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ARTICLE *in* ANALYTICAL CHEMISTRY · NOVEMBER 1997

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Direct Determination of Oxygen by HPLC. 1. Basic Principles of a Sensitive and Selective Oxygen Sensor

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The basic principles of a novel, versatile, sensitive, and selective oxygen-sensing assay are presented in this paper. For the first time, liquid chromatography with electrochemical detection (at the hmde) has been used for the determination of oxygen. All factors concerning optimization of the chromatographic separation conditions and electrochemical detection with respect to direct determination of oxygen even in complex biological samples are discussed. Due to the combination of a chromatographic technique with amperometric detection, a high selectivity can be achieved. A direct and linear relationship between the oxygen concentration in the sample and the reduction current was verified in a large concentration range from saturation down to trace level oxygen concentrations. The novel oxygen-sensing assay provides a much higher sensitivity compared to conventional oxygen sensors. In principle, O_2 concentrations down to 4.5×10^{-9} mol L^{-1} O_2 (corresponding to a signal-to-noise ratio of 3) can be detected. Precision was determined by repeated measurements ($n = 6$) of air-saturated solutions (2.5×10^{-4} mol L^{-1} O_2, 20 °C, 920 mbar) which yielded relative standard deviations of lower than 0.2%.

Oxygen can be considered to be one of the most reactive molecules in nature, playing an important role in many fields, such as chemical and biochemical reactions, physiology, and medicine. The determination of oxygen is of considerable importance in environmental and biomedical analysis as well as analytical chemistry, and many different types of analytical methods have been designed for the study and determination of oxygen in gases, liquids, and biological samples. We were interested in finding a highly versatile and sensitive analytical method that might permit direct determination of oxygen in complex samples such as biological cells and tissue media, with high selectivity in a large concentration range. One of the applications we had in mind was the determination of oxygen in tumor cells, because knowledge of the oxygenation status in tumor cells may be important for predicting tumor response to therapy.[1,2] Methods used for the determination of oxygen are based on very different detection principles,[3] ranging from chemical methods pioneered by Haldane and Winkler, manometric methods introduced by Warburg, and more sophisticated spectroscopic techniques such as ESR, NMR, cryospectroscopy, and near-infrared spectroscopy.[1] Optical O_2 sensors[4,5] based on optical quenching of a dye or a transition-metal complex by molecular oxygen and electrochemical sensors, such as galvanic Hersch-type sensors,[6] zirconia-type sensors,[7] and amperometric Clark-type sensors[8−13] have been applied more or less successfully to the determination of oxygen in biological systems. However, the use of many of these methods mentioned above is restricted, because they suffer from disadvantages in practicability, reliability, or general applicability especially to determinations of very low oxygen concentrations.[1] Some assays are rather time consuming and some suffer from the need for expensive technical equipment or lack of sensitivity and selectivity, especially in cases where the analytical signal is indirectly related to the oxygen concentration of the sample (e.g., ^{1}H NMR spectroscopy).

At first sight, among all analytical methods, electrochemical sensors such as amperometric Clark-type oxygen electrodes seemed to be the most promising devices for the determination of oxygen in biological systems.[8−13] They are commercially available, are relatively low cost, and provide an almost instantaneous, continuous readout of oxygen activity. Sensitivity of amperometric oxygen sensors ranges from 2.8×10^{-4} mol L^{-1} obtained for an oxygen-saturated solution (at 20 °C and 1013 mbar) to $\sim 5.0 \times 10^{-7}$ mol L^{-1}. However, these oxygen-sensing devices are also prone to some systematic errors. First, due to the electrochemical measuring principle, all amperometric oxygen sensors consume oxygen during the measurement, at rates that depend on the applied polarizing voltage, size of the cathode, and pO_2.[8−12] This leads to progressive depletion of dissolved oxygen in the medium during the course of a measurement. Another disadvantage is that the measurement of a membrane-covered oxygen sensor is limited by the rate of diffusion of oxygen to the cathode. Due to matrix elements, bacteria, yeasts, and molds in

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(1) Stone, H. B.; Brown, J. M.; Phillips, T. L.; Sutherland, R. M. *Radiat. Res.* **1993**, *136*, 422−434.
(2) Marshall, R. S.; Koch, C. J.; Rauth, A. M. *Radiat. Res.* **1986**, *108*, 91−101.
(3) Battino, R.; Clever, H. L. *Chem. Rev.* **1966**, *66*, 395−463.
(4) Peterson, J. S.; Fitzgerald, R. V.; Buckhold, D. K. *Anal. Chem.* **1984**, *56*, 62−67.
(5) Xu, W.; Kneas, K. A.; Demas, J. N.; DeGraff, B. A. *Anal. Chem.* **1996**, *68*, 2605−2609.
(6) Ogino, H.; Asakura, K. *Talanta* **1995**, *42*, 305−310
(7) Böttcher, H.; Engel, S.; Fürst, P. *Anal. Biochem.* **1995**, *230*, 149−153.
(8) Haller, T.; Ortner, M.; Gnaiger, E. *Anal. Biochem.* **1994**, *218*, 338−342.
(9) Rasmussen, H. N.; Rasmussen, U. F. *Anal. Biochem.* **1993**, *208*, 244−248.
(10) Gnaiger, E., Forstner, H. *Polarographic Oxygen Sensors*; Springer: Berlin, 1983.
(11) Linek, V.; Vacek, V.; Sinkule, J.; Benes, P. *Measurement of Oxygen by Membrane-Covered Probes*; Wiley: London, 1988; p 184.
(12) Lessler, M. A. In *Methods of Biochemical Analysis*; Glick, D., Ed.; Wiley: New York, 1982; pp 175−199.
(13) Gooding, J. J.; Hall, E. A. H. *Electroanalysis* **1996**, *8*, 407−413.

