A System for the Evaluation of Aerial Disinfectants[1]

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A great number of chemical compounds have been proposed as aerial disinfectants (Bourdillon *et al.*, 1948; Wells 1955), and opinions of their value differ widely. The absence of a common testing procedure and method of expressing results may account for these differences. It was the opinion that an evaluation procedure analogous to that involved in determining "phenol coefficients" for *in vitro* disinfectants would be a valuable contribution to the search for a suitable aerial disinfectant. This study, started some four years ago, proposes a standard procedure for this purpose.

The most noteworthy prior contribution to the development of a procedure for the evaluation of aerial disinfectants is incorporated in the detailed report of the Aerosols Panel of the British Disinfectant Manufacturers' Association (Berry *et al.*, 1949). The procedure recommended by the Aerosols Panel consists of atomizing a bacterial suspension into a closed room, then introducing chemical vapors and determining the rate of disappearance of the airborne bacteria with a slit sampler. Our proposed procedure differs from that of the Aerosols Panel in almost every respect. For this reason, and because of the numerous variables involved, the experimental materials and methods are described in some detail.

Materials and Methods

The test culture. The investigations were carried out with cultures of *Serratia marcescens* strain ATCC 274. As the existence of a number of variants within this strain has been reported (Bunting, 1940), extreme care was taken to differentiate the culture as received from the Culture Collection. It was found that the primary form is characterized by circular, thin, smooth, red-carmine to orange-red colonies on nutrient agar when incubated at 20 C. (It should be noted that a few pink and a very few white colonies were isolated from the stock culture.) After isolating the primary form of *S. marcescens* strain ATCC 274, subsequent transfers were made into 60-ml volumes of 0.3 per cent beef-extract broth and incubated at 30 C. Five serial transfers at regular intervals of 45 to 48 hr were made before using the culture in tests. Thereafter the test culture was maintained in broth, exhibiting cultural stability through at least 45 serial transfers. Broth test cultures were reconstituted from stock nutrient agar butt tubes every 30 to 60 days, these being stored at 5 C with a surface overlay of mineral oil. The use of broth cultures 45 to 48 hr old provided a physiologically and numerically stable population (50 to 100 $\times$ 10^7 per ml) for daily use in aerosol tests.

All media were standard Difco preparations (Difco Laboratories), and all water used was distilled first and then passed through a mixed-bed, ion-exchange column (IRA 400 and IR 120).[2]

Control of temperature and humidity. All tests were conducted in a large insulated room in which the temperature and dewpoint of the air could be adjusted and controlled within $\pm$0.5 C. Although studies were made under a variety of conditions of temperature and humidity, it was found that 20 C and relative humidities of 25 and 80 per cent constituted representative conditions for the testing of aerial disinfectants.

In the case of effective aerial disinfectants, it is unnecessary to make any correction for the death of airborne bacteria under the control conditions because this loss is small compared to that caused by aerial disinfectants. However, in order to be certain that the response of the test organism to aerial dispersion remained constant, the aerosol was monitored at regular intervals in the absence of chemical vapors under standard conditions.

Performance of the Test

The production of the standard bacterial aerosol. (See figure 1 for details of equipment.) In producing the standard bacterial aerosol, approximately 100 ml of broth culture are transferred to a 250-ml aspirator bottle, which serves as a reservoir, being connected to a No. 40 DeVilbiss all-glass atomizer. Both the atomizer and the reservoir are placed in a water bath held at 10 C during operation. To minimize concentration effects within the atomizer, a siphon pump is connected to the atomizer-reservoir system, thereby continually mixing the culture in the atomizer with that in the reservoir.

The bacterial aerosol is formed from the broth culture by operating the atomizer with 6.4 L per min of carefully

[1] These studies were aided in part by National Institutes of Health grant-in-aid, RG-2771. A partial account of this work was presented at the 56th annual meeting of the Society of American Bacteriologists, Houston, Texas, May 1956.

[2] Rohm and Haas Co., Chicago, Illinois.

