AN HISTOCHEMICAL AND MORPHOLOGICAL INVESTIGATION OF THE RELATION BETWEEN THE GROWTH-PROMOTING PROPERTIES OF AGING TETRAHYMENA GELEII (STRAIN W) AND LESION INDUCTION IN THE CHORIOALLANTOIS

By

Frederick Otto Marzke

AN ABSTRACT

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

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ABSTRACT

A semi-quantitative histochemical determination of the distribution of alkaline and acid phosphatase, lipase, peroxidase, DNA, fat, glycogen, and urates in Tetrahymena geleii (strain W) reared in various media was made at 72-hour intervals throughout the life of the cultures. An investigation was then conducted to ascertain any correlation between the presence of these materials and the growth-promoting properties of the various suspensions of T. geleii W subsequent to inoculation onto the chorioallantois of 12-14-day old pedigreed White Leghorn embryos. The histochemical and morpholcgical characteristics of chorioallantoic lesions induced by T. geleii W were compared with those of lesions induced by croton oil, strain 12 lymphomatosis, embryonic grafts and various suspending media.

Alkaline phosphatase and lipase activity in T. geleii W reared in tryptone was at a maximum in 288-hour cultures; acid phosphatase activity, in 144-hour cultures; glycogen deposition, in 432-hour cultures; and fat and phospholipid deposition, in 504-hour cultures. DNA activity was cyclic but was at a maximum in 72, 144 and 360-hour cultures. No positive reaction for peroxidase or urates was obtained. The addition of vitamins to the tryptone media inhibited alkaline phosphatase and decreased lipase activity but tended to increase DNA and acid phosphatase activity and the deposition of glycogen. Diffusion

complicated the histochemical localization of both alkaline and acid phosphatase, and a negative reaction for the former was attributable to a complete diffusion of the enzyme or its catalyzed end-products. Calcium and magnesium increased alkaline and acid phosphatase activity and the deposition of glycogen but decreased the deposition of fat. Magnesium also counteracted the inhibitory effect of vitamins on alkaline phosphatase activity. The role of the investigated substances in the physiology of T. geleii W could not be definitely determined, but alkaline phosphatase may be concerned with DNA metabolism and the transfer of nutrient materials across the cell membrane while acid phosphatase may play a role in carbohydrate synthesis.

All T. geleii W suspensions, inorganic salt solutions and other inocula utilized for the study of lesion induction in the chorioallantois, with the exception of Ringer's solution, possessed growth-promoting properties to some degree. A direct relationship existed between the age of the culture of T. geleii W and the extent of epithelial proliferation and size of induced chorioallantoic lesion. Fat deposition was at a maximum in these aged T. geleii W and in areas of pronounced epithelial growth which suggested that fat synthesis may play an important role in cellular proliferation. T. geleii W cultures induced maximal proliferation in the epithelial tissues of the chorioallantois while inorganic salt solutions produced a more pronounced stimulation of mesodermal tissues. Alkaline and acid

phosphatase and lipase all appeared to aid in the transfer of nutrient materials across the vascular membranes of the induced lesions while alkaline phosphatase also appeared to aid in the metabolism of fibrillar proteins. Suspension of T. geleii W in physiological saline reduced its lesion-inducing properties. No histochemical or morphological characteristic of the induced lesion could be utilized for identification of the induction agent.

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A THESIS

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Outline of Studies:

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INTRODUCTION

Robertson (1923) maintained that protozoa released autocatalytic materials which stimulated growth. Mast and Pace (1946) demonstrated that cultures of Chilomonas paramecium produced a substance which was not a B vitamin but which in low concentrations accelerated growth but in high concentrations retarded it. Other living cells, including those of higher animals, have also been found to elaborate substances which facilitate growth. Duran-Reynals (1929) demonstrated that the addition of testicular extracts enhanced the pathogenicity of a neurovirus. Margoliash, Tennenbaum and Doljanski (1948) noted that the reduction in growth-stimulation by the chicken heart subsequent to dialysis was not accompanied by the appearance of growth substances in the dialyzate itself. Webb and Loofbourow (1947) found that the supernatant from ultra-violet damaged cells exhibited higher concentrations of biotin, folic acid, nicotinic acid, pantothenic acid and riboflavin and possessed greater growth-promoting properties than supernatants from undamaged cells. Loofbourow, Oppenheim-Errera, Loofbourow and Yeats (1947) found that enhanced growth-promoting properties of suspensions of damaged cells could be correlated with an increase in the amount of nucleotides and nucleosides. Linser and Kaindl (1951) believed that growth-inhibiting or promoting materials were adsorbed by molecules

present in the protoplasm and converted into a part of the living substance. These materials must fit available spaces in order to stimulate growth and must not accumulate to excess or growth inhibition may occur.

Fennell (1951) demonstrated that the growth-promoting materials in Tetrahymena geleii (strain W) induced proliferative lesions in the chorioallantois of the chicken embryo and investigated the relationship between age of culture, lesion induction and concentration of possible growth-promoting substances. As very little information is available concerning the effect of protozoan growth-promoting materials on vertebrate tissues, this present investigation is a continuation of that study. It has as its objectives: (1) a semi-quantitative histochemical estimation of the distribution of alkaline and acid phosphatase, lipase, peroxidase, fat, glycogen, urates and DNA at various intervals during the life of cultures of T. geleii W; (2) a clarification of the role of these materials in the physiological processes of aging T. geleii W; and (3) an ascertainment of any correlation between the presence of these substances and the growth-promoting properties of T. geleii W subsequent to inoculation onto the chorioallantois of the chicken embryo.

MATERIALS AND METHODS

All histochemical studies were made on specimens of Tetrahymena geleii (strain W) cultured at 20-30°. Two basic culture media were used: (1) tryptone made with 15 gm. Bacto-Tryptone (Difco Laboratories, Detroit, Michigan) and 1 gm. KH_2PO_4 in 1,000 cc. distilled water; (2) vitamin-enriched tryptone made in an identical manner as the preceding but enriched with the following vitamins: 1 mg. riboflavin, 1 mg. thiamine, 100 micrograms nicotinic acid and 0.5 micrograms biotin. Additional media were prepared in which either 0.448 gm. of $CaCl_2$ or 1.07 gm. $MgCl_2$ was added to each of the basic solutions.

Cultures of organisms were established by inoculating a 125 cc. Ehrlenmeyer flask containing 75 cc. of the sterile culture medium with 1 cc. of a sterile heavily populated stock culture. In most experiments tests were made with organisms 72 hours subsequent to inoculation and at 72-hour intervals thereafter throughout the life of the culture. In all investigations age of culture refers to hours subsequent to inoculation. Histochemical studies were made both on T. geleii W suspended in the original culture medium and on specimens suspended in physiological saline for 30 minutes subsequent to removal from the culture medium.

Alkaline and acid phosphatases were localized by the methods of Gomori (1941) and lipase by the method of Gomori (1946). Peroxidase was ascertained by methods of both McJunkin and Armitage (Glick 1949). The deposition of glycogen was studied by means of the Feulgen-Bauer reagent (Bensley and Bensley 1938). Desoxyribonucleic acid was identified by the method of Bensley and Bensley (ibid.) The Hollandē modification of the Courmont-André method was utilized for testing the presence of urates (Glick op. cit.). The deposition of phospholipids was determined by the Smith-Dietrich reaction (Lison 1936) although the reliability of this test has been questioned (Cain 1950). Aqueous suspensions of cells were stained with Sudan IV to ascertain the amount of fat at various ages.

Estimates of enzymatic activity and deposition of fat and glycogen in T. geleii W were made by measurements of the length of the cells and the approximate area of the positive reaction subsequent to the histochemical tests. With the exception of desoxyribonucleic acid, the following formula was used for establishing a comparative index of activity:

$$\text{Activity factor} = \frac{L_2 P}{L_1 T}$$

in which L_1, L_2, P and T represent the average length of the cells, the approximate area of the positive reaction, the total number of positive cells and the total number of cells counted, respectively. The term glycogen/ or fat/cytoplasm

ratio is used in the discussion on fat and glycogen deposition, but it was ascertained in the same manner as the activity factor for enzymes. However, on all graphs the term activity factor is used synonomously with glycogen and fat deposition. Each factor or ratio was determined after a count had been made of the total number as well as the total positive cells in 10 microscopic fields (600X) and after the average total length and length of positive area of 10-15 cells on each slide had been measured with a micrometer. All fields and cells were selected at random. It should be emphasized that this activity factor is an approximation, but a similar method was devised and used by de Robertis (1949) in a study of thyroid tissues in vertebrates. In the estimation of nuclear desoxyribonucleic acid an activity factor was established on intensity of the staining reaction. A negative reaction was given a value of 0; a light nuclear staining reaction, a value of 1; a medium reaction, a value of 2; and a heavy reaction, a value of 3. Each of these values was then multiplied by the total number of nuclei placed in each category, and the sum of these products for each slide divided by the total number of nuclei counted. Each activity factor for DNA was obtained by the classification and counting of all nuclei in 10 randomly selected microscopic fields (600X).

Pedigreed White Leghorn hatching eggs were used exclusively for the study of lesion induction by T. geleii W.

Organisms used for inoculation of the chorioallantois were washed in two changes of physiological saline. After each washing the cells were centrifuged and the supernatant removed. A suspension was then made of a 1-10 dilution of the concentrated washed organisms in physiological saline and left for 2 hours to several days. The chorioallantois of 12-14-day old embryos was then inoculated with 0.05 cc. portions of the suspension by the artificial air sac method of Burnet (1936). A few eggs were inoculated with organisms suspended in the original culture solution. Also in several experiments the organisms were heated for one-half hour at 50° C. prior to inoculation. Other inocula were prepared in which Ringer's solution or 0.02N HCl or 0.005N CaO in physiological saline was substituted for physiological saline as the washing and suspending medium.

Some embryos were also inoculated with 0.05 or 0.1 cc. portions of a 1:4 saline dilution of strain 12 lymphomatosis mince or 0.05 cc. portions of a 0.05-0.25 per cent croton oil emulsion in 0.02N NaOH in physiological saline.

Approximately 16 square millimeter sections of embryonic heart, head ectoderm or liver washed in normal saline were grafted onto the chorioallantoic membrane through a triangular window cut into the shell. The section of shell was then replaced and sealed with scotch tape. The embryonic tissue was maintained in saline at either room temperature or heated for 15 minutes at 50° C. prior to grafting.

In each series of experiments, 0.05 cc. portions of all solutions used as the suspending medium for T. geleii W and other inocula were used as controls.

Lesions were fixed in acetone for the histochemical localization of enzyme activity. Acid and alkaline phosphatase, lipase and desoxyribonucleic acid activity and the deposition of glycogen were determined by the methods utilized for T. geleii W. Lesions were fixed in acetic-bichromate to ascertain the site of fat accumulation (Bensley and Bensley, op. cit.). All morphological investigations were made with lesions fixed in Zenker-formol and stained with haemotoxylin-eosin or haemotoxylin-eosin azure.

RESULTS

1. HISTOCHEMICAL PROPERTIES OF TETRAHYMENA GELEII W.

All histochemical tests were at least duplicated and were conducted with specimens of T. geleii W obtained from various culture media 24 to 576 hours subsequent to inoculation. In all tests control slides were stained. Results in these experiments represent trends in activity rather than a quantitative estimation of the activity at definite hours, as variations may exist in the activity at identical hours in the duplicated studies, but the trend in activity usually remains fairly constant.

ALKALINE PHOSPHATASE

Localization and variation with age of culture. Alkaline phosphatase activity in young (24-hour) unwashed cells reared in tryptone was characterized by small black spherical entities widely scattered throughout the cell but most heavily concentrated near the nucleus or posterior end of the organism (Fig. 1). At 72 hours these discrete particles disappeared and the positive area was concentrated in the vicinity of the nucleus. A diffuse grayness noted throughout the cytoplasm of many cells was not considered as definitely positive. Subsequent to 144 hours, there was still phosphatase activity in the vicinity of the nucleus, but the most highly concentrated

site occurred in the posterior end of the cell (Figs. 2, 3 and 4). The tendency for the phosphatase to be concentrated in this area remained fairly constant until the age at which enzymatic activity disappeared except for occasional spherical senescent cells in which the phosphatase activity was located in the center of the organisms. The initial addition of vitamins alone to the tryptone solution did not materially alter the localization of alkaline phosphatase (Fig. 9). There was a tendency for any nuclear phosphatase to decline in activity in organisms washed in physiological saline.

A study of Text-figure I indicates that in specimens of T. geleii W cultured in tryptone alkaline phosphatase activity increased slightly between 72 and 288 hours at which time a maximum activity factor of 0.05 was obtained. A rapid decline then occurred and subsequent to 360 hours of age all activity disappeared. The initial addition of vitamins to the tryptone solution tended to induce maximum activity earlier (72 hours) and to delay the complete cessation of any visible reaction. With the exception of 360-hour cultures, enzymatic activity was greater in the organisms reared in tryptone than in those reared in vitamin-enriched media. It is evident from Text-figure II that alkaline phosphatase activity was higher in organisms in which washing in saline was omitted.

Effect of the addition of vitamins to the culture media. Early in the course of this study it became apparent that the cultures of T. geleii W were becoming increasingly short-lived and less productive. It was to counteract this trend

TEXT-FIGURE I

Relation between age and alkaline phosphatase activity in T. geleii W. All specimens were suspended in physiological saline for 30 minutes prior to fixation and staining. Activity factor determined by formula given under materials and methods.

○ - T. geleii W reared in tryptone;

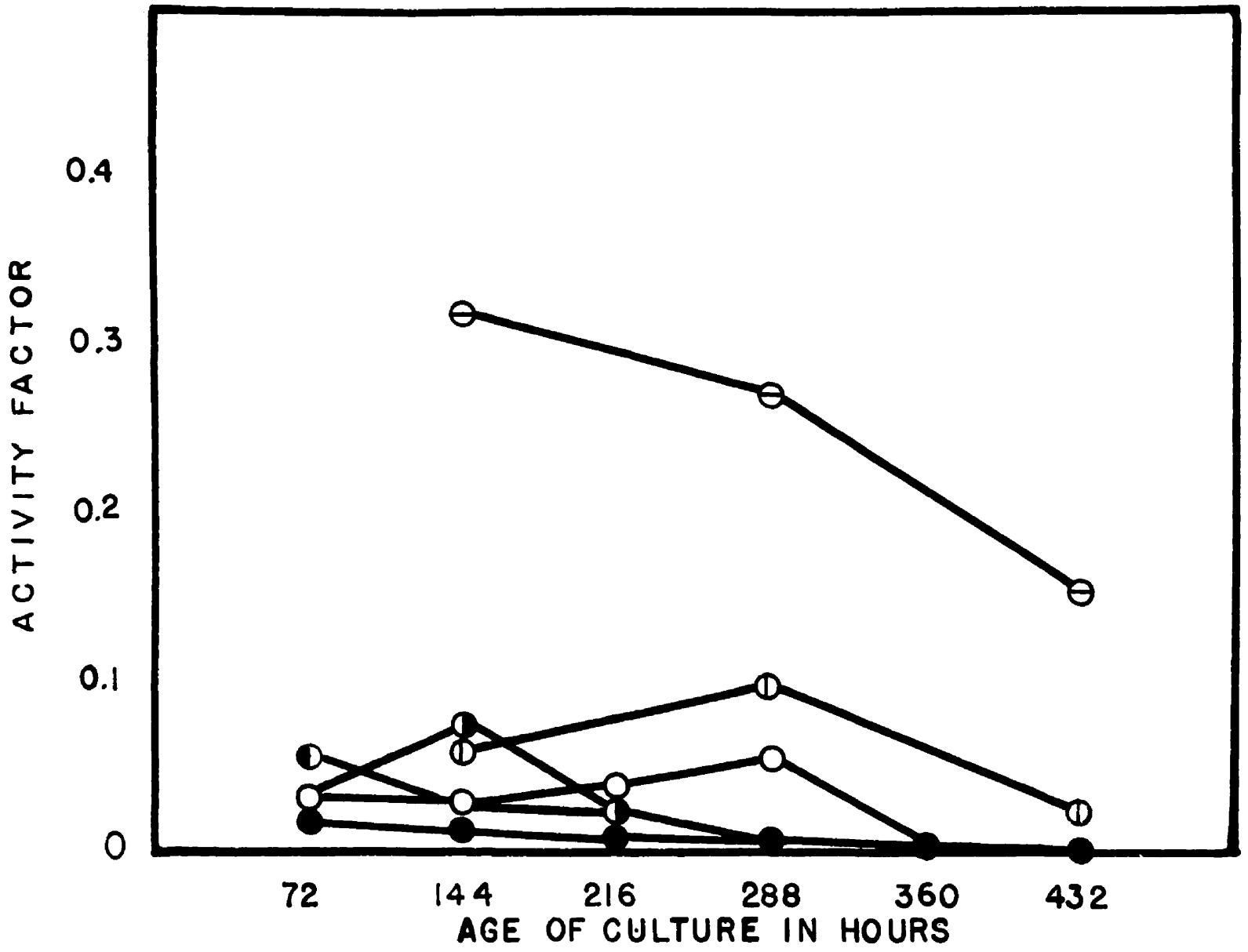
⊖ - T. geleii W reared in calcium chloride-enriched tryptone;

◐ - T. geleii W reared in magnesium chloride-enriched tryptone;

● - T. geleii W reared in vitamin-enriched tryptone;

◐ - T. geleii W reared in vitamin and magnesium chloride-enriched tryptone; and

◑ - T. geleii W reared in vitamin and calcium chloride-enriched tryptone.


ACTIVITY FACTOR
0.4
0.3
0.2
0.1
0
72
144
216
288
360
432
AGE OF CULTURE IN HOURS

TEXT-FIGURE II

Relation between age, suspension in physiological saline and the histochemical properties of T. geleii W reared in vitamin-enriched tryptone. Activity factor determined by formula given under materials and methods.

○ - Alkaline phosphatase activity in T. geleii W suspended in physiological saline;

● - alkaline phosphatase activity in T. geleii W suspended in original culture medium;

◐ - acid phosphatase activity in T. geleii W suspended in physiological saline;

⊖ - acid phosphatase activity in T. geleii W suspended in original culture medium;

◐ - glycogen deposition in T. geleii W suspended in physiological saline; and

◑ - glycogen deposition in T. geleii W suspended in original culture medium.

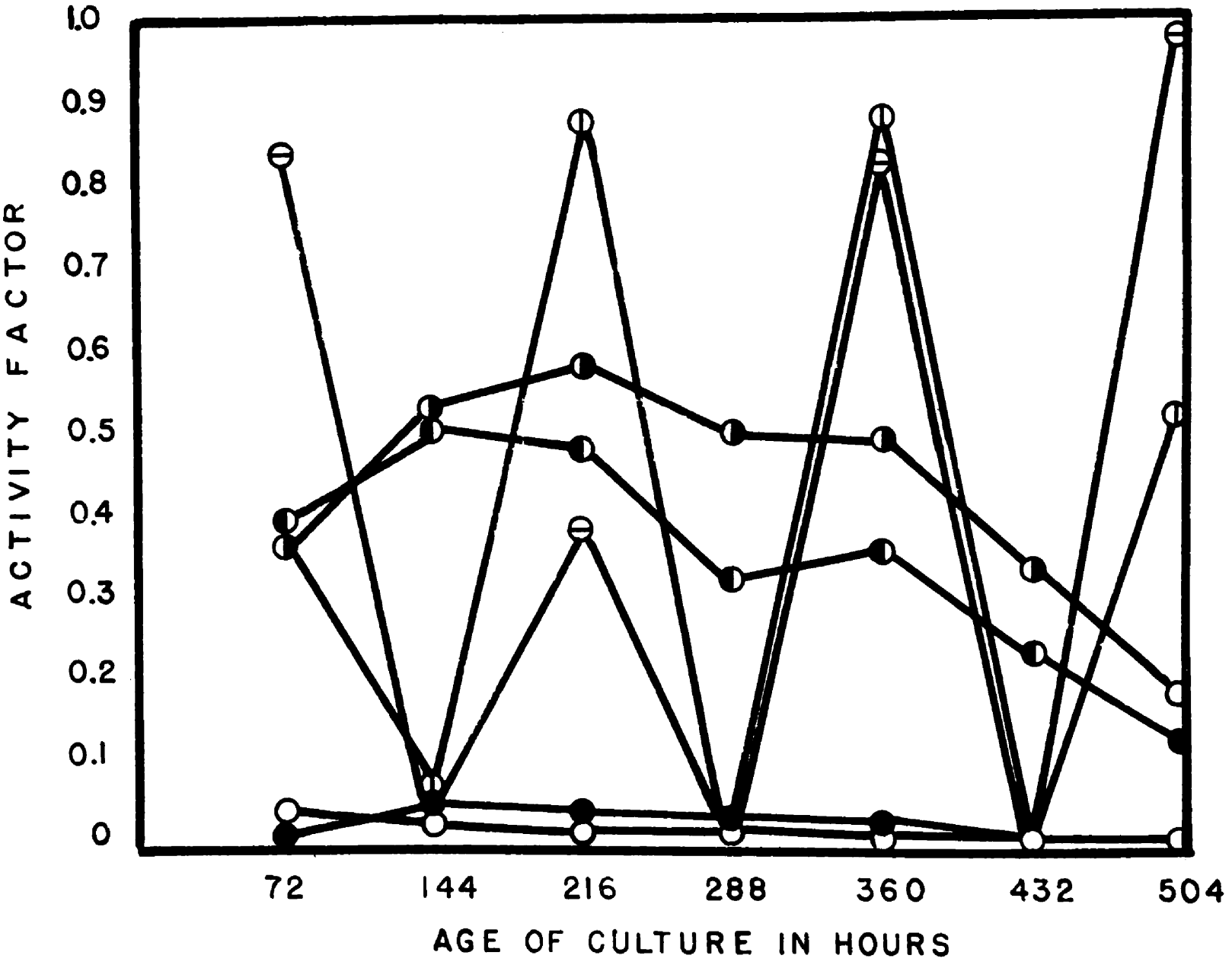

ACTIVITY FACTOR
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
72
144
216
288
360
432
504
AGE OF CULTURE IN HOURS

TABLE I

Alkaline Phosphatase Activity in Specimens of *T. geleii* W Reared in Tryptone and Vitamin-enriched Tryptone

Transfer No.	Days Subsequent to Inoculation into Initial Vitamin-enriched Culture Medium	Culture Medium	Age of Culture in Days[1]	Enzyme Reaction
1	0	Vitamin enriched	3-15 18-21	++[2] -
2	18	"	3-24	-
8	145	"	3-15	-
9	165	"	3-30	-
10	188	"	3-24	-
11	209	Tryptone	3	-
12	212	"	3	++
13	223	"	3 6-21	+ ++
17	264	Vitamin enriched	3	++
19	272	"	6	++
20	278	"	6	+
21	286	"	6-27	+
22	292	"	6-15	+
23	299	"	7	+
24	306	"	7-14	++
25	313	"	7-14	++

TABLE I (Cont.)

