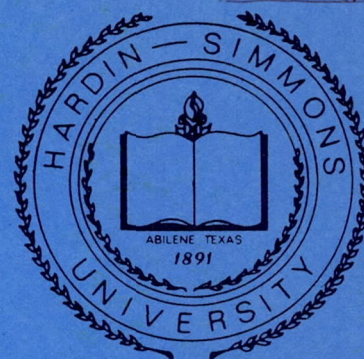


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Semiannual Progress Report No. 9
July 1, 1976 — June 30, 1977

RESPONSE OF SELECTED MICROORGANISMS TO EXPERIMENTAL PLANETARY ENVIRONMENTS

SCIENCE RESEARCH CENTER
Hardin-Simmons University
Abilene, Texas

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Progress Report No. 9 of Planetary Quarantine Activities
July 1, 1976 - June 30, 1977

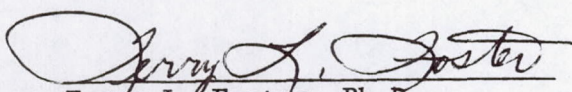
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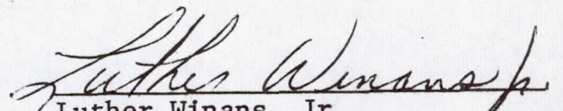
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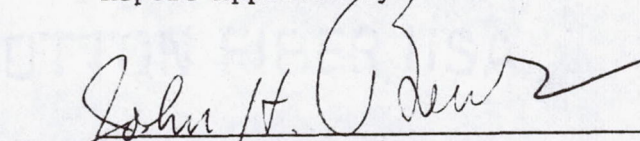
Science Research Center
Hardin-Simmons University
Abilene, Texas 79601

Report Prepared and Submitted by


Terry L. Foster, Ph.D.
Principal Investigator


Luther Winans, Jr.
Assistant Professor of Research

Report Approved by:


John H. Brewer, Ph.D.,
Director, Science Research Center

June, 1977

FOREWORD

[This ninth progress report summarizes work performed for the National Aeronautics and Space Administration by the Science Research Center at Hardin-Simmons University supported by NASA Grant NGR 44-095-001, and covers the period July 1, 1976 - June 30, 1977.]

This report includes the isolation of two organisms which appear to be utilizing PH_3 anaerobically. [These are now being investigated to verify this capability.] Other experiments have demonstrated that PH_3 is not toxic to the organisms being investigated. Another investigation is described to demonstrate reduction of PO_4^{-3} or utilization of PO_4^{-3} as the final H^+ acceptor.

Also included in this report is a reevaluation of the slide-culture technique with Nomarski interference contrast microscopy. This system was used to demonstrate growth of psychrotrophic, omnithermal, and hardy Bacillus spp. in a simulated Martian environment. [Other investigations with the omnitherms have demonstrated the presence of temperature-dependent cellular constituents, and plans are described to identify these.]

A new task of our research on improved or new techniques of anaerobic microbiology associated with space hardware describes detailed evaluation of the Brewer anaerobe jar/GasPak system, a new procedure to possibly grow aerobes and anaerobes simultaneously, [a new culture medium to differentiate obligate from facultative anaerobes, and a procedure to quantitate O_2 sensitivity of anaerobes.]

The NASA Technical Officer for this grant is Richard S. Young, NASA Planetary Programs, Code SBL, Washington, D.C.

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ANAEROBIC PHOSPHOROUS METABOLISM

In previous reports we have described the anaerobic utilization of phosphite and hypophosphite as related to potential terrestrial contamination of Jupiter. These results were presented at the 19th COSPAR meeting, accepted for publication in Life Science and Space Research, and another manuscript has been prepared for submission to the journals.

Of more relevance to contamination of Jupiter is our attempt to isolate microorganisms capable of utilizing phosphine (PH_3) anaerobically. These attempts are still in progress and will be described below.

The procedures and results of the phosphite work were presented at a Texas Branch-American Society for Microbiology meeting. The paper was of special interest to Dr. Orville Wyss, Department of Microbiology, University of Texas at Austin because he had worked on a similar problem for a number of years. After discussing this with him, we have agreed to perform a cooperative study on phosphorous metabolism. In this new investigation we are attempting to isolate organisms which can utilize phosphite or phosphate anaerobically as a final hydrogen acceptor. A study of this type can have several objectives. If reduction of phosphate continues to completion, phosphine would be evolved as the final product. Such a reaction could help explain "marsh flares" and "will-o-the-wisp" phenomena. When the evolved PH_3 contacts O_2 it will ignite spontaneously producing the flares or lights, and a phosphoric acid cloud forms immediately. This cloud could explain the "will-o-the-wisp." This type of reaction has not yet been demonstrated in our

laboratory as we have only recently begun this work, but by using our phosphorous assay procedures, we hope to demonstrate this phenomenon. Dr. Wyss is working with certain types of bacteria, and we are working with others in an attempt to demonstrate reduction of phosphate to phosphite, then hypophosphite, and finally phosphine. These procedures will be briefly outlined below.

This investigation will also make a significant contribution to origin of life studies by better explaining phosphorous metabolism in a primitive Earth atmosphere. Although there are no recorded natural deposits of phosphite, it has been postulated that during the development of Earth, deposits of phosphite may have existed. Because of the anaerobic environment at that time, organisms may have possessed the ability to use phosphate or phosphite as their final hydrogen acceptor, with the ultimate production of phosphine. If this was true, some present-day organisms may have retained the genetic capability for performing these reactions.

If we could indeed demonstrate anaerobic utilization of PH_3 as a phosphorous source and reduction of phosphate to PH_3 , this would be a description of a "phosphorous cycle." Cycles for elements such as nitrogen, carbon, and sulfur are well documented and utilized; however, such a cycle for phosphorous has not been described.

A. Anaerobic Utilization of Phosphine

We have employed procedures similar to those used in the phosphite work in an attempt to isolate organisms capable of utilizing PH_3 anaerobically. In this study various soil samples and pure cultures have been used. Numerous different configurations have been used to introduce PH_3 as a phosphorous source into our anaerobic systems. The system which appears to be most successful is to introduce PH_3 under pressure into a stainless steel cylinder which

contains distilled water. This is then added in equal volume to double strength phosphate-free basal medium to give a final PH_3 concentration of approximately 75-85 ppm P as PH_3 . This system has proven to be reproducible, and all manipulations can be performed anaerobically.

The hypophosphite-utilizing Bacillus sp. isolated earlier has been used extensively in the studies because it is only one step removed from performing the desired reaction. To date, we have not been successful in demonstrating this reaction. Similar investigations with other inocula have resulted in the isolation of a pseudomonas-like organism and another Bacillus sp. which appear to be utilizing PH_3 . Investigations are currently underway to verify this, and those results will be included in our next report.

B. Reduction of Phosphate or Phosphite

In an attempt to isolate organisms capable of utilizing phosphate or phosphite anaerobically as the final hydrogen acceptor, we have prepared several culture media of different formulations. Basically these are chemically defined media without nitrates or sulfates and with phosphate or phosphite. Ammonia is added as a nitrogen source and cysteine as a sulfur source. Because many organisms can ferment glucose with pyruvic acid serving as a final hydrogen acceptor, we also use media with citrate, lactate, or succinate as carbon source. These media are inoculated and incubated anaerobically, most of them in anaerobe jars with GasPaks to provide an atmosphere with gaseous hydrogen available.

At present we are concentrating primarily on members of the Bacillus and Pseudomonas genera in an attempt to get strict aerobes to grow anaerobically by using phosphate as the final hydrogen acceptor. During incubation the cultures are monitored for changes in turbidity and pH, and we are attempting to

isolate a sample that will have increased turbidity with little or no change in pH. If this can be done, we will then perform detailed phosphorous analyses to monitor phosphorous conversions during cultivation.

