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THE CYTOCHROME *b* AND FLAVIN CONTENT AND PROPERTIES OF THE O_2^--FORMING NADPH OXIDASE SOLUBILIZED FROM ACTIVATED NEUTROPHILS

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NADPH-dependent O_2^--generating activity was extracted and partially purified from guinea pig polymorphonuclear leukocytes. The most active preparation generated 202.8 nmol O_2^- / min per mg protein. This activity was 30-fold higher than that of extracts from resting cells, indicating that the activated state of the oxidase was retained after solubilization. The solubilization and purification of the enzyme activity were followed by a parallel solubilization and purification of cytochrome *b*. Spectroscopic studies showed that solubilized cytochrome *b* has an E_m of −245 mV and binds CO to about 30%. Cytochrome *b* was reduced by NADPH in anaerobiosis at a low rate and was rapidly reoxidized by air. A correlation was found between the inhibition of O_2^- formation caused by the SH reagent *p*-chloromercuribenzoate and the alterations induced by this compound on the E_m of cytochrome *b*. These observations strongly support the participation of cytochrome *b* in the catalytic activity of the solubilized NADPH oxidase. The enzyme preparations contained FAD, which was found to be associated both with NADPH oxidase and with diaphorase activities. The fraction with the highest O_2^- forming activity contained FAD and cytochrome *b* in a ratio of about 0.5:1. The participation of FAD in the electron transport from NADPH to O_2 is supported also by the inhibitory effect exerted by quinacrine on O_2^- formation.

Introduction

During phagocytosis and when stimulated with membrane-perturbing agents, polymorphonuclear leukocytes undergo a dramatic stimulation of their respiration, with the formation of several products of O_2 reduction (O_2^-, H_2O_2, $OH^\cdot$ and $^1O_2$) which play an important role in the bactericidal activity of these cells [1–4]. The key event of this respiratory burst is the activation of a membrane-bound oxidase that preferentially uses NADPH as substrate [2,5]. In recent years evidence has accumulated that a flavoprotein and a low-potential cytochrome *b* could be involved in this enzymatic activity. The Triton-solubilized O_2^--forming activity of human neutrophils is stabilized by the addition of FAD [6] and is inhibited by FAD analogues [7]. The implication of cytochrome *b* in the oxidase activity is suggested by the fact that it is lacking in patients affected by the X-linked form of chronic granulomatous disease, where pyridine-nucleotide-dependent O_2^- formation is absent [8]. Moreover, Cross et al. [9] showed cytochrome *b* reduction in the membrane fraction of pig neutrophils and this cytochrome has many properties of an oxidase. Similar results have been

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Abbreviations: PMA, phorbol-myristate acetate; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*′-tetraacetic acid; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

reported by Gabig et al. [10] utilizing a subcellular particulate fraction from human neutrophils. In spite of this, our knowledge on the nature of the oxidase and on the relationships between cytochrome *b*, FAD and the catalytic activity is scanty, due to the fact that most studies have concerned particulate fractions and not the isolated enzyme. Attempts which have been made to purify this oxidase have been hampered by the extreme instability of the enzyme activity following detergent solubilization [11,12]. The present study utilizes a deoxycholate-extracted and partially purified preparation of NADPH oxidase from guinea pig neutrophils, obtained with a procedure that preserves a high and relatively stable O_2^--forming and NADPH-oxidizing activity. The functional properties of the cytochrome *b* of this preparation and the ratio of cytochrome *b*/flavin have been also investigated.

Materials and Methods

Cell preparation. Guinea pigs were injected intraperitoneally with 50 ml of sterile 0.9% NaCl containing 1% sodium caseinate and the cells (more than 90% neutrophils) were collected after 12 h with two peritoneal lavages. The cell suspension was filtered through gauze, centrifuged for 10 min at $500 \times g$, freed from contaminating erythrocytes by hypotonic lysis and finally suspended in Krebs-Ringer phosphate buffer, pH 7.4, containing 5 mM glucose and 0.5 mM $CaCl_2$.

