SHERIDAN, CHARLES LINWOOD. A Developmental Study of Regeneration in Dugesia tigrina. (1969). Directed by: Dr. Laura G. Anderton. pp. 65.

This study was undertaken to determine the source of cells used to regenerate lost body parts in Dugesia tigrina. Decapitated flatworms were treated with colchicine to arrest cell division after the worms had regenerated for 24, 48, 72, 96, and 120 hours. Histological study and counts of mitotic cells revealed that the cells dividing to rebuild the lost head were cells of the fixed parenchyma and not neoblasts, embryonic free cells of the mesenchyme, as had been assumed by other researchers.

A DEVELOPMENTAL STUDY OF REGENERATION IN *DUGESIA TIGRINA*

by

Charles Linwood Sheridan, III

A Thesis Submitted to
the Faculty of the Graduate School at
The University of North Carolina at Greensboro
in Partial Fulfillment
of the Requirements for the Degree
Master of Arts

Greensboro
April, 1969

Approved by

Laura G. Anderton
Thesis Adviser

APPROVAL SHEET

This thesis has been approved by the following committee of the Faculty of the Graduate School at the University of North Carolina at Greensboro.

Thesis Adviser — Laura G. Anderson

Oral Examination Committee Members — Paul E. Lutz

Edward McCrady IV

Eugene E. McDowell

April 29, 1969
Date of Examination

ACKNOWLEDGMENTS

The writer wishes to acknowledge his sincere gratitude to Dr. Laura G. Anderton for her interest in the research, patience in the lengthy experimentation, and valuable suggestions in the completion of this thesis.

TABLE OF CONTENTS

TABLES

FIGURES

I. INTRODUCTION AND REVIEW OF LITERATURE

The phylum Platyhelminthes embraces three classes of flatworms. Only one class, the turbellarians, which includes the planarians, has representatives that are free living and possesses powers of regeneration. This morphological-physiological phenomenon has made them the subject of voluminous research in North America and in Europe. This paper deals with regeneration in the planarian Dugesia tigrina.

The planarian body, organized on the organ-system level, is constituted primarily of loosely-packed cells known as mesenchyme or parenchyma cells. It is the cells of the mesenchyme and their role or roles in regeneration about which there is so much controversy. Two theories are propounded as to the source of the cells in blastema formation. One theory states that the cells of the blastema are local in origin; that is, they are contributed by the tissue immediately behind an excision and that the cells are provided by dedifferentiation of the tissue in this locale.

Flickinger (1964), working on this assumption, conducted experiments with isotopically-labelled worms serving as donors for grafts made to unlabelled planarians. He concluded from his data that extensive migration of reserve cells into the blastema does not occur, but his evidence "did support the concept of local origin of the blastema cells."

According to the second theory, the blastema is derived from unique cells of the mesenchyme migrating to the regeneration site from more distant areas of the body. These special, undifferentiated, embryonic-like cells are believed to retain their capacity to differentiate into specific cell types capable of participating in the morphogenetic process. Dubois (1949) has described these cells by selectively staining with green methyl pyronin (after Pedersen). She further described the movement of the neoblasts through the parenchyma in Dendrocoel lacteum. From this work her theory of neoblast movement has had general widespread acceptance. More recent research by Betchaku (1967) takes issue with Dubois on how the neoblasts arrive at the wound area. He postulated that necrosis of injured cells of the fixed parenchyma created pockets devoid of any cells within the tissue. Uninjured neoblasts, interspersed among the fixed parenchyma, are released into the void created by the necrosing cells. In this way the neoblasts are juxtaposed with the regeneration area.

It is difficult to make a general statement covering all the species of planarians and their relatives in other genera. The power to regenerate is comparative; some genera possess a high ability to regenerate and others are poorly endowed. Many papers have been written about research done comparing the ability of a species to regenerate with the number of neoblasts found in the parenchyma. Curtis and Shulze (1924) compared Euplanaria maculata (Dugesia tigrina), Phagocata gracillis, another fresh water species, and Dendrocoel

lacteum.

Lender and Gabriel (1960) have repeated the experiments of Dubois using the same stain and staining techniques on Dugesia lugubrius. Their findings in this species paralleled those in Dendrocoel lacteum used by Dubois. Brønstedt (1955) traced the general regenerative process in stages, giving excellent descriptions of each phase as a basis for normal regeneration.

It is of interest that much of the previous work done in the regenerative area on planarians has been almost wholly concerned with neoblasts being constant embryonic stock which purportedly activate and maintain regeneration blastemas. Woodruff and Burnett (1965) made a fresh approach to the problem. They argued that neoblasts are not necessarily persistent embryonic stock cells. They proposed that the neoblasts could be formed by dedifferentiation of specialized intestinal gland cells as well as by division of pre-existing neoblasts.

The literature cited above indicates that there is general agreement that neoblasts are distinctive cells that persist in the parenchyma of the turbellarians, but agreement stops at this point. It is commonly assumed that neoblasts play a role in regeneration but whether the neoblasts that are of local origin as proposed by some workers are the same neoblasts that migrate through the mesenchyme is unclear. In view of conflicting ideas presented in the literature, it seems likely that the term neoblast has more than one meaning. Thus, the central purpose of this study was to identify

or attempt to identify cells that participate in or are responsible for blastema formation and eventual replacement of parts after surgical removal.

II. MATERIALS AND METHODS

A. Selection of Experimental Animals

In this vicinity two species of planaria are available, the common brown Dugestia tigrina and the black species Cura foremanii. To select which species would make the best experimental animals 12 worms of each species were bisected into anterior and posterior halves. The time required to regenerate the missing parts in the two species were compared. A comparison was also made between the regeneration time required for the anterior halves and the posterior halves. From the data obtained from these experiments, discussed under results and conclusions, Dugesia tigrina was selected for continued experimentation (Fig. 1).

Fig. 1. *Dugesia tigrina*.
Note that left eyespot is on another visual plane because of the position of the animal.

B. Culturing and Feeding

With the exception of a few dozen planarians collected in local streams and ponds, all the animals used in the experimental work were obtained from Carolina Biological Supply Company, Burlington, North Carolina. An approximate total of 475 worms were used in all the experiments.

Cultures of the animals were maintained in filtered stream water with a mean pH of 6. Stocks were kept in eight inch glass fingerbowls with no more than 25 animals confined to a single bowl. The water was changed twice a week.

Both stock and experimental animals were kept at 20°C in a constant temperature box, the temperature recommended by the supply house and by previous experimenters. To occlude light, to which they are negatively phototaxic, photography cloth was kept over and around the housing bowls.

Enchytraeus, small white annelids, were fed to the planarians once a week. They proved to be satisfactory not only from a nutritional standpoint, but also because of the convenience of maintaining a sanitary bowl culture. Those portions of enchytraeids that were not consumed could be lifted from the bowl and the Dugesia could remain in the water in which they had just been fed.

C. Method of Study

It was decided that the entire gross process of regeneration in Dugesia tigrina should be observed and followed before undertaking any investigation to determine if free cells of the parenchyma are responsible for the regeneration phenomenon. This decision was reached because of the mountainous literature comparing the form, rate, and structure of regeneration in the various orders, families, and genera of the phylum. Before seeking the role of the cells that constitute the Dugesia body, it was felt that the various cell types should first be identified, and the tissue composed of these cells should also be identified. Toward this end mature planarians, starved for one week, were sectioned, smeared, and stained. In all the succeeding work, the animals were deprived of food for at least seven days before they were used as experimental animals.

The planarians were removed from the culture medium with a camel's hair brush and placed on a paraffin disc. When the animals became extended, a transverse cut was made with a sharp single-edged razor blade between the head and the pharynx, and a second section was made behind the pharyngeal area, dividing the worm into three equal sections. The sections were transferred to a slide and smeared. The smears were washed with amphibian Ringer's solution (0.07%), flooded with Wright's stain, and buffered with Wright's buffer. The smears were washed in distilled water and allowed to dry.

They were then studied by microscopy.

Experiments were continued in pursuit of an effective way to mark the cells in Dugesia. At the same time, a study was begun of the healing pattern in Dugesia and also the way reconstruction was accomplished.

The subjects were selected so that each was of equal size and sectioned into anterior and posterior halves, each half isolated in Petri dishes with sufficient water to cover it. The sections were kept under surveillance for healing and growth patterns and rates.

D. Detection and Identification of Regenerative Cells

1. Colchicine Treatment

Colchicine is known to arrest cells in mitosis at metaphase. To identify the cells that divide and participate in the regenerative process, decapitated planarians were subjected to colchicine solution of two concentrations.

In the first experiment, 10 animals were placed in a solution containing 1000 ml of filtered stream water to 20 mg of colchicine (.002% solution) immediately after decapitation. They remained in the colchicine solution for 30 hours. An equal number of control animals were placed in filtered stream water.

In the second experiment with colchicine, 10 beheaded Dugesia were placed in natural medium (stream water) to begin regeneration. After 96 hours in the natural medium, the animals were transferred to a colchicine medium of 10 mg. of colchicine to 1000 ml of stream water (.001% solution). After 18 hours of exposure to .001% colchicine, five of the specimens were moved back to natural medium. Six hours after they were placed in the natural medium, two of the five animals were sacrificed, stained, and mounted for microscopic study. The other three members continued their regeneration in natural medium.

The remaining five specimens were given 30 hours exposure to .001% colchicine. One of these animals was fixed, stained, and

studied for mitotic cells. The remaining four were allowed to continue reconstruction in natural medium.

From this point, the remainder of the experimentation was confined to cell division inhibition by using a single concentration of colchicine and a single exposure time. Following treatment with colchicine, staining and identification of cellular constituents of the dugesian body were made, locations and concentrations of the constituents were determined, and counts of mitotic figures were made.

2. Histological Study Using Ehrlich's Hematoxylin Stain.

A group of 30 Dugesia were decapitated and placed in filtered stream water. In groups of five, the animals were transferred at 24-hour intervals, beginning at 24 hours and continuing through 120 hours (5 days), to a .001% colchicine medium. At the end of six hours exposure to colchicine, the planarians were fixed and stained in Ehrlich's hematoxylin and sectioned. Some of the microtome sections were cut on a transverse plane and some on a saggital plane. In each case, the sections were cut at four microns. The mounted sections were counterstained with eosin and studied under a light microscope.

