

## EVIDENCE FOR THE ADDITION OF THE SUPEROXIDE ANION TO THE ANTI-OXIDANT *n*-PROPYL GALLATE IN AQUEOUS SOLUTION

D.J. DEEBLE, B.J. PARSONS and G.O. PHILLIPS

Research Division, North East Wales Institute of Higher Education, Connah's Quay,  
Deeside, Clwyd, CH5 4BR, U.K.

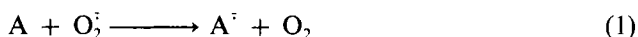
(Received July 21st 1986)

*n*-Propyl gallate reacts with the superoxide radical anion in aqueous solution ( $k = 5.1 \times 10^5 \text{ mol}^{-1} \text{ dm}^3 \text{ s}^{-1}$ ). The spectrum of the transient species so formed has been measured (absorbance maximum at 550 nm,  $\epsilon = 1360 \text{ mol}^{-1} \text{ dm}^3 \text{ cm}^{-1}$ ). Electron or H atom transfer processes as well as proton abstraction have been excluded as possible mechanisms, and it is proposed that an addition reaction takes place.

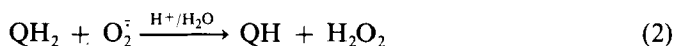
KEY WORDS: Superoxide, anti-oxidant, *n*-propyl gallate, pulse radiolysis.

### INTRODUCTION

The superoxide anion ( $\text{O}_2^-$ ) can be formed in oxygenated organic systems and is thought to be able to lead to serious peroxidative damage in living tissues and to the development of rancidity in foods. As a consequence of its role in inducing these deleterious effects, the chemistry of superoxide anions has received considerable attention. The superoxide anion acts as a strong base and can abstract protons from organic molecules, the resulting anions may then react with oxygen.<sup>1</sup> In aqueous solution the superoxide anion has been shown to participate in one-electron transfer reactions. The reduction of metal ions and their complexes by  $\text{O}_2^-$  is well documented.<sup>2</sup> Organic solutes can also be reduced by reaction with  $\text{O}_2^-$ ,



where A represents the organic solute, reactions of this type have been shown to occur in aqueous solutions with tetra-nitromethane<sup>3</sup> and various quinone derivatives.<sup>4</sup> The reduction of  $\text{O}_2^-$  in aqueous solution by hydroquinones<sup>5</sup> ( $\text{QH}_2$ ),



has been known for some time and more recently similar reactions involving other di-hydroxy benzenes have been reported.<sup>6</sup>

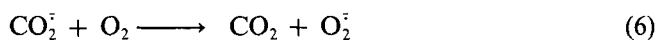
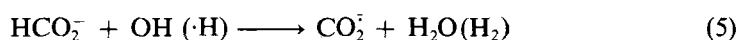
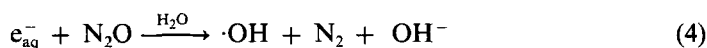
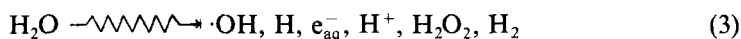
E.S.R. studies<sup>7</sup> indicate that in the absence of water  $\text{O}_2^-$  adds to dimethyl formamide to produce a peroxy radical, however the presence of even trace amounts of water prevents this reaction. In aprotic solvents a number of such  $\text{O}_2^-$  nucleophilic addition reactions have been reported for carbonyl containing compounds.<sup>8,9</sup> In the present paper data is presented which suggests that  $\text{O}_2^-$  reacts with the food anti-oxidant *n*-propyl gallate, I,(3,4,5,tri-hydroxy propyl benzoate) in aqueous solution by nucleophilic addition.

