LOCALIZATION, SUBSTRATE SPECIFICITY, AND THE EFFECT OF INHIBITORS ON ALKALINE PHOSPHATASES OF *TETRAHYMENA GELEII* W

By

Edward F. Degenhardt

AN ABSTRACT

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

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THESIS

Edward F. Degenhardt

THESIS ABSTRACT

Organisms used for experimentation were cultured in bacteria-free tryptone, tryptone-$MgCl_2$, vitamin-enriched tryptone, vitamin-enriched tryptone with added $MgCl_2$, and tryptone-citrate solutions with added $MgCl_2$. Control tests were utilized in all experiments. Phosphatase was demonstrated in various substrate solutions, e.g., glycerophosphate, muscle adenylic acid, yeast adenylic acid, adenosine triphosphoric acid, glucose-1-phosphate, and creatine phosphate with the calcium-cobalt procedure of Gomori (1952). Results obtained with some substrate solutions were checked against those obtained with sodium alpha-naphthyl acid phosphate substrate solution (azo-dye procedure).

(1) Validity of the calcium-cobalt procedure was established; (2) results obtained with inhibitors and activators and various substrate solutions suggest that multiple phosphomonoesterases exist in Tetrahymena; and (3) activity values were established for phosphomonoesterases under various experimental conditions.

Type of culture medium and prolonged culture of organisms in specific media affected phosphatase activity, i.e., in vitamin-enriched tryptone, citrate buffered (0.01M) tryptone with added $MgCl_2$, phosphatase activity (calcium-cobalt procedure) was inhibited. Increased concentration of citrate

(0.04M) suppressed the calcium-cobalt reaction. Positive azo-dye reactions were obtained in organisms for 0.02M and 0.04M citrate buffered tryptone to which $MgCl_2$ was added and also in organisms from vitamin-enriched tryptone solutions. Prolonged culture of organisms in tryptone solutions increased the intensity and area of staining reaction with the calcium-cobalt procedure. Organisms from tryptone solutions which exhibited pronounced phosphatase activity and preformed phosphates with the calcium-cobalt procedure were negative for azo-dye phosphatase.

Inhibitor experiments were carried out using semicarbazide, 0.002M; sodium arsenate, 0.001M; KCN, 0.01M; sodium glycocholate, 0.006M; HCl, 0.01N; citrate buffered solutions (pH 5.0), 0.2M; glycine, 0.25M; NaCl, 0.01M; saline, H_2O_2, and distilled water (80° C.). Complete inhibition was obtained with semicarbazide, sodium citrate, and HCl. Activation of ATPase was induced by KCN while enzymatic reactions in all other substrate solutions were inhibited to a greater or lesser degree. Sodium arsenate and H_2O_2 caused no apparent inhibition of G-1-Pase or Cr-Pase but did inhibit glycerophosphatase, A-5-Pase, A-3-Pase, ATPase. Sodium glycocholate did not inhibit G-1-Pase while occasional inhibition of Cr-Pase occurred. Glycine inhibited glycerophosphatase, A-5-Pase, and Cr-Pase to a greater degree than ATPase and G-1-Pase. Saline and NaCl induced slight or no inhibition of glycerophosphatase, A-5-Pase, A-3-Pase, and ATPase.

Hot distilled water (80° C.) inhibited all enzymatic reactions.

Reactions for phosphatase were usually located in the posterior ends of cells. Nuclear staining reactions (calcium-cobalt procedure) were believed to be artifacts. However, occasional nuclear reactions with ATP substrate, under certain conditions, appeared to represent true enzymatic reactions.

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INTRODUCTION

The ciliated protozoa have been used to a limited extent for histochemical studies. Alkaline phosphatase was first localized in specimens of Colpidium campylum (T. geleii) by Sullivan (1950). He maintained that enzymatic activity was limited to the nucleus and that cytoplasmic reactions were dependent on migration of the enzyme from the nucleus into the cytoplasm. Elliott and Hunter (1951) demonstrated that T. geleii contained an enzyme capable of splitting phosphate from 5-nucleotides. The enzyme involved was confined to the cell and not released into the surrounding medium. Fennell (1951) found positive phosphatase reactions in the cytoplasm of T. geleii and attributed this activity to both preformed phosphate and phosphatase. Bowman (1952) found phosphatase activity in both the nucleus and the cytoplasm of T. geleii. Mugard (1953) studied alkaline phosphatase activity in specimens of Ophryoglena and found that enzymatic activity was absent in starved animals; upon ingestion of food, phosphatase activity became observable in the vicinity of the food vacuoles. Thereafter, phosphatase activity appeared in the macronucleus, micronucleus, and cytoplasm. Fennell and Marzke (1954) demonstrated that, in specimens of T. geleii cultured in tryptone solution, cytoplasmic alkaline phosphatase increased from a minimum (24 hour cultures) to a maximum

(288 hour cultures) and then rapidly decreased to zero (360 hour cultures).

Alkaline phosphatase has been extensively studied in vertebrate tissues. Danielli (1946) made a critical study of the calcium-cobalt technique of Gomori (1939) and Takamatsu (1939) for the localization of alkaline phosphatase. He concluded that the positive reactions obtained with the technique indicated the site of alkaline phosphatase activity. Gomori (1952) found under certain experimental conditions that both false-positive and false-negative reactions were obtained with this technique. Danielli (1950) stated that the Gomori technique suffered from a deceptive simplicity, and one using the procedure should have a good knowledge of physics and chemistry.

Johansen and Linderstrom-Lang (1951, 1952) evaluated the Ca-cobalt technique mentioned previously. They concluded that the method was not dependable since (1) calcium phosphate has a tendency to form supersaturated solutions; (2) the time required (0.1-2.5 seconds) for the phosphate to reach a critical concentration was sufficient for diffusion of phosphate into adjacent cellular areas where precipitation occurred on preformed crystal nuclei; and (3) the enzyme was uniformly distributed in cells. Gomori (1952) and Gomori and Benditt (1953), after a repetition of the Johansen-Linderstrom-Lang experiments, concluded "there is no experimentally demonstrable tendency to supersaturation under the conditions of

the correctly performed histochemical tests (calcium concentration not less than 0.05%, pH not less than 9.3), nor do preformed crystal nuclei have any effect on the results". He concluded his discussion with this statement: "Furthermore, the thesis that phosphatase is distributed evenly (or at random) among all cells or even within one cell body cannot be accepted."

Martin and Jacoby (1949) superimposed strongly alkaline phosphatase-positive sections of liver tissue upon sections in which phosphatase was not histochemically demonstrable. Positive reactions were obtained in the underlying tissue. They were unable, on the basis of their experiments, to say whether or not positive reactions were due to diffusion of the enzyme or to an enzyme activator. Yokoyama, Stowell, and Mathews (1951), who superimposed enzyme active duodenum sections on inactivated liver sections, believed that positive reactions in the nuclei of the latter were due to diffusion of the enzyme from enzyme active tissue into the inactivated tissue sections. Danielli (1953) performed essentially the same type of experiment by superimposition of phosphatase-active kidney sections on inactive liver sections. He believed that positive reactions in the underlying tissue were dependent on either diffusion of phosphatase itself or an activator substance. Novikoff (1951) presented evidence to show that Ca phosphate was adsorbed on nuclei and concluded that the Ca-cobalt technique was not reliable for localization of phosphatase. Gomori (1951) stated that

secondary adsorption of Ca phosphate by nuclei did occur and that in most cases nuclear staining was an artifact.

For additional details concerning nuclear phosphatases consult Chevremont and Firket (1953) who have extensively reviewed and summarized the literature in this field of investigation.

Menten, Junge, and Green (1944a, 1944b) and Mannheimer and Seligman (1948) employed the azo-dye method for demonstration of phosphatase. The Gomori (1952) azo-dye method was based on the supposition that naphthol liberated from sodium alpha-naphthyl acid phosphate would couple with an azo-dye (Diazo Blue B salt) to form an insoluble purple precipitate.

Characterization and localization of various phosphorylating enzymes was investigated by Baengwyn-Davies et al. (1950, 1952). They used glycerophosphate, glucose-1-phosphate, fructose-6-phosphate, glucose-6-phosphate, muscle adenylic acid, yeast adenylic acid, creatine phosphate, and adenosine triphosphate as substrates along with various enzyme activators and inhibitors. They concluded, on the basis of their research, that multiple phosphomonoesterases could be demonstrated in fresh frozen rat tissues. Gomori (1949) utilized nineteen different phosphate-containing substrates at different hydrogen ion concentrations. He was unable to distinguish phosphatases other than the common non-specific alkaline and acid varieties with 18 of the substrates; how-

ever, one substrate (p-chloranilidophosphate) did present a different enzymatic distribution in the acid range.

Herman et al. (1950) employed various substrates and inhibitors and demonstrated three major groups of phosphatase enzymes, i.e., cytoplasmic phosphatases (groups I and II) and nuclear phosphatases (group III).

Studies on the role of phosphatase enzymes in growth and development have been made by numerous investigators. Moog (1946) found that alkaline phosphatase increased in chicken embryos of 48 to 96 hours incubation, decreased between 96 and 144 hours, and then increased again between 144 and 240 hours incubation. Johnson and Bevelander (1947) found that presence of alkaline phosphatase was correlated with proliferation and differentiation of all cells that were concerned with feather development. Bevelander and Johnson (1949) demonstrated abundant phosphatase activity in ameloblasts during matrix formation and the subsequent calcification of enamel. Karczmar and Berg (1951) observed differences between alkaline phosphatase associated with the larval limb of Amblystoma and that functional in limb ontogeny and regeneration. Flavia and Pearson (1954) found phosphatase activity was reduced in rapidly growing tumors and in tumor areas which were especially anaplastic. Clark et al. (1950) found that phosphatase activity in humans increased between the sixth and ninth decades of life.

The function of phosphatase in cell metabolism has been treated by Krugelis (1946a), Kamen and Spiegelman (1948), Rothstein and Meier (1949), Bradfield (1950), Gold and Gould (1951), Danielli (1951), Moog and Wenger (1951), and Lesher (1952).

It is evident from this review of the literature on phosphatases that the function of phosphatases, the action of enzyme inhibitors, relative enzymatic activity in various substrate solutions, and the existence of multiple phosphatases is still open to question. Questions have also arisen concerning the reliability of the Gomori technique for demonstration of alkaline phosphatase in cell nuclei.

It is the purpose of this study to: (1) test the reliability of the Gomori technique for demonstration of alkaline phosphatase in cell nuclei; (2) ascertain whether or not, on the basis of enzyme inhibitors, localization, and staining intensity, multiple phosphatases exist in T. geleii; and (3) show the activity value for phosphatase in specimens of T. geleii from various ages and types of culture media.

MATERIALS AND METHODS

Specimens of T. geleii (strain W) used in the following experiments were cultured in bacteria-free (1) tryptone solution; (2) tryptone-$MgCl_2$ solution; (3) vitamin-enriched tryptone solution; (4) vitamin-enriched tryptone solution with added $MgCl_2$; and (5) tryptone-citrate solution with added $MgCl_2$.

Tryptone solution (Bacto-tryptone, Difco Laboratories, 15 gm.; KH_2PO_4, 1 gm.; and 1 liter glass-distilled water) was used as an experimental medium and also for the maintenance of stock cultures. Repetitions of experiments were made with organisms that were maintained in vitamin-enriched solutions prior to inoculation of experimental cultures (tryptone, tryptone-$MgCl_2$, etc.). Tryptone-$MgCl_2$ solutions were made by the addition of 1.07 gm. $MgCl_2$ to the basic medium. Vitamin-enriched tryptone solutions were made by the addition of 1 mg. riboflavin, 1 mg. thiamine hydrochloride, 1 microgram nicotinic acid, and 0.5 microgram biotin to the basic medium. Vitamin-enriched tryptone solution with added $MgCl_2$ was made in the same manner as vitamin-enriched tryptone medium except that 1.07 gm. of $MgCl_2$ was added. The final culture medium (tryptone-citrate solution with added $MgCl_2$) was varied slightly by replacing the KH_2PO_4 buffer present in the basic medium with a 0.01M sodium citrate buffer (2.06

gm. sodium citrate and 0.63 gm. citric acid per liter of culture solution). Magnesium chloride was then added to this solution in the amount of 1.07 gm. In other experiments, either 4.72 gm. of sodium citrate and 0.84 gm. of citric acid, or 8.44 gm. sodium citrate and 1.68 gm. of citric acid was substituted, i.e., 0.02M and 0.04M citrate buffer, respectively, for the 0.01M buffer. The final pH before sterilization was set at 5.5.

Organisms used in all experiments were cultured in 125 ml. Erlenmeyer flasks. Bacteria-free cultures of T. geleii were established by the transfer of 1 ml. of bacteria-free culture solution, in which organisms were abundant, to each of fifteen 125 ml. Erlenmeyer flasks which contained 75 ml. of sterile culture solution.

