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Analysis of asexual reproduction Dugesia dorotocephala

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ANALYSIS OF ASEXUAL REPRODUCTION IN *DUGESIA DOROTOCEPHALA*

A Dissertation

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Doctor of Philosophy

by

Harvey Dee Mecham

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19 April 1972
Date

Typed by Allie Mecham

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INTRODUCTION

Planaria (class Turbellaria) as an experimental organism offers an exceptional opportunity to investigate, in vivo, cellular control mechanisms. This organism has not been used extensively in developmental biology. Recent research using planaria has been limited to memory transfer experiments by psychologists and little is known of the basic physiology of this animal.

In an attempt to unravel cell control processes, studies have been conducted using tissue cultures at one end of the organizational scale and mammalian systems at the other. Most of our information concerning cellular control comes from the tissue culture studies which have been extrapolated to explain observations in multicellular organisms. Many of these inferences are clouded by various interacting systems of the more complex whole animal. In order to make successful extrapolations from tissue culture to higher biological systems, it seems advantageous to understand more about simple organisms.

The planaria, a simple metazoan, has not developed the complex structures of higher organisms and therefore offers some of the advantages of tissue culture while maintaining a normal cellular interrelationship. This makes it possible to study cellular processes by techniques similar to those used in tissue culture and at the same time maintain intercellular communication.

Child (1941) proposed that the control of asexual reproduction or fission is a type of intercellular communication which involves a metabolic gradient. This process in the species Dugesia dorotocephala occurs by transverse fission at a point posterior to the pharynx (Curtis, 1902). The transverse fission mechanism, therefore, is a tool by which elucidation of the mechanisms of intercellular communication is possible. Such systems have been demonstrated in many biological systems such as the chalones proposed by Bullough (1965) as hormonal feedback controls. However, such control mechanisms have never been substantiated in association with the fission process of planaria (Pedersen, 1958).

The purpose of this research was to (1) establish optimum growth conditions for planaria similar to those used for tissue-culture maintenance so that planaria may be used for biological assay with consistant, reproducible results and (2) to demonstrate and isolate molecular classes associated with intercellular communication which control the fission process by stimulation and/or inhibition.

LITERATURE REVIEW

Planaria are the highest organisms on the phylogenetic scale in which fission is observed (Hyman, 1951). Though fission has been studied since 1895, little is known about control of the fission process as indicated in the excellent review compiled by Brondsted (1969).

Zacharias (1895) was the first to observe and describe transverse fission. In a long series of papers from 1910-1941 Child studied control mechanisms of planarian asexual reproduction (Child 1910, 1911, 1913, 1924, 1932, 1941). Since that time very little new or definitive information has been added to the literature on this subject.

In 1932 Child stated, "It has long been known that in planarian species which undergo fission, removal of the head will frequently induce fission even in animals of small size which very rarely or never undergo fission in nature. When the head is removed fission does not usually occur at once but only after several days when the new head has developed sufficiently so that the animal becomes active again". (Italics added)

On the basis of respiration studies, Child proposed an anterior to posterior metabolic gradient to explain his observations and concluded that the head controls fission. Buchanan's

(1938) demonstration that extracts of planaria heads stimulate head regeneration has been accepted as support of Child's metabolic gradient hypothesis (Brondsted, 1969).

It is interesting to note Child's observation that decapitation accelerates fission is now included in the "it is well known that" category (see Brondsted, 1969). However, the factors contributing to stimulation by decapitation have not been defined. Few experiments have been conducted to corroborate or refute the initial conclusions of Child.

Kanatani (1957a, 1957b and 1957c) thought that exudates from the body surface have an inhibitory effect on fission. Exudates were collected from containers of intact planaria and added to other containers of decapitated animals. The result was the production of supernumerary eyes, disintegration of the head and, in some animals, a general retardation of the fission rate.

Kanatani also determined that a large, nondialyzable substance was able to promote fission. This material, produced by the planaria, was effective only after removal, by dialysis, of other substances present in the culture medium. He believed that inhibition of fission is due to an accumulation of ammonia in the culture medium (Kanatani, 1958a, 1958b). This assumption was based on the fact that (1) ammonia is the chief excretory product of invertebrates, and (2) the addition of ammonium chloride to culture medium produces both supernumerary eyes and inhibition of fission. Kanatani concluded that the large molecular weight substance which enhances fission is produced by the planaria and is assumed to be antagonistic to ammonia.

In 1964 Kanatani extended his characterization of the fission stimulating compound. The material was heat-stable at 100°C for 30 minutes and acetone insoluble. He found that the fission-promoting activity was present in an extract of the head region, but in lesser amounts in the middle and tail regions. However, his experimental technique did not provide unequivocal proof of a stimulator and he continued to assume that ammonia is the inhibitor. This differs from the conclusion of Shefield (1970) who found a high molecular weight inhibitor.

Vowinckel, Wolfson and Marsden (1970) presented evidence that a heat-stable factor of low molecular weight which was in the culture medium would induce spontaneous head loss. Because the animals became less sensitive with time after feeding, they proposed that this factor was associated with some digestive activity. This substance builds up in the crowded culture and may be part of a feedback control mechanism relating fission rate and population density. The possibility that mucous slime-trails produced by the planaria are instrumental in this feedback control has been proposed by Best, Goodman and Pigon (1969).

Many researchers have associated the brain or neurosecretory system with the control of fission (Morita and Best, 1965; Lentz, 1967; Vowinckel, 1970b; Grasso, 1966; Bingham, 1968). Nentwig (1971) observed migration of neurosecretory cells in the ventral nerve and proposed that the entire nervous system is involved in control of fission and not just the cephalic ganglia as Child had proposed.

MATERIALS AND METHODS

Experimental Organisms

Dugesia dorotocephala, identified according to Hyman (1951) and Hyman and Jones (1959) were used exclusively during this study. These animals were obtained from the Dahl Company under the catalog name Dugesia tigrina. They were collected from streams which drain into San Francisco Bay at Berkeley, California.

Depending upon the season, the planarian collections were obtained from different streams which were within 5 miles of one another. The supplier noted (personal communication) that the streams differ extensively in turbitity and speed and do not unite before entering the bay. This is significant if genetic differences in isolated races, which maybe physiologically distinct, contribute to the experimental variations which have been noted.

Culture Maintenance

The planaria were kept in a low ionic strength medium described by Henderson and Eakin (1959) presented in Table 1.

Table 1. Low ionic strength medium used for incubation of planaria.

Salt	Concentration
$CaCl_2$	1×10^{-3} M
NaCl	1×10^{-4} M
KCl	1×10^{-6} M
EDTA*	1×10^{-6} M

*Ethylene-diamine-tetra-acetate

According to the authors EDTA is used as a precautionary measure against divalent cations. The pH was adjusted to neutrality with 0.1 M NaOH although this may not be necessary since planaria are capable of adjusting the pH (Saum, Andes, Park and Jenkins; 1969).

The animals were maintained in a constant temperature room at 24°C. Evaporation of fluid from the open containers reduced the temperature of the incubation medium to 20°C.

Stock cultures were kept in white plastic containers (dish pans) 25 cm x 33 cm with the fluid maintained at a depth of no less than 10 cm. The animals were fed at least weekly with frozen beef liver, then rinsed with tap water, and the container cleaned upon completion of feeding then clean medium was added.

The cultures were kept in constant, indirect light at less than 0.5 foot-candles. However, the animals were subjected to increased light (about 24 foot-candles) for approximately one hour three times per week when they were being tended.

Preparation of Tissue Extract

The tissue extract, unless otherwise noted, was obtained from homogenized Dugesia dorotocephala. Control of the size of the animals used was limited to exclusion of any animals which were sexual, that is exceptionally large and/or had a gonopore. The planaria were washed at least twice in distilled water, then cooled in an ice bath. The chilled tissue was homogenized in Henderson-Eakin solution with a size B Thomas Tissue Homogenizer while suspended in an ice bath. Homogenized tissue was then centrifuged ($4^{o}C$) at 10,000 RPM for 20 minutes in a Sorvall Superspeed RC-2 centrifuge with an SS-34 rotor.

Quantitative Determinations

Quantitative estimations on the concentrations of extract were made by protein determinations. Protein content was determined by a micro-modification of the Lowry method (Lowry, 1951) standardized with bovine serum albumin of known concentration. Two-tenths ml of known and unknown protein solutions were allowed to react for 10 minutes at room temperature with 2.0 ml of an alkaline-copper solution (2% $NaHCO_3$, o.1 M NaOH, 0.5% $CuSO_4$ and 1% sodium tartrate). Two-tenths ml of Folin-reagent (phosphomolybolic-phosphotungstate) was added and the solution incubated at room temperature for at least 45 minutes. The colored compound was analysed spectrophotometrically at 750 mu. Protein concentrations were determined by the formula:

$$\gamma \text{ protein} = \frac{(\text{O.D. unknown} - \text{O.D. blank})\ (\gamma \text{ protein in standard})}{(\text{O.D. standard} - \text{O.D. blank})}$$

All concentrations of "active" components (purified fractions of the tissue extract) were determined by a dilution equivalent value of the initial extract. Following each fractionation it was assumed that the procedure would result in almost all of the "active" material being in a single fraction.

Filtration Chromatography

Bio-gel is a polyacrylamide bead manufactured by Bio-Rad Company. This material is used for filtration chromatography and responds very well at low ionic strengths.

Bio-gel P-10, which has a molecular weight separation range between 500 and 15,000, was employed. The dry beads were hydrated in standard Henderson-Eakin solution at $4^{o}C$ for at least 24 hours before being degassed by vacuum. A slurry of the swollen P-10 gel was poured into a column 9.8 cm x 55 cm and packed according to standrad procedures specified by the manufacturer. The column was then washed with Henderson-Eakin solution overnight at a flow rate of 3 ml per hour using a 75 cm hydrostatic head. Compounds to be separated were layered on the top of the semi-dry gel bed, washed into the gel and eluted with Henderson-Eakin solution at pH 7.0.

Estimations of molecular weights of the eluted fractions were made by calculating the partition coefficient between the liquid and gel phase (Kav) as described by Determan (1969). The molecular weight relationships were verified by chromatography of insulin, cobalamin and tyrosine.

Detection

The column effluent was analyzed with a Uviscan III continuous flow ultraviolet detector (Buchler Inst. Co.). The U.V. absorption at 280 nm was recorded on two recorders (Photovolt 43 and Sargent-Walch SRG) connected in parallel with a 10:1 resistance ratio between them.

DEAE Separation

DEAE-Sephadex was hydrated in NaCl and kept at 4°C for at least 24 hours. Each hydrated sephadex solution was poured into a column with 10:1 height to diameter ratio. The pH was adjusted to 6.0 by continual washes of the eluting salt solution at the initial molarity which was either 0.05 M, 0.01 M, 0.005 M or 0.001 M. The sample to be analyzed was added to the semi-dry bed surface, washed into the gel and eluted with 20 ml of the same molarity salt solution (pH 6.0). The pH was then increased by 0.5 increments and this process repeated until the final washing was completed at pH 8.0 for each column of different ionic strengths.

Desalting

If the extracts were in other than the standard Henderson and Eakin solution (Henderson and Eakin, 1959) they were desalted. This process was accomplished at room temperature with the polyacrylamide Bio-gel P-2 (Bio-Rad) which has a molecular weight operating range from 100 to 2,000. The hydrated gel was poured into a column 8.5 cm in diameter to a height of 3.0 cm. The sample was

eluted with Henderson-Eakin solution at a flow rate of 2.0 ml per minute and the effluent fraction collected was from 39 ml through 99 ml. Bovine serum albumin and tyrosine were chromatographed to verify that the column separated all high molecular weight material from the low molecular weight salts within the collected volume.

Biological Assay

The biological assay was based on the number of fissions which could be induced or retarded in the decapitated planaria in comparison with control cultures. Animals used in the assay were selected from the stock culture five days after their last feeding. They were between 10 mm and 20 mm in length and only those with mature, pointed tails and well-formed heads were used. Animals that were wider than 2mm and/or had a gonopore were considered sexual and were excluded (Jenkins, 1970).

The assay animals were incubated individually in plastic petri dishes, either 10 mm x 35 mm or 15 mm x 60 mm. Each petri dish contained either 2.5 ml (small dish) or 5 ml (large dish) of the appropriate extract. Unless otherwise indicated, all extracts were assayed at 100 ug protein per ml in Henderson-Eakin solution.

When planaria were decapitated the cut was made just posterior to the auricles with a razor blade held in a hemostat. Care was taken to assure uniformity of the cuts (Leavitt, 1969). All decapitations took place in the petri dish containing the extract being assayed.

The covered petri dishes were incubated at 20°C in a high humidity (60%) chamber which was kept dark except during daily counting. Upon completion of the assay the animals were returned to a stock container and, after one month of regenerative growth, were used in further experiments.

RESULTS

Environmental Conditions

Planaria were examined at various temperatures to determine the optimum temperature necessary to obtain maximum fission. At temperatures below 20°C the onset of fission is delayed but the fission rate is not appreciably altered (Fig. 1). Animals cultured (five per plate) at 20°C begin to fission two days after decapitation whereas at 18°C the onset of fission is delayed to day four. At 16°C this delay extends to day six and at 14° fission does not begin until the seventh day after decapitation. When the incubation temperature is reduced below 20°C to 12°C there is no decrease in the total number of animals capable of fissioning.

Temperatures above 20°C do not alter the latent period between decapitation and onset of fission, although there is a reduction in the total number of fissions (Fig. 2). At 20°C a maximum of eighty percent of the planaria fissioned, at 22°C sixty-four percent fissioned, at 24°C forty-six percent fissioned, fifteen percent fissioned at 28°C and at 32°C all animals were dead 24 hours after decapitation.

Light

The sensitivity of planaria to fluorescent light is shown in figure 3. No attempt was made to correlate the light wave length and viability. When non-decapitated animals were subjected to a

Fig. 1. Post-decapitation fission pattern (five animals per plate) associated with low incubation temperatures 14 ○ , 16 □ , 18 ◇ and 20 ▽ degrees centigrade.

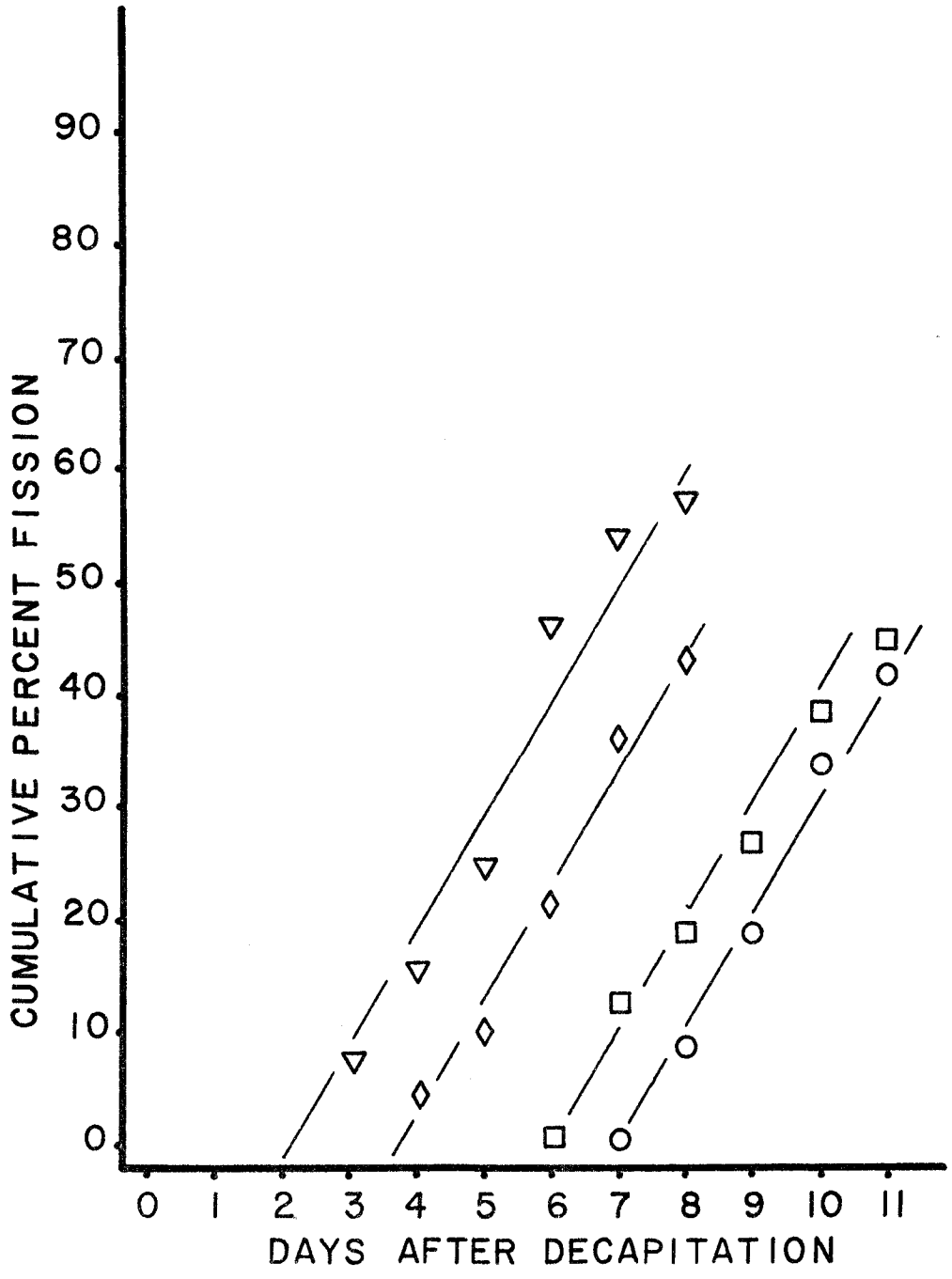

CUMULATIVE PERCENT FISSION
90
80
70
60
50
40
30
20
10
0
0 1 2 3 4 5 6 7 8 9 10 11
DAYS AFTER DECAPITATION

Fig. 2. Post-decapitation fissionability of planaria incubated five per plate, associated with high incubation temperatures 20.5 ○ , 22 □ , 24 ▽ , 28 ◇ degrees centigrade.

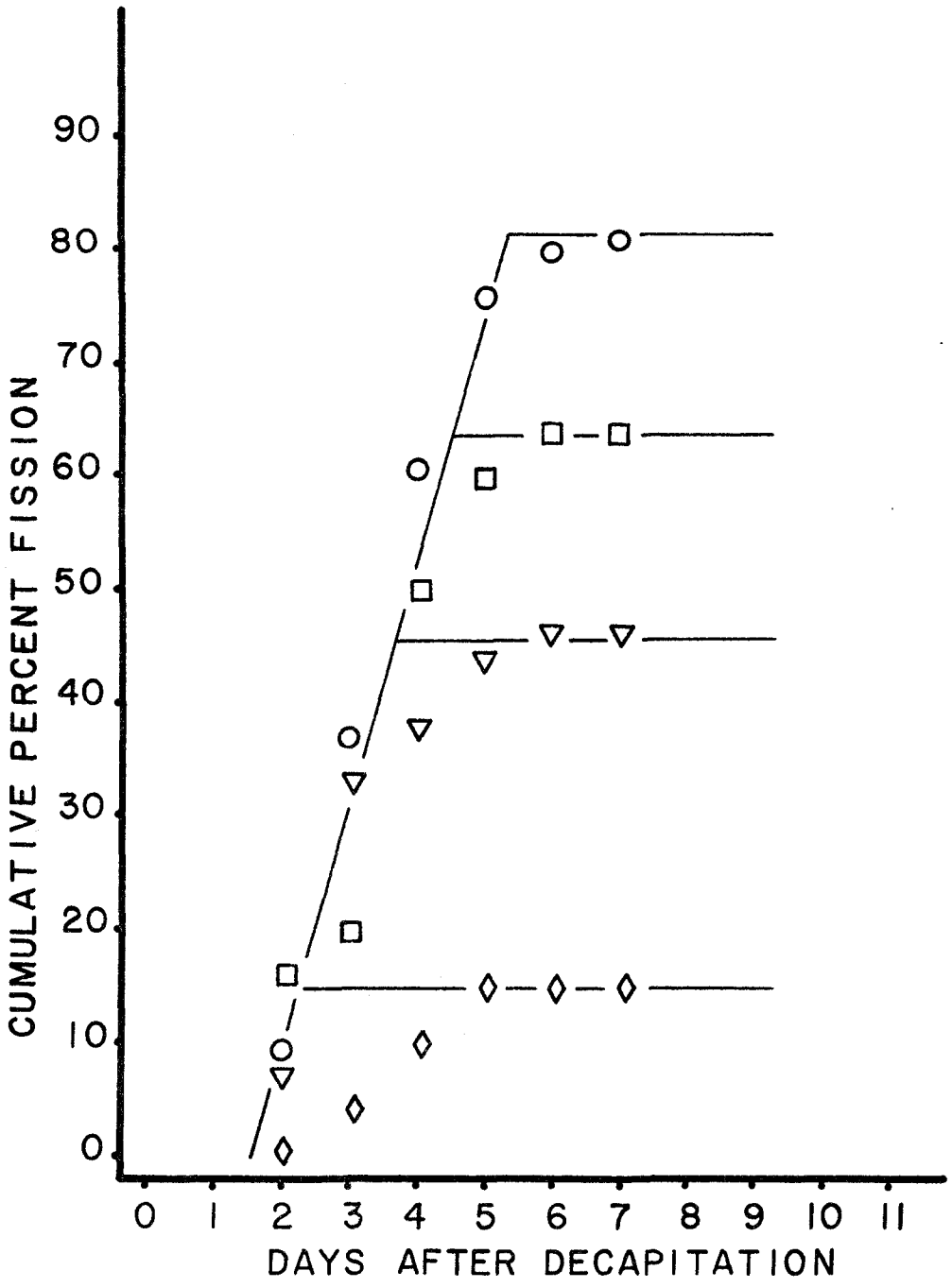
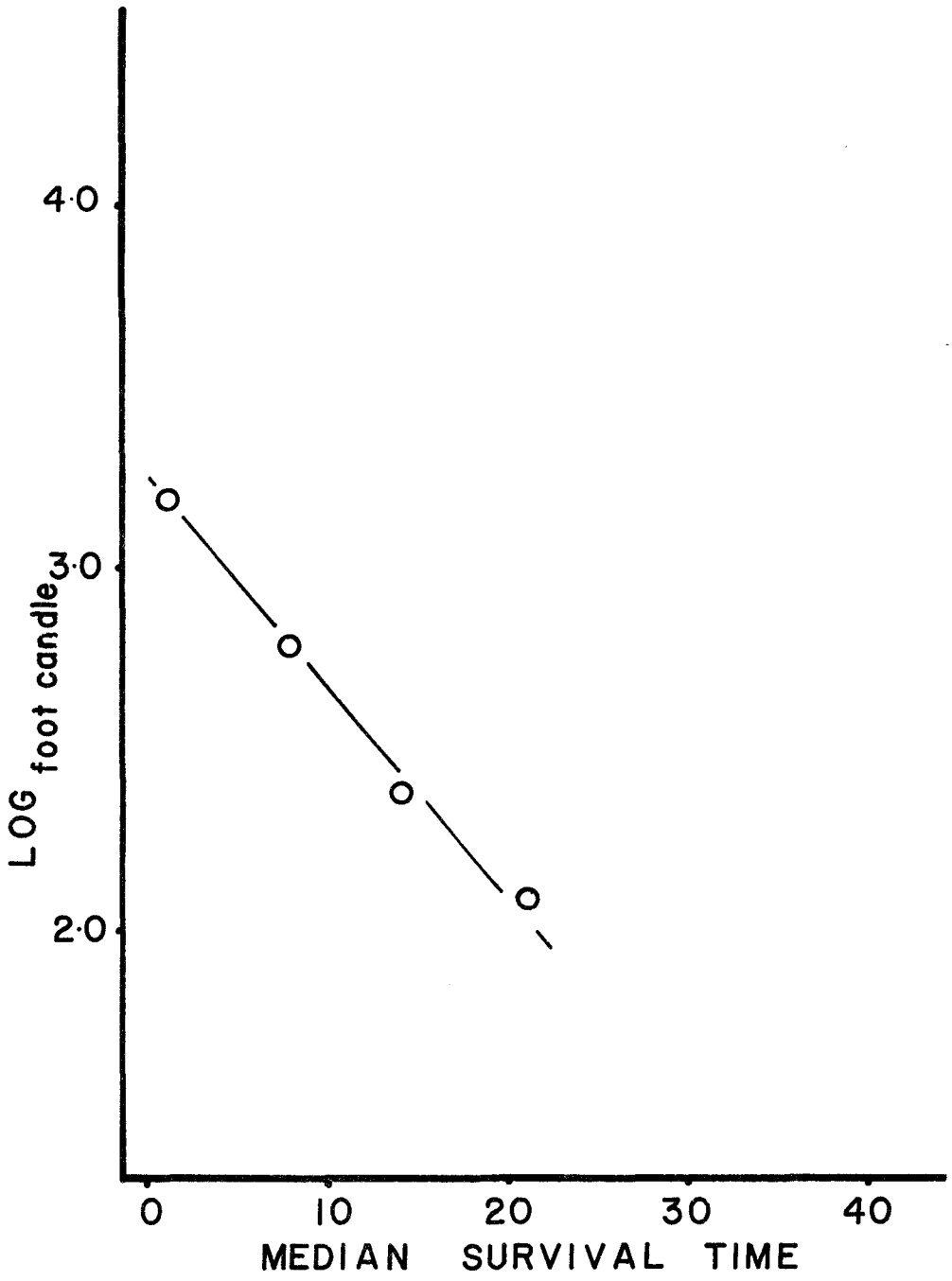

CUMULATIVE PERCENT FISSION
90
80
70
60
50
40
30
20
10
0
0 1 2 3 4 5 6 7 8 9 10 11
DAYS AFTER DECAPITATION

Fig. 3. Effect of continuous exposure to light. Dose of illumination required to kill fifty percent by date (days).


4·0
3·0
2·0
LOG foot candle
0
10
20
30
40
MEDIAN SURVIVAL TIME

light intensity of 1700 foot-candles, one hundred percent mortality was observed by the fifth day. Survival time was lengthened as the light intensity was decreased. An extrapolation of the log plot of these data indicates that normal room lighting would cause fifty percent death by day twenty if continuous exposure were permitted. Table 2 indicates that both decapitated and non-decapitated animals are equally sensitive to light.

Antibiotics

The sensitivity of the fission process to the antibiotic penicillin is indicated in figure 4. An inverse relationship was found between the concentration of penicillin in the culture medium and the number of fissions induced by decapitation.

The control group, containing no penicillin in the medium, showed sixty-three percent fission by day five, whereas 23 units per ml of penicillin, added to the medium, permitted only forty percent of the animals to fission. Ninety-five units of this antibiotic further reduced this number to ten percent. These data demonstrate that 100 units of penicillin (the amount used by many investigators as a bacteriostatic agent in planarian cultures) completely suppresses decapitation-induced fission. Penicillin concentrations above 100 units produce an increase in mortality (Fig. 5). At 1500 units of this antibiotic sixty percent of the treated animals were dead by the fifth day.

Table 2. Light sensitivity of decapitated D. dorotocephala as expressed by cumulative percent fission on days following decapitation.

Days	Decapitated		Non-decapitated	
	Light[1]	Dark[2]	Light[1]	Dark[2]
0	0	0	0	0
1	0	0	0	0
2	-10	0	-5	+5
3	-35	+20	-55	+5
4	-45	+50	-70	+10
5	-60	+55	-80	+15
6	-65	+60	-85	+15
7	-65	+60	-90	+15

[1]Cumulative percent mortality due to high light intensity (approximately 500 foot-candles).

[2]Cumulative percent fission while in indirect low intensity light (approximately 5 foot-candles).

