APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Nov. 1978, p. 700-704
0099-2240/78/0036-0700$02.00/0

Vol. 36, No. 5
Printed in U.S.A.

Surface Kinetic Test Method for Determining Rate of Kill by an Antimicrobial Solid

A. J. ISQUITH* AND C. J. McCOLLUM
Health and Environmental Research, Dow Corning Corporation, Midland, Michigan 48640

Received for publication 10 August 1978

An antimicrobial-surface kinetic test which maximizes probability of cell-to-surface contact has been developed. The measurement of rate of kill by a nonleaching antimicrobial surface is based on the number of surviving bacterial cells at specific times of exposure to various amounts of total treated surface area of test substrate. This method gives information for direct comparison of rate of kill for a variety of antimicrobial surfaces in terms of rate of kill per square centimeter of surface area. Data obtained by this method can also give valuable dose response application information as an indication of the exponential efficiency of concentration in terms of treated surface area.

Recent publications from this laboratory (4, 13) described the creation of durable antimicrobial surfaces by the application of a cationic alkoxysilane, 3-(trimethoxysilyl)propyldimethyloctadecyl ammonium chloride, to a spectrum of surfaces. In contrast to other antimicrobial agents for which durable residual-surface treatments have been claimed (1, 8, 12), the activity of the organosilicon-treated surface was not ascribed to slow-release, solution-active chemicals. Both radioisotope and bioassay procedures demonstrated that the agent was not released from the surface.

Standard methods for determining bactericidal activity of antimicrobial agents in terms of rate of kill per concentration of compound in solution are well established (2). Similar methods for determining dose response relationships for active surfaces are not. Because of the apparent catalytic nature of substrate treated with the cationic alkoxysilane, i.e., because there is no consumption of the chemical during microbial kill, a procedure used to measure kinetics of chemical catalysts was modified in an attempt to obtain a sensitive method for determining rate of reaction and obtaining dose response curves for biologically active material. This communication describes the development and use of the method.

MATERIALS AND METHODS

Preparation of resting-cell suspension. *Escherichia coli* B (ATCC #23226) was harvested from the logarithmic phase of growth in nutrient broth by centrifugation at 15,000 rpm for 15 min in a refrigerated Sorval RC2-B. Harvested cells were suspended in sterile physiological saline and washed three times by centrifugation. The washed cell pellet was suspended in fresh sterile saline and allowed to equilibrate at room temperature for 30 min to deplete endogenous metabolites. The resultant resting-cell suspension was diluted further in sterile saline to the desired cell concentration. Optical density and pour plate techniques were employed to measure concentration of cells.

Preparation of treated surface. Antimicrobial-surface test material was prepared by treatment of a type of commercial silica (Min-u-sil; Pennsylvania Glass Sand Corporation) which has a 10μ mean particle size and contains a surface area of 11.0 cm^2/mg with alkyl chain ^{14}C-labeled $(CH_3O)_3Si(CH_2)_3{}^+N(CH_3)_2C_{18}H_{37}Cl^-$ to a level of seven molecular layers. Surfaces were washed by centrifugation, and the supernatant fluid was assayed for soluble, unbonded alkoxysilane. At the level of treatment used, no unbound material was detected by either ^{14}C analysis or bioassay procedures in the decanted supernatants. By this procedure, ^{14}C analysis of the treated Min-u-sil showed 11.62×10^{-10} mol of organosilane per mg of sample.

Test procedure. The "surface kinetic test" was performed by the precise addition of 100 cm^2 of treated Min-u-sil to 2 ml of *E. coli* B resting-cell suspension in a screw-cap tissue culture tube (15 by 125 mm). The contents of the inoculated and sealed tube were blended in a Vortex mixer for 5 s, placed at 37°C, on a Fisher Roto Rack, and rotated through 360° at 29 rpm. Individual tubes were removed at specific time intervals, diluted to a concentration of 20 to 200 cells per ml in Letheen Broth (Difco Laboratories) to inactivate the cationic treatment, and pour plated in Letheen agar. Viable cell counts were made for each sampling time in triplicate after incubation for 24 h at 37°C. Inoculum control tubes consisting of resting cell suspensions, as well as inoculated, untreated Min-u-sil control tubes, were included in each experiment.

