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New biosensor for superoxide radical used to evidence molecules of biomedical and pharmaceutical interest having radical scavenging properties

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Abstract

A superoxide dismutase biosensor was used to determine the antioxidant properties of scavenger molecules and the antiradical activity of healthy and diseased human kidney tissues; this biosensor is based on the use of the enzyme superoxide dismutase (SOD), which is physically entrapped in a kappa-carrageenan gel membrane, and of a transducer consisting of an amperometric hydrogen peroxide electrode. Several compounds with scavenging properties were tested, including some commercial drugs. © 2000 Elsevier Science B.V. All rights reserved.

Keywords: Superoxide dismutase; Biosensor; Free radical; Scavengers

1. Introduction

The determination of free radicals is performed mainly by ESR [1], by chemiluminescence [2], or by certain semiquantitative colorimetric tests [3,4]. In recent years also some electrochemical methods have been developed [5–9], based in particular on electrochemical sensors [10,11] and biosensors [12–14]. These devices are generally inexpensive, easy to build and use. In some cases they can even operate in situ. We recently approached the problem starting from the determination of oxygen free radicals, in particular the superoxide radical, assembling several new kinds of electrochemical sensors and biosensors suitable for this purpose: firstly, [15] a voltammetric system based on the detection of reduced cytochrome c; this system was also applied to developing a suitable amperometric carbon paste electrode; secondly [16] two potentiometric sensors (one classical selective membrane sensor and the other a solid state field effect transistor sensor), based on selective membrane entrapping benzylidenephenylnitrone with potentiometric detection. More recently [17], we studied two different kinds of biosensors to determine superoxide radicals obtained by coupling a transducer consisting of an amperometric gaseous diffusion electrode for oxygen, or another amperometric electrode for hydrogen peroxide, with su-

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peroxide dismutase enzyme immobilised in kappa-carrageenan gel. Both the sensors showed a good response to the superoxide radical. We consider that the SOD/H_2O_2 biosensor is now comparatively mature both from an engineering and an operative point of view. As a first application of this biosensor aimed at studying in vitro the effects on the superoxide radical of molecules considered as radical scavengers, the response of the biosensor to the superoxide radical both in the presence and absence of three very common scavengers (ascorbate, glutathione, cysteine) was investigated. In this paper we performed an exhaustive study of biosensor response over the whole pH range and a new standardisation of the biosensor. We then studied the scavenging properties of other important molecules such as melatonin, β-carotene and acetylsalicylic acid as scavenger molecules of great biomedical and pharmaceutical interest, including also pharmaceutical formulations containing acetylsalicylic acid.

In addition, we addressed the problem of the experimental evaluation of any differences in the defence against free radicals (that is, in practice, the antiradical properties) of human kidney tissues both healthy and diseased, following a completely original approach involving the use of the superoxide dismutase biosensor described in this paper. The preliminary results obtained in this regard are also illustrated.

2. Experimental

2.1. Reagents and apparatus

Kappa-carrageenan, xanthine oxidase (XOD) from buttermilk 0.39 $U \cdot mg^{-1}$ supplied by Fluka (Buchs, Switzerland); sodium carbonate provided by Merck (Darmstadt, Germany); superoxide dismutase (SOD) E.C. 1.15.1.1, from bovine erythrocytes 4400 $U \cdot mg^{-1}$, xanthine (2,6-dihydroxy purine) sodium salt supplied by Sigma (Norfolk, MO, USA). Cellulose acetate supplied by Aldrich (Steinheim, Germany); hydrogen peroxide, potassium chloride, potassium phosphate, sodium hydroxide and all other reagents of analytical reagent grade were supplied by Carlo Erba (Milan, Italy).

A model 4000-1 electrode supplied by Universal Sensors Inc. New Orleans, LA, USA, connected to an Amel model 631 differential electrometer (Milan, Italy) coupled to an Amel model 868 analog recorder was used. An Amel model 551 potentiostat was used as potentiostatic power supply, and also used to convert the current signal into a tension signal.

Examined drugs: aspirin (500 mg of acetylsalicylic acid per tablet) and aspirin containing vitamin C (400 mg of acetylsalicylic acid and 240 mg of ascorbic acid per tablet) were obtained from the Italian market.

