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EXAMINATION OF THE OXIDASE FUNCTION OF THE *b*-TYPE CYTOCHROME IN HUMAN POLYMORPHONUCLEAR LEUCOCYTES

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The spectral properties of a particulate fraction of human polymorphonuclear neutrophils capable of oxidizing NADPH were studied before and after depletion of myeloperoxidase by KCl treatment. Difference spectra (dithionite reduced minus oxidized) at 77 K of non-extracted particles showed peaks of a *b*-type cytochrome at 556, 527 and 425 nm and of myeloperoxidase at 636 and 474 nm. Extraction of myeloperoxidase led to a 4–5-fold increase in the size of the cytochrome *b* peaks. In non-extracted particles, the CO-reduced spectra at 77 K revealed a typical CO-reduced myeloperoxidase complex with new peaks at 625–630 and 462 nm, and a limited shift of the Soret band of reduced cytochrome *b* from 425 to 424–423 nm. The same shift was observed for cytochrome *b* in extracted particles. Photoirradiation of the CO-dithionite-reduced particles resulted in a back shift of the CO-reduced peaks to their original positions in the reduced spectrum. Concomitantly, the size of the peaks both for myeloperoxidase and cytochrome *b* was increased, indicating photoreduction. Cytochrome *b* and myeloperoxidase in neutrophil particles were poorly reduced by NADPH; reduction occurred upon photoirradiation. FAD and FMN added to particles in the presence of NADPH were photoreduced concomitantly with cytochrome *b*. Addition of phorbol myristate acetate to intact neutrophils in the presence of glucose resulted in CO- and cyanide-insensitive respiration, accumulation of O_2^-, and also in reduction of cytochrome *b*. The lag required to reach the steady-state production of O_2^- was equal to the lag required for cytochrome *b* to reach a plateau of reduction. The data are consistent with the idea that cytochrome *b* in neutrophils might belong to a branched pathway that is not rate-limiting in the cyanide-resistant respiration of the neutrophils.

Introduction

The increase in oxygen uptake by polymorphonuclear neutrophils during phagocytosis is caused by the activation of a NADPH oxidase complex, located in plasma membranes. This enhanced respiration is cyanide-resistant; it generates a one-electron reduction product of oxygen, the superoxide anion O_2^- (see Refs. 1–5). Much attention has recently been paid to be a *b*-type cytochrome located in plasma membrane of neutrophils. A number of experimental data have been reported which were interpreted as evidence that the *b* cytochrome is the terminal oxidase in the cyanide-resistant respiration of neutrophils. (1) The *b* cytochrome of neutrophils has a mid-point potential at neutral pH of −245 mV [6,7], a value close to that of the O_2/O_2^- couple, i.e., −330 mV [8,9] or −160 mV [10]. (2) It is reduced by NADPH, the physiological source of reducing equivalent for the cyanide-resistant respiration of neutrophils, and

Abbreviations: Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid; Mops, 4-morpholinepropanesulphonic acid.

reduced cytochrome *b* is readily reoxidized [11,12]. (3) In its reduced form, it binds CO, and the CO-reduced cytochrome *b* complex is photodissociable [6]. (4) It is localized in the plasma membrane and the specific granules of neutrophils [13]. (5) It is incorporated into the membrane of phagocytic vacuoles during phagocytosis [14]. (6) It is absent or in an aberrant form in neutrophils from some patients with chronic granulomatous disease [15]. (7) Activation of human neutrophils by phorbol myristate acetate results in reduction of cytochrome *b* [16]. (8) During the granulocytic differentiation in the human HL-60 myeloid leukaemia cell line, there is a correlation between the appearance of the respiratory burst and the appearance of cytochrome *b* [17]. In spite of these data, the function of cytochrome *b* as an intermediate of the NADPH oxidase complex is still in question. For example, Hamers et al. [18] were unable to detect any reaction of reduced cytochrome *b* with CO; further, they could separate NADPH oxidase from cytochrome *b* by sucrose gradient centrifugation of neutrophils membrane fragments without loss of oxidase activity. Gabig et al. [12] found that the generation of O_2^- by the cyanide-resistant respiration was a 1000-times faster than cytochrome *b* reduction in anaerobiosis. Since the above discrepancies might be due in part to differences in experimental conditions, a critical analysis of the redox components in intact and fragmented neutrophils was desirable. This was the aim of the present work. The data reported here indicate that cytochrome *b* behaves as a branched oxidase.

Materials and Methods

Heparin was obtained from Choay Laboratories. Dextran T 500 from Pharmacia, Ficoll, phorbol myristate acetate, ferricytochrome *c* (horse heart grade VI), superoxide dismutase, Mops and Hepes from Sigma, NADPH, FMN and FAD from Boehringer, sodium metrizoate (hypaque) from Nyegaard, sucrose from BDH, sodium dithionite from Merck, KCN and all other reagents from Prolabo. Phorbol myristate acetate was dissolved at 2 mg/ml in dimethyl sulfoxide. High-purity gases were from Air Liquide, Grenoble, France.

Preparation of intact neutrophils and neutrophil particles

Veinous blood from human volunteers was drawn on heparin (25 I.U./ml blood). In routine preparations, 200 ml of blood was used. To allow sedimentation of erythrocytes, the blood was diluted with one third of its volume of an NaCl solution containing 2 g Dextran T500 per 100 ml of 0.9% NaCl, and poured into 100-ml siliconized cylinders, kept at 37°C. Sedimentation of red cells was virtually complete after 45 min. The supernatant containing the leucocytes was recovered. 20-ml fractions were layered onto 10 ml of a mixture of 5.6% Ficoll (w/v) and 9.6% sodium metrizoate, $d = 1.077$, in 50-ml propylene tubes, and centrifuged at $400 \times g$ for 30 min at 20°C. After careful elimination of the supernatant by aspiration, the pellet of purified neutrophils was resuspended in 6 ml of cooled distilled water, and the suspension was gently stirred for 30 s to lyse the remaining contaminant erythrocytes. Isotonicity was reestablished by addition of 2 ml of 3.5% NaCl, and the suspension was centrifuged for 10 min at $400 \times g$ at 20°C. This step was repeated once. The erythrocyte-free neutrophils were suspended in a phosphate-buffered saline medium made of 2.7 mM KCl/136.7 mM NaCl/1.5 mM KH_2PO_4/8.1 mM Na_2HPO_4 (phosphate-buffered saline medium) at the final concentration of about $3 \cdot 10^8$ cells/ml; 95–98% of the cells were found to be neutrophils by differential counting; of these, more than 95% were viable by the test of Trypan blue exclusion. Contamination by platelets was less than one platelet for 1000 cells.

