ARCTIC AEROMEDICAL Laboratory

THE EFFECT OF LOW TEMPERATURES ON THE SURVIVAL OF AIRBORNE BACTERIA

PROJECT NUMBER 8-7958

LADD AIR FORCE BASE

A L A S K A

THE EFFECT OF LOW TEMPERATURES ON THE SURVIVAL OF AIRBORNE BACTERIA

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I. SUMMARY

Results are reported for studies on various airborne bacteria under varying conditions of relative humidity at low temperatures. A complete description is given of the special aerosol cylinders devised for carrying out these studies. Employing airborne bacterial particles dispersed from beef broth cultures, extensive studies are reported for Serratia marcescens (ATCC 274, primary form), and Escherichia coli (ATCC 10536) for the range of temperatures 80°F to -40°F. Preliminary studies are reported for Serratia indica (ATCC 4003), Micrococcus pyogenes var. aureus (ATCC 6538) and Micrococcus pyogenes var. albus (ATCC 9491). It is concluded that the experimental bacterial particles studied are very similar to those arising from natural causes and that the findings are generally applicable to many naturally occurring airborne bacteria. The very low death rate observed for the experimental bacterial aerosols at low temperatures is consistent with previous reports of the presence of viable bacteria in the upper atmospheres.

It is further concluded that although the death rate of airborne bacteria is very small at low temperatures, the factors which determine the fate of these organisms are similar to those operating at higher temperatures. In general, it is suggested that the composition of the non-living material associated with the airborne bacterial particle is the most important single factor in determining the fate of such organisms.

II. INTRODUCTION

This report covers investigations carried out at the Georgia Institute of Technology during the period March, 1954 through May, 1956 under the terms of the

subject contract. The primary objective of these studies was to determine in the laboratory the effect of low temperatures on the viability of certain selected bacteria when the bacteria are suspended in the air. It was planned to study the following variables: (a) temperature, (b) relative humidity, (c) bacterial species and (d) suspending medium from which the bacteria are dispersed. The first three variables have been investigated; limitations of funds prevented completion of the study of the fourth.

During the period covered by the subject contract, eight Quarterly Reports have been prepared and submitted to the Arctic Aeromedical Laboratory. The current report is the Final Report under the terms of the contract and presents an orderly condensation of the investigations accomplished and a synthesis and interpretation of the various results obtained.

III. HISTORICAL BACKGROUND

Although living microorganisms have been shown to be present in the air under conditions of low temperatures, little is known about the history or condition of existence of such organisms. Pady and Kelly (12) recovered viable bacteria from polar air; in summarizing the results of studies on microorganisms of the upper atmosphere, Mitchell, Timmons and Dorris (10) indicated that living organisms are almost universally present in the upper atmosphere.

The presence of spores in the upper atmosphere under conditions of low temperature is not unexpected. However, vegetative forms are not usually considered hardy enough to survive for long in the aerial state, especially under such adverse conditions as exist in the upper atmosphere. The problems encountered in the successful freeze-drying of viable vegetative forms (11) suggests that special conditions are required to explain the presence of living bacteria in the upper atmosphere. However, the very fact that viable bacteria are found at high altitudes indicates that the

life expectancy of these organisms must be very great. Realizing this, and considering the probability that airborne organisms can successfully initiate diseases (16), the potential significance of the problem is increased.

The difficulties accompanying studies on airborne bacteria under natural conditions of low temperature, and the inherent limitations of such studies, prompted the consideration of laboratory studies directed toward an understanding of the conditions which permit the survival of bacteria in the air at low temperatures. It was felt that the results of these studies would not only aid in understanding the probable fate of airborne microorganisms under natural conditions, but would also contribute to a better understanding of the mechanisms of response of microorganisms to physical insults.

The results of prior work (1) on the fate of airborne bacteria at ordinary temperatures had shown that laboratory investigations on bacterial aerosols require special facilities, and that extreme care is necessary if accurate and reproducible results are to be obtained. This experience was invaluable especially since it was found that even at low temperatures relative humidity can be a significant factor in determining the fate of airborne bacteria. The design of equipment for the study of bacterial aerosols, in which relative humidity can be controlled at low temperatures, represents a major item in the planning and execution of studies on the effect of low temperatures on the survival of airborne bacteria.

IV. MATERIALS AND METHODS

A. Equipment

Although much of the equipment used in these researches was developed prior to their initiation and is described in detail elsewhere (1), such description as is necessary to the understanding of the operation is included in this report. In addition, the system for the study of bacterial aerosols at low temperatures developed in the course of these investigations is described in detail.

1. Aerosol cylinder system

The system developed for the study of bacterial aerosols at low temperatures consists of a pair of low pressure oxygen cylinders, which are referred to as aerosol cylinders. These are made of stainless steel, 12 inches in diameter, have a straight cylindrical midsection 12 inches in length and are 24 inches in overall length, the end sections being dome-shaped. The internal volume of each cylinder is approximately 35 liters. Air connections between each cylinder are made of 5/8-inch diameter smooth copper tubing having no abrupt bends or turns.

The pair of cylinders is housed in an insulated box in which the temperature can be controlled to as low as -100°F by the use of dry ice. A general view of the cylinders and auxiliary items is shown in Figure 1. On the left in this figure the tempering box (for adjusting the air used in the cylinders to the desired dewpoint) can be seen; in the center of the figure is the cylinder box (with the lid opened); to the right is the prechamber box (housing the equipment for the production of the standard bacterial aerosol). A close-up of the cylinders is shown in Figure 2.

The operation of the aerosol cylinder system is indicated in the schematic drawing in Figure 3. At the upper left of Figure 3 is shown the vacuum connection at the exit of the system, where a 1.0-cfm critical orifice is used to maintain a uniform flow rate through the system. The air entrance is shown just beneath the exit. The entering air is forced out of the tempering box by a blower, through the coils inside the insulated box housing the cylinders (to insure temperature equilibration), and then joins the aerosol from the prechamber (entering from the upper right of Figure 3). The aerosol from the prechamber is forced into the system by compressed air, passing through a loop of Tygon tubing† before joining the tempered air. The two

†A thick-walled plastic tubing. When the wet aerosol cloud was originally introduced into the cold conditions in the cylinder box a snow formed in the air which resulted in the clogging of the system. Using the plastic tubing, the excess moisture in the aerosol cloud forms heavy particles of ice on the surface of the plastic instead of in the air. Thus the air passing through the system remains free of snow.

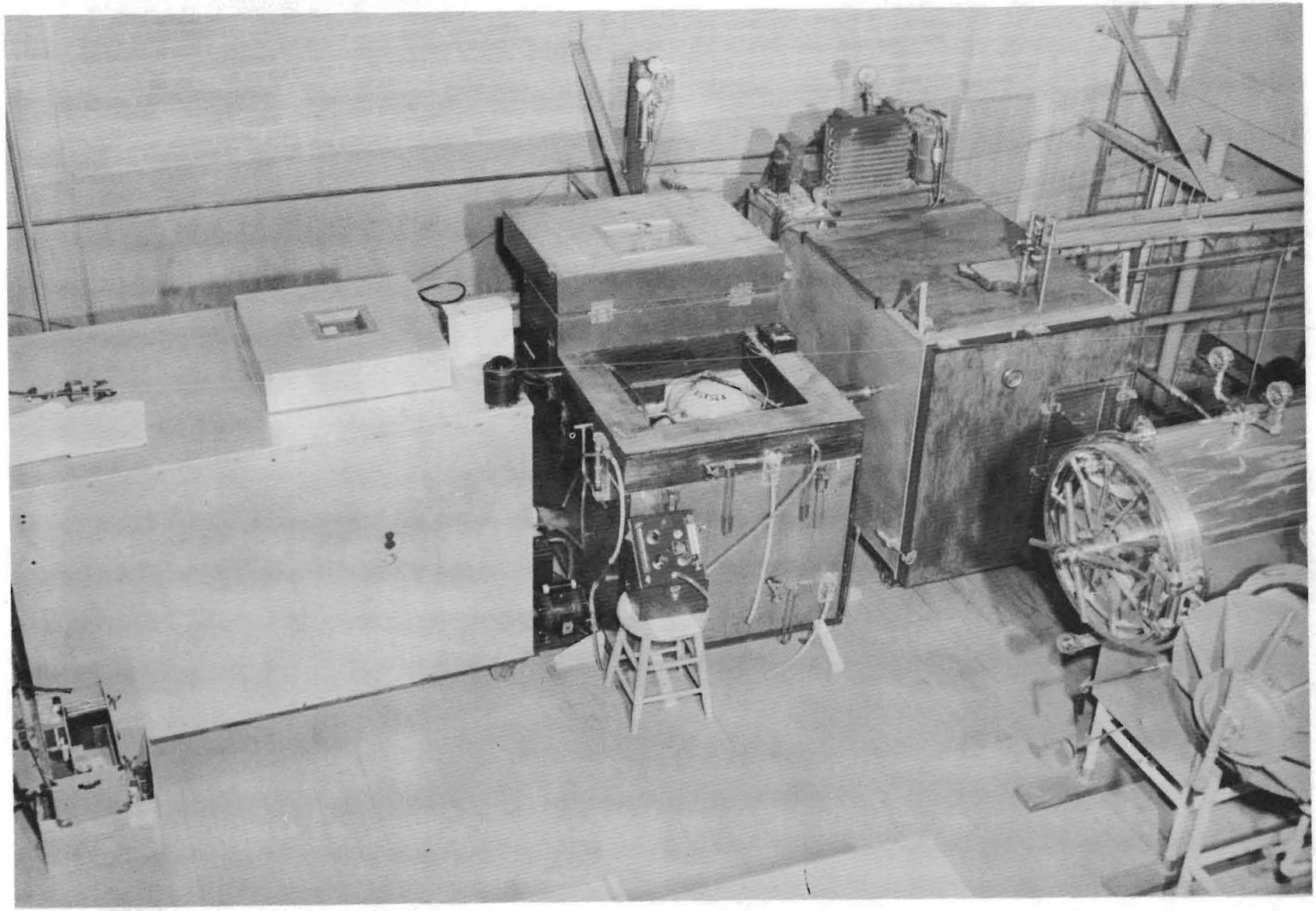

Figure 1. General view of cylinder system.

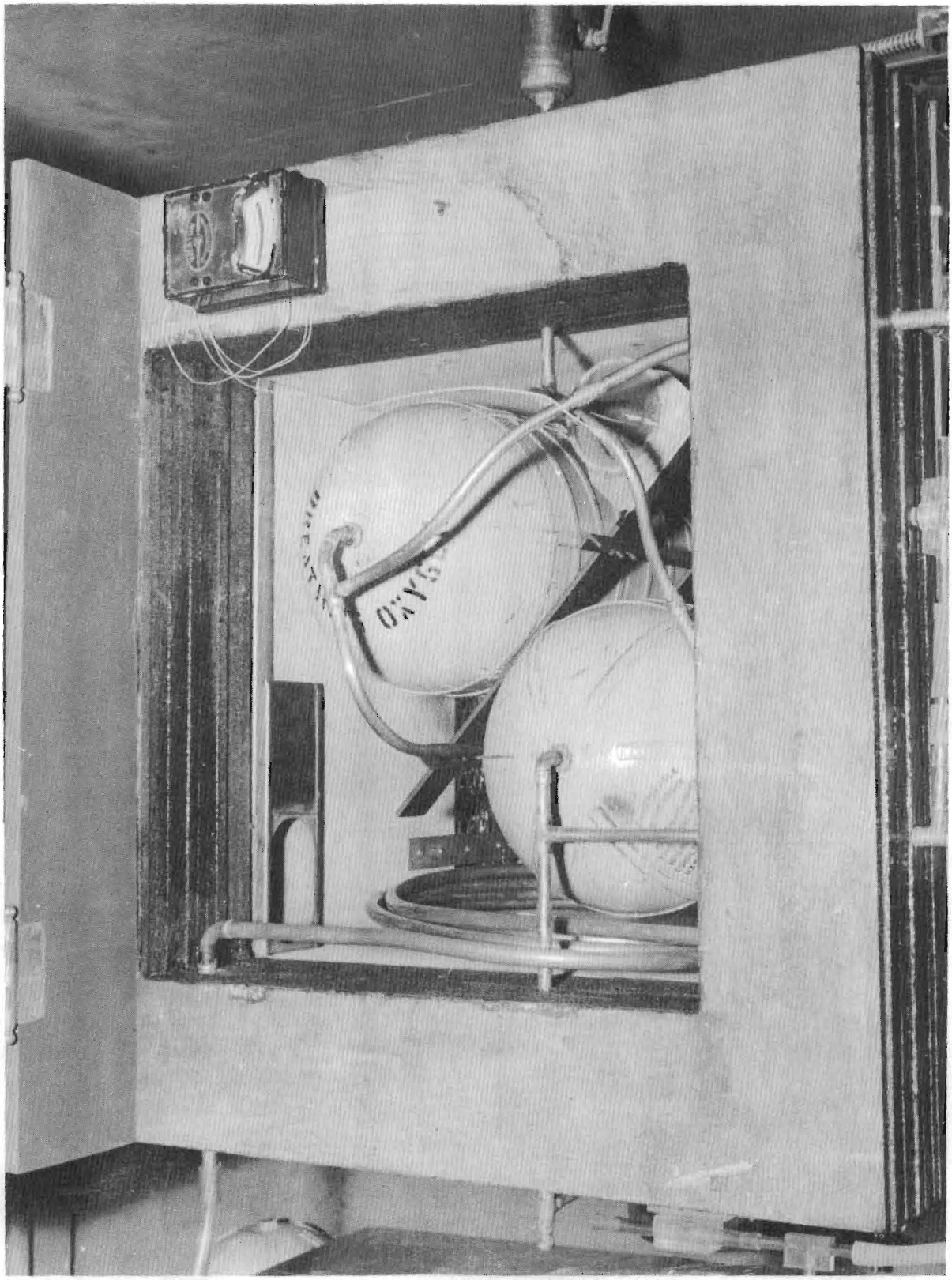

Figure 2. Close-up of cylinders in low-temperature box.

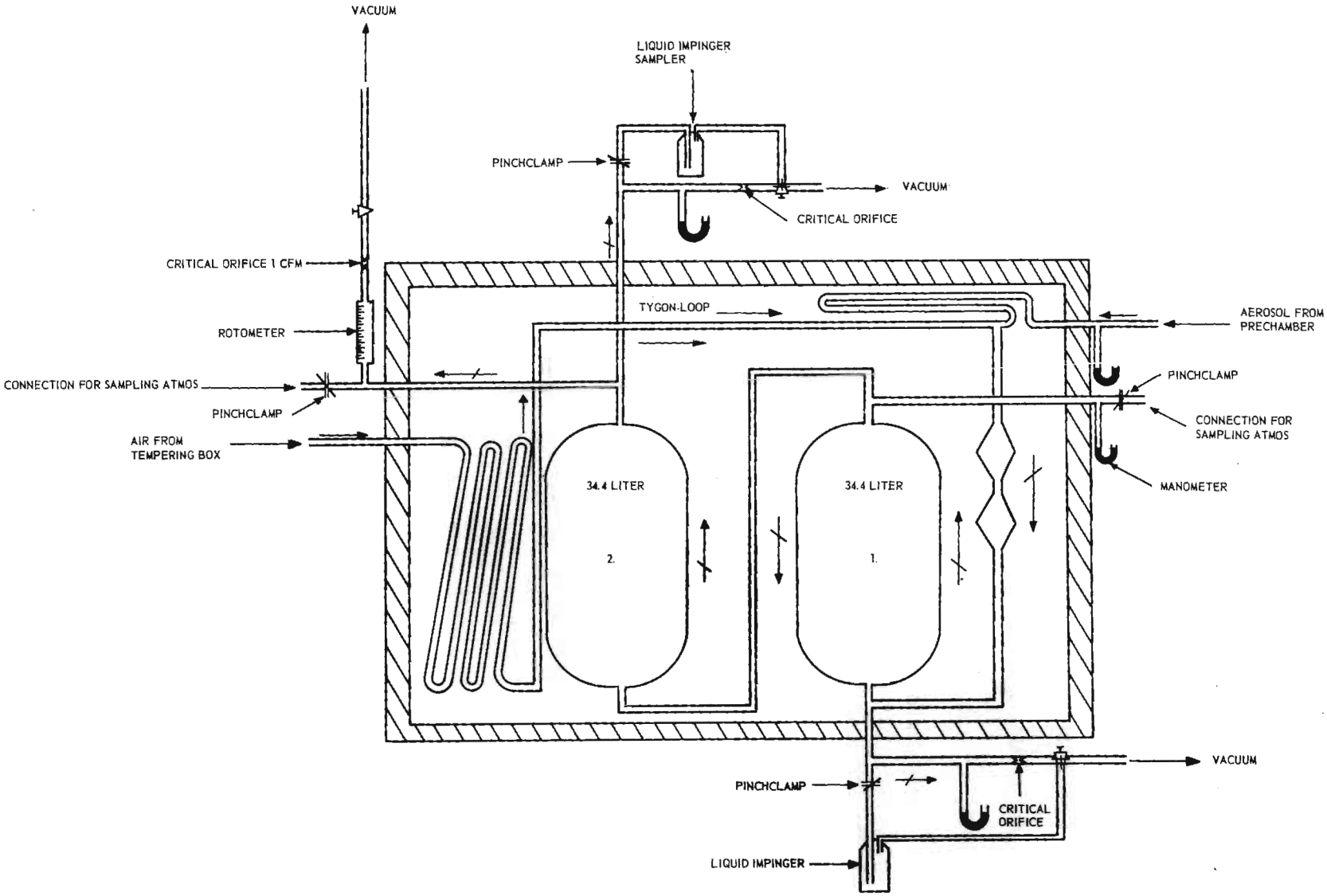

Figure 5. Schematic drawing of the aerosol cylinders.

