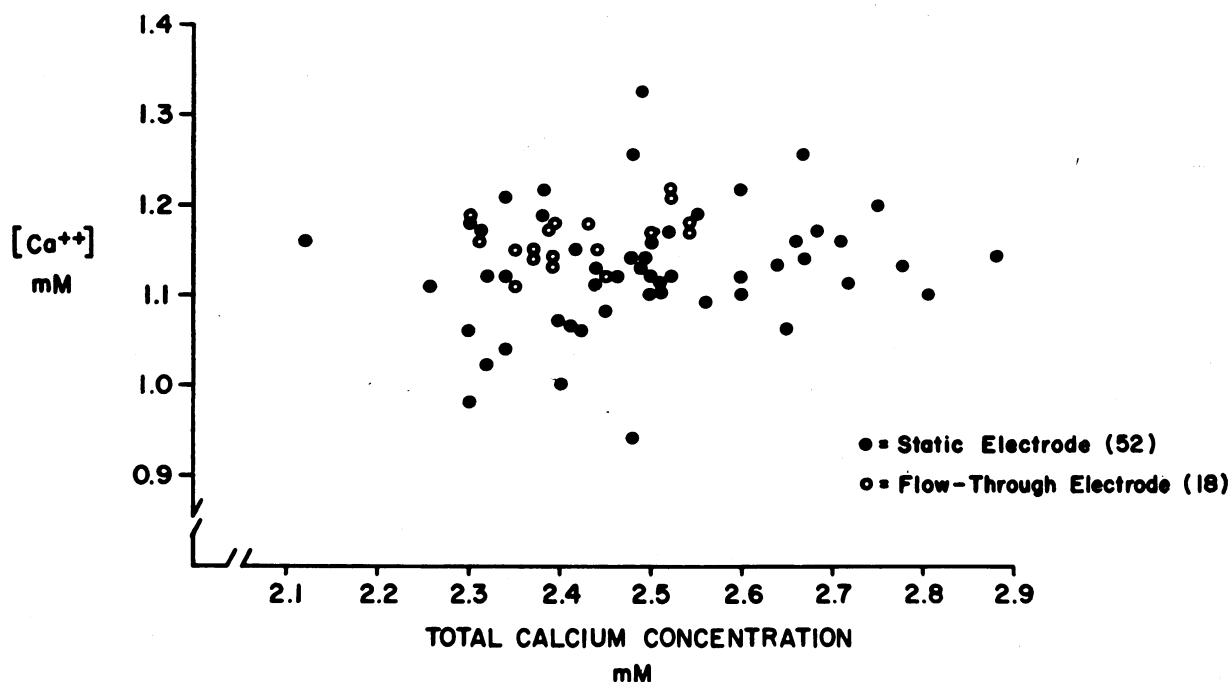


FIGURE 8 Relation between ionized and total calcium concentrations in 52 subjects studied with static-type electrodes (●) and 18 subjects studied with flow-through electrodes (○). Note the apparent lack of correlation; $[Ca^{++}]$ values were within a rather narrow range irrespective of total calcium levels.

increments of 10.0 mM citric acid were serially added and final pH adjusted to 7.0 by addition of NaOH. Calculated total ionic strength for each solution was about 0.15 mole/liter. Upon eight serial additions of citrate (0.20–1.67 mmoles/liter), observed $[Ca^{++}]$ decreased progressively (Fig. 6). pK' values, calculated for each point in the left-hand side of Fig. 6, varied from 3.34 to 3.48, with a mean pK' of 3.40. This value is somewhat higher than in most reported studies in which pK s of about 3.2 have been obtained (52).

Effects of freezing. Ionized calcium was measured in serum from seven normal subjects before and after freezing for 24–72 hr (Table II). No significant differences were observed. Upon freezing of ultrafiltrates, however, a decrease in $[Ca^{++}]$ was frequently observed; some samples became cloudy and occasional precipitates were noted. It is concluded that $[Ca^{++}]$ may be reliably determined in previously frozen sera but not in ultrafiltrates previously frozen. The apparent lack of precipitation in serum may reflect solubilizing properties of the serum proteins.

Serum ionized vs. total calcium. Ionized $[Ca^{++}]$ and total calcium $[Ca]$ concentrations were compared in 91 sera from 60 normal subjects. Of these, 72 studies in 52 subjects were made with static electrodes; the remainder were made with the more recently obtained flow-through electrode. As shown in Fig. 7, serum $[Ca]$

varied from 2.12 to 2.88 mmoles/liter (mean: 2.477 ± 0.286 [2 SD] mmoles/liter). Absolute variability in ionized calcium was only about one-half that of total calcium, with a range of 0.94–1.33 mmoles/liter. Percentage variability was thus similar for ionized and total calcium. Over-all mean serum $[Ca^{++}]$ for both static and flow-through electrodes was 1.136 ± 0.126 (2 SD) mmoles/liter. Thus, 95% of normal subjects would be expected to lie in the range: 1.01–1.26 mmoles/liter.

$[Ca^{++}]$ measurements were made in 11 subjects with both static-type and flow-through electrodes, with an intervening period of 1–10 months. Mean values for the two electrodes were almost identical: 1.169 ± 0.05 (2 SE) mmoles/liter and 1.161 ± 0.012 mmoles/liter, respectively. For all studies with the flow-through electrode (19 studies in 18 subjects), mean $[Ca^{++}]$ was 1.163 ± 0.014 (2 SE) mmoles/liter, which was similar to the mean value of 1.127 ± 0.019 (2 SE) mmoles/liter obtained with static electrodes in 52 subjects.

Small differences in $[Ca^{++}]$ values for the two types of electrodes might be expected, due to pH and temperature effects. Thus, static electrode $[Ca^{++}]$ values were obtained at 37°C and at the original whole blood pH; flow-through $[Ca^{++}]$ values were at 25°C and at the pH of serum. For all studies, average blood pH was 7.34 ± 0.01 (2 SE), while for separated serum it was 7.42

TABLE III
Variability of Replicate Calcium Determinations

	Ionized calcium (electrode)				
	Serum			Total calcium (EDTA)	
	Static electrode (47)	Flow-through (17)	Ultrafiltrate (static electrode) (16)	Serum (83)	Ultrafiltrate (16)
			<i>mM</i>		
Mean	1.14	1.16	1.17	2.50	1.52
±2 SD	0.09	0.03	0.07	0.03	0.06

No. given in parentheses denotes number of studies.

±0.01. From observed pH effects on $[Ca^{++}]$ (Fig. 9, below), this difference would be expected to yield $[Ca^{++}]$ values with the flow-through electrode about 3% lower than with static electrodes. However, from ultracentrifugation studies of Loken et al. (22), free $[Ca^{++}]$ is about 5–6% higher at 25°C than at 37°C at this pH. Since the pH and temperature effects are opposite in direction, $[Ca^{++}]$ values with the flow-through electrode would thus be expected to be about 2–3% greater than with the static electrode, almost exactly the difference observed.

As shown in Fig. 8, there was no apparent relationship between serum ionized and total calcium concentrations. For the 52 static electrode studies, the calculated least squares regression was: $y = 0.92 + 0.08x$ ($r = 0.18$) whereas for the 18 flow-through electrode studies it was: $y = 0.77 + 0.16x$ ($r = 0.48$). The somewhat higher correlation coefficient with the flow-through electrode, which has lower duplicate variability than the static electrode (Table III), suggests that change in total calcium may, in fact, be associated with small changes in ionized calcium which are beyond the limit of detection with the electrode. Much larger sample numbers would be necessary to establish or rule out such a possibility. We conclude that there is no *apparent* relationship between the two. The discrepancy between serum $[Ca^{++}]$ and $[Ca]$ reflects variation in serum proteins (mainly albumin) concentrations. Thus, as will be shown below, variation in serum total calcium was almost quantitatively accounted for by a corresponding variation in protein-bound calcium.

