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Superoxide generation by the respiratory chain of tumor mitochondria

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O_2^- generation by the succinate oxidase segment of the respiratory chain of mitochondria and submitochondrial particles from hepatoma 22a and hepatoma Zajdela has been studied with the use of the Tiron method. In the presence of succinate, superoxide generation is induced by antimycin, 2-*n*-4-hydroxyquinoline *N*-oxide or funiculosin, and is inhibited by mucidin, myxothiazol or cyanide. The rate of O_2^- generation in the antimycin-inhibited state is maximal at the [succinate]/[fumarate] ratio of 1:10 and diminishes at more positive and more negative redox potentials. These characteristics of O_2^--generation are the same as observed earlier in submitochondrial particles from normal tissues. Accordingly, the mechanism of superoxide production is suggested to be the same in tumor and normal mitochondria, namely, autoxidation of the unstable ubisemiquinone in the ubiquinol-oxidizing centre *o* of cytochrome bc_1 complex. With respect to the rate of O_2^- generation, the hepatoma mitochondrial membranes are approximately twice as active as bovine heart submitochondrial particles and exceed those from rat liver by more than one order of magnitude.

Introduction

Superoxide radicals play an important though yet not completely understood role in cell physiology. O_2^--producing and scavenging (or utilizing) reactions are likely to be carefully balanced in the cell to maintain an optimal level of radicals.

It has been established that in most tumors the cytoplasmic Cu- and Zn-superoxide dismutase activity is low as compared to the normal tissues [1–10]. The same may hold also for the mitochondrial Mn-isoenzyme [5,11,12]. Therefore it would be interesting to evaluate the O_2^--generation in the tumors.

Membrane-bound redox chains are likely to be a major source of H_2O_2 and O_2^- radicals in the cell [13]. In particular, the respiratory chain of mitochondria is involved in the superoxide production, the radicals being formed at the level of ubiquinone (for a review, see Ref. 14). The mechanism of this reaction in mitochondria from the normal tissues may consist in autoxidation of the unstable ubisemiquinone generated in the so-called centre *o* of the Q-cycle under the conditions when cytochrome *b* reoxidation via centre *i* is inhibited [15–18].

O_2^- production by the mitochondrial respiratory

Abbreviation: Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid.

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chain of several hepatomas was described earlier [5,11,19]. Although the mechanism of the reaction was not studied in detail, uncoupled tumor mitochondria were reported to be capable of superoxide generation in the absence of antimycin (see Table III in Ref. 5), which is in contrast to experiments with the preparations from normal tissues and may be indicative of a different mechanism of radical production. However, those results were obtained with the aid of the adrenaline method [20], notorious for many artifacts [21–23], and the control experiments presented were not sufficient for valid conclusions.

In recent years, the Tiron assay proved a simple and reliable tool for investigations into $O_2^{\cdot}$ generation by various membrane-bound redox chains [16,18,24–29].

Here we describe $O_2^{\cdot}$ production by the respiratory chain of mitochondria and submitochondrial particles from two ascites hepatomas as studied by the Tiron method. The results obtained show that the tumor mitochondria generate $O_2^{\cdot}$ radicals actively by the same mechanism as those from the cardiac tissue. There is, however, some $O_2^{\cdot}$-independent KCN-sensitive oxidation of the Tiron catechol by the tumor mitochondria which may account for the antimycin-independent '$O_2^{\cdot}$ generation' as measured by the adrenaline oxidation method in [5].

Materials and Methods

Chemicals. Tiron (1,2-dihydroxybenzene-3,5-disulfonate, Na_2 salt) 'pure for analysis' grade was from Reachim; aqueous solutions of the compound were prepared before experiments. Funiculosin was a gift from Dr. P. Bollinger (Sandoz, Basel), myxothiazol was kindly donated by Dr. W. Trowitsch (Gesellschaft für Biotechnologische Forschung, Braunschweig) and mucidin was obtained from Dr. V. Musilek (Institute of Microbiology, Academy of Sciences of Czechoslovakia, Prague). Other chemicals were commercial products largely from Sigma, Serva and BDH.

Preparations. Hepatoma 22a and hepatoma Zajdela ascites tumors were grown in male C3HA mice and random-bred albino rats, respectively. The cells were collected on the 7th day after intraperitoneal transplantation.

Mitochondria were isolated from the tumor ascites cells essentially according to Ref. 30 and the purity of the preparaitons was checked by electron microscopy. Mitochondria from rat liver and bovine heart were prepared as described in Refs. 31 and 32, respectively, with minor modifications. Ultrasonic Mg, ATP, succinate-submitochondrial particles were obtained from mitochondria essentially as described in Ref. 33; significantly, Mn^{2+} had to be omitted from the media to avoid interference of the metal ions with the EPR measurements.

Assays. Protein was measured with the use of the Folin reagent. $O_2^{\cdot}$ generation by the respiratory chain of mitochondria and submitochondrial particles was assayed by EPR spectroscopy with Tiron as superoxide scavenger as described earlier [16,18]. Measurements were made in a Varian E-4 spectrometer at 25 °C.

Unless indicated otherwise, the experiments were carried out as follows. To 0.25 ml of the basic incubation medium (0.5 M sucrose, 50 mM KCl, 20 mM Hepes (pH 7.5), 3 μM rotenone and 1 μm carbonyl cyanide *m*-chlorophenyl hydrazone) Tiron, mitochondria (or submitochondrial particles) and other reagents were added, as indicated in the figure legends; aliquots of the mixture were transferred into a flat quartz cell for EPR spectroscopy of liquid aqueous samples (total volume, approx. 100 μl; the actual operative volume, approx. 20 μl) and spectra were recorded one after another in the $g = 2.00$ region until the height of the EPR signal reached a steady-state level.

The EPR spectroscopy conditions: the clystron frequency, 9.13 GHz; modulation frequency, 100 kHz; modulation amplitude, 0.5 G; microwave power, 10 mW; scan rate, 5 G/min; time constant, 0.3 s; receiver gain, $6.3 \cdot 10^3$.

It has to be mentioned that, as found in the course of this work, the $O_2^{\cdot}$-generating activity of submitochondrial particles in the presence of antimycin and excess succinate can increase significantly upon prolonged storage (e.g., several weeks) of the frozen preparations. At the same time, the activity of the samples measured at the optimal [succinate]/[fumarate] ratio of approx. 0.1 (see Results, see also Ref. 34) kept nearly constant. According to our preliminary observations, the

storage of the frozen submitochondrial particles may modulate the redox-dependence of $O_2^{\cdot}$ generation reported in Ref. 34. Since virtually all of the measurements of $O_2^{\cdot}$ generation in the bc_1 site of the mitochondrial respiratory chain in the literature had been performed in the presence of excess succinate, i.e., at low E_h, the effect revealed here might have been a potential source of the scatter in the results reported. All the experiments described in this paper were carried out with freshly isolated mitochondria or submitochondrial particles.

Results

Experiments with Hepatoma 22a

Fig. 1A shows the results of a typical experiment on the $O_2^{\cdot}$ generation by submitochondrial particles, from hepatoma 22a as measured by the Tiron method. When antimycin and succinate are added to the particles an intensive EPR signal is observed (spectrum 4) and this effect is almost completely inhibited by cyanide (spectrum 5) in agreement with the results obtained with submitochondrial particles from bovine heart and rat

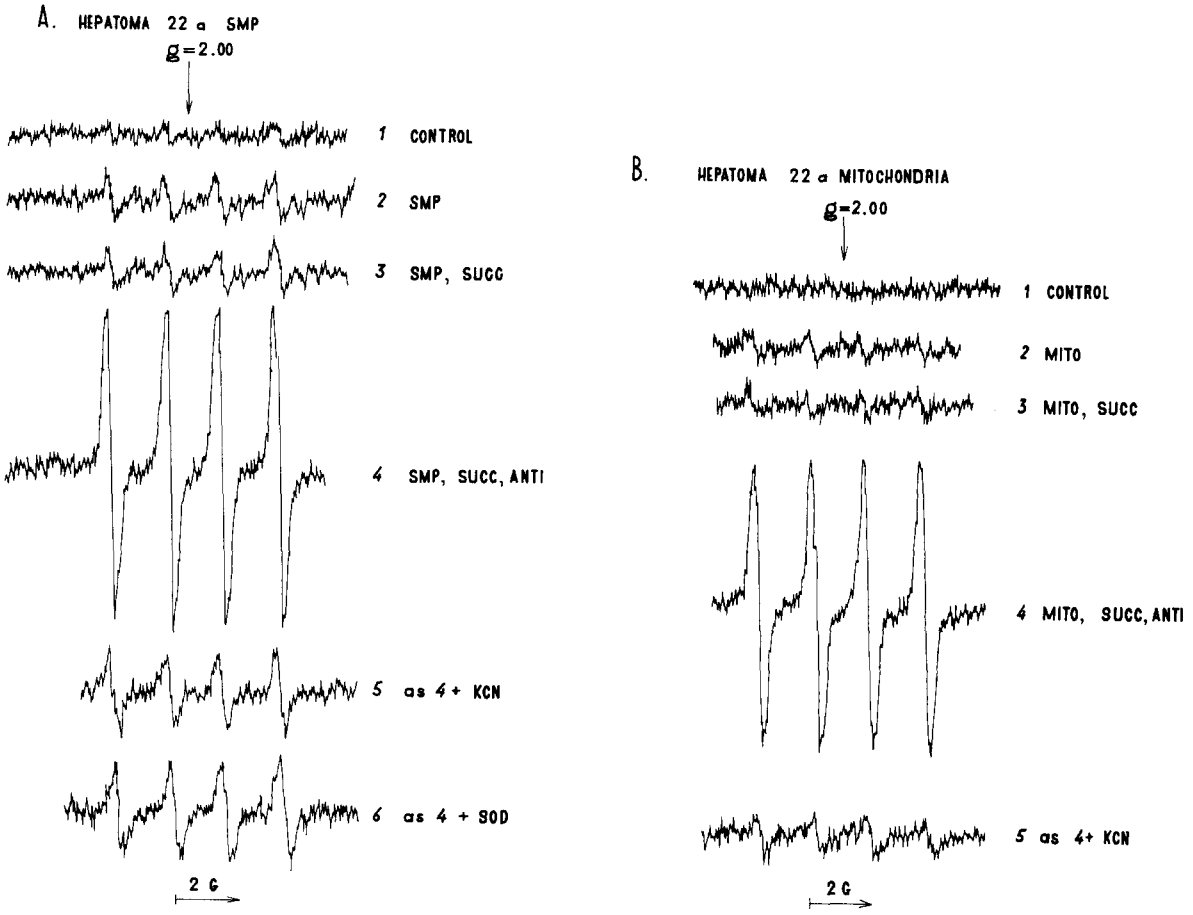

Fig. 1. Generation of superoxide radicals by the respiratory chain of submitochondrial particles (SMP) (A) and mitochondria (MITO) (B) from hepatoma 22a. (1) The EPR spectra of the basic medium (see Materials and Methods) supplemented with 10 mM Tiron; (2) the same + 0.4 mg protein/ml of submitochondrial particles (A) or mitochondria (B); (3) as (2) + 10 mM succinate (SUCC); (4) as (3), but in the presence of antimycin (ANTI) (0.5 μg/mg protein); (5) as (4) + 4 mM KCN; (6) as (4), but in the presence of superoxide dismutase (SOD) (120 μg/ml).

liver [16,18]. Superoxide dismutase strongly inhibits the antimycin + succinate-induced accumulation of the Tiron semiquinone (spectrum 6), which confirms $O_2^{\cdot}$ involvement in the Tiron radical generation.

Notably, a small EPR signal of the Tiron semiquinone is observed already upon hepatoma 22a submitochondrial particles addition to the aerobic Tiron containing buffer (Fig. 1, spectrum 2). This effect does not depend on succinate (Fig. 1, spectrum 3) and is not sensitive either to superoxide dismutase or to site 2 inhibitors such as antimycin, mucidin or myxothiazol (not shown). This background $O_2^{\cdot}$-independent generation of the Tiron radicals was even more pronounced with the preparations from hepatoma Zajdela (see below, Fig. 3) but was not observed earlier with the bovine heart or rat liver submitochondrial particles [16].

The succinate-dependent generation of $O_2^{\cdot}$ radicals by the respiratory chain of hepatoma 22a submitochondrial particles can be induced not only by antimycin, but also by other bc_1-site inhibitors such as 2-nonyl-4-hydroquinoline *N*-oxide (HOQNO) and funiculosin (Table I). In agreement with Ref. 18, a somewhat higher Tiron radical concentration is observed with HOQNO than in the experiments with antimycin. On the other hand, funiculosin proved to be a considerably less active $O_2^{\cdot}$-generation stimulant in hepatoma submitochondrial particles, which is at variance with the data on bovine heart preparation where no significant difference between the effects of antimycin and funiculosin was found [18]. Accordingly, in our control experiments performed with bovine heart submitochondrial particles on the same day with the assays given in Table I, funiculosin induced the Tiron semiquinone EPR signal which was 80–90% of that observed with antimycin (data not included). The reason for the weaker effect of funiculosin on the $O_2^{\cdot}$-generation in hepatoma mitochondrial membranes is now under investigation.

Two other inhibitors of electron transfer "betweeen *b* and c_1 cytochromes" myxothiazol and mucidin do not induce $O_2^{\cdot}$ generation in the presence of succinate and inhibit the antimycin-induced superoxide production (Table I), acting like cyanide.

As shown recently by Ksenzenko et al. [34], the rate of H_2O_2 and $O_2^{\cdot}$ generation in the bc_1-site of the respiratory chain of bovine heart submitochondrial particles depends on the redox state of a hydrogen carrier, presumably ubiquinone. Here we have carried out a redox titration of superoxide production by hepatoma 22a submitochondrial particles with the succinate/fumarate couple. As shown in Fig. 2, the radical generation is maximal at the [succinate]/[fumarate] ratio 1 : 10 ($E_h = 54$ mV at pH = 7.5 [35]) and decreases both at lower and higher redox potentials. Such a bell-shaped redox dependence of the superoxide-generating activity is in good agreement with the earlier observations on bovine heart submitochondrial particles, where the maximal rate of $O_2^{\cdot}$ and H_2O_2 production was observed at [succinate]/[fumarate] = 1 : 5 [34]. Altogether, the above data comply with the results obtained earlier with bovine heart preparation [16,18,34].

Consequently, the mechanism of $O_2^{\cdot}$ generation in the hepatoma 22a mitochondrial respiratory chain is likely to be much the same as that proposed for mitochondria from normal tissues [16–18] based on the Q-cycle electron-transfer scheme [36] (see Fig. 5).

TABLE I

EFFECT OF bc_1-SITE ELECTRON-TRANSFER INHIBITORS ON THE $O_2^{\cdot}$ GENERATION BY HEPATOMA 22a SUBMITOCHONDRIAL PARTICLES IN THE PRESENCE OF 10 mM SUCCINATE

Basic conditions are as in Fig. 1A. Concentrations of the inhibitors (μg per mg of protein): antimycin, 0.5; funiculosin, 1; HOQNO, 1; mucidin, 0.5; myxothiazol, 0.5 submitochondrial particles were preincubated with the inhibitors for several minutes prior to the succinate addition. The height of the EPR signal in the presence of antimycin + succinate has been taken arbitrarily as 100.

Inhibitor added	Tiron radical EPR signal height
None	0
Antimycin	100
Funiculosin	50
HOQNO	135
Mucidin	0
Mucidin + antimycin	< 5
Myxothiazol	0
Myxothiazol + antimycin	< 5

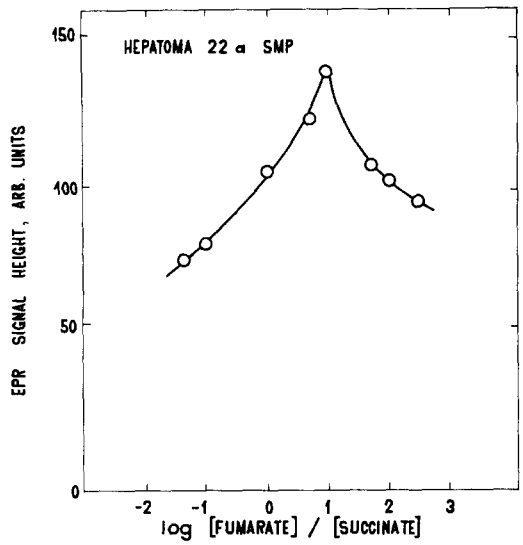

Fig. 2. Effect of the succinate/fumarate redox potential on the $O_2^{\cdot}$ generation by hepatoma 22a submitochondrial particles (SMP). The antimycin-inhibited submitochondrial particles (0.4 mg protein/ml) were incubated at indicated [succinate]/[fumarate] ratios in the basic medium with 10 mM Tiron and the steady-state levels of the Tiron radical were measured as in Fig .1; the ordinate gives the height of the EPR signal on the chart in mm at the spectrometer gain $6.3 \cdot 10^3$. Note that the rate of $O_2^{\cdot}$ generation is proportional to the second power of the Tiron radical concentration [42].

The Tiron method of $O_2^{\cdot}$ detection was reported earlier to be applicable to submitochondrial particles but not to intact mitochondria [16]. This could be a serious drawback of the assay in cases where only small amounts of mitochondria are available so that submitochondrial particles isolation may prove difficult. Here we found that the negative results obtained earlier with rat-liver mitochondria [16] may be specific for that tissue that is very rich in cytoplasmic superoxide dismutase [2,37,38], a significant amount of which is localized in the intermembrane space [2,37,39–41]. Indeed, with mitochondria from hepatoma 22a and hepatoma Zajdela, the succinate + antimycin-dependent $O_2^{\cdot}$ generation is no more difficult to measure with Tiron than in the submitochondrial particles from these mitochondria (Figs. 1B and 3). Accordingly, $O_2^{\cdot}$ generation by ischemic rat-heart mitochondria was measured recently by the Tiron method [29,42].

Experiments with hepatoma Zajdela

With mitochondria from hepatoma Zajdela, a considerable EPR signal of the Tiron semiquinone was observed upon aerobic incubation of the preparation with Tiron in the absence of succinate and antimycin (Fig. 3, spectrum 2). As described above for experiments with the hepatoma 22a mitochondrial membranes, the development of this

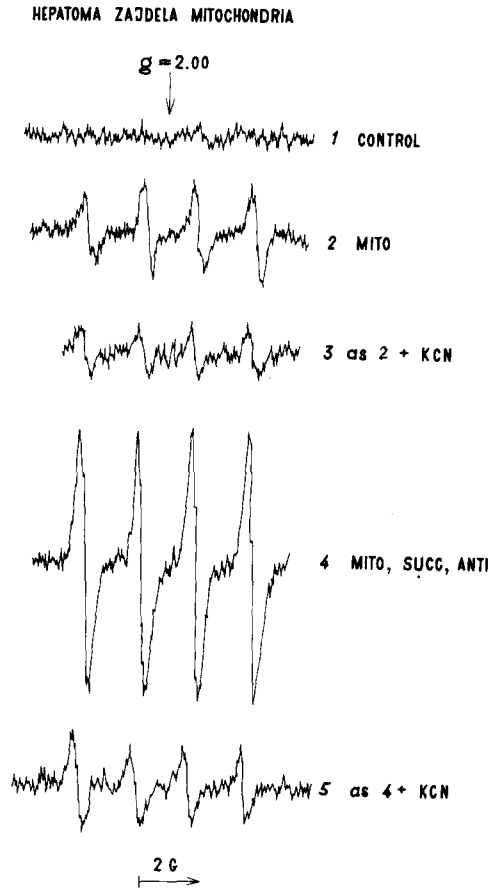

Fig. 3. Superoxide generation by hepatoma Zajdela mitochondria. The EPR spectra were recorded under the following conditions. (1) The basic medium with 10 mM Tiron; (2) the same + mitochondria (MITO) (0.4 mg protein/ml); (3) 4 min after addition of 4 mM KCN to (2); (4) as (2) + antimycin (0.5 μg/mg protein) and succinate (SUCC) (10 mM); (5) immediately after addition of 4 mM KCN to (4); besides the virtually instantaneous reversal of the succinate + antimycin-induced increment of EPR signal, one can see further diminution of the spectrum during the scan due to the slower suppression of the $O_2^{\cdot}$-independent background signal (cf. spectra (2) and (3) and see the text).

signal was not prevented by superoxide dismutase or inhibited by mucidin, neither was it sensitive to succinate addition (data not shown). However, the signal gradually disappeared upon incubation of mitochondria with cyanide (Fig. 3, spectrum 3). The same results were obtained with submitochondrial particles from hepatoma Zajdela (not shown). It appears likely that the preparations from this ascites tumor, and to a lesser extent from hepatoma 22a, are capable of oxidizing Tiron directly without involvement of $O_2^{\cdot}$ radicals. Whether this reaction is catalyzed by the terminal segment of the respiratory chain or by some KCN-sensitive contamination, e.g., hemoglobin, is not yet clear. Notably, the crude ascites cell preparations from hepatoma Zajdela contain more blood than those from hepatoma 22a, which may result in a higher hemoglobin and/or free heme contamination of the final membrane preparations.

As to the genuine $O_2^{\cdot}$ generation, the mitochondria from hepatoma Zajdela do not differ much from mitochondria or submitochondrial particles from other tissues. Fig. 3 shows that antimycin added to the succinate-supplemented mitochondria greatly enhances the back-ground EPR signal (spectrum 4). This effect of antimycin is reversed rapidly by cyanide (Fig. 3, spectrum 5) (the remaining background signal is further inhibited, but much more slowly) and in contrast to the background signal is inhibited by mucidin and prevented by superoxide dismutase (data not shown).

Evaluation of the $O_2^{\cdot-}$ generation rates

Simple kinetic considerations show that in the absence of side reactions the Tiron radical steady-state concentration (and, hence, the EPR signal height) should be proportional to the square root of the $O_2^{\cdot}$ generation rate [42].

As shown in Fig. 4, at a protein concentration below approx. 0.5 mg/ml, the plots of the EPR signal height vs. the square root of the protein concentration are indeed fairly linear for the preparations of mitochondria and submitochondrial particles assayed in this work, except possibly for the rat-liver submitochondrial particles. It can be seen from the data in Fig. 4 that the specific $O_2^{\cdot}$-generating activities of the hepatoma sub-

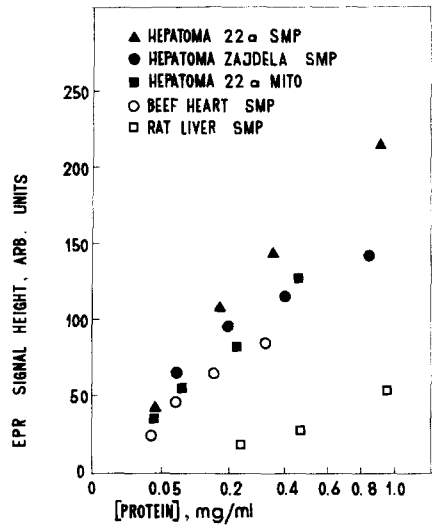

Fig. 4. Comparison of the $O_2^{\cdot}$-generating activity of mitochondria (MITO) and submitochondrial particles (SMP) from the normal and hepatoma tissues. $O_2^{\cdot}$ generation was monitored at various concentrations of the preparations in the presence of antimycin (0.5 μg/mg protein) and 10 mM succinate. Note that the abscissa axis is linear with respect to the square root of protein concentration.

mitochondrial particles in the presence of antimycin and succinate are higher than those in bovine heart and all the more so in rat-liver sub-

TABLE II

THE RATES OF $O_2^{\cdot}$ GENERATION BY THE MITOCHONDRIAL RESPIRATORY CHAIN FROM SEVERAL TISSUES IN THE PRESENCE OF SUCCINATE AND ANTIMYCIN

Conditions, see Fig. 4. The data from Fig. 4 have been recalculated into absolute rates of $O_2^{\cdot}$ generation with the use of the calibration plot from [43].

Preparation; protein concentration range	Specific rate of $O_2^{\cdot}$ generation (nmol/min per mg protein)
Rat-liver submitochondrial particles (240–940 μg/ml)	0.25 ± 0.07
Bovine heart submitochondrial particles (40–320 μg/ml)	2.5 ± 0.5
Hepatoma Zajdela submitochondrial particles (82–820 μg/ml)	4.1 ± 1.2
Hepatoma 22a submitochondrial particles (45–940 μg/ml)	5.7 ± 0.6
Hepatoma 22a mitochondria (45–460 μg/ml)	3.3 ± 0.2

mitochondrial particles. It is noteworthy that the high ionic strength treatment (0.12 M KCl), known to wash out membrane-adsorbed superoxide dismutase [2,40], did not increase the rates of $O_2^{\cdot}$ generation by the mitochondrial membranes. This observation makes unlikely membrane-bound superoxide dismutase interference with the measurements.

Upon completion of this work, a calibration of the Tiron method was carried out by Ledenev et al. [42,43] making it possible to estimate the absolute rates of the mitochondrial $O_2^{\cdot}$ production. A direct comparison of the Tiron radical spectra obtained in the present work (the data from Fig. 4) with the charts from Refs. 42 and 43 after correction for the different sensitivities of the EPR spectrometers used here and in Refs. 42 and 43, gives the specific $O_2^{\cdot}$-generation activity values listed in Table II.

Discussion

As reported originally in Refs. 1 and 2, the cytoplasmic Cu, Zn-superoxide dismutase activity is low in malignant tissues; the finding was interpreted as a consequence of cancer cell recapitulation to a more primitive anaerobic type of metabolism [1]. Those conclusions were first disputed by Oberley and Buettner [12], who claimed at that time (after Ref. 11) that it is the mitochondrial Mn-superoxide dismutase that is specifically absent from tumor cells. However, the presence of Mn-superoxide dismutase in mitochondria from many tumors has now been demonstrated by several researchers, including Oberley and co-workers [2,5,6,44]. At the same time, it has become generally acknowledged that the level of the cytoplasmic Cu,Zn-enzyme tends to correlate inversely with the tumor growth rate [4,5].

The ascites tumors studied in this work are no exception to this rule. The specific activity of Cu,Zn-superoxide dismutase is 25 units/mg of cytosolic protein for hepatoma 22a as measured in this work, and 17 units/mg of cytosolic protein for the Zajdela hepatoma [1,2]. These values obtained with the use of the xanthine oxidase/nitro Blue tetrazolium method [45] are 5–6-fold lower than the corresponding data for normal liver [1,2].

At the same time the experiments reported in this work show clearly that the respiratory chain of tumor mitochondria can generate $O_2^{\cdot}$ radicals actively. The rates of superoxide production by submitochondrial particles from the two ascites Hepatomas studied are 1.5–2-fold higher than in submitochondrial particles from bovine heart and exceed the activity of rat-liver preparation by more than one order of magnitude. Notably, the heart mitochondria are known to be one of the most active among various animal tissues as to H_2O_2 and $O_2^{\cdot}$ generation (reviewed in Ref. 46). $O_2^{\cdot}$ generation, although at somewhat lower rates, was reported previously for the mitochondrial membranes from several other tumors with the use of the adrenaline assay [5,11].

Hence it is likely that the low superoxide dismutase activity typical of most of the tumors is not accompanied by a corresponding decrease in the capability of mitochondria to generate $O_2^{\cdot}$. In addition, evidence for increased redox activity and oxygen-radical generation was reported earlier for nuclear membranes from several tumors studied in Refs. 23, 47 and 48.

It would be tempting, of course, to speculate on the basis of these observations that the balance between the $O_2^{\cdot}$-producing and utilizing reactions is altered in the tumour cells [1,2,12,47]. However, we feel that such speculations may be far reached indeed. The problem deserves a less naive approach and, probably, cannot be solved on the basis of in vitro studies only.

For instance, with respect to the actual rates of the mitochondrial $O_2^{\cdot}$ gneration, at least three additional factors should be taken into account: (i) actual O_2 concentration in tumors which are often hypoxic; (ii) mitochondrial respiratory chain content of the tumor cells, which may be lower than in normal cell (e.g., Refs. 49 and 50); and (iii) energization status (e.g., $\Delta\psi$-ΔpH relationships) of mitochondria in tumor cells in vivo, since this status is known to affect greatly the $O_2^{\cdot}$ generation rate is site 2 (see Ref. 15 and references therein).

As to the $O_2^{\cdot}$ scavenging, superoxide dismutase, though important, is not the only line of defence against oxygen radicals in the cell. As first suggested about 10 years ago [1,2], the tumours with a low superoxide dismutase activity may switch from the enzymatic to a more primitive chemical mechanism of oxygen radical detoxication as they accu-

mulate antioxidants [51] and "contain higher levels of radical reaction-terminating substances than normal cells" [44]. Therefore, in our opinion, the present and earlier in vitro studies on superoxide generation and superoxide dismutase activity in tumours certainly raise the question of $O_2^{\cdot}$ imbalance in malignant tissues but are hardly sufficient for providing the answer.

A second question which is much easier to discuss concerns the mechanism of $O_2^{\cdot}$ generation by the respiratory chain of tumour mitochondria in the presence of succinate. According to the results of some previous works, this mechanism might be different from that established for mitochondria from normal tissues. Thus, in Ref. 5 $O_2^{\cdot}$ generation in the absence of antimycin by the uncoupled mitochondria from several hepatomas was inferred on the basis of adrenaline oxidation assay. However, the authors in Ref. 5 did not report whether the antimycin-independent adrenaline oxidation was sensitive to superoxide dismutase, nor was the action of myxothiazol-type inhibitors on this reaction studied.

As shown in this work, preparations of the mitochondrial membranes from hepatoma Zajdela and to a lesser extent of hepatoma 22a are capable of direct oxidation of Tiron to semiquinone without involvement of $O_2^{\cdot}$ radicals. It is possible that the authors in Ref. 5 had encountered the same artifact, since Tiron and adrenaline are related compounds, i.e., both are substituted catechols and undergo similar redox reactions [52]. According to our observations, no true $O_2^{\cdot}$ generation by mitochondrial membranes from either normal tissues or hepatomas could be induced by succinate in the absence of antimycin or its analogs when measured by the adrenaline method, but significant initial absorbance drifts due to adrenaline oxidation were often registered upon the catechol aerobic incubation with the mitochondrial membranes (data not included).

Unlike Ref. 5, our results show that $O_2^{\cdot}$ generation by the respiratory chain of tumor mitochondria is very similar to the process studied earlier with normal tissue preparations. Thus, in the presence of succinate and oxygen, the effect is induced by the bc_1-site inhibitors such as antimycin, HOQNO and funiculosin which suppress cytochrome *b*-562 oxidation by ubiquinone in the ubiquinone-reducing centre *i* of the Q-cycle, whereas myxothiazol and mucidin known to block QH_2 oxidation to $QH^{\cdot}$ by FeS_{Rieske} [53] are inhibitory to $O_2^{\cdot}$ generation induced by antimycin, and so is cyanide.

The same observations have been made with respect to normal mitochondrial membranes [18] and, as discussed earlier, indicate $O_2^{\cdot}$ generation to originate in autoxidation of the unstable ubisemiquinone in centre *o* of the Q-cycle [15–18,54] (Fig. 5). The identity of the mechanisms of $O_2^{\cdot}$ generation by the normal and tumor mitochondrial respiratory chains is further supported by the very similar redox dependence of the process in the bovine heart and hepatoma submitochondrial particles.

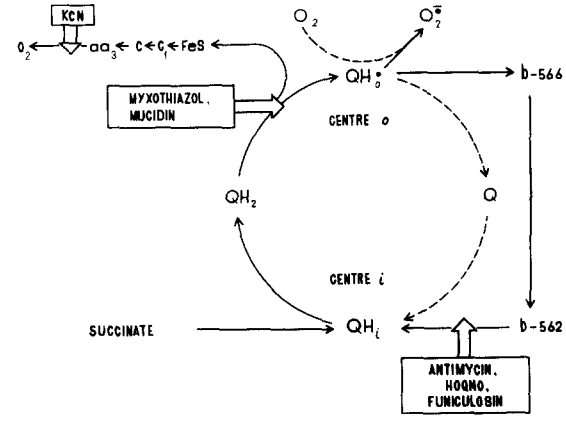

Fig. 5. The mechanism of the succinate-dependent $O_2^{\cdot}$ generation in the respiratory chain of tumor mitochondria. The scheme is based on the Q-cycle mechanism of electron transfer in coupling site 2 [36]. Solid lines trace the flow of reducing equivalents (e^- or H); dashed lines denote migration of the oxidized reactants (Q or O_2). Superoxide generation is assumed to be associated with autoxidation of the unstable ubisemiquinone ($QH_o^{\cdot}$) in QH_2-oxidizing centre *o* of the Q-cycle [15–18]. In the uninhibited state, $QH_o^{\cdot}$ formed upon oxidation of QH_2 by FeS_{Rieske} is rapidly oxidized to Q by heme *b*-566; hence, the steady-state concentration of $QH_o^{\cdot}$ is low and so is the rate of its autoxidation. When cytochrome *b* oxidation by Q via centre *i* is inhibited by antimycin, HOQNO or funiculosin, heme *b*-566 becomes highly reduced in the aerobic steady-state and can no longer serve as an electron acceptor for $QH_o^{\cdot}$. Consequently, autoxidation of the latter is greatly favoured. Inhibition of the QH_2 oxidation to $QH_o^{\cdot}$ would suppress $O_2^{\cdot}$ generation. The former reaction is directly inhibited by myxothiazol and mucidin and indirectly by KCN (in the presence of the latter FeS_{Rieske} becomes reduced and the rate of QH_2 oxidation in centre *o* decreases accordingly).

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