Transfer No.	Days Subsequent to Inoculation into Initial Vitamin-enriched Culture Medium	Culture Medium	Age of Culture in Days	Enzyme Reaction
26	320	Vitamin	7	-
27	327	"	7-14	-
28	334	"	7	+
29	341	"	14	-
30	355	"	14	-
31	367	"	7	-
32	369	"	7	+

[1]With the exception of those in which only one day is listed, tests were conducted in Transfers 1-22 at 3-day intervals and in Transfers 23-32 at 7-day intervals.

[2]+=Slightly positive; ++=Moderate to heavy positive reaction.

that vitamins were first added to the tryptone cultures in the amount stated under materials and methods. In the first series of histochemical studies subsequent to the addition of vitamins a slight but not significant decline in the level of alkaline phosphatase activity was apparent (Figs. 12 and 13). However, as noted in Table I, upon inoculation of subsequent series of vitamin cultures and conduction of additional series, all evidence of alkaline phosphatase activity disappeared (Figs. 14 and 15). This inhibition was at first believed to be attributable to factors other than the presence of the added vitamins, but investigations utilizing triple distilled water, fresh chemical solutions, etc., remained negative for alkaline phosphatase. After 10 transfers and approximately seven months' exposure to vitamins, the organisms were transferred to tryptone medium. Three days after this first transfer phosphatase activity was still inhibited, but after subsequent inoculations, activity again became visible.

After six transfers and approximately two months in the tryptone solutions the organisms were again transferred to media containing vitamins. Alkaline phosphatase activity was still visible after eight transfers and a two months' exposure in the vitamin-enriched media. On the ninth transfer inhibition of phosphatase activity again occurred. With the exception of a weakly positive reaction on the 11th transfer, complete alkaline phosphatase inhibition was maintained until

the 15th transfer in the vitamin-enriched media. Subsequent to this transfer the organisms have shown erratic results and have become alternately positive and negative. Although the presence of vitamins appeared to have an inhibitory effect on phosphatase activity, which could be counteracted by the addition of magnesium, future research is required to clarify existing results.

Effect of the addition of the magnesium or calcium ion to the culture medium. The magnesium ion has been widely described as an activator of alkaline phosphatase (Kabat and Furth 1941). The results obtained in this present investigation demonstrated that the addition of magnesium or calcium chloride to either of the basic culture media increased alkaline phosphatase activity, but that the calcium ion was the more effective (Text-fig. I and Figs. 5 and 7).

A definite shift in the site of alkaline phosphatase activity from the posterior towards the nuclear region of the cell occurred in organisms reared in vitamin-enriched tryptone to which magnesium chloride had been added (Fig. 10).

Calcium activation of phosphatase activity was more pronounced in organisms reared in tryptone and reached a maximum at 144 hours at which time the phosphatase activity factor increased from 0.03 to 0.32. Control slides from cultures containing magnesium or calcium chloride were negative (Fig. 6).

To determine the effect of the concentration of magnesium in the glycerophosphate buffer on alkaline phosphatase activity,

0.0137 gm. of magnesium chloride was added to each 650 cc. of the substrate. Increasing the concentration of the magnesium did not alter the site of phosphatase activity in organisms reared in tryptone (Fig. 8), but occasionally enhanced the reaction in cells from 72 to 144 hours of age. Eliminating magnesium from the buffer also did not alter the site of phosphatase activity but decreased the intensity of the reaction in 432-hour cultures.

In addition to its effect on alkaline phosphatase activity, magnesium chloride extended the life of the culture for 10-15 days.

Specificity of alkaline phosphatase activity. The shift in alkaline phosphatase activity from the posterior region towards the center of the organism (Fig. 10) subsequent to the addition of magnesium chloride to the vitamin-enriched media gave rise to the possibility that several substrate specific enzymes hydrolyzing phosphate esters might be present in T. geleii W. Enzyme inhibition tests were conducted in which 1 cc. of one of the following inhibitors was applied to a series of T. geleii W slides for 20 minutes prior to immersion in the phosphatase buffer: 0.02M semicarbazide, 0.025M oxidized or reduced glutathione and 0.01M sodium arsenate. Organisms were obtained from the various culture media 144 hours after seeding and from additional cultures every 144 hours thereafter.

All inhibitors completely inhibited enzyme activity in organisms reared in tryptone cultures (Fig. 17). Occasionally a diffuse grayness especially pronounced in the nucleus was visible. The addition of magnesium chloride to the tryptone culture medium enhanced this diffuse grayness but did not counteract the inhibition (Fig. 18). A slight positive reaction was visible in the distal end of a few cells from 432-hour vitamin-enriched cultures subsequent to exposure to sodium arsenate, but cells of all other ages were negative (Fig. 18). T. geleii W from vitamin-enriched cultures containing magnesium chloride exhibited a variation in alkaline phosphatase activity in accordance with the type of inhibitor used (Figs. 19-22). Semicarbazide and glutathione inhibited all activity, but a moderate to heavy reaction in the area between the nucleus and the posterior end of the cell was visible in 10-15 cells subsequent to exposure to sodium arsenate. From the preceding it was apparent that no definite conclusions could be drawn as to the existence of two separate enzymes on the basis of the inhibitor studies alone.

Diffusion of alkaline phosphatase. During the course of this study it also became evident that factors other than age, washing and concentration of the magnesium or calcium ion were operative in the localization of alkaline phosphatase. Pronounced activity was frequently visible in areas of highly concentrated cells but entirely absent in the more widely dispersed cells on the same slide. In addition, many slides

containing organisms which had not been washed in saline exhibited a heavy black extracellular precipitate subsequent to the histochemical localization of alkaline phosphatase. The aforementioned suggested that a diffusion of the enzyme or enzymatic end-products into the surrounding medium had occurred. Supernatant fluid from centrifuged cultures exhibited this black precipitate, but culture media not exposed to organisms were negative. In all tests cells were distributed as evenly as possible, but some congestion was always inevitable. On slides containing highly active organisms this difference in activity between concentrated and widely dispersed cells was not apparent. In general, however, the greater the concentration of the cells, the greater the phosphatase activity. This apparent difference may play a role in the high concentration of activity noted in 216-288-hour organisms, as it is at that age that the cultures are usually the most highly concentrated with organisms and the tendency towards diffusion may decrease.

ACID PHOSPHATASE

Localization and variation with age of culture. A heavy extracellular precipitate interfered with an accurate localization of the site of acid phosphatase activity in unwashed organisms. However, in most 72-hour cells reared in tryptone large discrete positive particles were visible thoughout the organism but heaviest along the sides and posterior end. A

few cells were visible in which the positive area completely filled the organism. In all cells the nucleus was usually the last to show a positive reaction. No restricted site for acid phosphatase activity was apparent at any age.

In washed cells from tryptone cultures acid phosphatase activity was most highly concentrated in the posterior end of the organism (Fig. 23). This remained the site of greatest activity until 288 hours at which time it shifted towards the nucleus (Fig. 25).

Highest activity in 72-hour unwashed organisms from vitamin-enriched cultures occurred in the vicinity of the nucleus. This positive area increased as the organisms aged and eventually filled the entire cell. After the cells had been suspended in physiological saline, acid phosphatase appeared to be most active in the posterior end of 72-hour cells (Fig. 24); but with an increase in the age of the culture, acid phosphatase activity shifted towards the nucleus (Fig. 26).

It is evident from Text-figures III and IV that the activity factor for acid phosphatase in washed organisms cultured in tryptone was at a maximum 144 hours subsequent to inoculation. This activity factor of 0.29 decreased to 0.06 during the next 72 hours but then gradually increased again to 0.18 at 432 hours. At 504 hours the activity factor declined to 0.05.

TEXT-FIGURE III

Relation between age and the histochemical properties of T. geleii W reared in tryptone. All specimens suspended in physiological saline for 30 minutes prior to fixation and staining. Activity factor determined by formula given under materials and methods.

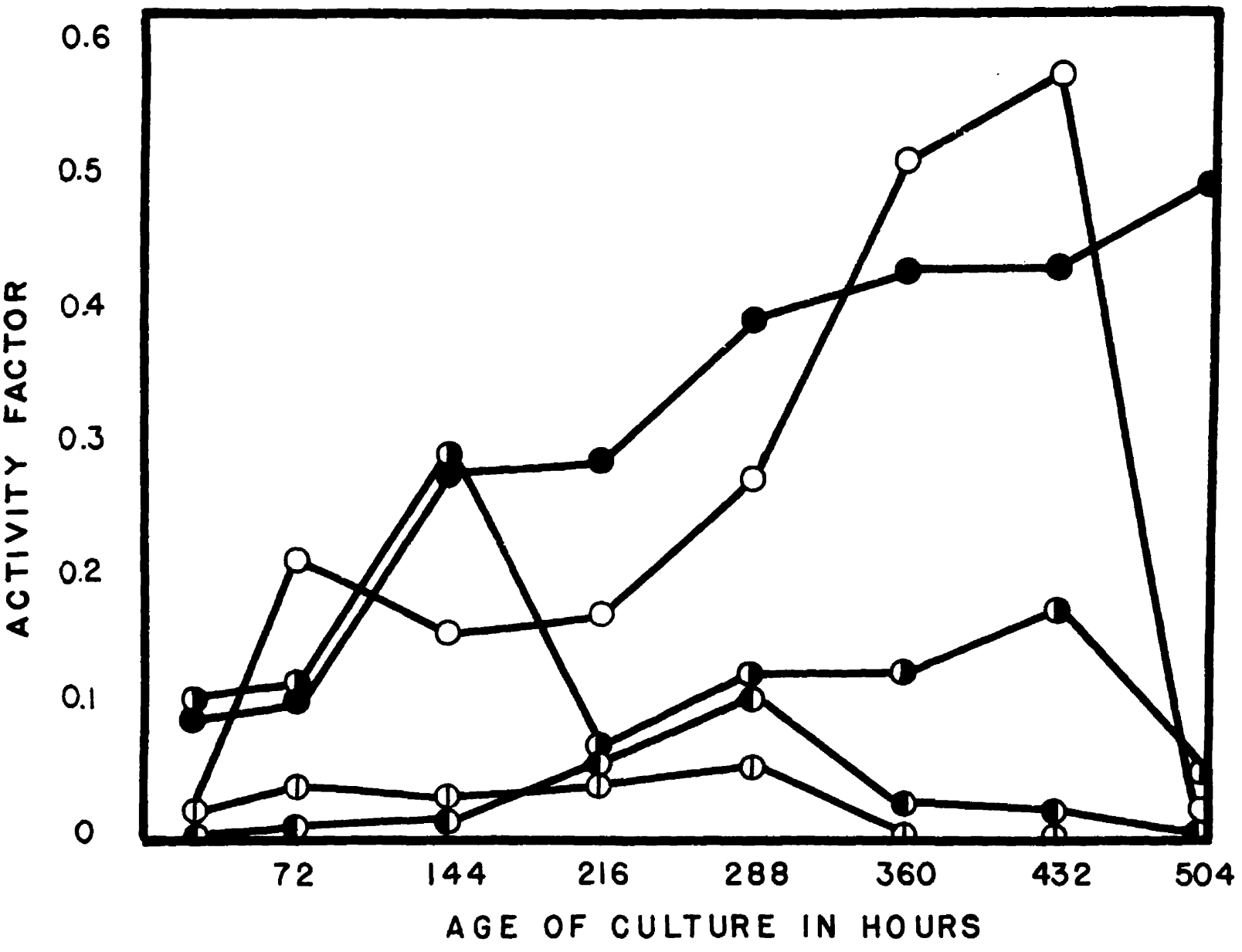
◐ - Alkaline phosphatase activity;

◑ - acid phosphatase activity;

◐ - lipase activity;

○ - glycogen deposition; and

● - fat deposition


0.6
0.5
0.4
0.3
0.2
0.1
0
ACTIVITY FACTOR
72
144
216
288
360
432
504
AGE OF CULTURE IN HOURS

TEXT-FIGURE IV

Relation between age and acid phosphatase activity in T. geleii W. All specimens were suspended in physiological saline for 30 minutes prior to fixation and staining. Activity factor determined by formula given under materials and methods.

● - T. geleii W reared in tryptone;

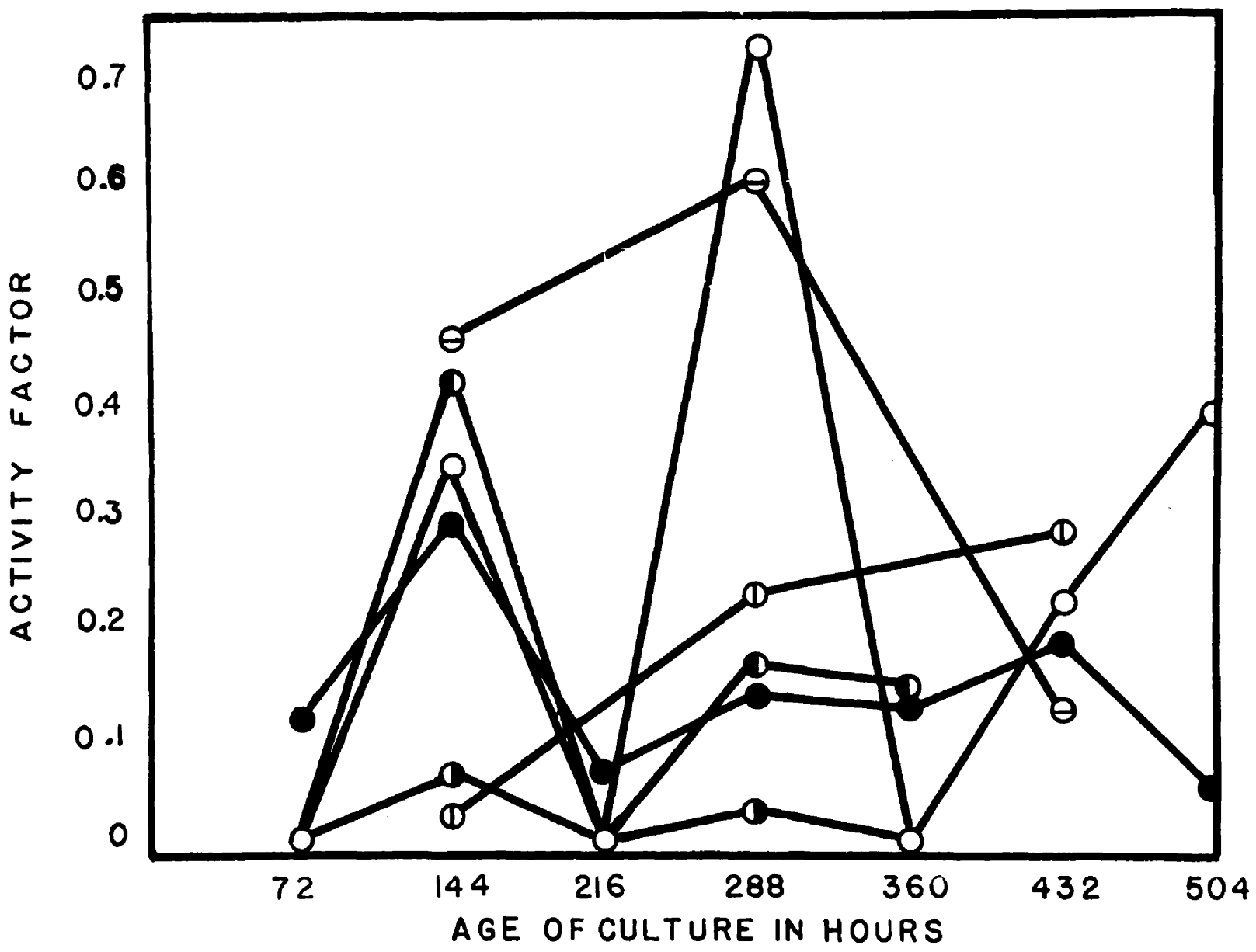
⊖ - T. geleii W reared in calcium chloride-enriched tryptone;

◐ - T. geleii W reared in magnesium chloride-enriched tryptone;

○ - T. geleii W reared in vitamin-enriched tryptone;

◐ - T. geleii W reared in vitamin and magnesium chloride-enriched tryptone; and

◑ - T. geleii W reared in vitamin and calcium chloride-enriched tryptone.


ACTIVITY FACTOR
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
72
144
216
288
360
432
504
AGE OF CULTURE IN HOURS

In vitamin-enriched tryptone an alternate rise and fall in activity appeared throughout the experiment (Text-fig. IV). No positive reaction was visible in cells from 72, 216 or 360-hour cultures, but at 144, 288, 432 and 504 hours acid phosphatase activity was greater than in cells not exposed to vitamins. The maximum difference in phosphatase activity between organisms cultured in tryptone and those cultured in vitamin-enriched tryptone occurred at 288 hours at which time the activity factor for acid phosphatase in cells from tryptone was 0.13 and from vitamin-enriched tryptone 0.72 (Text-fig. IV).

Washing organisms in physiological saline appeared to influence the activity factor for acid phosphatase to a greater extent than the factor for any other substance studied. The maximum effect occurred at 504 hours at which time the activity factor for unwashed cells reared in tryptone was 1.0 and for cells suspended in physiological saline 0.04 (Text-fig. V). In organisms from vitamin-enriched media, the effect of suspension in physiological saline was less pronounced (Text-figs. II and V).

Effect of the addition of the magnesium or calcium ion to the culture medium. The addition of calcium chloride to tryptone media increased acid phosphatase activity in all cells suspended in physiological saline with the exception of those aged 432 hours (Text-fig. IV). The maximum increase was obtained at 288 hours at which time the activity factor

TEXT-FIGURE V

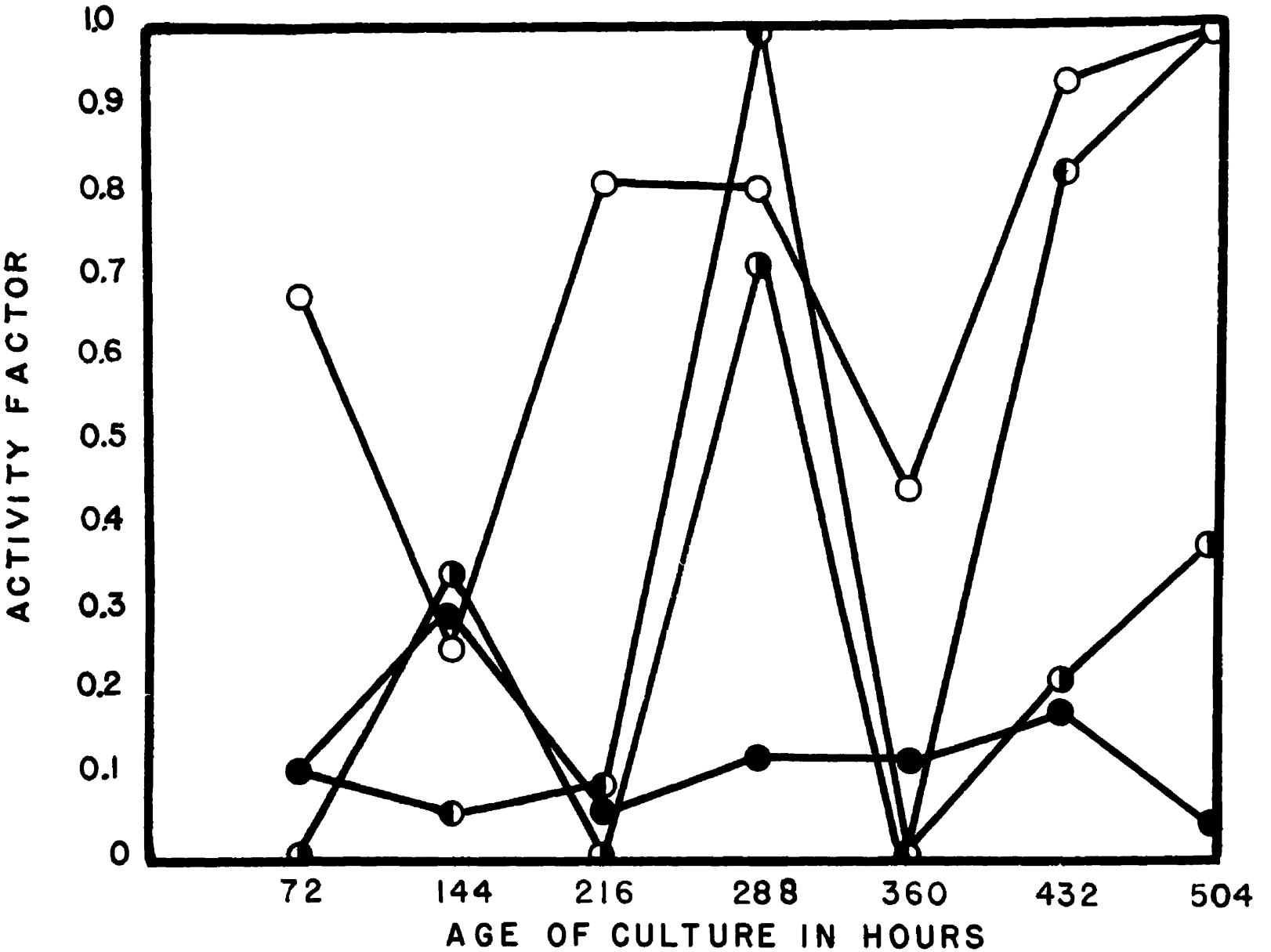
Relation between age, suspension in physiological saline and acid phosphatase activity in T. geleii W. Activity factor determined by formula given under materials and methods.

○ - T. geleii W reared in tryptone and fixed and stained immediately after removal from culture medium;

● - T. geleii W reared in tryptone and suspended in physiological saline for 30 minutes prior to fixation;

◐ - T. geleii W reared in vitamin-enriched tryptone and stained immediately after removal from culture medium; and

◑ - T. geleii W reared in vitamin-enriched tryptone and suspended in physiological saline for 30 minutes prior to fixation.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0.2
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0
ACTIVITY FACTOR
72
144
216
288
360
432
504
AGE OF CULTURE IN HOURS

increased from 0.13 to 0.60. The addition of calcium chloride to media containing vitamins decreased acid phosphatase activity (Text-fig. IV). The activation by magnesium chloride of acid phosphatase activity in organisms reared in tryptone reached a maximum at 432 hours at which time the activity factor increased from 0.18 to 0.28. In vitamin-enriched cultures the magnesium ion stimulated activity at 144 and 360 hours but decreased it at 288 hours. Nuclear acid phosphatase exhibited the maximum stimulation by the magnesium ion.

