1070

10 November 1968

DEVELOPMENT AND TEST OF

FLEXIBLE FILM COUPON STRIPS

FOR USE AS A SAMPLING TECHNIQUE

BI-MONTHLY PROGRESS REPORT NO. 2

Submitted to National Aeronautics and Space Administration, George C. Marshall Space Flight Center, Huntsville, Alabama 35812, in response to Contract No. NAS8-21443

Prepared by C. Aldridge
C. Aldridge, Microbiologist
Aerospace Medicine Department

Approved by G. J. Womack
G. J. Womack, Manager
Aerospace Medicine Department

Aerospace Medicine Department
Space and Missile Engineering Division

This report and data herewith were developed by McDonnell Douglas Corporation. ~~These ... intended only for distribution ... personnel of the ... and Space Administration.~~

MCDONNELL DOUGLAS ASTRONAUTICS COMPANY
EASTERN DIVISION

TABLE OF CONTENTS

LIST OF FIGURES AND TABLES

I. SUMMARY

Work under Contract NAS8-21443 has progressed through the first half of Phase II. Salient features of the work to date are as follows:

1. The flexible film coupon assay technique for microbiological monitoring has been shown to be equal to or superior to the stainless steel coupon methods approved by NASA as tested with aerobic microorganisms. To support this claim the following points have been established.
   - o Data from die-away studies in the Class 100 Clean Room indicate substantially less die-away on flexible film compared with stainless steel for seven microorganisms tested.
   - o Natural contamination assays for both materials in the same 1" x 2" format exposed in a Class 100 and Class 100,000 Clean Room indicate no superiority for the NASA approved stainless steel method.
   - o Aerosol studies indicate the flexible film coupons detect up to three orders of magnitude more microorganisms than stainless steel coupons under the same conditions.
   - o Studies under different conditions of relative humidity (35, 70, and 90 percent RH) reveal the superiority of the flexible film coupon because die-away is less on flexible film than stainless steel.
2. The flexible film coupon has been demonstrated to be useful as an adjunct or replacement for the stainless steel under the following conditions:
   - o Die-away studies on geometric forms simulating hardware in a Class 100 Clean Room have shown a definite difference in die-away with respect to orientation with the air flow. A solid cylinder, pyramid, cone, and sphere were used in these studies. Results indicate about one order of

of magnitude greater in die-away for organisms on top of an object versus the organisms on the bottom and out of the air flow.

- The 12" x 12" flexible film format is providing a measure of accuracy for the extrapolated estimates for square feet of surface sampled by the 1" x 2" stainless steel coupon method. Results to date indicate that the stainless steel method overestimates the microbiological load by about one order of magnitude for each square foot of surface.

Work is slightly ahead of schedule and no undue problems have been found or are anticipated.

II. INTRODUCTION

The flexible film assay concept was designed to improve the methodology needed for determining hardware microbiological contamination in modern clean room environments. This report presents data which demonstrate that the flexible film concept is expanding our understanding of the accuracy and limitations of data obtained with the presently accepted stainless steel coupons. These data are documenting the path to one of the primary goals of the project, the clarification of ways in which the flexible film may serve as either an adjunct to, or a replacement for the stainless steel assay method.

The current NASA approved method for assaying in clean room environments employs 1" x 2" stainless steel strips or coupons (Reference 1). These sterilized strips are exposed to the clean room environment for varying periods of time up to several months before removal for bioassay. Numbers of viable organisms obtained from these strips are extrapolated to give estimates for several square feet of hardware surface exposed to the same environment.

Problems develop with this technique because the microbial contamination level in a Class 100 type of clean room is quite low. Many strips may yield zero when bioassayed. Removal of such low numbers of contamination from the strips is difficult, even with sonication in a water bath. Extrapolation of these assay data employs large multiplication factors to arrive at equivalent square feet of hardware surface contamination. The resultant data are of questionable reliability.

In addition to the above disadvantages, there is an additional cost consideration. The steel strips require special cleaning procedures prior to reuse. The sonic water bath procedure required for maximum removal of microorganisms is also a time-consuming process which adds to the costs and limits the number of assays performed per unit time.

The flexible film technique was designed to overcome many of these disadvantages. Completely soluble in water, this type of coupon presents no problem with removal of microorganisms, and does not require the extra step of sonication necessary with stainless steel coupons. In addition, larger surfaces can be sampled in one step with coupons as large as one square foot.

Other advantages include the capability for determining distribution of microorganisms on the surface of the film. This can be accomplished simply by placing the film in contact with sterile nutrient material, such as tryptic soy agar, and incubating for at least 24 hours. This allows contaminants to grow *in situ*.

In July, McDonnell Douglas Astronautics Company, Eastern Division, was awarded a nine-month contract (NAS8-21443) to evaluate the flexible film. The results of the first two months of the Phase II effort are the subject of this report.

III. PROBLEM DEFINITION AND APPROACH

A. The Problem

The basic problem in the Phase I effort centered upon proof of merits of the flexible film coupon method as compared with the stainless steel coupon method.

The problem expands under the Phase II effort to include additional proof which was begun under Phase I, but now includes demonstrating ways in which the flexible film concept can either augment or replace the stainless steel concept.

B. The Approach

The general approach throughout the contract will be to conduct a series of tests designed to yield the following information:

1. A comparison between the microbial contamination buildup on similar sized gelatin and stainless steel strips over at least a six-week period of exposure in a clean room.
2. The statistical advantage of using sampling surfaces in excess of 2 square inches.
3. The relative die-off rates of known numbers of representative micro-organisms on gelatin and stainless steel.
4. The effect of geometric configurations on die-off relative to positioning in the laminar flow clean room.
5. Comparative aerobic and anaerobic microbial survival rates including spores and vegetative forms.
6. Reliability of the flexible coupon bioassay method under clean room conditions.

Specifically, the first 5 items were either started or completed during this two-month period of performance.

IV. RESULTS

A. Scheduling

The Phase II program covering the last two months of contractual obligations had scheduled the performance of the following tasks:

- Completion of die-away studies for various aerobic microorganisms in a Class 100 clean room.
- Continued testing to compare stainless steel assay coupons with the flexible film coupons under Class 100 Clean Room conditions, with emphasis on aerobic and anaerobic spores.
- Distribution and die-away on geometric forms in the Class 100 Clean Room environment.
- Continued studies of the 12" x 12" flexible film coupons versus 1" x 2" format.
- Evaluation of techniques for the attachment of the flexible film coupons to hardware.

