The superoxide-generating oxidase of leucocytes

NADPH-dependent reduction of flavin and cytochrome *b* in solubilized preparations

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(Received 5 April 1984/Accepted 11 July 1984)

An NADPH-dependent $O_2^{\cdot-}$ -generating oxidase was solubilized from phorbol 12-myristate 13-acetate-activated pig neutrophils by using a mixture of detergents. Recovery of oxidase was approx. 40%. The extract contained cytochrome *b*-245 (331 pmol/mg of protein) and FAD (421 pmol/mg of protein); approx. 30% of each was reduced within 60 s when NADPH was added to anaerobic incubations. Three different additives, quinacrine, *p*-chloromercuribenzoate and cetyltrimethylammonium bromide, strongly inhibited $O_2^{\cdot-}$ generation; they also inhibited the reduction by NADPH of cytochrome *b* at the same low concentrations. In the presence of *p*-chloromercuribenzoate cytochrome *b* reduction was strongly inhibited and flavin reduction was less inhibited. A detergent extract prepared from non-stimulated neutrophils also contained flavin and cytochrome *b*, but its rate of $O_2^{\cdot-}$ production was less than 1% of that from activated cells; its initial rate of cytochrome *b* and flavin reduction was low, although the state of reduction at equilibrium was similar to that of extracts of activated cells. Even in the non-activated cell extract the reduction of flavin and cytochrome was made fast and complete when Methyl Viologen was added to the anaerobic incubations. The oxidase was temperature-sensitive, with a sharp maximum at 25°C; temperatures above this caused loss of $O_2^{\cdot-}$ generation, and this coincided with loss of the characteristic cytochrome *b* spectrum, indicative of denaturation of the cytochrome. The cytochrome *b* formed a complex with butyl isocyanide (close to 100% binding at 10 mM); butyl isocyanide also inhibited the oxidase activity of stimulated whole neutrophils (22.5% inhibition at 10 mM). Photoreduced FMN stimulated O_2 uptake by the oxidase. The results support a scheme of electron transport within the oxidase complex involving NADPH, FAD, cytochrome *b*-245 and O_2 in that sequence.

Polymorphonuclear leucocytes (neutrophils) form part of the mammalian host defence system against microbial infection. As they phagocytose and kill bacteria there is a burst of O_2 uptake, which is accompanied by the release of reactive products of O_2 reduction ($O_2^{\cdot-}$, H_2O_2 , $OH^{\cdot}$ and, perhaps, 1O_2), which are likely to participate in the killing process (Baldridge & Gerard, 1933; Rossi & Zatti, 1964; Babior, 1978). Additional toxic species may be formed from these activated O_2 products, the best established being OCI^- , formed in neutrophils by the myeloperoxidase-catalysed reaction between H_2O_2 and Cl^- (Klebanoff, 1967). The first event in the chain of reactions leading to production of these toxic radicals and ions is likely to be the reduction of O_2 to $O_2^{\cdot-}$; H_2O_2 and $OH^{\cdot}$

can be readily formed from $O_2^{\cdot-}$ by dismutations and an iron-catalysed Fenton reaction respectively. In the present paper we describe experiments that help to characterize the $O_2^{\cdot-}$ -generating oxidase, which is known to require NADPH as electron donor (Rossi & Zatti, 1964).

The plasma membrane of human neutrophils contains a cytochrome *b* (Segal & Jones, 1979a) of very low oxidation–reduction potential ($E_{m,7.0}$ is -245 mV; Cross *et al.*, 1981), sufficiently low for its involvement in the reduction of O_2 to $O_2^{\cdot-}$ [$E_{m,7.0}$ calculated from the data of Wood (1974) as -160 mV]. The cytochrome is reduced when anaerobic suspensions of intact neutrophils are stimulated (Segal & Jones, 1979b) and is oxidized with a t_1 of 4.7 ms when partly reduced cytochrome

