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SPECTROPHOTOMETRIC DETERMINATION OF H_2O_2 IN MARINE WATERS WITH LEUCO CRYSTAL VIOLET

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Summary—H_2O_2 in marine waters may be determined by measuring the absorbance of crystal violet (CV^+), formed by the oxidation of leuco crystal violet (LCV) by H_2O_2 in the presence of the enzyme horseradish peroxidase, at 592 nm at pH 4. The detection limit and the precision of the method are about 0.02 μM and $\pm 1\%$ (at a concentration of 0.03 μM), respectively. The results obtained by this method and by a widely used fluorimetric method agreed with each other well. After color development, samples together with the sub-samples for constructing the calibration curve may be stored for up to 5 days before their absorbances are measured without significantly changing the estimated concentration of H_2O_2.

H_2O_2 has been found rather ubiquitously in a wide range of concentrations in natural waters. In ground water, the concentration is low, usually less than 50 nM.[1] In the surface oceans, its concentration ranges from less than 10 to several hundred nM.[2,3] In lakes, estuaries and rivers, higher concentrations, reaching several μM, have been reported.[4,5] In rain water, the concentration is even higher, ranging from several μM to tens of μM.[5–8] H_2O_2 can act both as an oxidizing and a reducing agent and the resulting reactions may affect the speciation of some trace elements in natural water.[9–14] A number of analytical methods, including those that are based on iodometric titration,[15] mass spectrometry,[16] spectrophotometry,[17] chemiluminescence[18] and fluorimetry,[1] have been reported for the determination of H_2O_2 in aqueous solutions. However, few of these methods have been evaluated for the determination of H_2O_2 in the variable matrices and at the low concentrations found in natural waters. Mottola *et al.*[19] reported that in the presence of the enzyme horseradish peroxidase (HRP), leuco crystal violet [4,4′4″-methylidynetris (*N*,*N*-dimethylaniline)], or LCV, may be oxidized by H_2O_2 to form crystal violet ({4-{bis[*p*-(dimethylamino)phenyl] methylene}-2,5-cyclohexadien-1-ylidine} dimethylammonium ion), or CV^+. CV^+ absorbs strongly at 590 nm. Thus, by measuring the absorbance of the CV^+ formed, H_2O_2 in pure aqueous solutions may be determined in concentrations ranging from 1.8 to 15 μM. Mottola *et al.*[19] also reported that, relative to the concentration of H_2O_2 consumed in the reaction, the molar absorptivity is 75,000M^{-1} cm^{-1}. Based on this molar absorptivity, this method may have a lower detection limit so that it may be adequate for the determination of the sub-μM concentrations of H_2O_2 in natural waters. Previous studies[19,20] also indicate that, at their reported concentrations, the other constituents of marine waters should not interfere with this analytical scheme. We have explored the possibility of applying this method to the determination of H_2O_2 in marine waters and the results are reported here.

EXPERIMENTAL

Reagents and apparatus

All chemicals used were of reagent grade.

Standard 0.01M H_2O_2 solution: a 1-ml portion of a 30% (w/w) solution of H_2O_2 was diluted to 1000 ml. This solution was standardized by an iodometric titration of the tri-iodide, formed by the reaction between H_2O_2 and excess iodide under acidic conditions, with thiosulfate.[21]

pH 4 buffer: a 68.0-g portion of KH_2PO_4 was dissolved in 490 ml of distilled deionized water. The pH was adjusted to about 4.1 with concentrated H_3PO_4 (85%). The solution was diluted to 500 ml.

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1 m*M* leuco crystal violet (LCV): a 0.374-g portion of LCV (Eastman Kodak Co., Cat. 3651, MW = 373.54) was dissolved in 1 l. of 0.06*M* HCl. This reagent was stored in the dark at room temperature. It is stable for at least 0.5 year.[20,22]

200 purpurogallin units (p.u.)/ml horseradish peroxidase (HRP): a 10-mg portion of HRP (Sigma, P-8250, 200 p.u./mg) was dissolved in 10 ml of distilled deionized water and stored at 4°C in a refrigerator. The catalytic effect of this reagent on the reaction between H_2O_2 and LCV can be maintained for at least 2 weeks.

'H_2O_2-free' marine water: a 0.5-g portion of powdered MnO_2 was added to 500 ml of a sample of marine water, with a salinity of 23, collected off the shore of Norfolk, Virginia. (Salinity is expressed in the practical salinity scale in a non-dimensional unit. One unit is approximately equivalent to 1 g of salt in 1 kg sea-water.[23]) The mixture was stirred for 2 h and then filtered through a 0.45 μM filter (Millipore, Type HA). The filtrate was free of H_2O_2. Alternatively, H_2O_2 in a sample of marine water was removed by storing it in the dark in a refrigerator for at least a week.