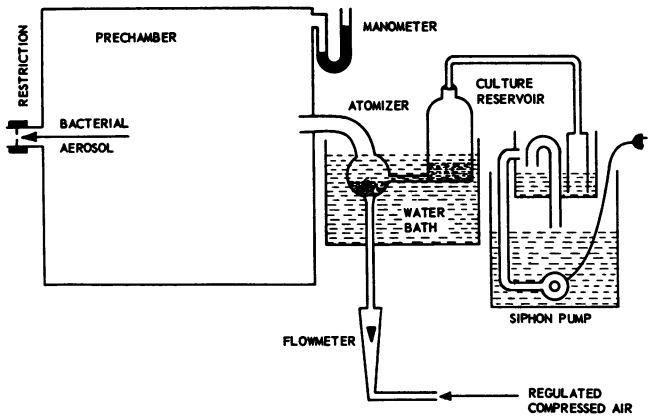

FIG. 1. Equipment for the production of the standard bacterial aerosol. A constant backpressure is maintained within the prechamber by the restriction at the exit. The siphon pump mixes the contents of the atomizer and the reservoir by varying the resistance to this backpressure.

regulated compressed air. This aerosol is classified by passage through a 33-cm cubical prechamber where the larger particles settle out, resulting in a cloud of not less than 90 per cent of single-bacterium-carrying particles, and no particles carrying more than two bacteria. These particles are approximately equivalent in size to spheres of 1.8 μ in diameter at 25 per cent relative humidity, and 2.3 μ at 80 per cent. Data as to the size and composition of the particles in this aerosol were obtained in a 4-foot cubical chamber by taking air and settling samples simultaneously and through the use of an electron microscope in examining the settled particles.

Diluting the standard aerosol. (See figure 2 for details of equipment.) The standard bacterial aerosol passes from the prechamber and is drawn into the cylinder system together with approximately 22 L of air of the desired temperature and dewpoint. An aerosol cloud is often difficult to disperse, and uniform dilution of bacterial aerosols is not consummated easily. For the rate of flow encountered here, the illustrated cone mixers are an effective solution to this problem. The cones are formed from two 6-inch, 60-degree metal funnels, soldered mouth to mouth. At the entrance to each cone is a restriction, the first being 0.375 inches in diameter, the second 0.25 inches. There is also a restriction of 0.25 inches in diameter at the exit of the second cone. This mixing system is employed both to dilute the standard bacterial aerosol and also to mix the diluted aerosol with the chemical vapors.

With the exception of the restrictions in the cone mixers, all other tubes in the aerosol cylinder system are of 0.5-inch internal diameter copper tubing. There are no abrupt bends or turns. Connections are made with refrigeration fittings, either solder or flare.

Production of the chemical vapors. (See figure 2 for details of equipment.) The compound under test is vaporized in a small chemical-vapor generator consisting of a weighed midget impinger, housed in an

