Nitric Oxide Involvement in *Hydra vulgaris* Very Primitive Olfactory-Like System

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Hydra feeding response is a very primitive olfactory-like behavior present in a multicellular organism. We investigated the role of nitric oxide (NO) in the induction and control of hydra feeding response. Under basal conditions, hydra specimens produce detectable amounts of nitrite \((\text{NO}_2^-)\), the breakdown product of NO. When hydra were incubated with reduced glutathione (GSH), the typical activator of feeding response, an increase of basal NO production was observed. This effect was inhibited by glutamic or \(\alpha\)-aminoacidic acids, two GSH antagonists, which block GSH-induced feeding response, and by the NO synthase (NOS) inhibitor \(\lambda\)-NAME. Moreover, we found that hydra possess a calcium-dependent (but calmodulin-independent) NOS isoform. By using exogenous NO donors and NOS inhibitors, we demonstrated that NO stimulus can participate both in triggering tentacular movements and in recruiting neighbor tentacles during hydra feeding response. By using dbt\(^2\)-cGMP, an analog to cGMP, we observed that the NO effect was independent of cGMP pathway. Our results strongly implicate NO involvement in hydra very primitive feeding behavior, thus confirming its preservation throughout evolution.

Key words: nitric oxide; NO synthase; cyclic GMP; hydra; feeding response; primitive olfactory-like model; chemosensory system

Nitric oxide (NO) is an unstable nitrogen radical spontaneously degrading into nitrates and is generated by the conversion of L-arginine (L-Arg) into citrulline, through the NO synthase (NOS) enzyme. In mammalian neurons, a constitutive \(\text{Ca}^{2+}\)-dependent (cNOS) isoform is activated by the glutamatergic pathway stimulation, as after and consequent to a raised cytosolic \(\text{Ca}^{2+}\) influx (Garthwaite, 1991; Snyder and Bredt, 1991). NO can exert its biological activity via the stimulation of soluble guanylate cyclase (Garbers, 1992), thus leading to an increase in cGMP. More recently, we have observed for the first time that, surprisingly, the NO–cGMP pathway is present in the freshwater coelenterate *Hydra* (Colasanti et al., 1995), the most primitive organism possessing a nervous system. Hydra is a sessile predator whose tentacles are armed with the typical, characteristic stinging capsules of the coelenterates called nematocysts. When a prey accidentally touches a tentacle, a typical feeding response is activated. Hydra feeding response, which can be considered the most primitive olfactory-like behavior present in a multicellular organism, is a complex behavioral phenomenon consisting of tentacle writhing and mouth opening. Loomis (1955) determined that the reduced glutathione (GSH) outflow from the prey when pierced by tentacle nematocysts is the physiological activator of hydra feeding response. GSH is thought to interact with a specific receptor, the presence of which in hydra tissues was demonstrated by radioligand binding methods (Venturini, 1987) and has been recently characterized, solubilized, and partially purified (Bellis et al., 1991, 1992, 1994). However, no data are available concerning the cellular localization of GSH receptor. Hydra feeding response occurs a few seconds after GSH addition, reaches its peak within few minutes, and gradually disappears in ~10 min. Different structural GSH analogs, i.e., glutamic or \(\alpha\)-aminoacidic acids, which bind to but do not activate GSH receptors, are able to competitively inhibit GSH activity in eliciting the feeding response (Lenhoff, 1981).

Despite the obvious interest for the study of a primitive chemosensory system as a simple olfactory-like model, little is known about the molecular mechanisms regulating hydra feeding response. Previous reports have indicated that the cytosolic \(\text{Ca}^{2+}\) influx (Lenhoff, 1981) and the glutamatergic system (Venturini, 1987) are both involved in the induction of feeding response and that calcium ionophore A23187 enhances this response (Venturini et al., 1988). Recently, it has been reported that the interneuronal messenger NO seems to play a central role in the processing of olfactory information in invertebrates (Gelperin, 1994). In particular, a behavioral role for NO in chemosensory activation of feeding in a mollusk has been demonstrated (Elphick et al., 1995). In this paper, we have investigated on NO involvement in both induction and control of hydra feeding response.

