Characterization of Sodium-Selective Optode Membranes Based on Neutral Ionophores and Assay of Sodium in Plasma


Plasticized poly(vinyl chloride) membranes that incorporate a highly lipophilic sodium-selective neutral ionophore (ETH 4120) and novel H⁺-selective chromo-ionophores, lipophilic isologs of Nile Blue, are used in the competitive binding of Na⁺ and H⁺ for a reversible, optical determination of sodium activities in buffered solutions at different selected pH values. These optode membranes are used to assay total sodium concentrations in pH-buffered human blood plasma.

Additional Keyphrases: chromo-ionophores • electrolytes • ion-selective electrodes compared

In European clinical chemical laboratories, analyses for total sodium and potassium concentrations account for nearly 20% of the data output. Today, total sodium and potassium concentrations are the most frequently requested analytes (1) and generally are determined with ion-selective electrodes (ISEs) (2, 3) or flame photometry. When ISEs are built into so-called mainframe analyzers, they require a special compartment, special service, and special calibration, because most clinical analyses are spectrophotometric. To make the assay of sodium and potassium more adaptable, many have attempted to develop optical techniques for the determination of these ions. The classical complexometric–spectrophotometric method has been exploited with the use of specially designed chromogenic compounds added directly to the sample solution (4). Other methods described for clinical applications have been based on enzymatic reactions (5) or on photometric dry chemistry (6). However, reversible chemical optical sensors (optodes) would be highly advantageous, because they would not consume expensive reagents with each assay.

Optodes involving negatively charged chromophoric crown ether ligands exhibit modest selectivities (7), but more efforts have been devoted to the application of electrically neutral ionophores, which are the selective compounds in the widely used ISEs. The selective characteristics reported for optical sensors based on neutral ionophores in combination with fluorescence indicator dyes are not fully satisfactory (8, 9); such sensors have not reached the state of development to allow quantification of sodium in blood plasma samples.

Recently, the fundamentals of a new class of optode membrane were set forth (10–12). Owing to simplicity of use, well-established membrane technology, and appropriate optical properties, plasticized poly(vinyl chloride) (PVC) was selected as the membrane matrix. In the same bulk membrane, conventional ionophores, selective for the ion of interest, were combined with chromionophores, the absorption spectra of which undergo drastic change upon complexation with a second sort of ions, e.g., H⁺. Introduction of neutral, highly basic, and lipophilic H⁺-selective chromo-ionophores of the oxazine type (13, 14) has allowed the realization of many optode systems selective for different cations (13–15) and anions (16). The selectivities of these systems correlate nearly perfectly with those of the corresponding ISEs.

The cation-selective membranes operate on the basis of a neutral-ionophore-mediated cation-exchange mechanism (11). They incorporate two competitive electrically neutral ionophores, selective for sodium and hydrogen ions, respectively, together with lipophilic anionic sites, which determine the total desired concentration of cation complexes in the membrane phase. Based on this mechanism, cation-selective optode membranes for assays of total calcium and potassium have been successfully applied to human blood plasma (15, 17). Here we report on sodium ion-selective optode membranes based on the same principle. Various neutral, sodium-selective carriers used in solvent polymeric membranes of ISEs are available, which at least partly satisfy the specifications required for measurements in blood serum samples (2, 18–20). Membranes with the ionophore ETH 2120 and the more lipophilic isolog ETH 4120 seem to meet all requirements (21). We focused on the highly lipophilic carrier ETH 4120, because the lipophilicity of the membrane components turned out to be the most decisive factor with respect to the lifetime of these thin, optical-sensing layers (22).

When used for the assay of total sodium concentrations in pH-buffered samples of human blood plasma, the results obtained with optodes with an optimized membrane composition showed an excellent correlation with ISE measurements.

Materials and Methods

Apparatus

The pH values were determined with a pH glass electrode (Orion Ross Model 8103) and a Model SA 720 Orion pH meter (Orion Research AG, Uetikon am See,
The measuring cells containing the two optode membranes were introduced into a conventional double-beam spectrophotometer (Model 555; Perkin-Elmer, K"unacht, Switzerland). For all measurements, we used an optical bandwidth of 2 nm.

