THE GROWTH OF PARAMECIUM IN VARIOUS CULTURE MEDIA

By

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INTRODUCTION

Protozoa combine the properties of a single cell with those of metazoan organisms. This observation has led to an increasing use of protozoa in physiological research. One such organism that has been used by many investigators as an experimental animal is Paramecium.

The reports of various investigations pointing out the difficulties encountered in raising paramecia in a bacteria-free medium, and the indications of changes in morphology due to environmental factors seem to make paramecia a promising organism for physiological research.

The culturing of Paramecium in a bacteria-free medium, in order to more accurately ascertain the nutritional requirements of these organisms, was found to be desirable in order to determine the effects of blastogenic agents. No investigator has reported a medium that satisfactorily supports paramecial growth when totally free of bacteria. Thus the study of the effect of blastogenic agents necessarily included a consideration of the problem of nutritional requirements of these organisms.

As a result of these considerations, the first part of this problem was confined to a study of the nutritional requirements of Paramecium, and this was followed by a study of the effect of blastogenic agents.

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REVIEW OF THE LITERATURE

I. THE CELL-FREE MEDIUM

The protozoans most difficult to culture are the Holozoic forms. Many attempts have been made to culture ciliates in bacteria-free media but the reports are contradictory owing to the manifold nature of the problem.

Peters [1] reported a successful culture of Colpidium colpoda in ammonium-glycero-phosphate medium but his work was not verified by other investigators, and he reported bacteria in his media.

Lwoff[2] found that Nitrogen in the form of nitrates, NH_4 salts, amino acids, or silk and tryptone would not maintain the growth of Glaucoma Piriformis in bacteria-free cultures. Peptone of muscle and yeast autolysate produced growth over many passages. The same investigator also isolated in a liquid medium a pure culture of glaucoma piriformis. When he attempted to cultivate other ciliates in peptone media, he did not succeed.

Loeffer[3] obtained a pure culture of Paramecium bursaria free from bacteria in a dextrose-tryptone medium. This case differs from all others in that the organism has a symbiotic green alga. Loeffer has not reported growth of this species in pure culture when freed of the alga.

Glaser and Coria[4] reported the culture of Trichodina in sterile bouillon medium, but later reported that they did not thrive after numerous transfers. These authors attempted a bacteria-free culture of Paramecium caudatum in a complex medium of liver extract, yeast and kidney. Hetherington[5] repeated the same and found slow growing

bacteria in the medium. He showed that Paramecium caudatum died in one hour in the 0.5% sterile liver extract, but that after the addition of yeast extract and kidney, they lived for 3 hours. He found the kind of bacteria hardest from which to free Paramecium looked like cilia, and needed incubation for more than 3 days to show colonies; also they did not cloud the liquid media, even after considerable periods, and were remarkably adherent to the paramecia.

Hargitt and Fray[6] sterilized paramecia by repeated washings and then introduced them into infusions containing a single known bacterium, and later into a mixture. They found that Bacillus subtilis supported fair growth of paramecium, but concluded that a mixture of several kinds formed a more suitable diet. Leslie[7] used 30 species of bacteria standardized as to age and amount used and tested their food properties for Paramecium multi-nucleatum. Of these 12 species supported good growth, with a division rate of 1.2 - 1.7; the rest were poor, having a division rate as low as 0.1 - 0.5.

Colpoda steinii, Glaucoma scintilleus and Glaucoma ficaria were cultured on dead bacteria or dead yeast and dead flagellates, but Hetherington[8a] and Phelps[8b] reported negative results with Colpidium, Paramecium and Euplotes. Leslie (1939) tried dead bacteria as a source of food for Paramecium without success while Glaser and Coria[4] obtained cultures of Paramecium in media containing either dead yeast or dead bacteria, only after the addition of sterile rabbit kidney and liver extract.

Hall[9] reported that none of the ciliates he worked with will grow in pure cultures of gelatine dextrose, but that on the addition of pimelic acid to this medium he was able to carry Colpidium campylum

through 6 transfers.

In 1941, Johnson and Stanley Baker[10] reported yeast juice as a medium for Paramecium. Their cultures had been passed through 18 transfers over a period of 10 months. The best fission rate was 0.5 per day.

In 1942, Medes, Grace and Anna K. Stimson[11] reported the inadequacy of autoclaved hay infusion for supporting the growth of Paramecium multi-nucleatum, even after the addition of the B complex vitamins. Passing the medium through Berkefeld filter prolonged the life of paramecia up to 30 hours. Similar results, they reported, were obtained with synthetic media of salts, dextrose, amino acids and crystalline vitamins.

II. EFFECTS OF CARCINOGENIC AGENTS

Mottram[12] subjected paramecia to 5 "blastogenic agents": polycylic hydrocarbons, gamma radiations, ultraviolet, heat and cold. Occasionally abnormal cells resulted which on reproduction in the absence of these agents produced populations of cells varying from apparently normal to very abnormal monsters. According to this author, proneness to abnormality was transmitted rather than any special form of abnormality and this transmission could be carried on indefinitely. The first change preceding abnormal cells was a change in the cytoplasm which interfered with cleavage leading first to delays and later to failure to cleave. After failure to cleave the cell reorganized itself into a single cell having a double constitution; the subsequent division of these doubles gave rise to the polymorphic abnormal populations resembling the cells of which tumors are produced in metazoa.

Irving et Al[13] employed methyl cholanthrene (0.1% - 1%),

Scharlach red (Ponceau 3B) (0.1% - 1%) and 3:4 benzpyrene (0.1% - 1%) suspended in rich culture of paramecium caudatum. Inoculation was made at regular intervals for the experimental as well as the control cultures. Swellings, vacuolizations and blisters appeared in the pellicle. They did not find monsters, however. Organisms exhibiting these abnormalities, even when isolated in normal media, became sluggish and misshapen and soon died. Organisms growing in methyl cholanthrene exhibited abnormalities within 30 - 32 days regardless of the concentration. But only a few organisms were effected. Survival following this treatment averaged 5 - 8 days. Benzpyrene proved to be more effective, giving results in 12 - 14 days, and these abnormal organisms survived more than 5 days. The number of organisms effected was considerably greater. Cytological studies of abnormal organisms that were fixed and stained gave no indication that either the macronucleus or micronucleus were affected.

