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CHARACTERIZATION AND QUANTIFICATION OF THE PEROXIDASE IN HUMAN MONOCYTES

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Summary

1. The properties of the peroxidase in human monocytes have been compared with those of myeloperoxidase in human granulocytes and with those of isolated human myeloperoxidase.

2. The optical difference spectra of the reduced minus the oxidized states of monocyte and granulocyte homogenates show peaks at 472 and 635 nm that are identical with those observed in the spectrum of reduced isolated myeloperoxidase.

3. Electron paramagnetic resonance spectra of monocytes, granulocytes and isolated myeloperoxidase show a rhombic high-spin haem iron signal with $g_x = 6.90$ and $g_y = 5.07$.

4. Monocyte and granulocyte lysates show a peroxidative reaction with *ortho*-dianisidine and require the same optimal substrate concentrations.

5. Monocyte and granulocyte homogenates, as well as isolated myeloperoxidase, catalyse the oxidation of iodide by H_2O_2. This reaction has an acid pH optimum of 4.5—5.5.

6. Ingested zymosan particles are iodinated by monocytes as well as by granulocytes.

7. On the basis of these observations we conclude that human monocytes contain myeloperoxidase.

8. The concentration of myeloperoxidase in monocytes is about $1.1 \cdot 10^{-17}$ mol per cell. Granulocytes contain three times this amount of myeloperoxidase per cell.

Abbreviations: EPR, electron paramagnetic resonance; PBS, phosphate-buffered saline; MEM, minimal essential medium (Eagle).

Introduction

Human granulocytes contain a peroxidase, known as myeloperoxidase. This peroxidase has optical absorbance [1—3] and electron paramagnetic resonance (EPR) spectra [4,5] that are different from those of other peroxidases [6]. Myeloperoxidase can be detected in granulocyte suspensions with a sensitive spectrophotometer [7,8] and with EPR at low temperature [5,9]. The amount of myeloperoxidase present in human granulocytes, as measured by its enzymic activity [10], was $5 \cdot 10^{-17}$ mol per cell. When the EPR signal of isolated myeloperoxidase was compared with that of granulocytes an amount of $1.9 \cdot 10^{-17}$ mol per cell was found [5]. It has been suggested that myeloperoxidase plays a role in the killing of microorganisms via a reaction that involves H_2O_2 and a halide [11]. As a model of this system in vitro, iodination of ingested zymosan particles by phagocytic cells has been used [12].

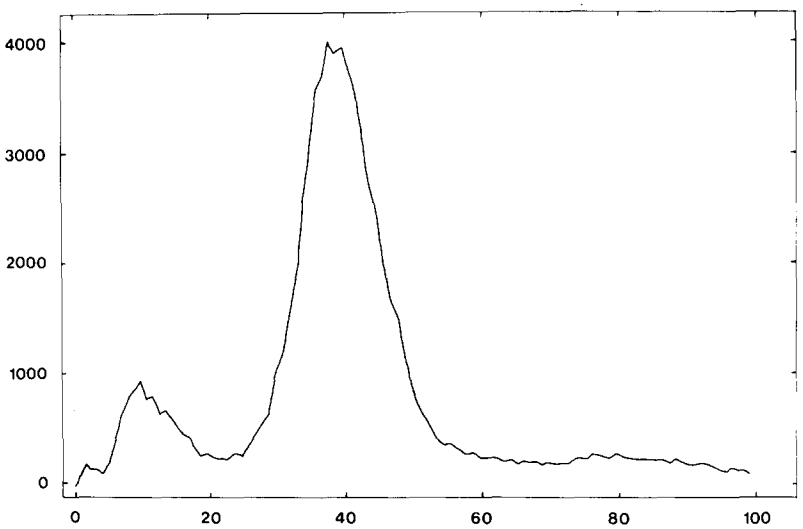
Human monocytes, like granulocytes, are active in the host defence against microorganisms. Monocytes contain a peroxidase that cross-reacts with an antiserum against granulocytic myeloperoxidase [13]. In patients with granulocytes that lack myeloperoxidase, monocytes show neither peroxidase activity [12] nor cross-reactivity towards anti-myeloperoxidase [13]. These observations suggest that human monocytes and granulocytes contain a strongly related peroxidase.

In this paper we describe spectroscopic and enzymic methods that may be used to compare the properties of the monocyte peroxidase with those of myeloperoxidase. The results show that monocytes contain myeloperoxidase.

Materials and Methods

Granulocytes were isolated from fresh human blood (treated with the anticoagulant ACD, acid citrate dextrose, U.S.P. Formula A) as described previously [14]. In short, the blood was centrifuged over Ficoll-Isopaque (ρ = 1.078 g/cm^3), and the cell pellet, containing erythrocytes and granulocytes, was treated with isotonic $NH_4Cl/KHCO_3$ to lyse the red cells [15]. The granulocytes were suspended in 140 mM NaCl buffered with 5.8 mM sodium phosphate (pH 7.2) (PBS), and counted electronically with a Coulter Counter, model ZF. Morphological differentiation showed that the preparations contained more than 95% granulocytes, the other cells being lymphocytes.

Monocytes were isolated from fresh human blood as described recently [16]. In short, after Ficoll-Isopaque (ρ = 1.078 g/cm^3) centrifugation of the blood, the interface cell suspension, containing lymphocytes, monocytes and some erythrocytes, was treated with isotonic $NH_4Cl/KHCO_3$ to lyse the red cells. Thereafter, the cell suspension was kept in glass Petri dishes (90 min at 37°C) for adherence of the monocytes. After removal of the non-adherent cells, the monocytes were scraped from the dishes with a piece of silicone rubber and suspended in Eagle's Minimal Essential Medium (MEM) (Gibco, Grand Island, N.Y.) buffered with 25 mM Tris · HCl (pH 7.4 at 37°C) (MEM-Tris), supplemented with 5% foetal calf serum (Gibco Bio-Cult, Glasgow, Scotland). The preparations contained 82 ± 11% monocytes (mean ± S.D., n = 52), the contaminating cells being lymphocytes and less than 3% granulocytes, as determined



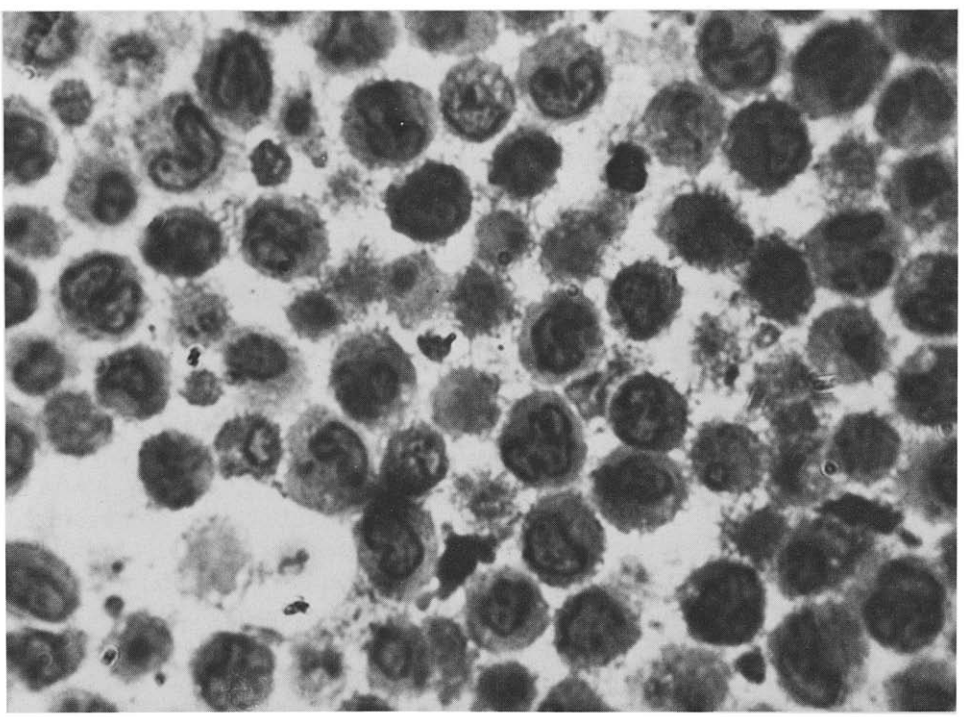


Fig. 1. Purity of monocyte preparations. Upper: Size distribution profile from a Coulter Counter, model ZF, equipped with a pulse-height analyzer (Channelyzer, model C-1000, Coulter Electronics). Abscissa: amplitude of the change in resistance expressed as channel number of the pulse-height analyzer. Ordinate: number of pulses per channel. A sample of 0.2 ml monocytes ($5 \cdot 10^6$ cells/ml) was diluted with 20 ml of particle-free PBS and introduced into a Coulter Counter with a 100 μm orifice. The attenuator was set at 2, the aperture at 16 and the threshold at 10. The lower threshold of the Channelyzer was set at 10 and the window width at 100, and the size distribution profile was recorded. In order to determine the percentage of monocytes the lower threshold switch of the Channelyzer was set at the minimum between the two peaks (channel 22). The total count between this channel and 99 was integrated and expressed as percentage of the total count between channels 0 and 99. One hundred thousand cells were counted. There were 87% monocytes. Lower: Morphologic appearance. The cells were spun down (1000 X g, 10 min, room temperature), and the cell pellet was fixed in 5 ml Karnovsky fixative, post-fixed with OsO_4, dehydrated with ethanol of increasing concentrations and embedded in Epon. Sections of 0.5 μm thickness were stained with Toluidin Blue. Magnification: X560. Granulocytes are not observed. Percentage of monocytes in this preparation, determined as described above, was 92%.

by electronic sizing [17] and microscopic examination. Electronic sizing was performed with a Coulter Counter, model ZF, equipped with a pulse-height analyzer (Channelyzer, model C-1000, Coulter Electronics). In the size distribution profiles so obtained two populations of mononuclear leukocytes could be distinguished (Fig. 1 upper). The % of large cells was considered to indicate the fraction of monocytes, since these cells had been shown by Loos et al. [17] to display intracellular lysozyme activity, phagocytosis, and kidney-shaped nuclei (Fig. 1 lower).

Myeloperoxidase was purified from human blood leukocytes as will be described elsewhere [18]. The ratio A_{430nm}/A_{280nm} of these preparations was 0.6—0.8.

Absorbance spectra were recorded on an Aminco-Chance DW-2 spectrophotometer. Cell lysates were prepared by suspension of granulocytes (10—100 · 10^6/ml) or monocytes (10—50 · 10^6/ml) in PBS, addition of 1% (w/v) cetyl trimethylammonium bromide and subsequent homogenization with a motor-driven Potter homogenizer at 0°C [19]. Sodium dithionite ($Na_2S_2O_4$) was used as reducing agent in the sample cuvette. For the determination of the concentration of isolated myeloperoxidase, an absorbance coefficient of 89 $mM^{-1} \cdot cm^{-1}$ at 430 nm was used [20,21]. In turbid suspensions the concentration of myeloperoxidase was calculated from the reduced minus oxidized difference spectrum at 472 nm. Since the absorbance difference at 472 nm in reduced minus oxidized myeloperoxidase is 84% of the intensity of the peak at 430 nm (not shown), an absorbance coefficient of 75 $mM^{-1} \cdot cm^{-1}$ was used in the calculations.

EPR experiments were carried out as described previously [5]. Granulocytes (3—12 · 10^8/ml) or monocytes (2—4 · 10^8/ml) were suspended in PBS.

Oxidation of *ortho*-dianisidine was measured on a Vitatron spectrophotometer at 470 nm and 25°C. Cells in PBS were lysed by addition of 0.2% (w/v) Triton X-100. The reaction medium contained lysates of granulocytes (5 · 10^3/ml) or monocytes (12 · 10^3/ml), with 1.54 mM *ortho*dianisidine and 0.133 mM H_2O_2 in 10 mM sodium phosphate buffer (pH 6.2). Oxidation of iodide was measured on a Cary 17 spectrophotometer at 350 nm and 25°C. Cells in 50 mM sodium phosphate buffer (pH 7.4) were frozen/thawed once and homogenized. The reaction medium contained homogenates of granulocytes (11 · 10^3/ml) or monocytes (25 · 10^3/ml) with 2 or 20 mM KI, 0.3 mM H_2O_2, 200 mM Na_2SO_4 and 50 mM sodium acetate buffer or 50 mM sodium phosphate buffer of different pH values. A correction was made for the auto-oxidation of iodide by H_2O_2 in the absence of cells. This latter process was independent of the pH.

Incorporation of ^{125}I into acid-precipitable material was measured as described by Weening et al. [22]. The cells were suspended in MEM-Tris/5% foetal calf serum. Granulocytes were used at 4 · 10^6/ml and monocytes at 2 · 10^6/ml.

Results and Discussion

Fig. 2 shows the absorbance difference spectra (reduced minus oxidized) of isolated myeloperoxidase (trace A), of granulocyte homogenates (trace B), and of monocyte homogenates (trace C). These spectra show similar peaks at 472

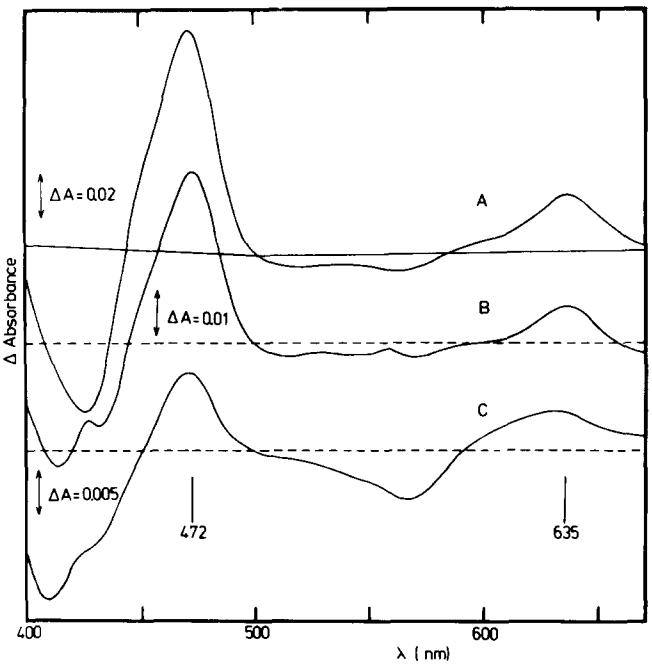
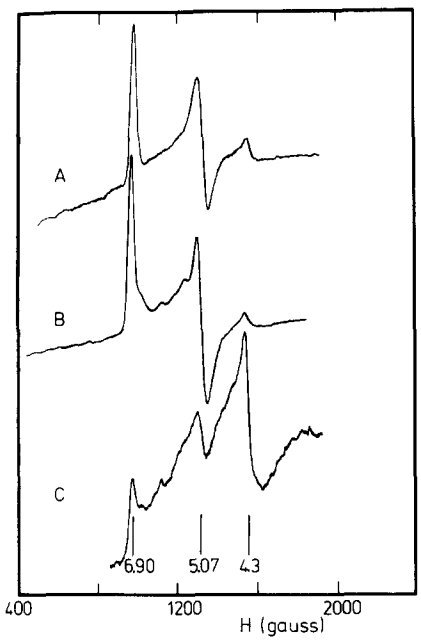


Fig. 2. Difference spectra of reduced minus native myeloperoxidase, reduced minus resting granulocytes, and reduced minus resting monocytes. A, isolated myeloperoxidase, 1.3 μM in 100 mM sodium phosphate buffer (pH 7.4); B, granulocytes, $18 \cdot 10^6$ per ml in PBS; C, monocytes, $10 \cdot 10^6$ per ml in PBS.

and 635 nm. These peaks are absent from the absorbance spectrum of lymphocyte homogenates. The absorbance minimum at 565 nm seen in the monocyte homogenates can be ascribed to the presence of the Phenol Red in Eagle's Minimal Essential Medium that was used in the preparation of monocytes.

Fig. 3 shows the low-field region of the EPR spectra of isolated myeloperoxidase and of granulocyte and monocyte suspensions. All spectra show a rhombically distorted haem iron signal with $g_x = 6.90$ and $g_y = 5.07$, characteristic for myeloperoxidase [4,5]. These signals were not observed in lymphocyte suspensions. In addition, monocyte suspensions (trace C) exhibited a more intense signal at $g = 4.3$ which can be ascribed to non-haem iron. In the high-field region of the EPR spectrum of isolated myeloperoxidase a minor low-spin species ($g_x = 2.56$, $g_y = 2.31$, $g_z = 1.81$) was observed (cf. ref. 18) which was, however, not detectable in the spectra of monocytes and granulocytes.

The enzymic properties of the peroxidase in monocytes and granulocytes were studied with H_2O_2 as substrate and *ortho*-dianisidine as electron donor. In lysates of both cell types, maximal velocity was obtained with 0.135 mM H_2O_2 and 1.54 mM *ortho*-dianisidine. Higher concentrations of either H_2O_2 or the dye inhibited the reaction. Since the molar absorbance coefficient of *ortho*-dianisidine was dependent on pH, it is not possible to use *ortho*-dianisidine to study the enzymic activity of myeloperoxidase as a function of the pH. Therefore, *ortho*-dianisidine was substituted by iodide in the assay. Fig. 4 shows that, in the oxidation of I^- by H_2O_2, isolated myeloperoxidase, as well as granulocyte and monocyte homogenates, has an optimal pH of 5.5.



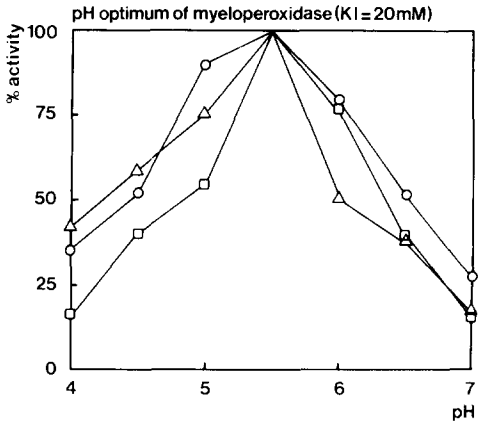


Fig. 3. EPR spectra of isolated myeloperoxidase, granulocytes and monocytes. A, isolated myeloperoxidase, 14.3 μM in 50 mM sodium phosphate buffer (pH 7.2); B, granulocytes, $12 \cdot 10^8$ per ml in PBS; C, monocytes $3.5 \cdot 10^8$ per ml in PBS. Conditions of EPR spectroscopy: frequency, 9.318 GHz; microwave power, 10 mW; modulation amplitude, 16 G; scanning rate, 500 G $\cdot$ min^{-1}; time constant, 1.0 s; temperature, 14 K. Gain in B was half that in A and gain in C was twice that in A.

Fig. 4. Oxidation of iodide by isolated myeloperoxidase, granulocytes and monocytes as a function of pH. ○———○, isolated myeloperoxidase ($5 \cdot 10^{-10}$ M); 100% activity = 0.75 pkat/10^{-12} mol myeloperoxidase. △———△, granulocytes (10^4 cells per ml); 100% activity = 2.48 pkat/10^5 granulocytes. □———□, monocytes ($2.5 \cdot 10^4$ cells per ml); 100% activity = 0.74 pkat/10^5 monocytes. Incubation conditions: 20 mM KI, 0.3 mM H_2O_2, 200 mM Na_2SO_4, 50 mM sodium uptake acetate buffer (pH 4—6) or 50 mM sodium phosphate buffer (pH 6—7). The cells were isolated from the same sample of blood.

Monocytes, like granulocytes, are capable of zymosan iodination [12,23,24]. Fig. 5 shows that both cell types exhibited the same pattern in this reaction, only the rate of iodination being different.

From these experiments it is evident that the peroxidases in human monocytes and granulocytes have the same physical (EPR and optical spectra) and enzymic properties (with *ortho*-dianisidine and iodide as electron donors) as isolated myeloperoxidase. Moreover, both cell types showed the same biological reaction (iodination of ingested particles), in which myeloperoxidase is thought to play a role. Therefore, we conclude that the peroxidases in human monocytes and in human granulocytes are identical. Although the monocyte preparations were contaminated with granulocytes, this contamination was so small (less than 3% granulocytes) that it cannot account for the amount of myeloperoxidase present in the monocyte preparations.

The concentration of myeloperoxidase in monocytes and in granulocytes can be calculated from the optical spectra by using the absorbance coefficient (75 mM$^{-1} \cdot$ cm^{-1}) for the absorption band at 472 nm (see Materials and Methods). It is also possible to calculate the concentration of myeloperoxidase

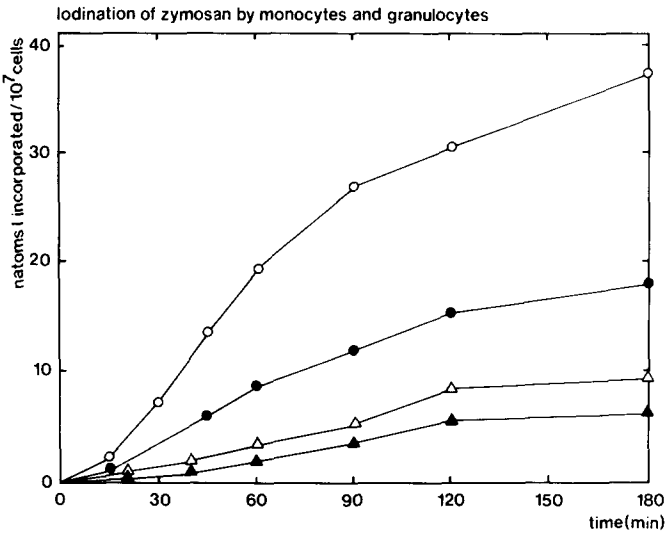


Fig. 5. Iodination of zymosan by granulocytes and monocytes. Granulocytes ($4 \cdot 10^6$ per ml) were incubated with 1.5 mg zymosan per ml (○———○) or 1 mg zymosan per ml (●———●); monocytes ($2 \cdot 10^6$ per ml) were incubated with 1.5 mg zymosan per ml (△———△) or with 1 mg zymosan per ml (▲———▲). The cells were isolated from the same sample of blood.

in monocytes and granulocytes from the EPR spectra by comparison of the intensity of the peak at $g = 5.07$ with that of isolated myeloperoxidase in a spectroscopically determined concentration. In addition, the concentration can be calculated by comparison of the initial velocities of the oxidation of *ortho*-dianisidine. The results are summarized in Table I. All three methods give similar values for the amount of myeloperoxidase present in monocytes ($1.1 \cdot 10^{-17}$ mol/cell) and in granulocytes ($3.4 \cdot 10^{-17}$ mol/cell).

From the rate of oxidation of iodide, catalysed by isolated myeloperoxidase and monocyte and granulocyte homogenates, a myeloperoxidase concentration of $1.0 \cdot 10^{-17}$ mol per monocyte and of $3.3 \cdot 10^{-17}$ mol per granulocyte can be calculated (Fig. 4), in good agreement with Table I. Thus, our results show that monocytes contain about one-third of the myeloperoxidase found in granulocytes. This is in agreement with the results of Baehner and Johnston [23].

The iodination experiments cannot provide information about the amount of myeloperoxidase in monocytes or granulocytes because this process is dependent on several additional factors, e.g. rates of particle ingestion and of H_2O_2 formation. Both the latter functions proceed at a considerably slower rate in monocytes than in granulocytes [16].

TABLE I

MYELOPEROXIDASE CONCENTRATION IN HUMAN MONOCYTES AND GRANULOCYTES

Monocyte and granulocyte concentrations are expressed in mol $\cdot 10^{-17}$/cell (mean ± S.E.M.). Number of experiments is indicated in parentheses.

Method	Monocytes	Granulocytes
Optical spectra	0.9 ± 0.1 (n = 5)	3.5 ± 0.1 (n = 7)
EPR spectra	1.1 ± 0.1 (n = 5)	3.2 ± 0.2 (n = 10)
Oxidation of *o*-dianisidine	1.3 ± 0.1 (n = 10)	3.4 ± 0.2 (n = 9)

As shown by us and in agreement with a previous report [24], myeloperoxidase catalyses the oxidation of I^- by H_2O_2. This reaction is inhibited by 0.3 mM NaN_3 and by 3 mM aminotriazole. At a KI concentration of 20 mM, the optimum of the iodide oxidation is at pH 5.5, as shown in Fig. 4. However, the pH optimum shifts to 4.5 when the iodide concentration is lowered to 2 mM. With other concentrations of iodide or H_2O_2, the optimal pH for this reaction remains between 4.5 and 5.5 (not shown). Moreover, Klebanoff has reported that the iodination of bacteria by a system containing partially purified canine myeloperoxidase, H_2O_2 and iodide is favoured by a pH lower than 6 [11]. The acid pH optimum of these reactions is consistent with the lysosomal localization of myeloperoxidase.

Acknowledgements

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