Fig. 4. Inhibition of decapitation-induced fission due to penicillin at different concentrations (units) per ml of incubation medium. (Cumulative fission by the 5th day post-decapitation)

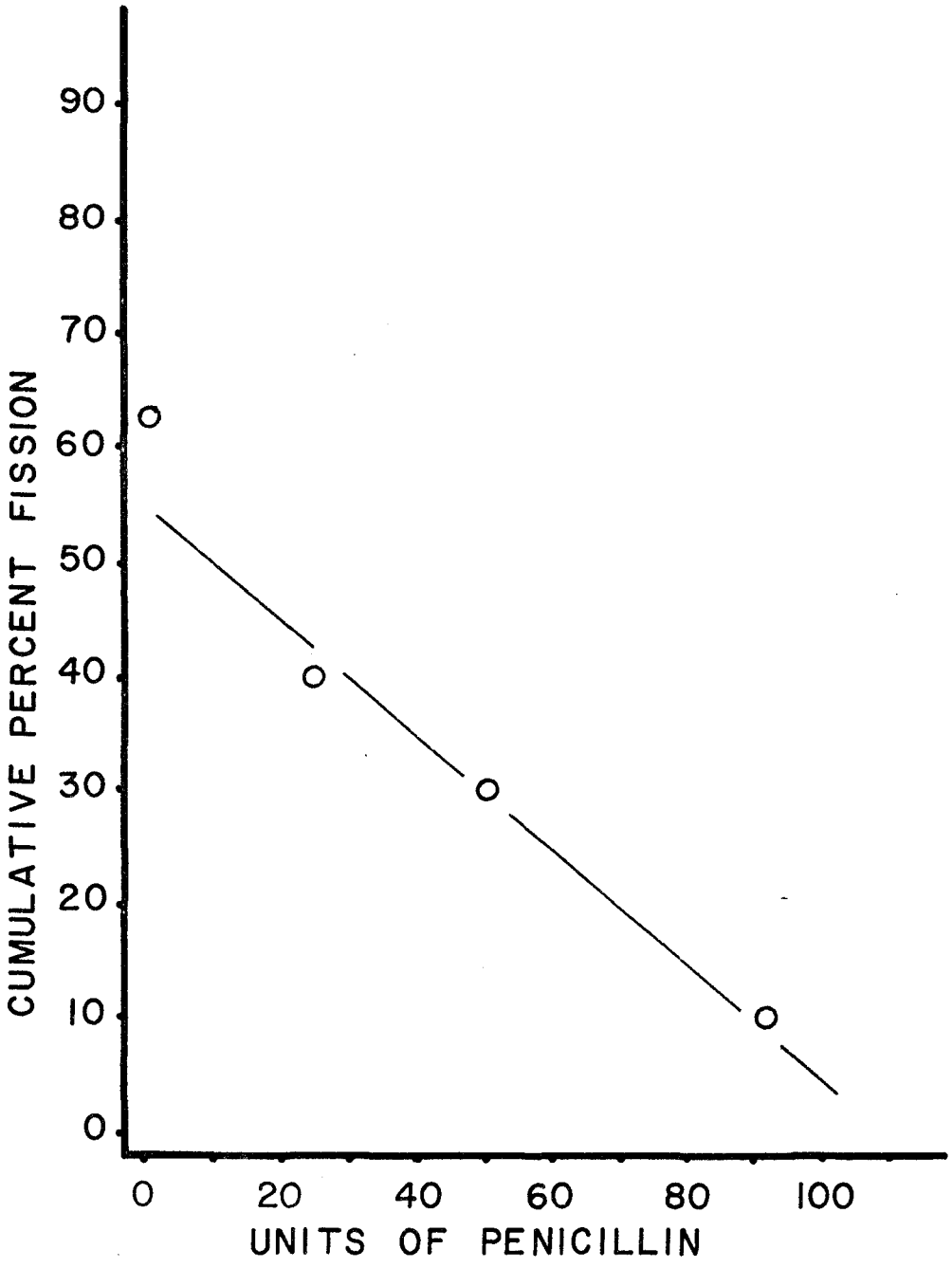
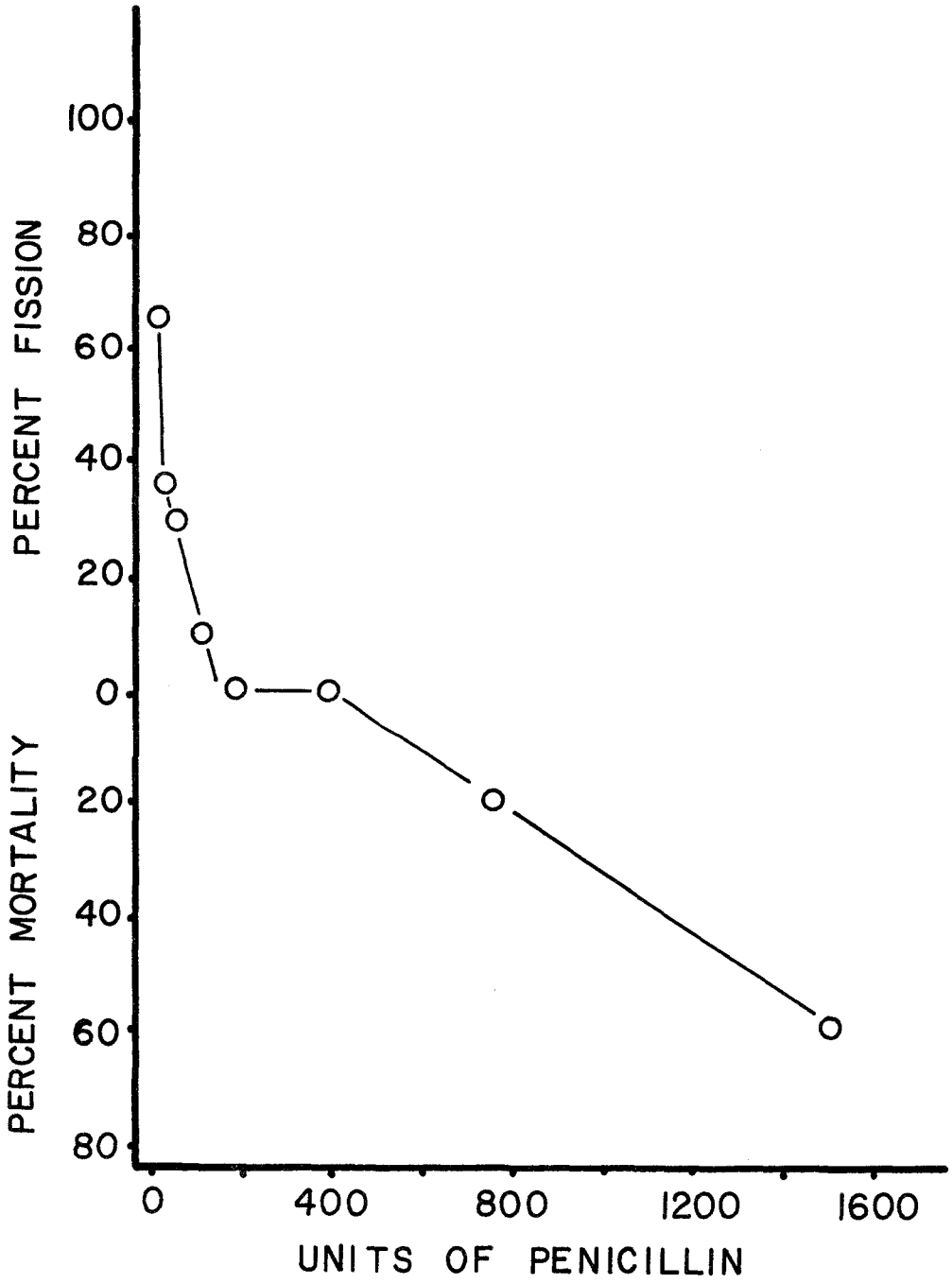

CUMULATIVE PERCENT FISSION
90
80
70
60
50
40
30
20
10
0
0
20
40
60
80
100
UNITS OF PENICILLIN

Fig. 5. Effect of penicillin on decapitation-induced fission and viability of decapitated planaria incubated individually. Ordinate is the percent mortality and percent fission by the fifth day post-decapitation. Absissa is the units of penicillin per ml of incubation medium.


PERCENT FISSION
100
80
60
40
20
0
PERCENT MORTALITY
20
40
60
80
0
400
800
1200
1600
UNITS OF PENICILLIN

Feeding

The fission rate of decapitated planaria was predictable during the summer of 1971 during which time experimental variability was within five percent of the cumulative mean of all control experiments. Later a marked change was observed (fall 1971) in which the fission rate became irregular and generally lower. The non-decapitates were beginning to fission at a higher than normal rate and in some cases as high as the decapitated animals. A group of 50 non-decapitated animals were incubated daily (one animal per plate) in an attempt to determine what was causing this pattern change (Fig. 6). The percent fission per day among these non-decapitates was irregular from the last of October through the middle of November at which time the fission rate became more constant.

When the fission rate among non-decapitates decreased in November, there was an increase in the number of cocoons produced by the stock cultures. This occurence decreased during December and ceased by the first of January 1972. Simultaneous with cocoon production, there was a significant increase in the number of sexual animals (more than 2mm wide, lethargic and exhibiting a gonopore).

Spontaneous fission rate in non-decapitated animals was compared with the fission rate induced by decapitation (Fig. 7). The population in which fission is induced by decapitation is more synchronous (fissions occur during a shorter time period) than those which fission spontaneously.

Further differentiation between fission induced by decapitation and spontaneous fission can be made by determining the

Fig. 6. Daily spontaneous fission rate (isolation-induced) associated with season. Ordinates is the cumulative percent fission per day of 1100 individually incubated planaria.

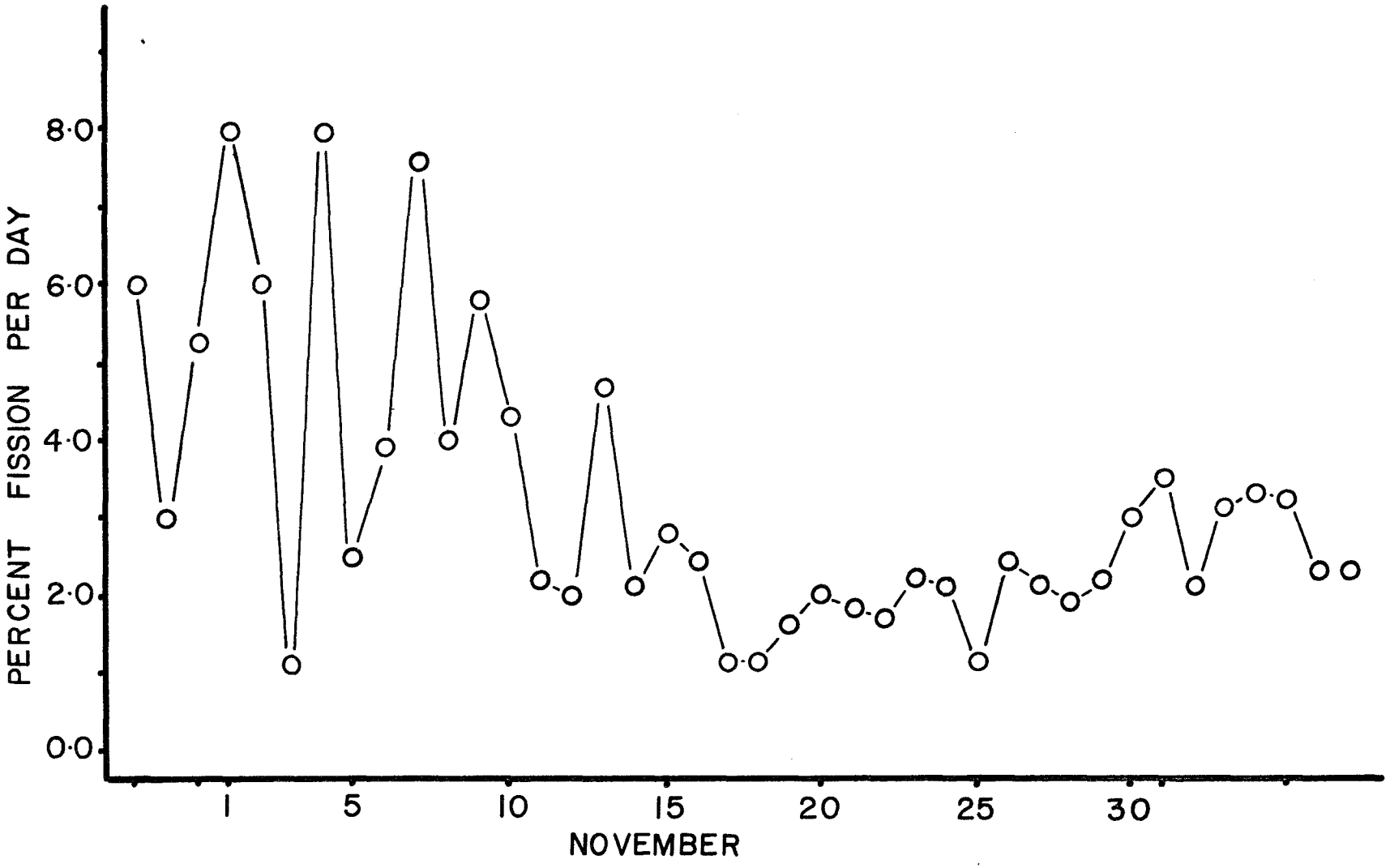
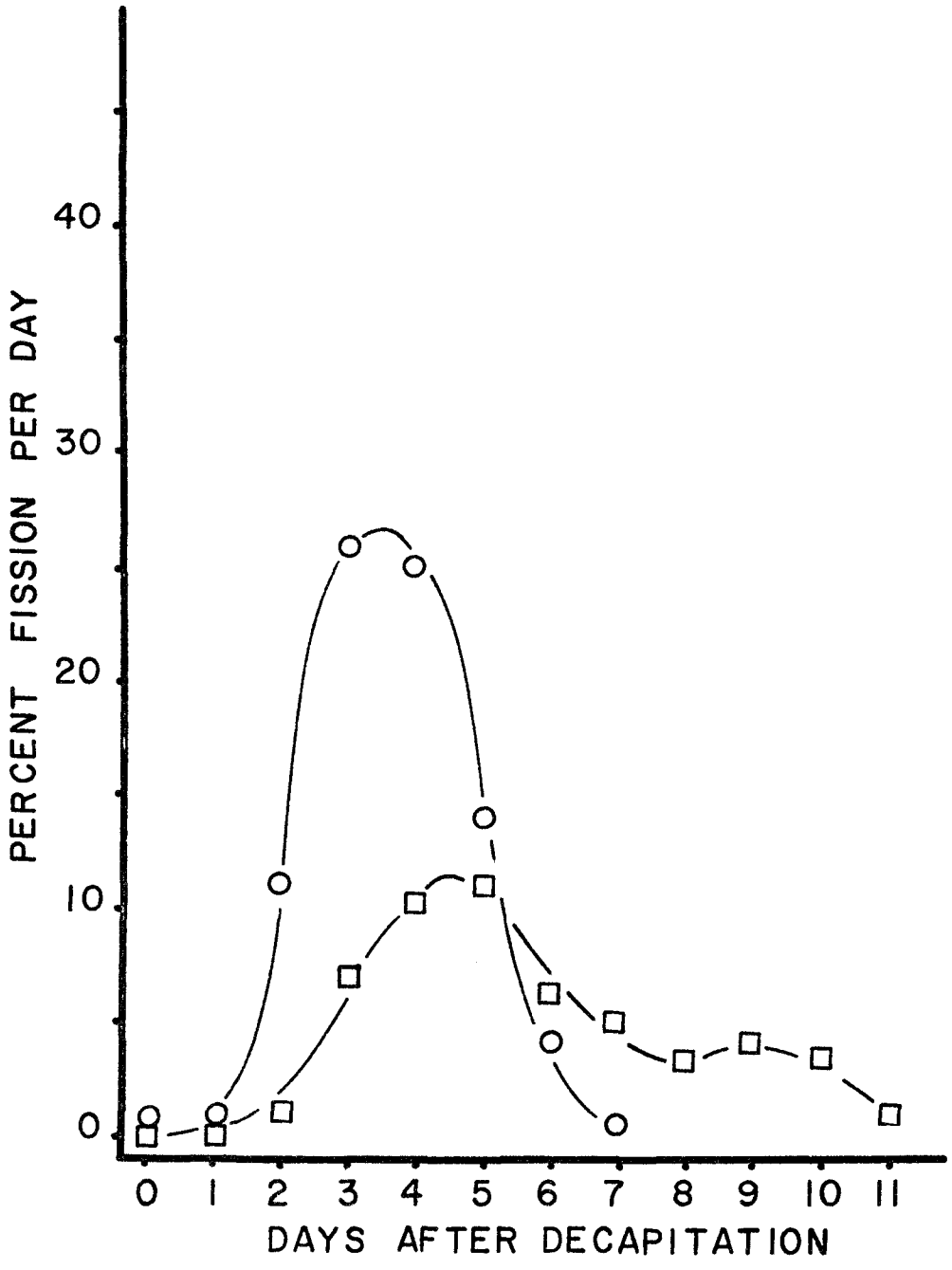

PERCENT FISSION PER DAY
8·0
6·0
4·0
2·0
0·0
1
5
10
15
20
25
30
NOVEMBER

Fig. 7. Comparison of fission mode which was induced by decapitation ○ and spontaneous fission in non-decapitates □ which were incubated individually.


PERCENT FISSION PER DAY
40
30
20
10
0
0 1 2 3 4 5 6 7 8 9 10 11
DAYS AFTER DECAPITATION

theoretical time of fission-onset. A line, derived by the method of the least square (Spiegel, 1961) using data points beginning the second day after the observed onset of fission up to either the termination of the experiment or the end of the log phase, is extrapolated to the absissa or base. The point of intersection of this line and the base, for decapitated animals is, with few exceptions, between day 1.5 and day 3 (Fig. 8). This point is in contrast to the non-decapitated group in which the intersection is day zero.

Intersection of the least square line and the absissa seems to be more consistant than the observed day of fission-onset. Even when the observed fission rate of non-decapitates is greater than decapitates the least square line of the non-decapitates intersects at day zero, when the first two days of observed fission are excluded (Fig. 9).

The feeding routine was identified as a major factor which contributed to spontaneous fission in non-decapitated planaria. Prior to day two (Fig. 10) the animals were fed on Mondays, Wednesdays and Fridays. During this time (November 13 through December 10) the average fission per day in the population of 1100 animals was three percent (Fig. 6). However, beginning December 10 (day 3 Fig. 10) the interval between feedings was progressively lengthened up to seven days. The alteration in feeding routine was associated with a greater variation in the percent spontaneous fission per day. The previous range was between 1.5 and 3.0 percent with little daily fluctuation. However, during this period of lengthened feeding interval the fission ranged from 0.8 to 5.0 percent.

Fig. 8. Comparison of fission rate in decapitated ○ versus non-decapitated planaria □ which were incubated individually.

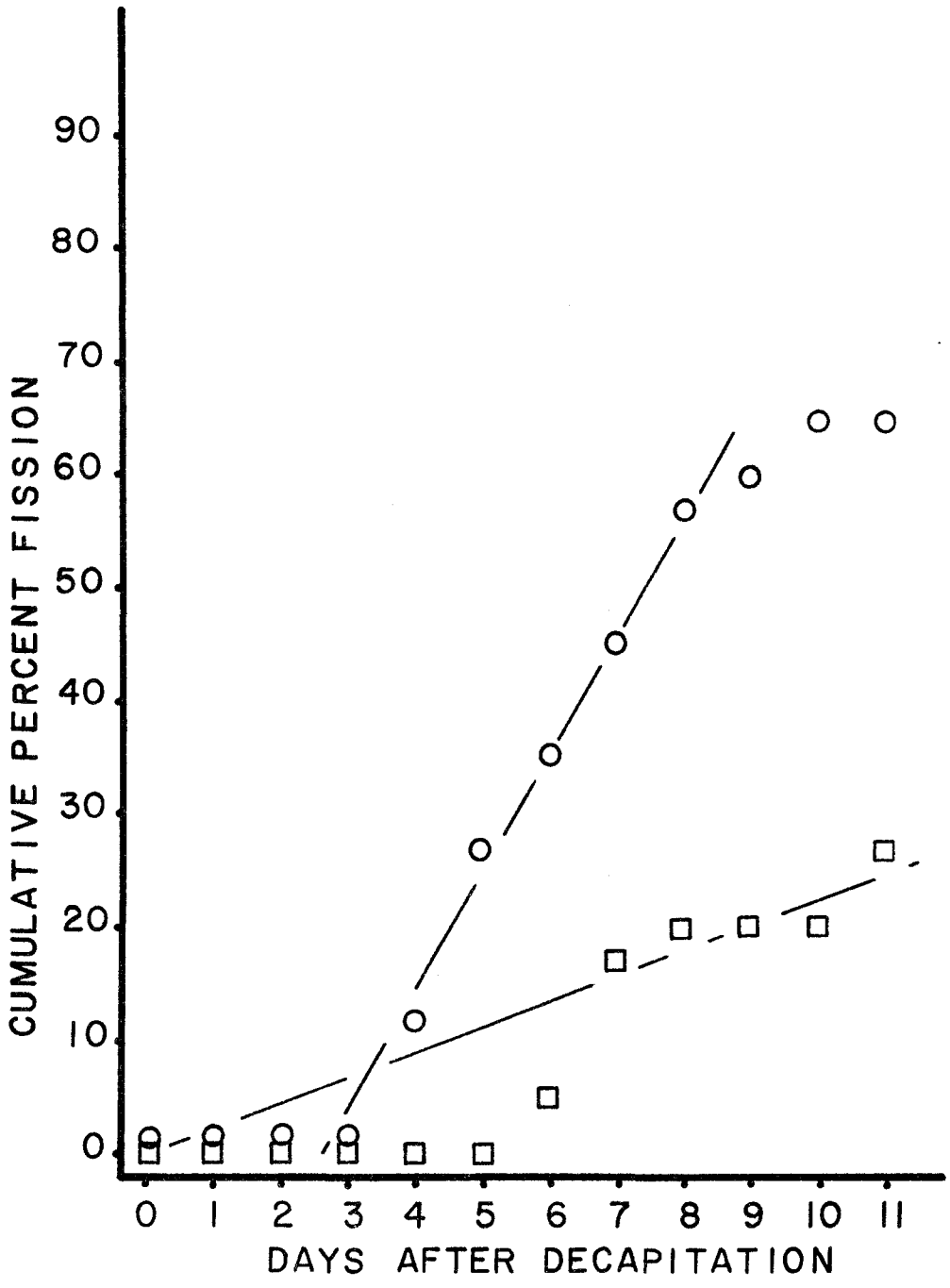

CUMULATIVE PERCENT FISSION
90
80
70
60
50
40
30
20
10
0
0 1 2 3 4 5 6 7 8 9 10 11
DAYS AFTER DECAPITATION

Fig. 9. Fission observed in non-decapitates O corrected by the method of the least-square ▽. Correction involved making $\bar{Y}$ equal to zero, in the formula:

$$Y = \bar{Y} + \left(\Sigma\ XY \,/\, \Sigma\ X^2 \right) X$$

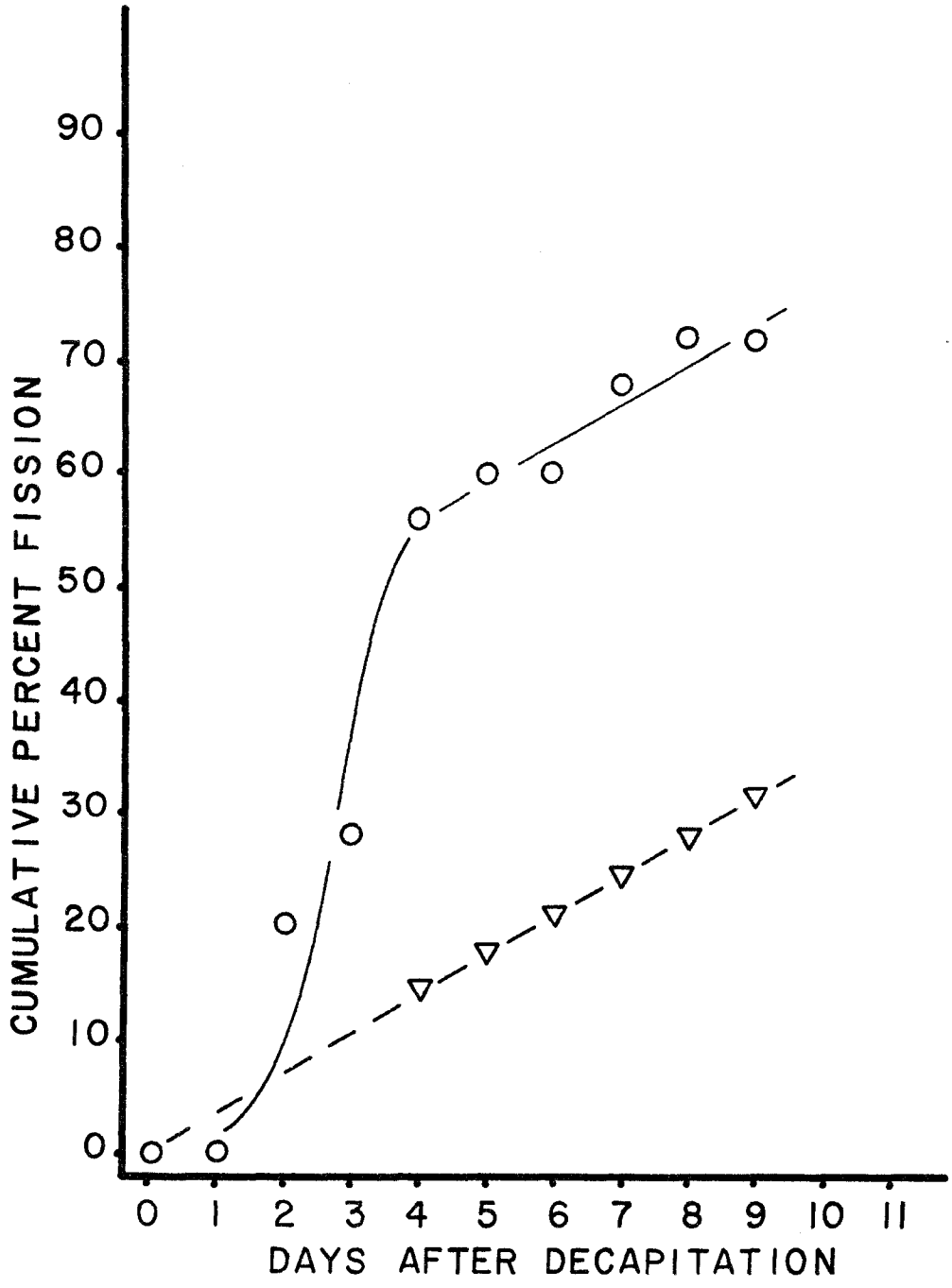
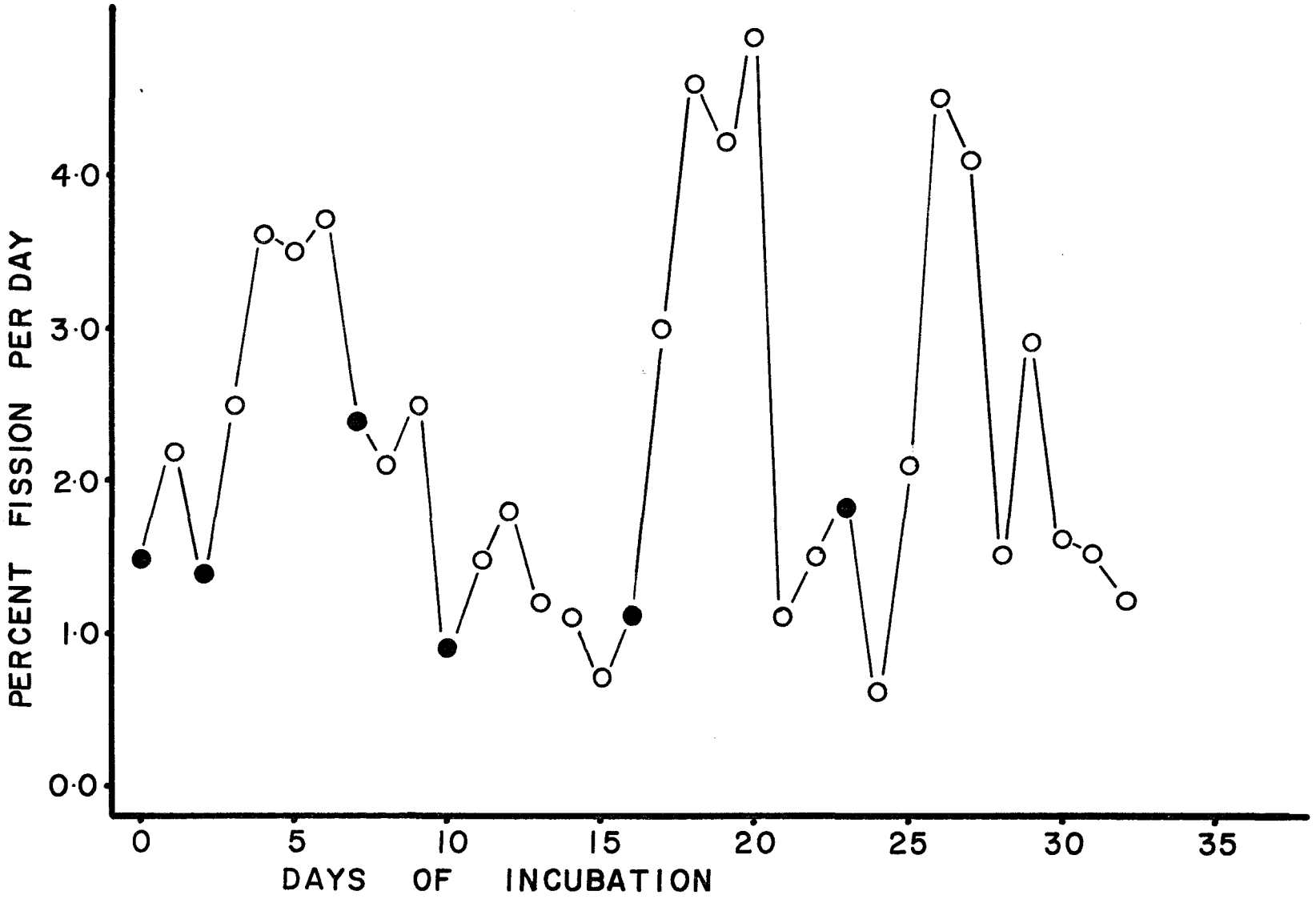

CUMULATIVE PERCENT FISSION
90
80
70
60
50
40
30
20
10
0
0 1 2 3 4 5 6 7 8 9 10 11
DAYS AFTER DECAPITATION

Fig. 10. Correlation of feeding sequence and spontaneous (isolation-induced) fission ○ . Day of feeding ● .


PERCENT FISSION PER DAY
4·0
3·0
2·0
1·0
0·0
0
5
10
15
20
25
30
35
DAYS OF INCUBATION

When the stock planaria were fed on day 10, then incubated individually, a fission peak occured two days later on day 12 (Fig. 10). This pattern was repeated in association with feedings on day 16 and day 23. Further demonstration of this relationship between feeding and the onset of spontaneous fission in non-decapitated animals is shown in figure 11. These data compare the percent fission on the fifth day after isolation of the animals, with the number of days between feeding and isolation. Of the animals which were incubated individually on the same day they were fed, ninety percent had fissioned by the fifth day. This is compared with an average of fourteen percent cumulative fission when fed 3 or more days prior to isolation.

Prolonged fasting (40 days) did not alter the fission rate of non-decapitated animals as compared with non-fasted controls (Fig. 12). However, when animals fasted 40 days were fed the fission rate was promptly stimulated. These non-decapitated animals which were starved and then fed one day prior to the experiment had a fission rate increase approximately two and one-half times greater than the control (experimentals 7.2 percent per day; controls 2.8 percent per day). Decapitation (Fig. 13) did not alter the rate difference (4 percent per day) only the absolute values (experimental 10 percent per day; control 6 percent per day).

Fig. 11. Decrease in spontaneous fission as a function of time after feeding. Cumulative percent fission on the fifth day after isolation of non-decapitated planaria is the ordinate. Absissa is the days after feeding before isolation of the animals.

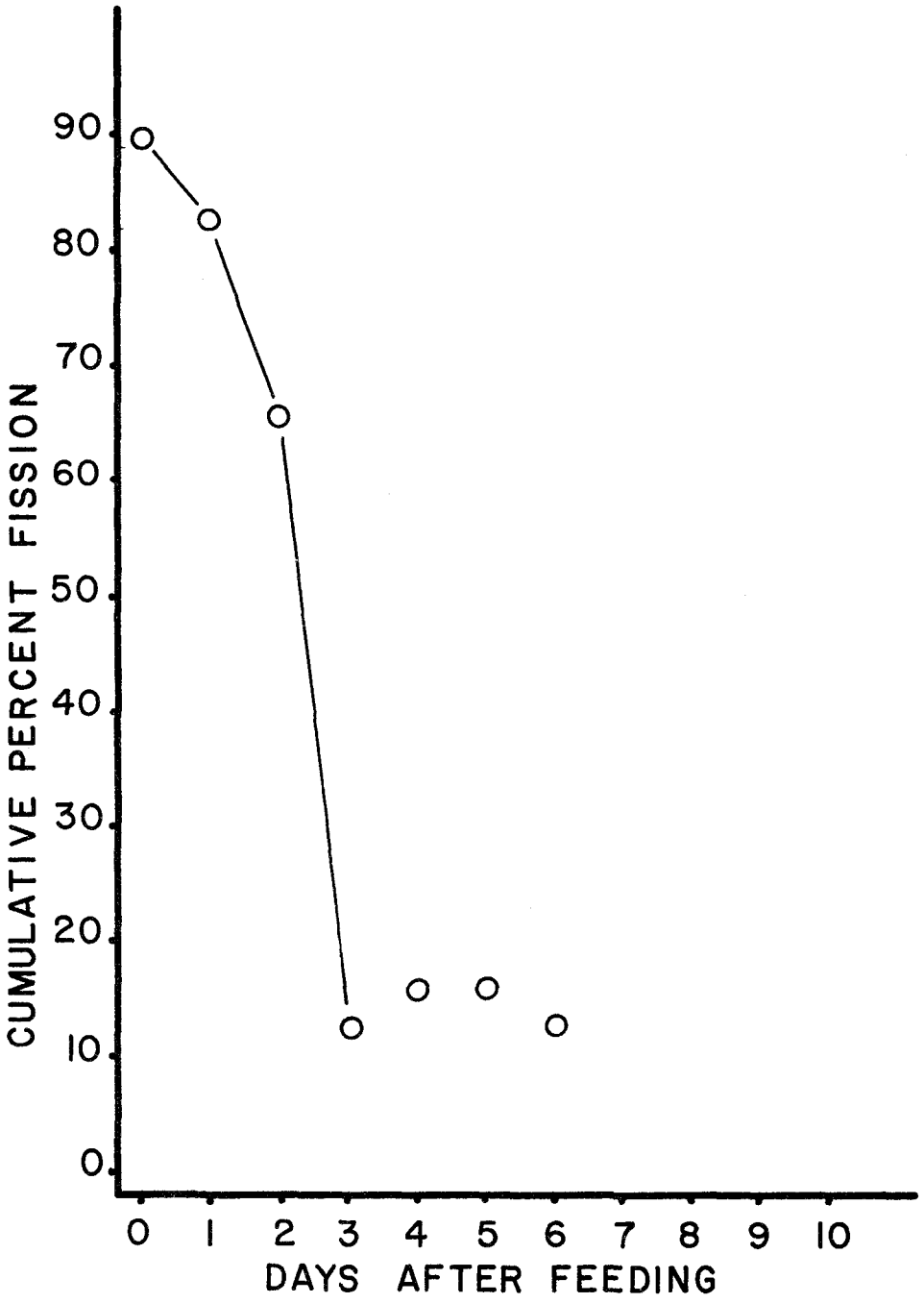

CUMULATIVE PERCENT FISSION
90
80
70
60
50
40
30
20
10
0
0 1 2 3 4 5 6 7 8 9 10
DAYS AFTER FEEDING

Fig. 12. Influence of feeding on non-decapitated planaria individually incubated, which have been fasted 40 days □ and then fed 1 day prior to the experiment ○ and non-fasted but fed controls ▽ .

