Free Rad. Res. Comms., Vol. 17, No. 2, pp. 97–107
Reprints available directly from the publisher
Photocopying permitted by license only

SUPEROXIDE ANION SCAVENGING CAPACITY MEASURED BY A POLAROGRAPHIC METHOD. COMPARISON WITH A COLOURIMETRIC METHOD

N. DARMON, Y. FERNANDEZ, A. PERIQUET and S. MITJAVILA*

Institut de Physiologie, 2 rue François Magendie, 31400 Toulouse, France

(*Received January 2, 1992; in final form April 8, 1992*)

A polarographic method to assess the scavenging capacity of a molecule for $O_2^{\cdot}$ is proposed. This method is based on the fact that $O_2^{\cdot}$ is not detected by the Clark electrode and that a scavenger competes with spontaneous dismutation of $O_2^{\cdot}$. So, the reduction of O_2 into $O_2^{\cdot}$ and the decomposition of H_2O_2 by catalase, releasing O_2, show a biphasic kinetic. Various kinetic parameters can be used to calculate the nmol of $O_2^{\cdot}$ scavenged and also supply data on the reaction mechanisms (oxidation or reduction of $O_2^{\cdot}$) involved in scavenging. This method presents several other advantages: scavenging capacity can be assayed without added indicators which themselves behave as scavengers (as demonstrated for NBT), the presence of scavengers which interfere with the $O_2^{\cdot}$ generating system (xanthine–xanthine oxidase) does not invalidate the measurements made.

KEY WORDS: Polarography, nitroblue tetrazolium, superoxide, free radical scavenger.

INTRODUCTION

Oxygen radicals are at the origin of oxidative stress and an increasing interest is being shown in therapy, food preserving and cosmetics for methods allowing the screening of compounds having a scavenger activity against these radicals. Among them, superoxide anion ($O_2^{\cdot}$), resulting from the univalent reduction of O_2, is considered as being the first step leading to oxidative stress. Currently, the optional methods for $O_2^{\cdot}$ evaluation are ESR and various colourimetric methods based on stable products formed through the reduction or the oxidation of an indicator "i" by $O_2^{\cdot}$.[1] The defect of the ESR method is the lack of sensitivity due to the steady state of the radical formation. Also, spin trapping techniques are not exempt from criticism.[2] The superoxide dismutase (SOD)-inhibitable component of the reduction (or oxidation) of "i" by $O_2^{\cdot}$ followed with colourimetric methods is widely used to assay $O_2^{\cdot}$. In this case "i" acts as a scavenger and competes only with the spontaneous dismutation of $O_2^{\cdot}$. The situation becomes rather complicated when "i" is used for measuring the scavenger capacity of a molecule with regard to $O_2^{\cdot}$. Many types of interference are described between "i" and the compounds of the reactional medium. So, nitroblue tetrazolium (NBT) or ferricytochrome C can be reduced by some other chemicals[3,4] or by an intermediate in the "phenazine methosulphate–NADH" $O_2^{\cdot}$ generating system.[5,6] It was therefore attractive to define the scavenging capacity of

* To whom correspondence and reprint requests should be addressed.

a compound in the absence of indicators. Polarographic oxygen measurement is used in different steps of oxidative stress. It is widely used in studies on peroxidation[7] or on the effect of compounds such as inhibitors of peroxidation[8] and has been applied to the assay of $O_2^{\cdot-}$.[9,10]

We propose here a polarographic method based on biphasic kinetics: reduction of O_2 into $O_2^{\cdot-}$ then decomposition of H_2O_2. The parametric analysis of the kinetics in the absence or presence of an $O_2^{\cdot-}$ scavenging molecule indicates the fraction of O_2 reduced to $O_2^{\cdot-}$ and also supplies data on the fate of $O_2^{\cdot-}$ (oxidation, reduction and/or dismutation). It thus becomes possible to not only quantify the scavenging capacity of a molecule but to gain access to the mechanisms involved in the scavenging process observed. The validity and the advantages of the method proposed are demonstrated using a xanthine–xanthine oxidase (X–XO) system for $O_2^{\cdot-}$ production and comparing the results with a classical colourimetric method using NBT.

MATERIALS AND METHODS

Chemicals

Trolox was purchased from Aldrich Chem. Co. (Strasbourg, France). Other compounds were from Sigma Chem. Co. (La Verpillière, France). All the buffers and aqueous media were treated with Chelex-100 resin and were air-saturated.