Tittler and Mildred Kobrim[14] exposed the culture of Paramecium caudatum to methyl cholanthrene, scharlach red, and 3:4-benzpyrene. In all experiments abnormalities appeared in the culture; these were swellings, vacuolizations, and blister formations regardless of the agent used. The order of potency is first benzpyrene, second methyl cholanthrene and lastly scharlach red.

M. Wolman[15] used methyl cholanthrene, benzpyrene and dibenzanthracene at an optimal concentration of 1:3 000 000 - 1:200 000 in Paramecia cultures, and observed profliferation effects. Four non-carcinogenic compounds like chysene and diphenyl anthracene have no such effect in any concentration used.

The investigations on the effect of carcinoganic compounds on

tissues in tissue culture first gave negative results. A Russian (16) investigator cultured rat tissue in benzpyrene for a month without any pathologic change appearing. The tissue later degenerated. Another Russian(16) investigator used methyl cholanthrene on cultured chick mesenchyme and found no sign of malignancy but a retardation of growth and disrupted mitosis.

Later on, more positive results began to appear. Creech (17) found cell proliferation in cultures of mouse fibroblasts by adding 0.001 mg. per cc methyl cholanthrene. In 10 times the concentration, growth was retarded. Non-cancerogenic compounds like desoxycholic acid caused a decrease in cell proliferation. A precocious separation of chromosomes in the prophase and metaphase was observed in carcinogenic media.

Chick fibroblasts were used to study the effect of carcinogenic hydrocarbons.(33) Colloidal suspensions were prepared by adding 1 cc. of a 1:1000 solution of benzpyrene in absolute alcohol, or 0.1 cc. of a similar concentration of benzanthracene (non-carcinogenic), dibenzanthracene and methyl cholanthrene in acetone to 10 cc. of boiling Ringer's solution. The final concentration of benzpyrene in the experimental explants was 1:40 000, and of the second group of hydrocarbons 1:400 000. The amount of alcohol and acetone in the culture (2.5% and 0.01% respectively) are considered insufficient to cause any decrease or abnormalities in growth. Cellular abnormalities appeared after the third transfer. More compactly arranged mass of cells with aberrations in cells and nuclei appeared. General enlargement of cells occurred frequently, sometimes cells appeared having 10 times the size of normal fibroblasts. The cytoplasm of many cells became finely vacuolated.

Degenerative process in the cytoplasm gave rise sometimes to bilobed or multilobed nuclei as a result of mechanical fragmentation of nuclear substance. In isolated instances, cells in benzpyrene cultures contained a network of small cytoplasmic vacuoles and about 10 distinct nuclei of various sizes without other evidence to indicate the presence of regressive phenomena.

Minor abnormalities of mitotic figures as chromosomal aberrations or clumping, appeared to occur more frequently after the addition of the hydrocarbons than in control cultures or in explants to which non-specific substances were added. Tripolar mitoses were observed regularly. A single tetrapolar mitosis was discovered in one culture of methyl cholanthrene, while hyperchromatic mitoses in the prophase stage with a probable increase in the number of chromosomes were observed in two cultures treated with this agent.

PROCEDURE AND TECHNIQUE

(A). The organism.

The organism used in this work was Paramecium caudatum. The original stock was taken from the A.U.B. campus. By successive isolation and purification a pure line - a clone, was established from which further stocks were made. See Fig. (3, a) for size and shape.

(B). The stock culture.[18]

Various media were used for the stock culture but the simplest and least troublesome were hay infusions and wheat cultures. Boiling water was poured over chopped hay and the infusion left overnight in an ice box. This was filtered and buffered by 0.2% K_2HPO_4. In the case of wheat, 100 grains in 100 cc. of water were infused overnight, then the grains were crushed and the fluid filtered and used without pH adjustments. Or the wheat grains were left intact and remained to decay in the medium.

(C). The sterile media.

Yeast Juice. 500 g. of Bakers' yeast (Palestine manufacture) were mixed with 500 g. of washed sand and ground to a homogeneous mass. Then 200 g. of diatomaceous earth was mixed with it and the whole thing ground again. The final mass was wrapped in a cloth and placed under a powerful mechanical press and the juice (about 50 cc.) was sterilized by passing through Berkefeld filters thus avoiding destruction of the growth - promoting substances by heat sterilization. The juice was diluted 10 times before use.

Liver Extract. Fresh minced pieces of calf or rat liver were infused in saline in the incubator overnight. The infusion was filtered with cotton to remove big particles. The fluid was then heated to 60°C.

to precipitate the proteins, and then refiltered. The filtrate was sterilized by Berkefeld or Zeitz filters. The result gave a fluorescent pinkish fluid (due to riboflavin).

Wheat Extract. This was prepared like number 2 of the stock culture media with the addition of sterilization by filtering. The fluid was pale yellow and was kept for several months without clouding.

(D). Bacteriological sterilization of Paramecium.[19]

Obtaining paramecia free from bacteria for experimental purposes was in itself a problem of great difficulty. Parpart used to transfer single animals by means of sterile pipettes through 5 - 10 succwsive washings of sterile water contained in sterile depression slides, the latter being enclosed in petri dishes. According to this author, after the 10th wash only 5 out of 26 animals were sterile. This method was tried many times and found very inconvenient for two reasons: first, there is so much exposure to contamination due to too much transfer from vessel to another. Second, it often happened after half the process had been reached, that the animal was killed or lost during manipulation, and the whole process had to be retarded.

Glaser and Coria[4] described the 'migration reaction' method. This was done by means of sterile glass pipettes in which the paramecia were made to travel vertically through sterile media to the top thereby being freed from their contaminants. Some investigators devised a wash - bottle that works on the same principle. The disadvantages of the migration in a pipette are two: first, while the tip of the pipette is being sealed (after the organisms have been introduced) an air bubble often arises and passes through the column of water upwards polluting it. This may happen in the second or third passage and spoil many hours of laborious work. Second, for an unknown reason not all paramecia are

negatively geotropic, or at least, often they do not exhibit this reaction. It even happens that after their first swim upward, in the second transfer to a fresh pipette, they refuse to rise to the surface. The wash - bottle eliminates the danger of air bubbles because it has a stopcock, see Fig. No. 1. A simple bent tube with a stopcock in one arm can serve the same purpose.

