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THE RESPIRATORY BURST IN PHAGOCYTIC LEUKOCYTES

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INTRODUCTION

One of the properties shared by granulocytes and macrophages is that of undergoing a dramatic increase in respiration during phagocytosis or following the interaction with certain membrane perturbing agents. This "respiratory burst" results in the production of large quantities of superoxide anion (O_2^-) and hydrogen peroxide (H_2O_2) and, indirectly, of hydroxyl radical ($OH^\cdot$) and singlet oxygen ($^1O_2$) (1 - 8). The phenomenon, first described as an increment in oxygen uptake in 1933 (1), has been extensively investigated during the last 20 years. In spite of this, a number of problems remain to be clarified, such as the nature of the triggering events at the level of the plasma membrane, the enzymatic basis of the stimulation of respiration, the mechanism of oxygen reduction, the mechanisms of enzyme activation, deactivation and inactivation, and the relationships to other responses (secretion, movement, arachidonic acid cascade), etc.

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The recent appearance of excellent reviews (9 - 15) and the vastness of the subject relieve one of the obligation of giving a complete survey and free one to present only some facts and problems concerning the enzymatic basis of the burst and the relationships between the activation of the primary oxidase and the oxygen balance in intact cells.

THE PRIMARY OXIDASE

At present all authors agree that an oxidase that utilizes reduced nicotinamide adenine nucleotides as substrate is involved in the activation of respiratory metabolism in all the phagocytic cells studied. The disagreements or doubts concern the identification of the physiological substrate (NADH or NADPH), the mechanism of its oxidation, the involvement of other cofactors such as cytochrome *b* (16), the subcellular localization of the oxidase, and the process of its activation.

The reasons for these disagreements are very complex (14). There is a general agreement on the criteria for the recognition of an enzyme responsible for the respiratory burst (Table I). Bases on these criteria, some hypotheses concerning the involvement of myeloperoxidase (3), of amino acid oxidase (17), or of ascorbate oxidase (18) have been ruled out. On the contrary, based on the same criteria, two enzymes are currently believed to be involved: a "soluble NADH oxidase" that can be measured in the supernatant of homogenates of guinea pig and

TABLE I. Criteria for the Recognition of the Enzyme Responsible for the Respiratory Burst

1.	*The enzyme must consume oxygen and produce* O_2^- *and* H_2O_2
2.	*The activity must be increased in homogenates or in cell fractions derived from stimulated cells*
3.	*The degree of activation of the enzyme should correlate with the intensity of the respiratory burst of intact cells*
4.	*The* K_m *for the substrate should fall in the range of the physiologic concentration of that substrate*
5.	*The inhibition profile of the enzyme should be similar to that of the respiratory burst of intact cells*
6.	*The enzyme activity should account for the net oxygen consumption by intact cells*
7.	*CGD cells are expected to lack the enzyme or the enzymatic activity*

TABLE II. Methods and Conditions Used for the Assay of NAD(P)H Oxidase

A. Source of enzyme(s)	*Cells in resting state or activated by different stimuli and for different times* *Disruption of the cells by different procedures (Potter type homogenizer, sonication, freezing and thawing, vigorous pipetting)* *Whole homogenate in sucrose* *Whole homogenate in KCl* *20-30-100,000 g supernatants* *20-30-100,000 g pellets (postnuclear or total)* *Cell fractions obtained by different methods of fractionation* *Extracts*
B. Conditions	*Acid, neutral or weakly alkaline pH* *Presence of KCN, NaN_3 or other inhibitors* *Buffers: Phosphate, HEPES* *Concentration of substrate: from 0.025 to 2 mM* *Others: presence of manganese, sucrose, ethanol, catalase*
C. Methods of assay	*Oxygen consumption* *NAD(P)H disappearance (directly)* *$NAD(P)^+$ formation (indirectly and directly)* *O_2^- formation (SOD-sensitive cyt c reduction)* *O_2^- formation (SOD-sensitive NBT reduction)* *H_2O_2 formation* *Reduction of artificial electron acceptors*

human granulocytes in isotonic KCl (19, 28, 29), and a membrane-bound NADPH oxidase (6, 7, 9, 22, 25).

It is likely that the main reasons for disagreement stem from the methods and the conditions employed for the assay of the enzymatic activity, and from the source of the enzymatic preparation (different cell types, different state of their activation, nature of the stimulating agents).

TABLE III. Contribution of a Chain Reaction to the NAD(P)H Oxidizing Activity of Cell-Free Particles from Phagocytosing Guinea Pig Granulocytes

pH	NADPH (mM)	Total activity[a]	Chain reaction	
			Absolute[a]	% of total
5.5	1.0	72.7	58.6	80.6
7.0	1.0	42.0	22.1	52.6
5.5	0.15	22.9	10.1	44.1
7.0	0.15	16.0	0.7	4.4
	NADH (mM)			
5.5	1.0	38.7	26.2	67.8
7.0	1.0	37.0	19.3	52.1
5.5	0.15	2.1	0.32	15.2
7.0	0.15	5.2	0.32	6.2

[a]nmoles O_2 consumed /2.5 min/mg protein.

Oxygen consumption was measured at 37°C with a Clark oxygen electrode connected with a plastic chamber (27). The assay medium contained 65 mM Na/K phosphate buffer, 170 mM sucrose, 2 mM NaN_3 and NAD(P)H. The reaction was started by addition of 0.5 to 1.5 mg protein. The volume was 2 ml. The contribution of the chain reaction in the NAD(P)H oxidizing activity was estimated by the inhibition of O_2 consumption caused by 20 µg/ml of superoxide dismutase (26).

The Mechanism of NAD(P)H Oxidation Related to the Conditions of the Assay

Table II summarizes the methods and the conditions more frequently used for the measurement of the NAD(P)H oxidase activity. We have compared the main methods of assay in cell-free particles from phagocytosing guinea pig granulocytes (26). The results can be summarized as follows: (1) at least three components of the NAD(P)H oxidizing reactions can be identified, a univalent reduction of O_2, a divalent reduction of O_2, and a chain reaction, probably initiated by O_2^-; (2) the rate of these reactions and their reciprocal relations vary depending on the experimental conditions (pH, substrate concentration). Table III reports the percentage contribution of the chain reaction in the oxygen consumption in the presence of NADPH and of NADH by cell-free particles from stimulated guinea pig granulocytes. It can be seen that the importance of this reaction varies greatly depending on the experimental conditions. For example, its contribution is very relevant (ex-

pressed as absolute values or percentage) at acidic pH and at high substrate concentrations. At neutral pH and at concentration of substrates approaching the physiological ones, the contribution of chain reaction is practically negligible.

These findings are worthy of some comments.

1. Some of the discrepancies on the kinetic properties of the NAD(P)H oxidase can be explained by the difference in the conditions and methods of the assay used. For example, a pH optimum of 5.5 has been reported for the activity measured as O_2 consumption or NADPH oxidation (27 - 29) and a pH optimum of 7.0 when the activity has been measured as O_2^- generation (30 - 32). From the data presented above it is clear that at acidic pH the chain reaction accounts for most of the oxygen consumed and of NADPH disappearance. Thus the assay of the oxidase at this pH might give erroneous information.

2. When the kinetic properties of the oxidase are to be studied or when its activity must be compared with the respiration of intact cells, it is advisable to choose conditions in which the chain reaction is not operative. The best are: pH 7.0 with 0.15 m*M* substrate by measuring the formation of O_2^- or the consumption of O_2.

3. When the experimental goal is only the detection of the activity of the enzyme, as, for example, in different cell fractions separated with various methods of fractionation, anyone of the procedures can be used. In some cases it might be quite useful to favor the chain reaction. For example, when the activities of the true enzymatic reaction (the univalent reduction of O_2) are low, the detection of a difference in the activity between preparations from resting and from activated cells might be facilitated by using conditions where the rate of the chain is high. In some cases, the use of manganese, which is known to greatly potentiate the chain reaction (33, 34), might be very useful.

The Activity of the "Soluble" and of the Membrane-Bound Oxidase(s) in Guinea Pig Granulocytes

Since 1964 we have shown, by using different conditions of assay (acid and neutral pH, presence and absence of manganese, different concentration of substrates) and by measuring the oxygen consumption or the NAD(P)H disappearance (6, 7, 13, 27, 35 - 37), that in granulocytes and in macrophages of many mammalian species the primary oxidase is a membrane-bound NADPH oxidase.

Recently Karnovsky's group (21) has extracted and characterized from guinea pig neutrophils an oxidase specific for NADH, with K_m of 0.4 m*M*, pH optimum at 4.5 to 5.0, inhibited by a variety of nucleotides and polyvalent anions. This enzyme is

TABLE IV. Activity and Kinetic Properties of the NAD(P)H-Dependent O_2^- Production by Cell-Free Particles and Supernatants from Resting and PMA-Activated Guinea Pig PMN[a]

	Resting cells		PMA-activated	
	Supernatant	Cell-free particles[b]	Supernatant	Cell-free particles
0.15 mM NADH	0.77 ± 0.22	0 - 1.47	1.31 ± 0.17	18.79 ± 6.4
1.0 mM NADH	1.25 ± 0.45	0 - 1.55	1.88 ± 0.13	53.40 ± 16.3
0.15 mM NADPH	0.66 ± 0.29	0 - 0.45	0.74 ± 0.12	45.70 ± 8.4
1.0 mM NADPH	0.74 ± 0.36	0 - 0.69	0.92 ± 0.23	65.25 ± 15.6
V_{max} NADH	1.46 ± 0.28	--	1.57 ± 0.57	62.5 ± 18.7
V_{max} NADPH	0.73 ± 0.38	--	1.32 ± 0.6	54.0 ± 18.0
K_m NADH	0.178 ± 0.07 mM	--	0.116 ± 0.03 mM	0.64 ± 0.11 mM
K_m NADPH	0.016 ± 0.016 mM	--	0.017 ± 0.004 mM	0.057 ± 0.01 mM

[a]Assay system: 50 mM HEPES (pH 7.0), 150 µM cytochrome c, NADH or NADPH. The reference cuvette contained also 30 µg of SOD. Temperature 37°C. Values of NAD(P)H oxidase activity are given as nmoles O_2^-/min/mg protein. The data are means ± SD of 5 experiments.

[b]Due to the high variability, the data are expressed as range of activity.

believed to function as a peripheral membrane protein in association with cytoplasmic surface of the cell membrane and to have the potential to account for all the increase in O_2 uptake observed during phagocytosis of a wide variety of particles (21). We have reinvestigated the matter by measuring the oxidase in HEPES medium and at pH 7.0, as O_2^- forming activity in supernatants, and in 100,000 *g* cell-free particles from resting and PMA-treated guinea pig granulocytes of peritoneal exudates. The cells were disrupted in alkaline KCl, a condition that has been shown to solubilize the NADH oxidase from the membrane (15, 38, 39).

The results (Table IV) are as follows:

1. The supernatants of resting granulocytes contain a very low O_2^--forming activity both in presence of NADH and of NADPH; the pH optimum is between 7.0 and 7.5; the K_m is 0.18 m*M* for NADH and 0.016 m*M* for NADPH.

2. The enzymatic activity and the kinetic properties do not vary in supernatants of PMA-treated granulocytes.

3. The cell-free particles from resting granulocytes have also a very low activity both with NADH or NADPH as substrates. Because of the wide scatter of experimental data due to a very low activity and to the turbidity of assay systems, the K_m values for both the substrates have not been measured. The feeling is that the oxidase activity is substantially in a dormant state in resting guinea pig granulocytes, as it has been shown in human neutrophils (40).

4. The oxidase activity of cell-free particles from PMA-treated granulocytes is very high both with NADH and NADPH. The K_m is 0.6 m*M* for NADH and 0.05 m*M* for NADPH. Similar results have been obtained by measuring the oxidase as oxygen consumption and NAD(P)H disappearance.

We have also compared the effect of various inhibitors on the oxidase(s) of supernatants and of cell-free particles of PMA-stimulated granulocytes, and on the stimulation of respiration in intact cells. The oxidases of supernatants and of cell-free particles, in presence of both NADH or of NADPH, and the respiratory burst induced by PMA are insensitive to cyanide, azide, and rotenone. Furthermore, the respiratory burst of intact cells as well as the oxidase activity, with NADPH, of supernatants and of cell-free particles and the oxidase activity with NADH of cell-free particles are inhibited by 0.1 and 1 m*M* sulfhydryl reagent *N*-ethylmaleimide (NEM) (Table V and Fig. 1). On the contrary the NADH oxidase of supernatants is stimulated by *N*-ethylmaleimide.

A brief comment is appropriate. These results seem to us very strong evidence that neither NADH nor NADPH oxidase of supernatants of guinea pig granulocytes take part in the mechanism responsible for the increased respiratory activity of these cells. In fact, these activities do not fulfill the requirement

TABLE V. Effect of Cyanide and N-Ethylmaleimide on NAD(P)H-Dependent O_2^- Production by Supernatants and Cell-Free Particles from PMA-Activated Guinea Pig Granulocytes

Additions	Supernatant		Cell-free particles	
	NADH	NADPH	NADH	NADPH
None	100	100	100	100
0.5 mM KCN	85	91	90	87
0.1 mM NEM	188	71	--	--
1 mM NEM	179	36	17	23

[a]*Assay system: see Table IV.*

listed in Table I. In particular their activity (1) is very low, (2) is not increased in the cell fraction derived from stimulated cells, (3) is not adequate to account for the respiratory burst of intact cells, (4) is activated by NEM in presence of NADH while the respiration of intact cells is inhibited.

We do not know whether the oxidation of NADH and of NADPH by supernatants is performed by a unique or by different enzymes. The different effects of some inhibitors might favor the second possibility. Also we do not know whether the activity (or the activities) found in the supernatants is (or are) due to enzyme(s) solubilized from the membrane. Some differences in K_m and in the sensitivity to inhibitors might be an indication against this possibility. It is likely, however, that changes of some properties are due to solubilization.

The data presented up to now seem to us a good confirmation of the results that we (6, 7, 27, 35, 36) and others (9, 22 - 25, 40) have published in the last 20 years. In other words, we have shown here that the O_2^--forming activities bound to some membranous structures of the granulocytes are involved in the respiratory burst. In fact this NAD(P)H oxidase fulfills the requirements listed above (Table I). The comparison between the values of K_m and the concentration of NADH and of NADPH inside the cells (Table VI) clearly indicate that the physiological substrate is NADPH. A more complete survey of the evidence in favor of the role of NADPH as substrate has been published elsewhere by us (7, 13) or by others (9, 11).

The Primary Oxidase in Macrophages

Our knowledge of the enzyme(s) responsible for the respiratory burst in mononuclear phagocytes is rather poor. We are investigating this problem by measuring the "soluble" and the

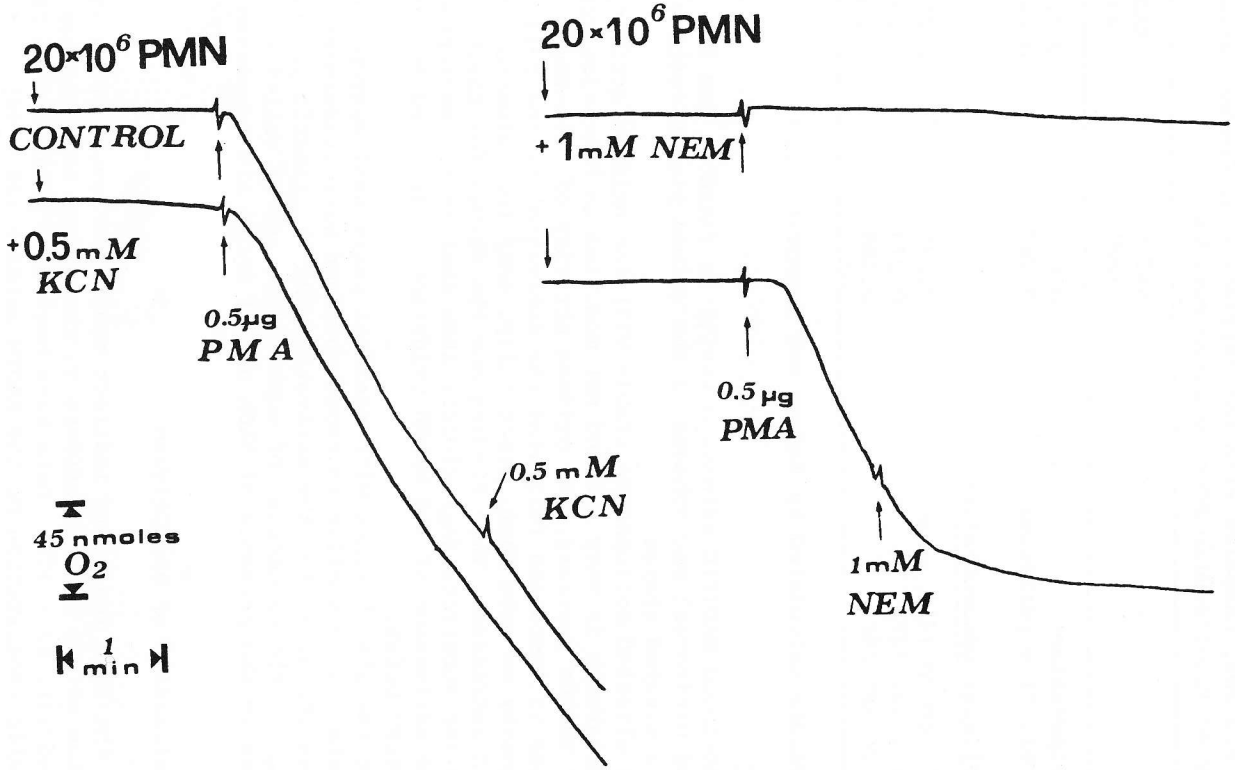

Fig. 1. Effect of cyanide and N-ethylmaleimide (NEM) on the stimulation of oxygen consumption by PMA in guinea pig PMN.

TABLE VI. K_m for NADPH and NADH with Respect to the Oxidase Activity of Cell-Free Fractions from PMA-Activated Guinea Pig PMN, Compared with the Intracellular Concentration of Reduced Nicotinamide Adenine Dinucleotides

	NADPH (mM)	NADH (mM)
K_m of supernatant	0.017	0.116
K_m of cell-free particles	0.057	0.64
Intracellular concentration		
Sbarra's group (41)	0.136	0.172
Rossi's group (27)	0.273	0.076
Frei's group[a] (42)	0.100	0.160

[a]Values calculated by Badwey and Karnovsky (21).

membrane-bound NAD(P)H oxidase activity in resident and in elicited peritoneal macrophages of the guinea pig in resting and in activated states.

In elicited macrophages (Table VII) the oxidase activity of supernatants is very low and not modified in PMA-stimulated cells. On the contrary, the oxidase activity of cell-free particles is increased following the activation of the cell. It is worthy to note that, practically, only the oxidation of NADPH is increased. This finding and the values for their avidity for substrate (K_m) clearly show that in the macrophage only the activation of the NADPH oxidation is involved in the respiratory burst.

The results obtained with resident peritoneal macrophages are substantially similar to those obtained with granulocytes as far as the activity, the activation and the kinetic properties (K_m) of the oxidase(s) of supernatant and of cell-free particles in the presence of NADH and of NADPH are concerned.

The Localization of the Oxidase

In the last few years indirect and direct evidence on localization of the primary oxidase on the plasma membrane has increased (31, 43 - 47). Data have been also published on intracellular localization of the NADPH oxidase (48 - 50). It is likely that the discrepancies depend on the methods of fractionation of cell homogenates, on the different state and degree of activation of the cells, and on the methods of assay of the oxidase.

TABLE VII. NAD(P)H-Dependent O_2^--Forming Activity of Supernatants and Cell-Free Particles from Resting and PMA-Activated Guinea Pig Elicited Peritoneal Macrophages[a]

	NADH (mM)		NADPH (mM)	
	0.15	1	0.15	1
Supernatant				
From resting cells	1.0 ± 0.3	2.1 ± 0.7	0.74 ± 0.1	0.95 ± 0.2
From PMA-activated cells	1.1 ± 0.3	1.9 ± 0.6	1.1 ± 0.1	2.0 ± 0.4
Particles				
From resting cells	2.7 ± 0.6	6.3 ± 0.9	0.79 ± 0.2	2.5 ± 0.5
From PMA-activated cells	3.1 ± 1.0	7.9 ± 1.7	13.1 ± 1.9	21.3 ± 2.7
K_m for NAD(P)H of particles from PMA-activated cells	0.633 ± 0.11 mM		0.087 ± 0.005 mM	

[a]Assay system: 65 mM Na/K phosphate buffer (pH 7.0), 170 mM sucrose, 150 μM cytochrome c, NADH or NADPH, and 2 mM N_2N_3, in a final volume of 1 ml. The reference cuvette contained also 30 μg of SOD (24, 32). The reaction was started by the addition of a suitable amount of enzyme. Temperature 37°C. Values of NAD(P)H oxidase activity are given as nmoles O_2^-/min/mg protein. The means of four experiments ± SEM are reported.

We have reinvestigated the problem (50) by using PMA-activated guinea pig granulocytes and macrophages and different methods of fractionation of cell homogenates (isopycnic equilibration, rate zonal centrifugation in continuous sucrose gradient, rate zonal sedimentation in discontinuous sucrose gradient). The results (not shown here) demonstrate that the distribution of the activated oxidase follows that of 5'-nucleotidase and of acid pNPPase, enzymes that are markers of the plasma membrane.

We are now measuring the oxidase in the membrane of the isolated phagosomes from phagocytosing guinea pig granulocytes and elicited peritoneal macrophages. We are performing this investigation in order to make clearer the localization of the O_2^--forming enzyme and also to understand whether the activation of the oxidase takes place only in the invaginated portion of the plasma membrane that is in the wall of phagosomes, or in the entire plasma membrane. Preliminary results, of which one example is reported in Table VIII, seem to indicate that the activated NADPH oxidase is located only in the portion of the plasma membrane that forms the phagosomes. In fact the enrichment of the oxidase in the phagosomal fraction, expressed by the relative specific activity, is much higher than that of 5'-nucleotidase, the typical marker of the plasma membrane. This localization of the activated O_2^--forming enzymes is a strategic one for the intravacuolar use of the radical and of hydrogen peroxide.

RELATIONSHIP BETWEEN THE ACTIVITY OF NADPH OXIDASE AND THE RESPIRATORY METABOLISM OF INTACT CELLS

One of the requirements to be fulfilled for the recognition of an enzyme responsible for the respiratory burst is that its activity should account for the extra oxygen consumption by intact cells. When we deal with this matter we are faced with a number of problems. The main ones are the mechanisms of the activity of the oxidase on one hand, and the complexity of the reactions involved in the oxidative metabolism of the phagocytes, that is, in the O_2 balance of intact cells, on the other.

The Stoichiometry of the Oxidase Reaction

On the basis of the overall reaction of the oxidase ($2\ O_2 + NADPH \longrightarrow 2\ O_2^- + NADP^+$) postulated by Babior *et al.* (24), for every nmole of NADPH oxidized 2 nmol of O_2^- are pro-

TABLE VIII. Relative Specific Activities of Marker Enzymes and of NADPH Oxidase in Fractions Isolated from the Homogenate of Elicited Peritoneal Macrophages Phagocytosing PMA-Coated Latex Beads

	5'-Nucleotidase	Glucose-6-phosphatase	β-Glucuronidase	NADPH oxidase
Phagosomes	2.6	2.6	2.1	13.2
Intermediate fraction	1.8	1.7	0.4	3.3
Pellet	1.9	1.6	0.9	2.0

duced. Under the conditions employed in the experiments reported in Table IV we have found 0.4 - 0.8 nmol of O_2^- produced for 1 nmol of NADPH or of NADH oxidized. This defective measurement of O_2^- could be interpreted to mean (1) that there are two pathways of oxygen reduction, a univalent one with formation of O_2^-, and a divalent with direct formation of H_2O_2; or (2) that there is only the univalent pathway but part of O_2^- is formed in a crevice of the enzyme or in a site not fully accessible to cytochrome *c*.

I shall now present some evidence against the first possibility, at least as far as the oxidation of NADPH is concerned. The results reported in Fig. 2 show, in fact, that the addition of deoxycholate (DOC) increases the production of O_2^- by cell free particles in presence of NADPH 2- to 3-fold. The detergent increases the oxidation of NADPH only slightly (+20%). Therefore, under these conditions the ratio: O_2^- produced versus NADPH oxidized approximates the theoretical one. These results indicate that the oxidation of NADPH *in vitro* by cell-free particles involves mainly an univalent reduction of O_2 with formation of O_2^-. Thus the oxidase activity should be assayed in presence of DOC in order to make a comparison with the extra respiration of intact cells. Alternatively, the values of NADPH-oxidase should be corrected by a factor calculated on the basis of the insufficient recovery of O_2^- with respect to the theoretical value. In our case the factor varies between 5 and 2.5.

The Oxygen Balance of Whole Cells

The complexity of the oxygen balance has been extensively discussed by us (51) and by others (14).

Briefly, the measurements of respiration on whole cells refer to net oxygen uptake and give no indication of the total amount of oxygen actually turned over. The net oxygen consumption is the result of several reactions: reduction to O_2^- catalyzed by the oxidase, on one side, and reactions of dismutation of O_2^- and of degradation of H_2O_2, on the other (Table IX).

In the reaction of dismutation and in the catalytic degradation of H_2O_2 part of the oxygen consumed originally is recovered. Then the ratio between the extent of reaction 1 (that is the amount of O_2^- formed) and the net oxygen consumption varies depending on the extent of the oxygen returned, that is, mainly through the agency of the degradation of H_2O_2. We have shown (51) that in guinea pig granulocytes the ratio of O_2^- formed to net O_2 consumed is 2.6. This means that in these cells the activity of the NADPH oxidase measured after breaking the cells would be adequate to account for the respiratory

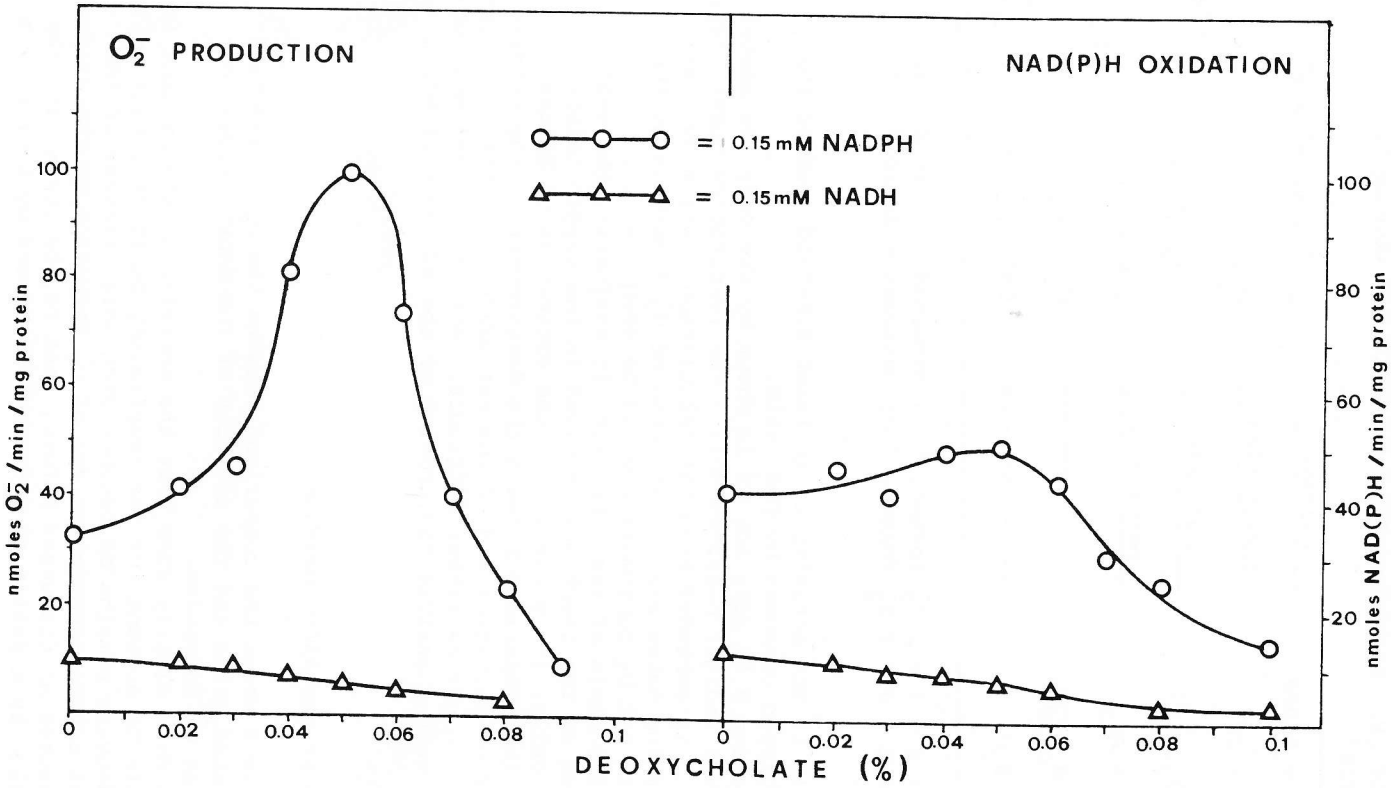

Fig. 2. Effect of deoxycholate on NAD(P)H oxidation by cell-free particles from PMA-activated PMN. O_2^- production was measured as SOD-inhibitable cytochrome c reduction. NAD(P)H oxidation was measured by following the decrease in absorbance at 340 nm.

TABLE IX. Reactions Involved in Oxygen Consumption in Whole Cells[a]

1.	$2\ O_2 + NADPH$	$\xrightarrow{oxidase}$	$2\ O_2^- + NADP^+ + H^+$
2.	$2\ O_2^- + 2H^+$	$\xrightarrow{dismutation}$	$H_2O_2 + O_2$
3.	$2\ H_2O_2$	$\xrightarrow{catalase}$	$2\ H_2O + O_2$
4.	$H_2O_2 + RH_2$	$\xrightarrow{peroxidase}$	$2\ H_2O + R$
5.	$O_2^- + X_{oxid}$	$\longrightarrow$	$O_2 + X_{red}$
6.	$O_2^- + H_2O_2$	$\longrightarrow$	$OH^\cdot + OH^- + O_2$

[a] *1 + 2 + 3 = 2 O_2^- formed, ½ O_2 consumed = Ratio 4 : 1.*
1 + 2 + 4 = 2 O_2^- formed, 1 O_2 consumed = Ratio 2 : 1.

burst only if its activity is at least 2.6-fold greater than the net oxygen consumed by the cells.

Reaction 6 is very low as is shown by the very low amount of hydroxyl radical measured during the respiratory burst (52). Reaction 5 is excluded from the calculation. Assuming that this reaction takes place, the ratio of O_2^- formation by the oxidase to net O_2 consumption would be even greater.

On the basis of the complexity in evaluating the real occurrence of the reactions involved in the oxygen balance in whole cells, it is clear that the estimation of adequacy based on the comparison between the measurement of the oxidase on cell-free particles and of the net oxygen consumption in intact cells might be rather difficult. With this consideration in mind, we now analyze the problem of the adequacy of NADPH oxidase.

Adequacy of the NADPH Oxidase

Table X shows the comparison between the O_2 consumption of activated cells and the activity of the NADPH oxidase expressed as O_2^- formation.

The data clearly show that the activity of the oxidase is inadequate to account for the respiratory burst of intact cells. The inadequacy remains or becomes even more relevant if the values of oxygen consumption and of O_2^- formation by the oxidase are corrected as discussed before, that is, by correcting the first value by a factor of 2.6, and the second by 5 or by 2.5.

TABLE X. Comparison Between O_2 Uptake by Intact Guinea Pig Granulocytes and NADPH Oxidase Activity of Cell-Free Particles

	Experimental	*Corrected*
Net O_2 uptake (nmoles/min/10^6 cells)	*4.7 ± 1.35*	*12.2*[a]
NADPH oxidase (nmoles O_2^-/min/cell-free particles from 10^6 cells)	*0.766 ± 0.140*	*3.8*[b] *1.9*[c]

[a]*Experimental (4.7) × 2.6, that is the ratio O_2^- formed/O_2 consumed (see reference 51). 12.2 are nmoles of O_2^- actually formed for a net O_2 consumption of 4.7 nmoles.*

[b]*Experimental (0.766) × 5 (stoichiometry O_2^-/NADPH 0.4 instead of 2.0).*

[c]*Experimental (0.766) × 2.5 (stoichiometry O_2^-/NADPH 0.8 instead of 2.0).*

This inadequacy could be explained in different ways. One is that, besides NADPH oxidase, other enzymes or other respiratory systems are involved in the respiratory burst. Another reason for the inadequacy is that we underestimate the oxidase activity because the enzyme is inactivated or deactivated during the procedures of cell disruption and the time of homogenization. It is known in fact that an inactivation of the oxidase takes place during the burst (53) and that proteolytic processes on the plasma membrane are activated by homogenization (54). Thus, if the oxidase is measured soon after the activation has taken place, and by using a procedure that allows complete accessibility of the substrates to the enzymatic site and of cytochrome *c* to the O_2^- generating site, one would expect that the actual activity of the enzyme could be fully revealed. This expectation has been verified by using the experimental model described in Fig. 3.

The respiratory activity of intact cells stimulated by PMA was measured as O_2^- production. During the linear part of the respiration, the cells were lysed and their respiratory activity was abolished by adding deoxycholate (DC). At this point, NADH 1 m*M* or NADPH 0.15 m*M* were added. The addition of NADPH induces an instantaneous resumption of O_2^- production at a rate similar to or even higher than that of the stimulated cells. After the addition of NADH the O_2^- production also resumed, but at rates much lower with respect to that of the

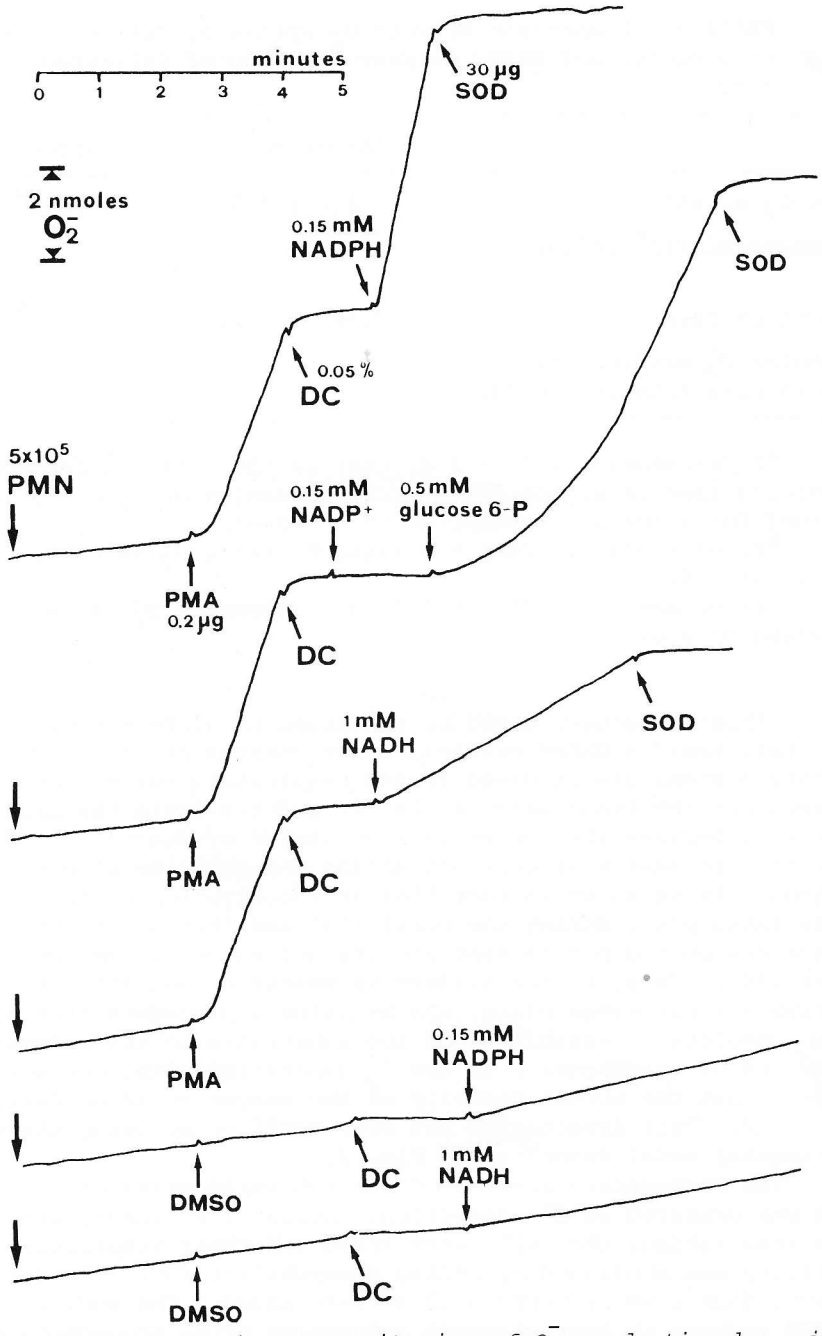

Fig. 3. Continuous monitoring of O_2^- production by guinea pig PMN and by the NAD(P)H oxidase activity of the same cells lysed with deoxycholate (DC). Assay mixture: 5 × 10^5 PMN incubated in 1 ml KRP containing 0.5 mM $CaCl_2$, 5 mM glucose, 2 mM NaN_3 and 150 µM cytochrome c. Temperature 37°C.

stimulated cells. DC has no stimulatory activity on NAD(P)H oxidase of resting cells.

Similar results have been obtained by measuring both the respiratory burst of intact cells before the addition of DC, and the oxidase activity after the addition of DC, as O_2 consumption with Clark oxygen electrode.

The validity of this experimental approach, that at the same time avoids the inactivation and allows a good substrate-enzyme interaction and a physiological fate of the intermediates, is also shown by the data presented in Fig. 4. It can be seen that the rate of respiration resumed after the addition of NADPH strictly parallels the extent of the activation of the respiratory metabolism of intact cells induced by different doses of PMA.

These results indicate that the activity of the primary oxidase that oxidizes NADPH, when measured in appropriate conditions, is adequate to account for the respiration of whole cells.

CONCLUSION AND SUMMARY

In conclusion the findings presented here are, in our opinion, convincing evidence that the primary oxidase mainly responsible for the respiratory burst in guinea pig phagocytes is an enzyme associated with the plasma membrane, whose physiological substrate is NADPH and which fulfills all the required criteria. In fact this oxidase (1) consumes O_2 and produces O_2^- and H_2O_2 after dismutation of O_2^-, (2) is activated in cells stimulated by phagocytosis or by soluble membrane-perturbing agents, (3) has a degree of activation that correlates with the intensity of the respiratory burst of intact cells, (4) has a K_m that is in the range of the physiological concentration of the substrate, (5) has a profile of inhibition by various compounds similar to that of the burst of intact cells, (6) accounts for the net oxygen consumption of the intact cell.

Furthermore, the site where the activated enzyme works, during phagocytosis, seems to be the portion of the membrane that forms the endocytic vacuole.

The results presented here confirm also the existence of an enzyme that oxidizes NADH in the supernatant of leukocytes homogenized in isotonic, alkaline KCl as described by others (19, 28, 29). This oxidase consumes O_2 and forms O_2^- and H_2O_2, but does not fulfill the criteria required for an involvement in the respiratory burst. In fact this enzyme (1) is not activated in stimulated cells, (2) has a K_m for the substrate higher than the physiological concentration of NADH inside the cells, (3) is

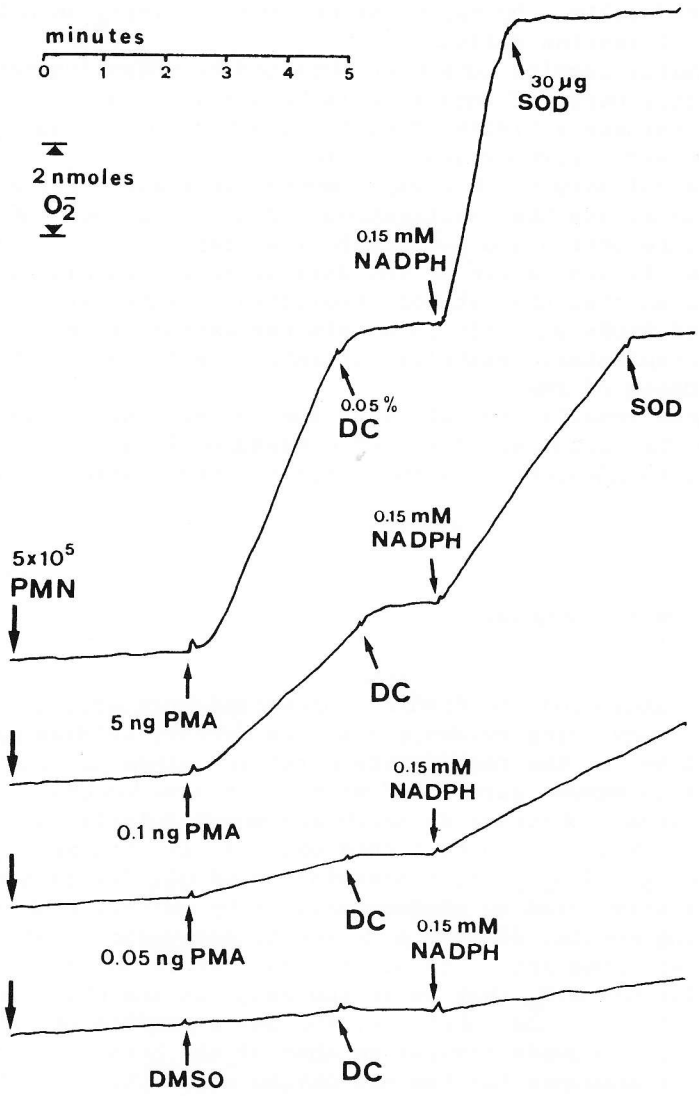

Fig. 4. Relation between O_2^- production by guinea pig PMN stimulated with various amounts of PMA and the corresponding NADPH oxidase activity after addition of deoxycholate. For the assay conditions see Fig. 3.

activated by a sulfhydryl reagent while the respiratory burst of intact cells is inhibited, (4) has an activity that is very low and does not account for the net oxygen consumption of intact cells.

We have also shown in this presentation the existence of a NADPH oxidase in supernatants of leukocytes homogenized in isotonic alkaline KCl. It is likely that this enzyme is different from that which oxidizes NADH, i.e., the one isolated by Karnovsky's group (19). In fact (1) the oxidase of Karnovsky is specific for NADH (19), (2) the K_m we have measured in supernatants for NADPH is one order of magnitude lower than that for NADH, (3) the activity with NADPH is inhibited by sulfhydryl reagents while that with NADH is activated.

In any case, the fact that the NADPH oxidase of supernatant has a very low activity that does not change upon stimulation of the cells with PMA indicates that this "soluble" NADPH oxidase also has nothing to do with the respiratory burst.

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