For activation by phorbol myristate, the cells were suspended in the phosphate-buffered saline medium supplemented with 10 mM glucose at 37°C, and phorbol myristate was added at the final concentration of 10 μg per 10^8 cells. After a 30 min contact at 37°C, activation was terminated by dilution with 30 vol. of ice-cooled phosphate-buffered saline medium. The cells were sedimented by centrifugation in a medium comprising 0.20 M sucrose/0.05 M Mops/0.002 M EDTA, pH 7.3 (standard sucrose medium). In the absence of activation, the neutrophils in the phosphate-buffered saline medium were directly transferred to the standard sucrose medium after centrifugation. The particulate fraction used in this work

was routinely prepared at 4°C from resting or activated neutrophils. The neutrophil suspension ($6 \cdot 10^8$ cells) in 2 ml standard sucrose medium was subjected to sonic irradiation in a Sonimasse apparatus (Annemasse, France) at maximum output at 4°C, twice for 15 s each time, each step of sonication being separated by a 1 min interval. The homogenate was centrifuged at $400 \times g$ for 10 min to eliminate unbroken cells. The supernatant was pipetted out and centrifuged at $100\,000 \times g$ for 1 h at 4°C. The pellet was resuspended in 2 ml of the standard sucrose medium. This crude membrane preparation was used immediately. Protein was assayed as described by Bradford [19].

Preparation of membrane fragments of neutrophils, depleted of myeloperoxidase. Membrane fragments with low myeloperoxidase content were obtained by two different methods. The first one was based on extraction of myeloperoxidase by KCl. The crude membrane preparation in the standard sucrose medium was diluted 10-times with the same sucrose medium. The suspension was supplemented with solid KCl to a final concentration of 2 M, and then left to incubate overnight at 4°C with stirring. In some assays, the final concentration of KCl was adjusted to values in the range 0.1–2 M. After centrifugation at $100\,000 \times g$ at 4°C for 1 h, the pellet was homogenized in the same volume of standard sucrose medium as that used at the beginning of the extraction, and assayed spectrophotometrically for cytochrome *b* and myeloperoxidase content.

The second method consisted of the fractionation of a $270 \times g$ supernatant homogenate by centrifugation on a discontinuous sucrose gradient [20] and the recovery of a fraction enriched in cytochrome *b* and depleted in myeloperoxidase. The purified neutrophils suspended in 0.30 M sucrose/0.02 M Mops/0.005 M $MgCl_2$ (pH 7.3) at a final concentration of $(2–5) \cdot 10^8$ cells/ml were homogeneized in a Dounce homogeneizer with a tightly fitted pestle. The number of strokes ranged between 50 and 100, homogeneization being stopped when 50–70% of the neutrophils had been broken, as checked under the optical microscope after coloration with Hayem reagent. The pH was maintained at 7.3, some acidification occurring during homogeneization. The homogenate was diluted 1.6-fold with the same sucrose/$MgCl_2$ medium as above. This was followed by a low-speed centrifugation at $270 \times g$ for 10 min at 4°C. 1 ml of the supernatant was layered on 10.5 ml of sucrose medium composed of five layers consisting of 2 ml 20% sucrose, 3 ml 32% sucrose, 2 ml 36% sucrose, 2 ml 42% sucrose and 1.5 ml 50% sucrose. Centrifugation was at $63\,000 \times g$ for 1 h at 4°C. The fraction enriched in cytochrome *b* was localized at the 20–33% sucrose interface; it was withdrawn and diluted in a buffer made of 0.01 M Mops/0.5 M KCl (pH 7.3) to lower the sucrose concentration to about 12%. The suspension was sedimented again by centrifugation at $100\,000 \times g$ for 1 h at 4°C and the pellet was resuspended in the standard sucrose solution. In agreement with the data of Mottola et al. [20] for bovine neutrophils, the gradient purified fraction obtained here was enriched about 15-times in alkaline phosphatase, 20-times in cytochrome *b* and 10-times in O_2^--generating activity; on the other hand, it was virtually devoid of myeloperoxidase. The enrichment in alkaline phosphatase, cytochrome *b* and O_2^--generating activity in the same fraction together with the elimination of myeloperoxidase indicated that cytochrome *b* is a component of the plasma membrane and could participate to the O_2^--generating respiration of neutrophils.

Spectrophotometric measurements. Difference spectra were recorded with a double-beam Perkin-Elmer 557 spectrophotometer with a baseline that could be programmed automatically. The scan speed for routine spectra was 60 nm per min; however, for accurate determination, especially in the case of the peaks in reduced and reduced/CO spectra, the scan speed was lowered to 12 nm per min. The spectral band width was 0.5 nm. The optical path of the cuvettes was 2 mm for spectral analysis at 77 K and 1 cm at 37°C. Reduction by dithionite was started by adding a preweighed sample of dithionite to the cuvette medium, pregassed with argon to reach a final concentration of 10 mM. Because of the strong buffering capacity of the standard sucrose medium, the pH was not modified. In some experiments, a mixture of glucose and glucose oxidase (40 mM and 2 mg, respectively) was added. For spectra at 37°C, the 1-cm cuvettes were hermetically sealed with gas-tight rubber stoppers, in which two needles were inserted. One of the needles was used for flushing

either argon or CO, the other for gas evacuation. All reagents were made anaerobic. When NADPH was used as reductant, the concentration was 300 μM. All further additions were made by gas-tight syringes. For CO difference spectra, the dithionite-reduced membrane fractions were bubbled with CO of purity greater than 99.9% at 4°C for 30 min under strict anaerobiosis; after 1 h at 4°C in the dark, the spectra were recorded. In the case of low-temperature spectra, the content of the 2-mm path cuvettes was frozen in liquid nitrogen and placed in an unsilvered Dewar flask, partially filled with liquid nitrogen in the cell compartment of the spectrophotometer [21]. Prior to spectrum scanning, the base line was recorded and stored in the memory of the spectrophotometer.

Photoirradiation. The light source was a 250 W Osram halogen lamp, placed at 7 cm from the cuvette containing the particle suspension. The frozen particle suspension in the 2-mm path cuvette was photoirradiated for periods of 10 s, separated by 1-min intervals during which the cuvette was plunged in liquid nitrogen. In the case of unfrozen particles, the 3-ml sealed cuvettes were photoirradiated in a cold air current for periods up to 15 min. The reference cuvette was maintained in the dark. Reduced photoirrediated minus oxidized spectra were recorded.

Respiratory activity. O_2^- generated by the oxidation of NADPH was assayed by reduction of ferricytochrome *c* at 30°C [22]. The medium consisted of 0.1 mM ferricytochrome *c* in 0.05 M Hepes buffer (pH 7.0)/2 mM KCN/1 mM sodium azide and the particle preparation (30–50 μg protein, final volume 1 ml). The reference cuvette was supplemented with a large excess superoxide dismutase (final concentration 1 μM). The reaction was started in both cuvettes by addition of 0.14 mM NADPH in 0.05 mM Hepes buffer (pH 7.0). The time-course of cytochrome *c* reduction was followed at 550 nm. For calculation the molar extinction coefficient of reduced cytochrome *c* was taken as 21 $mM^{-1} \cdot cm^{-1}$ [23].

