SINGLET OXYGEN GENERATION FROM THE PEROXIDASE-CATALYSED AEROBIC OXIDATION OF AN ACTIVATED $-CH_2-$ SUBSTRATE†

NELSON DURÁN and SANDRA T. FARIAS-FURTADO

Instituto de Química, Universidade Estadual de Campinas, C.P. 1170, CEP 13.100, Campinas, São Paulo (Brazil)

ADELAIDE FALJONI-ALARIO and ANA CAMPA

Universidade de São Paulo, São Paulo (Brazil)

JUAN E. BRUNET

Universidad Católica de Valparaíso, Valparaíso (Chile)

JUANITA FREER

Universidad de Concepción, Concepción (Chile)

(Received January 5, 1984; in revised form March 28, 1984)

Summary

Photon emission, emission spectra, the product distribution, the effect of D_2O and quenchers and steady state techniques were proposed to ascertain the involvement of singlet oxygen in a biological process. In particular, these experiments led to the conclusion that singlet oxygen is present in the reactions of malonaldehyde catalysed by horseradish peroxidase.

1. Introduction

There is strong evidence that electronically excited products as well as those that give bioluminescence are present in biochemical systems [1 - 3]. Indirect evidence includes energy transfer processes, quenching, kinetics and comparison with photochemical processes [4, 5], in addition to direct evidence provided by the emission spectra of excited products [6, 7]. The peroxidase-catalysed aerobic oxidation of substrates such as indole-3-acetic acid [8], 2-methylpropanal [5], propanal [6] and acetoacetate [9] generates ketones in their excited states [1, 10], but there are few cases where the important emitting species seems to be $^1O_2$ [7, 11, 12].

There is much contradictory evidence for the detection of $^1O_2$ in biological systems [12 - 14]. Four mechanisms have been proposed to explain the generation of $^1O_2$ from a hydroperoxide: a mechanism involving a

† Paper presented at the COSMO 84 Conference on Singlet Molecular Oxygen, Clearwater Beach, FL, U.S.A., January 4 - 7, 1984.

0047-2670/84/$3.00

zwitterion intermediate [15]; a mechanism involving a tetraoxide intermediate [16]; the recombination of activated oxygen species (*e.g.* hydroxy radicals, superoxide ion, H_2O_2 etc.) [13]; energy transfer from triplet ketonic compounds to triplet oxygen [17].

Although we are aware of the complexity of biological systems in applying the photochemical techniques that have been developed for pure organic solvents to detect $^1O_2$ [18], we describe here a systematic study of the detection of $^1O_2$ generated from the peroxidase-catalysed oxidation of malonaldehyde (MDA).

2. Materials and methods

Horseradish peroxidase (HRP) (type VI), superoxide dismutase (SOD), catalase sorbic acid, histidine, tryptophan, methionine, benzoate, guanosine, ethylenediaminetetraacetic acid, adenosine, cytosine, thymidine and bilirubin were obtained from Sigma Chemical Company. Cholesterol, ergosterol, dimethyl sulphoxide (Me_2SO) and eosin were from Merck. 1,4-diazabicyclo-[2.2.2]octane (DABCO) and D_2O (99.8%) were from Aldrich Chemical Company. (5,8-^{14}C)-guanosine was from New England Nuclear (Boston, MA).

The oxygen consumption was determined with a Yellow Spring Instruments model 53 oxygen monitor. The photon emission and emission spectra were measured with a Hamamatsu TV photocounter C-767 [5] or a Beckman model LS-100c liquid scintillation counter with the coincidence circuit turned off or with a Perkin–Elmer MPF-4 fluorescence spectrometer. Absorption spectra were recorded on a Zeiss DMR-21. Circular dichroism measurements were made with a Cary 60 spectropolarimeter equipped with a model 6001 CD attachment.

