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A Spectrophotometric Method for the Microdetermination of Periodate

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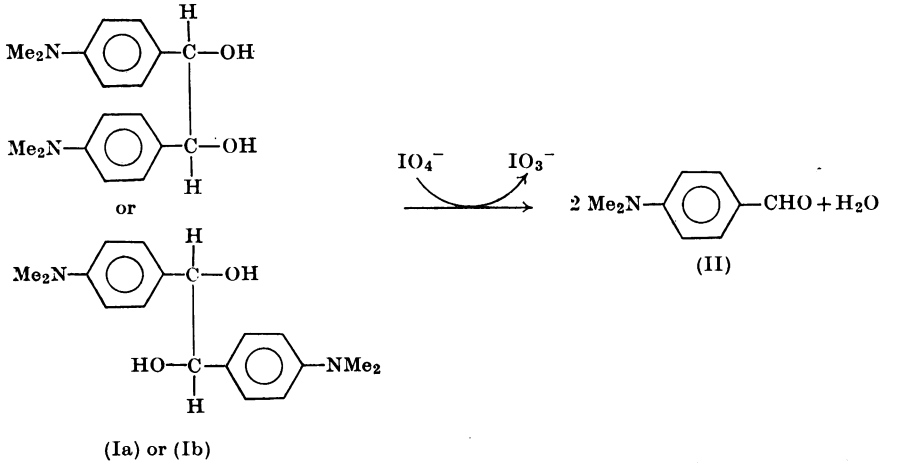
1. A method is described for measuring the concentration of periodate over the range 0·2–20 μM by adding 1,2-di-(*p*-dimethylaminophenyl)ethane-1,2-diol to a sample solution. Periodate cleaves this compound to form two molecules of *p*-dimethylaminobenzaldehyde, the extinction of which is then read at 352mμ.
2. The method has been used to follow the course of periodate oxidations of serine methyl ester, ribonuclease A and ribonuclease S-protein. Addition of the reagent stops further periodate reaction by reducing the remaining periodate to iodate.
3. The presence of protein does not interfere with the assay.

The need for a sensitive method to follow the oxidation by periodate of an *N*-terminal serine residue of a protein led us to consider reagents that would react with periodate to produce a chromophore. Dr I. Fleming of the Department of Chemistry, University of Cambridge, suggested the use of hydrobenzoins of the type 1,2-di-(*p*-dimethylaminophenyl)ethane-1,2-diol (I). This diol is oxidized by periodate in a classical Malaprade reaction, yielding two molecules of *p*-dimethylaminobenzaldehyde (II) (Scheme 1). The concentration of compound (II) in an assay solution may be determined from its extinction at 352mμ, since the absorption of isomers (Ia) and (Ib) at this wavelength is negligible (Figs. 1 and 2).

MATERIALS AND METHODS

Preparation of 1,2-*di*-(p-*dimethylaminophenyl*)*ethane*-1,2-*diol* (*I*). (1) Preparation of mixed isomers (Ia and Ib). The method of Clemo & Smith (1928) of reducing *p*-dimethylaminobenzaldehyde (II) with sodium amalgam in ethanol was used with minor modifications. Compound (I) has also been prepared from compound (II) by electrolytic reduction (Allen, 1950). An analytical grade of compound (II) was used without purification, since recrystallization from dilute HCl before use (Adams & Coleman, 1932) did not increase the yield or purity of the product. Compound (II) (52g.) was washed with ethanol (250ml.) into a round-bottomed flask that contained 4% sodium amalgam (772g.) prepared by the method of Holleman (1932). The mixture was boiled under reflux for 4hr. During this time the solution darkened from yellow to deep orange. The solution was then poured



(Ia) or (Ib)

Scheme 1.

into 1·8l. of 50% ice slurry and placed in a refrigerator overnight. The solid that formed (35·1g., 67% yield) was collected by filtration. It appeared heterogeneous, yellow and orange, and it contained both isomers of compound (I). These were separated by extracting the lower-melting isomer (Ia) from the dry solid with three 250ml. portions of diethyl ether.

(2) Recrystallization of the higher-melting isomer (Ib). The residue, after ether extraction, was dried and then dissolved in 70ml. of ethanol/g. by heating to boiling. The hot solution was filtered and allowed to cool slowly to 4°, when faintly coloured prisms crystallized. After 12hr. the crystals were collected (yield 8·2g.). After a further crystallization they possessed m.p. 178–179°, as found by Clemo & Smith (1928). They then possessed less than 1 part in 2000 of compound (II) as impurity (see below).

(3) Determination of contamination of compound (I) by compound (II). A small sample of compound (I) was dissolved in a solution of 0·1M-glycine in 0·05M-HCl, pH 2·55; after appropriate dilution its extinction was measured at 256mμ and at 352mμ. The molar ratio of compound (I) to compound (II) is given by the equation:

$$\frac{[\mathrm{I}]}{[\mathrm{II}]} = 20 \times \frac{E_{256} - 0{\cdot}12\,E_{352}}{E_{352}}$$

This equation follows from the three facts that at pH 2·55: (*a*) the molar extinction of compound (II) at 352mμ is 20 times that of compound (I) at 256mμ; (*b*) the extinction of compound (II) at 256mμ is 0·12 times its extinction at 352mμ; (*c*) compound (I) does not absorb appreciably at 352mμ (Fig. 1). A molar ratio of 2000 was taken to indicate an acceptable product.

(4) Recrystallization of the lower-melting isomer (Ia). Since most of the residual aldehyde (II) and coloured side products contained in the crude product are extracted by ether together with isomer (Ia), its purification is more difficult than that of isomer (Ib). The combined ether extracts were dried by rotary evaporation and recrystallized from 120 parts of light petroleum (b.p. 105°)–ethanol (9:1, v/v). Some coloured impurity settled out separately from the crystals and was rejected before further recrystallization. Four further recrystallizations were necessary before a product sufficiently free of aldehyde was obtained (final yield 7·3g., m.p. 118°; cf. Clemo & Smith, 1928).

Preparation of ribonuclease S-protein. Ribonuclease S-enzyme was prepared by the method of Richards & Vithayathil (1959). The S-protein was separated from the S-peptide by gel filtration in 10% (v/v) formic acid on a column (100cm. × 3cm. diam.) of cross-linked dextran (Sephadex G-75) and freeze-dried (Doscher & Hirs, 1967). It was then chromatographed on a column (36cm. × 1cm. diam.) of microgranular CM-cellulose (Whatman CM32) equilibrated and developed with a buffer of 0·105M-acetic acid and 0·105M-sodium acetate in 6·0M-urea. The minor fast peaks were discarded, and the main peak, which ran at 5 column volumes, was desalted by adsorption on the acid form of CM-cellulose, followed by displacing with 25% (v/v) acetic acid (R. Fields & H. B. F. Dixon, unpublished work).

Procedure for the determination of periodate. A measured volume of sample containing periodate is mixed with 2·0ml. of a 10mM solution of isomer (Ia) or isomer (Ib) in 0·1M-glycine in 0·05M-HCl. After 2min. the extinction at 352mμ is read. A similar reading is made on a solution of reagent without periodate. This blank reading rises slowly with time, at about 0·043/hr. with isomer (Ia). The difference in extinction between these solutions determines the concentration of periodate in the sample. If it is necessary to add the periodate in a large volume, the reagent solution may be made more concentrated so as to give a final concentration of 10mM and adequate buffering. For following periodate oxidations, samples are taken from the reaction mixture and from a blank solution that contains periodate but not the reductant under study. The difference in extinction between the two assay solutions is proportional to the amount of

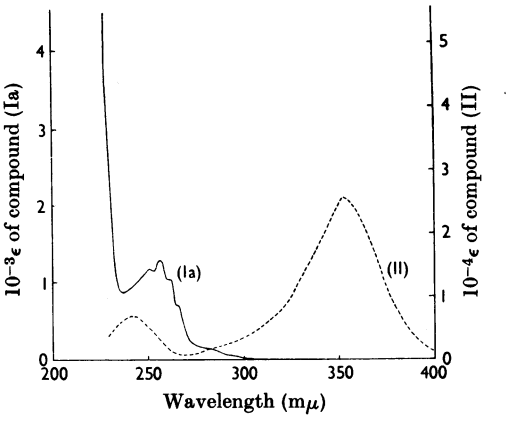


Fig. 1. Absorption spectra of 1,2-di-(*p*-dimethylaminophenyl)ethane-1,2-diol (Ia) and of *p*-dimethylaminobenzaldehyde (II) in 0·1M-glycine in 0·05M-HCl. ——, determined from 100μM-compound (Ia); ----, determined from 20μM-compound (II).

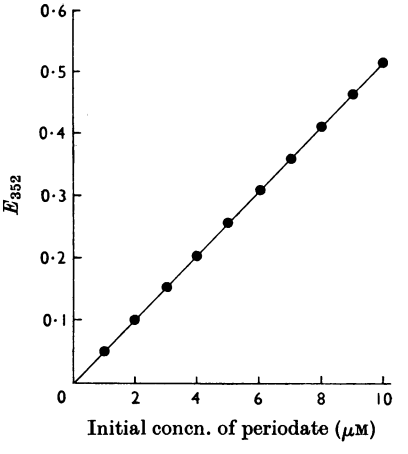


Fig. 2. Standard curve for periodate assay. Samples (1–10μl.) of 2·0mM-$NaIO_4$ were incubated for 2min. with 2·0ml. of 10mM-1,2-di-(*p*-dimethylaminophenyl)ethane-1,2-diol (Ia) in 0·1M-glycine in 0·05M-HCl (pH 2·55) before the extinction at 352mμ was read.

periodate consumed. Fig. 2 shows that a decrease of 1 μM in periodate concentration in the assay solution results in a decrease in extinction of 0·050 at 352 mμ.

RESULTS

Assay. Preliminary experiments showed that both isomers of the reagent (I) reacted with periodate without great change of rate over the pH range 1–7. The rate of decomposition of the reagent in the absence of periodate, as judged from the increase in extinction at 352 mμ, increased both with pH and with the concentration of some buffers. A glycine–hydrochloric acid buffer, pH 2·55, gave very little of this spontaneous reaction and was therefore used for further work. In making solutions of the reagent it was convenient to use a buffer of M-glycine in 0·5 M-hydrochloric acid, which was subsequently diluted tenfold. The isomer (Ib), though more stable in solution, proved harder to dissolve and solutions were filtered. Isomer (Ia) was used for most studies.

At pH 2·55 the second-order rate constant for the reaction of periodate with the reagent was found to be 21 M^{-1} $sec.^{-1}$ for the lower-melting isomer (Ia) and 9 M^{-1} $sec.^{-1}$ for the higher-melting isomer (Ib). Thus the half-life of periodate in a 10 mM solution of reagent is 6 or 15 sec., depending on which isomer of reagent is used. A satisfactory assay (Fig. 2) was developed with a final reagent concentration of 10 mM and a time of 2 min. for reaction to occur.

Oxidation of DL-*serine methyl ester.* The substrate was prepared from DL-serine, methanol and thionyl chloride by the method of Brenner & Huber (1953). To 10·0 ml. of 25 μM-DL-serine methyl ester hydrochloride in 1 mM-imidazole in 0·5 mM-hydrochloric acid, pH 6·95, at 20° was added 50 μl. of 10 mM-sodium periodate, to give an initial periodate concentration of 50 μM. An identical volume of periodate was added to 10·0 ml. of the imidazole–hydrochloric acid buffer as a control. Samples of 0·5 ml. were withdrawn at intervals and mixed with 1·5 ml. portions of 15 mM-reagent (Ia) in glycine–hydrochloric acid buffer, and the extinction at 352 mμ of the solution was read after 2 min. Fig. 3 shows the result: from the curve it appears that the apparent rate constant for reaction between periodate and serine methyl ester at pH 6·95 is 60 M^{-1} $sec.^{-1}$. The only check made, however, that second-order kinetics are followed here (cf. Bunton, 1959) is that $\log[(50-x)/2(25-x)]$, where x is the periodate consumed (μM), increases linearly with time.

Oxidation of ribonuclease S-protein. The conditions and procedures described above for DL-serine methyl ester were followed, with the exception that 3·0 mg. (0·26 μmole) of chromatographically homogeneous ribonuclease S-protein was dissolved in the buffer in place of the serine methyl ester hydrochloride. Fig. 4 shows the progress curve for this reaction, which indicates a rate constant of about 10^3 M^{-1} $sec.^{-1}$ for the fastest reaction.

Oxidation of ribonuclease A. To 0·050 ml. of

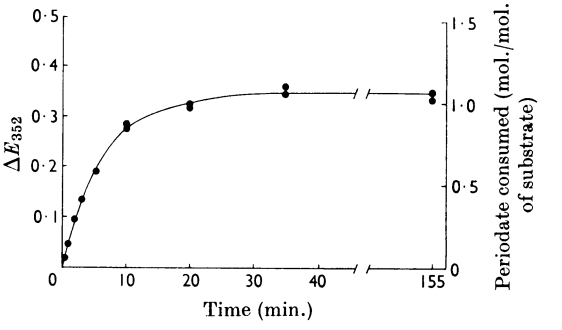


Fig. 3. Time-course of the oxidation of DL-serine methyl ester (25 μM) by $NaIO_4$ (50 μM) at pH 6·95 (see the text for details). Points are plotted as the difference in extinction at 352 mμ between a blank containing periodate and a solution containing both periodate and substrate.

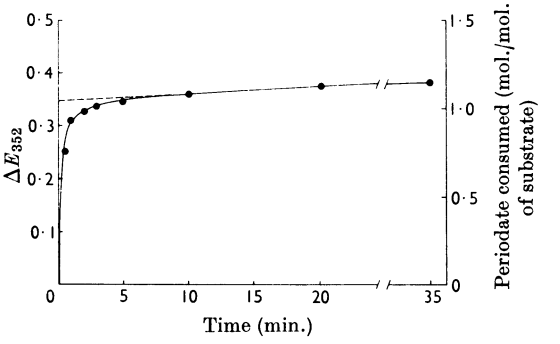


Fig. 4. Time-course of the oxidation of ribonuclease S-protein (26 μM) by $NaIO_4$ (50 μM) at pH 6·95, plotted as in Fig. 3.

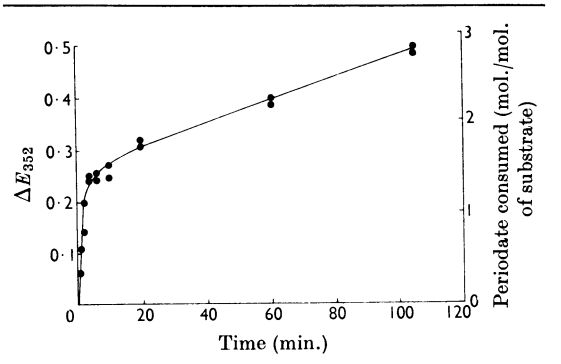


Fig. 5. Time-course of the oxidation of ribonuclease A (4 mM) by $NaIO_4$ (25 mM) at pH 7·15, plotted as in Fig. 3.

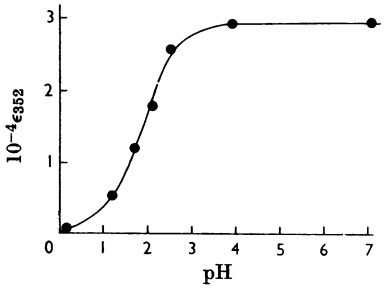


Fig. 6. Variation of the molar extinction coefficient of *p*-dimethylaminobenzaldehyde (II) at 352mμ with pH. Samples from a stock solution of compound (II) were combined with phosphate, citrate or glycine–HCl buffers or with dilute HCl solutions before reading the pH and extinction values. The line shows the titration curve for a p*K* of 1·8.

water containing 5·5mg. of ribonuclease A (0·4μmole) was added 0·050ml. of 50mM-sodium periodate in 0·25M-sodium phosphate buffer, pH 7·15. Samples (1·5μl.) were withdrawn by a microsyringe and injected into 2·0ml. of 10mM-reagent (I*a*) 2min. before the extinction at 352mμ was read. Fig. 5 shows that the initial rate constant is about 2000-fold smaller than that for ribonuclease S-protein.

