



Theses and Dissertations

1970-08-03

Asexual reproduction in planaria characteristics of the inhibitor(s)

Linda Gail Sheffield

Brigham Young University - Provo

Follow this and additional works at: <https://scholarsarchive.byu.edu/etd>

BYU ScholarsArchive Citation

Sheffield, Linda Gail, "Asexual reproduction in planaria characteristics of the inhibitor(s)" (1970). *Theses and Dissertations*. 7869.

<https://scholarsarchive.byu.edu/etd/7869>

This Thesis is brought to you for free and open access by BYU ScholarsArchive. It has been accepted for inclusion in Theses and Dissertations by an authorized administrator of BYU ScholarsArchive. For more information, please contact ellen_amatangelo@byu.edu.

ASEXUAL REPRODUCTION IN PLANARIA
CHARACTERISTICS OF THE
INHIBITOR(S)

A Thesis

Presented to the
Department of Zoology
Brigham Young University

In Partial Fulfilment
of the Requirements for the Degree
Master of Science

by

Linda G. Sheffield

August 1970

This thesis by Linda G. Sheffield is accepted in its present form by the Department of Zoology of Brigham Young University as satisfying the thesis requirement for the degree of Master of Science.

3 August 1970
Date

ACKNOWLEDGEMENTS

Grateful acknowledgment is made for the valuable suggestions and help given by the chairman of my advisory committee, Dr. A. Lester Allen and other members of my committee, Dr. Fred G. White, and Dr. James L. Farmer.

Financial support, laboratory space, equipment, supplies, and the experimental animals were supplied by the Department of Zoology, Brigham Young University.

TABLE OF CONTENTS

	Page
ACKNOWLEDGMENTS	iii
LIST OF TABLES	iv
LIST OF ILLUSTRATIONS	vii
INTRODUCTION AND LITERATURE REVIEW	1
MATERIALS AND METHODS	4
Care and Maintance of Stock Animals	4
Preparation of Homogenized Tissue	5
Head Homogenate Extract	
Whole Body Extract	
Preparation of Heated Extracts	6
Ammonium Sulfate Precipitation Tests	6
Preparation of Sephadex G-200 Column	7
Preparation of Diethylaminoethyl Cellulose (DEAE) Column.	9
Test Conditions	10
RESULTS	12
Normal Fissioning Pattern of Decapitated Planaria	12
Quantitative Differences in the Effects of Head Extract and Whole Body Extract	12
Effects of Adding Whole Body Extract at Different Times after Decapitation	20
Heat Treatment of Whole Body Extract	22
Ammonium Sulfate Precipitation Tests	29
Whole Body Extracts on Sephadex G-200 Column	31
Whole Body Extracts on DEAE Cellulose Anion Exchange	43
DISCUSSION	47

	Page
CONCLUSIONS AND SUMMARY	54
LITERATURE CITED	56

LIST OF TABLES

Table	Page
1. Comparison of quantitative differences between head and whole body extracts; May 5, 1970; one-hundred animals in each group	19
2. Protein loss of whole body extract after heating 15 minutes in water bath at each temperature; February 23, 1970; forty animals in each group	25

LIST OF FIGURES

Figure	Page
1. Normal fissioning pattern of one-hundred decapitated planaria reared in spring water; May 5, 1970	13
2. Normal fissioning pattern of one-hundred decapitated planaria reared in spring water; May 6, 1970	14
3. Comparison of quantitative differences in inhibitory activity of head and whole body extracts; May 6, 1970; one-hundred animals in each group	16
4. Comparison of quantitative differences in inhibitory activity of head and whole body extract; May 6, 1970; one-hundred animals in each group	18
5. Effect of adding whole body extract at different times after decapitation; June 20, 1969; twenty animals in each group	21
6. Heat inactivation of whole body extract after heating 15 minutes in water bath at each temperature; February 23, 1970; forty animals in each group	24
7. Heat inactivation of whole body extract; 20, 40, 60 and 120 minutes in water bath at 60° C.; April 15, 1970; number in () represents number of animals in each group.	27
8. Optical density of whole body extract before and after heat treatment at 60° C.; April 15, 1970	28
9. Effect of ammonium sulfate precipitation upon inhibitory activity of whole body extract; March 24, 1970; forty animals in each group	30
10. Elution curve of a whole body extract; Sephadex G-200 column (2.5 x 100 cm); 0.15 M NaCl; 5° C.; November 7, 1969	32

Figure	Page
11. Inhibitory activity of elution peak material; November 7, 1969; sixty animals in each control group; twenty animals in each experimental group.	34
12. Inhibitory activity of elution peak material; Peak I; November 7, 1969; sixty animals in control group; twenty animals in each experimental group	36
13. Inhibitory activity of elution peak material; Peak II; November 7, 1969; sixty animals in control group; twenty animals in each experimental group	38
14. Inhibitory activity of elution peak material; Peak III; November 7, 1969; sixty animals in control group; twenty animals in each experimental group	40
15. Apparent stimulatory activity of Peak I material concentrated with amicon pressure dialysis membrane (UM05) October 15, 1969; forty animals in control group; twenty animals in each experimental group	43
16. Elution curve of whole body extract; DEAE polycel anion exchange; 5° C. ; gradient reservoirs: 0.005M NaH ₂ PO ₄ and 0.1M NaH ₂ PO ₄ , 0.5M NaCl; February 3, 1970	44
17. Inhibitory activity of elution peak material from DEAE anion exchange column; February 7, 1970; one-hundred animals in each group	46

INTRODUCTION AND LITERATURE REVIEW

Fission, or the formation of a new individual by splitting off part of the maternal organism, is the only mode of asexual reproduction in planaria. Many investigators (Child, 1910, 1911, 1913, 1924, 1932; Vandel, 1921; Bingham, 1968; Kendall and Nachtwey, 1968; and Leavitt, 1969) have noted that fission is induced in asexual strains of planaria following decapitation. In many cases decapitation induces fission in animals which will not undergo fission in nature due to their small size. Observations by Wulzen (1916) indicated that the rate of fission in planarian worms was increased by a diet of pituitary extract and was inhibited by brain extract. Later work by Blumberg (1940) showed that there was an accelerated regeneration of posterior and anterior portions of Dugesia dorocephala grown in a beef pituitary medium.

Within the last two decades numerous experiments have shown that powerful inhibitors of regeneration are found in the body of the planarian. Experiments by Lender (1952, 1955, 1956, 1960) and Bingham (1968) showed that when decapitated planaria were reared in media containing head homogenate the regeneration of the brain was inhibited. Observations by Sengel (1959), Stephan (1967), and Ziller-Sengel (1967a, 1967b, 1967c) indicated that regeneration

of the pharynx was inhibited by a pharyngeal zone extract.

Asexual reproduction and regeneration are closely related, for asexual reproduction inevitably involves extensive regenerative processes. Within the animal kingdom the correlation between the two (regeneration and asexual reproduction) is so good that Caullery (see Needham, 1952) suggested that asexual reproduction is an evolutionary development from regeneration.

T. H. Morgan (1901) defined regeneration as both the replacement of lost parts as well as the new development of an organism from a piece of an adult or embryo. Regeneration in its most remarkable form has been recognized in fresh-water planaria for two centuries. As early as 1814 Dalyell (see Bronsted, 1955) wrote that planaria seemed to be "immortal under the edge of the knife." Other early workers such as Pallas, Shaw, Draparnaud, Johnson, Duges, Faraday, Darwin, Harvey, and Zacharias (see Bronsted, 1955) also observed this phenomenon in planaria.

Modern investigations in the field of planarian regeneration began with Morgan (1901) and Child (1910, 1911, 1913, 1924, 1932). Morgan was one of the first to put forth the idea that the new material which appears during the first stages of regeneration is totipotent. Child (1910) was the first to study regeneration on a physiological basis. He found that morphological differentiation could be ascribed to a distribution of quantitative differences in metabolism which he called a gradient. In more recent years the regeneration process

has been studied by Dubois (1948), Kolmayer and Dubois (1960), Wolff (1961), Flickinger and Coward (1964), Woodruff and Burnett (1965), Chandlbois (1965), Kohl and Flickinger (1966), Betchaku (1967), Bronsted (1968), and Hay (1968).

While exploring the observations of Wulzen (1916) and Bingham (1968), Leavitt (1969) found that a head homogenate extract of Dugesia dorotocephala inhibited fission in decapitated asexual strains of the same species. This inhibitory substance was found to be more concentrated in the cephalic region than in other areas of the body, and the optimum concentration for inhibition was shown to be equivalent to two heads per ml of culture medium. Leavitt's results also indicated that the inhibiting substance is not found in sexual strains of planaria.

On the basis of this known information I wanted to further characterize this postulated inhibitory substance through chromatography with Sephadex gel and DEAE cellulose columns, ammonium sulfate precipitation tests and heat inactivation tests. In addition I wanted to perform more extended investigations to determine if there is a quantitative difference between the inhibitory substance of a head homogenate extract and that of a whole body extract.

MATERIALS AND METHODS

Care and Maintenance of Stock Animals

All animals used in these experiments were of the species Dugesia dorotocephala as identified by Pennak (1953) and Hyman and Jones (1959) and were obtained from Dahl Biological Supply Company (Berkeley, California) under the species name of Dugesia tigrina. These planaria were easily grown in spring or river water as well as in a standard salt medium of $1 \times 10^{-3} \text{M}$ CaCl_2 , $1 \times 10^{-6} \text{M}$ KCl , $1 \times 10^{-4} \text{M}$ NaCl , and $1 \times 10^{-6} \text{M}$ disodium versenate (Henderson and Eakin, 1959). The stock animals were maintained in plastic containers filled with water from a spring found on the Brigham Young University grounds and were refrigerated at approximately 18°C .

The animals were fed and cleaned once a week. The containers were removed from the refrigerator and left at room temperature ($25\text{-}27^\circ \text{C}$.) during feeding and cleaning. One or two small pieces of fresh or frozen calf's liver was put into each container and left there for two to three hours. After feeding, the planaria would usually come to the water surface and at this time the food was taken out of the containers. Upon removal of the food the planaria and the containers were cleaned with tap water. The animals were then returned to spring water and put in the refrigerator at

18° C. Under these conditions the planaria remained healthy and reproduced slowly by fission.

Preparation of Homogenized Tissue

Head Homogenate Extract

Medium-sized planaria between 6 and 8 mm. were placed on a glass plate and, when extended, were decapitated with a stainless steel razor blade held in a hemostat. The cuts were made about 2 mm. posterior to the auricles according to the method outlined by Leavitt (1969).

After decapitation, the heads were placed in a Thomas Tissue Homogenizer in 5 ml. of sterile (autoclaved) spring water and homogenized with an electric motor at as slow a speed as possible. Frictional heat was kept at a minimum by chilling the homogenizer in an ice bath before and during the homogenization process.

The homogenized tissue was then placed in a Sorvall Superspeed RC-2 centrifuge at 0° C., allowed to stand for 20 minutes, and centrifuged for 20 minutes at 12,100 x g. After centrifugation the supernatant was carefully poured off and passed through a sterile 0.45 u filter. The precipitate was discarded. The extract was diluted to the appropriate concentrations with sterile spring water. All protein determinations were approximated with the Folin-Ciocalteu reagent (Lowry et al., 1951) and recorded as ug/ml.

Whole Body Extract

The whole body extract was prepared using the same procedure as that outlined for the head homogenate extract.

Preparation of Heated Extracts

The whole body homogenate extracts were prepared and filtered as previously described. The extracts were then placed in a water bath. Two experimental groups were set up. Group One extracts were heated at either 22° C., 40° C., or 60° C. for 15 minutes. Group Two extracts were heated at 60° C. for either 20, 40, 60 or 120 minutes. After heating, the extracts were centrifuged at 12,100 x g and 0° C. for 10 minutes. Protein determinations were made both before and after heat treatments by the Lowry method (Lowry et al., 1951) (Group One) and by optical density readings (Group Two) at 260 mu and 280 mu with a Beckman DB-G spectrophotometer. All protein determinations were made after the heated extracts had been centrifuged and cooled. The cooled extracts were then added to petri dishes containing decapitated planaria and refrigerated at 18° C. In both groups the number of planaria reproduced asexually was recorded each day.

Ammonium Sulfate Precipitation Tests

The whole body homogenate was prepared and the optical

density was measured as described above. Reagent grade ammonium sulfate was added to the homogenate as the solid salt according to the method outlined by Green and Hughes (1955). The extract was kept chilled in an ice bath and 5.63 gm. of ammonium sulfate was added to 18 ml of the extract to give 50% saturation. The ammonium sulfate was added slowly and with constant stirring.

After addition of the ammonium sulfate the mixture was allowed to set for 5 minutes. The extract was then centrifuged for 10 minutes at 12,100 x g and 0° C. The supernatant was poured off. Half of the supernatant was kept to run a test at 100% saturation and half was tested for inhibitory activity. The precipitate was saved and tested for inhibitory activity also.

The 100% saturation solution was prepared in the same manner as the 50% saturation solution; 3.92 gm. of ammonium sulfate was added to 10 ml of 50% saturated extract. Both the supernatant fraction and the precipitate were tested for inhibitory activity.

After dissolving the precipitates in sterile spring water, both the supernatant fractions and the precipitates were dialyzed against sterile spring water for 8 hours with frequent water changes. All fractions were diluted with sterile spring water to final volumes with the same optical density.