PROJECT NUMBER 8-7958

streams flow together and are mixed in the double-cone mixer shown on the right of Figure 3, just inside the insulated box. This mixer was developed for this experiment and consists of two pairs of six-inch, 60° metal funnels, soldered mouth-to-mouth (the top of the mixer can be seen in Figure 2). Between each pair a restriction is located so that a contraction-expansion system results, mixing the tempered air and the aerosol from the prechamber in a uniform manner. The uniformly diluted aerosol then passes through the two cylinders and out of the system into the vacuum line.

The total air-flow at the exit of the system is exactly 1.0 cfm.† The air carrying the aerosol from the prechamber is carefully regulated to exactly 6.4 L/min. The remainder of the air flowing through the system is supplied from the tempering box blower; careful regulation of this blower maintains the pressure within the aerosol cylinders at zero relative to the outside conditions (as indicated on the manometer at the first cylinder). This method of balancing a vacuum-pressure airflow is necessary because of the pressure drops encountered in the tubing and in the cone mixer, it being desirable to operate the cylinders at atmospheric pressure. It can be seen that this same system can readily be applied to the study of bacterial aerosols at reduced pressures such as exist in the upper atmosphere.

The insulated box in which the cylinders are housed is an Aminco Low Temperature Cabinet (American Instrument Company). Employing dry ice as a refrigerant, the temperature within this box can be controlled within ± 1°F. In order to achieve this degree of uniformity within all points of the cylinder system, blowers (not shown in Figure 3) are located near the cylinders in the box. A 16-point thermocouple recorder with sensing elements located on the various items of the system (including inside the exit) is used to assure temperature uniformity prior to operation of the system.

†One cubic foot of air equals approximately 28.31 liters.

In Figure 3 a total of four sampling positions are indicated, all being located outside the low-temperature box. The sampling position at the upper left of Figure 3 is for the taking of air samples for determining the dewpoint of the air flowing through the cylinder system. The one at the lower right of the figure is for sampling the diluted bacterial aerosol prior to entry into the first cylinder (inlet sample); at the upper right (indicated on diagram as connection for sampling atmosphere) is the position for sampling at the exit of the first cylinder, which is also the entrance to the second cylinder (mid-point sample); at the upper center is the position for sampling at the exit of the second cylinder (outlet sample). Two sampling positions are shown in detail in Figure 3, and consist of a vacuum connection, a 1.0 L/min. critical-orifice, and a 1.0 L/min. critical-orifice liquid impinger. This arrangement is necessary to maintain uniformity of operation. When not sampling exactly 1.0 L/min. is removed from the system across the critical orifice; during sampling the same amount is removed by switching over to the sampler.

2. Air samplers

The aerosol system described in this section is so designed that the linear velocity of the air in all parts of the system is always great enough to maintain airborne all of the bacterial particles introduced. For this reason, aerial samples only are taken. The air samplers used in this work are critical-orifice, liquid impingers similar to those used by Rosebury (13) and are illustrated in Figure 4. On the left of this figure can be seen the assembled sampler without any fluid; the critical-orifice† can be seen at the end of the longer glass tube. In

† A critical-orifice is one through which a definitely limited amount of gas can pass per unit of time because the rate of gas passage either is or approaches sonic velocity (a limiting velocity). This condition can be achieved when the ratio between the downstream and upstream pressures is less than the "critical pressure ratio," which is about 0.5 for air at room temperatures. Thus, with a vacuum pump of adequate capacity to operate at less than 0.5 an atmosphere across an orifice, the flow through that orifice will always be the same. By making the orifice of the proper size various flows can be achieved. In this work either 1.0 L/min. or 5.0 L/min. orifices are prepared by shrinking the end of the glass tubing to form the proper sized orifice.

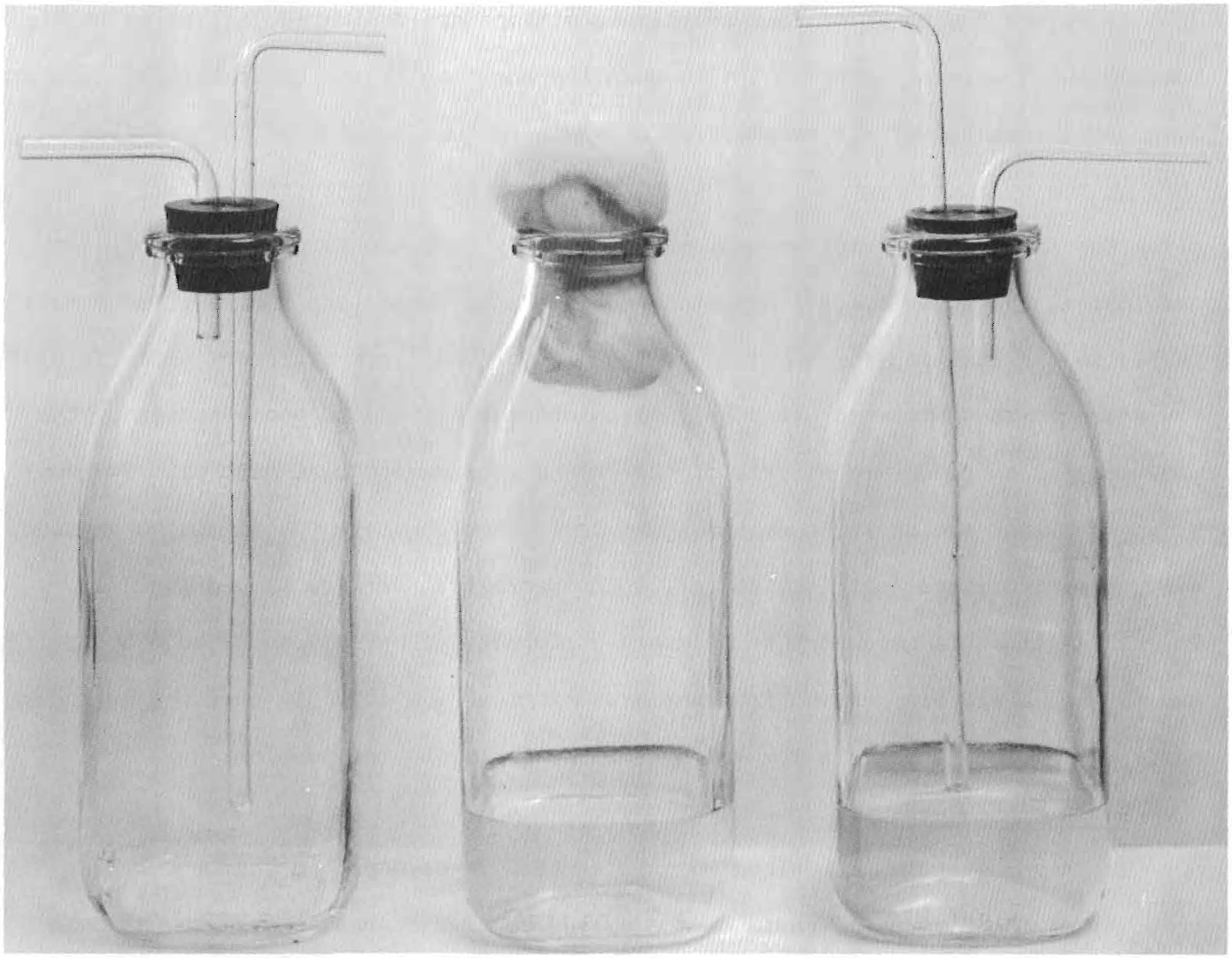

Figure 4. Critical-orifice liquid impingers.

the center of Figure 4 is the sample bottle (one-quart milk bottle) loaded with 200 ml of fluid ready for sterilization; on the right is the assembled sampler ready for use. The orifice tube, stopper, and vacuum connection are sterilized separately and inserted into the bottle just before use. When sampling, the shorter glass tube is connected to a vacuum line, and the tube with the orifice is connected to the proper sampling position. The air sample is thus drawn at sonic velocity into the liquid and the particulate matter is entrapped in the liquid, the air passing on through. Knowing the rate at which the sampler operates, the time of sampling, and the volume

of the sampling fluid, the results of plated aliquots of the sampling fluid can be converted into aerial concentration of viable bacteria per liter of air.

In this work two different fluids were used in the air samplers; either plain gelatin fluid or enriched fluid, generally referred to as brain-heart fluid. The latter was used in almost all of the work because with it almost twice as many viable organisms can be shown as with the plain gelatin fluid. The composition of the brain-heart fluid is as follows: brain-heart infusion (Difco Laboratories), 16 grams; gelatin (Pharmagel A, Pharmagel Corporation) 2 grams; dibasic sodium phosphate, anhydrous, 0.16 grams; distilled, deionized water to make one liter. The plain gelatin fluid is prepared in the same manner, deleting the brain-heart infusion. In each case, after adding one loopfull of AF antifoaming emulsion (Dow-Corning Company) to each 200 ml of fluid in the milk bottles, the bottles are plugged and sterilized.

The effectiveness of the critical-orifice liquid impinger with brain-heart fluid has been demonstrated in other work (1). However, the results of preliminary studies on the effect of dispersion media on airborne bacteria serve to show this effectiveness. These results can be seen in Tables I and II for studies in the aerosol chamber at 45 and 80 per cent relative humidity, respectively.

3. Auxiliary equipment for the aerosol cylinders

A schematic diagram of the tempering box and the prechamber box is shown in Figure 5. As previously stated, the function of the tempering box is to adjust the dewpoint of the air used in the cylinders. The relative humidity of the air in the cylinders is determined by the dewpoint of the entering air and the dewpoint of the air carrying the aerosol from the prechamber. Since the latter is always 40°F, the dewpoint of the tempered air must be correspondingly lower than that required for the desired relative humidity in the cylinders. For dewpoints down to -24°F this is accomplished by adjusting the suction pressure of the refrigerating compressor of the tempering box so that the cold plates of that box are at the temperature of the

required dewpoint. For lower dewpoints partially dried compressed air is forced through activated silica gel.

The exact dewpoint of the tempered air, and of the air within the aerosol cylinders is determined with the Alnor Dewpointer (Illinois Testing Laboratories). This instrument can be seen in the center foreground of Figure 1. The Dewpointer is one of the few instruments available for the accurate determination of very low dewpoints. The fact that the instrument must be manually operated is a considerable drawback because an operator must be continually on duty to monitor the dewpoint of the air during experimental studies.

TABLE I

Analysis of variation of critical-orifice impinger samplers used with the aerosol chamber at an atmosphere of 68°F and 45 per cent relative humidity†

Sampling interval minutes following start of run	Concentration of Organisms per liter of air: Sampling probe No. 2	Sampling probe No. 4	Sampling probe No. 8	Average	S.D.	S. E.
1.0 ml sampling fluid plated (counts 250-330)						
60-80	2800	2900	2700	2800	100	58
90-120	3300	3050	3050	3130	170	98
130-150	2950	2700	2800	2820	125	73
Average	3020	2880	2850	2920	195	65
0.10 ml sampling fluid plated (counts 26-43)						
60-80	3300	3300	2700	3100	346	115
90-120	3500	3650	3100	3420	282	94
130-150	3100	3450	3350	3300	180	60
Average	3300	3420	3050	3270	275	92

†Culture: S. marcescens, E-R/0-3/56 (ATCC 274) atomized as washed cells in an aqueous suspension of glycerol (0.375 per cent). Run made in A.M.

The prechamber box, as shown in Figure 5, is an insulated container operated at 40°F. Inside are the atomizer, reservoir and prechamber for the production of the standard bacterial aerosol. This portion of the system is operated continuously during experimental studies. A steady stream of airborne bacteria issues from the prechamber at exactly 6.4 L/min. At least 90 per cent of these particles contain only single bacteria, and none contain more than two, the larger particles having settled out in the prechamber, which is essentially a sedimentation classifier.

The contents of the prechamber box are shown in Figure 6, where can be seen the atomizer, reservoir and plastic prechamber. The oxygen cylinder beneath the pre-

TABLE II

Analysis of variation of critical-orifice impinger samplers used with the aerosol chamber at an atmosphere of 68°F and 80 per cent relative humidity†

Sampling interval minutes following start of run	Concentration of organisms per liter of air: Sampling probe No. 2	Sampling probe No. 4	Sampling probe No. 8	Average	S.D.	S.E.
1.0 ml sampling fluid plated (counts 410-650)						
60-80	5200	5000	4600	4930	300	176
90-120	5000	4900	5800	5230	490	280
130-150	5550	5550	5250	5450	173	100
Average	5250	5150	5220	5200	385	128
0.10 ml sampling fluid plated (counts 42-64)						
60-80	5900	5000	4750	5220	590	340
90-120	5200	5000	5800	5330	410	238
130-150	5300	5900	5700	5630	300	176
Average	5470	5300	5420	5400	440	147

†Culture: S. marcescens, E-R/0-3/56 (ATCC 274) atomized as washed cells in an aqueous suspension of glycerol (0.375 per cent). Run made in P.M.

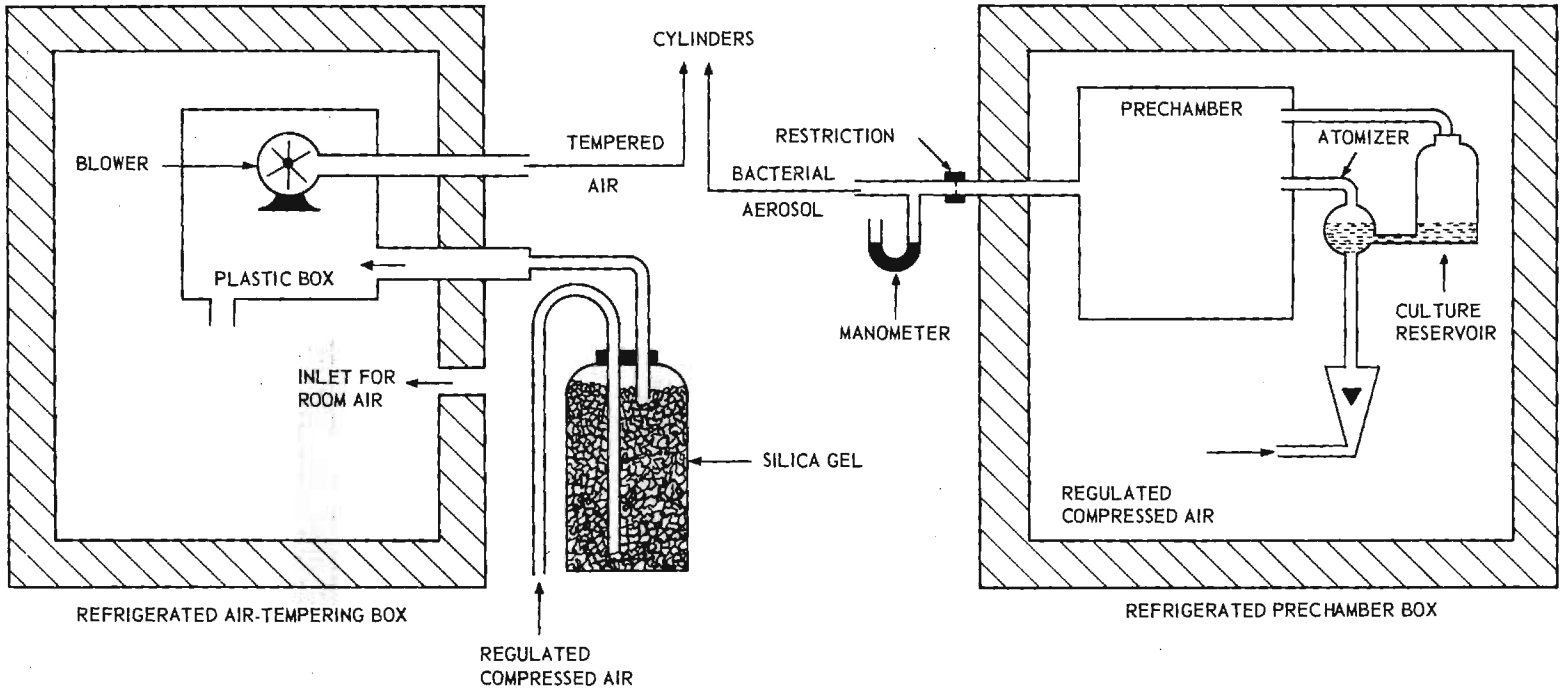

Figure 5. Schematic drawing of the air tempering box and the prechamber box.

