Br. J. Pharmacol. (1991), 102, 234–238

Identification of N-iminoethyl-L-ornithine as an irreversible inhibitor of nitric oxide synthase in phagocytic cells

T.B. McCall, [1]M. Feelisch, R.M.J. Palmer & [2]S. Moncada

Wellcome Research Laboratories, Langley Court, Beckenham, Kent BR3 3BS

1 The synthesis of nitric oxide (NO) from L-arginine by rat peritoneal neutrophils (PMN) and the murine macrophage cell-line J774 and the inhibition of this synthesis by N-iminoethyl-L-ornithine (L-NIO), N^G-monomethyl-L-arginine (L-NMMA), N^G-nitro-L-arginine (L-NNA) and its methyl ester (L-NAME) were investigated.

2 L-NIO was the most potent inhibitor in both types of cells while L-NMMA was less active. L-NNA and L-NAME had no significant effect in PMN and L-NNA produced only approximately 40% inhibition of the generation of NO in the J774 cells at the highest concentration tested (300 μM).

3 The inhibitory effect of L-NIO was rapid in onset, requiring 10 min pre-incubation to achieve its full inhibitory activity, while the other compounds required 20–60 min pre-incubation to achieve their full effect.

4 The inhibitory effect of L-NIO (10 μM) on intact cells could not be reversed by L-arginine (300 μM) but could be prevented by concomitant incubation with this compound (300 μM), while the effect of the other inhibitors could be reversed by a 3–5 fold molar excess of L-arginine.

5 The NO synthase from both PMN and J774 cells was cytosolic and NADPH- but not Ca^{2+}-dependent, with K_m values for L-arginine of 3.3 $\pm$ 0.8 and 4.2 $\pm$ 1.1 μM respectively.

6 L-NIO was the most potent inhibitor of the neutrophil and J774 enzymes with IC_{50} values of 0.8 $\pm$ 0.1 and 3 $\pm$ 0.5 μM respectively. Furthermore, the effect of L-NIO was irreversible. The other three compounds were less potent, reversible inhibitors.

7 The inhibitory effects of all these compounds were enantiomerically specific.

8 These data indicate that L-NIO is a novel, potent, rapid in onset and irreversible inhibitor of NO synthase in phagocytic cells. The rapid uptake of L-NIO compared with the other compounds indicates that phagocytic cells have different uptake mechanisms for L-arginine analogues.

Introduction

Neutrophils (PMN; McCall *et al.*, 1989; Salvemini *et al.*, 1989) and macrophages (Hibbs *et al.*, 1988; Marletta *et al.*, 1988; Stuehr *et al.*, 1989) synthesize nitric oxide (NO) from the amino acid L-arginine. This synthesis is inhibited by the L-arginine analogue N^G-monomethyl-L-arginine (L-NMMA; Hibbs *et al.*, 1987; McCall *et al.*, 1989; Salvemini *et al.*, 1989). However, in the PMN this compound only causes partial inhibition of synthesis following a long period of pre-incubation (McCall *et al.*, 1989), suggesting that L-NMMA is a weak inhibitor of the NO synthase in PMN or is only poorly taken up into these cells, or both.

Other L-arginine analogues, such as N-iminoethyl-L-ornithine (L-NIO), N^G-nitro-L-arginine (L-NNA) and its methyl ester (L-NAME) have recently been reported to be potent inhibitors of the NO synthase of adrenal glands (Palacios *et al.*, 1989), brain (Knowles *et al.*, 1990) and vascular endothelial cells (Rees *et al.*, 1990). Present evidence indicates that the NO synthase in endothelial cells and brain differs from that in PMN and macrophages. L-Canavanine, for example, inhibits NO synthesis in phagocytic cells (Hibbs *et al.*, 1987; McCall *et al.*, 1989), but does not affect the generation of NO by brain synaptosomes (Knowles *et al.*, 1989) or endothelial cells (Palmer & Moncada, 1989). Furthermore, L-homoarginine is a weak substrate in phagocytic cells (McCall *et al.*, 1989), but not in endothelial cells (Palmer & Moncada, 1989) or brain synaptosomes (Knowles *et al.*, 1989).

In the present study, we have examined the activity of these inhibitors of NO synthase on the release of NO from rat peritoneal PMN and from the murine macrophage cell line J774, activated with IFN-γ and lipopolysaccharide (LPS), as determined by a bioassay method using inhibition of platelet aggregation (McCall *et al.*, 1989). In addition, we have examined the potency of the inhibitors on the activity of NO synthase from these cells.

Some of these results were presented at the IUPHAR satellite symposium 'EDRF and EDRF-related substances' in Antwerp, Belgium, June 1990.

Methods

Preparation of human platelets and the detection of NO, by use of platelet aggregation as a bioassay, were carried out as previously described (McCall *et al.*, 1989).

Peritoneal PMN were elicited with oyster glycogen (0.2%) and harvested from male Wistar rats (200–250 g). A purified population of PMN (>95% pure) was prepared by Ficoll-Hypaque density gradient centrifugation. The 5% contaminating cells consisted of mast cells (2–3%) and mononuclear cells (2–3%). After hypo-osmotic lysis of erythrocytes, the PMN were resuspended at a final concentration of 1×10^7 cells ml^{-1} in Tyrode solution containing 1 mM Ca^{2+} and 5 μM indomethacin and maintained at 4°C. The PMN preparation was more than 98% viable as assessed by the uptake of Acridine Orange.

J774 cells (American Tissue Culture Catalogue T1B 67, page 231), cultured in stirrer bottles in RPMI 1640 containing 10% foetal calf serum, were stimulated with IFN-γ (150 u ml^{-1}) and LPS (10 μg ml^{-1}) for 18 h. Cells were then harvested by centrifugation, resuspended at a final concentration of 1×10^6 cells ml^{-1} as described for PMN and maintained at room temperature.

Bioassay of NO

Cells were added to indomethacin (5 μM)-treated platelets (1×10^8 platelets in 500 μl) in a Payton aggregometer (37°C,

[1] Present address: Schwarz Pharma 4019, Monheim, F.R.G.
[2] Author for correspondence.

900 r.p.m.) and incubated for 4 min before addition of a submaximal concentration of thrombin (20–40 mu ml^{-1}) to initiate aggregation. The response was monitored for 10 min. The anti-aggregating effect of phagocytic cells was expressed as a percentage (mean ± s.e.mean) of the aggregation induced by thrombin alone.

Preparation of NO synthase

Purified rat peritoneal PMN or activated and non-activated J774 cells, resuspended in HEPES buffer (0.1 M, pH 7.4) containing 100 μM dithiothreitol, were lysed by sonicating twice for 10 s and kept on ice. The lysate was then centrifuged at 105,000 *g* for 30 min at 4°C and the supernatant was incubated with AG50-X8 (Na^+ form; 100 mg ml^{-1} of supernatant) for 5 min at 4°C to deplete endogenous L-arginine.

Spectrophotometric assay for NO synthase activity

The generation of NO by cytosol was measured by difference-spectrophotometry according to the method of Feelisch & Noack (1987). This measurement is based on the quantitative oxidation of oxyhaemoglobin to methaemoglobin in aqueous solution by NO.

The cytosolic preparation was pre-incubated at 37°C in the presence of oxyhaemoglobin (5 μM) and NADPH (100 μM) for 5 min, prior to addition of a submaximally effective concentration of L-arginine (30 μM), to initiate NO generation by the enzyme. The initial rate of production of NO was determined as the difference in absorbance between 401 and 411 nm in a dual beam spectrophotometer (Shimadzu) and the results expressed as pmol NO min^{-1} mg^{-1} protein.

Materials

Oyster glycogen, indomethacin, superoxide dismutase (SOD), N^G-nitro-L-arginine methyl ester (L-NAME), NADPH, L-arginine, dithiothreitol (all Sigma), AG 50-X8 (Bio-Rad), RPMI (Gibco), foetal calf serum (Flow Labs.) human thrombin (Ortha Diagnostic Systems), Ficoll-Hypaque (Pharmacia), *Salmonella typhosa* lipopolysaccharide (Difco), recombinant murine IFN-γ (Genzyme), N-iminoethyl-L-ornithine (L-NIO), N^G-monomethyl-L-arginine (L-NMMA), N^G-nitro-L-arginine (L-NNA), prostacyclin (all Wellcome) were obtained as indicated. Human haemoglobin was prepared as described (Paterson *et al.*, 1976).

Statistics

Student's *t* test (two-tailed) for unpaired data was used to determine statistical significance, and $P < 0.05$ was taken as statistically significant.

Results

Phagocytic cells inhibited platelet aggregation in a cell number-dependent manner with maximal inhibition observed with 1×10^6 PMN and 1×10^5 activated J774 cells ($n = 3$ for each). The inhibitory activity of both cells was dependent on the generation of NO, since it was abolished by haemoglobin and potentiated by superoxide dismutase (SOD; McCall *et al.*, 1989).

Effect of L-arginine analogues on NO release by PMN and J774 cells

The synthesis of NO by PMN (1×10^6) and by J774 cells (1×10^5) was significantly inhibited by L-NIO and L-NMMA in a concentration-dependent and enantiomerically specific manner. In both types of cells L-NIO was more potent than L-NMMA as an inhibitor of NO synthesis (Figure 1). The inhibitory effect of L-NIO (100 μM) was rapid in onset in both

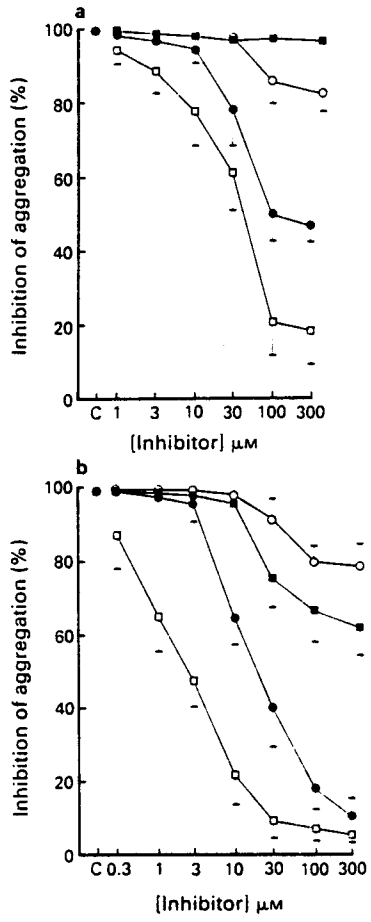

Figure 1 The effect of L-NIO (□), L-NMMA (●), L-NNA (■) and L-NAME (○) on the platelet anti-aggregatory activity of (a) 1×10^6 rat peritoneal PMN and (b) 1×10^5 activated J774 cells. The cells were pre-incubated with L-NIO for 10 min and with the other compounds for 50 min. Each point is the mean of 4–8 separate experiments with s.e.mean shown by vertical bars. C = control.

PMN and J774 cells, being complete within 10 min of pre-incubation (Figure 2). L-NMMA (100 μM), on the other hand, required 50 min of pre-incubation to achieve its full inhibitory activity on both types of cells.

The effect of L-NIO (10 μM) on the synthesis of NO by PMN and J774 cells was irreversible even when a 30 fold molar excess of L-arginine was added 10 min after incubation with the inhibitor ($n = 4$). However, the effect of L-NIO was prevented by concomitant incubation with a 30 fold molar excess of L-arginine ($n = 3$). In contrast, the effect of L-NMMA (300 μM) was fully reversed within 5 min with a 3 fold molar excess of L-arginine ($n = 4$).

The synthesis of NO by J774 cells, but not PMN, was significantly inhibited by L-NNA (Figure 1; $n = 4$). This effect of L-NNA on J774 cells was maximal within 20 min pre-incubation (Figure 2). L-NAME did not cause significant inhibition of NO synthesis by either cell type (Figure 1; $n = 3$–6).

Characteristics of NO synthase from PMN and J774 cells

The synthesis of NO by the cytosolic fraction from PMN and activated J774 cells in the presence of NADPH (100 μM) increased from 10 ± 4 and 12 ± 6 to 152 ± 22 and 181 ± 34 pmol NO min^{-1} mg^{-1} protein respectively ($n = 3$ for each) following addition of L-arginine (30 μM). The K_m for L-arginine of the enzyme from PMN was 3.3 ± 0.8 μM and from J774 cells was 4.2 ± 1.1 μM. The K_m for L-homoarginine of the enzyme from PMN and J774 cells was 15.4 ± 4.4 and

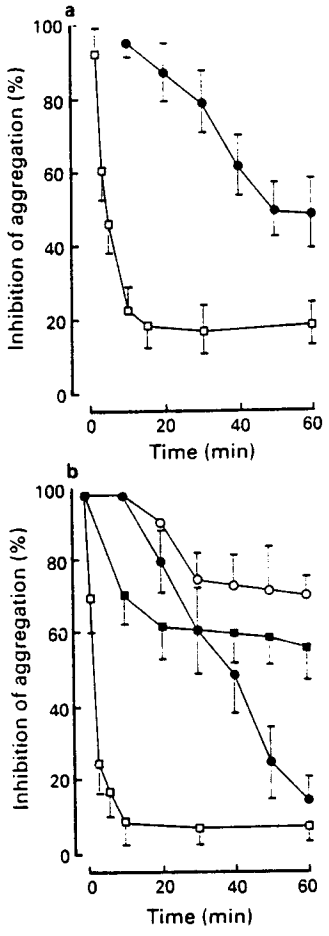

Figure 2 Time course of the effect of (a) L-NIO (□) and L-NMMA (●) on the anti-aggregatory activity of PMN and (b) of L-NIO (□), L-NMMA (●), L-NAME (○) and L-NNA (■) on that of activated J774 cells. All compounds were studied at 100 μM. Each point is the mean of 4–6 separate experiments with s.e.mean shown by vertical bars.

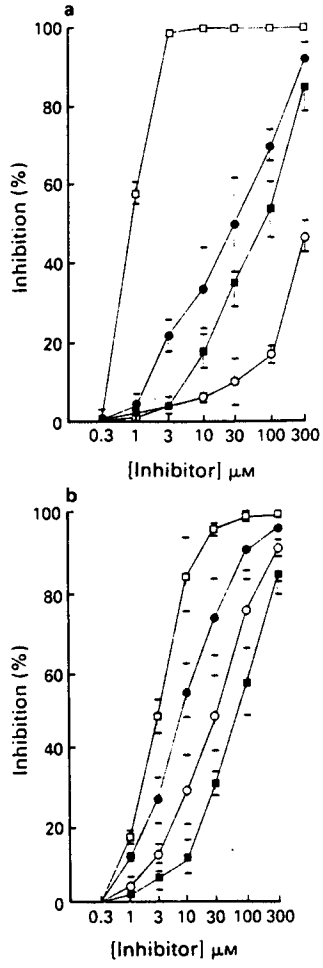

Figure 3 (a) Inhibition by L-NIO (□), L-NMMA (●), L-NNA (■) and L-NAME (○) of the activity of the NO synthase from PMN. Nitric oxide generation by the enzyme was initiated by addition of 30 μM L-arginine. Each point is the mean of 3–5 separate experiments with s.e.mean shown by vertical bars. (b) Inhibition by L-NIO (□), L-NMMA (●), L-NNA (■) and L-NAME (○) of the activity of the NO synthase from J774 cells. Each point is the mean of 3–8 separate experiments with s.e.mean shown by vertical bars.

13.8 ± 3.2 μM respectively. EGTA (100 μM) did not affect the activity of NO synthase from either PMN or J774 cells in the presence of $MgCl_2$ (1 mM; $n = 3$). The NO synthase from both cells was cytosolic, as enzymic activity was not detectable in the pellet following centrifugation at 105,000 *g* ($n = 3$). The cytosolic preparation from unstimulated J774 cells did not exhibit NO synthase activity ($n = 3$).

Inhibition of NO synthase from PMN and J774 cells by analogues of L-arginine

The NO synthase from PMN and J774 cells in the presence of 30 μM L-arginine was inhibited in a concentration-dependent manner by L-NIO, L-NMMA and L-NNA (Figure 3), with IC_{50} values as indicated in Table 1. The NO synthase from J774 cells was also inhibited in a concentration-dependent manner by L-NAME (Figure 3; Table 1) but this compound caused less than 50% inhibition of NO synthase from PMN at the highest concentration tested (300 μM; $n = 5$). The effects of the compounds were enantiomerically specific since their D-enantiomers did not alter enzyme activity ($n = 3$ for each).

Table 1 Potency of inhibitors of NO synthase from PMN and activated J774 cells in the presence of 30 μM L-arginine ($n = 3$–5)

	Inhibitor IC_{50} (μM)	
	PMN	*J774*
L-NIO	0.8 ± 0.1	3 ± 0.5
L-NMMA	30 ± 4	7.5 ± 1.2
L-NNA	80 ± 8	85 ± 9
L-NAME	<50% inhibition at 300 μM	28 ± 5

L-NIO = N-iminoethyl-L-ornithine; L-NMMA = N^G-monomethyl-L-arginine; L-NNA = N^G-nitro-L-arginine; L-NAME = N^G-nitro-L-arginine methyl ester.

The inhibitory effect of L-NIO (1 μM) on the NO synthase from PMN and from J774 cells (3 μM) was not reversed by L-arginine, even when a 300 fold molar excess was added 3 min after starting the incubation ($n = 4$). In contrast, the inhibitory action of equi-effective concentrations of L-NMMA, L-NAME or L-NNA was reversed by L-arginine (300 μM; Figure 4; $n = 3$–7).

Discussion

Rat peritoneal neutrophils and activated J774 cells inhibited platelet aggregation in a cell number-dependent manner and this activity was potentiated by SOD and inhibited by haemoglobin. Furthermore, the anti-aggregatory activity was inhibited by the L-arginine analogue L-NMMA, an established inhibitor of NO generation in the endothelial cell (Palmer *et*

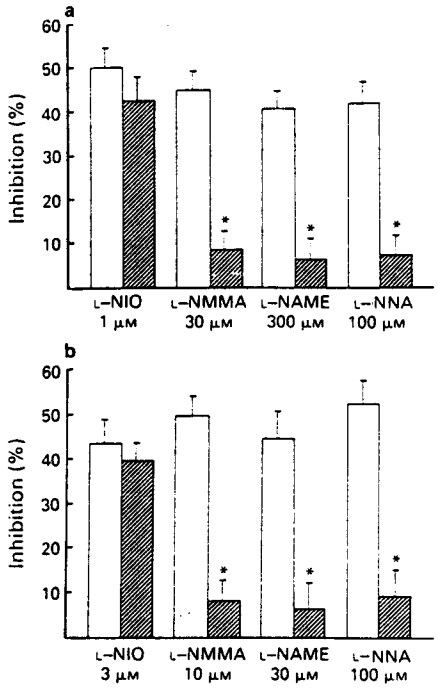

Figure 4 Reversal by L-arginine (300 μM; hatched columns) of the inhibition of NO synthesis in the presence of 30 μM L-arginine (open columns) by equieffective concentrations of L-NIO, L-NMMA, L-NAME and L-NNA in (a) PMN and (b) J774 cells. Each column is the mean from 3–7 separate determinations with s.e.mean shown by vertical bars.

al., 1988) and mouse peritoneal macrophages (Hibbs *et al.*, 1987). These data indicate that both rat peritoneal neutrophils and activated J774 cells synthesize NO.

L-NIO was the most potent inhibitor of the generation of NO in both cell types. In contrast, L-NNA only weakly inhibited NO synthesis by J774 cells and L-NAME had no significant effect on either cell. This profile of inhibition differs from that described in adrenal glands (Palacios *et al.*, 1989), brain (Knowles *et al.*, 1990), vascular endothelial cells (Rees *et al.*, 1990) and human platelets (Radomski *et al.*, 1990).

The effect of L-NIO was rapid in onset in both cell types, whereas L-NMMA required approximately 50 min preincubation before maximal activity was observed. The effect of L-NIO was prevented by concomitant incubation with L-arginine. These observations suggest that there are different mechanisms for uptake of L-arginine analogues in phagocytic cells. Since there is no evidence for substantial differences in the uptake of these compounds into endothelial cells or platelets (Rees *et al.*, 1990; Radomski *et al.*, 1990), further work is required to clarify the mechanisms underlying these variations in different cells.

Two different types of NO synthase have now been recognized. One is cytosolic, Ca^{2+}- and NADPH-dependent and generates NO as a transduction mechanism for stimulation of the soluble guanylate cyclase in vascular endothelium (Palmer & Moncada, 1989), brain (Knowles *et al.*, 1989), adrenal gland (Palacios *et al.*, 1989), platelets (Radomski *et al.*, 1990) and probably other tissues. The second type is present in macrophages, is cytosolic and NADPH- but not Ca^{2+}-dependent (Marletta *et al.*, 1988; Kwon *et al.*, 1989). Furthermore, in macrophages, the enzyme also requires tetrahydrobiopterin (Kwon *et al.*, 1989; Tayeh & Marletta, 1989) and is inducible, releasing NO as part of the cytotoxic functions of these cells (Hibbs *et al.*, 1988). Although circulating human and rabbit neutrophils release small amounts of NO (McCall *et al.*, 1990), it is likely that the enzyme in the cells used in the present study was also induced by the process of migration into the peritoneal cavity.

Our present results support the existence of two different NO synthases since, although both enzymes are inhibited by L-NIO and L-NMMA, the former is a more potent and irreversible inhibitor of the enzyme in phagocytic cells. Furthermore, L-NNA and L-NAME are only weak inhibitors of the NO synthase from phagocytic cells, in contrast to findings in endothelial cells. In addition all these compounds, including L-NIO, are reversible by L-arginine in endothelial cells.

In summary, we have identified L-NIO as a novel, potent and irreversible inhibitor of NO generation in phagocytic cells, which is rapidly taken up by intact cells. If L-NIO exhibits the same characteristics after administration *in vivo*, this compound may be a useful tool to investigate selectively the biological relevance of the production of NO by phagocytic cells.

References

FEELISCH, M. & NOACK, E.A. (1987). Correlation between nitric oxide formation during degradation of organic nitrates and activation of guanylate cyclase. *Eur. J. Pharmacol.*, **139**, 19–30.

HIBBS, J.B. Jr., VAVRIN, Z. & TAINTOR, R.R. (1987). L-Arginine is required for expression of the activated macrophage effector mechanism causing selective metabolic inhibition in target cells. *J. Immunol.*, **138**, 550–565.

HIBBS, J.B., TAINTOR, R.R., VAVRIN, Z. & RACHLIN, E.M. (1988). Nitric oxide: a cytotoxic activated macrophage effector molecule. *Biochem. Biophys. Research Commun.*, **157**, 87–94.

KNOWLES, R.G., PALACIOS, M., PALMER, R.M.J. & MONCADA, S. (1989). Formation of nitric oxide from L-arginine in the central nervous system. A transduction mechanism for stimulation of the soluble guanylate cyclase. *Proc. Natl. Acad. Sci., U.S.A.*, **86**, 5159–5162.

KNOWLES, R.G., PALACIOS, M., PALMER, R.M.J. & MONCADA, S. (1990). Kinetic characteristics of nitric oxide synthase from rat brain. *Biochem. J.*, **269**, 207–210.

KWON, N.S., NATHAN, C.F. & STUEHR, D.J. (1989). Reduced biopterin as a cofactor in the generation of nitrogen oxides by murine macrophages. *J. Biol. Chem.*, **264**, 20496–20501.

MARLETTA, M.A., YOON, P.S., IYENGAR, R., LEAF, C.D. & WISHNOK, J.S. (1988). Macrophage oxidation of L-arginine to nitrite and nitrate: Nitric oxide is an intermediate. *Biochemistry*, **27**, 8706–8711.

McCALL, T.B., BOUGHTON-SMITH, N.K., PALMER, R.M.J., WHITTLE, B.J.R. & MONCADA, S. (1989). Synthesis of nitric oxide from L-arginine by neutrophils. Release and interaction with superoxide anion. *Biochem. J.*, **261**, 293–296.

McCALL, T.B., PALMER, R.M.J., BOUGHTON-SMITH, N.K., WHITTLE, B.J.R. & MONCADA, S. (1990). The L-arginine: nitric oxide pathway in neutrophils. In *Nitric Oxide from L-arginine: a Bioregulatory System.* ed. Moncada, S. & Higgs, E.A., pp. 249–257. Amsterdam: Elsevier.

PALACIOS, M., KNOWLES, R.G., PALMER, R.M.J. & MONCADA, S. (1989). Nitric oxide from L-arginine stimulates the soluble guanylate cyclase in adrenal glands. *Biochem. Biophys. Res. Commun.*, **165**, 802–809.

PALMER, R.M.J. & MONCADA, S. (1989). A novel citrulline-forming enzyme implicated in the formation of nitric oxide by vascular endothelial cells. *Biochem. Biophys. Res. Commun.*, **158**, 348–352.

PALMER, R.M.J., REES, D.D., ASHTON, D.S. & MONCADA, S. (1988). L-arginine is the physiological precursor for the formation of nitric oxide in endothelium-dependent relaxation. *Biochem. Biophys. Res. Commun.*, **153**, 1251–1256.

PATERSON, R.A., EAGLES, P.A.M., YOUNG, D.A.B. & BEDDELL, C.R. (1976). Rapid preparation of large quantities of human haemoglobin with low phosphate content by counter-flow dialysis. *Int. J. Biochem.*, **7**, 117–118.

RADOMSKI, M.W., PALMER, R.M.J. & MONCADA, S. (1990). An L-arginine to nitric oxide pathway in human platelets regulates aggregation. *Proc. Natl. Acad. Sci., U.S.A.*, **87**, 5193–5197.

REES, D.D., PALMER, R.M.J., SCHULZ, R., HODSON, H.F. & MONCADA, S. (1990). Characterization of three inhibitors of endothelial nitric oxide synthase *in vivo* and *in vitro*. *Br. J. Pharmacol.*, **101**, 746–752.

SALVEMINI, D., DE NUCCI, G., GRYGLEWSKI, R.J. & VANE, J.R. (1989). Human neutrophils and mononuclear cells inhibit platelet aggregation by releasing a nitric oxide-like factor. *Proc. Natl. Acad. Sci., U.S.A.*, **86**, 6328–6332.

STUEHR, D., GROSS, S., SAKUMA, I., LEVI, R. & NATHAN, C. (1989). Activated murine macrophages secrete a metabolite of arginine with the bioactivity of endothelium-derived relaxing factor and the chemical reactivity of nitric oxide. *J. Exp. Med.*, **169**, 1011–1020.

TAYEH, M.A. & MARLETTA, M.A. (1989). Macrophage oxidation of L-arginine to nitric oxide, nitrite and nitrate: Tetrahydrobiopterin is required as a cofactor. *J. Biol. Chem.*, **264**, 19654–19658.

(Received July 13, 1990
Revised August 29, 1990
Accepted September 4, 1990)