Quantitative analysis of cell types during growth, degrowth and regeneration in the planarians Dugesia mediterranea and Dugesia tigrina

J. Baguñá and R. Romero
Dept. de Genètica, Facultat de Biologia, Univ. de Barcelona, Gran Via 585, B-7, Spain

Keywords: Turbellaria, *Dugesia,* cell types, growth, degrowth, regneration, maceration-technique

Abstract

A method of tissue maceration (dissociation) of planarian tissues into single cells was used to characterize the basic cell types in the planarians *Dugesia mediterranea* and *Dugesia tigrina,* and to determine the total cell number and distribution of cell types during growth, degrowth and regeneration.

Using this method, 13 basic cell types have been determined for both species. The total number of cells increases with body length and volume whereas the distribution of cell types is only slightly affected. Growth and degrowth occur mainly through changes in total cell number leaving cell distribution only moderately affected. During regeneration, an increase in neoblast density in the blastema followed later on by increases in nerve cells are the more significant changes detected.

These results are discussed in relation to mechanisms of cell renewal, blastema formation and maintenance of tissue polarity.

Introduction

The understanding of growth, degrowth and regeneration in planarians requires data on abundance and distribution of the basic cell types in the animal. Obtaining such data in histological sections is rather difficult because planarian parenchyma is a loosely organized tissue composed of several cell types with numerous and interdigitating processes that make it impossible to establish with certainty the cell limits. Moreover, neoblasts and nerve cells are difficult to distinguish using conventional histological techniques, gastrodermal and globet cells are difficult to count, and some cell types such as flame cells, muscle cells and pigment cells are almost impossible to recognize.

To overcome these difficulties we have used the method of maceration of planarian tissues into single cells. In such macerations each cell type is easily distinguishable and its abundance can be determined by counting.

In this paper we describe 13 basic cell types and the criteria for distinguishing them in the planarians *Dugesia mediterranea* and *Dugesia tigrina.* The total number of cells and the percentages of the different cell types for standard animals of different sizes are also described. Further, the distribution of cell types in different regions along the cephalo-caudal axis, and the changes that occur in total cell number and cell distribution in growing, starving and regenerating animals are described.

Material and methods

1. Material

The planarians used in this work belong to the asexual races of the species *Dugesia mediterranea* (Benazzi *et al* 1975) and *Dugesia tigrina* (Girard). The animals were reared in petri dishes and kept in

Hydrobiologia 84, 181–194 (1981). 0018-8158/81/0842-0181/$02.80.

filtered pond water at 17 °C in the dark. They were fed weekly on beef liver. All the experiments were done at 17 °C.

2. Methods

2.1. Total body volume determination (V)

The body volume of animals of different sizes for each species was determined according to Baguñà (1976a).

2.2. Maceration technique

The technique used is a modification of David's technique for *Hydra* (David 1973) and has been described elsewhere (Baguñà 1973, 1976a). Briefly, it consists in macerating whole animals in a solution containing methanol: glacial acetic acid: glycerol: distilled water (3:1:2:14) at 8–10 °C. After 24–48 h, the test tube is shaken very gently until a suspension of single cells is obtained. The cell suspension is fixed by addition of 0.1 volume of 20% formaldehyde and/or 0.1 volume of 1% osmium tetroxide and spread on microscope slides to dry on a leveled surface. To examine cells a drop of water and a coverslip were placed on the slide and examined by phase contrast optics (×40 and ×63 objectives).

2.3. Counting of total cell number (C)

The total number of cells for whole animals of different sizes was obtained by macerating a known number of identical animals (usually five) in a known volume of maceration solution and determining the cell concentration with an improved Neubauer cell counter (0.1 mm depth).

To estimate the individual variability in cell number, ten planarians of 5 mm and ten planarians of 7 mm of the species *Dugesia tigrina* were macerated individually and the total cell number determined. The mean cell number per planaria was found to be 314 100 ± 49 800 (standard deviation) for 5 mm long individuals, and 554 250 ± 69 600 for 7 mm long animals. Samples of five to ten animals of identical length were macerated together. The mean cell number per planaria was found to be 342 100 ± 8 240 and 523 350 ± 14 380 for 5 and 7 mm long animals respectively. All the experiments reported in this paper were done according to the last method.

2.4. Total number and distribution of cell types

Total number and distribution for each cell type were calculated from the total number of cells per animal (or region) and the percentage of a given cell type in a macerate of that animal (or region). The data presented are the average of ten experiments using five organisms per experiment.

Samples of planarians of the same length macerated individually and samples of 5–10 planarians macerated together were examined to determine the variability of different cell types among individual animals. The differences between both kind of samples were small, and were not reduced by pooling the organisms.

Though classification of most cell types in planarians is not difficult, there are cells difficult to assign to a specific cell type. Unfortunately such cells are not uncommon being mainly cells in the process of differentiation, a process enhanced by the high turnover of cells in planarians and by the fact that neoblasts are the stem-cells of most differentiated cell types. The percentage of these cells, labelled as 'unclassified' attains 10% in *Dugesia tigrina* and around 5% in *Dugesia mediterranea*. The higher value in the former is due to the difficulties in classifying flame, rhabdite and striped cells in this species as compared to their easy recognition in *Dugesia mediterranea*.

3. Experimental procedures

3.1. Degrowth experiments

One week-starved animals of 7 and 11 mm length of both species were kept fasting for three months at 17 °C. At different intervals in time five animals of each group were macerated together and the total cell number and total number and distribution of cell types measured.

3.2. Growth experiments

One week-starved *Dugesia tigrina* of 5 and 7 mm length were used. The animals were fed on an artificial mixture of sonicated beef liver and starch from the start of the experiment and every seven days during a period of three months. The artificial food was used because previous results (Romero, unpublished observations) showed a very slow digestion of raw beef liver by planarians that left many intact liver cells even three days after feeding.

At 1, 3, and 6 days after each feeding, five animals

of each group were removed, macerated together and their total cell number and total number and distribution of cell types measured.

3.3. Regeneration experiments

Animals of 11 mm length of both species, starved for a week, were used. The animals were cut at postcephalic level and the posterior part was kept regenerating in filtered pond water at 17 °C. At different intervals in time (1, 2, 3, 5, 9 and 15 days), five animals were killed and the blastema (B) and a narrow region behind it (Post-blastema, PB) were cut with a fine scalpel and macerated separately. The total number and distribution of cell types were then measured.

