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A Comparison of Different Criteria for Determining the Effects of Antibiotics on *Tetrahymena pyriformis* E.*

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SUMMARY. A study was made of the effects of Chloromycetin, Aureomycin and Terramycin on growth of *Tetrahymena pyriformis* E. Growth was measured by turbidimetry, cell count and cell volume. Data were analyzed to compare growth rates, time to attain half-maximal growth and growth after 4 days of incubation.

Antibiotic levels which yielded approximately 50% inhibition of optical density were as follows: (1) Aureomycin: 1.9×10^{-5}M at 4 days and 5.8×10^{-5}M for the number of days to reach half-maximal growth; 50% depression of growth rate was never attained since levels greater than 5.8×10^{-5}M were lethal; 4.9 to 5.8×10^{-5}M Aureomycin resulted in approximately 40% inhibition; (2) Chloromycetin: 12.4×10^{-5}M at 4 days and 23.2 to 31×10^{-5}M for growth rate and time to reach half-maximum; and (3) Terramycin: 1×10^{-4}M at 4 days, 1.5×10^{-4}M for growth rate, and 1.5 to 2×10^{-4}M for time required to reach the half-maximal point. The diverse results obtained are discussed.

Those concentrations of an antibiotic which showed approximately 50% inhibition of growth rate by turbidimetric measurement were then studied for their effect on number of cells during growth. Aureomycin was found to be 66% inhibitory at 4 days, but only 28 and 26% suppressive for growth rate and days needed to attain a half-maximal population, respectively. Chloromycetin reduced growth 82% at 4 days, inhibited rate of growth by 37% and suppressed time to reach the half-maximal level by 39%. Terramycin was calculated to be 50% inhibitory at 4 days, 16% suppressive for growth rate and inhibited the time to reach half-maximal population by 24%.

It is also demonstrated that Chloromycetin reduces the size of individual *Tetrahymena* cells during growth in the culture medium as well as during starvation in buffer.

The divers results reported in the literature on the influence of a given antibiotic on *Tetrahymena* are shown to be caused not only by strain and medium variability, but also, to a great extent, by differences in the methods of measurement and analysis employed. It is suggested that despite certain inherent errors, turbidimetric growth rate is the method of choice for measuring the influence of inhibitors upon total protoplasmic mass.

INVESTIGATIONS of the effects of antibiotics on protozoa have been performed in several laboratories in recent years. The ciliate, *Tetrahymena*, has been the experimental organism for many of these studies. The initial report in which this protozoan and others were observed for their response to the antibiotic substance, chloramphenicol (Chloromycetin), was made by Smith and his collaborators(19). Other investigators took up the work soon thereafter. Among these, Jirovec(11) tested a number of antibiotics including Chloromycetin, chlorotetracycline (Aureomycin) and oxytetracycline (Terramycin), the

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three compounds to be reported upon in this paper, for their ability to inhibit several protistans. Loefer (12,13) examined the action of Chloromycetin on growth of *Euglena* and *Tetrahymena* in 1950 and 1951, and Brown(3) used *Tetrahymena* to study the stimulatory effects of Aureomycin. Others have also employed protozoa in studies of antibiotic action. In attempting to correlate and evaluate the information reported by various investigators confusing results often are brought to light. This inconsistency seems to be caused primarily by the fact that different criteria for measurement and analysis of growth have been utilized by different researchers in the field.

The present report compares the effects of the three antibiotics mentioned on growth of *Tetrahymena* and treats the data in several different ways to elucidate the reasons for apparently controversial results. In addition, the method which the writer considers to be the best for evaluation of the influence of drugs upon increase in protoplasmic mass will be discussed.