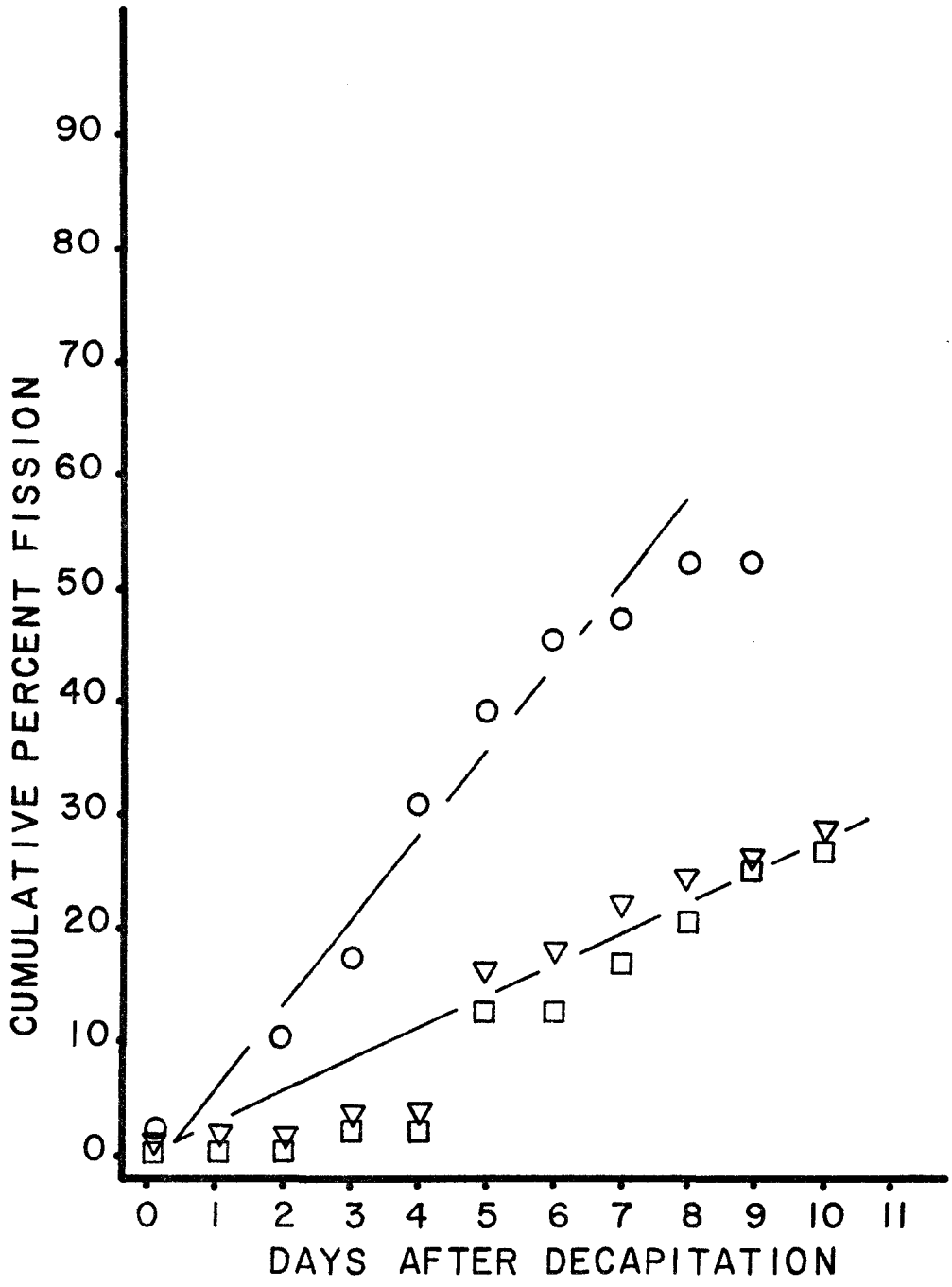
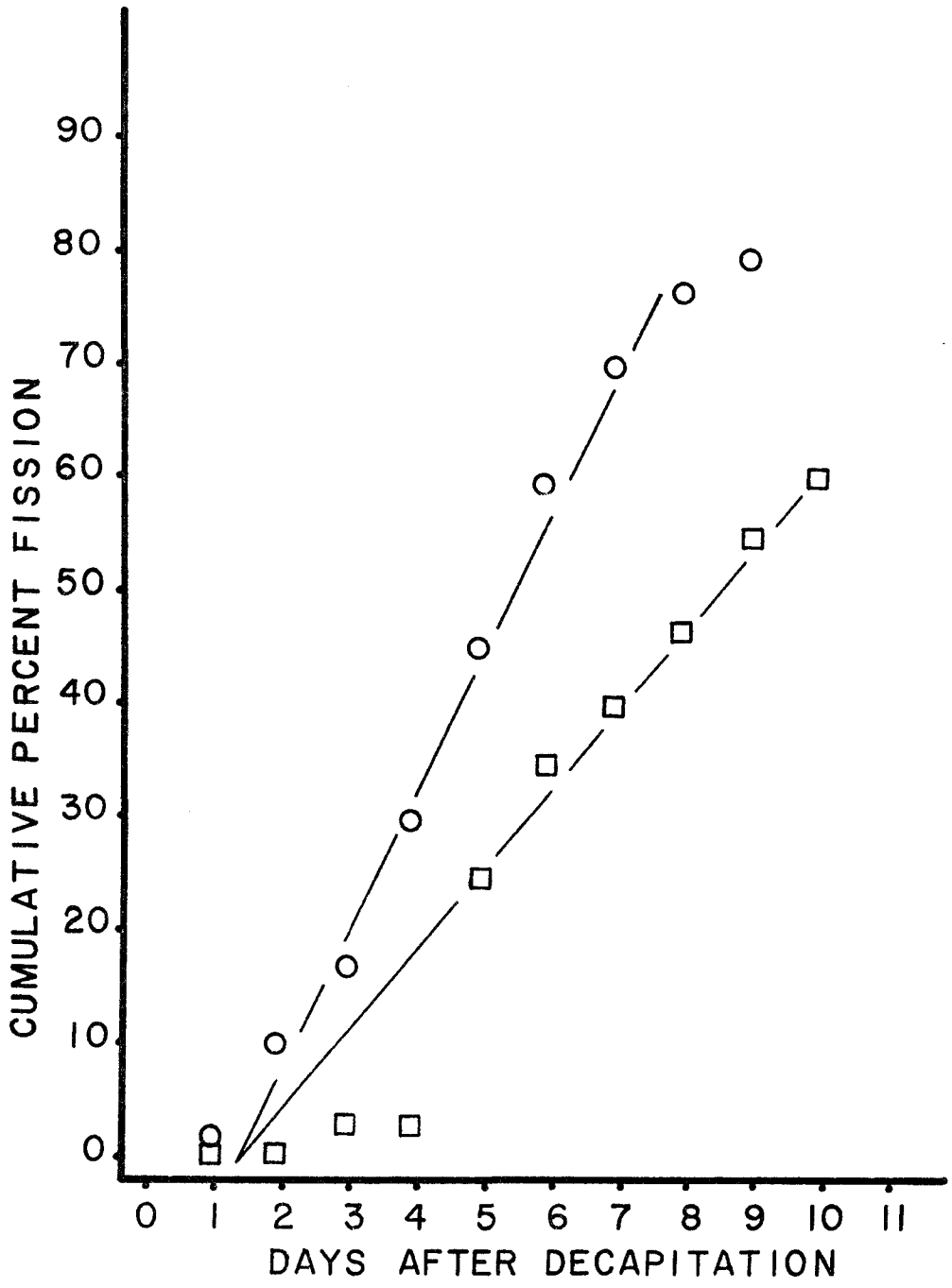

CUMULATIVE PERCENT FISSION
90
80
70
60
50
40
30
20
10
0
0 1 2 3 4 5 6 7 8 9 10 11
DAYS AFTER DECAPITATION

Fig. 13. Fission of decapitated animals fasted 40 days □ compared with those fasted 40 days then fed one day before decapitation ○ .


CUMULATIVE PERCENT FISSION
90
80
70
60
50
40
30
20
10
0
0 1 2 3 4 5 6 7 8 9 10 11
DAYS AFTER DECAPITATION

Animal Interactions

A comparison between decapitation-induced fission rates of isolated (one animal per culture dish) and grouped cultures (five or more animals per culture dish) strongly suggest an animal interaction mechanism. In our early experiments we found that non-decapitated animals isolated in groups of ten had a fission rate of less than two percent per day compared to a decapitation-induced fission rate of over eighteen percent per day between days three and six (Fig. 14). However, when the animals were incubated individually (one animal per plate) a fission rate in excess of five percent per day was observed for non-decapitates with sixteen percent per day among decapitates (Curve -0-0- Fig. 16).

The cumulative percent fission of the decapitated animals incubated in groups of ten was eighty percent by the seventh day (Fig. 14) compared to thirteen percent for the non-decapitated groups of ten. However, the isolated non-decapitates exhibit thirty-two percent fission by the seventh day (Fig. 15).

The effect of grouping decapitated animals (five animals per plate) is illustrated further in figure 16. Twenty percent had fissioned by day five when the decapitated animals were in cultures of five animals per plate compared to seventy-eight percent when cultured singly. In addition to the over-all suppression of fission observed in grouped cultures, there was a delay of 3.5 days in the mode of fission-onset (Fig. 17).

The suppression of fission associated with grouping is not the same as that associated with over-crowding (Fig. 18). When the

Fig. 14. Comparison of decapitation ○ versus non-decapitation □ when animals are in groups of ten per culture plate.

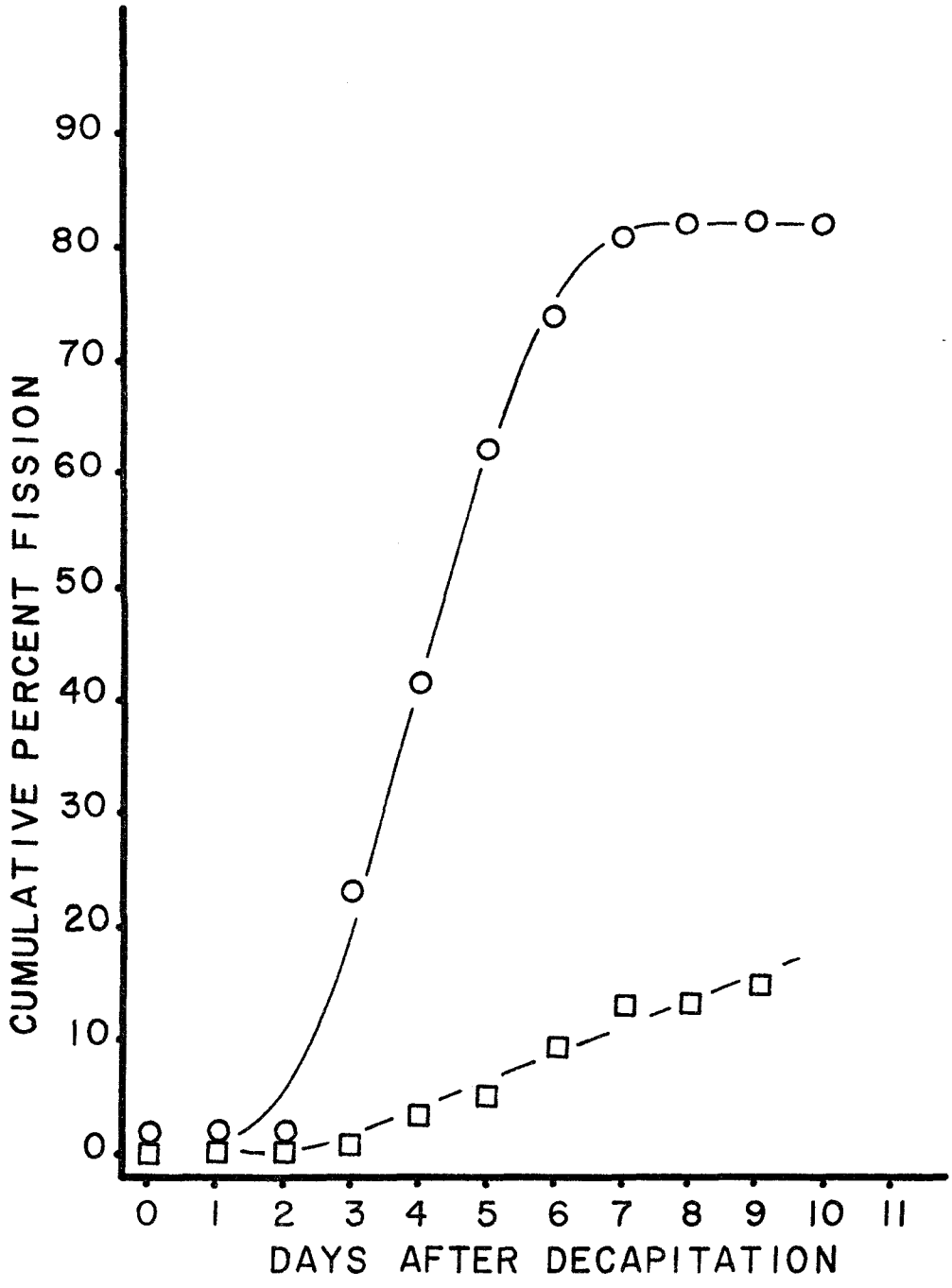

CUMULATIVE PERCENT FISSION
90
80
70
60
50
40
30
20
10
0
0 1 2 3 4 5 6 7 8 9 10 11
DAYS AFTER DECAPITATION

Fig. 15. Comparison of decapitates ○ , versus non-decapitates □ when animals are incubated one animal per culture dish.

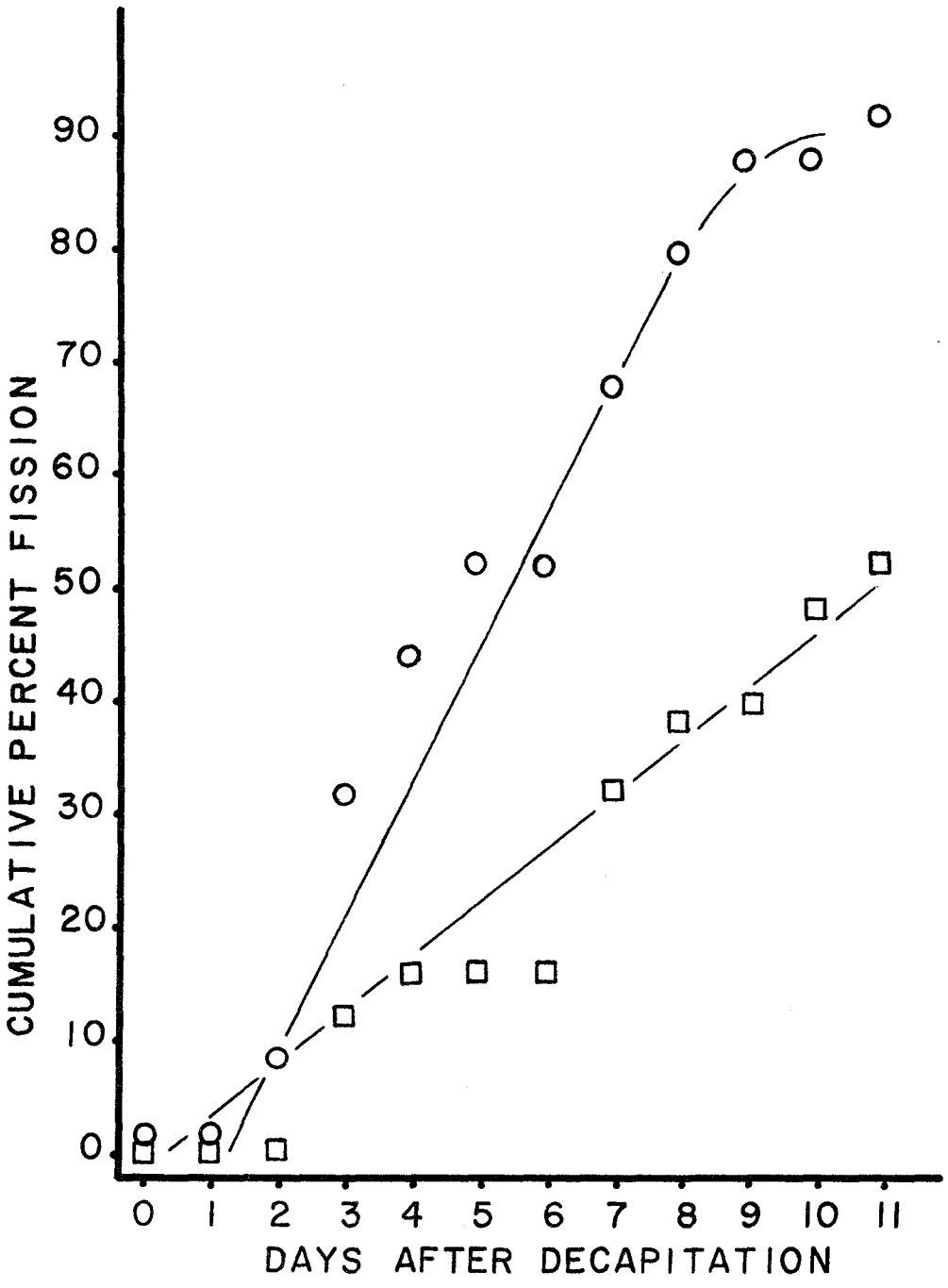

CUMULATIVE PERCENT FISSION
0
10
20
30
40
50
60
70
80
90
0 1 2 3 4 5 6 7 8 9 10 11
DAYS AFTER DECAPITATION

Fig. 16. Comparison of fission when animals are incubated one animal per culture plate ○ versus five animals per culture plate □ .

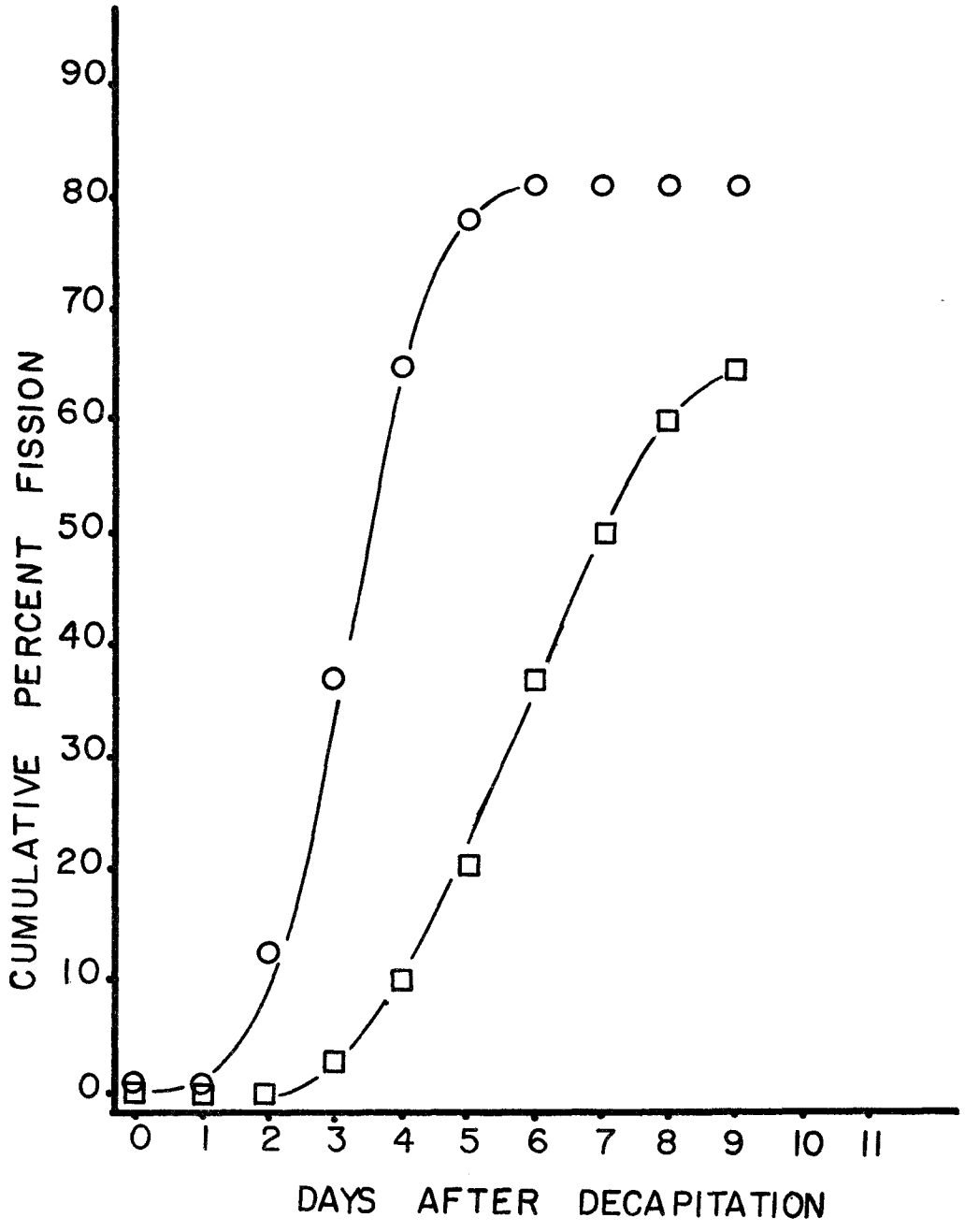

CUMULATIVE PERCENT FISSION
90
80
70
60
50
40
30
20
10
0
0 1 2 3 4 5 6 7 8 9 10 11
DAYS AFTER DECAPITATION

Fig. 17. Comparison of fission mode when animals are incubated one animal per culture plate ○ versus five animals per culture plate □.

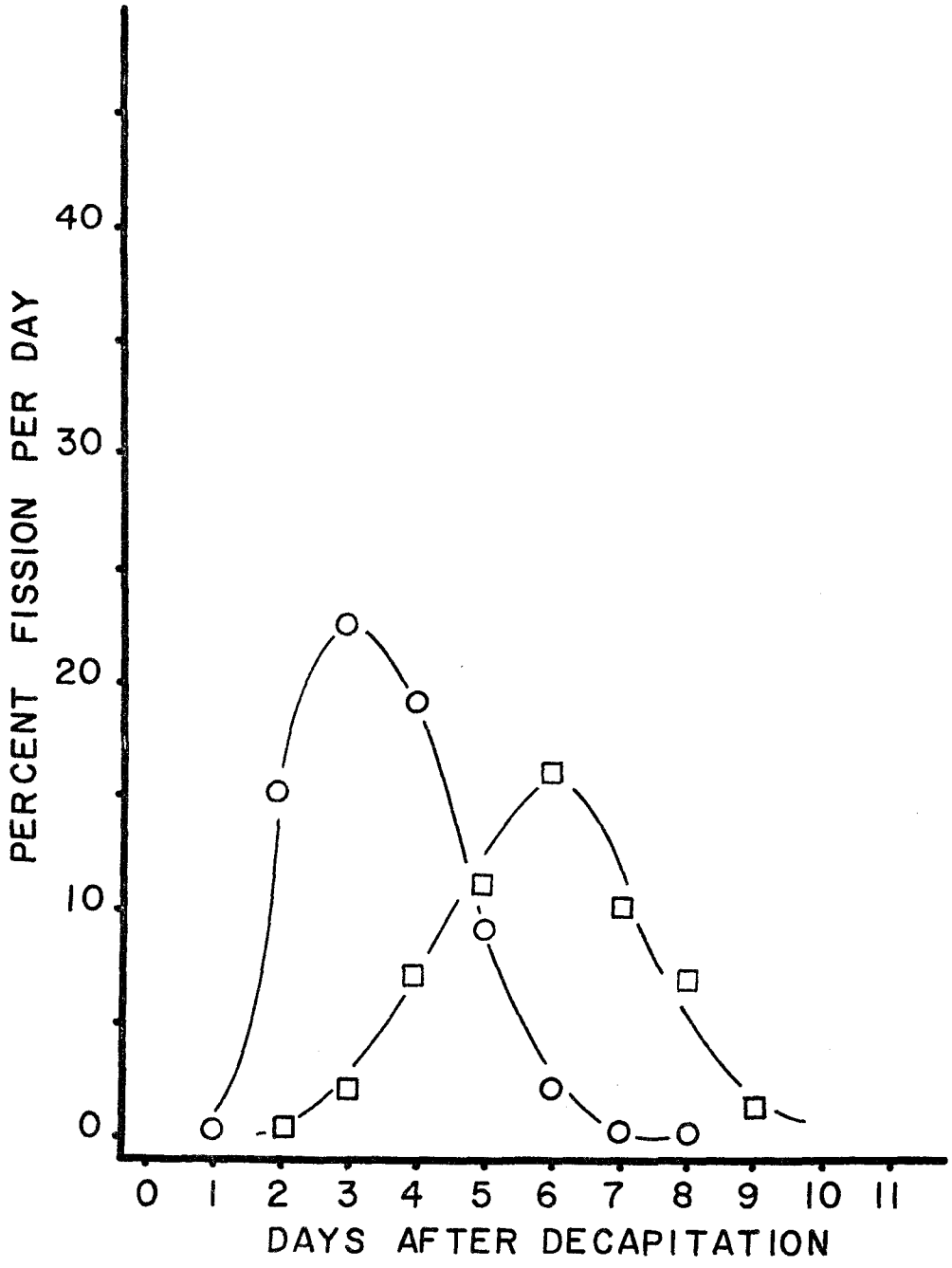
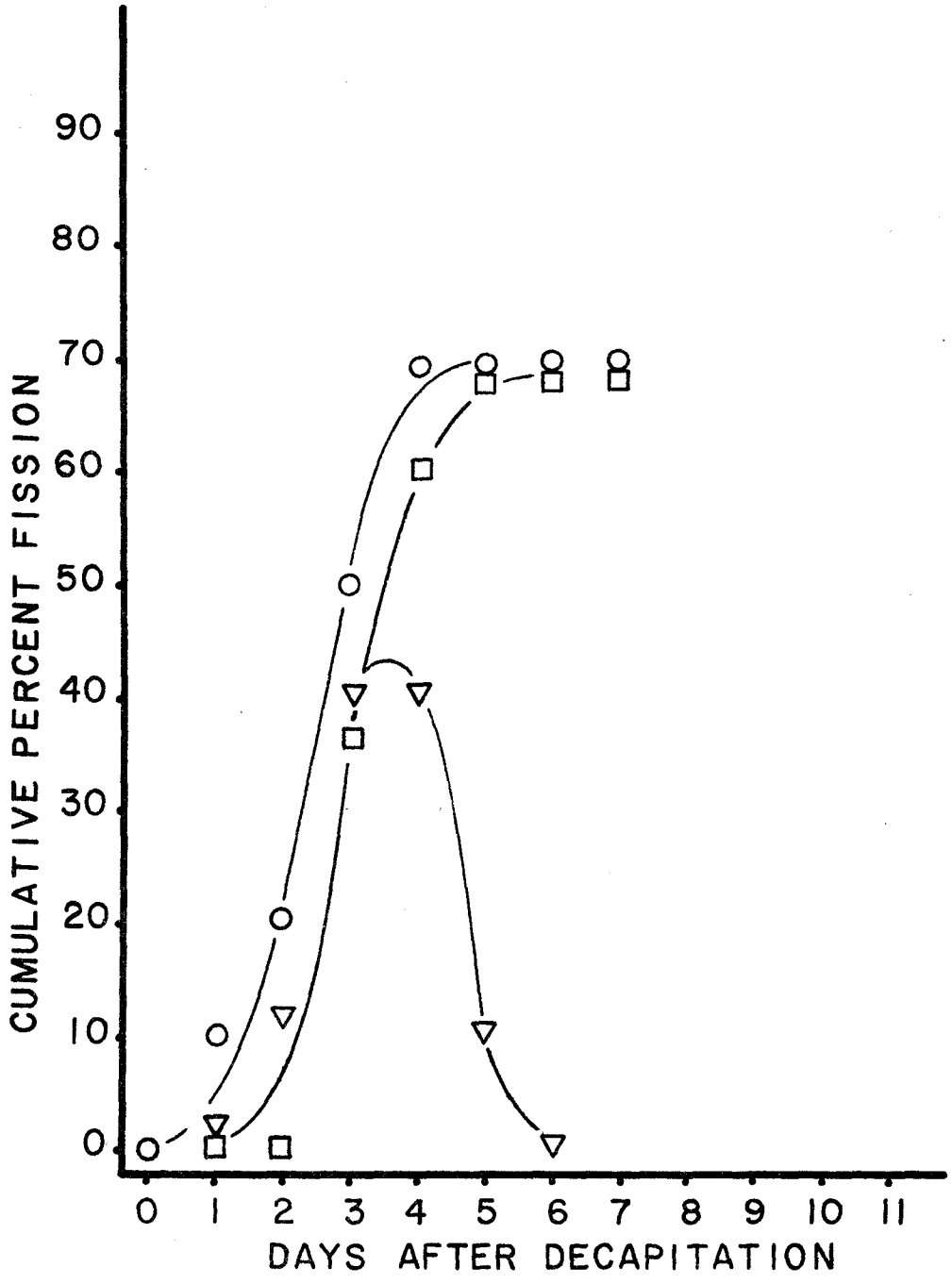

PERCENT FISSION PER DAY
40
30
20
10
0
0 1 2 3 4 5 6 7 8 9 10 11
DAYS AFTER DECAPITATION

Fig. 18. Effect of crowding in fresh Henderson-Eakin medium; one animal per 5 ml ○ , five animals per 10 ml (one per 2.5 ml) □ and five animals per 5 ml (one per 1 ml) ▽ .


CUMULATIVE PERCENT FISSION
90
80
70
60
50
40
30
20
10
0
0 1 2 3 4 5 6 7 8 9 10 11
DAYS AFTER DECAPITATION

animal density was high (five animals per 5 ml) death began on the fourth day of incubation but there was no increase in the latent period. Prior to the fourth day the fission rate had been about the same as the control group (one animal per 5 ml). At an intermediate animal density of 2.5 animals per 5 ml (five animals per 10 ml) there was no death observed by the sixth day and the cumulative fission was the same as the control group. When the animals were cultured individually the generation time was forty-five days as compared to two hundred days for animals cultured in groups of one hundred (Fig. 19).

