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First Progress Report

Aerosol Metabolism
Detection Tests

Contract No. 329380

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Naval Biomedical Research Laboratory
Naval Supply Center
University of California - Berkeley
Oakland, California 94625

Submitted by:

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

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Aerosol Metabolism Detection Tests

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 extra-terrestrial 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 micro-organism 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 glu-cose into a revolving tank designed to keep particulates air-borne 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.

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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 sub-

sequently 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}C glucose solution had lost radio-

    activity 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.

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2. Mixing of ^{14}C glucose and the bacterial sus-
pension 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 pro-
ducing radioactive gas indistinguishable
from that produced by metabolic activity
in the aerosol.

3. The extensive apparatus used for aerosol in-
put 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

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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.

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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 μCi/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 μCi/ml, 0.1%) were atomized separately and simultaneously for 6 minutes, whereupon the glucose atomizer was found to be without sufficient liquid volume

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for proper operation. The situation was quickly remedied
by addition of unlabeled glucose solution. The con-
centration was made up to 1% to facilitate the formation
of particulates of approximately 1 u diameter. Atomization
was continued for an additional 10 minutes using this glu-
cose solution which now contained approximately 30 $\mu Ci/$
ml of radioactivity. Samples were withdrawn from the
tank after 15 minutes, 2 hours, 1-hour and 24 hours.

EXPERIMENT NO. 3

^{14}C glucose (approximately 0.4 - 0.8 ml, of
30 $\mu Ci/ml$, 1%) was atomized into the tank. No bacteria
were aerosolized for this experiment. The ^{14}C glucose
solution was shown to contain little or no $^{14}CO_2$ 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.

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2. An analysis conducted in conjunction with
    Experiment No. 3 was done to test whether
    material in addition to $^{14}CO_2$ 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, indicat-
    ing 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 con-
    tained little dissolved $^{14}CO_2$). After
    establishment of this procedure (at the
    end of Experiment No. 1), little radio-
   activity remained on the filter pad.

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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 3×10^5 dpm in Experiment No. 1, 2.7×10^5 dpm in Experiment No. 2

