BIOCIDAL EFFECTS OF SILVER

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INTRODUCTION

The present study was intended to determine if silver ions can kill or inactivate microbial and viral agents in very pure water. The results were to be applicable to the design of future spacecraft water systems. Salts of silver were employed in many of these experiments, but silver from an electrolytic ion generator was used when possible.

Fairly extensive results of sampling previous prototypes and spacecraft water systems were available to guide our choice of test organisms. Many of the bacteria were laboratory or prototype strains, but some had actually been isolated from water systems. The viruses were selected very arbitrarily to represent several groups. There had been no previous effort to detect viruses which might be present in spacecraft water systems, but at least two of the groups represented here are detected fairly frequently in community wastewater.

Several features of the water systems, to which our results might be applied, have yet to be specified. The actual purity of the water in future systems, and the kinds of impurities which might occur, are unknown. The materials of which a system might be fabricated, the temperatures to which the water might be subjected, and the length of residence of water in the system before being consumed, all are subject to extensive changes. Therefore, we have tried to make our approach as general as possible. In addition to examining the broad aspects of silver sensitivity of bacteria and viruses, we have tried to identify significant variables which may influence the antimicrobial activity of silver ions.

Some open questions have been left in these areas, as well as in that of silver assay by neutron activation analysis (NAA). The weight of evidence indicates that silver ions do have potentially useful antimicrobial properties, but their true worth could only be determined in a real water system. Among the liabilities which we have observed, none would absolutely preclude the use of silver ions in this application.

PHYSICAL STUDIES

Assay System

The basic goal of the physical studies was to adapt and apply the neutron activation analysis (NAA) technique to the measurement of silver in samples from the biological experiments. This technique offers the theoretical advantages of high sensitivity and reproducibility, together with complete independence of the chemical state of the silver at the time that the assay is performed.

The NAA procedure for silver assay is carried out in the University of Wisconsin Nuclear Reactor Laboratory. Silver solutions to be analyzed are encapsulated in ~5 ml quantities in heat-sealed polyethylene vials selected for use in the pneumatic sample insertion system of the reactor. Irradiation takes place in a flux of approximately 5×10^{12} neutrons cm^{-2} sec^{-1}. The activated sample is counted with a solid-state gamma-ray spectroscopy system, and the resultant gamma-ray spectrum is read out on punched paper tape for subsequent hand or computer analysis.

The first nuclide selected for study was ^{108}Ag ($t_{1/2}$ = 2.4 min). Gamma ray peaks typical of the nuclide were selected, and a peak intensity evaluation was carried out by identifying the peak channel and the wings of the peak. A background level was then established for the spectral region in which the peak was located.

When the ^{108}Ag nuclide was used, irradiation took 10 min. One and a half min after the irradiation, the sample was counted for 200 sec of live time. After counting, the sample's spectrum was stripped with a "no silver" water sample spectrum. The net area under the curve is compared to one obtained with a standard silver sample of known concentration. The primary standard substance selected for this purpose was AgCN: its saturation value (220ppb of the whole compound, which is 180ppb of silver) is within our working range, and it is free of elements which are likely to interfere with NAA.

Several problems have been encountered, and most of them have been surmounted. At one time, we were losing many samples due to failures in heat sealing the vials. A Teflon-lined sealing iron was devised and has virtually eliminated this problem. Another problem was excessive background. Some of this could be reduced by careful cleaning of the outside of the vial to remove residues. An even greater gain was made when assay was based upon a different nuclide of silver, ^{110}Ag. The half life of this nuclide is shorter ($t_{1/2}$ = 24 sec) than that of ^{108}Ag, so irradiation was reduced to 72 sec. The shorter irradiation time suppresses the sodium background which had been a limiting factor in the precision of measuring ^{108}Ag. A quick-access carrier had to be built, so that there was only a 15 sec delay in getting the irradiated vial into the spectrometer.

Counting time was reduced to 1 min. Using the 659 keV gamma peak, the area which represents the "signal" is a factor of ten higher for ^{110}Ag analysis than for ^{108}Ag analysis (Table 2-1).

Table 2-1. Comparison of counts obtained with ^{108}Ag and ^{110}Ag at three levels of silver

Sample	Nominal Ag^+ Conc. (ppb)	^{108}Ag 633 keV peak counts	Background counts/channel*	^{110}Ag 659 keV peak counts	Background counts/channel*
1-0-1	150	750	-10	6110	-10
1-1-1	200	885	15	7720	-30
1-5-1	50	250	-10	2000	-40

*Post stripping background

The accuracy of the NAA technique described has been estimated at ± 10% or ± 10ppb, whichever is greater. Some of the polyethylene vials that we had been using had proved quite "noisy" and had degraded this performance somewhat, although at best a 10ppb solution would actually give a clear signal above background. We purchased a batch of low-oxygen vials which reduced the dead time in counting from 42% to 14%.

The rather high background activity arising from the presence of water in the silver samples is responsible for most of the uncertainty in the assay techniques. One approach to reducing this background problem, i.e., increasing the signal-to-noise ratio, is to surround the sample with a thermal neutron absorber during irradiation. The activitation of the silver is decreased less than that of the water because of the former's relatively large epithermal neutron absorption cross section.

A cadmium-covered pneumatic carrier ("rabbit") accomplished the desired background reduction but unfortunately proved impractical because of the radiation hazard associated with its high gamma activity. Boron-carbide was finally chosen as the best material for this purpose since its induced activity is primarily due to easily shielded alpha particles. The rabbit was machined from borated polyethylene containing boron carbide. The most significant result of its use was a three-fold reduction in background activity. This reduces the dead-time considerably, and consequently reduces the error associated with corrections for silver decay during this dead time.

A few problems are also associated with use of the boron carbide rabbit.

a) It is very difficult to machine B_4C, and we have made only one such rabbit.

b) The activity associated with a boron-carbide rabbit is greater than that of an ordinary rabbit. Heavy neoprene gloves must be worn.

c) The boron-carbide rabbit suffers some slight deformation under impact, and consequently must be used at slightly lower velocities in the pneumatic system.

Silver Standards

Clearly, the measurements being made are quite relative. The assigment of an absolute value to the silver content of any sample is done by comparison to a known standard. A report by Chambers et al. (J. Am. Water Works Assoc. 54:208, 1962.) had indicated that silver solutions were hard to store, so we selected AgCN as the standard, added an excess of it to deionized water, and let it form a saturated solution at room temperature. As was stated above, a saturated solution of AgCN at 20 C should contain 180ppb of silver. We began to suspect that the true silver content of the standard solution was higher.

An experiment was performed using $AgNO_3$ solution and a series of low power irradiations to determine the actual silver concentration in the standard cell. The standardizing solution for the experiment was 3.4×10^5 ppb $AgNO_3$. The standard was made up by weighing out 0.00171 g of $AgNO_3$ in a 2-dram polyethylene vial and adding 5 ml of distilled H_2O. The vial was heat sealed. A second solution of about the same concentration was prepared, and a set of concentrations were made by dilution steps. The vials were analyzed and their concentrations calculated based on the 3.4×10^5 ppb standard. The results are given in Table 2-2. The errors given in Table 2-2 reflect uncertainty in the actual value of No. 3 and error introduced by the power stepping procedure. It is apparent that the AgCN standard cell concentration is a factor of 3.6 ± 0.2 above the saturation value of 180 ppb. This discrepancy might be due to highly soluble silver compound impurities (1%) in the original AgCN powder. Since AgCN is so insoluble, a small amount of soluble impurity could easily double or triple the Ag ion concentration.

Table 2-2. Calibration of AgCN standard cell by comparison with $AgNO_3$ solutions prepared by stepwise dilution and assay

Sample	Power level, kw	Ag (ppb)
2	10	3.4×10^5
3	100	3.8×10^4
4	1000	$(3.3 \pm 0.2) \times 10^3$
5	1000	$(3.3 \pm 0.2) \times 10^2$
AgCN standard cell	1000	645 ± 40
1-2-1 NASA Ag ion generator, nominal 250 ppb	1000	290 ± 20

Two standard solutions of $AgNO_3$ were prepared on 23 February 1970 and assayed by NAA the next day. Portions of the same solutions were assayed by atomic absorption procedures at MSC. The results of the two sets of determinations differ by an almost constant factor, values reported from UW being 0.65 times those reported from MSC (Table 2-3). This suggests that the differences lie in the primary methods, rather than in the relative sensitivity of one technique or the other.

Table 2-3. Assay results for two $AgNO_3$ solutions in two laboratories

Sample	UW results	MSC results
SNM	110 ppb Ag	170 ppb Ag
SNN	378 " "	580 " "

The absolute values of our silver standards still have not been reconciled with those of NASA Houston. Following the results tabulated above, additional standard $AgNO_3$ samples were requested from MSC in early May. Despite a couple of reminders, these were never received.

Auxilliary standard calibration measurements were performed to try to resolve the discrepancy. The procedure used was an alternative to the customary "power-stepping" method described earlier. This alternate procedure involves full-power activitation of samples with a range of silver nitrate content, followed by counting at various source-detector distances which are greatest for the samples

of highest activity. These distances ranged from ~ 0 to ~ 10 cm. The alternate method revealed no inconsistancies in the power-stepping technique. In general its precision was inferior to the power-stepping technique because of the necessary large corrections for dead-time effects.

Ion Generator

Our experience with the electrolytic silver ion generator has been largely negative. The first of these arrived here during the first year's work. Its output of silver ions was below the predicted levels quite early. After some reworking at the Manned Spacecraft Center, it came back to us producing almost exactly half the predicted level of silver ions when operated at flow rates expected to produce 50 and 250 ppb. This may have been due to differences in the absolute values assigned to assays where and at MSC. More important, we prepared our own calibration curve for the silver output of the ion generator as a function of flow rate; and this, too, has eventually become a poor predictor. Operating the ion generator at throughput rates (with a small positive displacement pump) which were expected to yield a 250 ppb effluent eventually produced too little silver to be detectable above background.

It was rebuilt again in February of 1970 and carried back to Madison. When it was returned to service, meter readings were >10 μ amp. More fine, dark, granular deposits were found in the inner chamber. When these had been removed, it next appeared that one of the wires was shorted. The immediate area of the problem was potted in silicone sealant, and that appeared to solve the problem at the time.

Later difficulties accompanied a decrease in microamperage. Readings had ranged from 8.6 through 9.1 μ amp. during the month of March, though the neutron activation analyses indicated that the silver ion output of the generator was declining. On April 1, the meter reading fell to 7.2 μ amp. and continued at that figure through April 8. The chamber contained more deposits, which were removed. The new readings ranged from 5.6 through 6.4 μ amp. A series of eleven samples was collected at flow rates from < 1 to 11 ml min^{-1}. Each of the samples contained <50 ppb of silver. The problem was tentatively diagnosed as cell failure. As a result of a distress call to MSC, we received a "Tanner's Easy 'Do It Yourself' Silver Ion Generator Repair Kit." With it, we were able to replace the batteries and get the current up to a normal level (~ 9 μ amp). However, we continued to have trouble with chamber fouling.

Then our new Millipore "Super-Q" water purifier arrived, in pieces. Our Millipore representative had suggested that we set it up to operate in a recirculating mode, but not all of the necessary connectors were available from Millipore. After a good deal of blacksmithing, carpentry, and plumbing, we got it set up so that we had a

continuous supply of ~18 megohm water. We then attached our positive-displacement metering pump and the ion generator directly to it. The first thing that happened was that the chamber fouled again. This did not happen again after we took the Millipore filter off the ion generator and cleaned the electrodes with Brasso. The second thing that happened was that the current fell to 3.5 μ amp, apparently because the new water was so pure that its high resistance unbalanced the circuit.

We had been given the formula: $C = \frac{67i}{F}$, where C = silver concentration in ppb, i = current flow in μamp, and F = water flow in ml min^{-1}. At first, our only means of increasing C had been to reduce F. Eventually, this got to be a nuisance.

Mr. Roger Tanner at MSC suggested that we increase the voltage of the system by adding an external power supply and thereby increase i. This was done, but without the desired result. At i = 22μamp and F = 5 ml min^{-1}, we expected C = ~290 ppb. Three samples of the effluent, assayed by NAA, averaged 116 ppb. The electrodes were cleaned with Brasso, and the voltage was increased still more. At any reasonable rate of operation (i.e., predicted C < 1000 ppb), assays of the effluents still averaged < 50% of the predicted levels. In the range of predicted C > 1000 ppb, the outputs were a good deal below 50% of the predicted levels.

We then traded our ion generator for another. It arrived in September and has been tested at flow rates from 1.4 to 3.4 ml min^{-1} and at currents from 44 to 72 μamp. Silver yields, as measured by NAA, ranged from 10 to 25% of predictions. We were able to get silver concentrations high enough to use, by adjusting i and F to give very high estimated C. Still, the effluents had to be stored and assayed before use because even with the Super-Q water (which is of consistently high quality), yields under apparently identical conditions varied on a day-to-day basis by a factor of 5 or more. These effluents have been reasonably stable in storage.

Container Studies

The problem of silver ions complexing to containers was studied in a series of experiments using 250 ppb of silver propionate and soft glass, polystyrene, and polyethylene containers. A 250 ppb solution of silver propionate was prepared, and portions of this solution were transferred to containers of each material. Samples of the original solution and samples from each container were taken at intervals, sealed, and analyzed. There appears to be little effect for polyethylene and polystyrene and only a slight effect in glass after about 2 days (Table 2-4).

Table 2-4. Effects of storage in various containers upon the silver content of replicate solutions.

Time	Mother solution (ppb Ag)	Soft Glass (ppb Ag)	Poly-styrene (ppb Ag)	Poly-ethylene (ppb Ag)
0	250 ± 25			
0	260 ± 25			
0+		250 ± 25	250 ± 25	250 ± 25
5 min		280 ± 25	250 ± 25	250 ± 25
15 min		NPHS*	240 ± 25	260 ± 25
60 min		240 ± 25	250 ± 25	250 ± 25
97 min		240 ± 25	240 ± 25	240 ± 25
24 hr		240 ± 25	280 ± 25	240 ± 25
48 hr		210 ± 20	240 ± 25	230 ± 25
72 hr		220 ± 20	240 ± 25	240 ± 25

*NPHS = not properly heat sealed; sample lost due to vial leakage/

Further information was obtained by repeated sampling from styrene flasks containing silver propionate or ion generator effluent. These contained from 250 to 350 ppb Ag. Over sampling periods from 2 wk to 2 mo, no change in the silver concentration was detected. In another experiment, there was no measurable loss of silver with at least three transfers in disposable styrene pipettes. These results indicated that container losses were not likely to cause much difficulty in the conduct of these experiments.

Biological Samples

The silver used in the biological experiments has been either in the form of silver salts or of effluents from an electrolytic ion generator. In either case, problems were encountered in attempting to adjust the silver to the selected level. The propionate was stored at room temperature as a saturated solution. When the assay system had been corrected, we found that the silver content of diluted propionate solutions was sometimes far above what it should be. The error was attributed to finely divided, solid silver propionate in the saturation flask, which was being pipetted with the solution. It should have been possible to avoid this by filtering the saturated solution at 0.2 μm porosity just before the use dilutions were prepared, and there are good indications that this succeeded quite well.

Once we learned that the silver solutions were stable in storage (at least in styrene flasks) at use dilutions, we began to prepare the solutions well in advance of each experiment. It was made a matter of policy to have a NAA report back for each solution before it was used in a biological experiment. This prevented a great many unpleasant surprises. The level of silver in the solution was determined before the bacteria or viruses were added. Additional samples were taken of the bacteria or viruses without added silver and of the mixture at the end of each experiment. No suspension of bacteria or viruses was found to contain anything measured as silver by NAA unless we put it there. Concentrations of silver in the experimental samples did not differ from those in the "pre" samples by more than the usual sample-to-sample variation. The system outlined here appears adequate to the task of determining how much silver was present in each biological experiment, though there is still some lingering uncertainty about absolute values.

Silver Filters

Long ago, we did some work with viruses and Selas Flotronics fritted silver filter membranes. The results of the experiments will not be described here: what is important is that we did get some virus inactivation and did not know why. One possible reason was that silver ions were being leached from the filters; however, at that time we had no means of measuring the small amounts of silver that were involved. Now we do.

Five ml of deionized water were filtered through each of two Flotronics membranes. One was of nominal 0.45 μm porosity, and the other of 0.8 μm. These filtrates were determined to contain 59 and 92 ppb of silver, respectively, by NAA assay. This finding does not certainly explain the virus inactivation seen previously, but it's a start.

Summary of Physical Studies

Over the period of the contract, the reproducibility and sensitivity of NAA assay have been improved. The agreement in assays of paired samples has been especially close. The electrolytic ion generators have not performed reliably in our hands. However, the demonstration that silver suspensions could be stored successfully in polystyrene flasks took much of the uncertainty out of the process by permitting us to assay the silver before the experiment is performed.