Diffusion. Acid phosphatase or its catalyzed end products diffused readily from T. geleii W and resulted in the formation of a heavy extra-cellular precipitate during the histochemical localization of this enzyme. There was no evidence for a complete diffusion of acid phosphatase from any organism. Occasionally acid phosphatase activity was entirely absent, but undetermined factors other than diffusion appeared to be responsible.

LIPASE

Localization and variation with age of culture. Lipase activity in T. geleii W less than 216 hours of age and reared in tryptone was restricted almost entirely to the nucleus. However, a few cells exhibited slight activity in the distal end of the organism. In 288-hour cultures the positive sites of lipase activity became more widely dispersed but remained the most heavily concentrated in the vicinity of the nucleus (Fig. 27). Subsequent to 288 hours, lipase activity was again

restricted almost entirely to the nucleus. Neither suspending the cells in physiological saline nor adding vitamins to the culture medium shifted the site of lipase activity.

The first positive reaction for lipase was obtained in cells aged 144 hours (Text-fig. VI). The maximum activity factor for washed organisms reared in tryptone was 0.11 and for cells suspended in the original culture medium 0.04 (Text-figs. VI and VII). The former was obtained 288 hours and the latter 360 hours subsequent to seeding. The addition of vitamins to the tryptone medium decreased lipase activity and the activity factor never exceeded 0.03 (Text-fig. VI). Suspension in physiological saline appeared to have little effect on the amount of visible lipase activity.

Effect of the addition of the magnesium or calcium ion to the culture medium. The addition of either calcium or magnesium chloride to vitamin-enriched media shifted lipase activity from the nucleus towards the distal end of the cell. Calcium had little effect on the amount of activity in the vitamin-enriched cells, but magnesium increased the activity factor at 288 hours from 0 to 0.15 (Text-fig. VI).

Diffusion. All slides containing T. geleii W exposed to calcium chloride exhibited a heavy brown extracellular precipitate when stained for lipase activity. This may be attributed to either a diffusion of the enzyme or the presence of the calcium ion. In these present experiments it is believed that diffusion played only a minor role in the localization of lipase activity.

TEXT-FIGURE VI

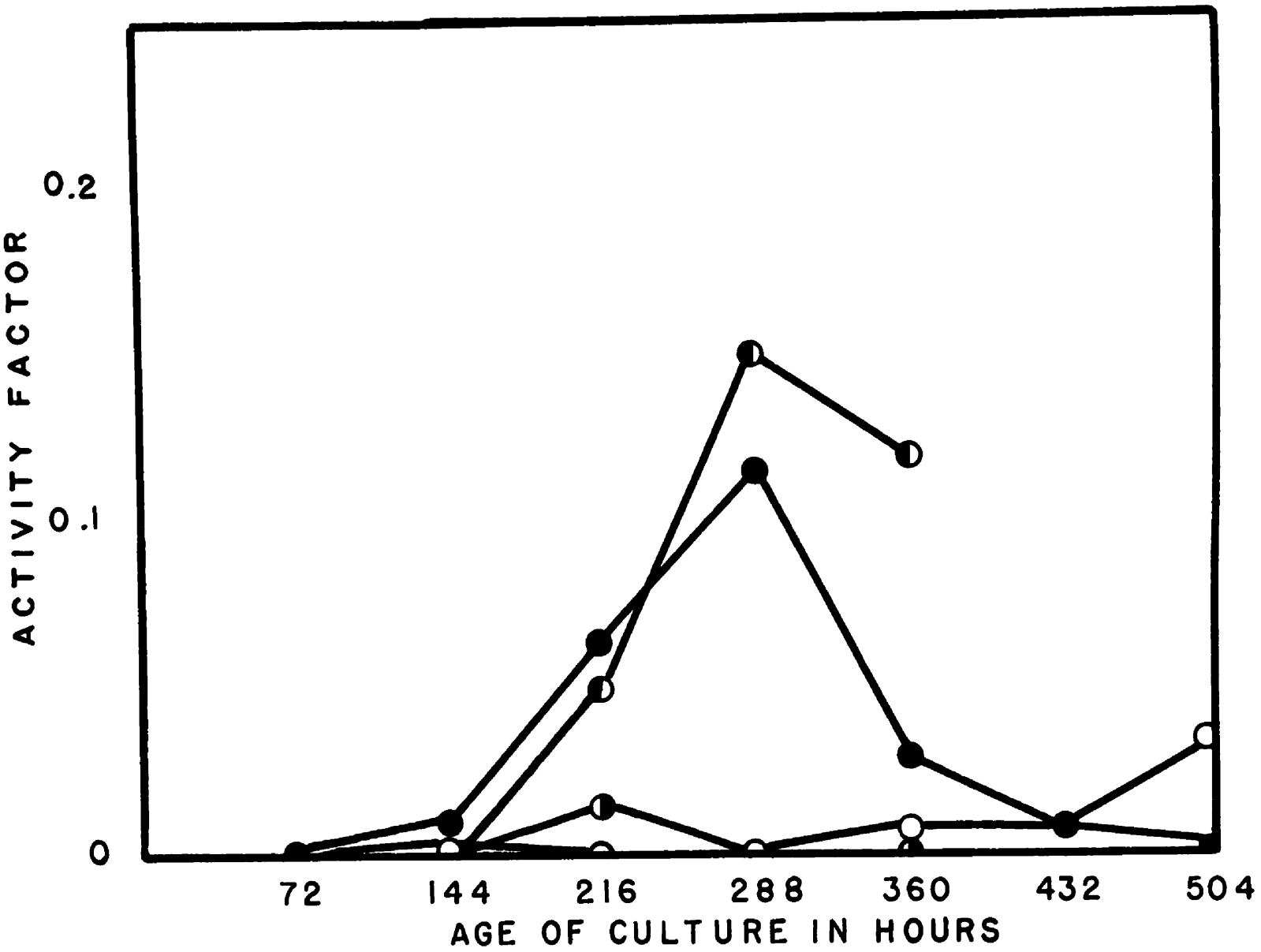
Relation between age and lipase activity in T. geleii W. All specimens were suspended in physiological saline for 30 minutes prior to fixation and staining. Activity factor determined by formula given under materials and methods.

● - T. geleii W reared in tryptone;

○ - T. geleii W reared in vitamin-enriched tryptone;

◐ - T. geleii W reared in magnesium chloride and vitamin-enriched tryptone; and

◑ - T. geleii W reared in calcium chloride and vitamin-enriched tryptone.


ACTIVITY FACTOR
0.2
0.1
0
72
144
216
288
360
432
504
AGE OF CULTURE IN HOURS

TEXT-FIGURE VII

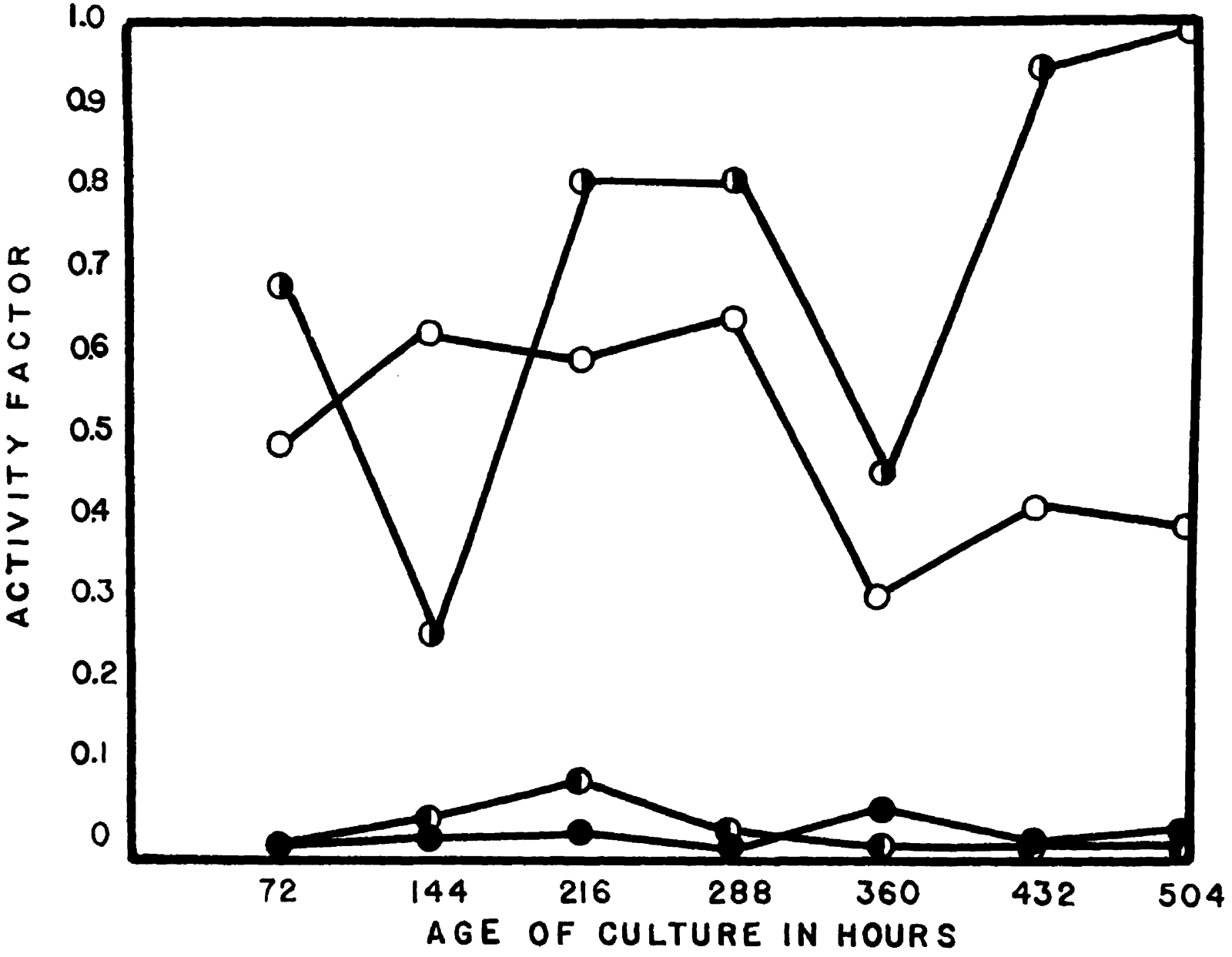
Relation between age of culture and the histochemical properties of T. geleii W reared in tryptone. All specimens stained immediately after removal from culture medium. Activity factor determined by formula given under materials and methods.

◐ - Alkaline phosphatase activity;

◑ - acid phosphatase activity;

● - lipase activity; and

○ - glycogen deposition.


1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
ACTIVITY FACTOR
72
144
216
288
360
432
504
AGE OF CULTURE IN HOURS

GLYCOGEN

Localization and variation with age of culture. In unwashed cells from tryptone cultures the highest glycogen concentration was visible between the nucleus and the pellicle of the organism (Figs. 30-33). The nucleus and proximal tip remained negative in all cells but those in which the entire organism exhibited a positive Feulgen-Bauer reaction. In 72-hour organisms suspended in physiological saline glycogen was located in scattered discrete sites which were most highly concentrated in the distal end of the cell. These sites enlarged and coalesced as the amount of glycogen increased, but deposition remained at a maximum in the posterior portions of the cell. The addition of vitamins to the culture medium did not alter the localization of glycogen.

Glycogen deposition in unwashed cells from tryptone cultures was at a maximum between 144 and 288 hours during which time the glycogen/cytoplasm ratio averaged approximately 0.61 (Text-fig. VII). The addition of vitamins decreased the glycogen/cytoplasm ratio (Text-fig. II). In organisms from tryptone cultures and suspended in physiological saline a maximum ratio of 0.57 was obtained at 432 hours (Text-figs. III and VIII). In these cells glycogen deposition was gradual between 72 and 288 hours but increased rapidly as it approached a maximum. Maximum glycogen deposition occurred earlier in organisms from vitamin-enriched cultures, and a glycogen/cytoplasm ratio of 0.57 and 0.53 was obtained at 216 and 360 hours, respectively (Text-fig. VIII).

In general, suspending cells in physiological saline decreased the glycogen/cytoplasm ratio. The maximum decline noted in cells from tryptone cultures occurred at 144 hours at which time the ratio decreased from 0.63 to 0.15 (Text-figs. III and VII). The maximum decline in cells from vitamin-enriched media occurred at 288 hours at which time the ratio decreased from 0.50 to 0.32 (Text-fig. II).

Effect of the addition of the magnesium or calcium ion to the culture medium. The addition of calcium chloride to vitamin-enriched tryptone increased the amount of visible glycogen in all cells studied (Text-fig. VIII). The addition of magnesium chloride increased glycogen deposition in all cells but those aged 216 and 360 hours. The glycogen/cytoplasm ratio in organisms from the magnesium-enriched media reached a maximum of 0.86 at 144 hours and in the calcium-enriched media it reached a maximum of 0.84 at 216 hours (Text-fig. VIII). The magnesium ion appeared to be more effective in increasing glycogen deposition in cells 144 hours of age or less and the calcium ion in increasing deposition in older cells.

Diffusion. Many slides containing cells which had been washed in saline exhibited an extracellular positive reaction for glycogen. Apparently handling of organisms during the washing process was an important factor in cytolysis and the escape of glycogen from the cell. No extracellular positive reaction was noted on slides of unwashed T. geleii W, and,

TEXT-FIGURE VIII

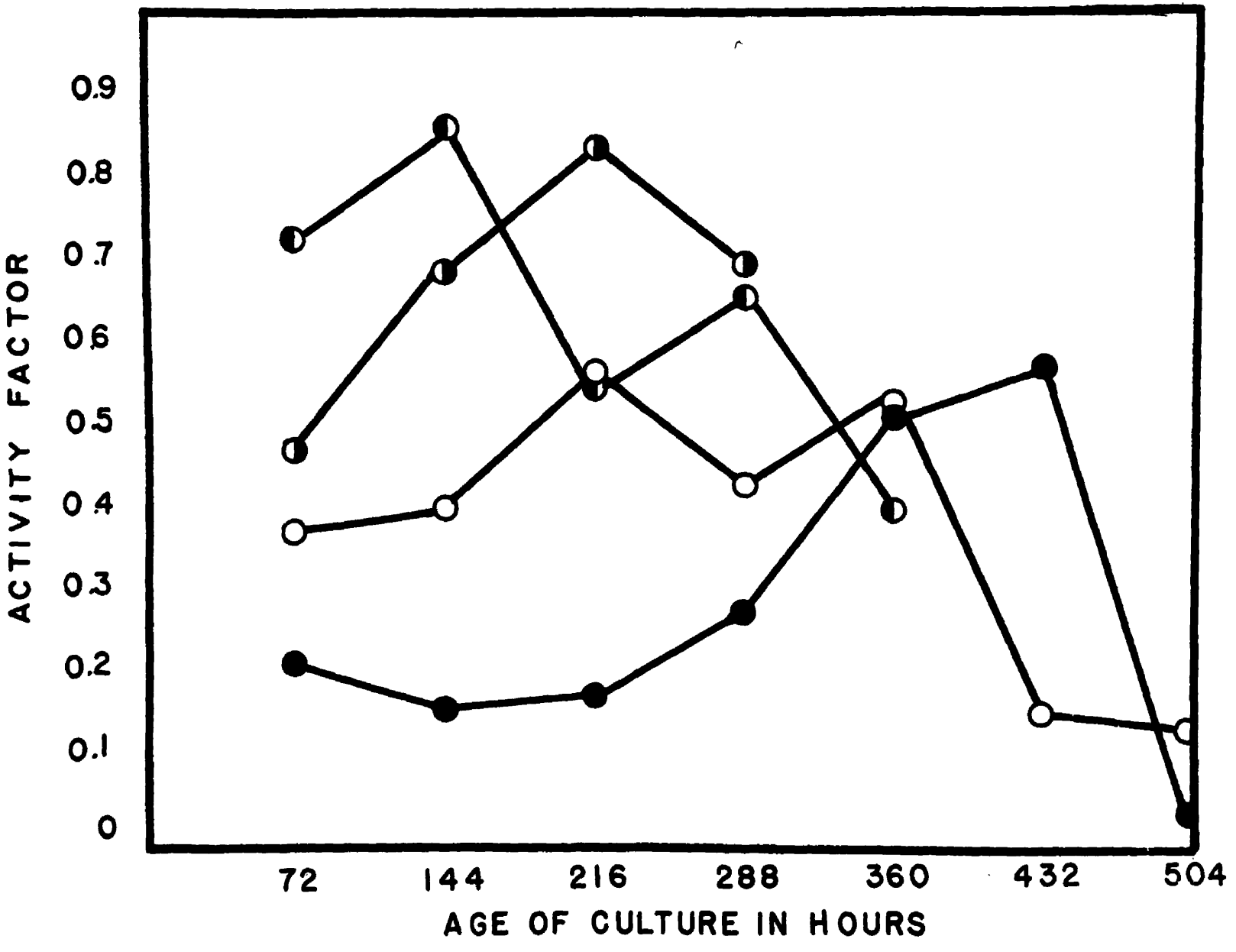
Relation between age and glycogen deposition in T. geleii W. All specimens were suspended in physiological saline for 30 minutes prior to fixation and staining. Activity factor determined by formula given under materials and methods.

● - T. geleii W reared in tryptone;

○ - T. geleii W reared in vitamin-enriched tryptone;

◐ - T. geleii W reared in magnesium chloride and vitamin-enriched tryptone; and

◑ - T. geleii W reared in calcium chloride and vitamin-enriched tryptone.


ACTIVITY FACTOR
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
72
144
216
288
360
432
504
AGE OF CULTURE IN HOURS

therefore, glycogen did not appear to diffuse naturally into the culture media.

FAT

Localization and variation with age of culture. All organisms were positive for fat with the exception of an occasional cell in the 72-hour cultures. The Sudan-positive area was first noted at the anterior end of the organism and consisted of distinct granules. After the cells had aged 216 hours, these granules coalesced to form a compact mass which eventually expanded distally to fill nearly the entire organism. Neither the addition of vitamins nor the washing of the organisms in saline prior to staining affected the distribution or amount of fat visible in T. geleii W.

An examination of Text-figure III indicates that the fat/cytoplasm ratio increased from 0.09 in 24-hour cells reared in tryptone and suspended in physiological saline to a maximum of 0.49 in organisms from 504-hour cultures. An extension to this curve would indicate a further rise in fat deposition as the cells aged.

Effect of the addition of the magnesium or calcium ion to the culture medium. The addition of calcium to tryptone media did not affect the localization or amount of fat in organisms from cultures 144 hours or less in age. After 144 hours, the fat/cytoplasm ratio decreased. An inhibition of fat production by magnesium was less pronounced.

Diffusion. No extracellular reaction for fat was visible on any slides and the suspension of the cells in saline did not materially alter the amount of fat present. Diffusion was not responsible for any variation which might occur in the fat/cytoplasm ratio.

PHOSPHOLIPIDS

Localization and variation with age of culture. The Smith-Dietrich test was used in all experiments on the determination of the presence of phospholipids in T. geleii W, and only discrete black particles as specified by Kaufmann and Lehmann (cited in Cain, 1950) were regarded as positive. The validity of this test has also been questioned by Cain (ibid.). No lipid/cytoplasm ratio was determined.

The first positive entities appeared in cells from cultures 504 hours of age. These spherical, randomly located particles, from one to seven in number, were noted in 5.1 per cent of the organisms (Figs. 34 and 35). At 576 hours of age 7 per cent of the cells contained positive granules, which were most heavily concentrated in the vicinity of the nucleus. Occasionally a gray or brown extracellular precipitate was visible, but this was not considered as evidence for diffusion of the phospholipids.

DESOXYRIBONUCLEIC ACID

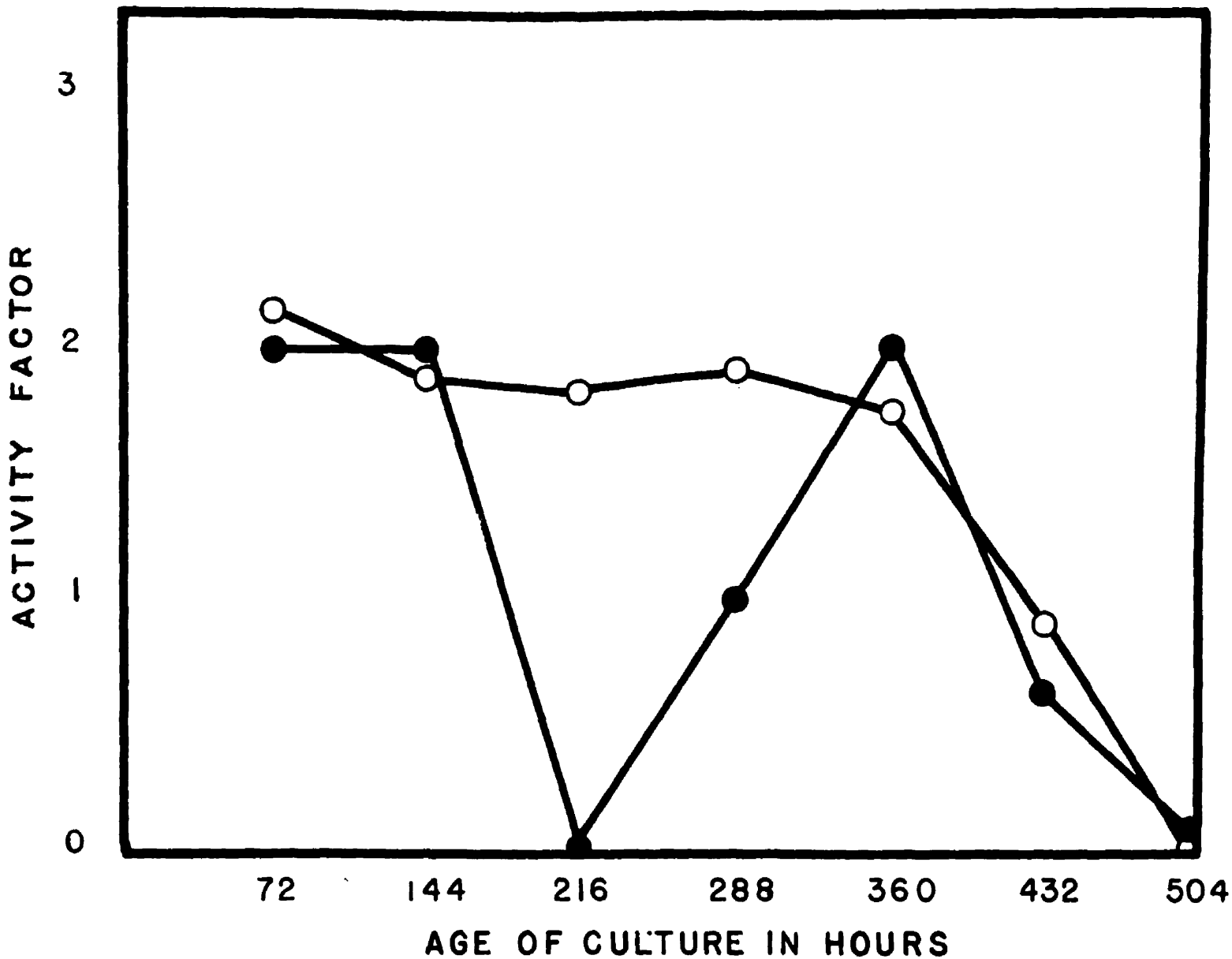
Localization and variation with age of culture. DNA activity was investigated by the Feulgen reaction as cited in Bensley and Bensley (1938).

