Establishing the Direct-Potentiometric "Normal" Range for Na/K: Residual Liquid Junction Potential and Activity Coefficient Effects

John D. Czaban, Alan D. Cormier, and Kenneth D. Legg

The observed reference ranges for sodium and potassium as determined by direct potentiometry vary from instrument to instrument, depending on the composition of the calibration standards. To resolve the existing confusion as to which reference intervals are most appropriately considered "normal," we propose a straightforward convention (based on plasma-water concentration units) in which the difference between direct (undiluted sample) and indirect (diluted sample) methodologies is accounted for by the volume displacement effect of proteins, lipids, and other dissolved substances in a typical plasma sample. Thus, the proposed reference intervals for sodium and potassium are approximately 7% greater by direct potentiometry than by procedures involving dilution. Data consistent with this convention can be obtained with a variety of aqueous-based calibrants, provided care is taken to minimize the errors resulting from activity coefficient and liquid junction potential effects. Additional experimental results are presented to show that these effects also account for the apparent suppression of the sodium ion concentration observed in the presence of bicarbonate ion.

Additional Keyphrases: reference interval • bicarbonate • ion-selective electrodes • plasma-water concentration units • physical chemistry of electrolytes • diluted vs undiluted samples

Ion-selective electrode analyzers are an important new tool for the measurement of electrolyte concentrations in whole blood, plasma, and serum, especially in the acute-care or "stat" laboratory, where rapid analyses are essential. However, because direct potentiometry represents a new technology, at least for electrolyte determinations, some confusion is being generated as the nuances of the technique are becoming better understood. A cooperative effort among clinicians, scientists, and manufacturers is necessary to resolve unanswered questions and to educate the clinician and laboratorian for the full utilization of direct potentiometry.

One important question to address is that of establishing the reference intervals for Na/K values expected in "normal" healthy individuals. Currently the suggested reference intervals vary from instrument to instrument (2-4) and reflect differing philosophies with regard to calibration of ion-selective electrodes for use with undiluted samples (5-9). This instrumental bias tends to be negligible for potassium (less than 0.3 mmol/L), but can be very large for sodium, exceeding 10 mmol/L in some cases. Furthermore, it is not generally recognized that direct potentiometry yields assay values on a concentration basis that differ from those obtained by indirect (diluted samples) techniques (10) and, as such, will not correlate exactly with results by the existing flame-photometric method. Therefore, it is no surprise that clinical users, eager to take advantage of the many unique features of direct potentiometry, are confused about the significance of the analytical results (11) and are somewhat frustrated in their efforts to implement this new technology for routine patient diagnosis.

In this paper we discuss comprehensively each of the factors pertinent to the establishment of the direct potentiometric reference intervals for sodium and potassium. We propose a straightforward and accurate calibration convention that can be used to eliminate the existing instrumental biases. This convention provides a manageable liaison with dilution-based techniques such as flame photometry, and can be adapted for other noncomplexed species dissolved in the plasma-water phase.

Theory

The Concept of Plasma-Water Concentration Units

The problem of establishing the most appropriate reference intervals for sodium and potassium can be considered a two-step process. The first is to use the existing data base obtained with conventional indirect techniques to define justifiable "target" concentration values for these ions in normal plasma. The second step is to develop suitable calibration standards that yield data consistent with these values.

The first step, defining the target values, is complicated by the fact that the concentration units derived from direct (undiluted sample) potentiometric analyses are not the same as those obtained by conventional indirect (diluted sample) techniques. Direct-potentiometric methods yield data on the basis of water volume, which does not include the volume displaced by the suspended or dissolved species. For biological samples the direct-potentiometric method is insensitive to the volume displaced by proteins, lipids, and other dissolved compounds and yields concentration data as millimoles per liter of plasma water. Indirect methods, including those involving ion-selective electrodes, present data on a total volume basis, with the concentration units expressed as millimoles per liter of plasma. Because of the dilution step, all assay values obtained by indirect measurements are sensitive to the volume occupied by the suspended and dissolved solids present in the sample. This distinction between concentration units is not significant for simple salt solutions, where the volume occupied by the dissolved salts is small (less than 0.5% for NaCl at 150 mmol/L); for biological samples, however, the difference is substantial and must be considered when attempting to define the reference values for direct potentiometry.

The volume occupied by each of the components of 100 mL of a typical normal plasma is as follows (12): water 93.1 mL, proteins 5.4 mL, lipids 0.6 mL, and crystalloids (soluble salts, sugars, etc.) 0.9 mL. The average water content for normal, healthy adults is strikingly constant at 93 mL per 100 mL of plasma (13, 14); however, it can vary substantially in patients' samples with abnormal concentrations of proteins and lipids. This variation may lead to misinterpretation of electrolyte status (e.g., pseudohyponatremia) when diluted-sample assay procedures are used. Waugh (12) and others (15-18) found that this problem could be overcome by expressing electrolyte

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data on a plasma-water concentration basis:

Plasma-water units = Total plasma units/f_{H_2O} \hspace{1cm} (1)

where f_{H_2O} equals the fractional water content of the sample. Data expressed on a plasma-water basis are generally considered a more reliable indicator of electrolyte status (1, 19); however, the inconvenience of having to measure or estimate the actual water content of the sample precludes this approach from becoming popular for indirect measurements. This step poses no problem for direct potentiometric measurements because plasma-water values are measured directly—which is an important advantage of direct potentiometry over indirect techniques.

We propose that equation 1 can be used to define the reference intervals for direct potentiometry based on values determined by flame photometry and the average water content for normal plasma (f_{H_2O} = 0.93). Thus, the target values for both sodium and potassium by direct potentiometry are approximately 7% greater than the total plasma concentrations for "normal" samples. Brand and Scott (20) used this approach to calculate plasma-water values in their comparison of concentration units for plasma electrolytes (Table 1). Accordingly, the typical normal range for shifts from approximately 136-144 (140 ± 4) mmol/L of plasma to 146-154 (150 ± 4) mmol/L of plasma water. The reference interval for potassium shifts from 3.2-4.8 (4.0 ± 0.8) mmol/L of plasma to 3.5-5.1 (4.3 ± 0.8) mmol/L of plasma water. 1

With equation 1 as the convention to define the target reference values, the next step is to formulate calibrants that yield data consistent with this convention.

