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Effect of certain nitrogen compounds on growth of *Chlorogonium* and *Chilomonas*[1].

By

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(With 5 figures in the text.)

Introduction.

Investigations on the group Euglenida (Dusi, 1931 and 1933) reveal that growth of certain species is greatly accelerated by particular amino acids, while others do not support growth at all. This peculiar utilization of nitrogen varies with the protozoan; indeed, some of them will not survive on single amino-acid nitrogen, but require a complete or partially hydrolyzed protein as a source of nitrogen. Lwoff (1932) has observed also that some amino acids are more favorable than others for the growth of *Polytoma uvella*, and specific growth acceleration by certain nitrogen compounds was also demonstrated for both *Haematococcus pluvialis* and *Chlamydomonas agloëformis* under similar conditions.

It was generally believed by the earlier investigators (Jacobsen, Artari, et al.) who worked with green flagellates, that growth was increased by certain amino acids, and similar claims were made for particular amides, all results having been based on qualitative observation, few of them made with bacteria-free cultures. The present investigation is to determine the relative accelerative effect of single amino acids and amides on growth of *Chlorogonium euchlorum*

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EHRBG., *Chlorogonium elongatum* DANGEARD and *Chilomonas paramecium* EHRBG. in bacteria-free cultures. Preliminary results were cited in abstracts (LOEFER, 1932 a and 1932 b). There are no quantitative growth determinations available for any forms representing these two orders of the Phytomastigoda. Such results are especially desirable in view of LWOFF's (1932) proposed physiological classification of microorganisms. The writer is greatly indebted to Prof. R. P. HALL for many helpful suggestions during the course of the investigation.

Material and methods.

The bacteria-free strains of *Chlorogonium* and *Chilomonas* are the same as those used in previous studies (LOEFER, 1934 a; 1934 b). Only flagellated forms of *Chlorogonium* have been observed in regularly transplanted cultures. The following basic media were used:

Medium A:	KH_2PO_4	0.5 grm.
	$MgSO_4$	0.1 „
	NaCl	0.1 „
	$FeCl_3$	trace
	Distilled water	1 liter
Medium B:	Same as above plus sodium acetate	2.5 grm.
Medium C:	Medium B plus sodium butyrate	2.5 grm.

Specific compounds to be tested for their effect on growth were added to portions of the basic medium; these were then tubed, sterilized by autoclaving and inoculated. The cultures were incubated at 28° C for varying periods of time, and counts were made with a SEDGWICK-RAFTER counting chamber and a WHIPPLE micrometer. Ratio of final and initial counts per milliliter (x/x_0) was determined as in previous experiments. p_H of the medium was determined colorometrically at the beginning and end of each experiment, and bacteriological tests for purity of the cultures were made at regular intervals during the course of the experimentation. A more detailed account of the technique employed is contained in an earlier paper (LOEFER, 1935).

The amino acids and amides used were obtained from the Eastman Kodak Company, Rochester, N. Y.; peptones, etc. from the Difco Laboratories.

Experimental results.

Preliminary studies were made to obtain a medium which would be most favorable for the growth and maintenance of stock cultures. To determine this, the following desiccated proteins were

made up in respective lots in 0.5 % concentration, using medium A as a base; Bacto-tryptone, Proteose-peptone, Bacto-peptone, Neopeptone, Bacto-protone, Bacto-yeast extract, Bacto-beef blood serum, Bacto-gelatin, Casein, Bacto-veal, Bacto-beef extract, Bacto-hemoglobin, Bacto-beef heart, Bacto-liver and Bacto-beef blood. After titration to p_H 6.8 all media was tubed, autoclaved, inoculated with *Chilomonas* ($x_0 = 675$) and incubated for 36 hours. At this time the average number of organisms per milliliter (x) in three to six tubes of each medium was determined. The remaining tubes of each set were incubated for a period of eight weeks, macroscopic observations on those which showed positive growth being recorded at four and eight weeks (table 1). Negative growth results were obtained with Bacto-beef extract, Bacto-hemoglobin, Bacto-beef heart, Bacto-liver and Bacto-beef blood.