## MATERIALS AND METHODS

In the present study all pulse radiolysis experiments were performed using the facilities at the Paterson Laboratories, Manchester, details of the set-up have been published elsewhere.<sup>10,11</sup> *n*-Propyl gallate and superoxide dismutase (from bovine erythrocytes) were obtained from the Sigma Chemical Co. Ltd. *t*-butyl hydroquinone (2-*t*-butyl 1,4 dihydroxy benzene) was purchased from the Aldrich Chemical Co. Ltd. All other chemicals were of the highest grade commercially available, formate solutions were made up either directly from sodium formate or from concentrated solutions made by neutralising formic acid with sodium hydroxide, no differences could be detected between these two methods of preparation. To exclude the possibility of any photolytic effects all solutions of the antioxidants were protected from light by aluminium foil. Experiments were always performed on freshly made up solutions. Triply distilled water was used throughout. Known concentrations of superoxide dismutase were made up by weighing, assuming a molecular weight of 32,000.

## RESULTS

*n*-Propyl gallate at a concentration of  $5 \times 10^{-4} \text{ mol dm}^{-3}$  was pulse irradiated in an aqueous solution,  $0.1 \text{ mol dm}^{-3}$  in sodium formate,  $2 \times 10^{-3} \text{ mol dm}^{-3}$  in phosphate buffer pH 7, saturated with a mixture of nitrous oxide and oxygen (4/1 by volume). Under these conditions superoxide anions are formed in high yield ( $G(\text{O}_2^-) = 6.5$ ) according to the following scheme,



Alternatively experiments were performed in  $\text{O}_2$  saturated formate solutions, under these conditions the solvated electrons are converted directly into  $\text{O}_2^-$ ,



All of these reactions (3–7) occur at near diffusion controlled rates<sup>12</sup> and are complete within a few microseconds after the pulse. At fairly long times (ms) after the pulse the buildup of a transient was observed, the spectrum for which is shown in Figure 1(a). The kinetics for this buildup were first order and the observed first order rate constant was directly proportional to the *n*-propyl gallate concentration (Figure 2). Thus *n*-propyl gallate must be involved in the rate determining step and from the plot of observed rate constant against *n*-propyl gallate concentration (Figure 2) the second order rate constant is  $5.1 \times 10^3 \text{ mol}^{-1} \text{ dm}^3 \text{ s}^{-1}$ . On generating superoxide anion radicals in the presence of *n*-propyl gallate and superoxide dismutase (SOD) it was found that the rate of buildup of the transient (absorbance maximum 550 nm) can be described by simple competition kinetics (Figure 3). The slope of the plot in Figure 3 gives a value of  $3.5 \times 10^8 \text{ mol}^{-1} \text{ dm}^3 \text{ s}^{-1}$  for the rate constant for the reaction of  $\text{O}_2^-$  with SOD, this value is somewhat lower than most published data

