Biochemical Pharmacology, Vol. 38, No. 9, pp. 1465–1469, 1989.
Printed in Great Britain.

0006-2952/89 $3.00 + 0.00
Pergamon Press plc

A METHOD FOR THE COMPARATIVE ASSESSMENT OF ANTIOXIDANTS AS PEROXYL RADICAL SCAVENGERS

V. M. DARLEY-USMAR,* A. HERSEY and L. G. GARLAND
Biochemical Sciences and Physical Sciences, Wellcome Research Laboratories, Beckenham, Kent, U.K.

(*Received* 30 *August* 1988; *accepted* 15 *November* 1988)

Abstract—Antioxidants which are peroxyl radical scavengers have been compared in a model of lipid peroxidation based on the oxidation of a suspension of linoleic acid initiated by a thermolabile azo compound. By analysing the effect of antioxidant concentration on linoleic acid peroxidation we have defined the constant k_{AH} which characterises the rate of the reaction of the antioxidant with the peroxyl radical. This allows quantitative comparison of the efficiency of different antioxidants as peroxyl radical scavengers. By using an initiation system which is not iron dependent we were able to show that the iron chelators desferrioxamine, BW A4C and U74500A are also peroxyl radical scavengers.

Lipid peroxidation is a self propagating reaction which can result in loss of membrane function and integrity [1, 2, 3]. Among the pathological conditions with which it has been associated are exposure of the cell to the redox cycling drug adriamycin® (doxorubicin hydrochloride)† and the hepatotoxic agent carbon tetrachloride [4, 5]. Peroxidation of the lipid in low density lipoproteins and the subsequent formation of foam cells from macrophages may play an important role in the pathogenesis of atherosclerosis [6]. In these examples the initiation of peroxidation is thought to occur by hydrogen atom abstraction from unsaturated lipids followed by reaction of the lipid radical with oxygen to form the peroxyl radical [2, 3]. The peroxidation reaction then continues to be propagated by this radical unless it is scavenged by a suitable antioxidant. Such compounds inhibit the peroxidation reaction and it is by this mechanism that vitamin E exerts its biological effects [7].

It has recently been recognized that a wide spectrum of therapeutic agents may have the capacity to scavenge peroxyl radicals and so act as chain-breaking antioxidants [8]. This property can be determined by pulse radiolysis but this involves the use of specialised equipment [8]. A simpler approach is to initiate peroxidation in a suspension of lipid membranes supplemented with iron and measure the formation of thiobarbituric acid reactive material [9]. This method suffers two drawbacks; firstly the method itself lacks specificity and secondly compounds which are iron chelators can, in principle, act to inhibit peroxidation by either iron chelation or by radical scavenging [10].

To overcome this problem we have adapted the TRAP assay, described by Ingold and co-workers [11–13], to allow comparison of different compounds as peroxyl radical scavengers. In this method peroxidation is initiated by use of a thermolabile azo compound which, on decomposition, forms radicals that are able to abstract hydrogen atoms from linoleic acid [11]. This assay has been used by other workers to determine the antioxidant capacity of serum samples and linoleic acid was used as carrier for the reaction [11]. By omitting the serum and increasing the linoleic acid concentration we have been able to measure peroxidation, in the absence and presence of antioxidant, by monitoring the oxygen uptake. By algebraic manipulation of the reaction scheme describing peroxidation in this system we have derived an equation which describes the effect of increasing antioxidant concentration on oxygen uptake. From this analysis and the resulting fit to the experimental data we have determined a composite rate constant, k_{AH}, which is characteristic of the rate of reaction of peroxyl radical with antioxidant. This then provides a simple method for comparing the ability of different antioxidants to scavenge peroxyl radicals.

MATERIALS AND METHODS

Peroxyl radical scavenging assay

Peroxidation of a suspension of linoleic acid (52 mM) in 50 mM phosphate pH 7.4 was initiated with 11 mM 2′2′-azobis-2-amidinopropane hydrochloride (ABAP) at 37° in the cuvette of an oxygen electrode [11]. Oxygen uptake was monitored for approximately 5 min and then the antioxidant added. The oxygen uptake due to the electrode was determined in a separate experiment and subtracted from the rates measured during the peroxidation of linoleic acid. The rate of oxygen uptake after addition of antioxidant was divided by the initial rate. This ratio was then determined for a range of antioxidant concentrations. Endogenous iron was removed by dialysis against the iron binding protein conalbumin as described in Ref. 14.

