Quantitative Treatment of the Kinetics of Free-Radical-Mediated Damage. Protection by Free-Radical Scavengers

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Dedicated to Professor *André M. Braun* on the occasion of his 60th birthday

Equations are derived to quantitatively describe the effect of a free-radical scavenger upon the rate of a radical-mediated process that senses the steady-state free-radical concentration. The dependence of the ratio R°/R (where R° is the rate of the process in the absence of additive) upon the additive concentration depends upon the type of reaction that determines the free-radical lifetime. Normal *Stern-Volmer*-like behavior is expected only when the lifetime of the radical in the absence of free-radical scavengers is determined by the concentration of the substance employed as the reporter of the free-radical concentration. These predictions are tested in a system comprised of 2,2′-azobis[2-methylpropanimidamide dihydrochloride) as the free-radical source, *c*-phycocyanin as the reporter molecule, and *Trolox* (=3,4-dihydro-6-hydroxy-2,5,7,8-tetramethyl-2*H*-1-benzopyran-2-carboxylic acid) tryptophan and 4-methoxyphenol as peroxyl-radical scavengers. The data obtained with *Trolox* show that it behaves as a nearly ideal free-radical scavenger. On the other hand, the data obtained with tryptophan and 4-methoxyphenol as scavengers show, when plotted according to the *Stern-Volmer* equation, a strong downward curvature. These results are explained in terms of *c*-phycocyanin bleaching by scavenger-derived free radicals.

Introduction. – There is great interest in developing procedures to test the reactivity of antioxidants (X) and/or free-radical scavengers towards free radicals of biological relevance. Among the experimental approaches to carry out these evaluations stand direct methods, where the rate of the reaction given in *Eqn. 1* is directly measured, and competitive methods, where it is evaluated how the added substrate protects a reference compound (C) from being degraded by exposure to a controlled free-radical source.

$$R^{\bullet} + X \rightarrow RH\ (\text{or } R^{-}) + X^{\bullet} \qquad (1)$$

The first approach is usually limited by the short lifetime of the radicals and can only be applied to fast-kinetics techniques [1] or by measuring the consumption of 'stable' free radicals, such as DPPH (=2,2-diphenyl-1-(2,4,6-trinitrophenyl)hydrazyl) or the radical cation obtained in ABTS (=2,2′-azinobis[3-ethyl-2,3-dihydrobenzothiazole-6-sulfonic acid) oxidation [2–4]. Fast-kinetics measurements require pulsed techniques and sophisticated instrumentation that is not available in most laboratories. On the other hand, stable-radical reactions can usually be followed with standard spectrophotometric equipment. Drawbacks of these methodologies are a frequently complex kinetics [5] and the use of free radicals whose properties and reactivity patterns are very different from those of biologically relevant radicals. Competition techniques have become then widely employed, particularly those involving peroxyl radicals. In these techniques, the following prerequisites are required *i*) a controlled free-radical source whose free-radical production rate is determined only by the temperature and the initiator concentration, and *ii*) a target molecule whose degradation by free radicals can be easily evaluated. AAPH (=2,2′-azobis[2-methylpropanimidamide] dihydrochloride) has been the most frequently employed free-radical source in aqueous solutions [6–9]. This compound produces, in presence of oxygen, peroxyl radicals at a convenient rate (in the order of μM/min) and is very little influenced by the presence of catalysts, such as transition-metal ions [10][11]. A variety of molecules have been employed as reactive targets [6–10]. In most studies, *Trolox* (=3,4-dihyro-6-hydroxy-2,5,7,8-tetramethyl-2*H*-1-benzopyran-2-carboxylic acid) is employed as reference free-radical scavenger.