MATERIALS AND METHODS

Tetrahymena pyriformis, strain E was obtained from Dr. A. M. Elliott who isolated the ciliate in 1932 as *Colpidium striatum* Stokes(5). Cultures were maintained in 1% tryptone-proteose-peptone medium as recommended by Slater(18). Transfers were made daily from 4-day-old stocks throughout the course of the investigation to insure the availability of organisms of a desired age at any time. Forty-eight-hour cultures from several tubes of stock medium were pooled in a centrifuge tube by approved aseptic methods, washed three to four times with sterile, redistilled water, and resuspended in redistilled water so that 0.2 ml. of suspension contained approximately 1500 ciliates to be used as the inoculum. This was subsequently pipetted into 5 ml. of sterile, tubed experimental medium. This was a modification of the basal synthetic medium of Elliott(7) as indicated in Table I. The essential changes were: (1) the use of 25 μg./ml. each of adenylic, cytidylic, guanylic acids and uracil in place of 100 μg./ml. of yeast nucleic acid; (2) replacement of protogen with a refined fraction called protogen A; and (3) omission of iron and copper salts because of the formation of a precipitate (probably of iron hydroxide)(10), and the precipitation of Terramycin in the presence of copper ions(16). Cultures were grown routinely in 15 x 125 mm. pyrex tubes in the dark at 25°C. The pH was set initially at 6.5.

TABLE I. Basal chemically defined medium for growth of *T. pyriformis* E.

Compound	mg./l.	Compound	mg./l.
L-Arginine	600	Na acetate	1000
L-Histidine	150	Glucose	1000
DL-Isoleucine	75		
DL-Leucine	75	Ca pantothenate	0.1
L-Lysine	150	Niacin	0.1
DL-Methionine	150	Pyridoxine • HCl	2
DL-Serine	300	Riboflavin	0.1
DL-Phenylalanine	150	Folic acid	0.01
DL-Threonine	300	Thiamine • HCl	1
DL-Tryptophane	150		
DL-Valine	150	Cytidylic acid	25
		Adenylic acid	25
KH_2PO_4	100	Guanylic acid	25
$MgSO_4 \cdot 7H_2O$	100	Uracil	25
$CaCl_2 \cdot 2H_2O$	50		
$MnCl_2 \cdot 4H_2O$	0.05	Protogen A*	1000 units/l.
$ZnCl_2$	0.05		

* Protogen A was generously supplied by Dr. E. L. R. Stokstad, Lederle Laboratories, Pearl River, N. Y.

Growth was measured in different experiments by cell count, average individual cell volume and turbidimetry. Cell counts were performed by a modification of the method described by Slater(18). Cell volume was computed from the formula for the volume of a prolate spheroid: $V = 4/3\ \pi\ ab^2$, where V = volume, a = major semi-axis and b = minor semi-axis. Measurements of length and width were made with a calibrated ocular micrometer in a 10X ocular combined with a 4mm. objective lens. Cells for observation were fixed in Bouin's and stained with eosin. Averages were obtained from measurements of 20 to 25 cells from each of two permanent clarite mounts; cells were surveyed as randomly as possible by scanning a slide with the aid of a mechanical stage and measuring only that cell which appeared nearest the center in every third field viewed. Optical densities of living organisms were measured turbidimetrically in a Lumetron, model 401, photoelectric colorimeter through a red (650 mμ) filter. The red filter was selected as a result of spectrophotometric studies made during growth of *T. pyriformis* in the presence of the antibiotics to eliminate the effect of color imparted to the medium by Aureomycin(10). "Tanning" of the medium caused by oxidation of Terramycin could not be prevented by $NaHSO_3$ as suggested by Seneca(16) because of the toxicity of the reducing agent for the ciliates. Another suggested reductant, ascorbic acid, was not employed because of the possible nutritional variability it might induce. This discoloration interfered with optical density readings although it did not seem to impair the potency of the drug. It was eliminated as a source of error by the simple expedient of centrifuging the cells out of the medium, washing with cold, distilled water, resuspending in distilled water and then measuring with the colorimeter.

Chloromycetin[1] (CM), Aureomycin[2] (AM) and Terramycin[3] (TM) were prepared as concentrated so-

[1] Chloromycetin was donated by Dr. G. Rieveschl, Parke, Davis and Co., Detroit, Michigan.