S0003-2700(97)00363-6 CCC: $14.00

biological samples, coating of the electrode membrane during the measurement may occur, leading to changes in the diffusion rate of oxygen to the cathode and to irreproducible and erroneous analytical signals especially during long-term studies. The temperature sensitivity of membrane-covered amperometric oxygen sensors represents a further problem. The entire reaction vessel, solution, membrane, and oxygen probe must be temperature equilibrated and the temperature must be maintained within relatively small limits (0.01−0.1 °C)[11,12] in order to obtain reproducible results. At any rate, calibration should be performed at the same temperature as determination of oxygen in a sample.[11,12] Apart from errors that are directly related to the measuring principle of the oxygen sensor, problems may be caused by the test chamber used for storage of the sample during measurements. Uncontrolled diffusion of oxygen due to leakage of the test chamber as well as absorbance or release of oxygen by the material constituting the chamber may lead to alteration of the oxygen concentration of the sample and to considerable errors in oxygen readings, particularly at very low oxygen tensions.

Since none of the conventional methods seemed to be completely appropriate for determinations of very low oxygen concentrations in complex samples, we began to work on the development of a new oxygen-sensing assay. The objective was to obtain an analytical signal with a higher sensitivity than obtained with conventional oxygen sensors, corresponding directly to the oxygen concentration in the sample, without the problem of permanent oxygen consumption due to the measuring principle and without the problem of interference from matrix elements or atmospheric oxygen. The detection principle of the analytical approach presented in this paper is basically similar to that of the amperometric oxygen sensor but relies on both the selectivity of liquid chromatography and the selectivity and sensitivity of electrochemical detection.

EXPERIMENTAL SECTION

Apparatus. The chromatographic system consisted of a low-pressure gradient pump (Model M 480, Gynkotek, Germering, Germany) in combination with a pulse-dampening device and an injection valve (Model 9125 or 7000, Rheodyne) with a 20 or 10 μL sample loop. The colums were 60 × 4 mm i.d. packed with 5 μm LiChrospher RP-8 (including precolumn) or 5 μm Eurospher RP-4 (including precolumn) and 60 × 2 mm i.d. packed with 5 μm Eurospher RP-4. An EG&G (Parc) amperometric detector (Model 400) in combination with a mercury LC electrode (Model LC-420, EG&G (Parc)) consisting of a three-electrode configuration with a mercury drop working electrode 303A, a silver/silver chloride (KCl) reference electrode, and a platinum wire auxiliary electrode was used for electrochemical detection. Recording of the chromatograms as well as quantitative data analysis was accomplished on a PC-based data system (Intel 486 DX/33 MHz, GynkoSoft, Version 4.22, Gynkotek).

Reagents. Buffer solutions were prepared by dissolving the appropriate amount of phosphate salts, $Na_3PO_4 \cdot 12H_2O$, $NaH_2PO_4 \cdot 2H_2O$, and Na_2HPO_4 (Fluka and Merck; highest purity) in high-purity water (Epure, Barnstead Co., Newton, MA). The pH value of the final solutions (ionic strength 0.01 mol L^{-1}) was controlled with a glass electrode and a calibrated pH meter. Methanol was obtained from Fluka (puriss pa, free of acetone). All mobile phases (phosphate buffer or mixtures of phosphate buffer and methanol) were filtered through 0.45 μm cellulose acetate filters (Millipore Corp.) before use. Unless otherwise stated, the mobile phase consisted of phosphate buffer (pH 7) and methanol (7:3, v/v). Helium 5.0 (Messer-Griesheim) and argon 5.0 (Messer-Griesheim) in combination with an oxysorb-scrubber (Messer-Griesheim) were used for deoxygenating the mobile phase and the sample.

Oxygen Removal. The mobile phase was refluxed at 60−80 °C, and helium gas was gently bubbled through the liquid.[14] Deoxygenation of the mobile phase was performed overnight (at a minimum eluent flow rate of 10−30 μL min^{-1}) and continued during the measurement. All plastic (Teflon) tubings were replaced by stainless steel capillaries. Sample deoxygenation was achieved by bubbling argon through the sample solution. The deoxygenated sample was transferred into the sample loop by pulling at the injection syringe (the injector being in load position) instead of direct injection.