The D_2O experiments were carried out as described earlier [19]. (5,8-^{14}C)-deoxyguanosine decomposition in the enzymatic system was analysed using the method of Cadet and Teoule [20]. The developed thin layer chromatography (TLC) plate (silica gel) (two-dimensional TLC; first, $CHCl_3$:CH_3OH:H_2O (4:2:1); second, ethyl acetate:isopropanol:H_2O (75:16:9)) was treated with 15 ml of 7% 2,5-diphenyloxaxole ether solution. The plate was then exposed to X-ray film at −80 °C for more than 2 weeks. The products were characterized by their chromatographic IR and nuclear magnetic resonance (NMR) behaviour. MDA was prepared by the method of Grabowski and Autry [21].

The standard reaction mixture was the following: MDA (10.4 mM); Mn^{2+} (3.13 mM); HRP (3.8 μM) in 0.2 M acetate buffer (pH 4.8).

3. Results and discussion

3.1. Correlations in O_2 consumption, product formation and photon emission

In the MDA–HRP–Mn^{2+}–O_2 system a correlation was observed between the product formation and the photon emission, but not between the product formation and the O_2 consumption (not shown). More recently, with many

enzymatic systems in which $^1O_2$ was generated there were also only correlations between the product formation and the photon emission [14]. In all these cases the O_2 consumption was much faster than the light emission and the product formation; this indicates accumulation of an intermediate, a fact which in turn suggests that the excited species may be formed outside the enzyme.

3.2. Conformational effect on photon emission and O_2 consumption

In the measurement of the O_2 consumption at different concentrations of Me_2SO–H_2O mixtures of the MDA–HRP–Mn^{2+}–O_2 system an increase in the peroxidase activity in the 1% - 5% range of Me_2SO was observed. This increase in the peroxidase activity is presumably related to conformational changes in the peroxidase as is observed in Fig. 1, where the variations in the circular dichroism spectra at 270 and 400 nm are shown. The band centred between 260 and 280 nm in the circular dichroism spectra of haemoprotein had been correlated with the spin state and the oxidation state of the iron. Then the variation observed in the Me_2SO–H_2O mixture could be related to a significant reorientation of the haem transition moment and/or to a conformational change leading to a displacement of aromatic side-chains in the haem environment [22].

Preliminary data have shown that in the MDA–HRP–Mn^{2+}–O_2 system $^1O_2$ is probably generated outside the enzyme [7], *i.e.* in the bulk solution. This has been confirmed since this system underwent a small activation followed by O_2 consumption, with a corresponding small increase in the emission. It is interesting to note that excited species such as $^1O_2$, which are generated outside the active site, are almost completely insensitive to peroxidase conformational modifications.

3.3. Photon emission: quenchers and enhancers

The emissions measured in our conditions are originated from "dimol" $O_2(^1\Delta_g\,^1\Delta_g)$. This emission was not affected by SOD (1700 units), catalase (125 units), 10 mM benzoate, 10 mM bicarbonate, 10 mM adenosine, 10 mM thymidine, 10 mM cytosine and 5 μM 9,10-dibromoanthracene-2-sulphonate, a well-known carbonyl emission enhancer. In contrast, the emission was quenched (by more than 50%) by 0.25 mM 3,4-dihydroxyphenylalanine, 10 mM histidine, 10 mM methionine, 10 mM guanosine and by 2.5 μM bilirubin, all excellent $^1O_2$ quenchers.

A small enhancement in the emission in the peroxidase–MDA system was observed in the presence of 10 mM DABCO. Enhancement (by a factor of 4 - 5) of the emission from the reaction system was observed in the presence of 10 μM eosin and in D_2O (4 - 5 times greater than that in H_2O).

The effect of guanosine and histidine on the emission of the MDA–HRP–Mn^{2+}–O_2 reaction was studied at concentrations that did not influence the rate of O_2 consumption. A Stern–Volmer plot of the observed quenching effect is presented in Fig. 2.

