INTERRELATIONSHIP BETWEEN OXYGEN CONSUMPTION, SUPEROXIDE ANION AND HYDROGEN PEROXIDE FORMATION IN PHAGOCYTOSING GUINEA PIG POLYMORPHONUCLEAR LEUCOCYTES

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Summary

The paper presents an experimental procedure for a simultaneous assay of oxygen consumption, O_2^- release and H_2O_2 accumulation at a very early stage of the respiratory burst that is induced by phagocytosis in guinea pig polymorphonuclear leucocytes. The main findings are as follows:

(a) The oxygen consumption that is measurable does not correspond to all oxygen that is reduced. The relationship between the actual oxygen consumed and the amount that is reduced depends on the fate of the intermediate products O_2^- and H_2O_2.

(b) O_2^- is measurable extracellularly by the reduction of cytochrome c. When cytochrome c oxidizes the extracellular O_2^-, molecular oxygen is formed. This fact is shown by a decrease of oxygen consumption. The molar ratio between the O_2^- detected and the oxygen given back is 1.

(c) The amount of O_2^- released from the cells accounts for only a small part of oxygen actually reduced.

(d) H_2O_2 is detectable only in the presence of NaN_3. In this condition almost all oxygen consumed is recovered in the form of H_2O_2. The molar ratio O_2/H_2O_2 is near unity. The amount of H_2O_2 derived from dismutation of O_2^- released is only an aliquot of the total H_2O_2 accumulated. Thus, most of H_2O_2 is derived from intracellular sources.

(e) In the absence of inhibitors of H_2O_2 degrading reactions, no detectable accumulation of peroxide occurs. Under these conditions, the main part of H_2O_2 formed is degraded in almost equal amount by catalase and myeloperoxidase, while only a small aliquot is degraded by NaN_3 insensitive reactions.

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Introduction

The process of phagocytosis in polymorphonuclear leucocytes (PMNL) is associated with a number of metabolic changes such as increased respiration, increased glucose oxidation through the hexosemonophosphate pathway (HMP) and increased production of hydrogen peroxide and superoxide anion[1–7]. A metabolic burst similar to that caused by phagocytosable material can be induced, *in vitro*, by producing a perturbation of the surface membrane of PMNL with a variety of agents, free insolution[8–15] or immobilized to non-phagocytosable substrates[16–18].

Many biochemical investigations have been aimed at the identification of the enzyme responsible for the respiratory burst. A granule bound NADPH oxidase has been identified in our laboratory and in others, which possesses properties that make it a reasonable candidate for the key enzyme of the increased oxygen uptake and of O_2^- and H_2O_2 production[19–27].

Other authors have postulated that the respiratory burst is secondary to enzymatic oxidation of NADH[28–30], that would be located at the level of the plasma membrane of PMNL[30–35].

Apart from the issue of the enzyme responsible for the respiratory burst, which has been reviewed elsewhere[4,23], many problems remain to be elucidated. The main ones are the mechanism of the reduction of oxygen, the amount of O_2^- formed, the fate of O_2^-, the amount of H_2O_2 generated and the mechanisms of its degradation.

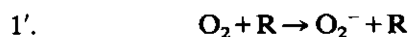
The main prerequisites that are necessary to address these problems is to find out the optimal conditions for a simultaneous determination of oxygen consumption as well as O_2^- and H_2O_2 production.

In this paper, optimal conditions for these measurements are reported. With this procedure we have been able to correlate, at a very early stage in the metabolic stimulation of guinea pig PMNL, the measurable oxygen uptake, O_2^- released and H_2O_2 accumulated. On the basis of the experimental findings and of those theoretically expected from the stoichiometries of the reactions involved in O_2^- formation, O_2^- dismutation and H_2O_2 degradation, some indications have been obtained on the relative amount of O_2^- released, on the fate of O_2^-, on the sources of H_2O_2 production, on the mechanisms of its degradation, and on the localization of the O_2^- and H_2O_2 generating system(s).

In order to make clear the strategy of the experimental approaches, the results and the calculations presented in the paper, it is necessary to indicate the reactions involved in oxygen consumption, in O_2^- formation, O_2^- dismutation and H_2O_2 degradation and the theoretical stoichiometries of the above reactions.

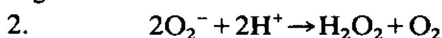
The steady-state rate of O_2 consumption and of generation of O_2^- and H_2O_2 depends on the activity level of the O_2 reductase and on the rate at which O_2^- and H_2O_2 are utilized in the cell and in the surrounding medium. On the assumption that the reduction of O_2 essentially proceeds via a one-electron pathway, the first step catalyzed by an oxidase, is the following:

1. $O_2 + RH \rightarrow O_2^- + H^+ + R^{\cdot}$



1'. $O_2 + R \rightarrow O_2^- + R$

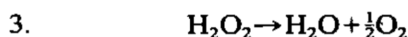
Reactions 1 and 1' have not yet been defined precisely, although sufficient evidence has been provided that RH is indeed NADPH[19–27]. Once formed, O_2^- dismutates according to the following reaction:



2. $2O_2^- + 2H^+ \rightarrow H_2O_2 + O_2$

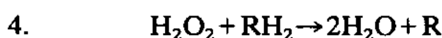
This reaction can proceed spontaneously, with a rate constant at PH 7.4 of about 1×10^5 $M^{-1}\,sec^{-1}$, or can be catalyzed by superoxide dismutase (SOD), with a rate constant of 2×10^9 $M^{-1}\,sec^{-1}$ [46].

