NASA CR 66548
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A STUDY OF ASEPTIC MAINTENANCE BY PRESSURIZATION

PREPARED UNDER CONTRACT NAS 1-7166
FOR
NATIONAL AERONAUTICS AND SPACE ADMINISTRATION

GENERAL ELECTRIC

REENTRY SYSTEMS

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MISSILE AND SPACE DIVISION

RE-ENTRY SYSTEMS DEPARTMENT

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SUBJECT: CONTRACT NAS 1-7166
REPORT ENTITLED "A STUDY OF ASEPTIC MAINTENANCE BY PRESSURIZATION"

REFERENCE: NASA CONTRACTOR REPORT NO. 66548

Gentlemen:

The subject report is transmitted in accordance with instructions of the Contracting Officer, National Aeronautics and Space Administration, Langley Research Center, Langley Station, Hampton, Virginia, under Contract NAS 1-7166.

Sincerely yours,

GENERAL ELECTRIC COMPANY

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

SUMMARY

SUMMARY

A study of aseptic maintenance by pressurization has been conducted for the Langley Research Center of the National Aeronautics and Space Administration under contract NAS 1-7166. In the course of the study, a theoretical analysis of the characteristics of gas flow through microscopic holes in thin membranes demonstrated that a pressure differential across a membrane separating two quiescent gas chambers should prevent migration of microorganisms through a single microscopic hole against that pressure gradient. Preliminary experimental verification of the theoretical analysis was obtained for holes ranging from 19 μ to 228 μ in diameter in .012 and .030 inch thick membranes of polypropylene and aluminum. Spores of Bacillus subtilis var. niger were presented by gravity to the hole from an aerosol above the membrane, and were captured in the event of passage through the hole on agar medium in a tube located directly below.

Forty-nine tests with pressure differentials ranging from 0.25 inches to 5.0 inches of water resulted in total exclusion of microorganisms from the detection medium. However, experimental conditions producing turbulence or high flow rates in either the aerosol or detection chambers resulted in penetration of spores against pressure differentials of 0.5 and 5.0 inches of water by mechanisms not yet explained. No differences could be detected in the exclusion or penetration of microorganisms as a result of the variables involved, namely,

a - Hole size

b - Membrane thickness

c - Membrane material.

The similarity of behavior of the polypropylene and the aluminum membranes indicates that electrostatic charge accumulation did not significantly affect the performance of the system.

During the study, some difficulty was experienced with extraneous contamination giving the appearance of penetration of holes by microorganisms migrating against the pressure gradient. Diagnostic tests have proved that procedural flaws existed and require that one discount growth in the detection chamber in tests run prior to revision of the test procedures.

The experimental data substantiate the theory that very low pressure differentials effectively exclude passage of microorganisms against the pressure gradient through microscopic holes as long as the atmosphere on either side of the hole is quiescent. Flow disturbances on either side of the membrane may increase the pressure differential required to prevent passage of the aerosol through the hole.

The larger holes, (100 μ or greater) examined in the course of this study are readily detectable by state-of-the-art gas leak detectors, even against the background of diffusion through materials anticipated in the biological isolator suit sytem (BISS). It appears, therefore, that protection against passage of microorganisms thru microscopic holes by differential pressures offers a practical solution to assuring the integrity of the BISS, assembly/sterilizer, or sterilization canister if the influence of the flow disturbances described above can be overcome.

INTRODUCTION

1. INTRODUCTION

1.1 BACKGROUND

The scientific requirement to maintain a biological quarantine of the planets until the existence and nature of living forms on these extraterrestial bodies is determined requires the sterilization, and maintaining the sterility, of the planetary entry vehicles to be used in the explorations. Conventional methods of sterilization employed by commerce and the pharmaceutical industry suggest that the entry vehicle be enclosed in a canister and the combination be heat sterilized. This approach imposes two obligations on the engineering community.

First, the canister used to maintain the sterility of the planetary entry vehicle must provide a perfect barrier, after sterilization, against contamination from dust, microorganisms and other viable contaminants. The canister, however, being subjected to the rigors of manufacture, assembly and sterilization plus the requirements of depressurization and separation, may have microscopic holes present. These holes provide a route for recontamination of the entry vehicle.

Second, either the adjustment, repair and checkout of the entry vehicle be accomplished without violating lander sterility, or the heat sterilization cycle must be repeated. The assembly/sterilizer concept proposes a facility which would permit opening the canister and subsequent repair and checkout of the entry vehicle in a sterile environment. This facility, like the canister itself, is subject to the probability of microscopic holes permitting the passage of viable contaminants thru wall, joints and door seals and the recontamination of the entry vehicle.

Supplemental means therefore are required to protect against the passage of microorganism thru small holes to assure the sterility maintenance of a planetary entry vehicle. Accordingly, NASA programs for planetary exploration contemplate the use of pressurization to totally exclude microorganisms from the canister and the assembly/sterilizer. In each application, internal pressurization to a few

inches of water above the ambient atmospheric pressure has been projected to protect against admission of microorganisms through accidental or unintentional microscopic holes in the microbial barrier.

The control of air movement has been employed for many years in industrial and hospital practices to assist in excluding microorganisms from closed or semi-closed systems. In most applications, it was desired to maintain a low population density of microorganisms in a room area, or prevent the escape of potential pathogens from a room or enclosure. By adjusting air pressure in individual rooms or enclosures, control of the microbial population densities has been achieved (1, 2). In developing techniques to maintain asepsis in closed systems, investigators adopted the use of a positive pressure differential to achieve an outward gas flow through any leak to insure the biological integrity of the system. Thus gnotobiotic animal laboratories and aseptic filling facilities in pharmaceutical houses use differential pressure to assist in excluding microorganisms from sterile systems. The success of such facilities in maintaining sterility of a system has been reported. However, the actual benefit accrued from maintaining a differential pressure (Δ P) across a barrier had not been established, nor had the magnitude of the Δ P required been determined (3).

The program described herein was directed at this problem, the Δ P required for the sterility maintenance of a planetary entry vehicle. The data evolved, however, will be applicable to many situations where it is desired to exclude or contain microorganisms.

An investigation of feasibility of sterile insertion using gnotobiotic techniques with flexible film isolators has been conducted (4). However, the study in reference (4) was aimed at determining the penetration of microorganisms against a positive pressure gradient with holes in vertically oriented surfaces.

Since the holes investigated were not oriented to be susceptible to the effect of gravity deposition, it did not represent as stringent a challenge to the pressure gradient as the AMP Program.

Preliminary theoretical calculations which were performed in preparation for this program indicated that pressurization will be an effective active means for preventing transmission of microorganisms through small holes through which gas flow is laminar. It was not known for large holes, whether the laminar flow would be established in short lengths due to pronounced entrance and exit effects which produce countercurrent molecular or particulate migration in the region of the boundary layer. In addition, the irregular shapes and dimensions of holes found in the practical situations make theoretical analysis very difficult. For this reason, analytical and experimental evaluation of simplified cases was required.

The intent of the present program was to screen a matrix of pressure differentials and hole dimensions thereby establishing regimes for more extensive testing to gain a high degree of confidence in the pressurization technique for protecting sterile chambers. It also evaluated the simplified case of near-cylindrical holes experimentally and analytically.

1.2 PROGRAM FORMULATION

The AMP Program was formulated to evaluate the influence of several parameters on the transmission of airborne microorganisms through small holes. The parameters of particular interest included: hole diameter, thickness of membrane, pressure differential, and electrostatic charge. In addition to providing data relative to the effectiveness of pressure differentials in excluding microorganisms, the study evaluated a method for detecting the passage of microorganisms through such a membrane.

A viable culture technique was used for this determination. Lyophilized spores were employed to obtain maximal viability and minimal clumping of the aerosol, thereby achieving potentially ultimate sensitivity. The aerosol was presented to the hole by gravity, the terminal velocity of a falling microbe exceeding other velocity vectors and thereby presenting the greatest challenge. The membrane was interposed in a horizontal plane between the aerosol and the detection chamber so that the hole was centrally located above the detection medium (Figure 5). As a result, microorganisms transgressing the hole were deposited on the culture medium by gravity. The volume of the detection chamber was large enough to facilitate pressure control and minimize air velocity, but small enough to permit rapid deposition of spores on the agar surface (See Table I). This experimental design afforded maximal sensitivity as a single viable spore entering the chamber could be detected.

The pressure differentials across the membrane were maintained by supplying sterile air to the detection chamber. To this end, Millipore filters were employed in the supply and manometer lines to insure a sterile gas supply.

A high population of aerosolized spores (ca 8 x 10^9) was employed to present a severe challenge to the system so that short test periods could be used to obtain meaningful results. After several stages of experiment development, a 72 hour exposure period with the hole open to the aerosol was found suitable and provided reliable data.

TABLE I SETTLING RATES OF AIRBORNE PARTICLES

Diameter of Particles (Microns)	Velocity of Settling	
	Feet per Minute	Inches per Hour
0.1	0.00016	0.115
.2	.00036	.259
.4	.0013	.936
.6	.002	1.44
.8	.005	3.60
1.0	.007	5.04
2.0	.024	17.3
4.0	.095	68.4
6.0	0.21	
8.0	0.38	
10	0.59	
20	2.4	
40	9.5	
60	21.3	
80	37.9	
100	59.2	
200	352	
400	498	

NOTE: This table has been compiled from "Size and Characteristics of Air-Borne Solids", by W.G. Frank, published in the Smithsonian Meteorological Tables. Rates are for particles in the shape of spheres, having a specific gravity of 1.0 and settling in air at a temperature of 70°F. Although the spores used in this study were ellipsoidal, the data above afford a reasonable approximation of expected settling rates for adequate experiment design.

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3. "Hospital Use of Sterile Patient Isolators" Contamination Control, 5, (1966), p. 40.

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ANALYTICAL CONSIDERATION

2. Analytical Consideration

Introduction

In the experimental investigation of bacterial contamination and, specifically, bacterial penetration of small openings, it is of interest to discover whether such penetration is possible on the theoretical grounds. For this reason an analytical study was performed of various postulated modes of motion of single bacterial spores and of the gas flow through small holes under the conditions of the experiments. Thus the transport and motion of spores due to concentration gradient (diffusion), due to gravity (settling), and due to thermal motion (Brownian motion) were investigated; the flow of gases through the membrane holes were calculated for each experimental membrane thickness, hole diameter, and pressure drop, and detailed calculations were made of velocity profiles for two types of jets issuing from the hole outlet, the laminar jet and the turbulent jet.

2.1 Diffusion

The laws of diffusion in gases are well known. If these laws are applied to the bacterial spores some insight should be gained as to the diffusion rates of such spores when dispersed in air. For the purpose of numerical demonstration of the phenomena in question, the following parameters are assumed:

Circular opening — 10 microns diameter, 50 microns long, gas (air) at one atmosphere and room temperature, spores — 1.0 micron diameter spheres of 1.0 g/cc density, and spore concentration — 10^9 per g-mol of air. Using the above values, the mass of a single bacterium is

$$m_b = 5 \times 10^{-13} g,$$

and the average mass of air "molecule",

$$m_a = 5 \times 10^{-23} g.$$

The diffusion coefficients are inversely proportional to the square root of mass.

Thus,

$$\frac{D_b}{D_a} = \sqrt{1.0 \times 10^{-10}} = 1.0 \times 10^{-5}.$$

This indicates that the diffusion rate of a one micron bacterial spore is only 1.0×10^{-5} that of air. For equimolar, steady state counterdiffusion of air the diffusion rate is

$$N_a = \frac{D_a}{RTL}(p-p_i)$$

where

N_a is the diffusion rate of air, g-mols/sec-cm^2

D_a is the diffusion coefficient of air, 0.2 cm^2/sec at 25°C

R is the gas constant, 82 cc-atm/g-mol-°K

T is the absolute temperature, °K

L is the tube length, 50 microns

p and p_i are partial pressures of the diffusing species

($p = 1$ atm, $p_i = 0$).

Substituting the proper values in the above equation,

$$N_a = 1.64 \times 10^{-3} \text{ g-mols/sec-cm}^2.$$

Then for 10 micron diameter openings the diffusion rate of air is

1.29×10^{-9} g-mols/sec, or

7.7×10^{14} molecules/sec.

