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Polarographic Assay and Intracellular Distribution of Superoxide Dismutase in Rat Liver

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1. A polarographic assay of superoxide ($O_2^{-\cdot}$) dismutase (EC 1.15.1.1) activity is described, in which the ability of the enzyme to inhibit $O_2^{-\cdot}$-dependent sulphite oxidation, initiated by xanthine oxidase activity, is measured. The assay was used in a study of the intracellular distribution of superoxide dismutase in rat liver. Both cyanide-sensitive cupro-zinc dismutase (92% of the total activity) and cyanide-insensitive mangano-dismutase (8%) were measured. 2. Rat liver homogenates contained both particulate (16%) and soluble (84%) dismutase activity. The particulate activity contained both types of dismutase, whereas nearly all the soluble dismutase was a cupro-zinc enzyme. The distribution pattern of mangano-dismutase was similar to that of cytochrome oxidase and glutamate dehydrogenase, indicating that the enzyme was probably present exclusively in the mitochondria. 3. Superoxide dismutase activity in the heavy-mitochondrial (M) fraction was latent and was activated severalfold and largely solubilized by sonication. Treatment of the M fraction with digitonin or a hypo-osmotic suspending medium indicated that most of the cupro-zinc dismutase was located in the mitochondrial intermembrane space, whereas the mangano-enzyme was located in the inner-membrane and matrix space. 4. A small amount of dismutase activity appeared to be present in the nuclei and microsomal fraction, but little or no activity in the lysosomes or peroxisomes. 5. The results are discussed in relation to the intracellular location of known $O_2^{-\cdot}$-generating enzymes, the possible role of superoxide dismutase activity in intracellular H_2O_2 formation, and to current views on the physiological function of the enzyme.

Since the isolation of the copper protein haemocuprein from bovine erythrocytes (Mann & Keilin, 1938*a*), similar proteins have been isolated from several mammalian tissues, including liver (Mann & Keilin, 1938*b*; Mohamed & Greenberg, 1953), brain (Porter & Folch, 1957) and heart (Keele *et al.*, 1971). For many years these copper proteins were thought to have no apparent enzymic function, until McCord & Fridovich (1969*a*) showed that the erythrocyte protein catalysed the dismutation of superoxide free radical anions ($O_2^{-\cdot}$) according to the following reaction:

$$2O_2^{-\cdot} + 2H^+ \rightarrow O_2 + H_2O_2$$

The copper enzyme also contains zinc (Carrico & Deutsch, 1970) and is now referred to as cupro-zinc superoxide dismutase.

A second type of mammalian superoxide dismutase, containing manganese but devoid of copper or zinc, has been found in chicken liver mitochondria (Weisiger & Fridovich, 1973*a*), mostly present in the matrix space (Weisiger & Fridovich, 1973*b*). The two types of dismutase can be distinguished by the action of cyanide, which inhibits the cupro-zinc enzyme, but has no effect on the mangano-enzyme (Rotilio *et al.*, 1972*a*,*b*; Weisiger & Fridovich, 1973*a*).

Superoxide dismutase activity is now known to be present in a wide variety of mammalian cells and micro-organisms (Beauchamp & Fridovich, 1971; Fridovich, 1974). In view of the presence of superoxide dismutase in all the aerobic cells examined and in aero-tolerant anaerobic bacteria devoid of catalase, McCord *et al.* (1971) proposed that the physiological function of superoxide dismutase is to protect oxygen-metabolizing cells against the potentially harmful effects of superoxide ions formed by the univalent reduction of oxygen during enzymic activity.

The present paper describes a polarographic assay of superoxide dismutase activity and the use of the assay in quantitative measurements of the intracellular distribution of the cupro-zinc and mangano-dismutase enzymes in rat liver. The results indicate that superoxide dismutase activity is present in at least three intracellular compartments, namely the matrix space of mitochondria, the inter-membrane space of mitochondria and the cytosol. In recent years, several enzymes and enzyme systems of mitochondria, the microsomal fraction and the soluble fraction of

mammalian cells have been shown to generate superoxide ions during catalytic activity (Fridovich & Handler, 1961; Massey *et al.*, 1969). It is concluded that the intracellular distribution of dismutase activity in rat liver is well adapted to enable the enzymes to serve as protective agents against the effects of superoxide ion formed in several different cell compartments.

Materials and Methods

Reagents

The following compounds were obtained from Sigma Chemical Co., St. Louis, Mo., U.S.A.: alumina C_γ gel (aged), cytochrome *c* (type III, from horse heart), glucose 6-phosphate, β-glycerophosphate, Triton X-100, xanthine (sodium salt) and xanthine oxidase. Glucose 6-phosphate dehydrogenase, hexokinase, NADH, $NADP^+$, oxaloacetate and triethanolamine hydrochloride were from Boehringer Corp., London W5 2TZ, U.K. Crystallized bovine plasma albumin was obtained from Armour Pharmaceutical Co., Eastbourne, Sussex, U.K. Other reagents were from BDH, Poole, Dorset, U.K. AnalaR-grade reagents were used whenever possible. Aqueous solutions were freshly prepared in glass-distilled water.

Enzyme preparations

Experiments were performed on adult male albino rats. Non-perfused livers from exsanguinated rats were rinsed and blotted six times by using fresh 50ml portions of 0.25M-sucrose containing 5mM-Tris–HCl, pH7.4. Liver (8g) was then fractionated by the method of de Duve *et al.* (1955). In the experiment shown in Fig. 4, the heavy mitochondrial fraction was further fractionated after sonication by the following procedure. The fraction, containing about 50mg of protein/ml, was diluted 1:10 (v/v) with 0.1M-KCl–20mM-Tris–HCl, and treated for 30s in an MSE 100W sonicator tuned to maximum power output. A portion of the sonicated fraction was centrifuged at 6000*g* for 10min to sediment heavy granules (HG fraction). The heavy-granule supernatant was centrifuged at 100000*g* for 60min to yield a sediment of light granules (LG fraction) and a supernatant (S). The sediments were resuspended in 0.25M-sucrose.

