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STUDIES ON THE NATURE AND ACTIVATION OF O_2^--FORMING NADPH OXIDASE OF LEUKOCYTES. IDENTIFICATION OF A PHOSPHORYLATED COMPONENT OF THE ACTIVE ENZYME

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Highly active superoxide (O_2^-)-forming NADPH oxidase was extracted from plasmamembranes of phorbol-12-myristate-13-acetate-activated pig neutrophils and was partially purified by gel filtration chromatography. Oxidase activity copurified with cytochrome b_{-245} in an aggregate containing phospholipids and was almost completely separated from FAD and NAD(P)H-cytochrome *c* reductase. A polypeptide with molecular weight of 31,500 strictly paralleled the purification of NADPH oxidase, suggesting that it is a major component of the enzyme. The enzyme complex was then dissociated by high detergent and salt concentration and cytochrome b_{-245} was isolated by a further gel filtration chromatography, with a 147 fold purification with respect to the initial preparation. The cytochrome b_{-245} showed a 31,500 molecular weight by SDS electrophoresis, indicating that it is actually the component previously identified in the partially purified enzyme. The 31,500 protein was phosphorylated in enzyme preparations from activated but not from resting neutrophils, suggesting that phosphorylation of cytochrome b_{-245} is involved in the activation mechanism of the O_2^--forming enzyme responsible for the respiratory burst in phagocytes.

Key words: Leukocyte metabolism, Neutrophil, NADPH oxidase, Cytochrome b_{-245}, Superoxide, Protein phosphorylation.

Abbreviations used: PMA, phorbol-12-myristate-13-acetate; PMSF, phenylmethansulphonyl fluoride; SDS, sodium dodecyl sulfate; EDTA, ethylendiaminotetraacetic acid; EGTA, ethylene glicol-bis (β-amino-ethyl ether), N,N,N′,N′-tetraacetic acid.

INTRODUCTION

The production of superoxide anion (O_2^-) and of other oxygen metabolites by neutrophils forms part of the mechanism by which these cells kill microorganisms and tumour cells[1,2]. The enzymatic system responsible for O_2^- forming activity is a plasmamembrane-bound NADPH oxidase which is dormant in resting cells but becomes activated when neutrophils are exposed to phagocytosable particles or to suitable stimuli[3,4,5]. In spite of the central role played by this enzyme in host defence system, its structure and activation mechanism remain to be clarified. Complete characterization

of NADPH oxidase has been hampered by rapid loss of activity following detergent solubilization[6,7,8,9]. A series of evidences indicates that various components such as flavoproteins, a low potential cytochrome *b* (cytochrome b_{-245}) and possibly quinones are involved in the enzymatic activity catalyzing the transport of electrons from NADPH to oxygen. The participation of FAD in the enzymatic activity has been suggested on the basis of indirect evidences[8,9,10,11], but this participation has been questioned by the recent demonstration in our laboratory that the content of FAD in the partially purified enzyme is very low[12]. The involvement of cytochrome b_{-245} has been proposed on the basis of kinetic studies[13,14,15] and of the lack of cytochrome b_{-245} spectrum in neutrophils affected by chronic granulomatous disease, where O_2^- formation does not take place[16].

Previous attempts from this laboratory led to the isolation from guinea pig[17,18,19] and pig[12] neutrophils and from guinea pig macrophages (manuscript in preparation), of an enzymatic complex endowed with high NADPH-dependent O_2^- forming activity and containing substantial amount of phospholipids and of cytochrome b_{-245}. The present paper reports an improved procedure for the isolation of active NADPH oxidase from plasmamembrane of pig neutrophils. The procedure allowed

a) the identification of cytochrome b_{-245} as a polypeptide with apparent molecular weight of 31,500,
b) the demonstration of the involvement of cytochrome b_{-245} as the major component of the oxidase,
c) the new finding that the activation of the oxidase is associated with the phosphorylation of this cytochrome b_{-245}.

MATERIALS AND METHODS

Materials

Ficoll 400 and Octyl Sepharose CL-4B were purchased from Pharmacia Fine Chemicals, Uppsala, Sweden. Dextran (M_r 300,000) was purchased from Serva Feinbiochemica, Heidelberg, West Germany. PMA, PMSF, di-isopropyl phosphorofluoridate, cytochrome *c* (type VI), NADPH (type III), NADH (type III), reactive Red 120-Agarose, Heparin-Agarose, Triton X-100, Lubrol PX and sodium deoxycholate were purchased from Sigma Chemical Co., St. Louis, MO, U.S.A.; sodium deoxycholate was recrystallized from ethanol. Ultrogel AcA34 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.). Zwittergent 3-12 detergent and Zwittergent 3-16 detergent were purchased by Calbiochem-Behring, San Diego, CA, U.S.A. All other reagents were the highest grade available.

Activation of Neutrophils and Isolation of a Plasmamembrane-Rich Subcellular Fraction

Neutrophils from 8 liters of pig blood (30–40 × 10^9) were isolated, treated with 3 mM-di-isopropyl phosphorofluoridate and activated with PMA (1 μg/ml) for 5 min at 37°C as previously reported[12]. After centrifugation, the cells were suspended in ice-

cold 40 mM-Tris/HCl, pH 7.4, 10 mM - EDTA, 1 mM - $MgSO_4$, 2 mM - NaN_3 and 2 mM - PMSF (buffer A) containing 10% sucrose and sonicated with 3 pulses of 5 seconds at 150 watts (Labsonic 1510, B. Brown). All the subsequent steps were performed at 0–4°C. Nuclei and unbroken cells were discarted by centrifuging at 1100 g for 10 min. 15 ml aliquots of the postnuclear supernatant were layered on discontinuous gradients formed by 10 ml of 50% sucrose and 5 ml of 20% sucrose in buffer A and centrifuged at 100,000 g for 60 min. The band between 20% and 50% sucrose (membrane fraction) was collected and stored overnight at −70°C. Once thawed, the 20–50% membrane fraction was treated with two volumes of buffer A containing 0.45 M - NaCl (final concentration of NaCl = 0.3 M) and centrifuged at 10,000 g for 10 min. The pellet was discarded and the supernatant centrifuged at 100,000 g for 90 min. The NaCl - washed membranes pellet was suspended in 50 mM - sodium phosphate buffer, pH 8.0, containing 20% (v/v) - glycerol, 2 mM - EGTA, 1 mM - $MgSO_4$, 2 mM - NaN_3, 2 mM - PMSF (buffer B) at a protein concentration of about 5 mg/ml.

Where the phosphorylation of proteins was investigated, neutrophils were labelled with $^{32}P_i$ before the activation. Cells (350×10^6/ml) were incubated in 30 mM - sodium Hepes buffer, pH 7.4, containing 110 mM - NaCl, 5 mM - KCl, 0.6 mM - $CaCl_2$ and 10 mM - glucose (buffer C) supplemented with 0.11 mC_i/ml of $^{32}P_i$ for 90 min at 37°C under continuous shaking. After centrifugation and washing, the cells were suspended in the same buffer D and activated with PMA as above described. Resting cells were treated only with an equivalent volume of dimethylsulphoxide.

