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INTRODUCTION

The NADPH oxidase is the enzyme, or enzymatic system, responsible for the respiratory burst of phagocytes (granulocytes, monocytes, macrophages). During phagocytosis, or when stimulated by soluble factors, phagocytes undergo to a dramatic alteration of their oxidative metabolism, the main feature of which is the increase of oxygen consumption, of glucose catabolism through the hexose monophoshate shunt, of the glutathione cycle activity, of the production of intermediate of oxygen reduction (1-5). These intermediates (O_2^-, H_2O_2, $OH^{\cdot}$, $^1O_2$) are utilized as powerful weapons against invading bacteria, viruses and tumor cells (6-10). Figure 1 shows that a strict interrelationship between the various reactions involved in the respiratory burst exist and that the NADPH oxidase is the «motor» of the overall phenomenon. The NADPH oxidase is completely silent in resting cells but it is activated after the interaction of specific membrane receptors with suitable soluble or particulate ligands. The enzyme is membrane-bound and during phagocytosis is selectively activated in the portion of the plasmamembrane that interacts with the phagocytosable particle (11).

The central role of NADPH oxidase in the phagocyte metabolism has been firstly pointed out by the group of prof. F. Rossi in the 60'thies (12) and at present is universally accepted (13-15). A simple experiment clearly illustrates the quantitative importance of the enzyme in the O_2^- production (figure 2). The addition of phorbol myristate acetate (PMA) to granulocytes causes an abrupt increase of O_2^- production, which is revealed as superoxide dismutase (SOD)-inhibitable cytochrome c reduction. The detergent deoxycholate stops the reaction, owing to the cell lysis, but the addition of NADPH induces an istantaneous resumption of the O_2^- production at a rate similar or even higher than that of the intact cells. A similar effect can be obtained by adding $NADP^+$ plus glucose 6 phosphate, which, in the presence of the glucose 6-phosphate dehydrogenase of the cell lysate, form NADPH. These results demonstrate that the rate of O_2^- production by NADPH oxidase and the rate of NADPH generation by the hexose monophosphate pathway account for all the O_2^- production by the intact cells.

