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Biochemical Method for Chlorine Dioxide Determination

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Chlorine dioxide is a disinfectant used worldwide. In this article, a new enzymatic method for the determination of chlorine dioxide has been developed. This rapid spectophotometric assay is able to detect from 0.2 to 4 mg/liter of chlorine dioxide. The method is based on the capacity of horseradish peroxidase to decolorize reactive yellow 17 in the presence of chlorine dioxide. The effects of several compounds on the assay have been determined. Except sodium hypochlorite, no interference was detected with 18 compounds including chlorides, sulfates, carbohydrates, amino acids, proteins, and organics. The biochemical method is faster and easier than the previous volumetric, amperometric, and colorimetric methods which are laborious and time-consuming.

Chlorine dioxide (ClO_2)[2] has been recently recommended as general purpose disinfectant (1–3). Chlorine dioxide is used for the disinfection of drinking water (4, 5), for the effluent treatment of bleaching plants in pulp mills (6, 7), and as a selective oxidizing agent for undesirable industrial compounds (8), and has been proposed as seawater disinfectant for aquaculture purposes (9). Although ClO_2 is recognized as a stable compound, it loses its disinfection properties in drinking water after only 10 min (2, 4). In addition to the susceptibility differences among bacteria species, the loss of disinfection activity is dependent on water quality (mainly on organic matter content) which determines the demand of chlorine (DOC). For this reason, Medema *et al.* (4) have recommended ClO_2 concentrations from 0.5 to 1.0 mg/liter for drinking water. Thus, it is important to monitor ClO_2 concentrations in routine determinations. Existing methods for ClO_2 determination, volumetric, amperometric, and colorimetric, are laborious and lack speed, sensitivity, or specificity (10–12). The titrimetric–spectrophotometric procedure introduced by Wheeler and Lott (13) using several pH indicators appears suitable over a concentration range of 0.05 to 2.5 mg/liter; however, some interferences may arise from other components in the sample and the deviations are greater at large concentrations.

In this article a new and rapid enzymatic method for chlorine dioxide determination is presented.

MATERIALS AND METHODS

Chemicals. Horseradish peroxidase (EC 1.11.1.7, donor: H_2O_2 oxidoreductase) type X, bovine serum albumin, glucose, glycerol, sodium dodecyl sulfate, Tween, amino acids, *N,N*-diethyl-*p*-phenylenediamine sulfate salt (DPD), and phenol were obtained from Sigma Chemicals Inc. (St. Louis, MO). Sodium chlorite, methanol, and ethanol were purchased from Merck (Darmstadt, Germany). Mineral salts were obtained from J. T. Baker (Phillipsburg, NJ). Reactive yellow FGRL (reactive yellow 17, C.I. 18852) and other dyes were obtained from BASF (Germany).

Reagents. The reagents used were reactive yellow solution, 1 mg/ml in water and horseradish peroxidase, 40 μM. The concentration of peroxidase solution was determined by using an extinction coefficient of 91,000 $M^{-1}\,cm^{-1}$ at 403 nm (14). The standard solution of chlorine dioxide was prepared according to Clesceri *et al.* (12) by using a gas-generating and -absorbing system. Chlorine dioxide gas is produced by the reaction of sulfuric acid and sodium chlorite. Chlorine dioxide concentration of the final solution was determined by three methods: (i) Spectrophotometrically by using an extinction coefficient of 1450 $M^{-1}\,cm^{-1}$ at 360 nm (15), (ii) by the titrimetric method with ferrous ammonium sulfate and DPD as indicator (10, 12), and (iii) by the iodometric method, in which ClO_2 releases free iodine from a KI solution acidified with H_2SO_4. The liberated iodine is titrated with a standard solution of sodium thiosul-

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[2] Abbreviations used: ClO_2, chlorine dioxide; DOC, demand of chlorine; DPD, *N,N*-diethyl-*p*-phenylenediamine sulfate salt.

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fate, with starch as indicator. The stock solution of ClO_2 was kept in the dark.

Procedure. Fifty microliters of reactive yellow solution (1 mg/ml) is added to a spectrophotometer cell containing 1 ml of water sample. The sample is dissolved in 60 mM phosphate buffer, pH 6.0, before performing the assay, and protected from light. The reaction is started by adding 20 μl of 40 μM horseradish peroxidase. The mixture is allowed to react for 15 min in the dark and then the mixture absorbance at 410 nm (A_i) is recorded. A control mixture containing peroxidase and reactive yellow in buffer is processed in the same way as the sample and its absorbance is determined (A_0). Then, the decrease of absorbance ($\Delta A_{410} = A_0 - A_i$) is calculated.