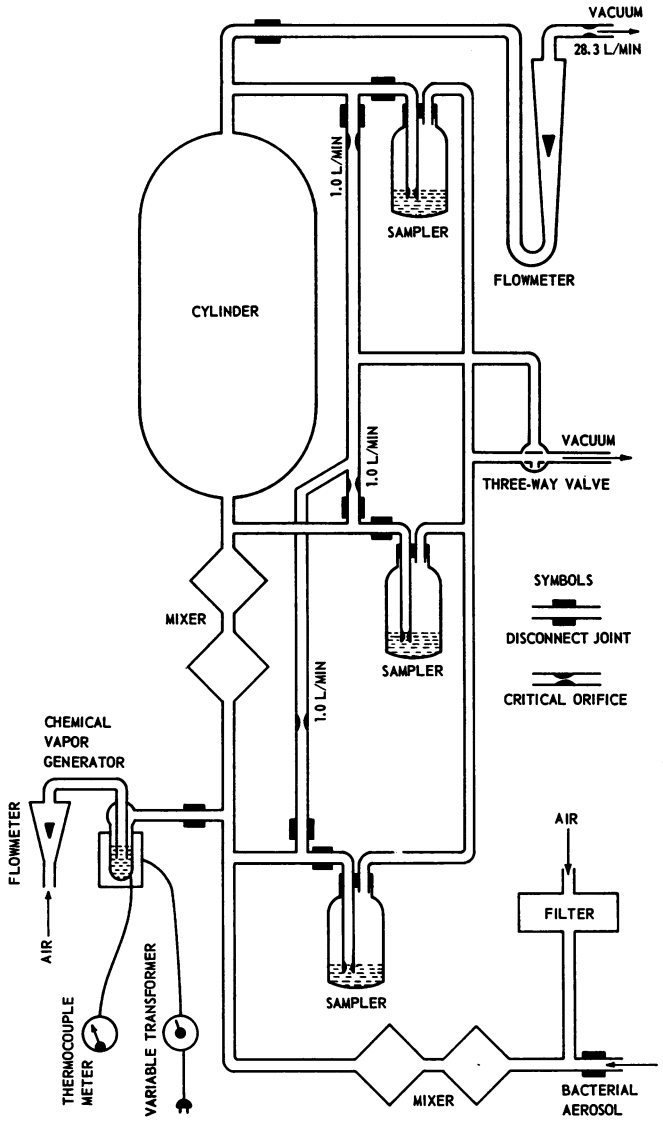

FIG. 2. Aerosol cylinder and accessory equipment for testing aerial disinfectants. All tubes carrying the aerosol are made of 0.5-in internal diameter copper tubing.

electrically heated jacket. The temperature of the compound is determined with a thermocouple and microammeter, and adjusted as desired (usually at 110 C) with a variable transformer connected to the heating jacket. Dry air is run through the midget impinger at flows of 200 to 500 cc per min so that the amount of compound vaporized per minute is slightly more than sufficient to achieve saturation of the total airflow through the system. The concentration of compound in this air is estimated from the weight-loss data during the timed test. Because the entire system is of smooth, nonporous materials and is held at a uniform temperature, losses of the chemical vapors are negligible, and the nominal and actual concentrations are in good agreement.

The aerosol cylinder. (See figure 2 for details.) The diluted bacterial aerosol and chemical vapors are mixed in a cone mixer, and then pass into the aerosol cylinder.

This cylinder is a low-pressure, nonshatterable oxygen cylinder such as used by aviators during high-altitude flights. It is made of stainless steel, 12 inches in diameter, has a straight cylindrical midsection 12 inches in length, and is 24 inches in over-all length, the end sections being dome-shaped. The internal volume of the cylinder is approximately 35 L. When air is passed through the cylinder at 28.3 L per min, the measured detention time is 3.5 min. The airflow through the cylinder is held constant by means of a critical orifice inserted in the vacuum line. A flowmeter in this same line provides a visual check on the operation of the system.

After passing through the aerosol cylinder, the mixture of air, bacterial aerosol and chemical vapors is drawn into the vacuum pump (Hytor)[3] and is flushed down the water drain. After the completion of a test, the cylinder is disconnected from the rest of the system, cleansed with live steam, and air-dried. In practice (although not shown in figure 2) two identical cylinders are mounted on a single stand and connected to the system in such a manner that the turning of two valves changes operation from one cylinder to the other. In this way testing can be carried on without interruption.

The samplers. (See figure 2 for details of equipment.) In the aerosol cylinder system, air samples are taken simultaneously at three points; prior to the introduction of the chemical vapors into the system, at the inlet to the cylinders, and at the exit of the cylinder. This procedure is repeated three times for a single test. To maintain uniform operating conditions, equivalent amounts of air are withdrawn from the system when the samplers are not in use. The change-over to sampling is controlled by a single three-way valve. The samplers are 1.0 L per min critical orifice liquid impingers, similar in principle, but different in design, from those used by Rosebury (1947). They consist of calibrated critical orifice tips formed by constricting the end of 0.25-inch internal diameter soft glass tubing. The tubing is inserted into a stopper fitted into a one-quart milk bottle containing 200 ml of sampling fluid. An outlet tube is also fitted into this stopper and is connected to the vacuum line, thus drawing the required sample through the fluid for the specified time of 5 min. An enriched sampling fluid is prepared as follows: brain-heart infusion, 16 g; gelatin (Pharmagel A)[4], 2 g; dibasic sodium phosphate anhydrous, 0.16 g; distilled, deionized water, to make one liter. With the enriched sampling fluid, approximately twice as many viable organisms have been found as when a plain gelatin fluid similar to that used by Rosebury (1947) was employed. After adding one loopful of AF antifoaming emulsion[5] to each 200 ml in the milk bottles, the bottles are plugged and sterilized. The impinger tips are sterilized separately and inserted immediately prior to use.