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Figure 1

Decrease in Numbers of Aerosolized <u>Serratia</u> <u>marcescens</u>
During Two Separate Tank Experiments
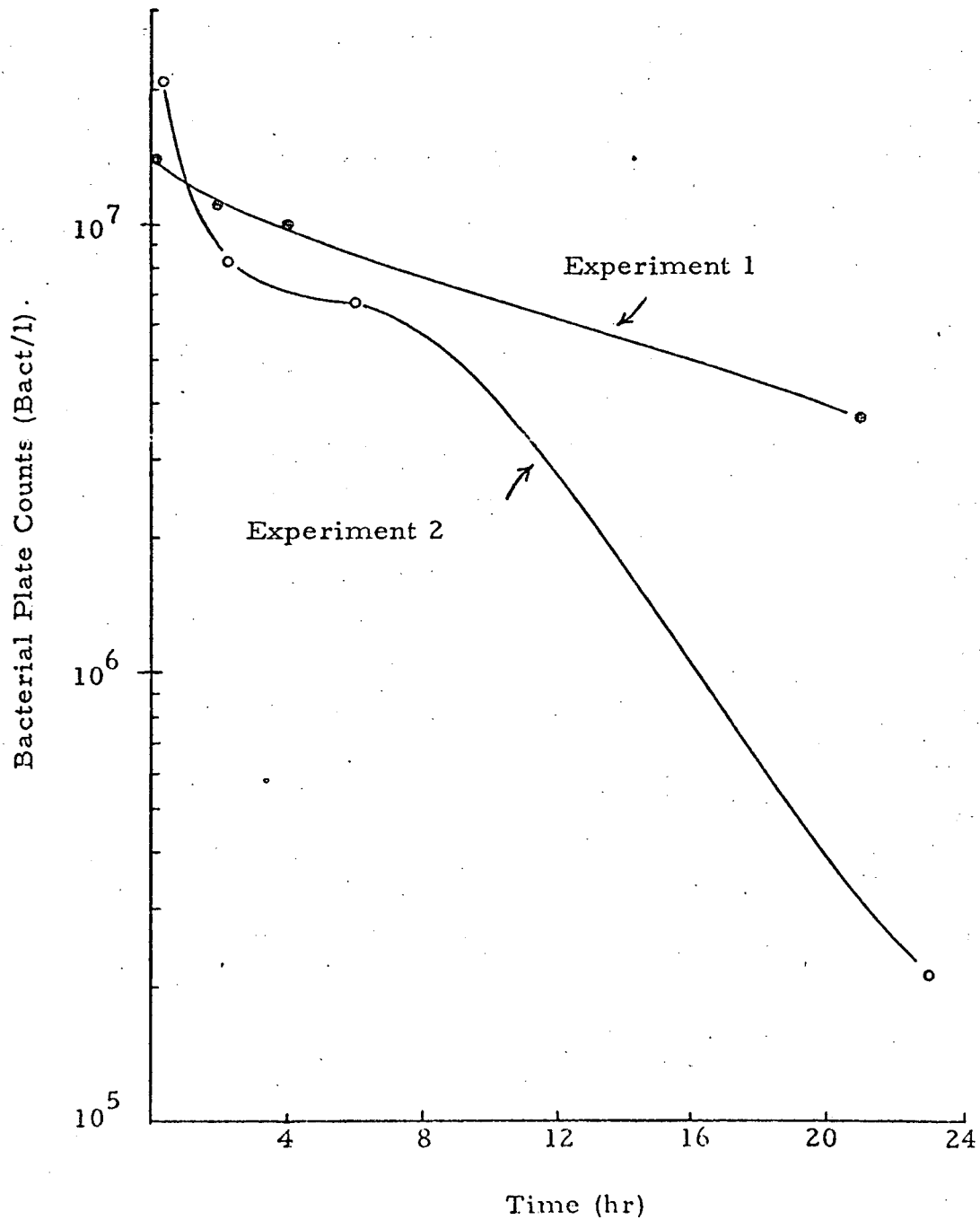
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and 2.0×10^5 dpm in Experiment No. 3.
The total amount recovered from each
liter withdrawn contained approximately
10% - 30% of this amount.

On the basis of these results, the following

recommendations are made for future studies regarding
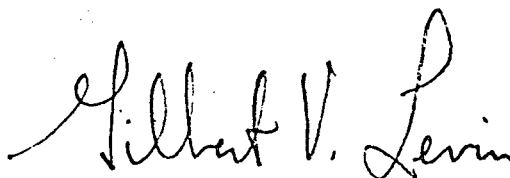
aerosol metabolism detection:

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 $\mu Ci/ml$) 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 care-
   fully 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
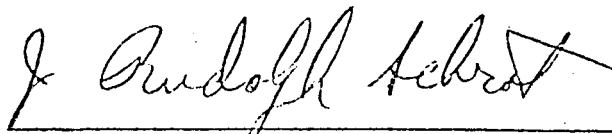
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sample constituents (^{14}C glucose, ^{14}CO$_2$, bacteria containing ^{14}C). Leakage at any point in the sample train must be prevented. All trapped radioactivity should be acidified to determine if the material is ^{14}CO$_2$ or some other form 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

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Test for Metabolism or Growth of Microorganisms in Aerosol

Experimental Protocol

December 15, 1972

I.   Materials and Supplies

   A.   Culture

   Growth Medium: 0.5% glucose in 0.1 M KH_2PO_4, 0.3 ml concentrated NH_4OH in 100 ml distilled water, pH 7.0 - 7.2.

   Solution: M/F filtered immediately after mixing.

   Inoculum: S. marcescens.