This phase of our phosphorous metabolism work is in its initial stages, and we have nothing to report at this time. Results of these investigations will be included in our next report.

C. Phosphine Toxicity

Because PH_3 is a toxic gas several people felt that its presence in Jupiter's atmosphere may serve as a decontamination agent to an interplanetary spacecraft that might enter this atmosphere. With this in mind, we performed experiments to determine if PH_3 would kill, inactivate, or prevent multiplication of various bacteria.

In an early experiment we streaked cultures of E. coli, Clostridium tetanomorphum, C. sporogenes, Bacillus coagulans, B. laterosporus, B. sp. HH-66 (omnitherm), B. sp. 8-26 (hardy), and B. sp. (hypophosphite utilizer) onto duplicate TSA plates and placed these into anaerobic atmospheres. One set was placed under a nitrogen atmosphere, the other, under a PH_3 atmosphere. These were incubated for 48 h at 32°C . All cultures showing growth in N_2 showed growth in PH_3 . The only observable difference was that the colony sizes of some cultures in PH_3 were slightly reduced. However, these results indicate that PH_3 is not toxic and cells can undergo reproduction in this atmosphere.

In another series of experiments we inoculated known concentrations of various organisms into either TSB + yeast extract or phosphate-free basal medium (described in Report #8). Phosphine water was added to some of these to give an available PH_3 concentration between 75-105 ppm P as PH_3 . Controls were identical to experimentals, but set up under a N_2 atmosphere, and all

vials were incubated at 32°C. Replicate vials were removed periodically and pour plate counts were performed to monitor the effect of PH₃ on changes in the bacterial populations.

The results of three such experiments are shown in Table 1. The first experiment was simply used to determine toxicity of PH₃ on E. coli and spores of B. brevis. No nutrients were available; therefore growth could not occur. After 6 h exposure, there was no significant decline in populations exposed to PH₃ as compared to controls exposed to phosphate-buffered saline. As before, this indicates that PH₃ is not toxic to these vegetative cells or spores.

The second experiment employs our hypophosphite-utilizer and B. coagulans with sufficient nutrients to allow growth. As can be seen, the hypophosphite-utilizer was evaluated twice and shows significant growth in the presence of PH₃, increasing 3 logs in one experiment and 6 logs in the other. The control (N₂ atmosphere) showed a faster increase (48 h) than the experimental (96 h and 72 h). This could be explained by the organism's becoming acclimatized to the PH₃ atmosphere. B. coagulans showed a slight population increase which occurred slightly faster in the N₂ atmosphere. The results of this experiment demonstrate that PH₃ is not toxic to these organisms and does not appear to interfere with reproduction.

In the final experiment we used a mixed VAB soil sample which has been used in many other investigations. As can be seen, populations did not increase, but neither did they show a decline in the presence of PH₃. The samples in TSB with no PH₃ showed substantially increased populations.

As a result of these experiments, one conclusion which can be drawn is that lack of growth appears to be more a function of our phosphate-free basal medium and not PH₃ inhibition. As a result of these experiments, we are now formulating and evaluating chemically defined media to use as a

Table 1. Response of various bacterial populations to an atmosphere of PH₃

			Cell counts per ml ¹ at various times (h)					
Organism	Medium	Atmosphere	0	2	4	6		
<u>B.brevis</u> spores	PH ₃ /H ₂ O ²	PH ₃	8.7x10 ³	3.5x10 ³	4.9x10 ³	3.0x10 ³		
<u>E.coli</u>	PH ₃ /H ₂ O	PH ₃	1.1x10 ⁸	4.3x10 ⁷	4.3x10 ⁸	2.5x10 ⁶		
<u>B.brevis</u> spores	Buffered Saline	Anaerobic	1.9x10 ⁴	-	1.5x10 ⁴	1.1x10 ⁴		
<u>E.coli</u>	Buffered Saline	Anaerobic	1.2x10 ⁸	1.4x10 ⁸	-	5.7x10 ⁷		
			0	24	48	72	96	120
<u>B.sp.</u> ³	TSB	N ₂	6.3x10 ⁸	2.3x10 ⁹	1.8x10 ¹¹	-	3.0x10 ¹¹	3.0x10 ¹¹
<u>B.sp.</u>	TSB	PH ₃	6.3x10 ⁸	8.6x10 ⁸	7.2x10 ⁸	7.6x10 ⁸	1.1x10 ¹¹	2.9x10 ¹¹
<u>B.sp.</u>	TSB	PH ₃	9.4x10 ⁵	9.4x10 ⁵	2.0x10 ⁶	2.4x10 ¹¹	4.1x10 ⁹	8.0x10 ⁹
<u>B.coagulans</u>	TSB	N ₂	4.3x10 ⁴	4.7x10 ⁵	5.6x10 ⁶	7.2x10 ⁶	4.4x10 ⁶	3.7x10 ⁶
<u>B.coagulans</u>	TSB	PH ₃	4.3x10 ⁴	5.7x10 ⁴	9.1x10 ⁵	1.5x10 ⁶	9.0x10 ⁵	1.0x10 ⁶
			0	6	12	24	36	48
VAB ⁴ soil	Phosphate-free Medium	PH ₃	5.0x10 ⁴	4.9x10 ⁴	4.9x10 ⁴	4.1x10 ⁴	4.3x10 ⁴	6.0x10 ⁴
VAB ⁴ soil	TSB	Anaerobic	5.0x10 ⁴	-	5.1x10 ⁴	8.9x10 ⁷	1.5x10 ⁷	1.6x10 ¹¹

1. All counts are an average of 3 samples plated in duplicate (6 plates)
2. Phosphine water solution at 75-105 ppm P
3. Hypophosphite-utilizing Bacillus sp.
4. Vehicle Assembly Building, Kennedy Space Center

phosphate-free medium in these investigations. We will attempt to develop a medium which will support more prolific growth of the organisms under study.

OMNITHERMS

Our recent reports have described the isolation and partial characterization of a group of organisms which we call omnitherms. These isolates are all Bacillus sp. and possess the ability to grow over a temperature range of from 3°C to 55°C. At least five of 28 have been shown to survive the Viking dry-heat cycle without loss of the omnithermal characteristic, whereas others have lost the ability to grow at the upper or lower limits after storage on Cystine Trypticase Agar (CTA) at room temperature and refrigeration temperature.

As indicated in Report #8, investigations on these isolates have been decreased in order to more effectively study other tasks. Two investigations of the omnitherms will be included here. Some of the results of this task were presented at the 19th COSPAR meeting in Philadelphia, and the paper was accepted for publication of the next volume of Life Sciences and Space Research. These organisms shall be maintained on stock culture and be made available to other researchers who might wish to study them in more detail.

A. Growth Curves and Generation Times

Various media were evaluated for their ability to support growth of omnitherms at 30°C, 32°C, and 55°C, and it was decided to use Trypticase Soy Broth (TSB) in this study. Spore suspensions of the omnitherms were inoculated into TSB and incubated for 24 h at 32°C. One-tenth ml inocula were introduced into 3 sets of 5.0 ml TSB for incubation at the three experimental temperatures. Replicate samples were removed periodically, and turbidity was read on a

Klett-Summerson colorimeter. The 32°C samples were read hourly for 48 h, and the 55°C and 3°C samples were read daily for 21 days. Growth curves for each isolate were drawn but are not included in this report. Instead, generation times were calculated at the three different temperatures and are shown in Table 2. As can be seen, not all isolates grew sufficiently to calculate generation times at all three temperatures. This is attributed to loss of omnithermal characteristics due to spore storage in 95% ethanol. We have seen this happen after other forms of storage and have demonstrated that with repeated subculture at the three temperatures, the organism can be induced to again demonstrate this characteristic. In other words, this is a temporary and reversible character loss. Another possible explanation for lack of growth at all three temperatures is preincubation of the samples at 32°C. To perform a more thorough study of generation times, it would have been advisable to preincubate samples at 3°C, 32°C, and 55°C prior to inoculation into TSB. This would triple the size of the experiment and may be performed in the future.

