Measurement of Ionized Calcium in Serum with Ion-Selective Electrodes: A Mature Technology That Can Meet the Daily Service Needs

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This article reviews key advances in ion-selective electrode technology that have made potentiometric measurements of ionized calcium (Ca\(^{2+}\)) reliable and precise. Our use of two second-generation Ca\(^{2+}\) analyzers (Radiometer ICA1 and NOVA 8) made possible uninterrupted service as volume increased to 31,640 patient tests in 1985. The lower results on the NOVA 8 were adjusted upwards to match those of the ICA1 to give identical results. Both analyzers were evaluated under working conditions of high volumes and multiple operators to establish downtime, electrode life, and costs. We have classified all Ca\(^{2+}\) analyzers into first-, intermediate-, and second-generation instruments, the better to understand their differences. Results for large numbers of patients' sera were shown to be systematically different when any two analyzers were compared. These differences are the consequence of each manufacturer's unique choices of the following: (a) the matrix of the calcium calibration solutions, (b) the type and configuration of the reference electrode, and (c) the salt-bridge solution. Elimination of each analyzer's biases will require agreement on a reference system that defines the accuracy of Ca\(^{2+}\) measurements on serum, plasma, or whole blood. The sound analytical performance of today's second-generation Ca\(^{2+}\) analyzers has allowed us to exploit the inherent superiority of Ca\(^{2+}\) over total calcium (Ca\(_T\)) measurements in the daily care of patients. We report on the preference of Ca\(^{2+}\) over Ca\(_T\) by physicians at our hospital since the introduction of second-generation Ca\(^{2+}\) analyzers. Therefore, we state unequivocally from our very satisfactory experience over the past five years that Ca\(^{2+}\) is a clinical laboratory test whose time has come!

The measurement of ionized calcium (Ca\(^{2+}\)) in serum was ordered 10 times more often than that of total calcium (Ca\(_T\)) by the medical staff at Hartford Hospital\(^{3}\) in February 1986. As shown in Figure 1, this preference by our physicians for Ca\(^{2+}\) measurements became clear about a year after its introduction as a daily service test five years ago. The experience gained in providing 31,000 Ca\(^{2+}\) measurements per year and in helping colleagues to interpret the meaning of results on individual patients is the reason for our advocacy of this basic test in the evaluation of calcium metabolism. This article will review the following topics:

I. Rationale for measuring Ca\(^{2+}\) vs Ca\(_T\)
II. Classification and availability of Ca\(^{2+}\) analyzers
III. Experience with two second-generation Ca\(^{2+}\) analyzers
IV. Analysis of the clinical utility of Ca\(^{2+}\) at our hospital

While the analytical reliability of Ca\(^{2+}\) measurements is the primary focus of this article, we must also emphasize the importance of pre-analytical factors (position, time, and meals) and post-analytical considerations (age, limits, and expected values) that must be taken into consideration to make the interpretation of Ca\(^{2+}\) results meaningful to the physician.

I. Rationale for Measuring Ca\(^{2+}\) vs Ca\(_T\)

The separation of calcium into a diffusible fraction and a non-diffusible protein-bound fraction was reported by Rona and Takahashi in 1911 (2). When McLean and Hastings initiated their classical studies on the meas-

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**Note:**

1. Nonstandard abbreviations: Ca\(^{2+}\), ionized calcium; Ca\(_T\), total calcium; E, liquid-lieuid junction potential; FY '85, fiscal year 1985; ICA1, Radiometer Model ICA1 Ca\(^{2+}\) analyzer; ICU, intensive care unit; ISE, ion-selective electrode; Nova 8, Nova Biomedical Inc., Model 8 Ca\(^{2+}\) analyzer.

2. Hartford Hospital is a tertiary-care 885-bed general hospital with a full complement of teaching programs and a wide range of medical-surgical services that include pediatrics, trauma, oncology, obstetrics/gynecology, psychiatry, and cardiovascular/transplantation surgery programs for the kidney, heart, and liver.

3. Hartford Hospital.

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**Fig. 1.** Ionized calcium was ordered 10 times more often than total calcium by the medical staff at Hartford Hospital in February 1986 (period 56).

A period is 28 days, and 13 periods make a fiscal year (FY), which runs from Oct. 1 to Sept. 30. FY '82 includes periods 1–13, FY '84 from 14 to 26, FY '85 from 27 to 38, FY '85 from 4 to 22, and FY '86 from 23 to 55.

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CLINICAL CHEMISTRY, Vol. 32, No. 8, 1986 1437
measure of Ca$^{2+}$ with the frog heart-muscle bioassay in the early 1930's, the diffusible fraction had been further subdivided into "free" calcium ions and calcium complexed to anions (3). Their experimental observations left no doubt that Ca$^{2+}$ was the "biologically active" fraction of primary physiological and clinical importance in blood and other body fluids (4). Subsequent studies on healthy individuals and on patients with parathyroid disorders showed unequivocally that the parathyroid glands regulated the concentration of Ca$^{2+}$ and established the central role of Ca$^{2+}$ in human calcium metabolism (5). While McLean and Hastings advocated Ca$^{2+}$ over Ca$\text{r}$ measurements in patients, they discouraged the use of the bioassay method in the clinical laboratory. This paradox was resolved by their providing a nomogram for calculating Ca$^{2+}$ from Ca$\text{r}$ and total protein. However, they noted that this estimation of Ca$^{2+}$ was based on several assumptions and was only a first approximation, because "other variables, including pH, temperature, albumin to globulin ratio, magnesium, and citrate, are known to influence the ionization of calcium in these fluids" (4). Neither this original nomogram nor later ones that replaced total protein with albumin and added pH have been wide use for calculating Ca$^{2+}$ in clinical laboratories (6).

