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Bud induction in decapitated *Hydra attenuata* by 5-azacytidine: a morphological study

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SUMMARY

The effect of 5-azacytidine (5-azaCR) on head regeneration and budding in hydra are reported. *Hydra attenuata* were exposed to various doses of 5-azaCR for 48 h and then decapitated and cultured. Head regeneration and bud formation were observed for 12 days after decapitation. Untreated control hydra regenerated heads within 7 to 8 days of decapitation with a budding index of 0·2. Buds invariably arose in the normal budding zone (below the gastric region).

In the group treated with 0·8 mM-5-azaCR, 9 days after decapitation head regeneration was seen in only 13 % of animals, and an average of two buds per hydra were formed, most of which were in the vicinity of the distal end. Induction of budding was also seen in the animals that regenerated heads.

In animals exposed to 1 mM-5-azaCR three main types of responses were observed 9 days after decapitation. (1) 44 % of the animals regenerated normal heads; about half of them developed at least one bud and these buds originated in the budding zone. (2) 17·5 % of the animals developed abnormal, long hypostome-like structures with single or bifurcated tentacles at their tips. There were at least two buds per animal and they were invariably at abnormal sites. (3) 32 % of the animals failed to regenerate heads, although they developed two buds. 87 % of these buds originated in abnormal sites of the body column and a large number (72 %) did not detach even by the 12th day after decapitation.

Both 5 and 10 mM of 5-azaCR were toxic to the animals; the survivors formed large globe-shaped heads. Bud induction was seen in 60 % and 28 % of animals in the 5 and 10 mM groups, respectively.

These observations demonstrate that 5-azaCR induces bud formation in hydra at doses that inhibit head regeneration. This bud induction might be due to a specific expression of gene products responsible for bud formation.

INTRODUCTION

5-azacytidine (5-azaCR) is a cytidine analogue with an aza group at position 5 of the pyrimidine ring. When this analogue is incorporated into cellular nucleic acids it inhibits their enzymic methylation at position 5 of cytosine. In recent years, the growing interest in the role of enzymic DNA methylation in gene regulation has prompted studies on the effects of 5-azaCR on cell functions and cell differentiation. It has been demonstrated that 5-azaCR incorporation into genomic DNA leads to DNA hypomethylation (Jones & Taylor, 1981; Maharajan, Tosi, Pratibha

Key words: hydra, regeneration, bud induction, 5-azacytidine.

& Scarano, 1982). Moreover, in several cell systems, 5-azaCR-induced hypomethylation of genomic DNA is often associated with specific activation of previously silent genes (for a review, see Riggs & Jones, 1983), which results in the expression of their products, and in morphologic changes at the cellular level. Experimental studies have demonstrated the differentiation of muscle cells, adipocytes and chondrocytes in strain 10T½ fibroblasts exposed to 5-azaCR for a short period (Constantinides, Jones & Gevers, 1977; Constantinides, Taylor & Jones, 1978; Taylor & Jones, 1979). This differentiation does not occur in untreated fibroblasts. 5-azaCR induces differentiation also in macrophages, Friend cell leukemia, melanocytes and T lymphoma cells (Boyd & Schrader, 1982; Creusot, Acs & Christman, 1982; Christman, Mendelson, Herzog & Schneiderman, 1983).

Developmental processes in hydra provide a simple system by which to probe the role of cell proliferation and cell movement in cell differentiation and pattern formation. Two well-studied developmental processes in hydra are the formation of buds and the regeneration of hypostome and tentacles. Whereas cell proliferation plays a minor role, if any, in these processes, a key role is played by cell movement or reorientation of cells followed by cell differentiation (Park, Ortmeyer & Blankenbaker, 1970; Webster, 1971). Both head regeneration and bud formation in hydra occur within 4 to 5 days. This suggests that gene expression in hydra might be easily inducible without elaborate preparatory cell cycles. If so, inductors of specific gene expression, such as 5-azaCR, should exert major effects on regeneration and budding. Despite these attractive possibilities, the effects of agents that alter gene expression have seldom been studied in hydra.

In the present experiments, we have studied the effects of 5-azaCR on head regeneration and on bud formation in *Hydra attenuata*. We observed that 5-azaCR either inhibits head regeneration in a dose-specific manner, or induces abnormal head regeneration. More important, 5-azaCR induces development of buds in hydra immediately after decapitation, irrespective of the stage of head regeneration. This is the first report in which 5-azaCR is shown to induce formation of morphologically complete organism such as buds in hydra.

