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Composition of partially purified NADPH oxidase from pig neutrophils

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The superoxide ($O_2^{\cdot -}$)-forming enzyme NADPH oxidase from pig neutrophils was solubilized and partially purified by gel-filtration chromatography. The purification procedure allowed the separation of NADPH oxidase activity from NADH-dependent cytochrome *c* reductase and 2,6-dichlorophenol-indophenol reductase activities. $O_2^{\cdot -}$-forming activity was co-purified with cytochrome *b*-245 and was associated with phospholipids. However, active fractions endowed with cytochrome *b* were devoid of ubiquinone and contained only little FAD. The cytochrome *b*/FAD ratio was 1.13:1 in the crude solubilized extract and increased to 18.95:1 in the partially purified preparations. Most of FAD was associated with fractions containing NADH-dependent oxidoreductases. These results are consistent with the postulated role of cytochrome *b* in $O_2^{\cdot -}$ formation by neutrophil NADPH oxidase, but raise doubts about the participation of flavoproteins in this enzyme activity.

The production of superoxide ($O_2^{\cdot -}$) radical ions from neutrophils appears to be due to the specific activation of a membrane-bound NADPH oxidase, which is inactive in resting cells (Rossi & Zatti, 1964; Patriarca *et al.*, 1971; Hohn & Lehrer, 1975; Babior *et al.*, 1976). Much information on the nature of this enzyme has been accumulated in recent years, as a consequence of studies on isolated membranes (Cross *et al.*, 1981; Babior *et al.*, 1981; Sloan *et al.*, 1981; Gabig *et al.*, 1982), on solubilized and partially purified preparations of oxidase (Tauber & Goetzl, 1979; Babior & Peters, 1981; Light *et al.*, 1981; Wakeyama *et al.*, 1982; Bellavite *et al.*, 1983*a*; Gabig & Lefker, 1984) and on patients affected by chronic granulomatous disease, a hereditary condition where the $O_2^{\cdot -}$ formation by phagocytes is lacking (Segal *et al.*, 1978; Tauber *et al.*, 1983; Gabig, 1983). There is a substantial agreement that NADPH oxidase contains FAD (Babior & Peters, 1981; Light *et al.*, 1981; Cross *et al.*, 1982*b*; Michell, 1983) and a cytochrome *b*-245 (Cross *et al.*, 1981; Gabig *et al.*, 1982; Borregaard *et al.*, 1982; Bellavite *et al.*, 1983*a*; Morel & Vignais, 1984) and that it needs phospholipids for optimal activity (Gabig & Babior, 1979). Other authors indicated also ubiquinone as a possible oxidoreduction component (Crawford & Schneider, 1982, 1983), but this was not confirmed by others (Cross *et al.*, 1983). However, true evidence of the participation of individual components in the $O_2^{\cdot -}$-generating system is lacking, because attempts to isolate and purify the oxidase in an active state have been unsuccessful. The main reason for this is the marked instability of the enzyme activity on detergent extraction (Patriarca *et al.*, 1974; Tauber & Goetzl, 1979; Babior & Peters, 1981; Light *et al.*, 1981; Bellavite *et al.*, 1983*a*; Gabig, 1983). Moreover, the respiratory burst of neutrophils is reversible when the stimulatory agent is removed (Rossi *et al.*, 1983), indicating that the return of the NADPH oxidase to the resting state can easily occur.

In spite of many attempts made in recent years in our laboratory (Bellavite *et al.*, 1983*a*,*b*; Serra *et al.*, 1983), we could not achieve complete purification. However, in the course of these studies, we were able to solubilize the $O_2^{\cdot -}$-forming enzyme from pig neutrophils with minimum loss of activity and to obtain a partially purified preparation by gel-filtration chromatography. The analysis of such a preparation, with special regard to cytochrome *b*, FAD, quinone and phospholipid content, is reported in the present paper.

Abbreviation used: DCIP, 2,6-dichlorophenol-indophenol.

Experimental

Materials

Ficoll 400 was purchased from Pharmacia Fine Chemicals, Uppsala, Sweden. Dextran (M_r 300000) was purchased from Serva Feinbiochemica, Heidelberg, West Germany. Phorbol-12-myristate-13-acetate, phenylmethanesulphonyl fluoride, di-isopropyl phosphorofluoridate, cytochrome *c* (type VI), DCIP, NADPH (type III), NADH (type III), Lubrol PX and sodium deoxycholate were purchased from Sigma Chemical Co., St. Louis, MO, U.S.A. Sodium deoxycholate was recrystallized from ethanol (MacLennan, 1971). Ultrogel AcA-22 was purchased from LKB Produkter, Bromma, Sweden. Human CuZn superoxide dismutase was a gift from Dr. J. V. Bannister (Department of Inorganic Chemistry, University of Oxford, Oxford, U.K.). Ubiquinone-10 was a gift from Dr. W. T. Griffiths (Department of Biochemistry, University of Bristol, Bristol, U.K.). All other reagents were the highest grade available.