In this work a new method was used for clarifying the paramecia. It was based on the principles of centrifuging and filtering and was developped from attempts to use one of these principles in freeing the paramecia, when it was found that neither alone could do the job satisfactorily. Glass tubes, 8 cm. x 1⅛ cm., with a large hole at the bottom were especially made to fit into the centrifuge cups (see Fig No. 2). Five layers of hard filter paper were pressed tightly by a glass rod in the bottom of the glass tubes. (The glass tubes were previously stoppered and sterilized in the oven, and the filter paper was soaked overnight in 95% alcohol.)

The contaminated organisms were concentrated by previous centrifuging and a small drop was introduced into the first sterile tube. This was filled with sterile tap water and centrifuging started thereby forcing the water out from the filter paper and washing the paramecia from adhering bacteria. At intervals of 3 minutes slow centrifuging a period of few minutes rest is allowed. Almost all the water was forced from the tube and then fresh sterile tap water was added before the animals dried. This detail ensured complete change of the water. The paramecia were transferred to a second tube and the process repeated, then to a third and so on. Sterility tests after the 6th tube showed absence of bacteria.

(E). Staining.

(E). Staining.[20]

Several techniques were used each serving a specific purpose. Best results for chromatin material were by (Heidenhain's) Hematoxylin after hot Schaudinn's fixative, destained by picric acid. The animals were stained either in centrifuge tubes or on cover slips. A convenient way of keeping the organisms from falling off the cover slips during manipulation was to drop a thin layer of colloidon on the surface of the slip. This did not interfere with the staining process.

For the carcinogenesis experiments the following techniques were used:

a. Whole mounts to show abnormalities in form: Schaudinn's and ironhematoxylin.

b. Whole mounts to spot possible changes in the silver line system: Klein's dry silver method.

c. For sections to spot possible changes in mitochondria: osmic acid fixation with Rigaude's hematoxylin, or Altman's stain.

d. For sections to show neuromotor apparatus: Zemker's fixative and Mallory's triple stain.

Sectioning was done in bulk. Fixing and dehydrating was done in centrifuge tubes. Imbedding was by using frog's intestines, or by capsules. Sections were cut at 3 and 7 micra.

(F). The control of the various factors involved.

According to Phelps the factors involved in protozoological work are: light, temperature, gases, food concentration, humidity, density of organisms, and H-ion concentration.

By keeping the organisms in the incubator temperature and humidity, as well as light were kept more or less constant.

All media were kept at pH 6.8 - 7. Both the electric and colorimetric methods were used for checking the hydrogen ion concentration.

(G). Sterilization of equipment.

Glassware was dry sterilized in the oven at a maximum temperature of 150°C. As mentioned, media were sterilized by filtering. Autoclaving was used for water and equipment that were needed in the sterile experiments. These instruments were all put in a closed chamber before use. The chamber was sprayed with phenol to sterilize the air. The hands of the operator were washed with mercuric chloride before insertion in the sleeves and a side hood was constructed into which an alcohol burner was kept for flaming during the experiment. The best method for obtaining sterile slides was to place a clean dry depression slide in a clean petri dish and sterilize the whole in the oven.

(H). Rates of growth in terms of division.[21]

The rate of growth was determined from counts of organisms. The hemocytometer counting was found more satisfactory than a calibrated pipette. But the success of this counting depended on very vigorous shaking for 1 minute prior to counting.

The number of divisions per day can be determined from the following formula

$$\frac{\log_{10} Q}{\log_{10} 2}$$

where Q is the ratio of the number at any day to that of the previous day.

EXPERIMENTS AND RESULTS

Exp. (1).

The salt content of the medium. The purpose of the experiment was to ascertain which of the following two salt solutions was more suitable for the organism:

Solution I. (22, a)		Solution II. (22, b)	
$MgSO_4$	360 mg.	KNO_3	0.5 g.
$Ca(NO_3)2$	200	KaCl	0.1
Na_2SO_4	200	$MgSO_4$	0.1
KNO_3	80	K_2HPO_4	0.5
KCl	65	$Ca_3(PO_4)2$	0.1
NaH_2PO_4	16.5	$FeCl_3$	a trace
$MnSO_4$	4.5	H_2O	1. litter.
$Fe_2(SO_4)3$	2.5		
$ZnSO_4$	1.5		
H_3BO_3	1.5		
KI	0.75		
H_2O	1. litter.		

The pH of each solution was 6.8 - 7.

The test was based on comparison of the number and vitality of organisms in the solutions with that in tap and distilled water. Cultures were made in 8 depression slides , a pair of them for each solution, another pair for tap and similarly for distilled water. These were placed in petri dishes and labeled. A drop of the proper solution was placed in each of the respective slides. To serve as food, a drop of 0.1% peptone was added to every slide. One single paramecium was placed in each depression and incubated at 24°C. The table below shows the results.

	Sol. I	Sol. II	Tap water	Distilled water
Nov. 17	1 param.	1 param.	1 param.	1 param.
Nov. 18	2	2	0	2
Nov. 18	A second passage started; one organism in each.			

	Sol. I.	Sol. II.	Tap water	Distilled water
Nov. 20 666	5 param.	4 param.	24 param.	28 param.
Nov. 21	16 - 30	7	35	Many

N.B. A drop of water was added to each of the depression slides the night of November 20.

From the table it seems solution I was better than solution II, but both tap and distilled water were better suitable for the reproduction of the paramecia. It should be noted that dilution of the salt solutions by adding water on November 20 enhanced multiplication in those media. Further experimentation should be carried on the salts changing one at a time in order to determine the value of each separately.

Exp.(2). The use of yeast juice as a medium.(10) Sterile yeast juice was prepared as described. Depression slides sterilized in petri dishes were served a drop each of the medium and one paramecium inoculated into each depression. All work was done under the sterile chamber. The next morning all organisms were dead and a cloudy area marked bacterial infection. The paramecia were next washed clean before the experiment was repeated, but again they died. Since the pH was within the viable range, it appeared that some toxic substance was introduced in the preparation of the extract.

Exp. (3). The use of liver and wheat extracts as media.(4) The media were prepared and sterilized as described. Operations were the same as described in Experiment 2, with the additional detail that media as well as organisms were introduced into the depression slides by sterile

5 cc. syringes instead of by pipettes. More than one organism was introduced in this way which does not effect the result since the ratios of the numbers at first and at the end were compared. The two media were tried together as well as separately. The table below will show the results. The paramecia were washed bacteria free before inoculation, but after a few days of passage in the media sterility tests showed infection and the experiment stopped. However the number of bacteria was very low, there was no clouding and no fouling of the fluid.

