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Accurate Measurement of Near-Micromolar Oxygen Concentrations in Aqueous Solutions Based on Enzymatic Extradiol Cleavage of 4-Chlorocatechol: Applications to Improved Low-Oxygen Experimental Systems and Quantitative Assessment of Back Diffusion of Oxygen from the Atmosphere[1]

Hirofumi Nakajima, Tetsuo Ishida,[2] Hiroyuki Tanaka, and Kihachiro Horiike

Department of Biochemistry, Shiga University of Medical Science, Seta, Ohtsu, Shiga 520-2192

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An enzymatic method for measuring the O_2 concentrations of aqueous solutions was developed by involving 4-chlorocatechol and catechol 2,3-dioxygenase from *Pseudomonas putida*. With this system, the amount of O_2 in a sample solution can be measured as the amount of 5-chloro-2-hydroxymuconate semialdehyde formed through the enzyme reaction. The product was stable and its anion exhibited strong absorption around 380 nm (molar absorption coefficient of 4.3×10^4 M^{-1} cm^{-1}, p*K* value of 5.4). A sensitive HPLC method involving a BioAssist Q column was developed to individually quantify the products derived from 4-chlorocatechol and catechol. When the O_2 concentration in a sample solution sealed in a vial was lowered from the air-saturation level by means of the amount enzymatically reacted with a known amount of catechol, the concentration of remaining O_2 could be successfully measured by the HPLC method. We developed devices through which reagents could be added to solutions sealed in cuvettes or the vessel of an oxygen electrode system under a flow of argon. By applying these devices, the submicromolar O_2 concentration of an anoxic solution and the back diffusion of O_2 from the atmosphere could be directly determined for the first time. The K_m values of the dioxygenase and an ascorbate oxidase for oxygen were also determined to be 7.2 (at pH 7.5) and 114 μM (at pH 6.5), respectively, at 25°C.

Key words: anaerobic reaction, catechol 2,3-dioxygenase, HPLC analysis, L-ascorbate oxidase, oxygen concentration.

Oxygen is a common substrate for many enzymes that support diverse forms of cellular metabolism. The biological functions of these enzymes (oxidases and oxygenases) are directly affected by a decrease in the intracellular O_2 concentration, depending on their K_m values for oxygen. In mammalian tissues, the cellular O_2 concentration is estimated to be 10–40 μM under normal oxygen tension (for reviews, see Refs. *1* and *2*). In hypoxic cells, the O_2 concentration decreases to near-micromolar levels (0.1–5 μM) and mitochondrial respiration becomes oxygen-limited (*3–5*, and for a review, see Ref. *6*). On the other hand, aerotolerant bacteria play very important roles in natural processes under low-oxygen conditions (*7, 8*). These bacteria have developed mechanisms to sense near-micromolar levels of O_2 and to handle the aberrant production of reactive oxygen species. Understanding the mechanisms by which cells sense oxygen and respond to changes in the intracellular O_2 level is, therefore, a central goal of current biology. Many O_2 sensor enzymes from various sources have recently been identified (*9–16*).

For further understanding of the molecular mechanisms of cellular systems for sensing O_2, it is important to determine the kinetic properties of relevant enzymes as to oxygen. For this purpose, we need a simple yet rigorous method for preparing reaction mixtures containing known amounts of O_2, and one for directly measuring the O2 concentrations of sample solutions. Many methods have been developed for experiments in which the oxygen concentration is the major variable (*17–26*). Most of these methods require calibration for relating the signal with the O_2 concentration, and for calibration, stoichiometric reactions of organic substrates with O_2 catalyzed by oxidases or oxygenases are often used. These enzymes are sometimes used to directly detect O_2 by measuring the products enzymatically formed. However, it is still difficult to accurately measure O_2 concentrations of 0.01–5 μM. It is equally difficult to prevent the back diffusion of oxygen from the atmosphere into the low-oxygen solution in a sealed vessel upon the addition of reagents and/or withdrawal of the sample.

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[2] To whom correspondence should be addressed. Fax: +81-77-548-2157, Phone +81-77-548-2158, E-mail teishida@belle.shiga-med.ac.jp

Abbreviations. AO, L-ascorbate oxidase, CHMSA, 5-chloro-2-hydroxymuconate semialdehyde, *I*, ionic strength; MPC, catechol 2,3-dioxygenase from *Pseudomonas putida* mt-2 (metapyrocatechase); TCA, trichloroacetic acid

Catechol dioxygenases catalyze the fission of the catecholic ring of one substrate molecule with insertion of both atoms of one dioxygen molecule (for a review, see Ref. *27*). The reactions are irreversible and proceed completely in the direction of the ring opening of catecholic substrates. No reactive oxygen species are released from the active sites of the enzymes during the reaction. Various catechol derivatives are commercially available in a pure form. They are easy to dissolve in water and only very slowly auto-oxidize in air-saturated solutions, except at extremely high pH values. Catechol dioxygenases and their substrates are therefore best suited for oxygen assaying

There are two types of catechol dioxygenases (*27*). Intradiol dioxygenases utilize non-heme ferric iron, show broad absorption in the visible region, and cleave the benzene ring between the hydroxyl groups to give products which show relatively strong absorption in the UV region. Extradiol dioxygenases utilize non-heme ferrous iron, show no absorption in the visible region, and cleave the benzene ring adjacent to the hydroxyl groups to give products which show very strong absorption in the visible region. Extradiol dioxygenases are better suited for oxygen assaying than intradiol dioxygenases, because extradiol-cleaved products exhibit stronger absorption in the visible region than intradiol-cleaved ones, and thus the former are more easily and sensitively detectable than the latter. However, extradiol dioxygenases have not been utilized for direct measurement of the oxygen concentration of a sample solution, mainly because they are rapidly inactivated in air-saturated buffer due to the auto-oxidation of the ferrous iron at the active site.

