The head activator is released from regenerating *Hydra* bound to a carrier molecule

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***Hydra* forced to regenerate a head releases head activator and head inhibitor during the first hours after cutting to induce head-specific growth and differentiation processes. Analysis of the size distribution demonstrated that the head-activator peptide is co-released with (a) large molecular weight carrier molecule(s) to which it is non-covalently bound. The carrier-bound head activator is fully active on *Hydra* indicating that a carrier does not hinder the interaction with receptors. In contrast to this the head inhibitor is released in its naked, low molecular mass form. The association or non-association with a carrier molecule results in marked differences in biological properties. The head activator has a short range of action, but a long half-life, the head inhibitor has a global range of action, but a short half-life. These results provide a plausible explanation why two antagonistically acting substances, although they are released from the same site and simultaneously nevertheless can give rise to a well-defined temporal and spatial pattern of differentiation as occurs, for example, during head regeneration in *Hydra*.**

Key words: pattern formation/*Hydra* head regeneration/head activator/head inhibitor/carrier molecule

Introduction

Head formation in *Hydra* is controlled mainly by two substances, head activator (HA) and head inhibitor (HI) (Schaller *et al.*, 1979). The two substances have their highest concentrations in the head region of the animal, including tentacles and hypostome and they decrease in concentration towards the foot region (Schaller and Gierer, 1973; Hoffmeister and Schaller, 1985). HA is needed for the induction of head-specific growth and differentiation processes, whilst HI acts antagonistically (reviewed in Schaller and Bodenmüller, 1985). In the process of head regeneration both substances are released more or less simultaneously. HI is released from 0 to 1 h after cutting and has a short half-life of ~1 h. HA is released from 0 to 4 h after cutting and has a longer half-life of ~3 h. In normal *Hydra* both molecules are products of nerve cells which release their secretory contents into the intercellular space. The release of both HA and HI is under the control of HI which acts as a release-inhibiting factor, probably pre-synaptically, for both substances. If disinhibition occurs, for example by removing an existing head, both substances are released (Kemmer and Schaller, 1984). Based on their molecular masses — HA is a peptide consisting of 11 amino acids, HI is a small hydrophilic molecule of <500 daltons — it is expected that they should have very similar diffusion properties. To explain why HI diffuses fast and over great distances and therefore exerts its effects over the whole animal,

whereas HA acts locally on the tissue directly surrounding the release site, we argued that due to its hydrophobic nature HA may stick more easily to neighbouring cells than HI (Kemmer, 1984; Kemmer and Schaller, 1984). Here we present evidence that HA, but not HI, is bound to (a) large carrier molecule(s) when it is released from head-regenerating *Hydra*. The carrier prevents diffusion of HA over long distances thereby providing a much better explanation for a slower diffusion and therefore local action of HA.

Results

HA is carrier-bound when released from head-regenerating Hydra
HA and HI are released extensively from a piece of tissue forced to regenerate a head (Schaller, 1976; Kemmer and Schaller, 1984). To further increase the amount released we made use of a morphogenetic mutant of *Hydra*, a non-budding, multi-headed *Hydra* (Lenhoff, 1965), which contains at least 10 times more HA than normal *Hydra* and also more HI (Schaller *et al.*, 1977). The higher HI content is reflected biologically in a larger inhibited area i.e. the gastric region of this mutant becomes oversized. Once away from the inhibitory influence of the head, the high HA content leads to an over-induction of heads as shown in Figure 1. This mutant is therefore very suitable for studying HA and HI release.

To study in what form HA and HI are released, we attempted to concentrate the medium cautiously, and subsequently analyse a possible association and co-release with a carrier molecule by separating the molecules according to size. The size distribution should indicate whether the molecule is released in its free form or bound to some larger structure.