Results

1. Maceration and identification of basic cell types

Using the maceration technique, planarian cells have morphological characteristics which help to identify most of them. It should be pointed out that 5 to 10% of the cells cannot be properly classified because they share characteristics of more than one cell type or because they are in the process of differentiation being between a neoblast and some terminal differentiated cell type.

We give now a description of the cell types as well as the criteria to identify them in phase contrast optics.

Neoblasts (Figure 1a). Small cells (8–15 μm), rounded or pear-shaped, with a large nucleus, scanty cytoplasm and usually a short process of 5–20 μm that may contain a lipid droplet. All these characteristics agree with the cell type described by light and electron microscopy.

Nerve cells (Figure 1b). Though several nerve cell types have been described with light and electron microscopy in planarians, they are morphologically so similar in phase contrast that we include them in a single group. Nerve cells have a small cell body (8–20 μm) and long (up to 500 μm), thin and branched processes, some of them beaded. The nucleus is large and a prominent nucleolus is usually present. Some nerve cells present vacuoles and/or lipid droplets in the cytoplasm near the cell body. The nerve cells are unipolar (50–60%), bipolar (20–30%) and rarely multipolar (less than 10%).

Epidermal cells (figure 1c). Regular-sized cuboidal or slightly columnar cells (20–40 μm long, 20–30 μm wide). Epidermal cells from the ventral size bear cilia at their distal end whereas cells from the dorsal side do not have them being taller and narrower. There is a small nucleus at the basal end with highly condensed chromatin. The cytoplasm is usually filled with cigar-shaped bodies or rhabdites alligned parallel to the proximo-distal axis. The basal end is usually highly indented.

Fixed parenchyma cells (Figure 1d). Highly polymorphic cells of large size (up to 100 μm in diameter) with numerous processes generally lost during the maceration procedure. Nucleus and cytoplasm pale in phase contrast optics and filled with vacuoles, intracellular spaces and lipid droplets of different sizes, numerous mitochondria and occasionally pigment granules.

Gastrodermal cells (Figure 1e). Tall, columnar cells (60–80 μm long, 15–20 μm wide) with the proximal end highly indented. Nucleus of medium size, placed in the proximal half of the cell and usually with a nucleolus. Cytoplasm filled with a heterogeneous group of food vacuoles and lipid droplets, highly refractile. During starvation the droplets and vacuoles disappear progressively.

Goblet or club cells (Figure 1f). Elongated cells (40–60 μm long, 10–15 μm wide) with a narrow and indented proximal end and a wider and rounder distal end filled with vacuoles separated by thin strands of cytoplasm. The nucleus, rather small, is usually obscured by the vacuoles.

Rhabdite cells (Figure 1g). Middle-sized, round or rectangular cells (30–40 μm long, 20–30 μm wide) with a variable number of rhabdites in different stages of formation. Nucleus with highly condensed chromatin. It is found in the parenchyma and it is presumed to be the precursor of the epidermal cells. Only described for *Dugesia mediterranea.*

Muscle cells (Figure 1h). Big, elongated cell (up to 150–200 μm long, 5–10 μm wide) with a central