The H_2O_2 formed can be degraded by different mechanisms, a catalase type or a peroxidase type. The first one, catalyzed by catalase, an haeme-enzyme sensitive to NaN_3, forms H_2O and gives back $\frac{1}{2}O_2$ for each molecule of H_2O_2:



3. $H_2O_2 \rightarrow H_2O + \frac{1}{2}O_2$

The second mechanism can be catalyzed by myeloperoxidase, an haeme-enzyme sensitive to NaN_3, or by a NaN_3 insensitive glutathione peroxidase. In both cases, H_2O_2 is utilized as an oxidant and the products of the reaction are H_2O and an oxidized compound:



4. $H_2O_2 + RH_2 \rightarrow 2H_2O + R$

Assuming that these reactions occur during the activated metabolism of phagocytosing PMNL, it is necessary to stress the following points:

a. The oxygen consumption which is measurable does not correspond to all the oxygen that is reduced to O_2^- since this compound does not accumulate.

b. The stoichiometric relationships between O_2^- formed as an intermediate and the actual oxygen consumed, depends on the mechanism of H_2O_2 degradation and on the amount of H_2O_2 accumulated.

b.1 If all H_2O_2 is degraded by catalase in the sequence of reactions 1, 1', 2, 3, two O_2^- will be formed but the actual oxygen consumed will be $\frac{1}{2}O_2$. In this case, for each oxygen molecule actually consumed by the cell, four O_2^- are produced.

b.2 If all H_2O_2 is degraded by peroxidase type mechanism in the sequence of the reactions 1,1', 2, 4, two O_2^- will be formed but the actual oxygen consumed by the cells will be one O_2. In this case, for each oxygen molecule actually consumed two O_2^- are formed.

b.3 A situation similar to that of point b.2, occurs when H_2O_2 accumulates.

b.4 Since in the cell all mechanisms for H_2O_2 degradation can be operative, the actual

stoichiometric relationship between the measurable oxygen consumed and the O_2^- formed as intermediate can vary between 1:4 and 1:2. This variation depends on the relative importance of the various reactions for H_2O_2 degradation. The variable factor in the ratio O_2/O_2^- is O_2 while the amount of O_2^- formed does not change.

The O_2^- formed in reaction 1, 1′ is partially released from the cell[6,7,33,51] and undergoes dismutation in the extracellular medium. When an oxidant as cytochrome c, which does not enter into the cell is present, the extracellular O_2^- is oxidized to molecular oxygen:

5. $2O_2^- + 2Fe^{+++}$ cytochrome c $\rightarrow 2O_2 + 2Fe^{++}$ cytochrome c

The rate constant of this reaction at neutral pH is about 1×10^6 M^{-1} sec^{-1} [47,55], and it is prevented by the presence of SOD, which intercepts O_2^- thereby catalyzing its rapid dismutation to H_2O_2 according to reaction 2. Therefore, when cytochrome c is added to phagocytosing PMNL and reaction 5 takes place in extracellular medium, the amount of O_2^- released is measured as reduced cytochrome c with a stoichiometry of 1:1. Furthermore, since reaction 5 is associated with O_2 formation, for every O_2^- oxidized and for every cytochrome c reduced one O_2 is given back.

It is worthy of note that with cytochrome c only the O_2^- released from the cells is measured, while the total amount of O_2^- formed during the activated metabolism can be only indirectly calculated.

Materials and Methods

Animals.

Albino guinea pigs, weighing 400–500 g, from Istituto Zooprofilattico delle Venezie (University of Padova) were used.

Leucocytes.

Polymorphonuclear leucocytes were obtained from guinea pig peritoneal exudates. The exudate was elicited by an intraperitoneal injection of 50 ml of sterile 1% sodium caseinate solution in 0, 9% NaCl. After 14 hours the exudate was collected and the cells were harvested by centrifugation. After lysis of the contaminating red cells by a brief hypotonic shock, the leucocytes were centrifuged again for 7 min at 200 g and resuspended in calcium-free Krebs-Ringer-phosphate buffer, pH 7.4 (KRP). Suspensions containing less than 90% PMNL were discarded.

Measurement of oxygen consumption, superoxide anion and H_2O_2.

Oxygen consumption was measured polarographically with a Clark oxygen electrode (Yellow Spring Inst. Co., OH) as previously described[10]. The assay medium contained 2 ml of KRP, 5 mM glucose, 0.5 mM $CaCl_2$, $1-2 \times 10^7$ cells and heat killed serum opsonized *B. mycoides* (bacteria: cell ratio, 100:1).

Superoxide anion and H_2O_2 were measured on aliquots of the reaction mixture withdrawn from the electrode vessel immediately after the recording of oxygen uptake was stopped.

Superoxide anion was determined by the SOD inhibitable cytochrome c reduction[7,36,37], according to reaction 5 presented in the introduction. Cytochrome c (grade VI, from horse heart, Sigma Chemical Co., St. Louis, Mo) was added to the electrode vessel just before addition of bacteria. At the end of oxygen consumption recording, an aliquot of the reaction mixture was quickly transferred into an "Eppendorf" microtube containing 30 μg of SOD (from bovine blood, Truett Labs. Dallas, Tex) to prevent further cytochrome c reduction, and centrifuged at 8,000 g for 30 sec in an "Eppendorf" table centrifuge. The supernatant (0.5 ml) was then diluted five fold and the absorbance at 550 nm was measured with a Beckman DU2 spectrophotometer. The absorbance values obtained were corrected relative to the total amount of cytochrome c in the incubation mixture, as determined from the increase in absorbance at 550 nm upon addition of Na dithionite. The amount of O_2^--dependent reduction of cytochrome c was calculated from the difference in absorbance between cytochrome c reduced in the absence of SOD and the cytochrome c reduced in the presence of SOD, by using an extinction coefficient of 21.1 mM^{-1} cm^{-1} (reduced minus oxidized)[38].

Hydrogen peroxide was measured fluorimetrically by the decrease of scopoletin fluorescence in the presence of horse radish peroxidase (HRP) (Type VI, Sigma Chemical Co., St.

