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CASTLE
RITTER PFAUDLER CORPORATION

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FINAL REPORT

THE EVALUATION AND REFINEMENT OF A MATHEMATICAL MODEL FOR THE STATISTICAL DETERMINATION OF INTERNAL MICROBIAL CONTAMINATION OF SPACECRAFT MATERIALS

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ABSTRACT

Many attempts have been made to determine viable microbial numbers in solid materials. Such methods were, for the most part, unsuccessful, or the estimates were invalid. The philosophy of extreme particle size reduction for microbial recovery lead to very low recoverable percentages, and this method would tend to overlook small contamination levels. The physical means for reducing particle size of products would also place undo stress on the microbes because of the heating, grinding, etc. By applying simple laws of probability, this report indicates that an exposure of known geometrical surfaces to microbial nutrients will allow the growth of discriminate colonies from the exposed surface. Furthermore, simple fragmentation procedures, producing particles of rather large volume, may be sufficient to provide meaningful estimates of in-solid microbial levels by adding statistical considerations, such as particle size and surface area distributions.

The data in this report indicates that the reliability and efficiency of estimating microbial in-solid levels are as good as or better than most surface contamination estimating methods.

It is felt that the proposed model is valid. For the most part, the results which did not agree with the expected were so radically different that the assumption must definitely relate to procedural problems. The application of this method would also point out specific problem areas.

INTRODUCTION

The recovery of viable microorganisms occluded within solid materials has been recognized as extremely important to the fulfillment of spacecraft sterilization.

Microorganisms occluded in solid materials are not only difficult to detect, but also are very likely to have greater resistance to dry heat than surface contamination (2-10).

Numerous investigators (8-9,12-15,21-25) have used methods for particle reduction such as grinding, breaking, drilling, milling, and sawing to recover organisms in solid materials.

Rather than sawing, milling, etc. it was proposed in this study to investigate simple cleavage and crushing techniques for the detection of entrapped microorganisms. The advantages of the application of such techniques in combination with probability estimates resides in the freedom from the need to obtain extreme size reduction of materials under assay. Indeed extreme size reduction is undesirable because it complicates the problem. This approach promised furthermore, to accomplish assaying more rapidly with a much higher level of confidence and to provide optimum data for total burden estimation.

THEORY

Assume that an object of some uniform matrix is a one cubic centimeter cube containing 10^6 viable spores uniformly dispersed. Assume also that its external surfaces will be sterilized prior to size reduction. For calculation purposes the bacterial spore is considered a particle of one micron size. (This is the average spore size). If this block could be fragmented into particles of one micron size, 10^{12} individual particles would result of which 10^6 would be the bacterial spores.

The probability of randomly choosing one particle out of 10^{12} and finding it viable would be 10^{-6} ($P=10^6 \div 10^{12}$). If this block were cleaved into two equal cross sections, theoretically two non-sterile areas of 10^8 square microns each would be exposed, exposing the equivalent only of 2×10^8 individual particles of one micron size. It is fair to assume that the exposed spores will distribute evenly on either of the two newly exposed surfaces. The chances are excellent, therefore, that the number of exposed particles of the specified size will be at least 10^8 or higher (since exactly smooth surfaces are usually not obtained on cleavage). Exposing in this manner at least 10^8 one micron particles, produces a chance of $C = 10^8 \times 10^{-6} = 100.$, and the probability is a certainty that at least one viable particle will grow if both of these half cubes are placed separately in or on a culture medium.

If now, for example, the same block contains only 100 viable spores instead of 10^6, one cleavage will present a probability of finding a single viable particle by the same single cleavage method of $P = 10^8 \times 10^{-10} = 10^{-2}$, or one chance in one hundred. However, fragmenting the block into at least 100 fragments would again increase the probability to a certainty or to a >1:1 chance of finding a single viable particle. Assuming uniform size, 100 fragments of a 1 cc block would require each fragment to be of $10mm^3$ in size, having an "effective" surface area of about 5×10^6 square microns. The "effective" surface area allows for equal distribution of "exposed" spores between the 6 mating surfaces.

In applying this model, it is not assumed necessary that the particles produced be of uniform size, but rather be of some uniform distribution from which a reasonable estimate of surface area can be made for use in the calculation.

Using the data presented by Reed (25) and applying the model in analysis to this data, an excellent agreement is obtained in most cases. In making these calculations the particle size was assumed to be of uniform distribution over a narrow range. Any error made in such an assumption would have led to overestimating the exact number, a safety factor in the application of this principle to total burden determination.

The calculations were made as follows:

As stated before, the probability P_M of finding a single viable particle of one micron size from the assumed 10^{12} unit particles (of one micron size) contained in 1 cubic centimeter block is $P_M = 10^{-6}$.

There are 6×10^4 exposed unit surfaces on each fragment of 100 micron size, but because external sterilization has been applied prior to crushing, only the newly exposed surfaces are counted and because of mating surface distribution 10^4 average effective unit particles exposed. Therefore, since there are 10^6 fragments, we would expect to find 10,000 viable spores in the total fragmented mass of 1 cc when all 10^6 fragments are tested for viability by colony counting procedures. This agrees very well with the data reported by Reed (Table 1). In like manner, taking fragments of 1,000 micron size and applying the same procedure, it is calculated that Reed should have found 1,000 exposed bacterial spores. This is what was found in several instances. In the same manner 150-micron size fragments should have produced a viable count of 6,760 particles. This agrees very closely with the reported values. Considering all of the variables associated with Reed's reported work, it is statistically very significant that such close agreement could be obtained between the calculations and the actual test

results.

We attempted in this study to question each step of technique which may lead to error. For example, some investigators have used the standard sterility test media, thioglycollate broth in determining internal sterility. This approach may be invalid since recently it was found that aerobic organisms held in the low oxygen tension portion of this media will not grow (11). Organisms released by grinding, milling, etc. may still adhere to material in which they were encased, consequently (depending on density of the material) be held in the low oxygen tension portion of thioglycollate broth. Since our test organism is aerobic, we did not use thioglycollate, but rather trypticase soy broth.

Ethylene oxide decontamination of the exterior portion of solid materials has also been a common method among researchers investigating interior contamination. We have found that some materials are permeated by ethylene oxide and internal contamination of space craft components could have been grossly underestimated.

Considerable effort was spent to find a water insoluble, organic solvent soluble solid material to which viable spores could be easily added, and subsequently recovered. The necessity of water insolubility is obvious - organisms would not be easily released when the solid was added to the media.