Depression		1st day	2nd day	3rd day	4th day	5th day	6th day
No. 1	5% Liver	Alive	Alive	Alive	Numer.	Numer.	Numer.
2		Alive	Alive	Alive	V.Numer	V.Numer	V.Numer
No. 1	0.5% Liver	Alive	Alive	Few	Few	None	None
2	plus 0.5%	Alive	Alive	Excell.	D r i	e d u	p
3	wheat	Alive	Alive	Numer.	Numer.	Exeell.	Excell.
4		Alive	Alive	Few	Numer.	Excell.	Excell.
No. 1	0.5% Liver	Alive	Numer.	Few	None	None	None
2		Alive	Numer.	Few	None	None	None
3		Alive	Excell.	Few	Few	Few	Few
4		Alive	Excell.	Excell.	Numer.	Numer.	Numer.
5		Alive	Alive	None	None	None	None
No. 1	5% Wheat	Alive	None	None	None	None	None
2		Alive	None	None	None	None	None
3		Alive	None	None	None	None	None
4		Alive	None	None	None	None	None

Exp. (4).
The same medium as Experiment 3 with the addition of vitamin C, and with complete sterility of paramecium.

In this experiment, the complete sterility of paramecium was achieved and the same medium of liver and wheat extract was used for the first three days. The Paramecia were alive, but did not reproduce appreciably. On the addition of sterile vitamin C, they increased rapidly for a few days. Further work is necessary to determine if this medium is complete.

Exp. (5).
The effect of Ultra-violet on paramecium. This and the following experiments were designed to show the effect of carcinogenic compounds. Ultra-violet and heat are included since they were shown by Mottram to have similar effects.

The experimental culture was irradiated from 5 to 10 minutes daily at a distance of 10 to 20 cm. with a high intensity mercury lamp.

1st day In the experimental culture there were 2380 organisms per cc. as compared to 620 with which the control culture started.

½ week later There were 2030 organisms per cc. in the experimental and 3100 in the control. On this day a new passage was started by a second transfer.

New passage Started with 54 cc. in the experimental and 256 in the control.

5th day Two forms appeared, the one much larger than the other. This dimorphism of the experimental culture did not appear so early in the control, which indicates that it might have been enhanced in the experimental culture by the ultra-violet radiation. No conjugants in either culture appeared.

7th day Diversity of form in the paramecia of the experimental culture was very apparent. Some large broad paramecia were about three times as big as the slender dwarfs. The occurrence of large forms and of dwarfs in the control was approximately half that found in the experimental cultures. There were 1220 organisms per cc. in the experimental and 1360 in the control.

A few of the large organisms, the medium and the dwarfs were isolated. From one of each a new line was derived by vegetative reproduction in a week's time. At first all organisms in the same culture were alike; i.e. large ones only in the culture of the large parent and vice versa. But at the end of the week all forms (large, medium and dwarg) could be seen in each of the cultures. This polymorphism must be due to individual differences, age and food only, since pure lines were used, unless during endomixis internal constitutional changes took place. No conjugation was seen.

Third Passage. It started with 264 per cc. in the experimental and 300 in the control. Irradiated 15 minutes daily for a month. Many sluggish forms began to appear. Blisters on the surface of the orgamisms took the form of a conical 'bud' at the edge of the anterior end (Fig. 3, c - e). Many had these buds, and a few developped very large swellings in the middle of the body. Many morphological variations appeared, as shown in Fig. 3, i - m. Vacuolizations in the body were abundant.

In this table are summarized the counts:

Date	Number per cc.		
	Exper.	Control	
Dec. 10	2380	620	
" 17	2030	3100	
Dec. 17	54	256	New passage
" 24	1220	1360	
Dec. 31	264	300	New passage
	Killed by overdosage		

See Graph 1.

In the first week the culture did not show any change in number whereas in the control the rate of division was log (3100 divided by 620)by log2=2.322A marked acceleration is shown in the growth of the experimental culture during the second week. The ratio being 4.54 as compared to 2.402 of the control. It can be said that with non-lethal dosages there is an enhancement of cell division.

Vitality. There is a very decided lowering of vitality of the irradiated forms manifested in very sluggish serving motion. About one tenth of the Paramecia were of this type.

Form. Accompanied with low vitality of some of the forms, there was a dwarfness of shppe and size reaching almost one third the normal.

A very striking series of deformities other than that of size reduction were often observed. Some that could be isolated were mounted and stained, but not all. Fig. 3 shows some of the bizzare forms encountered.

Two other manifestations of degeneration were seen:

Extreme vacuolization of the cytoplasm, eruptions of the pellicle seen as blisters on the periphery, and buds or protrusions of the cytoplasm at the poles or near the middle. Usually these buds were an indication of a small break in the pellicle, but one rare form had a swollen sphere half as big as the animal, arising from the middle. (See Fig. 3)

The silver line system. Study of silver mounts was made to detect possible changes in the pellicle, basal granules and other portions of the silver line system, but no obvious changes were seen.

Exp. (6).

The effect of heat on paramecium. The experimental and the control cultures were raised in stender dishes. The experimental was put

under a 50 w. lamp every night and returned to the incubator next day. The temperature under the lamp rose to 35°C. or more. A week later this method was discarded, the culture was put continuously in an incubator at 37°C.

First passage: The experimental culture had 1100 organisms per cc. and the control 840.

A week later: The experimental count rose to 3330 per cc. Unfortunately the control culture was very tight and all organisms suffocated.

Second passage: 38 organisms per cc. in the experimental as compared to a 100 in the control.

3 days later: In the experimental culture great accumulation and crowding of organisms; no conjugation.