Louis, Mo)[39,40] or colorimetrically with the ferrithiocyanate method[41]. Scopoletin (7-hydroxy-6-methoxy-coumarin) emits a blue fluorescence when excited with light at 350 nm wavelength (emission 460 nm). In the presence of H_2O_2 it is oxidized by HRP yielding a loss in fluorescence, which is directly proportional to the peroxide concentration in the medium. Aliquots (10–100 μl) of cell-free supernatants obtained by rapid centrifugation (30 sec at 8,000 g) of samples withdrawn from the electrode vessel were immediately transferred into a UV cuvette containing KRP (3 ml), 2.5 μM scopoletin and 0.166 μM HRP and the decrease of fluorescence intensity was measured with a CGA spectrophotofluorimeter (Florence, Italy). Appropriate H_2O_2 standards were prepared before each experiment using an extinction coefficient (230 nm) of 81 cm^{-1} M^{-1} [6,42].

H_2O_2 measurement with the colorimetric method was performed on 1 ml aliquots of the reaction mixture withdrawn from the electrode vessel and transferred into a tube containing trichloroacetic acid (10% final concentration). After removal of precipitated protein by centrifugation, 0.4 ml of 10 mM ferrous ammonium sulphate and 0.2 ml of 2.5 M potassium thiocyanate were added to 2 ml of the supernatant. The absorption of the red ferrithiocyanate complex formed in the presence of H_2O_2 was measured at 480 nm in a Beckman DU2 spectrophotometer and compared with appropriate H_2O_2 standards.

With the two methods employed, determinations of H_2O_2 on standard H_2O_2 solutions as well as on samples of the experimental mixtures gave results which matched each other very closely.

$^{14}CO_2$ production from glucose.

The method has been described in a previous paper[43]. 1-^{14}C-glucose was purchased from the Radiochemical Center (Amersham, England).

Experimental

Studies of optimal experimental conditions to determine oxygen consumption, superoxide anion and hydrogen peroxide.

From the data so far presented by others[6,7,33,51] it is difficult to correlate precisely oxygen consumption with O_2^- and H_2O_2 production by PMNL during the respiratory burst associated with phagocytosis. This difficulty derives from the fact that each measurement was performed under different conditions of incubation and on different samples.

We briefly describe the scheme of the experimental model we have used to measure O_2^- and H_2O_2 in the same reaction mixture, where oxygen consumption has been recorded with a Clark oxygen electrode: (1) Measurement of O_2 consumption during phagocytosis and, after a suitable time withdrawal of samples for H_2O_2 measurements; (2) measurement of O_2 consumption during phagocytosis in the presence of cytochrome c and sampling for assays of cytochrome c reduction and H_2O_2; (3) measurement of O_2 consumption during phagocytosis in the presence of cytochrome c and SOD and sampling for assays of cytochrome c reduction and H_2O_2. Polarographic traces of oxygen consumption in one typical experiment are reported in Figure 1.

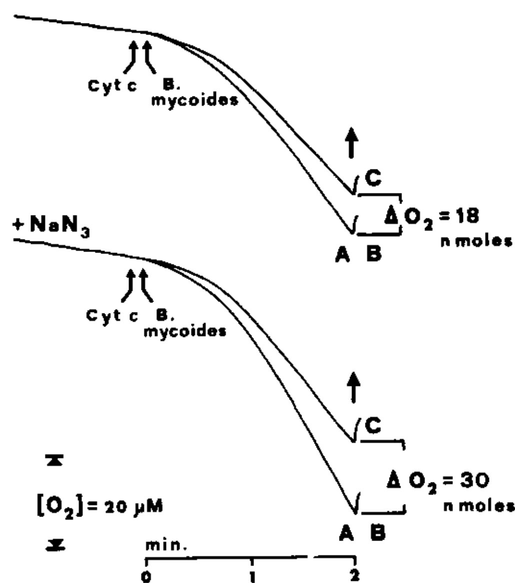


Fig. 1. Clark oxygen electrode measurement of the effect of cytochrome c and cytochrome c plus SOD on the stimulated oxygen consumption of guinea pig PMNL. Assay medium: 2×10^7 cells in 2 ml of KRP containing 5 mM glucose, 0.5 mM $CaCl_2$ and 2 mM NaN_3 where indicated. Bacteria : cell ratio = 100 : 1. Temperature 37 °C. A: control. B: plus cytochrome c (0.2 mM) and SOD (35 μg/ml). C: plus cytochrome c. At the points indicated by the arrows the reaction mixture was withdrawn from the electrode vessel for O_2^- and H_2O_2 measurements. A and B are represented by the same trace.

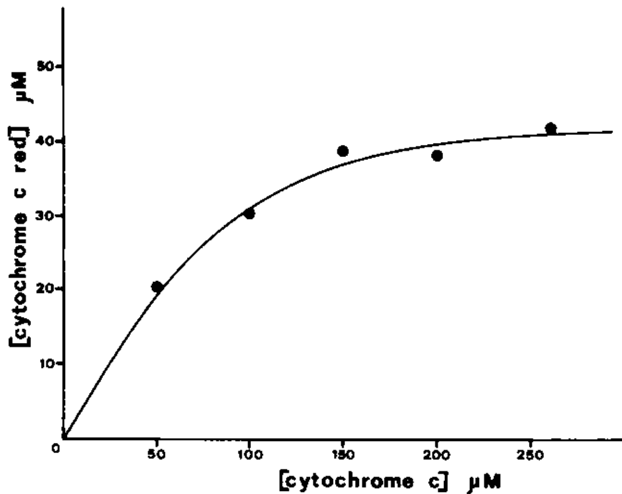


Fig. 2. Cytochrome c reduction by O_2^- as a function of cytochrome c concentration. For experimental conditions see Figure 1.

As for O_2^- determinations, Figure 2 shows that SOD-inhibitable cytochrome c reduction is a function of cytochrome c concentration. In all the subsequent experiments the saturating concentration of 200 μM was used.

Figure 3 shows that both SOD-inhibitable reduction of cytochrome c and the oxygen consumption increase linearly with cell concentration within a range of 0.5×10^7 to 1.5×10^7 cells/ml.