Cell activation and preparation of subcellular particles. Maximum activation of neutrophils was achieved by incubating $2 \cdot 10^7$ cells/ml in Krebs-Ringer-phosphate containing 5 mM glucose, 0.5 mM $CaCl_2$ and 0.2 μg/ml phorbol-myristate acetate (PMA) for 90 s at 37°C. The incubation was stopped by the addition of ice-cold Krebs-Ringer-phosphate and the cells were immediately centrifuged at $1000 \times g$ for 8 min, suspended in 0.34 M sucrose ($4 \cdot 10^8$ cells/ml) buffered with 10 mM Tris-HCl, pH 7.0, and containing the protease inhibitor phenylmethylsulfonyl fluoride (2 mM). Cells were homogenized with a Potter-type homogenizer at 4°C until 90% of cells were disrupted. The homogenate was centrifuged at $250 \times g$ for 8 min in order to sediment nuclei and unbroken cells. The supernatant was collected and centrifuged at $100\,000 \times g$ for 30 min. The resulting pellet (fraction 1) was suspended in 5 mM potassium phosphate buffer, pH 8.0, containing 20% (v/v) glycerol and 1 mM EGTA, at a protein concentration of 4–5 mg/ml.

Extraction of NADPH oxidase. NADPH oxidase was extracted with deoxycholate from cell-free particles and a partially purified preparation was obtained by ultracentrifugation of the extract. Sodium deoxycholate recrystallized from ethanol [13] was added to cell-free particles (fraction 1) at a final concentration of 0.3%. The mixture was kept at 0°C for 20 min under magnetic stirring. The deoxycholate-treated fraction 1 was centrifuged at $100\,000 \times g$ for 60 min and the resulting supernatant (fraction 2) was collected. This supernatant was chromatographed on Sephadex G-25 in order to eliminate the excess of deoxycholate and the resulting preparation (fraction 2A) was centrifuged at $100\,000 \times g$ for 14 h, and a final supernatant (fraction 3) and a pellet (fraction 4) were obtained. This pellet was suspended in 5 mM potassium phosphate buffer, pH 8.0, containing 20% glycerol and 1 mM EGTA.

Assay of NADPH oxidase and of NADPH-cytochrome c reductase. Assays were carried out in a Perkin Elmer 576 spectrophotometer at 22°C. The cuvette contained 50 mM Hepes, pH 7.0, 110 mM KCl, 25 mM NaCl, 1 mM diethyltriaminepentaacetic acid, 2 mM NaN_3, 0.1 mM cytochrome *c* and 150 nmol of NADPH. The final volume was 1 ml. The reaction was started by the addition of enzyme and the absorption change at 550 nm was monitored. The reduction of cytochrome *c* (maximal rate) was calculated using an extinction of 21.1 $mM^{-1} \cdot cm^{-1}$ [14]. NADPH-cytochrome *c* reductase (diaphorase) activity was measured as reduction of cytochrome *c* after the addition of 30 μg/ml of superoxide dismutase. O_2^- production by NADPH oxidase was measured as the rate of cytochrome *c* reduction in the absence of superoxide dismutase minus the rate of the diaphorase activity.

Spectrophotometry. Absorption spectra at room temperature and at 77 K were determined in a spectrophotometer constructed using a Hilger D330 monochromator with a reciprocal dispersion of 2.6 nm/mm, used with a slit width of 0.02 mm.

Determination of flavins and cytochrome b. FAD

and FMN were assayed by the method of Faeder and Seigel [15]. Flavins were extracted by heating samples in a 100°C waterbath before the fluorescence assay. The low-potential cytochrome *b* was assayed by reduced-minus-oxidized difference spectroscopy using $\Delta E_{559-540\,nm} = 21.6\ cm^{-1} \cdot mM^{-1}$ [16].

Potentiometric titrations. The mid-point oxidation reduction potential of cytochrome *b* was determined by titration in an anaerobic cuvette at pH 7.0 in the presence of a mixture of mediators [9]. Reduction was achieved by injection of sodium dithionite solutions, oxidation by injection of potassium ferricyanide solutions and spectra were constantly recorded.