Absorbances and absorption spectra were recorded with a Beckman Model 5230 double beam spectrophotometer.

The pH was measured with an Orion Model 701A Digital Ionalyzer.

Procedures

A 40-ml sample was pipetted into a 50-ml volumetric flask, 4 ml of the pH 4 buffer and 0.5 ml of the 1 m*M* LCV were added. The solution was mixed and then 0.1 ml of the HRP solution added. It was diluted to volume, the solution mixed and allowed to stand for 5 min. Its absorbance was measured at 592 nm with a 1-cm cell at concentrations above 1 μM and with a 10-cm cell at lower concentrations, with distilled deionized water as the reference. At concentrations above 1 μM, the concentration in the sample was calculated from a calibration curve constructed by standard additions to a sample of similar composition. At concentrations below 1 μM, the concentration of the sample was calculated from a calibration curve constructed with 'H_2O_2-free' marine water of similar composition with various known amounts of added H_2O_2 or by internal standard additions to each sample. The reagent blank determined and in a sample of 'H_2O_2-free' water of similar composition and was corrected for each sample.

RESULTS AND DISCUSSION

Effect of pH

Crystal violet undergoes consecutive protonation as pH decreases[23] according to the equilibria:

$$HCV^{2+} = CV^{+} + H^{+}, \quad pK = 2.39;$$

$$H_2CV^{3+} = HCV^{2+} + H^{+}, \quad pK = 0.91.$$

The different protonated forms of crystal violet have different absorption spectra. The absorption maxima of CV^+, HCV^{2+} and H_2CV^{3+} are located at 590, 635 and 434 nm respectively.[19,22,24] Since the molar absorptivity of CV^+ at its absorption maximum is the highest among the three forms of crystal violet, it is used for the determination of H_2O_2. The equilibrium constants suggest that CV^+ should be the predominant species (>90%) at pH values higher than 3.4.[19] However, as the pH increases, the excess LCV in the sample precipitates and gives rise to a turbidity blank. Thus, the pH of the final solution must be carefully controlled to insure that, on the one hand, CV^+ predominates, and, on the other hand, LCV does not precipitate.[19] In order to ascertain the optimal pH for the method, the pH of a sample of surface sea-water (salinity = 33), collected off the coast of Virginia Beach, VA, was adjusted to values between 2 and 7 with 1*M* HCL and/or 1*M* NaOH. Then, LCV, (LCV + HRP), or, (LCV + HRP + 0.4 μM of H_2O_2) were added in mole ratios as described in 'Procedures' and the spectrum of the solution between 400 and 700 n*M* was recorded. A sample of distilled deionized water was treated similarly in the presence of LCV, HRP and 0.4 μM of added H_2O_2. In the absence of H_2O_2, the absorbance at 592 nm remained low and rather constant below pH 4.7. Above pH 4.7, the absorbance increased abruptly and the solution became noticeably turbid as the precipitation of LCV occurred (Fig. 1). The presence of HRP in addition to LCV gave rise to higher absorbance. However, the shape of the curve remained the same. In the presence of H_2O_2, below pH 3, the absorbance was less than 0.05 above 450 nm and there was no well defined absorption peak in either sea-water or distilled deionized water (Fig. 2). At pH 3, an absorption maximum at around 590 nm became conspicuous. There were also two shoulders at 550 and 640 nm. Above pH 3, the absorbance at the absorption maximum at 592 nm increased while the absorbance at the shoulder at 640 nm decreased. The shoulder at 640 nm became undis-

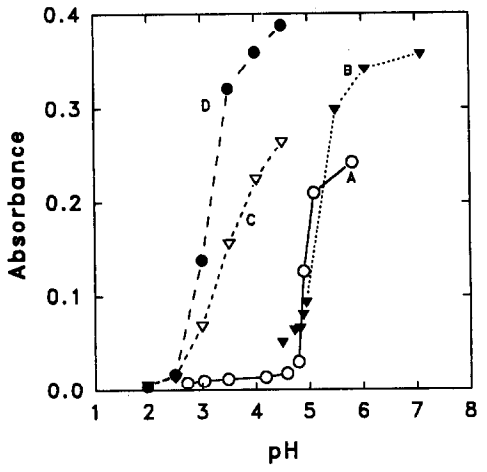

Fig. 1. The effect of pH on the absorbance at 592 nm of (A) LCV in sea-water; (B) LCV + HRP in sea-water; (C) LCV + HRP + 0.4 μM H_2O_2 in sea-water; and (D) LCV + HRP + 0.4 μM H_2O_2 in distilled deionized water in 10-cm cells.