For localization of alkaline phosphatase in T. geleii from various culture solutions (tryptone solution, tryptone-$MgCl_2$ solution, etc.), tests were made 72 hours subsequent to inoculation and every 72 hours thereafter until organisms disappeared from the culture solutions.

Test organisms were obtained from the cultures by transfer of approximately 12 ml. of the culture solution (cultures were then discarded) to a 15 ml. centrifuge tube. The tube was centrifuged at 1500 r.p.m. for not longer than five minutes. Eleven milliliters of the supernatant were removed with a pipette. The organisms were left suspended in 1 ml. of culture medium. The cellular concentrate was withdrawn

from the centrifuge tube using a 1 ml. pipette. A drop (0.1 ml.) of this cellular concentrate was then placed on each clean albumen or gelatin coated slide and allowed to dry in air. The slides were transferred to 50 ml. of either cold C.P. or U.S.P. acetone (5° C.) or 85 per cent alcohol in a coplin jar and left for 3 to 36 hours (usually 24 hours) for fixation. The slides were removed from the coplin jar, hydrated to water, and stained, using one of the Gomori Ca-cobalt tests for alkaline phosphatase (Gomori, 1952). They were then dehydrated, cleared in xylene, and mounted in balsam.

The test for glycerophosphatase was run as described by Gomori (1952). Tests for 5-nucleotidase were run as described by Gomori (1952) using 20 mg. of muscle adenylic acid as a substrate. This technique was also utilized when yeast adenylic acid and adenosine triphosphoric acid were used as substrates. In tests using creatine phosphate (calcium salt) and glucose-1-phosphate (potassium salt), the same technique was used except the pH of the substrate mixture was adjusted to 9.4 instead of 8.3. The Ca-cobalt technique was checked in most instances by substitution of sodium alpha-naphthyl acid phosphate (azo-dye method of Gomori, 1952) for the Gomori substrate solution.

Slides were incubated in the following substrates for two hours: muscle adenylic acid, yeast adenylic acid, adenosine triphosphoric acid, creatine phosphate, and glucose-1-

phosphate; they were incubated for one hour in sodium glycerophosphate. When sodium alpha-naphthyl acid phosphate was used as a substrate, the incubation time was five to ten minutes. The incubation temperature in all cases, except when sodium alpha-naphthyl acid phosphate was used, was held at 37° C.± 1° C. The incubation temperature for the latter was 20° C.±2° C.

In inhibition experiments the slides were exposed for 15 minutes to an inhibitor mixture prior to immersion in the substrate solution. Final concentration of inhibitors was: sodium arsenate (0.001M and 0.01M), semicarbazide (0.002M and 0.02M), citrate buffer (0.2M, 0.01M, and 0.0001M, pH 4.5-5.0), HCl (0.1N), KCN (0.01M), H_2O_2 (1 ml. 30 per cent H_2O_2 made up to 50 ml. with distilled water), NaCl (0.01M), saline (0.14M), sodium glycocholate (0.006M), and glycine (0.25M). Water used for inactivation of enzymes was heated to 80° C.; slides on which organisms were mounted were immersed in it and left 15 minutes.

In other experiments $MgCl_2$ was left out of the substrate mixture in order to ascertain the enzyme activating effect of this salt.

In control tests either glycerophosphate was omitted from the incubation mixture or distilled water was used as a replacement for the substrate salts.

Estimation of enzymatic activity was made using a subjective rating scale. Reactions were given values ranging from 0-6: zero, no reaction; 1, very weak reaction (few positive cells, etc.); 2, light reaction; 3, moderate reac-

tion; 4, heavy reaction; 5, very heavy reaction; and 6, extremely heavy reaction (reactive area large, heavy black precipitates,all cells positive). Criteria used for ascertainment of reaction values were: (1) the intensity of the staining reaction; (2) the relative concentration of the reaction granules; (3) the area of the reaction; and (4) the relative number of positive cells (few, most, all). This estimate also involved all the cells on the slide rather than individual cells or groups of cells. The study involved approximately 1,000 slides; more than one reading per slide was made, and an average was taken.

It should be noted that this estimate of phosphatase activity is in no sense a quantitative value. It does, however, indicate trends in enzymatic activity and does provide a means of qualitative comparison. Subjective rating scales for the estimation of phosphatase activity similar to this were used by Maengwyn-Davies et al. (1950, 1952) and Wachstein and Meisel (1954).

RESULTS

Organisms used for experimentation were obtained from various ages of cultures subsequent to seeding. The age of cultures ranged from 72 to 1,080 hours. In most experiments culture age varied between 72 and 360 hours.

1. A comparison of the Ca-cobalt and azo-dye techniques for localization of alkaline phosphatase activity. Doyle et al. (1951) described the chemical aspects of the staining reaction in the Ca-cobalt procedure of Gomori (1939). Reaction products obtained with the azo-dye method were described by Novikoff (1952). The azo-dye method of Gomori (1952) was used to check the validity of localization of phosphatase with the Ca-cobalt technique. Both procedures are indirect methods for localization of enzymatic activity.

In the Ca-cobalt method, inorganic phosphate is liberated from a phosphate ester by the enzyme, and it is precipitated as Ca phosphate within the cell. Subsequent treatment of the precipitate with cobalt and ammonium sulfide transforms the colorless Ca phosphate into a dark brown or black precipitate at the sites of enzymatic activity.

In the azo-dye method, the organic portion of the molecule is utilized for demonstration of enzymatic activity. Phenol is liberated from a phosphate ester (sodium alpha-naphthyl acid phosphate) by enzyme action. A coupling of the

phenol with the azo-dye produces an insoluble purple precipitate in enzyme active areas of the cell.

Three generalized types of precipitates were obtained in specimens of T. geleii with the Ca-cobalt technique, i.e., (1) fine granular black precipitates; (2) an intermediate precipitate (fine granules plus a variable number of larger black granules); and (3) a large vesicular precipitate.

1. Fine granular precipitates were usually encountered in specimens from young cultures of organisms. Figure 1 shows specimens from a 144 hour tryptone-$MgCl_2$ culture. Individual granules which were stained bluish-black were barely resolvable with a high power lens system (X400). They were usually arranged to form a narrow oblique band at the posterior end of the cells. In other individuals the fine granules were randomly or uniformly distributed in the cytoplasm.

Organisms from this same 144 hour culture when stained with the azo-dye technique exhibited a precipitate which differed in several respects from the precipitate described in the preceding paragraph. The azo-dye precipitate was dark purple in color. A concentration of precipitate at the posterior end of the cell was the exception rather than the rule. In some specimens the anterior end of the precipitate was adjacent to the nucleus, but the posterior end terminated several microns anterior to the posterior tip of the cell (fig. 2). In other specimens the precipitate was restricted to one or more longitudinal bands. Precipitates

concentrated posterior to the nucleus, including the longitudinal band-precipitates, exhibited a variable number of large purple granules which were invested by fine purple granules. The larger cytoplasmic granules (two or more) in other individuals were randomly distributed in the cytoplasm.

2. The intermediate type of precipitate, obtained with the Ca-cobalt procedure, is illustrated in figure 3. Specimens were obtained from a 140 hour tryptone-$MgCl_2$ solution. It is evident that the precipitate consisted of a compacted mass of large and small black granules. In most specimens the enzyme active area was restricted to the posterior end of the cells, but in some organisms precipitates were either uniformly or randomly distributed in the cytoplasm. Compacted precipitates were sufficiently extensive to cover the posterior one-fourth of the cytoplasm.

A comparison of the Ca-cobalt reaction (fig. 3) with the azo-dye reaction (fig. 4) in specimens from the same 140 hour culture, shows that the intracellular phosphatase active area was located in essentially the same area of the cell, i.e., in the majority of organisms both Ca-cobalt and azo-dye precipitates were concentrated in the posterior portion of the cell. The comparison also shows that the extent of the phosphatase active area was essentially the same with both procedures. It is also evident in figure 4 that a few specimens exhibit a precipitate that has a tendency to be arranged in longitudinal bands.

3. Large granular Ca-cobalt precipitates were identified in specimens cultured in tryptone, tryptone-$MgCl_2$, and vitamin-enriched tryptone with added $MgCl_2$ solutions. Spherical vesicles, principal components of the precipitate, had an average diameter of three microns. They occurred singly or in clumps ranging in number from three to six or more (fig. 5). There was a tendency for vesicles to be concentrated at the posterior end of the cells, but in some specimens they were arranged in a linear series. In other specimens they were uniformly or randomly distributed in the cytoplasm. Staining reactions were variable with the Ca-cobalt technique. Range of color varied from light gray to dark black. Gray vesicles were homogeneous in appearance, and the darker vesicles exhibited variable amounts of black particulate matter uniformly distributed within the vesicle. A comparison of vesicular particulate materials within vesicles with the fine particulate matter in the posterior ends of cells showed that from a morphological point of view they were indistinguishable.

Large granular precipitates were produced by experimental procedures. Specimens of T. *pelcii* cultured in tryptone-$MgCl_2$ solution were concentrated at the bottom of a 15 ml. centrifuge tube by centrifugation at about 1500 r.p.m. All but 1 ml. of solution (in which specimens of *Tetrahymena* were concentrated) was removed with a pipette. Organisms tested for alkaline phosphatase at this stage in the proced-

ure exhibited positive reactions (fig. 6). The centrifuge tube was left for five minutes subsequent to the addition of 14 ml. of 0.01M citrate buffer (pH 5.1). Organisms were again concentrated by centrifugation, and then 14 ml. of the citrate buffer was removed with a pipette. Organisms that were left in the centrifuge tube were vigorously shaken, and then they were transferred along with 1 ml. of citrate solution to 75 ml. of bacteria-free tryptone-$MgCl_2$ solution in a 125 ml. Erlenmeyer flask. Specimens of T. geleii prior to transfer to tryptone-$MgCl_2$ solution did not show either vesicles or phosphatase activity.

After two hours incubation in the Erlenmeyer flask, specimens of T. geleii were removed from the tryptone-$MgCl_2$ solution, affixed to slides, transferred to cobalt nitrate solution, and left for five minutes prior to immersion in ammonium sulfide. An examination of the preparation showed an abundance of intracellular and extracellular vesicles (fig. 7). Phosphatase activity ranged from a weak (gray vesicles) to a strongly positive reaction (black vesicles). It is evident from these observations that positive phosphatase reactions can be obtained in Tetrahymena when incubation in substrate solution is omitted.

2. A comparison of glycerophosphatase and muscle adenylic acid phosphatase activity in specimens of T. geleii cultured in tryptone solution. Control slides were utilized in all experiments. Careful consideration was given to positive

reactions that were in part dependent on preformed phosphates. Thus, the results given in the following paragraphs actually represent trends in phosphatase activity.

It is evident in text-figure 1 that phosphatase activity in glycerophosphate (pH 9.4) increased from about 1.5 (72 hour cultures) to a maximum of 3.0 (144 hours), and then decreased to zero (360 hours). Phosphatase activity increased again to reach a second maximum of about 2.5 (504 hours), and then it decreased to about zero at 648 hours. A third maximum (3.0) was observed in specimens from 792 hour cultures (fig. 8). Phosphatase activity then gradually decreased to nearly zero in 1,080 hour cultures.

It was apparent late in these experiments that cells maintained in tryptone solutions for approximately six months exhibited increased amounts of preformed phosphates (fig. 9) with an average value of 5.0-6.0 (text-fig. 1). Under these conditions enzyme inhibitors were utilized for making a distinction between false-positive reactions and true phosphatase reactions.