Isolation of neutrophils

Fresh pig blood, made incoagulable with 0.8% sodium citrate and 20mM-EDTA, was transferred to plastic cylinders, and dextran was added at the final concentration of 2.5% (w/v). After sedimentation of erythrocytes, the supernatant was centrifuged at 500*g* for 10min. Contaminating erythrocytes were lysed by suspending the cell pellet in 0.2% NaCl for 1min. Hypo-osmotic and hyperosmotic (1.2% NaCl) solutions employed for the haemolytic treatment contained 2mM-EDTA and 0.5i.u. of heparin/ml. Usually two to four cycles of hypo-osmotic treatment were necessary in order to remove all erythrocytes. Leucocytes were suspended in Krebs–Ringer phosphate buffer (122mM-NaCl/4.8mM-KCl/1.2mM-$MgCl_2$/17mM-sodium phosphate buffer, pH7.4) containing 5mM-glucose, 2mM-NaN_3, 2mM-EDTA and 0.5i.u. of heparin/ml (KRP medium), at the concentration of about 10^9 cells/ml. Any macroscopic aggregates and fibrin clusters were removed by filtration on gauze. Leucocyte suspension was layered on a Ficoll solution (5.7% Ficoll 400, 9% sodium diatrizoate, 0.2mM-EDTA) and centrifuged at 500*g* for 20min at room temperature. The pellet, containing more than 95% pure neutrophils, was suspended in ice-cold KRP medium at a concentration of about 10^9 cells/ml and treated with 3mM-di-isopropyl phosphorofluoridate for 5min. Neutrophils were then washed twice and finally suspended in KRP medium.

Cell activation and preparation of subcellular particles

Neutrophils were activated by exposing the cell suspension (5×10^7 cells/ml) to 1μg of phorbol-12-myristate-13-acetate/ml for 5min at 37°C with stirring. Preliminary experiments demonstrated that this treatment caused the maximum possible activation of the respiratory burst of neutrophils. The incubation was stopped by addition of an excess of ice-cold KRP medium and immediately centrifuged. After this point, all experimental steps were performed at 0–4°C. Non-activated (resting) neutrophils were centrifuged without previous exposure to phorbol-12-myristate-13-acetate. The pellet was resuspended in 10mM-Tris/HCl buffer, pH7.0, containing 0.34M-sucrose, 2mM-NaN_3, 2mM-EDTA and 1mM-phenylmethanesulphonyl fluoride, and sonicated with two to four bursts of 10s at 100W, until 90% of cells were disrupted. The homogenate was centrifuged at 400*g* for 10min in order to sediment nuclei and unbroken cells. Then 2vol. of the supernatant was layered on 1vol. of 10mM-Tris/HCl buffer, pH7.0, containing 0.68M-sucrose, 2mM-NaN_3, 2mM-EDTA and 1mM-phenylmethanesulphonyl fluoride and centrifuged at 100000*g* for 1h. The pellet, containing the subcellular particles, was suspended in 10mM-sodium phosphate buffer, pH8.0, containing 20% (v/v) glycerol, 1mM-phenylmethanesulphonyl fluoride, 2mM-NaN_3, 1mM-EGTA and 1mM-$MgSO_4$ (glycerol/phosphate buffer) at a concentration of 10mg of protein/ml.

Solubilization of subcellular particles and gel filtration

To the subcellular particles 0.4% Lubrol PX and 0.4% sodium deoxycholate were added. The mixture was kept at 0°C for 10min under magnetic stirring, then sonicated with three bursts of 20s at 100 W. The detergent-treated particles were centrifuged at 100000*g* for 1h. Then 10ml of the 100000*g* supernatant was immediately applied to an Ultrogel AcA-22 column (3.8cm × 16cm) equilibrated with glycerol/phosphate buffer. Fractions of volume 4ml were collected at a flow rate of 1ml/min.

Assays

Assays of enzyme activity were carried out with a Perkin–Elmer 576 double-beam spectrophotometer at 22°C. Reference and sample cuvettes contained 50mM-Hepes [4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid]/NaOH buffer, pH7.0, 1mM-diethyltriaminepenta-acetic acid, 2mM-NaN_3, 0.15mM-NADPH or 0.15mM-NADH and the appropriate electron acceptor. The final volume was 1ml. NAD(P)H–cytochrome *c* reductase activities were measured in the presence of 80μM-cytochrome *c* and of 60μg of superoxide dismutase. The reaction was started by adding the enzyme to the sample cuvette and an equal volume of glycerol/phosphate buffer to the reference

cuvette, and the absorption increase at 550nm was monitored. Cytochrome *c* reduction was calculated by using the absorption coefficient $21.1\,mM^{-1}\cdot cm^{-1}$ (Van Gelder & Slater, 1962). NAD(P)H oxidase activity was measured as $O_2^{\cdot-}$ production by the rate of cytochrome *c* reduction measured in the absence of superoxide dismutase minus the rate of NAD(P)H–cytochrome *c* reductase activity (Babior *et al.*, 1976). NADH–DCIP reductase activity was measured in the presence of 60 μM-DCIP and of 60 μg of superoxide dismutase, and the reaction was started by adding the enzyme to the reference cuvette and an equal volume of glycerol/phosphate buffer to the sample cuvette. Since DCIP is decolorized on reduction, the enzyme-catalysed reaction in the reference cuvette caused a net increase of the differential absorbance, which was recorded at 600nm. DCIP reduction was calculated by using the absorption coefficient $16.1\,mM^{-1}\cdot cm^{-1}$ (Dawson *et al.*, 1969) and is expressed in terms of e^-/min, on the basis that 1mol of DCIP is reduced by 2equiv. of e^-.

