STOICHIOMETRIC RATIOS OF REACTIVE OXYGEN SPECIES PRODUCTION BY POLYMORPHONUCLEAR LEUKOCYTES[1]

Adriana G. Prat, Mónica Galleano, Andrea S. Llera and Alberto Boveris*
Institute of Biochemistry and Biophysics - School of Pharmacy and Biochemistry - University of Buenos Aires - Junín 956 - 1113 Buenos Aires - Argentina

The stoichiometric ratios between O_2 uptake, O_2^- and H_2O_2 production were determined in polymorphonuclear leukocytes (PMN) stimulated by phorbol myristate acetate (PMA) and N-formyl-methionyl-leucyl-phenylalanine (FMLP). Polarographic measurements of O_2 consumption, and spectrophotometric determinations of O_2^- (superoxide dismutase-inhibitable cytochrome c reduction) and H_2O_2 (complex II [H_2O_2-horseradish peroxidase] formation) production in rat and human PMN, showed approximate ratios of 1:2:1 and 1:1:0.5 for PMA- and FMLP-stimulated PMN, respectively. The results indicate that when PMA is used as stimulus, both O_2^- and H_2O_2 are released to the extracellular milieu. When PMN are exposed to FMLP, O_2^- and H_2O_2 are released at different ratio, suggesting that part of them is being released to a different space than the extracellular and probably metabolized by the PMN. Differences between both stimuli were also found in the spectral analysis of the chemiluminescence from PMA- and FMLP-stimulated human PMN. In both cases, emission bands at 610 ± 20, 535 ± 15 and 420 ± 20 nm were identified. In the case of FMLP-activated PMN a prominent emission band at 550-580 nm was also observed. The main emission bands indicate the production of $^1O_2$ and carbonyl groups in the excited state. The minor difference observed between both spectra indicates that the production of a given excited state depends on the environment and stimuli to which PMN are challenged.

Keywords: leukocytes; reactive oxygen species; chemiluminescence.

INTRODUCTION

It is well known that, when polymorphonuclear leukocytes (PMN) are exposed to soluble or particulate stimuli, there is a large increase in cyanide-insensitive oxygen consumption, known as respiratory burst[1, 2]. The reactive oxygen species produced during this process are responsible for the bacterial action of PMN[3, 4]. It has also been reported that bacteria containing large amounts of catalase and superoxide dismutase (SOD) will resist the attack by PMN[5, 6]. The enzymatic system capable of taking up oxygen during the respiratory burst is the NADPH-dependent membrane-bound flavoprotein, known as NADPH oxidase or superoxide synthetase[7-9], dormant or inactive in non-stimulated cells. The activated oxidase catalyzes the one-electron reduction of oxygen to superoxide anion (O_2^-). The dismutation of O_2^- leads to the formation of hydrogen peroxide (H_2O_2), and both in turn may generate hydroxyl radical ($OH^\cdot$), hypochlorous acid and eventually singlet oxygen ($^1O_2$)[10, 11]. However, the molecular mechanisms by which these reactive species are generated are not completely clear. Moreover, it has been suggested that H_2O_2 could be produced directly by the NADPH oxidase under certain conditions[12 - 14].

Stimulation of PMN is also associated with the emission of chemiluminescence[15, 16]. Little is known about the identity of the excited species responsible for photon emission. The present study provides information suggesting that different excited species may be formed during the respiratory burst depending on the stimuli being used, in this case, phorbol myristate acetate (PMA) and N-formyl-methionyl-leucyl-phenylalanine (FMLP). The different response to these two agents is also reflected in the different stoichiometry for O_2, O_2^- and H_2O_2 obtained in each case.

MATERIALS AND METHODS

Isolation of polymorphonuclear cells

Human blood was collected from healthy human donors in conical tubes containing a mixture of dextran (6.5% in saline) and sodium citrate (3.8% in distilled water) in a relation 2:1 followed by hypotonic lysis of erythrocytes. Purified neutrophils were isolated using a Hypaque-Ficoll gradient[17]. Leukocytes were then washed and resuspended in 10 mM phosphate buffer, 145 mM NaCl, 5 mM glucose, pH 7.4 (PBS). Cells were quantified in a Neubauer chamber and the suspension usually contained 4-8 x 10^6 cell/ml. Integrity of plasma membrane was assayed by Trypan Blue exclusion, being the percentage of viable PMN larger than 97%. Rat polymorphonuclear cells were obtained from intraperitoneal exudates of animals that were injected i.p. with 1 ml of 2% starch 8-10 h before the extraction. The extraction was done by punction of the intraperitoneal cavity. Cell suspensions containing more than 90% of viable PMN were washed and resuspended in PBS.

