

## Subcellular localization of the superoxide-forming enzyme in human neutrophils.

B Dewald, ... , J T Curnutte, B M Babior

*J Clin Invest.* 1979;63(1):21-29. <https://doi.org/10.1172/JCI109273>.

### Research Article

The subcellular distribution of the superoxide ( $O_2^-$ )-forming enzyme in human neutrophils was investigated. Cells were activated by phorbolmyristate acetate or by opsonized zymosan, and were then fractionated by zonal-rate sedimentation at two different speeds. At high speed, the specific granules were resolved from the azurophilic and the membrane fraction, while at low speed, the azurophilic granules could be separated from fast-sedimenting particle aggregates. Under both conditions, the major portion of the  $O_2^-$ -forming activity (60--70% of the total) was found to be associated with the membrane fraction which was characterized by the presence of alkaline phosphatase, alkaline phosphodiesterase I, and acid aryl phosphatase. No significant  $O_2^-$ -forming activity was found in either specific or azurophilic granules. Some activity was present in the fastest sedimenting fractions which, as shown by electron microscopy, were heterogeneous and contained aggregated material which included membrane fragments. These fractionation results provide strong additional support for the current view that the activable  $O_2^-$ -forming system is localized in the plasma membrane of human neutrophils.

Find the latest version:

<https://jci.me/109273/pdf>



# Subcellular Localization of the Superoxide-Forming Enzyme in Human Neutrophils

BEATRICE DEWALD and MARCO BAGGIOLINI, *Research Institute Wander Ltd., Sandoz Research Unit, Wander Ltd., CH-3001 Berne, Switzerland*

JOHN T. CURNUTTE and BERNARD M. BABIOR, *Tufts University School of Medicine, New England Medical Center Hospital, Department of Medicine, Boston, Massachusetts 02111*

**ABSTRACT** The subcellular distribution of the superoxide ( $O_2^-$ )-forming enzyme in human neutrophils was investigated. Cells were activated by phorbol myristate acetate or by opsonized zymosan, and were then fractionated by zonal-rate sedimentation at two different speeds. At high speed, the specific granules were resolved from the azurophilic and the membrane fraction, while at low speed, the azurophilic granules could be separated from fast-sedimenting particle aggregates. Under both conditions, the major portion of the  $O_2^-$ -forming activity (60–70% of the total) was found to be associated with the membrane fraction which was characterized by the presence of alkaline phosphatase, alkaline phosphodiesterase I, and acid aryl phosphatase. No significant  $O_2^-$ -forming activity was found in either specific or azurophilic granules. Some activity was present in the fastest sedimenting fractions which, as shown by electron microscopy, were heterogeneous and contained aggregated material which included membrane fragments. These fractionation results provide strong additional support for the current view that the activable  $O_2^-$ -forming system is localized in the plasma membrane of human neutrophils.

## INTRODUCTION

On exposure to suitable stimuli, neutrophils undergo what is termed the "respiratory burst", a coordinated group of metabolic events which result in the generation of microbicidal agents by the partial reduction of oxygen (1, 2). The biochemical basis for the respiratory burst appears to be the activation of a flavoenzyme, dormant in resting cells, which catalyzes the reduction of oxygen to superoxide ( $O_2^-$ )<sup>1</sup> with

*Received for publication 19 May 1978 and in revised form 20 September 1978.*

<sup>1</sup>Abbreviations used in this paper:  $O_2^-$ , superoxide; PMA, phorbol myristate acetate; PBS, phosphate-buffered saline.

NADPH as the electron donor (3):  $2 O_2 + NADPH \rightarrow 2 O_2^- + NADP^+ + H^+$ .  $O_2^-$  then undergoes a series of secondary reactions which lead to the production of  $H_2O_2$ ,  $OH^\cdot$ , and perhaps other reactive oxidants. One or more of these oxidants is employed by the neutrophil for the destruction of ingested microorganisms.

Previous studies with homogenates from activated neutrophils have shown that the  $O_2^-$ -forming enzyme responsible for the respiratory burst is particle-bound and is pelleted at 27,000 g for 20 min (4). By electron microscopy, this fraction consists of plasma membrane fragments, azurophilic and specific granules, and occasional mitochondria. We have investigated the subcellular distribution of the  $O_2^-$ -forming activity with zonal centrifugation. We describe here experiments that show that the  $O_2^-$ -forming enzyme is a constituent of the membrane fraction of human neutrophils.

## METHODS

Reduced pyridine nucleotides, ferricytochrome *c* (Type VI), zymosan, and  $O_2^-$  dismutase were purchased from Sigma Chemical Co., St. Louis, Mo.  $O_2^-$  dismutase was also obtained from Truett Laboratories, Dallas, Tex. Phorbol myristate acetate (PMA) was from Consolidated Midland Corp., Brewster, N. Y.; 4-methylumbelliferyl-2-acetamido-2-deoxy- $\beta$ -D-glucopyranoside and 4-methylumbelliferone were from Koch-Light Laboratories Ltd., Colnbrook, Buckinghamshire, England; 4-nitrophenyl phosphate (disodium salt, 6 H<sub>2</sub>O), Perhydrol, *o*-dianisidine, and sucrose for density gradient centrifugation were from Merck AG, Darmstadt, West Germany; Triton X-100 was from Packard Instrument Co. Inc., Downers Grove, Ill.; *p*-nitrophenyl thymidine-5'-monophosphate (sodium salt) was from Calbiochem, San Diego, Calif.; and Macrodex (dextran 70, 6% in saline) was from Pharmacia Ltd., Uppsala, Sweden. Diaminobenzidine·4 HCl was purchased from Serva Ltd., Heidelberg, West Germany.

All solutions were made in double-distilled water. Two solutions of phosphate-buffered saline (PBS) were used: (a)  $Ca^{++}$ ,  $Mg^{++}$ -free PBS (composition: 137 mM NaCl, 2.7

mM KCl, 8.1 mM  $\text{Na}_2\text{HPO}_4$ , 1.5 mM  $\text{KH}_2\text{PO}_4$ , and (b) PBS which contained in addition to the above 0.9 mM  $\text{CaCl}_2$  and 0.49 mM  $\text{MgCl}_2$ .

Neutrophils were prepared from 150 ml whole blood obtained from normal volunteers by the method described previously (5). All steps after dextran sedimentation were performed at 4°C except when noted. The isolated cells were suspended in  $\text{Ca}^{++}$ ,  $\text{Mg}^{++}$ -free PBS at a concentration of  $10^8$  cells/ml. Granulocytes from two to three donors were pooled for zonal sedimentation experiments.

**Activation of neutrophils by PMA.** A stock solution of PMA in dimethylsulfoxide at a concentration of 2 mg/ml was prepared and stored in small aliquots at -60°C. Before use, the stock solution was diluted with PBS to give a concentration of 10  $\mu\text{g}/\text{ml}$ . One part of neutrophil suspension and 2.6 parts of PBS were preincubated separately in a rotating water bath (160 rpm) at 37°C for 10 min. After mixing, preincubation was continued for 5 min. Meanwhile, the diluted PMA solution was brought to 37°C. Activation was started by adding 0.4 parts of PMA solution to the neutrophil suspension. After 3 min, the reaction was stopped by diluting twofold with ice-cold  $\text{Ca}^{++}$ ,  $\text{Mg}^{++}$ -free PBS. The cells were centrifuged immediately at 400 g for 3 min at 4°C and resuspended in a small volume of 0.34 M sucrose adjusted to pH 7.4.

**Activation of neutrophils by zymosan.** Zymosan particles were opsonized as described by Cheson et al. (6), and finally suspended in PBS at a concentration of 10 mg/ml. Equal volumes of neutrophil and zymosan suspensions were pre-warmed separately at 37°C for 5 min and then mixed. Phagocytosis was allowed to proceed for 2 min and was stopped by addition of ice-cold  $\text{Ca}^{++}$ ,  $\text{Mg}^{++}$ -free PBS. The cells were centrifuged and resuspended in 0.34 M sucrose, pH 7.4, as above.

**Fractionation technique.** The cells were suspended in either 6 or 12 ml of 0.34 M sucrose, pH 7.4, depending upon the gradient volume chosen for zonal centrifugation, and homogenized at 0°C in a motor-driven glass homogenizer with a Teflon (E. I. Du Pont de Nemours & Co., Inc., Wilmington, Del.) pestle (7). A postnuclear supernate was prepared by centrifugation of the homogenate at 250 g for 10 min. Because the neutrophil subcellular particles tend to aggregate in sucrose solution (8), salt was added immediately after preparation by diluting with an equal volume of 0.308 M NaCl in 0.34 M sucrose. The suspension thus obtained was used as the starting material for gradient centrifugation. Zonal rate-sedimentation was performed in a B-XIV rotor (MSE 59144) operated by a MSE SS-65 ultracentrifuge (Measuring & Scientific Equipment, Crawley, Sussex, England). In some experiments, the initial conditions were identical with those described previously (9, 10) except that all sucrose solutions contained 0.154 M NaCl. For most experiments, a specially designed adapter<sup>2</sup> was used which reduces the volume of the standard B-XIV rotor from 650 ml to 272 ml. The initial conditions in the modified rotor were as follows: an 80-ml overlayer which consisted of 0.25 M sucrose in 0.154 M NaCl; a 10-ml sample in 0.34 M sucrose which contained 0.154 M NaCl; and a gradient made of eight 20-ml portions of 0.45, 0.5, 0.55, 0.6, 0.65, 0.7, 0.75, and 0.8 M sucrose in 0.154 M NaCl which rested on a cushion of 60% sucrose. The rotor was filled and emptied at a rate of 20 ml/min while rotating at 2,000 rpm. Sedimentation was carried out for 15 min at either 13,500 or 6,500 rpm as indicated. The rotor speed was

<sup>2</sup> Baggiolini, M., B. Dewald, and U. Bretz. 1978. A volume adapter for use in a B-XIV zonal rotor. Submitted for publication.

checked with a stroboscopic flash (Touro-Strob "Picrostrob", Mayer and Wonisch, 5760 Arnsberg, West Germany).

**Biochemical assays.** Protein was determined according to Miller (11). Peroxidase (10), lysozyme (12), and alkaline 4-nitrophenyl phosphatase (13) were assayed as described previously. Lactate dehydrogenase was measured by the method of Bretz et al. (14), except that a NADH concentration of 0.16 mM was used. Acid 4-nitrophenyl phosphatase was assayed at pH 5.3 with a substrate concentration of 5 mM essentially as described by Bretz and Baggiolini (10). N-acetyl- $\beta$ -glucosaminidase was determined in an incubation mixture which contained 0.05 M sodium citrate buffer, pH 5.0, 0.5% Triton X-100, 5 mM 4-methylumbelliferyl-2-acetamido-2-deoxy- $\beta$ -D-glycopyranoside, and sample. Incubation was carried out at room temperature for 30 min and was stopped by adding 3 ml of a 0.05 M glycine-NaOH buffer, pH 10.4. Liberated 4-methylumbelliferone was measured fluorimetrically. Alkaline phosphodiesterase I was assayed as described by Edelson and Erbs (15) with minor modifications. Incubation mixtures contained, in a total volume of 0.6 ml, 3.3 mM *p*-nitrophenyl thymidine-5'-monophosphate, 0.067 M glycine-NaOH buffer, pH 9.6, 1.3 mM zinc acetate, 0.033% Triton X-100, and enzyme. After incubation at 37°C for 3 h in the dark, the reaction was stopped by addition of 0.4 ml 0.2 N NaOH. As in the case of mouse peritoneal macrophages (15), alkaline phosphodiesterase I of human neutrophils was zinc-dependent. It was completely inhibited by 0.3 mM EDTA, and its activity was fully restored by subsequent addition of 1.3 mM zinc acetate. A possible contribution by alkaline phosphatase was excluded by showing that alkaline phosphodiesterase I activity was not affected by 0.5 mM tetramisole, which inhibits alkaline phosphatase by >95%.  $\text{O}_2^-$  production was determined according to method B of Curnutte et al. (16), except that 0.065 M potassium phosphate buffer, pH 7.0, was used, and the reference cuvet contained 60  $\mu\text{g}$   $\text{O}_2^-$  dismutase. In 7 of 12 experiments, the particulate material was concentrated before determination of the  $\text{O}_2^-$ -forming enzyme. An aliquot of each gradient fraction ( $\approx 80\%$  of the total vol) was centrifuged at 100,000 g for 45 min, and the pellet was resuspended in a small volume of 0.34 M sucrose, pH 7.4. For the calculation of recovery, sample activity was determined in an aliquot of the postnuclear supernate which was treated in the same way. In the remaining experiments,  $\text{O}_2^-$  production was assayed directly in aliquots of the fractions that were obtained from the gradient. Similar distribution profiles of  $\text{O}_2^-$ -forming activity were obtained with both methods.

**Electron microscopy.** Aliquots of gradient fractions (1.0 ml) were processed for electron microscopy according to the random-sampling technique of Baudhuin et al. (17). The pellicles obtained were washed with 0.1 M sodium cacodylate buffer and then reacted for peroxidase (18). Further processing and electron microscopy were according to standard techniques (10).

## RESULTS

**Activation of the  $\text{O}_2^-$ -forming enzyme.** In most experiments, neutrophils were activated by brief exposure to PMA (1  $\mu\text{g}/\text{ml}$ ). By this treatment, the  $\text{O}_2^-$ -forming activity of postnuclear supernates increased, on average, about six-fold over the resting levels (Table I). The specific activities of a number of marker enzymes in postnuclear supernates from PMA-activated cells are also shown. Similar values were obtained

TABLE I  
Specific Activities of Enzymes in the Postnuclear Supernate

Enzyme	PMA treatment	Assay conditions			Specific activity
		Temperature	pH	Substrate concentration	
		°C		mM	
O <sub>2</sub> <sup>-</sup> -dependent cytochrome <i>c</i> reduction	-	25	7.0	0.16	1.2 (2)
	+				7.3±3.6 (6)
Lactate dehydrogenase	+	25	7.5	0.4	1324±414 (5)
Alkaline phosphatase	+	20-22	9.75	1.25	17.1±7.6 (6)
Acid 4-nitrophenyl phosphatase	+	20-22	5.3	5.0	112.3±10.1 (5)
Alkaline phosphodiesterase I	+	37	9.6	3.3	0.8±0.2 (3)
Lysozyme	+	25	6.0	—	51.2±6.3 (5)
N-Acetyl-β-glucosaminidase	+	20-22	5.0	5.0	14.6±4.1 (5)
Peroxidase	+	20-22	5.5	0.07	1,860±600 (6)

Except for lysozyme, values given mean specific activity in mU/mg protein ± SD with the number of preparations in parentheses. One unit is defined as the amount of enzyme which changes 1 μmol of substrate/min under the conditions given (Methods). The value for lysozyme denotes equivalents in μg of crystalline hen egg-white lysozyme.

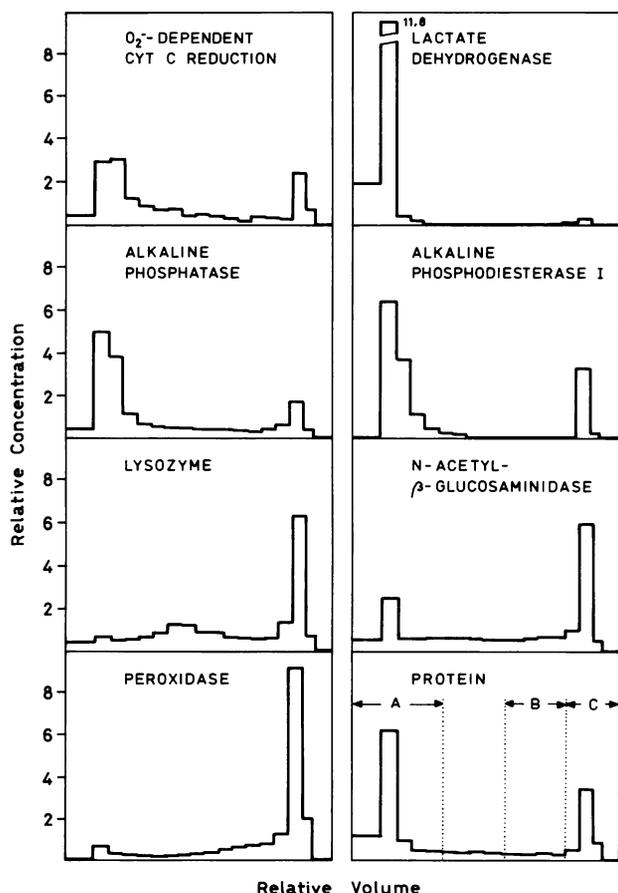


FIGURE 1 Fractionation of subcellular components of PMA-activated human neutrophils by zonal sedimentation at 13,500 rpm for 15 min. The graphs are normalized distribu-

with resting cells, thus indicating that the PMA treatment affected the O<sub>2</sub><sup>-</sup>-forming system only. PMA is known to induce release of specific granule contents (19, 20). Because activation was very short, only minor amounts of lysozyme were released. Characteristic vacuole formation (21, 22) was seen on electron micrographs. In some instances, we also activated neutrophils with zymosan. In our case, however, zymosan was not considered to be a convenient stimulus as the formation of phagosomes could render the interpretation of fractionation experiments unnecessarily complicated.

**Subcellular fractionation.** Postnuclear supernates from PMA-activated neutrophils were first fractionated by zonal sedimentation under conditions which assured optimum resolution of azurophil and specific granules from each other and from fractions that contained membranes (10). The results obtained are shown in Fig. 1. The distribution of azurophil and specific granules is represented by the histogram of

tion histograms as a function of the volume collected. The radial distance increases from left to right. The ordinate is the concentration in the fraction relative to the concentration that corresponds to uniform distribution throughout the gradient. The O<sub>2</sub><sup>-</sup>-dependent cytochrome *c* reduction was measured in original fractions which were collected from the gradient (Methods). The percentage recoveries were 68 for the O<sub>2</sub><sup>-</sup>-producing enzyme, 85 for lactate dehydrogenase, 90 for alkaline phosphatase, 62 for alkaline phosphodiesterase I, 149 for lysozyme, 72 for N-acetyl-β-glucosaminidase, 103 for peroxidase, and 81 for protein. A, B, and C in the right graph, bottom row, designate the portions of the gradient used for calculation of the enzyme distributions which are presented in Table II. Cyt, cytochrome.

lysozyme, which is a constituent of both particles. Most of the azurophil granules were found at the outer limit of the gradient, as demonstrated by the distribution of peroxidase, while specific granules were enriched in the middle of the gradient. Alkaline phosphatase and the thiol-sensitive acid aryl phosphatase (not shown), both previously identified as components of the membrane fraction (10), and alkaline phosphodiesterase I, a plasma membrane marker in other cells (15, 23), were all well separated from the markers of the two granule populations. The membrane fraction, however, was only slightly resolved from the sample zone as indicated by the distribution of the cytosol marker, lactate dehydrogenase. In this type of fractionation, the distribution of the PMA-activated,  $O_2^-$ -forming activity was always bimodal, as seen in Fig. 1. A major portion of the total activity was found on top of the gradient, with a distribution similar to that of the two membrane markers, alkaline phosphatase and alkaline phosphodiesterase I; on average, however, about one-fourth of the activity (Table II) was recovered at the cushion along with the bulk of the azurophil granules. Only minor amounts of  $O_2^-$ -forming activity were found to migrate with the specific granules in the middle of the gradient. To separate the membranous material from the cytoplasmic constituents, aliquots of the fractions were centrifuged at 100,000  $g$  for 45 min (Methods). The  $O_2^-$ -forming enzyme, together with the membrane markers, was recovered in the pellet. This resulted in a four- to sevenfold increase in specific activities.

These results strongly suggest that the  $O_2^-$ -forming enzyme is associated with membrane fragments. To gain more information about the localization of that portion of  $O_2^-$ -forming activity that was recovered

at the cushion, similar experiments were performed at 6,500 rpm, which corresponds to about one-fourth of the centrifugal force originally applied. The results of one of these low-speed experiments are presented in Fig. 2. As shown by the distribution of peroxidase, the azurophil granules were now recovered in the middle of the gradient, at a position occupied by the specific granules in the experiments discussed above (Fig. 1). This is confirmed by the distribution of lysozyme, whose location shows in addition that under these conditions azurophil and specific granules were not completely resolved from each other. The bulk of the  $O_2^-$ -forming activity was again found on top of the gradient. As shown in Fig. 2, its distribution histogram is very similar to that of the two membrane phosphatases. All three profiles are well resolved from that of the azurophil granule marker, peroxidase. Even under low-speed conditions, about one-sixth of the total  $O_2^-$ -forming activity was still recovered at the cushion (Table II). This fraction also contained some peroxidase and alkaline phosphatase. The high sedimentation rate and the heterogeneous morphology (*vide infra*) indicate that this fraction consists mainly of aggregated material.

In both types of sedimentation experiments, a remarkable coincidence was observed between the distribution of the  $O_2^-$ -forming enzyme and that of the membrane markers: alkaline phosphatase, alkaline phosphodiesterase I, and acid aryl phosphatase (23). The  $O_2^-$ -forming activity was almost fully resolved from the specific granules at high speed, and from the azurophil granules at low speed. In Table II, the average relative amounts of  $O_2^-$ -forming activity, alkaline phosphatase, and peroxidase recovered in selected zones of the gradients (designated A, B, and C,

TABLE II  
Distribution of  $O_2^-$ -Forming Activity, Alkaline Phosphatase, and Peroxidase after Rate Sedimentation of Postnuclear Supernates from PMA-Stimulated Neutrophils

Rotor velocity	Activity measured	Percent of total activity*			
		A	B	C	A + B + C
<i>rpm</i>					
13,500	$O_2^-$ -dependent cytochrome <i>c</i> reduction	60.1±8.4	4.9±3.1	25.5±12.8	90.5±5.5
	Alkaline phosphatase	76.0±7.5	6.1±1.1	10.3±4.9	92.4±3.1
	Peroxidase	8.5±1.8	21.4±4.3	64.0±2.1	93.9±1.6
6,500	$O_2^-$ -dependent cytochrome <i>c</i> reduction	68.8±8.6	11.6±4.1	16.4±8.3	96.8±3.4
	Alkaline phosphatase	76.5±4.2	15.9±3.0	3.6±1.6	96.0±1.9
	Peroxidase	14.7±2.3	62.3±4.9	15.3±3.6	92.3±1.9

\* Percent of total activity recovered from the gradient. Mean values±SD from four experiments at both rotor velocities. A, B, and C represent the three gradient zones indicated in Fig. 1 (13,500 rpm) and Fig. 2 (6,500 rpm).