McLean and Hastings' brilliant work led many investigators to try to develop chemical and physical separatory techniques to measure this "active" free-calcium fraction in blood (7, 8), but few of these methods were used in clinical service laboratories. The need for quantitative calcium measurements in blood was served by a variety of Ca$^{2+}$ methods (9). Dependence on Ca$\text{r}$ as the sole means of evaluating calcium status in patients started to change in 1967 after the publication of Ross's paper entitled "calcium selective electrode with liquid ion-exchanger" (10). During the 1965-70 period when the first ion-selective electrode (ISE) was in the early stages of development, Moore explored the clinical applications of Ca$^{2+}$ measurements in patients (8, 11-13). His work showed the feasibility of making rapid ISE measurements of Ca$^{2+}$ directly in serum, plasma, or whole blood. The calcium ISE of the electrochemical cell, like frog muscle, responded only to "free" calcium ions in the blood; it was not subject to the variability of calcium bound to albumin or complexed to anions. Moore concluded that the measurement of Ca$^{2+}$ by ISE was more useful in clinical medicine than that of Ca$\text{r}$.

This affirmation of McLean and Hastings' findings on the utility of Ca$^{2+}$, coupled with the availability of a Ca$^{2+}$ analyzer [Orion 99-20, Orion Research Inc., Cambridge, MA 02139) stimulated many clinical laboratory scientists and physicians to enter this field in the 1970's. Subsequently, when Robertson and Marshall (7) reviewed the literature on Ca$^{2+}$ a decade later in 1981, they noted that 200 of their 238 references were recent reports on the clinical applications of Ca$^{2+}$ measurements made by ISE direct potentiometry. The majority of the authors were enthusiastic about the clinical utility of Ca$^{2+}$ measurements in spite of problems involving analytical performance, standardization, sample handling, and reference values. Robertson and Marshall concluded that assay of Ca$^{2+}$ "provides the most relevant biological and chemical information for both the clinician and the research worker, but difficulties with methodology often make interpretation of data problematical." The key advances in ISE technology to be discussed below had not yet been introduced into clinical laboratories when Robertson and Marshall reached this conclusion in 1980.

II. Classification and Availability of Ca$^{2+}$ Analyzers

Ross's 1967 publication stimulated several chemistry groups to synthesize and compare the properties of some new compounds as liquid-membrane sensors with high selectivity for calcium ions. The major research and development contributions that have been progressively incorporated into Ca$^{2+}$ analyzers are as follows:

(a) development of more selective and more reliable liquid-membrane electrodes (14-16);
(b) incorporation of the ion-exchanger and solvent into polymeric membranes, usually polyvinyl chloride (PVC), to provide greater structural stability and flexibility (14, 15); and
(c) manufacture of semi-automated Ca$^{2+}$ analyzers that simultaneously measure pH and Ca$^{2+}$ in miniature electrochemical cells that maximized signal to noise ratio by unique electrode geometry, special liquid–liquid junction dynamics, and advanced microprocessor technology.

It is now clear that the two types of liquid-membrane sensors used in today's commercial analyzers—namely, the improved organophosphate and the neutral carrier—give a nearly identical Nernstian response to changes in Ca$^{2+}$. However, while voltage response may be similar, the final Ca$^{2+}$ results are often different because each analyzer has its own unique systematic bias* (19-22). These biases are due mainly to (a) the dissimilar matrix of the calibrator solutions and (b) the variations in the reference electrodes, especially in respect to their salt-bridge solutions and the dynamics occurring at the liquid–liquid junction during the 60-90 s of the measurements.

It is no longer possible to discuss Ca$^{2+}$ measurements by ISE devices without first identifying the analyzer(s) in order to differentiate between the instruments introduced before 1978 and those introduced afterwards. Ca$^{2+}$ analyzers have been or are now produced by eight original equipment manufacturers, with facilities located in Austria, Denmark, England, Finland, Japan, and the U.S.A. Table 1 classifies the past, present, and "soon-to-be-available" instruments into first-, intermediate-, and second-generation Ca$^{2+}$ analyzers.

First-generation analyzers were introduced between 1967 and 1978, but most of them have now been discontinued or replaced with newer models. Our initial experience with Ca$^{2+}$ measurements by ISE direct potentiometry started in 1971 with the purchase of an Orion 99-20. When this system was functioning properly, we were able to confirm Moore's findings that Ca$^{2+}$ results did indeed give more meaningful clinical information than Ca$\text{r}$ (23, 24). Unfortunately, we were forced to discontinue offering Ca$^{2+}$ measurements for patient care because the Orion 99-20 system was not rugged and precise enough for continuous daily use in testing. Our

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* We have reported on the various methods used for service calcium measurements at Hartford Hospital in an article entitled "Calcium Measurements at Hartford Hospital 1959-1982. The Integration of Service, Research, and Education within the Clinical Chemistry Laboratory." This paper appeared in the Hartford Hospital Bull 1982:34:43-55, and is available by written request to Dr. Bowers.