3. Mitotic Cell Index Study

The serially-arranged slides and sections of the previous study were restudied under an Olympus Elgeet microscope at 1500 X

magnification for cells in mitosis. For each of the worms in the five-group series, 900 cells were counted in groups of 100 cells, proceeding from the anterior, the site of regeneration, to the posterior end. In each of the 100 cells counted, the region of the body in which the count was made was noted and recorded. The cells in mitosis and the mitotic stage were tabulated. A total of 4,500 cells was counted and studied for each 24-hour stage of regeneration.

4. Histological Study Using Polychrome Stain

The five remaining worms of the beginning total of 30 were also allowed to regenerate, one for each 24-hour period up to and including 120 hours. At the end of each regenerating period, the animals were subjected to colchicine (.001%) for six hours. Taken from the colchicine, the worms were fixed, embedded and sectioned. The slides were hydrated and the tissue stained with polychrome stain (according to Lehman, 1965). This staining procedure is primarily for detecting cell division because the chromosomes of the mitotic cells stain green.

III. RESULTS

A. Selection of Experimental Animals

A close record of the time needed to reconstitute the lacking half was kept (Table I). The anterior part of 11 brown planarians, Dugesia tigrina, completed the task before their black counterpart did. The anterior section of the twelfth animal disintegrated. As Table I indicates, the black planarians varied as much as two days behind the Dugesia tigrina in growth rate; however, two of the black anterior sections were lost. The posterior sections followed a similar pattern of regrowth with all 12 of the halves regenerating a head while two of the black posterior halves necrosed.

In both the brown and the black planarians, the anterior sections regenerated a tail at a more rapid rate than the posterior sections reconstructed a head. This demonstrated clearly at the outset that a gradient exists along the antero-posterior axis (Child, 1941). As indicated earlier, the data provided by these observations culminated in Dugesia tigrina being selected as the laboratory experimental animals.

Table 1. Comparison of regeneration time of *Dugesia tigrina* and *Cura foremanii*.

Dugesia tigrina								*Cura foremanii*							
Mean days after Section							HEADS	Mean days after Section							
1	2	3	4	5	6	7		1	2	3	4	5	6	7	
	X						Blastema			X					
			X				Eyespots						X		
				X			Pigmentation					X			
				X			Auricles						X		
				X			Mature Head							X	
							TAILS								
		X					Blastema				X				
					X		Pigmentation						X		
					X		Mature Tail							X	

B. Experiments Using Smear Technique and Wright's Stain.

The cells of the ventral epidermis were found to be ciliated and the nucleus most often was located in the center of the cytoplasm. Many of the cells were found to be dividing. Cells of the body from the epidermis inward were diverse in shape, size, and density. Although this method of fixation and staining did not allow great detail to be studied, it was sufficient to emphasize the high degree of cellular differentiation, and the knowledge gained from the procedure formed a basis for more intensive study by a different technique which is discussed later in this paper.

C. Healing and Growth

When the planarians were cut into anterior and posterior sections, the perimeter of the wound where the section was made contracted and pulled the epidermis together much like a draw string purse. This was particularly evident because the pigment spots were pulled closer together, giving the appearance of a continuous dark band around the wound. The severed ends remained in this state until the beginning of blastema formation.

The first evidence of a blastema, or a regeneration bud, usually was a cone-shaped mass of cells that protruded beyond the margin of the cut, most often centered in the cut area, and separated from the old portion of the body by the pigmented band. The initial cells of the bud contained no pigment, remaining translucent and had an undifferentiated appearance. As the blastema aged, it spread from its original central position to both the right and the left until the complete margin of the cut had a clear layer of cells extending all the way across the wound.

At this time two changes became noticeable in the regenerating worms. First, the gathered in or puckered appearance at the site of the cut appeared to relax, although a distinct margin between the new and the old growth still existed. Secondly, as the regenerate aged, small dark skips appeared in the once translucent new growth, much smaller than the melanophores which appeared in the complete regenerate. This would appear to be the first visible sign of

differentiation in the new growth. The blastema appearance varied in the time of formation from 24 hours to 48 hours, and the anterior section of a bisected worm produced a blastema before the posterior section.

D. Eye Development

Following the appearance of pigment in the blastema regenerating a head, several melanophores located in the older section of the worms just behind the margin where the blastema and the "parent" worm joined became more intense in color. Often these appeared in groups of threes, but more often in twos. The size of the melanophores apparently had little or nothing to do with the change. Frequently the intensified pigment spots appeared little more than pin points of color, but the deepening of color content caused them to become conspicious; too, their location on the edge of the margin coincided with the normal location of eyespots.

Section 1 of each worm produced suspected eyespots at the end of four days (approximately 96 hours) after sectioning; at five days (approximately 118 hours) these sections definitely had developed eyes where the suspected precursor melanophores appeared, but the ocular pits were not present. The section from the pharyngeal cut to one-half the distance to the tail, Section 2, produced deeply colored spots at the anterior end behind the developing blastema on the fifth day after sectioning. Section 3, slower in blastema formation, did not produce the presumed rudimentary eyespots until six days after sectioning.

It has long been established that eye development in planarians is primitive. The eyes do not form an image but are sensitive to light, and the animals recede from strong light.

To test the presumptive eyespots in the regenerates, Section I of each animal was subjected to a light test when the presumptive eyespots appeared on the fourth day of reconstruction. Each of the sections was placed in a bright light (a fluorescent desk lamp) and their reactions noted. None of the sections tried to escape the light. This appeared to indicate that the presumed eyespots were not light sensitive. On the fifth day, however, all of the five sections gave a negative phototrophic response, indicating the eyes were functional. Sections 2 and 3 gave the same responses one and two days later, respectively (Table 2). It can be stated that morphologically the eye structure is present on the fourth, fifth, and sixth day, respectively, but physiological differentiation does not occur until one day later.

Fig. 2. Sections used for eye regeneration after removal of head.

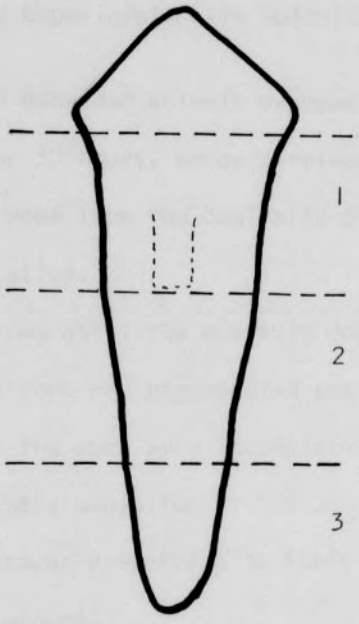


Table 2. Natural regeneration of eyespots from suspected malanophores.

Section level*	Number of sections	Number with eyespots						
		2	3	4	5	6	7	8
		days after sectioned						
1	5	0	X					
2	5			0	X			
3	5					0	X	

*The numbers refer to the various levels shown in Fig. 2 .
0-Structural differentiation of eye.
X-Physiological differentiation of eye.

E. Preliminary Experiments with Colchicine

Of the first 10 beheaded animals exposed to a .002% colchicine solution for 30 hours, seven survived to be transferred to plain medium. One week from the beginning of the experiment, four animals remained alive.

From the beginning until the eleventh day after the experiment was started, four survivors had regenerated small, underdeveloped, unpigmented heads, and the eyes were incompletely formed. In contrast, the control animals exhibited at the end of six days completely regenerated heads in proper proportion to their body size, and the eyes were normally developed.

In the second group of animals, those exposed to .001% colchicine, one-half (5) was given 18 hours exposure. In each of these animals the regeneration product was below normal in all ways as compared to untreated animals in a parallel stage of development. In the cells of the two sacrificed animals, no mitotic figures were detected. Data obtained from later experiments proved that peak cell division was past at this time. The remaining three animals of this group completed their regeneration but the entire process required three days beyond the five to six required for normal, untreated worms.

The five Dugesia treated with colchicine for 30 hours also displayed retarded reconstruction. One animal was fixed, stained, and studied for mitotic cells, but none were detected. Of the remaining four animals, two produced a normal head, but the time of accomplish-

ment was extended beyond that for untreated animals. The two other worms followed quite a different regrowth pattern in restoring their heads. Observed six days later, one of the worms had died, but the body remained intact. From the dorsal side of the dead worm at the level of the pharynx two small stalks projected caudad. Microscopic observation showed that at the tip of each stalk were two microscopic eyespots. At the anterior end of the animal, the body tapered into an undeveloped true head (Fig. 3).

The last worm of the 30-hour series remained vigorous, although it also exhibited an excrescence. The anomaly was a single slender stalk growing from the dorsal side opposite the pharyngeal region. The excrescence terminated in a finely pointed spine located off center of the stalk-end with eyespots at the base of the spine (Fig. 4). Although the experiment was repeated several times under the same circumstances, it was impossible to duplicate the results.

Fig. 3. Dugesia with two supernumerary heads produced after 30 hours of exposure to colchicine.