Preparation of Sephadex G-200 Column

The sephadex G-200 bed was prepared according to Technical

Data Sheet 6 (Pharmacia Chemicals, Inc., Uppsala, Sweden). The column used was the Sephadex column K25/100 with a diameter of 2.5 cm and a length of 100 cm. The column was mounted and checked for vertical alignment with a carpenter's level.

After packing, the column was equilibrated by allowing the eluant (0.15 M NaCl) to pass through the bed for 2 to 3 days. Homogeneity of the packing was tested by running Blue Dextran 2000 (Sephadex) through the column.

Sodium chloride was added to the whole body extract to give a final concentration of 0.15 M NaCl. The sample (3-5 ml) was carefully layered on the surface of the bed and allowed to settle in.

A mariotte reservoir was mounted at a 10 cm hydrostatic head and with a flow rate of 10-12 ml per hour. Eight milliliter aliquots were collected with an automatic fraction collector. The effluent fractions were examined at 260 m μ and 280 m μ with the spectrophotometer, and the curve was found to be divided into three peaks. The peak samples were collected and concentrated with a Diaflow Ultrafilter model 202 and Amicon pressure dialysis membrane UM10 (except for one peak-Fig. 14). After concentrating the peak fractions, they were dialyzed against sterile spring water for 24 hours. The fractions were then diluted to the appropriate concentrations and tested for inhibitory activity. All chromatography and subsequent operations were carried out in a cold room at 5°C.

To determine the approximate molecular weights of the

material in the peaks the Sephadex column was equilibrated according to the method outlined by Andrews (1966). The following marker proteins were used: (1) Blue Dextran 200 (Sephadex), molecular weight 500,000; (2) urease, molecular weight 484,000; (3) bovine serum albumin, molecular weight 69,000; (4) penicillinase, molecular weight 30,000 to 35,000; and (5) egg albumin lysozyme, molecular weight 17,000.

Preparation of Diethylaminoethyl Cellulose (DEAE) Column

The procedure for setting up the DEAE cellulose columns was adapted from the method outlined by Sober et al. (1955). The anion exchange cellulose DEAE polycel was used. After the DEAE cellulose had been adjusted to pH 7.0 by the addition of 0.005M NaH_2PO_4 , it was washed several times with the buffer. Nonsedimenting material (fines) was removed by decanting. The clear supernatant was always used for the final determination of pH. The DEAE was poured as a slurry into a glass column (30 x 1.5 cm) and packed under a pressure of 3 to 4 lbs. with nitrogen gas. The packed column was washed with several column volumes of 0.005M NaH_2PO_4 .

The whole body extract was dialyzed for 24 hours in 0.005M NaH_2PO_4 . After equilibration the sample was washed onto the column with several 1 ml portions of 0.005M NaH_2PO_4 . A solution

of 0.005M NaH_2PO_4 was then run through the column until the effluent coming off the column gave a constant reading at 280 mu. A Polystaltic pump (made by Buchler Instruments, Inc.) was used to pump the eluant from the reservoir to the column. The flow rate was adjusted to 2 ml per minute. The eluant was a linear gradient varying from 0.005M NaH_2PO_4 to 0.5M NaCl plus 0.1M NaH_2PO_4 .

Effluent fractions were collected in 6 ml aliquots and examined at both 260 mu and 280 mu. The material was divided into four peaks and dialyzed for 24 hours against sterile spring water. The water was changed approximately every 8 hours. All four peak fractions were tested for inhibitory activity.

Test Conditions

To determine if the planaria were most susceptible to the inhibitory activity immediately after decapitation three experimental conditions were used. In Experiment One the planaria were decapitated and immediately put into whole body extract diluted to a concentration of either 15 ug/ml or 34 ug/ml. In Experiment Two the planaria were decapitated and left in spring water. After 6 hours they were put into whole body extract diluted to a concentration of 14 ug/ml. In Experiment Three the decapitated planaria were left in spring water for 24 hours before being put into medium containing whole body extract diluted to a concentration of 21 ug/ml.

To test the quantitative differences in the effects of whole

body and head extracts four different concentrations of each were compared. The extracts were diluted with sterile spring water to either 100, 75, 50, or 25 ug/ml. Each concentration was tested for inhibitory activity.

The diluted extracts were put into plastic petri dishes, 15 ml. per dish, with ten decapitated planaria in each dish. In the spring water controls the decapitated planaria were grown in sterile spring water, and in the inhibitor controls the planaria were reared in diluted whole body extracts at a concentration of 100 ug/ml. This concentration was found to be optimal for inhibiting fission without killing the planaria. The experiments, which were carried out at 18°C, ran from 12 days to two weeks. By this time the rate of fission in the spring water controls had begun to plateau to a constant value. The animals were observed daily and the number of animals undergoing fission was recorded. All of the results were designated by the beginning date of the experiment.

RESULTS

Normal Fissioning Pattern of Decapitated Planaria

The data from typical spring water controls showing the normal fissioning pattern of decapitated planaria is represented in Figures 1 and 2. Decapitation is usually followed by a three to four day lag. After the lag period the fission rate increases until the sixth or seventh day when the peak (13 animals in this experiment) is reached. From days eight to twelve the fission rate decreases to zero.

Quantitative Differences in the Effects of Head Extract and Whole Body Extract

A comparison of the effects of head and whole body extracts is represented in Figures 3 and 4 and Table 1. At the end of a twelve-day period 96% of the spring water controls have undergone fission. At a concentration of 25 ug/ml 82% of the animals in both head extract and whole body extract have reproduced. With protein concentrations of 50 ug/ml and 75 ug/ml 58% of the planaria reared in head extract reproduced and 56% of the planaria reared in whole body extract reproduced. At a concentration of 100 ug/ml the head extract inhibited fission completely (0% reproduction) and the whole body extract allowed only 3% reproduction.

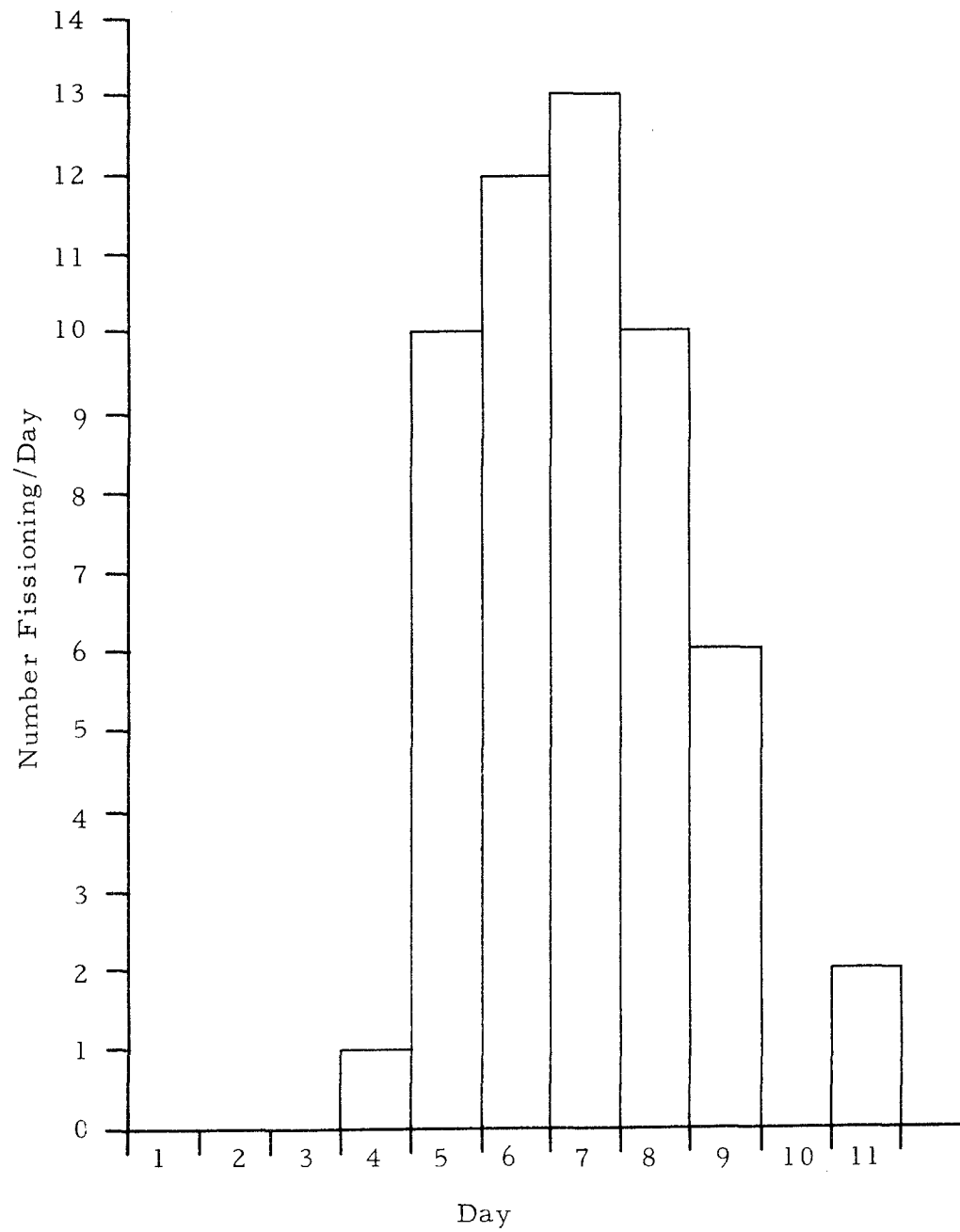


Fig. 1. Normal fissioning pattern of one-hundred decapitated planaria reared in spring water; May 5, 1970.

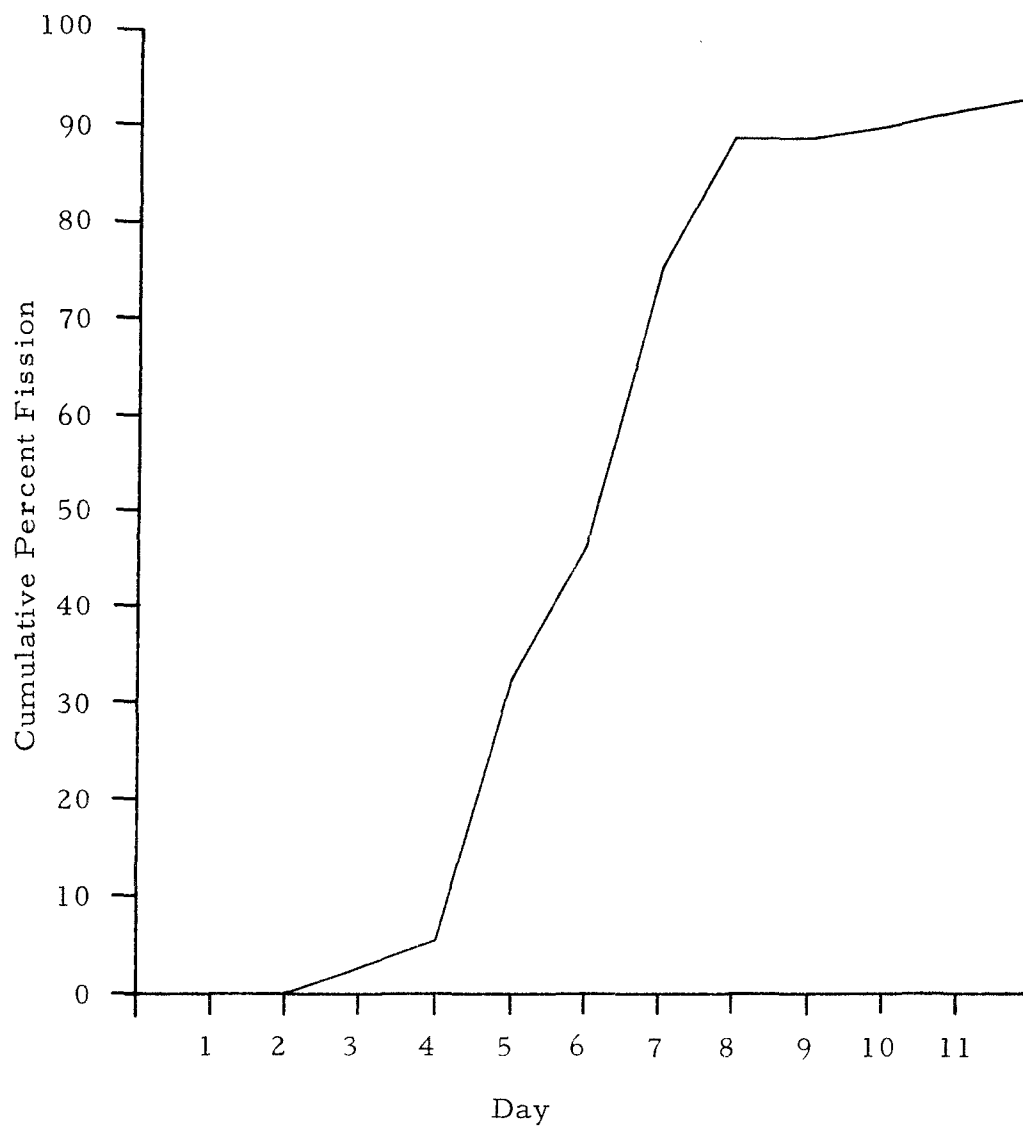


Fig. 2. Normal fissioning pattern of one-hundred decapitated planaria reared in spring water; May 6, 1970.

