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A Study of the Effect of Penicillin and Dihydrostreptomycin on Protozoa with Special Reference to the Problems of Bacteria-Free Culture and Speciation in Euglena

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A Study of the Effect of Penicillin and Dihydrostreptomycin on Protozoa with Special Reference to the Problems of Bacteria-Free Culture and Speciation in Euglena

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Among the many phenomena observed in studies with streptomycin and penicillin, the apparent resistance of certain protozoa to antibiotics is of interest to the protozoologist. If this resistance exists in the case of all protozoa, then it should be possible to develop a technique involving antibiotics which can be used for obtaining bacteria-free cultures of any protozoan. The concentrations of penicillin and streptomycin used by various authors in attempts to effect isolation of specific protozoans are presented in Table 1.

Speaking of sensitivity of bacteria to streptomycin, Waksman (1949) states that "within genera, there is considerable variation in susceptibility to streptomycin between species;". It follows then, that such variations may exist within the many genera of Protozoa and possibly within the genus Euglena. If such is the case, these variations between Euglena species may be valid taxonomic characteristics to add to those already in use in species determination.

The purpose of the present study is to: (1) determine if streptomycin and penicillin can be used to effect bacteria-free cultures of Euglena, (2) determine whether or not differences in sensitivity to streptomycin and penicillin exist among certain species of Euglena and if these variations can be used as valid diagnostic characteristics for speciation.

Table 1 lists individuals who have worked with antibiotics attempting to obtain bacteria-free cultures of protozoans.

Materials and Methods

Dihydrostreptomycin sulphate (Merck, lot No. 842 and No. 1173) and penicillin G sodium crystalline (Merck, lot No. 220, No. 551, and No. 617) were used in the following experiments with species of Euglena. Both penicillin and dihydrostreptomycin were put into solution in distilled water and then added to culture media. Concentrations used in all experiments were determined by methods which paralleled those advocated by Merck in bulletins I-162 and I-162A.

Table I

Concentration of Antibiotics in Which Various Protozoans Will Survive as Given by Various Authors

Author	Organism	Concentration of antibiotic organism will live in*	Time	Effected Isolation
Adler and Pulvertaft, 1944	*Trichomonas vaginalis*	250 units/cc. P	Not given	Yes
Johnson et al., 1945	*Trichomonas vaginalis*	5,000 to 10,000 units/cc. P	60 Hours	Yes
Knoll and Howell, 1946	*Endamoeba histolytica*	5-10,000 Oxford units/cc. P	3 Hours	No
Morgan, 1946	*Trichomonas foetus*	300 Oxford units/cc. P	48 Hours	Yes
		10,000 Oxford units/cc. P	Not given	Yes
Williams and Plastridge, 1946	*Trichomonas foetus*	100 Oxford units/cc. P	Not given	Yes
		1,000 units/cc. S	Not given	Yes
		100 units/cc. S	Not given	Yes
		100 units/cc. S and P	Not given	Yes
Quisno 1946	*Trichomonas vaginalis*	25 units/cc. S	10 Hours	Yes
Balamuth and Wieboldt, 1946	*Entomoeba histolytica* (cysts)	100-20,000 units/cc. S	48-96 Hours	Yes
Jacobs, 1947	*Endamoeba histolytica*	100 units/cc. P	Through 20 transplants 24-48 Hours	Yes
Balamuth and Wieboldt, 1947	*Entamoeba histolytica* (trop.)	10,000 units/cc. S	48 Hours	No
Seaman, 1947	*Colpidium campylum*	5,000 units/cc. P	12 Hours	Yes
Spingarn and Edelman, 1947	*Endamoeba histolytica*	1,000 to 3,000 units/cc. S	33.7 days	No

*P represents penicillin, S represents streptomycin.

Table I—Continued

Author	Organism	Concentration of antibiotic organism will live in*	Time	Effected Isolation
Spingarn and Edelman, 1948	*Endamoeba histolytica*	1 to 10 mg./cc. S	Positive Culture	No
		1,250 units/cc. P	Positive Culture	No
Pfeiffer, 1948	*Euglena gracilis*	20-16, 360 units/cc. S	240 to 384 Hours	No
Provasoli, 1948	*Euglena gracilis*	100 microgm./cc. S	4 days	No
		or 40 microgm./cc. S	15 days	No
	Euglena gracilis var. *bacillaris*	100 microgm./cc. S	4 days	No
		or 40 microgm./cc. S	15 days	No
	Euglena gracilis var. *urophora*	100 microgm./cc. S	4 days	No
		or 40 microgm./cc. S	15 days	No
Shaffer and Ryden, 1949	*Endamoeba histolytica*	1,000 Oxford units/cc. P and 1,500 units/cc. S	Through 2-3 48 hour transplants	Yes
	Trichomonas vaginalis	1,000 Oxford units/cc. P and 1,500 units/cc. S	Through 70 48 hour transplants	Yes
	Trichomonas hominis	1,000 Oxford units/cc. P and 1,500 units/cc. S	Not given	No
	Endamoeba coli	1,000 Oxford units/cc. P and 1,500 units/cc. S	6 48 hour transplants	No
Wagtendonk and Hackett, 1949	*Paramecium aurelia*	3,200 units/cc. P	48 Hours	Yes

*P represents penicillin, S represents streptomycin.

