First Progress Report

Aerosol Metabolism
Detection Tests

Contract No. 329380

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Naval Biomedical Research Laboratory
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BIOSPHERICS INCORPORATED
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Enclosure 1 to Annual Report, W-13,450,
Studies on possible propagation of microbial
contamination in planetary clouds.
Dimmick, R.L. and M.A. Chatigny.
15 January 1973
Continuing scientific exploration of outer space has resulted in much data concerning the physical and chemical atmospheres of the planets and stars. Some cosmologists believe that some of these atmospheres may be capable of providing an environment for native life forms which live out their life cycle completely "airborne." Some extraterrestrial atmospheres are so compatible with tolerances and needs of terrestrial microbes that it has been speculated that these atmospheres may be liable to infection by microorganism shedding scientific probes.

Since this contamination could impact native abiogenic or biogenic evolutionary patterns, researchers at the University of California proposed that a series of experiments be conducted to test the possibility that bacteria may survive, metabolize and reproduce in airborne droplets.

The approach was to aerosolize bacteria and $^{14}$C glucose into a revolving tank designed to keep particulates airborne and to monitor for metabolically produced $^{14}$CO$_2$. Biospherics Incorporated was retained to supply technical expertise in radiorespirometric methods, and, during several visits to Naval Biomedical Research Laboratory (NBRL), designed and conducted the experiments described herein.
SITE VISIT NO. 1

On 27 through 29 November 1972, Dr. Gilbert V. Levin and Mr. George J. Topol met with Mr. Mark Chatigny and Drs. R. Dimmick and H. Wolochow at NBRL. Equipment and facilities were inspected. It was felt that available sampling equipment was adequate. Hence, no hardware fabrication was planned. The design of experiments was discussed in detail. An experimental protocol (see Appendix I) was subsequently drawn up by Dr. Levin.

SITE VISIT NO. 2

On 4 January 1973, Dr. J. R. Schrot of Biospherics met with Mr. Chatigny and Dr. Dimmick of NBRL and the experimental phase was initiated per the protocol. Dr. Levin joined Dr. Schrot at NBRL on 5 January 1973 and the procedure was scrutinized in the light of results obtained. Several technical difficulties were encountered:

1. The $^{14}\text{C}$ glucose solution had lost radioactivity during storage, probably because of contamination. Also, losses during injection into the tank were such that higher levels of radioactivity than originally planned were clearly needed.
2. Mixing of $^{14}$C glucose and the bacterial suspension in the atomizer prior to aerosolization was viewed as a possible source of false positive results. Even though this contact period was brief, considerable metabolic activity could occur in the liquid phase producing radioactive gas indistinguishable from that produced by metabolic activity in the aerosol.

3. The extensive apparatus used for aerosol input and sampling could serve as surfaces for bacterial activity in deposited films of the medium. Bypassing some of this apparatus appeared to be desirable and possible.

4. It was felt that the addition of baffles to the tank would be desirable to assure uniform mixing of the relatively small injection volume.

5. The rinse regimen prescribed in the protocol was found to dilute the radioactivity too much for subsequent counting.

6. Liquid scintillation counting efficiencies for cocktails used were not known. The Unisol
cocktail was also not suitable for aqueous solutions. The liquid scintillation instrument (Packard Tri-Carb) required recalibration for the proposed use.

SITE VISIT NO. 3

From 23 through 26 January 1973, Drs. Patricia Ann Straat and J. R. Schrot conducted experiments at NBRL in cooperation with Drs. R. Dimmick and H. Wolochow and, Mr. Gard Forrster of NBRL. Dr. Straat remained on site and continued these experiments until 31 January 1973.

Attempts were made to resolve the problems described. The following modifications were implemented:

1. Fresh stocks of glucose were obtained and the isotopic level was verified. An adequate specific activity was selected for the final mixture.

2. Separate atomizers were used for bacterial culture and for the glucose solution. Simultaneous atomization of cells and $^{14}$C glucose was conducted and the two aerosol streams were mixed in a one-inch diameter by four-foot length of copper tubing.
3. Much of the installed input mixing apparatus was bypassed.

4. Baffles were installed in the tank.

5. A series of determinations was performed to establish counting efficiency for each of several counting systems used. These efficiencies are given in Table 1 of Appendix II. All counting was done on a Beckman two-channel liquid scintillation counter equipped with a cesium external standard.

Three separate experiments were conducted as follows:

**EXPERIMENT NO. 1**

Bacterial cells and $^{14}$C glucose (approximately 1.3 ml of 55 $\mu$Ci/ml, 0.1%) were atomized separately, but simultaneously. Fill time - 10 minutes. Samples were withdrawn from the tank after 10 minutes, 2 hours, 4 hours and 21 hours. The protocol established in Appendix I was followed in other respects.

**EXPERIMENT NO. 2**

Bacterial cells and $^{14}$C glucose (approximately 0.3 - 0.5 ml, 55 $\mu$Ci/ml, 0.1%) were atomized separately and simultaneously for 6 minutes, whereupon the glucose atomizer was found to be without sufficient liquid volume
for proper operation. The situation was quickly remedied by addition of unlabeled glucose solution. The concentration was made up to 1% to facilitate the formation of particulates of approximately 1 μ diameter. Atomization was continued for an additional 10 minutes using this glucose solution which now contained approximately 30 μCi/ml of radioactivity. Samples were withdrawn from the tank after 15 minutes, 2 hours, 1-hour and 24 hours.