From Table 2 it can be seen that the omnitherms are characteristically slow growers at extreme temperatures. The generation times at 3°C are consistent with that described for numerous psychrotrophic organisms, and the generation times at 32°C are consistent with other mesophiles. However, the 55°C generation times are much longer than those reported for thermophiles, but they are consistent with our earlier data describing 3-10 day incubation to detect turbidity in these cultures.

B. Pyrolysis Gas-Liquid Chromatography of Some Omnitherms

In an attempt to determine if there is any obvious correlation of chemical composition between the omnitherms, several of them were sent to the JPL-PQ laboratory at Cape Canaveral for analysis in their pyrolysis

Table 2. Generation times of Bacillus spp. which possess the ability to grow at 3°C, 32°C, and 55°C.

<u>Isolate Number</u>	<u>Generation time (h) at</u>		
	<u>3°C</u>	<u>32°C</u>	<u>55°C</u>
A-28	*	2.9	32.7
AA- 6	*	5.2	18.5
AA-10	86.4	5.2	68.6
BB- 1	30	0.7	7.2
C-16	80	4.6	62.6
G-20	62.6	6.0	26.0
G-28-2	127.0	5.2	190.0
G-33	33.3	4.3	54.2
G-33II-1	39.1	3.1	108.0
G-38A	113.7	4.2	48.0
G-38B	136.0	5.8	37.9
G-38C-2	74.4	6.6	62.4
GG-30	34.0	4.3	153.6
GG-41	34.0	3.8	31.0
GG-44	50.4	3.8	110.4
H-86-1	45.6	6.2	40.8
HH- 1	38.4	1.8	31.0
HH-34	31.2	4.6	33.6
JJ-30A	96.0	5.4	40.8
JJ-30B	48.0	1.6	72.0

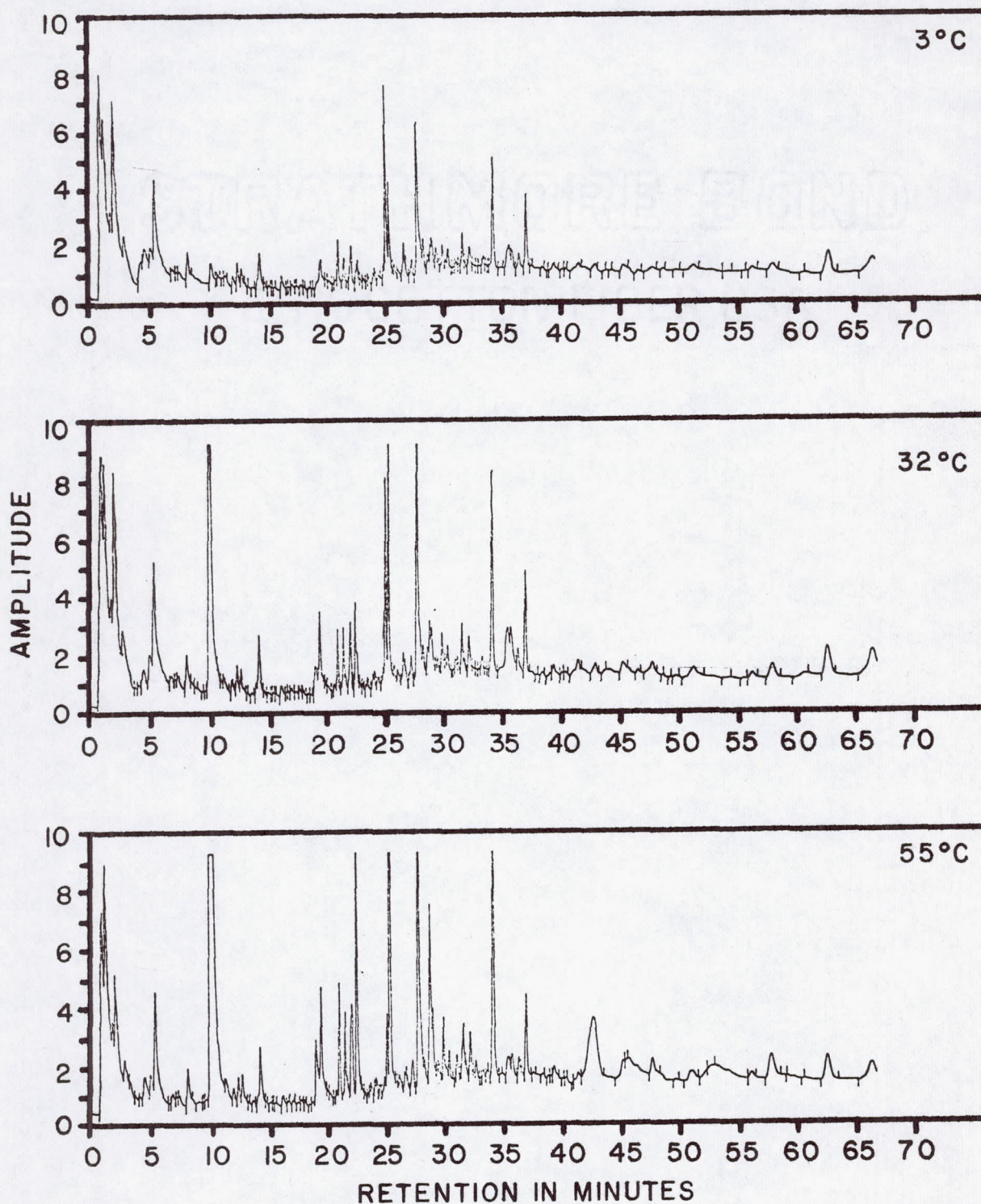
*Insufficient growth to calculate generation time.

gas-liquid chromatography apparatus (PGLC). The procedures and applications of this analysis are well documented in their JPL reports and in Appl. and Env. Microbio. 32:306,1976.

Selected omnitherms were grown on Millipore filters on TSA plates at 3°C for 7-10 days, 32°C for 24 h, and 55°C for 6-10 days. These were removed from the filters and introduced into the PGLC unit following the accepted procedures of the JPL-PQ lab. Some of the samples were run in duplicate to verify repeatability, but lack of time prevented duplicate runs of all samples. Of the samples run in duplicate, the chromatographs were identical, thus indicating that the procedure is efficient and repeatable. These duplicate controls were run at the beginning, middle, and end of our experiment. In all, the JPL-PQ lab ran 16 chromatographs for this work.

Although this was only a small experiment to better describe the omnitherms, some interesting results were obtained which probably warrants further investigation. The most significant results of this experiment are related to a tentative recognition of chemical components which appear to be temperature dependent. This is exemplified in Figure 1 which shows chromatograms of isolate C-16R grown at 3°C, 32°C, and 55°C. Upon careful examination of these chromatograms, there appear to be various regions which change as temperature changes. With isolate C-16R the peak with a retention time of 24 minutes shows a significant increase in concentration as incubation temperature increases from 3° to 55°C. Simultaneously, the peak at 24.5 min. almost totally disappears with increasing temperature. The same phenomenon is seen at the peaks with a retention time of approximately 27 minutes. The one at 27 min. decreases appreciably with increasing temperature while the peak at 27.4 min. increases with increasing temperature. The peaks at 31, 35.3, 36.4, 39.5, and 46.4 min. show increases with increased temperature,

FIGURE 1. PYROCHROMATOGRAMS OF *BACILLUS* SP. C-16R AFTER INCUBATION AT 3°, 32°, AND 55°C



the one at 31 min. being especially increased. The peaks at 30.5, 31.6, and 38.5 decrease as temperature increases.