H_2O_2 was measured in samples of the incubation mixture with both the scopoletin and the ferrithiocyanate methods. When cytochrome c was present only the latter method was adopted. The non reliability of the former method under these conditions is due to the fact that ferrocytochrome c, generated from the interaction of ferricytochrome c with O_2^-, competes with scopoletin as hydrogen donor for oxidation by H_2O_2 and HRP[44,45]. In fact, the addition of scopoletin and HRP to an aliquot of the supernatant of the suspension of phagocytosing PMNL, containing cytochrome c reduced by O_2^- during the respiratory burst, induces a fast decrease in absorbance at 550 nm, thus indicating that an oxidation of reduced cytochrome c instead of scopoletin takes place.

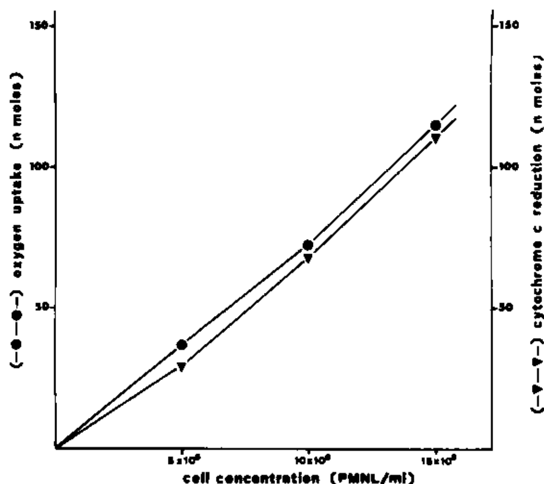


Fig. 3. Oxygen consumption and cytochrome c reduction by O_2^- as a function of varying granulocyte concentration. For experimental conditions see Figure 1.

Figure 4 shows that the SOD-inhibitable reduction of cytochrome c, oxygen consumption, as well as H_2O_2 production in the presence of NaN_3, are all linear for about 180 seconds. Therefore, we selected 120 seconds as the optimal incubation time for all subsequent experiments.

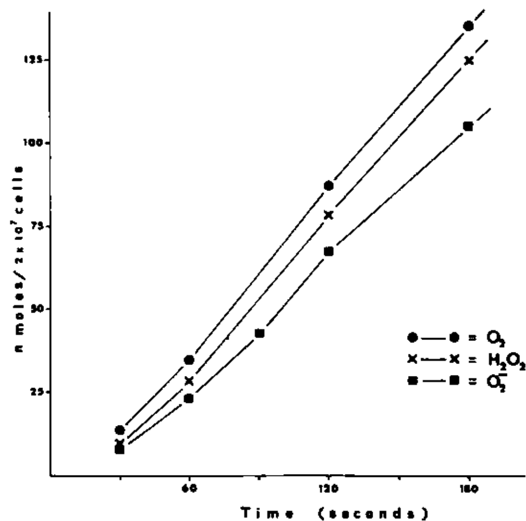


Fig. 4. Time course of oxygen consumption, cytochrome c reduction by O_2^- and H_2O_2 production by phagocytosing PMNL in the presence of 2 mM NaN_3. For experimental conditions see Figure 1.

Relationship between oxygen consumption and production of superoxide anion and hydrogen peroxide in polymorphonuclear leucocytes.
The results of simultaneous determinations of oxygen consumption, O_2^- and H_2O_2 in phagocytosing PMNL are reported in Tables 1 and 2. First of all it should be pointed out that cytochrome c, SOD, and cytochrome c plus

TABLE 1 - OXYGEN CONSUMPTION, SUPEROXIDE ANION RECOVERY AND HYDROGEN PEROXIDE ACCUMULATION IN PHAGOCYTOSING GUINEA PIG POLYMORPHONUCLEAR LEUCOCYTES[a]

	nmol/2 min/2x10^7 cells[b]			
	oxygen uptake		O_2^--dependent cytochrome c reduction	hydrogen peroxide production
Control	72.7 ±10.6 (5)			< 2
+ SOD 70 µg	66.0 ±12.8 (4)	NS		< 2
+ cyt.c 0.2 mM	56.3 ± 8.8 (6)	P<0.02	65.5 ±15.5 (6)	< 2
+ cyt.c 0.2 mM + SOD 70 µg	72.2 ± 8.0 (6)	NS		< 2

[a]The data are differences due to phagocytosis. The values for resting cells are: oxygen consumption 18.7 ±3.2 nmol/2 min/2x10^7 (3), O_2^--dependent cytochrome c reduction 5.4 ±4.1 nmol/2 min/2x10^7 cells (5), the addition of SOD, cytochrome c and cytochrome c plus SOD did not modify these values.

[b]Means ±S.D. of the number of experiments indicated in parenthesis. The significance of the difference of means with respect to control was calculated according to Student "t" test.

The experimental conditions are described in the text.

TABLE 2 - OXYGEN CONSUMPTION, SUPEROXIDE ANION RECOVERY AND HYDROGEN PEROXIDE ACCUMULATION IN PHAGOCYTOSING GUINEA PIG POLYMORPHONUCLEAR LEUCOCYTES IN THE PRESENCE OF NaN_3[a]

	nmol/2 min/$2x10^7$ cells[b]		
	oxygen uptake	O_2^--dependent cytochrome c reduction	hydrogen peroxide production
Control	94.5 $\pm$15.8 (5)		84.8 $\pm$ 7.4 (5)
+ SOD 70 µg	88.6 $\pm$ 9.8 (5) NS		81.4 $\pm$ 9.9 (5) NS
+ cyt.c 0.2 mM	64.0 $\pm$14.0 (5) $P<0.02$	63.7 $\pm$14.2 (5)	59.9 $\pm$11.1 (3) $P<0.02$
+ cyt.c 0.2 mM + SOD 70 µg	94.5 $\pm$18.3 (5) NS		77.4 $\pm$ 7.0 (3) NS

[a]The data are differences due to phagocytosis. The values for resting cells are: oxygen consumption 16.2 $\pm$2.8 nmol/2 min/$2x10^7$ cells (4), O_2^--dependent cytochrome c reduction 5.2 $\pm$3.9 nmol/2 min/$2x10^7$ cells (4), H_2O_2 production 3.8 $\pm$2.8 nmol/2 min/$2x10^7$ cells (3) and the addition of SOD, cytochrome c and cytochrome c plus SOD did not modify these values.