**EXPERIMENT NO. 3**

\(^{14}\text{C} \) glucose (approximately 0.4 - 0.8 ml, of 30 μCi/ml, 1%) was atomized into the tank. No bacteria were aerosolized for this experiment. The \(^{14}\text{C} \) glucose solution was shown to contain little or no \(^{14}\text{C} \text{CO}_2 \) at the time of aerosolization. Samples were withdrawn from the tank after 10 minutes, 3 hours, 6 hours and 22 hours.

Results of these experiments are shown in Tables 2 through 4 of Appendix II. They may be summarized as follows:

1. The radioactive samples collected by the bubbler show variable and inconsistent results. This in part can be attributed to leaks within the collection system.
2. An analysis conducted in conjunction with Experiment No. 3 was done to test whether material in addition to $^{14}\text{CO}_2$ would pass through the membrane filter. The digest containing trapped radioactivity was acidified and gettered with a KOH-saturated filter pad. After 90 minutes, most of the radioactivity remained in solution, indicating that most of the bubbler sample is not carbon dioxide but, rather, may represent glucose which passed through the filter.

3. During the course of these experiments, it was established that four rinses were necessary to remove all soluble glucose from the membrane filter. (Separate tests determined that these rinses contained little dissolved $^{14}\text{CO}_2$). After establishment of this procedure (at the end of Experiment No. 1), little radioactivity remained on the filter pad.
4. No labeled release activity was detected on the particles remaining on the membrane filter.

5. Impinger data from Experiments No. 1 and 2 indicate a steady fallout of bacterial particles with time. However, as shown in Figure 1, the rate appears to differ in the two experiments.

6. It is apparent that a significant percentage of the total radioactivity recovered is present in the bubbler sample. However, as was shown, little, if any, could be ascribed to carbon dioxide. Total counts recovered in the bubbler sample represent approximately 50% of the total counts recovered in the sampling.

7. If all added radioactivity was distributed evenly into the 500 liter capacity of the tank, each liter withdrawn should contain approximately $3 \times 10^5$ dpm in Experiment No. 1, $2.7 \times 10^5$ dpm in Experiment No. 2.
Figure 1

Decrease in Numbers of Aerosolized Serratia marcescens During Two Separate Tank Experiments
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and $2.0 \times 10^5$ dpm in Experiment No. 3.

The total amount recovered from each liter withdrawn contained approximately 10% - 30% of this amount.

On the basis of these results, the following recommendations are made for future studies regarding aerosol metabolism detection:

1. Quantitation of tank input should be carefully determined. At the onset of any series of experiments, a sufficient volume of $^{14}$C labeled glucose (30 - 50 μCi/ml) should be prepared to allow for repeated use. An aliquot of this solution should be monitored for radioactive content as distinguished from dissolved $^{14}$CO$_2$. Atomizer output and loss to input apparatus should be carefully established.

2. Procedures for the quantitative sampling from the drum need to be established. Sample volume measurements as well as the efficiency of various traps, impingers and membranes need to be precisely known for the various
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sample constituents ($^{14}$C glucose, $^{14}$CO$_2$, bacteria containing $^{14}$C). Leakage at any point in the sample train must be prevented. All trapped radioactivity should be acidified to determine if the material is $^{14}$CO$_2$ or some other form of radioactive material such as $^{14}$C glucose.

3. Methods to ensure adequate cleaning of the apparatus between experiments need to be established.

4. Attempts should be made to distinguish between metabolism occurring in aerosols versus that which may occur on wetted surfaces and in suspensions of bacteria and $^{14}$C glucose.

Respectfully submitted,

Gilbert V. Levin, Ph. D., Principal Investigator

J. Rudolph Schrot, Ph. D., Senior Research Microbiologist
APPENDIX I -

Experimental Protocol
I. Materials and Supplies

A. Culture

Growth Medium: \(0.5\%\) glucose in \(0.1\text{ M} \ K\text{H}_2\text{PO}_4\),

0.3 ml concentrated \(\text{NH}_4\text{OH}\) in 100 ml distilled water, \(\text{pH} 7.0 - 7.2\).

Solution: M/F filtered immediately after mixing.

Inoculum: \(S. \text{marcescens}\).

Growth and Preparation: Culture grown 18 hours @ \(30^\circ\text{C}\) on shaker. Spin down cell pack at room temperature, rinse in nonradioactive glucose solution and resuspend in one-half original volume in growth medium less glucose.

Objective: Population of \(10^{10}\) cells/ml.

B. Radioactive Glucose Working Stock Solution

NBL stock, \(^{14}\text{C}\)-glucose solution, \(S. \text{a.} = 335 \text{ mCi/mmol} ; 1.76 \text{ mCi/mg} ; \text{rad. conc.} = 500 \mu\text{Ci/ml} ; \text{mol. wt.} = 190; 99\%\) pure as of December 1970.