Here we developed a sensitive method to determine the O_2 concentrations of aqueous solutions using recombinant *Pseudomonas putida* catechol 2,3-dioxygenase (an archetypical extradiol dioxygenase) and 4-chlorocatechol, as the substrate (*28, 29*). The enzymatic reaction is shown in Fig. 1. The product, 5-chloro-2-hydroxymuconate semialdehyde (CHMSA), was stable and exhibited very strong absorption around 380 nm. A HPLC method involving a strong anion exchanger was developed to quantify nanomolar levels of CHMSA in reaction mixtures even with coexisting large amounts of the products derived from catechol. An aluminum-sealed vial with a rubber septum is a vessel that is technically easy to use for low-oxygen experiments, such as that we utilized in our previous work to examine anaerobic porphyrin biosynthesis (*30*) In the present study, we quantitatively assessed the back diffusion of O_2 into a low-oxygen solution sealed in a vial. The O_2 concentration could be successfully controlled at a near-micromolar level when the injection port of a cuvette or the vessel of an oxygen electrode system was continuously purged with argon. A method for calibration of a Clark oxygen electrode in the 2–20 μM region is also described.

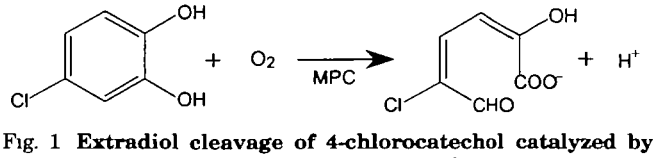

Fig. 1 **Extradiol cleavage of 4-chlorocatechol catalyzed by catechol 2,3-dioxygenase.** In the presence of an excess amount of 4-chlorocatechol, the product, 5-chloro-2-hydroxymuconate semialdehyde, is enzymatically formed in equimolar amounts as to dioxygen. Catechol 2,3-dioxygenase from *Pseudomonas putida* mt-2 (metapyrocatechase, MPC) was used in this study

MATERIALS AND METHODS

Materials—Catechol, 4-chlorocatechol, 3-methylcatechol, 4-methylcatechol, and 3-methoxycatechol were purchased from Tokyo Kasei. FAD and L-ascorbic acid were obtained from Nacalai Tesque L-Ascorbate oxidase (AO, EC 1.10.3.3) from *Cucurbita* sp was purchased from Wako Pure Chemical Industries. All other chemicals were of analytical grade.

Recombinant catechol 2,3-dioxygenase from *Pseudomonas putida* mt-2 (metapyrocatechase, MPC, EC 1.13.11 2) was expressed in *Escherichia coli*, purified to homogeneity, and stored in a crystalline form in the presence of 10% acetone as described previously (*28*). MPC stock solutions were prepared by dissolving the crystalline MPC preparation (specific activity ≥ 300 U/mg) in a minimum amount of 50 mM HEPES (pH 7.5). The enzyme concentrations of the stock solutions were always higher than 40 mg/ml, and after preparation their specific activities remained over 100 U/mg for several weeks. When further dilution of the stock solutions was necessary, it was done just before use to minimize enzyme inactivation (*28*). One unit of MPC activity is defined as the amount of the enzyme that produces 1 μmol of 2-hydroxymuconate semialdehyde per min under the following standard assay conditions; 200 μM catechol in air-saturated 50 mM HEPES (pH 7 5, ionic strength I = 0.15 M) at 25°C. The enzyme concentration was determined spectrophotometrically by using a molar absorption coefficient of 43,830 M^{-1} cm^{-1} at 280 nm and a subunit molecular weight of 35,000.

AO stock solutions were prepared by dissolving the commercial lyophilized powder (2,000 U, 150–300 U/mg) in 1.0 ml of 50 mM sodium phosphate (pH 7.5), and stored at 4°C.

Spectroscopic Characterization of Extradiol-Cleaved Products Derived from Catechol Derivatives—Spectroscopic experiments were performed with a Shimadzu UV-2200 spectrophotometer or a Shimadzu UV-3100 PC spectrophotometer. Substrate solutions (20 μM) were prepared in 20 mM HEPES (pH 7.5, I = 0.15 M). An aliquot (3.0 ml) of each air-saturated solution was placed in a 3-ml cuvette and then 0.5 μl of a 6.4 mg/ml MPC solution was added. Absorption spectra of the products were then repeatedly obtained for 6 h at 25°C.

The absorption spectra of 5-chloro-2-hydroxymuconate semialdehyde (CHMSA) in extreme pH regions were obtained as follows. A CHMSA solution (10.4 μM, 3.0 ml) in 5.0 mM HEPES (pH 7 5) was prepared in a cuvette as described above. The pH of the solution was increased over 10 by the addition of 10 μl of 14 M NaOH, and then decreased below 2 by the addition of 15 μl of concentrated HCl, and finally returned to neutral by the addition of aliquots of 14 M NaOH.

The following buffers were prepared for the respective pH regions: glycine/NaOH (pH 8 5–9.5), citrate/Na_2HPO_4 (pH 3.0–8.0), Tris/HCl (pH 7.5–9.0), HEPES/NaOH (pH 6.5–8.5), and MES/NaOH (pH 5.5–7 0). The concentration of all the buffers was 50 mM, and the ionic strength was always adjusted to 0.15 M with NaCl. A CHMSA stock solution (1.11 mM) was prepared in 5 mM HEPES (pH 7.5, I = 0.15 M). The stock solution (10 μl) was diluted with 1.0 ml of each of the buffers (final CHMSA concentration, 11.0

μM) and then the respective spectrum was measured at 25°C. The pH dependence of the absorption value at 380 nm of CHMSA (A) was analyzed according to the following equation.

$$A = \left(\frac{1}{1 + 10^{pH - pK}}\right)(A_1 + 10^{pH - pK} \cdot A_2) \quad (1)$$

where A_1 and A_2 are the absorbance values at 380 nm of the fully protonated and fully anionic CHMSA, respectively, and K is the dissociation constant of CHMSA.

Chromatographic Analyses of Extradiol-Cleaved Products—Solutions (0.2 ml) containing various amounts of CHMSA and/or 2-hydroxymuconate semialdehyde were enzymatically prepared. An aliquot (10 μl) of 60% trichloroacetic acid (TCA) was added to each mixture to inactivate MPC instantaneously, and then 10 μl of an FAD solution (25–500 μM, depending on the amount of CHMSA in the sample) was added as an internal standard. After centrifugation at 12,000 ×g for 5 min, an aliquot of the supernatant (5–20 μl) was injected onto a Bioassist Q column (Tosoh; 4.6 × 50 mm) preequilibrated with 0.2 M Tris-HCl (pH 8.3) containing 0.1 M NaCl. The column was developed with a linear increase in the NaCl concentration, from 0.1 to 0.46 M, in 6 min in the Tris buffer. The flow rate was 0.9 ml/min. The absorbance at 380 nm was continuously monitored. The column was reactivated by washing with the buffer containing 1.0 M NaCl for 2 min before the next run. A Shimadzu LC-10AS pump and a Shimadzu SPD-M10Avp diode array detector were used for HPLC analyses.