TEXT-FIGURE IX

Relation between age and desoxyribonucleic acid activity in T. geleii W. All specimens fixed and stained immediately after removal from the culture medium. Activity factor determined by formula given under materials and methods.

● - T. geleii W reared in tryptone; and

○ - T. geleii W reared in vitamin-enriched tryptone.


ACTIVITY FACTOR
3
2
1
0
72
144
216
288
360
432
504
AGE OF CULTURE IN HOURS

A positive Feulgen reaction in specimens of T. geleii W was restricted to the nucleus (Fig. 28). Maximum DNA activity in unwashed organisms from tryptone cultures was noted 72, 144 and 360 hours subsequent to inoculation (Text-fig. IX). No activity was visible at 216 hours and after 360 hours the Feulgen reaction decreased in intensity until most of the organisms were negative at 504 hours.

Organisms reared in vitamin-enriched tryptone exhibited maximum DNA activity 72 hours subsequent to seeding (Text-fig. IX). Between 72 and 144 hours a slight decrease in activity occurred and then remained constant until the cells had aged 360 hours. After 360 hours another decline occurred and all activity disappeared 504 hours subsequent to inoculation.

Suspending organisms in physiological saline prior to staining produced no significant alteration in the intensity of the Feulgen reaction in cells reared in tryptone. DNA activity in organisms reared in vitamin-enriched media was decreased by the washing process and reached a maximum at 216 hours at which time the activity factor decreased from 1.85 to 0.2 (Text-fig. X). A cyclic rise and fall in DNA activity occurred in all cells except unwashed organisms from vitamin-enriched tryptone (Text-figs. IX and X).

Effect of the addition of the magnesium or calcium ion to the culture medium. The addition of calcium or magnesium chloride to vitamin-enriched tryptone induced minimal DNA activity at 144 and 360 hours and a maximum reaction at 216

hours (Text-fig. X). This is the inverse of that noted in organisms from solutions in which calcium or magnesium was omitted.

Diffusion. No evidence of diffusion occurred on any slides stained for DNA.

Relation between intensity of staining reaction and size of nuclei. According to Bradfield (1950) fluctuations in nuclear staining for DNA may be due to changes in nuclear volume. Approximate measurements made of nuclear area in the course of this study indicated that, in general, the smaller the nuclei, the greater the intensity of staining. This would indicate that in the smaller nuclei the DNA was more highly concentrated.

Many nuclei were irregularly shaped, but all areas were calculated as the product of the length and the width. Subsequent to the measurement of 30 cell nuclei, the average area of those placed in categories 1, 2 and 3 (see materials and methods) was 59, 54 and 31 square microns, respectively. According to these results the average area of the lightest staining nuclei was approximately twice that of the heaviest staining ones, while no significant difference was apparent between the light and medium staining nuclei. The difficulty in classifying the nuclei into the latter two categories may account for this small difference.

TEXT-FIGURE X

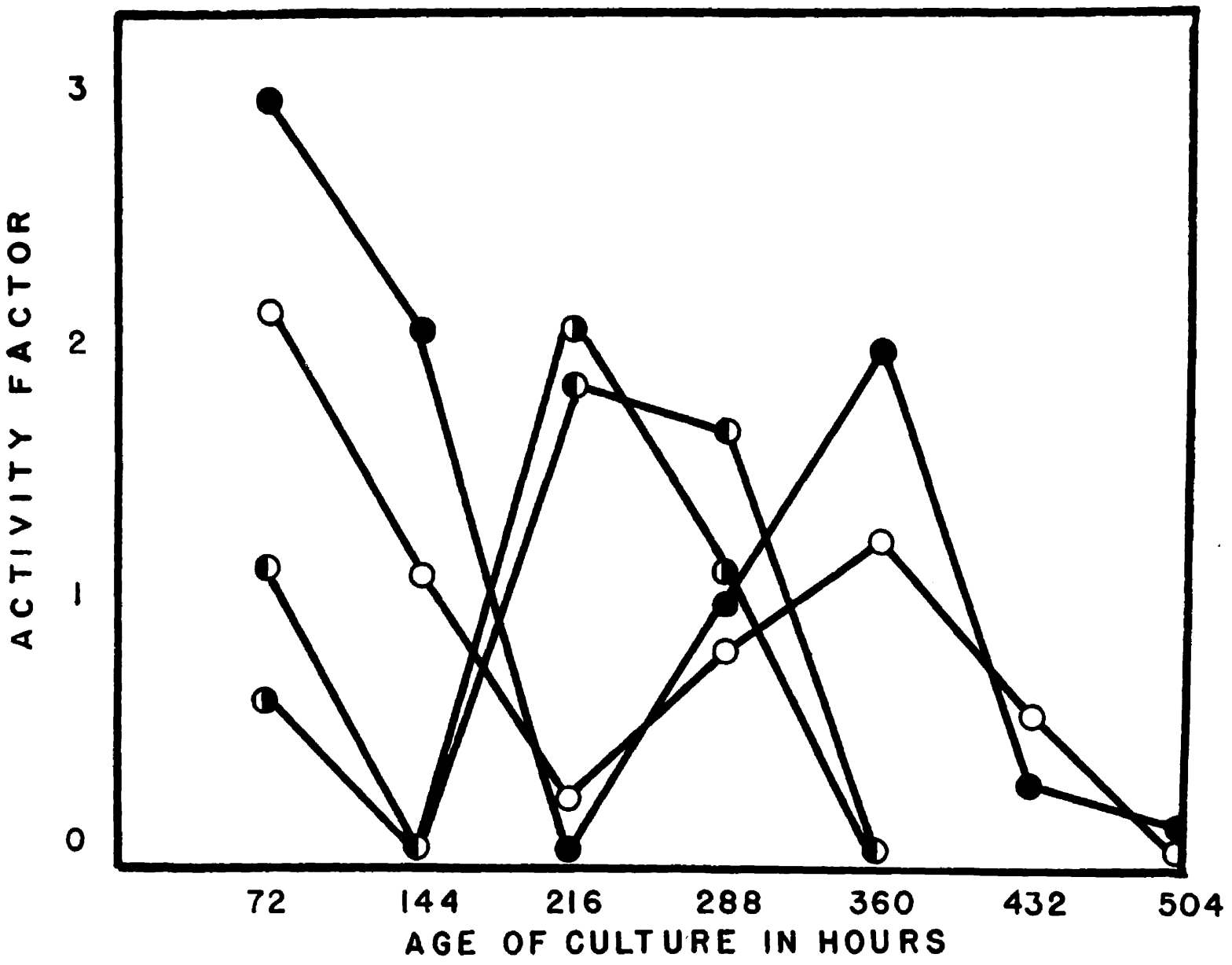
Relation between age and desoxyribonucleic acid activity in T. geleii W. All specimens were suspended in physiological saline for 30 minutes prior to fixation and staining. Activity factor determined by formula given under materials and methods.

● - T. geleii W reared in tryptone;

○ - T. geleii W reared in vitamin-enriched tryptone;

◐ - T. geleii W reared in vitamin and magnesium chloride-enriched tryptone; and

◑ - T. geleii W reared in vitamin and calcium chloride-enriched tryptone.


ACTIVITY FACTOR
3
2
1
0
72
144
216
288
360
432
504
AGE OF CULTURE IN HOURS

PEROXIDASE

No positive reaction for peroxidase was visible on any slides of T. geleii W.

URATES

No positive reaction for urates was obtained.

VARIATION IN SIZE OF CELL WITH AGE AND FIXATION

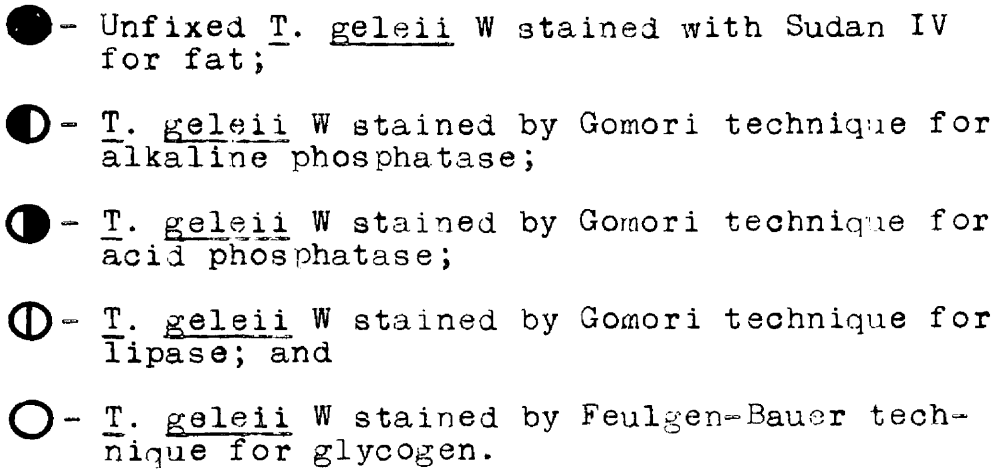
It is evident from Text-figure XI that specimens of T. geleii W reared in vitamin-enriched tryptone decreased in average length as the culture aged. Both fixation and the staining technique tended to decrease the size of the cell.

With the exception of organisms aged 360 and 504 hours, unfixed cells, those stained for fat, exceeded all cells in length. Organisms of the former age stained for lipase and of the latter age stained either for lipase or acid phosphatase were longer than unfixed cells of the same age. A maximum average length of 77 microns was exhibited by unfixed organisms 144 hours of age and a minimum of 38 microns by organisms 504 hours of age and stained by the Feulgen-Bauer reaction for glycogen.

In general, the average length of all fixed cells increased between 72 and 144 hours subsequent to inoculation. From 144 to 288 hours of age the length usually remained at a maximum and then gradually decreased until a minimum length was reached at 504 hours. An exception was noted in the cells stained for alkaline phosphatase, as a decline in length

TEXT-FIGURE XI

Relation between age, histochemical technique and length of T. geleii W reared in vitamin-enriched tryptone and fixed and stained immediately after removal from culture medium.



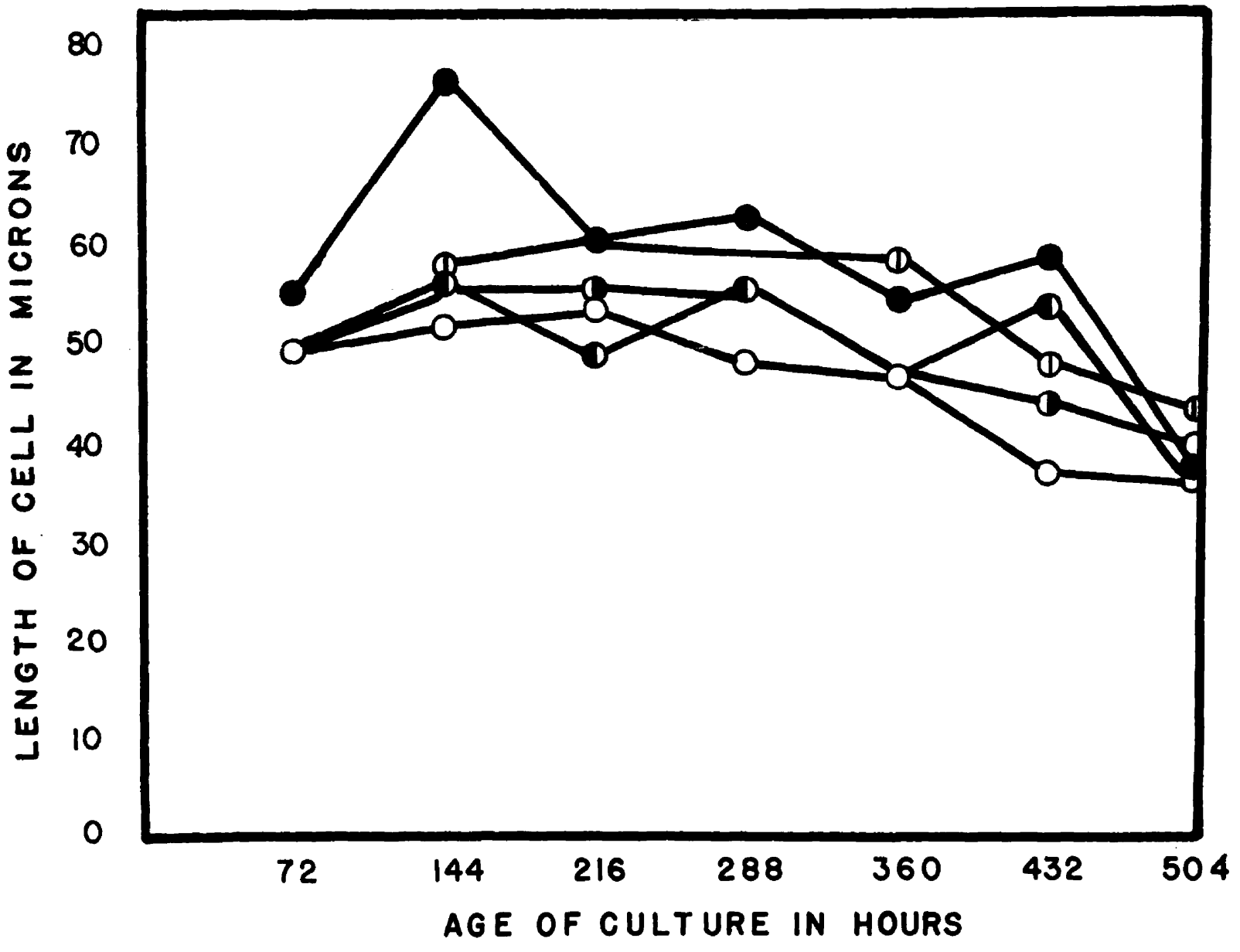
● - Unfixed T. geleii W stained with Sudan IV for fat;

◐ - T. geleii W stained by Gomori technique for alkaline phosphatase;

◑ - T. geleii W stained by Gomori technique for acid phosphatase;

◫ - T. geleii W stained by Gomori technique for lipase; and

○ - T. geleii W stained by Feulgen-Bauer technique for glycogen.


LENGTH OF CELL IN MICRONS
80
70
60
50
40
30
20
10
0
72
144
216
288
360
432
504
AGE OF CULTURE IN HOURS

occurred between 144 and 216 hours of age and a pronounced increase between 360 and 432 hours. The unfixed cells followed the same general trend as those stained for alkaline phosphatase.

INTERRELATIONSHIP OF ACTIVITY TRENDS IN BIOLOGICAL SUBSTANCES INVESTIGATED

In unwashed organisms from tryptone cultures acid phosphatase activity and glycogen deposition closely paralleled one another (Text-fig. VII). At 144 hours, however, an exception was noted as acid phosphatase activity decreased as glycogen deposition increased. The deposition of glycogen increased slightly between 360 and 504 hours while acid phosphatase rapidly approached a maximum activity factor of 1.0. Fat deposition increased gradually with the increase in the age of the culture. Alkaline phosphatase activity reached a maximum at 216 hours at which time acid phosphatase was also highly active and glycogen deposition near a maximum. In cells from cultures aged more than 360 hours acid phosphatase activity increased rapidly while any visible reaction for alkaline phosphatase disappeared entirely. Lipase activity was at a maximum at 360 hours at which time a rapid decline in acid phosphatase activity and the deposition of glycogen was apparent. Acid phosphatase activity exceeded that of alkaline phosphatase in all organisms. DNA activity was at a maximum 72, 144 and 360 hours subsequent to seeding and at a minimum at 216 and 504 hours (Text-fig. IX). In cells more

than 72 hours of age DNA activity bore an inverse relationship to glycogen deposition and acid and alkaline phosphatase activity (Text-figs. VII and IX). In older cells a slight parallel between lipase and DNA activity was apparent.

In general, the activity factor for acid phosphatase in unwashed cells exceeded that for glycogen deposition. Subsequent to suspension in physiological saline this interrelationship was reversed (Text-figs. III and VII). Nevertheless, in washed organisms a parallel between acid phosphatase activity and glycogen deposition was still apparent (Text-fig. III). At 144 hours acid phosphatase activity increased as glycogen deposition decreased, but in all cells 216 hours of age or older a parallel increase in the amount of both occurred. Acid phosphatase activity and glycogen deposition approached a maximum as fat deposition increased, but disappeared rapidly at the time fat deposition approached its maximum. Both lipase and alkaline phosphatase were at a maximum in cells 288 hours of age and decreased at the time acid phosphatase activity and glycogen and fat deposition were approaching a maximum. Alkaline phosphatase activity exceeded lipase activity in cells 144 hours or less in age, but in the older cells lipase became more active. Glycogen deposition exceeded fat deposition in cells 72, 360 and 432 hours of age, but in other cells the activity factor for fat usually exceeded that of all substances studied. DNA activity in washed cells appeared to be inversely related to alkaline

phosphatase and lipase activity, but the relationship was not as pronounced as that noted in the unwashed cells (Text-figs. III and X).

Acid phosphatase in unwashed organisms from vitamin-enriched tryptone exhibited a cyclic rise and fall in activity (Text-fig. II). In each period the activity factor increased rapidly from approximately 0 to a maximum and then declined to 0 again. The activity factor for glycogen deposition increased gradually to 0.58 between 72 and 216 hours and then declined to 0.18 at 504 hours. DNA and alkaline phosphatase activity and glycogen deposition followed parallel trends (Text-figs. II and IX). However, the activity factor for alkaline phosphatase did not exceed 0.04.

Suspension in physiological saline decreased the maximum peaks in the cyclic rise and fall in acid phosphatase activity in cells 216 to 360 hours in age (Text-fig. II). Glycogen deposition was decreased but maintained the same general trend as in the unwashed cells. Alkaline phosphatase activity practically disappeared in cells 216 hours of age or older while lipase activity increased slightly between 288 and 504 hours. Glycogen deposition and alkaline phosphatase activity appeared to decrease as lipase activity and fat deposition increased. The cyclic behavior of acid phosphatase did not appear to be directly correlated with the activity of any of the substances studied. Alkaline phosphatase and DNA activity decreased between 72 and 216 hours, but no additional parallel trend was noted (Text-figs. II and X).

TEXT-FIGURE XII

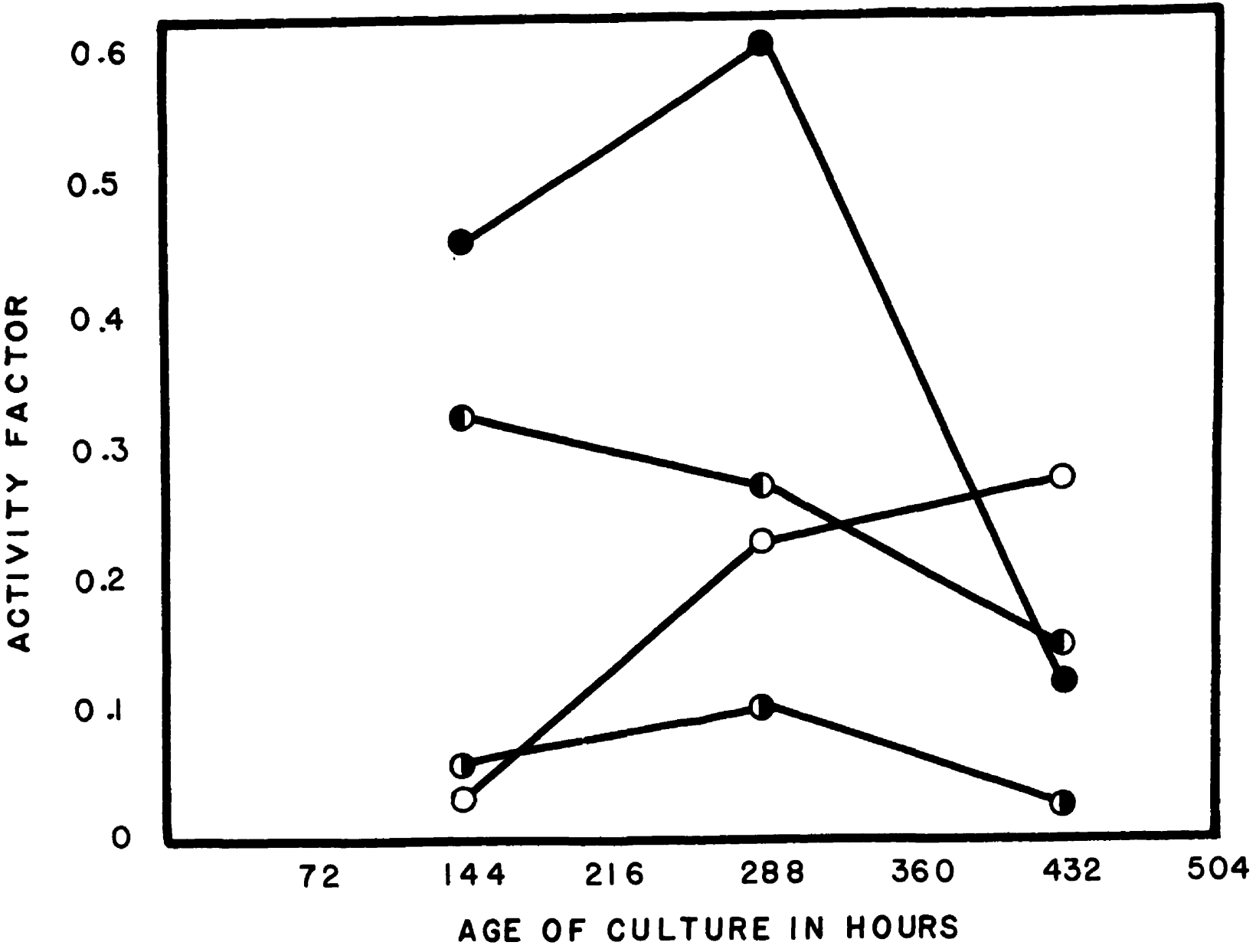
Relation between age and the histochemical properties of T. geleii W subsequent to the addition of calcium or magnesium chloride to the culture medium. All specimens reared in tryptone and suspended in physiological saline for 30 minutes prior to fixation and staining. Activity factor determined by formula given under materials and methods.

