

Ecological Application of Antibiotics as Respiratory Inhibitors of Bacterial Populations¹

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Two terrogenous and four marine bacterial isolates were treated with six antibiotics and antibiotic combinations. Comparisons made between responses of cells in early and late logarithmic and stationary growth phases indicated variable sensitivity to the agents. Bacteria in stationary and late log-phase cultures exhibited the greatest resistance, whereas the early log-phase cells exhibited greatest antibiotic susceptibility. We conclude that the tested antibiotics cannot be used for ecological purposes to delineate bacterial respiration in mixed microbial communities.

Respiration has been used as an activity index for both entire communities and their constituent populations (L. R. Pomeroy and R. E. Johannes, Deep-Sea Res. Oceanogr. Abstr. 15:381). Studies of microbial respiration in situ have been made after treatment with a variety of antibiotics with the assumption that bacterial respiration is blocked, but that of eukaryotes and blue-green algae remains relatively unaffected (7, 15; K. L. Smith, Ph.D. thesis, University of Georgia, Athens, 1971). Change in ¹⁴CO₂ evolution has been used as an index of drug effectiveness in clinical specimens incubated with the agent and ¹⁴C-labeled glucose (3, 4).

These approaches to assaying the activity of populations ignore the lack of information concerning the mode of action of antibiotics on different cell types. Common antibiotics used in field studies are chloramphenicol (CM), streptomycin sulfate (SM), neomycin sulfate (NEO), and penicillin G (PEN). The primary modes of action of each antibiotic have been defined. CM, SM, and NEO inhibit protein synthesis at the ribosomal level, whereas PEN inhibits production of cell wall material (6, 12). They have been reported as marine bacterial growth inhibitors by several authors (2, 11, 16). Their effects on bacterial respiration appear to be indirect.

In this paper, we examine the effect of these four antibiotics on respiration of pure cultures of marine and terrogenous bacteria. Additional experiments were performed on water samples from the Sapelo Island, Ga., estuary.

MATERIALS AND METHODS

Bacteria. Stock cultures were maintained in culture tubes at 26 C and transferred at 5-day intervals.

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Vibrio natriegens, *Pseudomonas perfectomarinus*, *Escherichia coli* and *Bacillus subtilis* were obtained from the Department of Microbiology's stock culture collection (University of Georgia). Marine isolates 911C and 5216 were collected by William Sottile from Sargasso Sea water samples. 911C is a gram-positive rod, and 5216 is a gram-negative rod, presumptively identified as a *Vibrio* species. All of these bacteria grew adequately at 26 C.

Media. The marine isolates *V. natriegens* and *P. perfectomarinus* were grown in a broth consisting of 0.1% proteose peptone, 0.1% yeast extract, and 1,000 ml of artificial sea water (10), with pH adjusted to 7.4 with 1 M NaOH. *E. coli* and *B. subtilis* were grown in 0.8% nutrient broth and made to volume with deionized water, with pH adjusted to 7.3 with 1 M NaOH. All media were from Difco Laboratories (Detroit, Mich.).

Antibiotics. The antibiotics used singly and in combination were: CM, SM, PEN, NEO, PEN-SM, and NEO-SM. Solutions were made to 2 mg of antibiotic per ml, stored at 4 C, and replaced by fresh stock every 3 days. Final concentrations of 50 mg of antibiotic per liter (7, 14) were maintained during respiration determinations. All dilutions of solid antibiotics were made with deionized water, and potency was assured by periodic assays with cultures with known susceptibility. All antibiotics were from Sigma Chemical Co. (St. Louis, Mo.).

Culture methods. Inocula from stock cultures of bacteria were introduced into 100 ml of medium and shaken at 100 rpm and 26 C. After 12 h, a 5-ml sample was pipetted into 45 ml of appropriate medium in a side-arm flask. Growth was initiated by shaking this flask at 100 rpm at 26 C. Turbidity was measured at intervals with a Klett-Summerson photoelectric colorimeter with an approximately 660-nm wavelength filter.