MATERIALS AND METHODS

Hydra attenuata were obtained from Prof. P. Tardent, University of Zurich, and were grown in our laboratory, according to the method of Loomis & Lenhoff (1956) with slight modifications. Hydra were kept at 18 ± 1°C in a medium consisting of 1 mM-$NaCO_3$, 1 mM-$CaCl_2$ and $1{\cdot}25 \times 10^{-2}$ mM-EDTA in distilled water. Animals were fed on alternate days. Only young animals without signs of budding were used in this study. Buds detached from these animals on a single day were collected and cultured for a week. Hydra of the same size without buds and with tentacles of normal length were then selected from the culture.

For preliminary experiments to assess drug toxicity, the animals were cultured individually in 35×10 mm plastic Petri dishes in 3 ml medium and were fed as above. They were observed daily under a stereomicroscope, for behaviour, feeding habits and bud formation. The toxic effect of 5-azaCR was graded: (1) high, (2) moderate and (3) low, depending on the response of the hydra. High corresponded to death, moderate to an impaired feeding response, and low to failure to contract or extend in response to stimulation. To study the effect of 5-azaCR on regenerates, animals of a single group were first cultured in 90×15 mm plastic Petri dishes in

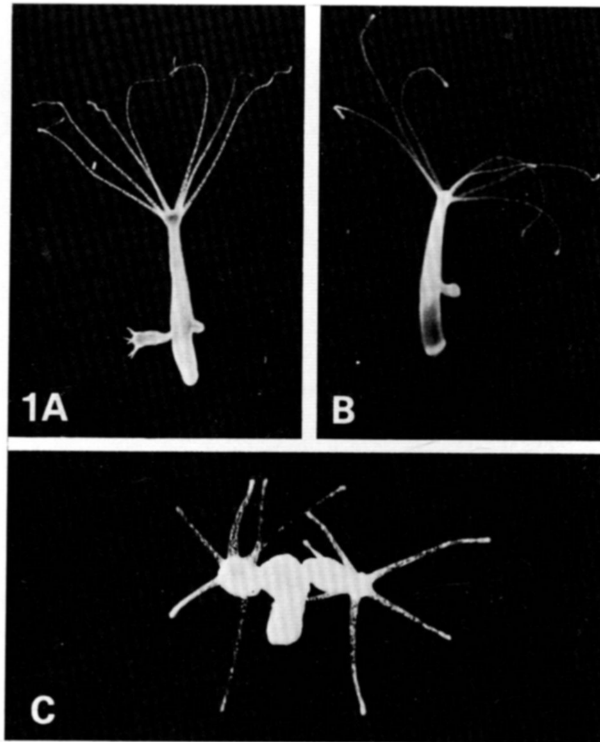


Fig. 1. (A) A typical control hydra with two buds; (B) buds formed in control regenerates arise exclusively in the budding zone; (C) absence of head regeneration, and formation of two buds opposite to each other gives a hammer-shaped regenerate.

30 ml medium containing the desired concentration of the analogue for 24 h. These animals were fed, washed and cultured in fresh medium containing the same concentration of 5-azaCR for a further 24 h. They were then washed and decapitated. The cut was effected immediately below the tentacle whorl. Decapitated animals were cultured individually in 35×10 mm plastic Petri dishes with 3 ml of medium without the analogue, and were not fed during the experimental period. These animals were observed daily under a stereomicroscope to record the formation of hypostomes and tentacles, the length of the tentacles, the formation of buds, and their site and the time of their detachment. The average number of buds per animal at any given time was called bud index. The total number of animals reported in a given group is not constant (on different days) because some animals were used for histology and others died.

A stock solution of 5-azaCR (Calbiochem) was made in distilled water immediately before being added to the culture medium to make up the final concentrations.

RESULTS

(A) *Studies on intact hydra*

Budding in intact hydra takes 4–5 days. The process of bud initiation and hydranth morphogenesis takes 3–4 days. The buds are formed laterally and exclusively at the budding zone, proximal to the gastric region (see, Fig. 1A) with a bud index of 0·3. However the time of detachment of the bud varies. These results are in agreement with those of Shostak & Kankel (1967), and Otto &

Campbell (1977). 5 and 10 mM-5-azaCR was toxic, and the animals died after 3 days of continuous exposure. On short exposures (up to 3 days), 1 mM-5-azaCR was non-toxic to the hydra. However, the buds formed in these animals had not detached after 6 days from their formation. Prolonged exposure (for 4 and more days) had a low toxic effect on the animals. 5-azaCR below 1 mM had no significant effect on the behaviour of hydra or on bud formation.

