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Determination of Ionized Calcium in Serum That Has Been Exposed to Air

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We examined changes in ionized calcium concentration in serum after its exposure to air. Samples with total protein concentrations ranging from 50 to 90 g/liter were equilibrated with CO_2 in nitrogen (5/95, by vol) or CO_2 alone, to produce pH values of 7.0 to 8.0. Ionized calcium was then measured with an Orion flow-through electrode system. Curves relating pH and ionized calcium concentration had statistically identical slopes regardless of protein concentration. A factor was derived, based on pH change, for correcting values for ionized calcium in serum exposed to air, and its validity was confirmed by comparing corrected values for samples allowed to stand at ambient temperature (23 °C) without anaerobic precautions with values initially obtained on anaerobic aliquots of the same samples.

Additional Keyphrases: *effects of protein concentration, pH, and temperature • correction factor for use with air-exposed serum • normal range*

The decreased calcium ion concentration that accompanies the increase in pH when blood is exposed to air is well recognized (*1–3*). Therefore anaerobic conditions are customarily used when ionized calcium is determined in serum, to obviate pH changes. Schwartz et al. (*4*) reported that the logarithm of the value for ionized calcium is linearly related to pH between 7.0 and 7.7. They also found that the ionized calcium concentration of refrigerated serum previously exposed to air could be returned to its original value by equilibrating the serum with an appropriate CO_2 atmosphere, to restore the pH of the serum to its original value. Hinkle and Cooperman's (*5*) study also demonstrated that ionized calcium concentration varied directly with p_{CO_2} and inversely with blood pH. Radde et al. (*6*) showed that calcium ion concentrations decrease and pH increases when whole blood is exposed to air for longer than 5 min, although they claimed no significant effect for shorter exposures.

The foregoing investigations suggested that a correction factor could be derived for ionized calcium values obtained from serum collected under non-anaerobic conditions. Here, we describe a method in which such a correction factor is used and the proof of the validity of the factor.

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Materials and Methods

Ionized calcium in serum was determined with a flow-through ion-selective electrode (Orion Research, Inc., Cambridge, Mass.; Model 99-20) connected to a digital pH meter Model 801 (Orion). This meter being connected to the ion-selective electrodes, it was not available for measuring pH, which was measured with a Corning Digital 110 Expanded Scale pH meter.

Reagents

Triethanolamine HCl, 1 mol/liter. Dissolve 18.6 g of triethanolamine HCl in 100 ml of water.

Trypsin, crystalline.

Stock calcium standard. Weigh out 500 mg of reagent-grade $CaCO_3$ and transfer it to a 100-ml volumetric flask. Add 4 ml of 3 mol/liter HCl and allow the mixture to stand until the $CaCO_3$ is dissolved. Add about 70 ml of water and adjust the pH to 6.0 with an ammonium acetate solution (500 g/liter). Dilute to 100 ml with water and mix. 1 ml = 50 μmol of Ca^{2+}.

Stock Ca^{2+}/Na^+ standards. Standards containing 0.5, 1.0 and 2.0 mmol of Ca^{2+}/liter, and also 150 mmol of Na^+/liter, are prepared by using the appropriate amounts of stock calcium standard and sodium chloride.

Working standards. To 25 ml of each stock Ca^{2+}/Na^+ standard, add 75 μl of triethanolamine HCl and 15 mg of trypsin, dissolve completely, and adjust the pH to 7.4 ± 0.05 with 0.4 mol/liter KOH. Refrigerated, working standards are stable for five days. *Bring to room temperature before use.*

Procedure

Equilibrate and calibrate the electrode as follows: Fill three 1-ml disposable capped syringes with working standards and a fourth syringe with a sample of clear serum. Cap the tips of the syringes until the serum Ca^{2+} concentration is measured. Alternately, pump through the electrode the 0.5 mmol Ca^{2+}/liter working standard, the serum, 0.5 mmol Ca^{2+}/liter working standard, the serum, etc., recording millivolt readings at exactly 2 min until the difference in successive standard readings is less than ±0.2 mV. Repeat for the other two working standards. Draw a point-to-point calibration curve on two-cycle semilogarithmic graph paper. We did not use a least-squares method to obtain the best fit because values obtained by use of a fitted curve are less precise as a result of sodium error (*7*). The difference in potential between the 0.5 and 2 mmol/liter standards should be 12 mV or greater.

Blood is drawn in the usual manner into a plain "Vacutainer" tube and allowed to clot for about 1 h without removing the stopper. After centrifugation, aliquots of serum for pH are withdrawn through the stopper with a disposable plastic syringe and needle. Using an expanded scale pH meter, determine and record the pH of the patient's serum to the nearest 0.01 unit at room temperature. Immediately aspirate serum again through the stopper into a 1-ml disposable plastic syringe for ionized calcium measurement, avoiding introduction of air bubbles. Promptly cap the syringe, to minimize contact with air. The sample is stable in the capped syringe for at least 4 h. If the sample cannot be assayed during this period, the pH must be redetermined. Pump the unknown sample through the electrode and record the voltage reading at exactly 2 min.

Recheck the standards that give voltage readings on either side of the value for the unknown, recording the readings for the standards at exactly 2 min.

Repeat these steps until the reading for the unknown varies by less than ±0.2 mV.

From the standard curve, determine the Ca^{2+} concentration in the unknown.

The pump lines are filled with any working standard after the samples are assayed.

In this method, Ca^{2+} is determined at ambient temperature (23 °C) and at the pH of the serum (pH_x) as received in the laboratory. The value obtained under these conditions is corrected to that at any desired pH (pH_y) by use of the following equation (see under *Results*):

$$Ca^{2+}\ pH_y,\ mmol/liter = Ca^{2+}\ pH_x,\ mmol/liter + [0.515\ (pH_x - 0.17 - pH_y)]$$

where $Ca^{2+}\ pH_y$ = concentration of Ca^{2+} at 37 °C and pH_y

$Ca^{2+}\ pH_x$ = concentration of Ca^{2+} at 23 °C and pH_x

(i.e., the concentration value obtained in the above procedure)

pH_x = pH of serum at 23 °C at time of analysis

pH_y = pH of serum at 37 °C at time specimen was drawn

The factor 0.17 is introduced to correct pH_x for the difference in temperatures—i.e., 37 °C and 23 °C—used for the two pH measurements.[1] Assuming the original pH of the blood to be 7.4 at the time it was drawn can lead to errors for Ca^{2+} as large as about 0.2 mmol/liter at the extremes of pH encountered physiologically. It is therefore essential that the original pH be accurately known.

Results

We studied the effect of pH on the ionized calcium concentration in sera with protein concentrations ranging from 50 to 90 g/liter and with pH values ranging from 7 to 8, produced by equilibrating the sera with CO_2/N_2 (5/95 by vol) or CO_2 only (Table 1). Table 2 gives equations of regression lines relating changes in serum ionized calcium to pH at the various protein concentrations. Analysis of these equations revealed that the slopes were not different statistically. We thus

Table 1. Change with pH of Concentration of Ionized Calcium in Sera with Various Protein Concentrations

	Ca^{2+} (mmol/liter) at total protein concentrations (g/liter)				
pH	50	60	70	80	90
8.0	1.06	0.95	0.97	1.02	0.97
7.8	1.18	1.07	1.07	1.24	1.08
7.6	1.30	1.14	1.22	1.31	1.13
7.4	1.35	1.26	1.34	1.50	1.38
7.2	1.44	1.33	1.42	1.55	1.52
7.0	1.52	1.45	1.50	1.61	1.62

Table 2. Equations for Regression Lines Relating Serum Ionized Calcium to pH at Various Protein Concentrations

Protein concn, g/liter	Equation
50	$y = 4.66 - 0.445x$
	$\gamma = 0.99$
	$S_{y,x} = 0.025$
60	$y = 4.595 - 0.45x$
	$\gamma = 0.99$
	$S_{y,x} = 0.015$
70	$y = 5.345 - 0.545x$
	$\gamma = 0.99$
	$S_{y,x} = 0.025$
80	$y = 5.73 - 0.53x$
	$\gamma = 0.97$
	$S_{y,x} = 0.055$
90	$y = 6.25 - 0.51x$
	$\gamma = 0.98$
	$S_{y,x} = 0.055$

In these equations, y is Ca^{2+} concentration at pH_y, x is the corresponding pH value, γ is the correlation coefficient, and $S_{y,x}$ is the standard deviation of the regression line.

[1] The decrease in serum pH caused by a decrease in temperature has been reported to be 0.012/°C (*8*). Hence, 0.012 (37 − 23) = 0.17.

decided to pool all the data, which resulted in the following equation:

$$y = 5.15 - 0.515x,\ \gamma = 0.90, \text{ and } S_{y,x} = 0.09$$

(In this regression equation, y is the Ca^{2+} concentration at pH_y, x is the corresponding pH value, γ is the correlation coefficient, and $S_{y,x}$ is the standard deviation of the regression line). This formed the basis for the calculation as given under *Procedure*. The factor thus derived was tested on serum samples separated anaerobically from blood collected from 24 individuals. Both ionized calcium and blood pH were determined and the samples were than allowed to stand at ambient temperature (23 °C), without anaerobic precautions, for 24 h. Ionized calcium was again determined and corrected back to the original blood pH, as we have described. The concentration range of these samples was 1.19–1.36 mmol/liter. Statistical treatment of the data resulted in a value for t of −0.3596 (critical t, 2.0739), thus confirming the validity of the correction factor.

Sera from six individuals with abnormal albumin/globulin ratios or low inorganic phosphorus concentrations were examined in an identical manner to those from normal persons. For all these, results obtained by use of the correction factor were 3% greater than those found under anaerobic conditions, but the small number of abnormal samples makes it impossible to draw a valid conclusion.

The between-run precision of the method for normal sera is approximately ±1% for 95% confidence limits. A stability study indicated that sera stored at 30 or 4 °C were stable for as long as six days: sera frozen at −20 °C gave reproducible results for as long as eight days.

We also studied the effect of temperature on the ionized calcium determination itself. For 10 low-to-normal and 10 normal-to-high samples we performed the determinations at ambient temperatures and at 37 °C. In the latter case the pump and electrodes were placed in an incubator kept at 37 ± 1 °C. Standards and sera, sealed in tuberculin syringes, were equilibrated at 37 °C for 30 min before the analysis and the electrode cables were attached to the pH meter by routing them through the overhead vent of the incubator. The results indicate that the values obtained at 37 °C are about 4% smaller than those obtained at ambient temperatures. The differences ranged from 0.01–0.09 mmol/liter for samples with ionized calcium concentrations of 1.13–2.36 mmol/liter.

By use of nonparametric statistics, the normal range, based on results for 36 men and 34 women, all apparently normal and healthy, age 20–50 years, was 1.21 to 1.39 mmol/liter. There was no statistically significant difference in the results obtained from the men when compared to those from the women. There was no skewness in the distribution of the results.

Discussion

Anaerobic conditions have heretofore been considered essential if results for ionized calcium were to be accurate. It was primarily this requirement that restricted the use of the flow-through electrode, even after various technical modifications and improvements (*7*). The practice of restoring the original pH with a CO_2/air mixture (*4–6*) makes the collection requirements less stringent but is time consuming and cumbersome as compared with our method. Our results for the determination of ionized calcium at 23 and 37 °C confirmed other reports (*7–10*) that results are 5 to 6% higher at 25 than at 37 °C. Moore (*7*) reported that Ca^{2+} can be accurately determined in sera that have been frozen 72 h but that values were often decreased in frozen ultrafiltrates of serum. Radde et al. (*6*) stated that blood can be stored for 2 h at room temperature and that plasma can be frozen or refrigerated without a measurable change in calcium ion activity in either case. Our data clearly demonstrate a much longer stability. This fact, in combination with the pH correction factor of the method presented, makes reliable ionized calcium determinations generally available and is especially helpful to physicians who are not close to laboratories with sophisticated equipment.

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