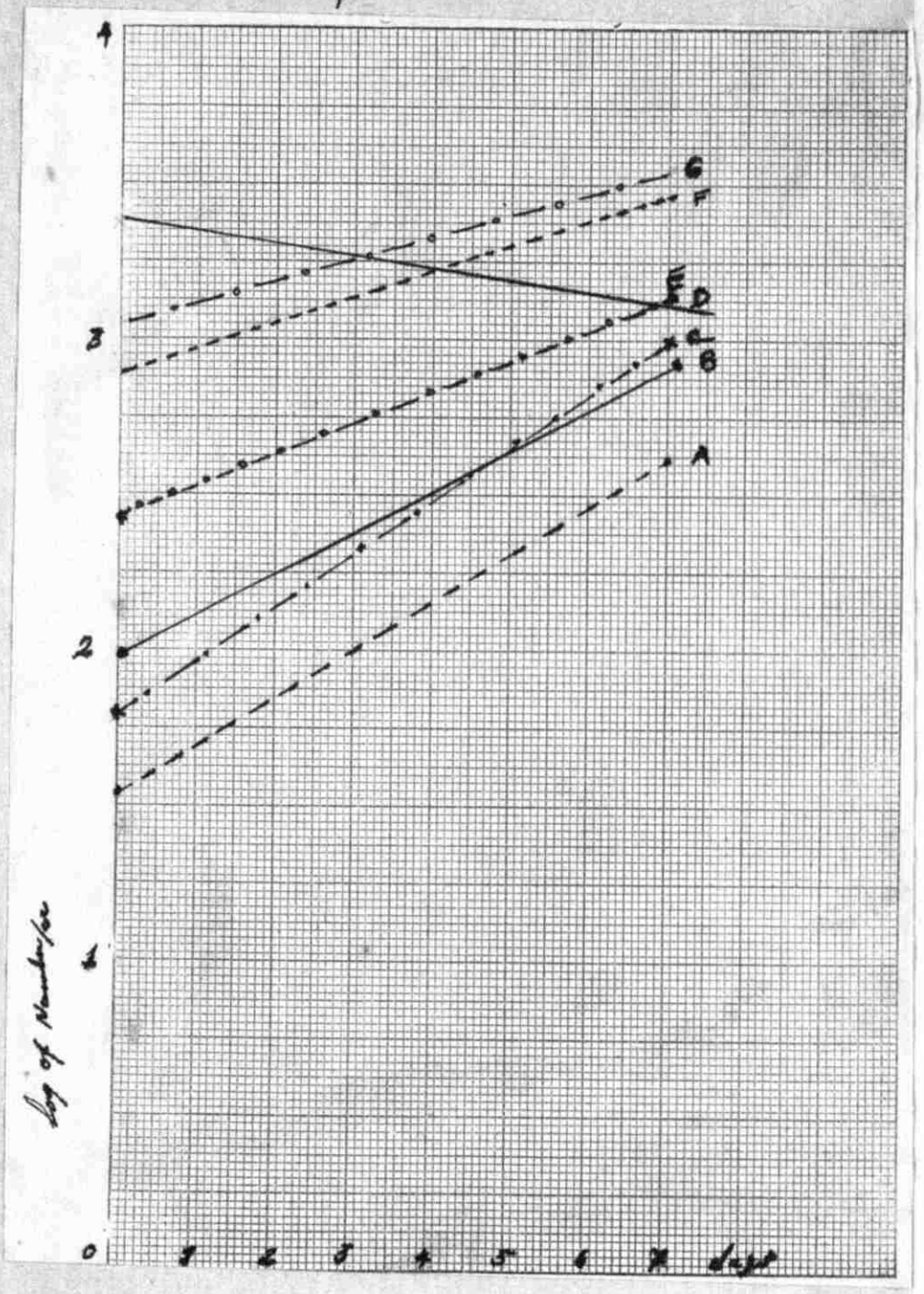
Fourth day: Sluggish and very fat forms appeared. Round things like cysts or recently dead organisms were seen at the bottom. The control forms appeared healthier but less concentrated.

Fifth day: Polymorphic forms as seen in the previous experiment: large giants and slender forms. Similar forms in the control. The counts were 592 in the experimental and 840 in the control.

Third passage: Many conjugation forms. At 39°C. many forms were killed so the temperature was restored to 37°C. Abnormal forms similar to those seen in the ultraviolet culture, but less abundant. (See Graph 1.)

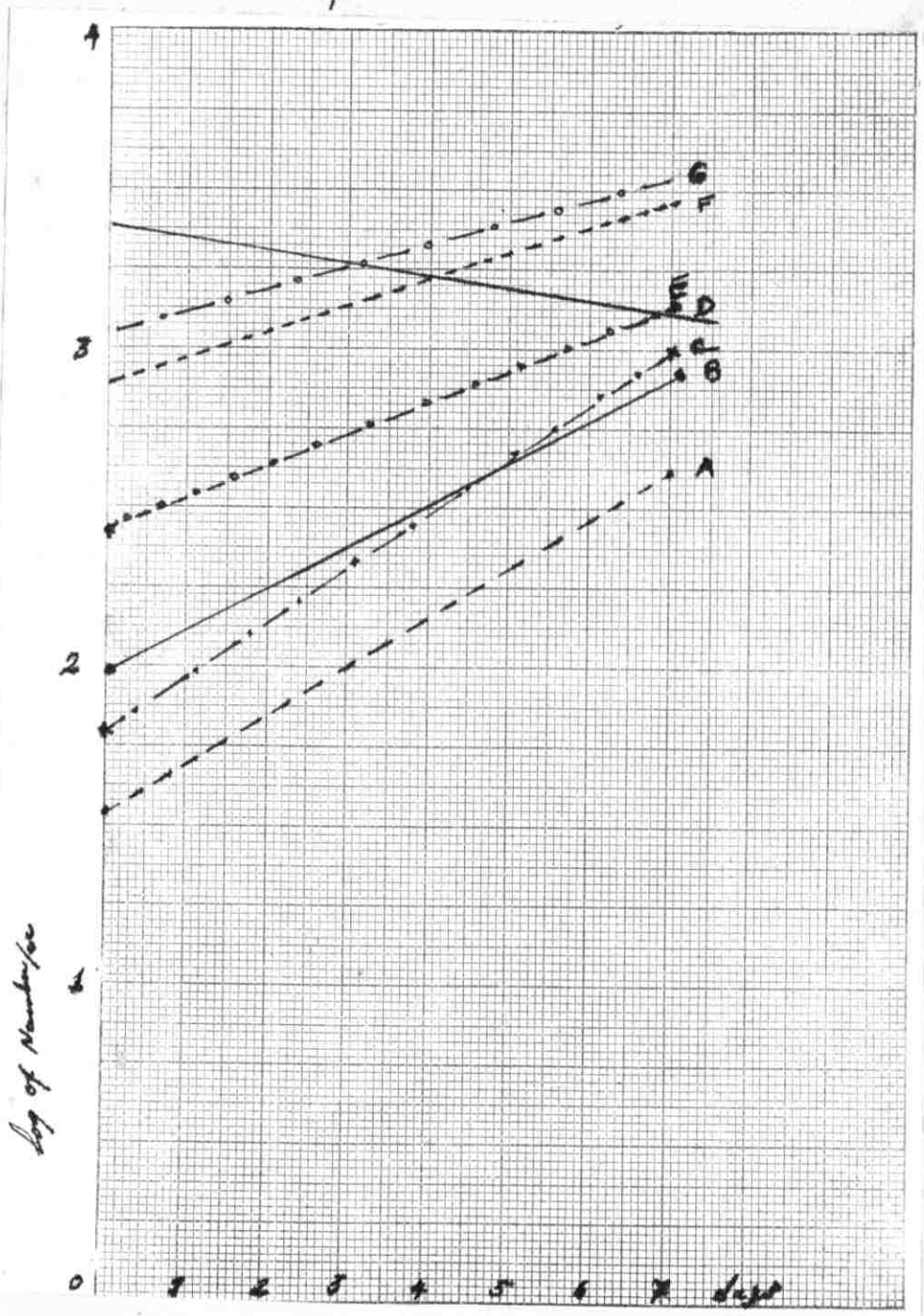
The rate of division in the experimental culture was log (592 divided by 38) divided by log 2 = 3.94 as compared to 3.05 in the control. Slides stained with silver method showed the cytosomal