Determination of relative respiratory uptake rates. A Clarke polarographic electrode connected to an oxygen monitor (Yellow Springs Instruments, Yellow Springs, Ohio), recorder (Rustrak, Instruments, Manchester, N. H.), and circulating water bath (Yellow Springs Instruments) were employed for

respiratory measurements. Samples (5 ml) were placed in the culture chamber and stirred at 100 rpm to ensure balanced oxygen uptake. Changes in total oxygen consumption (TO_2) were obtained by reading directly from the monitor scale over 10-min measuring periods.

Growth curves and cell number determinations. Turbidity was determined at 30-min intervals with a side-arm flask containing 50 ml of culture. At hourly intervals, 0.1-ml samples were withdrawn, appropriately diluted in sterile growth medium, and spread on plates. When direct microscope counts were made, a culture sample was placed in a Petroff-Hauser chamber for direct counting.

Experimental design. Measurements were obtained in triplicate for the respiration of samples of bacterial cells in both logarithmic and stationary growth phases. Cultures were grown to the desired phase of growth as determined by correlation of turbidity with viable count estimates. For stationary-phase cultures, three samples of 4.9 ml each were removed and placed in test tubes. Cultures in this growth phase were not diluted because of their tendency to resume log-phase growth. Volumes were adjusted to 5 ml with stock antibiotic for the experimental sample. Culture samples lacking antibiotic were made to volume with 0.85% saline for nutrient broth, and artificial sea water for the proteose peptone-yeast extract medium. Logarithmic-phase cultures were diluted 1:4 with the appropriate diluent. The tubes were mixed and then incubated for 120 min on a 100-rpm shaker at 26 C. After incubation, the tubes were mixed and assayed for differences in turbidity that would reflect growth changes due to the presence of the antibiotics. The samples were then placed in the measuring chamber with control assays both before and after the experimental samples. Respiration occurring during incubation for 10 min was measured, changes in oxygen consumption being recorded both on tape and as monitor readings.

Calculation of relative oxygen uptake values. Change in oxygen content was calculated for a 10-min period. A mean value was determined for the controls (\bar{c}); the difference between this and the experimental value (e) was used to represent inhibition if negative, stimulation if positive, in sign. Percent change from the control mean was calculated by the following equation:

$$100 \times \frac{e - \bar{c}}{\bar{c}} = \text{TO}_2$$

Percent change in turbidity and cell number for the samples after incubation was obtained in the same way.

Statistical analysis of data. The variance for two samples, N_1 and N_2 , was equal to:

$$\frac{(N_1 - 1)S_1^2 + (N_2 - 1)S_2^2}{N_1 + N_2 - 2}$$

The t value used in the small sample test of differences between two means was equal to:

$$\frac{D}{S \left(\frac{1}{N_1} + \frac{1}{N_2} \right)^{1/2}}$$

where N equals number of data points, S (standard deviation of the mean) equals $(S^2)^{1/2}$, and D (difference between means) equals $\bar{x}_1 - \bar{x}_2$. Statistical procedures follow those of Sokal and Rohlf (15). If t is greater than $t_{0.025}$, then the means are significantly different at the 95% confidence level.

Field data. Water samples were taken during various tidal periods from the estuary at Sapelo Island, Ga. Particulate material was concentrated 25- to 50-fold by passage through 0.45- μm glass-fiber filters. Concentrates were split into control and experimental volumes, incubated as for pure cultures, and placed in respirometer chambers. Antibiotics were added at the same concentration used for pure cultures. TO_2 was measured over a 3-h period at hourly intervals. No carbon sources were added to the concentrates.

RESULTS

Antibiotic potency tests. The minimum inhibitory concentrations of antibiotics were determined for the six bacterial isolates used. The concentrations required for growth inhibition were all less than the experimentally used 50 mg/liter.

Growth curves. Growth curves were determined for all bacteria used in respirometry measurements. In each case, turbidity readings in Klett units were plotted against viable plate counts. Each curve showed a linear region for cells in logarithmic growth phase. The plots of Klett units versus \log_{10} cell numbers showed no such area for stationary-phase cultures. Bacteria grown for respirometry measurements were judged in logarithmic or stationary phase by change in turbidity and by reference to these initial curves.

