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Enzymatic Basis of the Respiratory Burst of Guinea Pig Resident Peritoneal Macrophages

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The enzymatic basis of the respiratory burst induced by phorbol myristate acetate in guinea pig resident peritoneal macrophages was studied. The following evidence suggests that a membrane-bound oxidase that preferentially uses NADPH was responsible for the activation of the oxidative metabolism: a) The supernatant of homogenates of resting macrophages oxidized NADH and NADPH with the subsequent formation of O_2^-. The activity with both the substrates was very low and did not change in the supernatant of homogenates of activated cells. b) The 100,000 x *g* pellet of the homogenates of resting macrophages also oxidized both NADH and NADPH with the subsequent formation of O_2^-. The activity was markedly increased in the cell-free fraction of homogenates of activated macrophages. The increment and the activity was much higher with NADPH than with NADH as a substrate. c) The Km for NADPH was 0.030 mM and the Km for NADH was 0.355 mM. The Vmax were 46.7 nmol O_2^- per min per mg protein with NADPH and 22.8 nmol O_2^- per min per mg protein with NADH. d) The oxidase was insensitive to azide, cyanide, antimycin and rotenone and was sensitive to the sulfhydryl reagent PCMBS. e) A direct correlation was found between the degree of activation of the respiratory metabolism of intact macrophages and the extent of the activation of the oxidase. The rate of the oxidase of cell lysates in the presence of NADPH as substrate could account for the respiratory burst of intact macrophages.

INTRODUCTION

The interaction of phagocytic cells with opsonized particles and certain soluble stimuli induces a series of metabolic changes commonly referred to as the respiratory burst. This includes increased oxygen consumption with the subsequent generation of oxygen-reactive species (O_2^-, H_2O_2, $OH\cdot$ and singlet oxygen) and an enhancement of glucose oxidation through the hexose monophosphate pathway (1, 3, 7, 13, 16, 17, 20, 22, 24, 26, 31, 35, 38, 42, 44, 50).

The identity of the enzyme primarily responsible for this respiratory activation remains a matter of some controversy. There is a general agreement that an oxidase active on reduced pyridine nucleotides is primarily involved, but many discrepancies exist concerning the nature of the substrate, the kinetic properties and the subcellular localization of the enzyme. It has been shown in our (8, 33, 34, 39, 41-43, 49) and in other (2, 9, 10, 14, 19, 21, 23, 24, 27, 36, 46) laboratories that in granulocytes and in some types of macrophages the changes of the oxidative metabolism are due to the activation of an oxidase which preferentially uses

NADPH as substrate and which is measurable in the particulate fraction of cell homogenates. Other authors have suggested that a soluble NADH oxidase is the key enzyme responsible for the respiratory burst, at least in guinea pig granulocytes (4, 6, 18, 28, 29). The discrepancies can be due to the differences in the methods used for the assay of the enzymatic activity and in the type of phagocytic cell investigated.

To our knowledge, the nature of the oxidase responsible for the respiratory burst in guinea pig resident peritoneal macrophages has never been investigated. In this paper we present studies of the NAD(P)H oxidase activity of guinea pig resident peritoneal macrophages both at rest and stimulated by phorbol myristate acetate (PMA). These studies provide evidence that the enzyme responsible for the increase in oxygen uptake and in O_2^- and H_2O_2 production by peritoneal resident macrophages of guinea pigs is an oxidase that preferentially uses NADPH as a physiologic substrate.

MATERIALS AND METHODS

Cell Collections. Macrophages were harvested from male guinea pigs (300 to 500 g) by 4 peritoneal lavages with 50 ml of sterile 0.9% NaCl. The cell suspension was centrifuged at 400 x *g* for 7 min. The contaminating erythrocytes were lysed by treating the cells with hypotonic NaCl solution for 60 to 90 sec. After another centrifugation, the cells were suspended in Krebs-Ringer-phosphate buffer (pH 7.4) containing 0.5 mM $CaCl_2$ and 5 mM glucose (KRP). From each animal, 12 to 20 x 10^6 cells were usually collected. Differential counts were done on May-Grünwald and Giemsa stained smears.

Electron Microscopy. Cell suspensions were fixed in 1.5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4) for 10 min at 4 C. To visualize the peroxidatic reaction product, the cytochemical procedure described elsewhere (47) was used.

Metabolic Assays with Intact Cells. Oxygen consumption by resting and activated cells was measured at 37 C with a Clark oxygen electrode connected with a plastic chamber as previously described (33). Superoxide anion production by macrophages was quantitated by continuous spectrophotometric assay of the superoxide dismutase (SOD)-inhibitable reduction of cytochrome c (2, 10). Assays were performed in a Perkin Elmer 576 spectrophotometer. Both reference and sample cuvettes contained 150 μM ferricytochrome c, 2 mM NaN_3 and 5 x 10^5 resident peritoneal macrophages in KRP. The reference cuvette also contained 30 μg of SOD. The volume was 1 ml and the temperature was 37 C. The PMA, dissolved in dimethylsulfoxide (DMSO), was used as the stimulatory agent at a final concentration of 0.5 μg/ml.