Variability of serum $[Ca^{++}]$ and $[Ca]$ within a given individual was studied in 15 subjects (39 studies) over a 2 yr period. The average interval between studies was 7.6 months. Serum $[Ca^{++}]$ varied only about 6%; mean $[Ca^{++}]$ was 1.13 ± 0.01 (2 SE) mmoles/liter, and the average difference between replicate studies was 0.07 ± 0.03 mmole/liter. Absolute variability in serum total calcium was about three times that of ionized calcium: Mean $[Ca]$ for all studies was 2.52 ± 0.06 (2 SE) mmoles/

liter with an average difference between repeated studies of 0.23 ± 0.06 (2 SE) mmole/liter.

Replicate variability. An estimate of the relative accuracy of both electrode (ionized) calcium and EDTA (total), calcium measurements was obtained by analysis of variance for duplicate measurements and expressed as the 95% confidence limit for the "true" value in any given sample (Table III). For example, for a single electrode value of 1.14 mmoles/liter in serum with the static electrode, the 95% confidence limit for the true value in that sample (± 2 SD) would be between 1.05 and 1.23 mmoles/liter. Replicate variability with the flow-through electrode was one-third that of the static electrode and was comparable with that for total calcium by the EDTA method.

pH effects on ionized calcium. It is well known that alkalosis may precipitate hypocalcemic tetany and that the binding of calcium to serum proteins increases with increasing pH (15, 21, 22). The static electrode and CO_2 chamber have allowed, for the first time, a direct study of such pH effects on $[Ca^{++}]$. The effect of pH on serum $[Ca^{++}]$ was investigated in 52 subjects over the range 6.8–7.8 pH. Similar studies were also made in ultrafiltrates from 16 normal subjects. A representative study is shown in Fig. 9.

As expected, there was a pronounced pH effect in serum, with a linear or slightly sigmoid decrease in $[Ca^{++}]$ with increasing pH. The pH effect presumably results from competition between Ca^{++} and H^+ for negative sites on serum proteins and was considerably accentuated in residual "bag serum," i.e., upon concentration of the serum proteins. The pH effect was virtually instantaneous and was completely reversible.

Less consistent pH effects were also observed in ultrafiltrates, sometimes almost identical with those in corresponding sera, as in the subject shown in Fig. 9. This was quite unexpected and has since been studied rather extensively by Dr. Joel Jacknow in our laboratory, as will be noted below.

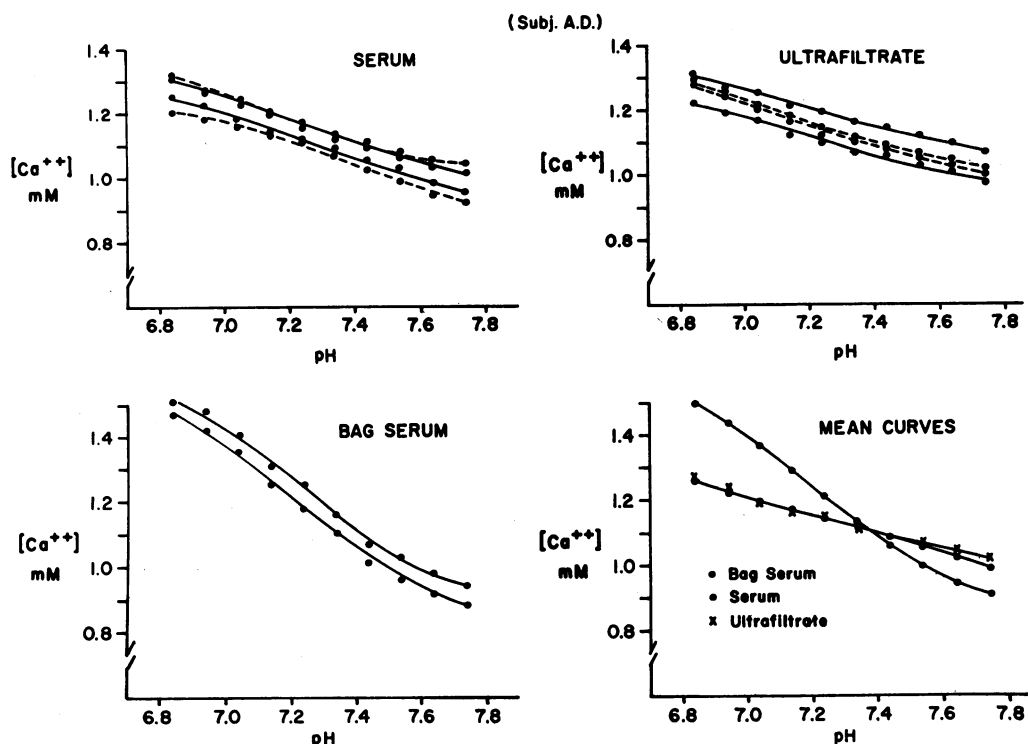


FIGURE 9 Ionized calcium concentration as a function of pH in serum, ultrafiltrate, and bag serum from a normal subject. In serum and ultrafiltrate, each $[Ca^{++}]$ curve was bracketed by measurements in standard $CaCl_2NaCl$ solutions; the four curves therefore represent duplicate runs. (The range of variability corresponds to about 1 mv.) The two curves for bag serum represent a single run bracketed by standards. Note that mean curves for serum and ultrafiltrate were virtually identical, while an accentuated pH effect was observed in residual bag serum; i.e., upon concentration of the serum proteins.

Components of serum total calcium. It is well-recognized that serum $[Ca]$ represents the sum of three distinct fractions: (a) protein-bound calcium $[CaProt]$, (b) diffusible calcium complexes $[CaR]$, and (c) ionized calcium $[Ca^{++}]$. Total ultrafiltrable calcium, representing both $[Ca^{++}]$ and $[CaR]$, has been widely used as an index of serum $[Ca^{++}]$.

Ultrafiltrates of serum were obtained from 27 subjects (29 studies) with the following results: mean serum $[Ca]$ was 2.47 ± 0.03 (SE) mmoles/liter and mean ultrafiltrate $[Ca]$ was 1.49 ± 0.02 mmoles/liter. Average $[CaProt]$ was thus 0.98 mmole/liter and 60.5% of serum $[Ca]$ was ultrafiltrable, a figure in general agreement with previous studies (9-11, 15, 21, 25, 26). Average serum pH at the time of ultrafiltration was 7.42 ± 0.005 . $[Ca^{++}]$ levels in sera and ultrafiltrates were almost identical with means of 1.16 and 1.17 mmoles/liter, respectively. Mean $[CaR]$ was 0.34 ± 0.02 mmole/liter. Of the total ultrafiltrable calcium, the ionized fraction varied considerably (range: 66.7-90.2%) with a mean of 76.7%. Total ultrafiltrable calcium was thus not a reliable index of serum ionized calcium. Resulting

fractions of serum total calcium are summarized in Fig. 10.