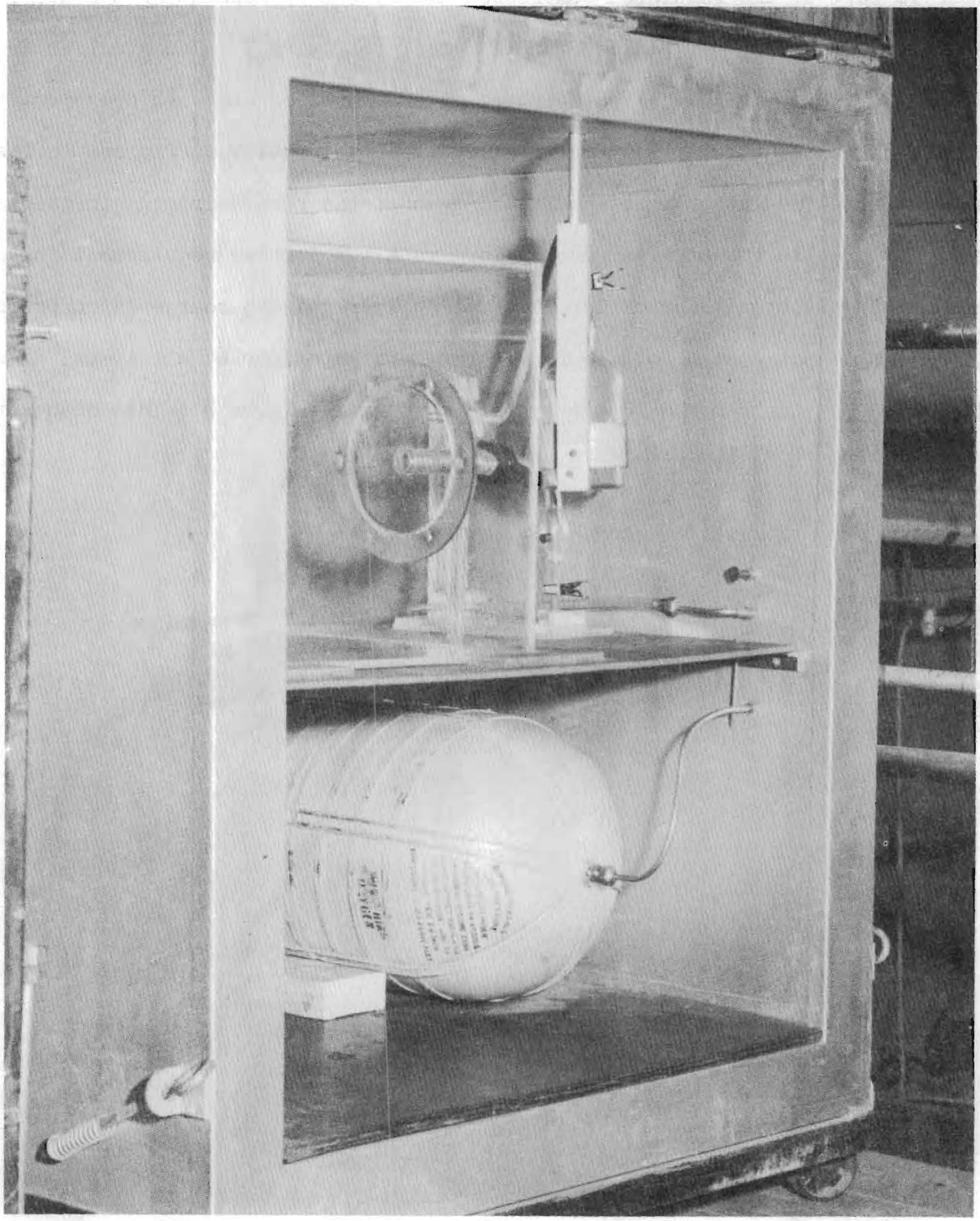

Figure 6. Prechamber box with lid open.

chamber is part of the regulated compressed air system for operating the atomizer. The atomizer and reservoir can be seen in detail in Figure 7. The atomizer is an all-glass DeVilbiss No. 40, which operates with compressed air. In operation the reservoir holds about 150 ml of bacterial culture, a quantity sufficient to last an entire day. The larger cross-sectional area of the reservoir aids in maintaining a constant level in the atomizer during long runs. A further requirement for constancy of operation of the atomizer is a carefully regulated source of compressed air, enabling exact control of the flow through the atomizer at all times. A schematic drawing of the compressed air system is shown in Figure 8. This elaborate

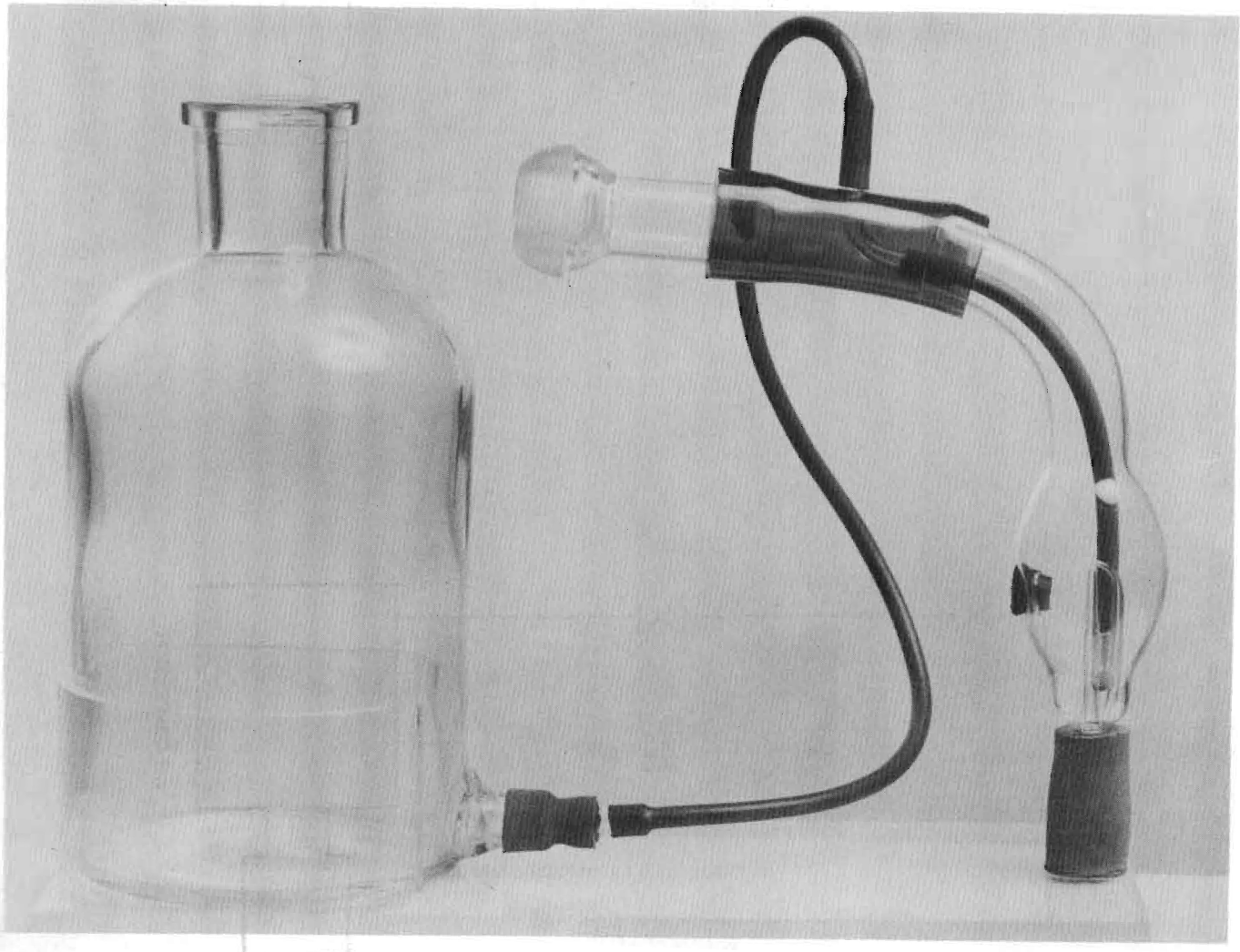

Figure 7. Close-up of reservoir and atomizer.

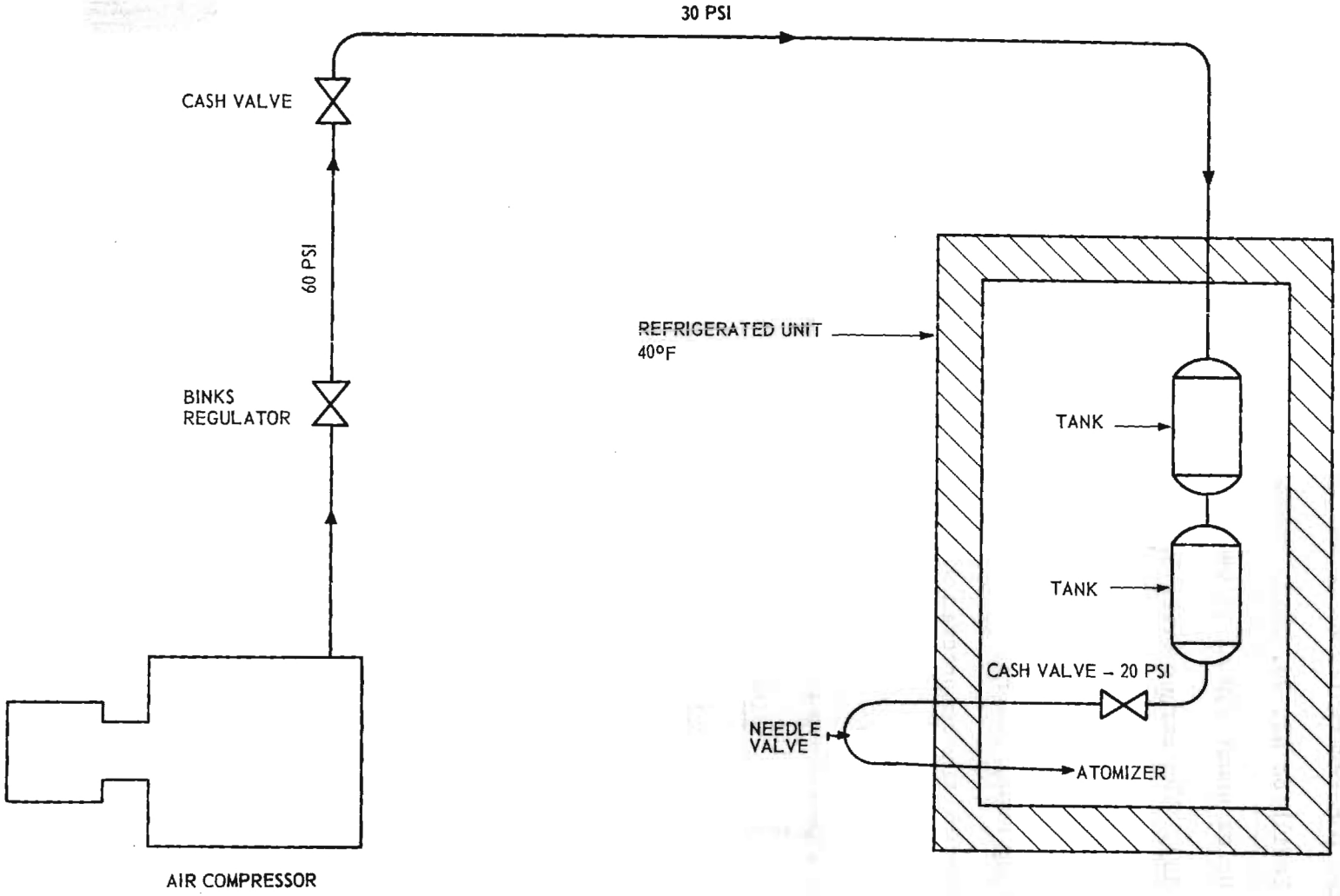

Figure 8. Schematic drawing of the regulated compressed air system.

system is required for the maintenance of a uniform and constant output of the bacterial aerosol.

4. Uniformity and reproducibility of operation of the cylinder system

Uniformity and reproducibility are essential characteristics of any experimental system. In order to determine the degree of uniformity of results obtainable, the aerosol cylinder system was operated continuously during an entire day under the same conditions. Samples were taken intermittently for four hours during periods lasting either 5 or 15 minutes. The results of this series of tests **are**

TABLE III

Results obtained from multiple samples from the aerosol cylinders at 16°F and 55 per cent relative humidity

Run number	Inlet concentration viable bacteria per liter		Outlet concentration viable bacteria per liter	
	5-minute samples	15-minute samples	5-minute samples	15-minute samples
1	65,500	65,000	63,300	60,000
2	83,000	73,000	76,000	74,000
3	78,500	68,500	71,500	53,500
4	88,000	75,000	72,000	62,500
5	79,000	66,500	74,000	65,500
Average ± S.D.†	78,200 ± 8400	69,600 ± 4100	71,300 ± 4800	63,100 ± 7800
Coefficient of variation†† (per cent)	10.7	5.9	6.8	12.3

†Standard deviation:

$$S.D. = \sqrt{\frac{\Sigma \left[\left(\frac{\Sigma x}{n}\right) - x\right]^2}{n - 1}}$$

Where x is the individual result, and n is the number of results. The term $\left(\frac{\Sigma x}{n}\right)$ is the average.

††Coefficient of variation: $C.V. = \frac{S.D.}{\left(\frac{\Sigma x}{n}\right)} \times 100.$

shown in Table III. The analysis of the data shown in this table indicates that, for a system employing a biological agent, the uniformity of operation is quite acceptable. It should be pointed out that the variations indicated in Table III include all variations due to sampling errors, plating, counting, and diluting, in addition to whatever non-uniformity actually results from the operation of the system itself.

The reproducibility of operation from day-to-day is illustrated by the data in Table IV, where are shown the results of three different runs. In each case the numbers shown at the inlet to the cylinders have been compared to the numbers actually issuing from the prechamber. The variation between runs, and that between the inlet concentration and the prechamber concentration appears to be negligible. The agreement between prechamber concentration and inlet concentration indicates that there is no significant loss of airborne bacteria in this portion of the system. The fact that in later studies, under conditions where no appreciable death rate was operable, the numbers of viables at the outlet were found to be identical with the numbers at the inlet to the cylinders indicates that no significant loss of airborne particles occurs within the system itself. This ability to account for all of the

TABLE IV

Comparison of inlet and prechamber concentrations of the aerosol system†

Run number	Inlet concentration		Prechamber concentration
	Viable bacteria per liter	Multiplied by air dilution factor	Viable bacteria per liter
1	96,500	540,000	473,000
2	86,500	474,000	570,000
3	95,500	535,000	475,000
Average	92,800	516,000	506,000

†For these tests the aerosol cylinders were at 22°F and 50 per cent relative humidity, the prechamber being at 40°F for all tests.

organisms which enter the system is valuable proof of the efficiency of operation and accounts for the high degree of reproducibility associated with the system.

The entire system was further checked by operating the atomizer with a sterile solution of beef extract broth instead of the usual culture of organisms. Samples were taken in the usual manner, and not one single organism was shown on a single plate, indicating that no significant errors from external contamination are associated with any part of the entire system.

5. Equilibration time of the cylinders

In order to convert data for the concentration of viable organisms per liter of air into viability data in terms of time, the factor of time is introduced through the use of the equation for the biological die-away, k.

$$k = \frac{\log n_o - \log n_1}{t}$$

where n_o is the number of viables originally present, and n_1 is the number of viables present after the lapse of the time, t, expressed in minutes. For the aerosol system described here, the lapsed time between sampling positions is the 99 per cent equilibration time of the cylinders which is the time required for 99 per cent of the air-aerosol mixture entering the cylinders to pass through them.

