SCIENCE PAPERS AND DISCUSSIONS

RESISTANCE OF CRYSTALLINE SUBSTANCES TO GAS STERILISATION

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THE manufacture of thermolabile drugs in a sterile form has always presented a difficult technical problem, requiring special apparatus and procedures. The maintenance of aseptic areas, the training and constant supervision of staff, the rigorous application at every stage of bacteriological testing, are examples of these problems, and may result in the rejection of batches because of contamination and their return for reprocessing or abandonment. The introduction of a "cold" sterilising process would, therefore, be a valuable addition to present aseptic techniques, and published reports on various bactericidal gases have suggested that such treatments have been successful. During the course of investigations on sterilisation by ultra-violet radiation we observed that on some occasions crystallised materials which had been contaminated with a liquid suspension of bacterial organisms and subsequently dried were more difficult to sterilise than materials contaminated with dry micro-organisms. As the majority of thermolabile drugs manufactured for pharmaceutical use are of a crystalline nature, it was thought that an investigation of this difference might have practical significance when applied to gas sterilisation. In general, it was found that materials crystallised from a bacteriologically contaminated liquor were more resistant to a gas exposure technique than similar materials contaminated with a bacterial "dust". This suggested the possibility of the inclusion of organisms within substances crystallised from such liquors, and so escaping contact with the sterilising gas. The phenomenon of included foreign matter in crystals is well known. Bentivoglio observed the inclusion of "rows of minute cavities filled with solution" in her work on the rate of growth of magnesium ammonium sulphate crystals[1] and similar observations were also made by Bunn[2]. There is, however, no reference in the literature to suggest that organisms may be so included and remain viable, with the exception of a report by Seriakowski[3] which mentions the survival of viable organisms in crystals of a phosphate growing in broth cultures. The experiments recorded here were designed to show that viable spores can survive within crystals and that such inclusion affects the sterilising ability of gaseous agents by preventing contact between organisms and agent.

EXPERIMENTAL

An initial series of experiments was planned to establish methods for preparation, contamination and sterilisation of crystals. As a crystalline

material, Rochelle salt was chosen. It is readily soluble, easily crystallised, non-hygroscopic and has no action on the proteins in the broth or agar media used. The method of gas sterilisation described in the work by Bullock and Rawlins[4] in which the action of formaldehyde and air on *B. subtilis* spores was investigated in detail, appeared to be suitable for our purpose, and with modifications was adopted.

Comparisons of Resistance to Sterilisation

Preparation of surface-contaminated crystals. A solution of two parts of Rochelle salt in one part of water was autoclaved, cooled slightly, and then poured into a covered Pyrex dish and allowed to crystallise. Crystals of suitable size were then transferred to filter paper to dry; these were selected for regularity in size and shape, and freedom from adhering small crystals and aggregates. Tests for the presence of viable bacteria were made on a 10 per cent. representative sample of the number produced at each crystallisation. Each crystal was dissolved in 10 ml. of Bacto Difco nutrient broth and incubated at 30° C. for 17 hours. If no growth occurred, the broth was subcultured into a similar broth and incubated for the same time and at the same temperature. If, again, no growth occurred, the original broth was then inoculated with a suspension of *B. subtilis* spores to check that the lack of growth was not due to bacteriostasis. It was established that crystals grown and tested in this way did not show the presence of viable organisms on any occasion. The test organism used for contamination was *B. subtilis* (NCTC 3110). The organism was grown for 48 hours at 37° C. on modified agar slopes in Roux bottles and spores were collected by washing from the surface with sterile water and glass beads. After washing and centrifuging the spores were suspended in water. These suspensions were heated at 60° C. for 30 minutes, to kill vegetative organisms, and after examining microscopically, were filled into ampoules and freeze dried. The number of viable organisms in the resulting powder was approximately 10^8/g. This was estimated by preparing suitable dilutions in agar pour plates using a layering technique to prevent surface spread as demonstrated to us by Mr. G. E. Davies. This powder was used to contaminate the exterior surfaces of the crystals previously produced. Sufficient spore powder to give a concentration of about 10^6/g. was added to a weighed number of crystals in a bottle, and the bottle was then placed on a roller mill for one hour. A 10 per cent. representative sample of the crystals from each batch produced was tested bacteriologically to ensure that the desired contamination had taken place.