Materials. Cytochrome *c*, type VI; NADPH, type III; phorbol 12-myristate 13-acetate (PMA) were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Superoxide dismutase was a gift from Dr. J.V. Bannister. Other compounds were the highest grade available.

Results

Preparation of NADPH oxidase

Table I shows that the treatment of cell-free particles from PMA-activated neutrophils with 0.3% deoxycholate resulted in extraction of proteins, of NADPH oxidase activity and of NADPH-cytochrome *c* reductase (diaphorase) activity. With this procedure, 44.5% of proteins and 37.9% of NADPH oxidase were solubilized, so that the specific activity of the oxidase slightly decreased. Conversely, the specific activity of diaphorase increased in the solubilized preparation. Fraction 2 was then chromatographed through Sephadex G-25, in order to eliminate the excess of

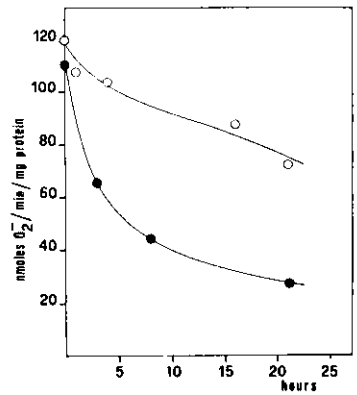

Fig. 1. Stability of NADPH oxidase activity. The extract containing 0.3% deoxycholate (●) and the extract after G-25 chromatography (○) were maintained at 4°C and assayed for O_2^- formation at time intervals.

TABLE I

PROTEIN CONTENT, NADPH-DEPENDENT O_2^- PRODUCTION AND DIAPHORASE ACTIVITY IN VARIOUS FRACTIONS OBTAINED FROM PMA-TREATED GUINEA PIG NEUTROPHILS

Cell-free particles (fraction 1) from PMA-activated neutrophils were treated with 0.3% deoxycholate and various fractions were prepared and assayed for proteins, NADPH oxidase and diaphorase activity. Values of recovery represent protein and activity recovered from $2.5 \cdot 10^8$ neutrophils. Data represent mean (±S.E., where given) of three experiments.

Fraction	Protein recovered in fraction (mg)	O_2^- production (nmol O_2^-/min)		Diaphorase (nmol cytochrome *c* reduced/min)	
		Spec. act. (per mg)	Activity recovered in fraction	Spec. act. (per mg)	Activity recovered in fraction
1	4.47 ± 0.25	132.4 ± 16.2	591.8	4.1 ± 1.5	18.3
2	1.99 ± 0.17	113.0 ± 17.2	224.8	7.0 ± 0.6	13.9
% of 1	44.5	—	37.9	—	75.9
2A	1.83 ± 0.16	106.8 ± 27.1	194.5	6.8 ± 1.7	12.4
3	1.00 ± 0.05	4.9 ± 0.6	4.9	10.3 ± 1.2	10.3
% of 2A	54.9	—	2.5	—	83.1
4	0.76 ± 0.09	202.8 ± 51.7	154.1	4.1 ± 1.9	3.1
% of 2A	41.8	—	79.2	—	25.1

deoxycholate. This step was necessary because deoxycholate interferes with the measurement of flavin fluorescence at pH 2.6. Moreover, the NADPH oxidase activity in the absence of deoxycholate was much more stable than in its presence (Fig. 1), thus allowing investigation over a longer time. Following ultracentrifugation of fraction 2A, most of the NADPH oxidase activity sedimented and was recovered in fraction 4 (Table I). The specific activity of oxidase in fraction 4 was about 2-fold that in fraction 2, indicating that this procedure separates high molecular weight complexes containing the enzymatic activity from other proteins of lower molecular weight solubilized by deoxycholate. The diaphorase activity remained in the supernatant (fraction 3) and was therefore separated from oxidase.

The activity of the enzyme in fraction 4 obtained from resting neutrophils was 6.5 ± 4.3 nmol O_2^-/min per mg protein (mean $\pm$ S.E. for three experiments), that is, 30-fold lower than that obtained from activated cells. This indicates that the functional properties of the oxidase are retained after solubilization.