If the cylinders were performing aerodynamically as diffusion chambers, the approximate equilibration time per cylinder would be 5.5 minutes (14). However, because of the parabolic profile of the velocity front through a cylinder, it was expected that the equilibration time would be shorter than that calculated for a diffusion chamber. The actual time was determined for each cylinder by following the build-up or disappearance of water vapor in the cylinders with a previously calibrated thermal conductivity cell. The determined equilibration time for each cylinder was found to be approximately 3.5 minutes and for two in series, 7.0 minutes. In order to verify this for actual operating conditions with bacterial aero-

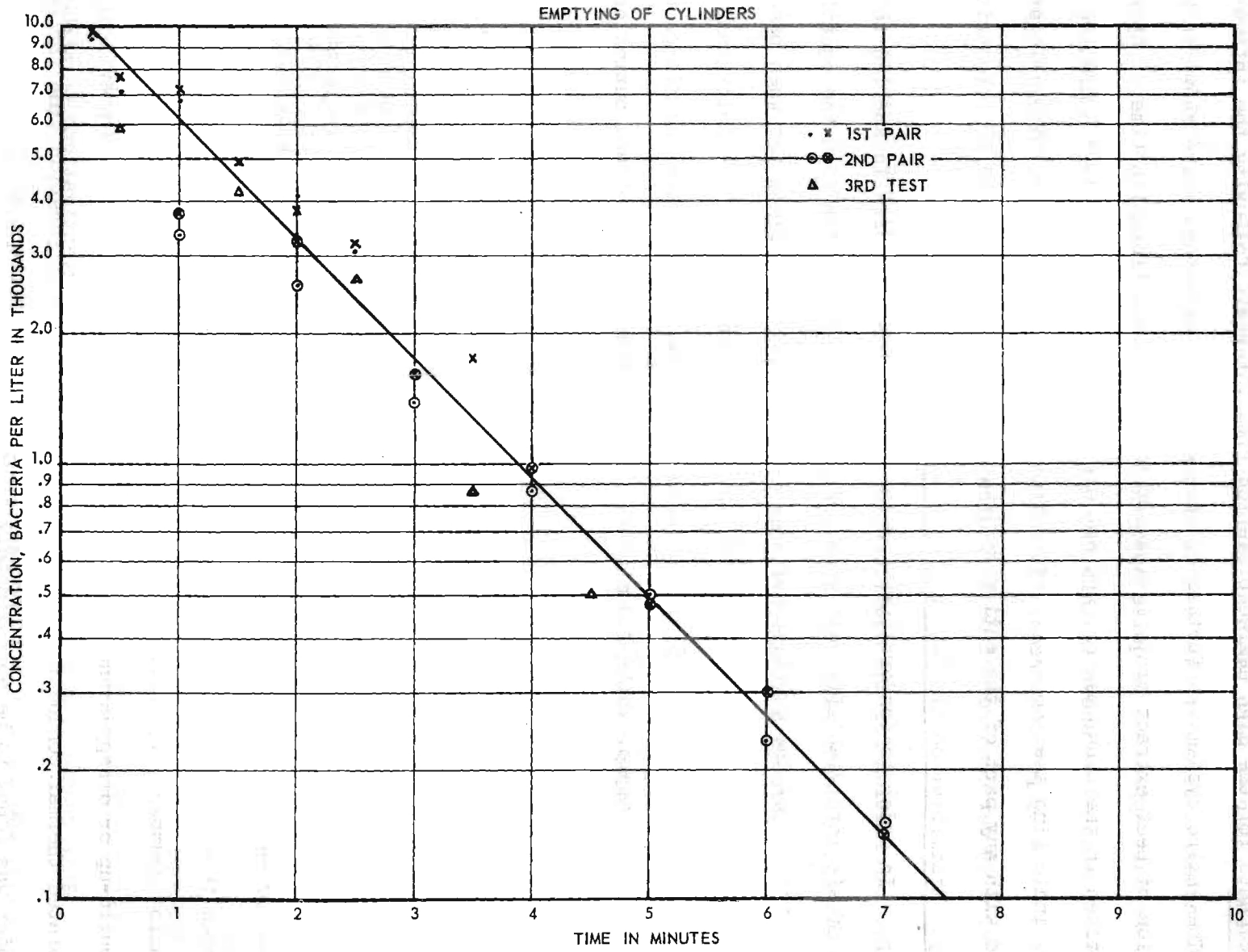

Figure 9. Emptying curve for the cylinders.

sols, tests were carried out in the assembled system using an aerosol of Serratia marcescens. Samples were taken at various time intervals following the introduction of the bacterial aerosol; the results of the several experiments are shown in Figure 9. These data appear to be quite homogeneous and an equilibration time of approximately 7.0 minutes is shown in this figure; that is, approximately 7.0 minutes is required for a 99 per cent reduction in the initial concentration. Shown logarithmically, a 99 per cent reduction is indicated by the change of two full logarithmic cycles.

The information for the equilibration time of the cylinders is applied to the determination of death rates by using a time of 3.5 minutes for t in the equation k when passage through a single cylinder is involved, and 7.0 minutes when passage through the two cylinders in series is involved. Thus, between the inlet sample and the mid-point sample, 3.5 minutes lapses, and the same between the mid-point and the outlet sample, while 7.0 minutes lapses between the inlet and outlet samples.

In order to determine biological die-aways in the manner described above, it is essential that the dynamically flowing system always be operated in an exactly uniform and reproducible manner. This requirement necessitates the somewhat elaborate equipment described for the study of airborne bacteria at low temperatures. The uniformity and reproducibility of operation of this system has been verified repeatedly over a period of some 18 months, and the results more than justify the extreme precautions taken to achieve this end.

6. The aerosol chamber

This chamber was developed during the earlier investigations on airborne bacteria (1), and has proven to be extremely useful in present studies, both as a means of verifying the validity of the results obtained in the aerosol cylinders and

for making certain studies on the nature and composition of the experimental bacterial aerosols used in these investigations.

The aerosol chamber is a 4-foot cube constructed of tempered Masonite; the internal surfaces were finished with several coats of a white alkyd resin enamel, each coat having been hand rubbed to a mirror finish. There are no internal projections in the chamber other than the air diffuser which is 5 inches in diameter and projects from the ceiling into the chamber about 2 inches. The chamber is located in a heavily insulated workroom into which about three changes per minute of tempered air are circulated from a control room where the temperature and dewpoint of the air is controlled within ± 1°F. The details of the chamber are shown schematically in Figure 10. During the dynamic runs, the main air stream of the chamber (60 cfm) is drawn from the workroom through filter A and is metered by orifice C, passing through Anemostat D (Anemostat Corporation, and donated by that company's local representative) into the chamber, and then is discharged outside the workroom through filter F. The pressure within the chamber is balanced to that of the surrounding room with the by-pass damper arrangement, G, the pressure being read on the manometer, H.

The standard bacterial aerosol issues from the classifying prechamber (6.4 L/min.) and is mixed with the main air stream in the turbulence created at the orifice meter, C. It is then uniformly distributed throughout the chamber by the Anemostat air diffuser.

Air samples are taken from any of the ports indicated for that purpose, including inlet and outlet to the chamber. In addition, settling samples are taken through the ports located at the bottom of the chamber. These ports are machined from one-inch diestock and have two air-tight seals, one of which consists of a sliding stainless steel shutter used for sealing the sampling port from the chamber, the other being a spring-loaded door which is used to seal the sampling port

from the outside room air. Through the use of these sampling ports it is possible to expose settling plates for any desired length of time and insert fresh plates as needed without interrupting the uniform flow conditions existing inside the chamber.

The accuracy and reproducibility of the results obtained using the aerosol chamber have been indicated in earlier reports. Some indication of the levels of uni-

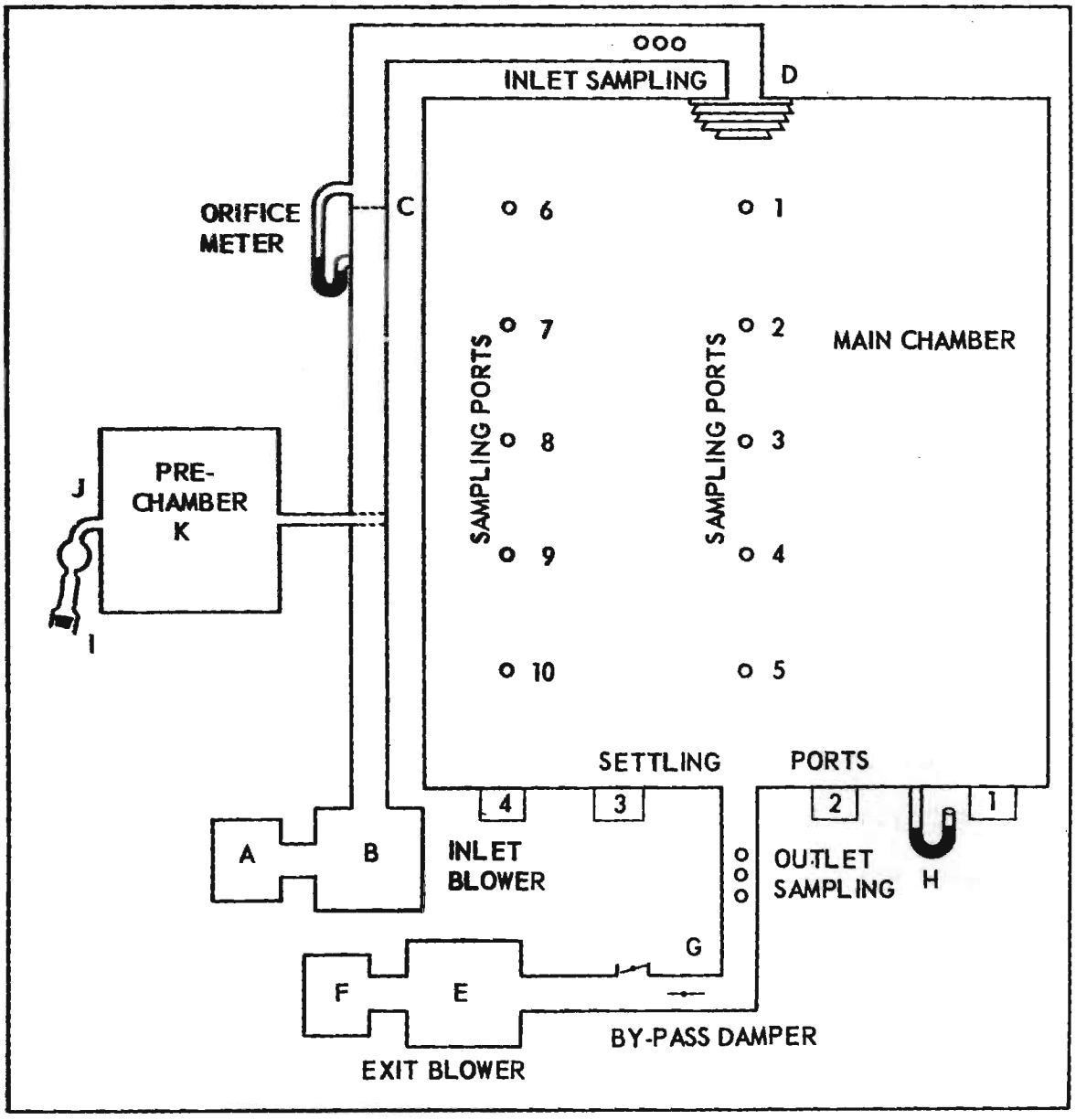

Figure 10. Schematic drawing of the aerosol chamber.

formity encountered in the air of the chamber is seen in the data illustrating the operation of the air samplers (Tables I and II). The levels of uniformity among the settling samples are illustrated in Table V. These data were obtained during preliminary studies on the effect of composition of the dispersion medium on the survival of airborne bacteria.

Static runs are made in the chamber following the achievement of dynamic equilibration by closing sliding valves located at the inlet and outlet of the chamber; simultaneously stopping the blowers and diverting the aerosol stream to throw-away. Because of the problem of volume losses caused by withdrawal of air samples, settling samples only are taken during static runs.

A view of the interior of the workroom in which the chamber is located is shown in Figure 11. In Figure 12 is shown a view of the side of the chamber with samplers in position, and also the atomizer, prechamber, and auxiliary equipment for the operation of the aerosol chamber. At the left of Figure 12 can be seen a pair of aerosol cylinders, complete with mixers. These are similar in design to those used in the low-temperature work, and have been used in the evaluation of aerial disinfectants (6).

B. Bacteriological methods

1. Methods and techniques

Of fundamental importance in the use of various bacterial genera and species in aerobiological work is the maintenance of a culturally stable organism which is characterized by a constant response to standard test conditions. The volume of variants within a strain may have marked effect on the consistency of results. Various organisms may have different degrees of stability of the contained variants and frequent tests should be made to the reference atmospheric condition to monitor the possible emergence of a variant of greater or less viability under these conditions.

a. General. The primary test organism, Serratia marcescens, was selected on the basis of natural occurrence, nonpathogenicity, relatively simple nutritive requirements, and other characteristics which facilitate the use of this bacterium without extraordinary procedures and techniques. These general considerations formed

TABLE V

Representative data† for settling plates exposed consecutively for one-minute periods during continuous dynamic operation of the aerosol chamber at 68°F and relative humidity of 45 and 80 per cent

Sampling interval minutes following start of run	Colony counts per plate				Avg.	S.D.	S.E.
	Port No. 1	Port No. 2	Port No. 3	Port No. 4			
A.M. Run at 45 per cent relative humidity							
61-64	162	150	190	175	169	17	8.5
65-69	180	189	214	183	190	16	8.00
70-74	155	231	160	235	195	41	20.5
75-79	155	201	216	203	193	26	13.0
80-84	176	182	171	176	181	7	3.5
85-89	161	185	182	167	174	10	5.0
Average	165	189	189	189	183	22.6	4.6
S.D.	9	26	23	25			
S.E.	4.0	11.5	10.0	11.0			
P.M. Run at 80 per cent relative humidity							
181-184	463	400	430	417	435	28	16.5
185-189	440	400	435	414	433	22	13.0
190-194	394	485	403	418	425	36	21.0
195-199	466	466	509	433	468	30	17.5
200-204	457	464	477	470	467	9	5.0
205-209	460	443	455	487	461	18	10.5
209-212	500	468	470	421	465	33	19.0
Average	454	447	454	437	448	30	5.7
S.D.	32	35	33	26			
S.E.	12.5	13.5	12.5	10			

†S. marcescens, E-R/O-3/56 (ATCC 274) atomized as washed cells in an aqueous suspension of glycerol (0.375 per cent).

