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STUDIES ON THE NADPH OXIDATION BY SUBCELLULAR PARTICLES FROM PHAGOCYTOSING POLYMORPHONUCLEAR LEUCOCYTES

EVIDENCE FOR THE INVOLVEMENT OF THREE MECHANISMS

PAOLO BELLAVITE [a], GIORGIO BERTON [a] and PIETRO DRI [b,*]

[a] *Istituto di Patologia Generale, Università di Padova, sede di Verona and* [b] *Istituto di Patologia Generale, Università di Trieste, Trieste (Italy)*

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Summary

1. The NADPH-oxidizing activity of a 100 000 × *g* particulate fraction of the postnuclear supernatant obtained from guinea-pig phagocytosing polymorphonuclear leucocytes has been assayed by simultaneous determination of oxygen consumption, NADPH oxidation and O_2^- generation at pH 5.5 and 7.0 and with 0.15 mM and 1 mM NADPH.

2. The measurements of oxygen consumption and NADPH oxidation gave comparable results. The stoichiometry between the oxygen consumed and the NADPH oxidized was 1 : 1.

3. A markedly lower enzymatic activity was observed, under all the experimental conditions used, when the O_2^- generation assay was employed as compared to the assays of oxygen uptake and NADPH oxidation.

4. The explanation of this difference came from the analysis of the effect of superoxide dismutase and of cytochrome *c* which removes O_2^- formed during the oxidation of NADPH.

5. Both superoxide dismutase and cytochrome *c* inhibited the NADPH-oxidizing reaction at pH 5.5. The inhibition was higher with 1 mM NADPH than with 0.15 mM NADPH.

6. Both superoxide dismutase and cytochrome *c* inhibited the NADPH-oxidizing reaction at pH 7.0 with 1 mM NADPH but less than at pH 5.5 with 1 mM NADPH.

7. The effect of superoxide dismutase at pH 7.0 with 0.15 mM NADPH was negligible.

* To whom correspondence should be addressed.

8. In all instances the inhibitory effect of cytochrome *c* was greater than that of superoxide dismutase.

9. It was concluded that the NADPH-oxidizing reaction studied here is made up of three components: an enzymatic univalent reduction of O_2; an enzymatic, apparently non-univalent, O_2 reduction and a non-enzymatic chain reaction.

10. These three components are variably and independently affected by the experimental conditions used. For example, the chain reaction is freely operative at pH 5.5 with 1 mM NADPH but is almost absent at pH 7.0 with 0.15 mM NADPH, whereas the univalent reduction of O_2 is optimal at pH 7.0 with 1 mM NADPH.

Introduction

The activation of an oxidase is regarded as the event responsible for the increase in O_2 consumption and in O_2^- and H_2O_2 production by polymorphonuclear leucocytes during phagocytosis and when challenged with appropriate stimulatory agents [1—3]. This enzymatic activity oxidizes, in a cell-free system, NADPH, which is the physiologic electron donor, with production of O_2^-, H_2O_2 and $OH^\cdot$ [1,3—19]. The localization of the enzymatic activity has been reported to be either in the granules (azurophilic or granules with higher density than the azurophilic ones) [20—22] or in membrane fragments [23—25].

The assay of NADPH oxidase activity in cell-free systems of granulocytes is currently performed with different methods such as the spectrophotometric disappearance of NADPH [3,4,8,10,11], the oxygen consumption [3,8,14,18], the measurement of $NADP^+$ formation [13,15,17,18] and the production of O_2^- [12,15]. The results obtained with these methods do not always agree with each other; the main discrepancies concern the optimal pH of the enzyme, its affinity for the substrate, the extent of its activation with respect to the enzyme of resting cells and its subcellular localization.

In this paper, the mechanism of the NADPH oxidation by a $100\,000 \times g$ pellet of postnuclear supernatants of homogenates from phagocytosing guinea-pig polymorphonuclear leucocytes has been re-evaluated by comparing three methods of assay (oxygen consumption, O_2^- generation and NADPH disappearance) under different pH conditions and substrate concentrations.