RESULTS

By the test procedure described above, solid

surfaces prepared by immobilization of 3-(trimethoxysilyl)propyldimethyloctadecyl ammonium chloride on Min-u-sil were found to be active against resting-state *E. coli* B cells (Table 1). Within 15 min, a 99% reduction in viable bacterial count was achieved in cells exposed to 50 cm^2 of active surface per ml suspended in sterile saline. When a standardized inoculum of between 1,000 and 8,000 cells is used, the rate of kill ($-k$) appears to be a first-order rate process commonly used to quantitate the exponential death of microorganisms (5). This process is described by the equation $S_t = S_0e - kt$, where S_t = surviving cells at time (t), S_0 = initial number of organisms at zero time, and $-k$ = rate of exponential death when $\log_e S_t$ is plotted against time. In Fig. 1, the surface activity rate can be described as $-k = [(\log_e S_t - \log_e S_0)/\text{time}] = 0.283$. Therefore, $\log_e$ reduction per square centimeter per minute = 0.283/50 = 0.0057.

The surface activity rate of kill ($-k$) of Min-u-sil treated with 3-(trimethoxysilyl)propyldimethyloctadecyl ammonium chloride was found to be a function of treated surface area, with $\log_e$ bacterial cell reduction per minute increasing with increasing surface area (Table 2). By using a constant initial inoculum level (S_0), one obtains a graded concentration effect of total treated surface area (Fig. 2; Table 3).

Therefore, a statistically significant ($P < 0.05$) response (determined by Student's t test) which correlated well at each concentration level at similar times of exposure was achieved.

The relationship of response time to area of antimicrobial surface (dose) was determined from the data in Table 2. The time (t) to kill 50% of the initial number of viable cells was calculated for each surface area (A). A sigmoid curve is obtained (Fig. 3) when $\log_t$ is plotted against $\log_A$ indicating that the change in exponential effectiveness below 25 and above 100 cm^2/ml decreases significantly at this inoculum level. The regression line in the linear portion of the curve results in a rate of decrease (x) in response time with increasing surface area of 0.781. $x = (\log_{t-a})/\log A$, where $a = \log_t$ at zero surface area.

In the linear portion of the curve, $A_2{}^x t_2 = A_3{}^x t_3 = A_4{}^x t_4 = 36.4$. Therefore, ${}_{25}\int^{100} A^x t = K$ (con-

TABLE 1. *Survival of E. coli in the presence of active Min-u-sil surfaces*

Time (min)	Viable cells/ml			% Reduction
	Control[a]	Control[b]	Expt[c]	
0	3,750	3,750	3,750	0
5	4,370	3,900	1,600	63
15	5,000	3,700	29	99
30	4,200	4,300	1	99.9
45	4,100	3,900	0	>99.9
60	3,500	4,400	0	>99.9
75	4,200	4,400	0	>99.9
90	3,600	4,900	0	>99.9
120	3,600	4,600	0	>99.9
24 h	5,250	6,000	0	>99.9

[a] Inoculum control in 2 ml of physiological saline.
[b] Untreated control: 10μ Min-u-sil, 50 cm^2/ml.
[c] Active surface: 10μ Min-u-sil, 50 cm^2/ml, treated with 3-(trimethoxysilyl)propyldimethyloctadecyl ammonium chloride.

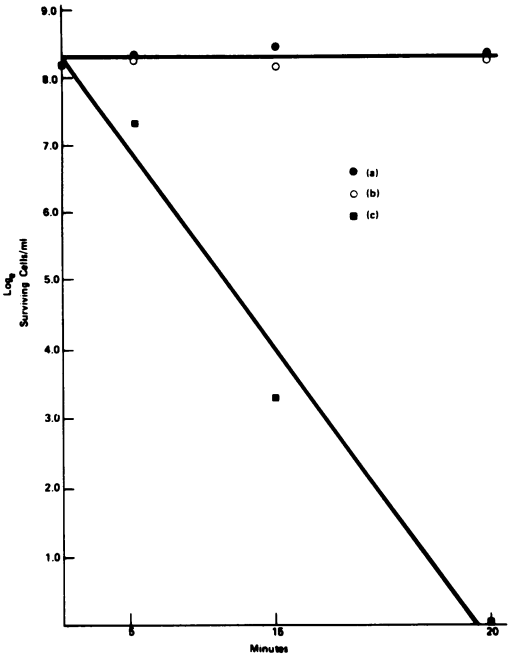

FIG. 1. *Rate of kill of E. coli B in the presence of: (a) physiological saline, (b) 10μ Min-u-sil, 50 cm^2/ml, or (c) 10μ Min-u-sil, 50 cm^2/ml, treated with 3-(trimethoxysilyl)propyldimethyloctadecyl ammonium chloride.*

