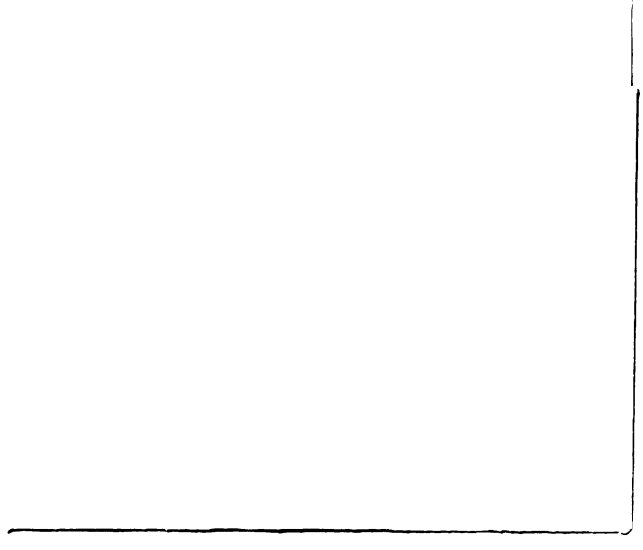
THE RELATION BETWEEN AGE, LIPID CONCENTRATION, AND GROWTH IN *TETRAHYMENA GELEII* W, WITH REFERENCE TO HISTOCHEMICAL IDENTIFICATION OF THE LIPIDS

Thesis for the Degree of M. S.

MICHIGAN STATE UNIVERSITY

Joseph G. Engemann

1956



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By

Joseph G. Engemann

AN ABSTRACT

Submitted to the School of Graduate Studies of Michigan State University of Agriculture and Applied Science in Partial Fulfillment of the Requirements for the Degree of

MASTER OF SCIENCE

Department of Zoology

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Approved Richard O. Fennell

Joseph G. Engemann

THESIS ABSTRACT

Specimens of Tetrahymena geleii W were grown in bacteria-free tryptone, tryptone and vitamins, tryptone citrate, and casein with yeast extract. Some were grown at different hydrogen ion concentrations; some were grown with increased surface area.

Cultures were usually grown at approximately 75°F. Lipids were visualized with Sudan black B. Control slides were extracted with pyridine before staining. An activity factor which represents the average length of cell (percentage) occupied by lipid was used to evaluate results.

It was found that very old cultures normally exhibit a decrease in lipid deposits following the steady increase in lipid deposition noted by other workers. Depletion of the media is suggested as the principal cause of decrease. With changed media and/or conditions, variations occurred in the normal pattern of lipid deposition. Thus transfer to tryptone and tryptone citrate decreased lipid production and growth. A higher pH resulted in increased fat deposition, a lower pH, in less fat deposition. Changing the surface area/volume ratio of tryptone and vitamin cultures resulted in pronounced changes in growth and lipid deposition. With decreased surface area there was decreased growth and decreased lipid deposition. However, in tryptone solution and

tryptone citrate solution no noticeable effect was produced on growth or lipid production by changed surface area/volume ratio. In casein with yeast extract, organisms produced abundant lipid at an early age.

In general, conditions which resulted in good growth also resulted in abundant lipid deposits.

By synchronizing the cells, i.e., treating them so that more are in the same phase of mitotic division at the same time than are under normal culture conditions, it was found that there is a decrease in lipid deposits preceding division. This was interpreted as being primarily caused, not by decreased synthesis at that time, but by an increased utilization of lipid during division. It is suggested that the decrease is a reflection of the use of lipids in cell membranes. This may also explain the faint generalized staining observed in controls extracted in pyridine and stained with fat stains.

Lipid deposits were largely neutral fat with some unsaturation, as determined by Nile blue sulfate and osmic acid staining. However, phospholipid was found in cells from casein with yeast extract solution while it was absent from others of the same age. No sterols, vitamin A, or carotenoids could be identified histochemically.

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INTRODUCTION

A review of literature of Tetrahymena presents a meager knowledge of their lipids. Prior to 1945 studies in which fats were observed in Tetrahymena were from cultures containing another organism which presumably influenced the type of fat found (Wilber, 1942). Subsequent studies have been conducted, often with only one media and organisms of one age class. However, Fennell (1951) demonstrated that, in Tetrahymena geleii, age could be a factor governing both amount and type of fat, young cells had fat in the apical end only while fat-positive granules filled the entire cell in many older specimens; most lipids were neutral fat, the rest being positive for fatty acid. Zinger (cited by Seaman, 1955) noted the formation of cytoplasmic sudanophilic globules following ingestion of starch and protein in Glaucoma (Tetrahymena).

Mast (1938) used Colpidium striatum as a source of fat when he studied the digestion of fat in Amoeba proteus. He found that the colpidia stained with nile blue had pink fat globules. The pink reaction indicated that the globules were neutral fats. The colpidia came from a culture contaminated with a fat-rich mold. Wilber (1942), when using C. striatum for a similar experiment with Pelomyxa carolinensis, found that colpidia from a bacterized culture had fat globules composed of fatty acid.

Harding (1937) conducted a series of starvation and feeding experiments on the ciliate Glaucoma using bacteria as food. His camera lucida drawings show more than a proportional reduction of fat with decrease in size during starvation, but after 25 days of starvation some fat was still present. His drawings indicate that twelve hours subsequent to feeding the level of fat returns to pre-starvation levels. Observations of his drawings indicate that those with food vacuoles tend to have the fat distributed throughout the cell more than in those cells lacking food vacuoles.

Pace and Ireland (1945) noticed the localization of the fat globules in the anterior end of Tetrahymena geleii. They also found that specimens from cultures grown in a pure oxygen atmosphere were smaller and had less fat due to rapid division and a high oxidative metabolism. When specimens were grown in an atmosphere of high carbon dioxide content they had little fat and were small because in this case they had lost some of their power of synthesis.

McKee et al. (1947) made biochemical determinations of the lipids from four to five day cultures of Tetrahymena geleii grown in 1.5% proteose peptone at 25°C. They found that the lipid fraction represented from 15 to 20% of the cellular solids. Of the lipid, 75% was free fatty acid, 15% neutral fat, and the remainder was a neutral steroid-like compound. The fatty acids had an average chain length of 22-24

carbon atoms and about half were saturated. They were able to crystalize myristic acid from the fatty acid fraction. Wilber and Seaman (1948) using microchemical analyses on Colpidium campylum from fat free culture media found fatty acid and phospholipid but no cholesterol. They concluded that the colpidia had synthesized the lipid from protein. Seaman (1949) found that the amount of fatty acid and phospholipid per 1,000 cells of C. campylum remained constant during the first seven days of growth. Seaman (1950) was able to detect sterols in Tetrahymena. He found them to make up about 0.05% of the lipids. Later Seaman (1950a) found that T. geleii could synthesize fatty acid from acetate and that carbohydrate was not a necessary precursor during this synthesis.

Ryley (1952) found that T. pyriformis in a salt solution exhibited an endogenous respiration which suggested the oxidation of fat or protein rather than carbohydrate.

Fennell and Marske (1954) made use of an activity factor in describing fat deposition in T. geleii. They found a gradual increase of fat in cells from tryptone solution up to 504 hours. Marske (1953, thesis) probably has the most information regarding lipids of Tetrahymena in relation to age of culture. He found a slight decrease in the lipids of older cultures due to addition of calcium and magnesium ions. He found that organisms from aged cultures (504 and 576 hours) gave positive reactions for phospholipid. Further, the addition of vitamins

to the culture solution had little effect on lipid synthesis in T. geleii nor did washing the organisms with saline prior to staining.

Mazia (1956) maintains that studies of cell mechanisms should show their relation to cell life history. Scherbaum and Zeuthen (1954) have provided a convenient means of studying the relationship of mechanisms to cell history in Tetrahymena with their method of synchronizing cell division.

Kidder and Dewey (1951) have pointed out the suitability of Tetrahymena for the study of cellular metabolism. Since Tetrahymena can be grown axenically, a thorough understanding of its metabolism may provide clues to widespread metabolic phenomena. Thus a further study of its lipids is in order.

The literature on Tetrahymena is somewhat confusing since this species is often referred to under the generic names of Glaucoma and Colpidium (Corliss, 1954). Kudo (1954) lists descriptions of three species of Tetrahymena, four of Colpidium and one of Glaucoma without discussing the synonomy. Perhaps it is not a problem of synonomy but one of improper identification. Reference is made to them as they are in the papers cited but Colpidium campylum, Glaucoma piriformis, and Tetrahymena pyriformis can be considered as probably identical to Tetrahymena geleii in the majority of cases.

Yasuda and Bloor (1932) have shown lipids to be present in greater than normal quantities in cancerous tissue. McKee et al. (1947) have shown that lipids from Tetrahymena exhibit

some antibacterial effect. They also found that peritonitis developed in mice when they injected a preparation of lipids from Tetrahymena intraperitoneally. Cellular infiltration and necrosis of the skin was induced in the mice when they injected it subcutaneously. Fennell (1951) demonstrated the effectiveness of alcohol-chloroform soluble materials from aged Tetrahymena geleii in the production of lesions in the chorioallantois of the chick. In view of the preceding studies it seems that any increase in knowledge of the relation of lipids to the living cell would be a valuable contribution.

It is hoped to further elucidate the relationship that exists between cell age, culture age, and lipids in Tetrahymena geleii W cultured in various media under selected conditions. The lipids occuring in Tetrahymena will be identified insofar as is possible with the histochemical tests available. The effects of varying treatment will be discussed in the light of results presented here and in the literature.

MATERIALS AND METHODS

Stock cultures of Tetrahymena geleii W used for experimentation were maintained in bacteria-free, vitamin enriched tryptone solution. Media used for experimental series were: (1) tryptone solution; (2) vitamin enriched tryptone solution; (3) tryptone citrate solution with added $MgCl_2$; (4) vitamin enriched tryptone solution with pH adjusted with KOH and KH_2PO_4; and (5) casein with yeast extract. Stock cultures and the first tryptone and vitamin series were run in 75 ml. of media in 125 ml. pyrex Erlynmeyer flasks at room temperature. Others were grown in 10 ml. of media in 16 x 150 mm. pyrex test tubes. Test tubes were usually positioned vertically. Some series were inclined at an angle of 60° from the vertical. Unless otherwise specified, cultures were grown in a constant temperature room. Range of room temperature was from 71° to 80°F. The range in most experiments was between 75° and 77°F.

Tryptone media was made using 15 gm. Difco Bacto-tryptone and 1 gm. monobasic potassium phosphate (KH_2PO_4) per liter of glass distilled water.

Vitamin enriched tryptone was prepared in essentially the same manner as tryptone media with 1 mg. riboflavin, 1 mg. thiamine, 100 ug. nicotinic acid and 0.5 ug. biotin added per liter of media.

Tryptone-citrate solution was made with 15 gm. Difco Bacto-tryptone, 2.06 gm. sodium citrate, 0.63 gm. citric acid and one liter of glass distilled water. Magnesium chloride was added to give a concentration of 1.07 gm. $MgCl_2$ per liter of media.

Vitamin enriched tryptone with adjusted pH was made by omitting the standard amount of KH_2PO_4 and substituting varying amounts of KH_2PO_4 and KOH, keeping the total K^+ molarity of the media at 0.020 M. Three series were run with the pH adjusted to 6.35, 6.73, and 7.40 by making 150 ml. of each containing 15 ml. of 0.2 M. KH_2PO_4; 10 ml. of 0.2 M. KH_2PO_4 and 5 ml. 0.2 M. KOH; and 5 ml. 0.2 M. KH_2PO_4 with 10 ml. 0.2 M. KOH respectively. The H^+ concentration was ascertained prior to sterilization. A Beckman model H2 pH meter equipped with standard electrodes was used for all pH determinations.

Casein media were prepared using vitamin free casein purchased from Sheffield Farms Co., Norwich, New York. Casein media was made using the proportions of 10 gm. casein, 1 gm. K_2HPO_4 and 0.1 gm. $CaCl_2$ per liter of glass distilled water. Casein with yeast extract was made in essentially the same manner except $CaCl_2$ was omitted and 1 gm. Difco yeast extract was added per liter. All media was sterilized for approximately 20 minutes at 15-20 lbs. pressure. Axenic cultures were established by transferring one loop of organisms to a bacteria free culture solution.

Optical density and/or cell counts were used as a measure of growth. The former was measured with a Klett-Summerson photoelectric colorimeter equipped with a red 66 filter. An adapter capable of receiving 16 mm. x 150 mm. test tubes was made to replace the standard Klett tube adapter. Optical density readings were taken by inserting cultures of organisms in the colorimeter. A standard or blank of culture media was first measured for optical density. Optical densities given in subsequent pages are based on increase in optical density of culture solutions. Cultures were agitated so as to distribute the Tetrahymena relatively evenly through the media above the cellular debris on the bottom without disturbing the bottom debris. Readings were then taken as rapidly as possible since the Tetrahymena tend to stratify or form patterns which cause optical density readings to fluctuate (Elliott, 1949). At the end of the series of readings the colorimeter was checked with either the standard or the distilled water blank and readings were repeated after restandardization if a marked change in the colorimeter setting had occurred. Since optically matched tubes were not used, readings of all tubes in a series were taken at the beginning. The pyrex label was always directed toward the front of the colorimeter.

Cell counts were usually made subsequent to fixation of cells with formalin. In some instances organisms were killed with heat fixation (50°C.). An average of three counts was

taken. Each count was made using a hemocytometer in the same manner as a white blood cell count. Dilution was usually one part culture solution to one part formol-calcium solution.