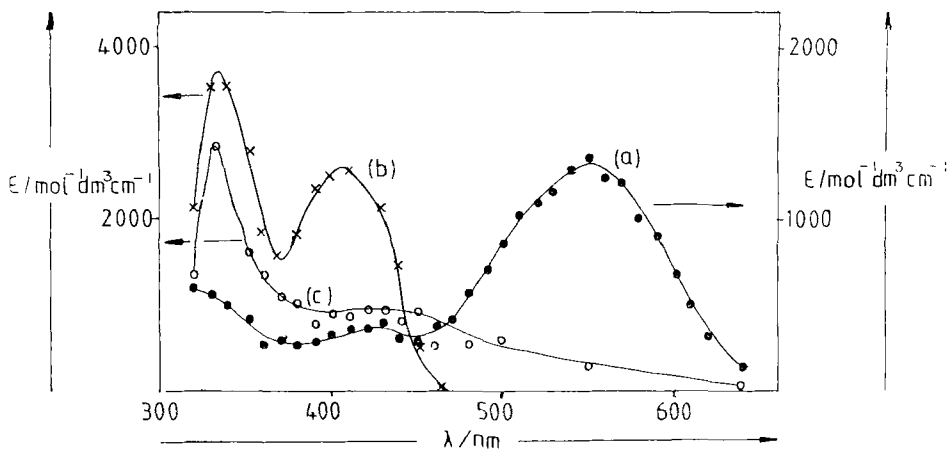


FIGURE 1. Transient spectra. (a) (●), transient formed by reaction of  $O_2^-$  with *n*-propyl gallate,  $0.1 \text{ mol dm}^{-3}$  sodium formate,  $2 \times 10^{-3} \text{ mol dm}^{-3}$  phosphate buffer pH 7,  $5 \times 10^{-4} \text{ mol dm}^{-3}$  *n*-propyl gallate, saturated with a mixture of nitrous oxide and oxygen (4/1 by volume), 5.4 Gy/pulse, 8.5 ms after pulse,  $\epsilon$  based on  $G(n\text{-propyl gallate} + O_2^-) = 6.5$ . (b) (x) *n*-propyl gallate phenoxyl radical (semiquinone),  $0.05 \text{ mol dm}^{-3}$  sodium azide,  $2 \times 10^{-3} \text{ mol dm}^{-3}$  phosphate buffer pH = 7,  $2 \times 10^{-5} \text{ mol dm}^{-3}$  *n*-propyl gallate, nitrous oxide saturated, 2.8 Gy/pulse, 6 s after pulse,  $\epsilon$  based on  $G(\text{phenoxyl radical}) = 6$ , corrected for the H atom adduct (see below). (c) (○), electron adduct of *n*-propyl gallate,  $1.0 \text{ mol dm}^{-3}$  *t*-butanol,  $2 \times 10^{-3} \text{ mol dm}^{-3}$  phosphate buffer pH 7,  $5 \times 10^{-4} \text{ mol dm}^{-3}$  *n*-propyl gallate, nitrogen flushed, 5.5 Gy/pulse, 9  $\mu\text{s}$  after pulse,  $\epsilon$  based on  $G(\text{electron adduct}) = 2.8$ , a correction has been made for the H atom adduct, using  $G(\text{H adduct}) = 0.6$  and the spectrum of the H atom adduct determined at pH 2.<sup>18</sup>

$(\sim 2 \times 10^9 \text{ mol}^{-1} \text{ dm}^3 \text{ s}^{-1})$ .<sup>2</sup> Low values have been obtained by other workers<sup>13</sup> and have been ascribed to the fact that the enzyme sample contained some inactive material. From our value for the rate constant for the reaction of SOD with  $O_2^-$  it can be calculated that the enzyme sample is only 20% active, this estimate was confirmed by directly monitoring the decay of  $O_2^-$  (280 nm,  $0.2 \text{ mol dm}^{-3}$  formate,  $2 \times 10^{-3} \text{ mol dm}^{-3}$  phosphate pH 7,  $O_2$  saturated) in the presence of SOD. Thus SOD and *n*-propyl gallate both react directly with  $O_2^-$  and the rate determining step in the buildup of the transient absorbing at 550 nm is the direct reaction of  $O_2^-$  with *n*-propyl gallate.

A major problem in studying such relatively slow reactions as that observed here ( $k < 10^3 \text{ s}^{-1}$ ), is that low concentrations of impurities having high reactivities with one of the reactants (in this case  $O_2^-$ ) could interfere. An obvious candidate for such impurities would be transition metal ions, consequently concentrated solutions of the various components were analysed for Mn, Fe, Cu, Cr (thought to be the most likely possible contaminants) by atomic absorption spectroscopy. These results indicated that any metal ion impurity was  $< 10^{-6} \text{ mol dm}^{-3}$  under all conditions used. Recrystallisation of *n*-propyl gallate from methanol/water had no effect on transient buildup or yield. Proton n.m.r. and mass spectrometry measurements on *n*-propyl gallate indicate impurities of less than 1%. As a final check, the transient absorbance at 550 nm (conditions as for Figure 1) was measured as a function of dose, the results are compiled in Table I and show that any impurity would have to be present at  $> 30 \text{ mol}\%$  *n*-propyl gallate in order to be responsible for the observed buildup. Such high levels of impurity in the *n*-propyl gallate would have been easily detected by n.m.r./mass spectrometry. In order to ensure that the observed transient was not due