(B) *Head regeneration and budding*

Controls

In control animals, regeneration of tentacles, preceded by hypostome formation, was observed 4 h after decapitation. After 48 h, at least two distinct rudiments of tentacles were seen per animal. Three days after decapitation, all control animals had tentacles (an average of four per animal), and by 9 days, the head regeneration was complete and there was an average of six fully grown tentacles (Table 1). On the 6th day after decapitation, the bud index ranged from 0·15 to 0·20. The entire process of bud initiation, growth and detachment in these animals usually took 3–4 days, and never more than 5 days. The position of buds was normal, i.e. lateral in the body column, below the gastric region (Fig. 1B). These findings are in accordance with an earlier report (Otto & Campbell, 1977).

5-azaCR-treated hydra

0·1 and 0·5 mM-5-azaCR. No significant effect on the process of regeneration or on budding was observed in hydra treated with 0·1 mM-5-azaCR (Table 1). 0·5 mM-5-azaCR inhibited head regeneration, and increased the bud index. These effects

Table 1. *Head regeneration and budding in control and 5-azaCR-treated hydra*

		Regeneration		Budding			
Days after decapitation	No. of hydra studied	No. of hydra showing regeneration	Average no. of tentacles per hydra	No. of hydra showing budding	Total no. of buds	Bud index	Buds detached
			Control:				
3	19	19	4·5	2	2	0·11	—
6	19	19	5·1	3	3	0·16	2
9	19	19	5·6	0	0	0	3
			0·1 mM-5-azaCR:				
3	44	44	4·8	7	7	0·16	—
6	44	44	5·9	5	7	0·16	3
9	44	44	6·1	0	8	0·18	8
			0·5 mM-5-azaCR:				
3	22	22	0·2	20	27	1·23	0
6	22	22	3·4	21	31	1·41	4
9	22	22	4·8	20	33	1·50	6

were found as early as 3 days after decapitation. By 9 days after decapitation, the average number of tentacles was 4·8 and the bud index was 1·55. Of these buds, 10% were found on the distal cut end, and the rest were in the normal budding zone.

0·8 mM-5-azaCR. Inhibition of head regeneration was prevalent in this group, and tentacles regenerated in only 12·5% of animals (Table 2). Before decapitation (within 48 h of treatment), bud formation was observed in 35% of animals. These buds originated from the normal budding zone of the parent. 6 days after decapitation bud induction was obvious. These hydra had a bud index of 1·65, and this increased to 2·9 by 12 days after decapitation (Table 2). The average number of buds formed in this group was at least 10 times higher than that observed in control regenerates. Most of the buds arising after decapitation originated in abnormal positions, i.e. laterally and very close to the presumptive head. When two buds formed laterally in the same animal, they were on opposite sides, which resulted in a hammer-like shape (Fig. 1C). In these animals, buds showed a marked delay in detaching from the parent. The first bud detachment was seen as late as 8 days after decapitation. Even the buds formed before decapitation in the normal budding zone showed delayed detachment. 14 days after decapitation, 58% buds were still undetached; they represented 69% of the buds found in abnormal positions.

1 mM-5-azaCR. Hypostome and tentacle regeneration in animals treated with 1 mM-5-azaCR were severely affected (Table 3). The effects observed 9 days after decapitation can be divided into the three types: 44% of hydra had at least three tentacles each; 35% displayed no regeneration of tentacles (Fig. 2A) and 20·6% animals had only one centrally placed tentacle (Fig. 2B). These patterns of regeneration became more evident 12 days after decapitation. Among the animals that had a normal number of tentacles, two distinct groups were discerned: one group (24% of the total) had no buds at all, and the other (21% of the total) had formed one bud per animal. 12 days after decapitation there was a slight reduction in the number of animals that had a single tentacle because some animals lost their tentacles. The mechanism underlying this process is not clear. 17·5% of the

Table 2. *Effects of 0·8 mM-5-azaCR on head regeneration and budding in hydra*

		Regeneration		Budding			
Days after decapitation	No. of hydra studied	No. of hydra showing regeneration	Total no. of tentacles	Total no. of buds	Bud index	Buds in abnormal site	Buds detached
3	20	1	3	18	0·90	11	0
6	20	3	10	38	1·90	31	0
9	16	2	12	43	2·69	36	9
12	16	2	12	46	2·88	39	15

Table 3A. *Effects of 1 mM-5-azaCR on head regeneration and budding in hydra*

Days after decapitation	No. of hydra studied	Tentacle regeneration*			Buds							
		Normal number	One tentacle	No tentacles	Total no. of buds	Bud index	Buds in abnormal sites	No. of buds per animal				Buds detached
								3	2	1	0	
3	63	2	2	59	24	0·38				1		1
6	63	23	13	22	84	1·33	61	6	22	22	13	1
9	63	28	11	20	86	1·37	62	3	22	22	15	3
12	63	27	7	25	93	1·48	59	4	14	26	19	26

* Abnormal hydra are not included.