The sodium concentrations of the plasma samples assayed by the optode membranes were compared with values established by ISEs in a Hitachi 737 discrete microchemistry analyzer (Hitachi Ltd., Instrument Division, Tokyo 100, Japan) and by flame atomic emission spectroscopy (FAES) in an IL-443 analyzer (Instrumentation Laboratory SPA, I-20128 Milan, Italy). The total calcium concentration was evaluated by the o-cresolphthalein complexone method, also performed with the Hitachi 737.

Reagents

Aqueous solutions were prepared with doubly quartz-distilled water and salts of the highest purity available. For membrane preparation, we used PVC (high M₂), sodium tetraakis[3,5-bis(trifluoromethyl)phenyl]borate [NaTm(CF₃)₂PB], bis(2-ethylhexyl)sebacate, bis(1-butylpentyl)adipate (BBPA), triis(2-ethylhexyl) phosphate, bis(1-butylpentyl)decane-1,10-diyl diglutarate (ETH 469), and tetrahydrofuran obtained from Fluka AG (Buchs, Switzerland). The synthesis of ETH 2439 and ETH 5350 is described by Seiler (13), the synthesis of ETH 4120 by Gehrig et al. (21).

Buffer solutions. The buffer solutions used for recording the calibration curves were pH 4.8 acetate buffer (50 mmol/L magnesium acetate adjusted with 1 mol/L acetic acid) for optode membrane I, and a pH 7.6 Tris buffer (0.1 mol/L Tris adjusted with 0.1 mol/L HCl) for optode membrane II (23). To determine the selectivity coefficients, we used different pH 5.5 acetate buffers (per liter, 50 mmol of magnesium acetate and 10 mmol of the respective metal ion acetate adjusted with 1 mol/L acetic acid) containing various concentrations of sodium. For the reproducibility and the stability measurements, we used pH 5.9 acetate buffer (50 mmol/L magnesium acetate adjusted with 1 mol/L acetic acid).

The plasma samples were diluted with 50 mmol/L magnesium acetate buffer adjusted with 0.1 mol/L hydrochloric acid to pH 4.9. The ionic strength, I, of this buffer is nearly identical to the physiological ionic strength (I = 0.15).

Blood plasma samples. Specimens were collected in heparinized Vacutainer Tubes (14 units/mL, corresponding to 109.2 mg/L standard heparinate; Becton Dickinson, Rutherford, NJ 07070) by the University Hospital in Zürich. An aliquot of the plasma was frozen and stored at temperatures below −20 °C for four days.

Calibrators and solutions for the standard addition method. The calibration function was evaluated with two commercially available calibration solutions for ISEs: an aqueous "high" standard (no. 646 911) with ion concentrations of Na⁺ 120 mmol/L, K⁺ 3 mmol/L, and Cl⁻ 80 mmol/L. For the working standard, we prepared an aqueous solution (100 mmol/L) of sodium chloride. In a first trial, we standardized the measurements with the optode membrane by using the commercially available solutions.

Quality-control samples. We selected the following two quality-control samples with assigned mean values and confidence intervals for single values determined by reference laboratories: Precinorm U (lot no. 162 486; Boehringer Mannheim GmbH) with an assigned mean Na⁺ value of 118 mmol/L (111−125 mmol/L) for flame photometry and 120 mmol/L (113−127 mmol/L) for ISEs (Hitachi 737 analyzer); and Precipath U (lot no. 159 589; Boehringer Mannheim GmbH) with assigned values of 135 mmol/L (127−143 mmol/L) for flame photometry and 136 mmol/L (128−144 mmol/L) for ISEs.