Oxygen uptake

Oxygen consumption was determined polarographically with a Clark electrode at 37ºC[18]. The PMN were assayed in

1. This work was supported by grants from the Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET) and University of Buenos Aires.

2. Present address of Dr. A. G. Prat: Renal Unit, Massachusetts General Hospital, Charlestown, MA 02129, USA.

PBS containing 1 mM $CaCl_2$, with constant gentle stirring. Results are given in nmol O_2/min/10^7 cells.

Superoxide anion production

The rate of superoxide anion production was measured by the superoxide dismutase (SOD)-inhibitable cytochrome c reduction[19]. The reduction of 10 mM ferricytochrome c was monitored at 550-540 nm (E = 19 mM^{-1} cm^{-1}) in a 356 Hitachi-Perkin Elmer double-beam spectrophotometer. The reaction medium was PBS containing 1 mM $CaCl_2$. The results are expressed in nmol O_2^-/min/10^7 cells.

Hydrogen peroxide generation

The assay used for the determination of H_2O_2 production was based on the formation of horseradish peroxidase H_2O_2 enzyme-substrate complex II[20]. This complex was measured spectrophotometrically at 417-402 nm (E = 50 mM^{-1} cm^{-1}) in a 356 Hitachi-Perkin Elmer double-beam spectrophotometer. The reaction medium was PBS containing 1 mM $CaCl_2$. The final concentration of horseradish peroxidase (HRP) in the cuvette was 0.2 μM. The accuracy of the method was estimated using mixtures of glucose and glucose oxidase as internal standards as previously described[20]. Hydrogen peroxide measurements were also performed in the presence of SOD (40 μg/ml). The results are given in nmol H_2O_2/min/10^7 cells.

Chemiluminescence measurements

Chemiluminescence was measured in a 3320 Packard Tricarb liquid scintillation counter in the out-of-coincidence mode using glass vials[21]. The cells (2-5 x 10^6 cells/ml) were suspended in PBS containing 1 mM $CaCl_2$. The photoemission of PMN was measured before (basal) and after (post-stimulation) stimulation with PMA or FMLP. Counting intervals were 30 sec and results are given in cpm. Acetate filters were used for the spectral analysis[22]. The percentages of chemiluminescence were corrected by filter transmittance and spectral response of the photomultiplier.

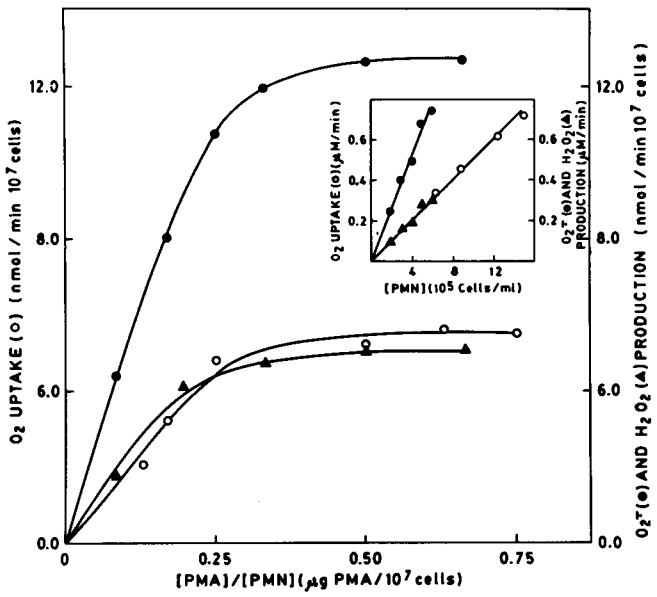

Figure 1. Dose-response curves of O_2 uptake (open circles) and O_2^- (filled circles) and H_2O_2 (filled triangles) released by PMA-stimulated rat PMN. The cell concentration was usually 4-8 10^6 cells/ml resuspended in PBS. Numbers express nmoles/min/10^7 cells. Inset: Linear relationship between the parameters measured and PMN concentration at saturating levels of stimulus (0.5 μg PMA/10^7 cells).