Linearity Test/Calibration Procedure. Solutions containing different oxygen concentrations were prepared from air-saturated water at different temperatures. Highly purified water was stirred vigorously in a beaker, in order to achieve air saturation, while the temperature of the surrounding water bath was slowly increased from 0 to 80 °C. Cooling beyond room temperature was achieved via ice/water slushes. Temperature control was in the order of ±0.2 °C. Repeated measurements were performed by injecting an aliquot of 10 μL of air-saturated water and registering the resulting reduction current versus time. A linear calibration plot was obtained by plotting the current values against the concentration of oxygen in air-saturated solutions at different temperatures, which were calculated from the semiempirical eq 4 (see Results and Discussion).

Details on the Derivation of Equation 4. The temperature dependence of the Bunsen coefficient (β') is described in literature[18] by semiempirical, polynomial equations ($\beta' = a - bT + cT^2$), which are given only for small temperature ranges:

$$T = 0-30\ ^\circ\text{C} \qquad \beta' = 0.04890 - 0.0013413T + 0.0000283T^2 - 0.000000295347T^3$$

$$T = 30-50\ ^\circ\text{C} \qquad \beta' = 0.02608 - 0.00345(T - 30) + 0.0000043(T - 30)^2$$

$$T = 50-70\ ^\circ\text{C} \qquad \beta' = 0.02090 - 0.0001595(T - 50) + 0.00000155(T - 50)^2$$

$$T = 60-80\ ^\circ\text{C} \qquad \beta' = 0.01946 - 0.0001335(T - 60) + 0.00000205(T - 60)^2$$

Several polynomial approximations based on these semiempirical equations were performed. The best fitting between the solubility data obtained from the semiempirical equations (see above) given for different temperature intervals and the data calculated by a mathematical function over the whole temperature

(14) Bratin, K.; Kissinger, P. T.; Bruntlett, C. S. *J. Liq. Chromatogr.* **1981**, *4*, 1777−1795.

(15) Southampton Electrochemistry Group, Eds. *Instrumental Methods in Electrochemistry*; Ellis Horwood Series in Physical Chemistry; Wiley: New York, 1985; Chapter 4, p 139.

(16) Postlethwaite, T. A.; Hutchison, J. E.; Murray, R. *Anal. Chem.* **1996**, *68*, 2951−2958.

(17) Kissinger, P. T.; Heinemann, W. R. *Laboratory Techniques in Electroanalytical Chemistry*, 2nd ed.; Marcel Dekker: New York, 1996; Chapter 27.

(18) Pietsch, E. H., Kotowski, A., Eds. *Gmelin Handbuch der Anorganischen Chemie*, Sauerstoff, 8th ed.; VCH: Heidelberg, 1958.

range (0−80 °C) was achieved by a polynomial equation of the fourth degree (eq 4). For convenience, eq 4 was used for the calculation of the concentration of oxygen in aqueous solutions in all further calibration experiments, because it is valid over a large temperature range and yields sufficiently accurate solubility values.

RESULTS AND DISCUSSION

Oxygen can be reduced electrochemically along several pathways, depending on the potential and the reaction conditions applied, namely, the two-electron reduction to hydrogen peroxide and the four-electron reduction to water (or in basic media, to HO_2^- and to OH^-, respectively).[11,15,16] In general, oxygen is considered a nuisance in liquid chromatography with reductive electrochemical detection, because it is well-known that oxygen reduction can pose severe problems. High background currents, increased baseline noise, and a huge tailing oxygen peak overlapping other chromatographic peaks may be the consequence if oxygen is not thoroughly removed from mobile phase and sample solution.[14] It was this observation that led to the idea that reduction of oxygen, which is the basic measuring principle of the amperometric oxygen sensor, might also be used for the development of a sensitive chromatographic oxygen sensor. Surprisingly, the use of liquid chromatography for the direct determination of oxygen has never before been reported in literature. Usually, only gas chromatography (GC) will be taken into account when talking about the determination of gases by chromatographic methods. However, gas chromatography is highly sensitive to interference of matrix elements and moreover suffers from the lack of suitable detectors, which are comparably sensitive and as inexpensive as the electrochemical detector. Therefore, GC was considered a highly inconvenient method for the determination of oxygen in complex samples. The advantages of combining a liquid chromatographic system with electrochemical detection seemed to be most promising compared to GC or simply using a membrane-covered amperometric oxygen sensor. First, separation of the sample solution from the detector cell prevents progressive depletion of oxygen in the sample as a consequence of the measuring principle (of, e.g., amperometric oxygen sensors) and renders the analytical signal insensitive to temperature changes in the sample. Second, the use of a chromatographic column eliminates the problem of interfering matrix elements. Third, due to the flow of the mobile phase, dissolved oxygen is transported to the detector cell cathode by convection, which is a faster process than diffusion through a membrane. Thus, a higher sensitivity can be gained.

