

## 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.<sup>1-6</sup> 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.<sup>7-12</sup> Early studies indicated that this oxygen metabolic pathway is insensitive to cyanide and therefore is independent of mitochondrial respiration.<sup>10-12</sup> 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.<sup>13-19</sup> 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.

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# 1. 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 I.

The increased respiration of phagocytosing cells is accompanied by other metabolic modifications including: (1) increase of glucose uptake<sup>30</sup> and catabolism through hexose monophosphate pathway (HMP),<sup>21</sup> (2) decrease of the NADPH:NAD<sup>+</sup> ratio<sup>22</sup> and GSH:GSSG ratio,<sup>23</sup> (3) production and release of hydrogen peroxide,<sup>24</sup> of superoxide anion,<sup>25</sup> of hydroxyl radical,<sup>26</sup> of singlet oxygen,<sup>27</sup> and (4) emission of photons of light (chemiluminescence).<sup>28</sup> Coincident with the activation of these systems, the intracellular pH undergoes rapid acidification, followed by gradual alkalization, due to extrusion of H<sup>+</sup> through a receptor-activated Na<sup>+</sup>/H<sup>+</sup> antiport.<sup>29-32</sup> All these biochemical events are strictly interrelated and take place almost simultaneously.<sup>33</sup> 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<sub>2</sub><sup>-</sup>, then to H<sub>2</sub>O<sub>2</sub> through the dismutation reaction:



It has been calculated that only a minor portion of O<sub>2</sub> that undergoes reduction can be recovered as O<sub>2</sub> or H<sub>2</sub>O<sub>2</sub> 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.<sup>34-40</sup>

The extent of these phenomena and the stoichiometries of O<sub>2</sub>:O<sub>2</sub><sup>-</sup>:H<sub>2</sub><sup>+</sup> vary according to the experimental conditions, the cell type, and the stimulant employed. Challenge of neutrophils with maximum doses of opsonized zymosan or of phorbol-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<sup>6</sup> cells.<sup>18</sup> 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.<sup>31,41</sup> Blood monocytes are more active than differentiated macrophages.<sup>42</sup> Particular types of tissue macrophages, such as liver Kupfer cells, appear to be almost totally impaired in their oxidative response.<sup>43</sup> Release of O<sub>2</sub><sup>-</sup> and H<sub>2</sub>O<sub>2</sub> by pulmonary alveolar macrophages is very low, due to the high efficiency of their scavenger systems.<sup>44</sup> Under appropriate stimuli such as inflammation, endotoxins, or γ-interferon, resident macrophages may mature 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.<sup>45-50</sup> Interestingly, a phagocytosis-associated respiratory burst has been described even in protozoa such as the amoebae.<sup>51,52</sup>

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;<sup>53</sup> chemotactic peptides trigger an almost instantaneous increase of O<sub>2</sub> consumption that often shows biphasic kinetics;<sup>54,55</sup> phorbol esters cause a progressive and irreversible activation;<sup>56</sup> activation by sodium fluoride shows a very long lag time.<sup>57</sup> The metabolic activation is reversible upon removal of the activator, which can be accomplished by washing the cells,<sup>57</sup> by displacing the ligand from receptor with competitive substances,<sup>58,59</sup> or by oxidative inactivation of the activator itself.<sup>54</sup> Once deactivated, the oxidative metabolism can be reactivated by a second stimulus,<sup>54,60</sup> but under particular conditions a "desensitization" of the system may occur.<sup>61-63</sup> 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 accumulated during the activation phase.<sup>64-66</sup>

Considerable advances in the knowledge of the res-

Table I. Stimulants of the Phagocyte Oxidative Metabolism

Bacteria	Opsonized zymosan
Latex particles	N-formyl-peptides
Oil droplets	Pyrogen
IgG-coated surfaces	Ca <sup>2+</sup> ionophores
Immune complexes	Cytochalasins D and E
Complement fragments C5a, C567	Phospholipase C
Fatty acids	Concanavalin A
Tumor necrosis factor	Leukotriene B <sub>4</sub>
Phorbol myristate acetate	Anti-leukocyte antibodies
Sodium fluoride	Low-Na <sup>+</sup> incubation buffer
Diacylglycerol	Platelet activating factor

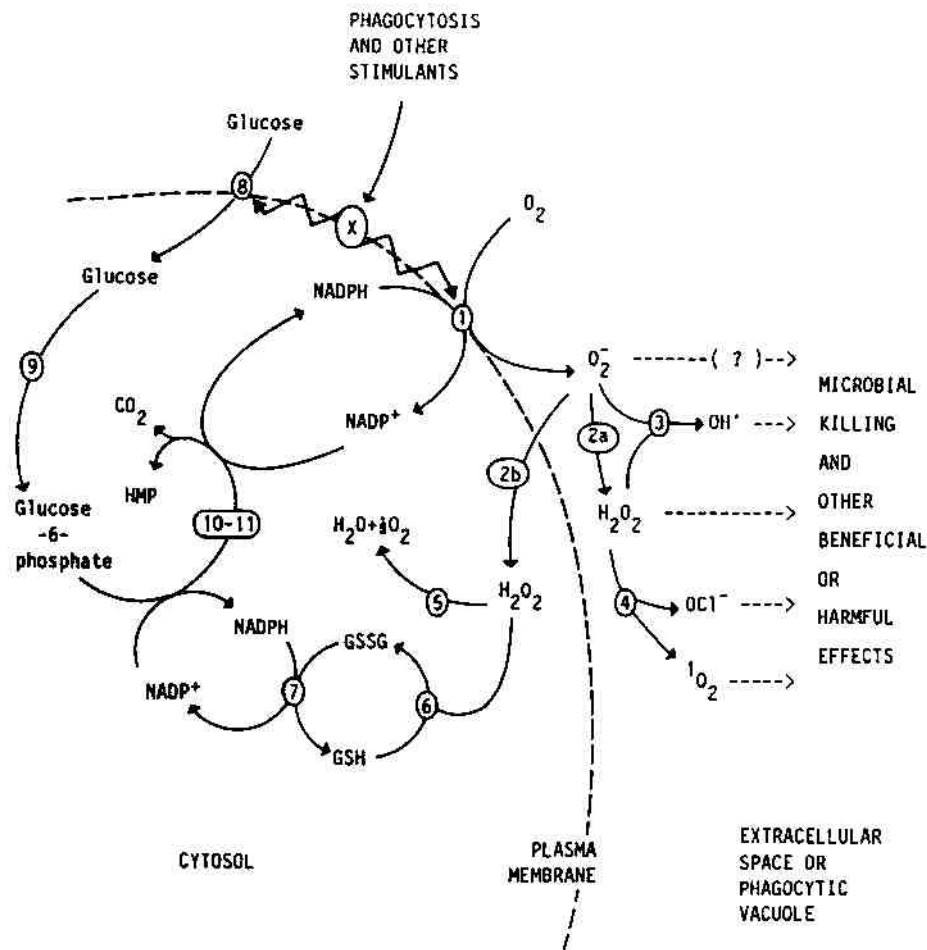


Fig. 1. Metabolic pathways of activated phagocytes. Perturbation of plasmamembrane (X) by various stimulants (see text) leads to activation of the primary oxidase (1) and of various correlated enzymatic and nonenzymatic systems. (2a) spontaneous dismutation; (2b) superoxide dismutase; (3) Haber-Weiss 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.<sup>67-72</sup> For the biochemist and the geneticist, 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 by 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,<sup>101-103</sup> amino acid oxidase,<sup>104</sup> myeloperoxidase,<sup>105</sup> NADPH oxidase,<sup>53,106,107</sup> and NADH-NBT reductase,<sup>108-110</sup> have been proposed as the respiratory burst enzyme.

Table 2. Defects of Phagocyte Oxidative Metabolism

Type of Defect	Reference Number
<i>Hereditary diseases</i>	
Chronic granulomatous disease	67-72
Glucose-6-phosphate dehydrogenase deficiency	73
Glutathione peroxidase deficiency	74
Mo1-glycoprotein deficiency	75
<i>Acquired defects</i>	
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.<sup>13,16,17,111-113</sup> Awaiting a definite clarification, from time to time the term NAD(P)H oxidase was also used.<sup>114-117</sup>

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  $\text{NADP}^+:\text{NADPH}$  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  $\text{NADP}^+$ . Theoretically, these reactions could be catalyzed by NADPH/ $\text{NAD}^+$  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  $\text{NADP}^+$  production sufficient to sustain all the glucose oxidation through the HMP.<sup>16</sup> Therefore, the HMP appears to be directly linked to oxygen consumption through generation of  $\text{NADP}^+$  by NADPH oxidase. This is in agreement with the observation that during phagocytosis the intracellular concentration of NADPH decreases and that of  $\text{NADP}^+$  increases, while the  $\text{NADH}:\text{NAD}^+$  ratio does not significantly change.<sup>22,118</sup> A significant contribution to NADPH consumption in phagocytosing cells is provided by the

glutathione 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.<sup>119-121</sup>

#### B. Identification of the oxidase in subcellular fractions

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

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

The activated oxygen consumption and the  $\text{O}_2^-$  production of intact neutrophils and macrophages is accounted for by the oxygen-consumption and  $\text{O}_2^-$  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).<sup>123,124</sup> After stimulation the  $\text{O}_2^-$  production increased until a linear rate was attained (trace 1). At this point the cells were lysed with deoxycholate and the  $\text{O}_2^-$  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  $\text{NADP}^+$  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)<sup>125</sup> demonstrated that both the NADPH-dependent  $\text{O}_2^-$  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,<sup>126</sup> while the deficiency of other enzymes in this disease is controversial.<sup>127-129</sup> The absence of NADPH-dependent  $\text{O}_2^-$  formation in CGD could be due to (1) lack of the oxidase or of one of its components, (2)



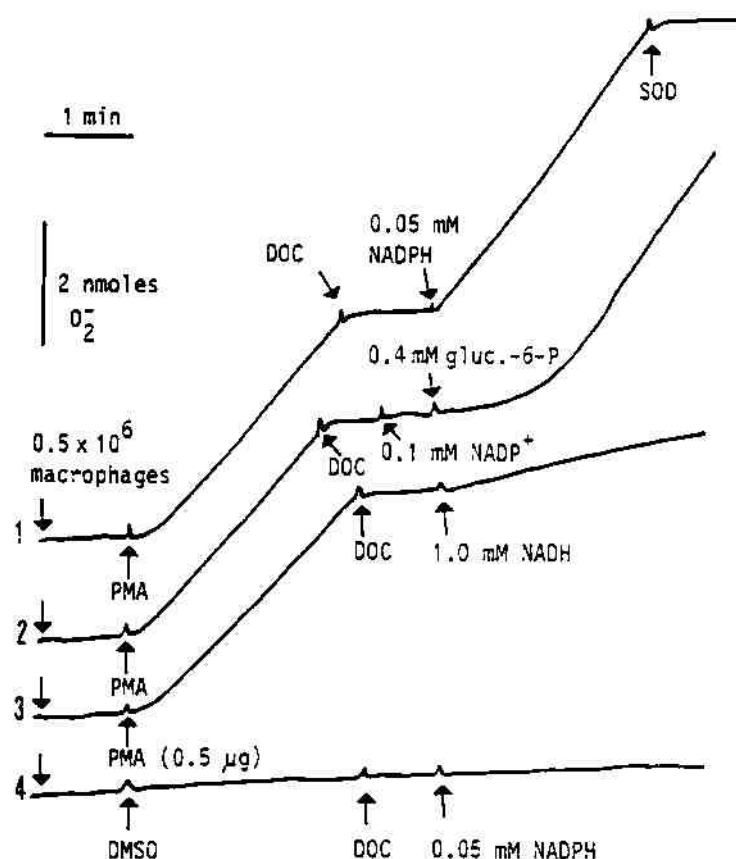


Fig. 2. Spectrophotometric recording traces of  $O_2^-$  production by guinea pig peritoneal macrophages and by the cell lysate in the presence of NADPH and NADH.  $O_2^-$  was measured as ferricytochrome c reduction at 37°C in a double beam spectrophotometer. Both the reference and the sample cuvette 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.,<sup>124</sup> 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.<sup>130</sup>

### III. MOLECULAR STRUCTURE 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.<sup>131-144</sup> 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.<sup>131,141,142</sup> 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,<sup>132,134,145-150</sup> 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.<sup>131</sup> The NADPH-dependent superoxide generating activity was extracted with deoxycholate and was partially stabilized in solution with glycerol, ethylene glycol and  $\text{Me}_2\text{SO}_4$ . 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 ( $\text{Mr} > 300,000$  daltons) and the other with apparent  $\text{Mr}$  of 150,000. However, the mean specific activity of the two peaks was very low, ranging from 1.2 to 2.4  $\text{nmol O}_2^-/\text{min}/\text{mg}$  protein. The solubilized extract was also subjected to isoelectric focusing and exhibited a peak of activity with  $\text{pI}$  of 7.6-8.3 and 0.95  $\text{nmol O}_2^-/\text{min}/\text{mg}$  protein. The possible presence of heme or flavin cofactors in these preparations was not investigated in this work.

Autor and Hoffmann<sup>133</sup> 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  $\text{Mr}$  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.<sup>137</sup> The enzyme was localized in the gel by NADPH-specific nitro blue tetrazolium (NBT) reduction and exhibited a  $\text{Mr}$  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  $\text{O}_2^-$ -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.<sup>16</sup> 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  $\text{O}_2^-$  but may also generate  $\text{O}_2^-$  through nonenzymatic reactions.<sup>151,152</sup>

Sakane et al.<sup>135,136</sup> extracted from membranes of guinea pig polymorphonuclear leukocytes a NADPH-cytochrome  $c$  reductase capable of forming  $\text{O}_2^-$  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  $\text{Mr}$  of 400,000, but when analyzed by SDS-polyacrylamide gel electrophoresis it gave a major band with  $\text{Mr}$  of 87,000. It is conceivable that the higher  $\text{Mr}$  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  $\text{nmol}/\text{mg}$  protein) and its  $\text{O}_2^-$ -generating activity in the presence of menadione had an optimal  $\text{pH}$  of 7.0-7.4, and a  $K_m$  for NADPH and for NADH of 25  $\mu\text{M}$  and 230  $\mu\text{M}$ , respectively. The sensitivity to several inhibitors was similar to that of NADPH oxidase in cell-free particles. The enzyme also expressed 2,6-dichlorophenolindophenol (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  $\text{O}_2^-$  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  $\text{O}_2^-$  by a flavoprotein in the presence of menadione is an experimental artifact.<sup>153-155</sup>

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.<sup>138-140,144,147,148</sup> 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<sub>2</sub><sup>-</sup> forming activity from PMA-activated neutrophils of the guinea pig.<sup>140,147,148</sup> 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 prepara-

tions 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.<sup>138,144</sup> 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*<sub>558</sub>. 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<sub>2</sub><sup>-</sup>-forming activity (up to 572 nmoles O<sub>2</sub><sup>-</sup>/min/mg protein) and cytochrome *b* (up to 1.9 nmoles/mg protein). This peak contained also phospholipids

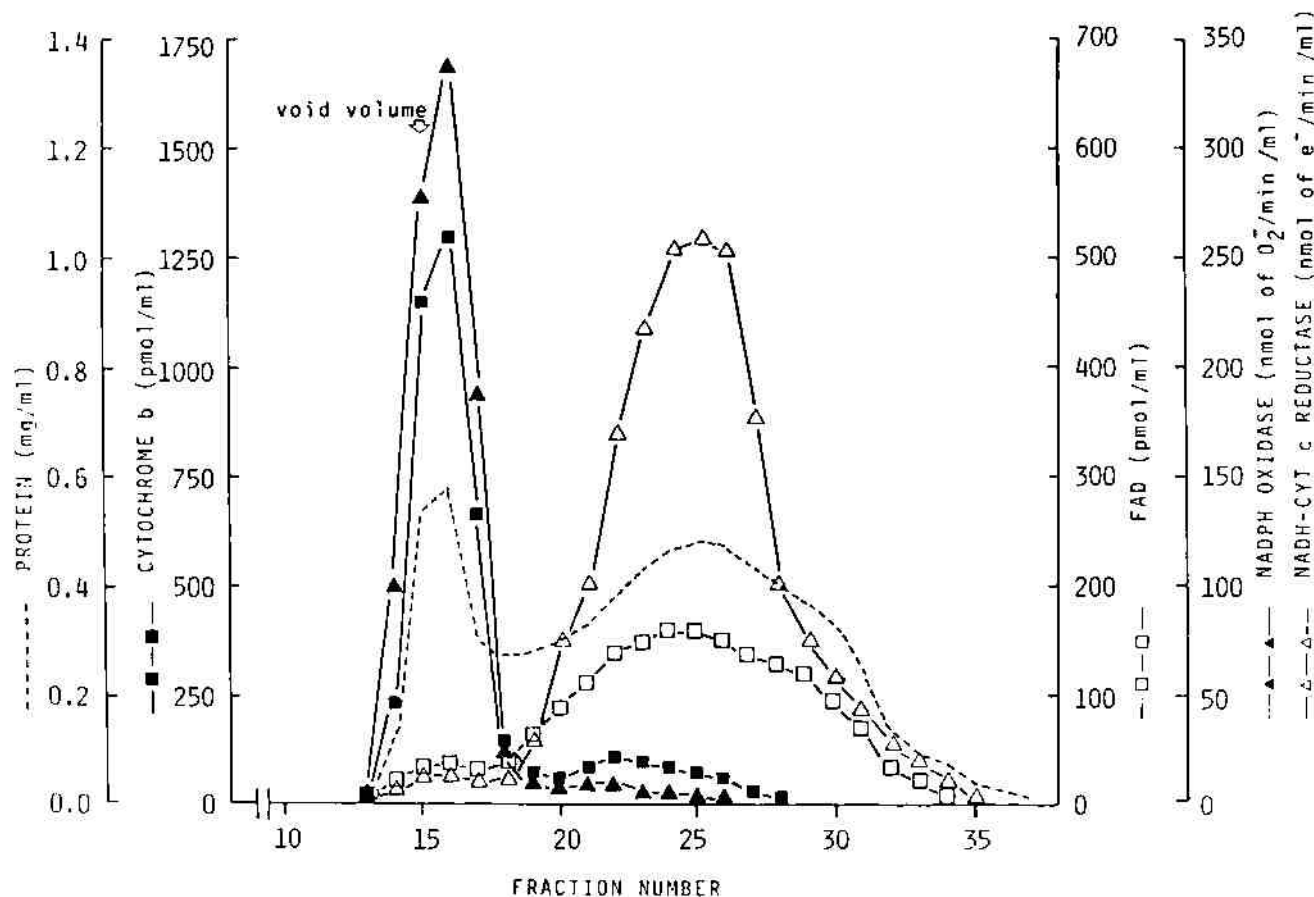


Fig. 3. Gel filtration of the solubilized extract from the plasma membranes of PMA-activated pig neutrophils. Membranes were suspended in glycerol-phosphate buffer (50 mM sodium phosphate buffer, pH 8.0, containing 20% glycerol, 2 mM EGTA, 1 mM MgSO<sub>4</sub>, 2 mM NaN<sub>3</sub>, 2 mM phenylmethanesulphonyl fluoride) and solubilized with 0.4% Lubrol-PX and 0.4% deoxycholate. 4 ml of the extract (protein = 3.3 mg/ml; cytochrome *b*<sub>558</sub> = 2.31 nmoles/ml; FAD = 1.03 nmoles/ml; NADPH oxidase = 601 nmoles O<sub>2</sub><sup>-</sup>/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.,<sup>144</sup> with permission).

( $\approx 0.3$  mg/mg protein) and very little FAD ( $\approx 0.05$  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*.<sup>144</sup>

Similar procedures of isolation were adopted for preliminary investigations of the oxidase from guinea pig peritoneal macrophages.<sup>139</sup> 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 ( $E_{m,0}$ ) of  $-247$  mV, similar to that of neutrophil's cytochrome ( $-245$  mV).<sup>156</sup> 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.<sup>142</sup> and Glass et al.<sup>143</sup> 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  $\mu$ moles  $O_2^-$ /min/mg protein) and this activity was elicited in phagocytosing 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  $\approx 150,000$  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 pro-

duction 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.<sup>140,144</sup> We therefore repeated the procedure of purification by dye-affinity chromatography and we looked at the possible presence of heme chromophores.<sup>157</sup> 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<sup>142</sup> 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*.<sup>143</sup> 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  $\mu$ g 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 ( $\approx 0.1$  nmoles/mg protein).<sup>72</sup> 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 Doussi re and Vignais,<sup>141</sup> 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  $\mu$ M, 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



paper regards the marked inactivation of the enzyme activity induced by detergent extraction and by fractionation procedures. Even if the final enzyme preparation was endowed with some residual O<sub>2</sub><sup>-</sup>-forming activity, it is conceivable that the inactivation was due to removal of some other component in the presence of which the activity would have been much higher. One could speculate that the protein of Mr 65,000, which is the only polypeptide present in the preparation of Doussi re and Vignais,<sup>141</sup> corresponds to one of the subunits found by other investigators in their oxidase preparations.<sup>142,143</sup> Interestingly, Doussi re and Vignais concluded that "one cannot entirely exclude that this molecule is the core protein with a redox center of an oligomeric complex which would contain additional redox components capable of reacting rapidly with O<sub>2</sub> to give O<sub>2</sub><sup>-</sup>. Possible candidates are a flavo-protein and, less probably, the *b*-type cytochrome. In our preparations these putative redox components could be present in spectroscopically undetectable amounts, but their turnover would be high enough to account for the rapid production of O<sub>2</sub><sup>-</sup>."

The results thus far published concerning the attempts of purification of NADPH oxidase are summarized in Table 3. It can be seen that some results from different laboratories are in substantial agreement (i.e. the molecular mass of 150,000 of the whole en-

zyme, the presence of subunits of 65,000 and of 32,000, the absence of quinones), while others are different or even opposite. This situation is a further suggestion that the system is formed by several components, which are loosely assembled so that different extraction and purification methods can dissociate them in a different fashion. At present it is difficult to exclude the participation of individual components or cofactors because the apparent lack of flavin or of heme in a preparation could be due either to sensitivity limits of the assays (the reported protein concentration of the most purified fractions was often very low) or to loss during some experimental steps. Clearly, further efforts are required in order to optimize the purification procedures and, most important, to compare the results of different laboratories in standard conditions. Perhaps only reconstitution experiments will definitely solve the problem of the participation of constituent subunits in the catalysis of the O<sub>2</sub><sup>-</sup>-forming activity. Until now, nobody has succeeded in this aim.

Besides the purification story, a series of indirect evidence and of kinetic studies on both normal and CGD neutrophils has provided additional insights into the composition of NADPH oxidase. These studies are the object of the following paragraphs which report the evidence both in favor and against the involvement of single cofactors in the oxidase system.

Table 3. Molecular Properties of NADPH Oxidase as Indicated by Purification Studies

Cell Type	Mr of Enzyme Complex	Mr of Subunits	Presence of			Reference Number
			Cytochrome <i>b</i>	FAD	Quinone	
Human neutrophils	≈150,000 (and >300,000)	n.r.	n.r.	n.r.	n.r.	131
Rat alveolar macrophages	n.r.	52,000	n.r.	n.r.	n.r.	133
Guinea pig neutrophils	150,000	70,000	n.r.	n.r.	n.r.	137
Guinea pig neutrophils	400,000	87,000	n.r.	Yes	n.r.	136
Guinea pig neutrophils	High-Mr complex	32,000	Yes	n.r.	n.r.	140
Pig neutrophils	High-Mr complex	n.r.	Yes	Little	No	138
Pig neutrophils	>350,000	31,500	Yes	Little	n.r.	144
Human neutrophils	n.r.	65,000	No	Yes	n.r.	142
Human neutrophils	≈150,000	67,000 48,000 32,000	No	Yes	No	143
Human neutrophils	n.r.	n.r.	Yes	Little	n.r.	157
Bovine neutrophils	n.r.	65,000	No	No	n.r.	141
Guinea pig peritoneal macrophages	n.r.	n.r.	Yes	Little	n.r.	139

Note: n.r. = not reported.

### B. Cytochrome $b_{558}$ : role and properties

All the phagocyte types express a membrane-bound  $b$ -type cytochrome with unique properties.<sup>158,159</sup> This hemeprotein was discovered by Hattori<sup>160</sup> and by Shinagawa et al.<sup>161</sup> and was called cytochrome  $b_{-245}$  from its peculiar midpoint potential,<sup>156</sup> which is much lower than that of most  $b$ -cytochromes. Others recommended the name cytochrome  $b_{558}$ , from the  $\alpha$  peak of the reduced-oxidized spectrum.<sup>167</sup> Here the original name cytochrome  $b_{558}$ <sup>161</sup>—or simply cytochrome  $b$ —will be used. As judged from potentiometric titrations, the low-potential cytochrome  $b$  is the predominant chromophore in the membranes of neutrophils<sup>134,148,163</sup> and macrophages.<sup>139</sup> According to some authors,<sup>164,165</sup> cytochrome  $b$  is also present in specific granules of neutrophils and translocates to the cell membrane during the cell activation process (see Section V).

The evidence suggesting that cytochrome  $b_{558}$  is a primary component of the  $O_2^-$ -forming system is as follows: (i) Patients affected by the X-linked form of CGD have no cytochrome  $b$  in their leukocytes.<sup>72,167–172</sup> Moreover, variant cloned macrophage cell lines defective in  $O_2^-$  production exhibit structural or functional abnormalities of cytochrome  $b$ .<sup>173</sup> (ii) Hybridization of monocytes from a cytochrome  $b$  negative CGD patient with monocytes from a cytochrome  $b$  positive CGD patient reconstitutes the  $O_2^-$ -forming activity.<sup>174</sup> (iii) During myelocyte differentiation, the cytochrome  $b$  appears in parallel with the development of the ability to perform the respiratory burst.<sup>175,176</sup> (iv) Cytochrome  $b_{558}$  has the very low midpoint potential ( $Em_{7.0}$ ) of  $-245$  mV,<sup>156</sup>  $-235$  mV,<sup>134</sup>  $-225$  mV,<sup>162</sup>  $-247$  mV.<sup>139</sup> This property suggests that the cytochrome could be involved in the reduction of  $O_2$  to  $O_2^-$ ,<sup>149,156</sup> since the  $Em_{7.0}$  for the couple  $O_2/O_2^-$  at  $p_{O_2} = 1$  atm. is of about  $-330$  mV, as recalculated by Wood.<sup>177</sup> (v) Cytochrome  $b$  in the membrane behaves as a terminal oxidase, since it binds carbon monoxide,<sup>140,161,173,178</sup> it is reduced in anaerobiosis,<sup>179–181</sup> and it is rapidly reoxidized by oxygen.<sup>178</sup> The anaerobic reduction does not occur in neutrophils from cytochrome  $b$  positive CGD patients, indicating that in this variant of the disease the cytochrome does not function as electron carrier.<sup>179</sup> (vi) Pyridine and imizadole, which bind to heme of cytochrome  $b_{558}$  as judged by the spectral changes they induce, inhibit the respiratory burst in intact neutrophils and NADPH oxidase in lysates.<sup>182</sup>

In apparent conflict with the previously reported data, two lines of evidence have questioned the involvement of cytochrome  $b_{558}$  in the oxidase activity, the first one regarding the reduction of cytochrome  $b$  by NADPH, the second one regarding the binding of carbon monoxide. As far as the first question is concerned, it has been observed that the rate of anaerobic

reduction of cytochrome  $b$  by pyridine nucleotides, in subcellular particles,<sup>149,163,183,184</sup> or solubilized enzyme<sup>134,147</sup> is very slow and does not account for the rate of  $O_2^-$  generation by the same preparation. This discrepancy would argue strongly against any model including the participation of cytochrome  $b$  as an obligatory electron carrier in the  $O_2^-$ -forming reaction.<sup>185</sup> It was therefore suggested that the oxidase works as a branched electron transport chain, where only part of the electrons that flow from NADPH to oxygen pass through the cytochrome  $b$ .<sup>163</sup> A relevant clarification of this problem came from the work of Cross et al.<sup>186</sup> They were able to determine the rate at which cytochrome  $b$  is reduced by NADPH under aerobic conditions, based on the extent of reduction at the steady state and of the rate of oxidation by  $O_2$ . It appears that the cytochrome  $b$  reduction in aerobiosis is much faster than in anaerobiosis and is very close to the measured overall rate of NADPH dependent  $O_2^-$  production. These results demonstrate that oxygen is apparently required for rapid electron flow into the oxidase complex, and that cytochrome  $b$  is kinetically competent to act as part of the  $O_2^-$ -generating complex.

The second controversial question regards the binding of CO to the cytochrome  $b_{558}$ . The formation of a CO complex, consistent with the proposed role as a terminal oxidase, was demonstrated by many investigators,<sup>147,156,161,162,173,187</sup> but not by others.<sup>188,189</sup> Morel et al.<sup>183,184</sup> observed only limited changes in cytochrome  $b$  absorption spectrum of CO-treated neutrophil membranes, indicative of a poor reactivity to CO. This is in agreement with the data of Cross et al.,<sup>178</sup> who estimated that the concentration of CO giving 50% binding is 1.18 mM, much higher than the  $K_m$  for  $O_2$  of the oxidase, which is of 0.01–0.03 mM.<sup>183,190–191</sup> The question was complicated by the demonstration that the respiration of activated neutrophils is not affected by CO, even at very low concentrations of  $O_2$  in the assay mixture.<sup>184,188</sup> Assuming that the cytochrome  $b$  is a CO-reacting pigment and is the terminal oxidase, it is difficult to explain why CO does not inhibit the respiratory burst. Therefore, one is faced with the following dilemma, which was well expressed by Morel and Vignais:<sup>184</sup> "Either cytochrome  $b$  is the terminal oxidase, but it is not CO-sensitive; or cytochrome  $b$  is sensitive to CO and cannot be the only terminal oxidase".

A possible explanation of these discrepancies could be found in the paper of Iizuka et al.<sup>182</sup> By using low-temperature (77°K) absorption spectroscopy of whole cells, the authors provided convincing evidence that cytochrome  $b_{558}$ , at least *in situ*, does not form a complex with CO (it should be noted that the studies reporting evidence of CO binding were done on subcellular fractions or solubilized enzyme). According to

these authors the absence of CO binding does not rule out a function as an oxidase, but only argues against the formation of a cytochrome-oxygen complex. In fact, the possibility of a transfer of electrons to O<sub>2</sub>, not by way of the oxygenated form, but through the heme edge of reduced cytochrome *b* could be taken into consideration.<sup>182</sup> The present status of knowledge of cytochrome *b* function does not allow any conclusion about this particular aspect. The anomalous behaviour with respect to CO binding must be added to the list of unusual properties of phagocytes' cytochrome *b*<sub>558</sub>.

Additional characteristic features of cytochrome *b* that merit mentioning are the following: (i) the  $\alpha$  band of the spectrum scanned at 77°K shows a fine structure with two components at 558 nm and at 556 nm.<sup>182</sup> Only the peak at 558 nm emerges upon stimulation of neutrophils in anaerobiosis. These data could suggest the presence of two heme cofactors with distinct properties. (ii) Electron spin resonance of cytochrome *b* reveals an atypical spectrum that could indicate the presence of some nonheme metal.<sup>161,162,193</sup> (iii) The midpoint potential exhibits a pH-dependency with a change of approximately 25 mV per pH unit.<sup>156</sup> The  $E_{m7.0}$  was modified from -245 mV to -180 mV by 1 mM *p*-chloromercuribenzoate, suggesting a structural or functional role for -SH groups.<sup>147</sup>

A number of groups have attempted the purification and characterization of cytochrome *b* from neutrophils.<sup>144,162,187,193-196</sup> Unfortunately, even in this field the various reports were often controversial. The first claim of a purification of cytochrome *b*<sub>558</sub> from human neutrophils was from Segal's group.<sup>193</sup> The cytochrome was isolated from neutrophils of patients with chronic myeloid leukemia (their blood contains high concentration of neutrophils, which aren't much different from the normal ones), by a procedure involving solubilization with Triton N-101, hydrophobic chromatography and final purification on heparin-agarose column eluted with NaCl gradient. The most purified fractions had a specific content of 11-16 nmoles cytochrome *b*/mg protein, representing a 140-200-fold purification. On SDS-polyacrylamide gel electrophoresis, the purified material gave a broad band with Mr of 68-78,000. Subsequent studies from the same group<sup>194</sup> showed that cytochrome *b* is a glycoprotein and cleavage of carbohydrates changed the Mr to 55,000.

Pember et al.<sup>162</sup> reported the purification of cytochrome *b*<sub>558</sub> from bovine neutrophils by using phase partitioning with Triton X-114, followed by hydroxylapatite- and hydrophobic-chromatography. Phase partitioning demonstrated that cytochrome *b* is an integral membrane protein, in accord with another report.<sup>197</sup> The final specific content was 20 nmoles cytochrome *b*/mg protein, and SDS-urea gel electro-

phoresis showed three principal protein bands with Mr of 14,000-12,000-11,000.

Lutter et al.<sup>187</sup> purified cytochrome *b*<sub>558</sub> from human neutrophils by solubilization with Triton X-100, followed by chromatography on Blue Sepharose and Sephacryl S-300. The specific content was 5.3 nmoles/mg protein. The apparent molecular mass, estimated by gel filtration, was 235,000 daltons, while SDS-gel electrophoresis indicated a Mr of 127,000.

In our laboratory a method for purification of cytochrome *b*<sub>558</sub> from pig neutrophils was developed.<sup>144</sup> We extracted the cytochrome *b* component from the partially purified and active oxidase (see above) by dissociation of the proteolipid complex with KCl, deoxycholate and Triton X-100. The activity was lost, but the heme spectrum was still detectable, so that cytochrome *b* could be purified through gel filtration chromatography on Ultrogel AcA34 carried out in the presence of KCl and detergents. The cytochrome-*b* containing fractions were eluted in the included volume of the gel filtration column, with an apparent molecular mass of 170-230,000 daltons and a specific content of 10.3 nmoles/mg protein. No flavin was associated with purified cytochrome. SDS-electrophoretic analysis showed one definite band with Mr of 31,500 and a light and broad protein smear at 90-100,000 daltons. We therefore suggested that the 31,500-daltons peptide, previously shown to be a component of the oxidase complex,<sup>140</sup> actually belongs to cytochrome *b* or is one of its subunits. Subsequent attempts to improve the purification of the 31,500 daltons peptide were unsuccessful and invariably resulted in copurification of protein bands around 100,000 daltons (P. Bellavite, unpublished experiments). It is therefore conceivable that these high molecular weight components also belong to cytochrome *b*, and this could partially reconcile our data with those from other laboratories.<sup>187,193</sup>

Recently the cytochrome *b*<sub>558</sub> was purified from human neutrophils by Parkos et al.<sup>195,196</sup> by solubilization with octylglucoside, followed by affinity chromatography on wheat germ agglutinin and then on heparin ultrogel. The nondenatured protein had a molecular weight of 202,000 daltons. SDS-gel electrophoresis revealed a broad band with Mr of 91,000 and a lower molecular weight species with Mr of 22,000. Cleavage of *n*-linked carbohydrates caused a change of Mr of the larger species to 48,000 daltons. The authors were also able to immunoprecipitate the hemoprotein with specific antisera.

The literature data on the structure of the hemoprotein component of NADPH oxidase are summarized in Table 4. A striking correspondence of the results from several investigators can be seen as regards the molecular weight estimated in nondenaturing conditions,



Table 4. Molecular Properties of Cytochrome  $b_{558}$ 

Cell Type	Mr in Nondenaturing Conditions	Mr in SDS-Electrophoresis	Reference Number
Human neutrophils	Aggregate	68–78,000	193
Human neutrophils	n.r.	55,000*	194
Bovine neutrophils	n.r.	14–12–11,000	162
Human neutrophils	235,000	127,000	187
Pig neutrophils	≈200,000	31,500	144
Human neutrophils	202,000	91,000 22,000 48,000*	195,196

\*After enzymatic removal of carbohydrate.  
Note: n.r. = not reported.

i.e. about 200,000 daltons. Even if this value includes the bound detergent and residual lipids, it indicates either the presence of a relatively large protein (with respect to other  $b$ -type cytochromes),<sup>198</sup> or association of subunits. The latter seems to be the case, as judged by the variety of polypeptide bands that has been described on SDS-electrophoretic analysis. The identification of the cytochrome  $b$  structure by SDS-gel electrophoresis was very difficult due to a number of factors, including dissociation of heme from apoprotein, possible proteolytic cleavage due to the abundance of proteases in neutrophils, easy aggregation of the cytochrome<sup>193</sup> and differences among animal species and purification procedures employed by the various laboratories.

The preliminary amino acid analysis of purified cytochrome  $b$  was also reported<sup>162,187,194</sup> and is comparatively shown in Table 5. It can be seen that there is a substantial agreement between the three groups, with marked discrepancy for only a couple of amino acids (Pro and Tyr). The finding of similar amino acid composition is encouraging, because it suggests that the

same cytochrome was purified by separate groups, even if the SDS-electrophoretic analysis gave controversial patterns.

Additional work is required for unequivocal characterization of this cytochrome with its unusual structure and behaviour. Probably protein sequencing of candidate subunits, gene cloning and production of specific monoclonal antibodies will clarify any questions and facilitate the comparison of results obtained in different laboratories.

### C. Flavoprotein

Besides the data derived from purification experiments (see above), a series of studies carried out on crude enzyme preparation have produced important insights on the possible role of a flavoprotein in the NADPH oxidase system. Assuming either the involvement of cytochrome  $b_{558}$  as the terminal oxidase of an electron transport chain, or the direct formation of  $O_2^-$  by the oxidase, participation of a flavoprotein would be theoretically obligatory, since no redox transfer between the two-electron donor NADPH and the one electron acceptor cytochrome  $b$  (or  $O_2$  in the case of direct formation of  $O_2^-$ ) could be performed without an intermediate electron carrier.<sup>163</sup>

The first demonstration of a flavin cofactor requirement for the  $O_2^-$ -forming enzyme came from experiments showing that NADPH oxidase activity of cell-free particles assayed in the presence of Triton X-100 required FAD.<sup>199</sup> Other cofactors (FMN, riboflavin, ADP, AMP) were without effect. The FAD-requirement for NADPH-dependent  $O_2^-$ -formation was confirmed in preparations solubilized with Triton X-100,<sup>132,200</sup> with Tween 20,<sup>146</sup> but not with deoxycholate.<sup>131,147</sup>

Additional evidence suggesting the participation of a flavoprotein is as follows. (i) According to Light et al.,<sup>134</sup> the enzyme activity is inhibited by micromolar

Table 5. Preliminary Amino Acid Composition of Cytochrome  $b_{558}$ 

Hydrophilic Amino Acids	Reference Number			Hydrophobic Amino Acids	Reference Number		
	187	162	194		187	162	194
Asx	9.0	7.4	8.2	Pro	5.4	14.1	1.4
Glx	9.8	5.5	9.0	Gly	9.1	6.8	9.4
His	1.5	3.3	4.9	Ala	8.0	6.6	9.2
Lys	5.7	4.6	5.6	Val	5.3	5.5	7.2
Arg	5.1	4.2	5.7	Met	0.9	1.4	1.7
Ser	8.5	6.2	5.8	Ile	4.4	5.8	6.1
Thr	5.0	8.6	5.1	Leu	8.5	9.2	10.3
				Tyr	9.4	3.0	3.5
				Phe	4.4	4.2	5.6
Total hydrophilic	44.6	39.8	44.3				

Note: Values are mole percent.



concentrations of 5-carba-5-deaza-FAD, a flavin analogue that is only competent for two-electron transfers. On the basis of this finding the authors suggested that this analogue completely displaces residual FAD from the apoenzyme and that the physiological oxidant of the flavoenzyme is a one-electron acceptor. However, it must be pointed out that other interpretations of the effect of 5-deaza-flavin are possible, because this compound appears to be a nicotine analogue as well as a flavin analogue.<sup>201</sup> (ii) According to Cross et al.,<sup>149</sup> addition of NADPH to anaerobic preparations of solubilized NADPH oxidase caused reduction of FAD with kinetics similar to those of cytochrome *b* reduction. The initial rates of reduction of each prosthetic group were higher in preparations from PMA-treated cells as compared with those from unstimulated cells. (iii) Gabig and Lefker<sup>202</sup> were able to resolve the flavoprotein from cytochrome *b* by differential centrifugation of solubilized membrane extracts. The resolved flavoprotein was anaerobically reduced by NADPH and was rapidly reoxidized upon addition of oxygenated buffer. The cytochrome *b* was anaerobically reduced by NADPH in the intact particulate oxidase, whereas reduction did not occur in the cytochrome resolved from the flavoprotein. (iv) Electron spin resonance studies on both stimulated and resting neutrophil membranes<sup>203</sup> showed formation of flavin free radical with spectrum at  $g = 2.004$ , with a peak to peak width of 19 G, suggesting that the redox intermediate is a neutral semiquinone. The authors could also calculate the  $E_{m7.0}$  of the couples  $FADH_2 \rightleftharpoons FADH^\cdot$  and  $FADH^\cdot \rightleftharpoons FAD$ , which were  $-256$  mV and  $-304$  mV, respectively. Interestingly, the flavin free radical was generated by reduction with dithionite in both resting and activated membranes, while it was generated by NADPH reduction only in active preparations. (v) O<sub>2</sub><sup>-</sup> generation by NADPH oxidase is inhibited by antibodies to an homologous liver flavoprotein (NADPH-cytochrome P450 reductase).<sup>204</sup> (vi) In neutrophils of some CGD patients, a deficiency of flavoproteins has been found.<sup>205-207</sup> According to Borregaard and Tauber,<sup>197</sup> the defect would be in regard only to a flavoprotein species that is present in the specific granule fraction of unstimulated cells and is functionally associated with cytochrome *b*<sub>558</sub>. Larger statistics were published by Bohler et al.<sup>171</sup> and by Ohno et al.,<sup>172</sup> who found a decrease of FAD content in 4 of 28 and 4 of 25 CGD patients studied, respectively. Both groups noted that most of cytochrome *b* negative patients had normal amounts of FAD, all CGD patients with reduced FAD also lacked cytochrome *b* and all CGD patients with normal amounts of cytochrome *b* had normal FAD. The simultaneous deficiency of both flavoprotein and hemeprotein raises the question of the mechanism by which a presumed single gene defect

(see Section III.E.iv) results in a dual protein abnormality. The possibility has been suggested that the oxidase is actually a flavocytochrome *b* where the defect in CGD may touch the common apoprotein, the heme or the FAD.<sup>171</sup> The hypothesis is attractive, but is in conflict with other evidence indicating that flavoproteins and cytochrome *b* can be physically dissociated by detergents.<sup>197,202</sup> Until further characterization of the genetic background of CGD syndrome and of the genetic NADPH oxidase gene(s) expression, transcription and translation is accomplished, any explanation must remain speculative.

Taken together, the above data provide overwhelming evidence implicating a flavoprotein in the NADPH oxidase of activated neutrophils. The only criticism that may be raised to the studies regarding the properties of the flavoprotein is that the investigations have been done on crude subcellular particles or detergent extracts and not on purified enzyme. Of course, this was often an obligatory choice due to technical problems or to scarcity of material. However, while cytochrome *b*<sub>558</sub> represents the predominant and perhaps the only cytochrome species of neutrophil membranes, a variety of flavoprotein species are present in the particulate fractions of neutrophils and can be extracted by detergents.<sup>138,146,197,208,209</sup> Among these flavoproteins there are oxidoreductase enzymes which have nothing to do with the true respiratory burst enzyme.<sup>138,209,210</sup> Therefore, the finding of FAD reduction in membranes or in crude detergent extracts does not demonstrate that reduced flavoproteins belong to NADPH oxidase. Similarly, the finding of a FAD:cytochrome *b* ratio ranging from 0.5:1 to 2:1 in membranes or phagocytic vacuoles<sup>197,202,205,206,211</sup> does not demonstrate that this is the actual stoichiometry in the oxidase complex.

Indirect evidence that these criticisms on the study of flavoproteins in unpurified fractions are well-grounded derivatives from the observation that in CGD patients the flavoprotein deficiency is, where present, only partial, while the cytochrome *b* deficiency is total.<sup>171,172,197,205</sup>

In the course of our studies on pig neutrophil oxidase, we analyzed the flavin content of enzyme preparations.<sup>138</sup> The main findings were as follows: (i) membranes and crude detergent extract from pig neutrophils contain flavoproteins (containing FAD and not FMN) in a ratio of 1:2 with respect to cytochrome *b*; (ii) most of the flavoproteins of the membrane extract may be chromatographically dissociated from NADPH oxidase activity and cytochrome *b*; (iii) the flavoproteins, which may be separated from NADPH oxidase activity and cytochrome *b*, have NADH- and NADPH-dependent cytochrome *c* reductase activity and 2,6-dichlorophenolindophenol (DCIP) reductase activity (disphorase activities). Probably these activities are not involved in the respiratory burst enzyme, as judged from their dif-

ferent substrate specificity (NADH instead of NADPH) and different  $K_m$  for NADPH.<sup>212</sup> (iv) the ratio FAD:cytochrome *b* in the partially purified and active enzyme complex is about 1:20. We advanced two possible explanations for these rather unexpected findings.<sup>138,213</sup> First, the oxidase would be composed of one flavoprotein surrounded by many cytochrome *b* molecules. A similar model was proposed for explaining the nonequimolar concentration of flavoprotein and cytochrome P450 in liver microsomes.<sup>214</sup> The second possibility is that the small amount of FAD found by us in the oxidase complex actually represented a contamination by NAD(P)H diaphorases due to incomplete purification. If this is the case, one should conclude that NADPH-dependent  $O_2^-$  formation may be catalyzed by a FAD-independent pathway. The latter hypothesis seems to agree with some results of Doussière and Vignais (see Section III),<sup>141</sup> but contrasts with all the above reported evidence in favor of participation of FAD and does not explain how a two electron donor (NADPH) reduces a one electron acceptor (the heme group or  $O_2$  in the case that  $O_2^-$  is formed). Theoretically, such a function could be accomplished by other electron carriers involving either thiyl and phenoxyl free radicals,<sup>215</sup> or metal atoms such as iron, molybdenum or copper.<sup>216</sup> The inhibitory effect of sulfhydryl reagents<sup>132</sup> and of bathophenanthroline sulfonate,<sup>139,148</sup> a lipophilic iron chelating agent, is compatible with this possibility, which at present cannot be excluded.

In conclusion, most evidence is strongly in favor of the flavoprotein nature of the NADPH oxidase and therefore of the participation of a FAD-containing NADPH dehydrogenase in the catalysis. However, some indications coming from purification studies point out that the situation is probably more complex than that predicted by an equimolar FAD:cytochrome *b* ratio.

#### D. Quinones

In analogy with the mitochondrial respiratory chain, quinones have been implicated in the hypothetical electron transport chain responsible for  $O_2^-$  production, where they could mediate the electron transfer between flavoprotein and cytochrome *b*, which are embedded in the lipid milieu of the membrane. However, the experimental evidence supporting this hypothesis is controversial, since apparently clear-cut data both in favor and against the participation of these electron carriers have been published.

Presence of quinone, identified as ubiquinone-10 ( $Q_{10}$ ), was discovered in lipid extracts of human neutrophils.<sup>217,218</sup> The concentration of ubiquinone (0.06 nmoles/mg protein) was close to a 1:1 stoichiometry with cytochrome *b*.<sup>219</sup> Similar values (0.1 nmoles/mg protein) were reported by Crawford and Schneider,<sup>220</sup>

who subsequently found enrichment of ubiquinone in phagolysosomes, possibly due to translocation from a ubiquinone-rich granule pool.<sup>221</sup> Gabig and Lefker<sup>222</sup> analyzed the content of electron carriers in neutrophil membranes, where they discovered FAD, ubiquinone 10 and cytochrome *b*<sub>558</sub> in a 1.3:1:2 molar ratio. 60% of quinone was reduced by NADPH under anaerobic conditions, irrespective of whether the membranes were obtained from stimulated or from resting cells. Since cytochrome *b* reduction by NADPH could only occur in the membranes from stimulated neutrophils, the authors proposed a model where the activation of the oxidase consisted in the "unblocking" of electron flow from  $Q_{10}$  to the cytochrome *b*, thus allowing the terminal oxidase component to reduce oxygen.

On the other hand, Cross et al.<sup>223</sup> showed that the ubiquinone in neutrophils is almost exclusively associated with mitochondria, and Lutter et al.<sup>211</sup> did not find any ubiquinone-10 in neutrophil cytoplasts (neutrophil subcellular fractions which are devoid of nuclei and of organelles but contain an intact, activatable oxidase system). No quinone could be extracted from partially purified and active NADPH oxidase.<sup>138,143</sup> Finally, Green and Beck (personal communication) depleted neutrophil membranes of ubiquinone-10 by pentane extraction, but the NADPH-dependent  $O_2^-$  formation was not significantly altered by this treatment.

The above data lead to the embarrassing alternative: are the findings of quinones due to the contamination of the preparations by other quinone-rich organelles, or are the negative results due to sensitivity of the assays and/or inefficient extraction? Of course, we agree upon the former explanation, since our analysis of partially purified enzyme was negative,<sup>138</sup> but one must acknowledge that the alternative explanation cannot at present be excluded.

The second line of debate about the role of quinones concerns the effect of these compounds or of their inhibitors on NADPH oxidase activity. Ubiquinone was proposed as a possible electron carrier on the basis of the stimulatory effect exerted by short-side-chain quinones (ubiquinone 5 and duroquinone) on the oxygen consumption by both intact cells and homogenates in the presence of NADPH.<sup>220</sup> Oxygen metabolism was inhibited by a number of compounds with ubiquinone-like structures, such as Juglone, quercetin, and nordihydroguaiaretic acid (NDGA).<sup>220</sup> Furthermore, membranes and phagocytic vesicles of neutrophils contain NADPH-dependent quinone reductase activity.<sup>217,224,225</sup>

In our opinion, the above reported effects of quinones are too indirect to be considered as positive evidence of the involvement of these electron carriers in  $O_2^-$  production. The inhibitors could have other unrelated targets, as has been shown for NDGA, quer-

cetin and menadione.<sup>226-229</sup> The NADPH oxidase activity, which was stimulated by duroquinone,<sup>220</sup> exhibited kinetic properties (K<sub>m</sub> for substrate) much different from these which are characteristic of the respiratory burst enzyme. Finally, the addition of exogenous quinones to membrane preparations that contain diaphorase enzymes is expected to divert electrons from the normal acceptors towards molecular oxygen.<sup>153,154</sup> In conclusion, the involvement of certain quinones in the phagocyte NADPH oxidase cannot be ruled out, but should be carefully reevaluated.

#### E. Other candidate, components of the oxidase

Additional factors or proteins possibly involved in the structure or function of the enzyme have been suggested on the basis of the following experimental findings:

(i) A role for phospholipids was first suggested by Gabig and Babior,<sup>132</sup> who showed that phosphatidylethanolamine (but not phosphatidylcholine or phosphatidylserine) is able to stimulate O<sub>2</sub><sup>-</sup> formation by the Triton X-100 solubilized enzyme. The NADPH oxidase extracted with deoxycholate contained considerable amounts of phospholipids,<sup>140,144</sup> mainly phosphatidylethanolamine and phosphatidylcholine. In addition, cytochrome *b*<sub>558</sub> was shown to be an integral membrane protein, which is large enough to span the lipid bilayer.<sup>195-197</sup> All these data are consistent with a structural requirement of phospholipids for O<sub>2</sub><sup>-</sup> forming enzyme. The possibility that the lipid milieu also plays a role in the triggering of the oxidase will be discussed in Section V.

(ii) Cross and Jones<sup>230</sup> reported that a novel inhibitor of O<sub>2</sub><sup>-</sup> production from the solubilized oxidase (diphenylene iodonium, DPI) specifically bound to a protein of 45,000 daltons. The binding was competitively inhibited by NADPH. Since DPI also abolished the NADPH-dependent reduction of both cytochrome *b*<sub>558</sub> and flavoprotein in anaerobiosis, the authors suggested that the site of action of DPI is at the level of (or possibly before) the flavoprotein, and that the flavoprotein contains a polypeptide with Mr of 45,000.

(iii) A possible component of the oxidase was described by Umei et al.,<sup>231</sup> who used the 2',3'-dialdehyde derivative of NADPH as an affinity labeling reagent of NADPH oxidase. The analogue competitively inhibited NADPH-dependent O<sub>2</sub><sup>-</sup> formation by solubilized enzyme preparations, and the inactivation was protected by excess NADPH, indicating that the compound did effectively interact with the binding site of the enzyme. The labeling of the dialdehyde-derivative treated oxidase was carried out by irreversible reduction with sodium cyanoboro [<sup>3</sup>H]hydride. SDS-gel

electrophoresis of the labeled enzyme allowed the identification of a major peak of radioactivity with Mr of 66,000. It is conceivable that the 66,000 daltons polypeptide is the NADPH-binding component of the oxidase system. It is worth pointing out that a protein with the same molecular weight was found by others in purified oxidase preparations.<sup>141-143</sup> Unfortunately, Umei et al.<sup>231</sup> could not demonstrate whether their labeled protein contained flavin and heme because the method dissociated any putative cofactor, if present. According to a preliminary report from the same group,<sup>232</sup> the NADPH analogue bound to the 66,000 daltons protein in CGD patients with both X-linked and autosomal recessive forms, indicating that the NADPH-binding domain is intact in this disease. The identification of a protein with Mr of 65,000 as the NADPH-binding component of the enzyme was also recently reported by Doussi re et al.<sup>233</sup>

(iv) An interesting approach to the nature of the respiratory burst enzyme was followed by the Orkin's group,<sup>234,235</sup> who identified a gene locus involved in CGD on the short arm of the X chromosome. The gene was cloned and found to be defective in one patient with X-linked CGD and abnormally transcribed in another three. The nucleotide sequence of complementary DNA clones predicted a translation product of 468 amino acids with an estimated Mr of 54,000. In the amino acid sequence, four potential *N*-linked glycosylation sites and one extensive hydrophobic segment were evident, indicative of a membrane-bound glycoprotein. The authors<sup>235</sup> suggested that the protein (designated as "X-CGD protein") is an essential component of the oxidase system of the phagocyte, but they couldn't find significant homology to other known sequences. The amino acid composition was also different from that reported for purified cytochrome *b*<sub>558</sub>. So it could be another redox component of the oxidase system or a protein involved in the process of synthesis and assembly of the oxidase. Afterwards, others pointed out that the predicted X-CGD sequence contained a region similar to the heme-binding region of cytochrome P450.<sup>236</sup> This observation seems to revive the possibility that the X-CGD protein is actually the cytochrome *b*<sub>558</sub>. Certainly the genetic approach will make important contributions to the understanding of NADPH oxidase in the near future.

#### IV. MECHANISM OF CATALYSIS OF FREE RADICAL FORMATION

Owing to the uncertainties about the structure of NADPH oxidase, any model of the reaction catalysis is still hypothetical. Assuming that the oxidase is a multicomponent system, enzymatic activity is the re-



sult of the three distinguishable events, i.e. formation of enzyme-NADPH complex, electron transfer throughout the elements of the complex, interaction with  $O_2$  to give  $O_2^-$  and  $H_2O_2$ . The available knowledge on these points is reviewed in the present chapter. Furthermore, some general properties of the reaction mechanism will be outlined.

#### A. NADPH binding

The probable identification of the NADPH binding site on a protein with Mr of 65–66,000 has been mentioned in the preceding section. Elegant experiments showed that the NADPH binding site of the oxidase is located on a portion of the oxidase extending into the cytoplasm, while the rest is buried in the lipid bilayer.<sup>237,238</sup> This portion of the oxidase was explored by nonpenetrating inhibitors, which were effective only on subcellular fractions but not on intact cells. The inhibition exerted by *p*-chloromercuribenzoate<sup>237</sup> also indicated that the portion of the enzyme which is located on the inner surface of the membrane contains important –SH groups, while the inhibition exerted by cibacron blue<sup>238</sup> suggested that the NADPH-binding site has a dinucleotide fold.

The affinity for the substrate NADPH ( $K_m$ ) ranges from 0.015 to 0.2 mM, according to cell type and experimental conditions.<sup>122,123,132,239–242</sup> The  $K_m$  for NADPH may increase as a consequence of the manipulation and the storage of the enzyme.<sup>143</sup>

Since NADPH oxidase may be activated and deactivated upon cell stimulation (see Section I), the possibility that modification of the NADPH-binding site was important in regulation of the functional level of the catalysis was investigated. Early studies demonstrated that after initiation of phagocytosis the kinetic parameters of NADPH change, with an increase of affinity for substrate so that the expression of enzyme activity would be allowed at physiological concentrations of NADPH.<sup>112,121,243</sup> These studies were carried out by measuring oxygen consumption or  $NADP^+$  production at acidic pH. By assaying the enzyme activity at neutral pH as superoxide production, or  $NADP^+$  production in the presence of SOD, such an effect couldn't be demonstrated, possibly because the  $O_2^-$ -formation of unstimulated NADPH oxidase is almost undetectable and as a consequence the  $K_m$  is not measurable.<sup>117,244,245</sup> It has been suggested that in the case of the enzyme in resting membranes, the redox center is completely shielded by some mechanism and cannot come in contact with NADPH.<sup>203</sup> The  $O_2^-$ -forming activity assayed on membranes of cells which were subjected to different stimulants or to different doses of

the same stimulant exhibited marked differences in maximal velocity, but about the same  $K_m$  for NADPH.<sup>245,246</sup> These results suggest that the activation of NADPH oxidase is an all-or-nothing event at the molecular level, and the activation of the respiratory burst in neutrophils is due to the recruitment of increasing numbers of enzyme units which enter into the play. Once activated, the single enzyme would work with a fixed  $K_m$  for NADPH.

A molecular defect of the NADPH-binding site of the oxidase has been identified in a rare variant of CGD.<sup>129,247,248</sup> Neutrophils of these patients produce superoxide at a rate that is significantly higher than zero and give a slight positivity at the NBT test. The disease in this variant is clinically mild. It has been shown that the defect of  $O_2^-$  production is associated with a markedly altered  $K_m$  for NADPH (1 mM in the patients compared with 0.05–0.08 mM in controls). It remains to be explained why in this CGD variant the leukocytes, whose molecular defect would be at the level of NADPH binding component of the oxidase, lack cytochrome  $b_{558}$  heme spectrum. Two possibilities exist, i.e. either the cytochrome *b* also carries the NADPH binding site, or the neutrophils are affected by multiple molecular lesions of the oxidase complex.

The NADPH binding site could be relevant in the regulation of macrophage oxidative functions. In fact, differences in the kinetic properties of NADPH oxidase have been found in macrophages at different states of immunological activation.<sup>249</sup> Resident macrophages, which produced little  $O_2^-$  in response to PMA, were compared with corynebacterium-parvum-activated macrophages, which produced much more  $O_2^-$  in response to the same dose of PMA. While the two macrophage populations had a similar content of cytochrome  $b_{558}$  and a similar number of receptors for PMA, corynebacterium-parvum-activated macrophages had a NADPH oxidase with a  $K_m$  much lower than that of resident macrophages. Similar differences of enzyme kinetic parameters were found by others on different types of mononuclear phagocytes with variable ability to produce oxygen free radicals.<sup>250–252</sup> Even the treatment of human monocyte-derived macrophages in culture with  $\gamma$ -interferon caused a decrease of  $K_m$  for NADPH and an increase of the extent of the respiratory burst.<sup>253</sup>

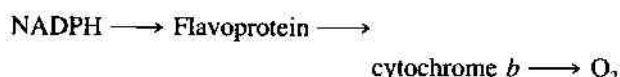
#### B. Electron transfer between the components

On the basis of the available information on the structure and the kinetic properties of the NADPH oxidase, a series of hypothetical models of the mechanism of electron transfer between NADPH and  $O_2$  may be



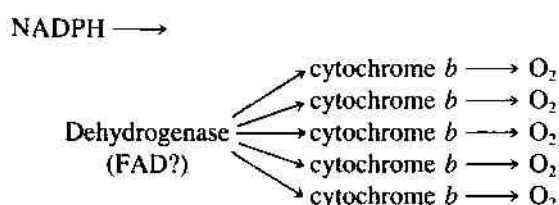
proposed:

#### Model 1.



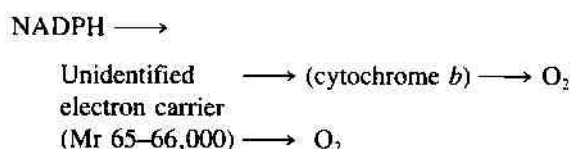
This model<sup>186,203,230,254</sup> suggests that the reducing equivalents are taken up by a flavoprotein acting as NADPH-cytochrome *b*<sub>558</sub> oxidoreductase. Probably a FAD-semiquinone intermediate is involved in the reduction of the obligate one-electron acceptor cytochrome *b*. The cytochrome is the terminal oxidase of the electron transport chain.

#### Model 2.



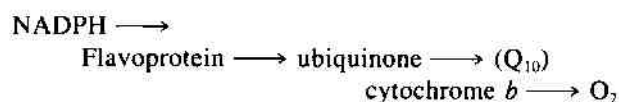
This is a variant proposed by us on the basis of the low FAD:cytochrome *b* ratio found in the partially purified oxidase.<sup>138,213</sup> If this is the case, the flavoprotein would have a faster turnover than the heme protein and would be able to reduce a number of neighbouring cytochrome *b* molecules. Alternatively, the excess of cytochromes could indicate that only a few of them are functionally coupled with the dehydrogenase and undergo cyclic oxido-reduction.

#### Model 3.



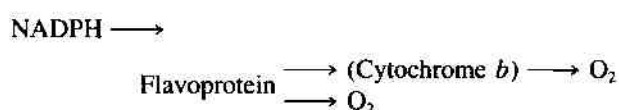
In this model, the NADPH dehydrogenase function would be performed by a component with Mr of 65–66,000 whose prosthetic group, if present, was not identified.<sup>141,231,233</sup> Since Doussi re and Vignais<sup>141</sup> did not find heme spectra in their purified preparations, the possibility of O<sub>2</sub><sup>•−</sup> formation bypassing cytochrome *b* could be also considered. These authors, however, did not exclude the possibility that in the whole membrane the reaction proceeds through cytochrome *b* at a faster rate.

#### Model 4.



Similar models have been proposed by several authors.<sup>218–222,224</sup> As it has been discussed above, the involvement of quinones in the electron transport chain is controversial but, at present, cannot be excluded, at least for the oxidase *in vitro*. Assuming that ubiquinone participates in the catalysis, its position in the sequence of the chain could also be between cytochrome *b* and oxygen.

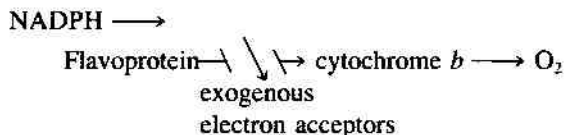
#### Model 5.



A branched-electron transport chain was proposed on the basis of the slow kinetics of cytochrome *b* reduction by NADPH in anaerobiosis.<sup>134,163</sup> If this is the case, the flavoprotein would act as a NADPH:O<sub>2</sub> oxidoreductase, i.e. as a classical oxidase, as well as NADPH:cytochrome *b*<sub>558</sub> oxidoreductase. For a discussion on this particular point, see Section III. A hypothetical model of NADPH oxidase considered as a single flavoenzyme with some similarity to glutathione reductase has also been proposed.<sup>255</sup>

Whatever the precise sequence of the electron-transport chain may be, the problem arises of whether the flavoprotein component is specific for the oxidase system or belongs to other oxido-reductases of the neutrophil membrane. One such flavoprotein is cytochrome *b*<sub>5</sub> reductase,<sup>197,209</sup> but this enzyme cannot be involved in reduction of cytochrome *b*<sub>558</sub> both because it is specific for NADH and because it has a subcellular localization different from that of the cytochrome *b*<sub>558</sub>-associated flavoprotein.<sup>197</sup> Furthermore, NADH diaphorase activity was chromatographically resolved from NADPH oxidase.<sup>138,144,210</sup> The cytochrome *b*<sub>5</sub> reductase of human neutrophils was purified by Tauber et al.<sup>208</sup> and exhibited a Mr of 45,000. A NADH-dehydrogenase of bovine neutrophils with Mr of 17,500 was recently purified and characterized by Nisimoto et al.<sup>256</sup> The functions of these oxidoreductases of neutrophil membranes are unknown, but they could include proton translocation or reduction of lipid peroxides which are expected to be produced in activated neutrophil membranes.<sup>256</sup>

It was proposed that the activity of the flavoprotein component of NADPH oxidase complex may be detected as NADPH-diaphorase activity, i.e. as NADPH-dependent reduction of exogenous dyes such as 2,6-dichlorophenolindophenol (DCIP), ferricyanide, or ubiquinone ( $Q_1$ ).<sup>224,225,257,258</sup> According to this hypothesis, these acceptors would intercept the electron flow from the dehydrogenase to cytochrome *b* and, as a consequence, would inhibit oxygen consumption and superoxide production:



This model entails that dye reduction by the flavoprotein is insensitive to superoxide dismutase. If this is the case, the NADPH-dependent, SOD-insensitive diaphorase would reveal the flavoprotein moiety of the enzyme complex and would be an effective device for understanding the mechanism of the electron transport chain.

A contention on this subject has developed in the literature. It was reported that subcellular fractions from stimulated but not from resting cells catalyze SOD-insensitive DCIP reduction and that DCIP inhibits  $\text{O}_2^-$  production.<sup>224,257</sup> DCIP reductase appeared to be absent in CGD patients.<sup>257</sup> Others showed that DCIP reductase activity of phagocytic vesicles is specific for NADPH ( $K_m$  of 25  $\mu\text{M}$ ), is inhibited by flavin analogues and is enhanced by FAD when extracted with detergents.<sup>258</sup> It was therefore suggested that the NADPH-dependent DCIP reductase activity is catalyzed by a flavin-containing component of the  $\text{O}_2^-$ -forming system. Subsequently, it was reported that phagocytic vesicles catalyze NADPH-dependent, SOD-insensitive, reduction of  $Q_1$ , with optimal pH and  $K_m$  values identical to those of the superoxide-forming oxidase.<sup>225,259</sup>

Contrary to these findings, early work of Gabig and Babior<sup>132</sup> showed that no diaphorase activities are associated with NADPH oxidase. We therefore reinvestigated the matter by using our preparations of partially purified NADPH oxidase, which were almost devoid of unspecific diaphorases.<sup>138,212</sup> We compared cytochrome *c* reduction and DCIP reduction both in the presence and in the absence of SOD and we found that (i) most of cytochrome *c* and DCIP reduction was inhibited by SOD and by anaerobiosis, indicating that it was carried out by superoxide; (ii) the SOD-insensitive (diaphorase) activities were very low as compared with the  $\text{O}_2^-$ -forming activities; (iii) only the SOD-sensitive reduction was increased in enzyme preparations from

PMA-activated cells; (iv) DCIP did not significantly inhibit NADPH-dependent oxygen consumption measured in the presence of SOD (the SOD was included to hinder a pseudo-inhibition due to oxygen given back during the reduction of the acceptor: dye ox +  $\text{O}_2^- \rightarrow$  dye red +  $\text{O}_2$ ). Based on this, we concluded that the preferential and perhaps obligate acceptor of the oxidase is molecular oxygen.

Following our paper, the problem was reinvestigated by the two groups whose work was in conflict with our data.<sup>224,225</sup> It was therefore established that DCIP and cytochrome *c* are actually inefficient in intercepting the electron flow from the oxidoreductase complex<sup>260</sup> and that NADPH-dependent  $Q_1$  reduction is SOD-sensitive, providing that the dismutase is present in sufficiently high concentration.<sup>261</sup> These results are substantially in agreement with ours and confirm the statement that molecular oxygen is the preferential electron acceptor in the NADPH oxidase. Therefore it appears that the enzyme is "protected" from the withdrawal of electrons, which are fully utilized for  $\text{O}_2^-$  formation, in keeping with the role of the oxidase in the phagocyte function.

On the other hand, it has been unequivocally shown that in particular conditions (very high concentration of ferricyanide<sup>260</sup> or high concentration of  $Q_1$  under strict anaerobiosis)<sup>262</sup> a SOD-insensitive reduction of these acceptors could occur. However, this observation does not mean that the electrons are taken up from the flavoprotein, because it is more conceivable that the reduction of electron acceptors takes place at the terminal oxidase site, where these compounds could compete with oxygen for reducing equivalents.<sup>262</sup>

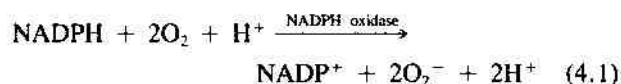
Taken together, the above considerations indicate that the putative flavoprotein moiety of the oxidase cannot be studied as a NADPH-dye reductase because the particular structure of the enzyme complex does not allow easy leakage of electrons.

A second approach to the investigation of electron transfer into the oxidase was provided by the analysis of kinetics of NADPH-dependent reduction of cytochrome  $b_{558}$  and of FAD. These studies were conducted on membranes or crude extracts and therefore are affected by the limitations previously exposed (Section III. C). Nevertheless, some findings are of interest: (i) reduction of cytochrome *b* may be driven by photo-reduced flavin;<sup>178,183</sup> (ii) *p*-chloromercuribenzoate, which reacts with cytochrome *b*, inhibits reduction of cytochrome *b* more than reduction of flavoprotein, suggesting that the cytochrome follows the flavin in the sequence of the putative chain;<sup>149</sup> (iii) the rate of aerobic NADPH-dependent cytochrome *b* reduction in an active preparation (up to 12.9 nmoles/s/mol cytochrome *b*) is close to the rate of  $\text{O}_2^-$  production (up

to 13.0 nmoles O<sub>2</sub><sup>-</sup>/s/mol cytochrome *b*), indicating that the overall electron flux from NADPH to O<sub>2</sub> passes through the cytochrome;<sup>186</sup> (iv) titration of stimulated cell membranes in anaerobiosis with NADPH leads to equilibration at -267 mV, a value which seems to be a middle potential between that of the low potential flavin (-280 mV) and cytochrome *b* (-245 mV).<sup>203</sup> Albeit indirect, all these data provide new insights on the relationships between flavoprotein and cytochrome *b* in the O<sub>2</sub><sup>-</sup>-forming chain.

### C. Oxygen reduction

Most evidence supports the cytochrome *b*<sub>558</sub> as the site of oxygen reduction, even if the controversial data on CO binding awaits a clarification (see Section III B). The final product of the electron transport chain is O<sub>2</sub><sup>-</sup>, which is released on the outer side of the plasma membrane and diffuses into the extracellular environment or into the phagocytic vacuole where it undergoes dismutation and/or reaction with other targets. The overall reaction of the oxidase:<sup>240</sup>



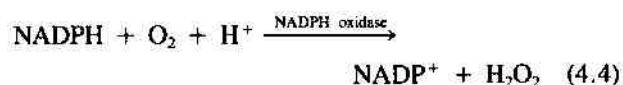
predicted an NADPH:O<sub>2</sub><sup>-</sup> stoichiometry of 1:2, which was confirmed by subsequent studies.<sup>40,117,145,146,265,266</sup> In this respect, it is worth pointing out that the phagocyte NADPH oxidase is the sole biochemical system which is "professionally" addressed to univalent oxygen reduction, while other oxidases usually form either H<sub>2</sub>O<sub>2</sub>, or both H<sub>2</sub>O<sub>2</sub> and O<sub>2</sub><sup>-</sup>, or H<sub>2</sub>O.<sup>216</sup>

It was reported that NADPH oxidase also produces H<sub>2</sub>O<sub>2</sub><sup>122,267</sup> and OH<sup>•</sup>.<sup>268</sup> However, we and others have shown that H<sub>2</sub>O<sub>2</sub> production may be generated by a nonenzymatic chain reaction which is operative at acidic pH and at quite high substrate concentrations.<sup>263,269-271</sup>



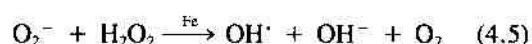
Whether this chain reaction works in intact cells is unknown, but since phagocytes contain a lot of SOD in their cytosol,<sup>34,35</sup> this series of reactions should be prevented.

We have demonstrated the occurrence of divalent reduction of oxygen to H<sub>2</sub>O<sub>2</sub> even in the absence of the chain reaction, i.e. at neutral pH and at low NADPH concentration.<sup>271</sup>



However, in these experiments we used subcellular particles (100,000 × *g* pellet from postnuclear supernatants). When the particles were treated with deoxycholate in order to lyse any aggregate and to break closed vesicles, the ratio of NADPH oxidized to O<sub>2</sub><sup>-</sup> formed approximated the theoretical one of 1:2.<sup>40</sup> Recently Green et al.<sup>272</sup> showed that fresh solubilized enzyme exhibits a univalent reduction of O<sub>2</sub> to O<sub>2</sub><sup>-</sup>, while during storage the stoichiometry changes towards a divalent reduction with formation of H<sub>2</sub>O<sub>2</sub> instead of O<sub>2</sub><sup>-</sup>.

As far as OH<sup>•</sup> is concerned, the formation of this radical by NADPH oxidase was clearly demonstrated.<sup>268,273</sup> However, OH<sup>•</sup> formation required addition of H<sub>2</sub>O<sub>2</sub> and Fe<sup>3+</sup>-chelates, suggesting that the radical was not a primary product of the oxidase but a product of a secondary Haber-Weiss reaction.<sup>274</sup>



The apparent *K<sub>m</sub>* for oxygen of the oxidase was reported to be from 10 to 30 μM.<sup>184,190,191</sup> Interestingly, Edwards et al.<sup>191</sup> showed that the *K<sub>m</sub>* for oxygen measured on intact cells decreases after stimulation of the respiratory burst. The authors suggested that this modification may be functionally relevant since considerable oxygen gradients exist in tissues, and at sites of inflammation oxygen concentration can be less than 12 μM.<sup>275</sup>

### D. Other general properties of the NADPH Oxidase activity

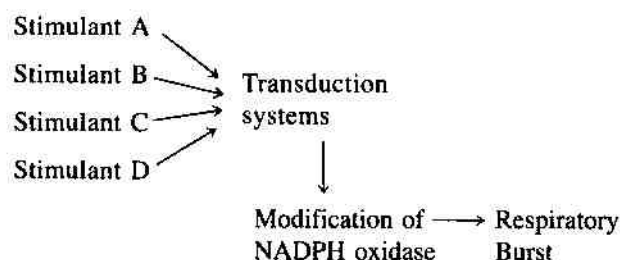
The catalytic mechanism of the NADPH oxidase reaction was also investigated by the effect of particular inhibitors. A list of the compounds that have been shown to affect the NADPH oxidase activity is reported in Table 6. The list includes many inhibitors, which have been already mentioned with regard to their effect on particular components of the system. Owing to the difficulty in the interpretation of the effect of exogenous compounds, only generic informations on the properties of the enzyme may be drawn from these studies: (i) the inhibitory effect of strong detergents indicates either that an assembly of various components is required for the activity or that the lipids in whom the enzyme is embedded play an important regulatory role; (ii) the effect of *p*-chloromercuribenzoate, of sodium thiomalate and of mersalyl suggest that the activity is dependent on free sulfhydryl groups, but this is not a peculiarity for an oxidoreductase; (iii) the inhibition by EDTA and the reversal of inhibition by Mg<sup>2+</sup> indicates that this divalent cation is required for optimal expression of catalytic activity.<sup>278,283</sup> It was

also reported that besides  $Mg^{2+}$ ,  $Ca^{2+}$  also enhances the NADPH oxidase activity<sup>277,283,284</sup> but this latter effect was not observed by others.<sup>148,278</sup> (iv) On the basis of the inhibition by calmodulin binding drugs such as trifluoperazine, it was proposed that calmodulin is essential for the function of NADPH oxidase,<sup>148,281</sup> but the specificity of calmodulin antagonists is questionable.<sup>285</sup>

## V. ACTIVATION MECHANISM

The fact that NADPH-dependent  $O_2^-$  formation is triggered only in cells engaged in phagocytosis or otherwise stimulated has attracted the attention of investigators for a long time. However the activation mechanism of the oxidase remained a mystery until few years ago, when the biochemical events involved in the stimulus-response coupling in phagocytes began to be clarified.

The change of the electron transport chain from an inactive to a functioning state is linked to a complex series of modifications occurring in the membrane and in the cytosol of phagocytes, following the binding of external stimulants to their receptors. The ligand-receptor interaction triggers a cascade of biochemical events—known as the transduction systems—involving calcium entry and mobilization from intracellular stores, phospholipid hydrolysis, transmembrane ionic fluxes, cyclic nucleotide variations, protein phosphorylation, intracellular translocation of enzymes, activation of proteinases and of GTP-binding proteins. Such a series of modifications is essential for coupling of external signals with the final modification of the effector enzyme that leads to its activation:



This scheme implies that different stimulatory agents are capable of activating the same NADPH oxidase through a series of reactions, which may be in common. On the other hand, the activation of the respiratory burst is only one of the multiple functions that are triggered by a stimulant in phagocytes. The transduction systems are then involved in the intracellular

Table 6. Inhibitors of NADPH Oxidase Activity

Compound	Reference Number
Quinacrine and other flavonoids	138,149,276
EDTA	277
Batophenanthroline sulfonate	139,149
5-carba-deaza FAD	134
Quinone analogues	220,227
Pyridine, imidazole, butyl isocyanide	149,182
Diphenylene iodonium	230
Gold sodium thiomalate, thiomalic acid, mersalyl	141,279
p-chloromercuribenzoate	131,237
Cibacron blue	238
Vitamin E	280
Trifluoperazine	148,281
Detergents	141,144,258,282

diversification of the signal in order to reach various targets:



The multiplicity of stimulants, transduction systems and targets makes their interrelationships a very complicated network. In addition, much evidence indicates that some stimulants also generate inhibitory signals, others may bypass one or more steps of the transduction pathways, and others may have different effects according to the concentration (for recent reviews on this matter see Refs.<sup>286,287</sup>).

Since the knowledge of the transduction apparatus is relevant for the understanding of the final modification leading to the activation of the respiratory burst enzyme, a survey of the principal systems involved is presented here.

### A. Calcium entry and mobilization

One of the early molecular events elicited by extracellular ligands is the rise in  $Ca^{2+}$  concentration of the cytosol ( $[Ca^{2+}]_i$ ), due both to increase of influx and to rapid mobilization from intracellular membraneous stores. Several lines of evidence have suggested that intracellular calcium changes may be important for the activation of NADPH oxidase: (i) the stimulation of the respiratory burst by  $Ca^{2+}$  ionophores A23187 and X537A;<sup>288</sup> (ii) the inhibitory effect of intracellular cal-



cium antagonists;<sup>289-291</sup> (iii) the stimulatory effect of Ca<sup>2+</sup> and calmodulin on NADPH oxidase activity.<sup>277,292</sup> However, a direct causal relation of [Ca<sup>2+</sup>]<sub>i</sub> increase with the activation of the oxidase is far from being proved. In fact, (i) the inhibitors could have other non-specific effects. For example, the calcium antagonist Verapamil is also an inhibitor of protein kinase C;<sup>285,293</sup> (ii) increase of intracellular free calcium by other calcium mobilizing agents (PMN 7C3) is not a sufficient event for inducing the respiratory burst;<sup>294,295</sup> (iii) the respiratory burst may be activated independently of Ca<sup>2+</sup> changes by PMA,<sup>296</sup> by phospholipase C,<sup>297</sup> and by FMLP, providing that the cells are pretreated with other stimulants.<sup>298</sup> Thus, it seems that the increase of [Ca<sup>2+</sup>]<sub>i</sub> is important for some biological responses (e.g. movement, translocation of intracellular components, activation of phospholipase A<sub>2</sub> and of arachidonic acid cascade) but is not always necessary for the final step of activation of NADPH oxidase.

### B. Phospholipid hydrolysis

Shortly after ligand-receptor interaction, a sequence of modifications involving the lipid composition takes place on the cell membrane: (i) the hydrolysis of phosphatidylinositol 4,5-bisphosphate by phospholipase C with formation of inositol 1,4,5 triphosphate (IP<sub>3</sub>), of diacylglycerol, and an increase of phosphoinositide turnover;<sup>299</sup> (ii) the hydrolysis of various phospholipids by phospholipase A<sub>2</sub>, with formation of lysophospholipids, arachidonic acid and then prostaglandins and leukotrienes. Lysophospholipids may be reacylated by fatty acids derived from triglycerides;<sup>300-302</sup> (iii) the hydrolysis of phosphatidylcholine by phospholipase C, with formation of phosphorylcholine and diacylglycerol.<sup>303-305</sup>

All these reactions are the source of important molecules such as IP<sub>3</sub> and diacylglycerol, which act as second messengers for Ca<sup>2+</sup> release and protein kinase C activation. Furthermore, phospholipid breakdown, reacylation processes and lipid methylation may modify the physical properties of the membrane bilayer, with obvious consequences on the function of enzymes and transport systems.<sup>306-309</sup> A stimulus-dependent modification of lipid properties in neutrophil membranes was demonstrated with fluorimetric methods by Romeo et al.<sup>310</sup>

The relationship between these events and the activation of NADPH oxidase is suggested by the finding that the respiratory burst may be activated by exogenous phospholipase C,<sup>297,311</sup> diacylglycerol,<sup>312</sup> fatty acids,<sup>59,313,314</sup> detergents,<sup>315,316</sup> and is prevented by the phospholipase inhibitor bromophenacylbromide.<sup>317</sup> It

is conceivable that the phospholipase product diacylglycerol is the physiological signal for activation of protein kinase C,<sup>287,318</sup> which, in turn, appears to be implicated in the phosphorylation of NADPH oxidase components (see below). Even fatty acids may activate protein kinase C,<sup>319</sup> while it can not be excluded that they are directly implicated in the control of NADPH oxidase function. It has been recently reported that macrophages stimulated by PMA, zymosan and bacterial lipopolysaccharide specifically incorporate myristic acid into membrane proteins.<sup>320</sup> Therefore, protein acylation could be an important intermediary in the activation process.

That the state of the lipid bilayer of the plasma-membrane exerts some control over the O<sub>2</sub><sup>-</sup>-forming enzyme is shown by the effect of *cis*-polyene fatty acids.<sup>59,313,321</sup> Insertion of various fatty acids into the membrane of neutrophils results in activation of superoxide release, which is well correlated with their melting point. The lower the melting point is (which is a function of the capability of increasing the membrane fluidity), the higher the O<sub>2</sub><sup>-</sup> release.

On the other hand, in particular experimental conditions (calcium-depleted neutrophils pretreated with low doses of PMA or concanavalin A and then stimulated with FMLP) the respiratory burst can be dissociated from the phosphatidylinositol hydrolysis.<sup>298,322,323</sup> Therefore the possibility of a phosphoinositide-independent transduction pathway must also be postulated.

Most receptors are coupled with stimulation of phospholipase C activity through an intermediate represented by GTP-binding proteins, whose function is specifically blocked by pertussis toxin.<sup>324</sup> Pertussis toxin is able to inhibit the respiratory burst by opsonized particles, chemotactic peptides, leukotrienes, or platelet-activating factor, while it does not inhibit the stimulation by concanavalin A, ionophore A23187, PMA and diacylglycerol, suggesting that these latter agents bypass the GTP-binding protein-step.<sup>286,325</sup>

### C. Cyclic AMP variations

A short transient rise in cAMP concentrations is observed within 1 min of the stimulation of neutrophils by some agents.<sup>326</sup> This increase in cAMP may activate type A protein kinases and glycogen metabolism, necessary for energy supply, but probably is uninvolved in NADPH oxidase activation system. In fact dibutyl cAMP and PGE, which induce increase of cAMP, do not activate, but instead inhibit, the respiratory burst.<sup>327</sup> Probably the rise in cAMP triggers inhibitory feedback pathways through inhibition of phospholipase C by protein kinase A.<sup>318,327-329</sup>

#### D. Protein phosphorylation

Phosphorylation and dephosphorylation of specific proteins is thought to be important in the regulation of a variety of cells responsive to external stimuli.<sup>330,331</sup> There is increasing evidence that this mechanism operates also in neutrophils for the activation of NADPH oxidase. In fact, (i) activation of the respiratory burst is an energy-requiring process;<sup>332</sup> (ii) the receptor of the potent stimulants PMA and diacylglycerol is the  $\text{Ca}^{2+}$ -phospholipid dependent protein kinase C itself;<sup>333,334</sup> (iii) the respiratory burst is associated with translocation of protein kinase C from cytosol to plasma membrane<sup>335-337</sup> and phosphorylation of membrane proteins.<sup>338,339</sup> (iv) inhibitors of protein kinase C (isoquinoline sulfonamide, retinal, sfinganine) block the respiratory burst;<sup>55,340,341</sup> (v) purified brain protein kinase stimulates NADPH oxidase activity of membranes in a cell-free system<sup>342</sup> (vi) some CGD patients exhibit a defect of phosphorylation in their neutrophils.<sup>343</sup>

The intracellular targets of stimulus-associated phosphorylation process have been investigated by autoradiography of electrophoretic separation of proteins from [ $^{32}\text{P}$ ]-labeled neutrophils,<sup>312,338,339,344-350</sup> and macrophages.<sup>351,352</sup> Starting from the paper of Schneider et al.,<sup>344</sup> who first showed increased  $^{32}\text{P}$ -labeling of four polypeptides, the list of phagocyte phosphoproteins became much longer and now it includes at least 20 species, which are distributed both in the cytosol and in the membrane. Unfortunately, most of them have not been identified yet. The candidate proteins whose phosphorylation may be involved in the activation of NADPH oxidase are the following:

(i) Polypeptides with Mr of 64,000, 21,000, and 50,000 have been implicated because their phosphorylation is strictly associated with respiratory burst activation, as far as dose-dependence, time course and inhibitor effects are concerned.<sup>339,347,348,350</sup> The 21,000 daltons protein is probably the myosin light chain,<sup>353</sup> suggesting a link between cytoskeleton functions (movement of membrane proteins, translocation processes and so on) and oxidase activation. The protein with Mr of 66,000, which has been suggested to be the NADPH-binding domain of the oxidase, is not phosphorylated upon stimulation.<sup>232</sup>

(ii) Polypeptides with Mr of 44-48,000 are phosphorylated upon stimulation in normal neutrophils, but not in neutrophils from autosomal recessive CGD patients.<sup>343,354,355</sup> The identity of these proteins is unknown. Since in this variant of the disease the neutrophils have normal amounts of cytochrome *b* and of flavoproteins, probably the defect is not in the oxidase itself but in some step of its activation system.

The relationship between the phosphorylation of these proteins and the respiratory burst activation was recently investigated in normal neutrophils.<sup>356</sup> With all stimulants and inhibitors tested the kinetics of the two phenomena corresponded. Therefore, the phosphorylation of these proteins which are defective in CGD appears to be a common trigger for activation of the burst.

(iii) The protein with Mr of 32,000 that was identified by us as the component which copurifies with the oxidase and belongs to cytochrome *b*<sub>558</sub> (see Section III) is markedly phosphorylated upon cell stimulation.<sup>144,349,357</sup> Figure 4 shows the densitometric analysis of autoradiographs of membranes and partially purified NADPH oxidase from both resting and PMA-activated pig neutrophils. The 32,000 daltons band is by far the most frequently represented phosphoprotein of the active oxidase complex. Increased  $^{32}\text{P}$  incorporation into this protein was also observed in the enzyme from cells stimulated with opsonized zymosan (P. Bellavite, in press). In a cell-free system, phosphorylation of the 32,000 daltons polypeptide was stimulated by  $\text{Ca}^{2+}$ , phosphatidylserine and PMA, indicating that it is due to protein kinase C.<sup>349</sup>

Preliminary demonstrations that cytochrome *b*<sub>558</sub> becomes phosphorylated in activated human neutrophils were reported by two laboratories,<sup>358,359</sup> but until now they haven't been illustrated in full papers.

Even in the case of the protein kinase C hypothesis, there is experimental evidence that this transduction system may be bypassed. In fact, the  $\text{O}_2^-$  release by neutrophils stimulated by FMLP and opsonized zymosan is relatively insensitive to protein kinase C inhibitors.<sup>301,360,361</sup> Moreover, concanavalin A and leukotriene B<sub>4</sub> activate the respiratory burst without inducing translocation of protein kinase C from cytosol to the plasma membrane.<sup>362</sup> These data suggest that PKC-independent pathways exist for the agonist-stimulated respiratory burst.<sup>360</sup>

#### E. Proteases

The respiratory burst is blocked by serine-protease inhibitors such as L-1-tosylamide-2-phenylethyl chloromethyl ketone (TPCK), *N*-benzoyl-L-tyrosine ethyl ester (BTEE) and diisopropyl fluorophosphate (DFP).<sup>363,367</sup> This fact has been taken as evidence for participation of proteolytic mechanisms in the triggering events leading to the activation of NADPH oxidase.

Washida et al.<sup>366</sup> and Dri et al.<sup>367</sup> have also described a stimulatory effect of TPCK on oxidative metabolism of guinea pig neutrophils and peritoneal macrophages, but this effect was exerted at concentrations higher than

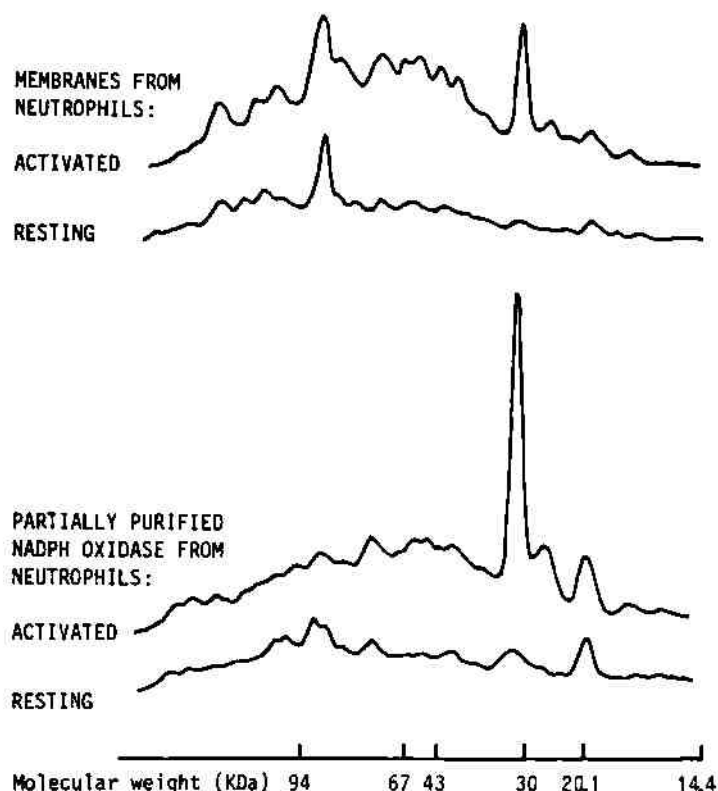


Fig. 4. Densitometric traces of autoradiographs of SDS-electrophoretic gels of neutrophil proteins. Pig neutrophils were labeled with  $^{32}\text{P}$ i and divided into two batches, only one of which was activated by treatment with  $1\text{ }\mu\text{g/ml}$  PMA. Membranes were then isolated by sucrose density gradient centrifugation and NADPH oxidase was partially purified through Ultrogel AcA34 as described.<sup>144</sup> Membrane and oxidase preparations from both resting and activated cells were subjected to SDS electrophoresis on a linear polyacrylamide (4–20%) gradient slab gel. The gel was desiccated, exposed to autoradiography, and the film was analysed by densitometric scanning.

those required for the inhibition of the PMA-activated respiratory burst.

The protease activities, which are possibly involved in the transduction system are similar to the chymotrypsin-type rather than to the trypsin-type, and appear to be located at the plasma membrane.<sup>364</sup> In order to identify the candidate protease, Kudoh et al.<sup>368</sup> used a [ $^3\text{H}$ ]DFP-affinity labeling technique. They could isolate a membrane protein with an Mr of 40,000, which became labeled on stimulation of neutrophils with antigen-antibody complexes. Although its biochemical entity was not identified, the authors suggested that this protein is the most probable candidate for a putative stimulus-activated serine protease.

It is difficult to establish a link between proteolytic events and activation of the oxidase. We have not found gross differences in the protein pattern of enzyme preparations from PMA-activated as compared with resting neutrophils, but the proteolytic modification could have been electrophoretically undetectable.<sup>144</sup> Markert et al.<sup>142</sup> showed different electrophoretic patterns of resting and PMA-activated neutrophils oxidase, and this would be consistent with a proteolytic cleavage of

the inactive enzyme to yield the active form. However, the authors found FAD only in the preparations from activated cells. So the observed differences of the protein pattern could be ascribed to different composition of the two preparations, possibly due to the procedure of enzyme isolation, which could have allowed extraction and purification of the flavoprotein from the activated material only.

Assuming that a protease is involved in the activation mechanism, its target could belong to some step of the transduction system. It has been recently shown that a serine proteinase of human neutrophils becomes associated with the plasma membrane in the presence of micromolar concentrations of  $\text{Ca}^{2+}$ . Binding to the membrane results in activation of the proteinase, which then catalyzes limited proteolysis of protein kinase C to produce a form that is fully active in the absence of  $\text{Ca}^{2+}$  and phospholipid.<sup>369</sup> It is tempting to speculate that the effect of protease inhibitors on the respiratory burst could be due to the interference with such a proteolytic activation of the kinase. In any case, every hypothesis based only on the effect of inhibitors must wait more direct validations.<sup>370</sup> In fact, it cannot be



excluded that the effect of chloromethylketones is due to the direct inhibition of protein kinase C<sup>371</sup> or of neutrophil sulfhydryl groups.<sup>372</sup>

#### F. Membrane depolarization

The excitation of oxidative metabolism of phagocytes is associated with early changes in Na<sup>+</sup> and K<sup>+</sup> fluxes and a depolarization in membrane potential, but the precise causal relationship between the two phenomena is at present unclear.<sup>373-376</sup> The most impressive evidence of a role of cell depolarization in the triggering of O<sub>2</sub><sup>-</sup> formation is the absence or marked impairment of membrane potential responsiveness in CGD neutrophils stimulated with either chemotactic peptides or PMA.<sup>377,378</sup> This defect has been described in both autosomal recessive and X-linked forms of the disease, while normal depolarization was found in a rare CGD variant where the NADPH oxidase appeared to be normally activated but malfunctioning due to abnormally high K<sub>m</sub> for substrate.<sup>129,247</sup> Lack of membrane depolarization in CGD is not secondary to the absence of O<sub>2</sub><sup>-</sup> production, because the respiratory burst can be inhibited by absence of oxygen, but the depolarization cannot.<sup>70</sup> Chemotaxis, phagocytosis and degranulation are not grossly impaired in CGD,<sup>13</sup> indicating that Na<sup>+</sup> and K<sup>+</sup> fluxes are involved in transduction events proximal to the final modification of the oxidase.

On the other hand, a direct coupling between membrane potential changes and respiratory burst activation does not appear to occur, because when the cells are depolarized by Na<sup>+</sup>-K<sup>+</sup>ATPase inhibitors, by monovalent cation ionophores, or by replacing external Na<sup>+</sup> with K<sup>+</sup>, they do not exhibit a respiratory burst in the absence of stimulants.<sup>379</sup> Moreover, membrane potential changes may be stimulated by concentration of PMA lower than that necessary for activation of oxidative metabolism.<sup>380</sup> Thus, depolarization is not in itself sufficient to trigger the oxidase and some stimulus-specific mechanism is required. Probably ionic events play a "permissive" or regulatory role on the activation mechanism. Consistent with the latter conclusion are the observations that the neutrophils from guinea pig exudates, which have been previously exposed to chemotactic factors, exhibit a spontaneous respiratory burst when they are suspended in Na<sup>+</sup>-free, high K<sup>+</sup> buffered solution,<sup>381</sup> and that human neutrophils incubated in Na<sup>+</sup>-free medium show enhanced respiratory response to FMLP.<sup>379,383</sup>

#### G. The "primed" state of the oxidase

A very interesting phenomenon, which is relevant for the mechanism of NADPH oxidase activation, is

the potentiation of the respiratory burst response to a stimulant by prior exposure of the cells to a sub-stimulatory dose of an heterologous stimulant.<sup>244,384-388</sup> Such a "priming" effect, which has been described with several stimulants, could have a physiological effectiveness in inflammation and defenses against infections. In fact, cells which have been in contact with very low concentrations of inflammation mediators such as complement fragments and leukotrienes, or with bacterial products such as endotoxins and chemotactic peptides, may be predisposed to a higher release of toxic oxygen metabolites. Recently Berton et al.<sup>389</sup> showed that pretreatment with  $\gamma$ -interferon enhances the oxidative metabolism of human neutrophils.

The molecular bases of the priming phenomenon remain to be established. According to McPhail et al.,<sup>390</sup> the oxidase appears to be regulated at three different levels: inactive, primed, and active. The signal for priming is separated from that for final activation of the oxidase and according to Grzeskowiak et al.<sup>298</sup> and Rossi et al.,<sup>391</sup> the priming effect may occur independently of phosphoinositide hydrolysis and Ca<sup>2+</sup> responses.

#### H. Final modification(s) of NADPH oxidase leading to its activation

The outcome of the biochemical events involved in the transduction systems is a modification of the oxidase complex from inactive (or primed) to catalytically active state. As inferred from the knowledge of the multiple pathways of activation, it is conceivable that the triggering of the enzyme is due to one or more of the following events: (i) phosphorylation of cytochrome *b*, (ii) phosphorylation of a regulatory protein, (iii) modification of lipid composition of the enzyme environment, (iv) conformational changes dependent upon membrane potential. As a consequence of these events the reciprocal interaction of the components of the enzyme system and the interaction with NADPH would be facilitated, and the transfer of electrons from the dehydrogenase site to the O<sub>2</sub> binding site would be allowed.

There is increasing evidence that the electron transport chain becomes assembled at the moment of the stimulation. In fact, treatment of neutrophils with *N*-hydroxysuccinimide, a bifunctional cross-linking agent, abolishes their ability to undergo activation, without inhibiting the O<sub>2</sub><sup>-</sup> formation.<sup>392</sup> Cleavage of the cross-linker by reduction of the disulfide bonds of the cell surface restores the activability of neutrophils.<sup>393</sup> Moreover, Manara and Schneider<sup>394</sup> have provided evidence that the activation of the respiratory burst and the granule-membrane fusion are concomitant events.

It has been reported that part of cytochrome *b*<sub>558</sub> translocates from granules to the plasma membrane during the activation.<sup>165,395,396</sup> The cytochrome appears to be incorporated in the membrane of phagocytic vacuoles,<sup>169</sup> where the NADPH oxidase is selectively activated.<sup>397,398</sup> However, according to other reports,<sup>164,181,399</sup> most of cytochrome *b* is associated with the plasma membrane before the stimulation. In any case, even assuming that cytochrome *b* translocates to the membrane in stimulated cells, this phenomenon does not appear to be essential for the oxidase activation. In fact, (i) cytoplasm, which are devoid of granules, are normally activatable;<sup>211</sup> (ii) granule release may be pharmacologically dissociated from O<sub>2</sub><sup>-</sup> formation<sup>400-402</sup> (iii) the extent of degranulation and of respiratory burst are not proportional in different conditions of cell stimulation.<sup>395</sup> Therefore the pool of cytochrome *b*, which is already present on the membrane of resting cells, is sufficient for the triggering of the respiratory burst. It is conceivable that the transfer of additional cytochrome *b* from an intracellular pool to the membrane is a concomitant event that may increase the efficiency or the duration of the oxidase activity.

Last but not least, we must mention an interesting phenomenon, which could get new insights to the final events involved in the activation mechanism of the oxidase. In 1984, two independent groups<sup>403,404</sup> showed that NADPH dependent O<sub>2</sub><sup>-</sup> formation is activatable in cell-free system by fatty acids. This was the first convincing demonstration that the intactness of the cell is not obligatory in order to stimulate the respiratory burst enzyme. Of course the availability of an *in vitro* model of enzyme activation has been a milestone in these studies and now many laboratories are interested in this system.<sup>404-410</sup> The most relevant findings published so far are as follows: (i) in the presence of NADPH, O<sub>2</sub><sup>-</sup> is generated by homogenate of neutrophils and macrophages upon the addition of arachidonic acid, other long-chain unsaturated fatty acids, SDS and lithium dodecyl sulphate; (ii) the activity has kinetic properties and inhibitor sensitivity identical to the activity triggered in intact cells; (iii) besides NADPH, the simultaneous presence of three components for the triggering of O<sub>2</sub><sup>-</sup> formation is required: the particulate fraction (membranes plus granules), the cytosolic fraction and the fatty acids (or detergents); (iv) probably the factor in particulate fraction is cytochrome *b*<sub>558</sub> as judged by purification studies<sup>411</sup> and by the colocalization in subcellular fractions;<sup>408,412</sup> (v) the cytosolic factor has not been identified yet, although it has been suggested that it could be the NADPH-dehydrogenase component of the system.<sup>411</sup> The molecular weight of the cytosolic factor ranges from 10,000 to 270,000 daltons according to different

laboratories;<sup>412,413</sup> (vi) the activation *in vitro* seems to be independent of protein kinase C, because it does not require Ca<sup>2+</sup> and phospholipids, is insensitive to protein kinase inhibitors and the molecular weight of the cytosolic cofactor is different from that of the protein kinase.<sup>403,405,410,412</sup> However, phosphorylation events cannot be excluded because the activation exhibits a dependency on ATP.<sup>412</sup> Possibly a GTP-binding protein may also be involved, because GTP potentiates the stimulatory effect of arachidonic acid;<sup>409</sup> (vii) the activation by arachidonic acid does not occur in homogenate from CGD neutrophils, thus confirming that the *in vitro* system discloses the true respiratory burst oxidase.<sup>403</sup> In the X-linked form, the defect resides in the membrane fraction, while the cytosol is able to activate normal membranes in the presence of arachidonic acid.<sup>406</sup> In a female CGD patient (presumably with the autosomal recessive form) a defect of cytosolic cofactor has been reported.<sup>413</sup>

It remains to be clarified whether the cytosolic cofactor necessary for the reconstitution of the active oxidase in a cell-free system is an actual component of the electron transport chain or is a protein with regulatory or enzymatic (protease, kinase, phospholipase) function. In the first case, the hypothesis that the final event of the activation sequence is the association of the flavoprotein with cytochrome *b*<sub>558</sub> will be reinforced, if not definitely proved. However, it should be pointed out that the flavin:cytochrome *b* ratio does not appear to change in the enzyme from activated cells as compared with that from resting cells,<sup>138,211</sup> suggesting that the association of the two putative components consists in a functional coupling instead of a structural assembly.

Even the role of unsaturated fatty acids or SDS in this system has to be investigated, considering that it is not simply linked to their detergent properties (many other detergents are not effective in the *in vitro* system)<sup>405</sup> and that the O<sub>2</sub><sup>-</sup> production requires the continuous presence of these compounds in the assay mixture. Probably fatty acids and SDS cause a quite selective perturbation of the proteolipid complex of the oxidase, which facilitates the functional interaction of cytosolic and membrane-bound factors.

### 1. Summary of the Activation Mechanism

How could one draw some synthetic conclusion from such a large body of information on the phagocytes' transduction systems? In general, it is out of the question that there are multiple pathways of activation and that a particular system could be utilized by one stimulant and be bypassed by another one. It is also probable that with most stimulants and especially with

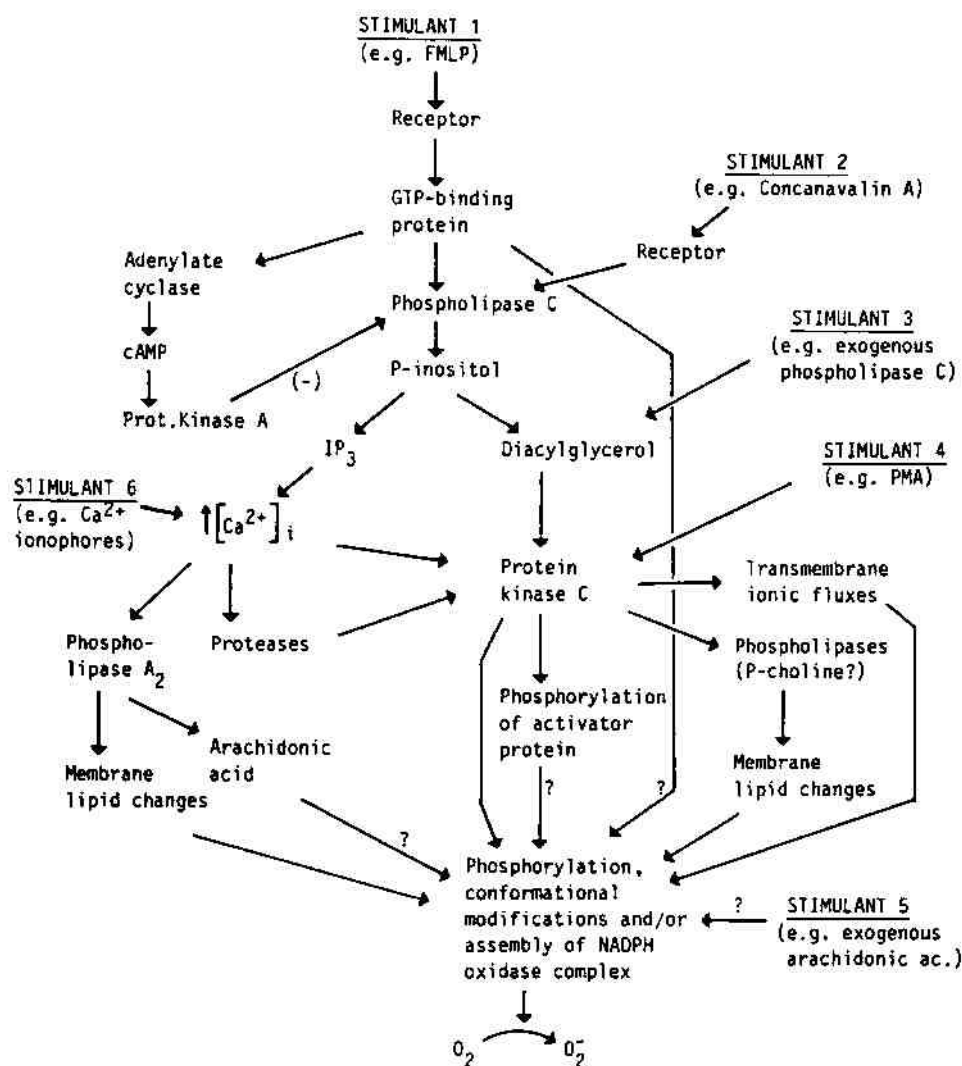


Fig. 5. Possible pathways for activation of NADPH oxidase. For explanation see text. IP<sub>3</sub> = inositol 1,4,5-triphosphate; P-inositol = phosphatidylinositol; P-choline = phosphatidylcholine.

those acting through membrane receptors, several transduction pathways are sequentially activated and function in a synergistic way. The various events must, however, converge towards a final change of the NADPH oxidase, because the same enzyme is eventually activated by different stimulants.<sup>246</sup>

A tentative model of the possible pathways for NADPH oxidase activation and of their interrelationships is shown in Figure 5. The scheme has been constructed on the basis of the above discussed data and therefore it must be regarded as a working hypothesis and not as a conclusive settlement. So, many effects

indicated by arrows in Figure 5 are only postulated, albeit not contradicted by experimental findings. This area will undoubtedly be the object of future research efforts.

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*Note added in proof.* Recently, the Segal's (*Nature* 326:88–91; 1987 and *Nature* 327:720–721) and Orkin's (*Nature* 327:717–720; 1987) groups have shown that human neutrophil cytochrome *b* has two components with Mr of 76–92,000 and 22,000 and that the X-linked CGD gene codes for the high-molecular weight component of the cytochrome.



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