THE ENZYME RESPONSIBLE FOR THE RESPIRATORY BURST IN ELICITED GUINEA PIG PERITONEAL MACROPHAGES

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SUMMARY. The enzymatic basis of the respiratory burst induced by phorbol myristate acetate in elicited peritoneal macrophages of the guinea-pig has been studied. The following evidence suggests that a membrane-bound oxidase that preferentially uses NADPH as substrate is the main enzyme responsible for activation of the oxidative metabolism: (1) The supernatant of postnuclear fractions of resting macrophages oxidises NADH and NADPH with formation of O_2^-. The activity with both substrates is very low and does not change in the supernatant obtained from activated cells. (2) The cell-free particles of resting macrophages also oxidise both NADH and NADPH with formation of O_2^-. The activity of the cell-free particles from activated macrophages does not change when NADH is the substrate. By contrast, the activity of the cell-free particles from activated cells is markedly increased when NADPH is the substrate. (3) In cell-free particles from activated macrophages the K_m for NADPH is about one order of magnitude lower than that for NADH and the V_{max} with NADPH is double that with NADH. (4) The NADPH oxidase of cell-free particles is insensitive to azide, cyanide, antimycin A and rotenone and is sensitive to the sulphydryl reagent PCMB. All these drugs have the same effect on the respiratory response of intact macrophages. (5) A direct correlation is found between the degree of activation of the respiratory metabolism of intact macrophages and the extent of activation of the NADPH oxidase. A new approach designed to measure the activity of the oxidase soon after the activation of the enzyme has taken place, shows that the NADPH oxidase can account for the respiratory burst of intact macrophages.

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PHAGOCYTIC cells exposed to phagocytosable particles and certain soluble stimuli undergo a series of metabolic changes commonly referred to as "respiratory burst". This includes an increased oxygen consumption with generation of oxygen reactive species (O_2^-, H_2O_2, $OH^.$ and singlet oxygen) and an enhancement of glucose oxidation through the hexose–monophosphate pathway (Baldridge and Gerard, 1933; Sbarra and Karnovsky, 1959; Iyer, Islam and Quastel, 1961; Rossi and Zatti, 1964; Paul and Sbarra, 1968; Zatti, Rossi and Patriarca, 1968; Gee et al., 1970; Allen, Stjernholm and Steele, 1972; Babior, Kipnes and Curnutte, 1973; Drath and Karnovsky, 1975; Homan-Müller, Weening and Roos, 1975; Klebanoff and Hamon, 1975; Root et al., 1975; Johnston, Lehmeyer and Gutrie, 1976; De Chatelet, 1978; Dri et al., 1979).

The intermediates of oxygen reduction are involved in the killing of bacteria (Johnston et al., 1975; Klebanoff, 1975; Klebanoff and Hamon), fungi (Sasada and Johnston, 1980), protozoa (Murray and Cohn, 1979; Murray et al., 1979; Nathan et al., 1979a) and tumour cells (Edelson and Cohn, 1973; Clark and Klebanoff, 1975; Clark et al., 1975; Nathan et al., 1979b and c). Furthermore they have been recently correlated with the induction of tissue damage at the inflammatory site (McCord, 1974; Salin and McCord, 1975; Sacks et al., 1978) and with the amplification and modulation of the inflammatory response (Levine et al., 1976; Tedesco, Tincani and Patriarca, 1977; Ohmori et al., 1979).

There is general agreement that the respiratory burst of phagocytes is due to the activation of an oxidase active on reduced pyridine nucleotides, but many discrepancies exist concerning the nature and the subcellular localisation of the enzyme.

It has been shown in our laboratory (Rossi and Zatti, 1964; Zatti and Rossi, 1965; Rossi and Zatti, 1966; Patriarca et al., 1971; Rossi, Romeo and Patriarca, 1972; Romeo et al., 1973b; Patriarca et al., 1975; Rossi, Zabucchi and Romeo, 1975; Bellavite et al., 1980) and other laboratories (Iyer et al.; Selvaray and Sbarra, 1967; Paul et al., 1972; Babior Curnutte and Kipnes, 1975; Curnutte, Kipnes and Babior, 1975; De Chatelet et al., 1975; Hohn and Lehrer, 1975; Babior, Curnutte and McMurich, 1976; Iverson et al., 1977; Cohen, Chovaniec and Davies, 1980; Kakinuma and Kaneda, 1980), that in granulocytes and some types of macrophages the enzyme responsible for the respiratory burst preferentially uses NADPH as substrate and is measurable in the particulate fractions of cell homogenates. Other authors postulate that a soluble NADH-oxidase would be the key enzyme which induces the activation of the oxidative metabolism, at least in guinea-pig granulocytes (Evans and Karnovsky, 1961; Karnovsky, 1962; Baehner, Gilman and Karnovsky, 1970; Karnovsky, 1973; Badwey and Karnovsky, 1979; Badwey, Curnutte and Karnovsky, 1979).