Diffusible calcium complexes $[CaR]$. McLean and Hastings (5) found 0.15-0.34 mmole/liter of the serum total calcium could not be accounted for as $[Ca^{++}]$ or $[CaProt]$; similar results were later obtained by Morrison, McLean, and Jackson (13). Neuman and Neuman (17) have given 0.3 mmole/liter as the estimated value for $[CaR]$ in normal serum. More recent studies by Walser (26), however, have yielded values of about 0.16 mmole/liter, using murexide for measurement of $[Ca^{++}]$. Rose and associates (18, 20, 28) have reported values somewhat lower than those of Walser. Some of these differences may be related to the methods employed. Thus, if we had used AAS for measuring $[Ca]$, rather than EDTA, our $[CaR]$ value would have been about 0.28 mmole/liter. Similarly, ultrafiltrate $[Ca^{++}]$ values reported for murexide are higher (0-0.3 mmole/liter) than those obtained here with the electrode. This would yield $[CaR]$ values proportionately lower than those obtained from electrode data.

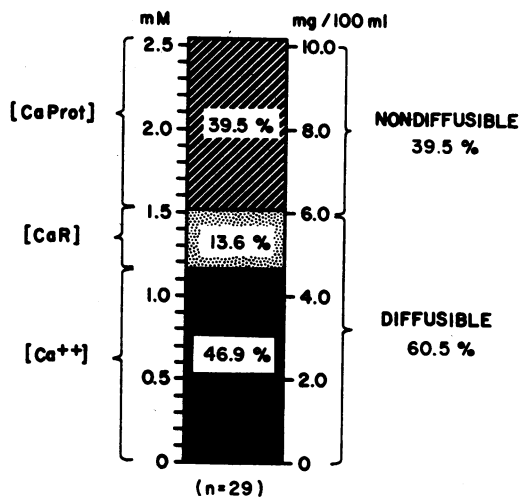


FIGURE 10 Components of serum total calcium obtained from ultrafiltration data in 29 normal subjects. Indicated percentages are the mean values for the group.

This problem has been studied by Dr. Jacknow in our laboratory, as noted elsewhere (1). Five aqueous solutions were prepared, each containing 1.5 mM CaCl_2 and 150 mM NaCl: (a) 1.0 mM phosphate, (b) 1.0 mM lactate, (c) 0.12 mM citrate, (d) 25 mM bicarbonate, and (e) a mixture of all of these. From resulting $[\text{Ca}^{++}]$ values, average concentrations for the respective cal-

cium complexes were 0.01, 0.02, 0.08, and 0.14 mmole/liter, with a sum of 0.25 mmole/liter. At a normal serum $[\text{Ca}^{++}]$ level of 1.15 mmole/liter, the sum would be about 0.21 mmole/liter, or about 0.07 mmole/liter less than that observed by AAS in our normal subjects. While this difference may reflect ultrafiltration error, it appears that a calcium sulfate complex must also be considered. Nakayama and Rasnick (53) have recently obtained K_a' for $\text{CaSO}_4 \cdot 2 \text{H}_2\text{O}$ of 5.9×10^{-8} . At a normal serum $\text{SO}_4^{=}$ level of 0.5 mmole/liter, a calcium sulfate complex of about 0.08–0.1 mmole/liter might be expected. This could account for the above difference between ultrafiltration and pure solution values.

Calculations indicate that the dissociation of each of the above complexes should not vary with pH over the pH range compatible with life, and this was experimentally confirmed in these solutions, i.e., no pH effect was observed. Calculations do indicate, however, that above about pH 6.9 ultrafiltrates are probably supersaturated with calcium carbonate and perhaps also hydroxyapatite. As noted above, the pH effect was often similar to that in native serum; in other instances, pH curves were virtually flat below pH 7.3, with a rapid decrease in $[\text{Ca}^{++}]$ above pH 7.3–7.6, suggesting precipitation. While unmeasured pH-dependent polyelectrolytes may also be present, we believe the observed pH effect in ultrafiltrates most likely represents a solubility

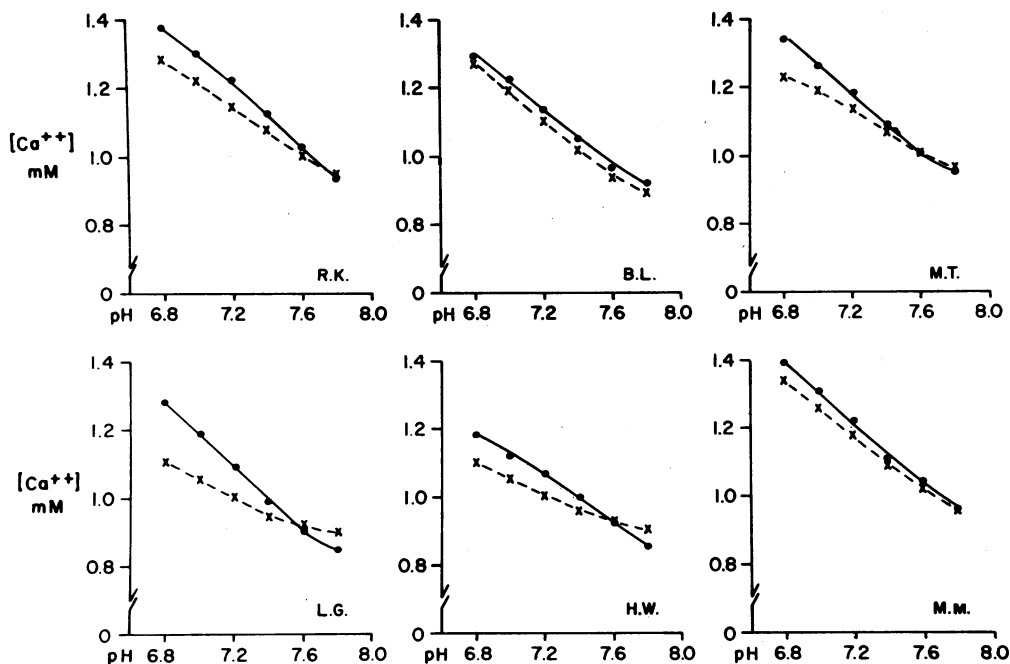


FIGURE 11 Comparison of ionized calcium concentrations in serum (●) and heparinized whole blood (x) as a function of pH in six normal subjects. Note that below pH 7.6, whole blood $[\text{Ca}^{++}]$ was less than corresponding serum $[\text{Ca}^{++}]$ in all subjects.

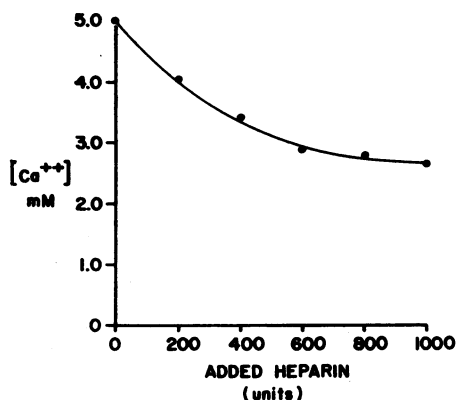


FIGURE 12 Effect of heparin on ionized calcium concentration in aqueous solution. To 1.0 ml of standard solution containing 5.0 mM CaCl_2 and 150 mM NaCl was added 0.02 ml increments of a commercial sodium heparin solution containing 10,000 U/ml. Data were obtained with static-type calcium electrode.

(kinetic) problem related to variable precipitation of certain calcium complexes.