The need for a solid that was soluble in an organic solvent was based on the fact that an alternate assay method would lend verification to results obtained by simple cleaving or crushing. The alternate assay method consisted of a membrane filter technique similar to that described by Sokolski and Chichester (27). Also the organic solvent had to be non-toxic to the test organism.

Although dissolution of solids cannot be the primary way of assaying spacecraft materials, we felt that for model test materials we should use this approach for verification and comparison of the internal bacterial count.

MATERIALS AND METHODS

I. Test Microorganism.

Bacillus subtilis var. niger spores were heat-shocked at 65°C for 30 min. and were used as inocula for starter cultures in casein acid digest medium of the following composition: casein acid hydrolyzate powder (General Biochemicals, Inc., Chagrin Falls, Ohio), 10.0 g; yeast extract (Difco), 5.0g; glucose, 5.0g; KH_2PO4, 5.0g; $CaCL_2 \cdot 2H_2O$, 0.66g; $MnSO_4 \cdot H_2O$, 0.03g; tap water, 1,000 ml. The medium was filtered, adjusted to pH 7.0, and sterilized in steam at 121°C for 20 min.

After shaking for 24 hours at 32°C, the sporulated culture was kept at 45°C overnight to allow autolysis of vegetative cells. The spores were then harvested by centrifugation, washed eight times in distilled water, and checked

by phase microscopy to ascertain absence of debris. As ascertained by standard plate counting technique, 100 ml. of Bacillus subtilis var. niger spores at 10^{10}/ml was obtained. This suspension was stored at refrigeration temperature and used throughout the Mathematical Model program.

II. Materials.

A variety of materials were investigated. For one reason or another, most of them were rejected for use in the Mathematical Model program. A listing of these materials follows, describing the problems associated with them.

A. Dental investment compounds. Solids are formed by mixing a powder with water contaminated with spores and allowed to set. The internal count could not be verified because they could not be dissolved after the material solidified.

1. Diolite, Cristobolite, and Grey investment formed soft porous solids which tended to break up when placed in media. Since organisms contained within them could easily grow out, these materials were rejected.
2. Super-die formed a hard solid, although the internal count could not be verified as stated previously.

   This solid was evaluated as to the ease of Ethylene oxide penetration. Exposure of 1 cubic centimeter cubes to 1200 mg ethylene oxide/liter, 40% RH, 130°F, reduced the viable count 2 logs as

compared to untreated controls.

The assay procedure consisted of crushing the solid with a sterile hammer, waring blending the crushed material in sterile distilled water for five minutes and plating the slurry in plate count agar using standard plate counting techniques. Although this assay method did not remove all occluded organisms, the method should be valid for comparing ethylene oxide treated and untreated material.

This material was dropped from further consideration after obtaining inconsistent results. Super-die tended to partially deteriorate at times leaving colloidal particles that appeared as growth.

B. Materials that solidify by catalyzation.

1. Styrene monomer (polymerized with methyl ethyl ketone peroxide) killed the spores that were added as determined by membrane filtration. The catalyzation process was implicated since complete recovery of spores was obtained when spores in dissolved polystyrene were assayed in a similar manner.

Other methods were investigated as possible ways of obtaining spores in polystyrene. They are as follows:

a. Polystyrene was softened to a liquid like state with acetone. The excess acetone is decanted off

and replaced with a measured amount containing the Bacillus subtilis var. niger spores. The resulting emulsion was then poured into cube molds (Fig.1) and allowed to harden to room temperature for several days. Spores, however, tended to settle before hardening.

b. In the second method, polystyrene was dissolved in toluene. Then a toluene suspension of Bacillus subtilis var. niger spores was added. The solution was then placed in isopropyl alcohol in order to precipitate the polystyrene. These methods were inexact, time consuming and spore recovery, erratic.

2. Methacrylate monomer was polymerized by known methods. However, we experienced difficulties in controlling the rate of polymerization. This material was dropped early in our investigation. However, by following recently published methods of Angelotti, et. al. (2), a solid probably could be formed which would be ideal. Angelotti et. al. obtained high recovery levels of spores in methacrylate polymer.
3. "Clearmount and Quickmount" were also investigated. However, no solvent could be found to completely dissolve them. These materials are used for mounting specimens.
4. Silicone rubber (General Electric RTV-615A) is catalyzed by a tin catalyst. This compound appeared to be ideal as to the ease of adding spores and forming a rather

flexible solid. However, cleavage of 1 cc cubes from 9.2×10^3 spores/cube to 3.5×10^6 spores/cube showed no recovery.

Silicone rubber was not inhibitory to Bacillus subtilis var. niger. The spores were possibly killed in the catalyzation process or the exposed spores cannot grow out when partially encased in this material.

C. Relatively low melting point compounds. The purpose here was to find compounds to which spores could be added while the compounds were molten. If the compound hardened sufficiently upon solidification for cleaving and would not be inhibitory to Bacillus subtilis var. niger, then it was to be used in this study.

1. Cement/stik (medium) Fisher Scientific Company was insoluble or partially insoluble in a wide range of solvents tested, although the manufacturer stated that it is alcohol soluble.
2. Varno-Cement (E.H. Sargent & Co.) seemed at first an ideal material; however, we were unable to find a solvent in which it was completely soluble. Alcohol was claimed to be a solvent but it was only partially soluble in this solvent.
3. Picein Cement (E.H. Sargent & Co.) seemed to be an ideal model material at first. It was completely

soluble in toluene. Spores could be added in the molten state at 80°C and spores were recovered with a membrane filter technique equal to what had been added. However, this compound was extremely inhibitory to Bacillus subtilis var. niger in both solid and liquid medium.

Other spore formers were tested to determine whether they were inhibited by Picein Cement. The following organisms were inhibited by Picein Cement: Bacillus coagulans, Bacillus megatherium, Bacillus subtilis 5230. The following organisms were not inhibited by Picein Cement: Bacillus cereus, Bacillus subtilis WC18.

This compound would very likely serve as an ideal material for the mathematical model. However, at the time we did not want to change organisms.

An important point was brought out by the experiments with Picein Cement - that materials to be tested for the presence of bacteria should be also tested with a variety or organisms to determine whether or not the compound is at least somewhat inhibitory.

4. Paloja (Dreyfus Company) is composed primarily of chicle which is used as a base in chewing gum. This material could make an interesting compound since spores can be added to it. However, none of

the solvents tested were capable of completely dissolving it.

5. Low melting point plasticizers were found in Modern Plastics Encyclopedia*and Plasticizers by D.N. Buttrey.**

The compounds listed in Table 2 were investigated. Of these compounds only two were considered in depth, Saniticizer 3 (n-ethyl para sulfonamide) and Aroclor 2565 (Chlorodiphenyl) made by Monsanto Company.