TO_2 and Klett unit measurements. Because TO_2 readings represented change due to both growth and respiratory effects, turbidity readings were obtained to reveal cell lysis that might occur. In both logarithmic and stationary growth phases, decreases in Klett units were concomitant with decreases in TO_2 for treated samples. This cytolytic effect was further examined by direct microscope observation. In most log-phase cultures examined, there was evidence of considerable cell lysis in the presence of antibiotics. Marine and terrogenous bacteria treated with PEN and PEN-SM yielded sphaeroplasts as well as lower total direct counts than control cultures. The other antibiotics produced only lytic effects, which contributed to observed decreases in turbidity.

Individual antibiotic effects on respiration.

In Fig. 1 and 2, data on the responses of the six bacterial cultures in log and stationary phases are shown. Significant stimulation or inhibition of oxygen uptake refers to values in relation to the zero on the Y-axis of the plots. Means and 95% confidence intervals are shown for three data points. Of 36 log-phase data sets, 15 showed significant inhibition of TO_2 , whereas the remainder did not statistically differ from zero. No single antibiotic exhibited a greater effect than any other, and no uniform bacterial effect was found. Mean values for inhibition varied from 7 to 90% for the 15 significant

responses, 13 of which were found in the range 30 to 75%. None of the stationary-phase cultures was affected, indicating resistance of cells in this growth state to respiratory changes. The variability in size of confidence intervals was due to use of cells with different growth rates in the stationary-phase calculations and the incorporation of early- and late log-phase values in logarithmic-phase determinations.

Comparative logarithmic and stationary growth phase effects. Estimates for differences between mean values at the 95% confidence level were made for log- and stationary-phase

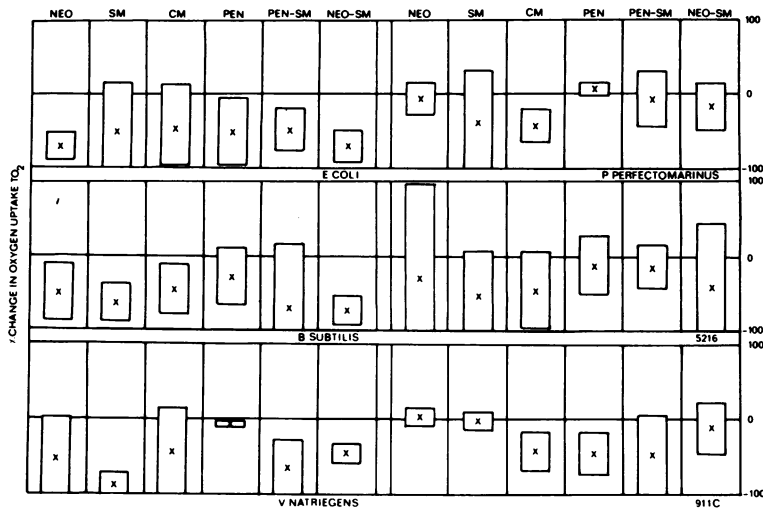


FIG. 1. Population respiratory responses of logarithmic-phase bacteria to antibiotic action with means and 95% confidence limits for data sets containing three data points each.

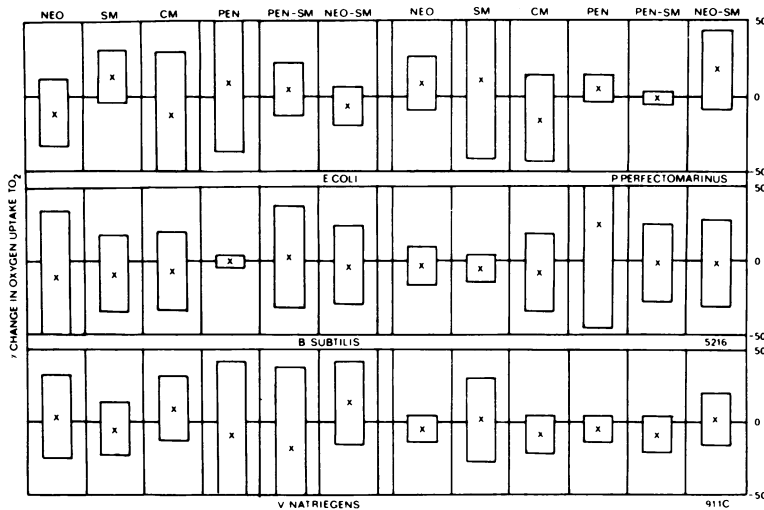


FIG. 2. Population respiratory responses of stationary-phase bacteria to antibiotic action with means and 95% confidence limits for data sets containing three data points each.

cells (Table 1). Of 36 TO_2 data sets, 22 had significantly different means. In 14 TO_2 sets, log- and stationary-phase cells did not differ in their antibiotic response.

Comparative early and late logarithmic growth phase responses. Differences in antibiotic susceptibility were noticed for early and late log-phase cultures. Comparisons were made between these stages with oxygen uptake values selected from all measurements of antibiotic susceptibility in log-phase cultures. Because individual antibiotics did not vary significantly in their effects on the bacteria tested, results were pooled to yield data sets for SM, CM, PEN-SM, and NEO-SM (Table 2). All TO_2 values showed significant differences in the susceptibility of cells in early and late log phase. Relative respiratory indices revealed differences in susceptibility of cells in different periods of the log phase.

Tests between means at 95% confidence levels were also performed for late log- and stationary-

phase cultures. The results for experiments involving four antibiotics (Table 3) showed no significant differences for population oxygen uptake response. A comparison was made between early log- and stationary-phase cells. All results of *t* tests between means (Table 4) showed significant differences at the 95% confidence level for four antibiotics.

Examination of antibiotic effects over an entire growth cycle. Because of the differences between early and late log-phase responses to the antibiotics, a detailed examination of respiration effects by SM was undertaken for an entire growth cycle. For *V. natriegens*, inhibition was greatest in the log phase and decreased continuously over the entire growth cycle until in the stationary phase there was actually some respiration stimulation (Fig. 3).

Field data. The effect of NEO, SM, and NEO-SM was a linear decrease in oxygen uptake relative to the control values (Fig. 4).

DISCUSSION

Effects of individual antibiotics on log- and stationary-phase bacterial cultures. The TO_2 of log-phase cells seemed related more to species characteristics than to nature of the antibiotic used. No consistent pattern of inhibition by a single agent was observed. Hargrave (7) exposed bacteria in the fresh water system he investigated to 50 mg each of SM and NEO per liter for preincubation periods of 1 to 3 h, and assumed uniform inhibition of bacterial respiration. Smith (14) found that respiration was inhibited from 75 to 95% in marine sediments treated with 50 mg of SM per liter and preincubated for

TABLE 1. Estimated differences between means for log- and stationary-phase TO_2 data

Organism	Fraction of data sets with significantly different means ^a
<i>B. subtilis</i>	6/6
<i>E. coli</i>	5/6
<i>V. natriegens</i>	4/6
<i>P. perfectomarinus</i>	2/6
911 C	3/6
5216	2/6

^a All *t* tests are for a 95% confidence interval.

TABLE 2. Estimated differences between means for early and late log-phase TO_2 data

Antibiotic	Growth phase	N ^a	$t_{0.025}$	Calculated <i>t</i> ^b	Significance (+ or -)
SM	Early log	8	2.23	6.03	+
	Late log	4			
CM	Early log	6	2.36	2.74	+
	Late log	3			
PEN-SM	Early log	5	2.23	4.95	+
	Late log	7			
NEO-SM	Early log	3	2.36	8.32	+
	Late log	6			

^a Number of data points.

^b Calculated *t* is tested at the 95% confidence level.

TABLE 3. *Estimated differences between means for late log- and stationary-phase TO₂ data*

Antibiotic	Growth phase	N ^a	$t_{0.025}$	Calculated t^b	Significance (+ or -)
SM	Late log	4	2.14	1.71	-
	Stationary	12			
CM	Late log	3	2.23	1.98	-
	Stationary	9			
PEN-SM	Late log	7	2.09	1.16	-
	Stationary	15			
NEO-SM	Late log	6	2.12	1.69	-
	Stationary	12			

^a Number of data points.

^b Calculated t is tested at the 95% confidence level.

TABLE 4. *Estimated differences between means for early log- and stationary-phase TO₂ data*

Antibiotic	Growth phase	N ^a	$t_{0.025}$	Calculated t^b	Significance (+ or -)
SM	Early log	8	2.10	9.83	+
	Stationary	12			
CM	Early log	6	2.16	10.33	+
	Stationary	9			
PEN-SM	Early log	5	2.10	6.40	+
	Stationary	15			
NEO-SM	Early log	3	2.16	7.91	+
	Stationary	12			

^a Number of data points.

^b Calculated t is tested at the 95% confidence level.

4 h. The current observations suggest that uniform suppression of bacterial respiration should not be assumed for marine or terregeous bacteria incubated for 2 h with comparable SM and NEO concentrations. The use of pure cultures may jeopardize acceptance of this conclusion by not approximating natural conditions, but Cviic (2) found similar variability in the growth responses of marine bacteria to SM in both artificial and natural sea water samples. Berland and Maestrini (1) also reported bacterial species differences in responses to CM, NEO, PEN, and SM.

Of 36 log-phase TO₂ measurements in the current study, 21 did not reflect significant

antibiotic effects. None of the stationary-phase cultures responded, indicating their, at least, short-term resistance to a number of potential metabolic disturbances. The effects observed by Smith (Ph. D. thesis, 1971), Smith et al. (14), and Hargrave (7, 8) could have been due to inhibition of some species, partial inhibition of all the bacteria, blockage of eukaryotes, or any combination of the above.

Effect of growth state. Antibiotic susceptibility in bacteria appears greatest in log-phase populations. Thus, many more log-phase values were significantly different from zero than were stationary-phase values. Comparisons of means at the 95% confidence level indicated that a

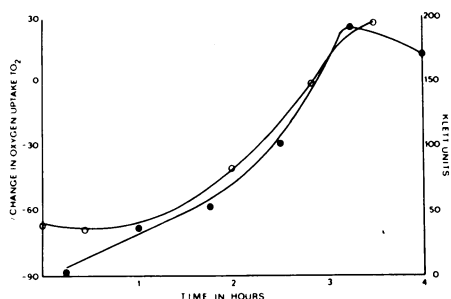


FIG. 3. Population respiratory responses and turbidity plotted against time for a growth cycle of *V. natriegens* treated with 50 mg of SM per liter. Symbols: O, represent turbidity readings; and ●, represent percent change in TO_2 .

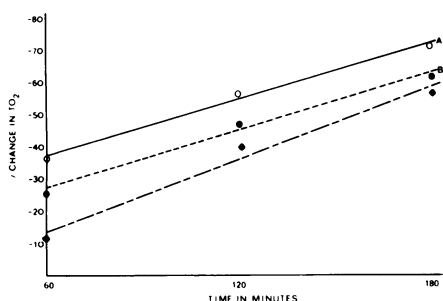


FIG. 4. Antibiotic responses of microorganisms plotted against time for water concentrates from the Sapelo Island, Georgia, estuary. Symbols: ◆, ebbing tide sample treated with 50 mg of NEO per liter; O, (A), flood-tide sample treated with 50 mg of SM per liter; ●, (B), ebbing tide sample treated with 50 mg of SM per liter.

majority of log- and stationary-phase data sets were significantly different from each other. Results for TO_2 varied with species, *P. perfectomarinus* and marine bacterium 911C showing fewest significant growth phase differences in antibiotic response. These results are consistent with the findings of Berland and Maestrini (1), who examined the growth effects of PEN, SM, NEO, and CM on a variety of marine bacterial species and found susceptibility to vary within the same genus.

The logarithmic growth phase is a uniform state of exponential growth. However, cells in late log phase are entering the stationary phase, with no real distinction possible between the end of the former and the beginning of the latter. Results of *t* tests for mean differences between late log- and stationary-phase cell responses to antibiotics suggest that no real differences exist. Differences between cells in early log and stationary phase were significant, however. Poyton (13) reported higher oxygen

consumption and heat evolution rates for early than later log-phase algal cells (*Prototheca zopfii*). In the present study, higher oxygen consumption rates were also seen during early log phase. Cells in this part of the growth cycle have the highest rate of biosynthetic activity of any portion of the growth cycle. The reason that the respiratory inhibition by antibiotics is greatest during this period may be that the production and activity of respiratory enzymes in these cells is greatest and, thus, interference with protein synthesis would have the maximum effect.