Gastric regions from the multi-headed mutants were excised and subdivided into as many small slices as possible. Each such slice regenerated one or more heads. For each experiment ~3000 such slices were cut and left to regenerate at a density of 3 regenerates per ml. After 3 h the medium was collected and filtered through paper. At each step an aliquot was removed for direct analysis with and without methanol extraction and subsequent Seppack separation of the HA from HI. The medium was concentrated either by lyophilization or by Diaflo ultrafiltration through an UM-10 membrane. UM-10 has a cut-off at 10 000 daltons and therefore separates molecules smaller than 10 000 daltons from larger ones. The concentrated retentate or the redissolved lyophilized medium were centrifuged at 10 000 g for 15 min to remove particulate material and applied at 4°C to a Sephacryl S-300 column equilibrated with 25 mM ammonium bicarbonate, pH 7.6. The collected fractions were lyophilized and stored frozen for biological or radioimmunological assay. HI content was analysed in a biological assay using the inhibitory effect of HI on bud outgrowth (Schaller *et al.*, 1979). HA concentration — or HA-like immunoreactivity — was determined in a radioimmunoassay (RIA) using the antibody 12/5 and [¹²⁵I]Tyr11-HA as tracer (Bodenmüller *et al.*, 1986a). The antibody 12/5 requires an intact amino-terminal portion of HA with pyroglutamic acid as amino-terminal amino acid (Schaller *et al.*,

Fig. 1. Multi-headed morphogenetic mutant of *Hydra viridissima*, in which head formation is dominant over bud and foot formation due to the overproduction of head factors, especially of HA but also of HI, over the respective foot factors.

1984) and does not react with carrier-bound HA, as it is recovered, for example, from mammalian blood (Roberge *et al.*, 1984; Roberge, 1985).

In Table I the results obtained from several regeneration experiments are summarized. In general we found that most of the HA (>90%) was carrier bound and only a small amount was in the free form (<10%). Carrier-bound HA was detectable in the RIA only after methanol extraction, not directly. This demonstrated that carrier-bound HA was retained by the UM-10 membrane and after lyophilization eluted from an S-300 column predominantly in a molecular mass range of >200 kd (Figure

Table I. Distribution of bound HA and HI in different separation steps

Separation	Endogenous HA determined in RIA (% of total applied)	Endogenous HI determined in bioassay (% of total applied)	Added tritiated HA (% of c.p.m. applied)
Regeneration medium without separation	0.5–2	100	100
Regeneration medium after methanol extraction	95–100	100	100
Diaflo eluate	10	92	95
Diaflo retentate after methanol extraction	90	8	5
S–300			
fraction 21–30*	(50–)90	0	0
fraction 31–40*	10(–50)	0	0
fraction 41–60	1–10	100	100

*HA was detectable in the RIA only after methanol extraction

Fig. 2. Gel filtration of medium collected from head-regenerating *Hydra* on a Sephacryl S-300 column equilibrated with 25 mM ammonium bicarbonate, pH 7.5, at 4°C (total volume 40 ml, fraction size 15 drops or 0.94 ml). Fractions were collected and either assayed directly or lyophilized in batches of 10, extracted with methanol and purified over Seppak C₁₈ cartridges. The solid line indicates the position of HA as discovered by the RIA after methanol extraction. (a) Medium concentrated by lyophilization. [¹²⁵I]Tyr¹¹-HA was added before application to the column (—▲—▲—▲). (b) Medium concentrated by Diaflo ultrafiltration. [³H]HA was added to the regenerating *Hydra* (○····○).

2a). In some experiments, where the medium was concentrated by ultrafiltration without concomitant increase in salt concentration, the apparent molecular mass of the bound HA shifted to a somewhat smaller value (Figure 2b). We therefore assume that different degrees of aggregation of the carrier(s) are possible. In all cases the carrier-bound HA was well separated from free HA.

From the carrier(s) HA could be released by methanol or organic solvent extraction, indicating that the HA peptide is bound

Fig. 3. Binding of an iodinated HA-BSA conjugate to carrier molecules released during head regeneration from *Hydra*. Medium was collected from dishes which contained *Hydra* regenerating heads (lane 1) and from dishes containing non-regenerating animals (lane 2). The medium was lyophilized, redissolved in water and spotted on nitrocellulose paper. The medium from 25, 50 and 100 regenerating or non-regenerating animals was spotted twice each. Since the protein content of the lyophilized medium was so minute that it was not measurable, a defined amount of bovine serum albumin was added to each sample to ensure identical protein concentration. After incubation overnight with the HA-BSA conjugate, the spots were visualized either by autoradiography, or they were cut out and counted directly in a γ -counter.

Table II. Stimulation of interstitial cell mitosis by HA bound to its high mol. wt carrier

Concentration of HA as determined in the RIA	Total number of cells counted	Cells in mitosis (#)	Mitotic index (%)	Increase over control (%)
None	5027	151	2.92 $\pm$ 0.27	0
1.5×10^{-12} M	4542	187	3.95 $\pm$ 0.32	35
0.5×10^{-11} M	4028	188	4.46 $\pm$ 0.11	52
1.5×10^{-11} M	3990	197	4.70 $\pm$ 0.16	61

to its carrier(s) by non-covalent linkage. This endogenous HA behaved like synthetic HA on molecular-sieve columns, on Sep-pack C₁₈ cartridges, on h.p.l.c. and in the RIA using the antibody 12/5.

In some experiments radioactively labelled HA was added both in its monomeric and dimeric state, to monitor unspecific absorption, chromatographic behaviour and rebinding. Table I and Figure 2b show that tritiated HA added immediately after cutting the slices behaved in all steps as expected from its molecular mass. Ninety-five percent of the counts were recovered in the flow-through of the UM-10 Diaflo ultrafiltration membrane in accordance with a 20-fold concentration of the medium. The remaining 5% of the radiolabel eluted from the S-300 column in the low molecular mass region. This result was obtained both if the HA was monomerized prior to application or used as dimer. We interpret this to mean that HA does not attach randomly to large molecules and that exchange with endogenous HA at the carrier(s) does not occur at this relatively dilute concentration (10^{-11} M) of tritiated HA. It also indicates that the binding of endogenous HA to the carrier(s) is relatively stable.

In Figure 2a monomeric [¹²⁵I]Tyr11-HA (100 000 c.p.m.) was added to the lyophilized medium prior to application to the S-300 column. At this concentration (10^{-9} M) and with this HA analogue no exchange with the endogenous HA was observed and all counts eluted in the salt region.

To study rebinding we made use of a solid phase binding assay in which the medium after lyophilization was spotted in increasing concentrations onto nitrocellulose filters. The protein content of the medium was so low that it was not measurable. To

ensure identical conditions, a constant amount of bovine serum albumin was added to each spot. Subsequently the nitrocellulose filters were incubated overnight with an HA conjugate, where HA was kept monomeric by coupling it over the ϵ -amino group of Lys7 to bovine serum albumin, and where the radiolabel was introduced by iodinating the tyrosines of the bovine serum albumin. Figure 3 shows that this ligand was bound to the *Hydra* medium in a concentration-dependent manner. The binding was only observed with medium collected from head-regenerating animals, no binding occurred with medium collected from non-regenerating animals.

Biological activity of carrier-bound HA

Earlier experiments indicated that HA released from regenerates into the medium is biologically active without any pre-treatment (Schaller, 1976). In the above experiments we had found that very little HA (<10%) is in the free form. To assay whether carrier-bound HA is biologically active, the high molecular weight fraction of the S-300 column was assayed directly on *Hydra*. As a fast biological assay we used the effect of HA to stimulate cell division in *Hydra*. Whole *Hydra oligactis* were incubated for 1.75 h in increasing concentrations of the S-300 fraction. The mitotic index of interstitial cells was determined in macerates of cells. Table II shows that increasing concentrations of the S-300 fraction led to an increase in mitotic index, indicating that carrier-bound HA was biologically active, and that it was bound as a monomer.

HI is not released with a carrier molecule

To investigate whether HI is also carrier bound, all steps of the above purification procedure were analysed for their content of HI. HI was assayed either directly or after methanol extraction in the water fraction of Sepapak C₁₈ cartridges. Table I shows that in contrast to HA, HI always behaved as expected of a molecule of <500 daltons. It was recovered quantitatively in the flow-through of the UM-10 Diaflo ultrafiltration. The small amount present in the retentate eluted on S-300 together with the salt fractions. No inhibitory activity was recovered from the high molecular mass fractions of S-300 as is also evidenced by the results presented in Table II where the presence of HI would have led to a decrease in mitotic activity.

Discussion

To create spatial and temporal patterns during development, gradients of diffusible substances have been postulated as the most likely substrates for realization. Starting from equilibrium conditions a slight advantage in activation leads by self-stimulation or autocatalysis to induction. This induction, e.g. for a head, should be a local event. To limit the induction locally, the inducing substance should have a restricted range of action. The spreading of the induction over a wider area is in addition prevented by an antagonistically acting inhibitory principle which in the simplest case is an inhibitory molecule with, in theoretical models, a 10^3 times faster rate of diffusion than the inducing principle (Gierer and Meinhard, 1972; Kemmner, 1984). In *Hydra* we have found that for head or foot formation an inducing and an inhibitory molecule can be isolated, respectively (reviewed in Schaller and Bodenmüller, 1985). Contradictory to predictions the molecular sizes of inducers and inhibitors are very similar. All four substances have molecular masses in the range of 500–1000 daltons implicating similar diffusion properties. However, in *Hydra* too, the inherent biological action of the inducers is local, that of the inhibitors is global. During head regeneration only the tissue close to the cut is induced to head-

specific growth and differentiation, not so the tissue at a greater distance. Likewise an existing structure such as a head inhibits the development of a similar structure nearby influencing negatively almost the whole animal (MacWilliams, 1982, 1983a,b). We have found that during regeneration both the activating and the inhibiting substance are released more or less simultaneously. The inhibitor has a very short half-life of $\sim 1/2$ h, the activator of ~ 3 h (Kemmer and Schaller, 1984). In experiments trying to explain the differences in spatial and temporal action of the two substances we discovered that the substance responsible for head induction in *Hydra*, HA, is released bound to a high molecular weight carrier molecule(s). For the substance inhibiting head formation, HI, no such carrier molecule was detectable. Depending on the type of pre-treatment these HA carrier molecules eluted at different positions. We assume that either several carrier molecules exist or more likely that different degrees of aggregation of one carrier molecule are possible. The association of HA to the carrier(s) seems to be very stable, since almost no free HA was detectable either after several hours of ultrafiltration or after lyophilization which results in a drastic increase in salt concentration. The carrier-bound HA was biologically active, which we interpret to mean that the carrier(s) did not prevent interaction with the receptor and that HA was bound in its monomeric state, since only the monomer is biologically active (Bodenmüller *et al.*, 1986b). In *Hydra* HA starts to be active at a concentration of 10^{-13} M. We have shown (Bodenmüller *et al.*, 1986b) that under physiological conditions HA once dimerized is stable at 10^{-13} M. This would imply that HA after release from its carrier(s) has to find its receptor fast to prevent inactivation by dimerization. The binding of HA to carrier(s) would therefore have several consequences: it would ensure that the diffusion range of HA as compared to HI is limited; it would represent a safe protection against degradation by enzymes (Roberge *et al.*, 1984) and it would also prevent dimerization. Protection from degradation by enzymes or by dimerization and a restricted range of diffusion are therefore plausible consequences of the association or co-release of HA with (a) carrier molecule(s), and offer a plausible explanation why the head induction in *Hydra* is local, whereas head inhibition extends over the whole animal.

Materials and methods

Animals

Hydra attenuata were used as normal or wild-type *Hydra*. A non-budding mutant of *H. viridissima*, originally isolated by Lenhoff (1965), which overproduced HA (Schaller *et al.*, 1977), was used as a donor to study the co-release of HA with carrier molecule(s). A strain of *H. oligactis* which among other morphogenetic abnormalities had an increased content of interstitial cells arrested in G2 phase of the cell cycle (Hoffmeister, 1985), was used to study the action of HA on interstitial cell mitosis. All animals were cultured in a medium consisting of 1 mM CaCl_2 , 0.1 mM MgCl_2 , buffered with 5 mM NaH_2PO_4 , pH 7.6, at 19°C. Animals were fed daily and washed 6–8 h after feeding.

Biological assays

As a standard assay for HI its effect on inhibiting bud outgrowth was used (Schaller *et al.*, 1979). Large well-fed *H. attenuata* without buds were selected, the heads cut off at time 0 to induce budding, and incubated in increasing concentrations of HI. Inhibition of bud outgrowth was determined at hourly intervals starting from 5 to 9 h after cutting. A 50% inhibition was defined as one biological unit (BU).

As a standard biological assay for HA its effect on stimulating cell division in *Hydra* was used. For that purpose *H. oligactis* without buds were incubated in increasing concentrations of HA for 1.75 h. Six animals were dissociated into cells by maceration in 200 μl of 7% acetic acid and 7% glycerol, fixed in 2% formaldehyde and spread on gelatin-coated slides (David, 1973). The mitotic index of big interstitial cells in nests of one and two was determined. With synthetic monomerized HA a 50% increase was obtained at a concentration of 10^{-12} M.

RIA for HA

HA concentration was measured in an RIA using the polyclonal rabbit antiserum 12/5 at a dilution of 1:30 000 and [^{125}I]Tyr11-HA (4000 c.p.m. per tube) as tracer and competing with increasing concentrations of synthetic HA (Bodenmüller *et al.*, 1986a). A competition was achieved with 10–20 fmol of HA. HA bound to the antibody was separated from free HA by precipitation with 13.7% polyethylene glycol.

HA and derivatives

Synthetic HA was purchased from Bachem, Switzerland. It was monomerized by incubation in 1 M $(\text{NH}_4)_2\text{SO}_4$ and diluted in 1% bovine serum albumin prior to use (Bodenmüller *et al.*, 1986b). Radiolabelled HA was obtained by tritiating 4 I-Phe11-HA, (20 Ci/mmol) or by iodinating Tyr11-HA (~ 1000 Ci/mmol), both synthesized by E. Escher (Bodenmüller *et al.*, 1986a). For rebinding studies an HA conjugate was used where 5–10 HA molecules were coupled by glutaraldehyde to bovine serum albumin (Schaller *et al.*, 1984) which was subsequently radiolabelled with iodine by the chloramin T method.

Purification procedures

HA and HI were extracted with 90% methanol, evaporated and applied in water to Seppak C_{18} cartridges (Waters). The flow-through contained the HI. After washing with 10 ml of distilled water and 10 ml of 20% methanol, HA was eluted with 10 ml of 80% methanol. For Diaflo ultrafiltration an Amicon cell was used with the UM 10 membrane filter which retains molecules of > 10 000 daltons. To separate free HA from carrier-bound HA Sephacryl S-300 (Pharmacia) columns were used, equilibrated with 10 mM ammonium bicarbonate. The same buffer was used as eluent. HA identity was checked on C_8 reverse-phase h.p.l.c. columns (LiChrosorb, 5 μm particle size) with a linear gradient from 20 to 40% acetonitrile in 0.1% trifluoroacetic acid (Schaller and Bodenmüller, 1981).

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References

- Bodenmüller, H. and Roberge, M. (1985) *Biochim. Biophys. Acta*, **825**, 261–267.
- Bodenmüller, H., Escher, E., Zachmann, B. and Schilling, E. (1986a) *Int. J. Peptide Protein Res.*, in press.
- Bodenmüller, H., Schilling, E., Zachmann, B. and Schaller, H.C. (1986b) *EMBO J.*, **5**, 1825–1829.
- David, C.N. (1973) *Wilhelm Roux's Arch. Dev. Biol.*, **171**, 259–268.
- Gierer, A. and Meinhardt, M. (1972) *Kybernetik*, **12**, 30–39.
- Hoffmeister, S. (1985) Doctoral thesis, University of Heidelberg.
- Hoffmeister, S. and Schaller, H.C. (1985) *Wilhelm Roux's Arch. Dev. Biol.*, **194**, 453–461.
- Kemmer, W. (1984) *Differentiation*, **26**, 83–90.
- Kemmer, W. and Schaller, H.C. (1984) *Differentiation*, **26**, 91–96.
- Lenhoff, H. (1965) *Science*, **148**, 1105–1107.
- MacWilliams, H.K. (1982) *J. Theor. Biol.*, **99**, 681–703.
- MacWilliams, H.K. (1983a) *Dev. Biol.*, **96**, 217–238.
- MacWilliams, H.K. (1983b) *Dev. Biol.*, **96**, 239–257.
- Roberge, M. (1985) Doctoral thesis, University of Heidelberg.
- Roberge, M., Escher, E., Schaller, H.C. and Bodenmüller, H. (1984) *FEBS Lett.*, **173**, 307–313.
- Schaller, H.C. (1976) *Wilhelm Roux's Arch. Dev. Biol.*, **180**, 287–295.
- Schaller, H.C. and Bodenmüller, H. (1981) *Proc. Natl. Acad. Sci. USA*, **78**, 7000–7004.
- Schaller, H.C. and Bodenmüller, H. (1985) *Biol. Chem. Hoppe-Seyler*, **366**, 1003–1007.
- Schaller, H.C. and Gierer, A. (1973) *J. Embryol. Exp. Morphol.*, **29**, 39–52.
- Schaller, H.C., Schmidt, T., Flick, K. and Grimmelikhuijzen, C.J.P. (1977) *Wilhelm Roux's Arch. Dev. Biol.*, **183**, 207–214.
- Schaller, H.C., Schmidt, T. and Grimmelikhuijzen, C.J.P. (1979) *Wilhelm Roux's Arch. Dev. Biol.*, **186**, 139–149.
- Schaller, H.C., Bodenmüller, H., Zachmann, B. and Schilling, E. (1984) *Eur. J. Biochem.*, **138**, 365–371.

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