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# PLASMA MEMBRANE AND PHAGOSOME LOCALISATION OF THE ACTIVATED NADPH OXIDASE IN ELICITED PERITONEAL MACROPHAGES OF THE GUINEA-PIG

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SUMMARY. The subcellular distribution of the NADPH oxidase of guinea-pig peritoneal-elicited macrophages was investigated. Postnuclear supernatants obtained from PMA-stimulated macrophages were fractionated in discontinuous sucrose gradients. The NADPH oxidase was found to be enriched at the interface between 20 and 34 per cent. sucrose. This interface was also enriched in 5'-nucleotidase, a plasma membrane marker and in glucose-6-phosphatase and NADPH-cytochrome *c* reductase, two endoplasmic reticulum markers. The distribution in the gradient of  $\beta$ -glucuronidase, a marker of lysosomes and of succinate dehydrogenase, a marker of mitochondria was clearly different from that of NADPH oxidase and of the markers of plasma membrane and of endoplasmic reticulum.

These results indicated that in stimulated-elicited macrophages the NADPH oxidase is associated with a membrane fraction. With the fractionation technique employed it was not possible to clarify whether the oxidase is located in the plasma membrane or in the endoplasmic reticulum. In order to clarify this matter the isolation of phagosomes was performed. NADPH oxidase was found to be enriched in the phagosomal fraction. Phagosomes were also found to be enriched in the plasma membrane marker 5'-nucleotidase. Glucose-6-phosphatase, a marker of endoplasmic reticulum, and  $\beta$ -glucuronidase, a marker of lysosomes were not enriched in the phagosomal fraction. The results obtained clearly suggest that the activated NADPH oxidase of peritoneal elicited macrophages of guinea pig is located in the plasma membrane.

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In the previous paper (Berton *et al.*, 1982) it has been shown that the stimulation of the respiratory metabolism in peritoneal-elicited macrophages of the guineapig is due to the activation of an oxidase that uses NADPH as substrate and reduces oxygen with the formation of  $O_2^-$ . The kinetic properties of this enzyme are similar to those of the oxidase described in human and guinea-pig granulocytes (Babior, Curnutte and McMurrich, 1976; Cohen, Chovaniec and Davies, 1980; Kakinuma and Kaneda, 1980) and in guinea-pig peritonealresident macrophages (Bellavite *et al.*, 1980).

The subcellular localisation of the oxidase in all the phagocytic cells so far investigated is controversial (Segal and Peters, 1977; Iverson *et al.*, 1978; Dewald *et al.*, 1979; Tauber and Goetzl, 1979; Cohen *et al.*; Rossi *et al.*, 1980). A clarification of this matter is necessary in order to understand the mechanism of its activation and the significance of the respiratory burst. The oxidase is in fact the main factor responsible for the generation of superoxide anion ( $O_2^-$ ), hydrogen peroxide ( $H_2O_2$ ), hydroxyl radical (OH<sup>•</sup>) and singlet oxygen ( $^{1}O_2$ ) that the phagocytic cells use as weapons against invading microorganisms (Klebanoff, 1975) and tumour cells (Clark and Klebanoff, 1975; Nathan, 1980).

The intensity and the effectiveness of these actions depend on equilibrium between the formation, the degradation and the discharge in the phagocytic vacuole, or, outside the cell, of the intermediates of the oxygen reduction. The equilibrium is linked to the activity of the oxidase, to the action of the enzymes catalysing the dismutation of  $O_2^-$  and the degradation of  $H_2O_2$  and to the site of production of  $O_2^-$  and the other reactive species.

We have investigated the last point by fractionating homogenates of macrophages stimulated by PMA and by isolating phagosomes. The results presented in this paper indicate that the oxidase activated by PMA or by phagocytosis is located in the plasma membrane.

### MATERIALS AND METHODS

# Collection, activation and homogenisation of macrophages

Peritoneal-elicited macrophages were obtained from guinea-pigs as previously described (Berton *et al.*). Macrophages were then stimulated with PMA and homogenised by the procedure previously described (Berton *et al.*). After homogenisation an aliquot of the total homogenate was withdrawn for enzymatic assays, the remainder was centrifuged for 5 min. at 400 g to remove nuclei, cell debris and intact cells. The postnuclear supernatant was used as the starting material for sucrose density gradient centrifugation.