Cytochrome *b* was measured by reduced-minus-oxidized difference spectroscopy by using the absorption coefficient ($\varepsilon_{559}-\varepsilon_{540}$) $21.6\,mM^{-1}\cdot cm^{-1}$ (Cross *et al.*, 1982*a*).

Flavins were extracted by heating the sample at 100°C for 5min before precipitating the protein with ice-cold 0.6M-$HClO_4$. After centrifugation at 100000*g* for 2h, the supernatant was adjusted to pH7.5 with 5M-KOH and with addition of Na_2HPO_4 to the final concentration of 0.1M. The white precipitate ($KClO_4$) was removed by centrifugation at 1000*g* for 10min. FAD and FMN were assayed fluorimetrically by the method of Faeder & Siegel (1976).

Ubiquinone-10 was extracted with light petroleum (b.p. 50–60°C) as described by Redfearn (1967) and was measured by u.v. absorption at 275nm after purification by reverse-phase high-pressure liquid chromatography on a 200mm Apex-ODS silica (pore size 5 μm) column eluted with acetonitrile/diethyl ether (4:1, v/v), with ubiquinone-10 as standard. Standard ubiquinone-10 was determined by oxidized-minus-reduced difference spectroscopy by using the absorption coefficient ($\varepsilon_{275,ox.}-\varepsilon_{275,red.}$) $12.25\,mM\cdot cm^{-1}$ (Redfearn, 1967).

Proteins were measured, after trichloroacetic acid precipitation, by the method of Lowry *et al.* (1951), with albumin as standard.

Phospholipid determination was carried out after extraction with chloroform/methanol (2:1, v/v). Phosphate was determined by the method of Bartlett (1959).

Results

Solubilization of NADPH oxidase, cytochrome b and FAD

Solubilization of membrane-bound enzymes is often complicated by loss of catalytic activity, and NADPH oxidase in particular is known to be unstable on detergent extraction. Preliminary experiments were therefore performed in order to optimize the solubilization procedure. The best results (i.e. maximum solubilization with minimum enzyme inactivation) were obtained by treating the subcellular particles, suspended in glycerol-containing buffer, with a mixture of 0.4% Lubrol PX and 0.4% sodium deoxycholate. As shown in Table 1, in these conditions the treatment of subcellular particles with detergent caused a slight increase in the specific activity of the $O_2^{\cdot-}$-forming enzyme, due probably to a better accessibility of substrates and of cytochrome *c* to the

Table 1. *Solubilization of NADPH oxidase, of cytochrome b, of FAD and of proteins from subcellular particles of phorbol-12-myristate-13-acetate-activated neutrophils*

Subcellular particles were treated with 0.4% Lubrol PX and 0.4% deoxycholate as described in the Experimental section and were centrifuged at 100000*g* for 1h. NADPH oxidase activity and cytochrome *b*, FAD and protein contents of various fractions were assayed as described in the Experimental section. 'Specific' refers to enzyme activity or cytochrome *b*, FAD or protein content/mg of protein. 'Total' refers to total activity or content obtained in the fraction from 10^9 neutrophils. The '%' values concern the total activity or content of the fraction with respect to the total activity or content of subcellular particles (treated). The means ± S.E.M. for three separate experiments are reported.

	Subcellular particles	Subcellular particles (treated)		100000*g* supernatant			100000*g* pellet		
	Specific	Specific	Total	Specific	Total	%	Specific	Total	%
NADPH oxidase (nmol of $O_2^{\cdot-}$/min)	41.3±7.2	58.6±15.0	621.2	54.9±14.9	323.9	52.1	29.9±12.1	170.4	27.4
Cytochrome *b* (pmol)	112.4±8.3	125.3±20.1	1328.2	121.9±19.9	719.2	54.1	85.4±6.0	468.8	36.6
FAD (pmol)	79.2±11.7	80.5±10.5	853.2	107.1±15.6	631.9	74.0	54.8±6.6	312.3	36.6
Protein (mg)	–	–	10.6±0.7	–	5.9±0.8	55.7	–	5.7±0.8	53.8

solubilized enzyme. No modifications of cytochrome *b* and of FAD content were caused by detergents. After ultracentrifugation, the supernatant contained about 50% of protein, of NADPH oxidase and of cytochrome *b*. The total recovery of enzyme activity (supernatant + pellet) was 79.5%, indicating a small loss of activity. The supernatant contained 74% of the FAD present in subcellular particles. Solubilized FAD was protein-bound, i.e. in the form of flavoprotein, since after chromatography of the 100000*g* supernatant on Sephadex G-25 at least 85% of FAD was co-eluted together with the protein peak in the void volume (results not shown).