The diffusion rate of bacteria will be smaller by the factor

$$1.0 \times 10^{-5} \times \frac{1.0 \times 10^{9}}{6 \times 10^{23}} \text{ or } 1.7 \times 10^{-20}$$

reflecting the fact that the diffusivity of bacteria is 1.0×10^{-5} smaller than that of air and that one gram-mol of air contains 1.0×10^{9} spores. The number of bacteria expected to diffuse through the opening in question is then

$7.7 \times 10^{14} \times 1.7 \times 10^{-20} = 1.3 \times 10^{-5}$ spores/sec, or

1.1 spores/24 hrs.

2.2 Settling

Settling due to the gravitational force is an another type of motion which may cause penetration of biological barriers through small holes under favorable geometry, i.e., the barrier in the horizontal plane with spore-laden gas column above it. Under these conditions it is of interest to calculate the settling velocity of the spore and the minimum pressure drop across the barrier to prevent its penetration by the spore.

At 1 atmosphere and 25°C, one may readily calculate the terminal velocity of a settling particle by applying Stoke's law:

$$u = gd^2 \frac{\rho - \rho_a}{18\mu}$$

where, in c.g.s. units

u is the terminal particle velocity, cm/sec

g is the acceleration due to gravity, 980 cm/sec^2

d is the bacterial diameter, 1×10^{-4} cm

ρ is the bacterial density, 1.0 g/cm^3

μ is the viscosity of air, 1.8×10^{-4} poises.

Employing the above values, the terminal velocity of a bacterium is

$$u = 980 \times (1 \times 10^{-4})^2 \times 1.0/(18 \times 1.8 \times 10^{-4})$$

$$= 3.0 \times 10^{-3} \text{ cm/sec.}$$

Assuming bacterial spore concentration of 10^9 spores/g-mol of air above a membrane with a 10 micron diameter hole, this terminal velocity would result in penetration of the membrane at a rate of 1.1×10^{-4} spores/sec or 10 spores/24-hr day.

For a 10 micron diameter, 50 micron long hole, the gas flow at this velocity is governed by the Hagen-Poiseuille equation

$$\Delta p = 32 \frac{uL\mu}{d^2}$$

where

Δp is the pressure drop

μ is the viscosity of air

d is the hole diameter

L is the hole length.

Employing the value of spore terminal velocity, $u = 3.0 \times 10^{-3}$ cm/sec calculated above, and the given hole size

$$\Delta p = 32 \times 1.8 \times 10^{-4} \times \frac{5 \times 10^{-3}}{10^{-6}} \times 3.0 \times 10^{-3}$$

$$= 8.6 \times 10^{-2} \text{dynes/cm}^2$$

$$= 3.5 \times 10^{-5} \text{inches } H_2O.$$

Thus negligible pressure drop should be required to prevent membrane penetration by bacterial spores.

2.3 Brownian Motion

In addition to settling due to gravity and diffusional flow microbial spores of about one micron size may undergo random thermal motion commonly known as Brownian motion. From the principle of equipartition of energy at equilibrium the spores will have the same average energy as the surrounding gas molecules. This energy equals 3/2 kT ergs/molecule or particle. From statistical mechanics consideration it may be demonstrated that the energy of molecules (particles) is normally distributed with the

standard deviation equal to $\sqrt{3/2}\ kT$. If this energy is essentially the kinetic energy, then

$$\frac{1}{2} mu^2 = \frac{3}{2} kT, \text{ and } u = \sqrt{\frac{3kT}{m}},$$

where

k is Boltzmann constant, 1.38×10^{-16} ergs/°K

T is absolute temperature, °K

u is velocity, cm/sec

m is particle mass, gms.

Thus the average energy per spore at room temperature (25°C) is

$$E = \frac{3}{2} kT = 6.2 \times 10^{-14} \text{ergs}$$

with the standard deviation

$$\text{s.d.}(E) = \sqrt{\frac{3}{2}}\, kT = 5.0 \times 10^{-14} \text{ergs.}$$

One micron spore of assumed density of 1.0 has mass

$$m = \frac{\pi}{6} \times (10^{-4})^3 = 0.52 \times 10^{-12} \text{gms.}$$

Its average velocity is

$$u = \sqrt{\frac{3kT}{m}} = 0.48 \text{ cm/sec.}$$

At seven standard deviations from the average only one spore in a population of 10^{12} spores is expected to have energy in excess of

$$\left(\frac{3}{2} + 7\sqrt{\frac{3}{2}}\right) kT = 10.2\ kT = 4.2 \times 10^{-13} \text{ergs,}$$

with the corresponding velocity

$$u = 1.26 \text{ cm/sec.}$$

By Stoke's law the surrounding air offers resistance to the spore motion

$$F = 3\pi\mu u d,$$

where

F is the retarding force

μ is the viscosity of air

u is the spore velocity

d is the spore diameter.

Equating spore deceleration to the force F

$$m \frac{du}{dt} = -3\pi\mu u d$$

gives spore velocity

$$u = u_o \exp(- \frac{3\pi\mu d}{m} t)$$

from which the distance traveled by the spore is

$$x = \frac{u_o m}{3\pi\mu d} (1 - \exp(- \frac{3\pi\mu d}{m} t)).$$

Substituting for t from the equation for spore velocity gives

$$x = \frac{m}{3\pi\mu d} (u_o - u).$$

The maximum distance traveled by a spore ($u = 0$) for the initial velocity $u_o = 0.48$ cm/sec, corresponding to the average kinetic energy of the spore, is

$$x = \frac{0.52 \times 10^{-12}}{3\pi \times 1.85 \times 10^{-4} \times 10^{-4}} \times 0.48 = 1.45 \times 10^{-6} \text{cm},$$

and one spore in 10^{12} is expected to travel a distance of

$$x = 3.8 \times 10^{-6} \text{cm}.$$

This distance is only a small fraction of the spore diameter.

The above analysis indicates that Brownian motion of spores whose diameter is on the order of one micron is practically non-existent and may be safely disregarded in discussion of spore motions.

Of all the spore motions those due to settling are most pronounced. For this reason a barrier in the horizontal position presents a more challenging case to bacterial penetration than in any other orientation.

2.4 Analysis of Gas Flow

2.4.1 Flow through holes

Under the conditions of the experiments performed in this study the gas flow through the membrane holes belongs to the laminar regime (Hagen-Poiseuille type of flow) and the head loss may be expressed by the formula

$$F_1 = 32 \frac{Lu\mu}{D^2\rho}$$

where

L is the membrane thickness

u is the gas velocity

μ is the gas viscosity

D is the hole diameter

ρ is the gas density.

To this head loss must be added fluid head loss due to the contraction of flow from the large gas reservoir, where gas velocity is essentially zero, to the flow conditions in the hole itself. This head loss is given by the expression

$$F_2 = K \frac{u^2}{2},$$

where

$$K = 0.4\left(1.25 - \frac{A_2}{A_1}\right)$$

and

A_1 is the cross-sectional area of the inlet reservoir,

A_2 is the cross-sectional area of the hole.

In our case $\frac{A_2}{A_1} \approx 0$

and, therefore,

$$K = 0.4 \times 1.25 = 0.5$$

giving

$$F_2 = 0.25u^2.$$

Combining these two head losses

$$\frac{\Delta p}{\rho} = F_1 + F_2 = \frac{32L\mu\mu}{D^2\rho} + 0.25u^2,$$

where

Δp is the pressure drop across the membrane

gives the following expression for gas velocity

$$u = 2\left[\sqrt{\left(\frac{32L\mu}{D^2\rho}\right)^2 + \frac{\Delta p}{\rho}} - \frac{32L\mu}{D^2\rho}\right]$$

Table II lists gas velocities, volumetric flow rates (Q), and Reynolds numbers for all the combinations of membrane thickness, hole size, and pressure drop employed in the experimental investigations.

The above expression is valid when the flow is fully developed into parabolic flow which is characteristic of the laminar flow.

TABLE II
AIR FLOW CHARACTERISTICS

Hole Dimensions		ΔP=0.5" H_2O			ΔP=2.0" H_2O			ΔP=5.0" H_2O			
Thickness, Mils	Diameter Microns	u_{ave}, cm/sec	Q, cc/sec	Re	u_{ave}, cm/sec	Q, cc/sec.	Re	u_{ave}, cm/sec	Q, cc/sec	Re	L/D
12	19	24.6	6.97×10^{-5}	0.299	98.4	2.79×10^{-4}	1.20	246	6.97×10^{-4}	2.99	16.0
12	21	30.1	1.04×10^{-4}	0.404	120.2	4.16×10^{-4}	1.61	300	1.04×10^{-3}	4.03	14.5
12	31	65.5	4.94×10^{-4}	1.30	261	1.97×10^{-3}	5.18	649	4.90×10^{-3}	12.8	9.8
12	48	156	2.82×10^{-3}	4.80	614	1.11×10^{-2}	18.9	1488	2.70×10^{-2}	45.7	6.3
12	99	609	4.69×10^{-2}	38.6	2020	1.55×10^{-1}	128	4050	3.12×10^{-1}	256	3.1
30	50	68.1	1.34×10^{-3}	2.18	272	5.34×10^{-3}	8.68	674	1.32×10^{-2}	21.6	15.2
30	90	218	1.39×10^{-2}	12.6	846	5.38×10^{-2}	48.7	2000	1.27×10^{-1}	115	8.5
30	100	268	2.10×10^{-2}	17.1	1020	8.01×10^{-2}	65.4	2360	1.85×10^{-1}	151	7.6
30	109x139	397	4.72×10^{-2}	31.2	1440	1.71×10^{-1}	114	3140	3.73×10^{-1}	247	6.2
30	117x141	427	5.49×10^{-2}	35.0	1540	1.98×10^{-1}	126	3300	4.24×10^{-1}	270	6.0
30	115x145	433	5.65×10^{-2}	35.8	1550	2.02×10^{-1}	128	3330	4.35×10^{-1}	275	5.9
30	190	824	2.33×10^{-1}	100	2480	7.02×10^{-1}	302	4680	1.33	568	4.0
30	228	1040	4.24×10^{-1}	152	2870	1.17	418	5150	2.10	751	3.3

Schiller* has shown both, theoretically and experimentally, that such parabolic flow is established a number of channel diameters downstream from the entrance. This distance is a function of the Reynolds number $Re = \frac{Du\rho}{\mu}$ of the flow. Specifically,

$$\frac{x}{D} = 0.029\ Re$$

where

x is the channel length.

At low pressure drops ($\Delta p = 0.5$ in H_2O) and small hole sizes ($D \leq 50$ microns) parabolic flow is established very near the entrance, at larger pressure drops and for larger hole sizes the parabolic velocity distribution is either established quite a distance from the inlet or not at all. To obtain complete solutions for these latter cases would require numerical integration of the governing equations which were derived with a number of simplifying assumptions, whose validity would also have to be analyzed. As some errors in the calculation of the relatively large velocities are more easily tolerated than of the small velocities the flows through large holes and at large pressure drops were also calculated using the above equations, with an understanding that the calculated values may be somewhat too low.

In gas flow through holes the velocity at the wall is zero and the velocity profile for the laminar flow is given by the expression

$$u = u_{max}\left(1 - \frac{y^2}{R^2}\right)$$

*Schiller, L., Forsch. a.d. Geb. Ing.-Wesen, No. 248 (1922).

where

u_{max} is the gas velocity along the axis

y is the radial distance

R is the hole radius.

At a distance of one micron from the hole wall in a 19 micron diameter hole the gas velocity is

$$u = 0.2u_{max} = 0.4u_{ave},$$

and in a 50 micron diameter hole the gas velocity is

$$u = 0.08u_{max} = 0.16u_{ave}.$$

An inspection of velocity data listed in Table 1 shows that any spore near the wall of either 19 or 50 micron hole (and, by an easy calculation, of the holes of all the sizes listed) would be rapidly swept out of the hole by the gas stream whose velocity one micron from the wall is several orders of magnitude greater, even at a pressure drop of 0.5 inch of water, than the settling velocity of the spore previously calculated to be 3×10^{-3} cm/sec.