Sudan black B was used for demonstration and localization of lipid in a method modified slightly from Baker (1944)(cited in Gatenby and Beams, 1950). Cells were usually concentrated by centrifugation then dried on albumin-glycerin coated slides. They were then fixed in formol-calcium. One control slide was placed in pyridine at room temperature for one-half to two hours. After rinsing in distilled water, both were upgraded to 70% alcohol then stained in a saturated solution of Sudan black B in 70% alcohol for seven minutes. They were then passed through 70% alcohol, three changes of 50% alcohol, 30% alcohol, and water before mounting in glycerogel.

Nile Blue sulfate was used to distinguish neutral lipid from acidic lipid. Concentrated Nile Blue sulfate was prepared by the method given in Conn (1929). The concentrated Nile Blue was diluted with distilled water when used. Slides fixed in formol-calcium were left in dilute Nile Blue sulfate solution (ten drops of Nile Blue added to 50 ml. distilled water) for 12 to 24 hours or longer.

Osmic acid was used for staining unsaturated fats. Both unfixed and dried suspensions of Tetrahymena were covered with a 0.5% solution of osmic acid for a period of 15 minutes before mounting in glycerogel.

The technique of Menschik (1953) was used for phospholipid determination. It was varied slightly by using the Nile Blue sulfate at 50°C. instead of 60°C. The Weigert-Smith-Dietrich test was also used for phospholipid determination as described by Gatenby and Beams (1950). The control slide was fixed in formol-calcium and extracted in pyridine before completing the regular staining procedure. The Schultz reaction was used for cholesterol. Glycerol dichlorhydrin was used for vitamin A determination as described by Penketh (1948).

The activity factors for fat synthesis were ascertained by the method of Fennell and Marzke (1954), i.e., activity factor $= \frac{L_1 P}{L_2 T.}$ L_1, the length of positive area; L_2, average length of cells; T, total cells counted; and P, total positive cells.

For synchronization of cell divisions the method of Scherbaum and Zeuthen (1954) was used. This method results in a higher than normal number of cells being in the same phase of division at the same time.

RESULTS

Series I. This series was grown in 75 ml. of a tryptone and vitamin solution at room temperature. A 1 ml. inoculum of a 10 day old tryptone and vitamin culture was used. It is evident in text-figure 1 that lipids, with the exception of the 6th day, gradually increased until the 21st day, after which there was a rather rapid decline. An examination of the slides shows the decrease on the 6th day is only apparent due to a lesser length of lipid area with much heavier concentration in this area. Thus the period of increase is more steady than the activity factor indicates in this instance. Figs. 1-7 indicate the general anterior localization previously described by other workers. Also shown is the aggregation of the smaller droplets into fewer larger lipid droplets in cells with greater amounts of fat. Note how in some cases (Fig. 4) large droplets have largely severed non-lipid cytoplasmic connections inward from the cell membrane. Also visible in Fig. 4 is lipid material that apparently has been extruded from the cells; this is frequently observed in cells with abundant lipid.

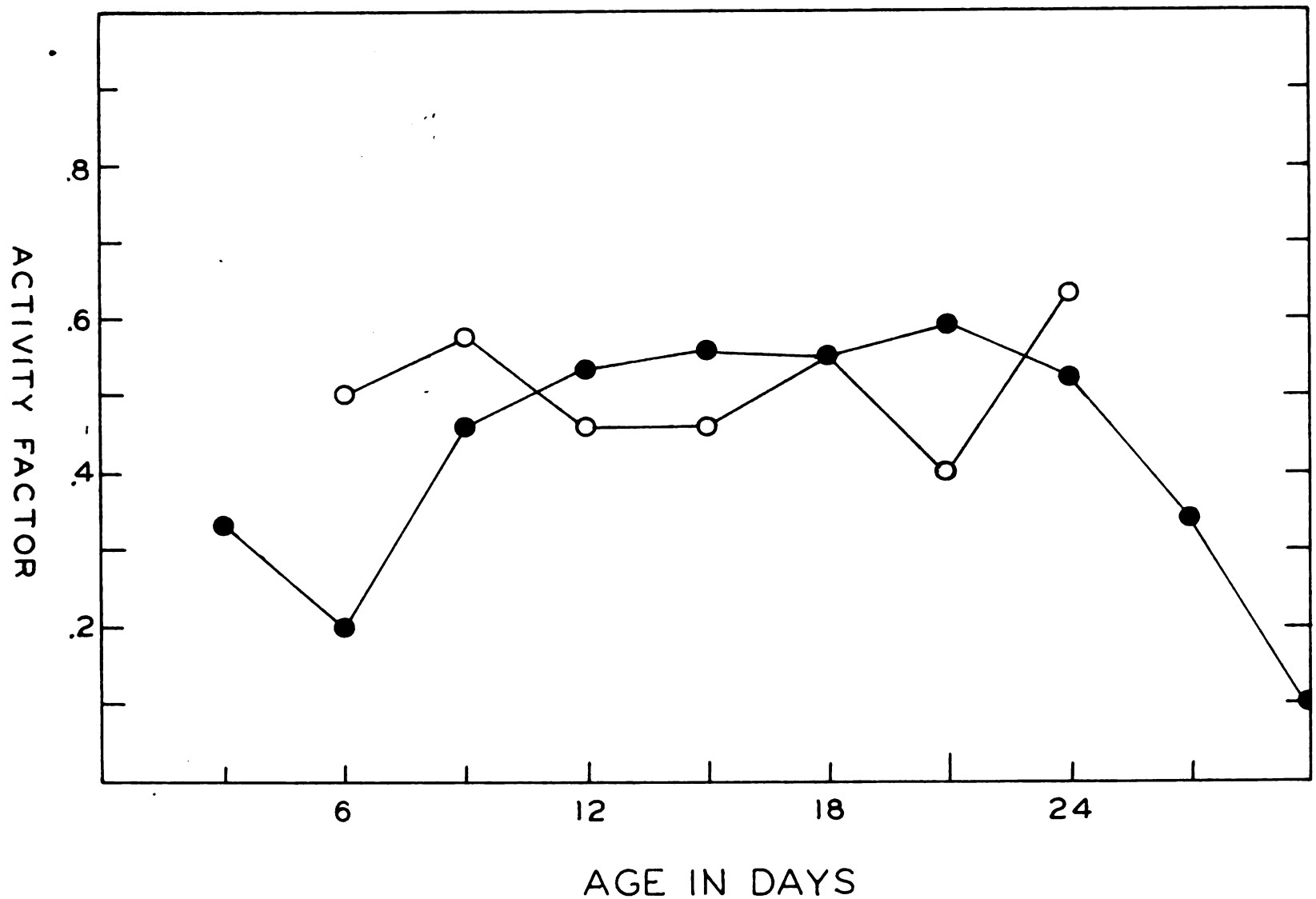
Series II. This series was similar to series I except that they were grown in a constant temperature room, and the inoculum was 1 loop of a 12 day old tryptone and vitamin

TEXT-FIGURE 1

Relation between culture age and lipid activity factor in specimens of T. geleii W of series I, cultured in tryptone and vitamins, and series XII, cultured in casein with yeast extract. Activity factor ascertained from slides stained with Sudan black B.

●, T. geleii W, series I, cultured in tryptone and vitamin media. 1 ml. inoculum into 75 ml. media; and

○, T. geleii W, series XII, cultured in casein with yeast extract. 1 loop inoculum into 10 ml. media.


ACTIVITY FACTOR
.8
.6
.4
.2
6
12
18
24
AGE IN DAYS

culture. Text-figure 2 shows the relationship between the lipid activity factor, pH, and optical density for this series. Note the rapid increase in pH until maximum optical density is reached, then a reduced rate until a stable pH is reached on the 14th day. Note also the rough correlation between pH and lipid activity factor. The lipid activity factor is zero until after the logarithmic growth phase (text-fig. 2 and fig. 8). From table 1 it can be seen that there is a drop in numbers following the peak in optical density. This initial drop is followed by a gradual decline in numbers. At about the time of the initial drop an accumulation of debris can be observed on the bottom of the culture flask.

Some fluctuation occurred in temperature after about the 8th day. Prior to that the temperature had been at 77°F. Temperatures for series I had been considerably lower, probably no higher than 73°F. and sometimes in the low 60's.

A comparison of text-figure 1 and text-figure 2 shows series I to have a higher lipid activity factor except on the 6th day. Comparison of figs. 1-7 with figs. 8-12 indicated series I has a consistently greater amount of intracellular lipid. Cellular debris is often high in lipid content as is seen in fig. 12.

Series III, IV, V, and VI. Series III was similar to series II, except the inoculum was one loop of a 15 day

TEXT-FIGURE 2

Relation between culture age, lipid activity factor, and pH of culture for T. geleii W, of Series II. 1 loop inoculum in 75 ml. tryptone and vitamin solution in 125 ml. Erlynmeyer flasks. Activity factor ascertained from slides stained with Sudan black B.

● , lipid activity factor;

O , optical density; and

⊕ , pH of culture.

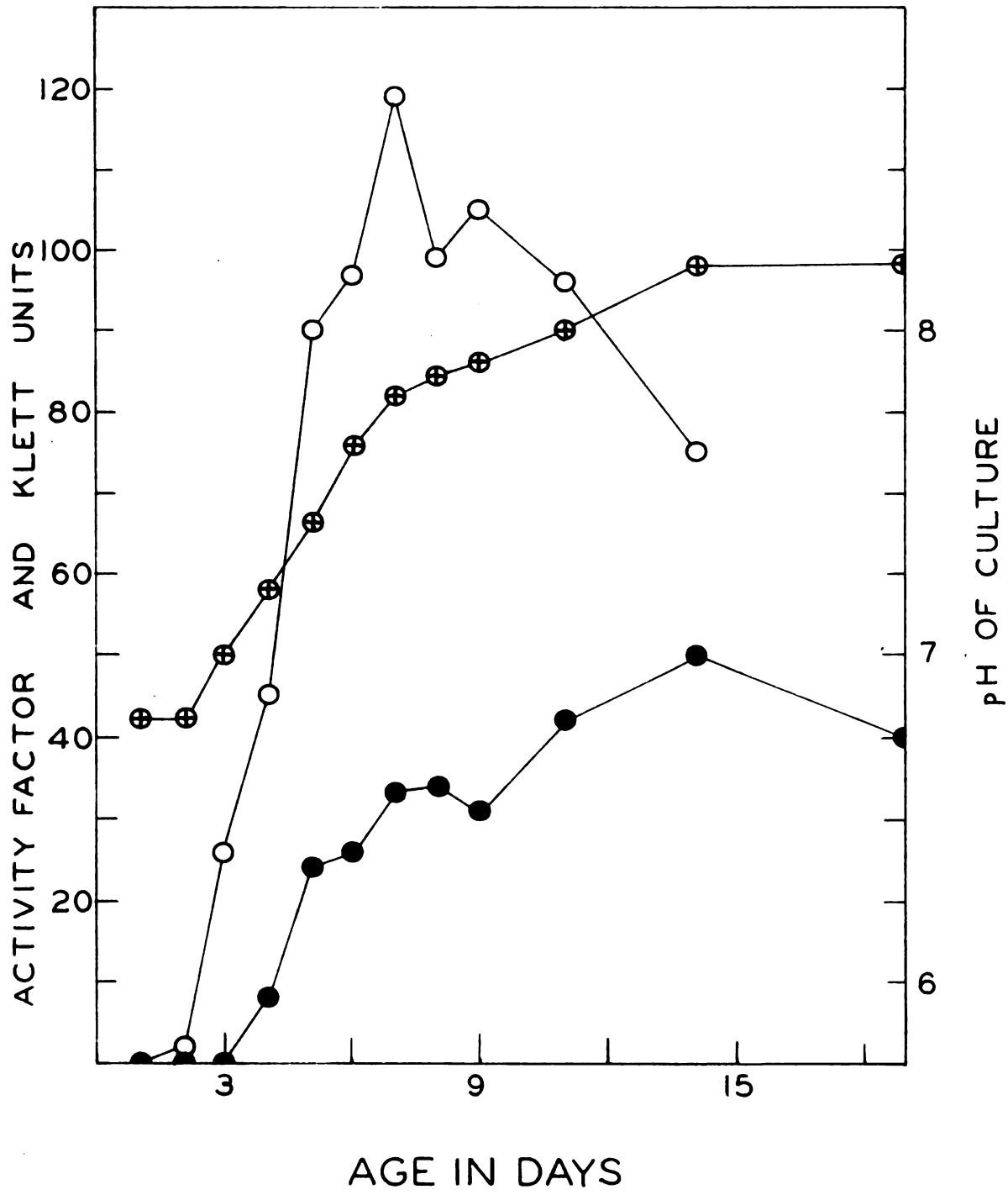

120
100
80
60
40
20
ACTIVITY FACTOR AND KLETT UNITS
8
7
6
pH OF CULTURE
3
9
15
AGE IN DAYS

TABLE 1

OPTICAL DENSITY, CELL COUNT (1,000 CELLS/ML.), pH, AND LIPID ACTIVITY FACTOR FOR CULTURES OF TRYPTONE AND TRYPTONE AND VITAMINS