Table II shows that the activity is sensitive to inhibition by *p*-chloromercuribenzoate, an inhibitor of thiol groups, and by quinacrine, a flavin inhibitor. Addition of FAD to the assay did not increase the maximum rate of the reaction. Nonetheless we have observed that in the presence of FAD the reaction is more linear and the spontaneous loss of activity is delayed (Fig. 2).

TABLE II

EFFECT OF VARIOUS COMPOUNDS ON THE NADPH OXIDASE ACTIVITY

NADPH oxidase (fraction 4 from PMA-activated neutrophils) was prepared and assayed for O_2^- production in the absence and in the presence of the indicated compounds. Data represent mean $\pm$ S.E. of three experiments.

Compound added	Final concentration	O_2^- production (%)
None	—	100
p-Chloromercuribenzoate	1 mM	1.5 ± 0.9
Quinacrine	1 mM	17.3 ± 8.2
FAD	50 μM	106.0 ± 10.5

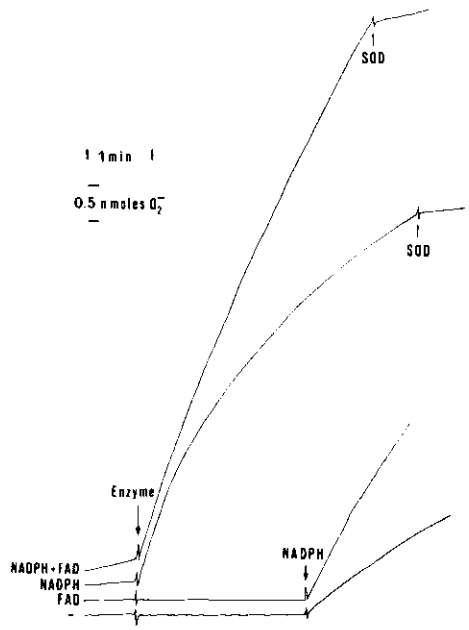

Fig. 2. Kinetics of O_2^--forming activity in the absence and in the presence of FAD. The assay was carried out as described in Methods. Concentrations of the reagents: NADPH, 0.15 mM; FAD, 0.05 mM; superoxide dismutase (SOD), 30 μg/ml; enzyme, 11 μg/ml protein of fraction 4 from PMA-activated neutrophils.

Flavin and cytochrome b content of the enzyme preparations

The preparations obtained during the extraction of NADPH oxidase contained variable amounts of flavins and of cytochrome *b* (Table III). About 40% of cytochrome *b* was extracted by deoxycholate and following ultracentrifugation it sedimented in fraction 4, with a 2-fold increase of concentration with respect to proteins. The pattern of solubilization and the distribution were very similar to those of oxidase activity (see Table I). Measurement of flavin content of the various fractions demonstrated that 50% of flavins present in fraction 1 are recovered in fraction 2A and that they are represented mostly by FAD. After ultracentrifugation of fraction 2A, 51.2% of flavins were found in fraction 3 and 42.6% in fraction 4. As a consequence, the ratio flavin/cytochrome *b* decreased from 0.98 in fraction 2A to 0.42 in fraction 4.

Spectroscopic properties of cytochrome b

Fig. 3 shows the absorption spectra of the pre-

TABLE III

FLAVIN AND CYTOCHROME *b* CONTENT OF VARIOUS FRACTIONS OBTAINED FROM PMA-TREATED GUINEA PIG NEUTROPHILS

Total recovery is given as pmol of flavins or cytochrome *b* recovered from $2.5 \cdot 10^8$ neutrophils. Data represent mean ± S.E. of three experiments.

