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Tissue protection against oxidative stress

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Abstract. We used an enhanced luminescence technique to study the response of rat tissues, such as liver, heart, muscle and blood, to oxidative stress and to determine their antioxidant capacity. As previously found for liver homogenate, the intensity of light emission (E) of tissue homogenates and blood samples, stressed with sodium perborate, is dependent on concentration, and the dose-response curves can be described by the equation $E = a \cdot C/\exp(b \cdot C)$. The b value depends on the antioxidant defence capability of the tissues. In fact, it increases when homogenates are supplemented with an antioxidant, and is correlated with tissue antioxidant capacity, evaluated by two previously set up methods both using the same luminescence technique. Our results indicate that the order of antioxidant capacity of the tissues is liver > blood > heart > muscle. The a value depends on the systems catalysing the production of radical species. In fact, it is related to the tissue level of hemoproteins, which are known to act as catalysts in radical production from hydroperoxides. The equation proposed to describe the dose-response relation is simple to handle and permits an immediate connection with the two characteristics of the systems analysed which determine their response to the pro-oxidant treatment. However, the equation which best describes the above relation for all the tissues is the following: $E = \alpha \cdot C/\exp(\beta \cdot C^{\delta})$. The parameter δ assumes values smaller than 1, which seem to depend on relative amounts of tissue hemoproteins and antioxidants. The extension of the analysis to mitochondria shows that they respond to oxidative stress in a way analogous to the tissues, and that the adherence of the dose-response curve to the course predicted from the equation $E = a \cdot C/\exp(b \cdot C)$ is again dependent on hemoprotein content.
Key words. Oxygen radicals; enhanced luminescence; tissue antioxidants; oxidative stress.

In previous studies we used an enhanced luminescence procedure to determine both the response to oxidative stress[1], and the antioxidant capacity of rat liver homogenate[2]. Our results showed that oxidative stress response depends on homogenate concentration (C). When this increases, the light emission (E), in the presence of a suitable oxidant, initially increases and then decreases rapidly, reaching negligible values at concentrations of about 5 mg/100 ml. The curve equation [$E = a \cdot C/\exp(b \cdot C)$] is that which should be expected from a system in which radical intermediates, involved in the reaction leading to light emission and produced with linear dependence on concentration, are intercepted with exponential dependence on concentration[1]. The study of a suitable model system showed that the action of well-known cell enzymatic and nonenzymatic antioxidant systems is supported by that of systems, such as the hemoproteins, which produce oxygen radicals[3–6], but are also able to react with them, particularly at high concentrations[5,7,8]. The actions of two kinds of system show a different dependence on concentration. When the iron ligand concentration is high, the emission-concentration curve is best described by a different equation, $E = \alpha \cdot C/\exp(\beta \cdot C^{\delta})$. It is, then, possible to think that the course of the emission-concentration curve can supply information on the relative amounts of the substances able to interact with the radicals produced by themselves, and of the substances able to intercept the radicals released in solution, so preventing their reaction with detector molecules[1]. Although hemoproteins have been reported in various subcellular sites, the greatest concentration of such substances is in the mitochondria. Furthermore, an examination of the pertinent literature clearly shows that the mitochondrial cytochrome content is different in different tissues[9–13].

The main objective of this research was to obtain indications of the relative capacity of different tissues both to produce radicals and scavenge them, and determine a relation with mitochondrial hemoprotein content. To this end we have determined the responses to the oxidative stress of blood and homogenates and isolated mitochondria of some tissues. The tissue responses were related to mitochondrial protein content and cytochrome oxidase activity, as an index of the amount of respiratory complexes. The mitochondrial responses were related to their cytochrome oxidase activity and

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cytochrome content. Furthermore, the methods set up for determination of the antioxidant capacity on liver homogenate were extended to homogenates and mitochondria of the other tissues.

Materials and methods

Animals. The experiments were carried out on 2-month-old male rats of a Wistar strain (Morini, San Polo D'Enza, Italy). All animals were raised at a room temperature of 24 ± 1 °C, with an artificial lighting cycle (LD 8–20 h), and fed the same diet (Mil-Ratti, Morini).

Tissue preparation. The animals were sacrificed by decapitation while under ether anesthesia. Blood samples were collected, and livers, hearts and muscles (gastrocnemious) were rapidly excised and placed into petri dishes containing ice-cold isolation medium (IM) consisting of 125 mM KCl, 2 mM EDTA, 15 mM Tris, pH 7.4. The heart great vessels and valves were trimmed away and the ventricles and atria were cut open and rinsed free of blood. Muscles and livers were freed from connective tissue. After the tissues were weighed, 20% (w/v) homogenates were prepared with a Potter-Elvehjem homogenizer set at a standard velocity (500 rpm) for 2 min either in IM or in a modified Chappel-Perry medium consisting of 1 mM ATP, 100 mM KCl, 5 mM $MgCl_2$, 1 mM EDTA, 5 mM EGTA and 50 mM Hepes buffer, pH 7.4. Aliquots of the homogenates were removed to determine response to oxidative stress, antioxidant capacity and cytochrome oxidase activity.