Reaction mixtures (2.7 ml) containing various amounts of catechol (0–288 μM) were prepared in air-saturated 50 mM HEPES (pH 7.5, I = 0.15 M). Each of the mixtures was added to a 3.0-ml vial (Maruemu), and the vial was sealed with a rubber septum and an aluminum cap by means of a clamp. A head space of 0.5 ml was left in each vial. MPC (2.0 μl, 100 μg) was added to each solution with a gas-tight syringe through the septum and the vial was inverted several times for mixing. After 5.0-min incubation at room temperature, an aliquot (75 μl) of 4-chlorocatechol (12.6 mM in H_2O) saturated with argon was added to each sample (final concentration, 340 μM). The vials were inverted several times and further incubated for 5 min. The MPC in each vial was inactivated by the addition of 250 μl of 30% TCA (air-saturated). The vials were then opened and an aliquot (0.2 ml) of each reaction mixture was transferred to an Eppendorf tube. FAD (10 μl, 0.5 mM) was added to each tube as an internal standard. After centrifugation, the supernatants (5.0 μl each) were subjected to HPLC analyses as described above.

Spectroscopic Determination of Absolute Amounts of Dioxygen—A reaction mixture containing 4-chlorocatechol (96.5 μM, 3.0 ml) was sealed in a spectrophotometer cell with a Teflon-silicone septum (GL Sciences). A stopper for the cell was made from a silicone plug by cutting with a cork borer, as shown in Fig. 2. The cap of the cell tightly fitted into the space in the stopper. Three stainless steel tubes pierced the stopper. One tube (tube 1 in Fig. 2) allowed the argon gas to exit and was also used as an injection port through which reagents were added with a gas-tight syringe. Tube 2 further pierced the septum of the cell until its end was 2–3 mm above the surface of the reaction mixture, and through this tube argon entered the cell at such a flow rate that the gas stream penetrated about 2 mm below the surface of the solution. The space in the stopper was purged with argon through tube 3, and the flow rate was adjusted so that the total exit flow through the injection port was 0.25 liter/min. Short stainless tube 4 pierced the septum to allow the argon gas to exit from the cell into the space in the stopper. The mixture was stirred at the rate of 150 run/min with a rod-shaped stirring bar and an acrobat stirrer (M&S Instruments Trading) to attain complete mixing. The absorbance at 380 nm was continuously monitored.

Kinetics with an Oxygen Electrode—Figure 3 schematically shows the oxygen electrode system used in the present study. A glass reaction vessel had a side hole through which an oxygen electrode (Yellow Springs Model 5331) was inserted. An O-ring was used to seal the dead volume. A tapered ground-glass stopper was placed in the central hole of the chamber. The stopper had a thin hole in the center through which reagents were added with a gas-tight syringe. A reaction mixture (2.9 ml) completely filled the vessel, and was continuously stirred at a constant rate with a stirring bar. The reaction vessel, the oxygen electrode, and the stirring plate were all put in a box with a minimum volume, and during experiments the box was continuously purged with argon (3 liters/min) through an inlet tube near the bottom. The box had holes through which the tubes for a circulating bath, and the cords of the electrode

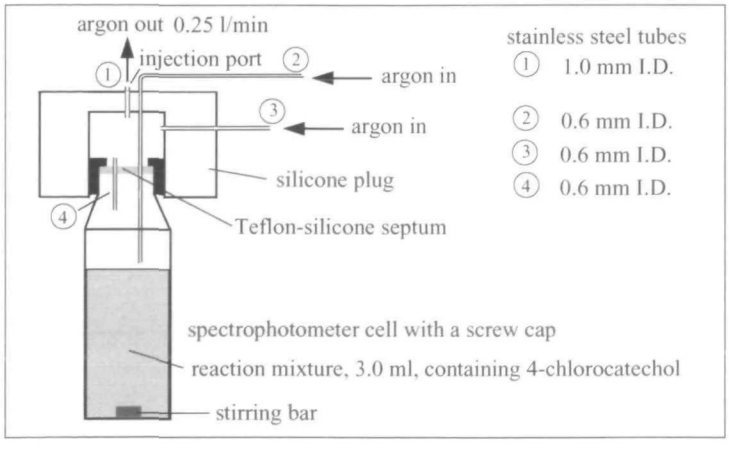

Fig. 2 **A device for purging oxygen from a reaction mixture in a glass cuvette and for preventing the back diffusion of oxygen into the cuvette.** A silicone plug with a space was attached to the cap of a septum-sealed cuvette. The space was continuously purged with argon through tube 3. To purge oxygen from the reaction mixture, argon was allowed to flow into the cuvette through tube 2, and the effluent gas passed into the space through tube 4. The total flow rate of argon through the injection port (tube 1) was 0.25 liter/min. The reaction mixture was continuously stirred as the rate of 150 run per min.

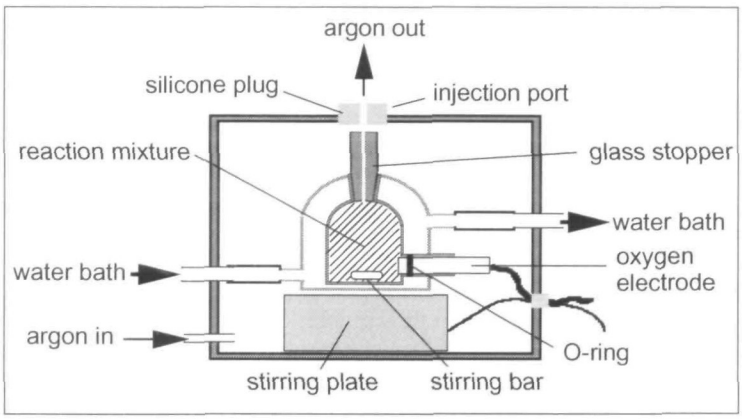

Fig 3 **A schematic diagram of a glass reaction vessel with an oxygen electrode and a box used for kinetic assays with low oxygen concentrations.** A hand-made box, just big enough to accommodate the reaction vessel, an oxygen electrode and a stirring plate, was continuously purged with argon during the experiments. Reagents were injected into the reaction mixture through thin holes in the silicone plug and glass stopper with a gas-tight syringe with the effluent flow of argon through the injection port

and the stirring plate were connected to the corresponding devices on the outside. The dead volumes of the holes were sealed with silicone plugs and grease. The outlet of the box was set just above the end of the stopper of the reaction vessel, and was fitted with a silicone plug with a thin hole. Argon gas exited through the hole in the plug. Reagents were added to the reaction mixture through the holes in the plug and the stopper with a syringe.