is mixed with air. Cytochrome *b*-245 is reduced by NADPH in isolated membranes (Cross *et al.*, 1982a; Gabig *et al.*, 1982) and in a soluble partly purified preparation of oxidase (Bellavite *et al.*, 1983). The cytochrome *b*-245 is associated with FAD in a ratio variously reported as 1:1 (Cross *et al.*, 1982b; Gabig, 1983) or 1:2 (Borregaard & Tauber, 1984). In some variants of the genetic disease chronic granulomatous disease, where the respiratory burst is absent, cytochrome is present but not reduced when intact neutrophils are stimulated (Segal & Jones, 1980; Segal *et al.*, 1983); in such cells the FAD/cytochrome *b* ratio is altered (Cross *et al.*, 1982b; Gabig, 1983). The normal respiratory-burst response can be restored to such cells by fusing them, *in vitro*, with cells from another group of chronic-granulomatous patients where the cytochrome *b*-245 is absent (Hamers *et al.*, 1984). The evidence suggests that the electron-transport system involves at least two proteins, cytochrome *b*-245 and an FAD-protein, each of which may be missing or altered in chronic granulomatous disease. We propose that these are organized to form an electron-transport pathway thus:

We describe here experiments that support this scheme, with the use of an optically clear oxidase solubilized from activated pig neutrophils.

Materials and methods

Isolation of neutrophils

Whole blood (about 4 litres) from the local abattoir was taken into EDTA in 0.9% (w/v) NaCl at a final concentration of 0.15% (w/v) to prevent coagulation, catering gelatin was added to give a final concentration of 1.25% (w/v) and the mixture was left to stand for about 1 h. The top layer, enriched in leucocytes, was sucked off and the cells were collected by centrifugation. The cells were washed once in saline containing 5 i.u. of heparin/ml and 1 mM-EDTA. The cells were subjected to three cycles of osmotic shock, to lyse any erythrocytes, by suspension for 30 s in 5 vol. of 0.2% NaCl containing 1 mM-EDTA and 5 i.u. of heparin/ml: iso-osmoticity was restored by addition of an equal volume of 1.6% NaCl containing 1 mM-EDTA and 5 i.u. of heparin/ml. Finally, when free of haemoglobin, the leucocytes were suspended in 0.9% NaCl containing 1 mM-EDTA and 5 i.u. of heparin/ml. The suspension was layered on to an equal volume of Ficoll (type F-P; Sigma Chemical

Co., Poole, Dorset, U.K.) and centrifuged at 400g for 20 min. The neutrophil pellet was collected, washed twice in 0.9% NaCl containing 1 mM-EDTA and 5 i.u. of heparin/ml and finally suspended in 50 ml of modified Krebs-Ringer buffer, pH 7.4, with Hepes [4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid] (50 mM) and NaH₂PO₄ (1 mM) in place of KHCO₃ and supplemented with 2 mM-glucose and heparin (5 i.u./ml).

Activation of neutrophils and preparation of soluble oxidase

The neutrophil suspension (50 ml) was made 1 mM with respect to di-isopropyl phosphorofluoridate (Sigma Chemical Co.) at 0°C. After 5 min the cells were washed twice with the modified Krebs-Ringer buffer and then incubated at 37°C in approx. 100 ml volume and stimulated by addition of 1 µg of phorbol 12-myristate 13-acetate (Sigma Chemical Co.)/ml. After 5 min stimulation the temperature was rapidly lowered to 4°C by the addition of frozen modified Krebs-Ringer buffer, and the cells were harvested by centrifugation. The cells were resuspended in 50 ml of disruption buffer, which has the following composition: sucrose (8.6%, w/v), MgSO₄ (4 mM), NaN₃ (1 mM), CaCl₂ (1 mM) and phenylmethanesulphonyl fluoride (5 µg/ml); chymostatin, leupeptin, antipain, pepstatin and 7-amino-1-chloro-3-L-tosylamidoheptan-2-one ('TLCK') (all supplied by Sigma Chemical Co.) were added at 1 µg/ml each. The suspension was sonicated by using a Branson Soniprobe (setting 4) for three 15 s bursts with 1 min cooling between treatments. The suspension was centrifuged at 500g for 7 min, and the postnuclear supernatant was collected and re-centrifuged at 100 000g for 30 min. The membrane pellet was suspended in approx. 50 ml of extraction buffer (modified from Markert & Babior, 1983): 0.25% (v/v) Lubrol PX, 0.25% (w/v) sodium deoxycholate (BDH Chemicals, Poole, Dorset), 20 mM-glycine, pH 8.0, 25% (v/v) glycerol, 1 mM-MgSO₄, 0.5 mM-CaCl₂, 1 mM-NaN₃ and the same mixture of proteinase inhibitors that was present in the disruption buffer. The suspension was sonicated three times for 20 s, with 1 min cooling between treatments, and centrifuged at 100 000g for 1 h. The clear supernatant contains about 40% of the total NADPH-dependent O₂^{·-}-generating activity of the postnuclear supernatant.

