(Biological Laboratory, University College, New York University.)

Effect of ethyl alcohol on the growth of eight protozoan species in bacteria-free cultures.

By

J. B. Loefer and **R. P. Hall.**

Introduction.

It is difficult, in view of the conflicting reports in the literature, to draw definite conclusions in regard to the effects of alcohol on the growth of Protozoa. Calkins and Lieb (1902) reported that in *Paramecium caudatum* the division rate is increased appreciably by dilute solutions of ethyl alcohol. With *Paramecium aurelia* and *Oxytricha fallax* Woodruff (1908) observed an accelerating effect in certain periods of the life cycle of these ciliates, and a decrease in division rate in other periods of the life cycle. Daniel (1909) found that alcohol accelerated division of one strain of *Stentor coeruleus*, although no significant effect was produced in a second strain of the same species. Bills (1924) concluded that ethyl alcohol prolonged the life of his "starving cultures" of *Paramecium*, and that such cultures might even "be restored to prosperity by the addition of suitable amounts of any alcohol". On the other hand, both Estabrook (1910) and Matheny (1910) reached the conclusion that growth of *Paramecium* is not accelerated by alcohol.

All of these investigations were carried out with bacterized cultures of Protozoa, and it is obvious that under such conditions it is impossible to control environmental factors as accurately as might be desired. For this reason, the writers have attempted to carry out a series of experiments to test the effect of differing concentrations of ethyl alcohol, using bacteria-free strains of *Chlorogonium*

elongatum, *Chilomonas paramecium*, *Euglena gracilis*, *E. deses*, *Astasia* sp., *Colpidium campylum*, *Glaucoma piriformis* and *Paramecium bursaria*. With the elimination of the bacterial factor, the results obtained should indicate, more accurately than has been the case heretofore, the effect of alcohol upon the growth of Protozoa.

Material and methods.

The bacteria-free strains of *Chlorogonium elongatum*, *Euglena gracilis* and *E. deses* were obtained several years ago from Prag, through the kindness of Professor E. G. Pringsheim. A strain of *Astasia* sp. was furnished us by Dr. T. L. Jahn, while the strain of *Chilomonas paramecium* used was the one originally isolated at Wood's Hole in 1932. The bacteria-free strain of *Paramecium bursaria* was isolated by Loefer (1934); cultures of *Colpidium campylum* and *Glaucoma piriformis* were obtained from strains maintained in this laboratory for several years.

The following media were used in the different experiments:

Medium A:	Bacto-tryptone (Difco)	5.0 gm
	KH_2PO_4	2.0 „
	Distilled water	1.0 liter
Medium B:	Bacto-tryptone (Difco)	5.0 gm
	$Ca(NO_3)_2$	0.2 „
	NaCl	0.2 „
	K_2HPO_4	0.02 „
	$MgSO_4 \cdot 7H_2O$	0.02 „
	$FeCl_3$	trace
	Distilled water	1.0 liter

By the addition of 95% alcohol aseptically to sterile distilled water in flasks, the following solutions of alcohol were prepared: 50, 40, 30, 20, 10, 5, 1, 0.5 and 0.25%. The stock medium was them made up and tubed in 8.0 cc amounts. One cc of sterile distilled water was added to each of the control tubes (0.0% alcohol). To each set of the remaining tubes, one of the different solutions of alcohol was added in 1.0 cc amounts. One tube of each set was incubated in order to determine bacterial sterility of the alcohol sclutions and media, while the remainder were inoculated (1.0 cc inoculum) from a stock culture of the appropriate organism. In the various sets of tubes the initial concentration of alcohol thus ranged from 0.0 to 5.0%.

Initial and final concentrations of organisms per cc were determined by means of the Sedgwick-Rafter counting-cell method (cf. HALL, JOHNSON and LOEFER, 1935; and LOEFER, 1936). In summarizing our results, growth is expressed as x/x_0 (ratio of final to initial concentration of organisms per cc).

Chilomonas paramecium.

Series I a. In this series, medium B was used with an initial p_H of 6.8. The tubes were inoculated from a stock dilution flask, the initial concentration being 296 organisms per cubic centimeter. The series was incubated for 45 hours in a water bath at 28° C., and then the tubes were fixed for counting. The results, expressed as x/x_0, are summarized in table 1. There was no evidence for acceleration of growth of this species by ethyl alcohol. In comparison with the control tubes, growth was decreased to some extent by concentrations of alcohol as low as 0.05% and was completely inhibited in 2.0% alcohol.

Series I b was started with a higher initial concentration (532) and was incubated for 43 hours at 28° C. The results (table 1) are similar to those obtained in series I a and also indicate that growth of *Chilomonas paramecium* is not accelerated by ethyl alcohol under the conditions of our experiments.

Chlorogonium elongatum.

Series II a. In this series the initial concentration of *C. elongatum* was 1320 per cubic centimeter, medium B being used at p_H 6.8. The cultures were incubated for 120 hours at room temperature and were then fixed for counting. The results are expressed as x/x_0 in table 1. The most interesting feature of the results is the comparatively high resistance of this flagellate to alcohol as compared with *Chilomonas paramecium*; the former species grew slowly in 3% and 4% alcohol, whereas no growth of the latter was observed in 2% alcohol.

Series II b was started with a lower initial concentration (306) and was incubated for 168 hours at room temperature. The results (table 1) were similar to those obtained in series II a. In neither case was there any evidence that growth of *Chlorogonium elongatum* is accelerated by alcohol, although some growth occurred in concentrations as high as 4%.

Table 1.

Series	Species	x/x_0 in different concentrations of ethyl alcohol									
		0.0 %	0.025 %	0.05 %	0.1 %	0.5 %	1.0 %	2.0 %	3.0 %	4.0 %	5.0 %
I a	*Chilomonas paramecium*	397	398	351	322	252	187	—	—	—	—
I b	*Chilomonas paramecium*	223	214	212	206	198	121	—	—	—	—
II a	*Chlorogonium elongatum*	21	21	22	21	19	17	12	5	3	—
II b	*Chlorogonium elongatum*	105	100	109	93	83	61	45	17	2	—
III a	*Euglena gracilis*	53	74	87	87	71	64	29	—	—	—
III b	*Euglena gracilis*	131	199	246	251	226	172	98	18	—	—
IV	*Euglena deses*	36	38	42	48	39	12	5	—	—	—
V	*Astasia* sp.	53	54	50	46	49	49	22	7	—	—
VI	*Glaucoma piriformis*	71	66	68	64	59	60	49	7	2	—
VII a	*Colpidium campylum*	119	122	115	111	101	105	38	13	—	—
VII b	*Colpidium campylum*	92	63	62	62	57	51	4	2	—	—
VIII	*Paramecium bursaria*	6.4	6.6	5.5	5.5	4.6	3.5	—	—	—	—

Euglena gracilis.

Series III a was started with an initial concentration of 363 in medium B at p_H 6.8, and was incubated for 120 hours at room temperature. Growth is expressed as x/x_0 in table 1. Contrary to the results obtained with *Chilomonas* and *Chlorogonium*, growth of *Euglena gracilis* was accelerated by concentrations of alcohol ranging from 0.025 to 1.0 %. Growth in 2 % alcohol was less than in the controls, and in 3 % alcohol there was a decrease in number.

Series III b was similar to series III a, except that the initial concentration was 195 organisms per cc and the cultures were incubated for 124 hours at room temperature. The results are summarized in table 1. In this series, as in the former, acceleration of growth occured in 0.025—1.0 % alcohol, while growth was decreased in concentrations of 2 % and 3 % and inhibited completely in 4 % alcohol.

Euglena deses.

Series IV was started in medium B at a p_H of 6.8 and was incubated for 254 hours at room temperature. The initial concentration was 210 per cc. Growth is expressed as x/x_0 in table 1, and in concentrations of 0.025 and 0.5 % alcohol was not significantly greater than in the controls, but in cultures containing 0.05 and 0.1 % alcohol there was definite acceleration of growth. It would seem, therefore, that growth of *Euglena deses* also is accelerated by ethyl alcohol but less markedly and over a narrower range of concentrations than in the case of *Euglena gracilis.*

Astasia sp.

In series V cultures were started in medium B at a p_H of 6.8 with an initial concentration of 548 organisms per cc and were incubated for 90 hours in a water bath at 28° C. The results (x/x_0) are summarized in table 1. It is apparent that growth of *Astasia* sp. was not accelerated by alcohol.

Glaucoma piriformis.

In series VI cultures were started with an initial concentration of 483 in medium B at p_H 6.8, and were incubated for 45 hours in a water bath at 28° C. From the results (table 1), it is obvious that growth of *Glaucoma piriformis* was not accelerated by alcohol under the conditions described.

Colpidium campylum.

In series VII a medium B was used at a p_H of 6.8. Cultures with an initial concentration of 328 were incubated for 45 hours in a water bath at 28° C. The results (table 1) indicate that growth of *Colpidium campylum* is not accelerated by alcohol.

Series VII b. Cultures were started with an initial concentration of 246 organisms per cc in medium A at p_H 6.0 and were incubated for 91 hours at room temperature. As in series VII a, the results (table 1) show no acceleration of growth by alcohol.

Paramecium bursaria.

Series VIII. Cultures were started with an initial concentration of 42 organisms per cc in medium B at p_H 6.8, and were incubated for 7 days at room temperature. The results (table 1) failed to indicate any acceleration of growth by alcohol. Growth of *Paramecium* was completely inhibited in 2 % alcohol.

Discussion.

In the series of Protozoa tested in the present investigation the lethal concentration of ethyl alcohol varied with the species. Most resistant were *Chlorogonium elongatum* and *Glaucoma piriformis*, both of which grew slowly in 4 % alcohol but failed to grow in a concentration of 5 %. *Chilomonas paramecium* and *Paramecium bursaria* were least resistant, since each failed to grow in 2 % alcohol. These findings do not differ markedly from results obtained previously with other species in bacterized cultures. For example,

Matheny (1910) reported that his cultures of *Paramecium* were weakened in 3% alcohol and died in concentrations greater than 3%. Similarly, Estabrook (1910) reported inhibition of growth in 3—5% alcohol.

In regard to the accelerating effect of low concentrations of alcohol on the growth of Protozoa, some investigators have obtained positive evidence, while others have reported negative results. Calkins and Lieb (1902) stated that the division rate of *Paramecium caudatum* was increased 30% or more in media containing 0.04—0.06% ethyl alcohol, and that the 'general vitality' of the cultures was improved. More recently, Bills (1924) found that starving cultures of *Paramecium* "can even be restored to prosperity by the addition of suitable amounts of any alcohol". Woodruff (1908), working with *Paramecium aurelia* and *Oxytricha fallax*, found that minute doses of alcohol (e. g., 1:2500) "will decrease the rate of division at one period of the life cycle and increase it at another period of the life cycle". Daniel (1909), on treating a strain of *Stentor coeruleus* with 1% alcohol, observed an acceleration of division "which resulted in cells of smaller size". With a second strain of the same species, no significant effect was observed. Negative results were obtained by Estabrook (1910), who stated that concentrations of 1% alcohol and less produced no effect on the division rate of *Paramecium*, while growth was retarded by concentrations of 2% and higher. Likewise, Matheny (1910) reported that 0.01—2.0% alcohol produced no effect whatever, and concluded that "there is no evidence that alcohol acts as a periodic or continued stimulus" to the growth of ciliates.

Our results, obtained with bacteria-free cultures of *Colpidium campylum*, *Glaucoma piriformis* and *Paramecium bursaria*, agree with the findings of Estabrook and Matheny in that they furnish no evidence that ethyl alcohol accelerates the growth of ciliates. However, we were able to detect inhibitory effects of alcohol in relatively low concentrations, and we are forced to disagree with Estabrook and Matheny in their conclusions that 1% and 2% solutions produce no effect on growth of ciliates.

The behavior of *Astasia* sp. and *Chilomonas paramecium* was similar to that of the ciliates investigated. Growth was decreased in relatively low concentrations of alcohol, and there was no evidence at all for acceleration of growth by alcohol. In the case of *Chlorogonium elongatum* there was likewise no acceleration of growth

by ethyl alcohol, although this species was distinctly less susceptible than the other two species.

Euglena gracilis and *Euglena deses* are particularly interesting in that both showed a definite acceleration of growth in low concentrations of alcohol. With the former species acceleration of growth was evident in concentrations ranging from 0.025 % to 1.0 %, the increase in growth ranging from about 20 % to more than 90 % as compared with the control cultures. Greatest acceleration was observed in 0.05—0.1 % alcohol. In the case of *Euglena deses* definite acceleration of growth was observed in 0.05 % and 0.1 % alcohol. With the related colorless euglenoid, *Astasia* sp., no acceleration of growth was observed, although the decrease in growth was relatively insignificant in concentrations ranging from 0.05 to 1.0 % alcohol. The results suggest that the two chlorophyll-bearing flagellates, *Euglena gracilis* and *E. deses*, utilize ethyl alcohol during growth in light, while the other species investigated are unable to do so.

The zoochlorella of *Paramecium bursaria* appears to be more resistant to alcohol than the host species, since free-living algae were often observed in cultures containing as much as 3 % alcohol after the host species had perished.

Summary.

The effect of ethyl alcohol (0.025—5.0 %) upon growth of the following species was determined: *Chilomonas paramecium*, *Chlorogonium elongatum*, *Euglena gracilis*, *E. deses*, *Astasia* sp., *Colpidium campylum*, *Glaucoma piriformis* and *Paramecium bursaria*. All experiments were carried out with bacteria-free cultures. Growth of *Euglena gracilis* was accelerated by concentrations of alcohol ranging from 0.025 to 1.0 %, and that of *Euglena deses* by 0.05 and 0.1 % alcohol. In the remaining species, there was no evidence for acceleration of growth by ethyl alcohol.

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