It is also evident in text-figure 1 that phosphatase activity in A-5-P (pH 8.3) was similar to enzymatic activity in glycerophosphate, i.e., maximum and minimum activities were evident. A comparison of enzymatic activity in the two substrate solutions shows that succeeding maxima in A-5-P, subsequent to 288 hours, became progressively lower, 3.5 at 288 hours, 2.0 at 432 hours, and 1.0 at 792 hours. Further-

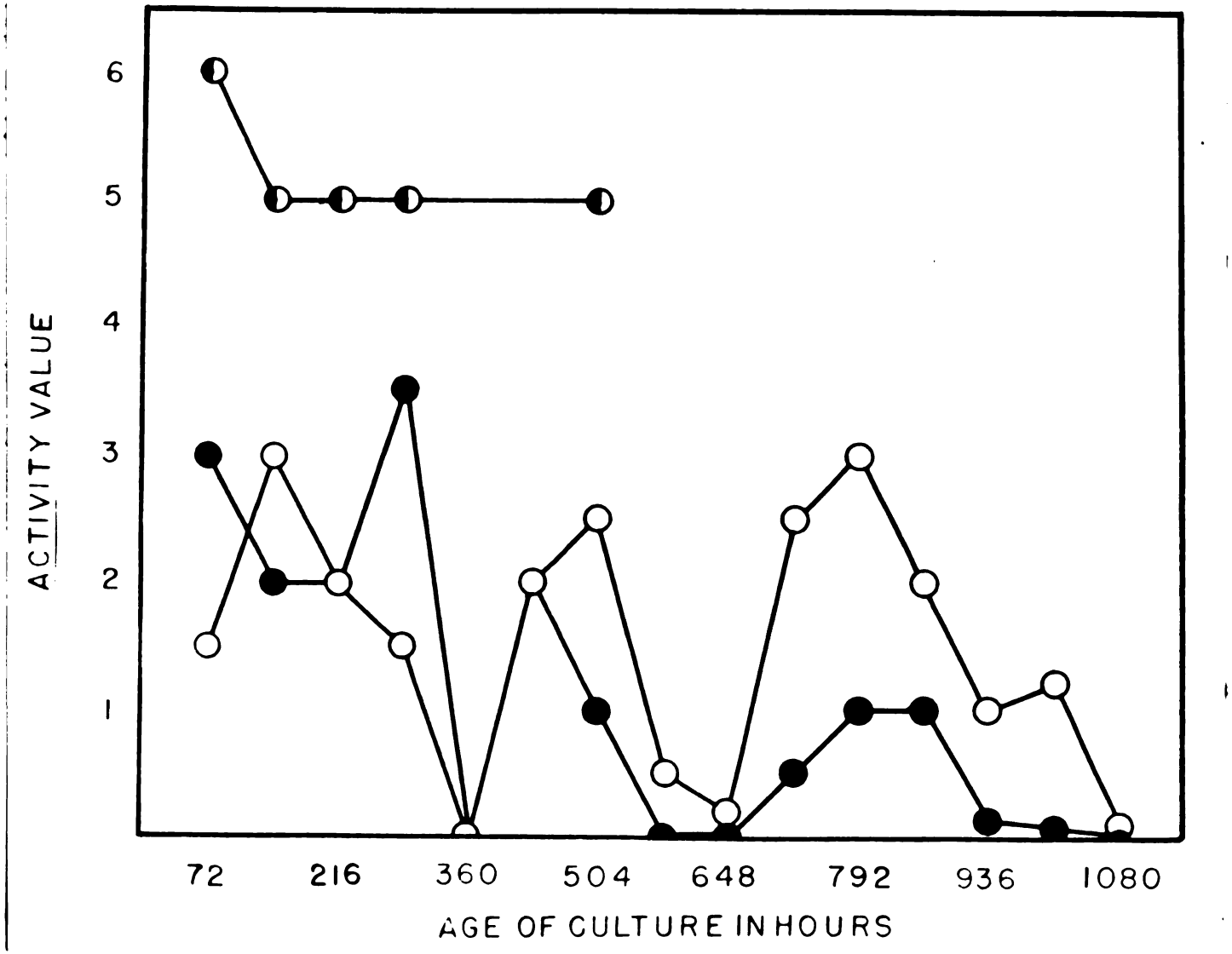
TEXT-FIGURE 1

Relation between culture age, glycerophosphatase activity, and A-5-Pase activity in specimens of T. geleii W cultured in tryptone media. Activity value ascertained as described under materials and methods.

◐, T. geleii W cultured in tryptone media for six months;

●, T. geleii W cultured in tryptone media and stained for A-5-Pase activity by the calcium-cobalt method of Gomori; and

○, T. geleii W cultured in tryptone media and stained for glycerophosphatase activity by the calcium-cobalt method of Gomori.


ACTIVITY VALUE
6
5
4
3
2
1
72
216
360
504
648
792
936
1080
AGE OF CULTURE IN HOURS

more, enzymatic activity decreased to about zero in A-5-P at 360, 576, and 936 hour cultures. On the other hand, glycerophosphatase activity decreased to zero at 360 and 1,080 hours. A comparison of figure 8 with figure 10 shows that maximum phosphatase activity in A-5-P substrate solution (288 hours) was higher than it was in glycerophosphate.

Text-figure 2 summarizes the results obtained with organisms cultured in tryptone-$MgCl_2$ solutions. A comparison of text-figure 1 with text-figure 2 shows that phosphatase activity in tryptone-$MgCl_2$ solution was higher than it was in tryptone solution. In the former, glycerophosphatase activity increased from about 3.0 in 72 hour cultures (the maximum activity in tryptone solution) to a maximum of about 5.0 in 360-432 hour cultures. Enzymatic activity then decreased to a minimum of about 1.0 as the age of the culture increased from 432 to 720 hours. Phosphatase activity increased again to reach a second maximum of 3.5 (792 hours), and then gradually decreased to 2.0 as the age of the culture increased to 1,080 hours.

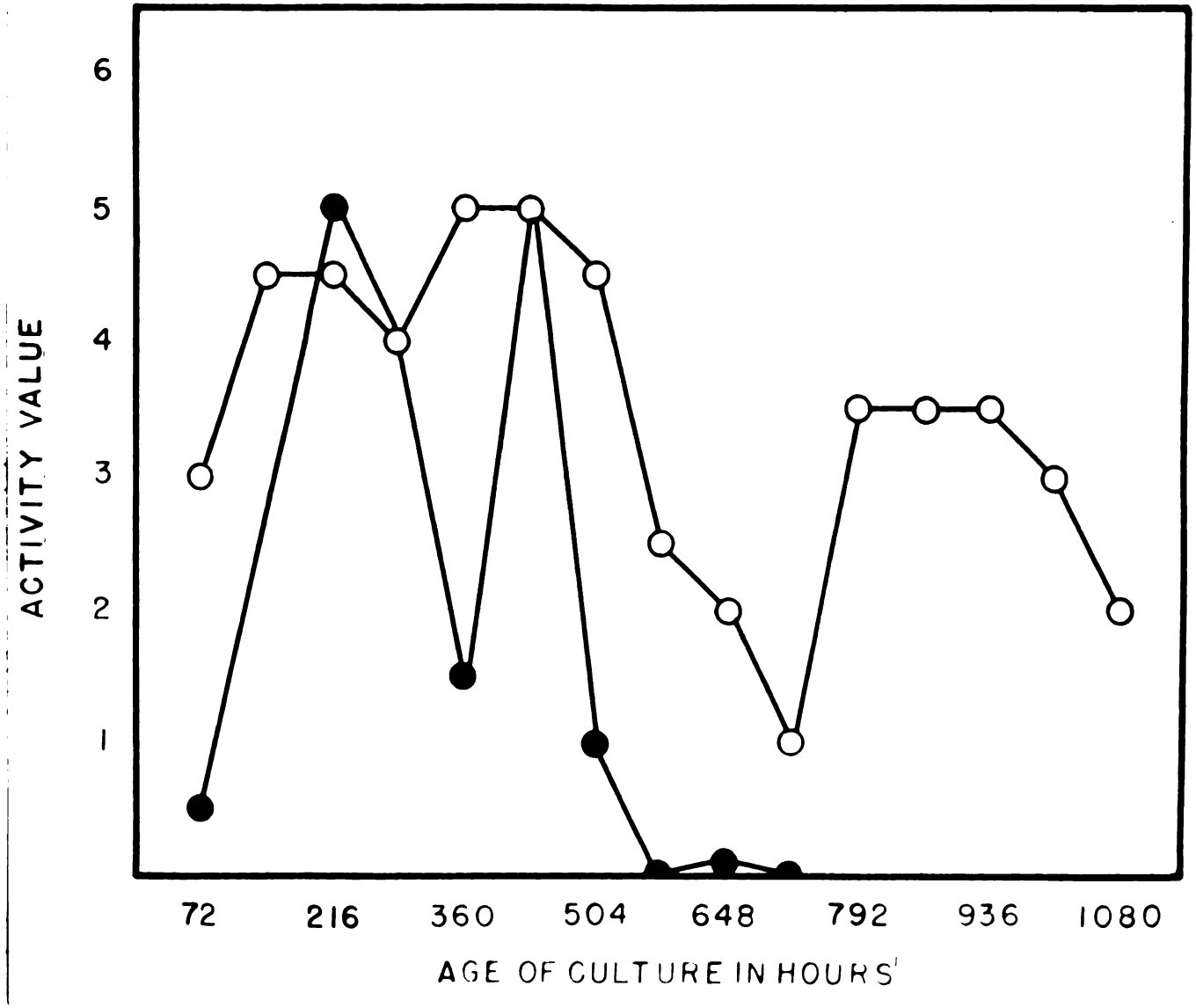
Text-figure 2 also shows that A-5-Pase activity was more erratic than that of glycerophosphatase in specimens cultured in tryptone-$MgCl_2$ solution. In the former, enzymatic activity was at a minimum of 0.5 at 72 hours. A-5-Pase activity then increased to a maximum of 5.0 at 216 hours (fig. 11), decreased to a second minimum of 1.5 at 360 hours, and then increased again to reach a second maximum of 5.0 at 432 hours. Minima of zero again occurred at 576 and 720 hours.

TEXT-FIGURE 2

Relation between culture age, glycerophosphatase activity, and A-5-Pase activity in specimens of T. pelcii W cultured in tryptone-$MgCl_2$ media.

○, Glycerophosphatase activity; and

●, A-5-Pase activity.


ACTIVITY VALUE
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AGE OF CULTURE IN HOURS

Results obtained with organisms cultured in vitamin-enriched tryptone solutions are summarized in text-figure 3. Phosphatase activity in glycerophosphate increased from 1.0 at 72 hours to a maximum of 3.0 at 288 hours, and then decreased to a minimum of 0.5 at 648 hours. It is evident in text-figure 3 that enzymatic activity in A-5-P tended to parallel enzymatic activity in glycerophosphate with the exception that in the former activity was lower. A-5-Pase activity in A-5-P increased from zero at 72 hours to a maximum of 2.5 at 144 hours, and then decreased to almost zero at 360 and 432 hours.

A comparison of text-figure 1 with text-figure 3 shows that, in general, total enzymatic activity was lower in organisms grown in vitamin-enriched tryptone solutions than it was in organisms from tryptone media. It also shows that in the former (vitamin-enriched tryptone solutions) enzymatic activity increased more uniformly to reach a maximum, and then it decreased to a minimum, whereas in tryptone solutions numerous maxima and minima were observed. A comparison of text-figure 2 with text-figure 3 shows that enzymatic activity was lower in cells grown in vitamin-enriched tryptone solutions than it was in organisms cultured in tryptone-$MgCl_2$ solutions.

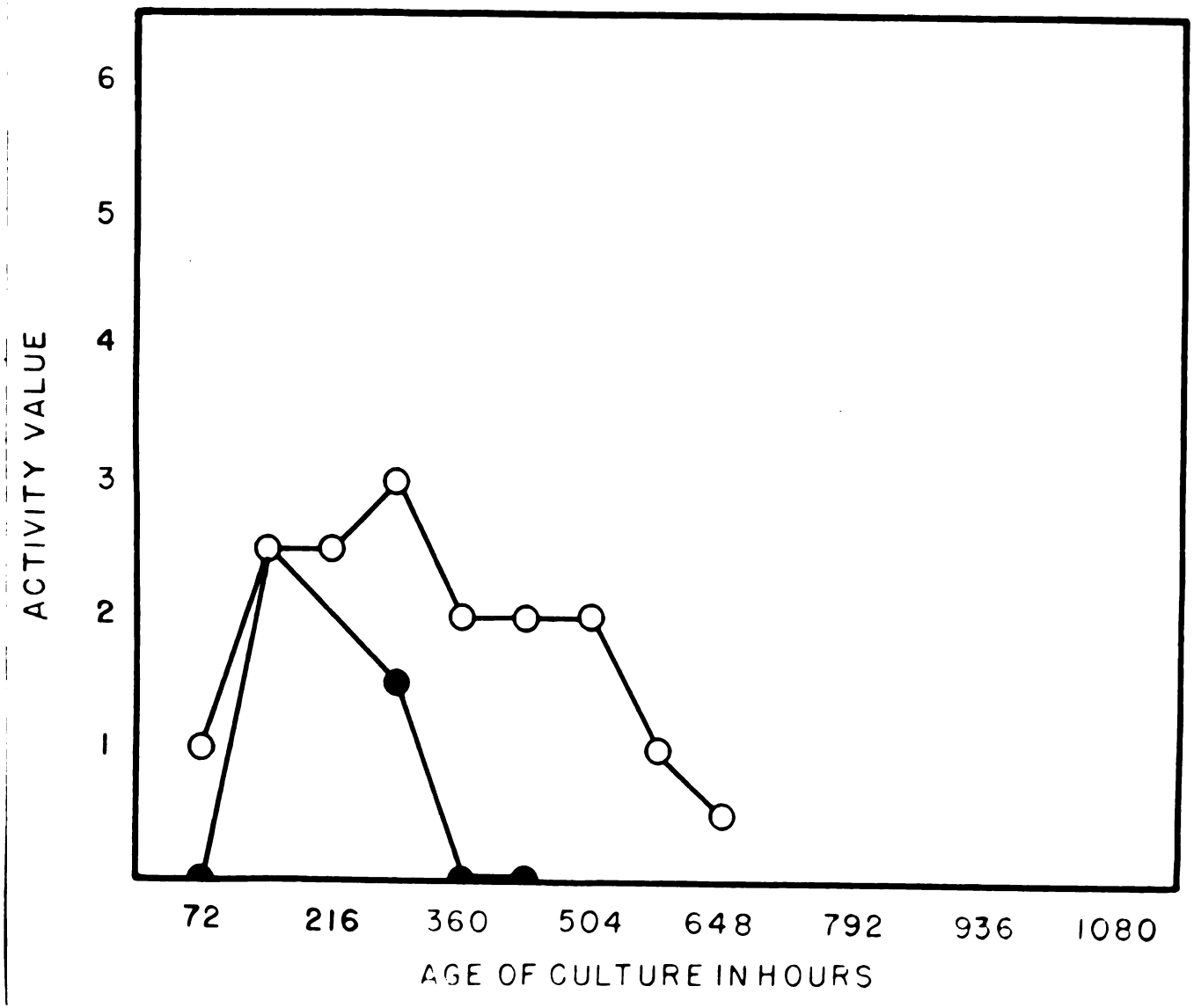
These results are in general agreement with those of Fennell and Harzke (1954) who found that strongly positive reactions (Ca-cobalt), which characterized organisms from

TEXT-FIGURE 3

Relation between culture age, glycerophosphatase activity, and A-5-Pase activity in specimens of T. geloii W cultured in vitamin-enriched tryptone media.

