Superoxide-forming NADPH oxidase preparation of pig polymorphonuclear leucocytes

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A phagocytic vesicle fraction with high NADPH-dependent superoxide-forming activity was obtained in large quantity from pig blood polymorphonuclear leucocytes, phagocytosing oil droplets in the presence of cyanide. The activity of the homogenate of the phagocytosing cells was 40 times that of the resting cells, and 70% of the activity in the homogenate was recovered in the phagocytic vesicle fraction. Essentially all of the superoxide-forming activity was extracted by repeated extraction with a mixture containing deoxycholate and Tween 20. The extract had a superoxide-forming activity of 1 $\mu\text{mol}/\text{min}$ per mg of protein with NADPH, and one-fifth of this with NADH, K_m values being similar to those of the vesicle fraction (40 μM for NADPH and 400 μM for NADH). A stoichiometric relationship of 1:2 for NADPH oxidation and superoxide formation was obtained, in agreement with the reaction $\text{NADPH} + 2\text{O}_2 \rightarrow \text{NADP}^+ + 2\text{O}_2^{\cdot-} + \text{H}^+$. The activity of the extract was enhanced 2-fold by the addition of FAD, suggesting that the flavin is a component of the enzyme system. The K_m value for FAD was 0.077 μM . The activities in both vesicle fraction and extract were labile even on refrigeration, but could be kept for several months at -70°C .

Polymorphonuclear leucocytes ingest and kill microbes, accompanied by a respiratory burst which leads to the formation of active oxygen metabolites such as H_2O_2 and superoxide anions ($\text{O}_2^{\cdot-}$). The production of the oxygen metabolites by the cells can be ascribed to the activation of a superoxide-forming system, which is dormant in the resting cells (DeWald *et al.*, 1979). The superoxide-forming system is considered to catalyse an initial one-electron reduction of oxygen to superoxide by a cyanide-insensitive reaction, followed by conversion of the radicals into H_2O_2 by dismutation (Curnutte & Babior, 1974). The question of whether the electron donor is NADH (Wilkinson *et al.*, 1975; Briggs *et al.*, 1975) or NADPH (Babior *et al.*, 1976; Iverson *et al.*, 1977; Cohen *et al.*, 1980) remains, but most investigators favour the NADPH-dependent superoxide-forming reaction (Nakamura *et al.*, 1981). A previous report from our laboratory also supported the dependency of the reaction on NADPH (Nabi *et al.*, 1979).

For further investigation of the biochemical basis of the respiratory burst, purification of the superoxide-forming system is necessary, but has not been done sufficiently, because of the difficulties in obtaining a particulate superoxide-forming preparation in large quantity and in solubilizing the activity.

At least two reports have appeared on the solubilization of the activity, but the yield was low (Gabig & Babior, 1979; Tauber & Goetzl, 1979).

In the present paper, we used pig polymorphonuclear leucocytes, which can be prepared in large quantity, and obtained a phagocytic vesicle fraction with high NADPH-dependent superoxide-forming activity. Essentially all of the activity was extracted with a mixture of detergents containing deoxycholate and Tween 20.

Experimental

Isolation of leucocytes

Polymorphonuclear leucocytes were obtained from pig blood. The blood (4.5 litres) was added to 0.5 litre of 5% (w/v) EDTA/0.9% NaCl and then to 1 litre of 3% polyvinylpyrrolidone (K 90) solution and mixed gently. The mixture was kept at room temperature for 30–40 min and the supernatant was separated from sedimenting erythrocyte aggregates by siphoning. Leucocytes in the supernatant were precipitated by centrifugation at 250 g for 10 min. Contaminating erythrocytes in the precipitate were lysed by the addition of 5 vol. of water. After 30 s, the suspension was made iso-osmolar by the addition of 10% (w/v) NaCl. The pellet obtained by