Following sampling, 1.0 ml aliquots of the fluid are plated, either directly or 1:10, in triplicate. The plating medium consists of: tryptone glucose extract agar, 24 g; sodium chloride, 5 g; anhydrous dibasic sodium phosphate, 2.5 g; deionized water, to make one liter. This medium promotes rapid growth so that colonies can be counted within 18 hours when incubated at 35 C.

From the time-volume relationships, the concentration of viable bacteria per liter of air in the aerosol system is calculated. To correct for evaporation losses the actual volume of fluid in the samplers is measured after sampling.

Method of reporting results. Using the concentration of viable organisms at the inlet (*in*), and at the outlet (*out*) of the cylinder, the die-away value (k) is determined for the effect of the chemical vapors during the detention time in the cylinder (3.5 minutes). Thus:

$$k = \frac{\log \text{in} - \log \text{out}}{3.5}$$

It should be noted that the k values so determined are for death alone, because the linear velocity through the aerosol system is great enough to maintain airborne all particles of the diluted standard aerosol (prepared by the method described in this paper). This is true for these particles, even though enlarged several times because of condensation of chemical vapors.

The value obtained from the sample taken just prior to the introduction of the chemical vapors constitutes a control or "blank" value.

All data are reported in terms of per cent of those obtained with the reference standards under the same conditions of temperature and relative humidity. At 20 C, triethylene glycol (representative of hygroscopic compounds) is the reference standard for 25 per cent relative humidity, and 2-ethylhexanediol-1,3 (representative of nonhygroscopic compounds) is the reference standard for 80 per cent relative humidity.

Results

In order to carry out testing of candidate aerial disinfectants, it is necessary to have a uniform and constant bacterial aerosol. The test culture, handled in the manner described, exhibited an average die-away in the aerosol cylinder of 0.030 (standard error of 0.0005 for 24 samples) and 0.008 (standard error of 0.0003 for 12 samples) under the standard conditions of 20 C, 25 and 80 per cent relative humidity, respectively. The numerical constancy of the bacterial aerosol is illustrated in figure 3. The effect of the siphon pump in maintaining this constancy is shown in table 1. The quality of re-

[3] The Nash Engineering Co., South Norwalk, Connecticut.

[4] Pharmagel Corporation.

[5] Dow-Corning Corporation, Midland, Michigan.

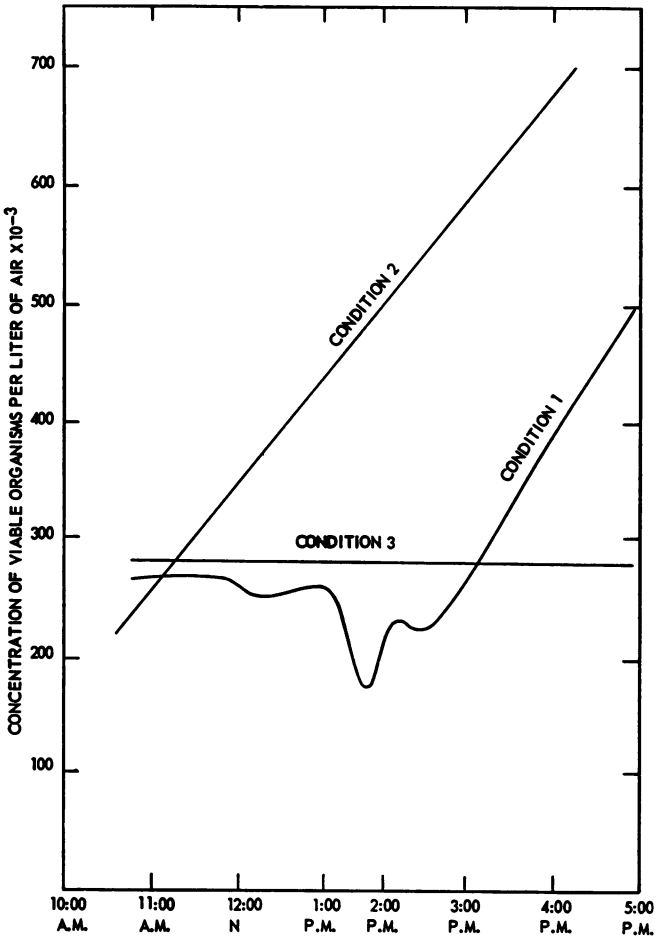

FIG. 3. Variation in the actual numbers of viable airborne organisms issuing from the prechamber during operation at 20 C, 60 per cent relative humidity. Condition 1: Atomizer air supply regulated to ±5 per cent; no siphon pump. Condition 2: Atomizer air supply regulated to ±0.1 per cent; no siphon pump. Condition 3 (conditions used in final tests): As condition 2; siphon pump added. The irregular response when operating under condition 1 was found to be due to fluctuation of large-scale use of compressed air.

producibility over a period of several months is shown in table 2.