The initial velocity of the MPC reaction was measured as a function of the O_2 concentration as follows. A reaction mixture (2.9 ml) containing 6.9 μg/ml AO, 20 μM 4-chlorocatechol, and 50 mM HEPES (pH 7.5, I = 0.15 M) was prepared in the vessel. The oxygen concentration was first reduced to a desirable level by the addition of aliquots of 10 mM L-ascorbate. The electrode output was then calibrated with the addition of 10 μl of L-ascorbate (10 mM). Just after the change in the output had been recorded, MPC (4 0 μl, 0.13 μg) was added to the mixture. The MPC reaction was monitored for 1–2 min, and then the final addition of L-ascorbate was performed to determine the zero [O_2] level of the electrode output. The initial O_2 concentration of the MPC reaction was obtained as the difference between the output at the initial part of the steady state reaction and that at zero [O_2], and by using the result of the calibration performed just before the addition of MPC.

The initial velocity of the AO reaction was measured as a function of the O_2 concentration as follows. A reaction mixture (2.9 ml) containing 4.3 μg/ml MPC, 0.8 mM L-ascorbate, and 50 mM MES (pH 6.5, I = 0.15 M) was prepared in the vessel. The oxygen concentration was first lowered to a desirable level by the addition of aliquots of 10 mM catechol. When the oxygen concentration increased higher than the air-saturation level (about 270 μM), the reaction mixture was flushed with pure oxygen. After calibration of the electrode output with the addition of 10 μl of catechol (10 mM), AO (4.0 μl, 0 13 μg) was added to the mixture. The AO reaction was recorded for 1–2 min, and then the final addition of catechol was performed to determine the zero [O_2] level of the electrode output.

RESULTS

Spectroscopic Properties of Extradiol-Cleaved Products Derived from Catechol Derivatives—MPC can catalyze the extradiol cleavage of many 3- or 4-substituted catechols (*28*). To find the most suitable substrate for O_2 measurement, we first compared the spectroscopic properties of the products derived from various catechol derivatives. The spectra of the products were measured at 25°C and pH 7.5 as a function of time The products from catechol, 4-chlorocatechol, 3-methylcatechol, 4-methylcatechol, and 3-methoxycatechol showed strong absorption with maxima at 375, 380, 387, 381, and 306 nm, respectively. As shown in Fig 4A, only the product from 4-chlorocatechol, 5-chloro-2-hydroxymuconate semialdehyde (CHMSA), did not show any time-dependent change in the absorption spectrum, whereas all the other products showed a slow but significant decrease in the absorption with time.

To examine the stability of CHMSA in a broad pH range, we titrated the product solution in 5 mM HEPES (pH 7.5) with a concentrated HCl solution and a 14 M NaOH solution. The absorption spectrum of CHMSA changed reversibly with an isosbestic point at 353 nm as the pH reached the extreme values of about 1 and 12. The spectra of both the fully protonated and fully anionic forms of CHMSA are shown in Fig. 4B. The effects of pH and buffer species on the absorption of CHMSA were examined in detail (Fig. 4C). The absorption spectrum of CHMSA showed no significant dependence on the buffer species used, whereas 2-hydroxymuconate semialdehyde, the product from catechol, showed weak but significant dependence on the buffer species (data not shown). The pH dependence of CHMSA absorption fitted well to Eq. 1 and the pK value was determined to be 5.39.

To examine the thermal stability of CHMSA, the absorption of CHMSA was measured at 76°C as a function of time for 1 h. The enzymatic conversion of 4-chlorocatechol into CHMSA was completed within a few seconds at 76°C with the addition of the same amount of MPC as used for the experiments performed at lower temperature. The absorption spectrum of CHMSA just after it was enzymatically formed was identical to that of that formed at 25°C. However, the maximum absorption at 380 nm decreased exponentially with a half-life of 123 min at 76°C To overcome the instability of CHMSA at high temperature, we rapidly cooled the reaction mixture to room temperature just after the enzyme reaction had finished. Because auto-oxidation of catechol and its derivatives was significantly rapid at high temperature, we first added a small aliquot of 0.1 M 4-chlorocatechol kept at room temperature to the buffers preincubated at 76°C, and then the reaction was started by the addition of MPC immediately after the addition of 4-chloro-

catechol.

Chromatographic Analyses of Extradiol-Cleaved Products—A mixture containing 1.0 μM CHMSA and 10 μM 2-hydroxymuconate semialdehyde was enzymatically prepared. After the addition of TCA and FAD (final concentration, 1.8 μM), and subsequent centrifugation, an aliquot (20 μl) of the supernatant containing 2.7% TCA was directly applied to a Bioassist Q column, which was preequilibrated with 0.2 M Tris-HCl (pH 8.3) containing 0.1 M NaCl. The column was developed with a linear NaCl concentration