Table 3B. *Relation between head regeneration and budding in 1 mM-5-azaCR-treated hydra*

Days after decapitation	No. of hydra studied	Tentacle regeneration			Budding						
		Normal number	One tentacle	No tentacles	Total no. of buds	Bud index	Buds in abnormal sites	No. of buds per hydra			
								3	2	1	0
9	63	15			0						
		13			14	1·0	2	0	1	12	0
			11		21	2·0	13	1	8	2	0
				20	38	2·0	33	2	13	5	0
12	63	18			0						
		10			10	1·0	2	0	0	10	0
			7		10	1·4	10	0	3	4	0
				24	44	1·8	39	4	11	10	0

animals that had a single, centrally placed tentacle on their distal end lacked normal hypostome. In place of the hypostome, these animals possessed a long fusiform structure at the end of which, a short tentacle was seen (Fig. 2C). These tentacles were sometimes bifurcated (Fig. 2D). Development of two hypostomes, each with a single tentacle (Fig. 2E), was a common abnormality in these animals.

Bud formation was observed before decapitation (within 48 h of treatment) in 7 of the 78 animals studied; all these buds originated in the budding zone. However, 72 % of the total buds formed by the 9th day after decapitation were in abnormal sites. As indicated earlier, 87 % of the buds formed in regenerates that possessed no tentacles originated in an abnormal position: they arose laterally, close to the presumptive head. Regenerates with a single, centrally placed tentacle usually had two buds per animal, and at least one, and generally both, buds were in an abnormal position (Fig. 2B,C). The average number of buds formed was 1·66, because 15 animals that had regenerated tentacles had no buds at all. The other regenerates, which had grown a normal number of tentacles, possessed a single bud each. The distribution, position and time of detachment of buds also varied

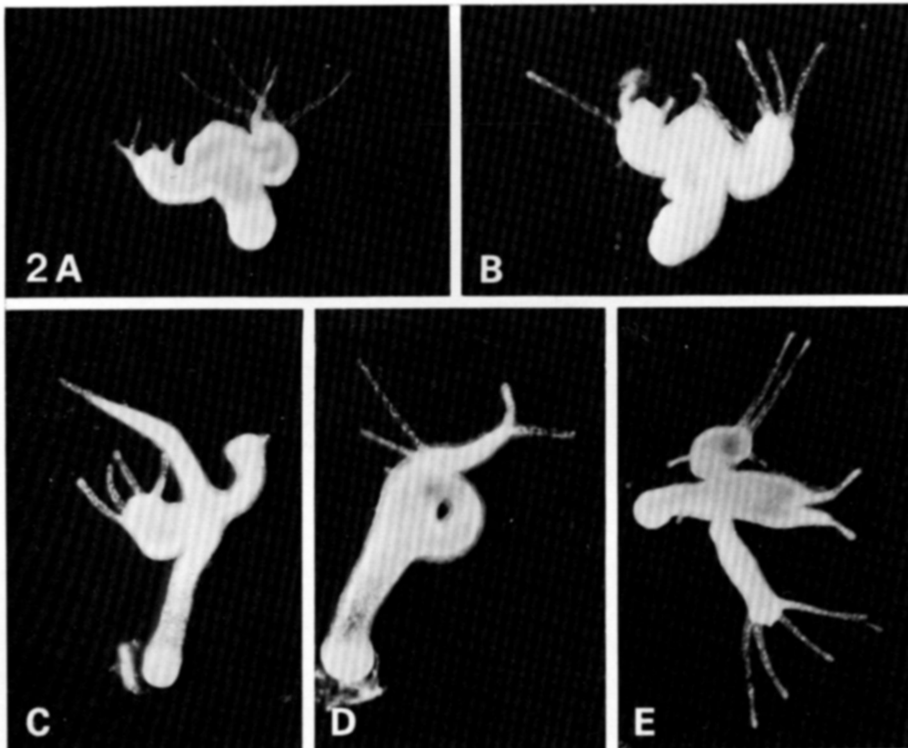


Fig. 2. 1·0 mM-5-azaCR-treated regenerates of hydra. (A) Hydra showing formation of a hypostome and two buds in its vicinity, but lacking tentacle regeneration; (B) a single tentacle arises centrally on the cut end and two buds are formed laterally close to the distal end; (C,D) formation of abnormally long hypostome ending in a single tentacle or bifurcated tentacles. (E) Regenerates with two abnormal hypostomes.