Cell Activation, Homogenization and Fractionation. Cell suspensions (2.5 x 10^6 cells per ml) were prewarmed at 37 C and were incubated with 0.5 μg per ml PMA for 90 sec under continuous stirring. Control cells were incubated under identical conditions with the exception that DMSO was added instead of PMA. The cells were then diluted 5-fold with ice-cold KRP and centrifuged at 800 x *g* for 5 min. Homogenization of the cells was done either by using a Potter type homogenizer with a teflon pestle driven by a motor or by sonication. When the former method was used the cells were resuspended in 0.34 M sucrose containing 1 mM $NaHCO_3$ at a concentration of 2 to 4 x 10^7 cells per ml. When sonication was used, the cells were suspended in ice-cold KRP at the same concentration and placed in a 10-ml plastic

tube. Sonication was performed at 0 C with 2 5-sec pulses using a MSE sonicator at the maximal intensity (150 w). The disruption of the cells was checked by light microscopic examination. The homogenates were centrifuged at 100,000 x *g* for 30 min in a refrigerated Spinco L50 ultracentrifuge. The pellet, which includes all the particulate subcellular structures, was resuspended in 0.34 M sucrose containing 1 mM $NaHCO_3$ at a concentration of 1.2 to 1.8 mg protein per ml. Both the resuspended pellets and the supernatants were used for biochemical assays.

Measurement of NAD(P)H Oxidase. Oxidase activity was assayed by measuring the O_2^- formation in the presence of NADPH or NADH. The method was based on the spectrophotometric determination of the superoxide-mediated cytochrome c reduction as originally described by Babior (2). The assays were done in a double-beam Perkin Elmer 576 spectrophotometer at 37 C. Both the reference and the sample cuvette contained 65 mM Na-K phosphate buffer (pH 7.0), 170 mM sucrose, 150 μM cytochrome c, NADH or NADPH (at the desired concentration) and 2 mM NaN_3, in a final volume of 1 ml. The reference cuvette contained also 30 μg SOD. The reaction was started by adding the enzyme (20 to 50 μg of protein) to both cuvettes, and the absorbance change at 550 nm was followed. Calculations of O_2^- production were based on an extinction coefficient of 21.1 $mM^{-1}cm^{-1}$ (reduced minus oxidized) (48).

Protein Determination. The method of Lowry was used to determine protein concentration. Bovine serum albumin was used as a standard (32).

Materials. Cytochrome c (type VI), SOD (type 1, from bovine blood), NADPH (type III), NADH (grade III), and PMA were purchased from Sigma Chemical Co. St. Louis, Mo. Other compounds of the highest available purity were obtained from commercial sources.

RESULTS

Characterization of Cell Preparations. The average cell composition of the cell suspensions used in this work was as follows: macrophages, 89%; lymphocytes, 4%; neutrophils, 1%; eosinophils, 4%; large cells which were judged to be exfoliated mesothelial cells, 2%. All the cells which were identified as macrophages actively phagocytosed serum-opsonized zymosan.

Another approach to cell identification was the electron microscopic cytochemistry of peroxidase. Phagocytic cells (macrophages, neutrophils and eosinophils) had positive reactions, while no reaction was seen in lymphocytes and mesothelial cells. In macrophages, the peroxidase activity was restricted to the endoplasmic reticulum, the nuclear envelope and the Golgi apparatus (Fig. 1). This pattern of distribution of peroxidase is considered typical for resident macrophages (11, 12).

Stimulation of Intact Cells by PMA. Table 1 and Figure 2 show that resident peritoneal macrophages exposed to PMA (0.5 μg/ml) underwent a rapid increase in O_2 consumption and in O_2^- production. By examination of the time course of O_2^- generation and of O_2 consumption it appeared that the stimulation took place a few seconds after the addition of the stimulatory agent and proceeded at a linear rate for at least 120 sec. The respiratory response induced by PMA is mostly insensitive to inhibitors of the mitochondrial respiratory chain. The nonpenetrating sulfhydryl reagent *p*-chloromer-curibenzensulphonate (PCMBS) caused a 20 to 30% inhibition (Table 3).