Stimuli

N-formyl-methionyl-leucyl-phenylalanine (FMLP) and phorbol myristate acetate (PMA) were dissolved in DMSO, which had no effect on PMN activation at the concentrations, about 1-3 ul/ml, used for these experiments.

Reagents

PMA, FMLP, HRP type IV, superoxide dismutase, dextran (MW 140 kD), Ficoll (MW 400 kD), Hypaque, cytochrome c type III and glucose were purchased from Sigma Chem.Co. (Saint Louis, MO).

RESULTS

Suspensions of either rat or human PMN were exposed to increasing concentrations of PMA and assayed for oxygen uptake and O_2^- and H_2O_2 production (Figs 1 and 2). Addition of PMA to rat PMN stimulated O_2 consumption, O_2^- and H_2O_2 production up to rates of 5.5, 12.5 and 5.0 nmol/min/10^7 cells, respectively, with PMA concentrations giving 50% of the maximal response of 0.14, 0.12 and 0.11 μg PMA/10^7 cells, respectively (Fig 1). The ratios of these three parameters at saturating concentration of PMA were linear with the number of cells (Fig 1, inset). When human PMA were exposed to PMA, the maximal rates of O_2 uptake, and O_2^- and H_2O_2 production were 3.0, 5.0 and 2.0 nmoles/min/10^7 cells, for ratios of 1.3, 1.6 and 1.9 μg PMA/10^7 cells, to reach half of the maximal response in each case (Fig 2).

Human PMN were also stimulated with FMLP and O_2 uptake, and O_2^- and H_2O_2 production were assayed (Fig 3). A great variability in rates of O_2 uptake and H_2O_2 production was observed among different cell batches due to individual differences and to the different decay of activity from the time of blood extraction to the time of the measurements[23]. The maximal rates of O_2 uptake, and O_2^- and H_2O_2 production were 5.6, 5.4 and 2.5 nmoles/min/10^7 cells, for ratios of 0.3, 0.4 and 0.35 nmoles FMLP/10^7 cells for half of the maximal response in each case (Fig 2).

The maximal rates of O_2 uptake were compared with the rates of O_2^- and H_2O_2 production (Table 1). Addition of SOD to either PMA- or FMLP-stimulated PMN did not alter the

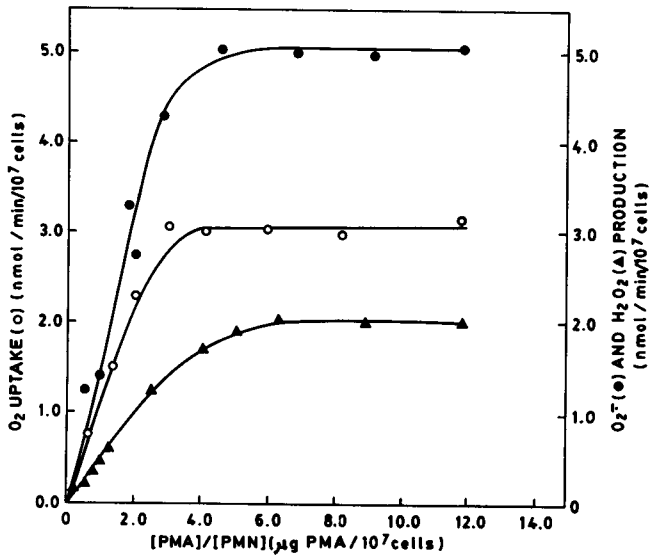

Figure 2. Dose-response curves of O_2 uptake (open circles) and O_2^- (filled circles) and H_2O_2 (filled triangles) released by PMA-stimulated human PMN. The conditions were as in Figure 1.

