Biochemical Pharmacology, Vol. 38, No. 17, pp. 2819–2823, 1989.
Printed in Great Britain.

0006-2952/89 $3.00 + 0.00

IN VITRO AND *IN VIVO* EFFECT OF MERCURY, LEAD AND CADMIUM ON THE GENERATION OF CHEMILUMINESCENCE BY HUMAN WHOLE BLOOD

O. GUILLARD* and R. LAUWERYS

Unité de Toxicologie Industrielle et Médecine du Travail, Université Catholique de Louvain, 30.54 Clos Chapelle-aux-Champs, 1200 Brussels, Belgium

(*Received* 10 *March* 1989; *accepted* 11 *March* 1989)

Abstract—A method based on the use of the luminogenic substance, luminol, was developed for measuring the respiratory burst induced by phorbolmyristate acetate in 4 μl of human whole blood. *In vitro*, the three cations, Hg^{2+}, Pb^{2+} and Cd^{2+}, inhibit the peak luminescence response at concentrations higher than those usually found in blood of subjects exposed to these metals. Cadmium metallothionein, however, in a concentration range similar to that found in whole blood of workers exposed to cadmium strikingly reduces the respiratory oxidative burst. In workers exposed to inorganic lead or mercury vapour, no reduction of the chemiluminescence response of whole blood was observed while a slight but statistically significant reduction was found in whole blood from cadmium exposed workers. If a similar effect occurs in lung macrophages, it might decrease the respiratory tract resistance to infection.

Phagocytosis and/or chemical stimulation of polymorphonuclear leukocytes (PMNs) and monocytes triggers a burst of oxidative metabolism which results in the formation of reactive oxygen species (ROS) such as superoxide anion (O_2^-), hydroxyl radical ($OH^\cdot$), hydrogen peroxide (H_2O_2) and singlet oxygen ($^1O_2$) [1]. The primary reaction is the reduction of O_2 to superoxide anion catalyzed by a plasma membrane NADPH oxidase, the cofactor being generated through the hexose monophosphate pathway. The generation of ROS is an important non specific defence mechanism against microorganisms [2].

The ROS can be quantified by the use of chemiluminogenic substances such as luminol or lucigenin. The resulting chemiluminescence (CL) is detected with a liquid scintillation counter or a luminometer [3]. Experimental studies have indicated that some heavy metals impair host resistance to infection [4, 5]. It has been suggested that this effect might result from metal interference with the microbicidal activity of polymorphonuclear leukocytes and macrophages, particularly from inhibition of the respiratory burst [6–8]. It should be noted, however, that the effect of metals on ROS production was only assessed *in vitro* and at concentrations far above those commonly encountered in blood of subjects exposed to heavy metals. We have therefore compared the *in vitro* and *in vivo* effect of lead, mercury and cadmium on ROS production by human leukocytes.

The CL is classically measured on purified leukocytes. This method is time-consuming and requires a large volume of blood. Whole blood can also be used [9–11] provided chemiluminescence quenching is prevented [12]. A study of the various parameters influencing CL has enabled us to develop a rapid and sensitive method using only 4 μl of whole blood. It offers the advantage of simultaneously measuring the respiratory burst of circulating PMNs and monocytes and may therefore be more representative of the *in vivo* situation than those utilising isolated cell systems.

MATERIALS AND METHODS

Apparatus. CL was measured with a LKB luminometer (model 1250) equipped with a digital printout set to 1 or 10 sec recording integral and connected to a chart recorder. The cuvette compartment was maintained at 37°.

Reagents. Except otherwise stated, all the chemicals were purchased from Merck (Darmstadt, F.R.G.).

All the solutions were prepared with purified water (specific resistance > 18 MΩ/cm) made sterile by filtration through a sterivex-GV-0.22 μm Millipore filter (Millipore Corp., Bedford, MA 0730).

The phosphate-buffered saline stock solution (PBS) contained per liter 1.36 mol of NaCl, 20 mmol KCl, 14 mmol KH_2PO_4 and 64 mmol $Na_2HPO_4.2H_2O$. Its pH was adjusted to 7.25. PBS Ca^{2+} Mg^{2+} was prepared by diluting ten-fold PBS with water containing per liter 0.88 mmol $CaCl_2$ $2H_2O$ and 0.24 mmol $MgCl_2$ $6H_2O$. The pH of this solution was 7.4. PBS Ca^{2+} Mg^{2+} containing D-glucose (PBS–GCa^{2+} Mg^{2+}) was prepared by adding 1 ml of 10% (w/v) D-glucose to 100 ml of the former solution. This solution was prepared daily.

Phorbolmyristate acetate (PMA) (Sigma Chemical, St Louis, MO) was used for initiating the respiratory burst; it was dissolved in dimethylsulfoxide (1 mg/ml) and 10 μl aliquots of this stock solution were stored at −20° in the dark. Before use, PMA was diluted with PBS Ca^{2+} Mg^{2+} to obtain a working solution containing 10 μg/ml which was kept at room temperature but protected from light by covering the

* Present address: Laboratoire de Biochimie et de Pharmacologie, Faculté de Médecine et de Pharmacie, 34 rue du Jardin des Plantes, 86034 Poitiers, Cedex, France.

tube with aluminium paper.

The stock luminol working solution was prepared daily and contained 2 mg of luminol (Sigma) and 8 μl of triethylamine (Sigma) per ml of PBS–G Ca^{2+} Mg^{2+}. After ultrasonication the solution was filtered through a Millipore filter (0.22 μm) and was stored in the dark at room temperature. Except otherwise stated, the working solution contained 2×10^{-3} M luminol in PBS–G Ca^{2+} Mg^{2+}.

Stock aqueous solution of $CdCl_2$ (10^{-1} M), $HgCl_2$ (10^{-2} M), $PbCl_2$ (10^{-2} M) were adjusted to pH 7.4. Two stock aqueous solutions of cadmium cysteine complex were prepared, one containing equimolar concentrations of $CdCl_2$ and L-cysteine (Cd–cysteine 1/1) and one containing L-cysteine at twice the molar concentration of $CdCl_2$ (Cd–Cysteine 1/2). Metallothionein bound cadmium was prepared as described by Bernard *et al.* [13]. The cadmium concentration in the complex was determined by flameless atomic absorption spectrometry. Appropriate dilutions of the various metal preparations were made in PBS G Ca^{2+} Mg^{2+}.

Study population. The control group included 21 clinically healthy subjects not occupationally exposed to heavy metals and whose age ranged from 22 to 52 years. The concentrations of lead, mercury and cadmium in whole blood were below 25, 1 and 0.1 μg/100 ml respectively. Groups of workers exposed either to lead (N = 25) or mercury (N = 16) or cadmium (N = 13) were also examined. The lead workers were occupied in a lead battery factory. Their age ranged from 23 to 50 years (mean: 33.5) and their mean duration of exposure was 10 years (range: 1–26). At the time of the survey the concentration of lead in blood (PbB) ranged from 34.8 to 76.5 with a mean of 60.3 μg/100 ml. The mercury workers were employed in a chlor-alkali plant. Their age ranged from 22 to 58 years (mean: 35.1) and their mean duration of exposure amounted to 7.4 years (range: 2.3–15). The average concentration (and range) of mercury in blood (HgB) and in urine (HgU) were 2.68 μg/100 ml (1.3–5.0) and 143 μg/g creatinine (71–315) respectively. The cadmium workers were occupied in a factory producing cadmium salts for 1 to 14 years. Their age ranged from 21 to 50 years. The average concentration of cadmium in blood (Cd–B) and in urine (Cd–U) were 0.47 μg/100 ml (0.06–1.25) and 4.21 μg/g creatinine (1–18.6) respectively.

Blood samples. Venous blood was withdrawn with a syringe and transferred in two tubes, one containing heparin used for CL measurement and the other containing EDTA used for differential leukocyte count. For the CL assay which was performed within 4 hr after blood withdrawal, 10 μl of heparinized whole blood was first transferred in a polypropylene tube and diluted with 0.990 ml of PBS.

Development of the CL procedure. With whole blood (4 μl) from normal subjects, the CL response induced by PMA was progressive and usually reached a maximum within 10 min (see also later, Figs. 3–5).

Figure 1 shows the effect of luminol concentration on peak CL response induced by PMA. The maximum activity was found with a luminol concentration of 2×10^{-3} M. The luminol solution must be prepared daily; a decreased CL response occurred when the solution was kept for more than 24 hr.