Activity of NAD(P)H Oxidase. The 100,000 x *g* pellets and the supernatants

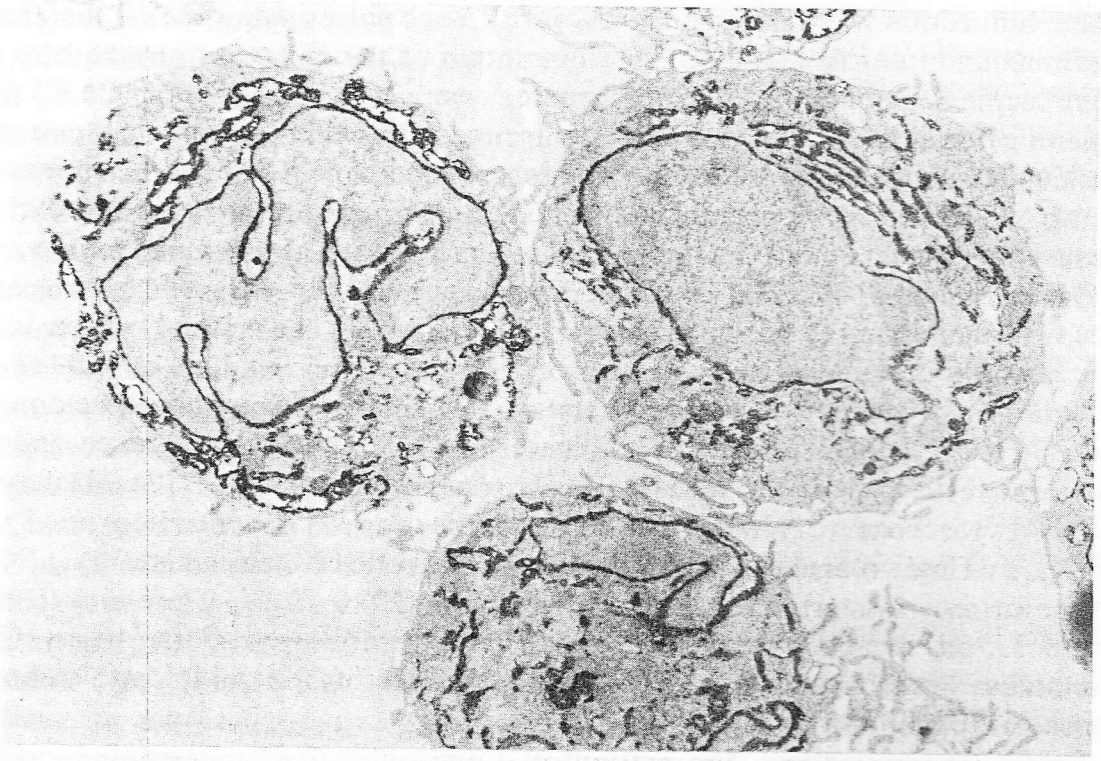

Fig. 1. Electron micrograph of guinea pig resident peritoneal macrophages incubated for the demonstration of peroxidatic reaction. Reaction product is present in the nuclear envelope and in the endoplasmic reticulum. x 6,000.

obtained from sonicates of resting and PMA-stimulated peritoneal resident macrophages were examined for their NAD(P)H oxidase activity by their ability to generate O_2^- in the presence of reduced pyridine nucleotides. The results reported in Table 2 show the following: a) the values of the oxidase activity of particles from resting cells were close to the lower limit of sensitivity of the assay method. The quantitation, therefore, was hard to perform with accuracy due to the wide scattering of the experimental data. For this reason, the range of the values obtained is given in the table. b) The activity of particles made from activated cells was much higher than that of particles from resting cells. The activity with NADPH was much higher than that with NADH at both substrate concentrations. c) The oxidase activity of the supernatants from both resting and stimulated cells was very low,

TABLE 1

Oxygen Consumption and Superoxide Anion Release by Resting and PMA-Treated Guinea Pig Peritoneal Resident Macrophages

Condition of Cells	O_2 Consumption	O_2^- release
Resting cells	1.71 ± 0.67[a] (7)	0.88 ± 0.58 (10)
PMA-treated cells	8.41 ± 2.1 (7)	9.08 ± 2.44 (10)

[a]Values (maximal rate) are expressed as nmol per min per 1×10^6 cells ± SD. The number of experiments is given in parentheses.