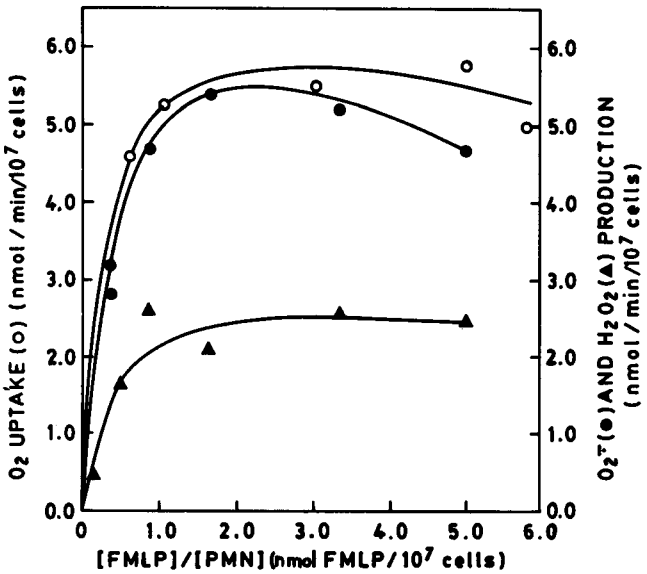

Figure 3. Dose-response curves of O_2 uptake (open circles) and O_2^- (filled circles) and H_2O_2 (filled triangles) released by FMLP-stimulated human PMN. The conditions were as in Figure 1.

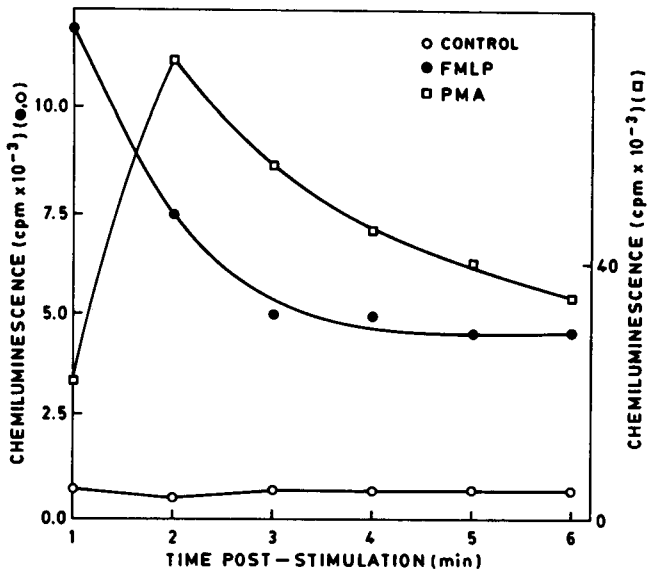

Figure 4. Time course of the emission of chemiluminescence of human PMN, control or stimulated with PMA (4 μg/10^7 cells) or FMLP (10 nmol/10^7 cells).

Table 1. Stoichiometric Ratios of the Rates of Oxygen Uptake, and Superoxide and Hydrogen Peroxide Production by Rat and Humam PMN Stimulated by PMA and FMLP.

PMN	Stimulus	Oxygen uptake	Superoxide anion	Hydrogen peroxide	Stoichiometric ratios
		(nmoles/min/10^7 cells)			
Rat	PMA	5.5±0.3	12.5±0.8	5.0±0.3	1:2.3±0.2:0.9±0.1
Human	PMA	3.0±0.4	5.0±0.5	2.0±0.2	1:1.7±0.3:0.7±0.1
Human	FMLP	5.6±0.4	5.4±0.3	2.5±0.2	1:1.0±0.3:0.4±0.1

rates of H_2O_2 production (not shown). This observation indicated that the O_2^- released to the extracellular media and thus, detected by the cytochrome c method, underwent spontaneous dismutation at a rate kinetically compatible with the rate of O_2^- production.

The stoichiometry calculated when human or rat PMN were stimulated with PMA indicated that all oxygen was being reduced to O_2^- which dismutated spontaneously to H_2O_2 (Table 1). If the stimulus was FMLP, however, the stoichiometry was different, indicating that other reactions (besides the spontaneous dismutation) could be taking place after the reduction of O_2 to O_2^-.

Since these oxidative reactions lead to the emission of chemiluminescence, it was reasonable to expect differences in the spectral properties of this emission as PMN were stimulated with the different agents. Human PMN exposed to FMLP reached maximal emission during mixing time (Fig 4). However, when PMA was the stimulus a lag time in emission was observed (Fig 4) similarly to the lag phase that was observed in both O_2 uptake and H_2O_2 production using the same stimulus. The spectral analysis of the 420-620 nm emission of PMN stimulated by FMLP and PMA shows some differences. In the range of 550-580 nm, the emission of PMA-stimulated cells was about 7% of that in the 520-550 nm range (Fig 5). With FMLP-stimulated PMN, the ratio between emissions at 550-580 to 520-550 nm was close to 50%, showing that some of the excited species involved in the process are different (Fig 5).