Fraction	FAD (pmol/mg protein)	FMN (pmol/mg protein)	Total flavins		Cytochrome *b*		Ratio total flavins : cytochrome *b*
			pmol/mg protein	total recovery	pmol/mg protein	total recovery	
1	106.7 ± 15.7	5.5 ± 0.19	112.2 ± 15.9	501.5	148.2 ± 12.9	662.4	0.76
2	132.0 ± 36.8	8.8 ± 0.4	140.8 ± 37.2	256.2	143.4 ± 8.8	260.9	0.98
3	113.1 ± 28.5	19.0 ± 6.8	132.1 ± 21.7	132.1	44.8 ± 10.7	44.8	2.95
% of 2A	—	—	—	51.6	—	17.1	—
4	137.4 ± 26.0	6.5 ± 0.3	143.9 ± 25.9	109.4	346.1 ± 14.2	263.0	0.42
% of 2A	—	—	—	42.6	—	100.8	—

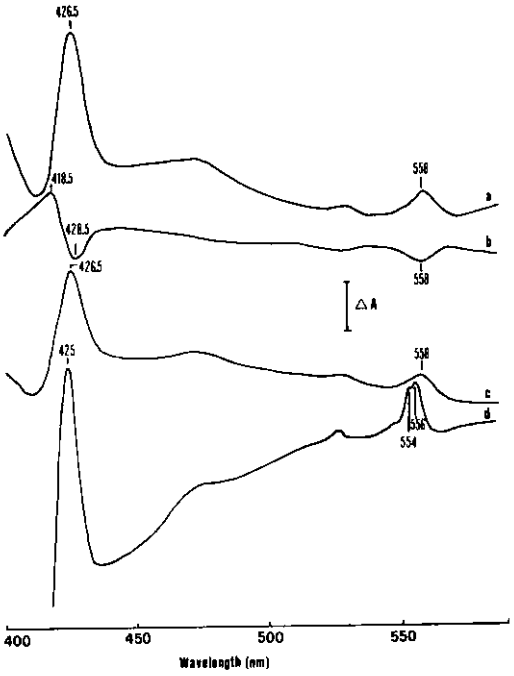

Fig. 3. Spectroscopic properties of the solubilized NADPH oxidase of guinea pig polymorphonuclear leukocytes. (a) Dithionite-reduced minus air-oxidized difference spectrum of solubilized oxidase (0.755 mg protein of fraction 4/ml) from activated leukocytes. (b) CO-difference spectrum of the same preparation used in (a); both cuvettes were reduced with sodium dithionite, CO was bubbled through the sample cuvette for 30 s, and the difference spectrum was recorded after 4 min. (c) Dithionite-reduced minus air-oxidized spectrum of solubilized oxidase (1.41 mg protein of fraction 4/ml) from non-stimulated leukocytes. (d) Spectrum of samples from (c) determined at 77 K. Buffer in each experiment was potassium phosphate, pH 8.0 (5 mM), glycerol (20%, v/v) EGTA (1 mM) and deoxycholate (0.1%, w/v). Absorbance marker = 0.005 in a and b; 0.01 in c and d.

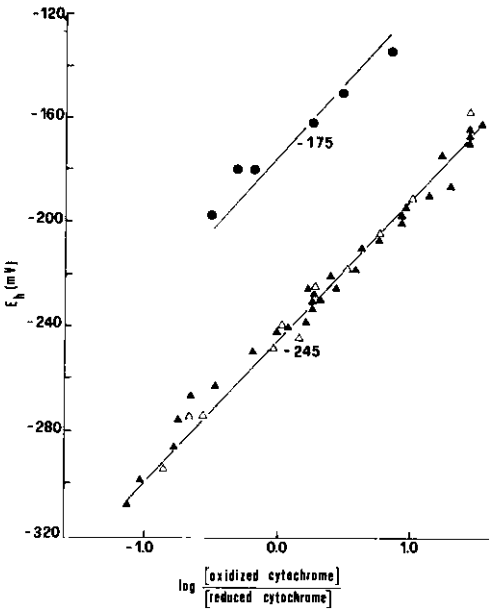

Fig. 4. Replot of data from potentiometric titration of the cytochrome *b* of solubilized oxidase of activated guinea pig leukocytes; the effect of *p*-chloromercuribenzoate. Titrations were carried out in 20 mM 4-morpholinepropanesulfonic acid, 100 mM KCl, pH 7.0, 20% (v/v) glycerol, and the extent of reduction of the cytochrome *b* was determined by repeated scanning of absorption spectra in a split-beam spectrophotometer, as described by Cross et al. [9]. Sodium dithionite and potassium ferricyanide solutions were used to lower and raise the E_h. ▲, reductive titration; △, oxidative titration; ●, titration following the addition of 1 mM *p*-chloromercuribenzoate (PCMB). $E_{m\,7.0}$ of oxidase cytochrome *b* = −245 mV; after treatment with PCMB $E_{m\,7.0}$ = −175 mV.