Graph 1.



A. & B. & G. from the heat Experiment:
G. is the experimental of the 1st passage.
A. is the experimental of the 2nd passage.
B. is the control of the 2nd passage.
ultra-violet.
D. is the experimental of the 1st passage.
C. is the experimental of the 2nd passage.
F. is the control of the 1st passage.
E. is the control of the 2nd passage.

Graph 1.



A. & B. & G. from the heat Experiment:
G. is the experimental of the 1st passage.
A. is the experimental of the 2nd passage.
B. is the control of the 2nd passage.
ultra-violet.
D. is the experimental of the 1st passage.
C. is the experimental of the 2nd passage.
F. is the control of the 1st passage.
E. is the control of the 2nd passage.

region and the pattern of the pellicle well but there was no universal change. In some cases the hexagonal pellicle was broken up and irregular.

Exp. (7).
Carcinogenic compounds. Paramecia were maintained under the effect of certain carcinogenetic compounds for a period of 4-5 weeks. They were subcultured twice a week and at the end of the period fixed and stained (whole and sections) for comparison with the normals. Observations on the organisms while alive in the experimental culture were also recorded.

The following are the compounds used in the cultures:

Culture No.	Compounds.
1	Benzidine;(from the chemistry department); 20 mg. per 100 cc; wheat grains used as food.
2	Alcohol, ethyl, 2% for control; wheat grains used as food.
3	Naphthyl amine acetate,(Chemistry Department); 170 mg. per 100 cc. Wheat food.
4	Diethyl dihydroxy stilbestrol emulsion; 20 mg. were dissolved in 5 cc. absolute alcohol(Biochemistry Department). Then the solution was added to boiling water; diluted 10 times. Wheat food.
5	The same as No. 4, but diluted 20 times.
6	Cholesterol emulsion (Biochemistry Department). 0.5 mg. of pure cholesteril was dissolved in 5 cc. of absolute alcohol at 78°C., and while stirring, was poured over 200 cc. hot water. It formed a milky emulsion. This stock solution contained 2.5% alcohol and 0.25% cholesterol. It was diluted 4 times before use; food wheat added.

Culture No.	Compounds
7	Butter yellow (N, N-dimethyl p-phenyl-azo aniline (Chemistry Department); 2 gms. were dissolved in 100 cc. olive oil. 10 gms of the latter were mixed with 50 gms. of finely ground rice starch and little water to form a cake. The organisms were cultured in tap water to which a bit of this cake was added.
8	The female sex hormone used in No. 4, but dissolved in olive oil and a starch cake made as in No. 7.
9	Methyl cholanthrene (Eastman Kodak Co. Rochester, N.Y.); 10 mg. were dissolved in 10 cc. acetone. From this 0.2 cc. were emulsified in 20 cc. boiling water; wheat grains used as food. Diluted 5 times before use.
10	Acetone medium used with wheat grains as food, for control.
11	A cake of starch and oil for control.

Results of the experiments of carcinogenesis:

1. Butter yellow. The organisms in this culture are darker in color , probably due to the pigment. They were all shorter than the normal with the posterior end much bulkier than the anterior. Sections of these paramecia show excessive vacuolization, many individuals being nothing more than vacuoles (Fig. No. 3, f.). No deviations were noted in mitochondria from the normal.

2. Although polymorphism was very troublesome in the other cultures, Methyl Cholanthrene culture (at least in the first passage) were all alike and never showed discrepencies in size. They are all larger than the average normal individual, exceeding about 15 mimra in length. Mallory's triple stain for connective tissue did not give good results with organisms from the methyl cholanthrene culture. It must be recalled that this

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compound may require a month to show pathological changes, and so far, these experiments have been carried on for two weeks only.

3. Silver impregnation for Benzidine, cholesterol, and female sex hormones cultures did not indicate any changes from the normal.

DISCUSSION and CONCLUSIONS

From previous work on the nutrition of ~~Paramecium~~, certain definite factors have been already determined.

1. That the nitrogeneous requirements were of the protein-cleavage products, like peptones. Many amino acids serve better than a single one (26).
2. That the addition of a sugar seems to accelerate growth (2).
3. In the abstract no. 7448 of Chemical Abstracts 1940, there is given the evidence for the synthesis of fats from carbohydrates.
4. Vitamins seem to be necessary, although their study is not complete (31). Ciliates need Thiamin, riboflavin and ascorbic acid; the need for other vitamins has not been determined.
5. It seems that autoclaved media do not promote the growth of Paramecium as do media sterilized by filtering, indicating heat labile compounds.

For making the cell-free medium, the author took into considerations these points as well as the feeding habits of the organism. Paramecia had to be first freed from all contamination whcih was a relatively easy procedure with the new method described.