The subcellular localisation of the enzyme responsible for the respiratory burst is controversial. Evidence has been presented showing, in stimulated granulocytes, a localisation in plasma membrane (Briggs et al., 1975; Goldstein et al., 1975; Segal and Peters, 1977; Dewald et al., 1979; Cohen et al.; Rossi et al., 1980) or an association with intracellular organelles (Iverson et

al., 1978; Tauber and Goetzl, 1979). According to Andrew *et al.*, (1978) in rabbit alveolar macrophages the oxidase is located in the endoplasmic reticulum.

The enzymatic basis of the respiratory burst of macrophages has been little investigated. This contrasts with the growing evidence that in macrophages the products of oxygen reduction play a key role in the killing of micro-organisms (Murray and Cohn; Murray *et al.*; Nathan *et al.*, 1979a; Sasada and Johnston) and of tumour cells (Nathan *et al.*, 1976b and c).

In this paper we present studies on the NAD(P)H oxidase activity in peritoneal inflammatory macrophages elicited by casein both at rest and stimulated with phorbol-myristate acetate (PMA).

The results provide evidence that the enzyme responsible for the respiratory burst in this type of macrophage is an oxidase that preferentially uses NADPH as physiological substrate. Another paper reports results on subcellular localisation of this oxidase.

MATERIALS AND METHODS

Cell collection. Guinea-pigs weighing 300–500 g were intra-peritoneally (i.p.) injected with 20 ml of 1 per cent. sterile casein solution in 0·9 per cent. NaCl. After 4 days, the animals were sacrificed and the peritoneum washed four times with 50 ml of 0·9 per cent. NaCl. The cells were collected by centrifugation at 250 g for 7 min. and the contaminating erythrocytes lysed by hypotonic shock (Romeo *et al.*, 1973b). Macrophages were resuspended in Krebs–Ringer phosphate buffer, pH 7·4, containing 0·5 mM $CaCl_2$ and 5 mM glucose (KRP). Differential counts were carried out on May–Grunwald and Giemsa-stained smears.

Phagocytosis. Macrophages (5×10^6 cells/ml) were incubated for 5 min. at 37°C with 10 μl of a Latex particle (0·81 μ) suspension of 30 mg/ml. Phagocytosis was stopped by the addition of an equal volume of ice-cold 3 per cent. glutaraldehyde in 0·1M sodium cacodylate buffer, pH 7·2. After fixation for 40 min. at 4°C, the cell suspension was centrifuged, washed three times in sodium cacodylate buffer and post-fixed for 60 min. at 4°C in 1 per cent. osmium tetroxide in cacodylate buffer. After dehydration in graded ethanols the pellet was embedded in Down epoxy resin 3RE (Lackwood, 1964). Ultrathin sections were doubly stained with uranylacetate and lead citrate (Venable and Coggeshall, 1965) and examined in a Philips EM 300 electron microscope.

Metabolic assay with intact cells. Oxygen consumption by resting and activated cells was measured at 37°C with a Clark oxygen electrode connected with a plastic vessel as previously described (Patriarca *et al.*,). 5×10^6 cells were used for each assay. Superoxide anion production was quantitated by continuous spectrophotometric assay of the superoxide dismutase inhibitable reduction of ferricytochrome *c* (Babior *et al.*, 1973; Curnutte *et al.*). Assays were performed in a Perkin–Elmer 576 spectrophotometer. Both reference and sample cuvette contained 150 μM ferricytochrome *c*, 2 mM NaN_3 and 5×10^5 macrophages. The reference cuvette contained also 30 μg of superoxide dismutase (SOD). The volume was 1 ml. The temperature 37°C. Phorbol myristate acetate (PMA), dissolved in dimethyl sulfoxide (DMSO) was used as the stimulatory agent

Cell activation and homogenization. Cell suspensions (5×10^6/ml) were pre-warmed at 37°C and then incubated for 90 s with 0·5 μg/ml of PMA under continuous stirring. Control cells were incubated under identical conditions with the exception that DMSO was added instead of PMA. The cells were then five-fold diluted with ice-cold KRP and centrifuged at 250 g for 7 min. The cell pellet was resuspended in 0·34M sucrose containing 1 mM $NaHCO_3$ at a concentration of 2×10^8 cells/ml. Homogenisation was carried out by using a Potter type homogeniser with a teflon pestle operated by a motor. The disruption of the cells was checked by light microscope examination. The homogenate was diluted 1:1 with 0·34M sucrose

containing 1 mM $NaHCO_3$ and 1·8 per cent. NaCl and centrifuged at 400 g for 5 min. to discharge nuclei, intact cells and cell debris. Postnuclear supernatants were centrifuged at 100.000 g for 20 min. in a refrigerated Spinco L50 ultracentrifuge (Beckman Instruments, Fullerton, California). The supernatant and the pellet obtained by the centrifugation at 100.000 g of the postnuclear supernatants were used for the study of the oxidase activity. Throughout the paper these two cell fractions will be indicated as supernatant and cell-free particles respectively.

Measurement of NADPH oxidase.