Derivation of an equation governing the dependence of oxygen consumption on antioxidant concentrations. The following simplified scheme has been derived as a means of comparing the peroxyl radical scavenging ability of different antioxidants and is similar to that described by other workers [15]. In the following scheme R = the initiator ABAP, LH = linoleic acid and AH = antioxidant:

* To whom correspondence should be addressed.

† Adriamycin is a registered trademark of Farmitalia Carlo Erba.

$$R_2 \xrightarrow{k_1} 2R^{\cdot}$$

$$R^{\cdot} + LH \xrightarrow{k'} L^{\cdot} + RH \quad \text{Initiation}$$

$$L^{\cdot} + O_2 \xrightarrow{k_2} LO_2^{\cdot}$$

$$LO_2^{\cdot} + LH \xrightarrow{k_3} L^{\cdot} + LO_2H \quad \text{Propagation}$$

$$LO_2^{\cdot} + LO_2^{\cdot} \xrightarrow{k_4} \text{Products Termination.}$$

If it is assumed that [R˙], [L˙] and [$LO_2^{\cdot}$] are at steady state it follows that:

$$\frac{d[L^{\cdot}]}{dt} = 0 = 2k_1[R_2] - k_2[L^{\cdot}][O_2] + k_3[LO_2^{\cdot}][LH] \quad (1)$$

$$\frac{d[LO_2^{\cdot}]}{dt} = 0 = k_2[L^{\cdot}][O_2] - k_3[LO_2^{\cdot}][LH] - 2k_4[LO_2^{\cdot}]^2 \quad (2)$$

and

$$\frac{d[O_2]}{dt} = -k_2[L^{\cdot}][O_2]. \quad (3)$$

Hence:

$$\frac{-d[O_2]}{dt} = 2k_1[R_2] + k_3[LH]\left(\frac{k_1[R_2]}{k_4}\right)^{\frac{1}{2}} \quad (4)$$

The lipid peroxidation reaction can be monitored by determining the rate of oxygen uptake as shown in Eqn (4).

In the presence of a chain-breaking antioxidant several reactions are possible [13, 15] but the major reactions by which these compounds inhibit peroxidation are assumed to be:

$$LO_2^{\cdot} + AH \xrightarrow{k_5} LO_2H + A^{\cdot}$$

$$A^{\cdot} + LO_2^{\cdot} \xrightarrow{k_6} LO_2A.$$

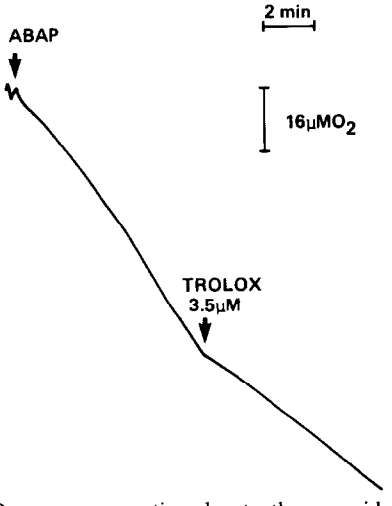

Fig. 1. Oxygen consumption due to the peroxidation of linoleic acid and the effect of the antioxidant trolox. Peroxidation of linoleic acid was initiated by ABAP and the O_2 monitored in an O_2 electrode. After 8 min trolox was added to give a final concentration of 3.5 μM.

From this it follows that each antioxidant molecule is able to scavenge two peroxyl radicals. It is assumed that the radical A˙ is unable to abstract hydrogen atoms from linoleic acid and the propagation reaction is inhibited so causing the rate of oxygen uptake to decrease. If, again, a steady state is assumed for [R˙] [L˙], [$LO_2^{\cdot}$] and also [A˙] it can be shown that:

$$\frac{d[O_2]}{dt} = 2k_1[R_2] + k_3\frac{[LH]}{2k_4}[-k_5[AH] + (k_5^2[AH]^2 + 4k_1k_4[R_2])^{\frac{1}{2}}] \quad (5).$$

