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FREE RADICALS AND SINGLET OXYGEN SCAVENGERS: REACTION OF A PEROXY-RADICAL WITH β-CAROTENE, DIPHENYL FURAN AND 1,4-DIAZOBICYCLO(2,2,2)-OCTANE.

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SUMMARY: The 'singlet oxygen scavengers'. 1,4-diazobicyclo(2,2,2)-octane (DABCO), diphenyl furan and β-carotene react rapidly with the organic peroxy-radical $CCl_3O_2^{\bullet}$. The absolute reaction rate constants k = 1.2 ± 0.2 x 10^7, 6 ± 2 x 10^7 at 1.5 ± 0.2 x 10^9 $M^{-1}s^{-1}$ respectively have been determined by pulse radiolysis. Comparison with other data suggest that other free radicals are also likely to react with these compounds; in the case of the hydroxyl radical and DABCO k = 1.25 x 10^9 $M^{-1}s^{-1}$ has been determined.

Interest in the role of reactive oxygen species in the initiation and progression of various types of tissue injury has increased rapidly in recent years (1,2). For example, superoxide anion radicals ($O_2^{-\bullet}$) have been implicated in the inflammatory process (3), in paraquat intoxication (4), and in haemolysis of erythrocytes (5) obtained from animals deficient in vitamin E; hydroxyl radicals in radiation-induced tissue injury (6), in bleomycin toxicity (7), and in NADPH-linked microsomal peroxidation (8), singlet oxygen in photosensitisation reactions (9), and in phagocytosis (10).

Much of the evidence for the involvement of $O_2^{-\bullet}$, $OH^{\bullet}$ and $^1O_2$ in biological systems is dependent on studies that have used 'selective scavengers', that is agents with a strongly preferential reactivity towards one or another of the reactive oxygen species mentioned above. This approach, however, has attracted a few cautionary statements (11).

In the case of singlet oxygen ($^1O_2$), the fact that a particular chemical or biological process is inhibited by the presence of β-carotene, diphenylfuran and/or 1,4-diazobiocyclo-(2,2,2)-octane (DABCO) has been used as

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a strong, sometimes the only argument for singlet oxygen involvement. We now report the direct observation of the rapid reaction of an organic peroxy-radical with these so-called 'selective scavengers' for singlet oxygen. Our results point very clearly to the need for caution in interpreting experimental results with complex biological systems based largely on inhibitions produced by these substances.

MATERIALS AND METHODS: Chemicals: β-carotene (Sigma), diphenylfuran (DPF, Eastman) and 1,4,diazobicyclo(2,2,2)-octane, (DABCO, Merck), were used without further purification. Carbon tetrachloride was of AnalaR grade and t-butanol was purified by recrystallization. Solutions of β-carotene (0-10μM) final concentration) were passed through Amicon microfilters before use, and the concentrations checked by absorption spectrophotometry.
Pulse radiolysis: the Brunel 4 MeV, 200 ns pulsed linear accelerator with its associated equipment for kinetic spectroscopy over the range 250-650 nm and computer analysis was used as described previously (12). Pulse doses were in the range 0.3-2.5 k.rads. Preliminary near infra-red studies with β-carotene in the region 650-1,100 nm were kindly undertaken by Dr.E.J.Land at the Paterson Laboratories, Christie Hospital Manchester.

All irradiated solutions contained carbon tetrachloride (0.1 M), were freshly prepared using air saturated t-butanol:water (1:1) as solvent. Under the conditions used the trichlormethyl peroxy-radical $CCl_3O_2^\bullet$ was formed within 1 μsecond (ca 3 μM per k.rad) (13,14).Its reactions with other substrates were followed spectrophotometrically either by observing the formation of the reaction products (diphenyl furan and DABCO) or the loss of the ground state absorption of the reacting substrate (β-carotene).

$$H_2O \xrightarrow{\text{radiation}} OH^\bullet + e_{aq}^- + H^+$$

$$OH^\bullet + \text{t-butanol} \longrightarrow \text{products (no change in absorption)}$$

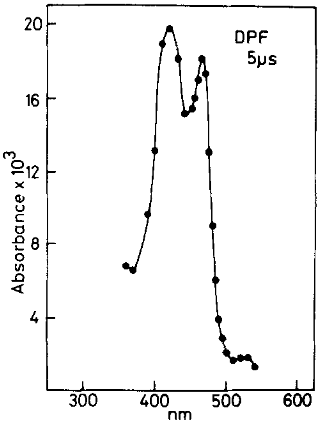
$$e_{aq}^- + CCl_4 \longrightarrow CCl_3^\bullet + Cl^-$$

$$CCl_3O_2^\bullet + O_2 \longrightarrow CCl_3O_2^\bullet$$

$$CCl_3O_2^\bullet + \text{substrate} \longrightarrow \text{products (change in absorption)}$$

RESULTS AND DISCUSSION: On pulse radiolysis of air-saturated aqueous solutions containing t-butanol (4 M) and CCl_4 (0.1 M), no change in absorption was apparent in the wavelengths range 300-600 nm. In the presence of diphenylfuran (4×10^{-3} M) strong transient absorptions (λ_{max} 410 and 465 nm) were observed (Figure 1). The absorptions were formed at an exponential rate, and further studies showed that the rate of formation was first order with respect to the diphenylfuran concentration corresponding to :

$$CCl_3O_2^\bullet + DPF \longrightarrow \text{Products} \qquad k = 6 \pm 2 \times 10^7\ M^{-1}s^{-1} \qquad (1)$$



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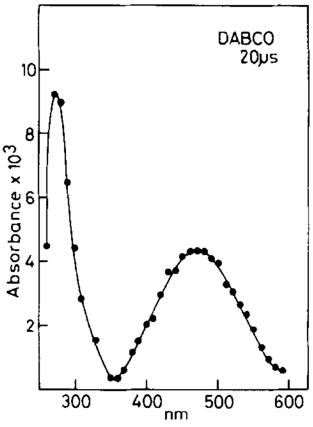

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Fig. 1 Transient absorption spectra observed 5 μseconds after pulse radiolysis of a neutral solution containing 0.1M CCl_4 and 5 x 10^{-3}M diphenylfuran.

Fig. 2 Transient absorption spectrum observed 20 μseconds after pulse radiolysis of a neutral solution containing 0.1M CCl_4 and 5 x 10^{-3}M DABCO.

Similar experiments with DABCO gave weaker absorptions at 270 and 460 nm; the spectrum was identical to that seen following the reaction of DABCO with $OH^\bullet$ (Figure 2). The rates of formation of these absorptions were proportional to the concentration of DABCO, corresponding to:

$$CCl_3O_2^\bullet + \text{DABCO} \longrightarrow \text{products} \qquad k = 1.2 \pm 0.2 \times 10^7\ M^{-1}s^{-1} \qquad (2)$$

On pulse radiolysis of β-carotene in 50% t-butanol:50% water containing 0.1 M CCl_4 a rapid bleaching of the ground state absorption of the β-carotene at 450 nm was observed. Kinetic studies were restricted by the very low solubility of the compound in our systems, and by its very high extinction coefficient. Studies with up to 5 μM β-carotene were carried out at 450 nm; with 5-10 μM the absorption changes were followed at 500 nm. The observed bleaching occurred exponentially with time (Figure 3) with the rate first order in β-carotene concentration corresponding to:

$$CCl_3O_2^\bullet + \beta\text{-carotene} \longrightarrow \text{products} \qquad k = 1.5 \pm 0.2 \times 10^9\ M^{-1}s^{-1} \qquad (3)$$

Clearly, the $CCl_3O_2^\bullet$ free radical reacts rapidly with DABCO, diphenylfuran and β-carotene. Although other studies (15) have shown that

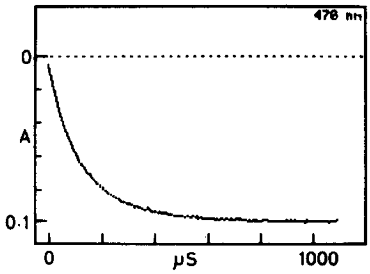

Fig. 3 Computer print-out of absorbance against time showing the loss of the ground state absorption at 470nm of β carotene (5 μM)following its reaction with $CCl_3O_2^{\bullet}$ (<u>ca</u> 1μM). Pulse dose 500 rads ($5Jkg^{-1}$), cell length 1.5 cm, 0.01M carbon tetrachloride.

$CCl_3O_2^{\bullet}$ is more reactive than $CHCl_2O_2^{\bullet}$, $CH_2ClO_2^{\bullet}$ and $CH_3O_2^{\bullet}$ towards phenothiazines, vitamin C and amino acids, the high rate constants obtained in this study suggest that other peroxy-radicals would also react with DABCO, diphenylfuran and β-carotene, although probably more slowly.

Using the pulse radiolysis thiocyanate competition method (16) we have also determined the rate constant for the reaction :

$$OH + DABCO \longrightarrow products \qquad k = 6.5 \pm 1.0 \times 10^9\ M^{-1}s^{-1}$$

a value slightly higher than $k = 1.25 \times 10^9\ M^{-1}s^{-1}$ previously reported (17) The low solubility in water of diphenylfuran and β-carotene prevents accurate pulse radiolysis studies on their reaction of $OH^{\bullet}$. However, as in the case of DABCO where the rate constants of reaction of $OH^{\bullet}$ and $CCl_3O_2^{\bullet}$ have been determined with a particular solute those for $OH^{\bullet}$ are higher. It is very likely therefore the rate constants for reaction of $OH^{\bullet}$ with β-carotene and diphenylfuran are higher than those for $CCl_3O_2^{\bullet}$:

$$OH^{\bullet} + diphenylfuran \longrightarrow products \qquad k > 10^8\ M^{-1}s^{-1}$$

$$OH^{\bullet} + \beta\text{-carotene} \longrightarrow products \qquad k > 10^9\ M^{-1}s^{-1}$$

The products of these reactions remain to be characterised. Early experiments indicate that in the case of $CCl_3O_2^{\bullet}$ and DABCO or diphenylfuran the absorbing products react readily with ascorbate, a property characteristic of many radical-cations (18). In the case of β-carotene preliminary studies in the near infra-red region show that the loss of β-carotene absorption observed at 450 nm corresponds in time with an increase in absorption at 950-1,000 nm., the known maximum of the β-carotene radical-cation (19). It is thus likely that in these instances, as in the case of the phenothiazines chlorpromazine and promethazine studied previously (15), $CCl_3O_2^\bullet$ reacts by an electron transfer mechanism :

$$CCl_3O_2^\bullet + \text{DABCO} \longrightarrow CCl_3O_2^- + \text{DABCO}^{\bullet+}$$

$$CCl_3O_2^\bullet + \text{diphenylfuran} \longrightarrow CCl_3O_2^- + \text{diphenylfuran}^{\bullet+}$$

$$CCl_3O_2^\bullet + \beta\text{-carotene} \longrightarrow CCl_3O_2^- + \beta\text{-carotene}^{\bullet+}$$

Whatever the mechanism however, the high rate constants of these reactions clearly confirm that in complex biochemical systems, evidence other than the fact that a particular reaction is affected by DABCO, diphenylfuran or β-carotene must be obtained before it can be concluded that singlet oxygen is the involved reactive species.

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REFERENCES

1. Oxygen Free Radicals and Tissue Damage. Ciba Foundation Symposium 65 (1979) ed. D.W.FitzSimons, Excerpta Medica, Amsterdam, Oxford, New York.
2. Biological Reactive Intermediates (1977) eds. Jollow, D.J., Kocsis, J.J., Snyder, R. and Vainio, H. Plenum Press, New York.
3. McCord, J.M. (1974) Science, 185, 529-531.
4. Bus, J.S., Aust, S.D. and Gibson, J.G. (1974) Biochem.Biophys.Res. Commun., 58, 749-755.
5. Fee, J.A. and Teitelbaum, H.D. (1972) Biochem.Biophys.Res.Commun. 49, 150-158.
6. Weiss, J. (1944) Nature (London) 153, 748-750.
7. Lown, J.W. and Sim, S. (1977) Biochem.Biophys.Res.Commun., 77, 1150-1157.
8. Fong, K. McCoy, P.B., Poyer, J.L., Keele, B.B. and Miscra, H. (1973). J.Biol.Chem., 248, 7792-7797.
9. Nilsson, R., Merkel, P.B. and Kearns, D.R. (1972) Photochem.Photobiol., 16, 117-124.
10. Allen, R.C., Stjernholm, R.L. and Steele, R.H. (1972) Biochem.Biophys.Res. Commun., 47, 679-684.
11. Discussion in Proc. Int. Conf. on Singlet Oxygen and Related Species in Chemistry and Biology (1978) Photochem.Photobiol., 28, 921-933.
12. Willson, R.L. in Biochemical Mechanisms of Liver Injury (1978) ed. T.F.Slater. Academic Press, London, New York and San Francisco. pp.124-224.
13. Packer, J.E., Slater, T.F. and Willson, R.L. (1978) Life Sciences, 23, 2617-2620.
14. Packer, J.E., Slater, T.F. and Willson, R.L. (1979) Nature, 278, 737-738.

15. Packer, J.E., Willson, R.L., Bahnemann, D. and Asmus, K-D. (1980) J.Chem.Soc.Perkin II, 296-299.
16. Adams, G.E., Boag, J.W., Currant, J. and Michael, B.D. (1965) in Pulse Radiolysis. eds. M.Ebert, J.P.Keene, A.J.Swallow and J.H.Baxendale. Academic Press, New York and London, pp.131-143.
17. Anderson, R.F. and Patel, K.B. (1978) Photochem.Photobiol. 28, 881-885.
18. Mahood, J.S., Packer, J.E., Searle, A.J.F., Willson, R.L. and Wolfenden, B.S. (1980) in Phenothiazines and Structurally Related Drugs: basic and clinical studies, eds. Usdin, Eckert and Forrest. Elsevier, North Holland, pp.103-106.
19. Dawe, E.A. and Land, E.J. (1975) J.Chem.Soc.Faraday I, 71, 2162-2169.