[b]Means $\pm$S.D. of the number of experiments indicated in parenthesis. The significance of the difference of means with respect to control was calculated according to Student "t" test.

The experimental conditions are described in the text.

SOD do not modify the rate of oxygen consumption by resting PMNL. Furthermore, SOD has not a significant effect on the stimulation of respiration by phagocytosis.

The addition of cytochrome c causes an apparent 22% decrease (16.4 nmoles) in the oxygen consumption of phagocytosing PMNL (Table 1). This effect is not due to an inhibition of the metabolic response of PMNL, since the stimulation of $^{14}CO_2$ production from 1-^{14}C-glucose (Fig. 5) is not modified.

The inhibitory effect on the oxygen consumption is associated with a reduction of 65.5 nmoles of cytochrome c, indicating that under these conditions 65.5 nmoles of O_2^- are released from phagocytosing PMNL. The molar ratio between the O_2^- detected (65.5 nmoles) and the inhibition of oxygen consumed (16.4 nmoles) is 3.99 : 1 (ratio O_2^- : O_2 Table 3). This means that for 4 nanomoles of O_2 reduced and released as O_2^- from phagocytosing cells, only 1 nmole of O_2 is actually consumed. The addition of SOD, which intercepts O_2^- catalyzing its rapid dismutation to H_2O_2, prevents both the reduction of cytochrome c and the decrease of oxygen consumption.

Data reported in Table 1 show that only traces of H_2O_2 are detectable, thus indicating

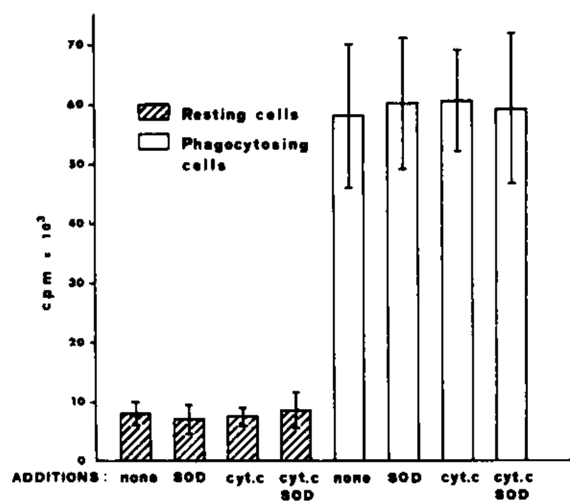


Fig. 5. Effect of cytochrome c and superoxide dismutase on $^{14}CO_2$ production from 1-^{14}C-glucose oxidation by guinea pig PMNL. The incubation mixture contained 2 ml of KRP, 0.5 mM glucose, 0.5 mM $CaCl_2$, 0.5 μCi of 1-^{14}C-glucose and 2×10^7 cells. Heat-killed opsonized B. mycoides were used as a phagocytosable particles. Bacteria : cell ratio = 100 : 1. The concentrations of SOD and cytochrome c were 35 μg/ml and 0.2 mM respectively. The mean ±S.D. of three experiments are reported as cpm/10 min/2×10^7 cells.

that H_2O_2 is rapidly degraded. Under these conditions, therefore, any relationship between O_2 consumed, O_2^- and H_2O_2 produced cannot be established.

In the presence of NaN_3 (Tables 2 and 3), which inhibits the degradation of H_2O_2 by catalase and myeloperoxidase the oxygen consumption by phagocytosing PMNL is higher than that in the absence of NaN_3 (94.5 versus 72.7 nmoles). It is likely that the difference of 21.8 nmoles corresponds to an increase in the amount of measurable oxygen consumption due to the inhibition of catalase breakdown of H_2O_2 (reaction 3).

Also, in the presence of NaN_3, cytochrome c produces an apparent decrease in oxygen consumption of 30.5 nmoles, which corresponds to 32.2% of the control value. In the same system, 63.7 nmoles of O_2^- are detected, an amount similar to that recovered in the absence of NaN_3. The molar ratio between O_2^- detected (63.7 nmoles) and the decrease of oxygen consumed (30.5 nmoles) is 2.09 : 1 (ratio O_2^- : O_2, Table 3). It means that for 4 nmoles of O_2^- released from phagocytosing cells, two nmoles of O_2 are actually consumed when H_2O_2 is prevented from being degraded. Also under these conditions, the addition of SOD completely counteracts the depression of oxygen consumption caused by cytochrome c.

Tables 2 and 3 show also data concerning the production of H_2O_2, which can be correctly measured due to the presence of NaN_3. Under these conditions almost all the oxygen consumed is recovered in the form of H_2O_2. The molar ratio between the oxygen consumed (94.5 nmoles) and H_2O_2 detected (84.8 n moles) is 1.11, thus indicating that 90% of H_2O_2 is prevented from being degraded by catalase or myeloperoxidase. It is worth pointing out that similar amounts of H_2O_2 were detected by using either the scopoletin or thiocyanate method. Since with the former method cell free supernatants and with the latter one supernatants obtained by deproteinization of cell suspensions were assayed, it means that all the H_2O_2 produced and accumulated is found outside the PMNL.

When in addition to NaN_3 also cytochrome c is present, the amount of H_2O_2 detectable is significantly decreased, because cytochrome c prevents O_2^- from being dismutated to H_2O_2.

