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Note

Spectrofluorimetric detection and measurement of hydroxyl radicals in periodate solution

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Alginate, polygalacturonate, hyaluronate, and other polyuronides and polyacetals undergo changes in aqueous periodate solutions, resulting in decreases in viscosity which are sometimes dramatic[1]. At least in the case of alginate, scission of the polymer chain occurs, possibly by two mechanisms, one of which could be of a specific, chemical nature[2]. In the more-general case, hydroxyl radicals ($HO^{\bullet}$) have been implicated[1]. The diagnosis of specific chemical scissions will be greatly eased by an understanding of the extent and importance of the free-radical-mediated breakdown.

The production of $HO^{\bullet}$ in IO_4^- solution was originally inferred from the observations that acrylamide polymerises, and hydroxylation of benzoate occurs, in strongly irradiated acid (pH 2) solutions[2]. These conditions are unlike those in which polyacetals were degraded[1], and the identification of 2-hydroxybenzoate depended on the rather unspecific colour reaction with Fe^{3+}. An attempt to demonstrate the presence of $HO^{\bullet}$ in IO_4^- solutions at nearly neutral pH and in normal lighting conditions by spectrophotometric assay of the hydroxylation of benzoate was unsuccessful[3], but has now been accomplished by using a fluorimetric assay. This paper examines aspects of IO_4^--generated $HO^{\bullet}$ production which are relevant to carbohydrate research.

EXPERIMENTAL

Determination of 2-hydroxybenzoate. — At pH <10, 2-hydroxybenzoate fluoresces[4]; at pH $\geqslant 10$, 3-hydroxybenzoate fluoresces with very similar quantum yields, and excitation and emission spectra. 4-Hydroxybenzoate has no appreciable fluorescence at either pH at the wavelengths used.

The fluorescence was almost completely quenched in the presence of 0.02M $NaIO_4$. By adding excess mannitol (0.04M) to convert IO_4^- into iodate, the fluorescence was restored to its original level. Blank solutions made by adding pre-mixed IO_4^- and mannitol to benzoate had no appreciable fluorescence. The fluorescence of sodium 2-hydroxybenzoate added to the blank solution was quantitatively that of

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the same amount of 2-hydroxybenzoate in distilled water. The addition of tetrahydrofuran or 1-propanol to these solutions did not affect the fluorescence.

Mixtures of 1% aqueous sodium benzoate (3 ml), 0.2M $NaIO_4$ (0.5 ml), and water (1.25 ml) were kept at room temperature for 2 h in normal daylight in Pyrex tubes held in a circular rack rotating at ~15 r.p.m., to ensure uniform illumination. The reaction was terminated by mixing rapidly with M mannitol (0.25 ml). After 10 min, the fluorescence was measured at 405 nm with an exciting wavelength of 300 nm, using a Farrand Spectrofluorimeter Mk 1, 1-cm quartz cuvettes, and 1-mm slit-widths.

Blank solutions were prepared by mixing M mannitol (0.25 ml) with 0.2M $NaIO_4$ (0.5 ml) and, after 10 min, adding 1% sodium benzoate (3 ml). Standard solutions containing sodium 2-hydroxybenzoate were prepared in the blank solution. After the fluorescence was measured, M NaOH (0.1 ml) was added to the sample and the fluorescence was re-measured immediately. Various amounts of 0.55M tetrahydrofuran and 0.5M 1-propanol (AR) were incorporated into the volume of 4.75 ml instead of water, for experiments on $HO^{\bullet}$ scavenging.

A linear dependence of fluorescence on IO_4^- concentration (0–0.02M) was observed, using the standard procedure.

For the greater part of the incubation time, there was a linear increase of fluorescence. However, appreciable formation of 2-hydroxybenzoate took place at the outset at a far more rapid rate (Fig. 1).