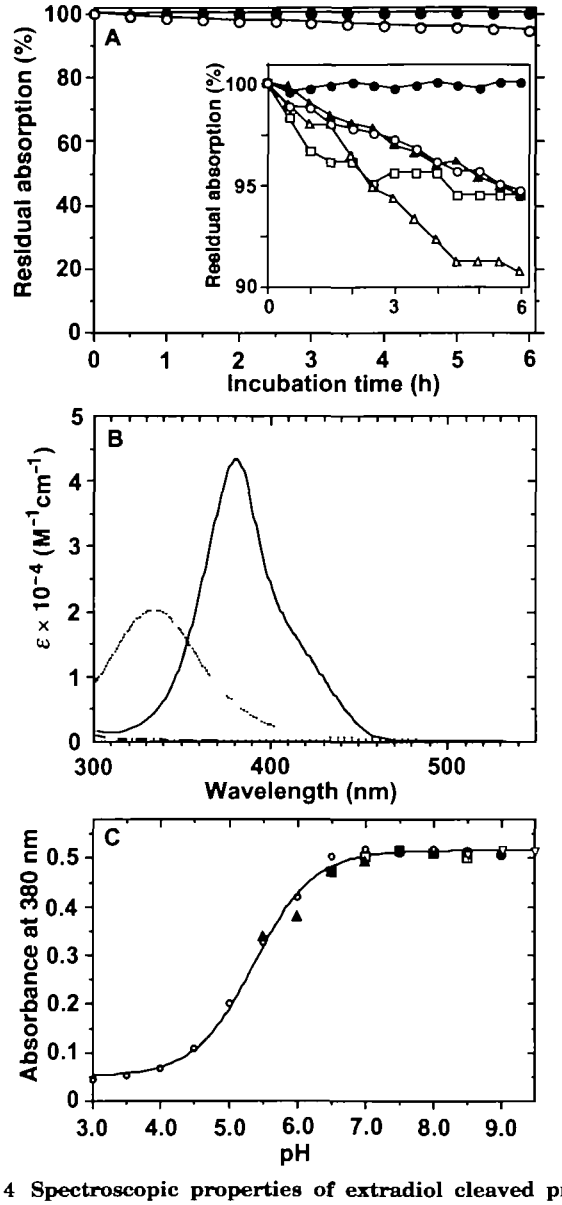

Fig 4 **Spectroscopic properties of extradiol cleaved products.** A. Time-dependent changes in the maximum absorption of the products derived from catechol derivatives (○, catechol, ●, 4-chlorocatechol; △, 3-methylcatechol, ▲, 4-methylcatechol; □, 3-methoxycatechol) in 20 mM HEPES (pH 7 5, I = 0 15 M) at 25°C The residual absorption (% of the initial level) was plotted against the incubation time. The ordinate is enlarged in the inset. B Absorption spectra of the fully anionic (solid line) and fully protonated (dotted line) forms of 5-chloro-2-hydroxymuconate semialdehyde (CHMSA) The absorption spectrum of 4-chlorocatechol is also shown (–·–) C: The effect of pH on the absorption of CHMSA. The absorption at 380 nm of CHMSA (11 μM) was measured at 25°C in various buffers. The buffer concentration and ionic strength were always 50 mM and 0 15 M, respectively The buffers were glycine/NaOH (pH 8.5–9.5, ▽), citrate/Na_2HPO_4 (pH 3.0–8.0, ○), Tris/HCl (pH 7 5–9 0, ●), HEPES/NaOH (pH 6 5–8 5, □), and MES/NaOH (pH 5 5–7 0, ▲) The solid line is the best simulation calculated according to Eq 1 with the following values pK = 5 39, A_1 = 0.050, and A_2 = 0 515

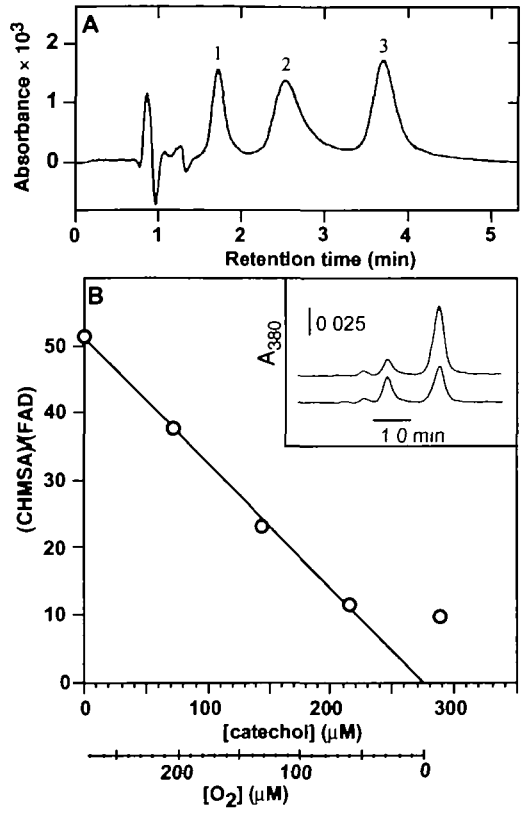

Fig 5 **Chromatographic analyses of extradiol-cleaved products.** A. A mixture (0 2 ml) containing 1 0 μM 5-chloro-2-hydroxymuconate semialdehyde and 10 μM 2-hydroxymuconate semialdehyde was enzymatically prepared in 50 mM HEPES (pH 7 4, I = 0 15 M) After the addition of 60% TCA (10 μl) and 40 μM FAD (10 μl), the mixture was centrifuged at 12,000 ×g for 5 min An aliquot of the supernatant (20 μl) was subjected to anion exchange chromatography on a Bioassist Q column as described under "MATERIALS AND METHODS." Peak 1, FAD, peak 2, 2-hydroxymuconate semialdehyde; peak 3, 5-chloro-2-hydroxymuconate semialdehyde. B· Reaction mixtures containing 0, 72, 144, 216, and 288 μM catechol were prepared in air-saturated 50 mM HEPES (pH 7 5, I = 0 15 M) Each mixture (2.7 ml) was sealed in a 3 0 ml aluminum-sealed vial to prevent access of air In the vials, the O_2 molecules in the reaction mixture were first reacted with the catechol molecules by the addition of MPC The remaining O_2 molecules were then reacted with an excess amount of 4-chlorocatechol. The resultant mixtures were subjected to HPLC analysis as described above. The ratio of the peak area of CHMSA and that of FAD, (CHMSA)/(FAD), was plotted against the catechol concentration. The inset shows typical chromatograms obtained (the upper and lower ones being for mixtures containing 144 and 216 μM catechol, respectively). A linear relation between the peak area ratio, (CHMSA)/(FAD), and the catechol concentration was found in the concentration range of 0–216 μM The fitted line intersects the horizontal axis at 274 8 μM. Supposing that the intersect gives the O_2 concentrations of air-saturated reaction mixtures, we calculated the O_2 concentrations of the catechol solutions after enzymatic O_2 consumption with a known amount of catechol The axis at the bottom shows subtraction of the catechol concentration from the air-saturated O_2 concentration of 274 8 μM

gradient in the same buffer (Fig. 5A). FAD, 2-hydroxymuconate semialdehyde, and CHMSA were eluted in that order, and the three peaks were mutually well separated. The peak area of 2-hydroxymuconate semialdehyde was nearly the same as that of CHMSA. This means that the recovery of 2-hydroxymuconate semialdehyde from the column was about 10-fold less than that of CHMSA, because 2-hydroxymuconate semialdehyde and CHMSA exhibit comparable strong absorption at 380 nm at pH 8 3. Since FAD was eluted as a sharp peak on the chromatography and showed a recovery comparable to that of CHMSA, we used FAD as an internal standard in the following experiments.

