From: MACROPHAGES AND LYMPHOCYTES, Part A
Edited by Mario R. Escobar and Herman Friedman
(Plenum Publishing Corporation, 1980)

O_2^- AND H_2O_2 PRODUCTION DURING THE RESPIRATORY BURST IN ALVEOLAR MACROPHAGES

F. ROSSI, G. ZABUCCHI, P. DRI, P. BELLAVITE and G. BERTON

Istituto di Patologia Generale
Trieste (Italy)

It is known that the process of phagocytosis in polymorphonuclear leukocytes and in macrophages is associated with a dramatic change in oxidative metabolism. This change includes: an increase in oxygen consumption, in O_2^- and H_2O_2 generation and in glucose catabolism through the hexose monophosphate pathway (HMP) (3,7,9,10,13,20-23,29,31,44-46,49,56). These metabolic changes are referred to as the "respiratory burst" of phagocytic cells.

A similar stimulation of oxidative metabolism is induced also when phagocytic cells are exposed to membrane perturbing agents such as endotoxins (51), phospholipase C (30), phorbol myristate acetate (35), concanavalin A (40), antileukocyte antibodies (48), inophores (39,50), chemotactic fragments of complement (15,52), etc.

In general terms it appears that the stimulation of the oxidative metabolism has a variable intensity depending on cell type, on the sources of phagocytic cells, on the animal species, on the type of stimulant used, and, in macrophages (29), with the state of activation.

An analysis of the various events of the respiratory burst shows the following situation: 1) All the phagocytic cells so far investigated present an increase in the oxygen consumption and in HMP activity. As regards the oxygen consumption, by and large, it may be said that polymorphs and inflammatory peritoneal macrophages show the greatest stimulation, whereas the lung macrophages show a moderate extrarespiration. 2) The present status of our knowledge of the other aspects of the respiratory burst

such as the mechanism and the amount of O_2^- and of H_2O_2 generation is not very clear. In general terms it may be said that the stimulation of the generation of these highly reactive compounds, and the amount that is measurable, presents a variability much greater than that of oxygen consumption and of HMP activity. The main reason of this variability is that O_2^- and H_2O_2 are intermediate products, which can be rapidly degraded by the cells.

Within this variability, two peculiar situations have been presented: the first one regards the polymorphs of chicken in which the phagocytic act would be accompanied by an increase in the respiration and in HMP activity but not in the production of O_2^- and of H_2O_2 (34).

The second situation is represented by alveolar macrophages of rabbit, rat and guinea pig where during the stimulation of the respiration no O_2^- and no, or only traces, of H_2O_2 would be produced (Table I).

In order to explain these results, the following hypotheses can be advanced: 1) The association between oxygen consumption and O_2^- and H_2O_2 production is not a general dogma of the metabolic burst of phagocytes. 2) In some type of cells only a very small part of oxygen consumed is reduced to H_2O_2. 3) In all the phagocytic cells the stimulation of oxygen consumption is associated with the general of O_2^- and H_2O_2 but in some cells these intermediates are degraded or utilized as fast as they are formed.

It is widely accepted that one of the purposes of the respiratory burst is to provide a battery of oxidizing agents that can be used in the phagocytic vacuole or in the extracellular environment for the killing and the destruction of microorganisms.

We have reinvestigated the respiratory burst of rabbit alveolar macrophages. In this paper, evidence will be provided that also in these cells the stimulation of O_2 consumption is associated with generation of O_2^- and H_2O_2.

MATERIALS AND METHODS

Preparation of the cells. Alveolar macrophages (AM) were obtained by tracheobronchial lavages from rabbits, 15 days after the intravenous injection of 10 mg of BCG (kindly supplied by Istituto Vaccinogeno Antitubercolare, Milano, Italy) suspended in 1 ml of physiological saline (37).

Polymorphonuclear leukocytes (PMNL) were obtained by injecting intraperitoneally 100 ml of 1% sterile sodium caseinate solution. The exudates were collected 14 hr later.

TABLE I

Reports of H_2O_2 and O_2^- Production by Alveolar Macrophages During the Respiratory Burst Induced by Phagocytosis

Authors	References	Species	O_2^-	H_2O_2
Paul et al.	(32)	rabbit	-	traces
Gee et al.	(13)	rabbit	-	traces
Klebanoff and Hamon	(24)	rabbit	-	traces
DeChatelet et al.	(9)	rabbit	no	-
		rabbit, BCG	no	-
Drath and Karnovsky	(10)	mouse	yes	-
		guinea pig	no	-
Gee and Khandwala	(12)	rabbit	no	-
Biggar et al.	(5)	rabbit	no	-
Bolen	(see ref. 29)	rabbit	-	traces
Tsan	(53)	rabbit	no	no
Biggar	(6)	rat	-	no
Lowrie	(25)	rabbit	yes	-

The cells were subjected to hypotonic treatment in order to lyse contaminating erythrocytes, and resuspended in Krebs Ringer phosphate buffer pH 7.4 (KRP) containing 5 mM glucose and 0.5 mM $CaCl_2$, and counted in an hemocytometric chamber.

Differential counts were carried out with May-Grunwald and Giemsa stained smears. On the average, alveolar lavages consisted of 87.6% macrophages, 8.5% lymphocytes and 3.9% polymorphs. The peritoneal exudates contained 93.5% polymorphs, 4.5% lymphocytes and 2.0% monocytes.

Metabolic assays. Oxygen consumption was measured polarographically with a Clark type oxygen electrode as previously described (38).

Superoxide anion was measured by the superoxide dismutase (SOD) inhibitable reduction of cytochrome c (2), on samples withdrawn from the electrode vessel, 4 min after the addition of the stimulatory agent. The experimental details have been described previously (11).

