Oxidation–reduction properties of the cytochrome *b* found in the plasma-membrane fraction of human neutrophils

A possible oxidase in the respiratory burst

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The oxidation–reduction midpoint potential of the cytochrome *b* found in the plasma membrane of human neutrophils has been determined at pH 7.0 ($E_{m,7.0}$) from measurements of absorption spectra at fixed potentials. In both unstimulated and phorbol myristate acetate-stimulated cells $E_{m,7.0}$ was -245 mV. Changes in pH affected the E_m of the cytochrome *b*, with a slope of approx. 25 mV/pH unit change. The $E_{m,7.0}$ of the haem group(s) of the membrane-bound myeloperoxidase of human neutrophils was found to be $+34$ mV. The plasma membranes contained no detectable ubiquinone, and no iron–sulphur compounds were detected by e.p.r. spectroscopy at 5 – 20 K. No flavins were detected by e.p.r. spectroscopy. The cytochrome b_{-245} was not reduced by added NADH or NADPH. Dithionite-reduced cytochrome b_{-245} formed a complex with CO, supplied as a saturated solution, which was dissociated with $26\mu s$ illumination from a xenon flash lamp, and the recombination with CO had a half-time of approx. 6 ms. Partly (80%) reduced cytochrome b_{-245} was oxidized by added air-saturated buffer with a half-time faster than 1 s at 20°C , a resolution limited by mixing time. These results are compatible with cytochrome b_{-245} acting as an oxidase.

Neutrophils form part of the host defence system against bacterial infection. They accumulate at sites of infection where they engulf, kill and digest microbes. Phagocytosis is accompanied by a marked increase in oxygen uptake (Baldrige & Gerard, 1933) that is not prevented by inhibitors of mitochondrial respiration, such as azide or cyanide (Sbarra & Karnovsky, 1959). This burst of oxygen uptake is important in bacterial killing and is accompanied by the production of reduced products of O_2 , namely superoxide ($\text{O}_2^{\cdot-}$) peroxide (O_2^{2-}) and hydroxyl radicals ($\text{OH}^\cdot$) [see Babior (1978) for a review and also Green *et al.* (1979)]. Neutrophils are also rich in granules containing myeloperoxidase, which fuse with developing phagocytic vacuoles and release their contents into them. In the rare condition of chronic granulomatous disease (Holmes *et al.*, 1967), this respiratory burst is absent from neutrophils and there is a severe, sometimes fatal, predisposition to bacterial infection, which suggests that this reduction of oxygen is important in the killing reactions. Although a variety of ‘oxidases’ has been proposed as the enzyme responsible for this burst of respiration, none has

withstood critical evaluation (Badwey *et al.*, 1979).

In previous papers we have shown that human neutrophils contain a cytochrome *b* (Segal & Jones, 1978) that is absent from many patients with chronic granulomatous disease (Segal *et al.*, 1978). This cytochrome *b* is located in the plasma membrane of the neutrophil (Segal & Jones, 1979), forms part of the membrane of the phagocytic vacuole (Segal & Jones, 1980a) and is reduced on stimulation of normal neutrophils but not on stimulation of cytochrome *b*-containing neutrophils from female patients (Borregaard *et al.*, 1979) who have the symptoms of chronic granulomatous disease (Segal & Jones, 1980b).

We believe it likely that this cytochrome *b* is a component in an electron-transport chain involved in the microbicidal oxidase system. The present paper reports the thermodynamic properties of this cytochrome in resting human neutrophils and in neutrophils stimulated by phorbol myristate acetate. We also describe the light-reversible binding of CO to the reduced cytochrome *b* and show that it can be reoxidized rapidly when mixed with oxygen. These are properties expected of an oxidase and suggest

that this cytochrome may itself transfer electrons to oxygen during the respiratory burst.