The standard curve is obtained by using dilutions of a chlorine dioxide solution, freshly prepared, in a range from 0 to 4 mg/liter in 60 mM phosphate buffer, pH 6.0. The chlorine dioxide concentration of stock solution is determined by both spectrometric and titrimetric methods.

Analytical methods. The DPD method according to Palin (10) was performed by titrating with a ferrous ammonium sulfate solution. Iodometric determination of chlorine dioxide was carried out by adding 0.5 g of KI in 100 ml of acidified sample. After 5 min, the liberated iodine was titrated with 0.001 N $Na_2S_2O_3$ solution with starch as the indicator. Activity of horseradish peroxidase was determined by guaiacol polymerization and monitored as the increase of absorbance at 470 nm (17). The reaction mixture contained 16 mM guaiacol and 1 mM hydrogen peroxide. Interferences to enzymatic method were estimated by comparing the assay results with, and without, different amounts of several compounds in phosphate buffer. Spectrophotometric data were obtained in a Beckman DU-650 spectrophotometer.

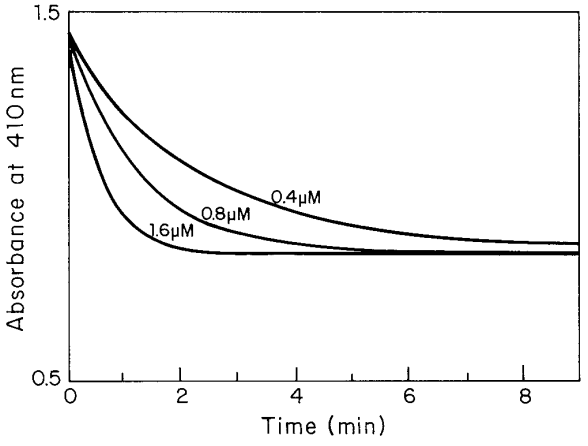

FIG. 1. Reaction kinetics of reactive yellow dye with different amounts of horseradish peroxidase. The reaction mixture contained 50 μg/ml of dye and 2.8 μg/ml chlorine dioxide.

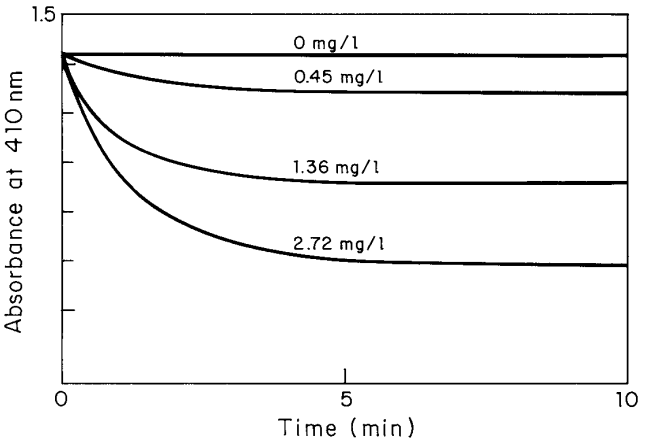

FIG. 2. Reaction kinetics of reactive yellow dye with different concentrations of chlorine dioxide. The reaction mixture contained 50 μg/ml of dye and 0.8 nmol/ml peroxidase.

RESULTS

Horseradish peroxidase is able to modify the reactive yellow dye in the presence of chlorine dioxide. We have assayed 26 other dyes, and reactive yellow 17 was selected because it produces decolorization in a visible-light band and no reaction was detected when peroxidase or chlorine dioxide was added alone.

Reaction rate is dependent on the amount of added peroxidase (Fig. 1) and, under our conditions (0.8 nmol/ml), after 15 min the reaction is completed. The extent of the reaction is independent of the peroxidase concentration and it is only dependent on the chlorine dioxide content (Fig. 2).