Solubilization and Partial Purification of NADPH Oxidase

NaCl-washed membranes were solubilized with 0.4% (v/v) Lubrol PX and 0.4% (w/v) sodium deoxycholate as described[12] and centrifuged at 100,000 g for 60 min. 4 ml of supernatant were then loaded on an Ultrogel AcA34 column (1.6×30 cm) equilibrated with buffer B containing 0.15% sodium deoxycholate and 0.15% Lubrol PX and eluted with the same buffer. Fractions of 1.5 ml volume were collected at a flow rate of 18 ml/h.

Glycerol Gradient of Partially Purified NADPH Oxidase and Isolation of Cytochrome b_{-245}

3 ml of partially purified NADPH oxidase from AcA34 chromatography were loaded on 12 ml linear gradient of glycerol (22–32% v/v) in buffer B supplemented with 0.15% sodium deoxycholate and 0.15% Lubrol PX and centrifuged at 131,000 g for 16 h with a Bekman SW 28 rotor[10]. Fractions containing cytochrome b_{-245} and NADPH oxidase activity obtained from 4 gradients were pooled, diluted with two volumes of buffer B plus 0.15% sodium deoxycholate and 0.15% Lubrol PX but without glycerol and centrifuged at 100,000 g for 3 h. The pellet was resuspended by sonication in 2 ml of 50 mM - sodium phosphate buffer, pH 8.0, containing 10% (v/v) glycerol, 1% Triton X-100, 0.5% sodium deoxycholate, 1 M - KCl, 1 mM - EGTA and 2 mM - NaN_3 (buffer D). The solution was stirred for 15 min, sonicated with two pulses of 5 seconds at 100 watts and centrifuged at 100,000 g for 3 h. The supernatant was then chromatographed through an AcA34 column (1.6×30 cm) pre-equilibrated with buffer C and eluted with the same buffer at a flow rate of 10 ml/h.

Fractions corresponding to the peak of cytochrome b_{-245} were pooled and Triton X-100, deoxycholate and KCl were removed by filtration on an AcA34 column run in the presence of 50 mM - sodium phosphate buffer, pH 8.0, containing 0.1 M - NaCl, 1 mM - EGTA and 2 mM - NaN_3. This procedure led to a more aggregated form of cytochrome b_{-245} which was pelleted by centrifuging overnight at 100,000 g. The final pellet was resuspended by sonication in buffer B.

SDS - Polyacrylamide Gel Electrophoresis and Autoradiography

Slab gels (13 × 20 × 0.1 cm) consisting of a linear polyacrylamide gradient (4 – 20%) in 40 mM - Tris/HCl buffer, pH 8.0, containing 1% SDS, 1 mM - EDTA and 20 mM - sodium acetate were prepared essentially according to Fairbanks *et al.*[20]. Samples were treated with 2% SDS and 2% (v/v) 2-mercaptoethanol and incubated for 1 h at 50°C in the presence of 2 mM - PMSF. Electrophoresis was run in a vertical apparatus with 40 mM - Tris/HCl buffer, pH 8.0, containing 1% SDS, 1 mM - EDTA and 20 mM - sodium acetate as electrode buffer for 20 min at 80 volt (constant) and for 7–8 h at 150 volt (constant). Gels were stained overnight with 45% methanol, 9% glacial acetic acid and 0.05% Coomassie blue R250 and destained in 10% ethanol, 7% acetic acid. For the autoradiography the gels were equilibrated for 1 h in 40% methanol, 7% acetic acid, essicated with a Gel slab Drier GSD - 4 (Pharmacia) and exposed to X - ray films (Kodak X - OMAT R) in a radiographic cassette for 2–3 days at −70°C. Densitometric profiles of the electrophoretic patterns were performed with an Ultoscan Laser Densitometer (LKB, Bromma, Sweden).

Assays

NADPH oxidase activity was measured as O_2^- generation and NAD(P)H cytochrome *c* reductase by monitoring the reduction of cytochrome *c* as in Reference[12].

Cytochrome b_{-245} was measured by reduced-minus-oxidized difference spectroscopy using the absorption coefficient ($\epsilon 559 - \epsilon 540$) 21.6 mM^{-1} cm^{-1} [14].

FAD was extracted as in reference[12] and assayed fluorimetrically by the method of Faeder and Siegel[21].

Proteins were measured by the method of Lowry modified for lipoproteins according to Markwell *et al.*[22] and by the fluorescamine method[23] where samples contained Triton X-100 that interferes with the Lowry's method.

Phosphate was determined by the method of Bartlett[24] after extraction of phospholipids according to Marche *et al.*[25] with methanol/chloroform/HCl 25% (1/2/0.1). High performance thin layer chromatography of phospholipids was performed according to Imai *et al.*[26].

RESULTS

Partial Purification of Active NADPH Oxidase

Extraction and gel filtration chromatography. Plasmamembranes of PMA - activated neutrophils were purified by sucrose density gradient centrifugation followed by a washing with 0.3 M - NaCl as described in Materials and Methods. The washing with NaCl removed about 50% of proteins from the membranes without

affecting the NADPH oxidase activity and the cytochrome b_{-245} content. Solubilization of the membranes was then performed with a mixture of deoxycholate and Lubrol PX, a "mild" treatment which preserves the NADPH - dependent O_2^- forming activity as previously reported in reference[12]. The solubilized extract was chromatographed through an Ultrogel AcA34 column equilibrated with buffer containing 20% (v/v) glycerol and 0.15% of both deoxycholate and Lubrol PX.

The elution profile of the gel filtration chromatrography is reported in figure 1. The first protein peak contained practically all the NADPH oxidase activity and cytochrome b_{-245}. This peak corresponded to the void volume of the column, indicating that it contained aggregated material with molecular weight of >350,000. This material contained also phospholipids (0.27–0.30 mg/mg of protein). A second broader peak appeared in the subsequent fractions. This peak was almost devoid of cytochrome b_{-245} and NADPH oxidase, while it contained most of FAD and of the diaphorase enzyme NADH cytochrome *c* reductase. Therefore the gel filtration chromatography indicated that the bulk of FAD present in the membranes and in the solubilized extract was not related with the NADPH oxidase/cytochrome b_{-245}/phospholipids active complex. On the other hand, a good correlation between FAD and NADH cytochrome *c* reductase was observed and this is in accord with the flavoprotein nature of this enzyme[27].