The oxidase activity was assayed by measuring the rate of O_2^- formation in presence of NADPH and NADH. The method was based on the spectrophotometric determination of the superoxide-mediated ferricytochrome c reduction as originally described by Babior *et al.* (1975). The assay was carried out 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, 100 μM ferricytochrome 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 to both cuvettes and the absorbance 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 oxidised) (Van Gelder and Slater, 1962).

Protein determination

The method of Lowry *et al.* (1951) was used with bovine serum albumin as standard.

Materials

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

RESULTS

Characterisation of cell preparation

Table I shows the average composition of the cell suspensions used in this work. Four days after i.p. injection of sodium caseinate, a cell population rich in macrophages and little contaminated by other cell types was obtained.

The electron microscopic examination showed that a high percentage of the macrophages were able to phagocytose Latex. In 5 min. most of the cells contained more than five Latex beads.

TABLE I

Characteristics of the peritoneal exudate of guinea-pigs 4 days after the injection of sodium caseinate

Total number of cells/guinea pig	mg proteins/ 10^6 cells	Macrophages (%)	Neutrophils (%)	Lymphocytes (%)	Phagocytosing macrophages (%)
$82 \cdot 5 \times 10^6 \pm 9 \cdot 9$ (10)	$0 \cdot 185 \pm 0 \cdot 011$ (10)	$96 \cdot 4 \pm 0 \cdot 9$ (10)	$3 \cdot 0 \pm 0 \cdot 9$ (10)	$0 \cdot 6 \pm 0 \cdot 25$ (10)	$94 \cdot 6 \pm 2 \cdot 2$ (4)

Peritoneal exudates were induced as described in Materials and Methods. The percentage of phago-cytosing macrophages was evaluated by electron microscopy. The average values ± SEM of the number of experiments shown in parentheses are reported.

The respiratory burst of intact cells

Fig. 1 and table II show that peritoneal-elicited macrophages exposed to PMA (0.5 μg/ml) undergo a rapid increase in O_2 consumption and O_2^- production. By examining the time course of the stimulatory response it appears that the increase in O_2^- generation and in O_2 consumption takes place a few seconds after the addition of the stimulatory agent and proceeds at a linear rate for at least 120 s. Both the increase in the O_2^- production and in the O_2 consumption are insensitive to inhibitors of the mitochondrial respiratory chain antimycin A and rotenone (fig. 1).

The activity of the NAD(P)H oxidase

The 100,000 g pellets and the supernatants obtained from homogenates of resting and PMA-stimulated macrophages were examined for their NADPH and NADH oxidase activity. The results reported in table III show that: (1) the enzymatic activity of supernatants from resting macrophages is very low in the

TABLE II

Respiratory burst of guinea-pig elicited peritoneal macrophages

	nmoles O_2/min./10^6 cells	nmoles O_2^-/min./10^6 cells
Resting macrophages	0.81 + 0.21 (10)	0.35 ± 0.23 (10)
PMA-stimulated macrophages	6.80 ± 0.58 (10)	7.70 ± 0.94 (10)

For experimental conditions see Materials and Methods. The means ± SEM of the number of experiments shown in parentheses are reported.
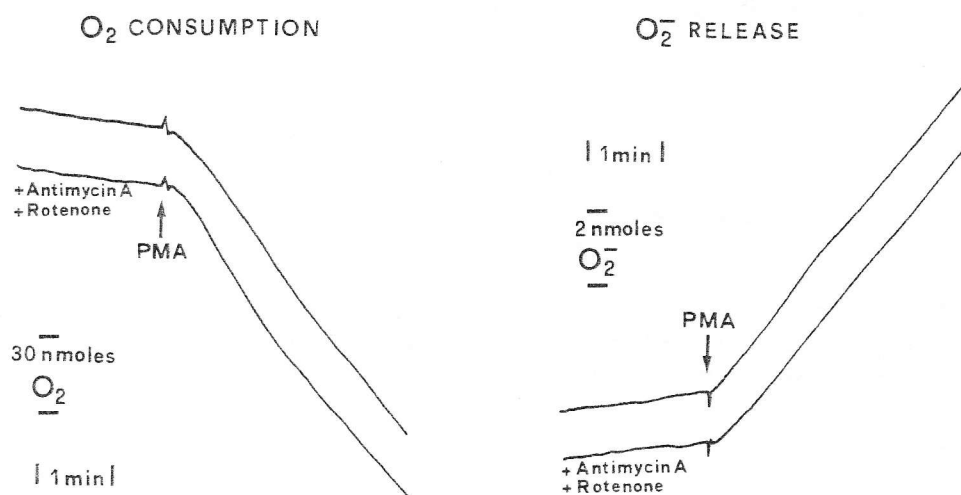
FIG. 1.—Polarographic traces of oxygen consumption and continuous spectrophotometric monitoring of O_2^- release by guinea-pig peritoneal-elicited macrophages stimulated by PMA. For conditions see Materials and Methods. Antimycin A (5 μg/ml) and rotenone (0.1 mM) were incubated with the cells 3 min. before the addition of PMA (0.5 μg/ml).