Figure 3 shows the pH effect on the assay. A complete reaction (100% response) is obtained from pH 4.5 to 6.5 after a 15-min reaction (Fig. 3a). This response is dependent on the enzyme activity. The pH affects both decolorization rate of reactive yellow with ClO_2 and peroxidase activity measured as guaiacol polymerization with hydrogen peroxide (Figs. 3b and 3c). The different activities showed different optimum pH. Peroxidase activity showed the highest rate at pH 6.0, while the highest decolorization rate was obtained at pH 5.0. However, it is possible to perform the assay at higher or lower pH, by increasing the reaction time, in order to allow for complete oxidation at lower enzyme activity. Chloride dioxide, which is a relatively stable radical in solution, is light sensitive. It reacts with light, at a quantum yield of 2 mol/Einstein, to generate $ClO^{\bullet}$ and $O^{\bullet}$ free radicals. These radicals react via a variety of well-defined pathways to produce Cl_2, ClO_2^-, ClO_3^-, and Cl^- (15, 16). Thus, it is important to maintain the ClO_2

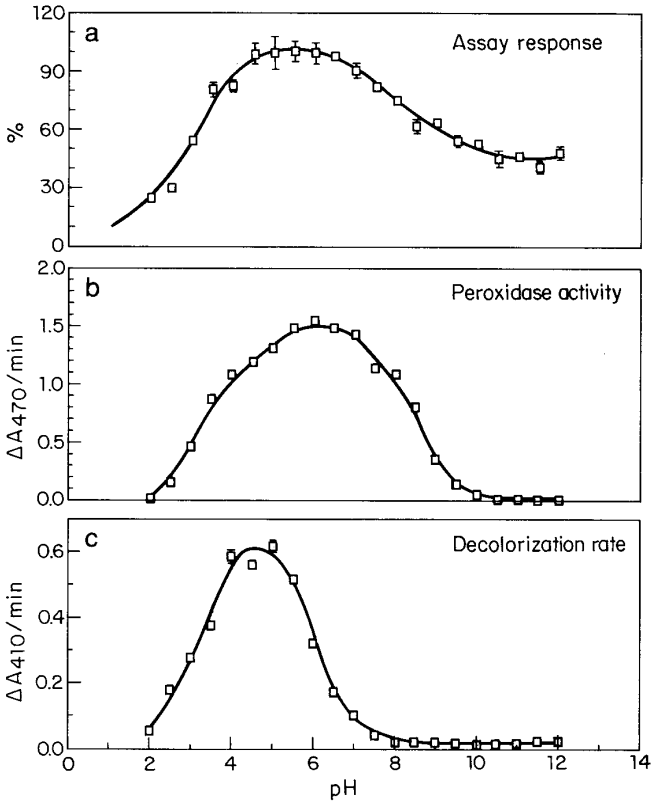

FIG. 3. The effect of pH on (a) chlorine dioxide determination by the biochemical method, (b) decolorization rate of reactive yellow in the presence of horseradish peroxidase and ClO_2, and (c) peroxidase activity as guaiacol oxidation in the presence of hydrogen peroxide. The buffer solutions used were 60 mM sodium acetate for pH 2–5, 60 mM sodium phosphate for pH 5.5–7.5, and 60 mM sodium bicarbonate for pH 8–12.

solutions in complete darkness. We found a constant ClO_2 loss of 3.06 ± 0.29% per minute, from pH 4 to 8 under light at room temperature. Nevertheless, under darkness at 4°C there was no significant ClO_2 loss (less than 1% per day).

A standard curve was obtained by using solutions containing from 0.5 to 4 mg/liter chlorine dioxide, freshly prepared. A perfect linear correlation was found (Fig. 4) by fitting the data on a linear regression with zero intercept:

$$\Delta A_{410} = (0.236 \pm 0.012)[ClO_2].$$

The correlation coefficient was 0.987 ± 0.008 for four replicate curves. ClO_2 concentrations as low as 0.2 mg/liter can be determined. Thus, the assay equation is:

$$\text{Chlorine dioxide (mg/liter)} = (\Delta A_{410})(4.23).$$

The effect of the presence of several compounds on this enzymatic method was determined. Table 1 shows only the values of the highest concentration of each compound, in which small interferences were detected.