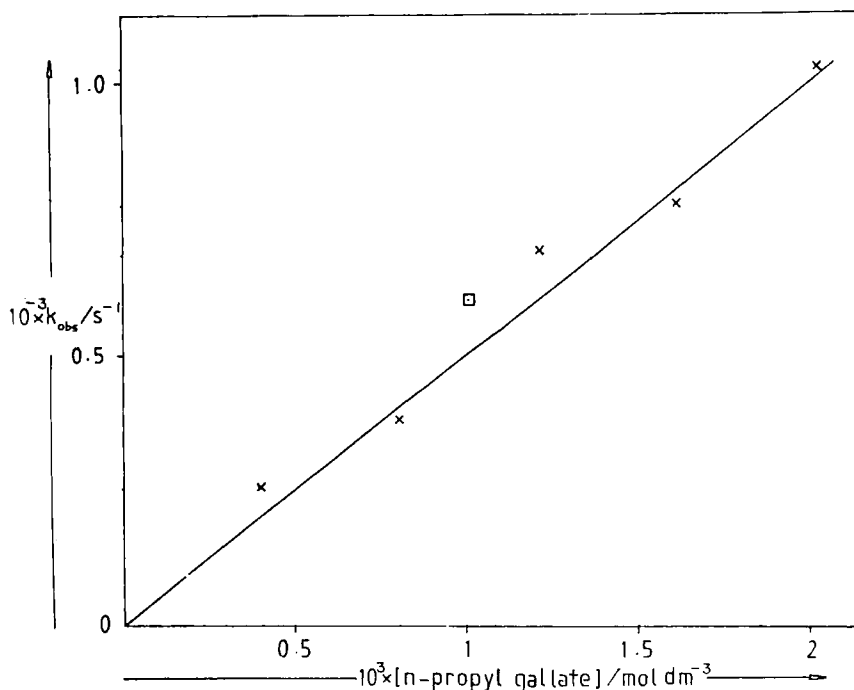
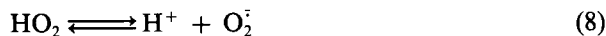


FIGURE 2 Dependence of the transient buildup at 550 nm on the concentration of *n*-propyl gallate.  $0.1 \text{ mol dm}^{-3}$  sodium formate,  $2 \times 10^{-3} \text{ mol dm}^{-3}$  phosphate buffer pH 6.9, oxygen saturated,  $3.5 \text{ Gy/pulse}$ .  $\square$ , as above but  $0.2 \text{ mol dm}^{-3}$  phosphate buffer pH 6.6, air saturated.

to some particular effect of the formate system, an experiment was performed in which the formate was replaced by  $0.5 \text{ M}$  *t*-butanol ( $\text{N}_2\text{O}/\text{O}_2$  4/1). Under these conditions *t*-butanol peroxy radicals are produced by irradiation in high yield, and these decay bimolecularly to give superoxide anion radicals with a *G* value around 2.<sup>14</sup> On pulse irradiating the *t*-butanol system the buildup of a transient, having an absorbance maximum at 550 nm, occurred with a rate constant identical to that found in the formate system. Changing from  $\text{N}_2\text{O}/\text{O}_2$  (4/1) mixtures to oxygen saturated solutions had no effect on the transient buildup rate constant.

When experiments were performed at pH 3 ( $0.1 \text{ mol dm}^{-3}$  formate,  $5 \times 10^{-4} \text{ mol dm}^{-3}$  *n*-propyl gallate, oxygen saturated) no transient absorbance was observable above 350 nm, at pH 4.6 a transient buildup was again apparent at 550 nm, although the yield was much lower than at pH 7. Superoxide is known to protonate, the p*K*<sub>a</sub> of the protonated form being 4.7,<sup>15</sup>



From the pH dependence of transient production it can be concluded that  $\text{HO}_2$  radicals are not reacting with *n*-propyl gallate to form the 550 nm absorbing transient.