Table I reports the specific activity or content and the purification factors of

TABLE I

NADPH dependent O_2^- forming activity, protein, cytochrome b_{-245} and FAD content of the preparations of NADPH oxidase at different stages of purification. The postnuclear supernatant from PMA-activated pig neutrophils was centrifuged on sucrose gradient and the plasmamembrane fraction was collected. After washing with NaCl, membranes were treated with 0.4% Lubrol PX and 0.4% deoxycholate and centrifuged at 100,000 g for 1 h. The supernatant (solubilized extract) was chromatographed through an Ultrogel AcA34 column and the fractions of the two main protein peaks obtained (see Figure 1) were pooled. The purification ratio is the ratio between the specific activity or content of the fraction and the specific activity or content of the postnuclear supernatant.

	Postnuclear supernatant	Washed membranes	Solubilized extract	First peak AcA34	Second peak AcA34
Protein (mg obtained from 10^{10} neutrophils)	331	8.4	5.4	1.2	4.7
NADPH oxidase					
Specific activity[1]	30.8	210	182	572	22
Purification factor	1	6.8	5.9	18.6	0.7
Cytochrome b_{-245}					
Specific content[2]	70.3	617.2	702	1,915	106
Purification factor	1	8.8	9.9	27.2	1.5
FAD					
Specific content[2]	35.6	290	312.5	50	330
Purification factor	1	8.1	8.8	1.4	9.2
NADPH cyt. c reductase					
Specific activity[3]	n.d.	5.2	8.1	4.6	12.6
NADH cyt. c reductase					
Specific activity[3]	n.d.	93	144	8.8	198

[1] nmoles O_2^-/min/mg protein
[2] pmoles/mg protein
[3] nmoles cytochrome c reduced/mg protein
n.d.: not determined

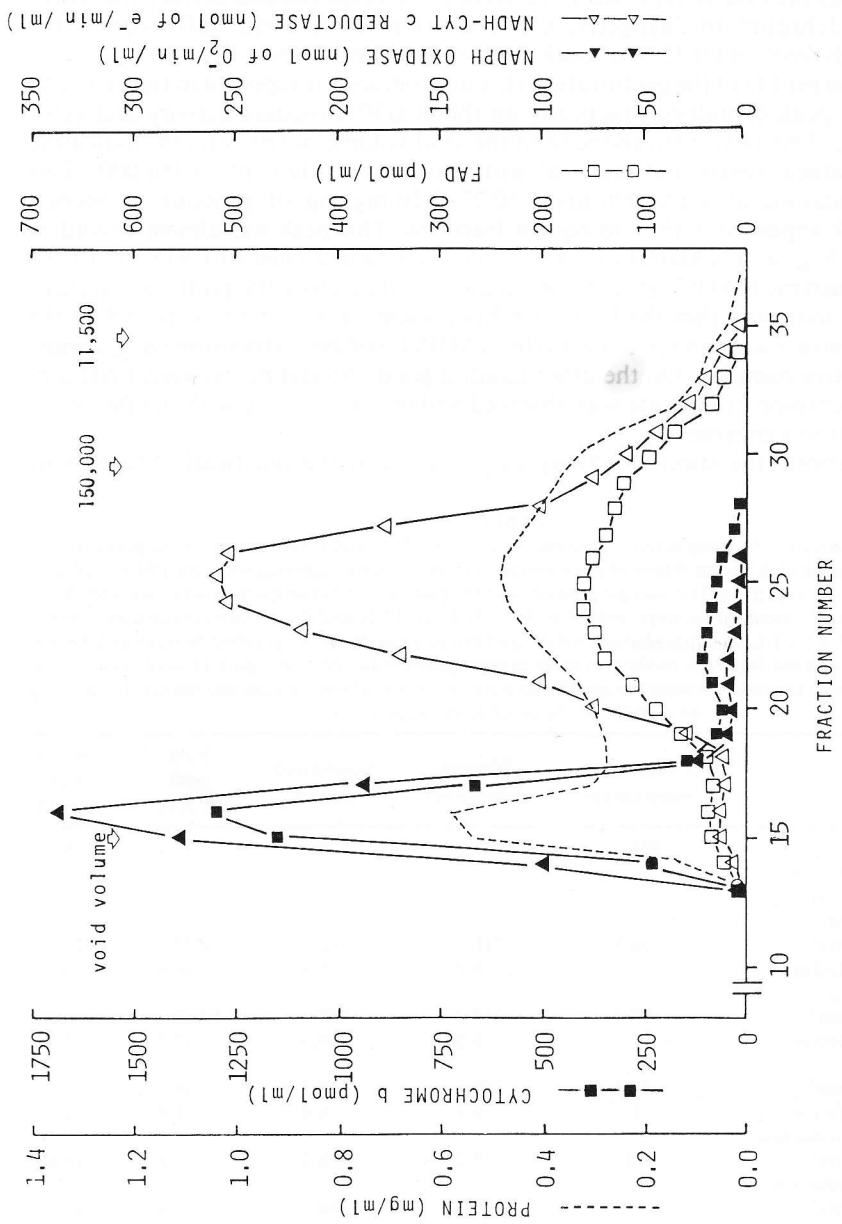

FIGURE 1 Gel filtration of the solubilized extract from plasmamembranes of PMA – activated pig neutrophils. The solubilized extract from NaCl – washed membranes was chromatographed through an Ultogel AcA34 column equilibrated with a buffer containing 0.15% deoxycholate and 0.15% Lubrol PX and 1.5 ml fractions were collected and assayed as described in Materials and Methods. The solubilized extract (4 ml) contained 3.3 mg protein/ml, 2.31 nmoles of cytochrome b_{-245}/ml, 1.03 nmoles of FAD/ml and the enzymatic activities were: NADPH oxidase, 601 nmoles of O_2^-/min/ml; NADH cytochrome *c* reductase, 475 nmoles cytochrome *c* reduced/min/ml. Void volume was determined with blue dextran (M_r ~ 2,000,000). Standards were alcohol dehydrogenase (M_r 150,000) and cytochrome *c* (M_r 11,500).

NADPH oxidase, of cytochrome b_{-245}, of FAD and of NAD(P)H cytochrome *c* reductase in the various fractions obtained at the different stages of the purification procedure. Data refer to postnuclear supernatant, washed membranes, solubilized extract and pooled fractions of the two main protein peaks obtained from gel filtration (first peak: fractions 14–17, second peak: fractions 18–36). NADPH oxidase specific activity increased 6–8 fold in the purification step from postnuclear supernatant to washed membranes and further 3 fold by the gel filtration chromatography. The specific content of cytochrome b_{-245} in the various preparations increased similarly to the specific activity of the oxidase, indicating a copurification of the cytochrome with the enzyme. The specific enzymatic activity and the specific content of cytochrome b_{-245} of the first peak were much higher than those previously published by others[7,8,28,29] and by us[12,17,18,19], indicating a significant improvement of the procedure.

FAD copurified with NADPH oxidase activity and with cytochrome b_{-245} up to solubilized extract of washed membranes, while it was almost completely separated from the oxidase activity and cytochrome b_{-245} by gel filtration chromatography. The main recovery of the FAD was in the second peak where also practically all NADH and NADPH cytochrome *c* reductase were present.