TABLE 2. *Rate of kill of E. coli B with time for various concentrations of antimicrobial surface*[a]

Surface area (cm^2/ml)		Response time (min)[b]	Log response time	($-k$) $\log_e$ reduction/min
A	$\log_A$			
12.5	1.0969	5.924	0.773	0.117
25.0	1.3779	5.163	0.713	0.133
50.0	1.6990	3.487	0.542	0.199
100.0	2.0000	1.751	0.243	0.396
400.0	2.6021	1.642	0.215	0.423

[a] Min-u-sil (particle size, 10μ) treated with 3-(trimethoxysilyl)propyldimethyloctadecyl ammonium chloride. Response time was defined as time required to reduce inoculum (10,000 cells per ml) by 50%.

stant). The surface area (dose) represents 1.66 $\times 10^{-11}$ mol/cm^2 per monolayer. Doubling of the surface area (dose) increases the antimicrobial effectiveness by a factor of 1.718.

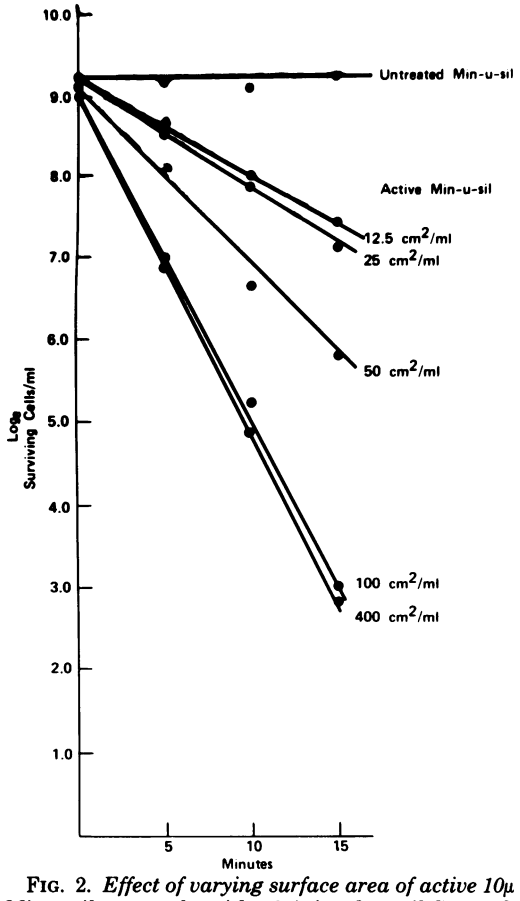

FIG. 2. *Effect of varying surface area of active 10μ Min-u-sil treated with 3-(trimethoxysilyl)propyldimethyloctadecyl ammonium chloride on the rate of reduction of viable E. coli B.*

The expected non-linearity at low concentrations, typical of dose response curves, was not due to tolerance. Positive growth was obtained after subculturing exposed bacterial cells to fresh growth media. Isolates from this culture were transferred three consecutive times and retested against freshly treated Min-u-sil. Activity against this isolate did not significantly differ from that in the initial test; therefore, the decreased rate ($-k$) of less than 25 cm^2/ml cannot be attributed to the development of resistance by *E. coli* B.

The decreased rate of kill could not be attributed to loss of treatment from the Min-u-sil. In separate experiments, Min-u-sil treated to the same level with ^{14}C-labeled 3-(trimethoxysilyl)propyldimethyloctadecyl ammonium chloride showed no loss of radioactivity to solution after extensive mixing (up to 24 h) in the surface kinetic test.

The response rate in Fig. 3, determined for increased amounts of Min-u-sil to confirm the function of limiting surface area per rate of kill, showed very little potentiation of activity above 100 cm^2/ml at this inoculum level.