The second point of major interest in a dynamic aerosol system concerns the characteristics of the chamber used for the detention of the aerosol, in this case the aerosol cylinder. The dynamic emptying characteristics of this cylinder are shown in figure 4. In this figure, the theoretical line represents the data which would have been obtained if the cylinder had exhibited the characteristics of a sphere. The variation from theoretical of the data obtained with water vapor and with the bacterial aerosol is as might be expected from the differences between the flow patterns through a cylinder and those in a sphere.

The effectiveness of the samplers employed is of great importance in studies such as this. The comparative effectiveness of several samplers is shown in table 3. These studies were carried out in a 4-foot cubical chamber under conditions of dynamic operation.

The results of detailed studies on a variety of different compounds indicated that among compounds having vapor pressures lower than that of water, relative humidity has a profound modifying effect on their ability as aerial disinfectants. It was found that these compounds can be grouped into two classes according to this effect; hygroscopic compounds, the effectiveness of which decreased with increasing relative humidity; and nonhygroscopic compounds, for which the reverse is true. Typical of these two classes are triethylene glycol and 2-ethylhexanediol-1,3. Representative information for the killing powers of these chemicals is shown in figure 5. Although not studied in detail, it has been reported by others (Lester *et al.*, 1949) that the killing power of triethylene glycol decreases markedly at relative humidities below 25 per cent.

The results of a study of a group of hydroxy compounds are shown in table 4. It can be seen that no

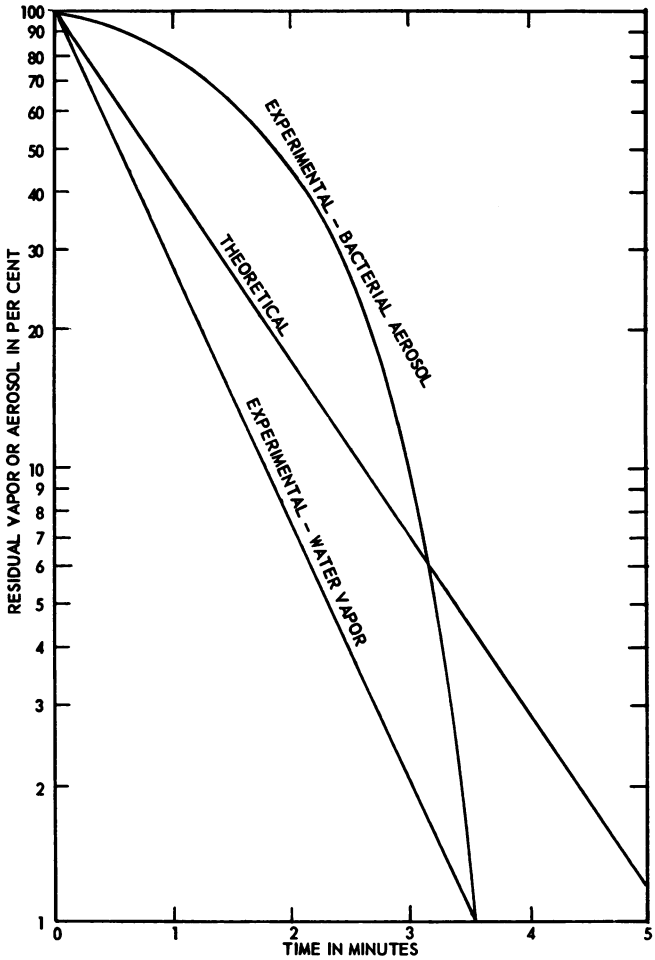

FIG. 4. The dynamic emptying characteristics of the aerosol chamber. The theoretical line is determined from the equation for per cent residual $= 100\left(e^{\frac{-ft}{v}}\right)$ where f is the air flow rate, v is the volume of the cylinder, and t is the time.

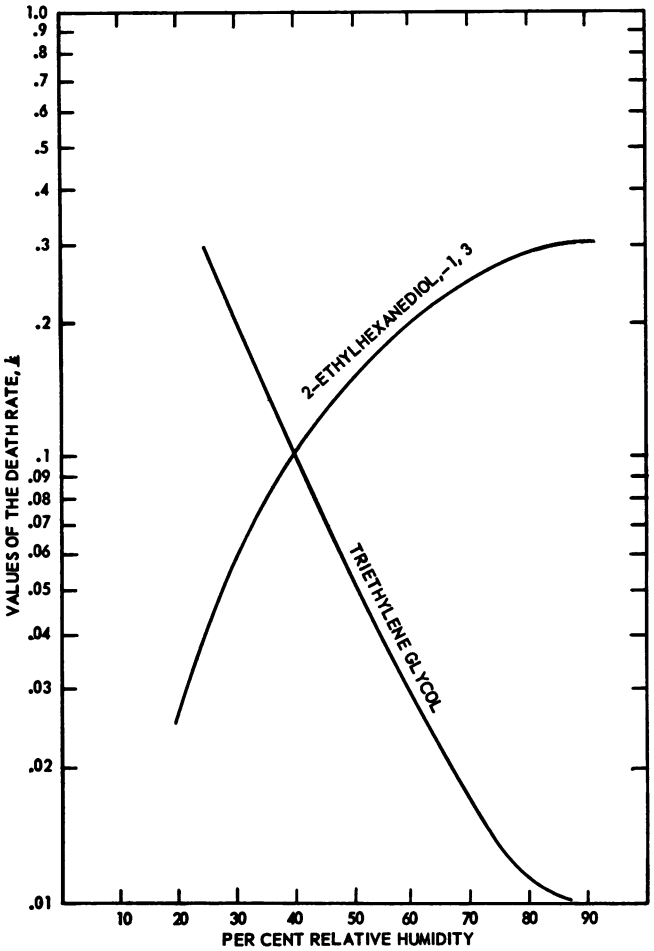

FIG. 5. The comparative effect of relative humidity on the aerial disinfection powers of hygroscopic and nonhygroscopic compounds. Triethylene glycol is representative of the former; 2-ethylhexanediol-1,3 of the latter.

single compound exhibits great effectiveness at both 25 and 80 per cent relative humidity.

DISCUSSION

The methods presented have proved a great aid in screening compounds as aerial disinfectants and in expressing the results in a manner which can be related readily to results obtained by others. However, it should be pointed out that the evaluation of disinfecting powers is not the sole criterion of a successful aerial disinfectant. The questions of economy, toxicity to humans, ease of use, and many others, must be answered first. Here the situation is analogous to the testing of disinfectants by *in vitro* techniques. In both instances, there is little to be gained by judging the ultimate worth of a compound until its disinfectant qualities have been evaluated. It has been through such evaluations that much of our present knowledge of the mechanisms and structure-activity relationships has been gained.

It must be realized that the physical implications inherent in an *in vitro* study and in an aerial study are radically different. *In vitro* studies involve an immediate contact between the bacteria and the environment into which the toxic material is placed. In aerial studies, the bacteria of interest normally are covered with nonliving material of animal origin. This nonliving material composes the immediate environment of the bacteria, and the chemical dissolved in the air is in contact with this immediate environment, but not in direct contact with the bacteria. This is the situation existing for the bacterial particles in the standard bacterial aerosol prepared as described herein. One important result of this difference is the apparently excessive rates of kill obtained for various fractions of aerial saturation

TABLE 1. *Effect of the siphon pump on the numbers of organisms in the atomizer following six hours of operation*

Operating Conditions	Number of Organisms/ml Fluid $\times 10^{-7}$					
	Initial culture		Atomizer at end of run		Culture reservoir at end of run	
	Total*	Viable	Total*	Viable	Total*	Viable
Pump off.....	190	97	348	152	204	100
Pump on.....	200	86	200	83	212	82

* Total counts made by enumerating all cells in a Petroff-Hauser counting chamber.

TABLE 2. *Average numbers of viable airborne organisms collected from the entrance to the aerosol cylinder at 20 C, in the absence of chemical vapors*

Per Cent Relative Humidity	Average Numbers* of Viable Organisms Per Liter $\times 10^{-3}$	Standard Error of Average	Number of Samples
25	810	24.5	43
80	588	19.5	69

* Corrected to a 100 $\times$ 10^7 per ml culture count. The significant difference between the numbers collected at 25 and 80 per cent relative humidity is presumably due to the initial survival of larger numbers when the rate of drying is very great.