Objective: Final aerosol in tank to contain 0.3 \(\mu\text{mol glucose/ml} @ 5.0 \mu\text{Ci/ml}\). To prepare working stock solution in 10.0 ml batches to yield objective glucose...
and radioactive concentrations after 1:1 dilution with culture during aerosolization:

1. Aseptically transfer 0.2 ml NBL stock glucose solution to 25 ml flask.
2. Add 0.3 ml sterile distilled water.
3. Add 9.5 ml of 0.54 μmoles/ml nonradioactive glucose solution.
4. Immediately filter through M/F into sterile 25 ml flask with cotton plug stopper.
5. Place flask on shaker at room temperature for two days to permit exchange of \( ^{14} \text{CO}_2 \) dissolved in solution.
6. Store at 4°C for use and label. (Solution contains 0.63 μmol/ml @ 10 μCi/ml).

C. Nonradioactive Glucose Working Stock Solution

Prepare 0.63 μmol/ml sterile glucose solution (membrane filtered) in 100 ml quantities, label and store at 4°C.

D. Nonradioactive Glucose Rinse Solution

Dilute nonradioactive working stock solution 1:1 immediately prior to use.

E. Aerosolization Apparatus

Use specially prepared nonrefluxing atomizer with short holdup (less than 30 sec.) and fluid output of 0.125 ml/min.
Fit with Teflon or Kel-F washers; use Tygon tubing and replace tubing after each run.

F. Sampling Equipment

- 0.45 μ, 25 mm membrane filters in sterile, disposable syringe adapter-type holders
- Planchets for 25 mm filters
- 25 mm nutrient pads
- 100 ml/sampling syringes
- Bead tube traps
- AGI 30 samplers

G. Counting

1. Radioactivity
   - Liquid scintillation counter
   - Sample vials
   - Counting cocktail
   - Hyamine hydroxide
   - KOH solution - 20%

2. Microbial
   - Colony counter and tally
   - Pour plates, pipets, etc.
   - DTSA
   - Sterile dilution water
   - Incubator

II. Procedure

A. Control
1. Turn on 500 liter tank and equilibrate to approximately 75 - 80% RH.

2. Heat culture to 70°C and maintain at that temperature for ten minutes.

3. Plate culture at three dilutions in duplicate on DTSA and incubate for counting.

4. Atomize culture and 14C-glucose working solution simultaneously through aerosolization apparatus into tank for eight minutes.

5. a. Immediately withdraw 100 ml volume from tank using syringe.

5. b. Attach sampling syringe to membrane filter apparatus and push sample through filter and attached bead trap tube containing 2 ml hyamine hydroxide solution.

5. c. Remove 1.0 ml hyamine hydroxide solution from trap tube and place into counting vial containing 9 ml counting cocktail and store for counting.

5. d. Transfer filter apparatus to syringe containing nonradioactive glucose rinse solution and pass 15 ml rinse solution in 5 ml increments through the membrane filter. Collect each 5 ml rinse fraction.
5. e. Place 0.1 ml of each fraction collected in
counting vial with 9.9 ml counting cocktail
and store for counting. Label "rinse 1," "rinse 2" and "rinse 3."

5. f. Open filter apparatus and place filter in
counting cocktail in sample vial and save
for counting. Label "O-time uptake."

5. g. Immediately take duplicate sample and
rinse in same manner. Acidify rinse water
to pH 4 and collect $^{14}$CO$_2$. Discard rinse water.

5. h. Transfer this filter to nutrient pad
moistened with nonradioactive glucose
rinse solution in planchet and invert
over duplicate planchet containing 0.2
ml 20% KOH solution. Label KOH plan-
chet "O-time evolved." Incubate at room
temperature and replace KOH planchet at
ten min, 1 hr, 5 hr, and 24 hr. At end of
respective incubations, wash 0.2 ml KOH
solution from exposed KOH planchet into
counting vials with 9.9 ml counting cocktail.
Label and hold for counting.
6. Immediately after step II A5. g. above and not more than ten minutes after step II A5. a. above, use AGI 30 to sample tank for one minute. Plate sample into DTSA at 3 dilutions in duplicate and incubate for standard viability count.

7. Repeat steps II A 5 and 6 at ten min, 1 hr, 5 hr and 24 hrs.

8. Count all samples.

B. Cleanup

1. Heat tank to $140^\circ F$ while pumping fresh, dry air through it at 50 l/min for 2 hr (bubble exhaust air through 20% KOH trap to remove $^{14}CO_2$ from exhaust gas).

2. At 1, 1.5 and 2 hr, remove 100 ml sample from tank with sampling syringe (rinse syringe five times with fresh air between samplings).

3. Discharge each sample from syringe through clean bead tube trap containing 2 ml hyamine hydroxide. Remove 1 ml from trap and add to sample vial containing 9 ml counting cocktail.