	Age in days	1	2	3	4	5	6	7	8	9	11	14	19
Series II	Optical density	0	2	26	45	90	97	119	99	105	96	75	
(75 ml. tryptone	1,000 cells/ml.		17	80	123	135	216	130	130	100	113	93	70
and vitamins)	pH of culture	6.8	7.0	7.0	7.0	7.4	7.65	7.0	7.05	7.9	8.0	8.2	8.2
	Lipid activity factor		.00	.00	.00	.04	.26	.33	.34	.31	.42	.50	.40

	Age in days	3	6	9	12	15	18	21	24	27	30	50	60
Series III	Optical density	23	45	37	34	35	33	34	32	35		26	23
(10 ml. tryptone	1,000 cells/ml.			34								72	35
and vitamins, erect)	pH of culture	6.9	7.2	7.3	7.3	7.4	7.5	7.45	7.5				
	Lipid activity factor	.01	.06	.15	.02	.19	.30	.20	.13	.19	.16		
Series IV	Optical density	22	93	53	47	49	39	43	40	39		20	16
(10 ml. tryptone and	1,000 cells/ml.			65								37	23
vitamins, slanted)	pH of culture	6.8	7.6	7.5	7.6	7.6	7.7	7.8	7.85				
	Lipid activity factor	.01	.07	.28	.31	.24	.29	.10	.02	.003	.03		
Series V	Optical density	12	32	12	5	3							
(10 ml. tryptone,	1,000 cells/ml.			27									
erect)	pH of culture	6.8	7.0	7.0	7.0	7.1	7.0						
	Lipid activity factor	.00	.00	.00	.00	.07							

tryptone culture, and test tubes of tryptone and vitamin solution were used (10 ml. culture solution in 16 x 150 mm. test tubes in an upright position). With the exception of the 12th day there is a gradual increase in the lipid activity factor until the 18th day, after which there is a gradual decline. Series IV was identical to series III, except the tubes were inclined at a 60° angle thus approximately doubling the surface for gaseous exchange. In series IV there was a rapid increase in fat until the 9th day when a moderately high level was reached; the activity factor then remained about the same until the 18th day, which is followed by a rather rapid decline. A comparison of series III and IV (text-fig. 3) shows the former to have a lower lipid level until the 18th day, after which it has a higher lipid level than series IV. Both series III and IV have less lipid than series II. In series IV pH increased more rapidly and maintained a higher level than in series III (table 1).

Series V was identical to series III except the vitamins were omitted. Text-figure 4 shows a low growth level and little increase in pH. A lipid activity factor greater than zero was obtained only on the 15th day when it was 0.07. On other days cells could be found with lipid, but they were not among those counted in the random technique used in obtaining the activity factor.

TEXT-FIGURE 3

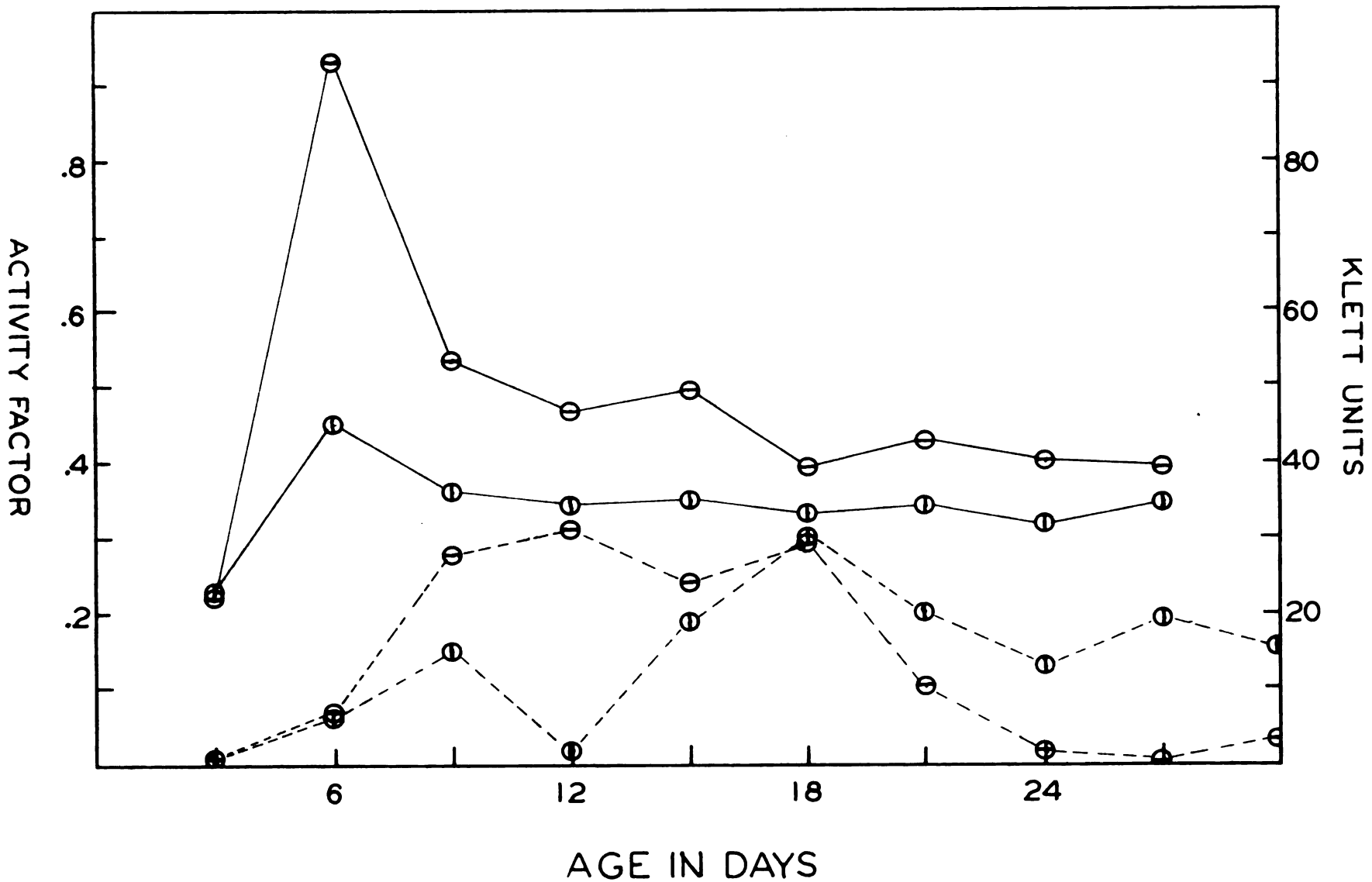
Relation between culture age, lipid activity factor and optical density of Series III, grown erect, and Series IV, inclined 60°. 1 loop inoculum in 10 ml. tryptone and vitamin solution in test tubes. Activity factor ascertained from slides stained with Sudan black B.

 , optical density, Series III (erect);

 , activity factor, Series III;

 , optical density, Series IV (inclined); and

 , activity factor, Series IV.


ACTIVITY FACTOR
.8
.6
.4
.2
KLETT UNITS
80
60
40
20
6
12
18
24
AGE IN DAYS

TEXT-FIGURE 4

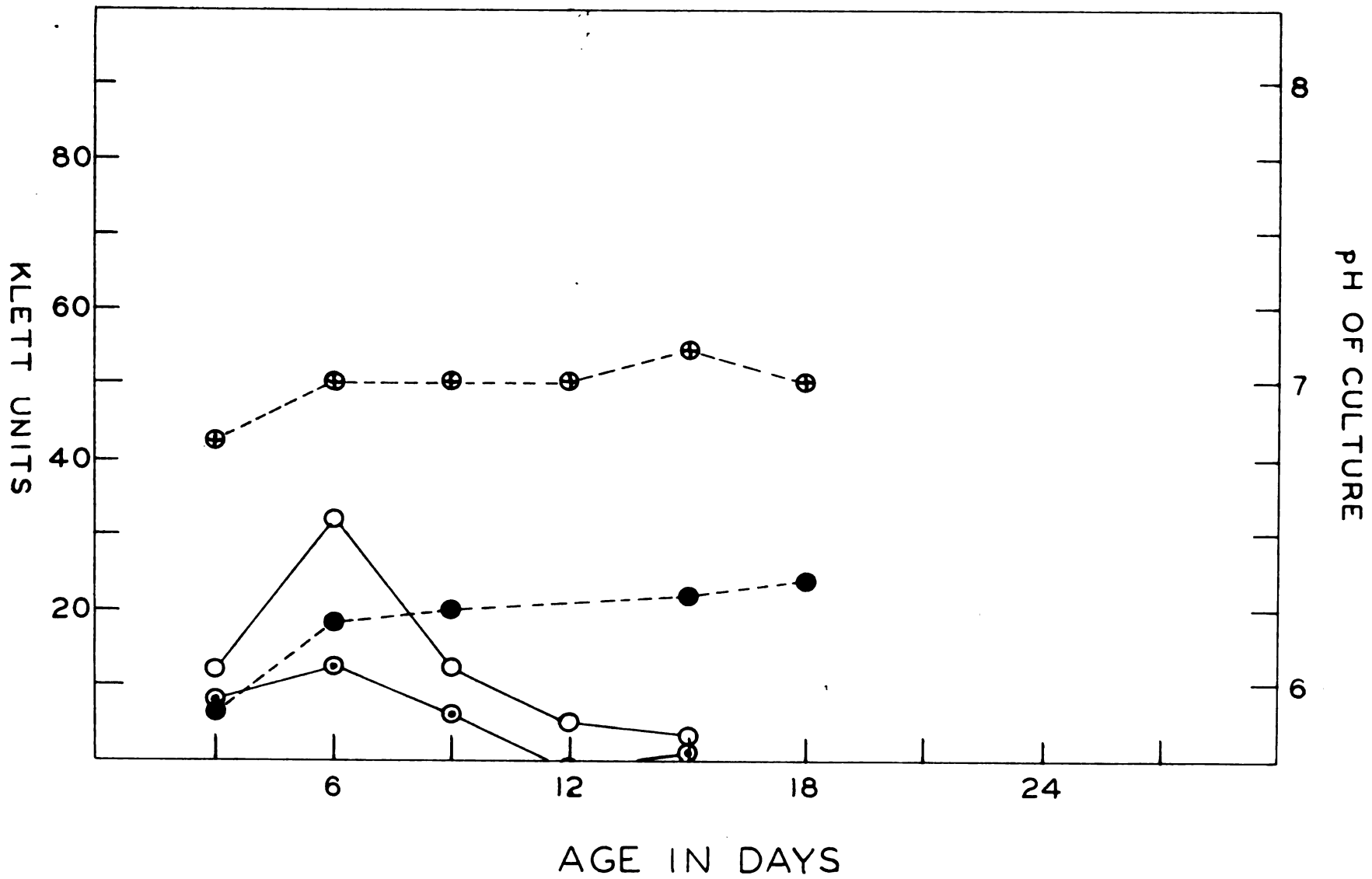
Relation between age, pH, and optical density of cultures of T. geleii W grown in tryptone solution (Series V) and tryptone citrate solution (Series VI).

⊕, pH, Series V (tryptone);

●, pH, Series VI (tryptone citrate);

○, optical density, Series V (tryptone); and

⊙, optical density, Series VI (tryptone citrate).


KLETT UNITS
80
60
40
20
8
7
6
PH OF CULTURE
6
12
18
24
AGE IN DAYS

Series VI, identical to Series III and V except tryptone citrate was used, gave results similar to series V. Even less growth as shown by optical density is observed (text-fig. 4). A different buffer at lower pH is involved, and the increase in pH is slightly greater than in series V, but much less than in series III or IV. As in series V a lipid activity factor was obtained on only one day. On the 12th day it was 0.005. Cells of series V and VI are small as is shown in figs. 25 and 26.

Series VII and VIII were repeats of V and VI respectively. However, some were slanted to see if the reduced surface area/volume ratio was responsible for the poor growth. No increase was observed in either case. There was some indication of a reduction in growth of the slanted tryptone citrate.

