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FREE RADICAL SCAVENGING ACTIVITY OF CARNOSINE

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The capacity of carnosine to decrease free radical-induced damage was evaluated using the oxidation of brain homogenates, the 2,2′-azobis-2-amidino propane-induced oxidation of erythrocyte ghost membranes, the radiation induced inactivation of horseradish peroxidase and the 2,2′-azobis-2-amidino propane-induced inactivation of lysozyme. Carnosine addition up to 17 mM did not produce any significant protection in either lipid peroxidation system, as assayed by the oxygen uptake rate. Carnosine addition reduces the intensity of the visible luminescence emitted, apparently due to a dark decomposition of the luminescent intermediates. Carnosine addition protects horseradish peroxidase and lysozyme from free radical mediated inactivation. The mean carnosine concentrations required to inhibit the inactivation rates by 50% were 0.13 mM and 0.6 mM for horseradish peroxidase and lysozyme, respectively.

KEY WORDS: Carnosine, free radical scavenger, lipid peroxidation, chemiluminescence, enzyme inactivation, hidroxyl radical

INTRODUCTION

Aerobic cells have developed different types of defensive mechanisms against active oxygen species whose formation can lead to deleterious reactions.[1,2] These cellular defences include protective metal ion chelating, chain breaking and repair processes.[2] Skeletal muscle and brain tissue exhibit a relatively active oxidative metabolism,[3] yet the concentration of the antioxidant vitamins E and C, as well as that of reduced glutathione, are rather low when compared with the levels in liver.[4,5] Carnosine (β-alanyl-L-histidine) was discovered at the begining of the century in skeletal muscle,[6] where it is present in the 1–20 mM range in most vertebrates. The dipeptide is also found at high concentrations in human muscles (2–20 mM), olfactory epithelium and bulbs (0.3 to 5 mM), and in different areas of the brain.[7,8] The biological role of carnosine and other related dipeptides has not been clearly established. However, several groups have proposed that their action as a defence mechanism against oxidative stress may represent a major function at cellular level.[9] This type of protective action has been suggested to by exercised by carnosine through a variety of mechanisms, including chelation of metal ions,[10,11] stabilization of the level of natural antioxidants such as vitamin E[9], elimination of pre-formed lipid peroxidation products,[9,12] interception of singlet oxygen[9,13–15] and inhibition of lipid peroxidation[9,11,15–18] and scavenging of oxidants generated by stimulated neutrophils.[19] However, recent work by Aruoma *et al.*[20] cast doubts on the capacity of carnosine to inhibit lipid

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peroxidation by chelating metal ions and/or by trapping oxygen free radicals. These authors reported that carnosine is a good scavenger of the hydroxyl radical, but it does not react with superoxide, hydrogen peroxide or hypochlorous acid at biologically significant rates. Also, carnosine, as well as homocarnosine and anserine, cannot bind iron ions in ways that interfere with 'site-specific' iron-dependent radical damage to deoxyribose, nor can they restrict the availability of Cu(II) to phenanthroline.[20] Furthermore, high concentrations of carnosine have only a rather weak inhibitory effect on iron-dependent lipid peroxidation, but the ability of carnosine to interfere with a commonly used version of the thiobarbituric acid test,[20,21] could lead to an overestimate of the antioxidant capacity of the dipeptide reported in several studies.[9,11,15] Considering these conflicting results and the physiological[6,8,22] and therapeutic[9,14,23,24] relevance of carnosine, we have carried out a study to assess the ability of carnosine to interfere with the free radical-mediated inactivation of horseradish peroxidase and lysozyme, and to inhibit lipid peroxidation either in a metal-dependent process (oxidation of brain homogenates[25]) or in a metal-independent system (red cell membranes supplemented with 2,2′-azobis-(2-amidinopropane) (ABAP)[26]). In the latter systems, the extent of lipid peroxidation was assay by the TBARS test, oxygen uptake and intensity of the emitted visible luminescence.[26,27]

MATERIALS AND METHODS

L-Carnosine, L-histidine, L-tryptophan, β-alanine, horseradish peroxidase type I (HRP), lysozyme and propyl gallate were obtained from Sigma Chemical Co. 2,2′-Azo-bis(2-amidinopropane)dihydrochloride (ABAP) was obtained from Polysciences, Warrington, P.A.