All glassware was rinsed with sulphuric acid-potassium dichromate cleaning solution, as suggested by Schoenborn (1949), rinsed eight times in tap water and twice in distilled water, and was air dried.

The Beckman pH meter was used for pH determinations.

Microscopic observations were made with a stereoscopic wide field microscope (10x eyepieces; 1x and 2x objectives) or a compound

microscope (10x eyepieces; 16 mm., 4 mm. and 1.8 mm. oil immersion objectives).

Euglena used for bacteria-free culture attempts were collected in quart or pint jars from roadside ponds, farmyard pools, and lakes of Dickenson County, Iowa. Materials were placed in the laboratory at Iowa Lakeside Laboratory where the following work was done.

After microscopic examination for species determination, the Euglena were concentrated and separated from most other protozoa by placing them in caster dishes illuminated from one side.

Measured volumes of the phototropically concentrated Euglena (0.005 to 0.01 ml.) were added to 0.5 ml. volumes of various concentrations of antibiotics. Penicillin solutions were made by serial dilutions of a 50,000 units per ml. solution with sterile glass-distilled water (pH 7.2 ± 2.0). Dihydrostreptomycin solutions were made by serial dilutions of a standard containing 250 microgram per ml. These solutions were placed in numbered depressions on a spot plate. The spot plates were kept in a moisture chamber except when being observed.

Hourly observations of motility were made using 20x magnification of the dissecting microscope. Approximation of per cent of total organisms motile (swimming freely) in each concentration was recorded. The same procedure was followed for observations of toxicity on other protozoa present in the wild cultures, from natural habitats.

Euglena remaining motile the following day were transferred with sterile pipettes from the spot plates to similar concentrations of penicillin and streptomycin in sterile culture media in pyrex test tubes. Culture medium used for bacteria-free culture attempts was similar to that used by Jahn (1935), 0.50 gm. NH_4NO_3, 0.50 gm. KH_2PO_4, 0.25 gm. $MgSO_4$, 1.0 gm. $NaC_2H_3O_2$, 3.0 gm. Proteose-peptone (Difco), and 1,000 cc. water. Observations for growth and contamination were made with dissecting microscope at three days and twenty days. Culture tubes were kept in a constant temperature water bath at 28°C. when not being observed. Range of penicillin concentrations was 12,500 units per ml. to 390 units per ml.; range of dihydrostreptomycin concentrations was 48 micrograms per ml. to 6 micrograms per ml.

In absence of a sterile room, all transfers to culture media were made in a box constructed to protect the media from air-borne contaminants, the air within being previously sprayed with water. Ster-

ility of culture medium (previously measured into test tubes, 8 ml. per test tube) and materials was accomplished by use of a pressure cooker at 9 pounds pressure for one hour.

Additional studies on sensitivity of Euglena to penicillin and streptomycin were continued at Drake University with aseptic species of Euglena obtained from Dr. Luigi Provasoli of Haskins Laboratories, New York, New York. The organisms were grown in the Provasoli medium namely: 1 per cent $NaC_2H_3O_2$, two per cent Trypticase (Baltimore Biological), and 1 per cent Thiopeptone (Wilson and Co., Chicago). This solution was adjusted to pH 6.0. Seven of the Euglena species obtained from Dr. Provasoli grew adequately for this study. These Euglena were: *Euglena gracilis*, Pringsheim; *E. gracilis* var. *bacillaris; E. gracilis* var. *bacillaris*, colorless; *E. gracilis* var. *urophora;* and unclassified Euglena designated *Euglena H, E. VI,* and *E. 9* by Dr. Provasoli.

Materials and medium were sterilized by autoclaving at fifteen pounds pressure for twenty minutes. Before autoclaving, 2.5 ml. of medium were placed in each test tube (kimble—screw cap type, 20 by 150 mm.) so that the addition of 0.5 ml. of antibiotic solution after autoclaving would produce the original culture medium concentration and the desired antibiotic concentration. This volume of 3.0 ml. was maintained in all toxicity experiments with the seven clones of Euglena obtained from Dr. Provasoli.

Additions of antibiotic solutions and inoculation of aseptic Euglena (with inoculating needle) were carried on in a sterile room. After inoculations (500 to 1000 Euglena per culture tube), culture tubes were placed in a constant temperature water bath at 24°C. and observed regularly for motility by means of 20x power of the dissecting microscope in the following manner. The test tube was shaken vigorously once and placed in focus under the dissecting microscope. When the initial movement of liquid ceased, twenty individuals were observed at random for motility. Those exhibiting typical euglenoid motility (swimming freely) were considered motile. The percent motile of those observed was considered representative of the motility of the culture.

