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Nitric oxide synthase activity in molluscan hemocytes

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Abstract The hemocytes of the freshwater snail *Viviparus ater* have nitric oxide synthase (NOS) activity, as demonstrated by [^{3}H]citrulline and nitrite+nitrate formation. The enzyme is NADPH dependent and is competitively inhibited by the mammalian NOS inhibitor N^G-monomethyl-L-arginine (K_i = 4.7 μM). The K_m for L-arginine is 2.5 μM. 70% of the total activity is observed at very low free Ca^{2+} concentration (3 nM). LPS treatment increased total NOS activity 2.4 fold. The activity is partly present in the non-soluble fraction of hemocytes (24% and 8% in non-stimulated and LPS-stimulated snails, respectively). An antiserum to the C-terminal synthetic pentadecapeptide of the rat cerebellar NOS inhibited the enzyme activity in a concentration-dependent manner. This is the first biochemical demonstration of the existance of NOS activity in molluscan hemocytes, the cells responsible for defence mechanisms.

Key words: Nitric oxide synthase; Molluscan hemocyte; Biochemical assay

1. Introduction

Nitric oxide (NO) is a molecular messenger with numerous functions, including regulation of vascular tone, cellular signaling in the brain, and the elimination of pathogens in a non-specific immune response [1–3]. Different NO synthase (NOS) isozymes have been purified from various mammalian tissues [4,5]. Three major isoforms have been cloned: a Ca^{2+}/calmodulin-dependent, cytosolic enzyme from the brain [6–9], an inducible, Ca^{2+}-independent NOS from activated macrophages [10,11], and a Ca^{2+}-dependent membrane-associated isoform from vascular endothelial cells [12,13]. However, some data suggest that there are more than three isoforms. Indeed, hepatocyte, chondrocyte and neutrophile NOS have peculiar properties [4,14,15]. The NOS so far characterized are all NADPH-dependent and require several co-factors: H_4biopterin (BH_4), FAD and FMN [10,11,16–19]. Both neuronal and inducible NOS are P450-type heme proteins [20–23]. NOS isozymes catalyze a two-step, monooxygenase-like oxidation of L-arginine [24] by means of an NADPH-dependent reductive activation of molecular oxygen [16] which is incorporated into both NO and L-citrulline [25,26]. At non-saturating concentrations of L-arginine and BH_4, oxygen activation catalyzed by the brain enzyme does not result in NO synthesis, but rather leads to the production of the superoxide anion and H_2O_2 [24].

NOS has been characterized from rodent, bovine or porcine tissue, as well as from several human cells, such as neutrophils, HL-60 cells and platelets [27,28]. The various NOS isoenzymes are very different from amino acid sequences, while the same isoenzyme is well conserved between mammalian species, suggesting either that the differentiation of the isoenzymes takes place before the evolution of mammalian species, or that the functional and structural differentiation between the various NOS isoenzymes is so high that no further major variability in the sequence is allowed.

Few data available on the NOS of non-mammalian animals. In the last few years, NOS activity has been shown to be present in the central nervous system of many advanced invertebrate phyla (annelids, arthropods and molluscs) [29,30]. Evidence for NOS activity in invertebrate hemocytes has been obtained by Radomski et al. [31] and Ottaviani and co-workers [32,33]. The study of NOS in invertebrates is a very useful way of understanding the evolution and the reaction mechanisms of the NOS isoforms, since many similarities between invertebrate and vertebrate immune/defence systems have been reported, suggesting that the origin of this system, as well as that of the nervous system, can be traced back to invertebrates [34,35]. To our knowledge, the present study is the first biochemical demonstration of the existence of NOS activity in molluscan hemocytes, indicating that this enzyme may have appeared very early in evolution and play an essential role in defence systems.

2. Materials and methods

2.1. Reagents

(6*R*)-5,6,7,8-Tetrahydro-L-biopterin dihydrochloride was purchased from Dr. B. Schircks Laboratories (Jona, Switzerland) and L-[2,3,4,5-^{3}H]arginine monohydrochloride (58 Ci/mmol) from Amersham (Buckinghamshire, England). The protein dye reagent and the ion-exchange resin AG50WX-8 were supplied by Bio-Rad (Milano, Italy), and calmodulin, glucose-6-phosphate dehydrogenase from yeast (EC 1.1.1.49) and glucose-6-phosphate by Serva (Heidelberg, Germany). Nitrate reductase from *Aspergillus* spp. (EC 1.6.6.2), lipopolysaccharide (LPS) from *Escherichia coli*, N^G-monomethyl-L-arginine (L-NMMA) and all other biochemicals were obtained from Sigma (Milano, Italy) and the anti-NOS polyclonal antiserum from Euro-Diagnostica (Ferring group, Sweden).

2.2. Snails

Adult specimens of *Viviparus ater* were collected in a freshwater canal near Modena (Italy) in spring and early summer. The animals were maintained at room temperature in dechlorinated freshwater for at least a week before experiments. Snail hemolymph was obtained by prodding the animal's foot and collected with a Pasteur pipette. Hemocytes were obtained by centrifugation at 600 × g. The cells were washed twice with snail saline solution [36], counted and collected by centrifugation. In order to evaluate the induced NOS activity, the snails were injected with 20 μg of LPS (4 U/μl) and hemolymph was collected 4 h after injection.

2.3. Determination of NOS activity

Hemocytes were homogenized with Ultra-Turrax (IKA-WERK, Germany) in 5 vols. of ice-cold solution containing 320 mM sucrose, 50 mM Tris, 1 mM EDTA, 1 mM dithiothreitol (DTT), 1 mM phenylmethylsulphonyl fluoride, 10 μg/ml soybean trypsin inhibitor,

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10 μg/ml antipain and 10 μg/ml bestatin brought to pH 7.0 at 20°C with HCl. The homogenate was centrifuged at 20,000 × g for 30 min at 4°C, and the supernatant was freed from low molecular mass compounds by Sephadex G-25 chromatography [37]. The protein fraction was eluted with a buffer containing 50 mM HEPES, pH 7.4, 1 mM EDTA, 1 mM DTT and the indicated above protease inhibitors.

NOS activity was assayed by following the conversion of radiolabeled arginine to citrulline. Standard reaction mixtures contained 50 mM HEPES, pH 7.4, 0.5 mM EDTA, 1.4 mM $CaCl_2$, 1 mM $MgCl_2$, 1 mM NADPH, 1 mM DTT, 12 mM L-valine, 1 mM citrulline, a variable amount of L-arginine, 80,000–100,000 cpm of purified L-[2,3,4,5-^{3}H]arginine monohydrochloride and 5–50 μl of Sephadex G-25 eluate in a finale volume of 100 μl.

After 30 min incubation at 37°C, [^{3}H]citrulline was quantified by liquid scintillation counting after separation from [^{3}H]arginine by cation exchange (AG50WX-8) [38]. The Na^+ form of AG50WX-8 was prepared by washing the $^+$H form of the resin with 1 M NaOH 4 times and then washing with H_2O until the pH was less than 7.5.

As reported by Bredt and Snyder [38], with thin layer chromatography, more than 95% of the radioactivity in the water eluates from AG50WX-8 columns, rechromatographed with three different solvents on Whatman no. 1 paper at 18°C, migrates as the authentic L-citrulline standard [39]. The solvents utilized as the mobile phase were: phenol:water:ammonia d 0.88 (160:40:1, w/v/v), methanol:water:pyridine (20:5:1) and *n*-butanol:pyridine:water (1:1:1).

The total NOS (Ca^{2+}-dependent and Ca^{2+}-independent activity) was determined by calculating the difference between the [^{3}H]citrulline produced in the presence and in the absence of 10 mM N^G-monomethyl-L-arginine (L-NMMA, an inhibitor of mammalian NOS) in standard reaction mixture. To evaluate the Ca^{2+}-independence activity, 6 mM EGTA was added to the standard assay mixture (free calcium concentration 20 nM). In some experiments, 6 mM EGTA was added to the standard mixture which contained 0.25 mM $CaCl_2$ instead of 1.4 mM $CaCl_2$ for 3 nM free Ca^{2+} concentration. The free calcium concentration was calculated following Fabiato [40]. Enzyme activity is given as pmol of [^{3}H]citrulline formed per min for each mg of protein in the cell extract.

L-[^{3}H]Arginine was purified by adding the radiolabel to 20 mM HEPES (2 ml), pH 5.5, to a 1 ml column of AG50WX-8 resin (prepared as above), washing the column with 8 ml of H_2O and eluting with 4 ml of 0.5 M ammonia. The eluate was freeze-dried and resuspended in 2% ethanol [41].

To determine the stoichiometry of nitrine + nitrate and citrulline, the standard reaction mixture was supplemented with an NADPH regenerating system (0.25 unit/ml glucose-6-phosphate dehydrogenase and 20 mM glucose-6-phosphate), and the incubation time was prolonged up to 3 h at 37°C. For nitrite + nitrate determination, nitrate was reduced by nitrate reductase (1.25 units/ml) in the presence of 2.5 mM NADPH for 1 h at room temperature [37]. Nitrite was determined according to Green et al. [42].

Formation of [^{3}H]citrulline and NO was linear with time for up to 1 h in the standard assay and for up to 3 h in the assay supplemented with an NADPH regenerating system. The NOS activity of the non-soluble fraction was determined by washing the hemocyte pellet twice with PBS, centrifuging at 20,000 × g for 30 min at 4°C and resuspending with the buffer used for Sephadex G-25 chromatography. Activity was calculated by the radiochemical method and expressed as pmol of [^{3}H]citrulline formed/min/10^6 cells.

Protein in cell extracts was determined according to Bradford [43], with BSA as standard. With the standard assay, the velocity was linear up to 10 μg of the total cell protein of the LPS stimulated snails. With 25 μM L-arginine, linearity was evident up to 150 μg/protein. The results obtained are expressed as means ± S.D. of at least four independent experiments performed in triplicate.

3. Results

Table I shows the formation of [^{3}H]citrulline and nitrite + nitrate in relation to incubation time, utilizing standard reaction mixtures containing 25 μM L-arginine and the NADPH regenerating system with and without 10 mM N^G-monomethyl-L-arginine. L-Citrulline and NO were formed in equimolar concentrations as a function of incubation time, suggesting that NOS activity is present in *V. ater* hemocytes.

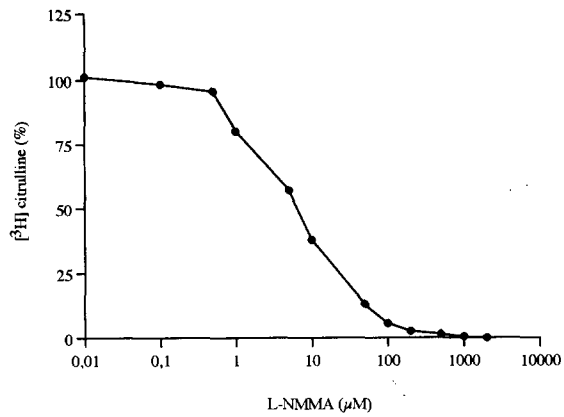

Fig. 1. Concentration-dependent inhibition of hemocyte NOS activity by L-NMMA in the presence of 1 μM [^{3}H]arginine. Data are expressed as percent inhibition of control (40.1 ± 3.2 pmol·min^{-1}·mg protein^{-1}). Each point is the mean of four independent experiments.

[^{3}H]Citrulline formation decreases with increasing concentrations of the NOS inhibitor N^G-monomethyl-L-arginine (Fig. 1). With 1 mM L-NMMA, almost complete inhibition was obtained (enzyme activity lower than 1%) at 1 μM substrate, whereas 4–5% of residual activity was maintained at 25 μM L-arginine. Thus, when substrate concentration was higher than 5 μM, L-NMMA concentration increased to 10 mM.

Fig. 2 reports the enzyme activity as a function of L-arginine concentration. In the same figure, NOS activity in the presence of two different concentrations of L-NMMA (2 μM and 6 μM) is also shown. Inhibition appears to be competitive for L-arginine. The K_m and K_i for L-arginine and L-NMMA are 2.5 ± 0.6 μM and 4.7 ± 1.1 μM, respectively. A V_{max} of 125.0 ± 8.0 pmol·min^{-1}·mg^{-1} was obtained with LPS stimulated hemocytes.

We also tested the inhibition of NOS activity in hemocyte extract with rabbit polyclonal antiserum to the C-terminal synthetic pentadecapeptide, FIEESKKDADEVFSS, of the rat cerebellar NOS [44]. As indicated in Table 2, NOS activity falls to 40% of the control in the presence of rabbit antiserum diluted 1:8. The inhibitory effect of the antiserum depends on dilution: with 1:40 and 1:400 dilutions, NOS activity decreases to 51% and 91% of the control. This effect is not seen with non-immune rabbit serum.

Table 3 shows the effect on [^{3}H]citrulline formation of the omission or addition of various co-factors. Whereas the omis-

Table 1
NOS activity in *V. ater* hemocytes: stoichiometric [^{3}H]citrulline and nitrite + nitrate formation

Time of incubation (min)	[^{3}H]Citrulline (nmol·mg protein^{-1})	Nitrite ± nitrate (nmol·mg protein^{-1})
60	6.7 ± 0.3	6.3 ± 0.4
120	12.9 ± 0.5	13.2 ± 0.6
180	18.4 ± 0.8	18.9 ± 1.1

Standard reaction mixtures containing 25 μM L-arginine and the NADPH regenerating system with or without 10 mM L-NMMA were prepared as detailed in section 2. Values are means ± S.D. of four independent experiments performed in triplicate.

Table 2
Inhibition of *V. ater* hemocyte NOS activity by rabbit antiserum to the C-terminal synthetic pentadecapeptide of the rat cerebellar NOS

Antibody and dilution	[^{3}H]Citrulline (pmol·min^{-1}·mg protein^{-1})
Control	42.3 ± 3.7
Anti-NOS (1:8)	16.8 ± 1.9 (39.7%)
Anti-NOS (1:40)	21.5 ± 2.5 (50.8%)
Anti-NOS (1:400)	38.7 ± 3.6 (91.5%)
Rabbit non-immune serum (1:8)	41.2 ± 4.3

A rabbit antiserum to NOS was included in standard reaction mixtures prepared as detailed in section 2, without substrate. After 5 min of incubation at 37°C, the reaction was started with 1 μM substrate. Values are means ± S.D. of four independent experiments performed in triplicate.

sion of NADPH decreases NOS activity to 35%, the adddition of FAD, FMN, BH_4 or calmodulin in the standard reaction mixture does not produce significant variations in [^{3}H]citrulline formation. NOS activity is significantly reduced by the addition of 6 mM EGTA, which decreases the free Ca^{2+} concentration to about 20 nM. The fall in the free Ca^{2+} concentration to about 3 nM does not further decrease the enzyme activity. The omission of Mg^{2+} results in no significant variation in [^{3}H]citrulline formation with respect to the control.

Table 4 presents the distribution of NOS activity in the soluble fraction and the pellet of LPS-stimulated and non-stimulated hemocytes. Stimulation with LPS increases the total NOS activity 2.4 fold. However, NOS activity in the pellet of LPS-stimulated cells does not rise, meaning that the distribution of activity between the soluble and pellet fractions varies considerably in non-stimulated (24% in the pellet) and LPS-stimulated hemocytes (8% in the pellet).

4. Discussion

Our results show that *V. ater* hemocytes present NOS activity comparable to that observed in mammalian cells. This is demonstrated by the strict relationship between [^{3}H]citrulline and nitrite + nitrate formation, by the inhibition of the reaction with the competitive inhibitor N^G-monomethyl-L-arginine and by the need for NADPH to provoke the full activity of the enzyme. Furthermore, a NOS polyclonal rabbit antiserum produced a concentration-dependent inhibition of the snail enzyme activity. The observed values of K_m for L-arginine and K_i for

Table 3
Effect of some cofactors on *V. ater* hemocyte NOS activity

Conditions	[^{3}H]Citrulline (pmol·min^{-1}·mg protein^{-1})
Standard	39.4 ± 4.1
NADPH omitted	13.8 ± 2.0
BH_4 (10 μM)	37.1 ± 3.9
Calmodulin (50 μg/ml)	39.9 ± 4.2
FAD (10 μM)	36.4 ± 3.7
FMN (10 μM)	38.7 ± 3.7
BH_4, FAD, FMN, (10 μM each)	35.9 ± 4.0
Ca^{2+} (≤ 3 nM)	28.6 ± 4.5
Ca^{2+} (≤ 20 nM)	29.8 ± 6.2
Mg^{2+} omitted	37.7 ± 3.9

Standard reaction mixtures containing 1 μM L-arginine were prepared as detailed in section 2. Values are means ± S.D. of four independent experiments performed in triplicate.

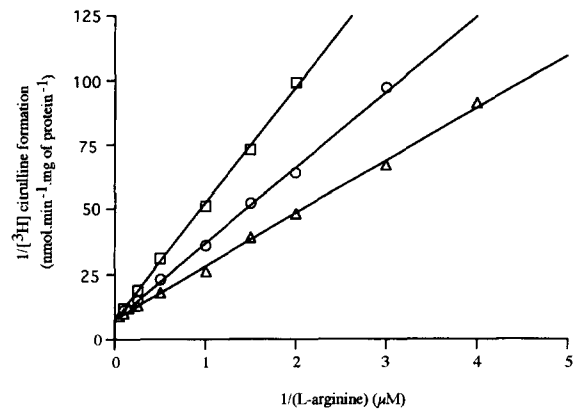

Fig. 2. L-Arginine concentration dependence of *V. ater* hemocyte NOS activity. The assay was performed using standard reaction mixtures with different amounts of L-arginine. [^{3}H]Citrulline formation in the presence of 2μM (○) and 6 μM (□) L-NMMA is also reported. Each point is the mean of four independent experiments.

L-NMMA were comparable to those seen in mammalian cell enzymes.

After removal of low molecular mass compounds by Sephadex G-25 chromatography, the NOS activity in the protein fraction did not increase when BH_4, flavins or calmodulin were added to the standard reaction mixture. It seems, however, unlikely that hemocyte NOS activity is dependent of these factors, which may be present in the Sephadex G-25 eluate in sufficient quantity to maximise enzyme activity. It should be noted that sometimes these factors remain bound to the protein fraction even after extensive purification of mammalian NOS [16,18,19]. This may also be the case for snail NOS, but further studies and a better characterization of the enzyme are needed to clarify this question.

Hemocyte NOS activity is significantly decreased at very low free calcium concentrations, but nevertheless more than 70% of the enzyme activity remains. In this respect, the Ca^{2+}-dependence of hemocyte NOS is similar to that of an inducible form of human hepatocyte NOS cloned by Geller et al. [14]. It has been suggested that this form, which is present in a wide range of tissues, may be a primitive, non-specific defence system against any type of invading organism.

The LPS stimulated snail hemocytes showed only a 2–3 fold increase in enzyme activity, while in stimulated mammalian cells a much more considerable rise in NOS activity was observed. It is possible that the non-LPS-treated snails were in a partially stimulated condition, given that they were collected from a wild environment and maintained in non-sterile water, and the hemocytes are the snail's most important defence system [45,46]. Also, LPS may not be the most effective inducible compound, as observed in some mammalian cells, in which greater responses have been obtained when combining two stimulating agents. It is further conceivable that there are two different isoforms, constitutive and inducible NOS, in these cells.

It should be noted that the NOS activity is inactivated by an antiserum obtained using the C-terminal synthetic pentadecapeptide of the rat cerebellar NOS isoenzyme as immunogen. This sequence is quite different in the various mammalian isoenzymes. Only 3, 8 or 3 amino acid residues appear to be

Table 4
Hemocyte NOS activity in the soluble fraction and in the pellet of LPS stimulated and non-stimulated *V. ater* snails

	[^{3}H]Citrulline (pmol·min^{-1}·10^6 cells^{-1})	
	Non-stimulated	LPS stimulated
Supernatant	47.9 ± 6.6	138.7 ± 15.1
Pellet	15.5 ± 4.8	12.3 ± 3.2
Total	63.4	151.0

Standard reaction mixtures containing 1 μM L-arginine were prepared as detailed in section 2. Values are means ± S.D. of four independent experiments performed in triplicate.

conserved in macrophage, hepatocyte and endothelial NOS, respectively, compared to rat cerebellar enzyme. Non of the fifteen amino acid residues appears in the same position in the four isoforms so far cloned, and no specific functions have been attributed to this C-terminal sequence. Nevertheless it would seem that a crossreactivity is still present between C-terminal sequences of rat cerebellar NOS and the NOS of the hemocytes of the *V. ater* snail.

From this preliminary characterization of the NOS activity of hemocytes, it appears that, although the snail enzyme(s) shares properties with the different NOS, it can not be identified with any of the enzymes so far studied and, in particular, not with the enzyme of mammalian phagocytic cells. It is in some ways similar to the hepatocyte isoenzyme studied by Geller et al. [14], which has been suggested to be a primitive non-specific defence system. However, further characterization is needed for a better evaluation of the similarities and the differences with respect to mammalian isoforms.

The demonstration that NOS activity is present in hemocytes of *V. ater* is relevant not only to our knowledge of the development of non-specific immunitary defence systems, but also to the evolution of the isoforms, the differences in multiple catalytic functions [25] and the possible relationship between the signaling and defence roles of NO. In this respect, it should be pointed out that at non-saturating L-arginine or BH_4 concentrations, the constitutive Ca^{2+}/calmodulin-dependent NOS from rat brain, but not the inducible Ca^{2+}-independent NOS from activated macrophages, catalyzes the formation of superoxide anions and idrogen peroxide [25], two oxygen reactive species utilized by phagocytic cells to eliminate invading organisms.

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References

[1] Moncada, S., Palmer, R..M.J. and Higgs, E.A. (1991) Pharmacol. Rev. 43, 109–142.
[2] Garthwaite, J. (1991) Trends Neurosci. 14, 60–67.
[3] Nathan, C. (1992) FASEB J. 6, 3051–3064.
[4] Förstermann, U., Schmidt, H.H.H.W., Pollock, J.S., Sheng, H., Mitchell, J.A., Warner, T.D., Nakane, M. and Murad, F. (1991) Biochem. Pharmacol. 42, 1849–1857.
[5] Mayer, B. (1993) Semin. Neurosci. 5, 197–205.
[6] Bredt, D.S. and Snyder, S.H. (1990) Proc. Natl. Acad. Sci. USA 87, 682–685.
[7] Mayer, B., John, M. and Böhme, E. (1990) FEBS Lett. 277, 215–219.
[8] Schmidt, H.H.H.W., Pollok, J.S., Nakane, M., Gorski, L.D., Förstermann, U. and Murad, F. (1991) Proc. Natl. Acad. Sci. USA 88, 365–369.
[9] Bredt, D.S., Hwang, P.M., Glatt, C.E., Lowenstein, C., Reed, R.R. and Snyder, S.H. (1991) Nature 351, 714–718.
[10] Stuher, D.J., Cho, H.J., Kwon, N.S., Weise, M.F. and Nathan, C.F. (1991) Proc. Natl. Acad. Sci. USA 88, 7773–7777.
[11] Hevel, J.M., Kimberly, A., White, K.A. and Marletta, M.A. (1991) J. Biol. Chem. 266, 22789–22791.
[12] Pollock, J.S., Förstermann, U., Mitchell, J.A., Warner, T.D., Schmidt, H.H.H.W., Nakane, M. and Murad, F. (1991) Proc. Natl. Acad. Sci. USA 88, 10480–10484.
[13] Lamas, S., Marsden, P.A., Li, G.K., Tempst, P. and Michel, T. (1992) Proc. Natl. Acad. Sci. USA 89, 6348–6352.
[14] Geller, D.A., Lowenstein, C.J., Shapiro, R.A., Nussler, A.K., Di Silvio, M., Wang, S.C., Nakayama, D.K., Simmons, R.L., Snyder, S.H. and Billiard, T.R. (1993) Proc. Natl. Acad. Sci. USA 90, 3491–3495.
[15] Charles, I.G., Palmer, R.M.J., Hickery, M.S., Bayliss, M.T., Chubb, A.P., Hall, V.S., Moss, D.W. and Moncada, S. (1993) Proc. Natl. Acad. Sci. USA 90, 11419–11423.
[16] Mayer, B., John, M., Heinzel, B., Werner, E.R., Wachter, H., Schultz, G. and Böhme, E. (1991) FEBS Lett. 288, 187–191.
[17] Bredt, D.S., Ferris, C.D. and Snyder, S.H. (1992) J. Biol. Chem. 267, 10976–10981.
[18] Schmidt, H.H.H.W., Smith, R.M., Nakane, M. and Murad, F. (1992) Biochemistry 31, 3243–4349.
[19] Hevel, J.M. and Marletta, M.A. (1992) Biochemistry 31, 7160–7165.
[20] White, K.A. and Marletta, M.A. (1992) Biochemistry 31, 6627–6631.
[21] Stuehr, D.J. and Ikeda-Saito, M. (1992) J. Biol. Chem. 267, 20547–20550.
[22] Klatt, P., Schmidt, K. and Mayer, B. (1992) Biochem. J. 288, 15–17.
[23] McMillan, K., Bredt, D.S., Hirsch, D.J., Snyder, S.H., Clark, J.E. and Masters, B.S.S. (1992) Proc. Natl. Acad. Sci. USA 89, 11141–11145.
[24] Klatt, P., Schmidt, K., Uray, G. and Mayer, B. (1993) J. Biol. Chem. 268, 14781–14787.
[25] Kwon, N.S., Nathan, C.F., Gilker, C., Griffith, O.W., Matthews, D.E. and Stuehr, D.J. (1990) J. Biol. Chem. 265, 13442–13445.
[26] Leone, A.M., Palmer, R.M.J., Knowles, R.G., Francis, P.L., Ashton, D.S. and Moncada, S. (1991) J. Biol. Chem. 266, 23790–23795.
[27] Schmidt, H.H.H.W., Seifert, R. and Böhme, E. (1989) FEBS Lett. 244, 357–360.
[28] Wright, C.D., Mulsch, A., Busse, R. and Osswald, H. (1989) Biochem. Biophys. Res. Commun. 160, 813–819.
[29] Elofsson, R., Carlberg, M., Moroz, L.L., Nezlin, L. and Sakharow, D. (1993) Neuroreport 4, 279–282.
[30] Moroz, L.L., Park, J.H. and Winlow, W. (1993) Neuroreport 4, 643–646.
[31] Radomski, M.W., Martin, J.F. and Moncada, S. (1991) Phil. Trans. R. Soc. Lond. B 334, 129–133.
[32] Ottaviani, E., Paemen, L.R., Cadet, P. and Stefano, G.B. (1993) Eur. J. Pharmacol. Environ. Toxicol. Pharmacol. Section 248, 319–324.
[33] Franchini, A., Fontanili, P. and Ottaviani, E. (1995) Comp. Biochem. Physiol. 110B, 403–407.
[34] Ottaviani, E., Caselgrandi, E., Bondi, M., Cossarizza, A., Monti, D. and Franceschi, C. (1991) Adv. Neuroimmunol. 1, 27–39.
[35] Stefano, G.B. (1992) Cell. Mol. Neurobiol. 12, 357–366.
[36] Ottaviani, E. (1983) Dev. Comp. Immunol. 7, 209–216.
[37] Werner-Felmayer, G., Werner, E.R., Fuchs, D., Hausen, A., Mayer, B., Reibnegger, G., Weiss, G. and Wachter, H. (1993) Biochem. J. 289, 357–361.
[38] Bredt, D.S. and Snyder, S.H. (1989) Proc. Natl. Acad. Sci. USA 86, 9030–9033.
[39] Smith, I. (1960) Chromatographic and Electrophoretic Techniques, William Heinemann Medical Books LTD, London.
[40] Fabiato, A. (1988) Methods Enzymol. 157, 379–417.

[41] Salter, M., Knowles, R.G. and Moncada, S. (1991) FEBS Lett. 291, 145–149.
[42] Green, L.C., Wagner, D.A. Glogowski, J., Skipper, P.L., Wishnok, J.S. and Tannenbaum, S.R. (1982) Anal. Biochem. 126, 131–138.
[43] Bradford, M.M. (1976) Anal. Biochem. 72, 248–254.
[44] Alm, P., Larsson, B., Ekblad, E., Sundler, F. and Andersson, K.E. (1993) Acta Physiol. Scand. 148, 421–429.
[45] Ottaviani, E. (1989) J. Moll. Stud. 55, 379–382.
[46] Franchini, A. and Ottaviani, E. (1990) J. Invertebr. Pathol. 55, 28–34.