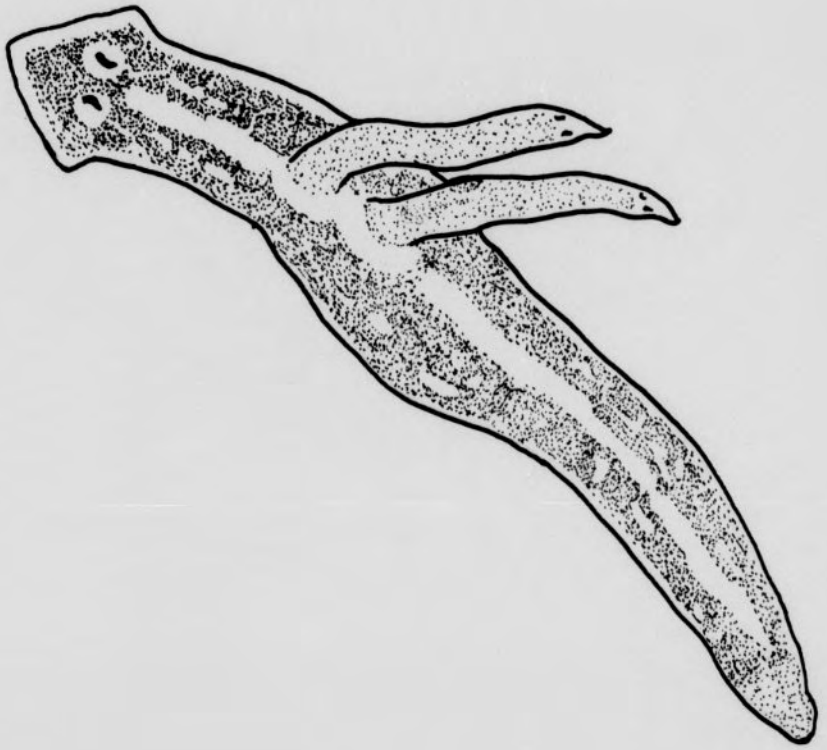
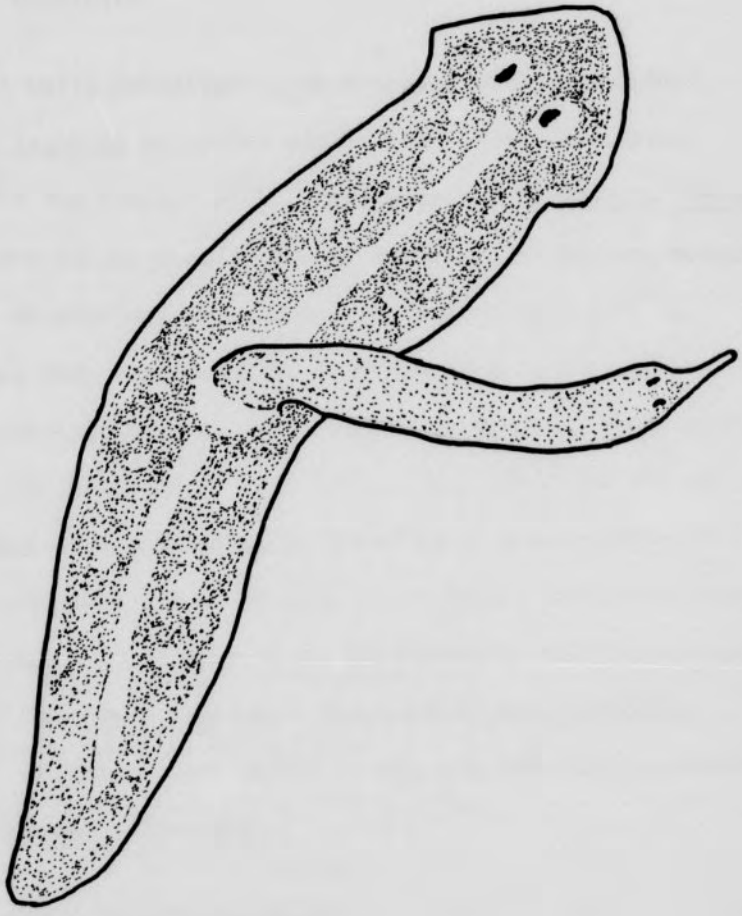


Fig. 4. Dugesia with one supernumerary head produced after 30 hours of exposure to colchicine.



F. Histological Study Using Ehrlich's Hematoxylin Stain.

1. Neoblasts

Neoblast cells described in dendrocoels by Dubois (1961) and in Dugesia lugubris by Lender and Gabriel (1961) have also been observed in the present work. The neoblasts in Dugesia tigrina observed had very large, usually ovoid, nuclei. The nuclei, measured with an ocular micrometer, had a diameter ranging from 9 to 11 microns and they were surrounded by a thin coat of cytoplasm of approximately one-half micron. The overall diameter of these cells varied from 10 to 12 microns. Some of the neoblast cells had an elongated nucleus with the cytoplasm tapering at one end (Fig. 5).

These cells appeared to have an affinity for cells like themselves; most often they were found in aggregates of two, three, and sometimes four. However, they were infrequently observed as an isolated cell. Whatever their distribution, the neoblasts were surrounded by the "fixed" parenchyma.

2. Fixed Parenchyma Cells

Fixed cells of the parenchyma are those cells composing the mesenchyme in which neoblast cells are distributed, and which make up the mass of the planarian body in which the organ systems are suspended. The fixed parenchyma was distinctly cellular in the Dugesia rather than syncytial; nevertheless, the cell membrane was

often difficult to delimit in areas where these cells were closely packed. The mean diameter of the fixed cells, as determined by ocular micrometry, was between three and four microns (Fig. 6).

3. Gastrodermal Cells

Gastrodermal cells composed the single layer epithelium of the gut. They were long, narrow cells of the columnar type and were highly vacuoled. Because the gut consists of many diverticula, these cells were observed throughout most of the parenchyma.

Fig. 5. Neoblast of *Dugesia tigrina* after staining with Ehrlich's hematoxylin stain. Note that pointer is on the nucleus of the neoblast.

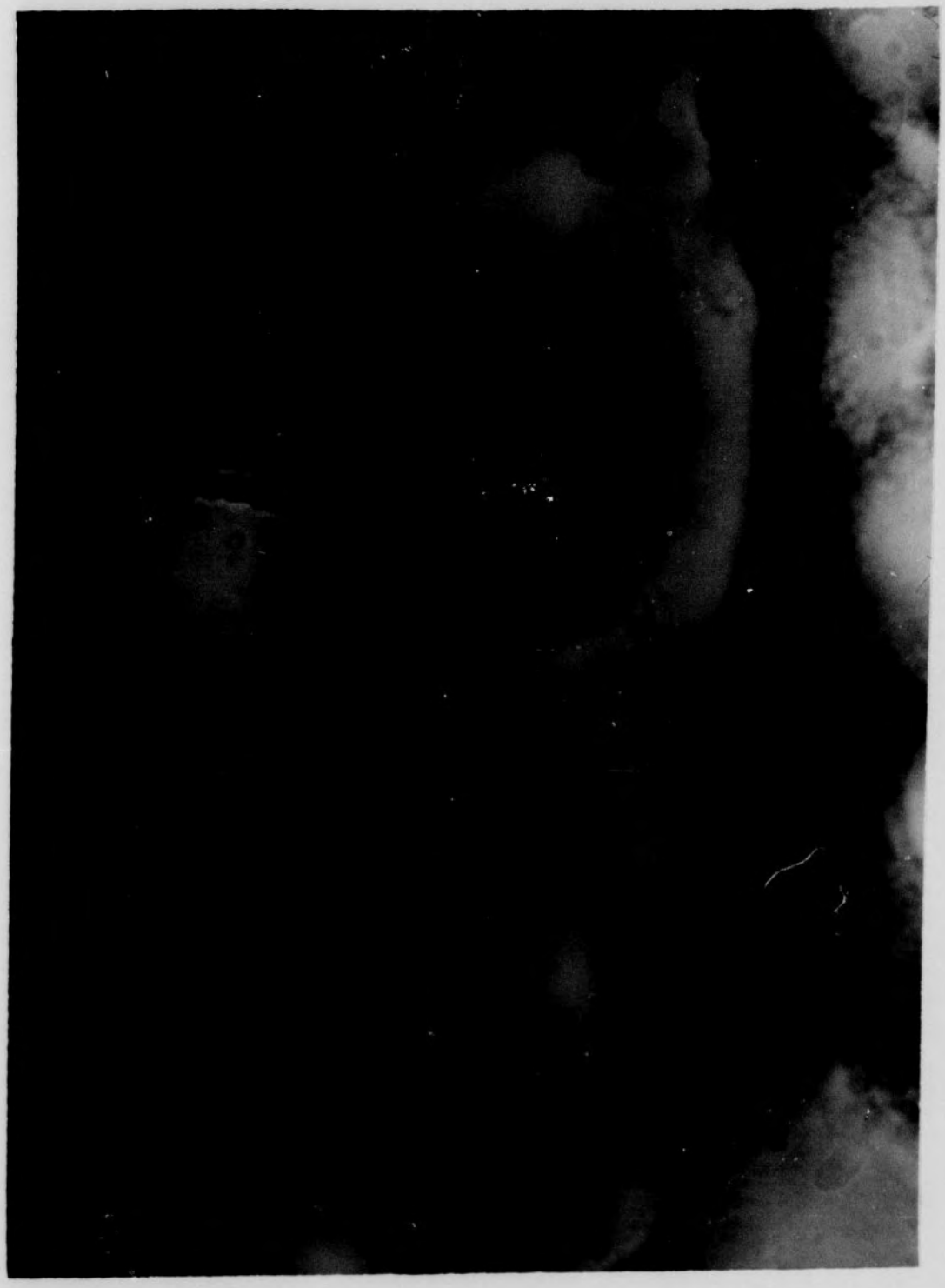
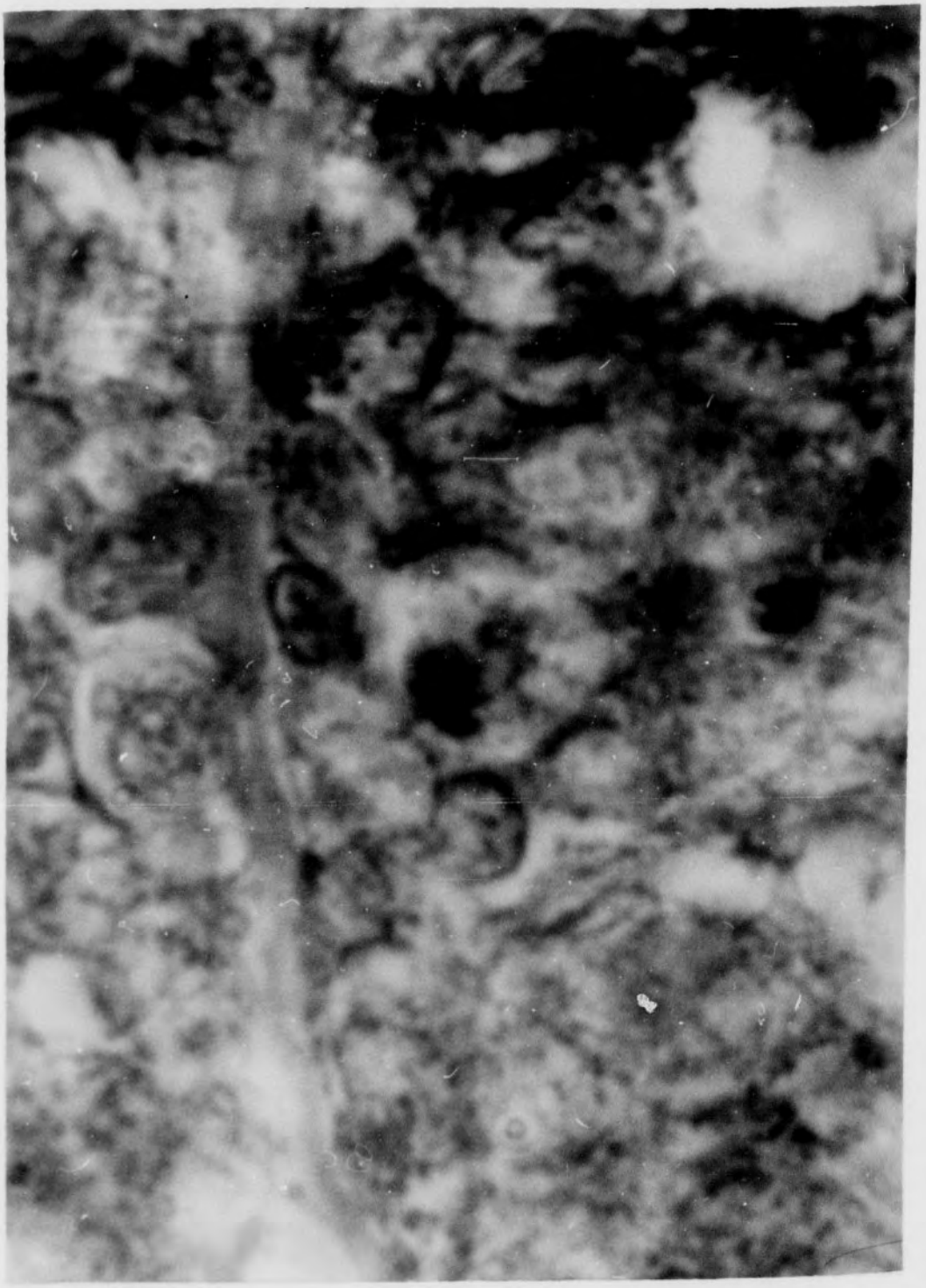


Fig. 6. Fixed parenchyma cells of *Dugesia tigrina* in saggital section after staining with Ehrlich's hematoxylin stain.



G. Mitotic Cell Study

1. The Twenty-four Hour Regenerated Worm

Table 3 shows the results of mitotic cells counted and their distribution in the 24-hour regenerated worm. In the 24-hour regenerate, the blastema was a minute aggregate of semi-transparent cells. At this time the epithelium had not formed over the blastema on a basal membrane that characterizes the body epithelium. The epidermal cells of the dorsal and ventral epidermis broke away from the intact epidermal sheet and streamed toward the center of the forming blastema. The cells became elongated and concave on the internal surface to fit over the arched blastema. At 24 hours these cells were few and were not in contact with each other; they were scattered randomly over the blastema substrate. The converging cells were morphologically similar to falling water drops. The regeneration bud consisted of very small, highly concentrated cells. The nuclei were surrounded by little cytoplasm, indicative of cells that had recently divided and that had not yet matured to their normal size. Within the 24-hour blastema no mitotic cells were observed. Neoblasts were singularly lacking in the blastema. However, in the region immediately behind the blastema a few cells of the fixed parenchyma were in a state of mitosis.

Although the number of cells in mitosis at 24 hours was low, they were found, except in the blastema, scattered throughout the body in the mesenchymal tissue (Fig. 7).

Table 3. Per Cent of cells in mitosis in the 24-hour regenerated *Dugesia tigrina*.

	Cell Count	Percent in Mitosis	Area
Worm #1	100	6	Head
	100	2	
	100	6	
	100	8	Pharynx
	100	12	
	100	14	
	100	8	Behind Pharynx
	100	8	
	100	6	Tail
Worm #2	100	6	Head
	100	9	
	100	7	
	100	10	Pharynx
	100	10	
	100	9	
	100	5	Behind Pharynx
	100	7	
	100	8	Tail
Worm #3	100	9	Head
	100	11	
	100	11	
	100	13	Pharynx
	100	10	
	100	12	
	100	7	Behind Pharynx
	100	7	
	100	6	Tail
Worm #4	100	5	Head
	100	4	
	100	4	
	100	4	
	100	10	Pharynx
	100	13	
	100	9	
	100	5	Tail
	100	1	
Worm #5	100	4	Head
	100	4	
	100	4	
	100	6	Pharynx
	100	12	
	100	9	
	100	5	Behind Pharynx
	100	5	
	100	6	Tail
Total	4,500	Average	7.48%

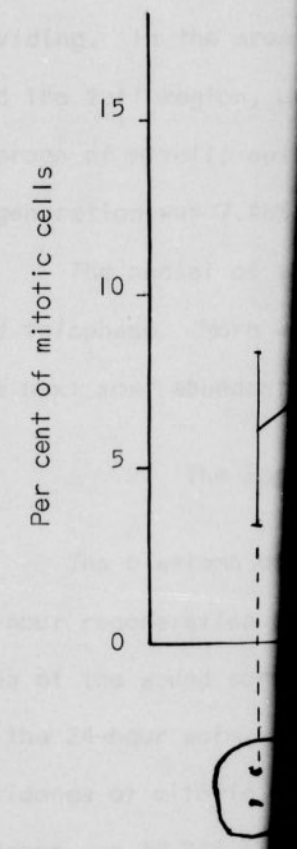
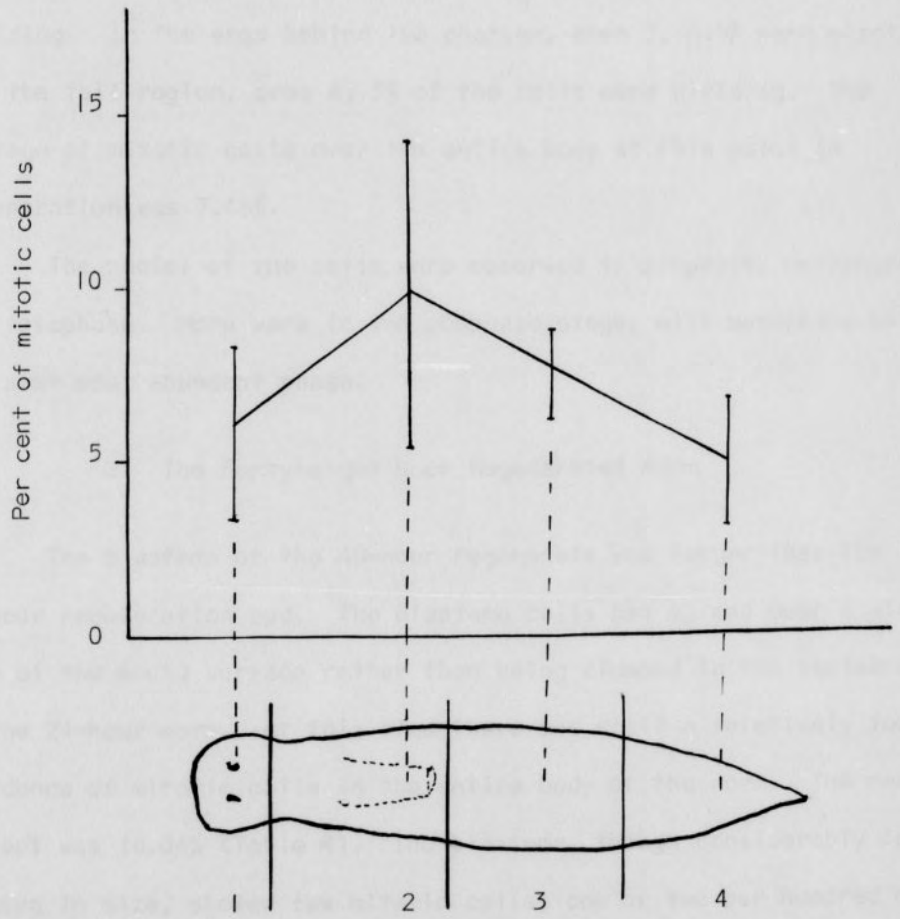


Fig. 7. Distributive percentages of mitotic cells in various body regions of the 24-hour regenerated Dugesia tigrina.


Per cent of mitotic cells
15
10
5
0
1
2
3
4

Neoblasts, concentrated more highly in the pharyngeal region, were mitotically inactive.

In the region behind the blastema, designated area 1 in Fig. 7, 6% of the cells were found to be dividing. The pharyngeal area, area 2, showed an increase in mitotic cells with 9.8% of the cells dividing. In the area behind the pharynx, area 3, 6.5% were mitotic, and the tail region, area 4, 5% of the cells were dividing. The average of mitotic cells over the entire body at this point in regeneration was 7.48%.

The nuclei of the cells were observed in prophase, metaphase, and telophase. More were in the prophase stage, with metaphase being the next most abundant phase.

2. The Forty-eight Hour Regenerated Worm

The blastema of the 48-hour regenerate was larger than the 24-hour regeneration bud. The blastema cells had spread over a wider area of the wound surface rather than being clumped in the center as in the 24-hour worm. At this time there was still a relatively low incidence of mitotic cells in the entire body of the worm. The mean percent was 18.04% (Table 4). The blastema, though considerably increased in size, showed few mitotic cells, one or two per hundred cells at best. The post-regenerate area showed 16.15% of each 100 cells dividing. The pre-pharyngeal and the pharyngeal sections, area 2, Fig. 8, became the region of most active cell division. Here the ratio of dividing to non-dividing cells was 20.59 to 100. The cells that had

Table 4. Per cent of cells in mitosis in the 48-hour regenerated *Dugesia tigrina*.

	Cell Count	Percent in Mitosis	Area
Worm #1	100	17	Head
	100	16	
	100	15	
	100	20	Pharynx
	100	20	
	100	26	
	100	7	Behind Pharynx
	100	18	
	100	17	Tail
Worm #2	100	15	Head
	100	16	
	100	13	
	100	26	Pharynx
	100	20	
	100	19	
	100	18	Behind Pharynx
	100	12	
	100	13	Tail
Worm #3	100	14	Head
	100	34	
	100	20	Pharynx
	100	17	
	100	18	
	100	19	
	100	17	Behind Pharynx
	100	15	
	100	21	Tail
Worm #4	100	13	Head
	100	16	
	100	12	
	100	21	Pharynx
	100	23	
	100	19	
	100	14	Behind Pharynx
	100	15	
	100	25	Tail
Worm #5	100	12	Head
	100	17	
	100	23	Pharynx
	100	19	
	100	20	
	100	20	
	100	19	Behind Pharynx
	100	21	
	100	22	Tail
Total	4,500	Average 18.04%	

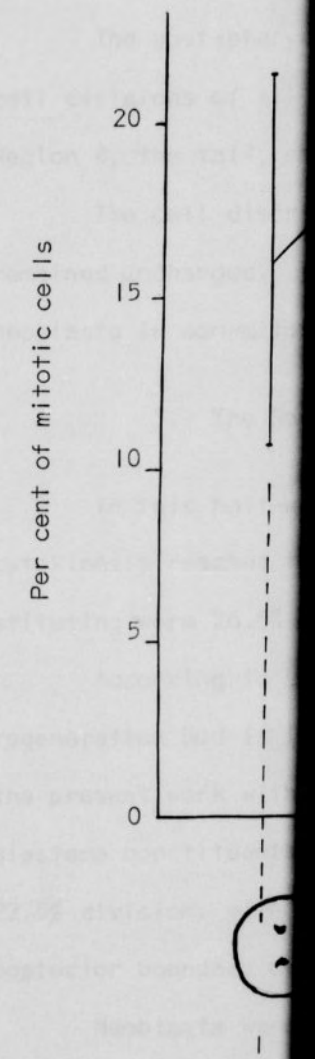
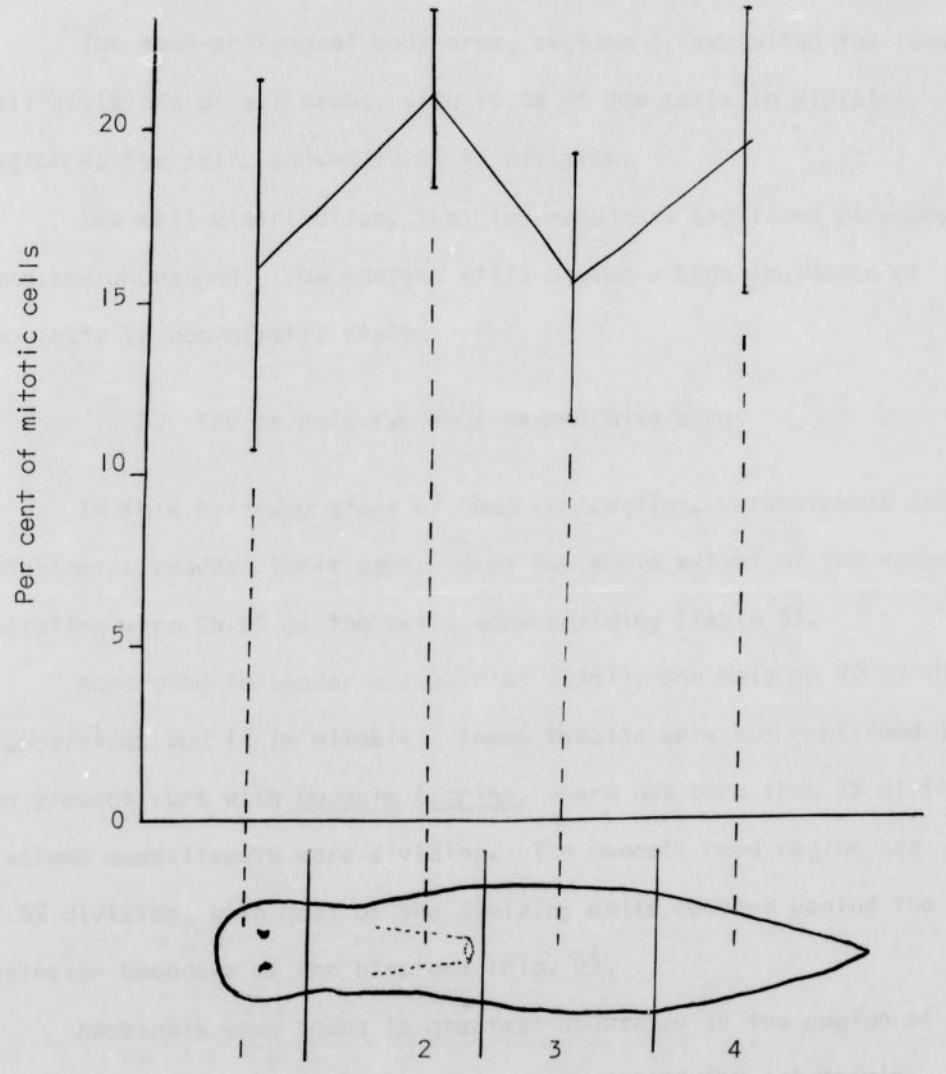


Fig. 8. Distributive percentages of mitotic cells in various body regions of the 48-hour regenerated Dugesia tigrina.


Per cent of mitotic cells
20
15
10
5
0
1
2
3
4

separated from the ectodermal epithelium to cover the bud were more orderly. The basal membrane to which they attach was still unformed, but the cells were more or less continuous. A great number of the covering epithelial cells were small, and the incomplete new epithelium was one cell in thickness.

The post-pharyngeal body area, section 3, exhibited the least cell divisions of all areas, with 15.6% of the cells in division. Region 4, the tail, showed 19.6% in division.

The cell distribution, that is, neoblasts and fixed parenchyma remained unchanged. The pharynx still showed a high incidence of neoblasts in non-mitotic states.

3. The Seventy-two Hour Regenerated Worm

In this half-way stage of head restoration, karyokinesis and cytokinesis reached their peak. Over the whole extent of the reconstituting worm 26.6% of the cells were dividing (Table 5).

According to Lender and Gabriel (1961), one cell in 20 in the regeneration bud is in mitosis. These results were not confirmed in the present work with Dugesia tigrina, where not more than 2% of the blastema constituents were dividing. The overall head region had 22.5% division, with most of the dividing cells located behind the posterior boundary of the blastema (Fig. 9).

Neoblasts were found in greatest abundance in the region of the pharynx concentrated in the mesenchyme around the gut margin. Their distribution was irregular with more of these cells appearing

Table 5. Per cent of cells in mitosis in the 72-hour regenerated *Dugesia tigrina*.

	Cell Count	Percent in Mitosis	Area
Worm #1	100	5	Head
	100	30	
	100	27	
	100	37	Pharynx
	100	30	
	100	30	
	100	80	
	100	28	Behind Pharynx
	100	29	Tail
Worm #2	100	20	Head
	100	30	
	100	35	Pharynx
	100	35	
	100	36	
	100	15	Behind Pharynx
	100	28	
	100	31	
	100	26	Tail
Worm #3	100	12	Head
	100	33	
	100	36	
	100	23	Pharynx
	100	33	
	100	31	
	100	18	Behind Pharynx
	100	34	
	100	19	Tail
Worm #4	100	19	Head
	100	28	
	100	34	
	100	31	Pharynx
	100	29	
	100	39	
	100	14	Behind Pharynx
	100	24	
	100	15	Tail
Worm #5	100	15	Head
	100	16	
	100	10	
	100	29	Pharynx
	100	26	
	100	17	Behind Pharynx
	100	19	
	100	22	
	100	20	Tail
Total	4,500	Average 26.62%	

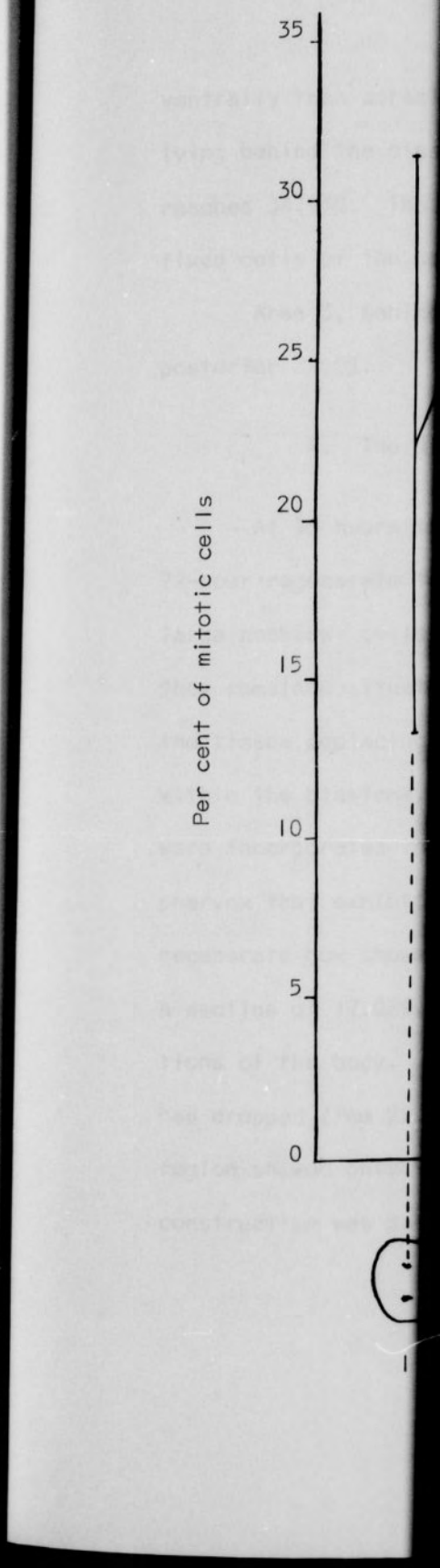
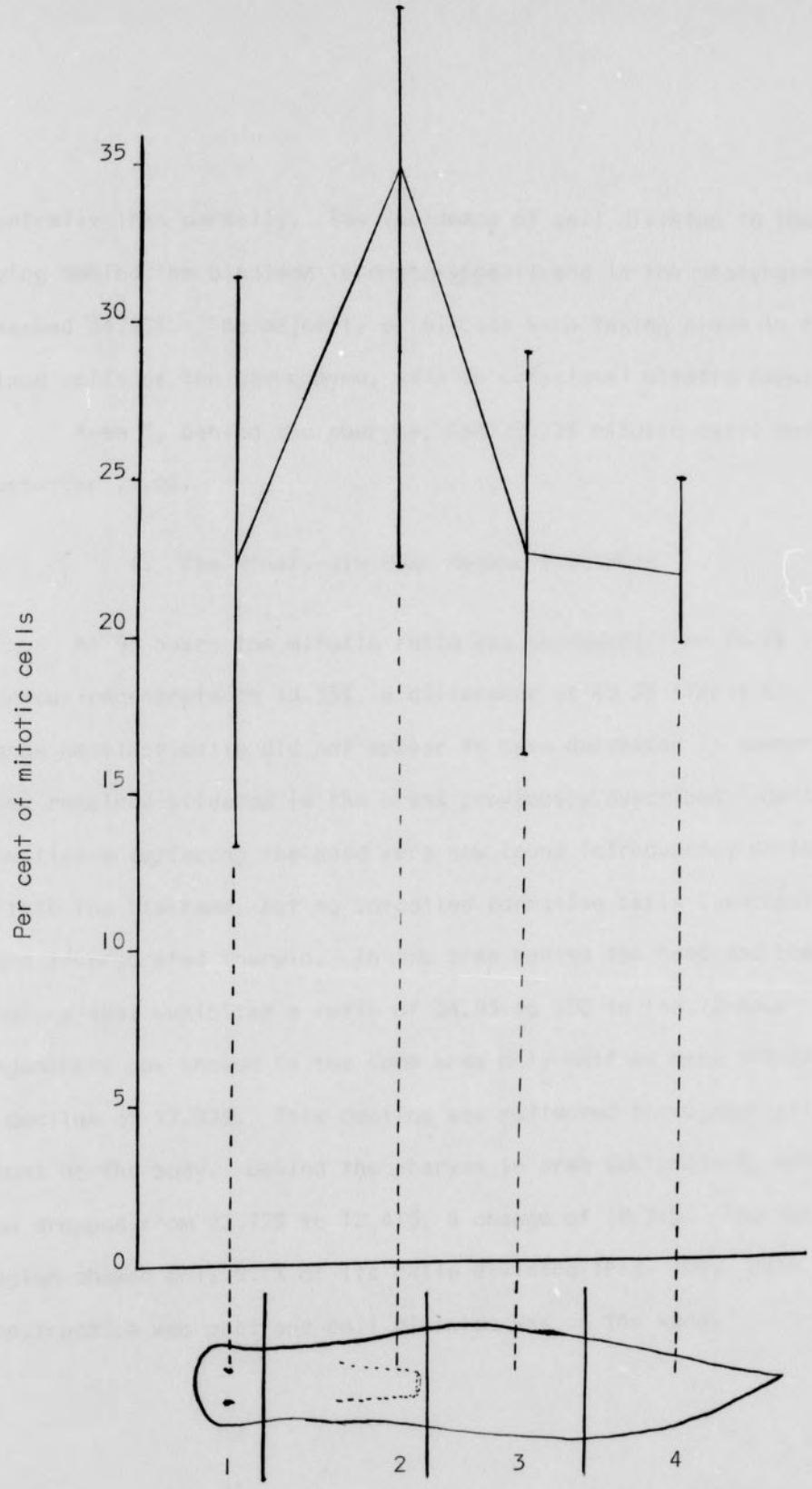


Fig. 9. Distributive percentages of mitotic cells in various body regions of the 72-hour regenerated Dugesia tigrina.


35
30
25
20
15
10
5
0
Per cent of mitotic cells
1
2
3
4

ventrally than dorsally. The incidence of cell division in the area lying behind the blastema (pre-pharyngeal) and in the pharyngeal area reached 34.93%. The majority of mitoses were taking place in the fixed cells of the parenchyma, with an occasional mitotic neoblast.

Area 3, behind the pharynx, had 22.72% mitotic cells and the posterior 22.0%.

4. The Ninety-six Hour Regenerated Worm

At 96 hours the mitotic ratio was decreased from 26.6% in the 72-hour regenerate to 13.33%, a difference of 13.3% (Table 6). The large neoblast cells did not appear to have decreased in number, and they remained situated in the areas previously described. Cells within the tissue replacing the head were now found infrequently dividing within the blastema, but no so-called formative cells (neoblasts) were incorporated therein. In the area behind the head and the pharynx that exhibited a ratio of 34.93 to 100 in the 72-hour regenerate now showed in the same area only half as many mitotic cells, a decline of 17.02%. This decline was reflected throughout all sections of the body. Behind the pharynx in area designate 3, mitoses had dropped from 22.72% to 12.41%, a change of 10.31%. The tail region showed only 9.1% of its cells dividing (Fig. 10). Peak reconstruction was past and cell division was on the wane.

Table 6. Per cent of cells in mitosis in the 96-hour regenerated *Dugesia tigrina*.

	Cell Count	Percent in Mitosis	Area
Worm #1	100	17	Head
	100	16	
	100	16	
	100	30	Pharynx
	100	24	
	100	17	Behind Pharynx
	100	15	
	100	15	
	100	13	Tail
Worm #2	100	18	Head
	100	17	
	100	20	Pharynx
	100	17	
	100	14	Behind Pharynx
	100	16	
	100	16	
	100	11	Tail
	100	12	
Worm #3	100	18	Head
	100	12	
	100	8	
	100	17	Pharynx
	100	22	
	100	17	
	100	12	Behind Pharynx
	100	16	
	100	13	Tail
Worm #4	100	8	Head
	100	13	
	100	15	
	100	11	Pharynx
	100	13	
	100	10	Behind Pharynx
	100	11	Tail
	100	8	
	100	5	
Worm #5	100	5	Head
	100	12	Pharynx
	100	14	
	100	7	Behind Pharynx
	100	7	
	100	4	
	100	2	Tail
	100	8	
	100	8	
Total	4,500	Average 13.33%	

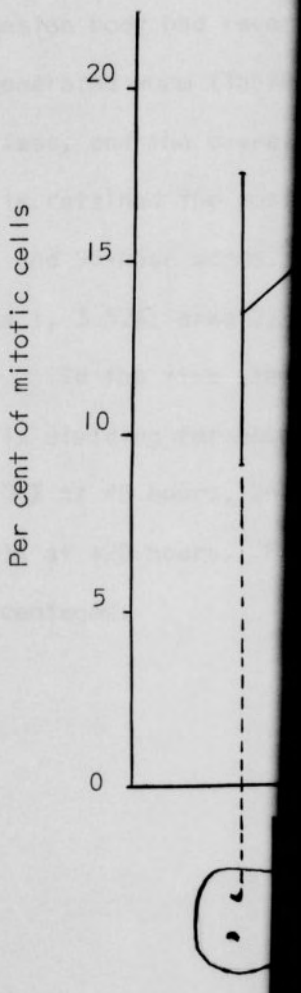
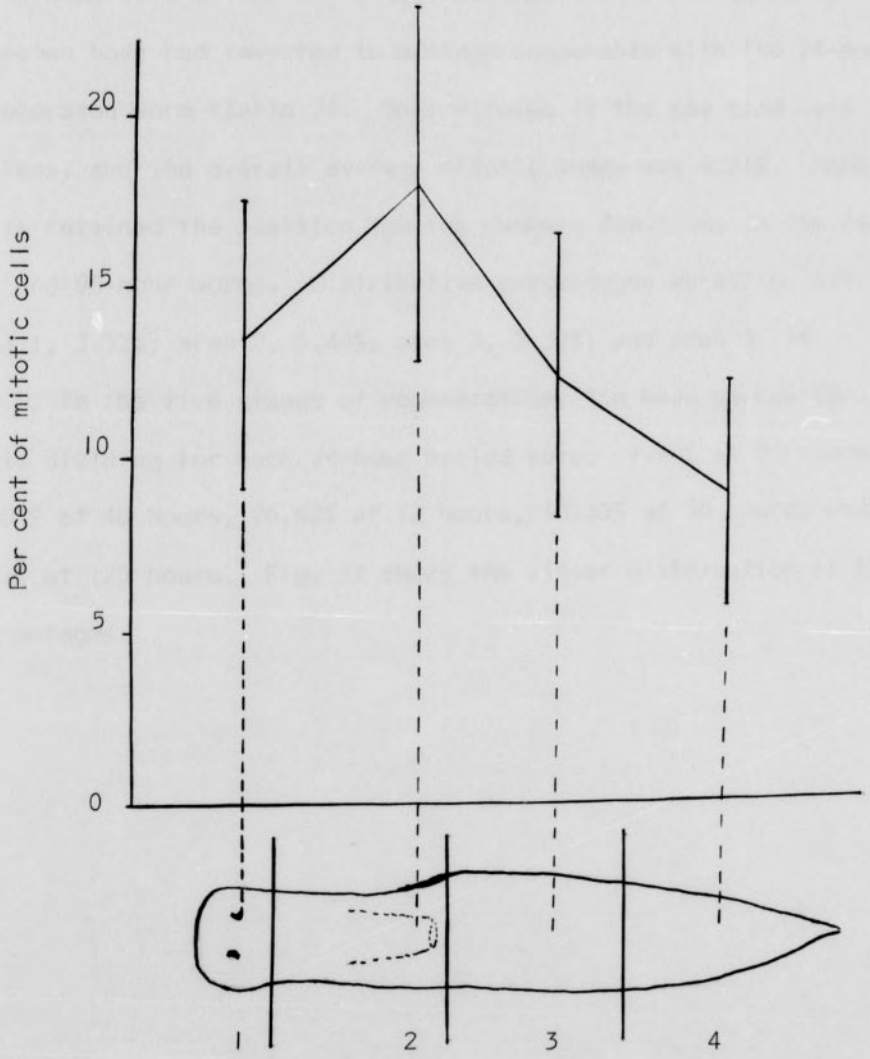


Fig. 10. Distributive percentages of mitotic cells in various body regions of the 96-hour regenerated Dugesia tigrina.


Per cent of mitotic cells
20
15
10
5
0
1
2
3
4

5. The One-hundred Twenty Hour Regenerated Worm

At the end of five days the regenerate had reached the stage of development where new growth had assumed the architecture of the adult head with all of its constituent parts. Histologically the dugesian body had reverted to a stage comparable with the 24-hour regenerated worm (Table 7). Cell mitoses in the new head were 3.52% or less, and the overall average mitotic index was 4.31%. Neoblastic cells retained the position and the numbers described in the 24, 48, 72, and 96-hour worms. Distributive percentages were(Fig. 11): area 1, 3.52%; area 2, 6.46%; area 3, 3.57%; and area 4, 3%.

In the five stages of regeneration, the mean percentages of cells dividing for each 24-hour period were: 7.48% at 24 hours, 18.04% at 48 hours, 26.62% at 72 hours, 13.33% at 96 hours, and 4.31% at 120 hours. Fig. 12 shows the linear distribution of these percentages.

Table 7. Per cent of cells in mitosis in the 120-hour regenerated *Dugesia tigrina*.

	Cell Count	Percent in Mitosis	Area
Worm #1	100	1	Head
	100	6	
	100	10	
	100	5	
	100	7	
	100	8	Pharynx
	100	11	
	100	7	
	100	8	
Worm #2	100	3	Head
	100	5	
	100	4	
	100	3	
	100	6	Pharynx
	100	14	
	100	11	Behind Pharynx
	100	2	Tail
	100	2	
Worm #3	100	4	Head
	100	1	
	100	4	
	100	2	Pharynx
	100	4	
	100	8	
	100	1	Behind Pharynx
	100	5	
	100	8	Tail
Worm #4	100	0	Head
	100	1	
	100	4	
	100	3	
	100	2	Pharynx
	100	6	
	100	3	Behind Pharynx
	100	3	
	100	4	Tail
Worm #5	100	2	Head
	100	0	
	100	4	
	100	6	Pharynx
	100	2	
	100	0	Behind Pharynx
	100	2	
	100	0	Tail
	100	2	
Total	4,500	Average	4.31%

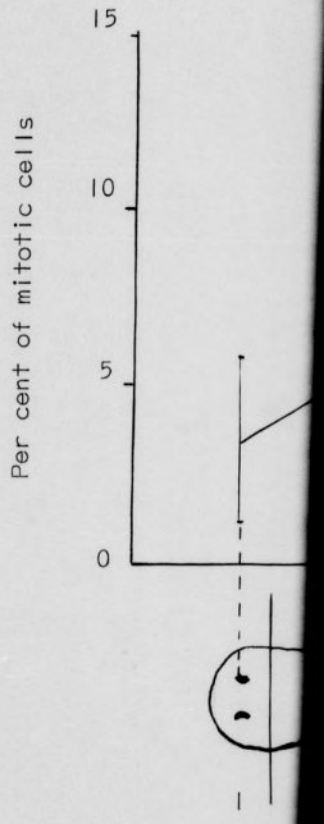
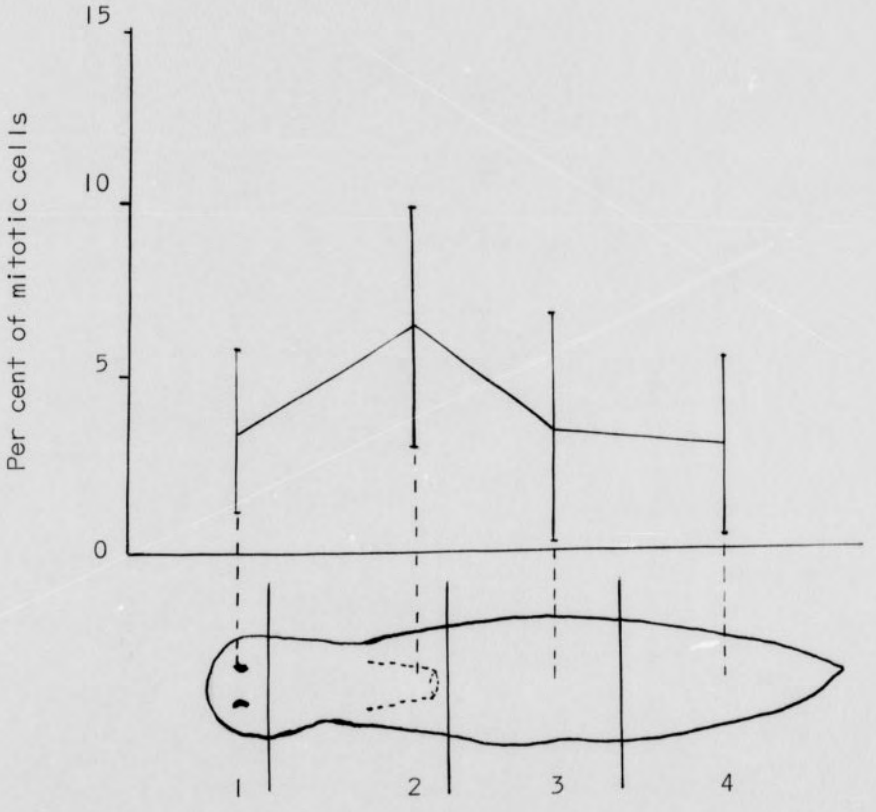


Fig. 11. Distributive percentages of mitotic cells in various body regions of the 120-hour regenerated Dugesia tigrina.


15
10
5
0
Per cent of mitotic cells
1
2
3
4

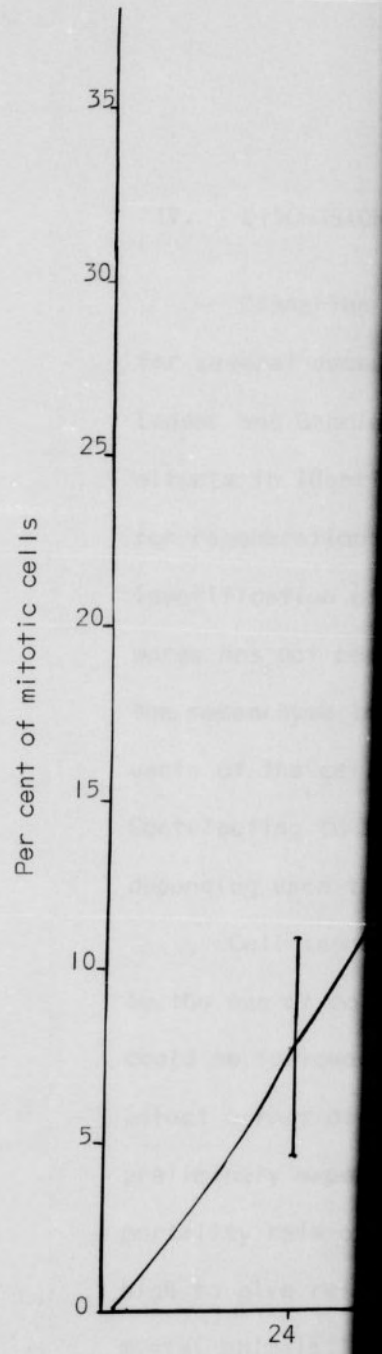
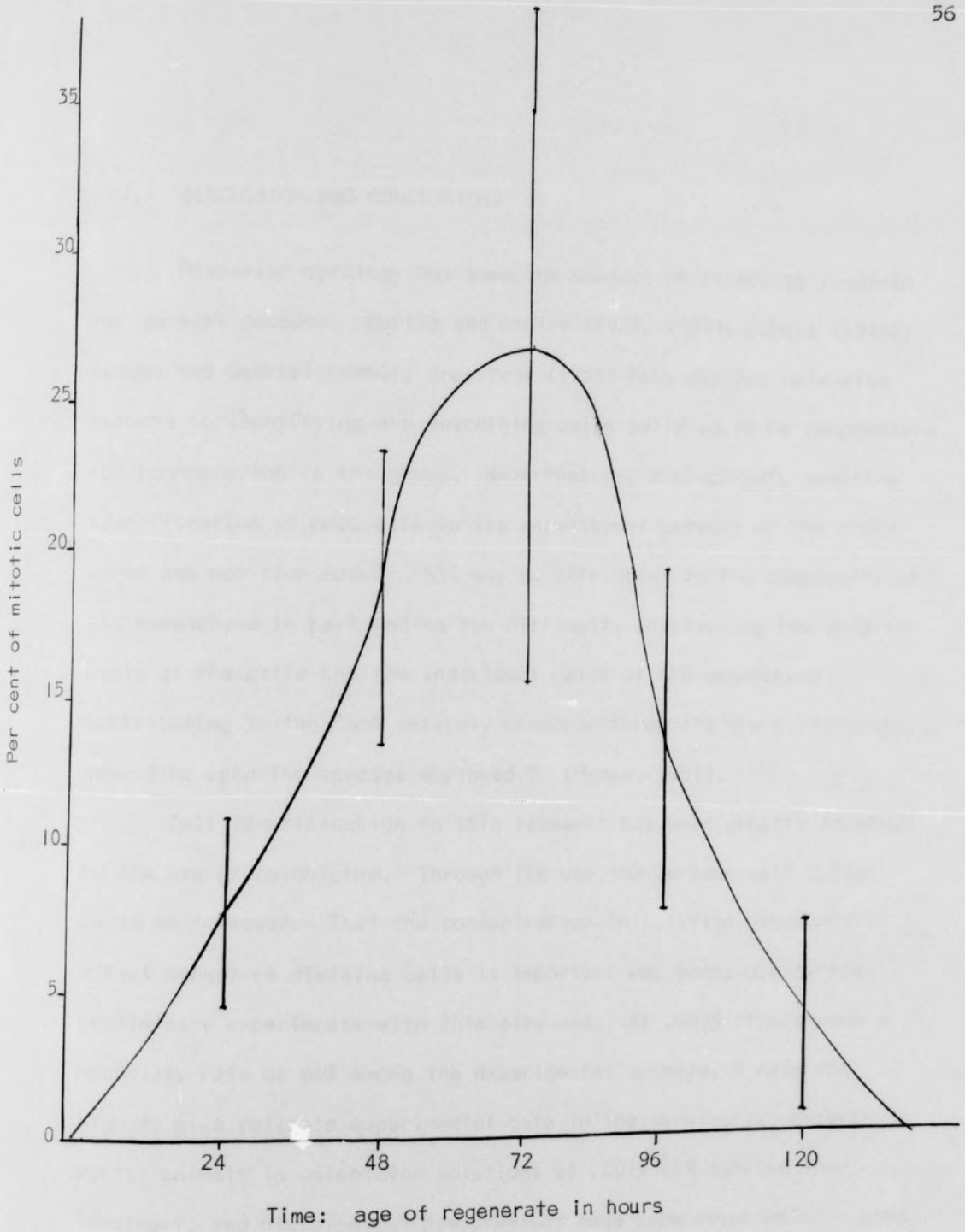


Fig. 12. Growth Curve of Dugesia tigrina.


35
30
25
20
15
10
5
0
Per cent of mitotic cells
24
48
72
96
120
Time: age of regenerate in hours

IV. DISCUSSION AND CONCLUSIONS

Planarian cytology has been the subject of intensive research for several decades. Curtis and Shulze (1924, 1934); Dubois (1949); Lender and Gabriel (1960); Brønstedt (1955) have devoted intensive efforts in identifying and describing cells believed to be responsible for regeneration in the group. Nevertheless, a clear-cut, positive identification of neoblasts in the mesenchymal network of the flatworms has not been made. This may be attributed to the complexity of the mesenchyme in part and to the difficulty in staining the constituents of the cells and the individual cells of the mesenchyme. Contributing to the "not entirely concordant results are differences... depending upon the species employed." (Hyman, 1951).

Cell identification in this research has been greatly enhanced by the use of colchicine. Through its use the various cell types could be followed. That the concentration in solution necessary to affect arrest of dividing cells is important was borne out by the preliminary experiments with this alkaloid. At .002% it produced a mortality rate of 60% among the experimental animals, a rate too high to give reliable experimental data in the survivors. Experimental animals in colchicine solutions of .001% did survive the treatment, and histological preparations made from these animals gave good evidence of cellular activity in the regenerating worms. However,

exposure time was as critical as the concentration. The supernumerary heads produced by 30 hours of exposure emphasized this point, albeit excrescent growths are not uncommon in Dugesia. This aspect of growth and development has been noted by Goldsmith (1940). The probable explanation of the above anomaly is that a metabolic pathway inhibited temporarily by the colchicine is reopened when the effect of the inhibiting agent becomes abated and the cells again resume their normal activity. Child (1941) noted that heads do not regenerate easily in areas near other heads because of the dominance and repression exerted by the first head. Colchicine probably destroys this apical dominance temporarily.

Neoblasts, one of the component cells of the parenchyme, are large cells with scant cytoplasm and macronuclei; however, this description differs from that of Lange and Gilbert (1968). These cells have been delegated as the builders in reconstruction of amputated parts. Much has been made of the migratory powers of these cells (Dubois, 1949). The data are based on irradiated transplants in which the neoblasts within the graft had been destroyed by X-ray and the host neoblasts migrated through the implant to establish the blastema. Dubois (1961) has also used cell orientation as evidence of their migratory ability. In her study of cephalic regeneration in Dendrocoel lacteum, she described neoblasts as elongated cells and that the plane of elongation appeared in the direction of their migration.

The completed histological study in this paper did not confirm these results. It was true that some neoblasts and also some cells of the fixed parenchyma appeared ovoid, but the plane of elongation was not only in the anterior direction, but some of both kinds of the elongated cells appeared oriented transversely to the long body axis and others in a dorso-ventral plane.

The difficulty in citing cell orientation as evidence of migratory power in neoblasts lies in the fact that tissues sectioned at five microns show neoblasts as part cells, not complete cells. Directional orientation on this basis would be at least questionable.

Betchaku (1967) has characterized the movement of neoblasts in vitro as amoeboid; however, collision with another neoblast produced an aggregate of these cells and then all movement stopped. Thus their manner of mobility in tissue culture excludes them from making extensive migrations in an animal in toto.

In the present work, in the intact parenchyma of a saggital section running the full length of the body, neoblasts were found most abundantly in the pharyngeal body region; others, but a few only, were scattered through the mesenchyme. It was this distribution observed in this study that placed their active migration in serious doubt. Secondly, in 30 sectioned worms neoblast cells were never found in the regeneration bud and only an occasional neoblast was found in the area immediately behind the reconstruction site. Thus evidence is presented here which indicates that neoblasts are not the cells that regenerate lost parts.

The fixed parenchyma cells or cells morphologically like fixed parenchyma cells were found dividing within the reconstituting organ, in the surrounding tissue, and throughout the general body area within the mesenchyme. Their presence within the regenerating parts and the total lack of neoblasts in the newly forming organs make these cells more than suspect as the source of material in regeneration. Other data accumulated in this work further substantiate the postulation that fixed parenchyma are the regenerative cells. Neoblasts in Dugesia have gigantic nuclei of 9 to 11 microns in diameter. When they undergo mitotic division it would be expected that they would produce two daughter cells with nuclei or 4.5 to 5.5 microns in diameter. However, cells scanned in the blastema had mean diameters of three to four microns, corresponding to the diameters of the cells of fixed parenchyma. This would preclude these cells as progeny of neoblasts.

It may be that neoblasts are the agents that in some way yet unknown trigger the regenerative process, either through hormonal control or enzymatic action, causing the fixed parenchyma cells to divide and become the basic cells of regeneration. Rather than actively migrating to the wound area to exert this influence, the neoblasts are probably brought into proximity of the injury by the contraction of the subepidermal muscles or they may be propelled to the area by the streaming epidermal cells which blanket the wound.

This study has further shown that cell division rate is a function of ability to regenerate. The results of the quantitative cell count in the reconstituting Dugesia tigrina at 24-hour intervals corroborates this statement. The animals used in the experiments were found to regenerate in five days (average), a period somewhat shorter than reported by other researchers. Twenty-four hours after decapitation the mean mitotic rate for the entire body was 7.48% based on a serial cell count in units of 100, nine units per worm. From this relatively low percentage, assuming that cell division is a normal phase in all animals, the rate of cell division increased rapidly up to 72 hours and peaked. From 72 hours to 120 hours, when regeneration was completed, mitosis declined. The decline percentages at parallel stages in time vary only 5% and 3%. Thus the growth rate of the regenerate correlated with time assumes a bell-shaped curve. On the left of the curve mitotic cells are directly proportional to growth, and on the right side of the curve mitosis is inversely proportional to growth.

Mitosis and regeneration are also a function of area. In all of the stages of regeneration, the pharyngeal area exhibited the highest mitotic rate. Averages for each of the areas, including the pharynx, were as follows:

Head	12.35%
Pharynx	17.94%
Behind pharynx	12.16%
Tail	11.74%

The percentages of cells in division in the various areas, head, pharynx, post-pharynx, and tail, are too inconclusive to pinpoint any area as the major contributor of fixed parenchyma in regeneration. However, the percentages appear to indicate a trend toward the pharyngeal area. It seems logical that a greater sampling may provide deviations between the areas that would be conclusive. This is an area in which further research should be pursued.

As a final conclusion, it should be noted that in the regenerative process, it is the fixed parenchyma that possesses a characteristic heretofore attributed to neoblasts. As part of the mesenchyme, overtly these cells appear not to be highly differentiated, and in this respect dedifferentiation for morphogenetic processes would be uncomplicated. Maintaining this potentiality, the fixed cells become completely totipotent.

V. SUMMARY

This study was undertaken to determine the source of cells used to regenerate lost body parts in the flatworm Dugesia tigrina. In the study the various cell types of the dugesian body were identified and the cells in mitosis were counted in units of 100 cells, nine units per worm, 4,500 cells per each stage of regeneration.

Mitosis was found to occur largely in the cells of the fixed parenchyma. The mitotic rate increased steadily from 24 hours to 72 hours after amputation and began declining from 72 hours to 120 hours when regeneration was completed.

Fixed parenchyma cells or cells morphologically like fixed parenchyma cells were found dividing in the regeneration blastema, in the adjoining areas, and throughout the body with a declining rate from the anterior to the posterior end.

Large neoblasts, "free" cells of the parenchyma, were also found in the mesenchyme, particularly in the pharyngeal area, but only occasionally one was found dividing. They were never found in the regeneration bud nor in the area immediately behind it.

From these results it can be concluded that fixed parenchyma rather than neoblasts are the source of cells for regeneration blastemas and for the eventual reconstruction of lost body parts.

VI. LITERATURE CITED

Betchaku, Teiichi. 1967. Isolation of planarian neoblasts and their behavior *in vitro* with some aspects of the mechanism of the formation of regeneration blastema. J. Exp. Zool. 164:407-434.

Brønstedt, H. V. 1955. Planarian regeneration. Biol. Rev. 30:65-126.

Child, Charles Manning. 1941. Patterns and problems of development. The University of Chicago Press. Chicago. 811 p.

Curtis, W. C. and L. M. Schulze. 1924. Studies upon regeneration. Formative cells of planarians. Anat. Rec. 29:105.

Curtis, W. C. and L. M. Schulze, 1934. Studies upon regeneration. I. The contrasting powers of regeneration in planaria *protocolyta*. J. Morph. 55:477-513.

Dubois, Françoise. 1949. Contribution à l'etude de la migration des cellules de régénération chez les planaries. Bull. Biol. Fr. 83:213-283.

Dubois, Françoise. 1961. Les cellules de régénération chez la planaire *Dendrocoelum lacteum*. Bull. Soc. Zool. Fr. 86:172-185.

Flickinger, R. A. 1964. Isotopic evidence for a local origin of blastema cells in regenerating planaria. J. Exp. Cell. Res. 34:403-406.

Hyman, L. H. 1951. The invertebrates platyhelminthes and rhynchocoela. McGraw Hill Co., New York. Vol. II.

Goldsmith, E. D. 1940. Regenerative and accessory growth in planarians. I. Initiation of the development of regenerative and accessory growths. Phys. Zool. 13:43-55.

Lange, C. S. and C. W. Gilbert. 1968. Studies on the cellular basis of radiation lethality. III. Measurement of stem-cell repopulation probability. J. Radiation Biol. 14:373-388.

Lehman, H. E. 1965. A cytological "screening" method for demonstrating general molecular groups in single tissue sections. I. Polychrome stain. J. Elisha Mitchell Sci. Soc. 81:89-90.

Lender, Th. and A. Gabriel. 1960. Sur la repartition des neoblastes de Dugesia lugubris (Turbellarie, Triclade) avant et pendant la régénération. C. H. Acad. Sci. 250:2465-2467.

Lender, Th. and A. Gabriel. 1961. Le comportment des neoblastes pendant la régénération de la planaire Dugesia lugubris (Turbellaire, Triclade). Bull. Soc. Zool. Fr. 86:67-73.

Woodruff, Lorraine S. and Allison L. Burnett. 1965. The origin of the blastema cells in Dugesia tigrina. Exp. Cell Res. 38:295-305.

SIKES, ELLEN M. A Fo
of Campbell College,
Through 1967 Directed
(1969) Directed by:

Statement of the Pro

The problem o
curriculum and to de
in the Business Depa
North Carolina.

Methods of Procedure

The data gath
use of a questionnai
to 441 business grad
398 completed questi
response of 91.7 per

Data

As a result o
ployment the majorit
teaching, and accoun
the graduates are no
ment, and 44.4 per c
needed in the future
accounting, economic
courses. Areas reco