By comparing the rate of oxygen uptake in the presence and absence of antioxidant at constant initiator and constant linoleic acid concentrations a composite rate constant can be evaluated. This allows the effectiveness of the compounds as peroxyl radical scavengers to be compared. By taking the ratio of Eqns (4) and (5) and simplifying the expression it can be shown that:

$$\frac{\text{The rate of oxygen uptake in the presence of antioxidant}}{\text{The rate of oxygen uptake in the absence of antioxidant}} = 1 - \beta\{(k_{AH}[AH]+1) - (k_{AH}^2[AH]^2+1)^{\frac{1}{2}}\} \quad (6)$$

where

$$\beta = \frac{k_3[LH]}{(4k_1k_4[R_2])^{\frac{1}{2}} + k_3[LH]}$$

and

$$k_{AH} = \frac{k_5}{(4k_1k_4[R_2])^{\frac{1}{2}}}.$$

β is independent of the nature of the antioxidant and the term k_{AH} which contains the rate constant k_5, therefore characterises the reaction of antioxidant with the peroxyl radical.

RESULTS AND DISCUSSION

Determination of the peroxyl radical scavenging properties of antioxidants. Figure 1 shows the consumption of O_2 with time when a suspension of linoleic acid was oxidised after initiation by ABAP. After the first few minutes of initiation of peroxidation the rate of oxygen uptake was linear with time and remained so until almost all of the available O_2 was consumed (result not shown). When an antioxidant such as trolox, a vitamin E analogue, was added the rate of O_2 consumption was decreased and remained constant at the lower rate (Fig. 1). The rate of O_2 uptake was determined for 2–3 min preceding the addition of antioxidant and for the same period of time after addition. Our analysis of these rates depends on the assumption of a steady-state over the time period of measurement. The fact that the rate of O_2 uptake is constant under these conditions supports the assumption that the reactions are proceeding under steady-state conditions. Increasing

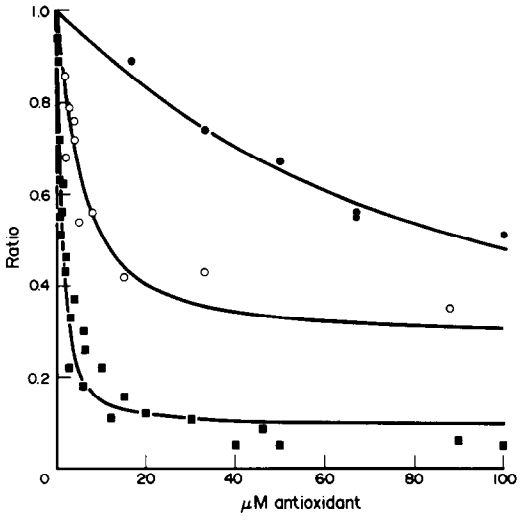

Fig. 2. Inhibition of peroxidation of a linoleic acid suspension in the presence of antioxidants. Peroxidation was initiated with ABAP and the rate of oxygen uptake measured for 5–10 min after which the antioxidants BHT (■), BW A4C (○) or desferrioxamine (●) were added. The ratio of O_2/min uptake after addition of antioxidant to the uninhibited rate was calculated and plotted against antioxidant concentration. The curves in the figure were fitted to the data points using Eqn (6).

Table 1. A comparison of different antioxidants as peroxyl radical scavengers

Antioxidant	k_{AH}/mol	β
BHT	0.75 ± 0.10	0.90 ± 0.02
Trolox	0.28 ± 0.03	0.96 ± 0.03
Desferr	0.01 ± 0.0006	0.85 ± 0.01
Desferr/Fe	0.0036 ± 0.0005	0.87 ± 0.04
U74500A	0.095 ± 0.021	0.80 ± 0.04
Nafazatrom	0.42 ± 0.05	0.94 ± 0.03
BW A4C	0.14 ± 0.02	0.72 ± 0.03
NDGA	0.27 ± 0.04	0.98 ± 0.05
Probucol	0.27 ± 0.06	0.71 ± 0.03

The constant k_{AH} is a measure of the radical scavenging ability of the antioxidant whereas β is a constant independent of the nature of the antioxidant. The constants were evaluated from Eqn (6) by an iterative fitting procedure in which the residual sum of squares was minimized. The values are reported with the SE and $P < 0.005$ for all fitted values.

Desferr = desferrioxamine, Desferr/Fe = the desferrioxamine 1:1 Fe complex. BHT = butylated hydroxy toluene, NDGA = nordihydroguaiaretic acid, BW A4C = *N*-(3-benzylcinnamyl)acetohydroxamic acid, U74500A = 21-[4-(3,6-bis(diethylamino)-2-pyridinyl)-1-piperazinyl]-16-methylpregna-1,4,9(11)triene-3,20-dione hydrochloride, probucol = 4,4′-[(1-methylethylidine)bis(thio)-bis[2,6-bis(1,1-dimethylethyl)phenol].

the antioxidant concentration progressively decreased the rate of O_2 consumption as shown for butylated hydroxy toluene (BHT), BW A4C and desferrioxamine in Fig. 2. Also shown in Fig. 2 is the fit of the data to Eqn (6) for the inhibition of oxygen uptake by the antioxidants (for the derivation of this equation see Materials and Methods).

The two constants which were evaluated from the model described above are k_{AH} which characterises the rate of the reaction of antioxidant with peroxyl radical and β which is antioxidant independent. The calculation of k_{AH} is necessary since the curves relating inhibition of O_2 uptake to antioxidant concentration (Fig. 2) are defined both by the antioxidant-dependent parameter k_{AH} and antioxidant-independent parameter β. These are reported in Table 1 for a number of antioxidants. The rate constant k_{AH} shows a range of approximately 200-fold for the compounds tested. The values reported in Table 1 for both k_{AH} and β were determined by an iterative fitting procedure and the SE reported in the table represent the significance of the fit for a given experiment. All fits were significant at a level of $P < 0.005$ or less. Thus, although the reaction scheme we have used does not include all the possible interactions which may occur in this chemically complex model and is therefore a simplification, it does appear to contain those steps which are important in determining the rate of incorporation of O_2 into linoleic acid. Trolox was tested in four different experiments and the standard error of the mean for k_{AH} and β was found to be 4% and 3%, respectively.

The structures of the compounds we have tested are shown in Fig. 3. It is well known that phenols are able to donate a hydrogen atom and thereby convert the peroxyl radical to a hydroperoxyl group [12]. The compounds studied here which fall into this category are BHT, probucol, NDGA, trolox and possibly nafazatrom which can tautomerise to a phenol. The iron chelators desferrioxamine and BW A4C contain hydroxamate groups which can donate a hydrogen atom resulting in the formation of a stable nitroxide radical [16, 17]. Interestingly, the Fe-desferrioxamine complex was also able to scavenge the peroxyl radical albeit less efficiently (Table 1). The mechanism for scavenging by U74500A is unknown but may be through electron donation [18]. It is interesting to note that the iron chelators desferrioxamine, U74500A and BW A4C can all act as peroxyl radical scavengers.

The ability of the iron chelators, desferrioxamine, U74500A and BW A4C to inhibit the O_2 uptake reaction could depend on chelation of endogenous iron present in the apparatus if this were making a significant contribution to either initiation or propagation of the peroxidation reaction (2). To test for this possibility we attempted to remove or immobilize iron in two different ways. Firstly, we used the iron chelators EDTA (ethylenediaminetetraacetic acid) or DPTA (diethylenetriaminepentaacetic acid) which are able to chelate iron but do not prevent the generation of free radicals. The rate of O_2 uptake in the absence of the chelators was 12.04 ± 1.43 μM O_2/min (mean ± SD, N = 6) and was not significantly altered by either 200 μM EDTA (12.52 ± 0.58 μM O_2/min N = 3) or 200 μM DPTA (11.56 ± 2.02 μM O_2/min N = 3). Secondly, we dialysed the phosphate buffer against the iron binding protein conalbumin. This process removes endogenous iron from laboratory reagents and buffers [14]. We found that the O_2