◑ - Alkaline phosphatase activity in T. geleii W reared in magnesium chloride-enriched tryptone;

◐ - alkaline phosphatase activity in T. geleii W reared in calcium chloride-enriched tryptone;

○ - acid phosphatase activity in T. geleii W reared in magnesium chloride-enriched tryptone; and

● - acid phosphatase activity in T. geleii W reared in calcium chloride-enriched tryptone.


0.6
0.5
0.4
0.3
0.2
0.1
0
ACTIVITY FACTOR
72
144
216
288
360
432
504
AGE OF CULTURE IN HOURS

All organisms reared in media containing magnesium or calcium chloride were suspended in physiological saline. The cells from tryptone cultures containing magnesium chloride exhibited maximum acid phosphatase activity at 432 hours and maximum alkaline phosphatase activity at 288 hours (Text-fig. XII). In organisms from cultures containing calcium chloride the maximum activity factor for the former occurred at 288 hours and for the latter at 144 hours. Acid phosphatase was more active than alkaline phosphatase in these cells.

In washed cultures containing both vitamins and magnesium chloride a correlation between acid phosphatase activity and glycogen deposition was again apparent (Text-fig. XIII). Both approached a maximum at 144 hours, declined in activity between 144 and 216 hours, increased between 216 and 288 hours and then decreased again between 288 and 360 hours. Lipase activity was absent in younger cells but between 216 and 360 hours it followed the same trend as acid phosphatase and glycogen activity. The alkaline phosphatase activity factor decreased gradually from 0.05 at 72 hours to 0 at 288 hours. A negative correlation between DNA activity and acid phosphatase activity and glycogen deposition was apparent in cells 288 hours or less in age (Text-figs. XIII and X). Lipase activity increased between 216 and 288 hours at which time DNA activity was decreasing.

Subsequent to the addition of calcium chloride to vitamin-enriched media glycogen deposition reached a maximum in

TEXT-FIGURE XIII

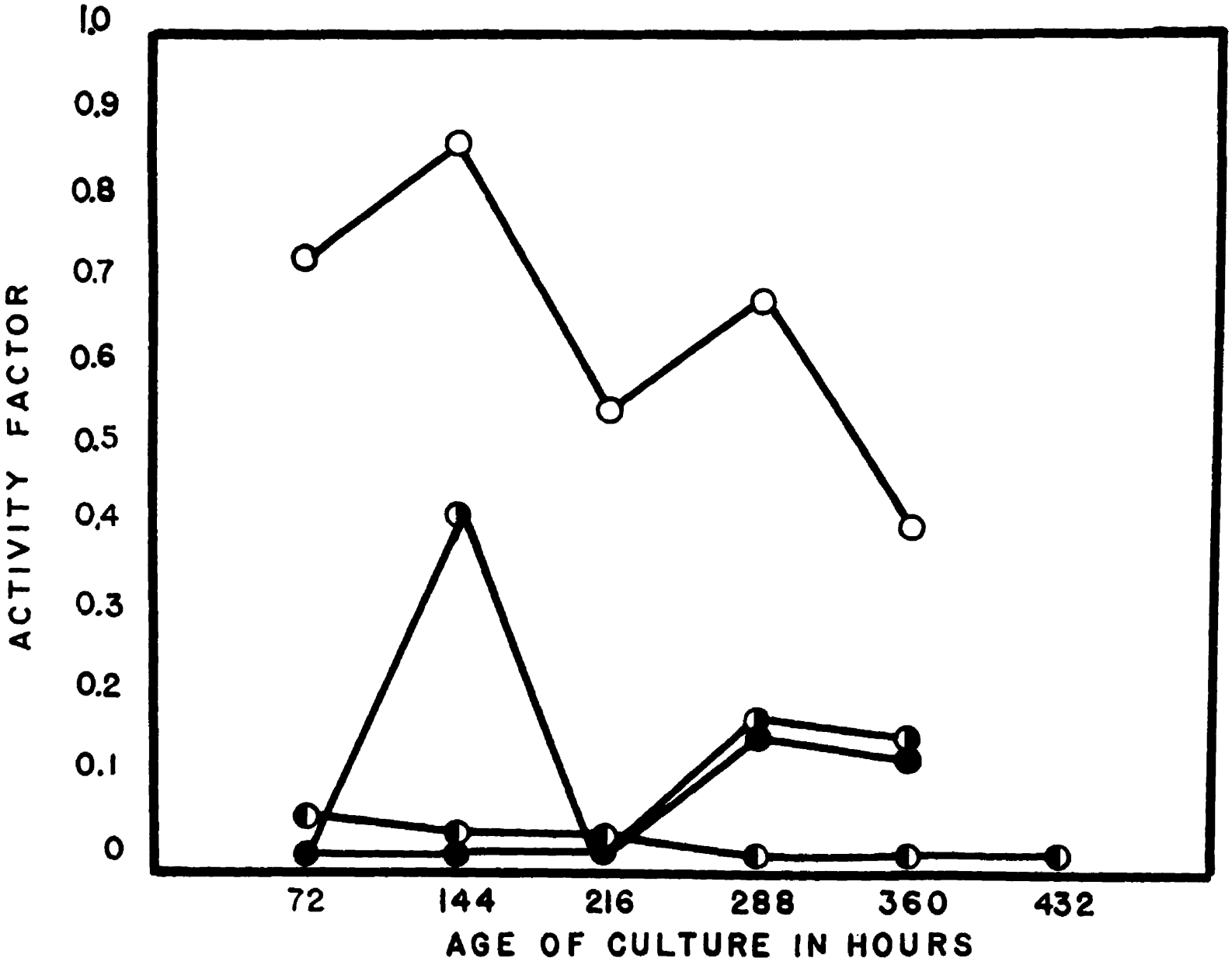
Relation between age and the histochemical properties of T. geleii W reared in vitamin and magnesium chloride-enriched tryptone. All specimens suspended in physiological saline for 30 minutes prior to fixation and staining. Activity factor determined by formula given under materials and methods.

◐ - Alkaline phosphatase activity;

◑ - acid phosphatase activity;

● - lipase activity; and

○ - glycogen deposition.


1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
ACTIVITY FACTOR
72
144
216
288
360
432
AGE OF CULTURE IN HOURS

TEXT-FIGURE XIV

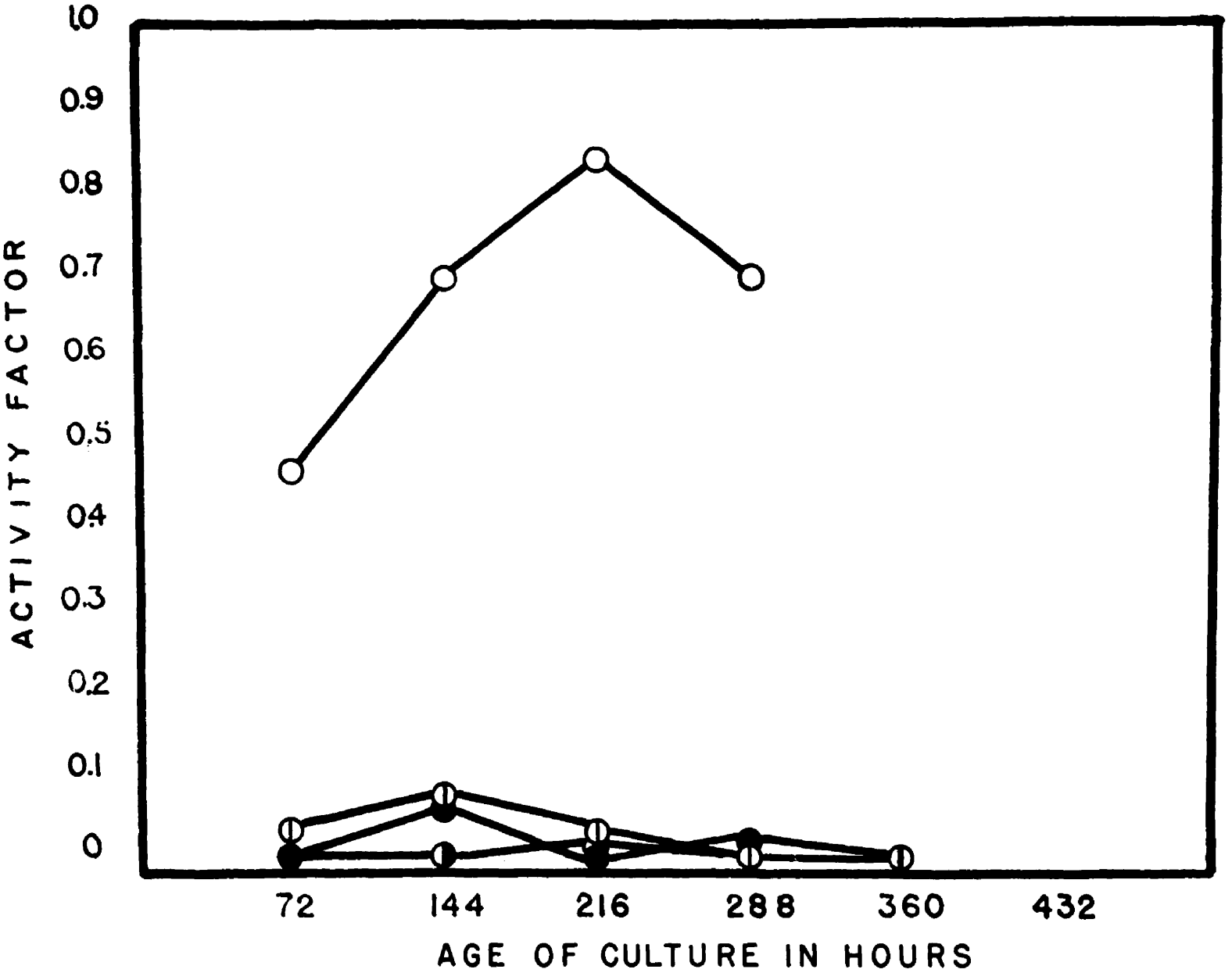
Relation between age and the histochemical properties of T. geleii W reared in vitamin and calcium chloride-enriched tryptone. All specimens suspended in physiological saline for 30 minutes prior to fixation and staining. Activity factor determined by formula given under materials and methods.

◐ - Alkaline phosphatase activity;

● - acid phosphatase activity;

◑ - lipase activity; and

○ - glycogen deposition.


1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
ACTIVITY FACTOR
72
144
216
288
360
432
AGE OF CULTURE IN HOURS

organisms aged 216 hours (Text-fig. XIV). There were no additional peaks in the activity curve. Both acid and alkaline phosphatase activity was at a maximum at 144 hours and lipase activity at 216 hours. In cells 72 to 144 hours of age DNA activity bore an inverse relationship to acid and alkaline phosphatase activity and glycogen deposition (Text-figs. XIV and X). Between 144 and 216 hours the same relationship between DNA and acid and alkaline phosphatase was apparent, but glycogen deposition paralleled that of DNA activity. The activity factor for glycogen deposition far exceeded that for any other substance.

2. LESION INDUCTION IN THE CHORIOALLANTOIS

Pedigreed White Leghorn hatching eggs were used exclusively for the study of lesion induction by protozoan cultures. T. geleii W were reared in the various culture media described under materials and methods and washed by centrifugation in two changes of physiological saline prior to inoculation onto the chorioallantois of 12-14-day old chicken embryos by the artificial air sac method of Burnet (1936).

Frequency of lesion induction. It is evident from Table II that at least 66.7 per cent of all embryos survived inoculation by the various suspensions of T. geleii W and other substances utilized in this study. The only exceptions occurred in embryos inoculated with croton oil or a heated suspension of unwashed T. geleii W in which a mortality of 100 and 75 per cent, respectively, was obtained. The maximum

TABLE

Chorioallantoic Lesions Induced by

Type of Inoculum	No of Embryos Injected	Per cent Survival of Embryos	Per cent Developing Lesions	Av Vol of Lesion mm^3	Av Max Height of Epithelium mm
T. geleii W 3-day cultures	27	85.2	56.5	60	D 0.139[3] V 0.130
7-9-day "	17	76.4	100	20	D 0.051 V 0.042
9-day " + 50°C	20	85	88.2	30	D 0.272 V 0.168
9-day " + 50°C (No saline)	4	25	100	297	D 0.073 V 0.030
9-day " in 0.005N CaO	10	80	87.5	46	D 0.161 V 0.073
12-14-day "	49	79.6	69.2	111	D 0.245 V 0.191
14-24-day " in 0.02N HCl	26	84.6	86.4	81	D 0.137 V 0.133
18-day "	47	80.9	68.4	118	D 0.140 V 0.135
18-20-day " with $MgCl_2$	33	93.9	51.6	25	D 0.122 V 0.155
18-20-day " with $CaCl_2$	37	94.6	68.6	29	D 0.142 V 0.070
24-day "	60	81.7	57.1	120	D 0.161 V 0.154

II

Specimens of T. geleii W and Other Inocula[1]

Per cent Lesions with Inclusions	Approx No of Inclusions[2]	Per cent of Lesions Showing Hyperplasia of Mesoderm + Epithelium				
		Mesoderm	M & E =	M > E	E > M	E >> M
83.3	0-+ Av 12+	22.2	44.4		11.1	22.2
--	--	37.5	37.5	25		
100	19-85 Av 52	18.2	36.3	18.2	18.2	9.1
0	--				100	
50	0-+ Av 0+		50		50	
40	0-25 Av 5	11.8	52.9		11.8	23.5
--	--	28.6	57.1		14.3	
50	0-13 Av 3	7.7	15.4	53.8		23.1
--	--	16.7		66.7	16.6	
--	--	20	33.3	40	6.7	
66.7	0-+ Av 15+	15.4	23.1	15.4	15.4	30.8

TABLE

Type of Inoculum	No of Embryos Injected	Per cent Survival of Embryos	Per cent Developing Lesions	Av Vol of Lesion mm^3	Av Max Height of Epithelium mm
0.05-0.25 croton oil emulsion	73	0	1.4	12	D 0.039 V 0.018
0.5-1.0 cc strain 12 lymphomatosis	14	78.6	54.5	13	D 0.024 V 0.063
0.02N HCl	3	66.7	50	90	D 0.032 V 0.013
0.02N NaOH	5	80	100	984	D 0.087 V 0.034
0.005N CaO	2	100	50	90	D 0.236 V 0.243
Physiological saline	18	77.8	57.1	28	D 0.155 V 0.202
Ringer's solution	6	66.7	0	0	--

1 Embryos exposed to 0.5 cc. portions of the various inocula unless strain 12 lymphomatosis 1:4 with physiological saline. All

2 Upper figure refers to range in number of inclusion bodies;

3 D-Dorsal epithelium; V-Ventral epithelium.

II (Cont.)

Per cent Lesions with Inclusions	Approx No of Inclusions	Per cent of Lesions Showing Hyperplasia of Mesoderm + Epithelium				
		Mesoderm	M & E =	M > E	E > M	E >> M
0	0	100				
0	0	33.3	66.7			
--	--		100			
0	0		25	50	25	
100	+		100			
0	0	20	40	40		
0	0					

otherwise specified. All T. geleii W were diluted 1:10 and inorganic salts dissolved in physiological saline.

lower figure to average number of inclusion bodies.

survival rate occurred subsequent to inoculation with 0.005N CaO or 18-20-day old T. geleii W reared in media containing calcium or magnesium chloride.

All embryos surviving inoculation with either 0.02N NaOH or 7-9-day old T. geleii W suspended in either the original culture medium and heated for one-half hour at 50° C. or in unheated physiological saline developed a chorioallantoic lesion at the site of inoculation. From 56.5 to 69.2 per cent of the embryos surviving exposure to unheated physiological saline suspensions of all other inocula of T. geleii W developed lesions. The suspension of 14-24-day old cultures in 0.02N HCl in physiological saline increased the rate of lesion induction to 86.4 per cent. The addition of calcium chloride to the culture medium did not materially alter the per cent of lesions developing subsequent to inoculation with 18-20-day old organisms, but the addition of magnesium chloride decreased the number induced to 51.6 per cent.

All concentrations of croton oil killed the embryos although one developed a lesion before death. The strain 12 lymphomatosis agent produced lesions in 54.5 per cent of the exposed embryos. With the exception of Ringer's and 0.02N NaOH, all solutions used for suspending T. geleii W produced lesions in approximately 50 per cent of the inoculated embryos. No lesions developed in any embryos inoculated with Ringer's solution.

Size of lesion induced. Subsequent to removal from the chorioallantois the length, width and depth of each lesion was measured in millimeters and the total volume used as an index for growth induction.

Lesions produced in these experiments ranged in average size from approximately 12 cubic millimeters in the chorioallantoic membranes inoculated with croton oil to approximately 984 cubic millimeters in those inoculated with 0.02N NaOH (Table II). The latter far exceeded in size lesions produced by any other inocula.

An examination of the effect of age of the T. geleii W culture on the size of lesion produced indicated that, in general, the older the culture the larger the lesion. An exception was noted in lesions induced by 7-9-day old cultures suspended in physiological saline, as they averaged only one-third the size of those induced by 3-day old cultures. The average size of lesion induced by 12-14-day old organisms was approximately twice that produced by 3-day cultures and six times that produced by 7-9-day old organisms. After 12 days the lesion-inducing properties of T. geleii W varied but slightly with an increase in age.

Suspending organisms in physiological saline decreased the lesion-inducing properties of T. geleii W. The average volume of lesion produced by 9-day old organisms suspended in the original culture medium and subjected to a temperature of 50° C. for one-half hour was 297 cubic millimeters. On

the other hand, the inoculation with organisms of the same age subsequent to suspension in physiological saline and subjection to heat produced an average lesion size of only 30 cubic millimeters. Similar aged cultures which remained unheated but suspended in saline induced lesions averaging 20 cubic millimeters. The latter would indicate that washing not heating was the principal factor involved in the difference in size of lesion produced. This effect of washing warrants further investigation in the future, as the results might be attributable to growth-inducing substances diffusing into the culture medium and being removed in the washing process, or it may indicate that the culture medium itself may have growth-promoting properties. No study was made of the effect of Bacto-tryptone on the chorioallantoic membrane.

In cultures 14 days of age or older the addition of magnesium or calcium chloride to the culture medium or HCl to the physiological saline utilized as a suspending medium decreased the lesion-inducing properties of T. geleii W. The addition of 0.005N CaO to the physiological saline used for suspending 9-day old unheated cultures of T. geleii W doubled the average size of lesion produced. Physiological saline alone induced an average sized lesion of 28 cubic millimeters while the addition of either 0.005N CaO or 0.02N HCl to the saline increased the size of lesion to approximately 90 cubic millimeters. Croton oil and the strain 12 lymphomatosis agent produced the smallest lesions in this study.

Ringer's solution was the only suspending medium to induce no lesions.

General morphological characteristics of induced lesions. Two types of lesions were induced by the inocula used in these investigations: (1) gray thickened areas in the chorioallantois and (2) umbilicated. The former was induced by the following inocula: 7-9-day old T. geleii W in physiological saline, cultures of 18-20-day old T. geleii W to which magnesium chloride had been added, 14-24-day old T. geleii W suspended in 0.02N HCl in physiological saline, croton oil and physiological saline. All other inocula induced both types of lesions. Umbilicated lesions usually exhibited the greater degree of hyperplasia of the various tissue elements and were frequently characterized by a complete destruction of the dorsal epithelium near the site of inoculation. Gray thickened areas tended to follow the course of the circulatory system and either varied but slightly from normal tissue or exhibited varying degrees of tissue proliferation. Hemorrhages were visible in many embryos, but no correlation was apparent between the type of inoculum and the amount of hemorrhaging.

It is also evident from Table II that lesions resulting from inoculation with croton oil were characterized by necrosis and slight mesodermal hyperplasia. Those induced by 0.02N HCl and 0.005N CaO exhibited both mesodermal and epithelial proliferation in equal ratios. All other inocula produced lesions characterized by mesodermal proliferation alone and in

conjunction with varying degrees of epithelial hyperplasia. No lesions were noted in which hyperplasia was restricted solely to the epithelium.

In general, maximum epithelial hyperplasia was induced by saline suspensions of T. geleii W and increased with an increase in the age of the culture. An exception was noted in the 18-day old cultures which produced lesions in which 23 per cent exhibited extreme proliferation of the epithelium but over 50 per cent exhibited the most extensive hyperplasia in the mesoderm. Maximum epithelial proliferation occurred in lesions produced by 24-day old cultures in which 30.8 per cent of the lesions exhibited a considerably more extensive hyperplasia of the epithelium than of the mesoderm. This epithelial hyperplasia varied from a simple increase in number of layers of cells to an extreme foliate type of growth. The addition of calcium or magnesium chloride to the culture medium or HCl to the saline used for suspension of the organisms decreased the amount of epithelial and stimulated the degree of mesodermal hyperplasia. The addition of CaO to physiological saline stimulated epithelial rather than mesodermal hyperplasia.

Unwashed 9-day old cultures subjected to 50° C. stimulated epithelial growth to a greater extent than mesodermal, but suspending the organisms in physiological saline prior to exposure to heat reversed this growth-promoting relationship.

Two-thirds of the lesions produced by strain 12 lymphomatosis virus showed equal hyperplasia of both mesodermal and epithelial tissues, while one-third of those examined exhibited hyperplasia only in the mesodermal elements. Physiological saline and most of the suspending media induced maximum hyperplasia in the mesoderm.