Oxygen uptake was measured amperometrically with a Clark type oxygen electrode (Y.S.I. 5331 oxygen probe, Yellow Spring Instrument, U.S.A.), equipped with a standard Teflon membrane. The oxygraphic cell was maintained thermostatically at 37°C. It contained 1.5 ml of phosphate-buffered saline medium. Calibration with dithionite indicated an O_2 concentration of 200 μM. The K_m O_2 values were calculated from oxygraphic traces between the point at which the traces deviated from linearity, and anaerobiosis in a 40 μM O_2 concentration medium. During the non-linear part of the traces, the recorder sensitivity was increased 20-fold and the expanded curves were analyzed in terms of rate of O_2 uptake by drawing tangents at different O_2 concentrations. Photoirradiation of the oxygraphic cell was performed with a 250 W Osram lamp, placed at 10 cm from the oxygraphic cell. The non-photoirradiated sample in the oxygraphic cell was protected from light by aluminium foil.

Results

The CO reactivity of dithionite-reduced pigments in a crude membrane fraction of neutrophils

The cyanide-resistant respiration of neutrophils is catalyzed by a specific respiratory chain whose components are localized in the plasma membrane. In the following, we examine the dithionite-reducible pigments, present in a crude membrane fraction of neutrophils, including plasma membrane.

The spectra of membrane fragments shown in Fig. 1 were taken at 77 K. The difference spectrum (dithionite-reduced minus oxidized) (spectrum A) revealed peaks at 556, 527 and 425 nm, which are typical of a *b*-type cytochrome, and peaks at 637 and 474 nm, that are assigned to myeloperoxidase. The identity of cytochrome *b* was corroborated by the hemochrome spectrum with a typical peak at 555 nm at 20°C, in accordance with the data reported by Segal and Jones [13]. Based on the reduced peaks, it could be calculated that the amount of myeloperoxidase in the crude membrane fraction was 30% of that present in a total homogenate of neutrophils, indicating that a large part of myeloperoxidase is not bound to membranes.

When the dithionite-reduced crude membrane fraction was reacted with CO, the peaks at 637 and 474 nm due to reduced myeloperoxidase were shifted to 625–630 and 462 nm, respectively (spectrum B). In the case of reduced cytochrome *b*, a slight shift of the Soret band from 425 to 423–424

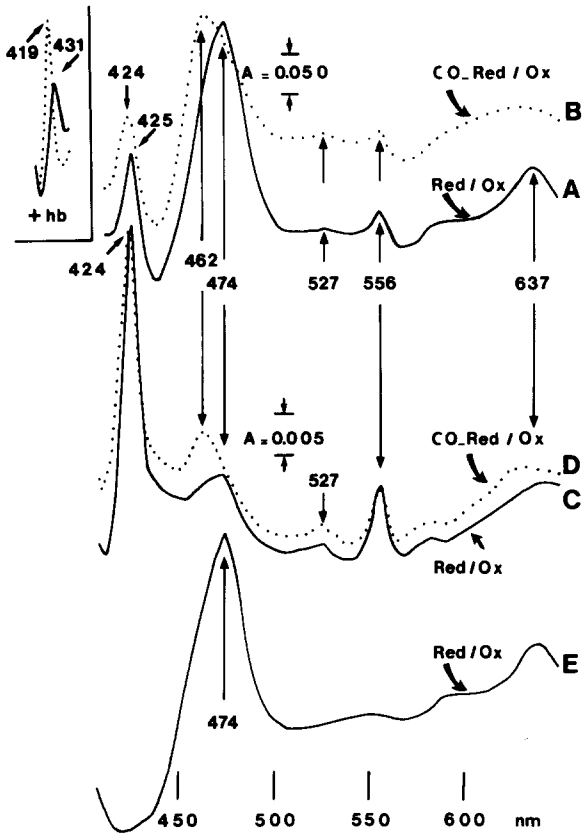

Fig. 1. Dithionite and CO-difference spectra of a membrane fraction of neutrophils at 77 K. Conditions of dithionite reduction and reaction with CO are described in Materials and Methods. 2-mm path cuvettes were filled with 0.5 ml of a suspension of neutrophil membrane fragments corresponding to 10 mg of protein (scans A and B), and 0.5 ml of a suspension of purified membrane, corresponding to 1 mg of protein (scans C and D). Scan A corresponds to the reduced-minus-oxidized spectrum, scan B to the CO-reduced-minus-oxidized spectrum of the crude membrane fraction not yet depleted of myeloperoxidase. Scans C and D correspond to the reduced-minus-oxidized spectrum and the CO-reduced-minus-oxidized spectrum, respectively, of a cytochrome-*b*-enriched fraction of neutrophil fragments recovered by gradient sucrose centrifugation (see Materials and Methods). Scan E corresponds to the reduced-minus-oxidized spectrum of partially purified neutrophil myeloperoxidase. Insert: Effect of contamination by hemoglobin. 0.9 nM of hemoglobin was mixed with 10 mg of the crude membrane fraction of neutrophils in 0.5 ml. The full line scan corresponds to the reduced-minus-oxidized spectrum and the dotted line to the CO-reduced-minus-oxidized.

nm was visualized on the spectrum after CO bubbling for periods as long as 30 min–1 h; no other modifications of the reduced cytochrome *b* spectrum could be detected.

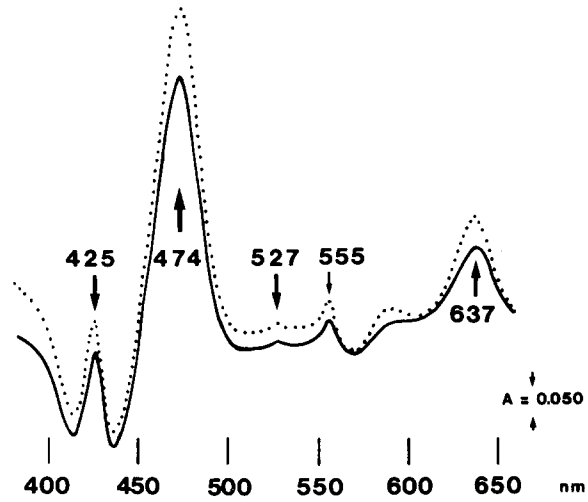

Fig. 2. Effect of photoirradiation on the dithionite-reduced-minus-oxidized pigments of a membrane fraction of neutrophils at 77 K. The experimental conditions were the same as in Fig. 1 (Scans A and B). Photoirradiation was performed for 10 s as described in Materials and Methods. ———, before irradiation; - - - - - -, after 10 s irradiation.