Generation of $O_2^{\cdot-}$

Superoxide was generated at 25°C by an enzyme method based on the oxidation of 62.5 nmol/ml xanthine (X) in 200 mM NaCl, 50 mM Tris-HCl buffer (pH 9 or pH 7.5) by xanthine oxidase (XO) (16.65 mU/ml or 30 mU/ml respectively).

$$X + 2O_2 + H_2O \xrightarrow{xo} \text{Uric acid} + 2O_2^{\cdot-} + 2H^+ \quad (1)$$

Part of the O_2 should also be reduced by a two-electron mechanism coupled to the oxidation of X[9,11] depending on pH, O_2 saturation and X concentration.

$$X + O_2 + H_2O \xrightarrow{xo} \text{Uric acid} + H_2O_2 \quad (2)$$

Determination of $O_2^{\cdot-}$

In the absence of scavenger, the $O_2^{\cdot-}$ generated undergoes immediate dismutation:

$$2O_2^{\cdot-} + 2H^+ \rightarrow H_2O_2 + O_2 \quad (3)$$

Polarographic determination of $O_2^{\cdot-}$ is based on the fact that $O_2^{\cdot-}$ is not detected by the Clark electrode whereas O_2 is. O_2 consumption was monitored with a Gilson KIC oxygraph, in a 2 ml reaction chamber. The polarographic kinetics include two phases (Figure 1). The first results from reactions (1) and (2), O_2 is therefore consumed. The $O_2^{\cdot-}$ generated is equivalent to, or lower than twice, the net O_2 consumption detected by the electrode. The second phase starts by the addition of 200 U/ml of catalase (Cat) decomposing H_2O_2 coming from the univalent or divalent

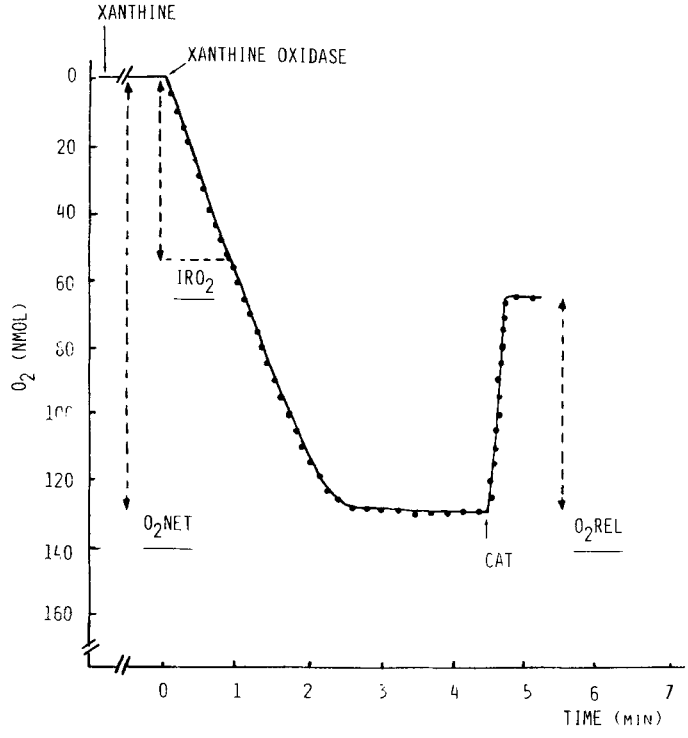


FIGURE 1 Typical plot obtained with a reaction chamber containing 2 ml of 200mM NaCl, 50 mM Tris-HCl, pH 9 at 25°C. The first phase of the polarographic kinetics started by the action of XO (16.65 mU/ml) on X (62.5 nmol/ml) and the second phase by the addition of catalase (200 U/ml). Control (———) and in the presence of 30 U/ml SOD (●●●).

reduction of O_2 according to:

$$H_2O_2 \xrightarrow{Cat} 1/2O_2 + H_2O \qquad (4)$$

The second phase gives rise to O_2 release equivalent to half the H_2O_2 produced or O_2 net consumed. The relative importance of univalent or divalent O_2 reduction does not modify the balance.

So, three parameters were defined for the kinetic study.

IRO_2: initial rate of oxygen consumption (nmol O_2/min),
O_2 net: net oxygen consumed (nmol O_2),
O_2 rel: oxygen released by catalase (nmol O_2).

