

# Superoxide Dismutase and Superoxide Radical in Morris Hepatomas<sup>1</sup>

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## ABSTRACT

Total superoxide dismutase (SOD) and manganese superoxide dismutase (Mn SOD) specific activities were measured in tissue homogenates and in isolated mitochondria from normal rat liver and three Morris hepatomas of different growth rates. Total SOD and Mn SOD specific activities were decreased in all tumor homogenates when compared to normal liver; the lowest activity was associated with the fastest growing tumor. These results are consistent with the hypothesis that total Mn SOD specific activity is decreased in all tumors.

The Mn SOD specific activity was similar to the total SOD specific activity of isolated mitochondria, indicating that mitochondrial SOD is almost entirely manganese containing. This activity was decreased in the fast- and medium-growth-rate hepatomas but was slightly increased in the tumor with the slowest growth rate when compared to liver. The normal or higher than normal mitochondrial Mn SOD specific activity indicates that decreased mitochondrial SOD specific activity is not a characteristic of all tumors.

Superoxide radical ( $O_2^{\cdot-}$ ) formation was measured in submitochondrial particles obtained by sonication of isolated mitochondria and subsequent washings to remove the SOD. The difficulty encountered in reducing the SOD activity suggests that at least part of the mitochondrial SOD might be associated with the mitochondrial membrane. In liver submitochondrial particles,  $O_2^{\cdot-}$  was formed only when succinate and antimycin A were used together, as substrate and inhibitor of the electron transport chain, respectively. In the hepatomas studied for  $O_2^{\cdot-}$  production (slow- and fast-growth rates), the formation of the radical was detected in the presence of succinate even when no inhibitor was present. Antimycin A stimulated the production of  $O_2^{\cdot-}$  in normal rat liver and slow-growth-rate tumor, but not in fast-growth-rate tumor submitochondrial particles. Reduced nicotinamide adenine dinucleotide did not lead to the production of  $O_2^{\cdot-}$  by normal liver or hepatoma submitochondrial particles.

Mitochondrial membrane damage was seen in micrographs of the medium- and fast-growing hepatomas. This could be a consequence of low mitochondrial SOD concomitant with a flux of superoxide, if the radical is produced *in vivo* by these mitochondria.

## INTRODUCTION

A few years ago, mitochondria were first recognized as an effective source of  $H_2O_2$ ; because production is maximal in the reduced states of the components of the respiratory chain,

$H_2O_2$  formation is thought to be associated with the respiratory chain or at equilibrium with it (3).

When malate-glutamate is used as the source of reducing equivalents,  $H_2O_2$  production is inhibited by rotenone; the rate of production of  $H_2O_2$  is maximal in the presence of antimycin A. From these 2 observations, it has been concluded that at least one of the members of the electron transport chain between the rotenone- and the antimycin A-sensitive sites reacts in its reduced form with molecular oxygen to produce  $H_2O_2$  (3).

SMP<sup>4</sup> are also effective sources of  $H_2O_2$  and at least under certain conditions,  $O_2^{\cdot-}$  is a precursor of  $H_2O_2$  (12). It has been found that the presence of SOD prevents the detection of  $O_2^{\cdot-}$  and stimulates the production of  $H_2O_2$ . In tumor mitochondria which contain little or no SOD, superoxide radicals but no  $H_2O_2$  are formed (6).

Superoxide is both an oxidant and a reductant and hence can modify a variety of biologically important molecules (8). Even though production of  $O_2^{\cdot-}$  represents only a small fraction of the total oxygen uptake (3), its potential physiological importance cannot be disregarded, since  $O_2^{\cdot-}$  and its reaction products are extremely reactive species (8). Peroxidative damage has been implicated as one of the principal causes of age-related damage to cells; peroxidative degradation of membrane lipids has been found to parallel steady-state concentrations of oxygen free radicals (19). The exposure of isolated mitochondria to an  $O_2^{\cdot-}$ -generating system caused changes in oxidative phosphorylations identical to changes observed in aging animals (18).

SOD catalyzes the dismutation of the superoxide radical into  $H_2O_2$  (8). Three distinct types of SOD's have been described. They all catalyze the same reaction and do so with comparable efficiency (16). Eukaryotes contain both a copper-zinc- and a manganese-containing enzyme.

Biological protection against  $O_2^{\cdot-}$  is afforded by the SOD's which often have a highly localized distribution (8). Specifically, several lines of evidence support the correlation between Mn SOD and oxygen toxicity. Mitochondrial SOD (manganese containing) has been suggested to have a protective function by preserving the molecular integrity of the oxygen-metabolizing cells against the by-products of this system (31). Stevens and Autor (31) arrived at this conclusion after experiments which showed that, in oxygen-treated tissue, the increased SOD enzymatic activity could be accounted for solely by an increase in the Mn SOD. Mn SOD is also induced in one species of fruit fly when it develops into an adult insect (7). In view of the low respiration of eggs, larva, and pupa, as compared with the extremely high rate of oxygen consumption in the active adult

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<sup>4</sup> The abbreviations used are: SMP, submitochondrial particles; SOD, superoxide dismutase (EC 1.15.1.1); Mn SOD, manganese superoxide dismutase; CuZn SOD, copper- and zinc-containing superoxide dismutase; HEPES, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; NBT, nitro blue tetrazolium; DETAPAC, diethylenetriaminepentaacetic acid.

that can give rise to a related increase in the superoxide concentrations, the increased protection afforded by a higher activity of mitochondrial SOD is easily understood.