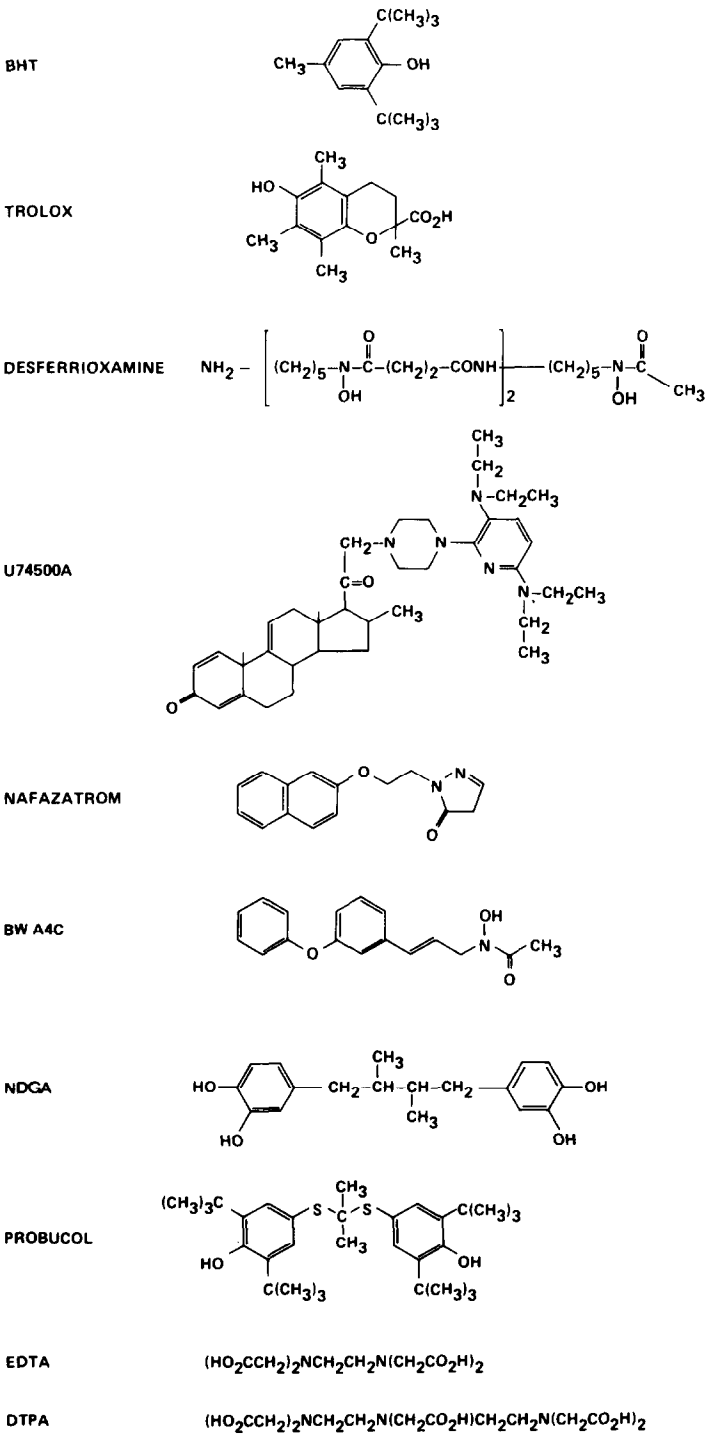

Fig. 3. Structures of compounds tested for peroxyl radical scavenging.

uptake after initiation in the conalbumin dialysed buffer was 13.8 ± 2.2 μM O_2/min (N = 6, mean ± SD) and for the undialysed buffer 12 ± 2.6 μM O_2/min (N = 6, mean ± SD). Since neither treatment affected the rate of linoleic acid oxidation it is unlikely that endogenous iron contributes to the peroxidation of linoleic acid in this system. It follows therefore, that any inhibitory effects of the iron chelators tested cannot be ascribed to their iron chelating properties.

It is interesting to note that BW A4C, nafazatrom and NDGA are all potent lipoxygenase inhibitors [19, 20]. It has been suggested that site-directed peroxyl radical scavenging in the lipoxygenase

enzyme may be an important mechanism for inhibition of this protein and our results would tend to support that view [21].

In summary we have described a system in which the efficacy of different antioxidants as peroxyl radical scavengers can be quantitated and compared. A further advantage is that, since the initiation of peroxidation is not iron-dependent, then the peroxyl radical scavenging of iron chelators can be studied without the added complication of iron binding.

Acknowledgements—We would like to thank Dr M. T. Wilson, Dept. of Chemistry, University of Essex and Mr J. Wood, Wellcome for their helpful discussions. We would also like to express our gratitude for the gifts of desferrioxamine from Ciba-Geigy pharmaceuticals, probucol from Merrell-Dow and U74500A from Prof. C. A. Gandolfi of Boehringer Biochemia Robin.

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