The ratio of viable to nonviable cells may alter adsorption-desorption kinetics and thus affect probability of contact with active sites as found in studies of conventional solution-active agents (6, 9). Additional tests at varying concentrations

TABLE 3. *Graded concentration effect of surface area*

Surface area (cm^2/ml)	$-k$	Correlation coefficient	Student's t
12.5	0.117	0.9485	2.9934
25.0	0.133	0.9243	15.978
50.0	0.199	0.9984	25.2199
100.0	0.396	0.9986	27.0036
400.0	0.423	0.9596	4.8247

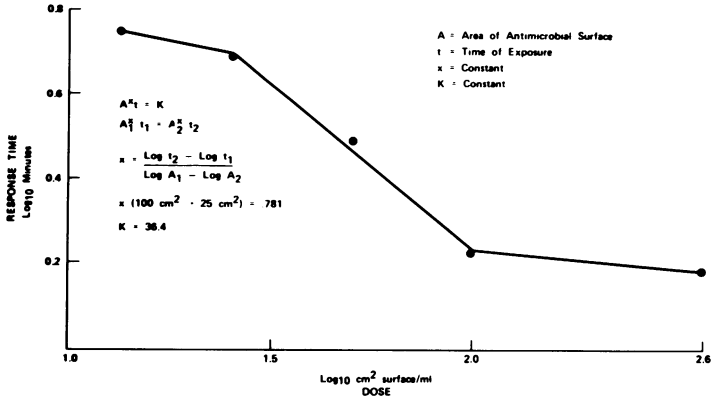

FIG. 3. *Dose response of 10μ Min-u-sil treated with 3-(trimethyoxysilyl)propyldimethyloctadecyl ammonium chloride.*

of initial bacterial challenge level (Table 4) showed that $-k$ is affected by total bacterial cells per milliliter per square centimeter of surface area. At a constant surface area of treated Min-u-sil (50 cm^2/ml), the rate of kill ($-k$) decreases with increasing concentration of initial bacterial inoculum. A preliminary standard curve (Fig. 4) was obtained from these data, indicating that the relationship can be described as the slope (m) when log S_0 (cells per ml at zero time) is plotted against $-k$ (rate of kill). In this range of S_0 (1,000 to 8,000 cells per ml), $m = -0.286$ and $t = 30.194$. A significant dependence of rate of kill on S_0 is observed in this initial inoculum range ($P < 0.05$). A standard treated surface of known activity should be included with each test of unknown antimicrobial surface to confirm this relationship. Additional tests at S_0 above 10^5 cells per ml showed very slight dependence of $-k$ on S_0. However, the sensitivity of the method was greatly reduced with a subsequent increase in experimental error. All of our rate calculations have been derived from the portion of the test (between 1,000 and 8,000 cells per ml) which fits first-order kinetics. However, one must recognize that the effects of cell population noted above decree that the overall process must be described as second order.

TABLE 4. *Effect of initial inoculum level (S_0) on the rate of kill ($-k$) of E. coli B at a constant concentration of active surface area*[a]

Surface area (cm^2/ml)	S_0 (cells/ml)	$\text{Log}_e S_0$	($-k$) (Log_e reduction/min)
50	1,321	7.1861	0.437
50	1,459	7.2855	0.413
50	3,988	8.2911	0.285
50	8,181	9.0096	0.208
50	8,243	9.0171	0.202

[a] Chemical used was 3-(trimethoxysilyl)propyldimethyloctadecyl ammonium chloride-treated 10μ Min-u-sil. See text for explanation of variables.

DISCUSSION

The organosilicon compound 3-(trimethoxysilyl)propyldimethyloctadecyl ammonium chloride is capable of creating highly active antimicrobial surfaces when durably affixed to a variety of materials. Although the activity of these solid surfaces can be described in terms of quantal data, it should be recognized that the expression of death rates of microorganisms, which are exponential in character, cannot be adequately described in terms of percentage (10).

The quantitative rate of kill observed when antimicrobial surfaces were incubated with resting-cell suspensions of *E. coli* B in a sealed rotating tube was found to be an exponential function when $\log_e S_t$ was plotted against time.

At a constant level of initial inoculum (S_0) and at various concentrations of antimicrobial surface area (in square centimeters per milliliter) and times of bacterial cell exposure (t), the rate of kill ($-k$) increases with increasing surface area.

A preliminary dose response curve was obtained for antimicrobial solid surfaces relating exponential effectiveness of the concentration of active material in terms of square centimeters of surface area.