TABLE 3 - RELATIONSHIP BETWEEN O_2 CONSUMPTION, O_2^- RECOVERY AND H_2O_2 ACCUMULATION IN PHAGOCYTOSING GUINEA PIG POLYMORPHONUCLEAR LEUCOCYTES

	$- NaN_3$			$+ NaN_3$		
	Control	+cyt.c	+cyt.c +SOD	Control	+cyt.c	+cyt.c +SOD
	A	B	C	A	B	C
O_2 uptake (nmoles)	72.7	56.3	72.2	94.5	64.0	94.5
ΔO_2 (A minus B)		16.4			30.5	
Percentage of control		-22.5%			-32.3%	
H_2O_2 production (nmoles)	<2	<2	<2	84.8	59.9	77.4
ΔH_2O_2 (A minus B)		-			24.9	
Percentage of control					-29.0%	
O_2^- release (nmoles)		65.5			63.7	
Percentage of total O_2^- formed[a]		34.5%			33.7%	
Ratio[b] $O_2^- : \Delta O_2$		3.99 $\pm$0.7 SD			2.09 $\pm$0.3 SD	
Ratio[c] $O_2^- : \Delta H_2O_2$					2.50 $\pm$0.8 SD	
Ratio[d] $O_2 : H_2O_2$				1.11 $\pm$ 0.2 SD	1.06 $\pm$ 0.4 SD	1.23 $\pm$ 0.3 SD
Percentage of H_2O_2 derived from extracellular dismutation of O_2^- (ΔH_2O_2x100/A_{NaN_3}=24.9x100/84.8)					29.4%	
Percentage of H_2O_2 from inside the cell					70.6%	

a: assuming that all O_2 consumed is first reduced to O_2^-.
b: theoretical ratio is 4 when H_2O_2 is destroyed by catalatic mechanisms, and 2 when H_2O_2 accumulates or is destroyed by non catalatic mechanisms.
c: theoretical ratio is 2 when H_2O_2 accumulates.
d: theoretical ratio is 1 when H_2O_2 accumulates.

Also with this condition, the molar ratio O_2 consumed: H_2O_2 detected is near unity. On the basis of stoichiometry of the dismutation reaction, for every 2 nmoles of O_2^- oxidized by cytochrome c, 1 nmole of H_2O_2 should be lacking. The experimental ratio ($O_2^- : H_2O_2$, Table 3) we have found is near the expected value of 2. The presence of SOD, which prevents O_2^- from oxidation by cytochrome c, brings again the amount of detectable H_2O_2 close to control values.

From the results reported in Tables 2 and 3, it can also be observed that the amount of H_2O_2 derived from the dismutation of O_2^- released from the cells is only an aliquot ($84.8 - 59.9 = 24.9$ nmoles) of the total H_2O_2 formed during the respiration of phagocytosing PMNL (84.8 nmoles). This indicates that at least 70% of H_2O_2 is derived from the inside of the cell or from O_2^- not accessible to cytochrome c.

A series of calculations can be made on the data reported in Tables 1 and 2 in the attempt to establish the stoichiometric relationships between O_2, O_2^- and H_2O_2 in phagocytosing cells. These calculations are reported in Table 3 and will be discussed in the next section.

Discussion

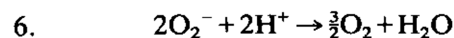
The experimental conditions we have employed allow us to establish good correlations between O_2, O_2^- and H_2O_2, the chemical species involved in the respiratory burst of PMNL. For this purpose, the determinations were based on 1) simultaneous measurements of oxygen consumption and recovery of O_2^- and H_2O_2, 2) short time assays, which permit evaluation of these parameters when the reaction rate is linear and 3) use of NaN_3, as an inhibitor of H_2O_2 degradation by catalase and myeloperoxidase.

The main findings reported in this paper and summarized in Table 3 will be discussed in three different sections.

Relationship between the decrease of oxygen consumption caused by cytochrome c and the release of superoxide anion.
The measurement of O_2^- is based on its ability to reduce a suitable electron acceptor[7,36,37]. Since the electron acceptor that has been used, i.e. cytochrome c, does not enter the cell, only the extracellular O_2^- is measured.

The data reported in Table 1 show that 65.5 nmoles of O_2^- are released by 2×10^7 phagocytosing PMNL/2 minutes. On the basis of the reaction 5, when extracellular O_2^- is oxidized, molecular oxygen is formed. Thus the addition of cytochrome c to O_2^- generating PMNL is expected to diminish the oxygen consumed, the effect being reversed by SOD. This has been verified. According to reaction 5, for every cytochrome c reduced one molecular oxygen is formed. However, the experimental finding shows that while 65.5 nmoles of cytochrome c are reduced only 16.4 nmoles of oxygen are given back, the molar ratio between extracellular O_2^- and O_2 lacking from the respiratory burst being 3.99 : 1. This finding can clarify the fate of extracellular released O_2^-.

The above ratio is twice that expected if, in the absence of the oxidant, O_2^- dismutates according to reaction 2 and H_2O_2 accumulates or is degraded by a peroxidase mechanism (reaction 4). Under these experimental conditions (absence of NaN_3), however, practically no H_2O_2 is formed in the medium of phagocytosing cells, suggesting that it is rapidly degraded. A ratio of 4 : 1 instead of 2 : 1, between the extracellular O_2^- and the O_2 lacking from the respiratory burst, is found if H_2O_2 formed in reaction 2 is degraded by a catalase mechanism (reaction 3). In fact on the basis of the sequence of reactions 1, 1′, 2, 3, one ends up with the following overall reaction:



6. $$2O_2^- + 2H^+ \rightarrow \tfrac{3}{2}O_2 + H_2O$$

Thus, with the experimental conditions presented here, the extracellular released O_2^- undergoes dismutation to H_2O_2 and the peroxide is degraded by catalase.

That an extracellular catalase type of degradation of H_2O_2 can indeed occur is supported by the observation that about 3% of the total cellular activity of catalase is found in the incubation media of resting and phagocytosing PMNL (unpublished). The presence of a small amount of cytoplasmic enzymes, such as lactate dehydrogenase, outside the cells, is a constant finding of our laboratory and of others[17,48,49]. This is probably due to some damage of a small number of PMNL and is not significantly modified by phagocytosis.