Table 1.
Growth of *Chilomonas paramecium* in 0.5 % concentrations of various nitrogen-containing media.

Culture medium	Number of organisms per cc after 36 hours	Growth after four weeks	Growth after eight weeks
Bacto-tryptone	123,100	good	good
Proteose-peptone	37,600	good	good
Bacto-peptone	11,400	moderate	good
Neopeptone	8,300	poor	poor
Bacto-protone	4,400	poor	poor
Bacto-yeast extract	99,800	good	good
Bacto-beef blood serum	70,000	good	good
Bacto-gelatin	24,900	good	good
Casein	33,400	moderate	moderate
Bacto-veal	20,000	moderate	moderate

The above results indicate that for *Chilomonas* maximum growth occurs at 36 hours in Bacto-tryptone, which contains considerable amino acid nitrogen. Growth is not as rapid after 36 hours in the media containing mostly unhydrolyzed proteins, e. g., casein and gelatin, but it is relatively good after four and eight weeks. Yeast extract and blood serum are also excellent media. Since similar results were obtained with *Chlorogonium* in this series of media, Bacto-tryptone was selected as a stock culture medium.

The high amino acid content of Bacto-tryptone suggested that growth of *Chlorogonium* and *Chilomonas* might be accelerated by certain isolated amino acids, as has been shown for other protozoa by earlier investigators. To test this hypothesis and to determine the relative effect of a number of such amino acids, the following experiments were performed.

Amino acids as nitrogen sources.

In the first two series the culture fluid (medium A, p. 75) contained no organic carbon compound, while in series III—IV sodium acetate was added (medium B, p. 75). The following amino acids (0.15 %) solution except for tyrosine, which was saturated at room temperature) were added to respective lots of the above mentioned media: glycine, dl-valine, l-leucine, dl-leucine, dl-isoleucine, dl-β-phenylalanine, l-tryosine and the compound asparagin. After inoculation the cultures were maintained at 28° C until counts were made.

Series I. *Chlorogonium euchlorum.*

The initial count was 3000 organisms per cc; results of two and four-day counts appear in figure 1. Asparagin, tryosine, l-leucine, isoleucine, glycine and valine produced the greatest amount of growth, while phenylalanine and a racemic mixture of leucine were less favorable. Growth in a mixture of all of these amino acids exceeded that in the most favorable of the single amino acids. p_H following inoculation was 7.2 and no change was observed after the four-day growth period.

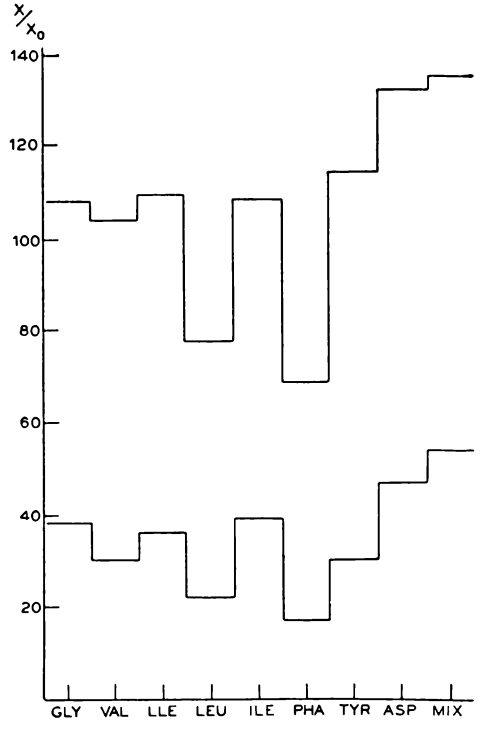

Fig. 1. *Chlorogonium euchlorum.* Effect of single amino acids on growth after two and four days. Glycine, gly; dl-valine, val; l-leucine, lle; dl-leucine, leu; dl-isoleucine, ile; dl-β-phenylalanine, pha; l-tyrosine, tyr; asparagin, asp; mixture, mix. x = final count of organisms per milliliter. x_0 = number of organisms per milliliter at the beginning of incubation.

Series II. *Chlorogonium elongatum.*

Figure 2 shows the results obtained with the more slowly growing species after three and six-day periods under the same conditions as in series I ($x_0 = 3000$). It would seem that relatively better growth of *Chlorogonium elongatum*

results with dl-leucine than with either l-leucine or isoleucine, while the tyrosine cultures showed greatly accelerated growth, differing in these respects from the previous experiment on *Chlorogonium euchlorum*.

As seen in figure 2, tyrosine produced a marked acceleration of growth after three days, but after six days this stimulating effect was relatively less. This difference is perhaps accounted for by the complete utilization of the small amount of tyrosine originally present (solubility = 0.04 % at 17° C) sometime between the third and sixth day. To show whether or not an excess of the other amino acids was present in the preceding experiments, a series of tubes containing phenylalanine, glycine and asparagin in 0.075 % concentration was inoculated (x_0 = 440), incubated (72 hours) and the results compared with those previously obtained. Results shown in figure 3 indicate the same relative growth effects as are found when a higher concentration (0.15 %) of the nitrogen compounds

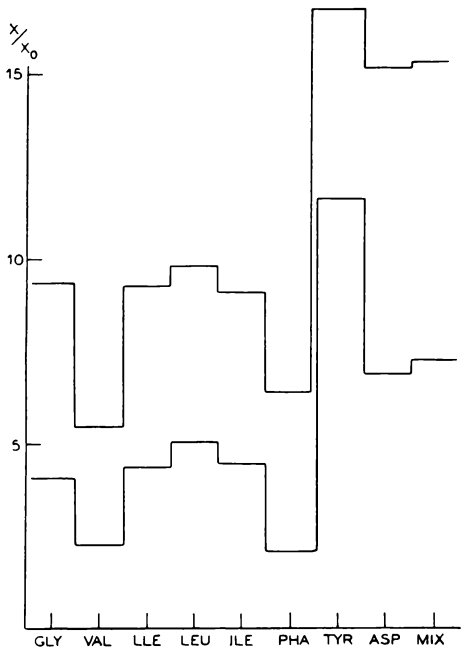

Fig. 2.

Chlorogonium elongatum. Growth on amino acids after three and six days. See figure 1 for legen.

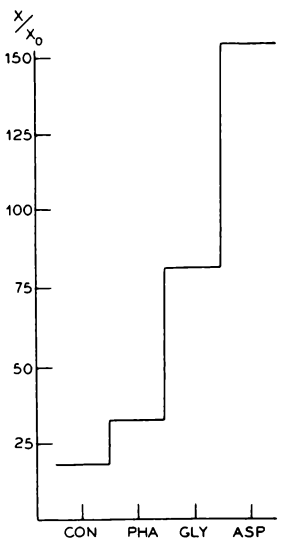

Fig. 3. *Chlorogonium elongatum*. Effect of certain nitrogen compounds in 0.07 % concentration after 72 hours incubation at 28° C. Con, control; pha, phenylalanine; gly, glycine; asp, asparagin. x_0 = number of organisms per milliliter at the beginning of incubation; x = number of organisms at termination of growth period.

are used. It is not surprising that these seemingly low concentrations are sufficient to provide a surplus for the organisms in view of the relatively short incubation periods. PRINGSHEIM's (1921) findings also show that glycine in 0.025 % concentration supports good growth of *Polytoma uvella*. The difference in growth between the controls (medium A without added amino nitrogen) and phenylalanine is indicative of the utilization of this compound as a nitrogen source.

Series III. *Chlorogonium euchlorum.*

Medium B, containing an organic carbon source, was used as a base for this and all following amino acid series. l-aspartic (asa) and d-glutamic (gla) acids were added to the series. Initial p_H of the cultures was 6.7; x_0 was 530. Figure 4 shows growth after 56 hours. It was greatest in the Bacto-tryptone cultures, with the asparagin and aspartic acid media ranking second and third, respectively.

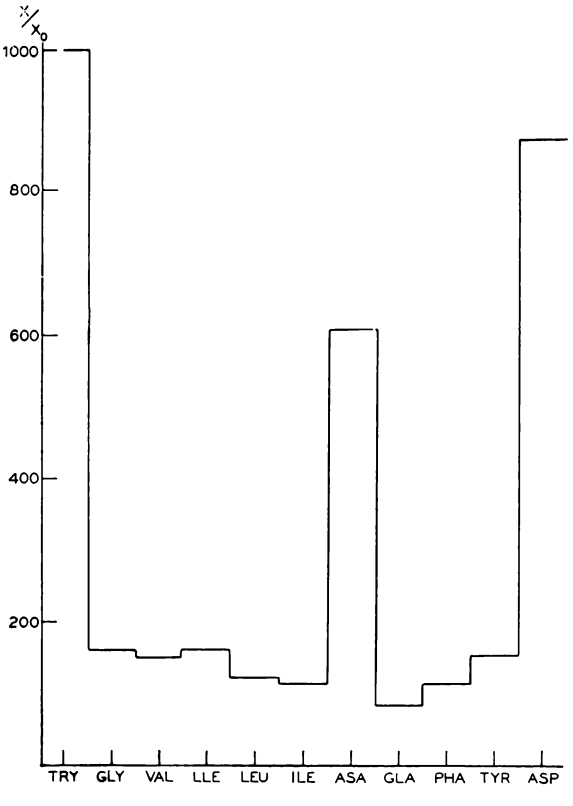

Fig. 4. *Chlorogonium euchlorum.* Growth with amino nitrogen after 56 hours. try, Bacto-tryptone; asa, aspartic acid; gla, glutamic acid. Other designations as shown in figure 1.

Series IV. *Chlorogonium elongatum.*

Media homologous to that in series III, also at p_H 6.7, was used in this experiment. After 84 hours incubation ($x_0 = 300$) growth of *Chlorogonium elongatum* had occurred as shown in figure 5. This species appears to use tyrosine and leucine to

good advantage, as well as aspartic acid and asparagin, differing in this respect from *Chlorogonium euchlorum* as shown in series III. Growth in Bacto-tryptone (try) was greater than in any of the other cultures.

Series V. *Chilomonas paramecium.*

Since *Chilomonas* could not be cultured on inorganic media containing either ammonium salts or nitrate (LOEFER, 1934), it seemed possible that it might utilize certain of the single amino acids for growth. Media as in series III and IV at p_H 6.7 was inoculated($x_0=56$) and incubated as previously. After 84 hours the organisms in Bacto-tryptone media had increased almost 1700 times as compared with 100 times for the amino acid and control (without nitrogen) cultures, there being no significant differences between the several amino acid cultures and controls. The final counts for several tubes of each set were determined after seven days, but additional growth had occurred only in the Bacto-tryptone cultures. The remaining single amino acid cultures were incubated for several weeks longer, but there was no evidence of additional growth, indicating that the single amino acids tested were incapable of supporting growth.

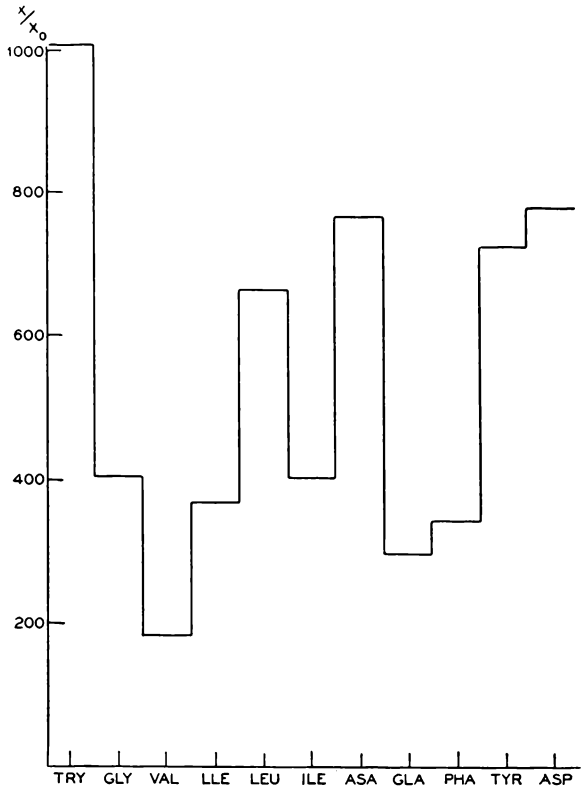

Fig. 5. *Chlorogonium elongatum.* Growth with amino nitrogen after 84 hours. See figure 4 for legend.

Series VI. *Chilomonas*, *Chlorogonium euchlorum*, *Chlorogonium elongatum*.

Since growth of *Chilomonas* in all of the single amino acid media was so nearly uniform, it seemed probable that the increase which took place in these cultures was due to utilization of nitrogen which had been transferred to the tubes with the original inoculum, rather than to utilization of the single amino acid in each case. In order to check this, medium B (without any nitrogen) was made up at p_H 6.9. It was divided into three equal portions. Asparagin (0.2 %) was added to the first portion. Bacto-tryptone (0.3 %) was added to a second lot, while the third was tubed as such to serve as a control. After autoclaving, a number of tubes of each were inoculated with each of the three forms, *Chilomonas* (x_0, 403), *Chlorogonium euchlorum* (x_0, 863) and *Chlorogonium elongatum* (x_0, 905), respectively. Counts were made after 30 and 60 hours incubation, and comparative results of the latter appear in table 2. No changes in p_H were observed.

Table 2.

Medium	*Chilomonas* x/x_0 after 60 hrs.	*Chlorogonium euchl.* x/x_0 after 60 hrs.	*C. elongatum* x/x_0 after 144 hrs.
1. Asparagin	87	152	106
2. Bacto-tryptone	357	168	115
3. Control (no nitrogen)	93	85	57

It appears that asparagin is not utilizable as a nitrogen source for *Chilomonas* inasmuch as growth is no better than in the control (medium B). By analogy with the preceding series it may be assumed that the amino acids of that series were not utilized for growth. On the other hand, growth of the green forms is distinctly accelerated by asparagin. The nitrogen utilized for growth in the control was probably that introduced with the 1 cc inoculum from the stock culture. The seemingly greatly increased growth in the medium which before inoculation contained no nitrogen is accounted for by the stimulating influence of sodium acetate, as was shown in an earlier investigation (Loefer, 1935).

Amides as nitrogen sources.

A series of amides including acetamide, propionamide, butyramide and valeramide (0.25 % concentration) were added to medium C (p_H 6.7). Different sets of culture tubes containing these compounds

were sterilized, inoculated, incubated, sampled and counted as in previous experiments. Since semi-final and final counts were in agreement in every case, only final growth determinations are tabulated for comparison (table 3).

Series I and II. *Chlorogonium.*

The initial count of *Chlorogonium euchlorum* in series I was 863 organisms per cc. Preliminary and final counts were made after 30 and 60 hours of incubation. Growth was greatest in the tubes which contained no amide, indicating that none of these compounds was of value as a nitrogen source. No growth was recorded for propionamide, while cultures which contained Bacto-tryptone showed considerably more growth than the controls.

After an inoculation of 900 *Chlorogonium elongatum* per milliliter, the cultures of series II were incubated for 144 hours. Propionamide also appeared to be lethal to this species. Slightly less growth was evident in acetamide than in the control cultures; growth is relatively less in butyramide and still less in valeramide. Even after six days there was evidence of increased growth only in the Bacto-tryptone cultures.

Table 3.
Effect of amides.

Medium	*Chilomonas* x/x_0 after 60 hrs.	*Chlorogonium euchl.* x/x_0 after 60 hrs.	*C. elongatum* x/x_0 after 144 hrs.
Bacto-tryptone	538	339	280
Control (no amide)	163	155	147
Acetamide	151	118	134
Propionamide	0	0	0
Butyramide	0	91	112
Valeramide	0	87	84

Series III. *Chilomonas.*

The initial count was 403 flagellates per milliliter. Counts after 30 and 60 hours of incubation showed that growth occurred only in acetamide, but to no greater extent than in the controls. *Chilomonas* appears to be more sensitive than *Chlorogonium* to the toxic action of butyramide and valeramide. Only propionamide was lethal to the green forms, while all except acetamide of the series tested were lethal for *Chilomonas.*

It appears from table 3 that for all three species acetamide is the least toxic of this series of compounds. The amides used were more or less toxic in every case, although acetamide, butyramide

and valeramide were not lethal to *Chlorogonium*. The nitrogen necessary for the growth noted was probably obtained from the Bacto-tryptone introduced with the inoculum.

Discussion.

It was shown in an earlier paper (Loefer, 1934 c) that *Chlorogonium* can utilize inorganic nitrogen in the form of either nitrates or ammonium salts. However, growth is greatly accelerated when organic nitrogen-containing compounds are added to the medium, whether in the form of single amino acids, or complete or partially hydrolyzed proteins. These results are quite in conformity with the earlier findings of Jacobsen (1910) who concluded that *Chlorogonium euchlorum* was primarily a mixotrophic form, but could be grown autotrophically as well. He found that asparagin, glycine and glucosamine were particularly effective in promoting growth. Artari (1913) showed that asparagin, glycine and alanine were favorable to growth of *Chlamydomonas ehrenbergii*, while both Pringsheim (1921) and Lwoff (1932) found that *Polytoma uvella* thrives on certain amino acids as nitrogen sources. In short, the Chlamydomonadida as a group appear to utilize nitrogen in the form of single amino acids. The possible bearing of this fact on the phylogeny of these forms is pointed out later.

Results obtained for the two species of *Chlorogonium* investigated indicate that they differ from each other as previously pointed out (Loefer, 1932 b). The chief difference is in their rate of growth, *Chlorogonium euchlorum* showing a consistently higher division rate than *Chlorogonium elongatum*. It is true also that leucine and tyrosine as compared with the other amino acids produced relatively better growth of *Chlorogonium elongatum* than they did in the case of the faster growing species. Whether or not the specific differences indicated would have been evident had the amino acid cultures been subinoculated a number of times, remains to be determined.

Notable species differences with respect to amino acid utilization have been recorded by Dusi (1931 and 1933). This investigator found that it was possible to distinguish six species of *Euglena* by their growth on a series of isolated amino acids. *Euglena pisciformis* did not grow with any single amino acid as a source of nitrogen, in which respect it is comparable to *Chilomonas* (cf. preceding results). Best growth of *Euglena anabaena* was obtained with phenylalanine, while growth of the other members of the series was relatively less

favorable with this nitrogen source. Other nutritional peculiarities of *Euglena gracilis*, *Euglena stellata*, *Euglena deses* and *Euglena klebsii* were pointed out by DUSI and referred to in LWOFF's (1932) proposed classification of microorganisms. Present findings indicate that *Chlorogonium* is similar to *Euglena gracilis*, *Euglena stellata*, *Euglena deses* and *Euglena klebsii*, and different from *Euglena pisciformis* with respect to growth with phenylalanine, which was one of the least favorable of the amino acids tested for growth of *Chlorogonium*.

The apparent growth of *Chilomonas* on single amino acid media which was observed in preliminary experiments (LOEFER, 1932 a) was probably not due to the direct utilization of the single amino acid, but rather to the nitrogen which had been introduced from the stock medium with the inoculum. The failure of *Chilomonas* to grow on single amino acids in tubes of the same lots of media as those in which accelerated growth of *Chlorogonium* had resulted, as is shown in the present experiments, indicates that nutrition of *Chilomonas* is more like that of the ciliates that that of the Phytomonadida and Euglenida (except *Euglena pisciformis*). LWOFF's (1932) findings on *Glaucoma piriformis* show that mixtures of certain amino acids are inadequate for growth and that nitrogen in a more complex form (peptids or peptones) is of vital importance. BOND (1933) reported that asparagin and tyrosine were inadequate nitrogen sources for *Colpidium campylum*. Using the same strain of *Colpidium campylum*, as well as a bacteria-free strain of *Colpidium striatum*, ELLIOTT (1935) found the same to be true for the single amino acids glycine, dl-valine, dl-leucine, phenylalanine and l-tyrosine, as well as asparagin. This investigator, however, found that best growth was correlated with an abundance of amino acids in the medium, and he also demonstrated the production of a gelatinase by both species. Such an enzyme has also been demonstrated for *Leptomonas ctenocephali* (M. LWOFF, 1929), *Glaucoma piriformis* and *Polytoma uvella* (A. LWOFF, 1932). Although *Chilomonas* did not liquify 12 % nutrient-gelatin, the elaboration of a gelatinase is indicated by the fact that good growth occurred in Bacto-gelatin (0.5 %) after a four-week culture period. Moderate growth also occurred in casein after four weeks. Table I shows that maximum growth of *Chilomonas* (after 36 hours) took place in a medium containing an abundance of amino acids (Bacto-tryptone). After four weeks, growth in this medium was no better than with Bacto-gelatin, which fact would indicate that enzymatic hydrolysis of the gelatin was probably taking place. Increased growth with Bacto-peptone

and good growth in Proteose-peptone and blood serum after eight weeks also indicate the elaboration of enzymes. No cytological evidence, such as VOLKONSKY (1930) obtained for the direct utilization of polypeptid nitrogen in *Polytoma uvella*, has as yet been obtained for *Chilomonas*.

It is interesting to note that both aspartic acid and asparagin are most effective in accelerating the growth of *Chlorogonium*, but not that of *Chilomonas*. Certain plant physiologists (cf. MAXIMOV, p. 226) believe that asparagin in plants may be analogous to urea in animal organisms. Ammonia, otherwise toxic to the organism, is utilized for synthesis of asparagin. These investigators have shown that there is an actual synthesis of asparagin at the expense of other amino acids during germination of certain seeds. Ammonia is later split off and used for protoplasmic synthesis. The occurrence of a similar process in *Chlorogonium* would account for the relatively better growth with asparagin and aspartic acid than resulted with the other single amino acids which were tested. On the other hand, nitrogen nutrition of *Chilomonas* appears to be fundamentally different. Another interesting fact concerning these forms which may have a bearing on their phylogeny is, that growth of *Chlorogonium* is accelerated by arabinose and xylose, while the effect of these pentoses on the growth of *Chilomonas* is negative (LOEFER, 1935).

Summary.

A series of proteins were tested for their growth-promoting properties on *Chlorogonium* (*euchlorum*, *elongatum*) and *Chilomonas paramecium*. After 36 hours most abundant growth had occurred in Bacto-tryptone, Bacto-yeast extract and Bacto-beef blood serum. After four weeks growth was also good in Proteose-peptone and Bacto-gelatin, while Casein, Bacto-veal and Bacto-peptone supported moderate growth.

The results of the amino acid experiments on *Chilomonas* indicate that this form is unable to utilize as a source of nitrogen any of the single amino acids tested. Since Bacto-tryptone, which supports excellent growth, contains complex nitrogen in addition to a relatively large number of the known amino acids, it can only be concluded that the single amino acids tested are not adequate nitrogen sources under the conditions of the experiments. Growth of *Chlorogonium* on the other hand, is appreciably increased by certain amino acids, especially aspartic acid. Difference in growth rate is the most

important difference between *Chlorogonium elongatum* and *Chlorogonium euchlorum* in all the media tested.

The influence of asparagin on growth of *Chlorogonium* is marked, although it caused no acceleration of growth in *Chilomonas* cultures. The possible bearing of this fact on phylogeny is pointed out. None of the amides, corresponding to the lower fatty acid series, was effective in accelerating growth of either *Chlorogonium* or *Chilomonas*.

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