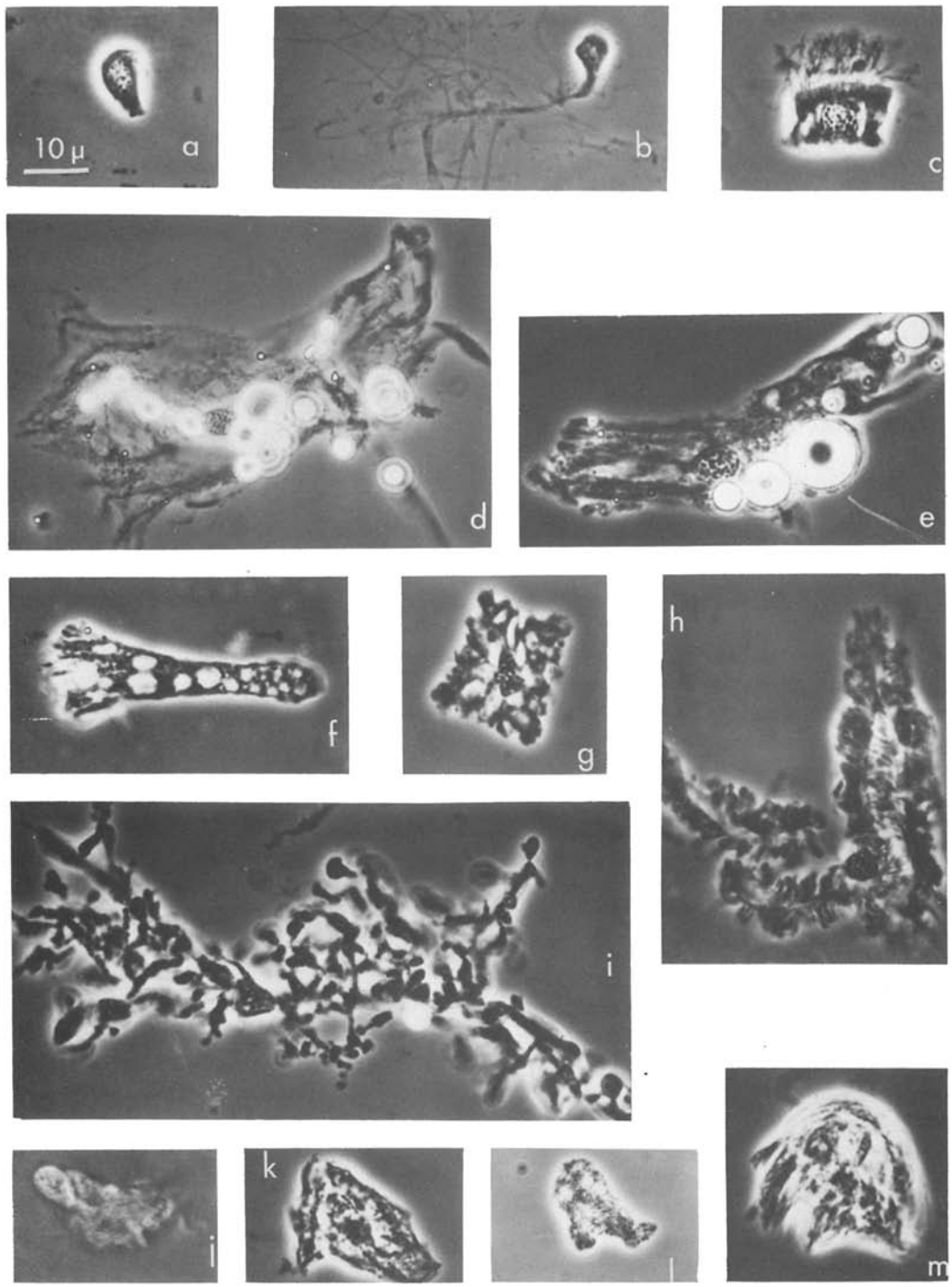

Fig. 1a–m. Basic cell types in the planarians *Dugesia mediterranea* and *Dugesia tigrina.* (a) neoblast; (b) nerve cell; (c) epidermal cell; (d) fixed parenchyma cell; (e) gastrodermal cell; (f) goblet cell; (g) rhabdite cell; (h) muscle cell; (i) pigment cell; (j) flame cell; (k) basophilic cell; (l) striped cell; (m) acidophilic cell. All photographs ×640.

fibrilar stem flanked with a double fringe of cytoplasmic extension filled with mitochondria. Large nucleus with highly condensed chromatin situated in a large cytoplasmic extension linked to the stem by a thin neck.

Pigment cells (Figure li). Large and highly branched cells (up to 100–130 μm in diameter) with the cytoplasm filled with pigment granules. The cytoplasmic processes are highly branched ending with bulbous terminals. Large nucleus, usually centrally located, with highly condensed chromatin.

Flame cells (Figure lj). Elongated cells (20–30 μm long, 8–15 μm wide) with a nucleus placed in the basal end and a centrally located tuft of long cilia. Cytoplasm scanty and homogeneous without inclusions or vacuoles. Only described for *Dugesia mediterranea.*

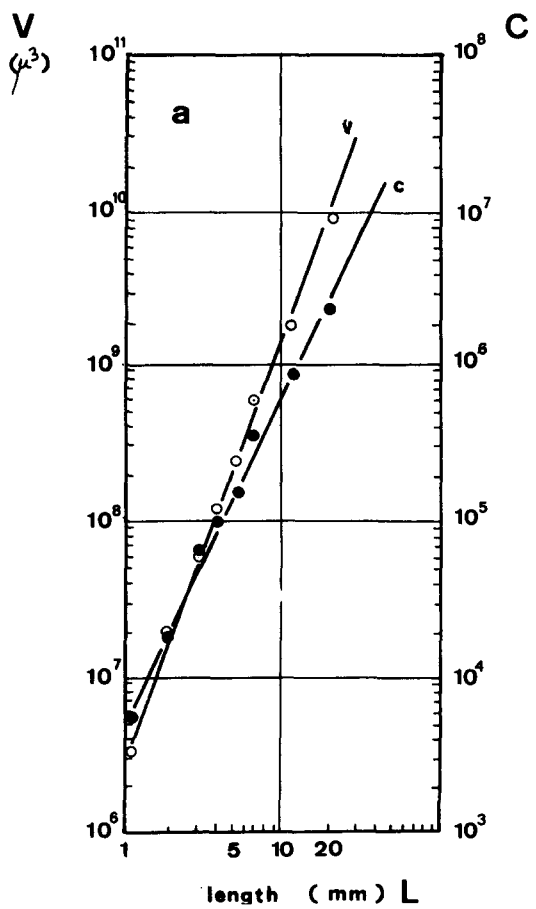

Fig. 2. Total body volume (V) (fixed state) and total cell number (C) as a function of body length (L) in *Dugesia mediterranea.*

Basophilic cells (Figure lk). Middle-sized to large cells (up to 50–70 μm in diameter) of variable form bearing some processes of different length which are usually lost during the maceration procedure. Cytoplasm filled with large and polymorphic granules heterogeneous in density giving a foamy appearance to the cell. Nucleus of middle size, often barely visible.

Striped cells (Figure 11). Cells of regular size (20–30 μm in diameter), ovoid to rectangular, with a cytoplasm very rich in endoplasmic reticulum arranged in concentric rings around the nucleus giving a striped appearance under phase contrast optics. The nucleus is small (6–8 μm) with finely dispersed chromatin. Most probably these cells are not terminal differentiated cells but they are in the process of differentiation from neoblasts to several types of parenchyma gland cells (rhabdite, basophilic, acidophilic, . . .). Only described for *Dugesia mediterranea.*

Acidophilic cells (Figure 1m). Middle sized cells (30–60 μm in diameter), usually oval or pear-shaped. Cytoplasm homogeneous, highly refractile in phase optics, and filled with numerous granules. Nucleus generally not visible.

2. Total body volume (V) and total cell number (C) as a function of the animal body length (L)

2.1 The 'standard' planarian

Since feeding conditions and temperature may affect cell number and cell distribution in planarians, we define a 'standard' planarian as an animal of a given length starved for a week at 17 °C. This is the period of starvation most commonly employed in regeneration and growth experiments.

2.2. The relationship between length (L), volume (V) and total cell number (C)

The relationship between the length (L) of a planarian with the total body volume (V) (fixed state) and the total cell number (C) for both species is shown in Figures 2 and 3. For similar-sized animals the total number of cells is higher in

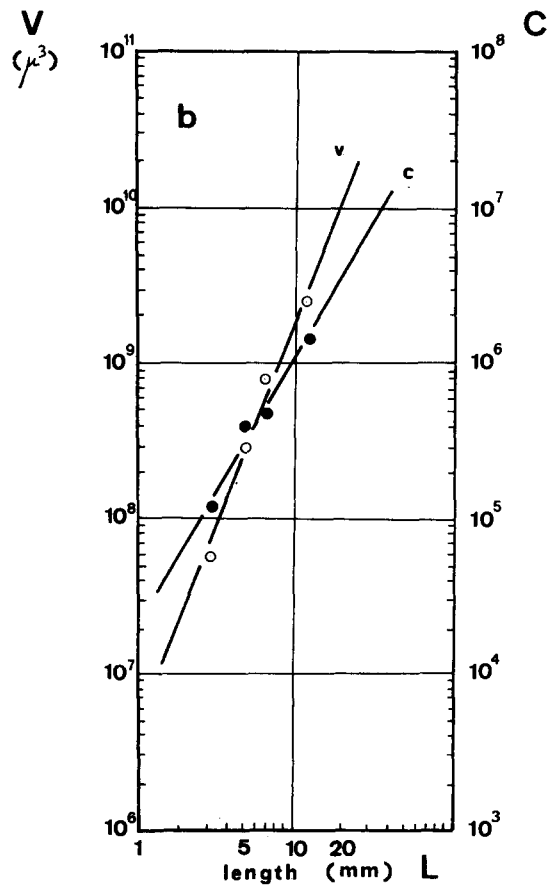

Fig. 3. Total body volume (V) (fixed state) and total cell number (C) as a function of body length (L) in *Dugesia tigrina.*

Dugesia tigrina than in *Dugesia mediterranea.* Since the body volumes are rather similar, the mean cell size (V/C) in *Dugesia tigrina* is lower than in *Dugesia mediterranea.* In both species the total number of cells rises with length at a slower rate than it does with total body volume, this trend being slower for *Dugesia mediterranea.* This means that a longer animal has in general a lower density since the mean size of its cells (V/C) is greater than that of a smaller animal. Correcting for the degree in tissue shrinkage (due to fixation) along the three body axis (Lange 1967) the live volume could be as much as eight times those given for fixed tissue. This means that the mean cell size of a 16 mm long *Dugesia mediterranea* is about 29×10^3 μm^3 (equivalent to a cuboidal cell of 31 μm each side) as compared to a volume of 12×10^3 μm^3 (equivalent) to a 23 μm sided cuboidal cell) for a 4 mm long animal.

However, this increase in body volume due to hypertrofy of cells is small compared to the hyperplasia or increase in cell number. Hence, most of the increase in body volume must have been due to cellular proliferation.

3. Total number and distribution of cell types in standard planarians

The total number and distribution of the 13 basic cell types in standard *Dugesia mediterranea* of, 4, 7, 11 and 16 mm in length, and ten basic cell types in standard *Dugesia tigrina* of 5, 7 and 11 mm in length, are shown in Tables 1 and 2 respectively.

It is interesting to note the decrease in neoblast

Table 1. Distribution of cell types in standard individuals of *Dugesia mediterranea* of different size.

		Body length (mm)			
Cell type		4	7	11	16
Neoblast	%	32	28	23	18
	#	35 200	95 200	190 900	324 000
Nerve	%	20	16	11.5	8
	#	22 000	54 400	95 400	144 000
Epidermal	%	14	13	13	11
	#	15 400	44 200	107 900	198 000
Fix. Parq	%	11.5	15	20.5	25
	#	12 650	51 000	170 000	450 000
Acidoph	%	4.5	5.5	6.5	9
	#	4 900	18 700	53 900	162 000
Basoph	%	2.5	4	5.5	8
	#	2 700	13 600	45 600	144 000
Pigment	%	1	1.5	1.5	1.5
	#	1 100	5 100	12 400	27 000
Muscular	%	2	2.5	2.5	2
	#	2 200	8 500	20 750	36 000
Gastrod	%	2.5	4	5.5	6
	#	2 700	13 600	45 600	108 000
Globet	%	0.5	1	1	1.5
	#	550	3 400	8 300	27 000
Flame	%	1	1	1	1.5
	#	1 100	3 400	8 300	27 000
Rhabdite	%	2.5	2	2	2
	#	2 700	6 800	16 600	36 600
Striped	%	1	1.5	1.5	1.5
	#	1 100	5 100	12 400	27 000
'Unclass.'	%	5	5	5	5
	#	5 500	17 000	41 500	90 000
Total Cell Number (C)		110 000	340 000	830 000	1 800 000

% percentage of total cells
# total number

Table 2. Distribution of cell types in standard individuals of Dugesia tigrina of different size.

Cell type		Body size (mm) 5	7	11
Neoblast	%	34	32	28
	#	115 600	166 400	372 400
Nerve	%	23	22	21
	#	78 200	114 400	279 300
Epidermal	%	13	11.5	10
	#	44 200	59 800	133 000
Fix. Parq	%	8	10.5	15
	#	27 200	54 600	199 500
Acidoph	%	3	3	4
	#	10 200	15 600	53 200
Basoph	%	2.5	3	3.5
	#	8 500	15 600	46 550
Pigment	%	1.5	1.5	1.5
	#	5 100	7 800	19 950
Muscular	%	1.5	1.5	1
	#	5 100	7 800	13 300
Gastrod	%	3	4	5
	#	10 200	20 800	66 500
Globet	%	0.5	1	1
	#	1 700	5 200	13 300
'Unclass.'	%	10	10	10
	#	34 000	52 000	133 000
Total Cell Number (C)		340 000	520 000	1 330 000

% percentage of total cells
# total number

density with the animal's growth; this trend is more evident in *Dugesia mediterranea* than in *Dugesia tigrina* and could be of importance in explaining the differences in the regenerative abilities of animals of different length. On the other hand there is a significant decrease in nerve cell density during growth in *Dugesia mediterranea* though this trend is not statistically significant for *Dugesia tigrina.* Both results agree with measures made at histological level (Lange, 1967, for *Dugesia polychroa;* Bagunà, 1973, 1976a, and unpublished results, for *Dugesia mediterranea).* There is too, for both species, a decrease in epidermal cell density during growth; this result is in agreement with the decrease in the ratio surface/volume as long as the animal increases in length.

Conversely, there are increases in cell percentages for the fixed parenchyma cells, both gland cells, and gastrodermal and goblet cells, the rest of the cells being mainly unchanged. Of special interest are the increases in fixed parenchyma and gastrodermal cells because as body volume increases an even greater increase in the relative volume or percentages of cells and tissues involved in food digestion and transport is necessary in organisms like planarians that lack an efficient transport system (Baguñà & Ballester 1978). This trend is evident for both species though more marked for *Dugesia mediterranea.*

Although actually present, flame, rhabdite and striped cells of *Dugesia tigrina* were difficult to visualize and count; therefore we placed them as 'unclassified' cell types. In *Dugesia mediterranea* (as in *Dugesia polychroa,* Baguñà unpublished results) those cells are easily seen and counted, though no statistically significant variations have been found between organisms of different length.