Preparation of mitochondrial fractions. The homogenates were freed from debris and nuclei by centrifugation at 500 *g* for 10 min. Crude mitochondrial fractions were isolated by centrifugation of the resulting supernatants at 12,000 *g* for 10 min. The mitochondrial pellets were resuspended in the respective homogenization media and centrifuged at the same sedimentation velocity. Mitochondrial preparations were washed in this manner three times before final resuspension in the homogenization medium. Aliquots of the mitochondrial fractions were used to determine protein and cytochrome content, cytochrome oxidase activity, response to oxidative stress and antioxidant capacity.

Protein content. The mitochondrial protein content was determined, upon solubilization in 0.5% deoxycholate, by the biuret method[14] with bovine serum albumin as standard.

Cytochrome oxidase activity. Tissue homogenates and mitochondrial suspensions were diluted in the modified Chappel-Perry medium so that the preparations contained per ml either 100 mg of tissue or 0.2 mg of mitochondrial protein. Lubrol PX (Sigma Chimica, Milano, Italy) was used to unmask enzyme activity. Cytochrome oxidase activity was determined polarographically at 25 °C, using a Gilson glass respirometer equipped with a Clark oxygen electrode (Yellow Springs Instrument, Ohio, USA), by the procedure of Aulie and Grav[15] modified by Barré et al.[16].

Cytochrome content. Mitochondria were solubilized by addition of suitable aliquots of 20% (v/v) Triton X-100 in 1.0 M potassium phosphate buffer, pH 7.4, to 1 ml of suspensions. The volume was made to 2 ml with the above buffer and then to 10 ml with isolation medium. Samples in the reference cuvette were oxidized by the addition of a few crystals of potassium ferricyanide and those in the experimental cuvette were reduced by the addition of a few milligrams of sodium dithionite. The difference spectra of cytochrome were recorded using a Hitachi (Model U-2000) double-beam spectrophotometer. Cytochrome contents were calculated by using the wavelength pairs and extinction coefficients as given by Estabrook and Holowinsky[17].

Blood hemoglobin content. The determination of hemoglobin content of the blood samples was performed by using the hematologic analyser Cellanalyzer CA 480 (Delcon, Novate Milanese, Italy).

Response to oxidative stress and antioxidant capacity of tissues. Response to oxidative stress and antioxidant capacity of tissues was determined as previously described[1,2]. In brief, samples of 10% (w/v) homogenates and of 10% (v/v) blood were obtained by diluting the 20% homogenates with equal volume of 30 mM Tris containing 0.2% Lubrol at pH 8.5, and the whole blood with nine volumes of 16.7 mM Tris containing 0.11% Lubrol at pH 8.5. When required the dilution was performed with the above solutions containing 4.0 mM or 1.0 mM desferrioxamine (Sigma). Several dilutions of samples with and without desferrioxamine, up to a tissue concentration of 0.002%, were prepared with 15 mM Tris, pH 8.5. The assays were performed in microtiter plates by using reagents and instrumentation of the Amerlite System (Johnson & Johnson, Cinisello B., Italy). Enhanced chemiluminescence reactions were initiated by addition of 250 µl of reaction mixture to 25 µl of the samples. The reaction mixture was obtained by dissolving a tablet containing substrate in excess (sodium perborate) and signal generating reagents (sodium benzoate, indophenol and luminol) (Amerlite Signal Reagent Tablets) in buffer at pH 8.6 (Amerlite Signal Reagent Buffer). The plates were incubated at 37 °C for 30 s under shaking and then transferred to a luminescence analyser (Amerlite Analiser), which supplied the emission values as percentages of the emission of a standard constituted by 25 µl of a solution of 22 ng/ml horseradish peroxidase. Such values were used to fit dose-response curves using Fig. P Program (Biosoft, Cambridge). The best fit of data to equation $E = a \cdot C / \exp(b \cdot C)$ supplied the b values for the homogenates or blood samples and for their mixtures with desferrioxamine. The solution of a system of equations, relating such values to the antioxidant concentra-