The composition of the medium finally reached, gave the organisms a variety of proteinaceous and carbohydrate material of both animal and vegetable origin . The water-soluble vitamins were found in the liver extract,(but vitamin C was added). The latter was found to promote division considerably. Nucleic acids were provided by the extracts, so that the medium contained

the most essential nutrients except the fat soluble vitamins. The mineral content of tap water seems to be adequate enough, but a more detailed study of the salt solution is needed. Sterile starch particles if added help to stimulate the formation of food vacuoles. More time is needed and more passages must be made before the final adequacy of this medium can be determined. The fat-soluble vitamins should be added as an alcoholic emulsion.(2% alcohol has no toxic effect as found in this work). If this medium should not be capable of supporting indefinite growth of Paramecium, then there must be unknown compounds that it gets from feeding on living forms. It should be noted that autoclave-killed microorganisms do not support Paramecium. It would be instructive to support organisms killed by means other than heat.

Since the problem of a bacteria-free medium was not completed, the ordinary infusions were used as media in the carcinogenesis experiments.

Several carcinogens were used in the media. Ultra-violet rays and heat are considered to have comparable effects as the carcinogenic compounds. In all cases observations and subcultures were made at least once a week, and at the end of a period of 3 weeks, fixed mounts and sections were made by various techniques for study. The cultures of the ultra-violet and heat remained 2 months however.

There is apparent confirmation of the work of Mottram in the case of ultraviolet and heat cultures. The major effect of irradiation seems to be on the pellicles, which seem to break at various points resulting in blisters and cytoplasmic protrusions like buds. A second abnormality was elongation of the anter-

end into a neck-like structure. This must have been due to unequal growth or delayed cell division. This promises fruitful investigation, and these forms must be isolated and studied more carefully for mitotic aberrations. A third type of morphological variation, shown in fig 3,i-m,might have arisen as a result of both of factors land 2.

The Paramecia in the butter yellow and methylcholanthrene cultures were studied in closer observation than those in the other carcinogens. There is a change in size, but unless a statistical study is made , it can't be stated in quantitative terms. Excessive vacuolization was very common. In about one fourth of the organisms the body contained from 6-12 large vacuoles. Many showed, in addition to a few small vacuoles, a huge one at the posterior middle of the body with a diameter almost equal to that of the organism. Such vacuolisation was not encountered in the mounts of the control and seems to warrant consideration as a sign of cell degeneration.

The determination of the specific action of the carcinogen in the cell is of prime importance. The site of action in the cell organelles must be determined. Since it was shown (32) that the lipoid granules absorb more carcinogen than the nucleus, the mitochondria should be studied to show possible modification form the normal. This means a thorough knowledge of the normal cytology must be achieved, and a mastery of sectioning and staining techniques.

Once the above task is accomplished, then we may try to induce by some metabolite of Paramecium the same change that was induced by foreign chemical compounds.

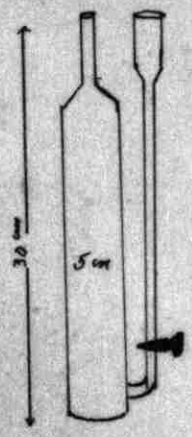
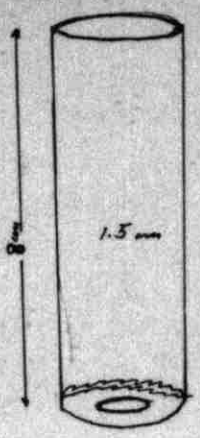


Fig 1. Wash-Bottle



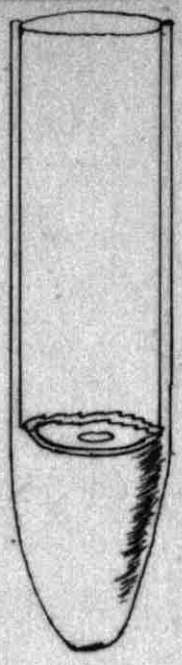


Fig 2. Centrifuge-filter tube

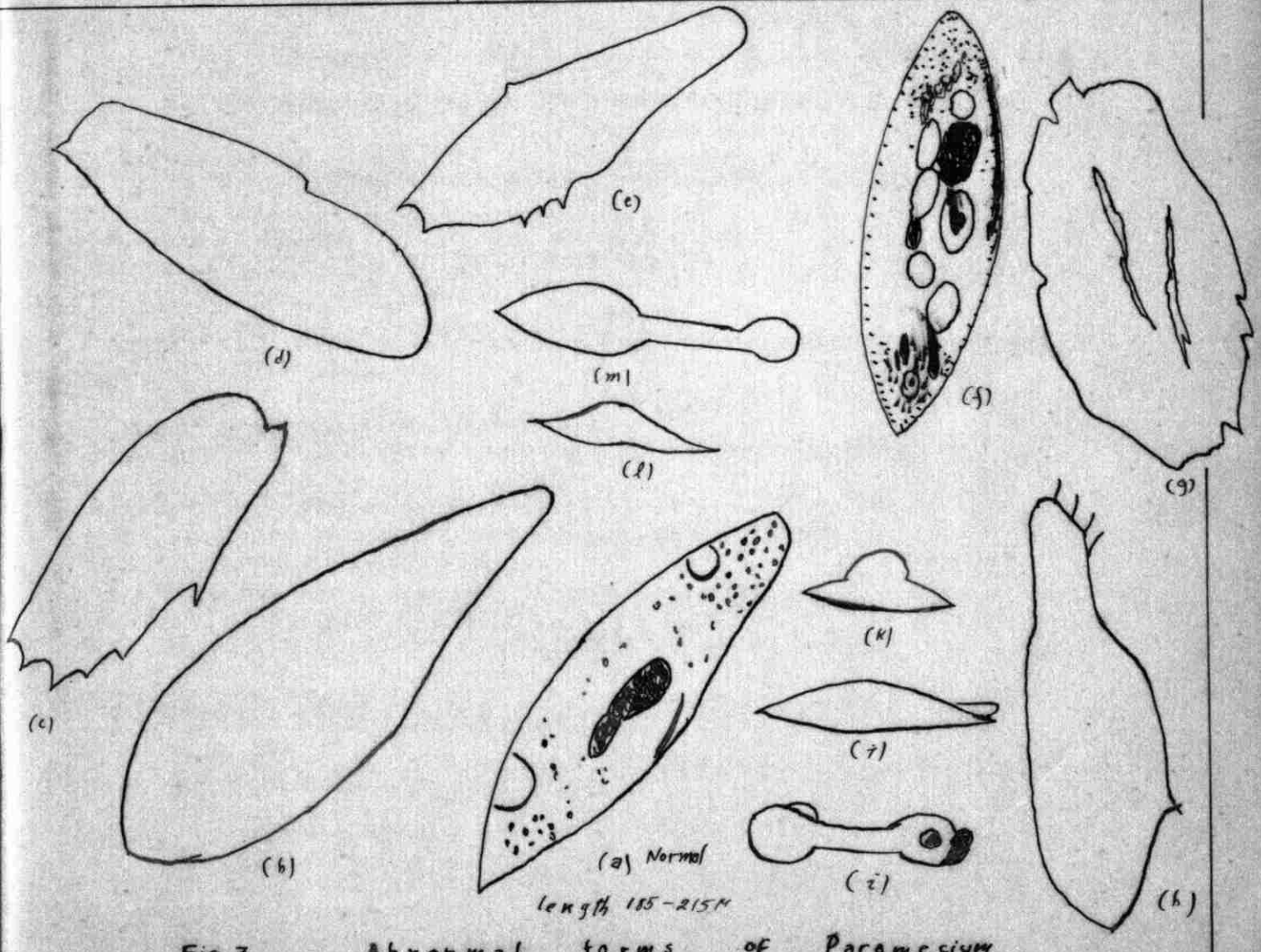
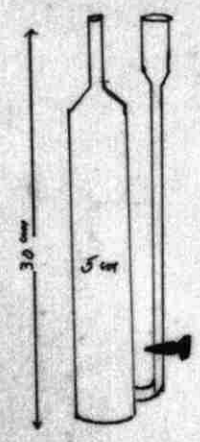