At present, however, no data are available concerning the interaction between GSH receptors and the NO pathway. Therefore, we decided to approach this problem using GSH antagonists in a study aimed at clarifying the role of the NO–cGMP pathway in the feeding response induced by either GSH or food using NOS inhibitors, exogenous NO donors, and a cGMP analog. MATERIALS AND METHODS

Materials. Oxyhemoglobin (oxyHb) was prepared from commercial bovine hemoglobin (HB; Sigma, Milan, Italy) by reduction with a 10-fold excess sodium hydrosulphite (Aldrich, Milan, Italy), followed by gel filtration on prepacked G-25 columns (Pharmacia, Uppsala, Sweden) and equilibration in air; authentic NO solution (NOsol) was obtained by a 30 min bubbling of distilled and deoxygenated water with \(>99.5\)% pure NO gas at 4°C. This stock solution is in the low millimolar range (~2 mM at 25°C). GSH was from Merck Italia (Milan, Italy). Sulfanilamide, \(N-(1-\)
activity was assayed incubating 340 μM, which treatments and doses were unknown. For each observation, 10 of Hydra attenuata specimens were grown in 1 mM CaCl₂, Hydras were treated with GSH or nauplia for 60 min (3 specimens per minute showing the typical feeding response (tentacle curling and mouth opening) under a stereomicroscope. For each experiment, one group of 10 specimens treated with GSH alone was used as control. Each point represents mean ± SEM of 10 experiments.

Analysis of nitrite levels. Nitrite (NO₂⁻) was determined by the Griess reaction, according to the method of Tracey (1992). Briefly, 170 μl of a solution containing 1 mM CaCl₂, 100 mM NaHCO₃, and 0.05 M sodium acetate buffer (pH 5.8), by using a commercial specific inhibitor (D-NAME; 0.01 to 0.32 nmol/ml) treatment increases basal NO₂⁻ levels, this effect being abolished by L-NAME (100 μM). Results are expressed for NO₂⁻ in nmol · ml⁻¹ · 60 min⁻¹. Also, glutamic acid (Glu; 10 μM) or α-aminoacidic acid (AAD; 10 μM) was used to inhibit NO₂⁻ production. Finally, nauplia increase basal NO₂⁻ levels, this effect being abolished by L-NAME (100 μM). Results are expressed for NO₂⁻ in nmol · ml⁻¹ · 60 min⁻¹, and each bar represents mean ± SEM of three experiments. Observation of differences between basal and GSH or nauplia, between GSH and GSH + NAME, between nauplia and nauplia + L-NAME, p = 0.01 between basal GSH and GSH + AAD.

RESULTS

Analysis of nitrite levels

Under basal conditions, hydra specimens produced detectable amounts of NO₂⁻, the breakdown product of NO, in a range of 0.18–0.20 nmol · ml⁻¹ · 60 min⁻¹ (Fig. 1), as measured by the Griess reaction (Tracey, 1992). A treatment of hydra with GSH (2.5 μM), the typical inducer of hydra feeding response, caused a significant (p = 0.001) increase in NO₂⁻ levels (0.19 ± 0.01 to 0.52 ± 0.02 nmol/ml). This effect was reduced by the specific NOS inhibitor N⁶-nitro-L-Arg methyl ester (L-NAME). In fact, when hydra were injected with L-NAME (100 μM) into the gastric cavity and preincubated for 1 hr, the GSH-induced NO₂⁻ levels were significantly (p = 0.001) decreased (0.52 ± 0.02 ± 0.08 ± 0.00 nmol/ml; see Fig. 1), this effect being reversed by excess L-Arg (200 μM; 0.08 ± 0.00 to 0.57 ± 0.03 nmol/ml). On the contrary, the D-isomer of NAME (100 μM) was inactive (Fig. 1). To test the interaction between GSH receptors and NO production, we examined the role of GSH antagonists that inhibited GSH-induced feeding response (see Table 2) on hydra NO₂⁻ release. In fact, when hydra were preincubated with glutamic acid (10 μM) or α-aminoacidic acid (10 μM), the GSH-induced NO₂⁻ production was significantly reduced (0.52 ± 0.2 ± 0.16 ± 0.01 and 0.32 ± 0.05 nmol/ml, respectively). Finally, nauplia (300), the physiological activators of hydra feeding response, increased basal NO₂⁻ levels (0.19 ± 0.01 to 0.65 ± 0.04 nmol/ml), as shown in Figure 1. This effect was abolished by preincubation of hydra with L-NAME (100 μM; 0.65 ± 0.04 to 0.05 ± 0.005 nmol/ml; Fig. 1). We verified that nauplia (300), either whole or injured, in the absence of hydra did not release NO, as determined by measuring nitrite levels (data not shown).