All tumor cells measured to date have lowered amounts of Mn SOD specific activity (21); the assay is not sensitive enough to detect the enzyme in many fast-growing tumors that have been studied. Regenerating liver tissue displays an activity of Mn SOD similar to that in nondividing liver (20). In contrast, CuZn SOD specific activity has usually, but not always, been found to be reduced in tumors (21).

In view of the above facts, we thought that it would be interesting to know more about the role of Mn SOD in tumors and the possible correlation with tumor growth rate. Inasmuch as Mn SOD is easily induced, we also wanted to know if the low activity found in tumor mitochondria could be accounted for by the absence of its substrate, the superoxide radical. In addition, we sought a correlation between Mn SOD specific activity, superoxide formation, and mitochondrial membrane damage.

## MATERIALS AND METHODS

### Animals and Tumors

Buffalo rats from Simonsen Laboratories, Gilroy, Calif., were used in the study and were maintained on food and water *ad libitum*. The tumors were implanted s.c. in both hind legs in the laboratory of Dr. H. P. Morris, Howard University, Washington, D. C. Slow-growing 9618A, medium-growing 5123D, and fast-growing 7288ctc Morris hepatomas were used in these experiments. Rats bearing these tumors were sacrificed at 179, 46, and 25 days, respectively, from the time of implantation of the tumors. At this time, the tumors were usually 2 to 3 cm in diameter and relatively free of hemorrhagic and necrotic areas.

### Isolation of Mitochondria

Tumor mitochondria were isolated by the method of Sordahl *et al.* (28). The isolation medium contained 0.25 M sucrose, 1 mM Tris-HCl, 0.1 mM EDTA, and 1% bovine serum albumin. A pool of 22 tumors obtained from 11 rats was used to isolate mitochondria from each hepatoma line. Electron microscopy was performed in the last mitochondrial pellet to check the purity of the preparation and the ultrastructure of the mitochondria.

Liver mitochondria were isolated by the same procedure as tumor mitochondria in a medium that contained 0.25 M sucrose, 1 mM Tris-HCl, 1 mM EDTA, and no bovine serum albumin. Normal rat liver mitochondria were isolated from a pool of 4 livers from each age group (2, 2.5, 4.5, and 6.5 months old). Isolated mitochondria were used to perform the enzymatic assay for SOD activity and to obtain SMP. A small aliquot was fixed in 3% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.2) for electron microscopy.

### Preparation of SMP

The suspended mitochondria were sonicated at maximum output of a Biosonic IV sonicator (Brownhill), in 15-sec bursts for a total of 3 min, while on ice. The SMP thus obtained were subsequently centrifuged at 48,000  $\times g$  for 50 min in a refrigerated Sorvall RC-5B centrifuge. The pellet was resuspended in 50 mM HEPES-0.25 M sucrose buffer, pH 7.5. The

centrifugation and resuspension was repeated twice to remove the SOD that could interfere with the  $O_2^{\cdot -}$  assay.

### Tissue Preparation for SOD Assay

**Homogenates.** The rats were killed by cervical dislocation when the tumors were approximately 2 cm in diameter. Four tumor-bearing animals were used from each hepatoma line. The average of 4 livers taken from rats of ages 2, 2.5, 4.5, and 6.5 months was used as control. The common bile duct was carefully removed, and all tissues were washed thoroughly in phosphate buffer to remove contaminating blood. All tissues were homogenized and assayed separately. Homogenization was performed in 4 volumes of 50 mM potassium phosphate buffer (pH 7.8) by a motor-driven Teflon-pestle homogenizer, followed by sonication at maximum output of the Biosonic IV; the homogenates were sonicated in 1-min bursts for a total of 5 min, on ice. Protein concentration was measured by the method of Lowry *et al.* (13); these sonicated crude homogenates were stored frozen until the assay was performed.

**Isolated Mitochondria.** SOD determinations on the isolated mitochondria were performed after freezing and thawing once, followed by 4  $\times$  15-sec sonication on ice, while the protein concentration was 10 mg/ml.

### SOD Assay

Enzymatic assay for SOD specific activity was performed using the xanthine-xanthine oxidase assay (2). NBT was used as a detector of  $O_2^{\cdot -}$ ; the NBT reductase activity of tumor tissue was not detectable at the protein concentrations used for the assay. The initial rate of reduction of NBT is diminished in the presence of SOD because the enzyme scavenges the superoxide radical. One unit of the enzyme is defined as the amount of enzyme that decreases this initial rate to 50% of its maximal value for the particular tissue being assayed. The assay mixture contained 50 mM potassium phosphate buffer (pH 7.8), 1 mM DETAPAC, 1 unit of catalase,  $5.6 \times 10^{-5}$  M NBT,  $10^{-4}$  M xanthine, and  $10^{-3}$  unit of xanthine oxidase. The Mn SOD was assayed in the presence of 5 mM cyanide; the tissue was incubated at room temperature for 30 min in the cyanide mixture to ensure complete inhibition of the CuZn SOD (21). The reduction of NBT was followed at 560 nm in a Cary recording spectrophotometer; the rate of increase in absorbance was 0.02/min in the absence of SOD. All data are expressed as units of SOD per mg of protein. Tissues were checked for xanthine oxidase-inhibitory activity by following the production of uric acid at 290 nm. Xanthine oxidase in the presence of either tumor tissue or normal tissue produced this product at the same rate.

### Determination of Superoxide Radicals

The inhibitory effect of SOD on  $O_2^{\cdot -}$ -induced oxidation of epinephrine to adrenochrome (17) was used to demonstrate the mitochondrial generation of this oxygen species. The formation of adrenochrome was followed at 480 nm in a Cary spectrophotometer; the absorption coefficient applied was  $4.02 \text{ mM}^{-1} \text{ cm}^{-1}$  (9). SOD was obtained from Diagnostic Data, Inc., Mountainview, Calif. It is 98% pure CuZn SOD isolated from bovine liver and has a specific activity of 3300 units/mg protein, as assayed by the cytochrome *c* method.

The standard assay consists of 300  $\mu\text{l}$  containing 5 mg SMP

per ml suspended in HEPES-sucrose buffer, added to 2.7 ml of HEPES-sucrose buffer that contained DETAPAC and epinephrine. The concentrations used were 50 mM HEPES (pH 7.5), 0.25 M sucrose, 1 mM DETAPAC, 1 mM epinephrine, and 0.5 mg protein per ml. As substrates, 3 mM succinate or 13  $\mu$ M NADH was used. As inhibitors, 2.5  $\mu$ g antimycin A per ml or 2  $\mu$ M rotenone was used. All preparations were carried out on ice; of the 3 ml prepared as above, 1 ml was incubated at 37° for 15 min, 1 ml was kept on ice to be used as blank, and the last ml received 50  $\mu$ l (4  $\mu$ M final concentration) of SOD and was also incubated. After incubation, all tubes were centrifuged at 48,000  $\times$  g for 5 min, and the change in absorbance was measured in the supernatant. To subtract any superoxide-independent oxidation of epinephrine, the change observed in the presence of SOD was subtracted from the change observed in its absence. In the case of succinate plus antimycin A, the ratio of superoxide dependent to total formation of adrenochrome was about 0.9. This indicates that most of the adrenochrome formation was due to superoxide. For some of the preparations that produced superoxide at very low rates, this ratio might be lower (about 0.5), but this was hard to verify because of the statistical fluctuations at this low rate of superoxide production.

## RESULTS

**SOD Activity in Whole Homogenates.** Total SOD and Mn SOD specific activities were decreased in all hepatomas studied (Table 1); this diminution is correlated with the growth rate of the tumors, with the lower activities being found in the fastest growing hepatomas. The percentage of Mn SOD specific activity to total SOD specific activity was found to be around 25% in the normal rat liver and in the slow-growing hepatoma 9618A, while in the medium- and fast-growing hepatomas this percentage was less than 15%.

The significance of tumor-specific reduction of enzyme activity alterations was evaluated according to Student's *t* test and is shown in Table 1. Because there were large differences in the standard deviation among groups and because no statistical tests are valid unless it is assumed that the standard deviation is the same for all groups, a pooled standard deviation, equal for all groups, was calculated. The standard error of the difference between groups was then calculated, using this pooled standard deviation.

**SOD Activity in Isolated Mitochondria.** Total SOD and Mn SOD specific activities measured in isolated mitochondria from normal liver did not differ significantly, demonstrating that mitochondria contain (Table 2) almost entirely Mn SOD. This had been shown before to be the case (27). Mn SOD specific activity was significantly increased in the slow-growing Morris hepatoma 9618A, but this increase might be due to differences in the purity of the mitochondrial preparations from liver and hepatoma 9618A; that this is the case can be deduced by comparing the ratio of Mn SOD to total SOD in the isolated mitochondria. This ratio is higher in the slow-growing hepatoma than in the normal liver, indicating a mitochondrial preparation free of contamination. This was corroborated by electron micrographs taken from these preparations. In general, there is good agreement between the Mn SOD to total SOD ratio in the isolated mitochondria, and the purity of the preparation as estimated from the electron micrographs; in the 5123D tumor,

Table 1  
SOD specific activity in whole sonicated homogenates

The specific activity of total SOD and Mn SOD in all hepatomas is significantly different from control liver activities with  $p < 0.01$  according to 2-tailed Student's *t* tests with 10 d.f. The CuZn SOD activities were obtained by subtraction of Mn SOD from total SOD; the errors were calculated using propagation of error theory.

	Units/mg protein			
	Total SOD	Mn SOD	CuZn SOD	Mn SOD/ total SOD
Liver	200 $\pm$ 18 <sup>a</sup> (4) <sup>b</sup>	52 $\pm$ 9 (4)	148 $\pm$ 20	0.26 $\pm$ 0.10
9618A <sup>c</sup>	116 $\pm$ 11 (4)	31 $\pm$ 3 (4)	85 $\pm$ 11	0.27 $\pm$ 0.07
5123D	60 $\pm$ 12 (3)	6.0 $\pm$ 0.5 (3)	56 $\pm$ 12	0.10 $\pm$ 0.07
7288ctc	40 $\pm$ 2 (3)	5.5 $\pm$ 0.9 (3)	34 $\pm$ 2	0.14 $\pm$ 0.06

<sup>a</sup> Mean  $\pm$  S.E.

<sup>b</sup> Numbers in parentheses, number of determinations.

<sup>c</sup> 9618A, slow-growing Morris hepatoma; 5123D, medium-growth-rate Morris hepatoma; and 7288ctc, fast-growth rate Morris hepatoma.

Table 2  
Total and Mn SOD specific activity in isolated mitochondria

Errors represent S.E. in the control and S.D. in the hepatomas. Different parameters were used to estimate errors because, in the control liver, mitochondria were isolated from 4 rats from each age group and then SOD was measured. Thus, a population of rats was involved and S.E. should be used. For the case of isolated liver from the hepatomas, mitochondria from several tumors were pooled and SOD was measured several times from the same pool. In this case, there is no population and S.D. should be used. Level of significance of the differences between the control liver and the hepatomas is shown in parentheses. In the case of normal liver, there were 4 age groups. SOD activity was measured 2 or 3 times in each age group, so that a total of 10 determinations were made.

	Units/mg protein			No. of determi- nations
	Total SOD	Mn SOD		
Control	105 $\pm$ 19	87 $\pm$ 16		10
9618A	122 $\pm$ 15 (no difference)	118 $\pm$ 19 ( $p < 0.05$ )		4
5123D	61 $\pm$ 27 ( $p < 0.05$ )	22 $\pm$ 10 ( $p < 0.01$ )		3
7288ctc	19 $\pm$ 18 ( $p < 0.01$ )	13 $\pm$ 13 ( $p < 0.01$ )		4

this ratio is low, indicating contamination by cytoplasmic CuZn SOD or a loss of Mn SOD from broken mitochondria.

The specific activity of SOD in isolated mitochondria is also a function of the growth rate of the tumor, similarly to what was seen in the whole homogenate. In this case, however, the SOD level of the slow-growth hepatoma overlaps with the level found in liver.

**Ultrastructure.** Electron microscopy of freshly prepared mitochondrial suspensions from normal liver revealed preparations with intact outer membranes and abundant cristae (Fig. 1a); they were estimated to be between 80 and 90% pure; this is in agreement with the ratio of Mn SOD to total SOD in this preparation. The mitochondria from the slow-growth hepatoma 9618A are also well preserved, and judging from the micrograph, they are close to 90% pure (Fig. 1b).

Mitochondrial damage was the most striking characteristic noted in the mitochondria from hepatoma 5123D (Fig. 1c). We do not know if these mitochondria are inherently damaged, or if this is a result of our isolation procedures. We had previously experimented with isolation procedures for mitochondria from mouse H6 hepatoma and discovered that we always see damaged mitochondria, no matter what the isolation procedure. Mitochondrial membrane damage has already been reported for mitochondria from medium- and fast-growing Morris hepatomas (24). The micrograph taken from this tumor shows very abnormal mitochondria with broken membranes. The mito-

chondria from the fast-growing tumor are also less well preserved than the ones from the slow-growing one (Fig. 1d); judging from the picture, 40 to 50% purity was assigned to this preparation and a value of 72% was obtained from the mean of the enzymatic assays performed (Table 2). This value is highly variable, so we might be overestimating the amount of Mn SOD as compared to the total; another possible explanation is that the vesicles seen in the micrograph might be derived from the mitochondria and still contain the matrix enzymes. In any case, the values obtained from the enzymatic assay had errors sufficiently large to include the values as judged from the electron micrographs.

**O<sub>2</sub><sup>-</sup> Production.** For the comparison of electron transfer rates in different spans of the respiratory chain, NADH and succinate, which provide electrons that enter the respiratory chain at different sites, were used as substrates. Rotenone, which blocks the electron transport chain before coenzyme Q, and antimycin A, which blocks between the cytochromes *b* and *c*, were used as inhibitors. Both had previously been shown to stimulate superoxide production in H6 hepatomas (22). The experiments described were performed with mitochondria at least partially freed from SOD by ultrasonic treatment and subsequent high-speed centrifugation (Table 3). Because of the high SOD activity obtained with Morris hepatoma 5123D, superoxide production was not measured in this tumor. The difficulty of removing the SOD from the broken mitochondria suggests that Mn SOD may be associated to some degree with the mitochondrial membrane.

A 2-way analysis of variance was used to test the overall difference among groups (liver, slow-growth tumor, fast-growth tumor), and no statistical difference was found in the rates of production of O<sub>2</sub><sup>-</sup> between the groups. A Duncan test was performed to check the difference of each treatment from each group against every other treatment from all groups. The level of significance used was 0.05. To test whether the values were different from zero (*i.e.*, whether O<sub>2</sub><sup>-</sup> was truly being produced), a random set of numbers was taken from all previous determinations the values of which were such that, when the standard deviation was subtracted from the mean, the value would be negative. This provided us with a group called "zero," with a random standard deviation the magnitude of which was similar to the standard deviation found in the assay. Each treatment in this group was assigned with 3 random values, and the Duncan test was performed.

Only succinate and not NADH supported the formation of superoxide radicals (Table 4). The same amount of NADH had previously been shown to produce superoxide in H6 hepatoma (22); obviously, higher concentrations of NADH might cause the production of O<sub>2</sub><sup>-</sup> in the present system. Using the above-described statistical test to see if superoxide was being produced, it was found that antimycin A was necessary for the detection of superoxide radicals with the SMP obtained from the normal liver, contrary to the results obtained with the slow- and fast-growth tumor in which O<sub>2</sub><sup>-</sup> was detected even in the absence of the inhibitor. However, antimycin A stimulated the production of O<sub>2</sub><sup>-</sup> in normal liver and the slow-growth tumor but not in the fast-growing one. All preparations produced O<sub>2</sub><sup>-</sup> in the presence of succinate plus antimycin A.

The fast-growing tumor produces O<sub>2</sub><sup>-</sup> at a rate only one-third of the rate found with the control liver SMP or the slow-growing tumor; this might be due, at least in part, to the

Table 3  
SOD specific activity of washed SMP

	Total SOD activity (units/mg)
Normal liver	5.0
Slow-growth tumor 9618A	6.0
Medium-growth tumor 5123D	60.0
Fast-growth tumor 7288ctc	<2.0

Table 4  
Rate of formation of O<sub>2</sub><sup>-</sup> by SMP

Treatment	nmol of O <sub>2</sub> <sup>-</sup> / min/mg SMP protein		
	Liver	9618A hepatoma	7288ctc hepatoma
Succinate + antimycin A	1.31 ± 0.36 <sup>a</sup> (4) <sup>b</sup>	1.57 ± 1.52 (5)	0.53 ± 0.39 (6)
Succinate + rotenone	-0.01 ± 0.21 (4)	0.22 ± 0.39 (3)	0.59 ± 0.25 (6)
Succinate	0.37 ± 0.44 (4)	0.55 ± 0.38 (3)	0.33 ± 0.27 (7)
No substrate, no inhibitor	0.25 ± 0.35 (4)	0.0 ± 0.0 (3)	0.18 ± 0.20 (4)

<sup>a</sup> Mean ± S.D.

<sup>b</sup> Numbers in parentheses, number of determinations performed from each pool of tissue SMP.

impurities of these preparations, because the rates are calculated per mg of submitochondrial protein.

## DISCUSSION

Three independent studies on the subcellular distribution of SOD in rat liver have indicated that the manganese enzyme accounts for 7 to 8% of the total activity present and that it is apparently located exclusively in the matrix of the mitochondria (23, 25, 33). Two of these reports (25, 33) demonstrate that 15% of the SOD is sedimentable, and that one half of this activity is found in the matrix of the mitochondria, while the other half is found in the intermembrane space. Both studies also agree in that the matrix enzyme contains manganese; the other report (23) shows that 18% of the total SOD is cyanide sensitive; it also says that 13% of the total activity is found in the mitochondria and that this fraction is almost entirely manganese containing. Tyler (33) concludes that his results do not exclude the possibility of small amounts of dismutase located in other organelles besides mitochondria. On the other hand, there is one report (34) of a study in which no Mn SOD was found in liver extracts. Our results indicate that the Mn SOD can account for 26% of the total SOD specific activity of normal rat liver (Table 1). This is probably because we are assaying the whole homogenate; *i.e.*, we are not discarding any fraction.

Specific Mn SOD activity of the homogenate is decreased in the slow-growing hepatoma, but mitochondrial Mn SOD specific activity is not decreased. This can signify one of 3 things: reduced number of mitochondria; presence of an inhibitor; or decreased extramitochondrial Mn SOD.

The reduction in the number of mitochondria in tumors appears to be a function of the growth rate of the tumor (1, 10) and a medium- and a slow-growth tumor have been shown to have normal levels of cytochrome oxidase, a mitochondrial marker (11). Moreover, the number of mitochondria in the particular slow-growth-rate hepatoma we have studied (9618A) has been shown to be normal (10). For these reasons, an explanation of our data based on the reduced mitochondrial number in the slow-growing hepatoma is unlikely.

We assayed specifically for the presence of an inhibitor of Mn SOD in the tumor homogenate by adding slow-growth tumor homogenate to isolated mitochondria from normal liver and 9618A hepatoma; we could not detect decreased SOD activity, ruling out the possibility of a repressor to explain our data.

We can roughly estimate the amount of mitochondrial Mn SOD specific activity that we should measure if all Mn SOD were localized in the mitochondria from the activity found in the whole homogenate of normal liver ( $52 \pm 9$  units/mg) and the ratio of mitochondrial protein to total protein (0.30 to 0.33). This is around 160 to 170 units/mg, but in fact in the isolated mitochondria from normal liver we have measured only about 50% of that amount ( $87 \pm 16$  units/mg), indicating that in normal rat liver only 50% of the Mn SOD is found in the mitochondria. However, there are other possibilities; the sensitivity of our enzymatic assay for SOD could vary, depending on the fraction being assayed. Still another possibility would be that we are overestimating the amount of Mn SOD in the total homogenate, due to incomplete inhibition of CuZn SOD in the presence of 5 mM cyanide. This has been discussed before (21) and does not seem to be the case.

If Mn SOD is decreased in the slow-growing hepatoma homogenate but not in the mitochondrial fraction, as our data show, and if there is extramitochondrial Mn SOD as our estimates show for the normal rat liver, then, in the 9618A hepatoma the extramitochondrial fraction of Mn SOD must be reduced. We could not detect Mn SOD activity in the supernatant of a  $17,000 \times g$  centrifugation after homogenization; this excludes the possibility of soluble Mn SOD. This indicates that in the slow-growing tumor Mn SOD has to be reduced in some cellular structure other than the mitochondria. We measured the SOD activity in the pellet of a  $17,000 \times g$  centrifugation after homogenization to test this possibility (this pellet contains the mitochondria); it was found that, in the normal liver, 75% of the total SOD (222 units/mg) was Mn SOD (166 units/mg) in this fraction; while in the slow-growing hepatoma this percentage was 45% (250 units of total SOD per mg protein and 112 units of Mn SOD per mg protein); this is a finding very hard to explain if we assume that there is only intramitochondrial Mn SOD and that both tissues have similar amounts of Mn SOD in their mitochondria. This conclusion is reinforced by the fact that, if we assume that the ratio of mitochondrial protein to total protein is 0.30 to 0.33, then in all the hepatomas the amount of total Mn SOD can be accounted for by that found in the mitochondria. This is in contrast to the result found in normal liver.

We can tentatively conclude then: (a) that in rat liver there is extramitochondrial Mn SOD, contrary to what had previously been reported (15); (b) that in the slow-growing tumor 9618A, this fraction is smaller than in normal liver; and (c) that the extramitochondrial Mn SOD is not found in the cytoplasm.

McCord *et al.* (15) have mentioned that in normal human liver Mn SOD is also found in the nucleus, and the possibility arises that it is in this fraction that Mn SOD is typically reduced even in the "minimal-deviation" Morris hepatomas. A very recent report points toward confirmation of these results (14). The carp RBC do not contain mitochondria but, contrary to RBC from other species, they do contain nuclei and they were found to contain Mn SOD. Measurements of Mn SOD activity in isolated nuclei should be performed to corroborate this

possibility. To summarize, we can say that the mitochondria matrix contains almost exclusively Mn SOD, but Mn SOD is apparently not exclusively a mitochondrial enzyme. If the deductions from our work are true, namely, that there is an extramitochondrial form of the Mn SOD and that this form is lower or absent even in the slower growing tumors, this could be exploited as a therapeutic tool (21, 22). Tumor cells, being lower in Mn SOD, would be more susceptible than normal cells to fluxes of superoxide radical.

Previous work in our laboratory had demonstrated the presence of Mn SOD in regenerating liver (20) and in fetal tissue in mice,<sup>5</sup> although vastly lowered amounts of Mn SOD were found in fetal liver. The latter observation may indicate that diminished Mn SOD is not an exclusive characteristic of tumors, although more data in this area are needed. The present study gives more credibility to the hypothesis that all tumor cells have lowered amounts of Mn SOD when compared to their differentiated normal cell counterparts. Even the slow-growing, differentiated Morris hepatomas have lower Mn SOD than does normal liver tissue. This is an impressive finding, because many other parameters have been thought to be characteristic of tumors, but when a slow-growing Morris hepatoma was measured this parameter was equal to that of normal liver and thus not characteristic of tumors. We also found a strong correlation between the specific activities of both CuZn and Mn SOD and growth rate (and degree of differentiation) of the tumors. Thus, the levels of SOD seemed to have some connection with the degree of malignancy in the Morris hepatomas. It should be noted that in our earlier studies we found no Mn SOD activity in transplantable tumors (21). In the present study, we do find Mn SOD activity in transplanted rat tumors, but it is very much diminished compared to homologous normal tissue. Thus, all tumors examined to date have lowered Mn SOD activity, but it is not always zero.

Our results confirm the hypothesis that particles from normal rat liver mitochondria do produce superoxide radicals at the level of the second phosphorylation site, as already known for mitochondria of other species (6, 26, 29). A reduced form of the ubiquinone appears to be responsible for the reduction of  $O_2$  to  $O_2^{\cdot-}$  (5). In our system, NADH does not support the formation of superoxide radical, but in a similar system (3) NADH seems to support  $H_2O_2$  formation; this would indicate that in this system  $O_2^{\cdot-}$  does not seem to be an intermediate in the formation of  $H_2O_2$  when NADH is used as substrate; this is not unique, since other pathways have been described that produce  $H_2O_2$  without the participation of  $O_2^{\cdot-}$  as an intermediate (4). However, NADH may be ineffective simply because the concentration used was too low ( $13 \mu M$ ). Higher concentrations of NADH might cause the production of measurable superoxide. Both  $H_2O_2$  and  $O_2^{\cdot-}$  may be formed in our system, but the assay for  $H_2O_2$  could be more sensitive than that for  $O_2^{\cdot-}$ , making the  $O_2^{\cdot-}$  undetectable while the  $H_2O_2$  is detected.

As demonstrated with this work,  $O_2^{\cdot-}$  can be produced in normal and tumor mitochondria when some components of the electron transport chain are fully reduced (SMP supplemented with succinate and antimycin A). All tumor mitochondria were capable of producing  $O_2^{\cdot-}$ , although superoxide production was low as compared to results obtained with SMP prepared from a mouse liver tumor H6 hepatoma (data not shown). SMP

<sup>5</sup> L. W. Oberley, unpublished observations.

of this tumor produced  $O_2^-$  at a rate of 7 nmol/mg/min in the presence of succinate and antimycin A; in the presence of NADH and antimycin A, the rate was half-maximal.

There is an important question related to the extent to which the mitochondrial generation of  $H_2O_2$  and  $O_2^-$  occurs under physiological conditions. It is possible to estimate that mitochondrial production of  $H_2O_2$  might be close to 2% of the total liver oxygen uptake (3). However, direct measurements in support of that possibility are still lacking. Mitochondria seem to constitute the main intracellular locus of origin of  $O_2^-$ ; they would produce as much as 24 nm  $O_2^-$  /min/g of rat liver, under the conditions used by Boveris (3), a rate that would account for about 75% of the total cellular production of  $O_2^-$  (3). Mitochondrial formation of  $O_2^-$  and  $H_2O_2$  are proportional to oxygen tension. Oxygen intermediates and hydroperoxides may constitute part of a chemical system able to sense oxygen tension in the tissue (3).

As mentioned before, mitochondrial SOD is manganese containing and Mn SOD is an inducible enzyme. Assuming that the substrate of this enzyme, superoxide radical, is responsible for the induction of the enzyme, we have demonstrated that the reason for the low SOD activity in most tumor mitochondria is not the lack of ability to produce  $O_2^-$ ; this is because the slow- and fast-growing tumor mitochondria were able to produce  $O_2^-$  at rates comparable to normal liver. However, we have not demonstrated that the radical is produced *in vivo*, but only that SMP prepared from isolated mitochondria have the ability to form  $O_2^-$  when supplemented with adequate substrates.

The highly damaged mitochondria of the fast- and medium-growing tumors could be explained by a decreased activity of mitochondrial Mn SOD and a normal flux of  $O_2^-$ . Membrane damage can result by exposure to  $O_2^-$  itself or to the products of its spontaneous dismutation, such as hydroxyl radical. Slow-growing Morris hepatomas have normal levels of Mn SOD and thus have normal mitochondria. The opposite argument, that mitochondrial membrane damage could result in loss of the mitochondrial enzyme, does not seem to be the case because no Mn SOD was detected in the cytoplasmic fraction of the medium-growing tumor, where highly damaged membranes were detected. However, it is still possible that mitochondrial membrane damage may lead to conformational changes and, hence, inactivation of this protein. This would be a reasonable possibility if we would know that the mitochondrial enzyme is associated with the mitochondrial membrane; however, this is still unknown at this point, although the difficulty we had to remove the SOD activity from mitochondria points in this direction. We do not at present know if tumor mitochondria are damaged *in vivo* or are simply damaged during isolation. If the latter is true, it would indicate an increased susceptibility to damage in tumor mitochondria. Both points of view have recently been presented. Stocco and Hutson (32) have shown that tumor mitochondria are more fragile than liver mitochondria and can be easily damaged during isolation. On the other hand, Springer (30) has characterized mitochondria from intact tissue of 16 human epithelial lines. Mitochondrial pleomorphism was expressed slightly by normal lines, to variable degrees by lines derived from nonmalignant tissues of cancerous organs, and to a much greater extent by all lines derived from malignant tissues. Hypertrophied mitochondria and longitudinal cristall arrangement were found in almost all the malignant lines, but not in any lines derived from nonmalignant tissues of cancerous

organs or from normal tissue. These data suggest that mitochondria from tumors are damaged before isolation. In any case, a difference between normal cell and tumor cell mitochondria is suggested. The question naturally arises as to the consequences of this difference. One possibility is that abnormal mitochondria may lead to the high glycolysis of tumors. This theory could explain why slow-growing Morris hepatomas show normal glycolytic rates, since they have normal levels of mitochondrial Mn SOD and apparently normal mitochondria.

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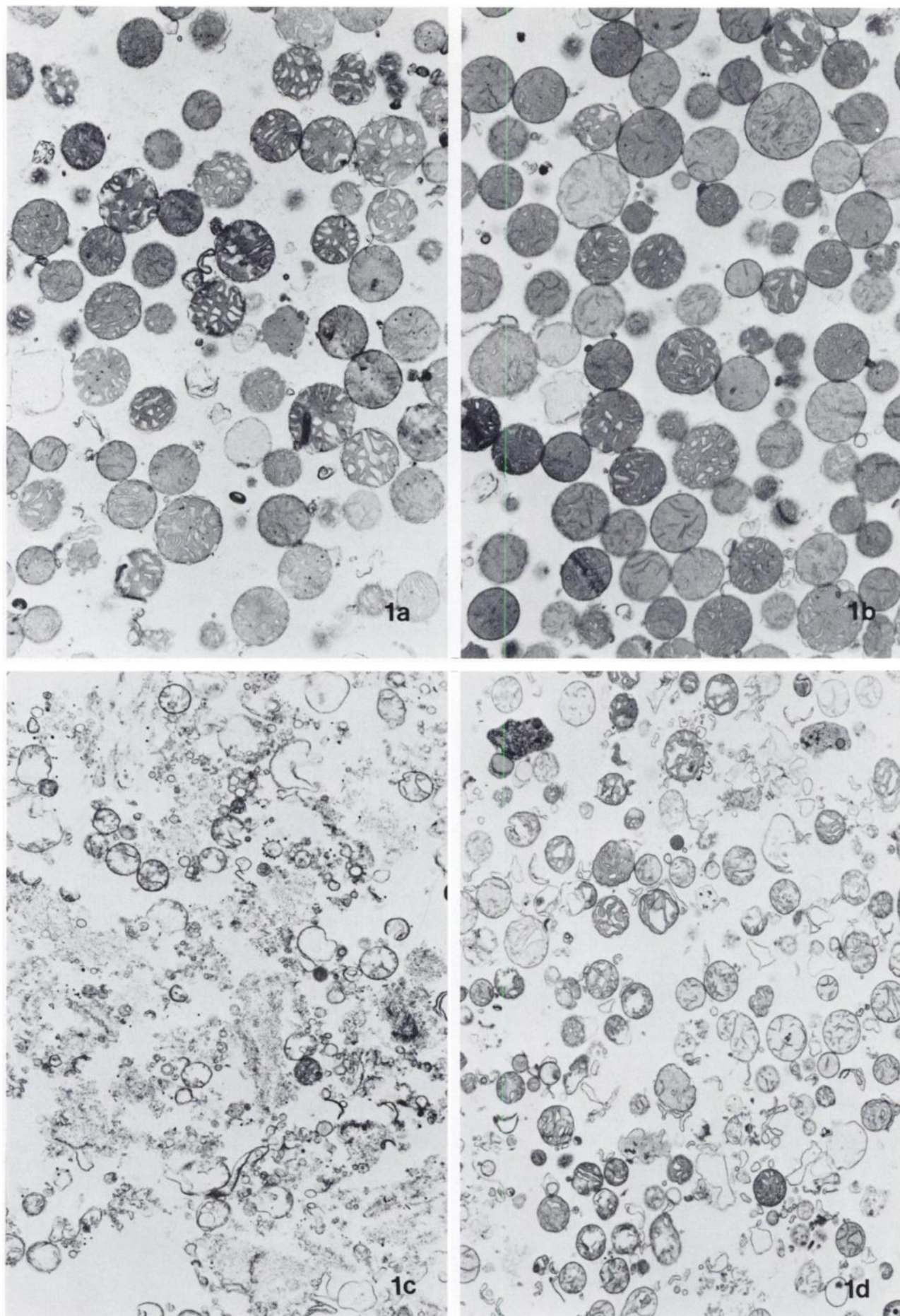
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**Fig. 1.** Electron micrographs of mitochondria isolated from normal and tumor liver tissues. *a*, isolated mitochondria from normal rat liver; *b*, isolated mitochondria from slow-growth-rate Morris hepatoma 9618A; *c*, isolated mitochondria from medium-growth-rate Morris hepatoma 5123D; *d*, isolated mitochondria from fast-growth-rate Morris hepatoma 7288ctc ( $\times 20,000$ ).