Maximum height of dorsal epithelial growth was obtained by inoculation with 9-day old washed T. geleii W subjected to heat, 13-14-day old T. geleii W in physiological saline and 0.005N CaO. Minimal proliferation was induced by the strain 12 lymphomatosis agent, croton oil, 0.02N HCl and 7-9-day old T. geleii W suspended in physiological saline. An examination of Table II indicates that, in general, the average height of maximum growth was greater in the dorsal (ectodermal) than in the ventral (endodermal) epithelium. The only exceptions noted were in those lesions produced by T. geleii W cultures to which magnesium chloride had been added and by the strain 12 lymphomatosis agent, 0.005N CaO or physiological saline. In many lesions the endodermal epithelium exhibited more extensive proliferation than the ectodermal epithelium although the height of the latter may be the greater. Investigations indicated that the most active site for growth stimulation was to be found in the endodermal or ventral epithelial tissues and that the growth-stimulating substances may diffuse through the dorsal epithelium to induce their maximum effect upon the endoderm. In

several lesions outpocketings were visible ranging in size from small knobs to large mushroom-like growths. These outpocketings were chiefly mesodermal in origin with no extensive proliferation of the dorsal epithelium.

Maximum mesodermal cellularity was visible in lesions produced by unwashed 9-day old organisms subjected to 50° C. for one-half hour. In most lesions the site of greatest fibroblast proliferation was to be found in the ventral half of the lesions adjacent to the endodermal epithelium. Pronounced fibroblast activity was also visible adjacent to the dorsal epithelium. These active sites tended to increase in concentration and area as the age of the T. geleii W used in the inoculum increased.

The endodermal epithelium was frequently vacuolated, the significance of which was unknown but may be an indication of fatty degeneration. Also in lesions stained with haemotoxylin-eosin a blue and a brown precipitate were frequently noted. The former occurred along capillaries and in areas in which necrotic tissue was present and was heaviest in lesions produced by 3-day old T. geleii W. The latter was restricted to the epithelial nests described in a subsequent section and may represent the breakdown product of some of the blood constituents.

A positive correlation appeared between the age of the T. geleii W cultures used for inoculation and the amount of necrosis visible in the induced lesion. Congestion was

heaviest in lesions induced by cultures of T. geleii W aged from 3-9 days. Lymphocytes and macrophage-like cells were visible in most lesions. The former were at a maximum in lesions produced by 3-day old T. geleii W and the latter in those induced by 14-24-day old cultures. Vascular endothelial cells were in the process of capillary formation in the larger lesions. Neutrophils were noted only in lesions induced by 0.02N NaOH.

No distinct characteristics could be isolated to differentiate histologically the lesions produced by the various inocula used in this study with the exception of those induced by croton oil in which extensive necrosis was visible. Variation was as extreme among lesions produced by the same inocula as between those induced by different ones.

Morphological characteristics of an individual lesion induced by T. geleii W. A microscopic examination of a lesion induced by 3-day old T. geleii W and stained with haemotoxylin-eosin revealed pilose projections in areas of maximum proliferation of the dorsal epithelium. Adjacent to this area deep pits were visible which divided the epithelium into broad-based foliate projections with truncate distal surfaces. The more distal the epithelium to the site of maximum proliferation the less convoluted and hyperplastic it became. The nuclei of the cells forming the most extensive epithelial growth were smaller than those in the less proliferative areas. In the latter the cells were segmentally arranged but in the

former the pattern was irregular. All epithelial cells had thin nuclear membranes and very little chromatin. The outer surface of the dorsal epithelium stained an intense blue.

Proliferating fibroblasts, mesenchymal cells and a few eosinophils were visible beneath the basement membrane of the dorsal epithelium. Proximal fibroblasts arranged themselves obliquely or perpendicular to the proliferating ectoderm but those more distal tended to lie parallel to the surface. Whorls of cells, surrounded by numerous red blood corpuscles, were visible but could not be identified. Aggregates of lymphocytes or other small blue staining cells were dispersed throughout the lesion.

The central part of the lesion became less cellular and an increase in fibrinous material noted. Numerous macrophages filled with a pale brown substance were visible. The latter was one of the first cells to appear subsequent to the onset of the proliferative process. A few scattered lymphocytes and eosinophils were present in this area of the mesoderm.

The ventral portion of the lesion increased in cellularity and fibroblast activity was at a maximum. The latter appeared to be walling off necrotic areas which were visible along the ventral surface. Hemorrhage, congestion, eosinophils and lymphocytes were present near areas of necrosis.

Morphological characteristics of embryonic grafts. All embryonic liver grafts exhibited pronounced necrosis, were

adherent to the chorioallantois and had established a connection to the blood supply. The embryonic heart and head ectodermal tissues were not adherent but had induced cellular proliferation of the chorioallantois. All liver grafts were hemorrhagic and heavily infiltrated with lymphocytes. Subsequent to staining with haematoxylin-eosin a heavy blue precipitate was visible along the ventral surface of the graft adjacent to the chorioallantois. This area exhibited pronounced alkaline phosphatase activity, a heavy concentration of actively proliferating fibroblasts and was the site of peculiar tubule formation. The latter may be precursors of epithelial nests which were at a maximum in the chorioallantois adherent to embryonic grafts.

All areas of embryonic liver grafts were intensely sudanophilic, but acid phosphatase activity was restricted to several small circumscribed portions of necrotic tissue. The Feulgen reaction for DNA was most intense in the proliferating blood cells.

The chorioallantoic lesion adjacent to all embryonic grafts was similar to that induced by the various inocula used in this study.

Epithelial nests. All inocula appeared to stimulate the formation of nests of cells (Figs. 36 and 43). A microscopic examination of the various lesions indicated that these aggregates of cells resulted from stimulation and outpocketings of the ventral (endodermal) epithelium. Apparently either

growth-promoting substances were capable of diffusing through the dorsal portion of the chorioallantois to produce their maximum effect upon the ventral surface (Fig. 42) or a greater capacity for growth is actually inherent in this area of the membrane. The majority of nests were in close proximity to the ventral surface, but in occasional lesions exhibiting extreme proliferation of mesodermal elements, nests were found adjacent to the dorsal epithelium (Fig. 36). The average maximum number of nests was 28 and was produced by unheated suspensions of 7-9-day old T. geleii W. A slight decline in the number produced occurred as the age of the culture used for inoculation increased.

Cells constituting the epithelial nests were larger and more vacuolated than those in the surrounding vicinity. Occasionally nests were visible in which a distinct lumen had been formed and in which cellular aggregates similar to blood cells were apparent. A definite determination of this tissue could not be made.

A comparative examination of the epithelial nests produced by the aforementioned inocula and the squamous cell carcinomas which developed in chickens treated with methylcholanthrene (Duran-Reynals 1952) revealed a superficial similarity between the two. However, no keratinization was visible in the former at the stage of development in which it was studied.

Inclusion bodies. Fennell (1951) noted intranuclear and cytoplasmic inclusion bodies in the epithelial tissues of

lesions induced by cultures of T. geleii W. He postulated that the combination of preformed calcium salts with the fat which accumulated as the organisms aged could produce calcium soaps which would be insoluble in water and might assume the characteristics of the visible inclusion bodies.

In this study intranuclear and cytoplasmic inclusion bodies were also visible in lesions stained with haemotoxylin eosin-azure and were most heavily concentrated in the basal cells of the dorsal epithelium. They were most numerous in moderately hyperplastic areas (Figs. 37 and 39). Spherical entities similar to inclusion bodies were also frequently visible in unabsorbed inocular remnants of T. geleii W and could not be distinguished from the intranuclear and cytoplasmic inclusion bodies. In some areas large aggregates of these particles could be found either on the periphery of the lesion (Fig. 40) or in a lumen present in the lesion itself. These extracellular bodies were at a maximum in lesions produced by 3-day old T. geleii W. Whether these particles were identical to inclusion bodies could not be definitely ascertained, but morphologically they could not be separated.

All lesions produced by 0.005N CaO and heated saline suspensions of 9-day old T. geleii W contained inclusion bodies, while none were visible in lesions induced by 9-day old unwashed T. geleii W, croton oil, strain 12 lymphomatosis agent, 0.02N NaOH and Ringer's or normal saline solution. The maximum number of cellular inclusion bodies occurred in

lesions induced by 0.005N CaO and 24 and 3-day old cultures of T. geleii W, respectively. Organisms from 12-18 days of age induced lesions containing the minimum number of inclusion bodies. The preceding results tend to support the possibility of a relationship between fat, calcium and the production of inclusion bodies.

Histochemical properties of induced lesions. All lesions were positive for fat with the exception of one-third of those produced by inoculation with 9-day old T. geleii W suspended in physiological saline and subjected to 50° C. for one-half hour. Fat was most highly concentrated in dorsal epithelial tissues exhibiting maximum proliferation (Figs. 38 and 44), and along the ventral epithelium and surface in areas of necrotic tissue or inocular fragments. Epithelial nests were frequently highly positive for fat (Fig. 45). Intensely sudanophilic epitheloid cells were scattered throughout the mesoderm of most lesions, and fat droplets were occasionally visible in arteries and veins.

No alkaline phosphatase activity was visible in lesions induced by 18-20-day old cultures of T. geleii W to which magnesium chloride had been added, in 25 per cent of those produced by 14-24-day old cultures in 0.02N HCl and in 33.3 per cent of those induced by physiological saline or 18-20-day old cultures of T. geleii W to which calcium chloride had been added. In all other lesions a positive reaction was obtained. Alkaline phosphatase was most highly concentrated

in the ventral half of the lesion along veins, arteries and capillaries and in proliferating fibroblasts (Fig. 41). Mesenchymal cells in the center of the lesion frequently exhibited a slightly positive reaction. A diffuse grayness was usually apparent throughout the epithelial tissue, including the epithelial nests, but was not considered a positive reaction for alkaline phosphatase.

No acid phosphatase activity could be detected in any lesions induced by 7-9-day old cultures of T. geleii W, in 50 per cent of those induced by either 14-24-day old cultures suspended in 0.02N HCl or 18-20-day old cultures to which calcium chloride was added or in 66.7 per cent of those produced by physiological saline. Acid phosphatase activity occurred in sites similar to those of alkaline phosphatase with the exception of the mesodermal fibroblasts and mesenchymal cells in which alkaline phosphatase was usually present but acid phosphatase entirely absent. The reaction for alkaline phosphatase was more intense than that for acid phosphatase.

Lipase activity was visible in all lesions except those produced by 3-day old cultures of T. geleii W suspended in normal saline and 9-day old cultures suspended in saline and subjected to 50° C. for one-half hour prior to inoculation. Lipase was active in the same sites as alkaline phosphatase, but the reaction was less intense.

A positive Feulgen-Bauer reaction was obtained in all lesions studied with the exception of that produced by croton

oil. This reaction was restricted to the dorsal epithelium and epithelial nests. This reaction was not eliminated by saliva and may be due to some polysaccharide other than glycogen from which aldehydes could be released.

Desoxyribonucleic acid was present in the nuclei of all cells but was most heavily concentrated in the actively proliferating blood and epithelial cells and in peripheral fibroblasts of the mesoderm.

With the exception noted in the discussion on glycogen (Feulgen-Bauer reaction) all control slides were negative.

DISCUSSION

1. HISTOCHEMICAL PROPERTIES OF TETRAHYMENA GELEII W.

Data presented in the preceding experiments were difficult to duplicate possibly due to a heterogeneity of the cultures, variations in temperature or to uncontrollable environmental factors. No definite conclusions could be drawn as to the function of the various substances investigated in T. geleii W for no distinct correlations were demonstrable. However, certain possible relationships were noted, and it is interesting to speculate on the functional role of these substances in the physiology of this organism.

Junquiera (1950) maintained that acid phosphatase might be involved in carbohydrate synthesis in vertebrates. In tryptone cultures of T. geleii W 216 hours in age or older the curves for glycogen deposition and acid phosphatase activity were similar if the organisms were stained immediately upon removal from the culture medium. This suggested that acid phosphatase might play a role in glycogen deposition. However, in organisms reared in vitamin-enriched media acid phosphatase frequently exhibited a cyclic activity curve; i.e., it increased from approximately zero to a maximum and then decreased to zero again. Glycogen deposition, on the other hand, was maintained at an approximately constant rate. This suggested that if acid phosphatase played

a role in glycogen deposition in these organisms, excess end products of dephosphorylation occurred with increased enzymatic activity and inhibited acid phosphatase activity. Eventually the end products of the reaction might be depleted and acid phosphatase activity again increased to a maximum. In rapidly growing cells, such as those exposed to vitamin-enriched tryptone, the end products of enzymatic activity may build up rapidly. In other cultures of T. geleii W no correlation was apparent between acid phosphatase activity and glycogen deposition.

Wichterman (1953) believed that fat under anaerobic conditions could form as an end product of carbohydrate metabolism in paramecium. Results of this study indicated that the curve for fat and glycogen deposition was similar in organisms reared in tryptone and suspended in physiological saline prior to fixation. In young cells the amount of fat and glycogen was relatively low, but as the cultures aged there was a corresponding increase in both. Eventually the organisms became completely filled with fat, and a rapid decline in glycogen deposition was observed. This suggested that the synthesis of glycogen may be closely related to that of fat, and that on completion of the latter the supply of the former becomes depleted either through utilization in the formation of fat or through a reduced requirement by senescent cells. However, the chemical interrelationships in the formation of these compounds in T. geleii W are not clarified at this time.

Wislocki and Dempsey (1945) maintained that in vertebrate tissues alkaline phosphatase played an important role in the synthesis of glycogen. If alkaline phosphatase was important in carbohydrate synthesis in T. geleii W, it may have been functional in organisms from tryptone cultures but not in cells which have become adapted to vitamin-enriched media.

Rothstein and Meier (1949) maintained that yeast surface phosphatases played no direct role in either metabolism or in the mechanisms of phosphate uptake, but that they made available to cells substances in the medium which ordinarily could not be utilized. Many authors (Gomori, 1941, and Maengwyn-Davies, Friedenwald and White, 1952, etc.) have demonstrated that in vertebrate tissues the duodenum, arteries and capillaries exhibited maximum alkaline phosphatase activity. This has led to the conclusion that one of the functions of alkaline phosphatase is the absorption and transfer of organic substances across cell membranes. Alkaline phosphatase activity in T. geleii W reared in tryptone was confined, in general, to the distal end of the cell adjacent to the pellicle which suggested that one function of alkaline phosphatase in this organism was also concerned with an increase in the availability and transfer of nutrients into the cell.

All visible alkaline phosphatase activity disappeared in T. geleii W which had become adjusted to vitamin-enriched culture media. Therefore, this enzyme may also be involved in the release of materials essential for the synthesis of

certain vitamins. Most of the vitamins used in this study regulate carbohydrate metabolism, and the disappearance of alkaline phosphatase may be correlated with a vitamin-activation of other enzymes associated with the synthesis of carbohydrates and other metabolic processes essential for rapidly growing cells. The presence of a negative reaction for alkaline phosphatase does not eliminate the possibility of the enzyme being present in either an inactive state or in too minute amounts to be detected by the Gomori technique.

Pritchard (1947) and Danielli (1948) believe that phosphatase is concerned with the metabolism of nucleic acid, nucleotides and nucleoproteins which are involved in protein synthesis and cell division. Brues et al (1944) found that both nuclear alkaline phosphatase and the rate of desoxynucleotide turnover increased in the liver of the rat subsequent to partial hepatectomy. Danielli and Catcheside (1945) and Krugelis (1948) showed that alkaline phosphatase was restricted to the Feulgen-positive bands of Drosophila salivary chromosomes which also contained the maximum concentration of desoxynucleotides. Malmgren and Heden (1947) noted that increased growth and protein synthesis were closely associated with increased polynucleotide content in both bacteria and metazoa. DeRobertis and Schmitt (1948) found that acid phosphatase occurred in areas of axons in which there was evidence for the presence of neurofibrils and suggested a relationship between acid phosphatase and the synthesis of protein.

In unwashed specimens of T. geleii W reared in vitamin-enriched media alkaline phosphatase was absent but DNA activity was maintained at a fairly constant level until the cells were 360 hours of age. In specimens from tryptone cultures in which DNA activity was cyclic, alkaline phosphatase activity approached a maximum at 360 hours at which time DNA activity disappeared. This suggested an inverse relationship between DNA and alkaline phosphatase activity in which the latter may catalyze the conversion of DNA into nucleotides, nucleosides, etc. Acid phosphatase also appeared to play a role in DNA turnover. In organisms from tryptone cultures and suspended in physiological saline the activity curve for DNA and acid phosphatase were similar, and in vitamin-enriched media in which DNA turnover may be rapid, acid phosphatase was highly active. The former suggested that acid phosphatase may aid in the synthesis of DNA.

On the other hand, DNA activity in unwashed organisms from tryptone solutions bore an inverse relationship to acid phosphatase activity. The shift in location of acid phosphatase from the posterior end towards the nucleus of the cell and the subsequent decrease in DNA activity may implicate acid phosphatase in the breakdown of DNA. Although both acid and alkaline phosphatase appeared to be involved in DNA metabolism, their role cannot be clarified at this time. Approximate measurements of nuclear size made in the course of this study indicated that, in general, the smaller the nucleus

the greater the staining intensity for DNA, which may alter the estimation of DNA activity made on the basis of staining reaction alone.

Kabat and Furth (1941) maintained that the magnesium ion activated alkaline phosphatase. In these present experiments, calcium was even more effective than magnesium in the activation of phosphatase. Both ions tended to increase glycogen deposition and the life of the culture but decrease fat deposition. The decrease in the latter by addition of magnesium to the culture solution may have resulted from a greater utilization of the stored lipids by the rapidly growing cells. The shift in alkaline phosphatase activity from the distal end towards the nucleus in cells from magnesium and vitamin-enriched culture media may be correlated with the increased division rate and DNA turnover which must occur in rapidly growing cells.

Maengwyn-Davies, Friedenwald and White (1950) and Newman, Feigen, Wolf and Kabat (1950) showed that it was possible to differentiate histochemically substrate specific enzymes hydrolyzing a number of phosphate esters. This suggested that in these present experiments the shift in alkaline phosphatase activity from the distal end of the cell towards the nucleus upon addition of vitamins and magnesium chloride to the culture medium might be attributable to the presence of several enzymes. The results obtained in the experiments on inhibition, however, suggested that the shift was a manifestation of two different functions of the same enzyme and

that a non-specific alkaline phosphatase was probably present in T. geleii W. Further investigation utilizing other concentrations of inhibitors and an increased number of substrates is essential before definite conclusions may be drawn.

LeDuc and Dempsey (1951) and Martin and Jacoby (1949) demonstrated that the phenomenon of diffusion complicated the histochemical reaction for alkaline phosphatase in vertebrate tissues. Diffusion was also a prominent factor in the localization of both alkaline and acid phosphatase in T. geleii W. A negative alkaline phosphatase reaction was obtained and attributed entirely to the diffusion of the enzyme or the inorganic phosphate resulting from the enzymatic catalysis of the dephosphorylation of hexose phosphates, etc. Acid phosphatase unlike alkaline phosphatase diffusion was never complete due to a higher degree of activity for the former. An extracellular equilibrium was readily established and diffusion curtailed before catalysis was complete.

Lipase appeared to be concerned with the hydrolysis of fat in T. geleii W, as the addition of magnesium to the culture medium enhanced lipase activity and decreased fat deposition. Moreover, a decrease in lipase activity occurred after the cells aged 360 hours during which time fat deposition rapidly approached a maximum. The shift in location of lipase activity from the nuclear region to the distal end of the cell subsequent to the addition of calcium or magnesium to the culture medium cannot be explained at this time unless fat hydrolysis is excessive in rapidly growing organisms and

lipase aids in the transfer of the end products of this reaction across the cellular membranes.

Peroxidase may have been present in T. geleii W in an inactive state, but it could not be demonstrated. This suggested that no biological oxidations in T. geleii W require catalysis by peroxidase.

Purine catabolism in T. geleii W does not appear to result in the formation of urates or uric acid but is probably accomplished through other chemical pathways.

2. LESION INDUCTION IN THE CHORIOALLANTOIS

All salt solutions, suspensions of T. geleii W and other inocula utilized in the experiments on lesion induction, with the exception of Ringer's solution, possessed growth-promoting properties to some degree. Apparently most foreign materials will stimulate cellular proliferation in the chorioallantois either by irritation or by release of quantities of normally occurring growth-promoting materials. Lesion induction by protozoan cultures undoubtedly involved both of these processes; i.e., an irritation by the suspending medium and the release of growth-promoting substances by the organisms themselves. These substances accumulated as the organisms aged and reached a maximum when the cells became senescent (24-day old cultures). Fennell (1951) demonstrated that suspensions of these senescent specimens exhibited abundant amounts of neutral fat, fatty acids, calcium salts and a high degree of phosphatase activity. The

largest lesions in the investigation were produced by 0.02N NaOH and must be attributable to either an inflammatory reaction or the release of growth-promoting factors by the necrosed tissue as NaOH would undoubtedly not occur in nature in this concentration and could not be considered a normally occurring growth-promoting factor.

In these present experiments it was noted that during the age interval in which the lesion-inducing properties of T. geleii W were at a maximum the fat and glycogen content of the organisms were also approaching a maximum. Glycogen, however, frequently disappeared rapidly when the cells had become nearly filled with fat. Acid phosphatase also ordinarily increased in activity during this period. The microscopic examination of the lesions induced by these senescent cells revealed that maximum fat deposition occurred in areas of maximum epithelial proliferation. It, therefore, appeared that the role of lipids in the proliferative process may have been underestimated. The corresponding appearance of maximum fat concentration in areas of greatest epithelial proliferation and in T. geleii W at the height of its lesion-inducing power indicated that although lipids may not be the dominant factor inducing growth their possible role in this process should not be overlooked. Acid phosphatase and glycogen also increased in T. geleii W during the period in which it manifested its maximum growth-inducing properties and may play an indirect if not dominant role in the proliferative

process. Experiments with organisms suspended in physiological saline emphasized the possible role of the phosphatases or other enzymes in growth induction as many of them diffused readily from specimens of T. geleii W and could be materially reduced in the washed organisms prior to inoculation onto the chorioallantois. Unwashed cultures of T. geleii W produced lesions larger than any induced by cultures suspended in physiological saline. Apparently the omission of washing conserved the phosphatases or other substances conducive to lesion induction. However, the culture medium itself may possess some growth-stimulating properties.

Neither the histochemical nor the morphological investigations of the induced lesions revealed any characteristics by which the induction agent could be accurately identified. Extensive variations existed between lesions produced by the same inocula. However, a tendency was noted for certain types of inducing agents to be specific in their stimulation of certain chorioallantoic tissues. In general, entities inducing maximum epithelial growth appeared to be concentrated in inocula prepared from protoplasm and appeared to increase in concentration as the age of the protoplasm used for inoculation increased. On the other hand, maximum mesodermal proliferation was stimulated by inorganic salt solutions and other suspending media. The addition of metallic ions to protozoan cultures reduced the protoplasmic effect on the epithelial tissues. The effect of metallic ions on mesodermal

tissues may be attributable to an alteration in the concentration of the extracellular fluid of the mesodermal connective tissue.