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* We have now compared five other analyzers with the ICA1 and find they all give essentially a parallel response, although lower by 0.02 to 0.04 mmol/L. These have included the NOVA 2, 7, and 8, the AVL 980, and a prototype of the Ciba–Corning Model 634.
major problem seemed to be lot-to-lot changes in the quality of both the liquid ion-exchanger and the nitrocellulose membranes. In retrospect, we expected too much of an early ISFET technology that was undergoing major changes and frequent field modifications, as subsequent reports have documented (8, 25–27).

Intermediate-generation Ca^{2+} analyzers were introduced from 1978 until 1982 and all used the neutral carrier sensor of Simon (see ref. 18). The classification of "intermediate" is used to identify the lack of a concurrent pH measurement at 37 °C. Consequently, these intermediate Ca^{2+} analyzers do not have the ability to normalize Ca^{2+} to pH 7.4 (Ca^{2+}_7.4) as many workers have recommended (7, 8, 24, 28–30).

All second-generation Ca^{2+} analyzers listed in Table 1 make simultaneous Ca^{2+} and pH measurements at 37 °C on every sample under the semi-automatic control of a microprocessor which internally calculates the Ca^{2+}_7.4 and then displays Ca^{2+}, pH, and Ca^{2+}_7.4 results. The importance of these simultaneous pH and Ca^{2+} measurements is shown in the bottom half of Figure 2, the distribution of pH values for 250 hospitalized adult patients who had Ca^{2+} requested in mid-December 1985. Note that 31% (14% low, 17% high) of these venous-serum samples had a pH value that fell beyond the reference limits of 7.35 and 7.45. By plotting the actual Ca^{2+} at the measured pH and then connecting to the Ca^{2+}_7.4 value by a line, one can see a two-dimensional representation of the alternative ways of reporting Ca^{2+}. The question of which of these parameters need to be reported to physicians is unresolved (29–32).

Table 2 lists the operational and analytical performance characteristics of four second-generation Ca^{2+} analyzers. The trend in the recently developed Ca^{2+} analyzers is towards smaller sample size, quicker display of results, and reduced overall cycling time (33, 34).

III. Experience with Two Second-Generation Ca^{2+} Analyzers

The Radiometer ICA1: In September 1981, we evaluated the Radiometer ICA1 [Radiometer A/S, Emdrupvej 72, DK-2400, Copenhagen, Denmark] and found it rugged and easy to operate and maintain. The small sample volume, only 120 μL, was advantageous for testing sera from premature and full-term newborns. Although the use of serum requires time for clotting and centrifugation, we evolved a strong preference in our routine service testing for serum over...
Fig. 2. Serum ionized calcium: NOVA 8 vs Radiometer ICA1
A concurrent pH with ionized calcium measurements is needed to make a meaningful interpretation of results. The distribution of venous pH values measured in 250 ionized calcium samples drawn on hospitalized patients shows (bottom) that 31% exceeded the reference limits of 7.35 and 7.45. The actual ionized calcium at its original pH is plotted (+) and connected by an arrow to its corrected value to pH 7.4.8. There is currently no consensus that supports the use of the normalized Ca⁺²⁺ value vs the actual Ca⁺²⁺ value.

heparinized plasma because its use eliminated clogging by microclots and avoided the spurious lowering of Ca⁺²⁺ from heparin binding. Selectively permitting the collection of heparinized whole blood samples in special circumstances, we were also able to provide 5-min "ultra-stat" responses during cardiovascular and transplant operations, even on very small children and babies. However, falsely low Ca⁺²⁺ values are caused whenever heparin is used (23). Larson et al. (31) have reported on their successful use of special heparinized capillary tubes containing CaCl₂ to compensate for Ca⁺²⁺ binding (Radiometer/Type D951); however, they noted overestimation at low (0.5 mmol/L) and underestimation at high (2 mmol/L) concentrations. Our experience was less favorable because microclots were much too frequent.

As workloads grew to 35–40 tests/day (1000 tests/28-day period in Figure 1), we noted that recalibration with aqueous reference materials and repeat values on serum became less reproducible. This poorer performance was attributed to "protein build-up" owing to the heavy volume of patient testing. Intensified cleaning and preventative maintenance did not improve the reproducibility, but addition of a surfactant to the rinse solution did. Further changes in all plastic parts in contact with serum and a new sodium formate bridge solution also helped to improve performance, even with a steadily increasing workload.