As already mentioned the superoxide anion can act as a strong base and abstract protons from organic compounds, in some instances the resulting anion reacts with oxygen.<sup>1</sup> In aqueous solutions the anions of acids such as *n*-propyl gallate, with p*K*<sub>a</sub>'s greater than 9 could be expected, (taking a value of  $10^9 \text{ mol}^{-1} \text{ dm}^3 \text{ s}^{-1}$  for the rate

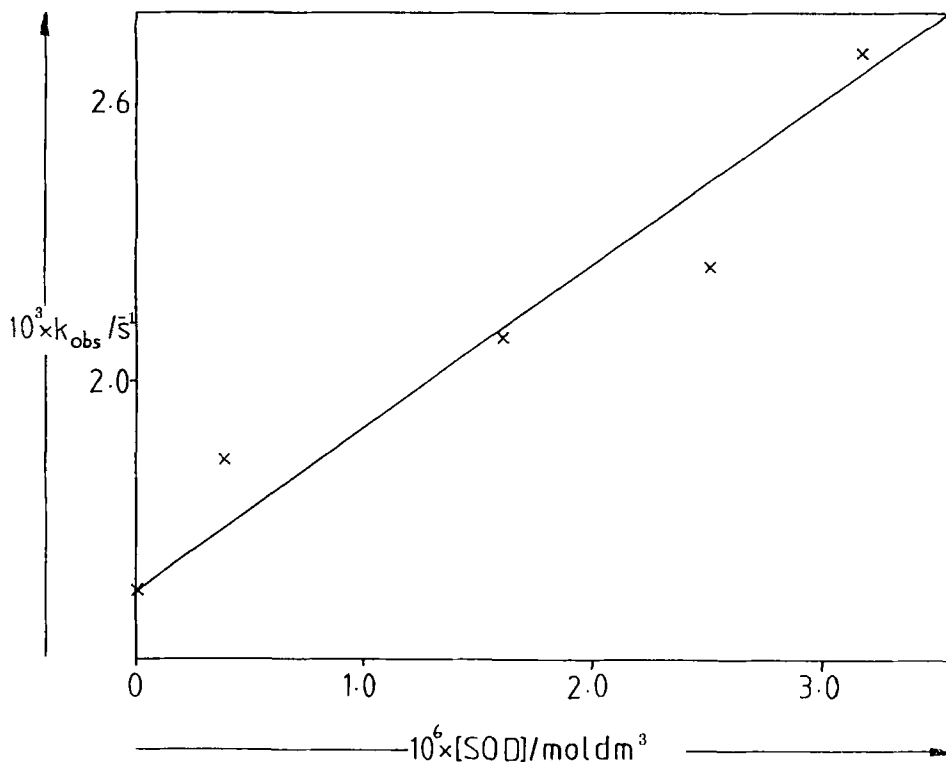


FIGURE 3 Influence of superoxide dismutase on the rate constant for the transient buildup at 550 nm.  $0.2 \text{ mol dm}^{-3}$  formate,  $2 \times 10^{-3} \text{ mol dm}^{-3}$  phosphate pH 7,  $4 \times 10^{-3} \text{ mol dm}^{-3}$  *n*-propyl gallate, saturated  $\text{N}_2\text{O}/\text{O}_2$  (4/1), 5.5 Gy/pulse.

TABLE I

Transient absorbance as a function of dose.  $5 \times 10^{-4} \text{ mol dm}^{-3}$  *n*-propyl gallate,  $0.5 \text{ mol dm}^{-3}$  formate,  $2 \times 10^{-3} \text{ mol dm}^{-3}$  phosphate pH 7,  $\text{O}_2$  saturated, 2.5 cm pathlength, 3.5 ms after pulse

Dose/Gy	6.4	9.9	21	107	286	534
$10^5 \times \text{initial } [\text{O}_2^\cdot]^\text{a} / \text{mol dm}^{-3}$	0.5	0.6	1.4	7	19	35
$10^2 \times \text{absorbance at 550 nm}$	0.9	1.5	3.3	15	24	26
Ratio [ <i>n</i> -propyl gallate]/ $[\text{O}_2^\cdot]$	0.009	0.013	0.027	0.14	0.38	0.70

<sup>a</sup>Calculated assuming  $G(\text{O}_2^\cdot) = 6.5$ .