centrifugation at 250 *g* for 10 min was suspended in 0.9% NaCl. Polymorphonuclear leucocytes were separated from platelets and mononuclear cells by Conray–Ficoll method described by Böyum (1968). The polymorphonuclear leucocytes were suspended in phosphate–Ringer solution (119 mM–NaCl, 4.7 mM–KCl, 1.2 mM–MgSO₄, 0.75 mM–CaCl₂, 15 mM–NaH₂PO₄/Na₂HPO₄, pH 7.4) and kept in ice until use. The yield of leucocytes was 1.6×10^{10} – 3×10^{10} cells, and the proportion of the cell types shown by May–Giemsa staining was 82% neutrophils, 14% eosinophils, 0.8% monocytes and 4% lymphocytes.

Preparation of oil droplets

Oil droplets were prepared by modifying the method of Stossel *et al.* (1971). Paraffin oil was replaced by 1,2,3,6-tetrahydrophthalic acid diisodecyl ester and serum albumin by immunoglobulin G. The tetrahydrophthalic acid ester (0.67 ml) was added to 2.2 ml of the phosphate Ringer solution, pH 7.4, containing 5 mg of pig immunoglobulin G/ml, and the mixture was sonicated with a Branson sonifier for 90 s at 4 A at 4°C.

Preparation of phagocytic vesicles

Phagocytic vesicles were prepared by modifying the method of Stossel *et al.* (1971). Polymorphonuclear leucocytes were preincubated in polycarbonate centrifuge tubes (2.5×10^9 – 3.5×10^9 cells per tube) with 12 ml of the phosphate Ringer solution, pH 7.4, and 2 mM–glucose at 37°C for 5 min. Then KCN was added to a final concentration of 1 mM and incubated with 2.9 ml of the oil droplets for 5 min. The reaction was terminated by the addition of 15 ml of ice-cold 0.9% NaCl. The cells were separated from the non-ingested oil droplets by centrifuging at 150 *g* for 10 min and washed with 25 ml of ice-cold 0.9% NaCl and then with 15 ml of ice-cold distilled water. The washed cells were suspended in 8 ml of ice-cold 0.34 M–sucrose buffered with 5 mM–Hepes [4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid], pH 7.5 (buffered 0.34 M–sucrose), and homogenized by ten strokes of a Teflon pestle in a Potter–Elvehjem homogenizer with a tight clearance. The homogenate was centrifuged for 1 h at 76 500 *g* at 4°C with buffered 0.25 M–sucrose solution layered on it. Vesicles filled with oil droplets floated to the top of the 0.25 M–sucrose solution and were thus washed with the sucrose solution. The floating pellet was removed and was dispersed with a glass pestle in a Potter–Elvehjem homogenizer in 4 ml of the buffered 0.34 M–sucrose (vesicle fraction). The sediment at the bottom of the tube was similarly dispersed (sediment fraction). Cytosol proteins remained in the 0.34 M–sucrose solution (cytosol fraction).

Extraction of NADPH oxidase

The vesicle fraction was mixed with detergents, for extraction of NADPH oxidase. The mixture consisted of the vesicle fraction (0.7–1.0 mg of protein/ml), 0.5% (w/v) sodium deoxycholate, 0.5% (v/v) Tween 20, 25% (v/v) ethylene glycol and 0.17 M–sucrose in 50 mM–glycine buffer, pH 8.5. Lowering the protein concentration led to poor yield of the extraction. The mixture was vigorously stirred with a vortex mixer and placed in ice for 30 min. Centrifugation of the mixture at 117 000 *g* with a swing-out rotor (Hitachi RPS 40) for 1 h at 4°C gave three fractions: floating oil layer, sediment and a clear solution with high NADPH oxidase activity between them.