Gel filtration of the solubilized extract

The solubilized preparation was filtered through an Ultrogel AcA-22 column, and the chromatographic profile shown in Fig. 1 was obtained. Proteins (Fig. 1*a*) were separated into two regions: a smaller peak was eluted in the void volume, whereas most of the proteins appeared as a large peak in the included volume. When the absorbance at 280nm of the fractions was measured (Fig. 1*b*), the peak eluted in the void volume appeared to be relatively greater than the protein peak measured with the Lowry method (Fig. 1*a*). This indicates the presence of other non-protein compounds absorbing in the u.v. These components were identified as phospholipids by measuring the phosphorus content. The first peak of gel filtration contained 1.34mg of phospholipid/mg of protein, a value much higher than that of the second peak (0.10mg of phospholipid/mg of protein) and that of the 100000*g* supernatant (0.15mg of phospholipid/mg of protein).

Fig. 1(*c*) shows that NADPH oxidase activity was eluted as a major peak in the void volume and that a smaller peak of activity was retained by the resin. A similar chromatographic behaviour was exhibited by cytochrome *b* (Fig. 1*e*), with the only difference that the second peak was relatively greater than the second peak of oxidase activity.

Superoxide dismutase-insensitive NADPH-dependent cytochrome *c*-reducing activity (NADPH–cytochrome *c* reductase, Fig. 1*d*) was very low in all fractions, and it was mostly represented in the second peak. The activities of superoxide dismutase-insensitive NADH-dependent cytochrome *c* reductase and DCIP reductase were much higher and easier to measure than the corresponding activities measured with NADPH as substrate. The elution profile of one of these diaphorase enzymes is reported in Fig. 1(*f*): the bulk of NADH–DCIP reductase activity was found in the included volume of the chromatographic fractionation, but a small contamination by this enzyme was still present in the first peak. A similar profile was obtained by measuring NADH–cytochrome *c* reductase activity (results not shown).

Enzyme activities, cytochrome b and FAD content of the two peaks separated by gel filtration

In the subsequent chromatographic experiments after gel filtration all the fractions were divided into two groups, according to the two main peaks measured as absorbance at 280nm, and the various samples were pooled. For example, in the experiment shown in Fig. 1 the pool of the first peak would have been composed of fractions 11–16, the pool of the second peak of fractions 17–40. Table 2 reports the enzyme specific activities and the cytochrome *b* and FAD specific content of the two peaks compared with those of the 100000*g* supernatant loaded on the column. NAD(P)H-dependent $O_2^{\cdot -}$-forming activity present in the first peak was purified 4–5-fold with respect to the 100000*g* supernatant. As expected, the activity was much higher with NADPH than with NADH as substrate. A very similar purification was observed as regards the cytochrome *b*, so that the oxidase/cytochrome *b* ratio in the partially purified fraction remained very similar to the ratio in the unfractionated supernatant. NADPH–cytochrome *c* reductase activity of the first peak increased only 1.5-fold, whereas NADH–cytochrome *c* reductase and NADH–DCIP reductase activities markedly decreased. Since the exclusion limit of Ultrogel AcA-22 resin is of about 1.2×10^6 daltons, these results indicate that the chromatography allowed the separation of high-molecular-mass complexes, enriched in phospholipids, NADPH oxidase activity and cytochrome *b*, from oxidoreductase enzymes that are in a more soluble form and are retained by the resin. Unlike the $O_2^{\cdot -}$-forming enzyme, these oxidoreductases use NADH as preferential electron donor.

Unexpected results were obtained on measurement of the flavin content. The first peak contained little FAD, so that its specific content decreased from 117.5pmol/mg of protein in the 100000*g* supernatant to 35.7pmol/mg of protein in the fractions where NADPH oxidase activity and cytochrome *b* concentration were found to be increased. As a consequence, the cytochrome *b*/FAD ratio increased from 1.13:1 to 18.95:1 and the NADPH oxidase/FAD ratio increased from 0.31:1 to 4.20:1. In contrast, the NADH–cytochrome *c* reductase/FAD ratio remained almost constant, indicating a similar distribution in the chromatographic separation. Tables 1 and 2 do not report the values of FMN determination because this flavin cofactor was present only in trace amounts (less than 10–20% of total flavins) and then not in all preparations tested, so that its