BACTERIOLOGICAL STUDIES

Preliminaries

The initial experiments were done with two model species: Escherichia coli and Staphylococcus aureus. These were grown in shake cultures in trypticase soy broth and enumerated by pour-plating in the same medium plus 1.5% agar. Cell suspensions were sedimented in the centrifuge and washed twice with deionized water at the start of each experiment. Nothing that assayed as silver by NAA has ever been reported in a control bacterial suspension in these experiments. We wanted a starting count of ~ 10^4 cells ml^{-1}. Both the agar medium and the deionized water in the dilution blanks in these first experiments contained 500 ppm of sodium thioglycollate.

Three experiments were done with E. coli. The first two employed silver propionate. Cell populations were quite stable at room temperature in the absence of added silver. The silver killed the cells. The process was not precisely exponential, but there was no indication that killing would not ultimately be complete. The extinction times (10^{-4} killing) might have ranged from < 2 hr to ~ 4 hr at 50 ppb of silver and from < 1 hr to ~ 2 hr at 250 ppb. Silver from the electrolytic ion generator was used in the third experiment, and the probable extinction times were ~ 4 and ~ 2 hr again at 50 and 250 ppb, respectively.

S. aureus was challenged with silver propionate in one experiment. Extinction times (10^{-4} killing) appeared to be ~ 2 hr at 50 ppb and ~ 1 hr at 250 ppb. The cell population was again quite stable in the silver-free cell control at room temperature.

We also did one experiment to determine if 250 ppb silver, tested undiluted, was capable of inhibiting E. coli colony formation in the pour plates. We found that the silver was not inhibitory, even in the absence of added sodium thioglycollate. On the other hand, fewer colonies were formed when the normal level (500 ppm) of thioglycollate was present in the medium, and fewer still if 1000 ppm were added.

Enumeration of Primary Test Species

Cultures of Achromobacter metalcaligenes, Achromobacter mucosa, Alcaligenes fecalis, Flavobacterium aquatile, Flavobacterium halmephilum, and Pseudomonas aeruginosa were obtained from the American Type Culture Collection. Each was propagated in trypticase soy broth at 36C except for F. aquatile, which was grown in Taylor's M-5 (sodium caseinate) medium at 30 C. Cells were enumerated by pour plating with the medium in which the cells were grown, plus 1.5% agar. We were concerned that silver in the cell suspensions, carried over into the pour plates, might continue to exert an effect. This would have nullified (or greatly complicated) rate

measurements where killing in the original cell suspension was treated as a function of time.

Sodium thioglycollate was tested for ability to neutralize silver rapidly. It was added to dilution blanks; but, since some samples were to be tested undiluted, it was also added to the agar media at levels of 500 (1x) and 1000 (2x) ppm. These were compared to no thioglycollate in pour plating cell suspensions in the presence of silver propionate. Silver levels were approximately 500 ppb, or twice the highest which was to be used experimentally, because of some uncertainties in silver assay at the time that these tests were done. Only F. aquatile was inhibited by silver in the absence of thioglycollate, presumably because trypticase soy broth with agar neutralized the silver, while Taylor's M-5 medium did not (Table 3-1). On the other hand, thioglycollate was strongly inhibitory to A. metalcaligenes and totally inhibitory to F. aquatile at the 1x level. The conclusion was that sodium thioglycollate did not do any good under these conditions, and in some cases might do a great deal of harm. It was used in pour plating in some of the experiments to be described below but was discontinued completely when the last of the results in Table 3-1 had been obtained.

Table 3-1. Effects of silver propionate and of sodium thioglycollate in the agar medium upon colony formation in pour plates

Test species	Ag (ppb)	Thioglycollate in agar (ppm)		
		0	500	1000
A. metalcaligenes	0	254*,242	161,160	15,25
	560	226,262	109,108	16,17
F. aquatile	0	352,314	0,0	0,0
	570	109,102	0,0	0,0
F. halmephilum	0	76,87	84,92	58,54
	550	74,87	67,57	64,72
P. aeruginosa	0	259,269	256,261	290,294
	410	258,266	255,250	235,219

*Number of colonies in one plate

APT (All-purpose, Tween) broth (Difco) was used to grow cells, and (with 1.5% agar) to enumerate them by spread plating, in later experiments. A survey of several reagents as alternatives to sodium thioglycollate in neutralizing silver indicated that APT broth was as effective as any of those tested. Thereafter, APT broth served as the diluent for samples as well as the growth medium and the assay medium.

Kinetics of Killing from 10^4 Cells ml^{-1}

The primary test species listed above were suspended to an initial level of ~ 10^4 cells ml^{-1} and challenged. Silver propionate was used in most of the experiments; silver from the ion generator was used in some but was far below the levels (50 and 250 ppb) for which the experiments were designed. All reactions were carried out at room temperature. Experiments in this and subsequent sections will generally be reported in alphabetical order by species name, rather than in the exact order in which they were performed.

A. metalcaligenes, in its first experiment, was reacted with levels of silver which were later assayed (by NAA) at 0, 100, and 560 ppb. The kinetic data were not useful, perhaps because of the adverse effects of the thioglycollate in the agar medium. The thioglycollate was omitted in the second experiment: 10^5 cells ml^{-1} were reacted with approximately 0,50, and 250 ppb of silver. They died at the rate (determined by linear least squares) of -0.38 hr^{-1} in the absence of silver, and killing surpassed 10^{-5} in first hour when silver was present.

A. mucosa did not yield very informative kinetic data, either. From an initial level of ~$10^{4.1}$ cells ml^{-1}, the logarithmic rate of death was estimated at -0.036 hr^{-1} for the first 24 hr at room temperature in the absence of silver. The first 10^{-4} of killing in suspensions containing silver (140 and 790 ppb) appeared to have occurred within the first hour, but an occasional colony or two appeared in tests of later samples.

A. fecalis gave results which were anomalous in different ways. Starting at a level of ~$10^{4.1}$ cells ml^{-1}, cell death in the absence of silver was at -0.027 hr^{-1} during the first 3 hr. At this rate, the $\log_{10}$ (cells ml^{-1}) at 24 hr should have been 3.47 but was found to be <0.7. Killing in the presence of silver (100 and 540 ppb) appeared to be more rapid at the lower level during the first hour, but no viable cells were detected thereafter. Some further results with this species are reported in a later section.

F. aquatile was a problem to enumerate because it had been shown that silver could carry over significantly into the pour plates and that addition of 500 ppm sodium thioglycollate to the agar medium would suppress the organisms completely. Thioglycollate was used in the dilution blanks only, as a compromise. This was no help when undiluted samples were tested, but it seemed the best that could be done at the time. The cell count in the absence of silver increased initially but had begun to decrease by 3 hr. No viable cells were detected at 24 hr. This is the kind of result one would expect of an aggregated suspension of labile cells. Killing by silver (80 and 570 ppb) was rapid during the first hour; 1 or 2 colonies grew from undiluted samples at 3 and 4 hr, though not at 24 hr.

F. halmephilum also showed an initial rising cell count in the absence of silver, and no viable cells (tested at 10^{-2} dilution) at 24 hr, in the first experiment. Killing in the presence of silver (110 and 550 ppb) was extremely erratic. In experiment 2 the silver-free suspension showed a very consistent death rate of -0.073 hr^{-1} for the first 6 hr at room temperature. This indicated that the $\log_{10}$ (cells ml^{-1}) at 24 hr should be 2.32, but it was found to be < 1.70. Killing in the presence of silver (80 and 330 ppb) was again very erratic. Cell death in the absence of silver was also seen in experiment 3, this time at a rate of -0.137 hr^{-1}, including the 24 hr sample. Starting at ~10^5 cells ml^{-1}, killing in the presence of silver (50 and 310 ppb) was rapid during the first hour; but residual, viable cells were detected as late as 24 hr.

P. aeruginosa, in the first experiment, gave relatively orderly results. Cells without silver appeared to die at a rate of -0.056 hr^{-1} (Fig.3-1). The measured kill rates at 72 and 410 ppb were approximately -0.88 and -1.46 hr^{-1}, respectively, which are the slowest seen among experiments in this section. Some points were missed in experiment 2 because agar in some of the pour plates did not solidify. The very slightly positive slope (+ 0.0075 hr^{-1} for 24 hr by linear least squares) for the 0ppb suspension probably does not differ significantly from zero, but it certainly is not negative. The silver solutions (140 and 750 ppb) appeared to have killed to the extent of 10^{-4} in the first hour. There was generally less than one viable cell per milliliter at 2, 3, and 4 hr; but only one of these samples was completely negative.

Extinction Time as a Function of Temperature

Since the kinetic data for suspensions which initially contained ~ 10^4 cells ml^{-1} were often difficult to interpret, we decided to try 5-log death time (t_{10-5}) as a comparative statistic when determining the effect of temperature upon cell killing. We planned to begin with ~10^4 cells ml^{-1} and to determine the time required to reach 10^{-1} cells ml^{-1} by collecting viable cells on 0.45 μm porosity filters and incubating these on agar medium to permit colony formation. As much as 100 ml could readily be sampled at once by this method. We feared, however, that neutralizing substances in the agar might reach the cells too slowly to arrest the action of silver promptly when the sample was taken. Two possible answers came to mind: adding thioglycollate to the sample or rinsing free silver ions through the filter with an additional volume of sterile, deionized water.

F. aquatile was used as the model in a three-factor experiment. The factors were silver (0 or 250 ppb), sodium thioglycollate (0 or 500 ppm), and rinsing (none or 100 ml of deionized water after the sample). The only forthright finding was that F. aquatile is quite intolerant of even brief exposures to thioglycollate. Further work with F. aquatile was deferred until another sampling method could be devised.

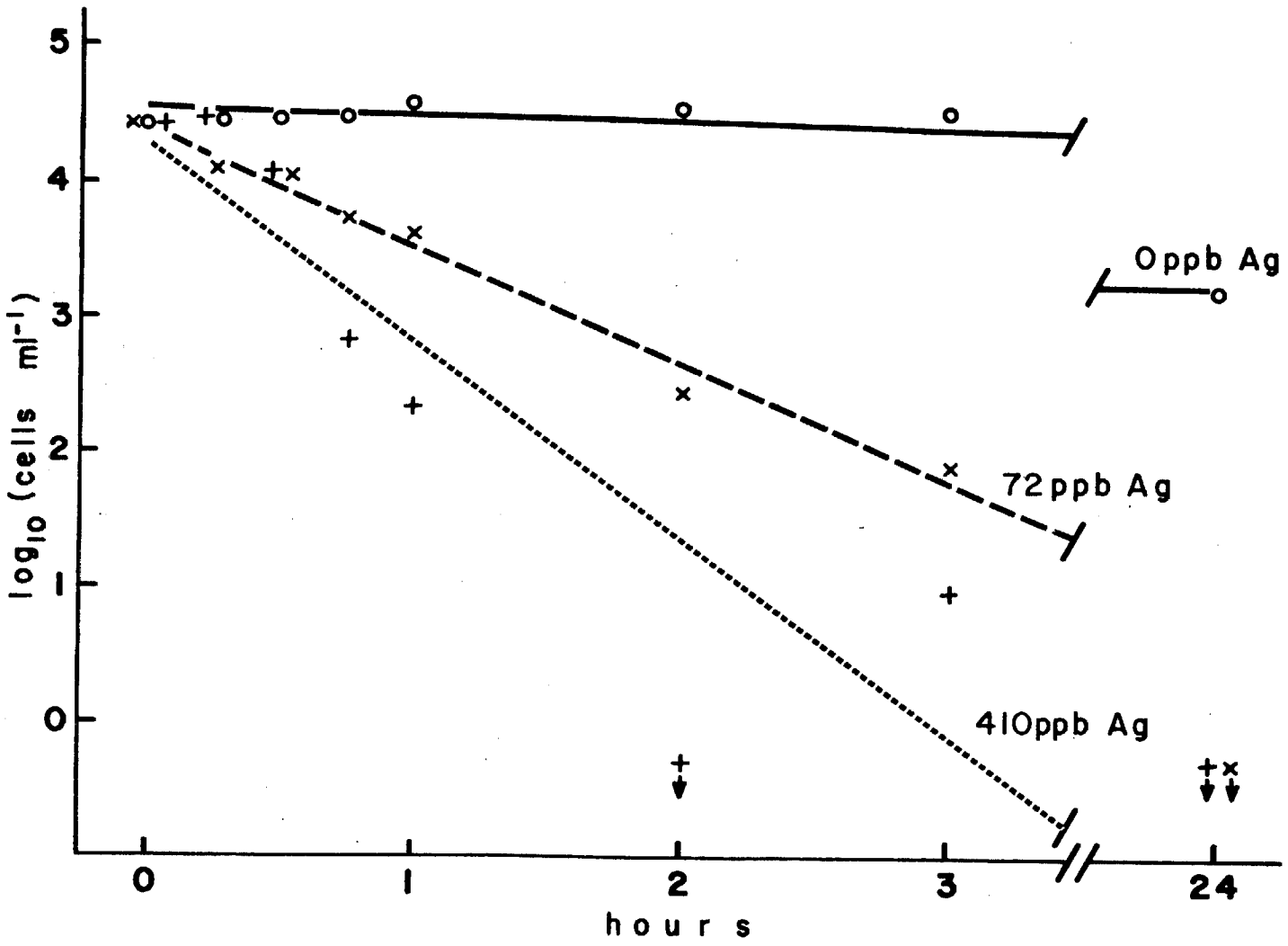

Fig. 3-1. Pseudomonas aeruginosa experiment 1

P. aeruginosa served as the model in a similar experiment, except that the filter porosity was 0.22 μm. Colony formation, in the absence of silver and other factors, was approximately as efficient on the filters as in pour plates (145 and 138 colonies on filters vs. 138 and 160 colonies in 2 plates). The combination of thioglycollate with rinsing seemed to do the best job of mitigating the silver effect (Table 3-2). This combination was selected for use in further experiments, though it may have been somewhat deleterious in the absence of silver ions.

Table 3-2. Effects of three factors upon colony formation on filters by P. aeruginosa

Thioglycollate	Rinse	Silver 0ppb	Silver 250ppb
−	−	145*,138	97,87
−	+	101,111	76,82
+	−	133,143	109,96
+	+	69,110	116,123

*Number of colonies on one filter

F. halmephilum, tested in this way, gave results which indicated that temperature exerts a significant effect upon t_{10}-5 (Table 3-3). It also appeared that F. halmephilum might be somewhat more resistant to silver than P. aeruginosa under these conditions and that t_{10}-5 might be somewhat dependent upon initial cell count. Some results to be reported below appear to support these notions at a higher level of certainty.

Table 3-3. Effects of silver propionate upon bacteria at 3 temperatures, expressed as t_{10}-5

Test species	Initial cell count	Ag^+ level	Temperature 14-15C	24C	36C
F. halmephilum	~$4x10^2$ cells ml^{-1}	50ppb	>>3 hr	>>3 hr	~2 hr
		250ppb	>3 hr	~2 hr	1-2 hr
	~10^3 cells ml^{-1}	50ppb	>>5 hr	>5 hr	~ 3 hr
		250ppb	~5 hr	~4 hr	~ 3 hr
P. aeruginosa	~$4x10^4$ cells ml^{-1}	50ppb	>>3 hr	>3 hr	~ 1 hr
		250ppb	1.5-2 hr	1.5-2 hr	~ 1 hr

Silver Source

A further experiment was intended to determine the effect of silver source upon t_{10}-5 for P. aeruginosa at various temperatures. Starting at ~8 x 10^3 cells ml^{-1}, all combinations of temperature and silver level were included for the ion generator silver, but only the points reported as inequalities in Table 3-3 were determined for silver propionate. Every t_{10}-5 for ion generator silver was shorter than its counterpart observed with silver propionate (Table 3-4). Little was made of this at the time, because the differences were not great and some might be attributable to the difference in initial cell numbers in the two experiments.

Table 3-4. Effects of silver propionate and ion generator silver upon P. aeruginosa at different temperatures, expressed as t_{10}-5

Silver		Temperature		
Source	Level	14-15C	24C	36C
Propionate	50ppb	5-6 hr	3-4 hr	—
Ion generator	50ppb	4-5 hr	3 hr	0.5- 1 hr
	250ppb	~ 1 hr	1-2 hr	<0.5 hr

Higher cell numbers were used in later experiments. Two test species were added: Flavobacterium sp (IIb) and another organism designated as group IIIa. These had been isolated from spacecraft water systems and supplied to us from the Manned Spacecraft Center. Their designations are based upon a taxonomic system used by the National Communicable Disease Center. An inquiry to NCDC yielded no more information about them than that group IIIa has not been assigned to a genus. Another equally obscure organism (group IIIb) was received with these from MSC but experiments on this organism have produced no results worth reporting. All cell counts in this series of experiments were obtained by spread plating on APT agar.