Isolate B-10-11 was treated in exactly the same manner as C-16R. Although the chromatograms are not shown, they do show similarities to C-16R. The peaks at 24 min. show similar but less obvious changes as C-16R. The peak changes at 27 min. are virtually identical to that of C-16R, with the peak at 27.4 min showing drastic increases with increasing temperature. The peak at 31 min. increases from 3° to 32°C but not at 55°C. In addition, the peak at 28.5 decreases with increasing temperature.

Isolate GG-26 was run only with incubation at 3° and 32°C, but it shows the same pattern of peak changes at 27 and 31 min.

From this limited data, the results strongly suggest that two areas of the chromatograms, 27 and 31 min., are temperature dependent. The peak at 24 min. may also be involved in a similar manner. It would be of interest to investigate this response in more detail, incorporating a larger number of our omnitherms. It would also be advisable to determine the peak responses with these organisms incubated at 5°C intervals. If these changes can be substantiated the next task would be to identify the chemicals showing temperature dependence and attempt to explain the mechanisms by which organisms are capable of growing at different temperatures. Because one current hypothesis is that temperature dependence is associated with the cell membrane, it would be of interest to know if the peaks in question are also associated with the cell membrane. This could be determined quite easily by growing the organisms at various temperatures, disrupting them, isolating cell membrane components, and running them through PGLC.

SLIDE-CULTURE TECHNIQUE

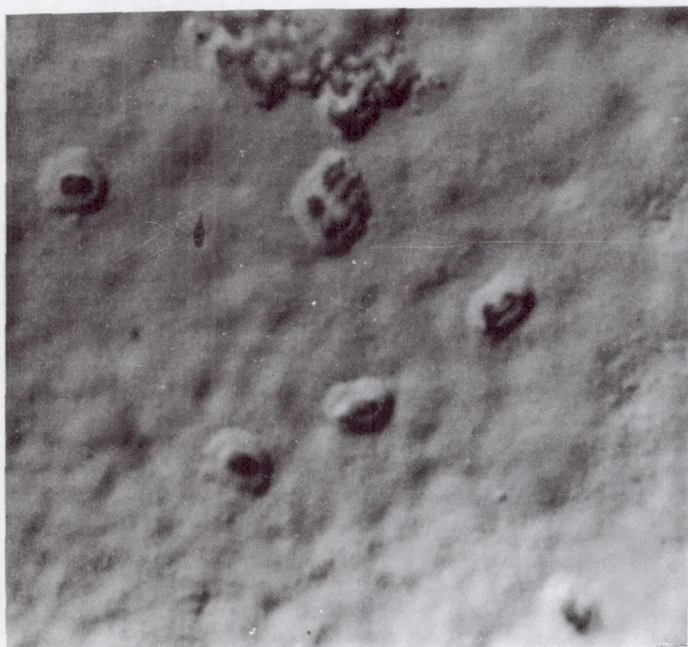
In earlier work we have described the use of the slide culture technique to demonstrate growth of microorganisms in experimental Martian environments (see Reports 3 and 4). At that time, we emphasized the ease and rapidity of this technique in demonstrating bacterial growth under various experimental conditions.

Recently we have again been utilizing this procedure for rapid detection of growth. Detailed procedures will not be given here, but these can be found in earlier reports (3 and 4). Basically, the process involves agar-coating slides with the desired culture medium and inoculating with the desired organism. We use 8-chambered tissue culture slides (Lab Tek No. 4808) and coat these with various media, some of which contain 0.1% formaldehyde as negative controls. These are then dried in a dessicator, under vacuum, or in a 45-60°C oven and stored in a dessicator prior to use. They are then inoculated and subjected to the desired experimental conditions.

In a recent study spores of representative omnitherms, psychrotrophs, and hardy organisms were inoculated onto TSA-coated slides, placed into vials, and subjected to an experimental Martian environment consisting of 99.9% CO₂ + 0.1% O₂ at 7 mb pressure. These were incubated in a freeze-thaw cycle of -65°C for 16 h and 20°C for 8 h. Sample vials were removed every 24 h after the vials had been at 20°C for 4 h. The slides were examined microscopically using Nomarski differential interference contrast microscopy on a Zeiss universal research model microscope with a 63x objective and magnification capability to 1500x.

All organisms investigated showed obvious growth under these conditions, beginning in 24 h. An example of a 3 day growth sequence of one of these isolates is shown in Figure 2 . As can be seen, cell numbers increase with time, and microcolony development is quite pronounced at 72 h. These results demonstrate that spores of omnitherms, psychrotrophs, and hardy organisms will germinate and vegetative cells will reproduce in this experimental environment. The results also demonstrate that the slide-culture technique is a rapid and dependable method for demonstration of bacterial growth and/or spore germination under various environmental conditions. Nomarski differential interference contrast microscopy is also shown to be an extremely reliable procedure for demonstrating growth on agar-coated slides. Even with light passing through the agar, resolution is very good, and cellular morphology is quite distinct. As compared to phase-contrast microscopy, Nomarski contrast microscopy is far superior for this type of work.

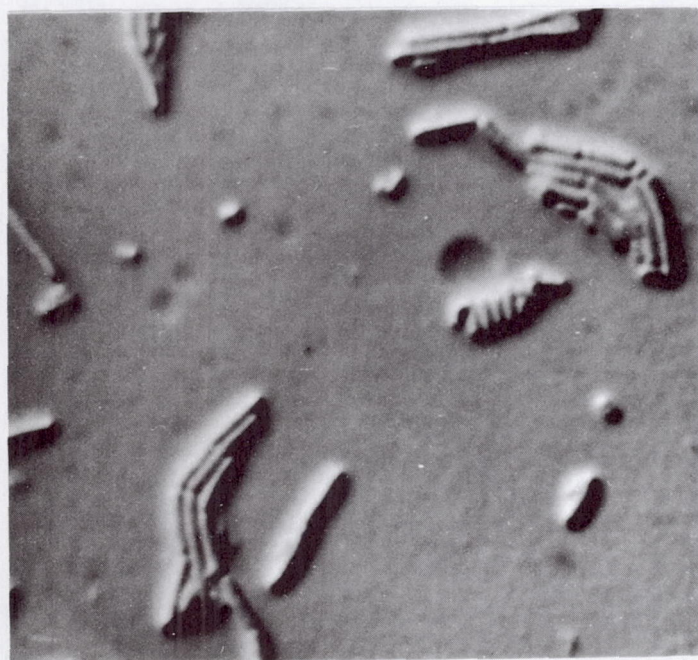
Using the slide culture technique with Nomarski microscopy, we have recently begun an investigation prompted by J. R. Wilkins et al of Langley Research Center (Appl. and Env. Microbiol. 32:294-297, 1976). In this paper, they described instrumentation to determine the effect of gravity on colony formation in soft agar. Their results show that colonies of E. coli produce a halo colony in the vertical plane. We are attempting to use our procedures to demonstrate that this halo formation is a result of cellular organization as cell division occurs. These workers performed a similar study in 1972 in which they used phase-contrast microscopy. We anticipate that the superior resolution of Nomarski interference microscopy will provide more detailed explanations of the mechanism of cellular division and arrangement in an increased gravitational field.