Results and Interpretation of Data

The average effects of penicillin (30,000 to 10,000 units per ml.) on motility of sterile Euglena as calculated from six experiments is shown by composite graphs in figures 1, 2, 3, 4, and 5 of plate I. The Euglena used were: *Euglena gracilis; E. gracilis* var. *bacillaris;*

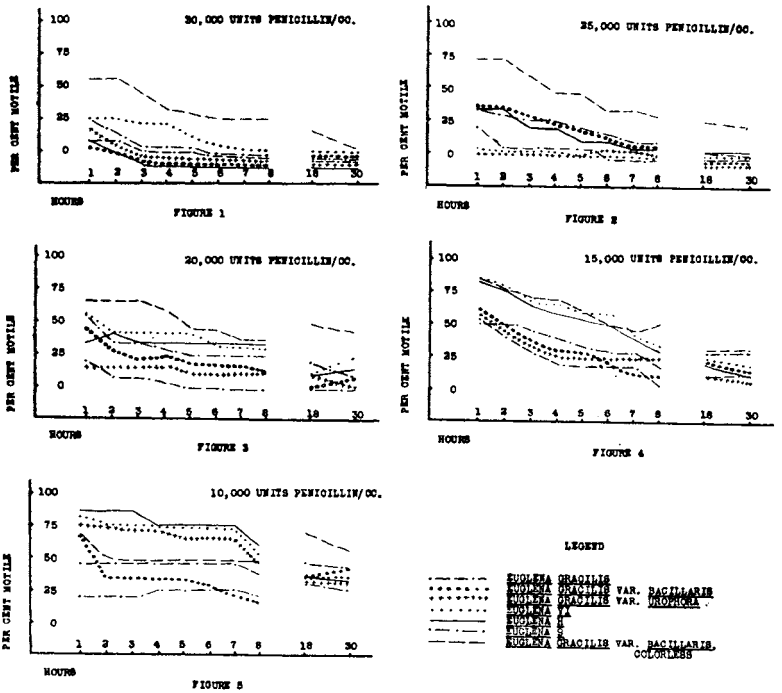
E. gracilis var. *bacillaris*, colorless; *E. gracilis* var. *urophora*; *E. VI*; *E. 9* and *E. H* in unbuffered Provasoli medium. Observations of motility were made with a dissecting microscope following inoculation of the Euglena into 3 ml. of the desired penicillin concentration and Provasoli medium (initial pH 5.7±0.3). Culture tubes were incubated at 24°C. in a constant temperature water bath. In controls motility was observed to be maintained at 75 per cent.

Under these conditions as shown in figure 1, motiltiy of the Euglena was reduced to below 10 per cent in 5 hours in 30,000 units penicillin per ml. (with the exception of *Euglena gracilis* var. *bacillaris*, colorless which was slightly over 25 per cent motile). Also complete loss of motility of all except *Euglena gracilis* var. *bacillaris*, colorless was observed at 18 and 30 hours of exposure to this concentration.

At 25,000 units penicillin per ml., as shown in figure 2, motility of Euglena was reduced to below 25 per cent in 5 hours (*Euglena gracilis* var. *bacillaris*, colorless, being slightly over 25 per cent

PLATE I

EFFECT OF PENICILLIN G SODIUM ON MOTILITY OF EUGLENA



motile). Complete loss of motility of all Euglena species except *Euglena gracilis* var. *bacillaris*, colorless; *E. H*; and *E. 9* was observed after exposure of 30 hours.

At 20,000 units penicillin per ml., as shown in figure 3, all Euglena were reduced to 0 to 45 per cent motile in 5 hours and 0 to 25 per cent motile in 18 hours (with the exception of *Euglena gracilis* var. *bacillaris*, colorless which was approximately 50 per cent motile at 18 hours.

Figure 4 is a composite graph of effects of 15,000 units penicillin per ml. on Euglena in this sterile medium. At 5 hours exposure, average motility of each of the given Euglena varied from 20 to 60 per cent, and at 18 hours varied from 15 to 30 per cent. At no time during the 30 hours exposure did the average motility (as determined from six experiments) of any Euglena fall to zero per cent.

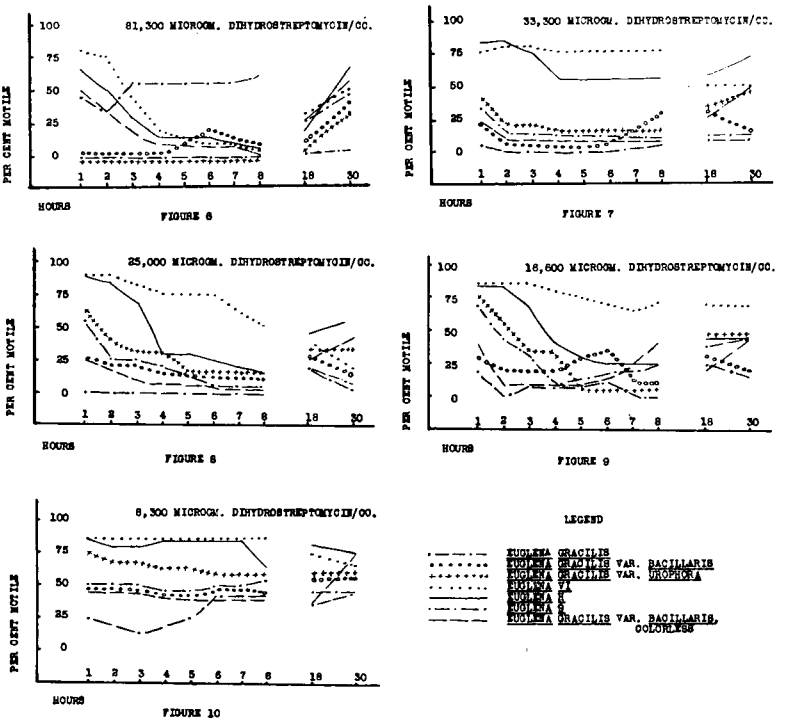
At 10,000 units penicillin per ml., average motility of each Euglena was between 30 and 75 per cent at 5 hours and 30 to 70 per cent at 18 hours. This is shown in figure 5. At no time during the six experiments did motility of any Euglena fall to zero per cent at this concentration.

Care must be taken in interpreting composite charts of this type. As shown in figure 3 for example, the antibiotic concentration of 20,000 units penicillin per ml. had least effect on *Euglena gracilis* var. *bacillaris*, colorless, but in two of the six experiments at this dilution, *Euglena H* and *Euglena VI* were equally motile. Also, although in figure 3 *Euglena gracilis* appeared as non-motile at 20,000 units penicillin per ml., this was not true in two of the six experiments at 4 through 30 hours of exposure.

Observations of survival and growth of Euglena (aseptic cultures) in penicillin G sodium in culture tubes inoculated eleven days before, showed motility and growth of all the seven Euglena species in 10,000 units penicillin per ml. In the higher concentration of 15,000 units penicillin per ml., *Euglena gracilis* exhibited no motility or growth at 11 days whereas the remaining six Euglena did. At 20,000 units penicillin per ml. after 11 days *Euglena gracilis* var. *bacillaris* and *Euglena VI* showed growth but no motility while *Euglena gracilis* var. *bacillaris*, colorless; *E. gracilis* var. *urophora; E. 9* and *E. H* showed both motility and growth. At 25,000 units penicillin per ml. *Euglena gracilis* var. *bacillaris*, colorless and *E. 9* showed growth but no motility and the remainder of the seven Euglena exhibited no growth and no motility. In the highest con-

PLATE II

EFFECT OF DIHYDROSTREPTOMYCIN ON MOTILITY OF EUGLENA



centration used (30,000 units penicillin per ml.) neither growth nor motility were exhibited by the seven species of Euglena.

Figures found on plate II are composite graphs of four experiments showing the average effects of concentrations of dihydrostreptomycin (8,300 to 81,300 microgram per ml.) on motility of seven aseptic Euglena species. The Euglena are: *Euglena gracilis; E. gracilis* var. *bacillaris; E. gracilis* var. *bacillaris*, colorless; *E. gracilis* var. *urophora; E. 9; E. VI;* and *E. H.* Euglena were inoculated into 3 ml. of desired streptomycin concentration in Provasoli medium, incubated at 24°C. and observed for motility with dissecting microscope for a 30 hour period.

Effects of 81,300 micrograms dihydrostreptomycin per ml. on sterile Euglena species are shown in the composite chart of figure 6. Motility of all except *Euglena 9* was below 25 per cent from 4 to 8 hours.

Effects of 33,300 micrograms dihydrostreptomycin per ml. on sterile Euglena is given in figure 7. The motility of all the Euglena

except *Euglena VI* and *Euglena H* was 25 per cent or lower from 2 to 8 hours exposure, but *Euglena gracilis* var. *urophora* and *Euglena gracilis* var. *bacillaris*, colorless, increased motility to above 25 per cent at 30 hours exposure

In figure 8, effects of 25,000 micrograms dihydrostreptomycin per ml. are shown. Motility of all except *Euglena VI* was observed to be below 25 per cent at 8 hours exposure, but at 18 hours, *Euglena 9*, *Euglena gracilis* var. *urophora*, and *E. gracilis* var. *bacillaris*, colorless, were over 25 per cent motile.

Effects of 16,600 micrograms of dihydrostreptomycin per ml. are shown in figure 9. At 8 hours exposure, motility of all except *Euglena VI* (70 per cent) was under 30 per cent and at 18 hours under 50 per cent.

Figure 10, shows effects of 8,300 micrograms dihydrostreptomycin per ml. on motility of Euglena. Average motility of all Euglena, as shown in the composite chart, was above 25 per cent at 8 hours. At 18 hours, motility of all Euglena in all experiments at this concentration for the 30 hours observed was above zero per cent.

The motility and growth of seven sterile Euglena species twelve days after inoculation into culture tubes containing dihydrostreptomycin concentrations of 8,300 to 81,300 micrograms per ml. was observed. Culture tubes examined were previously observed during first 30 hours of exposure for motility and recorded in composite graph form on plate II. *Euglena gracilis* is the only one of the seven aseptic Euglena which did not show indications of survival by motility and growth in the concentrations of dihydrostreptomycin used (81,300 units per ml., 33,300 units per ml., 25,000 units per ml., 16,600 units per ml., and 8,300 units per ml.).

Loss of color was observed in all Euglena cultures exposed to dihydrostreptomycin in concentrations of 81,300 to 8,300 micrograms per ml. This loss of color was apparent macroscopically as growth of the cultures became dense.

In bacteria-free culture experiments with wild cultures of Euglena and solutions of penicillin and dihydrostreptomycin alone and combined, protozoans, primarily Euglena, that had survived 24 hours in antibiotic concentrations in spot plates were transferred to sterile test tubes containing similar antibiotic concentrations in Jahn's medium. After three days incubation at 28°C. it was observed that the original inoculated test tubes containing dihydrostreptomycin alone in the concentrations used (48 to 6 micrograms per ml.) con-

tained bacterial contaminants. In all nine tests in which the penicillin concentration was 390 units per ml., bacterial contaminants were present, whereas in the higher concentrations of penicillin (12,500 to 781 units per ml.) a mold contaminant only was observed in 1 test out of 12 after 3 days and in 4 tests out of 12 after 20 days, with Euglena in isolated form at 20 days in 2 tests out of 12 in the concentrations of 12,500 to 781 units penicillin per ml.

In the test tubes containing penicillin (12,500 to 781 units per ml.) combined with dihydrostreptomycin (48 to 6 micrograms per ml.), mold contaminant was observed in 3 tests out of 12 after 3 days and 8 tests out of 12 after 20 days. Euglena in isolated form was observed after 20 days in 3 tests out of 12.

In addition to Euglena cultures isolated, cultures of *Colpidium sp.* were mold and bacteria-free after 3 and 20 days in the original inoculated test tube with penicillin concentrations of 12,500 to 781 units per ml. and in the test tubes with penicillin (12,500 to 781 units per ml.) combined with dihydrostreptomycin (48 to 6 micrograms per ml.).

Loss of color of the Euglena cultures was observed of Euglena exposed to dihydrostreptomycin in Jahn's medium.

Later experiments done in a sterile room showed less frequent bacterial or mold contamination. In these later experiments, washing the Euglena in a drop of sterile water on sterile agar under a petri dish cover was added to the procedure immediately prior to inoculation into the sterile antibiotic solution in test tubes.

Initial tests to determine resistance of wild cultures of *Euglena velata* to the antibiotics made use of the spot plate with serial dilutions of the antibiotic in sterile water and observation with 20x power of dissecting microscope. Motility was used as the criterion of survival.

Effects of penicillin on motility of *Euglena velata* (wild culture) were determined by ten experiments using the spot plate method. At 50,000 units penicillin G sodium per ml., no motility of the Euglena was observed after one hour. At 25,000 units penicillin per ml., motility was reduced to zero per cent in 5 hours in all experiments. Complete loss of motility was not observed in 12,500 units penicillin per ml. or in lower serial dilutions to 1,500 units per ml. during the 24 hours observed.

Other experiments with potassium salts of penicillin G showed that the effects of the two salts of penicillin G (potassium and sodium) on *Euglena velata* (wild culture) were nearly identical.

Effects of dihydrostreptomycin on motility of *Euglena velata* (wild culture) were observed in 9 experiments using the above mentioned spot plate procedure. In 195 micrograms dihydrostreptomycin per ml., motility of *Euglena velata* was reduced to zero per cent in 5 hours and remained so for the 24 hour period. After 24 hours in 97.6 micrograms per ml. and lower concentrations (to 6 micrograms per ml.) some motility was observed throughout the 24 hour period, with well above 25 per cent motile at 24 hours exposure to 12 micrograms per ml.

Five experiments with effects of combinations of dihydrostreptomycin and penicillin (25,000 units penicillin per ml. with 197 micrograms streptomycin per ml., to 1,500 units penicillin per ml. with 6 micrograms streptomycin per ml.) on motility of *Euglena velata*, indicate no increase in toxicity over the toxicity of penicillin alone.

Then after 24 hours in antibiotic solutions in spot plates, organisms remaining alive were transferred to test tubes for attempts at obtaining bacteria-free cultures. Having determined by the spot plate method concentrations of antibiotics in which the *Euglena velata* would survive, Euglena were transferred from the spot plate solutions to identical concentrations of antibiotics in sterile test tubes.

Observations of the effects of antibiotics on motility of various protozoans (wild culture) were made during July and August using the spot plate method. *Euglena rubra* and *Urostyla trichogaster* were observed motile at 25,000 units penicillin per ml. after 4 hours exposure. Also *Euglena granulata, E. oblonga, E. sanquinea, E. velata, Pandorina morum, Spirostomum teres* and *Trachelomonas sp.* were observed motile after 4 hours in 12,500 units penicillin per ml. *Euglena granulata* was observed motile in 48 microgram of dihydrostreptomycin per ml. after 4 hours, *Euglena rostrifera* in 390 microgram per ml., *Euglena obtusa* in 1,562 microgram per ml. and *Euglena pisciformis* in 25,000 micrograms per ml. Also in $CaCl_2$ salt of streptomycin, *Euglena rubra* was observed motile after 4 hours in 24 microgram per ml. *Euglena fundoversata* in 49 microgram per ml.; *Euglena granulata, E. pisciformis*, and *E. rostrifera* in 781 micrograms per ml.; *Leptocinclis sp.* in 195 microgram per ml.; and *Euglena spirogyra* in 6,250 microgram per ml. Other species of Euglena and protozoa survived the mentioned concentrations that were not identified and were not included in the present study.

Discussion

Motility-toxicity of *Euglena gracilis; E. gracilis* var. *bacillaris; E. gracilis* var. *bacillaris*, colorless; *E. gracilis* var. *urophora; E. 9; E. VI; and E. H* in given dihydrostreptomycin and penicillin concentrations respectively are presented in composite graphs of plate I and plate II. For the most part, resistance is about the same for all the species studied. However, *Euglena gracilis* var. *bacillaris*, colorless, could be distinguished from the other Euglena in 30,000 and 25,000 units penicillin per ml. This is possible since this Euglena remained above 25 per cent motile for 8 hours in these concentrations, whereas the other six Euglena did not. *Euglena VI* remained above 50 per cent motile for 8 hours in certain dihydrostreptomycin concentrations (figures 7, 8, and 9) and was relatively more resistant to the antibiotic than the other six Euglenae. Further differences in toxicity to the Euglena of antibiotics were exhibited by effect on motility and growth as observed after eleven days exposure. *Euglena gracilis* is immediately seen to be characterized by failure to exhibit growth and motility in 15,000 units penicillin per ml., or in any of the dihydrostreptomycin concontrations at 11 and 12 days of exposure respectively, whereas the other six Euglena exhibited motility under these conditions. Lack of motility of *Euglena gracilis* var. *bacillaris, E. VI*, and *E. gracilis* var. *bacillaris*, colorless in 20,000 units penicillin per ml. at 11 days, could be considered distinguishing characteristics for these species of Euglena in distinguishing them from the other four Euglena.

The possibility that some Euglena characteristically exhibit temporary loss of motility when first exposed to antibiotics is seen when interpreting changes in average motility of certain Euglena in penicillin and dihydrostreptomycin solutions recorded in composite graphs in plates I and II. In penicillin concentrations, in all except a very few cases, Euglena lost motility as time of exposure progressed. *Euglena 9*, however increased motility from 20 per cent at 1 through 3 hours to 30 per cent at 4 through 7 hours exposure in 10,000 units penicillin per ml.

In dihydrostreptomycin concentrations we find a general increase in average motility at 30 hours as compared with motility at 5 hours exposure as shown in plate II. Temporary loss of motility is seen of two species of Euglena (*Euglena gracilis* in 8,300 and 16,600 micrograms per ml.; *Euglena 9* in 81,300 micrograms per ml.).

While growth and motility of Euglena at a certain time of exposure and concentration of dihydrostreptomycin or penicillin, as

described above, could be used for species determination, it seems unwise to use these criteria unless a clear line of demarcation exists, such as the sensitivity of *Euglena gracilis* to dihydrostreptomycin at 12 days. Within the genus Euglena, criteria of species appears to be primarily based on morphology as indicated in the articles of L. Johnson (1944), Jahn (1946) and Pringsheim (1947). In isolated cases where minor morphological differences exist, differential in toxicity to antibiotics may be of taxonomic value. However, when the effect of dihydrostreptomycin or penicillin on motility or growth of Euglena is used as a diagnostic character for species determination, it is necessary to recognize the existence of a narrow range of concentration of antibiotic which exerts toxic effects on all species of Euglena and also the existence of variability in the sensitivity of each Euglena species to a given antibiotic concentration at a given time of exposure.

The survival of *Euglena velata* in various penicillin concentrations permitted elimination of susceptible bacteria and mold from wild Euglena cultures. With spot plate tests, Euglena were found to survive 12,500 units penicillin per ml., so this and lower concentrations of 6,250, 3,125, 1,620 and 781 units per ml. were chosen for bacteria-free culture attempts. Isolation was effected with these above mentioned penicillin concentrations in 3 days at 28°C. Combination of these same penicillin concentrations with dihydrostreptomycin also effected isolation of *Euglena velata.* The fact that dihydrostreptomycin alone did not inhibit bacterial growth may be attributed to the presence of bacteria resistant to the concentrations of antibiotic used (48 to 3 microgram per ml.) under the conditions used.

Since all the aseptic Euglena obtained from Provasoli, except *Euglena gracilis*, survive in 15,000 units penicillin per ml. and dihydrostreptomycin concentrations of 81,300 micrograms per ml. respectively, one would expect that bacteria susceptible to these concentrations could be eliminated from cultures of these Euglena.

Survival of various protozoans in penicillin and streptomycin solutions as given by various authors and recorded in table 1, can be compared to survival of Euglena in antibiotic solutions observed in the present investigation. In this table the highest concentration of penicillin used to effect sterilization appears to be 10,000 Oxford Units per ml. This concentration was used by Johnson *et al.* (1945) with *Trichomonas vaginalis* and Morgan (1946) with *Trichomonas foetus.* The lowest penicillin concentration apparently recorded for isolation of a protozoan was 100 units per ml. by Jacobs (1947) with

Endamoeba histolytica. In the present investigation, however, concentrations below 781 units penicillin per ml. did not eliminate bacteria.