   Growth and Preparation: Culture grown 18 hours @ $30^\circ C$ 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}C-glucose solution, S.a. = 335 mCi/m mol; 1.76 mCi/mg; rad. conc. = 500 μCi/ml; mol. wt. = 190; 99% pure as of December 1970.

   Objective: Final aerosol in tank to contain 0.3 μmol glucose/ml @ 5.0 μCi/ml. To prepare working stock solution in 10.0 ml batches to yield objective glucose

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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 $^{14}CO_2$ dissolved in solution.

6. Store at $4^{\circ}C$ 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 $4^{\circ}C$.

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.

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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

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A. Control

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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 ^{14}C-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.

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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 planchet "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.

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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°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. **18**

APPENDIX II -

Tables

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Table 1

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

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

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

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Table 2

Results from Experiment No. 1 Designed to
Detect Aerosol Metabolism

0.1% glucose, 55 μCi/ml, 1.3 ml (approximately 300,000 dpm/1)

Time	Filter		Rinse		Bubbler		Bacteria
	cpm	dpm**	cpm	dpm	cpm	dpm	(cells/1)
10 min.	257	390	4,850	26,400	7,064	30,400	1.4 x 10^7
20 min.		(2 x 1 ml)	4,450	17,800	1,253	25,500	
		(rinse)					
1 hr., 50 min.	802	1,220	10,400	45,450	1,398	4,030	1.1 x 10^7
2 hr.		(2 x 1 ml)	6,100	26,300	2,079	4,350	
		(rinse)					
4 hr.	1,645	2,500	21,300	90,100	4,280	9,200	1.0 x 10^7
4 hr., 10 min.		(2 x 1 ml)	12,600	53,600	8,764	27,400	
		(rinse)					
21 hr.		(3 x 2 ml)	4,600	40,000	1,607	3,500	3.7 x 10^6
		(rinse)					
21 hr., 10 min.	383	535	4,650	40,700	1,112	2,400	
21 hr., 20 min.	230	302	5,100	45,600	1,989	4,260	

*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.

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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.

Time	Filter		Rinse		Bubbler		Bacteria
	cpm*	dpm**	cpm	dpm	cpm	dpm	(cells/l)
15 min.			2,500	22,000	1,354	2,940	2.1 x 10^7
25 min.	202	306	4,600	38,700	2,444	5,150	
Approx.			4,460	38,000	1,700	3,680	8.3 x 10^6
2 hr.	199	302	3,718	35,000	1,036	2,200	
Approx.			5,200	44,500	3,280	3,280	6.7 x 10^6
6 hr.	151	229	3,800	32,300	2,860	2,860	
Approx.			2,100	17,900	1,380	3,000	2.1 x 10^5
24 hr.	146	222	1,594	14,000	1,357	2,920	

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

 *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.

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Table 4

Results from Control Experiment No. 3

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

Time	Filter		Rinse		Bubbler	
	cpm*	dpm**	cpm	dpm	cpm	dpm
10 min.			864	7,400	180	390
20 min.	41		514	4,200	272	590
Approx.	33	-	620	4,830	772	1,680
3 hr.	51	-	374	2,940	1,233	2,690
Approx.	130	150	376	2,900	825	1,800
6 hr.	52	-	446	3,580	625	1,360
Approx	174	218	335	2,610	790	1,720
22 hr.	50		375	2,900	662	1,440
	-		-	-	762	1,660
	-	-	-	-	664	1,440

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.

15 JANUARY 1973

STUDIES ON POSSIBLE PROPAGATION OF MICROBIAL CONTAMINATION

IN PLANETARY CLOUDS

by

R. L. Dimmick

and

M. A. Chatigny

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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 plants. Current work is directed at demonstration of aerial metabolism under near optimal conditions and tests of propagation in simulated Jovian atmospheres.

APPROACH

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 $NH_3:H_2O$, and probably $NH_3:H_2S$ 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 $NH_3:H_2O$ 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° C are postulated to exist. At these temperatures, pyrolysis occurs, and CH_4, H_2O 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 μm particle of density 1.1 would be about 5 years. that is:

$$\frac{Nt}{No} = e^{-kt}$$

where N = number of time t

No = number of time o

t = time, and

k = $\frac{V}{H}$

where V = Stokes terminal velocity

H = 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 (η),

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

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

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

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

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^{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.

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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 $^{14}CO_2$ 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 $^{14}CO_2$.

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 $^{14}CO_2$ 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 H_2S and 2 ml of H_2O. 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 anerobic forms that might have been in the population and, at the same time, inhibited the anerobic 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.

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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

1. Davis, N. S., G. J. Silverman and W. H. Keller. 1963. Combined effects of ultrahigh vacuum and temperature on the viability of some spores and soil organisms. Appl. Microbiol. 11:(13):202-210.

2. Dimmick, R. L. and Ann Akers (Eds.) 1969. An Introduction to Experimental Aerobiology. John Wiley and Sons, New York.

3. Divine, Neil. 1971. The planet Juipter. NASA SP-8069, 90 pp.

4. Foster, J. W. and R. H. Davis. 1966. A methane-dependent coccus, with notes on classification and nomenclature of obligate, methane-utilizing bacteria. J. Bacteriol. 91:1924-1931.

5. Greenspan, J. A. and T. Owen. 1967. Jupiter's atmosphere; its structure and composition. Science 156:1489-1494.

6. Goldberg, L. J., H. M. S. Watkins, E. E. Boerke and M. A. Chatigny. 1958. The use of a rotating drum for the study of aerosols over extended periods of time. Am. J. Hyg. 68:85-93.

7. Lasker, B. M. 1963. Wet adiabatic model atmospheres for Jupiter. Astrophys. J. 138:709-719.

8. Lewis, J. S. 1969. The clouds of Jupiter and the NH_3, H_2O and $NH_3:H_2S$ systems. Icarus 10:365-378.

9. Lewis, J. S. 1969. Observability of spectroscopically active compounds in the atmosphere of Jupiter. Icarus 10:393-409.

10. Lippincott, E. R., R. V. Eck, M. O. Dayhoff and C. Sagan. 1967. Thermodynamic equilibria in planetary atmospheres. Astrophys. J. 147(2):753-764.

11. Loew, G. H., M. S. Chanda and S. Chang. 1972. A molecular orbital and chemical study of animoacetonitrite: A possible prebiotic peptide precursor. J. Theor. Biol. 35:359-373.

12. Marquis, R. E. and C. E. Zobell. 1971. Magnesium and calcium ions enhance barotolerance of streptococci. Arch. fur Mikrobiol. 79(1):80-92.

13. Molton, P. and C. Ponnampruna. 1972. Survival of common terrestrial microorganisms under simulated Jovian conditions. Nature 238(5361): 217-218.

14. Morelli, F. A., F. P. Fehlner and C. H. Stembridge. 1962. Effect of ultrahigh vacuum on Bacillus subtilis var. niger. Nature 196:106-107.

15. Peek, M. B. 1958. The physical state and chemical composition of the interior, p. 215-221, and Physical conditions, chemical composition and temperature, p. 227-235, (Chaps. 26 and 28) In: B. M. Peek, The Planet Jupiter, Faber and Faber, London.

16. Ponnamperuma, C. 1965. A biological synthesis of some nucleic acid constituents, p. 221-236; discussion, p. 236-242, In: The Origins of Prebiological Systems, (S. W. Fox, Ed.), Academic Press, New York.

17. Ponnamperuma, C. and N. W. Gabel. 1968. Current status of chemical studies on the origin of life. Space Life Sciences 1(1):64-96.

18. Ponnamperuma, C. and F. H. Woeller. 1964. Differences in the character of C_6 to C_9 hydrocarbons from gaseous methane in low-frequency electric discharges. Nature 203(4942):272-274.

19. Saslaw, W. C. and R. L. Wildey. 1967. On the chemistry of Jupiter's upper atmosphere. Icarus 7:85-93.

20. Studier, E. H. 1966. Studies on the mechanism of ammonia tolerance of the guano bat. J. Exptl. Zoo. 163:79-85.

21. Siegel, S. M. and C. Giumarro. 1965. Survival and growth of terrestrial microorganisms in ammonia-rich atmospheres. Icarus 4:37-40.

22. Siegel, S. M. and C. Giumarro. 1966. On the culture of a microorganism similar to the precambrian microfossil Kakabekia umbellata barghoorn in NH$_3$ - rich atmospheres. Proc. Nat. Acad. Sci. U.S. 55:349-353.

23. Silverman, G. J., N. S. Davis and W. H. Keller. 1964. Exposure of microorganisms to simulated extraterrestrial space ecology from -190° C to + 170° C. p. 372-384 In: Life Sciences and Space Research II, (M. Florkin and A. Dollfus, Eds.) North Holland Publ. Co., Amsterdam, Holland.

24. Strobel. D. F. 1969. The photochemistry of methane in the Jovian atmosphere. J. of the Atmos. Sci. 26(I):906-911.

25. Stoecker, R. R. 1971. Survival of blue-green algae under primitive atmospheric conditions. Space Life Sci. 3(1):42-45.

26. Wildt, R., H. J. Smith, E. E. Salpeter and A. G. W. Cameron. 1963. The planet Jupiter. Physics Today 16:19-25.

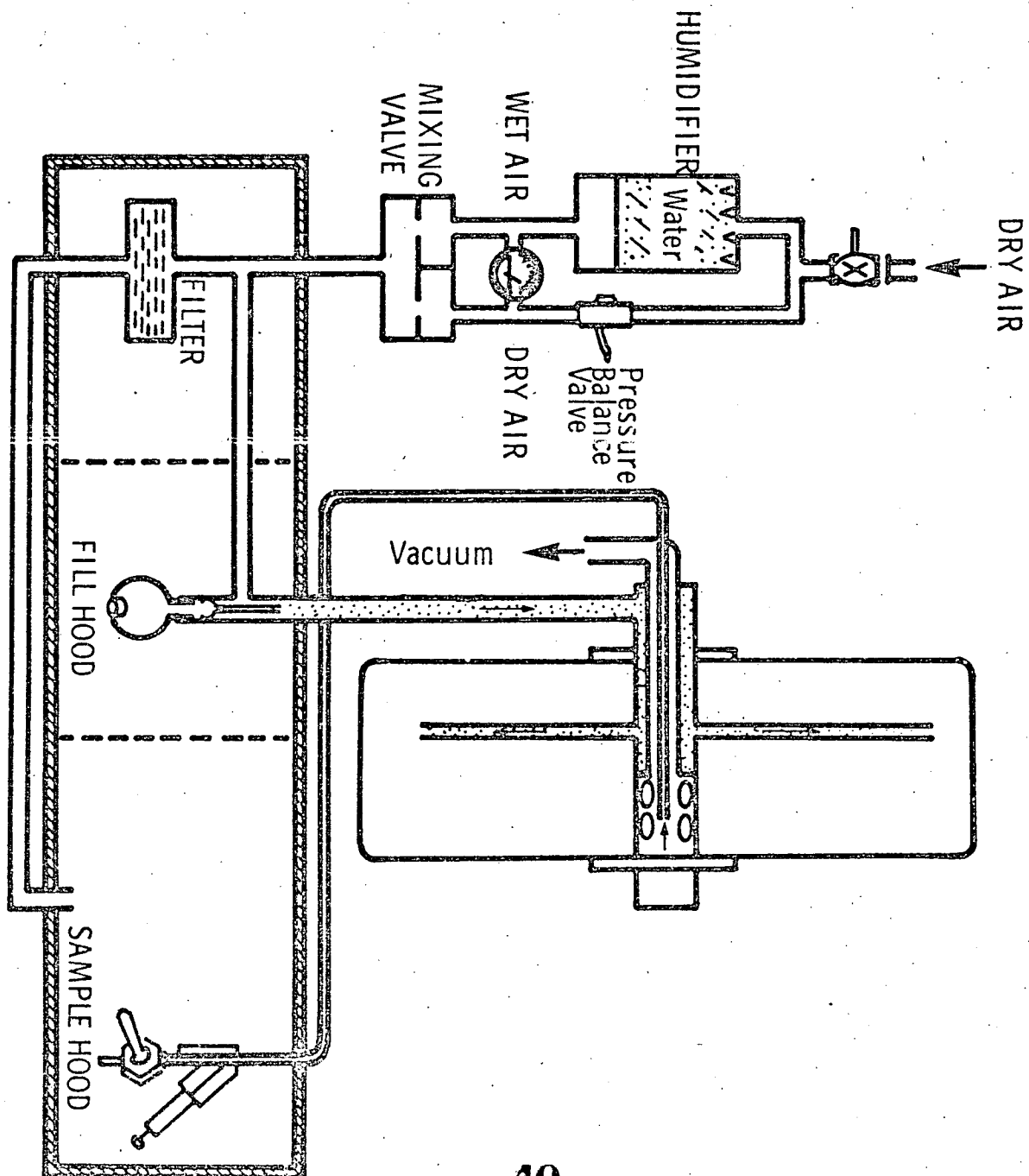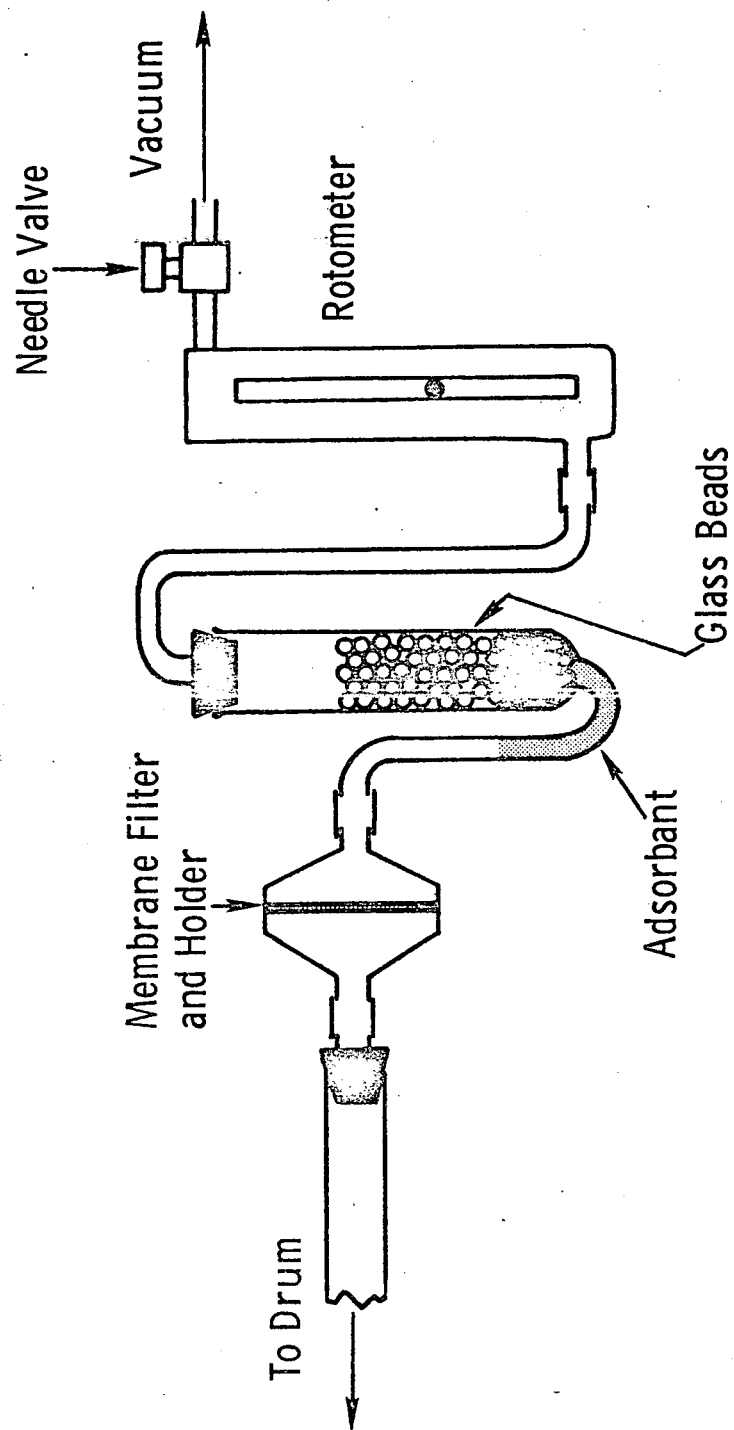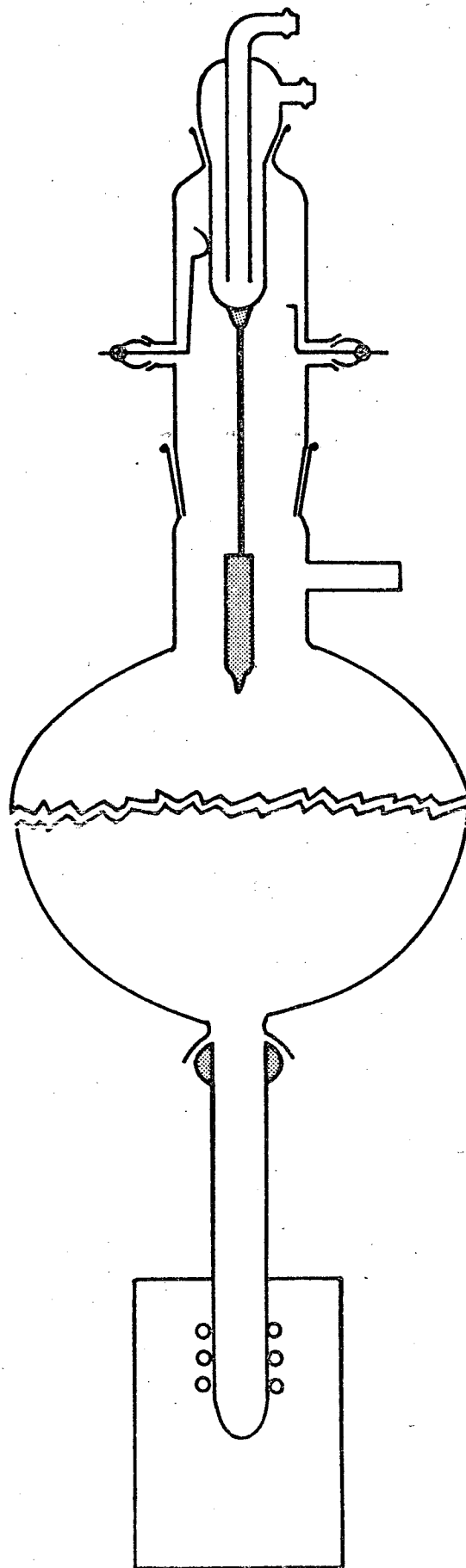27. Woeller, F. and C. Ponnamperuma. 1969. Organic synthesis in a simulated Jovian atmosphere. Icarus 10:386-392.

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FIGURE 1.   ROTATING DRUM SYSTEM

FIGURE 2

BUBBLER SAMPLER FOR COLLECTION OF CO_2

Vacuum

Needle Valve

Rotometer

Glass Beads

Membrane Filter
and Holder

Adsorbant

To Drum

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JUPITER CHAMBER 42

FIGURE 3