4. Antero-posterior distribution of cell types in 'standard' planarians

Table 3 gives the distribution of cell types in each region (1 to 6 along the body. 1: head; 2: post-cephalic region; 3: prepharyngeal region; 4: pharyngeal region minus pharynx; 5: post-pharyngeal region; 6: tail; Phx: pharynx) for a standard 11 mm long *Dugesia mediterranea.* Results for different sized *Dugesia mediterranea* and for *Dugesia tigrina* are rather similar, not being included for the sake of clarity. Because of difficulties to classify, pharyngeal cells are also not included though its total cell number was counted.

From the data in Table 3 we can draw the following conclusions: (a) the head (region 1) of the organism, is, as expected, high in nerve cells and also in neoblasts. Parenchyma and gastrodermal cells, especially in longer animals, are barely represented. (b) the tail (region 6) show similar characteristics to the head though the density in neoblasts and nerve cells is lower. Compared to the middle regions (2–4) parenchymal and gastrodermal cells have lower densities. We have not detected morphological differences between head and tail nerve cells.
(c) the middle region of the body (regions 2 to 4) show a uniform distribution of cell types. Neoblasts and nerve cells are at their lowest levels, and

Table 3. Distribution of cell types in different regions of a standard 11 mm long *Dugesia mediterranea.*

		Body region							
Cell type		1	2	3	4	5	6	phx	Total
Neoblast	%	28.5	23	20	19	21.5	24	-	23
	#	31 350	33 350	30 000	22 800	26 875	21 600		179 400
Nerve	%	20	11.5	6.5	6	8	10	-	11
	#	22 000	16 675	9 750	7 200	10 000	9 000		85 800
Epidermal	%	20.5	11.5	10.5	10.5	10.5	21	-	14
	#	22 550	16.675	15 750	12 600	13 125	18 990		109 200
Fix. Pareq	%	10.5	23.5	26.5	22	23	19	-	20.5
	#	11 550	34 075	39 750	26 400	28 750	17 100		159 900
Acidoph	%	4.5	5	8	13.5	9	6.5	-	7.5
	#	4 950	7 250	12 000	16 200	11 250	5 850		58 500
Basoph	%	2	5.5	6	7.5	5	2	-	5
	#	2 200	7 975	9 000	9 000	6 250	1 800		39 000
Pigment	%	1,5	1	1	1	1	1.5	-	1
	#	1 650	1 450	1 500	1 200	1 250	1 350		7 800
Muscular	%	2.5	2	2.5	2.5	2.5	1.5	-	2
	#	2 750	2 900	3 750	3 000	3 125	1 350		15 600
Gastrod	%	1.5	6.5	7.5	7	7.5	5	-	5.5
	#	1 650	9 425	11 250	8 400	9 375	4 500		42 900
Globet	%	0.5	1	1.5	1.5	1.5	1	-	1
	#	550	1 450	2 250	1 800	1 875	900		7 800
Flame	%	1	1.5	1	1	1	1	-	1
	#	1 100	2 175	1 500	1 200	1 250	900		7 800
Rhabdite	%	2.5	1.5	1.5	2	2	1.5	-	2
	#	2 750	2 175	2 250	2 400	2 500	1 350		15 600
Striped	%	1.5	1.5	1.5	1.5	1.5	1	-	1.5
	#	1 650	2 175	2 250	1 800	1 875	900		11 700
'Unclass'	%	3	5	6	5	6	5	-	5
	#	3 300	7 250	9 000	6 000	7 400	4 500		39 000
Total Cell Number ($\times 10^3$)		110	145	150	120	125	90	35	780

- Data not measured
phx pharynx
% percentage of total cells
# total number

parenchyma and intestinal cells attain their highest levels. The other cell types do not precent variation that are statistically significant.

5. The effects of degrowth on cell number and cell distribution

The effects of degrowth by starvation on cell number and cell distribution are shown in Figure 4. For the sake of clarity we only show the results for 7 mm long *Dugesia tigrina,* other body lengths giving similar results.

There is an increase in cell number during the first week (up to the third day) due to cell proliferation after feeding (Baguñà 1974; Romero & Baguñà, unpublished data). A week after, the cell number levels off and from then on decreases steadily reaching a number of 60 000 cells three months later. The final length and cell number are those typical of a 2 mm long organism.

The changes in the cell distribution brought about by starvation are much less extreme. Comparing the percentages at the start and at the end of the experiment, there are slight, but statistically

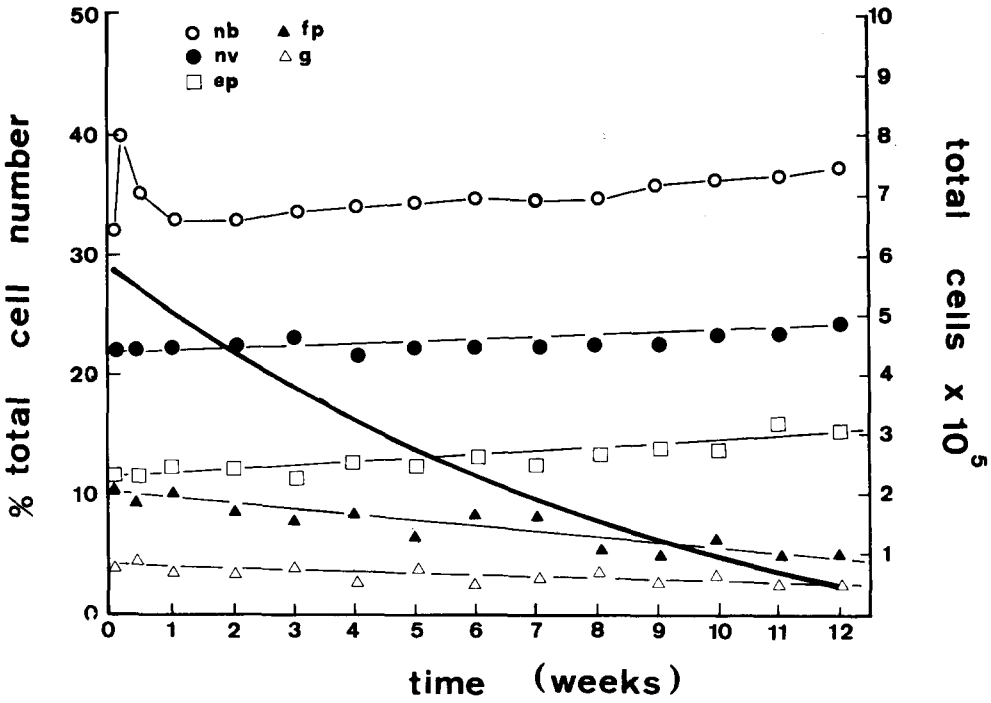

Fig. 4. Effect of degrowth by starvation on total cell number (thick line) and distribution of the main five cell types in *Dugesia tigrina*.

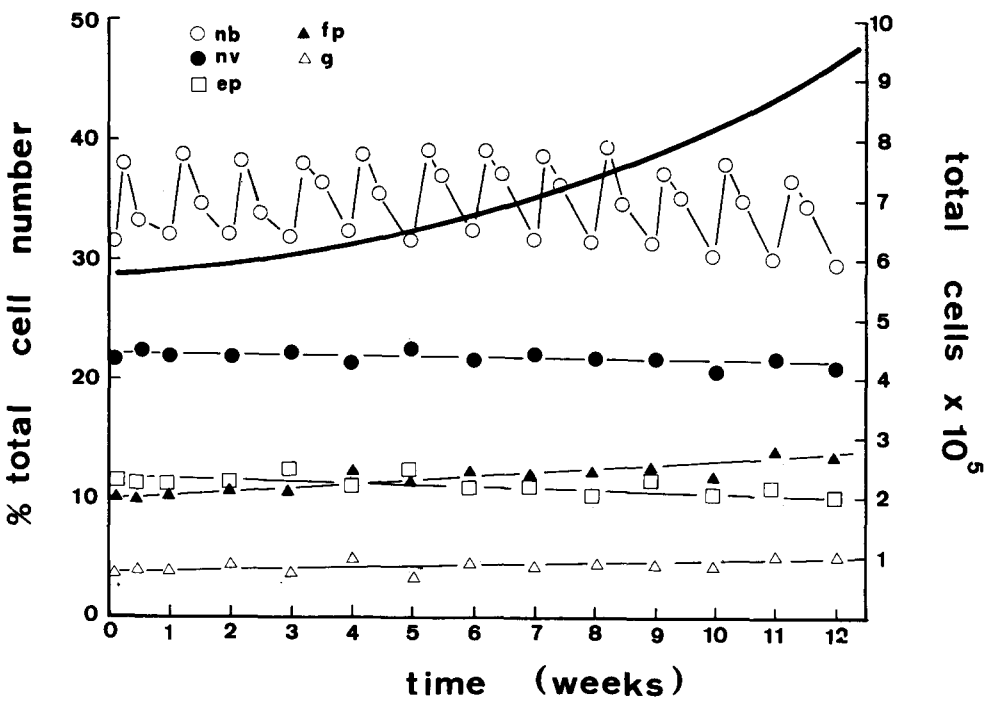

Fig. 5. Effect of growth by feeding on total cell number (thick line) and distribution of the main five cell types in *Dugesia tigrina*.

significant, increases in neoblasts and epidermal cells and a significant decrease in fixed parenchyma cells. Other changes in cell types are not statistical significant.

Overall, these results suggest that the effect of starvation is very marked in the number of cells per animal. We estimate that a 7 mm long starving *Dugesia tigrina* loses about 7 000 cells/animal/day when kept at 17 °C (Romero & Baguñà, unpublished data). The changes in cell composition are less severe. Apparently, during degrowth, there are adjustements in cell composition that make a 5 mm long starving organism very similar in cell composition to a 5 mm long growing animal. This could be relevant in explaining the 'rejuvenatory' effects brought about by starvation as has been suggested by several authors (see Brønsted 1969 and Reynoldson 1966 for references).

6. The effects of growth in cell number and cell distribution.

The effects of growth by feeding on cell number and cell distribution are shown in Figure 5. Only results for a 7 mm long starving *Dugesia tigrina* individuals are presented.

From the beginning of the experiment there is a continuous and steady increase in cell number. This increase is markedly steeper up to three days after feeding due to a sudden but transient burst of cell proliferation. This pattern repeats after each feeding. After three months an animal fed weekly has 950 000 cells, which is characteristic for a 9 mm long organism.

Growth by feeding results in only slight changes

Table 4. Cell distribution during head regeneration in *Dugesia mediterranea* (11 mm in length).

				Time (days)				
Cell type		0		1	2	5	9	15
Neoblast	%	23	B	50.5	59	47	39	33.5
			PB	25	34	37	34	29
Nerve	%	11.5	B	13.5	15.5	22.5	24	24.5
			PB	12	10.5	15.5	20	20
Epiderm	%	11.5	B	16.5	16.5	14	14	17.5
			PB	13.5	10.5	10.5	12	11
Fix. Parq	%	23.5	B	9	3.5	5	7	8
			PB	21.5	14	10	12	15
Acidoph	%	5	B	2.5	1.5	2.5	5	5
			PB	4.5	7	6	5.5	6
Basoph	%	5	B	3	1	1.5	2	2
			PB	5.5	6	4.5	3	4
Pigment	%	1	B	0.5	0	0	0	0.5
			PB	1.5	1	1.5	1	1
Muscular	%	2	B	1	1	1.5	2	2
			PB	2	2	2	2	2
Gastrod	%	6.5	B	0.5	0	0	0	0
			PB	3	4	2	2.5	2.5
Globet	%	1.5	B	0	0	0	0	0
			PB	1	1	0.5	0.5	0.5
Flame	%	1.5	B	0	0	0	0	0.5
			PB	1.5	1.5	1.5	1	1
Rhabdite	%	1.5	B	0	0	0	0.5	1
			PB	1.5	2	2	1.5	1.5
Striped	%	1.5	B	0	0	0	0.5	0.5
			PB	1.5	1.5	1	1	1.5
'Unclass.'	%	5	B	3	2	6	6	5
			PB	6	5	6	4	5

in cell composition that reverse the trend shown by starvation. There is a significant decrease in neoblast and a significant increase in fixed parenchyma cells. The cell composition at the end of the experiment is similar to the one of a standard 9 mm long animal. It is interesting to point out the weekly but transient increase in neoblast percentage due to cell proliferation. This increase levels off 3–4 days after feeding, being sustained in organisms fed twice weekly (Romero, unpublished results). These results reinforce again the suggestion that neoblasts are the only planarian cells endowed with mitotic power.