**ISE Calibration for Direct Potentiometry**

As illustrated in Figure 1, a conventional calibration curve for ion-selective electrode measurements consists of a plot of observed cell potential vs the logarithm of the analyte ion concentration in the calibration standards. Typically, because electrode response is linear over the physiological range of ion concentration, a two-point calibration procedure is sufficient. The midpoint or "balance" standard is chosen to have an analyte concentration (C_{b}) near the center of the range expected for the unknown samples, and defines the relationship between the absolute millivolt output of the electrochemical cell and analyte concentration. The "sloping" standard usually contains an analyte concentration (C_{a}) at or beyond one extreme of the unknown sample concentration range; in conjunction with the midpoint calibrant, this is used to characterize the sensitivity of the indicating electrode to changes in the analyte ion concentration. As will be discussed below, the cell potential observed in an undiluted solution is sensitive to the specific ionic matrix of that solution. Thus, the calibration line determined with simple aqueous-based salt solutions is typically offset from that obtained with a plasma-based matrix, and results in a shift in the reference interval observed for normal samples. In this paper we address the source and magnitude of this shift and identify a practical approach to minimizing its effect for sodium and potassium measurements in biological samples. Errors in the slope of the calibration line have little or no impact for normal samples and will not be discussed here. 2

The typical representation for an ISE electrochemical cell is

\[ \text{ISE} | \text{sample} | \text{salt bridge} | \text{reference electrode} \hspace{1cm} (2) \]

where the single line represents a solid-liquid interface and the double line represents a liquid-liquid interface. The potential observed for the cell includes contributions from the indicating electrode (E_{IS}), the reference electrode (E_{ref}), and the liquid junction (E_{j}), according to

\[ E_{\text{cell}} = E_{\text{ISE}} - (E_{\text{ref}} + E_{j}) \hspace{1cm} (3) \]

The primary term \( E_{\text{ISE}} \) is logarithmically related to the thermodynamic activity (\( a_{M^+} \)) of the ion being determined. According to the Nernst equation, this relationship for monovalent cations is

\[ E_{\text{ISE}} = E_{\text{IS}} + (\text{slope}) \log a_{M^+} \hspace{1cm} (4) \]

where \( E_{\text{IS}} \) is a constant representing the standard half-cell potential for the electrode, and (slope) is the standard Nernst factor (ideally 2.3 RT/ZF), which characterizes the sensitivity of the electrode (expressed in millivolts per decade change in activity). Slope values for monovalent cations are typically between 55 and 59 mV/decade at 25 °C.

All ion-selective electrodes respond to the activity or "effective concentration" of the analyte ion. The analytical concentration (\( C_{M^+} \)) and activity (\( a_{M^+} \)) units are related via a proportionality factor, the activity coefficient (\( \gamma_{M^+} \)), where:

\[ a_{M^+} = C_{M^+} \cdot \gamma_{M^+} \hspace{1cm} (5) \]

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1 Direct-potentiometric analyzers calibrated with serum-based controls (assayed by indirect methods) yield normal reference ranges coincident with the conventional total plasma values, because the water content for samples and calibrants are approximately equal.

2 In general, the slope observed for both salt-based and plasma-based calibration lines tend to be sub-nernstian because of changes in the activity coefficient as the ionic strength is varied. However, cation measurements by direct potentiometry, the liquid junction potential tends to change in such a way as to partly compensate for the activity coefficient effect. Thus, in both cases, the observed slopes tend to be closer to the ideal values obtained under conditions of constant ionic strength (7).

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**Table 1. Comparison of Electrolyte Concentrations in Normal Plasma**

<table>
<thead>
<tr>
<th>Electrolyte Concentration, mmol/L</th>
<th>Total Plasma</th>
<th>Plasma Water</th>
</tr>
</thead>
<tbody>
<tr>
<td>Na(^+)</td>
<td>140</td>
<td>150</td>
</tr>
<tr>
<td>K(^+)</td>
<td>4.0</td>
<td>4.3</td>
</tr>
<tr>
<td>Cl(^-)</td>
<td>100</td>
<td>107</td>
</tr>
</tbody>
</table>

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**Fig. 1. Conventional calibration plot for a direct potentiometric analyzer, establishing the relationship between the observed cell potential (E_{cell}) and analyte concentration C_{M+}.

**Error (%)**

**log C_{M+} (mmol/L PLASMA H_{2}O)\)**

<table>
<thead>
<tr>
<th>C_1</th>
<th>C_{OS}</th>
<th>C_{actual}</th>
</tr>
</thead>
<tbody>
<tr>
<td>C_2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>E_1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>E_2</td>
<td></td>
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<tr>
<td>E_3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>E_4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>E_5</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Substitution of equations 4 and 5 into equation 3 yields the overall observed cell potential:

\[ E_{\text{cell}} = E_{\text{ISE}} + [(\text{slope}) \log (C_{M^+} \cdot \gamma_{M^+})] - E_{\text{ref}} - E_i \]  

(6)

This expression can be written for both sample (s) and calibrant (c) solutions (the subscripts have been simplified for clarity):

\[ E_{\text{cell}} = E_{\text{ISE}} + [(\text{slope}) \log (C_s \cdot \gamma_s)] - E_{\text{ref}} - E_i(s) \]  

(7A)

\[ E_{\text{cell}} = E_{\text{ISE}} + [(\text{slope}) \log (C_c \cdot \gamma_c)] - E_{\text{ref}} - E_i(c) \]  

(7B)

The magnitude of the difference in the observed cell potential (\( \Delta E_{\text{cell}} \)) for the sample relative to that of the calibrant is a measure of the sample ion concentration. Subtracting equation 7B from equation 7A yields

\[ \Delta E_{\text{cell}} = (\text{slope}) \log \left( \frac{C_s \cdot \gamma_s}{C_c \cdot \gamma_c} \right) \]  

(8)

where \( \Delta E(s - c) \) equals the difference in the liquid junction potential between the sample and calibrant and is referred to as the residual liquid junction potential (see next section). The \( E_{\text{ISE}} \) and \( E_{\text{ref}} \) terms subtract out because they are the same for both sample and calibrant.