cernible while the shoulder at 550 nm remained present at pH above 4. The lack of a well defined absorption spectrum below pH 3 between 450 and 700 nm (Fig. 2) probably resulted from the fact that, although crystal violet was formed by the reaction between H_2O_2 and LCV, it existed primarily as H_2CV^{3+}, which does not absorb strongly at these wavelengths, at these low pH. The shoulder at 640 nm between pH 3 and 4 indicates that the concentration of HCV^{2+} became significant at these pH. Above pH 4, CV^+ predominated. As a result, its absorption maximum at 592 nm became the dominant feature in the absorption spectrum and the shoulder at 640 nm disappeared. Thus, the absorbance at 592 nm was negligible below pH 2.5 (Fig. 1). It increased rapidly between pH 2.5 and 3.5 in sea-water and between 2.5 and 4 in distilled deionized water. Then, it increased more gradually up to pH 4.5, the highest pH studied. Nonetheless, a plateau in the absorbance had not been reached at this pH. There was no evidence of the precipitation of LCV and the solution remained clear up to pH 4.5. Experiments at higher pH, when the precipitation of LCV would occur, were not attempted. Thus, the optimal pH for the method should be between 4 and 4.5, as suggested in previous studies,[19,20,22] when the absorbance of CV^+ approaches its maximum while the precipitation of LCV has not yet occurred. In the recommended procedure, the final pH of the sample is about 4.3.

Amount of horseradish peroxidase

The rate of an enzymatically catalyzed reaction, such as the catalytic oxidation of LCV to CV^+ by H_2O_2 in the presence of the enzyme HRP, is dependent on the concentration ratio of the enzyme and the reactant. In order to mini-

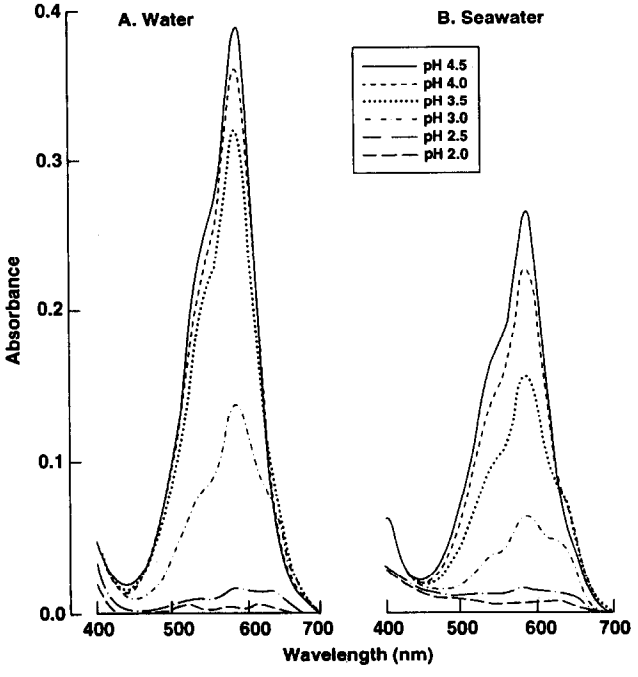

Fig. 2. The absorption spectrum of crystal violet formed by the oxidation of LCV with H_2O_2 in the presence of HRP in (A) distilled deionized water and (B) sea-water between pH 2 and 4.5.

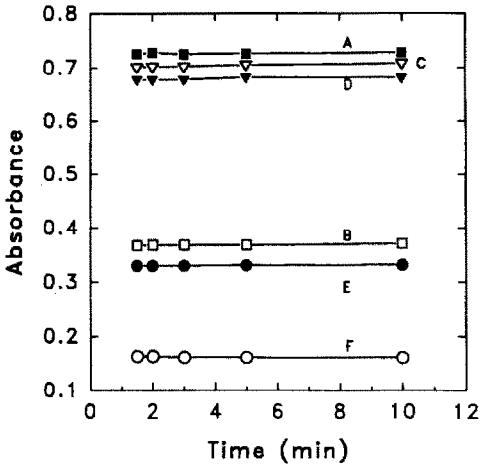

Fig. 3. Change in the absorbance of CV^+ with time at different molar ratio, R, of HRP/H_2O_2. The amount of HRP was fixed at 20 p.u. The concentration of H_2O_2 varied between 10 and 0.24 μM. (A) $R = 2$, 1-cm cells; (B) $R = 4$, 1-cm cells; (C) $R = 10$, 5-cm cells; (D) $R = 21$, 10-cm cells; (E) $R = 42$, 10-cm cells; (F) $R = 83$, 10-cm cells.