Table 3 not only demonstrates an animal interrelationship but illustrates (Fig. 20) an all-or-none type response among members of an incubation group. Animals in this experiment were incubated in groups of five per plate. At the end of eight days of incubation the fission patterns (number of fissions per plate per day) were grouped according to the number of animals (of the five per plate) which had fissioned.

The fission modes of each group and the controls are either at day four or day six. The control group, which was one animal per plate, had a fission mode at day 3.9. This value is similar to the mode (day 4.8) of the group in which all five (100 percent) fissioned. These values (control mode equal day 3.9 and five fissions by day eight, mode 4.8) are compared with groups in which one, two, or three of the animals fissioned with modes between day 6.2 and 6.7. The group in which four of the five animals fissioned has a bimodal curve with one mode at day 6.2 and the other at day 4.6.

Table 3. Demonstration of planaria intercommunication among animals which have been incubated in groups of 5 per petri dish.

Day[a]	Control[b]		Cumulative fissions per plate[c] 0		1	
	N[d]	%[e]	N[d]	%[e]	N[d]	%[e]
0	559	0.0	10	0.0	55	0.0
1	562	0.5	10	0.0	55	0.0
2	662	10.7	10	0.0	55	0.0
3	767	25.9	10	0.0	56	1.8
4	904	24.5	10	0.0	56	0.0
5	985	14.4	10	0.0	58	3.6
6	1008	4.1	10	0.0	61	5.4
7	1016	1.4	10	0.0	65	7.2
8	1020	0.7	10	0.0	66	1.8
Mode[f]	3.9		*		6.2	

[a]Days of incubation after decapitation.

[b]Control group had 1 animal per incubation plate.

[c]Cumulative fission per plate of 5 animals by day eight.

[d]Total number of animals.

[e]Percent fission per day.

[f]Day of maximum fission.

*Fission mode could not be determined.

Table 3. (continued)

Cumulative fissions per plate[c]							
2		3		4		5	
N[d]	%[e]	N[d]	%[e]	N[d]	%[e]	N[d]	%[e]
60	0.0	80	0.0	90	0.0	95	0.0
60	0.0	80	0.0	90	0.0	95	0.0
60	0.0	81	1.2	90	0.0	100	5.2
60	0.0	82	1.2	94	4.4	112	12.1
62	3.3	84	2.4	106	13.3	133	22.1
65	5.0	89	6.2	122	17.7	159	27.3
73	13.3	110	26.2	135	14.6	174	15.7
78	8.3	122	15.0	157	24.4	182	8.4
84	10.0	123	7.5	162	5.5	190	2.1
					4.6		4.8
6.7		6.6		6.2			

Fig. 19. Comparison of non-decapitated planaria which have been incubated individually O , and in groups of 100 □ with the same animal to fluid-volume ratio.

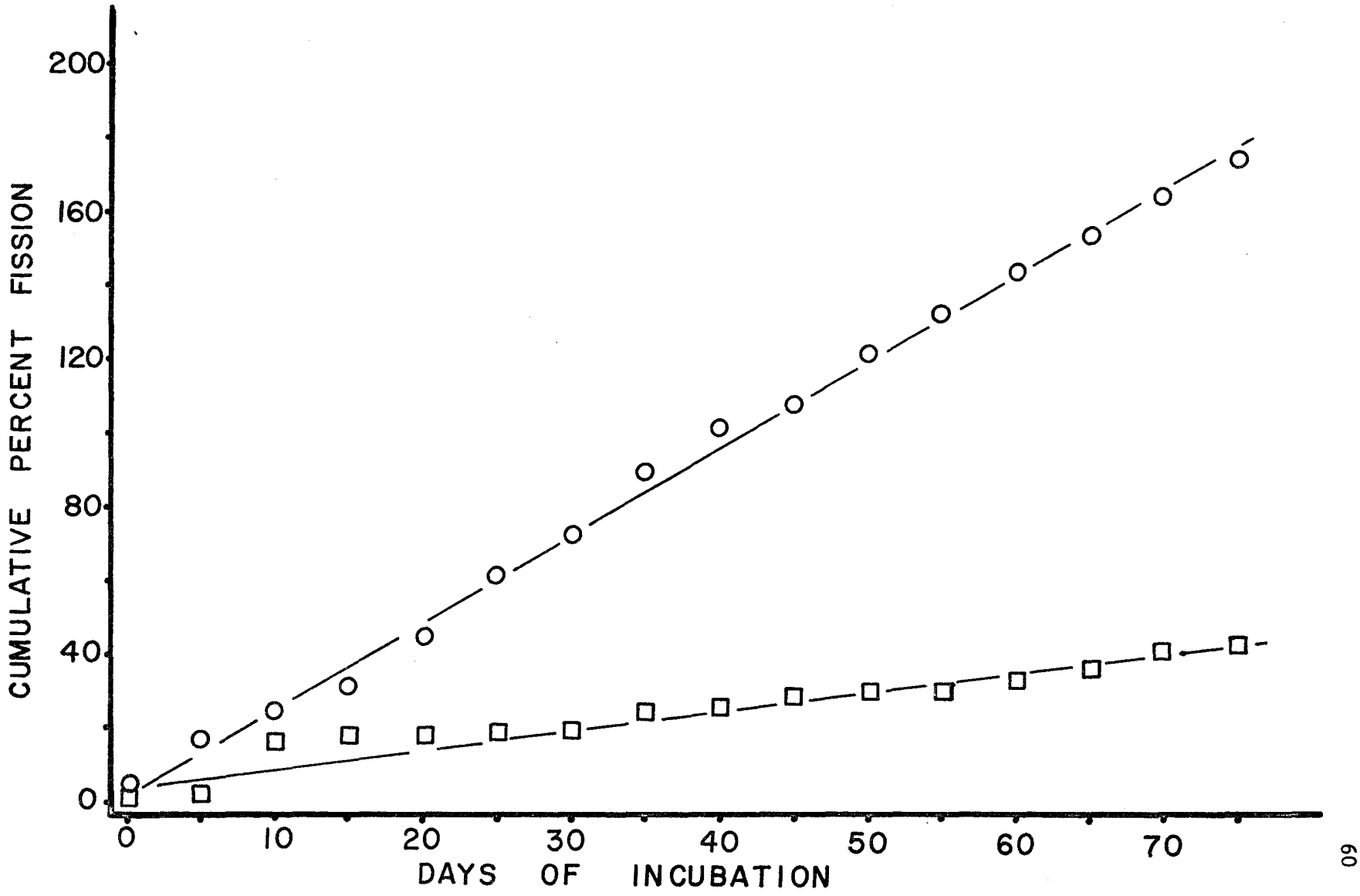
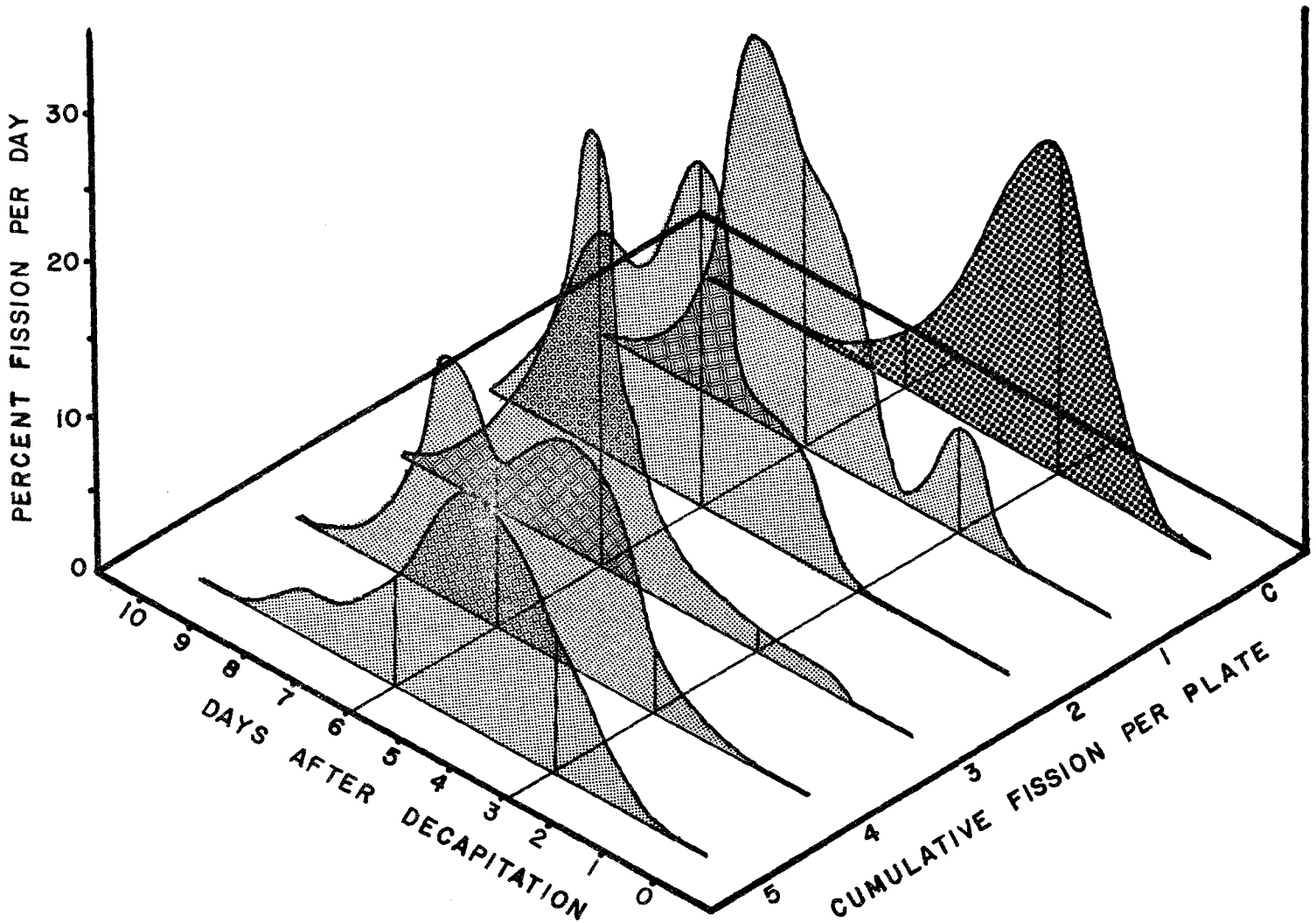

CUMULATIVE PERCENT FISSION
200
160
120
80
40
0
0
10
20
30
40
50
60
70
DAYS OF INCUBATION

Fig. 20. Graphic presentation of data in table 3 (animal intercommunication). Cumulative fissions by the eighth day after decapitation per plate of five animals in the experimental groups with a control groups of one animal per incubation plate.


PERCENT FISSION PER DAY
30
20
10
0
10 9 8 7 6 5 4 3 2 1 0
DAYS AFTER DECAPITATION
5 4 3 2 1 C
CUMULATIVE FISSION PER PLATE

The possibility of a diffusable substance influencing the fission process is seen in figure 21. When animals are cultured in medium in which animals had been previously grown for five days (conditioned medium) the fission process is accelerated. The time of fission-onset was approximately the same for animals in both experimental and control medium. However, the conditioned medium seemed to accelerate the fission rate. Eighty percent of the animals treated with the conditioned medium had fissioned by day two whereas twenty percent of the control group, in fresh medium, had fissioned by the same day. This acceleration is due to a stimulator factor in the conditioned medium which can be removed by ion-exchange chromatography.

The fission process is not only altered by a diffusable fraction of the culture medium but also by a factor related to the shape of the culture container or exposed surface. Figure 22 compares planarian fission when incubated in either petri dishes (15 mm x 16 mm) or test tubes (13 mm x 150 mm). In both groups the animals were cultured individually and the animal to fluid-volume ratio was the same (one animal per 5 ml). By the fifth day of incubation, animals in petri dishes had a cumulative percent fission of seventy percent whereas ten percent had fissioned in the test tubes.

Fig. 21. Effect of conditioned medium □ and non-conditioned medium ○ on the decapitation-induced fission process in planaria incubated individually.

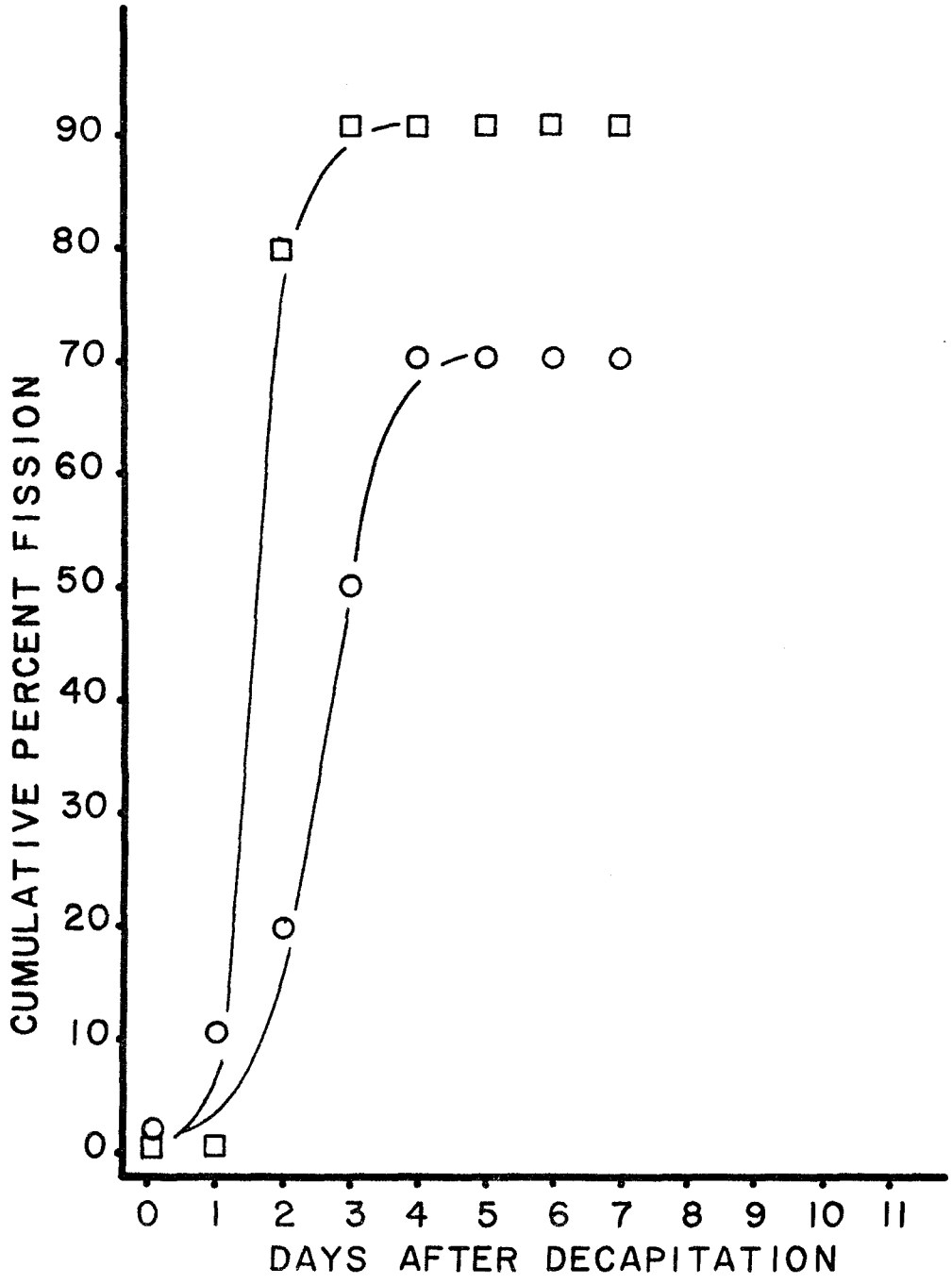
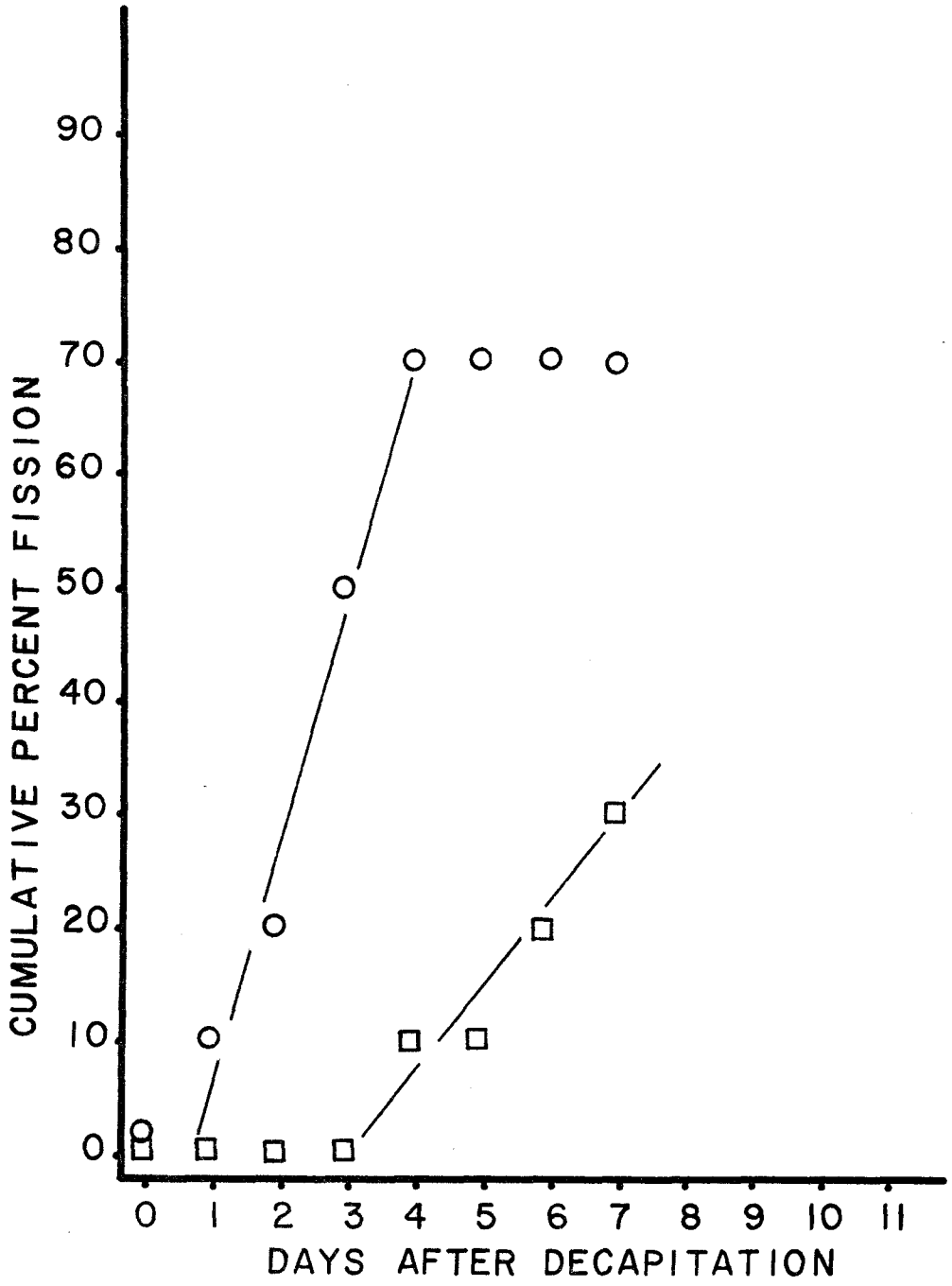

CUMULATIVE PERCENT FISSION
90
80
70
60
50
40
30
20
10
0
0 1 2 3 4 5 6 7 8 9 10 11
DAYS AFTER DECAPITATION

Fig. 22. Effect of incubation of one animal per culture unit in either petri dishes ○ or test tubes □ .


CUMULATIVE PERCENT FISSION
90
80
70
60
50
40
30
20
10
0
0 1 2 3 4 5 6 7 8 9 10 11
DAYS AFTER DECAPITATION

Intrinsic Control Of Fission

Figure 23 shows that decapitation induces fission in the asexual planaria and has no effect on the sexual race. Sexual animals were sectioned posterior to the auricles and anterior to the pharynx, and again mid-way between the tail and pharynx. The three resultant sections were allowed to regenerate for one month. After complete regeneration animals which had the normal morphology (well formed heads, pointed tails and less than 2 mm in width) were selected for analysis. Decapitation of these regenerated animals, which were originally sexual, resulted in forty-five percent fission by the tenth day, compared with five percent fission in non-decapitates which were also regenerates (Fig. 24).

A complex control of the fission process was indicated when asexual animals (one animal per plate) had undergone multiple fissions in both the parent and daughter zooids (Fig. 25, 26 and 27). Figure 25 shows a planaria which was decapitated and observed ten days later. The original decapitated animal "A" had given rise to zooid "B" and subsequently produced a second zooid "C". This sequence of zooid production is inferred from the maturity of the eyes, pharynx and tail of each animal in the dish. In another example (Fig. 26) the original decapitated planaria "A" had given rise to zooid "B" which in turn had produced its own daughter zooid "C". Animal "A" (Fig. 27) had produced zooids "B" and then "C". Zooid "B" is assumed to be more mature than zooid "C" based on the maturity of the eyes. Zooids "B" and "C" had produced zooids "D" and "E" respectively. This is concluded from the developmental stages of

Fig. 23. Comparison of decapitation-induced fission in sexual □ and asexual ○ planaria which were cultured one animal per petri dish in 5 ml medium.

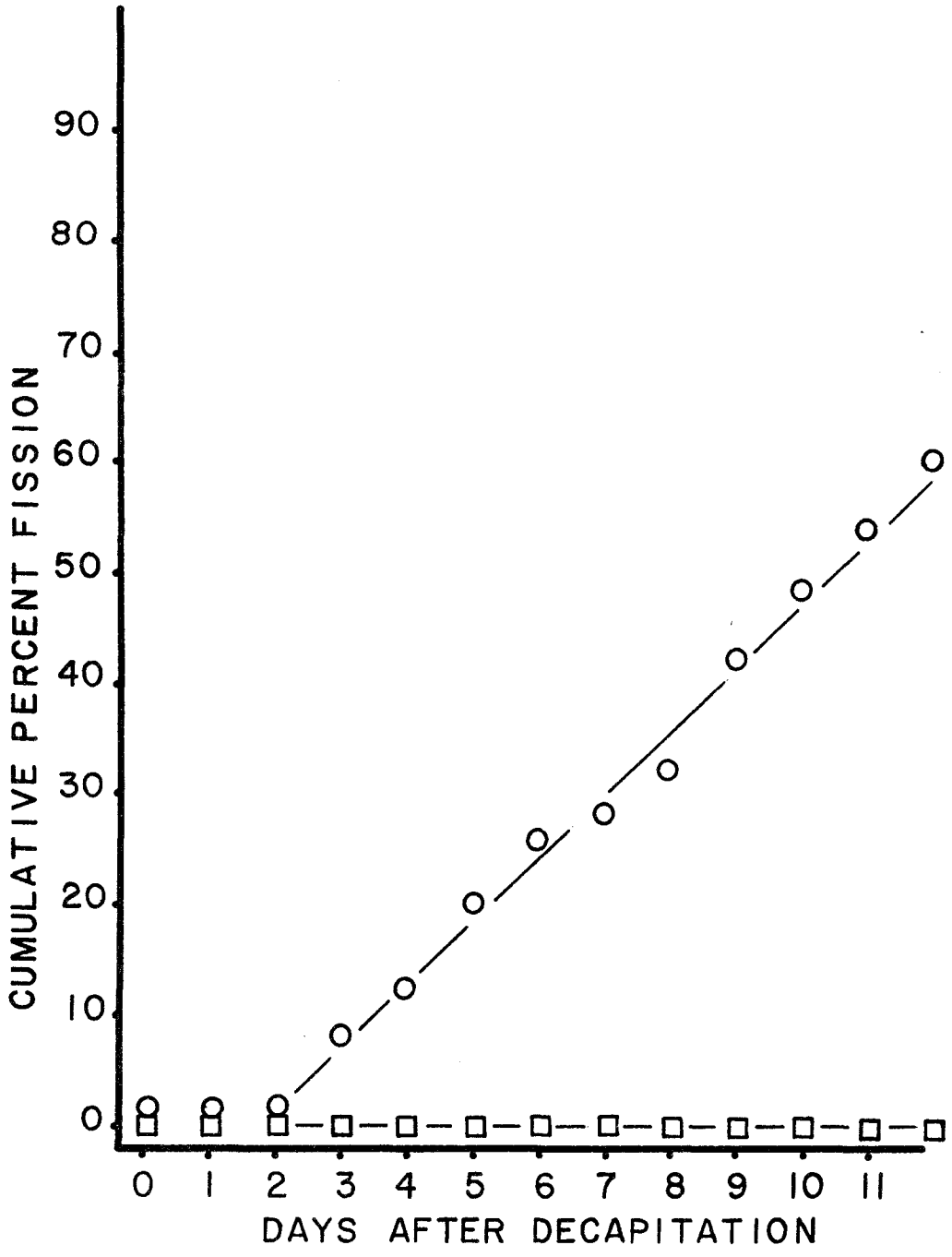

CUMULATIVE PERCENT FISSION
90
80
70
60
50
40
30
20
10
0
0 1 2 3 4 5 6 7 8 9 10 11
DAYS AFTER DECAPITATION

Fig. 24. Decapitation-induced fission in sexual planaria which were transected and allowed to regenerate before decapitation ○ and non-decapitated (regenerated) controls □ .

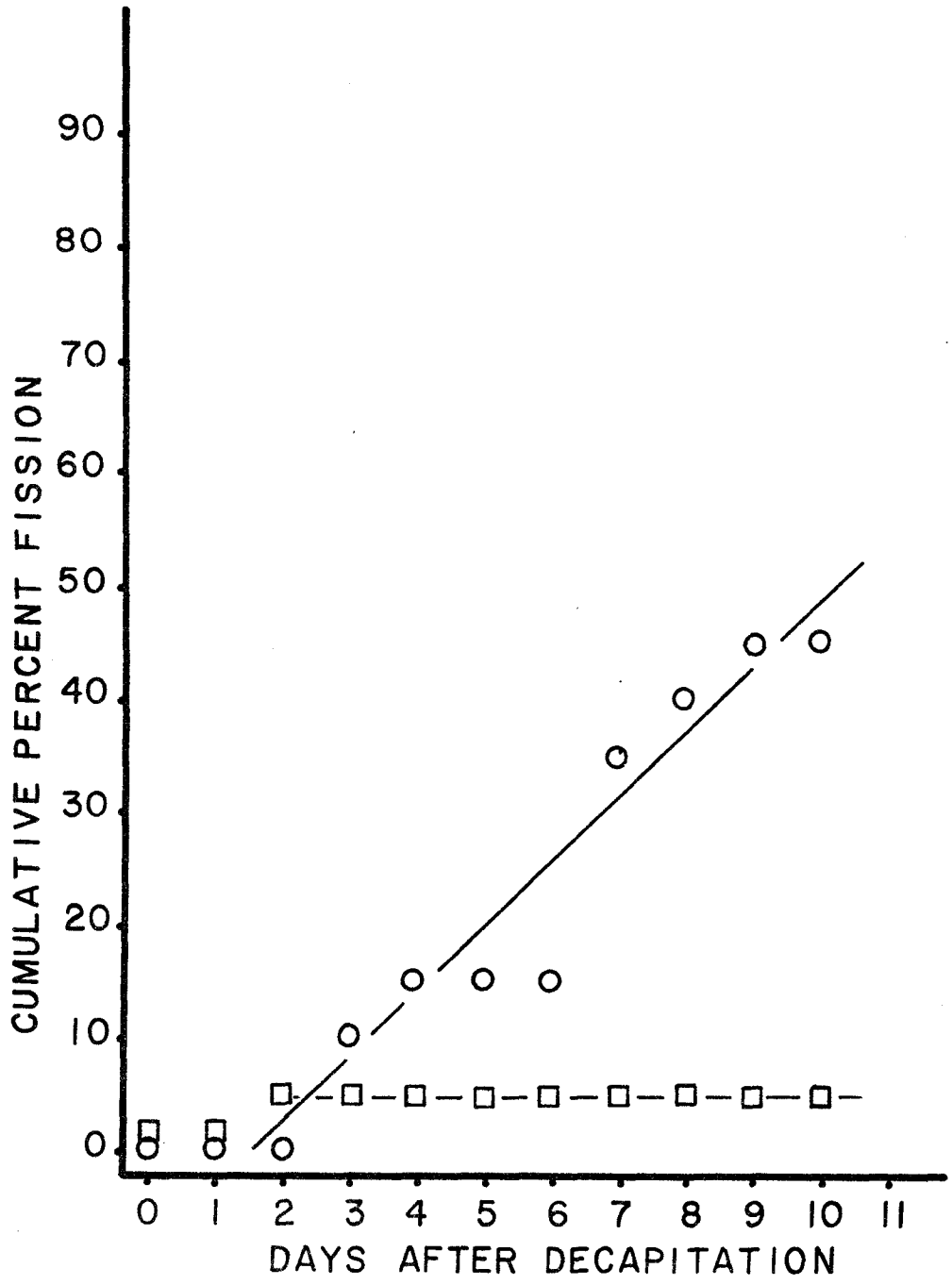

CUMULATIVE PERCENT FISSION
90
80
70
60
50
40
30
20
10
0
0 1 2 3 4 5 6 7 8 9 10 11
DAYS AFTER DECAPITATION

Fig. 25. Multiple fissions of a planaria ten days post-decapitation. Original decapitated planaria "A" has given rise to zooid "B" and then produced a second zooid "C".

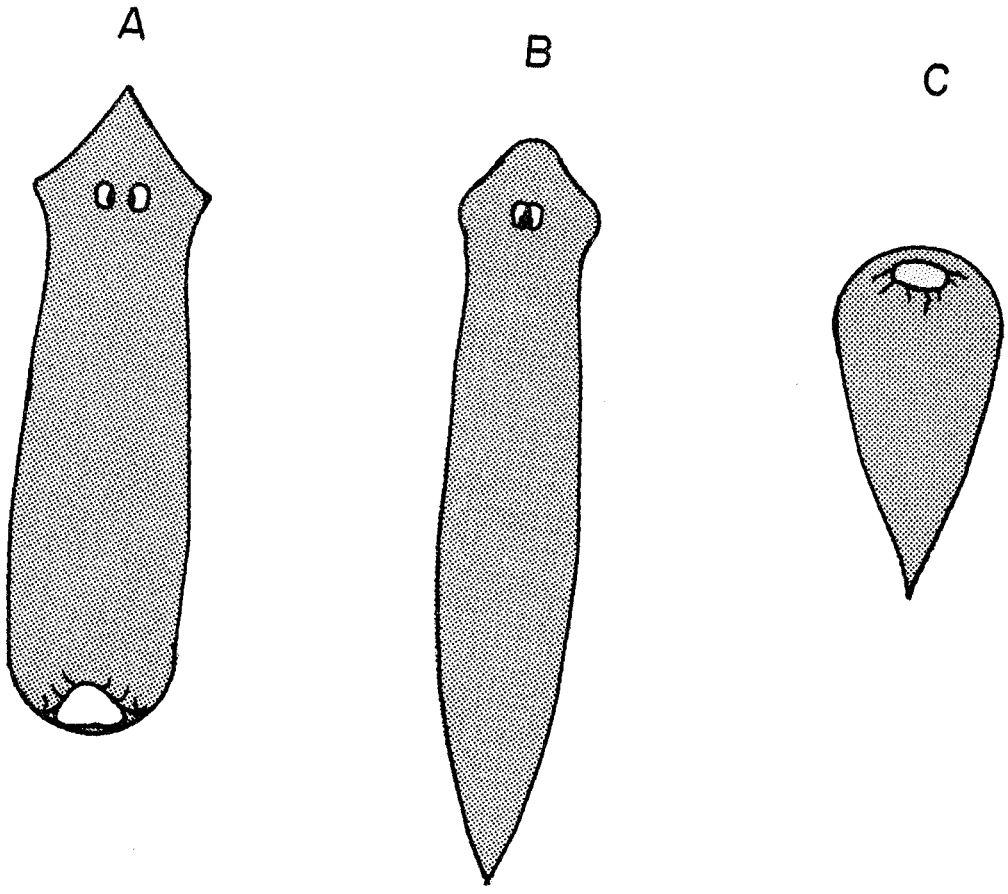

A
B
C

Fig. 26. Multiple fission of a planaria ten days post-decapitation. Original decapitated planaria "A" has given rise to zooid "B" which has produced its own daughter zooid "C".