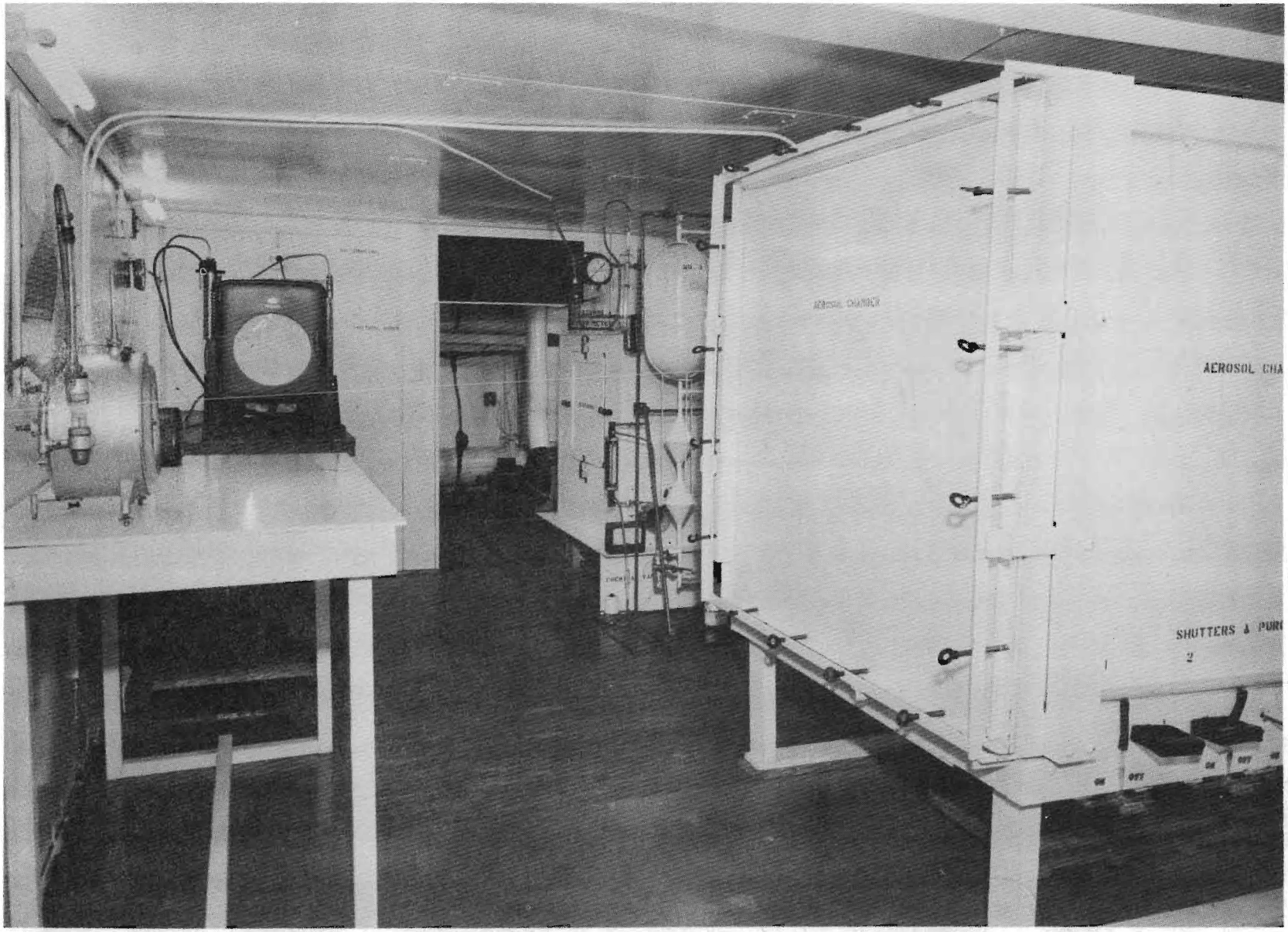

Figure 11. Interior view of insulated workroom.

the basis of selection of the other organisms used in this study. In addition to Serratia marcescens, ATCC 274, the bacteria S. indica, ATCC 4003, Micrococcus pyogenes var. aureus, ATCC 6538, M. pyogenes var. albus, ATCC 9491, and Escherichia coli, ATCC 10536 were used as airborne test organisms.

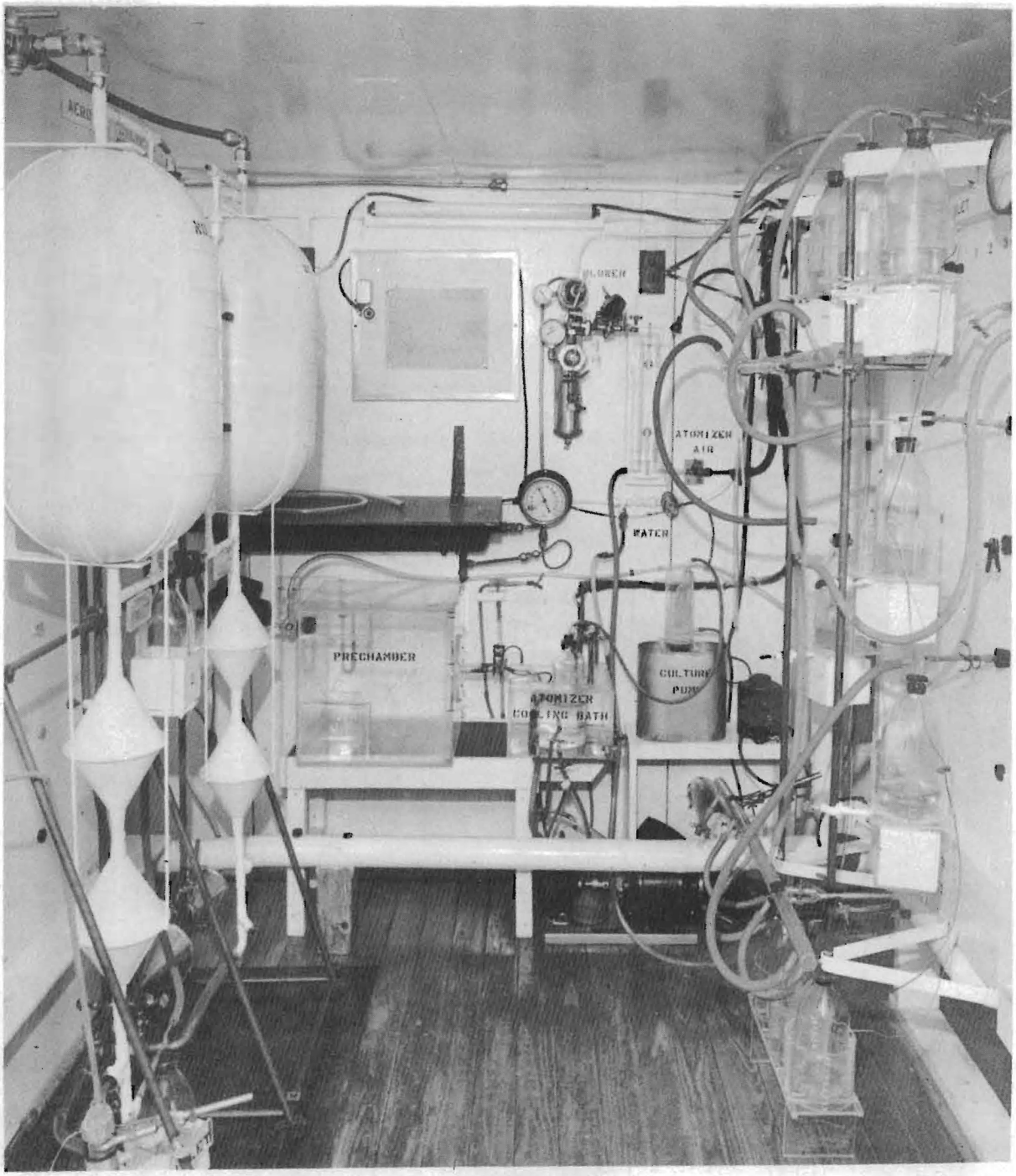

Figure 12. View of the side of the chamber and auxiliary equipment.

Experimental results indicated a more critical examination of the S. marcescens stock culture as essential for dependability of results from extended operational periods. The existence of several different colonial forms of strain No. 274 (6) was well recognized, and it had generally been assumed that, if a variant showed no appreciable percentage of any other variant, the culture was stable. However, it appears that even slight changes apparently related to cellular morphology may cause variation in resistance when the bacteria are suspended in the air. The selection of S. marcescens, ATCC 274, as the basic test organism in these studies, the ease of variant characterization by colonial chromogenicity, and the general use of S. marcescens by others in aerosol studies was sufficient to warrant corrollary studies of these variants as airborne organisms. The results afforded practical characterization of the test variant which was very important in maintaining a stable test organism.

The concept of a secondary environmental effect for airborne bacteria (6) places primary importance upon the composition of the immediate environment of the bacterial particle in determining the fate of the airborne bacterium. Beef-extract broth was selected as the dispersing medium because it is a common cultural medium and produces excellent growth. Though other media might possibly be more suitable, the primary emphasis of the study was not considered to be the selection of optimum media but rather the selection of suitable and useful media. The original concept of the state of the airborne organisms held by the personnel of this project was that the single organism in an airborne particle is covered by only a thin layer of material derived from the solids of the substrate from which it is atomized. However, evidence that the airborne bacterium is surrounded by a relatively thick layer of non-living residual substrate material which forms the actual immediate environment of the bacterium indicates that the actual response of the organism may be obscured by

effects peculiar to the presence of the accompanying material. This gives great importance to the nature of the substrate from which the organisms are atomized.

b. Culture of test organisms. Standard bacteriological procedures were adhered to in the production of the bacterial test organisms. The bacterial specimens were obtained from the American Type Culture Collection. Transfers were made into 0.3 per cent beef-extract broth, 60-ml volume, and incubated at 30°C. Serial transfers were subsequently made into beef-extract broth at regular intervals of 45 to 48 hours. Four or five transfers were made before using the culture in tests. Thereafter, the culture was maintained in beef-extract broth and transferred at the time intervals stated. Stock cultures were maintained in nutrient agar butt tubes with a surface overlay of mineral oil and storage in the refrigerator (5°C). Subsequent culturing of the microorganism in beef-extract broth, according to the routine method described, demonstrated the stability of the culture as determined by k_t values under standard conditions. All species of organisms were cultured as received from the American Type Culture Collection by this method with the exception of S. marcescens. The stock slant was differentiated into the variants present on the basis of colonial chromogenicity. The stock culture and variants, including the standard test variant "R", were placed in refrigerated storage according to standard procedure. The special treatment of S. marcescens will be dealt with in a separate section.

c. Plating media. Enumeration of viable bacterial cells were made according to routine bacteriological procedure using solid media. Nutrient agar was accepted as a generally standard medium suitable for the organisms being used, producing countable colonies of S. marcescens within 30 to 40 hours. However, as a result of testing several solid nutrient media, with the objective in mind of reducing the period of incubation of the bacteria before the counting of the colonies, a buffered, sodium chloride, tryptone-glucose-extract agar medium was developed which

reduced the necessary incubation time by one-half. The composition of the medium tryptone-glucose-extract agar (24 grams), sodium chloride (5 grams), anhydrous dibasic sodium phosphate (2.5 grams), and de-ionized water (1 liter). Inoculated agar plates were incubated at the temperature of 35° to 37°C. The two varieties of M. pyogenes grew quite well in this medium but the incubation time prior to the growth of countable colonies was about 12 to 16 hours longer than the other organisms used in these studies.

d. Plating technique. Upon sampling the aerosol cloud, the impinger solution was plated directly and/or serially diluted in sterile de-ionized water for subsequent plating. Aliquots (direct and/or diluted) of the sample were plated in triplicate for determination of viable cell numbers. The culture medium was maintained at 50°C in a water bath before being mixed with aliquots of the sample in Petri dishes of standard size (9 cm x 1 cm). After solidification of the agar, the plates were stored in an inverted position in the incubator at 35°C for approximately 18 hours (longer for M. pyogenes). After colonial development, the total colony count was made with the Quebec colony counter.

2. Study of variants of Serratia marcescens, ATCC 274

a. Differentiation of variants. The culture as received from the American Type Culture Collection was differentiated in the laboratory on the basis of colonial color and morphology. A beef-extract broth culture was made of the culture as received. Nutrient agar (2.3 per cent) plates were made from the broth culture grown for 48 hours at 30°C. Incubation of the plates was at 30°C for approximately 12 hours and then at 20°C for an additional 12-24 hours for pigment development. Typical colony forms of the primary variant (R) characterized by circular, smooth red-carmine to orange-red colonies were transferred into broth culture and incubated at 30°C. Five serial transfers at regular intervals of 45-48 hours were made before using the culture in aerosol tests. The test culture was continued at this temper-

ature and interval of transfer. The use of broth cultures 45-48 hours old provided cells in the late logarithmic phase of growth and a numerical density of 70-100 x 10^7 per ml. The other contained variants were similarly separated on the basis of colony pigmentation. These variants were blood red (BR), pink (P), and white (W).

b. Culture stability. The original culture (A-R) maintained over a period of several months by the procedure described previously provided an orange-red pigmented organism of approximately 0.7 to 1.0 micron in length. The stability of the response of the test bacterium to aerial dispersion was periodically affirmed under standard conditions of temperature and relative humidity (68°F and 65 per cent, respectively).

The primary concern with the variants of S. marcescens, ATCC 274, in relation to this investigation was the cultural characterization and maintenance of a standard bacterium as correlated with the standard atmospheric condition mentioned above. This requirement became apparent when, after months of stable performance, the culture (A-R) began to indicate changes, as evidenced by significant increases in k_t values--a reduced survival capacity under the standard atmospheric condition. Transfers were made into beef-extract broth from the previously inoculated nutrient agar stock slant which had been refrigerated (5°C) without mineral oil overlay. The static k_t of this undifferentiated culture A was determined to be 0.075 in the 4-$foot^3$ chamber. An entirely new specimen of S. marcescens, ATCC 274, was obtained and transferred directly into beef-extract broth without preliminary screening of the chromogenic variants. The static k_t value obtained with this culture was 0.034, a firm value which had been established over a period of months and accepted as correct response of the standard test culture.

A comparative study was made of the A-R culture in routine use, but showing erratic responses, and the newly received culture designated as E, undifferentiated as to variants. At the time of testing, culture E had been through five serial broth

transfers. Comparative media, both for the impinger samplers and settling plates, was employed to determine if this might be a factor influencing recovery. The results of the comparative tests are shown in Table VI. There are several significant points in these results: 1) the ratio of recovery of numbers of viable organisms in gelatin-brain heart/gelatin is greater for culture A-R, 2) plain gelatin impinger samplers from the chamber air show a greater number collected from culture E (all other comparisons of numbers do not show a significant difference), 3) the k_t values for culture A-R in the plain gelatin impinger solution is higher than in the enriched impinger solution, and the highest k_t values were obtained from the settling samplers, enrichment of the medium apparently having no effect. These results are interpreted as indicating that the culture A-R has undergone a deterioration of viability response to the standard test atmosphere. That this viability potential may be variably affected by the nature of the recovering environment is indicated from a consideration of the k_t values of 0.035, 0.050, and 0.078-0.067, each derived from a decreasing favorable environment following the inimical effect of the airborne state. Thus, the attenuated condition of the culture seems patently established.

c. Characterization of stable culture. Although culture E exhibited the same response under standard atmospheric conditions as that previously established (0.034), it was questionable whether such a culture would be inherently stable because of the chromogenic and nonchromogenic variants present. The variants were separated from the culture being used routinely (A-R) and from the newly received culture (E). The criterion of separation was the color of the colonies: BR (blood-red), R (orange-red), P (pink), and W (white). The procedure followed has been stated under the section Differentiation of variants. The distribution of these variants is shown in Table VII. The normal response of culture E, undifferentiated, and the distribution of colonial variants of A and E suggests a relation between the

TABLE VI

Comparative collection of culture A-R and culture E† in different impingement media expressed as concentration of viable organisms per liter of air††

	Inlet		Chamber		k_t Dynamic		k_t Static	
	Plain gelatin	Gelatin-brain heart	Plain gelatin	Gelatin-brain heart	Plain gelatin	Gelatin-brain heart	Nutrient agar	Nutrient agar-brain heart
Culture A-R (initial blank 37 x 10^7 cells/ml)†††								
Average	7676	16100	4318	10686	0.050	0.035	0.078	0.067
No. of samples	3	3	12	12				
S.D.	280	1150	563	1160				
S.E.	198	815	170	352				
C.V.	4	7	13	11				
Gel-B.H./Gel.		2.1		2.5				
Culture E†(initial blank 73 x 10^7 cells/ml)†††								
Average	8700	13600	5595	8698	0.038	0.039	0.043	0.039
No. of samples	3	3	11	11				
S.D.	1140	1630	1015	1720				
S.E.	810	1155	321	487				
C.V.	13	12	18	17				
Gel-B.H./Gel.		1.5		1.5				

† Undifferentiated as to variants (five transfers in broth from new stock slant).

†† Standard atmosphere

††† Values in table corrected to initial blank of 100 x 10^7 cells/ml.

distribution of variants and response to the airborne state. The emergence of the BR variant has been found to be significant in this respect and useful in monitoring the test culture for deteriorative changes. The relative stability of the different variants of culture E was followed through a number of serial broth transfers, the results of which are shown in Table VIII.

In order to determine the static k_t values, which show the greatest comparative differences, the variant isolates were tested in the aerosol chamber under the standard atmospheric condition. These results are tabulated in Table IX. The standard k_t is found within the range of response shown by E-R and E-P. The variant E-R was continued as the standard test organism, and, from 11 subsequent determinations, the static k_t value of 0.31 was obtained. The variant E-P was not used further because it was found that the more highly pigmented variants possess the greater cultural stability; Table VIII indicates the incipient differentiation of variants E-P at the sixth broth transfer.

Additional characterization of the stable test culture was sought in the study of possible morphological and dimensional variation by the use of phase and electron microscopy. Studies were made on E-R cells taken directly from the broth culture and glycerol-water solutions which were prepared to simulate conditions of relative humidity of the atmosphere. These size determinations were made with the phase contrast optical system. Other examinations of cell size were made with the electron microscope on cells sedimented on oil films spread of microscope slides, such deposited cells being removed to electron microscope grids by colloidin film-stripping technique. The results of these measurements are shown in Table X. Equivalent spherical diameter sizes of washed cells of S. marcescens derived from the ratio of the rate of cell sedimentation per minute per 1,000 cm^2 and the aerial concentration are reported in Table XI.