Whole blood $[\text{Ca}^{++}]$ and heparin effects. $[\text{Ca}^{++}]$ was determined in serum and simultaneously drawn heparinized whole blood from six normal subjects (Fig. 11). pH effects on $[\text{Ca}^{++}]$ in whole blood were roughly similar to those in corresponding sera. In all cases however, $[\text{Ca}^{++}]$ was less in whole blood than in serum at normal venous pH (7.3–7.4). Although the average difference was small (mean $\Delta = -0.045$ mmole/liter at pH 7.32), it was significant ($P < 0.01$). The effects of heparin on $[\text{Ca}^{++}]$ were therefore studied as follows:

Sodium heparin (10,000 U/ml) was added in 0.02 ml increments to an aqueous solution (1.0 ml) containing 5 mM CaCl_2 and 150 mM NaCl, with continuous monitoring of ionized calcium by the electrode. As shown in

Fig. 12, there was a progressive decrease in the observed $[\text{Ca}^{++}]$, amounting to about 45% reduction upon addition of 0.1 ml heparin. Similar addition of 0.05 ml and 0.2 ml, respectively, of heparin to two normal sera (1.0 ml) resulted in a 13% decrease and 45% decrease in ionized calcium, respectively. Thus, it appears that observed differences in $[\text{Ca}^{++}]$ in sera and whole blood can be accounted for by a calcium-heparin complex. Possible effects of erythrocytes on $[\text{Ca}^{++}]$ have not been studied, however.

Protein-binding studies. As noted in Fig. 8, there was no apparent relation between serum ionized and total calcium concentrations. It will now be shown that variation in serum $[\text{Ca}]$ was almost totally accounted for by corresponding variation in $[\text{CaProt}]$.

In the above ultrafiltration studies, serum proteins were measured in 21 subjects. In this group, mean serum $[\text{Ca}]$ was 2.47 ± 0.04 (SE) mmoles/liter and total ultrafiltrable calcium was 1.47 ± 0.03 mmoles/liter, yielding an average $[\text{CaProt}]$ of 1.00 ± 0.04 mmoles/liter. Mean serum $[\text{Ca}^{++}]$ was 1.16 ± 0.01 mmoles/liter and mean $[\text{CaR}]$ was 0.32 ± 0.03 mmole/liter. Average serum total protein concentration was 72.3 ± 1.1 g/liter, with mean albumin $[\text{Alb}]$ and globulin $[\text{Glob}]$ levels of 47.3 ± 1.1 and 25.0 ± 1.0 g/liter, respectively.

There was no correlation between $[\text{CaProt}]$ and serum globulin levels. As may be seen in Fig. 13, however, $[\text{CaProt}]$ appeared to be linearly related to $[\text{Alb}]$ by the function: $[\text{CaProt}] = 0.11 + 0.019 [\text{Alb}]$ ($r = 0.88$). Since mean $[\text{CaProt}]$ was 1.00 mmole/liter, the intercept on the vertical axis suggests that about 11% of $[\text{CaProt}]$ was bound to globulin. The extrapolation is quite long however, and this problem has been studied by another type of mathematical analysis, given in detail elsewhere (1), which indicates that about 81%

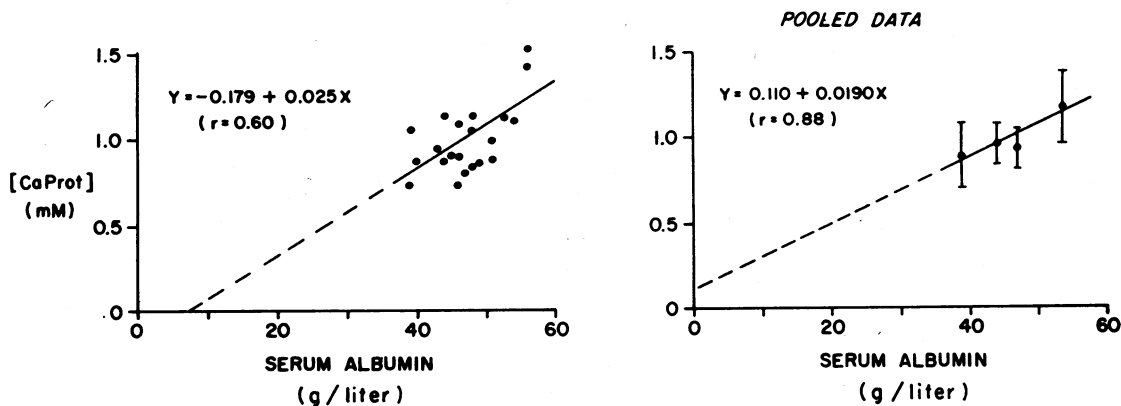


FIGURE 13 Relationship between protein-bound calcium $[\text{CaProt}]$ and serum albumin concentrations in 21 normal subjects. Individual subject data are given at the left. At the right, data were pooled by 5 g/liter increments in serum albumin. Vertical bars represent ± 2 SE.

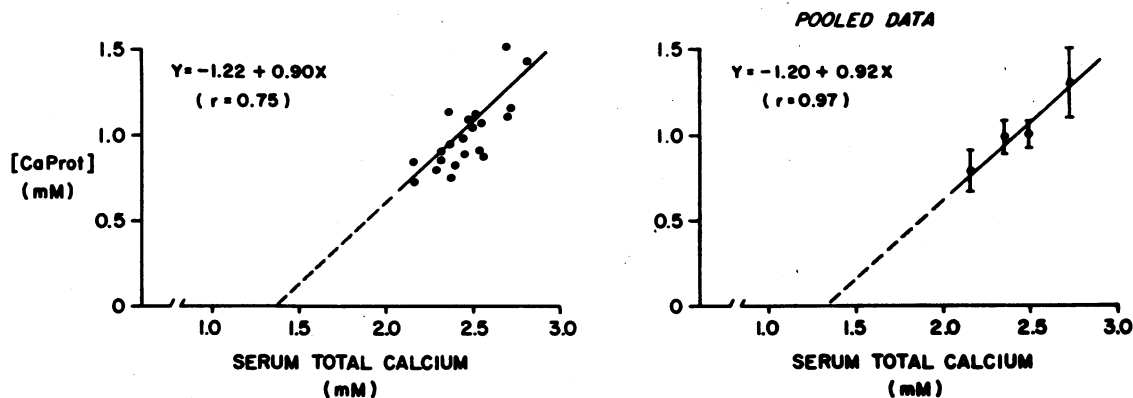


FIGURE 14 Relationship between protein-bound calcium [CaProt] and serum total calcium concentrations in 21 normal subjects. On the right, data were pooled by 5 g/liter increments in serum albumin. Vertical bars represent ± 2 SE. The slope of 0.92 indicates that change in serum total calcium was almost quantitatively accounted for by change in the protein-bound fraction.

of [CaProt] is bound to albumin and 19% to globulins in these normal subjects.

The relationship between [CaProt] and serum [Ca] was also linear, as shown in Fig. 14. The slope, 0.92, indicates that change in [Ca] was almost quantitatively accounted for by corresponding change in [CaProt]. Thus, serum total calcium was an indirect measure of the serum albumin level.

pH effects: nomograms for [CaProt]. In static electrode studies of serum (Fig. 9), it was noted that [Ca⁺⁺] decreased almost linearly with increasing pH. In the 52 normal subjects so studied, the following data were obtained: [Ca]_± = 2.505 mmoles/liter; [Ca⁺⁺]_± = 1.36 mmoles/liter at pH 6.8, and [Ca⁺⁺]_± = 0.94 mmole/liter at pH 7.8.

Thus at pH 6.8 an average of 54.3% of serum [Ca] was ionized, while at pH 7.8 it was 37.5%. This is virtually identical with the change in free calcium noted by Loken et al. (22) by ultracentrifugation. From this, we may surmise that a pH-induced decrease in [Ca⁺⁺] reflects a corresponding increase in [CaProt]. Stated another way, average change in serum [Ca⁺⁺] for a 1 U pH change was 0.42 mmole/liter. If we may apply this figure to the present group of 21 subjects in whom the serum proteins were also measured, 0.42 mmole/liter thus represents that change in [CaProt] produced at an average serum [Alb] of 47.3 g/liter and average [Glob] of 25.0 g/liter. The absolute change in [CaProt] induced by pH change would be expected to be proportional to existing protein levels, while the percentage change in [CaProt] would be expected to be constant.