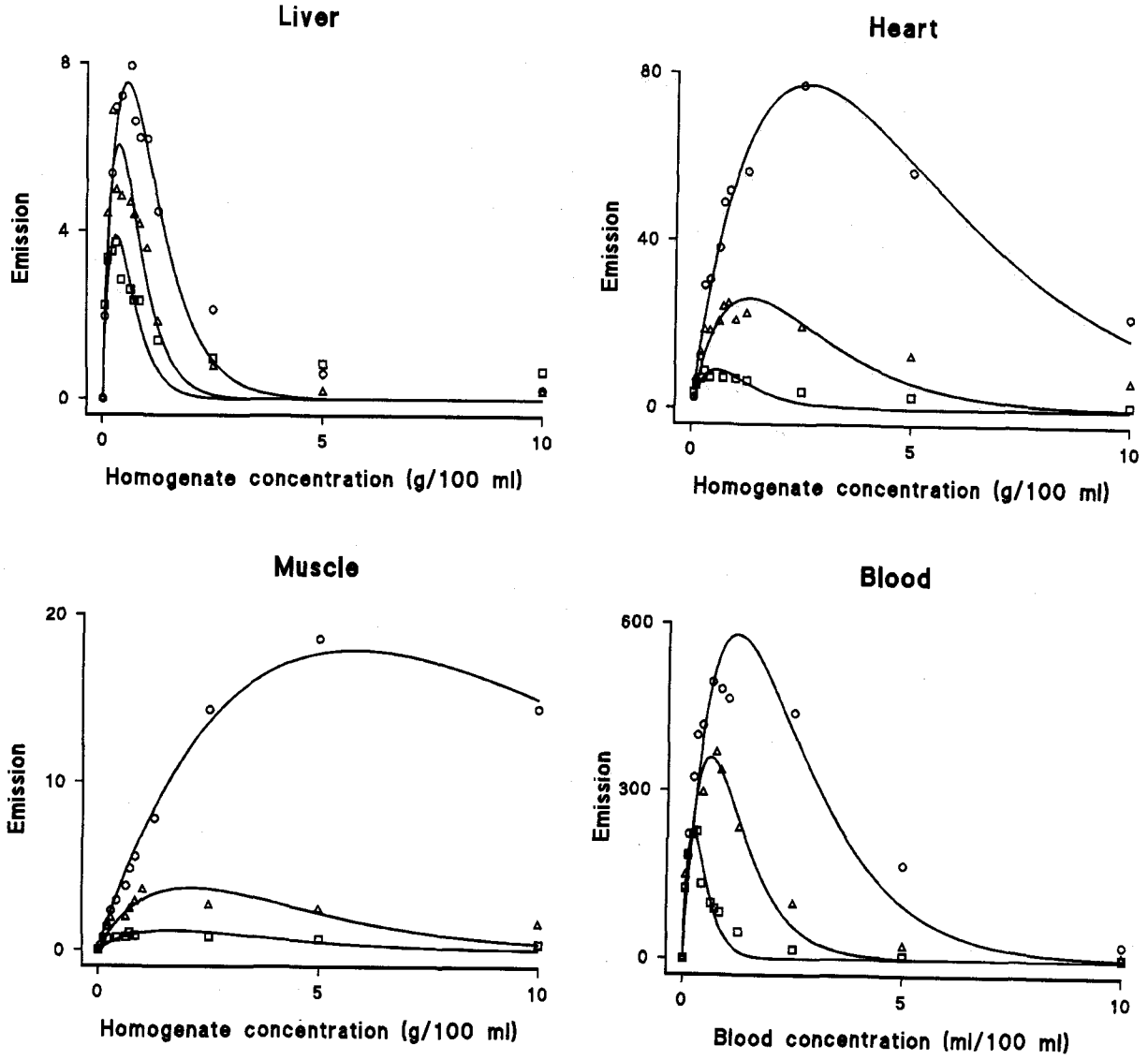

Figure 1. Effect of antioxidant supplementation on light emission from rat tissues. The experimental data are obtained modifying the concentration of: ○–○ = homogenate or blood samples; △–△ = samples plus 0.5 mM desferrioxamine; □–□ = samples plus 2.0 mM desferrioxamine. Emission is given in relative units with 100 units equivalent to the emission of 44 ng/ml peroxidase.

tion $b_{homo.} = K \cdot [Antioxidant_{homo.}]$ and $b_{mixture} = K \cdot ([Antioxidant_{homo.}] + [Antioxidant_{added}])$ allowed the level of antioxidants in homogenates and blood samples expressed in terms of the added antioxidant to be determined[2].

The antioxidant capacity of 10% (w/v) tissue homogenates, and 10% (v/v) blood samples, prepared as reported above, was also determined by a more rapid method based on enhanced luminescence technique[2]. In this case, 250 µl of the above reaction mixture were added to 10 µl of 110 ng/ml peroxidase plus 15 µl of desferrioxamine, at concentrations ranging between 0.01 and 3 mM, in 15 mM Tris, pH 8.5, or of buffer alone. Equal volumes of reaction mixture were also added to both 10 µl of 110 ng/ml peroxidase plus 15 µl of 10% homogenate samples (samples) and 10 µl of 15 mM Tris, pH 8.5, plus 15 µl of the same homogenate samples (blanks). The emission values obtained from the mixture of peroxidase and desferrioxamine were reported against the desferrioxamine concentration on logarithmic co-ordinates, supplying a standard curve. The differences between the emission values obtained from mixtures containing the samples and from those containing the relative blanks were referred to those of the standard curve, and this allowed the tissue antioxidant capacity to be expressed as equivalent desferrioxamine concentration.

Response to oxidative stress and antioxidant capacity of mitochondria. Several dilutions of the mitochondrial suspensions in a range of protein concentrations from 20 to 0.005 mg/ml were prepared with 15 mM Tris, pH 8.5. The determination of the response to oxidative stress and

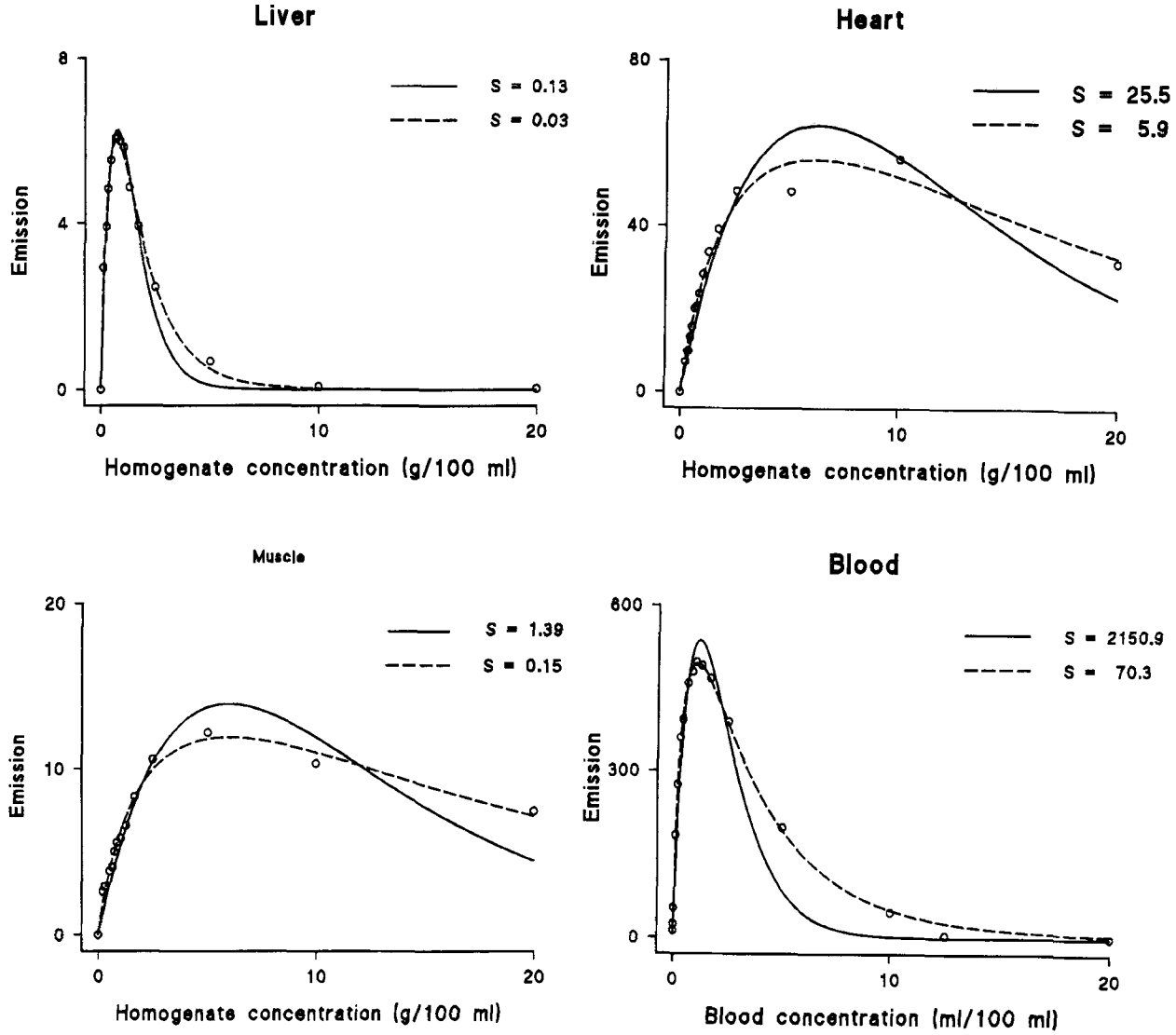

Figure 2. Curve fitting for light emission from tissue homogenates and blood of rat. Emission is given in relative units with 100 units equivalent to the emission of 44 ng/ml peroxidase. The curves are computed from experimental data using the equations: $E = a \cdot C/\exp(b \cdot C)$ (solid lines) and $E = \alpha \cdot C/\exp(\beta \cdot C^{\delta})$ (dashed lines). The degree of fit of curves to experimental data is inversely related to the ratio between sum of squares of the differences between theoretical and experimental values and number of such values (S).

antioxidant capacity of mitochondria was then performed by the same methods used for the homogenates.

Statistical analysis. The data obtained in eight different experiments are expressed as mean ± standard error in the tables and indicated by vertical bars in the figures. The standard errors of the values calculated as functions of experimentally determined quantities were computed according to Fenner as reported in Daniels et al.[18]. Data were analysed with a one-way analysis of variance method. When a significant F ratio was found, the Student-Newman-Keuls multiple range test was used to determine the statistical significance of differences between means. Probability values (p) < 0.05 were considered significant. In figures 1, 2 and 5 the results of at least six experiments are presented as sample curves.

Results

The results of the experiments performed on the liver (fig. 1) agree with those of the previous studies[1,2], showing that: *1*) the light emission is a function of homogenate concentration, $E = a \cdot C/\exp(b \cdot C)$, whose maximum, $E_{max} = a/e \cdot b$ is found at $C = 1/b$; *2*) the emission peak is lower and is found at lower concentration for homogenates supplemented with desferrioxamine, owing to a decreased a value and an increased b value; *3*) the effect on a and b values depends on desferrioxamine concentration.

The luminescence response to changes of concentration obtained for the other tissues exhibits analogous characteristics and can be described by the same equation (fig. 1). However, the degree of fit of the curves to

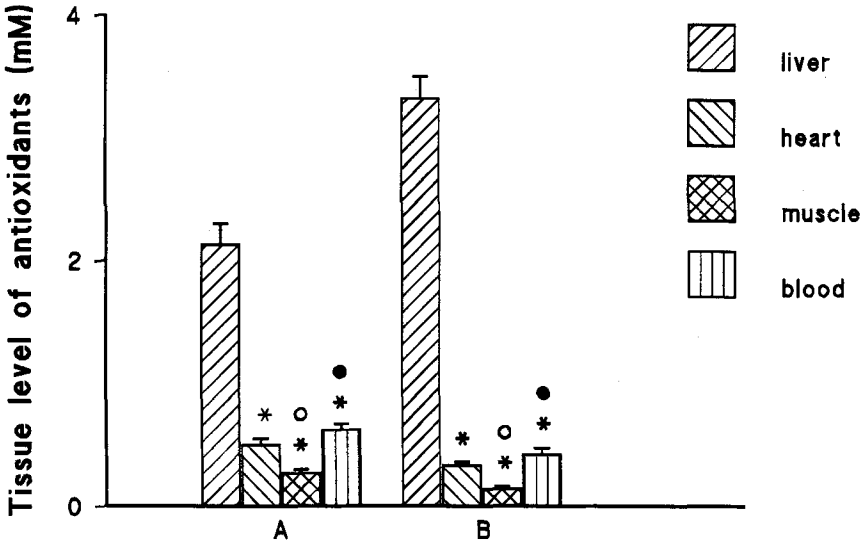

Figure 3. Values of antioxidant concentration of tissue homogenate and blood of rat. The values are determined by analysis of variations, with homogenate concentration, of light emission from both samples and samples supplemented with desferrioxamine (*A*); and use of standard curves obtained with peroxidase and desferrioxamine (*B*). The values are expressed as equivalent concentration of the antioxidant. *significant vs liver, ○ significant vs heart antioxidant level and ● significant vs muscle antioxidant level determined by the same method.

experimental data is lower than that found for liver (fig. 2). A better fit is obtained if the curves are described by a slightly different equation: $E = \alpha \cdot C/\exp(\beta \cdot C^{\delta})$ (fig. 2). The analysis of the function E shows that the emission maximum, found at $C = (1/\beta \cdot \delta)^{1/\delta}$, is given by $E_{max} = \alpha \cdot (1/\beta \cdot \delta)^{1/\delta} \cdot e^{-1/\delta}$. The emission maximum and the concentration at which such a maximum is found make the values $\alpha/e \cdot \beta$ and $1/\beta$ respectively if δ is equal to unity. This indicates that the pairs of parameters a, b and α, β illustrate the same characteristics of the systems analysed, in agreement with the experimental results showing that the differences among the various tissues in α and β are similar to those of a and b respectively (table 1).

The a (or α) value differences seem to reflect differences in mitochondrial protein, particularly in hemoprotein content. In fact, the values of mitochondrial protein mass, calculated by the cytochrome oxidase activity for both gram of tissue and milligram of mitochondrial protein, and the values of tissue cytochrome oxidase (table 2), indicate that both total mitochondrial protein and cytochromes are more abundant in heart and less abundant in muscle in comparison with liver, in substantial agreement with previous results[9–13,19,20]. The high value of a found for the blood is consistent with the high hemoglobin content of such a tissue (table 2). However, the parameter a also shows dependence on antioxidant level, as it decreases following antioxidant supplementation of the homogenates.

The parameter b values are 1.35, 0.18, 0.16 and 0.66 for

Table 2. Mitochondrial protein and cytochrome oxidase activity of tissues, and blood hemoglobin content.

Tissue	Mitochondrial proteins (mg/g)	Cytochrome aa_3 activity (μmol O/min/g)	Hemoglobin (mg/ml)
Liver	74.50 ± 3.8	81.21 ± 1.42	-
Heart	106.44 ± 9.6*	194.78 ± 16.16*	-
Muscle	23.35 ± 2.0*#	41.33 ± 3.11*#	-
Blood	-	-	120 ± 10

Data represent mean ± SEM of 8 experiments. *Significant ($p < 0.05$) vs. liver value; #significant ($p < 0.05$) vs. heart value.

Table 1. Parameters characterizing light emission from homogenates of rat tissues stressed with sodium perborate.

Tissue	Parameters					
	a	b	E_{max}	α	β	δ
Liver	25.1 ± 4.0	1.35 ± 0.10	6.8 ± 0.7	34.1 ± 6.3	1.81 ± 0.29	0.84 ± 0.07
Heart	34.9 ± 4.1	0.18 ± 0.02*	71.3 ± 0.8*	49.1 ± 5.7	0.65 ± 0.14*	0.66 ± 0.06*
Muscle	8.2 ± 1.3*	0.16 ± 0.01*	18.8 ± 2.1*#	22.2 ± 5.9	0.71 ± 0.25*	0.48 ± 0.06*
Blood	1046 ± 161*#, §	0.66 ± 0.05*#, §	583 ± 60*#, §	3525 ± 375*#, §	1.74 ± 0.16	0.55 ± 0.05*

Data represent mean ± SEM of 8 experiments. The relation between light emission and homogenate or blood concentration is described by the equations: $E = a \cdot C/\exp(b \cdot C)$ or $E = \alpha \cdot C/\exp(\beta \cdot C^{\delta})$. *Significant ($p < 0.05$) vs liver value, #significant ($p < 0.05$) vs heart value, § significant ($p < 0.05$) vs muscle value.

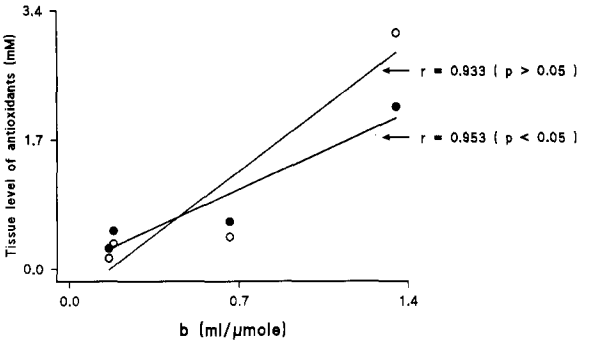

Figure 4. Level of antioxidants in tissue homogenate and blood of rat as function of b value. The level of tissue antioxidants is determined by analysis of light emission from both tissue samples and samples supplemented with desferrioxamine (●), or use of standard curves obtained with peroxidase and desferrioxamine (○). Equations of the curves are: $C = 0.003 + 1.44 \cdot b$ and $C = 0.40 + 2.40 \cdot b$ respectively.

liver, heart, muscle and blood respectively. The differences in the b (or β) value reflect differences in tissue antioxidant capacity. In fact, the order of antioxidant capacity, determined by two different methods, is equal to that of b value, i.e. liver > blood > heart > muscle (fig. 3). Furthermore, values of antioxidant capacity obtained with one of the methods are strongly correlated to b values (fig. 4).

As suggested by previous results obtained in model systems constituted by cytochrome c and by its mixtures with desferrioxamine[1], the δ value seems to depend on relative tissue levels of antioxidant system and hemoproteins. In fact, heart and blood exhibit values of the parameter δ lower than that of liver, in accordance with their lower concentrations of antioxidant systems and higher concentration of hemoproteins. The muscle exhibits the lowest δ value, in spite of its small content of mitochondrial proteins, probably because of the low content of antioxidant systems (table 1).

By analysing the response of the mitochondrial suspensions to sodium perborate oxidative stress, dependence of the light emission on protein concentration was found (fig. 5). In this case, though the best fit of data is obtained by the equation $E = \alpha \cdot C/\exp(\beta \cdot C^{\delta})$, the emission-concentration curve can be described by the equation $E = a \cdot C/\exp(b \cdot C)$ (fig. 5). Thus, a relation between the response to oxidative stress of the tissues and that of their mitochondria is readily seen, as the order of b value (liver > heart > muscle) is equal in both cases (table 1 and table 3). The same order is found for antioxidant capacity of the mitochondria (fig. 6). A different pattern is found for the values of the a parameter, that for the muscle mitochondria is higher than for liver (table 3). Also in this case, the differences are associated with analogous differences in cytochrome oxidase activity and cytochrome content (table 4). The order of the values of α and β is equal to that of a and b respectively, while the highest δ value is found for

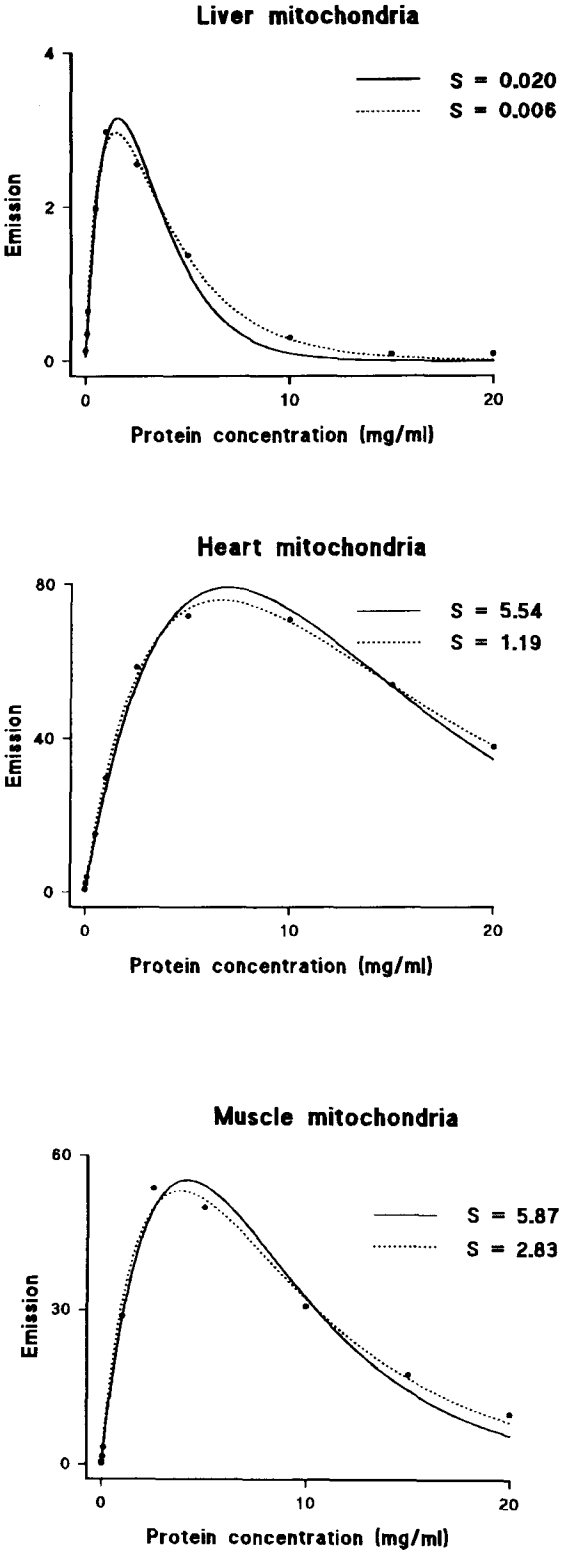

Figure 5. Curve fitting for light emission from mitochondria of rat tissues. Emission values, at different concentrations of mitochondrial proteins, are given as percentage of an arbitrary standard (44 ng/ml peroxidase). The curves are computed from experimental data by using the equations: $E = a \cdot C/\exp(b \cdot C)$ (solid lines) and $E = \alpha \cdot C/\exp(\beta \cdot C^{\delta})$ (dotted lines). The degree of fit of curves to experimental data is inversely related to the ratio between sum of squares of the differences between theoretical and experimental values and number of such values (S).

Table 3. Parameters characterizing light emission from mitochondria of rat tissues stressed with sodium perborate.

Tissue	Parameters					
	a	b	E_{max}	α	β	δ
Liver	9.2 ± 2.1	0.88 ± 0.11	3.8 ± 0.4	12.0 ± 1.6	1.53 ± 0.17	0.84 ± 0.08
Heart	37.4 ± 9.8*	0.29 ± 0.06*	47.4 ± 7.1*	48.0 ± 4.6*	0.65 ± 0.12*	0.43 ± 0.15*
Muscle	36.2 ± 11.7*	0.24 ± 0.03*	55.5 ± 7.9*	47.8 ± 5.1*	0.42 ± 0.12*	0.81 ± 0.17#

Data represent mean ± SEM of eight experiments. The relation between light emission and protein concentration of mitochondria is described by the equations: $E = a \cdot C/\exp(b \cdot C)$ or $E = \alpha \cdot C/\exp(\beta \cdot C^{\delta})$. *Significant ($p < 0.05$) vs liver values, #($p < 0.05$) significant vs heart values.

Table 4. Cytochrome oxidase activity and cytochrome content of mitochondria from rat tissues.

Tissue	Cytochrome aa_3 activity μmol O/min/g tissue	Cytochromes (nmol/mg mitochondrial protein)		
		a	b	$c + c_1$
Liver	1.09 ± 0.03	0.18 ± 0.01	0.26 ± 0.01	0.32 ± 0.01
Heart	1.83 ± 0.06*	0.49 ± 0.12*	0.52 ± 0.12*	0.58 ± 0.05*
Muscle	1.77 ± 0.11*	0.41 ± 0.09*	0.36 ± 0.09	0.56 ± 0.01*

Data represent mean ± SEM of 8 experiments. *Significant vs liver values.

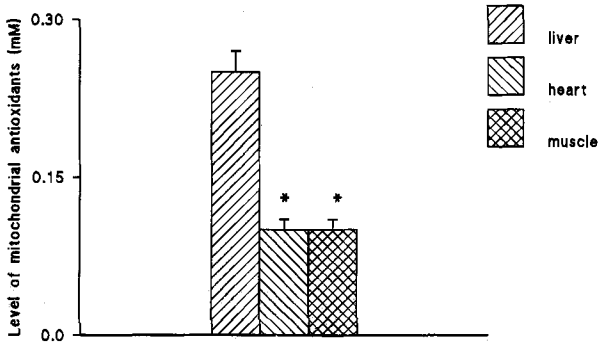

Figure 6. Values of antioxidant concentration in mitochondria of rat tissues. The values, determined by using standard curves with peroxidase and desferrioxamine, are expressed as equivalent concentration of the antioxidant. *Significant vs liver mitochondria antioxidant level.

liver mitochondria, which exhibits the lower cytochrome content and higher antioxidant concentration.

Discussion

Our results indicate that two systems coexist in the tissues: one produces free radicals and the other scavenges them. Tissue level indications of the two antagonist systems are supplied by the values of the constants a and b of the equation $E = a \cdot C/b \cdot C$, obtained measuring the luminescence response to changes of homogenate concentration.

Evidence that the a value depends on the level of substances inducing the radical production was previously obtained showing that such a value is increased by the addition of iron ligands to liver homogenates[1]. Other evidence is furnished by the present finding that tissues with higher mitochondrial protein content and cytochrome oxidase activity exhibit higher a values. However, the decrease, found after addition of desferrioxamine to homogenates, indicates that the a value also depends on the concentration of antioxidants. This dependence can be explained by considering that a preventative antioxidant[21] can bind metal ions in forms that will not generate reactive species, and its action should be the same as a decrease in concentration of the species catalysing radical production.