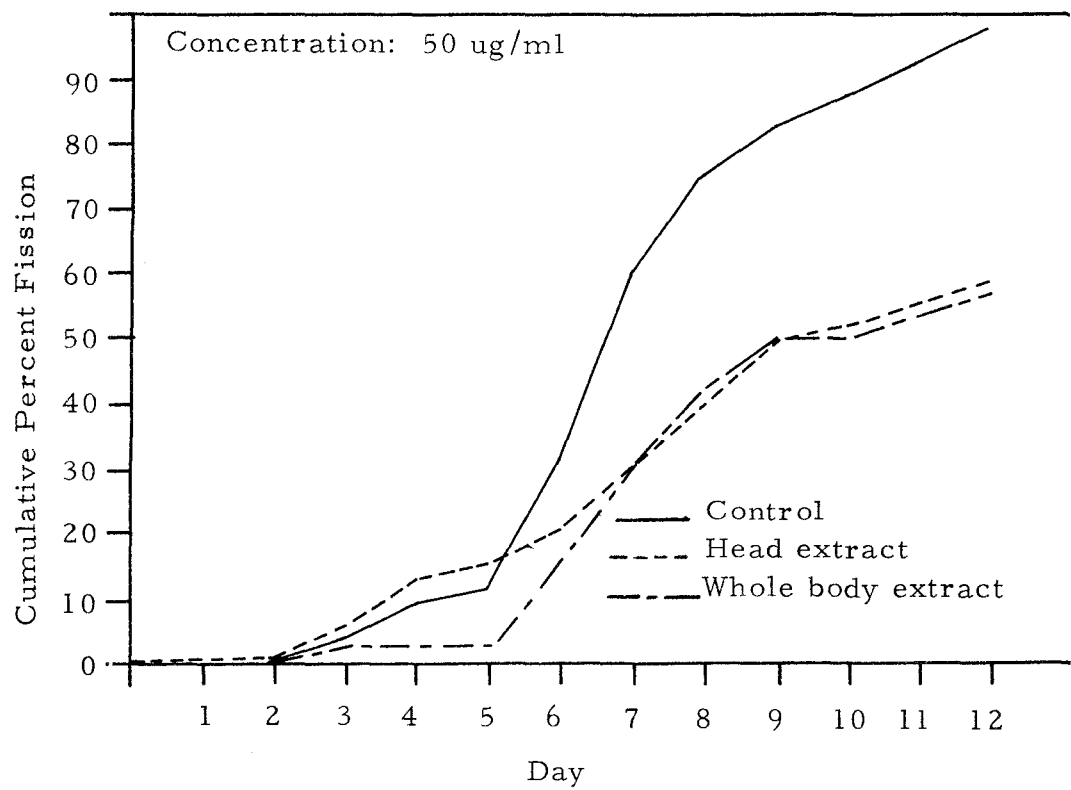
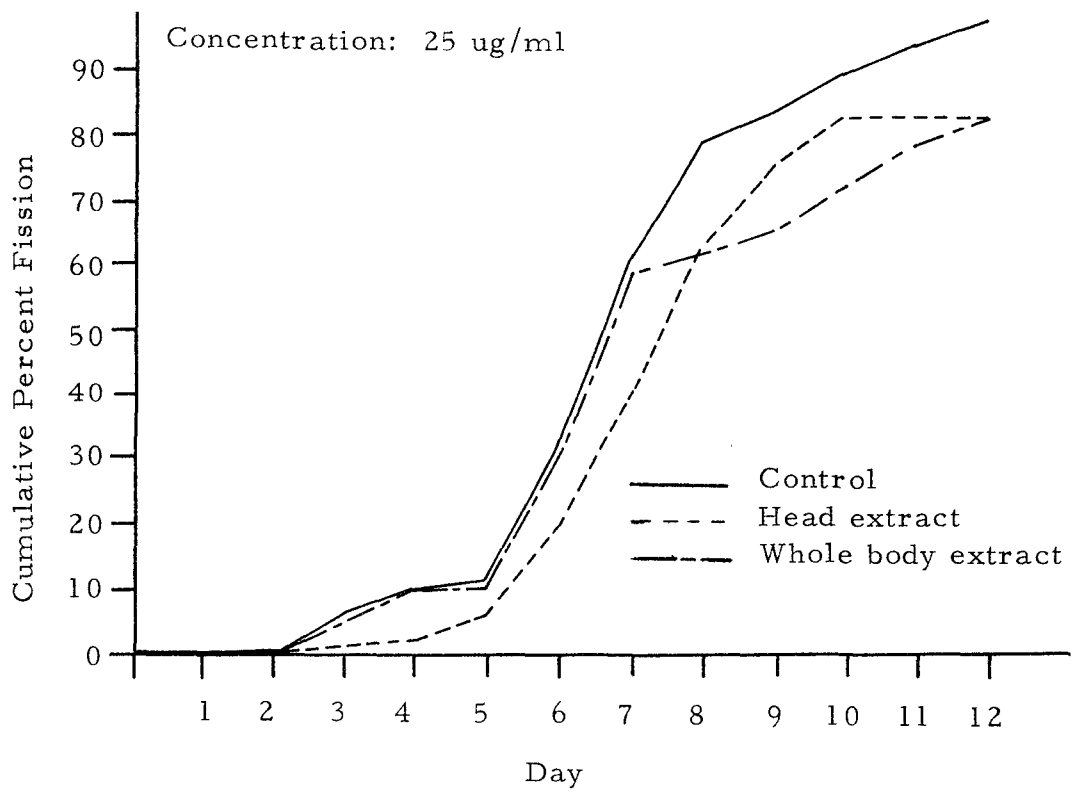


Fig. 3

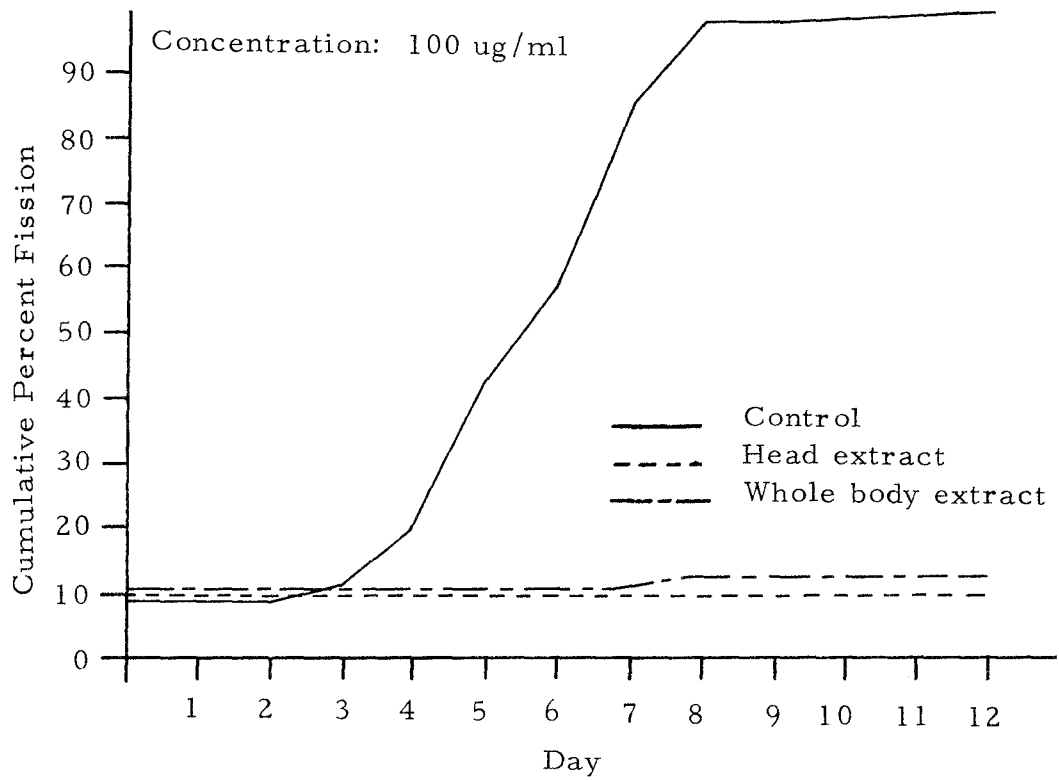
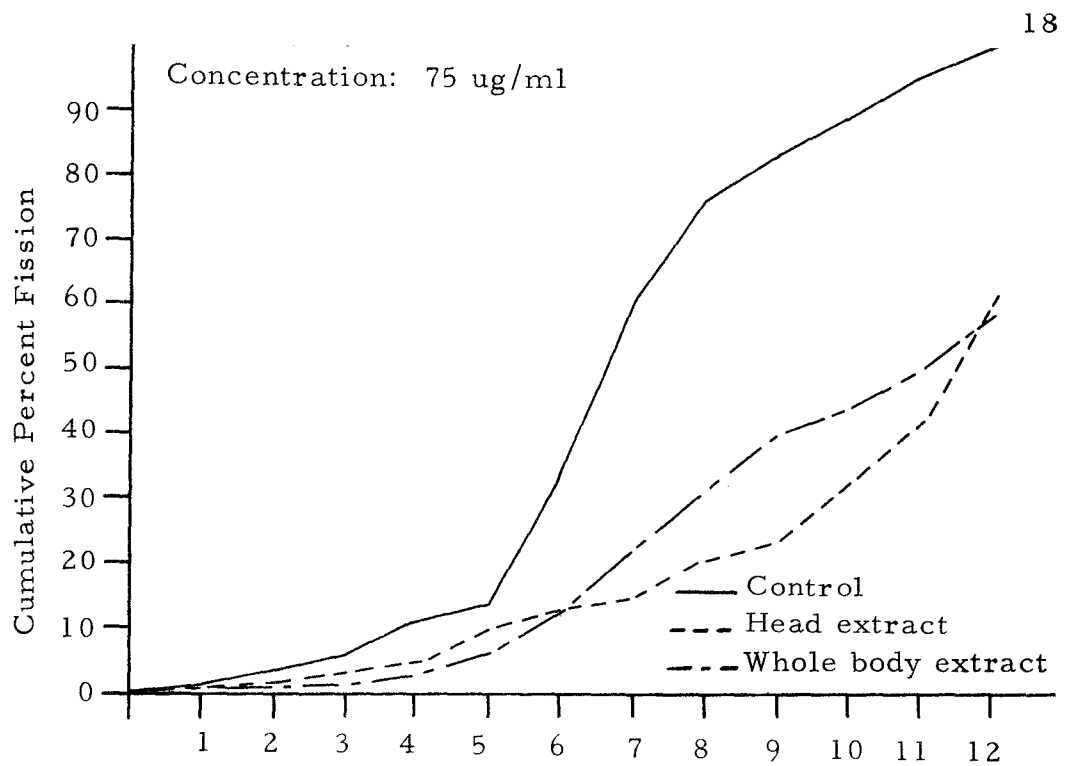


Fig. 4

Table 1. Comparison of quantitative differences between head and whole body extracts; May 5, 1970; one-hundred animals in each group.

Head Extract		Whole Body		Control
Protein conc. (ug/ml)	% Fission*	Protein conc. (ug/ml)	% Fission*	% Fission*
25	82	25	82	96
50	58	50	56	96
75	58	75	56	96
100	0	100	3	96

* Percent fission is defined as the number reproducing within 12 days.

The differences in inhibitory activity of head and whole body extracts did not vary more than 3% at the maximum in any group. Furthermore, it appears that the minimum concentration for maximum inhibition was a concentration of 100 ug/ml. of either a head extract or a whole body extract. Inhibitory activity showed a positive correlation with the concentration of the head and whole body extracts. To assure consistency in experimental procedures whole body extract at 100 ug/ml. was always used as the inhibitor control.

Effects of Adding Whole Body Extract at Different Times after Decapitation

Figure 5 shows the results of adding whole body extract immediately after decapitation, 6 hours after decapitation, and 24 hours after decapitation. By the termination of the experiment on day 9 the groups in which the extract had been added immediately had reproduced 60% (15 ug/ml.) and 30% (34 ug/ml.) while 90% of the control group had undergone fission. When the extract was added 6 hours (experiment two) after decapitation 70% of the animals reproduced (14 ug/ml.), and 80% reproduced if the decapitated planaria were allowed to grow in sump water 24 hours (experiment three) before adding the extract (21 ug/ml.).