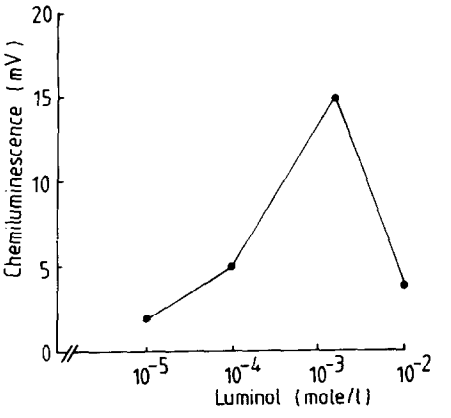

Fig. 1. Effect of luminol concentration on peak CL response induced by PMA in 4 μl human whole blood containing 25×10^3 PMNs and monocytes.

Table 1. Within-run precision of peak CL response induced by PMA in 4 μl whole blood from two subjects

Subject	PMNs + Monocytes (Number/μl)	CL response Mean* (mV)	SD (mV)	CV (%)
1	6250	14.34	0.63	4.39
2	14000	33.95	2.25	6

* 6 replicates.

It is known that the presence of glucose is necessary for ROS production [1]. We found that the CL response was amplified when the final concentration in the incubation system increased up to 0.1% and then leveled off.

CL activity of whole blood remained stable when the blood was kept at room temperature for 4 hr. Prewarming to 37° just before the assay was not necessary; a loss of activity was even found when the blood was kept at 37°.

These results lead us to select the following assay conditions for subsequent experiments. In a 3 ml luminometer polystyrene cuvette are successively added 200 μl of the luminol working solution, 200 μl of the PMA working solution, 200 μl of PBS–G Ca^{2+} Mg^{2+} and 400 μl of 100-fold diluted blood. The cuvette was immediately placed in the luminometer chamber kept at 37° and automatically agitated for 10 sec before starting CL monitoring. Each blood was analyzed in triplicate. Within-run precision was assessed by measuring the peak CL response in 6 replicates of two blood samples. The coefficients of variation did not exceed 10% (Table 1).

With the assay conditions described above, a linear correlation was found between peak CL response induced by PMA and the number of PMNs and monocytes in blood from 21 normal adult subjects (Fig. 2).

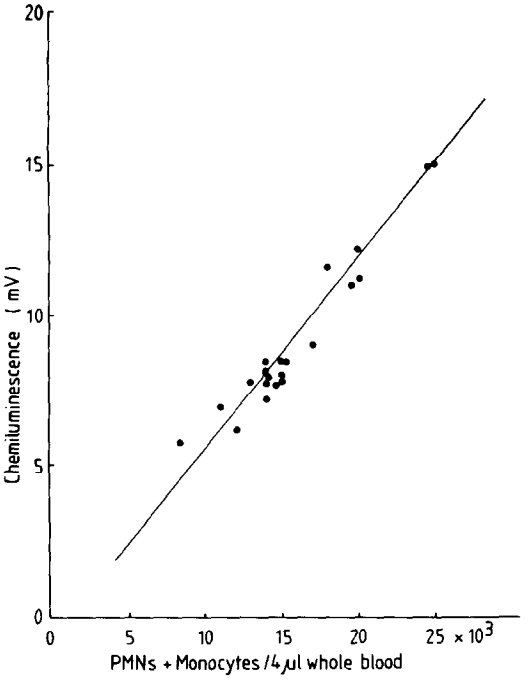

Fig. 2. Effect of PMN and monocyte concentration upon peak CL response induced by PMA in 4 µl human whole blood from 21 normal adults ($y = 0.603 \times 10^{-3} - 0.47$; $r = 0.97$; N = 21).

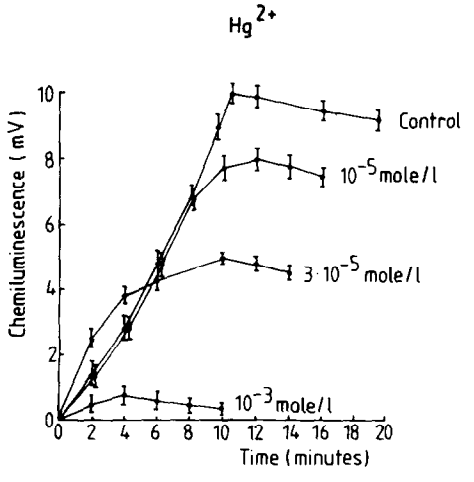

Fig. 3. Time- and dose-dependent inhibition of PMA stimulated chemiluminescence of human whole blood by $HgCl_2$. Each point and bar represent the mean ± SD of 3 replicates.