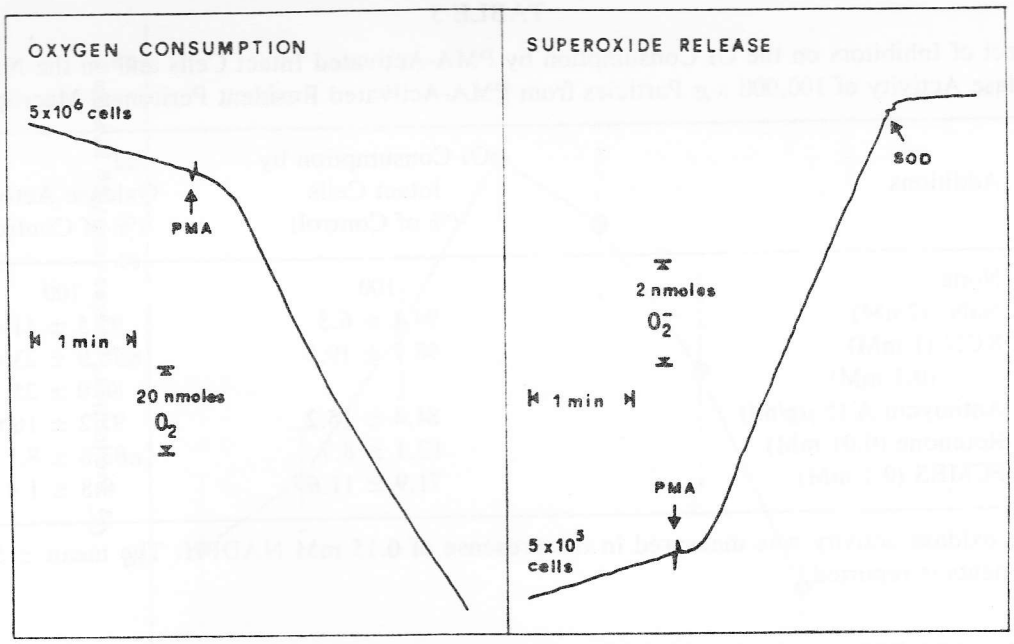

Fig. 2. Polarographic trace of O_2 consumption and continuous monitoring of O_2^- release by guinea pig resident peritoneal macrophages stimulated by PMA.

and, therefore, the same difficulties in quantitation pointed out above apply to particles from resting cells. However, it can be safely concluded that the oxidase activity of the supernatant from stimulated cells was not appreciably different from that of the supernatant from resting cells.

Experiments with supernatants and 100,000 x *g* particles obtained from cells homogenized in 0.34 M sucrose with a Potter type apparatus were also carried out. Neither the distribution of the enzymatic activity between particles and supernatant nor the specific activities differed from those found in the subcellular fractions obtained with sonication. The subsequent experiments were, therefore, performed

TABLE 2

NAD(P)H Oxidase Activities of 100,000 x *g* Particles and of Supernatant from Guinea Pig Resident Peritoneal Macrophages

Substrate		Particles from Resting Cells	Particles from PMA-Treated Cells
NADPH	0.15 mM	0 – 2.3 (8)[a]	55.7 ± 17.8 (10)
	1.0 mM	0 – 3.4 (4)	62.5 ± 27.0 (5)
NADH	0.15 mM	0 – 2.2 (8)	7.5 ± 2.6 (7)
	1.0 mM	0 – 3.2 (4)	19.6 ± 3.8 (5)
		Supernatant from Resting Cells	Supernatant from PMA-Treated Cells
NADPH	0.15 mM	0 – 1.3 (8)	0 – 0.95 (8)
	1.0 mM	0 – 3.8 (4)	0 – 2.5 (4)
NADH	0.15 mM	0.1 – 0.8 (8)	0 – 0.9 (8)
	1.0 mM	0 – 4.3 (4)	0.1 – 1.6 (4)

[a]Values are given as nmol O_2^- per min per mg protein ± SD. The number of experiments is given in parentheses.

TABLE 3

Effect of Inhibitors on the O_2 Consumption by PMA-Activated Intact Cells and on the NADPH Oxidase Activity of 100,000 x *g* Particles from PMA-Activated Resident Peritoneal Macrophages[a]

Additions	O_2 Consumption by Intact Cells (% of Control)	Oxidase Activity (% of Control)
None	100	100
NaN_3 (2mM)	94.1 ± 6.3	95.5 ± 11.4
KCN (1 mM)	98.7 ± 19.5	78.9 ± 23.6
(0.1 mM)		88.0 ± 25.1
Antimycin A (5 μg/ml)	84.4 ± 15.2	91.2 ± 16.6
Rotenone (0.01 mM)	82.1 ± 8.7	93.6 ± 8.1
PCMBS (0.1 mM)	71.9 ± 11.6	0.8 ± 1.4

[a]The oxidase activity was measured in the presense of 0.15 mM NADPH. The mean ± SD of 4 experiments is reported.

by using 100,000 x *g* particles from cell sonicates. This procedure had the advantage of rapidity and allowed use of a small number of cells.

Kinetic Properties of the Oxidase. The affinity of the NAD(P)H oxidase activity for the substrates was examined in the 100,000 x *g* particles from stimulated cells. The low activity of the corresponding fraction derived from resting cells did not allow accurate measurements.