The behavior of FAD and the analysis of its relationship with the behavior of NADPH oxidase and cytochrome b_{-245} raise the problem of the involvement of the FAD in the activity of NADPH oxidase system or, at least, question an involvement based on a ratio cytochrome *b*/FAD of 1:1 or 2:1[29,30].

Electrophoretic analysis. The SDS – polyacrylamide gel electrophoresis of the various purified fractions was then performed in order to identify the polypeptides that were progressively concentrated during the purification procedure. The rationale of this approach is that only the polypeptides showing an increase in concentration during purification of the oxidase could be considered as possible components of the enzyme. Figure 2 shows the densitometric profiles of the electrophoretic separation of polypeptides contained in the various fractions. The results were as follows:

1) The main finding was the behavior of the protein band (A) with a molecular weight of 31,500 which progressively enriched during the various steps of purification, from the postnuclear supernatant (trace 1) to the first peak from AcA34 chromatography (trace 4). The enrichment of this protein was consistently observed in eight separate experiments and in all the cases the enrichment paralleled the increase of the specific activity of the NADPH oxidase (table I). The 31,500 dalton protein became one of the major component of the partially purified enzyme after gel filtration.
2) Other two major proteins, band B and C, with apparent molecular weight of 42,000 and 59,000 daltons respectively, were found in the partially purified enzyme after gel filtration chromatography. However, the enrichment of these bands regarded only the last step, from the solubilized extract to the first peak of chromatographic separation, while from the membrane to the solubilized extract these bands decreased. Their behavior did not correlate with that of the specific activity of the oxidase.
3) The behavior of the other protein bands was variable, some remaining constant and others decreasing during the purification steps, without correlation with the changes of the NADPH oxidase specific activity.

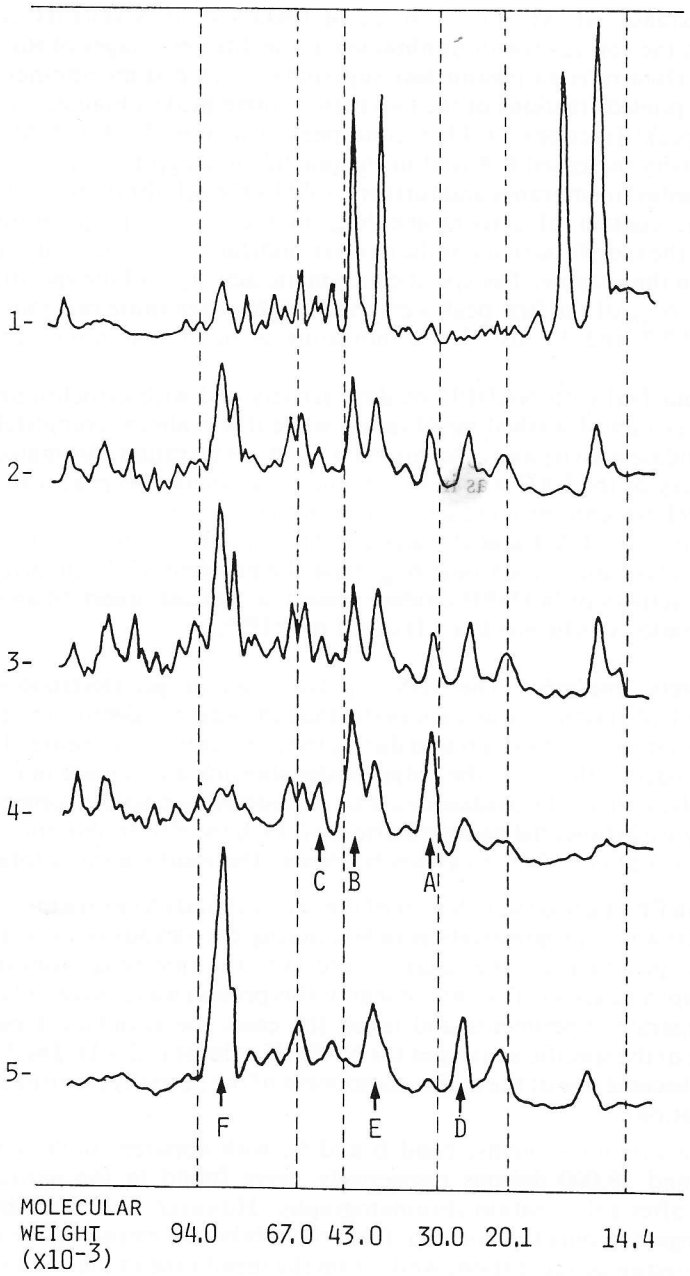

FIGURE 2 Densitometric profiles of SDS – polyacrylamide gel electrophoresis of the various stages of purification of NADPH oxidase. Trace 1: postnuclear supernatant. Trace 2: NaCl – washed plasmamembrane fraction. Trace 3: solubilized extract. Trace 4: first peak from AcA34 chromatography. Trace 5: fraction 15 from AcA34 chromatography shown in Figure 1.40 μg protein of each sample were subjected to electrophoresis.

4) Some protein bands, that is D, E, F, with molecular weight of 26,500, 38,000, 87,000 respectively, were prominent in a fraction of the second peak from AcA34 chromatography where NADPH oxidase activity was very low and where the NADH and NADPH cytochrome *c* reductase activity were high (trace 5).

The results strengthen the conclusion that among the major protein bands contained in the most active preparation, that with molecular weight of 31,500 is surely a component of the oxidase.

Attempts of Improving the Purification of Active NADPH Oxidase

The partially purified preparation, that is the first peak obtained by gel filtration chromatography, was then subjected to a series of procedures to improve the purification, but the results were disappointing. Briefly the hydrophobic chromatography through n-octylsepharose was unsuccessful because the enzyme did not bind to the resin. On the contrary, the enzyme was retained by the red-agarose resin, which should bind nucleotide-requiring enzymes[31], but it could not be eluted either by 10 mM - NADP, or 0.5 M - NaCl, or by pH changes of the eluting buffer. The enzyme was retained also by heparin-agarose, a resin used by others for the purification of human cytochrome b_{-245}[32], but when it was eluted by a NaCl gradient, the purification of oxidase and of cytochrome b_{-245} was minimal in terms of specific activity and specific content respectively.

Ultracentrifugation after a mild treatment with 0.4 M - NaCl, which removes proteins bound to the aggregates containing the activity, did not improve the purification, contrarily to what reported for the guinea pig enzyme in our previous paper[19]. It is likely that this discrepancy is due to the fact that the extrinsic proteins have been removed by the previous washing of the membrane with NaCl (see Materials and Methods).

These negative results led to the conclusion that the physico-chemical state of the enzyme complex and contaminating proteins, consisting of proteolipid aggregates, hampered a further purification by conventional methods. Attempts of purifying the active oxidase submitting to the above procedures a preparation treated with various disaggregating agents failed, since these treatments caused inactivation of the oxidase. The agents used for this purpose were: deoxycholate and Lubrol PX at concentration above 0.5%, 1% Triton X-100, 1 M - KCl and 1 M - NaCl, 0.4% Zwittergent 3-12 detergent, 0.4% Zwittergent 3-16 detergent.

All these results indicate that the aggregated state of the extract is a prerequisite for maintaining the enzyme in its native state. For this reason we decided to continue the purification in spite of the loss of NADPH oxidase activity and by measuring, during the subsequent steps, the content of cytochrome b_{-245}, whose spectrum showed a resistance to the experimental manipulation and treatment greater than that of NADPH oxidase activity. Practically this corresponded to the purification of cytochrome b_{-245} which is a true component of oxidase.

Further Purification of Cytochrome b_{-245}

The pooled fractions of the first peak eluted from Ultrogel AcA34 chromatography were loaded on a continuous glycerol gradient (22-32% in the presence of 0.15% deoxycholate and 0.15% Lubrol PX) and centrifuged at 131,000 g for 16 h. As shown

in figure 3 this procedure allowed the separation of a broad peak of protein (about 85% of the original preparation) containing cytochrome b_{-245} and oxidase activity from high density aggregate sedimented at the bottom of the gradient (15% of the original protein content). The recovery of the cytochrome b_{-245} in the gradient was about 90% of the original preparation and residual was at the bottom. On the basis of the glycerol concentration the cytochrome b_{-245} peak corresponded to a density of 1.114–1.128 g/ml. The recovery of NADPH oxidase was about 10–15% of the original preparation in the gradient and about 1% at the bottom, showing a great loss of activity. This decay of activity was not due to the centrifugation but to the time span needed for the centrifugation in the presence of detergents.

The fractions of the broad peak were pooled, diluted at 10% glycerol and centrifuged at 100,000 g for 3 h. Cytochrome b_{-245} sedimented at the bottom, indicating that it was still in highly aggregated form. The pellet was suspended in a buffer containing 1% Triton X-100, 0.5% deoxycholate and 1 M - KCl (buffer D, see Materials and Methods). This treatment abolished any residual oxidase activity and caused the dissociation of the aggregate, so that after further centrifugation at 100,000 g for 3 h, 75% of cytochrome b_{-245} was recovered in the supernatant. The supernatant was chromatographed through Ultrogel AcA34 equilibrated with buffer containing 1% Triton X-100, 0.5% deoxycholate and 1 M KCl, and the elution profile shown in figure 4 was obtained. Cytochrome b_{-245} was eluted as a broad peak in the included volume of the column, indicating an apparent molecular weight from 170,000 and 230,000. The cytochrome b_{-245} peak did not contained FAD and NADPH oxidase activity and was separated from a peak containing most of the other proteins.

Data of table II show the progressive purification of cytochrome b_{-245} from the first peak of the first chromatographic separation on AcA34 (27.2 fold) to the second chromatographic separation on AcA34 of disaggregated pool of glycerol gradient (147 fold).

TABLE II

Purification of cytochrome b_{-245} from partially purified NADPH oxidase. NADPH oxidase from AcA34 cromatography was subjected to centrifugation on glycerol gradient. Fractions of the glycerol gradient peak containing cytochrome b_{-245} were treated with Triton X-100/deoxycholate/KCl and centrifuged at 100,000 g for 3 h. The supernatant (Triton/deoxycholate/KCl extract) was rechromatographed on Ultrogel AcA34 in the presence of the same detergent mixture, as described in Materials and Methods. The fractions containing cytochrome b_{-245} (cytochrome b_{-245} peak, see Figure 4) were pooled and assayed as described in Materials and Methods. The purification ratio is the ratio between the specific activity or content of the fraction and the specific activity or content of the original postnuclear supernatant.

	First peak AcA34 (fig. 1)	Glycerol gradient peak	Triton/deoxycholate/ KCl extract	Cytochrome b_{-245} peak
Protein (mg obtained from 10^{10} neutrophils)	1.2	0.66	0.57	0.036
NADPH oxidase				
Specific activity[1]	572	24.1	0	0
Purification factor	18.6	0.8	0	0
Cytochrome b_{-245}				
Specific content[2]	1,915	2,233	2,481	10,324
Purification factor	27.2	31.7	35.3	147

[1,2] see notes to table I.