Experimental

Isolation of neutrophils

Neutrophils were isolated from buffy-coat residues (North West London Blood Transfusion Service, Edgware, Middx., U.K.) by dextran sedimentation of erythrocytes, centrifugation through Ficoll/sodium metrizoate and haemolytic lysis of residual erythrocytes as described previously (Segal *et al.*, 1980).

Separation of plasma-membrane fraction from neutrophils

A suspension of cells in RPMI 1640 medium (Flow Laboratories, Irvine, Ayrshire, Scotland, U.K.) was washed and homogenized in 0.34M-sucrose with a Dounce homogenizer. Samples (15 ml) were layered on to the surface of discontinuous gradients comprised of layers (14 ml) of sucrose densities 1.10, 1.17 and 1.21 g·ml⁻¹ in centrifuge tubes of a 3 × 65 ml swinging-bucket head of an MSE 65 centrifuge. The gradients were centrifuged at 23 000 (g_{av} , 70 000) rev·min⁻¹ for 11 h at 4°C and the plasma-membrane fractions collected at the interface between sucrose of densities 1.10 and 1.17 g·ml⁻¹ (Segal & Jones, 1979).

Activation of neutrophils

Portions of suspensions of neutrophils (approx. 5×10^8 cells in 5 ml of RPMI 1640 medium) were incubated at 37°C in a rapidly stirred incubation chamber containing a Clark-type oxygen electrode (Rank Brothers, Bottisham, Cambs., U.K.) for 2 min. Then 25 µg of phorbol myristate acetate (10 mg·ml⁻¹ stock solution in dimethyl sulphoxide) was added, and 2 min later, after the respiratory burst had been initiated, the cell suspension was added to 50 ml of ice-cold 0.9% NaCl. The plasma-membrane fraction from stimulated cells was collected as described above.

Spectroscopy

Reduced-minus-oxidized difference spectra and spectra at known fixed oxidation-reduction potentials were recorded with a split-beam spectrophotometer constructed in the University of Bristol Medical School Workshops (see Jones & Saunders, 1972). Oxidation-reduction potentials were measured in a stirred cuvette fitted with platinum and calomel (Hg₂Cl₂) electrodes under an atmosphere of argon (BOC, Brislington, Bristol 4, U.K.), as described by Dutton (1978). The buffer, at pH 7.0, was 100 mM-KCl/50 mM-Mops (4-morpholinepropane-sulphonic acid), and the following mediators were added to the suspension: phenazine methosulphate

(12.5 µM), phenazine ethosulphate (12.5 µM) (both from Sigma, Poole, Dorset BH17 7NH, U.K.), pyocyanine (6 µM) (K & K Laboratories; supplied by Kodak Fine Chemicals, Kirkby, Liverpool, L33 7UF, U.K.), 2-hydroxy-1,4-naphthoquinone (12.5 µM), anthroquinone 2,6-disulphonate (12.5 µM), anthroquinone (all from Aldrich Chemical Co., Gillingham, Dorset SP8 4JL, U.K., and 10 µl of a saturated solution in ethanol), 3,6-diaminodurene (12.5 µM) (Fluka A.G. Chemische Fabrik; supplied by Fluorochem, Glossop, Derbys. SK13 9NU, U.K.) and duroquinone (12.5 µM) (Sigma). The extent of reduction of the cytochrome *b* at different oxidation-reduction potentials (E_h) was calculated by measuring the height of the absorption band at 559 nm above a line joining the two troughs on each side of this band (see Fig. 1). The extent of reduction of myeloperoxidase was determined from the height of the absorption band at 640 nm above the baseline at 670 nm. Reductive titrations were carried out by injecting small amounts of sodium dithionite solution, and oxidative titrations by addition of small amounts of potassium ferricyanide solution.