Figure 3 shows the Lineweaver-Burk plots of O_2^- produced by the 100,000 x *g*

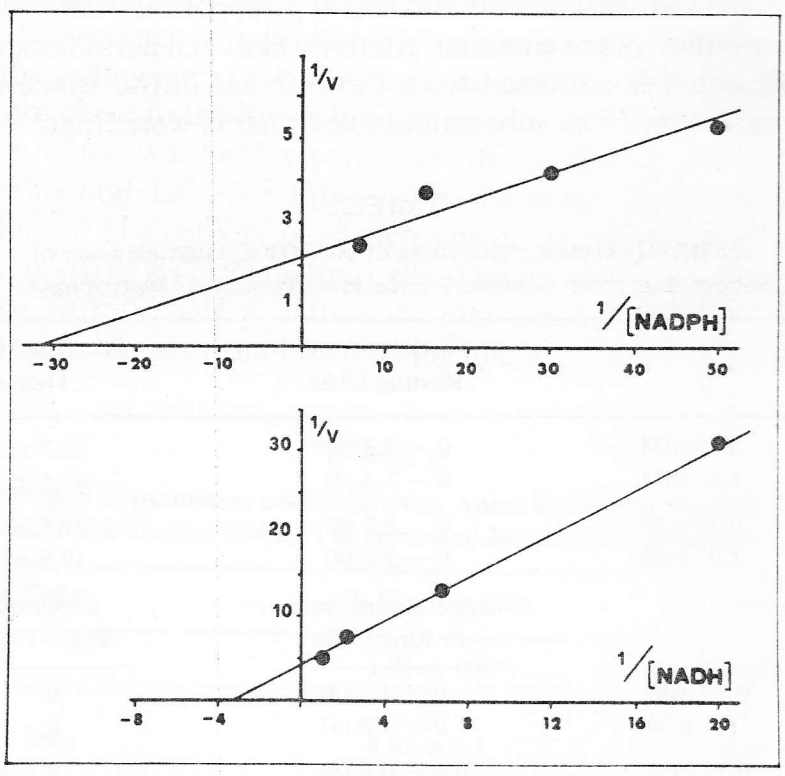

Fig. 3. Lineweaver-Burk plots of NADPH and NADH oxidase activities of the 100,000 x *g* particles obtained from PMA-activated resident peritoneal macrophages. The mean of 3 and 4 separate experiments with NADPH and NADH, respectively, is shown. Abscissas, mM^{-1}; ordinates, $(nmol\ per\ min\ per\ mg\ protein)^{-1} \times 10^2$.

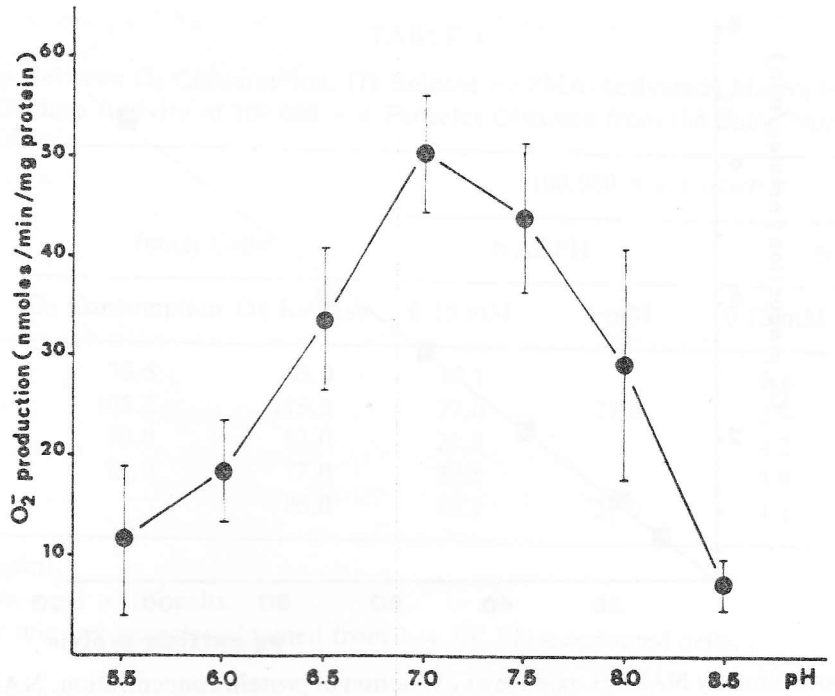

Fig. 4. pH dependence of the NADPH oxidase activity of 100,000 x *g* particles from PMA-activated resident peritoneal macrophages. NADPH, 0.15 mM. Results are expressed as mean ± SD of 3 separate experiments.

particles from PMA-stimulated cells as a function of pyridine nucleotide concentration. The values of the apparent Michaelis constant (Km) were 0.0305 ± 0.006 mM for NADPH and 0.355 ± 0.09 mM for NADH, and the maximum velocities were 46.7 ± 5.8 (SD) nmol O_2^- per min per mg protein with NADPH and 22.8 ± 6.0 (SD) nmol O_2^- per min per mg protein with NADH.

These results show that a particulate NAD(P)H oxidase was activated in resident peritoneal macrophages treated with PMA and that this oxidase preferentially used NADPH as a substrate. The pH optimum of this oxidase was ca. 7.0 (Fig. 4), and its activity was a linear function of protein concentration at least up to 100 μg per assay (Fig. 5).

Figure 6 shows the NADPH oxidase activity of 100,000 x *g* particles obtained from resident peritoneal macrophages stimulated with different concentrations of PMA. The data show that a direct correlation existed between the degree of activation of the respiratory metabolism of intact cells and the extent of the activation of the oxidase.

Effect of Metabolic Inhibitors on the NADPH Oxidase Activity. Table 3 shows that the oxidase activity of the 100,000 x *g* pellet of homogenates of PMA-stimulated resident peritoneal macrophages was practically insensitive to azide, cyanide, antimycin A and rotenone. These compounds were also ineffective on the oxidative metabolism of stimulated intact cells. On the contrary, the sulfhydryl reagent PCMBS markedly inhibited the enzymatic activity, indicating that SH groups are required for the enzyme function.