Upon photoirradiation at 77 K of the dithionite-reduced membrane fragments combined with CO, the peaks at 466 and 625–630 nm ascribed to the CO-reduced myeloperoxidase complex were shifted back to 474 nm and 637 nm, respectively, which is typical of photodissociation of any CO-reduced pigment complex to regenerate

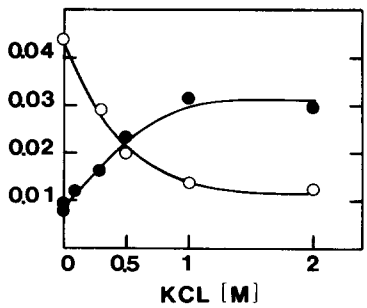

Fig. 3. Effect of increasing concentration of KCl on extraction of myeloperoxidase and the concomitant rise in absorbance of the dithionite-reduced cytochrome *b*. The data plotted on the figure concern the height of the Soret bands of cytochrome *b* (425–437 nm) (●———●) and myeloperoxidase (474–500 nm) (○———○).

the reduced pigment. A shift of the Soret band of reduced cytochrome *b* from 423 to 425 nm apparently occurred upon photoirradiation. Unexpectedly, the red shift caused by photoirradiation was accompanied by a 10–20% increase in the height of the shifted peaks, both for myeloperoxidase and cytochrome *b*. This effect of light was also found at 77 K with dithionite-reduced neutrophil fragments (Fig. 2). It means that a fraction of cytochrome *b* and myeloperoxidase remains oxidized in the presence of dithionite, and that this fraction is photoreduced. This will be discussed later.

The 1–2 nm blue shift of the Soret band of reduced cytochrome *b* after reaction with CO is the only manifestation that suggests that cytochrome *b* may combine with CO. The most trivial among the possible artifacts responsible for this minor shift was the presence of contaminant hemoglobin in neutrophils preparations [18]. In fact, hemoglobin at 77 K has a spectrum not very different from that of reduced cytochrome *b*; for example, the peak of hemoglobin at 429–431 nm is quite close to that of cytochrome *b* at 425 nm. The contribution of added hemoglobin (0.9 nM for 10 mg of the membrane fraction) to the spectrum of reduced neutrophils at 77 K is illustrated in Fig. 1 (insert). As shown in the spectrum, the Soret band of hemoglobin is shifted to 419 nm after reaction with CO. Even with 10-times less added hemoglobin, an unambiguous shoulder at 410 nm, reflecting the hemoglobin-CO complex, was observed. Contamination of neutrophils by hemoglobin can therefore be conveniently assessed by examination of the CO-spectrum; in the present experiment, contamination by hemoglobin was negligible.

The minor displacement of the Soret band of reduced cytochrome *b* after reaction of neutrophils with CO could also be explained on the basis of the spectral interference of reduced cytochrome *b* and myeloperoxidase. Preliminary assays had shown that myeloperoxidase could be selectively removed by KCl treatment of the crude membrane fraction of neutrophils. Extraction of myeloperoxidase as described in Materials and Methods led to a marked increase in the size of the peaks at 425, 527 and 556 nm, assigned to reduced cytochrome *b* (Fig. 1, spectrum C). Concomitantly with the progressive extraction of myeloperoxidase with increasing concentrations of KCl, the size of the peaks of cytochrome *b* was increased (Fig. 3). The response to CO remained, however, similar to that observed with the non-extracted membrane fragments, with the same maximal shift of the Soret band of cytochrome *b* in the CO-reduced spectrum from 425 to 423–424 nm (Fig. 1, spectrum D). The reverse experiment, which consisted in reloading myeloperoxidase-depleted neutrophils with purified myeloperoxidase, resulted in a decreased sharpening of the three peaks of cytochrome *b* (not shown).

Another way to eliminate the spectral interference between myeloperoxidase and cytochrome *b* was to separate the respective membrane fractions, in which each of the two species are localized by centrifugation on a discontinuous sucrose gradient. Cytochrome *b* was preferentially located in the membrane fraction at the 20–33% sucrose interface and myeloperoxidase was recovered at the 42–46% sucrose interface. The 20–33% sucrose fraction enriched in cytochrome *b* and largely depleted in myeloperoxidase (more than 90% with respect to the crude membrane fraction) behaved like the crude membrane fraction depleted in myeloperoxidase by KCl extraction, with respect to dithionite reduction and CO-reactivity. Based on a molar extinction coefficient of 21.7 $mM^{-1} \cdot cm^{-1}$ at 558 nm [18], it was calculated from dithionite-reduced spectra at room temperature that the crude membrane fraction of neutrophils obtained by centrifugation in sucrose gradient, enriched in cytochrome *b* and depleted of myeloperoxidase, contains 0.3–0.4 nM of cytochrome *b* per mg protein, depending on membrane preparations. The increase in absorbancy in the region of the Soret band of cytochrome *b* can be readily explained by the fact that reduced myeloperoxidase is characterized by a trough in the same region of the spectrum. The presence of cytochrome *b* and myeloperoxidase in the same membrane fraction therefore results in an artificial decrease of the Soret peak of cytochrome *b*.

That CO reacts with reduced cytochrome *b* is therefore possible; the spectral evidence is, however, tenuous; it is based essentially on the 1–2 nm blue shift of the Soret band after bubbling CO in the neutrophil suspension treated by dithionite.

Reduction of pigments in a membrane fraction of neutrophils in the presence of NADPH and other electron donors. Effects of light

If cytochrome *b* is an intermediate in the cyanide-resistant respiration of neutrophils, one can predict that it should readily be reduced by NADPH, since NADPH is the source of reducing equivalents for this type of respiration [24]. In the following assays, incubation of neutrophil membrane fragments with NADPH was carried out at 37°C, and the spectra were also recorded at 37°C.

When the membrane fraction was prepared from neutrophils that had been activated by phorbol myristate, a slight reduction (10% in 5 min and 15% in 15 min) of both cytochrome *b* and myeloperoxidase by NADPH was observed (not shown). There was no reduction with a membrane fraction from non-stimulated cells. This is in accordance with recent results obtained with a membrane preparation of human neutrophils [12].