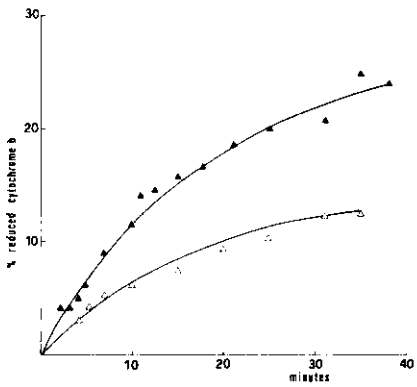

Fig. 5. Kinetics of reduction of cytochrome *b* of the solubilized oxidase of guinea pig leukocytes by NADPH. The oxidase from both activated (0.85 mg protein of fraction 4/ml) and resting (1.72 mg protein of fraction 4/ml) leukocytes was dissolved in 16 mM potassium phosphate, pH 7.0, containing 17% (v/v) glycerol, EGTA (1 mM) and glucose oxidase (1 mg/ml) and catalase (50 μg) as an oxygen-scavenging system. NADPH was generated by a mixture of 1.2 mM glucose 6-phosphate, 200 μM $NADP^+$ and 0.1 μl glucose-6-phosphate dehydrogenase, and the solution was placed in a stoppered cuvette. The extent of reduction of cytochrome *b* was determined from repeated absorption spectra in the region 500–600 nm, using $\Delta A_{559-540\ nm}$ [9]; 100% reduction of the cytochrome *b* was achieved by the addition of solid sodium dithionite. ▲, cytochrome *b* reduction by oxidase from activated cells; △, cytochrome *b* reduction by oxidase from resting cells.

paration with maximum oxidase activity (fraction 4). Dithionite-reduced minus air-oxidized difference spectrum (trace a) clearly shows a *b*-type cytochrome, which binds CO to about 30% at room temperature (trace b). These features resemble those of human neutrophil cytochrome *b* [9], indicating that the extent of CO-binding is not increased by solubilization. No obvious difference in the spectra of cytochrome between activated and resting (trace c) preparations is present. The low-temperature spectrum (trace d) shows a split of the α band like that found in cytochrome b_5 [17], although it is clearly different from cytochrome b_5 in E_m and CO-binding.

Potentiometric titration of the cytochrome *b* (Fig. 4) gave an $E_{m\,7.0}$ of −245 mV, similar to that of human neutrophil membrane [9] and of neutrophils from other animal species [16]. Therefore the deoxycholate treatment did not affect the E_m. Addition of 1 mM *p*-chloromercuribenzoate raised the $E_{m\,7.0}$ to −175 mV.

Fig. 5 demonstrates that cytochrome *b* is reduced by NADPH in anaerobiosis and that the reduction is faster in the activated than in the resting preparation. Nonetheless the rate of reduction is low. Maximal reduction was of 25% and was reached after 40 min with the activated enzyme. Cytochrome *b* oxidized rapidly when air was admitted (data not shown).

Discussion

The chemical nature and the properties of NADPH oxidase have been investigated on a solubilized and partially purified preparation obtained from guinea pig neutrophils. The specific activities of the NADPH oxidase isolated from resting and from PMA-treated cells were 6.4 and 202.8 nmol O_2^-/min per mg protein, respectively, indicating that the activation state of the enzyme is preserved by the procedure employed for isolation. The solubilized oxidase sedimented by centrifugation for 14 h at 100000 × *g*, indicating that it was extracted as a high molecular weight complex. This complex contains flavins and low-potential cytochrome *b*.

The preparation with the highest oxidase activity (fraction 4) contains flavins at a concentration of 143.9 pmol/mg protein and is characterized by a ratio flavin/cytochrome *b* of 0.42 : 1. The analysis of the flavin content of the enzyme preparations showed that over 90% of the flavin was FAD. The small amount of FMN present can be attributed to breakdown of FAD due to the assay method. The participation of FAD in the active enzyme complex is supported both by the finding of a substantial amount of FAD in all the active preparations and by inhibition studies with quinacrine (Table II) or FAD analogues, as reported by Light et al. [7]. Gabig and Babior [6] showed that the Triton-solubilized enzyme from human neutrophils needs FAD for optimal activity. The activity of our preparation was unaltered by addition of FAD (Table II), indicating that FAD remains associated with the enzyme during the extraction with deoxycholate. However, the O_2^- forming activity of fraction 4 is labile and rapidly loses activity in the assay medium at 22°C (Fig. 2). This is not due to the effect of products of the reaction, since the inactivation takes place

both in the presence and in the absence of NADPH. Since the spontaneous loss of activity is significantly prevented by FAD, it could be attributed to a progressive removal of FAD from the active complex at 22°C.

The solubilized preparation contains low-potential cytochrome *b* that was enriched by the isolation procedure to the same extent as the NADPH oxidase activity. Spectroscopic studies showed that the properties of the cytochrome, such as the $E_{m\,7.0}$ value of −245 mV and the binding of CO, are retained on solubilization with deoxycholate. The rate of cytochrome *b* reduction by NADPH (Fig. 5), although low, is higher in the enzymatic preparations obtained from activated leukocytes, and the reduced cytochrome *b* is rapidly oxidized by air. Taken together, the above observations are strongly in favour of the implication of the low-potential cytochrome *b* in the oxidase activity, as previously suggested [16,18]. The only datum in apparent contrast with this conclusion is the low rate of reduction of cytochrome *b* by NADPH in anaerobiosis, which does not account for the high rate of O_2^- production and NADPH oxidation. Perhaps slow reduction is an effect similar to that seen in cytochrome oxidase where reduction (in anaerobic conditions) is very much slower than the known rate of transfer of electrons from reduced donor to oxygen [19].

Treatment of fraction 4 with *p*-chloromercuribenzoate, an inhibitor of thiol groups, abolished the O_2^--forming activity (Table II) and raised the $E_{m\,7.0}$ to −175 mV. The function of cytochrome *b* as oxidase can be attributed to its characteristic E_m, which is markedly lower than that of other *b*-type cytochromes [9]. On the basis of our data, it is possible to suppose that the inhibitory effect of *p*-chloromercuribenzoate on the oxidase activity is due to the shift induced in the E_m. This would suggest also that one or more SH groups are important in the function of cytochrome *b*, either by direct involvement in electron transfer, or by playing a role in maintaining the conformation of the active site.

The ratio FAD/cytochrome *b* of 0.42 : 1 found in fraction 4 would indicate that the active oxidase complex contains at least 2 cytochrome molecules for 1 molecule of FAD. This is in contrast with the apparent coincidence of FAD and cytochrome noted by Cross et al. [20], where the ratio was 1 : 1 in membranes and 2 : 1 in phagosomes isolated from human neutrophils. This discrepancy can be explained in different ways. (i) It is possible that our preparation (fraction 4) contains active oxidase complexes with a ratio FAD/cytochrome *b* of 1 : 1, or even 2 : 1, plus other cytochrome *b* molecules not assembled with the active flavoprotein. (ii) Another possible explanation regards the difference in preparations used for the assays. The preparation of oxidase described in the present paper (fraction 4) is enriched in NADPH oxidase and practically devoid of diaphorase. The preparations used by Cross et al. [20] could possibly contain other membrane-bound flavoproteins not implicated in NADPH oxidase activity. We are working on a further purification of the solubilized enzyme to clarify better the structural aspects of the FAD- and cytochrome *b*-containing complex coupled with an NADPH-dependent O_2^--forming activity.

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