TABLE 3. *Comparison of various samplers at 20 C and 60 per cent relative humidity.*

Relative Per Cent Recovery of Viable Organisms from Chamber						
1.0 L/min critical orifice liquid impinger	Sieve samplers*		Greenberg Smith†	Midget impinger‡	1.5 cfm nozzle in G-S tube	1.5 cfm nozzle in milk bottle
	Single	Sum of 5 in series				
100	38	52	50	43	35	75

* Operated at 1.0 cfm, with funnel adapters (Wells, 1955)

† Operated at 1.5 cfm. Impinger made by Corning Glass Works.

‡ Operated at 0.3 cfm. Impinger made by Corning Glass Works.

Plain gelatin fluid in all liquid samplers; nutrient agar in solid surface samplers. Averages of three determinations each in 4-foot cubical chamber.

TABLE 4. *Physical properties and killing powers of compounds tested as aerial disinfectants at 20 C, 25 and 80 per cent relative humidity (RH)*

Chemical Compound	Aerial Saturation	Solubility in Water*	Death-Rate k at Aerial Saturation		Relative Effectiveness†	
			25 per cent RH	80 per cent RH	25 per cent RH	80 per cent RH
	mg/ft³ of dry air	*g/100 g*				
2-Ethylhexanediol-1,3	2.5	4.0	0.01	0.30	3	100
Triethylene glycol	0.25	M-H	0.30	0.01	100	3
Propylene glycol	9.5	M-H	0.25	0.01	83	3
Diethylene glycol-monobutyl ether	5.5	M-N	0.2	0.7	67	230
Propanediol-1,3	6.5	M-H	0.2	0.05	67	17
Hexanediol-2,5	3.5	M-N	0.15	0.4	50	134
2-Ethylhexanol	10	0.1	0.02	0.1	77	33
Ethylene glycol-monobutyl ether	140	M-N	0.1	0.3	33	100
Hexanedione-2,5	76	M-N	0.05	0.7	17	230
Octanol-1	16	0.05	0.01	0.2	3	67
Ethylene glycol	6	M-H	0.1	0.05	33	17
Lactic acid	0.55	M-H	ca 1	0.2	230	67
Dimethyl phthalate	1.3	0.5	0.01	0.06	3	20
Diethyl phthalate	0.9	—	0.01	0.04	3	13
Dibutyl phthalate	0.2	0.04	0.01	0.04	3	13
Diethylene glycol-monoethyl ether	37	M-N	—	0.8	—	270
Thiodiglycol	1.9	M-N	0.05	0.1	17	33

* The symbols used in this column are as follows: M indicates miscible with water in all proportions; H indicates hygroscopic; N indicates nonhygroscopic.

† Relative effectiveness is expressed as per cent of activity of reference compound; triethylene glycol being the reference compound at 25 per cent relative humidity, and 2-ethylhexanediol-1,3 at 80 per cent.

compared to conjectured equivalent *in vitro* concentrations (Wells, 1955). Our own experiences have confirmed this, but we find a regular pattern of response under these conditions. When completed, these findings will be offered for publication. The existence of a regular pattern of response to various fractions of aerial saturation constitutes a valid argument for the comparison of the relative effectiveness of aerial disinfectants at saturation concentration. It is suggested that the relative activity of a compound compared to that of a standard will be essentially the same, whether they are compared at saturation concentration or at some identical fraction of saturation. Saturation concentrations are preferable because they can be obtained with a minimum of equipment and effort.

The existence of a primary environment around the airborne bacteria is undoubtedly responsible for the difference in effectiveness of hygroscopic and nonhygroscopic compounds at various relative humidities. In the test procedure described herein, the typical airborne bacterial particle is composed of a single bacterium surrounded by beef-extract solids. These solids will hold a great deal more water at higher humidities than at lower humidities, and the net solubility in this material, for compounds having a vapor pressure less than that of water, will increase as the relative humidity increases. For nonhygroscopic compounds, the net result of increasing relative humidity is to increase the concentration of the compound in the airborne particle, with a resultant increase in killing power. On the other hand, the total water associated with a hygroscopic compound is determined by the humectant characteristics of the compound, that is, the greater the humidity, the greater the amount of water. The amount of hygroscopic compound in the airborne bacterial particle may increase as the relative humidity is increased, but the net concentration decreases, resulting in a decreased killing power. It is this difference in response to relative humidity that necessitates the use of a dual standard of reference in the evaluation of aerial disinfectants.

