Biochem. J. (1987) 242, 103–107 (Printed in Great Britain)

The inhibition by diphenyleneiodonium and its analogues of superoxide generation by macrophages

John T. HANCOCK and Owen T. G. JONES
Department of Biochemistry, University of Bristol Medical School, Bristol BS8 1TD, U.K.

Peritoneal macrophages were elicited in rats by using casein as a stimulus; when stimulated with phorbol 12-myristate 13-acetate (PMA) they produced $O_2^{\cdot-}$. Nearly 60% of the total cytochrome *b* had a low $E_{m,7.0}$ of −247 mV, typical of the cytochrome *b* component found in the NADPH-dependent $O_2^{\cdot-}$-generating oxidase of neutrophils. The rate of $O_2^{\cdot-}$ generation by macrophages was 1.23 mol of $O_2^{\cdot-}$/s per mol of cytochrome *b*. Treatment of intact macrophages with diphenyleniodonium (DPI) at 0.9 μM caused 50% inhibition of PMA-induced $O_2^{\cdot-}$ generation, with little effect on mitochondrial respiratory activity; KCN inhibited respiratory activity without affecting PMA-induced $O_2^{\cdot-}$ generation. A similar specificity of inhibition was found for di-2-thienyliodonium (50% inhibition of $O_2^{\cdot-}$ generation at 0.5 μM) and, at higher concentrations, for diphenyl iodonium. When macrophage suspensions were incubated with [^{125}I]DPI followed by autoradiography of SDS/polyacrylamide-gel-electrophoresis-separated polypeptides, radioactivity was most strongly associated with a band of M_r 45000, similar to that found in neutrophils [Cross & Jones (1986) Biochem. J. **237**, 111–116]. The $O_2^{\cdot-}$-generating oxidase of macrophages appears to have components in common with the NADPH oxidase of neutrophils, despite differences in activity. Its sensitivity to DPI suggests that selective prevention of radical generation by macrophages *in vivo* is possible.

INTRODUCTION

Macrophages in the tissues and granulocytes in the blood form part of the microbicidal armoury of mammals and contribute to the defence of the individual against infection. Both cell types produce activated oxygen species, such as superoxide ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2) and hydroxyl radicals ($OH^{\cdot}$), in response to phagocytic stimuli, and these participate in the killing process. The components of the superoxide-generating system are not completely established, but in polymorphonuclear leucocytes (neutrophils) it is apparent that the electron donor is NADPH (Rossi & Zatti, 1964) and that a low-potential cytochrome *b* (cytochrome *b*-245) is involved (Cross *et al.*, 1981, 1985) together with an FAD–protein (Light *et al.*, 1981; Cross *et al.*, 1983, 1985). Kinetic evidence (Cross *et al.*, 1985) suggests that these are arranged in the neutrophil plasma membrane so that electrons flow from NADPH to O_2 via FAD and cytochrome *b*. Recently it has been shown that DPI is a potent inhibitor of the neutrophil NADPH oxidase and binds specifically to a polypeptide (M_r 45000) of the plasma membrane (Cross & Jones, 1986). A peptide of similar M_r is phosphorylated when the NADPH oxidase of neutrophils is activated (Gennaro *et al.*, 1985), and is altered or absent from the neutrophils of patients with the autosomal recessive form of chronic granulomatous disease, where NADPH oxidase activity is absent (Segal *et al.*, 1985).

The superoxide-generating system of macrophages is less well characterized. NADPH acts as its electron donor (Romeo *et al.*, 1971; Tsunawaki & Nathan, 1984), and a low-potential cytochrome *b* has been found in macrophages from humans (Segal *et al.*, 1981), guinea pigs (Berton *et al.*, 1985) and mice (Berton *et al.*, 1986). We show in the present paper that the NADPH oxidase of macrophages, like that of neutrophils, is sensitive to inhibition by low concentrations of DPI and its analogues. The concentrations inhibitory to NADPH oxidase are lower than those that are required for inhibition of mitochondrial electron transport. This selective inhibition by DPI can be clearly demonstrated in macrophages, which, unlike neutrophils, have active mitochondrial respiration, and suggests that DPI and its analogues will be valuable tools in the characterization and, perhaps, control of superoxide-generating systems.