Let us consider a system comprising the free-radical source, the reactive target at a concentration [C], and the added scavenger at a concentration [X]. If R° is the rate of consumption of the target molecule in absence of additive, and R is the rate of consumption in the presence of additive, very simple steady-state considerations lead to the conclusion that *Stern-Volmer*-like behavior should be obtained when R°/R is plotted against the antioxidant concentration. In fact, this can be expected from the simple competition kinetics of *Eqn. 2*, where k_1 is the specific reaction rate constant for the reaction of the radicals with the added free-radical scavenger, and $(k_2\,[\mathrm{C}])^{-1}$ is the lifetime of the reactive radicals in the absence of additives. If *Eqn. 2* holds, a plot of R°/R *vs.* [X]/[C] should be linear, and its slope provides the value of (k_1/k_2). Accordingly, for a reference inhibitor, such as *Trolox* (T), *Eqn. 3* should hold, and the ratio of the slopes of the plots obtained employing the compound X $(\mathrm{Slope})_\mathrm{x}$ and the reference inhibitor $(\mathrm{Slope})_\mathrm{T}$ will be given by *Eqn. 4* and should be independent of the employed target molecule and its concentration.

$$R^\circ/R = 1 + k_1/k_2\,[\mathrm{X}]/[\mathrm{C}] \tag{2}$$

$$R^\circ/R = 1 + k_\mathrm{T}/k_2\,[\mathrm{T}]/[\mathrm{C}] \tag{3}$$

$$(\mathrm{Slope})_\mathrm{X}/(\mathrm{Slope})_\mathrm{T} = k_1/k_\mathrm{T} \tag{4}$$

In the present communication, we discuss when a simple equation such as *Eqn. 2* can be expected to hold, and test these predictions employing AAPH as free-radical source, c-phycocyanin as target molecule, and *Trolox*, 4-methoxyphenol, and tryptophan as free-radical scavengers.

Kinetics Scheme. – When a target molecule C is exposed to a free-radical source (AAPH) in the presence of a free-radical scavenger (X), the minimum set of reactions to be considered comprises the reactions of *Eqns. 5–8* [12][13]:

$$\mathrm{AAPH}\ (+\mathrm{O_2}) \rightarrow 2\ \mathrm{ROO^\bullet} \tag{5}$$

$$\mathrm{ROO^\bullet} + \mathrm{C} \rightarrow \text{bleaching} \tag{6}$$

$$\mathrm{ROO^\bullet} + \mathrm{X} \rightarrow \mathrm{X^\bullet} + \mathrm{ROOH} \tag{7}$$

$$2\ \mathrm{ROO^\bullet} \rightarrow \text{nonradical products} \tag{8}$$

In this scheme, and for simplicity, not considered were reactions of the radicals produced according to *Eqns. 6* and *7*, and the possible formation of alkoxyl radicals according to *Eqn. 8*. This reaction scheme predicts that the steady-state concentrations of the peroxyl radicals in the absence of additive, $(\mathrm{ROO}^\bullet)^\circ$, and in the presence of the additive, $(\mathrm{ROO}^\bullet)$, will be given by *Eqns. 9* and *10*, respectively, where R_5 is the rate of free radical production.

$$(\mathrm{ROO}^\bullet)^\circ = \{-k_6\,[\mathrm{C}] + ((k_6[\mathrm{C}])^2 + 4\,k_8\,R_5)^{0.5}\}/2\,k_8 \tag{9}$$

$$(\mathrm{ROO}^\bullet) = \{-(k_6\,[\mathrm{C}] + k_7\,[\mathrm{X}]) + ((k_6\,[\mathrm{C}] + k_7\,[\mathrm{X}])^2 + 4\,k_8\,R_5)^{0.5}\}/2\,k_8 \tag{10}$$

Since $R^\circ/R = (\mathrm{ROO}^\bullet)^\circ/(\mathrm{ROO}^\bullet)$, the ratio of rates will be equal to the ratio of the expressions given in *Eqns. 9* and *10*. This can be considered to be the general situation and shows that the effect of the additive is complex and does not conform, in general, to the simple *Stern-Volmer* equation. To see when this simple behavior holds, let us consider two limiting situations, obtained at very low and very high target-molecule concentrations [9][14].

Case A: Very Low C Concentration. In this limit, $R_8 \gg R_6$. This implies that most of the peroxyl radicals react by self-reactions, and hence *Eqn. 11* holds, and R° is given by *Eqn. 12*. In this limit, the rate of target-molecule degradation is a process that is first order in substrate [9][14]. Also, the order in AAPH is 0.5 [14]. The dependence of R°/R upon the scavenger concentration (X) is given by *Eqn. 13*.