The colourimetric method used is based on the fact that part of the $O_2^{\cdot}$ generated reduces NBT and leads to the formation of monoformazan. The concentrations of NBT used were between 600 μM and 75 μM. The changes in absorbance at 560 nm[12,13] were monitored continuously until the absorbance reached a stable maximum. The colour was read against a blank which did not contain XO.

Evaluation of the $O_2^{\cdot}$ scavenger capacity of a compound

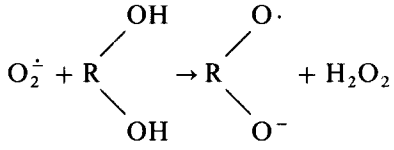
By polarography, the three parameters defined above were studied in the presence of a compound dissolved in 5 μl of water or DMSO added before XO. The parameters were modified in the presence of compounds having a scavenger effect on $O_2^{\cdot}$ and competing with dismutation. Analysis of the modifications allowed two situations to be distinguished.

When the scavenger oxidizes $O_2^{\cdot}$:

$$O_2^{\cdot} \rightarrow O_2 + e^- \tag{5}$$

the values of IRO_2, O_2 net and O_2 rel for the assay should be lower than those of the control.

When the scavenger reduces $O_2^{\cdot}$, for example to H_2O_2 :



(6)

(R is an aromatic ring)

the values of IRO_2, O_2 net and O_2 rel for the assay should be greater than those of the control.

In the two cases, the fraction of $O_2^{\cdot}$ scavenged can be quantified according to:

$$O_2^{\cdot} \text{ scavenged} = 2|O_2 \text{ net control} - O_2 \text{ net assay}|$$

In the presence of a scavenger, the variation of O_2 rel/O_2 net wirh respect to the control value gives additional information on the reaction mechanism.

By carrying out an additional experiment adding 30 U/ml of SOD to the medium before the XO, the $O_2^{\cdot}$ scavenger effect of the compound can be confirmed. Here, values obtained for the three parameters should, at least partially, return to control values. A return of O_2 net to control value without a return of O_2 rel and/or IRO_2 indicates that the studied compound scavenges $O_2^{\cdot}$ but also interferes with H_2O_2 or XO.

By colourimetry, the scavenger activity of a compound was expressed as the percentage of inhibition of NBT reduction with respect to the control.[12,13,14]

Consequences of the inhibition of XO activity by a scavenger

With the colourimetric method the inhibition of XO by a compound leads to a decrease in the reduction of NBT which could be mistaken for scavenging. So, additional spectrophotometric measurements of the uric acid produced are needed to confirm the absence of inhibition.

With the polarigraphic method, compounds acting as XO inhibitors decrease IRO_2. This variation is without any influence on the final scavenger activity and could be used as a measure of the inhibitory effect of a compound on XO.

To confirm the validity of this remark, we evaluated the formation of uric acid by measurement at 295 nm[15] in the same conditions of $O_2^{\cdot}$ generation. A correlation between the two types of measurements was made by comparing, for three compounds,

the concentration giving a 30% decrease of IRO_2 (D_{30} IRO_2) by polarographic method and of the initial rate of uric acid produced (D_{30} IRUA) by spectrophotometric measure.

RESULTS AND DISCUSSION

Polarographic scavenger quantification

Figure 1 gives typical kinetics for a control reaction obtained by the polarographic method at pH 9. The values of IRO_2 (nmol/min), O_2 net (nmol) and O_2 rel (nmol) are 53 ± 0.6, 127 ± 0.5 and 63 ± 0.3 respectively (means ± SEM of 40 results). The value of O_2 net is in agreement with reactions (1), (2) and (3) knowing that the amount of X present is the limiting factor. O_2 rel/O_2 net is close to 0.5 which corresponds to decomposition (reaction 4) of all the H_2O_2 formed. The addition of SOD to the medium had no significant effect on the values of IRO_2, O_2 net or O_2 rel. Similar kinetics were observed at pH 7.5 (results not shown).

The scavenging effect of some molecules was studied in parallel at pH 7.5 and pH 9. Table I illustrates the quantities of $O_2^{\cdot}$ scavenged by catechin and ferricytochrome C. In the presence of SOD, the quantity of $O_2^{\cdot}$ scavenged was strongly decreased in all cases confirming the trapping effect of the molecules. Scavenging was greater at pH 9 due to slower spontaneous dismutation.[9] So, all subsequent experiments were performed at pH 9.