Hydrogen peroxide was measured flurimetrically by the homovanillic acid (HVA) method. This procedure is based on the conversion of the non-fluorescent compound homovanillic acid to the highly fluorescent 2, 2'dihydroxy3,3'dimethoxydipheni15,5'diacetic acid, by horse radish peroxidase in the presence of H_2O_2 (17,18). In our hands, concentrations of H_2O_2 as low as 0.03 µM could be determined by this method. Three experimental procedures were employed: in the first one (method A) the H_2O_2 accumulated was measured on the supernatants of samples withdrawn from the electrode vessel, where oxygen consumption was being continuously recorded, 4 min after the addition of the stimulatory agent. Aliquots of such supernatants (10-100 µl) were added to a spectrofluorimetric cuvette containing in 2.5 ml of KRP, 0.02 mM HVA and 20 µg of HRP, and the increase of fluorescence was compared with appropriate standards of H_2O_2. The molecular extinction coefficient of H_2O_2 at 230 nm was determined on H_2O_2 solutions whose concentration was checked by titration with $KMnO_4$. The value obtained from four determinations was 0.0622 ± 0.00045 (SD) $cm^{-1}mM^{-1}$. In the second procedure (method B) HVA (0.8 mM) and HRP (20 µg/ml) were included in the incubation mixture, where oxygen consumption was being continuously recorded, and the fluorescence developed, indicating the H_2O_2 released and simultaneously trapped, was measured on the supernatants of samples withdrawn from the electrode vessel, 4 min after the addition of the stimulatory agent. In the third one, the kinetics of H_2O_2 release during the respiratory burst were carried out in a spectrophotofluirimetric cuvette, under continuous stirring, and the increase of fluorescence was followed with a CGA mod. DC3000 recording spectrophotofluorimeter.

Glucose oxidation: the rate of $^{14}CO_2$ production from 1-^{14}C-glucose was determined as described elsewhere (38).

Cell fractionation. The cells, sedimented and resuspended in 0.35 M sucrose ($300-400x10^6$/ml), were homogenized in a potter type glass homogenizer equipped with a teflon pestle, until more than 90% of the cells was broken as judged by light microscopic examination. The homogenate was then centrifuged at 100,000 g for 20 min and the pellet resuspended in 0.34 M sucrose to the original volume.

Enzyme assays. Superoxide dismutase activity was assayed according to the method of McCord and Fridovich (28), in the presence of 10 μM KCN in order to inhibit peroxidases and cytochrome c oxidase that would lead to an overstimulation of SOD activity (27).

Catalase activity was assayed by following the disappearance of H_2O_2 at 230 nm with an Hitachi-Perkin Elmer recording spectrophotometer was previously described (4).

Glutathione peroxidase and reductase activities were measured according to Gennaro et al. (14).

Peroxidase activity was assayed with the guaiacol test as described elsewhere (36).

RESULTS AND DISCUSSION

We began the investigation by comparing the various events of the respiratory burst of AM and PMNL.

The data of Table II show that the respiratory burst induced by phagocytosis in PMNL is associated with a substantial release of O_2^- and with a very small amount of H_2O_2 accumulated. On the contrary, the respiratory burst of AM is not accompanied by an accumulation of H_2O_2 while only traces of O_2^- are released. These results although in agreement with those reported by others (Table I) cannot be used as an evidence that AM do not produce H_2O_2. In fact in the experimental procedure employed, O_2^- was measured with cytochrome c present in the incubation medium, in such a way as the free radical is trapped as it is released from the cells. On the contrary, H_2O_2 was determined on samples withdrawn from the electrode vessel 4 min after the addition of bacteria. With this system only the aliquot of H_2O_2 that escaped the degradation was measured.

The reactions involved in oxygen consumption. Before describing the next data, in order to make clear the strategy of the experimental approaches and the results, it is worthy to indicate the reactions involved in O_2 consumption, in O_2^- and H_2O_2 formation and in H_2O_2 degradation (Fig. 1). Assuming that the reduction of O_2 essentially proceeds via a one electron pathway, in the first step (reactions 1, 1' of Fig. 1) O_2^- is formed. Evidences have been presented in our and in other laboratories that RH_2 is NADPH and that the reaction is catalyzed by an NADPH oxidase (20,1,8,19,33,47,55). O_2^- is a very reactive compound, that in the absence of oxidants, dismutates according to reaction 2. This reaction proceeds very fast either spontaneously with a rate constant of $1_9 \times 10^5\ M^{-1}$, or catalyzed by SOD with a rate constant of $2 \times 10^9\ M^{-1}\ sec^{-1}$ (27). The H_2O_2 formed can be de-

TABLE II

O_2 Consumption, O_2^- Release and H_2O_2 Accumulation by Phagocytosing PMNL and AM from Rabbit

	PMNL (3)			AM (5)		
	Rest	Phag	Phag - Rest	Rest	Phag	Phag - Rest
O_2	31.5±5.4	166.7±17.8	135.2±13.8	116.0±13.3	194.9±14.3	78.9±7.6
O_2^-	9.2±8.1	124.6±33.4	115.4±39.6	0	4.5± 2.2	4.5±2.2
H_2O_2	0	10.1±3.0	10.1± 3.0	0	0	0

The values are expressed as nmoles/4 min/$1.5x10^7$ cells ± S.E.M.
Assay medium: $1-2x10^7$ PMNL or AM in 2 ml of KRP pH 7.4 containing 0.5 mM $CaCl_2$ and 5 mM glucose. Opsonized B. mycoides were used as stimulatory agent (ratio cell/bacteria, 1/100).%= 37 C. O_2^- was measured by adding 400 µmoles of cytochrome c and, where required, 70 µg of SOD.
H_2O_2 was measured according to method A described in materials and methods.

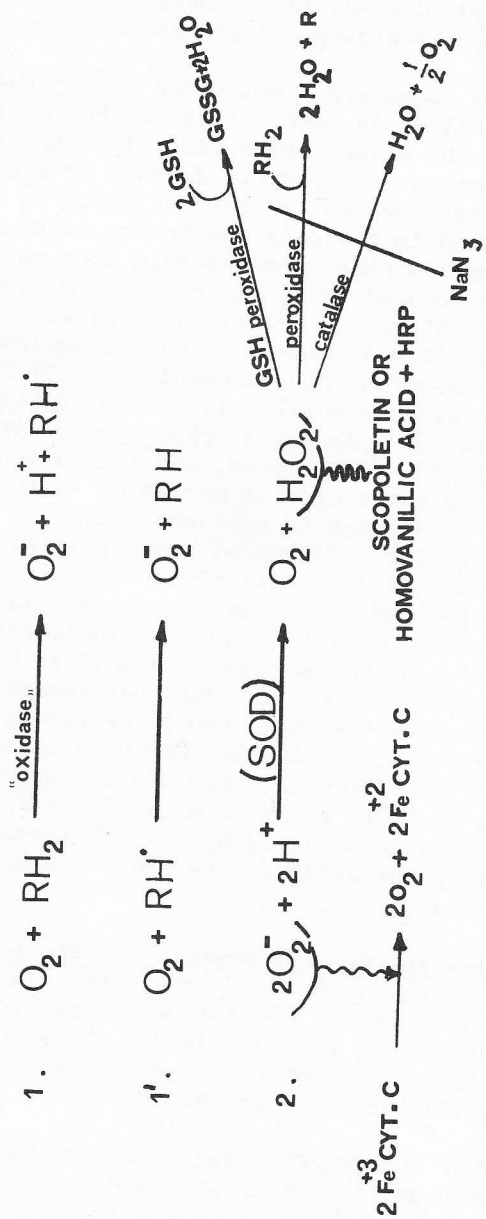

Fig. 1 - Scheme of the metabolic pathways involved in oxygen consumption, O_2^- and H_2O_2 production and degradation in phagocytes. The methods for O_2 and H_2O_2 measurement are also shown.