In the 21 subjects, average [CaProt] was 1.00 mmole/liter and average serum pH, at the time of ultrafiltration, was 7.42 \pm 0.01 (2 SE). The following equation

may therefore be written relating [CaProt] to [Albumin], [Globulin], and pH:

$$\begin{aligned}
 [\text{CaProt}] &= [\text{CaAlb}] \\
 &- \left[(0.42) \frac{[\text{Alb}]}{47.3} (7.42 - \text{pH}) \right] + [\text{CaGlob}] \\
 &- \left[(0.42) \frac{[\text{Glob}]}{25.0} (7.42 - \text{pH}) \right]. \quad (5)
 \end{aligned}$$

Estimation of [CaAlb], i.e., calcium bound to albumin, and [CaGlob], calcium bound to globulins can be obtained from the slope in Fig. 13: [CaAlb] = 0.019 [Alb]. Since average [Alb] in the 21 subjects was 47.3 g/liter, [CaAlb] = (0.019)(47.3) = 0.899 mmole/liter. Since average [CaProt] was 1.0 mmole/liter, the remainder (0.101 mmole/liter) represents [CaGlob], at an average [Glob] level of 25.0 g/liter. Thus, [CaGlob] = 0.101 = (z)(25.0) and z = 0.004 mmole/liter per gram. Equation 5 may thus be rewritten in terms of known quantities:

$$\begin{aligned}
 [\text{CaProt}] &= 0.019 [\text{Alb}] \\
 &- \left[(0.42) \left(\frac{[\text{Alb}]}{47.3} \right) (7.42 - \text{pH}) \right] \\
 &+ 0.004 [\text{Glob}] \\
 &- \left[(0.42) \left(\frac{[\text{Glob}]}{25.0} \right) (7.42 - \text{pH}) \right]. \quad (6)
 \end{aligned}$$

The correspondence between observed [CaProt] levels, determined by ultrafiltration, and those calculated by equation 6 was rather close. Thus in the 21 subjects, average calculated [CaProt] was 1.00 mmole/liter, exactly that observed experimentally, while the average error (without regard to sign) was 0.135 \pm 0.02 (SE)

mmole/liter or 13.5%. The constants 0.019 and 0.004 in equation 6 are derived in a different manner elsewhere (1) and were found to be 0.0167 and 0.0079, respectively. Use of the latter constants in equation 6 yielded almost identical results, however: Mean calculated [CaProt] was 0.99 mmole/liter and average error was 0.131 mmole/liter or 13.1%.

A nomogram is desirable, but this is not easily done with three measured variables and a constant fractional amplification. A simple solution was obtained as follows: If we assume that all protein-bound calcium is bound to albumin, then from observed mean values of: [Alb]_x = 47.3 g/liter and [CaProt]_x = 1.00 mmole/liter = [CaAlb]_x, the following is obtained:

$$[\text{CaAlb}] = 0.0211 [\text{Alb}] - \left[(0.42) \frac{[\text{Alb}]}{47.3} (7.42 - \text{pH}) \right]. \quad (7)$$

Equation 7 is readily expressed as a nomogram (Fig. 15). Using this nomogram, average calculated [CaProt] in the 21 subjects was 1.00 mmole/liter, exactly that observed experimentally, and average error was 0.136 mmole/liter or 13.6%. It can be seen that the effect of a 0.1 U pH change on [CaProt] was roughly equivalent to a 1.5 g/liter change in serum albumin. Finally, it should be noted that the nomogram was constructed for data obtained at 25°C. From data of Loken et al. (22), corresponding values in vivo would be about 5% higher.

Summary: the McLean-Hastings nomogram.—From the above electrode studies of [Ca⁺⁺] and ultrafiltration studies of [CaProt], the state of serum calcium in normal subjects may be summarized as follows: The 95% confidence interval for [Ca] was 2.19–2.76 mmoles/liter. Over this range in [Ca], serum [Ca⁺⁺] remained within rather narrow limits, 0.94–1.33 mmoles/liter. Serum [Ca⁺⁺] decreased about 4% for each 0.1 U increase in pH, reflecting the pH dependency of calcium binding by serum proteins. About 80% of [CaProt] appeared to be bound to albumin and 20% to globulins. Variation in serum [Ca] was accounted for by corresponding change in [CaProt]. As noted elsewhere (1), this change in [CaProt] was quantitatively that predicted by the mass-law equation:

$$K'_d = \frac{[\text{Ca}^{++}][\text{Prot}^{-n-2}]}{[\text{CaProt}^{-n}]} \quad (8)$$

confirming the fundamental postulates of McLean and Hastings (5). Their nomogram was based on the following equation at pH 7.35, assuming an [Alb]:[Glob] ratio of 1.8 and pK' = 2.22:

$$[\text{Ca}^{++}] = [\text{Ca}] - [\text{Prot}^{-}] - K' + \frac{\sqrt{4K'[\text{Ca}] + ([\text{Prot}^{-}] - [\text{Ca}] + [K'])^2}}{2} \quad (9)$$

where [Prot⁻] was estimated from protein titration data of Van Slyke, Hastings, Hiller, and Sendroy (54): [Prot⁻] (mmoles/liter) = 0.122 [Prot] (g/liter).

The electrode has yielded almost identical constants for K'_{CaProt} and for the 0.122 protein conversion factor. Thus, from studies given elsewhere (1), the electrode has yielded a pK' of 2.18 and about 8.4 negative sites available for calcium binding per albumin molecule. At a mol wt of 69,000, this corresponds exactly to the Van Slyke et al. (54) factor of 0.122 [Alb]. Also, our [Alb]:[Glob] ratio was 1.8 and average pH at the time of ultrafiltration was 7.42, allowing (within about 3%) a direct comparison between electrode serum [Ca⁺⁺] values and those predicted by the McLean-Hastings nomogram.

McLean and Hastings converted [Prot] in g/liter to g/kg H₂O by the formula: [Prot]_{g/kg H₂O} = (100 [Prot]) / (99.0 - 0.75 [Prot]_{g/liter}). To allow comparison of electrode and their predicted values, a similar conversion was made in our 21 subjects. Doing this, and using their value: pK' = 2.22, mean predicted [Ca⁺⁺] was 1.06 ± 0.01 mmoles/liter with an average error (without regard to sign) of 0.10 ± 0.01 mmole/liter, or about 8.5%. [Ca⁺⁺] was underestimated in all but 2 of the 21 subjects. If, however, [Prot] was expressed as g/liter of serum and the electrode value for pK' (2.18) was used, average predicted [Ca⁺⁺] was 1.15 ± 0.01 mmoles/liter, almost exactly that observed experimentally. Average error was 0.059 ± 0.01 mmole/liter or about 5.1%.

We conclude that, using appropriate constants, the McLean-Hastings nomogram gives a close prediction for serum [Ca⁺⁺] in the normal, which is another way of saying that binding of calcium appears to follow the mass-law equation and that variation in serum total calcium is accounted for by corresponding variation in serum protein (mainly albumin) concentrations. A refinement of the nomogram, to account for differences in pH and in albumin- and globulin-binding, might improve the prediction somewhat, but this would yield an algebraic nightmare and is probably not warranted since the electrode is now available for direct measurement of [Ca⁺⁺].