A comparison of results obtained in organisms cultured in tryptone and vitamin solution of series IX (pH 6.35, text-fig. 5) and series X (pH 6.73, text-fig. 6) with series XI (pH 7.40, text-fig. 7) shows the growth to be improved slightly by higher pH during the first three weeks, however, during the same period after the 3rd day the lipid activity factor shows a marked increase with increased pH. Note also the more marked rise in pH of series IX and X. Observe how the initial peak in lipid activity lags behind the peak in optical density. The first lipid peak is followed by a drop and then a second

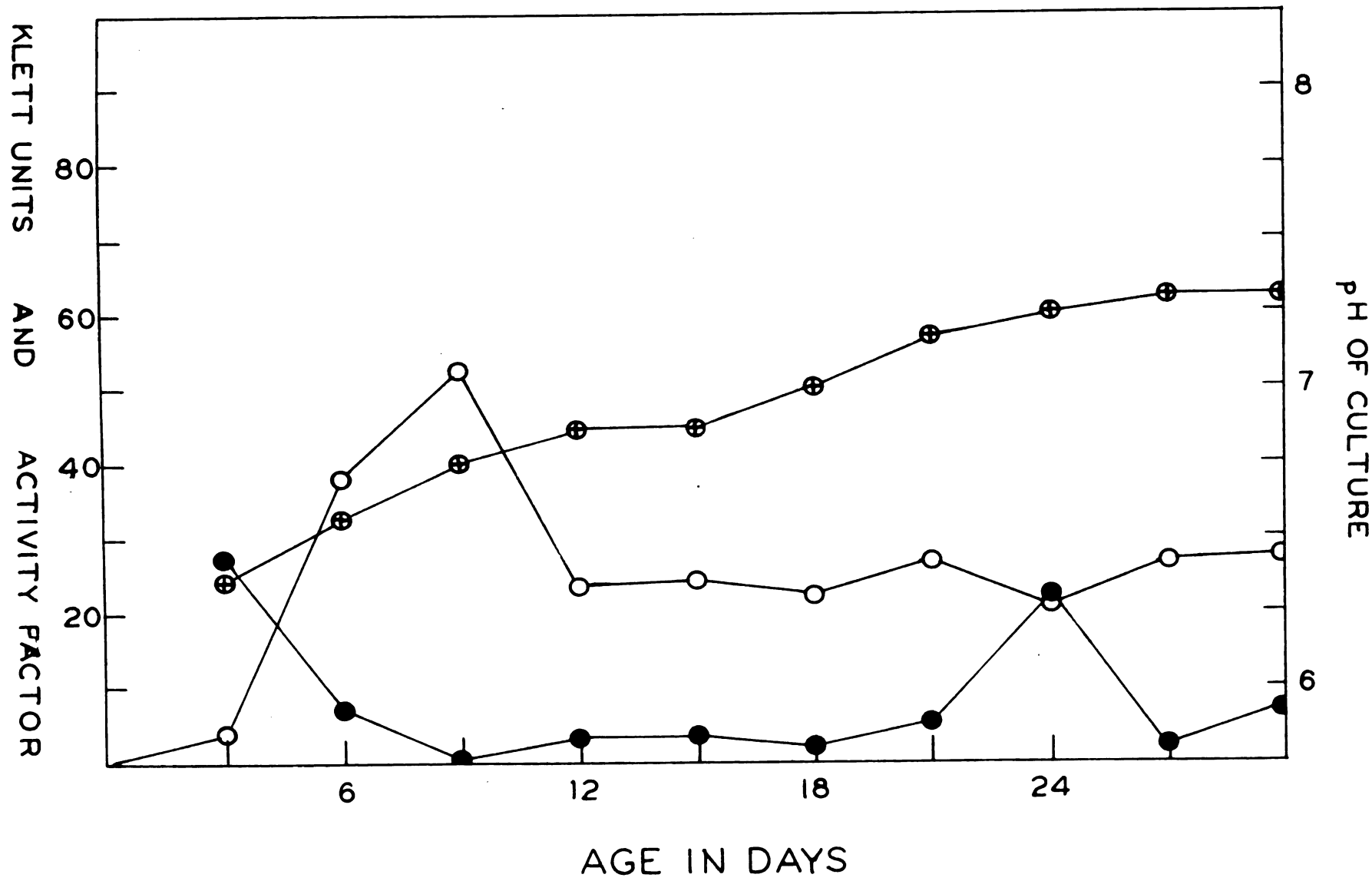
TEXT-FIGURE 5

Relation between culture age, lipid activity factor, optical density, and pH of Series IX (tryptone and vitamins with pH adjusted to 6.35). Activity factor ascertained from slides stained with Sudan black B.

●, lipid activity factor;

O , optical density; and

⊕ , pH.


KLETT UNITS AND ACTIVITY FACTOR
80
60
40
20
8
7
6
pH OF CULTURE
6
12
18
24
AGE IN DAYS

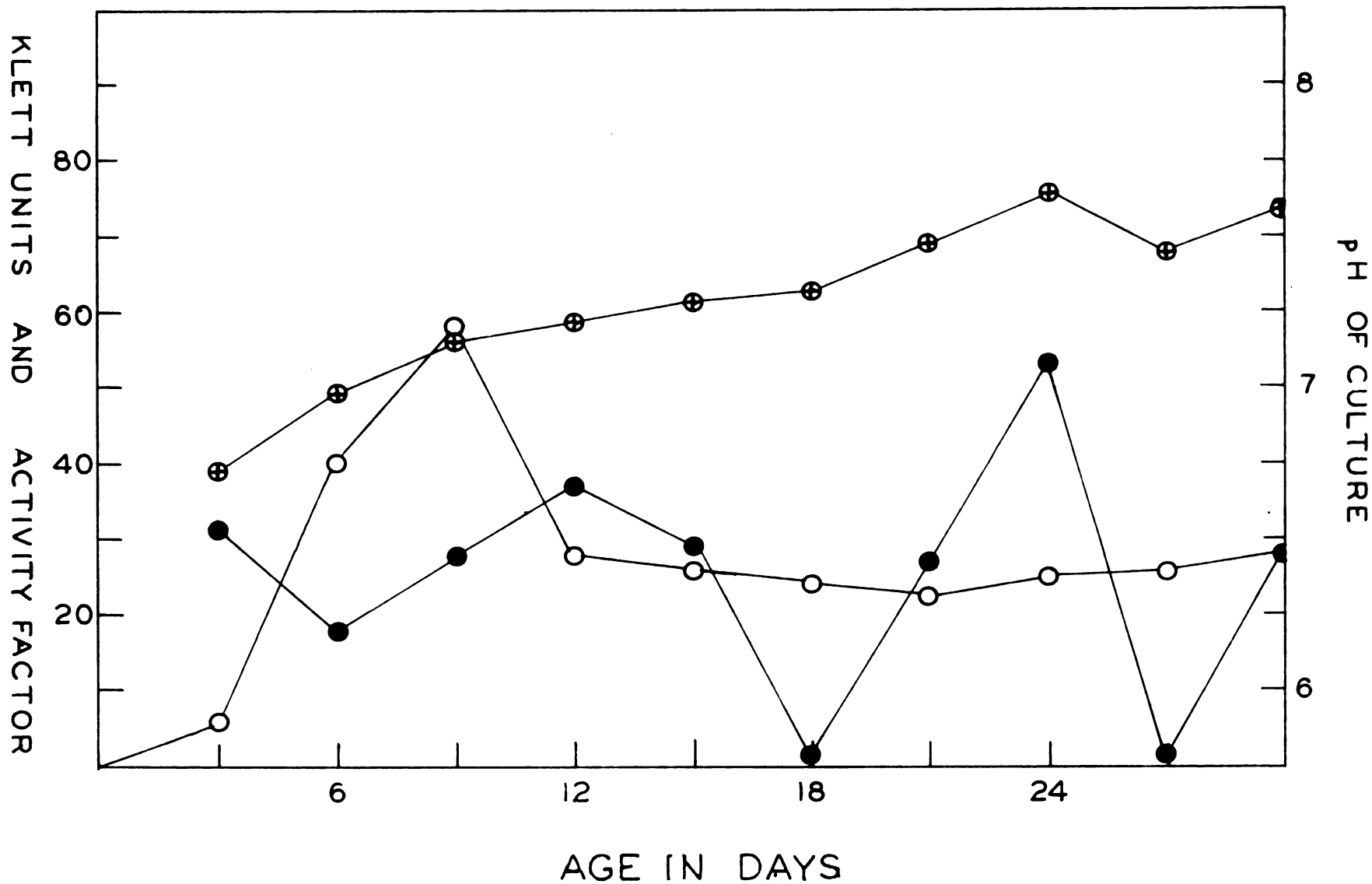
TEXT-FIGURE 6

Relation between culture age, lipid activity factor, optical density, and pH of Series X (tryptone and vitamins with pH adjusted to 6.73). Activity factor ascertained from slides stained with Sudan black B.

● , lipid activity factor;

O , optical density; and

⊕ , pH.


KLETT UNITS AND ACTIVITY FACTOR
80
60
40
20
8
7
6
pH OF CULTURE
6
12
18
24
AGE IN DAYS

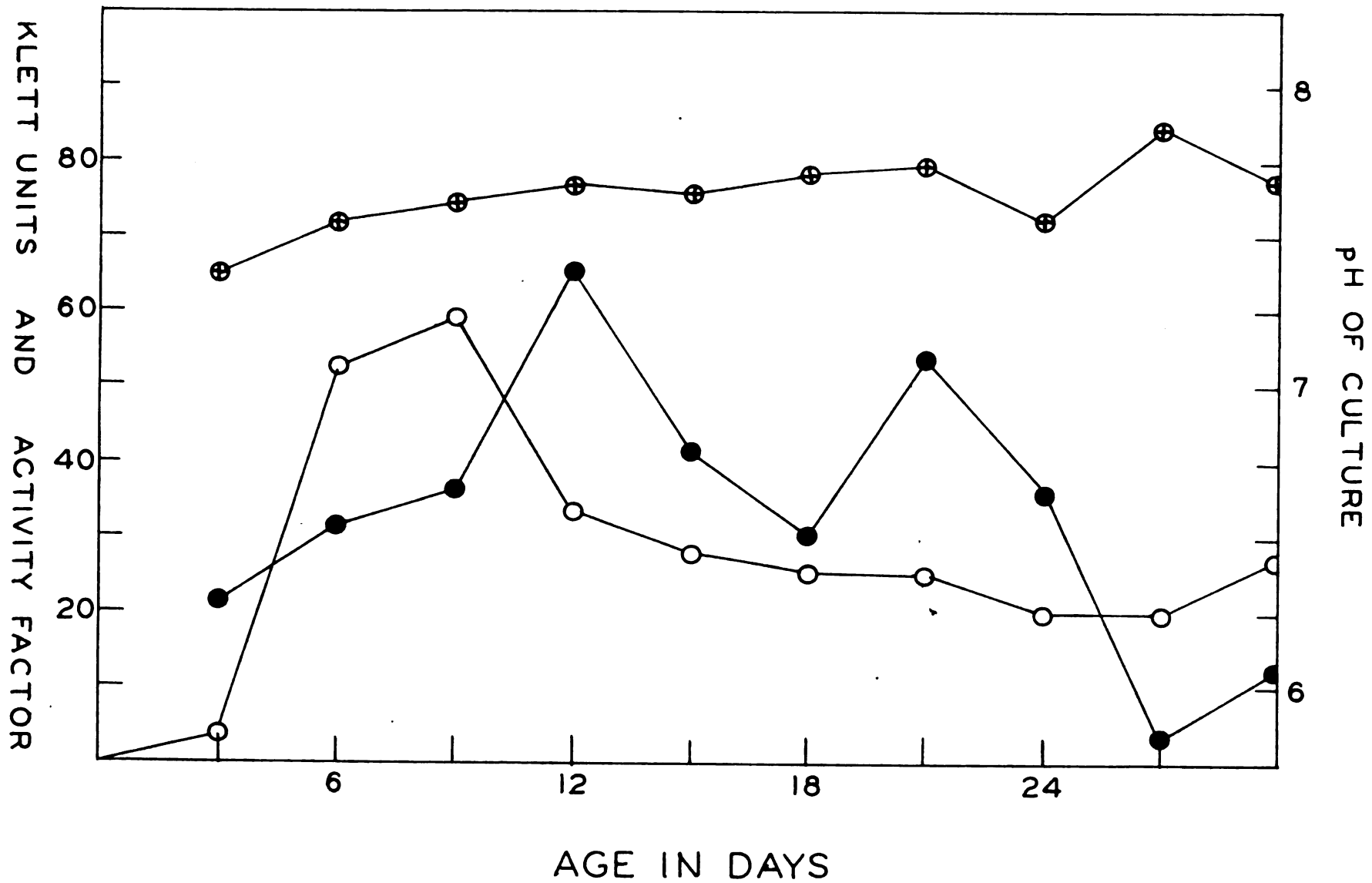
TEXT-FIGURE 7

Relation between culture age, lipid activity factor, optical density, and pH of Series XI (tryptone and vitamins with pH adjusted to 7.40). Activity factor ascertained from slides stained with Sudan black B.

● , lipid activity factor;

O , optical density; and

⊕ , pH.


KLETT UNITS AND ACTIVITY FACTOR
80
60
40
20
8
7
6
PH OF CULTURE
6
12
18
24
AGE IN DAYS

peak. There is an indication of this same phenomenon in series III and IV as shown in text figure 3.

Series XII. This series using casein plus yeast extract in test tubes was run following 3 transfers through the same media. From text-figure 1 note the high lipid level throughout the series. No optical density of value could be obtained because the culture solution had a milky turbidity at first that gradually cleared as the organisms grew. From table 2 note the presence of acidic lipid early in the culture life. This was a very pronounced reaction in contrast to most other reactions for acidic lipid.

Series XIII. This series of casein solution failed to support noticeable growth within the time it was observed.

Series XIV. The cells in this series from tryptone and vitamin solution were synchronized. During division they appeared to have less lipid than prior to division. Since those dividing were stained the next morning, while those fixed before division were stained the same day, it was felt the lower lipid amount might be due to a loss in the fixing solution. Hence another series was run.

Series XV. This was a repeat of series XIV. However, those fixed before division and those fixed following division were stained at the same time. Those fixed 15 minutes after cessation of synchronization treatment (before division) gave an activity value of 0.094. Cells fixed 90 minutes after

TABLE 2

HISTOCHEMICAL CHARACTERISTICS

	Age in days	4	5	6	7	8	9	11	14	19			
Series II	Neutral fat	*	**	**	**	**	**	**	***	**			
(75 ml. tryptone	Acidic lipid	**	**	**	*	*	*	*	*	*			
and vitamins)	Unsaturation	**		*			**	***	**				
	Age in days	3	6	9	12	15	18	21	24	27	30	50	60
Series I	Cholesterol	-	-	-	-	-	?	-	?				
(75 ml. tryptone	Phospholipid	-	-	-	-	-	-	-	-	-	-		
and vitamins)	Plasmal	-	-	-	-	-	-	-	-	-	-		
	Vitamin A	-	-	?	?	-	-						
Series III	Neutral fat	**	***	***		**	**	*	**	**		*	**
(10 ml. tryptone and	Acidic lipid	*	-	-		*	*	**	*	*	**		-
vitamins, erect)	Unsaturation		*	**				**		**			**
	Sudan black	**	**	**	**	**	**	**	**	**			
Series XII	Neutral fat	*	**	**	**	**	**	**	**				
(10 ml. casein and	Acidic lipid	***	***	**	*	*	*	*					
yeast extract)	Phospholipid					**							

Note: With few exceptions, all cultures were Sudan black positive, and these were positive for unsaturation except in a few questionable cases. All having fat gave some indication of neutral fat, and many appeared to have acidic lipid either separate or mixed in. No positive cholesterol, vitamin A, or carotenoid tests were ascertained in any culture tested for them.