Procedures

Optode membranes. The optode membranes consisted of 0.9 mg of NaTm(CF₃)₂PB, 15 mg of sodium ionophore ETH 4120, 36 mg of poly(vinyl chloride), 72 mg of plasticizer BBPA, and either 0.7 mg of chromo-ionophore ETH 2439 for membrane I, or 0.53 mg of chromo-ionophore ETH 5350 for membrane II. The membrane composition I, with various concentrations of ETH 4120, was used for determining the selectivity coefficients. Optode membrane III, the blank membrane for the reference cell, had the same composition as membrane I but omitted the chromo-ionophore ETH 2439. The membrane components were dissolved in 1.5 mL of freshly distilled tetrahydrofuran. By using a spin-on device, we cast two identical membranes, ~4 μm thick, on two glass plates that were later mounted into the measuring cell (13). This cell was introduced into a conventional spectrophotometer, where all measurements were made in the transmission mode. The absorbance values were taken at the absorbance maximum of the protonated form of the chromo-ionophore, 650 nm (±0.5 nm) for both optode membranes. At this wavelength, the logarithm of the molar absorptivities, log ε, of both chromo-ionophores was estimated at about 4.8 (assuming a density of 1 g/mL for both membrane phases).

Selectivity. Selectivity coefficients were determined by the fixed interference method, by a theoretical fit of the experimental absorbance values, assuming 1:2 complexes for sodium (12). The Na⁺ activities were calculated according to a Debye-Hückel formalism (24).

Measurements in human blood plasma. Two glass plates with the optode membranes I were mounted in a measuring cell especially adapted to measurements of samples in a flow-through system (22). The volume of the cell was 330 μL and the geometry permitted laminar flow. Two glass plates with membranes III were mounted in an identical reference cell, which was connected with tubing to the measuring cell and was a part of the flow-through system. Between samples, the measuring cell and reference cell were simultaneously flushed with the pH 4.9 acetate buffer. We prepared three samples for the standard addition procedure (25).
We diluted 250 µL of the blood plasma 20-fold with the pH 4.9 magnesium acetate buffer to yield the first sample, with unknown sodium concentration c_s. For sample 2, 50 µL of working standard was added to 250 µL of blood plasma; the mixture was diluted with 4.7 mL of the same buffer to get a total sodium concentration of c_s + c_p, resulting in an increase in the total sodium concentration over the original sample by 20 mmol/L. For the third sample, we diluted sample 2 1.3-fold with the buffer to yield a total sodium concentration of (c_s + c_p)/1.3. From these three concentrations and their corresponding absorbance values, we calculated c_s by equation 3 (see reference 25).

Results and Discussion

Characterization of the Optode Membranes

Response behavior. The response curve of this type of optode membrane is determined by the following ion-exchange reaction between the aqueous sample solution (aq) and the organic membrane phase (org):

$$K_{eq} = \frac{\text{Na}_L^+(\text{org}) + H^+(\text{aq}) + L(\text{org})}{\text{Na}^+(\text{aq})} \left(\text{HL}^+(\text{org}) + \nu L(\text{org})\right)$$ (1)

The corresponding equilibrium constant, $K_{eq}$, is characterized by the stability constants of the two ionophore complexes HL$^+$ and NaL$^{\nu+}$ (for 1:1 sodium:ionophore complexes) and by the ionic distribution coefficients of the involved cations.

Introducing the ratio, $\alpha$, of unprotonated chromo-ionophore relative to total chromo-ionophore, $L_T$, in the membrane, leads to the following relationship between concentration and absorbance (A) of the chromo-ionophore:

$$\alpha = \frac{[L]/L_T = (A - A_0)/(A_1 - A_0)}{1}$$ (2)

where $A_1$ and $A_0$ are the limiting absorbance values for $\alpha = 1$ (fully deprotonated ligand L) and $\alpha = 0$ (fully protonated ligand L), respectively. As pointed out earlier for this type of optode membrane, the absorbance response of the present system depends on the ratio of the corresponding ion activities ($a_{Na}/a_{HS}$) in a limited dynamic range, which is in clear contrast to the ISE response curves (12). By introducing chromo-ionophores having different complex formation constants, one can vary the overall equilibrium constant $K_{eq}$ and thus alter the working range of the optode membrane. Figure 1 shows two neutral lipophilic isologs of the Nile Blue type with different basicities (ETH 5350, ETH 2439 (13)), used as chromo-ionophores in the sodium-selective optode membranes presented here. Both Na$^+$-selective optode membranes, I and II, were equilibrated with pH-buffering solutions containing different concentrations of NaCl. For optode membrane I, incorporating ETH 2439, the absorption spectra in the visible range are given in Figure 2. Analogous absorption spectra were recorded for optode membrane II, incorporating ETH 5350, at pH 7.6. Through modifying the imino group substituent (see Figure 1), we obtained the expected change of the complex formation constant of the Nile Blue isologs. This is corroborated by the location of the dynamic range of the response curves in Figure 3.