Sterilisation of Surface-contaminated Crystals by Formaldehyde

Apparatus. The apparatus resembled that of Bullock and Rawlins[4] (Fig. 1). Two grade 2 sintered glass filters (C.1 and C.2) were connected to a Drechsel bottle containing glass beads (A) and a bubbler with a fine jet (B), both containing solution of formaldehyde A.R., and air filtered through a cotton wool plug, was passed through at controlled rates, *via* a gas flow-meter (E), by pressure or vacuum. To estimate the

concentration of formaldehyde in the air passing through the apparatus, a sintered glass bubbler (D) containing sodium metabisulphite solution was switched into the system when desired, and removed when a measured volume of formaldehyde and air mixture had been scrubbed. A second bubbler was found to be unnecessary.

Estimation of formaldehyde. The concentration of formaldehyde in the air was estimated by the method of Goldman and Yagoda[5]. Four litres of formaldehyde and air were scrubbed by means of the bubbler containing 100 ml. of 1 per cent. w/v sodium metabisulphite solution, when the formaldehyde formed a non-volatile complex with the bisulphite. After removal of the bubbler from the system, 10 ml. of bisulphite solution was placed in a 250 ml. glass-stoppered flask and titrated to a dark blue end-point with 0·1N iodine, using starch indicator. Excess iodine was then destroyed by addition of 0·05N thiosulphate, added dropwise from a burette, and 0·01N solution of iodine then added to give a faint blue end-point. Twenty-five ml. of a solution of sodium carbonate 80 g., glacial acetic acid 20 ml., and water 500 ml., was then added to decompose the bisulphite-formaldehyde complex and the liberated bisulphite titrated with 0·01N iodine to a faint blue end-point. (Each ml. of 0·01N iodine is equivalent to 0·15 mg. of H·CHO.)

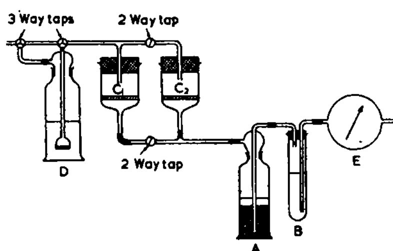

FIG. 1. Apparatus for subjecting the crystals to formaldehyde/air.

Method. It was shown in preliminary tests that with a flow rate of up to 800 ml. of air per minute through the apparatus, the concentration of formaldehyde in the air was about 2·4 mg. per litre (varying from 1·9–2·6 mg./litre). Contaminated crystals were exposed to these conditions and tested for the presence of viable spores by solution in broth and incubation as before. When no growth took place, inoculation with a *B. subtilis* spore suspension followed by incubation, produced growth, thus showing the absence of bacteriostasis due to residual formaldehyde. After five hours' exposure the crystals were sterile, and a six-hour exposure period was therefore used in subsequent experiments as a "standard sterilisation time".

Isolation of non-sterile crystals from solution. Sterile solutions of Rochelle salt were prepared and cooled to about 50° C. Diluted spore suspensions, prepared by suspending *B. subtilis* spores (freeze dried) in water, were added to give concentrations of 10^6/g. of solution. The solutions were then allowed to crystallise under aseptic conditions, crystals of about the same size as the sterile crystals previously prepared being removed and dried as before. To ensure that there was contamination of the crystals as distinct from the mother liquor, the individual crystals from weighed groups of ten were dissolved in sterile water, and suitable dilutions prepared in agar pour plates. It was found that the

contamination varied between 400–700 organisms/g. of crystal. Crystals prepared in this way are described as "internally contaminated".

Attempted sterilisation of internally contaminated crystals. Similar weights of internally contaminated crystals and externally contaminated crystals (as control) were placed in the two gas chambers, C_1 and C_2. The air supply or vacuum was adjusted to give the required flow rate and the formaldehyde concentration in the air passing through the apparatus was measured. During the exposure of the crystals the chambers were shaken frequently to expose all crystal surfaces. At the end of the "standard sterilisation time" of 6 hours, ten crystals were taken from each chamber, and tested by dissolving in broth and incubating. A series of eleven experiments with five batches of crystals were thus performed to enable a qualitative indication to be made of the possibility of bacterial spores surviving inside crystals and the results are shown in Table I.

TABLE I

RESISTANCE OF CRYSTALS TO STERILISATION

Number of crystals of each kind tested	276
External contamination: proportion of crystals found contaminated	5/276 (1·8 per cent.)
Internal contamination: proportion of crystals found contaminated	249/276 (90·2 per cent.)

This result shows a difference between the ability of formaldehyde to sterilise crystals merely surface contaminated, and those crystallised from a contaminated liquor. Although the contamination in the former is higher, the period for which both lots of crystals were exposed was sufficient to sterilise in all but 1·8 per cent. The suggestion is made that the spores in the latter instance are protected from contact with the gas by inclusion within the crystal mass. In order to obtain quantitative estimates of the extent to which such inclusion occurs, another experiment, using groups of graded crystals, was made. Ten crystals of each kind in different size ranges were exposed in the two chambers to the gas procedure as before. The crystals were then removed and plated in nutrient agar. Tests had shown previously that the presence of residual formaldehyde on the Rochelle salt did not inhibit colony development and there was no advantage in prolonging incubation time beyond 17 hours. Table II shows results obtained from the treatment of representative groups in each size range.

TABLE II

VIABLE SPORES AFTER EXPOSURE OF GROUPS OF 20 CONTAMINATED CRYSTALS TO FORMALDEHYDE GAS

Average weight of ten crystals g.	Contaminated externally		Grown in contaminated solution	
	Spores/g. before gas	Spores/g. after gas	Spores/g. before gas	Spores/g. after gas
4·5	$1 \cdot 1 \times 10^6$	2	450	70
1·3	$8 \cdot 6 \times 10^5$	3	500	63
0·27	$9 \cdot 2 \times 10^5$	0	680	93

The almost complete kill with the externally and heavily contaminated crystals, in contrast to the limited reduction of viable spores in those crystals isolated from contaminated solution, supported the conclusion drawn from the preceding experiment. Most of the contamination associated with crystals from a contaminated liquor was on the surface, and the amount of contamination, both internal and external, did not vary greatly with crystal size, provided that the conditions of growth were the same in each case. The resistant spores may be protected from the gas by lodgment in crevices or cracks in the crystal surface. Increasing the exposure time up to 80 hours did not improve the kill under the experimental conditions, as shown in Table III.

TABLE III

EFFECT OF PROLONGED EXPOSURE TO FORMALDEHYDE

Time of exposure (hours)	Spores/g. of crystals	
	Contaminated externally	Grown in contaminated liquor
0	10,000 approx.	—
5	0	3780
20	—	4210
40	—	3890
60	—	4470
80	—	4160

Influence of Rate of Crystallisation on Inclusion of Spores

The more rapidly a crystal is grown the more likely it is to contain inclusions of impurities. If this applies equally to inclusions of viable spores the chances of such inclusion taking place in a precipitation process typical of manufacturing practice are increased. The following experiments show the differences found by crystallisation from the same liquor by (*a*) normal growth as described already, and (*b*) a precipitation process. Aliquots of the cooled sterile and contaminated liquors prepared as previously described were poured into a slightly larger volume of 70 per cent. ethanol, previously sterilised by filtration. This mixture was stirred until crystallisation occurred and the crystals isolated by filtration using a No. 2 sintered glass funnel and vacuum under an aseptic screen. The crystals were then dried in closed sterile dishes at 37° C. When dry they were sieved through a 30 mesh sieve. This gave free flowing powders which did not cake further during the subsequent gas procedure. The uncontaminated material was tested for sterility and if found to be sterile was contaminated by mixing with spore powder. From another aliquot of the contaminated liquors, crystals were also grown by the normal slow growing process. These procedures provided, (i) rapidly grown (precipitated) small crystals from sterile mother liquors, (ii) crystals from (i) above externally contaminated with freeze dried spore powder, (iii) rapidly grown (precipitated) small crystals from contaminated mother liquors, and (iv), slowly grown larger crystals from the same contaminated mother liquor as in (iii).

A total viable bacterial count was then made on these crystals using groups of ten weighed large crystals and groups of 10 × 100 mg. samples

of crystalline powder, before and after exposure to gas, and the results are given in Table IV.

Obviously the method of exposing the crystalline powders to gas was sterilising the outside of the powder, and the small crystals grown rapidly were more heavily contaminated than the crystals grown slowly. Rapid crystal growth from bacterially contaminated liquors, therefore, increased the risk of internal contamination of the crystals.

TABLE IV
RATES OF CRYSTALLISATION
SPORES/G. IN CRYSTALS PREPARED BY VARIOUS METHODS

Rapid Crystallisation				Slow Crystallisation	
Externally contaminated		From contaminated liquor		From contaminated liquor	
Before gas	After gas	Before gas	After gas	Before gas	After gas
1×10^6 approx.	0	$1{\cdot}3 \times 10^6$	5840	855	96

TABLE V
AMOUNT OF CONTAMINATION

Initial count in liquor spores/g.	Powder after drying	
	Spores/g. before gas	Spores/g. after gas
10,000	8130	620
500	605	99
50	80	17
10	10	1·2

Amounts of contamination and spore survival. The amount of contamination met in manufacturing practice will be likely to be lower than those used in the previous experiments. It was, therefore, of interest to ascertain the degree of contamination in the final product, knowing the actual degree of contamination in the mother liquors. Table V shows the contamination in crystalline Rochelle salt powders before and after exposure to gas, after various initial counts in the mother liquors.

At 10 organisms/g. of mother liquor the final bacterial count on the gassed powder was only 1·2 organisms/g. This low count necessitated the plating technique being applied on a larger scale, and a full 10 g. of powder (in 100 mg. samples) was tested.

Survival of spores on storage. Although the evidence so far presented shows that *B. subtilis* spores can survive inside crystals, the possibility arises that they may not survive prolonged storage. The number of bacteria surviving in samples of Rochelle salt crystals and crystalline powder prepared from contaminated mother liquor was, therefore, investigated after a period of storage at laboratory temperature (Table VI). In all tests the samples were exposed to gas before making the viable count. Both samples were still heavily contaminated at the end of the storage period.

Other crystalline materials. The evidence so far presented relates only to Rochelle salt. A further series of rapidly crystallised water-soluble

TABLE VI

SURVIVAL OF SPORES IN CRYSTALS ON STORAGE

Material	Spores/g. Initially	Spores/g. After storage	Period of Storage in weeks
Crystals	450	470	21
Crystalline powder ..	813	540	16

powders were prepared from both sterile and contaminated mother liquor and examined in a similar way, using formaldehyde. (See Table VII.)

These results confirm that in many compounds crystallised from contamined mother liquor, spores are included within the crystal and remain protected from the gaseous sterilisation procedure.

TABLE VII

SURVIVAL OF SPORES IN CRYSTALLINE MATERIALS

			Viable spores/g.				
			Grown from contaminated liquor			Contaminated externally	
Substance	Precipitant	Size and shape of crystals (dimensions in μ)	Mother liquor (calc.)	Before gas	After gas	Before gas (calc.)	After gas
Hexamine	Acetone	Aggregates, rectangular plates mixed	1000	236	47	10^6	0
Ethylene diamine tartrate	Ethanol	Aggregates, needles and plates very mixed	100	96	15	10^6	0
Lactose	Ethanol	Aggregates of needles and rhomboids, bulk 40–60 long	1000	83	0·4 (2 in. 5 g.)	10^6	0
Glycine	Ethanol	Aggregates, oblong 90 long × 30 wide × 30	19,000	6200	125	10^6	0
Sodium diphosphate	Acetone	Aggregates, needles 130–180 long, 6–15 wide	10,000	10,000	1100	10^6	0
Hexamethonium bromide	Acetone	Aggregates rectangular tables 180–300 × 600	10,000	640	150	10^6	0
Sucrose	Evaporated from syrup	Aggregates of cubes, bulk 150–200 side	10,000	0	—	—	—
Chloral hydrate	Evaporated from ethereal solution	Aggregates no particular shape discernible, crystals in double pyramid	5000	0	—	—	—
Sodium chloride	Ethanol	Cubes, bulk about 250 side	10,000	0	—	—	—

Sterilisation with Ethylene Oxide

To confirm the phenomenon of viable spore inclusion in crystalline materials, it was thought desirable to use an alternative gas to formaldehyde, and ethylene oxide was investigated. Because of its explosive nature the method of exposure was different. The samples were placed in test tubes connected to a common gas inlet tube. The entire apparatus

was evacuated on a water-pump for 10 minutes, flushed with carbon dioxide, again evacuated on the water pump for 15 minutes and then filled with ethylene oxide at atmospheric pressure. After standing for the required period of time, the apparatus was evacuated on the water pump for 5 minutes and flushed with carbon dioxide before removing the samples. In the test in which extra moisture was added, an extra test tube containing water was connected to the gas inlet tube. Four samples of Rochelle salt were exposed simultaneously, and the results are given in Table VIII.

TABLE VIII

EXPOSURE TO ETHYLENE OXIDE

Material	Viable spores/g. of crystal			
	Initially	24 hours exposure	96 hours exposure	96 hours exposure with extra moisture
Sterile crystals dusted with freeze dried spores	1×10^6 approx.	—	53	110
Crystals grown from contaminated liquor	6000 approx.	5140	4870	2600
Sterile powder dusted with freeze dried spores	10×10^6 approx.	172	24	4
Powder crystallised from contaminated liquor	1×10^6 approx.	1960	6250	4490

While the maximum period of exposure to ethylene oxide was insufficient to sterilise surface-contaminated material, there was a reduction in the viable count. With both the large crystals and the crystalline powder prepared from contaminated mother liquor, the number of viable organisms/g. remained quite high, providing further evidence that viable spores are included in such materials.

Discussion

The results indicate that crystals contaminated on the external surface only can be sterilised by the described process using formaldehyde gas, but that crystals grown from contaminated liquors frequently include spores which are not sterilised by this process, even by extending the exposure time to 80 hours. It is probable that many are entrapped inside the crystal mass, and are, therefore, inaccessible to the gas. This theory is supported by the results of experiments in which ethylene oxide was the bactericidal gas and also by electron micrographs which show spore-like objects found on the fractured surfaces of contaminated crystals. (See Appendix.) It also appears that the more rapidly crystallisation takes place, the greater is the chance of inclusion of bacterial organisms. Furthermore, with *B. subtilis* spores, the organism is capable of surviving inside the crystal for at least five months. Most of this work was carried out with Rochelle salt, but experiments show that the inclusion phenomenon applies to many other materials. Exceptions were noted, in sucrose, chloral hydrate and sodium chloride crystals grown from contaminated liquors. The reason for this has not been investigated.

There is some evidence[1,2] that the possibility of the inclusion of small pockets of contaminated liquor in the crystals has to be considered. With these three substances it is known that the liquors possess strong osmotic properties, or even bactericidal powers on prolonged contact with spores. The inclusion effect in micro-crystals has not been investigated, although it seems obvious that, with small enough crystals, inclusion of a spore cannot take place. A 6-hour period was chosen for sterilising the exterior of contaminated crystals. In some subsequent experiments, occasional surface spores remained viable after this exposure period, but the numbers were not considered to affect the conclusions. An additional unexplained fact is that as shown in Table I; approximately 10 per cent. of the crystals grown from contaminated liquor did not contain viable organisms after exposure to formaldehyde gas, but subsequent quantitative tests did not result in a repetition of the observation. Comparatively heavy contamination was used in this work for convenience. Much less may be encountered in mother liquors during "clean" working as opposed to aseptic working, but the possibility still arises that crystals isolated from such liquors may be internally contaminated and, furthermore, the rapidity with which crystals are normally precipitated in manufacturing processes will increase this hazard. As a gas procedure cannot be relied upon to sterilise such crystals, the mother liquor must be sterile. Gas may, however, be useful in reducing external bacterial contamination, and thereby permitting a partial relaxation of aseptic precautions in the manufacturing stages after the sterile crystals have first been prepared. For these reasons, the control of gas procedures must be as rigid as control in normal aseptic processing, and it must not be assumed that exposure to gas can be compared with autoclaving or heat treatment. The design of the particular sterility test to be used is also of importance. Obviously if viable organisms inside crystals are to be detected the test must employ total solution of the solid, and sterility tests which involved the suspension of sparingly soluble solids in broth with subsequent subculturing should not be accepted as valid tests unless they indicate that the product is non-sterile. Whilst it has been shown that spores are protected from the sterilising effect of gases by inclusion in crystals, it is possible that other physical or chemical methods of sterilisation may be effective.

Appendix

Examination of Crystals by Electron Microscopy

Specimen crystals were prepared for examination by electron microscopy. *Internally contaminated crystals* were crystallised from Rochelle salt solution prepared to contain about 5 million spores/g. *Control crystals* were prepared by aseptic crystallisation from liquor filtered through sintered glass 5 on 3 and autoclaved.

B. subtilis *in Rochelle Salt*

Specimen preparation for the electron microscope. Since the crystals of Rochelle salt were too thick for direct examination in the transmission

type of electron microscope, they were viewed indirectly by means of a "replica" or "cast" of the surface formed in a suitable material and of sufficient thickness to be penetrated by the electron beam. A one-step negative replica technique was used in the form of an all-metal replica backed by a suitable polymer.

Experimental details. Heavily contaminated and uncontaminated, control crystals, 1 cm. in length and 0·5 cm. high, were selected, and scored around the outer surfaces at the centre with a sharp scalpel; this facilitated the splitting in half of the crystals. These pieces, with the

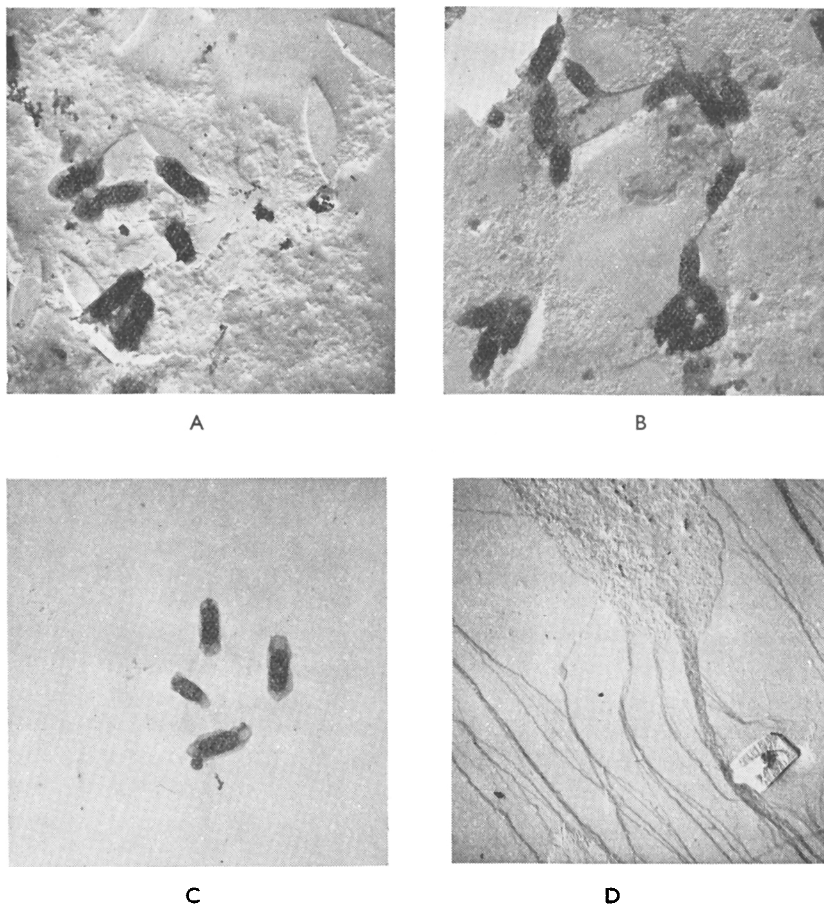

FIG. 2. Electron micrographs of crystal surfaces.

broken surfaces uppermost, were then placed on a microscope slide and chromium evaporated on to the surface *in vacuo* at a pressure of 10^{-5} mm. Hg. The angle of shadowing or evaporation was $\tan^{-1} 0{\cdot}2$. This surface was then coated with 0·25 per cent. nitrocellulose in amyl acetate, and when the solvent had completely evaporated the crystal was placed in a

petri dish. Water at 60° C. was carefully poured into the dish to the height of the crystal or until the film floated free, after which the crystal was removed immediately from the water before it completely dissolved. The replica was then washed twice in clean, distilled water to remove all traces of Rochelle salt, and mounted on 1/8 in. 250 mesh copper grids, examined, and electron micrographs taken in a Metrovick E.M. Type 2 at an electron magnification of × 2500.

Observations. On examination, pseudo-replicas had been produced since the majority of the bacteria had remained adhering to the metal-nitrocellulose film. Replicas from complementary broken surfaces showed aggregates of spores at fairly regular intervals throughout the specimen. To illustrate the imprisonment of bacteria within the crystal, areas where the spores were dispersed were selected and photographed. Specimens from the uncontaminated samples were thoroughly searched and no spores were observed.

Micrographs A, B and C are representative of broken crystal surfaces from Rochelle salt crystallised from liquor heavily contaminated with *B. subtilis* spores.

"D" is the main feature observed on many surfaces from sterile crystals.

Summary

1. Crystals grown from contaminated mother liquors may remain contaminated after exposure to bactericidal gases. It seems that these spores are included within the crystal.

2. This phenomenon occurs with a sufficiently large number of compounds to indicate a general occurrence.

3. Speed of growth of the crystals has been found to influence the number of spores included.

4. Implications on manufacturing processes have been discussed.

The authors wish to acknowledge the co-operation and help of Dr. A. R. Martin with the ethylene oxide experiments, Mr. W. E. Durrant and Mr. R. T. Leah, Dyestuffs Division, I.C.I., for preparation and production of the electron micrographs, and Mr. A. G. Fishburn for assistance in the preparation of this paper.

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DISCUSSION

The paper was presented by Mr. J. Cockton.

The Chairman asked whether the *B. subtilis* spores had been tested for resistance to formaldehyde, and whether there was any evidence that formaldehyde was taken up on the surface of crystals by absorption.

DR. W. MITCHELL (London) asked whether some of the organisms were sealed in the crystals and were therefore not accessible to the sterilising gas.

MR. J. A. MYERS (Bradford) enquired whether the authors would expect that calculi could contain embedded bacteria.

MR. J. COCKTON, in reply, said that the resistance of the *B. subtilis* spores to formaldehyde gas had not been measured. No evidence was found of adsorption of formaldehyde on the crystals. Removing the surface of the crystals in ethanol-formaldehyde, until half of the crystal weight had been dissolved showed viable spores still present. Electron micrographs seemed to suggest that the spores could be included within the matrix of the crystal. He saw no reason why spores should not be occluded in calculi.