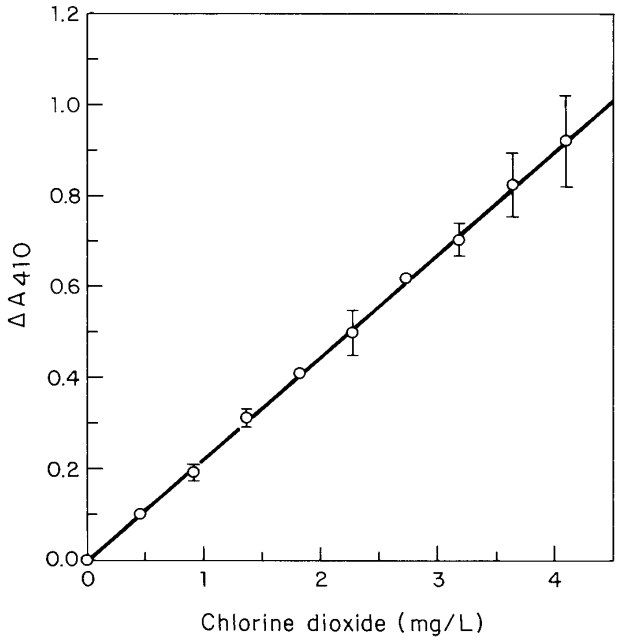

FIG. 4. Calibration curve for chlorine dioxide determination by the biochemical method. The reaction mixture contained 50 μg/ml of reactive yellow 17 and 0.8 nmol/ml of horseradish peroxidase. ΔA_{410} was obtained after a 15-min reaction.

TABLE 1

Interferences on the Chlorine Dioxide Determination by the Enzymatic Method

Compound	Concentration (M)	Method response (%)
None	—	100 (±6)
Acetate	1.0	99 (±7)
Arginine	1.0	132 (±8)
Citrate	1.0	81 (±1)
Ethanol	0.1	90 (±3)
Glucose	1.0	112 (±5)
Glycerol	1.0	121 (±5)
Glycine	1.0	132 (±4)
Hydrogen peroxide	0.1	102 (±3)
KCl	1.0	71 (±2)
K_2HPO_4	1.0	89 (±9)
Methanol	1.0	121 (±3)
$MgCl_2$	0.1	85 (±1)
NaCl	1.0	81 (±3)
NaClO	0.002	D[a]
Na_2SO_4	1.0	91 (±3)
$(NH_4)_2SO_4$	1.0	97 (±1)
Sodium dodecyl sulfate (SDS)	0.1	114 (±4)
Tween 80	0.1	97 (±4)

[a] D, dye decolorization.

TABLE 2

Comparison of Different Methods for the Determination of Chlorine Dioxide in Commercial Disinfectants

Product	Chlorine dioxide found (g/liter)			
	A_{360}	DPD	Iodometric	Biochemical
ClO_2 solution	0.18	0.18 ± 0.01[a]	0.19 ± 0.03	0.21 ± 0.02
Dichlor	ND	274 ± 18	269 ± 6	240 ± 9
Halox	ND	112 ± 2	117 ± 3	129 ± 5

[a] The standard deviation was obtained from four independent replicates.

At lower concentrations no interference could be found. Significantly high concentrations of these compounds, up to 1 M, were not able to affect the chlorine dioxide determination by the biochemical method. From 18 tested compounds, only sodium hypochlorite was able to affect the determination of chlorine dioxide. This may be due to the presence of free and active chlorine. Surprisingly, 0.1 M hydrogen peroxide has no effect on the chlorine dioxide determination, even if it is a substrate for peroxidase.

Determination of chlorine dioxide content in a stock solution was performed. The obtained data were compared with those obtained with the absorbance, DPD, and iodometric methods (Table 2). The values obtained with our new biochemical method are consistent with those obtained with the titrimetric methods. On the other hand two commercial preparations were tested. Dichlor, a commercial product, labeled as containing 10% (minimum) of active chlorine (equivalent to 190 g/liter of ClO_2), was shown to have 240 g/liter of ClO_2 or 12.6% active chlorine. Halox E-100 (Halox American, Burlingame, CA), claiming 6% of active chlorine, showed values of 129 g/liter of chlorine dioxide or 6.78% active chlorine.

DISCUSSION

We have developed a new spectrophotometric method for chlorine dioxide determination. This method is much easier and faster than volumetric, amperometric, and colorimetric methods, which are laborious and lack speed, sensitivity, or specificity (10–12).

This method is based on the catalytic activity of horseradish peroxidase. Monochlorodimedone has been transformed to dichlorodimedone by the action of horseradish peroxidase and sodium chlorite (18). Chlorite is disproportionated catalytically by horseradish peroxidase to form chlorine dioxide and chlorine ion (19), and the product chlorine dioxide is responsible for the chlorination of monochlorodimedone. The enzyme species formed seems to be compound II as shown by transient intermediate spectra (19). This is a direct oxidation of native horseradish peroxidase to compound II. Oxidation of compound II to compound I is performed by chlorine dioxide (20). Reaction of horseradish peroxidase at neutral pH produces mainly compound I and small amounts of compound II (20). Thus, in our case the compounds I and II may be involved in the reactive yellow decolorization. Other hemoproteins, such as hemoglobin, are able to modify catalytically phenols in the presence of sodium chlorate (21).

