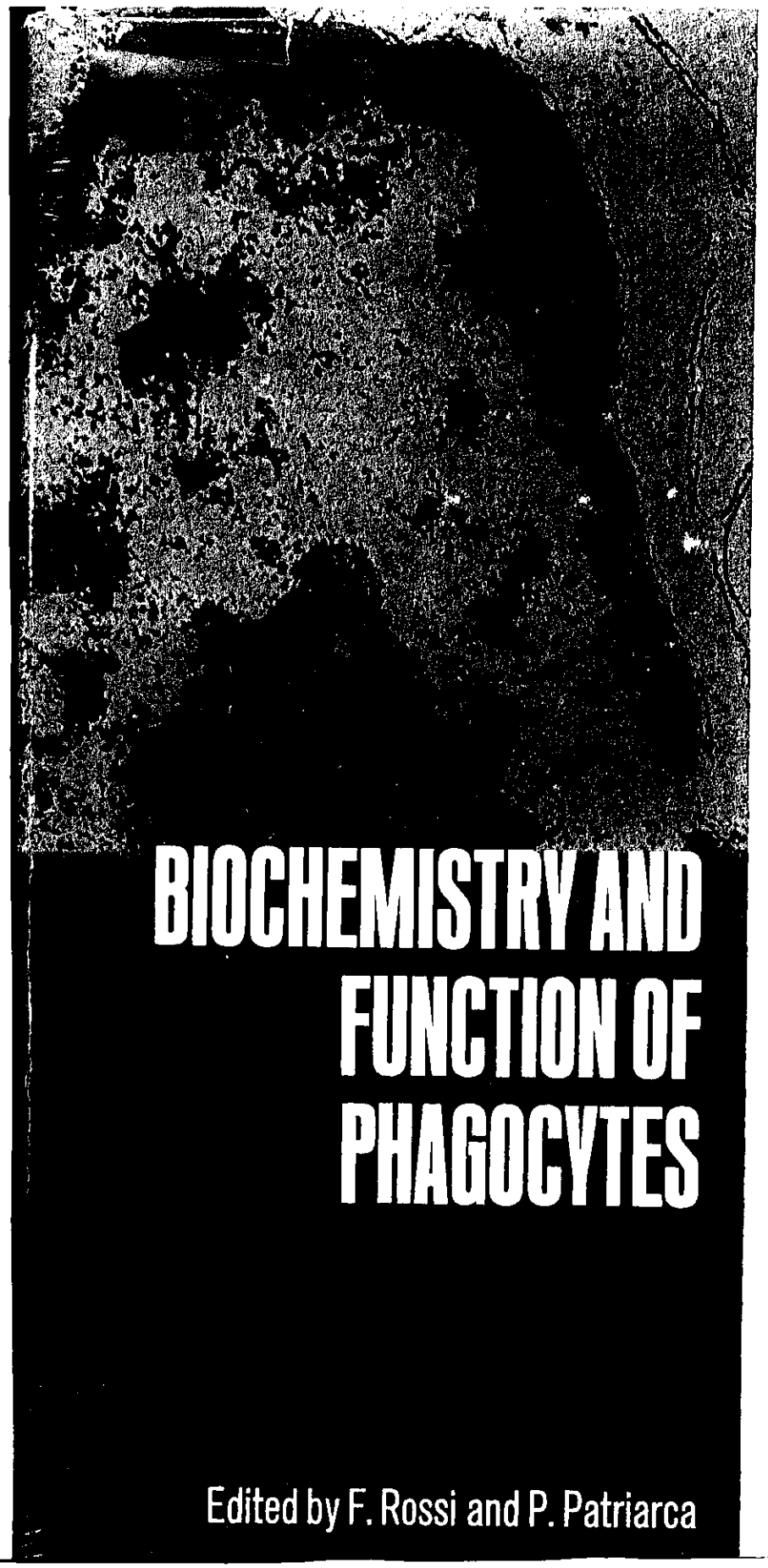

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THE RESPIRATORY BURST OF PHAGOCYTIC CELLS: FACTS AND PROBLEMS

Filippo Rossi, Paolo Bellavite, Giorgio Berton, Pietro Dri[+] and Giuliano Zabucchi[+]

Istituto di Patologia Generale dell'Università di Padova, sede di Verona, Strada Le Grazie, Verona (Italy) and [+]Istituto di Patologia dell'Università di Trieste

INTRODUCTION

The phagocytic cells have impressive capacities to respond to a variety of external stimuli with activation of random and oriented movement, with secretion of mediators and enzymes stored in cytoplasmic organelles, with production of new compounds and mediators, with changes of the quality and of the intensity of oxidative metabolism. The most impressive response is the production, in a very short time, of an enormous amount of weapons and projectiles that are used against viruses, bacteria, protozoa, tumor cells etc. These weapons are O_2^-, H_2O_2, $OH^\cdot$ and singlet oxygen and are intermediate products of a particular type of respiration, called "respiratory burst", which is induced by a perturbation of the plasma membrane of the phagocytes following the interaction with particulate matter and with a number of soluble factors[1-7].

In spite of a great number of investigations performed during the last twenty years, many aspects of the mechanisms responsible for the respiratory burst remain to be clarified, such as the role of the plasma membrane, the relationship with other responses, the nature of the enzymatic system responsible for the activation of the respiration and of the production of the oxygen radicals, the mechanisms of oxygen reduction, the nature of the substrate of the primary oxidase, the mechanism of activation and of deactivation, the relationship between the activity of the primary oxidase and the net

oxygen balance in whole cells, the fate of the intermediate products of oxygen reduction etc.

We shall analyse in this presentation some facts and problems concerning a) the enzymatic basis of the respiratory burst and b) the relationship between the activity of the primary oxidase and the oxygen balance of whole cells.

THE OXIDASE RESPONSIBLE FOR THE RESPIRATORY BURST

Table 1 presents the main hypotheses, advanced during the last twenty years, concerning the enzymatic basis of the activation of the respiratory metabolism of phagocytes.

There is a general agreement on the criteria for the recognition of an enzyme as responsible for the respiratory burst of the phagocytes (table 2). On the basis of these criteria the hypotheses concerning the involvement of myeloperoxidase[2], of aminoacid oxidase,[8] of ascorbate oxidase[9] have been ruled out.

Table 1. Enzymes or enzymatic systems proposed as responsible for the respiratory burst of phagocytes

MYELOPEROXIDASE	(Quastel's group)
"SOLUBLE" NADH OXIDASE	(Karnovsky's group)
MEMBRANE-BOUND NADH OXIDASE	(Segal)
MEMBRANE-BOUND NADPH OXIDASE	(Rossi's group Sbarra's group Hohn and Lehrer Babior's group De Chatelet's group Kakinuma's group Minakami's group)
AMINOACID OXIDASE	(Cline and Lehrer)
ASCORBATE OXIDASE	(De Chatelet)
NAD(P)H DEHYDROGENASE - CYT. b	(Segal)

Table 2. Requirements to be fulfilled for an enzymatic activity to be considered 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 Km 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 activity of the enzyme should account for the net oxygen consumption by intact cells
7. CGD cells are expected to lack the enzyme or the enzymatic activation

During the last twenty years evidences have been presented in our and in other laboratories that the burst is linked to the activation of a membrane bound oxidase that preferentially uses NADPH as substrate[4,5,6,10-15]. In our opinion these evidences are so strong that the controversy concerning the identification of the oxidase should be closed and the research should deal with the problems that remain to be clarified, such as the nature of the enzyme, the involvement of cytochrome b and of other cofactors, the solubilization and purification of the oxidase, the mechanism and the control of its activation.

In spite of this conviction we have been induced to reinvestigate the matter by two events. The first is the new emphasis that recently has been given to the involvement of a "soluble oxidase", specific for NADH, and measurable in the supernatants of homogenates of guinea pig and human granulocytes[16-20]. The second

event is the following: about one year ago we submitted for publication a paper showing that in guinea pig granulocytes treated with phorbol-myristate acetate (PMA) the activated NADPH oxidase is located on the plasma membrane. The paper was rejected on the basis of the following comment of one referee: "The choice of guinea pig as a source of neutrophils is very unfortunate. In this animal, more than in rabbit and human, the NADH oxidase contributes to the O_2^- formation. Therefore it is impossible to limit the study of guinea pig neutrophils to the NADPH oxidase".

The activity of the soluble and of membrane bound oxidase in guinea pig granulocytes

It is likely that the disagreements in the identification and characterization of the oxidase derive from the methods and the conditions employed for the assay of the enzymatic activity and from the sources of the enzymatic preparations. For these reasons we have measured the activity of the oxidase following the same experimental conditions used by Badwey and Karnovsky[34], that is: granulocytes (PMN) of guinea pig peritoneal exudates; supernatant and cell free particles of PMN homogenized in isotonic alkaline KCl; measurement of the oxidase as O_2^- formation (SOD sensitive cytochrome c reduction) in HEPES medium pH 7.0, in presence of NADH and NADPH.

The results reported in table 3 show that:

1. The supernatants of resting PMN contain a very low O_2^- forming activity both in presence of NADH and NADPH. The oxidase has the following characteristics: pH optimum between 7.0 and 7.5 (data not shown); a Km for NADPH one order of magnitude lower than that for NADH; an activity and kinetic properties that do not vary in supernatants of PMA activated PMN.
2. The cell free particles of resting cells have a very low oxidase activity both with NADH and NADPH as substrates. The values of the activity are around the lower limit of sensitivity of the assay method. The quantitation has been hard to be performed with accuracy due also to the turbidity of the assay system and hence to the wide scatter of the experimental data. For these reasons the range of the values is given in the table 3 and the Km has not been measured. From these results it can be concluded that the oxidase activity is substantially inactive in PMN in resting state. On the contrary the oxidase activity of cell free particles from PMA treated PMN is very high both with NADH and

Table 3. Activity and kinetic properties of the NAD(P)H-dependent O_2^- production by cell free particles and supernatants from sonicated and PMA-stimulated

Table 3. 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

	nmoles O_2^-/min/mg prot.			
	Resting cells		PMA-activated	
	Supernatant	Cell free particles+	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
Vmax NADH	1.46 ± 0.28	——	1.57 ± 0.57	62.5 ± 18.7
Vmax NADPH	0.73 ± 0.38	——	1.32 ± 0.6	54.0 ± 18.0
Km NADH	0.178 ± 0.07 mM	——	0.116 ± 0.03 mM	0.64 ± 0.11 mM
Km NADPH	0.016 ± 0.016 mM	——	0.017 ± 0.004mM	0.057 ± 0.01 mM

The data are means ± SD of 5 experiments.

+ Due to the high variability, the data are expressed as range of activity.

NADPH. This oxidase has the following characteristics: a pH optimum between 7.0 and 7.5; a Km for NADPH one order of magnitude lower than that for NADH; a Vmax with NADPH similar to that with NADH.

The comparison of the values of Km with the values of the intracellular concentrations of NADPH and NADH in guinea pig PMN (table 4) indicates that at the concentration of the substrates within the cells, the activity with NADPH is greatly favoured with respect that with NADH.

The activation of the oxidase of cell free particles parallels the degree of the activation of the respiratory metabolism of whole cells induced by stimulation with different doses of PMA. Fig. 1 reports the data on the activity in presence of NADPH.

We have also investigated the sensitivity to various inhibitors of the oxidase present in supernatants and in cell free particles of PMA-activated PMN. The data presented in table 5 show that:
1. The oxidase of supernatants and of cell free particles, both in

Table 4. Km for NADPH and NADH of the oxidase activity of cell free fractions from PMA activated guinea pig PMN compared with the intracellular concentrations of reduced nicotinamide adenine dinucleotides

	NADPH (mM)	NADH (mM)
Km of supernatant	0.017	0.116
Km of cell free particles	0.057	0.64
Intracellular concentration		
Sbarra's group 21	0.136	0.172
Rossi's group 22	0.273	0.076
Frei's group 23+	0.1	0.16

+ Values calculated by Badwey and Karnovsky 34.

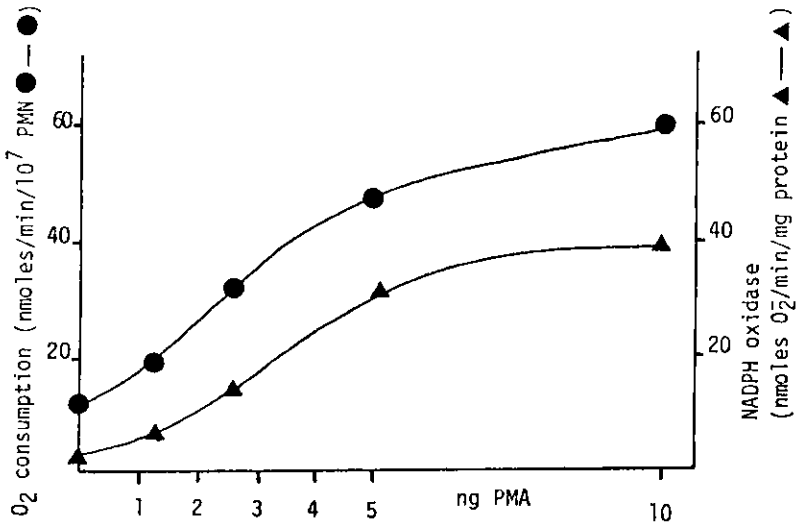


Fig. 1. Relationship between the activation of O_2 consumption by intact guinea pig PMN and the activation of the NADPH oxidase activity. O_2 consumption was measured in presence of different amounts of PMA and NADPH oxidase was measured as O_2^- forming activity of cell free particles obtained from the same cell suspensions.

presence of NADH and NADPH, are insensitive to cyanide, rotenone and NaN_3. 2. The oxidase of supernatants and of cell free particles are inhibited by ATP when NADH is the substrate and not inhibited when NADPH is the substrate. 3. The NADPH oxidase activity of supernatants and of cell free particles and the NADH oxidase activity of cell free particles are inhibited by appropriate concentrations of sulfhydrylic reagents N-ethylmaleimide (NEM) and p-chloromercurybenzoate (pCMB). On the contrary the NADH oxidase activity of supernatants is stimulated by NEM and also by pCMB 0.1 mM.

Fig. 2 shows the effect of various inhibitors on the respiratory activity of guinea pig PMN stimulated by PMA. It can be seen that the respiratory burst is insensitive to cyanide, rotenone and NaN_3, and inhibited by the penetrating sulfhydrylic reagent NEM and by high concentration of non-penetrating pCMB. This behaviour parallels that of the oxidase activity of cell free particles of PMA activated cells, while differs from that of the oxidase of supernatants which in presence of NADH is activated by sulfhydrylic reagents.

Table 5. Effect of inhibitors on the NAD(P)H-dependent O_2^- production by cell free particles and supernatant of PMA-activated guinea pig PMN

Additions			SUPERNATANT		CELL FREE PARTICLES	
			NADH	NADPH	NADH	NADPH
None			100	100	100	100
NaN_3	2.0	mM	80	93	100	104
KCN	0.5	mM	85	91	90	87
Rotenone	0.025	mM	88	111	96	100
ATP	0.5	mM	36	81	23	85
NEM	0.1	mM	188	71	--	--
NEM	1.0	mM	179	36	17	23
pCMB	0.1	mM	151	33	3	3.5
pCMB	1.0	mM	77	0	0	0

The results are expressed as percentage of O_2^- production in the control mixture which contained no inhibitor. NADH 1 mM, NADPH 0.15 mM.

A brief comment.

1. We do not know whether the activities found in the supernatants are due to enzymes solubilized from the membranes. Some differences in Km and in the sensitivity to inhibitors might be indications against this possibility. It is likely, however, that the changes of these properties are due to the solubulization.

We do not know also whether the oxidations of NADPH and of NADH by supernatants are performed by a single or by different enzymes. The different effect of some inhibitor might favour the second possibility.