7. Changes in cell distribution during anterior regeneration

Table 4 gives the distribution of cell types for blastema (B) and post-blastema (PB) during anterior regeneration in a 11 mm long *Dugesia mediterranea*. Only data for this length is shown for the sake of clarity and because other lengths gave similar results. The data at 0 days are the values for region 2 of an 11 mm long intact organism (see Table 3) and serve as an internal control.

The results obtained show a great increase in neoblast density in blastema due to cell proliferation (Baguñà 1976b) reaching up to 60% of total cell number at two days of regeneration. Later, neoblasts decrease in density as long as different cell types begin appearing or increasing in number. There is too a slight but significant increase in nerve cells at two days and especially from five days on, and an increase in epidermal cells due probably to epidermal contraction during wound closure. Conversely, fixed parenchyma cells, acidophilic and basophilic cells and gastrodermal and goblet cells decrease in percentage. This could be due either to cell lysis, cell dedifferentiation, or cell dilution by neoblast proliferation. Though the phenomenon of cell dedifferentiation could be accepted as a likely but unproven possibility, the unclear evidence backing it and the high rates of mitosis seen after cutting (Baguñà 1976b) suggest that the decreasing percentages of these differentiated cells are mainly due to cell dilution, and to a minor extent to cell lysis.

The results obtained for control regions (post-blastema, PB) show that besides slight increases in neoblast and nerve cell densities from the second day on and transient decreases in some differentiated cells (e.g. fixed and secretory parenchyma cells), most cell types remain mainly unchanged. However, it is interesting to point out that gastrodermal and goblet cells dissapear from the blastema at 1–2 days of regeneration while diminishing in density at post-blastema.

Discussion

1. The maceration technique and quantitative cell analysis in planaria.

The maceration technique employed here is a modification of David's technique to macerate *Hydra* tissues (1973). Planarians present some difficulties to macerate since they are not simple bilayered organisms and because their parenchyma is a loose arrangement of several cell types with long and intermingled processes. The modifications introduced (the use of methanol, lower temperatures, and longer periods of maceration) has enabled the preservation, to a reasonable extent, of most of the nerve and parenchyma cell processes.

Despite these difficulties, the maceration technique offers several advantages over standard histological and electron microscopy sections: (1) it provides single cell suspensions good for quantitative analysis of the different cell types; (2) the relative speed and ease of preparation make cell kinetic studies of growth and degrowth possible; (3) when ^{3}H-thymidine autoradiography in planarians becomes possible, maceration would be the method of choice to measure cell turnover of particular cell types in intact and regenerating organisms. Also, this method, when combined with proper histochemical staining methods, would allow a better classification of specific cell types.

2. Cell numbers and distribution

The number of cells per animal and, to a lesser extent, the cellular composition depend on the body length and on feeding regime. It is of interest to point out the decrease in neoblasts and nerve cells and the increase in parenchymal and gastrodermal cells as organisms increase in length. The early phenomena, as stressed by Lange (1968), could be relevant when trying to explain the

decreasing rates of regeneration as organisms increase in size.

The antero-posterior distribution of cell types show decreasing gradients for neoblasts and nerve cells, with a slight increase in tail regions. Data on neoblasts agree with published results from histological sections on *Dugesia polychroa* (Lange 1967). The decreasing gradient in nerve cell density could be related to the mechanisms that define polarity in intact and regenerating worms. Indeed, nerve cells have been identified as the source of morphogenetic factors that control polarity and regeneration in *Hydra* (Schaller & Gierer 1973). Although evidence for a similar mechanism operating in planarians is still lacking it still remains as a very likely possibility.

The high percentage of neoblasts found in intact worms of every length studied (20–35% of total cells) is rather surprising even if we consider them as the stem-cell of all differentiated cell types. In fact, most renewing cell systems (epidermis, gut epithelia, haemopoietic system, . . .) are functionally (and often structurally) divided in three main compartments: a small one composed of slowly-proliferating stem-cells, a bigger one composed of amplyfying and rapidly-proliferating predifferentiated cells, and a terminal compartment of functional differentiated cells. It is possible that, similarly to those systems, planarian neoblasts comprise two different populations: a small one representing uncommited multipotential cells (true neoblasts) and a bigger one representing cells in different stages of determination but retaining the capacity of division.

3. Quantitative cell analysis of growth and degrowth in planarians

Growth and degrowth in planarians are continuous processes whose balance depends on feeding and temperature. From a quantitative point of view crude estimates on the magnitude of increases and reductions in body volume and length have been considered by Abeloos (1930) and Reynoldson (1966) respectively. A better estimate have been given by Calow (1977) using plan area as an index of body size. The maceration technique used here has the advantages of looking at growth and degrowth as changes in cell number and of measuring the changes in cell type distribution during these processes.

In planarians, growth and degrowth occur by increase or decrease in cell number, though changes in the mean cell volume also occur, bigger animals having bigger cells. The decrease in cell number brought about by starvation (approx. 7000 cells/animal/ day for a 7 mm long *Dugesia tigrina* kept at 17 °C, Romero & Baguñà, unpublished data) results from more cells being lost by cell death (approx. 11 000 cells/animal/day) than produced by cell proliferation (approx. 4 000 cells animal day). Contrary to expectations, cell proliferation is maintained during starvation (Baguñà 1976a) probably because most tissues must be renewed for proper functioning and survival. This means that, besides epidermal and gastrodermal cells, most dead cells from the parenchyma must be re-cycled by living cells. This phenomenon, together with the use of stored energy (mainly fat and glycogen), must sustain the metabolic rate needed to maintain the proliferative rate for proper cell renewal.

The increase in cell number brought about by feeding is the result of more cells being born by cell proliferation than lost by cell death. Surprisingly, growth by feeding does not change significantly the death toll rate (Romero & Baguñà, unpublished data), and the increasing cell number is mainly the result of an increased cell proliferation rate caused by feeding (Baguñà 1974a). In summary, planarians are organisms in a continuous state of cell turnover. Since growth and degrowth affect cell type distribution only slightly, since neoblasts are the only cell type endowed with mitotic power, and because the cell death rate is maintained despite changing feeding regimes, we suggest that a fixed programme of cell determination and differentiation through cell lineage is operating throughout the organism's lifetime.