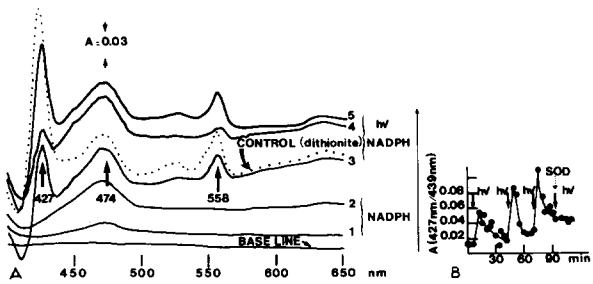

Fig. 4 (A). Enhancing effect of photoirradiation on the reduction of cytochrome *b* by NADPH at 37°C in a membrane fraction of neutrophils, depleted of myeloperoxidase at 37°C. The 1-ml cuvette of 1-cm path was filled under anaerobic conditions with the particulate fraction of neutrophils (7 mg protein), partially depleted of myeloperoxidase, in phosphate-buffered saline medium supplemented with glucose and glucose oxidase (see Materials and Methods). After addition of NADPH (final concentration 0.3 mM), spectra were recorded after 1 min and 5 min. At 7 min, the sample cuvette was withdrawn and photoirradiated for 5 min (see Materials and Methods) and its spectrum was immediately recorded (scan 3). The photoirradiated cuvette was left in its holder and another spectrum was recorded after 3 min (scan 4). The cuvette was again photoirradiated for 15 min and the spectrum recorded (scan 5). (B). Cycles of photoreduction. Effect of superoxide dismutase. A series of 2, 5, 15 min photoirradiation was carried out, showing the consequent photoreduction followed by reoxidation. Addition of superoxide dismutase (1 μM final concentration) (arrow) prevented photoreduction.

On the other hand, reduction by NADPH of cytochrome *b* in a plasma membrane fraction from horse neutrophils, either activated or not by PMA, was also reported [11].

In the course of these assays, it was found that reduction of cytochrome *b* by NADPH was markedly accelerated upon photoirradiation of membrane fragments from stimulated or unstimulated neutrophils. For a more accurate analysis of the effect of light on cytochrome *b*, the neutrophil fragments were first partially depleted of myeloperoxidase by KCl extraction, and then added to a medium, in which the oxygen tension was maintained low by bubbling with argon, followed by addition of glucose and glucose oxidase. Data in Fig. 4A and B illustrate a typical experiment of photoreduction of a crude membrane fraction, prepared from unstimulated neutrophils. Incubation of this fraction for 15 min at 37°C with 300 μM NADPH did not result in any reduction of cytochrome *b* (spectrum 1). However, there was some reduction of the residual myeloperoxidase by NADPH. Repetitive cycles of photoirradiation led to progressive reduction of cytochrome *b* (peak at 427 nm) and the residual myeloperoxidase (peak at 474 nm). No reduction occurred when NADPH was omitted. The extent of photoreduction of cy-

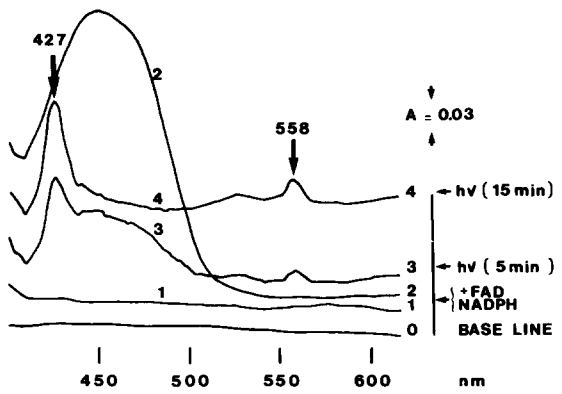

Fig. 5. Effect of FAD on photoreduction of cytochrome *b* in a myeloperoxidase-depleted particulate fraction of neutrophils at 37°C. The conditions are the same as in Fig. 4, except that sodium azide was added at the final concentration of 10 mM to block residual myeloperoxidase activity. The first spectra were recorded 2 min after addition of 300 μM NADPH (scan 1), and 5 μM FAD (scan 2). Then the sample cuvette containing NADPH and FAD was photoirradiated for 5 min (scan 3) and 15 min (scan 4).

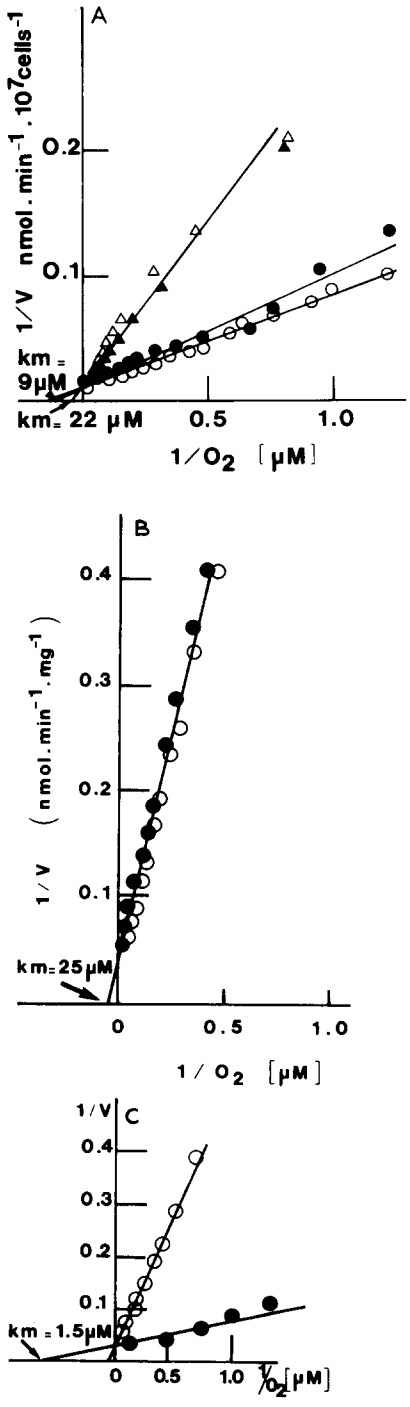

Fig. 6 (A). Effect of CO in the dark (●, ○) and light (▲, △) on the respiration of intact neutrophils activated by phorbol myristate. A 20 μl sample of the neutrophil suspension, argon- or CO-saturated, corresponding to $2 \cdot 10^6$ cells, was introduced in 1.5 ml of phosphate-buffered saline medium, partially saturated with argon or CO (saturation in CO, about 80%) and contained in an oxygraphic cell maintained at 37°C. The medium was supplemented with 30 mM glucose and 1 mM KCN. At the onset of the experiment, the O_2 concentration was 40 μM. Respiration was initiated by addition of 1 μg of phorbol myristate in 2 μl of dimethyl sulphoxide. Photoirradiation was performed as described in Materials and Methods. (Experiment carried out with Dr. Doussière.) ○, +CO; ●, −CO. (B). Effect of CO on the respiration of a particulate fraction of neutrophils preactivated by phorbol myristate. Same medium as in Fig. 6A. The neutrophil suspension (10^8 cells per ml) was activated by phorbol myristate (10 μg per 10^8 cells), and a crude membrane fraction of the phorbol myristate-activated neutrophils was prepared (cf. Materials and Methods). The oxygraphic assays were carried out with an aliquot of the membrane fraction (1 mg protein) in 1.5 ml of phosphate-buffered saline medium partially saturated with argon and CO, and supplemented with 1.6 mM NADPH (O_2 concentration was 40 μM). ○, +CO; ●, −CO. (C) Effect of CO on respiration of beef-heart mitochondria. The medium used was the standard sucrose medium (1.5 ml) supplemented with 10 mM succinate pH 7.4. (Experiment carried out with Dr. Doussière.) ○, +CO; ●, −CO.