The results show that the reaction of NADPH oxidation includes at least three components, namely: (i) an enzymatic generation of O_2^- (ii) a non-enzymatic chain reaction initiated by O_2^- and (iii) an apparently non-univalent reduction of O_2. The rate of each of the three components varies depending on the pH and the concentration of NADPH.

Materials and Methods

Preparation of leucocytes

Polymorphonuclear leucocytes were obtained from guinea-pig peritoneal exudates, elicited by an intraperitoneal injection of 1% sodium caseinate solu-

tion in 0.9% NaCl as previously described [26]. The cells were washed, freed from contaminating erythrocytes by brief hypotonic treatment and suspended in Krebs-Ringer phosphate buffer, pH 7.4, containing 0.5 mM $CaCl_2$ and 5 mM glucose.

Phagocytosis

Zymosan (Sigma Chemical Co., St. Louis, MO) was suspended in 0.9% NaCl to a concentration of 20 mg/ml and boiled for 10 min. After washing, it was incubated with fresh guinea-pig serum at a concentration of 25 mg/ml for 30 min at 37°C. After centrifugation and washing twice, this preparation of serum-treated zymosan was suspended in Krebs-Ringer phosphate buffer to a concentration of 20 mg/ml.

Suspensions of leucocytes ($15 \cdot 10^6$—$20 \cdot 10^6$ cells/ml) were prewarmed at 37°C and then 0.5 mg/ml of serum-treated zymosan was added. After 3—4 min incubation under stirring, the suspension was diluted with ice-cold Krebs-Ringer phosphate buffer and the leucocytes were collected by centrifugation at $200 \times g$ for 7 min.

Homogenization and fractionation of leucocytes

The packed cells were suspended in ice-cold 0.34 M sucrose, containing 1 mM $NaHCO_3$ ($30 \cdot 10^7$—$40 \cdot 10^7$ cells/ml) and were disrupted in a Potter-type homogenizer with a Teflon pestle until more than 90% of the cells were broken, as judged by light microscopic examination. The homogenate was then centrifuged at $400 \times g$ for 5 min to remove cell debris, unbroken cells and nuclei. The supernatant was centrifuged at $100\,000 \times g$ for 30 min and the pellet, resuspended in 0.34 M sucrose containing 1 mM $NaHCO_3$ was used for the assays. In each experiment, a small amount of $100\,000 \times g$ particles from resting cells was also prepared. The state of activation of the particles derived from phagocytosing cells was checked in each experiment by comparing their oxidase activity with that of particles from resting cells.

Biochemical assays

The rate of oxygen consumption by subcellular particles of polymorphonuclear leucocytes in the presence of NADPH was measured at 37°C with a Clark oxygen electrode, as previously described [8,26]. The assay medium contained 65 mM sodium phosphate/potassium phosphate buffer, pH 5.5 or 7.0, 170 mM sucrose, 2 mM NaN_3 and 1 or 0.15 mM NADPH (Boehringer-Mannheim). When required, 200 μM cytochrome *c* (Type VI, Sigma) and 20 μg/ml superoxide dismutase (Truett Laboratories, Dallas, TX) were also added to the assay mixture. The reaction was started by addition of 0.5—1.5 mg protein. The volume was 2 ml.

For the measurement of NADPH disappearance or of reduction of cytochrome *c*, aliquots of the mixture for the assay of oxygen consumption were withdrawn from the electrode chamber at suitable time intervals (including zero time) and were diluted ten times with ice-cold 0.1 M phosphate buffer, pH 8.0. After centrifugation at $8000 \times g$ for 2 min in an Eppendorf 3200 microcentrifuge, the supernatant was used for the determination of reduced cytochrome *c* or NADPH.

An extinction coefficient of 21.1 $mM^{-1} \cdot cm^{-1}$ (reduced minus oxidized) at 550 nm was used to calculate the reduction of cytochrome *c* [27]. The generation of O_2^- during the oxidation of NADPH was calculated from the difference in the amount of cytochrome *c* reduced in the absence and in the presence of superoxide dismutase.