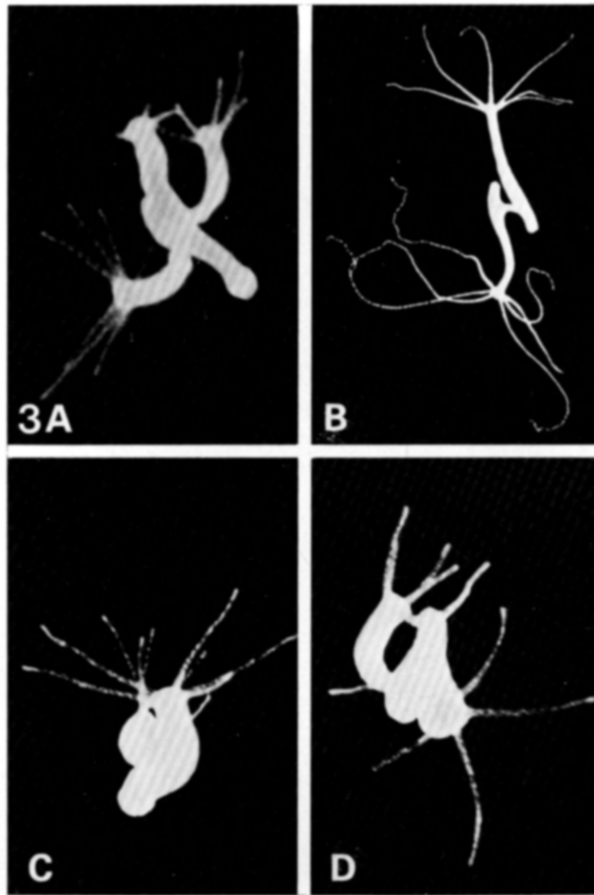


Fig. 3. In 1·0 mM-5-azaCR-treated hydra, buds developed at various sites of the body column after decapitation; mode of attachment of those buds also differed. (A) A regenerate developed three buds, one distally on the cut end and two laterally in the gastric region; (B) a bud formed in the normal budding zone of the regenerate is attached to the parent by a special growth and is oriented towards the opposite direction; (C,D) animals in which buds have completely fused to the body column of the parent.

widely (see Fig. 4). Remarkably, only 28 % of the buds had detached from their parents within 12 days of decapitation. A substantial number (84 %) of the detached buds had developed in the normal budding zone.

5 mM-5-azaCR. The effects of 5 mM-5-azaCR are summarized in Table 4. No regeneration of hypostomes or tentacles was seen in these animals. 3 days after decapitation, large globe-shaped heads without distinct differentiation (Fig. 5) appeared in 59 % of animals. Most of these hydra (86 %) were enclosed by a membrane-like structure (Fig. 5A,B). Initially, the globe heads are seen as small, blunt protrusions at the distal end of the regenerates. They soon dramatically increase in size and burst, so releasing cells into the culture. This results in a blunt,

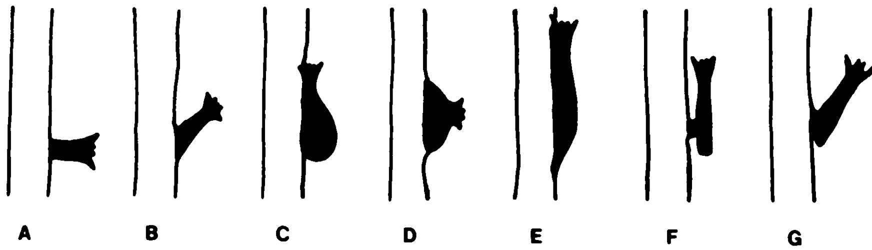


Fig. 4. Diagrammatic representation of different modes of bud attachment (to parents) in the 1·0 mM-5-azaCR-treated group. (A) Normal type of attachment, the bud axis is at right angles to the parent axis; (B) a large portion of the proximal part of the bud is attached to the parent. This mode of attachment resembles that of 'frustule' found in other coelenterates; (C) the bud axis is almost parallel to the parent and a substantial portion of the bud is attached to the body column of the parent; (D) a typical 'medusa' type of attachment; (E) the body column is completely fused to that of the parent; (F,G) sometimes the buds are attached to the parents by special extrastructures.

cylindrical animal. It seems that the growth of the globe heads is the result of syphoning off cells from the body column. 6 days after decapitation, 25 % of the animals that had lost their globe heads due to bursting, started regenerating tentacles and 10 days after decapitation, these animals had an average of five tentacles each (ranging from three to seven).

No budding was observed in the globe-headed animals until 4 days after decapitation. Thereafter only a few (10 %) animals showed budding. The buds were mainly at the distal end that had lost the globe head. By 10 days after decapitation 12 out of the 20 animals had buds. One animal had grown a bud from the basal disc. It should be mentioned that none of the growing buds on the head region developed tentacles in a normal position, nor had the buds detached from the regenerates even 15 days after decapitation.