2.4.2 Exit flow

Gas leaving a small circular opening issues as a jet. This jet may be either laminar or turbulent depending on the Reynolds number of the flow through the opening. For the jet to be turbulent the Reynolds number

$$Re = \frac{Du}{\nu}$$

where

D is the hole diameter

u is the average gas velocity through the hole

ν is the kinematic viscosity of the gas

must exceed 30.

Taking x and y as axial and radial coordinates, respectively, the governing system of differential equations for the laminar jet is

$$u \frac{\partial u}{\partial x} + v \frac{\partial u}{\partial y} = \nu \frac{1}{y} \frac{\partial}{\partial y} \left(y \frac{\partial u}{\partial y} \right)$$

and

$$\frac{\partial u}{\partial x} + \frac{\partial v}{\partial y} + \frac{v}{y} = 0,$$

where

v is the radial velocity.

Assuming a constant pressure in the exit chamber the flux of momentum in x direction is constant.

$$J = 2\pi\rho \int_0^\infty u^2 y dy = \text{const.}$$

where

J is the momentum flux

ρ is the gas density.

For the boundary conditions

$$v = 0 \text{ and } \frac{\partial u}{\partial y} = 0 \text{ at } y = 0$$

$$u = 0 \qquad \text{at } y = \infty$$

the solution for the axial and the radial velocities is

$$u = \frac{\nu}{x} \frac{2\gamma^2}{\left(1 + \frac{1}{4}\xi^2\right)^2}$$

$$v = \frac{\nu}{x} \gamma \frac{\xi - \frac{1}{4}\xi^3}{\left(1 + \frac{1}{4}\xi^2\right)^2}$$

where

$$\xi = \gamma y / x$$

and the constant of integration γ can be calculated from the given value of momentum.

$$J = 2\pi\rho \int_0^\infty u^2 y dy = \frac{16}{3} \pi\rho\gamma^2\nu^2$$

or kinematic momentum $K = \frac{J}{\rho}$,

$$K = 2\pi \int_0^\infty u^2 y dy.$$

In our case (parabolic velocity distribution)

$$u = u_{max}\left(1 - \frac{y^2}{R^2}\right) \qquad 0 \leq y \leq R$$

$$u = 0 \qquad y > 0$$

where

R is the hole radius

u_{max} is the maximum velocity

giving for the kinematic momentum

$$K = \frac{\pi}{3} u_{max}^2 R^2 = \frac{\pi}{12} u_{max}^2 D^2$$

and the expressions of the axial and the radial velocities

$$u = \frac{u_{max}^2 D^2}{32\nu x} \frac{1}{\left(1 + \frac{1}{4}\xi^2\right)^2}$$

and

$$v = \frac{u_{max} D}{8x} \frac{\xi - \frac{1}{4}\xi^3}{\left(1 + \frac{1}{4}\xi^2\right)^2}$$

with

$$\xi = \frac{u_{max} D}{8\nu} \frac{y}{x} .$$

For the turbulent jet, both the differential equations and the solutions are identical with one exception, namely, the kinematic viscosity of the laminar jet is replaced with the experimentally determined virtual kinematic viscosity

$$\epsilon = 0.0161\sqrt{K.}$$

In our case

$$\epsilon = 0.00824 u_{max} D.$$

The equations of solutions for the laminar and turbulent jets were programmed on the General Electric 605 computer and numerical solutions were obtained for a typical laminar jet (L = 30 mils, D = 50 microns, u_{ave} = 68 cm/sec, and Re = 2.18) and for a typical turbulent jet (L = 30 mils, D = 190 microns, u_{ave} = 4675 cm/sec, and Re = 568). The computer program in Fortran language and sample calculations are given in Appendix B.

The numerical results of the above calculations are shown in graph form in Figures 1, 2, 3, and 4. From these figures a conclusion may be drawn that the exit jets of air behave regularly, with axial velocity decreasing inversely with the distance from the opening and radial velocity being positive in the jet flare and negative in the regions in which air is drawn into the jet. The negative radial jet velocity occurs, however, some distance from the exit.

These results strongly suggest that bacterial spores would find an impenetrable barrier in the air jet, laminar or turbulent, to their approach even to the exit area of the membrane hole.