We prepared a series of air-saturated solutions (2.7 ml each) containing known amounts of catechol, where the catechol concentration of one solution was nearly the same as the O_2 level at air-saturation ([catechol] $\geq [O_2]_{air\text{-}saturated}$), and those for all other solutions were lower than that ([catechol] $< [O_2]_{air\text{-}saturated}$). We sealed them separately in 3.0 ml aluminum-sealed vials with rubber septa. With the addition of MPC, the oxygen molecules in each solution reacted with the catechol molecules available. If the back diffusion of oxygen into the catechol solutions from the air in the head space of the vials and the outside air is negligible, the O_2 concentration of each solution is enzymatically lowered exactly by the catechol concentration from the air-saturated O_2 level;

$$[O_2]_{remaining} = [O_2]_{air\text{-}saturated} - [catechol] \quad (2)$$

To measure $[O_2]_{remaining}$ (the concentration of the remaining O_2), the remaining oxygen was reacted with an excess amount of 4-chlorocatechol in the vials. After inactivation of MPC by the addition of TCA, the solutions were removed from the vials and subjected to HPLC analysis. The results are shown in Fig 5B Except in the case of the 288 μM catechol solution, there was a linear relation between the CHMSA peak area corrected as to the FAD peak and the catechol concentration. Because the amount of CHMSA formed is equimolar as to the available O_2, the linear relation obtained indicates the following equation:

$$[O_2]_{remaining} = a\ (CHMSA)/(FAD) \quad (3)$$

where (CHMSA) and (FAD) are the peak areas of CHMSA and FAD, respectively, and a is the slope of the fitted line (Fig. 5B). Combining Eqs. 2 and 3, we obtain the following relation:

$$a\ (CHMSA)/(FAD) = [O_2]_{air\text{-}saturated} - [catechol] \quad (4)$$

The intersection between the fitted line (Eq. 4) and the x-axis therefore gave the air-saturated O_2 concentration of 274.8 μM In the case of the 288 μM catechol solution, no oxygen was expected to remain, and therefore no CHMSA to be formed, because the catechol concentration was greater than the air-saturated O_2 concentration. However, a substantial amount of CHMSA (52.6 μM) was detected. This means that with the present experimental procedures atmospheric oxygen can rapidly enter sample solutions when the O_2 concentrations are low. The rate of back diffusion of O_2 from the atmosphere into the solution (2.9 ml) containing 21 μM O_2 sealed in the reaction vessel of the oxygen electrode system was 50 2 nmol/min when we did not use the box (see Fig. 3). This is consistent with the above result.

To assess the sensitivity of the present HPLC method, we prepared a series of solutions containing 0–1 μM CHMSA, which were enzymatically produced in air-saturated buffer containing 0–1 μM 4-chlorocatechol. The CHMSA solutions were analyzed by HPLC as described above. All chromatograms, and the relations between the CHMSA peak area and the CHMSA concentration are shown in Fig. 6. These results indicate that nanomolar O_2 concentration levels can be directly determined with the present HPLC method. It should be emphasized that the most difficult problem on the measurement of low-oxygen concentrations is now how to minimize the effect of O_2 diffusion from the air into samples during the assay procedures

Spectroscopic Determination of the Absolute Amounts of Dioxygen—Low-oxygen experiments require some effective measures to prevent the leakage of ambient oxygen into

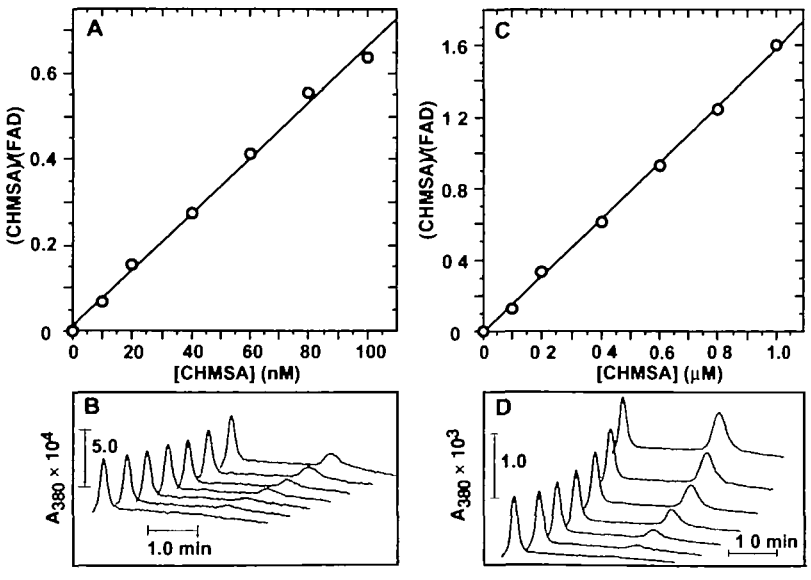

Fig 6. **Chromatographic quantification of submicromolar concentrations of 5-chloro-2-hydroxymuconate semialdehyde in reaction mixtures.** Reaction mixtures (0 2 ml each) containing various amounts of 5-chloro-2-hydroxymuconate semialdehyde (A, 0–0 1 μM; C, 0–1 0 μM) were enzymatically prepared in 50 mM HEPES (pH 7.4, I = 0 15 M) as described under "MATERIALS AND METHODS" After the addition of 60% TCA (10 μl) and 12.5 (A) or 50 (C) μM FAD (10 μl), and subsequent centrifugation, a 20 μl aliquot of each supernatant was applied to a Bioassist Q column, as described under "MATERIALS AND METHODS" The ratio of the peak area of CHMSA and that of FAD, (CHMSA)/(FAD), was plotted against the CHMSA concentration In B and D, the chromatograms obtained are shown in the concentration ranges of 0–0 1 μM and 0–1 0 μM, respectively

reaction solutions, as the experiments carried out in aluminum-sealed vials described in the previous section exemplify. Sealed vials with septa through which we can add reagents and withdraw aliquots of the sample with a gas-tight syringe are convenient to use and suitable for high-throughput experiments. It is therefore important to determine whether or not these vials are useful for near-micromolar oxygen experiments, and to develop a means to reduce the back diffusion of oxygen from the atmosphere.