○, Glycerophosphatase activity; and

●, A-5-Pase activity.


ACTIVITY VALUE
6
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1
72
216
360
504
648
792
936
1080
AGE OF CULTURE IN HOURS

tryptone solution, decreased to zero in organisms that were cultured in vitamin-enriched tryptone, i.e., strongly positive phosphatase reactions were obtained in organisms from subculture 1, weak positive reactions in subcultures 2-7, inclusive, and no reaction whatsoever in subculture 8 (56 days in vitamin-enriched media. Organisms were maintained in vitamin-enriched tryptone solutions until the Ca-cobalt reaction was no longer demonstrable. Specimens were then stained with the azo-dye procedure and strongly positive reactions were obtained for alkaline phosphatase (fig. 12). A comparison of figure 13 with figure 12 shows that alkaline phosphatase activity in specimens from tryptone-citrate solution with added $MgCl_2$ (0.02M) was localized in essentially the same area as it was in organisms from vitamin-enriched tryptone.

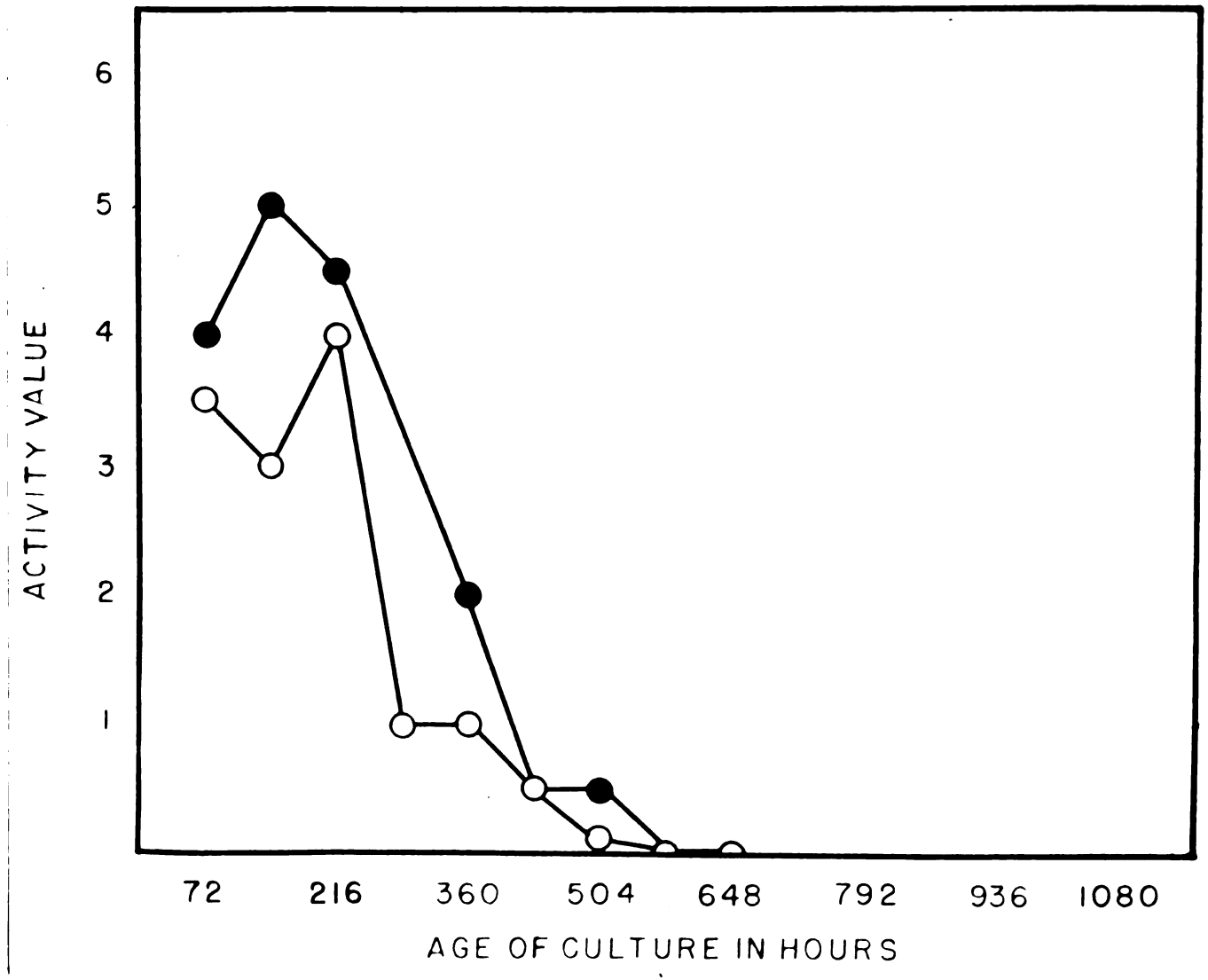
Text-figure 4 summarizes the results obtained with organisms cultured in vitamin-enriched tryptone solutions with added $MgCl_2$. It is evident here that enzymatic activity in glycerophosphate substrate solution increased from 3.5 at 72 hours (with a slight drop to 3.0 at 144 hours) to a maximum of 4.0 at 216 hours, rapidly decreased to 1.0 at 288 hours, and this was succeeded by a gradual decrease to zero at 576 and 648 hours. It is also evident that enzymatic activity in A-5-P was higher than it was in glycerophosphate. In A-5-P enzymatic activity increased from 4.0 at 72 hours to a maximum of 5.0 at 144 hours, decreased rapidly to 0.5 and zero at 432 and 576-648 hours, respectively.

TEXT-FIGURE 4

Relation between culture age, glycerophosphatase activity, and A-5-Pase activity in specimens of T. gelcii W cultured in vitamin-enriched tryptone media with added $MgCl_2$.

○, Glycerophosphatase activity; and

●, A-5-Pase activity.


ACTIVITY VALUE
6
5
4
3
2
1
72
216
360
504
648
792
936
1080
AGE OF CULTURE IN HOURS

A comparison of text-figure 4 with text-figures 1, 2, and 3 shows that enzymatic activity in both glycerophosphate and A-5-P drops much more sharply in vitamin-enriched tryptone solutions with added $MgCl_2$ than in the other experimental culture media. It is also evident (text-fig. 4) that enzymatic activity was initially higher in vitamin-enriched tryptone with added $MgCl_2$ than it was in tryptone, tryptone-$MgCl_2$, and vitamin-enriched tryptone media.

Results obtained with organisms from tryptone-citrate solutions with added $MgCl_2$ are summarized in text-figure 5. Enzymatic activity in glycerophosphate substrate solution rose from a minimum of 1.0 at 72 hours to a maximum of 2.0 at 144 hours, decreasing to a second minimum of 1.5 at 216 hours. Enzymatic activity reached a second maximum of 2.0 at 288 hours. The activity of the enzyme in A-5-P was almost zero in organisms from all ages of culture. Enzymatic activity was absent at 72 hours. It increased to about 0.1 at 144 hours and 216 hours, and then it decreased again to zero at 288 hours.

A comparison of text-figure 5 with text-figures 1, 2, 3, and 4 shows that maximum glycerophosphatase activity was lower and total A-5-Pase activity was lowest in tryptone-$MgCl_2$ solution in which the concentration of citrate buffer was 0.01M.

3. A comparison of yeast adenylic acid phosphatase, adenosine triphosphatase, glucose-1-phosphatase, and creatine phosphatase activity in tryptone solutions and in tryptone-citrate solutions with added $MgCl_2$. It is clearly evident from a comparison of text-figure 5 with text-figure 6 that phosphatase activity was higher in all substrate solutions (A-3-P, etc.) in organisms obtained from tryptone media. It can be seen that enzymatic activity in A-3-P was almost absent in organisms from tryptone-citrate solution with added $MgCl_2$ (text-fig. 5), while in tryptone (text-fig. 6) A-3-Pase activity was at a maximum of 3.5 in 72 hour cultures. This was followed by a minimum of 1.5 at 144 hours, a second maximum of 3.0 at 216 and 288 hours, and a subsequent decrease to zero at 360 hours.

Enzymatic activity in ATP substrate solution uniformly increased in organisms from 72 hours to 288 hours in tryptone-citrate solutions with added $MgCl_2$. Activity at 72 hours was at a minimum of 0.5, gradually increased to 1.5 at 144 and 216 hours, and then increased to a maximum of 2.0 at 288 hours. The opposite reaction was evident in cells grown in tryptone solutions. The activity was at a maximum of 4.5 at 72 hours (fig. 14); this was followed by a gradual decrease to zero at 360 hours.

Enzymatic activity in G-1-P substrate solution is shown in text-figure 5 by maxima of 2.5 at 72 and 288 hours, and minima of 2.0 at 144 and 216 hours. The opposite effect is

TEXT-FIGURE 5

Relation between culture age and enzymatic activity in specimens of *T. saleii W* cultured in tryptone-citrate solution (0.01M, pH 5.5) with added $MgCl_2$.

◑, G-1-Pase activity;

◒, Cr-Pase activity;

○, glycerophosphatase activity;

◒, ATPase activity; and

●, A-5-Pase, A-3-Pase activity.


ACTIVITY VALUE
6
5
4
3
2
1
72
144
216
288
AGE OF CULTURE IN HOURS

TEXT-FIGURE 6

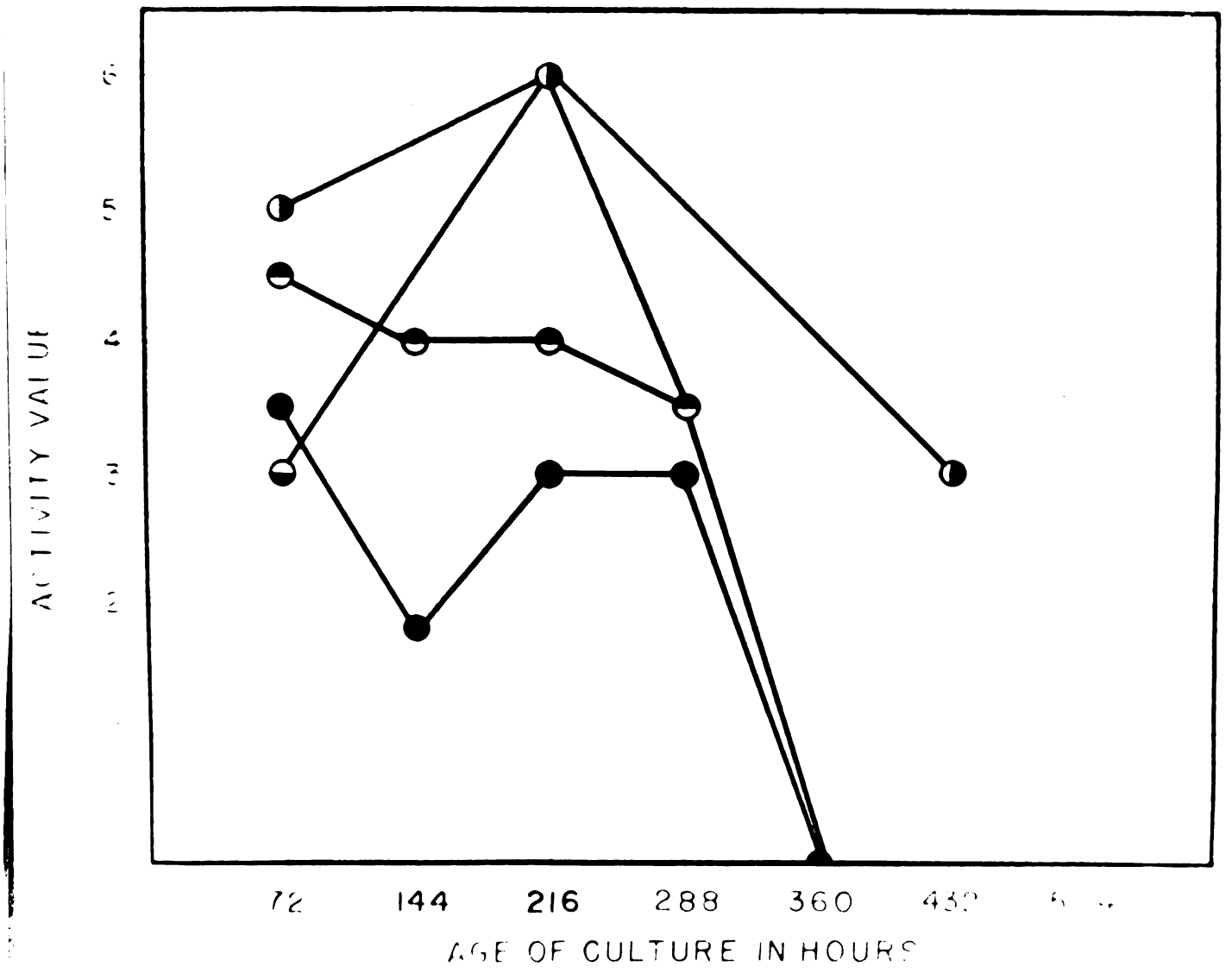
Relation between culture age and enzymatic activity in specimens of T. geleii W cultured in tryptone media.

