[6] See, for example, top of page 9, *J. of Symbolic Logic*, **6**.

[7] Compare middle of page 158, *Ibid.*, **7**.

[8] *Am. Math. Mo.*, **44**, p. 70.

[9] *J. of Symbolic Logic*, **7**, p. 1. The inconsistent system contains N7′ in place of N7. However, whether the replacement of N7′ by N7 would affect the derivation of the Burali-Forti contradiction is not immediately obvious.

[10] Middle of page 228, *J. r. angew. Math.*, **160**.

[11] If we want to obtain N7′ instead of N7, just delete the clause "and all the bound variables in ϕ are small variables" in ZZ7.

[12] Compare the proof of *231, ML, page 171.

[13] T1 can be proved by ZZI and ZZ1 in the usual manner. Cf., e.g., ML, page 170, *223.

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THE CULTURE OF PARAMECIUM AURELIA IN THE ABSENCE OF OTHER LIVING ORGANISMS

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The growth requirements of ciliated protozoa are very complex, and little accurate work on this has so far been possible since only a few species have been cultivated in the absence of other living organisms. Sterile cultures of *Tetrahymena geleii*,[1] *Colpoda duodenaria*[2, 3] and *Paramecium multimicronucleatum*[4] have been successfully established. *T. geleii* is thus far the only ciliated protozoan which has been cultured in a synthetic medium in the absence of natural proteins or peptones.[5] In addition to an as yet unidentified "plasmoptyzate factor," presumably of protein nature,[3] *C. duodenaria* requires thiamin, pyridoxin and nicotinic acid in the medium.[6, 7] The growth requirements of *P. micronucleatum* have not yet been established. It has been reported that this organism requires a heat-labile and a heat-stabile factor, both present in yeast press juice and in a bacterial plasmoptyzate.[8]

P. aurelia is routinely grown in a lettuce infusion inoculated with *Aerobacter aerogenes*.[9] While this procedure is satisfactory for general purposes, a standardization of this culture medium would be desirable. This requires the establishment of a culture of *P. aurelia* in a medium devoid of other living organisms, which would sustain the growth of this ciliate at a satisfactory fission rate. After such a medium is developed, a study can be made of the specific growth requirements of this organism.

Sterilization of P. aurelia.—Two different procedures were used to free *P. aurelia* from other living organisms: (*a*) electrical transport of the ciliates against a continuous flow of a sterile agar solution, and (*b*) exposure of *P. aurelia* to penicillin solutions.

(*a*) *Sterilization by Electromigration:* The electromigration tube is shown in figure 1. The tube and the Erlenmeyer containing the wash fluid (NaCl 0.0005 g., KH_2PO_4 0.008 g., $MgCl_2.7\ H_2O$ 0.0035 g., $FeCl_3.6\ H_2O$ 0.006 g., $CaCO_3$ 0.0005 g., agar 1.0 g., lettuce infusion 10 ml., distilled water to make 1 liter) were sterilized separately by autoclaving. Immediately before use the two parts were connected asceptically, and the tube was filled by opening stopcocks *A*, *B* and *C*. The flow of the liquid through the

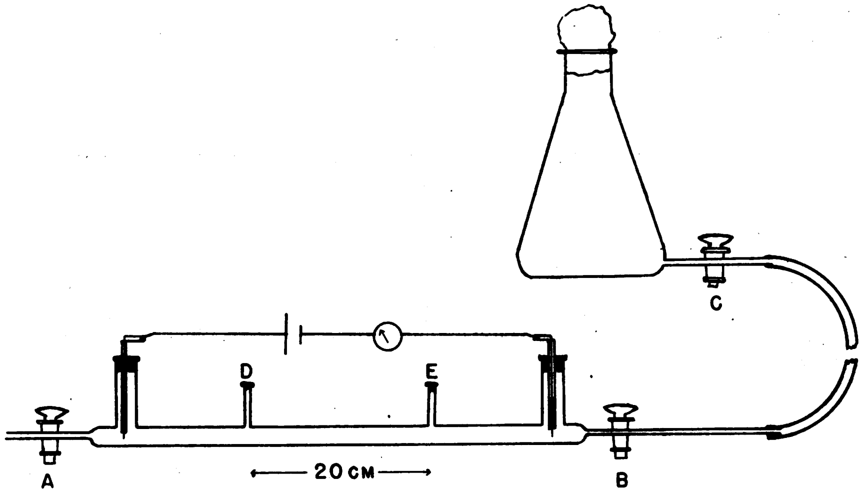

FIGURE 1
Electromigration tube.

tube was adjusted to a rate of 0.6 ml. per minute. The electrodes were connected to a 9-volt battery, thereby producing a current of 0.1 to 0.2 ma. A suspension of *P. aurelia* was injected into side arm *D*. The average migration time of the ciliates from *D* to *E* was about 27 minutes. When the first *P. aurelia* appeared at the side tube *E*, they were removed by means of a sterile syringe and placed in sterile culture fluids of different composition. At the same time samples were distributed to various culture media in order to check for sterility. Subsequent samples of *P. aurelia* can be removed from the tube at point *E* until all of the wash fluid is exhausted. From 30 to 50 ciliates can be recovered from each sample. Under the described conditions about 70 per cent of the samples are sterile.

(*b*) *Sterilization with Penicillin:* Seaman[10] was the first to use penicillin as an agent for the sterilization of protozoans. A wild culture of *C.*

campylum was sterilized by treatment with 5000 units of penicillin per ml. of a 5 per cent proteose peptone broth for 12 hours. In preliminary experiments it was found that *P. aurelia* cannot tolerate concentrations of penicillin higher than 3200 units per ml.

Approximately 20 *P. aurelia* were washed through nine 1-ml. volumes of sterile lettuce infusion, and the small number of organisms which were recovered from the washing process were grown in lettuce infusion inoculated with *A. aerogenes* until the population had reached a concentration of 2000 animals per ml. These paramecia, contaminated by the presence of only a single organism, were then transferred to a solution of 1 per cent yeast autolyzate (Basamin-Busch, kindly furnished by the Anhaeuser Busch Company) saturated with $CaCO_3$, and containing 3200 units of penicillin G-sodium salt per ml. (obtained through the courtesy of Dr. J. M. McGuire of Eli Lilly and Company). This solution had been sterilized by filtration through a sintered glass bacterial filter. After 48 hours in this medium, the organisms were washed through 5 2-ml. volumes of sterile lettuce infusion. The *P. aurelia* from the last wash were then transferred to various media. At the same time samples were distributed to various culture media in order to test for sterility.

(*c*) *Sterility Tests:* *P. aurelia,* treated in either of the two ways described under (*a*) or (*b*), were tested for the presence of contaminating organisms. Aliquots of 0.5 ml. of culture medium to which the organisms had been transferred after the sterilization procedure (taken immediately after the transfer), and aliquots of 0.5 ml. of the culture medium after *P. aurelia* had been in this medium for 5 days were inoculated in the following media: (1) yeast beef agar (Difco), (2) 0.5 per cent yeast extract (Difco), (3) 0.5 per cent yeast extract to which 0.5 per cent sucrose had been added, (4) 0.5 per cent beef extract (Difco), (5) nutrient agar (Difco) to which 0.5 per cent sucrose had been added. The tests were incubated at three different temperatures, 20°, 27°, 37°, under both aerobic and anaerobic conditions. If no contamination occurred, as observed by the absence of cloudiness in the liquid media or absence of colonies on the solid media, in three weeks, the originally isolated *P. aurelia* were considered to be free of any contamination. The organisms were independently tested for sterility by Dr. L. S. McClung of the Department of Bacteriology of Indiana University who confirmed our observations.

Culture in Sterile Media.—Of the various media tried, only the following were able to sustain growth of *P. aurelia:*

(*a*) Equal volumes of 0.5 per cent yeast autolyzate (Basamin-Busch) sterilized by filtration through a sintered glass bacterial filter, and an autoclaved lettuce infusion (20 minutes, 15 pounds), which had been inoculated with *A. aerogenes* 24 hours previous to autoclaving.

(*b*) Equal volumes of 0.5 per cent yeast autolyzate, sterilized by auto-

claving (20 minutes, 15 pounds) and an autoclaved lettuce infusion which had been inoculated with *A. aerogenes* 24 hours previously.

The organisms would multiply in this medium at an average fission rate of 1.7 fissions per day (table 1). The culture has been carried through more than 30 successive transfers. At this time no contamination could be detected by any of the methods mentioned under (*c*). Thus far it has not been possible to make any substitutions for either the particular brand of yeast autolyzate or for the autoclaved bacterial suspension. Various other brands of dried yeast preparations and preparations of fresh yeast were tried but none of these could support the growth of *P. aurelia*. Addition of a bacterial autolyzate in 1 *M* glycine[11] did not allow *P. aurelia* to survive for more than 10 days. No fission took place in this interval. Ultrasonic preparations of an Aerobacter suspension, followed by sterilization by filtration failed to yield preparations which, when added to the yeast autolyzate, would sustain the growth of the ciliates. When either the lettuce infusion or the autoclaved bacterial culture was omitted, the medium became incapable of supporting the growth of the ciliates; similar results were obtained when the three-component medium (described under (*a*) or (*b*) was filtered through a sintered glass bacterial filter. Rapid death resulted when the autoclaved lettuce–Aerobacter infusion was the only constituent of the medium.

TABLE 1

GROWTH RATE OF *Paramecium aurelia* IN A STERILE MEDIUM

NO. OF 48-HR. TRANSFERS	INITIAL NO. OF *P. aurelia*	NO. OF *P. aurelia* AFTER 48 HRS.	FISSIONS PER DAY
1	81	563	1.4
2	56	371	1.4
3	37	272	1.4
4	85	680	1.5
5	56	681	1.8
10	22	350	2.0
15	51	690	1.8
20	41	524	1.8
25	74	1170	2.0
30	15	216	1.9

Discussion.—In view of the accumulated knowledge of the nutrition of exacting species of protozoans,[12, 13] it is surprising that *P. aurelia* is capable of growing and multiplying in a medium containing only heat-stabile components. However, the fact that the sterile medium permits only a maximal growth of 1.7 fissions per day, as compared with 4 to 6 fissions per day in a lettuce–Aerobacter infusion, suggests that heat-labile factors may be necessary for optimal growth. The complex nutritional requirements of *P. aurelia* are evident from the fact that a three-component medium is required for sterile growth. It should now be possible to fractionate the

different components into their constituents and thus arrive at a synthetic medium for this organism. All three components of the medium, yeast extract, lettuce infusion and the autoclaved bacterium *A. aerogenes*, contribute essential factors for the nutrition of *P. aurelia*. The observation that this organism will not grow in a medium composed of the three essential components, but filtered through a bacterial filter seems to indicate that some particles of large size are also needed for its growth. However, it might be possible that some essential nutrilite is so firmly bound to the bacterial cell constituents that it had not been liberated in solution by the methods used.

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[1] Lwoff, A., *C. R. Acad. Sci. (Paris)*, **176**, 928 (1923).

[2] Taylor, C. V., and van Wagtendonk, W. J., *J. Cellular Comp. Physiol.*, **17**, 349 (1941).

[3] van Wagtendonk, W. J., and Taylor, C. V., *Ibid.*, **19**, 95 (1942).

[4] Johnson, W. H., and Baker, E. G. S., *Science*, **95**, 333 (1942).

[5] Kidder, G. W., *Ann. N. Y. Acad. Sci.*, **69**, 99 (1947).

[6] Tatum, E. L., Garnjobst, L., and Taylor, C. V., *J. Cellular Comp. Physiol.*, **20**, 199 (1942).

[7] Garnjobst, L., Tatum, E. L., and Taylor, C. V., *Ibid.*, **21**, 211 (1943).

[8] Johnson, W. H., and Tatum, E. L., *Arch. Biochem.*, **8**, 163 (1945).

[9] Sonneborn, T. M., and Dippell, R. V., *Physiol. Zoöl.*, **19**, 1 (1946).

[10] Seaman, G. R., *Science*, **106**, 327 (1947).

[11] Maculla, E. S., and Cowles, P. B., *Ibid.*, **107**, 376 (1948).

[12] Hall, R. P., *Protozoa in Medical Research*, University of Columbia Press, 1941, pp. 474–516.

[13] Hall, R. P., *Vitamins and Hormones*, Vol. 1, 1943, p. 249.