Spectroscopy

Absorption spectra were recorded on a rapid-scanning spectrophotometer constructed with the use of a Hilger D330 monochromator. Signals recorded at 0.05 nm intervals at 12-bit resolution were processed in an ITT 2020 microcomputer fitted with a floppy-disc drive unit. Spectra were

displayed on a visual-display unit, and hard copies were taken by using an *X-Y* plotter. The computer programs used were devised by Mr. James N. Sturgis of this Department.

Spectra under anaerobic conditions

Samples were added to the main well of a Thunberg cuvette. Appropriate reactants were pipetted into the side arm and the assembly was repeatedly (20 times) evacuated and flushed with N_2 . The N_2 was made O_2 -free by flushing through a solution containing proflavin hemisulphate (224 mg/ml), Methyl Viologen (270 μ g/ml) and EDTA (7.5 mg/ml) in 250 mM-sodium phosphate buffer, pH 6.5, which was activated by light during use (Sweetzer, 1967). Anaerobiosis was tested with the use of myoglobin as an indicator; incubations were at 14°C.

Determination of $O_2^{\cdot-}$ production

NADH- or NADPH-dependent production of $O_2^{\cdot-}$ was measured as the superoxide-sensitive rate of reduction of added cytochrome *c*, with the use of a dual-wavelength spectrophotometer at 25°C (Cross *et al.*, 1982a) set at 550 nm minus 540 nm.

Determination of flavin content

The reduction state of flavins was assayed in oxidized-minus-reduced difference spectra by using $\Delta\epsilon_{450-500} = 11.3 \text{ mM}^{-1} \cdot \text{cm}^{-1}$ (Dawson *et al.*, 1969). The values obtained were confirmed in separate experiments where the total flavins were extracted and assayed by fluorescence (Faeder & Seigel, 1976).

*Determination of cytochrome *b*-245*

The cytochrome was assayed by reduced-minus-oxidized difference spectroscopy by using $\Delta\epsilon_{559-540} = 21.6 \text{ mM}^{-1} \cdot \text{cm}^{-1}$ (Cross *et al.*, 1982a).

Protein assay

The method of Bramhall *et al.* (1969) was used.

Determination of oxidation-reduction potentials

The method, based on measurement of absorption spectra at known oxidation-reduction potentials (Dutton, 1978), was described by Cross *et al.* (1981). The accuracy of the electrode-voltmeter assembly was checked by using a hydrogen electrode at pH 4.0.

Results

The soluble oxidase prepared from membranes of activated pig neutrophils had the properties shown in Table 1. The rates of $O_2^{\cdot-}$ generation (365 nmol/min per mg of protein) were higher than

Table 1. *Properties of the superoxide-generating oxidase solubilized from activated pig neutrophils*

Neutrophils were activated, extracted with detergent and analysed as described in the Materials and methods section.

Property	Assay value
Protein content	1.0 mg/ml
NADPH-dependent $O_2^{\cdot-}$ generation*	365 nmol/min per mg of protein
K_m (NADPH) for $O_2^{\cdot-}$ generation	45 μ M
K_m (NADH) for $O_2^{\cdot-}$ generation	460 μ M
Cytochrome <i>b</i> content	331 pmol/mg of protein
Flavin content (FAD)	421 pmol/mg of protein
$E_{m,7.0}$ (cytochrome <i>b</i>)†	-238 mV
Stability	
Half-life at 4°C	32 h
Half-life at 25°C	2.5 min

* In separate experiments with a different extract it was found that the rate of NADPH-dependent O_2 uptake, measured with an oxygen electrode, was the same as the rate of $O_2^{\cdot-}$ production.

† In order to determine $E_{m,7.0}$, deoxycholate was first removed by dialysis.

those of whole cells (around 130 nmol/min per mg of protein) or postnuclear supernatants. In extracts prepared by using the same procedures, but with unstimulated cells as starting material, the rate of NADPH-dependent $O_2^{\cdot-}$ production by the detergent-solubilized extract was only 2.0 nmol/min per mg of protein. As repeatedly reported for the $O_2^{\cdot-}$ -generating system of neutrophils, the activity of the oxidase in disrupted cells was unstable at room temperature.

The absolute oxidized spectrum of the extract shows a broad absorption band, centred around 450 nm, due to flavin, which is lost after dithionite reduction (Fig. 1). There is in addition a more intense absorbance at 417 nm due to the absorbance of oxidized cytochrome *b* (Fig. 1). The addition of NADPH to an anaerobic preparation caused a substantial reduction of the cytochrome, seen by increased absorbance at 559, 521 and 427 nm, together with a loss of absorbance at 450 nm due to reduction of the flavin (Fig. 2). Potentiometric titration showed that less than 8% of the total cytochrome was reduced at oxidation-reduction potentials above -100 mV, indicating that contamination by cytochrome *b*₅ or mitochondrial cytochromes *b* was not significant in these extracts. The $E_{m,7.0}$ was found to be -238 mV, slightly higher than found for the cytochrome in intact membranes.

The generation of $O_2^{\cdot-}$ by the soluble oxidase was sensitive to low concentrations of the inhibitors *p*-chloromercuribenzoate, quinacrine and

Fig. 1. Spectroscopic properties of the solubilized superoxide-generating oxidase

Spectra were recorded as described in the Materials and methods section. Spectrum *a*, absolute absorbance spectrum of the air-oxidized enzyme (2 mg of protein/ml). Spectrum *b*, absolute absorbance spectrum of the mixture used for spectrum *a* after the addition of a few crystals of $\text{Na}_2\text{S}_2\text{O}_4$. Spectrum *c*, dithionite-reduced-minus-air-oxidized difference spectrum.

Fig. 2. Reduction of solubilized flavin and cytochrome *b* by NADPH

The methods used to record spectra under anaerobic conditions are described in the Materials and methods section. Spectrum *a*, difference spectrum of the solubilized oxidase 80 min after the addition of 1 mM-NADPH, minus the spectrum before the addition of NADPH. Spectrum *b*, difference spectrum after the addition of a few crystals of $\text{Na}_2\text{S}_2\text{O}_4$ to the mixture used to give spectrum *a*.

cetyltrimethylammonium bromide (Fig. 3). *p*-Chloromercuribenzoate is known to react with cytochrome *b*-245, since it causes a 65 mV increase in its $E_{m,7.0}$ (Bellavite *et al.*, 1983); quinacrine is an inhibitor of flavin-linked reactions (see, e.g., Wakeyama *et al.*, 1983), and cetyltrimethylammonium bromide, an ionic detergent, is, at low concentration, an inhibitor of the NADPH-dependent oxidase of pig leucocytes (Wakeyama *et al.*, 1983).

Fig. 3. Effect of inhibitors on the superoxide-generating activity of the solubilized oxidase

NADPH-dependent production of $\text{O}_2^{\cdot-}$ was measured by the superoxide dismutase-sensitive reduction of cytochrome *c*, as described in the Materials and methods section, in the presence of 100 μM -cytochrome *c* in 50 mM-triethanolamine hydrochloride, pH 8.0, 0.5 mM-NADPH and the appropriate concentration of inhibitor. The inhibitor concentration range 0–1.0 shown on the abscissa represents 0–4 mM-quinacrine ($\square$) or 0–0.01% cetyltrimethylammonium bromide ($\circ$) or 0–20 μM -*p*-chloromercuribenzoate ($\bullet$). The reaction was started by the addition of enzyme to give a final protein concentration of 50 $\mu\text{g}/\text{ml}$.

Reduction of the flavin and cytochrome components of the oxidase

Concentrations of the inhibitors that gave close to maximum inhibition (Fig. 3) were selected for use in experiments where the rates of reduction of cytochrome *b* and flavin were measured after the addition of NADPH to the oxidase under anaerobic conditions (Fig. 4). The traces show that a substantial proportion of the cytochrome *b* (24%) and of the flavin (34%) is reduced as soon as measurement can be made (about 1 min). The time taken to record the first measurement is, unfortunately, long, because the Thunberg cuvette had to be removed from the spectrophotometer so that NADPH could be tipped from the side arm. This initial fast burst of reduction was followed by a slow phase that eventually resulted in almost complete reduction of flavin and about 60% reduction of the *b* cytochrome (still slowly increasing after

Fig. 4. Effect of inhibitors on the enzymic reduction of (a) cytochrome *b* and (b) flavin

The enzyme (2mg of protein/ml) was incubated anaerobically in the presence of 1 mM-NADPH as described in the Materials and methods section, and spectra were recorded at the indicated time points. Inhibitors were used, where indicated, at the following concentrations: *p*-chloromercuribenzoate, 12.5 μ M (○); cetyltrimethylammonium bromide, 0.005% (□); quinacrine, 5 mM (■); control (no inhibitor) (●). The extent of reduction at each time point was calculated as a percentage of the total of cytochrome or flavin reduced after addition of $\text{Na}_2\text{S}_2\text{O}_4$ at the end of the experiment, as described in the Materials and methods section.

120 min). Cetyltrimethylammonium bromide and quinacrine strongly inhibited the rate of reduction of cytochrome *b*. The rate of flavin reduction could not be measured in the presence of quinacrine, but it was inhibited by cetyltrimethylammonium bromide. *p*-Chloromercuribenzoate inhibited the extent and rate of cytochrome *b* reduction though having less effect on flavin reduction (Fig. 4b).

Reactivity of extract from non-stimulated neutrophils

Control, non-phorbol-treated, cells were subjected to the same fractionation and solubilization procedures that were used to extract the active NADPH-dependent oxidase. The kinetics of reduction of the flavin and cytochrome *b* components of this extract are shown in Fig. 5, where they are compared with those of the active oxidase. The initial rates of reduction of each prosthetic group are diminished in extracts from unstimulated cells, although after about 30 min the eventual extents of reduction are greater. The addition

Fig. 5. Kinetics of the enzymic reduction of cytochrome *b* and flavin in the presence of Methyl Viologen and in enzyme prepared from resting cells

The methods used are described in the legend to Fig. 4. The Figure shows the NADPH-dependent reduction of flavin (▲) and cytochrome *b* (△) in the presence of 12.5 μ M-Methyl Viologen, and the NADPH-dependent reduction of flavin (●) and cytochrome *b* (○) in the solubilized enzyme prepared from unstimulated cells.

of Methyl Viologen, as a mobile electron carrier, resulted in the almost complete reduction of both flavin and cytochrome (Fig. 5).

Temperature-sensitivity of the oxidase

The activity of the soluble $\text{O}_2^{\cdot-}$ -producing oxidase was strongly influenced by the temperature of assay (Fig. 6). A maximum was found at 25°C but at increased temperatures activity declined very sharply. Spectroscopic examination of the extract showed that on incubation at temperatures above 25°C the characteristic spectrum of cytochrome *b* was lost, presumably as haem became detached from the apoprotein. It was not possible to measure the effects of temperature on enzyme activity and on absorption spectra under identical conditions, but both properties appeared to be sensitive to the same range of temperature.

Ligand binding by cytochrome *b*-245

The low-potential cytochrome *b* of the neutrophil oxidase has the typical absorption spectra of a hexa-co-ordinate low-spin haem, yet it does form a CO complex, resembling bacterial cytochrome *o* in this. The affinity for CO is low (Cross *et al.*, 1982a), binding to cytochrome *b* being incomplete even in saturated CO solutions, and so it is not possible to correlate CO inhibition of oxidase activity with the formation of the CO complex of the reduced cytochrome. The affinity of the cytochrome *b* for another haem ligand, butyl isocyanide, is such that almost complete conversion into the isocyanide complex is seen when 10 mM ligand is mixed with

Fig. 6. Effect of temperature on the NADPH-dependent superoxide-generating activity of the solubilized oxidase. NADPH-dependent generating activity was determined by the superoxide dismutase-sensitive reduction of cytochrome *c* as described in the legend to Fig. 3. A 1 ml volume of reaction buffer was preincubated in the thermostatically controlled cuvette holder of the spectrophotometer, and the reaction was initiated by the addition of 25 μ l (50 μ g of protein) of soluble enzyme.

Fig. 7. Reaction of cytochrome *b*-245 with *n*-butyl isocyanide

The dithionite-reduced solubilized enzyme (2.0 mg of protein/ml) was treated by the addition of 10 mM-*n*-butyl isocyanide, and after 10 min the difference spectrum was recorded against an untreated dithionite-reduced preparation. The proportion of the cytochrome binding *n*-butyl isocyanide was determined by using $\Delta\epsilon_{575-559} = 21.6 \text{ mM}^{-1} \cdot \text{cm}^{-1}$ for the (reduced-plus-butyl isocyanide)-minus-reduced spectrum. Spectrum *a*, dithionite-reduced-minus-oxidized spectrum; spectrum *b* (dithionite-reduced-plus-butyl isocyanide)-minus-reduced spectrum.

reduced solubilized oxidase (Fig. 7). The reaction is slow, requiring about 10 min for maximum binding, presumably because butyl isocyanide, like CO, displaces some natural ligand in a rate-limiting step. In separate experiments it was found that 10 mM-butyl isocyanide inhibited the oxidase acti-

Fig. 8. Photostimulation of O_2 uptake by the solubilized oxidase

O_2 uptake was measured, in a Clark-type oxygen electrode, in 50 mM-triethanolamine hydrochloride buffer, pH 8.0, containing 5 μ M-FMN and 5 mM-EDTA in 1.5 ml. O_2 uptake was determined in the dark and in conditions of illumination by a 1500 W tungsten lamp at 20°C. After determination of the rate of O_2 uptake by the photoreduction and auto-oxidation of FMN, solubilized enzyme was added (120 μ g of protein/ml) and the new rate of O_2 consumption determined.

vity of intact phorbol-stimulated neutrophils by 23%. At 20 mM-butyl isocyanide 35% inhibition was observed, indicating that ligand binding to the haem of cytochrome *b* would prevent oxidase activity.

Photostimulation of O_2 uptake by solubilized oxidase

In a previous paper (Cross *et al.*, 1982b) we have shown that, when a homogenate of activated neutrophils is illuminated, under the conditions used to cause photoreduction of flavins, the cytochrome *b*-245 becomes more reduced. The addition of soluble oxidase to photoreduced FMN caused a great increase in O_2 consumption (Fig. 8), which we interpret as arising from the transfer of electrons from FMNH_2 either directly to cytochrome *b*-245 or indirectly via FAD associated within the cytochrome *b*. Reduced FMN is itself auto-oxidizable and gives a background rate of O_2 consumption.

Discussion

There is a variety of evidence implicating FAD in the NADPH oxidase of activated neutrophils. The addition of FAD to Triton X-100-solubilized

extracts of activated human neutrophils stimulated NADPH-dependent $O_2^{\cdot-}$ production (Babior & Kipnes, 1977), and this led to the suggestion that the enzyme was a flavoprotein. There have been reports of similar stimulation by FAD of solubilized oxidase from pig neutrophils (Wakeyama *et al.*, 1982), and Bellavite *et al.* (1983) noted that the addition of FAD prolonged the linear phase of $O_2^{\cdot-}$ generation without increasing its maximum rate. Analogues of FAD inhibited NADPH-dependent $O_2^{\cdot-}$ generation by human leucocyte preparations (Light *et al.*, 1981). It has also been shown that in some types of chronic granulomatous disease where electron flow to cytochrome *b* is impaired the FAD/cytochrome *b* ratio is altered (Cross *et al.*, 1982*b*; Gabig, 1983).

The rate of reaction of chemically reduced cytochrome *b*-245 with air is fast in membranes from unstimulated leucocytes (Cross *et al.*, 1982*a*), although in such preparations the *b* cytochrome is not reduced by added NADPH. Cholate extraction of activated leucocyte membranes separates a flavoprotein with NADPH-linked dye reductase activity from a flavin-free non-NADPH-reducible auto-oxidizable cytochrome *b* (Gabig & Lefker, 1984). Reduction of the cytochrome *b* may be driven by photoreduced flavin (Cross *et al.*, 1982*a*). The published experimental evidence, although indirect, strongly supports an electron-transport scheme where NADPH reduces cytochrome *b*-245 via a flavoprotein. Reduced cytochrome *b*-245 may then react directly with O_2 to form $O_2^{\cdot-}$. Our optically clear soluble oxidase preparation has permitted experiments that strongly support this scheme. Although initial rates of cytochrome *b* reduction could not be measured in our system, the extent of cytochrome *b* reduction at 60s in this preparation is the greatest yet recorded, possibly because of treatment with a membrane permeable inhibitor of proteinases (di-isopropyl phosphorofluoridate) before activation and disruption (see the Materials and methods section). The initial rate of reduction of cytochrome *b*-245 was much higher in the extracts of activated cells, although eventually the extent of reduction of the cytochrome in the extracts of non-stimulated cells exceeded that of activated cells, as the systems approached equilibrium (Fig. 5).

Quenching of the flavin fluorescence by detergent prevented the use of fluorescence in kinetic studies, but the absorption band at 450nm of oxidized flavin (Fig. 1) could be used. Absorbance changes due to the cytochrome *b* reduction did not interfere significantly, since the cytochrome and the flavin could be shown to have different kinetics of reduction. Not all the flavin or cytochrome was rapidly reduced (Fig. 4), even under strictly anaerobic conditions. It is possible that the slowly

reacting components are not correctly associated: the activation process may change the interactions of an NADPH-flavoprotein-cytochrome *b* complex such that electron transfer between these components is favoured but not all the components present in the cell may be activated. It is known that a substantial amount of cytochrome *b*-245 is associated with the granule fraction as well as with the plasma membrane (Segal & Jones, 1979*a*; Borregaard *et al.*, 1983), and the granule fraction may avoid immediate activation. The stimulatory effect of added Methyl Viologen on the reduction of both flavin and cytochrome *b* indicates that interactions between the bulk of these components and NADPH is limited in a way that is overcome by a mobile mediator or that some reduced intermediate form that inhibits electron flow may accumulate under anaerobic conditions. Alternatively, incomplete reduction of these prosthetic groups could occur if active oxidase components become deactivated during extraction. This latter explanation is less likely, because even in intact neutrophils cytochrome *b* reduction on stimulation is slow and incomplete (Cross *et al.*, 1982*a*).

The inhibitors of $O_2^{\cdot-}$ production (Figs. 3 and 4) support our postulated electron-transport sequence. It is possible to add *p*-chloromercuribenzoate to diminish the rate of cytochrome *b* reduction at concentrations that have much less effect on flavin reduction, and quinacrine, the flavin analogue, inhibits the rate of cytochrome *b* reduction. Both flavin and cytochrome reduction are inhibited by cetyltrimethylammonium bromide at the same concentration found necessary for the inhibition of $O_2^{\cdot-}$ generation.

A coincidence of inhibition of $O_2^{\cdot-}$ production and alteration of the properties of cytochrome *b* is found on increasing the temperature of incubation (Fig. 6) or after the addition of butyl isocyanide. The reactivity (although slow or incomplete) of the cytochrome with both CO and isocyanide suggests that the binding of the sixth ligand to the haem is relatively weak. It is possible that this property facilitates the reaction of the cytochrome with O_2 .

This work has been supported by Grants from the Wellcome Trust and Medical Research Council. We are also grateful to Mr. James Sturgis for his advice on computer programming.

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