Examination of culture A-R, which had evidenced an abnormal response to the standard atmospheric condition, showed a wide variation in cell size, ranging from 1 to 2 microns in length and 0.7 to 1.0 micron in width to 15 microns in length and

TABLE VII

Percentage distribution of colony forms in cultures "A" and "E"

Number of broth transfers	Culture	Blood red	Red	Pink	White	Number of colonies
100	"A"	15.7	79.7	4.3	0.3	1,000
20	"E"	0	98	1.8	0.2	1,500

TABLE VIII

Stability of culture "E" variants in serial broth transfer

Number of broth transfers	Culture	Per cent of various colony forms				Number of colonies
		Blood red	Red	Pink	White	
1	E-BR	87.5	1.3	10.4	0.8	2,200
	E-R	0	100.0	0	0	1,200
	E-P	0	0	99.2	0.8	2,000
	E-W	0	0	0.2	98.8	2,000
6	E-BR	7.9	87.7	3.2	1.2	3,800
	E-R	0	99.7	0.1	0.2	11,000
	E-P	0.1	8.5	89.8	1.6	3,000
	E-W	0	0	1.0	99.0	5,000
45	E-BR	Discarded				
	E-R	0	100.0	0	0	1,200
	E-P	0	80.3	13.4	6.3	1,193
	E-W	0	0	10.0	90.0	1,112

1.0 to 1.5 micron in width. Many of the longer filamentous cells showed loci of increased density to the electron beam of the electron microscope. Culture E showed a more homogeneous population with cells having lengths ranging from 1.0 to 2.5 microns and widths ranging from 0.7 to 1.0 micron. Electron micrographs of this culture disclosed a uniform intracellular density.

The separation of the variants from culture A and culture E provided a more detailed examination of the cell types as correlated with colony pigmentation. Electron photomicrographs of third-isolate variants from nutrient agar surface colonies show a significant morphological and size variation among the variants of culture A. These variants also appear quite different from the corresponding variants of culture E which does not exhibit a correspondingly wide range of cellular differentiation among its variants. The pictures of the variants of culture A are shown in Figure 13; those of culture E are shown in Figure 14.

TABLE IX

Static k_t rates, airborne variants of S. marcescens at 68° F and 65 per cent relative humidity

Number of broth transfers	Colony forms: Blood red		Red		Pink		White	
	A†	E	A	E	A††	E	A	E
3	-	0.042	0.005	0.017	-	0.035	0.005	0.039
6	-	0.053	0.054	0.036	-	0.037	0.008	0.047
7	-	0.049	0.010	0.021	-	0.023	0.005	0.054
8-19	-	-	-	0.031	-	-	-	-

† Too few airborne to obtain values

†† After two isolations, cultures lost the pink color, became predominantly red and were discarded.

TABLE X

The size of S. marcescens[†] cells--determined by direct measurement

	Phase contrast microscopy. 1.17μ diameter spheres for calibration. Broth culture. Average of 21 measurements.	Electron microscope. Airborne cells collected on oil film. Internal calibration. Average of 21 measurements.		Phase contrast microscopy. Cells suspended in glycerol water to simulate relative humidity. Calibrated grid. Average of 17 measurements.	
		Air at 20 % RH	Air at 90 % RH	Equiv. 20 % RH	Equiv. 90 % RH
Average dimensions (microns)	1.1 x 0.5	1.15 x 0.62	1.08 x 0.67	1.5 x 0.4	1.5 x 0.5
Diameter of equivalent sphere (μ)	0.75	0.87	0.90	0.71	0.83

[†]ER/0-9/1: Primary form from stock culture stored under oil at 5°C.

The correlated findings of morphology, cell dimension, and chromogenicity, together with the static k_t determinations, form the basis for monitoring the test culture and enable a direct recovery of the test culture at such times when deterioration may be indicated. Storage of the variants in nutrient agar butts with mineral oil overlay at 5°C has proven satisfactory

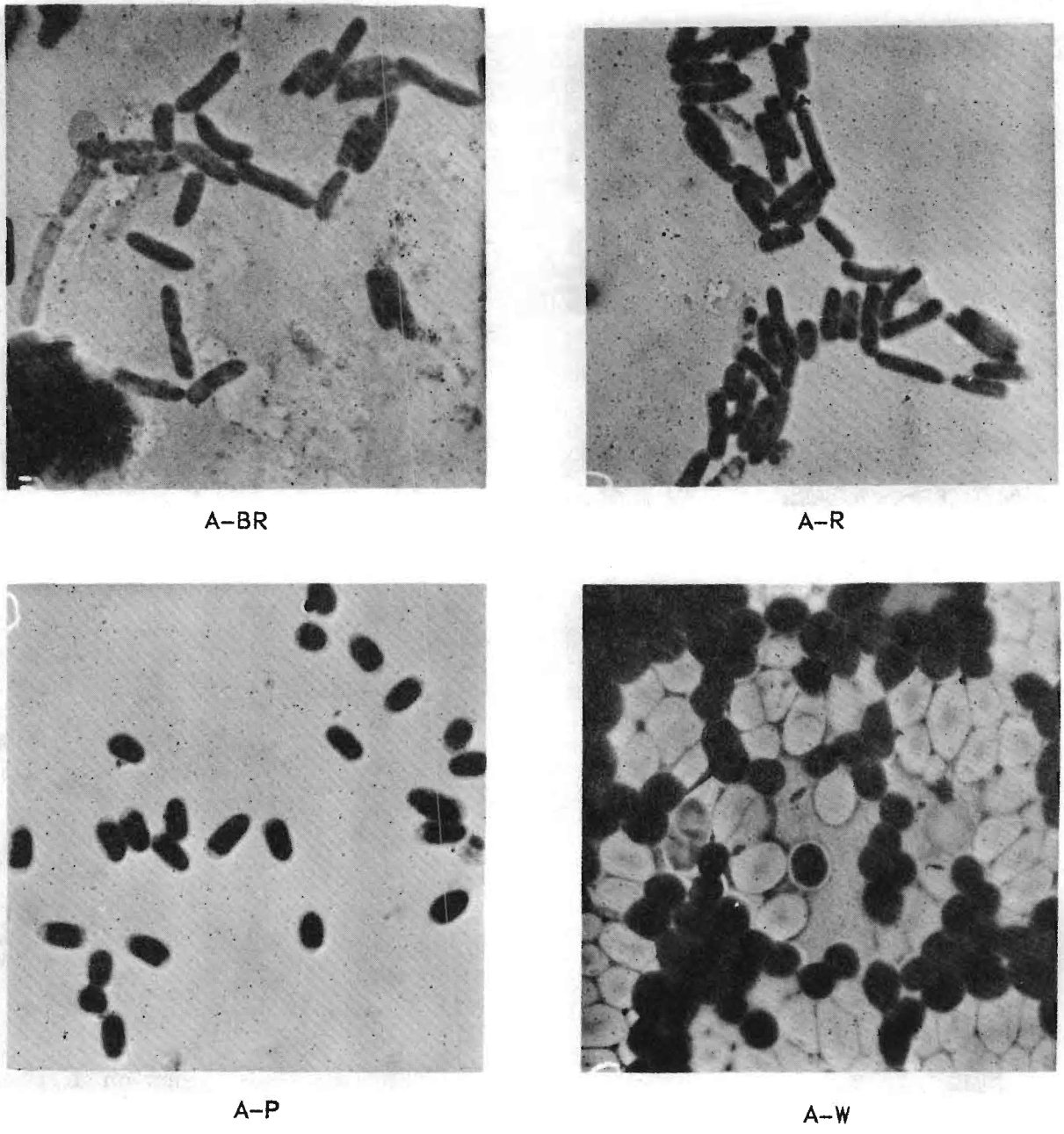

Figure 13. Electron microscope pictures of Serratia marcescens (ATCC 274), color variants of culture A.

3. Special procedures

Cognizance of the important function of the nature of the material composing the airborne particle as produced from a beef extract culture indicated further examination of the behavior of the airborne cell without adhering material, and also the effect of various other materials substituted for the beef extract.

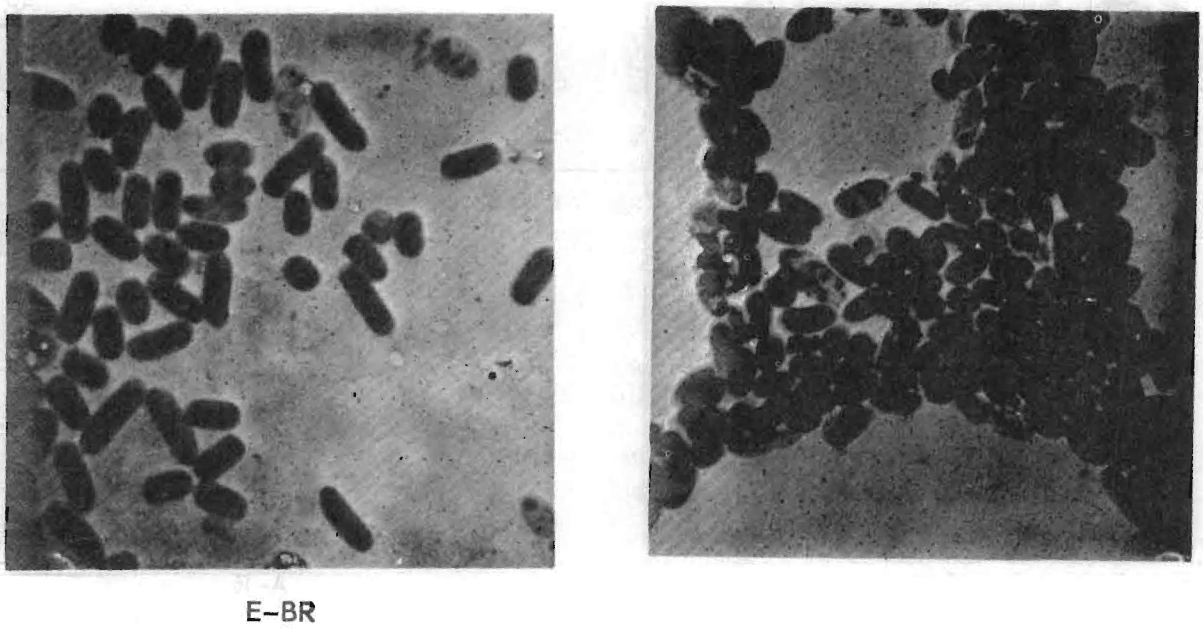

Figure 14. Electron microscope pictures of Serratia marcescens (ATCC 274), color variants of culture E.

TABLE XI

The average diameters of airborne washed S. marcescens† cells as determined from settling rates in the aerosol chamber at 68°F

(Dynamic runs)

Relative humidity, per cent							
16		25		58		88	
Average diameter ± S.D.	n	Average diameter ± S.D.	n	Average diameter ± S.D.	n	Average diameter ± S.D.	n
0.61 ± 0.21μ	8	0.56 ± 0.16μ	8	0.69 ± 0.22μ		0.41 ± 0.24μ	8

†ER/0-9/1: Primary form from stock cultures stored under oil at 5°C.

TABLE XII

Viability of washed cells in deionized water compared to broth cultures at 80° F

Culture type	Hours lapsed time following washing and suspension in water	Viable count per ml x 10^{-7}		Recovery of viable washed cells from broth cultures (per cent)
		Washed cells in water	Original broth culture	
S. marcescens†	0	46	56	60††
	3	47	78	
	4	47	67	
E. coli†††	0	36	51	70††††
	5.5	40	42	

† E-R/0-9/1--primary form from stock cultures stored under oil at 5°C.

†† Average of five experiments.

††† ATCC 10536.

†††† Average of three experiments.

a. Washed cells. Using a standard beef extract broth culture (45-48 hours old), 5-ml portions are transferred to the surface of a millipore filter (MF filters, Millipore Filter Corporation, Waterton 72, Mass.), the fluid is filtered by suction, and the residue is immediately washed with 25 ml of sterile deionized water, the water being passed through the filter by suction. Because the residue from 5 ml of culture causes the MF filter to begin to clog, a fresh MF filter is used for each 5 ml of culture. The MF filters with the washed cells are then placed in a sterile dilution bottle, a volume (equal to the volume of the original culture employed) of sterile deionized water is added, and the cells resuspended in this water by violent agitation. Any large particles are then removed by filtering through a sterile fritted filter (grade C), and the filtrate containing the washed cells is then atomized in the same manner as the broth cultures.

The viability of these washed cells resuspended in deionized water was determined over a period of hours and compared to aliquots of the original culture. The results of these tests are shown in Table XII and indicate that the viability of the washed cells is unaffected. In this table the recovery of cells by this procedure is between 60 and 70 per cent. As no effort was made to remove the cells from the surface of the MF filters except by agitation of the suspending water, and since these filters are known to have a high surface attraction, it is assumed that the balance of the cells remained adherent to the surface.

b. Particle charge as a factor in settling sample variations. The large variation in the distribution of sedimented cells from an aerosol of washed cells, shown in Table XIII, indicates a sharp deviation from the normally excellent homogeneously dispersed cloud produced from the beef-extract broth cultures. A study of the cell charge was made because the washed cells having virtually no ionizable materials at their surface, a random distribution of charges would result, and, since the particles atomized from the beef-extract broth would contain definite, although

small, amounts of ionizable materials, a more definitely charged cloud would be produced. Aerosol clouds having no net charge are extremely difficult to disperse and dilute, and a cloud having a definite and strong net charge is virtually self-dispersing. With the assistance of the Micromeritics Group at Georgia Tech, measurements were made on bacterial aerosols produced by atomization from beef-extract broth and also on those produced from washed cells in deionized water. Each determination consists of 100 individual particle observations on the behavior of single particles in an electrically charged field of high potential. Visualization of directional movement of the particle was made through an optical system for forward scattering dark-field illumination, each particle appearing as a bright speck. The results of these measurements are shown in Table XIV. It was concluded that the primary cause of the erratic dispersion was the presence of small clumps of inadequately mixed aerosol clouds and that the presence of these was due to the tendency of aerosol clouds having no net charge to resist dispersion. This irregular dispersion of the

TABLE XIII

Effect of particle charge on aerial and settling sample variation at 68° F. S. marcescens, E-R, in aerosol chamber

Type of Cells	RH	Average concentration per liter	Coefficient of variation	Average number per settling plate	Coefficient of variation
Washed cells	35	2,000	7	169†	136
	88	2,790	6.5	43†	90
Cells in 0.375 per cent glycerol	45	2,920	6	183 ††	12
	80	5,200	7	448 ††	7

† 5 minute sample.

†† 1 minute sample.

washed cells would not appear in the aerial samplers because of the large numbers of cells collected in such samples.

c. Effect of contrasting dispersion media. For further study of the influence of the immediate environment of the airborne bacterial cells of S. marcescens, two different and contrasting dispersion media were used. The first medium was the routine 45 to 48-hour old beef-extract broth culture to which sodium chloride was added in an amount equalling a 0.1 per cent solution. The second medium was a dispersion of washed cells in a 0.375 per cent aqueous solution of glycerol. Glycerol was chosen as a dispersion medium because of the similarity of its equilibrium moisture characteristics to those of beef-extract solids (Table XV). Sodium chloride was added to the beef broth to increase the crystalloid content of the resultant particles. If the lethal effect of low relative humidity on the airborne bacterium is caused by loss of water from the particle with attendant conditions of cellular dehydration, the results from the two different dispersion media would be expected to be the same.

The results shown in Table XVI indicate a very good correlation of response of the two media to changes in relative humidity as shown by the equivalent spherical diameters of the particles. These experimental values are very close to the

TABLE XIV

Distribution of charged particles in aerosols of S. marcescens,† atomized from broth cultures or from deionized water suspensions of washed cells

Washed cells			Cells from broth culture		
Positive	Negative	Neutral	Positive	Negative	Neutral
46	47	7	70	16	14

† ER/0-9/1: Primary form from stock cultures held under oil at 5°C.

derived values for the theoretical response of the particles to varying relative humidity.

The physical behavior of the two dispersion media, salinized culture and aqueous solution of glycerol, are within acceptable limits of agreement. There is an indicated difference, however, in the viability response of the cells resulting from the two media as shown in Table XVII. Whereas the k values for the salinized culture indicate a more lethal response at the lower relative humidity, the washed cells in the aqueous glycerol solution show a k response of a magnitude with the

TABLE XV

Equilibrium moisture content of beef-extract solids and glycerol at various humidities

Relative humidity (%)	Beef extract solids (20°C)†		Glycerol (20-100°C)††	
	Water content (%)	Solids content (%)	Water content (%)	Glycerol content (%)
20	11	89	5	95
30	13	87	8	92
40	18	82	14	86
50	28	72	20	80
60	34	66	26	74
70	36	64	35	65
80	46	54	48	52
90	62	38	67	33
95	75	25	83	17
98	86	14	95	5

An Investigation of the Effects of Minute Quanities of Chemical Vapors on Airborne Bacteria, Final Report, Project B-106, 15 January 1956.