mize the reaction time, and thus the time for the analytical procedure, it would be desirable for the reaction to be as rapid as possible. The absorbance of CV^+ formed was followed with time in the presence of the recommended amount of HRP (20 p.u.) and varying concentrations of H_2O_2 between 0.24 and 9.6 μM. The resulting ratio of HRP to H_2O_2 in these solutions ranged from 2 to 83 p.u. of HPR/μg H_2O_2. A constant absorbance was observed between 1.5 and 10 min of reaction time at all ratios of HRP to H_2O_2 (Fig. 3). The shortest reaction time attempted, 1.5 min, was the amount of time required for adding and mixing the reagents without allowing for any additional time for the reaction to proceed. Mottola *et al.*[10] suggested that a ratio of HRP to H_2O_2 of 20 be used in the reaction scheme. Our data indicate that the reaction reached completion within 1.5 min even at much higher or lower ratios. A reaction time of 5 min was chosen to insure that the reaction had reached completion before the absorbance was measured. The concentration of H_2O_2 in most natural waters is usually less than 10 μM. Thus, in the recommended procedure, the ratio of HRP to H_2O_2 will be maintained at values above 2. At higher concentrations, the analytical scheme may need to be modified slightly by using a larger volume of HRP or by diluting the sample before it is analyzed. The latter approach is preferred since, in the former case, the absorbance of the sample will exceed 1 even when a 1-cm cell is used.

Salt effect

The molar absorptivity of CV^+ relative to the concentration of H_2O_2 was determined at salinities between 0 and 33 in a sample of surface sea-water (salinity = 33) that had been diluted with distilled deionized water in various ratios. The molar absorptivity was about 89,000 $A.cm^{-1}.mol^{-1}$ of H_2O_2 in distilled deionized water. This value is slightly higher than the value of 75,000 $A.cm^{-1}.mol^{-1}$ of H_2O_2 reported by Mottola *et al.*[19] It is close to the value of 100,000 $A.cm^{-1}.mol^{-1}$ of CV^+ reported by Cigén[24] and suggests that the reaction between LCV and H_2O_2 may be a 1:1 reaction. The molar absorptivity decreased with increasing salinity (Fig. 4). The decrease was more pronounced between salinities of 0 and 15 where the molar absorptivity decreased from 89,000 to 74,000 $A.cm^{-1}.mol^{-1}$ or a decrease of about 17%. Between salinities of 15 and 26, the molar absorptivity further dropped to 70,000 $A.cm^{-1}.mol^{-1}$, or an additional decrease of 6%. Above a salinity of 26, there was no detectable change in molar absorptivity with increasing salinity. Thus, when the concentrations of H_2O_2 in samples with a large range of salinities are to be measured, such as those collected from an estuary, calibration curves must be constructed at multiple salinities to cover the range of salinities encountered. In the open oceans, variations in salinity are small enough, between 32 and 36, so that a correction for changes in salinity would not be necessary.

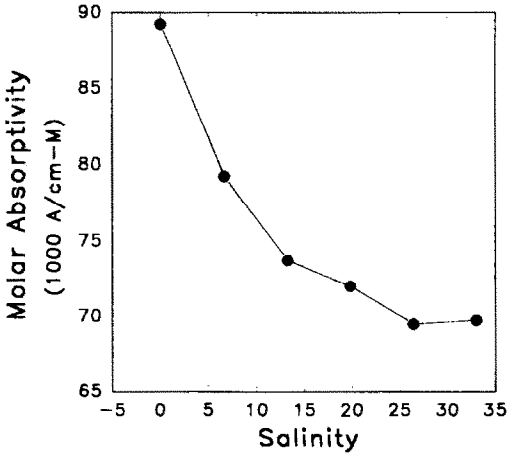

Fig. 4. The effect of salinity on the molar absorptivity of CV^+ relative to the concentration of H_2O_2.