If the ionic strength of the solution is kept constant by the pH buffer, the activity coefficient of Na$^+$ can be assumed to be constant. Furthermore, if measurements are performed at constant pH, as well as in the linear part of the response curve, then one can derive, to a first approximation, a linear relationship between the absorbance, A, and the logarithm of the sodium concentration, $\log c_{Na}$:

$$A = A_B + s \cdot \log c_{Na}$$ (3)

where $A_B$ and $s$ are the intercept and the slope, respectively.

Selectivity. A contraction of the dynamic range occurs when interfering ions compete with the primary ions for
complexation with the corresponding ionophores. In this case the activities have to be replaced by sums of selectivity-weighted activities (12), to a first approximation in analogy to the terms in the Nicolsky-Eisenman equation for ion-selective electrode potentials. Although a high concentration of anionic sites in the optode membrane is essential for a sufficiently large absorbance change (10-12), this may simultaneously influence the selectivity towards different interfering ions. A dependence of the selectivity on the ionophore/borate concentration ratio in the membrane has been found in the case of ISE membranes, if primary and interfering ions of different charges or if cation/ionophore complexes of different stoichiometries are involved (24). Such a dependence has now also been observed for the described optode membranes (Figure 4). Obviously, the best selectivities are obtained at high ionophore/borate concentration ratios. The selectivity coefficients for different physiologically relevant cations have been evaluated for both membranes with an 18-fold molar excess of ionophore over borate, according to the fixed-interference approach (see Table 1). For measurements in 20-fold-diluted blood plasma, the required physiological selectivity coefficients for sodium over potassium, magnesium, and lithium (in heparinized samples as prepared in Vacutainer Tubes) are fulfilled, whereas the selectivity over calcium obviously is not. The selectivity requirements are less severe if the calibration solutions contain mean ion concentrations as physiological background (see Table 1, right column).

The use of different plasticizers, e.g., BBPA, bis(2-ethylhexyl)sebacate, ETH 469, tris(2-ethylhexyl)phosphate, has no significant influence on the selectivity behavior.

Short-term reproducibility. The optical signals were highly reproducible, when the Na+-selective optode membrane I (incorporating ETH 2439) was exposed to repeated concentration step changes between 9 and 19 mmol/L in aqueous NaCl solutions (in pH 5.9 acetate buffer). The mean (SD) absorbance values obtained from the measured signals after 5 min were 0.1635 (0.0002) for the 9 mmol/L NaCl solution, and 0.1250 (0.0004) for the 19 mmol/L solution (n = 5 each). The precision of these absorbance values corresponds to a CV for the derived activity values of <1%. The response times of the present 4-μm-thick Na+-selective membranes are <30 s.

Lifetime. The absorbance signal at 650 nm for the optode membrane I in contact with a 9 mmol/L NaCl solution (pH 5.9 acetate buffer) was recorded for 6 h. From these absorbance values (n = 7), we calculated a mean absorbance of 0.1636 (SD 0.0002), which corresponds to the stability of the spectrophotometer.

Unfortunately, the lifetime is significantly shortened when the optode membranes are in contact with blood plasma (see below), because of a gradual extraction of membrane components into the sample solution. Because of the smaller thickness of optode membranes, a high lipophilicity of the components is more important.

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**Figure 3.** Absorbance values at 650 nm of optode membranes I and II as a function of log(\(a_{Na}^+/a_{Na}^-\)) in appropriate buffer systems. The Na\(^+\) activities were calculated according to a Debye-Hückel formalism (24). The hydrogen ion activities were estimated with a pH glass electrode.

**Figure 4.** Influence of the ionophore/borate concentration ratio of the optode membrane I on the selectivity coefficients, log \(K_{Na}^{opt}\) (fixed interference method).