DISCUSSION

Babior[4] suggested that oxygen taken up by stimulated PMN is primarily converted to O_2^-. It was known that oxygen uptake by PMN led to H_2O_2 formation[24] although this product may arise from either two one-electron transfers or one two-electron transfer. Actually, it has been suggested that, under some conditions, NADPH oxidase could generate O_2^- or H_2O_2[12-14]. When purified NADPH oxidase was used, for instance, it was capable of conducting both one- and two-electron transfer steps in O_2 reduction[13]. In fact, there are biochemical examples of both possibilities. Peroxisomal formation of H_2O_2 seems to occur quantitatively through two-electron transfers without generation of O_2^-[25]. On the other hand, mitochondrial formation of H_2O_2[26] appears as the consequence of two one-electron transfers and dismutation of the O_2^- formed as intermediate species. Moreover, microsomal formation of H_2O_2 seems to occur in both ways simultaneously; about one half by two one-electron transfers and the other half by one two-electron transfer[5, 21]. Up to the reports by Makino *et al.*[27] and Loschen[28], there was no clear data concerning the mechanism by in which O_2^- and H_2O_2 were formed in PMN. The present study agrees with them in that O_2^- is primarily generated and constitutes the stoichiometric precursor of H_2O_2 in the respiratory burst of activated neutrophils. The rates of O_2^- and H_2O_2 production enable us to calculate the extracellular steady-state of O_2^- compatible with the observations reported

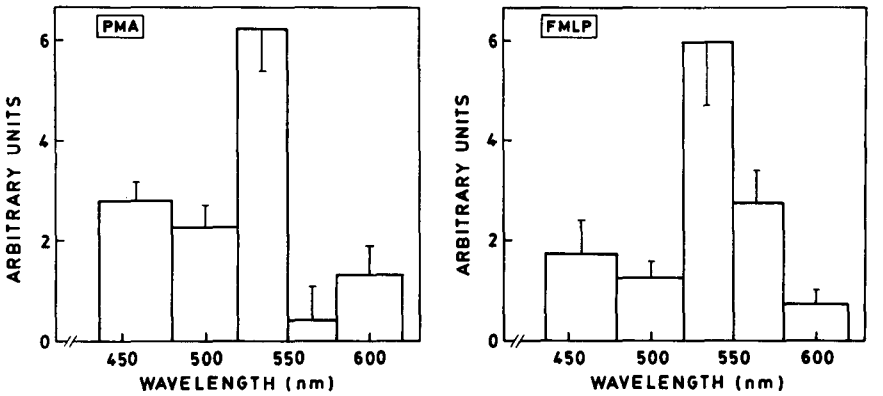

Figure 5. Spectral analysis of the chemiluminescence emitted by PMA- or FMLP-stimulated PMN. The partial spectral analysis was carried out using filters as described in Materials and Methods.

here. Hydrogen peroxide is produced by 10^7 PMN cells/ml at pH 7.4 and incubated with 3.6 μg PMA at a rate of 2 nmol/min. Since H_2O_2 is mainly produced from spontaneous O_2^- dismutation, considering that superoxide dismutase (SOD) had no effect in the rate of H_2O_2 production, the reaction: $O_2^- + HO_2. \longrightarrow H_2O_2 + O_2$ with a reaction constant of 6×10^5 M^{-1} sec^{-1}[29] and with $[HO_2.] = [O_2^-]/160$ considering a pK of 4.8 for protonation of O_2^- yield a steady state concentration of O_2^- of 2.9 μM, a concentration that justifies its bactericidal properties since this level is about 10^5 times higher than intracellular levels[5]. This extracellular steady state concentration of O_2^- will be reached in about 40 sec at the normal rate of 4.0 nmol O_2^-/ min of stimulated PMN.