Reaction mechanism of $O_2^{\cdot}$ scavenging

Two molecules were chosen to test the possibilities offered by the polarographic method to identify the scavenging mechanism.

TABLE I
Influence of pH on O_2^- scavenged (nmol) determined by the polarographic method

Addition	pH 7.5	pH 9
Catechin		
10 μM	0.0 ± 0.00	12.0 ± 0.00
10 μM + SOD	ND	0.0 ± 0.00
100 μM	0.0 ± 0.00	52.0 ± 2.30
100 μM + SOD	ND	7.3 ± 1.76
1000 μM	13.3 ± 1.66	114.7 ± 4.05
1000 μM + SOD	6.0 ± 2.31	28.0 ± 2.90
Ferricytochrome C		
10 μM	0.0 ± 0.00	12.7 ± 1.33
10 μM + SOD	ND	0.0 ± 0.00
100 μM	36.0 ± 3.05	72.0 ± 2.00
100 μM + SOD	0.0 ± 0.00	2.0 ± 1.15

At pH 9 the composition of the reaction media is the same as in Figure 1. At pH 7.5 the concentration of XO is 30 mU/ml. The results are means ± SEM of three experiments.
ND: not determined.

-Oxidation of $O_2^{\cdot}$

NBT is a known example of a scavenger since it is used in colourimetric techniques as an indicator to detect production of $O_2^{\cdot}$. It acts by oxidizing $O_2^{\cdot}$ into O_2. Table II shows that the presence of 100 μM NBT decreases IRO_2, O_2 net and O_2 rel with respect to the control; the ratio O_2 rel/O_2 net close to 0.5 is comparable to that of the control. These results agree perfectly with reactions (1), (2), (3), (4) and (5). In these conditions, the quantity of $O_2^{\cdot}$ scavenged was 50 nmol. Adding SOD to the medium re-established control values.

-Reduction of $O_2^{\cdot}$

In the presence of 100 μM catechin (Table II), IRO_2 is not greatly increased. A clear increase of O_2 net with respect to the control is observed and the quantity of $O_2^{\cdot}$ scavenged is 52 nmol. O_2 rel is also higher than in the control and the ratio O_2 rel/O_2 net is similar to the control. This is again compatible with reactions (1), (2), (3), (4) and (6) indicating the reduction of $O_2^{\cdot}$ into H_2O_2 as already described for diphenols.[16] Addition of SOD also leads to control values being re-established.

Particular cases of modifications of the polarographic kinetics

Analysis of the three kinetic parameters gives access to the possible existence of particular mechanisms: we observed variations unlike those previously described firstly for the ratio O_2 rel/O_2 net and secondly for IRO_2.

-Oxidation of $O_2^{\cdot}$ by ferricytochrome C

In the presence of 100 μM ferricytochrome C, which acts like NBT oxidizing $O_2^{\cdot}$ into O_2, the first phase of the polarographic kinetics (Table II) was qualitatively identical with that of NBT (decrease of IRO_2 and O_2 net with respect to the control). The amount of $O_2^{\cdot}$ oxidized was 72 nmol. However, the value of O_2 rel/O_2 net is lower than 0.5. This could be attributed to the partial reoxidation of ferrocytochrome C by H_2O_2[17] but, considering the low concentrations of H_2O_2 in our experimental conditions, such a possibility appears to be rejected.[11] A further possibility would be the partial persistence of a Cyt Fe^{2+}–O_2 intermediate after reduction of Cyt Fe^{3+}.

-Interference of a compound with XO

Kinetic parameters obtained with higher concentrations of catechin (1000 μM) are also given in Table II. Some characteristics of the kinetics are similar to those established at 100 μM catechin: increase of O_2 net and O_2 rel with respect to the control and the ratio O_2 rel/O_2 net close to 0.5. The amount of $O_2^{\cdot}$ scavenged was 114.7 nmol. However, there was a drop in IRO_2 with respect to the control. The inhibition of XO at this concentration of catechin is explained more fully in Figure 2. In the presence of SOD, only O_2 net and O_2 rel almost returned to control values. The plot obtained without SOD is the resultant of the $O_2^{\cdot}$ scavenging effect of catechin and its inhibiting effect on XO. These effects explain the slight accentuation of the decrease of IRO_2 in the presence of SOD. The same type of result was found with low concentrations of quercetin (results not shown) in agreement with reports describing the strong inhibiting effect of flavonols on XO.[15,18] Table III shows the correlation between the concentrations of three compounds (including allopurinol,