B. Die-away Studies

1. Flat Surfaces in Class 100 Clean Room - The previous bi-monthly report had presented data to show that the die-away for a variety of microorganisms was less on the flexible film coupon than on stainless steel under Class 100 Clean Room conditions. (Reference 2) However, due to unexpected die-away rates, dilutions were missed, and some of the data had to be repeated. All of this has now been accomplished. Test organisms were *Staphylococcus aureus*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Corynebacterium diphtheriae*, *Staphylococcus epidermidis*, and vegetative cells and spores of *Bacillus subtilis* var. *niger*. Except for the

case of spores, the trend toward greater survival on flexible film versus stainless steel was clear. Spores, however, demonstrate only slightly better survival on flexible film. As would be expected, the two gram negative organisms, P. aeruginosa and E. coli, were similar in survival rates.

After 68 hours in the Class 100 Room, the surviving vegetative cells of B. subtilis were not significantly reduced from the start of the test, and results were similar for both coupon materials.

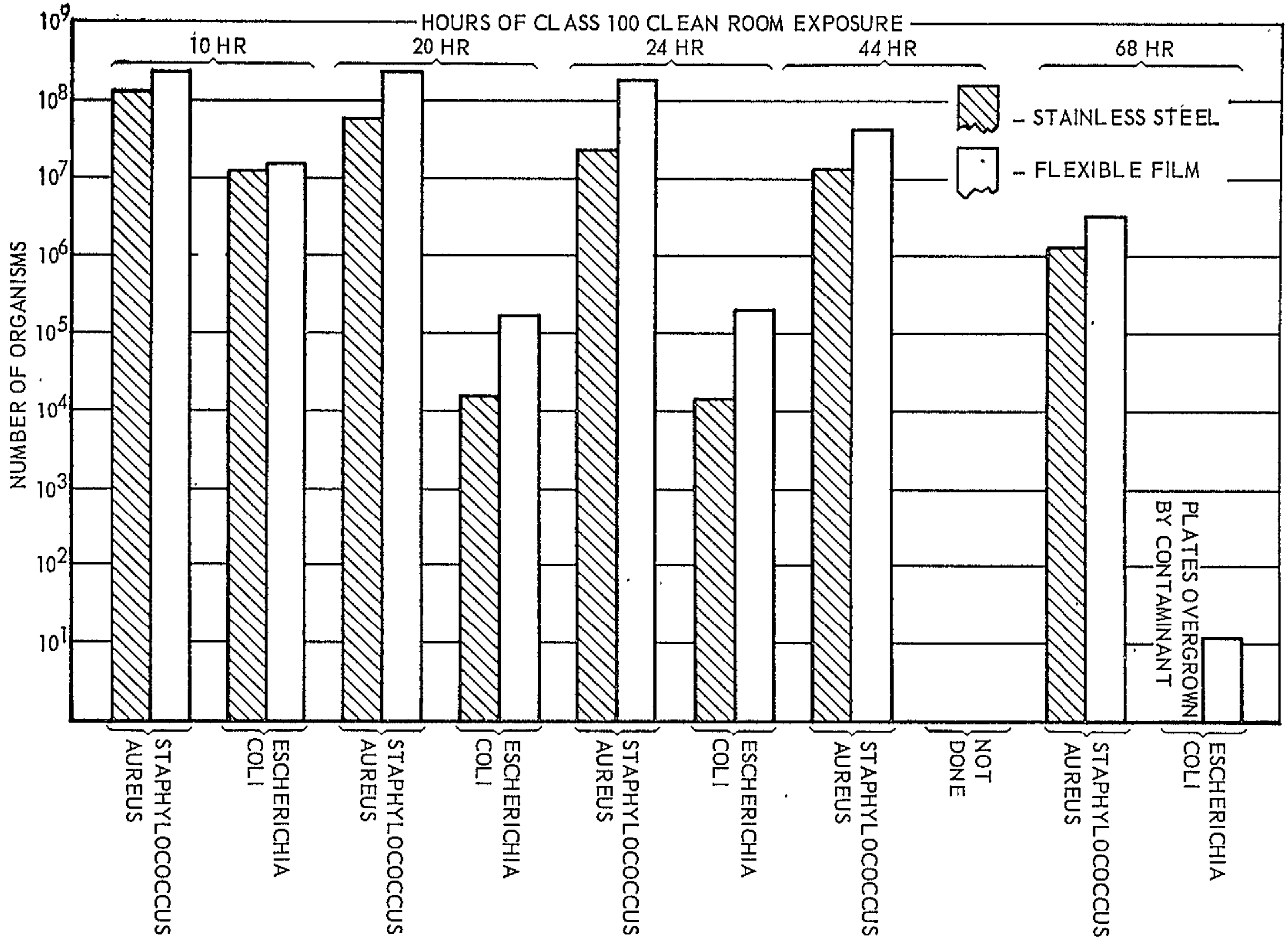
Results of S. aureus and E. coli die-away are presented in Figure 1. As can be seen from these data, the die-away on the flexible film is consistently less than on the stainless steel coupons under these conditions.

To acquire these data, the two coupon materials were attached to flat stainless steel trays as previously described in Bi-Monthly Progress Report No. 1. The bacterial cultures were harvested after 24 hours incubation at 37°C, and washed with sterile buffered saline. To achieve homogeneous suspensions of organisms, all washed resuspended cells were shaken vigorously and sonicated for 2 minutes prior to pipetting onto the coupons.

One might logically question whether the death rates for living cells pipetted onto the coupons are the same as naturally occurring living contamination which settles on the coupons from the air. To test this possibility, aerosols of microorganisms were generated in a glovebox, and trays containing alternating coupons of both materials were exposed to the aerosol for 30 minutes. To achieve consistent results and to eliminate errors due to contamination from the surface of the tray, each coupon was wrapped in perforated aluminum foil, making certain that each coupon had the same number of pre-punched 1/4 inch perforations. Thus, after aerosol exposure, each coupon could be handled for assay and if the coupon moved over the

FIGURE 1

DIE-AWAY COMPARISON BETWEEN TWO MICROORGANISMS ON STAINLESS STEEL VERSUS FLEXIBLE FILM COUPONS IN CLASS 100 CLEAN ROOM ENVIRONMENT



MCDONNELL DOUGLAS ASTRONAUTICS COMPANY
EASTERN DIVISION

surface of the tray on which it rested, it did not pick up additional organisms which settled on the tray during aerosol exposure.

The results of these tests were so grossly different from the expected, that it was requested that the test be repeated many times by different technicians. After over 25 different tests by three technicians, it was clear that it is not possible to expose stainless steel strips and flexible film coupons to an aerosol of living organisms for 30 minutes and achieve even approximately similar numbers of organisms on the two different materials. In every case, the numbers of organisms detected on flexible film coupons was one to three orders of magnitude greater than for stainless steel coupons. Results of some of these tests are presented in Table 1.

As can be seen from the data, if the aerosol method is used to seed the two different types of coupons, then the flexible film coupon has significantly more organisms on its surface as compared with the stainless steel coupons and therefore the die-away rates are difficult to compare because the two types of coupons do not start equal. An investigation was made to determine why flexible film coupons lying on the same tray alternately with stainless steel coupons and exposed to the same aerosol always assay to have at least one order of magnitude more living cells than stainless steel.

One could speculate that several factors account for the difference. One could logically assume that under the conditions of relative humidity (about 75%) during the aerosol exposure, the die-away on flexible film was less than stainless steel. If this biological decay is responsible for the difference, then living spores which are resistant to biological decay or die-away should yield nearly the same numbers for both types of coupons. Results in Table 1 confirm this hypothesis.

TABLE 1
RESULTS OF SIMULTANEOUSLY EXPOSING STAINLESS STEEL AND FLEXIBLE FILM COUPONS TO BACTERIAL AEROSOLS FOR 30 MINUTES PRIOR TO ASSAY

ORGANISM – ESCHERICHIA COLI

	NUMBER OF TRIALS (DIFFERENT AEROSOLS)						
	1	2	3	4	5	6	7
AVG OF 3 COUPONS STAINLESS STEEL	5.5×10^3	2.4×10^2	1.2×10^3	1.3×10^3	2.3×10^3	3.1×10^3	3.1×10^3
AVG OF 3 COUPONS FLEXIBLE FILM	4.6×10^4	1.4×10^5	1.3×10^5	3.1×10^5	2.4×10^5	6.8×10^4	2.8×10^4

ORGANISM – MICROCOCCUS SPECIES

	NUMBER OF TRIALS	
	1	2
AVG OF 3 COUPONS STAINLESS STEEL	7.8×10^2	8.4×10^2
AVG OF 3 COUPONS FLEXIBLE FILM	8.5×10^5	2.6×10^5

ORGANISM – SPORES OF (BACILLUS) SUBTILIS VAR NIGER

	NUMBER OF TRIALS	
	1	2
AVE OF 3 COUPONS STAINLESS STEEL	2.0×10^5	2.6×10^5
AVG OF 3 COUPONS FLEXIBLE FILM	3.2×10^5	4.5×10^5

To test the die-away under different conditions of relative humidity, die-away studies were performed under relative humidity conditions of 35, 70, and 90%. Results are presented in Table 2.

Inspection of the data in Table 2 confirm that the die-away on the two types of materials is very sensitive to relative humidity conditions.

An experiment was then performed which was designed to test some of these hypotheses in combination. Escherichia coli cells harvested after 24 hours of incubation at 37°C were aerosolized for 35 minutes. Three stainless steel coupons, three flexible film coupons, and three stainless steel coupons coated with sterile glycerin were exposed to the aerosol for 30 minutes and immediately assayed.

Stainless Steel	Flexible Film	Glycerin Coated Steel
Average of 3 = 1003 cells	Average of 3 = 250,000	Average of 3 = 245,000

These data suggest that glycerin tends to dehydrate the cells which settle upon it and protect the cells from the high humidity, and that much the same thing happens on the flexible film, which contains both gelatin and glycerin.

2. Geometric Form Die-away Studies - The purpose of these studies was to determine the effect of die-away of spatial orientation of various geometric forms with respect to the air flow in a Class 100 Clean Room environment.

The forms chosen were a pyramid, a cone, a solid cylinder, and a sphere, Figures 2, 3, and 4. After 4 hours of incubation at 37°C, the cells were harvested, washed in saline, and resuspended in saline. The suspension was sonicated for 2 minutes to achieve homogeneity, and equal amounts were pipetted onto flexible film coupons. The coupons were taped by the corners to the various geometric forms. Three locations on each geometric form were chosen. On the sphere, the top (north pole), the equator, and the south pole were chosen as test sites. For the cone

TABLE 2
EFFECT OF RELATIVE HUMIDITY ON FLEXIBLE FILM VERSUS STAINLESS STEEL COUPONS

ALL COUPONS IN EACH TEST SERIES EXPOSED TO ENVIRONMENTAL CONDITIONS SIMULTANEOUSLY

ORGANISM	TIME OF EXPOSURE TO RH ENVIRONMENT	NUMBERS OF ORGANISMS SURVIVING					
		35% RH		70% RH		90% RH	
		SS*	FF!	SS	FF	SS	FF
ESCHERICHIA COLI	30 MIN	2.4×10^2	1.4×10^5	5.5×10^3	4.6×10^4	1.2×10^3	6.3×10^3
	2 HR	0	1.2×10^5	8.1×10^2	4.1×10^4	1.3×10^3	6.1×10^3
	6 HR	0	4.9×10^4	3.0×10^1	7.0×10^3	1.4×10^5	6.0×10^3
SPORES OF BACILLUS SUBTILIS VAR NIGER	30 MIN			2.0×10^5	3.2×10^5		
	3 DAYS			2.4×10^4	2.5×10^5		
	10 DAYS			2.3×10^4	1.4×10^5		
	14 DAYS			7.4×10^3	1.3×10^5		
MICROCOCCUS SPECIES	30 MIN	8.4×10^2	2.6×10^5	7.8×10^2	8.5×10^5		
	24 HR	1.0×10^2	2.9×10^5	3.3×10^1	5.5×10^4		
	96 HR	1.2×10^1	5.4×10^3	1.1×10^1	1.2×10^3		

*SS = STAINLESS STEEL COUPONS
! FF = FLEXIBLE FILM

FIGURE 2
TITANIUM SPHERE USED IN CLASS 100 CLEAN ROOM DIE-AWAY STUDIES FOR GEOMETRIC FORMS. DIAMETER OF SPHERE IS 2 FEET.

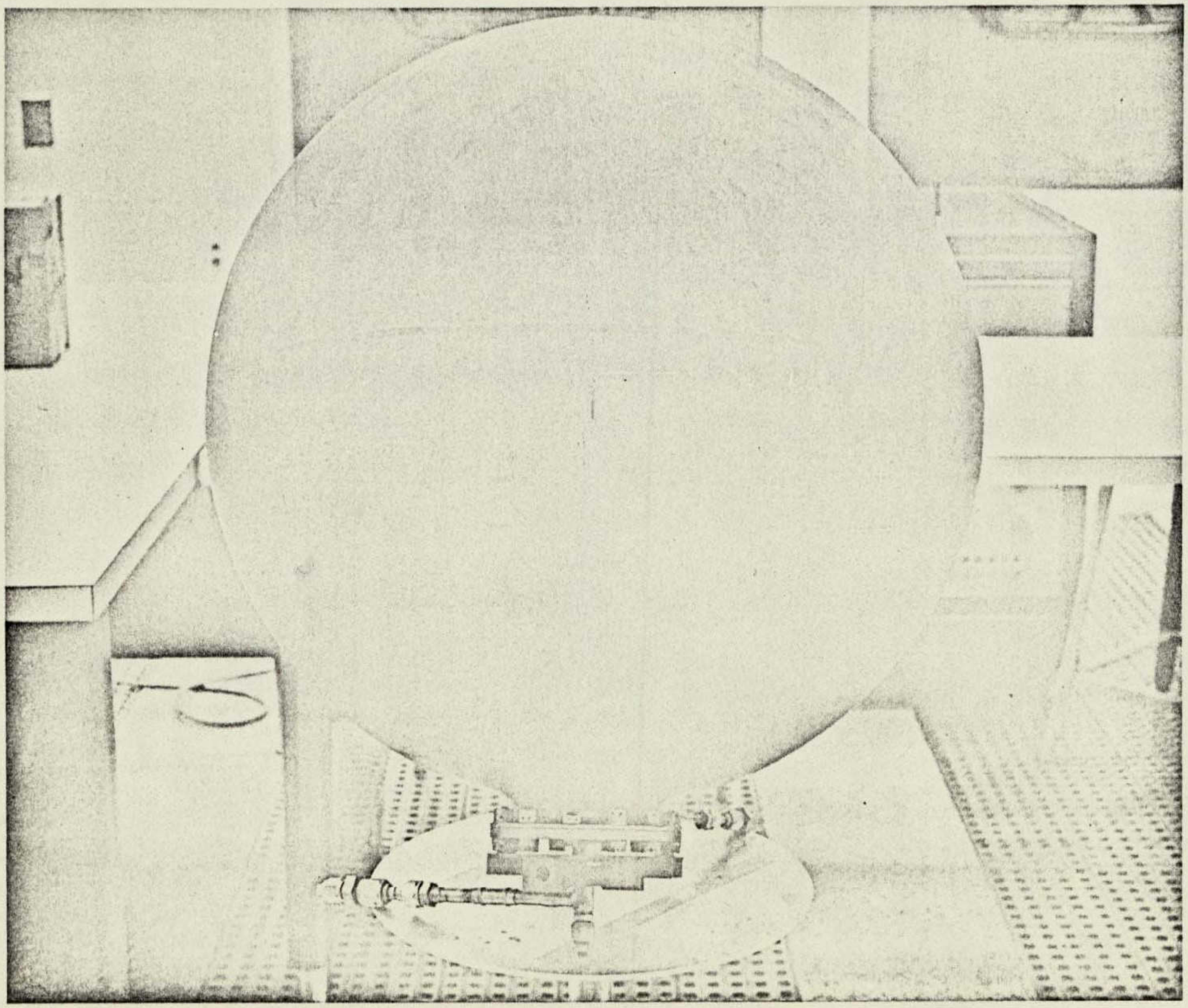


FIGURE 3
CONE USED IN CLASS 100 CLEAN ROOM DIE-AWAY STUDIES.
BASE OF CONE IS 3 FEET IN DIAMETER.

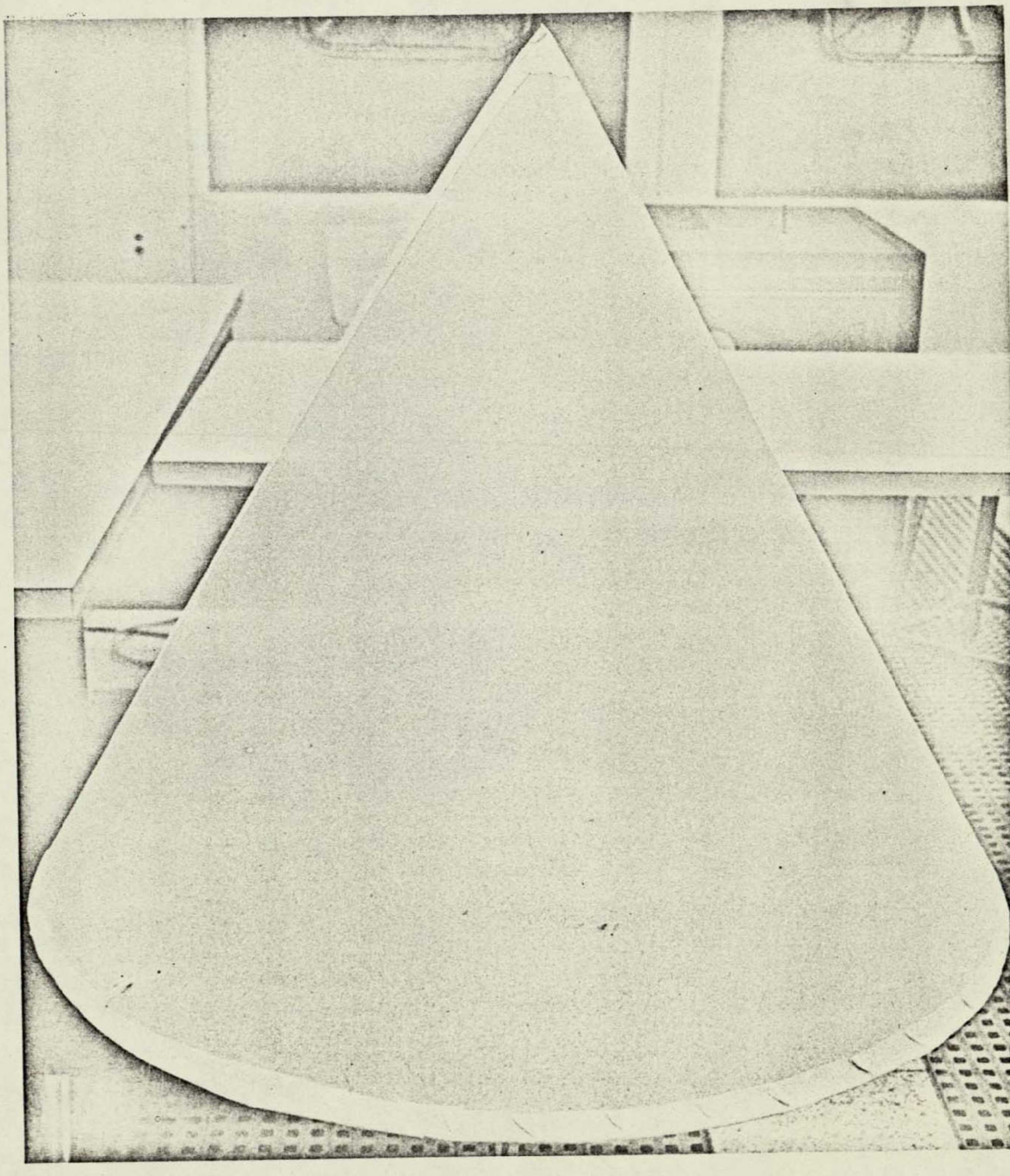
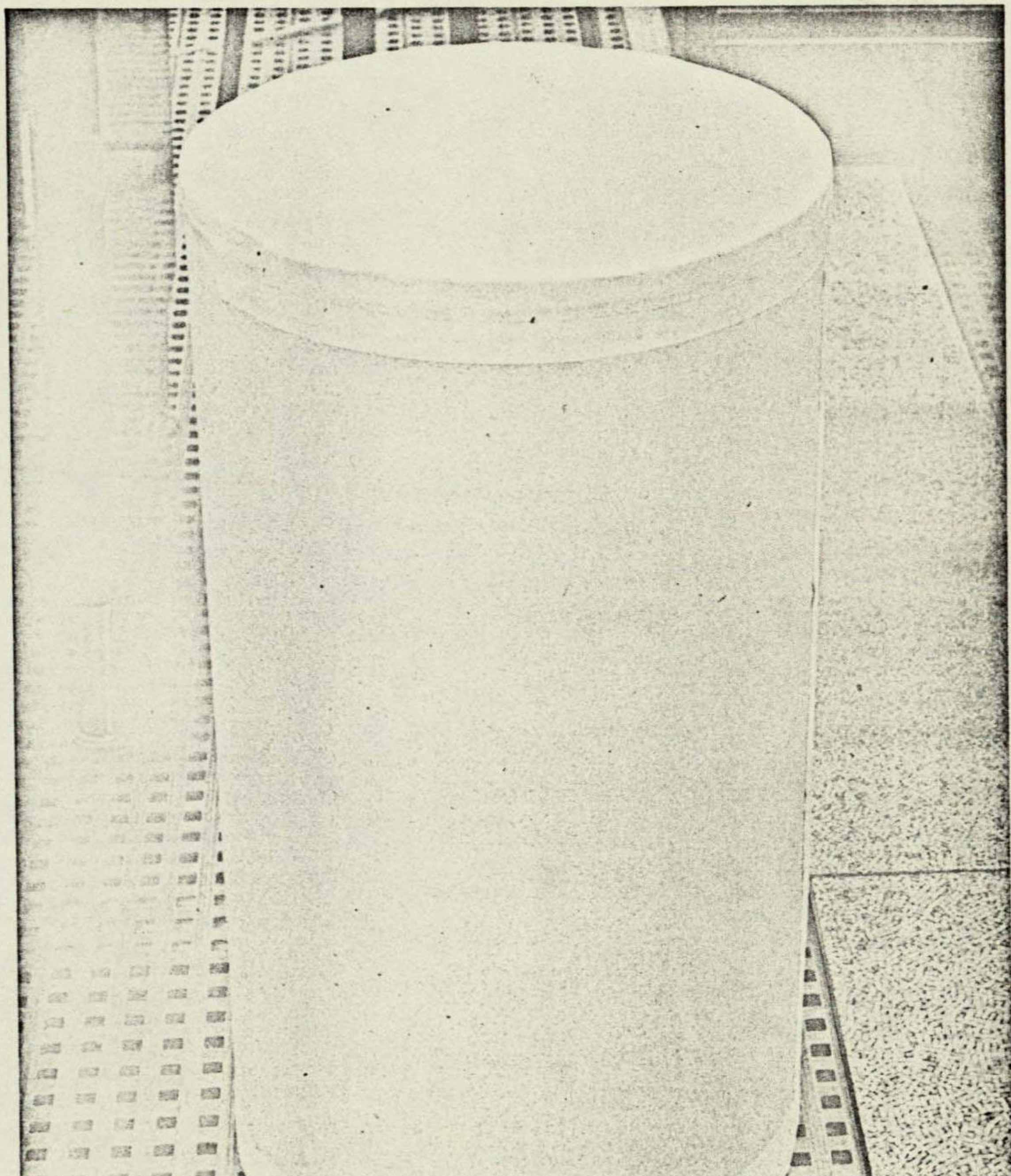


FIGURE 4
SOLID CYLINDER USED IN GEOMETRIC FORM DIE-AWAY STUDIES IN CLASS 100 CLEAN ROOM.
CYLINDER IS 1½ FEET IN DIAMETER AND 3 FEET HIGH.



and pyramid a site near the top, the edge, and the bottom were chosen. For the solid cylinder which was placed vertically to the air flow during these tests, a site on the top, on the middle of the vertical edge, and the bottom were chosen.

Results are quite decisive in all cases, as can be seen in data presented in Figures 5, 6, 7, and 8. These data indicate that the orientation with respect to air flow is very important. Die-away at the top of the form is greater in every case than for the protected area underneath.

Although each coupon at each site on any individual form had been seeded with approximately the same number of organisms, after a few days in the Class 100 Clean Room environment the difference between numbers of organisms detected at the bottom of the geometric form and those at the top directly in the air flow varied from 2.8×10^6 for the sphere to a minimum difference of 2.1×10^5 for the cylinder.

These data are quite striking and are being repeated with spores of <u>Bacillus subtilis</u> var. <u>niger</u> and <u>Clostridium sporogenes</u>.

In addition to die-away studies, geometric forms have also been covered with 12" x 12" flexible film coupons and microbial fall-out determined at each site. Results are not conclusive in that so few microorganisms are present, but thus far no microorganisms have been detected on those coupons not directly in the air flow.

C. <u>Large Format versus Small Format Coupons</u>

The previous progress report had indicated that studies with the 12" x 12" flexible film coupon had revealed that the estimate for one square foot of contamination obtained by assaying 6 of the 1" x 2" stainless steel coupons was conservative (too high) by at least one order of magnitude. These studies have since been extended from the Class 100 Clean Room environment to include data from a Class 100,000 Clean Room. These data are presented on page 21.

FIGURE 5
CLASS 100 CLEAN ROOM
GEOMETRIC FORM DIE-AWAY STUDIES
VERTICAL SOLID CYLINDER

ORGANISM – STAPHYLOCOCCUS AUREUS
EACH DATA POINT AVERAGE OF 3 PLATE COUNTS. ASSAYS MADE FROM FLEXIBLE FILM ORIGINALLY SEEDED WITH SAME NUMBER OF ORGANISMS. COUPONS THEN PLACED AT 3 LOCATIONS ON GEOMETRIC FORM

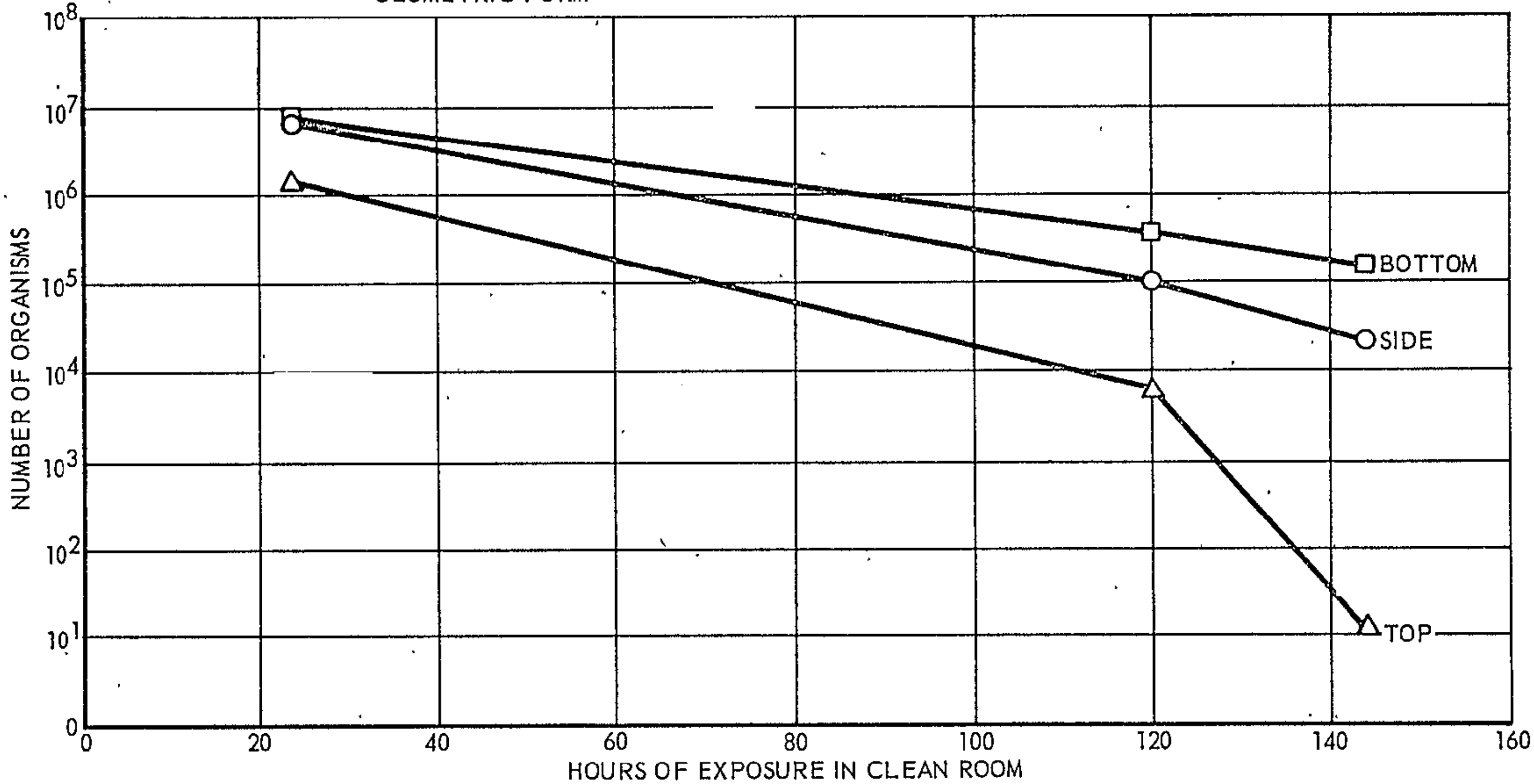


FIGURE 6
CLASS 100 CLEAN ROOM
GEOMETRIC FORM DIE-AWAY STUDIES

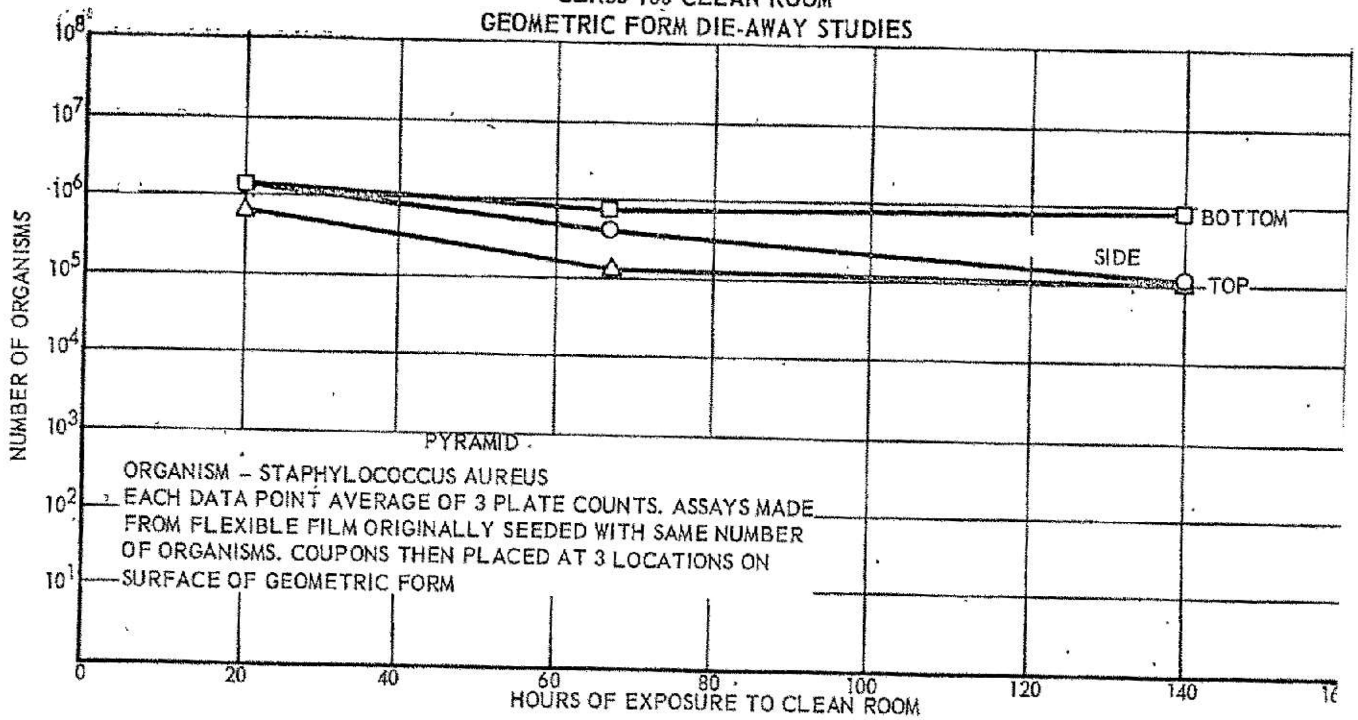


FIGURE 7
CLASS 100 CLEAN ROOM
GEOMETRIC FORM DIE-AWAY STUDIES

CONE

ORGANISM – STAPHYLOCOCCUS AUREUS
EACH DATA POINT AVERAGE OF 3 PLATE COUNTS. ASSAYS MADE FROM FLEXIBLE FILM ORIGINALLY SEEDED WITH SAME NUMBER OF ORGANISMS. COUPONS THEN PLACED AT 3 LOCATIONS ON SURFACE OF GEOMETRIC FORM

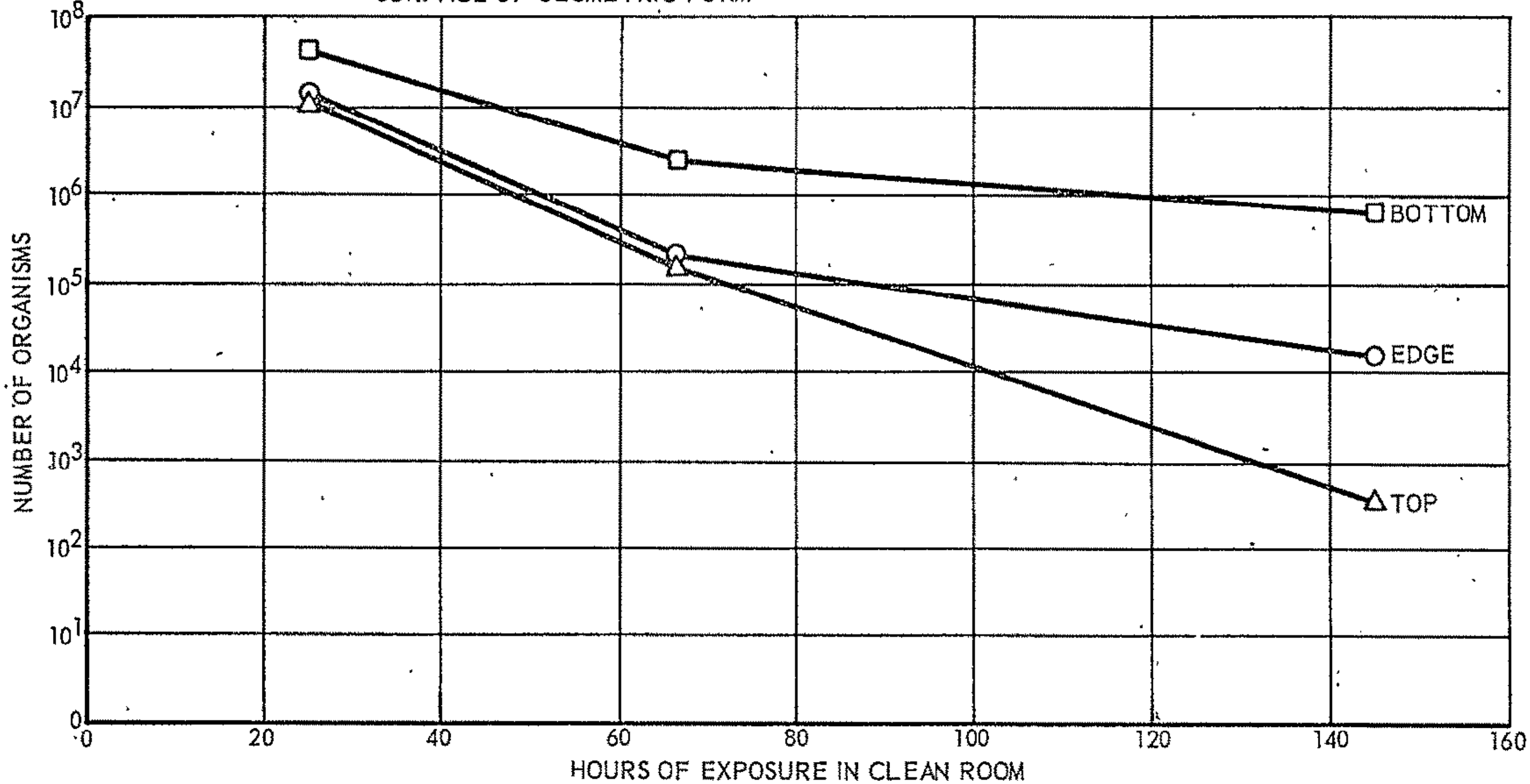
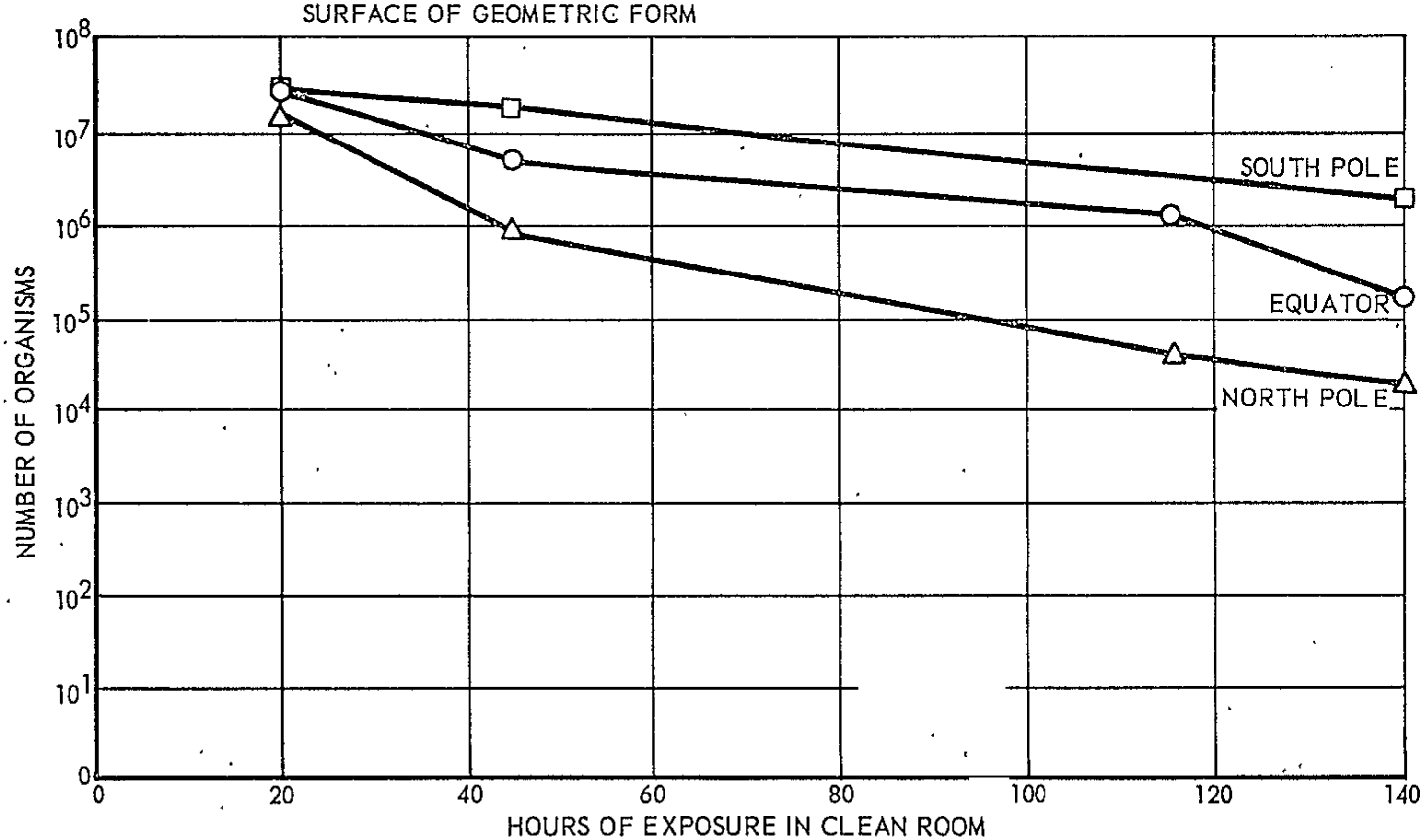


FIGURE 8
CLASS 100 CLEAN ROOM GEOMETRIC FORM
DIE-AWAY STUDIES

SPHERE

ORGANISM – STAPHYLOCOCCUS AUREUS
EACH DATA POINT AVERAGE OF 3 PLATE COUNTS. ASSAYS MADE FROM FLEXIBLE FILM ORIGINALLY SEEDED WITH SAME NUMBER OF ORGANISMS. COUPONS THEN PLACED AT 3 LOCATIONS ON SURFACE OF GEOMETRIC FORM



Days of Exposure to Class 100,000 Clean Room / Microbiological Contamination Expressed in Number of Colonies	5	8	10
Raw Data for 6 Stainless Steel Strips	3	11	21
Calculated for 1 Sq. Ft. from Above	180	864	1296
Actually Detected on 12" x 12" Flexible Film Coupon	3	20	24

The purpose for collecting data in the Class 100,000 Clean Room was to gain the statistical advantage of increased numbers of microorganisms rather than to rely completely upon the small numbers obtained in the Class 100 Clean Room environment.

D. Anaerobe and Spore Compatibility Studies

Having collected data with aerobic organisms, the emphasis is being shifted to anaerobe and spore compatibility studies. Anaerobic spores die-away and compatibility studies have begun and will continue for at least four more weeks. Organisms studied are *Clostridium sporogenes*, *Clostridium histolytica*, and *Clostridium roseum*. All three organisms are anaerobic spore formers. In addition to the above, naturally occurring anaerobic contaminants detected on flexible film coupons will also be studied on a comparative basis with results obtained from stainless steel coupons.

V. DISCUSSION AND CONCLUSIONS

The data presented in this report are exceptionally satisfying from a microbiological standpoint. In a field where one must become accustomed to dealing with dynamic and labile living systems, the microbiologist becomes suspicious of comparative experimental results which have only a small margin of difference.

The comparative data from die-away studies on flexible film versus stainless steel coupons in the Class 100 Clean Room demonstrate a clear superiority for the flexible film.

To those who may object to the validity of die-away studies performed by seeding the coupons with a saline suspension of microorganisms, the data from the aerosol exposure studies is even more convincing evidence of flexible film superiority. These data show that the number of organisms detected by the flexible film is from one to three orders of magnitude greater than with the stainless steel coupons exposed at the same time under the same conditions.

One of the objectives of the program is to determine usefulness of the flexible film either as an adjunct to or a replacement for the currently accepted 1" x 2" stainless steel coupons for microbiological assay. This report demonstrate one important point with regard to that objective. By comparing estimates of microbial load per square foot of surface obtained by the NASA approved stainless steel coupon assay method with actual results obtained with 12" x 12" flexible film, a very good measure of accuracy can be obtained. The data show the estimate tends to be at least one order of magnitude too high. It has always been accepted that the stainless steel coupon method would yield estimates per square foot of surface that were conservative (too high). However, until the advent of the flexible film concept which allows one or more square feet of surface to be dissolved and assayed, no real handle could be placed on the actual degree of conservatism.

These statements may appear paradoxical and at first inspection may seemingly contradict some of the data presented. Therefore some clarification is in order.

The currently approved method of assay employs one percent peptone which will support growth and cell division of bacteria. Fifty milliliters of the sterile peptone is placed in a sterile capped bottle. The bottles are transported to the assay area where sterile forceps are used to harvest six steel coupons, each of which is placed in a separate bottle. The bottles of peptone plus coupons are sonicated and assayed.

There are several opportunities for errors to be introduced in this procedure. Because one percent peptone supports growth, bacterial cell division can increase the numbers of organisms originally introduced. Insecure lids can allow contamination to be introduced into the peptone solution during storage prior to use.

Furthermore, because the number of aerobes detected is determined by plating 10 milliliters of peptone solution and multiplying by a factor of 5 to account for the original 50 milliliters, a random contaminating organism which landed on the plate during assay suddenly becomes five organisms. Then when the calculations are made to extrapolate to one square foot the error becomes 5 times 12 or 60 organisms!

The point to be made in all of these permutations is that the flexible film coupons studied in the same format as the stainless steel coupons, and assayed with peptone under NASA standard conditions, yields data indicating the microbiological contamination is at least as high as that detected by stainless steel coupons, and under certain conditions the yield is much higher. Therefore, the flexible film has been demonstrated to be comparable with the stainless steel coupons when used in the 1" x 2" format.

However, by expanding the flexible film coupon to the 12" x 12" format, which cannot easily be done with the stainless steel coupons, and eliminating the errors introduced by the standard method, the advantage of real data over extrapolated data is realized.

The same set of circumstances surrounds the die-away studies on the various geometric forms. Because the flexible film can more easily conform to irregular surfaces, it is easier to collect such data than with stainless steel coupons. Furthermore, in natural contamination studies the 12" x 12" format flexible film coupons would yield more realistic data than the results of assay from several smaller stainless steel coupons.

It has been assumed by most workers in the field that the differences in die-away such as those demonstrated by the data from the geometric forms with respect to orientation with the air flow in the Class 100 Clean Room would be negated because under real conditions the assembled hardware would be rotated. These data will be useful in answering such questions as "how much rotation is necessary?" and "how often should the hardware be rotated to achieve maximum die-away of the microbial contamination?"

VI. FUTURE TESTING

The Phase II program continues on schedule. During the next two months, the following tasks will be performed:

Die-away studies and compatibility studies for anaerobes on flexible film versus stainless steel coupons.

Die-away for aerobic and anaerobic spores under Class 100 Clean Room conditions on flexible film versus stainless steel.

Distribution of microorganisms on 12" x 12" versus 1" x 2" coupons.

VII. REFERENCES

1. NASA NHB 5340.1, Standard Procedures for the Microbiological Examination of Space Hardware. August 1967.
2. Development and Test of Flexible Film Coupon Strips for Use as a Sampling Technique, Bi-Monthly Progress Report No. 1, 10 September 1968.