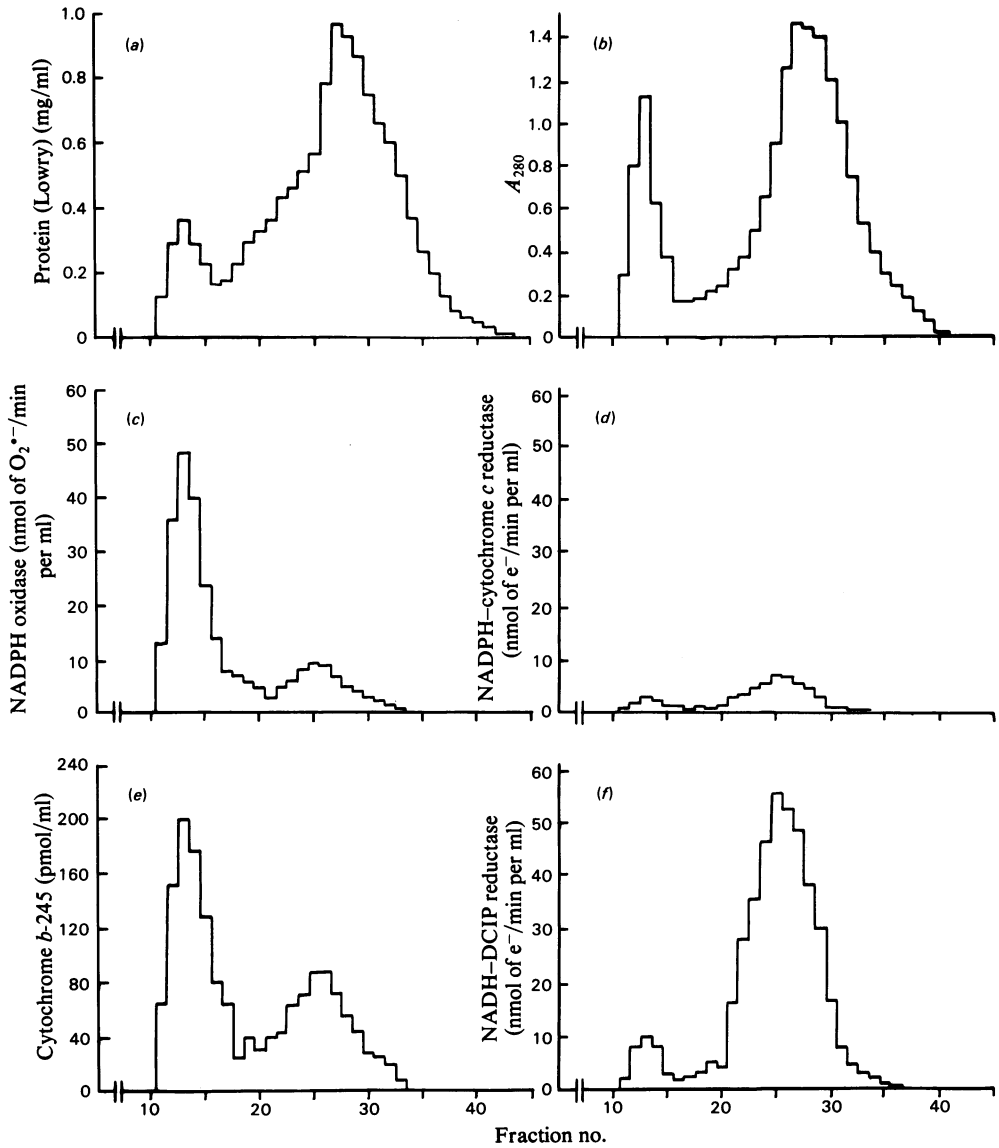

Fig. 1. *Gel filtration of the solubilized extract of subcellular particles from phorbol-12-myristate-13-acetate-activated pig neutrophils*

A 10ml portion of the 100000*g* supernatant was chromatographed on an Ultrogel AcA-22 column, and 4ml fractions were collected and assayed as described in the Experimental section. The 100000*g* supernatant contained 6mg of protein/ml and 750pmol of cytochrome *b*/ml, and the enzyme activities were: NADPH oxidase, 220nmol of $O_2^{\cdot -}$/min per ml; NADPH–cytochrome *c* reductase, 64nmol of e^-/min per ml; NADH–DCIP reductase, 280nmol of e^-/min per ml.

presence could be attributed to breakdown of FAD during the extraction procedure. In order to check if the finding of a small amount of FAD in the first peak was due to incomplete extraction, in some experiments 0.5% deoxycholate was added during the heat-extraction of flavins. No increase of FAD measured in the first peak was caused by the detergent, indicating that the presence of phospholipids does not interfere with the extraction procedure.

In some experiments the proteinase inhibitor phenylmethanesulphonyl fluoride was omitted from the glycerol/phosphate buffer and the flavin extraction was carried out after the treatment of the samples with 5mg/ml of trypsin at 37°C for 1h. Trypsin treatment did not significantly change the

Table 2. *NAD(P)H oxidase, NAD(P)H–cytochrome c reductase and NADH–DCIP reductase activities and cytochrome b, flavin and protein contents in various fractions obtained from phorbol-12-myristate 13-acetate-activated neutrophils*
The 100000*g* supernatant from phorbol-12-myristate-13-acetate-activated neutrophils was chromatographed on an Ultrogel AcA-22 column, and the fractions of the two main protein peaks were pooled as described in the text. Assays were carried out as described in the Experimental section. Specific activities and contents are expressed as follows: NADPH oxidase and NADH oxidase, nmol of $O_2^{\cdot -}$/min per mg of protein; NADPH–cytochrome *c* reductase, NADH-cytochrome *c* reductase and NADH–DCIP reductase, nmol of e^-/min per mg of protein; cytochrome *b* and FAD, pmol/mg of protein; protein, mg of protein recovered in fraction from 10^9 neutrophils. Recovery values concern the sum of total activity or content of the first and second peaks with respect to total activity or content of 100000*g* supernatant. The means±S.E.M. for four separate experiments are reported.