Assay of superoxide formation

The NAD(P)H-dependent superoxide-forming activity was measured by the reduction of acetylated cytochrome *c* in the presence of reduced nicotinamide coenzymes as described by Kakinuma & Minakami (1978). A cuvette containing 1 ml of reaction mixture, consisting of 100 μ M–acetylated cytochrome *c*, 50 mM–potassium phosphate buffer (pH 7.5 for the homogenate or the vesicle fraction and pH 8.0 for the extract) and either 0.5 mM–NADPH or 1.5 mM–NADH, was placed in a thermostatically controlled cuvette holder of a Hitachi 556 dual-wavelength spectrophotometer at 20°C. The assay was started by the addition of a sample and the absorbance change at 550 nm in reference to 540 nm ($A_{550} - A_{540}$) was followed on a recorder. The superoxide-forming activity was calculated from the initial velocity of the reduction of acetylated cytochrome *c* by using a molar absorption coefficient of $19.1 \times 10^3 \text{ M}^{-1} \cdot \text{cm}^{-1}$ and was corrected by subtracting the rate in the presence of superoxide dismutase.

Assays of oxygen consumption and NADPH oxidation

Oxygen consumption was measured with a Gilson 5/6 oxygraph equipped with a Clark-type electrode (Yellow Springs Instrument Co., Yellow Springs, OH, U.S.A.). The reaction mixture consisted of 54 μ g of protein of the vesicle fraction or 15 μ g of protein of the extract, 0.5 mM–NADPH, 650 units of catalase, 25 units of superoxide dismutase, and 50 mM–potassium phosphate buffer (pH 7.5 for the vesicle fraction and pH 8.0 for the extract) in 1.8 ml at 20°C. The full scale of the recorder was set to correspond to 28 μ M–O₂. NADPH oxidation was measured spectrophotometrically at 340 nm at 20°C. The reaction mixture consisted of 0.125 mM–NADPH, 50 mM–potassium phosphate buffer (the pH as indicated above) and either the vesicle fraction (8 μ g of protein) or the extract (4 μ g of

protein). The reaction was started by the addition of a sample, for both assays. The activities were represented by the initial rates.

Other enzyme assays and chemical determinations

Activities of myeloperoxidase and alkaline phosphatase were assayed as described by Michell *et al.* (1970) and that of β -glucuronidase as described by Hirschhorn *et al.* (1967). Glucose 6-phosphate and 5'-nucleotidase were assayed by measuring the release of P_i as described by Nordlie & Arion (1966) and Michell & Hawthorne (1965) respectively. Succinate-cytochrome *c* reductase and lactate dehydrogenase were assayed as described by Stotz (1955) and Bergmeyer & Bernt (1974) respectively. Protein concentration was measured by the method of Lowry *et al.* (1951) after the oil had been removed by centrifugation at 250 *g* for 10 min. The protein in the extract was measured after precipitation of proteins with 25% (w/v) trichloroacetic acid overnight to avoid interference by detergents. Phospholipid was determined by the method of Folch *et al.* (1957) and Bartlett (1959) by extracting the sample twice with 20 vol. of chloroform/methanol (2:1, v/v), and phosphorus in the chloroform layer was determined after wet ashing with H_2SO_4 . 1,2,3,6-Tetrahydrophthalic acid diisodecyl ester used for preparing oil droplets did not interfere with the measurement.

Reagents

Acetylated cytochrome *c* was prepared as described previously (Minakami *et al.*, 1958). Superoxide dismutase was prepared from bovine erythrocytes by the method of McCord & Fridovich (1969). Immunoglobulin G was purified from pig serum by the method of Peterson & Sober (1962). Iothalamic acid (Conray) was a generous gift from Daiichi Pure Chemical Co., Tokyo, Japan. Bovine

liver catalase was purchased from Boehringer, Mannheim, Germany, and Ficoll 400 was from Pharmacia, Uppsala, Sweden. 1,2,3,6-Tetrahydrophthalic acid diisodecyl ester was obtained from Tokyo Kasei Co., Tokyo, Japan. NADPH and NADH were from Oriental Yeast Co., Tokyo, Japan. Detergents of the Tween series, Triton X-100 and cetyltrimethylammonium bromide were from Wako Pure Chemical Co., Osaka, Japan, and cholate and deoxycholate were from Difco Laboratories, Detroit, MI, U.S.A. Detergents of the Emalgen and the Brij series were gifts from Kao-Atlas Chemicals, Tokyo, Japan. Other reagents were of analytical grade.

Results

Superoxide-forming activity in homogenates

Homogenates of polymorphonuclear leucocytes, both resting and phagocytosing oil droplets, were compared for their activities of forming superoxide in the presence of reduced nicotinamide coenzymes. As shown in Table 1, the NADPH-dependent superoxide-forming activity in the homogenate of the phagocytosing cells was about 40 times that of the resting cells. Moreover, the activity in the homogenate of the phagocytosing cells was essentially resistant to cyanide, whereas that of the resting cells was sensitive to cyanide, suggesting that the latter reaction may be catalysed by haem proteins, probably by myeloperoxidase, and that the cyanide-insensitive activity responsible for the respiratory burst was absent from the resting cells. The enhancement of the NADH-dependent reaction by phagocytosis was not so strong as that of the NADPH-dependent reaction.

Addition of cyanide during phagocytosis was effective for obtaining the cells with high superoxide-forming activities, as shown by Cohen *et al.*

Table 1. *Enzyme activities in homogenates of pig blood leucocytes*

Leucocytes were incubated for 5 min at 37°C with oil droplets (phagocytosing) or without them (resting) in the presence of 1 mM-KCN, and homogenized. The values in parentheses were those of the cells incubated without KCN. Values represent the means $\pm$ S.D. for three independent experiments.

	Specific activities (mmol/min per mol of phospholipid P)			
	Resting		Phagocytosing	
$O_2^{\cdot -}$ formation (NADPH)	3.2 $\pm$ 0.3	(2.6 $\pm$ 0.2)	123 $\pm$ 12	(56.8 $\pm$ 5.0)
$O_2^{\cdot -}$ formation (NADH)	4.5 $\pm$ 0.7	(3.6 $\pm$ 0.5)	42.7 $\pm$ 5.1	(20.9 $\pm$ 1.9)
5'-Nucleotidase	5.0 $\pm$ 0.2	(5.2 $\pm$ 0.2)	4.8 $\pm$ 0.3	(5.0 $\pm$ 0.3)
Myeloperoxidase	2450 $\pm$ 180	(2400 $\pm$ 120)	2420 $\pm$ 120	(2390 $\pm$ 50)
β -Glucuronidase	0.78 $\pm$ 0.02	(0.76 $\pm$ 0.02)	0.78 $\pm$ 0.03	(0.76 $\pm$ 0.04)
Alkaline phosphatase	2850 $\pm$ 30	(2850 $\pm$ 40)	2670 $\pm$ 30	(2800 $\pm$ 10)
Glucose 6-phosphatase	890 $\pm$ 10	(880 $\pm$ 6)	893 $\pm$ 3	(880 $\pm$ 7)
Succinate-cytochrome <i>c</i> reductase	321 $\pm$ 7	(303 $\pm$ 3)	317 $\pm$ 13	(297 $\pm$ 13)
Lactate dehydrogenase	5150 $\pm$ 90	(5110 $\pm$ 40)	5090 $\pm$ 90	(5060 $\pm$ 60)

(1980). NaN_3 (1 mM) also similarly enhanced the superoxide-forming activities, whereas antimycin A (0.1 mM), 2,4-dinitrophenol (0.1 mM) and rotenone (30 pmol/mg of protein) were not effective.