The concentration of NADPH was determined enzymatically using oxidized glutathione and glutathione reductase. The optical density of an aliquot of the supernatant was read at 337 nm before and after the addition of 1 mM oxidized glutathione and 0.5 units/ml of glutathione reductase (Boehringer-Mannheim). Variations in the relative concentrations of the two redox states of cytochrome *c* had no effects on the *A* readings at 337 nm (from which the NADPH oxidation was calculated), since at this wavelength the oxidized and reduced forms of cytochrome *c* have an isosbestic point.

Protein was determined by the method of Lowry et al. [28] with bovine serum albumin as standard.

Results

Comparative results of NADPH oxidase assayed with different methods

The NADPH oxidase activity of the 100 000 × *g* pellet of the postnuclear supernatants from phagocytosing polymorphonuclear leucocytes was assayed as oxygen uptake, NADPH disappearance or O_2^- generation at two different pH values and two NADPH concentrations. The results reported in Table I show that: (1) when measured as oxygen consumed or as NADPH oxidized, the oxidase activity is higher at pH 5.5 than at pH 7.0, particularly at 1 mM NADPH concentration; (2) the oxygen consumption is stoichiometric (1 : 1) to the NADPH oxidized in all the conditions; (3) when assayed as O_2^- generation, the oxidase activity is higher at neutral than at acid pH at both NADPH concentrations and (4) the oxidase activity expressed in terms of O_2^- generation is always lower than when expressed as oxygen consumption or NADPH oxidation.

The variability of the values of the NADPH oxidase measured in different conditions and, particularly, the discrepancy in the results expressed as oxygen consumption or NADPH oxidation on one hand and as O_2^- generation on the other, suggest a role of O_2^- as an intermediate.

TABLE I

OXYGEN CONSUMPTION, NADPH OXIDATION AND SUPEROXIDE ANION PRODUCTION BY PARTICLES FROM PHAGOCYTOSING POLYMORPHONUCLEAR LEUCOCYTES

The experimental conditions are described in Materials and Methods. The values are given as nmol/mg protein per 2.5 min. The mean of four experiments ± S.E.M. are reported.

NADPH (mM)	pH 5.5			pH 7.0		
	O_2 consumed	NADPH oxidized	O_2^- detected	O_2 consumed	NADPH oxidized	O_2^- detected
1	72.7 ± 9.7	84.3 ± 6.4	9.4 ± 2.0	42.0 ± 4.0	45.5 ± 4.0	22.1 ± 4.5
0.15	22.9 ± 1.2	23.6 ± 0.5	7.6 ± 1.7	16.0 ± 2.9	19.7 ± 5.4	12.4 ± 3.4

Reactions involved in the oxidation of NADPH

For a more detailed investigation of the role of O_2^-, the effect of superoxide dismutase and/or cytochrome *c* on both the oxygen consumption and the NADPH oxidation has been exploited. The rationale of this approach is based on the ability of these two compounds to remove O_2^- with two different mechanisms: superoxide dismutase catalyzes the dismutation of O_2^- ($2O_2^- + 2H^+ \rightarrow H_2O_2 + O_2$), and cytochrome *c* oxidizes O_2^- to molecular oxygen (ferricytochrome *c* + $O_2^- \rightarrow$ ferrocytochrome *c* + O_2).

The protocol of a typical experiment at pH 5.5 with 1 mM NADPH is shown in Fig. 1. The addition of superoxide dismutase causes a strong decrease in oxygen consumption (trace 2), indicating that the O_2^- produced is subsequently involved in other reactions, thus giving rise to a chain reaction. Marked inhibition of oxygen consumption is caused also by cytochrome *c* (trace 3). The greater inhibitory effect of cytochrome *c* with respect to superoxide dismutase is due to the fact that in the dismutation reaction half the O_2^- is converted to H_2O_2 and, hence, oxygen is consumed, whereas the reaction of O_2^- with cytochome *c* does not cause oxygen consumption. In fact, in the presence of both cytochrome *c* and superoxide dismutase (which prevents O_2^- from reacting with cytochrome *c*), the inhibition of O_2 consumption equals the inhibition caused by superoxide dismutase alone (trace 4).

Table II reports the mean of the results of four such experiments (panel A). By applying appropriate calculations, at least three components of the NADPH oxidizing reaction can be identified (panel B). One is given by a 'chain reaction' which corresponds to the inhibition by superoxide dismutase of the oxygen consumption or the NADPH oxidation. This aliquot is calculated from the difference between the values of line 1 (total activity) and those of line 2. A second aliquot, referred to as 'residual oxygen consumption', corresponds to the amount of oxygen consumption left after the addition of cytochrome *c* (line 3). The third aliquot corresponds to the superoxide dismutase-inhibitable cytochrome *c* reduction and is accounted for by the 'univalent reduction' of

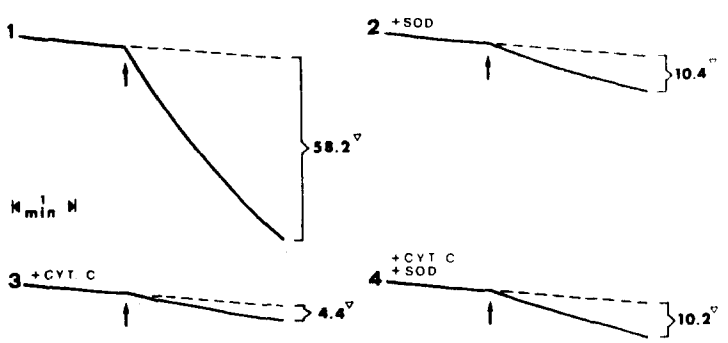

Fig. 1. Polarographic recording traces of the NADPH-oxidizing activity of 100 000 × *g* particles from phagocytosing polymorphonuclear leucocytes in the absence and in the presence of superoxide dismutase and of cytochrome *c*. Assay medium: 65 mM sodium phosphate/potassium phosphate buffer, pH 5.5, 170 mM sucrose, 1 mM NADPH, 2 mM NaN_3 and, where indicated, 20 μg/ml superoxide dismutase (SOD) and 200 μM cytochrome *c*. Volume, 2 ml, temperature, 37°C. 1 mg of 100 000 × *g* particles from phagocytosing polymorphonuclear leucocytes was added, where indicated by the arrows. ▽: nmol O_2/mg per 2.5 min.

TABLE II

COMPONENTS OF THE NADPH-OXIDIZING ACTIVITY OF PARTICLES FROM PHAGOCYTOSING POLYMORPHONUCLEAR LEUCOCYTES CALCULATED ON THE BASIS OF THE EFFECT OF SUPEROXIDE DISMUTASE AND OF CYTOCHROME *c*

For assay conditions see Fig. 1. The values are given as nmol/mg protein per 2.5 min. The mean of four experiments ± S.E.M. are reported.

Additions	A			B		C
	O_2 consumed	NADPH oxidized	cyto-chrome *c* reduced	Calculations	Results	% of total
1. None	72.7 ± 9.7	84.3 ± 6.4		Total activity (as O_2 or NADPH) = line 1	O_2 = 72.7 NADPH = 84.3	100 100
2. Superoxide dismutase	14.1 ± 1.9	14.8 ± 3.9		Chain reaction (as O_2 or NADPH) = line 1—line 2	O_2 = 58.6 NADPH = 69.5	81 82
3. Cytochrome *c*	9.2 ± 2.6	24.7 ± 7.8	22.6 ± 2.2	Residual O_2 consumption (as O_2 consumed) = line 3	O_2 = 9.2	13
4. Cytochrome *c* + superoxide dismutase	13.9 ± 1.9	28.2 ± 7.2	13.2 ± 1.2	Univalent reduction (as O_2^- detected) = cyt *c* reduced on line 3—line 4 (as O_2 consumed) = ½O_2^- detected	O_2^- = 9.4 O_2 = 4.7	 6

oxygen (cytochrome *c* reduction shown in line 3 minus cytochrome *c* reduction shown in line 4). In terms of oxygen consumed, this aliquot corresponds to half the O_2^- generated, according to the dismutation reaction of O_2^-.