[2] Aureomycin, as the hydrochloride, was supplied by Dr. S. M. Hardy, Lederle Laboratories, Pearl River, N. Y.

lutions in redistilled water so that 0.2 ml. of concentrate when dispensed into 5 ml. of medium would yield the desired final concentration. It was found unnecessary to predissolve any of the antibiotic compounds in organic solvent as was done by Jirovec(11). Although the hydrochlorides of AM and TM are strongly acidic, no pH adjustment was necessary at the concentrations employed in inhibition studies. In survival experiments, however, in which high concentration of the drugs was used, appropriate pH adjustments were made. The antibiotics were sterilized through all-glass filters with fritted discs of ultra-fine porosity. After filtration, the usual aseptic techniques were observed in handling the solutions. The concentrated solutions were stored by freezing when they were to be retained for periods longer than 3 or 4 days. This precaution prevented oxidation of TM and virtually eliminated decomposition of AM.

RESULTS

Survival limits. In order to establish the effective concentration range for inhibition studies, the lethal doses of the antibiotics were delimited. Lethality was determined by microscopic observation and by transferring from suspected dead cultures to fresh, antibiotic-free medium and observing for growth. In this series of experiments, the drugs were prepared by dissolving them directly in the medium instead of distilled water in order that higher antibiotic concentrations might be attained. The pH of the medium was adjusted after the addition of a drug, but prior to passing it through the filter for sterilization. The fatal doses of the antibiotics for *Tetrahymena* are tabulated (Table II). It is shown that lethality depends upon length of exposure as well as concentration of the inhibitor; both 7.8 x 10^{-3} M and 6.2 x 10^{-3} M CM kill the ciliates, but the higher concentration acts more rapidly. It can also be seen from these data that AM is the most and CM the least potent protozoicide of the three compounds tested. This relationship is maintained also at inhibitory levels.

TABLE II. Survival of *T. pyriformis* E in basal medium in the presence of antibiotics.

Molarity	Effect*	Time
	Chloromycetin	
7.8×10^{-3}	L	3 hours
6.2×10^{-3}	L	6 hours
4.7×10^{-3}	—	4 days
15.6×10^{-4}	—	30 days
	Aureomycin†	
9.8×10^{-5}	L	1 day
7.8×10^{-5}	L	3 days
	Terramycin	
5×10^{-3}	L	6 hours
4×10^{-3}	L	2 days
3×10^{-3}	L	3 days
1×10^{-3}	L	4 days
4×10^{-4}	—	12 days

* L = lethal; — = no growth, but cells still viable.

† *T. pyriformis* E apparently is far more sensitive to AM than either strains GL (Jirovec, 1951) or W (Cooley, 1954). These strains are reported not to succumb until a concentration of the order of 2×10^{-4} M AM is attained.

[3] Terramycin, as the hydrochloride, was obtained through the courtesy of Dr. H. Seneca, Chas. Pfizer and Son, Brooklyn, N. Y.

TABLE III. Inhibition of growth of *T. pyriformis* E by antibiotics.

Antibiotic	Concentration $\times 10^{-5}$ M	Concentration μg./ml.	% inhibition† At 4 days	% inhibition† Growth rate	% inhibition† Days to reach ½-max.
Control	0	0	0	0	0
Chloromycetin	<3.1	10.0	0	0	0
	3.9	12.5	26	5	13
	7.8	25.0	37	15	—
	12.4	40.0	50*	33	29
	15.6	50.0	68	41	36
	23.2	75.0	74	48*	44
	31.0	100.0	75	52*	47*
	62.0	200.0	76	78	—
	78.0	250.0	92	100	—
Aureomycin	<0.5	2.5	0	0	0
	1.0	5.0	17	5	15
	1.9	10.0	49*	5	25
	2.9	15.0	63	24	32
	3.9	20.0	75	37	38
	4.9	25.0	86	38	44
	5.8	30.0	89	39*	47*
Terramycin	<1	5.0	0	0	0
	2	10.0	10	17	13
	5	25.0	23	10	20
	10	50.0	54*	30	32
	15	75.0	58	60*	45*
	20	100.0	83	76	55*
	40	200.0	—	100	—

* Approximately 50% inhibitory values.