TABLE III

Activity of the NAD(P)H oxidase in the supernatants and cell-free particles of guinea-pig-elicited peritoneal macrophages

Substrate	nmoles O_2^-/min./mg protein	
	Particles from resting cells	Particles from PMA-stimulated cells
0·15 mM NADPH	0·79 ± 0·2	13·1 ± 1·9
1·0 mM NADPH	2·5 ± 0·5	21·3 ± 2·7
0·15 mM NADH	2·7 ± 0·6	3·1 ± 1·0
1·0 mM NADH	6·3 ± 0·9	7·9 ± 1·7
	Supernatant from resting cells	Supernatant from PMA-stimulated cells
0·15 mM NADPH	0·74 ± 0·1	1·1 ± 0·1
1·0 mM NADPH	0·95 ± 0·2	2·0 ± 0·4
0·15 mM NADH	1·0 ± 0·3	1·1 ± 0·3
1·0 mM NADH	2·1 ± 0·7	1·9 ± 0·6

For experimental procedure and assay of NAD(P)H oxidase see Materials and Methods. The means of four experiments ± SEM are reported.

presence of both NADPH and of NADH. The activities do not change appreciably in the supernatants obtained from PMA-treated cells. (2) The oxidase activities of cell-free particles from resting macrophages are also very low. The activity is higher in the presence of NADH at both substrate concentrations. (3) The enzymatic activity of cell-free particles from PMA-activated macrophages does not change with respect to that of cell-free particles from resting macrophages when NADH is the substrate. On the contrary the activity of cell-free particles from PMA-treated cells is greatly stimulated when NADPH is the substrate. These results clearly indicate that the oxidase found in the supernatants is not involved in the respiratory burst of intact cells. For this reason the subsequent studies were performed only on the activity of cell-free particles.

Kinetic properties of the activated NAD(P)H oxidase

Fig. 2 reports the Lineweaver–Burk plots of O_2^- produced by cell-free particles from PMA-stimulated cells as a function of pyridine nucleotide concentration. The values of the apparent Michaelis constant (K_m) are 0·087 ± 0·005 mM (SEM) for NADPH and 0·633 ± 0·11 mM (SEM) for NADH and the maximum velocities (V_{max}) are 22·0 ± 3·2 (SEM) nmoles O_2^-/min/mg prot. with NADPH and 10·8 ± 2·8 (SEM) nmoles O_2^-/min/mg prot. with NADH.

The results so far presented show that in peritoneal-elicited macrophages stimulated with PMA a particulate oxidase which uses NADPH as substrate is activated.

Other properties of the activated NADPH oxidase of cell-free particles

As shown in fig. 3 the NADPH-dependent O_2^- production of cell-free particles from PMA-stimulated cells have a pH optimum in the range of 7–7·5.
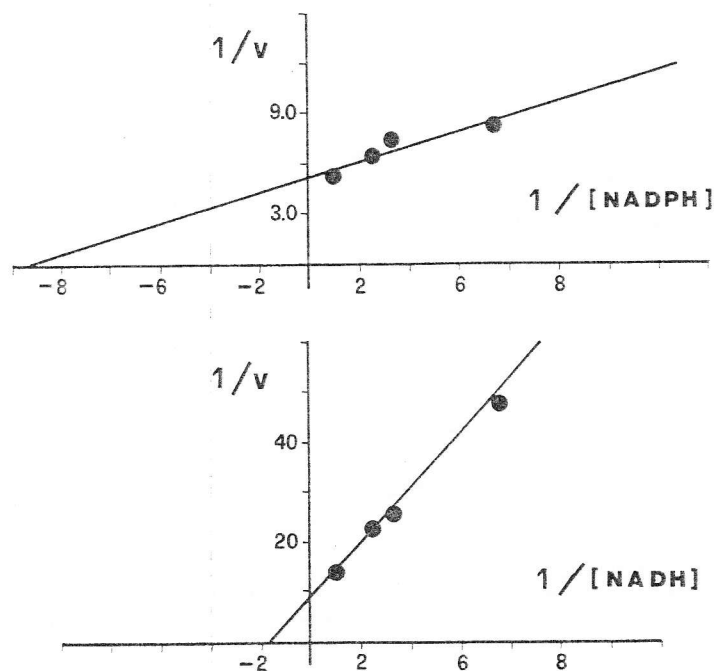
FIG. 2.—Lineweaver–Burk plots of NADPH and NADH oxidase activities of the cell-free particles obtained from PMA-activated peritoneal-elicited macrophages. The means of four separate experiments are reported. Abscissas: (millimolarity)$^{-1}$; Ordinates: (nmoles/min/mg prot)$^{-1} \times 10^2$.
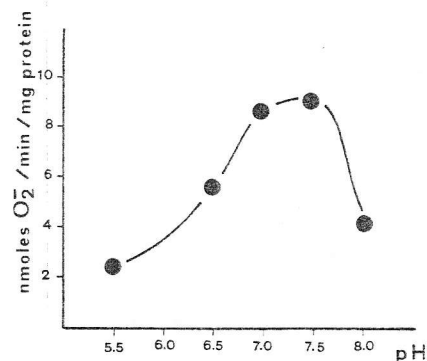

FIG. 3.—pH dependence of the NADPH oxidase activity of cell-free particles from PMA-activated peritoneal-elicited macrophages. NADPH 0·15 mM. The mean of two separate experiments is reported.