The endodermal epithelium and the ventral half of the mesodermal tissues exhibited the maximum growth stimulation and enzyme activity. The endodermal cells may normally be active sites of enzyme secretion which would aid in the transport of nutrient materials and would account for the strongly positive reaction visible in the ventral portions of the individual lesions. On the other hand, an apparent diffusion of enzymes or catalyzed end products from the dorsal to the ventral surface of the lesion was noted and the subsequent increased ventral growth may be attributable to the increased concentration of the diffused growth-promoting materials. It appeared, however, that all tissues of the chorioallantois were stimulated equally and that the growth differential of the ventral surface was normally greater than that of the dorsal. The increased amount of phosphatase activity present in the ventral half of the lesion might also have been a manifestation of induced stress, as Moog (1952) found that phosphatase in embryonic tissue was normally increased at times of stress.

Acid and alkaline phosphatase were visible in all lesions with the exception of several induced by physiological saline or protozoan cultures to which HCl, $MgCl_2$ or $CaCl_2$ had been added. The heaviest concentrations of both of the phosphatases and lipase were visible in the walls of arteries, veins and

capillaries of the induced lesions. It appeared, therefore, that in rapidly growing tissues these enzymes were either increased to enhance the transfer of nutrient materials across the vascular membranes or to catalyze the metabolism of substances present in the blood stream.

The presence of alkaline phosphatase in the mesodermal fibroblasts suggested a possible role in the metabolism of fibrillar proteins and the subsequent walling off of areas of active proliferation. Bradfield (1950) suggested that alkaline phosphatase may be concerned with the liberation of nucleic acid and protein from the nucleoprotein complex. Fell and Danielli (1943) noted that collagen formation in healing wounds was associated with a marked increase in alkaline phosphatase activity. Alkaline phosphatase activity was more pronounced in the induced lesion than in the induction agent and disappeared from T. geleii W when the latter's growth-induction properties were at a maximum. This suggested that alkaline phosphatase might act as a growth inhibitor by stimulating the formation of fibrillar proteins to wall off the irritants and growth-inducing materials. On the other hand, acid phosphatase was highly active during the period in which growth promotion by T. geleii W was pronounced and indicated that its role in various physiological processes may be important in growth promotion.

Glycogen appeared to be absent in all induced lesions. A positive Feulgen-Bauer reaction was obtained in the epithelial tissues of all lesions. However, the reaction was

not eliminated by saliva and was attributed to a mucopolysaccharide or other substance from which aldehydes could be released. In the induced lesions the glycogen may be utilized as rapidly as it is formed, so that it could not be detected by ordinary histochemical techniques. In embryonic tissue glycogen is ordinarily most highly concentrated in the yolk sac.

The localization of DNA activity suggested an interrelationship between alkaline phosphatase activity and DNA turnover in the proliferating tissue. Both were especially pronounced in the vascular tissue and peripheral fibroblasts and may be essential in the formation of cytoplasmic desoxyribonucleotides.

The calcium ion which activated most of the enzymes investigated in this study and decreased fat production in older T. geleii W also decreased the lesion-inducing properties of the senescent cultures. In young cultures calcium appeared to increase lesion induction. The effect of calcium on fat production may partially account for its inhibitory effect on lesion induction.

Croton oil at all concentrations was too powerful an irritant, and a detailed comparative study of lesion-induction by carcinogenetic agents and protozoan cultures could not be made. The small number and size of lesions induced by the strain 12 lymphomatosis agent was undoubtedly due to either an inherent resistance of the inoculated eggs or a loss in virulence of the viral culture.

Ringer's solution was the only inocula to simulate the normal extracellular environment. No inflammatory or growth-induction processes were stimulated. In all future research Ringer's solution should be used as the suspending medium.

Epithelial nests which were common to all lesions studied appeared to be invaginations of the ventral epithelium. Occasional nests were noted which contained lumens in which blood cells could be detected. The extreme morphological differences between these nests and the circulatory system eliminated any possible correlation between the two. A superficial similarity between these nests and the squamous cell carcinomas produced by Duran-Reynals (1952) with methylcholanthrene suggested that growth stimulation by carcinogenetic agents and protozoan cultures might encompass similar processes. Further investigations are required before any conclusions can be drawn.

Intranuclear and cytoplasmic inclusion bodies noted in many of the lesions could not be distinguished morphologically from those visible in viral infections. Other aggregates of morphologically similar entities were present on the periphery and in lumens of several lesions. All aforementioned bodies were most numerous in lesions produced by calcium oxide or 24-day old specimens of T. geleii W. This supports the hypothesis suggested by Fennell (1951) that inclusion bodies may be calcium soaps produced by the combination of preformed calcium salts with fat. No investigation was made to determine

the presence of any virus within the inocula, but as the virus requires living cells, it would be difficult to explain the production of these bodies by a CaO solution.

SUMMARY

1. Cultures of T. geleii W were stained every 72 hours throughout the life of the culture for alkaline and acid phosphatase, lipase, peroxidase, DNA, fat, glycogen and urates. A semi-quantitative determination of the amount of activity for each substance was made at each interval. The organisms were reared in tryptone and vitamin-enriched tryptone alone or in combination with magnesium or calcium chloride to ascertain the effect of the culture medium on the histochemical properties of T. geleii W. Histochemical tests were conducted both with organisms suspended in the original culture medium and in physiological saline.

2. No definite conclusions could be drawn as to the functions of the various substances investigated in T. geleii W as no distinct correlations were demonstrable. However, certain possible relationships were noted.

3. Alkaline phosphatase activity in T. geleii W reared in tryptone was most highly concentrated in the distal end of the cell and reached a maximum in cultures aged 288 hours. Alkaline phosphatase may play a role in DNA metabolism, vitamin synthesis and the transfer of nutrients across the cell membrane of T. geleii W. It may also play a role in the synthesis of carbohydrates in young T. geleii W. Inhibition tests indicated this phosphatase was non-specific.

4. A heavy extracellular precipitate interfered with an accurate localization of acid phosphatase activity in organisms suspended in the original culture medium. However, in cells reared in tryptone and suspended in physiological saline acid phosphatase activity was highly concentrated in the distal end of organisms less than 288 hours in age. In older cultures acid phosphatase activity shifted towards the nucleus. Maximum activity occurred at 144 hours. Acid phosphatase may play an important role in glycogen synthesis. Cyclic curves in activity suggested a possible relationship between acid phosphatase and DNA metabolism.

5. Lipase activity was most highly concentrated in the vicinity of the nucleus of organisms reared in tryptone and reached a maximum 360 hours subsequent to seeding. Lipase appeared to be concerned with the hydrolysis of fat in T. geleii W.

6. Glycogen deposition was heaviest between the nucleus and pellicle of T. geleii W and in organisms reared in tryptone reached a maximum at 432 hours.

7. A positive reaction for fat first appeared in the proximal tip of 3-day old T. geleii W and gradually expanded distally as the cells aged. Fat deposition reached a maximum in senescent cells.

8. Phospholipids were dispersed throughout T. geleii W. and first appeared in cells aged 504 hours.

9. DNA activity was restricted to the nucleus of T. geleii W and exhibited cyclic changes in concentration. An

inverse relationship existed between the size of the nucleus and the intensity of the Feulgen reaction.

10. No positive reaction for peroxidase or urates was visible in T. geleii W.

11. The addition of thiamine, nicotinic acid, riboflavin, and biotin to tryptone culture medium inhibited alkaline phosphatase and tended to decrease lipase activity and glycogen deposition in T. geleii W, but increased DNA and acid phosphatase activity. The addition of magnesium chloride to the culture medium counteracted the inhibitory effect of vitamins on alkaline phosphatase activity.

12. Suspension of T. geleii W in physiological saline decreased alkaline and acid phosphatase activity and the deposition of glycogen.

13. Calcium and magnesium chloride added to the culture medium increased alkaline and acid phosphatase activity and the deposition of glycogen but decreased the deposition of fat. In vitamin-enriched media these salts shifted the location of alkaline phosphatase and lipase activity.

14. The phenomenon of diffusion complicated the histochemical localization of alkaline and acid phosphatase. Complete diffusion of alkaline phosphatase occurred in widely dispersed cells.

15. Suspensions of T. geleii W in physiological saline and other salt solutions were prepared and used for inoculation of the chorioallantois of 12-14-day old pedigreed White Leghorn embryos. Croton oil, the strain 12 lymphomatosis

agent, embryonic grafts and the various suspending media were used as chorioallantoic inocula and their lesion-inducing properties compared with those of protozoan cultures.

16. All inocula utilized in these experiments, with the exception of Ringer's solution, possessed growth-promoting properties to some degree.

17. Two types of lesions were induced: (1) umbilicated and (2) gray thickened areas in the chorioallantois.

18. Lesion induction appeared to involve two processes: (1) an inflammatory reaction and (2) the presence of normally occurring growth-promoting substances.

19. A direct relationship existed between the age of the culture of T. geleii W and the size of the induced lesion.

20. Lesion-inducing properties of T. geleii W were at a maximum when acid phosphatase activity was increasing and fat and glycogen deposition approaching a maximum.

21. T. geleii W. cultures stimulated maximal proliferation of epithelial tissues while inorganic salt solutions induced a maximal proliferation of mesodermal tissues. A direct relationship existed between the extent of epithelial proliferation and the age of T. geleii W used for inoculation.

22. Suspension of T. geleii W in physiological saline prior to inoculation onto the chorioallantois reduced the size of lesion induced.

23. Maximal fat deposition was visible in areas of maximal epithelial proliferation and suggested that fat synthesis may play a role in cellular proliferation.

24. The heaviest concentration of alkaline and acid phosphatase and lipase activity was noted in walls of arteries, veins and capillaries of induced lesions. These enzymes may play a role in the transfer of nutrient materials across vascular membranes.

25. Alkaline phosphatase activity in mesodermal fibroblasts might be related to fibrillar protein metabolism and a subsequent inhibition of growth.

26. Intranuclear inclusion bodies were visible in induced lesions and an investigation of these bodies supported the hypothesis of Fennell (1951) that they may be calcium soaps.

27. No histochemical or morphological characteristic of the induced lesions could be utilized for the identification of the induction agent.

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PLATE I

Alkaline phosphatase activity in specimens of T. geleii W suspended in culture medium. All figures X800. Micrometer scale insert: 1 space = 0.01 mm.

Fig. 1. 24-hour cells reared in tryptone;

Fig. 2. 144-hour cells reared in tryptone;

Fig. 3. 216-hour cells reared in tryptone;

Fig. 4. 432-hour cells reared in tryptone;

Fig. 5. 288-hour cells reared in tryptone fortified with $CaCl_2$;

Fig. 6. 288-hour cells reared in tryptone fortified with $CaCl_2$ (control);

Fig. 7. 288-hour cells reared in tryptone fortified with $MgCl_2$; and

Fig. 8. 288-hour cells reared in tryptone fortified with $MgCl_2$ and exposed to an increased amount of $MgCl_2$ in the phosphatase buffer.

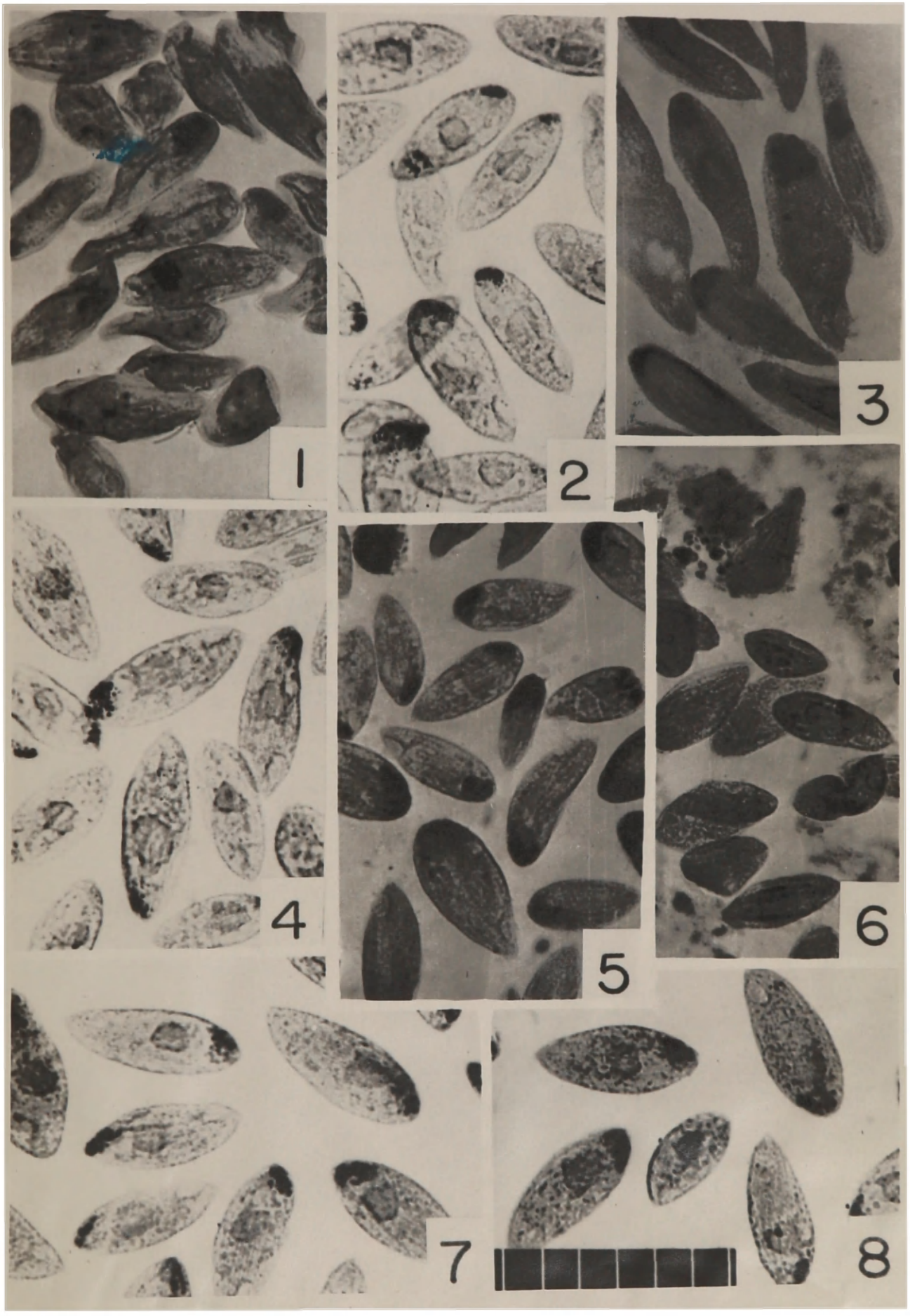
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PLATE II

Alkaline phosphatase activity in specimens of T. geleii W suspended in culture medium. All figures X600. Micrometer scale insert: 1 space = 0.01 mm.

Fig. 9. 288-hour cells reared in vitamin-enriched tryptone;

Fig. 10. 288-hour cells reared in vitamin and magnesium chloride-enriched tryptone;

Fig. 11. 288-hour cells reared in magnesium chloride-enriched tryptone;

Fig. 12. 72-hour cells reared in the initial riboflavin-enriched tryptone;

Fig. 13. Maximum phosphatase activity after first transfer to vitamin-enriched tryptone, 216-hour cells;

Fig. 14. 72-hour cells from 2nd transfer to vitamin-enriched tryptone; and

Fig. 15. 360-hour cells from 2nd transfer to vitamin-enriched tryptone.

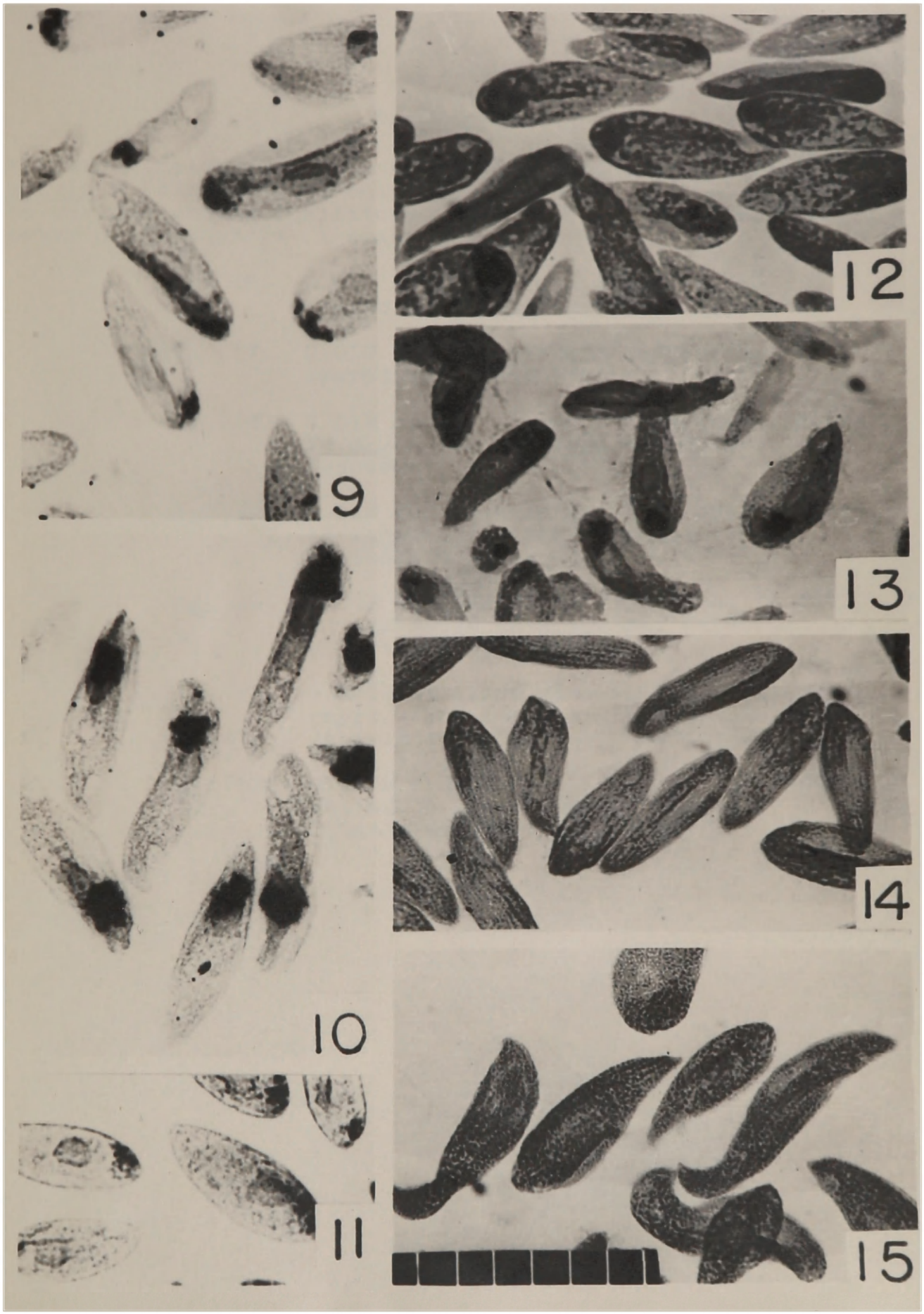
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PLATE III

Alkaline phosphatase activity in unwashed specimens of T. geleii W from 288-hour cultures after a 20-minute exposure to various enzyme inhibitors. All figures X600. Micrometer scale insert: 1 space = 0.01 mm.

Fig. 16. 0.01M sodium arsenate (specimens reared in vitamin-enriched tryptone);

Fig. 17. 0.01M sodium arsenate (specimens reared in tryptone);

Fig. 18. 0.01M sodium arsenate (specimens reared in magnesium chloride-enriched tryptone);

Fig. 19. 0.01M sodium arsenate (specimens reared in magnesium chloride and vitamin-enriched tryptone);

Fig. 20 0.02M semicarbazide (specimens reared in magnesium chloride and vitamin-enriched tryptone);

Fig. 21. 0.025M oxidized glutathione (specimens reared in magnesium chloride and vitamin-enriched tryptone); and

Fig. 22. 0.025M reduced glutathione (specimens reared in magnesium chloride and vitamin-enriched tryptone).

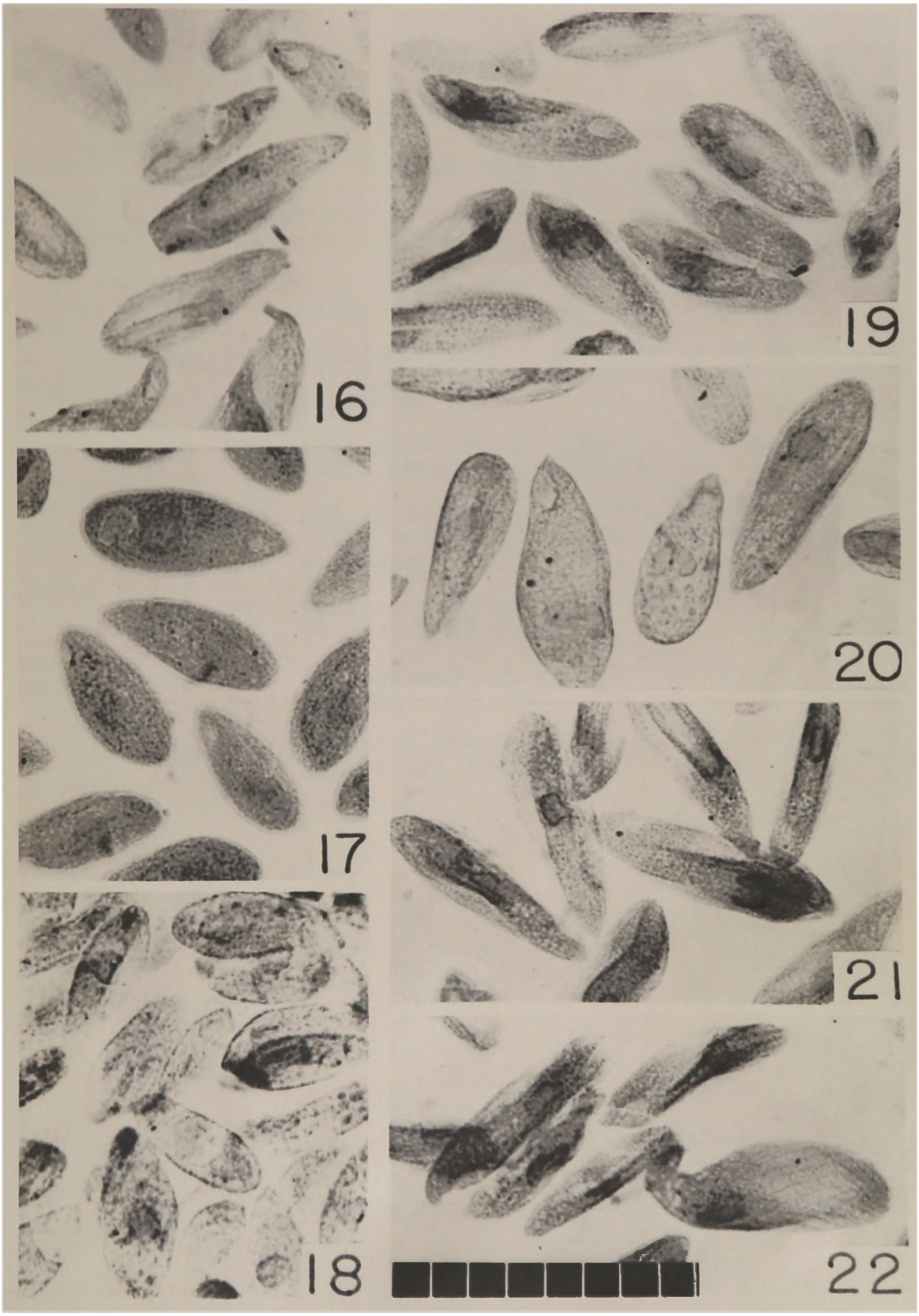
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PLATE IV

Histochemical reactions in specimens of T. geleii W. Specimens in Figs. 23-26 suspended in physiological saline and in Figs. 27-29 in the culture medium. All figures X800. Micrometer scale insert: 1 space = 0.01 mm.