Differences in the amount of antibiotic required to eliminate bacteria from cultures of parasitic protozoans as compared with free-living protozoans are no doubt due to the dissimilarities in bacterial flora associated with the organisms (as bacteria also vary in their susceptibility to antibiotics).

Combination of streptomycin with penicillin was used by several authors to isolate protozoan cultures. Schaffer and Ryden (1949) used the two antibiotics (1,000 Oxford Units with 1,500 units streptomycin per ml.) for isolation of *Trichomonas vaginalis*, *Endamoeba histolytica* and *Trichomonas hominis*. Williams and Plastridge (1946) used 100 units of each antibiotic per ml. to inhibit bacteria sufficiently to permit growth of *Trichomonas foetus*. Concentrations of the antibiotics combined, which eliminated bacteria and mold from Euglena in the present study, were 12,500 units penicillin with 48 micrograms dihydrostreptomycin per ml. to 1,562 units penicillin with 6 micrograms dihydrostreptomycin per ml.

Use of streptomycin alone for effecting isolation of protozoans has been used by several authors. Williams and Plastridge (1946) effected isolation of *Trichomonas foetus* with 1,000 units per ml., and Balamuth and Wieboldt (1946) effected isolation of *Entamoeba histolytica* with 100 to 20,000 units per ml. Forty-eight units of dihydrostreptomycin per ml. were insufficient to eliminate bacteria from wild cultures of *Euglena velata* in the present study.

When comparing sensitivity of protozoans to antibiotics as given by many authors and in results of this study, it is essential that one realize variations which exist in conditions of the experiments of the different workers. Culture medium, pH and incubation temperature are chosen to best fit the needs of the specific protozoan. Many workers, however, fail to record the specific conditions of their experiments. This omission makes their data difficult to interpret correctly since activity of the antibiotics has been found to be modified by varied factors. Pratt and Dufrenoy (1949) have listed the following as factors of this type: number of organisms present, pH of medium, presence of inhibitors or enhancers, and the type of antibiotic. Future workers in this area should not overlook these factors.

It is suggested, therefore, that variations in results of effects of streptomycin on wild cultures of *Euglena velata* and aseptic cultures of *Euglena gracilis* and other aseptic Euglena may be due not

only to species differences, but variation in method, media and source of cultures.

The pH of various ponds from which Euglena were collected may be important in maintaining activity of the streptomycin, and additional work should be done to see how the original pH of the media will change the effect of streptomycin on Euglena.

It is possible that the pH in which the seven sterile Euglena were living in the various dihydrostreptomycin concentrations was such that the antibiotic was not in its most active form. (Initial pH of solutions of dihydrostreptomycin plus Provasoli medium was 5.75 ± 0.1). Pratt and Dufrenoy (1949) stated: "Streptomycin is most effective in media which is neutral or somewhat alkaline in reaction." This would appear to tie in with results of studies of dihydrostreptomycin on *Euglena velata* in which the medium was more alkaline and the Euglena were killed in lower concentrations on the antibiotic.

Variations in use of washing technique in isolation experiments by various authors is readily observed in surveying the literature in this area. Wagtendonk and Hackett (1949) and Seaman (1947) washed protozoa in sterile solutions before and after penicillin treatment. Adler and Pulvertaft (1944), Quisno (1946), and Morgan (1946) inoculated from subcultures or added the antibiotic directly to the organism in the culture medium and therefore did not use washing of the protozoa to aid in effecting isolation. Original methods used in this study did not include washings of the protozoa before inoculation into the culture media as it was considered desirable to find a method which did not require tedious washing procedures. Later experiments made use of a simple washing technique of allowing the Euglena to migrate phototropically in large drops of sterile water on sterile agar before inoculation into the antibiotic solutions. Less frequent bacterial and mold contamination resulted from adding this step to the original procedure.

Seaman (1947) isolated the free-living protozoan *Colpidium campylum* and stated that "preliminary test must be made to ascertain the length of time the organism will survive in a given concentration of penicillin" if his method is to be used to obtain sterile cultures of most protozoa. The spot plate method introduced in the present study can be used for preliminary tests in determining toxicity of antibiotics to protozoans, and is particularly suitable as a rapid method of determination.

Provasoli et al. (1948) reported loss of chlorophyll from *Euglena gracilis*, *E. gracilis* var. *bacillaris*, and *E. gracilis* var. *urophora* with

streptomycin and stated: "There was a smoothly progressive bleaching of the cultures with increasing duration of exposure and increasing concentration of streptomycin." From this statement and the present study, in which bleaching of Euglena was observed, it would appear that bleaching is no indication of toxicity.

Several authors have attempted to use antibiotics other than streptomycin and penicillin for isolation of protozoa. Johnson et al. (1945) used thyrothricin with penicillin, but reported it to be lethal to *Trichomonas vaginalis* in concentrations used. Williams and Plastridge (1946) stated that clavacin, gramicidin, and actinomycin killed *Trichomonas foetus*. It is possible that new antibiotics may be discovered which may be even more effective than penicillin and streptomycin in eliminating contaminants from protozoan cultures.