-, negative; ?, doubtful negative; *, weak positive; **, positive; and ***, strongly positive.

synchronization treatment was stopped contained dividing cells and had an activity value of 0.0395. This appeared to fit the situation as observed, although the lipid is so diffuse and slight as to be almost indistinguishable in figs. 33 and 34. As a further check a transection was counted on each slide, that before division contained 66.6% cells positive for lipid, that during division only 34%. It seems apparent that during division following synchronization there is a decrease in histochemically demonstrable intracellular lipid of cells of T. geleii W grown in tryptone and vitamin solution. Observations on dividing cells as they are occasionally found tend to bear this out. They frequently have no lipid or less lipid than the culture average although no records were kept due to the infrequency of observations of dividing cells. It was also noted that distribution of lipid, in dividing cells having lipid, may occur in various manners. The first observation of such was a cell in which all lipid was going to the anterior daughter cell; figs. 12 and 13 may show cells in which this is occurring. Subsequent observations have shown cells in which most observable lipid is going to the posterior daughter cell, also cells in which the distribution is about equal as in fig. 32.

Histochemical observations (table 2). All series showed at least some lipid by Sudan staining if growth occurred. Unmasking treatment did not appear to demonstrate additional lipid. Cells stained with Sudan black B were usually darker

than unstained cells even in areas negative for lipid. Occasionally a few crystals would precipitate in the cells as is probably the case in figs. 25 and 26. In no cases were lipids known to be demonstrated within the nucleus. The general graying effect of Sudan black B persisted in the controls following pyridine extraction. This graying frequently disclosed the nucleus to be a more clear area as in fig. 35.

In most cases lipid positive cultures were positive for unsaturation by osmic acid staining. However, this reaction was usually rather faint and could not be ascertained when small amounts of tiny diffuse droplets were the only lipid present.

Lipids present were predominately neutral fats as shown by Nile Blue sulfate staining. Frequently a purplish cast was noted indicating some admisture of acidic lipids. Series XII was strongly positive for acidic lipid in the early part of the series. Series XII also gave a good reaction for phospholipid (fig. 30); positive droplets were fewer than total lipid droplets and were scattered throughout the cell. Negative reactions were obtained in all cases that tests were made for plasmal, steroids, carotenoids, and vitamin A.

Further observations. It was noted that when transfers were made from old casein and yeast extract to fresh solutions it took a week or two for cultures to develop an observable growth. When a buffered series was prepared with K^+ molarity

of the phosphate buffer set at 4/100 in the 75 ml. of tryptone and vitamin media, it took over a month for the 1 loop inoculum to develop to an observable number of cells. When optical densities of some cultures are taken daily and compared with identical cultures on which only one reading is taken, there is frequently a significant difference especially under more favorable culture conditions.

DISCUSSION

Elliott and Hogg (1952) and McCashland (1955) have shown that Tetrahymena can be acclimatized and cultured in various types of media. Subsequent to acclimatization intracellular lipid deposition was closely correlated with age of the culture solution, i.e. deposition increased as the age of the culture increased (Fennell, 1951; Marzke 1953, thesis; and Fennell and Marzke, 1954). Marzke (1953, thesis) found a high level of lipid deposition in organisms cultured in tryptone solution with essentially no change upon the addition of vitamins.

Observations made in this study demonstrated that growth and lipid deposition were retarded by transfer to tryptone solution. On the other hand, continued culturing in tryptone and vitamin solution resulted in the maintenance of high levels of growth and lipid deposition. Obviously, several interpretations can be given to these results; (1) the tryptone may have been deficient in some essential nutrient, possibly vitamins, while there may have been vitamin storage in the case of high lipid production in tryptone; (2) in the latter case there may have been synthesis of vitamins, since Seaman (1953) has shown that Tetrahymena can synthesize vitamins; (3) since in the case of high lipid production they had been repeatedly cultured in tryptone solution, they may have been acclimatized to it; and (4) there may have been a genetic change. The

importance of vitamins in growth is well known. Their relation to lipid synthesis is less well known. For further details concerning the role of thiamine in formation of acetate from pyruvate and the role of acetate as a precursor of fatty acids, consult Haurowitz (1955).

Factors other than vitamin concentration of the culture media may be involved in the rate of synthesis of lipids. A survey of results presented in this investigation shows that conditions which promote good growth usually result in abundant lipid deposition. In addition to the effect of vitamins it was shown in text-figure 3 that increasing the surface area of the tryptone and vitamin culture solution resulted in an earlier increase in lipid deposition and optical density. The improved growth is in accord with the observations of Elliott et al. (1952). The lower lipid values in the series with less surface area might be predicted from the results of Pace and Ireland (1945), but when the relationship is reversed later in culture life a further explanation is needed. Probably more nutrients remained for the later synthesis of fat since the poorer growth early in culture life required less nutrient material.

Maximum growth precedes maximum deposition of lipid in cells grown in tryptone and vitamin media. This might be due to change in the substrate, in the organism, or both. Obvious changes in the media are the depletion of nutrients, release of materials into the media by living and/or dead cells (with

or without resultant changes), and concentration by evaporation. Change in the organism itself is also a possibility.

Depletion of nutrients is probably not a direct factor in lipid increase. It could be a factor if an essential for normal growth and division were depleted while materials necessary for fat synthesis became relatively more abundant.

Release of materials into the substrate by living and/or dead cells is probably a factor. Seaman (1949) has shown that in cultures of Tetrahymena there is an increase in fatty acid in the culture fluid until the maximum number of cells is reached. This is followed by a decrease and a second increase in fatty acids. We saw in fig. 12 that lipid can be set free by broken cells. Ingestion of this material could influence lipids present. Eckstein (1929) has shown that diet can influence body fat in the rat. Logenecker (1941) cites papers showing direct deposition of ingested fatty acids. Mast (1938) and Wilber (1942) have shown that fat can be digested to increase stored fat in amoebae. Formation of a selective inhibitor might produce a lipid increase. Seaman (1950 a) found that fluoride inhibited carbohydrate production without effecting lipid production from acetate by Tetrahymena. Nardone and Wilber (1950) have shown that nitrogenous wastes in Colpidium are largely in the form of urea early in culture life and after the fourth day is only in the form of ammonia. From the second to the fourteenth day the amount of ammonia in the culture fluid constantly increased. This is probably a major factor

in the increasing pH of cultures graphed in text-figures 2,4, 5,6, and 7. That the relationship between pH and lipid production is significant is indicated by results of series grown at varying pH and graphed in text-figures 5,6, and 7. It is possible that higher lipid levels in this case are due to better growth. This is probably partially the case, but results graphed in text-figure 3 show growth differing much more with less effect on the lipid produced. Thus it is probable that the increased pH of older cultures is a factor in the increased amounts of lipids in older cells. Concentration by evaporation may have been a factor; but it was less than 1% a day.

Changes within the organism may be permanent or temporary. That changes with culture age are not mainly the result of genetic change is indicated by the constancy of results within any given group of similar cultures. That there is a change in the metabolic pattern is indicated by the growth curves and increase then decrease in cell numbers. Thus Ormsbe (1942) found a decreased oxygen consumption by Tetrahymena geleii after the exponential growth phase was passed. It is to be expected that with the decreased growth rate in aged cultures the energy available to the cell is insufficient for maximum protoplasmic synthesis, and as a consequence fat deposition may be increased. Degenhardt (1955) has shown that phosphotase activity is decreased by prolonged culturing in tryptone solution and the storage of inorganic

phosphate is enhanced. Meyerhof and Green (1950) maintain that phosphatase is concerned with the transfer of phosphate from high energy compounds to low energy compounds, e.g., from phosphopyruvate to glycerophosphate. Krugelis (1946), who reaches essentially the same conclusion, finds that two phosphodiesterases act upon nucleic acids making nucleotides vulnerable to the action of a phosphomonoesterase which liberates organic phosphate and provides energy for the cell.

An interesting point is brought up by the reduction of fat positive cells during cell division as was noted in the synchronization experiment. That is the role of lipids in the cell. Lipids are thought to have an important role in cell membranes (Haurowitz, 1955). Parpart and Dzieman (1940) have given evidence indicating the importance of lipids in the red cell membrane. If such is the case, it is to be expected that during cell division there would be an increased demand for lipids for the formation of new membranes. This seems to be the case since cell requirements for life only are probably much lower as is indicated by the starvation experiment of Harding (1937). The apparent reduction in lipid could be a result of mechanical dispersal throughout the cell; since the individual particles in this series were so fine this could not be ascertained. A possible means of dispersal, cyclosis, has apparently not been investigated in protozoans grown axenically. Cohen (1949) has used Sudan black B (a fat specific

stain) for staining chromosomes. Since chromosomal duplication precedes division, this too might draw upon fat reserves.

Further indication of the presence of lipid in cell membranes and interfaces is the staining reaction pointed out in fig. 35. Since such staining persists in controls, it is probably the result of bound lipids if it is lipid staining. That it is not a fine precipitate is indicated by the light colored nucleus. The alcoholic rinses after staining would be expected to have their strongest washing action on the more exposed cytoplasm giving a reverse in the actual results if it were a precipitate. Thus the concept of lipids forming a part of cell membranes and interfaces is supported in two ways by the present study; i.e., by the decrease in observable lipid during division and by the generalized staining reaction.

Depletion of lipid during division may be a factor in the lower lipid values early in culture life when cells are dividing more actively.

Due to lack of evidence, no conclusions could be reached concerning the mode of distribution of lipid in dividing cells having abundant lipid. Because of the transverse fission and the anterior localization of the fat, it was suspected that this might be the method of producing cultures with cells having widely varying amounts of lipid. Thus the cell that had repeatedly divided from the anterior end might be expected to have more lipid. A statistical approach might reveal such an occurrence in lieu of cytological observations. This might

explain the variation in cells as in fig. 14. No attempt was made to see if dead cells averaged more fat than living cells as casual observation seemed to indicate. If so it would indicate that lipid accumulation in some way interfered with life processes, maybe only mechanically as was pointed out in fig. 4. Kidder and Dewey (1949) have shown that oleic acid is progressively inhibitory as concentration is increased in cultures of Tetrahymena. However, Griffen and McCarten (1949) have shown that in amoebae low concentrations of oleic acid are inhibitory, but even lower concentrations are stimulatory. Pollock et al. (1949) have isolated diptheroids requiring oleic acid for growth.

Lipid decrease in older cultures is probably due largely to depletion of the media. Cells tend to become smaller, rounder, with less lipid as is also illustrated by Harding's (1937) starvation experiments. The previously mentioned mode of division might also account for it if lipid did distribute in that manner and was toxic, since dividing cells have been observed in very old cultures. The former explanation has more evidence since Ryley (1952) found that Tetrahymena can exhibit an endogenous respiration which indicates the oxidation of fat or protein rather than carbohydrate.

Histochemical aspects. That substances recorded as lipid are actually such is quite well substantiated by the control slides extracted with pyridine. Baker (1946) uses pyridine as a lipid solvent in his control for phospholipid determination. Sudan black is specified by Cain (1950) and Lison (1953) as

the stain for lipids in general. The close correlation between Sudan black results and osmic acid results (table 2) is not surprising since Bensley and Bensley (1938) point out that it is a test for unsaturation, and Hilditch (1949) states that no natural fat has been found that does not contain some of the unsaturated oleic acid.

Nile blue is of some use in distinguishing neutral fats from acidic lipid (Cain, 1947) and shows histochemically demonstrable lipids in Tetrahymena to be mostly neutral fats. This when compared with the results of Seaman (1949) indicates that dispersed lipids are largely acidic lipids while stored lipids are largely neutral fats. Since he found the level of fatty acid and phospholipid (the principal acidic lipids) to be constant during the first seven days following inoculation and since this study shows that lipid is usually increasing during this period, the neutral fat reaction is probably accurate. McKee et al. (1947) found 75% of the lipid of 4-5 day cultures to be fatty acid. Holter and Zeuthen (1948) found that in Chaos chaos the minimum lipid content of the cytoplasm is considerably higher than visible fat. This is probably the case in Tetrahymena. Further, the visible phase is largely neutral fat, the other, largely acidic lipid. From the nature of monomolecular films as discussed by Deuel (1951) it is probable that fatty acids (acidic lipids) would be better suited to their formation. Fatty acid films might explain the general cytoplasmic graying caused by Sudan black and the blue cytoplasmic staining of Nile blue sulfate.

The absence of carotenoids is not surprising since they are largely plant pigments.

The absence of positive sterol tests in the face of its presence in Tetrahymena, demonstrated by McKee et al. (1947) and Seaman (1950), is explained by the insensitivity of the Schultz reaction (Reiner, 1953) employed here histochemically.