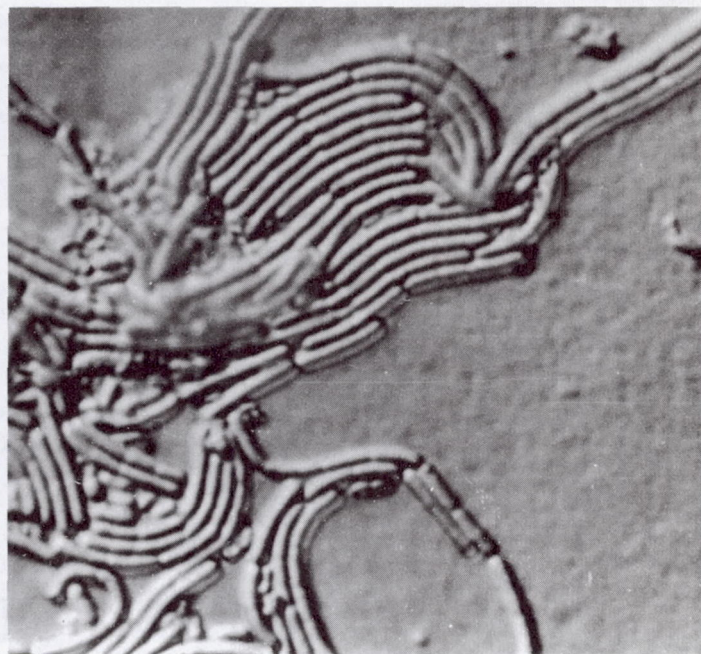
0 HR



24 HR



48 HR



72 HR

FIGURE 2. NOMARSKI INTERFERENCE CONTRAST PHOTOMICROGRAPHS (1500X) OF A PSYCHROTROPHIC BACILLUS SP. AFTER INCUBATION IN A SIMULATED MARTIAN ENVIRONMENT OF 99.9% CO₂ + 0.1% O₂ AT 7MB AND INCUBATED FOR 16H AT -65°C AND 8H AT 20°C.

The results of the work on response of omnitherms, psychrotrophs, and hardy organisms in an experimental Martian environment were presented recently at the national meeting of the American Society for Microbiology. Because the investigation on the effect of gravity on cellular division have only recently been initiated, no results are available at present. These will be presented in our next report.

TECHNIQUES OF ANAEROBIC MICROBIOLOGY

Because of the anaerobic environments of most of the planets and the need to know more about the response of terrestrial microorganisms in anaerobic environments, we have recently initiated a research task of investigating techniques of anaerobic microbiology. The primary objective of this task is to improve or develop new techniques of anaerobic microbiology, especially as applied to space-related microbiology.

A. Evaluation of the Anaerobe Jar/GasPak System (BBL)

The Brewer anaerobe jar with a cold catalyst and the GasPak (BBL) hydrogen-carbon dioxide generating package is used routinely in many laboratories as the anaerobe system of choice. The literature demonstrates it to be reliable, fast, and easy to use. In our search of the literature we failed to find investigations which monitor environmental changes in the anaerobe jar as it is being used. Most investigators simply evaluate the jar on the basis of its ability to support growth of the more oxygen-sensitive anaerobes. We decided to monitor environmental changes in the anaerobe jar as a baseline for further investigations.

For this investigation several large Brewer anaerobe jars (GasPak 150-BBL) were modified to accept an oxygen probe (Beckman 39590). These were connected to an oxygen analyzer, temperature monitor (Beckman 100800) which in turn was connected to a strip-chart recorder. Prior to each use, the oxygen probe was calibrated against atmospheric oxygen and oxygen-free nitrogen (Matheson Gas Products). This system provided continuous monitoring of

oxygen concentration and temperature during operation of the anaerobe jar. The probes were positioned at various locations in the jar during operation, but results presented here will be those with the probes suspended just above the bottom of the jar. A mercury manometer was attached to the vent nipple of the jar to provide continuous monitoring of pressure changes. It was necessary to read the mercury manometer visually or attach it to a kymograph. This will soon be replaced with a pressure transducer which can be continuously monitored on the strip-chart recorder.

At the beginning of each experiment the catalysts were replaced with fresh catalysts. Most of the experiments were performed with empty jars, but some used jars which contained TSA plates or TSB. This was done to evaluate the effect of oxygen dissolved in the medium. Six replications of each experimental procedure were performed to verify the repeatability of the procedures and to evaluate the effect of repeated use on catalyst activity.

The first experiment was performed to evaluate catalyst activity, GasPak activity, and demonstrate repeatability of procedures. Figure 3 shows a pressure increase beginning at 2 min. and reaches a peak at 8 min. This pressure increase is due to release of H_2 from the GasPak. As H_2 is generated, it begins reacting with O_2 to form H_2O and the O_2 level begins decreasing rapidly beginning at 6 min. Although it is not shown on the graph, temperature at the bottom of the jar begins increasing slowly at about 8 min. This rapid depletion of O_2 and H_2 results in a corresponding decrease in pressure, ultimately resulting in a negative pressure from 11.5 min. to 16 min. During this time CO_2 begins to be evolved from the GasPak, H_2 continues to be released, and the pressure again increases to a second peak at 24 min. Because of continued depletion of H_2 and O_2 , pressure again drops until the O_2 is eliminated (between 30-35 min.), and a slight positive pressure is maintained in the jar due to CO_2 and excess H_2 .

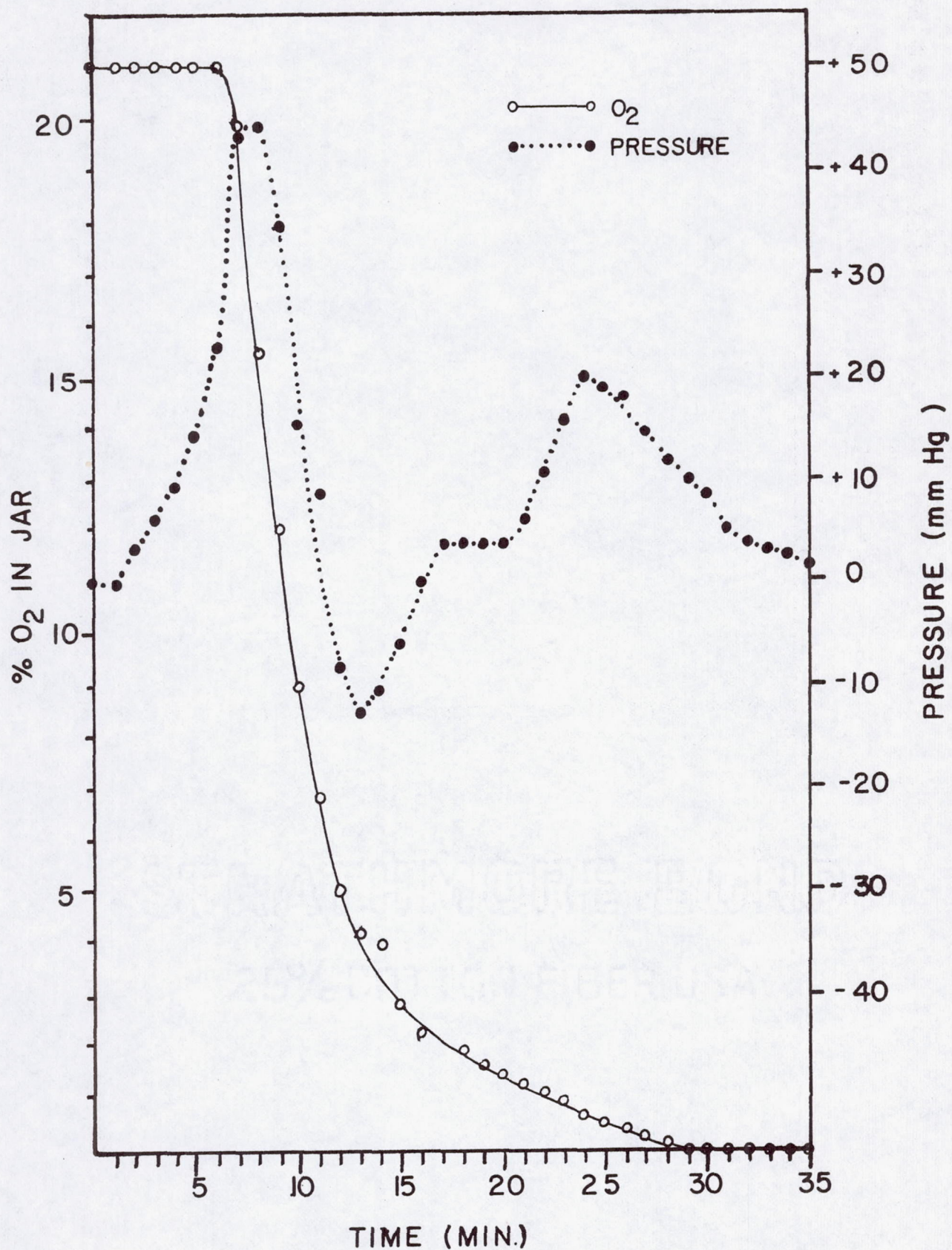


FIGURE 3. OXYGEN AND PRESSURE CHANGES IN A BREWER ANAEROBE JAR-GASPAK SYSTEM (BBL) AT ROOM TEMPERATURE.