Taking the antilog and solving for the concentration \( (C_s) \) of \( M^+ \) in the sample yields:

\[ C_s = C_c \cdot \exp_{10} \left( \frac{\Delta E_{\text{cell}}}{\text{slope}} \right) \cdot \frac{\gamma_c}{\gamma_s} \cdot \exp_{10} \left( \frac{\Delta E_1}{\text{slope}} \right) \]  

(9)

the exact equation relating the sample ion concentration to the observed change in the cell potential between sample and calibrant. Thus, to calculate the sample concentration, five parameters must be known:

- the concentration of the calibrant \( (C_c) \)
- the difference in the observed cell potential (\( \Delta E_{\text{cell}} \))
- the electrode slope (slope)
- the ratio of the activity coefficients (\( \gamma_c/\gamma_s \))
- the residual liquid junction potential (\( \Delta E_1 \))

Of these, \( C_c \) is known, and \( \Delta E_{\text{cell}} \) and (slope) are experimentally measured. However, \( \gamma_c/\gamma_s \) and \( \Delta E_1 \) are not known and cannot be readily determined for each sample. This poses no problem when performing indirect potentiometry because, at high dilution ratios, the activity coefficients and liquid junction potentials tend to be fixed by the diluent, with the result that the last two terms in equation 9 both tend toward unity. In this latter instance equation 9 simplifies to

\[ C_s = C_c \cdot \exp_{10} \left( \frac{\Delta E_{\text{cell}}}{\text{slope}} \right) \]  

(10)

which can be readily used to calculate the true sample ion concentration.

In the case of direct potentiometry, the activity coefficient and liquid junction potential terms do not necessarily drop out, and use of the simplified expression (which all commercial analyzers use) results in an error in the calculated sample concentration (see Figure 1). On a percentage basis the error is defined as

\[ \% \text{ error} = \frac{C_{\text{true}} - C_{\text{actual}}}{C_{\text{true}}} \times 100 \]  

(11)

where \( C_{\text{true}} \) equals the observed sample concentration calculated according to the simplified expression (equation 10), and \( C_{\text{actual}} \) represents the true sample concentration calculated according to the exact equation (equation 9). Substitution of equations 9 and 10 into equation 11 yields

\[ \% \text{ error} = \left( \frac{\exp_{10} \left( \frac{\Delta E}{\text{slope}} \right)}{\gamma_c} \right) \left( \frac{\gamma_c}{\gamma_s} \right) - 1 \times 100 \]  

(12)

which relates the percentage of error in the observed sample concentration to the mismatch of the activity coefficients and liquid junction potentials between sample and calibrant. This equation is a useful tool for understanding the effects of the liquid junction potential and activity coefficients on direct potentiometry, and is used throughout this paper to illustrate and predict these effects.

It should be noted that, whether related to the activity coefficient or the liquid junction potential, both types of errors are manifested in the experimentally measured quantity \( \Delta E_{\text{cell}} \) and correspond to a percentage error in the calculated sample concentration. For small errors (less than 5 mV) a useful approximation for monovalent ions is that the percent error in analyte concentration is equal to fourfold the millivolt error.\(^3\)

This relationship explains why the problems of direct potentiometry are so much more noticeable for sodium than for potassium. On a percentage basis, the reference interval for sodium is small [(±4 mmol/L of plasma water + 150 mmol/L of plasma water) x 100% = ±2.7%], and corresponds to less than a 0.7-mV change in the observed cell potential. As a result, errors of a few tenths of a millivolt in the sodium measurement appear to be very significant. On the other hand, the range for potassium is larger [(±0.8 mmol/L of plasma water + 4.3 mmol/L of plasma water) x 100 = ±20%], and corresponds to a change of about ±5 mV. In this case small errors tend to be much less significant. Thus for much of the work and discussion in this paper we emphasize sodium measurements and neglect potassium.

**Calculation of Activity Coefficients and Liquid Junction Potentials**

Theoretically, the absolute value of the molar activity coefficients (\( \gamma_{M^+} \)) for a single ion in solution is impossible to calculate. However, in moderately dilute solutions, an approximate value can be calculated with the Debye–Hückel law (21):

\[ -\log \gamma_M = \frac{A \cdot Z^2 \sqrt{I}}{1 + Bd \sqrt{I}} \]  

(13)

where \( A \) and \( B \) are constants equal to 0.5115 mol\(^{-1/2}\)L\(^{1/2}\) and 3.291 cm\(^{-1}\)mol\(^{-1/2}\)L\(^{1/2}\), respectively, at 25 °C (21); \( Z \) is the charge on the ion \( M \); and \( d \) is the ion size parameter, expressed in nanometers (22). \( I \) is the total ionic strength, defined as \( I = 1/2 \Sigma C_i Z_i^2 \), where \( C_i \) is the molar concentration of the ion (i) with charge \( Z_i \).

Note that the Debye–Hückel law takes into account the primary effect of ionic strength on the activity coefficient but does not model the smaller secondary effects of specific ion–ion interactions between \( M \) and other ions present. Thus, the calculated activity coefficient for sodium is the same regardless of whether the solution contains NaCl or some other monovalent sodium salt such as NaHCO\(_3\). In reality, the true activity coefficients for ions in solutions of ionic strength greater than approximately 0.1 mol/L do depend on the specific composition of the test solution (23, 24), and small differences are expected, e.g., when other anions are substituted for chloride in a NaCl solution. Despite this limitation, equation 13 provides a good approximation of the activity coefficient for solutions of moderate ionic strength and can be used to predict trends, especially within a series of solutions where the changes in the ionic strength are not severe.

In this work we calculated the activity coefficients for normal plasma by using the plasma-water concentration

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\(^3\) From equation 10, \( \Delta E_{\text{cell}} = (\text{slope}) \log (C/C) \). Therefore, a 1-mV error in \( \Delta E_{\text{cell}} \) corresponds to \( C/C = 1.04 \) (i.e., 4% error) when the slope of the electrode is 59 mV/decade.
values shown in Table 2. We assumed that the proteins present in switzenion form did not contribute significantly to the total ionic strength (R. G. Bates, personal communication). However, we did assign a net negative charge of approximately 20 mmol/L to the proteins, to balance the positive charges of the plasma cations (25).