The output from the split-beam spectrophotometer could be displayed on an *x-y* recorder to record spectra or on a *y-t* recorder with a

Fig. 1. Oxidized-minus-reduced difference spectra of human neutrophil membranes (2.3 mg of protein/ml) at fixed potentials

Details of added mediators are given under 'Spectroscopy'. The reference cuvette contained an identical sample of membranes without added mediators or reductant. The light-path was 10 mm. Addition of potassium ferricyanide to the reference cuvette caused no further oxidation of haem compounds.

full-scale-pen-response time of 1 s to display rates of change in absorbance at a fixed wavelength.

*Flash photolysis of cytochrome *b*-CO complexes*

Dithionite-reduced samples of neutrophil plasma membrane suspended in a 3 ml fluorimeter cuvette in 100 mM-KCl/50 mM-Mops, pH 7.0, were saturated with CO gas (BDH Chemicals Ltd., Poole, Dorset BH12 4NN, U.K.). The cuvette was stoppered tightly and placed in the sample position of a dual-wavelength spectrophotometer, fitted with two photomultipliers and built with two Applied-Physics high-radiance monochromators and aligned so that one photomultiplier is opposite each measuring beam. Rapid changes in absorbance were fed to a linear differential amplifier, the output stored in a transient recorder (Datalab 905) and then fed into a PDP8 computer that was programmed for use as a signal averager. CO was displaced from the complex by flash illumination with a Xenon lamp (snaked filament, 2 kV operating voltage, powered by a discharge of 3 μ F capacitor, giving a flash of 26 μ s half-bandwidth; T. W. Wingent, Milton, Cambridge CB4 4AY, U.K.), connected to the base of the cuvette with a fibre optic. The photomultipliers were protected with Kodak Wratten no. 16 and Corning blue glass 9782 filters (F. Knotty Precisions Optical Instruments, London W.6, U.K.) and the actinic lamp output with a Kodak Wratten no. 35 filter. Measurements of CO displacement and recombination were made at 559 minus 540 nm by using low-intensity measuring beams to diminish background displacement of CO. Control measurements made with high-intensity measuring beams gave no response to flash activation.

Results

In order to investigate the role of plasma-membrane cytochrome *b* in human neutrophil electron-transport systems it is useful to determine its oxidation-reduction midpoint potential ($E_{m,7.0}$) and whether other possible electron donors or acceptors are present in the same membrane. We have found that small changes in the baseline of the membrane difference spectrum occur over the course of a potentiometric titration and that these could be allowed for if measurements of the extent of cytochrome *b* reduction were made from difference spectra recorded over the wavelength region of the α - and β -band absorption. Some such spectra are shown in Fig. 1 and the results are plotted in Fig. 2(a) in the usual form. It can be seen that there is a very good fit to the Nernst equation for a one-electron transfer reaction, i.e. $n = 1$ in the equation:

$$E_h = E_m + \frac{RT}{nF} \ln \left(\frac{[\text{oxidized cytochrome } b]}{[\text{reduced cytochrome } b]} \right)$$

and the midpoint potential ($E_{m,7.0}$) is -245 mV. A similar titration was carried out on plasma membranes prepared from phorbol myristate acetate-stimulated cells and gave the same midpoint potential found in unstimulated cells, -245 mV (Fig. 2b). Clearly stimulation of the plasma membrane to induce the oxygen burst has no effect on the thermodynamic properties of the cytochrome.

Cytochromes of the *b*-type are often involved in proton-translocating and energy-conserving regions of mitochondrial or bacterial electron-transport systems and much attention has been given to the effect of pH on the midpoint potential of such cytochromes. Thus in ox heart submitochondrial particles it is known that, in the range pH 6.4–8.2 the midpoint potential of cytochrome *b* declines by 59 mV for an increase in pH of 1 unit (Straub & Colpa-Boonstra, 1962). We have found that cytochrome *b* of neutrophil membranes has a pH-dependent midpoint potential over the range pH 5.0–9.0 (Fig. 3) with a change of E_m of approx. 25 mV per pH unit, although the presence of more than one dissociable group with a pK in the region of the pH titration can make interpretation of such curves complex (see, for example, Pettigrew *et al.*, 1978).