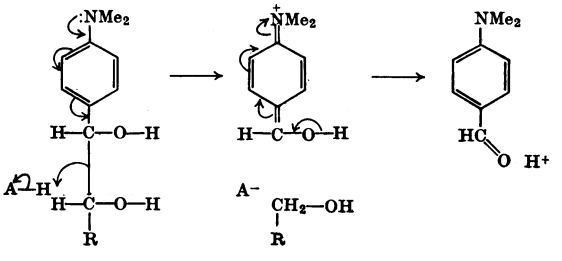
DISCUSSION

The assay described above can be used to determine the course of a periodate reaction with down to about 1mμmole of sample. This sensitivity depends on the high molar extinction coefficient of *p*-dimethylaminobenzaldehyde, which has a value of about 30000 at 352mμ in its unprotonated form. This extinction falls with pH, exhibiting p*K* 1·8, as shown in Fig. 6. The relationship shown here may be used to standardize the assay, or, if standard solutions of periodate are used for this, to estimate the amount of periodate required to give a desired extinction at a given pH. The very low p*K* in comparison with typical aromatic amines emphasizes the extent of interaction between the amino and aldehyde groups through the ring, a major feature of the chemistry of this compound.

Both isomers of the reagent have very low solubilities in water in the unprotonated form. At pH values higher than that used for the assay they break down more quickly to the aldehyde. It is thus convenient to use the reagent well below the p*K* values of its dimethylamino groups (4·5–4·7). It is nevertheless possible to use the reagent at a higher pH, since solutions below about 5mM do not rapidly form a precipitate if their pH is raised from acid values. The dihydrochlorides of the reagent isomers may be prepared and are freely soluble in water, but we have found it more convenient to dissolve the free base in an acidic buffer.

Table 1. *Increase in extinction at 352mμ in 3hr. of a 1mM solution of isomer (Ia) in various buffers*

	Concn. of buffer components (M)				
pH	Glycine	HCl	Acetic acid	Sodium acetate	Increase in E_{352}
2·57	0·1	0·05	—	—	0·017
2·46	1·0	0·5	—	—	0·021
2·47	0·1	0·05	1·0	—	0·025
3·69	—	—	1·0	0·1	0·360
4·70	—	—	0·1	0·1	0·175
4·72	—	—	1·0	1·0	0·650
5·71	—	—	0·1	1·0	0·350



Scheme 2.

The mechanism of breakdown of the reagent into *p*-dimethylaminobenzaldehyde has not been studied. Nevertheless the fact that it is much slower at pH 2·55 than at pH 3·7 or higher, and that at higher pH values it increases with the acetic acid concentration (Table 1), suggests that breakdown may be due to an attack by a general acid on a molecule of the reagent that carries at least one unprotonated amino group. The mechanism shown in Scheme 2 is therefore possible.

The extremely rapid uptake of periodate by ribonuclease S-protein (about 10^3 M^{-1}sec.$^{-1}$) is of interest. Periodate is known to attack 2-amino-alcohols 10^2–10^4 times faster than the corresponding vicinal diols (cf. Barlow, Guthrie & Prior, 1966), but it is not clear why the reaction should be much faster than that of serine methyl ester. No study has been made of the relative p*K* values of the amino groups in these two cases.

Several other sensitive methods for determining periodate exist, e.g. spectrophotometry of the periodate ion at 222·5mμ (cf. Dixon & Lipkin, 1954), polarography (Breck, Corlett & Hay, 1967) and various techniques based on the microanalysis of oxidation products. None of these techniques is as sensitive as the one described in the present paper, nor as convenient to use when protein or nucleic acids are being studied. The presence of buffer and

reagent in the assay solution are mild conditions unlikely to damage the oxidized or partly oxidized product, or to render its isolation difficult. Thus, after determination of the course of periodate reaction with ribonuclease S-protein, the combined assay solutions were concentrated and the partly oxidized S-protein was recovered by gel filtration.

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