In any case the results seems to us a strong indication that the oxidase (or the oxidases) of supernatants of guinea pig PMN is not involved in the respiratory burst of intact cells. In fact the activity a) is very low, b) is not modified upon activation of PMN

0.5 µg PMA

0.5 µg PMA

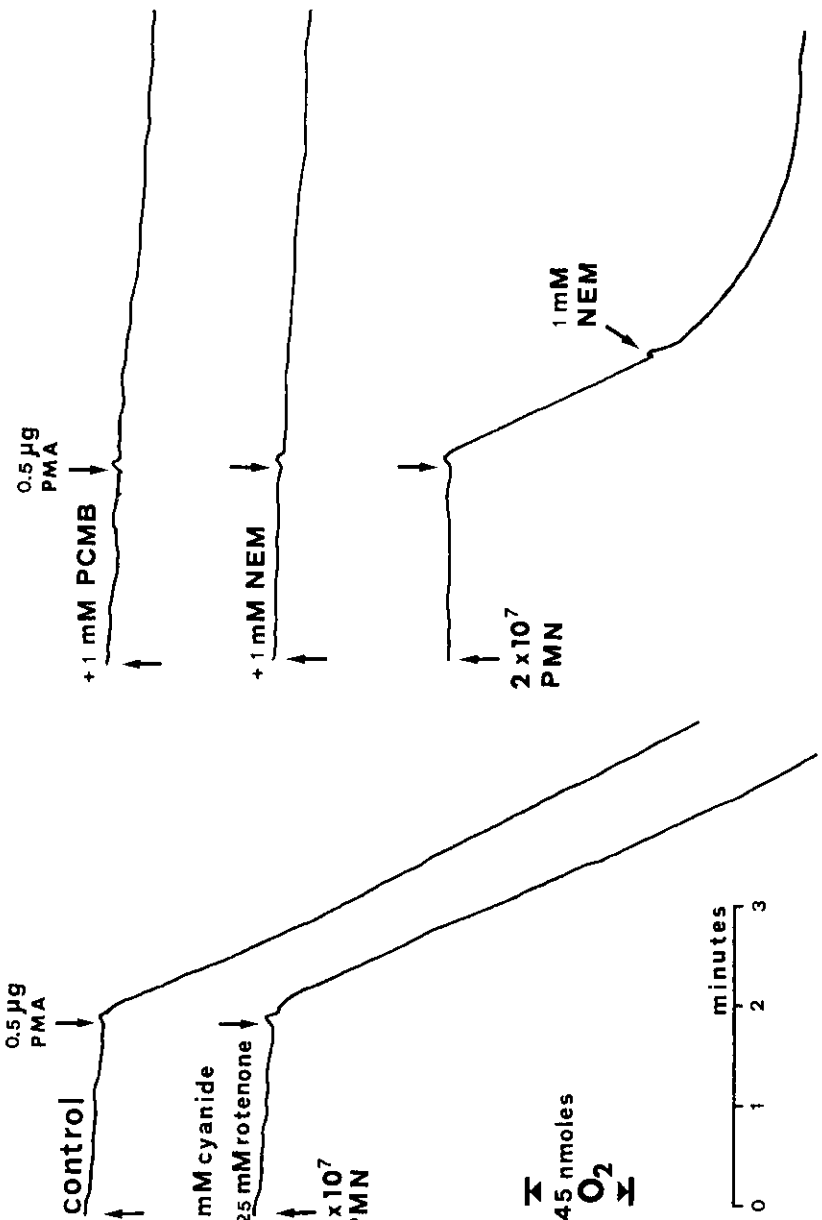


Fig. 2. Effect of inhibitors on the oxygen consumption by guinea pig PMN. Assay medium: 2 ml KRP containing 5 mM glucose and 0.5 mM $CaCl_2$.

by PMA, c) is inadequate to account for the net oxygen consumption by intact cells, d) in presence of NADH is activated by sulfhydrylic reagents whereas the respiration of the cells is inhibited.

2. The data presented here seems to us a strong confirmation of the results that we[4,6,10,11,24,35] and others[5,12,13,14,15] have published in the last twenty years. In other words we have shown that the O_2^- forming activity bound to some membranous structures of the granulocytes is involved in the respiratory burst. This enzyme fulfills the requirements listed above (table 2), and has more affinity for NADPH than for NADH. The comparison between the values of Km and the concentration of NADPH and NADH inside the cells (table 4) clearly indicates that the physiological substrate is NADPH. A more complete survey of the evidences in favour of the NADPH as substrate of the primary oxidase has been published elsewhere by us[6] and by others[5].

The primary oxidase in human PMN and guinea pig macrophages

We have investigated the activity of the oxidase present in supernatants and in cell free particles of resting and PMA stimulated human blood neutrophils, guinea pig peritoneal resident and casein elicited macrophages (data not shown). The results[24,25] clearly indicate that also in these cells the oxidase activity of supernatants both with NADH and NADPH is very low and does not change upon stimulation of intact cells with PMA. On the contrary the oxidase activity of cell free particles of PMA stimulated cells is greatly activated. In cell free particles of PMA-treated human granulocytes and of guinea pig peritoneal resident macrophages the degree of activation with NADPH and NADH, and the Km for NADH and for NADPH are similar to those of cell free particles of guinea pig granulocytes. On the contrary only the oxidase activity with NADPH is activated in cell free particles of PMA stimulated guinea pig peritoneal elicited macrophages.

The localization of the primary oxidase

In the last few years the number of indirect and direct evidence on the localization on the plasma membrane of the primary oxidase is increasing[26,27,28,29]. Data have been also published on intracellular localization of NADPH oxidase[30,31,32]. 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, on the methods of assay of the oxidase.

We have reinvestigated the matter[33] 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 (table 6) show that the distribution of the activated oxidase follows that of 5'-nucleotidase and acid pNPPase, enzymes marker of plasma membrane. These data confirm those obtained by Dewald et al.[29] on human granulocytes activated by PMA. We are now measuring the oxidase activity in the membrane of phagosomes isolated from phagocytosing guinea pig granulocytes and peritoneal elicited macrophages. Preliminary results, whose one example is reported in table 7, seems to indicate that during phagocytosis only the oxidase of the portion of the membrane that forms the phagocytic vacuole is activated. 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 enzyme is the more strategical one for the intravacuolar use of the oxygen radical and of hydrogen peroxide.

RELATIONSHIP BETWEEN THE ACTIVITY OF THE PRIMARY OXIDASE AND THE RESPIRATORY METABOLISM OF WHOLE 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 of whole cells. When we deal with this argument we are faced by a number of problems. The main are the mechanisms of the activity of the oxidase on one side, and the complexity of the reactions involved in the oxidative metabolism of phagocytes, that is in the O_2 balance of intact cells, on the other. We shall try to analyze these problems.

The reaction of NAD(P)H oxidation by cell free particles of leucocytes

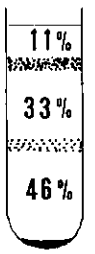
A. The NAD(P)H oxidase is measured in different conditions of pH and by using different procedures, the main being O_2 consumption, NAD(P)H disappearance, $NAD(P)^+$ formed, O_2^- production, H_2O_2 production. By comparing the activity of the oxidase expressed as O_2^- formation, O_2 consumption and disappearance of the substrate we have been able to show that more than one mechanism is operative

Table 6. Percent distribution of marker enzymes in the four fractions obtained by centrifugation on discontinuous sucrose gradient of postnuclear supernatants of guinea pig elicited peritoneal macrophages

	sucrose density (% w/w)				
Enzymes	20/34	34/40	40/45	PELLET	RECOVERIES
5'-nucleotidase	43.0 ± 13.0	15.6 ± 2.7	7.8 ± 2.2	9.2 ± 1.2	73.2 ± 14.0
Glucose-6 phosphatase	59.3 ± 18.0	46.5 ± 12.0	24.5 ± 7.5	5.0 ± 2.0	130.4 ± 35.0
NADPH-cyt.c reductase	27.0 ± 8.0	23.5 ± 4.0	13.8 ± 5.2	15.8 ± 1.0	76.2 ± 11.5
Succinic-dehydrogenase	13.3 ± 2.4	52.4 ± 18.5	15.8 ± 3.9	5.9 ± 2.8	85.5 ± 20.0
β-glucuronidase	6.8 ± 3.5	18.8 ± 4.2	22.8 ± 1.2	18.2 ± 2.1	62.0 ± 5.0
NADPH-oxidase	36.7 ± 5.3	24.0 ± 2.8	14.2 ± 5.3	12.1 ± 1.9	84.0 ± 7.5
Protein	20.6 ± 5.5	22.8 ± 3.4	17.6 ± 3.3	30.2 ± 6.2	84.0 ± 7.5

Percent distribution was calculated by taking 100,000g pellet of postnuclear supernatant as 100%. The average values of four experiments ± SEM are reported.

Table 7. Relative specific activities of marker enzymes and of NADPH oxidase in fractions obtained from guinea pig PMN that phagocytosed PMA-coated latex



	Acid pNPPase	5'nucleotidase	Myeloperoxidase	β-glucuronidase	NADPH oxidase
→ PHAGOSOMES	2.9	3.0	0.7	1.4	9.1
→ FRACTION 33/46	1.8	1.8	1.0	1.4	3.4
→ PELLET	1.5	1.1	0.8	1.1	1.5

in the oxidation of NAD(P)H and that these mechanisms vary by modifying the conditions of the assay[35]. Table 8 shows in fact that: 1) When measured as O_2 consumption or as NADPH disappearance, the enzymatic activity appears higher at pH 5.5 than at pH 7.0, particularly at 1 mM substrate. 2) In all the conditions the ratio be-

Table 8. O_2 consumption, NADPH oxidation and O_2^- production by cell free particles from phagocytosing guinea pig PMN

	NADPH (mM)	O_2 consumed	NADPH oxidized	O_2^- produced
pH 5.5	1.0	72.7 ± 9.7	84.3 ± 6.4	9.4 ± 2.0
" "	0.15	22.9 ± 1.2	23.6 ± 0.5	7.6 ± 1.7
pH 7.0	1.0	42.0 ± 4.0	45.5 ± 4.0	22.1 ± 4.5
" "	0.15	16.0 ± 2.9	19.7 ± 5.4	12.4 ± 3.4

The data are means of 4 experiments ± SEM. Values are nmoles/2.5 min/mg protein.

tween O_2 consumed and NADPH oxidized is near 1.0. 3) when assayed as O_2^- generation the oxidase activity is higher at neutral than at acid pH, at both NADPH concentrations. In any case it is always lower than when measured as NADPH oxidized or as O_2 consumed. The amount of O_2^- produced versus NADPH oxidized is much less than that theoretically expected that is 2 versus 1. Similar results have been obtained by using NADH as substrate.

The indications that in these conditions more than one mechanism is responsible for NAD(P) H oxidation have been verified by studying the effect of superoxide dismutase (SOD) and of cytochrome c (cyt. c) on the O_2 consumption and on NAD(P)H oxidation. The rationale of this approach is based on their ability to remove O_2^-, SOD by catalyzing the dismutation and cyt. c by oxidizing the superoxide to molecular oxygen. Table 9 reports the results of four experiments at pH 5.5 and 1 mM NADPH. It can be seen that the addition of SOD and of cyt. c causes a strong decrease in O_2 consumption and in NADPH oxidation. This means that O_2^- produced is subsequently involved in other reactions that oxidize NAD(P)H and consume oxygen and that the these reactions (chain reaction) are hindered when scavenger of O_2^- (in this case cyt. c) are present. By applying appropriate calculations to the data of table 9, at least three components of the NADPH oxidizing activity can be identified. 1. The first corresponds to the SOD inhibitable cyt. c reduction and represents the O_2^- forming activity, that is the reaction catalyzing the "univalent reduction of O_2". 2. The second corresponds to the amount of O_2 consumption that remains after the addition of cyt. c. This aliquot is referred as "residual O_2 consumed" and might represent either a divalent reduction of O_2 with direct formation of H_2O_2, or a formation of O_2^- in a space not accessible to cyt. c. 3. The third component is a "chain reaction" initiated by O_2^- and corresponds to the aliquot of O_2 consumption and of NADPH oxidation inhibited by SOD. It can be seen that at pH 5.5 with 1 mM NADPH most of the activity is contributed by the chain reaction.

Table 10 and table 11 report the data showing that the contributions of each of the three components vary depending on pH and on NADPH and NADH concentrations.

The rate of the "univalent reduction of O_2" increases by increasing the pH and decreases by lowering the substrate concentration. The so-called "residual O_2 consumption" is not significantly affec-

Table 9. Components of the NADPH-oxidizing activity of particles from phagocytosing polymorphonuclear leucocytes calculated on the basis of the effect of superoxide

Table 9. Components of the NADPH-oxidizing activity of particles from phagocytosing polymorphonuclear leucocytes calculated on the basis of the effect of superoxide dismutase and of cytochrome c.

Additions	A			B	C	
	O_2 consumed	NADPH oxidized	cyto-chrome c reduced	Calculations	Results	% of total
1. None	72.7±9.7	84.3±6.4		Total activity (as O_2 or NADPH) = line 1	O_2 = 72.7 NADPH = 84.3	100 100
2. Superoxide dismutase	14.1±1.9	14.8±3.9		Chain reaction (as O_2 or NADPH) = line 1 − line 2	O_2 = 58.6 NADPH = 69.5	81 82
3. Cytochrome c	9.2±2.6	24.7±7.8	22.6±2.2	Residual O_2 consumption (as O_2 consumed) = line 3	O_2 = 9.2	13
4. Cytochrome c + superoxide dismutase	13.9±1.9	28.2±7.2	13.2±1.2	Univalent reduction (as O_2^- detected) =cyt c reduced on line 3 − line 4 (as O_2 consumed) = $1/2$ O_2^- detected	O_2^- = 9.4 O_2 = 4.7	6

The values are given as nmol/2.5 min/mg protein. The mean of four experiments ± S.E.M. are reported.

Table 10. Components of the NADPH-oxidizing activity of particles from phagocytosing guinea pig PMN at different pH and substrate concentration

ASSAY CONDITIONS		ACTIVITIES ($nmoles\ O_2$/2.5 min/mg protein)				% OF TOTAL ACTIVITY		
pH	NADPH mM	Total activity	Univalent reduction	Residual oxygen consumption	Chain reaction	Univalent	Residual	Chain
5.5	1.0	72.7	4.7	9.2	58.6	6.5	12.6	80.6
7.0	1.0	42.0	11.0	11.8	22.1	26.3	28.1	52.6
5.5	0.15	22.9	3.8	7.2	10.1	16.6	31.4	44.1
7.0	0.15	16.0	6.2	6.9	0.7	38.7	43.1	4.4

O_2 consumption by the corresponding intact PMN during 2.5 min of phagocytosis was 99.0 ± 12.1 (SEM) nmoles O_2.

ted by pH changes and the concentration of the substrate. The "chain reaction" decreases by increasing the pH and by decreasing the substrate concentration. At pH 7.0 with 0.15 mM NAD(P)H the contribution of the chain reaction is 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[11,17,18] and a pH optimum of 7.0 when the activity has been measured as O_2^- generation[13,24,38]. From the data presented above it is clear that at acid 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 informations.

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.

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 favour 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 in which the rate of the chain reaction is high. In some cases the use of manganese, which is known to greatly potentiate the chain reaction[36,37], might be very useful.

B. On the basis of the overall reaction of the oxidase ($2O_2 + NAD(P)H \longrightarrow 2O_2^- + NAD(P)^+$), 2 nanomoles of O_2^- should be produced for one nanomole of NAD(P)H oxidized. In the experimental conditions employed in the experiments reported here we have found 0.4 - 0.8 nmoles of O_2^- produced for 1 nanomole of NAD(P)H oxidized (table 12).

Two hypotheses can be advanced to explain the defective stoichiometry: 1. There are two enzymatic pathways of oxygen reduction: an "univalent" with formation of O_2^- and a "divalent" with direct for

Table 11. Components of the NADH-oxidizing activity of particles from phagocytosing guinea pig PMN at different pH and substrate concentration

ASSAY CONDITIONS		ACTIVITIES (nmoles O_2/2.5 min/mg protein)				% OF TOTAL ACTIVITY		
pH	NADH (mM)	Total activity	Univalent reduction	Residual oxygen consumption	Chain reaction	Univalent	Residual	Chain
5.5	1.0	38.7	4.7	13.3	26.2	12.3	34.4	67.8
7.0	1.0	37.0	4.6	12.9	19.3	12.5	35.0	52.1
5.5	0.15	2.1	0.14	1.8	0.32	6.7	85.7	15.3
7.0	0.15	5.2	0.68	3.6	0.32	13.1	68.7	6.2

See note of table 10.

Table 12. Stoichiometric relationships between NAD(P)H oxidation and O_2^- production by cell free particles from PMA-activated guinea pig PMN

	NAD(P)H oxidation	O_2^- production
THEORETICAL	1	2
EXPERIMENTAL		
with NADH	1	0.4 - 0.8
with NADPH	1	0.5 - 0.8

Assay conditions: NAD(P)H 0.15 mM, pH 7.0.

mation of H_2O_2; 2. Only the univalent pathway is operative but part of O_2^- is formed in a site not accessible to cyt.c.

We shall present evidences against the first hypothesis at least as far as the oxidation of NADPH is concerned. The results are reported in fig. 3 show in fact that the addition of deoxycholate (DOC) increases 2-3 folds the production of O_2^- when cell free particles from activated PMN oxidize NADPH. The detergent increases only slightly the amount of NADPH oxidized. Therefore, in these conditions, the value of ratio O_2^- produced versus NADPH oxidized approximate the theoretical one. These results indicate that all the oxidation of NADPH by cell free particles involves mainly an univalent reduction of O_2.

Thus, the NADPH oxidase activity should be assayed at pH 7.0, in presence of 0.15 mM substrate and in presence of 0.05% DOC. Otherwise the values in absence of DOC should be corrected by a factor calculated on the basis of the insufficient recovery of O_2^- with respect to the theoretical. In our case the factor varies between 5.0 and 2.5.

THE OXYGEN BALANCE OF WHOLE CELLS

The discussion of this argument can be divided in two parts. The first concerns whether or not all the extrarespiration of intact cells involves a univalent reduction of oxygen, as we have

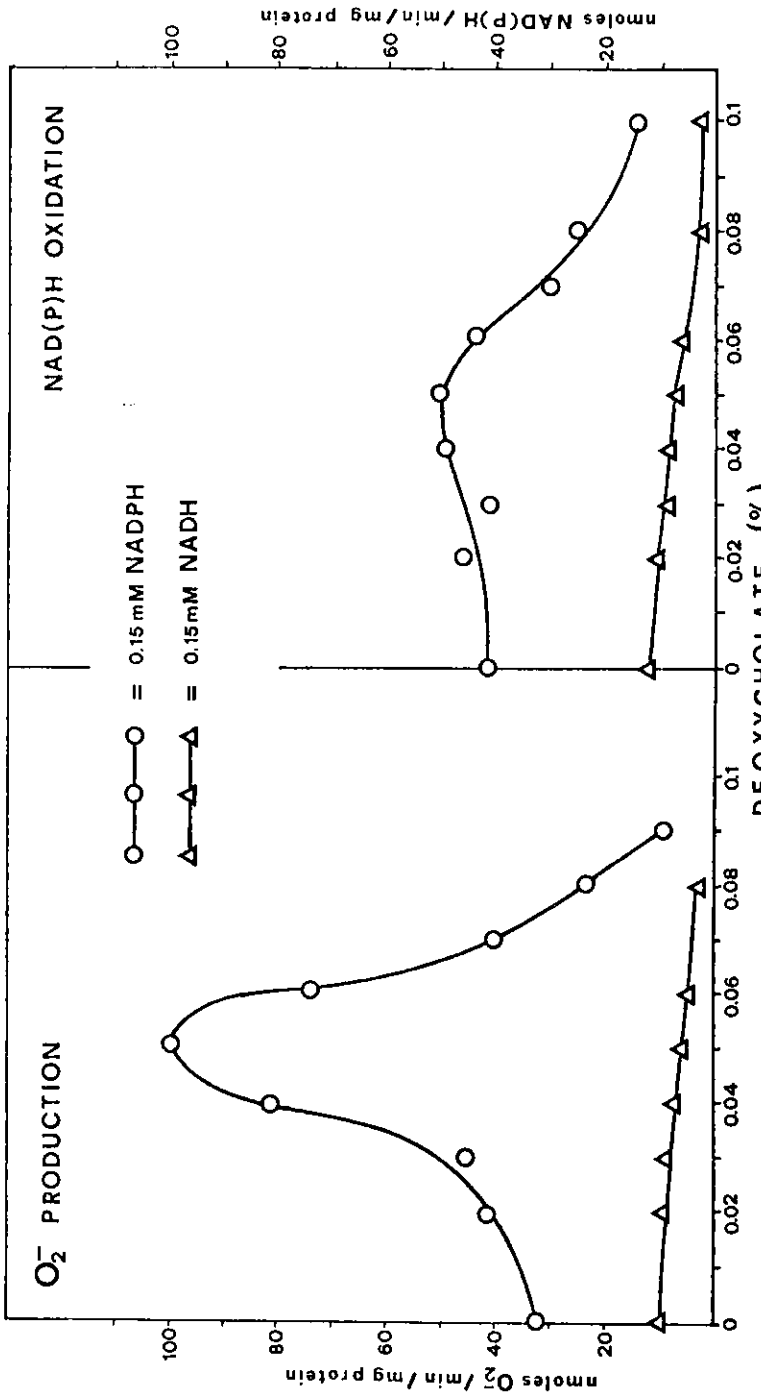


Fig. 3. Effect of deoxycholate on the NAD(P)H oxidation by cell free particles from PMA-activated PMN. O_2^- production was measured as SOD-inhibitable cytochrome c reduction NAD(P)H oxidation was measured following the decrease in absorbance at 340 mM.

shown to occur in cell free particles oxidizing NADPH at pH 7.0 and in presence of DOC. The second regards the complexity of the reactions underlying the net oxygen consumption of intact cells during the burst.

A. We shall present now data showing that, at least in appropriate conditions, all oxygen reduced during the burst passes through the formation of O_2^-.

It is known that during the respiratory burst only the O_2^- released from the cells is measurable. This measurement is based on the ability of O_2^- to reduce a suitable electron acceptor, such as cyt.c, that does not penetrate inside the cell (O_2^- + cyt.c ox. $\longrightarrow$ O_2 + cyt.c red.). Since in this reaction one molecule of O_2 is liberated for everyone molecule of O_2^- that reacts with cyt.c, the addition of this electron acceptor to a leucocyte suspension, in which O_2 is being consumed with formation of O_2^-, should lead to a decrease in the net oxygen consumption and this decrease should correspond to the amount of O_2^- that is released outside the cell. It has been shown in many laboratories that during the early stages of the respiratory burst induced by different stimuli the O_2^- release is only an aliquot of the O_2 consumed and, as a consequence, the inhibition of the respiration by cyt.c varies from 20 to 30%[39,40-42]. Many explanations have been given of this low recovery of O_2^- during the burst: the existence of more than one pathway for oxygen reduction; the production of O_2^- in a site not accessible; the discharge of O_2^- in the endocytic vacuoles where the amount of oxidized cyt.c becomes rapidly insufficient; the discharge of O_2^- inside the cell.

We have found that in some experimental conditions the addition of cyt.c completely inhibits the activation of respiration in termsof oxygen consumption. The fig. 4 shows the recording of the respiration of leucocytes stimulated by the ionophore A23187 in absence and in presence of cyt.c, and the data of table 13 reports the results of 8 experiments. That the inhibitory effect of cyt.c is due to the oxidation of O_2^- is demonstrated by the fact that the inhibition by cyt.c is removed by the addition of SOD that prevents O_2^- from reacting with cyt.c.

Results similar to those presented in fig. 4 have been obtained in our laboratory by using guinea pig PMN and Con A as stimulatory agents in presence of CB (data not shown). These data seem to us a strong evidence that all O_2 consumption passes through an univalent

Table 13. Effect of cytochrome c on the respiratory burst induced by A23187

	1	2	3
ADDITIONS	A23187	A23187 + cyt c	A23187 + cyt c + SOD
Extra O_2 consumed[+]	30.5 ± 4.7	3.0 ± 0.8	34.0 ± 5.5
Extra O_2^- detected[+]		77.4 ± 17.0	
O_2 taken off by cytochrome c	A) 1 minus 2 = 27.5		B) 3 minus 2 = 31.0
Ratio $\frac{O_2^- \text{ detected}}{O_2 \text{ taken off}}$	A) $\frac{77.4}{27.5} = 2.8$		B) $\frac{77.4}{31.0} = 2.5$

The data are means ± SEM of 5 experiments. [+]The values are given as nmoles/1.5 x 10^7 PMN/4 min.

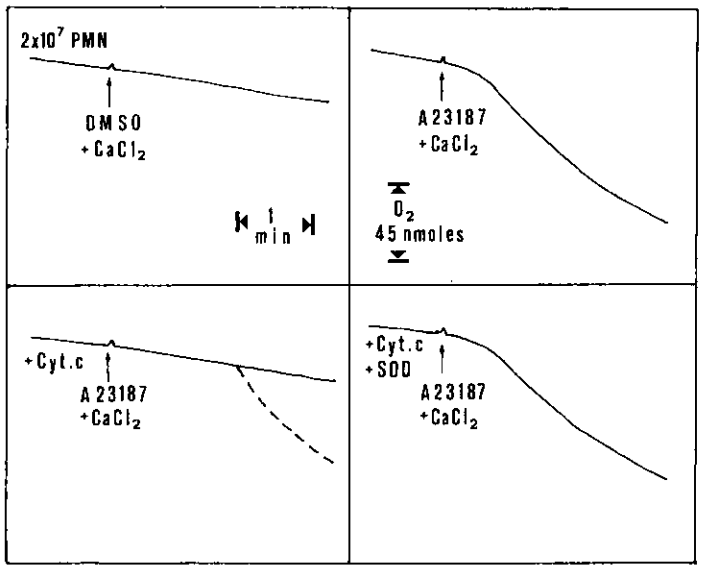


Fig. 4. Effect of cytochrome c on the oxygen consumption induced by the calcium ionophore A23187.

reduction and that all O_2^- formed is discharged outside the plasma membrane, at least in the early stages of the burst.

B. The complexity of the oxygen balance in whole cells has been extensively discussed by us[39] and by others[44]. The measurement of the extrarespiration in whole cells refers to net O_2 uptake, which is the result of several reactions: the reduction of O_2 catalyzed by the oxidase on one side and reaction of dismutation of O_2^- and of degradation of H_2O_2 on the other (table 14). Since in the reaction of dismutation and in the catalatic degradation of H_2O_2 part of O_2 is recovered, the amount of oxygen univalently reduced by the oxidase is greater than that measured as net O_2 consumed. The ratio between the extent of the reaction 1 and the net O_2 consumed varies depending on the extent of the O_2 given back, that is mainly on the mechanisms of H_2O_2 degradation. The reaction 6 is very low as it is shown by the very low amount of hydroxyl radical measured during the burst[45].

Table 14. Reactions involved in oxygen consumption in whole cells

1.	$2O_2 + NADPH$	$\xrightarrow{\text{oxidase}}$	$2O_2^- + NADP^+ + H^+$
2.	$2O_2^- + 2H^+$	$\xrightarrow{\text{dismutation}}$	$H_2O_2 + O_2$
3.	H_2O_2	$\xrightarrow{\text{catalase}}$	$H_2O + 0.5\ O_2$
4.	$H_2O_2 + RH_2$	$\xrightarrow{\text{peroxidase}}$	$2H_2O + O_2$
5.	$O_2^- + Xoxid.$	$\longrightarrow$	$O_2 + Xred.$
6.	$O_2^- + H_2O_2$	$\longrightarrow$	$OH^\cdot + OH^- + O_2$

1 + 2 + 3 = $2O_2^-$ formed, $\frac{1}{2}$ O_2 consumed = Ratio 4 : 1

1 + 2 + 4 = $2O_2$ formed, 1 O_2 consumed = Ratio 2 : 1.

The data of the table 13 allow us to derive some indications concerning the complexity of the reactions involved in the net oxygen consumption during the burst. We can infact calculate how much O_2^- is formed versus the net O_2 consumed in these particular experimental conditions. Furthermore, with this information we can understand whether or not in intact cell, beside the reactions reported in the table 14, some sort of chain reaction is operative.

Before to enter into the details of these problems it is worthy to point out two facts. The data of table 13 show that:

1. the ratio between the nanomoles of O_2^- oxidized by cyt.c and the nanomoles of O_2 not consumed due to the presence of cyt.c is 2.5 - 2.8. This means that in absence of this scavenger for one nanomole of O_2^- produced and released, only 0.4 nanomoles of O_2 are actually consumed.
2. During the respiratory burst, H_2O_2 that accumulates is usually much less than that expected, since the phagocytic cells are equipped with very efficient mechanisms for its degradation. The accumulation of the peroxide increases only when the main mechanism of degradation is inhibited by the addition of NaN_3 or KCN

or by the presence of a system that traps the H_2O_2 released as it is formed[39,43]. The calculations that we are presenting are valid whether, in absence of inhibitor and of scavenger or of trapping systems, all the intermediates of O_2 reduction are degraded. In the experimental conditions employed in this and in other circumstances[39,43], this has been verified: during the respiratory burst "in vitro" O_2^- and H_2O_2 originally discharged outside the cell or in the phagocytic vacuole are subsequently degraded by intracellular mechanisms since they equilibrate with the intracellular space.

We shall discuss now various hypotheses concerning the oxygen balance in whole cells during the burst. The hypotheses consist in different combinations of the reactions of table 14 and of chain reactions originated by O_2^- or by H_2O_2.

HYPOTHESIS n. 1. The oxidase forms O_2^-; O_2^- originates a chain reaction followed by a dismutation and by the degradation of H_2O_2 through catalatic and/or peroxidatic mechanisms

1. $NADPH + H^+ + 2O_2 \longrightarrow NADP^+ + 2O_2^- + 2H^+$

2. chain reaction:
   $2O_2^- + 2RH_2 \xrightarrow{+\,2H^+} 2RH^\cdot + 2H_2O_2$
   $2RH^\cdot + 2O_2 \longrightarrow 2R^+ + 2O_2^- + 2H^+$

3. $2O_2^- + 2H^+ \xrightarrow{\text{dismutation}} H_2O_2 + O_2$

4. $3H_2O_2 \xrightarrow{\text{catalase}} 3H_2O + 1.5\ O_2$

5. $3H_2O_2 + 3RH_2 \xrightarrow{\text{peroxidase}} 6H_2O + 3R$

In the case that these reactions occur in different combinations in whole cells (1+2+3+4; 1+2+3+5; 1+2+3 and 4 and 5 in different proportions), between 3.0 and 1.5 nanomoles of O_2 are actually consumed for 2 nanomoles of O_2^- initially generated by the oxidase or at the end of the reaction 1+2, with ratios O_2^-/O_2 between 0.66 and 1.33. These sequences do not account for the experimental ratio 2.5 - 2.8 (table 13). By increasing the lenght of the chain reaction the net O_2 consumed for the same initial amount of O_2^- generated would be higher. Thus, these sequences of reactions, as indicated in this hypothesis, do not occur in whole cells.

The experimental ratio O_2^-/O_2 does not change appreciably in presence of SOD (data not shown). This is a further evidence that a chain reaction, originated by O_2^- released, does not take place.

HYPOTHESIS n. 2. The oxidase forms O_2^-; O_2^- dismutates to H_2O_2; H_2O_2 initiates a chain reaction followed by the dismutation and by the degradation of H_2O_2 through catalatic or peroxidatic mechanisms

1. $NADPH + H^+ + 2O_2 \xrightarrow{\text{oxidase}} NADP^+ + 2O_2^- + 2H^+$
2. $2O_2^- + 2H^+ \xrightarrow{\text{dismutation}} H_2O_2 + O_2$
3. $2RH_2^{\cdot} + H_2O_2 \longrightarrow 2RH^{\cdot} + 2H_2O$; $2RH^{\cdot} + 2O_2 \longrightarrow 2R^+ + 2O_2^- + 2H^+$ (chain reaction)
4. $2O_2^- + 2H^+ \xrightarrow{\text{dismutation}} H_2O_2 + O_2$
5. $H_2O_2 \xrightarrow{\text{catalase}} H_2O + 0.5\ O_2$
6. $H_2O_2 + RH_2 \xrightarrow{\text{peroxidase}} 2H_2O + R$

In the case that these reactions occur in different combinations in whole cells (1+2+3+4+5; 1+2+3+4+6; 1+2+3+4 and 5 and 6 in different proportions), between 2.0 and 1.5 nmoles of O_2 are consumed for 2 nmoles of O_2^- initially generated by the oxidase or at the end of reactions 1+2+3, with a ratio O_2^-/O_2 between 1.0 and 1.33. These sequences do not account for the experimental ratio 2.5 - 2.8 (table 13) and therefore do not occur in whole cells.

When the respiratory burst of guinea pig granulocytes takes place in presence of azide, the ratio O_2 consumed versus H_2O_2 accumulated is near to the unity[39]. If a chain reaction initiated by H_2O_2 and independent of MPO is operative, the recovery of the peroxide should be much less than the oxygen consumed. Furthermore, assuming that the chain reaction is operative and mediated by MPO, the inhibition of this enzyme by the presence of NaN_3 or KCN should decrease the O_2 consumption. It is widely known that this is not the case. These are further evidences that a chain reaction initiated by H_2O_2 is not involved in the respiratory burst.

HYPOTHESIS n. 3. The oxidase forms O_2^-; O_2^- initiates a chain reaction with oxygen consumption and H_2O_2 formation; also H_2O_2 initiates a chain reaction followed by dismutation and by degradation of H_2O_2 through catalatic or peroxidatic mechanisms

1. $NADPH + H^+ + 2O_2 \longrightarrow NADP^+ + 2O_2^- + 2H^+$

2. $2O_2^- + 2RH_2 \xrightarrow{+\ 2H^+} 2RH^\cdot + 2H_2O_2$
   $2RH^\cdot + 2O_2 \longrightarrow 2R^+ + 2O_2^- + 2H^+$
   (chain reaction)

3. $4RH_2 + 2H_2O_2 \longrightarrow 4RH^\cdot + 4H_2O$
   $4RH^\cdot + 4O_2 \longrightarrow 4R^+ + 4O_2^- + 4H^+$
   (chain reaction)

4. $6O_2^- + 6H^+ \xrightarrow{\text{dismutation}} 3H_2O_2 + 3O_2$

5. $3H_2O_2 \xrightarrow{\text{catalase}} 3H_2O + 1.5\ O_2$

6. $3H_2O_2 + 3RH_2 \xrightarrow{\text{peroxidase}} 6H_2O + 3R$

In the case that these reactions occur in different combinations in whole cells (1+2+3+4+5; 1+2+3+4+6; 1+2+3+4 and 5 and 6 in different proportions) between 5.0 and 3.5 nmoles of O_2 are consumed for 2 nmoles of O_2^- initially generated by the oxidase (with a ratio O_2^-/O_2 between 0.4 and 0.57) or for 6 nanomole of O_2^- generated by the reactions 1+2+3 (with a ratio O_2^-/O_2 between 1.2 and 1.7). These sequences do not account for the experimental ratio 2.5 - 2.8 and therefore do not occur in whole cells.

HYPOTHESIS n. 4. The oxidase forms O_2^-; O_2^- dismutates to H_2O_2; H_2O_2 initiates a chain reaction followed by a chain reaction initiated by O_2^-; then O_2^- dismutates and H_2O_2 is degraded

1. $NADPH + H^+ + 2O_2 \xrightarrow{\text{oxidase}} NADP^+ + 2O_2^- + 2H^+$

2. $2O_2^- + 2H^+ \xrightarrow{\text{dismutation}} H_2O_2 + O_2$

3. $2RH_2 + H_2O_2 \longrightarrow 2RH^\cdot + 2H_2O$
   $2RH^\cdot + 2O_2 \longrightarrow 2R^+ + 2O_2^- + 2H^+$
   (chain reaction)

4. $2O_2^- + 2RH_2 \xrightarrow{+\ 2H^+} 2RH^{\cdot} + 2H_2O_2$ — chain reaction
   $2RH^{\cdot} + 2O_2 \longrightarrow 2R^+ + 2O_2^- + 2H^+$ — chain reaction

5. $2O_2^- + 2H^+ \xrightarrow{\text{dismutation}} H_2O_2 + O_2$

6. $3H_2O_2 \xrightarrow{\text{catalase}} 3H_2O + 1.5\ O_2$

7. $3H_2O_2 + 3RH_2 \xrightarrow{\text{peroxidase}} 6H_2O + 3R$

In the case that these reactions occur in different combinations in whole cells (1+2+3+4+5+6; 1+2+3+4+5+7; 1+2+3+4+5 and 6 and 7 in different proportions), between 4.0 and 2.5 nmoles of O_2 are consumed for 2 nmoles of O_2^- initially generated by the oxidase or at the end of the reactions 1+2+3+4 with a ratio O_2^-/O_2 0.5 - 0.8. These sequences do not account for the experimental ratio 2.5 - 2.8 and therefore do not occur in whole cells.

HYPOTHESIS n. 5. The oxidase forms O_2^-; O_2^- dismutates to H_2O_2; H_2O_2 is degraded by catalatic and/or by peroxidatic mechanism

1. $NADPH + H^+ + 2O_2 \xrightarrow{\text{oxidase}} NADP^+ + 2O_2^- + 2H^+$

2. $2O_2^- + 2H^+ \xrightarrow{\text{dismutation}} H_2O_2 + O_2$

3. $H_2O_2 \xrightarrow{\text{catalase}} H_2O + 0.5\ O_2$

4. $H_2O_2 + RH_2 \xrightarrow{\text{peroxidase}} 2H_2O + R$

In the case that these reactions occur in different combinations in whole cells (1+2+3; 1+2+4; 1+2 and 3 and 4 in different proportions, 1.0 or 0.5 nmoles of O_2 are consumed for 2 nmoles of O_2^- initially generated by the oxidase with a ratio O_2^-/O_2 between 2.0 - 4.0. This sequence could account for the experimental ratio 2.5 - 2.8.

Thus, on the basis of the data and of the calculation presented, one can conclude that the reactions involved in the oxygen metabolism are mainly those indicated in the last hypothesis. We have excluded from the calculation the reaction of oxidation of the O_2^- released ($O_2^- + X\text{-oxid.} \longrightarrow O_2 + X\text{-red.}$). Assuming that this reaction takes place, the amount of O_2^- oxidized must be rather low. This is

shown by the finding (not shown here) that when the calculation is made by using the values of the respiration in presence of SOD the ratio O_2^-/O_2 does not appreciably vary with respect that calculated using the values of the respiration in absence of SOD (table 13). We have also excluded the reaction between O_2^- and H_2O_2 with formation of $OH^\cdot$. Infact the data published by others[45] show that the production of this radical, probably through the Haber-Weiss reaction ($O_2^- + H_2O_2 \longrightarrow OH^\cdot + OH^- + O_2$), is very low with respect to that of O_2^- and H_2O_2.

ADEQUACY OF THE OXIDASE TO ACCOUNT FOR THE RESPIRATORY BURST

On the basis 1) of the reaction involved in oxygen balance in whole cells and 2) of the imperfect stoichiometry between O_2^- measured and NADPH oxidized (or O_2 consumed) when the oxidase activity of particles is measured, it is clear that the estimation of the adequacy of the primary oxidase runs the risk of being rather rough (or even wrong).

The data of table 15 show the comparison between the respiratory burst in intact guinea pig PMN and the NADPH oxidase activity of cell free particles. The values of column "experimental" clearly demonstrate that the activity of the oxidase measured as O_2^- formation is inadequate to account for the respiratory burst of intact cells. We have shown that this is partially due to an imperfect capture of O_2^- formed during the oxidation of NAD(P)H by cell free particles and that the measurement of the oxidase in presence of DOC increases the O_2^- measured with a stoichiometry O_2^-/NADPH oxidized near to the theoretical. The data of column "corrected" represent the values of O_2^- formation in intact cells and of O_2^- formation by the oxidase of cell free particles, obtained after homogenization and centrifugation of homogenates of the corresponding cells, corrected on the basis of the defective stoichiometry O_2^-/NADPH shown in table 12.

In spite of these corrections the activity of the oxidase, both in presence of NADPH and of NADH, remains inadequate to account for the rate of the extrarespiration of intact cells. This inadequacy could be explained in different ways. One is that, besides NADPH oxidase, other enzymes or other respiratory systems not involving reduced nicotinamide adenine nucleotides are responsible for the burst. Another reason is that we underestimate the oxidase activity because the enzyme is deactivated or inactivated during the procedures of cell disruption and homogenization. Thus, if the oxidase is mea-

Table 15. Comparison between the respiratory burst of intact cells by PMA (expressed as O_2 uptake and O_2^- release) and the activity of NADPH oxidase of cell free particles

		Experimental	Corrected
Whole cells	Net O_2 uptake[x]	4.7 ± 1.35	12.2 (a)
	O_2^- production[x]	6.5 ± 1.05	
NADPH oxidase of cell free particles as O_2^-[o]		0.766 ± 0.140	3.8 (b)
			1.9 (c)

x = nmoles/min/10^6 cells

o = nmoles O_2^-/min/cell free particles from 10^6 cells

a. Experimental (4.7) x 2.6, that is the ratio O_2^- formed/O_2 consumed (see reference 39). 12.2 are nmoles of O_2^- actually formed for a net O_2 consumption of 4.7 nmoles.

b. Experimental (0.766) x 5 (stoichiometry O_2^-/NADPH 0.4 instead of 2.0).

c. Experimental (0.766) x 2.5 (stoichiometry O_2^-/NADPH 0.8 instead of 2.0).

ured soon after the activation has taken place and by using a procedure that allows a 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 can be fully revealed. This expectation has been verified by using the experimental model described in fig. 5.

The respiratory activity of intact cells stimulated by PMA was measured as O_2 consumption with Clark oxygen electrode. During the linear part of the respiration, the cells were lysed and their respiratory activity was abolished by adding deoxycholate (DOC). At this point NADH 1 mM or NADPH 0.15 mM were added. The addition of NADPH induced a instantaneous resumption of O_2 consumption at a rate similar or even higher than that of the stimulated cells. After

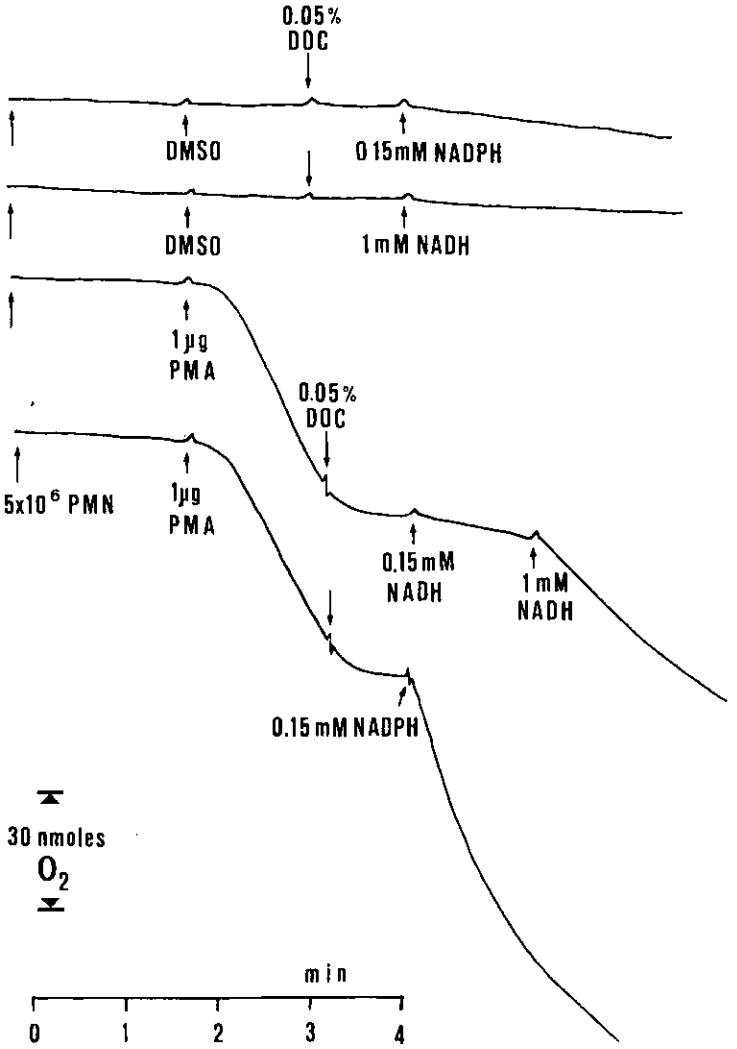


Fig. 5. Polarographyc traces of oxygen consumption by guinea pig PMN and by NAD(P)H oxidase after addition of deoxycholate (DOC). Assay medium: 2 ml KRP containing 5 mM glucose, 0.5 mM $CaCl_2$ and 2 mM NaN_3.

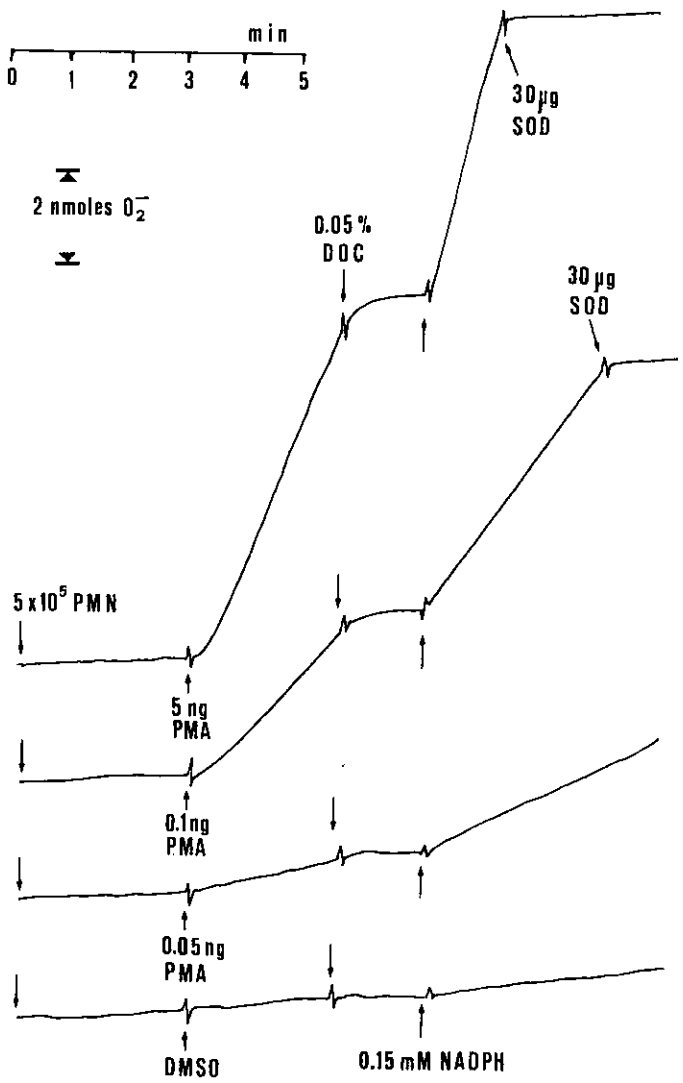


Fig. 6. Relation between the O_2^- production by guinea pig PMN stimulated with PMA and the corresponding NADPH oxidase activity after the addition of deoxycholate (DOC). Assay medium: 1 ml KRP containing 5 mM glucose, 0.5 mM $CaCl_2$, 2 mM NaN_3, 100 µM cytochrome c. 37°C.

the addition of NADH the O_2 consumption also resumed but at a rate much lower with respect that of the stimulated cells. DOC has not stimulatory activity on NAD(P)H oxidase activity of resting cells.

Similar results have been obtained by measuring as O_2^- formation both the respiratory burst of intact cells before the addition of DOC and the oxidase activity after the addition of DOC and NAD(P)H.

The validity of this experimental approach, that at the same time avoids the inactivation of the oxidase and allows a good substrate-enzyme interaction and a physiological fate of the intermediates, is also shown by the data presented in fig. 6. It can be seen that the rate of the 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

1. The so called "soluble" oxidase(s) are not involved in the respiratory burst of guinea pig and human granulocytes and of guinea pig peritoneal resident and elicited macrophages.

2. The activation of the oxidation of NADPH by a membrane bound NAD(P)H oxidase is the main mechanism responsible for the activation of the respiration of phagocytes.

3. The oxidase is inactive in resting cells and the activated form works on the plasma membrane.

4. More than one mechanism is operative in the oxidation of NAD(P)H by cell free particles in vitro. These mechanisms vary in relation to the conditions of assay (pH and concentration of substrate).

5. Under optimal conditions in vitro the enzymatic oxidation of NADPH practically involves the univalent pathway of oxygen reduction with stoichiometry of two nanomoles of O_2^- formed for one nanomole of NADPH oxidized.

6. Also in intact cells all O_2 is first univalently reduced to O_2^- and then discharged outside the cell or in the phagocytic vacuoles.

7. The main reactions involved in the O_2 balance in intact cells are the univalent reduction of O_2, the dismutation of O_2^- to H_2O_2 and the degradation of the peroxide through catalatic and peroxidatic mechanisms.

8. The total oxygen univalently reduced by the activated oxidase is 2-4 folds the net oxygen consumed by the cells, depending on the mechanism of H_2O_2 degradation.

9. All the rate of extrarespiration is accounted for by the rate of oxidation of physiological concentration of NADPH by the membrane-bound enzyme. This adequacy can be observed only under appropriate experimental conditions, because the high activity of the oxidase is not a permanent state.

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77).

DISCUSSION
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Dr. Minakami:

I would like to congratulate Prof. Rossi for his beautiful accomplishments. Our data, which will be presented tomorrow morning, agree quite well with your statement.

Dr. Rossi:

Thank you.

Dr. De Chatelet:

First I think I'll retract my talk scheduled for tomorrow. The stoichiometry demonstrated is surely outstanding. I still have an unpopular stance in believing the oxidase is present in a granule of the cell. I think there are several other interpretations of our data.

a) The enzyme may be located in a granule in the resting cell and translocated to the membrane by phagocytosis.
b) You are actually isolating phagosomes rather than plasma membrane. It is possible that there is very rapid degranulation into the phagocytic vacuole.

Would you care to comment on these possibilities?

Dr. Rossi:

Yes, it is possible that the presence of the enzyme in phagosomes is due to the translocation from the granules to the membrane of the vacuole or inside the vacuole. However, in this case, MPO or other enzymes bound to granules also should concentrate in the phagosomes. As you have seen from the results I have presented, this is not the case.

Furthermore, I say to you that we found that in resting PMN the oxidase is located in the granule fraction. However, it is likely that this activity corresponds to the oxidase activity of MPO. As you know, my old data, published many years ago, supported the hypothesis that the oxidase was located in the azurophilic granules. Now I am convinced that the enzyme is in the plasma membrane. Obviously we need further work in order to settle this matter.

Dr. Kakinuma:

In our recent study, we measured the rate of H_2O_2 generation by the particulate fraction isolated from phagocytosing polymorphonuclear leukocytes in the presence of NADPH by means of a spectrophotometric assay of cytochrome c peroxidase-H_2O_2 complex. We found that one mole of NADPH produces exactly one mole of H_2O_2 in the NADPH oxidation reaction by this fraction. Therefore, I support, one of your proposed several hypothetical reactions (hypothesis No. 5):

$$NADPH + H^+ + 2\ O_2 \xrightarrow{\text{oxidase}} NADP + 2\ O_2^- + 2\ H^+ \qquad (1)$$

$$2\ O_2^- + 2\ H^+ \xrightarrow{\text{dismutation}} H_2O_2 + O_2 \qquad (2)$$