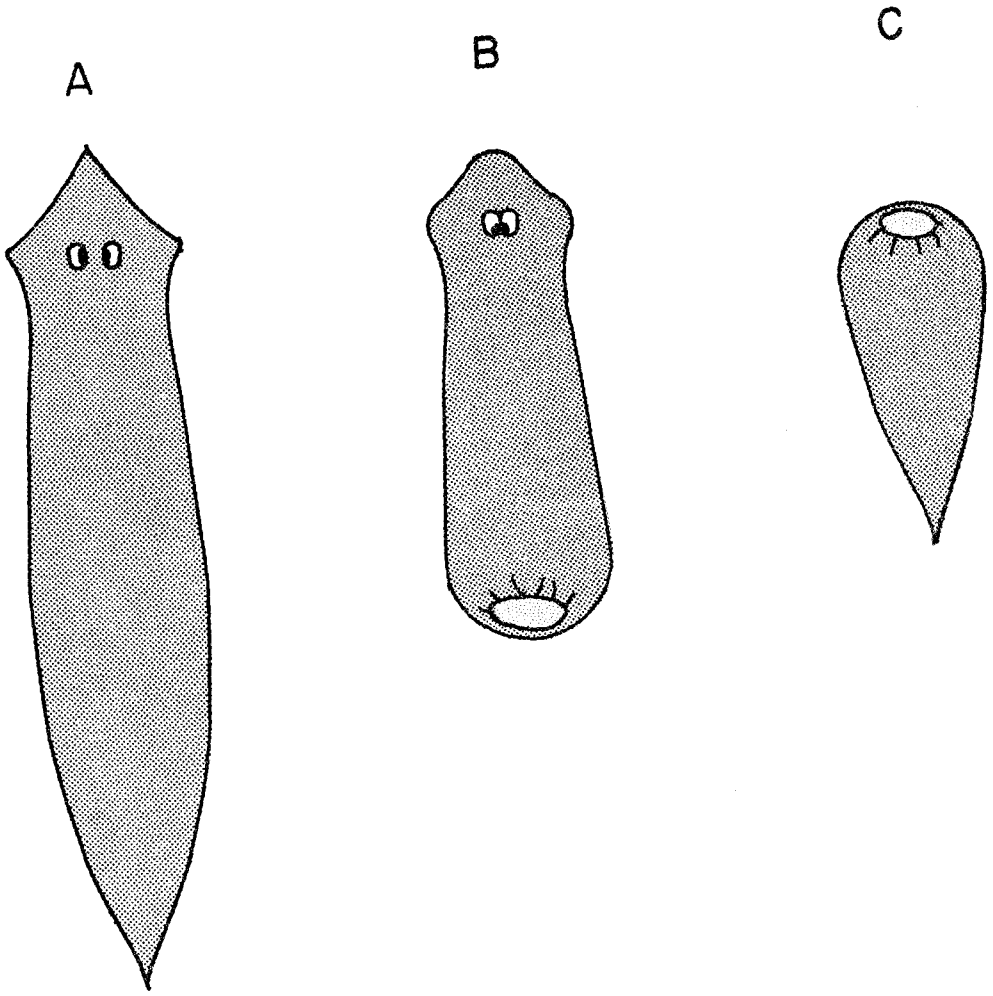
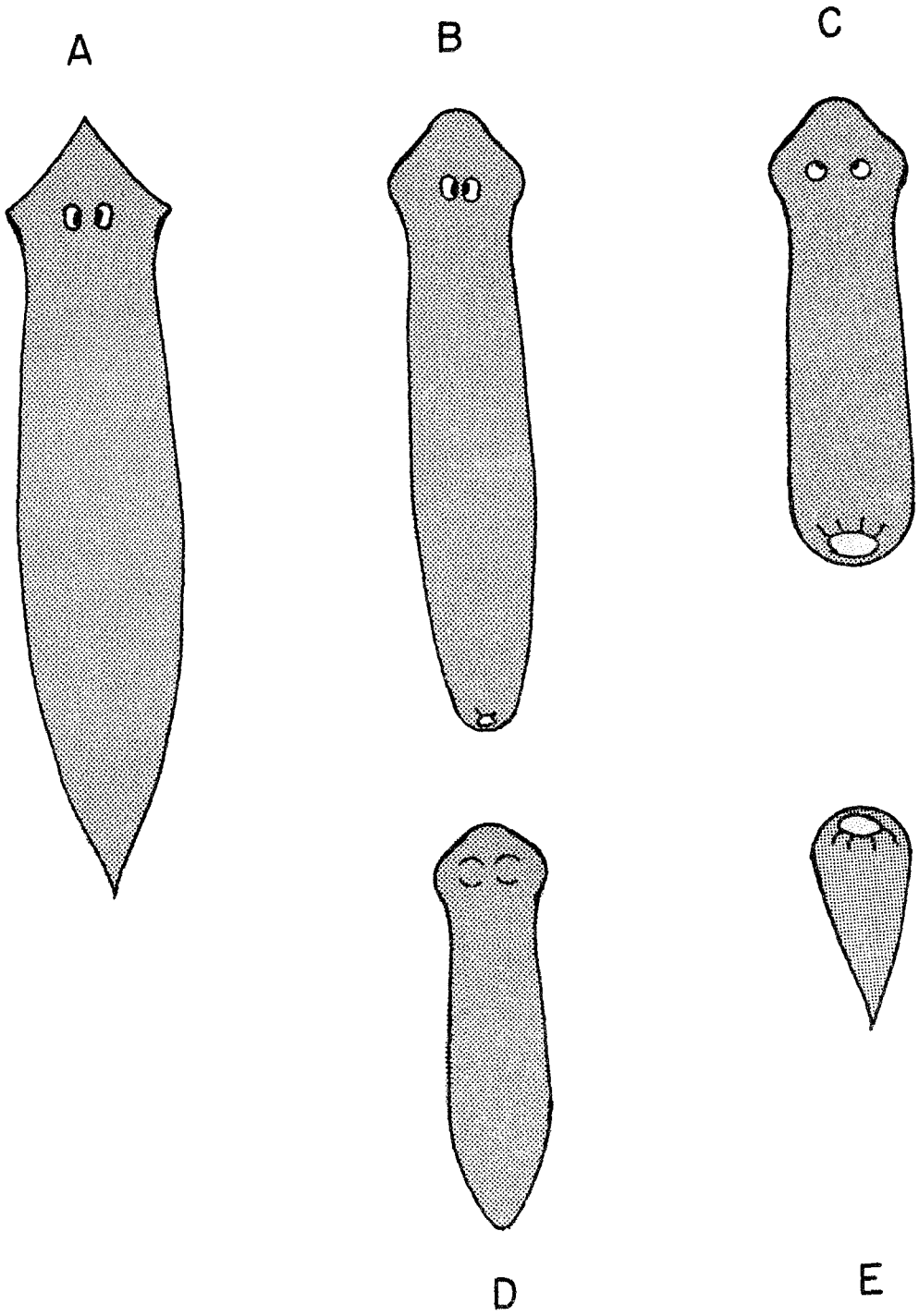

A
B
C

Fig. 27. Multiple fission of a planaria ten days post-decapitation. Original decapitated planaria "A" has given rise to zooid "B" and then to zooid "C". Zooid "B" then produced its own daughter zooid "D" followed by zooid "C" producing "E".


A
B
C
D
E

the blastemata at the posterior and anterior ends of "B" and "C".

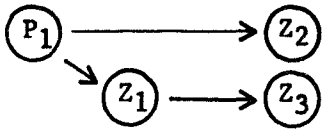
In another experiment the occurence of multiple fissions was quantitated. Table 4 presents data obtained from thirty decapitated planaria (P_1), all thirty produced a first zooid (Z_1). Twenty-six percent of the original animals (P_1) produced a second zooid (Z_2) within nine days after decapitation of P_1.

Ten days after decapitation of P_1, fifty percent of the first zooids (Z_1) had matured sufficiently to produce their own fission product (Z_3). Sixty-two percent of the original decapitated animals (P_1), whose offspring ultimately gave rise to a zooid (Z_3) had also produced a second zooid (Z_2). The time between production of these two offspring (Z_2 and Z_3) was less than two days.

Table 4. Quantitative analysis of multiple fission resulting from decapitation of Dugesia dorotocephala.

Generation*	Number	Percent[1]	Days
Parent animals (P_1)	30	100	
First zooid (Z_1)	30	100	
Second zooid (Z_2)	8	26	9[a]
Daughter zooids of first zooid (Z_3)	15	50	10[b]
Concordence of Z_2 production with Z_3**	5	62	2[c]

*Explanation of generation



**Second zooids (Z_2) whose parents (P_1) have also given rise to daughter zooid (Z_3) via zooid Z_1.

[1]Percent of possible animals.

[a]Average number of days required for production of Z_2 by P_1 after it (P_1) gave rise to Z_1.

[b]Average number of days required for Z_1 to produce Z_3.

[c]Maximum time difference between production of Z_2 and Z_3 when both had a common ancestor (P_1).

An aqueous extract obtained from whole planaria was examined at different concentrations (Fig. 28) to determine the effect on fission rate. Forty-eight percent of the animals which were incubated in 25 ug of extract per ml of Henderson-Eakin solution had fissioned by day five. Twenty-two percent of the animals in 100 ug per ml had fissioned and the control value was seventy-six percent by the same day.

Biological assay of planaria extract (100 ug per ml) showed cumulative fission ten percent below the control value (eighty-five percent) by day seven (Fig. 29). This lower fissionability is equivalent to the percent fission on the first day of fission-onset in the non-treated control group. In addition to the lowered fissionability, there is a change in both the fission rate and pattern. The extract-treated animals show accelerated fission (twenty-six percent) on the first day (control value ten percent), followed by an abruptly decreasing fission rate until the plateau value is seventy-four percent by day six. This alteration produced a bimodal fission curve which is characteristic of extract treatments (Fig. 30).

Assay of a single inoculation of planaria extract shows a 48 hour latent period following decapitation before fission-onset. When multiple inoculations of planaria extract (every 24 hours) are given to decapitated animals the length of the latent period is directly proportional to the number of inoculations (Fig. 31).

Fig. 28. Effect of planaria extract at different concentrations (ug protein per ml incubation medium) on decapitated planaria incubated (five per plate) immediately after extract preparation.

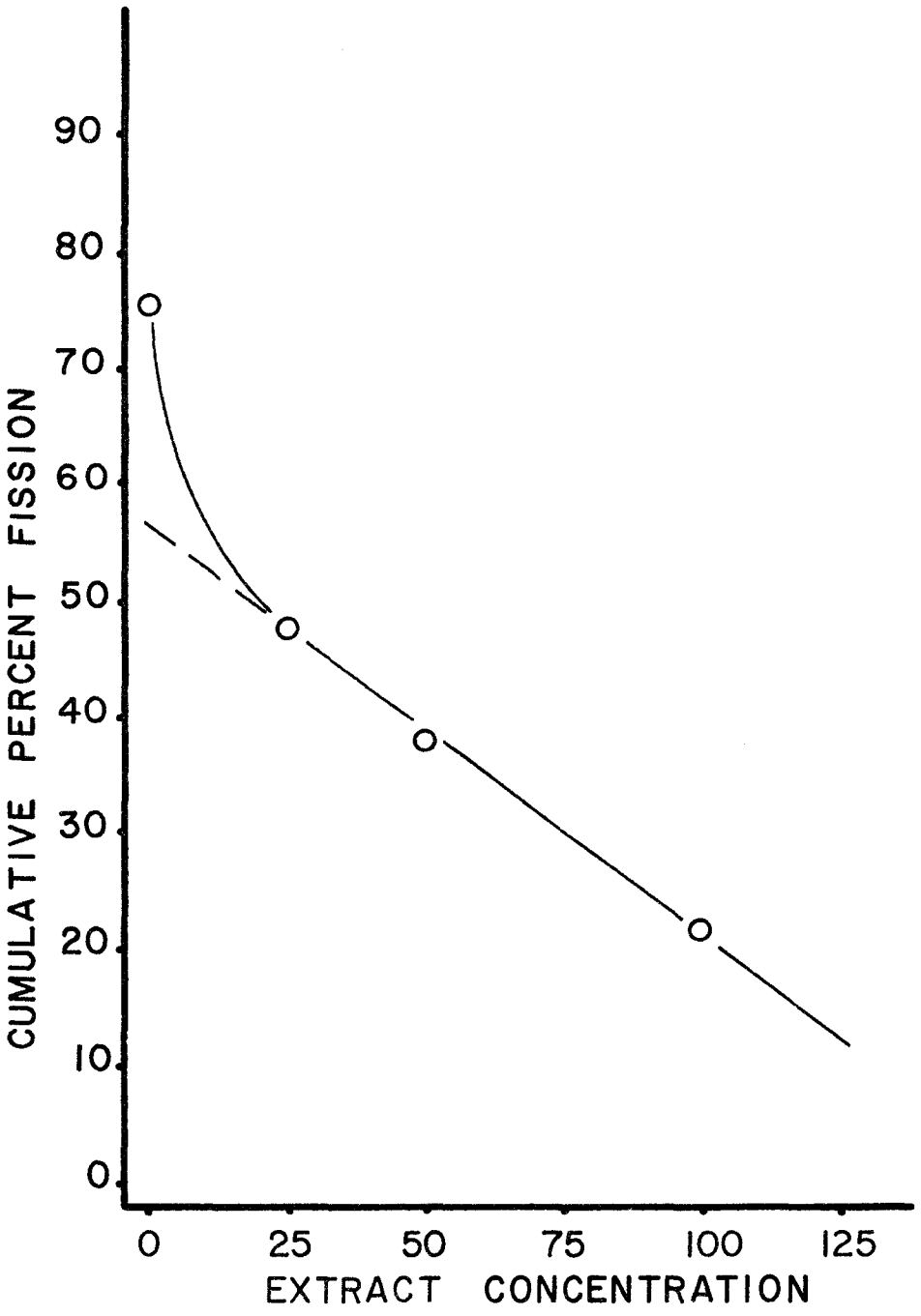

CUMULATIVE PERCENT FISSION
90
80
70
60
50
40
30
20
10
0
0
25
50
75
100
125
EXTRACT CONCENTRATION

Fig. 29. Summary effect of planaria extract (100 ug per ml) on decapitation-induced fission when incubated one animal per petri dish. Control total number at day zero equals 521 O , extract-treated number equals 222 □ .

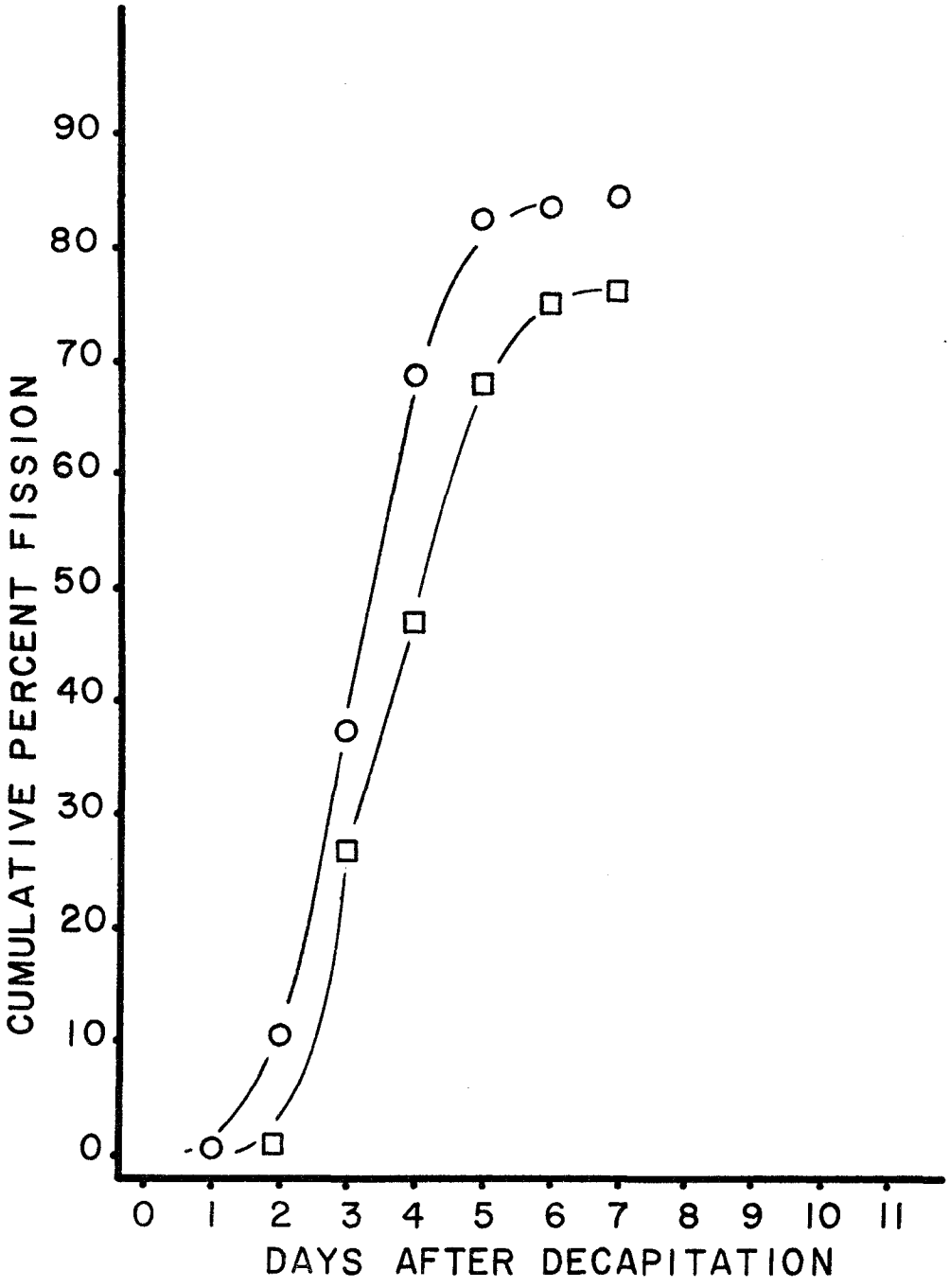

CUMULATIVE PERCENT FISSION
90
80
70
60
50
40
30
20
10
0
0 1 2 3 4 5 6 7 8 9 10 11
DAYS AFTER DECAPITATION

Fig. 30. Fission summary (fissions per day) of 699 planaria(on day zero) which were treated with whole planaria extract at 100 ug protein per ml.

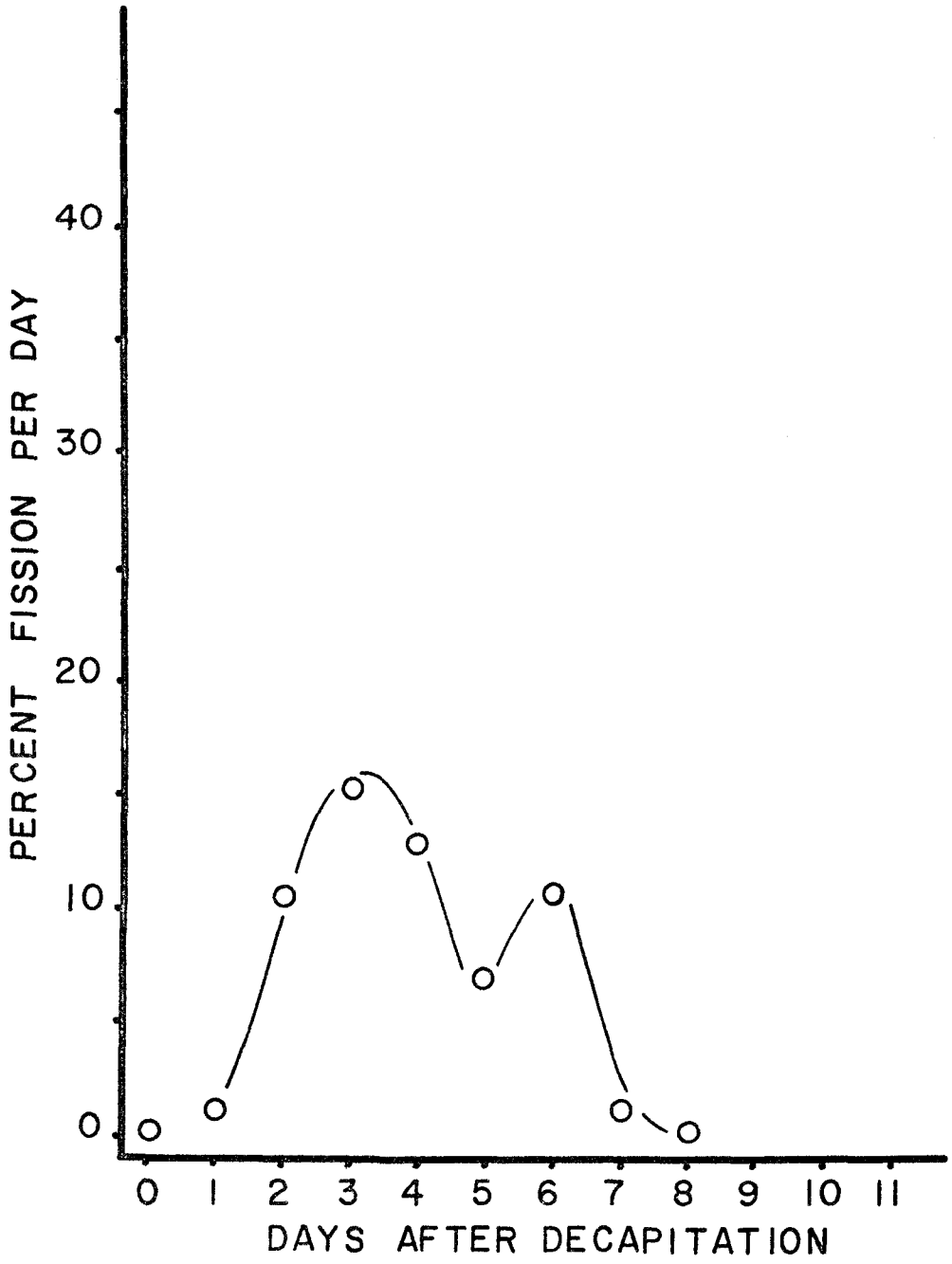
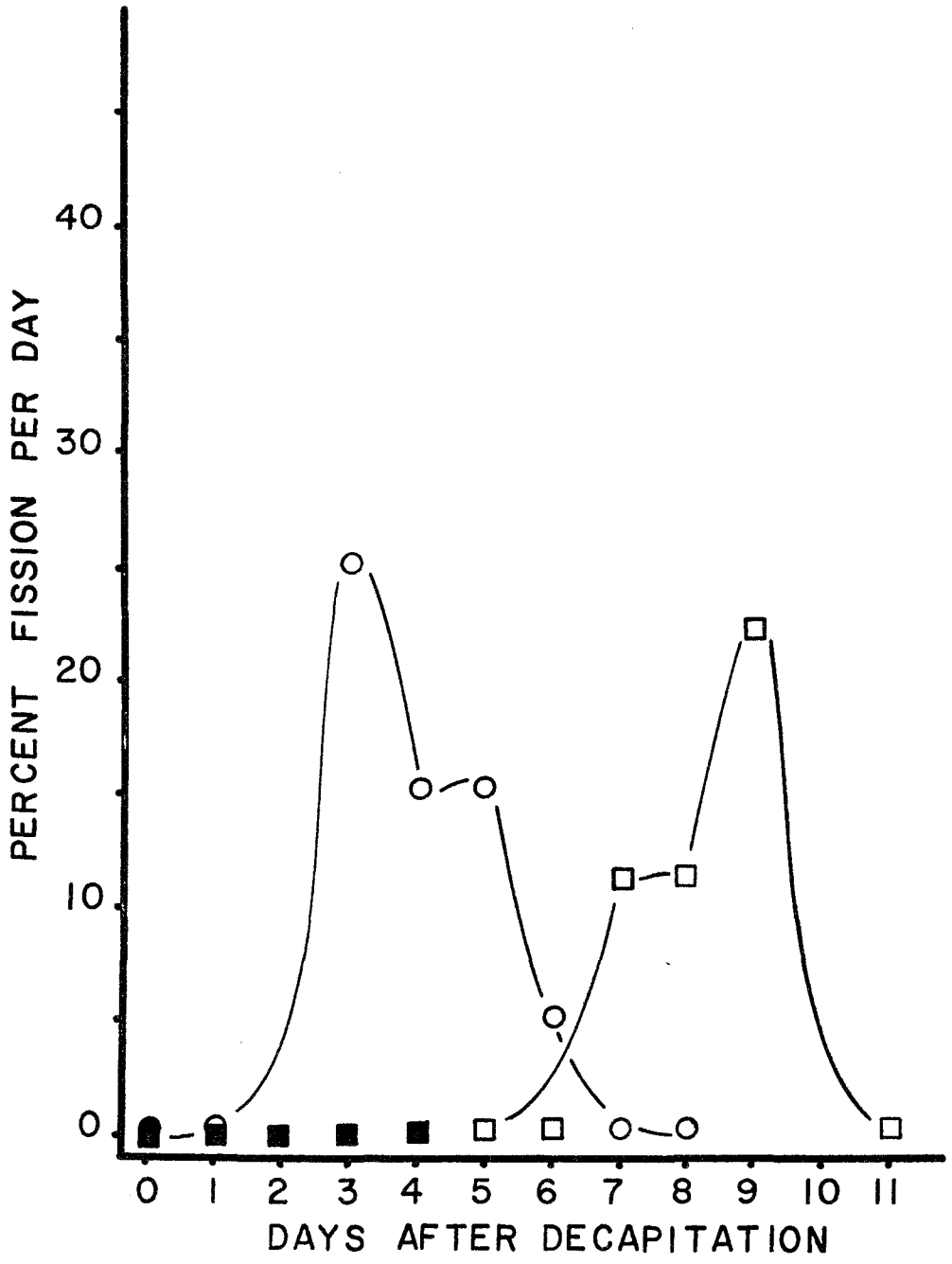

PERCENT FISSION PER DAY
40
30
20
10
0
0 1 2 3 4 5 6 7 8 9 10 11
DAYS AFTER DECAPITATION

Fig. 31. Comparison of a single inoculation (0 hours) of planaria extract ○ , with multiple inoculations (0, 24, 48, 72 and 96 hours) through day four □ , on decapitation-induced fission.


PERCENT FISSION PER DAY
40
30
20
10
0
0 1 2 3 4 5 6 7 8 9 10 11
DAYS AFTER DECAPITATION

Even after the protracted latent period the treated planaria still exhibit a bimodal fission pattern which appears 48 hours after the last extract dose.

Utilization of non-specific extracts such as casitone (Fig. 32), casamino acids (Fig. 33), chick plasma (Fig. 34) and chick embryo (Fig. 35) do not produce a bimodal fission pattern as does whole planarian extracts. However, yeast extract (Fig. 36) does produce a bimodal curve.

The inhibitory activity of the planaria extract was reduced when stored at 4^{o}C (Fig. 37). When the extract (100 ug per ml) was stored for 20 hours at 4^{o}C there was thirty-two percent fission by day five. Storage for 44 hours resulted in fifty-six percent fission. Extract which was used immediately after preparation resulted in a cumulative fission by day five of twenty-two percent.

Fig. 32. The effect of casitone (pancreatic digest of casein) on the decapitation-induced fission process when planaria are incubated at 100 ug per ml □ and 50 ug per ml ○ .

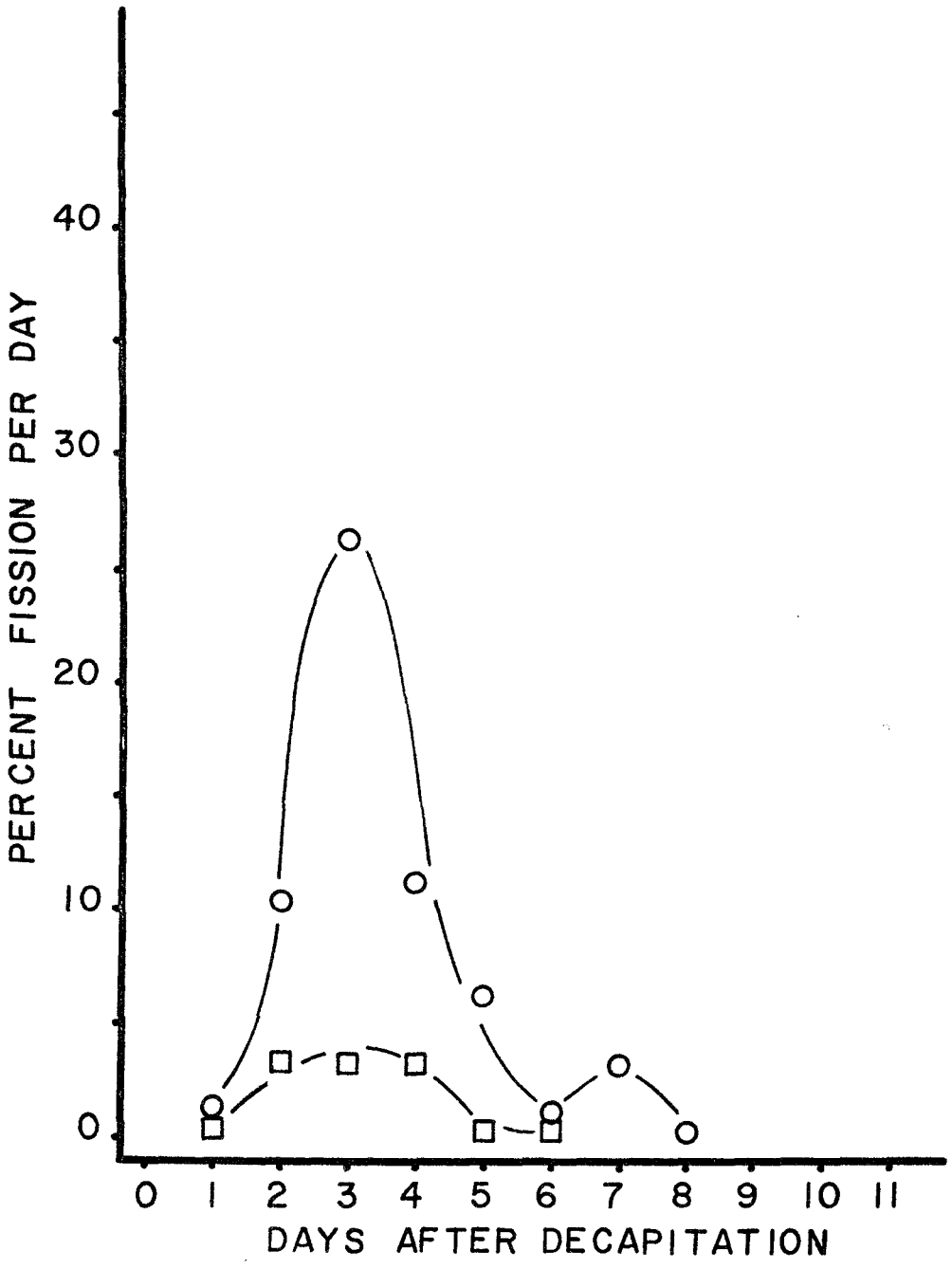

PERCENT FISSION PER DAY
40
30
20
10
0
0 1 2 3 4 5 6 7 8 9 10 11
DAYS AFTER DECAPITATION

Fig. 33. The effect of casamino acids (terminal hydrolysis of casein) on the decapitation-induced fission process when planaria are incubated at 100 ug per ml ○ and 50 ug per ml □ .