The two compounds selected as the standards in this procedure are easily obtainable as pure chemicals of commerce. Triethylene glycol is recognized as an aerial disinfectant, and especially pure grades are available for this purpose. The compound 2-ethylhexanediol-1,3 is used as an insect repellent, and pure grades are sold for this purpose. A point of more than passing interest is the fact that several classes of successful insect repellents have many desirable characteristics in common with successful aerial disinfectants. A recent insect repellent, 2-hydroxypropylcyclohexanol, has been reported to have aerial disinfectant properties equal to those of triethylene glycol (Grun, 1955).

Although it is to be hoped that the compounds selected for standards in the evaluation of aerial disinfectants will aid in the selection of other compounds more effective than the standards themselves, this does not detract from their usefulness as standards. The same argument applies to the test organism selected for these studies. *S. marcescens* strain ATCC 274 may not be the ideal test organism, but so much is known about the behavior of this strain that the use of a less well-known strain would necessitate a great deal of work. In any case, the test organism should be monitored at regular intervals under standard conditions in order to insure uniformity of response. The combination of standards of reference and uniformity of response of a standard test organism is required for the successful evaluation of aerial disinfectants on a uniform basis.

The procedures and techniques presented for evaluating aerial disinfectants were developed as the result of some two years of thorough study, and have been tested carefully over a period of another two years. The system as presented has been found useful and valuable. The equipment required for these procedures is relatively inexpensive; even the smallest shop is equipped to produce the nonstandard items required. The great-

est expense that might be encountered is represented by the large vacuum pump required to operate the system across a critical orifice at 28.3 L per min. A smaller pump would be suitable if properly ballasted to maintain a constant rate of flow through the system.

ACKNOWLEDGMENT

The guidance of J. M. DallaValle is gratefully acknowledged; without his original plans from which this work stemmed, none of these studies could have been carried out.

SUMMARY

A method is presented for the evaluation of aerial disinfectants. This method, properly applied, is capable of yielding reproducible results expressed in a manner which can be applied universally. The equipment required is relatively inexpensive and is fabricated from generally available materials.

The modifying effect of relative humidity on the activity of aerial disinfectants is taken into consideration in this system, and compounds are offered as standards of reference in light of this effect.

Methods are given for the production of a standard bacterial aerosol, as are the details of equipment for diluting this aerosol, mixing it with chemical vapors, and sampling the resultant mixture.

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A Broadened Concept of the Characteristics of *Streptomyces hygroscopicus*

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It is becoming increasingly apparent that marked variability abounds in a great many of the species of *Streptomyces* and, as a result of the indiscriminate granting of species status to numerous variants of already defined species, a needlessly complex taxonomic system has arisen. Burkholder and Sun (1954) have discussed criteria for speciation in *Streptomyces* and have stressed the need for a system of convenience in which a relatively few named species groups would be established. Hesseltine *et al.* (1954) emphasized the need for uniformity in methods of study and of reporting data relative to taxonomic studies, while Jones (1954) pointed out the need for a better understanding of the organisms themselves before progress can be made in comprehending variability in *Streptomyces*. Backus *et al.* (1954), in their study of variability in *S. aureofaciens*, and Duggar *et al.* (1954), studying the same organism as well as other species of *Streptomyces*, have made clear the necessity of examining a large assemblage of related forms as a prerequisite to establishing species boundaries. Our continuing interest in the fundamental problem of speciation in the genus *Streptomyces* prompted us to undertake the present study of variability in *S. hygroscopicus* (Jensen) (Waksman and Henrici) in an attempt to delimit more clearly the species boundaries of this organism.

While it is recognized that a majority of the cultures involved in this study are capable, under specific conditions, of elaborating one or more products which possess substantial and varied antimicrobial activity, it is beyond the province of this paper to discuss such products or activities. Furthermore, it is felt that neither the capacity of a culture to produce such products nor the nature of such products themselves