DISCUSSION

Recently developed ion-exchange calcium electrodes provide the first practical means for the direct determination of Ca⁺⁺ in biologic fluids. The present studies indicate that [Ca⁺⁺] may be readily and rapidly determined in serum, whole blood, and ultrafiltrates. *Relative* accuracy therefore appears to be quite satisfactory, as judged by low duplicate variability, reproducible [Ca⁺⁺] curves as a function of pH, high specificity for calcium ion, lack of response to anions and chelates of calcium, linear

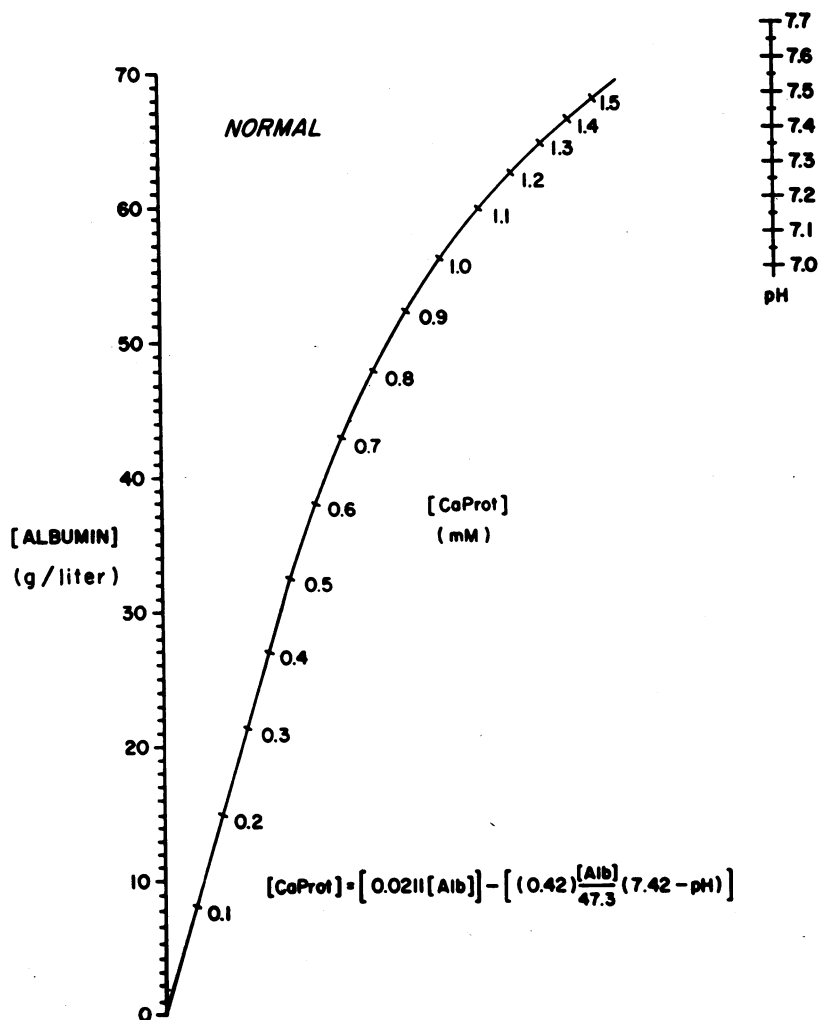


FIGURE 15 Nomogram for estimating protein-bound calcium levels [CaProt] in normal subjects. [CaProt] is obtained by connecting observed albumin and pH values with a ruler; [CaProt] is then read from the curve. Derivation of the equation is given in the text.

recovery studies, end-point detection in chelating titrations, and low variability within a given individual over extended periods of time.

Absolute accuracy is much more difficult to assess, for there is no independent method of direct corroboration. One must therefore consider possible sources and magnitude of error. There appear to be three main considerations: (a) electrode response to other ions or non-ionic constituents of plasma, (b) variation in calcium ion activity coefficients ($\gamma_{Ca^{++}}$) in biologic fluids as compared with calibrating standards, and (c) variation in residual liquid junction potentials (E_j).

The first of these would not appear to be of great importance because of the very high specificity for Ca^{++} over Na^+ , K^+ , and Mg^{++} . Absolute error resulting from

these ions is probably well below 5%. (The selectivity of the electrode varies somewhat with the relative concentrations of Ca^{++} and interfering cations [51]. As noted above [Fig. 5], it would appear advisable to determine the selectivity in studies of biologic solutions with $[Ca^{++}] < 5 \times 10^{-4}$ mole/liter.) At low pH, the calcium electrodes behave as pH electrodes; this is a problem only in gastric juice and perhaps some urines. As noted above, reproducible $[Ca^{++}]$ values have been obtained in gastric juice after alkalization with NaOH to pH 6-7 (44). It is possible that errors could be produced by uptake of plasma or ultrafiltrate constituents by the liquid ion-exchanger. While such a possibility cannot be completely excluded, at least three findings argue against it: (a) observed electrode $[Ca^{++}]$ values were within

5–10% of those for other methods; (b) $[Ca^{++}]$ values were very reproducible within a given individual over extended periods of time, presumably when other plasma constituents were variable; and (c) the EDTA data in Fig. 5 argue strongly against a significant potential of noncalcium origin. Upon addition of EDTA, $[Ca^{++}]$ fell to a very low level; error in apparent $[Ca^{++}]$ could be largely accounted for by sodium error.

Differences in ${}^7Ca^{++}$ in biologic fluids as compared with calibrating standards are quite possible, but again are probably of no great significance. Although there is no experimental means by which individual ion activities can be determined, Garrels (55) has found rather close agreement between ${}^7Ca^{++}$ values from electrode measurements and those from Debye-Hückel theory up to total ionic strength 0.3 mole/liter. The use of calibrating standards with total ionic strength similar to that of plasma should minimize ${}^7Ca^{++}$ differences. Nevertheless, serum proteins may have effects on water activity and apparent activity coefficients.

Variation in E_j , particularly in protein-containing solutions, must always be considered in electrode measurements using a reference (calomel) with liquid junction. As noted by Bates (56), such potentials will generally be small when μ is similar in unknown and standard. Dahms (57) has estimated E_j resulting from different mobilities of the inorganic ions to be below ± 0.1 mv and that due to negatively charged protein to be about 0.5 mv.

There is some evidence which suggests that variation in E_j was not very great in these studies. Thus, ultrafiltrates are essentially simple salt solutions and little difference in E_j as compared with standard $CaCl_2$ -NaCl solutions would be expected. That $[Ca^{++}]$ values were identical in serum and ultrafiltrates, as predicted from the generally accepted monoligand dissociation of calcium proteinate (1), suggests that E_j was similar in sera and ultrafiltrates. Also, we have shown previously that Na^+ activity in serum, as determined with sodium-selective glass electrodes, is almost identical with that of corresponding NaCl solutions after correction for serum water content (58). Nevertheless, a variation in E_j of 0.5 mv could produce an error of about 4% in apparent $[Ca^{++}]$.

It is of interest to compare electrode $[Ca^{++}]$ values with those obtained previously by other methods: (a) the frog heart (4–6), (b) rachitic cartilage bioassay (29), and (c) metal ion indicators (16, 18, 20, 23, 26–28, 30, 31). Each of these has yielded $[Ca^{++}]$ values of about 1.1–1.5 mmoles/liter (usually 1.2–1.3 mmoles/liter) in normal serum, rather close to the present mean electrode value of 1.14 mmoles/liter.