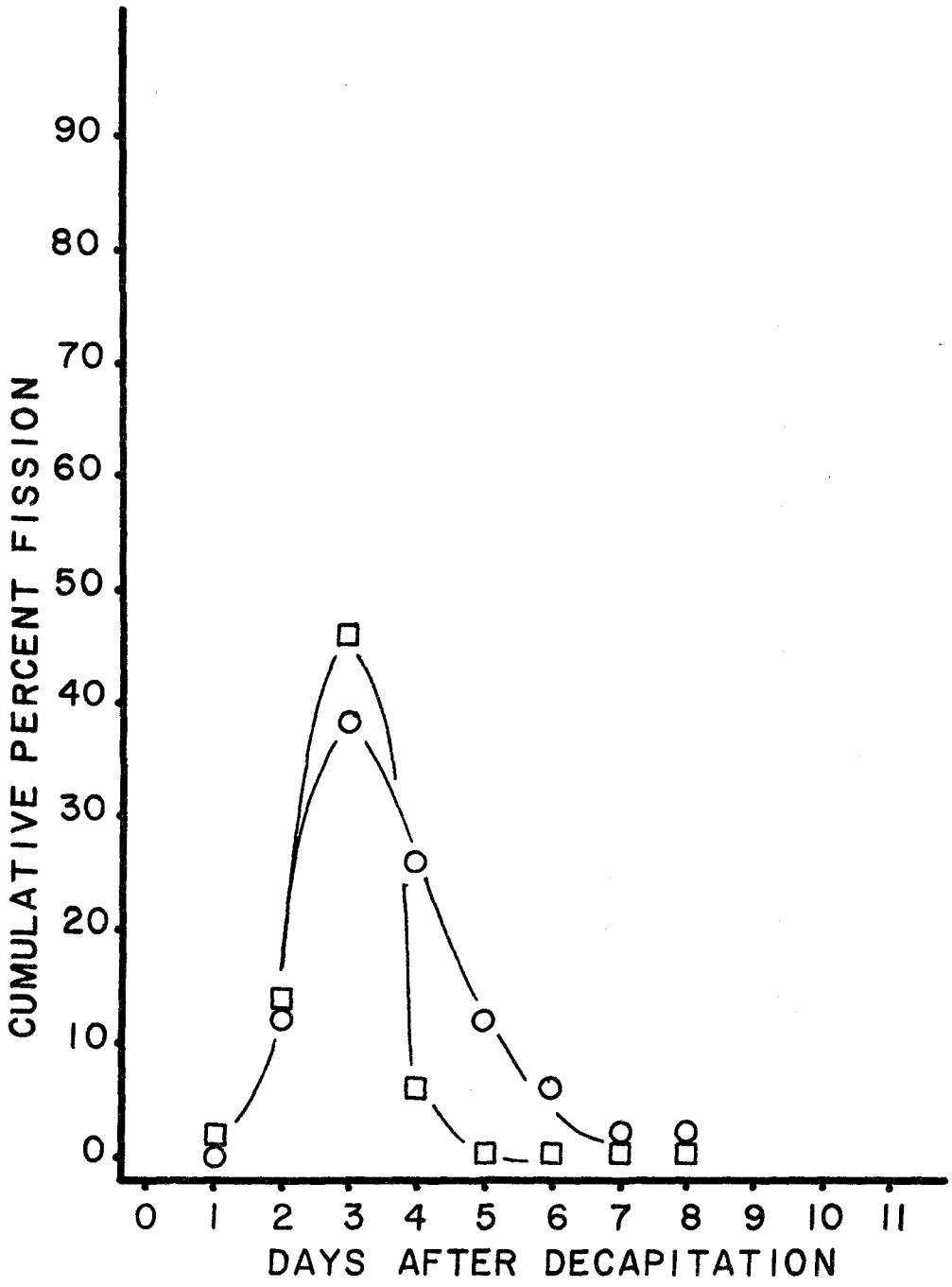

CUMULATIVE PERCENT FISSION
90
80
70
60
50
40
30
20
10
0
0 1 2 3 4 5 6 7 8 9 10 11
DAYS AFTER DECAPITATION

Fig. 34. The effect of chick plasma on the decapitation-induced fission process when planaria are incubated at 100 ug per ml ○ and 50 ug per ml □ .

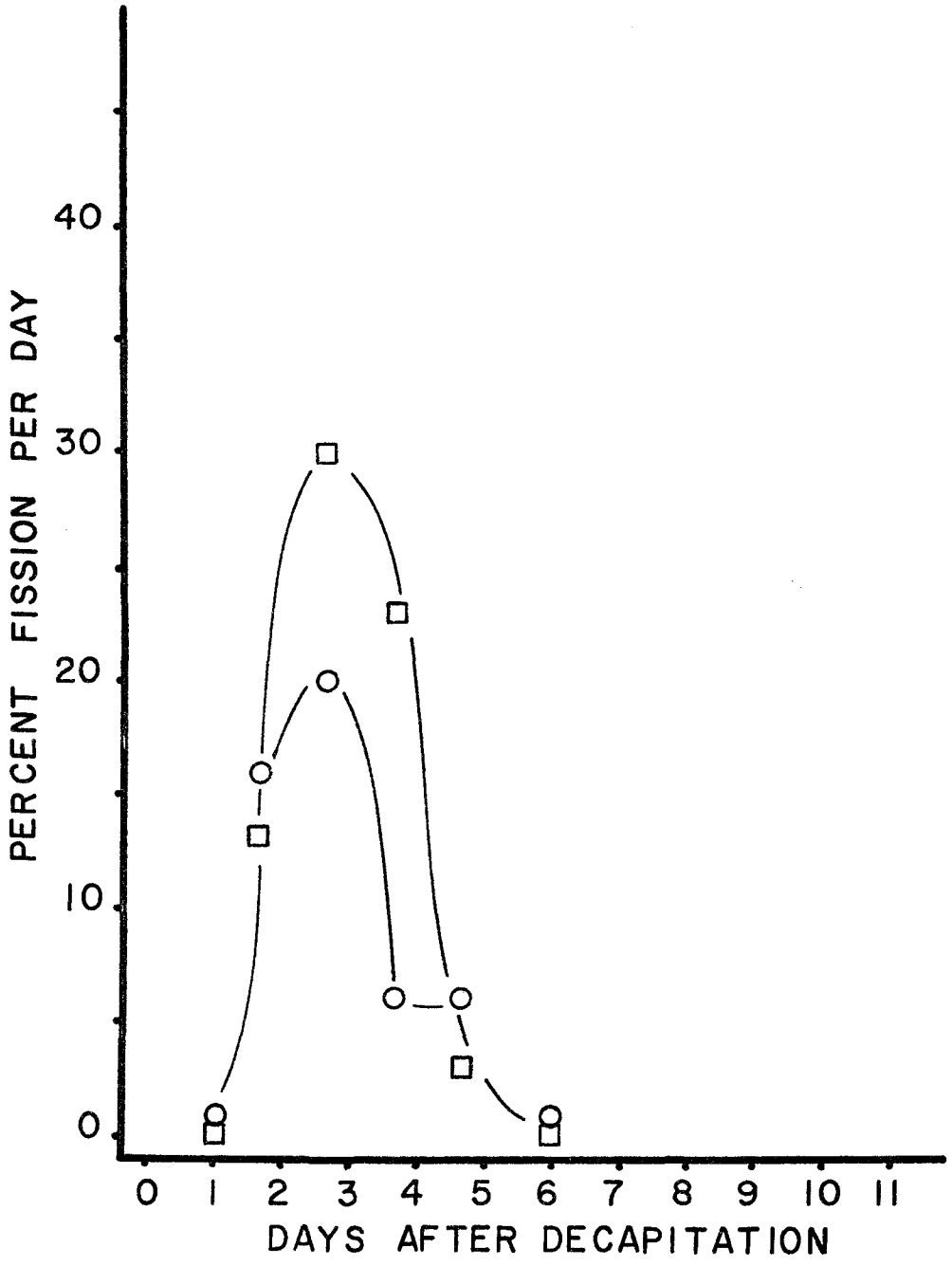

PERCENT FISSION PER DAY
40
30
20
10
0
0 1 2 3 4 5 6 7 8 9 10 11
DAYS AFTER DECAPITATION

Fig. 35. The effect of chick embryo extract on the decapitation-induced fission process when planaria are incubated at 100 ug per ml ○ , 50 ug per ml ▽ , and 25 ug per ml □ .

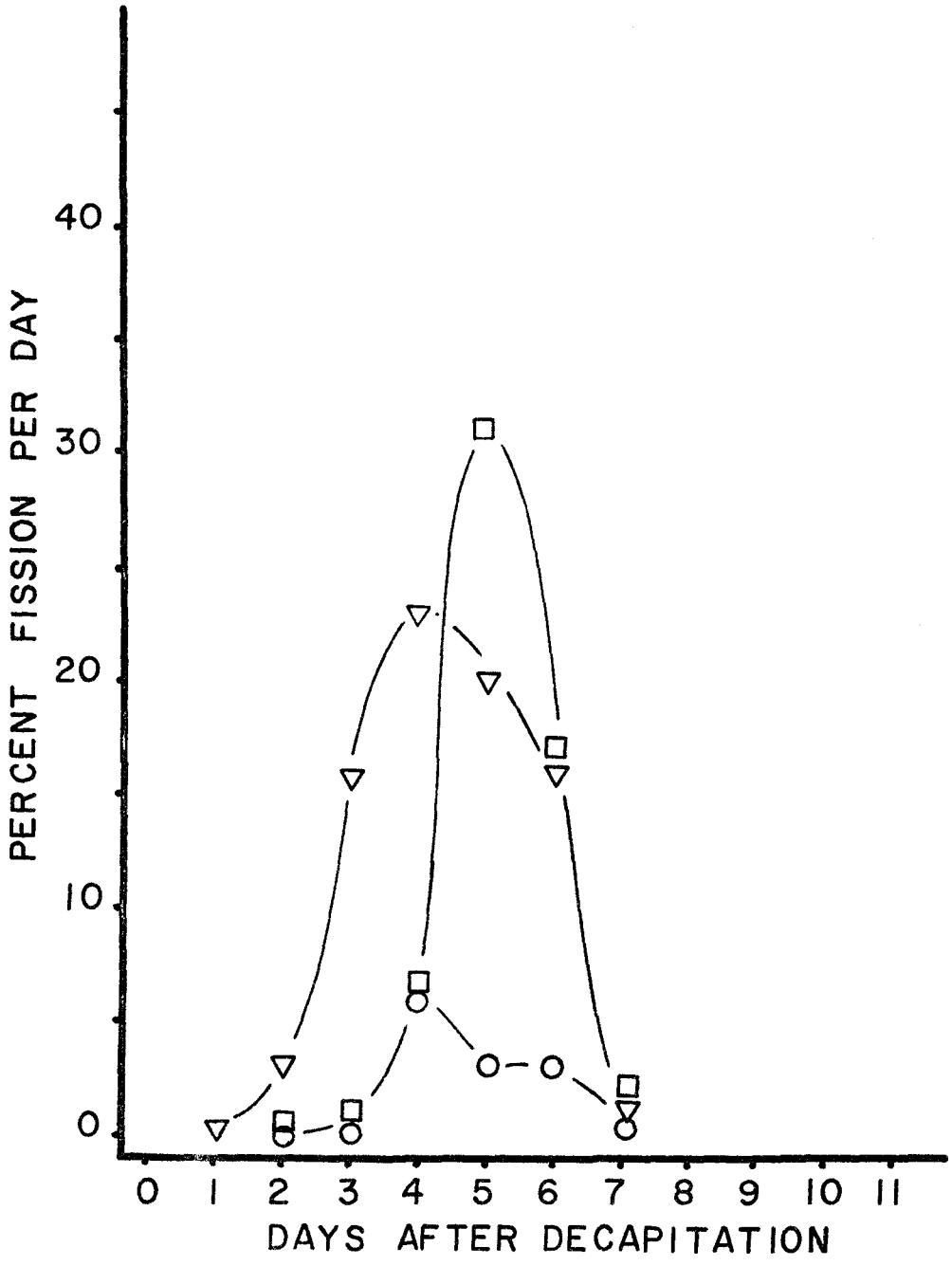

PERCENT FISSION PER DAY
40
30
20
10
0
0 1 2 3 4 5 6 7 8 9 10 11
DAYS AFTER DECAPITATION

Fig. 36. The effect of yeast extract on the decapitation-induced fission process when planaria are incubated at 100 ug per ml ○ and 50 ug per ml □ .

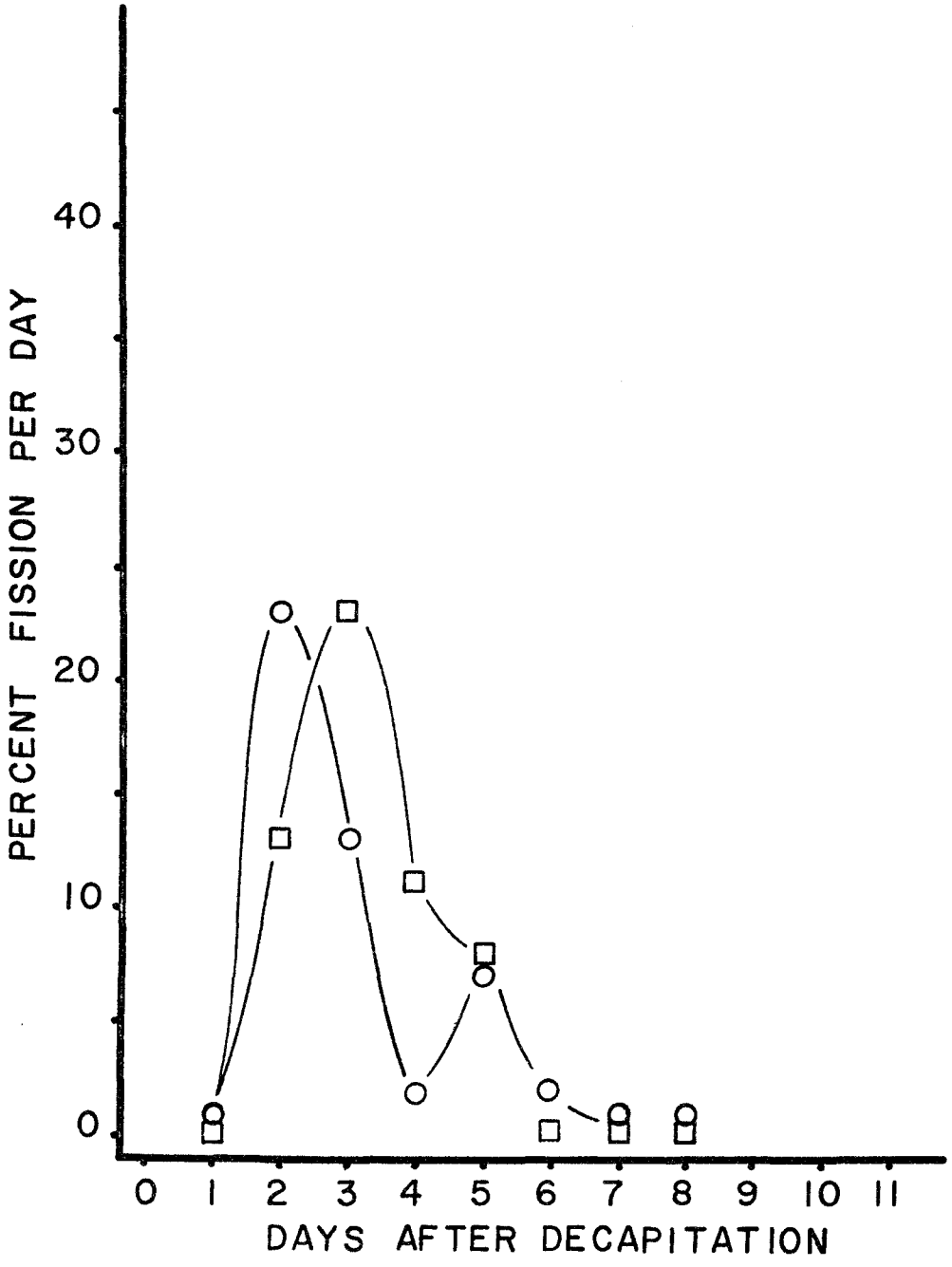

PERCENT FISSION PER DAY
40
30
20
10
0
0 1 2 3 4 5 6 7 8 9 10 11
DAYS AFTER DECAPITATION

Fig. 37. Inhibitory activity of planaria extract (ug protein per ml incubation fluid) which has been stored at 4°C for 0 hours ○, 20 hours □ , 44 hours ▽ and non-treated control ◇ .

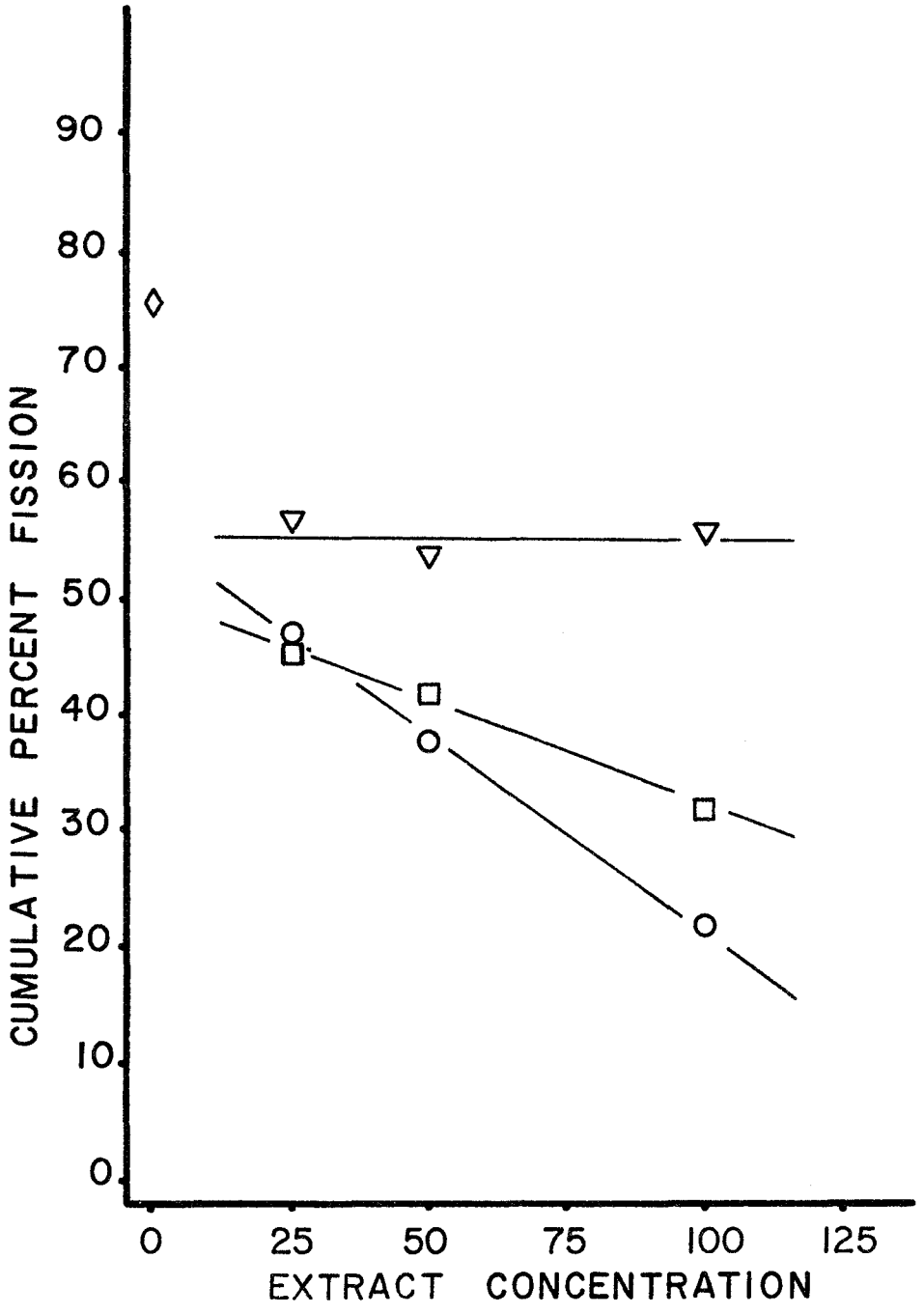
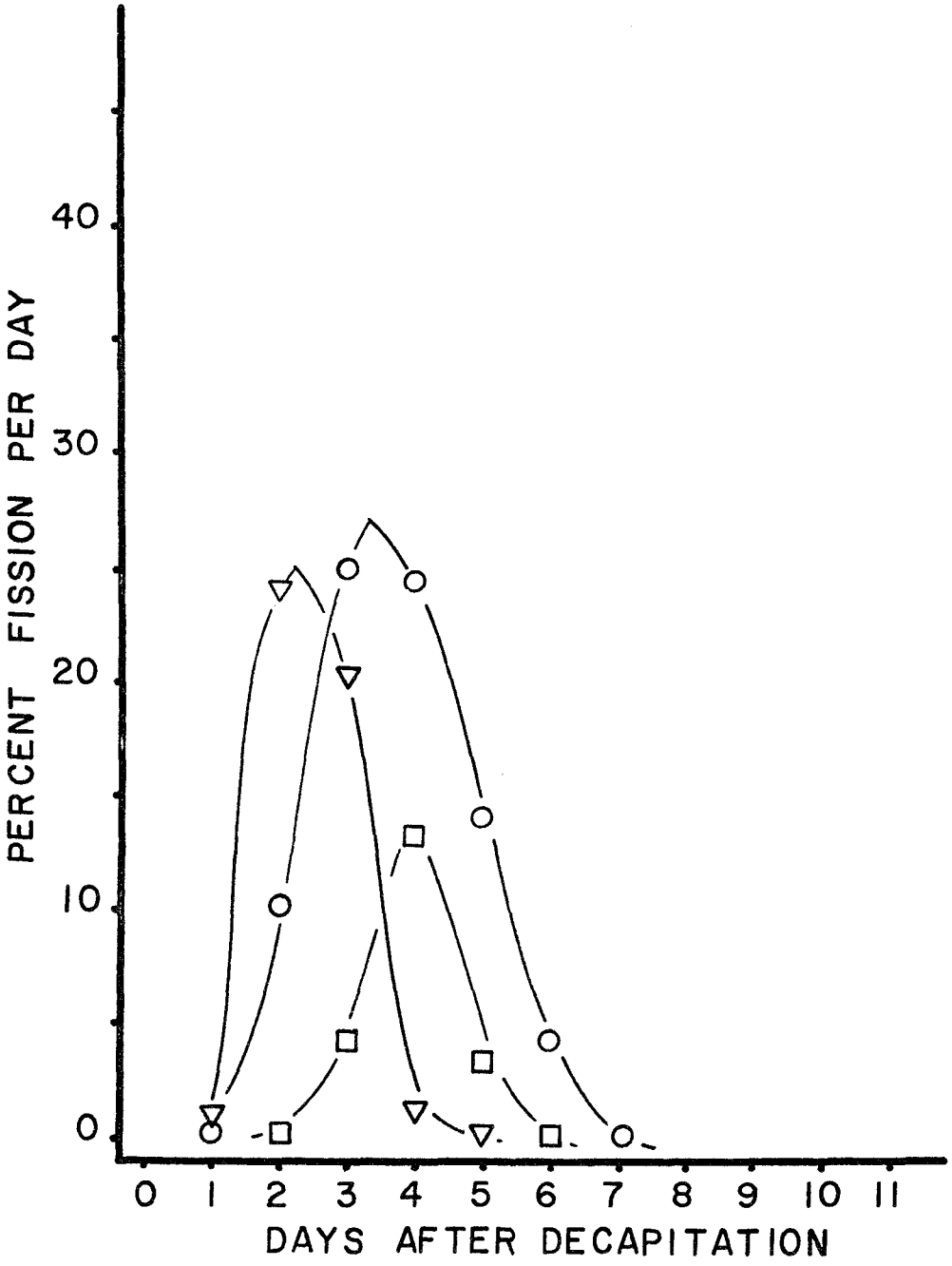

CUMULATIVE PERCENT FISSION
90
80
70
60
50
40
30
20
10
0
0
25
50
75
100
125
EXTRACT CONCENTRATION

Fig. 38. Appearance of a fission stimulator when whole planaria extract (100 ug per ml) is stored at $4^{\circ}C$ for 0 hours □ , 44 hours ▽ and control ○ .


PERCENT FISSION PER DAY
0
10
20
30
40
0 1 2 3 4 5 6 7 8 9 10 11
DAYS AFTER DECAPITATION

Characterization of Tissue Extract

The biological activity of tissue extract from whole planaria which was heated in a boiling water bath (96°C for 30 minutes) differed from that observed in either control (Henderson-Eakin solution) or non-heated planaria extract (Fig. 39). The fission pattern of the control was a typical gaussian distribution with the mode at day 3.5. The non-heated extract had a bimodal fission pattern with eight percent of the animals fissioning at day 2.5 and the rest at a mode of day six. However, when the animals were incubated in the heat-treated extract there was a fission mode on day two containing seventeen percent of the population (Fig. 39).

The data presented in figure 40 resulted from ultracentrifugation of the extract obtained from whole planaria. The assay results from the pellet (35,000 RPM for 45 minutes) and supernatant fluid (32,500 RPM for 17 hours) show the same cumulative percent fission as the control by day six. However, there was a delay in the fission-onset when animals were incubated in the supernatant fluid. By the third day after decapitation, twenty-eight percent of this group had fissioned compared to the control group with fifty-two percent. The fission rate from day three through day five was much greater than that observed in the control.

The data accumulated by incubation with a pellet obtained at 32,500 RPM for 17 hours demonstrates the presence of an inhibitory compound (Fig. 40). There was a decrease in both the rate and

Fig. 39. Effect of heated planaria extract on decapitation-induced fission. Non-heated extract ▩ , heated extract □ and control ▩ .

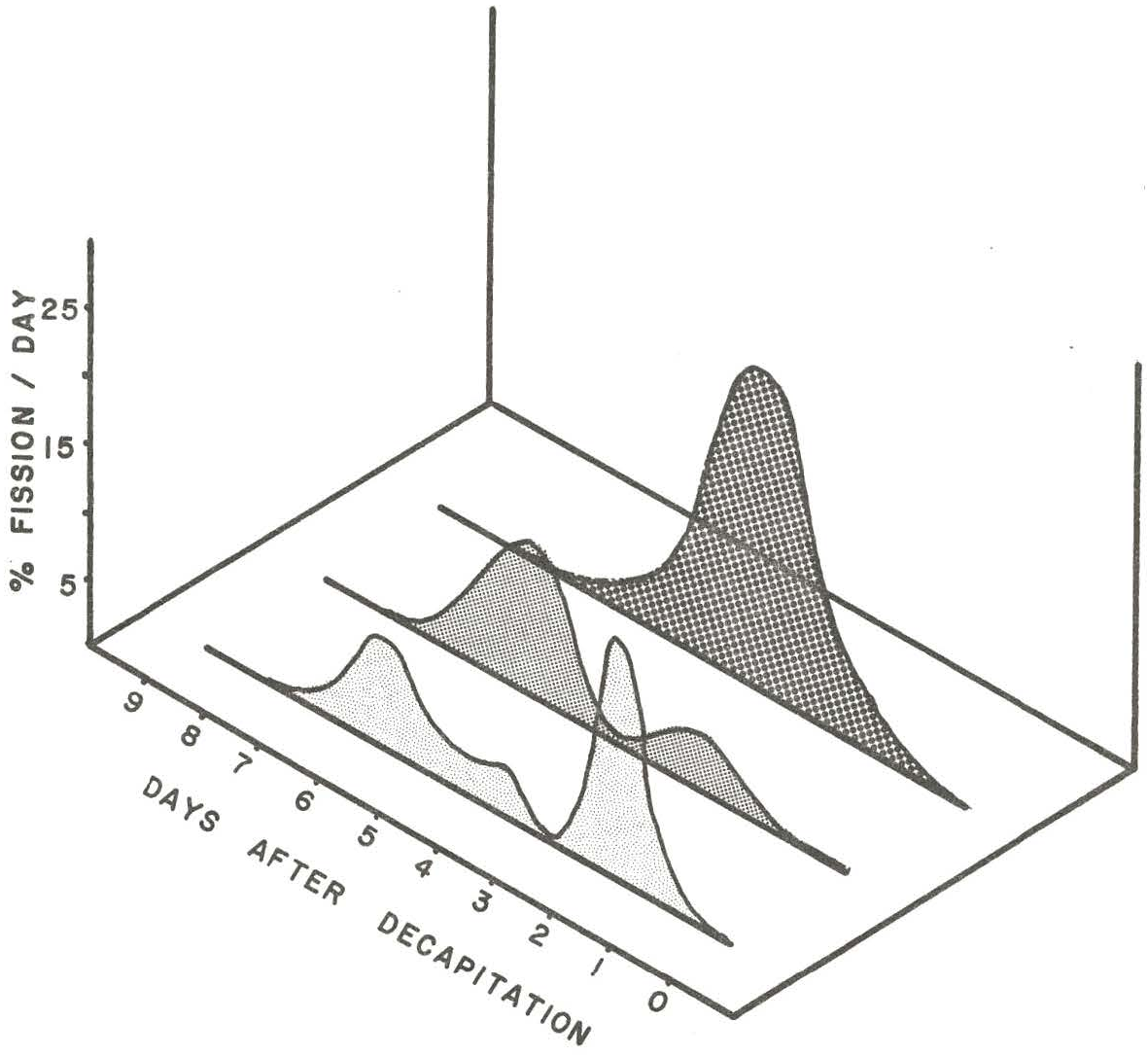
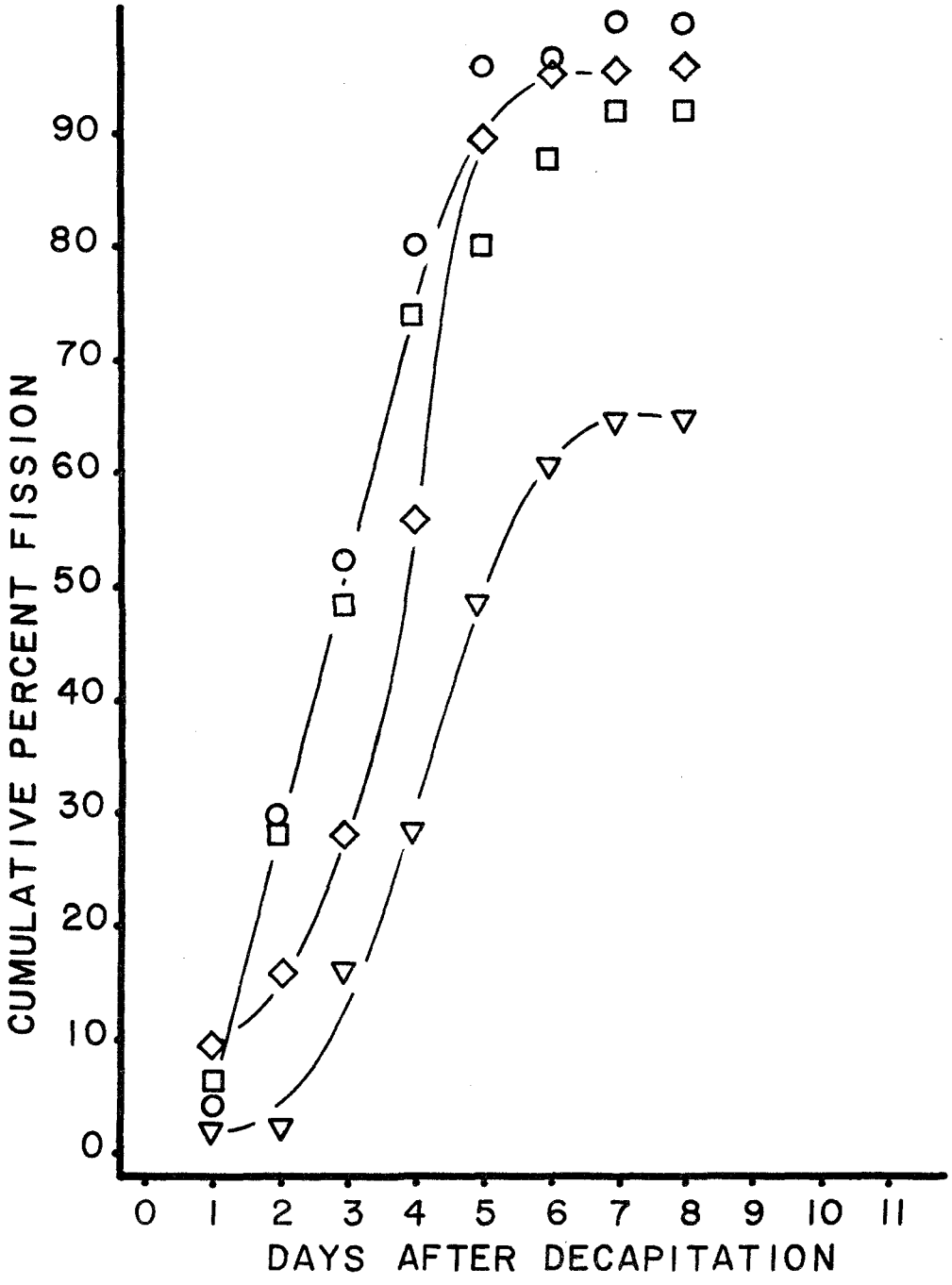

% FISSION / DAY
25
15
5
9
8
7
6
5
4
3
2
1
0
DAYS AFTER DECAPITATION

Fig. 40. Fission modifying effect of fractions obtained by ultra-centrifugation of whole planaria extract. Pellet obtained by 45 minutes at 35,000 RPM □, pellet obtained by 17 hours at 32,500 RPM ▽ supernatant fluid at the end of 17 hours at 32,500 RPM ◇ and control ○.