Within 5 days the group in which the extract was added 24 hours after decapitation had reached its peak of 80% fission and

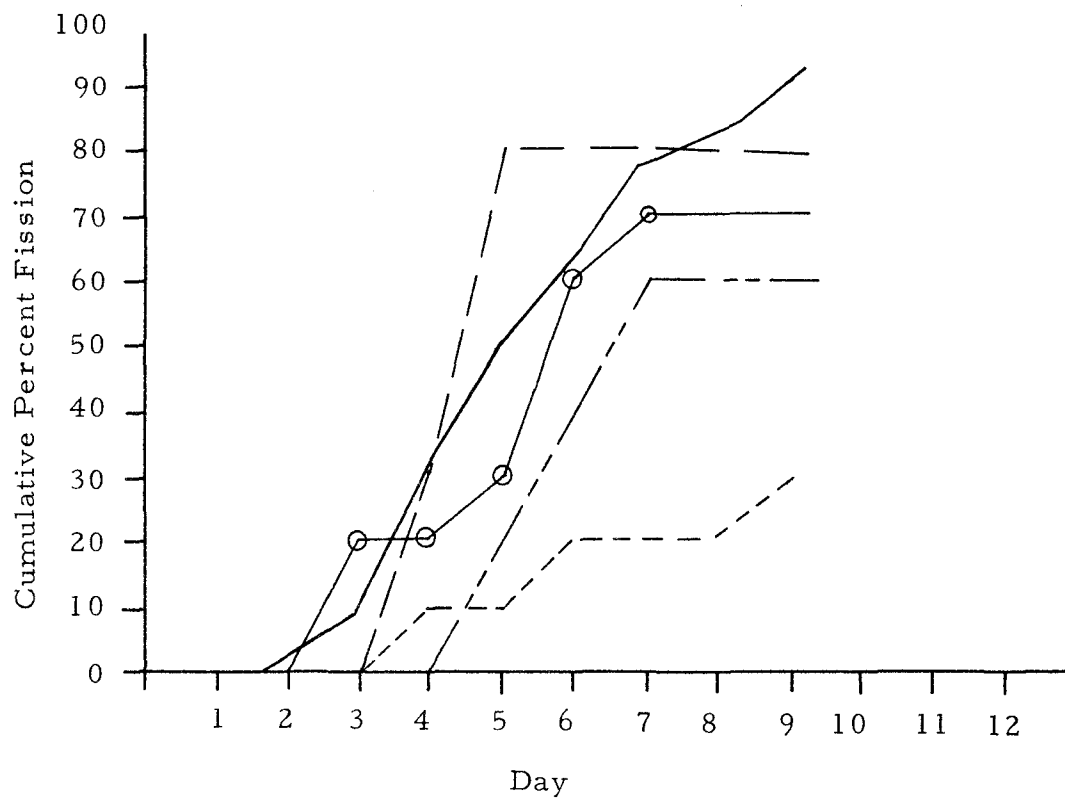


Fig. 5. Effect of adding whole body extract at different times after decapitation; June 20, 1969; twenty animals in each group.

————— Control
 - - - - - Immediately (15 ug/ml)
 - - - - - Immediately (34 ug/ml)
 ○ — ○ — ○ 6 hours (14 ug/ml)
 - - - - - 24 hours (21 ug/ml)

leveled off to a plateau for the remainder of the experiment. Both the 0 time group and the 6 hour group reached peak fission on day 7, two days later than the 24 hour group.

All of the experimental groups except the 6 hour group showed an increase in the lag period preceding the onset of fission. In the 24 hour group the lag was 3 days as compared to 2 days for the controls. The 0 time groups had a 4-day lag. A 6-hour delay in adding the extract caused no increase in the lag period. On day 3 the 6-hour group had a 10% increase in fission over the controls, 20% compared to 10%. Beginning with the fourth day the 6-hour group fell behind the controls and remained behind throughout the experiment.

Heat Treatment of Whole Body Extract

Two groups of experiments were run to test the effects of heat treatment upon the inhibitory activity of whole body extracts. In Group One the extract was heated in a water bath for 15 minutes at 22° C., 40° C. or 60° C. The results are presented in Figure 6 and Table 2. The points plotted represent the number of planaria which had undergone fission at the peak day of each respective group. This "peak day" is the day when the largest number of fission occurred and is represented as the apex of the bar graph (see Fig. 1). The spring water controls had a total of 15 (35%) animals undergoing fission on the peak day. In the 22° group only one planarian (2.5%) had fissioned; in the 40° C. group, two planaria (5%) fissioned; and

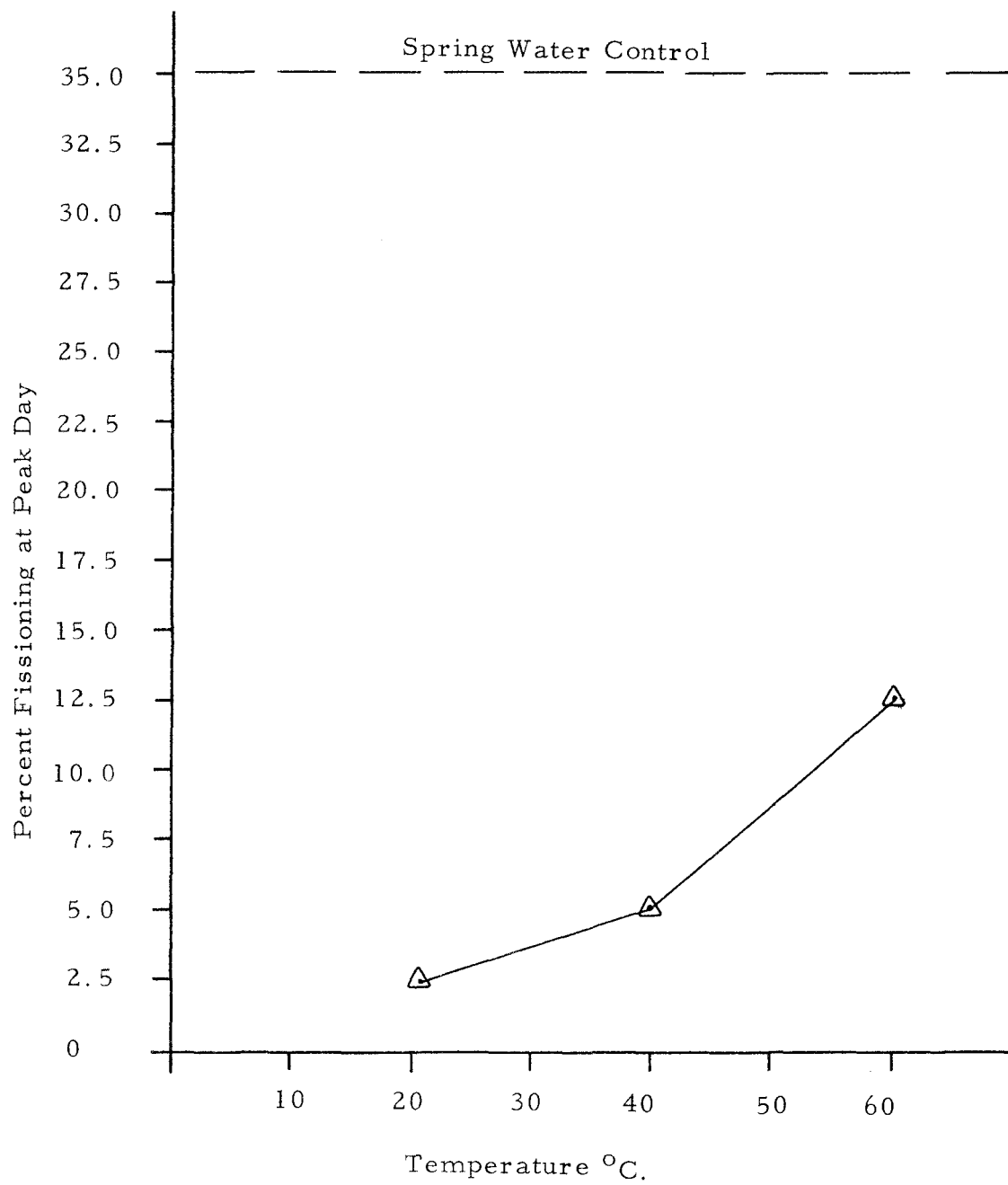


Fig. 6

in the 60° C. group, five planaria (12.5%) had fissioned. The increase in fission number on the peak day appeared to be directly related to both the increase in inactivation temperature and the decrease in protein concentration. The results presented in Table 2 indicated a correlation between the increase in temperature and the decrease in protein concentration. Heating for 15 minutes at 60° C. gave the greatest increase in percentage fission.

Table 2. Protein loss of whole body extract after heating 15 minutes in water bath at each temperature; February 23, 1970; forty animals in each group.

Temperature (° C.)	Protein Concentration (ug/ml) ¹
0	610 ^a
22	90
40	75
60	34

¹ Determined by Lowry Method

^a Estimated from a ten-fold dilution

In Group Two the temperature was kept constant and the time was varied. Figures 7 and 8 summarize the results. On the peak day of fission the control group had a total of seventeen animals (34%) undergoing fission, the 20 minute group had fourteen (14%), the 40 minute group had two (2%), the 60 minute group had three (6%), and the 120 minute group had five (10%). The optical density of the extract increased from .38 (280 mu) to 1.1 after 20 minutes, 1.2