4. Quantitative cell analysis of planarian regeneration

One of the main problems of planarian regeneration that remains is the never ending controversy over neoblast versus metaplasia in blastema formation. Supporters of the first theory ('neoblast theory') invoke the existence of a permanent population of reserve cells (neoblasts) that give rise to blastema formation through migration and proliferation (Wolff & Dubois

1947). The second theory ('dedifferentiation theory') claims that neoblasts do not exist at all and that the source cells for blastema are specialized cells that dedifferentiate to an undifferentiated state (Hay 1968; Coward 1969).

Recently, some attempts to bridge both theories have been advanced suggesting that both types of cells (neoblasts and dedifferentiated cells) can take part in blastema formation (Gremigni & Miceli 1980; Gremigni 1981). Though the technique used (chromosomal markers) offer new avenues for re-evaluating the problem of blastema formation in planarians, the results obtained are open to discussion and the conclusions formulated by the authors are questionable.

The results obtained using the maceration technique offer little help in solving this problem. From the results it is clear that neoblasts accumulate rapidly from the very beginning of regeneration. Since during this period (0–24 hours of regeneration) a burst of cell proliferation has been detected in both species (Baguñà 1976b for *Dugesia mediterranea;* Saló, unpublished data for *Dugesia tigrina)* it is sound to suggest that the increase in neoblast percentage is due to cell proliferation and not to cell dedifferentiation. Moreover, the decrease in percentage of some differentiated cells (fixed parenchyma cells, acidophilic and basophilic, gastrodermal and goblet cells), held as a proof of massive cell dedifferentiation, could be interpreted as well as a process of cell dilution by proliferating neoblasts. Indeed, post-blastema regions show very small changes in cell distribution, contrary to what is expected of a massive cell dedifferentiation near the wound.

It is also evident that from 2–3 days of regeneration neoblasts decrease in the blastema while differentiated cells begin to appear or increase in percentage. These results are in agreement with the first published results of a quantitative analysis of blastema cells with electron microscopy (Hori 1978). This work emphasizes the role of neoblasts as a replacement cells both in intact and regenerating animals, and stresses once more the lack of evidence for cell dedifferentiation in the blastema and regions behind it.

To summarize, from the results obtained using the maceration technique and from previous results of mitotic analysis during regeneration (Baguñà 1976b) we suggest that blastema formation in planarians is mainly due to local proliferation of neoblasts near the wound. Cell dedifferentiation, if actually present, would give only a limited percentage of blastema cells. The main problems of planarian regeneration will have a clear answer in the future if we can answer first these two key questions: (1) what is the role of the neoblasts in the intact worm?, and (2) from where do they come?

Abbreviations

nb: neoblasts
nv: nerve cells
ep: epidermal cells
fp: fixed parenchyma cells
g: gastrodermal cells

References

Abeloos, M., 1930. Recherches expérimentales sur la croissance et la régénération chez les planaires. Bull. Biol. Fr. Belg. 64: 1–130.

Baguñà, J., 1973. Ph. D. Thesis. University of Barcelona, Spain.

Baguñà, J., 1974. Dramatic mitotic response in planarians after feeding and a hypothesis for the control mechanism. J. Exp. Zool. 190: 117–122.

Baguñà, J., 1976a. Mitosis in the intact and regenerating planarian Dugesia mediterranea n.sp. I. Mitotic studies during growth, feeding and starvation. J. Exp. Zool. 195: 53–65.

Baguñà, J., 1976b. Mitosis in the intact and regenerating planarian Dugesia mediterranea n.sp. II. Mitotic studies during regeneration and a possible mechanism of blastema formation. J. Exp. Zool. 195: 65–80.

Baguñà, J. & Ballester, R., 1978. The nervous system in planarians: peripheral and gastrodermal plexuses, pharynx innervation and the relationship between central nervous system structure and the acoelomate organization. J. Morphol. 155: 237–252.

Benazzi, M., Baguñà, J., Ballester, R., Puccinelli, I & Del Papa, R., 1975. Further contributions to the taxonomy of the Dugesia lugubrispolychroa group, with description of Dugesia mediterranea n.sp. Boll. Zool. 42: 81–89.

Brønsted, H. V., 1969. Planarian Regeneration. Pergamon Press Ltd, London.

Calow, P., 1977. The joint effect of temperature and starvation on the metabolism of triclads. Oikos 29: 87–92.

Coward, S. J., 1969. Regeneration in planarians: some unresolved questions and problems. J. Biol. Psychol. 11: 15–19.

David, C. N., 1973. Quantitative method for maceration of Hydra tissue Wilhelm Roux' Archiv. 171: 259–268.

Gremigni, V., 1981. The problem of cell totipotency, dedifferentiation and transdifferentiation in Turbellaria. In: Schockaert, E. R. & Ball, I. R. Turbellaria. Proc. Third Int. Symp. Hydrobiologia. This volume.

Gremigni, V. & Miceli, C., 1980. Cytophotometric evidence for cell 'transdifferentiation' in planarian regeneration. Wilhelm Roux' Archiv 188: 107–113.

Hay, E. D., 1968. Dedifferentiation and metaplasia in vertebrate and invertebrate regeneration. In: Ed. Ursprung, H., The Stability of the Differentiate State. Berlin, Heidelberg, New York: Springer, pp: 85–108.

Hori, I., 1978. Possible role of rhabdite-forming cells in cellular succession of the planarian epidermis. J. Electr. Microsc. 27: 89–102.

Lange, C. S., 1967. A quantitative study of the number and distribution of neoblasts in Dugesia lugubris (Planaria) with reference to size and ploidy. J. Embryol. Exp. Morphol. 18: 199–213.

Lange, C. S., 1968. A possible explanation in cellular terms of the physiological ageing of the planarian. Exp. Geront 3: 219–230.

Reynoldson, T. B., 1966. Preliminary laboratory experiments on recruitment and mortality in triclad populations. Verh. int. Ver. Limnol. 49: 1621–1631.

Schaller, H. & Gierer, A., 1973. Distribution of the head-activating substance in Hydra and its localization in membranous particles in nerve cells. J. Embryol. Exp. Morphol. 29: 39–52.

Wolff, E. & Dubois, F., 1947. La migration des cellules de régénération et les facteur qui la provoquen chez les triclades. C. R. Acad. Sci. 224: 1387–1388.