CUMULATIVE PERCENT FISSION
0
10
20
30
40
50
60
70
80
90
0 1 2 3 4 5 6 7 8 9 10 11
DAYS AFTER DECAPITATION

cumulative percent fission through the seventh day. Sixty-two percent of the animals in this experimental group had fissioned compared to ninety-six percent of the controls. There was also a one day delay in the onset of fission.

The inhibitory compound suggested in figure 40 was further characterized with ultra-filtration (Fig. 41). Planaria extract was filtered first through a UM-50 membrane then a UM-10 membrane. This fractionated extract (UM-10 retained UM-50 filtrate) was used for biological assay at 100 ug per ml. The fission mode of the control group was day 3.5 compared to day seven for the experimentals. When the extract was filtered through a UM-50 membrane and not the UM-10, the fission pattern was the same in both the experimental and control groups.

The inhibitory material which passed through the UM-50 membrane and was retained by the UM-10 would have a molecular weight between 10,000 and 50,000 according to the specifications supplied by the manufacturer.

The inhibitory factor did not pass through a dialysis membrane when the extract obtained from whole planaria was dialized against the standard Henderson-Eakin solution (Fig. 42). The biological assays of the control and dialyzable material were very similar. However, fifteen percent of the animals incubated in solution containing the dialyzable material fissioned by the second day after decapitation compared with no fission in the control group. This dialyzable fraction appears to accelerate fission between day two and three, then again between day four and five. The non-dialyzable

Fig. 41. Effect of the planaria extract which has been fractionated with ultra-filtration on decapitation-induced fission. Extract which was UM-50 filtered and UM-10 retained □ , versus control ○ . Ultra-filtration was accomplished according to specifications provided by Amicon publication No. 403. Accordingly the Diaflo membrane UM-10 retains 95% of Myoglobin (M W 17,800) and 90% of Cytochrome C (M W 12,400). The UM-50 membrane retains 95% of Hemoglobin (M W 64,000) and 90% of Ovalbumin (M W 45,000).

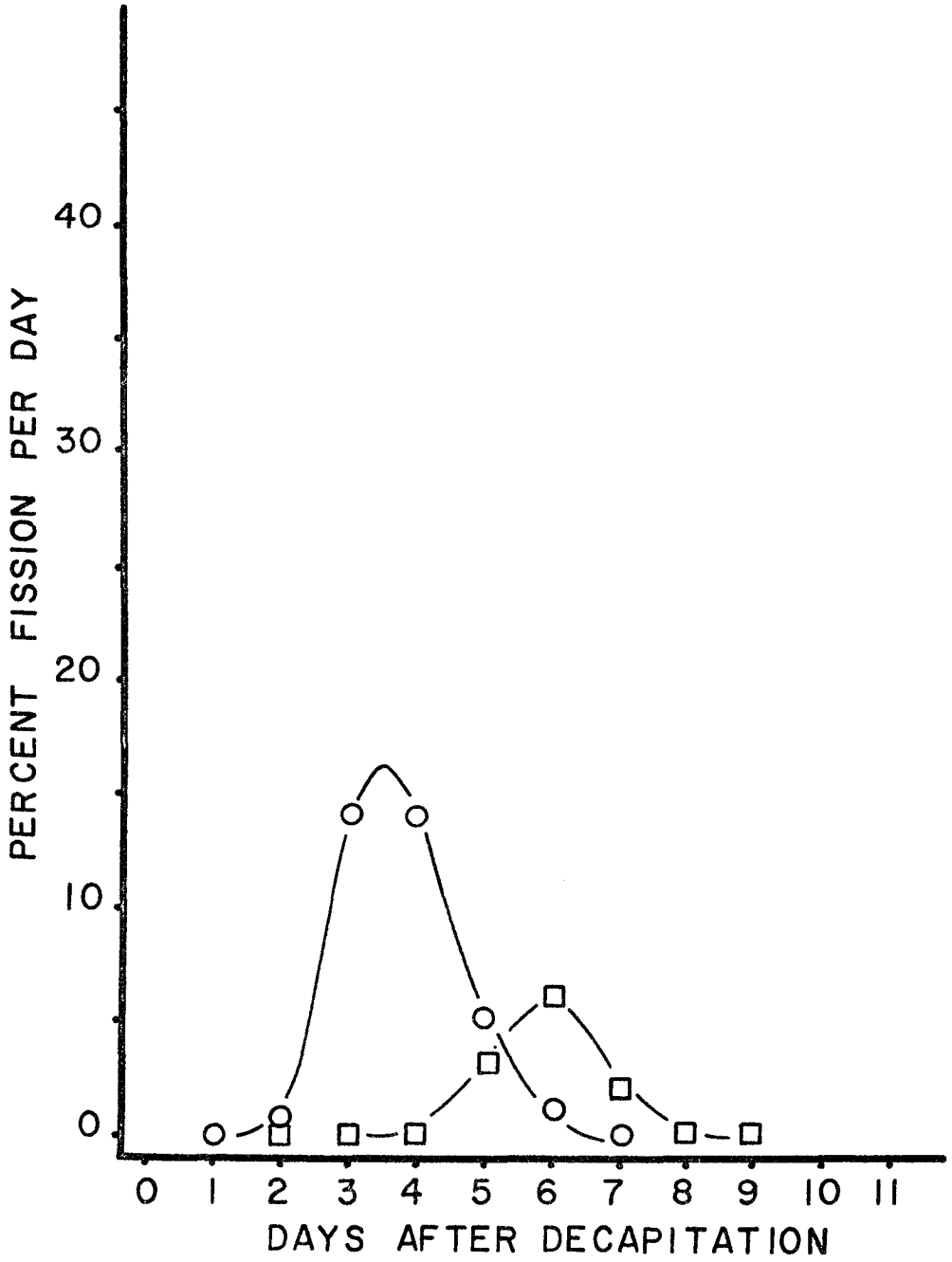
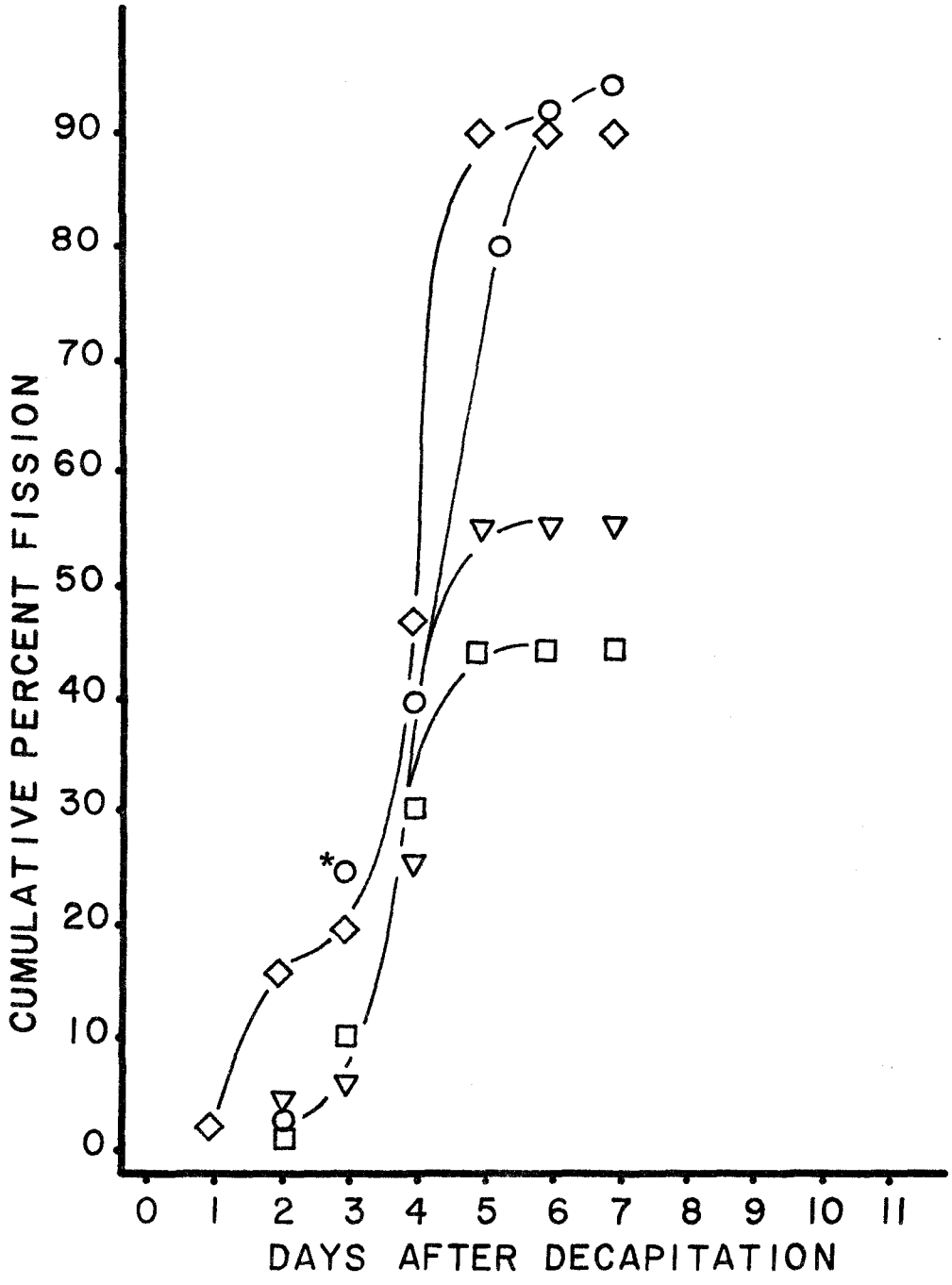

PERCENT FISSION PER DAY
40
30
20
10
0
0 1 2 3 4 5 6 7 8 9 10 11
DAYS AFTER DECAPITATION

Fig. 42. Effect of dialysis of whole planaria extract on decapitation-induced fission. Non-treated extract □ , extract dialyzable material ◇ , extract non-dialyzable material ▽ and non-extract treated control ○ .

*Anomalous point


CUMULATIVE PERCENT FISSION
0
10
20
30
40
50
60
70
80
90
0 1 2 3 4 5 6 7 8 9 10 11
DAYS AFTER DECAPITATION

extract-treated animals show fifty-five percent fission by day seven compared with forty-two percent in the non-dialyzed extract. The initial fission rate of these experimental groups (non-dialyzable extract and non-fractionated extract) are very similar with a ten percent difference in the fission plateau and a one day delay in fission-onset.

Heating the non-dialyzable fraction results in a slightly earlier fission-onset and a fifteen percent higher cumulative fission by day seven than its non-heated counterpart. Heating the dialyzable fraction did not alter either the cumulative fission or the latent period.

Further characterization of whole planaria extract was accomplished by filtration chromatography on Bio-gel P-10. The elution of whole planaria extract (Fig. 43) proceeds from higher molecular weights (Kav 0.00) through smaller molecules. Biological analysis of pooled fractions indicates (Table 5) that fission is retarded by the higher molecular weights (over 20,000). Fractions which were obtained towards the middle of the P-10 profile stimulated fission while the other fractions had no effect. The stimulator (Kav 0.82) spread from where it was initially observed (on a new column between 0.75 and 1.04 Kav) toward the trailing end as the column aged (0.92 through 2.09 Kav). The molecular weight of the stimulatory fraction was determined between 2500 and 3500 by rechromatography of the DEAE-purified stimulator (described later) on a calibrated P-10 column which was equilibrated with 0.1 M ammonium acetate (Fig. 44).

Table 5. Summary of biological activity of whole planaria extract which has been fractionated on Biogel P-10 (filtration chromatography).

Date	Fract.	K_{av}*			Percent Fission[1]	
		Min.	Max.	Peak[2]	Experimental	Control
27 Sept.	1	0.00	0.27	**	0	
	2	0.27	0.75	**	14	
	3	0.75	1.04	0.82	27	
	4	1.04	1.32	1.10	10	
	5	1.32	2.56	1.50	3	
						13
7 Dec.	1	0.00	0.18	**	20	
	2	0.18	0.46	**	10	
	3	0.46	0.84	**	25	
	4	0.84	1.25	0.95	35	
	5	1.25	1.65	1.30	35	
	6	1.65	2.08	1.75	25	
						13
15 Dec.	1	0.00	0.24	**	0	
	2	0.24	0.68	**	5	
	3	0.68	0.92	**	10	
	4	0.92	1.41	1.05	45	
	5	1.41	1.60	1.30	60	
	6	1.60	2.09	**	60	
						5

*$K_{av} = \frac{V_e - V_o}{V_t - V_o}$

**Peak was not present within fraction.

[1]Cumulative percent fission by the fifth day of post-decapitation incubation in extract fraction of whole planaria.

[2]Peak K_{av} is assumed on the basis of U.V. absorption.

Fig. 43. Elution profile (U.V. absorption) of whole planaria extract which has been fractionated (Table 5. 7 Dec.) on Biogel P-10 using standard Henderson-Eakin solution as the eluant.

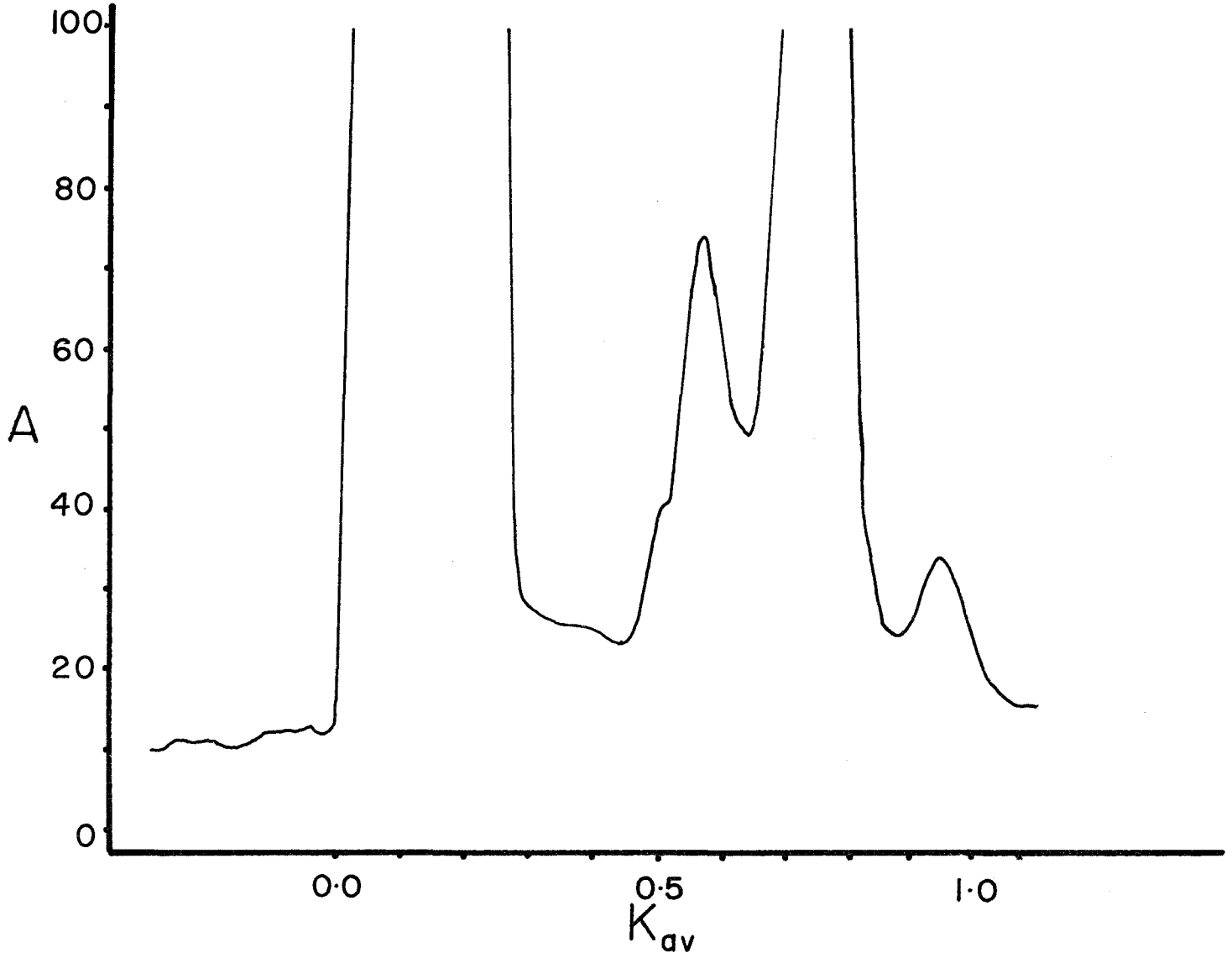

100
80
60
A
40
20
0
0·0
0·5
1·0
K_{av}

Fig. 44. Elution profile (U.V. absorption) of DEAE-purified stimulator which has been fractionated on Bio-gel P-10 using Henderson-Eakin solution plus 0.1 M ammonium acetate as the eluant. Insert ▦ is ten times absorbtion. Molecular weight calibration curve (-0-0-) was obtained using bovine serum albumin (M W 68,000), insulin (M W 11,460), vasopressin (M W 1050) and tyrosin (M W 181).

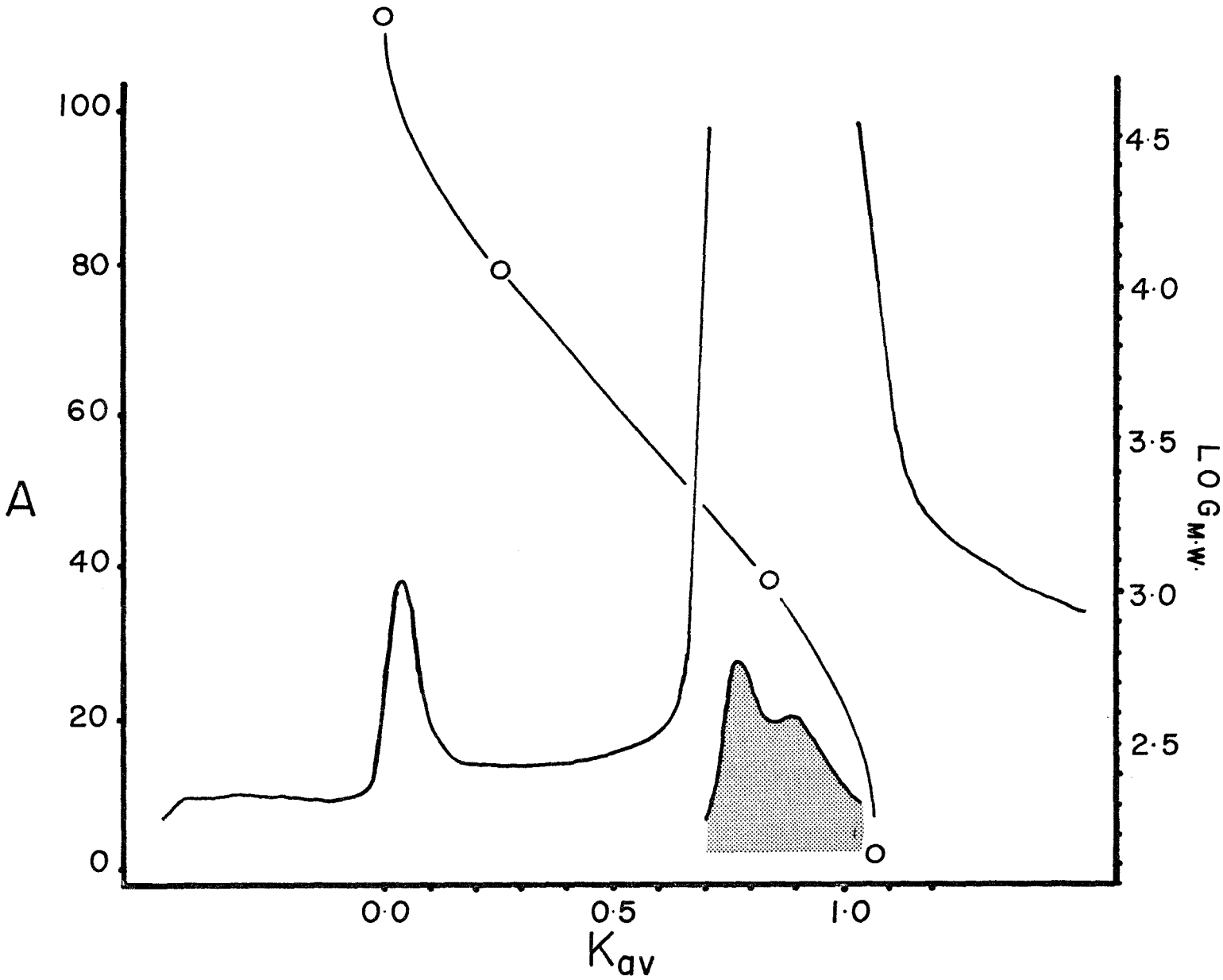
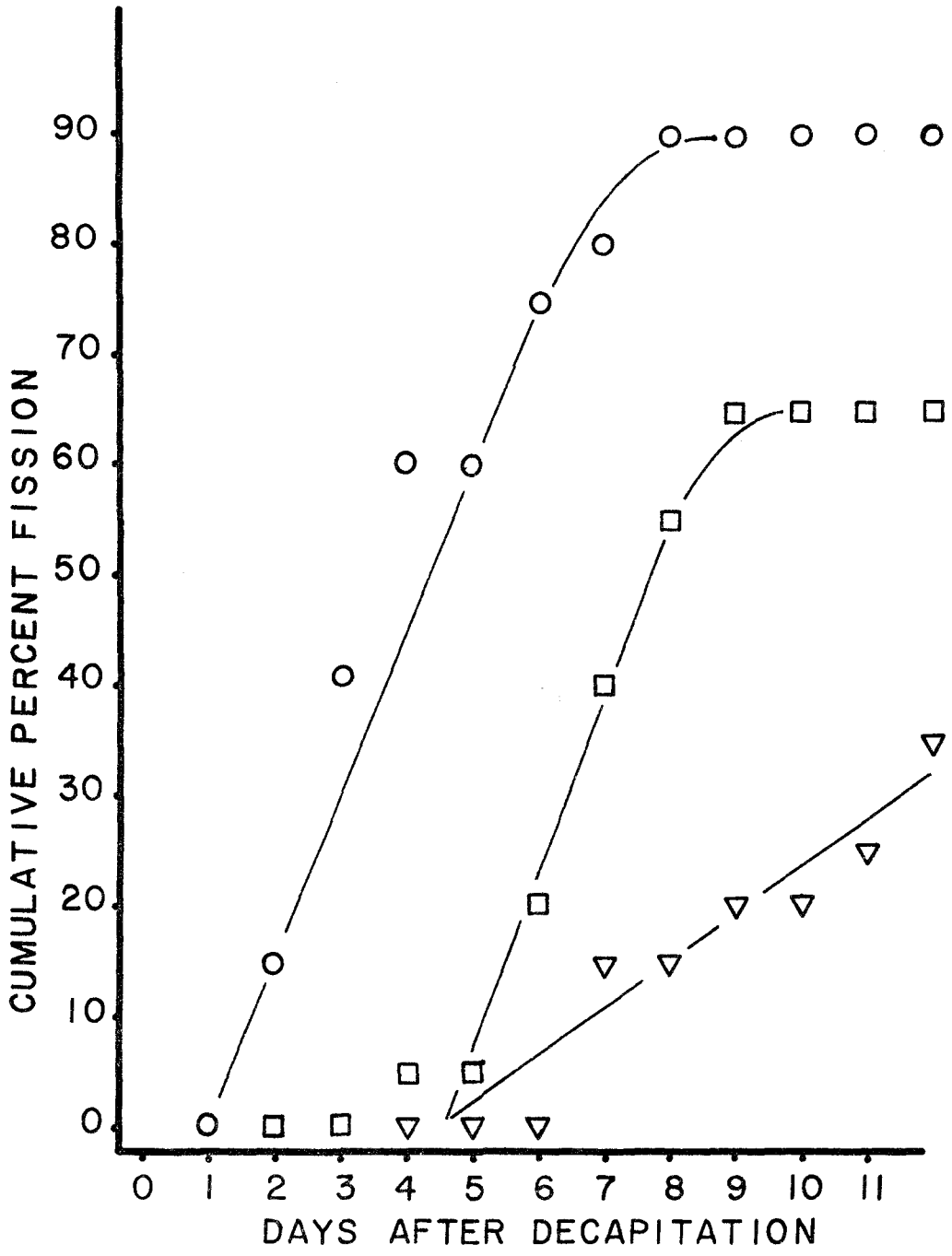

100
80
60
40
20
0
A
0·0
0·5
1·0
K_{av}
4·5
4·0
3·5
3·0
2·5
LOG M.W.

Fig. 45. Comparison of extract effect on decapitation-induced fission when whole planaria extract has been fractionated on Bio-gel P-10. Void volume fraction ▽ , mid-profile (K_{av} 0.82) fraction ○ and control □ .


CUMULATIVE PERCENT FISSION
90
80
70
60
50
40
30
20
10
0
0 1 2 3 4 5 6 7 8 9 10 11
DAYS AFTER DECAPITATION

A comparison between the void volume inhibitory material and the mid-profile (P-10 0.82 Kav) stimulatory fraction (Fig. 45) showed that the difference in cumulative fission continued through at least the thirteenth day after decapitation (sixty-nine percent versus ninety percent). In addition, fission had begun by the second day in these experimentals compared with day four in the controls.

Analysis of the void volume fraction (M W greater than 20,000) shows a significant retardation in the fission rate. The cumulative fission for this group by day nine was twenty percent compared to sixty-five percent for the controls. A line extrapolated to the base, by the method of the least square, indicates the fission process began at the same time in both experimentally inhibited and control groups.

The maximum difference between the control and the 0.82 Kav stimulatory fraction is plus fifty-two percent whereas the void volume material resulted in a depression of minus thirty-five percent (Fig. 46). The maximum stimulation (P-10 0.82 Kav fraction) occured by day five compared to maximum inhibition by day nine (P-10 void volume fraction). When the animals were incubated in non-fractionated planaria extract, both stimulation and inhibition are observed with a maximum deviation from the control of minus to plus ten percent.