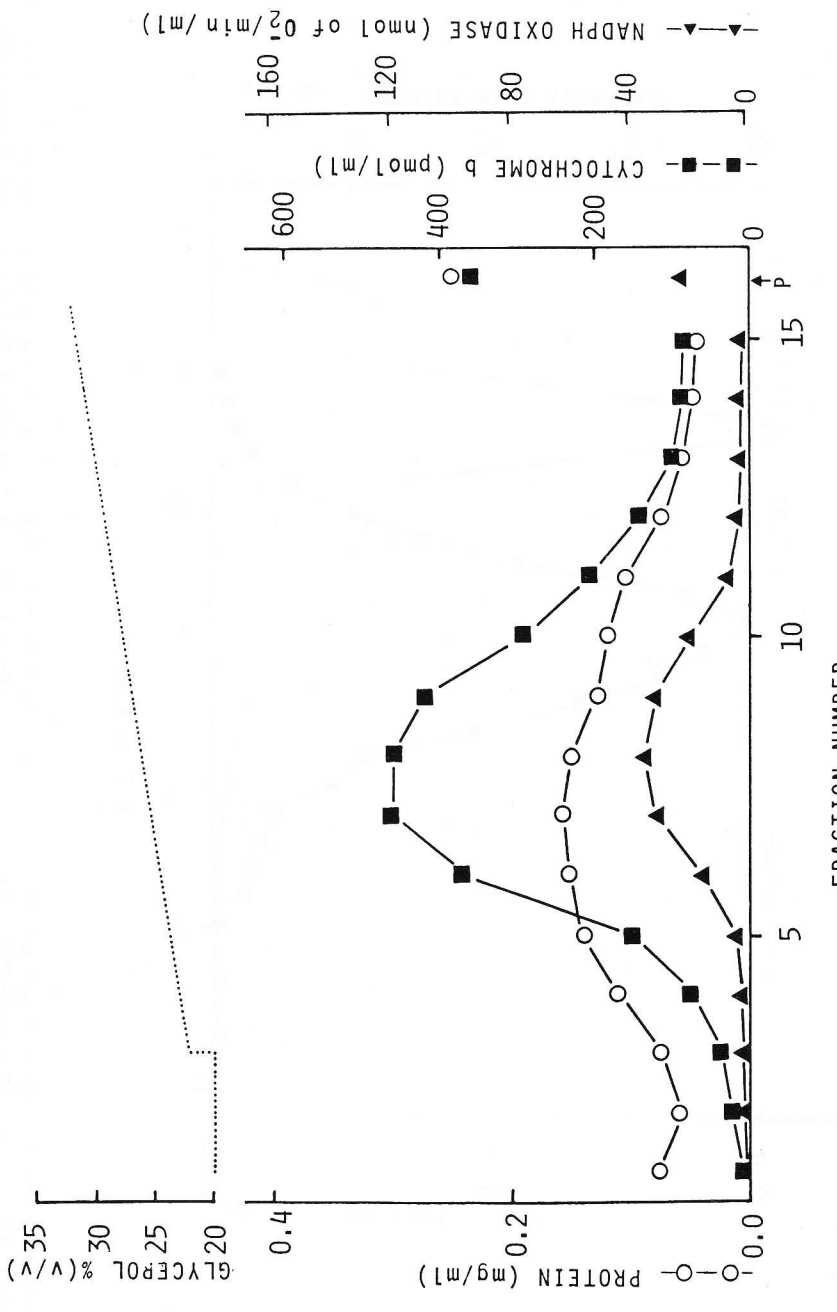

FIGURE 3 Glycerol density gradient centrifugation of partially purified NADPH oxidase. NADPH oxidase from AcA34 chromatography (3 ml, 0.56 mg protein/ml, 324 nmoles of O_2^-/min/ml, 1,084 pmoles of cytochrome b_{-245}/ml) was centrifuged on a 22–32% (v/v) glycerol gradient and fractions of volume 1 ml were collected and assayed as described in Materials and Methods. Fraction P is the pellet at the bottom of the glycerol gradient, which was resuspended in 1 ml of buffer B.

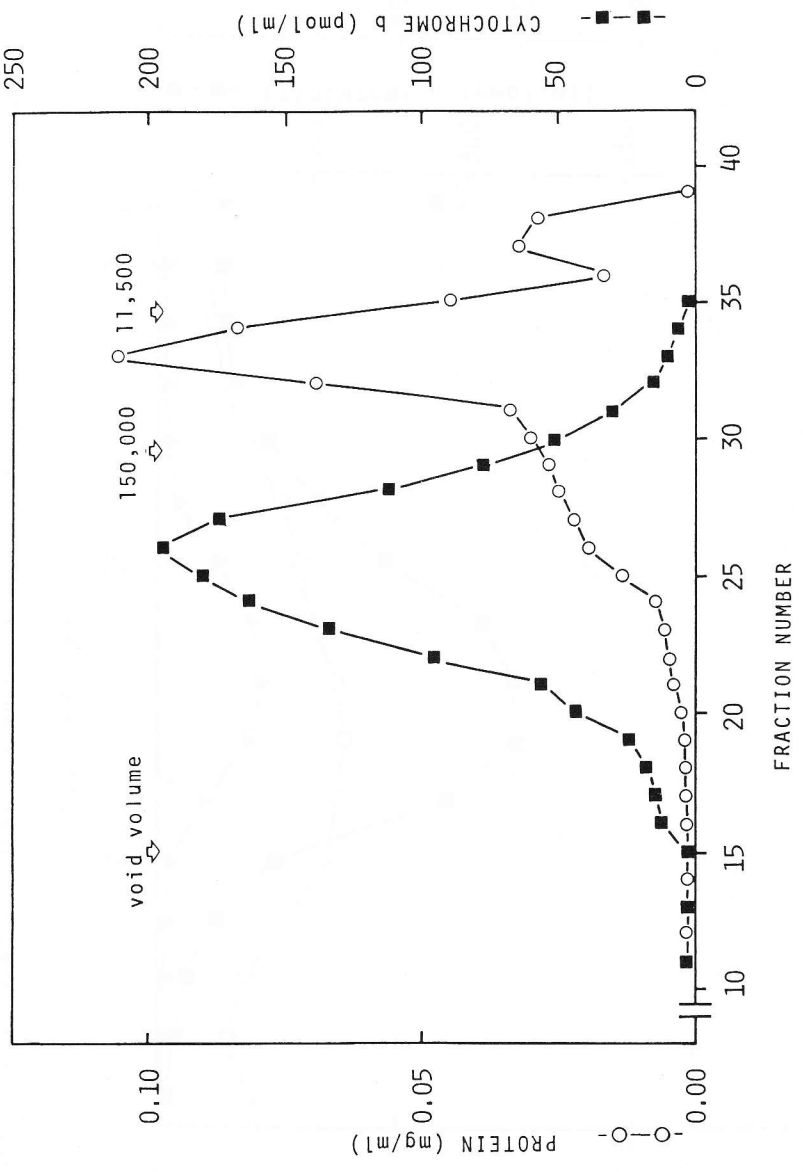

FIGURE 4 Isolation of cytochrome b_{-245} by gel filtration chromatography. A Triton X-100/deoxycholate/KCl extract (2 ml, 0.44 mg protein ml, 1,035 pmoles of cytochrome b_{-245}/ml) was chromatographed on an Ultrogel AcA34 column (1.6 × 30 cm) in the presence of 1% Triton X-100, 0.5% deoxycholate and 1 M KCl. Fractions of volume 1.5 ml were collected and assayed as described in Materials and Methods. Void volume was determined with blue dextran (M_r ~ 2,000,000). Standards were alcohol dehydrogenase (M_r 150,000) and cytochrome *c* (M_r 11,500).