2.2. Methods

2.2.1. Preparation of the kappa-carrageenan membrane

A hot (60–70°C) 2% w/w solution of kappa-carrageenan was prepared using distilled and de-ionised water; when a homogeneous phase was obtained, 5 ml of the solution were poured into a 5 cm diameter glass vial and allowed to gel at 5°C overnight. Five-mm diameter diskettes were then cut from the gel thus obtained, and immersed under gentle stirring in distilled de-ionised water containing the small amount of NaN_3 that would fit on a spatula tip as anti-bacterial agent. After 2 h stirring the membranes were removed and allowed to dry at a temperature of 5°C for 48 h; sufficiently dehydrated membranes were thus obtained, which could be used to physically immobilise the enzymes by rehydrating them with a small volume (50 μl) of enzymatic solution.

2.2.2. Immobilisation of the enzyme

Five mg of superoxide dismutase enzyme (4400 $U \cdot mg^{-1}$) were weighed out and dissolved in 50 μl of carbonate buffer 0.05 M at pH 10.2; this solution was then placed in a small vessel: a kappa-carrageenan membrane, prepared as described above, was then immersed in the solution, and allowed to stand at + 5°C overnight. The rehydrating membrane absorbed the entire solution and the enzyme was thus physically entrapped in the gel.

2.2.3. Assembly of the SOD/H_2O_2 biosensor

The assembly is illustrated in Fig. 1: the jelly-like membrane containing the immobilised enzyme is sandwiched between the cellulose acetate membrane of the electrode and a dialysis membrane and the whole assembly is fixed to the transducer by means of a rubber O-ring. In this case the transducer consists of an amperometric electrode for hydrogen peroxide comprising a Pt anode and an Ag/AgCl cathode; a constant potential of +650 mV is applied to the Pt anode. The absence of any possible interferents, such as ascorbic acid, is guaranteed by the semi-permeable cellulose acetate membrane [18].

2.2.4. Generation in situ of superoxide radical

The superoxide radical was in this case produced directly in solution as, in aqueous solution, it is not possible to prepare stable solutions of known titre of the radical. For the production in situ of the $O_2^{\cdot -}$ the classic reaction of Xanthine oxidation to uric acid by xanthine oxidase was used [19].

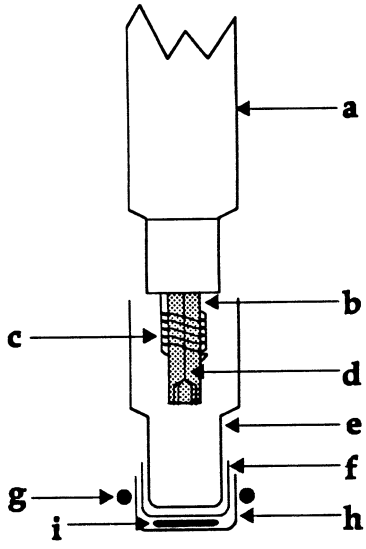

Fig. 1. Superoxide dismutase biosensor assembly, using amperometric hydrogen peroxide electrode as transducer. a, electrode body; b, dielectric; c, Ag/AgCl cathode; d, Pt anode; e, electrode plastic cap; f, cellulose acetate membrane; g, rubber O-ring; h, dialysis membrane; i, kappa-carrageenan membrane entrapping superoxide dismutase enzyme.

For this purpose the enzyme xanthine oxidase was added in solution. After ascertaining that the enzyme had dissolved completely, a fixed volume of a previously prepared Xanthine standard solution was added. This led to the production of $O_2^{\cdot -}$, according to reaction [20].

$$\text{Xanthine} + H_2O + O_2 \xrightarrow{\text{xanthine oxidase}} \text{Uric acid} + 2H^+ + O_2^{\cdot -}$$

2.2.5. Method and measurement

Superoxide dismutase enzyme catalyses the dismutation reaction of the superoxide radical with the release of O_2 and H_2O_2, according to the following reaction:

$$O_2^{\cdot -} + O_2^{\cdot -} + 2H^+ \xrightarrow{\text{superoxide dismutase}} H_2O_2 + O_2$$

the state of the enzymatic reaction is then monitored by the amperometric sensor for H_2O_2. The hydrogen peroxide produced is oxidised at the working platinum electrode polarised at +650 mV and thus generates a signal (nA) that is proportional to the concentration of the $O_2^{\cdot -}$ radical in solution.