Adequacy of the Oxidase to Account for the Respiratory Burst of Whole Cells. Table 4 shows a comparison of the maximal rate of O_2 consumption and of O_2^- release induced by PMA in intact macrophages and the NAD(P)H oxidase of 100,000 x *g* particles obtained from the same number of stimulated cells. The results

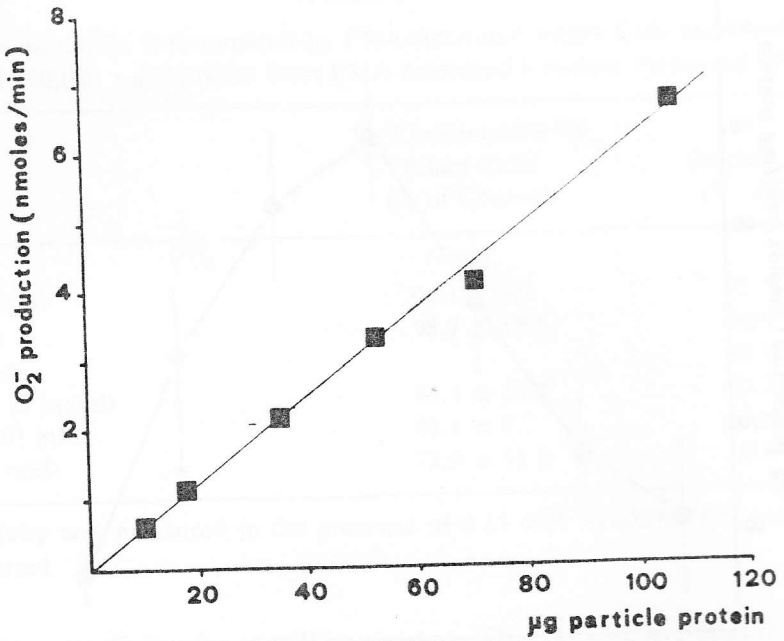

Fig. 5. O_2^- production by NADPH oxidase as a function of protein concentration. NADPH 0.15 mM.

show that the activities of the oxidase did not account for the respiratory burst of intact cells. This inadequacy may have been due to a) inactivation of the enzyme during the preparation of the cell fractions, b) inaccessibility of the O_2^- producing site to the substrate and/or c) cytochrome c, due, for example, to the formation of both inside-out and right-side-out vesicles during cell homogenization.

A new approach to compare the activation of cell metabolism and the NAD(P)H oxidase is reported in Fig. 7. The O_2^- formation by intact cells stimulated by PMA was recorded with a spectrophotometer. During the linear part of the O_2^- produc-

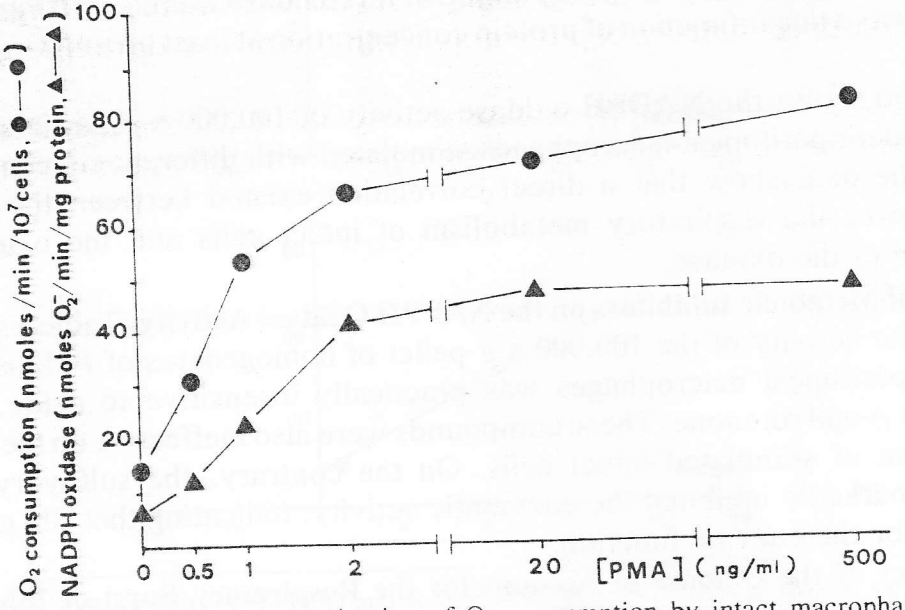

Fig. 6. Relationship between the activation of O_2 consumption by intact macrophages and th activation of the NADPH oxidase activity. O_2 consumption was measured in the presence of differen amounts of PMA and NADPH oxidase was measured as O_2^--forming activitiy of 100,000 x *g* particle obtained from the same macrophages. NADPH, 0.15 mM.

TABLE 4

Relationship Between O_2 Consumption, O_2^- Release by PMA-Activated[a] Macrophages and the NAD(P)H Oxidase Activity of 100,000 × *g* Particles Obtained from the Same Number of Cells

Experiment Number	Intact Cells[b]		100,000 × *g* Particles[c]			
			NADPH		NADH	
	O_2 Consumption	O_2^- Release	0.15 mM	1 mM	0.15 mM	1 mM
1	76.5	85.0	17.1		2.6	
2	105.2	119.0	27.0	27.0	2.0	5.5
3	90.0	83.0	21.5		4.2	
4	81.0	77.0	29.8		3.0	8.9
5		86.0	19.1	21.0	1.1	7.8

[a]PMA, 0.5 μg/ml.
[b]nmol per min per 1×10^7 cells.
[c]nmol O_2^- per min per particles obtained from 1×10^7 PMA-activated cells.

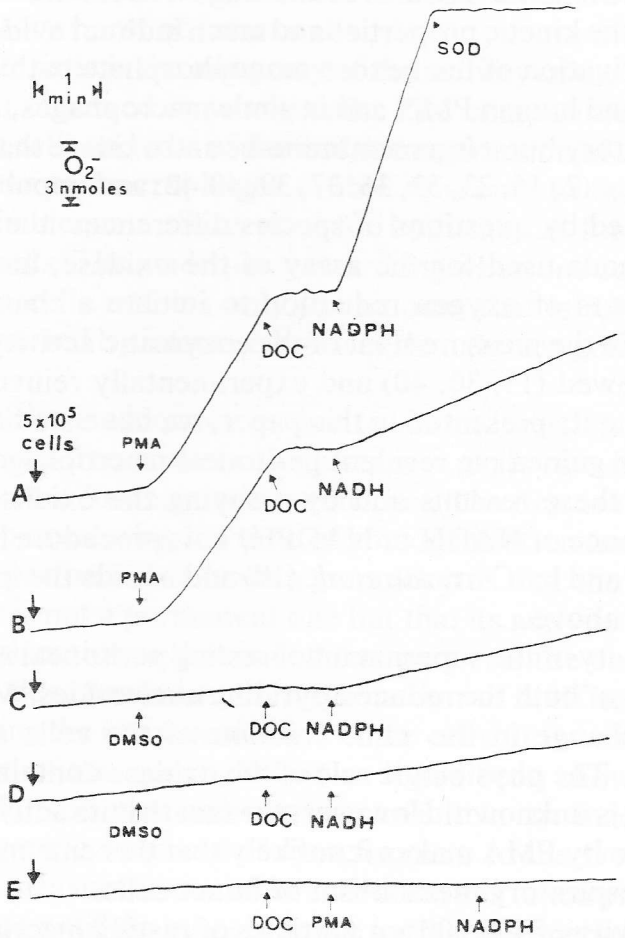

Fig. 7. Continuous monitoring of O_2^- production by intact resident peritoneal macrophages and by the NAD(P)H oxidase activity of the same cells lysed with DOC. Assay mixture: 5 x 10^5 macrophages incubated in 1 ml KRP containing 2 mM NaN_3 and 150 μM cytochrome c. Arrows: PMA, 0.5 μg; DMSO, 2.5 μl; DOC, 0.5 mg in 10 μl; NADPH, 0.15 μmol in 10 μl; NADH 1 μmol in 20 μl; SOD, 30 μg in 10 μl.

tion, deoxycholate (DOC) was added to the cuvettes in order to lyse the cells. The O_2^- generating activity was abolished by addition of DOC. At this point, NADPH or NADH were added to the cuvette. After addition of NADPH (trace A), there was an instantaneous resumption of O_2^- production at a rate which was higher than that of stimulated intact cells. With NADH the O_2^--generating activity was much lower than that of intact cells (trace B). Deoxycholate did not have a stimulatory effect on the NAD(P)H oxidase activity of resting macrophages as indicated by the appropriate controls (traces C, D, E).

These results indicate that the oxidation of NADPH, added at concentrations near those that are reported as physiologic for other types of phagocytic cells, can account for the respiratory burst in intact resident peritoneal macrophages. A possible inhibition of NADH oxidase by DOC was ruled out by studying the effect of the detergent on the NAD(P)H oxidase activity of the 100,000 x *g* pellet obtained from homogenates of PMA-stimulated cells.

DISCUSSION

The enzymatic basis of the respiratory burst that occurs in phagocytic cells during phagocytosis or following challenge with membrane-perturbing agents is still controversial. Two main hypotheses have been advanced. According to one of them, the activation of the respiratory metabolism of phagocytic cells is supported by the activation of a soluble flavin — containing NADH oxidase (4, 5, 28). A series of data concerning the kinetic properties and much indirect evidence concerning the linking with the activation of the hexose monophosphate pathway suggest that, at least in guinea pig and human PMN and in some macrophages, the enzyme responsible for the respiratory burst is a membrane-bound oxidase that uses NADPH as a physiologic substrate (2, 15, 23, 33, 36, 37, 39, 40-42; and unpublished). The matter has been complicated by questions of species differences, the type of phagocytic cell used, the methods used for the assay of the oxidase, and the ability of the intermediate products of oxygen reduction to initiate a chain reaction that can introduce artifacts in the measurement of the enzymatic activity. This problem has been recently reviewed (13, 30, 40) and experimentally reinvestigated (8).