constant for reaction of  $\text{OH}^-$  with the conjugate acid), to react with water to form the conjugate acid with pseudo first order rate constants greater than  $10^4 \text{ s}^{-1}$ , i.e. much larger than the rate of buildup observed in the present work. As anticipated a comparison of the spectrum of the transient observed here with the anion spectrum of *n*-propyl gallate (measured at pH 10.8 nitrogen flushed) definitely rules out the anion of *n*-propyl gallate as being the observed transient. However, the possibility exists that the anion could be formed by proton extraction and then react rapidly with oxygen to give the observed transient. This was ruled out by pulse irradiating an air saturated formate solution containing  $1 \times 10^{-3} \text{ mol}^{-1} \text{ dm}^3$  *n*-propyl gallate, and

0.2 mol dm<sup>-3</sup> phosphate buffer pH 6.6. Under these conditions reprotonation of the anion by the buffer should be rapid enough ( $k > 10^6 \text{ s}^{-1}$  to compete with any reaction of the anion with oxygen ( $k < 10^6 \text{ s}^{-1}$ ). Consequently the rate constant for transient buildup would be markedly higher in the presence of phosphate if the anion was formed and rapidly reacted with oxygen. The observed rate constant ( $\square$ , in Figure 2) is not appreciably altered by the presence of the buffer and so it is reasonable to conclude that proton abstraction by O<sub>2</sub><sup>-</sup> is not taking place.

The question now arises as to what type of reaction is occurring? A reaction analogous to reaction 2 would produce *n*-propyl gallate phenoxyl radicals, these radicals can be readily generated by reacting *n*-propyl gallate with azide radicals.<sup>16</sup> The spectrum of *n*-propyl gallate phenoxyl radicals was determined in this way and is shown in Figure 1(b) for reference. On producing the *n*-propyl gallate phenoxyl radicals in N<sub>2</sub>O/O<sub>2</sub> (4/1 by volume) saturated solutions containing azide ions, no spectral or kinetic (decay) changes were observable, when compared with the anoxic system. Thus, as recently reported for the  $\alpha$ -tocopherol phenoxyl radical,<sup>17</sup> the *n*-propyl gallate phenoxyl radical shows no measurable (i.e.  $k < 10^5 \text{ mol}^{-1} \text{ dm}^3 \text{ s}^{-1}$ ) reaction with oxygen. By comparing the spectrum for the observed transient (a) with that of the phenoxyl radical (b) as shown in Figure 1, it is evident that the reaction between O<sub>2</sub><sup>-</sup> and *n*-propyl gallate is not that given by equation (2) (the additional possibility of a rapid reaction between an *n*-propyl gallate phenoxyl radical formed in reaction (2) and oxygen to give the observed transient being obviated by the above mentioned stability of the phenoxyl radical in oxygen). At this point it is well worth mentioning that similar experiments have been performed with another food antioxidant, *t*-butyl hydroquinone, already reported to react in aqueous solution with O<sub>2</sub><sup>-</sup>,<sup>6</sup> the mechanism suggested being that shown in reaction (2). The transient spectra produced by the reaction of *t*-butyl hydroquinone with either O<sub>2</sub><sup>-</sup> or the azide radical are shown in Figure 4. The two spectra are identical, thus confirming that in this case the reaction of O<sub>2</sub><sup>-</sup> with *t*-butyl hydroquinone gives hydrogen peroxide and the *t*-butyl hydroquinone phenoxyl radical, i.e. reaction (2).

However, although electron transfer from *n*-propyl gallate to O<sub>2</sub><sup>-</sup> does not occur, the reverse reaction, electron transfer from O<sub>2</sub><sup>-</sup> to *n*-propyl gallate to yield oxygen and the *n*-propyl gallate radical anion (reaction (1)) has not yet been excluded. The spectrum of the radical anion of *n*-propyl gallate was measured and is also displayed in Figure 1(c). Here again it is quite apparent that the observed transient from the O<sub>2</sub><sup>-</sup> *n*-propyl gallate reaction is not the *n*-propyl gallate radical anion. On the contrary, as might be expected, oxygen reacts with the radical anion to produce superoxide and *n*-propyl gallate<sup>18</sup> ( $k = 3 \times 10^9 \text{ mol}^{-1} \text{ dm}^3 \text{ s}^{-1}$ ).