METHODS AND MATERIALS

Chemicals

DPI sulphate was synthesized as described by Collette *et al.* (1956) and was standardized by using $\epsilon_{264} = 15.1\ mM^{-1} \cdot cm^{-1}$ (Ragan & Bloxham, 1977). [^{125}I]DPI was synthesized as described previously (Gatley & Sherratt, 1976), but was converted into the acetate salt by the addition of barium acetate. Na^{125}I was obtained from Amersham International, Amersham, Bucks., U.K. Diphenyliodonium and di-2-thienyliodonium were generously given by Dr. F. Earley, Department of Biochemistry, University of Southampton, Southampton, U.K. Stock solutions of these inhibitors were made in ethanol. At the concentrations used the ethanol had no effect on the respiratory activity of the macrophages.

Stock solutions of PMA were made in dimethyl sulphoxide to a concentration of 1 mg/ml.

Preparation of macrophages

Intraperitoneal macrophages were elicited in male Wistar rats with a single intraperitoneal injection of 8 ml of 12% (w/v) sodium caseinate as described for mice by Tsunawaki & Nathan (1984) and Johnston *et al.* (1978).

Abbreviations used: DPI, diphenyleneiodonium; PMA, phorbol 12-myristate 13-acetate.

Then 48 h later the contents of the peritoneal cavities were were washed with two portions of phosphate-buffered saline containing 130 mM-NaCl, 2.6 mM-KCl, 8.1 mM-Na_2HPO_4, 1.4 mM-KH_2PO_4 and 0.4% sodium citrate. The cells were pelleted by centrifugation at 400 *g* for 15 min at 4 °C, washed and layered on to 60% Percoll (Sigma Chemical Co.), and centrifuged at 400 *g* for 15 min at 4 °C, and the cells at the interface were collected by aspiration. Contaminating erythrocytes were removed by hypo-osmotic lysis in 0.2% NaCl containing 1 mM-EDTA and 5 μg of heparin/ml; the iso-osmoticity was restored by addition of an equal volume of 1.6% (w/v) NaCl.

Cell purity was checked by the M+D Diff-Quik staining technique [Merz–Dade A.G.; from AHS (U.K.) Ltd., Didcot, Oxon., U.K.); the main contaminants were lymphocytes (approx. 10%); these did not contribute to $O_2^{\cdot-}$ generation or to the content of the low-$E_{m,7.0}$ cytochrome *b*. Neutrophil contamination was negligible.

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

Superoxide production was determined by the superoxide dismutase-inhibited reduction of cytochrome *c* as described previously (Cross *et al.*, 1982). The incubation mixture contained 100 μM horse heart cytochrome *c* (Sigma type III) in Krebs–Ringer buffer, pH 7.4 (Cross *et al.*, 1984), at 37 °C.

Determination of O_2 concentration

O_2 concentrations were determined with a Clark-type oxygen electrode at 37 °C.

Spectroscopy

Spectra were recorded on a rapid-scanning recording spectrophotometer with the facility for averaging, as described previously (Cross *et al.*, 1984). Potentiometric titrations were performed on the same spectrophotometer by using a method described previously (Cross *et al.*, 1981).

Protein determinations

Protein concentrations were determined as described by Bramhall *et al.* (1969) or as described by Bradford (1976), with bovine serum albumin as standard.

SDS/polyacrylamide-gel electrophoresis

SDS/polyacrylamide-gel electrophoresis and autoradiography of the gels was carried out as described by Cross & Jones (1986).