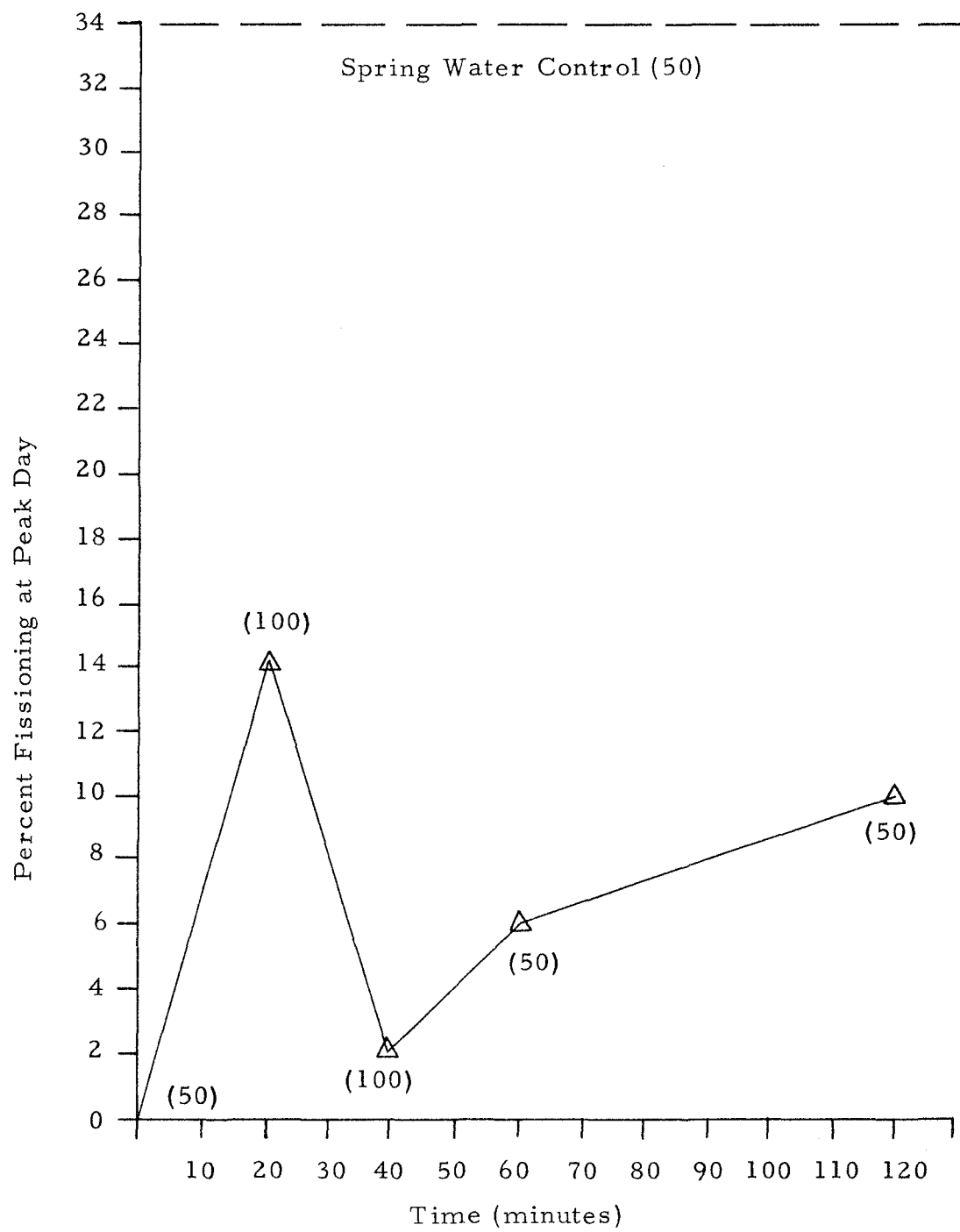


Fig. 7

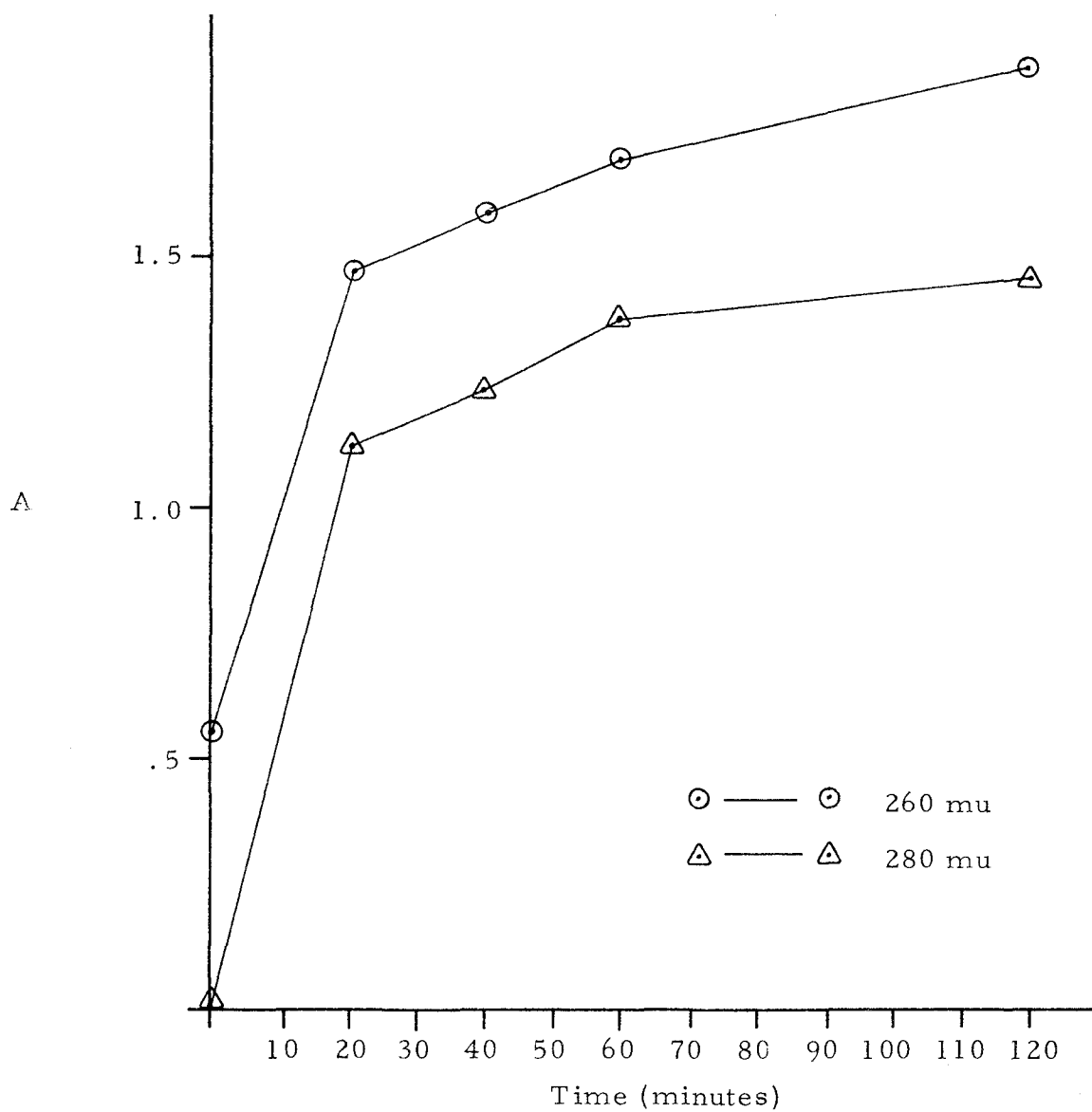


Fig. 8. Optical density of whole body extract before and after heat treatment at 60° C.; April 15, 1970.

after 40 minutes, 1.3 after 60 minutes, and 1.35 after 120 minutes. The increases in optical density at 260 mu paralleled those at 280 mu. Before heat treatment the optical density was .525. After 20 minutes at 60° C. the O. D. had increased to 1.4 and it continued to increase to 1.55 after 40 minutes, to 1.6 after 60 minutes, and to 1.7 after 120 minutes.

There appears to be a contradiction between the results obtained in Group One and Group Two. In Group One increasing the temperature resulted in a decrease in protein concentration. In Group Two, increasing the time of incubation appeared to cause an increase in protein concentration. Even though there was a discrepancy in the protein loss between the two groups the fissioning pattern of the two was similar. Increasing either the incubation temperature or the time of incubation resulted in an increase in fission.

Ammonium Sulfate Precipitation Tests

Ammonium sulfate was added to the extract to precipitate the protein. The data presented in Figure 9 shows that the inhibitory substance contained in whole body extract is precipitable with ammonium sulfate. In the 50% saturation supernatant, four planaria fissioned on the peak day and in the 100% saturation supernatant eight planaria fissioned on the peak day as compared to seven in the control group. With the 50% precipitate four animals fissioned on the peak day, and with the 100% precipitate no planaria fissioned.

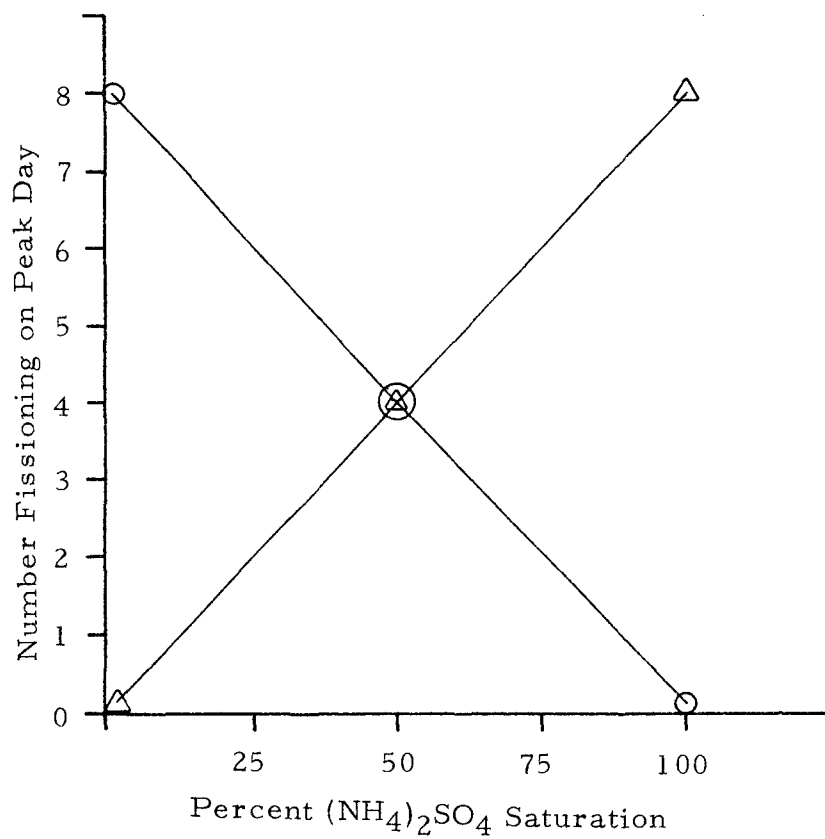


Fig. 9. Effect of ammonium sulfate precipitation upon inhibitory activity of whole body extract; March 24, 1970; forty animals in each group.

△ ——— △ Supernatant
○ ——— ○ Precipitate

Whole Body Extracts on Sephadex G-200 Column

When a whole body extract was put on a Sephadex G-200 column the elution curve showed the presence of three peaks as represented in Figure 10. Peak I is the most prominent and contained all material of molecular weight 325,000 or greater. The second peak contained molecules within the weight range of 25,000 to 325,000. All molecules of molecular weight less than 25,000 came off in Peak III.

Figures 11, 12, 13 and 14 summarize the results when the peak material was tested for inhibitory activity. All three peak fractions inhibited the fission rate of decapitated planaria, and the amount of inhibition was dependent on the concentration. It is interesting to note that a concentration of material as high as 15,000 ug/ml from peak I did not cause death even though this concentration did not completely inhibit fission. The material from peak II caused death at a concentration of 500 ug/ml or greater. In all cases the extract caused an increase in the fission lag period as compared to the controls.

In one experiment peak I was concentrated with an Amicon UM05 pressure dialysis membrane instead of a UM10. The UM05 filter retained anything of molecular weight 500 or greater, while the UM10 filter retained anything of molecular weight 10,000 or greater. The results of this experiment are shown in Figure 15.

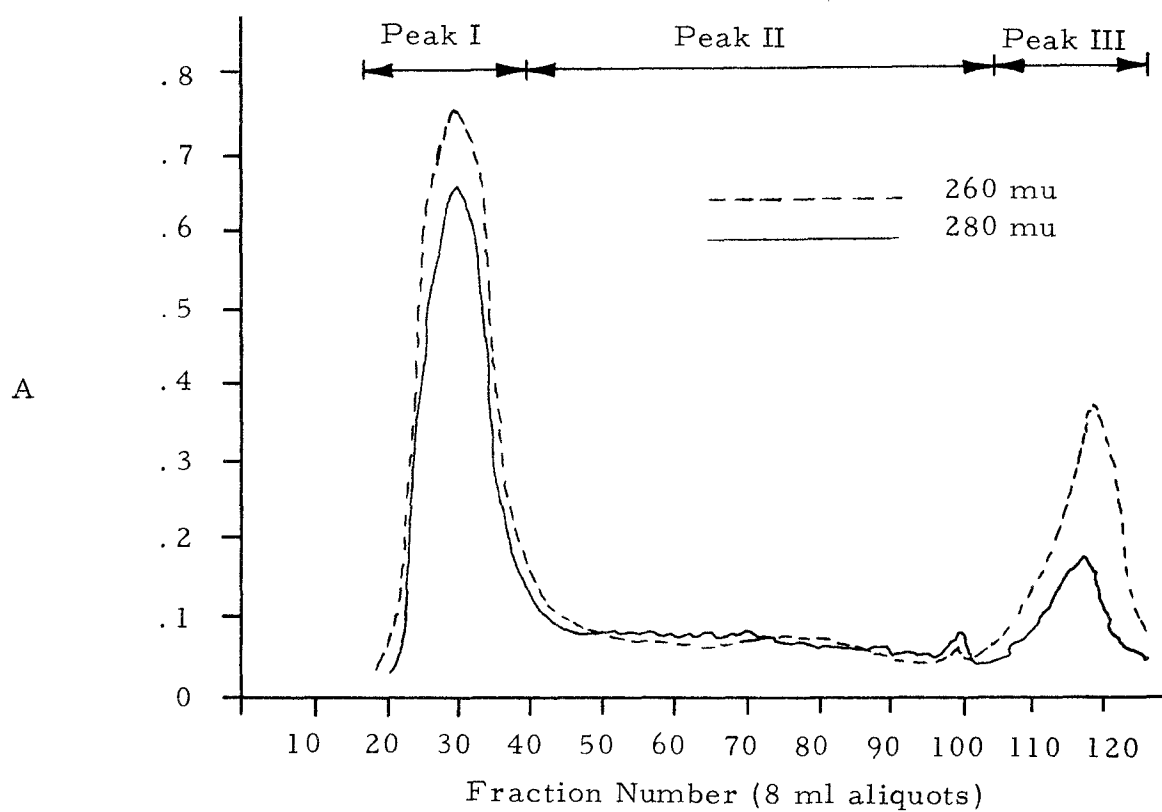


Fig. 10. Elution curve of a whole body extract; Sephadex G-200 column (2.5 x 100 cm); 0.15M NaCl; 5° C.; November 7, 1969.