TABLE II
Kinetic parameters and scavenging capacity at pH 9 of NBT, catechin and ferricytochrome C with respect to the controls

	Controls		NBT 100 μM + SOD		Catechin 100 μM + SOD		Catechin 1000 μM + SOD		Ferricytochrome C 100 μM + SOD	
IRO_2 (nmol O_2/min)	52.0 ±1.73	35.7 ±0.86	52.0 ±1.52	54.7 ±1.45	52.3 ±1.20	35.0 ±2.52	27.7 ±2.40	33.0 ±2.08	52.3 ±1.76
O_2 net (nmol O_2)	127.3 ±1.33	102.3 ±2.60	126.4 ±2.02	153.3 ±2.40	131.0 ±0.57	184.7 ±1.20	141.3 ±2.18	91.4 ±0.33	126.3 ±0.88
O_2 rel (nmol O_2)	63.0 ±1.00	51.7 ±0.88	62.0 ±1.00	76.0 ±1.15	65.7 ±0.66	93.0 ±1.73	70.0 ±2.00	36.0 ±0.57	63.0 ±0.00
$O_2^{\cdot}$ scavenged (nmol)		50.0 ±3.05	2.00 ±2.00	52.0 ±2.30	7.3 ±1.76	114.7 ±4.05	28.0 ±2.90	72.0 ±2.00	2.00 ±1.15

The composition of the reaction media is the same as in Figure 1.
The results are means ± SEM of three experiments.

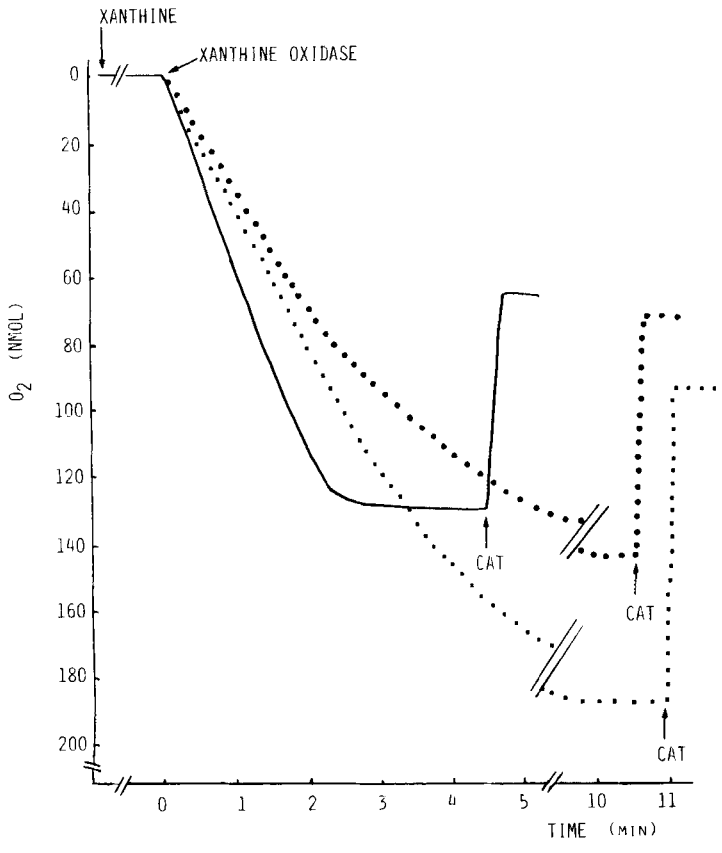


FIGURE 2 Typical effect of 1000 μM catechin on the biphasic polarographic kinetics obtained as described in Figure 1. Control (———), Catechin (■■■), Catechin + SOD (●●●).

TABLE III
Concentrations of different compounds causing at pH 9, a 30%, decrease in the initial rate of O_2 consumption (D_{30} IRO_2) and in the initial rate of uric acid production (D_{30} IRUA)

Compound	D_{30} IRO_2 (μM)[a]	D_{30} IRUA (μM)
Quercetin	0.40 ± 0.024	0.30 ± 0.022
Allopurinol	3.53 ± 0.176	5.16 ± 0.273
Catechin	840 ± 32.1	600 ± 25.2

The compositions of the reaction media are given in Material and Methods.