$$(\mathrm{ROO}^\bullet)^\circ = (R_5/k_8)^{0.5} \tag{11}$$

$$R^\circ = k_6\,(R_5/k_8)^{0.5}\,[\mathrm{C}] \tag{12}$$

$$R^\circ/R = 2\,(R_5\,k_8)^{0.5}/\{-k_7\,[\mathrm{X}] + ((k_7\,[\mathrm{X}])^2 + 4\,k_8\,R_5)^{0.5}\} \tag{13}$$

The general *Eqn. 13* shows that, in this limit, the ratio R°/R is only function of the X concentration, being independent of the target-molecule concentration. At very high X concentration (when $R_7 \gg R_8$), the ratio R°/R reduces to the expression given by *Eqn. 14*. A plot of R°/R *vs.* [X] has then the profile shown in *Fig. 1*. According to *Eqn. 13*, at very high X concentration, the data converge to a straight line of ordinate zero and slope given by $k_7/(R_5\,k_8)^{0.5}$.

$$R^\circ/R = (R_5\,k_8)^{-0.5}\,k_7\,[\mathrm{X}] \tag{14}$$

Case B: Very High C Concentration. In this limit $R_6 \gg R_8$, and all the peroxyl radicals interact with the target molecule. Moreover, *Eqn. 15* holds, and the bleaching rate becomes zero-order in the target molecule and first-order in AAPH concentration [14]. The ratio R°/R is, over the entire X-concentration range, given by *Eqn. 16*, which conforms to the simple *Stern-Volmer*-like relationship (*Eqn. 2*).

$$R^\circ = R_5 \tag{15}$$

$$R^\circ/R = 1 + (k_7/k_6)\,[\mathrm{X}]/[\mathrm{C}] \tag{16}$$

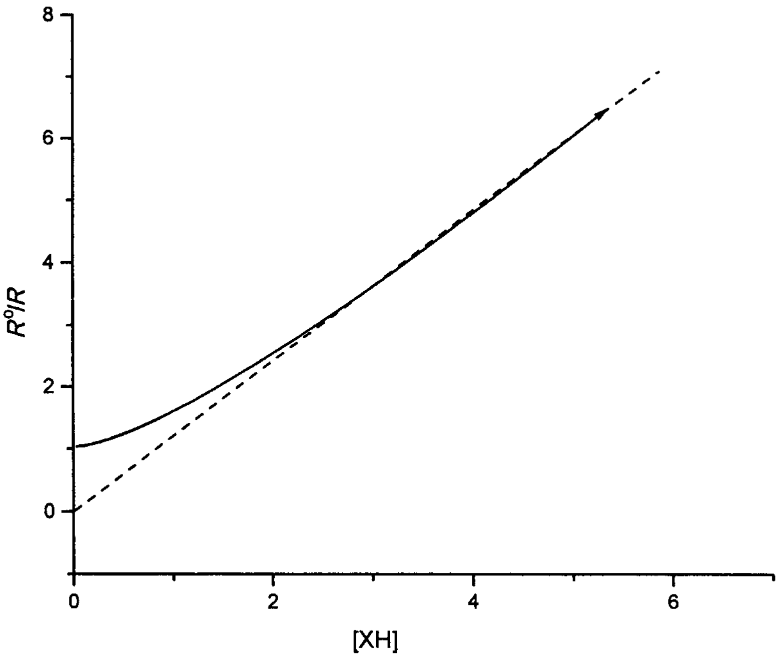

Fig. 1. *Dependence of the* R°/R *ratio upon the additive concentration for a system in which the bleaching of the substrate is in the first-order limit*

The above kinetics analysis shows that, only when the concentration of the target molecule is high enough as to reach the zero-order limit, a simple relationship between R°/R and the X/C concentration over the entire X range can be expected. However, irrespective of the substrate concentration, a linear relationship between R°/R and the X concentration can be expected when $R^\circ/R \gg 1$.

Materials and Methods. – C-Phycocyanin (c-Pc; *Sigma Chemical Co.*) from *Spyrulina* species, AAPH (*Wako Chemicals*), *Trolox*, and 4-methoxyphenol (*Aldrich*), and tryptophan (Trp; *Sigma Chemical Co.*) were employed as received. The consumption of c-Pc was followed spectrophotometrically. At low concentrations, the consumption was related to the decrease in fluorescence intensity (excitation 620 nm; emission 640 nm). At higher concentrations, the kinetics of the process was evaluated from the decrease in absorbance measured at 620 nm.

Results and Discussion. – The consumption of c-Pc, measured either by the decrease in fluorescence or absorbance of the bilin moiety, follows a first-order kinetics. The rate of the process is reduced by the addition of free-radical scavengers. Typical results derived from fluorescence measurements are shown in *Fig. 2*. The upward curvature observed in the presence of low concentrations of a very reactive free-radical scavenger, such as *Trolox*, can be explained in terms of its consumption [13]. *R* Values were then obtained from the initial slopes. In the particular case of *Trolox*, this precludes the evaluation of the rates at low additive concentrations, due to the short reaction times required for its almost complete depletion. Values of $R°/R$ obtained under conditions of nearly zero-order kinetics ([c-Pc] = 0.2 mg/ml) and first-order kinetics ([c-Pc] = 0.001 mg/ml) [9], plotted against the *Trolox* concentration, are given in *Fig. 3*. Noticeable features of these data are: *i*) A linear relationship between $R°/R$ and the scavenger concentration, which holds even at $R°/R$ values higher than 10 is compatible with the reaction scheme represented by *Eqns. 5–8*. Data at lower concentrations could not be obtained to determine with precision whether the line extrapolates to 1 or zero. *ii*) A smaller slope at higher c-Pc concentrations is compatible with the proposed reaction scheme.

From the slope of the plot obtained at high c-Pc concentration (0.2 mg/ml), it is concluded that $k_7/k_6 = 7.8$, if the rate of the bleaching reaction (*Eqn. 6*) is expressed in terms of the bilin groups concentration. This implies that *Trolox* is nearly eight times more reactive than the bilin moieties, and explains why it is extensively consumed prior to significant c-Pc depletion [13].

The data obtained with Trp and 4-methoxyphenol as free-radical scavengers, given in *Fig. 4*, present noticeable differences with those obtained with *Trolox*. Firstly, higher concentrations are required to protect c-Pc. This is compatible with the lack of induction times observed in *Fig. 2*. Second, both plots show a noticeable downward curvature, a behavior that is not compatible with the proposed reaction scheme. A plausible explanation of this curvature is the occurrence of a reaction such as that shown in *Eqn. 17*, in which a scavenger-derived radical can react with the bilin group of c-Pc. The occurrence of this reaction can also explain why the limiting value of $R°/R$ decreases when the c-Pc concentration increases. This last result reflects that the fraction of $X^•$ radicals engaged in reactions with c-Pc must increase when the concentration of this target molecule increases. This analysis shows that Trp and 4-methoxyphenol are not only rather poor free-radical scavengers, but also that their derived radicals retain the capacity to damage the target molecule. We consider that the data given in *Fig. 4,a*, provide clear evidence that Trp-derived radicals are able to damage biomolecules. This is in agreement with the proposal that the production of Trp-derived radicals can lead to the occurrence of chain reactions in the oxidation of proteins [14][15].

$$X^• + \text{c-Pc} \rightarrow \text{bleaching} \qquad (17)$$

The reaction scheme represented by *Eqns. 5–8* has been oversimplified to the extreme such that it does not consider the involvement of $ROO^•$ radicals in cross-termination steps and/or the fate of the radicals produced according to *Eqns. 6* and *7*. In the former reaction, a radical c-Pc$^•$ derived from c-Pc must be produced. The secondary reactions of these radicals can modify the observed bleaching rate. However, it must be considered that changes in the rate of the free-radical-removing steps can alter the observed rate by less than a factor of two. In particular, changing the dominant free-radical-removing step from *Eqn. 18* at low X concentrations to *Eqn. 19* and/or *20* at high X concentrations would increase the c-Pc consumption rate by a factor of ≤ 2. The possible effect of a change in the main free-radical-removing step cannot then explain the very large curvature of the plots given in *Fig. 4* and the results showing that $R°/R$ tends to plateau at high X concentrations.

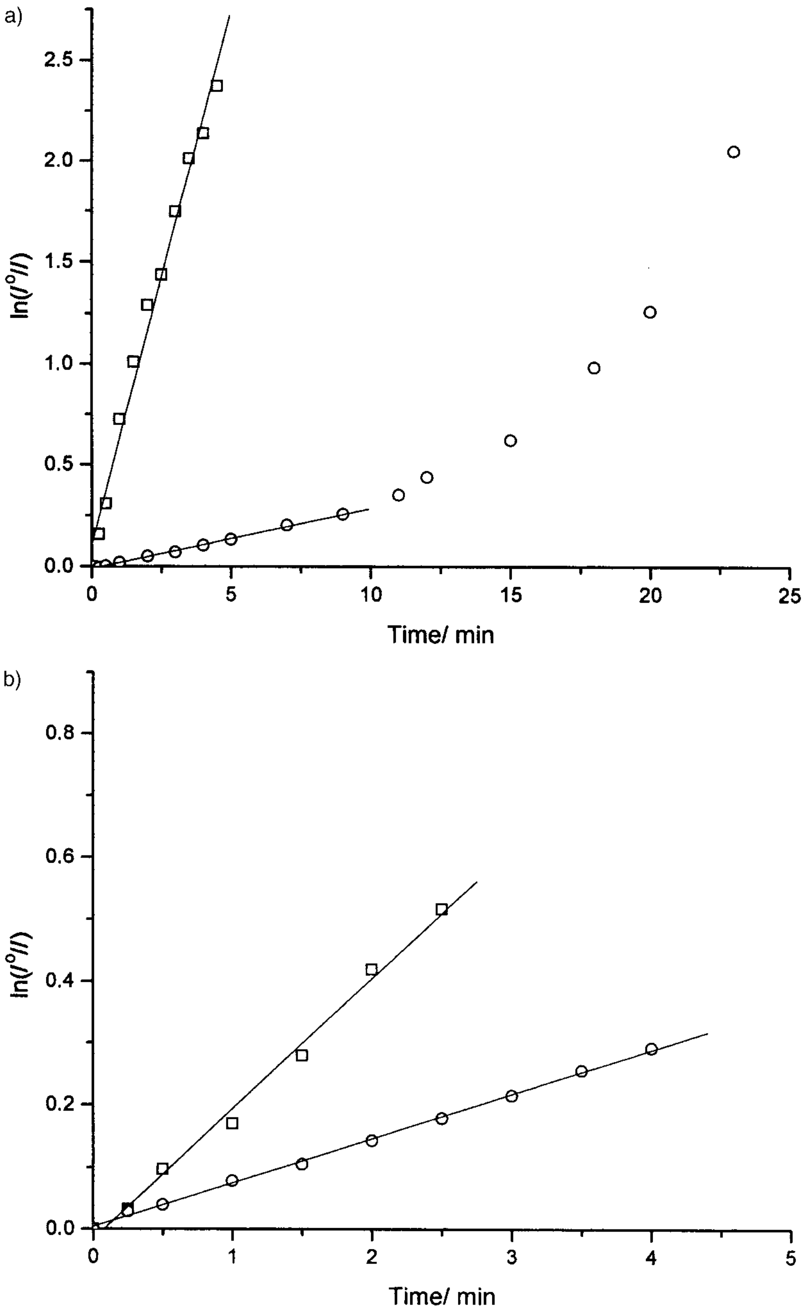

Fig. 2. *First-order plot for the bleaching of c-phycocyanin* (0.001 g/l): a) *in the absence* (□) *and in the presence* (○ *of added* Trolox (10 μM), b) *in the absence* (□) *and in the presence* (○) *of added tryptophan* (0.2 mM), *and* c) *in the absence* (□) *and in the presence* (○) *of added 4-methoxyphenol* (0.2 mM)

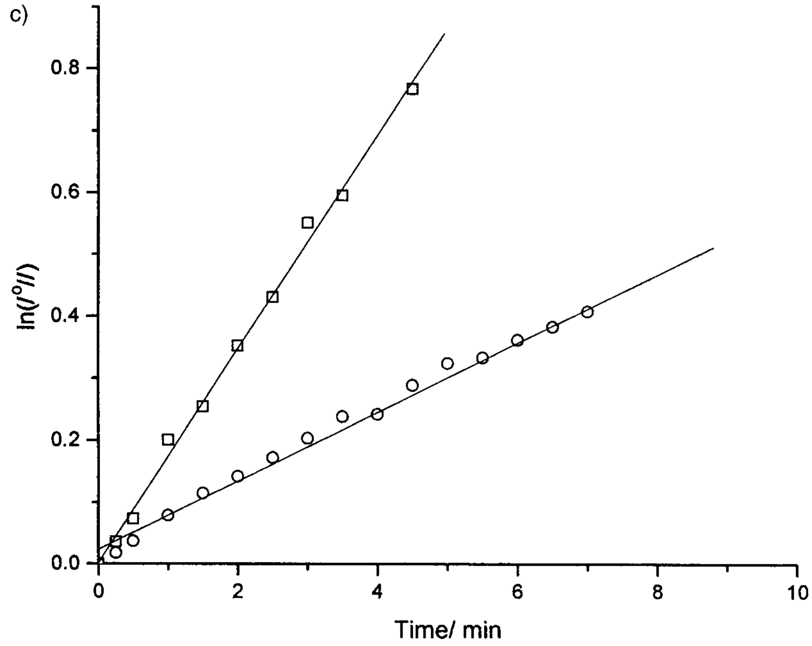

Fig. 2. (cont.)

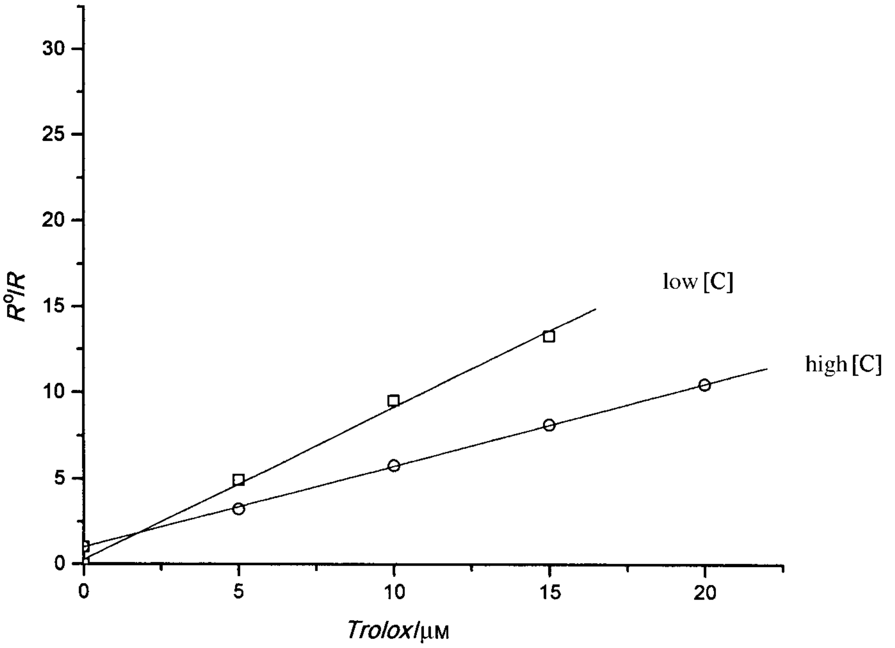

Fig. 3. *Change in* R°/R *values as a function of* Trolox *concentration*. Data obtained at low (□; 0.001 mg/ml) and high (○; 0.2 mg/ml) c-Pc concentrations

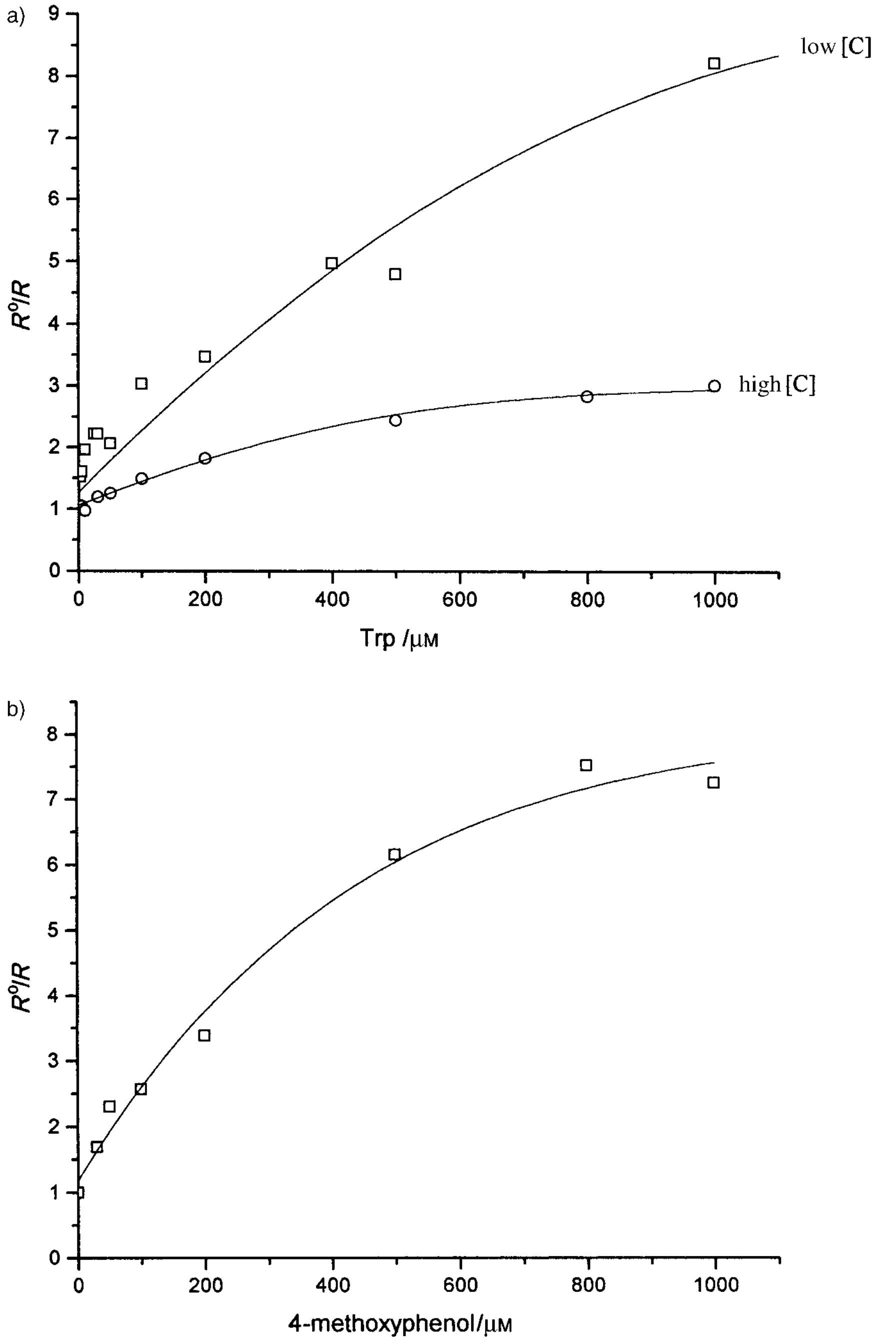

Fig. 4. *Change in* R°/R *values as a function of* a) *Trp and* b) *4-methoxyphenol concentrations.* Data obtained at low (□; 0.001 mg/ml) and high (○; 0.2 mg/ml) c-Pc concentrations.

$$\mathrm{ROO^{\bullet} + c\text{-}Pc^{\bullet} \rightarrow products} \tag{18}$$

$$\mathrm{X^{\bullet} + X^{\bullet} \rightarrow products} \tag{19}$$

$$\mathrm{X^{\bullet} + c\text{-}Pc^{\bullet} \rightarrow products} \tag{20}$$

It is difficult to obtain an estimate of k_7/k_6 from the data given in *Fig. 4* without making gross assumptions. However, comparison of the initial slopes obtained at low c-Pc concentration (were consumption of c-Pc by the X-derived radicals is minimal) with that obtained with *Trolox* under similar conditions provides a gross estimate of the relative efficiencies as scavengers of the added compound and the reference compound. The data obtained with 4-methoxyphenol (*Fig. 4,b*) would indicate that this compound is *ca*. 25 times less reactive than *Trolox*. The relevant point is that a comparison of the relative reactivity of both compounds is complex and that kinetics information can not be obtained if the effect of the additive is measured at a single concentration. The present discussion emphasizes the fact that to obtain meaningful kinetics data, it is necessary to know the quantitative relationship between the observed effect and the additive concentration. Furthermore, it shows that the effect of low additive concentrations could or could not be influenced by the target concentration, depending if its consumption is in the first- or zero-order kinetics limit.

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