graded by different mechanism that is catalatic, where $\frac{1}{2}O_2$ is given back for each molecule of H_2O_2, or peroxidatic, where a reduced compound is oxidized and H_2O is formed. The main enzymatic reactions for H_2O_2 degradation in phagocytic cells are catalyzed by catalase, peroxidase and glutathione peroxidase.

Assuming that the above reactions occur during the activated metabolism of AM as well as in PMNL, it is worthy to stress the following points: 1) the steady state rate of oxygen consumption and O_2^- and H_2O_2 generation and recovery depends on the activity level of the O_2^- reductase and on the rate at which O_2^- and H_2O_2 are released or utilized in the cell and in the surrounding medium. 2) The oxygen consumption that is measurable does not correspond to all oxygen that is reduced to O_2^- since this compound is not accumulated. The stoichiometric relationship between the actual oxygen consumed and O_2^- formed depends on the mechanism of H_2O_2 degradation and on the amount of H_2O_2 accumulated. 3) In many phagocytic cells, for example guinea pig, human, rabbit PMNL and in inflammatory peritoneal mononuclear phagocytes, part of O_2^- formed in reactions 1, 1', is released, and this aliquot undergoes dismutation in the extracellular medium or in the phagocytic vacuole. The amount of O_2^- that is released varies in different cell types. 4) In normal conditions of phagocytosis, that is when one allows that H_2O_2 follow its physiological fate, only a small aliquot of the peroxide is accumulated. 5) The amount of H_2O_2 that is accumulated can be increased by inhibiting the reations involved in its degradation. When impeded to be degraded H_2O_2 is released from the cells. 6) Only the amount of O_2^- and H_2O_2 that is released from the cells can be measured. For the first intermediate the SOD inhibitable reduction of cytochrome c or NBT can be used. The hydrogen peroxide is correctly measured by trapping as it is released or as it is formed by dismutation of O_2^-. This is achieved by using horseradish peroxidase (HRP) catalyzing the oxidation by H_2O_2 of compounds such as scopoletin or homovanillic acid.

Activities of the enzymes responsible for the formation and for the degradation of O_2^- and H_2O_2 in rabbit AM. In this group of experiments we try to answer the following questions: 1) is the NADPH oxidase the enzyme responsible for the reactions 1, 1' (Fig. 1) present in AM? 2) What is the activity of the enzymes involved in the degradation of O_2^- and H_2O_2?

Fig. 2 shows that the 20,000 g pellet of the postnuclear supernates of homogenates of resting AM, oxidize NADPH with formation of H_2O_2. This oxidizing activity is highly activated in the same fractions obtained from phagocytosing AM. This suggests that in these cells the enzymatic basis of the metabolic burst is similar to that described in PMNL (1,8,19,33,47,55). The

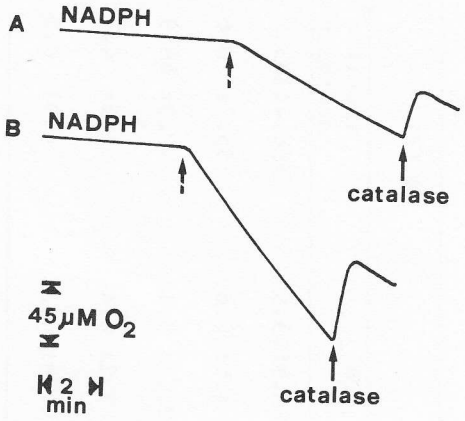

Fig. 2. Production of H_2O_2 during oxidation of NADPH by 20,000g sedimentable fractions isolated from resting (A) and phagocytosing (B) rabbit alveolar macrophages. Assay medium: 65mM Na, K-phosphate buffer pH 5.5, 125 mM sucrose, 0.5 mM $MnCl_2$, 1 mM NADPH. Final volume 2 ml T= 37 C. At the points indicated by broken arrows 80 μg of protein of 20,000g sedimentable fraction was added.

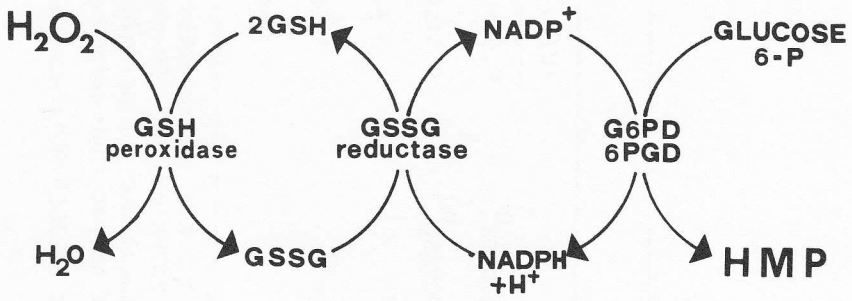

Fig. 3. Relationship between H_2O_2 degradation through the glutathione cycle and the hexose monophosphate pathway.