We also noted that the longevity of the Ca⁺²⁺ electrode tips decreased as workloads grew. As seen in Table 3, the interval between tip changes in the three heavily used service laboratory ICA1's ranged from nine to 52 days and averaged 25 days. In contrast, the tip-life in the less heavily used R&D instrument ranged from nine to 84 days and averaged 51 days. This difference in tip-life is to be expected, because each cycle causes minor leaching of the vital ion-exchanger and solvent from the PVC membrane. Thus, longevity is more a function of the number of cycles to which each tip is exposed rather than of the time in days. To examine this relationship in greater detail, we counted all cycles for Ca⁺²⁺ testing during February 1986 and found, as shown in Table 4, that only 44% of cycles represented reportable patient tests. Thus, the 31 640 patient tests of fiscal year 1986 translated to a grand total of about 70 000 cycles. We estimated from yearly purchase orders that we used 150 membranes and 50 electrode tips. These figures suggest that membranes need to be changed after 500 cycles and tips after 1500 cycles to be sure an ISE is fully functional. From these data on tip cycle-life and knowledge of yearly reagent and calibrator usage, we can make a reasonable estimate of the cost for expendable items for the ICA1 at our heavy volume of daily use. This cost is currently

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Table 2. Operational Characteristics of Ca⁺²⁺ Analyzers

<table>
<thead>
<tr>
<th>Feature</th>
<th>Radiometer ICA1</th>
<th>Nova Biomed. Model 8</th>
<th>Baker Inst. Analyte+2</th>
<th>Ciba-Corning Model 84**</th>
</tr>
</thead>
<tbody>
<tr>
<td>Microprocessor</td>
<td>yes</td>
<td>yes</td>
<td>yes</td>
<td>yes</td>
</tr>
<tr>
<td>Parameters reported</td>
<td>Actual Ca⁺²⁺</td>
<td>yes</td>
<td>yes</td>
<td>yes</td>
</tr>
<tr>
<td>pH at 37°C</td>
<td>yes</td>
<td>yes</td>
<td>yes</td>
<td>yes</td>
</tr>
<tr>
<td>Ca⁺²⁺ at pH 7.4</td>
<td>yes</td>
<td>yes</td>
<td>yes</td>
<td>yes</td>
</tr>
<tr>
<td>Sample vol, µL</td>
<td>120</td>
<td>350</td>
<td>250</td>
<td>35</td>
</tr>
<tr>
<td>Time to result, s</td>
<td>70</td>
<td>90</td>
<td>45</td>
<td>70</td>
</tr>
<tr>
<td>Total cycle time, s</td>
<td>180</td>
<td>90</td>
<td>75</td>
<td>90</td>
</tr>
<tr>
<td>Estimated max. samples/h</td>
<td>15-20</td>
<td>20-35</td>
<td>25-40</td>
<td>20-35</td>
</tr>
<tr>
<td>Calibrators</td>
<td>2 point</td>
<td>2 point</td>
<td>2 point</td>
<td>2 point</td>
</tr>
<tr>
<td>[Ca⁺²⁺], mmol/L</td>
<td>1.25:2.50</td>
<td>1.00:3.06</td>
<td>1.00:1.50</td>
<td>1.25:2.50</td>
</tr>
<tr>
<td>External controls, mmol/L</td>
<td>0.75:1.75</td>
<td>1.00:1.50</td>
<td>1.00:1.50</td>
<td>0.75:1.15</td>
</tr>
<tr>
<td>Precision (±1SD in mmol/L)</td>
<td>0.016</td>
<td>0.014</td>
<td>0.016</td>
<td>0.012</td>
</tr>
<tr>
<td>CV, %</td>
<td>1.3</td>
<td>1.2</td>
<td>1.4**</td>
<td>1.0**</td>
</tr>
<tr>
<td>Patient results vs ICA1</td>
<td>NA (100%)</td>
<td>97%</td>
<td>(97 ± 1%)</td>
<td>adjust</td>
</tr>
</tbody>
</table>

All measure samples of whole blood, plasma, or serum.
*NBI All are estimates by one of us (GNB Jr.).
**From reference 34.
**By comparison to Nova or Radiometer Ca⁺²⁺ analyzers in ref. 34.
**By Henderson equation from Sena et al. (22) and ref. 34.
**Ciba-Corning Instruments Inc., Medford, MA 02052.
Table 3. Longevity of Ca\textsuperscript{2+} Tips in Radiometer ICA1 (Sept. 27, 1984–March 18, 1985)

<table>
<thead>
<tr>
<th>Radiometer</th>
<th>Use</th>
<th>Membranes</th>
<th>Longevity of ISE tips, no. (and days in service)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ICA1-1</td>
<td>daily</td>
<td>14</td>
<td>6 (*, 14, 16, 16, 30, 41)</td>
</tr>
<tr>
<td>ICA1-2</td>
<td>daily</td>
<td>19</td>
<td>8 (*, 9, 20, 25, 26, 30, 42)</td>
</tr>
<tr>
<td>ICA1-3</td>
<td>daily</td>
<td>22</td>
<td>7 (12, 15, 21, 23, 28, 39, 52)</td>
</tr>
<tr>
<td>Totals, service only</td>
<td>daily</td>
<td>55</td>
<td>21 (excluding*, X = 25 ± 12)</td>
</tr>
</tbody>
</table>

*Longevity < seven days.

Table 4. Details of February 1985 Cycles on Three Radiometer ICA1 Service Instruments

| Patient’s serum sample testing | 2319 | 44% |
| Calibrators and aqueous controls | 2002 | 38% |
| Internal serum pool controls | 587 | 11% |
| Repeats on patient samples | 397 | 7% |
| Total cycles | 5305 | 100% |

In FY '86, we estimate from the usage of tips from Table 3 data that 50 tips will be required to produce the 31,600 patients’ serum cycles or 70,000 total cycles (above). This suggests that each calcium tip lasts for about 1400 analytical cycles.

The analytical performance of each of the three ICA1's used for patient testing at workloads averaging 70 patient tests/day (160 cycles/day) was monitored daily in FY '85 with our pooled human serum internal quality-control material (35). The standard deviation (SD) for Ca\textsuperscript{2+} each month ranged from 0.010 to 0.024 mmol/L at a mean concentration near 1.20 mmol/L. The median SD for all 36 months was ±0.016 mmol/L, giving a CV of 1.3% for our day-to-day long-term intralaboratory Ca\textsuperscript{2+} testing. Boink and Sprokholt recently reported (21) on the interlaboratory performance observed during a large survey of Ca\textsuperscript{2+} results involving 24 separate ICA1’s in European and American laboratories (including our four ICA1’s). The Ca\textsuperscript{2+} result for a bovine serum sample had a mean and SD of 1.069 and 0.026 mmol/L, respectively, and a CV of 2.5% for a network of 16 laboratories located in a wide geographical area.

To estimate the systematic bias of the ICA1 when measuring serum, we used the Henderson equation (36) to calculate the liquid–liquid junction potential (Ej) of the ICA1 calibrator and serum. The residual Ej suggested that results for patients’ serum samples are systematically low by 5.0%. Our experimental studies suggested that values for serum are 6.2% low as measured in the ICA1 (22).

The NOVA 8: In simultaneous comparisons of Ca\textsuperscript{2+} results on patients’ serum samples measured in the NOVA 8 (Nova Biomedical Inc., Waltham, MA 02254) and the ICA1, we found that the NOVA 8 consistently gave parallel but lower results by 0.02 to 0.03 mmol/L at all concentrations as plotted in Figure 3. Knowing that all analyzers have their unique biases, we simply adjusted the NOVA 8 outputs upwards electronically to give results that matched those of the ICA1 (see the table in lower right of Figure 3), because the reference intervals in this hospital were firmly established by four years of use of the ICA1’s. This "adjusted" NOVA 8 was placed in service alongside two ICA1’s during the summer of 1985 so that it might be evaluated under similar working conditions of heavy daily testing by many analysts. In so doing, we achieved a long-sought goal of redundancy for service testing with two second-generation Ca\textsuperscript{2+} analyzers, replacement parts, reagents, and calibrators supplied by totally independent U.S. and Danish sources. Furthermore, the 90-s cycle time of the NOVA 8's, as compared with 180 s for the ICA1, shortened the time needed to perform Ca\textsuperscript{2+} testing. The trade-off for this increase in throughput was a 350-μL sample volume for the NOVA 8 vs only 120 μL for the ICA1. However, sample volume has been a secondary consideration in adults, although this too is changing (37, 38), and is mitigated when an analyzer such as the ICA1 is available for smaller samples (e.g., from premature infants).

Our daily operational experience with the NOVA 8 showed that it required the same fastidious care and regular maintenance as all other ISE devices. We became aware of a subtle relationship between the plastic tubing and the calcium sensor ("inner element") that caused results to change as either or both components aged. Aqueous controls were relatively insensitive to these changes and we, like

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5 These expendable costs can be decreased if the laboratory makes the rinse solutions and calibrators. Labor costs vary from lab. to lab., but we estimate it is $0.50/cycle or about $1.00/charged patient test. Likewise, firm estimates of repair and capital costs that depend on test volume are quite varied. However, our current estimate is $0.10/cycle.
others (39), found it necessary to monitor the daily analytical performance with serum-based control materials in order to determine when to install and (or) accept a new "inner element" or to replace tubing and other components.

A long-term study of the longevity of the NOVA 8 "inner elements" is in progress and present data suggest that they should be replaced after 650 ± 50 cycles if a full response to all serum samples is to be assured. Our cost estimate for expendables for the NOVA 8 is $0.27 per cycle (exclusive of labor and capital costs) or about $0.45 per patient sample.8

The analytical performance of the NOVA 8 when used daily for patient testing at overall workload rates of 70 patient tests per day (160 cycles per day) was also monitored with our internal quality-control pool. The monthly SD for Ca2+ for seven months ranged from 0.010 to 0.023 mmol/L for a mean concentration near 1.20 mmol/L; the median SD was 0.014 mmol/L, giving a CV of 1.2%. The residual E8, calculated with the Henderson equation for the difference between Nova 8 calibrator and serum, was -6.5%; our experimental studies gave an identical value (22).

IV. Analysis of the Clinical Utility of Ca2+ on Our Wards

Basal conditions: For hospitalized patients, we request that the patient remain supine after an overnight fast until an anaerobic venous serum sample is drawn between 7 and 9 a.m. This deliberate choice of preparation, timing, and sampling defines the basal conditions and helps to avoid Ca2+ changes due to posture, diurnal variation, and recent food ingestion. The anaerobic sample can be held and centrifuged at room temperature for up to 4 h before changes in Ca2+ concentration are seen. For longer storage intervals of days or months, we hold serum off the clot at 4°C or -80°C, to minimize pH and other metabolic changes.