Consistent pH effects on serum $[Ca^{++}]$ have been directly demonstrated for the first time, undoubtedly

resulting mainly from pH-dependent binding by serum proteins. A pH effect on $[Ca^{++}]$ was not expected in ultrafiltrates, which are essentially protein free. At first we thought this might represent an electrode artifact, but no data could be obtained to support this assumption. Thus, pH effects have been observed in varying degree in all ultrafiltrates studied, but have not been seen in mixed standard solutions containing the most prevalent inorganic anions of serum. While the presence of unmeasured pH-dependent polyelectrolytes must be considered a possibility, the pH effect often seemed too great to be accounted for solely on this basis. Detailed calculations and careful studies by Dr. Jacknow in our laboratory suggest that the pH effect in ultrafiltrates largely represents precipitation of certain calcium salts. His calculations indicate that this is thermodynamically possible above pH 6.9; the kinetics might vary considerably, in which case a close correspondence of pH curves in serum and ultrafiltrate (Fig. 9) would be purely fortuitous.

The pH effect is presumably of importance in hypocalcemic tetany. pH effects in both serum and ultrafiltrates suggest that in interstitial fluid, alkalosis might diminish $[Ca^{++}]$ by both protein-binding and solubility effects. If hypocalcemic tetany is due to reduction in $[Ca^{++}]$ at nervous tissue, we may surmise from observed pH effects (Fig. 9) that such pH-dependent changes in $[Ca^{++}]$ are not great. Thus, a 0.1 U increase in serum pH resulted in an average decrease of only 0.04 mmole/liter in $[Ca^{++}]$ in the 52 subjects so studied.

Of particular note was the apparent lack of correlation between serum $[Ca^{++}]$ and $[Ca]$. Ionized calcium was in a rather narrow range, regardless of the total calcium level. (The observed $[Ca^{++}]$ range of 0.94–1.33 mmoles/liter corresponds, in negative log terms, to "pCa" values of 3.02–2.88). In addition, serum $[Ca^{++}]$ within a given individual varied only slightly over a several month period. Ultrafiltration studies indicated that variation in serum total calcium was almost entirely accounted for by variation in protein-bound calcium, in accordance with the mass-law equation. A serum $[Ca]$ level in the normal was therefore an indirect measure of the serum albumin level. The relative constancy of serum $[Ca^{++}]$ in the face of varying total calcium and protein is not observed upon dilution or addition of calcium to normal serum in a test tube (1). The present studies therefore lend strong support to a hormonal regulation of serum $[Ca^{++}]$ and provide some insight into the complex interplay of factors governing serum $[Ca^{++}]$ in man.

We wish to conclude with some notes of caution regarding these electrodes and point out some possible pitfalls in their use. First, the measurement is potentiometric; thus as with pH electrodes, small errors in

potential measurements generate rather large errors in apparent ion activity (or concentration). Since calcium binding by serum proteins and calcium solubility in other biologic fluids is pH dependent, sample pH should be closely regulated and loss of CO₂ prevented. For whole blood and plasma Ca⁺⁺, the common anticoagulants (citrate, oxalate, EDTA) cannot be used, owing to formation of rather strong calcium chelates; it now appears that heparin (Fig. 12) also should not be used unless its effect is quantified at the concentration employed. Na⁺ and K⁺ errors must be considered, respectively, in certain extracellular and intracellular fluids having very low Ca⁺⁺ concentrations. Mg⁺⁺ error must be considered in intracellular fluids and H⁺ error in any fluid with pH less than about 5.5. Insofar as the electrode itself is concerned, the commercial (Orion flow-through) electrode has the disadvantages of being rather expensive, requires periodic replacement of membranes, and has not yet been thermostated. However, as summarized previously (1), high quality data can be obtained in a variety of biologic systems if these factors are considered and appropriate precautions taken. We believe the electrode is a major step forward; [Ca⁺⁺] data, coupled with recent advances in hormone assays, should greatly enhance our present understanding of the physical chemistry and physiology of calcium metabolism.

ACKNOWLEDGMENTS

I gratefully acknowledge the able technical assistance of L. Anderson, D. Alvarez, S. Lipchus, and C. Benjamin.

This investigation was supported by U. S. Public Health Service Research Grants Nos. AM-07417 and AM-10307, National Institute of Arthritis and Metabolic Diseases. Dr. Moore is a recipient of Research Development Award No. K3-GM-11,386, National Institute of General Medical Sciences, U. S. Public Health Service.

REFERENCES

1. Moore, E. W. 1970. Studies with ion-exchange calcium electrodes: some applications in biomedical research and clinical medicine. In Symposium on Ion-Exchange Electrodes, January 1969. R. Durst, editor. National Bureau of Standards, Gaithersburg, Md. In press.
2. Moore, E. W. 1966. Ionized calcium in normal serum, ultrafiltrates, and whole blood directly determined by ion-exchange electrodes. *J. Clin. Invest.* **45**: 1047. (Abstr.)
3. Moore, E. W. 1968. Cation measurements in biological materials. In Conference on Bioelectrodes, June 1966. *N. Y. Acad. Sci.* **148**: 93.
4. McLean, F. C., and A. B. Hastings. 1934. A biological method for the estimation of calcium ion concentration. *J. Biol. Chem.* **107**: 337.
5. McLean, F. C., and A. B. Hastings. 1935. The state of calcium in the fluids of the body. I. The conditions affecting the ionization of calcium. *J. Biol. Chem.* **108**: 285.

6. McLean, F. C., and A. B. Hastings. 1935. Clinical estimation and significance of calcium-ion concentrations in the blood. *Amer. J. Med. Sci.* **189**: 601.
7. Rona, P., and D. Takahashi. 1911. Über das Verhalten des Calciums im Serum und über den Gehalt der Blutkörperchen an Calcium. *Biochem. Z.* **31**: 336.
8. Marrack, J., and G. Thacker. 1926. The state of calcium in body fluids. *Biochem. J.* **20**: 580.
9. Watchorn, E., and R. A. McCance. 1932. Inorganic constituents of cerebrospinal fluid. II. The ultrafiltration of calcium and magnesium from human sera. *Biochem. J.* **26**: 54.
10. Greenberg, D. M., and L. Gunther. 1930. On the determination of diffusible and non-diffusible serum calcium. *J. Biol. Chem.* **85**: 491.
11. Nicholas, H. O. 1932. Diffusible serum calcium by high pressure ultrafiltration. *J. Biol. Chem.* **97**: 457.
12. Dillman, L. M., and M. B. Visscher. 1933. The calcium content of ultrafiltrates of plasma and the influence of changes in hydrogen and bicarbonate ion concentrations upon it. *J. Biol. Chem.* **103**: 791.
13. Morison, R. S., R. McLean, and E. B. Jackson. 1937. Observation on the relation between ionized and total calcium in normal and abnormal sera and their ultrafiltrates. *J. Biol. Chem.* **122**: 439.
14. Hopkins, T., J. E. Howard, and H. Eisenberg. 1952. Ultrafiltration studies on calcium and phosphorus in human serum. *Bull. Johns Hopkins Hosp.* **91**: 1.
15. Toribara, T. Y., A. R. Terepka, and P. A. Dewey. 1957. The ultrafiltrable calcium of human serum. I. Ultrafiltration methods and normal values. *J. Clin. Invest.* **36**: 738.
16. Rose, G. A. 1957. Determination of the ionized and ultrafiltrable calcium of normal human plasma. *Clin. Chim. Acta.* **2**: 227.
17. Neuman, W. F., and M. W. Neuman. 1958. The Chemical Dynamics of Bone Mineral. University of Chicago Press, Chicago. 1-38.
18. Lloyd, H. M., and G. A. Rose. 1958. Ionized, protein-bound, and complexed calcium in the plasma in primary hyperparathyroidism. *Lancet.* **2**: 1258.
19. Prasad, A. S., and E. B. Flink. 1958. The base binding property of the serum proteins with respect to calcium. *J. Lab. Clin. Med.* **51**: 345.
20. Fanconi, A., and G. A. Rose. 1958. The ionized, complexed, and protein-bound fractions of calcium in plasma. *Quart. J. Med.* **27**: 463.
21. Prasad, A. S. 1960. Studies on ultrafiltrable calcium. *Amer. Med. Ass. Arch. Intern. Med.* **105**: 560.
22. Loken, H. F., R. J. Havel, G. S. Gordan, and S. L. Whittington. 1960. Ultracentrifugal analysis of protein-bound and free calcium in human serum. *J. Biol. Chem.* **235**: 3654.
23. Walser, M. 1960. Ion association. I. The effect of multivalent anions on the protein-bound and complexed calcium in serum. *J. Cell. Comp. Physiol.* **55**: 245.
24. Breen, M., and S. Freeman. 1961. Plasma calcium distribution in relation to parathyroid function. *Amer. J. Physiol.* **200**: 341.
25. Breen, M., and S. Freeman. 1961. The use of high centrifugal force in separating plasma calcium into its protein-bound and protein-free fractions. *Clin. Chim. Acta.* **6**: 181.
26. Walser, M. 1961. Ion Association. VI. Interactions between calcium, magnesium, inorganic phosphate, citrate