In following the O_2 level, it can be seen that 57% of the O_2 is eliminated after 10 min., 86.7% at 15 min., 93.8% at 20 min., 97.4% at 25 min., and the level falls below 0.1% after 28 min. at which time anaerobiosis is achieved. The anaerobic indicator used with the GasPak system begins showing anaerobiosis between 1-2% O_2 . From this data it can be seen that the reaction to remove O_2 from the system is more than 97% complete after 25 min. indicating that this is a dependable and fairly rapid system.

Six identical runs using the same catalyst were performed for this experiment. The results are summarized in Table 3. As can be seen, the general trend in O_2 reduction is the same, although the rate of decrease is slower each time, with one exception. After 35 min. the first two runs show O_2 levels below 0.1%, but runs 4-6 are increasingly slow and less efficient. Even though the indicator shows anaerobiosis in the last 4 runs, there appears to be residual O_2 ranging from 0.6 - 1.1% O_2 after 50 min., at which time the runs were terminated. This indicates that in order to achieve optimum success with the GasPak system, the catalysts should be changed after 1 or 2 uses and reactivated according to manufacturers' procedures.

Table 3 also shows pressure changes during the six runs of the anaerobe jar. This was performed to demonstrate the reliability of individual GasPaks, and all show a pressure increase during the first 5 min. Although there is individual variation in the GasPaks, they all show peak H_2 release in 8-12 min., followed rapidly by a decrease as H_2 and O_2 are reacting. The rapid initial reduction of O_2 in run 5 is probably due to the fact that H_2 was released more rapidly from the GasPak used in that run, thus allowing for earlier O_2 reduction. The final O_2 concentration of run 5 is consistent with slight loss of catalyst activity with repeated use.

Temperature changes in the anaerobe jar are also shown in Table 3. As expected the temperature increases as the $H_2 + O_2$ reaction proceeds, with

Table 3. Summary results of changes in O₂ concentration (% reduction of O₂), pressure (mm Hg), and temperature (°C) during 6 runs of the GasPak anaerobe system (BBL).

Time (min.)	<u>% Oxygen Reduction</u>					
	Run Number					
	<u>1</u>	<u>2</u>	<u>3</u>	<u>4</u>	<u>5</u>	<u>6</u>
5	0	1.4	0	.9	1.4	.48
10	57.1	27.4	27.0	23.8	40.5	12.1
15	86.7	77.4	66.8	67.1	73.3	53.6
20	93.8	92.0	79.6	81.4	85.2	73.9
25	97.4	98.3	89.1	88.6	91.4	82.1
30	99.9	99.9	93.8	92.9	94.3	88.9
35	99.9	99.9	95.7	94.8	94.3	91.3

<u>Pressure in mm Hg</u>						
5	+14	+ 4	+ 6	+ 7	+ 9	+ 5
8	+44	+15	+22	+17	+23	+15
10	+16	+24	+27	+19	+19	+22
12	- 4	+ 4	+15	+26	+ 9	+21
15	- 7	- 8	-15	- 5	- 3	+ 5
20	+ 3	+ 3	-13	- 8	+ 2	-18
25	+19	+13	+ 4	- 2	+ 4	- 7
30	+ 8	+12	+10	+ 5	+ 7	+11
35	+ 2	ND	+ 9	+ 4	ND	+ 6

<u>Temperature (°C)</u>						
0	ND	ND	23.0	23.0	23.5	21.0
8	ND	ND	26.0	23.5	23.7	22.0
13	ND	ND	27.2	24.0	24.0	23.0
26	ND	ND	29.0	27.0	26.0	25.0
36	ND	ND	28.0	27.0	26.0	26.0
40	ND	ND	28.0	26.5	25.5	26.0

maximum temperature being achieved shortly after the majority of O_2 has been reacted. As the reaction becomes less active, temperature in the jar again begins to decrease. Temperature changes obviously do affect pressure, but because the temperature changes were relatively small, it is felt that the pressure changes were due primarily to gas release and depletion. The effect of temperature on pressure changes in the jar is currently being investigated.

Much of our previous work dealt with psychrotrophic organisms, and in many experiments we isolated psychrotrophs anaerobically using the Brewer anaerobe jar with GasPak and cold catalyst. We observed very early that placing the anaerobe jar directly into a low temperature incubator will result in no color change in the indicator. For this reason it became standard procedure to leave the jars at room temperature for 30 min. prior to placing them at low temperature. On occasion we have also used the heated catalyst anaerobe jar, placing them immediately at low temperature, but leaving the heating element on for 30 min. Both procedures resulted in successful establishment of anaerobic environments.

To demonstrate the effect of low temperature incubation on reliability of the cold-catalyst jar, we ran a series of experiments as above, but placed the jars immediately into a $0^{\circ}C$ incubator. The results are shown in Figure 4. Not only is anaerobiosis affected, but the heat of the catalyst delays establishment of incubator temperature inside the jar. The temperature decreases from $21^{\circ}C$ to $4.0^{\circ}C$ in the first 50 min., but decreases to $0^{\circ}C$ only after another 3.3 hrs.

That H_2 is being generated by the GasPak is indicated by the O_2 reduction, although there is no increase in pressure. It appears that pressure increase due to H_2 and CO_2 is more than offset by pressure decrease due to lower temperature. Positive pressure is not achieved, and a constant negative pressure of -35mmHg is achieved after 75 min.

