THE USE OF ANTIBIOTICS FOR OBTAINING BACTERIA-FREE CULTURES OF EUGLENA

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Preliminary to studies of the physiology of *Euglena gracilis*, we attempted to obtain bacteria-free cultures of a clone isolated by Professor W. J. Kostir and maintained in the Protozoology Laboratory of the Ohio State University. Attempts to obtain bacteria-free cultures using the established technique of washing a few individuals in successive baths of sterile distilled water were not always successful and the procedure proved to be time consuming.

The difficult problem of obtaining bacteria-free protozoan cultures is now being attacked by the utilization of antibiotics. Reported investigations (Mahmoud, 1944; Morgan, 1946; Seaman, 1947; and others) have indicated success with penicillin and with streptomycin. However, because of the extensive studies of antibiotic resistance of bacteria (Miller and Bohnhoff, 1950), it appears highly improbable that the reported studies of protozoan isolations with single antibiotics or simple combinations represent a true sampling of the likely antibiotic resistances that may be expected.

Goodwin (1950) reported obtaining bacteria-free cultures of *Euglena gracilis* with the use of penicillin alone, and in combination with dihydrostreptomycin. Our attempts to repeat this work with the Kostir clone were unsuccessful.

MATERIALS AND METHODS

Stock clone cultures of *Euglena gracilis* have been maintained in 0.04 percent peptone (Arlington Chemical Co., Yonkers, N.Y.) solution in a one-to-one mixture of distilled and well-water. However, since this medium was found to be inadequate for maintaining euglena growth in the absence of bacteria, Hall's (1937) medium for pure (bacteria-free) cultures was used. This medium consists of: 0.5 gm of KNO_3, 0.5 gm of KH_2PO_4, 0.25 gm of $MgSo_4$, 0.1 gm of NaCl, a trace of $FeCl_3$, 5.0 gm of tryptone, and 1.0 liter of distilled water. A standard inoculum of the contaminated euglena culture was used, consisting of a single loopful containing 30–40 actively swimming euglenas.

Various combinations of the readily obtainable water-soluble antibiotics—penicillin, dihydrostreptomycin, aureomycin and bacitracin—were tried in an attempt to obtain bacteria-free cultures of *E. gracilis*. These antibiotics were added in varying concentrations and combinations to 10 ml of a three-week-old contaminated euglena culture. No bacteria-free cultures were obtained, possibly because of the large number of bacteria present. Bacitracin was found to be slightly toxic at all the concentrations used and therefore was abandoned.

When a standard inoculum of the source culture was streaked out on blood agar and on nutrient agar, two types of colonies were isolated. The one colony contained small Gram-negative rods while the other, a much larger colony, contained Gram-positive rods. No attempts were made to identify the contaminants further. Finding both Gram-positive and Gram-negative organisms indicated that in order to obtain pure cultures with antibiotics, a wide antibacterial spectrum would have to be provided.

Rather high concentrations of the antibiotics were used in order to make sure that the amount present was greater than the minimal effective concentration

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required for antibacterial action. Epps, Weiner, and Bueding (1950) successfully obtained bacteria-free cultures of *Ascaris lumbricoides* by using combinations of 1000 units per ml of penicillin, 200 micrograms per ml of aureomycin, 100 units per ml of bacitracin, 500 micrograms per ml of streptomycin, and 1.2 mg per ml of sulfadiazine. This concentration of sulfadiazine was found to be toxic to *Euglena gracilis* and was omitted in addition to bacitracin. In our final experiments the above concentrations of the antibiotics used were doubled. The antibiotics were diluted with sterile culture medium to obtain the desired concentrations in a final total of 5 ml of medium. Culture tubes, 15 by 85 mm were used. Table I summarizes the combinations and quantities of the antibiotics tested.

The experiment was conducted at 23 degrees Centigrade, and under fluorescent lighting of 220 foot-candles illumination for 14 hours a day. After 10 days the sterility of the cultures was checked by Gram-stained smears, by subculture into thioglycollate broth (a specified medium for the testing of sterility), and by direct microscopic examination of wet mounts. To test the viability of the euglenas, the test material was also inoculated into sterile euglena culture medium.

TABLE 1

Combinations and quantity of antibiotics tested.
μg = micrograms, u = units.

Culture	1	2	3	4	5	6	7
penicillin	2000 u/ml			2000 u/ml		2000 u/ml	2000 u/ml
dihydro-streptomycin		1000 μg/ml		1000 μg/ml	1000 μg/ml		1000 μg/ml
aureomycin			400 μg/ml		400 μg/ml	400 μg/ml	400 μg/ml

RESULTS

The bacterial resistances to the antibiotics are summarized in table 2. In the control culture, containing no antibiotics, Gram-positive and Gram-negative rods were found, with the Gram-positive rods in preponderance. In culture 1, containing penicillin, a preponderance of Gram-negative rods was present, but there were also noted a few unevenly stained, Gram-positive, long filamentous rods. There was a change in the morphology and the staining reaction of these Gram-positive rods. These changes were probably due to the action of penicillin, and indicated that the organisms were damaged to a certain extent. In culture 2, containing dihydrostreptomycin, no Gram-negative rods were found, but heavy growth of Gram-positive rods occurred. In culture 3, containing aureomycin, both Gram-positive and Gram-negative rods were present. In culture 4, containing penicillin and dihydrostreptomycin, some Gram-positive rods were found in filamentous arrangement. In culture 5, containing dihydrostreptomycin and aureomycin, only some Gram-positive rods were found. Culture 6, with penicillin and aureomycin, showed the presence of Gram-negative rods only. In culture 7, in which all three antibiotics were present, *no bacteria were found.*

After a period of 10 days, the control and test cultures, 1, 2, and 3, showed no viable euglenas. This may be explained by the more rapid and dense growth of the contaminating bacteria in Hall's medium, which has more than a ten fold increase of organic material over the peptone medium previously used.

In evaluating the viability of *Euglena* in cultures and sub-cultures from cultures 4, 5, and 7, the numbers of euglena after ten days growth was found to be approximately 4 percent of that of culture 6, which was the only one of this series of cultures not containing dihydrostreptomycin. The euglenas did not show any loss of chloroplasts in those cultures containing 1000 micrograms per ml of dihydrostrep-

tomycin, contrary to the results obtained by Provasoli, Hutner, and Schatz (1948). Perhaps this discrepancy may be accounted for by the fact that the culture medium contained no carbohydrates and that those cells that did bleach died soon after bleaching. This may account for the small number of organisms in cultures 4, 5, and 7, as compared with culture 6.

TABLE 2

Results of tests of bacterial resistance to antibiotics.

G+ = *Gram-positive, G— = Gram-negative.*

Culture	G+	G—
control	+	+
1 (penicillin)	+	+
2 (dihydrostreptomycin)	+	
3 (aureomycin)	+	+
4 (pen. and dihydrostrep.)	+	
5 (dihydrostrep. and aureo.)	+	
6 (pen. and aureomycin)		+
7 (pen., dihydrostrep., and aureomycin)		

CONCLUSIONS AND DISCUSSION

Penicillin, dihydrostreptomycin, aureomycin, bacitracin, and sulfadiazine were added, both singly and in combinations, to a clone culture of *Euglena gracilis* in attempts to obtain bacteria-free cultures. Successful pure (bacteria-free) cultures were obtained by the use of penicillin, dihydrostreptomycin, and aureomycin in combination. Bacitracin and sulfadiazine were found to be toxic to the *Euglena gracilis* strain tested.

Since both Gram-positive and Gram-negative bacteria were present, obtaining bacteria-free cultures proved to be more difficult than was the case in previously reported studies, except possibly for the study of Williams and Plastridge (1946). Indeed it is difficult to understand the success obtained in earlier studies using a single antibiotic unless factors other than antibiotic action were utilized. From the details of these reports it is difficult to judge what these factors might be, but it appears probable that in some cases at least (Seaman, 1947) washing out some of the bacteria during the isolation procedure may have played an important role.

In the development of antibiotic techniques for obtaining bacteria-free cultures it would certainly be profitable to exploit such methods as washing out at least some of the contaminating bacteria. The numbers of bacteria can also be controlled by such conditions as incubation temperature, inoculum size, pH (Zumstein, 1900), and organic content of the medium. Although the value of these methods has been recognized for some time, it is our own experience from these studies that by themselves they may not be adequate and that the use of antibiotics provides a valuable tool for obtaining bacteria-free protozoan cultures.

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