The explanation given above for the 3.99 : 1 ratio is further supported by the results obtained from experiments performed in the presence of Na-azide. With this inhibitor of haeme-enzymes in the medium, the ratio between the amount of extracellular O_2^- and the decrease in oxygen consumption caused by cytochrome c is 2.09 (ratio $O_2^- : O_2$, Table 3), which closely approaches the 2 : 1 ratio that would be expected if all O_2^- released is allowed to dismutate to H_2O_2 (reaction 2) and the peroxide is not degraded.

Hydrogen peroxide generation and pathways of its utilization.

Hydrogen peroxide can be correctly measured only in the presence of NaN_3, which inhibits most of the enzymes which catalyze its degradation. The total amount of peroxide that is accumulated corresponds to the oxygen consumed with a stoichiometry very near to unity in all experimental conditions (Tables 2 and 3). On the basis of the stoichiometry of reaction 2, the amount of H_2O_2 accumulated (84.8 nmoles) greatly exceeds the amount expected from the dismutation of the extracellularly recovered O_2^- ($\frac{1}{2} \times 63.7$ nmoles). The excess of 53.0 nmoles calculated by the difference between the total amount of H_2O_2 (84.8 nmoles) and H_2O_2 theoretically derivable from extracellular O_2^- (31.8 nmoles), matches very closely with the 59.9 nmoles actually measured when the extracellular dismutation is impeded by the presence of cytochrome c. This indicates that a relevant portion of H_2O_2 derives from other sources, i.e. from intracellular dismutated O_2^- or from two electron reduction of O_2. Similar results have been presented by ROOT and METCALF[51].

The present status of our knowledge does not permit us to draw any conclusion on those reactions involved in the formation of H_2O_2 which are independent of the extracellular dismutation of O_2^-. The only datum available is the ratio $O_2 : H_2O_2$ (Table 3), which also in the presence of cytochrome c is near unity, as it is expected either from the univalent reduction of O_2 followed by dismutation of O_2^- (reactions 1 + 1′ + 2) or from a divalent reduction of O_2.

Whatever the reaction(s) involved in H_2O_2 production may be, the present results permit a discussion of its degradation. PMNL contain at least three H_2O_2 utilizing enzyme systems, which are, catalase and myeloperoxidase, both sensitive to NaN_3, and glutathione peroxidase, which is insensitive to this inhibitor. An analysis of the relationship between H_2O_2 accumulation, oxygen consumption and increased respiration in the presence of NaN_3 allows an evaluation of the relative efficiency of these three systems. An oxygen consumption of 94.5 nmoles by PMNL that phagocytose in the presence of NaN_3 is accompanied by a degeneration of 84.8 nmoles of H_2O_2. Thus, the ratio between oxygen consumed and H_2O_2 produced is 1.11, which is slightly higher than the ratio of 1 that would be expected from reaction 2. The lower recovery of H_2O_2 as compared to oxygen consumption, in the presence of azide, may be ascribed to a partial utilization of H_2O_2 by glutathione peroxidase or by non-enzymatic pathways.

The relative efficiency of catalase and myeloperoxidase in degrading H_2O_2 can be inferred from the effect of NaN_3 on oxygen consumption. It is known that only the degradation of H_2O_2 by catalase (reaction 3) and not by myeloperoxidase (reaction 4) releases oxygen. Since the oxygen consumed in the presence of NaN_3 is 21.8 nmoles higher than that observed in its absence (Table 3), it is likely that this increment corresponds to the nmoles of oxygen liberated by the catalase degradation of 43.6 nmoles of H_2O_2. If this is the case, the difference between the total amount of H_2O_2 measured in the presence of NaN_3 (84.8 nmoles) and that degraded by catalase (43.6 nmoles), should be accounted for by the amount of H_2O_2 degraded by myeloperoxidase (41.2 nmoles). The conclusion that can be derived from these calculations is that under the conditions employed here only a small amount of H_2O_2 produced by guinea pig phagocytosing PMNL is utilized by NaN_3-insensitive pathways, while the main part is degraded in almost equal amount by myeloperoxidase and by catalase.

Relationship between released superoxide anion and totally generated superoxide anion.

It has suggested that during the respiratory burst in human PMNL most, and probably all, oxygen is first reduced to O_2^- before being

converted to H_2O_2[6,50]. Furthermore, there should not be other measurable sources for H_2O_2 production in these cells beyond that requiring an O_2^- intermediate[51]. It has been also calculated that O_2^- production by subcellular particles from activated human PMNL can account for a large fraction of the increment in oxygen consumption induced by phagocytosis[26]. Although further investigations are required to confirm that O_2 is all reduced to O_2^-, this assumption can be considered in that it helps in the understanding of the relationship between the amount of O_2^- released and that actually generated.

It has already been pointed out in the introduction that a factor of 4 or 2 (for experiments done in the absence or in the presence of azide, respectively) should be used to calculate the stoichiometry between the extracellular O_2^- and the cytochrome c-inhibitable oxygen uptake.

When H_2O_2 is allowed to accumulate (presence of NaN_3) $2O_2^-$ are generated for each O_2 consumed, according to the reactions 1, 1', 2. In this case, the ratio between the oxygen consumed and the O_2^- theoretically generated should be 1 : 2. The total O_2^- generated could be calculated by multiplying the oxygen consumed by a factor of 2, that is $94.5 \times 2 = 189$ (Tables 2 and 3). Then the 63.7 nmoles of O_2^- detected extracellularly correspond to 33.7% of the total O_2^- generated.