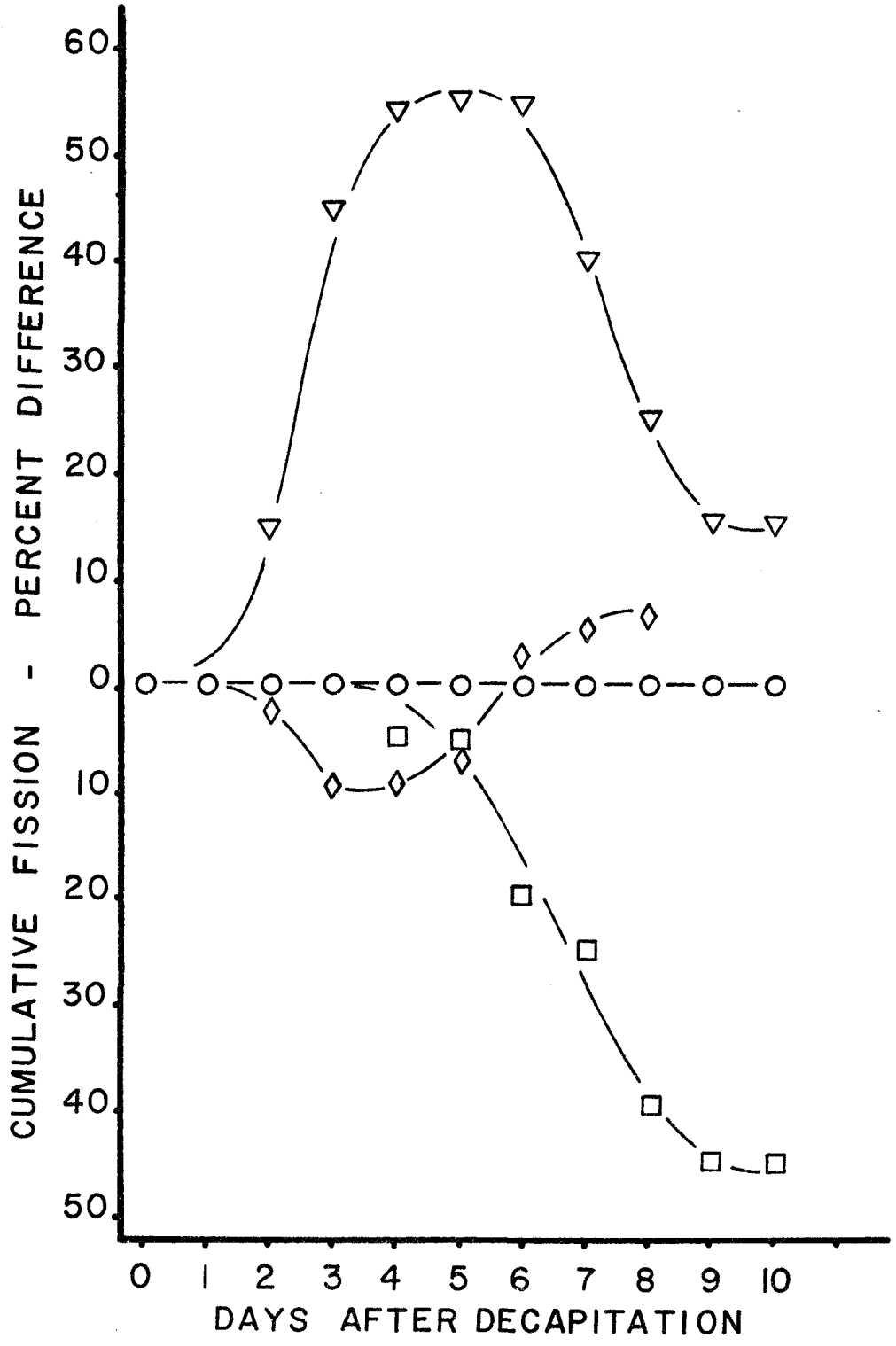
The P-10 0.82 Kav stimulator fraction was chromatographed on DEAE-Sephadex. Table 6 compares the biological activity of material eluted from the ion exchange resin (DEAE-Sephadex) at different pH and ionic strengths. At pH 7.0 the active material is attached at

Table 6. Cumulative percent fission of decapitated planaria on the fifth day after incubation in DEAE-purified fractions of the P-10 stimulator peak of whole planaria extract. (Assay at 50 ug per ml)

Molarity	pH				
	6.0	6.5	7.0	7.5	8.0
0.001	*	5	5	0	5
0.005	*	15	5	10	10
0.01	10	15	20	5	35
0.05	*	60	65	55	15

*Data not available.

Fig. 46. Comparison of stimulator and inhibitory factors which were isolated from whole planaria extract, then fractionated on Bio-gel P-10. Mid-profile (Kav 0.82) ▽ , void volume fraction □ , nonfractionated extract ◊ and control ○ .


CUMULATIVE FISSION - PERCENT DIFFERENCE
60
50
40
30
20
10
0
10
20
30
40
50
0 1 2 3 4 5 6 7 8 9 10
DAYS AFTER DECAPITATION

0.005 M and released from the ion exchange resin at 0.05 M. Twenty percent of the animals treated with the fraction obtained from the resin at 0.05 M underwent multiple fissions within ten days after decapitation which is indicative of significant stimulation.

DISCUSSION

Early in this study it became obvious that many environmental factors would influence both the rate of fission and the viability of planaria. Since few descriptions of the variables are available, it was necessary to establish "optimum" conditions as defined by maximum fission and survival.

Because of the potential unknowns involved in maintaining this organism in various modifications of "spring water" (Meyer, Meyer and Bueding, 1970; Vowinckel, Wolfson and Marsden, 1970; Jenkins, 1961) or conditioned "tap water" (Brown and Park, 1965; Pedersen, 1958; Rodrigues and Flickinger, 1971; Marsh, 1968) a synthetic medium was necessary. Both Betchaku (1970) and Henderson & Eakin (1969) have described defined solutions which were tried in this laboratory. The Betchaku solution (1970) was not satisfactory for maintaining planaria beyond one week. The Henderson-Eakin solution, however, has been successfully employed in maintaining all stocks and experimental animals for over two years with no apparent deleterious effects. We have verified that EDTA (at 10^{-6} M) has no detectable effect on the fission processes and is therefore included as a safeguard against divalent cations in the distilled water as described by the authors (Henderson and Eakin, 1961).

Light intensity was one of the most critical factors associated with viability of both decapitated and non-decapitated planaria (Table 2). It has been postulated that this sensitivity to light is

due to the eye spots (Marriott, 1958; Vowinckel, 1970b). Since Dugesia dorotocephala does not have eye spots this hypothesis cannot be used to explain the influence of light on the decapitates. Photo sensitivity of the epidermis is one possible explanation (Marriott, 1958).

We have ruled out the possibility that a temperature rise could account for the observed light-induced death and have further shown a direct relationship between the light intensity and death in this species (Fig. 3). We have not determined the most effective wave length although Marriott (1958) indicates that such a relationship exists.

It is recognized that in most biological systems the rate of activity is directly influenced by the environmental temperature. This has a greater effect on the metabolic processes in poikilothermic invertebrates than in warm-blooded animals. It would naturally follow, therefore, that the planaria would have an optimum temperature range for the fission process.

Incubation temperatures for Dugesia dorotocephala have been reported from 12°C (Sanders and Sanders, 1970) to 30°C Henderson & Eakin, 1961). However, this study indicates that 32°C and above results in death of the planaria within 24 hours. Temperatures between 14 and 20°C delays the time of fission-onset in the decapitated animals (five animals per plate) as the temperature is decreased (Fig. 1). At the lower temperatures, however, once the fission process begins the rate is the same as at 20°C. This similarity of fission rate indicates that the fission delay is not due to an

over-all reduction in the metabolic processes. Rather, it seems that this delay is due to a decreased sensitivity to the animal's intercommunication mechanism.

Temperatures from 20°C to 38°C does not hasten the fission-onset but decreases the fission rate (Fig. 2) possibly due to a deleterious effect on the metabolic processes. As the temperature is increased the number of animals which are capable of fission is reduced, and temperatures above 32°C are lethal. Since there is no alteration in the time of fission-onset it could be concluded that the animal intercommunication system is not modified. The optimum temperature selected for this study was 20.5°C based on the maximum number of animals fissioning with the shortest post-decapitation latent period. Whether or not this temperature would be optimum to other physiological processes has not been determined. Indeed, it appears that each process has an optimum temperature, such as development of the sexual apparatus (Vowinckel, 1970a and Jenkins, 1970a).

During the period from 1941 (Child, 1941) to 1968, (Bingham, 1968) very little interest was expressed in decapitation-induced fission. "Tail dropping" or decapitation-induced fission should have been further corroborated in the many studies of the process of head regeneration in planaria. Penicillin has been routinely used in most recent experiments to reduce bacterial infection (Coward, Flickinger and Goren, 1964). Our data, however, indicates rather conclusively that the routine concentrations of 100 ug per ml, penicillin will completely inhibit fission (Fig. 4). Concentrations above 100 ug per ml, in addition to inhibiting fission are lethal to the planaria (Fig. 5).

During the initial investigation period a regular feeding routine was established at weekly intervals, and a fast of five or six days was imposed before experimental homogenization or decapitation. The main purpose of the fast was to reduce the complexity of the internal milieu of the homogenate.

Due to a decrease in our stock population because of cannibalism, feeding was changed to three times per week (Monday, Wednesday and Friday). This resulted in an alteration of the fission rate (Fig. 6) which was eventually associated with the change in feeding routine (Fig. 10). The time of fission-onset among non-decapitated animals showed the greatest variation with latent periods from one day (Fig. 9) to six days (Fig. 8). In addition, the total percent of the population which had fissioned at the end of the log phase varied from twenty percent (Fig. 8) to fifty-five percent (Fig. 9). This latter value was very similar to that observed in many decapitation-induced fission experiments (Fig. 13).

In spite of the similarity in fission rate of both decapitation-induced fission and spontaneous fission in non-decapitates, it is possible to differentiate between the two groups by determination of the theoretical time of fission-onset. A line derived by the least square method, when extrapolated to the absissa, will intersect at a theoretical fission-onset point (Fig. 8). For all populations which have not been decapitated this intersection is at day zero even when the fission rate is as great during the log phase as the decapitated animals (Fig. 9). The theoretical fission-onset point for nondecapitated planaria (day zero) is compared to the decapitated animals in which the least square line intersects no earlier than day 1.5 (Fig. 8). This time varies depending upon the

group of animals being analysed.

When planaria were fed often (Monday, Wednesday and Friday) many animals in the stock culture were not eating. This suggested that feeding regularity approached ad libitum feeding which the animal would experience under natural environmental conditions. Feeding-induced variability in fission rate of non-decapitated animals could be explained on the basis of the number of animals within a culture which were eating at a given time. Fluctuations in fission rate which are initiated by feeding subside after three days; the rate assumes a stable, low value (ten percent) and is more linear with a theoretical fission-onset at day zero (Fig. 9). The feeding-induced fission is most noted when feeding is followed by isolation of the animal from group cultures (Fig. 11). If the animals are left in group cultures the stimulation of fission is not apparent and a slight fast prior to isolation will result in a delay in observed fission-onset (Fig. 8).

These observations suggest that at least two environmental conditions contribute to fission control: (1) the availability of food; and (2) the population density. In the natural environment (grouped animals) the post-feeding response is a delay in fission followed by an accelerated fission (five to ten days later) then return to a constant rate. The delay is more likely associated with animal interaction mediated by sensitivity to slime produced by other animals (McConnell, 1966) than to inhibition by digestive waste which results from the feeding processes (Vowinckel, Wolfson and Marsden, 1970).

Further indication that feeding is responsible for increased fission is seen when planaria are fasted for two weeks and four weeks. Extended fasting (40 days) does not usually alter the spontaneous fission rate (Fig. 12). Only when the animals are fasted, fed, then isolated is the spontaneous fission rate significantly increased (Fig. 12) as compared to the control groups. The effect of feeding-induced fission is minimized when the fasting period is shorter.

The fission pattern in decapitated planaria is much more uniform than that associated with spontaneous fission in non-decapitated animals and follows a gaussian distribution (Fig. 7). Further, prolonged starvation prior to decapitation does not reduce the fission rate nor the fission mode (Fig. 13). These observations suggest that the decapitation-induced fission process is the result of more explicit instructions (possibly of a genetic nature) to the fission machinery. These instructions may be associated with a cyclic process (biochemical sequence of events) in which fasting retards or inhibits the initiation of a particular sequence among non-decapitates.

In addition to feeding-induced fluctuations the fission rate varies depending on the population types. Planaria obtained in the spring, when decapitated, would fission in a very predictable manner throughout the summer. During the fall, however, the fission pattern changed. Fission-onset was accelerated by one day and the fission rate decreased from twenty-six percent per day (Fig. 15 during the log stage,days two through five) to twelve percent per day (days two through eight Fig. 8). The spontaneous fission rate

among non-decapitates increased (Fig. 6) from summer to fall. It was at this time that cocoon production began in the stock containers and continued for a short time (two months) then subsided, while spontaneous fission diminished. This suggests that spontaneous fission among non-decapitates is highest just prior to the onset of the sexual cycle. These data do not agree with those of Lender (1970) using Dugesia dorotocephala. He said that in autumn (period just prior to the sexual stage) the scissiparity cycle is longer and in January and February planaria are not scissiparous.

Possible explanations for the variations in fission are (1) genetic selection, (2) physiological change and (3) food type. The company (Dahl) which supplied the animals used in these experiments indicated that the planaria received in the early spring were obtained from a stream which was turbid due to spring run-off. Later in the season the animals were collected from another, slow-moving, stream which was approximately five miles away. These two streams were not connected which would suggest the possibility of species differences similar to those observed by Kenk (1966). These species differences are possible because the characteristics for taxonomic identification have not been resolved (De Bold, Thompson and Landrites, 1965) and may be more morphological (quantitative) than physiological (qualitative). This is exemplified by the fact that we obtain Dugesia dorotocephala under the name Dugesia tigrina.

Selection of asexual animals for biological assay, while the animals are in our laboratory, could increase the percentage of sexual animals in stock; thereby producing a change in "fission-

ability" associated with race differences.

Dugesia dorotocephala has been reported to exist in "physiological races". This species is supposed to be able to change from an asexual race to sexual race but not from sexual to asexual (Jenkins, 1970). Our observations, however, do not support Jenkins' statements on the irreversibility of the sexual animals. By using decapitation-induced fission as the criterion to define the asexual race (Fig. 23) we have shown that morpholactic regeneration of sexual planaria which have been cut in two or three sections will produce animals which are capable of fission induced by decapitation (Fig. 24). The state of physiological maturation is a very definite problem which needs more attention.

Not to be disregarded is the possibility that the accumulation of steroids through the feeding process causes a change in the physiological state of the animals. It has been shown (Meyer, Meyer and Bueding, 1970) that D. dorotocephala is unable to synthesize steroids from acetate. This supports the possibility that the supply of food (containing steroids) will permit a shift in the chemical balance which is expressed as a physiological race.

During the experimental excursion in which we examined the effect of feeding on the fission process we noticed that the stimulation of fission by decapitation was not as great at this time of year (fall 1971) as that which we had seen earlier (Fig. 14) or that reported by Bingham, (1968) Leavett, 1969) and Sheffield (1970). When decapitated and non-decapitated animals were compared early in

our research, one of the most striking differences was the rate of fission (Fig. 14). During the fission log phase of the decapitated animals (day two through six) the fission rate was eighteen percent per day, compared to two percent per day for non-decapitates. This difference in fission rate of sixteen percent per day indicates that decapitation stimulates fission. Later in the study differences between decapitates and non-decapitates (6.5 percent per day) were not as great as had been previously observed (Fig. 15). The only difference between these two experiments (Fig. 14 and Fig. 15) is the number of animals per culture dish. The earlier experiments (Fig. 14) had ten animals per petri dish and the later (Fig. 15) had one animal per petri dish. The animal to fluid-volume ratio was about the same in both. We noticed that the generation time was different; 200 days with grouped animals and 45 days when incubated individually (Fig. 19).

Experiments in which one animal per petri dish was compared with five animals per dish, it became obvious, in accord with the literature (Nentwig, 1971), that decapitated planaria will respond to population density in grouped incubation (Fig. 16). However, this modification is not as great as that observed among non-decapitates (Compare Fig. 14 with Fig. 15). Multiple animal incubations result in a delay in fission mode of three days (day 3 to day 6) when compared to one animal per incubation plate (Fig. 17). This delay (Fig. 18) differs from that which has been attributed to crowding (Kanatani, 1957a; Vowinckel, Wolfson and Marsden, 1970) and is influenced by the container shape (Fig. 22).

Data collected from decapitated animals incubated in groups of five indicate that the fission process, although retarded (compared to individually incubated animals) shows that a complex intercommunication system exists (Fig. 20, Table 3). Both stimulation and inhibition mechanisms would have to exist in order to fully explain the data. However, when the fission mechanism (stimulation or inhibition) has been initiated, all animals within the dish will respond accordingly. That is, it appears as though one animal can trigger fission in the other animals of that group. The result is an all-or-none fission pattern of grouped animals in one plate which is very similar to that in which animals are incubated individually.

One component (stimulator) of the intercommunication system is water soluble and is secreted into the culture medium (Fig. 21). Animals decapitated and then incubated in medium in which planaria had been previously grown (conditioned medium) have both a greater fission rate and total fission (ninety percent) than those incubated in fresh medium (sixty-five percent). Fission pattern variations due to animal interactions seem to have their basis in the genetic complement of the planaria.

Sexual organisms which have been described as a "physiological race" are genetically predisposed to depositing cocoons according to Jenkins(1963). Although fission has been observed in animals with a gonopore, it is extremely rare (Fig. 23). Further, decapitation-induced fission in the sexual race has not been seen in this laboratory. Whether this is a specific example of a genetic or physiological phenomenon is not known at this time. Although the literature indicates that it is a genetic manifestation, our data

suggests it is possibly a physiological expression (Fig. 24).

On numerous occasions multiple fissions were observed and seemed to indicate that the process is closely controlled by the animals' genetic complement. Many fissions are of the type illustrated in figure 25 in which the original anterior end produced a second zooid. This is the type of fission which would be expected if fission were a physiological function restricted to the mature animal. This hypothesis would require complete maturation of the head or reproduction control centers before fission (reproduction) could be initiated. However, in figure 26 the second zooid originated from the first zooid and not from the original anterior end. This pattern suggests the presence of a fission control mechanism in the daughter zooid (tail section) which became functional after separation from the parent and before complete maturation. The appearance of four daughter zooids (Fig. 27) from a single animal within ten days gives further support to an intrinsically controlled fission mechanism. In this example each daughter zooid has given rise to its own zooid even before the eyes are completely mature. An experiment designed to determine the frequency of such occurences (Table 4) was conducted with thirty decapitated animals. All thirty animals produced one zooid and eight of these produced a second zooid from the original head. The time required for this process averaged nine days, which is in agreement with Curtis (1901). Of the thirty daughter zooids, eight produced an additional zooid (Z_2, Table 4) in an average of ten days. Of these eight additional zooids, five of the original parents (sixty-two percent) produced a second zooid (Z_3, Table 4) within two days. These observations do not agree with

Curtis (1901), who stated that the frequency of head redivision was just twice that of the tail. In addition the concordance (sixty-two percent) suggests contrary to Curtis, that the fission process is regular. This supports the hypothesis that the genetic mechanism in the original parent and tail or "zooid" are in synchrony with respect to the fission mechanism.

Observations by Sengel (1959), Stephan (1967), and Ziller-Sengel (1967a, 1967b and 1967c) indicate that pharyngeal regeneration can be inhibited by incubation in a brei of pharynx. Lender (1952, 1955, 1956, 1960) has shown that incubation with planaria brain will inhibit brain regeneration of decapitated planaria. Further characterization lead Bingham (1968) to associate this retardation of brain development with control of asexual reproduction. At the present speculation centers around the possibility of a neurosecretory or neurohormonal compound responsible for fission control generally thought to be inhibitory (Grasso, 1965b, 1966b;Bondi, 1966; Liotti and Rosi, 1967; Liotti, 1968; Lender and Zghal, 1968, 1969; Lender, 1971). The focus of such a compound has been thought to be associated with the cephalic ganglia (Lender and Klien, 1961). However, Nentwig (1972) suggests that such a compound probably emanates from the entire nervous system. Wolff, Lender and Sengel (1964) explained Childs' theory of axial gradients and dominance by postulating the presence of specific regeneration-inhibitory substances which proceeded from the head region toward the tail.

Assuming that inhibitory compounds which specifically alter the fission process do exist, an experiment with an extract of the entire organism was conducted. That the effect of the whole-body

extract on the fission rate was specific was evidenced by the linear relationship which exists between concentration and fission (Fig. 28).

Because of the variable results, however, it was necessary to determine if there was a time-dependent change in the extract when stored. Figure 37 indicates that inhibitory activity is lost when the extract is stored at $4^{o}C$. It should by noted that this mixture still contained compounds, possibly non-specific, which decrease the planaria fissionability and is not modified with time. However, the significant feature is the labile compound. If the extract was kept frozen there was not the same decrease in fission rate. It was also noted that the fission mode was progressively increased (day 2) when the extract was kept at $4^{o}C$ (Fig. 38). This suggests the possibility of a stimulator type compound.

Twenty-five experiments in which extract at 100 ug per ml was used showed that inhibitory activity is lost within the first 48 hours (Fig. 29) after decapitation. The fission-onset is delayed 24 hours beyond the control. The initial fission rate is approximately the same as the non-treated control group. However, the extract treated group has a slight acceleration in the fission rate from day three to day five compared to the controls. The plateau of the extract-treated decapitates was approximately ten percent lower than the non-treated controls. This value is the same as the percent fission observed in the controls between days one and two. This indicates that those animals which would have fissioned during the first 24 to 48 hours (ten percent of the group) have not fissioned even after inactivation of the inhibitor. The over-all implication is that the genetic events which are inhibited by the extract begin about 24 hours before fission is observed. Further, once these events have

been inhibited they do not recover during the first cycle of decapitation-initiated fission. When the fission process is observed for at least two weeks, fissionability of the extract-treated animals reaches that of the controls. This observation suggests that extract inhibition isn't permanent but may retard a group of biochemical events beyond a "critical" time. This hypothesis is further supported by an apparent "synchrony wave" which has been associated with decapitation-induced fission (-0-0- Fig. 15).

Inhibition by planaria extract is apparently non-deleterious to the fission cycle. The fission profile of decapitated animals treated with planaria extract is typically bimodal (Fig. 30) and is quite different than the normal profile for non-treated controls (Fig. 17).

When multiple, daily inoculations of the frozen extract are given the post-decapitation latent period is lengthened (Fig. 31). When the daily inoculations are terminated and fission commences, the same "bimodal" time peak that is shown by the controls is observed. In spite of the fission time delay, the total number of animals fissioning was the same as the controls; indicating the fission apparatus has not broken down even when delayed as long as five days.

When the extract is kept at $4^{\circ}C$ for 24 hours prior to inoculation the over-all profile changes slightly (Fig. 38). Most significant is the time of fission-onset which is at least one day earlier than would be expected if the extract had been kept frozen. This again indicates the possibility of a stimulator which acts when the inhibitor has been inactivated.

When the planaria were treated with whole extract which was heated for 30 minutes at 96°C there was a definite increase in fission during the first day after decapitation (Fig. 39). This could infer the presence of a protein-stimulator complex, the stimulator activity increasing upon removal of the protein portion. Ultracentrifugation of the extract put the inhibitory factor in a pellet obtained at 32,500 RPM for 17 hours (Fig. 40).

Ultrafiltration was used to further characterize the whole planaria extract. The inhibitor material passed through a UM-50 membrane and was retained by a UM-10 membrane (Fig. 41). The molecular weight of the inhibitory fraction would be between 10,000 and 50,000. This inhibitory activity is noted only when both the UM-10 and UM-50 membrane are used. If the UM-50 membrane is used exclusively the biological assay of extract filtrate is about the same as that of the control. The implication of this finding is that a stimulatory molecule (M W less than 10,000) must be removed before the inhibitory action can be properly evaluated.

The fission mechanism seems to be more sensitive to stimulation than inhibition because almost complete removal of the stimulator molecule was necessary before inhibition was significant (Fig. 42). The non-dialyzable material which contains the inhibitory factor was not completely removed although owing to diffusibility of the stimulator, ninety-five percent was removed. This resulted in a fission pattern for both non-dialyzable and non-treated extract being the same. It is possible that the inhibitory molecule is complexed with the stimulator molecule, thereby preventing or failing to induce fission. When the non-dialyzable fraction was heated there was a decrease in the post-decapitation latent period (earlier

than control) which is similar to that induced by purified stimulator. This enhanced initial fission rate was lost after the third day (post-decapitation) indicating either a deactivation of the inhibitor-stimulator complex or a short period of sensitivity by the animal to the stimulator molecule.

When whole planaria extract was chromatographed on Bio-gel P-10 the typical profile (Fig. 43) resulted in five major UV absorbing peaks. The first peak is the void volume effluent which has a molecular weight greater than the retaining capacity of the gel beads (greater than 20,000). The void volume peak is rather complex indicating that there are several high molecular weight species. Three of the UV absorbing peaks, in the middle of the profile, represent molecular weights which are in the optimum working range of the Bio-gel P-10.

Biological assay of various fractions of the Bio-gel P-10 profile (Table 5) indicate that inhibitor and stimulator molecules are separated from one another by this technique. The inhibitory molecule is located in the void volume fraction which is of a molecular weight greater than 20,000 according to the manufacturer's specifications. A single inoculation of this fraction (void volume) did not result in a significant increase in the latent period (compared to the control) but decreased the fission rate (Fig. 45). In addition, the inhibitory molecule was not degraded by incubation with animals as previously demonstrated (Fig. 31). Whether the inhibition is due to lack of stimulator by complexing (with the high molecular weight inhibitor) or presence of a specific inhibitory molecule has not been investigated. A second fraction was found to be inhibitory (P-10 Kav 1.39 through 2.56 molecular weight less than 500).

This is probably non-specific inhibition because of the amino-acids and peptides which would be contained in this fraction as demonstrated in figure 31. Inhibition associated with this P-10 fraction was not consistant owing to the "sorbitive spreading" of the charged stimulator (P-10 Kav 0.75 through 1.04).

A molecular species observed in P-10 mid-profile (M W 2500 to 3500 (Fig. 44) was found to stimulate the decapitation-induced fission process (Table 5). It should be noted that this factor was spread from the point in which it was initially located toward lower molecular weights. This spreading is due to sorption to Bio-gel P-10 when operated at low ionic strengths.

The stimulator molecule decreased the decapitation-induced latent period when compared to the controls (Fig. 45). In addition, the fission rate was greater than the control value (most noted during the first three days after fission-onset). Also the number of animals induced to fission was thirty percent higher than the control value.

The fission modes of the stimulator-treated (0.82 Kav) and inhibitor-treated (void volume fraction) were compared with the control group (Fig. 46). The stimulator caused a fifty percent greater fission rate at its maximum activity (day five). This is compared to the inhibitory fraction which caused a forty-five percent decrease in fission by the tenth day after decapitation. These data indicate that each molecular type has a time of optimum activity. That is, the stimulator results in early fission while the inhibitor acts later by suppressing the fission sequence itself. It is interesting to compare these data with the activity modes of the non-fractionated

extract. The non-fractionated extract inhibits early while stimulating later (Fig. 46). These data seem to support the concept of a short-lived inhibitor and a long-lived stimulator.

Further purification of the Bio-gel P-10 mid-profile stimulator was accomplished using an ion exchange resin DEAE-Sephadex. The stimulator was completely absorbed to the resin at an ionic strength of 0.005 M and was removed when the molarity of the eluting buffer was increased to 0.05 M. This procedure resulted in a four fold increase in specific activity. In addition to an acceleration of fission it caused the planaria to undergo multiple fissions. One hundred percent of the decapitated planaria treated with the DEAE-purified stimulator produced one zooid by the seventh day of treatment and twenty percent had produced a second zooid by day twelve (Fig. 26). Many of these (ten percent) had produced three zooids by this time. These observations further support the hypothesis that the stimulator molecule is long-acting. The material is apparently not degraded upon utilization and accelerates the fission mechanism in the zooids.

SUMMARY AND CONCLUSIONS

Decapitation of the planaria Dugesia dorotocephala stimulates asexual reproduction, indicating that substances of cephalic origin may be the controlling factors.

Molecular classes were resolved, using filtration chromatography, which would stimulate or inhibit the fission process. Both of these classes, which were initially obtained by homogenization of the whole planaria, were active in the non-fractionated whole extract although to a lesser extent than when purified.

The inhibitor molecules are specific for the decapitation-induced fission process and not metabolic poisons similar to those which have been associated with crowding. The non-dialyzable inhibitor molecules are sedimented by centrifugation for 17 hours at 32,500 RPM and have a molecular weight between 20,000 and 50,000 as determined by ultrafiltration and gel chromatography. Further, the inhibitor molecules are heat labile and begin to lose the inhibitory properties in 24 hours of incubation with decapitated planaria. Fission inhibition can be maintained by replacement of the extract at 24 hour intervals with no deleterious effect to the animals' fission mechanism. Inactivation of the extract during incubation is not as extensive after fractionation of the crude extract as before. Non-fractionated whole planaria extract loses its biological activity when stored at $4^{o}C$; stability is maintained, however, if the non-fractionated brei is kept frozen. Storage inactivation results in the appearance of a

stimulation of the fission process as does short term heating (96°C for 5 minutes) which suggests the possibility that the inhibitor is a physiological complex between the heat labile protein and the stimulator.

The stimulator molecule is dialyzable and has a molecular weight between 2500 and 3500. This material is not heat labile and is not degraded by planaria. Purity and specific activity was increased by adsorption on DEAE-Sephadex at 0.005 M and removal was accomplished at 0.05 M. The stimulator (at higher specific activity) will decrease the generation time from 45 days to 5 days or less.

The decapitation-induced fission process is under intrinsic (gene) control as demonstrated by the concordance of fission of the second zooid of the original decapitated animal and the secondary zooid of a primary daughter zooid. Further, decapitation causes a post-decapitation latent period followed by a "fission wave". The percent of the population which contribute to the fission wave, described as stimulation, is dependant upon the physiological maturity of the animals. Sexual planaria are unable to respond to decapitation as asexuals, however, sexuals are capable of decapitation-induced fission after morpholactic regeneration.

A complex inter-animal communication mechanism suggests a population control system in which both inhibitory mechanism (which is dependant upon intimate contact) and a stimulatory mechanism participate. The stimulator factor will trigger a group of animals to fission in synchrony (all-or-none). Further, the difussible stimulator molecule which was isolated from whole planaria extract was shown to be secreted by the animal into the culture medium.

The medium (containing the stimulator) was then capable of accelerating decapitation-induced fission.

The fate of the non-diffusible inhibitor was not completely elucidated. It was thought to be present in the slime produced by the planaria. Whether its mode of action was removal of the fission-specific stimulator or a specific inhibitor was not shown.

The association of feeding in population control was not completely resolved although it does present a complex inter-animal communication system. The extent of this response is reduced by grouped post-feeding incubation of planaria. Stimulation associated with feeding is lost three to four days after feeding and may be associated with accumulation of steroid precursors through the liver. The head has been suggested as a receptor site for such intercommunication because of the relative response of decapitates compared with non-decapitates. This difference may however, be partly accounted for in the similarity of time of response to feeding (three days) and the post-decapitation latent period (two days).

Seasonal cycles were not investigated extensively although they may be additional factors contributing to population control. The fission process (in both decapitates and non-decapitates) was increased in late October following which cocoons were produced. The cocoon production ceased in the middle of January with a concurrent reduction in the fission rate of both decapitates and non-decapitates followed eventually by return to "normal" fission rate.

Although both temperature and light fluctuations were ruled out as causes for fission rate changes in the laboratory, both are

important factor. Temperature is particularly important because of the associated delayed animal response to the fission control mechanism through inter-animal communication (stimulator) as the temperature of incubation was reduced.

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ANALYSIS OF ASEXUAL REPRODUCTION

IN DUGESIA DOROTOCEPHALA

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ABSTRACT

Genetic control of decapitation-induced fission in planaria is mediated by stimulator and inhibitor molecules. Resolution of the two factors was accomplished by filtration chromatography of whole planaria extract. The stimulator (M.W. 2500 to 3500) was purified with ion exchange chromatography and increases the multiple fission rate. The inhibitor (M.W. 20,000 to 50,000) is non-dialyzable, heat labile and is inactivated unless kept frozen. Storage at 4°C results in a loss of inhibitory capacity with a concomitant stimulator increase, suggesting a protein-stimulator complex as the inhibitor.

In light of a demonstrated animal intercommunication system, the inhibitor-stimulator complex is hypothesized as part of a natural population control mechanism. The stimulator (long-acting) is released into the culture medium and induces an all or none response among other members of the population which are physiologically receptive. Fission inhibition (short-lived) is elicited only upon intimate animal contact and is possibly in the animal slime.

COMMITTEE APPROVAL

VITA

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