Glycerol, ACS Monograph Series, Carl S. Miner and N. N. Dalton (Ed.), Rheinhold Publishing Co., Chapter 7, 1953.

general range of the salinized culture but a response not correlated with relative humidity. These results would seem to indicate that dehydration of the particle is not a functioning mechanism operating lethally on the airborne cell.

V. RESULTS

The primary objective of this study was to determine the effect of low temperatures on the survival of airborne bacteria. In approaching this problem it was realized that the important variables in such a study are: the temperature and relative humidity of the air; the dispersion fluid from which the bacteria are atomized; the species and sub-species of organism studied. The first step in attacking this problem consisted of a detailed study of the effect of temperature and relative humidity on a single species of bacteria, employing a single dispersion medium. The organism selected was Serratia marcescens (ATCC 274, primary form), and the medium 0.3 per cent beef extract broth. The next step in the plan was to study other bacterial species, dispersed from beef extract broth under those conditions of temperature and humidity shown to effect major changes in the viability of airborne

TABLE XVI

Comparison of experimental and calculated particle size of salinized culture and aqueous glycerol

RH	NaCl-culture (0.1 per cent)†		Glycerol (0.375 per cent)††	
	Derived	Experimental	Derived	Experimental
25	2.02	1.89	---	---
45	--	--	2.14	2.24
80	2.21	2.64	2.31	2.58

† Calculated minimum particle size 1.95.
†† Calculated minimum particle size 2.03.

TABLE XVII

Response to relative humidity of S. marcescens, E-R/O-3/56, cells atomized from salinized (0.1 per cent) culture and as washed cells from an aqueous solution of glycerol (0.375 per cent)[†]

Atmosphere D.B. (°F)	Atmosphere D.P. (°F)	Atmosphere R.H. (°F)	Culture count (ml x 10^{-7})	(n)[††]	Average concentration of organisms per liter of air: Inlet	Average concentration of organisms per liter of air: Average chamber and outlet	Settling (min/1000 cm^2)	Rate of Fall (cm/min)	Equivalent spherical diameter (μ)	k
NaCl (0.1 per cent) added to culture										
68	30	25	73	9	11140	7500	4820	0.645	1.89	0.057
68	62	80	73	9	11700	9470	12000	1.26	2.64	0.018
Washed cells suspended in aqueous solution of glycerol (0.375 per cent)										
68	46	45	49[†††]	9	4000	3000	2820	0.91	2.24	0.025
69	63	80	49	9	6470	5191	6475	1.20	2.58	0.021

[†] Experiments performed with main chamber (4 ft^3) system.

[††] Number of replicate samples.

[†††] Cell count from washed cells suspended in aqueous solution of glycerol (0.375 per cent).

S. marcescens. This plan was applied to several species, studying only a few conditions of temperature and humidity in order to obtain data to verify or deny the conclusion that no real species difference was indicated at low temperatures. The final step in the plan was to investigate the effect of varying the composition of the dispersion media from which the bacteria were atomized, in an effort to evaluate the physical factors which may affect the fate of airborne bacteria. A few preliminary studies were carried out on this step; the results of these studies have been most helpful in adding to our knowledge of the nature and composition of experimental bacterial aerosols.

In order to present the results obtained in the most lucid manner, the majority of the data dealing with the effect of low temperatures on the survival of airborne bacteria are shown graphically. All die-away values were calculated as the average of at least three separate determinations; thus each set of experimental conditions required a minimum of 27 plate counts. Reporting such a multiplicity of results would have hindered rather than aided the process of interpretation and analysis.

The most significant results obtained during the course of these investigations are summarized in Figure 15. In this figure the values of the die-away, k, are plotted against relative humidity for the various ranges of temperature which were studied, for airborne Serratia marcescens (ATCC 274, primary form). With the exception of the single curve for washed cells at 68°F all the data for Figure 15 were obtained from studies on cells dispersed into the air from beef extract broth.

The information shown in Figure 15 encompasses some 41 individual points, each of which is the average of not less than 3 determinations. In general, a high degree of validity is attached to the values plotted in this figure. However, the minimum value of k which can be considered statistically significant is 0.007. Values of k of less than this cannot be distinguished statistically because of the

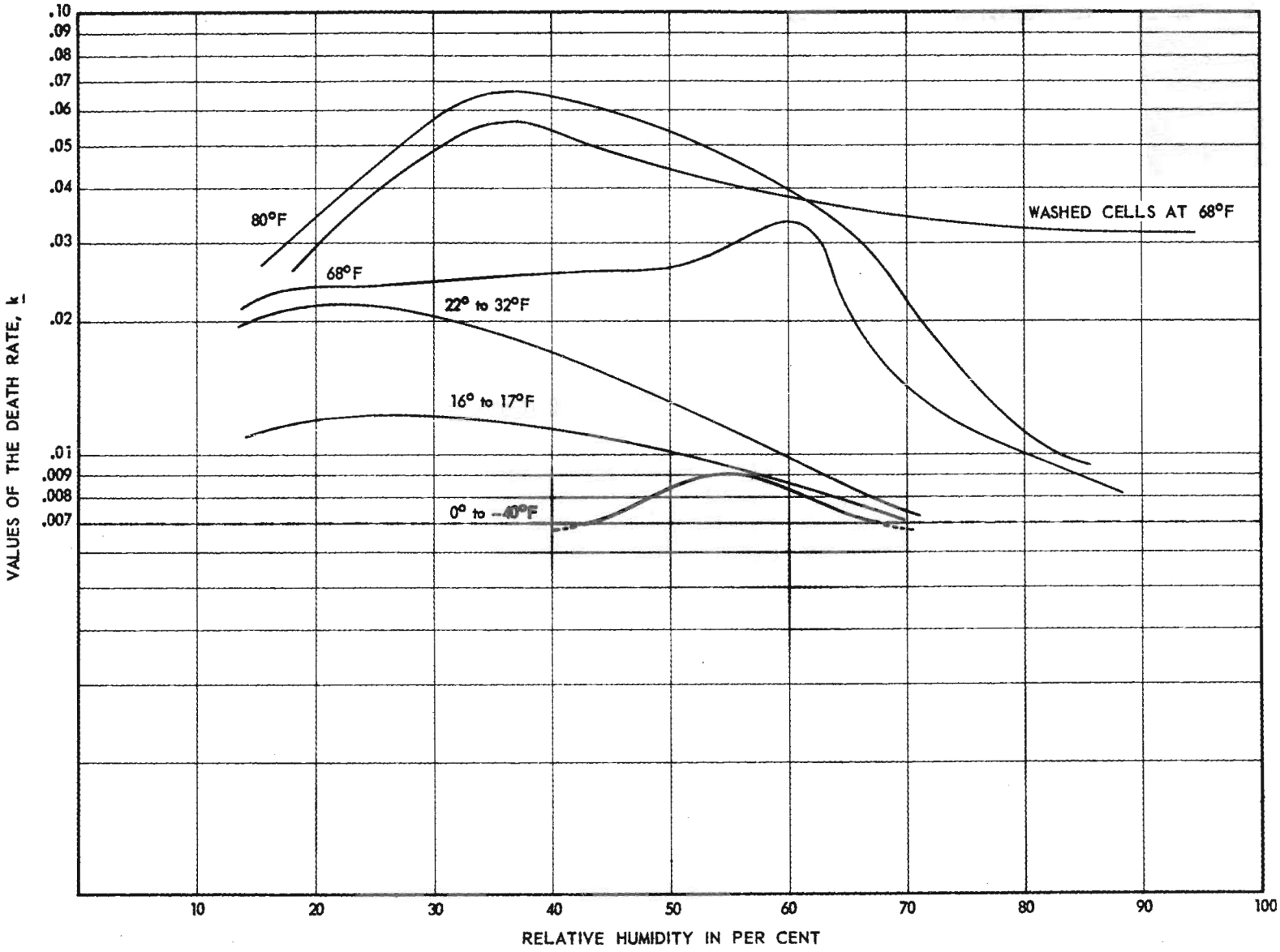

Figure 15. The effect of relative humidity at various temperatures on the death rate of airborne Serratia marcescens (ATCC 274, primary form).

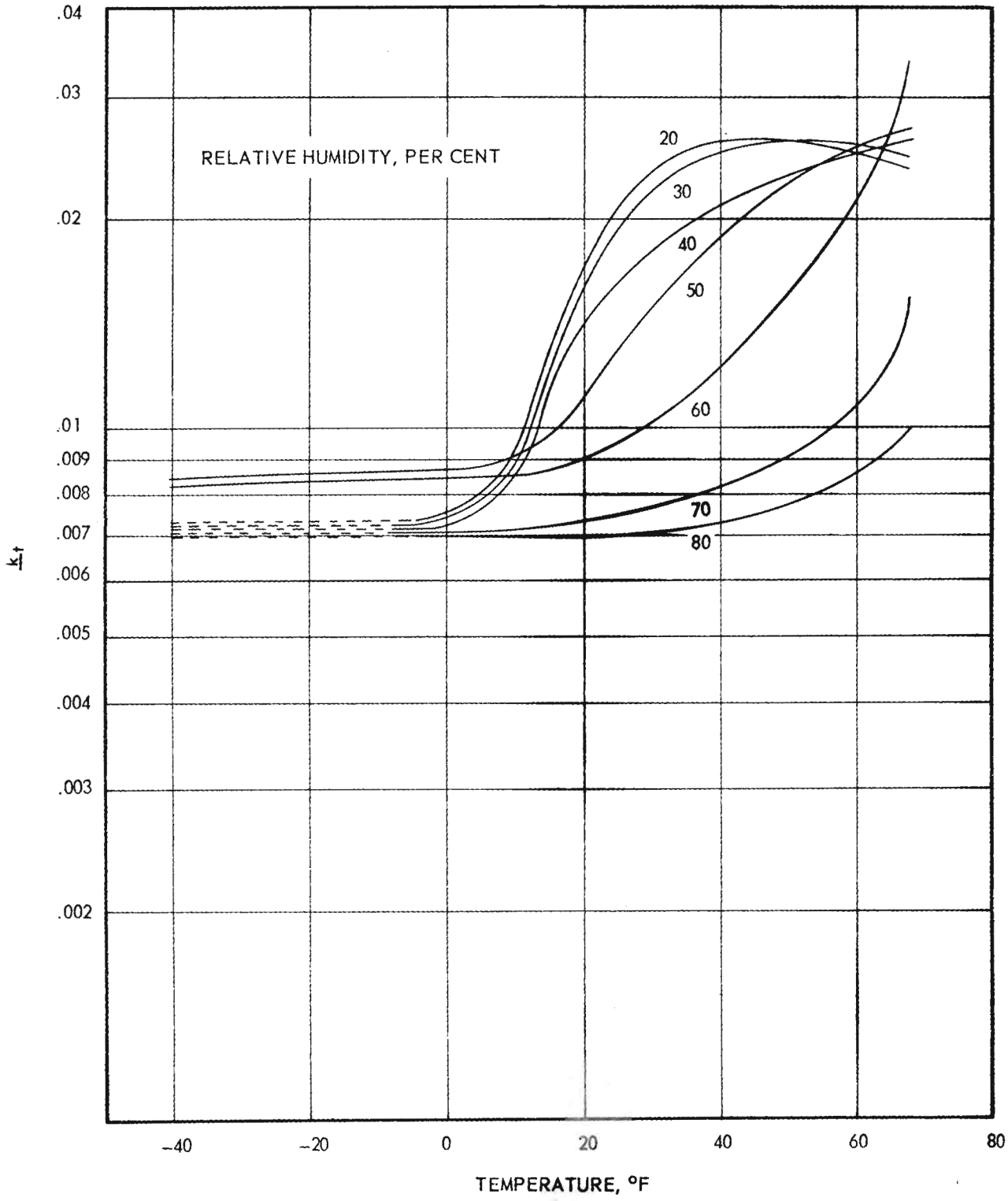

Figure 16. The effect of temperature upon the survival of airborne Serratia marcescens (ATCC 274, primary form) at various relative humidities.

limitations inherent in the method of determining the die-away. Thus for the temperature range 0° to -40°F and relative humidities below 40 per cent and above 70 per cent, nothing really quantitative is known about the death rate of the organisms, except that it is extremely small.

The effect of decreasing temperature upon the survival of airborne bacteria is even more clearly shown in Figure 16, where death rates are plotted against temperature for the various relative humidities. The curves shown in Figure 16 are essentially qualitative in nature, being derived from smoothed data taken from Figure 15, and are presented to show qualitatively the effect of decreasing temperature on the survival of airborne bacteria.

Compared to S. marcescens, none of the bacterial species studied showed a greater difference in response to the aerial state than did Escherichia coli. The detailed results of the studies carried out with this organism at 17°F are shown in Figure 17, both for cells dispersed from beef extract broth, and for washed cells dispersed from pure water. The curves shown in this figure illustrate two anomalies: in the first place, a larger death rate is indicated for the first cylinder than for the second (contrary to the work with S. marcescens, where little or no difference was found); and secondly, the death rate for the "unprotected" washed cells appears to be less than for the cells dispersed from beef extract broth. It had previously been indicated that certain bacteria when airborne (4) exhibit a "two-stage" die-away in the air, that is, for an initial short time a very high death rate was observed, followed by a lesser death rate for the longer periods of time following the initial high death rate. The evidence in Figure 17 appears to confirm this observation for E. coli. However, the unexpected finding of a higher death rate for the supposedly protected cells dispersed from beef broth, as contrasted to the supposedly unprotected cells dispersed from pure water, led to the investigation of both these phenomena at a higher temperature. The results of this investigation are

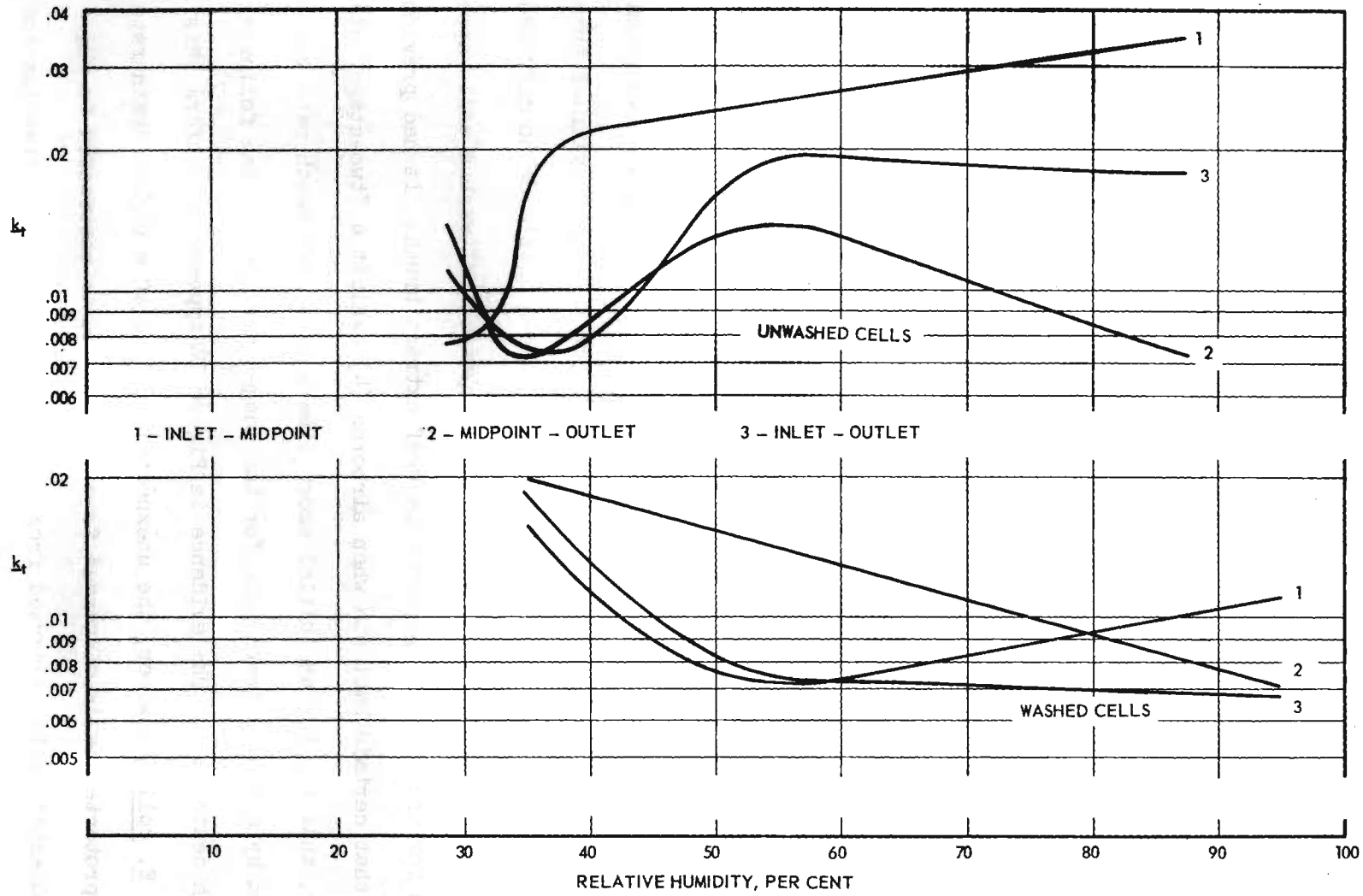

Figure 17. The effect of relative humidity at 17°F on the survival of airborne Escherichia coli (ATCC 10536), both for washed cells dispersed from pure water and for unwashed cells dispersed from beef extract broth for various times of exposure in the aerosol cylinders.