Fig 3 Abnormal forms of Paramecium



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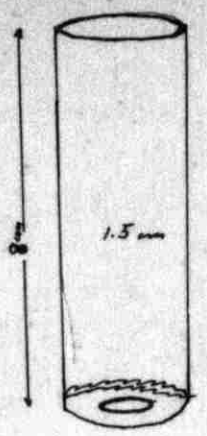

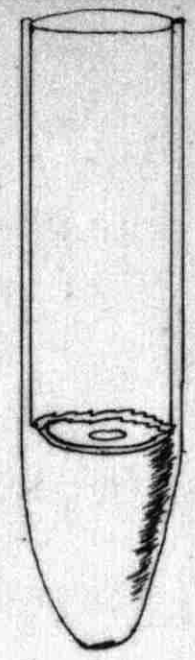

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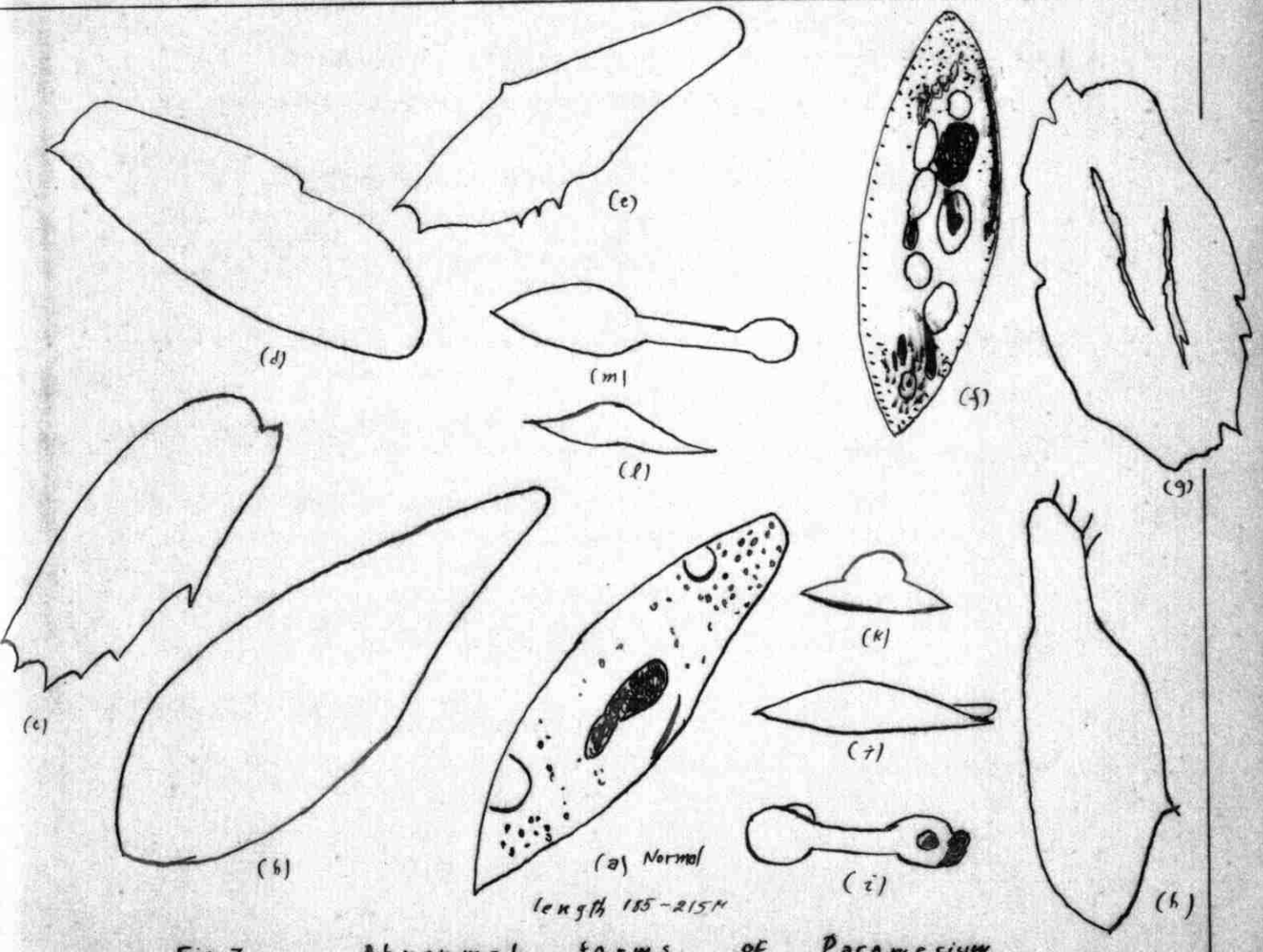

Fig 3. Abnormal forms of Paramecium

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