ESR analysis

Both optical and ESR spectroscopy of hemoglobin were used to probe the NO released by hydra. To do this, oxyhemoglobin (25
mM) was included in hydra supernatants before the addition of 2.5 mM GSH. Figure 2 shows the optical spectra of hemoglobin in supernatants from unstimulated (a) and GSH-stimulated (b) hydra. The spectral variations observed are consistent with the massive conversion of oxyhemoglobin (oxyHb) to methemoglobin (metHb). This conversion is typical of oxyHb reacting with NO (Kelmetal., 1988) and was not observed when hemoglobin was treated with GSH in the absence of hydra (data not shown). The corresponding low-temperature ESR spectra are shown in the inset of Figure 2. As can be seen, no signal is detected in the control sample from unstimulated hydra (a), whereas a resonance centered at  = 2.004 appears after treatment with GSH (b). This resonance can be safely ascribed to nitrosyl–hemoglobin (Hb-NO), which is formed either from NO reaction with the small fraction of reduced, deoxy-hemoglobin present under our conditions or, more likely, from a complex pathway leading from oxyHb to Hb-NO through formation of metHb (Kosaka and Shiga, 1996).

**Ca**^{2+}-dependent NOS activity

NO production was the result of a basal expression of NOS activity, as measured by a time-dependent [^{3}H]citrulline generation from [^{3}H]arginine (Fig. 3A). As shown in Figure 3B, in hydra homogenates [^{3}H]citrulline production was dependent on the presence of NADPH (94.6 ± 7.2 cpn x 10^{-3}/mg protein). Moreover, NOS activity of hydra homogenates incubated with 1 mM NADPH was inhibited by co-incubation with L-NAME (100 μM; 94.6 ± 7.2 to 49.54 ± 3.6 cpn x 10^{-3}/mg protein; Fig. 3B). This effect was reversed by excess L-Arg (200 μM; 44.54 ± 3.6 to 84.00 ± 5.93 cpn x 10^{-3}/mg protein), whereas D-NAME (100 μM) was inactive, as shown in Figure 3B.

In addition, the removal of Ca^{2+} by EGTA (2 mM) from the incubation medium greatly affected citrulline formation (94.6 ± 7.2 to 34.54 ± 6.36 cpn x 10^{-3}/mg protein), showing that NOS isoform was Ca^{2+}-dependent (Fig. 3B).

**Calmodulin-independent NOS activity**

Interestingly, we found that the NOS isoform was calmodulin (CaM)-independent. In fact, high concentrations of CaM inhibitors, such as W7 (50 μM) and trifluoperazine (TFF; 50 μM), do not alter NOS activity. In contrast, both W7 and TFF are able to strongly inhibit mouse brain Ca^{2+}/CaM-dependent [^{3}H]citrulline generation. Results are expressed as percent NOS activity with respect to the basal expression in NADPH-incubated hydra homogenates. Each bar corresponds to mean ± SEM of samples performed in triplicate; *p ≤ 0.001.

**Figure 3.** NOS activity in hydra homogenates. In A, a time-dependent [^{3}H]citrulline production is shown. B, Constitutively, hydra express NOS activity in the presence of NADPH, this expression being reduced by L-NAME (100 μM). Excess L-Arg (200 μM) reverses the L-NAME effect, whereas D-NAME (100 μM) is inactive. NOS isoform results in being Ca^{2+}-dependent. In fact, when resuspended in EGTA/Ca^{2+}-free buffer, NADPH-incubated hydra homogenates show a significant decrease in NOS activity. Data are expressed as the ratio between [^{3}H]citrulline production (cpn x 10^{-3}) and mg protein as assayed in the homogenates. In C the isoform of NOS is shown to be CaM-independent. In fact, CaM inhibitors, such as W7 (50 μM) and trifluoperazine (TFF; 50 μM), do not alter NOS activity. In contrast, both W7 and TFF are able to strongly inhibit mouse brain Ca^{2+}/CaM-dependent [^{3}H]citrulline generation. Results are expressed as percent NOS activity with respect to the basal expression in NADPH-incubated hydra homogenates. Each bar corresponds to mean ± SEM of samples performed in triplicate; *p ≤ 0.001.
Table 1. Exogenous NO donors are able to elicit an incomplete feeding response

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<th>SIN-1 (10 μM)</th>
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<th>NOsol (30 μM)</th>
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| OxyHb (10 μM)
|            | +            | +           | +             | -                 |
| L-NAME (100 μM) |             | +           | -             | -                 |

* The incomplete feeding response is represented by tentacle curlings identical to those elicited by GSH, but no mouth opening.

The NO donors used in this study included N-(6-aminohexyl)-5-chloro-1-naphthalene-sulfonamide hydrochloride (W7; 50 μM) and trifluoperazine (TFP; 50 μM), either alone or in synergism, did not alter NOS activity (Fig. 3C). In our experimental conditions, the efficiency of the above-mentioned CaM inhibitors was tested on mouse brain Ca2+/CaM-dependent NOS activity. As shown in Figure 3C, both W7 (50 μM) and TFP (50 μM) were able to strongly inhibit mouse brain [3H]citrulline generation.

Effect of exogenous NO donors on hydra feeding response

By using exogenous NO donors, we studied the role of NO in the control of hydra feeding response. In this respect, we found that 3-morpholino-sydnonimine (SIN-1) elicited an incomplete feeding response consisting in tentacular movements similar to those of GSH-induced feeding response (Lenhoff, 1981), but without the typical mouth opening (see Fig. 5D). When tested at concentrations of 0.03–100 μM, the effect of SIN-1 exhibited a bell-shaped profile. Tentacle curls were absent at low levels of SIN-1 (0.03 μM). They were observed to gradually occur up to 0.1 μM, then to rise sharply up to a peak of 1–10 μM. At higher SIN-1 concentrations, movements were seen to decline.

As shown in Table 1, an induction of an incomplete feeding response similar to the one observed after SIN-1 stimulation was also obtained by using NOS inhibitors like sodium nitroprusside (SNP; 20 μM) or authentic NO solution (NOsol; 30 μM). The NO donor-induced tentacle contraction was completely inhibited by oxyHb (10 μM), a trapping agent for NO, but not by l-NAME (100 μM; Table 1). Like GSH-induced feeding response, NOS-stimulated tentacle curls disappeared a few minutes after addition of NO donors and then slowly disappeared. After 15 min, all specimens exhibited a normal behavior.

Involvement of cGMP

Because NO exerts its biological activity by stimulating the soluble guanylate cyclase, the role of cGMP in this event was studied. As shown in Figure 4, a basal level of cGMP (117 ± 22 fmol per 10 specimens) was present. Treatment of hydra with GSH (2.5 μM) increased production of cGMP levels, a peak being observed after a 1 min treatment (from 117 ± 22 to 297 ± 27 fmol per 10 specimens; Fig. 4). This maximal effect was abolished by preincubation of hydra with l-NAME (100 μM) (from 297 ± 27 to 144 ± 31 fmol per 10 specimens), indicating that cGMP generation is attributable to NO synthesis. To test the role of cGMP in the induction of hydra feeding response, N3,2′-O-dibutylrylguanosine-3′,5′-cyclic monophosphate (dbt2-cGMP), a cGMP analog, was used. When hydra were incubated with 100 μM dbt2-cGMP alone, neither tentacle curls nor mouth opening was observed (Table 1).

Effect of NO inhibitors on hydra feeding response

In a typical GSH-induced feeding response, all tentacles were simultaneously curled, as shown in Figure 5B. A pretreatment of hydra with the NOS inhibitor l-NAME (100 μM) or with oxyHb (10 μM) for 1 hr did not alter the induction of tentacular movements of GSH-induced feeding response (Table 2). However, when pre-incubated with l-NAME (100 μM) for 1 hr, nauplia were able to induce curlings only in the tentacles directly touched by nauplia, whereas all the other untouched tentacles were at complete rest (Fig. 5F). The same effects were also obtained by co-incubating hydra specimens with oxyHb (10 μM) for 1 hr (Table 2).

DISCUSSION

NO production in hydra

In this study, we report that NO is involved in hydra feeding response, the most primitive olfactory-like behavior present in a multicellular organism. Hydra are able to release basal levels of NO, as determined by measuring nitrite, the NO breakdown product (Tracey, 1992). Nitrite production was also verified by fluorimetric assay according to the method of Misko (Misko et al., 1993) (data not shown). Optical and ESR spectroscopies further proved NO production. The conversion of oxyHb to metHb (see Fig. 2) provided strong evidence that NO had been selectively released by GSH-treated hydra, because NO is known to act as an oxidant on oxyHb (Kelm et al., 1988). The ESR result was even more clear-cut, in that a signal with features typical of nitrosyl-Hb appeared in Hb-containing hydra supernatants after the GSH stimulus. Note that the intensity of this latter signal was lower than the massive phenomenon observed by optical spectroscopy. This is not surprising because, under aerobic conditions, Hb is mostly in the oxy form, which converts to metHb in the presence of NO, and only a very small fraction escapes oxidation forming the nitrosyl adduct. This adduct, on the other hand, can also be
produced in a sequence as follows: after a binding of NO to metHb; autoxidation of the complex to Hb(II)NO; dissociation of NO; and binding of a second molecule of NO to reduced iron (Kosaka and Shiga, 1996). The basal production of NO was enhanced in the presence of GSH, the activator of hydra feeding response. This effect was abolished by the specific NOS inhibitor L-NAME. Excess L-Arg reversed the L-NAME effect, whereas the D-isomer of NAME was inactive, thus demonstrating that NO₂⁻ production was dependent on L-Arg metabolism. In addition, GSH-induced NO production was also decreased in the presence of glutamic or α-aminoacidic acids, two GSH antagonists, which bind to but do not activate GSH receptors (Lenhoff, 1981), showing that NO synthesis was attributable to GSH receptor activation. It is worth noting that the same compounds are able to competitively inhibit the activity of GSH in eliciting hydra feeding response (see Table 2). Finally, nauplia caused an increase of basal nitrite release, whereas nauplia alone did not produce nitrite. This effect was inhibited by L-NAME, thus demonstrating that also a physiological stimulus of hydra feeding response (Table 2) was able to activate the L-Arg–NO pathway.

**NOS expression in hydra**

We have observed that hydra constitutively express a NADPH/ Ca²⁺-dependent NOS activity, as verified by evaluating the conversion of [³H]arginine to [³H]citrulline. L-NAME was able to reduce significantly the *in vitro* NOS activity, whereas D-NAME was inactive. In addition, excess L-Arg reversed the effect of L-NAME, demonstrating that [³H]citrulline production was attributable to L-Arg metabolism. Because Ca²⁺ ions are not required for the binding of GSH to its receptor (Venturini, 1987), GSH may stimulate NOS by activating a receptor-linked calcium channel. Interestingly, hydra NOS appears to be CaM-independent. It should be pointed out that Ca²⁺-binding protein(s) other than CaM in hydra may account for NOS regulation.
It is very intriguing to note that a CaM-independent/Ca$^{2+}$-dependent NOS has been described already in the catfish taste organ (Huque and Brand, 1994) as well as in rat neutrophils (Yui et al., 1991). Although no evidence is available concerning the molecular evolution of NOS, our results suggest the hypothesis that the primitive NOS isoform as appearing throughout evolution may be a CaM-independent isoform.

**Role of the NO–cGMP pathway in hydra feeding response**

By using exogenous NO donors like SIN-1, SNAP, or authentic NO solution, we observed that an incomplete feeding response was elicited, consisting of tentacular movements similar to those of GSH-induced feeding response, but without the typical mouth opening. This effect was completely inhibited by oxyHb, a trapping agent for NO, and the NOS inhibitor L-NAME, thus demonstrating that the effect was attributable to exogenous NO.

Because NO is a potent inducer of soluble guanylate cyclase (Garbers, 1992), the role of cGMP in this experiment was analyzed. A basal level of cGMP was present in hydra, and GSH treatment was able to significantly increase cGMP production. This effect was abolished by L-NAME, showing that GSH-induced cGMP generation derives from NO synthesis. However, we observed that an analog to cGMP, dbt$^2$-cGMP, was unable to trigger tentacle curlings. It is worth noting that the same amount of dbt$^2$-cGMP was capable to make the GSH-induced hydra feeding response shorter (Colasanti et al., 1995), thereby demonstrating the efficiency of the cGMP analog. Thus, these results show that NO was sufficient to induce tentacular movements and that this process was independent of NO-stimulated cGMP production. The dissociation of the NO and cGMP pathways has been reported elsewhere for many processes (Brunner et al., 1995; Brune et al., 1996; Vallette et al., 1996). In many of these cases, this dissociation appears to be attributable to the redox state of NO (Stamler et al., 1992), even if in our context, other different mechanisms may be involved.

Again, along with other authors have indicated that factors such as cAMP, IP$_3$, and/or Ca$^{2+}$ can be important as primary mediators of the GSH-induced hydra feeding response (Cobb et al., 1980; Lenhoff, 1981; Venturini, 1987; Venturini et al., 1988). Our experiments show that NO does not seem to be a primary stimulus in eliciting feeding response. In fact, a pretreatment of hydra with NO inhibitors did not alter the tentacular movements of the GSH-induced feeding response. However, it is interesting to note that in GSH-induced feeding response, all tentacles are simultaneously curled because of all available receptors for GSH being stimulated. This massive stimulation can induce a cascade of the primary mediators in all tentacles, in a way likely to disguise the NO stimulus-induced effect. On the contrary, when a prey (i.e., nauplia) accidentally touches a tentacle, initially a certain amount of GSH is released locally, thus stimulating only the local GSH receptors present in that tentacle. In fact, the nauplia-induced hydra feeding response begins with the activation of the tentacle touched by nauplia and successively spreads to the neighboring tentacles. In these conditions, the movements of all of the nauplia-untouched tentacles were totally inhibited by the NO inhibitors, whereas the nauplia-touched tentacle still curled, thus demonstrating that NO inhibitors prevent the diffusion of the activator stimulus to the neighboring tentacles.

The present data suggest that in hydra, NO stimulus can participate in the triggering and coordination of tentacular curling, providing for the fast diffusion of a primary stimulus to the neighboring tentacles, regardless of any direct connection through synapses (tentacle recruitment). NO stimulus, however, is not sufficient for complete induction of the complex behavioral phenomenon.

Therefore, we propose that initially GSH induces the feeding response through mediators such as cAMP, IP$_3$, and/or Ca$^{2+}$, while successively, an increase of GSH-induced Ca$^{2+}$ levels is responsible for NO production, which in turn elicits the recruitment of neighboring tentacles. Finally, on a longer timescale, elevated NO-induced cGMP levels are able to trigger inhibition of the GSH-induced feeding response, as described elsewhere (Colasanti et al., 1995), presumably via cGMP-activated protein kinases. Taken together with data in the literature, our results are consistent with those reported for the mammalian olfactory system (Breer and Shephered, 1993). In the latter, in fact, the rapid and transient generation of pulses of cAMP and/or IP$_3$ is considered the primary reaction in olfactory signal transduction. However, high doses of odorant elicit a delayed and sustained elevation of Ca$^{2+}$ that is sufficient to initiate NO formation. NO is thought to induce the recruitment of neighboring cilia. Finally, the rise in NO-induced cGMP levels is supposed to trigger molecular mechanisms leading to olfactory inhibition (e.g., adaptation processes) (Breer and Shephered, 1993). It should be pointed out that, like NO, carbon monoxide (CO), another activator of soluble guanylate cyclase, may serve as an intercellular messenger in mammalian olfaction (Verma et al., 1993). In the near future, it may be very intriguing to verify the presence of a CO pathway in hydra and to study the role of CO in the hydra feeding response.

In conclusion, our results confirm that the NO pathway is highly preserved throughout evolution and that it is involved in the tentacle control of feeding response, i.e., in the most primitive model of an olfactory-like system present in a multicellular organism. Noteworthy is the conclusion that NO seems to play a similar activation–adaptation role both in a primitive chemoreceptorial mechanism of a coelenterate and in the sophisticated olfactory system of a mammal.

**REFERENCES**