In experiments performed in the absence of NaN_3, the ratio between oxygen consumed and O_2^- theoretically generated would lie between 1 : 2 and 1 : 4, depending on the relative involvement of the different mechanisms of H_2O_2 degradation (see point b.4 of the Introduction). Since as many H_2O_2 nmoles are produced as O_2 nmoles are consumed, 94.5 nmoles of peroxide are formed by 2×10^7 PMNL. The amount of peroxide which is degraded by catalase, that is 43.6 nmoles, accounts for 21.8 nmoles of oxygen consumed. For this amount of oxygen consumption the ratio $O_2 : O_2^-$ is 1 : 4 (see point b.1 of the Introduction). The remaining 50.9 nmoles of peroxide that are degraded by peroxidase mechanisms account for an oxygen consumption of 50.9 nmoles. For this quantity, the ratio $O_2 : O_2^-$ is 1 : 2 (see point b.2 of the Introduction). The total amount of O_2^- generated can be calculated by multiplying 21.8 by 4 and 50.9 by 2, that is 189 nmoles of O_2^- generated for 72.7 nmoles of oxygen consumed. Therefore the actual ratio between oxygen consumption and total O_2^- generated in the absence of inhibitors of catalase and myeloperoxidase is 1 : 2.6. In this case 65.5 nmoles of O_2^- detected extracellularly correspond to 34.6% of total O_2^- formed. Thus, both in the presence and in the absence of NaN_3 it may be calculated that, in spite of a difference in the measurable oxygen consumed, the same amount of O_2^- is actually generated and similar aliquots are released.

On the basis of these results, the data in the literature concerning the relationship between O_2^- generation and oxygen consumption by phagocytosing PMNL must be revised. Only two examples will be given here. In human phagocytosing PMNL an oxygen consumption of 1350 μ moles/10^{10} cells/hour and a production of O_2^- of 800–1000 μ moles/10^{10} cells/hour have been reported[50]. On the basis of these values it has been calculated that 60–70% of the oxygen consumed is recovered as O_2^- [6]. Apart from the difference between the conditions used to measure oxygen consumption and O_2^- release, a recalculation based on a ratio $O_2 : O_2^-$ intermediate between 1:2 or 1:4 (that is 1:2.6) indicate that only 23–28 per cent of the oxygen which is consumed during phagocytosis is detectable as O_2^- in extracellular medium. Other Authors[52] have measured the oxygen consumption and the release of O_2^- into the medium by both phagocytosing PMNL and macrophages of different species. They report that phagocytosing guinea pig PMNL consume 81 nmoles of oxygen/30 min/mg of cell protein, and release 29.2 nmoles of O_2^-/30 min/mg of cell protein. From these data it has been concluded that the O_2^- released during phagocytosis accounts for 36% of the oxygen consumption. Since in the absence of NaN_3 the actual stoichiometry between oxygen consumed and O_2^- produced in phagocytosing PMNL lies between an 1:2 and an 1:4 ratio, the correct percentage of the respiratory burst that can be accounted for by O_2^- release for the various types of phagocytes is at best half of that calculated by these authors.

In view of the results presented in this paper, it can be concluded that the O_2^- measurable extracellularly can account only for a small part

of both the oxygen consumed and the oxygen actually reduced during the respiratory burst.

Localization of the superoxide anion generating system/s.
The problem concerning the subcellular localization of the enzyme responsible for the respiratory increment of phagocytosing PMNL is still controversial[53]. Evidence has been presented by us and others[19,21,23,24,25,27,54] that an NADPH oxidase localized in the azurophilic granules of resting PMNL is the primary enzyme involved in the respiratory stimulation, and that this oxidase is likely to be responsible for the superoxide generation[22,26]. On the other hand, evidences have also been reported which indirectly support a plasmamembrane localization of the oxygen reducing systems[30–35].

Two sets of data presented in this paper can be discussed in relation to the problem. Firstly, a comparison between total O_2^- produced, as calculated from oxygen consumption, and extracellular O_2^-, shows that the latter is only a small fraction of the former. As a corollary, the decrease in oxygen consumption by cytochrome c is only 22% and 32%, in the absence and in the presence of NaN_3, respectively. If the O_2^- generating system were located in sites of the plasmamembrane wherefrom O_2^- might be easily released, a much higher recovery of extra cellular O_2^- and, consequently, a more marked decrease of oxygen consumption by cytochrome c would be expected. Secondly, cytochrome c causes a decrease in H_2O_2 production of 24.9 nmoles, an amount which is roughly stoichiometric to extracellular O_2^- (63.7 nmoles). Again a higher inhibition of H_2O_2 production would be expected if the H_2O_2 generating system were associated with the plasmamembrane and all the peroxide were derived from external O_2^-. The residual H_2O_2 found in the medium when cytochrome c is present (70% of the total H_2O_2 actually measured) originate, therefore, from intracellular sources.

The hypothesis concerning a granular localization of the O_2^- generating systems requires that the size of the extracellular portion of this radical is modulated by its intracellular dismutation, either spontaneous or catalyzed by SOD, by the extent of granule translocation within the cytoplasm, and by the rate of O_2^- diffusion across the plasmamembrane. The relative influence of these factors may vary in different cell types or in different experimental conditions. Data recently obtained in our laboratory, and not reported here, indicate that in phagocytosing human PMNL the extracellular release of O_2^- is much lower than that observed in guinea pig PMNL given the same oxygen consumption. Furthermore aging of guinea pig PMNL increases the percentage of O_2^- released as compared to total O_2^- generated and calculated as discussed above.

The data presented in this paper, although suggestive of an intracellular localization of the O_2^- generating system/s, do not rule out, however, a possible plasmemembrane location of this system. In fact, other possibilities might be considered: (1) that O_2^- is generated on a plasmamembrane site which is not accessible to cytochrome c, for example at the inner face of the membrane; (2) that the O_2^- generating system, physiologically located in the membrane of azurophilic granules, is transferred to the plasmamembrane of the phagosome by the process of fusion. In this case during phagocytosis two sources for O_2^- generation are operative, one associated with non-fused granules and the other with the plasmembrane; (3) that two O_2^- generating systems exist with different subcellular localization, one associated with the plasmamembrane and the other located in the granules; (4) that two enzymatic systems exist, with different activity, one associated with the plasmamembrane and generating O_2^- and the other localized in the azurophilic granules and catalyzing a bivalent reduction of oxygen with direct formation of H_2O_2.

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