Reference values: The Ca2+ limits for adults under basal conditions are from 1.17 to 1.29 mmol/L for the Radiometer ICA1's and the "adjusted" NOVA 8 analyzers (29). Younger men and older women tend slightly towards the higher limit, while younger women tend slightly towards the lower limit. Due to diurnal variation, which causes a peak in Ca2+ between 7 to 9 a.m., the values for specimens drawn at other times are slightly lower. However, the upright position causes shifts of fluids into the extravascular spaces of the lower extremities and the serum from blood drawn from an arm vein of an individual who is upright will consistently give a slightly higher Ca2+ value. For adults who are ambulatory prior to venipuncture, we find the upper reference limit (Ca2+ 99) is increased by 0.04 mmol/L over the pre/fasting value of 1.29 to 1.33 mmol/L.7 Food ingestion usually increases Ca2+, but this varies with intake and timing, and we have seen lower values in a few individuals (24).

The reference intervals for the different pediatric age groups vary greatly in respect to the relatively narrow adult range. Values <1.00 mmol/L are characteristic of prema-

ture infants, and the degree of hypocalcemia correlates with the gestational age (40). Healthy newborns show about the same mean value as adults during the first 24 postnatal hours, but with much wider limits. The mean and limit values move progressively higher during the succeeding two weeks. Given the rapid physiological shifts in Ca2+, water, and other electrolytes that can occur just before and after the birth of sick newborns, the lack of well-defined pediatric reference values has made difficult the clinical interpretation of Ca2+ results in premature and newborn infants. Values for young children continue to be somewhat higher than those for adults until after the pubertal growth spurts. The reference values merge with those for adults in the mid to late teens for females and in the late teens to early twenties for males.

Abnormal results in hospitalized patients: The importance of the simultaneous measurement of pH with Ca2+ in patients' samples has already been shown. The slight shift in the distribution to the higher pH values (see Figure 2) suggests a tendency to lose small amounts of CO2 during sample handling, thus lowering the calcium ion concentration in vitro from what it was in vivo. To offset this systematic bias toward an artificially lowered Ca2+, we routinely report to the wards only the Ca2+ 99, but all three values are recorded and made available when requested. The bold dashed line in Figure 2 represents the vector of changing Ca2+ values as CO2 is either lost or gained in a serum that starts at the physiological mean concentration of 1.23 mmol of Ca2+ per liter and a pH of 7.40. We would prefer to plot all three results on a report form such as that shown in Figure 2, but computerized formats currently limit the routine reporting to only the Ca2+ 99.

Table 5 summarizes the abnormal Ca2+ 99 results that were reported to the wards and intensive-care units of medicine, surgery, and pediatrics for the months of December 1983 and February 1985. Note that the abnormal values in outpatients were at overall rates of between 3 and 6%, as expected. However, we were surprised at the 39% (31% low, 8% high) abnormal rate for Ca2+ results on the inpatient wards in December 1983. Closer inspection of these later data reveals that the highest abnormal rates were as follows: Recovery Room at 69% (50% low, 20% high), Surgical ICU/C5W at 64% (64% low, 0% high), Renal Transplantation ward at 59% (52% low, 7% high), Medical ICU/C8W at 58% (52% low, 6% high), and Pediatric Newborns at 57% (39% low, 18% high). Most wards had many more low values than high ones and we believe that these low values, apart from pediatrics, are related to the following: (a) the very transient hypocalcemia associated with citrate binding of Ca2+ owing to use of multiple units of ACD blood, (b) the more-prolonged hypocalcemia associated with albumin changes in end-stage renal disease, malignancy, and malabsorption, and (c) as-yet-unexplained but frequently encountered low values associated with the "critically ill" patients as described by Drop and associates (41, 42). There is need for wider recognition of the severe Ca2+ hypocalcemia in these extremely sick patients and for better understanding of the help that calcium replacement therapy might give them.

Re-examination of the abnormal rates on the same wards in February 1985 showed 41% (31% low, 10% high) and except for the Surgical ICU/C5W showed just about the same proportions between low and high abnormalities. In retrospect, we now know that the 57% abnormal rate on the pediatric wards in December 1983 was distorted because the
much narrower reference values for adults were used when no reference limits for the pediatric age groups were available. When appropriate pediatric reference values were in place in February 1985, the overall abnormal rate on the pediatric wards fell to only 18% (8% low, 10% high).

The other noticeable findings seen in the December 1983 data were for the medical oncology wards, where patients are frequently admitted for the evaluation and treatment of severe hypercalcemia associated with malignancy. The abnormal rate for the oncology wards was 53% with abnormally high values predominating (48% high, 5% low). Lindemann and Mueller-Plathe have reported that Ca$^{2+}$ is twice as sensitive as C$_{a}$P for detecting hypercalcemia caused by osteolytic metastases (43). A study done in our private medical oncology services of 71 outpatients who were known to have metastatic malignancy to any site revealed that 23 of 71 patients (32%) had an abnormal Ca$^{2+}$ value (44). Fourteen of the 71 (20%) were hypercalcemic by Ca$^{2+}$ assay but only eight (11%) by C$_{a}$P. Nine of the 71 (13%) were hypocalcemic by Ca$^{2+}$ measurements, 19 (27%) by C$_{a}$P. All the additional 10 cases were associated with a low albumin concentration.