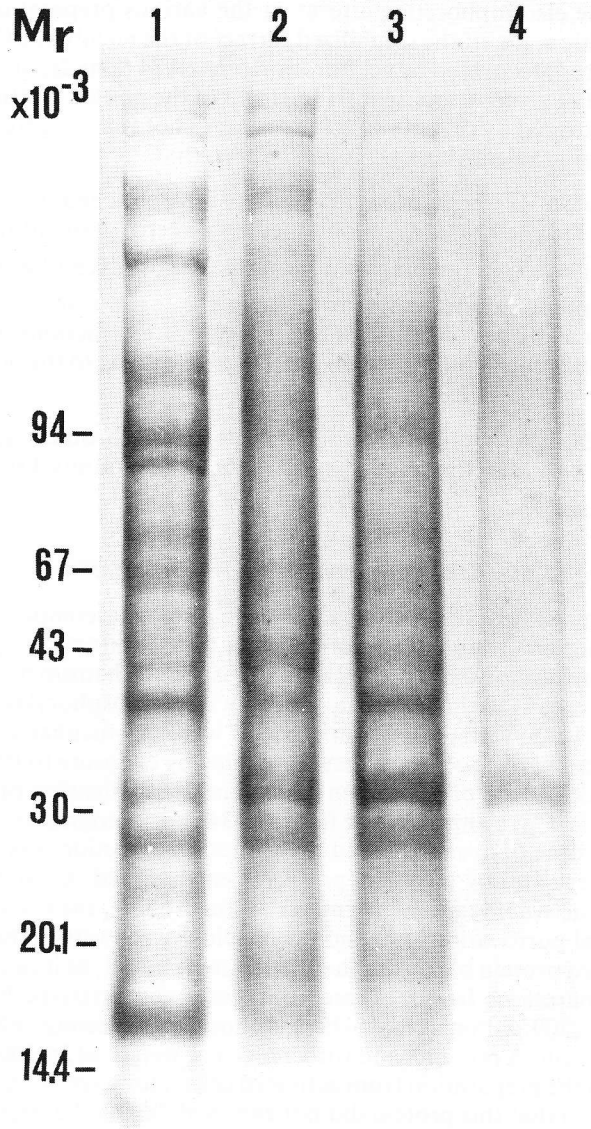

FIGURE 5 SDS - polyacrylamide gel electrophoresis of the different stages of purification of cytochrome b_{-245}. Lane 1: deoxycholate/Lubrol PX solubilized extract. Lane 2: partially purified NADPH oxidase from the first peak of AcA34 chromatography (Figure 1). Lane 3: cytochrome b_{-245} containing peak from the glycerol gradient (Figure 3). Lane 4: purified cytochrome b_{-245} from AcA34 chromatography (Figure 4). 50 μg protein were subjected to electrophoresis in lanes 1–3, while lane 4 contained 7.3 μg protein because of the scarcity of the material.

Figure 5 presents the electrophoretic patterns of the various preparations at different steps of purification, i.e. of the solubilized extract of the membrane (lane 1), of the first peak (see Figure 1) recovered by gel filtration on AcA34 (lane 2), of the pooled fractions (see Figure 3) of glycerol gradient (lane 3) and of the pooled fractions of the peak (see Figure 4) recovered by the gel filtration on AcA34 of the disaggregated preparation of the glycerol gradient (lane 4). It can be seen that

1) the protein with molecular weight of 31,500 progressively enriched from the lane 1 (solubilized extract) to the preparation obtained by glycerol gradient (lane 3),
2) many proteins are present in the preparations until that of glycerol gradient,
3) the most purified preparation (lane 4) containing the highest amount of cytochrome b_{-245} obtained by gel filtration of the disaggregated fractions of glycerol gradient contains only one definite protein band corresponding to the polypeptide of 31,500 daltons.

This finding clearly indicates that the band corresponds to cytochrome b_{-245} and that 31,500 is the molecular weight of the cytochrome b_{-245} or of its subunits dissociated by SDS.

Phosphorylation of the 31,500 daltons Protein in Active Preparations

The above reported experiments allowed the identification of a component of the NADPH oxidase of pig neutrophils that corresponds to cytochrome b_{-245}. Since a fundamental question regarding that NADPH oxidase is the mechanism by which this enzyme is turned on in activated cells, the possibility of a phosphorylation of the identified component was investigated. Granulocytes were pre-incubated with $^{32}P_i$, then divided in two aliquots, one of which was stimulated by exposure to PMA. Both activated and resting cells were processed until the partial purification of NADPH oxidase and cytochrome b_{-245} by mean of the first AcA34 chromatography. Complete purification of cytochrome b_{-245} as described in the preceding section was hampered both by the low recovery and by technical problems encountered in handling large amount of resting and activated radioactive material. In any case, the results demonstrated that even partial purification gave enough conclusive answers to the question of whether the identified protein band was phosphorylated. Infact, as it can be seen in Figure 6, enzyme preparations from activated cells were characterized by marked phosphorylation of 31,500 daltons band whereas those from resting cells showed negligible phosphorylation. A protein band with molecular weight of 26,500 appeared also phosphorylated in the preparation from activated cells. However, it has been previously shown (Figure 2) that this protein did not enriched during the step of partial purification of the NADPH oxidase in active state, but it greatly enriched in the second peak recovered from gel filtration chromatography on AcA34 which is the peak practically devoid of NADPH oxidase activity and rich in NADH and NADPH cytochrome *c* reductase. This finding support that this 26,500 daltons protein does not belong to the oxidase system.

Figure 6 also shows that non-protein ^{32}P phosphorylated material was also present in resting and active preparations. This material was identified as phospholipids by thin layer chromatography and the $^{32}P_i$ labelled species were essentially phosphatidylinositol-4,5-biphosphate, phosphatidylinositol-4-phosphate and phosphatidic acid (data not shown).

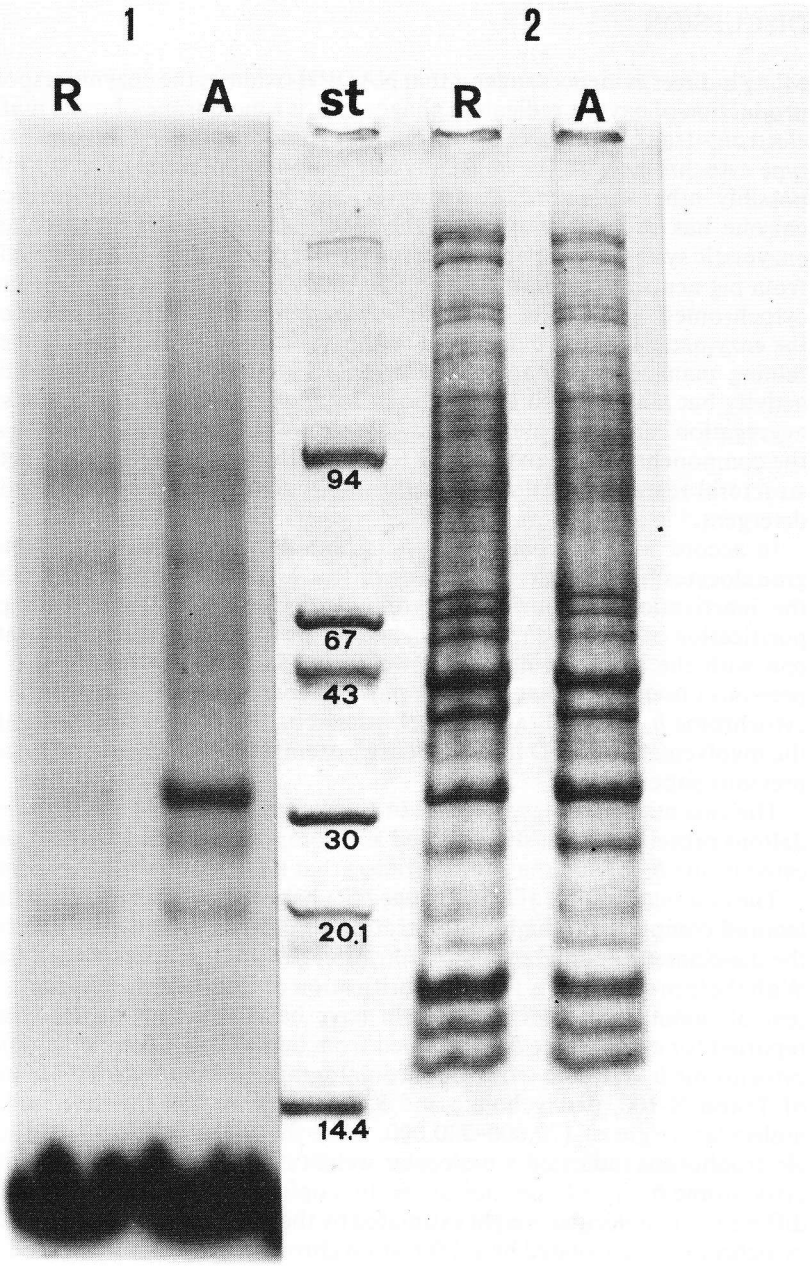

FIGURE 6 Autoradiography of phosphorylated proteins present in resting and PMA – activated partially purified NADPH oxidase. Neutrophils were labelled with $^{32}P_i$ and divided in two parts only one of which was treated with PMA. NADPH oxidase from both resting (R) and activated (A) cells was partially purified by AcA34 chromatography, subjected to electrophoresis and autoradiography as described in Materials and Methods. 1: autoradiography, 2: Coomassie stain, St: protein standards (Kdalton).