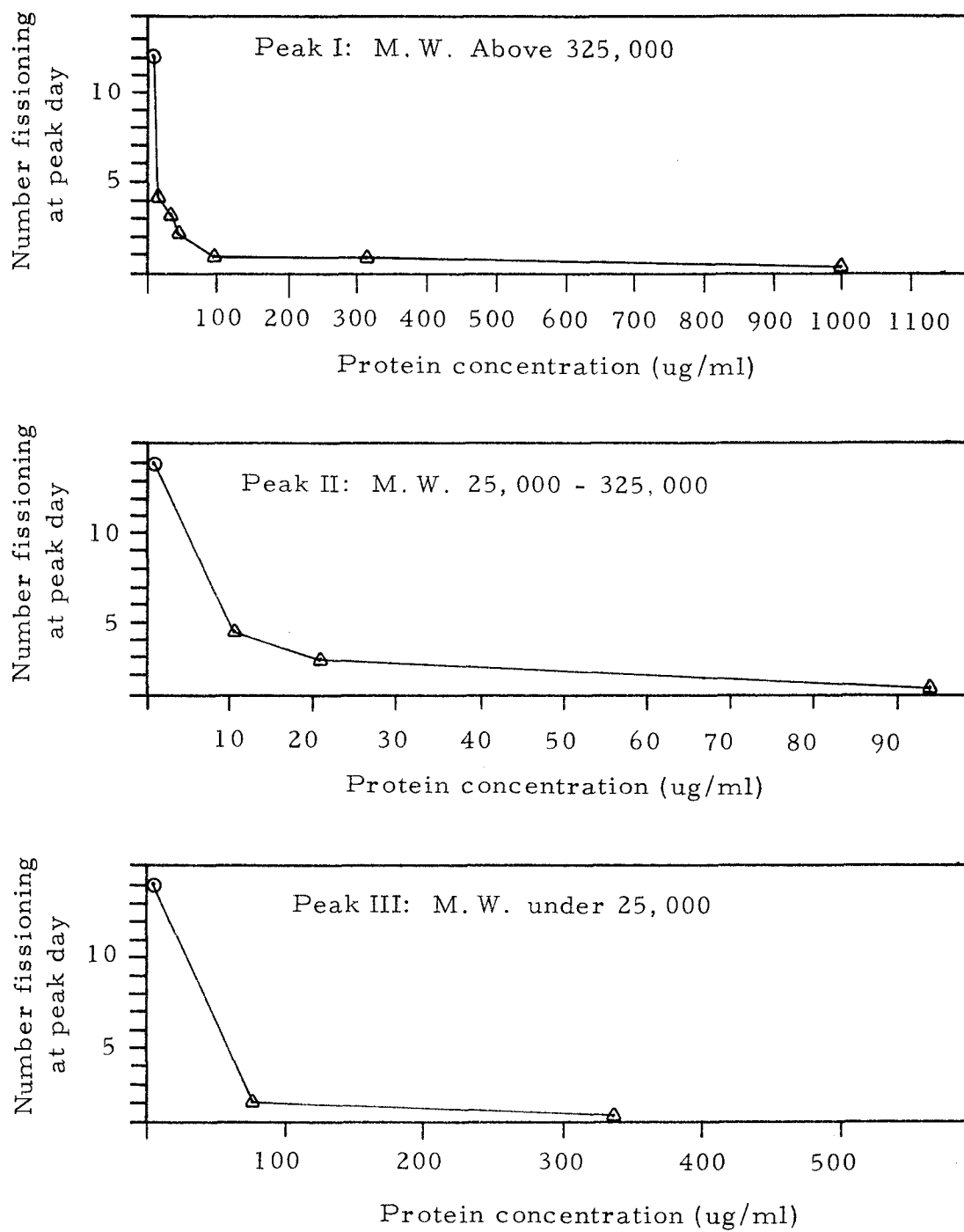


Fig. 11

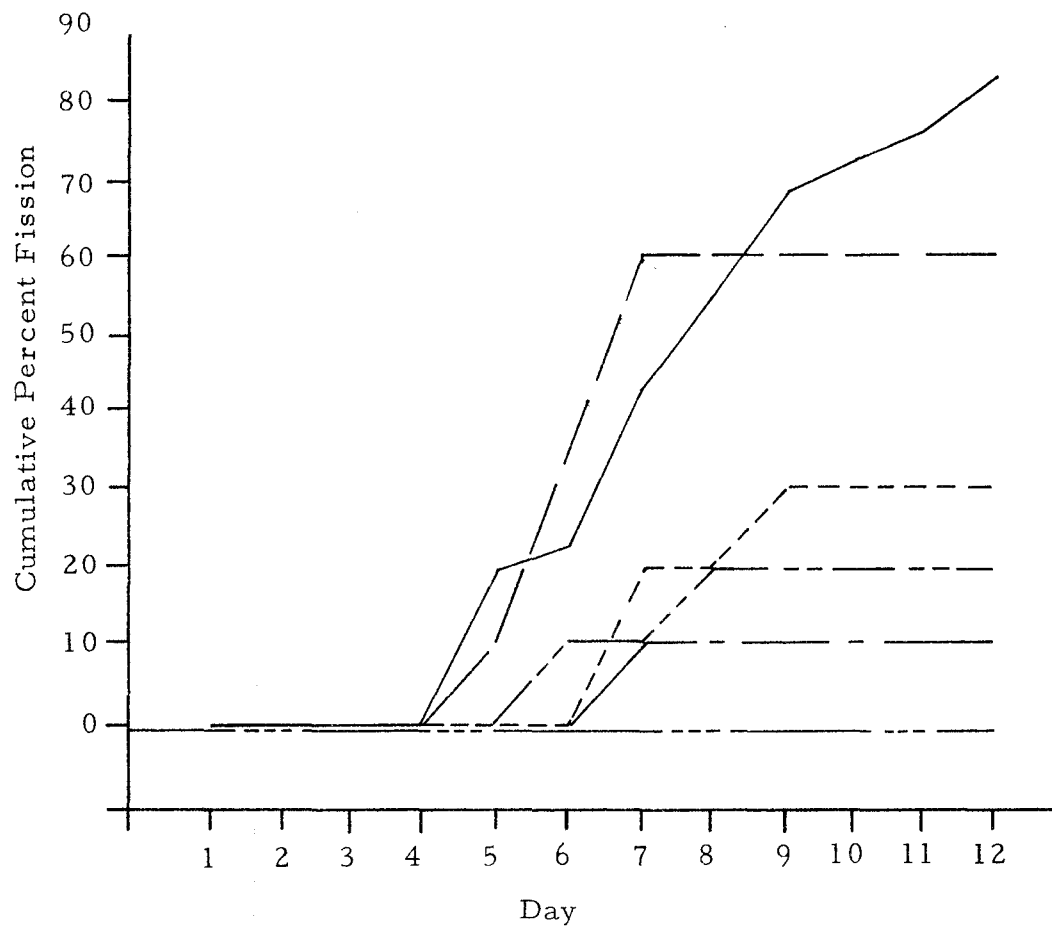


Fig. 12. Inhibitory activity of elution peak material; Peak I; November 7, 1969; sixty animals in control group; twenty animals in each experimental group.

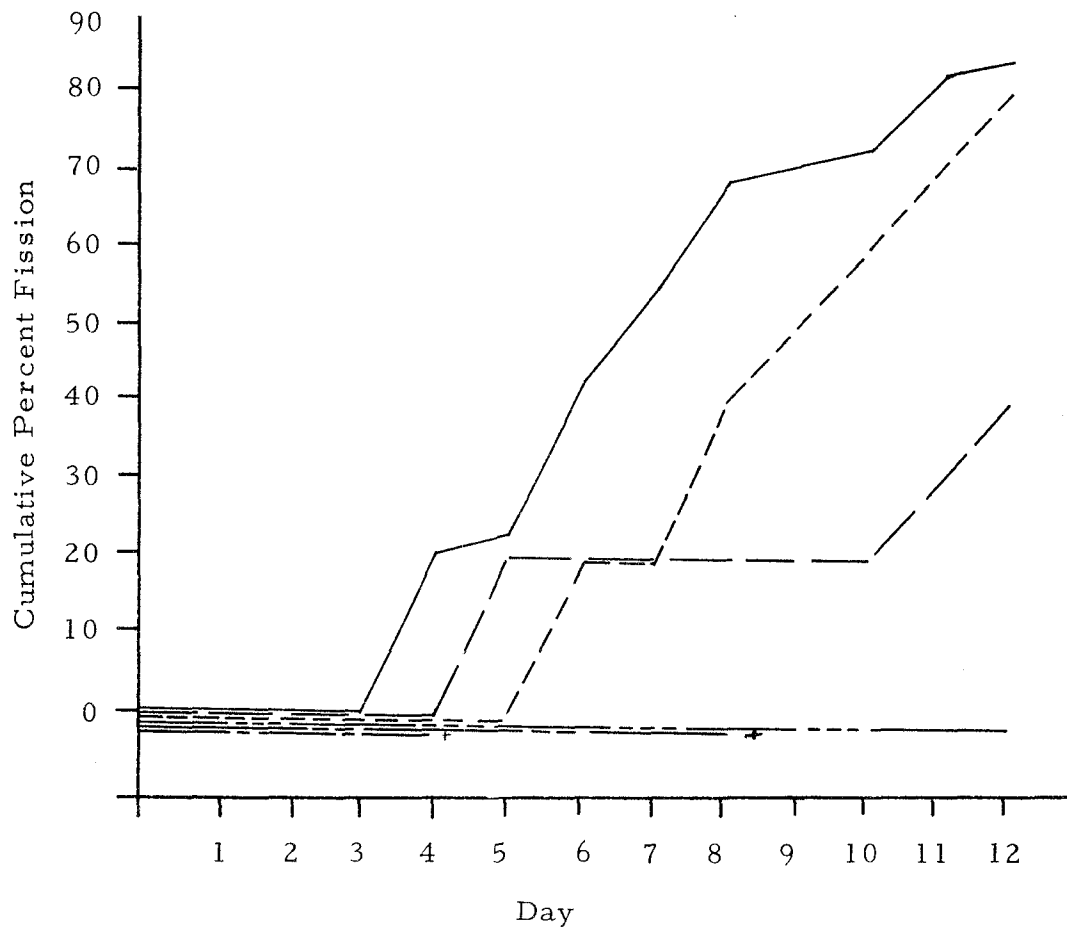


Fig. 13. Inhibitory activity of elution peak material; Peak II; November 7, 1969; sixty animals in control group; twenty animals in each experimental group.

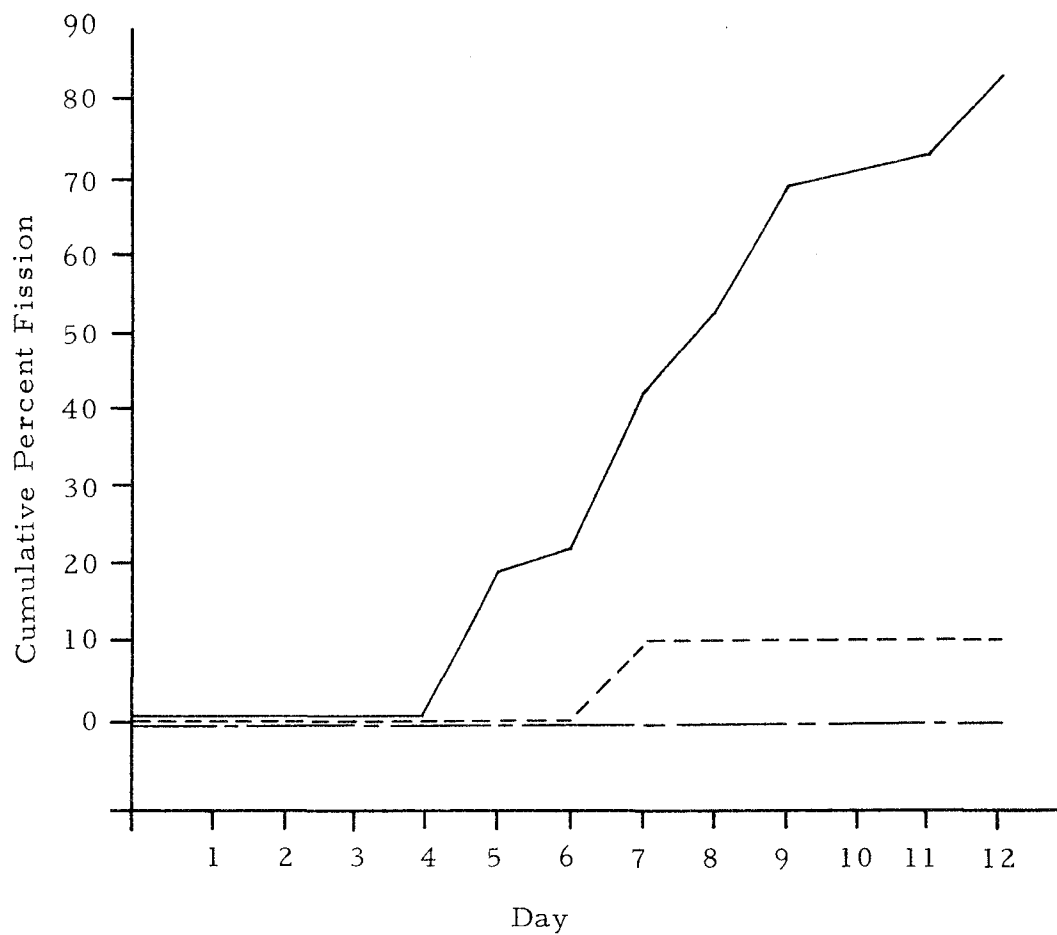


Fig. 14. Inhibitory activity of elution peak material; Peak III; November 7, 1969; sixty animals in control group; twenty animals in each experimental group.

None of the concentrations tested showed inhibitory activity. At the end of the experiment 85% of the spring water controls had fissioned, but with the extract at a concentration of 100 ug/ml 95% fissioned. At concentrations of 200 ug/ml and 400 ug/ml 90% of the planaria reproduced. There was no increase in lag period over the controls, and at 100 ug/ml the planaria began to fission a day earlier than the controls and continued ahead of the controls for the entire experiment. With all three concentrations there appeared to be a stimulatory effect, especially at the beginning of the experiment.

Whole Body Extracts on DEAE Cellulose Exchange

Figures 16 and 17 show the results of fractionation with DEAE cellulose. The material came off in four peaks. At the termination of the experiment the spring water controls had reproduced 100%. When tested for inhibitory activity peak I material (55 ug/ml) resulted in 95% reproduction, peak II material (83 ug/ml) 97% fission, peak III material (54 ug/ml) 100% fission, and peak IV material (32 ug/ml) 97% fission. Material from each peak increased the lag period; the peak I fraction caused an increase of 3 days over the controls, the peak II fraction a 2-day increase, the peak III fraction a 2-day increase, and the peak IV fraction a 2-day increase.