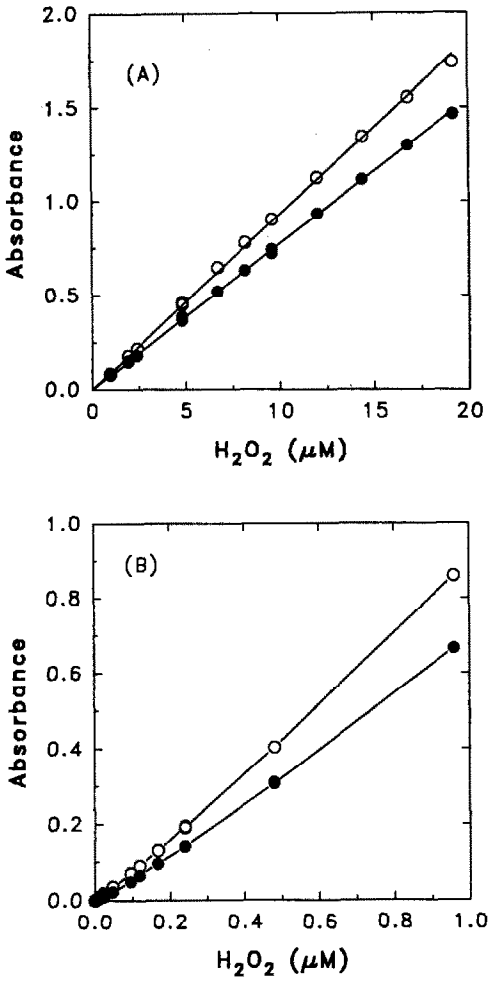

Fig. 5. The relationship between the absorbance of CV^+ at 592 nm and the concentration of H_2O_2 in distilled, deionized water and sea-water. (A) Between 1 and 20 μM of H_2O_2. Solid lines represent the best fit lines. (B) Below 1 μM of H_2O_2. ○, Distilled, deionized water; ●, sea-water.

Relationship between absorbance and the concentration of H_2O_2

The relationship between the absorbance of CV^+ and the concentration of H_2O_2, in sea-water and distilled deionized water, between 1 and 20 μM are shown in Fig. 5A. Absorbance was linearly related to concentration. This linear dynamic range is adequate for the determination of H_2O_2 in most natural waters. However, at closer scrutiny, a slight curvature in the relationship between absorbance and the concentration of H_2O_2 was found at concentrations below 1 μM (Fig. 5B). The relationship between 1 and 20 μM of H_2O_2 using 1-cm cells and below 0.5 μM, where the concentration of H_2O_2 in sea-water is usually found, using 10-cm cells are, respectively, as follows:

in distilled, deionized water,

1–20 μM:

$$A = 0.0929\ (\pm 0.0005)\ [H_2O_2],$$

$$r^2 = 0.9991;$$

<0.5 μM:

$$A = 0.813\ (\pm 0.008)\ [H_2O_2],$$

$$r^2 = 0.9974;$$

in sea-water,

1–20 μM:

$$A = 0.0773\ (\pm 0.0002)\ [H_2O_2],$$

$$r^2 = 0.9995;$$

<0.5 μM:

$$A = 0.617\ (\pm 0.008)\ [H_2O_2],$$

$$r^2 = 0.9942,$$

where the concentration of H_2O_2 is in μM. While the correlation coefficients were high in all cases, the slopes of the regression lines, when normalized to the same pathlength, were consistently smaller, by 10–20%, in the lower concentration range in both sea-water and distilled, deionized water. In this exercise, the data were forced fit through the origin. If intercepts were computed, the following relationships were found:

in distilled, deionized water,

$$1\text{–}20\ \mu M:\ A = 0.0920\ (\pm 0.0007)\ [H_2O_2] + 0.010\ (\pm 0.007),\quad r^2 = 0.9992;$$

$$<0.5\ \mu M:\ A = 0.834\ (\pm 0.010)\ [H_2O_2] - 0.006\ (\pm 0.002),\quad r^2 = 0.9983;$$

in sea-water,

$$1\text{–}20\ \mu M:\ A = 0.0767\ (\pm 0.0003)\ [H_2O_2] + 0.007\ (\pm 0.003),\quad r^2 = 0.9996;$$

$$<0.5\ \mu M:\ A = 0.641\ (\pm 0.010)\ [H_2O_2] - 0.007\ (\pm 0.002),\quad r = 0.9965.$$