Distribution in subcellular fractions

The distribution of the superoxide-forming activities in the homogenate of the phagocytosing cells is shown in Table 2. About 70% of the NADPH-dependent activity was found in the vesicle fraction, whereas 60% of the NADH-dependent activity was in the sediment fraction. The distribution of marker enzymes indicates that the sediment fraction contains mitochondria, lysosomes and microsomal fraction, hence reduced-nicotinamide-coenzyme-dependent superoxide-forming activities in the sediment fraction can be explained as those of microsomal fraction (Aust *et al.*, 1972) and of mitochondria (Takeshige & Minakami, 1979). The activities of glucose 6-phosphatase, which exists in the microsomal fraction of the cells (Klempner *et al.*, 1980), and succinate-cytochrome *c* reductase were not observed in the vesicle fraction, indicating that neither microsomal fraction nor mitochondria were responsible for the superoxide formation in this fraction. The presence of 5'-nucleotidase in the fraction indicates the involvement of the plasma membrane in the formation of the phagosomes, and the presence of myeloperoxidase and β -glucuronidase indicates the formation of phagolysosomes.

Change in the superoxide-forming activity during phagocytosis

Fig. 1 shows that the specific activity of the superoxide formation in the vesicle fraction in-

creased for the first 5 min and then decreased sharply with prolonged incubation. A similar decrease in the activity was observed in the homogenate. The amount of proteins in the fraction increased even after 5 min, indicating that the ingestion process was continuing, while the superoxide-forming activity decreased, probably by some inactivation process.

Extraction of the superoxide-forming activity

We tried to extract the NADPH-dependent superoxide-forming activity of the vesicle fraction by using various detergents (Table 3). Among the detergents used, the Emalgen series (including Triton X-100), the Brij series and cationic detergents inhibited the activity when they were added to the vesicle fraction, whereas bile salts enhanced the activity. Detergents of the Tween series neither enhanced nor inhibited the activity. By centrifugation of the vesicle/detergent mixture at 117 000 *g* for 1 h, a clear solution with high NADPH-dependent superoxide-forming activity could be obtained. The yield of the activity in the extract, however, was less than 20% when a single detergent was used, and better results were obtained by the combined use of detergents, one from bile salts and another from the Tween series. The optimal procedure for the extraction so far obtained was treatment of the vesicle fraction with 0.5% (w/v) deoxycholate, 0.5% (v/v) Tween 20, 25% (v/v) ethylene glycol and 0.17 M-sucrose in 50 mM-glycine buffer, pH 8.5, at about 1 mg of protein/ml. The conclusion was based on the results shown in Fig. 2, which gives the activity of the vesicle/detergent mixture and the yield of the activity in the extract, at various concentrations of Tween 20, deoxycholate and ethylene glycol, and

Table 2. *Distribution of enzyme activities in leucocyte subcellular fractions*

The cells were incubated with oil droplets in the presence of KCN and homogenized. Three fractions were obtained by centrifugation: one floating on the top (vesicles), one remaining in buffered 0.34 M-sucrose solution (cytosol) and one sedimenting to the bottom (sediment). Values are activities and amount of protein expressed as percentages of those in the homogenate (the means $\pm$ S.D. of three independent experiments).

	Activities and amount of proteins (% of those in homogenate)		
	Vesicles	Cytosol	Sediment
$\text{O}_2^{\cdot -}$ formation (NADPH)	71.1 $\pm$ 1.0	0.6 $\pm$ 0.1	24.0 $\pm$ 1.3
$\text{O}_2^{\cdot -}$ formation (NADH)	33.6 $\pm$ 1.6	0.7 $\pm$ 0.1	58.9 $\pm$ 1.4
5'-Nucleotidase	30.1 $\pm$ 1.5	10.8 $\pm$ 1.9	71.7 $\pm$ 3.8
Myeloperoxidase	15.7 $\pm$ 0.7	17.1 $\pm$ 0.2	61.3 $\pm$ 1.8
β -Glucuronidase	7.8 $\pm$ 1.1	14.1 $\pm$ 1.3	79.4 $\pm$ 2.2
Alkaline phosphatase	11.5 $\pm$ 0.7	28.6 $\pm$ 0.8	63.0 $\pm$ 0.8
Glucose 6-phosphatase	<0.1	23.6 $\pm$ 2.8	79.1 $\pm$ 5.9
Succinate-cytochrome <i>c</i> reductase	<0.1	16.0 $\pm$ 1.3	80.6 $\pm$ 3.0
Lactate dehydrogenase	<0.1	95.0 $\pm$ 2.3	11.4 $\pm$ 2.3
Protein	11.0 $\pm$ 1.2	52.3 $\pm$ 3.1	36.8 $\pm$ 2.4