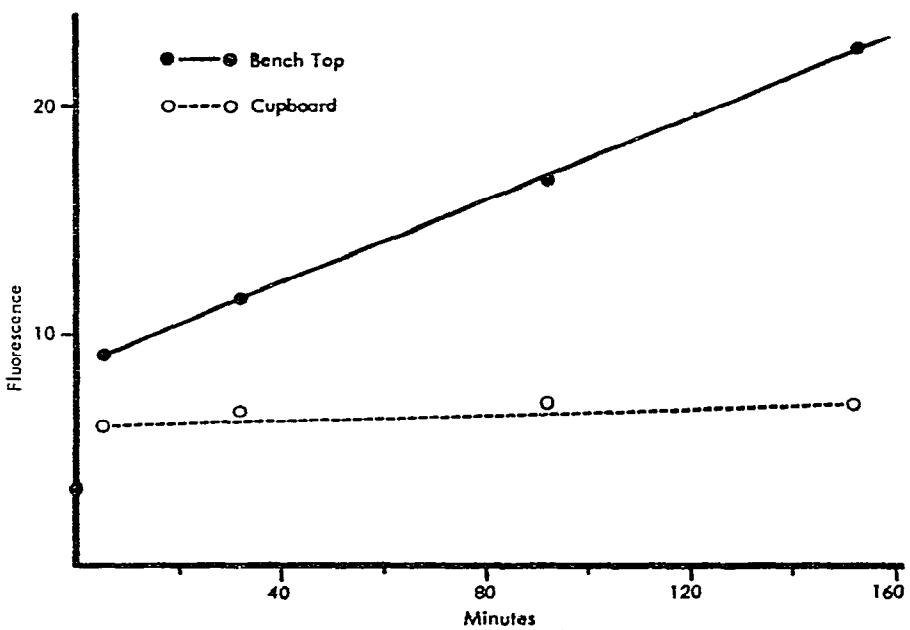

Fig. 1. Fluorescence (at pH 6) produced from 0.043M sodium benzoate containing 0.02M sodium periodate in normal daylight (— ● —) and in the dark (— O —), as a function of time.

Only small amounts of fluorescence were produced when the tubes were kept in the dark (Fig. 1).

Identification of the fluorescent material. — The excitation and emission spectra of the fluorescence produced from periodate-treated benzoate were practically identical with that of sodium 2-hydroxybenzoate at the same pH (6), with peaks at 307 and 405 nm, respectively. The fluorescence increased markedly on adding NaOH to pH ~11. This fluorescence faded in a few minutes, and the solutions became faintly yellow-brown. The ratio of the fluorescence at pH 6 to that additionally produced at pH 11 varied very widely between experiments, but was typically 3–4.

HO• Scavenging. — The incorporation of both 1-propanol (AR) and purified tetrahydrofuran into the standard procedure diminished the amount of fluorescent material produced (Table I). Control experiments showed no effect of either compound on the fluorescence of 2-hydroxybenzoate. Tetrahydrofuran which had not been distilled over ferrous sulphate markedly *increased* the amount of fluorescence (Table I).

TABLE I

SECOND-ORDER RATE CONSTANTS (k'') FOR THE REACTION OF HO• WITH 1-PROPANOL OR TETRAHYDROFURAN (THF), CALCULATED ON THE BASIS OF k (BENZOATE) = 3.3×10^9. COLUMN 3 (F_0/F Calc.) IS BASED ON k'' VALUES GIVEN IN COLUMN 6 AND ON THE ABOVE VALUE FOR k (BENZOATE)

$\frac{[THF]}{[Benzoate]}$[a]	F_0/F				
	Obs.[b]	*Calc.*	*Obs.*[c]	k'' (*Calc.*)	($\times 10^9$)
0.52	1.67	1.25	1.08	4.2	
1.05	1.84	1.50	0.96	2.64	
1.57	1.87	1.75	0.86	1.82	k'' THF (lit.), 1.6
1.98	1.95	1.95	0.82	1.55	
$\frac{[1\text{-}Propanol]}{[Benzoate]}$				k'' (*Calc.*)	
0.48	1.23	1.22		1.58	
0.98	1.23	1.42		0.80	k'' 1-Propanol (lit.), 1.5
1.43	1.28	1.64		0.65	
1.80	1.25	1.81		0.45	

[a][Benzoate] = 0.043M throughout. [b]For distilled tetrahydrofuran. [c]Laboratory reagent tetrahydrofuran.

DISCUSSION

The fluorimetric assay of 2-hydroxybenzoate is very specific. The detailed similarity of both emission and excitation spectra to those of authentic material leaves little doubt that the material fluorescing at pH 6, generated from benzoate in illuminated periodate solutions, is 2-hydroxybenzoate. The increase in fluorescence on raising the pH to 11 implies that there are other products as well (probably including 3-hydroxybenzoate, which does not fluoresce at pH 6). The lability of the

fluorescence in the conditions of the experiment prevented good comparisons with authentic material.

In principle, proof that hydroxylation is due to $HO^{\bullet}$ is available from a quantitative comparison of the amount of salicylate produced in the presence and the absence of competitive scavengers with known reaction rates (k_2) with $HO^{\bullet}$. Assuming that $HO^{\bullet}$ combines only with benzoate or competitor, and that the total $HO^{\bullet}$ production is the same in both instances, then, in the absence of competitor, $\{HO^{\bullet}\} = \{\text{salicylate}\} = k'[\text{benzoate}][HO^{\bullet}]$, where [ ] indicates concentrations and { } the total amount produced. In the presence of competitor, $F = \{\text{salicylate}\}$ and $F_0 - F = \{X\} = \{\text{salicylate}_0\} - \{\text{salicylate}\}$, where X is product of $HO^{\bullet}$ and competitor, F_0 is the fluorescence in the absence of competitor, and F the fluorescence in the presence of competitor.

$$\{X\} = k''[\text{competitor}][HO^{\bullet}]$$

$$\frac{\{\text{sal}\}}{\{X\}} = \frac{k'[\text{benzoate}][HO^{\bullet}]}{k''[\text{competitor}][HO^{\bullet}]}$$

$$\frac{F}{F_0 - F} = \frac{k'[\text{benzoate}]}{k''[\text{competitor}]}$$

$$\frac{F_0}{F} = \frac{k''[\text{competitor}]}{k'[\text{benzoate}]} \qquad (1)$$

Although the results show a marked inhibition of salicylate formation in the presence of both competitors, as expected for a $HO^{\bullet}$-mediated reaction, the linear relationship between F_0/F and [competitor]/[benzoate] predicted from equation *1* is not seen. Inhibition by both tetrahydrofuran and 1-propanol increases asymptotically as their concentration ratios increase. However, whereas the inhibition due to tetrahydrofuran at low concentration ratios is considerably stronger than that calculated from the literature values of k' benzoate (3.3×10^9) and k'' tetrahydrofuran (1.6×10^9), approaching the expected value at higher concentration ratios, the inhibition by 1-propanol is close to the calculated value at low ratios, but falls off considerably at higher ratios (Table I). It is possible that the simple assumptions behind equation *1* do not hold in these experiments. Both tetrahydrofuran and 1-propanol may interfere with the chain of events leading to salicylate at more than one point. The experiments performed with ordinary laboratory-reagent tetrahydrofuran, as compared with distilled material, suggest caution in this context. They imply that small amounts of the "peroxide inhibitor" (0.05% in the tetrahydrofuran) exert a potentiating effect on the hydroxylation of benzoate sufficient to outweigh the inhibiting effect of a large amount of tetrahydrofuran. The absence of a linear correlation between the figures in columns 2 and 1 in Table I might be due to trace contaminants, particularly in the 1-propanol.

Nevertheless, it is clear that inhibition by tetrahydrofuran and 1-propanol is of the right order, being within the uncertainty of the measurements of k' and k''. The evidence points decisively to the presence of HO• in periodate solution. The postulated role of HO• in the degradation of polymers in IO_4^- solutions is thus placed on a firmer basis.

In our laboratory, on the bench top, in indirect sunlight, the amount of HO• was sufficient to produce ~ 10 μM salicylate/h, in 0.02M $NaIO_4$ from 0.04M benzoate. The level of illumination and the periodate concentration were determining factors. The routine precautions of working in dim light and with low concentrations of IO_4^- ought to avoid most of the side reactions associated with HO•. It is clearly undesirable to use HO• scavengers uncritically, in view of the complications (*cf.* columns 3 and 4 in Table I).

The very simple, spectrofluorimetric technique described here ought to be of value in investigations of other reactions involving HO•.

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REFERENCES

1 J. E. Scott and M. J. Tigwell, *Carbohydr. Res.*, 28 (1973) 53–59.
2 J. E. Scott, M. J. Tigwell, C. F. Phelps, and I. A. Nieduszynski, *Carbohydr. Res.*, 47 (1976) 105–117.
3 M. C. R. Symons, *J. Chem. Soc.*, (1955) 2794–2796.
4 G. A. Thommes and E. Lehninger, *Anal. Chem.*, 30 (1958) 1361–1363.
5 M. Anbar and P. Neta, *Int. J. Appl. Radiat. Isotop.*, 18 (1967) 493–495.