PMA- and FMLP-stimulated PMN gave different stoichiometric ratios $O_2:O_2^-:H_2O_2$. The soluble stimulus PMA activates PMN without vacuole formation. The stoichiometry of O_2 uptake, O_2^- and H_2O_2 production, considering O_2 uptake as the unit, is approximately 1:2:1 (Table 1), which is consistent with a total release of O_2^- as primary product to the extracellular space. All oxygen taken up was recovered as O_2^- in the reaction medium in agreement with the postulated array of the NADPH oxidase as a transmembrane complex in which the active site is on the outer face of the PMN plasma membrane[30]. The reactions that explain these results are:

$$2\ O_2 \longrightarrow 2\ O_2^- \qquad (1)$$

$$2\ O_2^- + 2\ H^+ \longrightarrow H_2O_2 + O_2 \qquad (2)$$

Although there is an initial relationship $O_2:O_2^-$ of 1:1, the fact that O_2^- readily dismutates to H_2O_2 giving a molecule of oxygen back to the system (eq. 2), results in an actual ratio $O_2:O_2^-$ of 1:2 which is the value reported in Table 1.

Moreover, functioning of the oxidase implies a transmembrane electron flow in which the electron is delivered at the external face of the plasma membrane to the molecule. The operation should create a membrane potential, negative outside and positive inside. The recovery of two O_2^- per each O_2 used, as predicted for equations (1) and (2), indicates that there is no effective back diffusion of O_2^- through anion channels, in spite of the membrane potential, as described for the plasma membrane of red blood cells[31].

In the case of FMLP-stimulated PMN, the mechanism seems to be different as a consequence of the internal vacuole formation. In this case the stoichiometric ratio is 1:1:0.5 (Table 1). Assuming that all the O_2^- at the extracellular side is detected, this result (ratio $O_2:O_2^-$ of 1:1) appears to indicate that part of the O_2 consumed is reduced to O_2^- and released to a different space, probably, inside the cell. Moreover, the measured ratio $O_2^-:H_2O_2$ was 2:1 indicates that O_2^- produced at the extracellular space dismutates forming H_2O_2. The H_2O_2 produced in internal vacuoles may in turn be metabolized by myeloperoxidase, before it diffuses to the extracellular space.

Cheson *et al.*[32], offered an spectral analysis of the chemiluminescence of zymosan-stimulated PMN that appeared as "white" without prominent and definite bands in the range 480 to 640 nm, but with lower emission in the 420-470 nm range. Our results using PMA - and FMLP-stimulated PMN cells indicate that the emission spectra of stimulated phagocytes depend partially on the nature of the stimulus. We have identified emission bands at 420-480; 520-550 and 580-620 nm which are observed in PMN stimulated with both PMA and FMLP. The main emission band in both cases is the one at 520-550 nm. An interesting difference is observed in the case of FMLP-stimulated PMN where a prominent emission band appears at 550-580 nm. The 420 ± 20 nm band is consistent with the generation of excited carbonyl groups (aldehydes and ketones)[33], the 580-620 nm band with the dimol emission of singlet oxygen[34] and the 550-580 nm band with a compound of the structure R-CO*-CO-R[35]. The differences in the generated excited states may reflect the different reactions in which the precursors (O_2^-, H_2O_2 and $OH^\cdot$) become involved, in terms of their vicinity with cell membranes, targets and interaction with myeloperoxidase.

REFERENCES

1. Baldridge, C. W. and Gerard, R. W.; *Am. J. Physiol.* (1933), **103**, 235.
2. Klebanoff, S. J. (1992) In: Inflammation: Basic Principles and Clinical Correlates (Gallin, J.I., Goldstein, I.M. and Snyderman, R., Eds.) pp 541-588, Raven Press, Ltd., New York.
3. Curnutte, J. T. and Babior, B. M.; *J. Clin. Invest.* (1974), **53**, 1662.
4. Babior, B. M.; *N. Engl. J. Med.* (1978), **298**, 659.
5. Chance, B., Sies, H. and Boveris, A.; *Physiol. Rev.* (1979), **59**, 527.
6. Beaman, B. L.; Scates, S. M.; Moring, S. E.; Deem, R. and Misra, H. P.; *J. Biol. Chem.* (1983), **258**, 91.
7. Patriarca, P.; Cramer, R.; Moncalvo, S.; Rossi, F. and Romero, D.; *Arch. Biochem. Biophys.* (1971), **145**, 255.
8. Serra, M. C.; Bellavite, P.; Davoli, A.; Bannister, J. V. and Rossi, F.; *Biochim. Biophys. Acta* (1984), **4788**, 138.
9. Borregaard, N.; *J. Bioenerg. Biomemb.* (1988), **20**, 637.
10. Cagan, R. H., and Karnovsky, M. L.; *Nature* (1964), **204**, 255.
11. Klebanoff, S.; *J. Biol. Chem.* (1974), **249**, 3724.