With the experiments presented in this paper, we have studied this problem in a phagocytic cell, the guinea pig resident peritoneal macrophage, that has not been studied before for these reasons and by assaying the oxidase as O_2^- generating activity in the presence of NADH or NADPH. This procedure has been introduced by Babior *et al.* (2) and by Curnutte *et al.* (10) and avoids the interference with the artifacts discussed above.

The oxidase activity of the supernatant of resting peritoneal macrophages is very low in the presence of both the reduced pyridine nucleotides. Moreover, these low activities do not change in the same fraction of the cells after the metabolic activation by PMA. The physiologic role of the oxidase contained in the cytosol of these macrophages is unknown. However, the fact that its activity does not change after the stimulation by PMA makes it unlikely that this enzyme plays a role in the activation of the respiratory metabolism of intact cells.

The oxidase activity of 100,000 x *g* particles of resting macrophages is also very low with both NADH and NADPH. The enzymatic activity is greatly enhanced in the same fraction of PMA-stimulated macrophages, the enhancement being more striking when NADPH is used as substrate. Similar results were obtained years ago

in our laboratory with different methods of assay in 20,000 x *g* particles from guinea pig granulocytes (33, 39, 42) and subsequently by others in phagocytes of different species (2, 9, 14, 23).

These data suggest that the oxidation of NADPH by this particulate oxidase is the main enzymatic mechanism responsible for the respiratory burst. However, other characteristics of the enzyme must be examined before positive conclusions concerning its role in the activation of the respiratory metabolism of macrophages can be made. These characteristics are as follows: a) extent of activation, b) kinetic properties, c) sensitivity to inhibitors and d) adequacy to account for the metabolic activation of intact cells.

With regard to the first characteristic, we have shown (Fig. 6) that the extent of activation of the cell-free enzyme parallels the degree of the respiratory stimulation of intact cells induced by using different concentrations of PMA. As far as the kinetic properties of the enzyme are concerned, the Michaelis constant (Km) and the maximum velocity (Vmax) of the enzyme with NADH and NADPH clearly show that the preferential substrate is NADPH. In fact, the km for NADPH is one order of magnitude lower than that of NADH and the Vmax of NADPH is double that of NADH. The km values are similar to those found in our (33, 39) and in other (2, 9, 14, 19) laboratories for the membrane-bound oxidase of other phagocytes.

With regard to the third characteristic, the particulate NADPH oxidase of resident peritoneal macrophages is insensitive to azide, cyanide, rotenone and antimycin as is the respiratory burst of intact cells.

The fourth characteristic is the adequacy of the activity of the enzyme to account for the phenomenon observed in whole cells. The comparison of the rate of O_2^- production by whole cells and that of the 100,000 x g fraction of the homogenates of an equivalent number of macrophages (Table 4) seems to indicate that the enzymatic activity is inadequate to account for both the O_2^- formation and the O_2 consumption of the cells. The inadequacy is striking when one considers that the O_2^- measurable in whole cells reflects only the amount that is released and that the oxygen actually consumed by intact cells is less than the oxygen turned over by the enzyme (17, 40).

It is possible that the inadequacy is due to an underestimation of the rate of NADPH oxidase in cell-free particles with respect to its rate in intact cells. Two explanations can account for this underestimation. The first is that an inactivation of the oxidase takes place during the time of homogenization and centrifugation. It is known by studies performed in human granulocytes that the activation state of the NADPH oxidase is not a permanent one but that its activity decays in the time (25). The second explanation is that in cell-free particles the enzymatic activity is not fully detectable, due, for example, to formation of both inside-out and right-side-out vesicles during the homogenization of the cells. If the oxidase is located on the plasmamembrane of the macrophages, as it has been shown in granulocytes (unpublished; 15, 45), the phenomenon can result in an inaccessibility of the substrate to the enzymatic sites and of cytochrome c to the O_2^- produced. Therefore, if the oxidase is measured soon after activation has taken place and if inaccessibility of the substrate or of the cytochrome c to the O_2^--generating site is avoided, one would expect that actual activity of the oxidase can be fully revealed.

This expectation has been verified by using the experimental model described in Figure 7. In this model, DOC was used to lyse the cells after their activation by PMA

and to avoid vesicle formation due to its detergent properties. Under these experimental conditions, the NADPH-dependent O_2^- generating activity of the DOC-treated cells was higher than that of activated intact cells, while the NADH-dependent O_2^- generation was considerably lower. These results indicate that NADPH oxidase activity may account for the increased respiration of intact cells and that this activity may be considerably underestimated when assayed in cell-free particles. Therefore, the NADPH oxidase also fulfills the fourth requirement, i.e., the adequacy to account for the phenomenon in intact cells, necessary for one enzyme to be considered as the basis of the respiratory burst.

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