Wistar rats (Facultad de Medicina, Universidad de Chile) were kept on standard pellet diet *ad libitum* (Alimentos Balanceados S.A., Santiago). Rat brain homogenates and erythrocyte ghost membranes were prepared as previously described.[25,28]

Measurements of chemiluminescence intensities were carried out in a Beckman LS-315OP liquid scintillation counter in the out-of-coincidence mode using the narrow tritium iso-set module[29] at 30°C. The oxygen uptake was measured polarographically at 37°C with a Clark-Type platinum electrode.[30] Thiobarbituric acid reactive substance (TBARS) of lipid peroxidation were determined in brain homogenates and erythrocyte ghost membranes before and after 1 hour incubation at 37°C, according to Buege and Aust.[31]

Hydroxyl radicals were produced *in situ* by gamma irradiating samples with a ^{60}Co source for 15 minutes. Irradiation doses of 9.5 Gy/min were employed. For horseradish peroxidase (HRP), samples contained 1 μM HRP in 0.1 M phosphate buffer, pH 6.0. Horseradish peroxidase activity was measured by recording the increase in absorbance at 420 nm produced as a consequence of the oxidation of pyrogallol.[32]

Solutions of lysozyme (3.4 μM) and/or tryptophan (3.0 μM), with or without carnosine, were incubated at 45°C in PBS buffer (0.07 M phosphate, 0.017 M NaCl, pH 6.5) with 10 mM ABAP. Aliquots were withdrawn at different times and analyzed to obtain the tryptophan concentration and/or the remaining enzymatic activity. Lysozyme activity was evaluated from the lysis rate of *Micrococcus luteus (M. lysodeikticus)* measured by the decrease in absorbance at 436 nm.[33] Tryptophan modification was monitored by following the decrease of its fluorescence intensity (excitation at 295 nm, emission at 345 nm).

RESULTS AND DISCUSSION

Protection of HRP against inactivation by hydroxyl radicals

Inactivation of enzymes exposed to gamma irradiation is usually a consequence of their modification by hydroxyl radicals.[34] Experiments employing HRP as a target enzyme showed that its inactivation can be prevented by addition of carnosine, L-histidine or β-alanine. However, the efficiency of these additives is widely different. Results obtained for carnosine and β-alanine indicate that both additives, at high concentrations, are able to afford complete protection to the enzyme (Figure 1), a result that implies that the radicals produced by their interaction with the hydroxyl radicals are unable, under the present conditions, to inactivate the enzyme. The concentration of additive required to reduce the enzyme inactivation rate to one-half that observed in its absence (Q1/2) is 0.13 mM for carnosine and 4.5 mM for β-alanine. These results are in agreement with those reported by Aruoma *et al.*[20] for the inhibition of hydroxyl radical-dependent deoxyribose degradation, showing that carnosine is nearly 50 times more efficient that β-alanine. The protection afforded by L-histidine lies between that of carnosine and β-alanine. However, its behaviour appears to be complex, with the double reciprocal plot exhibiting a strong downward curvature (data not shown) that could indicate secondary reactions of the radicals produced after the hydroxyl radical/histidine interaction.

In conclusion, the results obtained in this system show that carnosine, in the mM concentration range, is able to completely prevent the inactivation of HRP mediated by hydroxyl radicals.