F. aquatile began to be killed by silver propionate only after the first hour (Table 3-5). By 3 hr, more cells had been killed by the propionate than the ion generator silver. This was the only result of its kind: each of the other test organisms had been killed to a greater extent at 1 hr and thereafter by the ion generator silver. The other test organisms showed a definite tailing in killing by ion generator silver at the end of the second or third hour. The difference at the end of the fourth hour was greatest with Flavobacterium sp. (IIb), least with group IIIa, and intermediate (at approximately 20-fold) in two experiments with P. aeruginosa.

Table 3-5. Relative effectiveness of ion generator and propionate silver in killing cells at room temperature

Test organism	Silver Source	Silver Level (ppb)	Sampling time (min) 0	30	60	90	120	180	240
F. aquatile	Generator	197	1.2×10^{7}*	–	4.1×10^{5}	–	1.2×10^{3}	5.8×10^{2}	–
	Propionate	187	1.1×10^{7}	–	1.2×10^{7}	–	1.3×10^{2}	7.5×10^{1}	–
F. sp. (IIb)	Generator	58	1.6×10^{6}	5.6×10^{5}	8.5×10^{3}	5.6×10^{3}	2.1×10^{3}	4.3×10^{2}	5.5×10^{2}
	Propionate	49	5.2×10^{6}	2.7×10^{5}	1.2×10^{5}	2.5×10^{5}	1.7×10^{5}	1.4×10^{5}	3.2×10^{4}
Group IIIa	Generator	51	2.7×10^{6}	–	1.6×10^{6}	–	9.1×10^{5}	7.8×10^{5}	6.5×10^{5}
	Propionate	51	2.5×10^{6}	–	2.5×10^{6}	–	1.5×10^{6}	1.9×10^{6}	7.6×10^{5}
P. aeruginosa	Generator	219	4.6×10^{7}	4.5×10^{6}	2.9×10^{5}	5.1×10^{4}	–	7.3×10^{4}	7.1×10^{4}
	Propionate	226	4.2×10^{7}	2.5×10^{7}	1.4×10^{7}	1.0×10^{7}	–	–	1.3×10^{6}
	Generator	246	6.5×10^{7}	–	4.8×10^{5}	–	6.0×10^{4}	2.1×10^{4}	1.9×10^{4}
	Propionate	246	4.0×10^{7}	–	1.4×10^{6}	–	8.0×10^{5}	3.3×10^{5}	4.7×10^{5}

*Cells ml^{-1}

We are hesitant to conclude from the data in Table 3-5 that ion generator silver will kill more cells than the same amount of silver as the propionate. It may be that the ion generator silver takes effect more rapidly, though the number of cells ultimately killed does not differ.

Initial Cell Load

The higher cell numbers used in the experiments just described had permitted more extensive comparisons than could be made by beginning at 10^4 cells ml^{-1}. However, it appeared that the number of cells which could be killed by a given quantity of silver was exceeded in some experiments with high cell numbers. For instance, the t_{10}-5 for F. halmephilum had been estimated at 2 hr at room temperature in 250 ppb silver (propionate), starting with 400 cells ml^{-1} (Table 3-3); in 200 ppb, with an initial cell count of 9×10^7, killing was only $10^{-2.3}$ in 3 hr. A further example was provided by P. aeruginosa at initial levels in excess of 10^4 and 10^7 cells ml^{-1}. Kill rate was a function of silver concentration at the lower cell level, and there was no indication of a "tail" in the kill curve (Table 3-6). At the higher cell level, a tail appeared within 90 min at 220 ppb of silver, and very little killing occurred at 58 ppb.

Table 3-6. Killing of P. aeruginosa by ion generator silver, as a function of silver level and of initial cell count

Experiment	Silver (ppb)	Time (min) 0	30	60	90	120
1	54	3.4×10^4*	1.3×10^4	1.1×10^3	5	< 5
	260	4.1×10^4	3.0×10^2	2.0×10^1	< 5	< 5
2	58	3.7×10^7	2.5×10^7	3.0×10^7	3.0×10^7	–
	220	4.5×10^7	4.5×10^6	2.9×10^5	5.0×10^4	7.0×10^4

*Cells ml^{-1}

Rapid killing of dense cell suspensions might still be achieved if higher silver concentrations were employed. A series of trials of ion generator silver with Flavobacterium sp. (IIb) is reported in Fig. 3-2. This organism appears to be more sensitive to silver than is P. aeruginosa, for there is extensive killing ($\sim 10^{-4.5}$) at 58 ppb. However, the kill rate is approaching zero at 4 hr in this silver suspension and has definitely declined within 1-2 hr at 220 and 250 ppb. Only at 370 ppb is the kill curve approximately linear over the entire range of observed values.

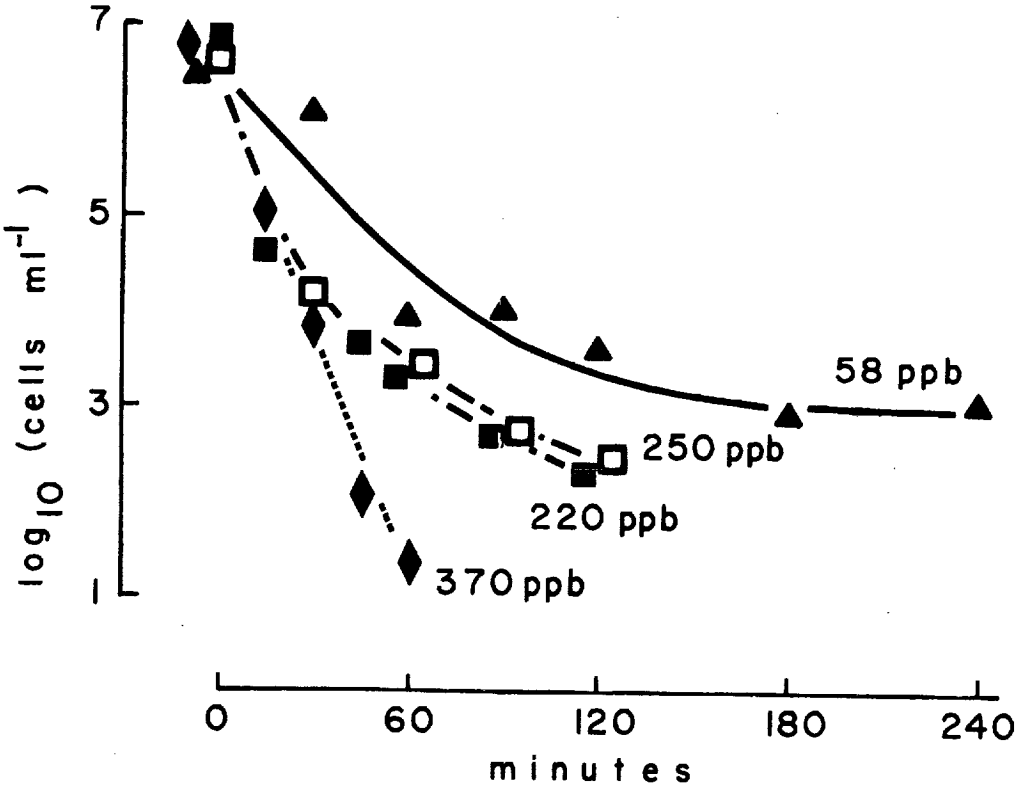

Fig. 3-2. Killing of large numbers of Flavobacterium sp. (IIb) cells by ion generator silver

A direct comparison of Flavobacterium sp. (IIb) death at three different initial cell loads, using a single level of silver, is shown in Fig. 3-3. The cells which were initially present at lower levels were killed more rapidly, as expected. This is presumably due to the higher initial level of silver per cell. It appears that 250 ppb of silver will kill, promptly, cells present initially at a level of 10^4 ml^{-1} or less. Higher initial levels than this seem quite improbable, except in instances of massive contamination, such as with feces.

Relative Silver Sensitivity

We wished to know which of our test cultures was most susceptible to the action of silver. Seven experiments which bear upon this question are summarized in Table 3-7. It is unfortunate that the starting concentrations of silver and of cells were not identical in all of these, but the conditions seem similar enough to permit comparisons. These and other experiments described elsewhere in this report suggest that F. halmephilum is the least sensitive to silver of the species compared. P. aeruginosa and group IIIa are comparable to each other and are followed by F. aquatile, Flavobacterium sp. (IIb), Achromobacter metalcaligenes, and Alcaligenes fecalis in apparent order of increasing sensitivity. The use of higher initial cell numbers facilitates this kind of comparison; it also leaves one to assume that a similar rank order would have resulted if the cell numbers had been in the range that has occurred in spacecraft water systems. Unfortunately, experimental conditions are almost never identical with the conditions under which the results are to be applied.

Table 3-7. Killing by ion generator silver of seven test bacteria

Test organism	Silver (ppb)	Time (min) 0	60	120	180	240
A. metalcaligenes	200	2.2×10^{7}*	$<10^{4}$	$<10^{3}$	5×10^{2}	3×10^{1}
A. facalis	200	6.8×10^{5}	$<10^{1}$	$<10^{1}$	–	–
F. aquatile	200	1.1×10^{7}	4×10^{5}	1.3×10^{3}	5×10^{2}	–
F. halmephilum	200	9×10^{7}	2.3×10^{7}	9×10^{5}	4.5×10^{5}	–
F. sp (IIb)	250	4.4×10^{6}	2.8×10^{3}	2.0×10^{2}	–	–
Group IIIa	250	4.3×10^{6}	5.6×10^{4}	7.3×10^{3}	2.5×10^{3}	2.2×10^{3}
P. aeruginosa	260	4.4×10^{7}	2.0×10^{6}	2.6×10^{4}	1.2×10^{4}	1.1×10^{4}

*Cells ml^{-1}

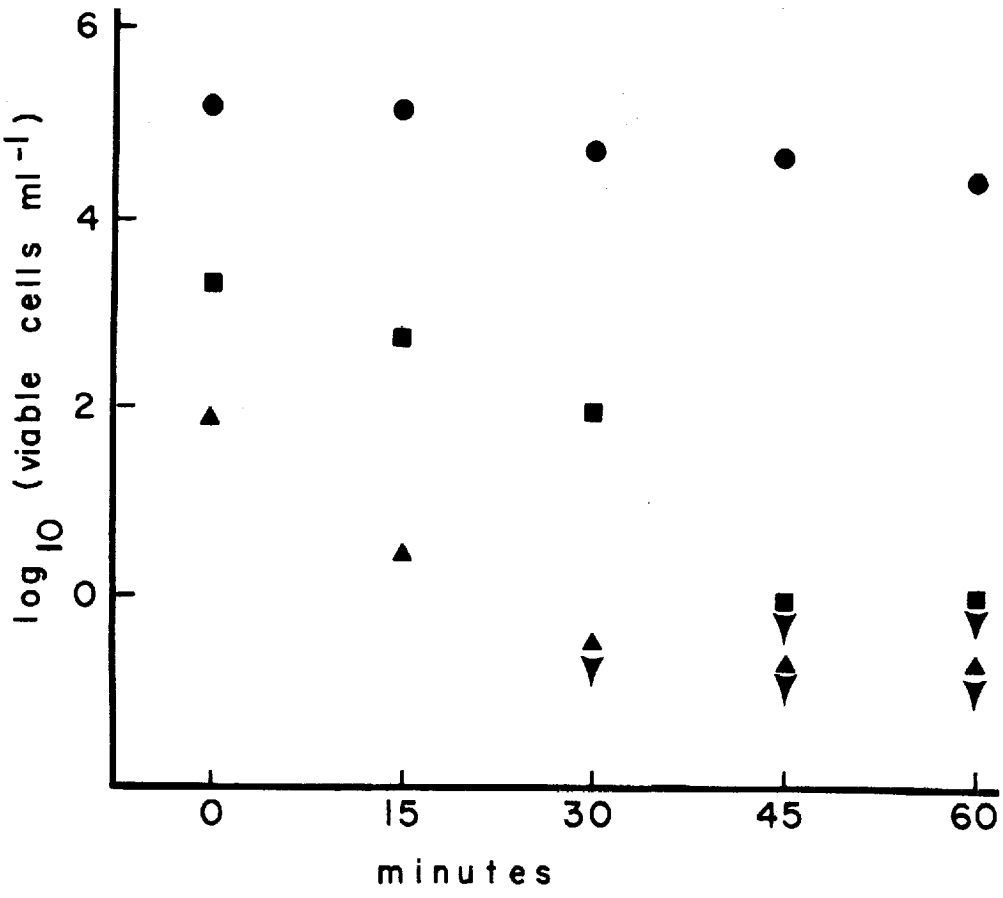

Fig. 3-3. Effect of initial cell number on the rate of killing Flavobacterium sp (IIb) by 250 ppb of silver

During the second year's work, additional species were tested for susceptibility to silver ions. These included Bacillus cereus, Micrococcus lysodeikticus, and Saccharomyces cerevisiae, as representatives of gram-positive spore formers, gram-positive non-spore formers, and yeasts, respectively.

Bacillus cereus was obtained as a suspension of spores which had been washed twenty times with deionized water. Cells grown from these in APT broth were washed three times in deionized water. Fig. 3-4 compares killing, by 240 ppb of ion generator silver, of spores and of vegetative cells harvested after 4 to 5 hr in culture. Samples were diluted in APT broth and plated on APT agar. The young, vegetative cells were extremely susceptible to silver, while the spores were totally resistant. Attempts to determine the effect of age upon the susceptibility of vegetative cells were unsuccessful because the cell suspensions showed a significant and increasing proportion of spores with increasing age.

Killing as a Function of Cell Age

Micrococcus lysodeikticus was grown and plated with trypticase soy medium, but samples were diluted with APT broth. Cells after 4 to 5, 24, and 72 hr in culture were washed three times with deionized water and exposed to 260 ppb of ion generator silver. The micrococci appear to be relatively resistant to silver, though the 72 hr cells were somewhat more sensitive than the others (Table 3-8).

Table 3-8. Effect of age of Micrococcus lysodeikticus cells upon killing with 260 ppb of ion generator silver

Reaction Time (hr)	Age of cells		
	4-5 hr	24 hr	72 hr
0	7.0×10^5*	1.3×10^6	6.6×10^5
1	8.7×10^4	6.0×10^3	4.6×10^4
2	2.1×10^4	1.0×10^3	1.9×10^3
3	6.0×10^2	3.0×10^2	1.0×10^2
4	4.8×10^2	4.0×10^2	$< 10^2$

*Viable cells ml^{-1}

Saccharomyces cerevisiae was grown in trypticase soy broth with 1% glucose added. Cells washed 3 times with deionized water were reacted with 260 ppb of ion generator silver. Samples were diluted in APT broth and plated on potato-dextrose agar, the pH of which had not been adjusted. The very young cells were quite sensitive, while those aged 24 and 72 hr were less so (Table 3-9). The problem seemed

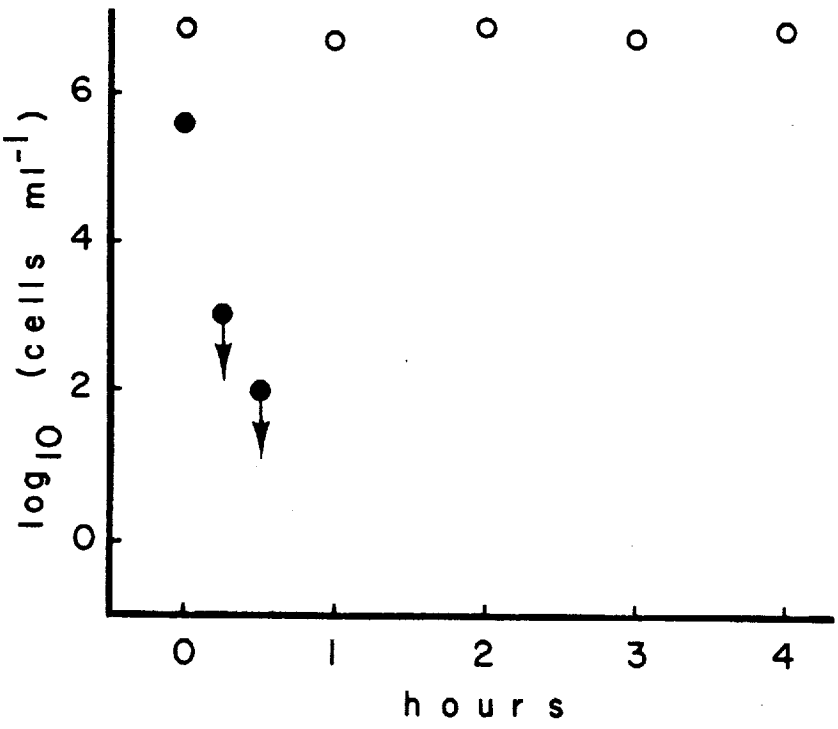

Fig. 3-4 Killing of Bacillus cereus spores (O) and vegetative cells (●) by 240 ppb of silver

to be one of sporulation in the older cultures. No yeast colonies were ever detected by direct plating of 0.1 ml from the reaction vessel. This indicates the inability of the plating medium (potato dextrose agar) to neutralize the silver ions and the extreme sensitivity of the yeast organism to small quantities of silver.