The clinical use of radiometry for antibiotic susceptibility tests should be re-examined in light of variability in respiratory bacterial drug responses due to species and growth state factors (3, 4). Inhibition of growth should not be assumed from short-term (1 to 4 h) metabolic studies, because susceptible cells can show little or no respiration inhibition over these time periods, and resistant populations could exhibit depressed respiration, yet increase in number with time.

Field study. If our experimental results on respiration inhibition of pure cultures can be extrapolated to the field (Fig. 4), existence of populations of early log-phase cells would account for the susceptibility to antibiotics. Late log- or stationary-phase bacteria should not be affected readily by antibiotics. If bacteria were in late log or stationary phases, the inhibition of respiration would have to be due to effects on sensitive eukaryotes. Foter et al. (5) and other authors have found that growth of various algae was inhibited over long incubation periods, 72 h to 4 days (96 h), in the presence of CM, SM, or NEO. There is a possibility that oxygen uptake by such organisms could be affected over shorter periods, but this has never been examined.

If any antibiotics can be used to separate bacterial respiration from that of the rest of a natural community, then the percent inhibition must be a reasonably constant value for a wide variety of species. Results in Fig. 3 show dramatically that the percent inhibition can be a continuously varying factor, dependent not only on growth phase per se but also on the position within a phase of growth. We conclude that CM, SM, NEO, and PEN cannot be used as presently suggested in the literature to delineate bacterial respiration in mixed microbial communities.

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LITERATURE CITED

1. Berland, B. R., and S. Y. Maestrini. 1969. Study of bacteria associated with marine algae in culture. II. Action of antibiotic substances. *Mar. Biol.* **3**:334-335.
2. Cviic, V. 1953. The bactericidal and bacteriostatical action of antibiotics on marine bacteria. *Acta Adriat.* **5**:137-162.
3. DeBlanc, H. J., Jr., P. Charache, and H. N. Wagner, Jr. 1972. Automatic radiometric measurement of antibiotic effect on bacterial growth. *Antimicrob. Ag. Chemother.* **2**:360-366.
4. DeLand, F. H. 1972. Metabolic inhibition as an index of bacterial susceptibility to drugs. *Antimicrob. Ag. Chemother.* **2**:405-412.
5. Foter, M. J., C. M. Palmer, and T. E. Maloney. 1953. Antialgal properties of various antibiotics. *Antibiot. Chemother.* (Washington, D. C.) **3**:505-508.
6. Hahn, F. E. 1967. Chloramphenicol, p. 308-330. *In* D. Gottlieb, and P. D. Shaw (ed.), *Antibiotics. I. Mechanism of action.* Springer-Verlag, New York.
7. Hargrave, B. T. 1969. Epibenthic algal production and community respiration in the sediments of Marion Lake. *J. Fish. Res. Bd. Can.* **26**:2003-2026.
8. Hargrave, B. T. 1970. The effect of a deposit-feeding amphipod on the metabolism of benthic microflora. *Limnol. Oceanogr.* **15**:21-30.
9. Lamanna, C., and M. F. Mallette. 1973. *Basic bacteriology: its biological and chemical background.* The Williams & Wilkins Co., Baltimore.
10. Lyman, J., and R. H. Fleming. 1940. Composition of sea-water. *J. Mar. Res.* **17**:341-346.
11. Oppenheimer, C. H. 1955. The effect of marine bacteria on the development and hatching of pelagic fish eggs, and the control of such bacteria by antibiotics. *Copeia* **1**:43-49.
12. Park, J. T., and J. L. Strominger. 1957. Mode of action of penicillin. *Science* **125**:99.
13. Poyton, R. O. 1973. Effect of growth rate on the macromolecular composition of *Prototheca zopfii*, a colorless alga which divides by multiple fission. *J. Bacteriol.* **113**:203-211.
14. Smith, K. L., Jr., K. A. Burns, and J. M. Teal. 1972. In situ respiration of benthic communities in Castle Harbor, Bermuda. *Mar. Biol.* **12**:196-199.
15. Sokal, R. R., and F. J. Rohlf. 1969. *Biometry: the principles and practice of statistics in biological research.* W. H. Freeman and Company, San Francisco.
16. Spencer, C. P. 1952. On the use of antibiotics for isolating bacteria-free cultures of marine phytoplankton organisms. *J. Mar. Biol. Ass. U. K.* **31**:97-106.