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Review Article

THE SUPEROXIDE-FORMING ENZYMATIC SYSTEM OF PHAGOCYTES

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Abstract—The formation of oxygen-derived free radicals by the phagocytes (neutrophils, eosinophils, monocytes and macrophages) is catalysed by a membrane-bound NADPH oxidase which is dormant in resting cells and becomes activated during phagocytosis or following interaction of the cells with suitable soluble stimulants. This enzyme is under investigation in many laboratories but its molecular structure remains to be clarified. Possible components such as flavoproteins, cytochrome b_{558}, and quinones have been proposed on the basis of enzyme purification studies, effects of inhibitors, kinetic properties and analysis of genetic defects of the oxidase. An extensive discussion of the evidence for the participation of these constituents is reported. On the basis of the available information on the structure and the catalytic properties of the NADPH oxidase, a series of possible models of the electron-transport chain from NADPH to O_2 is presented. Finally, the triggering mechanism of the respiratory burst is discussed, with particular reference to the stimulus-response coupling and the final modification(s) of the oxidase (phosphorylation, assembly, change of lipid environment, etc.) which are involved in its activation.

Keywords—Superoxide formation, NADPH oxidase, Phagocyte metabolism, Cytochrome b_{558}, Flavoprotein, Respiratory burst, Transduction systems, Chronic granulomatous disease

INTRODUCTION

The generation of oxygen-derived free radicals such as superoxide anion (O_2^-), hydroxyl radical ($OH\cdot$) and singlet oxygen ($^1O_2$) and of hydrogen peroxide (H_2O_2) represents one of the main systems by which phagocytes kill invading organisms and tumor cells and may cause other harmful effects in inflammatory processes.[1-6] These intermediates of oxygen reduction are formed by phagocytes (neutrophils, eosinophils, monocytes, and macrophages) during the engulfment of particulate matter or when the cells are stimulated by a variety of soluble compounds. The peculiar metabolic pathway of activated phagocytes is called "respiratory burst" because it was first described as a sudden increase of oxygen consumption occurring a few seconds after the interaction of the cell with the stimulatory agent.[7-12] Early studies indicated that this oxygen metabolic pathway is insensitive to cyanide and therefore is independent of mitochondrial respiration.[10-12] Following these observations, increasing efforts were addressed to the investigation of the phagocyte's respiratory burst.

In spite of the great body of information accumulated in the past 20 years, the nature of the free-radical generating system has yet to be clarified. While there is substantial agreement on the properties of the respiratory burst in intact cells and on its products and its function in host defense and inflammation, there is a series of newly generated questions concerning the structure of the enzyme(s) involved and the mechanism of its activation. Here the main body of knowledge regarding phagocyte metabolism will be summarized, consolidating detailed reviews which have been published on this topic.[13-19] Then, the most recent and often controversial reports on the nature of the enzymatic system which is responsible for the respiratory burst will be presented.

Born April 9, 1952 at Verona (Italy), Paolo Bellavite studied in Classic Liceo secondary schools, then took a medical degree and a specialization as hematologist at the University of Trieste. At present he is employed as Associate Professor of General Pathology at the University of Verona. His research area is the function, metabolism and pathology of leukocytes. He is member of the European Society for Clinical Investigation.

I. THE RESPIRATORY BURST OF PHAGOCYTIC CELLS

Oxidative metabolism was first studied in intact phagocytes. The increased oxygen consumption and free radical generation can be stimulated by a large series of phagocytozable and soluble substances capable of reacting with specific receptors present on the cell surface or by inducing other kinds of membrane modification. A representative list of the compounds and of the experimental conditions which activate the respiratory burst is reported in Table 1.

The increased respiration of phagocytozing cells is accompanied by other metabolic modifications including: (1) increase of glucose uptake[20] and catabolism through hexose monophosphate pathway (HMP),[21] (2) decrease of the NADPH:NAD^+ ratio[22] and GSH:GSSG ratio,[23] (3) production and release of hydrogen peroxide,[24] of superoxide anion,[25] of hydroxyl radical,[26] of singlet oxygen,[27] and (4) emission of photons of light (chemiluminescence).[28] Coincident with the activation of these systems, the intracellular pH undergoes rapid acidification, followed by gradual alkalinization, due to extrusion of H^+ through a receptor-activated Na^+/H^+ antiport.[29–32] All these biochemical events are strictly interrelated and take place almost simultaneously.[33] A scheme of these interrelationships is proposed in Figure 1.