tochrome *b* in the presence of NADPH (or NADH) attained values corresponding to nearly 60% of the reduction that could be attained by dithionite. When the light was turned off, the reduced peaks of cytochrome *b* returned to the oxidized state after a few min, half decrease occurring in 10 min (cf. Fig. 4B). When light was turned on again, reduction of cytochrome *b* started again. Most interestingly, addition of superoxide dismutase prevented photoreduction of cytochrome *b*, suggesting that O_2^- species might play a role in photoreduction of cytochrome *b*. It was checked that heat-denatured superoxide dismutase had no effect on photoreduction, indicating the specific effect of the enzyme [25].

From this set of experiments, it could be concluded that in the absence of photoirradiation, NADPH does not reduce cytochrome *b* significantly, whereas it does reduce myeloperoxidase. However, when the neutrophil fragments were exposed to light, cytochrome *b* and myeloperoxidase were reduced by NADPH. NADH, which is a less efficient electron donor for human neutrophils than NADPH [24], and also EDTA, which is an artificial reservoir of electrons [26], were able to photoreduce cytochrome *b*.

As shown in Fig. 5, natural photosensitizers like

FAD or FMN can act as intermediates in the photoreduction process of cytochrome *b* [27]. Addition of 5 μM FAD to a suspension of neutrophil fragments, previously supplemented with NADPH at 37°C, resulted in a spectrum characterized by the typical absorption band of the oxidized flavin residue in the 430–480 nm wavelength region (spectrum 2). Upon photoirradiation, the oxidized flavin peak was progressively resorbed, most likely due to the reduction of the flavin moiety; concomitantly, the peaks of reduced cytochrome *b* at 428 and 558 nm were progressively revealed (spectra 3 and 4). The same sequence of events occurred when FAD was replaced by FMN. Here again, addition of superoxide dismutase prevented reduction of cytochrome *b*.

Effect of CO and light irradiation on phorbol myristate-activated respiration in intact neutrophils

Phorbol myristate is known to stimulate the cyanide-resistant respiration of intact neutrophils that are incubated with glucose as substrate [2]. In the experiments to be described now, both respiration and cytochrome-*b* reduction were recorded, following addition of phorbol myristate to intact neutrophils; the effects of CO and light irradiation were also tested.

The data in Fig. 6A show that, in the absence of light and at different O_2/CO ratios, CO had virtually no effect on the rate of O_2-uptake by neutrophils supplemented with glucose and activated with phorbol myristate, even at very low concentrations of O_2 close to anaerobiosis in the oxygraphic assay. The K_m value for O_2 was close to 10 μM, irrespective of the presence of CO in the medium. The same results were obtained in the case of neutrophil fragments incubated with NADPH and CO (Fig. 6B). A comparative assay carried out with beef-heart mitochondria respiring with succinate as substrate (Fig. 6C) illustrates the drastic inhibitory effect of CO on mitochondrial respiration and the recovery of maximal respiratory activity after photoirradiation of the CO-inhibited mitochondria by photodissociation of the CO-cytochrome a_3^{2+} complex. The K_m for O_2 of heart mitochondria in this assay was about 1.5 μM, very close to values found in the literature, $K_m \approx 2.8$ μM [28] and significantly lower than the K_m O_2 for the cyanide-resistant respiration of neutrophils, 10 μM, as shown above.

Photoirradiation of intact neutrophils either in the presence or absence of CO led to an apparent competitive inhibition of respiration (Fig. 6A). This could well be explained by an acceleration of

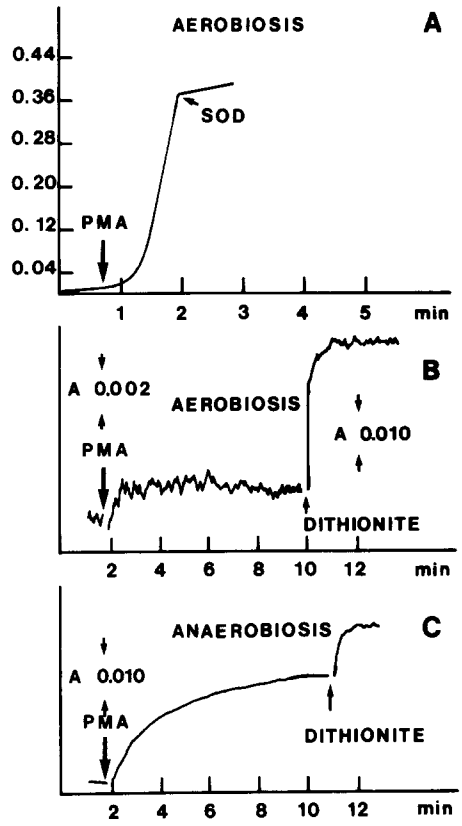

Fig. 7. Effect of activation of neutrophils by phorbol myristate on O_2^- production and cytochrome *b* reduction at 37°C. (A) O_2^- formation was assayed with 10^7 cells in 1.5 ml phosphate-buffered saline medium, supplemented with 10 mM glucose, after activation by 1 μg of phorbol myristate in 5 μl of dimethyl sulphoxide. Cytochrome *c* reduction was assayed as a test of O_2^- production; the near complete inhibition by superoxide dismutase testified that most of the cytochrome *c* reduction was caused by O_2^- (trace A). *A*, 550 nm. (B) Kinetics of cytochrome *b* reduction in neutrophils activated by phorbol myristate under forced aeration. A 3 ml cuvette was used and filled with 10^8 cells in phosphate-buffered saline. Trace amounts of H_2O_2 and catalase were added to provide the medium continuously with O_2. 10 μg of phorbol myristate were used. At 10 min, dithionite was added. ΔA, 427–410 nm. (C) Kinetics of cytochrome *b* reduction in neutrophil-activated by phorbol myristate under strict anaerobiosis (see Materials and Methods). Same amounts of neutrophils and phorbol myristate as in B. ΔA, 427–410 nm. At 10 min, dithionite was added.