Fig. 23. Acid phosphatase activity in 72-hour cells reared in tryptone;

Fig. 24. acid phosphatase activity in 72-hour cells reared in vitamin-enriched tryptone;

Fig. 25. acid phosphatase activity in 288-hour cells reared in tryptone;

Fig. 26. acid phosphatase activity in 288-hour cells reared in vitamin-enriched tryptone;

Fig. 27. lipase activity in 288-hour cells reared in tryptone;

Fig. 28. DNA activity in 288-hour cells reared in tryptone; and

Fig. 29. alkaline phosphatase activity in 144-hour cells reared in tryptone.

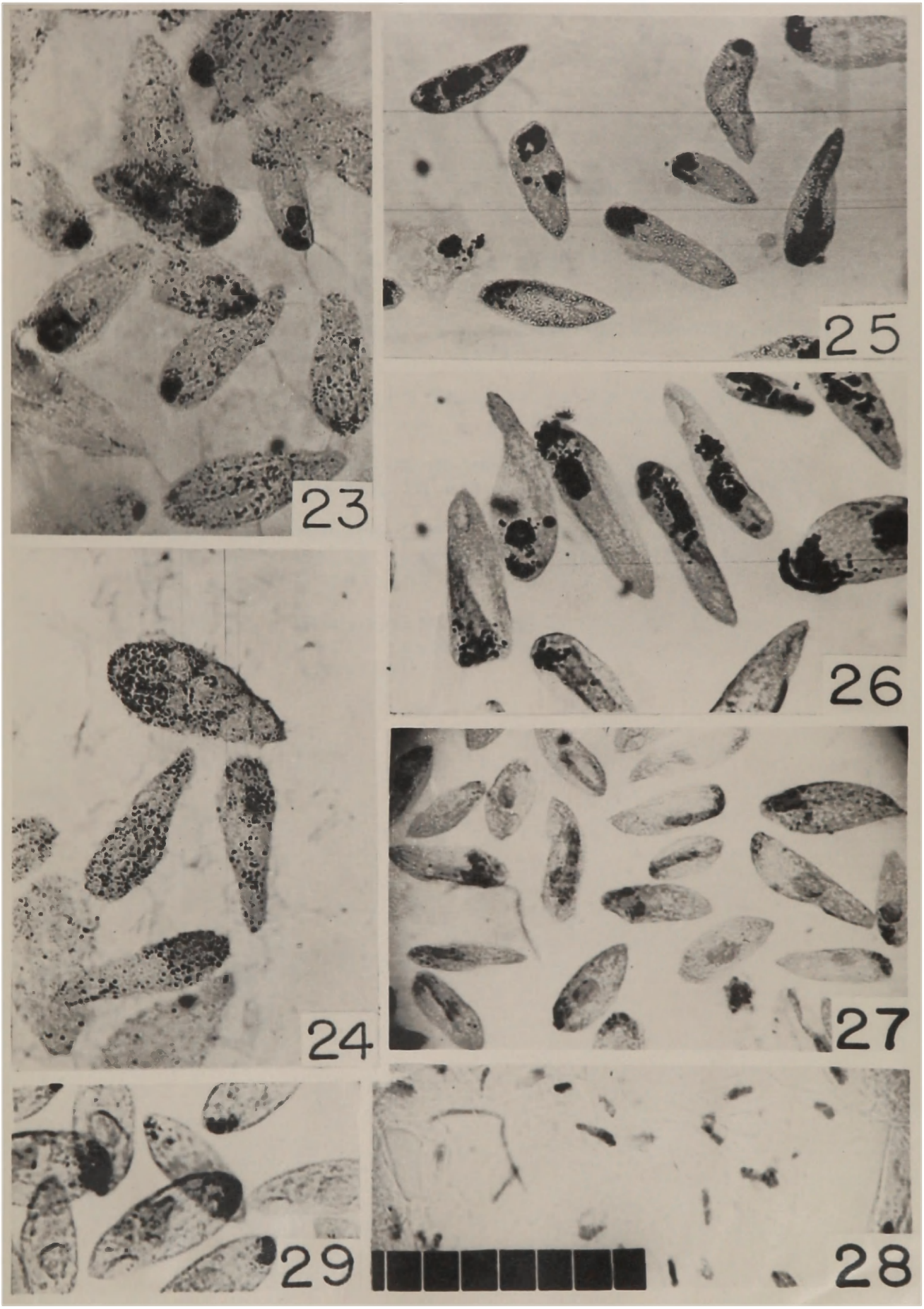
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PLATE V

Histochemical reactions in specimens of T. geleii W suspended in culture medium. All figures X600. Micrometer scale insert: 1 space = 0.01 mm.

Fig. 30. Glycogen deposition in 72-hour cells reared in tryptone;

Fig. 31. glycogen deposition in 288-hour cells reared in tryptone;

Fig. 32. glycogen deposition in 72-hour cells reared in vitamin-enriched tryptone;

Fig. 33. glycogen deposition in 288-hour cells reared in vitamin-enriched tryptone;

Fig. 34. phospholipid deposition in 72-hour cells reared in vitamin-enriched tryptone; and

Fig. 35. phospholipid deposition in 504-hour cells reared in vitamin-enriched tryptone.

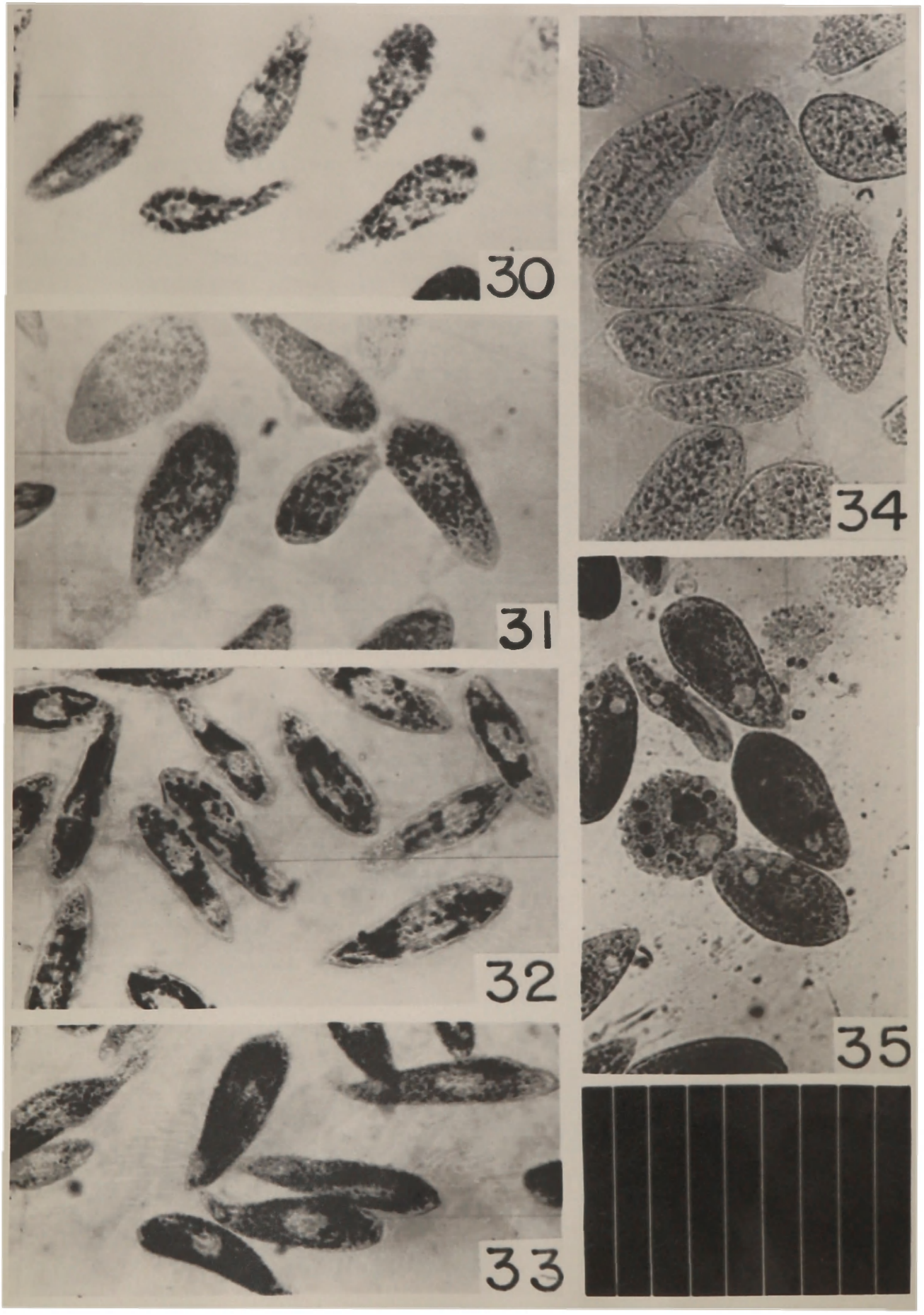
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PLATE VI

Chorioallantoic lesions induced by saline suspensions of T. geleii W. Lesions in Figs. 36, 37, 39 and 40 fixed in Zenker's fluid and stained with haemotoxylin eosin-azure. Lesions in Fig. 38 fixed in acetic-bichromate and in Figs. 41 and 42, in acetone. Figs. 36 and 42 X100. Figs. 37-41 X600. Micrometer scale insert: 1 space = 0.01 mm.

Fig. 36. Cross-section of chorioallantoic lesion showing epithelial nest formation induced by 18-day old T. geleii W;

Fig. 37. cross-section of epithelial nest induced by 18-day old T. geleii W;

Fig. 38. localization of fat in hyperplastic dorsal epithelium of chorioallantoic lesion induced by 14-day old T. geleii W;

Fig. 39. inclusion bodies in epithelium of chorioallantoic lesion induced by 3-day old T. geleii W;

Fig. 40. inclusion bodies in unabsorbed inocula of 3-day old T. geleii W;

Fig. 41. alkaline phosphatase activity in peripheral mesodermal fibroblasts of chorioallantoic lesion induced by 12-day old T. geleii W; and

Fig. 42. acid phosphatase diffusion in chorioallantoic lesion induced by 9-day old T. geleii W exposed to 50° C. for one-half hour.

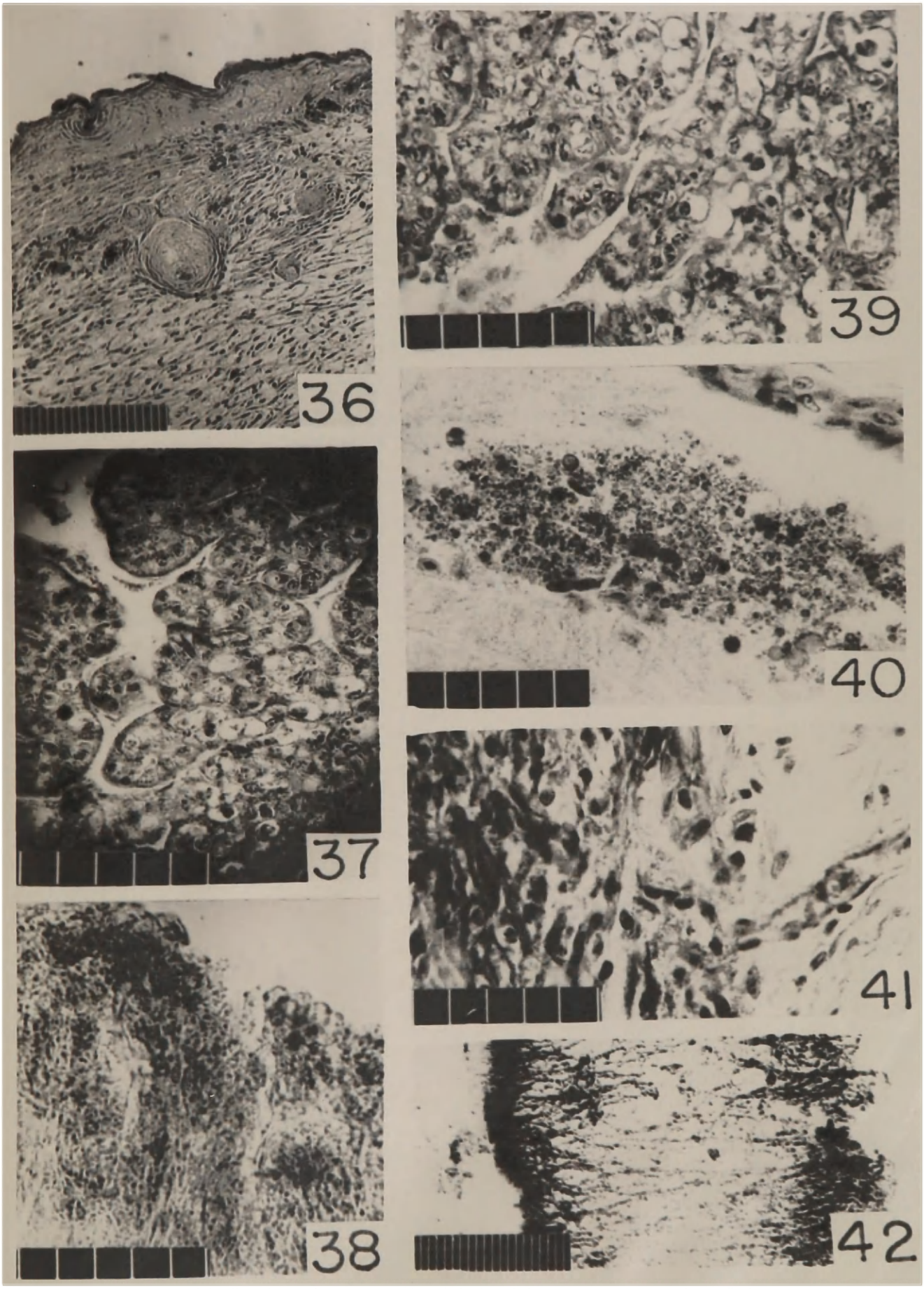
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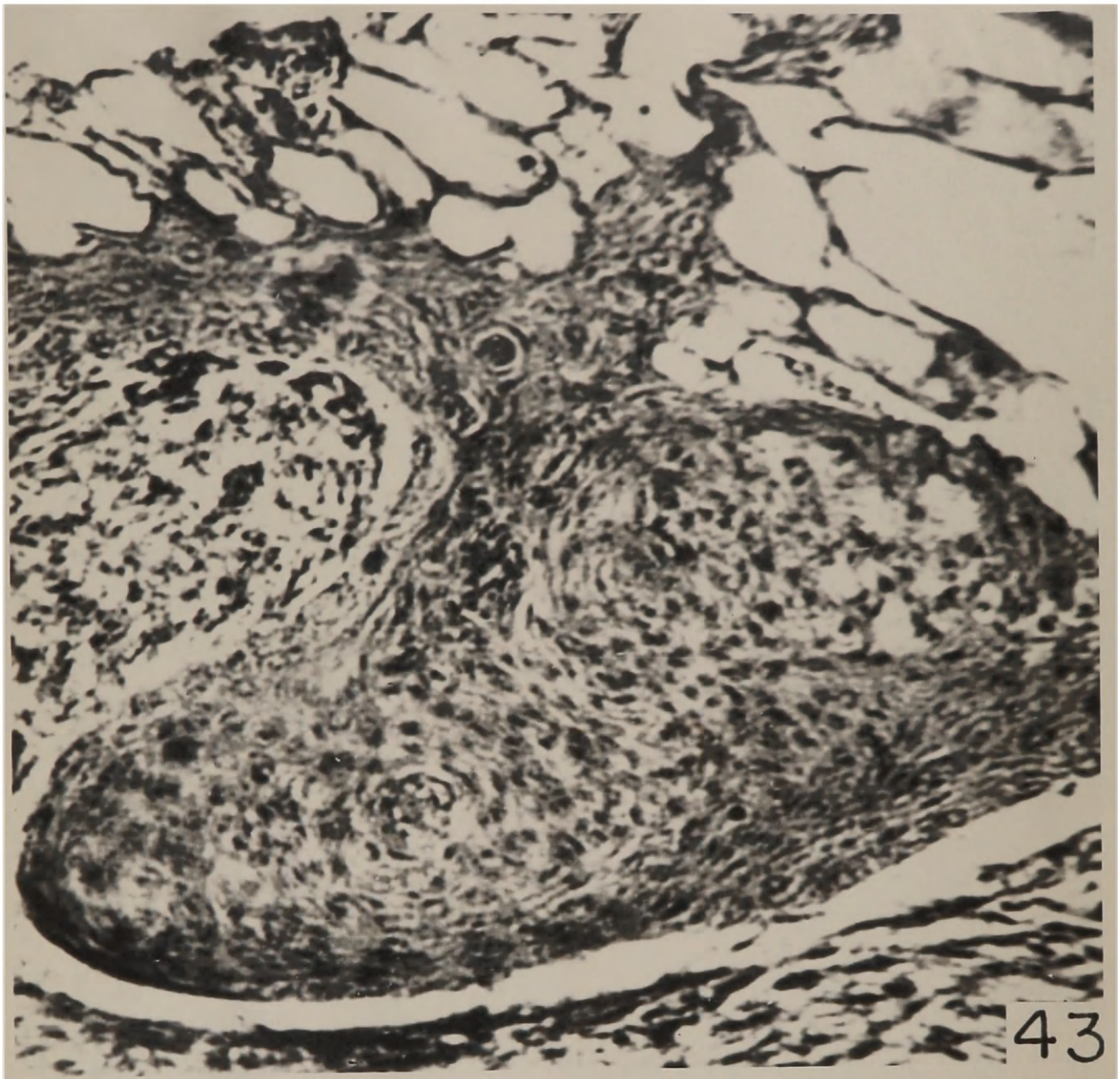
PLATE VII

Chorioallantoic lesions induced by saline suspensions of T. geleii W. Lesion in Fig. 43 fixed in Zenker's fluid and stained with haemotoxylin eosin-azure; Figs. 44 and 45 fixed in acetic-bichromate. All figures X1700. Micrometer scale insert: 1 space = 0.01 mm.

Fig. 43. Cross-section of ventral surface of chorioallantoic lesion induced by 3-day old T. geleii W showing formation of epithelial nests;

Fig. 44. fat deposition in dorsal epithelium of chorioallantoic lesion induced by 14-day old T. geleii W; and

Fig. 45. fat deposition in epithelial nests of chorioallantoic lesion induced by 14-day old T. geleii W.



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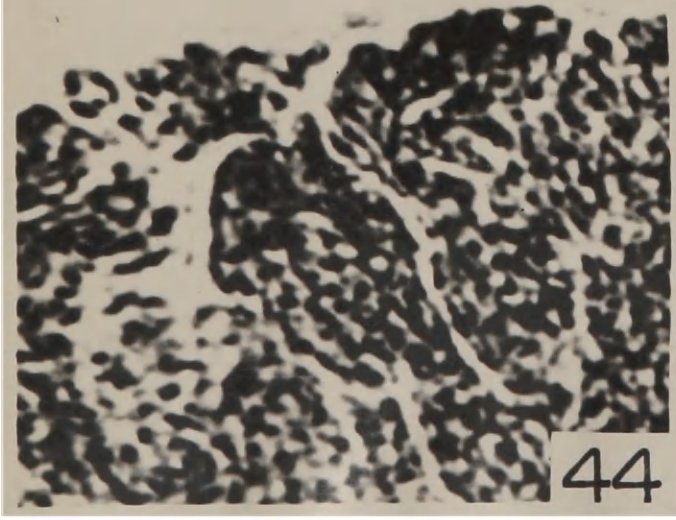

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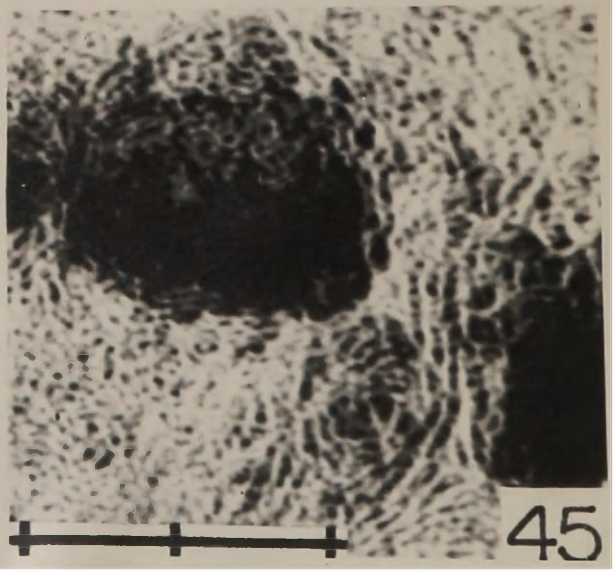

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