Virtually all the extra-oxygen consumption due to functional activation by phagocytosis is converted first to O_2^-, then to H_2O_2 through the dismutation reaction:

$$O_2^- + O_2^- + 2H^+ \longrightarrow O_2 + H_2O_2 \quad (1.1)$$

It has been calculated that only a minor portion of O_2 that undergoes reduction can be recovered as O_2^- or H_2O_2 in the extracellular environment, because phagocytes utilize the products in phagocytic vacuoles or destroy them through their powerful scavenger systems represented by superoxide dismutase, catalase, glutathione peroxidase, ascorbate and tocopherol.[34–40]

The extent of these phenomena and the stoichiometries of O_2:O_2^-:H_2^- vary according to the experimental conditions, the cell type, and the stimulant employed. Challenge of neutrophils with maximum doses of opsonized zymosan or of phorol-myristate-acetate (PMA, one of the most powerful soluble stimulatory agents) triggers an oxygen consumption (and a corresponding free-radical production) in the order of 5–10 nmol/min/10^6 cells.[18] It is a common experience of investigators working with these cells to observe 10–20 million neutrophils in the Clark-oxygraph, which consume all the oxygen of the solution in a few minutes. Considerable differences in the extent of the oxidative metabolism among the various mononuclear phagocytes have been reported.[31,41] Blood monocytes are more active than differentiated macrophages.[42] Particular types of tissue macrophages, such as liver Kupffer cells, appear to be almost totally impaired in their oxidative response.[43] Release of O_2^- and H_2O_2 by pulmonary alveolar macrophages is very low, due to the high efficiency of their scavenger systems.[44] Under appropriate stimuli such as inflammation, endotoxins, or γ-interferon, resident macrophages may maturate to cell types which are more active in bactericidal and cytotoxic functions. One of the characteristic modifications of "activated" or "inflammatory" macrophages is a higher production of oxygen-derived free radicals.[45–50] Interestingly, a phagocytosis-associated respiratory burst has been described even in protozoa such as the amoebae.[51,52]

The time-course of the respiratory burst varies according to the stimulant used. Some stimulants, such as phagocytozable particles, induce a progressive increase of respiration that accompanies the engulfing act and ceases when phagocytosis is completed;[53] chemotactic peptides trigger an almost instantaneous increase of O_2 consumption that often shows biphasic kinetics;[54,55] phorbol esters cause a progressive and irreversible activation;[56] activation by sodium fluoride shows a very long lag time.[57] The metabolic activation is reversible upon removal of the activator, which can be accomplished by washing the cells,[57] by displacing the ligand from receptor with competitive substances,[58,59] or by oxidative inactivation of the activator itself.[54] Once deactivated, the oxidative metabolism can be reactivated by a second stimulus,[54,60] but under particular conditions a "desensitization" of the system may occur.[61–63] Desensitization is stimulus-specific and is probably due to either down-regulation or uncoupling of the receptorial system. Different from deactivation is the termination of the respiratory burst taking place as a consequence of auto-inactivation of the enzymatic system that generates free radicals. Such an inactivation is due to toxic effects of hydrogen peroxide and myeloperoxidase, which are released and accumulate during the activation phase.[64–66]

Considerable advances in the knowledge of the res-

Table 1. Stimulants of the Phagocyte Oxidative Metabolism

Bacteria	Opsonized zymosan
Latex particles	*N*-formyl-peptides
Oil droplets	Pyrogen
IgG-coated surfaces	Ca^{2+} ionophores
Immune complexes	Cytochalasins D and E
Complement fragments C5a, C567	Phospholipase C
Fatty acids	Concanavalin A
Tumor necrosis factor	Leukotriene B_4
Phorbol myristate acetate	Anti-leukocyte antibodies
Sodium fluoride	Low-Na^+ incubation buffer
Diacylglycerol	Platelet activating factor

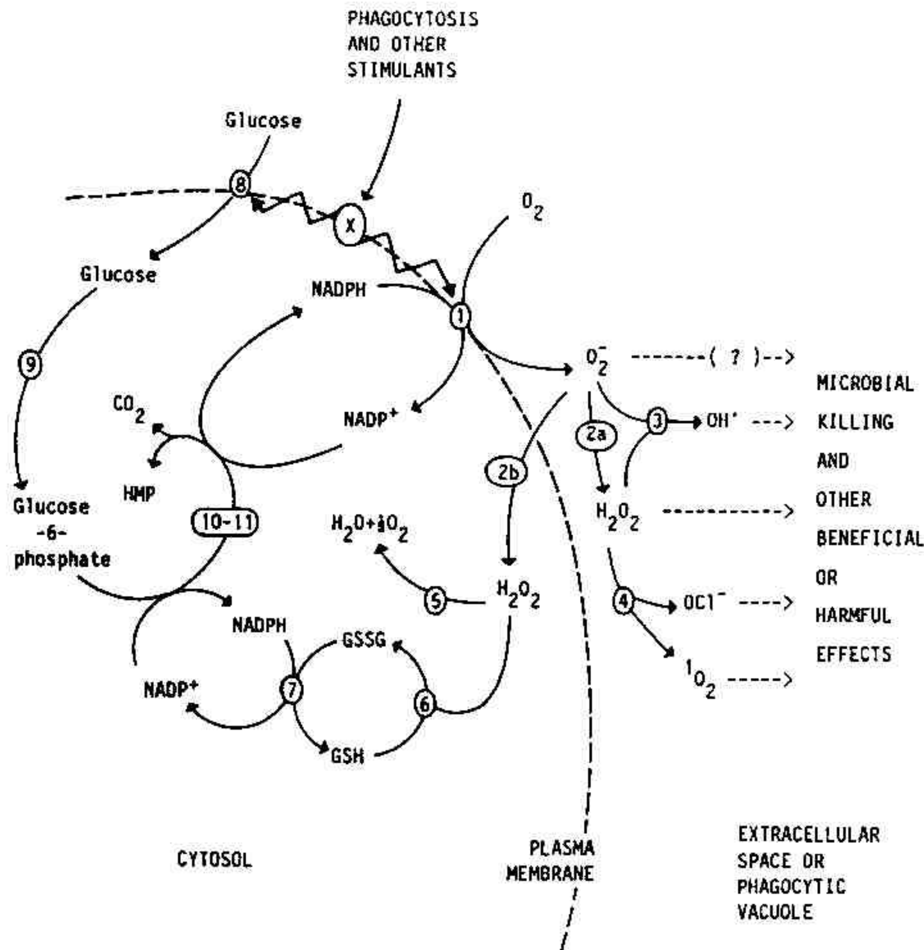

Fig. 1. Metabolic pathways of activated phagocytes. Perturbation of plasmamembrane (X) by various stimulants (see text) leads to activation of the primary oxidase (1) and of various correlated enzymatic and nonenzymatic systems. (2a) spontaneous dismutation; (2b) superoxide dismutase; (3) Haber-Weiss reaction; (4) myeloperoxidase; (5) catalase; (6) glutathione peroxidase; (7) glutathione reductase; (8) glucose transporter; (9) hexokinase; (10) glucose-6-phosphate dehydrogenase; (11) 6-phosphogluconate dehydrogenase; HMP = hexose monophosphate pathway.

piratory burst and of its mechanisms have been provided by studies on congenital and acquired defects of phagocytic metabolism (Table 2). The prototype of the congenital deficiencies is chronic granulomatous disease (CGD), a syndrome with many variants having in common the complete lack of superoxide and hydrogen peroxide generation by all types of phagocytes in the homozygous form.[67–72] For the biochemist and the genetecist, CGD is a formidable nature-experiment which is utilized for the investigation of the components of the respiratory burst enzyme. Unfortunately, patients affected by this disease (and also be severe glucose-6-phosphate dehydrogenase deficiency) suffer serious infections and often die in their early youth. Acquired cellular defects are milder in their expression and do not create severe clinical problems unless they are multiple or concomitant with leukopenia or with other immunodeficiencies. Table 2 also reports a list of drugs that are known to inhibit the respiratory burst. Some of them have a precise cellular target, while the effect of others has not been explained yet. It should be pointed out that many compounds that have been claimed to be inhibitory were tested only in vitro and at concentrations far above those commonly used in therapy.

II. THE ENZYMATIC BASIS OF THE RESPIRATORY BURST

The discovery of the peculiar oxygen metabolism of phagocytes opened a large debate on its biochemical basis. A variety of enzymes, such as NADH oxidase,[101–103] aminoacid oxidase,[104] myeloperoxidase,[105] NADPH oxidase,[53,106,107] and NADH-NBT reductase,[108–110] have been proposed as the respiratory burst enzyme.

Table 2. Defects of Phagocyte Oxidative Metabolism

Type of Defect	Reference Number
Hereditary diseases	
Chronic granulomatous disease	67–72
Glucose-6-phosphate dehydrogenase deficiency	73
Glutathione peroxidase deficiency	74
Mo1-glycoprotein deficiency	75
Acquired defects	
Burns	76,77
Viral infections	78,79
Malnutrition	80
Iron deficiency	81
Liver diseases	82–84
Type IV hyperlipoproteinemia	85
Anaesthetic agents	86–88
Nonsteroidal antiinflammatory drugs	89–93
Corticosteroids	94
Antimicrobial agents	95–98
Busulphan, methotrexate	99,100

Most of these indications were not confirmed by subsequent investigations, and NADH oxidase and NADPH oxidase remained the sole candidate enzymes. The debate over the true substrate (NADH versus NADPH) was quite lengthy and various arguments in support of each theory were reported.[13,16,17,111–113] Awaiting a definite clarification, from time to time the term NAD(P)H oxidase was also used.[114–117]

In recent years, unequivocal evidence established that the physiological substrate of the free radical generating oxidase is NADPH, although in the test tube the enzyme is also capable of oxidizing NADH. The main reasons for such a conclusion are as follows.

A. Coupling of the oxidase with HMP activity

The stimulation of HMP is supported by an increase of the $NADP^+$:NADPH ratio, i.e. by oxidation of NADPH. Assuming that the respiratory burst was due to NADH oxidase, the linkage between NADH oxidation and HMP activity would be provided by ancillary reactions, which oxidize NADPH to $NADP^+$. Theoretically, these reactions could be catalyzed by NADPH/NAD^+ transhydrogenase or by NADPH-dependent lactate dehydrogenase. It has been shown that the activity of these systems in phagocytes is too low to account for a $NADP^+$ production sufficient to sustain all the glucose oxidation through the HMP.[16] Therefore, the HMP appears to be directly linked to oxygen consumption through generation of $NADP^+$ by NADPH oxidase. This is in agreement with the observation that during phagocytosis the intracellular concentration of NADPH decreases and that of $NADP^+$ increases, while the NADH:NAD^+ ratio does not significantly change.[22,118] A significant contribution to NADPH consumption in phagocytozing cells is provided by the gluthatione cycle (see Fig. 1), which is an important peroxide-scavenging system. However, the HMP is activated even in conditions where the glutathione cycle is not operative, as in guinea pig neutrophils.[119–121]

B. Identification of the oxidase in subcellular fractions

The superoxide-forming activity is detectable in subcellular organelles (usually 27,000 × *g* pellet of postnuclear supernatants, or membranes purified through sucrose or Percoll gradients) prepared from phagocytozing cells. Both NADH and NADPH oxidase activities are triggered by phagocytosis or by soluble stimulants. The Km for the substrate of the NADPH oxidizing activity is close to the NADPH concentration in the cell, whereas the Km of the NADH-oxidizing activity is much higher, suggesting that the preferred physiological substrate is NADPH.[16,113,117,118,122]

C. Adequacy of NADPH oxidase to account for the respiratory burst

The activated oxygen consumption and the O_2^- production of intact neutrophils and macrophages is accounted for by the oxygen-consumption and O_2^- production catalyzed by NADPH oxidase. This concordance was demonstrated by a simple experiment where the respiratory burst of PMA-stimulated macrophages was monitored (Fig. 2).[123,124] After stimulation the O_2^- production increased until a linear rate was attained (trace 1). At this point the cells were lysed with deoxycholate and the O_2^- production ceased, but addition of NADPH restored the activity at a rate similar to that sustained by whole cells. A similar result was obtained by adding $NADP^+$ plus glucose-6-phosphate, which together with glucose-6-phosphate dehydrogenase provided by the cell lysate form a NADPH generating system (trace 2). NADH was less effective than NADPH (trace 3), and NADPH oxidase activity was undetectable in unstimulated cells (trace 4). This experiment (and similar results published by others)[125] demonstrated that both the NADPH-dependent O_2^- generating system and the NADPH-generating system of phagocytes are competent for supporting the respiratory burst.

D. Studies of genetic defects

The primary molecular lesion in phagocytes of patients with CGD lies in the NADPH oxidase system,[126] while the deficiency of other enzymes in this disease is controversial.[127–129] The absence of NADPH-dependent O_2^- formation in CGD could be due to (1) lack of the oxidase or of one of is components, (2)

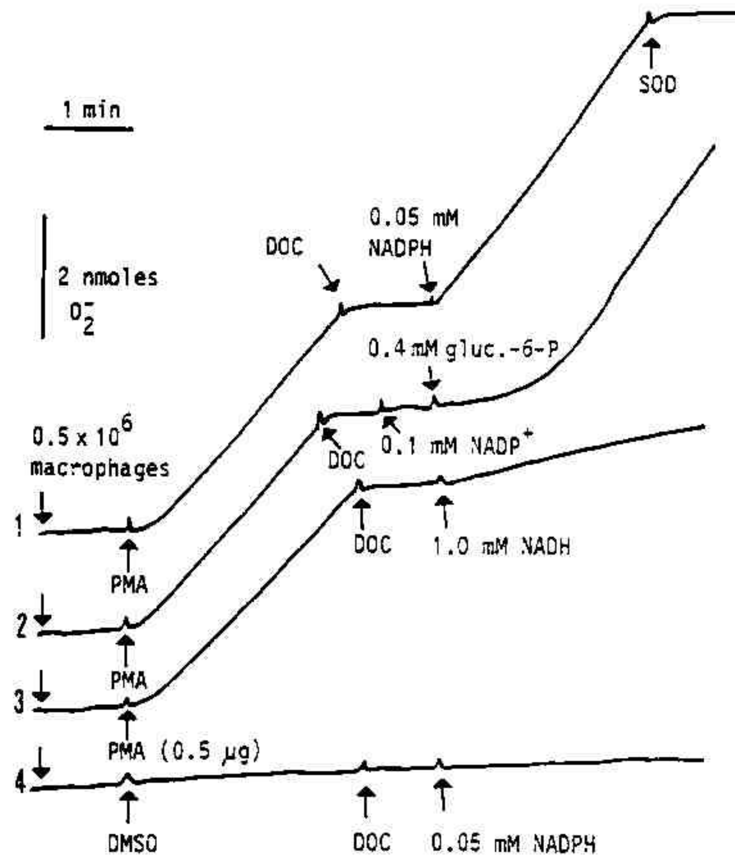

Fig. 2. Spectrophotometric recording traces of O_2^- production by guinea pig peritoneal macrophages and by the cell lysate in the presence of NADPH and NADH. O_2^- was measured as ferricytochrome *c* reduction at 37°C in a double beam spectrophotometer. Both the reference and the sample cuvette contained Krebs–Ringer–Phosphate buffer, pH 7.4, 0.15 mM cytochrome *c*, 2 mM NaN_3, macrophages and the additions indicated in this figure. The reference cuvette contained also 0.03 mg of superoxide dismutase (SOD). The final volume equaled 1 ml. DOC = sodium deoxycholate; DMSO = dimethylsulfoxide; gluc.-6-P = glucose 6-phosphate. (Modified from Berton et al.,[124] with permission).

structural abnormality, (3) defect of the activation system. The following sections of this review will show that all these possible defects have been found in variant forms of the disease.

Additional evidence in favor of the NADPH oxidase was provided by another genetic defect of leukocytes, the deficiency of glucose-6-phosphate dehydrogenase. Leukocytes of patients with severe expression of this disease cannot regenerate NADPH and as a consequence do not undergo a metabolic burst during phagocytosis.[130]

III. MOLECULAR STUCTURE OF NADPH OXIDASE

In spite of a great number of investigations performed during the past ten years, the structure and the components of the free-radical generating NADPH oxidase have yet to be completely clarified. Several laboratories, including ours, have attempted the extraction and purification of the enzyme from neutrophils and macrophages, in order to characterize its structure in molecular terms.[131-144] As a consequence of these studies, much information on the nature of NADPH oxidase has been accumulated, but the data reported by the various groups were often controversial. The main reason for the difficulties of purifying the oxidase lies in its extreme lability upon detergent extraction and upon manipulation by the usual purification methods.[131,141,142] Furthermore the NADPH oxidase is detectable only when the enzyme is in the activated state, and since little is known on the mechanism responsible for this state, it is practically impossible to reconstitute the activity once it has been lost.

According to the most accepted hypothesis, the NADPH oxidase activity is due to a membrane-bound multicomponent system in which a flavoprotein, a *b*-type cytochrome and possibly other cofactors (quinones) are involved. These factors would be assembled as an electron-transport chain capable of transferring reducing equivalents from NADPH to molecular oxygen with formation of O_2^-.

Most of the data supporting this hypothesis (which

will be detailed below) are based on indirect evidence, because until now nobody has been able to purify the complete system in a homogeneous and fully active form. Nevertheless, various groups have investigated the structure and the properties of individual components of the system, even if these components were devoid of enzymatic activity. This chapter deals with both the attempts to purify the enzyme in active form and the studies on the various putative components of the system.

A. Extraction and purification of NADPH oxidase

Several groups have extracted and characterized in solution the NADPH oxidase of neutrophils and macrophages,[132,134,145–150] but the first study reporting partial purification of the enzyme from subcellular particles of activated human neutrophils was published by Tauber and Goetzl in 1979.[131] The NADPH-dependent superoxide generating activity was extracted with deoxycholate and was partially stabilized in solution with glycerol, ethylene glycol and Me_2SO_4. From the methodological point of view, such a stabilization was very important and was adopted by almost all the following investigators. The solubilized enzyme was resolved by filtration through Sephacryl S-200 in two peaks, one of which appeared in the void volume (Mr > 300,000 daltons) and the other with apparent Mr of 150,000. However, the mean specific activity of the two peaks was very low, ranging from 1.2 to 2.4 nmol O_2^-/min/mg protein. The solubilized extract was also subjected to isoelectric focusing and exhibited a peak of activity with pI of 7.6–8.3 and 0.95 nmol O_2^-/min/mg protein. The possible presence of heme or flavin cofactors in these preparations was not investigated in this work.

Autor and Hoffmann[133] solubilized with deoxycholate the NADPH oxidase from rat pulmonary macrophages, both unstimulated and treated with PMA. Filtration of the solubilized preparation on a Sephacryl S-300 column yielded several peaks of activity. All the active peaks were electrophoresed on SDS-polyacrylamide gels and appeared to have in common two polypeptide bands with Mr of 53,000 and 57,000. However, the presence of other bands and the anomalous distribution of the activity in the column rendered this report inconclusive.