Search for additional electron-transport carriers

Analysis of the membranes failed to detect ubiquinone by standard techniques of extraction with acetone, chromatography on alumina, followed by t.l.c. on silica gel (Griffiths *et al.*, 1967). Model experiments showed that ubiquinone at half the concentration of the cytochrome *b* would have been readily detected. (We are grateful to Dr. W. T. Griffiths, Department of Biochemistry, University of Bristol, for performing the extraction and analysis.)

The search for iron-sulphur proteins by e.p.r. spectroscopy in the $g = 2.0$ region at temperatures between 5 and 20 K failed to detect any such compounds. Iron-sulphur proteins, if present in the membrane, are present at extremely low concentrations. No flavin semiquinone signal was observed in these experiments in either oxidized or reduced samples, although a clear signal was obtained from the cytochrome b_{-245} . (We are very grateful to Dr. M. C. W. Evans, Department of Botany and Microbiology, University College London, for performing this analysis.)

The burst of respiration has the same time course as the degranulation of the myeloperoxidase-containing azurophil granule in the vacuole (Segal *et al.*, 1980). In order to determine whether membrane-bound myeloperoxidase, which is a major component of neutrophil proteins, was a possible electron donor to the *b* cytochrome, we have determined its midpoint oxidation-reduction potential in a crude membrane pellet fraction (Fig. 4). The value $+34$ mV for the $E_{m,7.0}$ is very close to that

Fig. 2. Potentiometric titrations

(a) Membrane-bound cytochrome (cyt.) b from unstimulated neutrophils in 40 mM-Mops/100 mM-KCl, pH 7.0. The membranes were obtained from neutrophils that had not been stimulated. ●, Reductive titration with sodium dithionite; ○, oxidative titration with potassium ferricyanide. The line through the points is the theoretical curve for a one-electron-transfer reaction. The membrane concentration was 2.1 mg of protein/ml. Details of the

reported previously for the soluble enzyme from canine leucocytes [$E_{m,7.0} = +36 \text{ mV}$ (Harrison & Schultz, 1978)] and showed that the peroxidase haem group is unlikely to donate electrons to the cytochrome b , although it could possibly accept electrons from it. Other attempts to find the electron donor to the b cytochrome have not been successful. It is very poorly reduced in purified plasma

Fig. 3. Effect of pH on the midpoint redox potential (E_m) of the neutrophil cytochrome b

Details are given under 'Spectroscopy'. The membrane concentrations were approx. 2.0 mg of protein/ml. The following buffers were all 50 mM in 100 mM-KCl: pH 5.0, Tris/succinate; pH 6.0, Mes (4-morpholine-ethanesulphonic acid)/KOH; pH 7.0, Mops/KOH; pH 8.0 and 9.0, Tris/HCl.

added mediators are given under 'Spectroscopy'. (b) Membrane-bound cytochrome b from stimulated neutrophils in 50 mM-Mops/100 mM-KCl, pH 7.0. The membranes were obtained from neutrophils that had been stimulated with phorbol myristate acetate ($5 \mu\text{g} \cdot \text{ml}^{-1}$). ●, Reductive titration with sodium dithionite; ○, oxidative titration with potassium ferricyanide. The line through the points is the theoretical curve for a one-electron-transfer reaction. The membrane concentration was 1.9 mg of protein/ml. Details of the added mediators are given under 'Spectroscopy'.

Fig. 4. Potentiometric titration of myeloperoxidase haem, measured at the 640 nm absorption band in a crude neutrophil-membrane preparation

Details of added mediators are given under 'Methods'. The protein concentration was 7.0 mg of protein/ml. ●, Reductive titration with sodium dithionite; ○, oxidative titration with potassium ferricyanide. The line through the points is the theoretical curve for a one-electron-transfer reaction.