	Specific activity or content			
	100000*g* supernatant	First peak from gel filtration	Second peak from gel filtration	Recovery (%)
NADPH oxidase	36.6±6.1	150.0±30.2	9.5±1.9	57.6
NADH oxidase	10.5±2.3	58.6±18.3	1.0±0.9	55.7
NADPH–cytochrome *c* reductase	9.8±5.1	14.4±5.7	3.6±0.4	44.8
NADH–cytochrome *c* reductase	96.8±28.5	29.2±7.7	128.9±30.4	119.9
NADH–DCIP reductase	76.5±9.5	49.9±16.2	58.3±9.2	72.7
Cytochrome *b*	133.1±18.4	676.8±4.6	59.8±9.8	82.6
FAD	117.5±12.2	35.7±5.9	122.3±3.5	94.3
Protein	5.9±0.8	0.5±0.1	5.2±0.3	96.6
NADPH oxidase/cytochrome *b* ratio	0.27	0.22	0.16	
Cytochrome *b*/FAD ratio	1.13	18.95	0.49	
NADPH oxidase/FAD ratio	0.31	4.20	0.08	
NADH–cytochrome *c* reductase/FAD ratio	0.82	0.82	1.05	

Table 3. *NAD(P)H oxidase, NAD(P)H–cytochrome c reductase and NADH–DCIP reductase activities and cytochrome b, flavin and protein contents in various fractions obtained from resting neutrophils*
The 100000*g* supernatant from resting neutrophils was chromatographed on an Ultrogel AcA-22 column, and the fractions of the two main protein peaks were pooled as described in the text. Assays were carried out as described in the Experimental section. Specific activities and contents are expressed in the units indicated in Table 2 legend. The values of two separate experiments are reported.

	Specific activity or content		
	100000*g* supernatant	First peak from gel filtration	Second peak from gel filtration
NADPH oxidase	0.9–1.7	0.8–3.9	1.1–0.9
NADH oxidase	0.7–0.7	5.4–1.7	0.8–0.8
NADPH–cytochrome *c* reductase	4.4–9.1	10.4–8.4	4.3–5.7
NADH–cytochrome *c* reductase	71.6–119.5	31.7–38.9	98.8–117.8
NADH–DCIP reductase	55.2–65.5	21.1–26.9	39.3–55.1
Cytochrome *b*	97.3–127.0	698.8–934.5	51.7–65.3
FAD	89.1–94.4	31.5–51.7	82.8–88.0
Protein	8.3–9.4	0.6–0.5	9.3–9.7

amount of extracted FAD, indicating that flavin is non-covalently bound to the proteins.

Table 3 reports the results of two chromatographic fractionations carried out on preparations obtained from non-stimulated neutrophils. NAD(P)H oxidase activity was practically undetectable, whereas the rates of superoxide dismutase-insensitive cytochrome *c* reduction and DCIP reduction were in the same range as those measured in fractions obtained from phorbol-12-myristate-13-acetate-activated neutrophils. Cytochrome *b* and FAD distribution in the two peaks obtained from gel filtration did not change with respect to the distribution observed in Table 2.

Ubiquinone content

Fig. 2 shows the high-pressure-liquid-chromatographic profiles of extracts of subcellular particles and of the first peak obtained from Ultrogel AcA-22 chromatography. Ubiquinone content was very low, even in the subcellular particles (about 20pmol/mg of protein). For this reason in this experiment the highest possible sensitivity had to be used, causing the 'noise' present in the reported