SUMMARY

1. Various media were used to grow test specimens of Tetrahymena geleii W axenically. All had at least some specimens positive for lipid as indicated by Sudan black staining.

2. It was noted that lipid concentration decreases following the increase noted by other investigators. Depletion of the media is suggested as the major cause.

3. At near neutral pH, a higher pH will result in greater increase of lipid in tryptone and vitamin cultures. It is noted that this is probably a factor in the original rise in lipid since pH rises then too.

4. Culture conditions allowing for greater maximum growth tend to result in greater maximum lipid production. Factors which were found to decrease growth and lipid activity values were removal of vitamins from the media, decrease in surface area/volume ratio, and decrease in pH.

5. Lipid is present in lesser amounts during division than prior to division in cells of Tetrahymena geleii W grown in tryptone and vitamins and subjected to a synchronization treatment. Some ideas concerning the significance of this decrease in observable lipid are discussed.

6. Lipid is stored largely as neutral fat with some unsaturation present. However, varying the media may result in other lipid being present in greater amounts at some periods.

7. Steroids, plasmal, carotenoids and vitamin A were not identified with the tests used for them here at the times they were tested. This does not exclude their presence. Especially since it was shown that different media may provide cells having different lipid histochemistry. Further, lipid content may be changed more than growth by some culture conditions, hence it can be seen that histochemical characteristics may provide a useful tool in bio-assay work where growth is too insensitive an indicator.

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PLATE I

Lipid in cells from 75 ml. tryptone and vitamin cultures grown at room temperature following inoculation with a 1 ml. inoculum (series I).

Fig. 1. 3-day cells. Series I. Sudan black B. 530X.

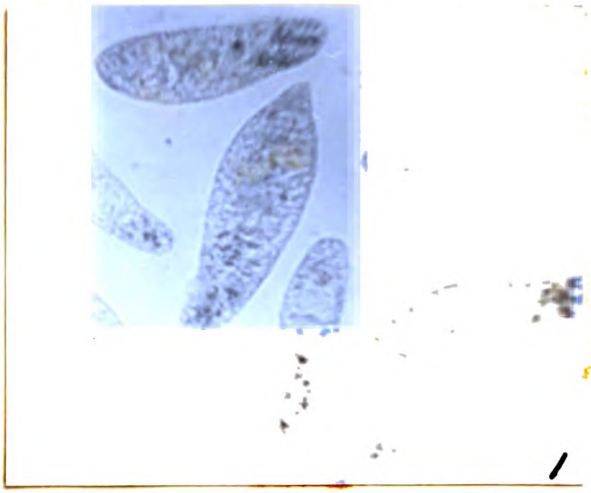
Fig. 2. 6-day cells. Series I. Sudan black B. 530X.

Fig. 3. 9-day cells. Series I. Sudan black B. 530X.

Fig. 4. 21-day cells. Series I. Sudan black B. 530X.

Fig. 5. 21-day cells. Series I. Control; stained with Sudan black B after pyridine extraction. 530X.

Fig. 6. 24-day cells. Series I. Sudan black B. 530X.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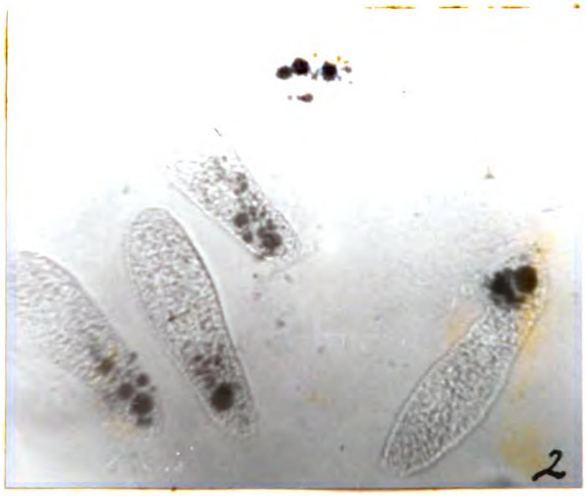
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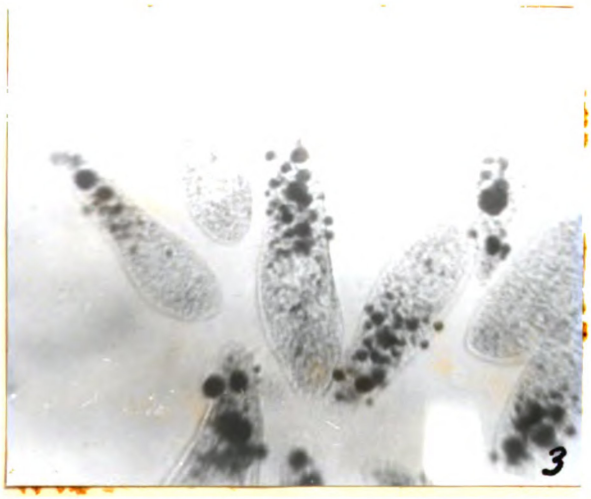
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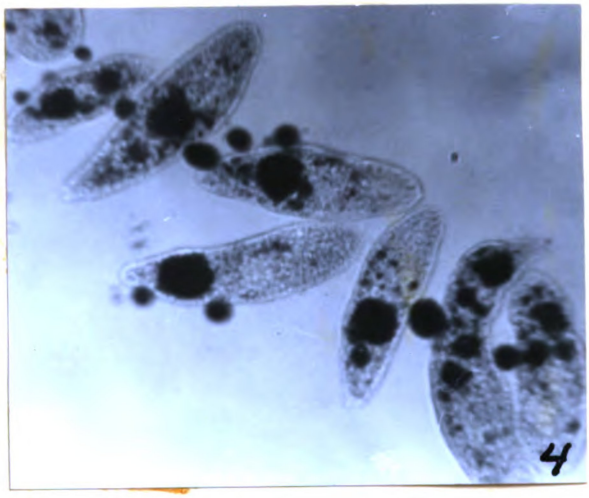
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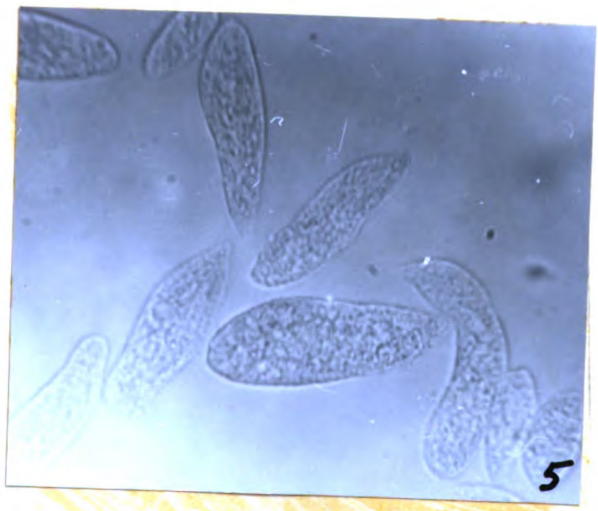
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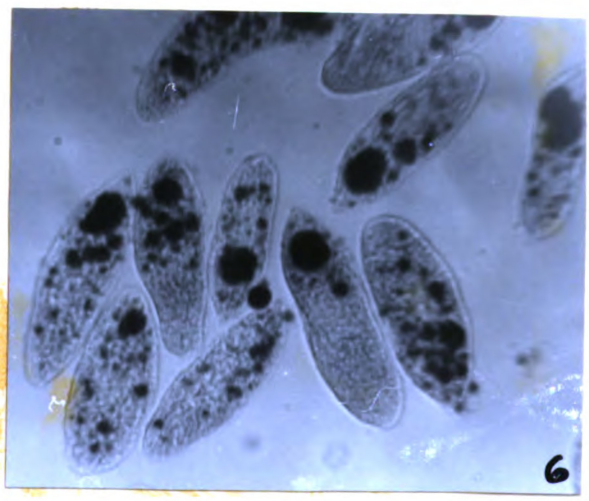
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PLATE II

Lipid in cells from 75 ml. tryptone and vitamin cultures inoculated with a 1 ml. inoculum (series I), and a 1 loop inoculum (series II). Series II grown in a constant temperature room.

Fig. 7. 30-day cells. Series I. Sudan black B. 530X.

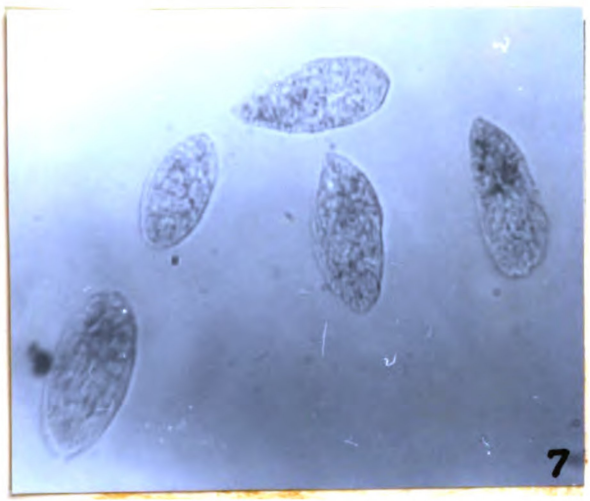
Fig. 8. 3-day cells. Series II. Sudan black B. 530X.

Fig. 9. 6-day cells. Series II. Sudan black B. 530X.

Fig. 10. 9-day cells. Series II. Sudan black B. 530X.

Fig. 11. 19-day cells. Series II. Sudan black B. 530X.

Fig. 12. Dividing cell, otherwise same as Fig. 11.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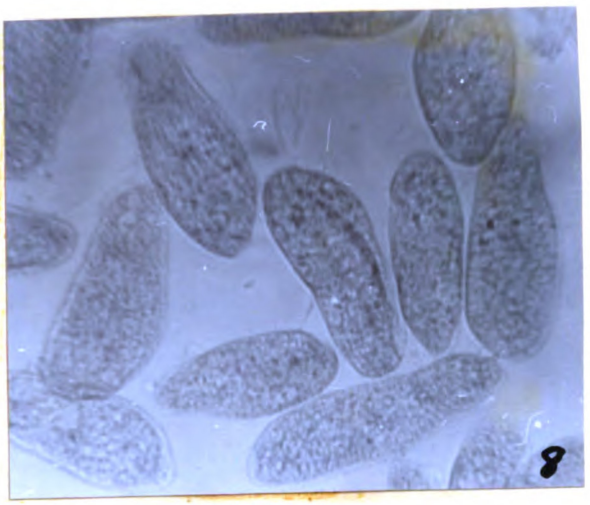
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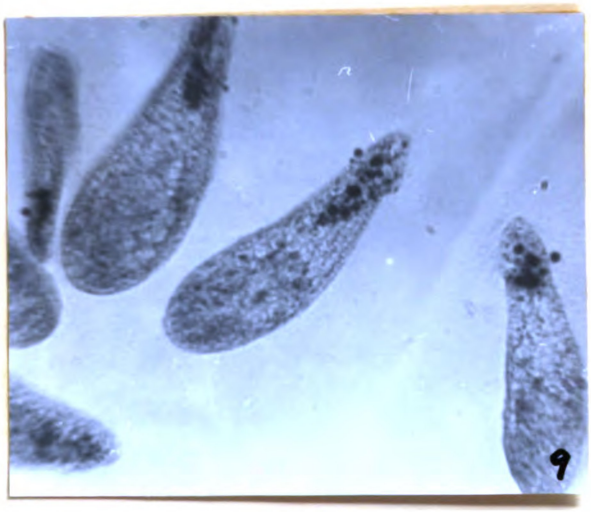
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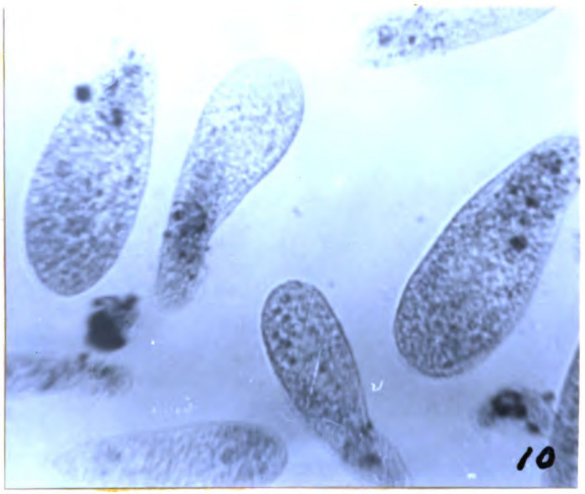
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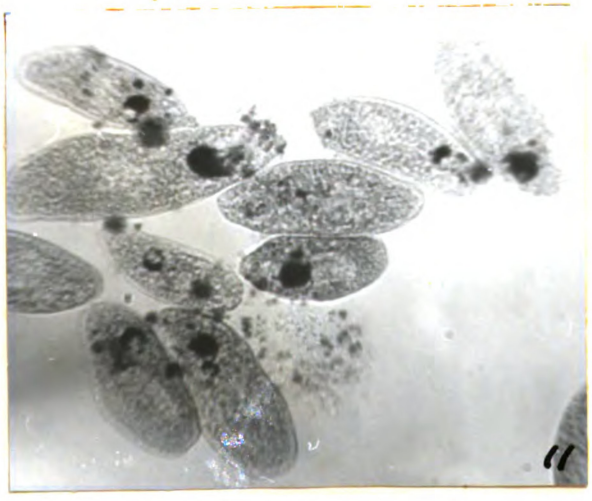
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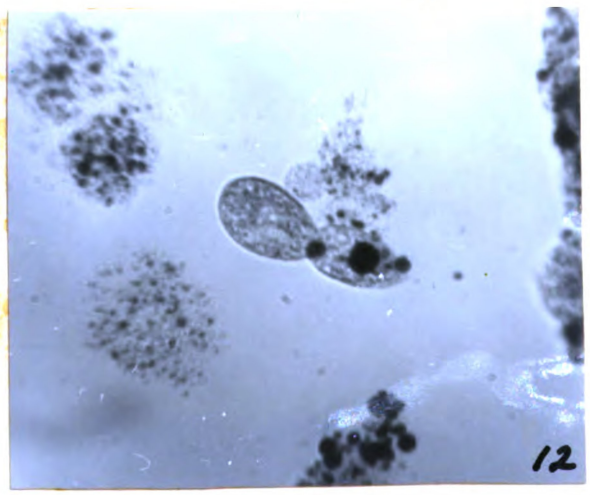
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PLATE III

Lipid in cells from 10 ml. Tryptone and vitamin cultures inoculated with a 1 loop inoculum and grown in a constant temperature room. Series III grown with tubes erect. Series IV tubes slanted 60° during incubation.

