Subcellular Distribution of Superoxide Dismutases in Human Neutrophils

INFLUENCE OF MYELOPEROXIDASE ON THE MEASUREMENT OF SUPEROXIDE DISMUTASE ACTIVITY

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We have identified two distinct pools of superoxide dismutase in fractions of human peripheral neutrophils obtained by the isopycnic fractionation of homogenates of the latter with linear sucrose gradients. Superoxide dismutase activity, observed with polyacrylamide gels impregnated with Nitro Blue Tetrazolium, was present in: (1) the mitochondrial fraction [density (ρ) 1.169 g/ml], containing the high-molecular-weight KCN-resistant enzyme, and (2) the cytoplasm fraction, containing the low-molecular-weight KCN-sensitive enzyme. Superoxide dismutase activity, observed with a quantitative assay involving cytochrome *c*, was present in: (1) the mitochondria, (2) the cytoplasm, and (3) the azurophil-granule fractions ($\rho = 1.206$ and 1.222 g/ml). No substantial enzyme activity was observed in specific-granule fractions ($\rho = 1.187$ g/ml) or in the membranous fraction ($\rho = 1.136$ g/ml) in either assay. The apparent superoxide dismutase activity observed in the azurophil granules with the cytochrome *c* assay was attributable not to true superoxide dismutase but to myeloperoxidase, an enzyme found solely in the azurophil granules. In the presence of H_2O_2 , human neutrophil myeloperoxidase oxidized ferrocytochrome *c*. Thus, in the cytochrome *c* assay for superoxide dismutase, the oxidation of ferrocytochrome *c* by myeloperoxidase mimicked the inhibition of reduction of ferricytochrome *c* by superoxide dismutase. When myeloperoxidase was removed from azurophil-granule fractions by specific immuno-affinity chromatography, both myeloperoxidase and apparent superoxide dismutase activities were removed. It is concluded that there is no detectable superoxide dismutase in either the azurophil or specific granules of human neutrophils. Mitochondrial superoxide dismutase, 15% of the total dismutase activity of the cells, occurred only in fractions of density 1.160 g/ml, where isocitrate dehydrogenase and cytochrome oxidase were also observed.

The purpose of the present study was to determine the subcellular location of superoxide dismutase within human neutrophils, especially in the granule fractions. With techniques developed in our laboratory, human neutrophil-granule classes can be separated from each other by isopycnic sucrose-density-gradient centrifugation, allowing us to study individual granule classes.

Superoxide anion ($O_2^{\cdot-}$), continually produced within prokaryotic and eukaryotic cells by enzymic reactions such as the oxidation of NADPH or xanthine (Fridovich, 1972), is toxic to the very same cells that produce it. To overcome the deleterious effects of $O_2^{\cdot-}$, both cells have evolved superoxide dismutases, enzymes that dispose of $O_2^{\cdot-}$ by dismutating it to H_2O_2 and O_2 . At least three common superoxide dismutases have been described in the literature (for a review see Fridovich, 1975). These are found in the cytoplasm of bacteria and in

mitochondria, in the cytoplasm of eukaryotes, and in the pericytoplasmic space of bacteria. The bacterial cytoplasmic enzyme and the mammalian mitochondrial enzyme are similar, contain Mn^{2+} and are uninhibited by KCN. The mammalian cytoplasmic enzyme contains Cu^{2+} and Zn^{2+} and is inhibited by KCN, and the bacterial pericytoplasmic enzyme contains Fe^{2+} .

Human neutrophils produce and excrete $O_2^{\cdot-}$ and possess both the $Cu+Zn$ - and the Mn -containing superoxide dismutases (Babior *et al.*, 1973; Salin & McCord, 1974). These findings have led to speculations as to the role of $O_2^{\cdot-}$ and superoxide dismutase in the bactericidal mechanisms of neutrophils. It has been proposed that the $O_2^{\cdot-}$ produced by neutrophils kills certain bacteria (Babior *et al.*, 1973). Exogenous superoxide dismutase in phagocytosing neutrophils inhibits bactericidal activity (Johnston *et al.*, 1975); moreover, some bacteria may protect

themselves against the bactericidal effects of $O_2^{\cdot-}$ with their own pericytoplasmic superoxide dismutase (Gregory & Fridovich, 1973).

In view of the present hypothesis of oxidative mechanisms and intraleucocytic killing, it would be expected that little or no superoxide dismutase is located in the neutrophil granules, in order to allow killing by $O_2^{\cdot-}$. On the contrary, if substantial amounts of superoxide dismutase were located in the granules, it would be difficult to imagine $O_2^{\cdot-}$ playing an important role in intraleucocytic killing. It was thus important to demonstrate the presence or absence of the enzyme in the granules of human neutrophils.

Methods and Materials

Granule preparations

Neutrophils were isolated from freshly drawn venous blood, from apparently healthy male and female donors, as described previously (Spitznagel *et al.*, 1974). These studies were approved by the Committee on the Protection of the Rights of Human Subjects at the University of North Carolina. All donors signed informed-consent forms before blood was drawn. Purified neutrophil suspensions contained 90–96% neutrophils, 0–2% lymphocytes, 0–2% monocytes and 3–8% eosinophils. The following modification of the method of Spitznagel *et al.* (1974) was used to obtain neutrophil-granule fractions. Supernatants (126g, 15 min) of homogenized neutrophils (80–85% disruption in 0.43M-sucrose) were made 25% with respect to sucrose and layered over linear 30–53% (w/w) sucrose gradients. The equivalent of approx. 10^9 neutrophils, containing about 35mg of protein, was layered on three gradients. Gradients were centrifuged under isopycnic conditions in a Beckman SW 25.2 rotor (r_{av} , 10.8 cm) at 2000 rev./min for 15 min, accelerated to 21000 rev./min and centrifuged for an additional 120 min at 22°C. Gradients were collected by upward displacement with 60% (w/w) sucrose, and collected in 1 ml fractions. Absorbances of these fractions were measured at 450 nm (1 cm-light-path cuvette) to locate subcellular fractions.

To obtain a mixed-granule fraction (free of cytoplasmic constituents) the 126g supernatant of homogenized neutrophils was differentially centrifuged at 20000g (Sorvall RC2-B centrifuge, HS rotor) for 20 min. The pellet, containing the azurophil and specific granules and some mitochondria, was resuspended in 0.34M-sucrose.

Extracts of granules were prepared by stirring 10–20mg of granule protein (equivalent to 1×10^9 – 2×10^9 neutrophils) with 5 ml of 0.1M-sodium acetate buffer, pH 4.0, containing 0.01M- $CaCl_2$, overnight at 4°C. The extract mixture was centri-

fuged at 20000g for 20 min at 4°C, the pellet re-extracted overnight, centrifuged (20000g for 20 min), and the two supernatants were combined and dialysed against phosphate-buffered saline (7.4g of NaCl, 0.285g of KCl, 0.29mg of $Na_2HPO_4 \cdot 7H_2O$, and 0.083g of KH_2PO_4 per litre of deionized water, pH 7.0). Dialysis was done with an Amicon MMC unit, over a UM-2 membrane.

Assay of superoxide dismutase activity

Three different assays were used. (1) The reduction of ferricytochrome *c* by $O_2^{\cdot-}$ was followed at 550 nm by the method of McCord & Fridovich (1969). Superoxide dismutase causes a decrease in the reduction of ferricytochrome *c*, proportional to the removal of $O_2^{\cdot-}$ by the enzyme. One unit is the amount of superoxide dismutase that inhibits the rate of reduction of cytochrome *c* by 50% (i.e. from a rate of 0.0250 absorbance unit/min to 0.0125 absorbance unit/min). This assay was linear from 10 to 60% inhibition (0.2–1.2 units) of initial cytochrome *c* reduction. Fractions containing enzyme activity were diluted to give inhibition within this range. For cytochrome assays involving KCN, ferricytochrome *c* (type III; Sigma Chemical Co., St. Louis, MO, U.S.A.) was further purified by passage through a column (1 cm $\times$ 30 cm) of Sephadex G-75 (with phosphate-buffered saline as eluent) to remove any contaminating superoxide dismutase (I. Fridovich, personal communication). Further purification of cytochrome *c* (type VI; Sigma) was not necessary. (2) The reduction of Nitro Blue Tetrazolium to formazan was measured at 560 nm, by using the method of Beauchamp & Fridovich (1971), or (3) a Nitro Blue Tetrazolium assay adapted for polyacrylamide gels was used as described by Salin & McCord (1974). This last assay allows one to distinguish easily between the high-molecular-weight Mn-containing mitochondrial superoxide dismutase and the low-molecular-weight Cu+Zn-containing soluble enzyme. All spectrophotometric assays were performed in an Acta V spectrophotometer (Beckman Instruments) at room temperature (23–26°C), in 1 ml 1 cm-light-path cuvettes, in the presence of 0.01% Triton X-100, unless otherwise indicated.

Other enzyme assays

The following enzymes were assayed: isocitrate dehydrogenase (EC 1.1.1.42; Leighton *et al.*, 1968); cytochrome *c* oxidase (EC 1.9.3.1; Wharton & Tzagoloff, 1967); glutamate dehydrogenase (EC 1.4.1.3; Leighton *et al.*, 1968); succinate dehydrogenase (EC 1.3.99.1; King, 1967); lysozyme (EC 3.2.1.17; Shugar, 1952); and myeloperoxidase (EC 1.11.1.7; Worthington Biochemical Corp., 1972). Myeloperoxidase ($A_{430}/A_{280} = 0.72$) was obtained

from human leukaemic leucocytes by acetate extraction, followed by molecular-sieve and Bio-Rex 70 chromatography by the method of Olsson *et al.* (1972), and it appeared homogeneous on polyacrylamide-gel disc electrophoresis. Homogeneous myeloperoxidase has an A_{430}/A_{280} of approx. 0.87. One unit of myeloperoxidase activity is defined as that causing an increase in A_{460}^{1cm} of 0.001 in a 1 ml reaction mixture, with *o*-dianisidine as the hydrogen donor.

To distinguish between soluble isocitrate dehydrogenase and mitochondria-associated NADP⁺-dependent isocitrate dehydrogenase, the individual sucrose gradient fractions were diluted with equal volumes of 0.15M-NaCl with rapid mixing and were centrifuged at 25000 rev./min (100000 $g_{av.}$) for 1 h in a Beckman SW 50.1 rotor. The supernatants were retained, and the pellets were resuspended in 0.34M-sucrose containing 0.02% Triton X-100.

For assays involving reduced cytochrome *c*, ferricytochrome *c* (1%) was reduced with a few crystals of NaBH₄ and diluted 1:100 in 0.05M-potassium phosphate buffer, pH7.8.

Protein

Protein content was determined by the method of Lowry *et al.* (1951), in the presence of 0.03% Triton X-100, with egg-white lysozyme as a standard. This concentration of Triton X-100 did not interfere with the Lowry assay. Lactoferrin was purified from human milk by the method of Querinjean *et al.* (1971).

Affinity chromatography

Rabbit anti-(human myeloperoxidase) antibody, purified from serum by (NH₄)₂SO₄ precipitation (Johnson & Holborow, 1973), was bound to CNBr-activated Sepharose 4B as directed by the manufacturer (Pharmacia, Piscataway, NJ, U.S.A.). This antibody gave a single precipitin line in an Ouchterlony (1958) plate when tested against neutrophil homogenates. The column (1cm×4cm) did not retain lactoferrin, lysozyme or bovine serum albumin. Samples were applied to the column with 0.1M-sodium borate buffer, pH8.0, containing 1M-NaCl. When quantities of myeloperoxidase approaching the capacity of the column were bound to the column at pH8, the enzyme could be quantitatively eluted from the column with either 0.1M-sodium acetate buffer, pH4.0, containing 1M-NaCl, or 0.1M-glycine/HCl buffer, pH2.8, containing 1M-NaCl, both buffers giving similar results. When quantities of myeloperoxidase sufficiently less (<50%) than the capacity of the column were bound to the column, recovery at acid pH was poor with either buffer. This was possibly due to the high affinity of the column or more likely to the noted instability of myeloperoxidase

in dilute solutions. Column samples were concentrated in an Amicon ultrafiltration apparatus over a P-10 membrane, which quantitatively retained both myeloperoxidase and superoxide dismutase.

Materials

Ferricytochrome *c* (type III or VI), xanthine, xanthine oxidase, Nitro Blue Tetrazolium, *o*-dianisidine and CNBr-activated Sepharose 4B were obtained from Sigma. Superoxide dismutase (EC 1.15.1.1; 3 units/ μ g) was obtained from Truett Laboratories, Dallas, TX, U.S.A. All other materials were reagent grade.

Results

Separation of subcellular components of neutrophils

We first fractionated subcellular components of neutrophils with differential centrifugation and tested for superoxide dismutase activity. Owing to the similar size and density of subcellular components, the fractions were substantially contaminated with unwanted components. To obtain cleaner subcellular fractions, we then separated neutrophil homogenates by isopycnic centrifugation.

Differential centrifugation of neutrophil homogenates yielded four fractions in addition to the initial cell homogenate (CH): (1) the pellet (126P) and (2) the supernatant (126S) from a 126g, 15min centrifugation of the homogenized neutrophils, and (3) the pellet (20000P) and (4) the supernatant (20000S) from a 20000g, 20min centrifugation of the 126S fraction. The 126P fraction contained whole cells, nuclei and aggregated cell debris; the 126S fraction contained granules, some mitochondria, cytosol enzymes and membranous material; the 20000P fraction contained granules and some mitochondria; the 20000S fraction contained the cytosol enzymes, membranous material and some mitochondria.

Isopycnic centrifugation of neutrophil homogenates through sucrose led to the separation of five particulate classes; thus five bands were observed in the linear sucrose gradients of the 126g supernatant of homogenized neutrophils (Fig. 1). The top three to four tubes (3–4ml) of the gradient contained the 'soluble' cytosol enzymes. Band 1 (tubes 5–7) was a membranous fraction ($\rho = 1.136$ g/ml) identified on grounds of density and the appearance of this fraction in electron micrographs (Spitznagel *et al.*, 1974). Band 2s (tubes 8–17) contained the mitochondria ($\rho = 1.160$ g/ml). Identification of this band is discussed below. Band 2f (tubes 18–30) contained the specific granules ($\rho = 1.187$ g/ml). It contained all of the lactoferrin of the neutrophil and about one-half of the lysozyme, but no myeloperoxidase. Band 3s

(tubes 31–37) contained the slow-migrating azurophil granules ($\rho = 1.206\text{g/ml}$). These granules contained no lactoferrin, about one-quarter of the cell's lysozyme, and about 60% of the cell's myeloperoxidase. Band 3f (tubes 38–46) contained the fast-migrating azurophil granules ($\rho = 1.222\text{g/ml}$). They contained slightly more lysozyme and slightly less myeloperoxidase than the slowly sedimenting azurophil granules. An absorbance profile (1 ml fractions) of a representative gradient is shown in Plate 1.

Distribution of superoxide dismutase activity qualitatively defined by Nitro Blue Tetrazolium-impregnated polyacrylamide gels

Owing to the considerable cross-contamination of neutrophil fractions obtained by differential centrifugation, data obtained from Nitro Blue Tetrazolium-stained polyacrylamide gels of these fractions were not conclusive as to the subcellular distribution of superoxide dismutase activity. Therefore the 126S fraction was isopycnicly fractionated through linear sucrose gradients. The particulate material from one or two fractions from the top of each gradient peak was concentrated by centrifugation, electrophoresed in polyacrylamide gels, and the gels were impregnated with Nitro Blue Tetrazolium to locate superoxide dismutase activity. Plate 2 shows two different superoxide dismutase activities in the gels. Mitochondrial Mn-containing superoxide dismutase activity appears as a wide band only in peak 2s (Plate 2, top arrow). It was observed only occasionally and faintly in the cytosol fractions and peak 2f, but never in peaks 1, 3s or 3f. The small amount of mitochondrial enzyme observed in the cytosol fractions was possibly released from the mitochondria during homogenization. The small amount observed in the specific-granule fraction (peak 2f) could be due to contamination of this fraction with mitochondria, owing to the close proximity of the bands. The Cu+Zn-containing enzyme was observed as a wide band only in the cytosol fractions (Plate 2, bottom arrow). The weak Cu+Zn-containing activity occasionally observed in peaks 1, 2s and 2f was possibly carried over from the cytosol fractions during gradient fractionation or was due to the adherence of small amounts of superoxide dismutase to the specific granules (peak 2f). The enzyme activity observed in peaks 3s and 3f is discussed below.

Distribution of superoxide dismutase activity quantitatively defined by the cytochrome c spectrophotometric assay

Table 1 shows the apparent superoxide dismutase activities observed with the cytochrome *c* assay in the fractions obtained by differential centrifugation of homogenized neutrophils. Apparent activity was

Table 1. *Apparent superoxide dismutase activity in neutrophil fractions obtained by differential centrifugation*

This is a representative experiment carried out with 2.4×10^8 neutrophils. Superoxide dismutase activity was measured by the cytochrome *c* assay. Abbreviations: CH, cell homogenate of neutrophils in 0.34M-sucrose; 126P and 126S, pellet and supernatant of a 126g, 15 min centrifugation of the cell homogenate; 20000P and 20000S, pellet and supernatant of a 20000g 20 min centrifugation of the 126S fraction. For details of the fractionation procedure, see the Methods section.

Fraction	Superoxide dismutase activity			Protein (mg)
	(units)	(% of CH)	(% of 126S)	
CH	204.0	100.0	—	12.30
126P	70.5	34.6	—	5.25
126S	97.0	47.5	100.0	7.12
20000P	78.0	—	80.6	3.00
20000S	70.5	—	72.9	4.87

Fig. 1. *Histogram of apparent superoxide dismutase activity in the linear sucrose gradient shown in Plate 1* Superoxide dismutase activity was measured by the cytochrome *c* spectrophotometric assay. The broken line indicates average of total superoxide dismutase activity divided by the 54 gradient fractions. 1, 2s, 2f, 3s and 3f refer to the tops of the peaks described in Plate 1.

observed in all fractions, with more than 50% of the enzyme activity recovered from the 126S fraction observed in the 20000P granule fraction. This was inconsistent with the low amount of activity seen with Nitro Blue Tetrazolium-stained polyacrylamide gels of this fraction (results not shown).

To identify the source of apparent superoxide dismutase activity in the 20000P granule fraction, cytochrome *c* assays were next performed on the isopycnic sucrose-gradient fractions. Fig. 1 shows apparent activity, in a fractionated sucrose gradient, located in the cytosol fractions, in the mitochondrial fraction (peak 2s), and in the azurophil granules

EXPLANATION OF PLATE I

Linear sucrose (30–53%, w/w) gradient of the 126g supernatant of homogenized neutrophils

(a) Photograph of gradient. (b) Absorbance (—) and sucrose (----) profiles of gradient. In (a) 15 mg of protein was applied to the top of the gradient. In (b) 54 fractions (1 ml each) were studied. Key to peaks: 1, membranous fraction; 2s, mitochondrial fraction; 2f, specific granules; 3s, light azurophil granules; 3f, heavy azurophil granules. For details of the experimental design, see the Results section.

EXPLANATION OF PLATE 2

Nitro Blue Tetrazolium-impregnated polyacrylamide gels of isopycnically separated neutrophil fraction
 Gels were loaded with 280, 250, 220, 335, 335 and 380 μ g of protein respectively for fractions CYT, 1, 2s, 2f, 3s and 3f. CYT, top three cytosol fractions from top of sucrose density gradient; other samples are explained in the legend to Plate 1. The top arrow indicates the position of the mitochondrial superoxide dismutase activity; the bottom arrow indicates the cytoplasmic enzyme activity. Electrophoresis was from top (-) to bottom (+). The dark line at the bottom of the gels is the tracking dye.

(peaks 3s and 3f). No significant enzyme activity was observed in the membranous (peak 1) or specific-granule (peak 2f) fractions. Heating the sucrose-gradient fractions in boiling water for 15 min abolished apparent superoxide dismutase activity. The cytoplasm and peak-1 pooled fractions together contained approx. 26% of the superoxide dismutase activity found in the whole gradient. Peaks 2s, 2f, 3s and 3f contained respectively 12, 6, 33 and 23% of the enzyme activity. (For the number of tubes contributing to each pooled fraction, see the Methods section.) Approx. 50% of the apparent superoxide dismutase activity was therefore located in the azurophil granules (peaks 3s and 3f). This was consistent with the enzyme activity observed in the 20000P granule fraction (Table 1).

It was surprising to find about one-half of the apparent superoxide dismutase activity in the azurophil granules (peaks 3s and 3f), since only occasional, barely discernible, bands of enzyme activity had been observed on Nitro Blue Tetrazolium-stained polyacrylamide gels of these fractions (Plate 2). Several hypotheses were proposed to explain the above discrepancy: (1) an inhibitor of superoxide dismutase was present in azurophils that co-migrated and was concentrated with the enzyme on the polyacrylamide gels and thus prevented its activity from being expressed; (2) a component of the azurophil granules was acting as a superoxide dismutase as detected only by the cytochrome *c* assay or specifically not detected by the Nitro Blue Tetrazolium-stained polyacrylamide-gel assay; or (3) a component of the azurophil granules was somehow affecting the cytochrome *c* assay, making it appear as if superoxide dismutase were present (e.g. oxidizing ferrocycytochrome *c* or inhibiting the oxidation of xanthine). The first hypothesis was apparently false, since bovine erythrocyte superoxide dismutase gave a clear band on Nitro Blue Tetrazolium-stained gels when co-electrophoresed with azurophil-granule fractions. For the second hypothesis, much work was done with different detergents and different gel and electrophoresis conditions, with only faint superoxide dismutase activity ever being observed in the Nitro Blue Tetrazolium-stained gels of the azurophil granules. Also, as described below, no other superoxide dismutase-like activity was observed in azurophil granules after removal of myeloperoxidase. There are no reports in the literature suggesting that there is indeed another type of superoxide dismutase in neutrophils. The third hypothesis was most tempting, considering the plethora of enzymes present in the azurophil granules.

A number of purified neutrophil-granule proteins were thus tested to observe their effects on the cytochrome *c* assay. Lactoferrin, lysozyme, myeloperoxidase or granule fractions from the sucrose

gradient did not affect the oxidation of xanthine to urate by xanthine oxidase (results not shown). Specific granules (as shown above), lactoferrin or lysozyme did not influence the cytochrome *c* assay for superoxide dismutase. It soon became apparent that myeloperoxidase was the azurophil-granule enzyme that influenced the cytochrome *c* assay.

Influence of myeloperoxidase and H₂O₂ on the cytochrome c assay

Purified human myeloperoxidase produced apparent superoxide dismutase in the cytochrome *c* assay. This activity was linear with myeloperoxidase concentration and time. In 20000P granule fractions or azurophil-granule fractions (peaks 3s and 3f), containing no true superoxide dismutase, about 1300 units of myeloperoxidase activity correlated with 1 unit of apparent superoxide dismutase activity. We suspected that this activity might be caused by the oxidation of ferrocycytochrome *c* by myeloperoxidase, either alone or in conjunction with H₂O₂. Therefore myeloperoxidase and H₂O₂ were tested for their ability to oxidize 0.01% ferrocycytochrome *c*. (During the cytochrome *c* assay, H₂O₂ is produced by the oxidation of xanthine by xanthine oxidase.) Concentrations of H₂O₂ below 1 μM or quantities of myeloperoxidase below 1000 units/ml did not oxidize ferrocycytochrome *c*. When combined, these concentrations of H₂O₂ and myeloperoxidase together oxidized ferrocycytochrome *c* linearly with time, H₂O₂ concentration and myeloperoxidase concentration (Table 2).

To determine if both myeloperoxidase and a superoxide dismutase might be present together in the azurophil granules, we performed affinity chromatography by using monospecific rabbit anti-

Table 2. Oxidation of ferrocycytochrome *c* by H₂O₂ and human myeloperoxidase

Myeloperoxidase activity was measured by *o*-dianisidine oxidation. Ferrocycytochrome *c* oxidation is described in the Methods section. For details of the experimental design, see the Results section.

Reagent	Myelo- peroxidase (units)	H ₂ O ₂ (μM)	Ferrocycytochrome <i>c</i> oxidation (A ₅₅₀ /min)
Myelo- peroxidase	250	—	0
	500	—	0
	1000	—	0
H ₂ O ₂	—	0.88	0.000
	—	8.80	0.0020
	—	88.0	0.0070
H ₂ O ₂ +myelo- peroxidase	250	0.88	0.0032
	500	0.88	0.0065
	1000	0.88	0.0110

(human myeloperoxidase) antibody coupled to Sepharose 4B. First, to test the specificity of the column, a solution containing 12.5 units of bovine erythrocyte superoxide dismutase (measured before addition of the myeloperoxidase) and 2000 units of myeloperoxidase was passed through the affinity column. Superoxide dismutase activity was measured spectrophotometrically with cytochrome *c*, and myeloperoxidase was measured by *o*-dianisidine oxidation. At pH 8 the myeloperoxidase was quantitatively retained by the column, whereas the superoxide dismutase was not (Table 3).

In the next experiment, an acid extract of a mixed-granule fraction (20000P), containing 20000 units of myeloperoxidase and 13.3 units of apparent superoxide dismutase activity, was passed through the affinity column. Table 4 shows that all of the apparent superoxide dismutase activity of the 20000P granule extract was due to myeloperoxidase, since both the latter and the former enzyme activity were retained by the column at pH 8. In a similar experiment, the superoxide dismutase activity of the cytosol fractions (6.8 units, tubes 1–4) was due to true neutrophil superoxide dismutase, despite the presence initially of 100 units of myeloperoxidase in these fractions (Table 4). The superoxide dismutase that was applied to

the column was not retained and was recovered in the pH 8 effluent.

Distribution of KCN-resistant superoxide dismutase and other mitochondrial enzymes

Mitochondrial superoxide dismutase is resistant to inhibition by KCN, whereas the soluble enzyme is not, as was stated above for the Nitro Blue Tetrazolium-stained polyacrylamide gels. Thus to locate the mitochondria in the isopycnic sucrose gradients, fractions from the top of each peak were assayed in the cytochrome *c* assay, in the presence or absence of 0.1, 0.5 and 1.0 mM-KCN; 90–100% of the superoxide dismutase activity in peak 2s was resistant to these concentrations of KCN. The superoxide dismutase activity in all other fractions tested was 50–85% inhibited by 0.1 mM-KCN, and 100% inhibited by 0.5 mM-KCN. Commercial bovine erythrocyte superoxide dismutase was 90% inhibited by 0.1 mM-KCN.

We measured the distribution of mitochondrial marker enzymes isocitrate dehydrogenase and cytochrome oxidase in the sucrose gradient fractions. Cytochrome oxidase activity was only in peak 2s fraction in the sucrose gradient and was dependent on 0.01% Triton X-100 for maximal expression. Isocitrate dehydrogenase was present in both the cytosol fraction (tube nos. 1–4) and the mitochondria (peak 2s) and was dependent on NADP⁺ for activity. No isocitrate dehydrogenase activity was observed with NAD⁺ in the cytosol or mitochondria-containing fractions. Since NADP⁺-dependent isocitrate dehydrogenase in mouse and rat liver is located mainly in the cytosol (Ernster & Navazio, 1956), we were concerned that the NADP⁺-dependent enzyme activity that we were observing in the mitochondrial fraction (peak 2s) might be due to the cytosol enzyme adhering to the outside of the mitochondria. To distinguish between true mitochondrial isocitrate dehydrogenase and the soluble cytoplasmic enzyme, individual sucrose-gradient fractions were centrifuged as described in the Methods section. The results (Fig. 2) show that samples from peak 2s contained only sedimentable isocitrate dehydro-

Table 3. *Separation of human myeloperoxidase from bovine erythrocyte superoxide dismutase on an anti-(human myeloperoxidase) affinity column*

Myeloperoxidase activity was measured by *o*-dianisidine oxidation; superoxide dismutase activity was measured by the cytochrome *c* assay. For details of the experimental design, see the Results section.

	Total enzyme recovered (units)	
	Myeloperoxidase	Superoxide dismutase
Starting material (human myeloperoxidase + bovine erythrocyte superoxide dismutase)	3000	12.5
pH 8 column effluent	0	12.6

Table 4. *Separation of human neutrophil myeloperoxidase and human superoxide dismutase on an anti-(human myeloperoxidase) affinity column*

Myeloperoxidase activity was measured by *o*-dianisidine oxidation; superoxide dismutase activity was measured by the cytochrome *c* assay. For details of the experimental design, see the Results section.

	Mixed-granule (20000P) extract			Cytosol fractions from sucrose gradient		
	Myeloperoxidase (units)	Superoxide dismutase (units)	Protein (μ g)	Myeloperoxidase (units)	Superoxide dismutase (units)	Protein (μ g)
Starting material	20000	13.3	385	100	6.8	799
pH 8 column effluent	0	0	540	0	7.2	540

Fig. 2. Distribution of NADP^+ -dependent isocitrate dehydrogenase in the sucrose density gradient shown in Plate 1

Gradient fractions were centrifuged at 75000g for 30 min, and the supernatant (—) and pellet (---) fractions measured for activity. For details of the experimental design, see the Results section.

genase, and samples from the cytoplasmic fractions contained only the non-sedimentable enzyme. Activity of the mitochondrial (sedimentable) enzyme was dependent on Triton X-100 for expression, whereas the cytosol (non-sedimentable) activity was not. No isocitrate dehydrogenase activity was observed in the specific (peak 2f) or azurophil (peaks 3s and 3f) granules, indicating little or no contamination of these fractions with mitochondria. In all fractions of the sucrose gradient, succinate dehydrogenase and glutamate dehydrogenase activities were too low to be measured by the techniques used.

Discussion

We have confirmed the presence within human neutrophils of the two commonly found eukaryotic superoxide dismutases and have shown them to be in two distinct subcellular locations, namely the mitochondria (15%), and the cytoplasm (85%). Beckman *et al.* (1973) and DeChatelet *et al.* (1974) were the first to describe superoxide dismutase in human neutrophils, first describing only the KCN-insensitive and then (Johnston & DeChatelet, 1974) the KCN-sensitive enzyme. Salin & McCord (1974) confirmed the presence of both enzymes in the human neutrophil. The above investigations were performed with neutrophil fractions obtained by differential centrifugation, and the above authors acknowledged that the heterogeneity of their fractions obscured the precise subcellular location of superoxide dismutase. Hence, it was necessary to separate more cleanly

the particulate fractions of neutrophils from each other.

By our previously published procedure (Spitznagel *et al.*, 1974), a membranous fraction, a mitochondrial fraction, the specific granules and two populations of azurophil granules were separated from each other and from the cytosol of neutrophils. With the quantitative cytochrome *c* assay, superoxide dismutase activity was undetectable in the azurophil- or specific-granule fractions of human neutrophils, after specific removal from them of myeloperoxidase by affinity chromatography. The faint bands of Cu+Zn-containing superoxide dismutase activity observed with granule preparations in Nitro Blue Tetrazolium-stained polyacrylamide gels might have been due either to a small amount of enzyme located in the granules and undetected by the cytochrome *c* assay, or to cytosol enzyme adhering to the granules during homogenization of the cells. The mitochondrial superoxide dismutase observed could have been due to residual mitochondria contaminating the granule preparations, or due to enzyme released from mitochondria and adsorbed on the granules during homogenization. We do know that myeloperoxidase (located in the azurophil granules) produces no achromatic band in the Nitro Blue Tetrazolium-stained gels.

We measured superoxide dismutase quantitatively with a system involving an $\text{O}_2^{\cdot-}$ -generating system (i.e. xanthine and xanthine oxidase) and an indicator, cytochrome *c*, that was reduced by $\text{O}_2^{\cdot-}$. Superoxide dismutase competes for $\text{O}_2^{\cdot-}$ with cytochrome *c* and so decreases the rate of cytochrome *c* reduction. However, measurement of superoxide dismutase activity in whole cells or their fractions must be performed with caution, owing to unrelated enzymes that may interfere with any part of this complex assay. Thus an azurophil-granule component, myeloperoxidase, oxidized ferrocycytochrome *c* in a reaction that required H_2O_2 . H_2O_2 is produced during the cytochrome *c* assay by the oxidation of xanthine. No azurophil-granule fraction affected the rate of oxidation of xanthine to urate by xanthine oxidase, nor did azurophil fractions reduce ferri-cycytochrome *c*. The literature shows that cytochrome *c* is often used as a substrate for peroxidases. Chance (1950) showed that dog verdo-peroxidase (i.e. myeloperoxidase) oxidized ferrocycytochrome *c* in the presence of H_2O_2 . Once myeloperoxidase was removed from the azurophil-granule fractions, they lacked superoxide dismutase activity. It was concluded that myeloperoxidase mimics superoxide dismutase in the cytochrome *c*-xanthine-xanthine oxidase assay.

Some investigators have used cytochrome *c* to measure the amount of $\text{O}_2^{\cdot-}$ produced by resting and phagocytosing leucocytes (Babior *et al.*, 1973; Drath & Karnovsky, 1975; Johnston *et al.*, 1975; Weening

et al., 1975). Interpretation of these results obtained with phagocytosing neutrophils is complicated, because, during phagocytosis, myeloperoxidase from azurophil granules partially degranulate into the extracellular fluid (Leffell & Spitznagel, 1974). Thus myeloperoxidase would be expected to oxidize the ferrocyanochrome *c* used to indicate the presence of O_2^{--} . In this case, O_2^{--} production would be underestimated. It appears that the estimation of O_2^{--} production by resting cells that have not regurgitated myeloperoxidase would be correct.

The lack of superoxide dismutase activity in the membranous fractions of human neutrophils is of considerable interest. Many reports have shown that O_2^{--} is produced and excreted by intact leucocytes (Babior *et al.*, 1973; Drath & Karnovsky, 1975; Johnston *et al.*, 1975; Weening *et al.*, 1975). Because of the great amount of O_2^{--} found outside of the neutrophils, it might be supposed that superoxide dismutase is not available to the outside of the cell, is membrane-bound in a form inaccessible to its substrate, or is not a membrane-bound enzyme at all. This last hypothesis is now substantiated by the fact that we did not find appreciable enzyme activity in the membranous fractions of the sucrose gradients.

The concentration of the cytosol superoxide dismutase within neutrophils was calculated to be approx. 5–10 μM , which compared with 35 μM for chicken liver cells (Weisiger & Fridovich, 1973) and 23 μM for rat liver cells (Tyler, 1975). Calculations were performed by assuming that 1.2×10^9 neutrophils (the amount obtained from 450 ml of blood) contained about 200 units of true superoxide dismutase and about 425 μl of intracellular water, and that the molecular weight and specific activity of the superoxide dismutase were the same as for the enzyme reported by Weisiger & Fridovich (1973). In the 20000S cytosol fractions, which contain no granules and thus no myeloperoxidase, the concentration of superoxide dismutase is approx. 6–12 units/mg of protein. Johnston *et al.* (1975) reported 2.4 units of enzyme activity/mg of protein for human neutrophil-cell homogenates that had been cleared by centrifugation at 30000g.

The location and identification of mitochondria within human neutrophils have been difficult because peripheral neutrophils are short-lived 'end' cells whose mitochondria have begun to degenerate. Energy for phagocytosis is obtained from anaerobic glucose metabolism, and the granulocyte has little or no detectable tricarboxylic acid cycle (Cline, 1975). We have now resolved a neutrophil fraction containing mitochondria. The distribution of the mitochondrial marker enzymes isocitrate dehydrogenase and cytochrome oxidase within the sucrose gradients correlates with the mitochondrial Mn-containing superoxide dismutase located in the fraction of the gradient of density 1.160 g/ml. West *et al.* (1974) have

reported, in morphological studies, the appearance of 'mitochondrial remnants' in their gradients of human neutrophil granules at a density of 1.165 g/ml.

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