Digitonin fractionation

Samples of the heavy mitochondrial fraction, suspended in 0.25M-sucrose containing 5mM-Tris–HCl, pH7.4 ('sucrose–Tris solution') and 0.5mg of bovine plasma albumin/ml, were incubated for 5min with various concentrations of digitonin by the method of Schnaitman & Greenawalt (1968). At the end of the incubation, the samples were centrifuged at 9500*g* for 10min, the supernatants collected, and the sediments were washed once and finally resuspended in sucrose–Tris solution.

In other experiments, the heavy mitochondrial fraction was treated with 12mg of digitonin/100mg of protein and separated into inner-membrane-matrix particles, outer-membrane particles and soluble protein by the differential centrifugation procedure of Schnaitman & Greenawalt (1968). The inner-membrane-matrix preparation was diluted to a protein concentration of 4–7mg of protein/ml and sonicated for 20s in an MSE 100W sonicator tuned to maximum power output. The sonicated preparation was separated into inner-membrane particles and soluble matrix protein by centrifuging at 104000*g* for 60min.

Hypo-osmotic treatment

Samples of the heavy mitochondrial fraction were diluted to a final concentration of 5–7mg of protein/ml in tubes containing a suspension medium consisting of 5mM-potassium phosphate buffer, pH7.4, 0.1mM-EDTA–NaOH, pH7.4, 0.5mg of bovine plasma albumin/ml, and either 0.25M-sucrose or 0.05M-sucrose. After incubation at 1°C for 8min, the tubes were centrifuged at 20000*g* for 20min. After collection of the supernatants of the two samples in separate tubes, the pellets obtained were washed once by resuspension in sucrose–Tris solution and centrifugation at 10000*g* for 10min. The supernatants were combined with the corresponding supernatants obtained from the previous centrifugation and the pellets were resuspended in sucrose–Tris solution.

Bovine erythrocyte superoxide dismutase was purified by the method of McCord & Fridovich (1969*a*). The material obtained after the acetone precipitation step was dissolved in water and dialysed overnight against water. The solution obtained was centrifuged at 5000*g* for 5min, and the supernatant containing the dismutase was further purified by fractional adsorption on alumina C_γ gel, fractional precipitation by acetone, and crystallization by the method of Mann & Keilin (1938*b*). The final product contained 0.34μg of copper/mg of protein and 3000 dismutase units/mg of protein when assayed by the method of McCord & Fridovich (1969*a*), in which one unit of dismutase activity is defined as the amount required to inhibit by 50% the rate of aerobic reduction of added cytochrome *c* during xanthine oxidase activity.

Protein was determined by the biuret method (Gornall *et al.*, 1949) on samples clarified with 0.2% (w/v) sodium cholate. The protein content of cell fractions was measured with samples of the dilute sonicated samples prepared for the dismutase assays (see below). Copper was measured by the biquinoline method of Griffiths & Wharton (1961).

Enzyme assays

Spectrophotometric assays were performed at 20–22°C. Polarographic assays were performed at 25°C by the method of Chappell (1964), a 3.2ml reaction mixture being used. Cytochrome *c* oxidase activity was measured by the method of Appelmans *et al.* (1955), monoamine oxidase activity by the method of Schnaitman *et al.* (1967), adenylate kinase by the method of Schnaitman & Greenawalt (1968); sulphite–cytochrome *c* reductase by the method of Wattiaux-De Coninck & Wattiaux (1971), and xanthine oxidase activity by the spectrophotometric method of Fridovich (1970). Acid phosphatase activity was measured by the method of de Duve *et al.* (1955), by using 20mM-triethanolamine–HCl buffer, pH6.5, instead of glycylglycine buffer. Glutamate dehydrogenase activity was measured at 340nm in a reaction mixture containing 0.05M-potassium phosphate buffer, pH7.4, 0.1mM-EDTA–NaOH, pH7.4, 5mM-α-oxoglutarate, 5mM-NH_4Cl, 0.4mM-KCN and 0.1mM-NADH. 6-Phosphogluconate dehydrogenase activity was measured at 340nm in a reaction mixture containing 0.1M-triethanolamine buffer, pH7.6, 3.6mM-6-phosphogluconate, 3.3mM-$MgCl_2$ and 0.42mM-$NADP^+$.

Catalase activity was assayed by a polarographic method (Rørth & Jensen, 1967). Just before assay, each subcellular fraction was diluted to a protein concentration of 1.5–2.0mg/ml in an ice-cold solution containing 30mM-potassium phosphate buffer, pH6.8, 0.1mM-EDTA–NaOH, pH7.4, bovine plasma albumin (1mg/ml) and 1% (v/v) Triton X-100. Samples of this diluted preparation (10–100μl) were then added to a reaction mixture containing phosphate buffer, EDTA, bovine plasma albumin and 1.7mM-H_2O_2. The slow rate of spontaneous release of oxygen recorded in the H_2O_2 solution before catalase addition ($k = 0$–$0.04\,min^{-1}$) was subtracted from the recorded rates of the enzyme-catalysed reaction. One unit of activity is defined as the amount of enzyme causing the destruction of 90% of the substrate in 1min in a volume of 50ml (Baudhuin *et al.*, 1964).

Assay of superoxide dismutase activity

The direct measurement of superoxide dismutase activity, by the method of pulse radiolysis (Rotilio *et al.*, 1972*a*; Fielden *et al.*, 1974), though applicable to cell fractions, requires specialized apparatus that is not generally available. Other assays of superoxide dismutase activity are indirect and are based on the ability of the enzyme to compete with various compounds for the $O_2^{-\cdot}$ generated either enzymically (xanthine oxidase) or photochemically. For example, superoxide dismutase inhibits the $O_2^{-\cdot}$-dependent reduction of cytochrome *c* or Nitro Blue Tetrazolium by the xanthine oxidase system and the photoreduction of Nitro Blue Tetrazolium (Beauchamp & Fridovich, 1971). These spectrophotometric assays have several disadvantages when applied to tissue fractions. The cytochrome *c* reductase assay cannot be readily applied to fractions containing cytochrome *c* oxidase and the first two assays depend on the measurement of small changes in extinction that are difficult to measure accurately in turbid suspensions. In all three assays, $O_2^{-\cdot}$-dependent and $O_2^{-\cdot}$-independent reductase activities are present to a variable extent. These disadvantages are avoided in an alternative assay developed in the present work, which uses the inhibition of $O_2^{-\cdot}$-dependent oxidation of sulphite ions (McCord & Fridovich, 1969*b*) as a measure of dismutase activity. Sulphite oxidation was measured polarographically in a reaction mixture containing 50mM-potassium phosphate buffer, pH7.4, 0.1mM-EDTA–NaOH, pH7.4, 50μM-xanthine, dismutase preparation (when present) and 16mM-Na_2SO_3 (dissolved in potassium phosphate–EDTA). The reaction was started by the addition of xanthine oxidase. A sample of stock xanthine oxidase preparation (usually 2–5μl) was added to catalyse oxygen uptake with a velocity constant $k = 0.5\,min^{-1}$. One unit of dismutase activity is defined as the amount of enzyme causing a decrease of 50% in the reaction velocity in a volume of 100ml. Just before assay, subcellular fractions were diluted tenfold in ice-cold 5mM-potassium phosphate buffer, pH7.4, and sonicated for 30s. Portions of dilute sonicated samples (up to 0.2ml) were assayed for dismutase activity. Similar samples had no effect on xanthine oxidase activity. Thus the observed inhibition of sulphite oxidation by the samples could not be attributed to an inhibition of the $O_2^{-\cdot}$-generating system. As described in the Results section, a small correction was made in the calculation of dismutase activity, to account for the inhibition of sulphite oxidation by sucrose present in the tissue fractions. The inhibitory effect of sucrose may be explained by the observation that several sugars act as scavengers for radicals produced during the aerobic oxidation of sulphite (Fuller & Crist, 1941; Asada & Kiso, 1973). The effect of sucrose alone was measured with a sample prepared from 0.25M-sucrose by a dilution and sonication procedure similar to that used with the tissue fractions. The combined rate of oxygen uptake observed in the absence of sulphite, owing to xanthine oxidase activity, and in the absence of xanthine, owing to liver sulphite oxidase activity, was measured and subtracted from the rate of sulphite oxidation observed in the presence of xanthine oxidase activity. The combined rate was 3–8% of the $O_2^{-\cdot}$-dependent sulphite-oxidation rate.

In some experiments, the effects of dialysis on the dismutase activity of the liver fractions were investigated. Dilute sonicated samples of the fractions were dialysed for 15h against 400vol. of 5mM-potassium phosphate buffer, pH7.4. Dialysis had no significant effect on the dismutase activity of the particulate

fractions, but removed a cyanide-insensitive inhibition of sulphite oxidation present in the soluble fraction, possibly due to the removal of simple endogenous compounds (e.g. ascorbate, cysteine) that inhibit the aerobic chain oxidation of sulphite (D. D. Tyler, unpublished work). For this reason, the results presented in Table 1 summarize the dismutase activity of non-dialysed particulate fractions and dialysed soluble fraction.

The amount of cupro-zinc dismutase activity in the liver fractions was estimated by measuring the effect of cyanide on their total dismutase activity (see the Results section).

Blood content of subcellular fractions

Superoxide dismutase is present in rat erythrocytes, which may contribute significant amounts to the enzyme activities present in liver homogenates (Castagna, 1965). The contribution of blood dismutase and catalase to the activity of liver fractions was therefore investigated. The average blood content of whole liver from four rats used in the fractionation experiments, measured by the method of Hohorst *et al.* (1959), was 0.044ml/g wet wt. The mean values of the dismutase and catalase activities of whole blood collected from the same animals were 27.8 and 18.4 units/ml respectively. These values represent about 0.9% of the total dismutase activity and 0.7% of the total catalase activity present in the liver samples used in the fractionation experiments, indicating that the contribution of blood enzymes to the total activity present in liver is very small. This conclusion is supported by results of experiments with livers fractionated after perfusion with 0.9% NaCl. The total dismutase and catalase activities of non-perfused and perfused livers were found to be similar. Non-perfused livers were preferred in fractionation experiments because they yielded subcellular fractions with less cross-contamination than did NaCl-perfused livers. The dismutase and catalase activities of the nuclear (N) and the nuclear+cytoplasmic (N+E) fractions (Table 1) were corrected for the amounts of the blood enzymes estimated to be present according to the blood content of the fractions.

Results

Assay of superoxide dismutase

The kinetics of sulphite oxidation initiated by the xanthine oxidase system were first order with respect to oxygen concentration. A reciprocal plot of sulphite-oxidation rate against the amount of liver fraction or erythrocyte dismutase preparation added to the system indicated that the inhibition of sulphite oxidation by dismutase was strictly competitive (Fig. 1). Similar plots have been presented by Misra & Fridovich (1972) showing that erythrocyte dismutase causes a competitive inhibition of the autoxidation of adrenaline.

The amount of erythrocyte dismutase containing one unit of activity in 100ml of reaction mixture,

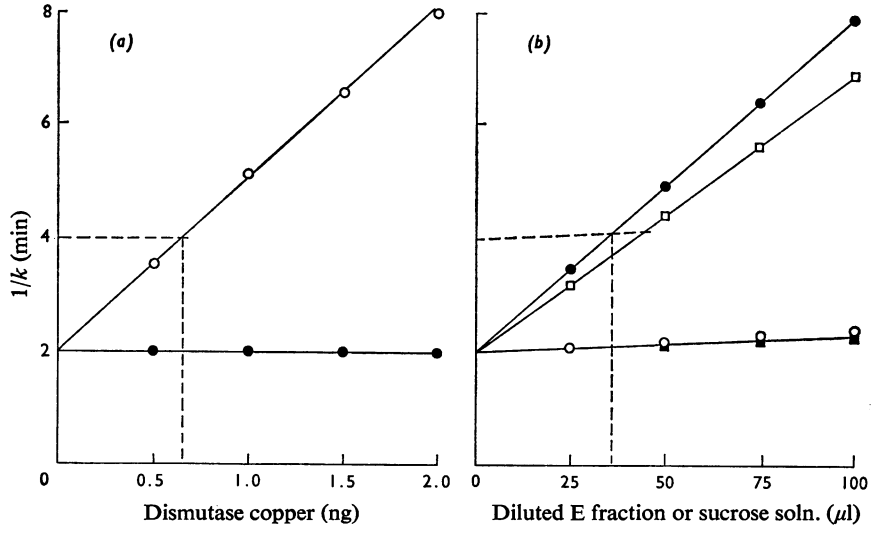

Fig. 1. *Assay of superoxide dismutase activity*

Samples were prepared for assay as described in the Materials and Methods section. (*a*), Activity of crystalline erythrocyte dismutase: ○, native enzyme; ●, enzyme boiled for 10min before assay. (*b*) Activity of E fraction: ●, E fraction; □, E fraction, preincubated at 60°C for 3min before assay; ○, E fraction, boiled for 10min before assay; ■, 25mM-sucrose.

based on the sulphite-oxidation assay, was found to contain about 17 units of activity according to the cytochrome *c* assay described by McCord & Fridovich (1969*a*). When non-dialysed liver fractions were assayed for dismutase activity, a small but significant inhibition of sulphite oxidation was caused by concentrations of sucrose similar to those added with the liver sample (Fig. 1*b*). The inhibitory effects of dismutase and sucrose on sulphite oxidation were additive. The amount of true dismutase activity present in each liver fraction was therefore obtained graphically by drawing a dashed line, from the ordinate value corresponding to 50% inhibition of the sulphite-oxidation rate, parallel to the line representing inhibition by sucrose alone. In the example shown in Fig. 1(*b*), one unit of dismutase activity in 3.2ml is given by the volume of dilute sonicated E fraction corresponding to the intercept of the dashed line and the line obtained from assay of the E fraction owing to dismutase plus sucrose inhibition. The abscissa value of the intercept, 36μl, shows that 3.6μl of E fraction contains one unit of activity in 3.2ml after the volume of the E fraction added has been corrected for the tenfold dilution of the assay sample. To measure the activity of dismutase preparations devoid of sucrose, the dashed line was drawn parallel to the abscissa (Fig. 1*a*). Fig. 1(*b*) also shows that the dismutase activity of the E fraction was largely resistant to brief incubation at 60°C, but was inactivated at 100°C. In this respect, the liver dismutase activity shows a heat-sensitivity similar to that found by McCord & Fridovich (1969*b*) with the erythrocyte enzyme, indicating that the observed inhibition of sulphite oxidation by the liver fraction is due to the presence of a protein catalyst.

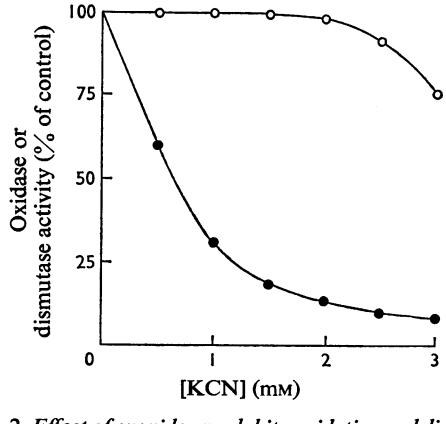

Fig. 2. *Effect of cyanide on sulphite oxidation and dismutase activity*

○, Sulphite oxidation initiated by xanthine oxidase activity with no dismutase present; ●, superoxide dismutase activity in the sulphite oxidation assay. The samples contained crystalline erythrocyte dismutase (4ng of copper).

Effect of cyanide on superoxide dismutase activity

Cyanide is a potent inhibitor of the cupro-zinc enzyme at pH10.5, but is less effective at lower pH values (Rotilio *et al.*, 1972*a*,*b*). Fig. 2 shows the effect of cyanide concentration on sulphite oxidation in the absence and presence of erythrocyte dismutase, at neutral pH values. The dismutase activity was inhibited by 86% by 2mM-cyanide. Similar results were obtained when the cytochrome *c* assay of McCord & Fridovich (1969*a*) was used instead of the polarographic assay. Higher cyanide concentrations caused a stronger inhibition of dismutase activity, but were not used because they also caused an inhibition of sulphite oxidation initiated by the xanthine oxidase system in the absence of dismutase (Fig. 2). The amount of cupro-zinc dismutase activity in liver fractions was estimated by measuring the dismutase activity in the absence and presence of 2mM-cyanide. Cupro-zinc dismutase activity was calculated from the results obtained, by assuming that the enzyme was inhibited by 86% in the presence of cyanide. For example, if the sample contained x dismutase units/ml in the absence of cyanide and y units/ml in the presence of cyanide, then the units of cupro-zinc dismutase activity present in the sample = $(x-y)/0.86$ units/ml.

Intracellular distribution of liver superoxide dismutase

The distribution of superoxide dismutase and of several reference enzymes in fractions isolated from rat liver homogenates is shown in Table 1 and Fig. 3. In most cases, the recovery of enzyme activities was between 90 and 110%. The total units of enzyme activity/g wet wt. of liver were similar to those reported previously, except for catalase activity, which was higher than that observed by Baudhuin *et al.* (1964), perhaps owing to differences in the assay conditions used. The distribution of the marker enzymes cytochrome oxidase (mitochondria), glutamate dehydrogenase (matrix space of mitochondria), sulphite–cytochrome *c* reductase (intermembrane space of mitochondria), catalase (peroxisomes), acid phosphatase (lysosomes), glucose 6-phosphatase (microsomal fraction) and 6-phosphogluconate dehydrogenase (soluble fraction) was similar to that described previously (de Duve *et al.*, 1955; Beaufay *et al.*, 1959; Baudhuin *et al.*, 1964; Wattiaux-De Coninck & Wattiaux, 1971; Lloyd-Davies *et al.*, 1972). Virtually all the cyanide-insensitive dismutase activity (mangano-form) was present in particulate fractions, whereas about 90% of the cupro-zinc enzyme was found in the soluble fraction. The distribution of

