SOME PROPERTIES OF A PHOTODYNAMIC PIGMENT FROM BLEPHARISMA*

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Blepharisma undulans, a ciliate protozoan, accumulates a reddish pigment when grown in the dark. If the blepharismas are first bleached by dim light they are not affected by brilliant visible light, but deeply pigmented individuals are killed by such exposure. This occurs in the presence of oxygen, but not in its absence (Giese, 1946). Photodecomposition is accompanied by a greatly increased oxygen consumption. Such oxygen consumption also occurs on illumination of the pigment extract even in the absence of live animals (Giese and Zeuthen, 1949).

The pigment released by maceration of a concentrated suspension of blepharismas is toxic to various protozoans in the dark (Giese, 1949), but when diluted it is harmless. The question arises whether even at low concentrations, but in bright light, it might be injurious because of its action as a photodynamic sensitizer. Recently it was found that the pigment in dilute solutions renders colorless protozoans susceptible to visible light. The results are described below as are also some of the other properties of the extracted pigment of blepharisma.

Materials and Methods

The various protozoans used in this study were cultured as previously described (Giese and Taylor, 1935; Giese, 1938, 1946) except that blepharismas were grown at pH 8.0 rather than at 7.0 since they multiplied faster in cultures at this pH. The protozoans were concentrated by gentle hand centrifuging at about ten times gravity. The pigment of blepharisma was extracted by exposure of a dense concentrate to boiling water for half a minute.[1] The bodies of the animals which still remained pink, were centrifuged off and only the reddish supernatant was used. In most experiments this extract was diluted before use but the concentration of the pigment could not be measured since it has not been purified. The pigment has been named zoopurpurin by Arcichovskij (1905).

The source of radiations for all photodynamic experiments was a GE 100 watt spotlamp. The protozoans were exposed at a distance of 17 inches at which point the

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[1] Other methods of extraction were used for absorption spectrum studies (see section 3).

intensity of the light was of the order of 750 foot-candles as determined with a photometer. In all cases the light was first passed through a layer of 5 to 6 inches of water to remove heat and then through a No. 3389 Corning filter to exclude ultraviolet and the short end of the visible spectrum. The cutoff for this filter is about 4100 A, with 50 per cent transmission at 4300 A. Absorption spectra were determined with a Beckman spectrophotometer. Only densities of arbitrarily diluted solutions could be measured and the relative absorption plotted, since the concentration of the pigment is not known. The characteristic absorption peaks were determined and the effects of pH, heat, or other treatment upon these were studied. The ozone was obtained from an ozonizer similar to that described by Yost and Russell (1944).

EXPERIMENTAL

1. Photodynamic Action of the Blepharisma Pigment, Zoopurpurin

If the pigment extracted from blepharisma by hot water treatment is diluted so that it is only slightly colored when observed against a white background, it has no effect whatsoever on paramecia in diffuse light or in the dark, even after many hours. However if the paramecia in the solution of the pigment are put in intense light they swell up, shorten, and widen just as they do when illuminated in a solution of rose bengal. Because the body cilia are immobilized before those of the oral groove, the paramecia rotate about the long axis, and, finally, clear vesicles appear on the surface into which the protoplasm squirts before the animals burst. The rate of cytolysis depends upon the concentration of pigment as seen in Fig. 1. When enough pigment is present, the photosensitized animals become stained slightly after injury.

The pigment acts as a photosensitizer not only on paramecium but also on other protozoans as well, thus *Colpidium colpoda, Stylonychia curvata, Frontonia leucas, Urocentrum turbo, Ameba proteus, Chilomonas paramecium,* and other protozoans were killed by exposure to light in the presence of the pigment. Frontonia is very susceptible, urocentrum almost as susceptible as frontonia, colpidium less so, and forms like ameba and chilomonas are quite resistant. Ameba is killed only in the higher concentrations of the pigment. In all cases the dilute zoopurpurin is not harmful if the protozoans are kept in the dark. Colpidia, like paramecia, become filled with pink food vacuoles as they eat the small particles of blepharisma still containing the pigment, but like the paramecia they seem to be unaffected by these in the dark.

Paramecium bursaria, containing zoochlorellae, is more resistant to the solutions of zoopurpurin in light than *P. multimicronucleatum.* Thus in a concentrated solution of the pigment in which paramecia of the latter species died in less than 2 minutes, those of the former were killed in 6. This difference might be due to a lesser sensitivity of *P. bursaria* to any type of photodynamic action. To test this, members of both species were illuminated in the presence of 1:80,000 rose bengal. *P. bursaria* proved even more sensitive than *P. multi-*

micronucleatum, the former dying in less than a minute whereas the latter died in somewhat over a minute. The greater resistance of *P. bursaria* to zoopurpurin must therefore be due to some factor other than a general high resistance to photodynamic action. Since the photosynthetic zoochlorellae in *P. bursaria*

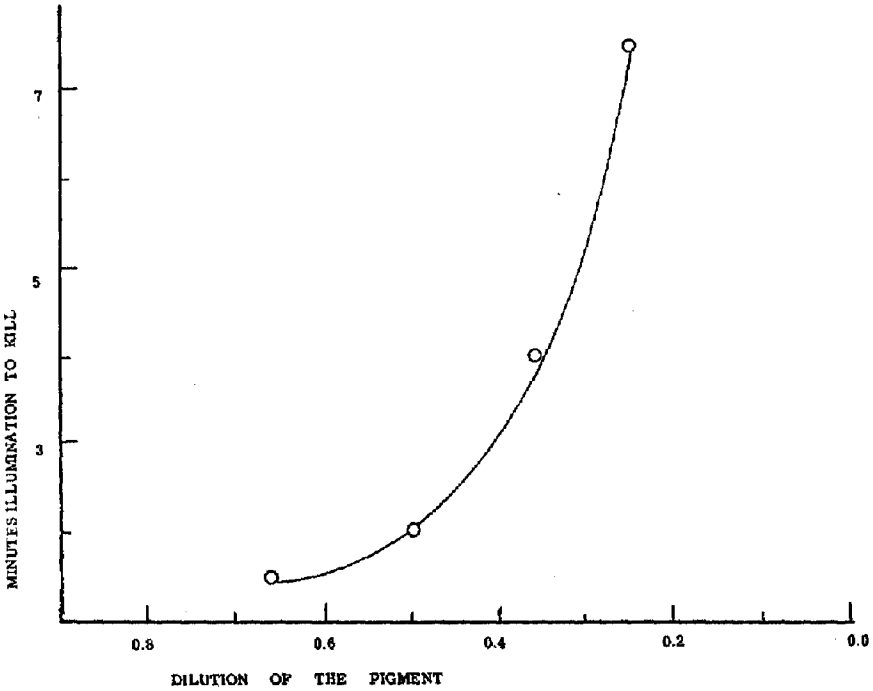

FIG. 1. Relation between exposure time to kill *Paramecium multimicronucleatum* and concentration of pigment from *Blepharisma undulans*. The pigment was extracted from 75 cc. of culture medium of a rich culture containing about 250,000 blepharismas. The 0.2 cc. of pigment extract containing this pigment was diluted to give the arbitrary concentration scale on which 1.0 is undiluted pigment. The intensity of the spotlight was 750 foot-candles; the light was passed through a water filter to remove heat and through a Corning filter No. 3389 to remove long ultraviolet and short visible light.

evolve oxygen during illumination, it is possible that the zoopurpurin is oxidized before it can exert its full destructive effect.

Blepharismas bleached by prolonged exposure to dim visible light (Giese, 1938) were placed in a spot of intense light in the presence of the most concentrated pigment extract from red specimens, to determine whether they would be affected by pigment of their own kind. Even with prolonged illumination—well over 2 hours—the bleached specimens were unaffected. Yet paramecia placed in the same high concentration of the pigment were killed

in 2 minutes in the light. It is possible that the pigment is so rapidly bleached by the intense light that it cannot produce its characteristic photodynamic effect on bleached blepharismas. To test this, paramecia were added among the blepharismas after half an hour exposure of the latter in the same pigment extract. The paramecia were killed in 5 minutes. Therefore while the pigment had been bleached to some extent, it still photosensitized paramecia. The resistance of bleached blepharisma to its own pigment must therefore be explained in some way other than lack of photosensitizer.

The possibility that the pigment penetrates into blepharisma slowly had to be considered. Bleached specimens were therefore added to the concentrated pigment extract 3 hours before illumination. Even after being exposed to the light for 4 hours they were not injured.

Perhaps blepharisma is just not susceptible to photodynamic injury. To check this, bleached specimens were added to 1:80,000 rose bengal. Whereas paramecia placed in the same solution were strongly affected after exposure to the intense light for 30 seconds and killed in 45, blepharismas were not affected until a lapse of 9.5 minutes and cytolyzed only after 11.5. In experiments in which samples of the two species were exposed together in the same dish, similar differential results were obtained. In still another experiment colpidia were exposed along with blepharismas with like results. It is therefore obvious that blepharisma is more resistant to photodynamic action with rose bengal than either of the other species.

Because the mechanism of photodynamic action[2] is unknown, it is difficult to say why blepharisma is so resistant to photodynamic action either in the presence of synthetic dyes or of its own pigment. However, recent work on photodynamic action in yeast (Freeman and Giese, 1952) indicates that the photosensitizing dye probably penetrates under the influence of light before it exerts its full effect. It is possible that blepharisma resists action by such dyes because of their slow penetration. When the natural dye is already inside, as in the deeply pigmented blepharismas, intense illumination results in death. But even in the latter case, death occurs only if the animals are subjected to intense illumination, in other words only when photooxidation occurs rapidly. Subjected to weak illumination, they are bleached without noticeable injury (Giese, 1938).

Further evidence of slow passage of blepharisma pigment through mem-

[2] Blepharisma pigment failed to sensitize frog sciatic nerve preparations to intense visible light. The action potential measured with an oscilloscope remained comparable to that of a control nerve. Since ultraviolet light also failed to change the potential, it was concluded that the connective tissue sheath covering the nerve probably screens the nerve fibers from the radiations. Therefore the negative results with the pigment cannot be considered significant. Attempts are now being made to unsheathe the nerve for these studies.

branes was obtained form experiments in which they were fed to other protozoans. Thus *Actinosphaerium eichhorni*, a carnivorous heliozoan, feeds upon blepharisma ravenously, a maximum of 20 vacuoles having been seen in a large animal. Several such animals filled with about six blepharismas each were exposed to the light from the spotlamp. In no case were they killed or visibly injured but in all cases they promptly evacuated the red food vacuoles. This would suggest either that the pigment escaped on illumination and rendered the vacuole "distasteful" to the heliozoan or that some toxic product was formed which had this effect.[3] Until actinosphaerium was illuminated, the pigment was confined within the food vacuole.

2. Solubility of Zoopurpurin

Arcichovskij (1905) showed that the pigment of *Blepharisma lateritium* is readily dissolved in ethyl alcohol and that neither carbon disulfide, benzene, petroleum ether, nor xylol removes it from alcohol. Only sulfuric ether successfully extracts it from alcohol. Emerson (1930) showed that the pigment of *B. undulans* was soluble in 95 per cent alcohol. Present experiments have also demonstrated the solubility of the pigment of the latter species not only in ethyl but also in methyl, normal butyl, and tertiary butyl alcohol but not in isoamyl or isopropyl alcohol. In all these cases the highly concentrated blepharismas at the bottom of a centrifuge tube, freed of as much water as possible, were treated with the reagent directly. When attempts were made to extract such a concentrate with a variety of other solvents, slight solubility was observed in dioxane and ethyl ether but none in carbon tetrachloride, benzene, toluene, chloroform, acetone, carbon disulfide, and petroleum ether. However, if the alcohol extract was dried, extraction was successful with many of the above relatively non-polar solvents such as toluene, xylene, 1,2-dichlorethane, chloroform, isoamyl alcohol, and ethyl acetate. However non-polar solvents such as benzene, carbon tetrachloride, and ether still dissolved the pigment sparingly and petroleum ether only slightly.

As is apparent from the photodynamic experiments with zoopurpurin, the latter is also water-soluble. Boiling in water or freezing (dry ice, or a longer time at 0°C.) releases part of the pigment bound inside the animal. Furthermore physical disruption releases it even without cooling or heating. The water extract is never as clear as the alcohol extract; light scattering from large

[3] Experiments carried out by one of my students, Nicholas Pappas, indicate that while *Blepharisma undulans* is eaten it does not serve as a fully adequate food for *Actinosphaerium eichhorni* even in relative darkness. In all seven series of trials the heliozoans died when fed these animals exclusively. At the same time in parallel cultures specimens fed on *Colpidium colpoda* divided more than once daily to produce thriving cultures. This suggests the inadequacy of blepharisma as a nutrient or the slow poisonous action of the pigment or some other constituent.

particles in the colloidal state is suggested. The data on solubility indicate that both hydrophobic and hydrophilic radicals are present in zoopurpurin. Since zoopurpurin is more soluble in lipoid solvents than in water, it probably contains more hydrophobic groups than hydrophilic ones.

3. Absorption Spectrum and Stability of Zoopurpurin

The absorption spectrum of the aqueous solution of blepharisma pigment possesses peaks at 6000 to 5700 (maximum 5800 A), 5400, 4900, and 3300 A.

TABLE I

Absorption Spectra of Zoopurpurin Subjected to Various Treatments

Solvent	Treatment	Maxima in A			
		1	2	3	4
Ethyl alcohol	Extraction	3300	4900	5400	5800
	Heated ½ min. in boiling water	3300	4900	5400	5800
	pH 3.3,	3500	4950	5500	5900*
	pH 11.0	3300	4900	5400	5800
Water	Frozen, pH 8.0	3300	4900	5400	5800
	Heated ½ min. in boiling water, pH 8.0,	3300	4850	5400	5800
	pH 3 (heat-extracted),	3300	4250 4800	5400	5800
	pH 11.0 (heat-extracted)	3300	Broad band	Broad band	5600–5800
	Aqueous solution of dried alcohol extract pH 9.0	3300	4900	5400‡	5800

* Note shift of all maxima by 100 A or more to the longer wave lengths.

‡ This band merged with the next one at 5800 A.

An alcohol extract has a similar absorption as seen in Table I and Fig. 2. It should be possible to detect effects of various agents on the pigment by determining their action on the absorption spectrum. Judged on this basis, boiling and freezing have little effect on the pigment as seen in Table I. Some samples of the pigment have been kept in alcohol in the refrigerator for over 10 years without apparent deterioration. A sample in water even if kept on ice rapidly loses color.

Mild acidification and alkalinization had little action on the absorption spectrum as seen in Table I. A slight shift towards longer wave lengths occurred with acidification. Strong alkali and acid destroyed the pigment.

Intense visible and ultraviolet light bleached the pigment in aqueous or alcohol solution as seen in Fig. 2. This bleaching was accompanied by strong ab-

sorption of oxygen (Giese and Zeuthen, 1949) and is therefore a photooxidation. Oxygen alone did not bleach the pigment but ozone bleached it rapidly and irreversibly. Hydrogen peroxide was ineffective.

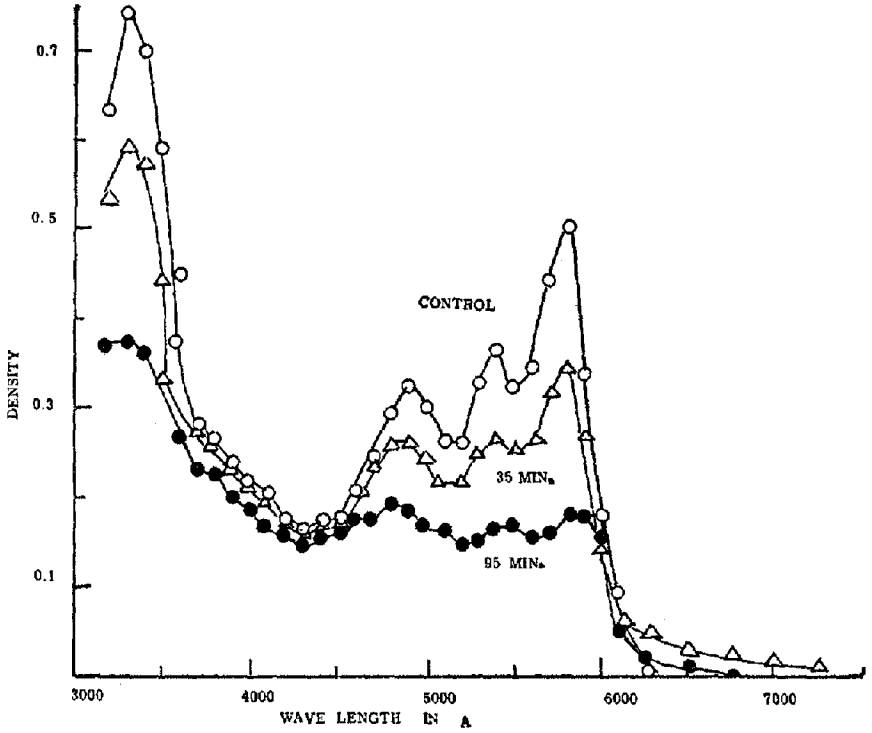

FIG. 2. Effects of light on the absorption spectrum of zoopurpurin in alcohol. Essentially similar results are obtained with the pigment in water except that the photodecomposition appears to be faster. The 35 minute exposure was through Corning No. 3389 filter to exclude short visible and long ultraviolet from the spot-lamp. For the next exposure the filter was removed, exposing the pigment to radiations over the span from 3100 A to the red end of the visible spectrum (7500 A). In some experiments the pigment was exposed to the radiations from a Sterilamp, which gives off about 85 per cent of its energy at λ 2537 A, with qualitatively similar results.

It is interesting to note that when intact blepharisma was exposed to ozone, the color of the animal remained unchanged until cytolysis had occurred. Long before this time blepharisma reacted to the ozone, characteristically by swimming slowly backwards (vacuole forward). After a while the front end began to cytolyze and fragment. Only when this had occurred was the pigment bleached. The uncytolyzed part of the blepharisma continued to swim and the pigment

remained red. Only when this part also cytolyzed was the internal pigment bleached. Some component of the protoplasm either prevented entry of ozone or its reaction with the pigment.

DISCUSSION

Relatively few ciliates possess pigment. Among these are several species of Blepharisma (Arcichovskij, 1905; Emerson, 1930), several species of *Stentor* (Lankester, 1873; Kudo, 1946), *Fabrea salina* (Donnasson and Faure-Frémiet, 1911), and *Holosticha rubra* (Wallengren, 1900). The loricas of various Folliculinids possess pigment, presumably formed by the protozoan (Kudo, 1946).

TABLE II

Absorption Spectra of Pigments of Ciliates

Source of pigment	Citation	Solvent	Approximate absorption maxima* in A
Blepharisma lateritium	Arcichovskij (1905)	Ethyl alcohol	4800–5100, 5150–5450, 5700–6000
Blepharisma undulans	Emerson (1930) This paper	Ethyl alcohol Ethyl alcohol	Below 4000, 4850, 5400, 5900 3300, 4900, 5400, 5800
Stentor coeruleus	Lankester (1873)	In the animal	A broad band from 4800 to shorter wave lengths, 5500 to 5600, 6600
Fabrea salina	Donnasson and Faure-Frémiet (1911)	Acidified ethyl alcohol	Broad band below 5100, 5300, 5890, 6870
Holosticha rubra	Giese (1952)	Ethyl alcohol	Broad band from 3400 to 5500

* Estimated from descriptive data on Fabrea and Stentor for which no curves have been published.

The pigments of blepharisma, fabrea, and holosticha have been studied in extract and the absorption spectra determined. The pigment of stentor has been studied to date only in the intact animal. The data are summarized in Table II. The pigments of stentor, blepharisma, and fabrea all show distinctive bands of absorption with maxima in similar regions of the spectrum. The absorption by the pigment of holosticha is somewhat different, perhaps indicating a different type of pigment. The extracted pigments are at least superficially similar in their solubility in solvents. In all cases the pigments are present as granules in the ectoplasm of the ciliates, perhaps precipitated in an insoluble form.

Arcichovskij (1905) called the pigment of blepharisma zoopurpurin, and with a spectroscope determined the three absorption bands in the visible spectrum. Emerson (1930) made a quantitative determination of the spectrum and corroborated the three maxima. A fourth maximum appearing at 3300 A in the long ultraviolet is reported in the present study. Spectrophotometric determinations were not made in the short ultraviolet since the pigment extract may contain colorless impurities which, while not interfering in the visible part of the spectrum, may absorb in the short ultraviolet.

Perhaps the most interesting feature of zoopurpurin is its uniqueness as an intracellular photodynamic sensitizer, deeply pigmented blepharismas being killed on exposure to bright visible light. Sensitization does not occur unless oxygen is present (Giese, 1946). Furthermore as the present study shows, the pigment is capable of sensitizing colorless protozoans as well. It is therefore comparable to other photodynamic sensitizers, such as the fluorescein dyes.

Of the photodynamic studies reported those with bleached blepharismas are difficult to interpret. Even in presence of high concentrations of extracted pigment and intense light, bleached blepharisma proved resistant. It must have a way of rendering innocuous its own pigment in darkness or light. Blepharisma is also much more resistant than paramecium or colpidium to illumination in a solution of the photodynamic dye rose bengal.

Zoopurpurin is not a large molecule since it diffuses through 4 per cent agar and 8 per cent gelatin at about the same rate as eosin. It seems to have both hydrophobic and hydrophilic groups judging from its solubility in various solvents. It does not diffuse out of living blepharismas in noticeable amounts since both paramecia and blepharismas grow at a normal rate in a mixed culture (Giese, 1949) yet paramecia are quickly injured by dilute extracts of macerated blepharismas even in the dark. It may be that the pigment always exists in bound or precipitated form in blepharisma. When applied in solution to the outside of blepharisma it may also be superficially precipitated, preventing it from entering the protoplasm in sufficient quantity to do harm.

The pigment is normally present in subpellicular ectoplasmic granules, about 2 mμ in diameter, regularly arranged in rows. Weisz (1950) has shown that the granules are basophilic and Feulgen-negative but stain with sudan black, indicating presence of lipids. The latter staining reaction occurs even after the zoopurpurin is extracted with acetone. The granules also stain with the "Nadi" reagents indicating presence of cytochrome oxidase. They stain with Janus green B suggesting that they are mitochondria. The role of zoopurpurin in this complex is unknown, but possibly it is a redox system. If so, it is high in the potential scale since oxygen and even hydrogen peroxide do not oxidize it. Oxygen is effective only when light is applied but ozone oxidizes it irreversibly and readily in the dark.

Experiments are needed to test adequately the purity of the washed alcohol extracts of zoopurpurin. Preliminary chromatographic experiments suggest a single band and a single material. The ultraviolet absorption spectra of purified extracts might help classify the pigment. It is doubtful whether by present culture methods sufficient quantities of pigment could be obtained for chemical analysis. Attempts to develop methods for pure culture or even large vat culture have so far been unsuccessful.[4]

SUMMARY

1. A pigment can be extracted from *Blepharisma undulans* by heat treatment of a concentrated suspension of the deeply pigmented animals.

2. In the presence of this pigment, various colorless protozoans are sensitized to light and killed if exposed long enough. The protozoans show a differential sensitivity, some being much more sensitive than others.

3. Bleached colorless blepharismas are not sensitized to their own pigment even after prolonged exposure in the most concentrated solutions available.

4. Blepharisma is also less sensitive than any of the protozoans tested to such photodynamic dyes as rose bengal.

5. The pigment is not extracted from wet blepharismas by non-polar solvents, but is readily extracted into such polar organic solvents as the alcohols.

6. When the alcohol extract is dried, the amorphous residue is readily soluble in a variety of organic solvents, but not in the most non-polar.

7. The pigment is highly stable in alcohol extract and has been kept in the dark for years.

8. The pigment is bleached by light in a photooxidation.

9. Absorption maxima are found at wave lengths 5800, 5400, 4800, and 3300 A, the latter being the largest. Similar peaks are found in alcohol and water solutions, although the heights are not exactly the same. In both alcoholic and aqueous solutions pH had an effect on the absorption spectrum. Heat has little effect but illumination with intense visible light or exposure to ultraviolet light bleaches the pigment with decreases in the characteristic peaks.

10. Preliminary absorption column experiments indicate a single pigment in the alcohol extract.

11. Experiments on the migration of zoopurpurin in agar and gelatin gels indicate that it diffuses at about the same rate as eosin and is therefore probably not a large molecule.

[4] Attempts were made to grow blepharisma in pure culture in the absence of bacteria without success. Experiments were also carried out by one of my students, Dr. Patrick H. Wells, to feed *Tetrahymena geleii* in pure culture to blepharisma in the absence of bacteria. While blepharismas ate the small ciliates voraciously in cultures containing bacteria, they were unable to survive on the ciliates alone in the absence of bacteria.

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