In the higher concentration range, the 't' statistic of the intercepts, which is the ratio of the regression coefficient to its standard error, indicates that the intercepts are within a factor of about 2 of its standard error and should be considered statistically insignificant. In the lower concentration range, the intercepts are about three times its standard error. The inclusion of an intercept in the linear relationship does not improve the correlation coefficients or change the slopes of the lines significantly. All these suggest that the assumption of no intercept may be a good one. Mottola *et al.*[19] reported that the relationship between the absorbance of CV^+ and the concentration of H_2O_2, between about 2 and 15 μM, intersected the abscissa near the origin rather than passing through the origin and thus had a small negative intercept. In our data, a small negative intercept was found only in the lower concentration range. Mottola *et al.*[19] speculated that the negative intercept may be caused by the presence of another reducing species that reduces H_2O_2 or the occurrence of a competitive reaction which converts CV^+ to a product with a lower molar absorptivity at 592 nm and this competitive reaction is faster at low concentrations of CV^+. The former mechanism was purely conjectural and our results do not support it. When the relationship between the absorbance of CV^+ and the concentration of H_2O_2 in sea-water and distilled deionized water at concentrations below 2 μM were determined repeatedly over a number of months, the relationship cannot be represented by an extension of the linear relationship at the higher concentrations to a non-zero intercept as implied by Mottola *et al.*[19] Instead, the relationships fall along a well defined and highly reproducible curve which may be approximated as linear lines in smaller concentration intervals. The reproducibility of these curves suggests that it is unlikely that the non-zero intercept of the linear line at higher concentrations can be explained by the presence of some unknown reducing substance in the sample since there is no reason to believe that the same amount and the same kind of reducing substances should be found repeatedly over a relatively long period of time. Cigén[24] reported that, in addition to protonation, CV^+ may also be involved in the following equilibria,

$$CV^+ + H_2O = HCVOH^+$$

and $CV^+ + OH^- = CVOH$.

If these reactions are favored at the lower concentrations of crystal violet under the mildly acidic condition, the conversion of CV^+ to a different form may be the more likely explanation of the observed curvature in the calibration curve. In theory, these side reactions may be minimized by using a lower pH in the reaction scheme. However, this approach cannot be taken since the pH must be maintained between 4 and 4.5 in the analytical scheme as discussed in an earlier section. Nonetheless, the well defined and reproducible relationship between the absorbance of CV^+ and the concentration of H_2O_2 below 1 μM indicates that H_2O_2 may be determined with this method at sub-μM concentrations if a proper calibration curve covering the range of concentrations of interest can be constructed.

The blank of this method was determined repeatedly at different time over a period of about two years. The average absorbance of the blank ranged from 0.017 to 0.021 and the standard deviation ranged from 0.0008 to 0.0036. The detection limit of the method, which is estimated as three times the largest standard deviation of the blank, corresponds to a sample that yields an absorbance of 0.011 with a 10-cm cell. The concentration of H_2O_2 in such a sample is about 0.02 μM.

Because of the slight curvature in the relationship between absorbance and the concentration of H_2O_2 at low concentrations, in order to maximize the precision of the method at these low concentrations, the calibration curve, at concentrations below about 1 μM, should be constructed either by internal standard additions to each sample or with sub-samples of similar composition from which the naturally occurring H_2O_2 has been removed. Two methods have been found to be effective for preparing 'H_2O_2-free' natural water. H_2O_2 in natural waters decomposes in the dark with a life-time of hours to several days.[3,5,25] Thus, 'H_2O_2-free' natural water may be prepared by storing it in the dark for at least a week. No detectable amount of H_2O_2 was found in samples treated in this manner. Alternatively, H_2O_2 may be removed by treating the water with manganese dioxide which greatly accelerates the auto-decomposition of H_2O_2.[26] When 500 ml of sea-water containing 0.3 μM of H_2O_2 was stirred with 0.5 g of powdered manganese dioxide, the half-life of H_2O_2 was found to be about 10 min at room temperature. In order to insure the complete removal of H_2O_2, natural

Table 1. Determination of H_2O_2 in sea-water by the spectrophotometric/LCV and the fluorimetric method*

Sample	Concentration of H_2O_2 (μM) LCV	Fluorimetry	H_2O_2 added (μM)
Sea-water	0.01_1	0.02_5	0
	0.02_1	0.03_4	
	0.02_4	0.03_2	
Average (μM)	0.02 ± 0.007	0.03 ± 0.005	
Sea-water with added H_2O_2	0.29_3	0.31_8	0.30
	0.29_3	0.29_6	
	0.29_0	0.30_8	
		0.32_4	
Average (μM)	0.29 ± 0.002	0.31 ± 0.012	
Recovery (μM)	0.27	0.28	
	91%	94%	
Sea-water irradiated with sunlight	0.26_9	0.33_6	0
	0.26_4	0.33_1	
	0.26_8	0.32_6	
		0.32_9	
Average (μM)	0.27 ± 0.003	0.33 ± 0.004	
H_2O_2 formed (μM)	0.25	0.30	

*Spectrophotometric/LCV method, this work. Fluorimetric method, Holm *et al.*[1]

waters are stirred with manganese dioxide for 2 h in the preparation of 'H_2O_2-free' natural waters. The calibration curves prepared with these two kinds of 'H_2O_2-free' sea-water were identical.

Precision and comparison with the fluorimetric method

A sample of surface estuarine water (salinity = 23) was collected from the lower Chesapeake Bay and stored in the laboratory under diffuse fluorescence light for nine days. The residual concentration of H_2O_2 in this sample was determined in triplicate by the spectrophotometric method described here and by the fluorimetric method which is based on the bleaching of scopoletin by H_2O_2 in the presence of HRP.[1] The latter method has been used widely for the determination of H_2O_2 in natural waters. Then, to one sub-sample of this water, a known concentration of H_2O_2 was added. Another sub-sample was placed under direct sunlight for 3 h so that H_2O_2 may be formed by the photo-oxidation of naturally occurring dissolved organic compounds.[8,9,27] The concentrations of H_2O_2 in both of these sub-samples were again determined repeatedly by both methods. The results are shown in Table 1. The precision of the spectrophotometric method ranged from about ± 0.002 to ± 0.007 μM. It corresponded to about $\pm 1\%$ at a concentration of 0.3 μM and $\pm 35\%$ at a concentration 0.02 μM. The precision of the fluorimetric method ranged between ± 0.004 and ± 0.012 μM. Thus, the precision of the spectrophotometric method is similar to or slightly better than that of the fluorimetric method. The concentration of H_2O_2 determined by using the spectrophotometric method was slightly lower than that obtained from the fluorimetric method. However, a t-test indicates that the difference in concentration was not statistically significant. In the sample with 0.3 μM of added H_2O_2, both methods recovered a similar amount, 0.27 and 0.28 μM or 91 and 94%, respectively, of the added H_2O_2. The recovery can probably be considered quantitative within the combined uncertainties in the concentration of H_2O_2 in the sample, in the sample with added H_2O_2 and in the amount of H_2O_2 added. The difference was larger and might be significant in the sample which had been irradiated with sunlight. Part of this difference may be caused by the dynamic nature in the production and decomposition of H_2O_2 in natural waters. A number of studies have clearly demonstrated that H_2O_2 is produced in natural waters that have been exposed to light and the amount of photochemically produced H_2O_2 can be detectable within an hour of irradiation.[3,5,9,27] Since the sub-samples from this sample were not analyzed simultaneously by these two methods, individual sub-samples could have been subjected to slightly different light and dark conditions. As a result, slightly different amounts of H_2O_2 might have been formed in different sub-samples.

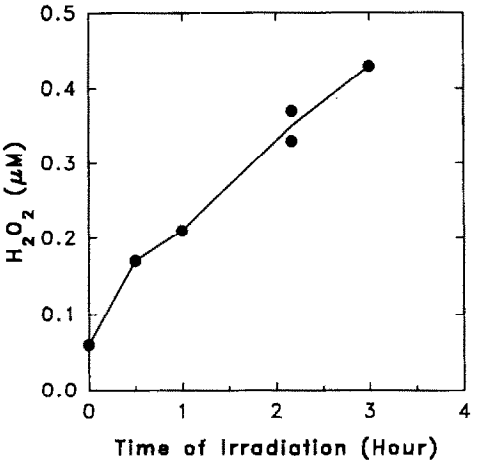

Fig. 6. Change in the concentration of H_2O_2 in a sample of estuarine water with time under irradiation with sunlight.

Application to the determination of H_2O_2 *in sea-water*

A sample of estuarine water (salinity = 23) was collected from the southern Chesapeake Bay off the shore of Norfolk, Virginia. It was filtered through glass fibre filters (1 μM nominal pore size) and then placed under direct sunlight for 3 h in the afternoon. The concentration of H_2O_2 in the sample was followed with time by the spectrophotometric method. Initially the water contained 0.06 μM H_2O_2. The concentration of H_2O_2 increased steadily with time, reaching 0.43 μM after 3 h (Fig. 6). Thus, the average rate of production of H_2O_2 was about 0.12 μM/hr. This rate of photochemical production of H_2O_2 is within the range that has been reported by other investigators for various types of natural waters.[3,9,27]

Stability of CV^+ *and sample storage*

After a sample of aged surface estuarine water (salinity = 22.3) had been exposed to sunlight for 3 h to form H_2O_2, the H_2O_2 formed was reacted with LCV to produce CV^+. Then, the sample was stored in the dark at room temperature and the absorbance of CV^+ at 592 nM was followed over time for 115 h. Simultaneously, known concentrations of H_2O_2 were added to sub-samples of this sample of aged sea-water which had not been exposed to sunlight. These sub-samples were similarly treated and stored. The absorbances of CV^+ in these sub-samples were similarly followed over time and were used for constructing the calibration curve at different storage times. Then, the concentrations of H_2O_2 at various times of storage were calculated from these calibration curves. The results are shown in Fig. 7. The initial absorbance of the sample was 0.158. It remained the same for the first hour. Thus, after color development, a sample may be stored for up to an hour before its absorbance is measured without significantly altering the results. After the first hour, the absorbance started to drop. The drop was most pronounced, at 0.0007 A/hr or 0.4%/hr, in the remaining hours of the first day of storage. Henceforth, the decrease in absorbance with time became progressively smaller. There was little discernible decrease in absorbance beyond 2 days of storage. Over the 115 h, the average decrease in absorbance was about 0.0003 A/hr or 0.2%/hr. Thus, while the color of CV^+ does fade with time, the rate of decrease in absorbance is small, well defined and quite predictable. The variations in the corresponding concentration of H_2O_2 were smaller than the variations in the absorbance when the sub-samples for constructing the calibration curve were similarly stored so that the fading of the CV^+ in the sample was compensated for by the fading of CV^+ formed in these sub-samples. The initial concentration of H_2O_2 in the sample was estimated to be 0.233 μM. The average concentration over the first 24 h was 0.232 ± 0.003 μM. This uncertainty is within the analytical uncertainty of the method. After 115 h of storage, the concentration was 0.227 μM. Thus, the average decrease in concentration can be estimated to be 0.0001 μM/hr or 0.04%/hr. However, if analytical uncertainties are taken into consideration, the concentration found after 115 h of storage should not be considered significantly different from the initial concentration. Thus, after CV^+

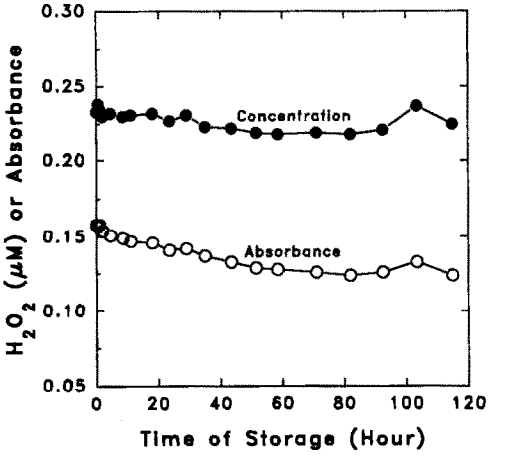

Fig. 7. Change in the absorbance of CV^+ (○) and the corresponding concentration of H_2O_2 (●) with storage time in the dark at room temperature.

is formed in a sample, it would of course be best to read the absorbance immediately. However, it is also possible to store the sample after color development and to measure the absorbance within a few days if sub-samples for the construction of the calibration curve are similarly stored. Presently, the most widely used method for the determination of H_2O_2 in natural waters is the fluorimetric method.[1] When this fluorimetric method is used, it is recommended that the samples should be analyzed immediately after they are collected. This requirements creates stringent logistical restrictions in a field sampling strategy. The possibility of storing the sample for later analyses is an important analytical advantage.

CONCLUSIONS

H_2O_2, down to sub-μM concentrations, in natural waters, including sea-water, may be determined spectrophotometrically by measuring the absorbance of CV^+, formed by the oxidation of LCV with H_2O_2 in the presence of HRP at pH 4. The reaction is almost instantaneous and independent of the ratio of HRP to H_2O_2 within the range of 2–83 p.u. of HRP/μg H_2O_2. A stringent control of pH is necessary since LCV precipitates at higher pH and other protonated forms of crystal violet may become dominant at lower pH. While correction for salt effect must be made at salinities below 26, such a correction is unnecessary at higher salinities as the molar absorptivity of CV^+ stays almost constant at these salinities. The relationship between the absorbance of CV^+ and the concentration of H_2O_2 has a slight curvature at low concentrations below about 1 μM. Thus, different calibration curves will be needed at concentrations above and below this value. The detection limit is about 0.02 μM and the precision at 0.3 μM is about $\pm 1\%$ and about $\pm 35\%$ at 0.02 μM. With proper precautions, once CV^+ is formed, a sample may be stored for up to several days before its absorbance is measured and the concentration of H_2O_2 is estimated. The precision and detection limit of this spectrophotometric method are comparable to those of the widely used fluorimetric method. However, in the latter method, samples must be analyzed immediately after sample collection.

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