Fig. 4 reports the NADPH oxidase activity of cell-free particles obtained from macrophages stimulated with different concentrations of PMA. The data show that a direct correlation exists between the degree of activation of the respiratory metabolism of intact cells and the extent of the activation of the oxidase.

This enzymatic activity is practically insensitive to inhibitors of mitochondrial respiratory chain, azide, cyanide, antimycin A and rotenone (table IV). All these compounds are also ineffective on the stimulated O_2 consumption of intact
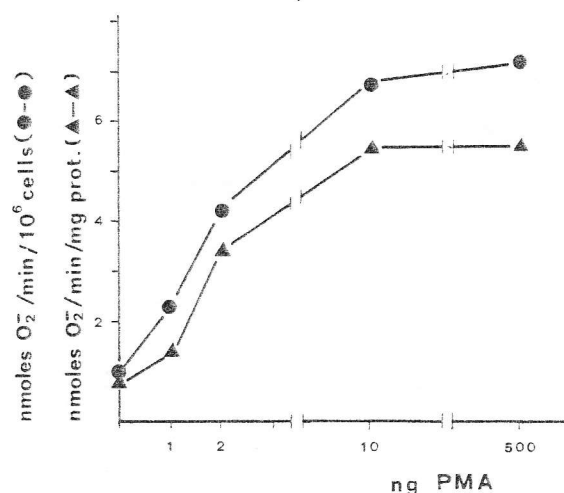
FIG. 4.—Relationship between the activation of O_2 consumption by intact macrophages and the activation of NADPH oxidase. O_2 consumption was measured in presence of different amount of PMA and NADPH oxidase was measured as O_2^--forming activity of cell-free particles obtained by centrifugation for 20 min. at $100.000 \times$ g of cells withdrawn from the electrode chamber and sonicated at $4°C$ with two 5-s pulses using a MSE sonicator at the maximal intensity (150 W).

TABLE IV

Effect of various inhibitors on the O_2 consumption of intact cells and on the NADPH-oxidase activity of cell-free particles of guinea-pig-elicited peritoneal macrophages

	O_2 consumption of intact cells	NADPH-oxidase activity of cell-free particles
Control	100	100
+ 2 mM NaN_3	103	98
+ 1 mM KCN	92	107
+ 5 μg/ml antimycin A	104	83
+ 0·01 mM rotenone	95	92
+ 0·1 mM pCMB	96	5
+ 1·0 mM pCMB	5	5

The values reported for the O_2 consumption by intact cells were obtained by monitoring the cell respiration of macrophages prewarmed for 3 min. at $37°C$ in the presence of the appropriate inhibitor after the addition of 0·5 μg/ml PMA. The NADPH-oxidase activity was measured in cell-free particles obtained from PMA-stimulated macrophages, in the presence of 0·15 mM NADPH. The reaction was started by adding the enzyme to the assay mixture (see Materials and Methods) pre-warmed at $37°$ and containing the appropriate inhibitor.

The average values of two experiments are reported.

cells. On the contrary, the sulphydryl reagent *p*-chloromercuribenzoate (PCMB) markedly inhibits the enzymatic activity, indicating that SH groups are required for the enzyme function. PCMB causes an inhibition of the respiratory response of intact cells only when used at high concentrations (1 mM). This is probably due to the fact that the site of NADPH oxidase with which the drug interacts is not easily accessible from outside the cell unless PCMB, which is a non-rapid penetrating agent (Webb, 1966), is added at high concentrations.

Adequacy of the oxidase to account for the respiratory burst of the whole cell

Table V shows the comparison between the intensity of the respiratory response induced by PMA in intact macrophages and the activity of the NADPH oxidase of cell-free particles obtained by the same number of stimulated cells. The results clearly indicate that the activity of the oxidase does not account for the respiratory burst of intact cells. This inadequacy may be due to an underestimation of the enzymatic activity because of inadequate accessibility of the substrate and/or of the cytochrome c to the O_2^--producing site of the enzyme. The data reported in table V show that this might be the case. In fact, when the oxidase is measured in the presence of deoxycholate (DOC) the activity of cell-free particles from PMA-stimulated cells increases about three-fold. This increase in the activity is not due to the activation by DOC, *per se*, of the NADPH oxidase since this detergent does not modify the activity of the oxidase of cell-free particles from resting cells (data not shown).

However, the NADPH oxidase activity remains inadequate to account for the respiratory burst of intact cells also when measured in presence of DOC. It is likely that the enzyme is deactivated or inactivated during the procedures of cell homogenization and fractionation. Therefore we have developed a new approach to compare the activation of cell metabolism and the NADPH oxidase. This approach has been designed to measure the activity of the oxidase soon after the activation has taken place, and in conditions that facilitate the accessibility of the substrate and of cytochrome c to the enzyme. The experiment is described practically in fig. 5. The O_2 consumption and the O_2^- formation by intact cells were measured. During the linear part of the cell respiration, DOC was added to the chamber in which the O_2 consumption was recorded or to the cuvettes of the spectrophotometer in which the O_2^--dependent reduction of cytochrome c was monitored. As shown by the traces, DOC, by lysing the cells abolished the respiratory burst of macrophages. The addition at this point of NADPH caused an instantaneous resumption of O_2 consumption or O_2^- production if the macrophages had been previously stimulated with PMA.

TABLE V

Relationship between the O_2 consumption and O_2^- release by intact cells and the NADPH oxidase of cell-free particles of guinea-pig-elicited peritoneal macrophages stimulated with PMA

nmoles O_2/min./10^6 cells	6.0 ± 0.34
nmoles O_2^-/min./10^6 cells	7.4 ± 0.76
nmoles O_2^-/min./particles deriving from 10^6 cells	0.54 ± 0.10
nmoles O_2^-/min./particles deriving from 10^6 cells in the presence of 0·05% Na-deoxycolate	1.7 ± 0.45

O_2 consumption and O_2^- release by intact cells were measured as described in Materials and Methods. NADPH oxidase was measured in the presence of 0·15 mM NADPH.

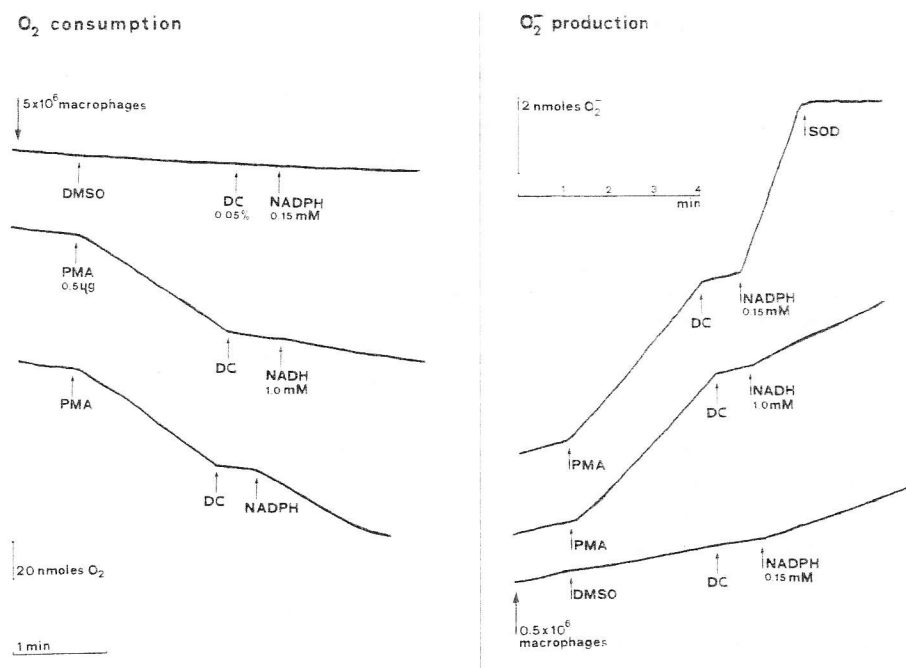The means of four experiments $\pm$ SEM are reported.

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O₂ consumption

O₂⁻ production

FIG. 5.—Continuous monitoring of O_2 consumption and O_2^- production by intact peritoneal-elicited macrophages and by the NAD(P)H oxidase activity of the same cells lysed with deoxycholate (DC).

On the contrary, by using resting macrophages, the addition of NADPH after DOC did not induce an increase in O_2 consumption or in O_2^- production. The addition of NADH after the lysis of PMA-stimulated macrophages did not cause the resumption of the O_2 consumption or O_2^- production.

Fig. 6 shows that the rate of the resumption of O_2^- production is a function of the concentration of the NADPH. Trace D of fig. 6, shows that the O_2^- production of the lysate of PMA-treated cells is linked to the activity of the reactions of the hexose monophosphate shunt for glucose oxidation. In fact, the NADPH can be substituted by $NADP^+$ and glucose-6-phosphate.

All these results clearly indicate that when the oxidase of PMA-stimulated cells is measured in appropriate conditions, its activity can account for the respiratory burst of intact macrophages.

DISCUSSION

It is widely known that one of the properties shared by granulocytes and mononuclear phagocytes is that of undergoing a dramatic activation of the respiratory metabolism associated with the production of oxygen reactive species such as O_2^-, H_2O_2, $OH\cdot$ and singlet oxygen. This phenomenon occurs during phagocytosis and following the interaction of the plasma membrane of the cell with a number of stimuli including lectins (Romeo et al., 1973a), fatty
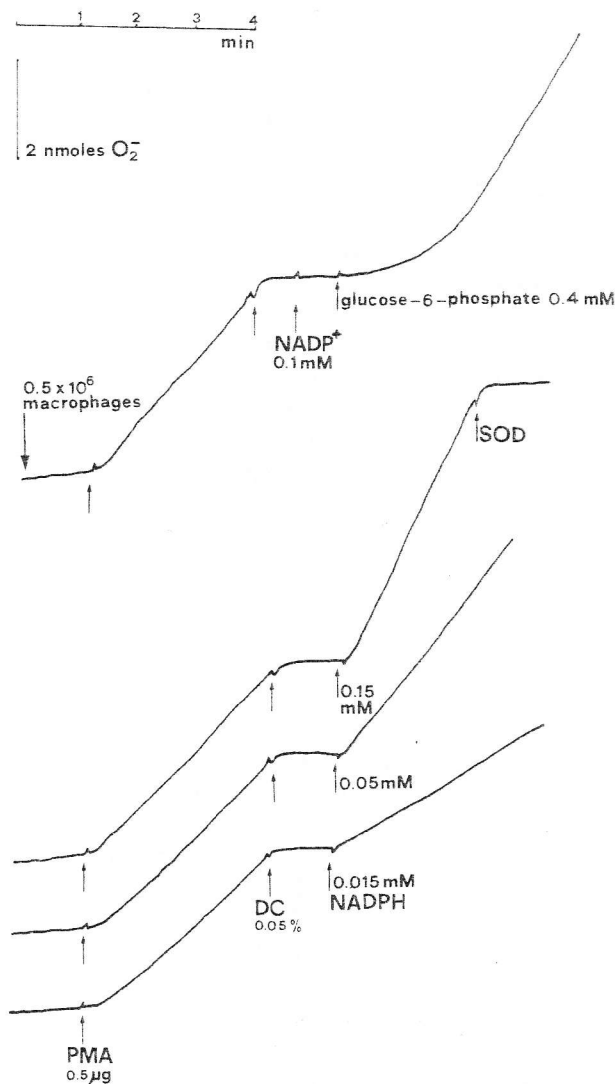
FIG. 6.—Continuous monitoring of O_2^- production by intact peritoneal-elicited macrophages and the NADPH oxidase activity of the same cells lysed with DOC in presence of different concentrations of NADPH.

acids (Kakinuma, 1974), activated fragments of complement (Goldstein *et al.*, 1975), chemotactic peptides (Becker, Sigman and Oliver, 1979), compounds which promote exocytosis (Romeo *et al.*, 1975) and others.

It seems natural that such a state of respiratory excitation is a common feature of the phagocytes engaged in different functions inside and outside the blood vessels, i.e. during chemotaxis, during the sticking to endothelial cells, during phagocytosis of infective agents or cellular debris in the inflammatory site, following contact with immunocomplexes or with agregating factors.

The functional meaning of this excited state is linked to the production of the highly reactive intermediates of oxygen reduction such as hydrogen peroxide and free radicals. The dangerous and the useful effects of these compounds have been reviewed by us elsewhere (Rossi *et al.*, 1979). Briefly they represent one of the strongest weapons by which the phagocyte, with and without phagocytosis, kills invading bacteria, viruses, mycoplasma, protozoa and tumour cells. The intermediates of the oxygen reduction represent in addition chemical messengers involved in various functions linked to the development and the modulation of the inflammatory response.

In spite of much information concerning the effects of the respiratory burst, the mechanism responsible for the activation of metabolism and the nature of the enzyme responsible for the generation of the intermediates of the oxygen reduction are poorly understood. The lack of knowledge is more marked with respect to the mononuclear phagocyte. The reasons for this depend on the fact that macrophages are an heterogenous population with a common origin but characterised by different degrees and type of differentiation.

We are investigating the enzymatic basis of the respiratory burst in various types of mononuclear phagocytes in order to find out the biochemical basis of the functions of these cells. The present paper reports on a group of data on the enzyme responsible for the respiratory burst in the inflammatory macrophages of the guinea pig. This is one of the more controversial problems in the biochemistry of phagocytosis.

Some authors believe that the respiratory metabolism of granulocytes is supported by a soluble NADH oxidase (Karnovsky, 1962; Badwey and Karnovsky, 1979; Badwey *et al.*, 1979). According to others, the enzyme responsible for the respiratory burst is a membrane bound oxidase that uses NADPH as the physiological substrate (Iyer *et al.*, 1961; Rossi and Zatti, 1966; Selvaray and Sbarra, 1967; Patriarca *et al.*, 1971; Babior *et al.*, 1975 and 1976; De Chatelet *et al.*, 1975; Hohn and Lehrer, 1975; Iverson *et al.*, 1977; Cohen *et al.*, 1980; Kakinuma and Kaneda, 1980; Bellavite *et al.*, 1980).