Subsequent experiments with Saniticizer 3 showed that recovery was obtained from whole uncleaved cubes. Since Saniticizer 3 was crystalline in nature it was felt that some of the spores were in the interstices of the crystalline structure. This structure was apparently porous enough to allow media penetration and subsequent outgrowth.

Aroclor 2565 was finally settled on as the test model material for the program. A detailed description of procedure concerning this compound will be given later in this report.

D. Miscellaneous solids.

a. Agar gel systems. Various percentages of agar in distilled water from 3% to 9% containing Bacillus subtilis var. niger spores were investigated to determine the suitability of a water based agar solid. Other materials such as hydroxyethyl cellulose and cabosil were added to the agar gel system to de-

* McGraw-Hill, Inc. New York, N.Y. 1967

** Buttrey, D.N., Franklin Publishing Co., Inc., Palisade, N.J. 1960

crease the permeability of the agar to nutrients and/or bacteria. The stimulus for this approach came from a paper by Miller and Davey (16) who found a decrease in bacterial multiplication with culture media plus agar when compared to culture media without agar. These investigators suggested that the decrease in bacterial multiplication may be due in part to a quasi-crystalline structure of the water around the agar in the gel state which reduces the fluidity of the water, the distribution of the bacteria, and the mobility of nutrient ions. However, growth was obtained from uncleaved agar cubes containing spores and this approach was abandoned.

III. Methodology with Aroclor 2565.

Aroclor 2565 was presterilized at 125°C for at least three hours. (In a series of experiments, Aroclor was sterilized with 1200 mg/l Ethylene oxide, 40% RH, 130°F for 16 hours. However, the Aroclor became sporicidal in this procedure and was discontinued). Spores were then added to the molten Aroclor by either adding predetermined amount of acetone spore suspension or adding pre-inoculated Aroclor disc and vigorously shaking.

The latter method of adding the spores became routine since violent acetone fumes were produced by the former method.

After vigorous shaking for 3 minutes the molten Aroclor was dispensed into 1 cc molds. Originally the mold we used was one made out of stainless steel, machined to give accurate 1 cc cubes (Figure 1). However, using this mold limited the number of cubes that could be made. Consequently, more 1 cc molds were made by vacuum forming 10 mil. polyethylene over cubes made in the original mold from 11% agar (Figure 2). Molds of almost any size and shape could be made with this procedure.

Assaying. ——— Representative cubes were taken, weighed and dissolved in toluene. This solution was then passed through a 0.25 micron membrane filter. The residual toluene was rinsed away by passing acetone through the filter. The filter was then "waring"-blended in sterile distilled water for 3 minutes followed by sonication at 10kc. Plating was then performed using standard plate counting procedures.

Cleaving. ——— The surfaces of cubes were sterilized by dipping the cubes in 2% peracetic acid for 2 minutes followed by 1 or 2 rinses in sterile distilled water. Cleaving was performed by simply placing a sterile single edged razor blade at the spot to be cleaved and tapping with a hammer.

Crushing.——— Crushing was accomplished by simply pounding the cubes with a sterile hammer. Sizing was accomplished by sieve shaker (Figure 3). Surface area determinations

were made from the size distribution. The cubes or pieces were placed either in trypticase soy broth or plate count agar. Incubation temperature was 23°C. (Previously some whole cubes were incubated at 37°C and growth occurred. Perhaps organisms were released when the cubes softened at that temperature). Incubation time was 5 days at 23°C in agar or 14 days at 23°C in broth.

The data generated by the agar overpour technique allowed a comparison of expected colony numbers (as derived from membrane filtration counts) with observed colony numbers that grew from cleaved surfaces.

Contamination was more readily detected by the agar overpour method since the colonies grew only from the cubes.

Crystalline material such as saniticizer produced higher colony levels upon cleavage than expected, especially from broken pieces. This indicated that contamination was readily released from between crystals.

RESULTS

Not all of Reed's (25) data is displayed in Table 1. In fact, only 50% of the applied data is shown. Of that data which is not displayed, most of it was reasonably close (similar to results one would expect from swabbing surfaces), except for the eccocoat IC2.

In our calculations, Reed's particle size diameter is taken as the side of a perfect cube to provide maximum surface area for the calculations, according to the following formula; $Ne = n \times P\mu \times As$; where Ne is the estimated colony count and (n) is either the number of fragments or the number of effective cleaved surfaces. $P\mu$ is the probability of finding a single particle viable of the total number of unit particles of 1 micron. There are 10^{12} unit particles per one centimeter cube. (As) is the "effective" exposed surface area. Since it is known that the specimens contained an inoculum of 10^{6} spores, the calculations were made to provide an estimate of the colony counts which one would expect under these circumstances by colony counting methods.

To determine estimates for viable microorganisms in solids one would have to work from plate count or viable culture tube counts backwards.

Table 3 shows generally poor recovery compared to the

following tables. In this case surface viability was determined specifically by a growth or no growth technique. This technique is more applicable to estimating counts by fragmenting solids, such as indicated in Table 1. For simple cleavage, Table 3 shows that colonies can actually be counted on the cleaved surface where growth occurs, and that this is a better method for determining numbers more accurately. However, in the countable range from 10^2- 10^4, the recoverability is within the same order as one would expect recovering viable bacteria from surfaces. The same is further implied in Table 4, which data is also plotted in Figure 4. For this particular case (single cleavage and counting surface colonies), the accuracies of the estimates seem to pass through an optimal contamination level of around 10^4 per cc, from overestimates to underestimates as the contamination level increases. The over all spread of counts is fairly wide, but within well-accepted limits as implied above. Since the estimates are all well within the order of magnitude, it would be acceptable from the standpoint of death time determinations, for example.

Colony counts are generally overestimated for the amalgum solids as shown in Table 5. In this case the higher than expected results of cleaved surfaces are close to the expected estimates, whereas the viability vs.non-viability method of estimating numbers produces less than the expected, but again generally far exceeding most surface detecting accuracies.

The additives may tend to reduce crystallinity thereby releasing more viable particles to the nutrients of the medium.

Use of the cleaved surface counting procedure (agar overpour method) would require less sampling to obtain meaningful statistical estimates which are much more accurate than the results which can be obtained by the simple viable vs. non-viable (broth technique) determinations of cleaved surfaces.

The data of Table 6 indicates that the fragments have a greater surface area to volume ratio than the theoretical cubes, and that this ratio increases as fragment sizes decrease.

DISCUSSION

In the preliminary consideration of Reed's Data (25), it would be statistically impossible where so many degrees of freedom are involved to get such close agreement between theory and experiment, as was displayed in Table 1, by pure chance. There were 3 different solid materials, 2 methods of fragmentation (Reed displayed 4), and 4 replicates in each case.

The relatively poor results of Table 3, and the results for the high contamination levels of Table 4, may reflect coincident counting (definitely implied by the method of Table 3), or by competition for nutrients, space, etc.

The probability method for estimating high levels of contamination will no doubt suffer from accuracy for the reasons discussed, using the single cleavage method. However, it may be better to use a fragmentation plus plate counting technique for high contamination levels as per Table 1.

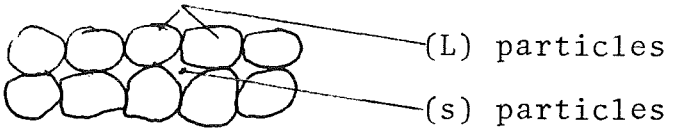
The overall range of results presented thus far (one graphical presentation Fig. 4), agrees with probability estimates well within an order of magnitude, and are, in the opinion of the author as good, and in some cases better, than the generally accepted methods for determining viable levels on solid surfaces.

The examination of Table 6 makes several things apparent: (A) The data is consistently uniform in the progression and distribution of the plate counts for the various seive sizes, which indicates a definite analyzable pattern. (B) The range of theoretical counts, as determined by the cube fragment method, are reasonably close to the actual range of counts in the larger particle sizes; however, the actual range of counts in the lower fragment sizes are consistently (excluding one case) higher than the theoretical. There are undoubtedly several explanations for this.

Normally, one would expect the opposite of what is actually the case, that the counts on the larger fragments would tend to bias toward higher levels than the smaller fragments because multiple colonies can be observed on the larger surfaces, whereas crowding would prevent discriminate colonies from forming on the smaller surfaces.

The estimation of "effective surface" area for large fragments must be higher than that for small fragments, depending on the ability to observe more than one colony per surface. Thus, "effective surface" area for cleaved surfaces are based on a bipartite system, such as was applied to the analysis of cubes or slices of cubes, whereas the "effective surface" of minute fragments (considered as cubes) are based on total surface area, and viable particles are considered as being shared by 6 mating pieces.

What may have caused the observed bias in counts is due to fragmentation non-uniformity. This possibility can be described by considering the following diagram:



The analysis of the fragmentation considered the particles as various sized cubes. To be more accurate would require a careful surface analysis, and a closer evaluation of size distribution and geometry. One possible method of determining the surface area of particles would be to use low pressure permeametry as described by Orr (19). The large (L) particles of the diagram more closely approximate the areas of the theoretical cubes, and would generally bias the results to lower actual than theoretical counts. However, the smaller (s) intersticial fragments would tend to have a higher effective surface area and bias the probability of detection, and therefore result in higher than theoretical counts. Analysis of (s) particles indicates that their surface areas are at least 1.75 times that of cubes of the same volume.

Where fragmented particles can be properly identified as to distribution, and surface areas, colony counting or the use of most probable number techniques will lead to reasonable estimates of viable contamination.

For sterility testing, it would be indeed fortunate if the bias such as is indicated in Table 6 would be toward higher than theoretical recovery. For the estimation of internal burden for space craft solids, it is also better to overestimate, since it will lead toward the application of sterilizing parameters which will provide additional confidence and safety. It is indeed better to use cleaved surfaces of known areas wherever it is possible, since this provides more reliable estimates of in-solid viable contamination.

The method could be made nearly universally applicable if the following guidelines are followed:

1. One must be familiar with the solid material in question:
    a) Degree of crystallinity
    b) Is it inhibitory to the contaminating microorganism?
    c) Determine the die off rate in the solid in question. This can be reasonably estimated by the same probability procedures.
2. A reasonable estimate of the surface area must be made. Small variations in surface area do not significantly effect the estimates. For fragments, particle sizing and distribution, it is indicated, will generally provide reasonable estimates of surface area.

3. The effect of cleavage on exposed surfaces could be critical if microgrooves or cracks result. This can be determined by the observation of the surface under a microscope. Probability estimates that are excessively high would indicate this as a possible condition. Other methods of cleavage or cutting, such as liquid N_2-freezing or slicing can be attempted to alleviate this condition.
4. Extremely high counts can also occur if the material is slightly soluble on the agar or in the media. Experimentation may show, in such a situation that the solid can be dissolved, and determinations could be made by filtering the dissolved solid using membrane techniques.
5. Very low numbers in undissolvable solids must be determined statistically by using many cleaved samples or by fragmentation and plate counting or by using liquid media and most probable number techniques.
6. Very high numbers in undissolvable solids can probably best be determined by use of fragmentation and plate counting techniques.
7. There will be an optimal efficiency contamination level, in the range of which best estimates will provide. From these data, materials, and methods, this range is between and including 10^2 and 10^4 spores per cc.

PROPOSED APPLICATIONS OF THE METHOD

1. Estimating viable contamination in solids. Particularly bacterial spores have increased resistance to heat in solid materials.

2. Sterility testing. (A) under ideal conditions using an adequate sample, the method could be made sensitive enough to detect as low a single viable particle per cc. (B) Where internal contamination of solid materials need to be sterilized test pieces of the particular solid may be fabricated incorporating high numbers of resistant spores. The method is then applied to assay the test pieces in lieu of actual parts or components.

3. Depth of penetration studies can be made meaningful by using test pieces of materials of interest for heat, irradiation, or permeating chemicals such as EtO. For example: (A) In-depth penetration of heat can be quite readily determined by the same mathematical process if the microorganisms near the external portion of the solid piece are killed to some depth leaving some internal volume with viable organisms. (B) A definite differentiation can be made between the situation described above and one in which the microorganisms are reduced in number but uniformly dispersed, such as might result from a random protective effect from the heat treatment. (Protection by occlusion in Crystals, 10). The same evaluation could relate to depth of penetration of ethylene oxide. All previous evaluations of in-solid con-

tamination which used EtO for surface decontamination are invalid since we have shown that EtO does indeed penetrate many such materials.

4. Grow-through studies or depth of penetration of viable organisms growing into materials could possibly be evaluated by this method.

5. Application in the statistical evaluation of release of viable organisms from solids breaking upon impact or by thermal stress of hot-cold cycles and uneven heating, by erosion or chemical elution over an extended period of time.

CONCLUSION

It is felt that the proposed model is valid. For the most part, the results which did not agree with the expected were so radically different that the assumption must definitely relate to procedural problems. The application of this method would also point out specific problem areas.

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Table 1

Number of Bacillus subtilis var. niger colonies recovered from pulverized solids (Reed, L.L., 1965, Ref. # 25). Compared with probability estimates calculated by the method of this report.

Fragmented Material	Particle Size	Probability Estimate (PE)	Colony Count (CC)	Pulverizing Method	% Recovery CC/PE x 100
Paraplast	150	6,760	5,750	Drill	85.0
			6,080	Drill	89.9
			6,680	Drill	98.8
			2,120	Drill	31.3
			6,990	Mortar & Pestle	103.4
			6,280	Mortar & Pestle	92.9
			5,750	Mortar & Pestle	85.0
Plaster of Paris	100	10,000	10,750	Drill	107.5
			10,070	Drill	100.7
			10,100	Drill	101.0
			8,200	Drill	82.0
			6,600a	Mortar & Pestle	66.0
			4,800a	Mortar & Pestle	48.0
			5,490a	Mortar & Pestle	54.9
			4,500a	Mortar & Pestle	45.0
Eccocoat IC2	1,000	1,000	1,580	Drill	158.0
			1,580	Drill	158.0
			150	Drill	15.0
			260	Drill	26.0
			1,220	Mortar & Pestle	122.0
			1,020	Mortar & Pestle	102.0
			46	Mortar & Pestle	4.6
			61	Mortar & Pestle	6.1

Table 2

Chemical Formula	Trade Name	Company	Solvent
tricyclohexyl citrate	Citroflex 6-R	Chas. Pfizer & Co.	
dimethyl isophthalate	Morflex X-1129	Chas. Pfizer & Co.	most organic solvents
diphenyl phthalate	-------	Monsanto Company	most organic solvents
chlorodiphenyl	Aroclor 2565	Monsanto Company	most organic solvents
chlorodiphenyl	Aroclor 4465	Monsanto Company	most organic solvents
chlorodiphenyl	Aroclor 5442	Monsanto Company	most organic solvents
n-ethyl para-toluenesulfonamide	Saniticizer 3	Monsanto Company	(50 parts/100 resin cellulose acetate)
hydroabietyl alcohol	Abitol Lot A1K-221	Hercules Inc.	esters, ketones, alcohols
dihydrobiethyl phthalate	Cellolyn 21 Lot A1E-771	Hercules Inc.	esters, ketones, alcohols
monoglyceride Type 18-06	Myverol 18-06	D.P.I. Eastman Kodak Co.	congeal pt. @ 70°C

Melting Point	Inhibition to *Bacillus subtilis* var. *niger*. Trypticase soy broth	Plate Count Agar
57°C	not inhibitory	very slightly inhibitory
66°-68°C	not inhibitory	very slightly inhibitory
69°C	not inhibitory	very slightly inhibitory
Softening pt. 66°-72°C	not inhibitory	not inhibitory
Softening pt. 60°-66°C	not inhibitory	very slightly inhibitory
Pour pt. 46°C	not inhibitory	not inhibitory
60°C	not inhibitory	not inhibitory
Viscosity 2 poises @ 80°C	(not tested)	(not tested)
Softening pt. 60°-70°C	(not tested)	(not tested)
70°C congeal pt.	-------	inhibitory

Table 3

SUMMARY OF PLATE COUNT AGAR OVERPOUR AND TRYPTICASE SOY BROTH RECOVERY [a]

Assay count/cm^3	Chance of Detecting Viability [b]	Expected% Recovery	Agar Recovery [c] (PerCent)	Broth Recovery [a] (PerCent)
8.15×10^2	0.08:1	8.		0/98 (1)
6.85×10^3	0.68:1	68		30/60 (50)
1.04×10^4	1.04:1	100	4/14 (64)	(67)
1.18×10^4	1.18:1	100	8/12 (64)	17/26 (65)
1.4×10^4	1.4 :1	100	-	58/77 (75)
2.38×10^4	2.38:1	100	7/14 (50)	18/24 (75)
2.62×10^4	2.62:1	100	9/12 (75)	5/24 (21)
3.72×10^4	3.72:1	100	8/11 (73)	20/24 (83)
4.68×10^4	4.68:1	100	9/15 (60)	12/25 (48)
6.02×10^4	6.02:1	100	13/14 (99)	12/22 (55)
7.55×10^4	7.55:1	100	11/11 (100)	
1.1×10^5	11:1	100	-	71/88 (100)
2.7×10^5	27:1	100	-	33/33 (100)

a. Recorded as number of positive growth/number cubes cleaved

b. Based on assay and probability

c. Recorded as number showing growth/number tested.

Table 4

SUMMARY OF AROCLOR CLEAVAGE EXPERIMENTS

WITH AGAR OVERPOUR TECHNIQUE

Assay count/cm^3	Expected [a] Cleavage counts/cm^2	Actual [b] Cleavage counts/cm^2	PerCent Recoverability $\frac{\text{Actual Count}}{\text{Expected Count}} \times 100.$
3.73×10^3	0.37	1.45	392%
1.04×10^4	1.04	0.71	68.3%
1.18×10^4	1.18	1.0	83.3%
2.38×10^4	2.38	0.93	39.0%
2.62×10^4	2.62	1.92	73.4%
3.29×10^4	3.29	1.0	30.4%
3.72×10^4	3.72	1.81	48.7%
4.68×10^4	4.68	0.83	17.8%
6.02×10^4	6.02	2.64	43.8%
7.55×10^4	7.55	7.45	98.6%
1.41×10^5	14.1	0.92	6.56%
2.55×10^5	25.5	8.93	35.7%
2.6×10^5	26.0	6.43	24.7%
3.47×10^5	34.7	12.8	36.9%
7.7×10^5	77.0	12.9	16.8%
9.7×10^5	97.0	19.54	20.2%
1.41×10^6	141.0	16.2	11.5%
1.41×10^6	141.0	52.0	36.8%
5.43×10^6	543.0	116.0	21.4%
8.06×10^6	806.0	124.5	15.5%
9.38×10^6	938.0	209.2	22.3%

a. Expected count is based on probability estimate

b. Average of at least ten cleavages

Table 5

SUMMARY OF AROCLOR - ADDITIVE CLEAVAGE EXPERIMENTS

WITH AGAR OVERPOUR TECHNIQUE

PerCent Additive	Assay Count/cm^3	Expected Cleavage Count/cm^2[a]	Actual Cleavage Count/cm^2[b]	PerCent Recoverability $\frac{\text{Actual Count}}{\text{Expected Count}}$	Agar Recovery
5.2% Charcoal	4.09×10^3	0.409	0.67	163%	4/12
5.0% Charcoal	1.16×10^4	1.16	1.42	122%	9/12
5.4% Charcoal	2.72×10^4	2.72	1.92	70.5%	8/12
5.1% Charcoal	5.37×10^4	5.37	7.91	147%	12/12
4.2% Charcoal	6.65×10^4	6.65	7.8	117%	12/12
4.2% Charcoal	7.55×10^4	7.55	6.83	90.5%	12/12
8.9% Avicel	2.1×10^4	2.1	3.5	166%	10/12
9.3% Avicel	6.6×10^4	6.6	6.75	102%	12/12
8.2% Avicel	6.8×10^4	6.8	6.67	98%	12/12
Average	4.37×10^4	4.373	4.83	110%	91/108

a. Based on probability estimates

b. Average of twelve cleaved cubes

* Cubes showing growth on their cleaved surfaces in agar over the no. tested.

TABLE 6

SUMMARY OF AROCLOR GRINDING AND SIZING EXPERIMENTS
FOLLOWED BY PLATE COUNTING

Assay Level/cm^3	% Additive	Sieve Size	Count/cm^3	Theoretical Count/cm^3
1.18×10^4	None	5.0 mm +	7.65	<3.5
		3.15 - 5.0 mm	--	5.6 ± 2.1
		2.0 - 3.15 mm	86.7	10.9 ± 3.1
		1.25 - 2.0 mm	377.4	19.5 ± 3.1
7.55×10^4	None	5.0 mm +	30.6	<23.0
		3.15 - 5.0 mm	37.4	36.0 ± 13.0
		2.0 - 3.15 mm	96.9	70.0 ± 21.0
		1.25 - 2.0 mm	328.1	125.0 ± 34.0
2.55×10^5	None	5.0 mm +	45.4	<76.5
		3.15 - 5.0 mm	104.6	121.3 ± 44.8
		2.0 - 3.15 mm	346.8	286.0 ± 120.0
		1.25 - 2.0 mm	868.7	421.0 ± 115.0
9.7×10^5	None	5.0 mm +	88.1	<291.0
		3.15 - 5.0 mm	302.6	460.5 ± 169.5
		2.0 - 3.15 mm	442.9	897.0 ± 267.0
		1.25 - 2.0 mm	967.3	1600.5 ± 436.5
6.65×10^4	4.2% Charcoal[1]	5.0 mm +	16.2	<20.0
		3.15 - 5.0 mm	37.7	31.5 ± 11.5
		2.0 - 3.15 mm	69.4	61.5 ± 18.5
		1.25 - 2.0 mm	164.7	110.0 ± 30
5.37×10^4	5.1% Charcoal[1]	5.0 mm +	23.8	<16
		3.15 - 5.0 mm	19.9	25.5 ± 9.5
		2.0 - 3.15 mm	78.4	49.5 ± 14.5
		1.25 - 2.0 mm	289.0	88.5 ± 24.5
7.55×10^4	4.2% Charcoal[1]	5.0 mm +	38.1	<23.0
		3.15 - 5.0 mm	30.5	36.0 ± 13.0
		2.0 - 3.15 mm	70.4	70.0 ± 21.0
		1.25 - 2.0 mm	223.7	125.0 ± 34.0
2.1×10^4	8.9% Avicel [2]	5.0 mm +	9.9	<6
		3.15 - 5.0 mm	21.1	10 ± 4
		2.0 - 3.15 mm	75.8	19.5 ± 5.5
		1.24 - 2.0 mm	263.3	34.5 ± 9.5
6.8×10^4	8.2% Avicel [2]	5.0 mm +	24.0	<20
		3.15 - 5.0 mm	20.0	32.0 ± 12.0
		2.0 - 3.15 mm	127.0	63.0 ± 19.0
		1.25 - 2.0 mm	294.1	112 ± 30.5
6.6×10^4	9.3% Avicel [2]	5.0 mm +	22.8	<20.0
		3.15 - 5.0 mm	38.3	31.5 ± 11.5
		2.0 - 3.15 mm	127.5	61.0 ± 18.0
		1.25 - 2.0 mm	361.3	$122.5 \pm 43.$

1. Nuchar, +30 mesh Industrial Chemical Sales, N.Y., N.Y.

2. Microcrystalline cellulose, FMC Corporation, Marcus Hook, Pa.

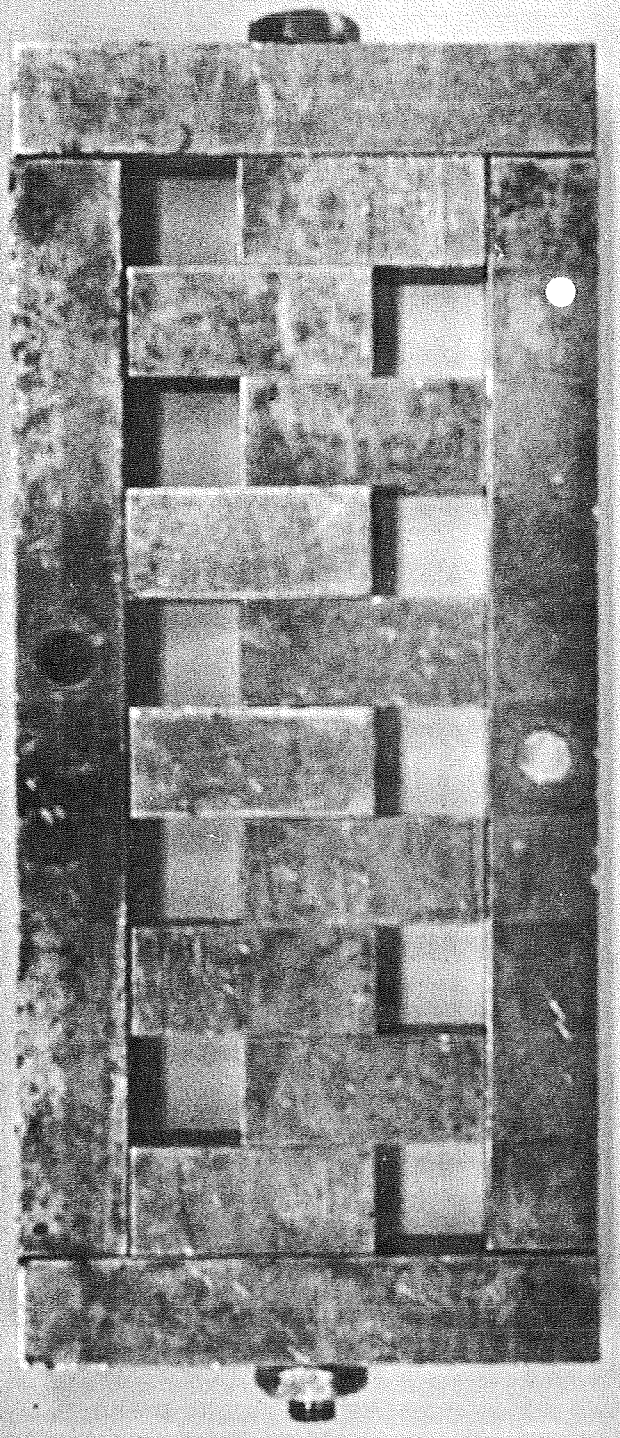

Figure 1. STAINLESS STEEL MOLD FOR FABRICATING ONE CENTIMETER SOLID CUBES.

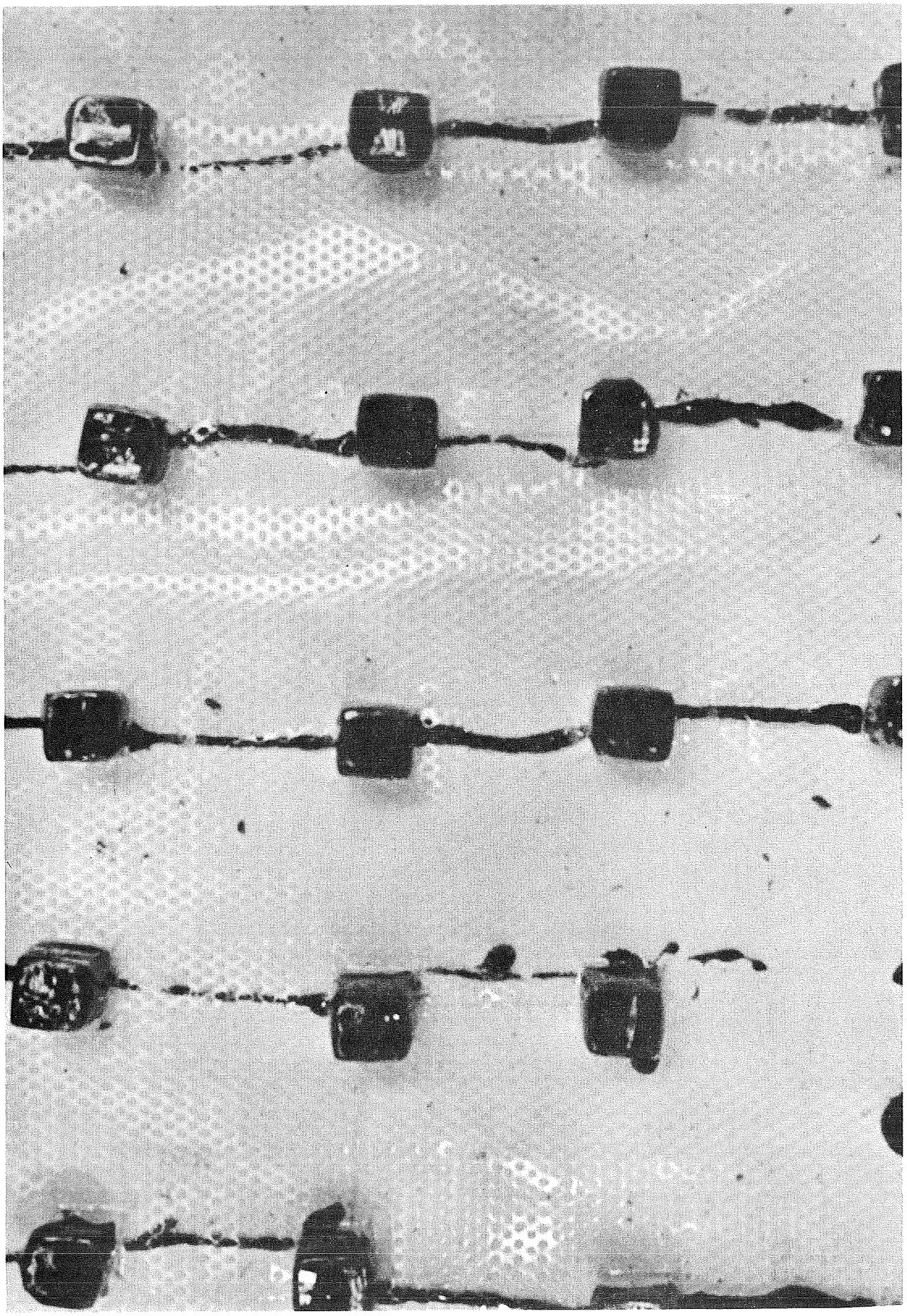

Figure 2. PLASTIC MOLD FOR FABRICATING LARGE NUMBERS OF ONE CENTIMETER SOLID CUBES.


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Figure 3. SIEVE SHAKER FOR DISCRIMINATING PARTICLE SIZE.

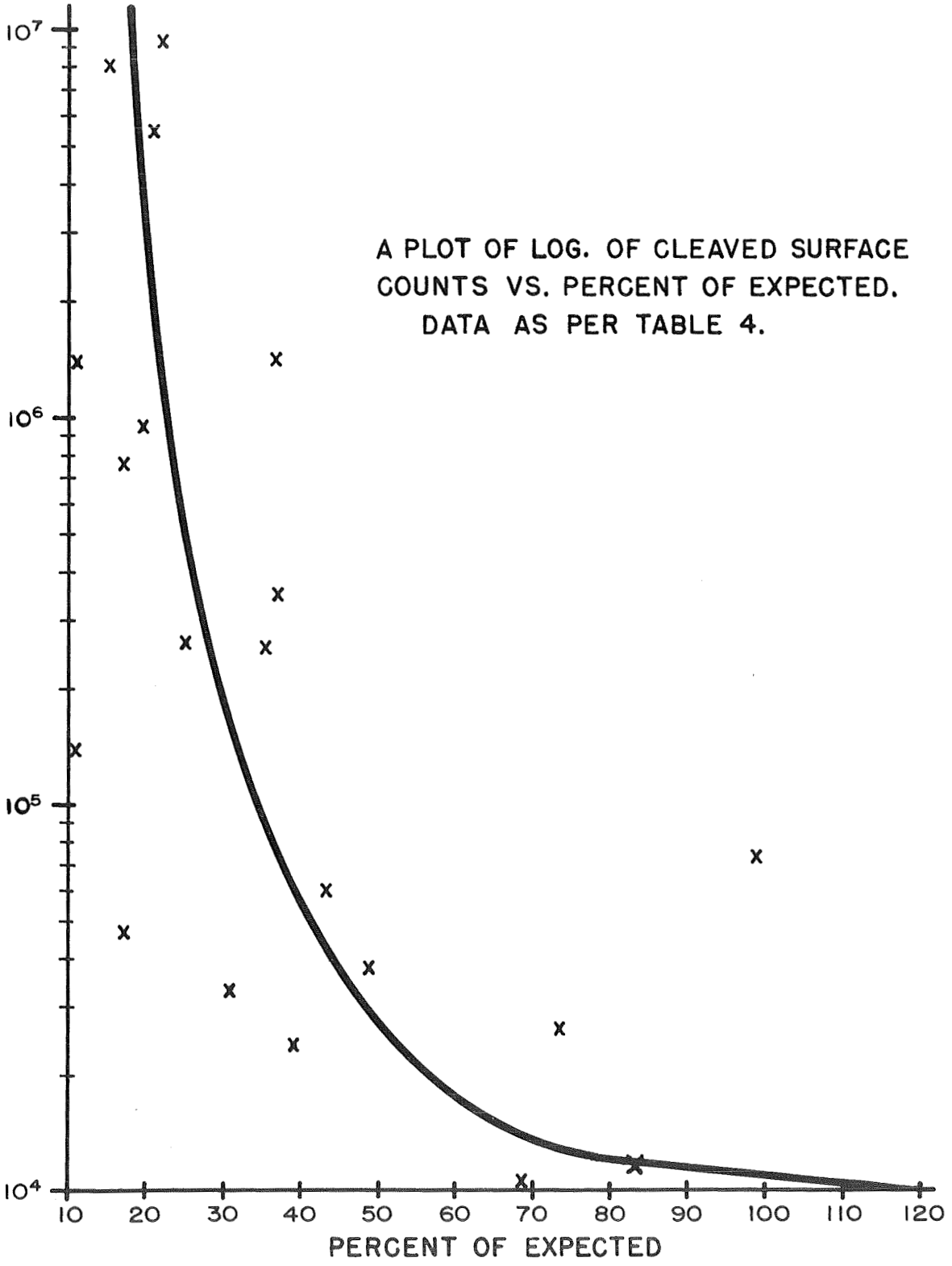
A PLOT OF LOG. OF CLEAVED SURFACE
COUNTS VS. PERCENT OF EXPECTED.
DATA AS PER TABLE 4.
10^7
10^6
10^5
10^4
10
20
30
40
50
60
70
80
90
100
110
120
PERCENT OF EXPECTED

FIG. 4

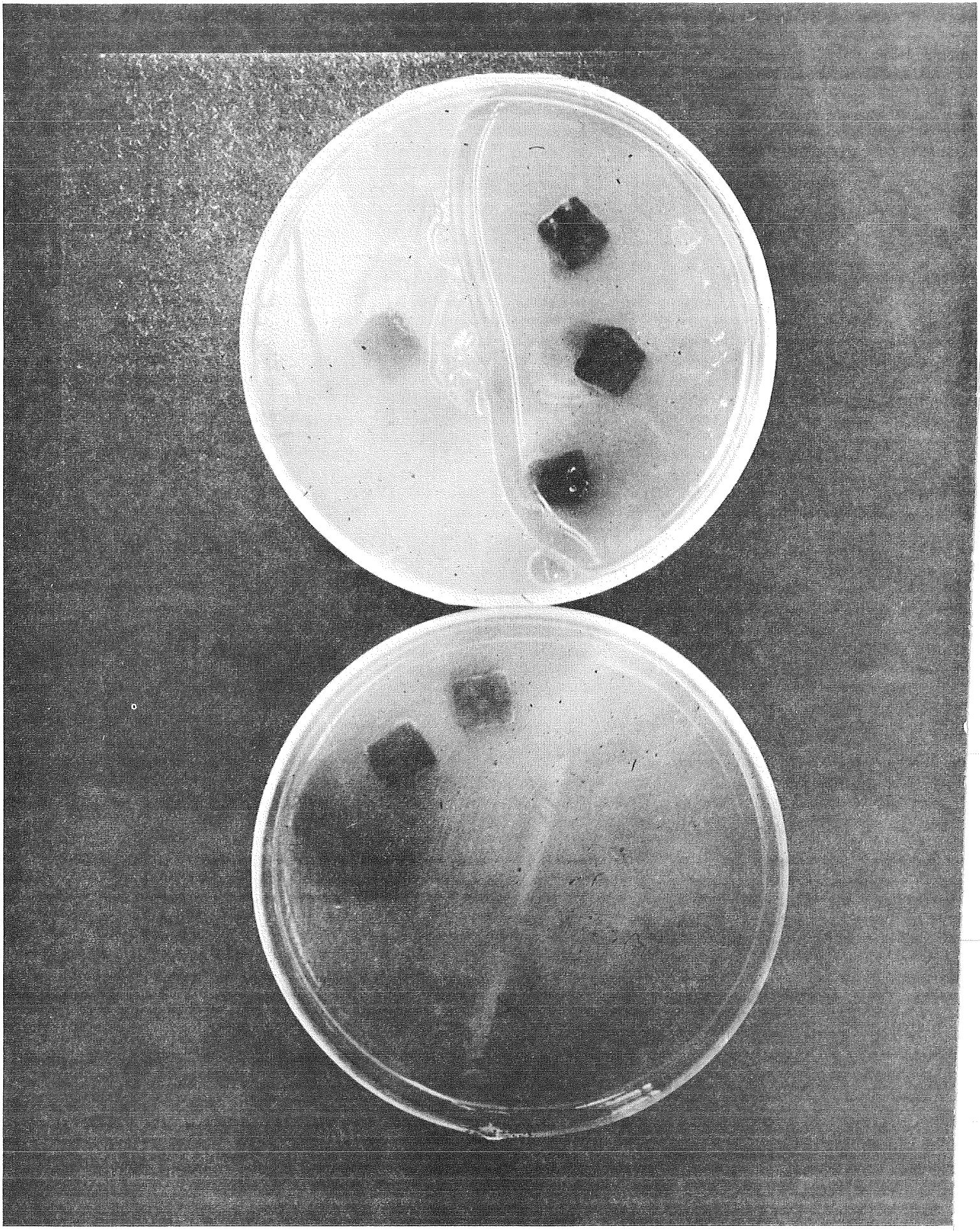

Figure 5. SOLID CLEAVED HALF IN AGAR SHOWING ALL COLONIES WHICH GREW FROM THE SOLID CLEAVED SURFACE.

Note: The cube halves are placed on the medium cleaved surface up, which is overlaid with more agar medium. Colonies grow beneath the agar overlay from the cleaved surface.