Fig. 5 Effect of latex particles opsonized with immunoglobulin G on oxygen uptake by human neutrophils

Neutrophils (1.2×10^8 cells $\cdot$ ml $^{-1}$) in RPMI 1640 medium containing heparin (5 units $\cdot$ ml $^{-1}$) at 37°C in the chamber of a Clark-type oxygen electrode. A, incubation commences; B, 1×10^{10} latex particles opsonized with immunoglobulin G added; C, solid dithionite added. Unstimulated rate of oxygen uptake = 0.67 nmol of O₂/10⁷ cells. Stimulated rate = 9.9 nmol of O₂/10⁷ cells.

membranes or crude homogenates, even under anaerobic conditions, by added NADH or NADPH. NADH or NADPH have been widely reported to be the source of electrons for the respiratory burst [see Babior (1978) for a review]. The same negative results were obtained in either control or phorbol-stimulated cells. It is possible that traces of contaminating oxygen could be present in the cuvette, causing oxidation of the *b* cytochrome or that although surface activation of the whole cell is necessary for reduction of the *b* cytochrome, the effects of activation are transient or labile on homogenization. We have previously shown that cytochrome *b* reduction does take place in whole cells that have been treated with phorbol myristate acetate under anaerobic conditions (Segal & Jones, 1980b).

Oxidase activity of neutrophils and neutrophil membranes

When intact neutrophils are stimulated, oxygen uptake is greatly increased and continues at a rapid rate until nearly all the dissolved oxygen is consumed (see Fig. 5). In five studies in which 2×10^8 cells suspended in 2 ml of RPMI 1640 buffer were mixed with 1×10^{10} latex particles opsonized with

immunoglobulin G, it was observed that the rate of oxygen consumption remained linear until 6.8 ± 2.3 nmol of oxygen remained. This suggests that the oxidase has an affinity for oxygen close to that of respiratory-chain oxidase. It has been found that an NADPH-dependent oxygen-reducing system producing O₂^{•−}, can be solubilized from membrane fractions of zymosan-stimulated neutrophils (Gabig & Babior, 1979; Babior & Kipnes, 1977; Tauber & Goldzl, 1979), and it is unlikely that this activity is located in the plasma-membrane fraction (Dewald *et al.*, 1979). We have investigated certain oxidase-type reactions catalysed by the plasma-membrane fractions that are likely to involve cytochrome *b*. Because we failed to find natural reductants for the cytochrome *b*, we titrated sodium dithionite into a stirred anaerobic cuvette gassed with oxygen-free argon to give stable 80% reduction of the cytochrome, as measured by the height of its α -absorption band. Small volumes of air-saturated buffer were injected into the cuvette from a side arm fitted with a rubber septum, and the rate of oxidation of the cytochrome *b* was measured (Fig. 6). The cytochrome was found to undergo rapid oxidation, and since measurements were

limited by the rate of mixing in the apparatus used, it was possible to conclude that at 20°C the $t_{\frac{1}{2}}$ for oxidation of cytochrome *b* was less than 1 s.

Haem proteins that react with oxygen are likely to

Fig. 6. Reoxidation of the neutrophil cytochrome *b* by oxygen

The trace shows the effect of the addition of 50 μ l of air-saturated buffer (50 mM-Mops/100 mM-KCl, pH 7.0) to a stirred cuvette containing a neutrophil-membrane suspension under an atmosphere of argon. The reaction volume was 5 ml and the protein concentration was 6.1 mg/ml. Approx. 80% of the cytochrome *b* was reduced by the addition of sodium dithionite, and the reoxidation was monitored by observing the decrease in A_{559} on the addition of air-saturated buffer. The inset shows the difference spectrum in the α -region of the *b* cytochrome before (—) and after (----) the injection of air-saturated buffer.