Fig. 13. 12-day cells. Series III (erect). Sudan black B. 530X.

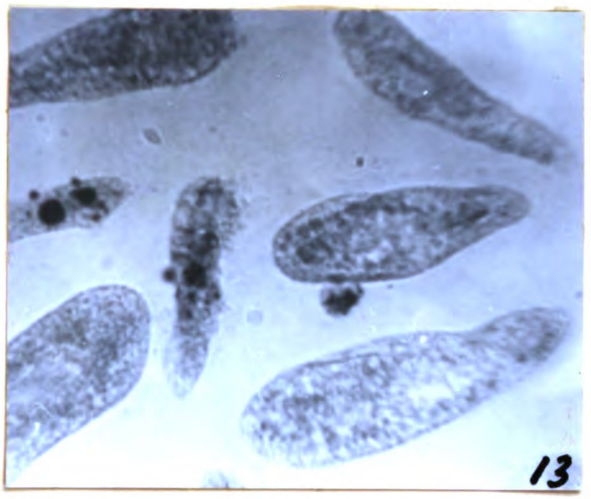
Fig. 14. 12-day cells. Series IV (slanted). Sudan black B. 530X.

Fig. 15. 18-day cells. Series III (erect). Sudan black B. 530X.

Fig. 16. 18-day cells. Series IV (slanted). Sudan black B. 530X.

Fig. 17. 24-day cells. Series III (erect). Sudan black B. 530X.

Fig. 18. 24-day cells. Series IV (slanted). Sudan black B. 530X.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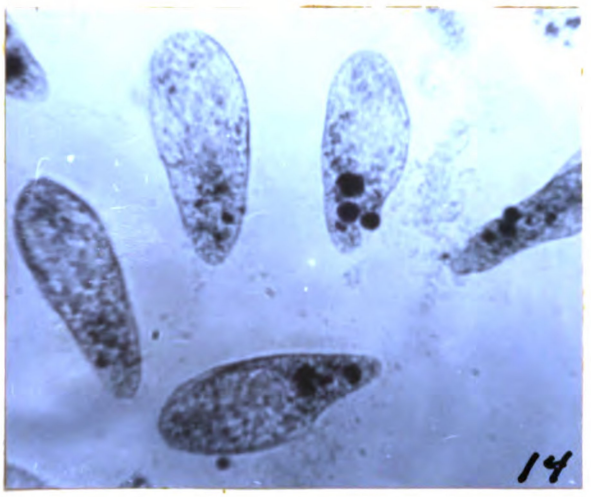
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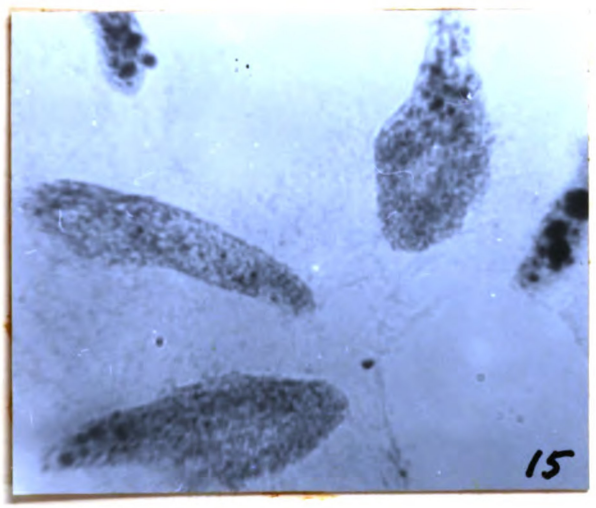
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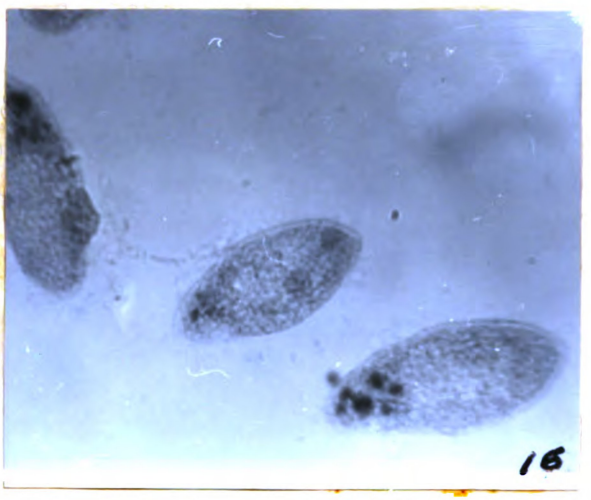
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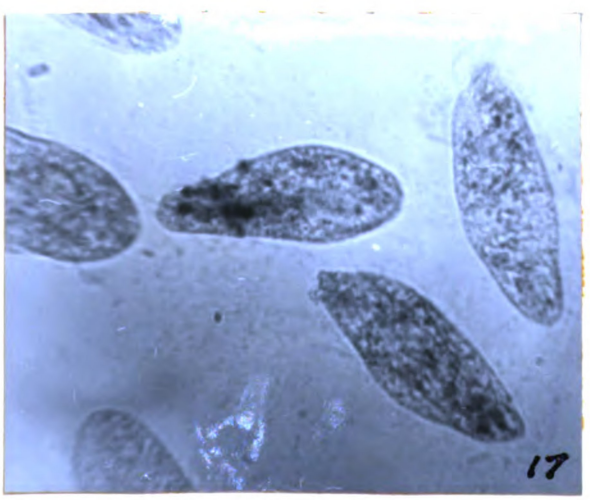
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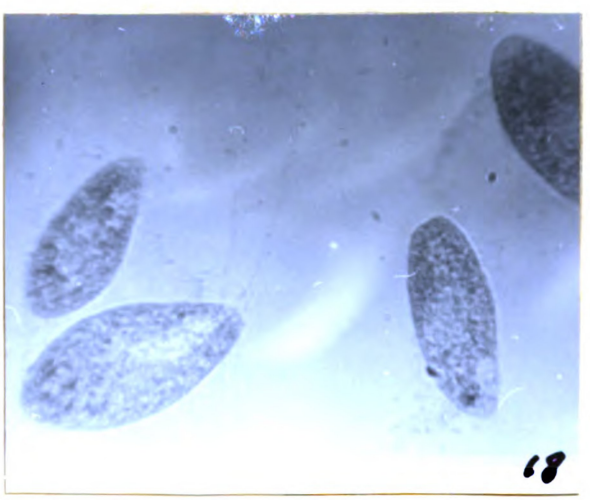
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PLATE IV

Lipid in cells from 10 ml. tryptone and vitamin solutions with pH adjusted. Cultures inoculated with a 1 loop inoculum and grown upright in a constant temperature room.

Fig. 19. 12-day cells. Series IX, pH 6.35. Sudan black B. 530X.

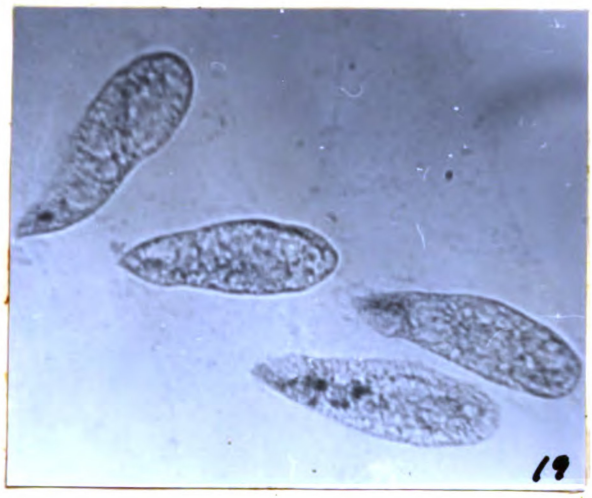
Fig. 20. 24-day cells. Series IX, pH 6.35. Sudan black B. 530X.

Fig. 21. 12-day cells. Series X, pH 6.73. Sudan black B. 530X.

Fig. 22. 24-day cells. Series X, pH 6.73. Sudan black B. 530X.

Fig. 23. 12-day cells. Series XI, pH 7.40. Sudan black B. 530X.

Fig. 24. 24-day cells. Series XI, pH 7.40. Sudan black B. 530X.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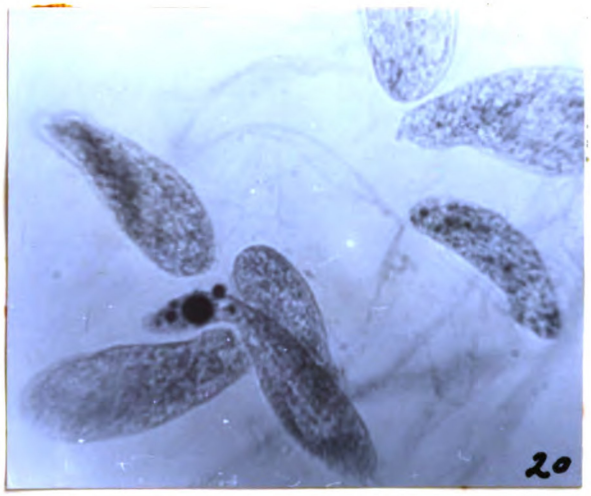
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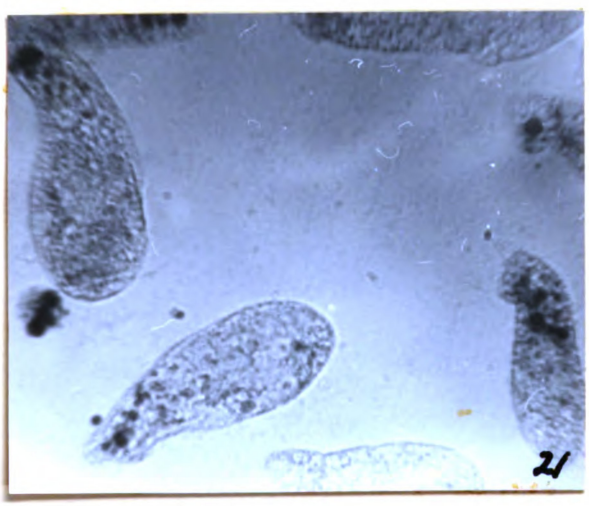
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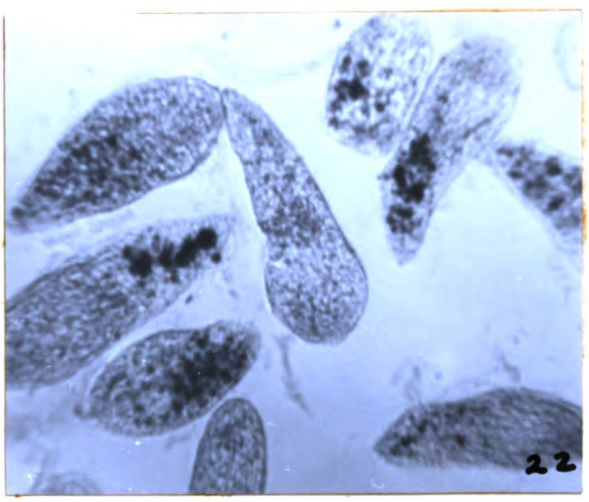
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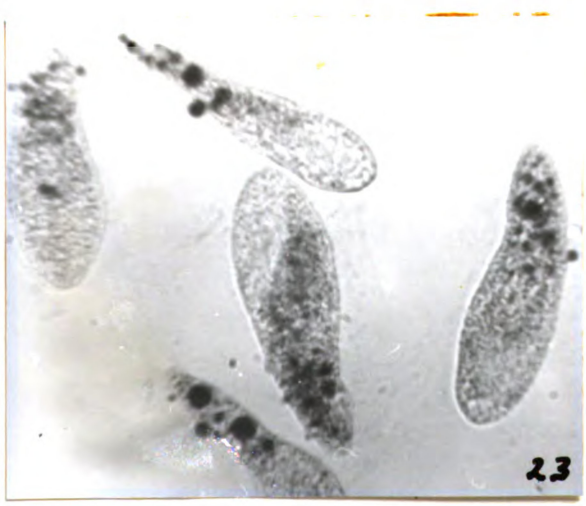
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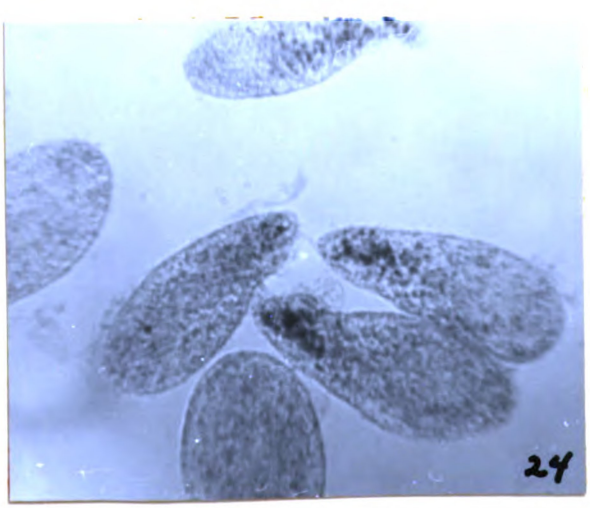
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PLATE V

Cells from 10 ml. cultures inoculated with an 1 loop inoculum and grown upright in a constant temperature room. Series V, tryptone; series VI, tryptone citrate; and series XII, casein with yeast extract.