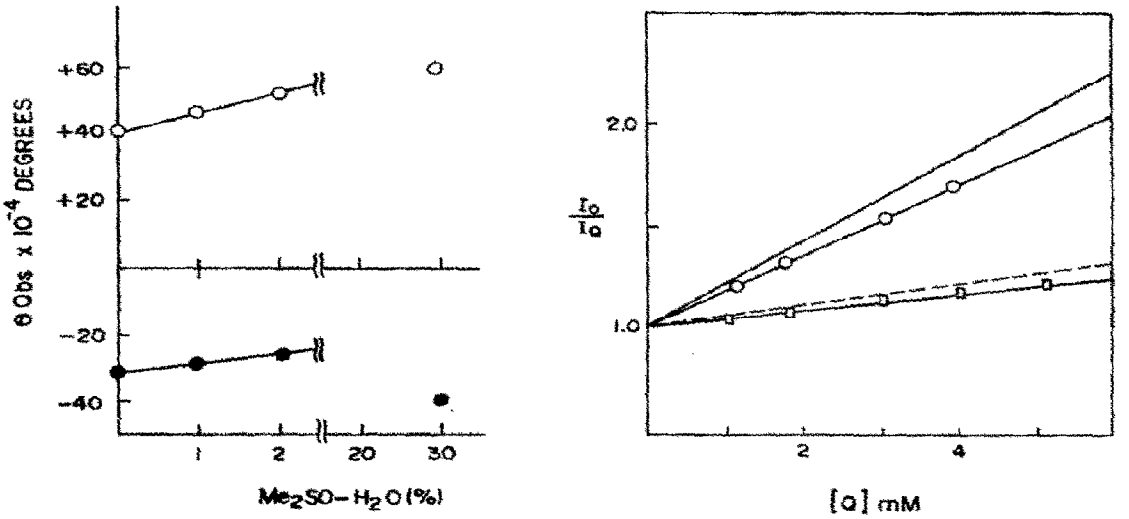

Fig. 1. Circular dichroism variations in peroxidase at different Me_2SO concentrations: ○, λ = 400 nm; ●, λ = 270 nm.

Fig. 2. Stern–Volmer plot for the quenching of the total light emission by guanosine (□) and histidine (○) in the MDA system (see Section 2): – – –, ———, theoretical curves using k_d ($5 \times 10^5\ s^{-1}$) and k_q ($0.86 \times 10^7\ M^{-1}\ s^{-1}$ and $5 \times 10^7\ M^{-1}\ s^{-1}$) for guanosine and histidine respectively.

For ready reference, the following equations for $^1O_2$ emission and quenching can be written:

$$\text{substrate} + O_2 \xrightarrow{\text{HRP}} O_2(^1\Delta_g)^* \quad (1)$$

$$O_2(^1\Delta_g)^* \xrightarrow{k_d} O_2(^3\Sigma_g^-) \quad (2)$$

$$O_2(^1\Delta_g)^* + O_2(^1\Delta_g)^* \xrightarrow{k_0} O_2(^1\Delta_g\,^1\Delta_g)^* \quad (3)$$

$$O_2(^1\Delta_g)^* + Q \xrightarrow{k_q} O_2(^3\Sigma_g^-) + Q^* \quad (4)$$

$$O_2(^1\Delta_g\,^1\Delta_g)^* \xrightarrow{k_{d'}} 2O_2 + O_2(^3\Sigma_g^-) \quad (5)$$

$$O_2(^1\Delta_g)^* + Q \xrightarrow{k_R} QO_2 \quad (6)$$

$$O_2(^1\Delta_g)^* + A \xrightarrow{k_A} AO_2 \quad (7)$$

$$O_2(^1\Delta_g\,^1\Delta_g)^* + Q \xrightarrow{k_Q} 2O_2(^3\Sigma_g^-) + Q^* \quad (8)$$

$$O_2(^1\Delta_g\,^1\Delta_g)^* \xrightarrow{k_f} 2O_2(^3\Sigma_g^-) + h\nu(640\ \text{nm}) \quad (9)$$

$$O_2(^1\Delta_g\,^1\Delta_g)^* + A \xrightarrow{k_{ET}} 2O_2(^3\Sigma_g^-) + A^* \qquad (10)$$

The following kinetic equation represents the quenching of $O_2(^1\Delta_g\,^1\Delta_g)$ species:

$$\frac{I_{dim}^0}{I_{dim}^Q} = \{1 + k_Q\tau'(^1\Delta_g\,^1\Delta_g)[Q]\}\{1 + (k_q + k_R)\tau^0(^1\Delta_g)[Q]\}^2$$

In our case $k_Q\tau'(^1\Delta_g\,^1\Delta_g) \ll k_q\tau^0(^1\Delta_g)$ presumably because of the very short lifetime of $O_2(^1\Delta_g\,^1\Delta_g)$. Then it is possible to apply the following equation:

$$\frac{I_{dim}^0}{I_{dim}^Q} = \{1 + (k_q + k_R)\tau^0(^1\Delta_g)[Q]\}^2$$

$$\frac{I_{dim}^0}{I_{dim}^Q} = 1 + 2(k_q + k_R)\tau^0(^1\Delta_g)[Q] + \{(k_q + k_R)\tau^0(^1\Delta_g)[Q]\}^2$$

At low concentrations of [Q] the plot is approximately linear:

$$\frac{I_{dim}^0}{I_{dim}^Q} = 1 + 2K_{SV}[Q]$$

In fact the initial plot is linear (Fig. 2) and the slopes $2K_{SV}$ (or $2(k_q + k_R)\tau^0$) for histidine and guanosine are 160 M^{-1} and 20 M^{-1} respectively. Since $k_q \approx 0.86 \times 10^7\ M^{-1}\,s^{-1}$ and $k_q \approx 5 \times 10^7\ M^{-1}\,s^{-1}$ for guanosine and histidine respectively [23, 24], $\tau^0 \approx 1.4 \times 10^{-6}$ s; this value suggests that the excited species in the presence of peroxidase has a lifetime similar to that of $O_2(^1\Delta_g)$ in H_2O ($\tau^0 \approx 2 \times 10^{-6}$ s [24]).

The quenching experiments with bilirubin also showed two different slopes (Fig. 3) and the value for the first slope ($2K_{SV}$) was 20 000 M^{-1}. Also if we calculate the lifetime for $^1O_2$ with $k_q = 2 \times 10^9\ M^{-1}\,s^{-1}$ the τ value is about 5×10^{-6} s, which is, within error, the lifetime of $O_2(^1\Delta_g)$.

It is interesting to note that in an experiment carried out by us using this method a $2K_{SV}$ value of about 188 M^{-1} for histidine was found in the lactoperoxidase–H_2O_2–Br^- system. $^1O_2$ was recently shown by its emission at 1270 nm [25] to exist in this system [26]. If we use the k_q value as $5 \times 10^7\ M^{-1}\,s^{-1}$ [24] the τ^0 value is 1.8×10^{-6} s.

These values suggest that $O_2(^1\Delta_g)$ is undoubtedly generated in the MDA–HRP–Mn^{2+}–O_2 system.

3.4. Product distribution

Cadet and Teoule [20] reported that guanosine reacts specifically with $^1O_2$. In fact, using (5,8-^{14}C)-deoxyguanosine in the MDA–HRP–Mn^{2+}–O_2 system we detected *N*(2-deoxy-D-*erythro*-pentosyl)urea ($R_f = 0.18$; see Section 2). The formation of this compound may be accounted for by the concerted 1,4-cycloaddition of $O_2(^1\Delta_g)$ across the 7,8 and 4,5 double bonds and subsequent decomposition of the resulting 4,8-endo peroxide as previ-

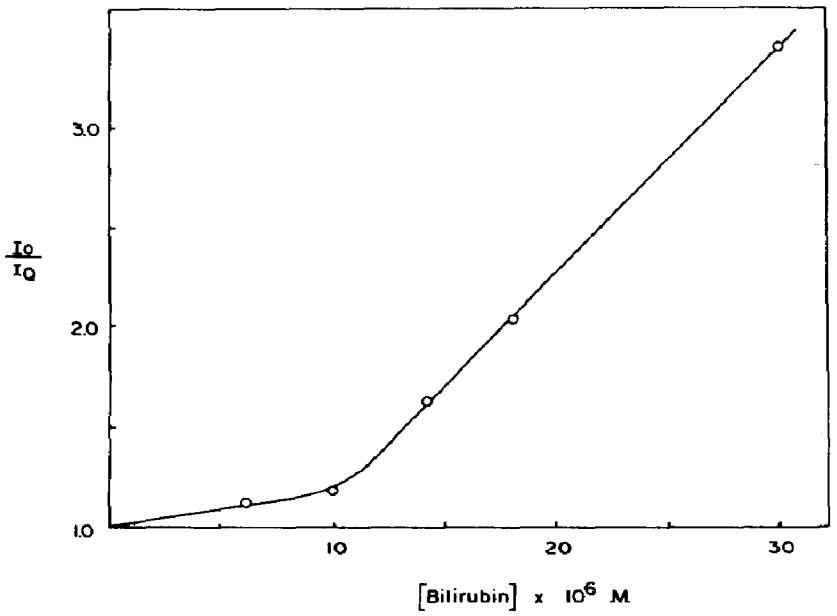

Fig. 3. Stern–Volmer plot for the quenching of the total light emission by bilirubin in the MDA system (see Section 2).

ously suggested by Matsuura *et al.* [27]. An unknown compound (ref. 20, compound 4 ($R_f = 0.23$)) and N^1(2-deoxy-β-*erythro*-pentofuranosyl)-N^2-formylurea ($R_f = 0.31$) were also detected. All these compounds were characterized by IR and NMR spectra.

In the MDA–HRP–Mn^{2+}–O_2 system cholesterol (20 μM) (a specific $^1O_2$ trap) was used in a detergent (BRIJ-35) and a 5% quenching of the total light emission was found. Ergosterol (1 μM concentration) in buffer quenched the total emission. No product analysis in these two cases was carried out.

3.5. Emission spectra

The emission spectrum from $^1O_2$ (dimol) is notoriously complex in the presence of proteins [28]. In the MDA–HRP–Mn^{2+}–O_2 system the light was so intense that it was possible to run the spectrum in a conventional fluorometer in H_2O [7] or in D_2O. In this case the principal emission peak is just above 700 nm and occasionally a smaller peak was observed at 630 nm (Fig. 4). The spectral distribution has also been determined with a highly sensitive spectrometer [5]. IR chemiluminescence with a maximum intensity near 1270 nm, which represents $O_2(^1\Delta_g)$ [25], is actually under study.

3.6. An attempt to apply the steady state techniques

Zweig and Henderson [29] gave a useful kinetic treatment for quenching $O_2(^1\Delta_g)$, which Bellus [30] and Foote [31] applied as a steady state technique to determine the rates of $^1O_2$ reactions. Essentially they described the amount of product AO_2 which is formed following eqns. (2), (4), (6) and (7).

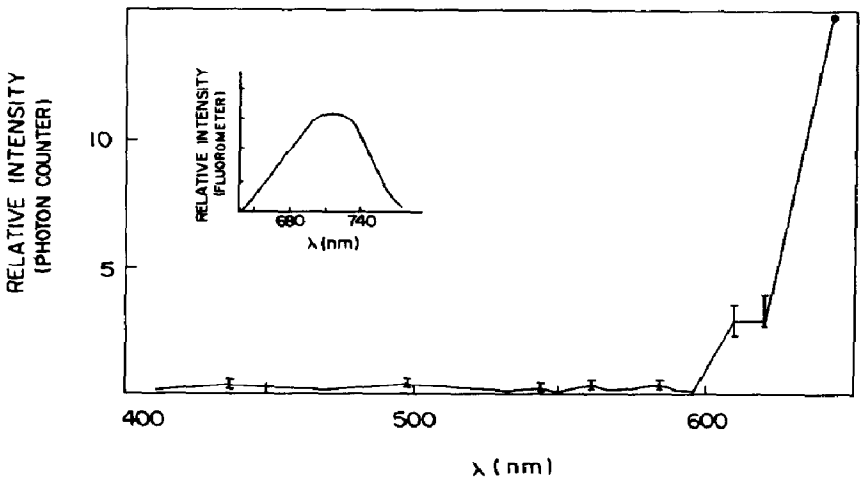

Fig. 4. Chemiluminescence spectrum of the MDA–HRP–Mn^{2+}–O_2 system determined with the filter spectrometer and chemiluminescence spectrum determined with the spectrofluorometer (inset).

The fraction of AO_2 formed is related to

$$[AO_2] = [^1O_2] \frac{k_A[A]}{k_A[A] + (k_q + k_R)[Q] + k_d}$$

and the reciprocal equation is

$$\frac{1}{[AO_2]} = \frac{1}{[^1O_2]} + \frac{(k_q + k_R)[Q] + k_d}{k_A} \frac{1}{[A]} \frac{1}{[^1O_2]}$$

where $[^1O_2]$ is the total amount of $^1O_2$ produced, [A] is the acceptor concentration, [Q] is the quencher concentration and k_q, k_R and k_A are the rate constants for quenching, reaction with Q and reaction with A respectively. If we consider the quenching of the photon emission by the bioenergized method [14] we have to use eqn. (9).

In this case $[AO_2]$ is proportional to $I_0 - I_{A,Q}$, where I_0 is the integrated photon emission in the absence of A and Q. Then if [Q] is constant and the amount of A converted into AO_2 is kept to less than 10%, a plot of $1/(I_0 - I_{A,Q})$ against $1/\{(k_q + k_R)[Q] + k_A[A]\}$ gives a straight line, as is shown in the following equation:

$$I_{A,Q} = [^1O_2] \frac{k_f[^1O_2]}{k_f[^1O_2] + (k_q + k_R)[Q] + k_d}$$

The reciprocal equation is

$$\frac{1}{I_0 - I_{A,Q}} = \frac{1}{k_f[^1O_2]^2} \left\{ (k_f[^1O_2] + k_d) + \frac{(k_f[^1O_2] + k_d)^2}{(k_q + k_R)[Q] + k_A[A]} \right\}$$

The intercept of this line is proportional to $(k_f[^1O_2] + k_d)/k_f[^1O_2]^2$ and the slope is $(k_f[^1O_2] + k_d)^2/k_f[^1O_2]^2$.

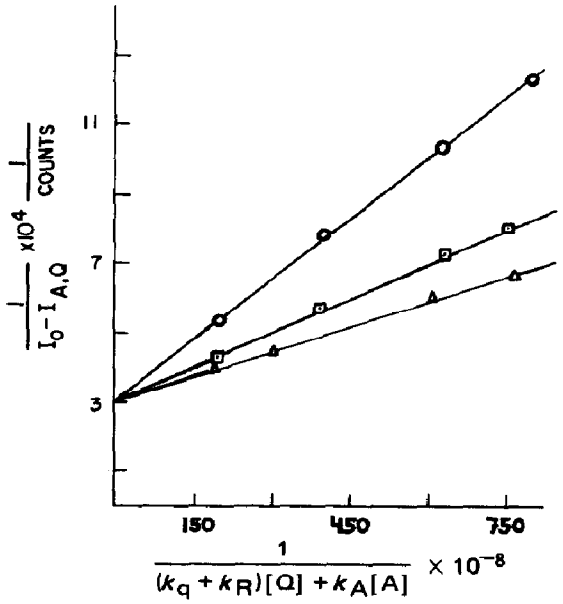

Fig. 5. Quenching of the bioenergized oxidation of histidine in the MDA–HRP–Mn^{2+}–O_2 system by guanosine: ○, 0.0 M; ⊡, 1.25 mM; △, 2.5 mM.

A series of such curves for several different quencher concentrations allows a distinction to be made between true physical quenching and other alternatives. The constancy of the intercept of the $I/(I_0 - I_{A,Q})$ *versus* $1/\{(k_q + k_R)[Q] + k_A[A]\}$ plot at different [Q] is diagnostic for $^1O_2$ quenching and implies that A and Q compete for a common intermediate [31]. The quenching of histidine oxidation by guanosine in the MDA–HRP–Mn^{2+}–O_2 system (Fig. 5) gave constancy in the intercept. This is an indication that the steady state equation used is a good fit to the observed values. The ratio of reactivity K_{SV}(histidine)/K_{SV}(guanosine) was about 6.0 in the photodynamic processes [24] (Table 1), and about 8.0 in the photobioenergized process.

TABLE 1

Quenching rate constants, and lifetime values for $^1O_2$ generated by the malonaldehyde–peroxidase–Mn^{2+}–O_2 system

System	*Quencher*	$k_q\tau^0$ (M^{-1})	k_q ($M^{-1}\,s^{-1}$)	$\tau^0 \times 10^6$ (s)
Photochemical	Histidine	—	5×10^7 [a]	2.0
MDA	Histidine	80	5×10^7	1.6
Photochemical	Guanosine	—	0.86×10^7 [b]	2.0
MDA	Guanosine	10	0.86×10^7	1.2
Photochemical	Bilirubin	—	2.0×10^9 [c]	2.0[d]
MDA	Bilirubin	10000	2.0×10^9	5.0

[a] From ref. 30.
[b] From ref. 24.
[c] From ref. 31.
[d] From ref. 23.

This implies that histidine and guanosine interact in similar ways in the photodynamic processes and in the photobioenergized process.

In conclusion, we suggest that the presence and role of $^1O_2$ in the HRP–MDA systems through the photobioenergized process can be ascertained in terms of combined data from photon emission, emission spectra, inhibitions by quenchers, product analyses, D_2O and DABCO effects, energy transfer to xanthenic dyes and kinetic analyses. In our work, we have successfully employed these experimental techniques for the detection of $^1O_2$ in many enzymatic processes in the dark.

Acknowledgments

This study was supported by the Conselho Nacional de Desenvolvimento Científico e Tecnológico, the Fundação de Amparo à Pesquisa do Estado de São Paulo, the Financiadora de Estudos e Projetos, the United Nations Educational, Scientific and Cultural Organization–United Nations Developing Programme (Brazil), Organization of American States (Binational Programme (Brazil–Chile)) and the Dirección de Investigaciones de la Universidad Católica de Valparaíso (Chile).

We thank Professor Pill-Soon Song, Professor G. Cilento, Professor E. Lissi and Professor F. Quina for suggestions and critical reading of the manuscript as well as for enlightening discussions. N.D. thanks the Guggenheim Foundation for a fellowship.

References

1 G. Cilento, *Photochem. Photobiol. Rev.*, *5* (1980) 199.
2 G. Cilento, *Acc. Chem. Res.*, *13* (1980) 225.
3 N. Durán, M. Haun and J. E. Brunet, *Rev. Latinoam. Quím.*, *12* (1981) 1.
4 N. Durán and G. Cilento, *Photochem. Photobiol.*, *32* (1980) 113.
5 E. J. H. Bechara, O. M. M. Faria Oliveira, N. Durán, R. C. de Baptista and G. Cilento, *Photochem. Photobiol.*, *30* (1979) 101.
6 M. Haun, N. Durán, O. Augusto and G. Cilento, *Arch. Biochem. Biophys.*, *200* (1980) 245.
7 C. Vidigal-Martinelli, K. Zinner, B. Kachar, N. Durán and G. Cilento, *FEBS Lett.*, *108* (1978) 266.
8 C. C. C. Vidigal, A. Faljoni-Alario, N. Durán, K. Zinner, Y. Shimizu and G. Cilento, *Photochem. Photobiol.*, *30* (1979) 195.
9 K. Takayama, M. Nakano, K. Zinner, C. C. C. Vidigal, N. Durán, Y. Shimizu and G. Cilento, *Arch. Biochem. Biophys.*, *176* (1976) 663.
10 G. Cilento, N. Durán, K. Zinner, C. C. C. Vidigal, O. M. M. Faria Oliveira, M. Haun, A. Faljoni, O. Augusto, R. C. de Baptista and E. J. H. Bechara, *Photochem. Photobiol.*, *28* (1978) 445.
11 M. P. de Mello, S. M. De Toledo, M. Haun, G. Cilento and N. Durán, *Biochemistry*, *19* (1980) 5270.
12 M. P. De Mello, S. M. De Toledo, H. Aoyama, H. K. Sarkar, G. Cilento and N. Durán, *Photochem. Photobiol.*, *36* (1982) 21.
13 N. I. Krinsky, in H. H. Wasserman and R. W. Murray (eds.), *Singlet Oxygen*, Academic Press, New York, 1979, p. 597.

14 N. Durán, in W. Adam and G. Cilento (eds.), *Chemical and Biological Generation of Excited States*, Academic Press, New York, 1982, p. 345.
15 D. F. Bowman, T. Gillant and K. U. Ingold, *J. Am. Chem. Soc., 93* (1971) 6555.
16 R. E. Kellogg, *J. Am. Chem. Soc., 91* (1969) 5433.
17 K. C. Wu and A. M. Trozzolo, *J. Photochem., 10* (1979) 407.
18 C. S. Foote, in W. S. Caughey (ed.), *Biochemical and Clinical Aspects of Oxygen*, Academic Press, New York, 1979, p. 603.
19 P. A. Srere, G. W. Kosicki and R. Lumry, *Biochim. Biophys. Acta, 50* (1961) 184.
20 J. Cadet and R. Teoule, *Photochem. Photobiol., 28* (1978) 661.
21 E. J. J. Grabowski and R. L. Autry, *Tetrahedron, 25* (1969) 4315.
22 N. Durán, J. Baeza, J. E. Brunet, G. A. Gonzales, C. P. Sotomayor and A. Faljoni-Alario, *Biochem. Biophys. Res. Commun., 103* (1981) 131.
23 C. S. Foote, in W. A. Pryor (ed.), *Free Radicals in Biology*, Academic Press, New York, 1976, p. 85.
24 C. S. Foote, in B. Rånby and J. F. Rabek (eds.), *Singlet Oxygen, Reactions with Organic Compounds and Polymers*, Wiley, New York, 1978, p. 135.
25 A. U. Khan, P. Gebauer and L. P. Hager, *Proc. Natl. Acad. Sci. U.S.A., 80* (1983) 5195.
26 J. Piatt and P. J. O'Brien, *Eur. J. Biochem., 93* (1979) 323.
27 T. I. Matsuura, I. Saito and S. Kato, in E. D. Bergmann and B. Pullman (eds.), *The Purines — Theory and Experiment*, Vol. IV, Israel Academy of Sciences and Humanities, Jerusalem, 1972, p. 418.
28 B. R. Anderson, T. F. Lint and A. M. Brendzel, *Biochim. Biophys. Acta, 542* (1978) 527.
29 A. Zweig and W. A. Henderson, Jr., *J. Polym. Sci., Polym. Chem. Edn., 13* (1975) 717.
30 D. Bellus, in B. Rånby and J. F. Rabek (eds.), *Singlet Oxygen, Reactions with Organic Compounds and Polymers*, Wiley, New York, 1978, p. 61.
31 C. S. Foote, in H. H. Wasserman and R. W. Murray (eds.), *Singlet Oxygen*, Academic Press, New York, 1979, p. 139.