The surface kinetic test method we have developed allows quantitative reproducible measurement of the activity of antimicrobial solid surfaces in terms of the well-defined kinetics already established for measurement of solution-active agents. To determine the dose response relationship by this method, one substitutes

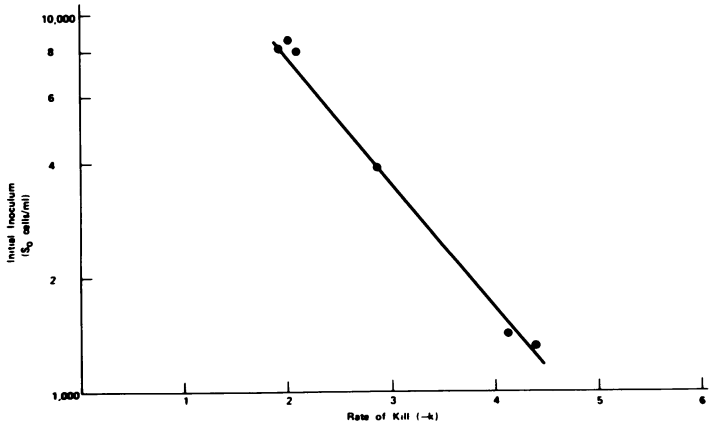

FIG. 4. *Standard curve for rate of kill of 10μ Min-u-sil (50 cm^2/ml) treated with 3-(trimethoxysilyl)-propyldimethyloctadecyl ammonium chloride in most sensitive range of E. coli B initial inoculum level.*

square centimeters of active surface area for parts per million or micrograms of drug per milliliter.

Although the idea of measuring the efficacy of solution-active agents on surfaces is not new (11), the quantitative measurement of rate of kill by solid surfaces which themselves serve as the active agent is new. If, as expressed by Klarmann et al. (7), the ideal method of control of microorganisms should involve creation on the exposed material surfaces of a persistent antibacterial potential (considerable effort has been expended on the development of such surfaces [3]), methods for the accurate assessment of this activity are needed. The surface kinetic test method described above measures this antimicrobial potential.

ACKNOWLEDGMENTS

We thank J. R. Malek for necessary chemical synthesis, and D. R. Bennett and J. L. Speier for valuable guidance and suggestions throughout these studies.

LITERATURE CITED

1. **Dunklin, E. W., and W. L. Lester.** 1959. Residual surface disinfection. II. The effect of orthophenylphenol treatment of the floor on bacterial contamination in a recovery room. J. Infect. Dis. **104**:41-55.
2. **Garrett, E. R.** 1971. Drug action and assay by microbial kinetics. Prog. Drug Res. **15**:271-352.
3. **Hoffman, R. K., S. B. Yeager, and S. Kaye.** 1955. A method for testing self disinfecting surfaces. Soap Chem. Spec. **31**:135-138, 163-165.
4. **Isquith, A. J., E. A. Abbott, and P. A. Walters.** 1972. Surface-bonded antimicrobial activity of an organosilicon quaternary ammonium chloride. Appl. Microbiol. **24**:859-863.
5. **Jawetz, E., J. L. Melnick, and E. A. Adelberg.** 1972. Review of medical microbiology, p. 78-86, 10th ed. Lange Medical Publications, Los Altos, Calif.
6. **Kahn, O.** 1945. Injury and death of bacteria by chemical agents. Biodynamicia, Normandy, Mo.
7. **Klarmann, E. G., E. S. Wright, and V. A. Shternov.** 1953. Prolongation of the antibacterial potential of disinfected surfaces. Appl. Microbiol. **1**:19-23.
8. **Lester, W., Jr., and E. W. Dunklin.** 1955. Residual surfaces disinfection. I. The prolonged germicidal action of dried surfaces treated with orthophenylphenol. J. Infect. Dis. **96**:40-53.
9. **Min Won, C., and E. R. Garrett.** 1973. Kinetics and mechanism of action of drugs on microorganisms. XVI. J. Pharm. Sci. **62**:1087-1092.
10. **Stuart, L. S.** 1968. Introduction to antimicrobial testing methods, p. 109-113. *In* C. A. Lawrence and S. S. Block (ed.), Disinfection, sterilization and preservation. Lea and Febiger, Philadelphia.
11. **Sykes, G.** 1967. Methods of testing disinfectants, p. 38-80. *In* Disinfection and sterilization, 2nd ed. J. B. Lippincott Co., Philadelphia.
12. **Vol'f, L. A.** 1965. Imparting antimicrobial properties to fibers. Tekst. Prom. (Moscow) **8**:9-11.
13. **Walters, P. A., E. A. Abbott, and A. J. Isquith.** 1973. Algicidal activity of a surface-bonded organosilicon quaternary ammonium chloride. Appl. Microbiol. **25**:253-256.