First of all we measured the NAD(P)H oxidase activity in the cytosol and in particulate fractions of resting and PMA-stimulated cells. The oxidase activity of the cytosol of resting elicited macrophages is very low in the presence of both pyridine nucleotides. Moreover this low activity does not change after the metabolic activation by PMA. The physiological role of the oxidase contained in the cytosol of this type of macrophages is unknown. We also do not know whether the oxidation of NADPH and NADH is performed by a single or by different enzymes. However, the fact that the activity does not change after stimulation by PMA makes it unlikely that this enzyme (or these enzymes) play a role in the activation of the respiratory metabolism of intact cells. The oxidase activity of cell-free particles of resting elicited macrophages is also very low both with NADH and NADPH. In the cell-free particles of PMA-stimulated macrophages the enzymatic activity is greatly enhanced only in the presence of NADPH. In this case also we do not know whether the oxidation of NADPH and NADH is performed by a unique enzyme or by different

enzymes. Whatever the case, the data clearly suggest that the physiological substrate for the enzyme activated by PMA is NADPH.

This activation can explain both the production of the intermediates of the oxygen reduction and, by forming $NADP^+$ with the modification of the ratio $NADPH/NADP^+$, the increase of glucose catabolism through the hexose-monophosphate pathway, that is one of the characteristics of the activated metabolism.

However, besides the rate, other characteristics of the oxidase must be examined before establishing its quantitative role in the excitation of the respiratory metabolism. They are as follows: (1) extent of activation, (2) kinetic properties, (3) sensitivity to inhibitors and (4) ability to account for the metabolic activation of intact cells.

With regard to the first point we have shown (fig. 4) that the extent of activation of the NADPH oxidase of cell-free particles 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 (K_m) and the maximum velocity (V_{max}) of the enzyme with NADH and NADPH clearly show that the preferential substrate is NADPH. In fact the K_m for NADPH is about one order of magnitude lower than that for NADH and the V_{max} with NADPH is double than that with NADH. The values of K_m are similar to those found in our (Patriarca *et al.*; Rossi *et al.*, 1972) and in other laboratories (De Chatelet *et al.*; Babior *et al.*, 1976; Cohen *et al.*) for the " membrane bound " oxidase of other phagocytes.

With regard to the third aspect it is worth pointing out the correlation between the effect of inhibitors on the NADPH oxidase and the respiratory burst of intact cells. In fact, the NADPH oxidase of cell-free particles is practically insensitive to azide, cyanide, rotenone and antimycin A as is the respiratory burst of intact cells. The sulphydryl reagent PCMB, markedly inhibits the enzymatic activity of cell-free particles and, in appropriate concentrations, also prevents the respiratory response of intact cells.

The last point that remains to be discussed is the adequacy of the activity of the enzyme to account for the phenomena observed in intact cells. The comparison between the respiratory metabolism of intact cells and the O_2^- production by cell-free particles (table V) seems to indicate that the enzymatic activity is inadequate to account for both the O_2^- generation and the O_2 consumption of the cells.

It is possible that the inadequacy stems from an underestimate of the rate of the NADPH oxidase in cell-free particles with respect to its rate in intact cells, due, for example, to inaccessibility of the substrate and/or cytochrome c to the O_2^--forming site of the enzyme. The fact that DOC increases the O_2^- production in activated cell-free particles about three-fold, indicates that this may be the case. However, the oxidase activity of cell-free particles of PMA-treated macrophages remains inadequate to account for the respiratory burst of intact macrophages even when its activity is measured in the presence of DOC, i.e. in the best conditions for revealing all the O_2^- production. It is likely that besides NADPH oxidase other enzymes or other respiratory systems

are involved in the activation of the respiratory metabolism in macrophages. Another possibility is that the oxidase is deactivated or inactivated by the procedures of cell disruption and during the time of cell homogenization and centrifugation. It has been shown by Jandl *et al.* (1978) that the activation of the NADPH oxidase of human granulocytes is not a permanent state and that within a few minutes of activation, the enzyme progressively loses its activity.

If this is the case one would expect that the real activity of the oxidase can be fully revealed if the oxidase is measured soon after the activation has taken place and in conditions allowing complete accessibility of the substrate and of cytochrome c to the site of O_2^- generation. This expectation has been verified by using the experimental model described in fig. 5. Under these experimental conditions the NADPH oxidase measured as rate of O_2^- generation and as O_2 consumption, fully account for the respiratory burst of intact cells measured as O_2^- generation and O_2 consumption respectively. These results clearly suggest that activation of the NADPH oxidase may be the major key mechanism responsible for the excited respiratory metabolism of macrophages. Similar results have been obtained in guinea-pig granulocytes (Rossi and Zatti, 1964; Rossi *et al.*, 1972) and in human granulocytes (Babior *et al.*, 1976) and recently in guinea-pig peritoneal resident macrophages (Bellavite *et al.*).

The molecular mechanism triggering the activation of the oxidase is practically unknown. A prerequisite to understanding this matter is the knowledge of the subcellular localization of the enzyme. This point has been investigated in our laboratory and the results are reported in the accompanying paper.

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