The formation and disappearance of membrane-like structures around the regenerates and of globe heads, and the formation of buds occurred within very short periods. Thus every few hours, the structure of the regenerates changed drastically.

10 mM-5-azaCR. Marked morphological changes were encountered in hydra exposed to 10 mM-5-azaCR. 24 h after decapitation, there was an accumulation of cells at the distal (cut) end that resulted in the formation of a large globe-shaped head (see Fig. 6A), without any sign of tentacle formation. A few animals did not show any morphological changes (Fig. 6B). Encapsulation of animals in membrane-like structures, loss of cells from animals and eventual death were common features of this group. Some of the globe heads were found to shrink gradually, probably due to a loss of cells. The sharp decrease in the total number of hydra was due to the death of animals. Only six animals had regenerated a normal number of tentacles by the 9th day after decapitation.

Since a substantial (56 %) number of animals died before the 9th day after decapitation, no bud index could be calculated. Bud formation was seen even

Table 4. *Effects of 5 and 10 mM-5-azaCR on head regeneration and budding in hydra*

			Regeneration		Budding				
Days after decapitation	No. of hydra studied	No. of hydra dead	No. of hydra showing regeneration	Total no. of tentacles	No. of hydra showing budding	Total no. of buds	Buds in abnormal sites	Buds detached	No. of hydra with globe head
				5 mM-5-azaCR:					
3	20	0	0	—	0	0	—	—	16
6	17	3	1	4	9	10	9	0	1*
9	14	—	4	21	12	16	5†	0	0
				10 mM-5-azaCR:					
3	43	8	0	—	9	9	0	0	32
6	35	16	0	—	12	15	7	0	8*
9	15	1	6	27	12	21	13	0	2

* Rest of the globe heads were burst.
† Site of others not distinct.

before the animals lost their globe heads (Fig. 6C). The number, position and the mode of attachment of the buds varied from animal to animal. Buds arose at all sites including the distal end of the unregenerated head (Fig. 6D) and foot (Fig. 6E). Later, buds were seen to arise from other buds (Fig. 6F). Abnormal growth without any distinct forms were observed in some animals (Fig. 6F,G,H). The mode of attachment of the buds to the parent also varied and some were attached indirectly through special intermediary structures (Fig. 6I).

DISCUSSION

Regeneration

Inhibition of head regeneration induced in hydra by 5-azaCR varies with the concentration used, but it is not proportionate to the dose (Tables 2, 3). Only the general effects of toxicity are dose dependent. This suggests that failure to regenerate is not due to a general metabolic inhibition brought about by 5-azaCR, but rather to specific inhibitory effect(s) exerted by 5-azaCR on the regenerative process. This is supported by the paradox that the most drastic inhibition of regeneration and the highest budding index are found in the same group of hydra, i.e. the group treated with non-toxic doses of 5-azaCR (Table 2). More important, most of the buds in treated hydra arise from the body column very close to or at the distal end. In addition, the formation of globe heads in hydra treated with 5 or 10 mM-5-azaCR is probably related to regeneration but completely lacking differentiation. Apparently the early events of regeneration, such as recruitment and reorientation of cells take place, whereas the differentiation processes required for normal structure (hypostome and tentacles) formation are lacking. However, further studies are required to understand the exact mechanisms of