[a]Assays in presence of SOD.

The values are means ± SEM of three results each obtained from experiments using six different concentrations of each compound.

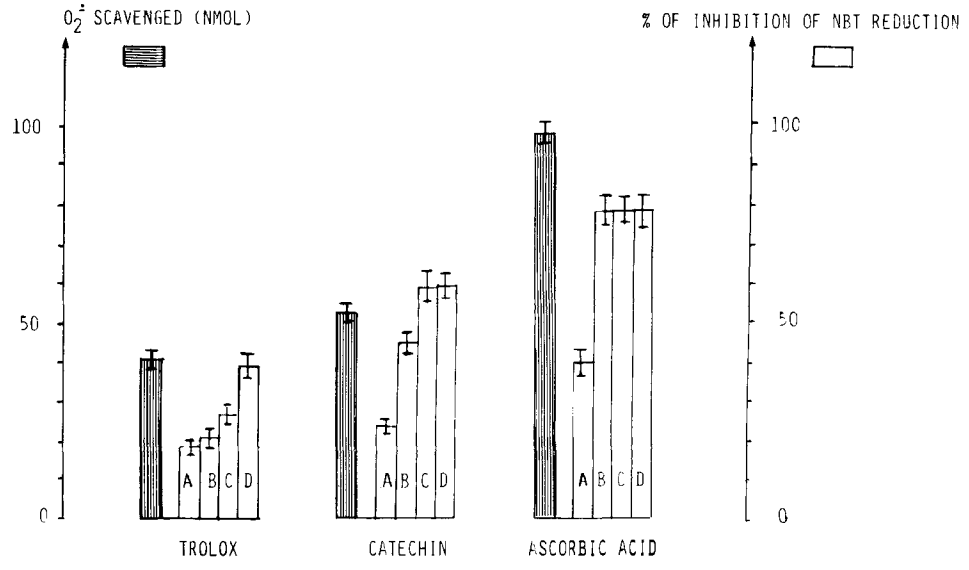


FIGURE 3 $O_2^{\cdot}$ scavenged and percentage of inhibition of NBT reduction by Trolox, catechin and ascorbic acid (100 μM). The NBT concentrations used are 600 μM (A), 300 μM (B), 150 μM (C) and 75 μM (D). Results are means $\pm$ SEM of three experiments.

a known inhibitor of XO) causing a 30% decrease in, firstly, IRO_2 (polarographic method) and, secondly, the initial rate of uric acid production (spectrophotometric method). In these conditions of $O_2^{\cdot}$ production, the polarographic method presents the advantage of enabling the simultaneous determination of the $O_2^{\cdot}$ scavenging effect and the inhibition of XO.

Scavenging capacity of a few molecules: comparison between the polarographic and colourimetric methods

It was checked that NBT reduction (colourimetric method) is inhibited by SOD in a dose-dependent manner (results not shown). The scavenging capacity was measured in parallel with the two methods (Figure 3) for three compounds which do not inhibit XO at the concentration used (100 μM). For the three scavengers studied, in absence of "i", the quantity of $O_2^{\cdot}$ scavenged increases in the order: Trolox < catechin < ascorbic acid. With the colourimetric method, the same order is observed but the highest inhibitions of NBT reduction are observed with the lowest concentrations of NBT. However, the concentration of NBT in the medium influenced its reduction yield in a different way for the three molecules studied. For Trolox, a weak scavenger, a decrease in the NBT concentration caused a steady increase in the percentage inhibition of the reduction while for ascorbic acid, a strong scavenger, the inhibition remained unchanged between 300 and 75 μM NBT. So, with the colourimetric method, the use of too high NBT concentrations can lead to the underestimation of the scavenger capacity of certain compounds.

The polarigraphic method for the quantification of $O_2^{\cdot}$ scavenging by molecules developed in this study presents numerous advantages with respect to the colourimetric method.

Obviously, any interaction between the coloured indicator and the molecule to be studied or the H_2O_2 produced is avoided. As with the other methods, the possibility of interaction between the studied molecule and the X–XO system or H_2O_2 is inevitable but with the polarographic method the detection and analysis of interference is facilitated.

Whereas the colourimetric method expresses the scavenging capacity of a molecule as a percentage of inhibition of NBT reduction, the polarographic method gives the value in nmol $O_2^{\cdot-}$ scavenged. Moreover, the use of SOD with the polarographic method allows the modifications observed to be attributed specifically to the $O_2^{\cdot-}$ scavenging effect of the molecule. This specificity cannot be checked with the colourimetric method.

Analysis of the polarigraphic kinetics reveals any inhibition of XO by a molecule without disturbing the measurement of its $O_2^{\cdot-}$ scavenging capacity. It, above all, allows the reaction mechanism involved to be identified notably whether oxidation or reduction of $O_2^{\cdot-}$ occurs during scavenging.

Acknowledgements

We are grateful to A. Belliure for typing the manuscript.

References

1. W. Bors, M. Saran, E. Lengfelder, C. Michel, C. Fuchs and C. Frenzel (1978) Detection of oxygen radicals in biological reactions. *Photochemistry and Photobiology*, **28**, 629–638.
2. E. Finkelstein, G.M. Rosen and E.J. Rauckman (1980) Spin trapping of superoxide and hydroxyl radical: Practical aspects. *Archives of Biochemistry Biophysics*, **200**, 1–16.
3. I. Fridovich (1987) Cytochrome C. In *Handbook of Methods for Oxygen Radical Research* (ed. R.A. Greenwald), CRC Press, Inc., pp. 121–122.
4. C. Auclair and E. Voisin (1987) Nitroblue tetrazolium reduction. In *Handbook of Methods for Oxygen Radical Research* (ed. R.A. Greenwald), CRC Press, Inc., pp. 123–132.
5. S.D. Picker and I. Fridovich (1984) On the mechanism of production of superoxide radical by reaction mixtures containing NADH, phenazine methosulfate, and nitroblue tetrazolium. *Archives of Biochemistry Biophysics*, **228**, 155–158.
6. U.M. Rao (1989) Source of superoxide anion radical in aerobic mixtures consisting of NAD[P]H, 5-methylphenazinium methyl sulfate and nitroblue tetrazolium chloride. *Free Radicals Biology and Medicine*, **7**, 513–519.
7. R. Ursini, M. Maiorino, P. Hochstein and L. Ernster (1989) Microsomal lipid peroxidation: Mechanisms of initiation. The role of iron and iron chelators. *Free Radicals Biology and Medicine*, **6**, 31–36.
8. E. Niki (1987) Antioxidants in relation to lipid peroxidation. *Chemistry Physics of Lipids*, **44**, 227–253.
9. I. Fridovich (1970) Quantitative aspects of the production of superoxide anion radical by milk xanthine oxidase. *Journal of Biological Chemistry*, **49**, 4053–4057.
10. C. Auclair, D. De Prost and J. Hakim (1978) Superoxide anion production by liver microsomes from phenobarbital treated rat. *Biochemical Pharmacology*, **27**, 355–358.
11. J.F. Turrens and J.M. McCord (1988) How relevant is the reoxidation of ferrocytochrome C by hydrogen peroxide when determining superoxide anion production? *FEBS Letters*, **227**, 43–46.
12. J. Robak and R.J. Gryglewski (1988) Flavonoids are scavengers of superoxide anions. *Biochemical Pharmacology*, **37**, 837–841.
13. C. Yuting, Z. Rongliang, J. Zhongjian and J. Yong (1990) Flavonoids as superoxide scavengers and antioxidants. *Free Radicals in Biology and Medicine*, **9**, 19–21.
14. A.I. Huguet, S. Manez and M.J. Alcaraz (1990) Superoxide scavenging properties of flavonoids in a non-enzymic system. *Z. Naturforsch*, **45c**, 19–24.
15. A. Bindoli, M. Valente and L. Cavallini (1985) Inhibitory action of quercetin on xanthine oxidase and xanthine dehydrogenase activity. *Pharmacological Research Communications*, **17**, 831–839.

16. B. Halliwell and J.M.C. Gutteridge (1989) *Free Radicals in Biology and Medicine*. Clarendon Press, Oxford.
17. P.L. Vanderwalle and N.O. Petersen (1987) Oxidation of reduced cytochrome C by hydrogen peroxide. Implications for superoxide assays. *FEBS Letters*, **210**, 195–198.
18. M. Iio, A. Moriyama, Y. Matsumoto, N. Takaki and M. Fukumoto (1985) Inhibition of xanthine oxidase by flavonoids. *Agricultural Biological Chemistry*, **49**, 2173–2176.

Accepted by Dr. C. Rice-Evans