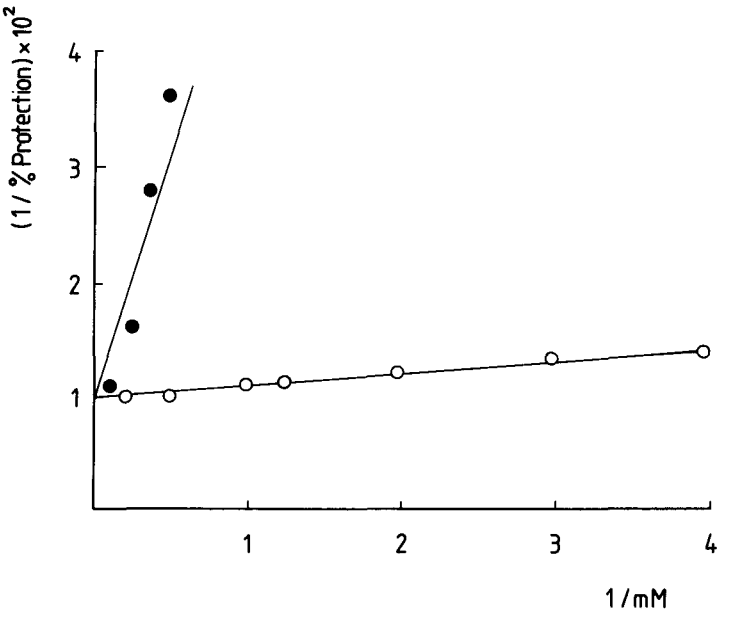

FIGURE 1 Dependence of the % of protection with the additive concentration shown as a double reciprocal plot for carnosine (○) and β-Alanine (●).

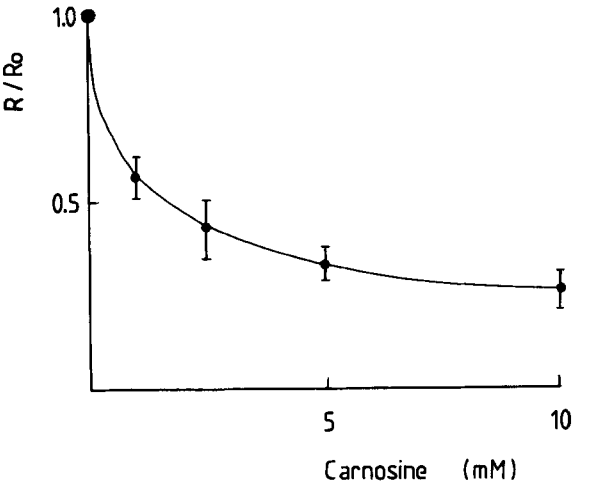

FIGURE 2 Effect of carnosine addition upon tryptophan modification rate induced by ABAP thermolysis at 40°C. The data are plotted as R/Ro when R is the inactivation rate in presence of carnosine, and Ro is the control inactivation rate. ABAP; 10 mM, Tryptophan: 3.0 μM.

Protection of enzymes against inactivation by radicals from ABAP thermolysis

Free radicals generated by thermolysis of ABAP in the presence of oxygen lead to enzyme inactivation.[32,35] Lysozyme and HRP inactivation is totally prevented by carnosine addition, if concentrations higher than about 5 mM are employed. The (Q1/2) value obtained employing lysozyme was 0.6 mM. In this system, L-histidine protection was also observed, with a (Q1/2) value of about 1 mM. These data indicate that the protection afforded by carnosine and L-histidine is relatively mild, since the efficient antioxidant propyl gallate, at a concentration of 3.4 μM, totally prevents the enzyme inactivation under similar experimental conditions.[35] Furthermore, the (Q1/2) for tryptophan is 9 μM, a result that indicates that this aminoacid is nearly 200 time more reactive than is carnosine towards the alkylperoxyl radicals.

Free tryptophan can also be modified by incubation with ABAP.[35] The rate of the process can be obtained from the initial slope of tryptophan fluorescence vs time plots. The rate of the process is reduced by carnosine addition (Figure 2). However, in agreement with the above considerations regarding the relative reactivity of tryptophan and carnosine towards alkylperoxyl radicals, the data of Figure 2 show that 1.6 mM carnosine is needed to reduce to one half the consumption rate of 3 μM tryptophan.

The results obtained in the present work indicate that carnosine is not particularly reactive towards alkylperoxyl radicals. However, given the high concentrations of carnosine present in several tissues and organs,[7,8] its capacity to interact with peroxyl radicals, might contribute to decreasing protein damage by reactive oxygen species.

Prevention of lipid peroxidation

The action of carnosine as a chain breaking and/or preventative antioxidant was evaluated in a metal ion-independent system (erythrocyte ghost membranes in the

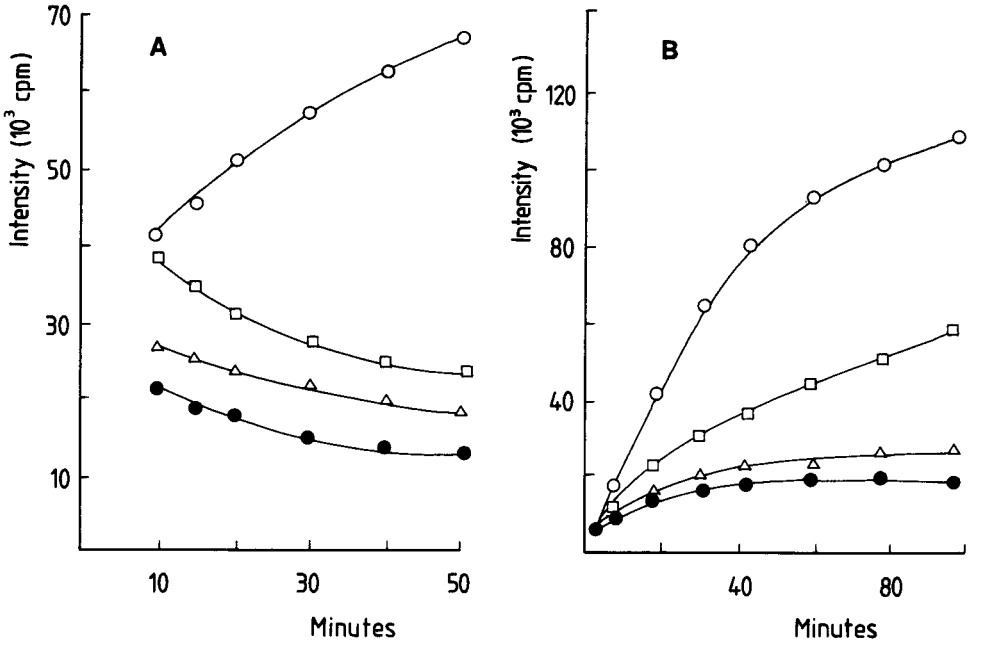

FIGURE 3 Luminescence intensity as a function of time during ABAP (10 mM) mediated erythrocyte membrane oxidation (A) or brain homogenates oxidation (B) in the presence of carnosine: 0 mM (○); 2 mM (□); 6 mM (△) and 12 mM (●).

presence of ABAP[26]), and in a metal ion-dependent system (brain homogenate oxidation[25]). Addition of mM concentrations of carnosine notably decreased formation of TBARS in both systems (data not shown). However, it has been demonstrated[20,21] that carnosine interferes with TBARS determination by the standard procedure, rendering these determinations invalid to assess antioxidant capacity. The antioxidant action of carnosine was then evaluated form its effect upon the rate of oxygen uptake and the emitted luminescence intensity. The results obtained were different for both parameters. Carnosine did not modify the rate of oxygen uptake in the erythrocyte ghost membrane-ABAP system when added up to 12 mM, whereas in the brain homogenate oxidation system, 17 mM carnosine decreased the oxygen consumption by nearly 38%. However, the increases in luminescence intensity associated with the erythrocyte membrane oxidation induced by ABAP[26] (Figure 3A), and with the brain homogenate oxidation (Figure 3B) are totally prevented by carnosine, at concentrations of 2 and 6 mM, respectively. In order to elucidate the apparent higher antioxidant efficiency of carnosine determined by measurements of light emission, a brain homogenate preparation preincubated for 60 minutes was supplemented with an excess (200 μM) of propyl gallate, and carnosine (12 mM) was added after a further 30 minutes (Figure 4). The added propyl gallate is enough to completely inhibit further lipid peroxidation, and the light emitted from the system can be adscribed to the formation of excited species in the decomposition of the lipid peroxidation products accumulated in the system.[36] The data given in Figure 4 show that carnosine addition elicits a further decrease in the observed luminescence, and