Table 3-9. Effect of age of Saccharomyces cerevisiae cells upon killing with 260 ppb of ion generator silver

Silver level (ppb)	Sampling time (hr)	Age of cells		
		4-5 hr	24 hr	72 hr
0	0	6.0×10^5*	1.9×10^3	1.4×10^5
	4	4.2×10^5	3.1×10^5	1.7×10^5
260	0	1.5×10^4	2.3×10^5	6.0×10^4
	0.5	5.0×10^1	3.0×10^3	5.0×10^3
	1	<10	3.6×10^3	3.9×10^3
	1.5	<10	2.4×10^3	6.7×10^3
	2	<10	2.4×10^3	9.6×10^3
	3	<10	2.9×10^3	6.6×10^3
	4	<10	2.8×10^3	5.3×10^3

*Viable cells ml^{-1}

Flavobacterium sp. (IIb) was also used to compare the silver sensitivity of cells of different ages. Cells were grown in APT broth and washed three times in deionized water. The washed cell suspensions were reacted with 260 ppb of ion generator silver. Samples were diluted in APT broth and plated on APT agar medium. The susceptibility of these cells was less than that observed previously (Table 3-10); we do not know why. The 72 hr cells appeared to be more silver-resistant than the younger cells.

Interaction of Cells and Silver

Silver complexing by cells. One would expect that the silver ions must associate with the cells in order for killing to occur. Still, this had not been demonstrated directly. The question is of more than theoretical concern: if the silver is irreversibly associated with the cell it kills, it will not be available to kill other cells which may be introduced later.

Table 3-10. Effect of age of Flavobacterium sp. (IIb) cells upon killing with 260 ppb of ion generator silver

Sampling time (min)	Age of cells		
	4-5 hr	24 hr	72 hr
0	3.2×10^{6}*	5.4×10^{6}	1.0×10^{6}
15	2.4×10^{6}	3.7×10^{6}	–
30	1.8×10^{6}	2.9×10^{6}	–
45	1.5×10^{5}	–	–
60	7.6×10^{4}	1.8×10^{5}	–
90	1.5×10^{4}	1.5×10^{4}	2.2×10^{5}

*Viable cells ml^{-1}

We had hoped that we could measure silver complexing by collecting the killed cells on a membrane filter and subjecting this to NAA. Some preliminary tests indicated that this might be possible: the control filters show no silver activity by NAA, though they tend to adsorb silver from solutions they filter. Further work showed that the signal-to-noise ratio was unfavorable in this system, so that NAA would not give us precise enough data for physical assay of silver complexing. Therefore, the only remaining possibility was biological assay.

Two experiments were performed which illustrate this possibility. In the first, 2 ml of Flavobacterium sp. (IIb) washed cell suspension were added to 18 ml of ion generator effluent to give a level of 250 ppb and sampled over a period of 120 min. Then another 0.2 ml of the same cell suspension was added, and sampling was continued for another 120 min. The added cells were definitely killed more slowly than the initial inoculum at zero time, but it is difficult to say that killing is slower than for the initial inoculum after 120 min (Fig. 3-5). That is, if the second segment of the curve were transposed downward, it would form a fairly smooth continuation of the first segment. The second experiment employed P. aeruginosa; and although the procedure was the same, the results were rather different (Fig. 3-5). The slope of the second segment of the curve is a distinct departure from that of the end of the first segment. There is also a "shoulder" at the beginning of this curve. This is usually thought to indicate that the cells in the suspension are aggregated, but there was no indication of this when another portion of the same suspension was added at 120 min. Though killing was definitely slower in the second segment, it did occur.

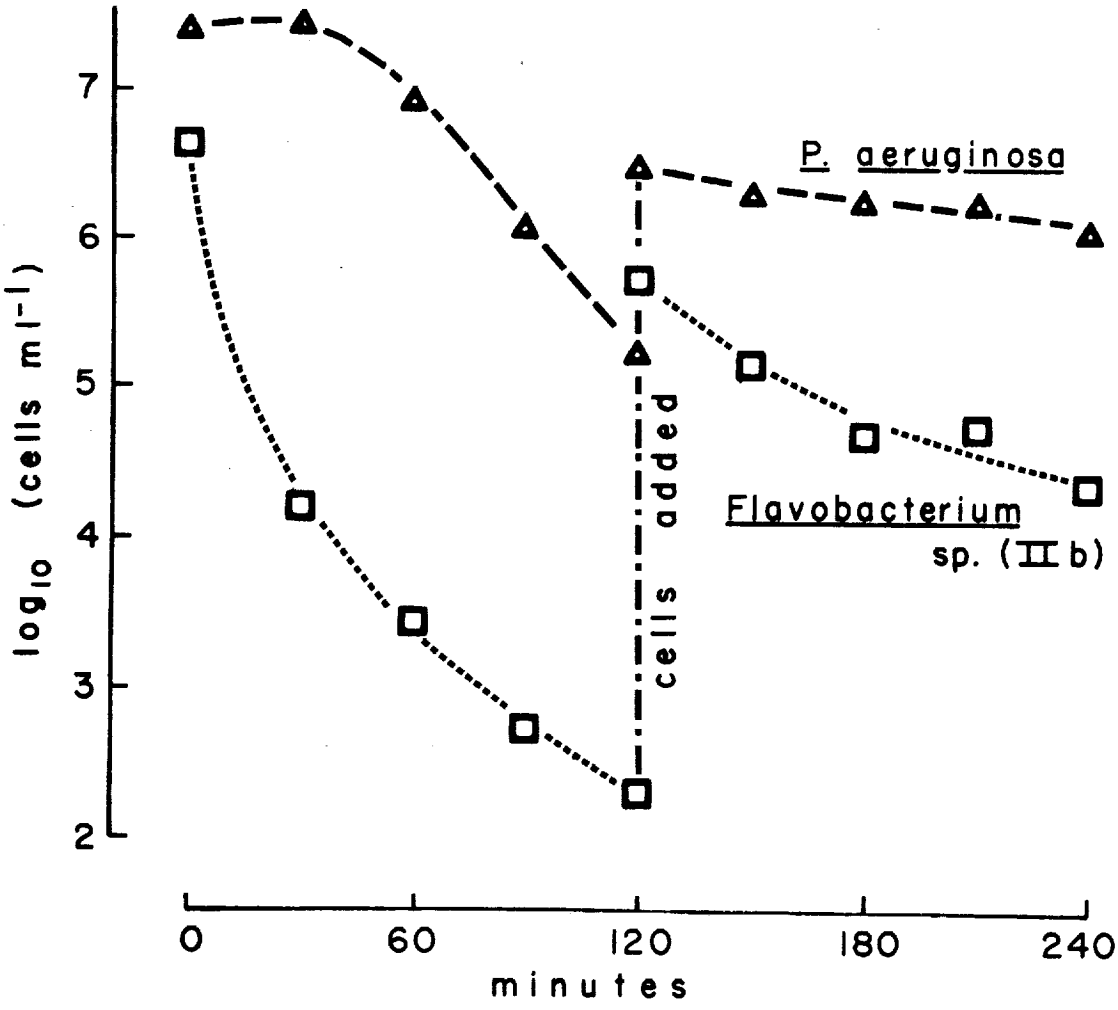

Fig. 3-5. Effects of adding live cells after many have been killed by silver

Cell aggregation. The aggregation of bacterial cells or of virus particles has been examined from time to time. We have usually been most concerned with the possible adverse effect of beginning an experiment with a highly aggregated suspension. In the present instance, suspensions of Micrococcus lysodeikticus were examined by darkfield microscopy after 30 min exposure to 0 or 250 ppb of silver. Large clumps of cells were obtained in the presence of silver, whereas the cells were in singles, pairs, or triples in the control suspension. The clumped suspension could not be reduced to a state comparable to the control by means of manual shaking. The clumps were large enough to be detectable without the microscope. The component cells were not obviously abnormal as seen with the darkfield microscope.

Reversibility of "killing." We wanted to know whether cells killed by silver could subsequently be revived. Two experiments with Flavobacterium sp (IIb) were done to test this, one with silver from the ion generator and one with silver propionate. After 4 hours reaction with 250 ppb of silver, cells from 8 ml of the suspension were washed twice in APT broth and resuspended to 8 ml final volume in APT broth. Assay of this suspension, compared to one of silver-treated cells which had not been washed, showed no revival in either experiment. Limited testing gave no indication of cell lysis as evidenced by leakage of protein or nucleic acid.

Selection for Silver Resistance

Having determined the sensitivity of an organism to silver, one would like to believe that this would remain relatively constant. However, we had seen "tails" on kill curves and not known whether to attribute them to depletion of the silver, or to the presence of a more resistant fraction, or both. If a resistant fraction were present, subculture after the more susceptible cells had been killed should select for this property.

A suspension of washed P. aeruginosa cells was used in a first experiment. This was treated with silver (260 ppb) for 120 min at room temperature and 0.25 ml of the remaining suspension was transferred to 9 ml of APT broth and shaken for 20 hr at 30C (Table 3-11). The cycle was then repeated: cells were again washed and challenged with 260 ppb of silver at room temperature. The degree of killing may have been somewhat greater in this second cycle. The change in cell count was positive in the third and fourth cycles. We have no explanation for these increases, but they do appear to be significant. We also noted that in plating cells that had been treated with silver, pinpoint colonies were often formed, indicating a late start or slow growth, and that such atypical colonies were absent in assaying the cells in cycles 3 and 4. Great selective pressure was involved in producing the silver-resistant derivative. Whether a comparable result is possible in a spacecraft water system is uncertain.

Table 3-11. Effect of repeated silver challenge - subculture cycles upon the silver susceptibility of P. aeruginosa

Cycle number	0 ppb Ag		260 ppb Ag	
	0 min	120 min	0 min	120 min
1	4.5×10^{7}*	4.3×10^{7}	4.5×10^{7}	3.5×10^{4}
2	2.8×10^{7}	2.2×10^{7}	1.5×10^{7}	2.1×10^{3}
3	5.3×10^{6}	6.9×10^{6}	4.7×10^{6}	1.5×10^{7}
4	1.3×10^{8}	1.4×10^{8}	1.5×10^{8}	6.1×10^{8}

*Cells ml^{-1}

Flavobacterium sp. (IIb) was used in a second experiment. Two cycles of selection were carried out, based upon 30 min at room temperature with 250 ppb of silver. The parent strain had shown more than 10^{-4} killing in 120 min at 250 ppb, whereas the derivative strain after two selection cycles was killed 10^{-2} at 260 ppb (Fig. 3-6). The parent strain of this organism was more sensitive to silver than that of P. aeruginosa. One cannot tell from these results whether the fact that the derivative strain of Flavobacterium sp (IIb) is not as resistant is due to this or to the decreased selective pressure which resulted from subculturing after just 30 min of silver challenge.

These experiments had to be done. Now that we have done them, we don't know what they prove. It would have been reassuring if we had not been able to select strains which were more silver-resistant than their parent strains. Still, we cannot be sure that the conditions under which this selection was performed are a valid analogue of those which might occur in a spacecraft water system. This point bears watching when future systems are being designed.

Water Purity

Chemical impurities. We have undertaken a limited survey of impurities which, if they occurred in water, might interfere with the antibacterial action of silver. The survey was begun with a number of common chemicals (mostly inorganic) and an arbitrarily chosen "impurity" (or additive) level of 100 ppm. All silver was derived from the ion generator and was diluted to 250 to 275 ppb. The model organism for these experiments has been Flavobacterium sp. (IIb).

The first tests indicated that salts containing the chloride anion were quite strongly antagonistic to silver ions (Table 3-12). This was not really expected: AgCl is soluble to the extent of

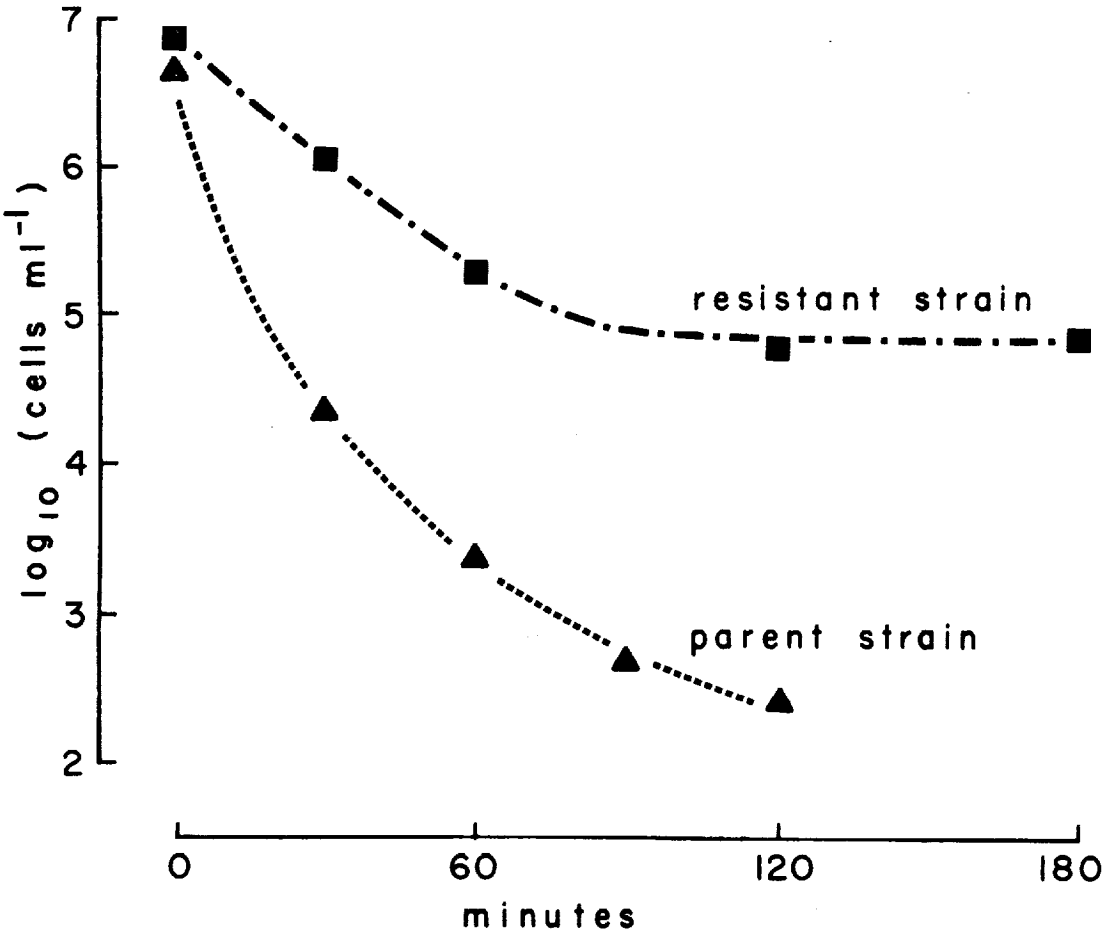

Fig. 3-6. Killing by silver of two strains of Flavobacterium sp. (II b)

1,130 ppb of silver at 20 C, which is far above our working range. There also seems to be an intermediate range in which the silver effect is reduced, but not blocked, by a few compounds. We were not sure what direct effects glycine and cysteine might have upon the bacteria: when they were tested without silver, they had no effect at all.

Table 3-12. Effects of various compounds, added at 100 ppm, upon the bactericidal action of silver on Flavobacterium sp. (II b)

Ag (ppb)	Additive	Molarity ($\times 10^{3}$)	Titer at 3 hr
0	–	–	5.37
250	–	–	3.81
	NaCl	1.7	5.51
	KCl	1.3	5.45
	$CaCl_2$	0.9	5.60
	$MnCl_2$	0.8	5.65
	$FeCl_3$	0.6	5.47
	Na_2SO_4	0.7	4.66
	$(NH_4)_2SO_4$	0.8	3.16
	KH_2PO_4	0.8	4.23
	Na_2HPO_4	0.7	< 3
	Glucose	0.6	3.47
	Glycine	1.3	4.38
	Cysteine	0.8	4.96

*In $\log_{10}$ (viable cells ml^{-1}) compared to a 0 hr titer of 6.49

The survey was then extended in a quest for other compounds which might be effective. This time, each additional compound was also tested without silver, to see if it had any antibacterial effect of its own. None of the additional tested compounds was antibacterial, but $MnSO_4$ showed a very strong antagonism for silver (Table 3-13). This was not the first Mn^{2+} compound tested, but the antagonistic effect of $MnCl_2$ (shown in Table 3-12) might have been attributed to the Cl^- ion. Enough other SO_4^{2-} compounds have now been tested to indicate that this anion has little or no effect, so

the Mn^{2+} ion was expected to prove to be the active portion of this compound.

Table 3-13. Effects of additional compounds, added at 100 ppm, upon Flavobacterium sp (II b) and on the antibacterial action of silver

Additive	Molarity ($\times 10^{-3}$)	Ag (ppb)	Titer* at 1 hr	Titer* at 3 hr
None	–	0	–	6.59
		250	< 3	< 2
$MnSO_4$	0.7	0	6.19	6.26
		250	6.24	6.26
$NaCO_3$	1.0	0	6.19	6.10
		250	< 4	< 3
$NaHCO_3$	1.2	0	6.19	6.14
		250	< 4	< 3
K_2SO_4	0.6	0	6.19	6.17
		250	< 4	< 3
$(NH_4)_2CO_3$	1.0	0	6.18	6.14
		250	3.30	< 2
K_2HPO_4	0.6	0	6.17	6.12
		250	< 4	< 3
$(NH_4)HCO_3$	1.3	0	–	5.90
		250	< 4	< 3

*In $\log_{10}$ (viable cells ml^{-1}), compared to a 0 hr titer of 6.74

A further extension of the survey has added both information and confusion. The Cr^{3+}, Fe^{2+}, and Fe^{3+} ions appear to be toxic to the cells (Table 3-14). Al^{3+}, and perhaps Cu^{2+} and Mg^{2+} afforded some protection against silver. A second trial failed to confirm the protection against silver by $MnSO_4$ (data not shown).

The chloride ion (Cl^-) had been shown consistently to inhibit the action of silver, at the concentrations tested. The concentrations were arbitrarily 100 ppm for all compounds, which is a relatively high level of impurity. We wanted to know how much Cl^- had to be present to show this effect. The test reagent was NaCl, since Na^+ seemed not to inhibit silver. We found that 1.7 mM (100 ppm) was the lowest significant concentration (Table 3-15). We have

shown that some solutes have qualitatively significant anti-silver activity, but that the presence of solute as such at as high a level as 100 ppm is not a total disaster.

Table 3-14. Effects of still more compounds, added at 100 ppm, upon Flavobacterium sp. (IIb) and on the anti-bacterial action of silver

Additive	Molarity ($\times 10^{-3}$)	Ag (ppb)	Titer* at 0 hr	Titer* at 1 hr	Titer* at 3 hr
None	–	0	6.05	–	6.11
KNO_3	1.0	0		6.09	6.24
		250		2.57	< 1
$CaSO_4$	0.7	0		5.11	5.54
		250		2.00	< 1
$FeSO_4$	0.7	0		< 2	< 2
		250		< 1	< 1
None	–	0	6.24	–	6.01
		250		1.69	< 1
$Cr_2(SO_4)_3$	0.3	0		< 2	< 1
		250		< 1	< 1
MnO_2	1.2	0		5.91	6.14
		250		< 1	< 1
$NiSO_4$	0.6	0		5.99	5.76
		250		< 1	< 1
$CoSO_4$	0.6	0		5.96	5.90
		250		< 1	< 1
$(NH_4)H_2PO_4$	0.9	0		5.83	6.00
		250		1.17	< 1
None	–	0	5.73	–	5.63
		250		1.84	< 1
$CuSO_4$	0.6	0		6.43	6.32
		250		2.81	1.47
$Al_2(SO_4)_3$	0.3	0		5.66	5.57
		250		5.66	4.59
$Fe_2(SO_4)_3$	0.3	0		3.89	< 1
		250		< 1	< 1
$MgSO_4$	0.8	0		5.65	5.90
		250		4.26	2.00

*In $\log_{10}$ (viable cells ml^{-1})

Table 3-15. Effects of various levels of Cl^- upon the bactericidal action of silver on Flavobacterium sp. (II b)

Ag (ppb)	NaCl (mM)	Titer* at 0 hr	Titer* at 1 hr	Titer* at 3 hr
0	0	6.53	–	6.30
250	0	6.74	< 3	< 2
	1.7		6.23	5.17
	0.17		3.47	< 1
	0.017		2.26	< 1
	0.0017		2.49	< 1

*In $\log_{10}$ (viable cells ml^{-1})

pH. Another "impurity" with which we had been led to be concerned was the H^+ ion. We had found that the pH of very pure water was quite erratic and was almost impossible to measure because the needle on the meter never stopped. The results shown in Tables 3-12 and 3-13 indicated that, if buffer were used to control the pH, it would not necessarily block the silver ions. The buffer system selected was 0.01 M NaH_2PO_4 - Na_2HPO_4. The results shown in Table 3-12 for 0.0008 M KH_2PO_4 had been somewhat suspicious, so controls were added to evaluate any possible effect of the buffer on anything other than H^+ ion potential. The results indicated that pH changes over the range of 5.4 to 8.2 had very limited influence upon the antibacterial activity of the silver (Table 3-16). Neither did the phosphates affect the bacteria or the silver, as far as could be determined from the results presented here. One can't be sure exactly what this proves, but it suggests that we don't need pH control to make the silver work.

Table 3-16. The pH of a silver suspension, as obtained with with 0.01 M NaH_2PO_4 – Na_2HPO_4, buffer has very little influence upon the silver's ability to kill Flavobacterium sp. (II b)

Buffer (M)	pH	Ag (ppb)	Titer* at 1 hr	Titer* at 3 hr
0	~ 7	0	–	5.41
		250	< 3	< 2
0.01	5.4	0	5.22	5.17
		250	3.17	< 2
	6.0	0	5.23	5.12
		250	3.78	< 2
	7.1	0	5.19	5.15
		250	< 3	< 2
	8.2	0	5.17	5.16
		250	< 3	< 2

*In $\log_{10}$ (viable cells ml^{-1}), compared to a 0 hr titer of 5.22

Metal contact surfaces were of interest because they are likely to be present in any water system and because they were known to be active in systems containing viruses (see Section 4— VIROLOGICAL STUDIES). The Al^{3+} ion had already been shown to protect Flavobacterium sp. (IIb) against silver (Table 3-14). A further survey was done with this same species, using 10 ml of fluid to ~ 10 cm^2 of exposed metal surface, and propionate as the silver source. Copper showed some toxicity to the organism, but none of these metals protected against the silver (Table 3-17).

Table 3-17. Selected metals do not protect Flavobacterium sp. (IIb) from the bactericidal action of silver

Metal present	Ag (ppb)	Titer* at 0 hr	1 hr	4 hr
None	0	6.45	—	6.46
	250		< 1	< 1
Copper foil	0		6.39	3.83
	250		< 1	< 1
Lead foil	0		6.34	6.43
	250		< 1	< 1
Tin foil	0		5.99	6.01
	250		< 1	< 1
Cadmium stick	0		6.04	5.93
	250		< 1	< 1

*In $\log_{10}$ (viable cells ml^{-1})

Fecal contamination was a matter for special concern because we had found that minute quantities of feces would protect some viruses from the action of silver. We wished to determine if feces were protective for bacteria. Flavobacterium sp. (IIb) with 0 or 250 ppb of ion generator silver was incubated with 0 or 0.1% sterile feces. In contrast to the results with poliovirus, there was no indication whatever of protection by feces in this experiment (Table 3-18).

Summary of Bacteriological Studies

Silver ions were shown to be capable of killing all of the microbial species tested. Only spores appeared to be oblivious to silver. There were significant differences in silver sensitivity among the various test species, but these were not great enough to be of practical concern. None of the selected model organisms was

a pathogen; however, there is no reason to believe that the pathogens would be any differently affected by silver ions.

We do not really know what the silver ions do to the cells, but the effect seems to be essentially irreversible. Some cells were seen to clump but apparently did not lyse. The age of the cells was not a critical factor. Repeated, intensive selection would yield bacterial strains of greater silver resistance, but we cannot tell whether the same might happen in a spacecraft water system.

Large numbers of cells were killed fairly rapidly by 250 ppb of silver ions. Silver added as the propionate salt or by electrolysis worked approximately equally well. The pH and the effects of temperature impose no significant limits upon the use of silver ions in this application. Feces, and certain metal contact surfaces, were without effect, but Cl^- and Al^{3+} ions were found to be strongly inhibitory to the antibacterial action of silver.

Table 3-18. Effect of feces upon killing of Flavobacterium sp. (IIb) by 250 ppb of ion generator silver

Silver (ppb)	Feces (%)	Titer* at 0 hr	Titer* at 4 hr
0	0.1	6.08	6.20
250	0	5.72	2.34
250	0.1	6.26	2.18

*$\log_{10}$ (viable cells ml^{-1})

VIROLOGICAL STUDIES

The viruses used in these experiments have been quite diverse and have been handled rather differently from each other. The enterovirus, myxovirus, poxvirus, reovirus, and rhinovirus groups are represented. Within a topic area in this section, the viruses will be considered in ascending order of silver sensitivity. The methods of propagation and assay of the viruses have been those described by Cliver and Herrmann (Health Lab. Sci. 6:5, 1969.), with the noted exceptions. Virus assays are expressed as plaque-forming units per milliliter (PFU ml^{-1}).

Basic Silver Sensitivity

Our first task was to determine if, and to what extent, the model viruses are inactivated by silver. The silver ions in these experiments (and others, unless indicated otherwise) were added as silver propionate. Stock solutions of the propionate were assayed repeatedly before being used with virus.

Vaccinia virus (representing the poxviruses) was propagated in primary rhesus monkey kidney (PMK) cells under L-15 medium plus 2% agamma calf serum. The titers observed under various agar media in PMK were all > 10^7 PFU ml^{-1}. A plaque was subcultured and grown up to prepare a virus stock. Some of this was cleaned by centrifuge clarification at 3,000 rpm for 30 min, concentrated by centrifugation at 8,000 rpm for 30 min, and resuspended in deionized water. The clean-up process was ~ 50% efficient, by comparison of the final titer to that of the stock virus suspension. Filtration of a 0 time, Oppb suspension at 450 nm resulted in a 74% decrease in plaque numbers; this may not be an ideal indicator of the degree of aggregation, but it is all we have. The levels of silver used were found to have been 0,600, and 2400 ppb. There was an apparent early decline in the titers of the suspensions which contained silver; but despite the tenfold excess of silver over the desired concentrations, there was no significant difference at 24 hr. That is, approximately the same proportion (5-10%) of virus "survivors" was present over a range of silver concentrations of 0-2400 ppb. Filtration of the 24 hr samples at 450 nm showed less aggregation among these than in the 0 time sample, so aggregation probably was not the basis on which these plaque-forming units escaped inactivation.

Virus for a second experiment was purified by washing in an ultrafilter (Amicon Diaflo), rather than by centrifugation. After 24 hr at room temperature with 250 ppb of silver there were 3.7×10^3 PFU ml^{-1} in a suspension, compared to 3.5×10^3 PFU ml^{-1} in a suspension from which silver had been omitted. Given the similarity of these titers, it seemed unlikely that longer contact times would accomplish any more. Still, we decided to try. The contact time was extended, and samples were taken at 0, 2, 5, and 7 days. Both the control (Oppb) and 250 ppb samples taken at 7 days had 4.9×10^3 PFU ml^{-1}, and the earlier samples differed within pairs by no more than 15%. Vaccinia appears to be completely resistant to silver under

the conditions of these experiments. If it is a valid representative of the poxvirus group (it often is used for this purpose), then the other members of the group may well share this property.

Influenza virus type A, strain PR 8 (representing the myxoviruses), was propagated in the allantoic sacs of embryonated chicken eggs. A sample of the stock was clarified at 5,000 rpm for 30 min and concentrated at 15,000 rpm for 30 min. The final suspension had a titer of ~ 1.4×10^5 PFU ml^{-1} and was ~ 80% aggregated as determined by filtration at 200 nm porosity. The original suspension was found to be > 90% aggregated by this criterion, but some of the aggregates were removed during clarification.

Virus from the same stock suspension, clarified and concentrated in the same way, was used in the first silver challenge experiment. Filtration at 300 nm porosity (0 time, 0ppb suspension) resulted in a 26% increase in plaque count, while a similar test with a 24 hr sample showed a 30% increase. Such increases are unusual among viruses in general and are usually taken to indicate that aggregates are breaking up at the filter surface. Since some aggregates are breaking up while others are almost certainly being retained by the filter, there is no absolute basis for estimating the degree of aggregation in these preparations. It does seem likely that the two were pretty much the same, however (i.e., little or no net change in degree of aggregation during 24 hr of incubation), and must certainly have been aggregated to > 26 to 30%. The results are shown in Fig. 4-1. Least squares estimates of the curve slopes are -0.0099 (0ppb), -0.029 (50 ppb), and -0.11 (250 ppb) hr^{-1}; fits are neither outstandingly good nor bad. Influenza virus appeared to be quite limited in its susceptibility to silver ions.

This finding has essentially been confirmed. Virus harvested from the allantoic sacs of infected, embryonated chicken eggs was washed with deionized water in an Amicon Diaflo apparatus (Model 200; XM100 membrane). This washed preparation showed a 50 to 60% titer loss in filtration at 300 nm porosity, suggesting that at least some of the virus was aggregated. The virus was reacted with 250 ppb of silver (as the propionate). Half of the samples were diluted in phosphate buffered saline (PBS), and the other half in PBS plus 10% agamma calf serum. This made no difference at all, so the data from corresponding samples were pooled (Fig. 4-1). The slope of the inactivation curve at 250 ppb was -0.11 hr^{-1}, exactly the same as that reported for the previous experiment. There are several possible reasons for this close correspondence. The available evidence seems to favor dumb luck. Though the silver sensitivity of the virus is slight, it certainly is not insignificant.

Our interest in aggregation lay in the possibility that aggregates might be significantly more difficult to inactivate than single virus particles. This was investigated, using 300 nm porosity filters. Filtration of a zero-time sample caused a 73%

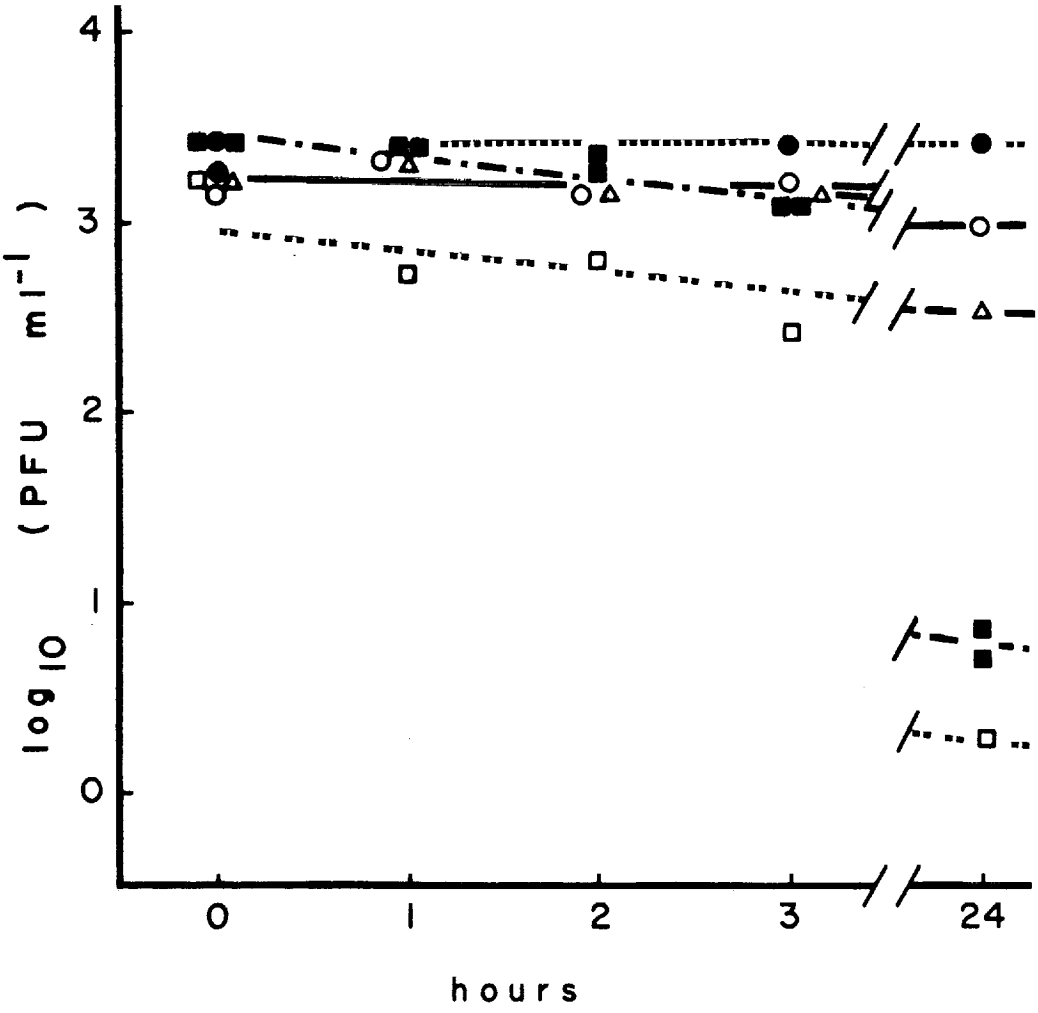

Fig. 4-1. Inactivation of influenza virus type A in the presence of 0 (circles), 50 (triangles), and 250 (squares) ppb of silver. Open symbols from experiment 1, filled symbols from experiment 2.

increase in virus titer, suggesting that aggregates were present and were breaking up at the filter. After 24 hr in deionized water, both the titers before and after filtration were down slightly. Filtration of a suspension after 24 hr in 150 ppb of silver (added as the propionate) caused no significant change in titer. This might mean that the remaining virus was less aggregated. It certainly does not suggest a higher degree of aggregation.

Enteroviruses were first represented by Coxsackie virus type B-3 (CB-3). It was propagated in primary rhesus monkey kidney (PMK) cells under L-15 medium plus 2% agamma calf serum. It was clarified at 16,500 rpm for 30 min and concentrated at 50,000 rpm for 120 min. In a preliminary trial, there had been no loss of titer with filtration at 50 nm porosity. However, the cleaned virus suspension used in the first silver challenge experiment lost 28%, which may be taken as a rough indication of the degree of aggregation.

The silver levels in this experiment were reported to have been 0, 72, and 410 ppb. The infectivity data were analyzed by the plaque technique. Inactivation rates were estimated at -0.0058 (Oppb), -0.19 (72 ppb), and -1.48 (410 ppb) hr^{-1}. This last rate may be used to predict that the time required for 10^{-5} inactivation (t_{10}-5) would be approximately 3.4 hr. These data are not directly comparable to those reported above for influenza A virus, but they do seem to suggest that CB-3 (and, implicitly, the other enteroviruses) is a good deal more silver-sensitive.

We wanted to be sure that the inactivation we were measuring was taking place in the reaction vessel, rather than in the tissue cultures. Still, we were hesitant to try strong chemical neutralizers for silver, for the tissue cultures in which the samples had to be tested might be injured by the neutralizing reagent. Either dilution or addition of serum seemed a likely way to arrest inactivation by silver. A preliminary experiment indicated that both were effective: dilution to 10^{-3} in phosphate-buffered saline plus 2% agamma chicken serum arrested the process promptly, and addition of 10% serum after 24 hr with 250 ppb of ion generator silver may even have reversed some of the inactivation. There was another innovation in this experiment: the CB-3 was washed in a Diaflo filter apparatus, rather than in the centrifuge. The suspension of tissue culture virus was placed in the filter holder, and deionized water was added from the top while the diluted tissue culture medium was being drawn through the filter and out of the bottom, with continuous stirring.

Results of a more extensive experiment led to a different interpretation. In the presence of 250 ppb ion generator silver, the addition of 10% serum at the time of sampling might preclude some undefined carry-over effect on CB-3 in samples taken at 0 and 2 hr (Table 4-1). The serum was without effect in samples taken at 4 and 24 hr, even though these were tested without dilution.

Table 4-1. Arresting silver inactivation of Coxsackie virus type B-3 by adding serum

Silver (ppb)	Serum* (%)	PFU ml^{-1} at 0 hr	2 hr	4 hr	24 hr
0	0	2.6×10^4	—	1.2×10^4	9.0×10^3
250	0	1.3×10^4	2.3×10^2	34	1
250	10	2.7×10^4	4.6×10^2	35	1

*Added at time of sampling

Rather recently, we became concerned with how precise a "dose response" could be expected of the enteroviruses. That is, we wished to know how important the level of silver ions was in determining the rate of virus inactivation. The first experiment compared 0,125, and 250 ppb of silver, using poliovirus type 1 (Po-1) as the test agent, with the results given in Table 4-2. We could not distinguish, given these data, whether the virus was insensitive to a two-fold difference in silver concentration or whether the inactivation rate was equal for this particular range of levels.

Table 4-2. Effect of silver concentration on inactivation of Po-1

Silver (ppb)	PFU ml^{-1} at 0 hr	2.5 hr	4 hr
0	1.5×10^4	1.4×10^4	1.5×10^4
125	1.6×10^4	2.4×10^2	44
250	1.6×10^4	2.1×10^2	45

A more closely-spaced series of silver levels was used in a further experiment. This time the silver was from the ion generator, and the virus was CB-3. There was somewhat less inactivation at 250 ppb (Table 4-3) than in the previous experiment, but the results were compatible otherwise. The present data indicate that silver concentration is no longer the rate-determining factor at levels of 150 ppb or more. We do not attach great importance to this precise figure, given the uncertainties of the silver assay. We do think it significant that the dose response of enterovirus inactivation to silver level may be expressed over only a limited range of silver concentrations.

Table 4-3. Effect of silver concentration on inactivation of CB-3

Silver (ppb)	PFU ml^{-1} at 0 hr	PFU ml^{-1} at 4 hr
0	1.7×10^4	1.9×10^4
50	1.6×10^4	1.3×10^4
100	1.5×10^4	1.7×10^3
150	1.7×10^4	1.5×10^2
200	1.8×10^4	1.3×10^2
250	1.7×10^4	1.6×10^2

Reovirus type 1 (representing the reoviruses) was propagated in PMK cells under L-15 medium plus 2% agamma calf serum and was assayed by the plaque technique in PMK with pancreatin overlay. It was clarified at 7,500 rpm for 30 min and concentrated at 50,000 rpm for 30 min. Filtration of the clean, silver-free suspension at 0 time at 100 nm (0.1 μm) porosity resulted in a loss of 84% in the plaque count, while filtration of a 24 hr sample caused a 72% loss of titer. This difference probably is not significant. The silver levels in a first experiment were found to have been 0, 580, and 2700 ppb, or roughly a factor of 10 greater than were intended. The virus appeared to have been quite rapidly inactivated. No residual plaque-forming units were detected in samples taken at 1 hr or later.

This told us little about the relative silver-sensitivity of the reoviruses. The question was held in abeyance until some time later, when reovirus was used as a model in studying the effects of impurities on silver inactivation. Data to be reported there indicate that type 1, at least, is probably more sensitive than the enteroviruses: more than 99% inactivation occurred within an hour with 250 ppb of silver.

Rhinovirus type 1A (representing the rhinoviruses) was propagated in WI-38 cells and was cleaned up by the same method as was described for CB-3. Filtration of a 0 time, 0ppb suspension at 50 nm porosity caused a 31% decrease in plaque count (determined in PMK cells), which may provide a rough estimate of the degree of aggregation. The levels of silver in the first experiment were reported to have been 0, 46, and 280 ppb. The 0 time, 0ppb virus suspension had slightly less than 10^3 PFU ml^{-1}, which was lower than we wanted by a factor of more than 10. The 0 time samples with silver had titers < 10 PFU ml^{-1}, which is approximately a 100-fold reduction (or more). The least squares slope for the 0ppb points was approximately -0.036 hr^{-1}. The suspensions which contained

silver were tested undiluted at 1,2,3, and 24 hr without detecting any residual infectivity. Even though the experiment was begun with less than the desired level of virus, it demonstrates that this rhinovirus is extremely sensitive to inactivation by silver ions.

Silver Source

CB-3 had been found to be inactivated by silver ions at a moderate rate. It was therefore selected as a first model agent for studies of another question: the relative effectiveness of silver propionate and of ion generator silver. CB-3 was cleaned up and incubated with silver from each of these sources at room temperature. Inactivation by silver was seen at 24 hr, but not during the first 3 hr of the challenge, in the first experiment. When this experiment was repeated, inactivation was demonstrable beginning with the 2 hr sample (Table 4-4). The inactivation at 3 hr seemed to be greater with silver from the ion generator, but by 24 hr the difference appeared insignificant. One might well be tempted to read more into the results of this experiment than is actually there.

Table 4-4. Inactivation of Coxsackie virus type B-3 with time, as a function of the source of the silver ions.

Silver Source	Silver Level	PFU ml^{-1} at			
–	0ppb	4.0×10^4	–	6.4×10^4	4.0×10^4
Ion generator	40	4.0×10^4	2.7×10^2	86	2.7
	240	5.0×10^4	1.6×10^2	89	< 1
Propionate	66	6.4×10^4	2.2×10^2	3.2×10^2	6.4
	190	7.3×10^4	2.2×10^2	1.2×10^2	1

Instead, we did three more comparative experiments. The model viruses in this series were Po-1, ECHO virus type 6 (EC-6), and CB-3. Had the data in Table 4-5 been represented graphically, they would have yielded a bewildering variety of curve shapes. The principal conclusion to be drawn, however, is that enterovirus inactivation is little influenced by silver source under these conditions.

Table 4-5. Inactivation of enteroviruses by 250 ppb of silver as a function of silver source

Virus type*	Silver source	PFU ml^{-1} at 0 hr	1 hr	2.5 hr	4 hr
Po-1	None	$1.4x10^4$	$1.3x10^4$	$1.2x10^4$	$1.4x10^4$
	Ion generator	$1.4x10^4$	$1.2x10^3$	$1.1x10^2$	$1.0x10^2$
	Propionate	$1.6x10^4$	$1.4x10^3$	$1.3x10^2$	72
EC-6	None	$1.9x10^4$	$1.9x10^4$	$1.8x10^4$	$1.7x10^4$
	Ion generator	$2.1x10^4$	$1.3x10^4$	$2.4x10^3$	50
	Propionate	$2.0x10^4$	$1.7x10^4$	$2.7x10^3$	50
CB-3	None	$1.1x10^4$	$1.1x10^4$	$1.1x10^4$	$1.2x10^4$
	Ion generator	$1.1x10^4$	$5.8x10^3$	$4.1x10^2$	$2.5x10^2$
	Propionate	$1.0x10^4$	$4.8x10^3$	$3.8x10^2$	$2.7x10^2$

*Po-1 = poliovirus type 1, EC-6 = ECHO virus type 6, CB-3 = Coxsackie virus type B-3

We did not attempt to confirm this conclusion with rhinovirus type 1A or vaccinia, since the one was inactivated too rapidly and the other apparently not at all. The reovirus was also neglected when we found quite strong indication that silver from the ion generator and propionate were equally effective against influenza virus (Table 4-6). We find in these data nothing which would lead us to prefer one of the silver sources to the other.

Table 4-6. Inactivation of influenza virus type A by 150 ppb of silver, as a function of silver source

Silver source	PFU ml^{-1} at 0 hr	1 hr	3 hr	24 hr
None	$6.5x10^3$	$7x10^3$	$7x10^3$	$8x10^3$
Ion generator	$5x10^3$	$5.2x10^3$	$2.3x10^3$	5
Propionate	$6x10^3$	$4.8x10^3$	$3.1x10^3$	5

Association of Silver with Virus

We hoped to determine what silver ions do to a virus. We had

found great differences in silver susceptibility among the viruses surveyed, and it seemed that this might be related to the ability of the ions to associate with the virus particles. This hypothesis was especially attractive because the two least susceptible viruses (vaccinia and influenza), among those tested, have lipid envelopes and might reject ions. The test should have involved "cleaning up" the virus suspension, incubating it with silver, and cleaning it up again for NAA assay. Centrifugation and washing on membrane filters were the available clean up procedures.

Vaccinia which had been washed in an Amicon Diaflo apparatus was incubated for 24 hr in suspensions of 0 and 250 ppb of silver. Again, no inactivation attributable to the silver was detected. Silver suspension (5 ml) with and without virus was centrifuged 1 hr at 3,000 rev/min. The sediment was resuspended in 5 ml of deionized water, centrifuged the same way, and resuspended in another 5 ml of deionized water. The suspension with virus assayed 461 ng of silver, and the one without virus had 0 ng. This suggested that nearly 20% of the silver had associated with virus.

Influenza virus, Po-1, and rhinovirus 1A were used in further, similar experiments. These viruses were recovered from the silver suspensions either by filtration with small-pore membranes or by very high speed (50,000 rev/min $\sim$ 275,000 xg) centrifugation. The signal-to-noise ratio in these experiments was very poor, and we never were able to demonstrate significant association of silver with the viruses. Inactivation of virus by silver was seen in each instance, but silver uptake could not be measured. The measured uptake of silver by vaccinia virus was not accompanied by inactivation. Therefore, we failed to demonstrate a correlation between the two phenomena.

Water Purity

We intended originally to work only with virus in very pure water (< 1 ppm total solids). However, when it appeared that we would not be able to demonstrate the mode of action of silver in inactivating virus, we decided to take a pragmatic look at the constraints on the process. Five gradations of water purity were used in determining the dependence of the silver activity on the absence of other substances. These included water from a Millipore "Super-Q" Purifier (~ 8 Mohm), from a Barnstead Bantam Mixed-bed Deionizer (1-2 Mohm), from our building's distilled water line, from the Madison tap system, and from the Ohio River near Pittsburgh.

Influenza virus type A is the least silver sensitive among the model viruses which were inactivated at all. Over the range of impurities present in our survey, the differences in silver inactivation were not significant (Table 4-7). Silver ions could as well have been used to disinfect tap water as ultrapure water contaminated with influenza virus.

Table 4-7. Water purity has little effect on inactivation of influenza virus type A by silver at 250 ppb

Water source	PFU ml^{-1} at 0 hr		PFU ml^{-1} at 24 hr	
	0ppb Ag	250 ppb Ag	0ppb Ag	250 ppb Ag
Super-Q	2.2×10^4	2.3×10^4	2.0×10^4	7
Deionizer	2.1×10^4	2.2×10^4	2.0×10^4	4
Tap	2.0×10^4	2.1×10^4	2.1×10^4	5

CB-3 gave similar results (Table 4-8). The degree of inactivation in 4 hr was comparable to that seen previously (cf. Table 4-5) for each grade of purity down to tap water. Ohio River water caused significant inhibition of the silver effect. The plaque counts for the 4 hr sample were too great at the dilutions tested; this is the reason for the uncertainty shown in the table value. The data indicated to our satisfaction that some inactivation due to silver had occurred, though decidedly less than 90%. The Ohio River water used here was potentially potable raw water which had had no treatment, other than settling, between the time of collection and the experiment.

Table 4-8. Only very impure water reduces inactivation of CB-3 by 250 ppb of silver

Water source	PFU ml^{-1} at 0 hr		PFU ml^{-1} at 4 hr	
	0ppb Ag	250ppb Ag	0ppb Ag	250ppb Ag
Super-Q	1.4×10^4	1.4×10^4	1.4×10^4	2.0×10^2
Deionizer	1.2×10^4	1.3×10^4	1.2×10^4	1.9×10^2
Tap	1.2×10^4	1.4×10^4	1.4×10^4	1.6×10^2
Ohio River	1.3×10^4	1.3×10^4	1.3×10^4	$\geq 2.8 \times 10^3$

Reovirus type 1 had been seen (in an experiment to be reported in a later section) to be inactivated more rapidly in the absence of silver than we thought appropriate. We incubated reovirus for 3 hr at room temperature in deionized, distilled, and tap water; phosphate-buffered saline; and M/15 phosphate buffer (pH 7.5). Among these, only the deionized water (from a mixed-bed cartridge in a Barnstead Bantam deionizer) caused significant inactivation. The loss in this instance was > 90% in 3 hr. A smaller, confirming experiment was done, with approximately the same result: inactivation in 3 hr in tap water (no silver) was 25%, in distilled water - 20%, and in deionized water - 92%.

We don't consider these figures to be accurate to two digits, but we think it is clear that the deionized water was uniquely antiviral. This was not the result of anything as simple as acidity: the pH of the deionized water was measured at 7.2 to 7.5. The effect was uniquely directed to reovirus. Every other model virus included in the project showed no inactivation when incubated with this deionized water. At about this time, our Millipore Super-Q apparatus was at last put into service. The cartridge in the Barnstead unit was changed also, since the previous cartridge had been used for several months. We then found that reovirus was not inactivated by water from either of these sources and that the action of silver on the virus was not affected, either (Table 4-9). We never did find the exact cause of the problem. It seems likely to have come with the build-up of microorganisms on the resins in the deionizer cartridge. Something non-ionic (we monitored all collections with a conductivity meter) in the effluents from this cartridge evidently would inactivate the reovirus. It is not surprising that reovirus was unusually susceptible to this: the coat protein of the reoviruses is unique in some ways and might, therefore, be affected differently than other viruses by the hypothetical inactivating substance.

Table 4-9. Water purity has little effect on inactivation of reovirus type 1 by 250ppb of silver

Water source	PFU ml^{-1} at 0 hr		PFU ml^{-1} at 3 hr	
	0ppb Ag	250ppb Ag	0ppb Ag	250ppb Ag
Super-Q	1.2×10^4	1.2×10^4	1.3×10^4	21
Deionizer	1.2×10^4	1.2×10^4	1.3×10^4	20
Tap	1.2×10^4	1.4×10^4	1.3×10^4	22

Rhinovirus type 1A was the only model agent, inactivation of which was inhibited by tap water. Given the rate at which silver acted upon this virus, we had to go to considerable lengths to demonstrate this effect. First, only 50 ppb of silver were used. Second, only the silver-free control suspensions were sampled at zero time because we had been unable to detect this virus in the presence of silver previously. Third, the silver-containing suspensions were sampled at 15 min and the controls at 30 min, and this concluded the experiment. Starting with just over 10^4 PFU ml^{-1} of virus in Super-Q, deionized, and tap water, no infectivity was detected after 15 min with 50 ppb of silver, except in the tap water ($\sim$ 16 PFU ml^{-1}). Inactivation in 30 min in the absence of silver was negligible. This indicates that our tap water was a little bit inhibitory to a little bit of silver, but not very.

Biological Impurities

The results reported above demonstrated that the water need not be ultrapure for silver to be active against viruses: so much for the impurities that might be present in the water originally. However, virus contamination, if it should occur, would result in the introduction of some different impurities. These are the biological (body) products in which the virus is shed by the infected individual.

Feces would be the natural suspending agent for the enteroviruses and reoviruses, which seem to be shed principally or exclusively from the intestines. Though CB-3 had been selected to represent the enteroviruses, it was not available to us as shed in feces. We did have frozen some feces from infants who had received trivalent oral poliovirus vaccine and were shedding one or more of the virus types. We had selected those specimens whose titer exceeded 10^6 PFU ml^{-1} but had not bothered to determine which types of polioviruses they contained. Poliovirus type 1 (Po-1), strain CHAT, produced in PMK cell cultures, served as the comparison. The feces were suspended at 1% ($^W/v$) in deionized water, and the virus from PMK cells was simply diluted 10^{-4} in deionized water or was washed in the Diaflo filter apparatus after dilution. The experimental results were not extremely orderly, but they did indicate that only washed virus was susceptible to inactivation by silver ions (Table 4-10).

Table 4-10. Effects of virus source and purity upon inactivation of poliovirus by silver ions

Virus		Silver	PFU ml^{-1} at	
Source	Purification	(ppb)	0 hr	4 hr
Feces	0	0	9.5×10^3	1.6×10^4
		250	9.5×10^4	1.4×10^4
PMK cells	0	0	5.9×10^4	5.9×10^4
		250	9.5×10^4	2.1×10^4
	+	0	3.5×10^4	9.5×10^4
		250	8.2×10^4	6.2×10^1

The experiment was repeated, except that two samples were taken from each suspension each time and were assayed independently. The agreement between duplicate assays was not good, but the results of the previous experiment were essentially confirmed (Table 4-11). The inability to inactivate tissue culture virus which had not been purified is significant only as it shows that our purification

process was necessary and adequate. There is no reason to believe that the inhibitory components of the tissue culture suspension are the same as those in feces.

Table 4-11. Effects of virus source and purity upon inactivation of poliovirus by silver ions — second experiment

Virus Source	Virus Purification	Silver level (ppb)	PFU ml^{-1} at 0 hr	PFU ml^{-1} at 4 hr
Feces	0	0	6.0×10^4, 7.3×10^4	3.6×10^4, 4.0×10^4
		250	8.0×10^4, 8.2×10^4	6.0×10^4, 7.3×10^4
PMK cells	0	0	4.0×10^4, 5.5×10^4	2.7×10^4, 6.0×10^4
		250	6.4×10^4, 5.5×10^4	3.0×10^4, 2.0×10^4
	+	0	3.0×10^4, 3.0×10^4	4.0×10^4, 2.0×10^4
		250	3.6×10^4, 2.0×10^4	6.0×10^2, 6.2×10^2

The effect of feces was not easy to explain. We had thought that the fecal virus might be aggregated, but neither sonic treatment nor mechanical agitation with glass beads raised the infectivity titer so as to confirm this. When feces were added to purified Po-1 plus silver, the virus was completely protected for 4 hr at nominal fecal concentrations from 10^{-1} to 10^{-7} (Table 4-12). Taken at face value, this finding indicates that the effect of silver ions upon Po-1 could be prevented by less than one weight unit of fecal solids per weight unit of silver.

A series of three experiments was intended to give some hint of what the feces were doing. The first suggested that Po-1 inactivated by silver might be reactivated by adding feces. The second indicated that feces, added later than the silver, probably just stopped the inactivation process quite promptly. The third indicated that feces at 10^{-7} concentration would protect Po-1 from as much as 2,500 ppb of silver added after the feces. The data from these experiments were not concise enough to permit conclusions to be drawn very firmly. Rather than just repeat the experiments, we changed tactics.

A series of four experiments was performed to get some idea of the character of the active component of the feces. Six ml of a 1% fecal suspension were dialyzed against 8 ml of 250 ppb silver

propionate solution for 24 hr at room temperature (two replicates). NAA assay indicated that the silver had essentially equilibrated through the dialysis tubing, as it had in two comparable assemblies from which feces had been omitted. Po-1 was then added to the 8 ml portion. In the control (no feces) assemblies, 99% inactivation was obtained within 4 hr. Only 18% (not significant) inactivation occurred in the others (fecal dialysate plus silver). In the second experiment, virus was incubated in fluids from both sides of the dialysis tube. This time, fecal inhibition of the silver effect was not complete, but the inhibitory component of the feces evidently had equilibrated through the dialysis tubing during the 24 incubation.

Table 4-12. Feces inhibit inactivation of Po-1 by 250 ppb of silver ions (two experiments)

Silver source	Feces(%)	PFU ml^{-1} at 0 hr	PFU ml^{-1} at 4 hr
Ion generator	10	5.3×10^4	5.6×10^4
	1	3.8×10^4	5.3×10^4
	0.1	3.6×10^4	4.4×10^4
	0.01	3.5×10^4	4.7×10^4
	0.001	5.2×10^4	4.9×10^4
	0	3.7×10^4	3.6×10^2
Propionate	0.001	1.9×10^4	1.6×10^4
	0.0001	1.5×10^4	2.0×10^4
	0.00001	1.8×10^4	1.9×10^4
	0	1.9×10^4	7.2×10^2

The results of the two experiments just described showed that some of the protective activity of feces would pass through a dialysis membrane. However, the starting concentration of these fecal suspensions (1%) had been 100,000 times greater than the lowest that we had shown would protect Po-1 from silver, so we didn't know how much of the activity was dialyzable. Six ml of a 2% fecal suspension were placed in a dialysis bag and dialyzed against 100 ml of deionized water in the refrigerator. The outer water was changed twice daily for 7 days. At the end of that time, the residue within the bag (which looked just as it had initially) was mixed with an equal volume of silver solution to give final concentrations of 1% feces (equivalent) and 250 ppb of silver. The titer of a Po-1 suspension with no silver or feces decreased 0.09 $\log_{10}$ in 4 hr at room temperature, while a suspension with 250 ppb

of silver decreased 1.53 $\log_{10}$. An equal level of silver inactivated 1.62 $\log_{10}$ of Po-1 in the presence of the fecal residue after a week's dialysis, whereas a comparable suspension of feces, held in the refrigerator for a week without dialysis, allowed only ~0.14 $\log_{10}$ of silver inactivation. This indicates that essentially all (>99.999%) of the protective substance in feces is dialyzable and, therefore, that most (on a weight basis) of the fecal solids are probably not active in protecting Po-1 from silver.

Each of these dialysis experiments had been done with quite high levels of feces, though feces at levels as low as 10^{-7} g ml^{-1} would protect against 250 ppb of silver. A further experiment was done in which the concentration of feces was initially 10^{-7} g ml^{-1}. Six ml of this suspension were placed inside of a small dialysis bag, and 8 ml of 250 ppb silver (as propionate) were placed outside the bag. After 24 hr at 5C, the fluids were collected and inoculated with poliovirus type 1 (Po-1). The same level of silver, in the control suspensions, inactivated > 99.5% of the virus in 4 hr at room temperature. The suspension from inside the tubing, which contained the feces and whatever of the silver had diffused in, inactivated 90% of the virus. The outer suspension, which contained the silver and whatever of the feces had diffused out, inactivated ~ 95% of the Po-1 in 4 hr. Thus the feces, under these conditions, prevented $\geq$ 1 $\log_{10}$ of inactivation on both sides of the dialysis tube. We conclude that the inside and outside suspensions had almost reached equilibrium during the 24 hr incubation.

We also tried agar as an "antidote" for silver, thinking that it might be useful in control suspensions in experiments with feces. Noble agar (Difco) was used initially because it is in quite common use in our laboratory. The results of four experiments with it have indicated that it affords protection erratically, and usually not to as low concentrations as feces. A further survey, using 0.1% concentrations of Difco Bacto and Purified agars, Oxoid Ionagar No.2, and potato dextrose agar, showed that all afforded significant protection. Significant silver inactivation occurred only in the presence of 0.1% Difco Purified agar and Oxoid Ionagar No.2. Both of these agars are supposed to have been extensively extracted in preparation, so it may be that some active substances had been leached out. A dialysis experiment, similar to the last described above, was done with 1% Noble agar and with 1% agarose suspensions instead of feces. The active component of each was apparently able to pass through the dialysis membrane and inhibit the silver ions on the other side. It was concluded that agar was too complex and variable a substance to be of help with the feces mystery.

Whatever the mode of action, it was very clear that feces at as low a concentration as 10^{-7} g ml^{-1} would protect poliovirus type 1 (Po-1) from 250 ppb of silver ions. We wished to know if the same

was true of other enteroviruses, so we did eight more experiments with poliovirus types 2 (Po-2) and 3 (Po-3), Coxsackie virus types A-9 (CA-9), B-2 (CB-2), and B-3 (CB-3), and ECHO virus type 6 (EC-6). All showed varying degrees of protection, except CB-3 in the first (but not the second or third) experiment (Table 4-13). One might conclude that this is what we get for trying to repeat an experiment. All of these experiments were done with the same silver propionate solution and the same fecal suspension, but in different weeks.

Table 4-13. Enteroviruses are protected from silver inactivation by various levels of feces

Virus type[1]	Initial titers (range)	Inactivated in 4 hr: Controls[2] 0ppb	Controls[2] 250ppb	Feces[3] ($g\ ml^{-1}$) 10^{-3}	Feces[3] ($g\ ml^{-1}$) 10^{-5}	Feces[3] ($g\ ml^{-1}$) 10^{-7}
Po-2	4.15-4.32[4]	0.11[5]	1.89	0.12	0.16	0.18
Po-3	3.90-4.00	0.08	2.04	0.05	0.56	1.64
CA-9	4.23-4.32	-0.02	3.42	0.86	0.69	0.78
CB-2	4.04-4.15	0.12	1.37	0.14	0.16	0.97
CB-3	4.34-4.40	0.25	1.86	1.77	1.83	1.75
"	3.98-4.08	0.27	1.39	0.50	0.49	0.66
"	4.34-4.45	0.06	1.98	1.14	1.44	1.66
EC-6	4.78-4.85	0.37	1.74	0.50	0.44	0.52

[1]Po-2 = poliovirus type 2; Po-3 = poliovirus type 3; CA-9 = Coxsackie virus type A-9; CB-2 = Coxsackie virus type B-2; CB-3 = Coxsackie virus type B-3; EC-6 = ECHO virus type 6

[2]Ag ion levels in ppb

[3]Each of these suspensions also contained 250 ppb of Ag ions

[4]$\log_{10}$ (PFU ml^{-1})

[5]Decrease in $\log_{10}$ (PFU ml^{-1})

Reovirus type 1 (Re-1) was reported in a previous section to be more silver-sensitive than the enteroviruses. That conclusion was based upon the results with 0% feces that are shown in Table 4-14. We also found that 0.1% feces was extremely protective against 50 ppb of silver, but only negligibly protective against 250 ppb. This is significant, for reoviruses are as likely to be shed in feces as are enteroviruses. This was the experiment alluded to previously, in which inactivation in the absence of silver appeared to

be excessive.

Table 4-14. Interaction of silver ions and feces with reovirus type 1

Silver (ppb)	Feces (%)	PFU ml^{-1} at 0 hr	1 hr	3 hr
0	0	1.2×10^4	1.1×10^4	1.9×10^3
	0.1	1.3×10^4	1.3×10^4	1.6×10^3
50	0	1.4×10^4	5.0×10^2	27
	0.1	1.4×10^4	5.0×10^3	1.9×10^3
250	0	1.4×10^4	60	< 10
	0.1	1.4×10^4	1.8×10^2	< 10

Rhinoviruses are not shed in feces. However, they are closely related to the enteroviruses (both belong to the picornavirus group), so it seemed that they might also be protected from silver by feces. We tried to include all possibilities in a single experiment. The initial level of silver was 50 ppb, so that some infectivity could be detected. The level of feces was 1%, which would be far more than enough to protect an enterovirus or reovirus. Another 500 ppb of silver were added at 15 min to test the durability of any apparent protection. Within the limits of experimental error, no protection was seen (Table 4-15).

Table 4-15. Interaction of silver ions and feces with rhinovirus type 1A

Silver added (ppb) at 0 min	15 min	Feces (%)	PFU ml^{-1} at 0 min	12 min	20 min	60 min
0	0	0	2.7×10^3	—	—	1.5×10^3
50	500	0	25	22	4	< 1
		1	28	23	11	< 1

Mucus is the body product in which the respiratory viruses are shed. If respiratory virus contaminated a water system, one would expect it to be accompanied by mucus. Bovine respiratory mucus, which had been boiled to remove nonspecific antiviral activity, had been used as a carrier for influenza virus in another study. When included here, it appeared to have no significant effect upon inactivation of influenza virus type A by silver ions (Table 4-16).

Table 4-16. Mucus does not protect influenza virus type A from silver ions

Silver (ppb)	Mucus (%)	PFU ml^{-1} at 0 hr	PFU ml^{-1} at 24 hr
0	0	2.1×10^4	2.4×10^4
250	0	1.9×10^4	1.5×10^2
	1	1.8×10^4	2.3×10^2
	10^{-1}	2.1×10^4	1.8×10^2
	10^{-2}	1.7×10^4	2.0×10^2
	10^{-3}	2.5×10^4	2.2×10^2
	10^{-4}	2.3×10^4	2.5×10^2
	10^{-5}	2.2×10^4	1.6×10^2

The other respiratory virus in the present study is rhinovirus type 1A. When an experiment similar to that reported in Table 4-16 was attempted with this agent, the mucus exerted a strong antiviral effect. We have been informed (Dr. George Kenny, University of Washington School of Medicine; personal communication) that there are polyanions in respiratory mucus to which type 1A is unusually sensitive, even for a rhinovirus. Po-1 was selected as a surrogate. Over the range of concentrations employed in the previous experiments (1% to 10-5%), bovine respiratory mucus had absolutely no effect upon the inactivation of Po-1 by silver. Since Po-1 was protected by miniscule quantities of feces, it appears that the mucus has no comparable active component.

Metal Surfaces

Assuming that the water in a system is pure initially, and that no impurities of the kinds just considered have been introduced, there are still container effects to be considered. Ultrapure water is quite an efficient solvent, and it seems to draw some impurity from virtually any surface with which it comes in contact. Water systems seem usually to be constructed principally of metal. Because we could not anticipate all of the alloy compositions and surface treatments which might be employed, we concentrated on tests with as pure metals as possible. The fluid volume in these experiments was 10 ml, and the available metal surface was adjusted to as near $10 cm^2$ as possible.

CB-3, the primary model enterovirus, was used in most of these experiments. The first included silver propionate (250 ppb of silver), but all others were done with Ag_2S (150 ppb of silver). A survey, which should be regarded more as qualitative than quantitative, is reported in Table 4-17. Only one metal was essentially inert: the lead had no direct effect upon the virus and little or

no influence upon inactivation of the virus by silver. The aluminum foil and the iron wire had little direct effect upon the virus, but they seemed to protect it significantly against silver ions. Tin foil and iron filings (0.5 g, rather than 10cm^2 of surface, in this instance) showed a direct antiviral effect which which was not additive with that of the silver. Copper, silver, cadmium, and magnesium showed a direct antiviral effect which appeared to be additive to that of the silver ions. These differences seemed to depend upon whether ions of the metal being tested were leached into solution, whether these ions had antiviral activity, and whether the metal surface would "plate out" silver ions from the suspension.

Table 4-17. Inactivation of CB-3 by silver ions proceeds differently in contact with different metals

Silver (ppb)	Metal present	PFU ml^{-1} at 0 hr	PFU ml^{-1} at 4 hr
0	None	1.7×10^4	1.5×10^4
	aluminum foil	1.6×10^4	1.7×10^4
250	None	1.8×10^4	1.2×10^2
	aluminum foil	1.6×10^4	6.1×10^3
0	None	1.1×10^4	1.1×10^4
	copper foil	1.2×10^4	29
150	None	1.2×10^4	3.1×10^2
	copper foil	1.1×10^4	15
0	None	1.2×10^4	1.3×10^4
	silver plate	1.3×10^4	1.9×10^2
	cadmium stick	1.3×10^4	2.9×10^2
	lead foil	1.2×10^4	1.3×10^4
	tin foil	1.2×10^4	2.9×10^3
150	None	1.2×10^4	1.5×10^2
	silver plate	1.3×10^4	45
	cadmium stick	1.3×10^4	62
	lead foil	1.4×10^4	3.3×10^2
	tin foil	1.3×10^4	2.7×10^2
0	None	1.1×10^4	1.2×10^4
	iron filings	1.2×10^4	2.1×10^2
150	None	1.3×10^4	3.9×10^2
	iron filings	1.3×10^4	2.0×10^2
0	None	1.2×10^4	1.3×10^4
	iron wire	1.2×10^4	1.1×10^4
150	None	1.2×10^4	1.6×10^2
	iron wire	1.2×10^4	3.7×10^3
0	None	1.6×10^4	1.5×10^4
	magnesium turnings	1.5×10^4	90
150	None	1.6×10^4	1.5×10^2
	magnesium turnings	1.5×10^4	< 1

Aluminum was a metal in which we were particularly interested. In line with the preceding hypothesis, we thought probably that the silver ions were plating on the aluminum surface (perhaps by an exchange reaction) rather than that aluminum ions had any direct protective effect upon the virus. This should mean that there would be little or no inactivation if the aluminum and the silver suspension had been reacted before the virus was added. Such was not the case. Aluminum foil pieces ($10cm^2$ total surface) were soaked in 10 ml of ultrapure water and in 10 ml of 150 ppb silver suspension for 4 hr. The CB-3 was not added to these until after the aluminum foil had been removed. The pretreatment with aluminum foil apparently had not affected the antiviral potency of the silver suspension (Table 4-18). We have no alternate hypothesis to explain this.

Table 4-18. Pretreatment with aluminum foil did not affect the antiviral activity of a silver suspension against CB-3

Silver (ppb)	Aluminum pretreatment*	PFU ml^{-1} at 0 hr	PFU ml^{-1} at 4 hr
0	0	1.1×10^4	1.3×10^4
	+	1.2×10^4	1.1×10^4
150	0	1.2×10^4	80
	+	1.3×10^4	73

*See text for details of pretreatment.

Metallic magnesium could be seen reacting with water in which it was immersed. We hoped that longer treatment would cause some "passivation" of the surface. It did not. Three days' soaking in water, with a water change daily, did not make the metal any less visibly reactive when immersed in fresh water or 150 ppb silver suspension. If anything, the antiviral effect was somewhat greater than that in the experiment reported in Table 4-17 (Table 4-19).

Table 4-19. Pre-soaked magnesium has a strong antiviral effect against CB-3

Silver (ppb)	Magnesium present	PFU ml^{-1} at 0 hr	PFU ml^{-1} at 4 hr
0	0	1.1×10^4	1.2×10^4
	+	1.0×10^4	< 1
150	0	1.2×10^4	60
	+	1.1×10^4	< 1

The interaction of two metals in the presence of silver ions was also of interest. Using 10cm^2 (total surface) of copper foil and of aluminum foil, we found that the aluminum protected against both the silver ions and (what we suppose to be) copper ions (Table 4-20).

Table 4-20. Aluminum mitigates the antiviral activity of silver and copper against CB-3

Silver (ppb)	Aluminum foil	Copper foil	PFU ml^{-1} at 0 hr	PFU ml^{-1} at 4 hr
0	0	0	1.2×10^4	1.3×10^4
		+	1.2×10^4	37
	+	+	1.1×10^4	5×10^3
150	0	0	1.3×10^4	82
		+	1.3×10^4	51
	+	+	1.2×10^4	1.0×10^4

The metals acted somewhat differently with silver suspension in tap water (Table 4-21). Aluminum showed some antiviral activity of its own and was less protective against silver. The performance of the copper probably did not differ significantly from that shown in Tables 4-17 and 4-20, but the lead showed somewhat more protection against the silver than it had previously. These differences probably are entirely due to the change of water: the results in Table 4-17 have been largely confirmed with another enterovirus.

Table 4-21. Inactivation of CB-3 by silver ions in tap water is also influenced by contact with metals

Silver (ppb)	Metal present	PFU ml^{-1} at 0 hr	PFU ml^{-1} at 4 hr
0	None	1.0×10^4	1.0×10^4
	Aluminum foil	1.1×10^4	2.7×10^3
	Copper foil	1.0×10^4	91
	Lead foil	1.0×10^4	1.0×10^4
150	None	1.0×10^4	29
	Aluminum foil	1.0×10^4	91
	Copper foil	1.0×10^4	6
	Lead foil	1.1×10^4	3.7×10^2

Po-1 was used as a secondary model enterovirus, with which some of the survey experiments reported in Table 4-17 were repeated (Table 4-22). It should be emphasized that these results are to be interpreted qualitatively. The only possible discrepancies between the two sets of results are those relating to metallic silver and cadmium. The effect of each of these against CB-3 was expressed directly, but it seemed also to be additive with the activity of the silver ions when the latter were present. This additive property was less apparent in the experiments with Po-1. Results with aluminum, copper, lead, and tin foils, as well as magnesium turnings and iron wire, are very similar to those obtained with CB-3. We are not quite sure what all of this proves: we know that ultrapure water tends to become impure very quickly when stored in contact with metals, and this may have a very significant influence on silver inactivation of a contaminating virus.

Table 4-22. Inactivation of Po-1 by silver ions is affected by contact with metals in much the same way as inactivation of CB-3

Silver (ppb)	Metal present	PFU ml^{-1} at 0 hr	PFU ml^{-1} at 4 hr
0	None	1.4×10^4	1.3×10^4
	Aluminum foil	1.5×10^4	1.1×10^4
150	None	1.4×10^4	47
	Aluminum foil	1.4×10^4	3.5×10^3
0	None	1.3×10^4	1.1×10^4
	Silver plate	1.3×10^4	60
	Copper foil	1.4×10^4	1.3×10^2
150	None	1.2×10^4	60
	Silver plate	1.2×10^4	55
	Copper foil	1.3×10^4	2
0	None	1.3×10^4	1.3×10^4
	Lead foil	1.4×10^4	1.1×10^4
	Magnesium turnings	1.4×10^4	40
150	None	1.4×10^4	55
	Lead foil	1.3×10^4	46
	Magnesium turnings	1.4×10^4	< 1
0	None	1.2×10^4	1.1×10^4
	Tin foil	1.1×10^4	9.1×10^2
	Cadmium stick	1.3×10^4	1.5×10^2
	Iron wire	1.3×10^4	1.1×10^4
150	None	1.2×10^4	30
	Tin foil	1.3×10^4	16
	Cadmium stick	1.2×10^4	14
	Iron wire	1.4×10^4	1.9×10^2

Summary of Virological Studies

Only vaccinia (representing the poxviruses), of the model viruses in these studies, appeared to be completely resistant to silver ions. The others tested, in order of increasing silver sensitivity, were influenza virus type A (representing the myxoviruses), several enteroviruses, reovirus type 1 (representing the reoviruses). and rhinovirus type 1A (representing the rhinoviruses). The time dimension for inactivation of these viruses ranged from days to minutes. The rate of inactivation was similar, whether the silver ions had been added by an electrolytic generator or as a soluble salt. The concentration of silver (in the range of 50 to 250 ppb) was not always the principal rate-limiting factor in inactivation of the virus. We were not able to measure the uptake of silver by the virus particle.

Extremely pure water was not necessary for viruses to be inactivated by silver ions. However, feces (or a dialyzable component of feces) were extremely effective in preventing silver inactivation of enteroviruses and, to a lesser extent, reovirus. Respiratory mucus did not show this sparing effect. A metal surface in contact with the silver and virus suspension may participate either by adding antiviral ions or by protecting the virus from the silver. The metals tested differed greatly in their effects, but lead was the only one that appeared to be inert.

DISCUSSION

The physical studies have shown us that the electrolytic silver ion generator generates silver ions (though not always) and that ion generator effluents and silver propionate solutions can be stored for long periods in polystyrene containers without measurable loss of silver activity. We have no idea why the ion generators have performed so erratically in our hands. The possibilities that water impurities, particulate contaminants, or inadequate voltage might be to blame have all been tested and discarded by now. When the Millipore Super-Q water purifier was put into service, we began to operate the ion generator continuously, as had been suggested to us. This did not seem to help.

Since our biological experiments have indicated that silver ions need not come from an ion generator to be effective, other methods of delivery might be considered. The water might be percolated through or around solid Ag_2S, for instance. This compound can be melted and presumably cast, it is 87% silver by weight, and its specific gravity is ~ 7.3. It is soluble to a silver level of 120 to 150 ppb at 20 C, and it might be gotten to higher levels by warming the water. Many other such possibilities surely exist.

We know a great deal more now about neutron activation analysis (NAA) for silver than we did at the outset. Certain limitations have become evident. For instance, there is no way to produce an absolute silver standard on which to base comparisons. We have had to rely upon consensus among two or more methods of measurement in assigning absolute values to our silver standards. Consensus among the various procedures used here has been reasonably good, but we were unable to resolve our differences with the measurements made at MSC. Whether we could have done so if they had sent us the samples we requested is now a moot question.

The NAA procedure used here is a comparator method: after the area under the 659 keV peak for each sample has been "stripped" with that from a zero-silver sample, the net areas under peaks for experimental samples are compared with one for a control containing a known amount of silver. A conventional sample submitted for NAA assay comprised a little bit of silver in (relatively) a great deal of water, and not much else. Here, background and "noise" problems stemmed from the sample vial and the water itself (as H_2O). Low-noise vials minimized the first of these. The noise from the water was reduced to some extent by absorbing thermal neutrons on their way to the sample (during activation), and it appeared that evaporating most of the water before sealing the vial might also have helped.

Our luck with unconventional samples was not as good. We worked with two general classes of unconventional samples - impure

water and filter membranes. In each instance, we could prepare a zero-silver sample for direct-comparison stripping. However, we could not prepare a comparable sample known to contain, say, 1,250 ng of silver. This was because of the uncertainty related to standards, even in pure water, and of the greater difficulty involved in trying to be sure that every impurity present in the experimental samples was equally represented in these "positive controls." It was this problem which led us to abandon the assay of filter membranes and other samples in which we had hoped to be able to measure the association of silver with bacterial cells or with virus particles. This was a great disappointment because it had seemed that, in every other respect, NAA would be the ideal (or only) means of doing such a measurement.

The bacteriological studies have indicated that organisms of the genera associated with spacecraft water supplies are killed by silver ions at room temperature. Bacterial concentrations in these experiments have ranged from initial levels of 10^4 to nearly 10^8 cells ml^{-1} in different experiments. Silver concentrations have been intended to be 50 or 250 ppb. It had already been noted that, at 10^4 cells ml^{-1} and 50 ppb of silver, there are $\sim 2.8 \times 10^{10}$ silver ions per cell. This is a commentary on the use of the term "oligodynamic." In the most extreme situation (10^4 cells ml^{-1} with 250 ppb of silver), if one estimates the dry weight of a bacterial cell at 2.5×10^{-13}g, there should actually be more than one silver ion in the system for every atom (other than those in water) in every bacterial cell. At the other extreme (10^8 cells ml^{-1} and 50 ppb of silver), the ratio of weight of silver to dry weight of cells should be approximately 0.002. These are, of course, only extremes within the constraints imposed by our chosen experimental conditions. Though the source of the bacteria that have been found in spacecraft water systems is not known, there is no reason to suppose that contamination occurs initially at a level of at least 10^4 cells ml^{-1}.

The range on which bacterial concentrations might vary is much more extensive than that for the silver ions, assuming that the bacteria can find any substrate on which to multiply in the spacecraft water system and are not immediately suppressed by the silver. They evidently have found an adequate substrate on occasions in the past, and the substrate has not been identified. Thus, while the silver might well affect the organisms' ability to use the substrate, the substrate might also influence the silver's ability to act upon the organisms. In the light of these considerations, it is surprising how limited a variety of results has been obtained. Viable cells were usually recoverable for at least an hour after the challenge was begun (at room temperature), regardless of the concentration of silver. Except for intensively selected cell strains which might have no counterpart in practice, any level of silver ions that we could measure killed at least some cells. Between these two extremes, there have been some significant differences.

The differences in susceptibility of bacterial cells are as yet totally unexplained. If we knew what silver ions did to the cells they kill, we might have some idea of how the selected strains could be resistant. No matter how rigorously the mode of action of silver might be approached, there will undoubtedly always be some lingering area of uncertainty which is dismissed as "species differences." The total resistance of spores to silver may not be a great problem. However, it does suggest that the silver ion concentration should be maintained at active levels at all points in the water system, so that cells resulting from delayed spore germination would still be killed.

The differences among viruses are more extreme than those among bacterial species. Of the two model viruses which had lipid envelopes, one (vaccinia) was completely resistant to silver, and the other (influenza) was relatively insensitive. The model reovirus and rhinovirus were inactivated very rapidly, and the enteroviruses were inactivated at an intermediate rate. The ratio of weight of silver to weight of virus is often very large. A picornavirus (enterovirus or rhinovirus) particle may have a mass of 7×10^6 daltons, and a plaque-forming unit might represent from 1 to 100 physical particles. This means that a suspension of 10^4 PFU ml^{-1} of picornavirus in 50 ppb silver may have a ratio of 4,000 to 400,000 g of silver per gram of virus.

There are many other ways that viruses might be transmitted among personnel aboard spacecraft than through the water system. In the case of the respiratory viruses, aerosol or so-called contact transmission seems more likely. Water is a possible vehicle for intestinal viruses, such as the enteroviruses and reoviruses. It is, therefore, a rather hopeful finding that viruses of both groups are inactivated fairly rapidly by silver ions. On the other hand, it is a matter of some concern that the enteroviruses, especially, are protected by such minute quantities of feces. It seems rather unlikely that enterovirus contamination would occur without at least some fecal material being present as well.

The finding that aluminum ions or foil may interfere with the antimicrobial action of silver ions is noteworthy. The aluminum foil was the kind one buys in the grocery store and had not been anodized or passivated, so our results just might be irrelevant. However, water from the Super-Q purifier seems to have extreme solvent properties (it got enough silver ions into it from silver foil to cause significant antiviral action), so passivation may not preclude contact effects with an aluminum surface. This suggestion may be supported (and one possible solution offered) by the finding that the aluminum foil did not protect virus against silver ions in tap water. That is, the system might behave more stably and predictably when charged with water of moderate, rather than extreme, purity. If valid, this suggestion might influence other aspects of the design of spacecraft water systems.

SUMMARY

Bacteria and viruses in very pure water were treated with silver ions (added as salts or by electrolysis of elemental silver). The silver in the suspensions was assayed by neutron activation analysis, using the 659 keV gamma peak emitted by the ^{110}Ag nuclide. Bacteria were enumerated by colony counts, and viruses were quantitated by the plaque technique in tissue cultures.

The bacteria tested were principally of the genera Achromobacter, Alcaligenes, Flavobacterium, and Pseudomonas. Each test species was killed by silver both at nominal 50 and 250 ppb concentrations, but some were apparently more susceptible than others. The rate of killing was found to vary with cell numbers, with silver level, and with water temperature. Spores of Bacillus cereus were not affected by silver ions, but the vegetative cells were killed rapidly. Silver apparently is bound to the cells during the killing process. Silver resistant strains of two species were obtained by a very rigorous selective process, but this occurrence may have no counterpart in practice. In general, silver ions appear to have broad and useful antibacterial activity. Chloride and aluminum ions, which interfere with the silver, must be held to extremely low levels.

Vaccinia was the only one of several model viruses which was not inactivated significantly by silver ions. Inactivation of agents from four other virus groups proceeded at rates measurable in from minutes to days. Several model enteroviruses were inactivated by silver but were protected by very minute quantities of feces. Silver ions in very pure water were inhibited by contact with metallic aluminum. The significance of this finding to the design of spacecraft water systems is not certain.

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