DISCUSSION

Many indirect evidences suggest that NADPH oxidase, the enzyme responsible for the production of oxygen radicals in phagocytes, is a membrane - bound multicomponent electron - transport system whose putative components are a flavoprotein[8,9,10,11], a *b* - type cytochrome with the unusually low midpoint potential of −245 mV[13,14,15,16] and possibly other cofactors[33,34]. However, the lack of complete purification of the enzyme has still now hampered the identification of the intimate nature of this enzymatic system. This paper reports a partial purification of active NADPH oxidase from pig neutrophils plasmamembrane followed by almost complete purification of cytochrome b_{-245}. A mild solubilization treatment was required in order to preserve the enzymatic activity, but this led to the isolation of a big proteolipid complex containing many contaminant proteins. Dissociation of this complex caused loss of activity but allowed high purification of cytochrome b_{-245}. The reasons why the disaggregation caused the inactivation are unknown. It could be due to the separation of the components of the oxidase, or to the removal of an activatory factor, or to the structural rearrangement of the active molecule(s) caused by delipidation or by the detergent.

In accord with our previous results obtained using an extract from guinea pig granulocytes[19], the results of the present study have shown that in all the steps before the inactivation the purification of NADPH oxidase was always paralleled by purification of the cytochrome b_{-245} and by the increase in the concentration of a protein with the apparent molecular weight of 31,500. The results also confirm our previous observation that partially purified enzyme contains very little FAD, with a cytochrome b_{-245}/FAD ratio of 38:1 instead of 19:1[12]. This finding, which questions the involvement of FAD in the oxidase system, has been extensively discussed in the previous paper[12].

The two main findings reported in this paper are the identification of the 31,500 daltons protein, previously indicated as a component of the NADPH oxidase, as the cytochrome b_{-245}, and the demonstration that it is phosphorylated during activation.

The best purification of cytochrome b_{-245} has been obtained by dissociating the proteolipid complex with high concentration of detergents and salts and by submitting the dissociated supernatant to gel filtration chromatography (figure 4 and table II). With these procedures, a 147 fold purification of cytochrome b_{-245} and a specific content of about 10 nmoles/mg protein have been achieved, values similar to those reported for cytochrome b_{-245} purified from human[32] and bovine[35] granulocytes. The cytochrome b_{-245} eluted from the chromatography column carried out in the presence of Triton X-100, deoxycholate and KCl (Figure 4) was characterized by apparent molecular weight of 170,000-230,000, while the analytical SDS - polyacrylamide gel electrophoresis indicated a molecular weight of 31,500. Binding of Triton X-100 to cytochrome b_{-245} and presence of residual lipids can partially explain such a marked difference in molecular weight extimated by the two methods. It is more likely that the cytochrome b_{-245} isolated by gel filtration chromatography is composed by some (less than eight) identical subunits of 31,500 daltons that are dissociated by SDS. Easy aggregation of hydrophobic membrane proteins and particularly of *b* - type cytochromes was observed[32,36]. Recently, Harper *et al.*[32] reported a molecular weight of 68-78,000 for human neutrophil's cytochrome b_{-245}, whereas Pember *et al.*[35] indicated three proteins of 14,000, 12,000 and 11,000 as component of cytochrome b_{-245} purified from bovine neutrophils. The reason of these discrepancies remains to

be investigated.

The results presented in this paper demonstrated that cytochrome b_{-245} is a major component of NADPH oxidase and that it corresponds to the 31,500 daltons protein band identified by electrophoresis. The fact that only this protein clearly enriched in partially purified preparations might suggest the hypothesis that the cytochrome b_{-245} is actually the NADPH oxidase. In other words, the NADPH oxidase would be an hemoprotein with cytochrome *b* - like spectrum. The hypothesis is circumstantial. The almost complete absence of flavoproteins in the partially purified enzyme could be in agreement with this possibility. However, the fact that the disaggregation of the proteolipid complex caused the loss of NADPH oxidase activity is typical of a multicomponent system. Furthermore, the above hypothesis requires a complete revision of the mechanism till now suggested for the electron transport from NADPH to O_2 via cytochrome b_{-245}[30,34,37,38,39].

The second relevant finding reported in this paper is the phosphorylation of the 31,500 daltons protein in the partially purified preparation from activated leukocytes. In the last few years indirect evidences have been obtained in favour of the hypothesis[40,41,42,43,44] that the transduction mechanism responsible for many leukocyte responses to external stimuli involves the activation of a Ca^{2+} and phospholipid - dependent protein kinase C and of protein phosphorylation. The activation of protein kinase C would be caused either directly, as in the case of PMA[45,46], and indirectly, that is via formation of diacylglycerol by increased breakdown of phosphoinositides[47], as occurs when the stimulant is a chemotactic peptide[48,49].

The phosphorylation of many cytosolic and membrane proteins associated with the activation of phagocytic leukocytes has been reported[50,51,52,53,54,55], but the data are so heterogeneous that a precise correlation between one or more phosphorylated proteins and the cell response has not been found. However, it is worthy pointing out that among the proteins phosphorylated in activated leukocytes some Authors have found also species with molecular weights ranging from 31,000 to 34,000[51,53,55]. Our finding that in the extract from PMA - activated neutrophils containing active NADPH oxidase the 31,500 daltons protein, that is the cytochrome b_{-245}, appears phosphorylated is relevant for two reasons. Firstly, it can be the conclusive evidence that the hypothesis of the involvement of protein kinase C in the transduction mechanism is correct. Secondly, it identifies the target of protein kinase whose phosphorylation is functional for the activation of the NADPH oxidase.

The mechanism by which the phosphorylation of the 31,500 protein changes the state of the NADPH oxidase remains to be investigated. One could speculate that phosphorylation induces a conformational change of the hemoprotein that facilitates either its functional relation with other components of the oxidase, or the transfer of electrons, or in the case that the hemoprotein be the oxidase, the interaction with the substrate NADPH. Alternatively, the phosphorylation could cause the removal, or the interaction with, a regulatory factor.

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