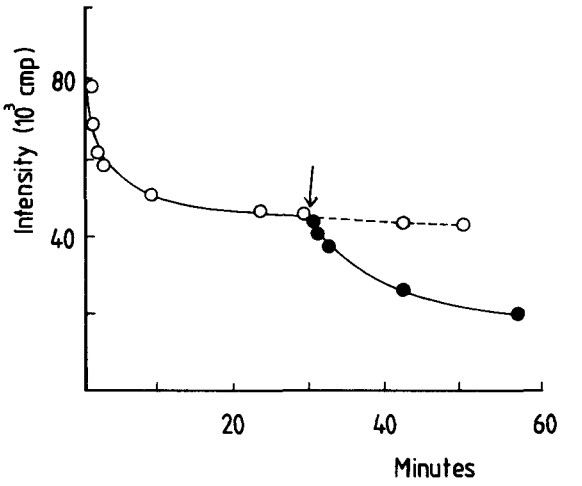

FIGURE 4 Luminescence of brain homogenate pre-incubated 60 minutes as a function of time elapsed after propyl gallate (200 μM) addition. (○) Results obtained in presence of propyl gallate. (●) Results obtained when 12 mM carnosine was added at the time indicated by the arrow.

that this decrease does not take place instantaneously. This behaviour can be ascribed to dark decomposition, by the additive, of the lipid peroxidation products that are precursors (catabolites) of the luminescent substances.[36] We conclude that the effect of carnosine upon the emitted luminescence is mainly due to its capacity to react with the non-radical luminescence precursors. It follows that the effect of carnosine upon the rate of lipid peroxidation cannot be evaluated from luminescence intensity measurements. The compounds that are precursors of the visible luminescence are not known, but its has been postulated that they might be dioxetanes and/or hydroperoxides.[37,38] In this regard, it is relevant to note that it has been reported that one of the main roles of carnosine in biological systems relates to its capacity to decompose pre-formed peroxide products generated during the lipid peroxidation process.[9,12]

In conclusion, carnosine does not prevent metal ion-independent oxidation of red cell membranes, and has only a small effect upon the brain homogenate oxidation rate, when employed in high concentration. These results confirm those reported by Aruoma *et al.*[20] showing that carnosine has a weak inhibitory effect at high concentrations in some (but not all) systems in which an iron-mediated lipid peroxidation occurs. In the brain homogenate system the weak effect observed could be due either to partial trapping of the chain carrying radicals to and/or a reduction of the initiation rate as a consequence of a weak iron ion-chelating capacity.[10,11] It is interesting to note that the behaviour of the dipeptide is sustantially different to that of its aminoacids. Histidine, when added in millimolar concentrations, has been reported to be a powerful pro-oxidant in Fe-dependent lipid peroxidation.[11] However, in the brain homogenate oxidation, addition of L-histine or β-alanine up to 12 mM did not produce any noticeable effect upon the emitted luminescence or the oxygen consumption rate. These differences can be accounted for by the high specificity of the response of metal mediated lipid peroxidation systems to the addition of metal chelators.[10,11]

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References

1. C.E. Cross, B. Halliwell, E.T. Borish, W.A. Pryor, B.N. Ames, R.L. Saul, J.M. McCard, and D. Harman (1987) Oxygen radicals and human disease. *Annals Internal Medicine,* **107,** 526–545.
2. H. Sies (1986) Biochemistry of oxidative stress. *Angewandte Chemie. International Edition,* **25,** 1058–1071.
3. B. Halliwell and J.M.C. Gutteridge (1990) The antioxidants of human extracellular fluids. *Archives of Biochemistry and Biophysics,* **280,** 1–8.
4. D.J. Kornbrust and R.D. Mavis (1972) Relative susceptibility of microsomes from lung, heart, liver, kidney, brain and testes to lipid peroxidation: correlation with vitamin E content, *Lipids,* **15,** 315–322.
5. C.R. Scriver, W. Nutzenadel and T.L. Perry (1983) Disorders of ß-Alanine and carnosine metabolism. In *The metabolic basis of inherited disease.* (eds. J.B. Stanbury, J.B. Wyngaarden, J.L. Goldstein and M.S. Brown) McGraw-Hill, New York, pp. 528–542.
6. A.A. Boldyrev (1986) The biological role of histidine-containing dipeptides. *Biokhimiya USSR,* **51,** 1930–1943.
7. K.G. Crush (1970) Carnosine and related substances in animal tissues. *Comparative Biochemistry and Physiology,* **34,** 3–30.
8. F. Margolis (1974) Carnosine in the primary olfactory pathway. *Science,* **184,** 909–911.
9. A.A. Boldyrev, A.M. Dupin, A. Ya. Bunin, M.A. Babizhaev and S.E. Severin. (1987) The antioxidative properties of carnosine, a natural histidine containing dipeptide. *Biochemistry International,* **15,** 1105–1113.
10. C.E. Brown (1981) Interactions among carnosine, anserine, ophidine and copper in biochemical adaptation. *Journal Theoretical Biology,* **88,** 245–256.
11. R. Kohen, Y. Yamamoto, K.C. Cundy and B.N. Ames (1988) Antioxidant activity of carnosine, homocarnosine and anserine present in muscle and brain. *Proceedings of the National Academy of Sciences,* **85,** 3175–3179.
12. A.M. Dupin, M. Bemananozara, S.L. Stvolinskii, A.A. Boldyrev and S.E. Severin (1987) Muscle dipeptides: Natural inhibitors of lipid peroxidation. *Biokhimiya,* **52,** 782–787.
13. A. Dahl, W.R. Midden and P.E. Hartman (1988) Some prevalent biomolecules as defenses against singlet oxygen damage. *Photochemistry and Photobiology,* **47,** 357–362.
14. M.A. Babizhayev (1989) Antioxidant activity of L-carnosine, a natural histidine-containing dipeptide in crystalline lens. *Biochimica et Biophysica Acta,* **1004,** 363–371.
15. A.A. Boldyrev, A.M. Dupin, M.A. Batrukova, N.I. Bavykina, G.A. Korshunova, and Yu.P. Shvachkin (1989) A comparative study of synthetic carnosine analogs as antioxidants. *Comparative Biochemistry and Physiology,* **94B,** 237–240.
16. A.M. Dupin, A.A. Boldyrev, Yu.U. Arkhipenko, and V.E. Kagan (1984) Protection of Ca^{2+} transport by carnosine against disturbances induced by lipid peroxidation. *Bulletin Experimental Biology and Medicine,* **98,** 1071–1073.
17. A.A. Boldyrev, A.M. Dupin, E.V. Pindel and S.E. Severin (1988) Antioxidative properties of histidine-containing dipeptides from skeletal muscle of vertebrates. *Comparative Biochemistry and Physiology,* **89B,** 245–250.
18. L.V. Chasovnikova, V.E. Formazyuk, V.I. Sergienko, A.A. Boldyrev and S.E. Severin (1990) The antioxidative properties of carnosine and other drugs. *Biochemistry International,* **20,** 1097–1103.
19. B.P. Sharonov, N.Ju. Govorova and S.N. Lyzlova (1990) Carnosine as a potential scavenger of oxidants generated by stimulated neutrophils. *Biochemistry International,* **21,** 61–68.
20. O.I. Aruoma, M.J. Laughton and B. Halliwell (1989) Carnosine, homocarnosine and anserine: Could they act as antioxidants *in vivo?. Biochemical Journal,* **264,** 1863–1869.
21. M. Salim-Hanna A.E. Lissi and L.A. Videla (1989) On the inhibitory capacity of carnosine. XVIII Jornadas Chilena de Química. Contribuciones Científicas y Tecnológicas. pp. 106–107.
22. C.L. Davey (1960) Significance of carnosine and anserine in straited skeletal muscle. *Archives Biochemistry and Biophysics,* **89,** 296–299.
23. P.R. Normark, Z.S. Edel, P.S. Leonov, D.I. Gimpelzon, M.O. Pasko, E.V. Boguslavskaya and N.I. Sokolovskaya (1940) Experience in application of carnosine in a pure state and in combination with