At the liquid–liquid junction formed between the sample and KCl salt bridge (equation 2), ions from the two solutions diffuse into each other in proportion to their respective concentration gradients. Different ions have different mobilities and diffuse at different rates. The chloride ion, for example, is one of the most mobile ions and diffuses faster than other biologically important anions. A charge separation occurs, related to the concentration gradients and differences in mobilities of the cations and anions in the two solutions, and results in a potential difference across the junction called the liquid junction potential. KCl is favored for the salt bridge because both ions are fast moving and have nearly the same mobility (Table 2). This match in mobilities tends to keep the charge separation and thus the liquid junction potential close to zero. The concentration gradient for the salt bridge solution is generally kept very high, to minimize the contribution of the sample ions to the total charge transfer across the junction. For example, the IUPAC operational definition for blood pH specifies that the KCl concentration used be at least 3 mol/L (26). In spite of this, small changes in the liquid junction potential do occur when samples of different composition come in contact with the KCl solution, e.g., when the calibrant solution is replaced by a plasma sample. The difference in the liquid junction potential is termed the residual liquid junction potential (27).

The true value of the liquid junction potential cannot be calculated exactly. However, Salling and Siggaard-Andersen (28) concluded that reliable results can be obtained for plasma when activities rather than concentrations are used in the Henderson equation:

\[
E_J = 59.2 \left( \frac{X_s - X_{\text{KCl}}}{Y_s - Y_{\text{KCl}}} \right) \log \left( \frac{Y_s}{Y_{\text{KCl}}} \right)
\]

(at 25 °C) where the subscripts refer to the sample (s) and salt bridge (KCl) and where

\[
X = \sum(C_+ \cdot \gamma_+ \cdot \lambda_+ \cdot Z_+) - \sum(C_- \cdot \gamma_- \cdot \lambda_- \cdot Z_-)
\]

\[
Y = \sum(C_+ \cdot \gamma_+ \cdot \lambda_+ \cdot Z^2_+) + \sum(C_- \cdot \gamma_- \cdot \lambda_- \cdot Z^2_-)
\]

\[\lambda_+ \text{ are the molarities of the positive and negative ions, respectively; } \gamma \text{ is the molar activity coefficient; } Z \text{ is the ionic}
\]

charge; and \(\lambda\) is the limiting equivalent ionic conductance (mho · cm²/ equivalent) (29).

In this work we assumed that \(\gamma_{\text{KCl}}\) and \(\gamma_{\text{Cl}}\) for the KCl salt bridge were equal to the mean molar activity coefficient (\(\gamma_{\text{KCl}}\)) interpolated from molal values (27). The equivalent conductance for the ionized protein in plasma, owing to its very large size, can be assumed to be zero (29).

Calculations to Identify Possible Calibrant Formulations

Perhaps the most obvious approach to the development of a midpoint calibrant consistent with the plasma-water convention would be to use a protein-based solution formulated to match "normal" plasma, as shown in Table 2. In this case, the residual liquid junction potential would equal zero and the ratio of activity coefficients would equal unity. Thus, the simplified Nernst equation (equation 10) could be used without error. In practice, however, the realistic considerations of reagent stability, cost, manufacturability, and quality control make the use of a protein-based calibrant unattractive. In fact, consideration of the error equation (equation 12) shows that the same result can be accomplished with simple aqueous-based calibrants, provided care is taken to eliminate the mismatch of the activity coefficients and liquid junction potentials between the standard and normal plasma.

Equation 12 shows that, in contrast to common belief, the activity coefficient and liquid junction potential of the calibrant need not match those of the sample; it is only necessary that the product of the error terms,

\[
\exp \left( \frac{-\Delta E}{\text{slope}} \right) \left( \frac{\gamma_s}{\gamma_d} \right)
\]

be unity. The activity coefficient and the ionic strength of the calibrant could conceivably be much different from that of the sample if the liquid junction potential error compensated for the difference in activity coefficients between the two solutions. This allows a great deal of freedom in choosing possible calibrant formulations. To better understand the importance of these effects, we calculated the percent error for sodium and potassium in normal plasma (Table 2), using equations 12–14 for various hypothetical midpoint calibrants. Two approaches were considered, both with a simple flame photometry-type calibrant (NaCl/KCl, 140/4 mmol/L) used as the baseline ISE calibrant. In the first, we predicted the error for a normal sample as alternative ions were substituted for chloride ion in the baseline calibrant. In the second, we predicted how the calibration offset would vary as additional inert salts were added to the simple NaCl/KCl calibrant. In all cases the total analytical concentrations of sodium and potassium were maintained constant at 140 and 4 mmol/L, respectively. In the first case, the ionic strength of the calibrant either remained constant at 144 mmol/L or increased, depending on the valency of the substituted anion. In the latter case, the ionic strength increased as additional salts were added. In these calculations we assumed that the slope of the indicating electrode was 58 mV/decade, and that a 3 mol/L KCl salt bridge was used.

Using the results of these calculations as a guide, we then selected one of the model systems for experimental studies with human plasma.

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4 The equivalent conductance (\(\lambda\)) for a charged species is proportional to its diffusion coefficient (D) according to the Nernst–Einstein relation \(\lambda = (RT/\pi DC)D (30)\). It is reasonable to assume that the equivalent conductivity for the net negatively charged protein is essentially zero in view of the fact that the diffusion coefficient for serum albumin (D = 6.1 × 10⁻⁷ cm²/s) and other proteins (31) is approximately 25-fold less than for common inorganic salts (D = 1.5 × 10⁻⁵ cm²/s) (21).
The Bicarbonate Effect—an Example of the Role of $E_j$ and $\gamma_{Na^+}$ in Direct Potentiometry

Previously, we predicted (32) that substitution of other anions for chloride in sodium chloride-based calibrant solutions would cause an apparent shift in the observed sodium concentration as determined by direct potentiometry. On the basis of calculations similar to those outlined above, we attributed the shift to changes in the activity coefficient and liquid junction potential. Recently, Coleman and Young reported (33) that mixtures of NaCl/NaHCO$_3$ containing a constant sodium concentration showed an apparent decrease in the observed sodium concentration as NaHCO$_3$ was substituted for NaCl in the solutions. They determined the sodium concentration by direct potentiometry with a commercial Na/K analyzer (NOVA 1; Nova Biomedical, Newton, MA 02164) equipped with a 2 mol/L KCl salt bridge. In analyzing the data, however, they ignored the shift in the residual liquid junction potential and activity coefficients, and concluded that the entire observed sodium suppression (−2.7%; HCO$_3^-$ = 25 mmol/L) was due to binding between sodium and bicarbonate ions. They concluded that more than 15% of the total bicarbonate in normal plasma is bound to sodium and potassium, and suggested that because of this binding the actual difference between results by direct potentiometry and flame photometry should be only 2−3%, not 7%, as predicted by equation 1. In a review (34) of this communication, we challenged the interpretation of the magnitude and mechanism responsible for the bicarbonate effect, citing our previous predictions and more recent experimental evidence to support our hypotheses. The details and results of our experiments are included here.