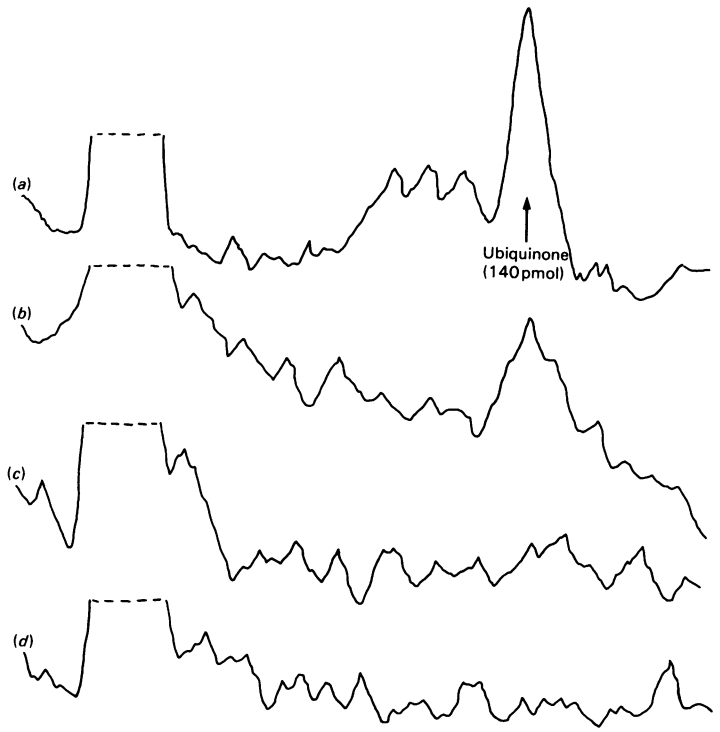

Fig. 2. *High-pressure-liquid-chromatography recording traces of ubiquinone analysis*
Light-petroleum extracts of ubiquinone-10 standard (140pmol) (*a*), of subcellular particles from activated neutrophils (4.4mg of protein, 460pmol of cytochrome *b*) (*b*), of the first peak from Ultrogel AcA-22 chromatography of resting preparations (1.0mg of protein, 643pmol of cytochrome *b*) (*c*) and of the first peak from Ultrogel AcA-22 chromatography of activated preparations (0.8mg of protein, 514pmol of cytochrome *b*) (*d*) were fractionated by high-pressure liquid chromatography as described in the Experimental section, and the absorbance at 275nm of the eluate was monitored.

traces. Ubiquinone in the first peak was undetectable. Considering the sensitivity limits of the method and the amount of sample extracted, it can be calculated that the ubiquinone/cytochrome *b* ratio in this fraction is certainly less than 1:20.

Discussion

In spite of the central role played by NADPH oxidase in the generation of toxic radicals by phagocytes, the structure and the components of this enzyme (or enzymic system) are not completely defined. The main reason for this is the lack of complete purification of the enzyme. At present, information on the nature of NADPH oxidase can be obtained by functional studies or by analysing partially purified enzyme as compared with non-purified preparations. This latter procedure was adopted in the present work.

Sound evidence of the participation of some component in the $O_2^{\cdot -}$-forming system can be obtained only by studying enzyme preparations that are endowed with their full $O_2^{\cdot -}$-forming activity. Care was therefore taken in order to solubilize and isolate the enzyme with minimum inactivation. Satisfactory results were obtained by subjecting the solubilized extract to gel-filtration chromatography on Ultrogel AcA-22 (Tables 1 and 2). The method allowed the isolation of an high-molecular-mass complex where the radical-generating oxidase was purified 4–5-fold with respect to the non-fractionated preparation (Table 2). Probably the high catalytic activity of this complex was preserved by the presence of phospholipids, which are necessary for the activity of NADPH oxidase (Gabig & Babior, 1979).

The peak containing the $O_2^{\cdot -}$-forming activity was separated by gel filtration from a second peak containing most of NAD(P)H–cytochrome *c* reductase and NADH–DCIP reductase activity (Fig. 1). This separation indicates that superoxide dismutase-insensitive reduction of electron acceptors such as cytochrome *c* and DCIP is catalysed by enzymes not involved in the $O_2^{\cdot -}$ formation. Two other observations support this conclusion: (*a*) the superoxide dismutase-insensitive oxidoreductase

activities use NADH as preferential substrate and (*b*) they are not activated in fractions obtained from phorbol-12-myristate-13-acetate-treated cells (compare Tables 2 and 3). The physiological role of these diaphorase enzymes in the neutrophil is not known.

Oxidase activity co-purified with cytochrome *b*, and this is a further indication that this cytochrome, whose particular feature is low midpoint potential ($E_{m,7.0} = -245$mV) (Cross *et al.*, 1981), is strictly related to the $O_2^{\cdot -}$-generating electron-transport system.

In addition to cytochrome *b*, it has been suggested that a quinone and FAD may be involved in the radical-generating oxidase. We therefore measured the amount of these cofactors in the partially purified preparations. The quinone analysis was not able to demonstrate the presence of quinone in the oxidase–cytochrome *b*–phospholipid complex. Traces of ubiquinone were found in the subcellular particles. The discrepancy with the results of other authors (Crawford & Schneider, 1982, 1983) may be explained in terms of purity of the preparation. As reported by Cross *et al.* (1983), the quantity of ubiquinone in neutrophils is very low (25.5pmol/mg of cell protein) and it is associated with the mitochondrial fraction. In the present investigation the isolation of neutrophils by a method that allowed us to obtain cell preparations at least 95% pure, the solubilization of cell-free particles and the gel filtration gave an active enzyme fraction practically free of quinones. This excludes the participation of these cofactors from the $O_2^{\cdot -}$-forming activity of neutrophils and agrees with the results obtained by Cross *et al.* (1983) with pure membrane fractions.