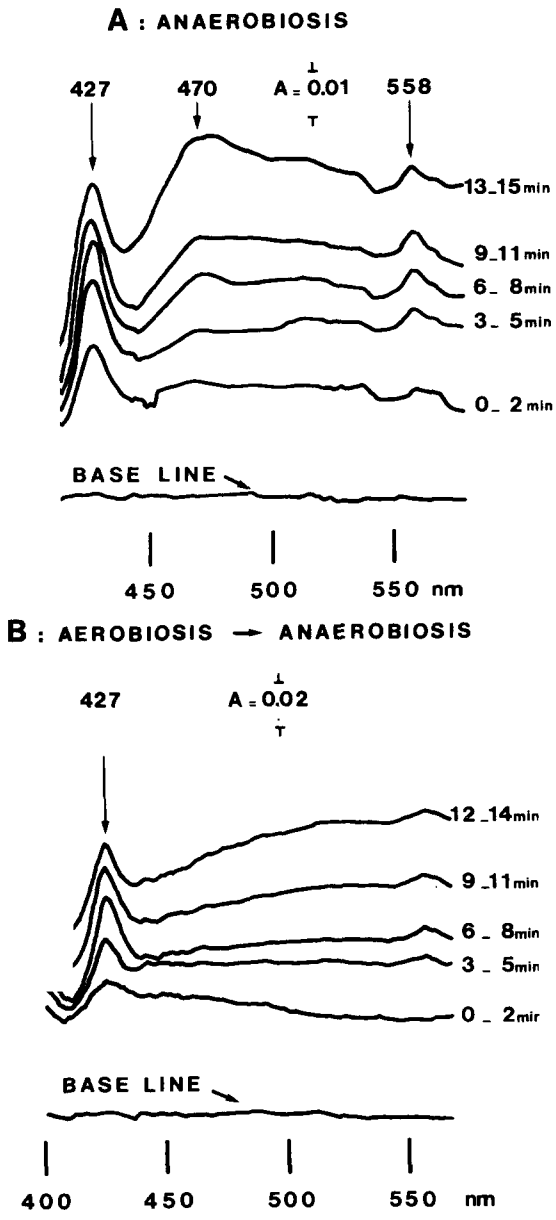

Fig. 8. Spectral modifications of neutrophils after activation by phorbol myristate at 37°C. (A) Strict anaerobiosis was obtained by bubbling argon in a phosphate-buffered saline medium, supplemented with glucose plus glucose-oxidase. The 3-ml cuvette was filled with 10^8 neutrophils in phosphate-buffered saline medium. The cells were activated by 10 μg of phorbol myristate. The spectrum of activated cells versus nonactivated cells is shown in the figure. (B) Transition from aerobiosis to anaerobiosis. The increased reduction of cytochrome *b* at 3 min, resulted from exhaustion of O_2 in the medium.

recycling of O_2 consumed, caused possibly by the light-induced decomposition of H_2O_2.

Kinetics of cytochrome b-reduction and O_2^- production in intact neutrophils activated by phorbol myristate

As shown in Fig. 7A, treatment of intact neutrophils by phorbol myristate in a glucose medium resulted in activation of O_2^- production. At 37°C the rate of O_2^- formation became steady after a 1-min lag. Reduction of cytochrome *c* in the presence of glucose was largely inhibited by superoxide dismutase, which indicates that the cytochrome *c* was reduced mainly by O_2^-. An oxygraphic assay carried out with the same preparation showed the same lag prior to establishment of a steady rate of O_2 uptake.

A parallel assay was performed for analyzing by spectrophotometry the kinetics of reduction of cytochrome *b* under conditions of forced aerobiosis, where O_2 was produced by addition of traces of catalase and H_2O_2 (Fig. 7B). Following addition of phorbol myristate to neutrophils, a plateau of reduction was reached after about 1 min, corresponding to a level of reduction of only 4–5% with respect to the dithionite-induced reduction.

A control assay carried out in strict anaerobiosis under comparable conditions (see Materials and Methods) (Fig. 7C) showed a slow increase in the level of reduction of cytochrome *b*, the maximal level of reduction being attained after 3 min. The plateau of reduction corresponded to 65% of the dithionite-induced reduction. It is clear, therefore, that the redox state of cytochrome *b* in neutrophils depends on the redox conditions of the medium, which is a criterion for cytochrome *b* being an electron carrier in the NADPH-O_2 respiratory chain. Moreover, the low level of reduction of cytochrome *b* in aerobiosis (less than 5%) makes cytochrome *b* a likely candidate to react with O_2. Not only was cytochrome *b* reduced under anaerobiosis upon addition of phorbol myristate to neutrophils, but also myeloperoxidase was (Fig. 8A). It must be stressed that in the absence of phorbol myristate, even under anaerobiosis, reduction of cytochrome *b* and myeloperoxidase remained negligible. Spectra in Fig. 8B show the transition from aerobiosis to anaerobiosis by exhaustion of O_2 in the medium

after respiration had been activated by phorbol myristate. An oxygraphic assay under similar conditions of medium and cell concentration showed that anaerobiosis was attained in 3 min.

Discussion

Is cytochrome b the terminal oxidase of the cyanide-resistant respiratory chain of neutrophils?

The most convincing evidence for the proposal that cytochrome *b* is the terminal oxidase of the cyanide respiratory chain in neutrophils is the reactivity of reduced cytochrome *b* with CO (see Introduction). However, this conclusion requires qualification. As shown in the present paper, after extensive bubbling of CO in a particulate fraction of neutrophils maintained in anaerobiosis in the presence of dithionite, the Soret band of reduced cytochrome *b* is shifted from 425 to 424–423 nm only, and the reduced peaks at 527 and 556 nm are hardly displaced. These results are in accordance with those reported [12]. The limited change in spectrum by CO treatment of reduced neutrophils may be indicative of a poor reactivity to CO, which is consistant with the high K_d value, 1.18 mM, of the neutrophil cytochrome *b* for CO. Even by the criterion of photodissociation, it was difficult to check whether the 1–2 nm shift of the Soret band of reduced cytochrome *b* observed upon bubbling of CO reflected the formation of a CO-reduced cytochrome *b* complex; in fact, upon photoirradiation of neutrophil fragments maintained in anaerobiosis with dithionite, and extensively bubbled with CO, an additional reduction of cytochrome *b* was observed which partly obscured the back shift that is expected to occur upon dissociation of the CO-reduced cytochrome *b* complex. Photoreduction of cytochrome *b* means that a fraction or a separate species of cytochrome *b* in the presence of dithionite is still oxidized. This puzzling observation of incomplete reduction of cytochrome *b* by dithionite recalls similar observations reported in the case of low-potential electron carriers, that were explained by contamination of dithionite with small amounts of bisulphite [29]. Photoreduction of the oxidized cytochrome *b* may be due to accumulation of molecular species with redox potential lower than that of cytochrome *b*. This will be discussed later.