**Discussion**

The experimental and clinical findings of McClean and Hastings demonstrated unequivocally in the mid-1930s that Ca$^{2+}$ rather than C$_{a}$P is of primary physiological and clinical importance (3-5). Over the past 50 years, numerous investigators have repeatedly confirmed and extended this central role of Ca$^{2+}$ (7, 8, 24, 45-47). Given the opportunity to order Ca$^{2+}$ as easily as C$_{a}$P determinations, physicians at our hospital have shown a preference for the use of Ca$^{2+}$ over C$_{a}$P, as shown in Figure 1. However, when Ca$^{2+}$ testing was first made available five years ago, physicians initially were reluctant to use the test because the new reference intervals appeared strange and the magnitude of the Ca$^{2+}$ abnormalities usually found in association with diseases of calcium metabolism were unfamiliar to them. Despite a special effort by the laboratory staff that made Ca$^{2+}$ assay readily available 24 hours a day in a way exactly parallel to pH and blood gas determinations, it took about a year for the volume of Ca$^{2+}$ testing to equal that of C$_{a}$P. In fiscal year 1985 we performed 31 640 Ca$^{2+}$ measurements on patients’ sera vs 69 150 C$_{a}$P determinations. This 5:1 ratio had increased to 10:1 in February 1986 at a time when total clinical chemical test volume was dropping slightly because of fewer inpatient admissions!

This recent successful experience in offering Ca$^{2+}$ as a routine daily service test stands in stark contrast to our prior failure in the 1970s. At that time we were forced to discontinue the test when the world’s only manufacturer of calcium ISE analyzers was unable consistently to deliver the vital ion-exchanger. In contrast, the technology behind today’s calcium ISE testing has matured to such a degree that eight original equipment manufacturers with facilities located in six different countries are producing Ca$^{2+}$ analyzers. Calcium ISE technology now rests upon a substantial body of scientific knowledge produced over the last two decades by a number of academic and industrial research groups in the United States and Europe (18). Translation of this ISE technology into meaningful Ca$^{2+}$ measurements on patients’ samples has required the integration of unique chemical ISE skills with mechanical and electronic engineering capacities within the industry (48). As a direct result of these industrial efforts, several state-of-the-art second-generation Ca$^{2+}$ analyzers are now available. At least two of these analyzers have been shown to be rugged and precise under the daily working conditions of very high

<table>
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<tr>
<th>Patient location</th>
<th>Data collected</th>
<th>Tests done</th>
<th>No. abnormal/Lo/Hi</th>
<th>% abnormal/Lo/Hi</th>
<th>No. repeats/Lo/Hi</th>
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<td>Feb '85</td>
<td>49</td>
<td>9/4/5</td>
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|                 |               |           |                    | 22/18/4         | 23/3/5

*Also includes ambulatory peritoneal-dialysis patients.
volume and continuous use by many analysts in our laboratory.

There now exists a substantial cadre of knowledgeable clinical laboratory scientists and physicians who are advocates of Ca²⁺. They have organized scientific meetings to exchange their ideas and experiences with colleagues and have also promoted lively interactions with their academic and industrial peers (49–54). These interactions have progressively refined our understanding of the basic ISE technology as it is found in today's Ca²⁺ analyzers and have continuously added to our clinical knowledge concerning the importance of Ca²⁺ measurements. This cadre of advocates is now asking the question: "Why do so few service laboratories offer Ca²⁺, given that the ISE technology is so dependable and the accumulated clinical experience with Ca²⁺ over Ca₇ measurements is so uniformly favorable?"

Some of our thoughts about that question are as follows:

First, the move to offer Ca²⁺ measurements in service laboratories must be stimulated by the physicians who deal with the clinical problems of calcium metabolism. However, Ca₇ has been the focal clinical test for many years and is readily available at a low cost as part of widely used chemistry screening profiles. Ca₇ is the normative test by current medical standards, and physicians are satisfied with the use of Ca₇, especially for outpatient evaluations, because it readily uncovers abnormally high values. Furthermore, the interpretation of results is backed by years of personal experience and many publications. In short, it is difficult to switch to Ca²⁺ because the Ca₇ is so entrenched, so readily available, and so well perceived by physicians because it serves their office needs well.

In contrast to outpatients, more and more of today's hospitalized patients are quite ill on admission because of delays due to DRG and other preadmission clearance hurdles and cost-containment policies. As a consequence, these sicker patients and their attending physicians need the immediate availability of accurate analytical information in order to establish the diagnosis quickly and initiate definitive therapy without delay (in part to remain within the predetermined average length of stay for Medicare and other third-party payers). In sicker patients, it is clear that Ca₇ measurement by itself is a poor indicator of the calcium status, even when quantified quickly with an accurate method. A patient's serum albumin concentration must also be known if the Ca₇ result is to be meaningfully interpreted. Many clinicians regularly do correct Ca₇ values for changes in albumin, but the effects of several other variables such as pH and various anions is not revealed by the Ca₇ determination. For example, the physiological hypocalcemia associated with citrate loads from multiple transfusions with "ACD" blood, or from an abnormally high lactate or bicarbonate concentration is of great importance in many critically ill patients, yet is obscured by Ca₇. In contrast, the ISE measurement of Ca²⁺ tracks the hypocalcemia when any anion complexes the free calcium.