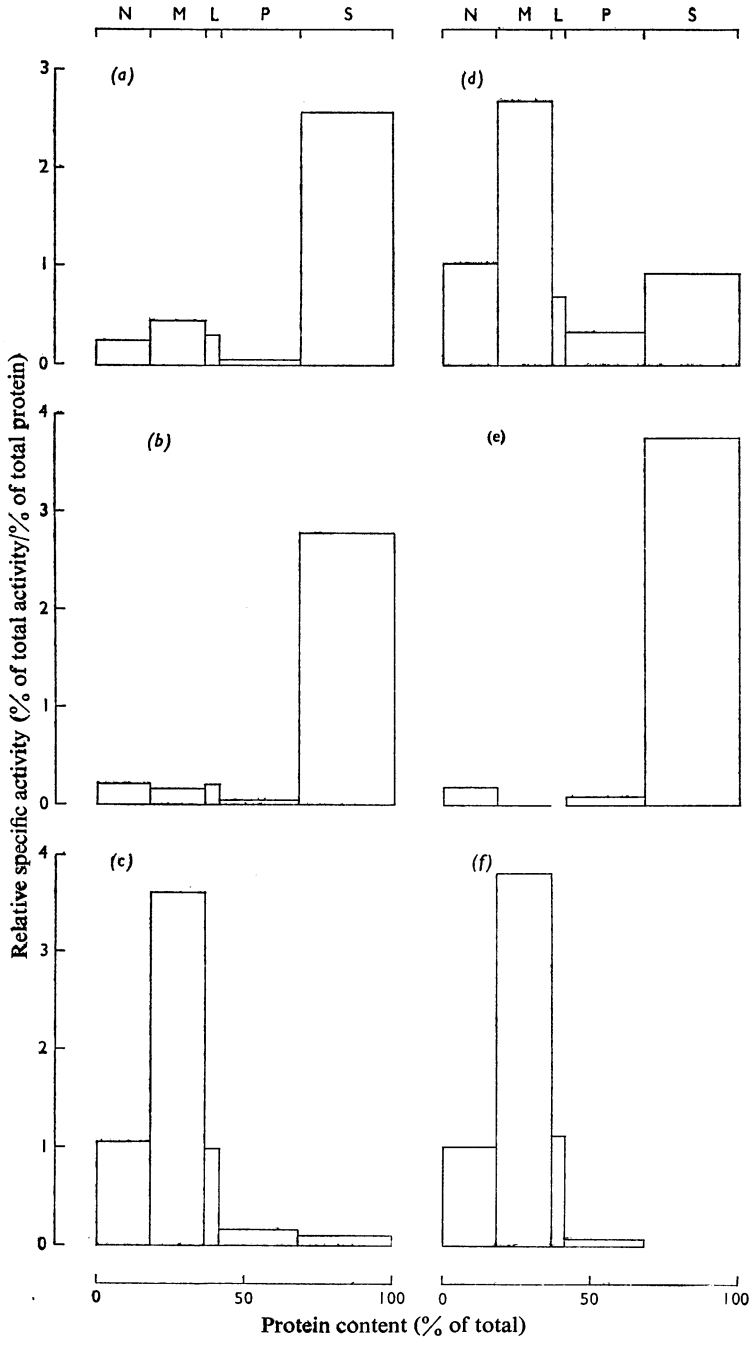

Fig. 3. *Distribution patterns of superoxide dismutase and marker enzymes*

(*a*) Total superoxide dismutase; (*b*) cyanide-sensitive dismutase; (*c*) cyanide-insensitive dismutase; (*d*) sulphite–cytochrome *c* reductase; (*e*) 6-phosphogluconate dehydrogenase; (*f*) cytochrome oxidase. Each fraction is represented separately on the ordinate scale by its relative specific activity (% of total recovered activity/% of total recovered protein). On the abscissa scale each fraction is represented by its protein content expressed as a percentage of total recovered protein.

Table 1. *Intracellular distribution of superoxide dismutase and some marker enzymes in rat liver*

Absolute values are given as units/g fresh wt. for enzymes and mg/g for protein. All values are presented as means±S.D. Abbreviations: E, cytoplasmic extract; N, nuclear fraction; M, heavy mitochondrial fraction; L, light mitochondrial fraction; P, microsomal fraction; S, final supernatant, assayed after dialysis for 15h (see the Materials and Methods section).

Enzyme	No. of expts.	Absolute values (E+N)	Percentage values N	M	L	P	S	Recovery (%)
Superoxide dismutase								
Total activity	7	129.4±14.3	4.4±0.5	7.9±1.2	1.9±0.7	2.4±0.9	81.3±3.9	97.9±5.2
Cupro-zinc form	7	119.3±11.5	3.2±0.5	3.3±0.9	1.0±0.2	1.5±0.7	87.7±4.2	96.7±5.1
Mangano form	7	10.3±3.5	20.3±4.5	61.1±4.7	3.5±1.6	3.6±2.0	3.6±1.0	94.1±7.2
Cytochrome oxidase	6	21.8±3.5	18.2±4.3	68.4±8.0	5.6±2.8	1.7±1.1	0	93.9±5.8
Glutamate dehydrogenase	3	12.7±3.2	20.8±3.6	70.7±8.1	4.1±1.1	1.3±0.7	2.0±0.4	98.9±5.0
Sulphite–cytochrome *c* reductase	3	16.8±4.5	19.3±7.5	48.2±9.3	3.5±0.8	9.4±3.4	30.0±4.2	110.4±12.6
Catalase	6	117.0±14.2	8.9±5.1	19.0±6.1	16.7±5.0	7.1±4.1	38.6±11.0	90.3±11.3
Glucose 6-phosphatase	3	13.2±3.8	7.6±1.2	8.3±4.2	5.7±1.5	70.2±9.3	1.4±1.2	93.2±6.1
Acid phosphatase	3	8.3±2.3	4.5±1.2	17.0±5.2	23.2±2.6	30.5±2.3	12.7±3.9	87.9±12.3
6-Phosphogluconate dehydrogenase	3	6.1±2.4	3.5±1.1	0.8±0.3	0	2.3±2.1	120.8±23.6	127.4±22.4
Protein	7	264.2±19.2	18.0±5.4	17.9±3.7	4.7±2.1	26.3±3.9	32.7±5.0	99.6±6.7

cyanide-insensitive dismutase activity was similar to that of cytochrome oxidase, suggesting that this form of dismutase is confined exclusively to the mitochondria (Fig. 3). Similar results were obtained when the distributions of cyanide-insensitive dismutase and glutamate dehydrogenase were compared (Table 1).

As shown below, most of the cupro-zinc enzymic activity of the heavy mitochondrial fraction was located in the inter-membrane space, for which sulphite–cytochrome *c* reductase serves as a marker enzyme. From the results presented in Table 1, the ratio of the average reductase activity and cupro-zinc dismutase activity was 6.0, 14.6, 3.5, 6.3, 0.34 for the N, M (heavy-mitochondrial), L (light-mitochondrial), P (microsomal) and S (final-supernatant) fractions respectively, suggesting that the N, L, P and S fractions contained more cupro-zinc dismutase activity than can be accounted for by the mitochondria, or enzymes liberated from disrupted mitochondria, present in the fractions.

Partial purification of the dismutase activity of the separate M, L and S fractions by the method of McCord & Fridovich (1969*a*), up to and including the acetone-precipitation step, yielded samples containing about 0.4μg of copper/mg of protein with 20–50% recovery of activity. These results support the conclusion that a cupro-zinc dismutase, similar to the erythrocyte enzyme was present in the fractions. No attempt was made to purify the cyanide-insensitive dismutase. The latter activity showed several features in common with the mangano-form of the chicken liver enzyme described by Weisiger & Fridovich (1973*a*), including inactivation by the organic solvents used to purify the cupro-zinc enzyme. The cyanide-insensitive dismutase of rat liver is therefore assumed to be similar to that found in chicken liver and is referred to in the present paper as the mangano-form of dismutase.

Latency of the M-fraction dismutase activity

The total dismutase activity present in the M fraction was increased about sevenfold by sonication. Most of the activity was released by this treatment in a non-sedimentable form (Fig. 4). In contrast, the dismutase activity of the other particulate fractions was increased less than threefold by sonication. These experiments indicate that most of the dismutase activity in the M fraction is enclosed by the membranes of particles present in the fraction and is not due simply to the activity of soluble dismutase adsorbed on their surfaces. In contrast, the low degree of latency in the other particulate fractions suggests that at least part of the extra cupro-zinc dismutase activity, not associated with the mitochondria of these fractions, may be due to adsorbed soluble-fraction dismutase.

Digitonin fractionation

The effect of digitonin on the release of enzymes present in the M fraction is shown in Fig. 5. Treatment with 2mg of digitonin/100mg of protein caused the release of most of the acid phosphatase activity of the fraction (Loewenstein *et al.*, 1970) without releasing much dismutase activity, indicating that little or no dismutase activity was associated with lysosomes present in the fraction. Most of the cupro-zinc dismutase was released by 5–10mg of digitonin/100mg of protein, together with the marker enzymes

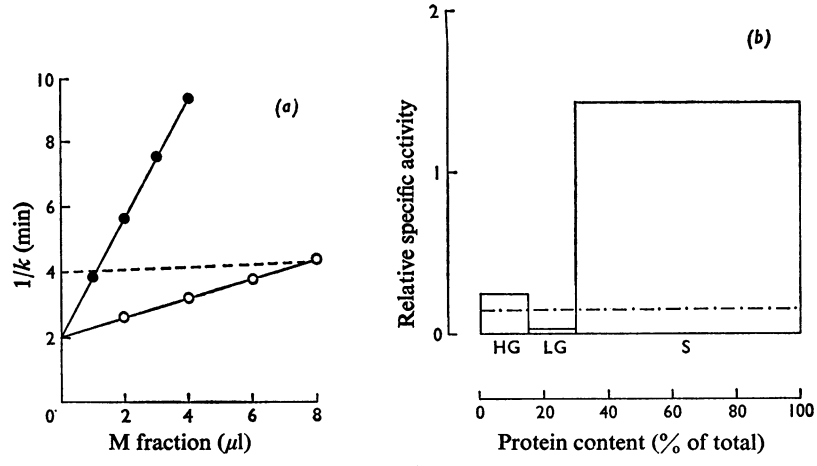

Fig. 4. *Latency of superoxide dismutase activity in the M fraction*

Assays were performed in 0.1 M-KCl and 0.1 mM-EDTA, pH7.4. (*a*) Effect of sonication on dismutase activity. One unit of dismutase activity in 3.2ml of reaction mixture is given by the abscissa values of the intercept between the solid lines and the dashed line drawn from the ordinate. ○, Untreated M fraction; ●, sonicated M fraction. (*b*) Distribution of dismutase activity in sonicated M fraction. Fractionation after sonication was performed as described in the Materials and Methods section. The horizontal broken line represents the relative specific activity of the M fraction before sonication. Abbreviations: HG, heavy granules; LG light granules; S, supernatant.

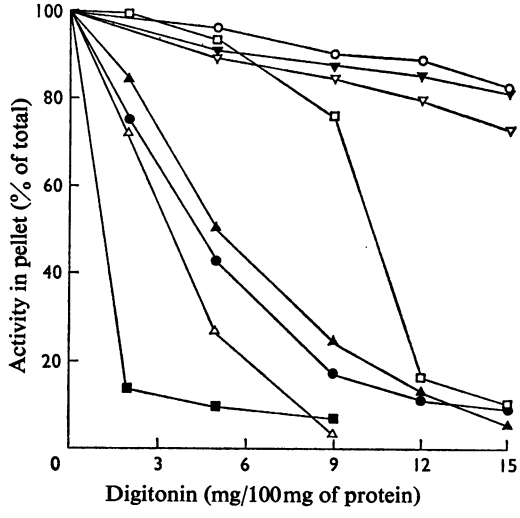

Fig. 5. *Release of M-fraction enzymes by digitonin*

The percentage of protein and enzymic activities sedimented by centrifugation after digitonin treatment is plotted against the concentration. ▽, Protein; ●, cyanide-sensitive dismutase; ○, cyanide-insensitive dismutase; ▼, glutamate dehydrogenase; ■, acid phosphatase; △, catalase; ▲, adenylate kinase; □, monoamine oxidase. The specific activities of adenylate kinase and monoamine oxidase in samples containing no digitonin were 0.46 and 0.01 μmol/min per mg of protein respectively.

from the peroxisomes (catalase) and the inter-membrane space of mitochondria (adenylate kinase). Between 90 and 100% of the dismutase activity released by digitonin treatment was recovered in the supernatant obtained after centrifuging the suspension. This result indicated that most of the cupro-zinc dismutase was present in either the peroxisomes or the inter-membrane space. The marker enzymes for the outer mitochondrial membrane (monoamine oxidase) and the matrix space (glutamate dehydrogenase) were released in response to even higher concentrations of digitonin in a manner similar to that described by Schnaitman & Greenawalt (1968). The release of mangano-dismutase activity was similar to that of glutamate dehydrogenase, confirming that this form of dismutase is located in the inner-membrane matrix of the mitochondria.

In other experiments, the M fraction was treated with a fixed amount of digitonin (12mg/100mg of protein) and separated into inner-membrane particles, outer-membrane particles, matrix-space protein and inter-membrane-space protein. The latter sample is contaminated with protein released from peroxisomes and lysosomes. The dismutase activities found in the various fractions are shown in Table 2. The results are consistent with those of Figs. 4 and 5 and indicate that most of the mangano-form is present in the matrix space, although a significant amount is also associated with the inner membrane.

Table 2. *Fractionation of digitonin-treated heavy mitochondrial fraction*

The heavy mitochondrial fraction was treated with 12mg of digitonin/100mg of protein and fractionated as described in the Materials and Methods section. The inter-membrane-space protein value does not include bovine plasma albumin added during the separation procedure. Results were similar with two other liver preparations. The values of enzyme activity and protein are expressed as a percentage of those present in untreated M fraction.

Sample	Superoxide dismutase: Cupro-zinc form	Superoxide dismutase: Mangano form	Monoamine oxidase	Adenylate kinase	Glutamate dehydrogenase	Protein	
Inner-membrane matrix							
Unfractionated	12.9	90.3	12.1	0	85.8	76.1	
Sonic pellet	1.6	11.1					18.1
Sonic supernatant	10.3	68.4					55.3
Outer membrane	0.9	1.4	61.3	4.2	1.2	7.5	
Inter-membrane-space protein	85.7	11.0	10.2	83.7	9.3	14.7	
Recovery (%)	99.5	102.7	83.6	87.9	96.3	98.3	

Table 3. *Release of enzymes from the heavy mitochondrial fraction by hypo-osmotic treatment*

For experimental details see the Materials and Methods section. The supernatant-protein values do not include bovine plasma albumin added during the preparation of the samples. Results were similar with two other liver preparations.

Enzyme	0.25M-Sucrose: Sediment	0.25M-Sucrose: Supernatant	0.25M-Sucrose: Recovery (%)	0.05M-Sucrose: Sediment	0.05M-Sucrose: Supernatant	0.05M-Sucrose: Recovery (%)
Superoxide dismutase						
Cupro-zinc form	93.2	5.1	98.3	18.1	77.9	96.0
Mangano-form	96.5	0	96.5	83.0	6.8	89.8
Adenylate kinase	93.5	2.1	95.6	1.2	87.4	88.6
Catalase	81.2	19.7	100.9	67.4	28.3	95.7
Glutamate dehydrogenase	97.8	0.6	98.4	92.0	3.5	95.5
Monoamine oxidase	99.8	0	99.8	102.3	0	102.3
Sulphite–cytochrome *c* reductase	95.3	4.2	99.5	6.9	84.3	91.2
Protein	96.7	2.1	98.8	92.3	7.1	99.4

Effect of hypo-osmotic treatment

The location of cupro-zinc dismutase present in the heavy mitochondrial fraction was investigated further by studying the release of enzymes caused by hypo-osmotic treatment. Incubation of M-fraction samples in 0.05M-sucrose caused an almost total loss of the inter-membrane-space enzymes adenylate kinase and sulphite–cytochrome *c* reductase (Wattiaux-De Coninck & Wattiaux, 1971), together with about 78% of the total cupro-zinc dismutase activity. In contrast, when the effects of incubation in 0.25M-sucrose or 0.05M-sucrose on the release of catalase were compared, hypo-osmotic treatment caused the specific release of only 15% of the total catalase activity (Table 3). These results suggest that little or no dismutase activity is associated with the peroxisomes.

Molar content of rat liver dismutases

The data of Table 1 (heavy mitochondrial fraction) indicate that the livers used in these experiments contained an average of 47.3mg of mitochondrial protein and 6.5 units of mangano-dismutase/g of liver. Assuming that the molecular weight and activity of the rat liver enzyme is similar to that of the chicken liver enzyme (Weisiger & Fridovich, 1973*a*), it may be calculated that rat liver mitochondria contain about 0.7mg or 8.8nmol of mangano-dismutase/g of mitochondrial protein. The dismutase content is therefore considerably below the cytochrome content (e.g. cytochrome *a* content, 200nmol/g of mitochondrial protein; Estabrook & Holowinsky, 1961), but may be similar to the content of NADH dehydrogenase, which is believed to be about one-tenth of the cytochrome *a* content (Klingenberg, 1968). If the water content of the matrix space of mitochondria is taken to be 0.8ml/g of mitochondrial protein (Klingenberg & Pfaff, 1966) then the mangano-dismutase concentration in the matrix space would be about 11μM. These values of the content and concentration of mangano-dismutase may be underestimated because the heavy mitochondrial fraction

is contaminated with other organelles that do not contain the enzyme.

Similar calculations can be made from data on the content of soluble cupro-zinc dismutase (107 units of dismutase activity/g of liver; see Table 1), by assuming that the molecular weight and activity of the rat liver enzyme are similar to those of the bovine erythrocyte and chicken liver supernatant enzymes. These calculations indicate that the soluble cupro-zinc dismutase content of rat liver is about 0.55mg or 17nmol/g of liver. If the cytosol water content is 0.7ml/g of liver, the average dismutase concentration would be about 24μM. The calculated content of soluble rat liver dismutase is in good agreement with the results of fractionation studies on rat liver supernatant carried out by Bremner *et al.* (1973). They found a cupro-zinc protein, identified as superoxide dismutase on a molecular-weight basis, that was present at a concentration of 2μg of copper/g of liver, which corresponds to a content of about 0.53mg of dismutase/g of liver.

A comparison between the calculated content of rat liver dismutase and the content of the chicken liver enzymes found by purification (Weisiger & Fridovich, 1973*a*) suggests that the dismutase contents of the two tissues are the same order of magnitude.

Discussion

Liver superoxide dismutases

The results presented in this paper demonstrate that rat liver cells contain two forms of superoxide dismutase, similar to those purified from chicken liver (Weisiger & Fridovich, 1973*a*). Although most of the rat liver dismutase activity is present in the soluble fraction *in vitro* and is therefore presumably free in the cytoplasm *in vivo*, a significant amount, about 15% of the total activity, is present in particulate form. The effects of sonication, digitonin fractionation and hypo-osmotic treatment indicate that the particulate cupro-zinc dismutase is present mainly in the mitochondrial inter-membrane space, whereas the cyanide-insensitive enzyme is confined to the mitochondrial inner-membrane and matrix space. These conclusions are supported by the results of Peeters-Joris *et al.* (1973), who have made similar observations by using a different method to assay dismutase activity. Earlier differing conclusions that the mitochondrial dismutase is a mangano-enzyme (Weisiger & Fridovich, 1973*a*) and that it is a cupro-zinc enzyme (Tyler, 1973) are thus resolved, and the later results obtained with chicken liver mitochondria (Weisiger & Fridovich, 1973*b*) and rat liver mitochondria are in good agreement.

Since superoxide dismutase tends to combine with other proteins (Keele *et al.*, 1971) the non-latent activity of the particulate fractions may be due to soluble-fraction dismutase adsorbed on the surface of the organelles. However, the present results do not exclude the possibility that small amounts of dismutase are truly located in other organelles besides mitochondria. For example, it may be misleading to draw conclusions about the enzyme content of the nucleus by using a nuclear fraction isolated in an aqueous medium, since this procedure is known to cause a pronounced loss of enzymes located in the nuclear sap (Georgiev, 1967). Weisiger & Fridovich (1973*a*) found at least three electrophoretically distinct forms of liver cupro-zinc dismutase. Since most enzymes that have a multiple intracellular distribution are present as different isoenzyme forms in different cell compartments (de Duve, 1971), it seems probable that two of these forms are dismutase isoenzymes, one present in the cytoplasm and the other in the inter-membrane space of mitochondria.

Superoxide and H_2O_2 formation in rat liver

Boveris *et al.* (1972) showed that all the major subcellular fractions of rat liver generated H_2O_2 and they gave estimates of the physiological rate of H_2O_2 production. By using their estimates, together with those of the intracellular cytosol dismutase concentration (see the Results section) and a recently revised estimate of the cupro-zinc dismutase rate constant (Fielden *et al.*, 1974), it is possible to calculate a very approximate intracellular superoxide concentration, by assuming that all the H_2O_2 formed arises from dismutase activity. If the extramitochondrial generation of H_2O_2 is 76nmol/min per g of liver (Boveris *et al.*, 1972), then the calculated superoxide concentration is 66pM. However, some liver H_2O_2-generating enzymes are known to form H_2O_2 without forming a superoxide intermediate that dissociates from the enzyme (e.g. urate oxidase and D-amino acid oxidase; Fridovich & Handler, 1961). Further, at neutral pH, only about 20% of the H_2O_2 generated during xanthine oxidase activity is formed with superoxide as an intermediate (Fridovich, 1970). Thus the calculated concentration certainly overestimates the actual physiological concentration. A similar calculation can be made by using the estimates of mitochondrial H_2O_2 production (12nmol/min per g of liver; Boveris *et al.*, 1972), dismutase concentration and the mangano-enzyme rate constant (Forman & Fridovich, 1973), and gives an estimated intramitochondrial superoxide concentration of about 8pM. Although these calculations are based on a number of assumptions and may not be very accurate, they do suggest that the dismutase activity of the liver cell is sufficient to maintain the superoxide concentration at a very low value.

Functions of superoxide dismutase

According to McCord *et al.* (1971), the general

Table 4. *Intracellular location of known $O_2^{-\cdot}$-generating enzymes in liver cells*

References: 1, Fridovich & Handler (1961); 2, Massey *et al.* (1969); 3, Sato (1967); 4, Aust & Pedersen (1972); 5, Strobel & Coon (1971).

Enzyme	Location	Reference
Cytochrome oxidase	Mitochondria	1
Lipoyl dehydrogenase	Mitochondria	2
NADPH-oxidation system	Endoplasmic reticulum	1, 3, 4
Mixed-function oxidase system	Endoplasmic reticulum	5
Xanthine oxidase	Cytosol	1
Aldehyde oxidase	Cytosol	1
Glutathione reductase	Cytosol	2

function of superoxide dismutase is to protect oxygen-metabolizing cells against the potentially toxic effects of superoxide ions generated during enzymic activity. It is therefore of interest to compare the intracellular distribution of dismutase with that of enzyme systems known to generate superoxide ions during their enzymic activity. The latter distribution is shown in Table 4 and indicates that $O_2^{-\cdot}$ ions are generated in all compartments of the liver cell where superoxide dismutase is present. Thus the distribution of superoxide dismutase is well suited to a protective function of the enzyme. The significance of an association between superoxide-forming enzymes and superoxide dismutase is supported by studies on rat skeletal muscle. This tissue, which was found to contain no xanthine oxidase activity, in agreement with Morgan (1926), contained only about 8% of the soluble cupro-zinc dismutase activity present in rat liver (D. D. Tyler, unpublished work).

Recent evidence suggests that $O_2^{-\cdot}$ ions are toxic to various biological structures including nucleic acids, proteins and lipid membranes (Lavelle *et al.*, 1973; Zimmermann *et al.*, 1973). Since the mitochondrial inner membrane is rich in haemoproteins that can catalyse lipid peroxidation (Tappel, 1955) and also has a high content of unsaturated fatty acids (Colbeau *et al.*, 1971) it is probable that one function of the dismutases located on each side of the membrane is to prevent peroxidation and disintegration of the membrane. Similar considerations apply to the bacterial plasma membrane of *Escherichia coli*, which is also in contact with two distinct forms of dismutase, located one on each side of the membrane (Gregory *et al.*, 1973). Although evidence of the toxic effects of $O_2^{-\cdot}$ ions is in accordance with the view of McCord *et al.* (1971) that 'the superoxide free radical is a commonly occurring but quite undesirable physiological species', the generation of $O_2^{-\cdot}$ ions in cells may be usefully concerned in mechanisms of aging and turnover of cell constituents, drug metabolism (Fried *et al.*, 1973) and bactericidal activity of leucocytes (Babior *et al.*, 1973). Thus the physiological function of superoxide dismutase may be not to destroy the product of an aberrant reaction between certain respiratory enzymes and molecular oxygen, but rather to regulate the concentration of a useful cell metabolite.

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