2.2.6. Detection of radical scavenging properties

The following protocol was used for the tests: the biosensor was allowed to stabilise in 20 ml of phosphate buffer solution 0.05 M, pH 7.5 containing 1.2 mg of XOD and 0.05 M of the selected scavenger in solution, under magnetic stirring, until the response of the biosensor became constant; at this stage, several additions of the xanthine to the solution contained in the cell were performed. A calibration curve was then recorded. The latter has been compared with the calibration curve obtained under the same experimental conditions, in the absence of the scavenger molecule.

The antioxidant properties of the scavenger molecules considered have been evaluated from the percent ratio of slope values of calibration graph, both in the absence and presence of each scavenger molecule in solution considered. The results have been presented as a histogram.

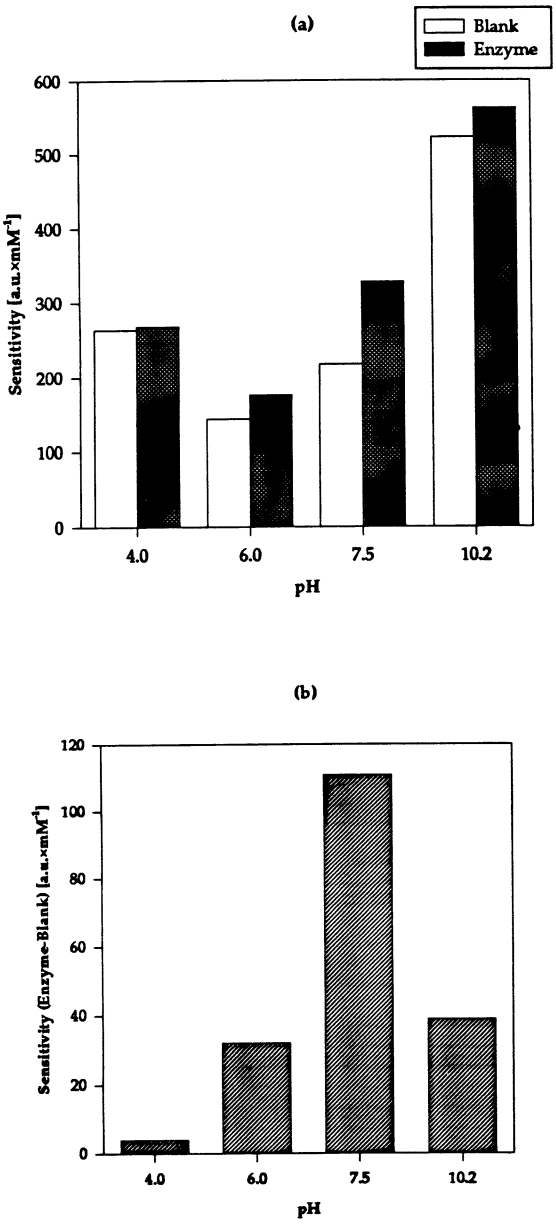

Fig. 2. (a) Comparison of the sensitivity (expressed as slope of the calibration curve) of the SOD/H_2O_2 biosensor (Enzyme) and that one of the H_2O_2 indicator electrode (Blank). (b) Trend of the sensitivity of the SOD/H_2O_2 biosensor (expressed as difference between enzyme biosensor and blank slope values at different pH) working in different aqueous buffer solutions: pH 4.0 acetate buffer, pH 6.0 phosphate buffer, pH 7.5 phosphate buffer, pH 10.2 carbonate buffer.

2.2.7. Tests on healthy and diseased tissues

The following protocol was used for the tests: the biosensor was allowed to stabilise in 20 ml of phosphate buffer solution 0.05 M, pH 7, under magnetic stirring, until the response was constant; at this stage 500 μl of a solution containing homogenised healthy or cancerous kidney tissue (0.5 g of tissue in 3 ml of distilled water, homogenised using a Janke & Kunkel (Germany) homogeniser) was added and the biosensor response, that is the current variation occurring after addition of the homogenised tissue, was then recorded. Before use the tissue was stored in freezer at −20°C.

3. Results

In previous research [17], for the purpose of selecting the working pH, account was taken above all of the pH value required by the reaction catalysed by the xanthine oxidase, which was used to produce the superoxide radical [19,20]: this reaction is generally performed at pH 10.2, in accordance with the results of Rotilio et al. [21], who performed the polarographic determination of superoxide dismutase at basic pH. On the other hand, the oxidation reaction of hydrogen peroxide itself is a pH-dependent redox process facilitated by basic pH values.

$$H_2O_2 \rightarrow O_2 + 2H^+ + 2e^-$$

Therefore, in the previous investigation [17] it was decided to operate at pH 10.2.

However, in a very recent study, Mesãros and co-workers [22] suggested pH 7.5 as an optimal working pH, and so a more detailed experimental investigation is now being carried out by us at the various pH values. To this end, in Fig. 2(a), a comparison is made of the sensitivity (expressed as slope of calibration curve) of the SOD/H_2O_2 biosensor and that of the blank due to the spontaneous dismutation of superoxide radical. The sensitivity of the biosensor is highest at a pH of about 10, but also the blank slope is very high, while Fig. 2(b) shows the trend of the sensitivity of the SOD/H_2O_2 biosensor (expressed as the difference between the biosensor and the blank

slope, at different pH), obtained using a different buffer solution. The highest value is observed at pH 7.5 according to Mesãros' suggestions, in addition this pH value is also closer to physiological values. On the basis of these results we performed a further full characterisation of the biosensor working at pH 7.5. Fig. 3 shows the mean calibration graph and Table 1 the main analytical data and the working conditions. The trend of the slope value and the linear range of the calibration graph of the SOD/H_2O_2 biosensor for superoxide radical, working in aqueous phosphate buffer solution at pH 7.5, during the lifetime of the biosensor (≥ 7 days), is shown in Fig. 4.

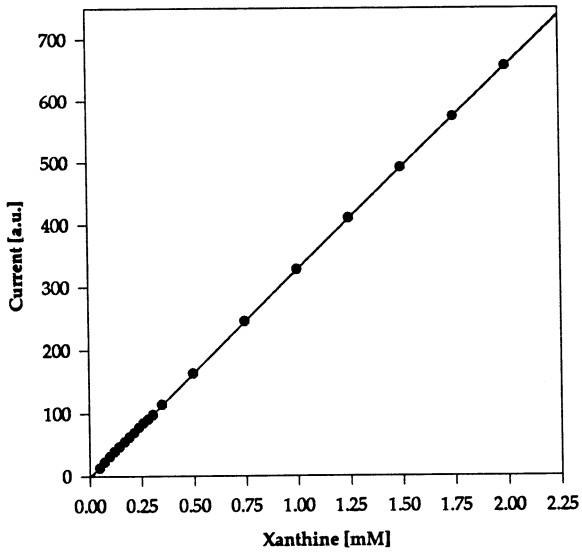

Fig. 3. Calibration graph of SOD/H_2O_2 biosensor.

Table 1
Working condition and main analytical data

Transducer	Amperometric hydrogen peroxide electrode
Biological system	Superoxide dismutase enzyme
Immobilisation	Physical entrapment in kappa-carrageenan gel
Buffer	Phosphate 0.05 M, KCl 0.01 M, EDTA 0.5 mM
pH	7.5
Temperature	25.0 ± 0.5°C
Substrate	Superoxide radical generated in situ by xanthine/XOD system
Equation of calibration curve (y = a.u.; x = [Xanthine] (mM)) (confidence interval: $t = 2.23$; $(1-\alpha) = 0.95$)	$y = (328.9 \pm 6.4)x + (-2.3 \pm 1.3)$
Correlation coefficient	0.9996
Linear range (mM)	0.02–2.0
Minimum detection limit (mM)	0.01
Precision (as RSD%)	≤ 5
Response time (s)	≤ 100
Time of analysis (min)	5
Lifetime (days)	≥ 7

After the response of the biosensor had been characterised completely, following the procedure explained in the previous section, the biosensor was used to evaluate the scavenging properties of several new compounds also of pharmaceutical interest, including two pharmaceutical formulations (aspirin and aspirin containing vitamin C), using the SOD/H_2O_2 biosensor working in phosphate aqueous buffer solution at pH 7.5. Good results were obtained, especially in the case of acetylsalicylic acid and of two pharmaceutical forms derived from it. (Fig. 5)

Lastly, a number of tests are under way in our laboratory to investigate the antiradical action of homogenised healthy or cancerous kidney tissue, comparing the responses given by the superoxide dismutase biosensor, working in aqueous solution, in the presence of healthy or cancerous tissues, respectively, and taking into account also the blank of the indicator electrode.

The histogram in Fig. 6 shows that the signal variation of the biosensor is significantly different after addition of homogenised healthy or cancerous kidney, which indicates that cancerous kidney tissue probably contains a greater amount of superoxide radical than healthy kidney. Its scavenging properties thus seem to be lower than those of healthy kidney tissue. Although still in the early stages, these experiments appear very promising.

4. Discussion

Comparing the data of the biosensor characterisation reported in Table 1 with those found when

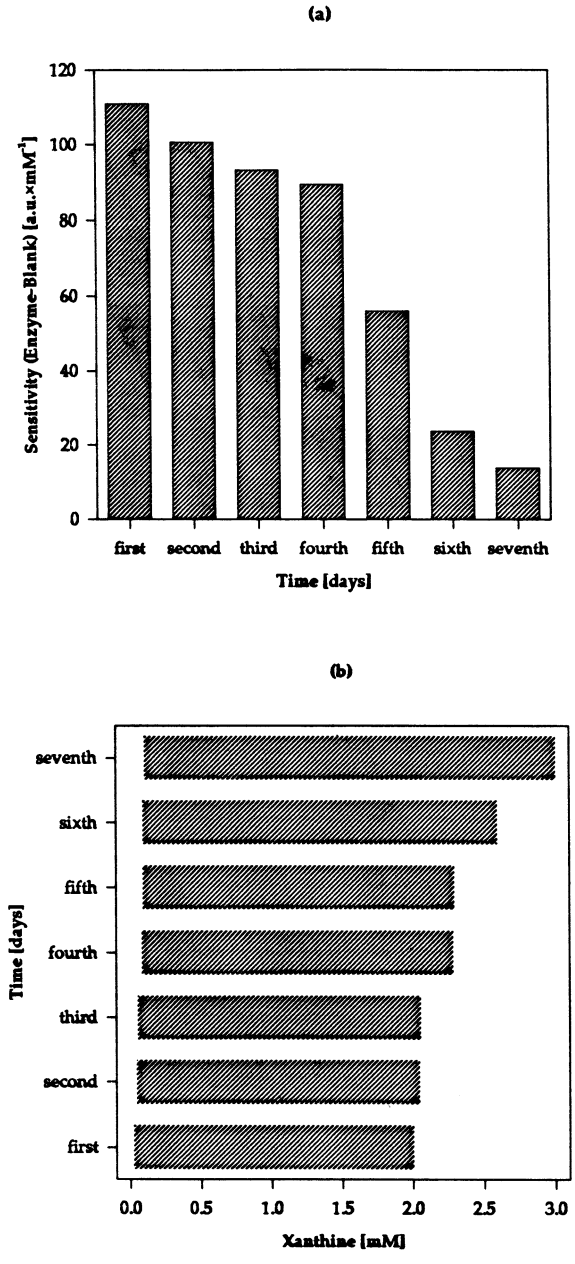

Fig. 4. Trend of (a) the slope and of (b) the linear range of the calibration graph of the SOD/H_2O_2 biosensor (as difference between enzyme biosensor and blank) for superoxide radical, working in aqueous phosphate buffer solution at pH 7.5, during the biosensor lifetime.

working at higher pH, as reported in a previous paper [17], the minimum detection limit and the linear range are seen to be practically the same, while the latter is shifted slightly at lower concentrations. The sensitivity (expressed as the difference between the slope value of the calibration graph for the biosensor and the blank, respectively) is higher for the biosensor working at pH 7.5 (Fig. 2). From the trend of the histogram reported in Fig. 4, the biosensor lifetime may definitely be said to be higher than 7 days, while sensitivity is higher on the first day of biosensor lifetime than on the following days under the working conditions used.

Using the biosensor, the effects on the superoxide radical of several molecules accepted as radical scavengers were studied in vitro. In particular the response of the biosensor to the superoxide radical in the presence and in the absence of each of several scavenger molecules of biochemical and pharmaceutical interest (Fig. 5) (ascorbic acid,

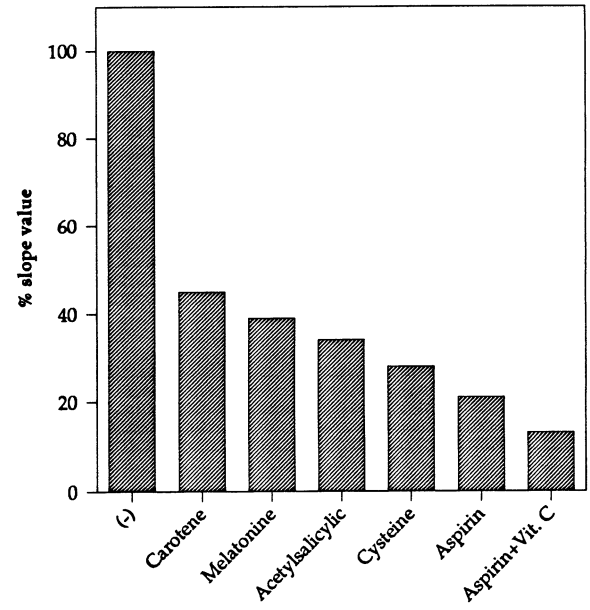

Fig. 5. Evaluation of scavenger properties of several compounds and drugs using the SOD/H_2O_2 biosensor working in aqueous phosphate buffer solution 0.05 M at pH 7.5. Behavior of the % sensitivity of the biosensor in the absence (−) and presence of different antioxidant compounds β-carotene and melatonin in saturated solution; acetyl salicylic acid and cysteine at a concentration of 0.05 M, while the two pharmaceutical preparations (aspirin and aspirin + vitamin C) at a concentration of 12.5 $g \cdot l^{-1}$.

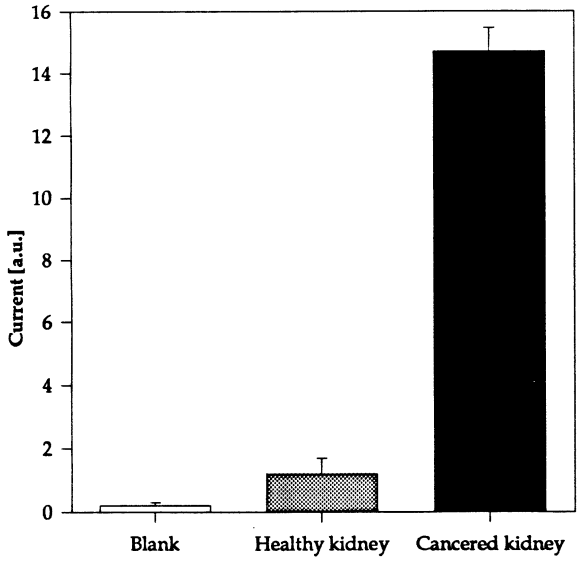

Fig. 6. Signal variation of the superoxide dismutase biosensor after addition of homogenised healthy and cancered kidney in aqueous solution. Each value is the mean of at least three determinations.

cysteine, β-carotene, melatonin, acetylsalicylic acid) was investigated. Results confirm the free radical scavenger function of compounds such as β-carotene, melatonin, acetylsalicylic acid, ascorbic acid and cysteine the action of which was easy to evidence not only in vivo (as reported in literature) but also in vitro. Furthermore, the potential of the proposed method as a fast and very simple way of determining the antioxidant properties of commercial drugs such as aspirin and aspirin containing vitamin C (Fig. 5) is also considered of great interest.

In conclusion, the SOD/H_2O_2 biosensor is, in our opinion, sufficiently mature both from an engineering and an analytical standpoint and appears of great interest for in vitro studies on possible radical scavenger molecules.

Lastly, as shown in the histograms in Fig. 6 the SOD biosensor gave a different signal according to whether the homogenised tissue in solution was healthy or cancerous. The experimental results show how the SOD electrode reveals a certain difference in the amount of superoxide radical contained in the healthy or cancerous kidney tissue.

The response was apparently proportional to the concentration of superoxide radical present in the tissue. The experimental results thus show that cancerous kidney tissue contains a greater amount of superoxide radical. On the other hand, in view of the short life span of this radical, it is probably the product of different chain reactions in which also other radical species participate. It would thus seem to be fairly reasonable to assert that, generally speaking, cancerous tissue contains a greater quantity of free radicals and not just of the superoxide radical. This is probably because the cancerous tissue contains a smaller quantity of scavengers or, more probably, of endogenous superoxide dismutase (SOD), which can directly block the superoxide radical, than healthy tissue. In conclusion, from these preliminary results it seems possible to conclude that the superoxide dismutase biosensor can be used both as an 'indicator' of the total content of radicals present in a tissue and to determine in practice the latter's antiradical capacity, i.e. the level of scavengers or endogenous SOD contained in the tissue itself.

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