Panel C of Table II shows the absolute and relative values of the three reactions at pH 5.5 with 1 mM NADPH. Under these conditions, most of the activity is contributed by the chain reaction (more than 80%). The remainder reflects the enzymatic oxidation of NADPH of which at least 30% proceeds via O_2^- formation.

Effect of pH and NADPH concentration on the reactions involved in NADPH oxidation

The experiments described in Fig. 1 and Table II have been repeated in different conditions, i.e., at pH 7.0 with 1 or 0.15 mM NADPH and at pH 5.5 with 0.15 mM NADPH. Table III reports the results of these experiments and Table IV shows the calculations made on the basis of these results, according to the procedure shown in Table II. The contribution of each of three aliquots to the NADPH-oxidizing activity varies depending on the experimental conditions. The rate of the chain reaction, as indicated by the inhibitory effect of superoxide dismutase, decreases by increasing the pH and by decreasing the substrate concentration. Particularly at pH 7.0 with 0.15 mM NADPH, the contribution of the chain reaction is negligible.

TABLE III

EFFECT OF SUPEROXIDE DISMUTASE AND OF CYTOCHROME *c* ON THE NADPH-OXIDIZING ACTIVITY OF PARTICLES FROM PHAGOCYTOSING POLYMORPHONUCLEAR LEUCOCYTES AT DIFFERENT pH AND NADPH CONCENTRATIONS

The experimental conditions are described in Materials and Methods. The values are given as nmol/mg protein per 2.5 min. The mean of four experiments ± S.E.M. are reported. Cyt., cytochrome.

	pH 5.5				pH 7.0			
	O_2 consumed	NADPH oxidized	Cyt. *c* reduced	O_2^-	O_2 consumed	NADPH oxidized	Cyt. *c* reduced	O_2^-
1 mM NADPH								
1. Control	72.7 ± 9.7	84.3 ± 6.4			42.0 ± 4.0	45.5 ± 4.0		
2. + superoxide dismutase	14.1 ± 1.9	14.8 ± 3.9			19.9 ± 2.9	20.9 ± 5.0		
3. + cytochrome *c*	9.2 ± 2.6	24.7 ± 7.8	22.6 ± 2.2	9.4	11.8 ± 2.5	32.0 ± 4.3	41.0 ± 6.7	22.1
4. + cyt. *c* + superoxide dismutase	13.9 ± 1.9	28.2 ± 7.2	13.2 ± 1.2	± 2.0	21.2 ± 3.8	34.1 ± 5.6	18.9 ± 3.5	± 4.5
0.15 mM NADPH								
1. Control	22.9 ± 1.2	23.6 ± 0.5			16.0 ± 2.9	19.7 ± 5.4		
2. + superoxide dismutase	12.8 ± 1.9	12.5 ± 1.4			15.3 ± 3.3	16.5 ± 4.1		
3. + cytochrome *c*	7.2 ± 1.0	16.3 ± 4.8	12.4 ± 1.7	7.6	6.9 ± 2.2	23.6 ± 9.4	25.9 ± 5.0	12.4
4. + cyt. *c* + superoxide dismutase	11.4 ± 1.2	11.0 ± 3.2	4.8 ± 0.1	± 1.7	13.9 ± 3.1	23.7 ± 8.9	13.5 ± 4.6	± 3.4

The rate of the so-called 'residual oxygen consumption', as indicated by the oxygen consumption left after the addition of cytochrome *c*, is not affected appreciably either by pH changes or NADPH concentration.

The rate of the 'univalent reduction' of oxygen, as indicated by the superoxide dismutase-sensitive cytochrome *c* reduction, increases by increasing the pH and decreases by lowering the NADPH concentration.

TABLE IV

COMPONENTS OF THE NADPH-OXIDIZING ACTIVITY OF PARTICLES FROM PHAGOCYTOSING POLYMORPHONUCLEAR LEUCOCYTES AT DIFFERENT pH AND SUBSTRATE CONCENTRATIONS

The values in Results are derived from the data of Table III and are expressed in terms of nmol O_2/mg protein per 2.5 min. For explanation, see the text.

Assay conditions		Results				% of total activity		
pH	NADPH (mM)	Total activity	Univalent reduction	Residual oxygen consumption	Chain reaction	Univalent	Residual	Chain
5.5	1	72.7	4.7	9.2	58.6	6.5	12.6	80.6
7.0	1	42.0	11.05	11.8	22.1	26.3	28.1	52.6
5.5	0.15	22.9	3.8	7.2	10.1	16.6	31.4	44.1
7.0	0.15	16.0	6.2	6.9	0.7	38.7	43.1	4.4

From the values reported in the right-hand columns of Table IV, it appears clear that the various experimental conditions used greatly influence the percent contribution of each of the three aliquots to the overall NADPH oxidation. The chain reaction is the most affected, both in terms of reaction rate and percent of total activity.

Discussion

A comparative evaluation of different methods of assaying the NADPH-oxidizing activity by subcellular particles from stimulated cells has revealed that the results vary according to the method used.

The measurement of oxygen uptake or of NADPH oxidation give comparable results, with a stoichiometry of 1 : 1.

When cytochrome *c* (both in the presence or absence of superoxide dismutase) is included in the medium, the NADPH oxidized exceeds the oxygen consumption. This may be due to anaerobic oxidation of NADPH by NADPH-dependent cytochrome *c* reductase present in the preparation.

When O_2^- generation is taken as a measure of the oxidase activity, the values are much lower than those obtained with the measurement of the oxygen consumption or the NADPH oxidized. This difference is due to the complex nature of the NADPH oxidase reaction assayed as oxygen uptake. In fact, in this paper we show that this reaction can be broken down to at least three components, namely, an enzymatic univalent reduction of oxygen, an enzymatic non-univalent reduction of oxygen, which has been referred to previously as residual oxygen consumption, and a non-enzymatic chain reaction. An analysis of the properties of each of these three components has been carried out under different experimental conditions, i.e., by varying the pH and the substrate concentration.

The univalent reduction accounts for the enzymatic generation of O_2^- and is measured as superoxide dismutase-inhibitable cytochrome *c* reduction. Production of O_2^- during the oxidation of NADPH by subcellular particles from activated polymorphonuclear leucocytes has been demonstrated by Patriarca et al. [11] and Babior et al. [12,15]. We show here that the rate of the univalent reduction of oxygen is higher at neutral than at acid pH and that it is affected slightly by changing the substrate concentration from 0.15 to 1 mM. These kinetic properties are in agreement with those reported by Babior et al. [15] for the human enzyme.

A second aliquot corresponds to the oxygen consumption which is detectable even in the presence of cytochrome *c*. The rate of this 'residual oxygen consumption' is not appreciably affected by changes in pH or NADPH concentration. This might be an indication that the enzyme responsible for this residual oxygen consumption differs from the O_2^- generating enzyme. It can be postulated that this enzyme calayzes a divalent reduction of O_2 according to the following reaction

$$NADPH + H^+ + O_2 \xrightarrow{\text{oxidase}} NADP^+ + H_2O_2 .$$

However, the fact that the residual oxygen consumption is attributable to

the same enzyme which produces O_2^- cannot be excluded. In this case it must be admitted that O_2^- is formed within a space inaccessible to cytochrome *c* which is large enough to allow some spontaneous dismutation in the time required for the radical to diffuse out of this space. The extent of diffusion would be modulated by pH conditions known to influence the rate of spontaneous dismutation [29]. At acid pH, where the dismutation rate is high, the ratio between the residual oxygen consumption and the univalent reduction should be higher than at pH 7.0. The results shown in Table IV demonstrate that this is the case under our experimental conditions. It is noteworthy that a similar explanation has been given by Fridovich [30] to account for the cytochrome *c*-insensitive oxygen consumption by the xanthine-xanthine oxidase system.

The inhibitory effect of superoxide dismutase on the NADPH oxidase activity, measured either as oxygen consumption or NADPH oxidation, indicates the existance of a chain reaction initiated by O_2^-:

$$\begin{aligned} &\rightarrow O_2^- + NADPH + 2\,H^+ \rightarrow NADPH^\cdot + H_2O_2 \\ &\quad NADPH^\cdot + O_2 \rightarrow NADP^+ + O_2^- + H^+ \end{aligned}$$

This 'chain reaction' is the third component of the NADPH oxidase activity. It is greatly influenced by pH and by the concentration of NADPH. Thus, when the NADPH oxidase activity is measured at pH 5.5 with 1 mM NADPH, the high activity is accounted for mostly by the chain reaction. On the contrary, at pH 7.0 with 0.15 mM NADPH, the contribution of the chain reaction is negligible and the activity measured is due almost completely to enzymatic reaction(s).

From the analysis of the mechanisms of the NADPH-oxidizing reaction the following conclusions can be drawn:

(1) Some of the discrepancies found in the literature on the kinetic properties of the NADPH oxidase can be explained by the different assay methods used. For example, a pH optimum of 5.5 has been reported for this activity measured as oxygen consumption [8,14,26] or NADPH oxidation [4,11,13, 16,18]. Instead, a pH optimum of 7.0 has been found when assaying the enzyme as O_2^- generation [15]. In this paper we have shown that at pH 5.5 the chain reaction accounts for most of the oxygen consumed. Therefore, this pH provides optimal conditions for the study of the properties of the chain reaction but not for the enzymatic part of the reaction.

(2) Since the components of the NADPH oxidizing reaction are influenced greatly by the experimental conditions used, it is important to carefully select appropriate procedures. For example, when the properties of the enzymatic reaction(s) are to be studied it is advisable to choose conditions in which the chain reaction is not operative. Such conditions are provided by the O_2^- assay at pH 7.0 or by oxygen consumption at pH 7.0 with 0.15 mM NADPH. The O_2^- assay, however, does not allow an evaluation of the residual O_2 consumption, which can be detected instead by using the oxygen consumption method. If the experimental goal is only the detection of the activation of the enzyme, any one of these procedures can be used. To this end, the use of conditions

which favor the chain reaction may make the detection of a difference in activity between preparations from resting and activated cells easier, particularly when the activities are low.

(3) It is widely accepted that the respiratory burst of polymorphonuclear leucocytes is due to the activation of a NADPH-oxidizing system [1,3—19]. The problem then arises of the physiological relevance in intact cells of the mechanisms described here for the oxidation of NADPH by subcellular particles. One of the basic requirements for assessing the physiological importance of an enzyme in the respiratory bust is that its activity is sufficient to account for the extra oxygen consumption of intact cells. The average value of the extra respiration of phagocytosing polymorphonuclear leucocytes used in the present experiments was 99.0 ± 12.1 S.E.M. nmol $O_2/4 \cdot 10^7$ cells per 2.5 min (8 experiments). The oxygen consumed by the NADPH oxidase activity of particles (1 mg particle protein is obtained from $4 \cdot 10^7$ leucocytes) accounts for the extra respiration of intact cells only under conditions where the chain reaction is fully operative. At first sight, this might suggest that a chain reaction functions in the cell also. However, the intracellular concentration of NADPH, 0.1—0.3 mM [8,13,32] and the presence of superoxide dismutase in the cell [33] are not expected to favor a propagation reaction initiated by O_2^-. If this is the case, the extra oxygen consumption should be sustained exclusively by the enzymatic component of the NADPH-oxidizing reaction, which is referred to in this paper as 'univalent reduction' and 'residual oxygen consumption'. However, the present results show that the rate of this enzymatic oxidation of NADPH does not account for the extra respiration of whole polymorphonuclear leucocytes. Since the activation of the NADPH oxidase is not a permanent state [34], it is likely that the enzymatic activity decays in the time required for homogenization and fractionation and, therefore, the activity of the particles does not reflect the levels of activation attained in intact cells.

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