Summary

1. Figures and materials indicating effect of penicillin G sodium and dihydrostreptomycin sulphate respectively on motility and growth of Euglena species are presented.

2. Although variations in sensitivity to penicillin or dihydrostreptomycin exist between species of Euglena, use of these effects as valid diagnostic characteristics for Euglena species is of limited value.

3. It is suggested that since all Euglena survive 24 hours in 10,000 units of penicillin G sodium per ml., susceptible bacteria could be eliminated from wild cultures of Euglena by exposure to this concentration of antibiotic.

4. Bacteria-free cultures of Euglena were not effected by the use of dihydrostreptomycin in this study. It is suggested that the lack of effectiveness of dihydrostreptomycin in eliminating bacteria from Euglena cultures was determined by the pH of the medium or presence of resistant bacteria.

5. It is suggested that workers in this area report conditions of experiments more completely as effectiveness of antibiotics is determined by many factors.

6. A spot plate method for initial determination of toxicity of antibiotics on protozoa is presented and suggested to be particularly applicable to rapid tests.

7. Loss of green color of Euglena was observed in all dihydrostreptomycin solutions (8,300 to 81,300 micrograms per ml.), but this loss of color is not a measure of the toxicity of the antibiotic to the Euglena.

Literature Cited

Adler, S., and Pulvertaft, R. J. 1944. Use of penicillin for obtaining bacteria-free cultures of *Trichomonas vaginalis*, Donne. Ann. Trop. Med., *38*: 188.

Balamuth, W. and Wieboldt, M. L. 1946. Action of streptomycin on *Entamoeba histolytica* in vitro. Jour. Parasitol., *32* (suppl.): 10.

Jacobs, L. 1947. Elimination of viable bacteria (with penicillin) from cultures of *Endamoeba histolytica* and subsequent maintainence of such cultures. Am. Jour. Hyg., *46*: 175, 172.

Jahn, T. L. 1935. Studies on physiology of Euglenoid Flagellates, VII. Arch. f. Protistenk, *86*: 258.

Jahn, T. L. 1946. The Euglenoid Flagellates. Quart. Rev. Biol. *21*(3): 246.

Johnson, G., Trussell, M., and Jahn, F. 1945. Isolation of *Trichomonas vaginalis* with penicillin. Science, *102*: 126, 127.

Johnson, L. P. 1944. Euglenae of Iowa. Trans. Am. Micr. Soc., *68*: 133.

Knoll, E. W., and Howell, K. M. 1946. Invitro effect of penicillin on *Endamoeba histolytica*. Arch. Path., *4*(6): 594.

Morgan, B. B. 1946. The use of antibodies in the purification of *Trichomonaas foetus* (Protozoan) cultures. Jour. Bact., *94*: 437.

Pfeiffer, A. V. 1948. Effect of streptomycin on *Endolimax nana* in vitro. Jour. of Parasitol., *34*(2): 145.

Pratt, R. and Dufrenoy, J. D. 1949. *Antibiotics*. J. B. Lippincott Co., Philadelphia, Pennsylvania, *1*: 86, 136.

Pringsheim, E. G. 1947. Taxonomic problems in Euglenineae. Biol. Rev. Cambridge Phil. Soc., *23*(1): 46.

Provasoli, L., Hunter, S. H., and Schatz, A. 1948. Streptomycin-induced chlorophyll-less races of Euglena. Biol. Bull., *95*: 260.

Quisno, R. A. and Foter, M. J. 1946. The use of streptomycin in the purification of cultures of *Trichomonas vaginalis*. Jour. Bact., *51*: 404.

Schoenborn, H. W. 1949. Growth of Protozoa in glassware cleaned with sulphuric acid-potassium dichromate solution. Physiol. Zool., *22*(1): 30.

Seaman, G. R. 1947. Penicillin as agent for sterilization of Protozoan cultures. Science, *106*: 327.

Shaffer, J. G., Ryden, F. W., and Frye, W. W. 1949. Studies on the growth requirements of *Endamoeba histolytica*. Amer. Jour. Hyg., *49*(2): 127.

Spingarn, C. L. and Edelman, M. H. 1947. The prolongation of viability of culture of *Endamoeba histolytica* by addition of streptomycin. J. Parasitol., *33*: 418.

Spingarn, C. L. and Edelman, M. H. 1948. The use of streptomycin in the cultures of *Endamoeba histolytica* from stools. Am. Jour. Trop. Med., *28*: 829.

Wagtendonk, W. J. van, and Hackett, P. L. 1949. The culture of *Paramecium aurelia* in the absence of other living organisms. Proc. Nation. Acad. Sci. U. S. A., *35*: 157.

Waksman, S. A. 1949. *Streptomycin*. The Williams and Wilkins Co., Baltimore, *1*: 92.

Williams, L. F., and Plastridge, W. N. 1946. Use of antibiotic substances for freeing *Trichomonas fetus* from bacteria. Jour. of Bact., *51*: 127.

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