bind CO. The dithionite-reduced plasma-membrane cytochrome *b* forms a complex with CO (Fig. 7) with a strong absorption band in the region around 420 nm. When the complex is briefly illuminated with a xenon flash lamp, filtered to transmit around this wavelength, the CO is dissociated and its rate of recombination can be measured at 559 nm, the wavelength at which the α -band of the free reduced cytochrome absorbs (Fig. 8). The change was found to be centred at 559 nm and was excited maximally by actinic light transmitted around 420 nm, but a complete action spectrum was not attempted. The $t_{\frac{1}{2}}$

Fig. 8. Flash photolysis of the cytochrome *b*-CO complex

Details are given in the Experimental section. The membranes (1.4 mg of protein/ml) were suspended in 50 mM-Mops/100 mM-KCl, pH 7.0, reduced with dithionite and saturated with CO. The light-path was 10 mm. The trace is an average for twenty experiments. The arrow indicates the time at which the xenon flash was triggered. The horizontal line corresponds to a time of 100 ms. The wavelength pair was 559 minus 570 nm.

Fig. 7. Reaction of carbon monoxide with the cytochrome *b* of neutrophil membranes

Details are given under 'Spectroscopy'. The membrane concentration was 2.65 mg of protein/ml. — shows the reduced-minus-oxidized spectrum (dithionite-reduced-minus-untreated); ---- shows the (dithionite reduced + CO)-minus-(dithionite-reduced) difference spectrum after 60 s exposure to a saturated solution of CO.

of recombination of the reduced cytochrome with CO was 6 ms; control experiments with myoglobin-CO complexes under the same conditions gave a $t_{\frac{1}{2}}$ of 2 ms, and a second-order rate constant close to published values (Antonini & Brunori, 1971) for myoglobin-CO recombination ($K = \sim 4.0 \times 10^5 \text{ M}^{-1} \cdot \text{s}^{-1}$). These in turn are similar to published values (Nicholls & Chance, 1974) for cytochrome oxidase-CO recombination ($K = \sim 1.0 \times 10^5 \text{ M}^{-1} \cdot \text{s}^{-1}$). The kinetics of the association of the membrane cytochrome with CO were very different from those of haemoglobin with CO. In separate experiments with haemoglobin solution it was found that the $t_{\frac{1}{2}}$ was 150 ms, showing that haemoglobin could be excluded as a major CO-binding pigment contaminant of our preparations.

Discussion

The cytochrome *b* that we have found in the plasma membrane of human neutrophils exhibits a midpoint potential markedly lower than that of any other *b*-type cytochrome reported in mammalian cells. The major cytochromes *b* of liver and heart mitochondria have $E_{m,7.2}$ of -30 mV and $+30 \text{ mV}$ (Lindsay *et al.*, 1972; Wilson & Dutton, 1970) and the cytochrome *b*₅ of the endoplasmic reticulum has $E_{m,7.0}$ around -12 mV when measured within the membrane (Strittmatter & Ball, 1952). This unusually low E_m ensures that cytochrome *b*₋₂₄₅ can be assayed without problems of contamination by cytochromes *b* from other membrane fractions, although in neutrophils, mitochondria and endoplasmic reticulum are relatively sparse (Bainton *et al.*, 1971). A cytochrome *b* found in the plasma-membrane fraction of rat liver cells has the properties of cytochrome *b*₅ (Jarasch *et al.*, 1979) and is clearly different from that described here.

The absence of cytochrome *b*₋₂₄₅ from a group of patients suffering from chronic granulomatous disease (Segal & Jones, 1978) has suggested that this plasma-membrane component is involved in the respiratory burst induced in neutrophils by membrane stimulation and its E_m is sufficiently low to suggest that it could reduce oxygen directly to O_2^- at a considerable rate. Neutrophils stimulated with phorbol myristate acetate respire at a rate that is variable but, at 37°C , from 8 to around $40 \text{ nmol of O}_2 \cdot \text{min}^{-1} \cdot 10^{-7} \text{ cells}$ (see, for example, Segal & Coade, 1978). The amount of cytochrome *b* reduced on stimulation of anaerobic neutrophils (10^7 cells) is approx. 0.1 nmol (Segal & Jones, 1980b). We observed (Fig. 6) that in an anaerobic suspension of plasma membranes, in which cytochrome *b*₋₂₄₅ is 80% reduced with sodium dithionite, the cytochrome *b* is oxidized by injected oxygen within the resolving time of the experiment (about 1 s) at 20°C . This minimum estimated rate is close to the rate of