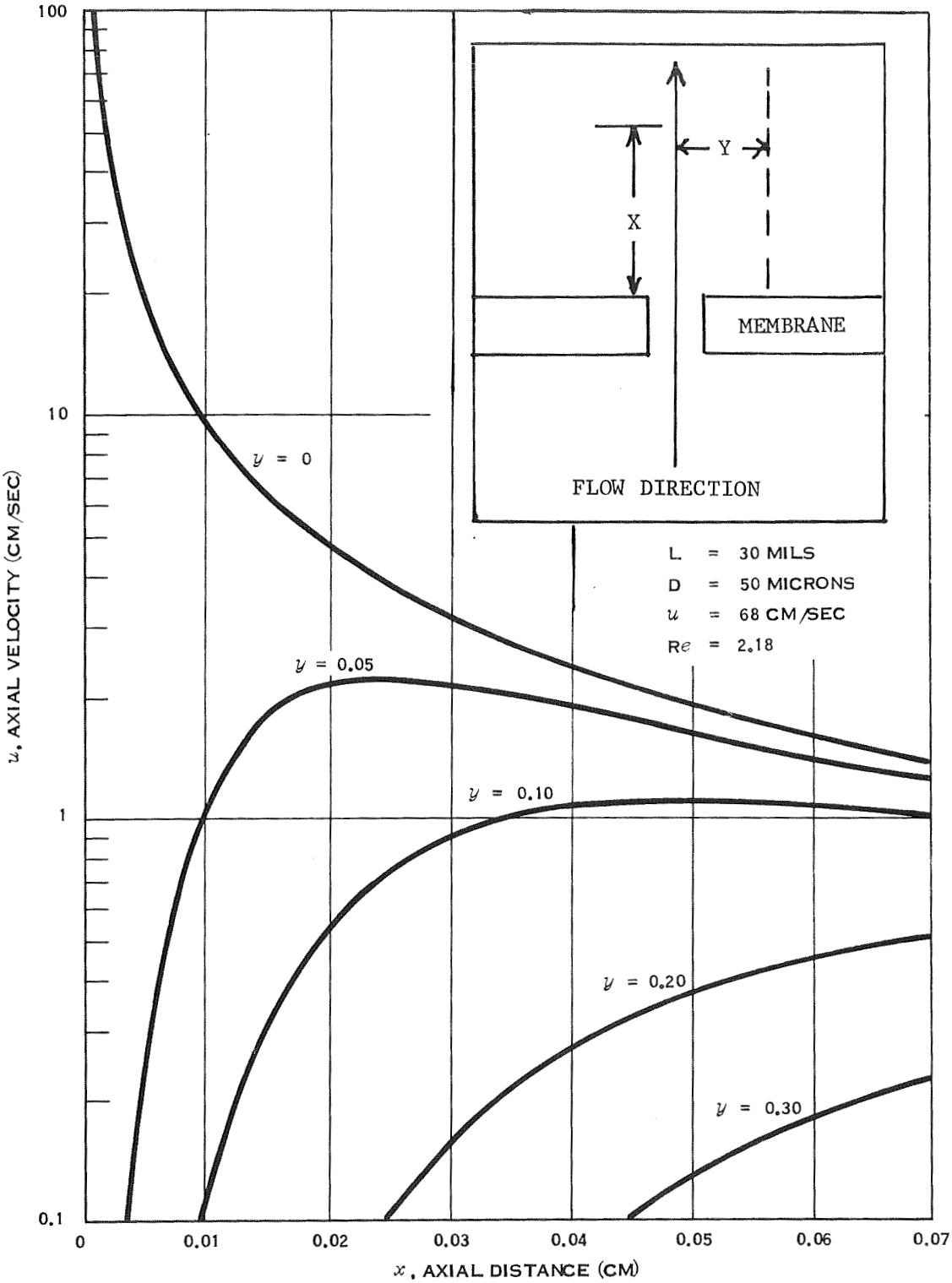


Figure 1. Axial Velocity Laminar Jet

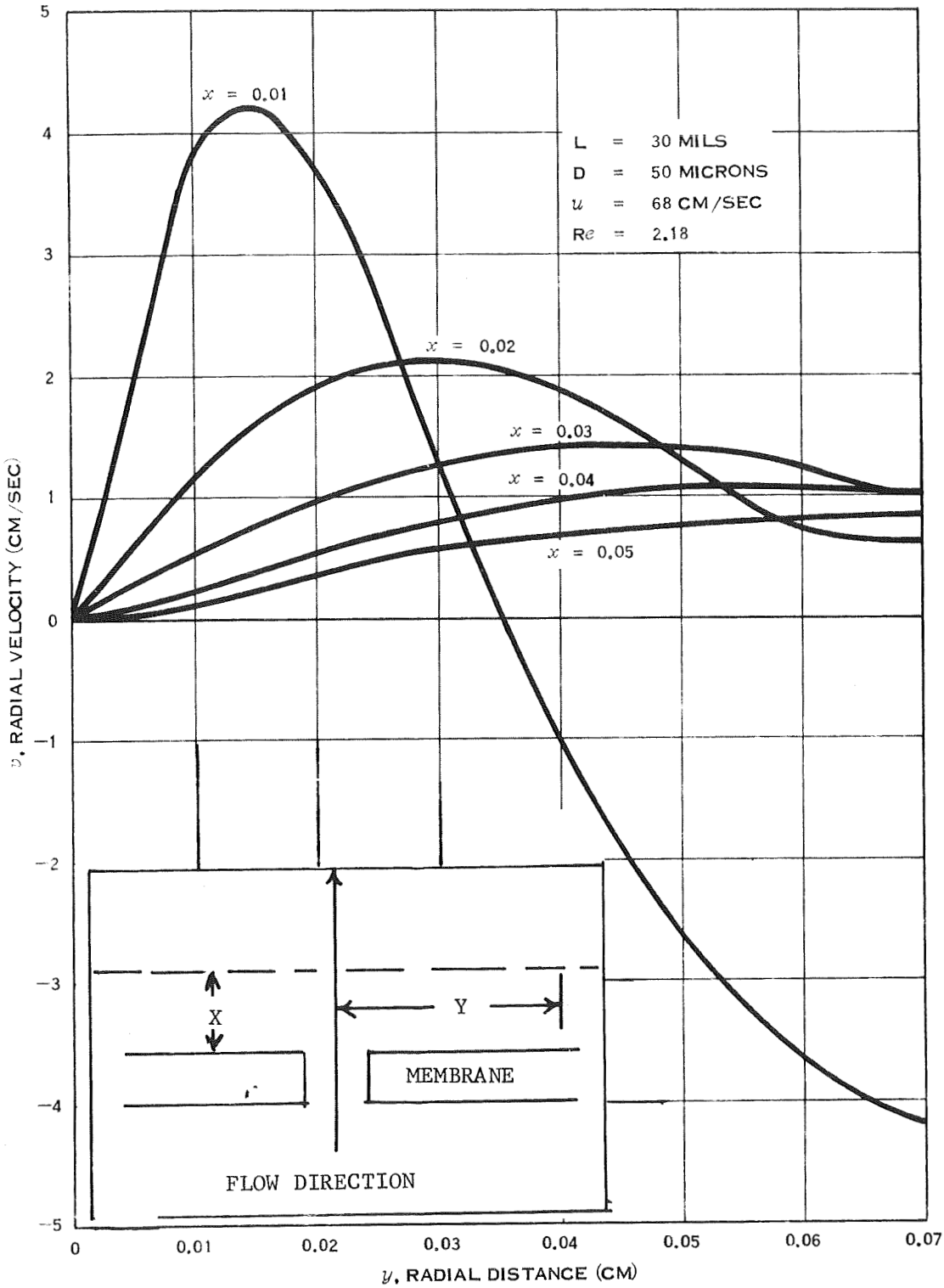


Figure 2. Radial Velocity Laminar Jet

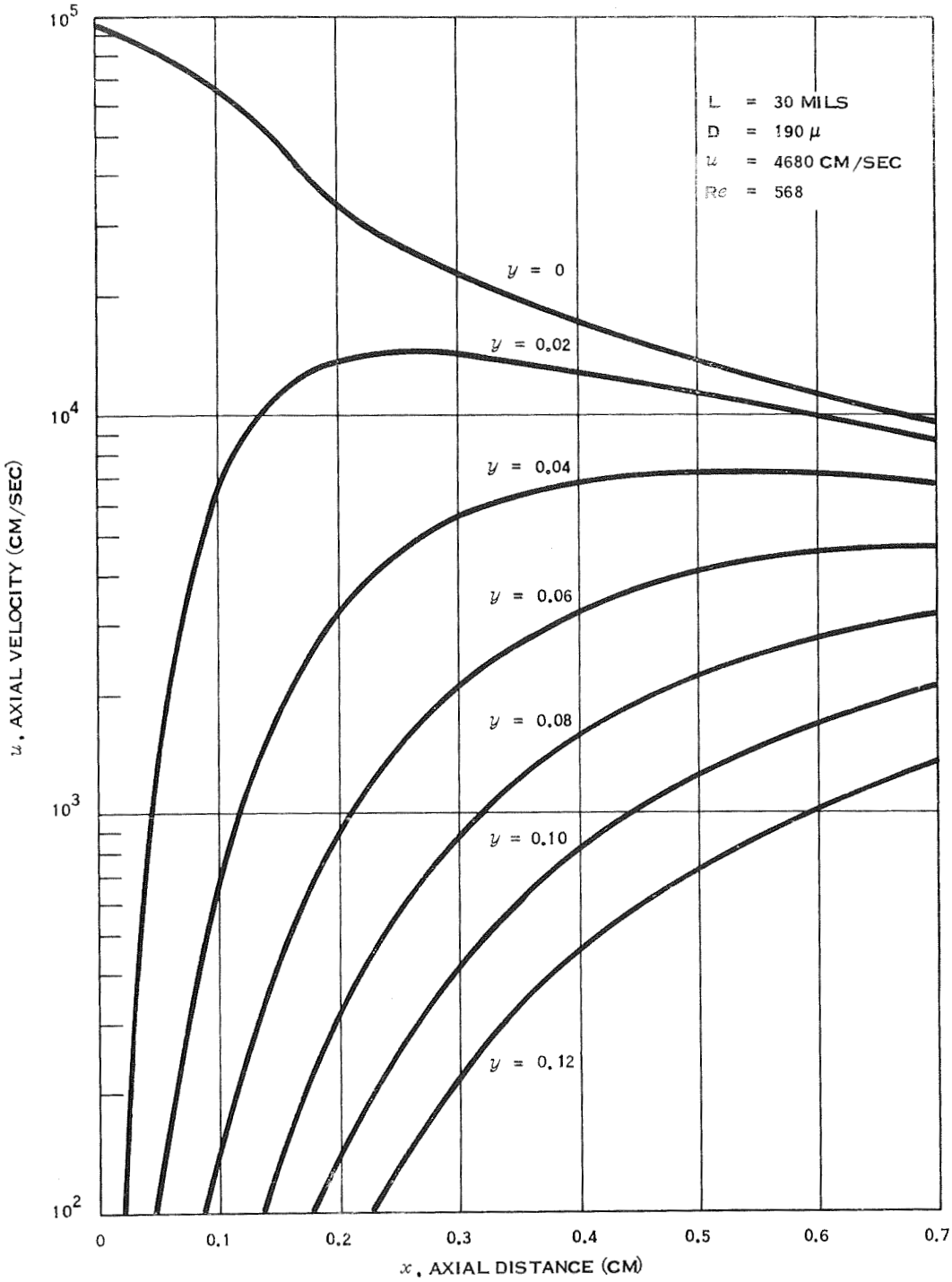


Figure 3. Axial Velocity Turbulent Jet

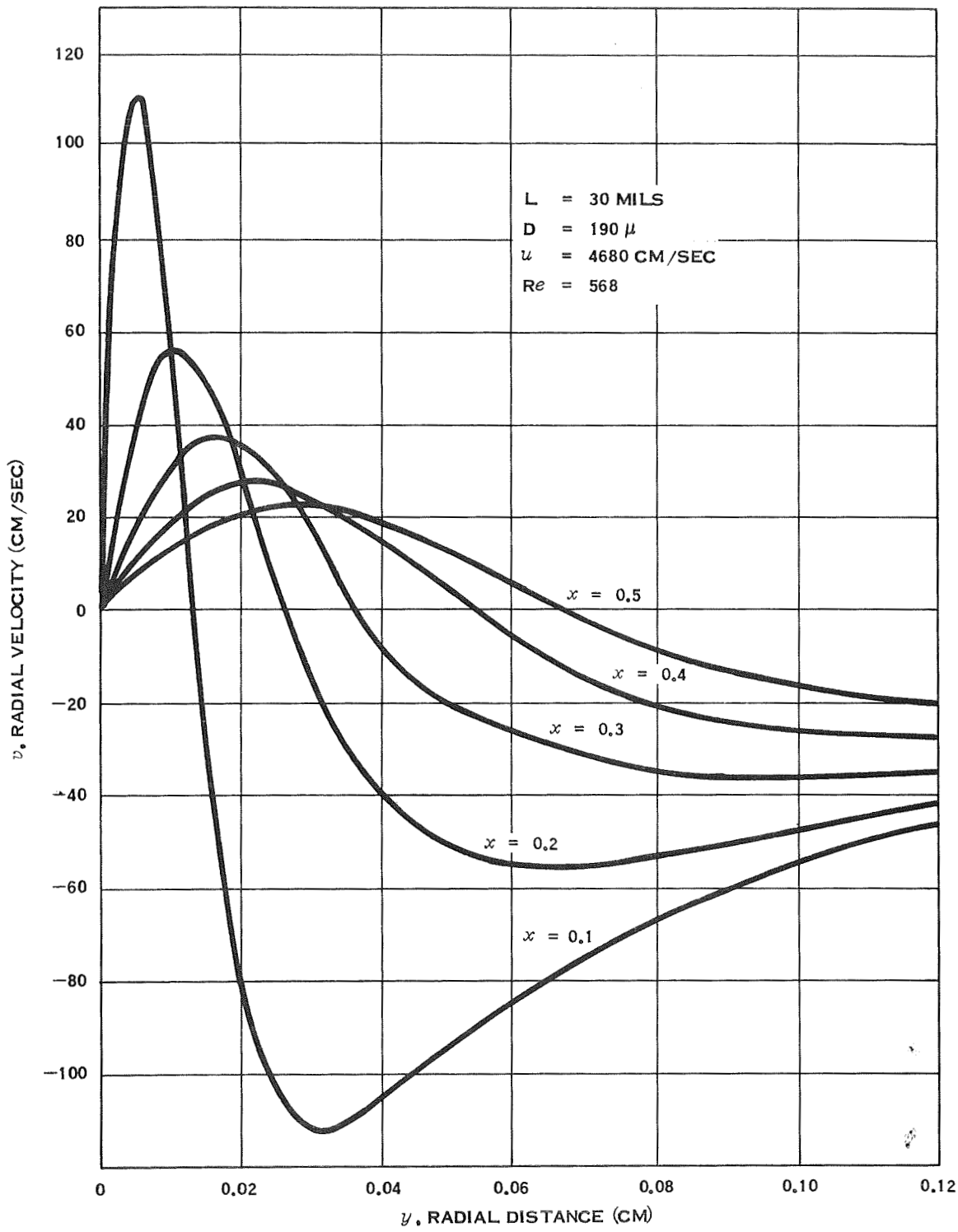


Figure 4. Radial Velocity Turbulent Jet

Conclusions

The analysis of bacterial spore motions and air flow through holes indicate that in the absence of air flow the spores may penetrate thin barriers through openings exceeding their size. Once, however, even small pressure drop (on the order of a fraction of an inch of water) is applied across such a barrier, no penetration by single spores can take place.

MATERIALS AND METHODS

3. Materials and Methods

3.1 Experimental Apparatus

A laboratory apparatus for determining the flow of microorganisms through various microscopic holes in membranes, and for controlling a range of differentail pressures was designed and two such units were fabricated. Each apparatus consisted of the following materials assembled as shown in Figure 5.

Aerosol Chamber

A 22 liter Glass Reaction Flask, Catalog No. K-61240, Kontes Glass Co., Vineland, N.J.

Chamber Clamp

Reaction Flask Clamp, Catalog No. K-61375, Kontes Glass Co., Vineland, N.J.

Membrane Holder

The membrane holders were machined from Lexan. The lower plate was 1.9cm thick and the upper .6cm thick. These were machined to fit the clamp with a 2.54cm diameter hole drilled centrally in each piece. Additionally, the 1.9cm piece had a 1.5mm hole drilled on its horizontal plane through the center and entire diameter of the piece. This was adapted to provide a source of air pressure on the membrane and a port for attachment of the manometer.

Agitating Fan

The fan was a defroster type, Sears Roebuck Co., Catalog Sales No. 28A78528, with rubber blades.

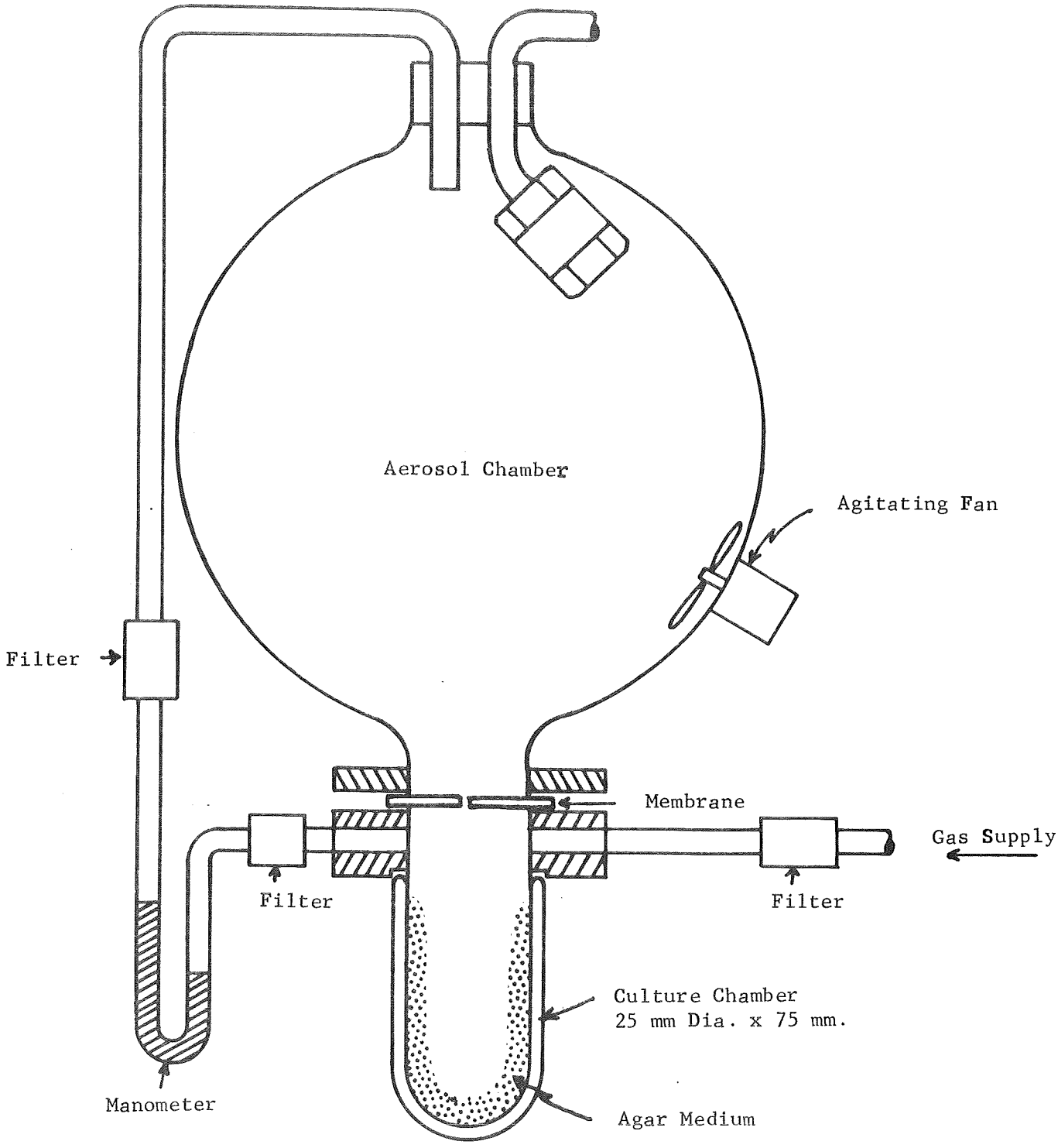


Figure 5. Experimental Apparatus

Filter Holder

The air filter holders used were Millipore Gas Line Filter Holder, Catalog No. XX40 025 00, Millipore Corp., Bedford, Mass.