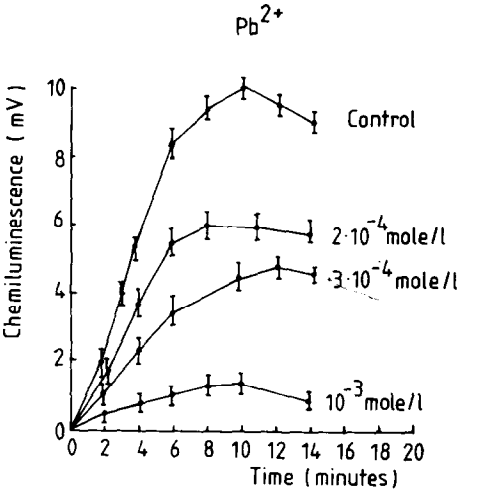

Fig. 4. Time- and dose-dependent inhibition of PMA stimulated chemiluminescence of human whole blood by $PbCl_2$. Each point and bar represent the mean ± SD of 3 replicates.

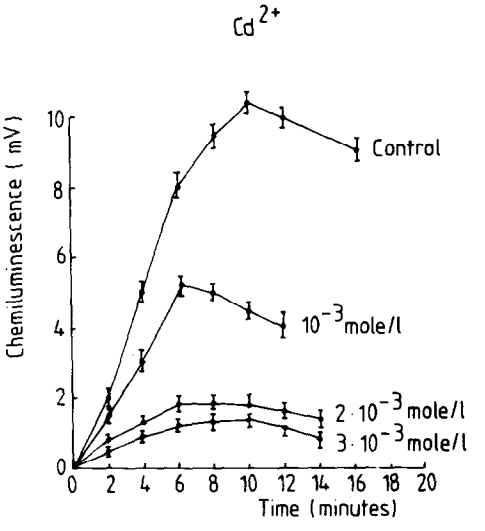

Fig. 5. Time- and dose-dependent inhibition of PMA stimulated chemiluminescence of human whole blood by $CdCl_2$. Each point and bar represent the mean ± SD of 3 replicates.

RESULTS

In vitro *effect of metals*

The effect of heavy metals on whole blood chemiluminescence induced by PMA was studied by adding aliquots of appropriate dilutions of the stock solutions in PBS G Ca^{2+} Mg^{2+} in the luminometer cuvette just before the diluted blood. The time-course of inhibition of whole blood respiratory burst by the three cations is shown in Figs. 3 to 5. Their effectiveness in inhibiting CL is in the order $Hg^{2+} > Pb^{2+} > Cd^{2+}$. Since incubation of the blood alone at 37° before PMA addition led to a significant loss of CL activity, the influence of the duration of metal preincubation with whole blood could not be studied. Since cadmium is transported in blood mainly bound to metallothionein (a low MW protein rich in cystein residues) [14], we have assessed the effect of cadmium–metallothionein and cadmium–cysteine complexes on whole blood chemiluminescence (Table 2). Cd–cystein complexes were not markedly more efficient in reducing the respiratory burst than $CdCl_2$ or even cysteine alone but cadmium–metallothionein produced 50% inhibition of the peak CL response at a concentration of 1.8×10^{-7} mol/l.

Table 2. *In vitro* inhibition by several cadmium compounds of peak CL response induced by PMA in 4 μl whole blood

Cadmium compound	ED_{50} (mol/l)
$CdCl_2$	$1.0\ 10^{-3}$
Cd–metallothionein	$1.8\ 10^{-7}$
Cd–cysteine (1/1)	$2.0\ 10^{-4}$
Cd–cysteine (1/2)	$5.7\ 10^{-4}$
L-cysteine	$6.1\ 10^{-4}$

ED_{50} is the concentration of metal which produces 50% inhibition of the response.
The blood contained 4.5×10^3 PMNs and monocytes/μl.