diathermia in the therapy of infections and rheumatoid polyarthritis. *Vrachebnoye delo, USSR,* **11/12,**741–744.
24. K. Nagai, T. Suda, K. Kawasaki and S. Mathnura (1986) Action of carnosine and ß-Alanine on wound healing. *Surgery,* **100,** 815–821.
25. J. Stocks, J.M.C. Gutteridge, R.J. Sharp and T.L. Dormandy (1974) Assay using homogenate for measuring the antioxidant activity of biological fluids. *Clinical Science in Molecular Medicine,* **47,** 215–222.
26. Y. Yamamoto, E. Niki, J. Eguchi, Y. Kamiya and H. Shimasaki (1985) Oxidation of biological membranes and its inhibition. Free radical chain oxidation of erythrocyte ghost membranes by oxygen. *Biochimica et Biophysica Acta,* **819,** 29–36.
27. E.A. Lissi, T. Caceres and L.A. Videla (1986) Visible chemiluminescence from rat brain homogenates undergoing autoxidation. I. Effect of additives and products accumulation. *Free Radical Biology and Medicine,* **2,** 63–69.
28. L.A. Videla, T. Caceres and E.A. Lissi (1988) Antioxidant capacity of desferrioxamine in the chemically – initiated lipid peroxidation of rat erythrocyte ghost membranes. *Biochemistry International,* **16,** 799–807.
29. L.A. Videla (1983) Assessment of the scavenging action of reduced glutathione, (+)-cyanidanol-3 and ethanol by the chemiluminescent response of the xanthine oxidase reaction. *Experientia,* **39,** 500–502.
30. R.W. Estabrook (1967) Mitochondrial respiratory control and polarographic measurement of ADP/O ratios. *Methods in Enzymology,* **10,** 41–47.
31. J.A. Buege and S.D. Aust (1978) Microsomal lipid peroxidation. *Methods in Enzymology,* **52,** 302–305.
32. E.A. Lissi, M. Salim-Hanna, M. Faure and L.A. Videla. 2,2′-Azo-bis-amidinopropane as a radical source for lipid peroxidation and enzyme inactivation studies. *Xenobiotica,* (in press).
33. D. Shugar (1952) The measurement of lysozyme activity and ultraviolet inactivation of lysozyme. *Biochimica et Biophysica Acta,* **8,** 302–303.
34. R.L. Willson (1982) Iron and hydroxyl free radicals in enzyme inactivation and cancer. In *Free radicals, lipid peroxidation and cancer.* (eds. D.C.H. McBrien and T.F. Slater), Academic Press, London, pp. 275–303.
35. E.A. Lissi and N. Clavero (1990) Inactivation of lysozyme by alkylperoxyl radicals. *Free Radical Research Communications,* **01,** 177–184.
36. E.A. Lissi, T. Caceres, S. Llesuy, L. Solari, A. Boveris and L.A. Videla (1989) On the characteristics of the visible chemiluminescence following free radical lipid peroxidation. *Free Radical Research Communications,* **6,** 293–301.
37. E. Cadenas, A.I. Varsavsky, A. Boveris and B. Chance (1981) Chance oxygen organic hydroperoxide induced chemiluminescence of brain and liver homogenates. *Biochemical Journal,* **198,** 645–654.
38. E. Cadenas and H. Sies (1984) Low-level chemiluminescence. *Methods in Enzymology,* **105,** 221–231.

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