TABLE III

Enzyme Activities in Rabbit PMNL and AM

	PMNL (6)		AM (7)	
	*U/10^6 cells	specific activity	*U/10^6 cells	specific activity
GSH-PEROXIDASE^0	0.62±0.19	36.5± 8.6	76.9±15.5	998.4±133.2
GSSG-REDUCTASE^0	0.51±0.07	30.0± 7.2	4.5± 0.6	55.7± 6.6
CATALASE^0	4.0 ±0.3	285.7±60.5	26.2± 3.1	340.3± 46.9
PEROXIDASE $^\square$	21.8 ±8.9	660.6±127.6	2.2± 0.4	21.5± 4.7
SOD$^\Delta$	0.13±0.03	2.6± 0.6	0.74± 0.12	4.31 0.5

The mean ± S.E.M. is reported. The number of experiments is given in parentheses.
* nmoles NADPH/min for GSH-peroxidase and GSSG-reductase, µmoles H_2O_2/min for catalase, nmoles tetraguaiacol/min for peroxidase. One unit of superoxide dismutase (SOD) is defined as the amount of enzyme that causes a decrease in the reduction of cytochrome c of 0.0125 OD/min.
^{0}Measured on 1000,000g supernatant, $^\square$ measured on 100,000g pellet, $^\Delta$measured on total homogenate.

results presented in Table III show that in AM, glutathione peroxidase, glutathione reductase, catalase and superoxide dismutase have activities much higher than those of PMNL. On the contrary, the peroxidase activity is higher in PMNL. From the pattern of the enzymatic activities, it appears that in comparison with PMNL, AM have very active mechanisms for H_2O_2 degradation. Since catalase has usually a low affinity for H_2O_2 and the activity of peroxidase is very low, it is likely that the main mechanism for H_2O_2 degradation in AM is linked to the activity of glutathione peroxidase coupled with that of glutathione reductase (glutathione cycle).

The utilization of H_2O_2 through the glutathione cycle is coupled with an increased activity of HMP, as shown in Fig. 3. On these basis the efficiency of the glutathione cycle can be investigated by measuring the effect of externally added H_2O_2 on $^{14}CO_2$ production from 1-^{14} C-glucose, that in these cells indicates the rate of glucose oxidation through the HMP.

The results reported in Fig. 4 show that the effect of exogenous H_2O_2 is much greater in AM than in PMNL. Furthermore, in AM the intensity of the stimulation of HMP activity by 0.05 mM H_2O_2 is very near to the maximal stimulation induced by phagocytosis.

This group of experiments indicate that in AM the respiratory burst is associated with H_2O_2 formation and that the peroxide is rapidly utilized as it is formed.

Attempts to increase the accumulation of H_2O_2. This was achieved by using two devices. The first one was that of inhibiting some of the reactions involved in H_2O_2 degradation, that is the heme enzymes catalase and peroxidase with NaN_3. The second way was that of trapping H_2O_2 as it is formed and released in NaN_3 treated alveolar macrophages, by adding HRP and homovanillic acid. The results presented in Table IV show that in the presence of NaN_3 (column II) the stimulation of O_2 consumption induced by phagocytosis in PMNL is associated with a marked accumulation of H_2O_2, while in AM, only traces of H_2O_2 are measurable. When the H_2O_2 trapping system is included in the incubation medium (column III) a definite amount of H_2O_2 becomes constantly measured during the respiratory burst of AM. By comparing the amounts of O_2 actually consumed and of H_2O_2 measured, it is evident that in AM the percentage of O_2 consumed, recovered as H_2O_2, is very low, while in PMNL is very high. These results clearly indicate that the main mechanism for H_2O_2 utilization involve reactions NaN_3-sensitive (catalase and myeloperoxidase) in PMNL, and reactions NaN_3-insensitive (glutathione peroxidase) in AM.

In subsequent experiments a continuous monitoring of H_2O_2

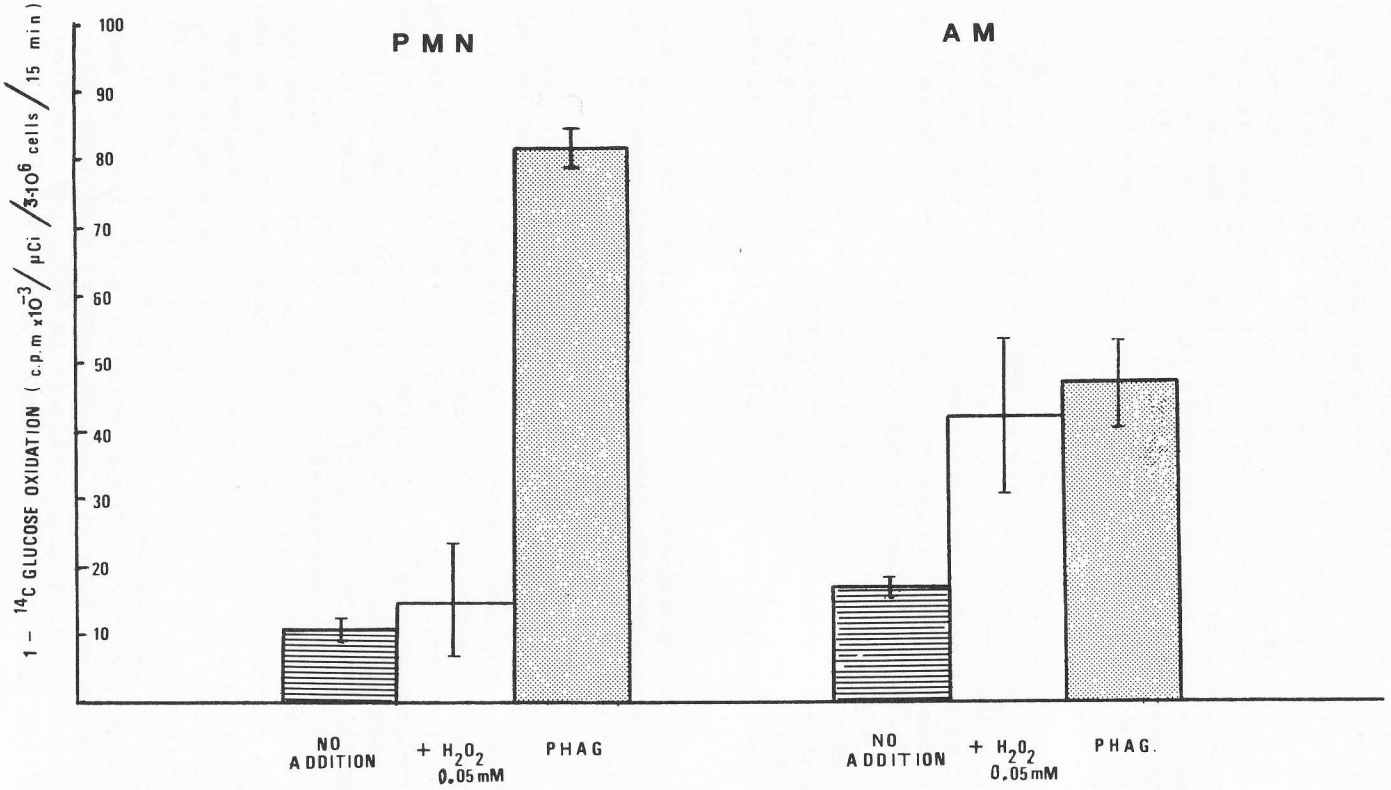

Fig. 4. Effect of bacteria and H_2O_2 addition on the activity of the hexose monophosphate pathway of rabbit PMNL and AM. Assay medium: $3x10^6$ cells in 1 ml of KRP pH 7.4 containing 0.5 mM $CaCl_2$, 0.2 mM glucose, 0.2 µCi of glucose-1-^{14}C. Opsonized B. mycoides was used as stimulatory agent (ratio cell/bacteria, 1/100). Vertical bars indicate S.E.M. T= 37 C.