- and protein in normal human plasma. *J. Clin. Invest.* **40**: 723.
27. Walser, M. 1952. The separate effects of hyperparathyroidism, hypercalcemia of malignancy, renal failure, and acidosis on the state of calcium, phosphate, and other ions in plasma. *J. Clin. Invest.* **41**: 1454.
 28. Lloyd, H. M., G. A. Rose, and D. Smeenk. 1962. The ability of plasma proteins to bind calcium in normal subjects, in patients with primary hyperparathyroidism both pre- and post-operatively, and in other hypercalcemic conditions. *Clin. Sci. (London)*. **22**: 353.
 29. Yendt, E. R., T. B. Connor, and J. E. Howard. 1955. In vitro calcification of rachitic rat cartilage in normal and pathological human sera with some observations of the pathogenesis of renal rickets. *Bull. Johns Hopkins Hosp.* **96**: 1.
 30. Etti, J., and S. M. Scoggan. 1959. Ionized calcium in biological media. *Nature (London)*. **184**: 1315.
 31. Walser, M. 1960. Determination of free magnesium ions in body fluids: Improved methods for free calcium ions, total calcium and total magnesium. *Anal. Chem.* **32**: 711.
 32. Fosbinder, R. J. 1929. A study of the calcium amalgam electrode in dilute aqueous solutions. *J. Amer. Chem. Soc.* **51**: 1345.
 33. Abrams, I., and K. Sollner. 1942. The structure of the colloidion membrane and its electrical behavior. *J. Gen. Physiol.* **26**: 369.
 34. Sollner, K. 1943. A new potentiometric method to determine cations and anions with colloidion and protamine-colloidion "membrane electrodes." *J. Amer. Chem. Soc.* **65**: 2260.
 35. Sollner, K., and R. Neihof. 1951. Strong acid type of permselective membrane. *Arch. Biochem. Biophys.* **33**: 166.
 36. Carr, C. W. 1955. Determination of ionic activity in protein solutions with colloidion membrane electrodes. *In Electrochemistry in Biology and Medicine*. T. Shedlovsky, editor. John Wiley & Sons Inc., New York. 266.
 37. Eisenman, G., D. O. Rudin, and J. U. Casby. 1957. Glass electrode for measuring sodium ion. *Science (Washington)*. **126**: 831.
 38. Moore, E. W. 1967. Hydrogen and cation analysis in biological fluids in vitro. *In Glass Electrodes for Hydrogen and Other Cations: Principles and Practice*. G. Eisenman, editor. Marcel Dekker, Inc., New York. 412.
 39. Truesdell, A. H., and C. L. Christ. 1967. Glass electrodes for calcium and other divalent cations. *In Glass Electrodes for Hydrogen and Other Cations: Principles and Practice*. G. Eisenman, editor. Marcel Dekker, Inc., New York. 293.
 40. Gregor, H. P., and H. Schonhorn. 1957. Multilayer membrane electrodes. *J. Amer. Chem. Soc.* **79**: 1507.
 41. Gregor, H. P., and H. Schonhorn. 1959. Multilayer membrane electrodes. II. Preparation and use in double concentration cells. *J. Amer. Chem. Soc.* **81**: 3911.
 42. Schonhorn, H., and H. P. Gregor. 1961. Multilayer membrane electrodes. III. Activity of alkaline earth salts in mixed electrolytes. *J. Amer. Chem. Soc.* **83**: 3576.
 43. Ross, J. W., Jr. 1967. Calcium-selective electrode with liquid ion-exchanger. *Science (Washington)*. **156**: 1378.
 44. Moore, E. W., and G. M. Makhlof. 1968. Calcium in normal human gastric juice: a 4-component model with speculation on the relation of calcium to pepsin secretion. *Gastroenterology*. **55**: 465.
 45. Moore, E. W., and J. W. Ross, Jr. 1965. NaCl and CaCl₂ activity coefficients in mixed aqueous solutions. *J. Appl. Physiol.* **20**: 1332.
 46. Harned, H. S., and B. B. Owen. 1963. *The Physical Chemistry of Electrolytic Solutions*. Reinhold Publishing Corporation, New York. 595.
 47. Klotz, I. M. 1950. *Chemical Thermodynamics*. Prentice-Hall Inc., Englewood Cliffs, N. J. 329.
 48. Robinson, R. A., and R. H. Stokes. 1959. *Electrolyte Solutions*. Butterworth & Co. (Publishers) Ltd., London. 438.
 49. Bachra, B. N., A. Dauer, and A. E. Sobel. 1958. The complexometric titration of micro and ultramicro quantities of calcium in blood serum, urine and inorganic salt solutions. *Clin. Chem.* **4**: 107.
 50. Gornall, A. G., C. J. Bardawill, and M. M. David. 1949. Determination of serum proteins by means of the biuret reaction. *J. Biol. Chem.* **177**: 751.
 51. Ross, J. W., Jr. 1966. *Calcium Ion Electrode Instruction Manual*. Orion Research, Inc., Cambridge, Mass. 10.
 52. Silen, L. G., and A. E. Martell. 1964. *Stability Constants of Metal-Ion Complexes*. Special Publication No. 17. The Chemical Society, London. 478.
 53. Nakayama, F. S., and B. A. Rasnick. 1967. Calcium electrode for measuring dissociation and solubility of calcium sulphate dihydrate. *Anal. Chem.* **39**: 1022.
 54. Van Slyke, D. D., A. B. Hastings, A. Hiller, and J. Sendroy, Jr. 1928. Studies of gas and electrolyte equilibrium in blood. *J. Biol. Chem.* **79**: 769.
 55. Garrels, R. M. 1967. Ion-sensitive electrodes and individual ion activity coefficients. *In Glass Electrodes for Hydrogen and Other Cations: Principles and Practice*. G. Eisenman, editor. Marcel Dekker, Inc., New York. 349.
 56. Bates, R. G. 1964. *Determination of pH*. John Wiley & Sons, Inc., New York. 275.
 57. Dahms, H. 1966. Automated electrochemical determination of inorganic blood constituents (Na⁺, K⁺, H⁺, Cl⁻). IBM Research Paper RC-1625, Watson Research Center, Yorktown Heights, N. Y.
 58. Moore, E. W., and D. W. Wilson. 1963. The determination of sodium in body fluids by the glass electrode. *J. Clin. Invest.* **42**: 293.