Fig. 1. Activation and inactivation of the NADPH-dependent superoxide-forming activity in the vesicle fraction

Leucocytes (2.5×10^9) in 12 ml of phosphate Ringer solution, pH 7.4, containing 2 mM-glucose were incubated at 37°C with (●, ■) or without (○, □) 1 mM-KCN, and 2.9 ml of the oil droplets were added at zero time. After the times indicated on the abscissa, the vesicle fractions were prepared from the 15 ml suspensions and the amounts of proteins (□, ■) and the NADPH-dependent superoxide-forming activities (○, ●) in the vesicle fractions were measured. Each point represents the mean of three independent experiments.

pH, with other conditions kept fixed. When the vesicle-detergent mixture was kept at 4°C for 1 h and centrifuged at 117000 *g* for 1 h, half of the activity was in the extract, but 40% remained in the floating layer and 10% in the sediment. By repeating the extraction four times, we obtained a clear preparation with a good yield of the activity ($96 \pm 3\%$, mean $\pm$ S.D., $n = 4$).

Catalytic properties

Table 4 shows kinetic parameters of the NAD(P)H-dependent superoxide-forming reac-

tions. K_m values of both vesicles and extract for reduced coenzymes were essentially the same, and the values for NADPH were about one-tenth of those for NADH. The activities of the extract were about 10 times those of the vesicle fraction. The addition of cyanide during phagocytosis enhanced the activity, but it did not affect the K_m values for the coenzymes. The activities in the presence of 0.5 mM-NADPH and 1.5 mM-NADPH were 1018 ± 11 and 235 ± 7 nmol/min per mg of protein ($n = 4$) respectively, whereas the activity in the presence of both substrates was 1024 ± 12 nmol/min per mg of protein ($n = 4$), suggesting that the NADPH- and NADH-dependent activities share a common pathway. The optimal pH of the vesicle fraction was 7.5, whereas that of the extract was 8.0. The activities in the extract were insensitive to cyanide.

The activities of the extract were enhanced about 2-fold by the addition of $1 \mu\text{M}$ -FAD, whereas those of the vesicle fraction were not affected. The apparent K_m value for FAD was $0.077 \mu\text{M}$. FMN at a similar concentration did not enhance the activity.

Stoichiometric relations

The ratio between superoxide formation and NADPH oxidation by both vesicle fraction and extract was 2:1 as shown in Table 5. The consumption of oxygen was essentially negligible in the presence of cytochrome *c* because of the regeneration of oxygen molecules. The consumption was variable in the absence of cytochrome *c*, owing to the dismutation of superoxide and catalytic reactions, but if it was measured in the presence of superoxide dismutase and catalase, the ratio of NADPH oxidation to oxygen consumption was 2:1. Thus superoxide formation follows reaction (1) and oxygen consumption follows the sum of the reactions (1)–(3) (reaction 4).

Stability of the superoxide-forming activity

Activities in both vesicle fraction and extract were extremely labile. The activity in the vesicle fraction was decreased to half in 2 min at 37°C. Thus the assay was performed at 20°C. The activity in the vesicle fraction was most stable at pH 7.5 and that in the extract at pH 8.5. They were more susceptible to acidic pH than to alkaline pH. The activity in the vesicle fraction decreased to half in 12 h at 4°C and that of the extract in about 24 h. At -20°C , the vesicle fraction could be kept for more than 1 week,

Fig. 2. Extraction of the NADPH-dependent superoxide-forming activity

The vesicle fraction was treated with detergents (at 0.7 mg of protein/ml) as described in the Experimental section, except that concentrations of Tween 20 (a), deoxycholate (b) and ethylene glycol (c), and pH (d), were varied as shown on the abscissae, and centrifuged. The activity of the vesicle fraction suspended in 0.34 M-sucrose solution (0.7 mg of protein/ml) was 60 nmol/min per ml. HEPES buffer was used for adjusting the pH between 7 and 8. Each point represents the mean of four experiments. ●, NADPH-dependent superoxide-forming activity of the extract; ○, yield (activity in the extract as a percentage of that in the vesicle/detergent mixture).

Table 3. *Extraction of NADPH-dependent superoxide-forming activities from the vesicle fraction*

The vesicle/detergent mixture, consisting of the vesicle fraction (0.7 mg of protein/ml), 1% detergent, 25% ethylene glycol, 0.17 M-sucrose in 50 mM-glycine buffer, pH 8.5, was centrifuged at 117000 *g* for 1 h. The NADPH-dependent superoxide-forming activities of both mixture and extract (supernatant) were assayed. When two detergents were used in combination, each concentration was 0.5%. Yield represents percentage of the activity in the extract to that in the mixture. The values are the means $\pm$ S.D. for three experiments.

	Specific activities (nmol/min per mg of protein)		Yield (%)
	Vesicle/detergent mixture	Extract	
Control	86.3 $\pm$ 1.3		
+ cholate	134 $\pm$ 4	253 $\pm$ 13	15.1 $\pm$ 1.1
+ deoxycholate	251 $\pm$ 7	267 $\pm$ 20	17.1 $\pm$ 1.2
+ Triton X-100	56.1 $\pm$ 5.2	97.4 $\pm$ 10	16.5 $\pm$ 0.9
+ Tween 20	95.0 $\pm$ 4.8	196 $\pm$ 12	22.7 $\pm$ 1.3
+ Tween 40	86.1 $\pm$ 8	151 $\pm$ 8	20.2 $\pm$ 1.1
+ Tween 60	77.7 $\pm$ 6.4	144 $\pm$ 14	18.9 $\pm$ 1.5
+ Tween 80	86.4 $\pm$ 5.7	125 $\pm$ 10	16.3 $\pm$ 0.8
+ deoxycholate + Tween 20	184 $\pm$ 3	1019 $\pm$ 27	50.4 $\pm$ 1.2
+ deoxycholate + Tween 80	151 $\pm$ 5	466 $\pm$ 11	43.2 $\pm$ 1.9
+ cholate + Tween 20	138 $\pm$ 7	257 $\pm$ 7	18.6 $\pm$ 1.0

Table 4. *Kinetic parameters of the superoxide-forming activities in the vesicle fraction and the extract*

The absorbance changes ($A_{550} - A_{540}$) were determined in reaction mixtures consisting of 50 mM-potassium phosphate buffer (pH 7.5 for the vesicle fraction and pH 8.0 for the extract), 100 μ M-acetylated cytochrome *c* and various concentrations of NAD(P)H (0.02–1.2 mM-NADPH or 0.1–2 mM-NADH) at 20°C in a total volume of 1 ml. The reaction was started by addition of the sample (15–30 μ g of protein of the vesicle fraction or 5–10 μ g of protein of the extract). Activities are expressed as nmol of superoxide formed/min per mg of protein. The values in parentheses were those for the samples from the cells incubated without KCN. The results represent the means $\pm$ S.D. for four independent experiments.

		Vesicle fraction		Extract	
		NADPH	NADH	NADPH	NADH
K_m (μ M)		35.1 $\pm$ 4.9	(36.5 $\pm$ 1.3)	38.8 $\pm$ 2.3	(37.0 $\pm$ 0.5)
		401 $\pm$ 15	(389 $\pm$ 11)	383 $\pm$ 22	(391 $\pm$ 24)
V_{max} (nmol/min per mg of protein)	NADPH	101 $\pm$ 7.6	(47.4 $\pm$ 2.4)	1086 $\pm$ 17	(478 $\pm$ 19)
	NADH	28.8 $\pm$ 2.2	(14.3 $\pm$ 1.8)	252 $\pm$ 16	(113 $\pm$ 11)

Table 5. *Stoichiometric relation of NADPH-dependent superoxide formation, NADPH oxidation and oxygen consumption*

The assay procedures were given in the Experimental section. The assays were performed at 20°C, that of superoxide formation by the reduction of acetylated cytochrome *c*, that of NADPH oxidation by the decrease of NADPH and the oxygen consumption polarographically in the presence of superoxide dismutase and catalase. Each value represents the mean $\pm$ S.D. for four experiments.

	Activities (nmol/min per mg of protein)		
	O ₂ ^{-•} formation	NADPH oxidation	O ₂ consumption
Vesicles	80.5 $\pm$ 1.9	41.0 $\pm$ 3.8	19.2 $\pm$ 0.3
Extract	988 $\pm$ 14	495 $\pm$ 20	231 $\pm$ 5.5

but the extract lost half of the activity in 4 days. The loss of the activities was not observed when kept at -70°C.

Discussion

The main aim of this paper is to describe a procedure to obtain highly active preparations of the NADPH-dependent superoxide-forming system of leucocytes in good yield. Several reports have appeared on the preparations with superoxide-forming activity, but none of them to date was as active as the preparations reported in this paper: for example, 51 (at 37°C) by Lew & Stossel (1981) for the vesicle fraction and 51 by Gabig & Babior (1979) for the extract, compared with about 100 for the vesicle fraction and about 1000 for the extract in

the present paper (activity expressed as nmol/min per mg of protein). The advantages of the present method are as follows. (1) Pig leucocytes, which could be obtained in large quantity, were used. (2) An oil-droplet preparation consisting of 1,2,3,6-tetrahydrophthalic acid di-isodecyl ester and immunoglobulin G was used, and phagocytosis was carried out in the presence of cyanide. Opsonization with immunoglobulin G stimulates the vesicle formation and enhances the yield of the vesicle fraction (Amano & Mizuno, 1980). The tetrahydrophthalic acid ester has suitable physical properties for separating the cells from the oil droplets and the vesicles from other particulate components. (3) Two types of detergents, anionic and non-ionic, were used in combination.

We have successfully extracted the superoxide-forming NADPH oxidase of leucocytes as an apparently clear solution, using a mixture of detergents, deoxycholate and Tween 20. The effectiveness of the mixture agrees with the statement of Tanford & Reynolds (1976) that mixed micelles containing both non-ionic and anionic detergents are good solubilizing agents and may imply that the NADPH oxidase requires an anionic hydrophobic environment for its activity. Deoxycholate, which has a lower critical micelle concentration than cholate, is more effective than the latter, and non-ionic detergents with high hydrophilic-lipophilic balance (HLB) number and a bulk conformation seem to be effective in the extraction.

The superoxide-forming NADPH oxidase can be regarded as responsible for the phagocytic respiratory burst of leucocytes. It is essentially absent from the resting cells, is localized in phagocytic vesicles and is insensitive to cyanide. The NADH-dependent activity always accompanied the NADPH-dependent reaction, but the K_m of the former was 10 times that of the latter, and the activity of the former was one-fifth of that of the latter, and both reactions seemed to be catalysed by a common system. The low K_m values and high V_{max} values of the preparations with NADPH, and consideration of intracellular concentrations of reduced coenzymes (0.1–0.2 mM: Baehner *et al.*, 1972; Selvaraj & Sbarra, 1967; Suzuki & Lehrer, 1980), led us to the conclusion that the preferential electron donor for the superoxide formation in the cells is NADPH. The activity of the extract was stimulated by the addition of FAD, in agreement with the observation of Babior & Peters (1981), though Tauber & Goetzl (1979) obtained a negative result. A *b*-type cytochrome was present in the vesicle fraction, in agreement with the report of Segal & Jones (1980), and a part of it could be found in the extract. Studies on both catalytic and molecular properties of this physiologically important enzyme system are required.

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