Before any measurement of oxygen in a real sample could be performed, the fulfillment of some basic requirements of the analytical system had to be verified. Reliable and reproducible measurements of the oxygen concentration in a sample can only be expected if interference from atmospheric oxygen and oxygen dissolved in the mobile phase can be excluded. Thus, it had to be tested whether the analytical apparatus was closed tight against atmospheric oxygen, so that no diffusion of oxygen into and out of the system could take place. This was achieved by replacing any plastic tubings or Teflon connections in the chromatographic system by stainless steel capillaries. Complete deoxygenation of the mobile phase was successfully accomplished by a similar procedure which had been described previously.[14] The degree of deoxygenation of the system is indicated by the height of background current and baseline noise and can be easily controlled. Deoxygenation was considered to be sufficiently effective if the background current reached values lower than 5 nA with a baseline noise lower than 5 pA. Another problem that remained to be solved was the question of sample injection. Whenever sample application was performed as usual by direct injection of the oxygen-containing sample into the sample loop of the injector via a syringe, irreproducible peak heights were obtained, in particular at low oxygen concentrations. This phenomenon was caused by uncontrolled diffusion of atmospheric oxygen into the syringe and dead volumes of the filler port during the injection process. However, this problem could be easily solved by reversing the injection process.[14] The filler port of the injector was connected directly to the sample solution, and the sample was tranferred into the sample loop by pulling at the injector syringe, which was kept in place during injection and during the course of the measurement. Thus, influx of atmospheric oxygen during the injection process could be avoided, and reliable and reproducible oxygen readings were obtained. An interesting phenomenon arises whenever the sample has been deoxygenated more thoroughly than the mobile phase. When the injected sample fraction passes the detector electrode, the reduction current decreases due to the lower oxygen concentration in the sample, leading to a negative peak (marked by an asterisk in Figure 3) with the same retention time as the chromatographic oxygen peak. This phenomenon can be completely avoided, however, if the very effective deoxygenation procedure described in the Experimental Section is applied to the mobile phase as well as to the sample. When it could be concluded that the system met all basic requirements of deoxygenation, the chromatographic and electrochemical detection conditions of the novel oxygen-sensing assay could be optimized with respect to the determination of oxygen in a sample, in order to obtain maximal selectivity and sensitivity.

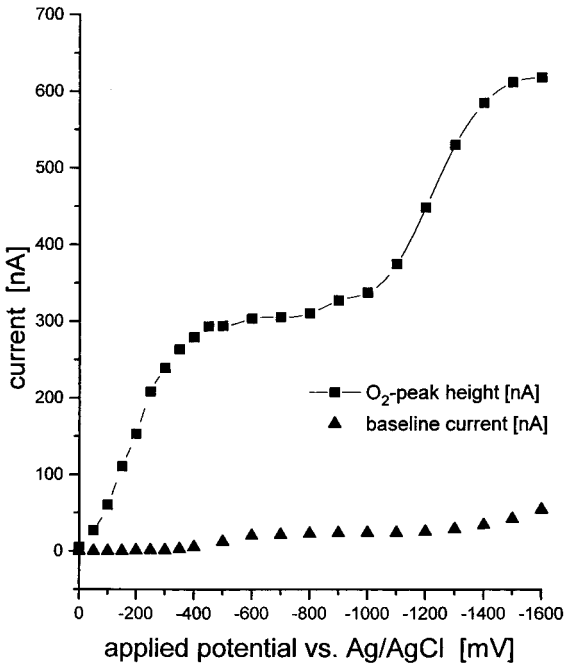

Figure 1. Influence of the detector potential on background current and oxygen peak height: column RP-4 60 × 4 mm i.d.; mobile phase, phosphate buffer (pH 7, I = 0.01 mol L^{-1})/methanol (7:3, v/v); flow rate, 0.80 mL min^{-1}; injection of 10 μL of air-saturated water; oxygen concentration, 0.30 × 10^{-3} mol L^{-1}.

Table 1. Retention Times of Oxygen at Different Chromatographic Conditions

column	mobile phase[a]	O_2 retention times (min)
precolumn PS-DVB (10 × 4 mm i.d.)	phosphate buffer pH 2.5/MeOH (4:6, v/v)	0.2[b]
Grom Eurospher RP-4ec (60 × 2 mm i.d.)	phosphate buffer pH 7/MeOH (7:3, v/v)	0.5
silica-100 (60 × 4 mm, i.d.) including precolumn	phosphate buffer pH 7	1.2
Eurospher RP-4ec (60 × 4 mm i.d.) including precolumn	phosphate buffer pH 7/MeOH (7:3, v/v)	1.2
Eurospher RP-8ec (60 × 4 mm i.d.) including precolumn	phosphate buffer pH 7/MeOH (7:3, v/v)	1.9
PS-DVB RP-18 (nonporous) (100 × 4 mm i.d.)	phosphate buffer pH 2.5	8
Eurospher RP-8ec (250 × 4 mm i.d.) including precolumn	phosphate buffer pH 2.5	10

[a] Flow rate, 0.8 mL/min; electrode, hanging mercury drop electrode; detection potential, −650 mV. [b] Coelution with injection peak.

Optimization of the experimental conditions of electrochemical detection comprised choice of working electrode material and applied potential. Mercury proved to be the most suitable material for detection at negative potentials in aqueous solutions due to its high overvoltage for hydrogen ion reduction and easily renewable surface. Contamination and poisoning of the electrode surface can be avoided by using a hanging mercury drop electrode and by applying a fresh mercury drop for each injection. Variation of the drop size revealed that medium and large mercury drops in comparison to small drops exhibit higher background currents and a higher tendency to vibrations, particularly at higher flow rates, because the detector cell design corresponds to the "wall-jet" principle.[17] This results in higher baseline noise. A small drop size (electrode area, 0.0101 cm^2), however, gave very good results as to signal-to-noise ratio and reproducibility of the current response. Thus, only small mercury drops were used for all further investigations.

Figure 1 shows the influence of the applied potential on the detector response. In the hydrodynamic voltammogram, it can be clearly seen that oxygen is reduced via two two-electron steps. Though the highest and thus most sensitive analytical signal would be expected at ∼−1.6 V, in the potential region of a four-electron transfer, the best results concerning both sensitivity and signal-to-noise ratio were obtained at potentials between −600 and −800 mV. At those potentials, a comparatively rapid stabilization of the baseline (after about 10 s) was observed after mercury drop dislodge, whereas at higher and at lower potentials stabilization of the baseline took much longer (between ∼30 and 60 s). Moreover, use of a lower detection potential provides higher selectivity of the oxygen detection, because the probability that other reducible compounds in the sample will interfere with oxygen detection decreases. Therefore, most future measurements were performed at a working electrode potential of −650 mV.

Unlike the usual chromatographic separations, in this case the chromatographic conditions (column and mobile phase) had to fulfill only two requirements: to retain matrix elements without too much retarding oxygen in order to prevent tailing of the chromatographic oxygen peak and to avoid time-consuming measurements. This is especially important if changes in oxygen concentration are studied during a long-term experiment. On the other hand, sufficient separation of the oxygen peak from the injection peak is essential for exact evaluation of the analytical signal. Pure phosphate buffer solutions or a mixtures of phosphate buffer pH 7 and methanol were tried as mobile phase. It took less time for the detector's response to come back to the baseline after mercury drop dislodge, and the stability of the baseline was higher, if a small amount of methanol was added to the mobile phase. The best results were obtained with a mobile phase consisting of buffer and methanol in a volume ratio of 7:3. A higher content of methanol (>30%, v/v) should be avoided in order to prevent coelution of matrix elements with oxygen. The flow rate of the mobile phase was varied between 500 and 2000 $\mu L\ min^{-1}$. In general, the detector signal increased with increasing flow rate, while the baseline noise remained almost constant up to a flow rate of 1500 $\mu L\ min^{-1}$, where a sudden increase of the baseline noise was observed. The best signal-to-noise ratio was obtained at flow rates between 800 and 1000 $\mu L\ min^{-1}$; therefore, these flow rates were chosen for all further measurements.

Several reversed-phase columns were tested with respect to the requirements mentioned above. The results are summarized in Table 1. In general, retention times for oxygen increased with increasing alkylation of the stationary phase and with decrease of the methanol content of the mobile phase. Retention times between 0.5 (Grom Eurospher RP-4ec, 60 × 2 mm i.d., without precolumn) in phosphate buffer (pH 7)/methanol (7:3, v/v) and 10 min (Eurospher RP-8ec, 250 × 4 mm i.d., including precolumn) in pure phosphate buffer (pH 7) were observed. The best results as to relatively short retention time and sufficient separation of the oxygen reduction peak from the injection signal were obtained with an Eurospher RP-4ec column (60 × 4 mm i.d., including precolumn) with a retention time of 1.2 min for oxygen. Figure 2 shows a typical chromatogram for oxygen determination by LC−EC.

It had to be determined whether the selected column and the new elaborated assay were selective enough to permit oxygen determination in complex biological matrixes. Interference of peptides, proteins and cell particles in the oxygen determination was investigated by measurements of a trypsin solution (1.0% in 0.1% EDTA; Flav Laboratories) and a liver cell homogenate, since both solutions consist of a very complex spectrum of matrix elements. For these selectivity studies a RP-8ec column and a mixture of phosphate buffer (pH 7) and methanol (7:3, v/v) was selected as mobile phase. The results are demonstrated in Figure 3. The chromatograms were recorded at very sensitive gains of the electrochemical detector in order to make visible any chromatographic peaks resulting from matrix elements interfering with oxygen. At this receiver gain, the top of the chromatographic oxygen peak (corresponding to an oxygen concentration of $\sim 3.0 \times 10^{-4}$ mol L^{-1}) is cut off. Under optimized conditions, deoxygenated trypsin solution and liver filtrate show the same chromatogram as pure, deoxygenated buffer solution and no peaks occur within the retention time region of oxygen at ∼2 min. All matrix elements are retained almost irreversibly on the chromatographic column. Thus, it can be concluded that under the experimental conditions applied the new assay is sufficiently

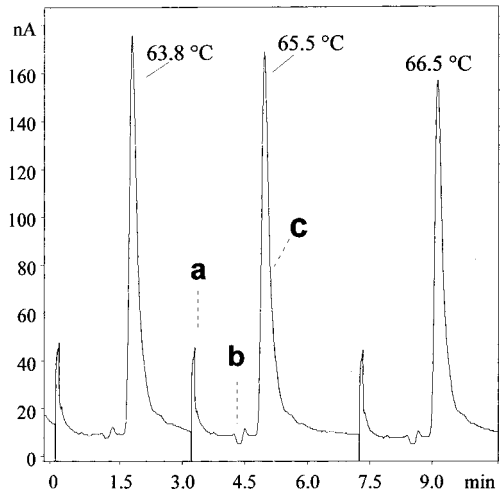

Figure 2. Typical chromatogram for the determination of oxygen by LC−EC: column, RP-4 60 × 4 mm i.d.; mobile phase, phosphate buffer (pH 7, I = 0.01 mol L^{-1})/methanol (7:3, v/v); flow rate, 0.80 mL min^{-1}; detector potential, −650 mV; injection of 10 μL of air-saturated water at different temperatures. (a) Stabilization of the baseline current after drop dislodge; (b) injection peak; (c) peak corresponding to the reduction of oxygen.

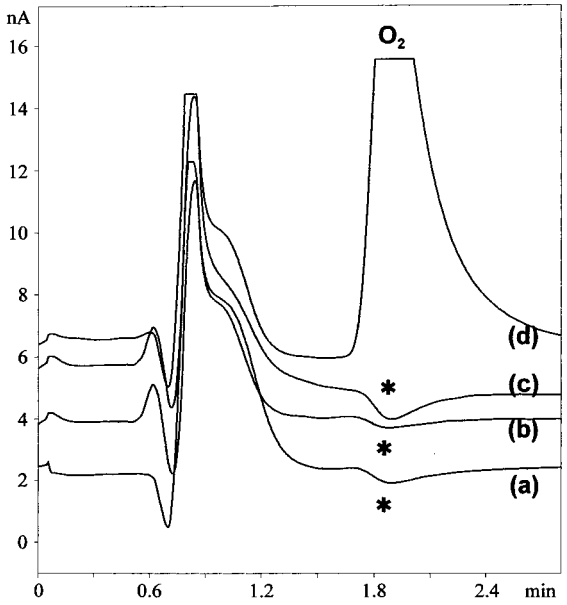

Figure 3. Selectivity studies. Injection of 10 μL of (a) pure, deoxygenated buffer solution, (b) deoxygenated trypsin solution, (c) deoxygenated liver cell homogenate, and (d) air-saturated water: oxygen concentration, 2.4 × 10^{-4} mol L^{-1}; column, RP-8 60 × 4 mm i.d.; mobile phase, phosphate buffer (pH 7)/methanol (7:3, v/v); flow rate, 0.80 mL min^{-1}; detector potential, −650 mV.

selective for oxygen determination even in complex biological media. The use of a precolumn, however, is highly recommended for measurements in complex samples to protect the analytical column against contamination. Precolumns permit at least 150 repeated measurements of complex samples before they have to be regenerated.

Furthermore, it had to be determined whether the sensor produced a reproducible and constant signal when the oxygen tension of the sample, pO_2 (partial pressure of oxygen at known temperature and barometric pressure), remained unaltered and whether it responded linearly to oxygen tension changes. Air, because of its constant composition, analyzed gas mixtures of known oxygen partial pressure, and 100% oxygen can be used for linearity tests of the sensor output at different partial pressures.[3,10,11] In general, Clark-type oxygen electrodes are calibrated at two concentrations, with 100% air-saturated aqueous solutions and at zero oxygen concentration.

Since amperometric oxygen sensors and the new oxygen-sensing LC−EC assay are based on a very similar detection principle, a linear relationship between reduction peak current and oxygen concentration in the sample was expected. However, the range of the linear response and the existence of a zero intercept had to be verified before a simple two-point calibration could be performed. In our experiments, we used the temperature dependence of the solubility of gases in liquids for the preparation of solutions with well-defined oxygen concentrations.

In many handbooks of physics and chemistry, solubility constants for oxygen in water can be found with varying degrees of accuracy.[3,10,11,18−20] It is possible to calculate the oxygen content of a solution (mg L^{-1}) with accuracy by use of the Bunsen coefficient (β'),[10,11,18] which is defined as the volume of gas (reduced to standard temperature and pressure: T = 0 °C, p = 1 atm = 1.013 25 bar = 101.325 kPa = 760 Torr) dissolved in a unit volume of solvent at standard partial pressure of the gas (p_{gas} = 1 atm = 1.013 25 bar = 760 Torr) at the specific temperature of the measurement. When the partial pressure of the gas above the solvent differs from the standard partial pressure, it is corrected to this pressure by Henry's law. The oxygen content in dry air is 20.94% (v/v), and if dry air is used for calibration, the partial pressure of oxygen, pO_2, is given by the formula pO_2 = 0.2094p_o [p_o is the barometric air pressure (mbar)]. The solubility of oxygen in water at a given temperature and pressure can be calculated according to the following equation:

$$\text{Sol } O_2 \text{ (mg L}^{-1}) = \frac{\beta' M_{O_2}}{V_{molar}} pO_2 = \beta' \times 1.43\, pO_2 \qquad (1)$$

where M_{O_2} = molecular mass of oxygen = 31.9988 g mol^{-1} and V_{molar} = molar volume = 22.413 83 L mol^{-1} at 0 °C and 1 atm.

If the solution is in equilibrium with an atmosphere of standard composition and saturated with water vapor at a total pressure, p_o, the partial pressure of water vapor, pH_2O, has to be taken into account when the solubility of oxygen in water is calculated. The dependence of the water vapor pressure, pH_2O, on temperature can be expressed by eq 2[11] and the partial pressure of oxygen has to be corrected according to eq 3:[10,11,18]

$$p_{H_2O} \text{ (mbar)} = 0.01 \times 10^{(10.09 - 1668/(228 + T\,(°C)))} \qquad (2)$$

where 0.01 is the conversion factor from Pa to mbar.

$$pO_2 \text{ (mbar)} = 0.2094(p_o - pH_2O) \qquad (3)$$

where p_o is the barometric air pressure.

Since the semiempirical equations describing the temperature dependence of β' have been published[18] only for relative short temperature ranges (see Experimental Section), we derived a

(19) Rump, H. H.; Krist, H. *Laborhandbuch für die Untersuchung von Wasser, Abwasser und Boden*, 2nd ed.; VCH: Weinheim, Germany, 1992; p 81.
(20) Nelson, G. O. *Gas Mixtures, Preparation and Control*; Lewis Publishers: Boca Raton, FL, 1993.

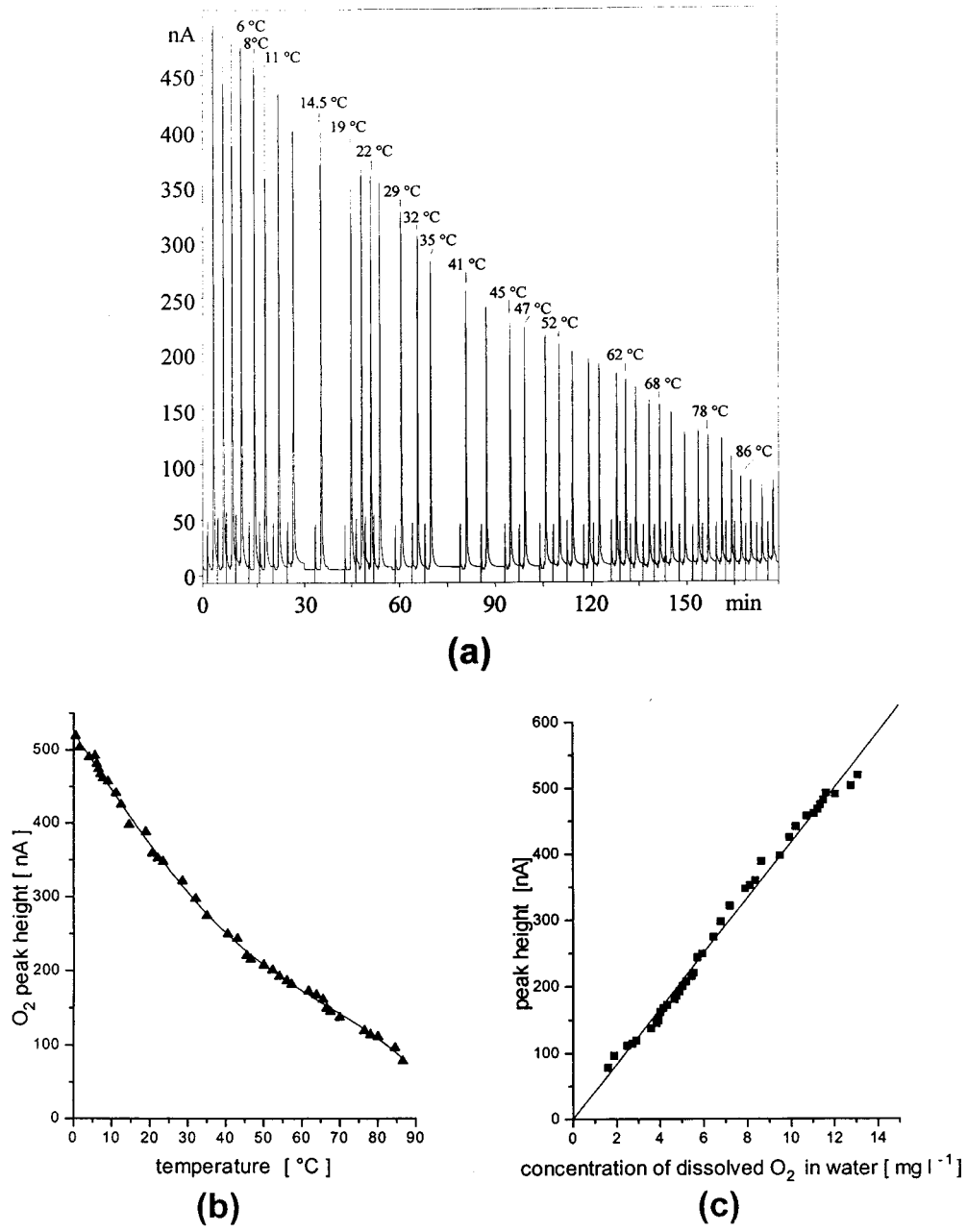

Figure 4. Demonstration of a calibration experiment: measurement of the solubility of oxygen in water at different temperatures. (a) Chromatograms; column, Nucleosil RP-4 60 × 4 mm i.d.; mobile phase, phosphate buffer (pH 7)/methanol (7:3, v/v); flow rate, 0.80 mL min^{-1}; detector potential, −650 mV; injection of 10 μL of air-saturated water. (b) Plot of the peak currents in (a) vs water temperature (c) Corresponding calibration curve ($y = a + bx$), i (nA) = −0.089 + 41.6C (mg L^{-1}) (s_a = 4.81; s_b = 0.623; $s_{y/x}$ = 13.01; r = 0.9968; n = 39).

mathematical function (eq 4) that allows the calculation of solubility values in a larger temperature range with sufficient accuracy.

$$\text{Sol } O_2 \text{ (mg } L^{-1}) = 0.2094(p_o - p_{H_2O}) \times 1.43(0.04873 - 0.001272T + 2.321 \times 10^{-5}T^2 - 2.202 \times 10^{-7}T^3 + 8.514 \times 10^{-10}T^4) \quad (4)$$

Solubility data for oxygen in aqueous solutions calculated by eq 4 are in good agreement with values obtained from tables reported in literature.[18−20] Therefore, in all calibration measurements, eq 4 was used to calculate the concentration of oxygen in air-saturated water at a given temperature. In Figure 4, a typical linearity test is demonstrated, including the chromatograms for several temperatures (Figure 4a), and corresponding plots showing the dependence of the reduction peak current on water temperature (Figure 4b) and concentration of dissolved oxygen in air-saturated water (Figure 4c). The calibration curve i (nA) = −0.089 + 41.6C (mg L^{-1}) is linear in the range from 2 to 14 mg L^{-1} O_2 in water with zero intercept and a correlation coefficient of 0.9968 (s_a = 4.81; s_b = 0.623; $s_{y/x}$ = 13.01; n = 39). Calculation of the oxygen concentration in water at a given temperature (according to eq 4) is based on the assumption that equilibrium exists between gas phase and liquid phase. However, since it takes rather long until equilibrium is fully established, most probably some of the measurements at higher temperatures were performed at nonequilibrium conditions. This accounts for the

small deviations of the calibration curve from exact linearity. Of course, calibration with air-saturated solutions at different temperatures is highly inconvenient, and this procedure will not be applied for quantification of oxygen in real samples. It will be shown in part 2 that the use of a special chamber and a novel sample application system in combination with LC−EC permits reliable calibration of the assay even at very low oxygen concentrations by simply injecting an aliquot of an oxygen standard solution. These experiments principally only served the purpose of demonstrating the linear relationship between reduction peak current and oxygen concentration. Precision was determined by repeated measurements ($n = 6$) of air-saturated water at room temperature (between 18 and 22 °C, corresponding to an oxygen concentration of $\sim 2.5 \times 10^{-4}$ mg L^{-1}). Reproducible signals were obtained with a relative standard deviation of lower than 0.2%.

CONCLUSIONS

Liquid chromatography with electrochemical detection provides a very versatile, selective, and sensitive tool for the detection of oxygen with relatively inexpensive and simple instrumentation, which exhibits several advantages compared to conventional oxygen sensors. The detector responds directly and quickly to changes in oxygen concentrations in the sample. Due to the short retention time of oxygen, repeated measurements are possible every 2−3 min, also permitting monitoring of dynamic oxygen release or consumption processes in a sample. A high selectivity even in complex samples can be achieved by deliberate choice of the chromatographic conditions. In contrast to other oxygen measurement systems, the analytical response of LC−EC detection is not affected by temperature fluctuations of the sample. Therefore there is no need for further calibration when temperature changes occur, as would be the case when a probe (e.g., Clark electrode) in direct contact with the sample solution is used. Moreover, the problem of oxygen consumption by the detector electrode leading to oxygen depletion in the sample during long-term measurements, or contamination of a membrane (as in case of membrane-covered oxygen sensors), is avoided.

In principle, the novel chromatographic oxygen-sensing assay permits reliable and reproducible oxygen readings from saturation down to trace level oxygen concentrations with an extreme sensitivity. Although only oxygen concentrations between 2 and 14 mg L^{-1} were actually determined in this study, a concentration of 4.5×10^{-9} mol L^{-1} O_2 is the lowest oxygen content that can be distinguished from zero. This value can be estimated from the sensitivity, b, of 1.33 mA L mol^{-1} obtained in the calibration plot (Figure 4c) and the peak-to-peak variation ($N_{p\div p}$), "noise", in the baseline signal, which amounts to 2 pA at a baseline current of 5 nA (corresponding to LOD $\sim 3N_{p\div p}/b$).[21] However, in order to obtain reliable measurements of trace level oxygen concentrations, it is absolutely necessary that the influence of atmospheric oxygen as well as oxygen release or consumption by materials constituting the measuring system is excluded. For this purpose, we developed a high-quality sample chamber together with a completely novel sample application procedure ("two-chamber-siphon technique") which permits reliable quantification of very low oxygen concentrations with the novel chromatographic oxygen-sensing assay. Special features of the new test chamber and sample application system will be presented in part 2.

Received for review April 4, 1997. Accepted July 31, 1997.[⊗]

AC970363Q

(21) Riley, C. M., Rosanske, T. W. Eds.; *Development and Validation of Analytical Methods*; Pergamon: Tarrytown, NY, 1996; p 56 ff.

[⊗] Abstract published in *Advance ACS Abstracts,* September 15, 1997.