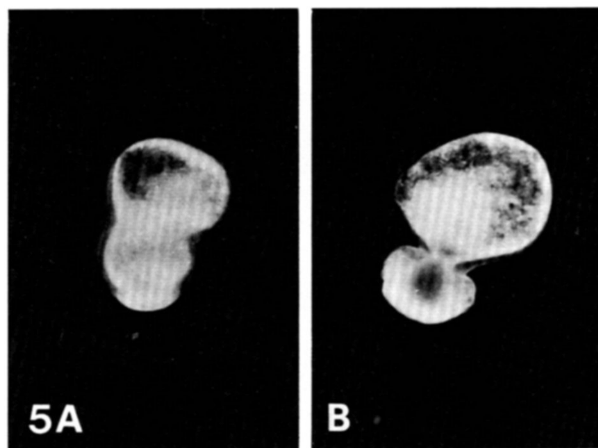
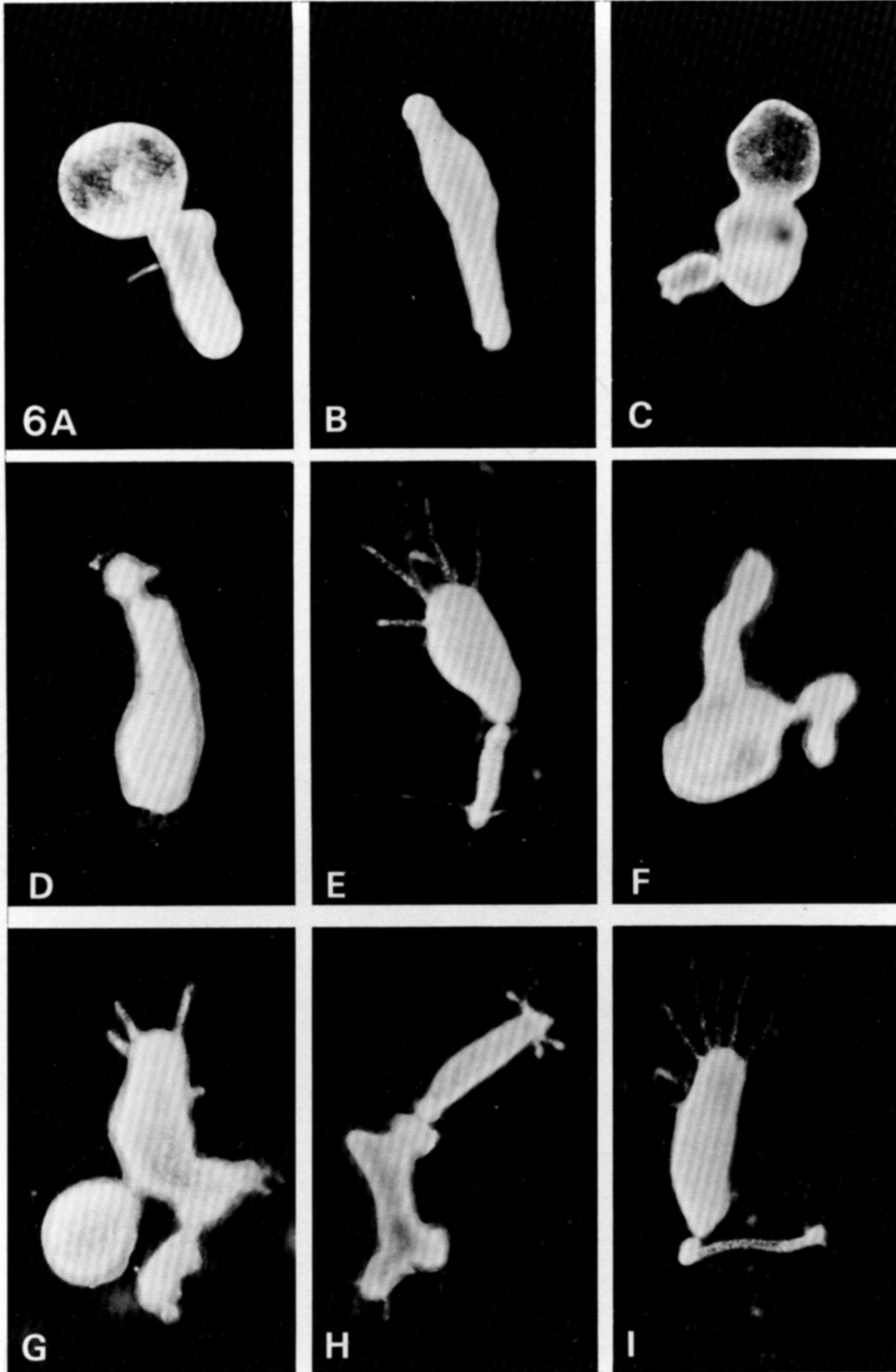


Fig. 5. Formation of globe-shaped heads in decapitated hydra exposed to 5 mM-5-azaCR. Formation of the globe heads begins as a small protrusion of the cut distal end (A) of the regenerate which gradually increases in size (B).

formation and loss of globe heads. Another example of abnormal head regeneration without normal differentiation, is the formation of long, hypostome-like structures at the distal end of hydra exposed to 1 or 5 mM-5-azaCR.



What is the mechanism underlying the specific inhibition of regeneration by 5-azaCR? 5-azaCR is a known inhibitor of DNA, RNA and protein synthesis in eukaryotic cells (Vesely & Cihak, 1978). Therefore the absence of a dose-proportionate inhibition of regeneration in 5-azaCR-treated hydra seems to exclude the involvement of inhibition of the synthesis of these informational molecules in the present context. A wide variety of chemicals, such as hydroxyurea, aphidicolin, colcemide and 5-bromouracil, severely inhibit mitosis, but do not affect head regeneration in hydra to the extent reported for 5-azaCR (Table 2) (Ham & Eakin, 1958; Eakin, 1961; Webster, 1967; De Petrocellis *et al.* unpublished data). Hence, the inhibition caused by a low dose of 5-azaCR is probably due to subtle changes in the expression of the genome at the transcriptional and/or translational level. Two likely candidates are hypomethylation of genomic DNA, which results in the expression of certain silent gene(s), and incorporation of 5-azaCR into RNA molecules, which interferes with the translation processes. A biochemical analysis will help to clarify the mechanism of action underlying the 5-azaCR-induced inhibition of regeneration in hydra.

Although the inhibition of regeneration caused by lower (0·8 and 1 mM) concentrations of 5-azaCR seems to be irreversible (up to 12 days after decapitation), the early inhibition of regeneration induced by higher concentrations is reversible in a substantial number of cases (Table 4). This reversibility is, in general, preceded by drastic morphological changes, such as the bursting of globe heads. In earlier experiments, where inhibition of head regeneration in hydra was obtained by treatment with lipoic acid, severance of the inhibited distal end resulted in the regeneration of a normal head (Eakin, 1961).

Budding

The results presented above clearly demonstrate that 5-azaCR induces bud formation in *Hydra attenuata.* In the 0·8 mM-5-azaCR-treated group, all animals formed buds within 12 days of decapitation, even though 87 % of the animals never regenerated heads and were thus unable to feed. We found that when the animal lacked a hypostome, the buds arose close to the distal end. This supports the findings of Burnett (1966), and Webster (1971) that the hypostome exerts an inhibitory effect on bud development. This inhibitory effect of the hypostome on the development of buds in its vicinity is confirmed by the fact that among the animals in which normal head regeneration took place, either no buds were formed or, if formed, they arose mainly in the budding zone (Table 3). This seems to suggest that the long conical structure formed distally on the head zone

Fig. 6. Hydra exposed to 10 mM-5-azaCR and then decapitated showed various types of abnormalities such as (A) development of globe heads, or (B) lack of obvious morphological reactions. Bud induction was prevalent in these animals irrespective of other morphological alterations; (C) a hydra with a globe-head forms a bud. (D) An animal which failed to regenerate a head developed a bud-like growth on the distal end. (E) Hydra showing a normal head developed a bud at an abnormal site, such as basal disc. Formation of several bud anlage-like structures was a common feature in these animals (F,G,H). (I) Some buds were attached to the parents by special structures.

(Fig. 2C,D) cannot be classified as an abnormal hypostome. Either it is not a hypostome or, if it is, it does not exert any bud inhibitory effects because in almost all cases at least one bud arose close to the putative head. Whereas in animals lacking head regeneration (due to 0·8 mM-5-azaCR treatment) buds arose mainly in the vicinity of the head zone, such was not the case at higher (5 and 10 mM) concentrations. In the latter groups, we have even observed the development of buds on the head (distally) in regenerates that showed apparently normal tentacle formation. This might indicate that, at higher concentrations, 5-azaCR interferes with the synthesis of the morphogens (Schaller, 1973; Kemmner & Schaller, 1984) normally produced in hydra, or that it renders the target cells in the vicinity non-responsive to the morphogen(s).

It is well known that there are no specific growth zones in hydra (Webster & Hamilton, 1972). This is confirmed by the fact that 5-azaCR can induce budding in any part of the body column including the basal disc. We might thus conclude that 5-azaCR induces budding *via* an active and direct process, and not by inhibition of anti-budding morphogens. Chemical induction of budding is a rare phenomenon. Chemicals that interfere with head regeneration in hydra, such as lithium chloride, lipoic acid, colcemide, hydroxyurea and aphidicolin, chloretone and colchicine, do not induce budding (Ham & Fakin, 1958; Eakin, 1961; Webster, 1967; De Petrocellis *et al.* unpublished data). Dithiothreitol (DTT), a protective agent for protein sulphydryl groups, on head regeneration, inhibits head regeneration in *Hydra littoralis* and enhances bud formation (48 to 78 %) in regenerates (Hicklin, Hornbruch & Wolpert, 1969). The exact mechanism of bud induction in the above experiment is not known. A functionally similar chemical agent, β-mercaptoethanol, was found not to induce budding in hydra (Descotilis-Heernu, Quertieri & Brachet, 1961). The 5-azaCR-induced budding in decapitated hydra reported in the present paper is far more uniform and striking than DTT-induced budding. It would be of interest to study if 5-azaCR could induce budding in a non-budding strain of *Chlorohydra viridissima* (Lenhoff, 1965), which is believed to owe its phenotype to a deficiency in the interstitial cell differentiative events necessary for bud initiation (Moore & Campbell, 1973). The above report that buds could be induced in *Chlorohydra viridissima* by grafting normal hydra tissue pieces containing interstitial cells, suggests the induction of budding in *Hydra attenuata* by 5-azaCR might involve the induction of differentiation events in the interstitial cells.

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