4. Examine the three samples for evidence of low plateau in radioactivity before proceeding to step II C.

C. Test

Repeat all steps in II A except omit step II A 2.
APPENDIX II -

Tables
Table 1

Counting Efficiencies for Various Systems Used for Aerosol Metabolism Detection Experiments
(beckman Two-Channel Liquid Scintillation Counter With ESR)

<table>
<thead>
<tr>
<th>Cocktail</th>
<th>Additions</th>
<th>App. ESR</th>
<th>Efficiency</th>
<th>Method Determination</th>
</tr>
</thead>
<tbody>
<tr>
<td>Toluene/MeOH</td>
<td>0.05 - 0.1 ml glucose solution</td>
<td>0.200</td>
<td>79%</td>
<td>$^{14}$C-Benzoic Acid</td>
</tr>
<tr>
<td>Toluene/MeOH</td>
<td>1.0 ml hyamine hydroxide</td>
<td>0.007</td>
<td>64%</td>
<td>$^{14}$C-Benzoic Acid</td>
</tr>
<tr>
<td>Toluene/MeOH</td>
<td>0.3 ml glucose solution</td>
<td>0.200</td>
<td>77%</td>
<td>$^{14}$C-Benzoic Acid</td>
</tr>
<tr>
<td>Toluene/MeOH</td>
<td>Filter pad, 5 drops KOH</td>
<td>0.200</td>
<td>65%</td>
<td>$^{14}$C-glucose spotted on pad</td>
</tr>
<tr>
<td>Toluene/MeOH</td>
<td>Millipore filter, soaked with glucose</td>
<td>0.200</td>
<td>66%</td>
<td>$^{14}$C-glucose spotted on pad</td>
</tr>
<tr>
<td>*Unisol Mixture</td>
<td>0.5 ml digest</td>
<td>0.500</td>
<td>92%</td>
<td>$^{14}$C-Benzoic Acid</td>
</tr>
<tr>
<td>*Unisol Mixture</td>
<td>1.0 ml digest</td>
<td>0.500</td>
<td>92%</td>
<td>$^{14}$C-Benzoic Acid</td>
</tr>
</tbody>
</table>

*Contained 1 ml Unisol tissue solubilizer, 10 ul Unisol complement, 0.5 ml methanol.
# Table 2

Results from Experiment No. 1 Designed to Detect Aerosol Metabolism

0.1% glucose, 55 mCi/ml, 1.3 ml (approximately 300,000 dpm/l)

<table>
<thead>
<tr>
<th>Time</th>
<th>Filter cpm</th>
<th>Filter dpm**</th>
<th>Rinse cpm</th>
<th>Rinse dpm</th>
<th>Bubbler cpm</th>
<th>Bubbler dpm</th>
<th>Bacteria (cells/l)</th>
</tr>
</thead>
<tbody>
<tr>
<td>10 min.</td>
<td>257</td>
<td>390</td>
<td>4,850</td>
<td>26,400</td>
<td>7,064</td>
<td>30,400</td>
<td>1.4 x 10⁷</td>
</tr>
<tr>
<td>20 min.</td>
<td></td>
<td>(2 x 1 ml)</td>
<td>4,450</td>
<td>17,800</td>
<td>1,253</td>
<td>25,500</td>
<td></td>
</tr>
<tr>
<td>1 hr., 50 min.</td>
<td>802</td>
<td>1,220</td>
<td>10,400</td>
<td>45,450</td>
<td>1,398</td>
<td>4,030</td>
<td>1.1 x 10⁷</td>
</tr>
<tr>
<td>2 hr.</td>
<td></td>
<td>(2 x 1 ml)</td>
<td>6,100</td>
<td>26,300</td>
<td>2,079</td>
<td>4,350</td>
<td></td>
</tr>
<tr>
<td>4 hr.</td>
<td>1,645</td>
<td>2,500</td>
<td>21,300</td>
<td>90,100</td>
<td>4,280</td>
<td>9,200</td>
<td>1.0 x 10⁷</td>
</tr>
<tr>
<td>4 hr., 10 min.</td>
<td></td>
<td>(2 x 1 ml)</td>
<td>12,600</td>
<td>53,600</td>
<td>8,764</td>
<td>27,400</td>
<td></td>
</tr>
<tr>
<td>21 hr.</td>
<td></td>
<td>(3 x 2 ml)</td>
<td>4,600</td>
<td>40,000</td>
<td>1,607</td>
<td>3,500</td>
<td>3.7 x 10⁶</td>
</tr>
<tr>
<td>21 hr., 10 min.</td>
<td>383</td>
<td>535</td>
<td>4,600</td>
<td>40,700</td>
<td>1,112</td>
<td>2,400</td>
<td></td>
</tr>
<tr>
<td>21 hr., 20 min.</td>
<td>230</td>
<td>302</td>
<td>5,100</td>
<td>45,600</td>
<td>1,989</td>
<td>4,260</td>
<td></td>
</tr>
</tbody>
</table>

*cpm = actual counts per minute in sample unadjusted for dilution factor and counting efficiency.

**dpm = calculated from dilution factor and counting efficiency of each system.
Table 3

Results from Experiment No. 2 Designed to Detect Aerosol Metabolism

0.1% glucose, 55 μCi/ml, 0.3 - 0.5 ml, fill time - 6 minutes; plus 1% glucose, 30 μCi/ml, fill time - 10 minutes.

<table>
<thead>
<tr>
<th>Time</th>
<th>Filter cpm</th>
<th>Filter dpm</th>
<th>Rinse cpm</th>
<th>Rinse dpm</th>
<th>Bubblor cpm</th>
<th>Bubblor dpm</th>
<th>Bacteria (cells/l)</th>
</tr>
</thead>
<tbody>
<tr>
<td>15 min.</td>
<td>202</td>
<td>306</td>
<td>2,500</td>
<td>22,000</td>
<td>1,354</td>
<td>2,940</td>
<td>2.1 x 10^7</td>
</tr>
<tr>
<td>25 min.</td>
<td>4,600</td>
<td>38,700</td>
<td>2,444</td>
<td>5,150</td>
<td>8.3 x 10^6</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Approx.</td>
<td>4,460</td>
<td>38,000</td>
<td>1,700</td>
<td>3,680</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2 hr.</td>
<td>3,718</td>
<td>35,000</td>
<td>1,036</td>
<td>2,200</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Approx.</td>
<td>5,200</td>
<td>44,500</td>
<td>3,280</td>
<td>3,280</td>
<td>6.7 x 10^6</td>
<td></td>
<td></td>
</tr>
<tr>
<td>6 hr.</td>
<td>3,800</td>
<td>32,300</td>
<td>2,860</td>
<td>2,860</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Approx.</td>
<td>2,100</td>
<td>17,900</td>
<td>1,380</td>
<td>3,000</td>
<td>2.1 x 10^5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>24 hr.</td>
<td>1,594</td>
<td>14,000</td>
<td>1,357</td>
<td>2,920</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Note: Starting 14C glucose contained negligible 14CO2.

*cpm = actual counts per minute in sample unadjusted for dilution factor and counting efficiency.

**dpm = calculated from dilution factor and counting efficiency of each system.
## Table 4

Results from Control Experiment No. 3

1% glucose, 39 μCi/ml, 0.4 - 0.8 ml. No bacteria were aerosolized.

<table>
<thead>
<tr>
<th>Time</th>
<th>Filter cpm*</th>
<th>Filter dpm**</th>
<th>Rinse cpm</th>
<th>Rinse dpm</th>
<th>Bubbler cpm</th>
<th>Bubbler dpm</th>
</tr>
</thead>
<tbody>
<tr>
<td>10-min</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>20 min</td>
<td>41</td>
<td></td>
<td>864</td>
<td>7,400</td>
<td>.180</td>
<td>390</td>
</tr>
<tr>
<td>Approx</td>
<td>33</td>
<td>-</td>
<td>620</td>
<td>4,830</td>
<td>772</td>
<td>1,680</td>
</tr>
<tr>
<td>3 hr.</td>
<td>51</td>
<td>-</td>
<td>374</td>
<td>2,940</td>
<td>1,233</td>
<td>2,690</td>
</tr>
<tr>
<td>Approx</td>
<td>130</td>
<td>150</td>
<td>376</td>
<td>2,900</td>
<td>825</td>
<td>1,800</td>
</tr>
<tr>
<td>6 hr.</td>
<td>52</td>
<td>-</td>
<td>446</td>
<td>3,580</td>
<td>625</td>
<td>1,360</td>
</tr>
<tr>
<td>Approx</td>
<td>174</td>
<td>218</td>
<td>335</td>
<td>2,610</td>
<td>790</td>
<td>1,720</td>
</tr>
<tr>
<td>22 hr.</td>
<td>50</td>
<td>218</td>
<td>375</td>
<td>2,900</td>
<td>662</td>
<td>1,440</td>
</tr>
</tbody>
</table>

Note: Starting $^{14}$C glucose contained negligible $^{14}$CO$_2$.

Acidified 22 hour digest in bubbler showed 737 cpm and 94 cpm evolved radioactivity. Therefore, little $^{14}$CO$_2$ is in bubbler.

*cpm = actual counts per minute in sample unadjusted for dilution factor and counting efficiency.

**dpm = calculated from dilution factor and counting efficiency of each system.
STUDIES ON POSSIBLE PROPAGATION OF MICROBIAL CONTAMINATION IN PLANETARY CLOUDS

by

R. L. Dimmick

and

M. A. Chatigny

ANNUAL REPORT

NASA Contract W-13,450
INTRODUCTION

Current U.S. planetary quarantine standards based on international agreements require consideration of the probability of contamination (Pc) of the outer planets, Venus, Jupiter, Saturn, etc. One of the key parameters in estimation of the Pc of these planets is the probability of growth (Pg) of terrestrial microorganisms on or near these planets. For example, Jupiter (and Saturn) appears to have an atmosphere in which some microbial species could metabolize and propagate. This study includes investigation of the likelihood of metabolism and propagation of microbes suspended in dynamic atmospheres. It is directed toward providing experimental information needed to aid in rational estimation of Pg for these outer planets. Current work is directed at demonstration of aerial metabolism under near optimal conditions and tests of propagation in simulated Jovian atmospheres.
Theory

Literature on the planetary composition and the atmospheric structure and dynamics of the planet Jupiter includes reports of some firm data, discussions of some theoretically valid concepts, and some speculative ideas. In brief, the most probable structure is that the planet has an atmosphere at least 1000 Km in depth (3), beyond which components may become liquid, or molecular structure may shift to the metallic state (5,15,26). A solid planetary surface, as on earth, is not very probable (5). Solids most certainly exist in the form of particles or crystals, and it is possible that very large masses of these can be formed by agglomeration to "float" in the atmosphere. The "red spot" may be such an agglomerate (8). Jupiter has a magnetic field, and a turbulent atmosphere with electromagnetic and thermodynamic storms (3). All thermal models indicate there is an atmospheric stratum about 80 Km thick where temperatures vary from 0°C to 100°C at pressures from about 2 to 50 earth atmospheres (3,15,26).

The planet consists of 97 - 99% hydrogen and helium in nearly equal masses. The remainder of the planet has been shown to contain mostly methane, ammonia and water with traces of neon (3,5,7).

The fact that there are patches of red, brown and white color in the Jovian atmosphere led to speculation about the presence of other elements. Unless the planet is very unusual, most other elements should exist there in some quantity, and the effective mass of minor elements could be large (8,9,10). Note that the average abundance of carbon (300 parts per million) on earth, classes it as almost a trace element. Since the Jovian atmosphere is highly
reducing, elements such as phosphorus or sulfur would exist as hydrides with appreciable vapor pressures (19).

Clouds of NH$_3$ NH$_3$:H$_2$O, and probably NH$_3$:H$_2$S exist just above the biological stratum (8). Rain undoubtedly occurs. The water, if pure, is probably saturated with ammonia (5,7). However Ponnaparuma and others have shown that a variety of organic compounds are formed by electrical discharges in methane-ammonia atmospheres. Many are soluble in water and could act as buffers to reduce the pH of NH$_3$:H$_2$O solutions. The quantity and extent of the organic substances vary with electrical discharge intensity and gaseous composition (11,16,17,18, 19,24,27).

As droplets fall through the biological stratum, the water will evaporate, leaving small, solid particles. Many will be carried aloft to act as condensation nuclei; others will fall into the lower areas where temperatures as high as 1000$^\circ$C are postulated to exist. At these temperatures, pyrolysis occurs, and CH$_4$, H$_2$O and NH$_3$ would be reformed. Prior to reaching pyrolytic temperatures, however, a variety of other degradation compounds could be formed; some materials would escape as vapor. Thus, the biological stratum is probably rich in organic substances both particulate and gaseous.

If that stratum were considered to be a stirred settling chamber, then the half-life (2) of a 2 $\mu$m particle of density 1.1 would be about 5 years. that is:

\[
\frac{N_t}{N_0} = e^{-kt}
\]

where \(N\) = number of time \(t\)

\(N_0\) = number of time \(0\)

\(t\) = time, and

\(k = \frac{V}{H}\)

\(\frac{-4}{3.7}\)
where \( V \) = Stokes terminal velocity

\[ H = \text{effective height of a volume of air} \]

and \( t_{\frac{1}{2}} = 0.693 \frac{H}{V} \), where \( t_{\frac{1}{2}} \) = half life in seconds.

Substituting the Jovian gravitational constant in Stokes formula and assuming no significant changes in viscosity (\( \eta \)),

\[ V = \frac{p d^2 g}{18.4\eta} \quad \text{or} \quad V = \frac{1.1(2 \times 10^{-4})^2 2500}{18 (180 \times 10^{-6})} \]

\[ = 3.4 \times 10^{-2} \text{ cm/sec} \]

then \( \frac{H}{V} = \frac{8 \times 10^6 \text{ cm}}{3.4 \times 10^{-2}} = 23.5 \times 10^7 \text{ sec.} \)

\( 0.69 \times 2.3 \times 10^8 = 1.6 \times 10^8 \text{ sec} = 5 \text{ years.} \)

Under these circumstances, any anaerobic microbial species that could properly collect and utilize the organic material to reproduce new individuals at least once every 2.5 years would maintain a stable population (21). Those species with shorter division times would increase in numbers.

Such anaerobic microbes would have to evolve a metabolic and mechanical system that permits wastes and electron donor molecules to be discarded while simultaneously storing elements such as S, P, Fe, Mg, Ca, Na, Cl and water molecules with great tenacity (9,12,13).

There are two major aspects of studies that might determine whether airborne growth in such atmospheres is possible.

1. One can attempt to demonstrate first metabolism and then reproduction in the airborne state under any atmospheric situation.

2. One can attempt to isolate and cultivate an earth-type microbe capable of utilizing electrically-formed compounds in a closed system simulating Jovian conditions. This research has been investigating both aspects.
Experimental

The aerosol apparatus (Fig. 1): Aerosols are contained in a 540 liter drum (6) rotating at 4 RPM held in a temperature-controlled room (±1 C). The drum and seals are air-tight and the non-rotating port contains ports for air inlet and outlet. The inlet side has provisions for mixing wet and dry air to allow desired humidity conditions to be established, as well as dual ports for insertion of aerosols.

Samples are obtained either by a standard AGI impinger, 12.5 liters per min. (for viability studies) or by a bubbler system, 100 ml per min., shown in Fig. 2 (for sampling radioactivity).

Aerosols are produced by a standard, twin fluid, peripheral refluxing atomizer known to produce particles of mass median diameters of near 2μm (2). In one instance, the jet was modified to permit two liquids to mix at a point near the air-jet exit.

The Jovian chamber (Fig. 3): The chamber consists of a glass vessel having a central, spherical volume of 3 liters, a vertical tube extending upward about 45 cm and a vertical quartz tube extending downward about 10 cm. The top tube contains a removable "cold finger" through which cold water can be circulated, and a set of electrodes, one (at ground potential) in contact with the finger.

The quartz tube is fitted into a fire-clay and asbestos cyclinder containing a nichrome heating element in contact with the tube. This arrangement allows the bottom of the tube to be heated to a bright, cherry-red color.

The cold finger terminates in a thin rod 30 cm long and ending in a hook. A glass filter paper strip 1.5 cm wide and 8 cm long, with a pointed
bottom, is affixed to the hook via a hole in the top of the strip.

The unit also contains a port allowing gas exchange and pressure monitoring, as well as a hole 2 mm in diameter covered with an RTV rubber seal through which a long needle can be inserted for sampling purposes. The entire unit is enclosed in a metal mesh container for protection against explosion, as well as to serve as a Faraday shield. A simple Tesla coil with an output of about 30 KV and operated 10 sec during each minute, provides an electrical discharge directed onto the grounded cold finger. Thus the organic compounds are formed at or near the surface of water collected on the cold finger, and the compounds are made available to microbes which can be planted on the filter strip.

METHODS AND RESULTS

Aerosol Studies

The first approach to determine whether airborne microbes can carry out metabolic functions was to add uniformly $^{14}C$ labeled glucose to a washed suspension of *Serratia marcescens* cells just prior to aerosolizing them into the chamber conditioned to a relative humidity of 80% at 22 °C. If metabolism occurred, then the cell content of labeled glucose should increase, then decrease, as a function of time, and $^{14}CO_2$ should be formed. This work is being done in collaboration with Biospherics, Inc. and a detailed report of their work is appended.

The first experiment was done with the dual-fluid atomizer. It failed because of the lack of effectiveness of that atomizing technique to produce an adequate $^{14}C$ level per liter of air; 95% of the glucose solution was lost, since material not atomized contained a high cell content and could not be re-used.
The second set of experiments was conducted with two atomizers, one containing bacteria and the other tagged glucose. The experiment depended on collision of bacteria with a tagged glucose particle, uptake of glucose and subsequent evolution of \(^{14}\text{CO}_2\) as a result of glycolysis. The two aerosols were mixed in a 2.5 ft, 1/2 inch diameter tube prior to injection into the chamber. A preliminary experiment wherein aerosols of cells and hypochlorite were mixed, indicated the particulate collision efficiency would be adequate.

The protocol was to establish an aerosol (0 time) and sample at 5 min., 2 hr., 4 hr., and 24 hrs. At each sample time 3 specimens were taken in rapid succession; an impinger (1 min. duration) and two bubblers (1 min. each).

The first bubbler was supplied with a sterile filter. The filter disk was washed, glucose was added, and the disk incubated in a closed chamber with a CO\(_2\) adsorbent to determine whether collected cells produced \(^{14}\text{CO}_2\).

The second disk was washed, digested and counted to determine the amount of glucose incorporated into cells. Assay of radioactivity was done with standard scintillation counting fluids and techniques.

Results were essentially inconclusive. Viability assays indicated the overall loss of living, airborne cells to be 1/2 log in 24 hrs. and this value includes loss of particles to walls. The total radioactivity collected per sample, a measure of airborne particulate matter, showed a decrease of about 1/3 log in 24 hrs. Thus, a slight loss of numbers of viable cells (biological decay) had occurred at 90% relative humidity.

As samples were assayed, it became evident that the filters, or filter holders, were leaking. A final run was made with glucose alone to determine
the percentage of particles lost through the filter. When ratios of the soluble fraction to that retained on the filter are compared statistically, there was a significant difference between samples in the first run and the glucose-only set \((t = 2.59)\), but not in the second run. We believe this may show some uptake of glucose by cells, and that—since the first run had a greater particulate concentration, and hence greater chance for particulate collision—it may be indicative of poor collision efficiency. The standard error of the mean was large, however, and we interpret this to mean that filter holders leaked rather than filter disks; if disks leaked, the ratios should have been more uniform. Regardless, the extent of \(^{14}\text{CO}_2\) evolution was marginal, if any.

**Jupiter Environment**

The first task was to isolate ammonia-tolerant anaerobes \((4,22,26)\). Guano samples, as well as Guano beetles, were obtained from the Frio bat caves in Texas, where often ammonia vapor is sufficiently high to be toxic to the human \((20,25)\). Samples of various local soils were also obtained.

Enrichment cultures were made by mixing the samples with distilled water and placing the mixtures in anaerobic jars with a small beaker holding 10 ml of 30% solution of ammonium hydroxide. The jar was evacuated 5 times to 1/2 atm, sequentially replacing each lost volume with nitrogen. Samples were removed after 5-10 days, plated on nutrient agar and plates were incubated as above.

Another method was to provide deep-poured tubes of nutrient agar, to overlay the agar with 1 ml of the ammonia solution, and to stab the sample through the solution and deep into the agar with an inoculating needle.

Almost all samples yielded mixed populations of bacteria that would grow anaerobically on nutrient agar in the presence of ammonia. Surprisingly,
the culture that grew most abundantly within two days was found in a soil sample obtained from a small garden near the entrance to the laboratory.

Cultures obtained in this way were sequentially transferred every three days for a period of 4 months. Colonies were deliberately intermixed for the purpose of stimulating synergistic growth.

A sample of the mixed population was inoculated onto a moistened filter strip, the strip was hung on the glass hook described above and placed in the Jovian chamber. The chamber was flushed 5 times with nitrogen and supplied with about 2% by volume of ammonia and methane gas plus about 50 ml of H₂S and 2 ml of H₂O. No hydrogen or helium was used in these initial experiments. The pressure was maintained at one atm.

Water at 5 C was circulated through the cold finger and the electric discharge was started. In about 30 minutes, sufficient moisture had been collected on the finger, and had run down the rod to the strip, to cause the strip to be saturated and liquid drops started falling into the heated quartz tube. After that time, moisture continuously circulated and the air mass was being mixed as a result of sudden rush of steam from the falling drops.

Several experiments were conducted in this manner. In the first experiment, the strip was removed after 24 hrs., placed on nutrient agar, and incubated in an anaerobic jar. Growth occurred after 4 days, indicating some cells had survived and had been held by the glass fibers. Organic matter was being formed because dark-brown material collected on the top of the quartz tube, and a red-brown coloration appeared in water droplets condensing on the side of the vessel.
In another experiment the strip was removed after 4 days, but no growth occurred after 4 additional days of anaerobic incubation on nutrient agar. The plate with the strip was then removed and allowed to incubate at room temperature on the bench. Abundant aerobic growth occurred after 3 days. This indicates that cells were held by the strip for at least 4 days and that the glass paper strip is a suitable holding matrix. It is evident that the environment, probably the electric discharge products, inhibited any obligate anaerobic forms that might have been in the population and, at the same time, inhibited the anaerobic metabolism of any facultative forms.

**DISCUSSION**

Metabolism, *per se*, is not necessarily proof that the cell can propagate. Yet without metabolism propagation is impossible. It is probably easier to demonstrate metabolism than division, for living processes may be very slow in airborne cells, and there is a limit to how long particulate matter can be held airborne on earth. Hence metabolism should be the first demonstrable activity. If it can be shown to happen, the rate should help determine how long the division process might require.

There are ways other than use of radioactive tracers for indicating whether metabolism has occurred. A change in the DNA protein ratio might be one; loss or gain of specific enzyme activity might be another. Deficient mutants capable of induced enzyme formation or of genetic cross-linkage might also be utilized.

The simulated atmosphere of Jupiter was not immediately lethal to common vegetative cells. Presumably, spores would be no less sensitive. However,
survival alone is not the important question; since survival is inherently limited. An important question is whether cells can propagate in atmospheres utilizing traces of organic material formed by electrical discharges. (Note that methane cannot be used as an energy source without oxygen (4)). The reason the question is important is that if microbes can propagate under these conditions, then if a single cell or more were released, the most reasonable Pc value is 1. Of course, many other factors are involved. Growth on a moist surface is certainly not equivalent to growth in a gas. The cell must be able to obtain and hold moisture, withstand high pH or regulate the pH of its envelope, create and dispose of a suitable energy sink, conserve essential elements and control waste products, to mention a few.

FUTURE WORK

The tracer experiments will be continued, but at increased humidity and at temperatures providing optimal conditions for metabolism. An effective filter holder will be obtained and the sampling procedures improved. At the same time, we will initiate parallel aerosol experiments using strains of bacteria capable of inducing enzymes and with other mutant strains produce identifiable daughter cells. This work will be done at conditions optimal for the microbial systems to be employed (variants of E. coli) as models.

The effect of the rotating drum configuration and speed of particle fallout will be examined to determine whether it is possible to increase efficiency. Present drum design allows aerosols of 1 μm particles to be suspended in useful quantities for nearly two weeks. It is possible that this could be doubled and aerosols could be studied for nearly a month; time enough, perhaps, to allow slow propagation mechanisms to become evident.
We will obtain cultures of spores found on and around the launch facilities and select anaerobic ones, if any (1,14). These will be tested for growth in the products formed in the Jupiter chamber. The influence of the electrical discharge duration, and of the gas composition on the ability of formed products to support growth will be examined.
LITERATURE CITED


FIGURE 1. ROTATING DRUM SYSTEM
FIGURE 2

BUBBLER SAMPLER FOR COLLECTION OF CO₂

Needle Valve

Vacuum

Rotometer

Glass Beads

Membrane Filter and Holder

Adsorbant

To Drum