Second: Another reason for slow acceptance of Ca²⁺ testing may be the fact that earlier Ca²⁺ analyzers have failed in too many clinical laboratories. We had a vivid memory of our earlier failure and in 1981 we were reluctant to offer the test again as a routine daily service. We knew from the literature that the second-generation Ca²⁺ analyzers were much improved, but we needed to make our own evaluations to be certain that these instruments functioned well under hard and continuous daily use by many analysts in our own service laboratory. Two Ca²⁺ analyzers have met that performance goal. Today, in 1986, manufacturers and their allied companies have worldwide marketing and service organizations to provide for the timely distribution of analyzers, reagents, and replacement parts needed for maintenance and repair. A half-dozen instruments are now exhibited at national meetings and, as shown in Table 2, the newly introduced analyzers require smaller sample volumes and respond quicker with shorter total cycle times. Together, Tables 1 and 2 suggest that competitive market forces are continuing to stimulate improvements in both the analytical performance and productivity of Ca²⁺ analyzers.

Third: All Ca²⁺ analyzers are plagued with the need to replace the vital Ca²⁺ ISE, which causes unpredictable downtime despite sound preventative maintenance. The problem of frequent electrode dysfunction and replacement of ISE's is characteristic of most types of ISE analyzers—such as pH/blood gas and Na/K analyzers—that must be online continuously without any interruption in vital service. We have emphasized some of the unique failure modes that are related to high volumes of patients' samples. However, the information that has helped us to understand and resolve some of these repetitive problems is a clear working knowledge of the wide variability in the lifetime to be expected in the key calcium ISE element. A few ISE sensors simply never work, a few more fail within the first few days, but most elements function well for hundreds of analytical cycles.

We have stressed the need for redundancy in instruments and supplies; we must stress that redundancy in well-trained personnel is also necessary. Knowledgeable technical personnel must be available to perform the testing and to oversee the quality of the results by assuring that analyzers are used and maintained correctly. By providing such redundancy of instrumentation and personnel, we have been able to offer Ca²⁺ services on a 24 h per day basis, without interruption, for the last four years.

Fourth, the introduction of Ca²⁺ testing may be slow because of the lack of agreement on standardization. Even a brief review of the reference values published for Ca²⁺ reveals that incompatibility of results is a serious problem. For example, the lower reference limit in the serum of adults of 0.96 mmol/L obtained with some first-generation analyzers as compared with the 1.12 to 1.17 mmol/L of second-generation Ca²⁺ analyzers not only suggests a non-Nernstian response with the early ISEs but also reflects inter-instrumental differences between the more recently introduced analyzers. Fortunately, very few of the earlier devices are still in service, and in the magnitude of the differences found between second-generation Ca²⁺ analyzers is only about ±0.03 mmol/L in our comparisons. These differences are due primarily to calcium binding to buffers in the calibrator solutions and (or) to the residual liquid–liquid junction potential between the calibrators and serum.

Groups in Europe (21, 50) and the U.S.A. (22) are actively working to develop reference methods and primary reference solutions to help eliminate these systematic errors in Ca²⁺ measurements. The need for agreements on a reference system for Ca²⁺ that defines the accuracy of these valuable measurements is now recognized (56–59).

At present, we seem to have a "catch 22" situation. Many physicians might like to request Ca²⁺ testing, yet have had little or no practical experience with Ca²⁺ as compared with Ca₇ because the test is not available. On the other hand, clinical laboratories do not offer the test because doctors do
Table 6. The Clinical Use of Ca$^{2+}$ vs Ca$_T$ [Modified from Toffaletti (47)]

A. Ca$^{2+}$ tracks the hypocalcaemia that is missed or misleading by Ca$_T$

1. Transfusions with citrated blood (62)
   a. Massive replacement with ACD blood
      - Transplantation surgery
      - Severe trauma or vascular rupture
   b. Exchange transfusions in newborns
   c. Ca$^{2+}$ critical to diagnosis and rational therapy

2. Critically ill patients (41, 42, 61)
   a. Often unrecognized after major surgery, trauma, sepsis, burns, pancreatitis, and multiple organ failure
   b. Ca$^{2+}$ critical to diagnosis and rational therapy

3. End-stage renal disease (48)
   a. pH and albumin changes after all calcium fractions unpredictably
   b. Profound protein losses (albumin in nephrosis)
   c. Regulation of dialysis baths and patients with Ca$^{2+}$

B. Greater diagnostic sensitivity and (or) specificity of Ca$^{2+}$

1. Hyperparathyroidism (45)
   a. Sensitivity 5–10% higher in recent studies
   b. Specificity high with concurrent C-terminal parathyrin elevation
   c. "Normocalcemic" hyperparathyroidism resolved

2. Malignancy (43–45)
   a. Twice as sensitive as Ca$_T$ for tumor-associated hypercalcemia
   b. High suspicion of neoplasm or granuloma if parathyrin normal
   c. True hypocalcemia detected, not one secondary to low albumin

3. Neonatal hypocalcemia (40)
   a. Physiological due to a relative hypoparathyroidism
      - Related to gestational age in small premature infants
      - Rapidly changes after birth, may not need treatment
   b. Sick newborns (tetany, seizures) need to be monitored

These same companies also paid for the travel costs that made it possible for Dr. Bowers to attend and present papers at the European meetings of the Working Group on Ion-Selective Electrodes at Copenhagen in 1982, at Oxford in 1984, and at Helsinki in 1985. Portions of this work were submitted by C. B. to the Department of Biology, University of Hartford, Hartford, CT 06117, as part of the requirement for a masters degree in biology.

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