TABLE IV

O_2 Consumption, O_2 Release and H_2O_2 Accumulation by Phagocytosing PMNL and AM from Rabbit

	I		II + NaN_3		III + NaN_3 + HRP + HVA	
	PMNL (3)	AM (5)	PMNL (5)	AM (6)	PMNL (3)	AM (6)
O_2	135.2±13.8 (31.5± 5.4)	78.9± 7.6 (116.0±13.3)	195.9±12.9 (21.3± 5.6)	76.2±14.0 (111.7±18.5)	203.6±29.4 (18.8± 5.4)	71.3±12.8 (101.3±12.9)
O_2^-	115.4±39.5 (9.2± 8.1)	4.5± 2.2 (0)	105.9±20.7 (9.2±8.1)	6.0± 1.5 (0)	---	---
H_2O_2	10.1± 3.0 (0)	0	179.3±17.2 (6.2± 2.7)	0.2± 0.1 (0)	163.0±25.2 (7.8± 3.5)	7.1± 1.9 (0)
%O_2 recovered as H_2O_2	7.4	0	91.5	0.26	80.0	10.0

The differences between phagocytosing and resting cells are reported. The values are expressed as nmoles/4 min/$1.5x10^7$ cells ± S.E.M. Resting values are given in brackets.
Assay medium: $1\text{-}2x10^7$ PMNL or AM in 2 ml of KRP pH 7.4 containing 0.5 mM $CaCl_2$ and 5 mM glucose. Opsonized B. mycoides were used as stimulatory agent (ratio cell/bacteria, 1/100). NaN_3, 2 mM. T= 37 C. O_2 was measured as described in Table II. H_2O_2 was measured according to method A (columns I,II) and B (column III), described in materials and methods.

production during phagocytosis in NaN_3-treated AM and PMNL was employed. This was achieved by recording the change of fluorescence during the oxidation of homovanillic acid by HRP. A typical experiment is reported in Fig. 5. In the experiments performed with AM, only a small amount of H_2O_2 is detectable when the reactants for H_2O_2 measurement are added some min after addition of bacteria (trace B'). In contrast, when the reactants for trapping the peroxide are present at the beginning of the respiratory burst, H_2O_2 is detectable after a lag time of about 3-4 min. In the experiments performed by using PMNL a marked amount of H_2O_2 is measurable either when the reactants are present at the beginning of the metabolic burst or some min after addition of bacteria. These results are in agreement with those of Table IV and strengthen the conclusion that in AM, H_2O_2 is rapidly utilized even when catalase and peroxidase are inhibited.

This group of results permit the following conclusions: 1) By using appropriate devices it is possible to increase also in AM the amount of H_2O_2 that is measurable. 2) In any case the amount of H_2O_2 detectable represents a very low percentage of the O_2 actually consumed.

Effect of cytochalasin B on the production of O_2^- and H_2O_2 during phagocytosis. The results presented above introduce the problem of whether or not the small percentage of H_2O_2 accumulated represents the total amount that is formed or the aliquot that escaped the intracellular degradation. If the latter is the case it could be possible to increase this aliquot. This result was, at least partially, achieved by using cytochalasin B (CB), a drug that is known to increase the release of lysosomal enzymes and of O_2^- in phagocytic cells during the respiratory burst (16,42,43,57). Table V illustrates the effect of CB on oxygen consumption, O_2^- and H_2O_2 release during phagocytosis in NaN_3-treated AM and PMNL. The data show that: 1) CB induces a decrease of the intensity of the stimulation of oxygen consumption both in PMNL and AM. This effect is related to the inhibition of the rate of phagocytosis as shown in our and in other laboratories (26,41,43) and as it has been controlled in these experiments. 2) CB has also a great effect on the release of O_2^- both in PMNL and AM. In fact while CB induces a decrease of the total amount of oxygen univalently reduced, as it is shown by the decrease of measurable oxygen consumed, the aliquot of O_2^- released in comparison with the actual oxygen consumed is markedly increased either in PMNL and in AM. 3) CB increases the amount of the peroxide recovered in the extracellular medium of phagocytosing AM either as absolute value or as related to the extrarespiration. In fact, the percentage of oxygen actually consumed recovered as H_2O_2 rises from 10.0 to 25.0% in the presence of CB. In other words, by using CB we can see that a consistent aliquot of the oxygen consumed by AM during the respiratory burst is recovered as H_2O_2. This aliquot

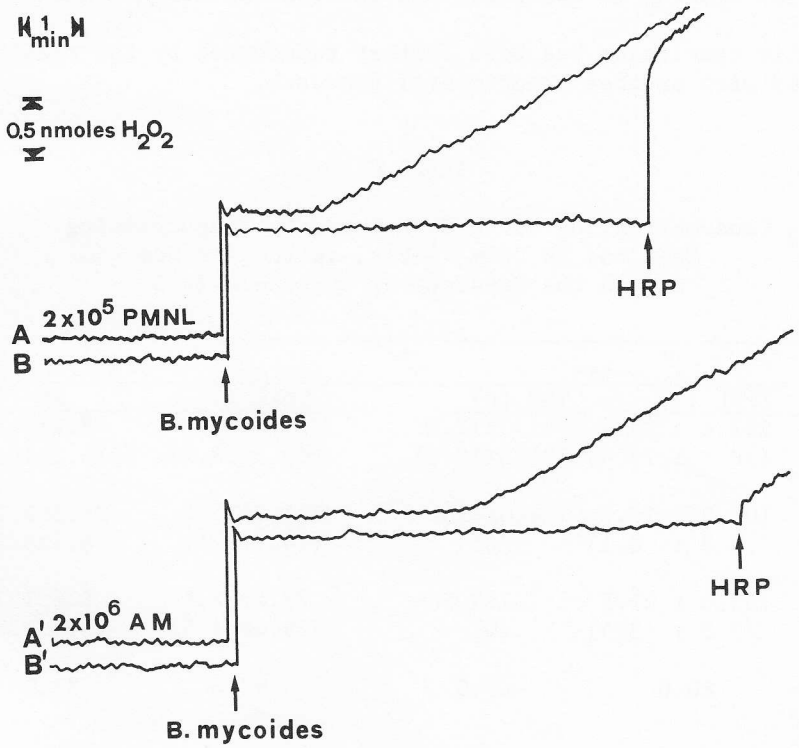

Fig. 5. Spectrophotofluorometric traces of H_2O_2 production by rabbit PMNL and AM during phagocytosis. Assay medium: 2.5 ml of KRP pH 7.4 containing 0.5 mM $CaCl_2$, 5 mM glucose, 2 mM NaN_3, 20 µM HVA and 20 µg HRP. A,A', complete system. B,B', HRP, omitted from the assay medium was added where indicated.

does not reach the values obtained in PMNL where 97.6% of oxygen consumed is recovered as H_2O_2 when CB is present.

Apart from the mechanisms by which CB causes these effects, that are in agreement with the results published by others (42, 43), the data presented in this group of experiments indicate that in AM the type of the metabolic burst is similar to that of granulocytes; that is, the univalent reduction of oxygen followed by formation of H_2O_2 is operative and substantial also in AM.

This conclusion has been further reinforced by the results obtained with another experimental approach.

TABLE V

O_2 Consumption, O_2^- and H_2O_2 Release by Phagocytosing PMNL and AM from Rabbit, in the Absence and in the Presence of Cytochalasin B

	-----		+ CB	
	PMNL (3)	AM (6)	PMNL (3)	AM (4)
O_2	203.6 ± 29.4 (18.8 ± 5.4)	71.3±12.8 (101.3±12.9)	79.3±8.7 (60.3±18.4)	38.1±5.7 (116.1±16.8)
O_2^-	105.9 ± 20.7 9.2 ± 8.1)	6.0±1.5 (0)	114.9±15.3 (24.7±0.4)	24.5±9.1 (6.2±3.1)
H_2O_2	163.0 ± 25.2 (7.8 ± 3.5)	7.1±1.9 (0)	77.4±28.8 (29.4± 4.5)	9.6±1.7 (3.6±2.6)
$\%O_2$ recovered as H_2O_2	80.0	10.0	97.6	25.0

The differences between phagocytosing and resting cells are reported. The values are expressed as nmoles/4 min/1.5×10^7 cells ± S.E.M. Resting values are given in brackets.
Assay medium: $1-2 \times 10^7$ PMNL or AM in 2 ml of KRP pH 7.4 containing 0.5 mM $CaCl_2$ and 5 mM glucose. Opsonized B. mycoides were used as stimulatory agent (ratio cell/bacteria, 1/100). NaN_3, 2 mM. T= 37 C. CB, 10 µg in 2 µl DMSO. In control experiments, 2 µl DMSO were added.
O_2 was measured as described in Table II. H_2O_2 was measured according to method B described in materials and methods.

Effect of concanavalin A and CB. In an attempt to get an insight into the effect of CB and in order to avoid the effect of this drug on the respiratory increment due to inhibition of the phagocytic act, we employed as a stimulatory agent the Concanavalin A (ConA). First of all we have studied the metabolic burst induced by ConA, evaluated as H_2O_2 formation, in NaN_3-treated AM in the presence and in the absence of CB. The results presented in a typical experiment (Fig. 6) show that: 1) When ConA is added to CB-treated AM (trace B) a very rapid and marked increase of fluorescence takes place, indicating that in this condition a substantial amount of H_2O_2 is released from stimulated cells. A similar effect is obtainable when CB is added after ConA (trace C).

The amount of H_2O_2 measured in CB-treated AM was unexpectedly high in comparison to that detected in the experiments presented above. This fact prompted further experiments in order to investigate the effect of CB on all the events of the respiratory burst induced by ConA. The data on the contemporaneous measurements of oxygen consumption, of O_2^- and of H_2O_2 reported in Table VI show that: 1) ConA alone induces in PMNL an increase of oxygen consumption associated with O_2^- and H_2O_2 formation and release. In AM ConA alone induces an increase of oxygen consumption which is associated with a small production and release of O_2^- and H_2O_2. The relationship between these three events are similar to that observed when the stimulation is induced by phagocytosis (Table IV). 2) Both in PMNL and AM, CB markedly enhances the activation of the oxidative metabolism triggered by ConA measured as oxygen consumed and O_2^- and H_2O_2 formation and release. These results clearly suggest that the increased amount of O_2^- and H_2O_2 detected is due to a double effect of CB. The first one is a potentiation of the stimulatory activity of ConA with increased formation of O_2^- and H_2O_2. The mechanisms by which CB induces this stimulation is at present unknown. It is worthy to point out that CB does not modify the amount of ^3H-labeled lectin bound to the surface of the cell (results not presented here). The second effect consists in an increased availability in the extracellular medium of both the intermediate products of oxygen reduction. The mechanism of this increased availability might be related to an increased permeability of the cell membrane to O_2^- and H_2O_2 or to an inhibition of some of the mechanisms of their degradation. The latter possibility is unlikely since we have seen that CB does not modify *in vitro* the activities of SOD, catalase, peroxidase and glutathione peroxidase and reductase.

Although we need further investigations to clarify the mechanisms by which CB causes this double effect, the results obtained with this experimental model, that is ConA plus CB, show that also in AM it is possible to obtain a respiratory burst in which the values of the various parameters (oxygen consumed,