◐, G-1-Pase activity;

◒, ATPase activity;

◓, Cr-Pase activity; and

●, A-3-Pase activity.


144
216
288
360
AGE OF CULTURE IN HOURS

shown in cells grown in tryptone where the results, when illustrated graphically, form an inverted "V". A minimum of 5.0 occurred at 72 hours followed by a maximum of 6.0 at 216 hours, succeeded by a second minimum of 3.0 at 432 hours (fig. 15).

It is evident in text-figure 5 that enzymatic activity in Cr-P substrate solution increased from 2.0 at 72 hours to a maximum of 2.5 at 144 hours, and then it decreased to a minimum of 2.0 at 216 hours. This was succeeded by a second maximum of 3.0 at 288 hours. It is also shown in text-figure 6 that Cr-Pase activity in tryptone rose from an activity value of 3.0 at 72 hours to a maximum of 6.0 at 216 hours (fig. 16), succeeded by a minimum of zero at 360 hours.

A comparison of text-figures 5 and 6 also shows that enzymatic activity in tryptone (text-fig. 6) was either represented by a curve that increased to a peak and then declined to a low level (G-1-P and Cr-P) or one in which there was essentially a continued lowering of enzymatic activity with culture age (ATP and A-3-P). Enzymatic activity in cells grown in tryptone-citrate solution with added $MgCl_2$ (text-fig. 5) is exhibited by curves essentially the reverse of those in text-figure 6, that is, there is a gradual rise in enzymatic activity in ATP and Cr-P substrate solutions, or there is a fall in enzymatic activity to minimal level which is followed by a rise in activity (G-1-P). The activity of A-3-Pase is here represented by minimal activity.

4. The relation between concentration of citrate buffer and phosphatase activity. Organisms from vitamin-enriched tryptone solutions with added $MgCl_2$ in which the concentration of citrate buffer was increased to 0.04M were used in this series of experiments.

Figure 13 shows that strongly positive alkaline phosphatase reactions were obtained with the azo-dye procedure in organisms from 144 hour cultures (216 hours subsequent to establishment of citrate buffered cultures). On the other hand, alkaline phosphatase activity was not demonstrated in organisms from this same culture with the Ca-cobalt procedure (fig. 17). Essentially the same results were obtained with organisms from 96 hour cultures (288 hours subsequent to establishment of citrate buffered cultures). The experiment was repeated again with organisms from a 96 hour culture (total time in citrate buffered solutions, 384 hours). Positive reactions were again obtained with the azo-dye procedure, and no reaction was obtained with the Ca-cobalt procedure although both A-3-P and ATP were substituted for glycerophosphate in the substrate solutions. Specimens incubated in the latter exhibited nuclei which were stained dark brown.

Stock cultures of organisms were maintained in 0.02M citrate buffered solutions by subculturing for 49 days, and then subcultures were established in which the concentration of citrate buffer was increased to 0.08M. Ninety-six hours

subsequent to seeding, negative reactions for alkaline phosphatase were obtained with both the azo-dye and Ca-cobalt procedures.

5. Phosphatase as related to inhibitor reactions, substrate specificity, and intracellular localization. Six phosphate-containing substrates were utilized at different hydrogen ion concentrations, e.g., sodium glycerophosphate, glucose-1-phosphate, potassium salt (G-1-P), and creatine phosphate, calcium salt (Cr-P), pH 9.4; muscle adenylic acid (A-5-P), yeast adenylic acid (A-3-P), and adenosine triphosphoric acid (ATP), pH 8.3.

In general, all substrates tested gave positive reactions which were usually restricted to the posterior ends of the cells. Part of this posterior activity was due to preformed phosphates as was shown by control slides. Hydrogen ion concentration of the substrate solution was an important factor in demonstration of preformed phosphates, i,e, the concentration of preformed phosphate was greater at pH 9.4 than at pH 8.3. No method was devised for removal of phosphate without destroying all phosphatase reactions along with the preformed phosphates. In all cases critical comparisons of control and substrate slides were made, and only those results are reported which demonstrated distinct differences between experimental and control organisms.

Various compounds known to cause inhibition or activation of phosphatase enzymes, Macgwyn-Davies et al. (1950,

1952) and Newman et al. (1950), were used in conjunction with the substrates. Organisms were left in the various inhibitor solutions for 15 minutes prior to immersion in substrate solution. Results obtained are presented in Table I which summarizes the effect of inhibitors and activators on the phosphomonoesterases of T. geleii. It is apparent that 0.002M semicarbazide, 0.2M citrate, pH 5, and 0.1N HCl completely suppressed all phosphatase activity. These inhibitors did not distinguish between false-positive and true enzyme reactions. This was not the case with the other inhibitors used. Potassium cyanide, 0.01M, tended to activate ATPase. It induced moderate inhibition of A-5-Pase, and caused slight or no inhibition of glycerophosphatase, G-1-Pase, and Cr-Pase. Its action on A-3-Pase was erratic. Sodium arsenate, 0.001M, caused less inhibition of glycerophosphatase, ATPase, G-1-Pase, and Cr-Pase than of A-5-Pase or A-3-Pase. Glycine, 0.25M, caused little reduction of G-1-Pase activity, slightly more inhibition of ATPase, and induced moderate to heavy suppression of glycerophosphatase, A-5-Pase, A-3-Pase, and Cr-Pase activity. Saline and 0.01M NaCl caused little or no reduction of enzymatic activity. Distilled water at 80° C. always induced inhibition of enzymatic activity but seldom completely suppressed positive phosphate reactions. H_2O_2 caused no inhibition of G-1-Pase and Cr-Pase; glycerophosphatase and A-3-Pase were inhibited to a greater degree than either ATPase or A-5-Pase. Sodium glycocholate, 0.006M,

TABLE I

THE EFFECT OF INHIBITORS AND ACTIVATORS ON THE PHOSPHOMONOESTERASES OF T. GELEII W

		Glycero-phosphatase	A-5-P ase	A-3-P ase	ATP ase	G-1-P ase	Cr-P ase
pH of substrate solution		9.4	8.3	8.3	8.3	9.4	9.4
Sodium arsenate	0.001M	0-1	1-2	0-2	0-1	0	0
	0.01M	2	4	---	---	---	---
Semicar-bazide	0.002M	4	4	4	4	4	4
	0.02M	4	4	4	4	4	4
KCN	0.01M	1	2	0-1-//	//	0-1	0-1
Sodium glyco-cholate	0.006M	---	---	---	---	0	0-1
Citrate buffer pH 5.5	0.2M	4	4	4	4	4	4
	0.01M	3	---	---	---	---	---
	0.0001M	1	---	---	---	---	---
HCl	0.1N	4	4	4	4	4	4
Glycine	0.25M	2-3	2-3	2-3	1-2	0-1	2-3
H_2O_2 (1 ml. 30 percent in 49 ml. H_2O)		1-2	1	1-2	0-1	0	0
Saline		0-1	0-1	0-1	---	---	---
NaCl	0.01M	0	0-1	0-1	0-1	---	---
$MgCl_2$		/0	/0	/0	/0	/0	/0
Distilled water 80° C.		3	3	3	3	3	3

TABLE I (Cont.)

Specimens of T. goleii mounted on slides which served as controls were normally positive for preformed phosphates with the Gomori Ca-cobalt technique. Organisms incubated in substrate solutions without prior immersion in inhibitor or activator substances also exhibited positive staining with this technique.

Explanation of Table number values is as follows: ---, no data available; 0, no inhibition; 1, slight inhibition; 2, moderate inhibition; 3, heavy inhibition; 4, total inhibition; ≠, activation; ≠0, no activation or inhibition.

caused no inhibition of G-1-Pase and little suppression of Cr-Pase activity. The addition of $MgCl_2$ to the various substrate solutions did not seem to inhibit or stimulate phosphomonoesterase activity. This was in contrast to the usually accepted fact that $MgCl_2$ is an activator of phosphatase enzymes.

Substrate specificity was demonstrated utilizing staining intensities and the presence or absence of enzymatic reactions. In general A-5-Pase and A-3-Pase gave lighter and less intense reactions than either glycerophosphatase or ATPase. It can be seen in text-figures 1-5 that if the reaction for glycerophosphatase was absent, the reaction for A-5-Pase was also absent. On the other hand, the reaction for glycerophosphatase did not seem to depend on the presence or absence of A-5-Pase. The behavior of A-3-Pase in this respect was essentially the same as A-5-Pase. ATP usually gave the best results of any substrate used, i,e, a good distinction between control slides and substrate slides could usually be made. ATPase activity was in most cases demonstrable even if the A-5-Pase and A-3-Pase reactions were absent (text-fig. 5). The reverse was not observed. When A-5-Pase and A-3-Pase were present, their activity was less than that of ATPase where direct comparisons were made (text-fig. 5-6). The ATPase reaction usually was less intense than the reaction for glycerophosphatase in tryptone-citrate solutions with added $MgCl_2$ (text-fig. 5).

The reactions for G-1-Pase and Cr-Pase tended to be higher than those obtained for ATPase and glycerophosphatase, and always higher than the reaction for A-5-Pase and A-3-Pase (text-figs. 5-6). In general there was a slight tendency for Cr-Pase to give a stronger reaction than G-1-Pase when the cells were cultured in tryptone-citrate solution with added $MgCl_2$ (text-fig. 5). When only tryptone was present in the culture medium, the reverse seemed true (text-fig. 6).

A differential location of enzymes attacking phosphomonoesters in T. geleii was not demonstrable in organisms from tryptone, tryptone-$MgCl_2$, vitamin-enriched tryptone, or vitamin-enriched tryptone with added $MgCl_2$. However, ATPase presented a different pattern of enzymatic activity in cells grown in tryptone-citrate solutions with added $MgCl_2$ (0.01M, pH 5.5), i.e., both nuclear and cytoplasmic activity was demonstrated.

6. Nuclear staining and diffusion artifacts observed in T. geleii. Nuclear staining was observed most frequently with glycerophosphate, G-1-P, Cr-P, and ATP, less frequently with A-5-P and A-3-P, and never with sodium alpha-naphthyl acid phosphate (azo-dye).

With few exceptions (noted below), organisms incubated in control solutions and in the various inhibitor preparations also exhibited nuclear staining. Cells subjected to 0.2M citrate buffer (pH 5) for 15 minutes and incubated in glycerophosphate for 24 hours gave only nuclear reactions (fig.18).

The nuclear reactions observed with ATP in cells cultured in tryptone-citrate solution with added $MgCl_2$ (pH 5.5) appeared to be of a different type and intensity than the other nuclear reactions (fig. 19). The nuclei were very dark and highly discernible. Cells from the same cultures incubated in A-5-P, G-1-P, and Cr-P substrate solutions (pH 8.3) did not show this same type of nuclear reaction. The nuclear reaction in ATP was demonstrated only in cells raised in culture media where the 0.01M citrate buffer (pH 5.5) was present. However, the reaction was not always observed. It was seen when cytoplasmic reactions were either positive or negative. Nuclear reactions other than the one just described seem to indicate the presence of diffusion artifacts.

It has already been mentioned that most specimens of T. geleii exhibited preformed phosphates. It should be noted, however, that in dividing cells preformed phosphates and enzymatic reactions were either entirely absent or a few faint-staining reaction granules were located near the nucleus. Posterior cytoplasmic reactions were never observed in dividing cells. A diffuse gray staining in the cytoplasm immediately beneath the pellicle in all organisms was assumed to be a diffusion artifact.