In vivo *effect of metals*

The CL response induced by PMA in whole blood from lead, mercury and cadmium workers is summarized in Table 3. Since the concentration of PMNs and monocytes (but not their ratio) was slightly different between the groups, the CL response was standardized for 2000 PMNs and monocytes. This was done on the basis of the regression line ($r > 0.90$) between the number of PMNs and monocytes and the CL response found in each group. A non parametric analysis of variance (Kruskal Wallis test) did not reveal any significant effect of lead and mercury exposure. However, a statistically significant reduction of ROS production in whole blood of cadmium exposed workers was found by comparison with that measured in control subjects.

DISCUSSION

Inorganic salts of three heavy metals, Hg^{2+}, Pb^{2+}, Cd^{2+} added to whole blood *in vitro* can inhibit the chemiluminescence induced by PMA. However, the concentration of metal required to produce 50% inhibition of the peak CL is quite high; it amounts to 2.8×10^{-5} M for Hg^{2+}, 2.8×10^{-4} M for Pb^{2+} and 10^{-3} M for Cd^{2+}, respectively. For Hg^{2+} and Cd^{2+}, our results are approximately 100 times higher than those reported by Malamud *et al.* [15] on isolated human PMNs. The relative ineffectiveness of these cations on whole blood is probably due to their binding to several non critical proteins. In agreement with our results, Malamud *et al.* [15] and Governa *et al.* [16] did not find any effect of Pb^{2+} on isolated PMNs CL at concentration of 3×10^{-6} and 5.75×10^{-5} M, respectively. Likewise, on isolated mouse peritoneal macrophages, Lison *et al.* [17] did not find a significant effect of non cytotoxic concentrations ($< 5 \times 10^{-6}$ M) of Hg^{2+} on superoxide anion production. Except in the case of acute mercury intoxication, the effective concentration of metals found in this study are never reached in the blood *in vivo* and therefore, the observed *in vitro* inhibition of whole blood respiratory burst by the three cations has probably no health significance. A striking reduction of respiratory oxidative burst, however, was found when whole blood was incubated with cadmium–metallothionein in a concentration range similar to that found in whole blood of workers exposed to cadmium [18]. Several hypotheses can be suggested to explain this effect; either the cadmium–metallothionein complex accumulated in leukocyte easily exchanges cadmium with one critical site involved in the metabolic pathway responsible for ROS production (e.g. with cysteine localised at the catalytic site of protein kinase C) [19] and thus prevents its activity or metallothionein binds other elements which may be essential for the production of ROS, or metallothionein acts as a scavenger of ROS [20] as do many thiol containing compounds.

In vivo, only cadmium seems to have an inhibitory effect on whole blood CL response induced by PMA. Although this observation is in agreement with the *in vitro* effect of Cd-metallothionein, the reduction of ROS production in whole blood of cadmium workers is rather small. Furthermore no negative correlation was found between the metal level in blood and/or in urine and the CL response.

In conclusion, the results of the *in vitro* and *in vivo* study on the effect of lead, mercury and cadmium do not support the hypothesis that environmental exposure to these metals may significantly impair host resistance to infection by reducing ROS production. Excessive occupational exposure to cadmium, however, may be associated with slight impairment of leukocyte respiratory burst. If a similar effect occurs in lung macrophages, it might decrease the respiratory tract resistance to infection. It would therefore be relevant to compare ROS production in macrophages collected by bronchoalveolar lavage in control and cadmium exposed workers.

Table 3. Peak CL response induced by PMA in whole blood from workers exposed to lead, mercury and cadmium

Groups	N	PMNs + Monocytes mean* (number/μl)	(% of control)	Ratio of PMNs to monocytes†	CL response mean* (mv/20000 PMNs + monocytes)	(% of control)
Control	21	3987	100	11.6	11.57	100
Lead	26	5546‡	139	11.6	11.24	97
Mercury	16	4265	107	11.1	10.18	88
Cadmium	13	4643	116	11.9	8.93†	77

* Geometric mean.
† Arithmetic mean.
‡ Significantly different from control value (Kruskal Wallis test; $P < 0.05$).

Acknowledgements—The authors are grateful to J. P. Buchet for his help in the statistical analysis of the data, to Dr A. Bernard for preparing the cadmium-metallothionein sample and to Dr H. Roels, J. P. Gennart and A. Ouled for providing urine and blood samples from workers exposed to mercury, lead and cadmium.