† Based on optical density measurements as described in text.

Although some of the antibiotic levels listed were not lethal, no growth occurred during the course of the experiment. Lower concentrations than those shown in Table II were inhibitory, but not fatal.

Inhibition studies. Growth inhibition studies were performed with antibiotic levels below those described in Table II in order to avoid undesirable delay in the initiation of growth. In the first set of experiments, growth was measured turbidimetrically in the presence of varied concentrations of an antibiotic and in its absence. AM was tested over the range: 2 x 10^{-7} to 5.8 x 10^{-5} M; TM was examined in concentrations ranging from 1 x 10^{-5} to 4 x 10^{-4} M; and CM was studied at levels from 3.1 x 10^{-6} to 7.8 x 10^{-4} M.

Growth inhibition was compared in several different ways: (1) percent of inhibition, based on optical density, was measured after 4 days of growth; (2) suppression of growth rate, also recorded in percent, was

TABLE IV. Comparative inhibition study: cell counts vs. optical density measurements.

Antibiotic	Concentration ($\times 10^{-5}$ M)	% inhibition: 4 days O. D.	4 days Counts	Growth rate, Logarithm of: O. D.	Growth rate, Logarithm of: Counts	½-maximal O. D.	½-maximal Counts
Chloromycetin	31.0	75	82	52	37	47	39
Aureomycin	4.9	86	66	38	28	44	26
Terramycin	15.0	58	50	60	16	45	24

based on the slopes of growth curves during exponential increase calculated from the logarithm of the optical density against time; and (3) inhibition of growth, based on the number of days required to reach half-maximal optical density, is also reported in percent. Table III shows the results of these experiments. Values marked by asterisks emphasize that different antibiotic levels are required to produce essentially equivalent amounts of inhibition. That these results are related to the analytical technique used is clearly demonstrated.

In a second series of experiments, cell counts were made during growth for one concentration of each of the antibiotics. In this study, too, results are reported as the percent of inhibition of growth rate, of time to reach half-maximal growth, and after 4 days of incubation. These data are compared with those obtained from optical density measurements (Table IV). The ensuing differences are notable and are conspicuously contingent upon the method of measurement. The information reported in Table IV was computed from data plotted in Fig. 1, which compares graphically the influence of the antibiotics on cell counts and optical densities.

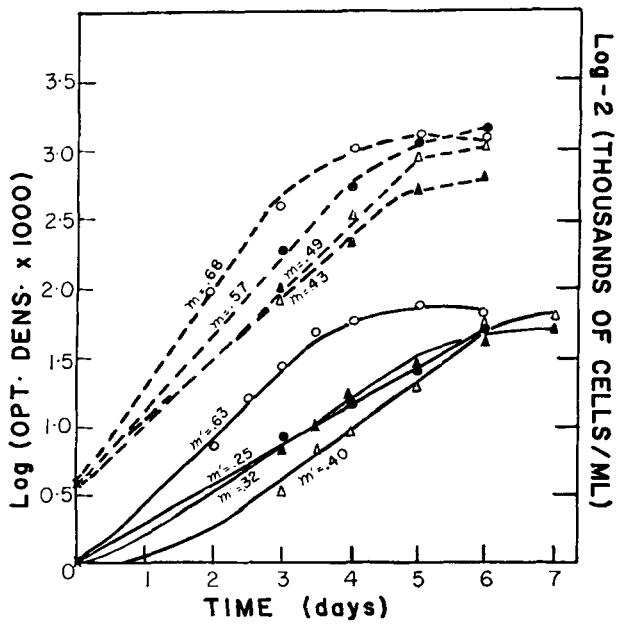

Fig. 1. Comparison of rates of growth from cell counts and optical density measurements in the presence of equal antibiotic concentrations. Solid lines = optical density measurements; broken lines = cell counts; m, m′ = slopes; ○ = control; ● = 1.5×10^{-4} M Terramycin; ▲ = 3.1×10^{-4} M Chloromycetin; △ = 4.9×10^{-5} M Aureomycin.

TABLE V. Size changes in the average *T. pyriformis* E cell in the presence of Chloromycetin (CM).

Dimension	During growth*: Hours after inoculation	Measurement —CM	Measurement +CM	During starvation†: Hours after inoculation	Measurement —CM	Measurement +CM
Length	48	41 μ	39 μ	¼	58 μ	58 μ
	72	49	41	12	45	44
	96	55	39	20	42	34
	120	59	43	44	36	29
Width	48	26 μ	28 μ	¼	27 μ	27 μ
	72	26	27	12	25	24
	96	26	25	20	22	21
	120	24	23	44	21	20
Volume‡	48	14700 μ^3	15900 μ^3	¼	22200 μ^3	22200 μ^3
	72	17800	15900	12	14500	13100
	96	19700	13300	20	11100	7800
	120	17500	12700	44	7900	6100

* 1% tryptone-proteose-peptone; pH 6.5; 25°C.; 12.4×10^{-5} M CM.

† 0.02 M phosphate buffer; pH 6.5; 25°C.; 62×10^{-5} M CM.

‡ Loefer(14) reports that the 48-hour volume of strain E is about 29,000 μ^3 (corrected for formula error). The discrepancy between his figures and the data reported here are likely the result of differences in culture conditions, past history of the culture and fixing and staining technique.

Cell size changes during growth and starvation were tested in the presence of CM only. The variations which occur in length, width and volume of an average *T. pyriformis* E cell during growth of a culture without CM and with 12.4 x 10^{-5} M CM are compared in Table V. These data were originally reported in abstract by Gross(9). It can be seen that controls increase in volume through 96 hours, then drop after the culture has entered the stationary phase. On the other hand, cells grown with CM decrease in volume after 72 hours. What occurs before the 48-hour reading or after 120 hours is not known.

A study of the influence of CM on cells in phosphate buffer (Table V) demonstrates that the antibiotic accelerates the rate of size reduction which normally occurs during starvation. The data show that it takes 44 hours of starvation to reduce the volume of an average control cell from 22,200 μ^3 to 7,900 μ^3, a de-

crease of 64%, while in the presence of 6.2 x 10^{-4} M CM, the same order of volume loss occurs in 20 hours. It may be inferred from these experiments, that not only are growth rate and number of cells inhibited by the antibiotic, but that size of individual cells is also affected. The fact that other antibiotics also influence the size of *Tetrahymena* has been shown by Blaszczynski and Nardone(1) and Blumberg and Loefer (2).

DISCUSSION

It is just as essential to recognize that varied results may be obtained when different criteria are used to measure growth as it is to consider culture media and strain differences as sources of irregularity. Various workers in the field have used a marked heterogeneity of criteria for measurement, analysis and definition of growth. In the writer's opinion, the definition of choice for describing growth of microorganisms is one which includes all possible changes which may occur within the cell mass during culture. Such a broad characterization can be readily appreciated when one considers that the growth process involves not only cellular proliferation and increase in cell size as Loefer (14) clearly pointed out, but also changes in protoplasmic density which occur as a culture ages. Therefore, it is felt that a method for measuring cellular mass during culture is one to be preferred.

That results obtained from studies of the effects of inhibitors on growth are governed by the methods of measurement and analysis employed, is shown in Tables III and IV. This fact is further emphasized by a comparative analysis of information in the literature in which CM, AM, and TM have been investigated for their effects on *Tetrahymena*.

In an early investigation of the biological effects of CM, Smith *et al.*(19) reported that no change in appearance or locomotion of *Tetrahymena* could be observed after 48 hours of exposure to 2.5 mg./ml. (7.8 x 10^{-3} M) of the antibiotic. Although the strain of the ciliate tested is not mentioned, a lack of effect at such a high concentration of the drug is difficult to understand in view of the investigations of Loefer(12,13), Jirovec(11), Blaszczynski and Nardone(1), Gross (8,9,10 and present paper) and Cooley(4). Even though these workers used various strains of the organism, there is general agreement that no growth occurs at the order of 1.5 x 10^{-3} M CM and that higher concentrations are lethal. In addition, Smith's claim that appearance of cells exposed to CM, both in buffer and in culture medium, do not change, is at variance with two of the reports(1,9) as well as with data presented in the present paper (Table V).

Jirovec(11) compared the actions of antibiotics on several protozoa, using as his indices for activity, the following: (1) toxicity, determined by comparing the number of living and dead organisms at selected intervals through 24 hours; and (2) inhibition, recorded as the number of motile organisms observed on the 10th and 20th days after inoculation. He demonstrated that the number of *Glaucoma piriformis* (*T. pyriformis* GL)(5) cells was 50% less than the controls after 10 days exposure to 4.9 x 10^{-5} M AM, but that at 20 days, twice as much AM was needed to produce an equal amount of inhibition. It is clear from these results, that the quantitative effect of the drug depends upon the time of measurement. Furthermore, Jirovec chose unfortunate times to take readings since 10 and 20 days are well into and past the stationary phase of growth of *T. pyriformis*. In this case, results are likely to be erratic, viz., the lack of agreement in his paper between control cultures from two separate experiments (Subtilin and AM).

An analysis of the cell count curves plotted by Loefer(13) shows a 60% depression of growth rate of strain H subjected to 100 μg./ml. (3.1 x 10^{-4} M) of CM. An equivalent concentration of the drug inhibits strain E by only 37% (Table IV). Further evaluation of Loefer's data shows a 90% inhibition in cell numbers if they are measured at 30 hours (peak of log growth). This is similar to the 4-day measurement for strain E (Fig. 1), which yields 82% less cells in CM-containing cultures than in controls. In these comparisons, the effect of strain difference and diversity due to the measuring technique can be recognized. Although inhibition at peak of log growth is similar for both strains, the same data show strikingly different results when inhibitions of growth rate are compared.

It is well known that the age of, and number of cells present in the initial inoculum can influence the cell count in the culture at any specified time thereafter. It is, therefore, felt that a definite time for reading as used by Dewey and her colleagues(6) to determine the effect of AM on *T. pyriformis* W and by Jirovec(11) is not a desirable method for quantitating environmental effects on growth. The method of cell counts, even when used to determine rate of division, is not a good measure of the action of an antibiotic or other inhibitor on growth, since it overlooks the influence that the drug may have on cell size. Besides, it has been pointed out by Loefer(14), Slater and Elliott (17) and Gross(9, Table V) that cell volumes normally undergo certain changes with increasing age of culture.

Although the technique of determining cell viability by application of the most probable number method advocated by Loefer and Mefferd(15) is admirably suited for toxicity studies in which the number of surviving cells is of interest, it does not appear to be ap-

plicable to inhibition studies for the several reasons mentioned above. The turbidimetric determination of growth rate in a colorimeter seems to hurdle these obstacles since all the factors involved in increase of optical density, such as number of organisms, volume of cells and protoplasmic density, which may be considered under the term growth, can be measured simultaneously. In addition, the rate of growth during exponential increase remains unaffected by the size of the inoculum used and furthermore, is considered to be more constant and reproducible than other growth indices. The limitations to this method pointed out by Loefer(14) are minimal during the log phase of growth since few dead or fragmented cells can be observed microscopically at this time. Additionally, the rapidity and ease of performing repeated measurements on the same culture during its growth cycle favors the use of this method. Therefore, in spite of certain inherent errors, the turbidimetric method would seem to be generally preferable to more restricted techniques like dry weight determination or cell count and volume for expressing the effects of environmental changes on cellular mass.

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