Subcellular fractionation. Postnuclear supernatants obtained from  $10^7$  PMA-stimulated macrophages were carefully layered by using a Pasteur pipette on preformed sucrose gradients in 13 ml polyallomer tubes. The gradients had been made by layering 2.5 ml of 45 per cent. (w/w) 3 ml of 40 per cent., 3 ml of 34 per cent. and 3 ml of 20 per cent. sucrose solutions all containing 1 mM NaHCO<sub>3</sub> and 0.9 per cent. NaCl. Gradients were centrifuged at 28,000 r.p.m. for 120 min. at 4°C with a Beckman SW40 Ti rotor (g max. 140,000, g min. 60,000). At the end of the centrifugation the material at the interfaces between 20 and 34, 34 and 40 and 40 and 45 per cent. sucrose was collected by a Pasteur pipette. The pellet at the bottom of the tubes was resuspended in 0.34M sucrose containing 1 mM NaHCO<sub>3</sub> and 0.9 per cent. NaCl. All the fractions obtained and the total homogenate were sonicated with an MSE sonicator (two 5 s pulses at 150 W) before the enzyme assays.

Coating of Latex particles with PMA. 50  $\mu$ l of a Latex (0.81  $\mu$ m) suspension of 30 mg/ml were added to 1 ml of calcium and glucose-free Krebs-Ringer phosphate buffer, pH 7.4 (KRP) containing 10  $\mu$ g of PMA and incubated for 20 min. at 37°C. At the end of incubation the suspension was five-fold diluted with ice-cold KRP and centrifuged at 3000 g for 20 min. in a refrigerated centrifuge (4°C). After three washes PMA-coated Latex was resuspended to the original concentration (30 mg/ml) and stored for no more than 4 days at 4°C. Stored PMA-coated Latex was washed again before use. The suspension of PMA-coated Latex did not contain soluble PMA. In fact, after sedimentation of Latex beads the supernatant did not stimulate the oxidative metabolism of macrophages.

Isolation of phagosomes. The procedure for the isolation of phagosomes was essentially that described by Segal, Dorling and Coadl (1980).  $2 \times 10^8$  macrophages in 15 ml of KRP were incubated for 2-3 min. at 37°C with 150 µl of PMA- coated Latex (4.5 mg) with continuous stirring. At the end of the incubation the cell suspension was five-fold diluted with ice-cold KRP and centrifuged at 250 g for 7 min. Appropriate controls by light microscopy examination on May-Grunwald stained smears showed that after 2-3 min. of incubation practically all the macrophages had engulfed more than five Latex beads. The pellet was washed again and finally resuspended in 11 per cent. (w/w) sucrose containing 1 mM NaHCO<sub>3</sub> and 0.9 per cent. NaCl at a concentration of  $1.5 \times 10^8$  cells/ml. The cells were disrupted as previously described (Berton et al.). An aliquot of the homogenate was withdrawn for enzymatic assays, the remainder was brought to a sucrose concentration of 46 per cent. sucrose with 66 per cent. sucrose containing 1 mM NaHCO<sub>3</sub> and 0.9 per cent. NaCl. 5-5.5 ml of total homogenate in 46 per cent. sucrose was placed at the bottom of 13 ml polyallomer tubes and layered with 5.5 ml of 33 per cent. and 1.5 ml of 11 per cent. sucrose solutions all containing 1 mM NaHCO<sub>3</sub> and 0.9 per cent. NaCl. The tubes were then centrifuged at 12,000 r.p.m. for 30 min. with a Beckman SW40 Ti rotor (g max. 24,000, g min. 10,000). At the end of the centrifugation the material at the interface between 11 and 33 and 33 and 46 per cent. sucrose was collected by a Pasteur pipette. The 46 per cent. sucrose band together with the pellet at the bottom of the tubes were diluted to 11 per cent. sucrose, centrifuged at 100,000 g in a refrigerated centrifuge and then resuspended in 11 per cent. sucrose. All the fractions obtained were sonicated with an MSE sonicator (two 5 s pulse at 150 W).

### Electron microscopy of gradient fractions and of phagosomes

Aliquots of sucrose density gradient fractions or of phagosomes were centrifuged at 100,000 g for 20 min. The pellets were fixed for 30 min. at 4°C in 2 per cent. osmium tetroxide in 0.1M sodium cacodylate buffer, pH 7.2. After washing, the fixed pellets were dehydrated in graded ethanols and left overnight in 0.4 per cent. uranyl acetate in 70° ethanol. Embedding was carried out in Dow epoxy resin 332 (Lackwood, 1964). Ultrathin sections were double stained with uranylacetate and lead citrate (Venable and Coggeshall, 1966) and examined in a Philips EM 300 electron microscope.

### Biochemical assays

NADPH oxidase was assayed as  $O_2^-$  generating activity by continuous spectrophotometric monitoring of superoxide dismutase inhibitable cytochrome *c* reduction as previously described (Babior, Curnutte and Kipnes, 1975). Both reference and sample cuvette contained 0.065m na/K phosphate buffer *p*H 7.0, 0.170m sucrose, 0.1 mm cytochrome *c*, 0.15 mm NADPH and 2 mm NaN<sub>3</sub>. The reference cuvette also contained 30  $\mu$ g of superoxide dismutase.

NADPH cytochrome c reductase was assayed by continuous spectrophotometric monitoring of cytochrome c reduction (Sottocase et al., 1967) in the presence of superoxide dismutase. The assay mixture was the same as that used for the measurement of NADPH oxidase except that the sample cuvette contained 30  $\mu$ g superoxide dismutase and in the reference cuvette NADPH was omitted.

Succinate dehydrogenase was assayed by continuous spectrophotometric monitoring of cytochrome c reduction (Sottocasa et al.). Both reference and sample cuvette contained 0.05 M Na-phosphate buffer pH 7.4, 0.1 mM cytochrome c and 0.5 mM KCN. The sample cuvette also

contained 3 mM sodium succinate. The volume was 1 ml. The temperature 37°C. The reaction was started by adding the enzyme.

 $\beta$ -glucuronidase was assayed by measuring the amount of phenolphtalein released by phenolphtalein  $\beta$ -glucuronide. The assay mixture was made of acetate buffer 0.4M pH 5.2, 0.075 per cent. Triton X-100 and 0.05 mM phenolphtalein  $\beta$ -glucuronide. The volume was 1 ml. The temperature 37°C. After 30 min. of incubation the reaction was stopped by adding 2 ml of a mixture made of 0.266M glycine buffer pH 10.7, 0.134M NaCl and 0.166M Na<sub>2</sub>CO<sub>3</sub>. The tubes were then centrifuged at 3000 g for 20 min. and the absorption at 550 nm read against appropriate blanks in which the enzyme had been omitted.

5'-nucleotidase was assayed by measuring the Pi released by 5'-AMP (Widnell and Unkeless, 1968). The assay mixture was made of 0.05M Tris-HCl buffer pH 8.0, 0.01M MgCl<sub>2</sub>, 0.01M Na/K tartrate and 0.01M 5'-AMP. The volume was 1 ml. The temperature was  $37^{\circ}$ C. The reaction was started by adding the substrate to tubes containing the reaction mixture and the enzyme which had been pre-warmed at  $37^{\circ}$ C. Tubes without substrate or without enzyme were run in parallel. After 30 min. the reaction was stopped by adding 2 ml of a mixture made of 6 vol. of  $1 \text{ N H}_2$ SO<sub>4</sub> containing 0.42 per cent. ammoniumheptamolybdate and 1 vol. of 10 per cent. ascorbic acid. The tubes were then maintained for 20 min. at  $45^{\circ}$ C and centrifuged at 3000 g for 20 min. The supernatants were read at 820 nm against appropriate blanks in which enzyme and substrate were omitted. The O.D. of tubes without enzyme and without substrate was subtracted from the O.D. of the samples. Glucose-6-phosphatase was assayed by measuring the Pi released by glucose-6-phosphate (Harper, 1965). The assay mixture was made of 0.05M citrate buffer pH 6.0 and 0.04M glucose-6-phosphate. The volume was 1 ml. The temperature was  $37^{\circ}$ C. Pi release was assayed by the same procedure as in the 5'-nucleotidase assay.

Proteins were measured with the method of Lowry et al. (1951) using bovine serum albumin as standard.