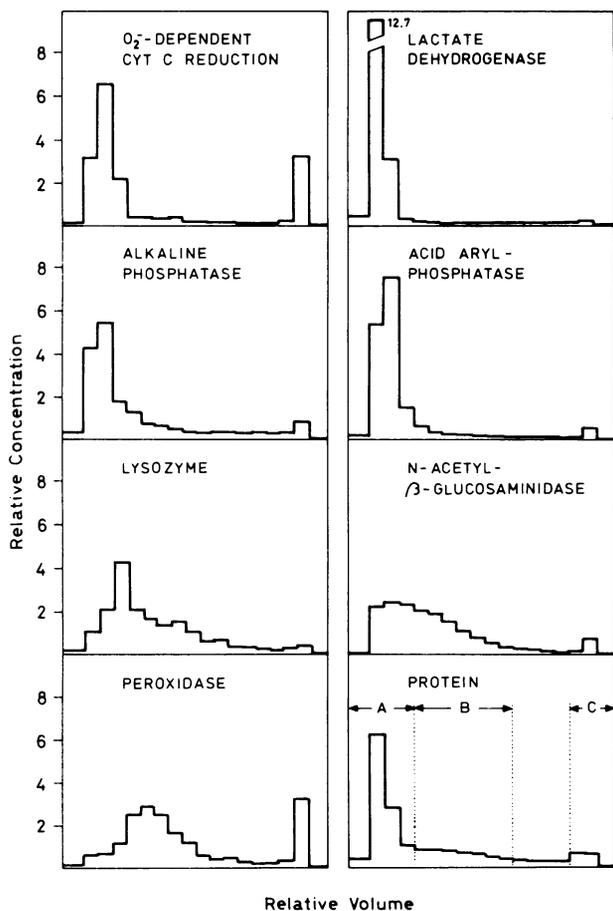


FIGURE 2 Fractionation of subcellular components of PMA-activated human neutrophils by zonal sedimentation at 6,500 rpm for 15 min. The results are presented as in Fig. 1. The  $O_2^-$ -dependent cytochrome *c* reduction was determined in the concentrated particulate material which was prepared from each fraction as described under Methods. The percentage recoveries were 57 for the  $O_2^-$ -producing enzyme, 101 for lactate dehydrogenase, 97 for alkaline phosphatase, 96 for acid aryl phosphatase, 88 for lysozyme, 99 for N-acetyl- $\beta$ -glucosaminidase, 122 for peroxidase, and 110 for protein. A, B, and C in the right graph, bottom row, designate the portions of the gradient used for calculation of the enzyme distributions which are shown in Table II. Cyt, cytochrome.

in Figs. 1 and 2) are presented. The values for the  $O_2^-$ -forming enzyme were similar to those of alkaline phosphatase in A and B under both experimental conditions, and were clearly different from those of peroxidase. Fraction C (material accumulated at the cushion) contained an appreciable fraction of the  $O_2^-$ -forming activity (25 and 16% in high and low speed experiments, respectively) which exceeded that of alkaline phosphatase. This may indicate that membrane fragments that contain the activated  $O_2^-$ -forming enzyme tend to form aggregates more readily.

Two experiments, one at 13,500 rpm and one at

6,500 rpm, were performed with zymosan-activated neutrophils. Distribution profiles were obtained which were almost identical with those described for the corresponding experiments with PMA-activated cells. No zymosan particles were found in the fast-sedimenting fractions as disclosed by electron microscopy, thus indicating that no phagosomal vacuoles were present in the postnuclear supernate used as starting material for fractionation. This does not preclude the possibility that the preparation contained phagosomal membranes derived from disrupted phagocytic vacuoles during homogenization. Such phagosomal fragments are likely to distribute like other membranes during zonal sedimentation. It is, therefore, not surprising that similar results were obtained with both activation procedures.

In a further experiment, we made a direct comparison of the subcellular distribution of components from activated and resting cells prepared from the same neutrophil sample. Neutrophils were purified and divided into two equal portions, one of which was activated with PMA. Postnuclear supernates from both portions were then fractionated under identical conditions in two consecutive runs at 6,500 rpm. In both experiments, the distribution profiles of all enzymes tested, except for the  $O_2^-$ -forming enzyme, were similar to those shown in Fig. 2. As expected, very low absolute activities of  $O_2^-$ -dependent cytochrome *c* reduction were found after fractionation of the postnuclear supernate from resting cells. Moreover, the relative distributions of the  $O_2^-$ -forming activities that were obtained in the two experiments differed markedly. While the sedimentation profile obtained after PMA activation of the cells was similar to that shown in Figs. 2 and 3, that obtained from resting cells had a trimodal distribution: approximately 48 and 33% of the total  $O_2^-$ -forming activity was found in the membrane and azurophil granule-containing fractions, respectively, and about 15% was recovered in the fast-sedimenting fractions near the cushion, together with 15% of the total peroxidase activity.

*Electron microscopy.* The ultrastructural appearance of purified granules and of the membrane fractions obtained from postnuclear supernates of human granulocytes by subcellular fractionation is well known (10). In the present study, electron microscopy was used mainly to establish the nature of the material that collected at the cushion even at low speed, and which was always associated with some of the  $O_2^-$ -forming activity. At low speed, this material was fully resolved from the largest particles of neutrophil postnuclear supernates, the azurophil granules. Fig. 3 shows the distribution profiles which were obtained in one of these experiments. Survey micrographs of three selected fractions, which are marked on the histograms of Fig. 3, are presented in Fig. 4. These micro-

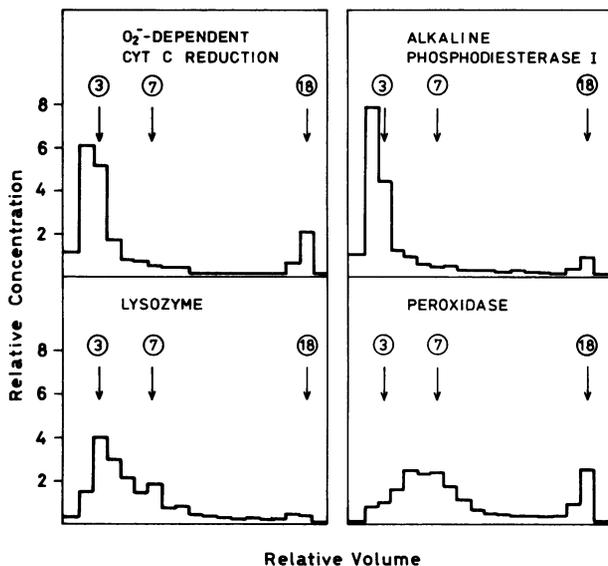


FIGURE 3 Fractionation of subcellular components of PMA-activated human neutrophils by zonal sedimentation. Conditions and graphs are as in Fig. 2.  $O_2^-$ -dependent cytochrome *c* reduction was determined in the original gradient fractions (Methods). The percentage recoveries were 83 for the  $O_2^-$ -forming enzyme, 81 for alkaline phosphodiesterase I, 84 for lysozyme, and 110 for peroxidase. The micrographs of fractions 3, 7, and 18 (arrows) are shown in Fig. 4. Cyt. cytochrome.

graphs show vertical sections through the particle pellicles which were obtained by the random-preparation technique of Baudhuin et al. (17), and allow, therefore, a representative assessment of the composition of the fractions processed. Fraction 18 (Fig. 4C) is very heterogeneous in appearance. It consists mainly of eosinophil granules (recognized by their characteristic peroxidase-negative crystalloid inclusions); membrane vesicles, often as components of heterogeneous clusters of particles; and apparently amorphous, electron-dense deposits of material which may have originated from disrupted nuclei. Some azurophil granules are also seen. Fig. 4B shows the composition of fraction 7, a sample from the zone of the peroxidase peak which has very little  $O_2^-$ -forming activity. As expected from the biochemical data, this fraction is enriched in azurophil granules. It is only slightly contaminated by other particles like mitochondria and membrane vesicles. As observed before (10), some azurophil granules appear partially extracted. Fig. 4A shows the composition of fraction 3, which belongs to the peak of  $O_2^-$ -forming activity. In accordance with the relative concentrations of the respective marker enzymes, this fraction is made up mainly of membrane fragments, which appear as morphologically empty vesicles of various sizes, and of specific granules with their characteristically uni-

form content of moderate electron density. Azurophil granules, very electron dense in appearance because of the peroxidase reaction product, and mitochondria are rare. The amorphous background material in the lower part of the pellicle is probably made up of constituents of the cytosol because fraction 3 is not fully resolved from the sample zone.

## DISCUSSION

Activation by stimuli such as PMA or contact with phagocytatable particles is a characteristic property of the  $O_2^-$ -forming enzyme of neutrophils. In this study, we report the subcellular localization of the activable enzyme in human neutrophils. We have used zonal rate-sedimentation, a well-established technique for subcellular fractionation of these cells (10), and have assayed for  $O_2^-$  dismutase-inhibitable, NADPH-dependent  $O_2^-$ -forming activity. The sedimentation profiles of the  $O_2^-$ -forming enzyme closely resemble those of alkaline phosphatase and acid aryl phosphatase, constituents of the membrane fraction of human neutrophils (10), and of alkaline phospho-

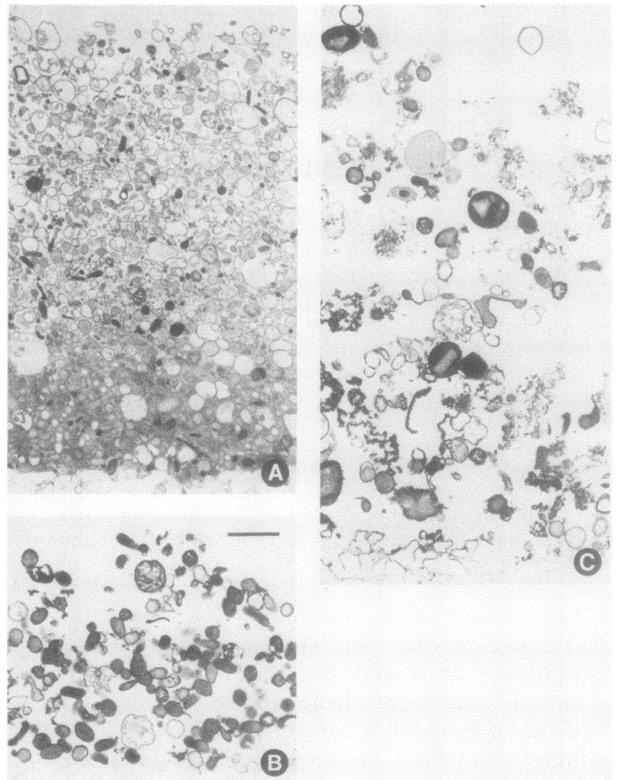


FIGURE 4 Survey electron micrographs of fractions 3 (A), 7 (B), and 18 (C) obtained in the zonal sedimentation experiment shown in Fig. 3. The filter face of pellicles (Methods) is at the bottom of the figures. All specimens were reacted for peroxidase. The bar in (B) corresponds to 1  $\mu$ m in all figures.

diesterase I, a plasma membrane enzyme in other cells (15, 23). These profiles are clearly resolved from those of both azurophil and specific granule markers. From these results, we conclude that the  $O_2^-$ -forming enzyme is a membrane constituent and is not present in either azurophil or specific granules. The fact that on the average, more  $O_2^-$ -forming activity than alkaline phosphatase was found in the fast-sedimenting, aggregated material (Table II) remains to be explained. The presence of some  $O_2^-$ -forming activity in eosinophil granules, which are present in the fast-sedimenting fractions from both the high-speed and low-speed gradients, cannot be ruled out entirely, but is unlikely, as no  $O_2^-$ -forming enzyme is present in the neutrophil granules.

In other cell types, alkaline phosphatase, acid aryl phosphatase, and alkaline phosphodiesterase I are considered as plasma membrane markers (23). Our fractionation data therefore support the hypothesis, based on functional considerations, that the  $O_2^-$ -forming enzyme is a constituent of the neutrophil plasma membrane. Definite proof of such a localization, however, must await purification of the plasma membrane fraction. In a first step, it has to be shown that the markers used are unequivocally associated with the plasma membrane in human neutrophils. Under the experimental conditions used, the membrane fraction is presumably heterogeneous. However, plasma membrane fragments are likely to represent the major component, because electron microscopy and morphometry<sup>3</sup> show that the plasmalemma is the most abundant membrane system in neutrophils. Some membrane fragments are expected to arise from PMA-induced vacuoles or from phagocytic vacuoles which are formed during zymosan ingestion. These vacuolar membranes are derived, at least in part, from internalized plasmalemma (22). Thus, the presence of  $O_2^-$ -forming activity in vacuolar membranes would not be inconsistent with a plasma-membrane localization of the enzyme.

Several earlier studies have suggested that the  $O_2^-$ -forming enzyme of the respiratory burst is located on the plasma membrane of the neutrophil. The cytochemical demonstration that  $H_2O_2$  was liberated at the surface of intact phagocytosing neutrophils led Briggs et al. to suggest the plasma membrane as a likely site for the respiratory burst oxidation reaction (24). Goldstein et al. (25) found significant diminution of  $O_2^-$ -forming activity by brief treatment of neutrophils with the nonpenetrating diazonium salt, *p*-diazobenzene sulfonic acid, before activation with concanavalin A. This result is in accord with the idea of a plasma membrane localization. The authors point out, however, that their finding could also be explained, without invoking

an exterior location for the  $O_2^-$ -forming system, by postulating that the diazonium salt interfered with some surface protein(s) responsible for activating the enzyme. Root and Metcalf (26) found that a non-penetrating  $O_2^-$  scavenger (ferricytochrome *c*) completely prevented the production of  $H_2O_2$  from  $O_2^-$  generated by neutrophils pretreated with cytochalasin B and then stimulated by bacteria. This indicates that the source of  $O_2^-$  had to be very close to the surface of the cell.

Besides these more indirect approaches, attempts have been made by several investigators to localize the enzyme responsible for the respiratory burst in subcellular fractions of neutrophils (27–29). Various, the activity has been attributed to the azurophil granule (27); to very dense particles distinct from azurophil granules which had hitherto not been described (28); and, depending upon the assay conditions, to the plasma membrane, the azurophil granule, or to several subcellular organelles (29). Comparison of these different results is complicated by the fact that in some cases (27, 29) resting neutrophils had been used, and that the methods for oxidase determination varied greatly. NADPH and NADH oxidase, as determined by the oxidation of the reduced pyridine nucleotide, were localized to the azurophil granules from resting cells by Segal and Peters (29), and to particles denser than azurophil granules from zymosan-activated cells by Iverson et al. (28). In both cases, activity was absent from membrane fractions. In the experiments of Iverson et al. (28), activity measurements were made with either gradient fractions that were dialyzed overnight against PBS, or with pooled fractions pelleted by centrifugation and resuspended in PBS. Under these conditions, it is possible that the authors were not measuring “activated” enzyme, because in our study, the  $O_2^-$ -forming activity of fractions prepared from zymosan- or PMA-stimulated cells which were homogenized in PBS rather than in 0.34 M sucrose, rapidly decreased to resting levels. Furthermore, the possibility arises that preparations were used for gradient centrifugation which contained the membrane fragments in a highly aggregated state. This could explain the occurrence of the NADPH oxidase in very dense particles. We could not confirm the results of Iverson et al. (28). In preliminary experiments in which we measured NADPH oxidase by following the decrease in OD at 340 nm,<sup>4</sup> we found a major portion of the activity to be associated with the membrane fraction on top of the gradient, while another portion was recovered from the fast-sedimenting fractions which contained the aggregated material. The distribution profile obtained in a rate sedimentation experiment at 6,500 rpm resembled

<sup>3</sup> Baggiolini, M., and B. Streit. Unpublished data.

<sup>4</sup> Dewald, B. Unpublished data.

that of the NADPH-dependent cytochrome *c* reduction. Segal and Peters (29), with preparations from resting cells, also determined NAD(P)H-dependent cytochrome *c* reduction. They found a complex distribution with activity in the plasma membrane, mitochondria, and the cytosol fraction. These results are not very different from our findings with resting neutrophils, when one takes into account that different fractionation systems were used. We also obtained a trimodal distribution, with low activity (in absolute terms), in the region of the gradient that contained the membrane fragments, azurophil granules, and agglutinated material (Results). However, when components of activated neutrophils were fractionated under the same conditions, the major portion of the total activity was always localized in the membrane fraction, as discussed above. Segal and Peters (29) described the distribution of yet another activity, a NADH-dependent nitroblue tetrazolium reductase, which they found to be associated with the plasma membranes. This enzyme exhibited no activity with NADPH. It therefore does not appear to be related to the O<sub>2</sub><sup>-</sup>-forming enzyme, which has a well known preference for NADPH (4), but rather resembles the NADH oxidase described by Briggs et al. (24).

Taken together, the fractionation data presented in this paper, and the results of experiments with undisturbed cells by Briggs et al. (24), Goldstein et al. (25), and Root and Metcalf (26), strongly support the hypothesis that the activable O<sub>2</sub><sup>-</sup>-forming enzyme of human neutrophils is associated with the plasma membrane. Such a localization has important functional implications. The phagocytic vacuole in which a microorganism is segregated is produced by internalization of plasma membrane, which forms the wall of the vacuole. The O<sub>2</sub><sup>-</sup>-forming enzyme, which is a constituent of this vacuole wall, is thereby placed in close apposition to the ingested organism. This spatial arrangement assures that the highly reactive and short-lived microbicidal products of the respiratory burst are delivered onto the target organism with maximum efficiency.

## REFERENCES

- Klebanoff, S. J. 1975. Antimicrobial mechanisms in neutrophilic polymorphonuclear leukocytes. *Semin. Hematol.* **12**: 117-142.
- Babior, B. M. 1978. Oxygen-dependent microbial killing by phagocytes. *N. Engl. J. Med.* **298**: 659-668, 721-725.
- Babior, B. M., and R. S. Kipnes. 1977. Superoxide-forming enzyme from human neutrophils: Evidence for a flavin requirement. *Blood.* **50**: 517-524.
- Babior, B. M., J. T. Curnutte, and B. J. McMurrich. 1976. The particulate superoxide-forming system from human neutrophils. Properties of the system and further evidence supporting its participation in the respiratory burst. *J. Clin. Invest.* **58**: 989-996.
- Curnutte, J. T., and B. M. Babior. 1974. Biological defense mechanisms. The effect of bacteria and serum on superoxide production by granulocytes. *J. Clin. Invest.* **53**: 1662-1672.
- Cheson, B. D., R. L. Christensen, R. Sperling, B. E. Kohler, and B. M. Babior. 1976. The origin of the chemiluminescence of phagocytosing granulocytes. *J. Clin. Invest.* **58**: 789-796.
- Hohn, D. C., and R. I. Lehrer. 1975. NADPH oxidase deficiency in X-linked chronic granulomatous disease. *J. Clin. Invest.* **55**: 707-713.
- Baggiolini, M., U. Bretz, and B. Dewald. 1978. Subcellular localization of granulocyte enzymes. In *Neutral Proteases from Polymorphonuclear Granulocytes—Physiology, Pathophysiology and Clinical Significance*. K. Havemann and A. Janoff, editors. Urban & Schwarzenberg, Inc., Baltimore, Md. In press.
- Baggiolini, M., J. G. Hirsch, and C. de Duve. 1969. Resolution of granules from rabbit heterophil leukocytes into distinct populations by zonal sedimentation. *J. Cell Biol.* **40**: 529-541.
- Bretz, U., and M. Baggiolini. 1974. Biochemical and morphological characterization of azurophil and specific granules of human neutrophilic polymorphonuclear leukocytes. *J. Cell Biol.* **63**: 251-269.
- Miller, G. L. 1959. Protein determination for large numbers of samples. *Anal. Chem.* **31**: 964.
- Baggiolini, M., J. G. Hirsch, and C. de Duve. 1970. Further biochemical and morphological studies of granule fractions from rabbit heterophil leukocytes. *J. Cell Biol.* **45**: 586-597.
- Bretz, U., and M. Baggiolini. 1973. Association of the alkaline phosphatase of rabbit polymorphonuclear leukocytes with the membrane of the specific granules. *J. Cell Biol.* **59**: 696-707.
- Bretz, U., M. Baggiolini, R. Hauser, and C. Hodel. 1974. Resolution of three distinct populations of nerve endings from rat brain homogenates by zonal isopycnic centrifugation. *J. Cell Biol.* **61**: 466-480.
- Edelson, P. J., and C. Erbs. 1978. Plasma membrane localization and metabolism of alkaline phosphodiesterase I in mouse peritoneal macrophages. *J. Exp. Med.* **147**: 77-86.
- Curnutte, J. T., R. S. Kipnes, and B. M. Babior. 1975. Defect in pyridine nucleotide dependent superoxide production by a particulate fraction from the granulocytes of patients with chronic granulomatous disease. *N. Engl. J. Med.* **293**: 628-632.
- Baudhuin, P., P. Evrard, and J. Berthet. 1967. Electron microscopic examination of subcellular fractions. I. The preparation of representative samples from suspensions of particles. *J. Cell Biol.* **32**: 181-191.
- Graham, R. C., and M. J. Karnovsky. 1966. The early stages of absorption of injected horseradish peroxidase in the proximal tubules of mouse kidney. Ultrastructural cytochemistry by a new technique. *J. Histochem. Cytochem.* **14**: 291-302.
- Estensen, R. D., J. G. White, and B. Holmes. 1974. Specific degranulation of human polymorphonuclear leukocytes. *Nature (Lond.)* **248**: 347-348.
- Goldstein, I. M., J. K. Horn, H. B. Kaplan, and G. Weissmann. 1974. Calcium-induced lysozyme secretion from human polymorphonuclear leukocytes. *Biochem. Biophys. Res. Commun.* **60**: 807-812.
- Repine, J. E., J. G. White, C. C. Clawson, and B. M. Holmes. 1974. Effects of phorbol myristate acetate on the metabolism and ultrastructure of neutrophils in chronic granulomatous disease. *J. Clin. Invest.* **54**: 83-90.
- White, J. G., and R. D. Estensen. 1974. Selective

- labilization of specific granules in polymorphonuclear leukocytes by phorbol myristate acetate. *Am. J. Pathol.* **75**: 45-60.
23. Solyom, A., and E. G. Trams. 1972. Enzyme markers in characterization of isolated plasma membranes. *Enzyme (Basel.)*. **13**: 329-372.
  24. Briggs, R. T., D. B. Drath, M. L. Karnovsky, and M. J. Karnovsky. 1975. Localization of NADH oxidase on the surface of human polymorphonuclear leukocytes by a new cytochemical method. *J. Cell Biol.* **67**: 566-586.
  25. Goldstein, I. M., M. Cerqueira, S. Lind, and H. B. Kaplan. 1977. Evidence that the superoxide-generating system of human leukocytes is associated with the cell surface. *J. Clin. Invest.* **59**: 249-254.
  26. Root, R. K., and J. A. Metcalf. 1977. H<sub>2</sub>O<sub>2</sub> release from human granulocytes during phagocytosis. Relationship to superoxide anion formation and cellular catabolism of H<sub>2</sub>O<sub>2</sub>: Studies with normal and cytochalasin B-treated cells. *J. Clin. Invest.* **60**: 1266-1279.
  27. Patriarca, P., R. Cramer, P. Dri, L. Fant, R. E. Basford, and F. Rossi. 1973. NADPH oxidizing activity in rabbit polymorphonuclear leukocytes: Localization in azurophilic granules. *Biochem. Biophys. Res. Commun.* **53**: 830-837.
  28. Iverson, D., L. R. De Chatelet, J. K. Spitznagel, and P. Wang. 1977. Comparison of NADH and NADPH oxidase activities in granules isolated from human polymorphonuclear leukocytes with a fluorometric assay. *J. Clin. Invest.* **59**: 282-290.
  29. Segal, A. W., and T. J. Peters. 1977. Analytical subcellular fractionation of human granulocytes with special reference to the localization of enzymes involved in microbicidal mechanisms. *Clin. Sci. Mol. Med.* **52**: 429-442.