Materials and Methods

Reagents

Plasma samples were obtained by centrifugation of blood drawn from apparently healthy adults and collected with sodium heparin anticoagulant. Salt solutions were prepared with analytical grade reagents (Analyzed Reagent; J. T. Baker Chemical Co., Phillipsburg, NJ 08865).

Apparatus

For the study on human plasma, we used a standard direct-potentiometric Na/K analyzer (System 502; Instrumentation Laboratory, Inc., Lexington, MA 02173), and for the bicarbonate study we used a modified version of the same instrument. In the latter case, we developed a special program for the on-board microprocessor to allow for manual operation with expanded millivolt resolution (0.01 mV) and large sample volume (0.8 mL). The standard calibration routine was bypassed and the millivolt data for each channel were recorded on the printer output. In both cases the sodium electrode was a capillary flow-through type fabricated with Na-1000 glass (Ingold Electrodes Inc., Andover, MA) and the potassium electrode was a neutral carrier flow-by type. The reference electrode was an Ag/AgCl type with a ceramic frit junction and a 3 mol/L KCl internal fill solution. Continuity between the reference electrode and the sample was maintained via a KCl salt bridge (3 mol/L unless otherwise noted) with an inverted-"T" open, static junction. The sodium electrode was preconditioned with ammonium bifluoride, 100 mM/buffer, to ensure a rapid response characteristic (35). The slopes of the electrodes were measured by using the standard instrument calibrants.

Indirect determinations of sodium and potassium were performed with a flame photometer (IL Model 343) with manual dilution. Samples were diluted 200-fold with lithium nitrate, 15 mM/L, as diluent/internal standard. We determined total protein, cholesterol, and triglycerides with a centrifugal analyzer (IL Model Multistat III).

Procedures

Human plasma study. To evaluate experimentally our calculations for the hypothetical calibrants, we substituted acetate ion for chloride ion. Using a series of NaCl/NaOAc solutions as the ISE midpoint calibrant, we analyzed (in duplicate) 10 normal human plasmas for sodium and potassium with the IL 502. The test calibrants were prepared with NaCl, NaOAc, and KCl to yield solutions containing a constant sodium and potassium concentration of 140 and 4 mmol/L, respectively, and various amounts of chloride (144, 84, 64, 44 mmol/L) and acetate (0, 60, 80, 100 mmol/L). Mg(OAc)$_2$ (4 mmol/L) was added to buffer the pH of NaOAc-free solutions and was included in all other calibrants to maintain the ionic strength at 156 mmol/L.

Each plasma sample was also analyzed (in duplicate) on a total plasma basis by flame photometry. Total protein, cholesterol, and triglyceride content were determined and used to estimate the fractional plasma-water volume ($f_{H_2O}$) for each sample according to the Waugh (12) equation:

$$f_{H_2O} = \frac{99.1 - 0.73 \text{[protein]} - 1.03 \text{[lipid]}}{100}$$

(15)

where the protein and lipid concentrations are in grams per 100 mL. The total lipid concentration was estimated as the sum of the cholesterol and triglyceride concentrations (12). The plasma-water-based assay value for each sample was then calculated from the flame-photometric assay result and the fractional water content according to equation 1.

The observed plasma-water-based concentrations obtained by direct potentiometry ($C_{obs}$) were compared with those derived from the indirect measurement ($C_{calc}$) and the percentage error was calculated according to

$$\text{error} = \frac{C_{obs} - C_{calc}}{C_{calc}} \times 100$$

(16)

The percentage error values for each test calibrant were averaged for the 10 plasma samples and plotted as a function of the NaOAc concentration in the calibrant.

Bicarbonate study. To investigate the mechanism responsible for suppression of sodium in the presence of bicarbonate, we analyzed a series of NaCl/NaHCO$_3$/KCl test solutions, using the direct potentiometer described above. The solutions were prepared with the total respective sodium and potassium concentrations held constant at 140 and 4 mmol/L, as verified by flame photometry. Starting with a composition of NaCl/KCl (140/4 mmol/L), we prepared a series of six solutions in which NaHCO$_3$ was substituted for NaCl in 10 mmol/L increments such that the total chloride concentration decreased from 144 to 94 mmol/L and the bicarbonate concentration correspondingly increased from 0 to 50 mmol/L. We used the observed millivolt data for the sodium channel to calculate the percent change in apparent sodium concentration ($C_{Na^+}$), as calculated with equation 10, for each of the chloride/bicarbonate mixtures relative to that of the pure chloride solution. For these calculations we used:

$$\text{% change in } C_{Na^+} = \left[\frac{\exp_{10} \left( \frac{\Delta E_{cell}}{\text{slope}} \right) - 1}{1} \right] \times 100$$

(17)

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*Editor's Note: In ref. 34 equation 12 was printed incorrectly (the minus sign on the $-\Delta E_j$ term was omitted). However, it was printed correctly in manuscripts sent to the reviewers and, therefore, the misprint has no bearing on the reply of Coleman and Young with regard to the sign of the residual liquid junction potential.*
where \( \Delta E_{\text{cell}} = E_{\text{cell}}(\text{HCO}_3^-/\text{Cl}^-) - E_{\text{cell}}(\text{Cl}^-) \). This equation was derived by substituting the simplified Nernst equation (equation 10) into the expression

\[
\% \text{ change in } C_{\text{Na}^+} = \frac{C_{\text{Na}^+}(\text{Cl}^-/\text{HCO}_3^-) - C_{\text{Na}^+}(\text{Cl}^-)}{C_{\text{Na}^+}(\text{Cl}^-)} \times 100
\]

(18)

where \( C_{\text{Na}^+}(\text{Cl}^-/\text{HCO}_3^-) \) and \( C_{\text{Na}^+}(\text{Cl}^-) \) equal the apparent concentration of sodium in the chloride/bicarbonate mixtures and the pure chloride solution, respectively. The experiment was performed twice, once with a conventional 3 mol/L KCl salt bridge and again with a 0.5 mol/L KCl salt bridge. In each case, the percent change in apparent sodium concentration (equation 17) was plotted as a function of the NaHCO3 concentration.

Results and Discussion

Error Calculations for Hypothetical Calibrants

Figure 2 shows how the predicted error for \([\text{Na}^+]\) in normal plasma varies as the matrix of baseline calibrant (NaCl/KCl, 140/4 mmol/L) is altered. Figure 2a presents the percent error for a series of hypothetical calibrants in which alternative sodium salts, e.g., NaOAc, have been substituted for NaCl. Figure 2b presents the percent error for a series containing added inert salts, e.g., MgSO4. On the basis of these results we make several important observations and conclusions:

1. As indicated by the y-intercept in Figure 2a, use of a simple NaCl/KCl (140/4 mmol/L) flame-photometric-type calibrant results in a predicted error of –3.1% in the sodium assay value. In this case both the activity coefficient and the residual liquid junction potential terms produce negative errors. Of the total error, approximately –1.4% is attributable to the mismatch in the sodium ion activity coefficient \( \gamma_{\text{Na}^+}(\text{plasma}) = 0.740 \) vs \( \gamma_{\text{Na}^+}(140/4) = 0.751 \) and reflects the difference in the ionic strength between normal plasma and the calibrant \( I(\text{plasma}) = 168 \text{ mmol/L of plasma water vs } I(140/4) = 144 \text{ mmol/L} \). The remainder of the error, –1.7%, is due to the residual liquid junction potential of 0.42 mV \( (E_j^{\prime}(\text{plasma}) = 1.27 \text{ mV vs } E_j(140/4) = 0.85) \). This relatively large residual liquid junction potential arises from the fact that approximately one-third of the total anion charges in plasma is accounted for by ions having a much lower ionic mobility than chloride \( (\lambda_{\text{Cl}^-} = 76.4 \text{ mho} \cdot \text{cm}^2/\text{Eq}) \) as shown in Table 2. Specifically, bicarbonate \( (\lambda_{\text{HCO}_3^-} = 44.5) \) is present at approximately 29 mmol/L of plasma water, and negatively charged protein \( (\lambda_{\text{protein}} \approx 0) \) is present at 21 mmol/L of plasma water. In comparison with the calibrant that contains only fast-moving chloride ions, the total anionic charge transfer across the liquid junction is substantially less for plasma. Thus, in accordance with the Henderson equation (equation 14), there is a marked difference in the liquid junction potential between plasma and the simple NaCl/KCl calibrant. Furthermore, additional calculations (not shown) indicate that this difference becomes more pronounced as the concentration of the salt bridge decreases. For example, the calculated residual liquid junction potential increases to 0.60 mV when a 2 mol/L KCl salt bridge is used (as it is on some commercial analyzers), and the total predicted sodium error increases to –3.8%.

Therefore, normal plasma containing a sodium concentration of 150 mmol/L of plasma water has a predicted assay value of only about 145 mmol/L of plasma water when a simple chloride-based standard is used as the ISE midpoint calibrant. This prediction is consistent with our own experimental evidence (see below) and accounts for the trend toward a lower than expected reference interval for sodium observed with analyzers making use of such calibrants. In these cases, the reference interval will appear to be only about 3% more than that obtained by flame photometry (11).6

2. As the apparent concentration of sodium in the midpoint calibrant decreases, owing to activity coefficient and liquid junction potential effects accompanying the alteration of the calibrant ionic matrix, the calculated sodium error for plasma shifts in a positive direction, crossing the zero-error axis at various points, depending on how the calibrant is being changed. Accordingly, any of the calibrant compositions that correspond to the intersection of the error line and the zero error axis are predicted to yield a reference interval for sodium consistent with the plasma-water convention described by equation 1. In other words, for these calibrants, the shift depicted in Figure 1 goes to zero.

3. In those cases where monovalent anions are substituted for chloride (Figure 2a), the slope of the error line is inversely proportional to the difference in mobility of the two ions. That is, the smaller the equivalent conductance of the anion, the

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6 Assay values for normal plasma obtained with commercial analyzers may also tend to be lower than expected because of dilution by the residual fluid in the sample inlet lines remaining from the previous instrument-flush cycle. Flush solutions (often the midrange calibrant) generally contain a lower sodium concentration (typically 140 mmol/L) than normal plasma (150 mmol/L of plasma water) and will cause a consistent negative error if contamination is present.
greater the slope, which increases according to the series: bicarbonate (λ = 44.5 mho - cm²/Eq.) < acetate (λ = 40.9) < trichloracetate (λ = 36.6) < dihydrogen phosphate (λ = 33). For these monovalent anions the error lines reflect only the shift in the liquid junction potential, because the activity coefficients, as calculated by the Debye–Hückel equation (equation 13), remain constant. However, substitution of chloride with a divalent anion increases the ionic strength, for example, for Na₂SO₄, 70 mmol/L, I = 210 mmol/L, and produces a steeper error line, owing to the additive effects of both the activity coefficient and the liquid junction potential.

4. Similar additive effects are observed in Figure 2b, which shows how the sodium error varies as inert salts are added to the baseline calibrant. In these cases the slopes of the error lines vary, depending on the interaction between the terms in equation 12 for the activity coefficients and the residual liquid junction potential. With monovalent salts such as the quartzary ammonium chlorides, the slope decreases as the mobility of the cation decreases, indicating that the expected positive shift in sample error due to increased calibrated ionic strength is partly compensated for by the decrease in the calibrant liquid junction potential. For example, the slope of the error line for tripropyl ammonium chloride (λ₂₃₄₅₆₇قطاع8 = 26.1 mho - cm²/Eq.) is less than that for tetramethyl ammonium (λ₂₃₄₅₆₇قطاع8 = 45.3), methyl ammonium (λ₂₃₄₅₆₇قطاع8 = 58.3), and ammonium (λ₂₃₄₅₆₇قطاع8 = 73.5) chlorides. With multivalent inert salts, such as magnesium acetate and magnesium sulfate, the slopes tend to be steeper, owing to the dominating effect of the decreasing activity coefficient with the rapidly increasing ionic strength.

5. The calculated error trends for potassium are very similar to those shown for sodium in Figure 2. In general, however, both the sodium and potassium error lines for a given calibrant formulation do not cross the zero error axis at the same point, indicating that the optimum calibrant composition for sodium differs from that for potassium. Fortunately, modest errors (0–5%) in potassium have relatively little impact on the interpretation of assay values and, therefore, in this work we optimized the calibrant for zero error in the sodium measurement.

Experimental Results with Human Plasma

These calculations indicate that a large variety of calibration systems could be used to achieve accurate calibration according to our plasma-water convention. After considering many practical requirements such as reagent purity, stability, pH, cost, and ease of manufacture, we chose to evaluate experimentally the calibrant system based on NaCl/NaOAc/KCl. The sodium and potassium plasma-water concentrations for 10 normal plasma samples were obtained via direct potentiometry and compared with plasma-water values derived from flame photometry according to equation 1. The analyses were repeated for a series of calibrant solutions containing various NaCl/NaOAc ratios, as described in the Procedures. The results for sodium are presented in Figure 3.

Clearly, the experimentally determined error trend was consistent with our theoretical calculations (Figure 2), and showed a positive shift in the assay error as acetate was substituted for chloride in the simple NaCl/KCl calibrant. Linear regression yielded a slope of 0.04% [Na⁺] per millimole of NaOAc ion and an intercept of −3.1% [Na⁺]; the correlation coefficient was 0.990. Using the regression parameters, we determined that zero error occurred at a NaCl/NaOAc ratio of approximately 60/80 mmol/L. Analysis of the potassium data obtained simultaneously yielded a slope of 0.03% [K⁺] per millimole of NaOAc, an intercept of −5.5% [K⁺], and a correlation coefficient of 0.80. At the optimum NaCl/NaOAc ratio determined for sodium, the observed potassium error was −3.2%, or 0.1 mmol/L of plasma water. In general, the potassium data showed more scatter than the sodium data; however, as expected, the resulting error was negligible (<0.2 mmol/L of plasma water) over the tested range of the chloride/acetate ratio.

Thus, accurate calibration (plasma-water basis) can be achieved without resorting to a protein-based control serum or a complex aqueous solution formulated to resemble the composition of plasma. Note that in this experiment Mg(OAc)₂ was added to the calibrant series to adjust the pH of the unbuffered NaCl/KCl baseline calibrant, and the sodium concentration was chosen to match that used for flame photometry (140 mmol/L). To further simplify the optimum calibrant, the magnesium salt can be eliminated and the sodium concentration increased to that of a normal plasma, namely, 150 mmol/L. These two modifications tend to shift the observed error in opposite directions such that the optimum NaCl/NaOAc ratio remains nearly constant. Thus, one could also use a simplified formulation of NaCl/NaOAc/KCl (65/85/4 mmol/L). The Bicarbonate Effect—Results with a NaCl/NaHCO₃/KCl Model System

Consistent with our expectations, we observed a decrease in the apparent sodium concentration as bicarbonate was substituted for chloride in a NaCl/KCl mixture. As shown in line A, Figure 4, the percent of sodium suppression increased as the bicarbonate concentration increased (data obtained with a 3 mol/L KCl salt bridge). To determine whether the effect was occurring at the sodium electrode (i.e., a change in the activity of Na⁺) or at the sample/salt bridge liquid junc-

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7 The Henderson equation predicts that for a series of salts containing a common anion, the junction potential will decrease as the mobility of the cation decreases (constant salt bridge composition).

8 This work was done in conjunction with the development of the IL System 502 Na/K Analyzer.

9 The experimental data in Figure 3 include a small positive shift from the Mg(OAc)₂ (4 mmol/L) present in each test calibrant. Based on the Mg(OAc)₂ line in Figure 2b, this shift is about 0.5%, which would produce an observed error for a NaCl/KCl (140/4 mmol/L) calibrant of about −3.6%.

10 In the previous calculations (Figure 2a), NaHCO₃ was substituted for NaCl in the calibrant and yielded a positive error trend. Here, the substitution is made for the sample, so that a negative error trend is expected.
tion, we repeated the experiment with an unconventionally dilute KCl (0.5 mol/L salt bridge (line B, Figure 4).

Linear regression with a forced zero intercept yielded a slope of −0.06% per millimole of HCO₃⁻ with a correlation coefficient of 0.998 for the 3 mol/L KCl salt bridge, and a slope of −0.12% per millimole of HCO₃⁻ with a correlation coefficient of 0.999 for the 0.5 mol/L KCl salt bridge. With bicarbonate at 25 mol/L (approximately the concentration in normal plasma), data obtained with the more concentrated salt bridge showed a total sodium suppression of about −1.6% (or −2.2 mmol/L); with the more dilute salt bridge, the effect was nearly twice as large, −3.0% (−4.2 mmol/L).

The observed dependence on salt-bridge concentration clearly indicates that the liquid junction potential is changing, as the slow-moving bicarbonate ion is substituted for the fast-moving chloride ion. To subtract that fraction of the total suppression due to the liquid junction potential effect, we used the Henderson equation (equation 14) to correct the data of lines A and B in the liquid junction potential and replotted the results as line C in Figure 4. This corrected line had a slope of −0.04% per millimole of HCO₃⁻ and a correlation coefficient of 0.996.

The data illustrated by line C are important for two reasons. First, the fact that both the 3 mol/L and 0.5 mol/L salt bridge experiments yield the same residual suppression validates the use of the Henderson equation for estimating the changes in the liquid junction potential as sample and salt bridge compositions are varied. Second, as predicted by equation 19 (below), the residual suppression, which is independent of the salt bridge concentration, can be attributed to a change in actual activity coefficient for sodium ion in the presence of bicarbonate ion relative to that in the presence of chloride ion. Thus, at 25 mmol of bicarbonate per liter, the sodium ion activity coefficient is decreased by about 1%. These results are consistent with our previous comments on the inability of the Debye–Hückel Law to characterize the secondary ion–ion interactions, which begin to be noticeable at ionic strengths above 0.1 mol/L.¹¹

Table 3 summarizes the liquid junction potential calculations and the individual contributions of the activity coefficient and liquid junction potential effects to the total observed sodium suppression. The calculations are as follows:

\[
\text{% change in } C_{Na^+} = \left[ \frac{1}{\gamma(\text{Cl}^-)} \cdot \exp\left( \frac{-\Delta E_1}{\text{slope}} \right) \right] \times 100 \quad (19)
\]

where \( \Delta E_1 = E_1(\text{Cl}^-/\text{HCO}_3^-) - E_1(\text{Cl}^-) \). The labels (\( \text{Cl}^-/\text{HCO}_3^- \)) and (\( \text{Cl}^- \)) refer to the chloride/bicarbonate mixtures and the pure chloride solution, respectively.¹²

The Question of Binding

As shown by line C in Figure 4, the actual suppression of the sodium activity by bicarbonate is very small. However, it is worthwhile to consider the mechanism by which this occurs, to address one of most subtle and difficult-to-resolve questions associated with direct potentiometry, that is, the question of sodium and potassium binding in plasma.

Researchers interested in understanding the properties of sea water have for many years been concerned with the problems associated with activity coefficient effects and electrolyte binding (36–38). While there is no consensus among the experts as to exactly which mechanisms are operating, two basic models have been proposed. Each can be used to account for the decreasing \( \gamma_{Na^+} \) in the preceding experiment.

According to the first model, the activity coefficient decreases with bicarbonate concentration simply because the specific ion–ion interactions between \( Na^+ \) and \( \text{HCO}_3^- \) are different from those between \( Na^+ \) and \( \text{Cl}^- \). In dilute solution, all monovalent anions appear to interact with the sodium ion to the same extent. However, in more concentrated solutions, differences in the ionic nature of the anions, e.g., in the surface vicinity, are significant.

¹¹ If an additional −1% error (to account for the unpredicted decrease in \( \gamma_{Na^+} \) due to bicarbonate in normal plasma) is added to the calculated error for the NaCl/KCl calibrant (y-intercept, Figure 2), the total predicted error is −4.1%. As explained in footnote 9, the observed value with plasma was −3.6% (3 mol/L KCl salt bridge).

¹² Equation 19 was derived by substituting

\[
C_{obs} = C_{actual} \cdot \frac{\gamma_{Na^+}}{\gamma_{Na^+}} \cdot \exp\left( \frac{-\Delta E_1}{\text{slope}} \right)
\]

(see equation 9) into equation 18. In this case, \( C_{obs} \) is the apparent concentration as defined by equation 10. In the derivation, \( C_{actual} \) and the terms due to the calibrant cancel out.
charge density, become significant. This model requires that even in moderately concentrated solutions ($I < 0.5$ mol/L) all of the sodium is present as free (unassociated) ions. The actual sodium ion activity, however, depends on the ionic strength, the water activity, and the specific interactions with the other ions.$^{13}$

The second model recognizes that the interactions mentioned in the first model play a role, but also suggests that at moderately high ionic strengths a new species (an "ion-pair") forms, corresponding to a sodium–anion complex. In theory, this species would have a unique set of molecular properties and, being uncharged, would not be sensed by an ion-selective electrode (36).

With both models, short-range interactions of the sodium ion with the anion are manifested experimentally as a shift in the activity coefficient. However, the distinction between the models is important because, if ion-pair formation were the dominating mechanism, the actual concentration of ionized sodium in normal plasma would be slightly less than the expected value of $150$ mmol/L of plasma water (Table 1).

In addition to the question of ion binding between sodium and electrolyte anions, some published reports suggest that sodium and (or) potassium are complexed with protein or a protein-related substance in plasma to a very small (less than 1%) extent (39–41). However, because the reported levels of association are so low, the interpretation of the observed effects is debatable (42). Furthermore, results of experiments intended to study these effects are sometimes confused by subtle and compensating phenomena.$^{14}$ For example, in their studies on the effect of adding albumin to dilute NaCl ($\sim 1$ mmol/L) solutions, Mohan et al. (43) postulated that residual traces of sodium in the protein were responsible for an unexpected positive shift in the observed sodium electrode potential. Review of their results, however, alternatively suggests the effect could have been due to $H^+$ interference accompanying the shift in pH when albumin (isoelectric point $\sim 4.9$) was added. [At low sodium concentrations some sodium glass electrodes exhibit $H^+$ sensitivity at pH $\leq 8$ (46),]

In view of the limitations of the existing theoretical models and the difficulties associated with the experimental measurements, quantitative resolution of the question of ion binding in biological samples will not likely be forthcoming in the near future. Ideally, the best approach to eliminate the problem is to calibrate on an activity basis, similar to that now used for pH measurements. Unfortunately, the difficulties encountered in developing multicomponent activity standards for electrolyte measurement would be very great (44). One reasonable and practical solution to the problem is to calibrate according to the plasma–water concentration convention described by equation 1. We propose that this convention be adopted to define the normal ranges for sodium and potassium in whole blood, plasma, and serum.

In conclusion, this work illustrates that, to understand the relationship between the reference intervals for sodium and potassium, as determined by direct and indirect methodologies, one must consider several subtle differences. In particular, because ion-selective electrodes respond to the concentration of ions in the plasma-water phase, due allowance must be made for the volume occupied by proteins, lipids, and other plasma components. Also, in contrast to ISE measurements of diluted samples, the roles of the liquid junction potential and the activity coefficient are indeed important and must be taken into account. The question of sodium and potassium binding to anions and other plasma species represents a complicated but important issue that certainly deserves further study, especially for abnormal samples. However, for the purposes of establishing the "normal" range, the significance of this effect is, at worst, quite small and can be neglected.

The proposed calibration convention (equation 1) provides a consistent and well-defined scheme to interrelate direct and indirect assay values for typical normal samples, which, if implemented universally, should eliminate the current confusion as to what reference intervals are most appropriate for direct potentiometry. Finally, both the theoretical and experimental data presented above show that one can calibrate accurately and simply with common salt solutions, if sufficient attention is paid to eliminating the errors associated with differences in the liquid junction potential and the activity coefficients between normal plasma and the midrange ISE calibrate.

We express our appreciation to Dr. Janet D. Vitiello for her valuable contribution to this work. Much of our understanding was derived from her careful review and consideration of the facts available during the initial stages of this work.

References