For all the biochemical assays appropriate controls were made to exclude interference by sucrose in which subcellular fractions were suspended.

### Materials

Cytochrome *c*, type VI, SOD, type I from bovine blood, NADPH, type III, and NADH, grade III, PMA, were purchased from Sigma Chemical Co. (St Louis, Missouri). Other compounds were obtained from commercial sources at the highest available state of purity.

## RESULTS

### *Subcellular fractionation of PMA-stimulated macrophages*

Tables I and II show the distribution of marker enzymes in the four fractions obtained by centrifuging postnuclear supernatants of PMA-stimulated macro-phages in discontinuous sucrose gradients.

As shown in table I the subcellular fraction at the interface between 20 and 34 per cent. sucrose is enriched in 5'-nucleotidase, an enzyme marker of plasma membrane in a variety of cells (De Pierre and Karnovsky, 1973) including peritoneal macrophages of the mouse and guinea-pig (Werb and Cohn, 1972; Chauvet, Anteunis and Robineaux, 1980) and in glucose-6-phosphatase and in NADPH-cytochrome *c* reductase, markers of endoplasmic reticulum.

The data reported in table I also show that the distribution of succinate dehydrogenase, a marker of mitochondria, and of  $\beta$ -glucuronidase, a marker of lysosomes, is clearly different from that of the markers of plasma membrane and of endoplasmic reticulum. Mitochondrial marker was, in fact found to be

	Total		1					Y	
	homogenate	20/34	34/40	40/45	Pellet	20/34	34/40	40/45	Pellet
(0	$0.56 \pm 0.07$	$2.2 \pm 0.5$	$0.87 \pm 0.2$	$0.49\pm0.2$	$0.49 \pm 0.1$	$3.6 \pm 0.5$	$1.4 \pm 0.2$	$0.8 \pm 0.2$	$0.8 \pm 0$
es Pi	$0.86 \pm 0.1$	$3.4 \pm 0.3$	$2.2 \pm 0.1$	$1.20 \pm 0.1$	$0.90 \pm 0.3$	$3.9 \pm 0.4$	$2.6 \pm 0.3$	$1.4 \pm 0.3$	$0.8 \pm 0$
toles	$8.90 \pm 1.2$	$17.8 \pm 2.6$	$14.2 \pm 2.3$	$10.0 \pm 2.8$	$8.0 \pm 2.0$	$2 \cdot 0 \pm 0 \cdot 1$	$1.7 \pm 0.4$	$1 \cdot 1 \pm 0 \cdot 3$	$0.8 \pm 0$
es es	$11.7 \pm 1.4$	$6.6 \pm 1.9$	$20.2 \pm 2.7$	$10.3 \pm 2.7$	$1.3 \pm 0.4$	$0.6 \pm 0.2$	$1 \cdot 7 \pm 0 \cdot 2$	$0.9 \pm 0.3$	$0.1\pm 0$
lein	$148{\cdot}0\pm21{\cdot}0$	$56.4 \pm 17.0$	$184.3 \pm 29.0$	$310 \pm 50$	$145 \pm 17$	$0.4 \pm 0.1$	$1.3 \pm 0.2$	$2.1 \pm 0.2$	$0.9 \pm 0$
6	$6.8 \pm 1.6$	$28 \cdot 1 \pm 6 \cdot 0$	$16.9 \pm 5.0$	$9.6 \pm 0.7$	$6.4 \pm 1.2$	$4.3 \pm 0.8$	$2.5 \pm 0.7$	$1.6 \pm 0.4$	$0.8 \pm 0$

# NADPH OXIDASE LOCALISATION

TABLE I

Specific activities of marker enzymes in the four fractions obtained by centrifugation of postnuclear supernatants of guinea-pig-elicited peritoneal macrophages stimulated with PMA in discontinuous sucrose gradient

Enzymes 5'-nucleotidase (µmoles Pi released/30 min./mg proteins) Glucose-6-phosphatase (µmoles Pi released/30 min./mg proteins) NADPH-cyt. c. reductase (nmoles cyt. c. reduced/min./mg proteins) %-glucuronidase (y phenolphtalein released/30 min./mg proteins) NADPH-oxidase (mmoles O<sub>7</sub>/min./mg proteins) \* Specific activity of the fractions/specific activity of total homogenate. The means of four experiments  $\pm$  SEM are reported.

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### TABLE II

Per cent. distribution of marker enzymes in the four fractions obtained by centrifugation on discontinuous sucrose gradient of postnuclear supernatants of guinea-pig-elicited peritoneal macrophages

Enzymes	20/34	34/40	40/45	Pellet	Recoveries
5'-nucleotidase Glucose-6-phosphatase NADPH-cyt. <i>c</i> reductase	$\begin{array}{c} 43.0 \pm 13.0 \\ 59.3 \pm 18.0 \\ 27.0 \pm 8.0 \end{array}$	$   \begin{array}{r} 15 \cdot 6 \pm 2 \cdot 7 \\     46 \cdot 5 \pm 12 \cdot 0 \\     23 \cdot 5 \pm 4 \cdot 0   \end{array} $	$7.8 \pm 2.2 \\ 24.5 \pm 7.5 \\ 13.8 \pm 5.2$	$9 \cdot 2 \pm 1 \cdot 2 \\ 5 \cdot 0 \pm 2 \cdot 0 \\ 15 \cdot 8 \pm 1 \cdot 0$	$\begin{array}{c} 73 \cdot 2  \pm  14 \cdot 0 \\ 130 \cdot 4  \pm  35 \cdot 0 \\ 76 \cdot 2  \pm  11 \cdot 5 \end{array}$
Succinic dehydrogenase β-glucuronidase NADPH-oxidase Protein	$\begin{array}{c} 13 \cdot 3  \pm  2 \cdot 4 \\ 6 \cdot 8  \pm  3 \cdot 5 \\ 36 \cdot 7  \pm  5 \cdot 3 \\ 20 \cdot 6  +  5 \cdot 5 \end{array}$	$52.4 \pm 18.5 \\ 18.8 \pm 4.2 \\ 24.0 \pm 2.8 \\ 22.8 + 3.4$	$\begin{array}{c} 15 \cdot 8 \pm 3 \cdot 9 \\ 22 \cdot 8 \pm 1 \cdot 2 \\ 14 \cdot 2 \pm 5 \cdot 3 \\ 17 \cdot 6 + 3 \cdot 3 \end{array}$	$5 \cdot 9 \pm 2 \cdot 8 \\ 18 \cdot 2 \pm 2 \cdot 1 \\ 12 \cdot 1 \pm 1 \cdot 9 \\ 30 \cdot 2 + 6 \cdot 2 \\ \end{cases}$	$\begin{array}{c} 85 \cdot 5 \pm 20 \cdot 0 \\ 62 \cdot 0 \pm 5 \cdot 0 \\ 84 \cdot 0 \pm 7 \cdot 5 \\ 84 \cdot 0 + 7 \cdot 5 \end{array}$

Per cent. distribution was calculated by taking 100,000 g pellet of postnuclear supernatant as 100 per cent.

The average values of four experiments  $\pm$  SEM are reported.

enriched at the interface between 34 and 40 per cent. sucrose and lysosomal marker between 40 and 45 per cent. sucrose.

As shown in table II the subcellular fraction at the interface between 20 and 34 per cent. sucrose contains a high percentage (40–60 per cent.) of the total activity of plasma membrane and endoplasmic reticulum markers applied to the gradient, and only 7 and 13 per cent. of the total activity of lysosomal and mitochondrial markers respectively.

The non-resolution of the two types of membrane was probably due to the method of cell homogenization used, which causes the disruption of plasma membrane in small fragments, of density similar to that of endoplasmic reticulum (De Pierre and Karnovsky). Electron microscopy shows, in fact, that the interface between 20 and 34 per cent. sucrose is mainly composed of small and empty vesicles (fig. 1).

The distribution of the NADPH oxidase in the gradient follows the same pattern of 5'-nucleotidase, glucose-6-phosphatase and NADPH-cytochrome c reductase. In fact, the subcellular fraction at the interface between 20 and 34 per cent. sucrose shows the highest relative specific activity and the highest percentage of the NADPH oxidase.

The data presented above indicate that in guinea-pig elicited peritoneal macrophages, the activated enzyme responsible for the respiratory burst is located in a membrane fraction. However, by using this type of homogenisation and fractionation, we were not able to distinguish whether the enzyme is located in the plasma membrane or in the endoplasmic reticulum.

In an attempt to clarify this point we decided to isolate phagosomes.

# Isolation of phagosomes

For the isolation of phagosomes macrophages were challenged with Latex particles previously coated with PMA. These particles, which, as shown by light microscopy examination, are easily phagocytosed by macrophages, were



FIG. 1.—Survey electron micrograph of the fraction obtained at the interface between 20 and 34 per cent. sucrose by centrifuging in discontinuous sucrose gradient homogenates of PMA-stimulated macrophages. (×23,900).

used because they induce a stimulation of oxygen uptake much greater than non-opsonised and opsonised Latex particles.

It is worth pointing out that the fractionation procedure employed does not allow a purification of different subcellular organelles and membranes, but it has been adapted to isolate a small number of phagosomes in rather pure form. The electron microscopy examination (fig. 2) shows, in fact, that the fraction at the interface between 11 and 33 per cent. sucrose is made up of large vesicles containing Latex beads with very low numbers of contaminating small vesicles and other organelles. By contrast, the material at the interface between 33 and 46 per cent. sucrose and the pellet is more heterogeneous, containing all the types of organelles, vesicles, membranes and phagosomes.

## TABLE III

# Distribution of marker enzymes in subcellular fractions obtained from phagocytosing guineapig elicited peritoneal macrophages

Specific activities*	5'-nucleotidase	Glucose-6-phosphatase	$\beta$ -glucuronidase	NADPH-oxidase
11/33 (Phagosomes) 33/46 Pellet Homogenate	$\begin{array}{c} 1 \cdot 72 \pm 0.65 \\ 0 \cdot 86 \pm 0.15 \\ 0 \cdot 73 \pm 0.09 \\ 0 \cdot 43 \pm 0.11 \end{array}$	$\begin{array}{c} 1.78 \pm 0.60 \\ 1.58 \pm 0.38 \\ 1.48 \pm 0.34 \\ 0.92 \pm 0.17 \end{array}$	$\begin{array}{c} 101 \cdot 0 \pm 38 \cdot 2 \\ 95 \cdot 1 \pm 25 \cdot 5 \\ 73 \cdot 3 \pm 2 \cdot 5 \\ 85 \cdot 5 \pm 5 \cdot 2 \end{array}$	$\begin{array}{c} 88 \cdot 5 \pm 37 \cdot 3 \\ 34 \cdot 4 \pm 9 \cdot 8 \\ 23 \cdot 1 \pm 2 \cdot 8 \\ 12 \cdot 0 \pm 1 \cdot 7 \end{array}$
Relative specific activiti	es	×		
11/33 (Phagosomes) 33/46 Pellet	$\begin{array}{c} 4 \cdot 60 \pm 1 \cdot 70 \\ 2 \cdot 20 \pm 0 \cdot 40 \\ 1 \cdot 80 \pm 0 \cdot 2 \end{array}$	$\begin{array}{c} 1 \cdot 70 \pm 0.30 \\ 1 \cdot 70 \pm 0.30 \\ 1 \cdot 70 \pm 0.40 \end{array}$	$1 \cdot 2 \pm 0 \cdot 6$ $1 \cdot 1 \pm 0 \cdot 4$ $0 \cdot 9 \pm 0 \cdot 04$	$7.3 \pm 3.0 \\ 3.0 \pm 0.7 \\ 2.1 \pm 0.4$

\* Specific activities are expressed as in table I. Recoveries were:  $110 \pm 11$  for 5'-nucleotidase,  $108.5 \pm 6.9$  for glucose-6-phosphatase,  $97.5 \pm 8.9$  for  $\beta$ -glucuronidase,  $94.3 \pm 20.4$  for NADPH-oxidase and  $114.1 \pm 14.4$  for protein.

Average values of five experiments ± SEM are reported.



FIG. 2.—Survey electron micrographs of the fractions obtained by centrifuging in discontinuous sucrose gradient homogenates of macrophages challenged with PMA-coated Latex particles, (a) interface between 11 and 33 per cent. sucrose (phagosomes); (b) interface between 33 and 46 per cent. sucrose; (c) pellet. (×11,500).

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Table III reports the biochemical profile of the phagosomal fraction, of the fraction located at the interface between 33 and 46 per cent. sucrose, of the pellet and of the total homogenate. The data show that the relative specific activity of 5'-nucleotidase and of NADPH oxidase is much higher in the phagosomal fraction than in total homogenate and in the other two fractions. By examining the values of the relative specific activity it can be seen that in the phagosomal fraction the plasma membrane marker 5'-nucleotidase is enriched about five-fold and the NADPH oxidase about seven-fold.

On the contrary, glucose-6-phosphatase, a marker of endoplasmic reticulum and  $\beta$ -glucuronidase, a marker of lysosomes, are not significantly enriched in the phagosomal fraction. These results clearly suggest that the activated NADPH oxidase is associated with the plasma membrane and not with the membrane of endoplasmic reticulum.

### DISCUSSION

In the last few years many attempts have been made in different laboratories and by using different methods of cell homogenisation and fractionation to show directly the localisation of the oxidase responsible for the respiratory burst in phagocytic cells. Most of the studies have been performed in granulocytes and the results obtained do not agree. Evidence has been presented in granulocytes of different species that the enzyme is located in azurophilic granules (Patriarca et al., 1973), in particles of higher density than that of azurophilic granules (Iverson et al., 1978), in plasma membrane (Segal and Peters, 1977; Dewald et al., 1979; Rossi et al., 1980) or in other organelles (Tauber and Goetzl, 1979). The only study on macrophages is that of Andrew et al. (1978) who have shown that the oxidase in alveolar macrophages would be located in endoplasmic reticulum. The histochemical technique applied to intact granulocytes and based on the detection of H<sub>2</sub>O<sub>2</sub> (Briggs et al., 1975) does not allow any conclusions about the localisation of the oxidase because the peroxide diffuses far from the site of its production. The results presented in this paper show that by using a fractionation on a discontinuous gradient of sucrose we have obtained a good separation of plasma membrane and membranes of endoplasmic reticulum from mitochondria and from other organelles. The assay of the oxidase in those fractions has shown that the activated enzyme is associated with the fraction containing plasma membrane and membranes of endoplasmic reticulum. Localisation only in the plasma membrane has been shown in the second step of the experiments presented here, i.e. the isolation of a pure fraction of phagosomes. This finding deserves some comment.

It is worth pointing out that this is the first reported attempt to associate NADPH oxidase with the membrane of phagosomes. The data presented in this paper would suggest an answer to this problem. By comparing in the phagosome fraction the relative specific activity of an enzyme marker of the plasma membrane (5'-nucleotidase), whose activity does not change during the burst, with that of the oxidase it can be seen that the latter is higher than the former. The higher concentration of the oxidase with respect to 5'-nucleotidase in the

plasma membrane may be an indication that the NADPH oxidase is activated only in the portion of the plasma membrane that forms the phagosome.

The presence of the activated oxidase on the plasma membrane and, mostly in that part that has been stimulated by contact with the particles, is relevant to the significance of the respiratory burst. The defence mechanism against invading micro-organisms and tumour cells is linked to the reactivity of the intermediates of the oxygen reduction  $O_2^-$ ,  $H_2O_2$ ,  $OH^{\bullet}$  and singlet oxygen. The plasma membrane is in the best strategical position for the oxidase to produce a number of lethal weapons that are immediately discharged into the phagosome or at the site of contact with other surfaces including tumour cells or other target cells.

We do not know whether the activated oxidase is present in the plasma membrane because it is transferred from other particles, where it is located in the dormant state, or whether the dormant oxidase is primarily located in the plasma membrane before being activated. An answer to this problem, which is relevant to the mechanism of activation, will need further investigation.

In conclusion the respiratory burst that occurs in inflammatory macrophages during phagocytosis or following the interaction with soluble stimuli is linked to the activation of an oxidase that uses NADPH as physiological substrate and that directly forms superoxide anion. These results, including those showing the kinetic properties of the oxidase, are similar to those obtained in granulocytes of different species (Rossi and Zatti, 1964 and 1966; Rossi, Romeo and Patriarca, 1972; Babior *et al.*, 1976). The enzyme is located in the plasma membrane of macrophages, presumably in the phagosomal portion or at the site of contact with a membrane perturbing agent, in the best position for the utilisation of the products of the enzyme.

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