RESULTS AND DISCUSSION

An oxidized-minus-reduced difference spectrum of rat peritoneal-macrophage homogenate reveals absorption bands around 428, 445, 559 and 604 nm, characteristic of mitochondrial cytochromes (Fig. 1*a*). The contribution of a low-potential cytochrome *b* to this composite spectrum was determined by measuring difference spectra between known low oxidation–reduction potentials (Fig. 1*b*), in this example between −159 mV and −343 mV. It was found that nearly 60% of the mixture of cytochromes *b*, measured at 559 nm minus 540 nm, was a component with mid-point potential more negative than −160 mV, giving a concentration of low-potential cytochrome *b* of 96 pmol/mg of protein. In separate experiments it was found by potentiometric

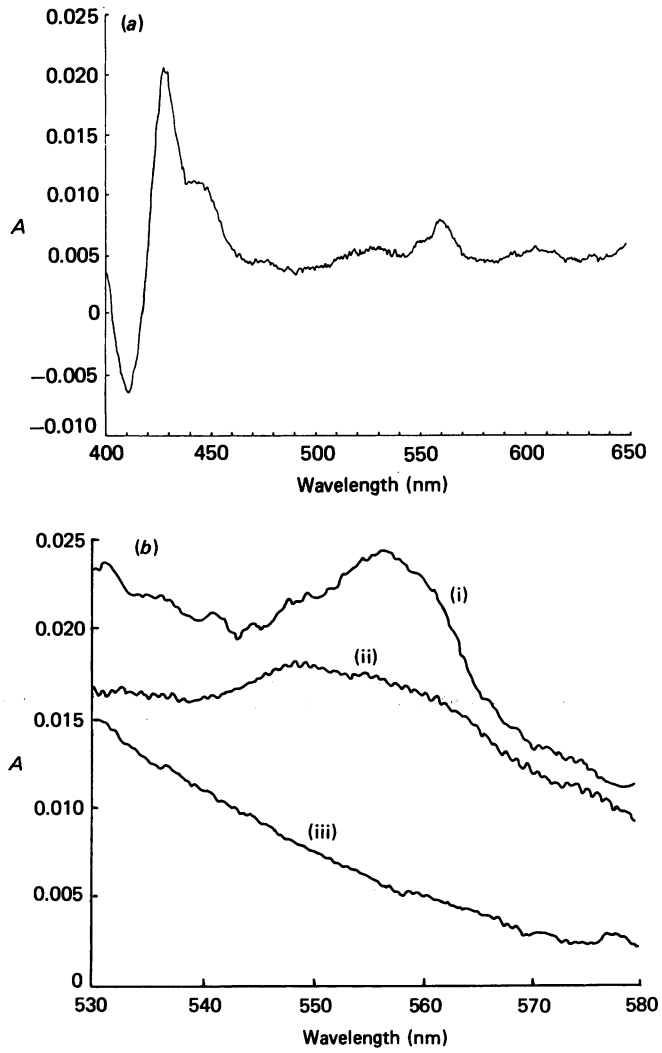

Fig. 1. Absorbance spectra of macrophage homogenates

Spectra were recorded on a rapid-scanning recording spectrophotometer. (*a*) Rat peritoneal macrophages were suspended in phosphate-buffered saline and disrupted by sonication (2 × 15 s at 50 W). Complete reduction was obtained by addition of a few crystals of $Na_2S_2O_4$; the oxidized spectrum was subtracted. The protein concentration was 7.1 mg/ml. (*b*) The membrane fraction was pelleted by centrifugation and resuspended in 50 mM-Mops/NaOH buffer, pH 7.0, containing 100 mM-KCl. The sample was made anaerobic by gassing with argon, and spectra were recorded in the presence of redox mediators: (i) recorded at $E_h = -343$ mV: (ii) recorded at $E_h = -159$ mV; (iii) recorded at $E_h = +380$ mV (Cross *et al.*, 1981). E_h was lowered by addition of $Na_2S_2O_4$ solution or raised by the addition of $K_3Fe(CN)_6$ solution. The protein concentration was 2.5 mg/ml.

titration that its mid-point oxidation–reduction potential at pH 7.0 ($E_{m,7.0}$) was −247 mV (details not shown). This is in reasonable agreement with values of −255 mV and −244 mV found by Berton *et al.* (1986) using Phenosafranin dye titration of mouse peritoneal macrophages and with the value of −245 mV for human and pig neutrophil cytochrome *b* (Cross *et al.*, 1981).

In pig neutrophils this low-potential cytochrome *b* behaves kinetically as a component of the NADPH

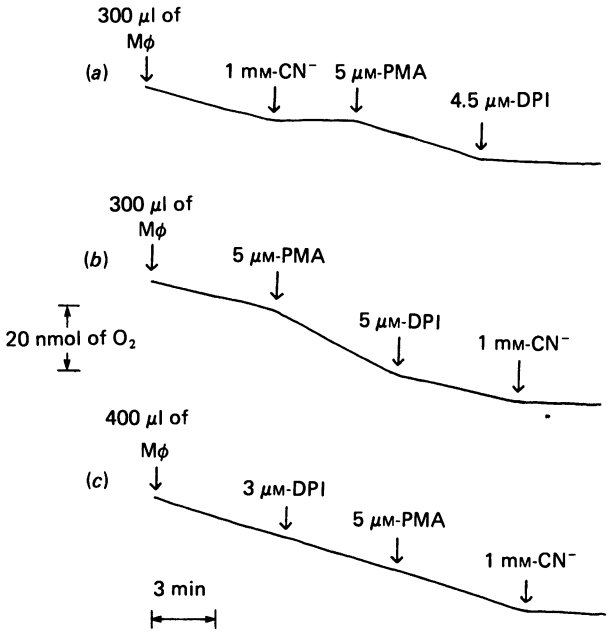

Fig. 2. Effects of DPI and CN^- on respiration and on the PMA-stimulated O_2 uptake of intact macrophages

O_2 uptake was recorded by using a Clark-type oxygen electrode with the macrophages suspended in Krebs–Ringer buffer. For experiments (*a*) and (*b*) the protein concentration was 0.68 mg/ml; the protein concentration in experiment (*c*) was 1.2 mg/ml. All additions indicated by arrows; Mφ indicates addition of macrophages.

oxidase transferring electrons between NADPH and O_2 (Cross *et al.*, 1985), and our results suggest that an NADPH oxidase of very similar composition is likely to be present in rat macrophages, although its rate of $O_2^{\cdot-}$ generation (1.23 mol of $O_2^{\cdot-}$/s per mol of cytochrome *b*) is much lower than that found for soluble pig NADPH oxidase (13.03 mol of $O_2^{\cdot-}$/s per mol of cytochrome *b*).

Selective inhibition by DPI of NADPH oxidase but not mitochondrial respiration

NADPH oxidase of neutrophils is inhibited by low concentrations of DPI (Cross & Jones, 1986). We show in Fig. 2 the effect of DPI upon O_2 uptake by intact macrophages. Mitochondrial respiration was first blocked by CN^-, and the NADPH oxidase was activated by the addition of a soluble stimulus, PMA. Addition of DPI inhibited this respiratory burst (Fig. 2*a*). If intact macrophages were stimulated with PMA the enhancement of O_2 uptake arising from NADPH oxidase was prevented by the addition of DPI; residual O_2 uptake due to the respiratory activity of mitochondria could then be abolished by the addition of CN^- (Fig. 2*b*). Cells that were pretreated with DPI did not give enhanced O_2 uptake when PMA was added, and their respiratory activity was abolished by CN^- (Fig. 2*c*).

In separate experiments added DPI (20 μM) prevented PMA-induced reduction of cytochrome *b* in anaerobic macrophage suspensions.

The effects of increasing concentrations of DPI on the NADPH oxidase and mitochondrial respiratory activity of macrophages are shown in Fig. 3. The PMA-stimulated O_2-uptake $O_2^{\cdot-}$ production was measured at

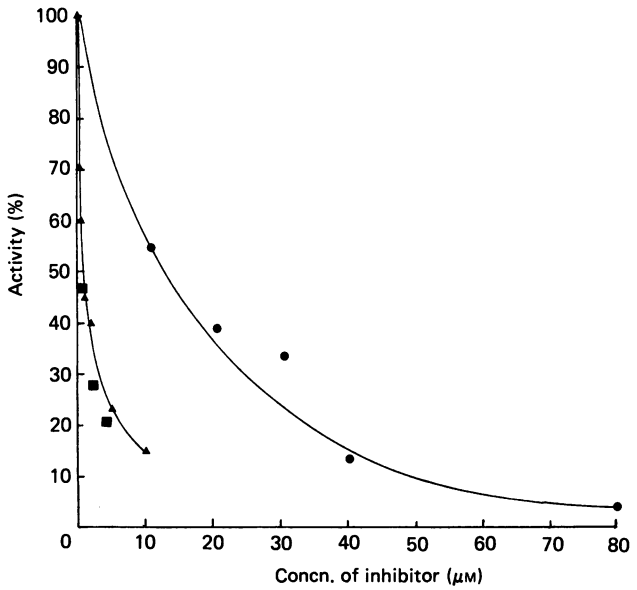

Fig. 3. Effects of increasing concentrations of DPI on respiration and PMA-stimulated O_2 production of intact peritoneal macrophages

Intact macrophages were suspended in Krebs–Ringer buffer and the production of $O_2^{\cdot-}$ was measured by the superoxide dismutase-inhibited reduction of cytochrome *c* after stimulation with 1 μM-PMA (▲). The protein concentration was 0.15 mg/ml. PMA-stimulated O_2 uptake was also measured as an indication of $O_2^{\cdot-}$ production at a protein concentration of 1.8 mg/ml (■). Also, the rate of mitochondrial respiration of intact macrophages suspended in Krebs–Ringer buffer was measured with an oxygen electrode (●); the protein concentration was 1.5 mg/ml.

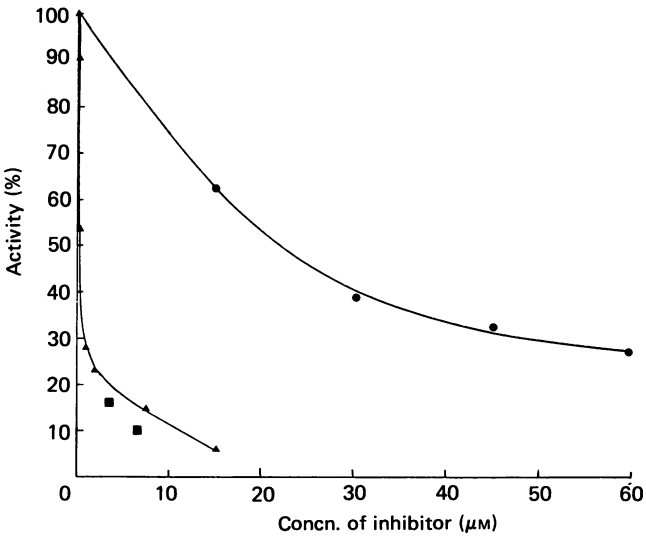

Fig. 4. Effects of increasing concentrations of di-2-thienyliodonium on respiration and the PMA-stimulated O_2 production of intact intraperitoneal macrophages

The procedure was the same as that for Fig. 3 except that di-2-thienyliodonium was substituted for DPI. The protein concentrations were 0.15 mg/ml (▲), 0.72 mg/ml (●) and 1.8 mg/ml (■).

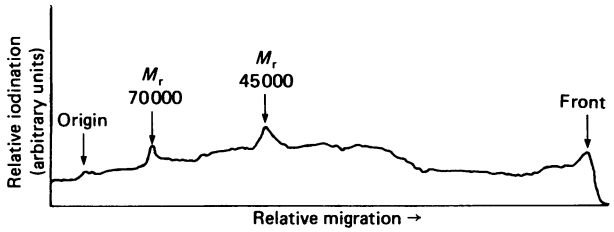

Fig. 5. Labelling of polypeptides by [^{125}I]DPI

Macrophages were suspended in phosphate-buffered saline and disrupted by sonication (2 × 15 s at 50 W). A 100 μg portion of the homogenate was incubated with [^{125}I]DPI for 10 min as described by Cross & Jones (1986), and the proteins were separated by SDS/polyacrylamide-gel electrophoresis. The developed gel was dried overnight and autoradiographed against Fuji-Rx film for 10 days, and the film was monitored with a gel scanner. After autoradiography the gels were rehydrated and stained in the conventional manner. The following M_r markers were run: bovine albumin (M_r 66000); egg albumin (M_r 45000); glyceraldehyde-3-phosphate dehydrogenase (M_r 36000); carbonic anhydrase (M_r 29000); trypsinogen (M_r 24000); trypsin inhibitor (M_r 20100; lactalbumin (M_r 14200).

high and low protein concentrations. There was a significant difference in the concentrations of DPI required for 50% inhibition of NADPH oxidase (0.9 μM) and for 50% inhibition of respiration (13 μM). There was little effect of changing the protein concentration at the ranges tested. Di-2-thienyliodonium was even more selective in its effects on NADPH oxidase (Fig. 4), with 50% inhibition at 0.5 μM compared with 50% inhibition of respiration at 21 μM. Diphenyliodonium was less potent as an inhibitor of O_2-uptake activity, but still favoured inhibition of NADPH oxidase activity (50% inhibition at 80 μM) over respiration (50% inhibition at 120 μM).

It was found in separate experiments that the enhanced uptake of O_2 by macrophages after treatment with PMA was accompanied by the production of an equivalent yield of $O_2^{\cdot-}$.

Binding of [^{125}I]DPI to macrophage peptides

The pattern of inhibition of NADPH oxidase and of the mitochondrial oxidase of macrophages by DPI resembled that found by Cross & Jones (1986) for the soluble NADPH oxidase of neutrophils and by Ragan & Bloxham (1977) for the inhibition of NADH:ubiquinone reductase of mitochondria. In both cases it was found that radiolabelled DPI could be used to label specific polypeptide components of these oxidase systems. We incubated suspensions of macrophages with [^{125}I]DPI at 20 °C for 10 min, and analysed the nature of labelling of the protein fraction by SDS/polyacrylamide-gel electrophoresis, followed by autoradiography. Radioactivity was most strongly associated with a polypeptide of M_r 45000 (Fig. 5). Radioactivity was also present in a faint band of M_r about 70000 and at the tracking dye front, presumably associated with lipid or with SDS^- anions (Ragan & Bloxham, 1977).

In a previous paper Cross & Jones (1986) showed that DPI was a very potent inhibitor of the soluble NADPH oxidase prepared from activated neutrophil plasma membranes. It prevented the reduction by NADPH of both the flavin and the cytochrome *b* components of the oxidase in aerobic conditions. The DPI covalently labelled a polypeptide of M_r 45000, and the extent of labelling was diminished by the addition of NADPH, suggesting that this polypeptide might be the flavoprotein electron acceptor from NADPH. Our experiments show that DPI is similarly effective against the PMA-stimulated respiratory burst of macrophages and labels a peptide of the same M_r. We can conclude that the NADPH oxidase of macrophages has two components in common with the NADPH oxidase of neutrophils, namely the cytochrome *b* with $E_{m,7.0}$ −245 mV and a polypeptide of M_r 45000.

The relative potency of the three inhibitors tested in our study of the macrophage respiratory burst mirrored that found by Cross (1987) for their effects on the solubilized NADPH oxidase of neutrophils, and is in agreement with our suggestion that the NADPH oxidases of the two cell types are very similar. Such compounds may prove useful when considering the design of anti-inflammatory drugs intended to act by diminishing the production of active oxygen species at inflammatory sites. Experiments with macrophages are likely to be valuable in distinguishing between anti-inflammatory effects, mediated by inhibition of the generation of oxygen radicals, and the known hypoglycaemic effects of these compounds, mediated through inhibition of mitochondrial NADH:ubiquinone reductase (Bloxham, 1979). For di-2-thienyliodonium the ratio of these inhibitory activities encourages the view that inhibitors of NADPH oxidase can be designed that have little effect upon mitochondria.

This work was supported by grants from the Medical Research Council and the Arthritis and Rheumatism Council. We are grateful to Dr. A. R. Cross for his advice and the gift of DPI and [^{125}I]DPI and to Dr. C. I. Ragan and Dr. F. Earley for the gift of iodonium compounds.

REFERENCES

Berton, G., Papini, E. Cassatella, M. A., Bellavite, P. & Rossi, F. (1985) Biochim. Biophys. Acta **810**, 164–173
Berton, G., Cassatella, M. A., Bellavite, P. & Rossi, P. (1986) J. Immunol. **136**, 1393–1399
Bloxham, D. P. (1979) Biochem. Soc. Trans. **7**, 103–106
Bradford, M. M. (1976) Anal. Biochem. **72**, 248–254
Bramhall, S., Noack, N., Wu, N. & Loewenberg, J. R. (1969) Anal. Biochem. **31**, 146–148
Collette, J., McGreer, D., Crawford, R., Chubb, F. & Sandin, R. D. (1956) J. Am. Chem. Soc. **78**, 3819–3820
Cross, A. R. (1987) Biochem. Pharmacol., in the press
Cross, A. R. & Jones, O. T. G. (1986) Biochem. J. **237**, 111–116
Cross, A. R., Jones, O. T. G., Harper, A. M. & Segal, A. W. (1981) Biochem. J. **194**, 599–606
Cross, A. R., Higson, F. K., Jones, O. T. G., Harper, A. M. & Segal, A. W. (1982) Biochem. J. **204**, 479–485
Cross, A. R., Jones, O. T. G., Garcia, R. & Segal, A. W. (1983) Biochem. J. **216**, 765–768
Cross, A. R., Parkinson, J. F. & Jones, O. T. G. (1984) Biochem. J. **223**, 337–344
Cross, A. R., Parkinson, J. F. & Jones, O. T. G. (1985) Biochem. J. **226**, 881–884
Gatley, J. S. & Sherratt, M. S. A. (1976) Biochem. J. **158**, 307–315
Gennaro, R., Floris, C. & Romeo, D. (1985) FEBS Lett. **180**, 185–190

Johnston, R. B., Godzik, C. A. & Cohn, Z. A. (1978) J. Exp. Med. **148**, 115–127
Light, D. R., Walsh, C., O'Callaghan, A. M., Goetzl, E. J. & Tauber, A. I. (1981) Biochemistry, **20**, 1468–1476
Ragan, C. I. & Bloxham, D. P. (1977) Biochem. J. **163**, 605–615
Romeo, D., Zabbuchi, G., Soranzo, M. R. & Rossi, F. (1971) Biochem. Biophys. Res. Commun, **45**, 1056–1062
Rossi, F. & Zatti, M. (1964) Br. J. Exp. Pathol. **45**, 548–559
Segal, A. W., Garcia, R., Goldstone, A. H., Cross, A. R. & Jones, O. T. G. (1981) Biochem. J. **196**, 363–367
Segal, A. W., Heyworth, P. G., Cockcroft, S. & Barrowman, M. M. (1985) Nature (London) **318**, 547–549
Tsunawaki, S. & Nathan, C. F. (1984) J. Biol. Chem. **259**, 4305–4312

Received 21 July 1986/1 September 1986; accepted 21 October 1986