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**Table 1.** Selectivity Coefficients \(K_{Na}^{opt}\) of the Na\(^+\)-Selective Optode Membranes for Interfering Ions Compared with Corresponding Potentiometric Selectivities \(K_{Na}^{pot}\) and Required Selectivities for Blood Sample Applications

<table>
<thead>
<tr>
<th>Ion</th>
<th>Optode I</th>
<th>Optode II</th>
<th>(\log K_{Na}^{opt})</th>
<th>(\log K_{Na}^{pot})</th>
<th>(a)</th>
<th>(d)</th>
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<tbody>
<tr>
<td>K(^+)</td>
<td>-1.2</td>
<td>-1.2</td>
<td>-1.4</td>
<td>-3.0</td>
<td>-2.7</td>
<td>-3.0</td>
</tr>
<tr>
<td>Li(^+)</td>
<td>-1.1</td>
<td>-1.1</td>
<td>-1.0</td>
<td>-3.0</td>
<td>-2.7</td>
<td>-3.0</td>
</tr>
<tr>
<td>Ca(^{2+})</td>
<td>-1.2</td>
<td>-1.4</td>
<td>-1.5</td>
<td>-3.0</td>
<td>-2.7</td>
<td>-3.0</td>
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<tr>
<td>Mg(^{2+})</td>
<td>-2.5</td>
<td>-3.0</td>
<td>-3.8</td>
<td>-3.0</td>
<td>-2.7</td>
<td>-3.0</td>
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* By fixed interference method; see text for composition of optodes.
* Sodium-selective electrodes (19).
* \(K_{Na}^{pot}\) by a physiological total calcium concentration of 2.7 mmol/L, Na\(^+\) 2.7 mmol/L, and Mg\(^{2+}\) 1.0 mmol/L; 20-fold dilution.
* 
* Required selectivity coefficient for a maximum interference of 1% by other cations (calibration by using a physiological total concentration of the following interfering ions: K\(^+\) 4.0 mmol/L, Ca\(^{2+}\) 2.3 mmol/L, and Mg\(^{2+}\) 0.8 mmol/L; 20-fold dilution).
* Heparinated blood plasma.
for optodes than for ISEs (22). A loss of components (e.g., of ionophore, chromo-ionophore, borate) not only results in a change of the response curve, but also may substantially affect the selectivity behavior of the optode membrane (see above).

Measurements in Blood Plasma

To adapt to the limited dynamic measuring range of optode membrane I (see Figure 3), the plasma samples were diluted 20-fold with pH 4.9 acetate buffer. In a first series of assays, total sodium concentrations in plasma samples were evaluated by a calibration procedure with commercially available calibration solutions, which unfortunately do not contain calcium. Total sodium values were calculated by a linear least-squares regression function of the absorbance vs log \( \frac{a_{Na}}{a_M} \). Compared with ISEs, the optode membranes gave a positive systematic error of +2 to +5 mmol/L for total sodium concentration. The insufficient selectivity over calcium is supposedly the main reason for these high readings, which were eliminated when the standard addition method was applied (see Materials and Methods).

Calibration. Twenty plasma samples with total sodium concentrations in the range of 83–153 mmol/L, corresponding to a difference in absorbance of 0.060, and two quality-control samples, were diluted and assayed with the standard addition method twice. We found a linear relationship between the absorbance values and the logarithm of the total sodium concentration for each sample. Owing to the instability of the membrane, the slope of the calibration curve decreased by 11% during the 10-h sample run. Therefore, for evaluating subsequent data on the serum samples, we added standards to each sample, resulting in a continuous recalibration.

Extraction of sodium. The extraction of sodium ions into the optode membrane causes a concentration change in the sample solution. The diluted blood plasma samples contain a total of about 2 μmol of Na\(^{+}\) in the 330-μL volume of the measuring cell and are in contact with a membrane segment of about 1.5 mm\(^2\). The amount of extracted sodium, which produces an absorbance change of 0.060 (equal to the total absorbance range of the blood plasma samples investigated), alters the total sodium in the sample by <0.1%. Of course, the change in the sodium concentration is even smaller for a flow-through system.

Quality control. Total sodium concentrations of the two quality-control samples, determined with the optode membrane I to be 135.0 ± 2.8 (n = 5) and 120.8 ± 2.4 mmol/L (n = 5), show excellent agreement with the assigned values (136 and 120 mmol/L) from the indirect ISE measurements. The deviations of the optically determined mean values from the assigned mean values were only −0.7% and +0.7%, respectively. The reproducibility of the five measurements of the quality-control samples varied by 2.0% at 135 mmol/L and 121 mmol/L.

Comparison of methods. Total sodium concentrations obtained by indirect potentiometry (x) and by the optode membranes (y) show an excellent correlation over the range investigated (Figure 5). This is equally well demonstrated by the nonparametric linear-regression method of Passing and Bablok (26), \( y = 0.00 + 1.00x \), and by the least-squares linear regression, \( y = (1.03 \pm 1.05) + (0.995 \pm 0.047)x \) \( (R^2 = 0.979, n = 20) \). The imprecision with respect to the regression line, \( S_{y,x} \), was ±3.31 mmol/L, or ±2.9% of the mean (113.4 mmol/L). The Cusum test does not refer to a significant deviation from linearity (27).

A comparison of the optode membranes (y) with FAES (x) by the method of Passing and Bablok gave \( y = -11.0 + 1.074x \), and by the least-squares linear regression was \( y = -(11.6 \pm 1.15) + (1.084 \pm 0.056)x \) \( (R^2 = 0.976, n = 20) \). \( S_{y,x} \) was ±3.6 mmol/L, or ±3.2% of the mean (112.2 mmol/L). An intercept of the same magnitude was found when FAES was compared with ISEs, whereas the imprecision of that comparison was smaller (\( S_{y,x} = ±1.3 \) mmol/L).

Basically, ISEs and optode membranes are measuring the same physicochemical characteristic and therefore demonstrate a close relationship. Compared with the automated methods (ISEs, FAES), the optode membranes are expected to show a greater imprecision of the results, mainly because of the manual proceeding.

According to recommendations for analytical variation (28), \( s_a = 0.5 \cdot s_b \), where \( s_b \) represents the intra-individual variation (26); thus, the analytical variation, \( s_a \), should ideally be <0.35%. However, this CV is not reached in present clinical tests, not even by fully automated analyzers. A CV of 0.7–1% for total sodium

<table>
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<tr>
<th>OPTODE Na(^{+}) [mM]</th>
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<tr>
<td>60</td>
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<td>120</td>
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Fig. 5. Comparison of methods: indirect ISEs of Hitachi 737 vs optode membrane I by the nonparametric Passing–Bablok (PB, with confidence limits shown by solid line) procedure as well as by the linear least-squares regression method (LSR, ---)
concentration by indirect methods is ordinarily acceptable.

Interferences. Investigations with icteric, hemolytic, and lipemic samples as well as with exogenous interfering compounds seem not relevant at this stage. A possible interference by a colored or turbid background is compensated by the reference cell arrangement, which, of course, is not valid for the insufficient selectivity towards calcium (see Table 1). The total calcium concentration, determined for each sample, ranged from 0.72 to 2.52 mmol/L for the 20 samples. Subsequent calculations show that the insufficient selectivity (log $K_{\text{Ca}^{2+}}^{\text{opt}} = -1.2$; see Table 1) induces an apparent increase in the total sodium concentration by 7.8 ± 1.0%. Although systematic error was taken care of by using the standard addition method, the insufficient selectivity may still contribute to the imprecision of the results. A calibration procedure with calibrators containing physiological concentrations of all possible interfering ions, including calcium, will certainly improve the precision of the results.

In conclusion, the optode membranes we have described allow the reversible quantification of total sodium concentration in human heparinized plasma with as high accuracy as indirect ISE measurements. Recalibration takes care of the instability of the optode membranes in contact with blood plasma for 10 h. Automation of the manual procedures should improve the precision of the results. Further increases in precision may be achievable by enhancing the membrane stability and reducing the interference from calcium.

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References