oxidation needed in whole cells if cytochrome *b*₋₂₄₅ is directly implicated in the respiratory-burst system in whole cells. The rate of cytochrome *b*₋₂₄₅ oxidation by oxygen may indeed prove to be much faster. The extremely rapid binding of CO to reduced cytochrome *b*₋₂₄₅ after flash illumination (Fig. 8) suggests that the haem prosthetic group has easy access to CO and therefore probably to O_2 , a property expected of an oxidase. A consideration of these properties, coupled with the evidence from the defective chronic-granulomatous-disease neutrophils has led us to suggest that it is likely that cytochrome *b*₋₂₄₅ is the oxidase responsible for the respiratory burst in neutrophils. A somewhat similar cytochrome *b*, $E_m = -175 \text{ mV}$, has been found in *Paracoccus denitrificans*; it too binds CO, is very poorly reduced by NADH and is thought to act as an oxidase (Henry, 1980).

Neutrophil plasma-membrane cytochrome *b*₋₂₄₅ is unaffected in its oxidation-reduction properties by stimulation of the intact neutrophil by phorbol myristate acetate, although regulation of this special respiratory metabolism by alteration of the E_m of the postulated oxidase is an attractive idea. Indeed, the 'switch' that initiates the respiratory burst remains to be elucidated, as does the function of the respiratory burst (see Badwey *et al.*, 1979). It is possible that the 'switch' is related to a change in the membrane potential of the plasmalemma, since ionophores can act as stimulators (Romeo *et al.*, 1975). Since cytochrome *b*₋₂₄₅ takes up protons on reduction and releases them on oxidation, it could act as a proton translocator, making the interior of phagocytic vacuoles more acid to facilitate the activity of lysosomal enzymes, which enter the phagosome by granule fusion. These possibilities remain to be investigated.

Analysis of the plasma-membrane fraction failed to reveal groups that might donate electrons to cytochrome *b*₋₂₄₅. Quinones and iron-sulphur proteins appear to be absent and, although there is some slight NADH- and NADPH-cytochrome *c* reductase activity present, no reduction of the *b*₋₂₄₅ cytochrome was caused by nicotinamide nucleotides, nor was the cytochrome reduced by nicotinamide nucleotides in isolated phagocytic vacuoles (Segal & Jones, 1978). Myeloperoxidase was not sufficiently reducing ($E_{m,7.0} = +34 \text{ mV}$, Fig. 4) to act as an electron donor, in contrast with horseradish peroxidase, which has a reported $E_{m,7.0}$ of -271 mV (Harbury, 1957). Opsonized zymosan-treated human neutrophils are reported to contain a plasma-membrane-bound flavin-requiring NADPH-dependent superoxide-generating system (Gabig *et al.*, 1978; Babior & Kipnes, 1977; Gabig & Babior, 1979). This activity can be solubilized in 0.8% (v/v) Triton X-100, but appears to be unstable. Its relationship, if any, to cytochrome *b*₋₂₄₅ is un-

certain; it is present in phorbol-stimulated cells but absent from latex-stimulated cells. We have found that the addition of FAD did not increase the steady-state extent of reduction of cytochrome b_{-245} induced by added nicotinamide nucleotides. Such low rates of cytochrome b reduction by NADH have been noted even in uncoupled mitochondria (e.g. Wikstrom, 1971), and show that barriers to redox equilibration can arise in apparently well-defined electron-transport systems and we can not be certain that reduced nicotinamide nucleotides are not the primary electron donors to cytochrome b_{-245} .

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