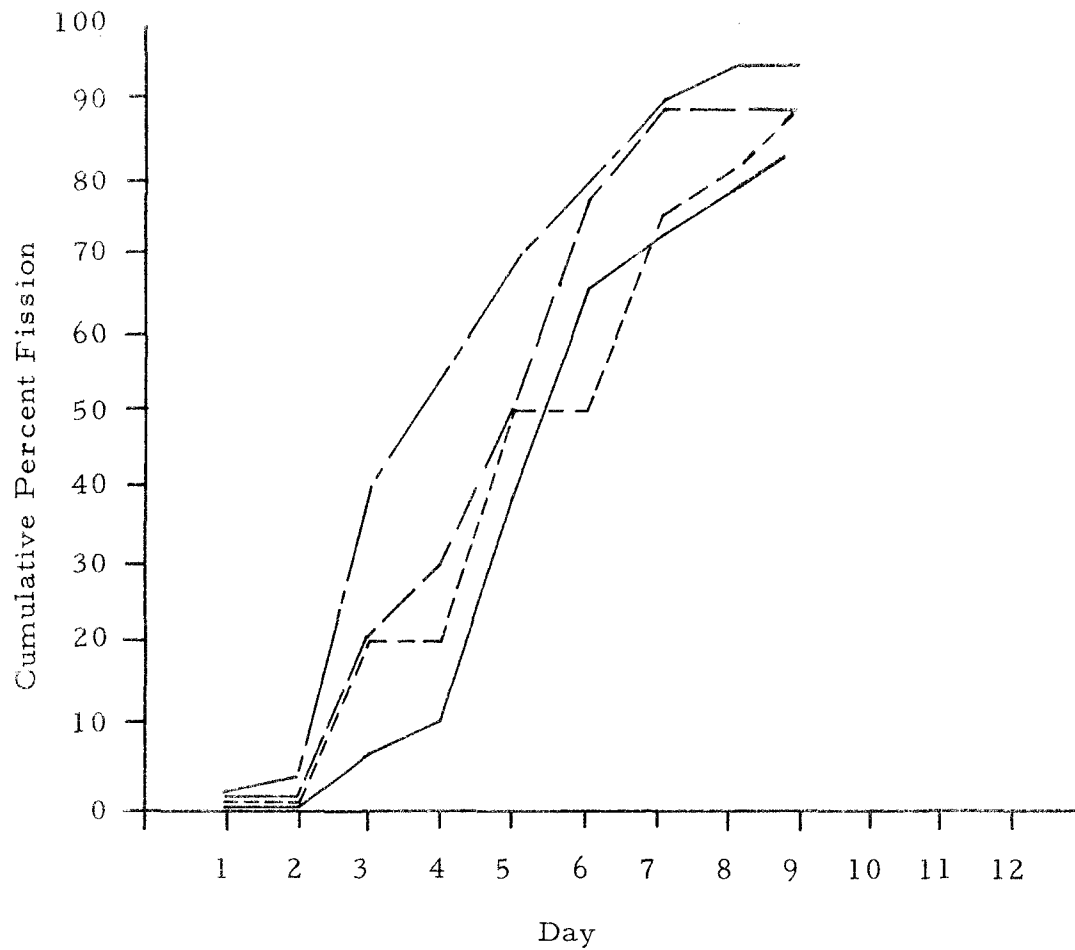


Fig. 15. Apparent stimulatory activity of Peak I material concentrated with amicon pressure dialysis membrane (UMO5); October 15, 1969; forty animals in control group; twenty animals in each experimental group.

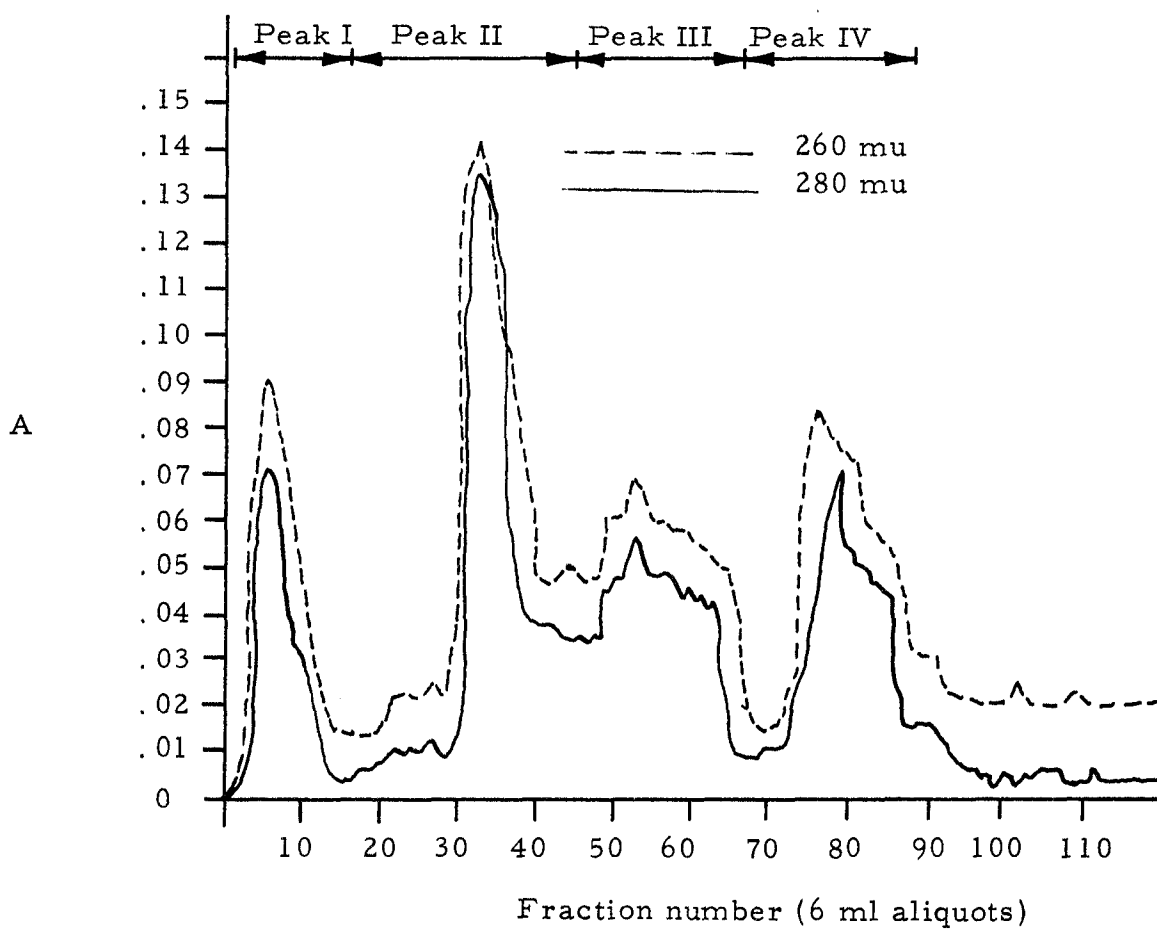


Fig. 16. Elution curve of whole body extract; DEAE polycel anion exchange; 5° C.; gradient reservoirs: 0.005M NaH₂ PO₄ and 0.1 M NaH₂ PO₄, 0.5M NaCl; February 3, 1970.

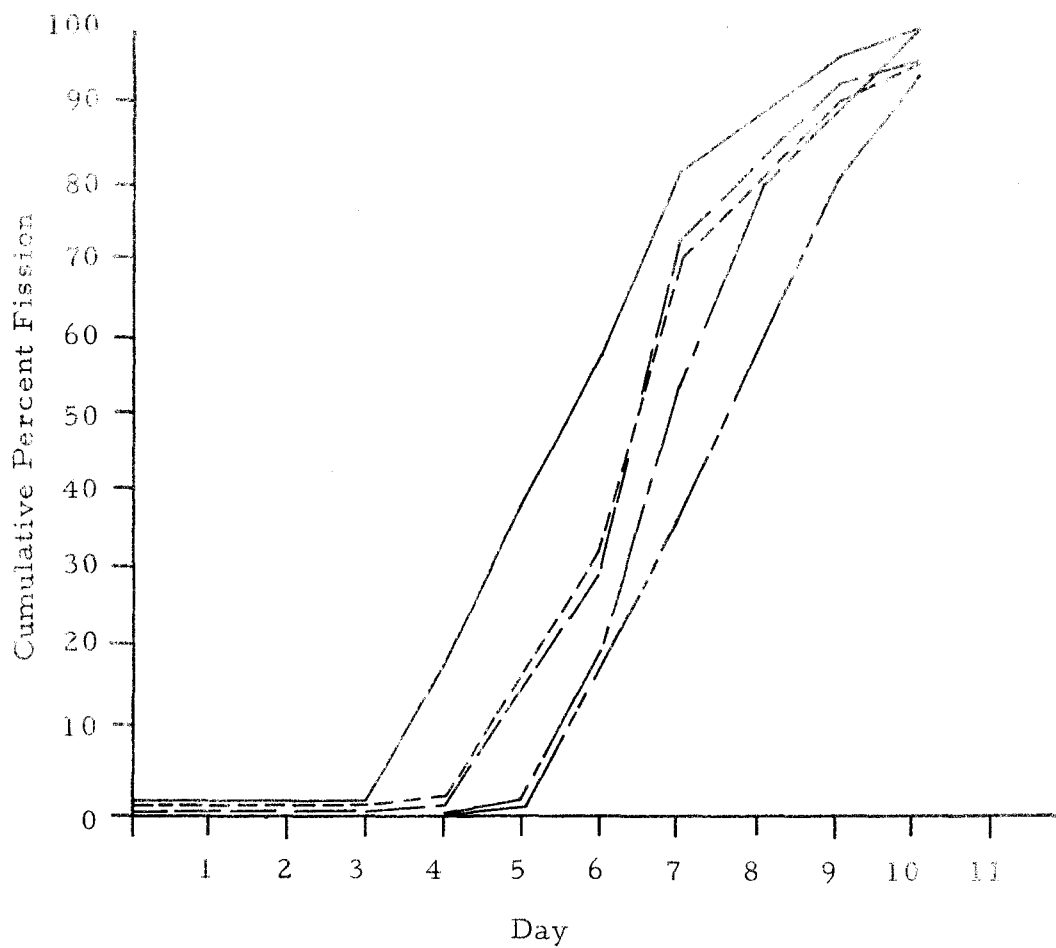


Fig. 17. Inhibitory activity of elution peak material from DEAE anion exchange column; February 7, 1970; one-hundred animals in each group.

DISCUSSION

Child (1910) was one of the first to do extensive investigations into the mechanisms involved in fissioning of planaria. He postulated that fission occurred when the posterior zooid became physiologically independent of the anterior zooid. This physiological isolation could be induced by removal of the head. It was not the removal of the head per se which induced fission, but rather the substitution of a young, partially developed organ (head ganglion) less capable of controlling the body than the original nervous system. He further postulated that a neural substance which originated in the cephalic ganglia diffused throughout the body of the animal, its influence decreasing in the posterior portions of the body. The lessening influence of the neural substance in the posterior regions would account for the isolation of the posterior zooid, and thus its eventual separation from the anterior part of the body.

While extending Lender's work (1952, 1955, 1956, 1960) on the inductive capacities of the brain, Bingham (1968) found that a head homogenate extract would inhibit fission in decapitated planaria as much as 60%. Leavitt (1969), in an attempt to study this inhibition phenomenon further, confirmed that a head extract would inhibit fissioning in decapitated planaria at a concentration equivalent to

2 heads per ml.

The results presented here agree with the results of Leavitt (1969). A head homogenate extract at a concentration of 100 ug/ml. inhibits fission completely (0% reproduction). A decrease in the concentration results in a corresponding increase in fission. Contrary to Child (1910) and Leavitt, the inhibitory substance does not appear to be only a cephalic neural substance. If the only substance which inhibits fission is a neural substance then a lower concentration of head extract should inhibit fissioning to the same extent as a higher concentration of whole body extract. Results from the experiments presented here indicate that this is not the case. Comparable concentrations of head extract and whole body extract inhibit equally.

There is the possibility that inhibition may be due to two different systems acting simultaneously. In the head extract the inhibition may be due to an inductive substance from the cephalic ganglia. When the head extract is added to the medium, the neural substance which had previously been removed by decapitation is put back into the system. This substance then acts to reestablish the anterior dominance, and thus inhibit fission even though the head has been removed.

The second possibility is that of auto-inhibition. When whole body extract at a concentration of 100 ug/ml. is used for rearing decapitated planaria this extract inhibits fission to the same degree as

a head extract at the same concentration. Addition of the whole body extract is adding the supposed cephalic neural substance as well as adding that region which includes the tail. According to Wolff (1961) each zone of the planarian strongly inhibits the differentiation of a homologous region. If fission involves a differentiation process, then rearing decapitated planaria in whole body extract could inhibit fission merely from the presence of the tail region in the extract. Work by Leavitt (1969) has indicated that a homogenate of tails will inhibit fission by 90%, a homogenate of the pharyngeal region will inhibit fission completely (100%), and a homogenate of heads will cause an 85% inhibition of fission. The protein concentration of each homogenate was not determined. Instead, a concentration equivalent to 2 regions per ml. was used. Furthermore, only 10 animals were used in each experiment. Experiments in this laboratory have shown that planaria are variable in their behavior to the extent that large numbers must be used in order to obtain a recognizable trend. Vowinckel (1970) has shown in his work that temperatures less than 20°C stimulate sexual reproduction and inhibit fission. Temperature effects may account for some of the variation in results reported in this laboratory. The planaria were reared in a temperature approximating 18°C, but thermometer checks at various intervals showed this temperature to vary 1°C in either direction. In addition the planaria were exposed to room temperature during cleaning, feeding, and counting. Even though Leavitt did not make protein

determinations, and the number of animals used was small, his results tend to support the hypothesis that the tail region exhibits an auto-inhibitory effect upon fission in decapitated planaria.

The presence of auto-inhibitory systems in planaria is not unusual. Reports by Lender (1952, 1955, 1956, 1960) and by Ziller-Sengel (1967a, 1967b, 1967c) indicate that brain regeneration and pharynx regeneration can be inhibited by extracts of the respective regions. This, they attribute to the inductive powers of an auto-inhibitory system.