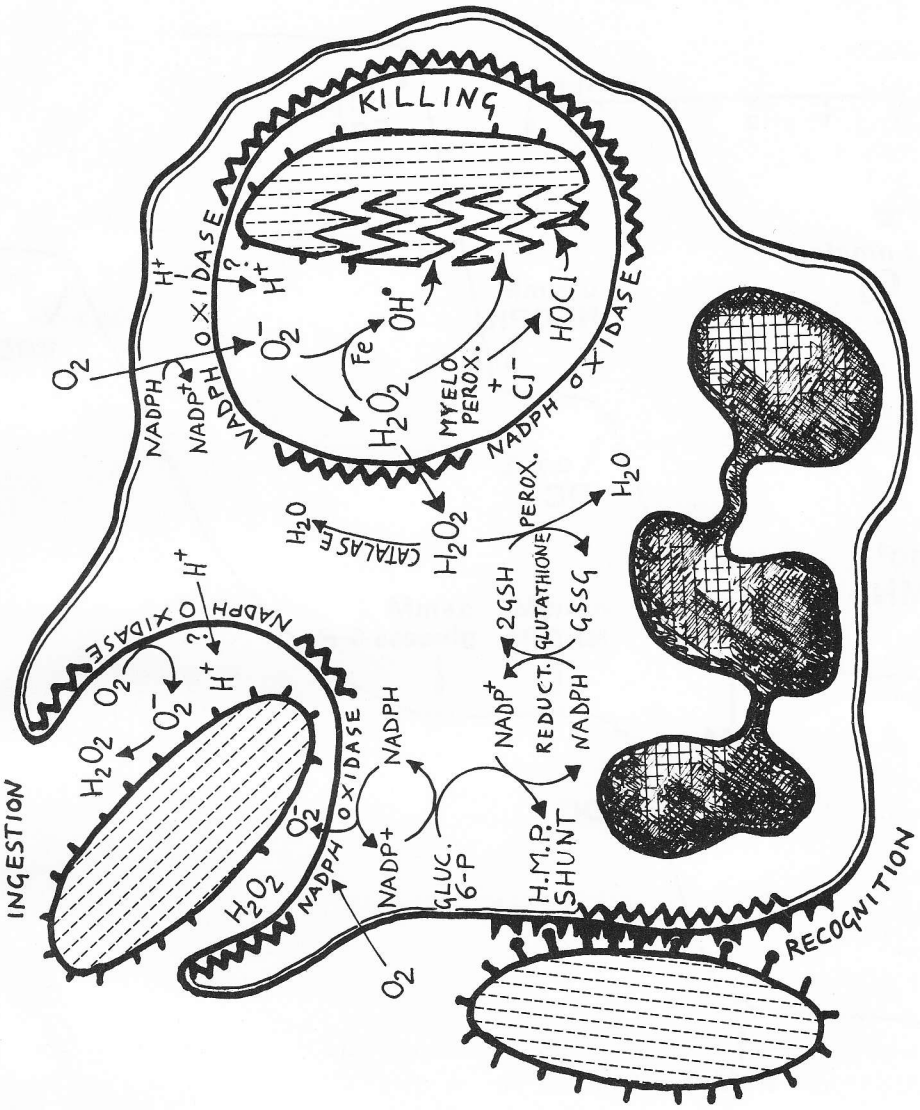

Fig. 1 – Oxidative metabolism of a granulocyte during phagocytosis.

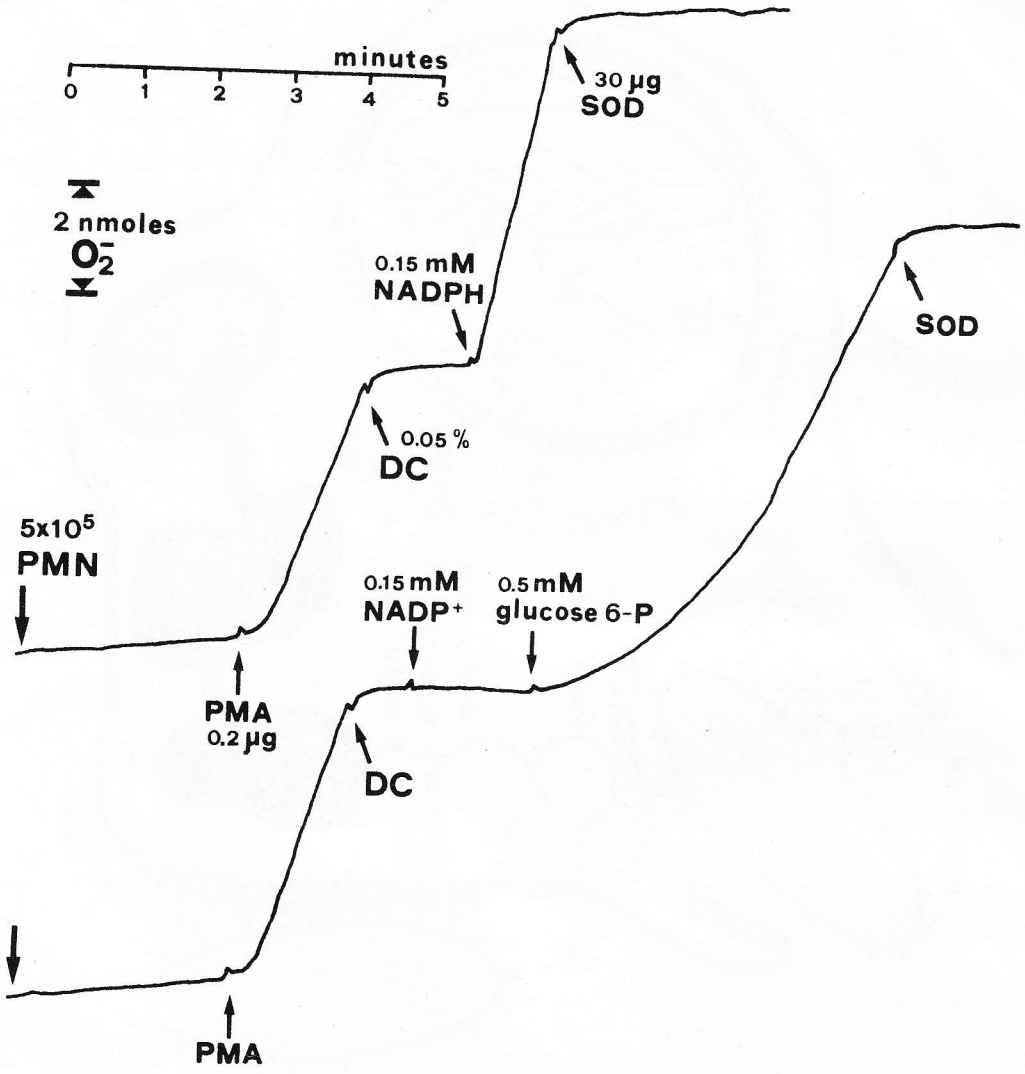

Fig. 2 – Kinetics of O_2^- production by granulocytes and by NADPH oxidase. Guinea pig granulocytes were incubated at 37°C in a spectrophotometer cuvette containing 1 ml of Krebs Ringer phosphate buffer, pH 7.4, 5 mM glucose and 80 µM cytochrome c. The increase of O. D. at 550 nm was recorded. PMA: phorbol myristate acetate; DC: sodium deoxycholate; SOD: superoxide dismutase.

Solubilization and isolation of NADPH oxidase

Most of the data here presented have been obtained in our laboratory by studying solubilized and partially purified preparations of NADPH oxidase. The protocol used in our laboratory for the isolation of the enzyme from guinea pig granulocytes is schematically reported in figure 3. The best conditions for the solubilization are obtained by treating the cell free particles (membranes + granules, fraction 1) with deoxycholate in the presence of EGTA and glycerol. The solubilized extract (fraction 2) is then rapidly passed through a column filled with Ultrogel Ac A22 resin and eluted with buffer without detergent. This procedure allows both the stabilization of the activity and a 2-3 fold purification of the enzyme. After gel filtration (fraction 3), the activity is associated with a high molecular weight complex (> 1,200,000 daltons) which can be further purified by isopychnic centrifugation on a glycerol gradient (fraction 4) and by treatment of the fraction 4 with salts. The final preparation (fraction 5) is composed by complexes containing a NADPH oxidase activity ranging from 50 to 200 nmoles O_2^- produced/min/mg protein. Further attempts to dissociate the complexes with strong detergents, urea, high ionic strenghts, were accompanied by complete loss of enzymatic activity. Even if the final active preparation is not completely pure, it can be advantageously used to investigate the nature and the properties of the NADPH oxidase.

Nature of the enzyme

In spite of a great number of investigations performed during the last twenty years, the structure and the components of the enzymatic system responsible for the activation of the respiration and for the production of free radicals are not well defined. The main reason of this is the lack of a complete purification of the enzyme, because of the difficulties caused by its extreme lability. Nevertheless, many informations on the nature of the enzyme have been recently accumulated as consequence of studies on isolated membranes (16, 17), on partially purified preparations of oxidase (18-21), on patients affected by chronic granulomatous disease, an ereditary condition where the NADPH oxidase activity is absent or abnormal (22-23).

Consistent evidence indicates that the NADPH oxidase activity is due to a multicomponent system in whom a flavoprotein, a b-type cytochrome and possibly other cofactors (quinones?) are involved.

The involvement of a cytochrome b in the activated respiration of phagocytes was suggested by the discovery that it is lacking in some cases of chronic granulomatous disease (22). Since that discovery the properties of this cytochrome have been intensively investigated (see table I), and these properties strongly indicate that cytoch-

Subcellular particles (membranes + granules) from PMA-activated granulocytes, suspended (5 mg protein/ml) in 10 mM KP buffer pH 8.0 + 1 mM EGTA + 2 mM NaN_3 + 20% glycerol

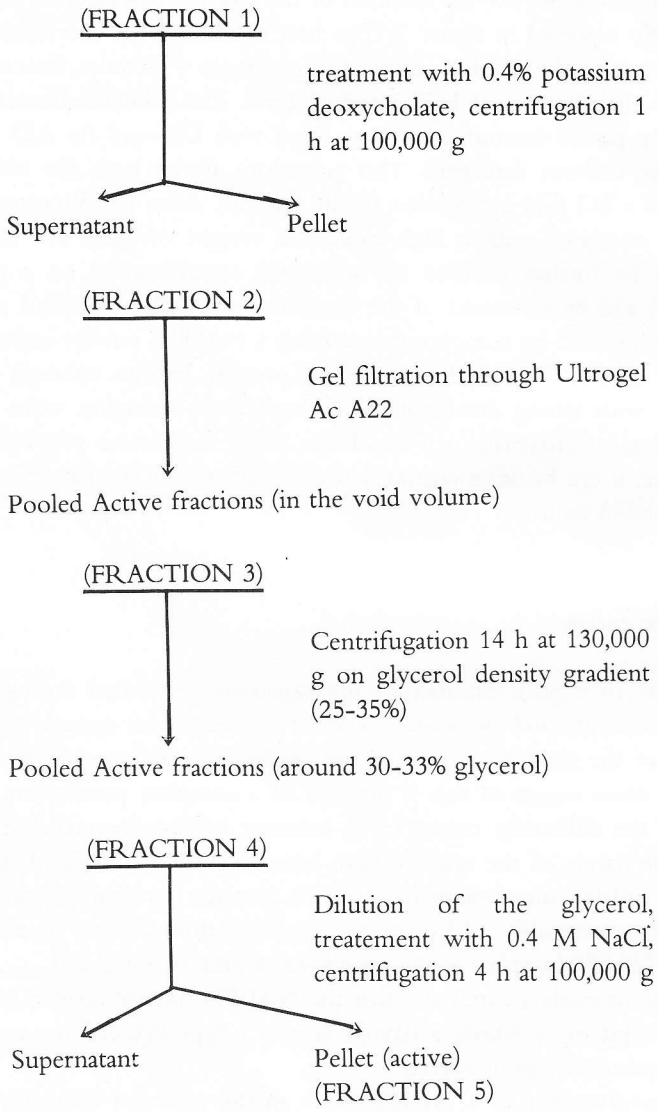

Fig. 3 – Procedure employed for the solubilization and partial purification of NADPH oxidase.

Table I

PROPERTIES OF THE CYTOCHROME B INVOLVED IN THE NADPH OXIDASE ACTIVITY OF PHAGOCYTES

1. Absorption peaks: oxidized: 412 nm; reduced α = 558 nm, β = 531 nm, γ = 427 nm
2. Low midpoint potential ($Em_{7.0}$): -245 mV.
   The $Em_{7.0}$ is modified by parachlomercuribenzoate and by Triton X 100
3. It is (slowly) reduced by NADPH in anaerobiosis and is reoxidized by O_2 with a t 1/2 of 4.7 ms.
4. It is reduced in granulocytes activated under anaerobic conditions
5. It binds CO, althought with lower affinity than O_2.
6. It is present in neutrophils, eosinophils, monocytes, macrophages, not in lymphocytes.
7. During cell differentiation, it appears in parallel with the development of the metabolic activation capacity.

rome b is an essential component of the oxidase. The midpoint potential ($Em_{7.0}$) is -245 mV (24), a value extremely low as compared with that of other b type cytochromes and a low Em is a prerequisite for a cytochrome which would reduce O_2 to $O_2^{-}\cdot$. We found cytochrome b in the solubilized and partially purified preparations of NADPH oxidase and its $Em_{7.0}$ resulted of exactly -245 mV. Interestingly, parachloromercuribenzoate (PCMB), a powerful inhibitor of the oxidase (see below), modifies the $Em_{7.0}$ to -175 and this could be the reason of the inhibition of the catalytic activity (20-21). Cytochrome b of the membrane and of the extracted enzyme is reduced by NADPH in anaerobiosis (though at slow rate) and is rapidly reoxidized by oxygen (24). Another important property is that the cytochrome b is reduced in the cells activated in anaerobiosis (25). During our purification procedure, a progressive increase of the specific content of cytochrome b was obtained (from 150 pmoles/mg protein in fraction 1 to about 1000-1300 pmoles/mg protein in fraction 5). This increase was paralled by an increase of NADPH oxidase activity, taking into account the spontaneous loss of enzymatic activity. Cytochrome b and oxidase activity are coeluted from the gel filtration and co-distribute in a single peak in the glycerol density grandient. Never we found enzyme activity in the absence of cytochrome b.

A role for a flavoprotein was proposed on the basis of stimulatory effect exerted by exogenous FAD added to the enzyme solubilized with triton X-100 (26) and of the inhibition of the activity by 5 carba - 5 - deaza FAD (18) and by quinacrine (20). The involvement of FAD should be theoretically obligatory to link a two electron donor (NADPH) with the formation of $O_2^{-}\cdot$ which occurs via one-electron pathway. Our enzyme preparations from guinea pig neutrophils contain flavoprotein (with FAD as prosthetic group), but the amount of the flavins relative to proteins do not increase during purification. Preliminary data show that the ratio cytochrome b/FAD progressively increases as the purification goes on. For example we found in the deoxycholate extract (fraction 2) a ratio of 1 and in the most purified preparation (fraction 5) a ratio

near 10. It is clear that the determination of the content of the flavin in the oxidase needs a better purification. The presence of contaminants, the loss of flavin from the oxidase apoprotein, the presence of other flavoproteins related to other enzymes (for example cytochrome c or quinone-reductases) are possible events that at present do not allow any conclusion.

Kinetic properties

The kinetic properties and the sensitivity to various compounds of the NADPH oxidase activity are summarized in table II. It is worth of note that the enzyme reduces the O_2 via one electron patway, with a stoichiometry of $2O_2$ produced/1 NADPH consumed (26). The Km for NADPH is one order of magnitude lower than that for NADH (27, 28). The pH optimum is 7.0-7.5 and the stoichiometry $2O_2$/1 NADPH is mantained in a broad range of pH (20).

Table II

PROPERTIES OF THE NADPH OXIDASE

1. Non active in cell in resting state, activated by phagocytosis and by other factors that trigger functional responses
2. Catalyzes the reaction
   $NADPH + H^+ + 2O_2 \rightarrow NADP^+ + 2O_2^- + 2H^+$
3. Optimum pH: 7.0 - 7.5
4. Km for NADPH 0.015 - 0.080 mM
   Km for NADH 0.5 - 1.0 mM
5. *Insensitive* to cyanide, rotenone, antimycin A, azide, myxothiazol, electron acceptors such as 2,6-dichlorophenol indophenol, nitroblue of tetrazolium, potassium ferricyanide.
6. *Inhibited* by parachloromercuribenzoate, quinacrine, trifluoperazine, cibacron blu, strong detergents, high ionic strenghts

The respiration sustained by the oxidase is insensitive to mitochondrial inhibitors and also to electron acceptors which can react with most of dehydrogenases (20, 26, 28). This indicates that the only possible electron acceptor of the NADPH oxidase is molecular oxygen, in keeping with the physiologic role of the enzyme.

The activity is markedly inhibited by the sulfhydryl reagent parachloromercuribenzoate (PCMB) indicating that thiol groups are essential for the catalysis. The inhibition exterted by quinacrine is in agreement with the involvement of the flavoprotein and that exerted by cibacron blue confirms the specificity of the binding site for nucleotides (21). The involvement of calmodulin in the mechanism of activation of the respiratory burst has been proposed on the basis of the inhibitory effect of trifluopera-

zine in intact cells (29). Trifluoperazine inhibits also the activity of the solubilized enzyme. Whether this inhibition is due to the involvement of calmodulin in the activity of NADPH oxidase or to other effects of this rather non-specific inhibitor (30) remains to be clarified.

The enzyme activity is very labile (18-20). This is consistent with the fact that the activation of the respiration in intact cells is reversible and rapidly ceases when the stimulatory agent is removed (31). The loss of activity is more rapid in the presence of detergents and of high ionic strenghts (21). This could be an indication either that many components are assembled in the active enzyme, and that the active conformation depends on the phospholipidic milieu of the protein (32). This latter point is confirmed by our experiments which demonstrate that the NADPH oxidase isolated from activated granulocytes is present in a complex containing phospholipids (about 1.8 mg phospholipids/mg protein).

Molecular weight

It is difficult to establish the molecular weight of the NADPH oxidase, since the enzyme has not so far purified. Two papers (19, 33) reported an apparent m. w. around 150,000 daltons, but the specific activities of the «purified» fractions were extremely low and no significant differences between resting and activated enzyme were demonstrated. As a conseguence there is no evidence that these isolated proteins are the true NADPH oxidase. Moreover, the determination of molecular weight with gel filtration or with non denaturing gel electrophoresis in the presence of Triton, as done in the two quoted papers, is not completely reliable.

With our procedure of partial purification we obtain a big lipoprotein complex whose molecular weight ($>$ 1,200,000 daltons) is clearly due to aggregation of various components. However, by analysing the SDS electrophoretic pattern of the various preparations obtained during the purification of the enzyme from guinea pig neutrophils (figure 4) it can be observed a progressive increase of a protein band of 32,000 daltons (band δ), whereas the other main protein bands (α, β, γ and ϵ) does not increase or even decrease. This could be an indication that this protein is related to the NADPH oxidase activity. Whether it corresponds to cytochrome b or both to cytochrome b and flavoprotein remains to be established. The fact that only one protein band is progressively enriched in the course of the purification could also infer the existence of a flavocytochrome b. If this is the case, this flavocytochrome, a unique polipeptide with both FAD and heme as prosthetic groups, would be the essential component of the NADPH oxidase.

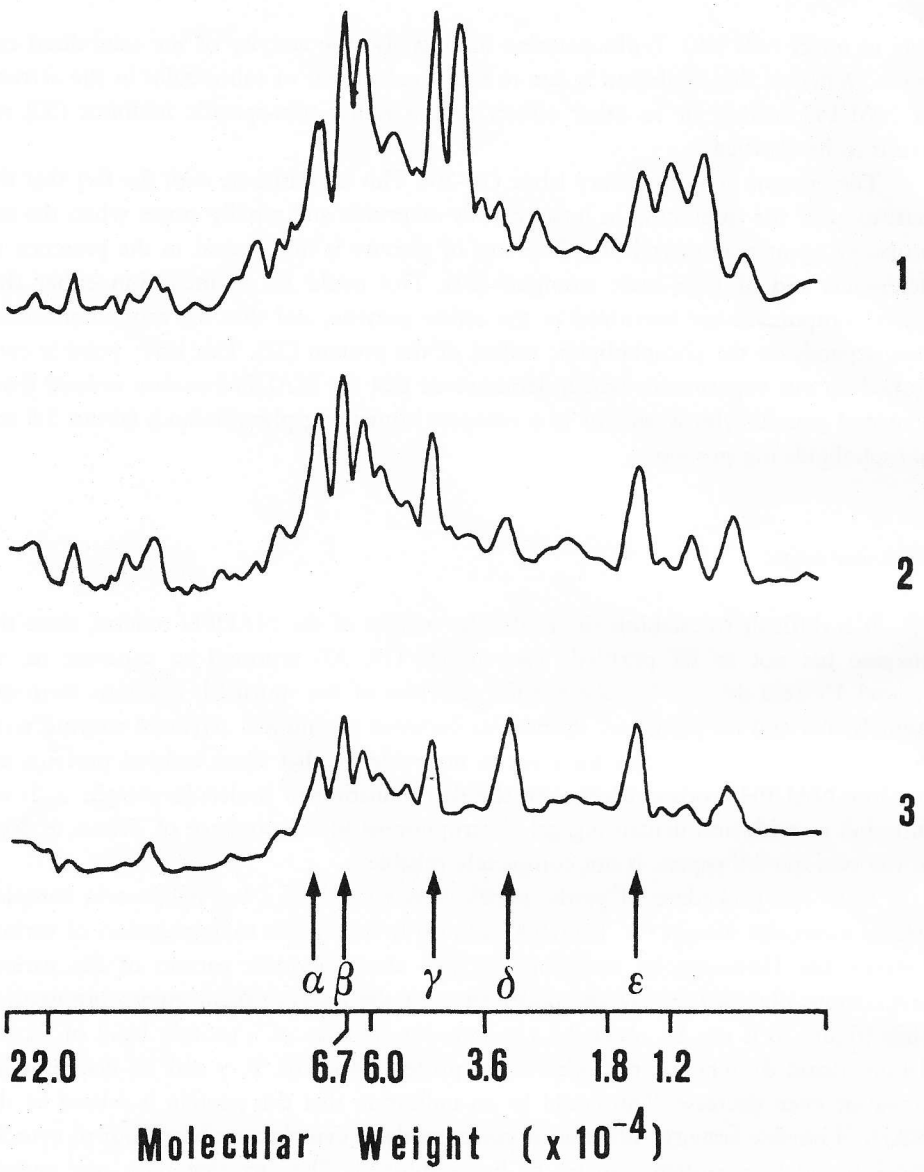

Fig. 4 – Densitometric scanning of polyacrylamide gel electrophoresis. Samples (15 μg protein) were treated with 1% SDS and loaded on slab gels (8x8 cm) consisting of a linearly increasing polyacrilamide concentration (4 to 30%). The running buffer was 40 mM Tris-HCl (pH 8.0) containing 20 mM sodium acetate, 2 mM EDTA and 1% SDS. Trace 1: solubilized extract (fraction 2); trace 2: active peak eluted from gel filtration (fraction 3); trace 3: active peak purified by glycerol density gradient (fraction 4).

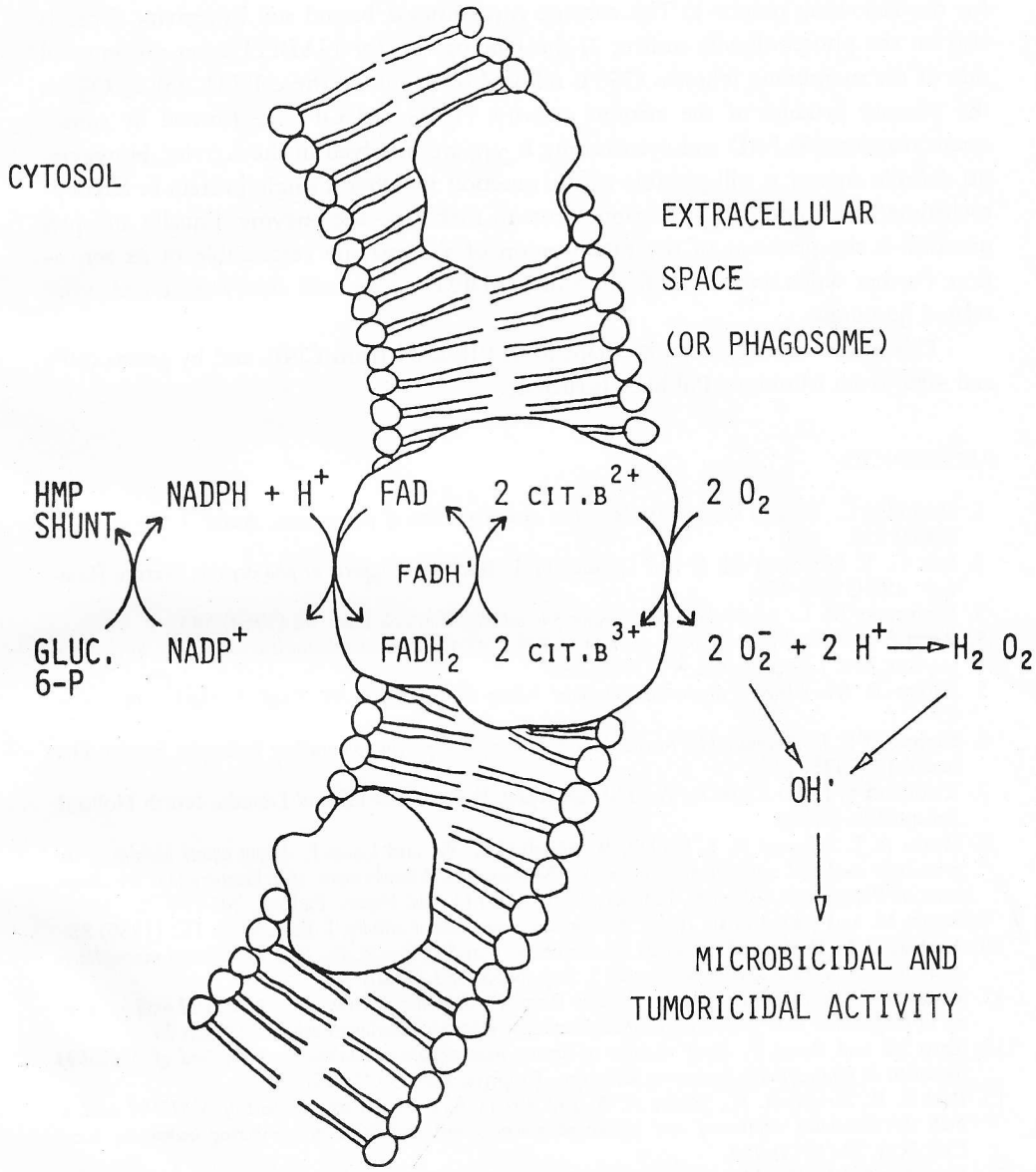

Fig. 5 – Schematic representation of the electron transport activity of NADPH oxidase.

CONCLUSION

On the basis of the present knowledge on the nature and on the properties of NADPH oxidase, the model shown in figure 5 can be proposed. This model accounts for the following points: 1) The enzyme is membrane bound and its activity depends also on the phospholipidic milieu; 2) the binding site for NADPH is on the internal side of the membrane whereas O_2^- · is released only outside the cell (34, 35); 3) O_2^- · is the primary product of the enzyme activity: H_2O_2 and $OH^{\cdot}$ are formed by subsequent reactions; 4) FAD and cytochrome b_{-245} are involved in the activity. However, no definite answer is still possible to the question whether a single protein (a flavocytochrome?) or a complex of various proteins makes up the enzyme. Equally an open question is the problem of the modification of the enzyme responsible of its activation. Further work on the purification of NADPH oxidase will clarify these and other related questions.

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