This method has almost no interference with high concentrations (up to 1 M) of several compounds (Table 1). At compound concentrations lower than those shown in Table 1 no significant interference could be detected. The compounds tested included chlorides, sulfates, phosphates, organic compounds, detergents, organic solvents, and ions, such as potassium, sodium, and magnesium. Ethanol, $MgCl_2$, SDS, and Tween at 1.0 M produced a precipitate, making the determination assay impossible. Hypochlorite ion affects seriously the ClO_2 determination. Very low hypochlorite concentrations ($<$1.7 mM) are able to decolorize the yellow dye without peroxidase. This nonenzymatic decolorization could be used as indicator for the presence of ClO^- ion. Nevertheless, the analysis of a sample containing ClO_2 and hypochlorite can be carried out by a conventional method for chlorine determination and using the pH-dependent reduction of chlorine species. The total available chlorine (free plus combined) is determined at pH 12 in which ClO_2 is converted to chlorite and chlorate. In a second titration, ClO_2 is estimated at pH 2 in the presence of KI (12).

In conclusion, the new biochemical method for determination of chlorine dioxide constitutes a valuable tool for routine determination in water treatment plants and other industrial processes. This method is much more rapid, less laborious, and less time-consuming than the titrimetric or amperometric methods. With this accurate method it is possible to detect from 0.2 to 4.5 mg/liter of ClO_2 and it is not affected by normal substances present in water. The biochemical determination of chlorine dioxide in commercial disinfectants and freshly prepared solutions showed consistent results when compared with other two titrimetric methods.

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REFERENCES

1. Whitmore, T. N., and Denny, S. (1992) *J. Appl. Bacteriol.* 72, 160–165.
2. Harakeh, S., Illescas, A., and Matin, A. (1988) *J. Appl. Bacteriol.* 64, 459–463.

3. Breg, J. D., Roberts, P. V., and Matin, A. (1986) *J. Appl. Bacteriol.* 60, 213–220.
4. Medema, G. J., Wondergem, E., van Dijk-Looyaard, A. M., and Havelaar, A. H. (1991) *Water Sci. Technol.* 24, 325–326.
5. Wondergem, E., and van Dijk-Looyaard, A. M. (1991) *Sci. Total Environ.* 102, 101–112.
6. Owens, J. W., Swanson, S. M., and Birkholz, D. A. (1994) *Chemosphere* 29, 89–109.
7. Wrisberg, M. N., and van der Gaag, M. A. (1992) *Sci. Total Environ.* 121, 95–108.
8. Wheeler, G. L., and Yau, F. (1976) *in* Proceedings of the Fourth Annual Industrial Pollution Conference of the Water and Wastewater Equipment Manufacturers Associations Inc. March 30–April 1, 1976, Houston, TX.
9. Puente, M. E., Vega-Villasante, F., Holgin, G., and Bashan, Y. (1992) *J. Appl. Bacteriol.* 73, 465–471.
10. Palin, A. T. (1967) *J. Inst. Water Eng.* 21, 537–547.
11. White, G. C. (1972) Handbook of Chlorination, Van Nostrand–Reinland, New York.
12. Clesceri, L. S., Greenberg, A. E., and Trussell, R. R. (1989) Standard Methods for Examination of Water and Wastewater, 17th ed., American Public Health Assoc., Washington DC.
13. Wheeler, G. L., and Lott, P. F. (1978) *Microchem. J.* 23, 160–164.
14. Herskovits, T. T. (1969) *Arch. Biochem. Biophys.* 130, 19–29.
15. Lippard, S. J. (1972) The Chemistry of Chlorine Dioxide, Progress in Inorganic Chemistry, Wiley-Interscience, New York.
16. Masschelin, W. J. (1979) Chlorine Dioxide, Chemistry and Environmental Impact of Oxychlorine Compounds. Ann Arbor Science Publ., Ann Arbor, MI.
17. Castillo, F. J., Penel, C., and Greppin, H. (1984) *Plant Physiol.* 74, 846–851.
18. Hollenberg, P. F., Rand-Meir, T., and Hager L. P. (1974) *J. Biol. Chem.* 249, 5816–5826.
19. Hewson, W. D., and Hager, L. P. (1979) *J. Biol. Chem.* 254, 3175–3181.
20. Hewson, W. D., and Hager, L. P. (1979) *J. Biol. Chem.* 254, 3182–3186.
21. Ander, P., Stalmasek, M., and Pettersson, B. (1992) *Appl. Microbiol. Biotechnol.* 36, 525–529.