The above data make the reactivity of the neutrophils cytochrome *b* with CO questionable; this leads in turn to question the ability of reduced cytochrome *b* to react with O_2, since it is generally accepted that any pigment that reacts with CO has the ability to react with O_2. However, reduced cytochrome *b* is readily reoxidized in a membrane fraction of neutrophils, half the oxidation being completed in 4.7 ms after mixing with an aerated buffer at room temperature [11]. Further, as shown here, the steady-state reduction level of cytochrome *b* in neutrophils, maintained in aerobiosis, is less than 5%, whereas in anaerobiosis it reaches 65%, which suggests but does not prove that cytochrome *b* is an O_2-reacting pigment. If this is the case, one has to admit that, although the CO-reactivity of a pigment is indicative of its oxidase function, there might exist exceptions, including the neutrophil cytochrome *b*. On the other hand, assuming that the 1–2 nm shift of the Soret band of cytochrome *b* in the presence of CO indeed reflects the CO-reactivity of this cytochrome functioning as an oxidase, it is difficult to explain why the cyanide-resistant oxidation of glucose by intact neutrophils stimulated by phorbol myristate is totally insensitive to CO, whereas the mitochondrial respiration under similar conditions is extensively inhibited by CO. One may, of course, argue that the cyanide-resistant oxidase of neutrophils has such a high affinity for O_2 that the CO-reduced cytochrome *b* complex is immediately reoxidized by traces of O_2. This is not the case; in fact, the affinity of the cyanide-resistant oxidase in neutrophils is 7-times lower than that of the mitochondrial cytochrome oxidase (K_m 10 μM vs. 1.5 μM). It is true, however, that O_2 competes favourably against CO, since the K_d for CO is 1.18 mM [11] and the K_m for O_2 is 10 μM (this paper); it remains that in oxygraphic assays, when O_2 is nearly exhausted and CO is present in the medium at subsaturating concentration, 80%, respiration should be inhibited compared to the control without CO, if cytochrome *b* were the only terminal oxidase. One is therefore faced with the following dilemma: either cytochrome *b* is the terminal oxidase, but it is not CO-sensitive, or cytochrome *b* is sensitive to CO and it cannot be the only terminal oxidase; in this case, it has to coexist with another terminal oxidase which would be CO-in-

sensitive. In this context, a branched pathway involving cytochrome *b* has been proposed [12].

Another observation reported here is that cytochrome *b* in membrane fragments of neutrophils previously activated by phorbol myristate is hardly reducible by NADPH, even in anaerobiosis in spite of the fact that NADPH is the source of reducing equivalents for the cyanide-resistant respiration. This result confirms a previous report [7]. It must be recognized, however, that sonication used to prepare membrane fragments of neutrophils could disturb a possible phorbol myristate-induced organization of the cyanide-resistant chain, resulting in lack of reactivity of cytochrome *b*.

That cytochrome *b* participates in respiration is clearly indicated by the different percentages of reduction of cytochrome *b* in phorbol myristate-activated neutrophils in response to the variation of the redox state of the medium. Another criterion in favour of cytochrome *b* being an intermediate of the NADPH oxidase system is the parallelism in the time-course of reduction of cytochrome *b* in aerobiosis, of O_2 uptake and O_2^- production in neutrophils activated by phorbol myristate. In all cases, a 1-min lag was required before a steady level was attained.

A flavin has been postulated to act as a cofactor in the cyanide-resistant respiratory chain of neutrophils [30,7]. This is consistent with the equivalence between the amounts of flavin and cytochrome *b* found in these cells [27]. In keeping with the idea that cytochrome *b* is a branched electron carrier, the NADPH oxidase could well have two pathways for directing electrons from NADPH to O_2, one of them being a flavoprotein and the other, cytochrome *b*, as previously suggested [7].

The photoreduction of cytochrome b in neutrophils

An unexpected finding was the marked reduction of cytochrome *b*, and also of myeloperoxidase when membrane fragments of neutrophils are exposed to light in the presence of NADPH or NADH or even an artificial reductant like EDTA; photoreduction was also observed in the case of neutrophils fragments not completely reduced by dithionite (see above). Flavin derivatives, like FMN and FAD, that are efficient photosensitizers, were also reduced when photoirradiated with polymorphonuclear neutrophils fragments, and their photoreduction was accompanied by a concomitant reduction of cytochrome *b*.

There are many reports in the literature about photoreduction of pigments and particularly of cytochrome in cells other than photosynthetic cells, and in simple systems as well. For example, it was reported [31,32] that the flavin mononucleotide radical, $FMNH^{\cdot}$, generated by flash photolysis of FMN in the presence of an electron donor like EDTA, was able to reduce rapidly cytochrome *c* in aqueous solution. Light-inducible photoreduction of cytochrome *b* in *Neurospora crassa* was also reported by Munoz et al. [33], in *Dictyostelium discoidium* by Poff and Butler [34] and in corn coleoptiles by Brain et al. [35] and Britz et al. [36].

In isolated respiratory multienzyme complexes from beef-heart mitochondria, the *b*-type cytochrome can be reduced in presence of flavin; it was postulated that the superoxide anion O_2^- is an intermediate in this reduction because the cytochrome *b* reduction was diminished by superoxide dismutase [37]. Since the main product of the cyanide-respiration in neutrophils is O_2^- [38], and superoxide dismutase inhibits the photoreduction of cytochrome *b* in membrane fragments of neutrophils, it is quite possible that, under appropriate conditions, O_2^- may be a reductant for cytochrome *b*, although in physiological conditions the reverse may be true. The likeliness of this hypothesis is strengthened by the fact that the redox potential of the O_2/O_2^- couple, -330 mV [9] or -160 mV [10] is close to that of cytochrome *b*, -245 mV [6].

Spectral interference between cytochrome b and myeloperoxidase

As shown in this paper, reduction of myeloperoxidase interfered with that of cytochrome *b* with either crude membrane preparations or intact neutrophils. The most likely explanation for an increase in the Soret band of cytochrome *b* when myeloperoxidase is extracted from neutrophil membranes is that the spectrum of myeloperoxidase interferes with that of cytochrome *b*. In fact, the reduced myeloperoxidase spectrum is characterized by a trough in the region of 420–430 nm; in other words, in the non-extracted membranes, the Soret band of reduced cytochrome *b* is artificially decreased by the con-

comitant reduction of myeloperoxidase. However, for the α and β peaks of cytochrome *b*, i.e., in the 520–560 nm region of the spectrum, there was no marked contribution of myeloperoxidase (cf. Fig. 1); this suggests that a redox interaction might well exist between cytochrome *b* and myeloperoxidase. In fact, in spite of the distinct localizations of cytochrome *b*, which is found in plasma membrane and specific granules, and myeloperoxidase which is present in azurophil granules, there are situations in which the two pigments may come in contact and share a common intermediate. This is the case during the process of phagocytosis, when the azurophil granules fuse with phagosomes. Membrane fusion may also occur as an artifact when neutrophils are homogeneized and membrane fractions are prepared.

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