Unexpectedly, the FAD content of the purified oxidase preparation was very low as compared with the cytochrome *b* content. Since no doubts on the presence of a flavoprotein in the system have been raised until now, this point deserves a special comment. First of all, we should rule out possible trivial explanations such as inadequate extraction and loss of flavin during the experiment. Inadequate extraction of FAD can be excluded for the following reasons. (*a*) The amount of FAD extracted from non-purified fractions was about 100pmol/mg of protein and the cytochrome *b*/FAD ratio was about 1:1. These values are in the same range as those previously reported by us and by others for crude cell fractions (Cross *et al.*, 1982*b*; Gabig, 1983; Bellavite *et al.*, 1983*b*). (*b*) Addition of deoxycholate during flavin extraction did not increase the amount of measured FAD. (*c*) Total recovery of flavins from gel-filtration chromatography was very good (Table 2). Loss of FAD from the enzyme during solubilization and during the chromatography can be excluded, because it should have been followed by loss of activity. A certain inactivation during the experimental steps was unavoidable, as indicated by the incomplete recovery of $O_2^{\cdot -}$-forming activity after gel filtration (Table 2). However, the decay of activity cannot be compared with the decrease of FAD concentration during purification. In fact, assuming that the cytochrome *b* is a structural component of the enzyme, the NADPH oxidase/cytochrome *b* ratio could be taken as a parameter of the functional integrity of the enzyme. As indicated by Table 2, this ratio only slightly decreased in the partially purified preparation with respect to the 100000*g* supernatant (0.22 and 0.26 respectively), whereas the cytochrome *b*/FAD and NADPH oxidase/FAD ratios changed by a factor of 15-fold. Moreover, the NADPH oxidase/cytochrome *b* ratio in the first peak of gel filtration was greater than in the second peak (Table 2 and Fig. 1), indicating that the inactivation affected the portion of the oxidase that was retained by the resin more than the portion that was excluded.

The most logical explanation of the distribution of FAD after gel filtration could be found by looking at the distribution of cytochrome *c* oxidoreductase and DCIP oxidoreductase. These enzymes underwent a chromatographic separation that was practically superimposable on that of FAD, and the NADH–cytochrome *c* reductase/FAD ratio remained constant in all the fractions. Since it is known that diaphorase enzymes are flavoproteins (Mahler & Cordes, 1969), it is conceivable that most of the FAD present in the various fractions is associated with these enzymes.

What about the small amount of FAD remaining in the first peak after gel filtration? It could be attributed either to a bimodal distribution of FAD in the column fractions, or to spreading of FAD of the included peak. Our data do not allow us to distinguish between these two possibilities, because the sensitivity of the flavin assay did not permit accurate measurements of the FAD content of each column fraction. However, since the distribution of superoxide dismutase-insensitive oxidoreductases is clearly bimodal (Fig. 1), it is conceivable that at least part of FAD present in the first peak is attributable to the small contamination by these flavoproteins. However, it cannot be excluded that a small portion of FAD is associated with the $O_2^{\cdot -}$-forming system. If this is the case, one should hypothesize that the NADPH oxidase of neutrophils is formed by a flavoprotein surrounded by a number (20 or more) of cytochrome *b* molecules. Such a system could work as proposed for the interaction between cytochrome *P*-450 and the cytochrome *P*-450 reductase, whose ratio was found to be 10–25:1 (Yang, 1977; Gut *et al.*, 1983). One could speculate that in neutrophil membrane

only a minimum fraction (5%) of the cytochrome *b* is bound to the flavoprotein in a functional complex, whereas the bulk of cytochrome *b* is a non-active (not bound) form. However, this does not mean that the activation process consists in the aggregation between a flavoprotein and a cytochrome *b* molecule, because our data (Tables 2 and 3) indicate that the FAD and cytochrome *b* contents in complexes isolated from resting cells and from activated cells are very similar.

Of course, the results presented in this paper do not exclude the alternative explanation, i.e. that NADPH oxidase does not contain FAD as prosthetic group. This conclusion contrasts with many reports suggesting the participation of a flavoprotein in the radical-generating oxidase (Babior & Peters, 1981; Light *et al.*, 1981; Cross *et al.*, 1982*b*; Michell, 1983). However, these reports are based, not on the analysis of purified enzyme, but on the effect of stimulatory (FAD) or inhibitory (FAD analogues, quinacrine) compounds (Babior & Peters, 1981; Light *et al.*, 1981; Bellavite *et al.*, 1983*b*), and the absolute specificity of these effects remains to be demonstrated. Cross *et al.* (1982*b*) reported a cytochrome *b*/FAD ratio of 1:1 or even of 0.5:1, but the data refer to whole membranes or phagocytic vesicles respectively. In a previous report from this laboratory (Bellavite *et al.*, 1983*b*) we indicated a cytochrome *b*/FAD ratio of 2.4:1, but on a much-less-purified preparation. More recently Gabig & Lefker (1984) 'resolved' the 'flavoprotein and the cytochrome *b* components of the NADPH-dependent $O_2^{\cdot -}$-generating oxidase', but, since they analysed non-purified preparations solubilized from membranes, it is impossible to say what portion of the solubilized FAD was due to the presence of other flavoproteins, different from the NADPH oxidase.

In recent work (Bellavite *et al.*, 1984) we obtained evidence that NADPH oxidase does not reduce DCIP, thus confirming data from another laboratory (Babior & Peters, 1981), and this is a very anomalous behaviour for a flavoprotein. In conclusion, the present study, based on partially purified preparation of oxidase, excluded the participation of quinones and raised many doubts on the flavoprotein nature of the oxidase. In any case, the cytochrome *b*/FAD ratio in the $O_2^{\cdot -}$-forming system should be much different from that previously reported. Probably the final answer will be given when completely pure and fully active oxidase will be available.

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