Filters

The filters used in the filter holder were Millipore MF Type GS with a mean pore size of .22μ.

Manometer

Solid plastic, vertical style with a range of 0-10" of water. Model No. 310, F.W. Dwyer Mfg. Co. Inc., Michigan City, Indiana.

Pressure Regulator

Range 0-60psi. Model No. 40, The Matheson Co., East Rutherford, N.J.

Aerosol Dispenser

The Aerosol Dispenser was fabricated from standard brass tubing connections. Figure 6 shows the Aerosol Dispenser with one end fastened to a stainless steel tube.

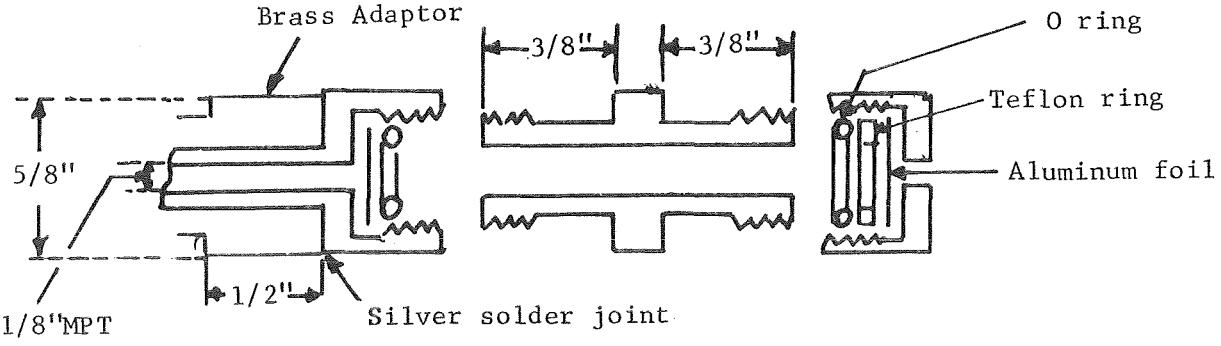


Figure 6. Aerosol Dispenser

The dispenser functions when a pressure of approximately 30 psig is supplied to the dispenser. Both aluminum foil seals rupture and an aerosol is discharged explosively. The dispenser is a minaturization of a design obtained from Mr. Carl Depeople of the U.S. Army Chemical Corps at Fort Detrick, Md., whose cooperation was gratefully appreciated.

Membranes

The membranes used were fabricated from .030 inch and .012 inch aluminum and polypropylene sheets. Initially, teflon and copper were selected for membranes as noted in the proposal. However, some difficulty was encountered with both materials. The copper dissipated the heat so readily that it was impossible to drill with a laser even when subjected to 8 or 9 exposures. The teflon was impervious to penetration with the laser on 8 or 9 exposures. In the teflon drilling operation, it was considered that the color might be influencing the lack of penetration and a colored piece of polyethylene was placed on both sides of the potential membrane. On actuation of the laser, however, holes were drilled on both sides of the polyethylene and the teflon surface was only etched.

To obtain a drillable material that was autoclavable, polypropylene and aluminum were used.

Microscopic holes were drilled using a Lear-Siegler LW-212 metal working laser with a Nikkon Compariton using a 6 millisecond pulse at 4 kilowatts, 2 pulses per hole.

Holes varying in size from 19 to 228 microns in diameter were produced for our determinations. Because the 2 pulses were required, some of the holes were oval in cross-section. Such holes were dimensioned by their maximum and minimum dimensions.

Measurement of Membranes

Micrometer calipers were used to determine the thickness of each membrane. The lateral dimensions of the membrane holes were determined by light microscopy using a standardized ocular micrometer.

Viable Culture Detector Tubes

Twenty-five by seventy-five millimeter culture tubes were washed in an aqueous solution of Alconox and rinsed in distilled or deionized water. Ten milliliters of Trypticase soy agar was placed in each tube and the tubes capped. The capped tubes were then autoclaved at 121°C and 15 psig for 15 minutes and removed from the autoclave. The detector tubes were then placed in a water bath set at 45°C and allowed to equilibrate. When the agar had cooled to 45°C, the tubes were rotated at an 80° angle until the medium hardened. The tubes were then cooled additionally by placing them in a refrigerator for approximately one hour to set the agar, and then incubated for 48 hours at 37°C to detect contaminants.

3.2 Biological Materials

Selection and Preparation of Aerosol

Viability of the culture through preparation and aerosol production is critical to the Viable Culture Technique. For this purpose, it appeared suitable to employ spores of a species of Bacillus subtilis var. niger. This was a logical choice because of its characteristic cultural morphology, stability to drying and storage, and general ease of handling. Spores used for this purpose were thoroughly cleaned to reduce clumping. The spores were then freeze dried in order to provide uniform distribution in an aerosol.

Preparation of Spores

Spores of B. subtilis var. niger were prepared as follows:

A starter culture was prepared by inoculating 50 ml. of sporulation medium consisting of nutrient broth supplemented with 0.0001 percent $MnCl_2.4H_2O$ in a 125 ml. flask from the stock culture, heat shocking at 80°C for ten minutes, and incubating at 35°C for twenty-four hours. One milliliter of the starter culture was then spread over each of approximately 15 large petri dishes containing a sporulation medium consisting of nutrient agar supplemented with 0.001 percent $MnCl_2.\ 4H_2O$. The plates were incubated at 37°C in a humidified atmosphere for seven (7) days, the point at which sporulation was found to be virtually complete. The spores were harvested by suspending the growth in distilled water. (The plates were refrigerated, water was poured over the plates, the growth scraped from the agar, and the resulting suspension recovered with a pipet.) The suspension was centrifuged at 4°C, resuspended in a sterile M/50 phosphate buffer, pH7. The spores were scrubbed with ultrasonics at 25 kilocycles per second for 10 minutes. The suspension was washed 10 times with sterile distilled water. The resulting spore suspension was virtually free of any debris or vegetative cells as determined by phase microscopy.

Ten milliliters of cell suspension were placed in a 100 ml. Florence flask, which was then placed in liquid nitrogen until the suspension was frozen. The flask was connected to a high vacuum line and dried for approximately 5 hours. Upon completion of the drying, the spores were pooled in a container with a cotton plug and placed in a dessicator over anhydrous $CaSO_4$. The aerosol was produced from lypholized preparations to limit clumping and to avoid the introduction of moisture. The particle size distribution of the lypholized spores was determined by microscopic measurements as shown in Table III.

TABLE III PARTICLE SIZING DATA

Diameter (microns)	Number Counted	Cumulative Percent of Total	Relative Volume (diameter^3 x number)	Percent of Volume	Cumulative Percent of Volume
1.0	56	28	56	.24	.24
1.4	14	35	38	.16	.40
1.9	42	56	287	1.21	1.61
2.7	30	71	590	2.48	4.09
3.8	25	83.5	1371	5.77	9.86
5.4	17	92	2676	11.27	21.13
7.7	8	96	3652	15.38	36.51
10.9	6	99	7770	32.72	69.23
15.4	2	100	7304	30.76	99.99
Totals	200		23744		

To summarize the data on this batch of lypholized dry spores,

MMD	mass median diameter	9.0
MASS	$<$5 microns	22%
CMD	count mediam diameter	1.7
NUMBER	$<$5 microns	90.0%
The viable spore count per gram		1.7×10^{11}

The method used for bacterial particle sizing is shown in Appendix A.

3.3 Test Protocol

3.3.1 Initial Test Procedure

The initial test procedure followed is shown graphically in Figure 7. The details in each step are explained below.

Preparation of the Experimental Apparatus

The membrane selected for the test to be run was examined microscopically to verify that the hole was open, and then was installed in the membrane and culture tube holder. A piece of teflon was placed over the hole and held in place with a piece of autoclave tape with strings attached to each

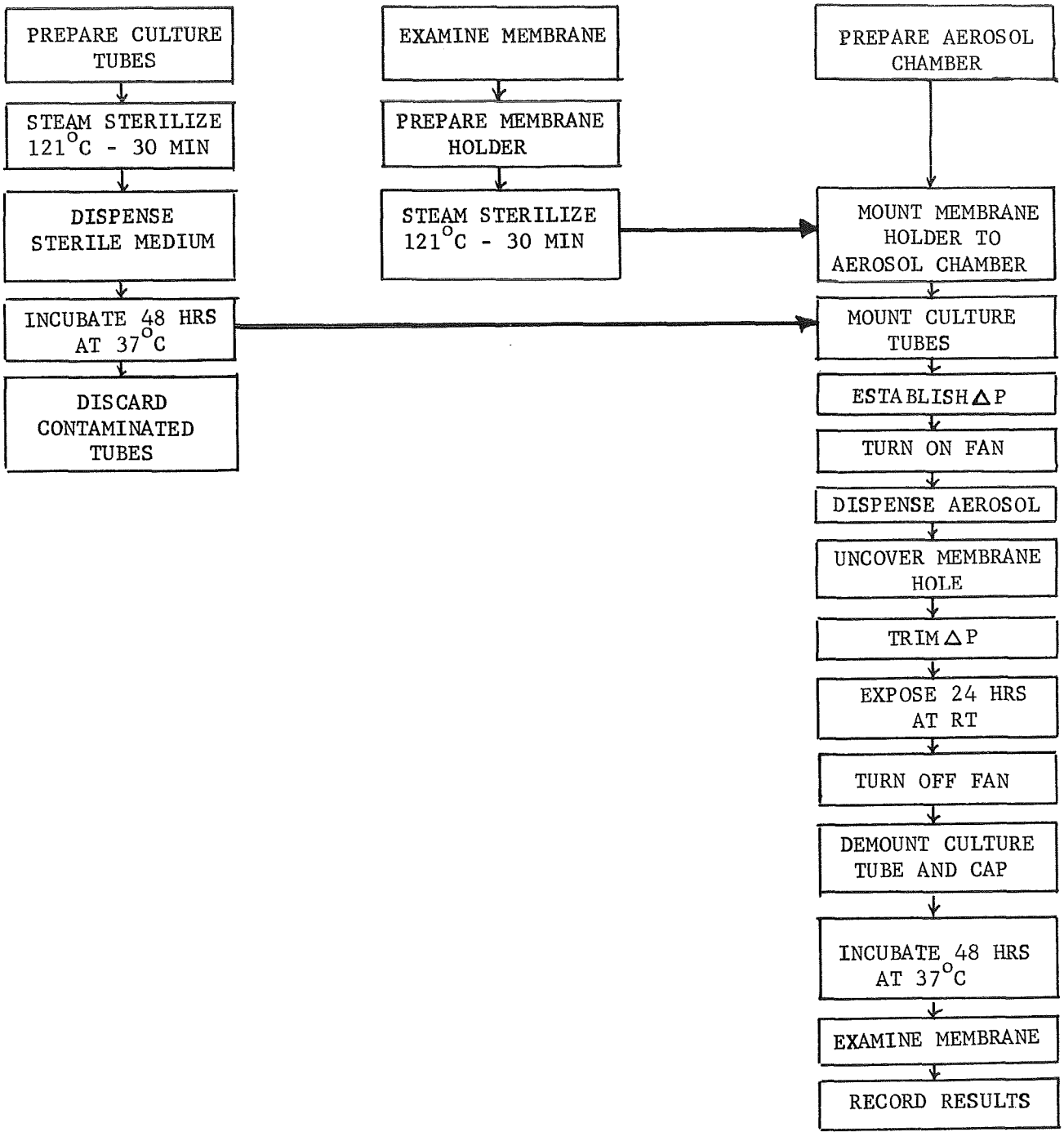


Figure 7. Initial Test Procedure

end. An empty 25 x 75mm tube was mounted to the lower side of the membrane holder. Tygon tubing was attached to each of the ports in the lower lexan disc. The membrane holder, filters, tubing and the aerosol dispenser were washed in an aqueous solution of Alconox and rinsed thoroughly in distilled or deionized water. After washing, the assembled membrane holder and filters were autoclaved at 121^{o}C and 15 psig for 15 minutes and dried.

The sterilized assembled membrane and chamber holder was brought into position near the bottom opening of the aerosol chamber. The strings, attached to the piece of autoclave tape covering the membrane hole, were fed through the top opening of the chamber. The holder was then clamped to the bottom of the aerosol chamber.

The filters, manometer and associated tubing were connected and a nitrogen source was connected to the upstream side of the aerosol dispenser line. An air source was connected to the upstream side of the line to the membrane holder as the final step in the equipment setup.

Aerosol Generation and Maintenance

The aerosol dispenser was opened at the exit end and loaded with 50 mg of lypholized spores having a viability of 1.7 x 10^{11}/gm. The aluminum foil and nut were replaced to retain the viable microorganisms. The rubber stopper containing the two stainless steel tubes was placed loosely in the top opening of the aerosol chamber. The dispenser was inserted into the chamber through the bottom opening and drawn up into position by pulling the steel tubing partially through the rubber stopper. The test plan originally called for sterilizing the aerosol chamber prior to each test run. Upon running the first tests, it was determined that large amounts of aerosol were being lost

through adhesion and electrostatic attraction to the aerosol chamber walls. It was postulated that this residual layering could be used to increase the aerosol population and sterilization between tests was discontinued. Subsequent counts taken on the aerosol indicated that an increase in the aerosol population was attained by this means.

When the test equipment had been assembled as described above, nitrogen, passed through a bacteriological filter at 30 psi, was introduced into the aerosol dispenser. The electric fan was activated. The aluminum foil, sealing both ends of the dispenser, ruptured under the gas pressure and the stored spores were ejected into the aerosol chamber. The resultant mixture was constantly agitated by the fan blades.

Installation of Detector Chamber and Exposure

After the rupture of the aluminum seal in the aerosol dispenser, the bottom portion of the culture chamber holder was flamed. The empty 25 x 75mm tube was removed and a previously prepared culture detector chamber containing agar was inserted aseptically. The air supply to the culture chamber was activated and adjusted to the desired pressure differential. Upon obtaining the pressure value desired, the autoclave tape blocking the topside of the membrane hole was removed by means of the strings passing through the top of the aerosol chamber. The air input was trimmed, as necessary, to maintain the differential pressure.

Initially, the test was run for 48 hours, but,as problems arose, this time was reduced to 24 hours to increase the number of tests run and hasten resolution of anomalous results. Upon completion of the test period, the aerosol chamber and attached membrane and culture chamber holder were inverted. The culture detector chamber was removed aseptically, capped, incubated for 48 hours, and finally examined for growth of B. subtilis var.

niger. An empty test tube was attached to the holder and the tygon tubing from the manometer to the aerosol chamber was disconnected at the chamber before sampling the residual aerosol.

Aerosol Sampling

At the termination of the exposure period of a given test, the aerosol was sampled and viable counts were made according to the following procedure to evaluate the stability of the aerosol. Ten milliliters of sterile water were added to a sterile glass impinger. The aerosol was passed through the sterile water at the rate of 1 CFM for ten minutes by means of a vacuum pump attached to the impinger outlet. Air in the aerosol chamber was continuously replaced.

Aliquots of the contents of the glass impingers were plated with appropriate dilutions to determine the viable count of the aerosol which was still suspended after the test period.

The results of the aerosol measurements taken after the testing period are shown in Table IV.

Membrane Examination

At the completion of each test, the membrane was removed carefully from the apparatus and examined microscopically to insure that the hole had remained open.

3.3.2 Test Procedure Revisions

As the experimental program progressed, experience with the apparatus dictated several procedural changes which were incorporated sequentially to overcome difficulties with sporadic contamination of the detection medium and the effects of non-quiescent gaseous atmospheres. The stages of the

TABLE IV: AEROSOL POPULATIONS AFTER COMPLETION OF TEST PERIODS

# of Apparatus	Aerosol Age (Days)	(Colonies/chamber) Total Count
#1	2	6.6×10^4
#2		2.8×10^4
#1	2	5.5×10^4
#2		4.2×10^4
#1	2	1.4×10^4
#2		9.2×10^3
#1	2	5.7×10^4
#2		6.1×10^3
#1	2	4.4×10^3
#2		3.8×10^4
#1	2	2.5×10^4
#2		3.7×10^3
#1	2	4.5×10^4
#2		1.5×10^4
#1	2	6.3×10^3
#2		7.2×10^3
#1	2	5.3×10^3
#2		3.4×10^4
#1	3	3.9×10^4
#2		1.8×10^5
#1	3	2.7×10^4
#2		2.6×10^5
#1	2	9.0×10^5
#2		9.4×10^4
#1	2	1.4×10^5
#2		7.5×10^4
#1	2	4.9×10^4
#2		9.0×10^4
#1	2	2.0×10^4
#2		8.9×10^3
#1	2	4.0×10^4
#2		5.4×10^4

program leading to these changes are described in Section 4.0, and the changes are only itemized here.

a. Fan Operation - The fan in the aerosol chamber was stopped prior to uncovering the hole in the membrane to eliminate the influence of high velocity particles.

b. Improved Gaskets were provided to obtain a bettwe seal between the membrane and its holder.

c. A Pressure Regulator was incorporated in the air supply line to insure a stable gas supply.

d. The Viable Culture Detector was assembled to the sterile holder and membrane on a laminar flow clean bench by a technician wearing sterile gloves.

e. Incubation of the assembled membrane - membrane holder - culture detector for 24 hours at 37°C prior to assembly to the aerosol chamber to detect initial contamination.

f. Enclosure of the test apparatus to limit distribution of the aerosol in the laboratory.

g. In Situ incubation of the culture tube on the apparatus and extension of the test time to 72 hours. The incubation consisted of 48 hours at room ambient temperature followed by 24 hours at 37°C.

The resulting test protocol is shown in Figure 8.

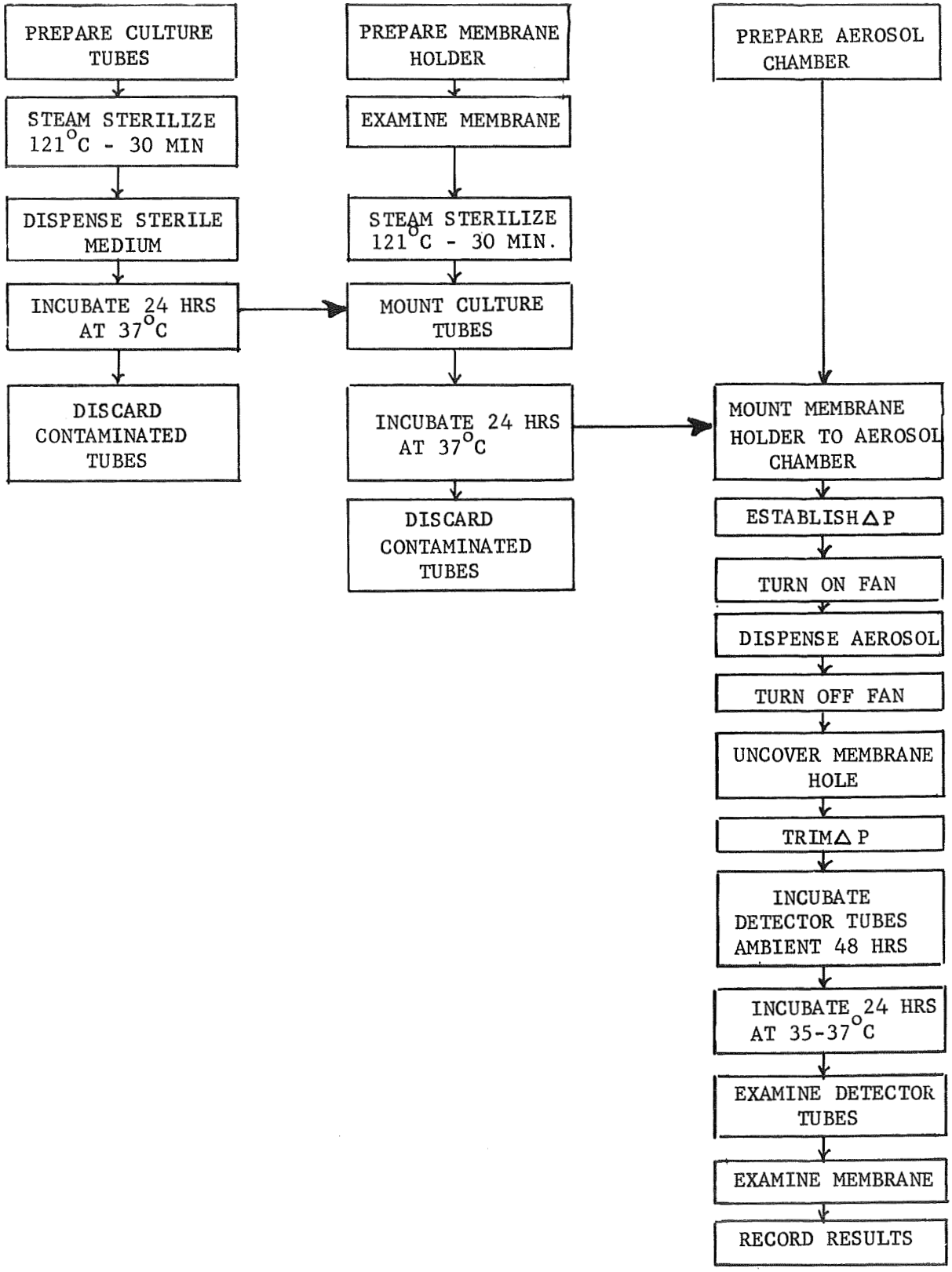


Figure 8. Final Test Procedure

RESULTS AND DISCUSSION

4.0 RESULTS AND DISCUSSION

The initial testing was conducted in accordance with the protocol shown on Fig. VII. The membranes tested, the differential pressures used and the results obtained are displayed on Table V.

The aluminum membrane containing a 90 micron hole was inadvertently clogged during a cleaning operation and had to be discarded. The polypropylene membrane with a 100 micron hole was substituted for subsequent tests. Profuse growth was observed in each instance. While no specific attempt was made to quantify the number of organisms transmitted, no visual difference was detectable in the magnitude of growth resulting from penetration of the aluminum or polypropylene membranes tested at 0.5" H_2O ΔP. This visual observation showed that a marked reduction in the number of spores occurred during the testing at 5.0 inches of water.

The appearance of profuse growth in the detector tubes during testing under 0.5" and 5.0" H_2O pressure differentials was unexpected since the analytical investigations suggested that penetration would be prevented by 3.5×10^{-5} inches H_2O. It was concluded that this growth was due to propulsion of the spore particles by the agitating fan and that the tests should be performed with a static aerosol.

The test procedure was modified to specify that the aerosol fan be stopped prior to removing the cover from the hole in the membrane. A series of tests were run on the polypropylene membrane with a 100 μ hole to evaluate the effect of this procedural change. The results using this modified test procedure confirmed the suspected influence of the agitating fan. In all tests conducted under a positive pressure differential, either no evidence of growth occurred or only an occasional colony in the detector tube. Tests were performed on other membranes to evaluate different holes sizes and membrane thicknesses. The results obtained in this phase are shown on Table VI.

TABLE V. DEVELOPMENT TEST RESULTS - FAN RUNNING

RESULTS					
MATERIAL	THICKNESS (MILS)	HOLE SIZE (μ DIA.)	ΔP (INCHES H_2O)		
			0	0.5	5.0
ALUMINUM	30	90	PROFUSE	PROFUSE	NO TEST
			PROFUSE	PROFUSE	NO TEST
POLYPROPYLENE	30	100	NO TEST	PROFUSE	PROFUSE
			NO TEST	PROFUSE	PROFUSE

TABLE VI. DEVELOPMENT TEST RESULTS - FAN OFF

TEST CONDITIONS

RESULTS

MATERIAL	THICKNESS (MILS)	HOLE SIZE (μ DIA.)	ΔP (INCHES H_2O)		
			0	0.5	5.0
POLYPROPYLENE	30	100	12	0 6 0 6 0	0 2 0 0 0 2 0 0
		50		0 0	
ALUMINUM	30	109x139	PROFUSE	6 1	0 0
		190	PROFUSE	0 6	0 0
	12	21		0 1 0 1	
		33		0 0	

During this test sequence, it was noted that the Lexan membrane holders had begun to craze and warp. It was suspected that the seal between the two Lexan holders and the membrane had been faulty. A redundant system of "O" rings was installed in the test apparatus to remedy this situation. No noticeable change was noted in the test results, however. Testing was continued with both the aluminum and polypropylene membranes with various size holes. Profuse penetration occurred only under zero differential pressure conditions with the agitating fan not operating during the test. Sporadic appearances of growth in the detection chambers occurred for all tests run at differential pressures of 0.5, 2.0, and 5.0 inches of H_2O on membranes with hole sizes ranging from 19 to 228 microns. The results obtained during this phase are shown in Table VII.

The test data obtained to this point (Tables VI and VII) suggested that a threshold may have appeared with polypropylene membranes between the 100 and 117 x 141 μ diameter holes and between 2.0 and 5.0 inches of water ΔP. The causes of the sporadic appearances of growth in other portions of the test matrix had not been identified, however. Blank membranes were employed as a diagnostic tool to determine if the sporadic growth observed could be attributed to contamination of the detector chamber.

In three of four tests as shown in Table VIII, contamination of the detector chambers occurred. It was evident, therefore, that the sporadic results previously observed could conceivably be attributed to such contamination.

When it was found that indications of growth occurred in the detector tube after the blank membrane tests, several equipment and procedural changes were incorporated into the test cycle. The major changes involved were:

1. Incorporation of a pressure regulator in the supply line.
2. Performing the assembly of the viable culture detector on a laminar flow bench.
3. Wearing of sterilized gloves during assembly and disassembly operations.

TABLE VII. DEVELOPMENT TEST RESULTS - FAN OFF

TEST CONDITIONS

FAN OFF - CONTINUED TESTING FOR FURTHER ANALYSIS OF GROWTH APPEARANCE

RESULTS

MATERIAL	THICKNESS (MILS)	HOLE SIZE (μ DIA.)	Δ P (INCHES OF H_2O)			
			0	0.5	2.0	5.0
POLY	12	99	PROFUSE	30 1 1		
	30	117x141		18-20 18-20	18 18	
		228		20 18		1 2
AL	12	19	PROFUSE	0	1	0
		21		0 1	1 1	

TABLE VIII. DEVELOPMENT TEST RESULTS - BLANK MEMBRANES

TEST CONDITIONS

RESULTS

MATERIAL	THICKNESS (MILS)	HOLE SIZE (μ DIA.)	ΔP (INCHES OF H_2O) 0.5
AL	12	0	1 3 0 2

4. Rigorous procedures for control of aerosol dissemination in the laboratory were instituted.

The data gathered after the incorporation of these modifications, shown in Table IX, suggested that the apparent threshold phenomena observed was not real, but an artifact caused by increasing laboratory contamination. The sporadic contamination persisted, but no large number of colonies occurred. Several tests in this sequence were conducted with a 72 hour exposure period instead of a 48 hour period to determine whether contamination was occurring during removal of the detector tube. The detector tubes were examined prior to removal from the membrane holder.

This testing showed that the viable detection medium was extremely sensitive and subject to contamination in the assembly and disassembly steps. Standard aseptic techniques practiced daily in industry were not adequate. More stringent controls were necessary to prevent pre and post test contamination. Accordingly, the test procedures were modified to provide incubation of the culture detector chamber assembled to the membrane holder for 24 hours at 37°C before attachment to the aerosol chamber. The exposure time was extended to 72 hours to permit in situ incubation of the culture detector chamber. A small water bath controlled at 37°C was first employed around the detector chamber for the entire 72 hours of incubation. However, tests at zero differential pressure demonstrated that condensation formed on the membrane and prevented the expected passage of spores to the culture medium. Therefore, subsequent tests employed 48 hours of ambient temperature incubation followed by 24 hours at 37°C, all in situ. The final test protocol is outlined in Figure VIII.

Thirty-four test attempts were started in accordance with the final procedure. In nine instances, microbial growth was noted in the detector tube after the 24 hour pre incubation period but prior to the assembly of the membrane holder to the aerosol chamber. Of these, four detector tubes indicated 1 or 2 colonies, four indicated 5 to 10 colonies and one showed 23 colonies growing.

As mentioned above, two tests at zero differential pressure were invalid because condensation on the membrane prevented the passage of microorganisms thru the membrane. The test results for the twenty-three valid tests are presented in Tables X and XI. The data is arranged sequentially and by test apparatus. It can be noted from Table X, that no growth occurred for six consecutive tests performed on apparatus #2. The results obtained on apparatus #1 indicated the 0.5" H_2O differential pressure was effective for the first three tests. Profuse growth, however, occurred during the next three tests. This growth was much greater than previously experienced with the 190 micron hole (see Tables VI and IX). An investigation was undertaken to identify the cause for these penetrations. Examination of the test apparatus showed that the seal between the detector tube and the detector tube holder was loose. A series of diagnostic tests was conducted, therefore, to demonstrate that extraneous leakage had occurred on apparatus #1 around the detector tube.

Initially, the 190 micron hole was placed in apparatus #2 and the 19 micron hole installed in apparatus #1. No penetration was observed in either test. The 190 micron hole was then reinstalled in apparatus #1 along with a tight fitting detector tube. Apparatus #2 was set up, using a 115 x 145 micron hole aluminum membrane, and an intentional leak was created around the detector tube. This was accomplished by adjusting the ΔP, prior to removing the hole cover, to 0.5 inches of water. The detector tube holder was then loosened to allow escape of air until no ΔP could be maintained. The air flow was then readjusted to reestablish 0.5 inches of water ΔP, and the hole was uncovered and <u>in situ</u> incubation was started. Seven colonies were noted in the detector tube of apparatus #2 and post examination revealed that the 115 x 145 micron hole was blocked. No growth was observed in apparatus #1. The tests were repeated, except the intentional leak was incorporated into apparatus #1 and not in apparatus #2. No growth was observed in either detector

TABLE IX- DEVELOPMENT TEST RESULTS -REVISED PROCEDURES

TEST CONDITIONS

1. RESTRICT DISSEMINATION OF AEROSOL
2. ASSEMBLY OF MEMBRANE HOLDER ON CLEAN BENCH
3. TECHNICIANS EMPLOY STERILE MASKS AND GLOVES
4. INCORPORATED PRESSURE REGULATOR IN AIR SUPPLY LINE

RESULTS

MATERIAL	THICKNESS (MILS)	HOLE SIZE (μ DIA.)	ΔP (INCHES OF H_2O)		
			0	0.5	2.0
POLY	30	228		2 0 0 (1) (0)	
	12	48	PROFUSE	1 0	0
		99	PROFUSE	3	0 0
AL	30	109x139	PROFUSE	0 (0)	
		115x145		0 8 0 (0) (2)	
		190	PROFUSE	(0) 1	

( ) INDICATE 72 HR. TEST CONDITION

TABLE X. TEST RESULTS WITH FINAL TEST PROCEDURE

APPARATUS #1				APPARATUS #2			
MAT'L	THICK	HOLE	GROWTH	MAT'L	THICK	HOLE	GROWTH
			0" ΔP				
Al	.030	190	40	POLY	.030	228	PROFUSE
			0.5" ΔP				
POLY	.030	117x141	0	POLY	.030	228	0
POLY	.030	117x141	0	POLY	.030	228	0*
Al	.030	190	0	POLY	.030	228	0
Al	.030	190	PROFUSE	Al	.012	19	0
Al	.030	190	PROFUSE	Al	.012	19	0
Al	.030	190	PROFUSE	Al	.012	19	0

* 0.25" H_2O Δ P

TABLE XI - DIAGNOSTIC TESTS

APPARATUS #1				APPARATUS #2			
MAT'L	THICK	HOLE	GROWTH	MAT'L	THICK	HOLE	GROWTH
			0.5" H_2O ΔP				
Al	.012	19	0	Al	.030	190	0
Al	.030 (SNUG FIT - TUBE TO HOLDER)	190	0	Al	.030	115x145 HOLE BLOCKED	7*
Al	.030	190 HOLE BLOCKED	0*	Al	.030	115x145	0
Al	.030	190 HOLE BLOCKED	0*	--	--	--	--
Al	.030	190	1**	Al	.030	115x145	>30**

* BUILT-IN LEAK - 0.5" H_2O ΔP

** BUILT-IN LEAK - 0.1" H_2O ΔP

tube, however, post examination showed that the 190 micron hole was blocked. The 190 micron hole membrane was retested in apparatus #1 with the same intentional leak. Again no growth occurred and the membrane hole was blocked. The two membranes were then tested with smaller intentional leaks. Growth occurred in both detector tubes and neither membrane blocked.

The results of these diagnostic tests confirmed that air leaks on the sterile side of the membrane contribute to the penetration of organisms thru a micron sized hole. It therefore was concluded that the three consecutive instances of growth occurring on the 190 micron hole (Table X) were the results of test apparatus leakage and did not constitute valid tests.

The experience gained throughout the test program has shown that the viable culture technique for detection of microorganisms is of the utmost sensitivity. Rigorous attention to contamination control beyond the usual scope of conventional aseptic techniques is required. The tests performed with blank membranes and the 25% rate of contamination experienced after instituting the pre test incubation clearly indicate that all indications of penetration experienced during quiescent testing were in question. It is evident that contamination was being introduced into the culture detection tubes during the tests documented on Tables VI, VII, VIII and IX by the test regimen itself.

It is further evident from the initial testing which was conducted with continuous operation of the agitating fan, and the last tests which created flow on the sterile side of the membrane, that non quiescent conditions influence microbial penetration against a differential pressure. Tests performed after completion of this contract confirm that microbial penetration will occur against 0.5" H_2O when the agitating fan is operated throughout the 72 hour test period. The specific influence of non quiescent conditions requires further investigation.

CONCLUSIONS

5. CONCLUSIONS

1.0 An experimental apparatus and test procedure to permit measurement of the counter current flow of microorganisms under quiescent conditions has been developed. The system employed is potentially sensitive to penetration of one viable microorganism. Rigid aseptic techniques are required to detect and eliminate extraneous contamination.

2.0 Gravitational settling is the most significant factor in quiescent atmospheres contributing the microbial penetration of holes. A small differential pressure gradient will effectively protect against penetration of microscopic holes by the spore settlement.

3.0 The application of the pressure differential concept to BISS, and Assembly Sterilizer and a Sterilization Canister appears feasible. The specific differential pressure to use is dependent upon the environmental conditions surrounding each item.

4.0 A 0.5" H_2O differential pressure should prevent penetration of microorganisms thru small holes if the flow on either side of the bio barrier is not disrupted by some other factor. Additional tests should be performed at 0.5" H_2O however to provide greater assurance in this pressure differential.

5.0 The 0.5" H_2O pressure gradient may not be adequate under non-quiescent environments. The effect of non-quiescence on both sides of the bio barrier must be studied individually and in combination prior to establishment of the required differential pressure.

6.0 The differential pressure level is not influenced by any of the variables studied, namely, hole sizes from 19 μ to 228 μ, membrane thickness of .012" and .020" and membrane materials of aluminum and polypropylene.

RECOMMENDATIONS

6. RECOMMENDATIONS

1.0 Additional testing under quiescent conditions should be undertaken to provide greater assurance in the effectiveness of 0.5" H_2O differential pressure.