REFERENCES

1. Bellavite P, The superoxide-forming enzymatic system of phagocytes. *Free Radical Biol Med* **4**: 225–261, 1988.
2. Babior BM, Oxygen-dependent microbial killing by phagocytes. *New Engl J Med* **298**: 659–668, 1978.
3. Allen RC, Mead ME and Kelly JL, Phagocyte oxygenation activity measured by chemiluminescence and chemiluminescence probing. In: *CRC Handbook of Methods for Oxygen Radical Research* (Ed. Greenwald RA) pp. 343–351. CRC Press, Boca Raton, 1985.
4. Hemphill FE, Kaerbele ML and Buck WB, Lead suppression of mouse resistance to *Salmonella typhimurium*. *Science* **172**: 1031–1032, 1971.
5. Cook JA, Hoffmann EO and DiLuzio NR, Influence of lead and cadmium on the susceptibility of rats to bacterial challenge. *Proc Soc Exp Biol Med* **150**: 741–747, 1975.
6. Loose LD, Silkworth JB and Warrington D, Cadmium-induced depression of the respiratory burst in mouse pulmonary alveolar macrophages, peritoneal macrophages and polymorphonuclear neutrophils. *Biochem Biophys Res Commun* **79**: 326–332, 1977.
7. Malamud D, Dietrick SA and Shapiro IM, Low levels of mercury inhibit the respiratory burst in human polymorphonuclear leukocytes. *Biochem Biophys Res Comm* **128**: 1145–1151, 1985.
8. Baginski B, Effect of mercuric chloride on microbicidal activities of human polymorphonuclear leukocytes. *Toxicology* **50**: 247–256, 1988.
9. De Chatelet LR and Shirley P, Evaluation of chronic granulomatous disease by a chemiluminescence assay of microliter quantities of whole blood. *Clin Chem* **27**: 1739–1741, 1981.
10. Lindena J, Burkhardt H and Dwenger A, Mechanisms of non-opsonized zymosan-induced and luminol-enhanced chemiluminescence in whole blood and isolated phagocytes. *J Clin Chem Clin Biochem* **25**: 765–778, 1987.
11. Tono-Aka T, Ueno N, Matsumoto T, Ohkawa M and Matsumoto S, Chemiluminescence of whole blood: a sample and rapid method for the estimation of phagocytic function of granulocytes and opsonic activity in whole blood. *Clin Immunol Immunopathol* **26**: 66–75, 1983.
12. De Sole P, Lippa S and Littaru GP, Whole blood chemiluminescence: a new technical approach to assess oxygen-dependant microbicidal activity of granulocytes. *Clinical Laboratory Automation* **3**: 391–400, 1983.
13. Bernard AM, Ouled A and Lauwerys R, The effects of low doses of cadmium-metallothionein on the renal uptake of β_2-microglobulin in rats. *Toxicol Appl Pharmacol* **87**: 440–445, 1987.
14. Webb M and Etienne AT, Studies on the toxicity and metabolism of cadmium–thionein. *Biochem Pharmacol* **26**: 25–30, 1977.
15. Malamud D, Dietrich SA and Shapiro IM, Low levels of mercury inhibit the respiratory burst in human polymorphonuclear leukocytes. *Biochem Biophys Res Commun* **128**: 1145–1151, 1985.
16. Governa M, Valentino M and Visona I, *In vitro* impairment of human granulocyte functions by lead. *Arch Toxicol* **59**: 421–425, 1987.
17. Lison D, Dubois Ph and Lauwerys R, In vitro effect of mercury and vanadium on superoxide anion production and plasminogen activator activity of mouse peritoneal macrophages. *Tox Lett* **40**: 29–36, 1988.
18. Bernard A and Lauwerys R, Effect of cadmium exposure in humans. In: *Handbook of Experimental Pharmacology* (Ed. Foulkes, EC) Vol. 80, pp. 135–177. Springer, Berlin, 1986.
19. Parker PJL, Coussens N, Totty L, Rhee S, Young E, Chen S, Stabel S, Waterfield MD and Ullrich A, The complete primary structure of protein kinase C—the major phorbol ester receptor. *Science* **233**: 853–859, 1986.
20. Thornalley PJ and Vasal M, Possible role of metallothionein in protection against radiation-induced oxidative stress. Kinetics and mechanism of its reaction with superoxide and hydroxyl radicals. *Biochim Biophys Acta* **827**: 36–44, 1985.