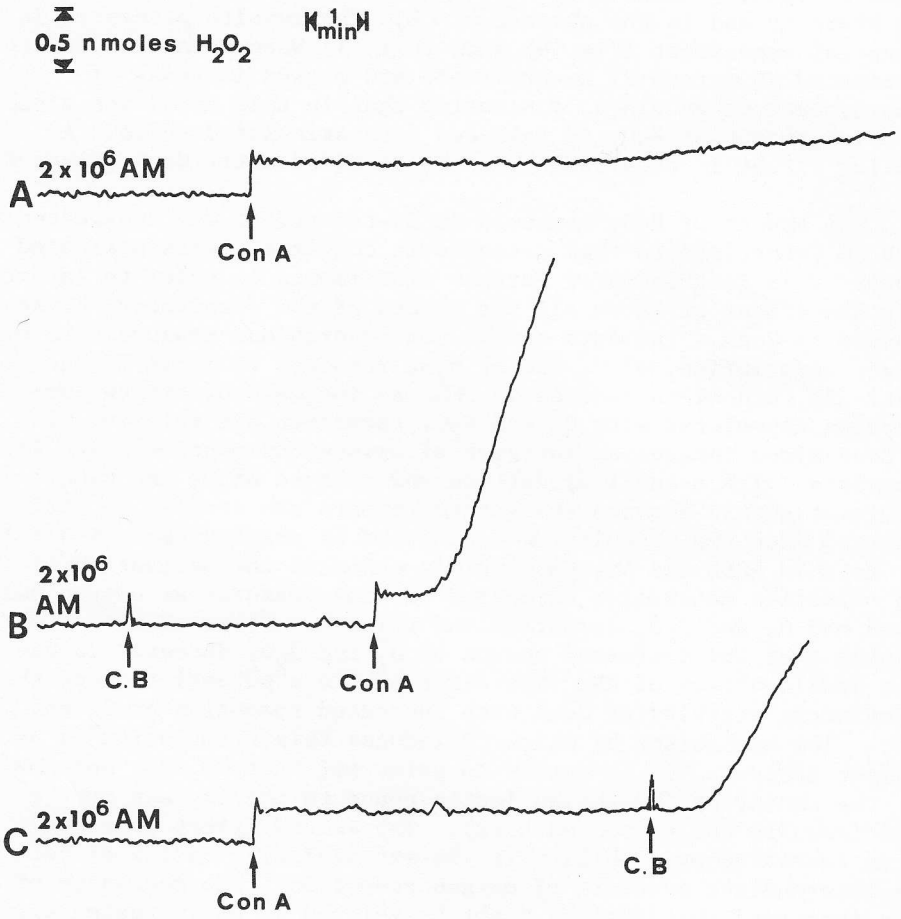

Fig. 6. Spectrophotofluorometric traces of H_2O_2 production by rabbit PMNL and AM stimulated by Con A, in the presence and in the absence of cytochalasin B. For experimental conditions see Fig. 5, Con A, 100 µg/ml. CB 12.5 µg in 2.5 µl DMSO. In A 2.5 µl DMSO were added.

O_2^- and H_2O_2 formed and released) and their quantitative relationship are in the range of the values obtained in the granulocytes of rabbit and of other mammalian species.

TABLE VI

O_2 Consumption, O_2^- and H_2O_2 Release by Rabbit PMNL and AM Stimulated by Concanavalin A in the Absence and in the Presence of Cytochalasin B

	Con A		Con A + CB	
	PMNL (3)	AM (4)	PMNL (3)	AM (4)
O_2	52.6±17.8 (18.8± 5.4)	24.3± 5.7 (101.3±12.9)	106.3±30.4 (60.3±18.4)	75.6± 6.9 (116.1±16.8
O_2^-	30.3±13.9 (9.2± 8.1)	3.7± 1.8 (0)	98.1±41.1 (24.7± 0.4)	48.4± 9.8 (6.2± 3.1)
H_2O_2	37.6±21.4 (7.8± 3.5)	2.1±1.5 (0)	93.4±42.7 (29.4± 4.5)	33.8± 2.2 3.6± 2.5)
$\%O_2$ recovered as H_2O_2	71.5	8.6	87.8	44.7

The differences between phagocytosing and resting cells are reported. The values are expressed as nmoles/4 min/1.5×10^7 cells ± S.E.M. Resting values are given in brackets.
The experimental conditions are described in Table V. Concanavalin A (100 µg/ml) instead of bacteria was used as stimulatory agent.

CONCLUSIONS

The results presented in this paper can be summarized as follows: 1) In rabbit AM the respiratory burst is associated with the generation of O_2^- and H_2O_2. 2) These intermediate products of oxygen reduction are not accumulated in physiological conditions because these cells are equipped with very efficient systems for their transformation and degradation. 3) The main mechanism for the degradation of H_2O_2 in these cells is represented by the glutathione cycle in agreement with the results presented by others (54). By using appropriate experimental conditions, the intermediate products of oxygen reduction can be released at high rate and recovered in the extracellular medium.

In another paper elsewhere in this volume, experiments performed with chicken PMNL, will be presented. By following the same line of experimental approach, we obtained evidence that also in these phagocytic cells the respiratory burst is associated with the production of O_2^- and H_2O_2.

Thus we are provided with experimental evidence that allows the following conclusions: 1) Among phagocytic cells so far investigated, examples do not exist where the stimulation of oxidative metabolism is not accompanied by the formation of O_2^- and H_2O_2. 2) The possibility of detecting these compounds depends on the great differences found among the various types of phagocytic cells with respect to the efficiency of the mechanisms for their degradation and, also, on the experimental strategy employed. 3) These differences in the efficiency and in the type of the reactions for the degradation of intermediate products of respiration suggest that the phagocytic cells are heterogeneous, even as regards the defensive role played by the respiratory burst.

ACKNOWLEDGEMENTS

P. Bellavite is recipient of a fellowship from "Fondazione Anna Villa Rusconi", Varese, Italy. Supported by grant No 77.01481.04 from the National Council of Research (CNR) of Italy.

REFERENCES

1. Babior, B. M., Curnette, J. T. and Kipnes, R. S., J. Clin. Invest. 56 (1975) 1035.
2. Babior, B. M., Kipnes, R. S. and Curnutte, J. T., J. Clin. Invest. 52 (1973) 741.
3. Baehner, R. L., Gilman, N. and Karnovsky, M. L., J. Clin. Invest. 49 (1970) 692.
4. Bellavite, P., Dri, P., Bisiacchi, B. and Patriarca, P., FEBS Letters 81 (1977) 73.
5. Biggar, W. D., Buron, S. and Holmes, B., Infect. Immun. 14 (1976) 6.
6. Biggar, W. D. and Sturgess, J. M., Infect. Immun. 19 (1978) 621.
7. Curnutte, J. T. and Babior, B. M., J. Clin. Invest. 53 (1974) 1662.
8. DeChatelet, L. R., McPhail, L. C., Mullikin, D. and McCall, C. E., J. Clin. Invest. 55 (1975) 714.
9. DeChatelet, L. R., Mullikin, D. and McCall, C. E., J. Lab. Clin. Med. 85 (1975) 245.
10. Drath, D. B. and Karnovsky, M. L., J. Exp. Med. 141 (1975) 257.
11. Dri, P., Bellavite, P., Bergon, G. and Rossi, F., Molec.