In summary, the observed transient results directly from a reaction between *n*-propyl gallate and the superoxide anion radical, this reaction is not an electron (or H atom) transfer. The only likely alternative mechanism is addition. A possible mechanism is tentatively outlined in Scheme 1, at this stage the precise site of addition is not known, although C-4 seems a reasonable choice in terms of nucleophilicity.

Such reactions have been proposed to take place in aprotic solvents with benzil<sup>9</sup> and dehydro ascorbic acid.<sup>19</sup> In these systems, the initial addition product (II in this case) is believed to rearrange, reaction (10) illustrates the equivalent rearrangement in the present system. Compound III will undergo further reactions, e.g. addition of oxygen, rapid protonation, elimination of OPr<sup>-</sup> followed by dimerisation or dismutation. Product analysis is being undertaken to elucidate the fate of III. It is also intended to

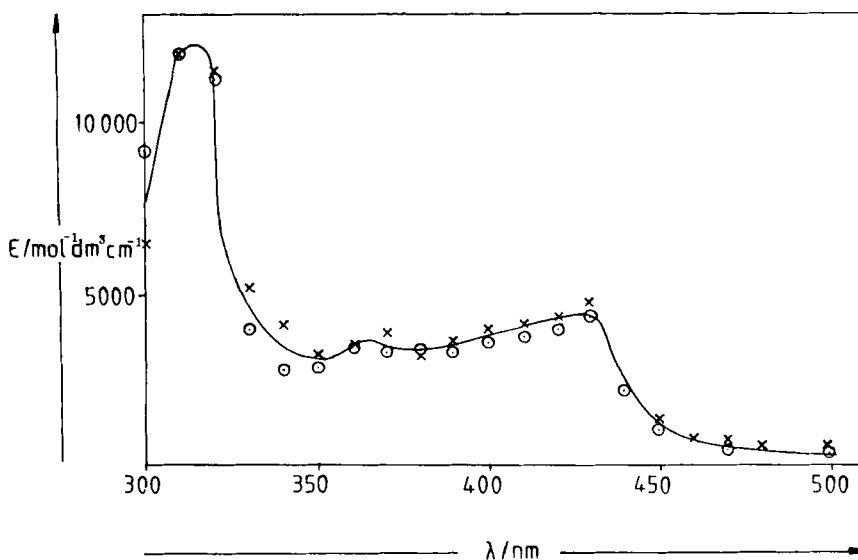
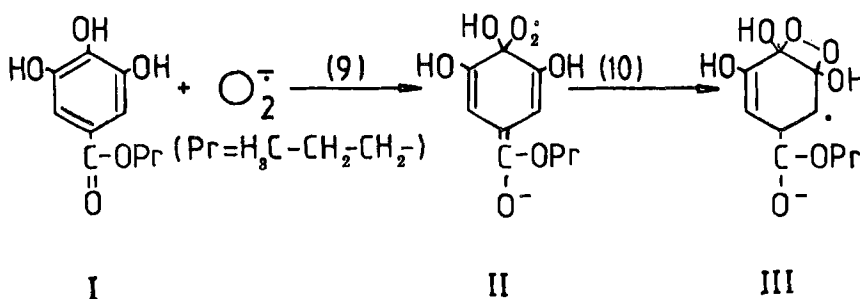