Evidence of a relation between b value and level of antioxidants in the liver homogenate was obtained demonstrating that the addition of scavengers increased the b value and decreased the E_{max} value[1]. The results of this study show that the phenomenon takes place for all the tissues (fig. 1). In addition, the antioxidant capacities of the different tissues are in strict correlation with their b values (fig. 5).

The values of both b and antioxidant level indicate that the different tissues are provided with differently effective antioxidant defense systems (fig. 4). Our evaluation of the overall antioxidant capacity of the different tissues seems to be correlated with the level of the components of the major antioxidant systems. In effect, in the rat the content of glutathione[22,23], vitamin E[22,24] and vitamin C[25], and the activity of glutathione peroxidase[23,24,26], glutathione reductase, superoxide dismutase and catalase[23,26], is much higher in liver than in skeletal muscle. Thus, the activity of the aforementioned enzymes in the rat heart is intermediate between those of liver and muscle[26]. No general pattern of antioxidant capacity of the blood in relation to that of the other tissues can be derived from available literature, as this reports data on either serum or erythrocyte antioxi-

dants. With regard to the mitochondrial antioxidant capacity, our findings seem to be corroborated by previous data indicating that the activity of superoxide dismutase and glutathione peroxidase are higher in liver than in the other tissues[26].

The analysis of model systems constituted by cytochrome c and by its mixtures with desferrioxamine showed that when the scavenger action was carried out either solely or prevalently by hemoproteins, the emission decreased more slowly with the concentration increase[1]. In this case the intensity of the light emission was represented by a more complex equation: $E = \alpha \cdot C/\exp(\beta \cdot C^{\delta})$. The coefficient δ had a value smaller than 1, which, however, increased when greater desferrioxamine concentrations were used. This led us to think that the parameters α and β have the same biological significance as the parameters a and b, while the parameter δ is an index of the relative importance of the 'normal antioxidants' and hemoproteins in trapping radicals. The results obtained with homogenates and mitochondria from tissues with different hemoprotein content confirm such an idea. Moreover, the analysis of the processes, which presumably take place in our experimental system, supplies indications of the meaning of the parameter δ. Such an analysis also shows that in vitro study of biological preparations in our experimental conditions supplies a realistic view of the phenomena happening in vivo.

It is well known that reactive oxygen species are generated at several subcellular sites[27,28], and particularly in the mitochondria[29-32]. An increase in mitochondrial O_2 consumption is followed by an increase in their rate of O_2^- radical generation[33-35]. In effect, in the intact mitochondria the superoxide ion is generated at a rate related to the rate of oxygen consumption[36]. The increase in O_2^- generation leads to an increase in superoxide dismutase production of H_2O_2 that, in turn, can generate hydroxyl radicals through the Haber-Weiss and Fenton reactions. Some doubt has been expressed about the possibility that Fenton reactions occur in vivo. However, evidence is now available that hemoproteins contained in the membranes act as peroxidases[37], which can initially form a ferryl species[38] that then decomposes to release $\cdot OH$[39].

In our system H_2O_2 is released by sodium perborate at a concentration higher than that actually available in vivo. However, the hydroxyl radicals or other reactive species are produced via the Fenton reaction catalysed by ligand-bound iron ions. The subsequent phases are probably similar for the in vivo and in vitro systems.

The hydroxyl radicals, formed at the point of metal binding, can interact with the ligand, giving rise to site-specific damage[40,41]. Actually, H_2O_2 breakdown on iron chelated by macromolecules in vivo is considered to be a general pathway of oxidative modification of proteins[42]. Some authors have demonstrated that the oxidation of enzymes by oxidase systems could be the first step of a multistep mechanism for enzyme degradation[43-45]. The oxidation of the proteins can lead to their degradation through a different mechanism involving the disruption of the haem ring. It has been shown in model systems that iron can be released from hemoglobin and myoglobin by excess hydrogen peroxide[46,47]. This phenomenon could explain the low δ values in preparations with high content of hemoproteins. In fact, the greater release of iron, a substance unable to retain the $\cdot OH$ produced, might give rise to a decrease of the amount of substances able to scavenge radicals without equally decreasing substances able to catalyse the radical production. In this case, the concentration of the antioxidants, unlike that of the pseudoperoxidases, should not increase in a parallel fashion with homogenate concentration.

Hydroxyl radicals can be released and then intercepted by scavenger systems. Some of the released radicals may, however, escape the scavengers and interact with membrane lipids, DNA, enzyme systems or ion channels, thus compromising important cellular functions. Analogous reactions take place when the radicals are released in solution. The only difference is that in our system the radicals can interact with detector molecules, thus giving rise to light emission. Therefore, the light emission can be considered as an index of oxidative stress in our preparations, just as oxidatively damaged lipid, protein cellular enzymes and DNA molecules are considered as indexes of oxidative stress in in vivo biological systems[48-51].

In conclusion, our results indicate that the enhanced luminescence technique opens a large range of applications, and can be used to study the oxidative stress response and the overall antioxidant capacity both in tissue homogenates and in cell organelles. Furthermore, such a study of a model system is able to supply information on the behaviour of the in vivo biological system, as the basic processes taking place in the two systems are substantially similar.

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