2.0 The influence of spore or particle velocity on the non sterile side of a bio barrier on the pressure differential required to prevent their passage through microscopic holes should be investigated.

3.0 The effect of gas flow on the sterile side of a bio barrier on the pressure differential should be studied.

4.0 The interaction between flow disturbances resulting from multiple holes close together should be examined.

5.0 The combined effects on all non quiescent conditions should be studied to determine whether these environments are additive.

6.0 Extensive testing for reliability information should be performed only after the differential pressure required for protection against high velocity particles and sterile side flow is established.

APPENDIX A

APPENDIX A

BACTERIAL PARTICLE SIZING

Introduction

The method most used for size analysis of bacterial particles is direct measurement with a light microscope with a calibrated ocular micrometer. To facilitate reporting the size of bacterial particles, it is usually assumed that all particles in a given sample have the same density and shape. To simplify further the determination of bacterial particle size, it is usually assumed that all the bacterial particles are spherical and the mean diameter need only be approximated. Although this is not a precise method, it provides the necessary type of information for all practical purposes.

Procedure - Particle Size (Dry Agent)

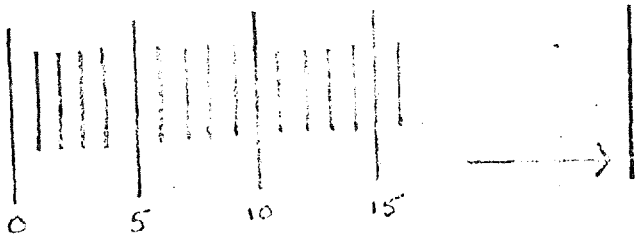
1. Clean 5 slides and cover slips.
2. Weigh 20 mg of agent into a clean beaker.
3. Add 20 ml of suspending fluid (200 parts n-butyl alcohol and 1 part Canada Balsam).
4. Magna-mix the solution for approximately 5 minutes.
5. While the solution is mixing, take a wooden applicator and place 1 or 2 drops on each slide.
6. Allow the slides to dry completely, about 2 hours.
7. Cover with cover slips.
8. Repeat about 4 times, in other words, 4 Reps.
9. Pick 2 best slides from each Rep, thus providing 10 good slides. Store slides in dessicator until ready to size.

Reading Slides

10. Use a 10 x 96 or 12.5 x 96 power scope, calibrated with oil emmersion.

11. Size 50 particles per slide.

12. Use the following type ocular micrometer:



13. Sweep field until 50 particles per slide are read.

14. Read 4 slides.

15. Sample calculations are attached. (See Table II)

16. Plot values on logarithmic probability paper. (Figure 9)

After 200 particles from the slides are counted, sized, and tabulated (Table III), the percent of the total number is calculated for each size and these percentages are then cumulated for determination of count median diameter. The total mass for each group is calculated by cubing the diameter and multiplying by the number of particles in that size group. The percent of the total mass is calculated for each size group and these percentages are then cumulated for determination of mass median diameter. The cumulated figures for number percent and mass percent are plotted against the corresponding size, as shown in Fig. 9 by reading the size corresponding to the 50 percent intercept, as shown on the graph (Fig. 9); the count median diameter or C.M.D. is determined as 1.7 microns; the mass median diameter (M.M.D.) is determined in the same manner as 9.0.

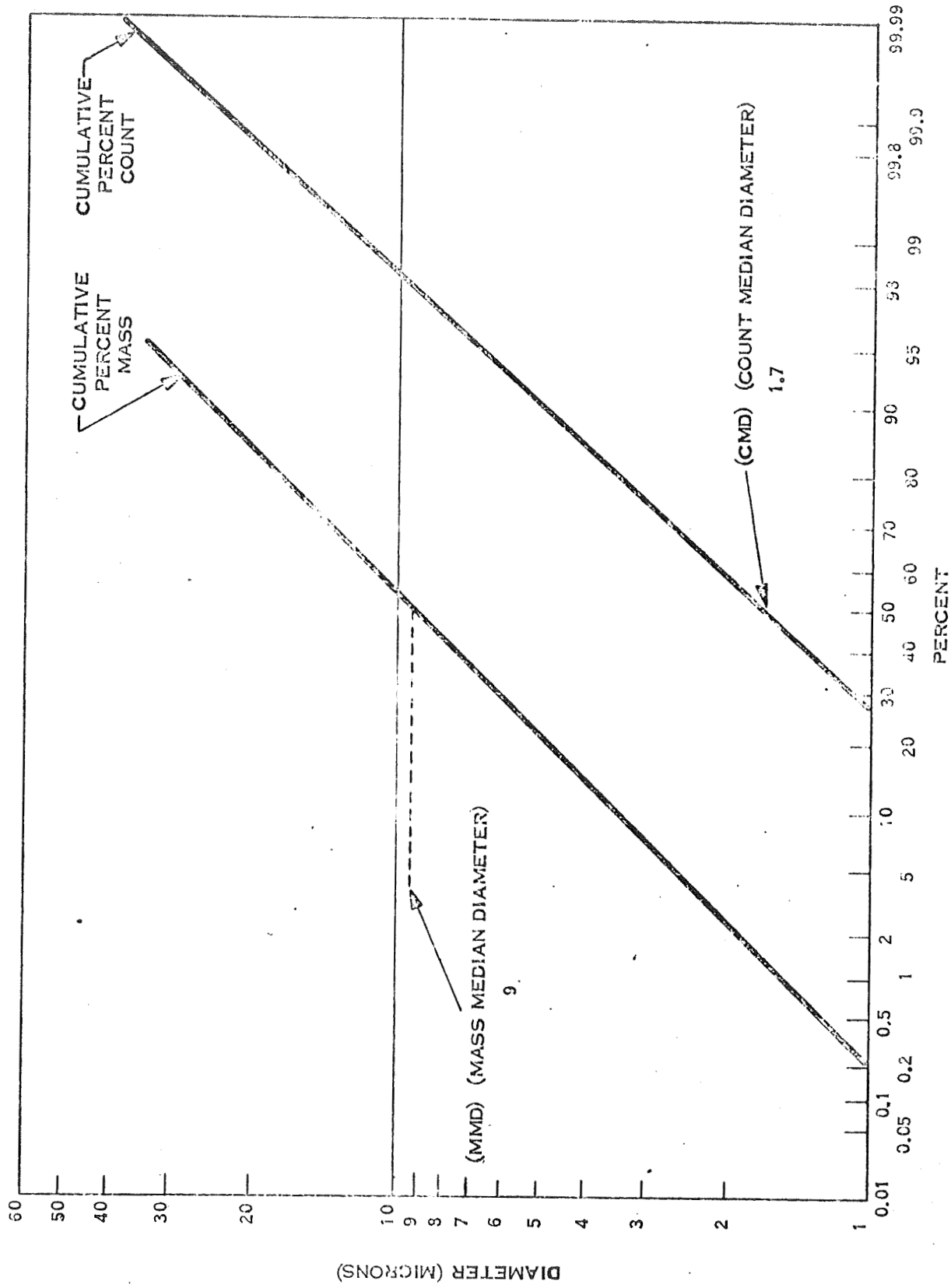


Figure 9. Bacterial Particle Sizing Graph

APPENDIX B

AIR JET COMPUTER PROGRAM AND SAMPLE CALCULATIONS

```
00010 *      A FORTRAN PROGRAM FOR THE CALCULATIONS OF THE
00020 *      LAMINAR AND THE TURBULENT JET VELOCITIES
00030        DIMENSION U(50), V(50), Y(50)
00040        PRINT 50
00050    200 FORMAT(" X0 ="I3," *X="E8.3,//)
00060        READ:UMAX,D,A,X0,X1,Y0,Y1,N,M,R,S
00070        PRINT 100, UMAX, D, A, X0, X1, Y0, Y1, N, M, R, S
00080        PRINT 250
00090        UMX =UMAX*UMAX
00100        B=.00824*UMAX*D
00110        N1=N+1
00120        M1=M+1
00130        NX=0
00140        DO 10 II=1,N1
00150        EYE=II-1
00160        X=X0+X1*EYE
00170        W=X*(R*A+S*B)
00180        DO 20 J=1,M1
00190        EJAY=J-1
00200        Y(J)=Y0+Y1*EJAY
00210        Z=.125*UMAX*D*Y(J)/W
00220        Z2=Z*Z
00230        T=(4.+Z2)**2
00240        V(J)=UMAX*D*Z*(4.-Z2)/(X*T*2.)
00250     20 U(J)= UMX*D*D/(2.*W*T)
00260        PRINT 200,NX,X
00270        NX=NX+1
00280        PRINT 300, (I,Y(I),U(I),V(I),I=1,M1)
00290        PRINT 400
00300    400 FORMAT (//)
00310     10 CONTINUE
00320    250 FORMAT("      K+1                YO+K*Y1                U"
00330 &      "                  V")
00340    300 FORMAT(I8,7X,3E15.3)
00350     100 FORMAT(" UMAX="E10.3," D="E10.3," A="E10.3,/
00360 &       " X0="E10.3," X1="E10.3," Y0="E10.3," Y1="E10.3,/
00370 &       " N="I3," M="I3,/
00380 &      " R="E8.1," S="E8.1,//)
00390     50 FORMAT(" THE ORDER OF INPUT IS: UMAX, D, A, X0, X1, "
00400 &      "YO, Y1, N, M, R, S",/
00410 &      " ALL INPUTS AND OUTPUTS ARE IN CM, SEC UNITS",///)
00420        STOP
00430        END

READY
```

```
$FORTRAN

THE ORDER OF INPUT IS: UMAX, D, A, X0, X1, Y0, Y1, N, M, R, S
ALL INPUTS AND OUTPUTS ARE IN CM, SEC UNITS

=9350.0.019.1.0.1.0.1.0..1.4.5.0.1

UMAX= 0.935E+04 D= 0.190E-01 A= 0.100E+01
X0= 0.100E+00 X1= 0.100E+00 Y0= 0.          Y1= 0.100E+00
N=   4 M=   5
R= 0.       S= 0.1E+01

     K+1                 Y0+K*Y1              U                   V
X0 +  0 *X=.100E+00

       1                 0.              0.674E+04          0.
       2                 0.100E+00       0.197E+01         -0.556E+02
       3                 0.200E+00       0.126E+00         -0.289E+02
       4                 0.300E+00       0.250E-01         -0.194E+02
       5                 0.400E+00       0.793E-02         -0.146E+02
       6                 0.500E+00       0.325E-02         -0.117E+02


X0 +  1 *X=.200E+00

       1                 0.              0.337E+04          0.
       2                 0.100E+00       0.142E+02         -0.476E+02
       3                 0.200E+00       0.983E+00         -0.278E+02
       4                 0.300E+00       0.198E+00         -0.191E+02
       5                 0.400E+00       0.631E-01         -0.144E+02
       6                 0.500E+00       0.259E-01         -0.116E+02


X0 +  2 *X=.300E+00

       1                 0.              0.225E+04          0.
       2                 0.100E+00       0.411E+02         -0.369E+02
       3                 0.200E+00       0.318E+01         -0.261E+02
       4                 0.300E+00       0.656E+00         -0.185E+02
       5                 0.400E+00       0.211E+00         -0.142E+02
       6                 0.500E+00       0.868E-01         -0.115E+02


X0 +  3 *X=.400E+00

       1                 0.              0.168E+04          0.
       2                 0.100E+00       0.797E+02         -0.259E+02
       3                 0.200E+00       0.712E+01         -0.238E+02
       4                 0.300E+00       0.151E+01         -0.178E+02
       5                 0.400E+00       0.492E+00         -0.139E+02
       6                 0.500E+00       0.204E+00         -0.113E+02


X0 +  4 *X=.500E+00

       1                 0.              0.135E+04          0.
       2                 0.100E+00       0.124E+03         -0.161E+02
       3                 0.200E+00       0.129E+02         -0.212E+02
       4                 0.300E+00       0.286E+01         -0.169E+02
       5                 0.400E+00       0.948E+00         -0.135E+02
       6                 0.500E+00       0.393E+00         -0.111E+02
```