FIGURE 4 Transient spectra from *t*-butyl hydroquinone. X, *t*-butyl hydroquinone phenoxyl radical formed by reaction with azide radicals,  $5 \times 10^{-2} \text{ mol dm}^{-3}$  sodium azide,  $2 \times 10^{-3} \text{ mol dm}^{-3}$  phosphate buffer pH 7,  $5 \times 10^{-5} \text{ mol dm}^{-3}$  *t*-butyl hydroquinone,  $\text{N}_2\text{O}$  saturated,  $40 \mu\text{s}$  after pulse, 2.3 Gy/pulse,  $\epsilon$  based on  $G(\text{phenoxyl radical}) = 6.0$ , transient formed on reaction of  $\text{O}_2^-$  with *t*-butyl hydroquinone.  $0.2 \text{ mol dm}^{-3}$  formate,  $2 \times 10^{-3} \text{ mol dm}^{-3}$  phosphate pH 7,  $1 \times 10^{-3} \text{ mol dm}^{-3}$  *t*-butyl hydroquinone,  $\text{O}_2$  saturated,  $400 \mu\text{s}$  after pulse, 6 Gy/pulse,  $\epsilon$  based on  $G(\text{O}_2^- + \text{t-butyl hydroquinone}) = 6.5$ .



investigate the effect of various ring substituents on the addition reaction, with the intention of learning more about the factors governing this interesting process.

In conclusion it is perhaps worth mentioning that the relatively high efficiency with which *n*-propyl gallate reacts with  $\text{O}_2^-$  may have some importance in its action as an anti-oxidant in foods.

### References

1. Sawyer, D.T. and Nanni, E.J. in *Oxygen and Oxyradicals in Chemistry and Biology*, eds. M.A.J. Rodgers and E.L. Powers (Academic Press, New York, 1981). p. 15-40.
2. Afanas'ev, I.B. *Russian Chemical Reviews*, **48**, 977 (1979).

3. Rabani, J., Mulac, W.A. and Matheson, M.S. *J. Phys. Chem.*, **69**, 53 (1976).
4. Patel, K.B. and Willson, R.L. *J. Chem. Soc., Faraday Trans., I*, 814 (1973).
5. Rao, P.S. and Hayon, E. *J. Phys. Chem.*, **79**, 397 (1975).
6. Bielski, B.H.J., Arudi, R.L. and Cabelli, D.E. in *Oxygen Radicals in Chemistry and Biology*, eds. W. Bors, M. Saron and D. Tait (de Gruyter and Co., Berlin, 1984) p. 1–13.
7. Symons, M.C.R., Eastland, G.W. and Denny, L.R. *J. Chem. Soc., Faraday I*, **76**, 1868 (1980).
8. Johnson, R.A. *Tetrahedron Lett.*, 331 (1976).
9. Sawyer, D.T., Stamp, J.J. and Menton, K.A. *J. Org. Chem.*, **48**, 3733 (1983).
10. Keene, J.P. *J. Sci. Inst.*, **41**, 493 (1964).
11. Butler, J., Hoey, B.M. and Swallow, A.J. *FEBS Letters*, **182**, 95 (1985).
12. Buxton, G.V. in *The study of fast processes and transient species by electron pulse radiolysis*, eds. J.H. Baxendale and F. Busi (NATO Advanced Study Institute 1981 – Capri, Italy) published by D. Reidel Publishing Co. 1982, p. 241–266.
13. Wardman, P. in *Radiation Biology and Chemistry* (Proceedings of the Association for Radiation Research. Winter meeting 1979) eds. H.E. Edwards, S. Navaratnam, B.J. Parsons, G.O. Phillips (Elsevier Scientific Publishing Co. Amsterdam, 189 (1979).
14. Schuchmann, M.N. and von Sonntag, C. *J. Phys. Chem.*, **83**, 780 (1979).
15. Bielski, B.H.J. *Photochem. Photobiol.*, **28**, 645 (1978).
16. Hoey, B.M. and Butler, J. *BBA*, 212 (1984).
17. Burton, G.W., Doba, T., Gabe, E.J., Hughes, L., Lee, F.L., Prasad, L. and Ingold, K.U. *J. Am. Chem. Soc.*, **107**, 7053 (1985).
18. Deeble, D.J. and Parsons, B.J. unpublished results.
19. Sawyer, D.T., Chiericato, G. and Tsuchiya, T. *J. Am. Chem. Soc.*, **104**, 6273 (1982).

**Accepted by Prof. H. Sies**