Fig. 25. 9-day cells. Series V, tryptone. Sudan black B. 530X.

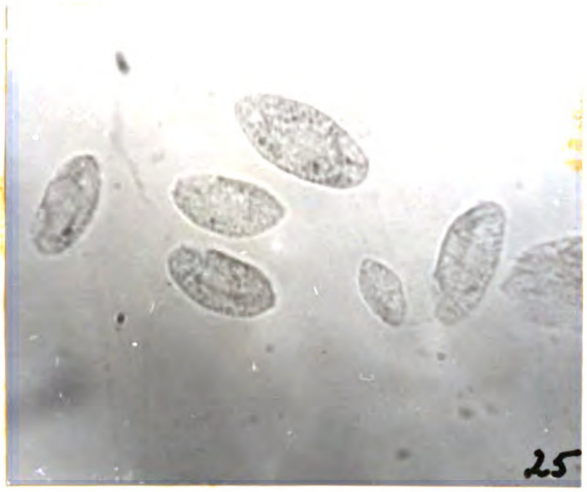
Fig. 26. 9-day cells. Series VI, tryptone citrate. Sudan black B. 530X.

Fig. 27. 9-day cells. Series XII, casein with yeast extract. Sudan black B. 530X.

Fig. 28. 9-day cells. Series XII, casein with yeast extract. Control, pyridine extraction followed by Sudan black B. 530X.

Fig. 29. 15-day cells. Series XII, casein with yeast extract. Sudan black B. 530X.

Fig. 30. 15-day cells. Series XII, casein with yeast extract. Weigert-Smith-Dietrich method for phospholipid. 530X.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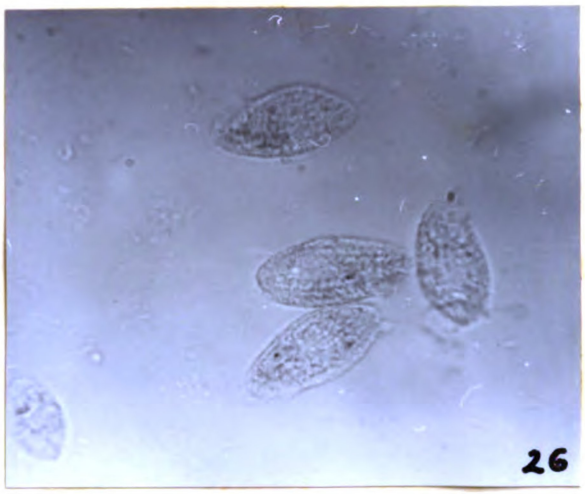
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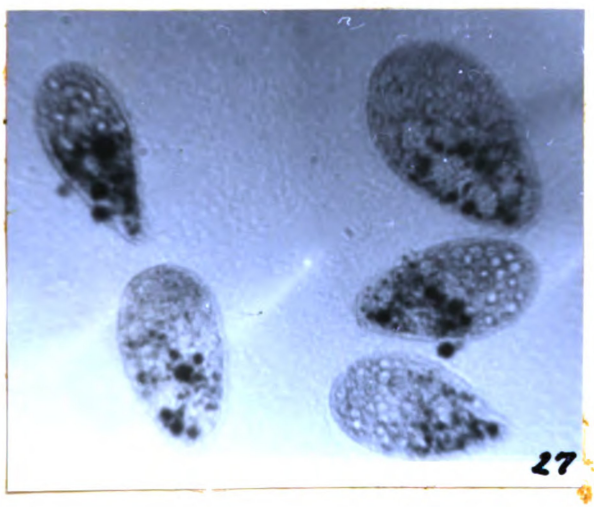
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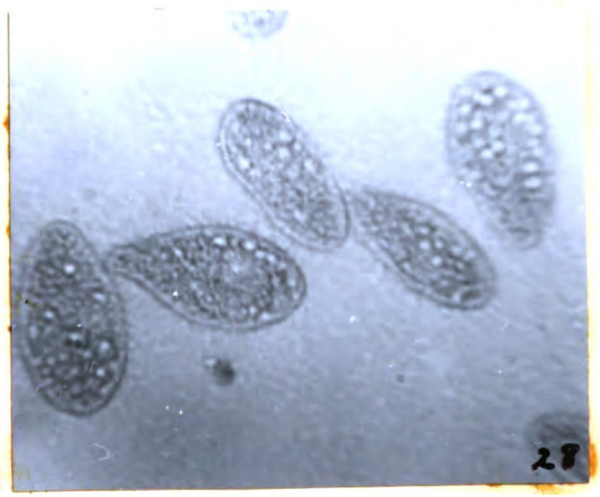
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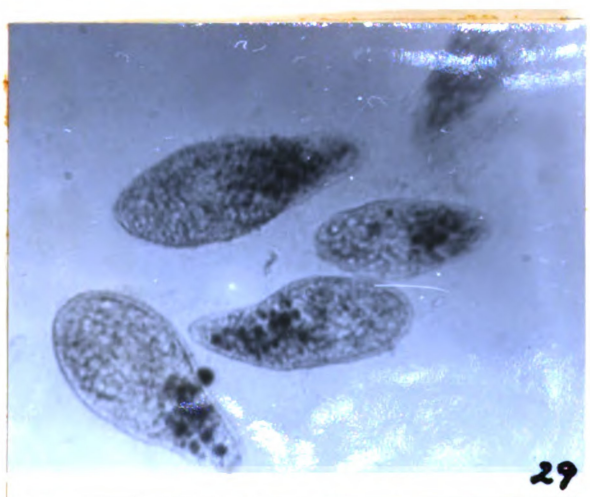
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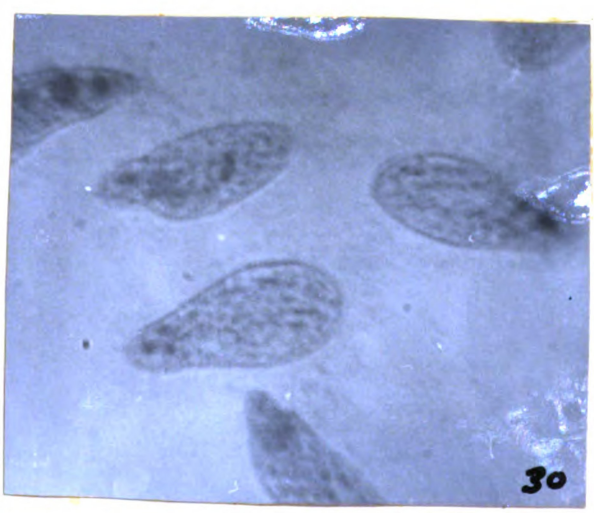
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PLATE VI

Lipid in relation to cell division (see also Fig. 12, Plate II). Cells of series XI from tryptone and vitamin cultures with pH originally set at 7.40. Cells of series XV were from a 75 ml. tryptone and vitamin culture inoculated with a 1 ml. inoculum and "synchronized" by the method of Scherbaum and Zeuthen (1954) when 2½ days old. Fig. 35 is a control slide of series IV, a slanted 10 ml. tryptone and vitamin series.

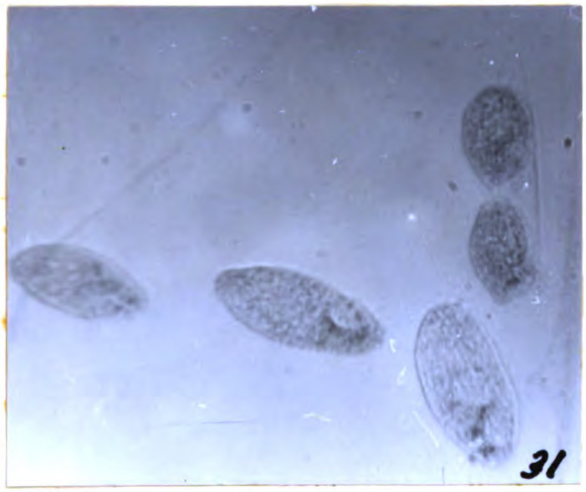
Fig. 31. 3-day cells. Series XI. Sudan black B. 530X.

Fig. 32. 3-day cells. Series XI. Sudan black B. 530X.

Fig. 33. Series XV cells fixed 15 minutes following "synchronization" treatment. Sudan black B. 530X.

Fig. 34. Series XV cells fixed 90 minutes following "synchronization" treatment. Sudan black B. 530X.

Fig. 35. 21-day cells. Series IV. Pyridine extraction followed by Sudan black B. 530X.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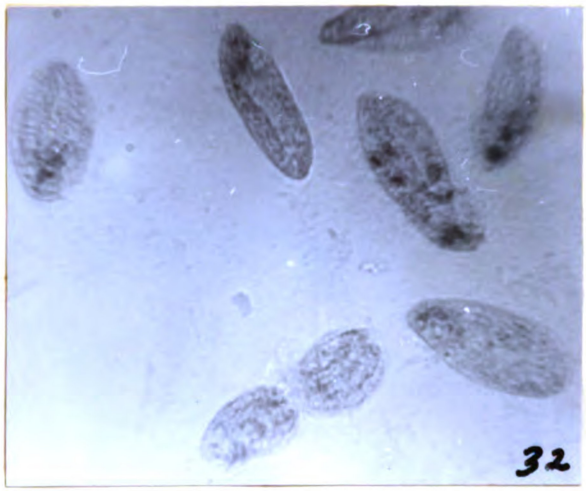
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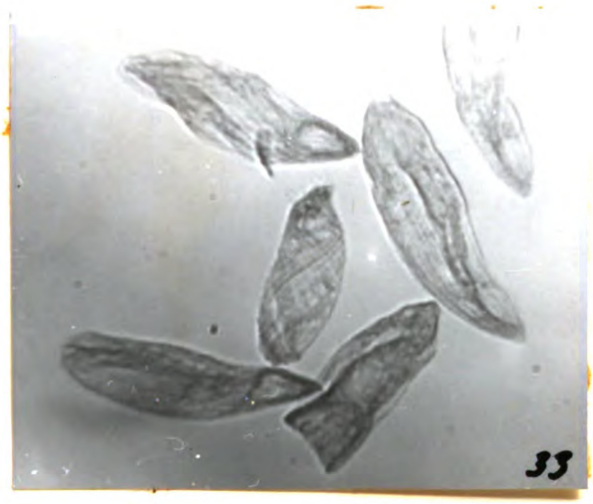
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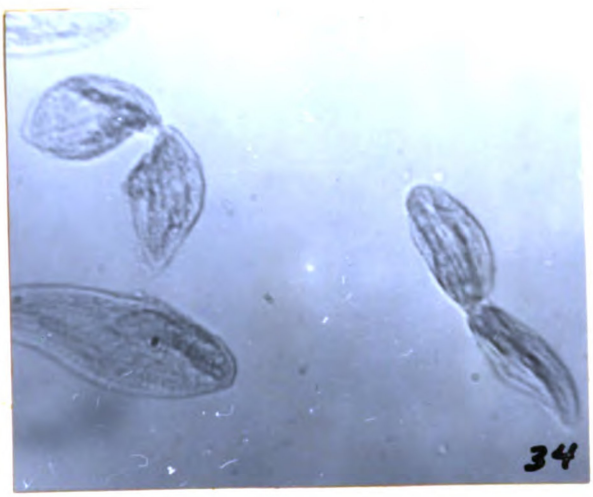
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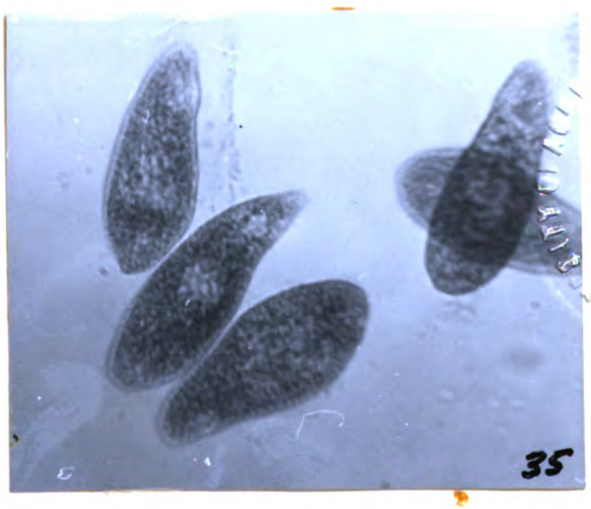
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