Cell. Biochem. (1978) in press.
12. Gee, J. B. L. and Khandwala, A. S., J. Reticuloendothel. Soc. 19 (1976) 229.
13. Gee, J. B. L., Vassallo, C. L., Bell, P., Kaskin, J., Basford, R. E. and Field, J., J. Clin. Invest. 49 (1970) 1280.
14. Gennaro, R., Schneider, C., de Nicola, G., Cian, F. and Roemo, D., Proc. Soc. Exp. Biol. Med. 157 (1978) 342.
15. Goetzl, E. J. and Austen,K. F., J. Clin. Invest. 53 (1974) 591.
16. Goldstein, I. M., Roos, D., Kaplan, H. B. and Weismann, G., J. Clin. Invest. 56 (1975) 1155.
17. Guilbaut, G. G., Brignac, P., Jr. and Zimmer, M., Anal. Chem. 40 (1968) 190.
18. Guilbaut, G. G., Kramer, D. N. and Hackley, E., Anal. Biochem. 39 (1967) 271.
19. Hohn, D. C. and Lehrer, R. I., J. Clin. Invest. 55 (1975) 707.
20. Iyer, G. J. N., Islam, M. F. and Quastel, J. H., Nature (London) 192 (1961) 535.
21. Karnovsky, M. L., Physiol. Rev. 42 (1962) 143.
22. Karnovsky, M. L., Lazdins, J. and Simmons, S. R., In: Mononuclear Phagocytes (Ed. R. Van Furth) Blackwell Scientific Publications, Oxford, England (1975) 423.
23. Klebanoff, S. J. and Hamon, C. B., J. Reticuloendothel. Soc. 12 (1972) 170.
24. Klebanoff, S. J. and Hamon, C. B., In: Mononuclear Phagocytes (Ed. R. Van Furth) Blackwell Scientific Publications, Oxford, England (1975) 507.
25. Lowrie, D. B. and Aber, V. R., Life Sciences 21 (1977) 1575.
26. Malawista, S. E., Gee, B. J. and Bensch, K. G., Yale, J. Biol. Med. 44 (1971) 286.
27. McCord, J. M., Crapo, J. D. and Fridovich, I., In: Superoxide and Superoxide Dismutase (Ed. A. M. Michelson, J. M. McCord and I. Fridovich) Academic Press, London (1977) 11.
28. McCord, J. M. and Fridovich, I., J. Biol. Chem. 244 (1969) 6049.
29. Nathan, C. F. and Root, R. K., J. Exp. Med. 146 (1977) 1648.
30. Patriarca, P., Zatti, M., Cramer, R. and Rossi, F., Life Sciences 9 Part I (1970) 841.
31. Paul, B. B. and Sbarra, A. J., Biochim. Biophys. Acta 156 (1968) 168.
32. Paul, B. B., Strauss, R. R., Jacobs, A. A. and Sbarra, A. J., Infect. Immun. 1 (1970) 338.
33. Paul, B. B., Strauss, R. R., Jacobs, A. A. and Sbarra, A. J., Exp. Cell Res. 73 (1972) 456.
34. Pennial, R. and Spitznagel, J. K., Proc. Nat. Acad. Sci. USA 72 (1975) 5012.
35. Repine, J. E., White, J. G., Clanson, C. C. and Holmes, B. M. J. Lab. Clin. Med. 83 (1974) 911.

36. Romeo, D., Cramer, R., Marzi, T., Soranzo, M. R., Zabucchi, G. and Rossi, F., J. Reticuloendothel. Soc. 13 (1973) 399.
37. Romeo, D., Zabucchi, G., Jug, M. and Rossi, F., Cell. Immunol. 13 (1974) 313.
38. Romeo, D., Zabucchi, G., Marzi, T. and Rossi, F., Exp. Cell Res. 78 (1973) 423.
39. Romeo, D., Zabucchi, G., Miani, N. and Rossi, F., Nature 253 (1975) 542.
40. Romeo, D., Zabucchi, G. and Rossi, F., Nature New Biol. 243 (1973) 111.
41. Romeo, D., Zubacchi, G. and Rossi, F., In: Movement, Metabolism and Bactericidal Mechanisms of Phagocytes (Ed. F. Rossi, P. Patriarca and D. Romeo) Piccin Medical Books, Padova (1977) 154.
42. Roos, D., Homan-Miller, J. W. T. and Weening, R. S., Biochim. Biophys. Res. Commun. 68 (1976) 43.
43. Root, R. K. and Metcalf, J. A., In: Movement, Metabolism and Bactericidal Mechanisms of Phagocytes (Ed. F. Rossi, P. Patriarca and D. Romeo) Piccin Medical Books, Padova (1977) 185.
44. Root, R. K., Metcalf, J. A., Oshino, N. and Chance, B., J. Clin. Invest. 55 (1975) 945.
45. Rossi, F., Romeo, D. and Patriarca, P., J. Reticuloendothel. Soc. 12 (1972) 127.
46. Rossi, F., Zabucchi, G. and Romeo, D., In: Mononuclear Phagocytes (Ed. R. Van Furth) Blackwell Scientific Publications, Oxford, England (1975) 441.
47. Rossi, F. and Zatti, M., Brit. J. Exp. Pathol. 45 (1964) 548.
48. Rossi, F., Zatti, M., Patriarca, P. and Cramer, R., J. Reticuloendothel. Soc. 9 (1971) 67.
49. Sbarra, A. J. and Karnovsky, M. L., J. Biol. Chem. 234 (1959) 1355.
50. Schell-Frederick, E., FEBS Letters 48 (1974) 37.
51. Strauss, B. S. and Stetson, C. A., J. Exp. Med. 112 (1960) 653.
52. Tedesco, F., Trani, S., Soranzo, M. R. and Patriarca, P., FEBS Letters 51 (1975) 232.
53. Tsan, M. F., Blood 50 (1977) 935.
54. Vogt, M. T., Thomas, C., Vassallo, C. L., Basford, R. E. and Gee, J. B. L., J. Clin. Invest. 50 (1971) 401.
55. Zatti, M. and Rossi, F., Biochim. Biophys. Acta 99 (1965) 557.
56. Zatti, M., Rossi, F. and Patriarca, P., Experientia 24 (1968) 669.
57. Zurier, R. B., Hoffstein, S. and Weissmann, G., Proc. Nat. Acad. Sci. USA 70 (1973) 844.