12. Hoffstein, S. T.; Gennaro, D. E. and Manzi, R. M.; *Inflammation* (1985), **9**, 425.
13. Green, T. R. and Wu, D. E.; *J. Biol. Chem.* (1986), **261**, 6010.
14. Follin, P. and Dahlgren, C.; *Biochem. Biophys. Res. Comm.* (1990), **167**, 970.
15. Allen, R. C.; Stejernholm, R. L. and Stelle, R. H.; *Biochem. Biophys. Res. Comm.* (1973), **47**, 679.
16. Kakinuma, K.; Cadenas, E.; Boveris, A. and Chance, B.; *FEBS Lett.* (1979), **102**, 38.
17. Boyum, A.; *Scand. J. Clin. Lab. Invest.* (1968), **21**, 77.
18. Estabrook, R. (1967) In: Methods in Enzymology (Estabrook, R. and Pullman, M.E. Eds.) Vol X, pp 41-47, Academic Press, New York.
19. McCord, J. M. and Fridovich, I.; *J. Biol. Chem.* (1968), **243**, 5753.
20. Boveris, A.; Oshino, N. and Chance, B.; *Biochem. J.* (1972), **128**, 617.
21. Boveris, A.; Fraga, C. G.; Varsavsky, A. I. and Koch, O. R.; *Arch. Biochem .Biophys.* (1983), **227**, 534.
22. Boveris, A.; Sanchez, R. A.; Varsavsky, A. I. and Cadenas, E.; *FEBS Lett.* (1980), **113**, 29.
23. English, D.; Roloff, J. S. and Lukens, J. N.; *J. Immunol.* (1981), **126**, 165.
24. Baehner, R. L; Gilman, N. and Karnovsky, M. L.; *J. Clin. Invest.* (1970), **49**, 629.
25. Masters, C. and Holmes, R.; *Physiol. Rev.* (1977), **57**, 816.
26. Boveris, A. and Cadenas, E.; *FEBS Lett.* (1975), **54**, 311.
27. Makino, R.; Tanaka, T.; Iizuka, T.; Ishimura, Y. and Kanegasaki, S.; *J. Biol. Chem.* (1986), **261**, 11444.
28. Loschen, G. (1986) In Superoxide and Superoxide Dismutase in Chemistry, Biology and Medicine (Rotilio, G., Ed.) pp 373-375, Elsevier Publishers B., Amsterdam.
29. Fee, J. A. and Valentine, J .S. (1977) In: Superoxide and Superoxide Dismutases (Michelson, A. M., McCord, J. M. and Fridovich, I., Eds.) pp 191-60, Academic Press, London.
30. Schirmer, R. H.; Schulz, G. E. and Untucht-Grau, R.; *FEBS Lett.* (1983), **54**, 1.
31. Lynch, R. E. and Fridovich, I.; *J. Biol. Chem.* (1978), **253**, 1838.
32. Cheson, B. D.; Christensen, R. L.; Sperling, R.; Kohler, B. E. and Babior, B. M.; *J. Clin. Invest* (1976), **58**, 789.
33. Faria Oliveira, O. M. M.; Haun, M.; Duran, N. O'Brien, P.; O'Brien, C.; Bechara, E. J. H. and Cilento, G.; *J. Biol. Chem.* (1978), **253**, 4707.
34. Khan, A. V. and Kasha, M.; *J. Chem. Phys.* (1963), **39**, 2105.
35. Zinner, K.; Vidigal-Martinelli, C.; Duran, N.; Marsaioli, A. J. and Cilento, G.; *Biochem. Biophys. Res. Comm.* (1980), **92**, 32.

This special issue of Química Nova is dedicated to Prof. G. Cilento on the occasion of his 70th birthday and is financed by the Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP).