Subcellular Localization of Superoxide Dismutase in Rat Liver

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The subcellular localization of superoxide dismutase was investigated in rat liver homogenates. Most of the superoxide dismutase activity is present in the soluble fraction (84%), the rest being associated with mitochondria. No indication for the occurrence of superoxide dismutase in other subcellular structures, particularly in peroxisomes, was found. Mitochondrial activity is not due to adsorption, since the sedimentable activity is essentially latent. Subfractionation of mitochondria by hypo-osmotic shock and sonication shows that half of the mitochondrial superoxide dismutase activity is localized in the intermembrane space, the rest of the enzyme being a component of the matrix space. In non-ionic media the matrix enzyme is, however, adsorbed to the inner membrane, from which it can be desorbed by low (0.04M) concentration of KCl. Superoxide dismutase activity was found in all rat organs investigated. Maximal activity of the enzyme is observed in liver, adrenals and kidney. In adrenals, the highest specific activity is associated with the medulla.

Superoxide dismutase is thought to play an important role in protecting cells against the toxic effect of the $O_2^{\cdot -}$ radical (McCord *et al.*, 1971; Gregory & Fridovich, 1973) and has been well characterized in a large variety of prokaryotic and eukaryotic cells. Studies on the subcellular localization in liver have been published by Weisiger & Fridovich (1973a,b) and by Rotilio *et al.* (1973). The occurrence of superoxide dismutase in the cytosol was established by the two groups of workers. The observations on the association of the liver enzyme to subcellular particles are, however, contradictory; the presence of superoxide dismutase in mitochondria was claimed by Weisiger & Fridovich (1973a,b), whereas the enzyme was found exclusively in the soluble fraction by Rotilio *et al.* (1973). Further, examination of the experimental evidence for the occurrence of superoxide dismutase in mitochondria shows that an association of the enzyme to particles such as lysosomes or peroxisomes has never been excluded. For the latter type of particles, this point deserves careful consideration; these particles are involved in H_2O_2 metabolism, and a preliminary report on the occurrence of superoxide dismutase in peroxisomes has been published (Tyler, 1973). The possibility of a peroxisomal localization places also some doubts on the intramitochondrial localization claimed by Weisiger & Fridovich (1973b), since the behaviour of peroxisomes in the fractionation system used by these authors is unknown.

The present study was undertaken to investigate the possible association of superoxide dismutase

with liver peroxisomes and also to provide an analysis of the distribution of the enzyme on a quantitative basis, by using fractionation techniques allowing clear discrimination between mitochondria, lysosomes and peroxisomes. A survey of the activity of superoxide dismutase in various organs from rat is also included. The findings reported here have been published in an abstract form (Peeters-Joris *et al.*, 1973).

Experimental

Fractionation of homogenates by differential centrifugation

The procedure described by de Duve *et al.* (1955) was followed for preparation of homogenates and for isolation of fractions by differential centrifugation, except that heavy and light mitochondrial fractions were recovered in a single fraction. Four fractions were thus isolated from the homogenate: a nuclear fraction (N), a large-granule fraction (ML), a microsomal fraction (P) and a final supernatant (S).

Analysis of the large-granule fraction

The automatic rotor designed by Beaufay (1966) was used for density equilibrium. It was loaded with 10ml of fraction ML, 32ml of a linear sucrose gradient and 6ml of a concentrated sucrose solution (density 1.32) as cushion. The procedure described by Leighton *et al.* (1968) was followed for centrifugation. Results are presented as histograms constructed as described by de Duve (1967).

Two systems were used to establish the submitochondrial localization of superoxide dismutase. In some experiments, mitochondria were swollen by the method of Parsons *et al.* (1966). The washed pellet from fraction ML was resuspended in 20mM-potassium phosphate buffer, pH 7.2, which contained 0.2g of bovine serum albumin/litre, and it was adjusted to a volume equivalent to seven times the amount of liver from which particles were isolated. This preparation was then analysed by density equilibration or was used for determination of soluble activity.

In other experiments, a large-granule fraction in 0.25M-sucrose was diluted fivefold with water, or in some cases with a solution of bovine serum albumin (20g/litre). The preparation was centrifuged at 3000000g-min (rotor 40, Spinco centrifuge), yielding a supernatant (IS) which contained the constituents of the intermembrane space. The pellet was resuspended in water, sonicated for 15s at 90W in a Branson Sonifier (type D-12, equipped with a microtip), and centrifuged at 3000000g-min. The supernatant (MA) contained the soluble matrix protein, whereas inner and outer mitochondrial membranes were recovered in the pellet (MB).

Enzyme determinations

Superoxide dismutase was measured by photochemical reduction of riboflavin as $O_2^{\cdot-}$ -generating system and inhibition of Nitro Blue Tetrazolium reduction to assay the enzyme (Beauchamp & Fridovich, 1971). The incubation medium contained in a final volume of 3 ml: 50mM-potassium phosphate buffer, pH 7.8; 45mM-methionine; 5.3 μ M-riboflavin; 84 μ M-Nitro Blue Tetrazolium (Sigma Chemical Co., St. Louis, Mo., U.S.A.); 20 μ M-KCN; 0.5g of bovine serum albumin/litre. The amount of superoxide dismutase added to this medium was kept below 1 unit of enzyme to ensure sufficient accuracy.

Tubes were placed in an aluminium-foil-lined box, maintained at 25°C and equipped with two 15W fluorescent lamps. To provide homogeneous illumination, it was found necessary to place tubes on a slowly rotating (0.5 rev./min) holder. Reduced Nitro Blue Tetrazolium was measured spectrophotometrically at 600nm after 10min exposure to light. Maximum reduction was evaluated in the absence of enzyme. Each sample was also incubated with an excess (40 units/ml) of bovine erythrocyte superoxide dismutase, purified as described by McCord & Fridovich (1969). This allowed a correction for Nitro Blue Tetrazolium reduction brought by reactions not involving the $O_2^{\cdot-}$ radical.

Enzyme activities were calculated from the inhibition of reduction by using a standard curve constructed by varying the amounts of homogenate. One unit of enzyme activity is defined as the amount of enzyme giving a 50% inhibition of the reduction of

Nitro Blue Tetrazolium (Beauchamp & Fridovich, 1971).

Superoxide dismutase displays latency in fraction ML. Partial inhibition of the particulate enzyme was observed in the presence of detergents such as digitonin or Triton X-100. Maximum activity was obtained by sonication of the preparations, before assay, in a Branson Sonifier (type D-12, equipped with a microtip), at 90W for 120s, in periods of 30s, with pauses to allow for dissipation of any heat produced.

Cytochrome oxidase was measured as described by Beaufay *et al.* (1974), except that 0.9g of Tween-80/litre (Société Générale de Produits Chimiques, Brussels, Belgium) and 0.3g of Triton X-100/litre (Rohm and Haas, Philadelphia, Pa., U.S.A.) were included in the reaction mixture. Units are defined as described by Cooperstein & Lazarow (1951).

For the determination of glutamate dehydrogenase, a modification of the procedure described by Leighton *et al.* (1968) was followed. Advantage was taken of the finding of Frieden (1959) that ADP promotes the association of the enzyme subunits necessary for maximum catalytic activity. The following procedure was adopted: 0.2ml of 2g of Triton X-100/litre was first mixed with 0.2ml of particle suspension to solubilize the enzyme; 1.4ml of a mixture containing 28.6mM-glycylglycine buffer, pH 7.7, 0.57mM-NaCl, 1.47mM-EDTA, 1mM-ADP and 2mM-NAD⁺ was then added. After a preincubation of 5min at 0°C, the samples were heated to 25°C and the reaction was started by addition of 0.2ml of 0.8M-sodium glutamate. The extinction at 340nm was recorded during a 5min period. A sixfold increase in enzyme activity over that reported by Leighton *et al.* (1968) was obtained by this method. One unit of enzyme activity reduces 1 μ mol of NAD⁺/min.

The method for the determination of adenylate kinase is a slight modification of the technique described by Schnaitman & Greenawalt (1968). The reaction mixture contained 20mM-glucose, 67mM-MgCl₂, 93mM-glycylglycine buffer, pH 8, 1mM-NADP⁺, 4mM-ADP, 0.6mM-KCN, 9 units of hexokinase/ml and 0.2 unit of glucose 6-phosphate dehydrogenase/ml. These purified enzymes (analytical grade) were purchased from Boehringer, Darmstadt, Germany. The mixture was kept at 0°C for about 2h, to exhaust contaminating ATP, and the reaction was started by the addition of 0.1ml of suitably diluted enzyme to 1.5ml of the mixture. As mentioned by Schnaitman & Greenawalt (1968) the enzyme is very labile at high dilution; hence the preparation was diluted with a solution containing bovine serum albumin at 16g/litre. This was found to be essential to avoid substantial inactivation. The extinction at 340nm was recorded during 5min. Enzyme activity is expressed as μ mol of ATP formed/min.

Table 1. *Fractionation of liver homogenate by differential centrifugation*

Absolute values are in mg/g of liver for protein, in units/g of liver for enzymes. They are given as the sum of the activities in the nuclear fraction and in the cytoplasmic extract. Distributions are expressed as percentages of the sum of recovered activities in all fractions. E, Cytoplasmic extract; N, nuclear fraction; ML, large-granule fraction; P, microsomal fraction; S, supernatant. Recoveries give the sum of activities in the fractions compared with the value for fractions E+N, as a percentage. Values are the means $\pm$ S.D. for three experiments.

Fractions	Absolute value (mg/g or units/g)	Distribution (%)				Recovery (%)
		N	ML	P	S	
Protein	190 $\pm$ 4.0	10.6 $\pm$ 4.1	29.4 $\pm$ 5.1	21.2 $\pm$ 2.1	38.8 $\pm$ 7.8	98.2 $\pm$ 5.2
Superoxide dismutase (total)	4178 $\pm$ 403	2.4 $\pm$ 0.9	12.6 $\pm$ 4.6	0.7 $\pm$ 0.5	84.3 $\pm$ 6.0	108.5 $\pm$ 1.3
Superoxide dismutase (sedimentable)	656 $\pm$ 63	15.3 $\pm$ 5.7	80.2 $\pm$ 29.3	4.5 $\pm$ 3.2	—	—
Cytochrome oxidase	16.9 $\pm$ 6.1	11.5 $\pm$ 7.4	79.4 $\pm$ 11.0	8.3 $\pm$ 5.5	0.8 $\pm$ 1.0	88.8 $\pm$ 20.4
Acid phosphatase	5.86 $\pm$ 0.43	9.1 $\pm$ 1.7	68.3 $\pm$ 3.7	12.2 $\pm$ 2.9	10.4 $\pm$ 2.0	97.8 $\pm$ 6.8
Catalase	44.2 $\pm$ 4.2	8.2 $\pm$ 1.8	59.0 $\pm$ 11.6	2.8 $\pm$ 2.5	30.0 $\pm$ 11.0	91.9 $\pm$ 3.7
Glucose 6-phosphatase	21.6 $\pm$ 3.2	15.9 $\pm$ 7.9	17.0 $\pm$ 2.9	64.7 $\pm$ 9.8	2.4 $\pm$ 0.5	87.7 $\pm$ 13.1

Fig. 1. *Isopycnic centrifugation of a large-granule fraction from Triton WR-1339-treated rats*

Density-frequency-distribution histograms of enzymes and protein after density equilibration in a sucrose gradient: (a) superoxide dismutase; (b) cytochrome oxidase; (c) protein; (d) acid phosphatase; (e) catalase. A large-granule fraction, containing the particles isolated from 5g of liver, was layered over a sucrose gradient, extending linearly from 1.15 to 1.27 in density. A cushion of density 1.32 was placed below the gradient. Centrifugation was performed at 35000 rev./min for 35 min. The percentage of activity in fraction ML and recovery with respect to the initial homogenate were respectively 13.3 and 93.3 for superoxide dismutase, 67.0 and 104.6 for cytochrome oxidase, 26.7 and 96.9 for protein, 45.8 and 104.3 for acid phosphatase, 71.6 and 100.2 for catalase.

Monoamine oxidase was measured as described by Baudhuin *et al.* (1964); glucose 6-phosphatase and acid phosphatase as described by de Duve *et al.* (1955), except that the incubation was performed over 20 h for the latter enzyme. For these three enzymes, one unit of enzyme activity yields 1 μ mol of the product of reaction/min, under the conditions described by the above authors.

Catalase was measured as described by Baudhuin *et al.* (1964). The unit of activity is the amount of enzyme that decreases the decadic logarithm of the H_2O_2 concentration by 1 unit/min, in a reaction volume of 50 ml.

Protein was measured by the method of Lowry *et al.* (1951), automated as described by Beaufay

et al. (1974). Bovine serum albumin was used as standard.

Results

Fractionation of liver homogenates

The distribution of superoxide dismutase in the fractions isolated from rat liver homogenates is presented in Table 1, together with similar data for marker enzymes. Most of the superoxide dismutase activity is recovered in the final supernatant, as observed by others in rat liver (Rotilio *et al.*, 1973) and in chicken liver (Weisiger & Fridovich, 1973a). However, the data show a bimodal distribution of the enzyme, with a second peak in the large-granule

Fig. 2. Isopycnic centrifugation of a large-granule fraction, after swelling of mitochondria

Density-frequency-distribution histograms of enzymes and protein after density equilibration in a sucrose gradient: (a) cytochrome oxidase; (b) glutamate dehydrogenase; (c) superoxide dismutase; (d) adenylate kinase; (e) monoamine oxidase; (f) protein. The medium of Parsons *et al.* (1966) was used for swelling mitochondria. The preparation, containing the particles isolated from 1.4 g of liver, was layered over a sucrose gradient extending linearly from 1.05 to 1.25 in density. A cushion of density 1.32 was placed below the gradient. Centrifugation was performed at 35000 rev./min for 35 min. The percentage of activity in fraction ML and recovery with respect to the initial homogenate were respectively 68.7 and 90.0 for cytochrome oxidase, 86.8 and 100.4 for glutamate dehydrogenase, 12.2 and 91.4 for superoxide dismutase, 45.1 and 83.4 for adenylate kinase, 63.4 and 104.8 for monoamine oxidase, 29.2 and 94.9 for protein.

fraction. This is clear when the distribution of sedimentable enzyme is calculated. It is, however, not possible with this type of experiment to know if the particulate enzyme is associated with mitochondria, lysosomes or peroxisomes, since those particles are all concentrated in the large-granule fraction.

Fractionation of the large-granule fraction

The intracellular localization of the superoxide dismutase found in the large-granule fraction was further studied by isopycnic centrifugation. To separate lysosomes from mitochondria and peroxisomes, 170 mg of Triton WR-1339 (Rohm and Haas) was injected intravenously to the rat, 3 days before death, as described by Wattiaux *et al.* (1963). Density distributions are presented in Fig. 1.

It is clear that superoxide dismutase has a distribution similar to the mitochondrial marker, cytochrome oxidase. Adsorption of soluble enzyme to mitochondria is unlikely. Indeed, it is hardly compatible with the latency of mitochondrial superoxide dismutase. When the enzyme from a large-granule fraction is measured in an iso-osmotic incubation medium (0.25 M-sucrose) to preserve the integrity of particles, it is found that the activity represents between 10 and 20% of the activity observed after disruption of granules by sonication. Further, we have verified that addition of 0.3 M-KCl to the suspension medium of a large-granule fraction does not solubilize mitochondrial superoxide dismutase.

The density distribution of superoxide dismutase does not show any evidence of a peroxisomal localization. It can be calculated from the data of Fig. 1 that, for the two fractions corresponding to the peak of the distribution of catalase, the latter enzyme is concentrated 13-fold with respect to superoxide dismutase. Taking into account the amount of these two enzymes present in the large-granule fraction and the contamination of the peroxisomal peak by mitochondria, it can be inferred that if any superoxide dismutase is associated to peroxisomes it represents less than 1% of the enzyme activity of the homogenate.

Localization of superoxide dismutase in mitochondria

Density distributions observed after swelling the mitochondria of a large-granule fraction in the medium of Parsons *et al.* (1966) are presented in Fig. 2. The distribution of adenylate kinase, which is localized in the intermembrane space, shows that the external mitochondrial membrane was disrupted. Under the conditions used for density equilibration (35 min at 35000 rev./min), soluble protein does not sediment appreciably; it is clear that adenylate kinase remains essentially in the top fractions of the gradient, corresponding to the sample zone before

centrifugation. Further, the distributions of cytochrome oxidase and glutamate dehydrogenase show that the inner membrane remained fairly intact: the two enzymes, used respectively as markers for the inner membrane and the soluble matrix protein, have similar distributions. Most outer mitochondrial membranes remain associated with the inner-membrane-matrix complexes, since the amount of monoamine oxidase found around a density of 1.11, where freed outer mitochondrial membranes equilibrate, is small.

The distribution pattern of superoxide dismutase in Fig. 2 is bimodal, half of the enzyme remaining in the upper fractions, like adenylate kinase, whereas the rest equilibrates at a density of 1.19, like cytochrome oxidase. In view of these results, two interpretations can be proposed: either we are dealing here with a double localization of the enzyme in mitochondria, or the distribution observed for superoxide dismutase can be explained by a secondary adsorption of soluble

Fig. 3. Solubilization of mitochondrial superoxide dismutase after disruption of the outer mitochondrial membrane and influence of KCl in the suspension medium

After swelling of mitochondria in the medium of Parsons *et al.* (1966), 0.3 M-KCl was added to a portion of the particle suspension. The two preparations, with and without KCl, were centrifuged at 300000g-min (rotor 40, Spinco centrifuge) and the activity was measured in the supernatant. Soluble activity is expressed as a percentage of the activity in the large-granule fraction.

Fig. 4. Localization of superoxide dismutase in submitochondrial fractions

(a) Superoxide dismutase; (b) adenylate kinase; (c) glutamate dehydrogenase; (d) cytochrome oxidase; (e) monoamine oxidase. Mitochondria from a large-granule fraction were fractionated, as described in the Experimental section, to separate the intermembrane space (IS) from soluble matrix (MA) and inner and outer membranes (MB). Relative specific activity (% enzyme activity/% protein) is expressed with respect to the initial large-granule fraction. Results are the average of two experiments.

intermembrane enzyme to mitochondrial outer or inner membranes. The second possibility deserves careful consideration, since it was observed with nucleases present within the intermembrane space of mitochondria (Baudhuin *et al.*, 1970).

To distinguish between these two possibilities, KCl was added to the suspension medium, after swelling of mitochondria. Fig. 3 shows that no desorption of superoxide dismutase occurred in the presence of 0.3M-KCl. Similar results were obtained after swelling mitochondria in 0.05M-sucrose. Further, latency of superoxide dismutase was still observed under conditions where complete solubilization of adenylate kinase was achieved. When the mitochondrial outer membrane is ruptured, free activity of superoxide dismutase is only 52% of the maximum activity.

At this point, it may be concluded that superoxide dismutase is associated partly with the intermembrane space and partly to either the matrix or the inner mitochondrial membrane. To localize the enzyme more precisely, mitochondria were subfractionated as described in the Experimental section to separate the constituents of the intermembrane space (IS), those of the soluble matrix protein (MA) and inner and outer membranes (MB). As shown in Fig. 4, half of the mitochondrial superoxide dismutase is found associated with the marker for the intermembrane space (adenylate kinase) and the rest remains in the fractions containing the markers for inner and outer membranes (cytochrome oxidase and monoamine oxidase). Adsorption is again a possibility, since the inner mitochondrial membrane was intact in the desorption attempts reported in Fig. 3. To verify this point, the

Fig. 5. Solubilization of residual mitochondrial superoxide dismutase, after elimination of intermembrane-space components

A large-granule fraction in 0.05M-sucrose was centrifuged at 3000000g-min (rotor 40, Spinco centrifuge) to eliminate intermembrane protein. The pellet was resuspended in water and sonicated. After addition of KCl in various amounts, the suspension was centrifuged again at 3000000g-min. Soluble activity is expressed as a percentage of the activity found in the sonicated preparation. ●, Glutamate dehydrogenase; ■, superoxide dismutase; ▲, cytochrome oxidase.

experiment reported in Fig. 4 was repeated, but KCl was added in various amounts to the particle suspension immediately after sonication, before the last centrifugation. In Fig. 5, the proportion of soluble activity, i.e. the distribution of superoxide dismutase between fractions MA and MB, is given as a function of KCl concentration. The distribution of the enzyme is changed completely by KCl; most of the enzyme is found soluble in KCl at a concentration of 0.04M or more. In view of the low concentration of KCl necessary to solubilize the enzyme, this component of the mitochondrial superoxide dismutase activity can be considered as associated with the matrix.

A double localization of mitochondrial superoxide dismutase is thus demonstrated by our results. This observation agrees with the results of Weisiger & Fridovich (1973b) on chicken liver. The enzyme activity is divided roughly equally between the matrix and the intermembrane space. We have also confirmed the observation of Weisiger & Fridovich (1973b) that the matrix enzyme is not sensitive to KCN, whereas both the cytosol enzyme and the intermembrane enzyme are inhibited by 1 mM-KCN (Fig. 6).

Fig. 6. Effect of cyanide on superoxide dismutase

Superoxide dismutase activities are expressed with respect to the preparations in the presence of 0.01 mM-KCN. At this concentration, KCN does not significantly affect the enzyme activities. ■, High-speed supernatant of the homogenate; ●, fraction IS; ▲, combined fractions MA and MB.

Superoxide dismutase activity in other tissues

A survey of the superoxide dismutase activity in various rat organs is presented in Table 2. Liver was found to be the richest in superoxide dismutase. Except for adipose tissue, the enzyme was found in all organs investigated, the adrenals showing a specific activity nearly equal to that of liver. This may be related to a possible role of superoxide radicals in auto-oxidation of adrenaline (Misra & Fridovich, 1972). In view of this observation, medulla and cortex were separated from bovine adrenals. Both on a wet-weight basis and on a protein basis, a threefold higher activity was found in the medulla. For four experiments, average activities $\pm$ s.d. were 3100 ± 402 units/g for the medulla and 1095 ± 297 units/g for the cortex; corresponding values for the specific activities were 28 ± 3.1 and 10 ± 3.4 units/mg of protein.

Discussion

The dual localization of superoxide dismutase in the soluble fraction and in mitochondria is clearly demonstrated by the combination of differential and isopycnic centrifugation. Our findings are in good agreement with the observations of Weisiger & Fridovich (1973a,b) on chicken liver, but the intracellular localization found here is in contradiction with the results published by Rotilio *et al.* (1973)

Table 2. *Superoxide dismutase activity in various organs of the rat*

In pancreas homogenates, superoxide dismutase activity is rapidly inactivated, even when the preparation is kept at 0°C. The value given was obtained in a single experiment by measuring the activity within 30 min of homogenization. Statistics refer to averages $\pm$ s.d., for three experiments.

Organ	Activity (units/g)	Specific activity (units/mg of protein)
Liver	4480 $\pm$ 1165	22.1 $\pm$ 6.2
Adrenal	1563 $\pm$ 1053	19.7 $\pm$ 5.0
Kidney	1342 $\pm$ 383	12.9 $\pm$ 3.0
Blood	785 $\pm$ 317	3.6 $\pm$ 1.0
Spleen	441 $\pm$ 95	4.7 $\pm$ 1.1
Heart	421 $\pm$ 109	8.6 $\pm$ 2.0
Pancreas	300	1.5
Brain	262 $\pm$ 68	2.8 $\pm$ 0.6
Lung	200 $\pm$ 55	3.1 $\pm$ 1.0
Stomach	140 $\pm$ 30	6.5 $\pm$ 2.2
Intestine	122 $\pm$ 43	2.5 $\pm$ 1.5
Ovary	112 $\pm$ 45	2.0 $\pm$ 0.8
Thymus	88 $\pm$ 42	1.3 $\pm$ 0.5
Fat	0	0

on rat liver. The latter authors give few details of their experimental procedure, but it appears that their assay system for superoxide dismutase did not take into account the mitochondrial enzyme. In our opinion, the latency of the mitochondrial enzyme is most probably responsible for this discrepancy. Unfortunately the composition of the reaction medium for superoxide dismutase assay is not given by Rotilio *et al.* (1973).

With respect to latency of superoxide dismutase, no data comparable with ours exist in the literature. However, in aerobic flagellates, where the enzyme is associated with hydrogenosomes, Lindmark & Müller (1974) have shown that superoxide dismutase is also latent. For particulate superoxide dismutase, latency should be most likely interpreted as a restriction to the penetration of $O_2^{\cdot-}$ -generating system or of Nitro Blue Tetrazolium, rather than as a slow penetration of the $O_2^{\cdot-}$ radical through the mitochondrial membrane.

At variance with the observation of Tyler (1973), no significant amount of superoxide dismutase could be detected in peroxisomes. It is worthwhile mentioning here that in spinach leaves Asada *et al.* (1973) were also able to separate, by gradient centrifugation, particulate catalase from superoxide dismutase in large-granule fractions. Further, as pointed out by these authors, in this material an association with mitochondria, in addition to the localization in chloroplasts, is not excluded by their results.

By comparison with cytochrome oxidase, and assuming that all the sedimentable superoxide dismutase is associated with mitochondria, our results

show that in rat liver the mitochondrial enzyme represents 16% of the activity of the homogenate. On average, superoxide dismutase activity of mitochondria was partitioned 56% in the intermembrane space and 44% in the matrix (13 experiments, s.d. $\pm$ 10%); these values correspond respectively to 9 and 7% of the activity found in the homogenate. Our calculation does not account for the 10% inhibition of the soluble and intermembrane enzyme at the KCN concentration used for the assay (see Fig. 6). A correction can be introduced, but is found to be small and within experimental error.

The survey of the activity in various organs confirms the wide distribution of the enzyme, liver displaying the highest specific activity of superoxide dismutase. Our value for superoxide dismutase in liver is in good agreement with that of Lindmark & Müller (1974), who measured the activity in rat liver with xanthine oxidase as $O_2^{\cdot-}$ -generating system and cytochrome *c* reduction for detection.

As shown by Weisiger & Fridovich (1973*b*), mitochondrial superoxide dismutases can be clearly differentiated by their sensitivity to cyanide. The cyanide-insensitive matrix enzyme is a manganoprotein, similar to the bacterial superoxide dismutase. Both intermembrane mitochondrial superoxide dismutase and cytosol enzyme have been shown to be sensitive to cyanide, and they are considered by Weisiger & Fridovich (1973*b*) to be a cuproprotein.

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