In the following experiment, we used a cuvette with a hand-made stopper (Fig. 2). A 4-chlorocatechol solution (96.5 μM, 3.0 ml) in the cuvette was gently flushed with argon for 30 min, and then MPC was added to the solution through the septum under an argon flow of 0.25 liter/min. Immediately after the addition of MPC, the absorbance at 380 nm due to CHMSA was continuously monitored (Fig. 7).

Just after 30-min purging with argon and the first addition of MPC, the absorbance at 380 nm of the solution was 0.030. Using a molecular absorption coefficient of 4.33×10^4 M^{-1} cm^{-1} for the CHMSA anion (Fig. 4B), the O_2 concentration was calculated to be 0.70 μM. The absorbance increased linearly at the rate of 0.0088/min. This means that the CHMSA concentration increased at the constant rate of 0.203 μM/min. In other words, oxygen diffused continuously into the solution at the rate of 0.61 nmol/min.

When 25 μl of air-saturated water was added, the absorbance increased by 0.0979 within 10 s, and then it resumed the constant increase of 0.0088/min. An absorbance change of 0.0979 corresponds to a 2.26 μM increase in the CHMSA concentration. This means that 6.84 nmol O_2 molecules were contained in 25 μl of the water air-saturated at room temperature. The O_2 concentration was calculated to be 273.6 μM, showing excellent agreement with the value of 274.8 μM obtained in the chromatographic experiments described above

After the addition of MPC, the O_2 concentration of the solution was maintained at a subnanomolar level by the strong oxygen-scrubbing ability of the 4-chlorocatechol-MPC system. When the same experiment was performed without using the stopper, the CHMSA concentration increased more than 10 μM immediately after 1 0 μl of MPC was added to the solution through the septum of the cuvette (data not shown).

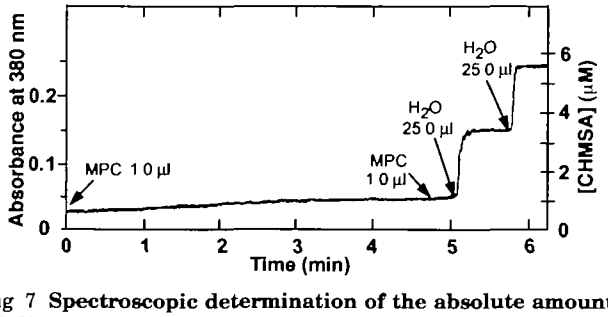

Fig 7 **Spectroscopic determination of the absolute amounts of dioxygen.** A reaction mixture (3 0 ml) containing 96 5 μM 4-chlorocatechol and 50 mM HEPES (pH 7 4, I = 0 15 M) was sealed in a cuvette with the device described in Fig 2 After 30-min purging with argon, MPC (1 0 μl, 50 mg/ml) was added to the mixture with a gas-tight syringe, and then the absorbance at 380 nm was continuously monitored At the times indicated in the figure, MPC (1 0 μl) and aliquots (25 μl) of air-saturated H_2O were further added to the reaction mixture with a gas-tight syringe. Argon was continuously allowed to flow at the rate of 0.25 liter/min throughout the experiment.

Kinetics with an Oxygen Electrode—A Clark-type oxygen electrode was used to examine the kinetic properties of MPC and AO for O_2. When we tried to measure the enzymatic O_2 consumption at O_2 concentrations of 10–50 μM,

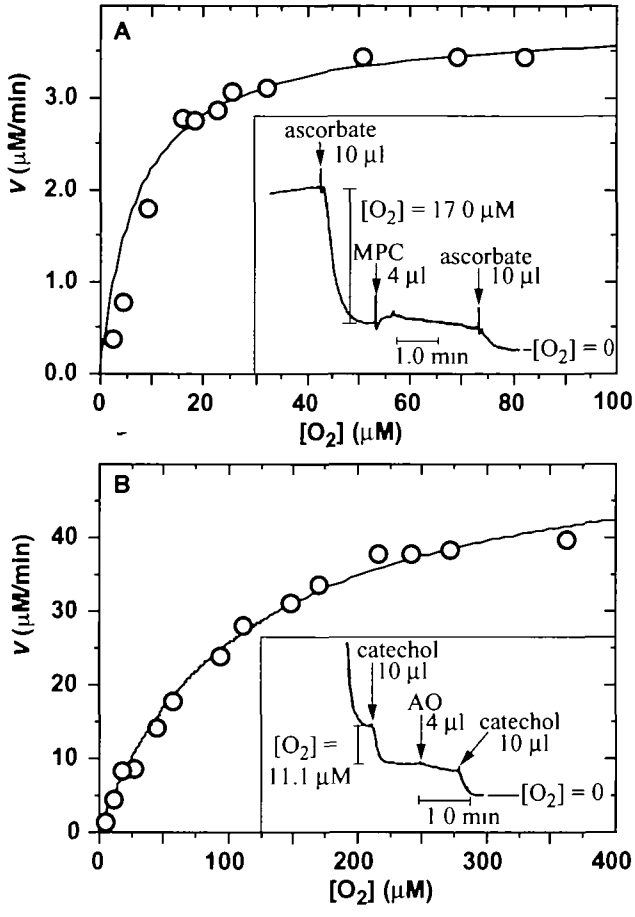

Fig 8 **Calibration of a Clark-type oxygen electrode and control of the oxygen concentration of a reaction mixture.** A: A reaction mixture (2 9 ml) comprising 6 9 μg/ml AO, 20 μM 4-chlorocatechol, and 50 mM HEPES (pH 7 5, I = 0 15 M) was prepared. The oxygen concentration of the reaction mixture was lowered by the addition of aliquots of 10 mM L-ascorbate. After calibration of the electrode output with the addition of 10 μl of L-ascorbate (10 mM), the MPC reaction was started by the addition of the enzyme (4 0 μl, 0 13 μg) The zero $[O_2]$ level of the electrode output was determined by the addition of excess amounts of ascorbate. The inset shows a typical record of the electrode output during one series of the experiments performed as described above. The solid line was drawn with apparent V_{max} and K_m values of 3 81 μM/min and 7 22 μM, respectively, for catechol 2,3-dioxygenase (MPC) B A reaction mixture (2 9 ml) comprising 4 3 μg/ml MPC, 0 8 mM ascorbate, and 50 mM MES (pH 6 5, I = 0 15 M) was prepared Oxygen concentrations of higher than 270 μM were obtained by flushing with pure oxygen. The oxygen concentration was decreased by the addition of aliquots of 10 mM catechol to the reaction mixture. After calibration of the electrode output with the addition of 10 μl of catechol (10 mM), the AO reaction was started by the addition of the oxidase (4 0 μl, 0.13 μg) The zero $[O_2]$ level of the electrode output was determined by the addition of excess amounts of catechol. The solid line was drawn with apparent V_{max} and K_m values of 54 4 μM/min and 114 μM, respectively, for L-ascorbate oxidase (AO)