The isolation of NADPH oxidase from guinea pig polymorphonuclear leukocytes by nondenaturing gel electrophoresis of Triton X-100 solubilized membranes was reported by Tamoto et al.[137] The enzyme was localized in the gel by NADPH-specific nitro blue tetrazolium (NBT) reduction and exhibited a Mr of 150,000 in nondenaturing conditions. Biochemical characterization of this enzymatic activity showed that it was trypsin-sensitive, insensitive to cyanide and inhibitable by sulfhydryl reagents. Addition of microsomal cytochrome b_5 caused a dose-dependent increase of O_2^--generating activity of the enzyme. On the basis of this finding the authors suggested that probably NADPH oxidase requires some cofactor for elicitation of its full activity expression. However, the NADPH oxidase activity of the purified preparations was not significantly increased in the enzyme isolated from activated cells as compared with the enzyme isolated from resting cells. Therefore the criteria for an oxidase to be considered the true respiratory burst enzyme were not completely fulfilled.[16] Moreover, it should be noted that the detection of free radical formation by NBT-staining procedures is not free of possible pitfalls, because it has been shown that this reagent is reduced by O_2^- but may also generate O_2^- through nonenzymatic reactions.[151,152]

Sakane et al[135,136] extracted from membranes of guinea pig polymorphonuclear leukocytes a NADPH-cytochrome *c* reductase capable of forming O_2^- in the presence of menadione. The enzyme was purified over 110-fold by gel filtration through Sephacryl S-300 and by affinity chromatography on 2′,5′-ADP-agarose. In the gel filtration it exhibited an apparent Mr of 400,000, but when analyzed by SDS-polyacrylamide gel electrophoresis it gave a major band with Mr of 87,000. It is conceivable that the higher Mr in nondenaturing conditions was an overestimate due to aggregation and/or to the presence of detergent bound to the protein. The reductase contained FAD (5 nmol/mg protein) and its O_2^--generating activity in the presence of menadione had an optimal pH of 7.0–7.4, and a Km for NADPH and for NADH of 25 μM and 230 μM, respectively. The sensitivity to several inhibitors was similar to that of NADPH oxidase in cell-free particles. The enzyme also expressed 2,6-dichlorophenolindephenol (DCIP)-reductase activity. On these bases, the authors suggested that the purified NADPH-cytochrome *c* reductase is the putative flavoprotein of the NADPH oxidase system responsible for the respiratory burst. However the requirement of menadione for O_2^- generation raises some doubt on the involvement of this enzymatic activity in the respiratory burst. In fact, since menadione can be reduced by a number of oxidoreductases and rapidly autooxidizes, it cannot be excluded that apparent generation of O_2^- by a flavoprotein in the presence of menadione is an experimental artifact.[153–155]

In our laboratory the purification of a free-radical generating NADPH oxidase that is active in the absence of exogenous cofactors and therefore endowed with all its putative components was pursued.[138–140,144,147,148] We could not achieve complete purification owing to the

instability of the activity in the presence of strong detergents, but the analysis of partially purified preparations provided information on the nature of this enzyme. In the first series of experiments we extracted with deoxycholate the NADPH-dependent O_2^- forming activity from PMA-activated neutrophils of the guinea pig.[140,147,148] The solubilized enzyme was purified by chromatography on Ultrogel AcA22, by isopycnic glycerol gradient centrifugation and by treatment with 0.4 M NaCl. The distribution profiles of the various fractionation procedures clearly showed that the purification of NADPH oxidase was accompanied by parallel purification of the low potential cytochrome *b*. Therefore, we suggested that oxidase activity and cytochrome *b* are closely associated. SDS-electrophoretic gel analysis of the enzyme samples at increasing purification states indicated that a protein band with Mr of 32,000 progressively increased in the preparations as the purification proceeded. This protein was absent in fractions of glycerol gradient, which were devoid of oxidase activity.

We carried out further studies on pig neutrophils, a particularly convenient source when large amounts of starting material are required.[138,144] The enzyme was extracted from membranes by treatment with a mixture of deoxycholate and Lubrol-PX and the solubilized extract was chromatographed through an Ultrogel AcA34 column. As it can be seen from the elution profile reported in Figure 3, the first protein peak contained mostly NADPH oxidase activity and cytochrome b_{558}. This peak corresponded to the void volume of the column, indicating that the enzyme was isolated as a high molecular weight complex (Mr > 350,000 daltons) containing O_2^--forming activity (up to 572 nmoles O_2^-/min/mg protein) and cytochrome *b* (up to 1.9 nmoles/mg protein). This peak contained also phospholipids

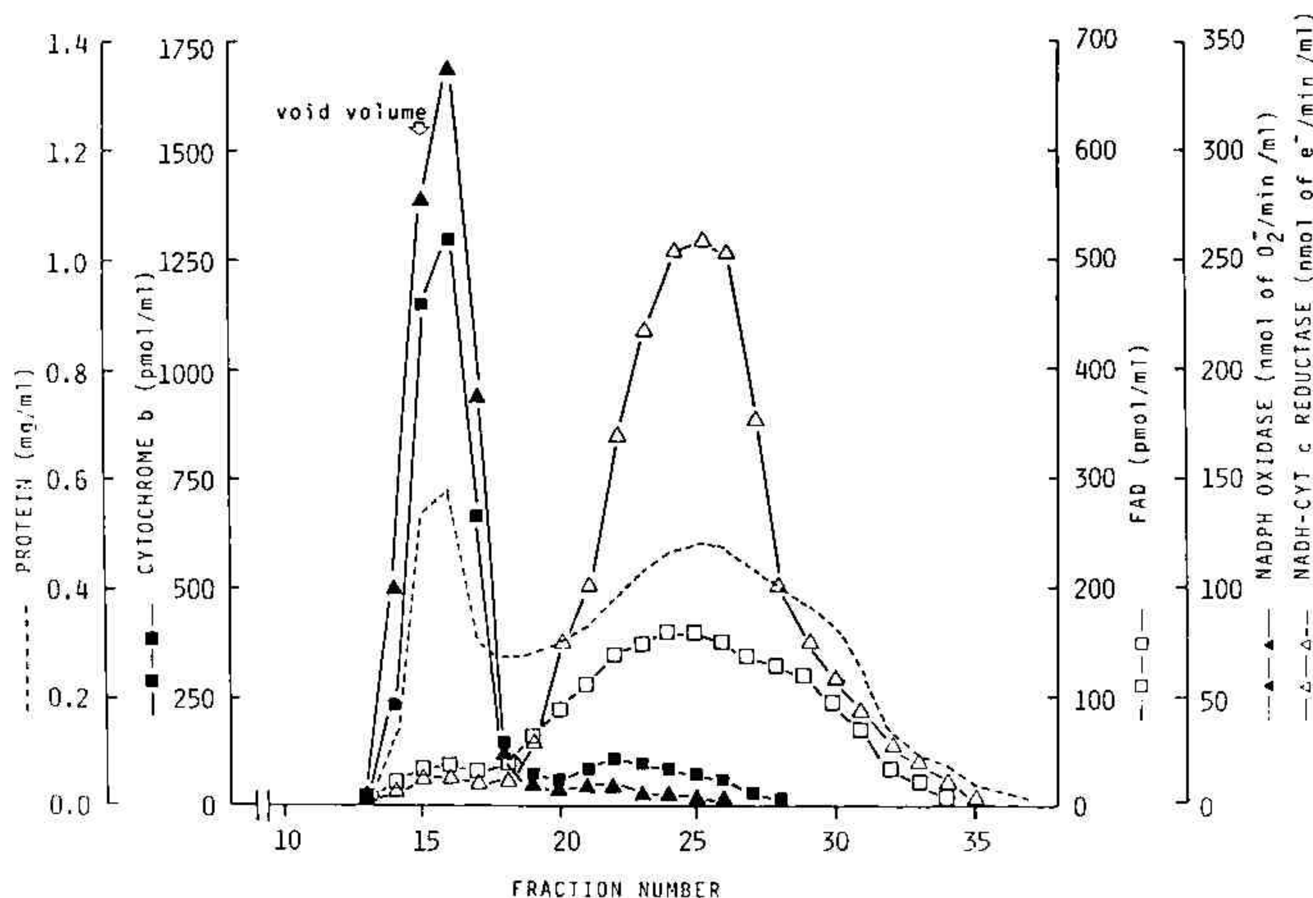

Fig. 3. Gel filtration of the solubilized extract from the plasma membranes of PMA-activated pig neutrophils. Membranes were suspended in glycerol-phosphate buffer (50 mM sodium phosphate buffer, pH 8.0, containing 20% glycerol, 2 mM EGTA, 1 mM $MgSO_4$, 2 mM NaN_3, 2 mM phenylmethansulphonyl fluoride) and solubilized with 0.4% Lubrol-PX and 0.4% deoxycholate. 4 ml of the extract (protein = 3.3 mg/ml; cytochrome b_{558} = 2.31 nmoles/ml; FAD = 1.03 nmoles/ml; NADPH oxidase = 601 nmoles O_2^-/min/ml; NADH cytochrome *c* reductase = 475 nmoles cytochrome *c* reduced/min/ml) were chromatographed through an Ultrogel AcA34 (LKB) column equilibrated with glycerol-phosphate buffer containing 0.15% Lubrol-PX and 0.15% deoxycholate and eluted with the same buffer. 1.5 ml fractions were collected at a flow rate of 18 ml/h. (From Bellavite et al.,[144] with permission).

(≈0.3 mg/mg protein) and very little FAD (≈0.05 nmoles/mg protein). A second broader peak was eluted in the included volume. This peak contained most of FAD and of the diaphorase enzyme NADH cytochrome *c* reductase. Therefore this experiment indicated that the bulk of FAD present in the membrane extract was not related with the NADPH oxidase-cytochrome *b*-phospholipids active complex, but was related with other flavoprotein oxido-reductases. Electrophoretic analysis of the active preparations from pig neutrophils showed that a polypeptide with Mr of 32,000 strictly paralleled the purification of the NADPH oxidase and of cytochrome *b*.[144]

Similar procedures of isolation were adopted for preliminary investigations of the oxidase from guinea pig peritoneal macrophages.[139] Owing to technical problems due to scarcity of starting material, the purification achieved with these cells was lower than that achieved with neutrophils, but the results indicated that the structure of the enzyme in the two phagocyte types is qualitatively similar. The NADPH oxidase complex isolated from macrophages contained cytochrome *b* (0.47 nmoles/mg protein) and FAD (0.09 nmoles/mg protein), in a ratio of 5.3:1. The cytochrome *b* was characterized by a midpoint potential ($Em_{7.0}$) of -247 mV, similar to that of neutrophil's cytochrome (-245 mV).[156] The solubilized and partially purified enzyme from macrophages was insensitive to mitochondrial inhibitors, was inhibited by *p*-chloromercuribenzoate and by bathophenanthroline sulfonate and was specific for NADPH as substrate.

Markert et al.[142] and Glass et al.[143] reported the purification of the "respiratory burst oxidase" of human neutrophils by dye-affinity chromatography on Red-agarose. The purified preparations were endowed with very high enzymatic activity (5–10 μmoles O_2^-/min/mg protein) and this activity was elicited in phagocytozing neutrophils. It is therefore conceivable that these authors have purified the "true" oxidase. The SDS-gel electrophoretic pattern of these preparation showed three major bands with Mr of 65–67,000, 48,000 and 32,000. Assuming that all these proteins are subunits of the enzyme, the overall molecular mass would be ≈150,000 daltons. The oxidase preparations contained a lot of FAD (20.4 nmoles/mg protein), whereas heme was present in minimal amount (0.1 nmoles/mg protein) and ubiquinone could not be detected. The authors gave two possible interpretations of these results: "1) the oxidase is a flavoprotein that requires neither quinone nor heme for the catalysis of O_2^- production, or 2) the oxidase is actually an electron transport chain from which we have purified the first component—i.e. the pyridine nucleotide dehydrogenase." Whichever the correct interpretation may be, the production of O_2^- by a flavoprotein in the absence of cytochrome *b* would seriously question the participation of this hemeprotein in the oxidase activity and would contrast with our finding of copurification of the cytochrome with the enzyme.[140,144] We therefore repeated the procedure of purification by dye-affinity chromatography and we looked at the possible presence of heme chromophores.[157] Our results indicated that these enzyme preparations contained cytochrome *b*, of which the absorption spectrum was disclosed by highly-sensitive spectrophotometric techniques. We therefore ascribed the reported lack of cytochrome *b* detection[142] to the sensitivity problems that could have affected the assays of diluted samples. In the second paper from the same group, the authors used a sensitive fluorimetric method for the measurement of heme and reached the conclusion that their preparations were devoid of cytochrome *b*.[143] However, careful inspection of their data reveals the presence of a substantial amount of heme associated with the oxidase. In fact, from the traces presented in Figure 2 of the quoted paper, it may be calculated, on the basis of the standard traces of the hemoglobin (which contains 4 heme/molecule), that the purified oxidase with 8.9 μg protein contained about 0.0039 nmoles of heme. Therefore, the specific cytochrome *b* content of that preparation was about 0.44 nmoles/mg protein, a value that is higher than that of whole cells (≈0.1 nmoles/mg protein).[72] In conclusion, the participation of cytochrome *b* as a cofactor in the human oxidase purified by dye-affinity chromatography cannot be ruled out.

The discrepancy between the literature data reporting high flavin and low cytochrome content with those reporting high cytochrome and low flavin content is remarkable. It would be desirable for other independent groups to repeat the procedures published by the various laboratories in order to clarify this important point regarding the components of NADPH oxidase.

In this context, surprising results were reported by Doussière and Vignais,[141] who purified a NADPH oxidase from bovine neutrophils by extraction with Triton X-100, ion exchange chromatography, gel filtration and isoelectric focusing. The final preparation was very pure, as judged from SDS-electrophoretic analysis, which showed only one major band with Mr of 65,000. The purified enzyme was devoid of both cytochrome *b* and flavin and still produced O_2^-. The K_m for NADPH was 30 μM, the activity was inhibited by the thiol reagent, mersalyl, and by cibacron blu, but not by cyanide. All these properties are compatible with a function in the respiratory burst. A major criterion of specificity that remains to be clarified is whether the activity was increased in preparations from stimulated cells. Another consideration concerning this important