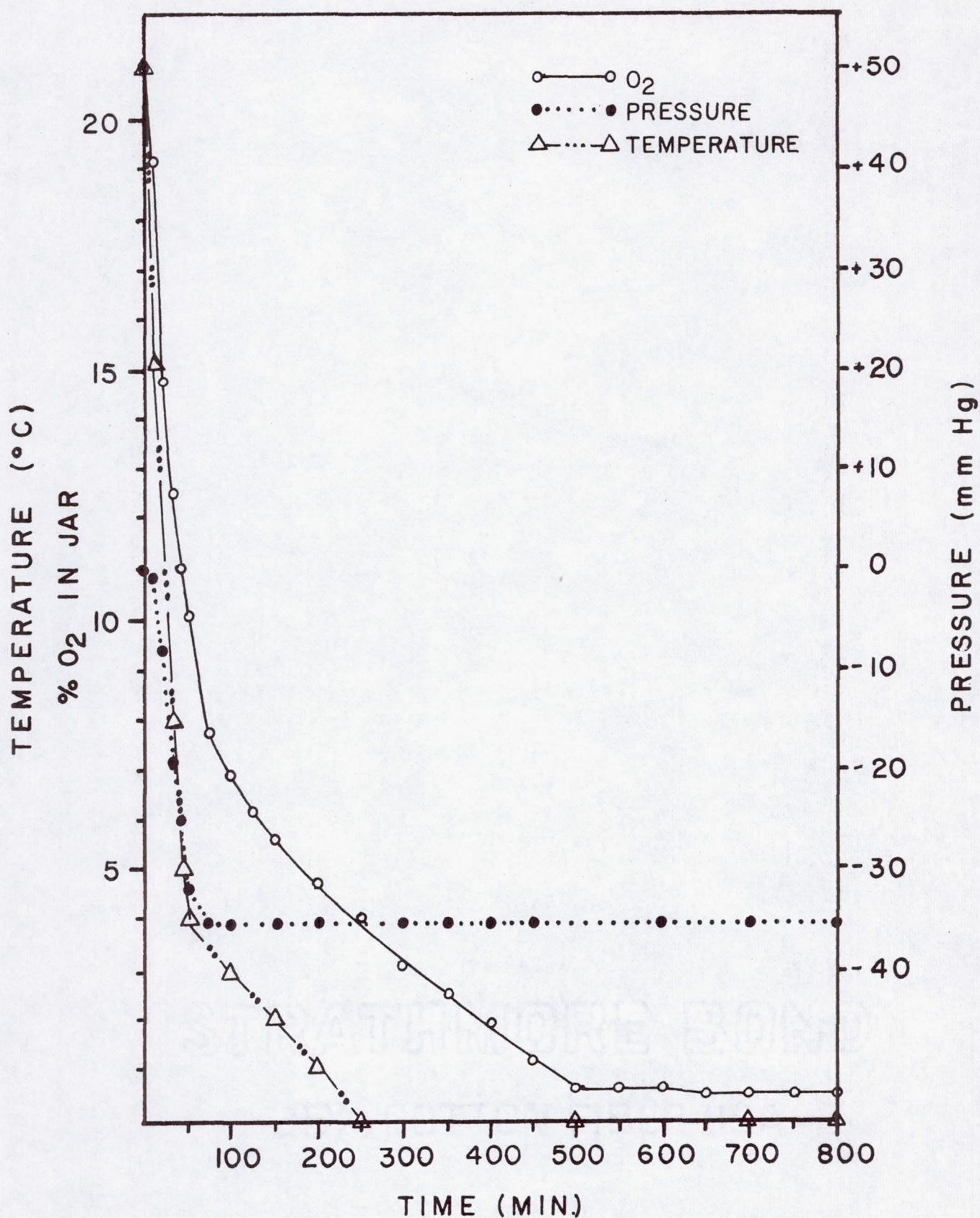


FIGURE 4. OXYGEN, PRESSURE, AND TEMPERATURE CHANGES
IN A BREWER ANAEROBE JAR-GASPAK SYSTEM (BBL) PLACED
IMMEDIATELY INTO A 0°C INCUBATOR.

Removal of oxygen occurs slowly with only 45.2% reduction in O_2 concentration after 35 min. This gradual decrease continues until it reaches minimum O_2 concentration after 8.3 hrs. At this point O_2 concentration is approximately 0.6%, or 97% of the O_2 has been removed. These results indicate that a great deal more time is required for the anaerobe jar to achieve anaerobiosis in the low temperature incubator. This prolonged time requirement would likely have a lethal effect on some anaerobes.

In another series of experiments the jars were prechilled to $10^{\circ}C$, assembled as per instructions, and placed at $4^{\circ}C$. Figure 5 shows a representative curve of these results. The O_2 concentration decreases to about 8% in 20 min., 7% in 35 min., and finally reaches 1.3% at 5 hrs. The temperature decreases from 10° to $6^{\circ}C$ in 35 min. and reaches $4^{\circ}C$ after 60 min. Because the jar was prechilled, there is less of a temperature change, and pressure changes follow a pattern similar to those in the experiments at room temperature. An obvious difference is that the pressure remains negative after the initial peak and drop, holding at about -23 mm Hg after 35 min.

Experiments performed by holding the jars at room temperature for 30 min. prior to placing them in the low temperature incubator are in progress, and results are not available at the writing of this report. These will be included in our next report along with results of other experiments including evaluation of the heated catalyst jar for psychrotrophic isolations, effect of presence of culture media on the removal of O_2 from the anaerobe jars, and others. We will also evaluate the procedure of using a replacement atmosphere such as N_2 to establish anaerobic conditions.

B. Use of Freon in an Attempt to Grow Anaerobes

In some of our work not related to NASA research, it was suggested to

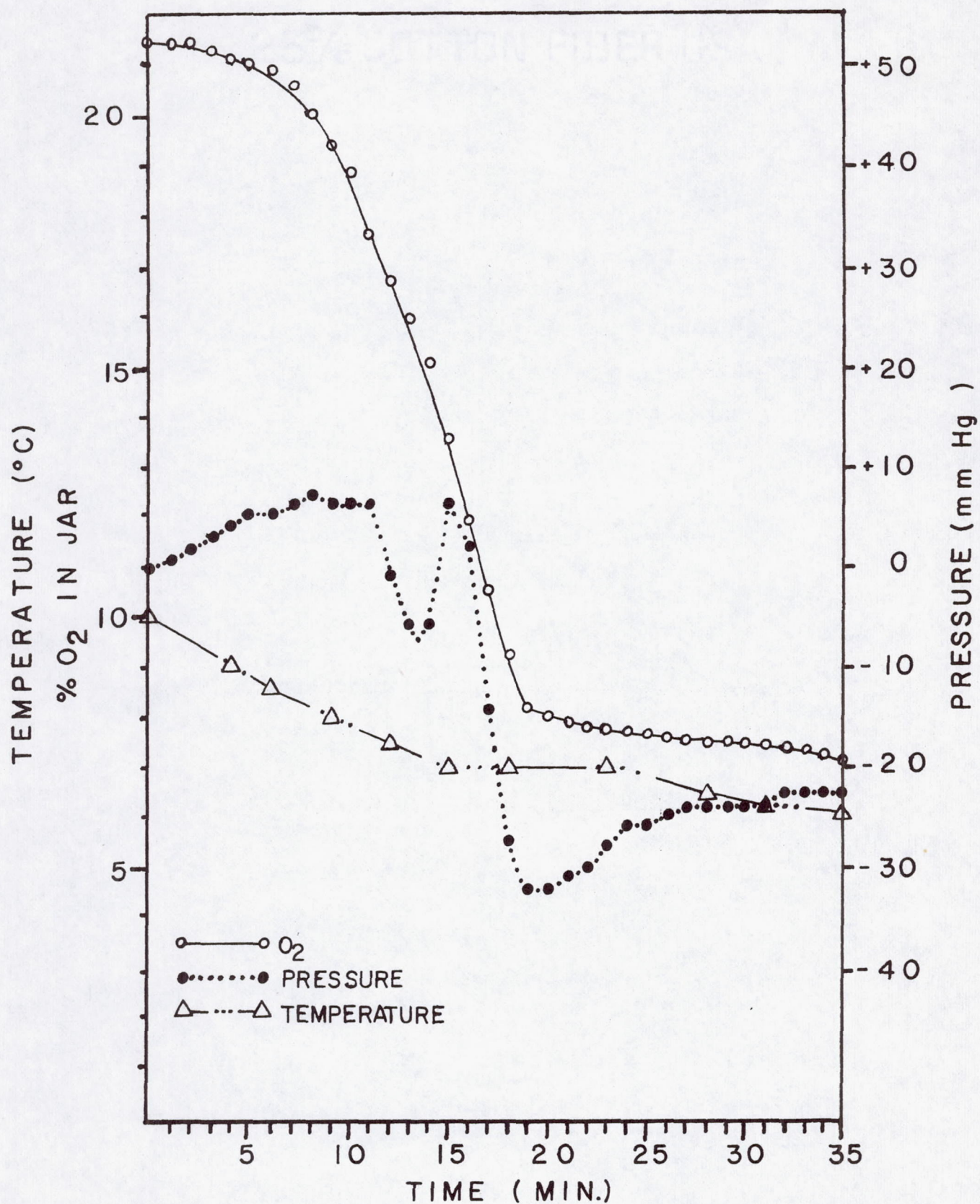


FIGURE 5. OXYGEN, PRESSURE, AND TEMPERATURE CHANGES IN A BREWER ANAEROBE JAR-GASPAK SYSTEM (BBL) PRECHILLED AT 10°C THEN PLACED INTO A 4°C INCUBATOR.

use Freon in an attempt to neutralize H_2O_2 thus allowing anaerobes to grow. It is known that the lethal effect of O_2 on many anaerobes is that they form H_2O_2 and have no system to neutralize this by-product. The same principle has stimulated other investigators to employ catalase in their culture media, and the results have been encouraging. With this in mind, we have recently initiated experiments to evaluate the use of Freon to allow aerobic growth of anaerobes. Because Freon is considered non-toxic, such a system would allow simultaneous growth of aerobes and anaerobes from a mixed sample. These investigations have only recently been initiated, but a preliminary report will be given here. Other data will be available for our next report.

The first experiments included use of a vented Brewer anaerobe jar as the vessel in which to establish the Freon atmosphere. Commercially available Freon 12 was obtained and used in many of these investigations. Replicate TSA plates were inoculated with C. novyi, C. perfringens, and Alcaligenes fecalis. One set of plates were placed into a standard GasPak anaerobe jar, one set in an anaerobe jar flushed with and maintained at 3 psi Freon 12, and one set incubated aerobically. After 48 h incubation at $32^{\circ}C$, these were examined, and the results were as follows: (1) Anaerobe jar - Indicator turned white - C. novyi, good growth; C. perfringens, good growth; A. fecalis, no growth; (2) Freon jar - Indicator almost white - C. novyi, good growth; C. perfringens, good growth; A. fecalis, good growth; (3) aerobic plates - no indicator - C. novyi and C. perfringens, no growth; A. fecalis, good growth. These early results are encouraging, and similar investigations are in progress. A similar experiment was performed with no catalysts in the Freon jar. The clostridia did not grow in this system. This and the fact that the indicator changed colors in the previous investigations indicates that if Freon is indeed allowing growth of anaerobes, it might be reacting with the O_2

to create anaerobic conditions. This is currently being investigated with our O₂ monitoring system.

Simultaneous investigations were performed with that of the preceding paragraph. One such experiment included bubbling Freon 12 through TSB and inoculating with C. novyi. No growth occurred initially, so this phase was repeated with the addition of 0.1% agar to the TSB. None of the flasks with TSB alone showed growth, but all flasks with 0.1% agar showed good growth. This indicates that Freon 12 may be reacting with O₂, and the 0.1% agar prevents convection currents in the medium, thus causing less O₂ to redissolve in the TSB. Another possible explanation is that the agar is causing the Freon to remain dissolved in the TSB.

In another investigation TSA was prepared, cooled to 45°C, and Freon 12 was bubbled through the molten TSA. This procedure resulted in intense foaming of the TSA, and the foam was allowed to subside prior to pouring plates. Another set of plates were prepared in which Freon was bubbled through the TSA prior to autoclaving. These and appropriate controls were inoculated with C. novyi and C. perfringens. The plates with Freon 12 added prior to autoclaving demonstrated no growth, and those with Freon added at 45°C showed very scant growth. As expected, this indicates that the solubility of Freon is decreased with increasing temperature and that most of the Freon escaped from the culture medium. In all of these experiments aerobic control plates showed no growth, but anaerobic control plates showed good growth of the clostridia.

In an attempt to determine the mechanism of action of Freon, an experiment was set up to determine the effect of Freon inactivation of H₂O₂. In this investigation duplicate sets of flasks of TSB were prepared. H₂O₂ was added to flasks of each set to give concentrations ranging from 10% to 0.06%.

One set had Freon bubbled through it, the other set was a control and had no Freon. These were inoculated with a catalase negative organism, and results showed growth in the flasks without Freon at H_2O_2 levels of 0.0% - 0.33%. Growth occurred in the Freon-treated flasks from 0.0% - 0.66%. Because of this slight difference and because of the earlier effect of agar on growth, this entire experiment was repeated with the addition of 0.7% agar. The results of this investigation indicated that the flasks without Freon again supported growth at H_2O_2 levels of 0.0% - 0.33%, the same as without agar. However, the Freon-treated flasks supported growth in H_2O_2 levels of 0.0% - 3.3%. These results indicate that Freon is causing inactivation of H_2O_2 . Because of the results, this investigation is currently being expanded to include use of several different microorganisms, both aerobic and anaerobic.

In an attempt to evaluate other Freons, Freon 113, which is liquid at room temperature, and Freon 11, which is liquid below $17^{\circ}C$, were subjected to the same experimental procedures as described above. The results with these materials were basically unsatisfactory, and, for now, future work will concentrate on the use of Freon 12 in our systems. If this proves to be reliable, it will provide a simplified system in which to grow aerobes and anaerobes simultaneously. We are also investigating the use of Freon as a transport and/or storage material for anaerobic organisms.

C. Evaluation of a Culture Medium to Differentiate Between Obligate and Facultative Anaerobes

In many procedures involving anaerobic microbial assay of space hardware, it is necessary to inoculate a sample containing mixed populations and incubate these anaerobically. During examination of growth, it is not possible to visually differentiate the obligate anaerobes from the facultatives. Therefore, it seems desirable to have a procedure to visually differentiate

obligates from facultative anaerobes. This will be especially true if the Freon system allows simultaneous growth of aerobes and anaerobes. To this end, a culture medium was designed and is being evaluated.

The medium in use is TSA with 1.0% starch, 0.1% yeast extract, and 0.1% KI added. Theoretically, the release of H_2O_2 by a catalase negative isolate on exposure to O_2 will convert the KI to I_2 which reacts with the starch to form a blue ring around the colony. In this way, most obligate anaerobes can be detected without testing their ability to grow aerobically. We have used this procedure on mixed populations. After anaerobic incubation, colonies were transferred to duplicate TSA slants for aerobic and anaerobic incubation, then plates were allowed to set aerobically. Blue rings did appear around the colonies which proved to be obligate anaerobes. Most aerobes produce H_2O_2 but degrade it by production of catalase before it can convert KI to I_2 .

In population investigations where the cultures do not have to be saved, this is a very quick procedure. We are now in the process of testing numerous different organisms to evaluate the efficiency of the medium, and these investigations are not yet completed. The procedure becomes more complicated when the cultures need to be saved because they must be transferred prior to the development of I_2 which is toxic. In the past we have worked on replicate-plating procedures, and this will be begun again if this medium proves to be practical.

Because this I_2 -starch system is indicative of H_2O_2 production, we are also attempting to use this procedure to measure oxygen sensitivity of anaerobes colorimetrically. If this can be done, it may be a quantitative procedure to rank the anaerobes as to their oxygen tolerance.

Other experiments concerning anaerobic microbiology of space hardware are in progress. These are in keeping with our proposal to improve techniques or basic knowledge of anaerobic microbiology for application in this field and will be described in our next report.

OTHER AREAS OF INVESTIGATION

Associated with the tasks described in this report, other topics of investigation are frequently required. We continue to maintain cultures of many organisms isolated from previous investigations. In doing this, we are attempting to determine a desirable storage medium for most of these isolates. To this end we have initiated a prolonged investigation on the use of diluted TSA as a maintenance medium. The results of this investigation will not be available for some time.

Because much of our work deals with bacterial population increases or decreases, we have written a computer program to statistically process our data. Very soon we should also have access to equipment to store data for immediate retrieval.

ACTIVITIES SINCE LAST REPORT

Since our last report activities of members of the Science Research Center have included the following:

- Two presentations at the 19th COSPAR meeting in Philadelphia,
- Two papers accepted for publication in Life Sciences and Space Research,
- One presentation at the Texas Branch- ASM-Fall, 1976,
- Two presentations at the National ASM-May, 1977,
- One paper accepted for publication in the August issue of Applied and Environmental Microbiology,

One chapter revision of the NASA publication Advances in Sterilization
and Decontamination to be published this summer, and

One section of a chapter in Advances in Sterilization and Decontamination.