substantial back diffusion of oxygen into the reaction mixture occurred (at 87 and 21 μM O_2, the diffusion rates were 24.7 and 50.2 nmol/min, respectively), and the drift of the electrode signal made exact determination of the initial velocities difficult. The noise of the electrode output obscured the signal with near-micromolar O_2 concentrations. We therefore used a hand-made box to reduce the back diffusion of oxygen, as described under "MATERIALS AND METHODS" and shown in Fig. 3. In our previous study (*28*), we carried out kinetic experiments with various O_2 concentrations (15–280 μM) established by equilibration with O_2-N_2 mixed gases. To determine K_m for oxygen more accurately, we varied the O_2 concentration over a wide range (2–80 μM) by means of AO-catalyzed reduction of O_2 to water with ascorbate. The oxygen electrode signal was calibrated just before the start of the MPC reaction by the addition of a known amount of ascorbate (usually 100 nmol). The zero O_2 level of the electrode output was determined by the final addition of an excess amount of ascorbate. A typical record of the electrode output is shown in the inset in Fig. 8A. By this method, we successfully obtained initial velocities for O_2 concentrations of 2–80 μM (Fig. 8A). The data fitted well to a hyperbola and the apparent K_m for oxygen with 20 μM 4-chlorocatechol was determined to be 7.22 μM.

The ascorbate-AO system is used to lower the oxygen concentration by precisely controlled amounts (*31*), as we used it for the MPC kinetics. However, the kinetic properties of AO for O_2 have not been examined in detail. To determine the K_m for oxygen of AO, we used the catechol-MPC system to lower the oxygen concentration in a controlled manner and to calibrate the oxygen electrode signal. The initial velocity was measured as a function of the O_2 concentration (4–370 μM) with 0.8 mM ascorbate (Fig. 8B). The inset shows a typical record of the electrode output for one series of experiments. The data fitted well to a hyperbola and the apparent K_m for oxygen was determined to be 114 μM.

DISCUSSION

In the present study, we have developed a method for measuring submicromolar O_2 concentrations in aqueous solutions. This method is based on the MPC-catalyzed extradiol cleavage of 4-chlorocatechol with dioxygen (Fig. 1). In the 4-chlorocatechol/MPC system, the amount of oxygen in a sample solution is measured as the amount of CHMSA formed through the enzyme reaction in the presence of an excess amount of 4-chlorocatechol. This method therefore requires no calibration, and is applicable to any experimental systems in which MPC can be active. CHMSA was found to be extremely stable and its anion exhibits strong absorption around 380 nm. These excellent properties of CHMSA enabled us to measure even 10 nM levels of O_2 for the first time. MPC shows micromolar level K_m values for both 4-chlorocatechol (2.4 μM) and O_2 (7.2 μM), and is active over a wide range of pH values (6–8.5). In addition, if necessary, a large amount of the enzyme can be used without affecting spectroscopic measurement of CHMSA, because MPC exhibits no absorption in the visible region The enzyme reaction therefore is completed within 10 s in a closed system such as an aluminum-sealed vial and a cuvette with a screw cap, and then MPC can be instantaneously inactivated with no effect on CHMSA by the addition of TCA before the transfer of the sample to an open system.

With the HPLC method developed in the present study, an aliquot of the supernatant (up to 20 μl) of a TCA-treated sample can be directly applied to a Bioassist column. On such chromatography, CHMSA and 2-hydroxymuconate semialdehyde eluted as individual peaks, the latter peak being about 10-fold lower than that of CHMSA. This means that in a closed system we can lower the oxygen concentration in a solution by precise equimolar amounts as to catechol, and that we can then measure the remaining O_2 concentration by the addition of an excess amount of 4-chlorocatechol and the following quantification of CHMSA formed. In fact, we could precisely control the O_2 concentration of a solution sealed in a vial in the range from the air-saturated level to about 60 μM (Fig. 5B).

Low oxygen experiments require the maintenance of a near-micromolar O_2 concentration in a sealed vessel, the prevention of O_2 leakage, and the anoxic addition of reagents and/or withdrawal of a sample. As the experiments described in the present study demonstrate, it is technically very difficult to carry out low-oxygen experiments in the open air On the other hand, the use of anaerobic glove boxes hampers easy handling, and does not necessarily prevent O_2 contamination because such boxes have large surfaces and spaces from which oxygen should be scrubbed. Quantitative detection of possible O_2 leakage is therefore helpful for improving experimental procedures for a low-oxygen experiment The 4-chlorocatechol/MPC system combined with the HPLC method for measuring CHMSA can be used for quantitative assessment of low-oxygen experimental systems.

We examined the products derived from five catechol derivatives in terms of stability at pH 7 5 (Fig. 4). Except for CHMSA, all the products examined showed a relatively slow decrease in absorption as a function of the incubation time. It has been reported that β-carboxy-muconic acid, the intradiol-cleaved product from protocatechuate, undergoes isomerization from the *cis,cis* isomer to the *cis,trans* isomer at pH values greater than 9 (*26*). The extradiol cleaved products possibly undergo the same kind of isomerization and the observed decrease in absorption may be due to slow isomerization Further experiments are needed to determine why only CHMSA is stable and the other four products show time-dependent decreases in absorption.

In conclusion, the present 4-chlorocatechol/MPC system is the most accurate and sensitive method for directly measuring the O_2 concentration of a solution. In fact, for the first time, we could accurately measure a submicromolar concentration of oxygen, quantify the slow back diffusion of 0.6 nmol/min into a solution containing a subnanomolar level of O_2, and improve low-oxygen experimental systems. Further studies are underway for application of the 4-chlorocatechol/MPC system to high-throughput experiments and continuous O_2 monitoring.

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