DISCUSSION

1. Ca-cobalt and azo-dye phosphatase. Gulland and Jackson (1938a) maintained that both phosphodiesterases and phosphomonoesterases existed in a variety of plant and animal tissues. Phosphodiesterases liberated phenol but did not split phosphoric acid from diphenylphosphate. These authors concluded that polynucleotidases, which split nucleotides from nucleic acids, were phosphodiesterases.

Krugelis (1946b) suggested that the nucleus and cytoplasm contained multiple alkaline phosphatases. Liberation of inorganic phosphate from substrate solutions was dependent on two reactions: (1) phosphodiesterases split nucleotides from nucleic acid; and (2) phosphomonoesterases hydrolyzed nucleotides to yield inorganic phosphate.

Accumulations of preformed phosphates in the tissues of higher vertebrate animals was reported by Bourne (1944). Elliott and Hunter (1951), who investigated phosphatase (5-nucleotidase) in T. geleii, demonstrated that during the growth cycle of this ciliate total phosphorus in the supernatant decreased while intracellular phosphate increased. Kamen and Spiegelman (1948) maintained that if "inorganic phosphate" was stored in organisms, it was probably derived from the breakdown of organic phosphates. Willmer (1942) believed that the accumulation of Ca phosphate in fixed cells was an artifact.

Results obtained in this study showed that the position of preformed phosphate ("inorganic phosphate") in T. pelsii was in essentially the same position as the purple precipitate which was obtained with sodium alpha-naphthyl acid phosphate substrate solution. This suggests that if phosphate storage occurred, its location was in essentially the same position as enzymes hydrolyzing organic phosphate compounds, i.e., in the posterior end of the cells.

Other observations made in this study suggest that only Ca-cobalt phosphatase (glycerophosphatase) was functional after prolonged culture of organisms in tryptone solutions. Furthermore, Ca-cobalt reactions were inhibited in organisms from citrate buffered (0.01M, pH 5.5) tryptone solutions, and they were completely suppressed when the concentration of citrate was increased from 0.01M to 0.04M. On the other hand, organisms from the 0.04M citrate-tryptone solutions exhibited strong positive reactions with the azo-dye technique. Repetitions of these tests approximately 304 hours subsequent to introduction of organisms into citrate buffered tryptone were positive for phosphatase with the azo-dye technique.

A discussion of the function of phosphatases can be established on the basis of presence or absence of enzymatic activity in organisms from various culture solutions (tryptone, tryptone-$MgCl_2$, vitamin-enriched tryptone). Fennell and Marzke (1954) also found that specimens of Tetrahymena

maintained in vitamin-enriched tryptone for several months did not exhibit phosphatase activity (Ca-cobalt technique). Observations made in this study showed that organisms from vitamin-enriched cultures exhibited abundant phosphatase activity with the azo-dye technique. Since it is well established that growth of Tetrahymena was accelerated by addition of vitamins to tryptone solutions, it seems reasonable to assume that vitamins supply some specific unit, i.e., possibly coenzymes, which makes it possible for azo-dye phosphatase to exert a role in the synthesis of protoplasm.

Meyerhof and Green (1950) demonstrated that "phosphatase reacts with phosphate acceptors as well as with phosphate donors; if no acceptor is present, the phosphate becomes directly liberated as inorganic phosphate; if an acceptor is present, the phosphate group is at first transferred from the higher to the lower energy levels before being split off."

In specimens in which both azo-dye and Ca-cobalt phosphatases were identified, the latter may be concerned with transphosphorylation, i.e., phosphate was transferred from a higher energy compound to a lower level compound. Furthermore, both Ca-cobalt and azo-dye phosphatase may cooperate in biological synthesis. However, Ca-cobalt phosphatase may function in the reverse manner in cells maintained in tryptone solutions for several months, i.e., it is concerned primarily with dephosphorylation. As a consequence, phosphate accumulates in the posterior end of the cell.

Observations described in the preceding pages indicated that naturally occurring substrates existed in T. geleii. Organisms were cultured in tryptone-$MgCl_2$ solution and then were washed with 0.04M citrate buffer (pH 5.1) for removal of preformed phosphates. Strongly positive phosphatase reactions were obtained in the posterior region of cells after resuspension in tryptone-$MgCl_2$ solution for two hours subsequent to immersion in cobalt nitrate and ammonium sulfide. It might be argued that positive reactions which were obtained with this procedure were dependent on diffusion of preformed phosphates from the culture solution into the cell. Other observations do not support this view. Organisms that were washed in citrate and transferred to Gomori glycerophosphate solution exhibited the same type of reaction but to a lesser degree.

It was shown in the preceding pages that specimens of T. geleii exhibited, after prolonged culture in tryptone solutions, an abundance of cytoplasmic vesicles. The Ca-cobalt procedure revealed that positive reactions in vesicles were dependent on glycerophosphatase since preformed phosphates were removed with citrate buffer prior to the staining procedure. The question arises as to the relationship between phosphatase-positive vesicles and the Ca-cobalt positive areas in the posterior end of the cells. The latter reaction was found to be dependent on both preformed phosphate and alkaline phosphatase. A comparison of the reaction

granules in the vesicles with those in the posterior end of cells revealed that from a morphological point of view they were indistinguishable. These observations strongly suggest that the heavy concentration of preformed phosphate in the posterior end of cells was dependent on alkaline phosphatase activity.

It is still open to question as to whether or not preformed phosphate is utilized by dividing cells. Observations made in this study lend support to this view. It was found that preformed phosphates migrated from the posterior end of the cell to a perinuclear position and ultimately disappeared entirely after transfer of organisms to fresh culture solution.

2. The relation of phosphatase activity to variations in culture media. The results described in the preceding pages for localization of glycerophosphatase in various culture media are in general agreement with those of Fennell and Marzke (1954). The use of A-5-P as a substrate further increased the validity of the evidence presented by these authors as it was now possible to compare phosphatase activity in organisms from various culture media in two different substrate solutions. The activity of A-5-Pase, although generally lower, tended to parallel that of glycerophosphatase. Decreased activity of A-5-Pase might be explained on the premise that $CaCl_2$ when added to the incubation medium acted as an inhibitor. Heppel and Milnoe (1951) found this was the case in their studies of bull seminal fluid and

lyophilized snake venom. They found that in the presence of $MgCl_2$ the reaction for A-5-Pase was inhibited by $CaCl_2$, and this inhibition could be overcome by the addition of more $MgCl_2$. Novikoff (1952) also observed that calcium ions strongly inhibited the action of A-5-Pase. In these experiments both $MgCl_2$ and $CaCl_2$ were present in the substrate mixture. Assuming that the results obtained by these workers are correct, the conditions for A-5-Pase inhibition were satisfied in these experiments. The possibility that hydrogen ion concentration was the controlling factor in lowered A-5-Pase activity cannot be ruled out since pH values used in these experiments were at optima for maximum enzymatic activity in mammalian tissues, i.e., pH 8.3 (A-5-Pase) and pH 9.4 (glycerophosphatase), Gomori (1952). Optimum hydrogen ion concentration for enzymatic activity in Tetrahymena may differ from that found in vertebrate tissues.

The conditions causing erratic A-5-Pase reactions when magnesium was added to the basic culture medium was not satisfactorily explained on the basis of $CaCl_2$ inhibition or unfavorable pH optima. It was evident that addition of magnesium ions to the basic culture medium (tryptone) did not have the same activating effect on A-5-Pase activity as it did on glycerophosphatase activity, i.e., glycerophosphatase activity reached a higher total level and was sustained for a longer period of time than was A-5-Pase activity. These results suggest, but do not demonstrate, that $MgCl_2$ is not

an efficient activator of A-5-Pase. Wachstein and Meisel (1952) and McManus et al. (1952) found that magnesium was not essential for maximum A-5-Pase activity, while Keppel and Wilmoe (1951) found that addition of $MgCl_2$ to glycine buffer induced pronounced activation of A-5-Pase.

It was observed by Fennell and Harzke (1954) that $MgCl_2$ added to the tryptone culture media increased glycerophosphatase activity. Results obtained in this study are in general agreement with these findings.

It was found during the course of this study that the addition of vitamins to tryptone-$MgCl_2$ solution increased A-5-Pase activity to a greater degree than glycerophosphatase activity, whereas the addition of vitamins alone decreased A-5-Pase activity.

Fennell and Harzke (1954) found that cells cultured in vitamin-enriched tryptone solutions exhibited decreased and ultimate inhibition of glycerophosphatase activity and that enzymatic activity was erratic after the addition of $MgCl_2$ to vitamin-enriched tryptone. Results reported here tend to bear out their observations in this respect. It would seem from observations made in this study that in vitamin-enriched cultures to which $MgCl_2$ was added, A-5-Pase was affected to a lesser degree than glycerophosphatase, although the reduction in A-5-Pase activity parallels, for the most part, that of glycerophosphatase. This suggests that perhaps vitamins along with $MgCl_2$ operate in some way to increase the activity of A-5-Pase more than that of glycerophosphatase.

The lowered activity of A-5-Pase and A-3-Pase after substitution of 0.01M citrate buffer (pH 5.5) for phosphate buffer in tryptone-$MgCl_2$ solution was attributed to the citrate buffer. Furthermore, activity of A-5-Pase and A-3-Pase in citrate buffered solutions was affected to a greater degree than glycerophosphatase activity, e.g., A-5-Pase and A-3-Pase did not show the gradual increase in activity as was the case with other phosphomonoesterases (glycerophosphatase, etc.). Increasing the concentration of citrate buffer from 0.01M to 0.08M completely inhibited glycerophosphatase reactions. It seems reasonable to assume that low concentrations of citrate (0.01M) had a tendency to provide a more stable acid culture medium than phosphate buffer. As a consequence, growth rate of organisms was maintained but at a lower level than it was in tryptone solution. Furthermore, omission of the phosphate buffer decreased phosphate concentration in the culture solutions. Under these conditions enzymatic activity was decreased to a greater degree in A-5-P and A-3-P substrate solutions than it was in the other substrate solutions used in this study.

Results presented in the preceding pages suggest that phosphatase reactions in T. <u>gondii</u> are directly correlated with the type of media used for the culture of organisms. It seems reasonable to assume that tryptone solutions contained suboptimum concentrations of accessory growth-promoting substances. In citrate buffered (0.04M) vitamin-enriched tryptone solutions transfer of phosphate from high energy

compounds to low energy compounds was inhibited and growth rate of organisms was retarded. Mann (1944) showed that in the mold Aspergillus niger the type of growth medium markedly affected such things as the rate of phosphate utilization, phosphate content, and growth, etc.

3. Enzyme inhibition and substrate specificity (multiple phosphomonoesterases. The utilization of inhibitor substances appears to make possible the differentiation of substrate specific enzymes hydrolyzing multiple phosphomonoesterases in T. geleii. It is significant to note that all enzymes were acid and heat labile. Kaengwyn-Davies et al. (1950, 1952) and Newman et al. (1950) made exhaustive studies on phosphatase localization and reaction to inhibitors and activators. On the basis of their findings, they concluded that multiple phosphomonoesterases are functional in mammalian tissues. Kunel (1946, 1950) demonstrated that the alkaline phosphatases of both the mammalian kidney and intestine differed in their sensitiveness to KCN and HCl inhibition. Fennell and Harzke (1954), using only glycerophosphate as a substrate and semicarbazide (0.02M), sodium arsenate (0.01M), and reduced and oxidized glutathione (0.025M) as inhibitors, were unable to reach a conclusion on the existence of several enzyme systems in T. geleii.

The inhibition or activation of phosphatase enzymes in T. geleii was the principal method used for making a distinction between them. However, intensity of the enzymatic reaction in specific substrates was also utilized. In

these experiments localization was not used as a criterion for classification of enzymes, since localized reactions in the posterior ends of cells were obtained with all substrate solutions. The activation of ATPase by KCN (0.001M) was reported by Stern et al. (1951). The ability of KCN (0.01M) to activate only ATPase in T. goleii supports the view that this enzyme is a distinct entity. ATPase activity was easily demonstrated, i.e., preformed phosphates did not occlude enzymatic activity and in many tests preformed phosphates were lacking altogether. On the basis of these observations, it appeared that either the concentration of ATPase was high, or specific activators of ATPase existed in Tetrahymena. Addition of $MgCl_2$ to the culture solution neither increased nor decreased ATPase activity. Bailey (1942) and DuBois et al. (1943a, 1943b) found that calcium was a specific activator of this enzyme in mammalian tissues. Since $CaCl_2$ was added to the substrate solution in these experiments, it is suggested that the Ca ion may have essentially the same activating effect on the ATPase of Tetrahymena. The presence of ATPase in Tetrahymena may have in vivo significance in that it releases phosphate from ATP, a substance containing high energy bonds. Thus, energy is made available for metabolic needs. It may also function as an activator of the substrates involved in glycolytic reactions. ATP has also been found to be identical with or intimately bound to myosin, the contractile element in mammalian muscle (DuBois, 1943b). Its

appearance in the nucleus may be associated in some way with cell division and overall body metabolism.

Variability of enzymatic activity in A-5-P and A-3-P substrate solutions, and the differential effects of inhibitors, indicated the presence of two different enzymes. However, on the basis of experiments performed during this investigation, no data was obtained that seemed conclusive enough to support this view. Exposure of organisms to sodium arsenate (0.001M) and KCN (0.01M) prior to immersion in A-3-P induced erratic staining reactions in specimens of T. geleii, i.e., results were seldom duplicated. On the other hand, organisms incubated in A-5-P subsequent to immersion in these inhibitors exhibited more uniform results. However, cells cultured in tryptone-citrate solutions with added $MgCl_2$ and incubated in A-5-P and A-3-P substrate solutions gave essentially similar staining reactions. Gomori (1949b), Reis (1951), and Koppel and Hilmoe (1951) believed enzymes which split A-5-P and A-3-P were distinct entities. Gulland and Jackson (1938b) found that under certain conditions both substrates could be split with equal ease by the same enzyme.

Differential enzymatic activity in G-1-P, Cr-P, and glycerophosphate along with the inhibitory effect of KCN (0.01M), glycine (0.25M), and sodium arsenate (0.001M) lend further support to the existence of multiple phosphomonoesterases in T. geleii. Enzymatic activity in G-1-P and Cr-P was not affected by either sodium arsenate (0.001M) or H_2O_2.

However, inhibition of glycerophosphatase activity was observed. HCN (0.01M) induced more inhibition of glycerophosphatase than either G-1-Pase or Cr-Pase. On the other hand, glycine (0.25M) caused more inhibition of glycerophosphatase and Cr-Pase than G-1-Pase.

The effect of these inhibitor substances on the phosphomonoesterases of T. <u>pelcii</u> appear to be due either to a combination of causes or in some instances to a single clearly discernible process. Complete phosphomonoesterase inhibition by acids and semicarbazide in solution seemed to be due to enzyme denaturization by hydrolysis. Preformed phosphate was soluble in acid solution and semicarbazide, resulting in completely negative control and substrate slides. On the other hand, partial reduction in staining activity appeared to be the result of chemical combination of the inhibitor with the enzyme, resulting in precipitation or inactivation of the enzyme involved. In other cases, a combination of the enzyme reaction product (phosphate) with the inhibitor resulted in an apparent reduction of enzymatic activity. A more thorough washing of organisms subsequent to immersion of the cells in the substrate solution reduced this type of inhibition. Hot water (80° C.) inhibition seemed to work by coagulation of the enzyme protein, thus reducing activity to that of the preformed phosphate present in the cell. Other enzyme inhibitions appeared to result from a combination of the above mentioned effects.

The ability of the phosphomonoesterases to resist these inhibitory reactions apparently was the reason for the specificity of the inhibitor-enzyme reaction. Activation of enzymatic activity seems to be the result of the activator material supplying a needed metallic ion to the enzyme.

Regardless of the mode of action of these inhibitors, the specific inhibitory effect produced by them on certain enzymes is a very excellent means of separating the various phosphomonoesterases when localization and staining intensity are of slight or no use. However, it was found that unless proper controls and techniques were maintained, the use of inhibitors with the Ca-cobalt procedure yielded conflicting results.

4. Nuclear enzymes. The specificity of the Gomori technique for demonstration of nuclear phosphatases has been questioned by various investigators; among them were Gomori (1951), Leduc and Dempsey (1951), Martin and Jacoby (1949), and Novikoff (1952). These investigators believed that nuclei have a special affinity for Ca phosphate. Furthermore, enzyme diffusion and the presence of nuclear activators cannot be ruled out. Danielli and Catheside (1945) and Willmer (1942) were apparently able to demonstrate nuclear enzymes by histochemical means, while Dounce (1943) used analytical procedures in the demonstration of phosphatase-positive nuclei.

It was pointed out in the results that nuclear staining could be demonstrated in E. galcii with all substrates used

except sodium alpha-naphthyl acid phosphate and that cells subjected to inhibitor substances frequently exhibited nuclear staining as did most control slides. These observations suggest that most nuclear staining observed in this ciliate was an artifact possibly due to the diffusion of Ca phosphate from sites of high enzymatic activity.

The lack of nuclear staining in the azo-dye procedure was pointed out by Gomori (1951) and Novikoff (1952). Experimental evidence presented in this paper is in complete agreement with their observations in this respect. Artifactual nuclear staining with the Ca-cobalt procedure, however, does not rule out the usefulness of this technique. Critical comparisons of nuclear staining in cells incubated in the substrates utilized in these experiments has led to the presumed demonstration of a non-artifactual nuclear reaction for ATPase under certain conditions, e.g., cultivation of organisms in tryptone-citrate solution with added $MgCl_2$ (0.01%, pH 5.5). Cells stained from this culture medium have at times shown pronounced nuclear staining as was shown in figure 19. This staining differed significantly from that obtained in cells reared in the same culture medium and incubated in the other substrate solutions, i.e., A-5-P, G-1-P, etc. The ATP reactions were darker than those obtained in these substrates, and ATP controls never exhibited nuclear staining approaching the degree of darkness obtained when cells were immersed in ATP solutions. It is believed that these re-

sults indicate specific nuclear ATPase activity under the conditions specified.

SUMMARY

1. Specimens of T. goleii used for studies of alkaline phosphatase were obtained from bacteria-free cultures ranging in age from 72 hours to 1,080 hours. In most experiments culture age varied from 72 hours to 360 hours. The techniques of Gomori (1952) were used throughout this study to demonstrate alkaline phosphatase.

2. Seven phosphomonoesters were used in these experiments: sodium glycerophosphate, muscle adenylic acid, yeast adenylic acid, adenosine triphosphoric acid, glucose-1-phosphate (potassium salt), creatine phosphate (calcium salt), and sodium alpha-naphthyl acid phosphate.

3. Three types of reaction granules were obtained by use of the Ca-cobalt method: (1) fine granular precipitates; (2) an intermediate type of precipitate; and (3) large granular precipitates containing small spherical vesicles. These could be produced experimentally.

4. It was possible to demonstrate a number of phosphomonoesterases active in the alkaline range: glycerophosphatase, ATPase, G-1-Pase, Cr-Pase, and A-5-Pase. Variable results were obtained with A-3-Pase. These enzyme systems could be differentiated from one another by their different responses to activators and inhibitors, and by differences in the relative intensities of their reactions. Localization

could not be relied upon to indicate intracellular differences in enzyme distribution.

5. Magnesium chloride did not appear essential for full phosphatase activity in specimens of T. galeii. However, KCN (0.01M) caused increased activity of ATPase.

6. The type and age of the culture media employed influenced alkaline phosphatase activity in specimens of T. galeii. Cells reared in tryptone-$MgCl_2$ media exhibited high glycerophosphatase activity throughout culture life. On the other hand, A-5-Pase activity was erratic. Replacement of the phosphate buffer present in tryptone media with a 0.01M citrate buffer (pH 5.5) and the subsequent addition of $MgCl_2$ reduced phosphatase activity. A-5-Pase activity was absent or nearly so. Glycerophosphatase activity in tryptone, tryptone-$MgCl_2$, vitamin-enriched tryptone, and tryptone-citrate media with added $MgCl_2$ was higher than that of A-5-Pase. However, the opposite effect was observed in vitamin-enriched tryptone solution with added $MgCl_2$.

7. In the absence of glycerophosphatase activity, no enzymatic reactions could be obtained for A-5-Pase and A-3-Pase. Glycerophosphatase activity did not appear to depend on the presence or absence of A-5-Pase or A-3-Pase.

8. Specimens of T. galeii contained preformed phosphates that were demonstrable with the Ca-cobalt technique of Gomori. Cells grown in tryptone solutions for six months or longer exhibited increased amounts of preformed phosphates. It was

suggested that this phenomenon was due to the lack of phosphate acceptors normally supplied by the vitamins. This resulted in the storage of phosphate within the cell. However, liberation of phosphate from cellular components immediately prior to fixation was not ruled out as a source of preformed phosphate.

9. Enzymatic activity in specimens of T. coleii appeared to be located in the posterior of the cell regardless of the substrate used. Azo-dye tests utilizing the organic portion of the sodium alpha-naphthyl acid phosphate molecule tended to confirm this location.

10. Observations made in this study indicated that Ca-cobalt phosphatase and not azo-dye phosphatase was functional in organisms cultured in tryptone solutions for six months or longer. However, organisms cultured in vitamin-enriched tryptone medium for extended periods of time exhibited abundant phosphatase activity with the azo-dye technique.

11. The conclusion was reached that most nuclear reactions for phosphatase obtained with the Ca-cobalt technique were artifacts, probably due to the affinity of the nucleus for Ca phosphate. However, demonstration of a nuclear reaction when ATP was used as a substrate was believed to represent a true enzymatic reaction.

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PLATE I

Alkaline phosphatase activity in specimens of T. geleii W. Figures 1, 3, 5, and 6 stained for glycerophosphatase using the Gomori Ca-cobalt technique; figures 2 and 4 stained for azo-dye phosphatase using the Gomori azo-dye technique. Magnification X570. Micrometer scale insert: 1 space = 0.01 mm.

Fig. 1. 144-hour cells cultured in tryptone-$MgCl_2$ solution;

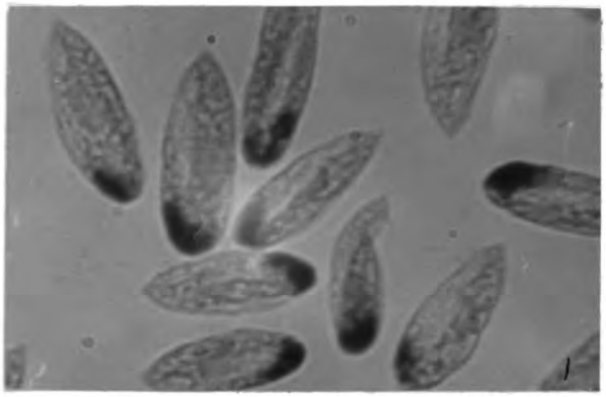
Fig. 2. 144-hour cells cultured in tryptone-$MgCl_2$ solution;

Fig. 3. 140-hour cells cultured in tryptone-$MgCl_2$ solution;

Fig. 4. 140-hour cells cultured in tryptone-$MgCl_2$ solution;

Fig. 5. 140-hour cells cultured in tryptone-$MgCl_2$ solution; and

Fig. 6. 144-hour cells cultured in tryptone-$MgCl_2$ solution.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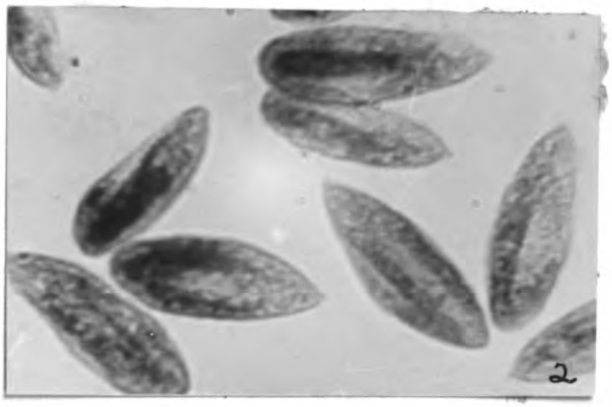
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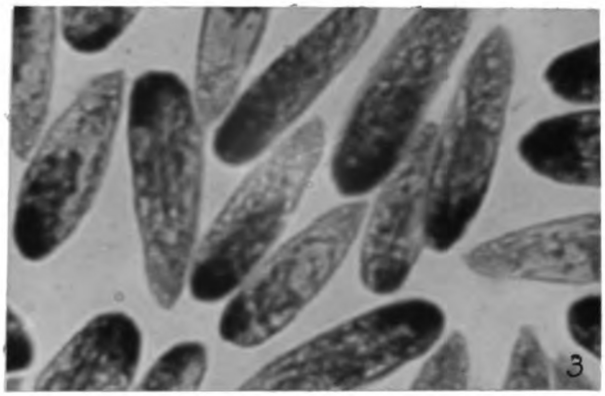
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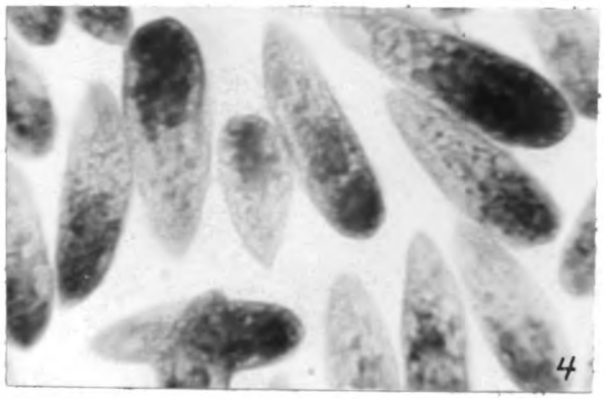
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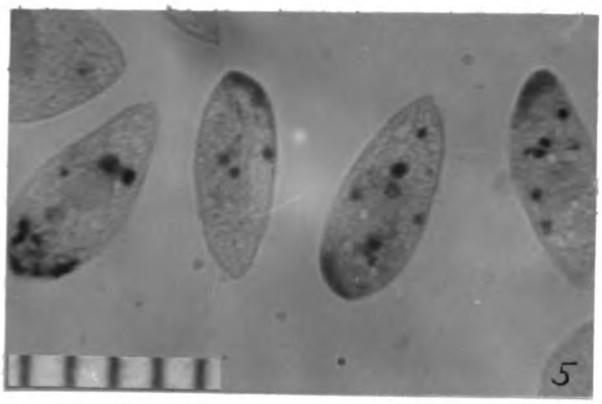
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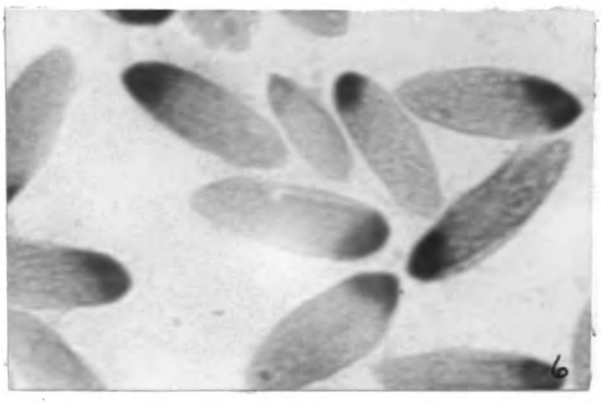
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PLATE II

Alkaline phosphatase activity in specimens of T. geleii W. Figures 7, 8, and 9 stained for glycerophosphatase, 10 and 11 for A-5-Pase, using the Gomori Ca-cobalt technique; figure 12 stained for azo-dye phosphatase using the Gomori azo-dye technique. Magnification X570. Micrometer scale insert: 1 space = 0.01 mm.

Fig. 7. 144-hour cells cultured in tryptone-$MgCl_2$ solution;

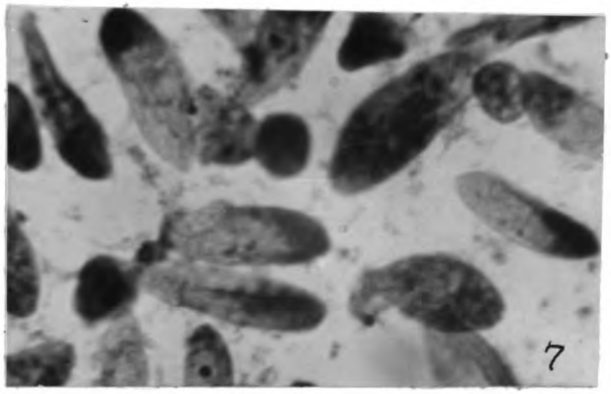
Fig. 8. 792-hour cells cultured in tryptone solution;

Fig. 9. 144-hour cells subcultured in tryptone solution for approximately six months;

Fig. 10. 288-hour cells cultured in tryptone solution;

Fig. 11. 216-hour cells cultured in tryptone-$MgCl_2$ solution; and

Fig. 12. 264-hour cells cultured in vitamin-enriched tryptone solution.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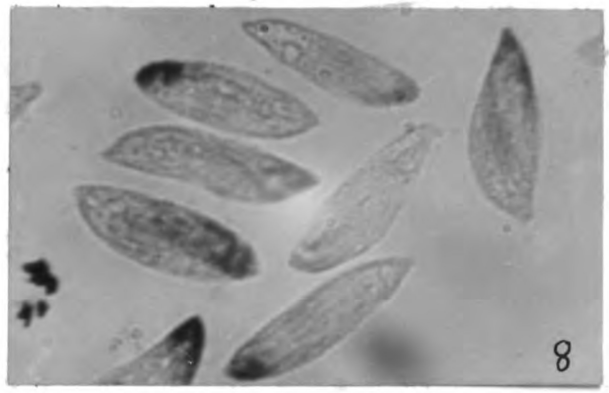
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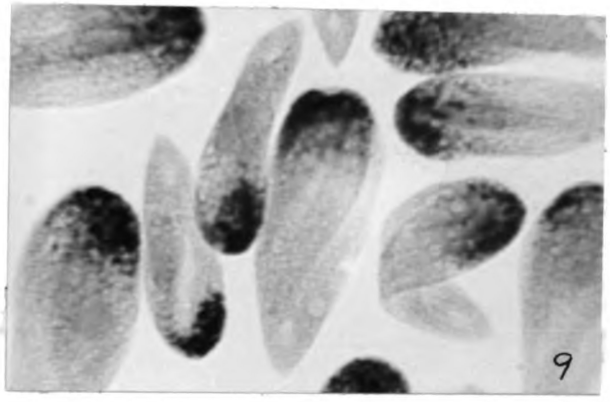
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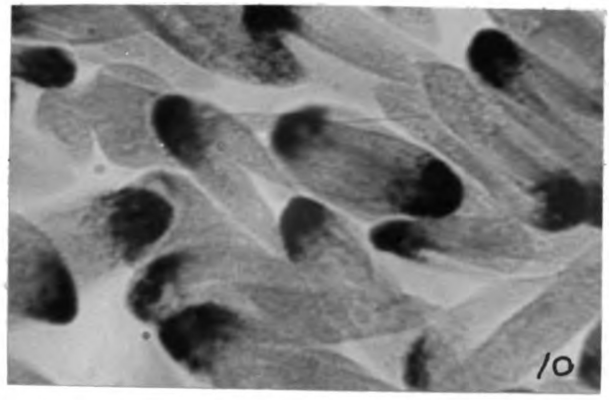
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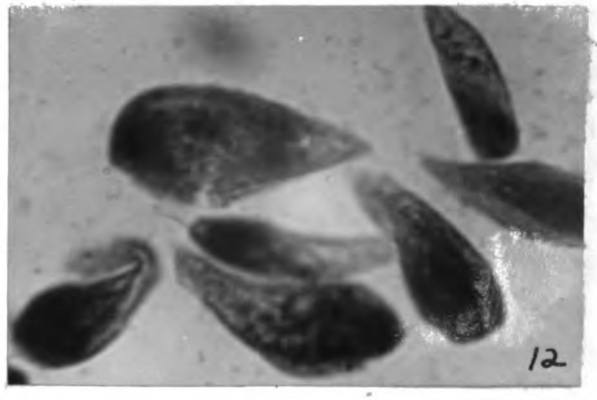
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PLATE III

Alkaline phosphatase activity in specimens of T. geleii W. Figure 13 stained for azo-dye phosphatase using the Gomori azo-dye technique; figures 14 and 19 stained for ATPase; 15, G-1-Pase; 16, Cr-Pase; 17 and 18, glycerophosphatase, using the Gomori Ca-cobalt technique. Magnification X570. Micrometer scale insert: 1 space = 0.01 mm.

Fig. 13. 144-hour cells cultured in tryptone-citrate solution with added $MgCl_2$;

Fig. 14. 72-hour cells cultured in tryptone solution;

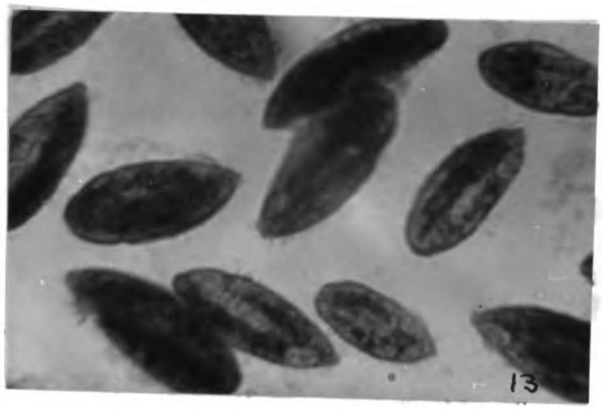
Fig. 15. 432-hour cells cultured in tryptone solution;

Fig. 16. 216-hour cells cultured in tryptone solution;

Fig. 17. 144-hour cells cultured in tryptone-citrate solution with added $MgCl_2$;

Fig. 18. 288-hour cells cultured in tryptone solution. Organisms immersed in 0.2M citrate buffer for 15 minutes prior to incubation in glycerophosphate for 24 hours; and

Fig. 19. 72-hour cells cultured in tryptone-citrate solution with added $MgCl_2$.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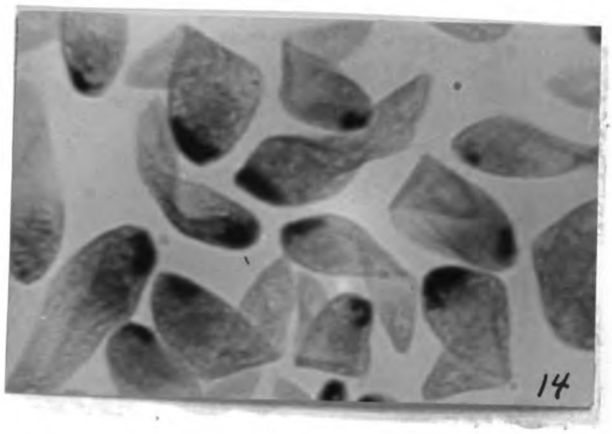
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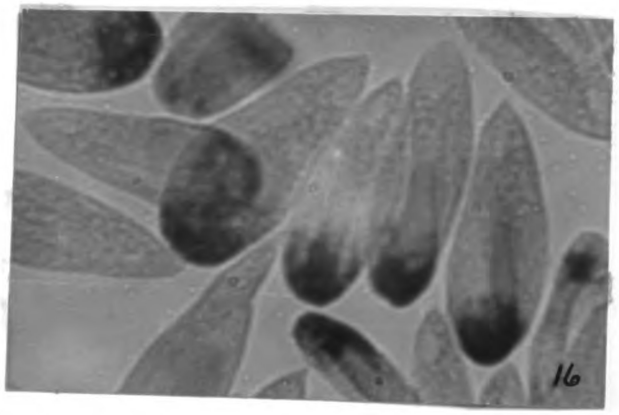
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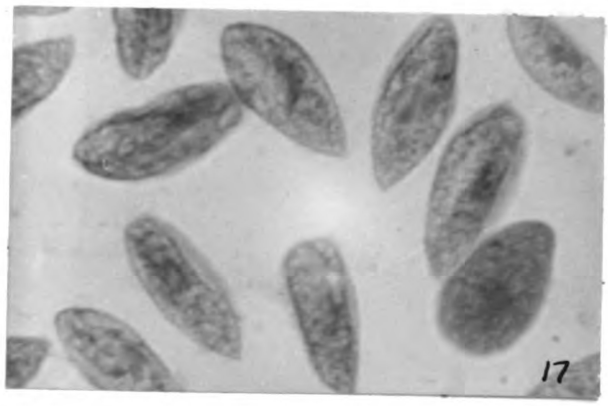
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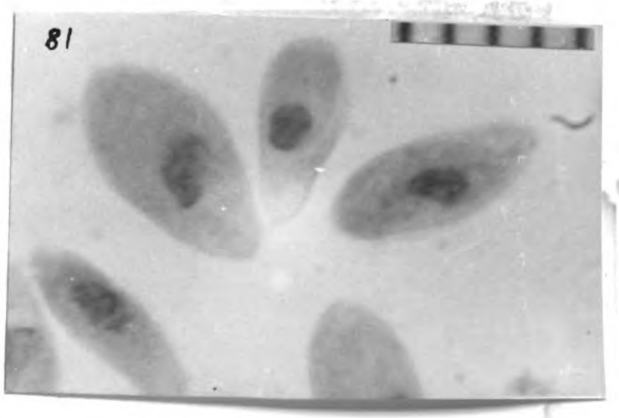
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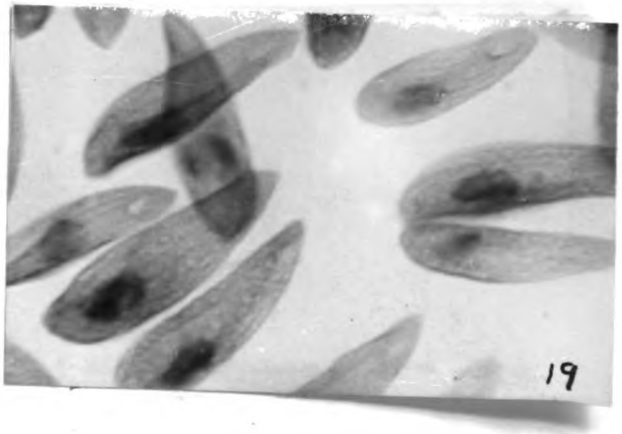
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