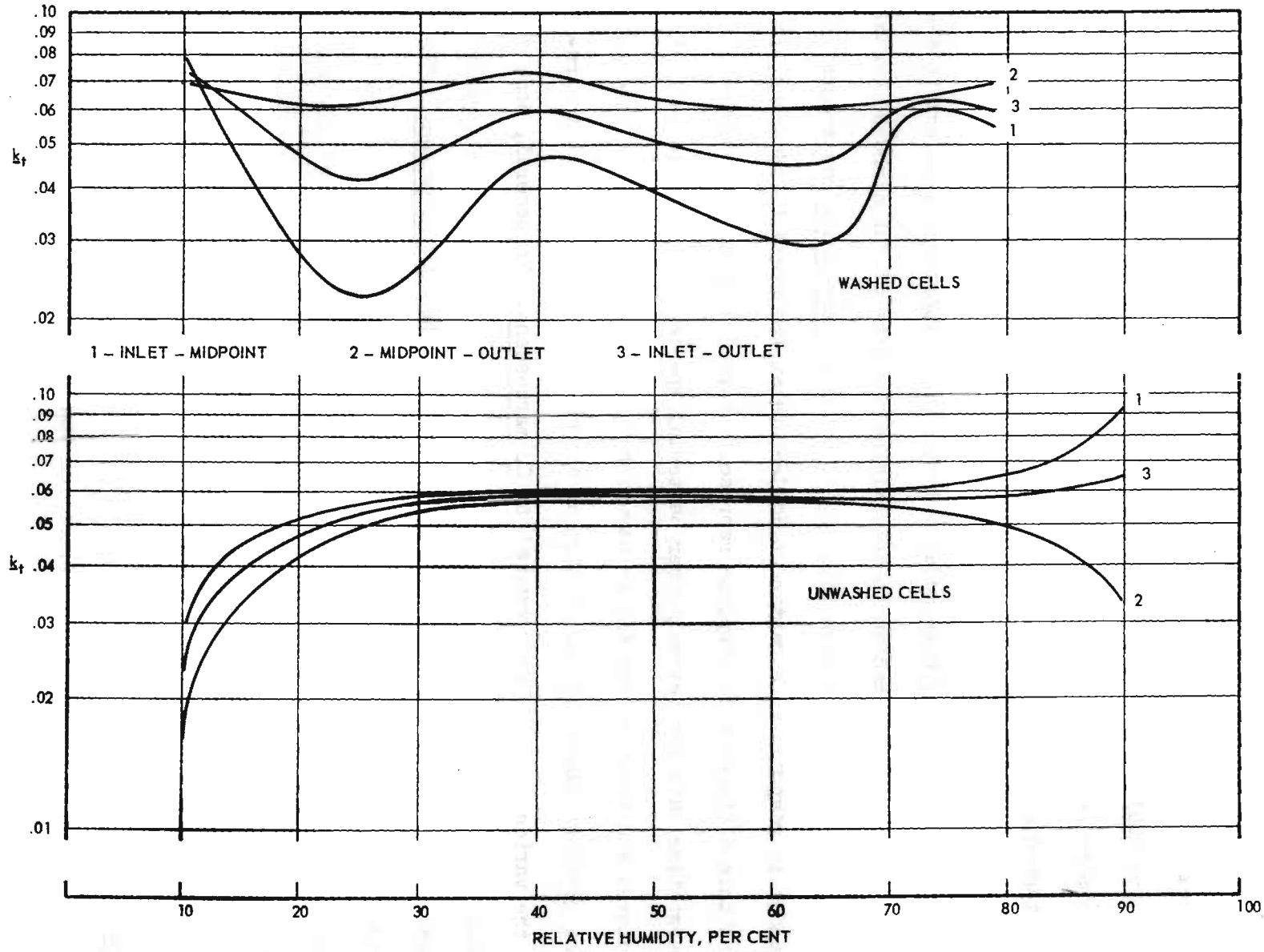

Figure 18. The effect of relative humidity at 68°F on the survival of airborne Escherichia coli (ATCC 10536), both for washed cells dispersed from pure water, and for unwashed cells dispersed from beef extract broth for various times of exposure in the aerosol cylinders.

shown in Figure 18 for both washed and unwashed cells of E. coli at 68°F for various relative humidities. Again, the results obtained are somewhat unexpected, the unwashed cells exhibiting a higher death rate than the washed cells, except at very low relative humidities; this finding being contrary to that for S. marcescens at the same temperature (see Figure 15). Furthermore, although the washed cells exhibit a two-stage die-away, the unwashed cells do not do so, except at very high relative humidities.

The data shown for E. coli have added weight to the importance attached to the composition of the dispersion medium in determining the fate of the airborne organism; just what species difference between this organism and S. marcescens may account for the differences in response is a matter of conjecture at the present. However, it is felt that this difference in response represents a real clue to the problem.

Representative data for several other bacterial species are shown in Table XVIII. The temperatures and relative humidities at which these studies were made were selected to yield the greatest amount of information as to possible differences in the responses of the various species as compared to S. marcescens. In general, none of them exhibit greater death rates under comparable conditions; however, several appear to be even more resistant to the rigors of the aerial state than S. marcescens. The very low values of the death rate obtained at temperatures of 0°F or lower for all of the organisms studied suggests that airborne organisms can exist for long periods of time at very low temperatures.

VI. DISCUSSION

A. Introduction

Interpretation of the results obtained from the study of the fate of airborne bacteria at low temperatures must take into consideration the nature and composition of the bacterial particle, and also give due consideration to the available

information as to the response of bacterial aerosols at ordinary room temperature. That is to say, for bacteria, the aerial state at low temperatures cannot be considered as an isolated and peculiar situation; those bits of information which indicate points of similarity or dissimilarity to other conditions of the aerial state can be expected to be of great value in the interpretation of results.

TABLE XVIII

Response of various organisms in the aerosol cylinders to various conditions of temperature and relative humidity†

Organism	Dry bulb (°F)	Relative humidity (%)	Die-away (k)
S. marcescens E-W/9-28††	32	22	0.016
	10	28	0.022
	- 1	34	< 0.007
	-10	37	0.008
S. marcescens A-W/9-1†††	34	25	0.007
	18	23	< 0.007
	10	28	< 0.007
S. indica	32	24	< 0.007
	32	31	< 0.007
	32	33	0.015
	20	37	< 0.007
	10	39	< 0.007
	0	45	0.013
E. coli	32	34	0.012
	20	30	< 0.007
	10	24	0.014
	- 1	33	0.009
m. pyogenes, var. albus	32	24	< 0.007
	20	31	< 0.007
	17	29	0.007
	17	35	< 0.007
	17	68	< 0.007
	10	68	< 0.007
m. pyogenes var. aureus	34	32	0.010
	10	39	0.011
	0	42	< 0.007

† Specific results not included in previous figures of this section.

†† White, non-mucoid variant of E culture (S. marcescens ATCC 274).

††† White, mucoid variant of A culture (S. marcescens ATCC 274).

In considering the fate of airborne bacteria at low temperatures the following variables have been considered important: relative humidity, temperature, bacterial species, and the composition of the medium from which the organisms are dispersed into the air. Although the composition of the dispersion medium is listed last among the variables, it has been found to be the most important of all, because it determines the nature and composition of the bacterial particle which forms the immediate environment of the airborne bacteria. In the following discussion, consideration of the nature and composition of the bacterial particle is an essential part of the discussion of the other variables.

B. The effect of relative humidity

On the basis of information derived from the studies reported here, and similar studies previously reported (1), it has been shown that the immediate environment of airborne bacteria is the non-living material associated with the particle, rather than the atmosphere surrounding the particle. That is to say, the atmosphere determines the physical response (water content, ice formation, etc.) of the non-living materials, and the response of the bacterium is to these changes effected in the non-living materials, rather than directly to the atmospheric changes. The nature and response to relative humidity of the materials in the dispersion medium are therefore of primary importance in determining the fate of airborne bacteria.

It has been shown that bacteria dispersed into the air from beef broth are covered with a relatively thick layer of beef extract solids. These solids are somewhat hygroscopic and the water content varies considerably with relative humidity (see Table XV). It is believed that it is the response of airborne bacteria to the varying water content of their immediate environment which is responsible for the characteristic death-rate, relative humidity response at room temperatures of airborne bacteria shown in this report (see Figure 15). This same response has been reported by other workers (3). Various explanations have been offered to account for the

higher lethal effect of intermediate humidities on airborne bacteria. At present most of these must be considered of doubtful value in interpreting this phenomenon. Certain information presented in this report (Table XVII) is contradictory to the most promising current concept (3), that an increased salt concentration is responsible for the phenomenon. At present it can only be stated that there appear to be a minimum of two separate effects operating; at very low and very high humidities these seem to be favorable to the survival of the bacteria, and at some intermediate humidity these favorable effects are at a minimum. This intermediate humidity appears to be related to concentration of water in the particle. However, the fact that a similar intermediate humidity-lethality is shown for washed cells (that is, having little or no non-living material in the particle) tends to negate this explanation, unless a very thin layer of non-living materials is assumed.

It should be noted that the general concept that the effect of relative humidity on the survival of airborne bacteria is the result of the interaction of at least two factors suggests a possible explanation for the varying location of the relative humidity at which the maximum lethal response is exhibited. If such factors arise from some indirect response to the physical effects of the atmosphere, then it is not unexpected that at different temperatures the interaction of two factors is not identical. As shown in Figure 15, an intermediate humidity of maximum lethality is demonstrated by each of the curves for the various conditions of temperature.

Until the factors involved are better understood, no real explanation can be offered for the varying effect of relative humidity on the survival of airborne bacteria. However, the concepts suggested here do form a framework upon which a general evaluation of the responses can be built. As temperature is decreased, the relative lethality of an intermediate humidity is diminished, indicating that some activity factor is involved. It would then follow that the shifts in location of the maximum lethal humidity exhibited at lower temperatures are expressions of the

chance lowering of one or the other of the more favorable factors associated with either very high or very low relative humidity. On the basis of this, it is suggested that a uniform and orderly response of the death-rate--relative humidity curve to changes in temperature, composition of the bacterial particle, and variations in species is not necessarily expected. The data at hand appear to substantiate this, as do the variations in data reported by others dealing with the response of airborne bacteria to relative humidity (2, 3, 7, 17).

C. The effect of low temperatures

Applying the above ideas to the probable situation existing in bacterial particles at low temperatures, changes in activity and the presence or absence of ice crystals in the particles seem to be of primary importance. Certainly as the temperature is decreased, a decrease in all kinds of activity, biological as well as physical, is anticipated. Thus, the observed decrease in death rate and effect of relative humidity (note Figure 16) with decreasing temperature can be rationalized. However, it was anticipated that ice formation in the bacterial particle would result in a very high death rate at those temperatures associated with crystallization. This so-called "zone of crystallization" (5) usually is found within the temperature range 30° to 10°F. Materials which can be transferred through this zone in sufficiently short time often vitrify, that is, pass from the liquid to solid state without crystallization (9). The vitreous state is not associated with lethality towards living cells, whereas the crystalline state is (9); although some results contradict this (15)

The information shown in Figure 15 illustrates the search made in attempts to locate a temperature at which some high degree of lethality would be exhibited, presumably associated with the presence of ice crystals in the airborne bacterial particles. The absence of such a condition of temperature in the expected range is further verified by the studies at 17°F with another organism (see Figure 17). It must be concluded that crystallization does not occur in the airborne bacterial particles to

any great extent, or that the crystalline state is not as inherently lethal as it is presently considered. The results obtained in the course of the present study (see Figure 16) conclusively show a direct relationship between temperature and death rate for airborne bacteria; the lower the temperature, the lower the death rate. Although the limitations of the method employed prevent the estimation of extremely small death rates, it is suggested that the information shown in Figure 16 indicates that, with the possible exception of certain relative humidities, the life expectancy of airborne bacteria at low temperatures is very long.

D. Species difference

In the present study a partial study was made of possible species differences involved in the response of airborne bacteria to low temperatures. Although some very interesting results were obtained at intermediate temperatures, for temperatures at 0°F or lower the observed death rates for all organisms studied were so small that the present method was incapable of distinguishing any differences. In general, no real difference was found between the response of the various organisms and that of the initial test organism (S. marcescens). The only organism which showed perceptibly less resistance to the airborne state than did S. marcescens was E. coli. However, this variation in resistance to the airborne state was not peculiarly characteristic of the low temperatures, both organisms exhibiting very small death rates under these conditions. At 68° and 17°F. the airborne E. coli (Figures 16 and 17) dispersed into the air from beef broth exhibited a considerably higher death rate than did S. marcescens under the same conditions (Figure 15). On the basis of this observation, it is suggested that a difference in death rates does exist at low temperatures for these two organisms; a more sensitive method than that given in this report would be necessary to verify or deny this.

Although no real information was obtained as to the significance of species variation in the survival of airborne bacteria at low temperatures, the differences

in response of S. marcescens and E. coli recommend these two organisms for use in further studies of this subject. It was originally felt that the nature of the surface of the organisms under study was a primary factor in determining the fate of airborne bacteria at low temperatures. Thus, it was expected that a mucoid variant would be especially long lived, because of the protective nature of the surface material adherent to the cells. However, it is now believed that the nature and composition of any non-living material in the bacterial particle serves this same purpose, and that species differences which are observed are not those peculiar to the airborne state, but are such as might be found under any condition.

E. Effect of varying the composition of the dispersion medium

The studies on E. coli discussed above are interpreted as indicating that at temperatures above or below freezing, beef extract solids do not form as favorable a medium for the survival of E. coli in the air as they do for S. marcescens. The washed cells of the latter exhibit a greater death rate than do those covered with broth solids; however, for E. coli the opposite is the case. As suggested above, this is believed due entirely to the difference in response of the organism to its immediate environment. Thus, in a more favorable immediate environment (formed from the dispersion medium), E. coli could be presumed to exhibit death rates more comparable to those exhibited by S. marcescens under the same conditions of temperature and relative humidity.

This observation, like several previously made in this section, points up the importance of the composition of the dispersion medium in determining the fate of airborne bacteria under any set of conditions. The variations in response effected by differences in temperature, relative humidity and species all are strongly modified by the composition of the dispersion medium. The similarity of composition and of response to relative humidity of the solids of beef extract broth and of human saliva (7) indicates that the information given here is generally applicable to many of the naturally occurring airborne bacteria.

VII. CONCLUSIONS

At low temperatures the death rate of airborne bacteria is markedly lower than that observed at ordinary room temperatures. However, the effect of various other factors on the fate of airborne bacteria at low temperatures is not dissimilar from that found at the higher temperatures; the difference appears to be one of degree rather than of kind. Within the limitations of the methods used, the data obtained indicate that relative humidity, organism studied, and composition of the bacterial particles are the significant factors under any condition of temperature, the last being most important.

Furthermore, the finding that the response of the airborne bacterium to various relative humidities is primarily determined by the composition of the non-living material in the airborne particle suggests that the responses of airborne organisms can be understood more thoroughly in terms of the response of the organism to this material.

The strong degree of similarity between saliva and the beef broth used in the preparation of experimental bacterial aerosols suggests that the findings of this report are applicable to many naturally occurring bacterial aerosols. The very small death rate observed for such bacteria under conditions of low temperature verifies the assumption that viable bacteria reported in the upper atmospheres must have a long life expectancy.

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