The possibility of a multiple-component inhibition system of asexual reproduction in planaria may aid in explaining the results obtained from the chromatography studies. Using a Sephadex column three peaks are obtained, and each one of the peaks exhibits inhibitory activity, depending on the concentrations tested. Even though all the peaks inhibit fission the action of each peak is not qualitatively the same. A concentration of material as high as 1500 ug/ml. from peak I inhibits fission completely but does not cause death. A whole body extract which has not been chromatographed causes death at concentrations above 1500 ug/ml. The material from peak II causes death at a concentration of 500 ug/ml. or greater. The possible existence of more than one inhibitory substance seems plausible in view of that fact that all three elution peaks inhibit fission in decapitated planaria.

The mechanism of action of the inhibitory substance is not

known. Leavitt (1969) found that planaria which had been reared in head extracts begin to undergo fission within three days after being transferred to spring water. That the inhibitory effect does not seem to be permanent tends to support the hypothesis that the inhibition is due to some inductive influence involving metabolism. The results from the experiments reported here show that there is a critical time when the planaria are more susceptible to the inhibitory activity of the whole body extracts. If the extract is added 24 hours after decapitation then there is only 30% inhibition of fission as compared to 70% inhibition when the decapitated planaria are immediately put in whole body extract at a concentration of 34 ug/ml. These results agree with those reported by Ziller-Sengel (1967b). If the pharynx of *Polycelis nigris* is excised 36 hours before the planaria are put in a pharyngeal extract then there is no inhibition of pharynx regeneration. This same extract inhibits pharynx regeneration 22% if planaria are put into the extract immediately after pharynx extirpation. More work is necessary to determine the characteristics of this critical time period. That the effect is not permanent and that a critical time is involved for maximum inhibition would tend to indicate that the mechanism involved is not a simple one. Furthermore it appears that the inhibitory substance acts in such a way as to increase the fission lag period. From experiments run thus far there is apparently no direct correlation between the amount of inhibition and the increase in the lag period.

Whatever the mechanism of inhibition the substance(s) appears to be protein in nature. The inhibitory material is water soluble and remains in the supernatant after centrifugation at 12,100 x g. Upon precipitation with ammonium sulfate the inhibitory substance(s) is found in the precipitate. After 100% saturation the supernatant contains no inhibitory activity and the precipitate inhibits completely.

The inhibitory material is partially inactivated by heat. Even though the activity is lost upon heating, the inhibitory substance(s) seems to be more stable than most proteins. Although there does appear to be a linear relationship between duration of heating and increase in fission, inhibitory activity is still retained after heating for 120 minutes at 60° C.

The inhibitory material is retained by a dialysis membrane. Fractionation of whole body extract with a Sephadex G-200 column results in three peaks. When peak I material is dialyzed with an Amicon pressure dialysis membrane UM05, the retained material appears to have a stimulating effect on the fission rate. If the elution peaks are dialyzed with an Amicon Pressure dialysis membrane UM10 the retained material from all three peaks exhibits inhibitory activity whose extent depends on the concentration. The UM05 membrane retains all material of molecular weight greater than 500, and the UM10 membrane has an exclusion limit of 10,000. It appears that the stimulatory substance is unmasked using the UM05

membrane. It is possible that this substance is in the molecular weight range of 500 to 10,000 since it does not appear to be retained by the UM10 filter. This stimulatory substance has only been demonstrated in dialysis experiments. More work is necessary to determine if there is such a substance. The inhibitory material seems to be greater than 10,000 molecular weight because it is retained by the UM10 membrane. There is also the possibility that the inhibitor is a relatively small, non-protein component which is bound very strongly and non-specifically to proteins.

The fractionation procedure with DEAE cellulose anion exchange columns appears to inactivate the inhibitory substance(s).

CONCLUSIONS AND SUMMARY

Head extracts and whole body extracts inhibit fission in decapitated asexual strains of Dugesia dorotocephala. There does not appear to be any difference in the effects of these two types of extracts run at comparable concentrations. A concentration of 100 ug/ml of either extract gives maximum inhibition without causing death. The percentage of inhibition is directly correlated with the concentration. The exact mechanism of action of the inhibitory substance(s) is not known. There is a critical period within 24 hours after decapitation when the planaria are more susceptible to the inhibition. After 24 hours the inhibition is slight (30%).

Fractionation of whole body extract with a Sephadex G-200 column results in three peaks. Peak I contains material of molecular weight 325,000 or greater; peak II contains material of molecular weight between 25,000 and 325,000; and peak III contains all material of molecular weight less than 25,000. Inhibition may be due to more than one inhibitory substance since the material from all three elution peaks exhibit inhibitory activity. There is also the possibility that the inhibitory substance cannot be resolved with the fractionation procedures used.

The inhibitory substance(s) is inactivated by heat. After

ammonium sulfate precipitation tests the activity is found in the precipitate. The fractionation procedure with DEAE anion exchange columns appears to inactivate the inhibitory substance(s). There seems to be a substance between 500 and 10,000 molecular weight which causes stimulation of fission in decapitated planaria.

LITERATURE CITED

- Andrews, P. 1966. Molecular weights of prolactins and pituitary growth hormones estimated by gel filtration. *Nature*, 5019: 155-157.
- Betchaku, T. 1967. Isolation of planarian neoblasts and their behavior in vitro with some aspects of the mechanism of the formation of regeneration blastema. *J. Exptl. Zool.*, 164: 407-434.
- Bingham, D. 1968. Inhibition of planarian regeneration. Unpublished master's thesis, Department of Zoology and Entomology, Brigham Young University, Provo, Utah.
- Blumberg, Th. T. 1940. Regeneration of *Euplanaria dorotocephala* with pituitary gland extract. *Proc. Soc. exp. Biol.* New York, 44: 117-119.
- Bronsted, H. V. 1955. Planarian regeneration. *Biol. Rev.*, 30: 65-126.
- Bronsted, H. V. 1968. Planarian regeneration. Pergamon Press, London.
- Chandlbois, R. 1965. Cell transformation systems in planarians, pp. 131-142. In V. Kiortsis and H. A. L. Trampusch, (ed.) *Regeneration in animals and related problems.* North-Holl.

Publ. Co., Amsterdam.

- Child, C. M. 1910. Physiological isolation of parts and fission in the planaria. *Arch. Entwmech. Org.*, 30: 159-205.
- Child, C. M. 1911. Studies on dynamics of morphogenesis, etc. I. Physiological dominance of anterior over posterior regions in the regulation. *J. Exp. Zool.*, 11:187-280.
- Child, C. M. 1913. Certain dynamic factors in experimental reproduction and their significance for the problems of reproduction and development. *Arch. Entwmech. Org.*, 35: 598-641.
- Child, C. M. 1924. *Physiological foundations of behavior.*
New York.
- Child, C. M. 1932. Experimental studies on a Japanese planarian. I. Fission and differential susceptibility. *Sci. Rep. Tohoku Univ.*, 7: 313-345.
- Dubois, F. 1948. Demonstration de la migration des cellules de regeneration des planaires par la methods des greffes et des irradiations combinees. *C. R. Acad. Sci.*, 226: 1316-1317.
- Flickinger, R. A. and S. J. Coward. 1964. Isotopic evidence for a local origin of blastema cells in regenerating planaria. *Exptl. Cell Res.*, 34: 403-406.
- Green, A. A. and W. L. Hughes. 1955. Protein fractionation on the basis of solubility in aqueous solutions of salts and

- organic solvents, pp. 67-90. In S. P. Colowick and N. O. Kaplan, (ed.) *Methods in enzymology*, Volume I. Academic Press, Inc., New York.
- Hay, E. D. 1968. Fine structure and origin of regeneration cells in planarians. *Anat. Rec.*, 160: 363-
- Henderson, R. F. and R. E. Eakin. 1959. Alteration of regeneration in planaria treated with lipoic acid. *J. Exp. Zool.*, 151(2): 175-187.
- Hyman, L. H. and E. R. Jones. 1959. Turbellaria, pp. 323-334. In W. T. Edmondson, (ed.) *Fresh-water biology*. John Wiley and Sons, Inc., New York.
- Kendall, K. and D. S. Nachtwey. 1968. Inhibition of regeneration of planaria by midlethal exposures to x-rays. U. S. Naval Radiological Defense Laboratory, San Francisco.
Reproduced by Clearinghouse for Federal Scientific and Technical Information, Springfield, Virginia.
- Kohl, D. M. and R. A. Flickinger. 1966. The role of DNA synthesis in the determination of axial polarity of regenerating planarians. *Biol. Bull.*, 131: 323-330.
- Kolmayer, S. and F. Dubois. 1960. Neoblasts et limitation du pouvoir de regeneration cephalizue la planaire Dendrocoelum lactrum. *J. Embryol. exp. Morph.*, 8: 376-384.
- Leavitt, L. H. 1969. Inhibition of asexual reproduction in planaria.

- Unpublished master's thesis, Department of Zoology and Entomology, Brigham Young University, Provo, Utah.
- Lender, T. 1952. Le role inducteur de cerveau dans la regeneration des yeux d'une planaire d'eau douce. Bull. Bio. Fr. Belg., 86: 140-215.
- Lender, T. 1955. Sur l'inhibition de la regeneration du cerveau de la planaire Polycelis nigra. C. R. Acad. Sci., 241: 1863-1865.
- Lender, T. 1956. L'inhibition de la regeneration du cerveau des planaire Polycelis nigra et Dugesia lugubris en presence de broyats de tetes ou de queues. Bull. Soc. Zool. Fr., 81: 192-199.
- Lender, T. 1960. L'inhibition specifique de la differenciation du ceryeau des planaires d'eau douce en regeneration. J. Embryol. exp. Morph., 8(3): 291-301.
- Lowry, O. H., N. J. Rosenbrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the folin phenol reagent. J. Biol. Chem., 193: 265-275.
- Morgan, T. H. 1901. Regeneration. Macmillan Co., London.
- Needham, A. E. 1952. Regeneration and wound healing. John Wiley Sons, Inc., New York.
- Pennak, R. W. 1953. Fresh-water invertebrates of the United States. The Ronald Press Co., New York.
- Sengel, C. 1959. La region caudall d'une planaire est-elle capable

- d'induire la regeneration d'un pharynx? J. Embryol. exp. Morph., 7: 73-85.
- Sober, H. A., F. J. Gutter, M. M. Wyckoff, and E. A. Peterson. 1955. Chromatography of proteins. 11. Fractionation of serum protein on anion-exchange cellulose. J. Am. Chem. Soc., 78: 756-763.
- Stephan, F. 1967. Resultats des excisions de pharynx chez les individus doubles de la planaire Dugesia tigrina. Bull. Soc. Fr., 92: 303-309.
- Vandel, A. 1921. Recherches experimentales sur les modes de reproduction des Planaires triclades paludicoles. Bull. Biol., 55: 343-518.
- Vowinckel, C. 1970. The role of illumination and temperature in the control of sexual reproduction in the planarian Dugesia tigrina (Girard). Biol. Bull., 138:77-87.
- Wolff, E. 1961. Migration et contacts cellulaires dans la regeneration. Exp. Cell. Res. 8: suppl: 246-255.
- Woodruff, L. S. and A. L. Burnett. 1965. The origin of blastemal cells in Dugesia tigrina. Exp. Cell Res., 38: 295-305.
- Wulzen, R. 1916. The pituitary gland. Its effect on growth and fission of planarian worms. J. Biol. Chem., 25: 625-633.
- Ziller-Sengel, C. 1967a. Sur le facteur inhibiteur de la regeneration du pharynx chez les planaires d'eau douce. Bull. Soc. Zool. Fr., 92: 295-302.

Ziller-Sengel, C. 1967b. Recherches sur l'inhibition de la
regeneration du pharynx chez les planaires. 1. Mise en
evidence d'un facteur auto-inhibiteur de la regeneration
du pharynx. J. Embryol. exp. Morph., 18: 91-105.

Ziller-Sengel, C. 1967c. Recherches sur l'inhibition de la
regeneration du pharynx chez les planaires. 11. Variations
d'intensite du facteur inhibiteur suivant les especes et les
phases de la regeneration. J. Embryol. exp. Morph.,
18: 107-119.

ASEXUAL REPRODUCTION IN PLANARIA
CHARACTERISTICS OF THE INHIBITOR(S)

Linda G. Sheffield

Department of Zoology

M. S. Degree, August 1970

ABSTRACT

A study was made of the postulated fission-inhibiting substance located in the cephalic region of Dugesia dorotocephala. Rearing decapitated planaria in four different concentrations (25, 50, 75 or 100 ug/ml) of head or whole body extract resulted in a positive correlation between the inhibition of fission rate and the concentration of extract. The difference in inhibitory activity between the two types of extracts was three percent at maximum.

The inhibitory substance was partially inactivated at 60° C. There was a direct correlation between the length of time that the extract was heated and the inactivation of inhibitor. The active substance was precipitable with ammonium sulfate. Fractionation of whole body extracts with a Sephadex G-200 column showed the presence of three peaks. The material from all three peaks exhibited inhibitory activity, possibly indicating the presence of more than one inhibitory substance. Fractionation procedures with DEAE anion exchange columns inactivated the inhibitory substance(s).

The mechanism of action of the inhibitory substance(s) is not known. The animals must be exposed to the extract within 24 hours after decapitation in order for maximum inhibition to be obtained.

COMMITTEE APPROVAL: