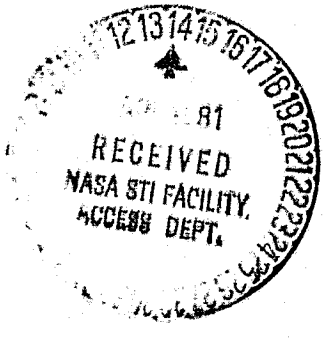
February, 1981

Final Report (No. 11)

RESPONSE OF SELECTED MICROORGANISMS TO EXPERIMENTAL PLANETARY ENVIRONMENTS

Science Research Center
Hardin-Simmons University
Abilene, Texas



RESPONSE OF SELECTED MICROORGANISMS TO EXPERIMENTAL PLANETARY ENVIRONMENTS

Final Progress Report No. 11 of
Planetary Biology and Protection Activities

June 30, 1979 - December 31, 1980

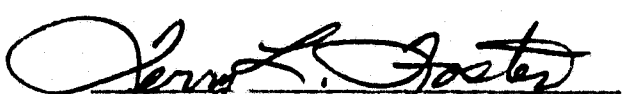
Supported by
NASA Grant NGR 44-095-001

Submitted to the
National Aeronautics and Space Administration
Washington, D.C.

by the

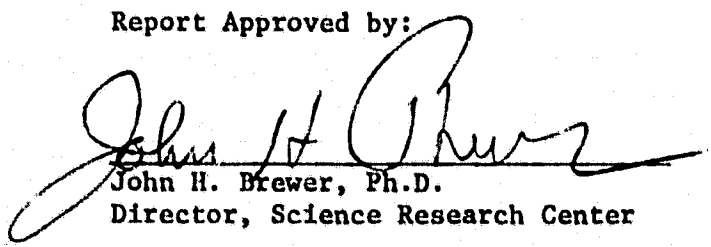
Science Research Center
Hardin-Simmons University
Abilene, Texas 79698

Report Prepared and Submitted by:



Terry L. Foster, Ph.D.
Principal Investigator

Report Approved by:



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

February, 1981

FOREWORD

This final progress report summarizes work performed for the National Aeronautics and Space Administration by the Science Research Center at Hardin-Simmons University and covers the period June 30, 1979 - December 31, 1980. It also provides a brief history of research activities from September, 1972 - December, 1980 and a summary of significant contributions made by this research effort.

One major emphasis of the last grant period was the continued evaluation of microbial response to various pressures of H_2, CH_4, H_2S, and NH_3. The bulk of this data was presented in Report No. 10 and the final data is included in this report in the form of a manuscript to be submitted for publication.

The major task investigated during this final grant period was the continued evaluation of new procedures for establishment of an anaerobic environment. Report No. 10 described new procedures being considered. One of these in particular was successfully completed by BBL Microbiology Systems. The new system is a new H_2/CO_2 generator envelope called GasPak II. This device has been extensively evaluated in our laboratory, and its use by NASA as an adaptable means for establishing anaerobic conditions is strongly recommended.

The NASA Technical Officer for this grant is NASA Code SBL, Planetary Biology and Protection, Washington, D.C.

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MICROBIAL RESPONSE TO VARIOUS PRESSURES OF HYDROGEN, METHANE, AMMONIA, AND HYDROGEN SULFIDE

In Report No. 10, the background of our work on the microbial response to various pressures of H_2, CH_4, NH_3, and H_2S was described and most of the results on this effort were presented. Since that report, the project has been completed and terminated and microbial response trends described in Report No. 10 were continued during the final phase of this project. The final results were presented at the National Meeting of the American Society for Microbiology in May, 1980. A manuscript of this work has been prepared and submitted for publication. The manuscript is included in this report as a final report on this task.

MICROBIAL RESPONSE TO VARIOUS PRESSURES OF HYDROGEN, METHANE, AMMONIA, AND HYDROGEN SULFIDE

T. L. Foster and C. D. Morgan

Science Research Center, Hardin-Simmons University, Abilene, Texas

At the recommendation of the Space Science Board, anaerobic and aerobic sporeformers and non-sporeformers were cultivated anaerobically in nutrient media under various pressures (up to 1800 psi) of pure H_2, CH_4, NH_3, and H_2S. Viability assays were performed periodically to determine growth, survival, or spore survival. Hydrogen up to 1800 psi demonstrated little or no suppression of growth with the possible exception of Bacillus coagulans at 1800 psi. The obligate anaerobes grew very well. Under CH_4 the obligate anaerobes again exhibited the most prolific growth, whereas the facultative anaerobes grew well except under higher pressures. Ammonia at low pressure was extremely toxic to all test organisms. At 100 psi all populations were killed within 24 hours except Staphylococcus aureus which survived for 72 hours and the Bacillus spp. which produced a surviving population of approximately 10^4 spores/ml. All populations in H_2S were killed within 24 to 48 hours except Proteus mirabilis which decreased to 10^2 cells/ml and the Bacillus spp. Spore survival studies of two months duration demonstrated that B. coagulans and B. pumilus survived under all experimental conditions. Clostridium novyi type B and C. sporogenes were killed rapidly in NH_3 and H_2S and demonstrated no sporulation. All positive control samples exhibited rapid population increases.

Running Title: Microbial Response to Various Gases

INTRODUCTION

In May, 1976 the Exobiology Panel of the Space Science Board submitted a document entitled "Recommendations on Quarantine Policy for Uranus, Neptune, and Titan" to the NASA Administrator (5). This document included recommendations about quarantine policy and included specific recommendations for suggested experiments to overcome "some glaring deficiencies in fundamental knowledge". They felt that such experiments would be of interest to planetary and earth-based biology and would be of significant assitance in determining the chances of growth and survival of microorganisms on outer planets. This type of information is vital to the United States international commitment to prevent biological contamination of planets under investigation by the United States space program (1). Such experiments would also provide fundamental knowledge concerning limits of microbial growth in various environmental parameters. Specific recommendations included in this document are as follows:

1. Determination of the upper limits of growth and spore survival in various hydrogen concentrations and pressures;
2. Determination of the upper limits of growth and spore survival in various concentrations and pressures of other gases likely to prevail in the envelopes of the outer planets, e.g. hydrogen sulfide, methane, and ammonia;
3. Search for microorganisms which can utilize phosphine as a sole source of phosphorous;
4. A survey of the sensitivity of microorganisms to phosphine;
5. A search for anaerobic halophiles and a determination of their tolerance to low water activity;

6. The tolerance of recently isolated anaerobic methane oxidizing bacteria to simulated Titan conditions should be investigated. An investigation should also be made of the microelement nutrient requirements of microorganisms at the lower temperature limits of growth; and
7. Further investigations on the growth of microorganisms in water droplets suspended in atmospheres should be undertaken.

These recommendations of the Space Science Board were published by Margulis, et al in 1977, and their summary statements concerning suggested experimentation were essentially the same as the seven mentioned above (4).

The objective of this study was to determine the effect of various pressures of H_2, CH_4, NH_3, and H_2S on growth or survival of selected microorganisms. The immediate application was to determine if microorganis can grow in pressures of the predominant gases of the proposed "biozone" of Jupiter and Saturn. In this investigation, pressure was limited to that of the proposed atmospheric zone of biological interest of Jupiter (2) instead of determination of the upper pressures of these gases at which growth occurred.

MATERIALS AND METHODS

Microorganisms --- Organisms used in this investigation were Bacillus coagulans (Cape Canaveral isolate), B. pumilus (Cape Canaveral isolate), Clostridium novyi type B, Fusobacterium necrophorum, Corynebacterium xerosis, Pseudomonas aeruginosa, Proteus mirabilis, Escherichia coli, Staphylococcus aureus. Because Jupiter possesses an anaerobic atmosphere, the potential

terrestrial contaminants must possess the capability of growing in the anaerobic environment which was used throughout this investigation. The strict aerobes (P. aeruginosa and B. pumilus) were included as controls for anaerobiosis.

Experimental gases --- All gases used in this investigation were certified pure or research quality gases from Matheson Gas Products (LaPorte, Texas). Samples were cultivated in high pressure, stainless steel cylinders (500 ml size, Matheson Gas Products) fitted with high pressure needle valves. All connectors, tubing, and valves were rated at a minimum of 2500 psi.

Pressurization of cultures --- Cultures were grown anaerobically for 24 to 48 hours in Trypticase Soy Broth (TSB, BBL Microbiology Systems, Cockeysville, Maryland). The stainless steel cylinders were half-filled with TSB, autoclaved for 20 minutes with valves open, and the valves were closed immediately upon removal from the autoclave. After the cylinders cooled, the pure culture was drawn into a sterile, air-tight syringe which was then connected to the cylinder by connectors which had been flushed with nitrogen. When the valves were opened, the vacuum pulled the sample into the cylinder. This was followed by 5.0 ml of sterile, prereduced TSB to rinse the needle valve. All cylinders were pressurized to 30 psi with the desired gas passed through in-line bacteriological filters, and the cylinders were shaken to distribute the organisms. Replicate samples were immediately removed, and counts were performed by standard plate count procedures for enumeration of the original population. The cylinders were pressurized to the desired pressure and incubated on a shaker at room temperature. Replicate samples were removed for enumeration every 6, 12, or 24 hours for the

duration of the experiment. Anaerobes were diluted and plated in prereduced media and incubated 48 to 72 hours prior to counting. Facultative anaerobes were incubated aerobically. Turbidity of all samples was monitored on a Klett-Summerson colorimeter, but results are not included because of close agreement with actual plate counts. In long-term experiments, the spore-forming organisms were inoculated into the high pressure cylinders as described above. Initially, samples were taken every 24 hours, then every 96 hours for at least two months. Spore assays were performed by heat-shocking the samples at 60°C for 30 minutes according to standard procedures (6).

Controls --- In an early study on survival of organisms at different pressures, known concentrations of selected organisms were deposited into sterile, stainless steel or plastic cups (7 x 7 mm) or coated onto sterile beads. These were air-dried under laminar flow and counts were again performed. Multiple samples were placed into different stainless steel cylinders and subjected to various pressures up to 2000 psi of hydrogen or nitrogen. These were stored at room temperature with samples being removed periodically for enumeration. One set was not pressurized but was stored under hydrogen as a viability control. Results of this study indicated no significant differences of survival between samples stored under nitrogen as compared to hydrogen and no significant differences in survival at the different pressures of either gas. On the basis of this work, it was decided to use nitrogen at 30 psi as a positive control for future work and a nitrogen control was included with every experimental set.

RESULTS

Growth and survival responses of the experimental organisms are presented in Figures 1-7. Each point is an average of 4 counts. Although all curves are not presented here, each assay was performed at least in duplicate. Duplicate and triplicate assays demonstrated the same trends as those presented in Figures 1-7. In all experiments, control cultures under pressurized N_2 exhibited prolific growth (greater than 3 logs in 48 hours). The aerobic control organisms (P. aeruginosa and B. pumilus) failed to grow in the anaerobic atmospheres and were killed rapidly in NH_3 and H_2S. Spores of B. pumilus survived in all atmospheres.

As a general rule, all organisms showed population increases under all pressures of H_2 with the exception of C. xerosis, which showed stable populations under all pressures, and B. coagulans, which grew at 500 and 1200 psi, but was stable at 1500 and 1800 psi. All organisms showed population increases or stable populations in CH_4, except C. xerosis, which produced a gradually declining population. All organisms were killed rapidly in NH_3 except B. coagulans, which produced a stable spore population. The anaerobic sporeformer did not produce a surviving spore population. All organisms were killed rapidly under H_2S except B. coagulans, which produced a stable spore population, and P. mirabilis, which declined initially but later demonstrated a slight population increase.

Spore survival studies of two-month duration demonstrated that B. coagulans and B. pumilus sporulated under all gases and that the spores survived for the duration of the experiment. C. novyi did not sporulate under any of the gases; therefore, was killed in the presence of NH_3 and H_2S.

CONCLUSIONS

Within the pressures investigated, H_2 and CH_4 exhibited virtually no toxic effect to the organisms tested. Ammonia and H_2S were generally toxic to vegetative cells at relatively low pressures. Although no ammonia-tolerant organisms were demonstrated, researchers at Ames Research Center (3) have isolated bacteria which grow in high concentrations of ammonia.

From these studies, it appears that the proposed concentrations of NH_3 and H_2S in the "biozone" of Jupiter and Saturn should be sufficient to prevent microbial contamination of their atmospheres. Although prolonged spore survival studies indicated survival of B. spp. spores for up to two months, these germinated only after removal from the experimental atmospheres.

ACKNOWLEDGEMENTS

This investigation was supported by the National Aeronautics and Space Administration under grant NGR 44-095-001.

LITERATURE CITED

1. COSPAR. Resolution 26. Fifth International Space Science Symposium. Florence, Italy, 1964. COSPAR Inform. Bull. No. 20, pg. 25-26.

2. Chang, S. and R. D. MacElroy. 1977. Possible nutrients and energy sources available to contaminating terrestrial organisms on Jupiter, pg. 89-93. In: R. Holmquist and A. C. Stickland, (eds.), Life Sciences and Space Research, Vol. XV, Pergamon Press, New York.

3. Deal, P. H. (NASA, Ames Research Center, Moffett Field, California, USA). Toxicity of ammonia for microorganisms. In Life Sciences and Space Research, Pergamon Press, New York.

4. Margulis, L., H. O. Halvorson, J. Lewis, and A. G. W. Cameron. 1977. Limitations to growth of microorganisms on Uranus, Neptune, and Titan. Icarus 30:793.

5. Mazur, P. (Chairman). 1976. Recommendations on quarantine policy for Uranus, Neptune, and Titan. Exobiology Panel. Space Science Board. Recommendations to the NASA Administrator.

6. National Aeronautics and Space Administration. 1968. NASA standard procedures for the microbiological examination of space hardware. NGB 5340.1A. National Aeronautics and Space Administration, Washington, D.C.

FIGURE 1: The response of Bacillus coagulans when subjected to various pressures of hydrogen, methane, ammonia, and hydrogen sulfide.

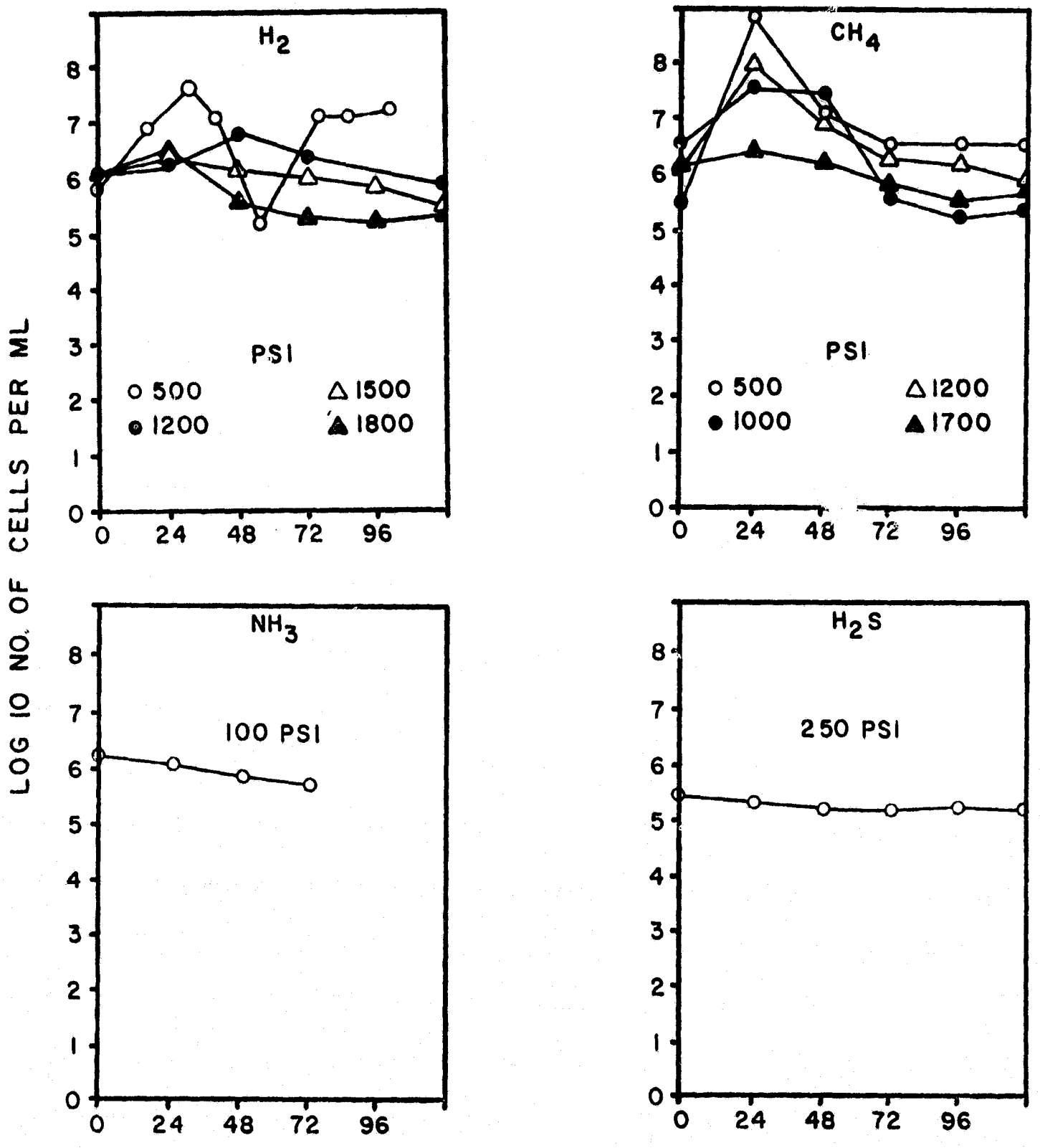


FIGURE 2: The response of Clostridium novyi when subjected to various pressures of hydrogen, methane, ammonia, and hydrogen sulfide.

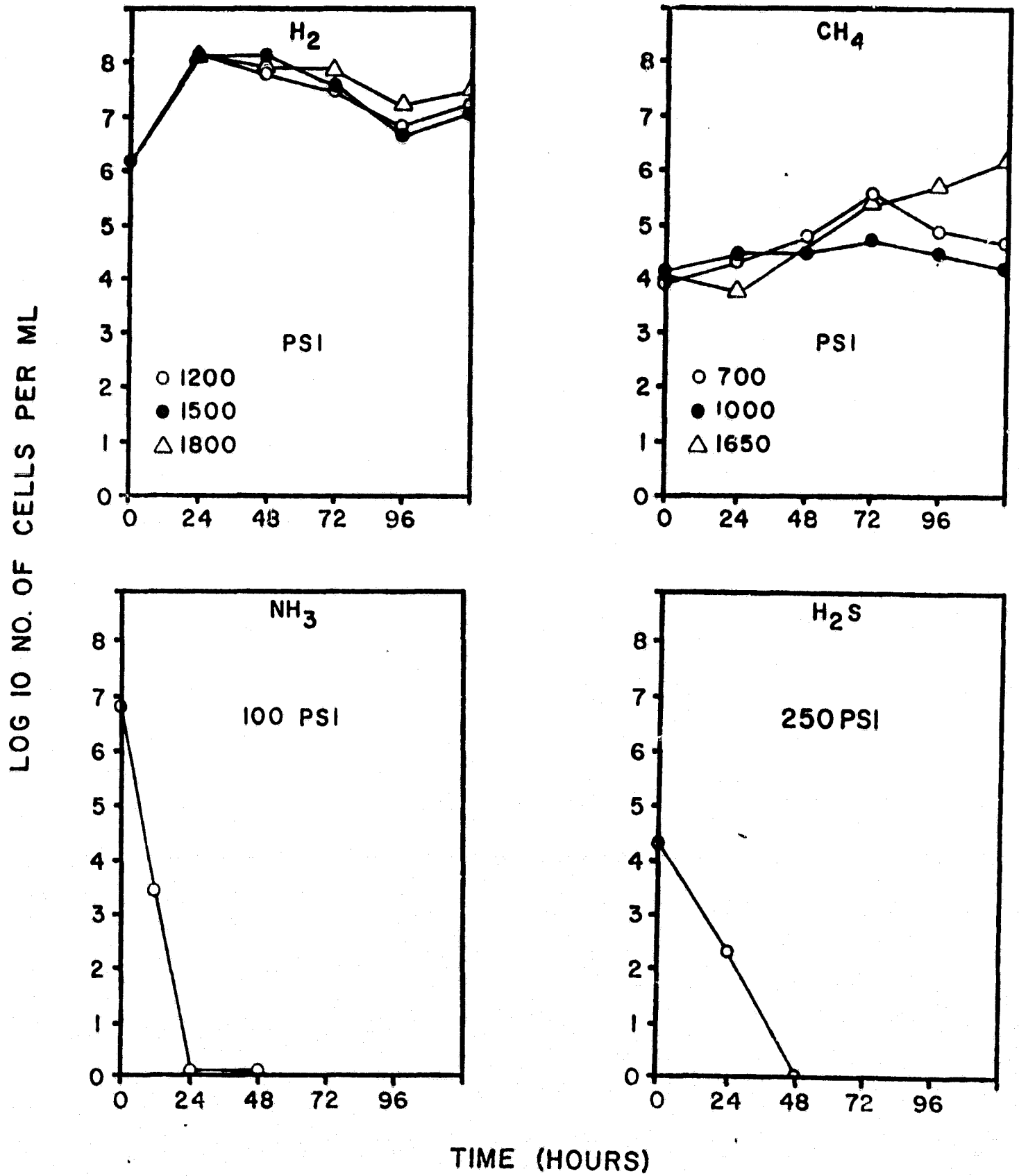


FIGURE 3: The response of Corynebacterium xerosis when subjected to various pressure of hydrogen, methane, ammonia, and hydrogen sulfide.

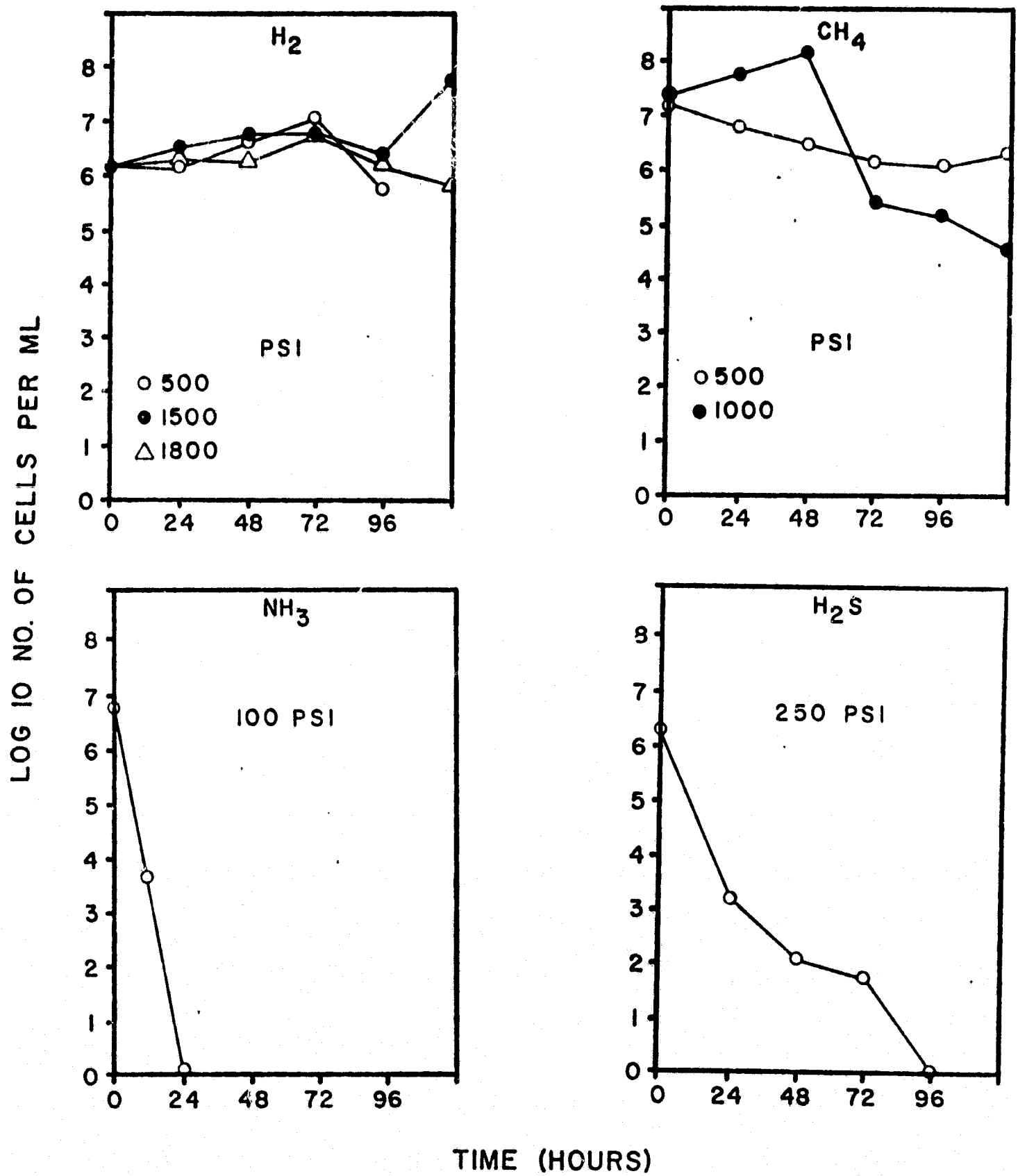


FIGURE 4: The response of Escherichia coli when subjected to various pressures of hydrogen, methane, ammonia, and hydrogen sulfide.

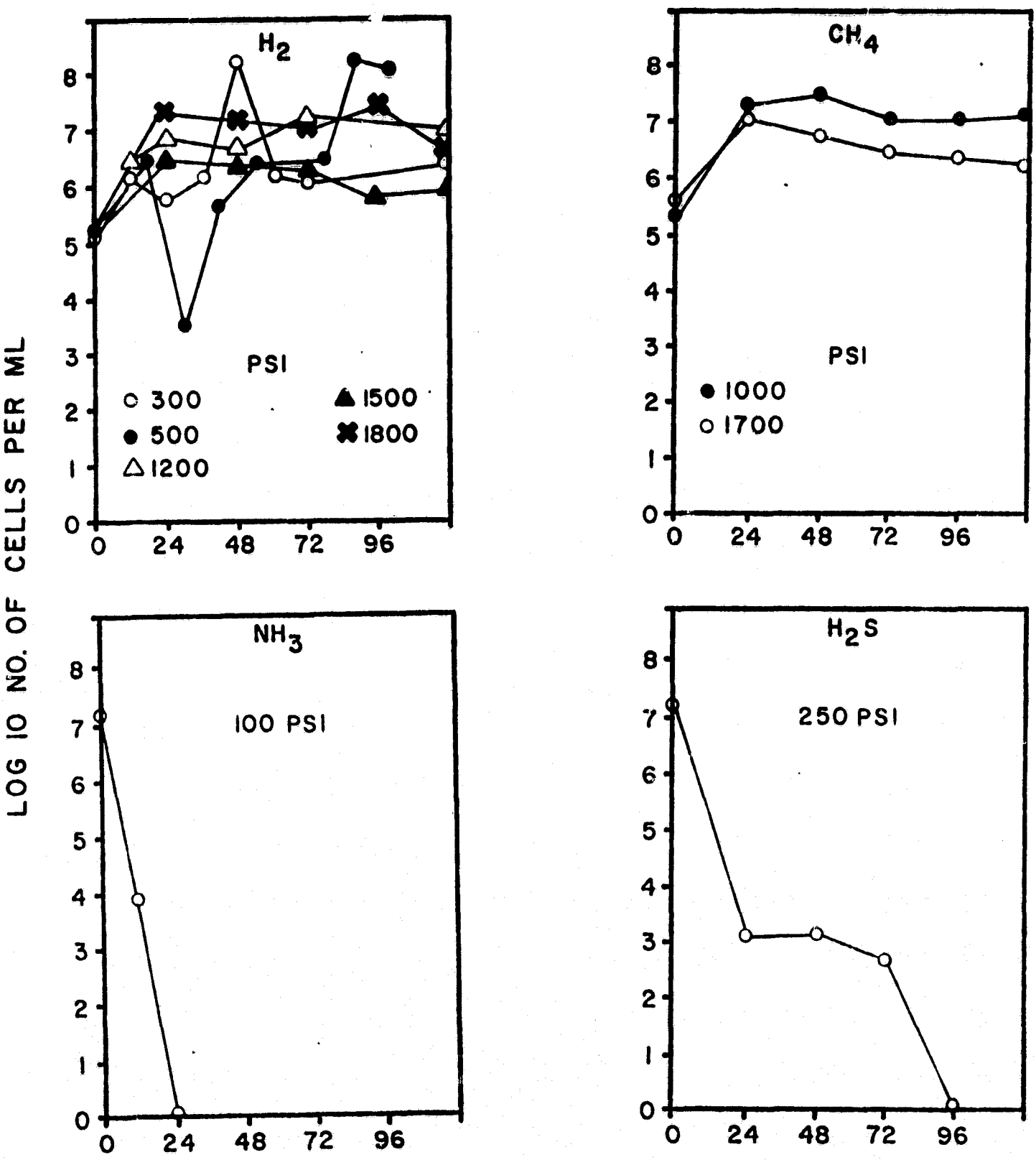
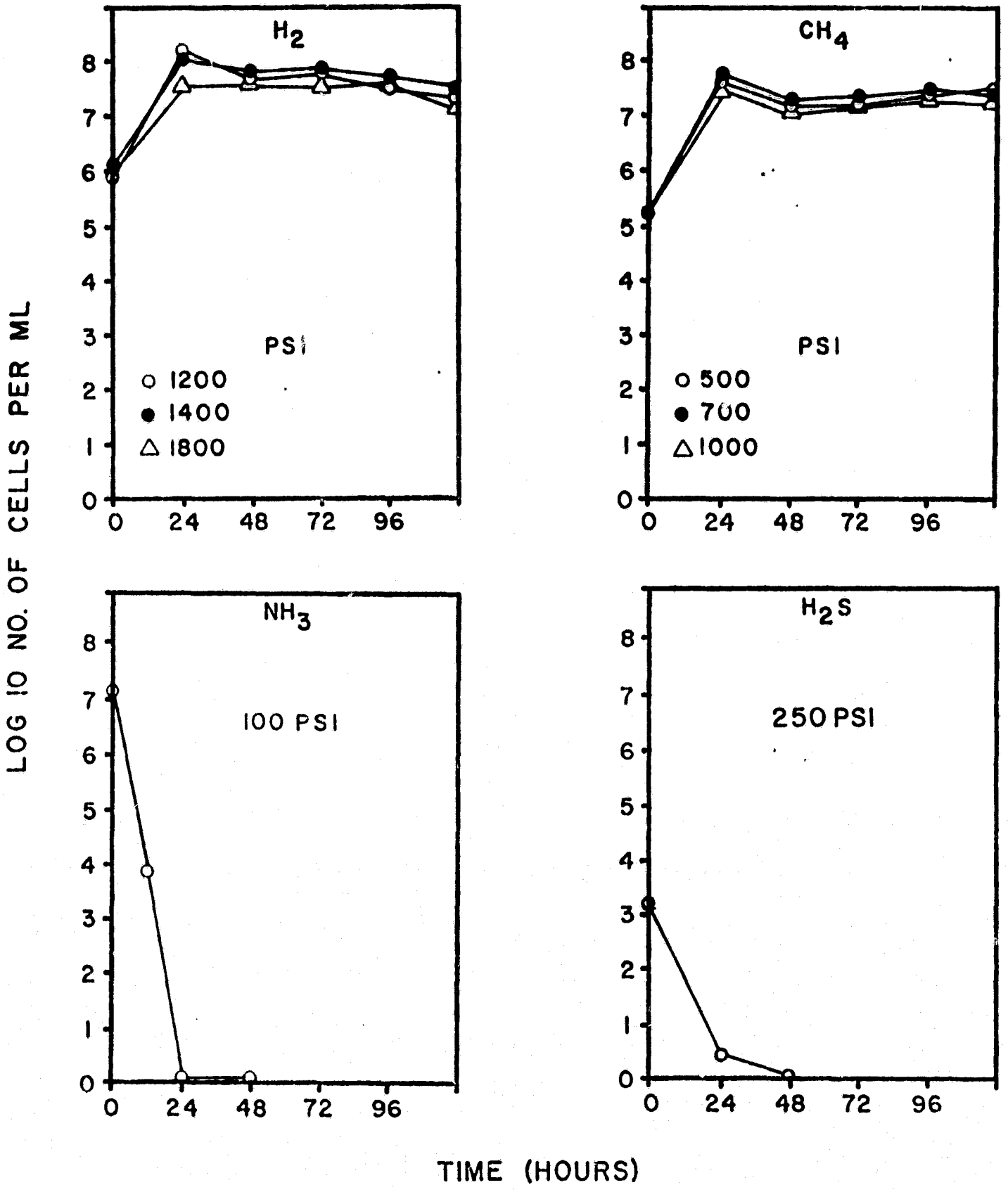


FIGURE 5: The response of Fusobacterium nechrophorum when subjected to various pressures of hydrogen, methane, ammonia, and hydrogen sulfide.



FIGUKE 6: The response of Proteus mirabilis when subjected to various pressures of hydrogen, methane, ammonia, and hydrogen sulfide.

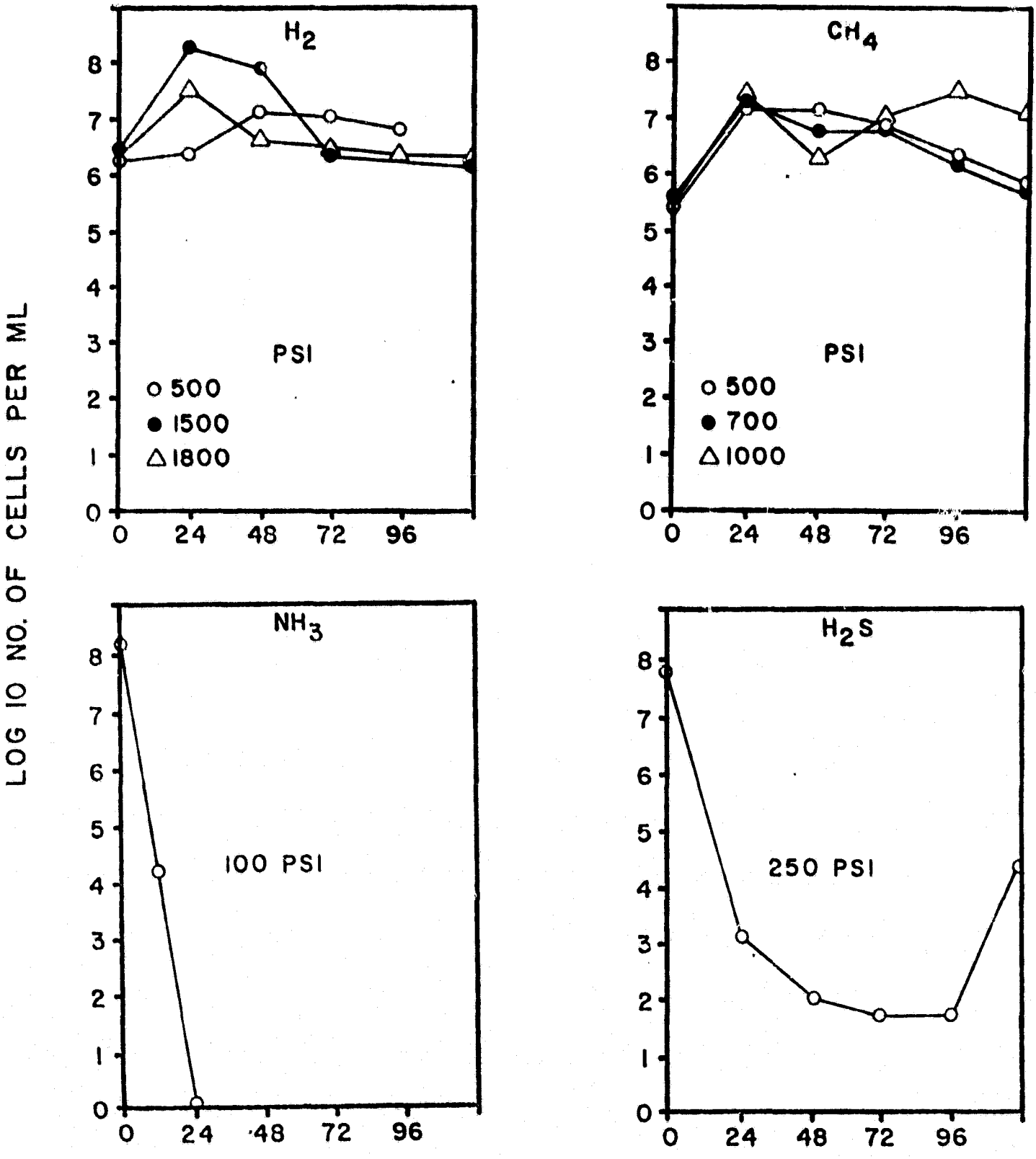
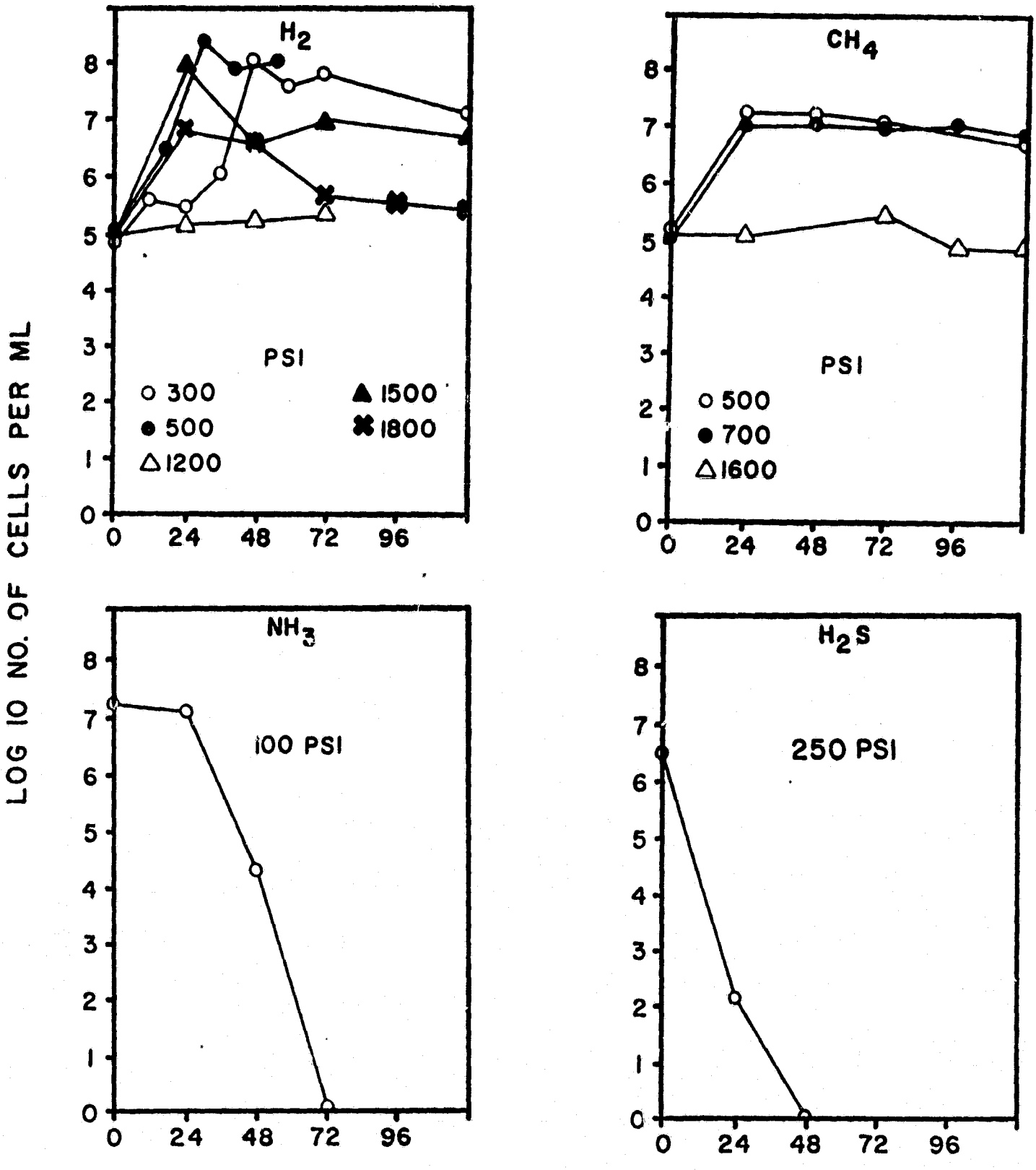


FIGURE 7: The response of Staphylococcus aureus when subjected to various pressures of hydrogen, methane, ammonia, and hydrogen sulfide.



Results from the preceeding manuscript and from the phosphine experiments in Report No. 10 provide experimental data useful to answering four of the seven points raised by the report of the Space Science Board to the NASA Administrator. These four recommendations have particular application to potential microbial contamination of Jupiter and/or Saturn. Based upon the research performed at the Science Research Center, microbial contamination of atmospheres of these planets is unlikely. If the NH_3 or H_2S does not restrict growth or kill the potential contaminants, inability to utilize PH_3 as a source of phosphorus will severely reduce the probability of microbial contamination.

ANAEROBIC MICROBIOLOGICAL PROCEDURES

IMPROVED GASPAK H_2/CO_2 GENERATOR

In Report No. 10, we described Dr. Brewer's involvement with development of an improved GasPak H_2/CO_2 generator for the establishment of anaerobic environments. Since that report, BBL Microbiology Systems, Cockeysville, Maryland, has introduced and begun selling the new device called GasPak II. This generator incorporates two significant improvements over the original GasPak. First, the catlyst is applied directly to the foil pak, thus providing a fresh palladium catalyst for each run. Secondly, the CO_2 generating tablet has been modified to produce at least 8% CO_2 in the jar. This modification was accomplished by using a reaction mixture which provides a final acid pH after the CO_2 and H_2 tablets have reacted, thus preventing reabsorption of CO_2. Complete details of the development of this new device are described in the following manuscript which has been submitted to Applied and Environmental Microbiology for consideration for publication.

DEVELOPMENT AND EVALUATION OF THE GASPAK II[R] HYDROGEN-CARBON DIOXIDE GENERATOR

J. H. Brewer, T. L. Foster, R. L. Garner, and L. Winans

Science Research Center, Hardin-Simmons University, Abilene, Texas

The GasPak II hydrogen-carbon dioxide generator was developed to provide a fresh catalyst during each run of the GasPak Anaerobe System[R] and to provide a higher CO_2 concentration than was normally achieved with use of the original GasPak[R]. Comparative investigations using GasPak and GasPak II generators demonstrate that the performance of GasPak II surpasses manufacturer's specifications and generally develops a lower O_2 concentration faster than the GasPak. The change in location of the cold catalyst resulted in no increased hazard during use of the GasPak II.

INTRODUCTION

A new hydrogen-carbon dioxide generator envelope was recently described by J. H. Brewer (1). In 1966, Brewer and Allgeier (2) described a safe, self-contained hydrogen-carbon dioxide generating system for establishment of an anaerobic environment for cultivation of microorganisms. Since that time, the GasPak system has become well established as a method for culturing anaerobic bacteria (3,4,5,9,11) and has provided a convenient means to allow

[R]GasPak II, GasPak Anaerobe System, and GasPak are trademarks of BBL Microbiology Systems, a Division of Becton-Dickinson Company, Inc., Cockeysville, Maryland.

Running Title: Development of GasPak II

more laboratories to perform this type of work. Recent increased awareness of anaerobes in infection has rekindled an interest in anaerobic methodology and numerous investigators have recommended or performed comparative investigations of various methods (4,6,8,9,10). This renewed interest has resulted in a continuing demand for a thorough understanding of methods employed in anaerobic microbiology. Because of the widespread use of the GasPak anaerobic system, it has been evaluated in various comparative studies and found to be generally reliable and simple to use. One disadvantage of the GasPak system is that when improperly used, the cold catalyst (2) is inactivated by H_2S and other sulfur compounds. If the user is unaware of this and fails to rejuvenate or change the catalyst, the system will not develop anaerobiosis. Another factor which remains controversial is the final concentration of CO_2. Ferguson, et al (5) concluded that the GasPak system provided an atmosphere of less than 4% CO_2 after overnight incubation. Results by Abramson, et al (Carbon Dioxide Requirements for Anaerobes, Presented at the National Meeting, American Society for Microbiology, Atlantic City, May, 1976) disagreed with the lower figure, but still were significantly lower than the 10% CO_2 that is advertised. The present investigation was undertaken to develop a system which provides a fresh catalyst during each run of the GasPak system and to develop a final CO_2 concentration of at least 7.5%. A second objective, after the developmental phase, was to fully evaluate the new system and compare it with the original GasPak generator. Even though the results demonstrate obvious decreases in O_2 concentration, it is not an objective of this paper to define when an environment becomes anaerobic. As a point of reference, however, Futter and Richardson (7) refer to $< 0.1\%$ O_2 as an acceptable level for anaerobiosis.

MATERIALS AND METHODS

Application of catalyst to foil --- Several methods for applying catalyst to the foil surface in such a way as to maintain catalytic activity were attempted. The actual method is proprietary information to be released only through BBL Microbiology Systems, Cockeysville, Maryland.

CO_2 tablet --- The composition of the original GasPak CO_2 tablet was modified by the addition of organic acid to provide a final acid pH to prevent reabsorption of CO_2.

Evaluation of GasPak II --- Several Brewer anaerobe jars (GasPak 100 and 150, BBL, Division of Becton, Dickinson, and Company) were modified to accept oxygen, temperature, and redox probes. The oxygen analyzer probe (#39553, Beckman Instruments, Fullerton, California) and temperature probe (#39590, Beckman Instruments, Fullerton, California) were arranged so that they could be positioned at various locations in the jar. They were then connected to oxygen analyzer, temperature monitors (#100800 and #0260, Beckman Instruments, Fullerton, California) which are capable of reading both gaseous and dissolved oxygen

and temperature. The analyzers were connected to recorders (SR 204 Heath/ Schlumberger Instruments, Benton Harbor, Michigan) for continuous monitoring of O_2 concentrations and temperature. The Eh probe was connected to a pH meter (Fisher Accumet) set to read in millivolts and calibrated with a 0.2% quinhydrone solution in pH 4.0 phthalate buffer. Vented anaerobe jars were used in this investigation, and the vents were connected to a mercury manometer for monitoring pressure while the anaerobe system was in operation. This arrangement provided capabilities for monitoring oxygen concentration, temperature, Eh, and pressure.

A variety of experimental arrangements were employed for evaluation of the anaerobe jar. The stated parameters were monitored when using the GasPak system at room temperature (20-21°C) and incubator temperature (32-35°C) with and without media in the jars. GasPak and GasPak II envelopes were run simultaneously with numerous different lots of both types being evaluated.

All jars were tested for leaks prior to use by establishing a positive pressure of 30 mm Hg with nitrogen or a vacuum at 30 mm Hg. These conditions were maintained for 24 hours with the pressure being checked periodically. Any jar showing indications of a leak was not used in this investigation. This same procedure was used at various times during the project to determine if leaks had occurred in the jars. Another procedure to detect leaks was to allow the jars to set for extended periods (3-5 days after anaerobiosis was established to determine if the oxygen concentration increased).

The oxygen probe was calibrated against atmospheric O_2 and O_2-free nitrogen (Matheson Gas Products, LaPorte, Texas). Its reliability was then evaluated by measuring the oxygen content of an analyzed gas mixture of 99.9% CO_2 + 0.1% O_2

(Matheson Gas Products). This process was performed before and after each run, and when the O_2 probe membrane was changed. The probes were positioned at various locations in the jars during each experimental run. When broth was included in the anaerobe jar, the probe was positioned to monitor atmospheric O_2 during some of the runs and dissolved O_2 during others.

Controls for these investigations included the use of anaerobic indicators (BBL, Cockeysville, Maryland) and Alcaligenes fecalis (microaerophilic control) and Clostridium novyi type B (anaerobic control). If the color of the indicator did not change from blue to white, if A. fecalis did grow, and/or if C. novyi did not grow, the results of that jar were not included in the experiment.

Repeated runs in the GasPak 100 and 150 jars demonstrated no appreciable differences. Therefore, unless otherwise noted, all of the following results will be based upon data collected from the GasPak 100 jars.

RESULTS

Comparative runs of GasPak and GasPak II have been performed on more than 400 of each type of generator and include samples from several different lots of each. Figures 1, 2, and 3 demonstrate typical comparative data of oxygen concentration, Eh, pressure, and temperature. These parameters in all cases are almost identical, with the GasPak II showing only a slight time lag for the pressure and temperature changes. Brewer and Allgeier demonstrated the slight vacuum phase in the jar (Figure 3) (2). This part of the cycle is beneficial in diffusion of O_2 out of the culture medium and is also characteristic of

GasPak II. The temperature reported in Figure 3 is air temperature in the jar with the probe tip about three inches from the top. The manufacturer of GasPak II (BBL, Cockeysville, Maryland) reports temperature on the surface of the envelope to reach a maximum of approximately 58°C (personal communication). The maximum air temperature of 35°C obviously will not interfere with bacterial growth.

Figure 2 demonstrates that the decrease in Eh is virtually identical with both types of gas generators. Figure 1 demonstrates that the decrease in O_2 concentration is essentially the same, reaching a level of 0.1% O_2 in less than one hour. Some of the more recent lots of GasPak II show faster increased pressure (generation of H_2) followed by a faster decrease in O_2 concentration, often achieving < 0.1% O_2 in approximately 30 minutes. This indicates that water is reaching the sodium borohydride tablet a little faster, thus speeding up the entire process of establishing anaerobiosis.

The GasPak generator is designed so that water reaches the sodium borohydride tablet first. The initial pressure increase of Figure 3, should, then, be due to H_2, and it reaches a maximum pressure in approximately 20 minutes. Temperature begins increasing and O_2 begins decreasing in approximately 10 minutes. Thus indicating that the catalyst is indeed being activated by the $H_2 + O_2$ reaction. At 20 minutes, the reaction progresses sufficiently to cause a decreasing pressure and corresponding increasing temperature as the H_2 and O_2 are removed from the atmosphere of the jar. At about 40 minutes, the reaction nears completion and the release of CO_2 causes a return to a positive pressure.

An early indication of successful function of the GasPak is the development of condensate on the wall of the anaerobe jar as a result of the reaction of

$H_2 + O_2$. Of 600 GasPak II's evaluated to date, the average time for condensation to appear was approximately 18 minutes. The time required for the anaerobic indicator to be reduced was approximately 2 hours at 35°C.

DISCUSSION

Numerous procedures for applying the finely-divided palladium-on-alumina catalyst to the foil surface of the GasPak were largely unsuccessful. Failure was usually based on inadequate binding to the foil or inactivation of the catalyst. However, two procedures, one of which is described in the materials and methods section, have proven to be most functional and applicable to commercial procedures. The availability of a fresh catalyst during each run of the anaerobe jar offers distinct advantages over the former system of changing the catalyst in the catalyst basket and provides greater flexibility of use, including use of GasPak II in glove boxes. This application requires only the measurement of volume of O_2 to be removed and calculation of how many GasPak II envelopes to use.

The original GasPak resulted in a solution with a final alkaline pH which reabsorbed CO_2 after several hours. The new formulation of the CO_2 tablet was designed to provide a final acid pH so that CO_2 would not be reabsorbed. This acid pH resulted in more complete reaction of the borohydride tablet thus providing an excess of H_2 in the early lots of GasPak II. This problem is currently being resolved, and the amount of borohydride is being reduced accordingly (BBL, personal communication).

Although GasPak II is an improvement over the original GasPak, it is in-

complete at the time. We have recently developed an anaerobic indicator for application onto the foil surface and are currently in the process of evaluation of this feature. Early results demonstrate that it is functional and possesses a shelf-life of at least two months when stored at 37°C. It has not been developed long enough to present shelf-life data at room or refrigerator temperature.

ACKNOWLEDGEMENTS

The developmental phase of this work was supported by BBL Microbiology Systems, and the evaluation phase was supported by the National Aeronautics and Space Administration under grant NGR 44-095-001.

LITERATURE CITED

1. Brewer, J. H. 1980. An improved H_2-CO_2 generator envelope for the GasPak anaerobic system. Abstracts of the annual meeting, ASM, Miami Beach, p97.

2. Brewer, J. H. and D. L. Allgeier. 1966. Safe self-contained carbon dioxide-hydrogen anaerobic system. Appl. Microbiol. 14:985-988.

3. Collee, J. G., B. Watt, E. B. Fowler, and R. Brown. 1972. An evaluation of the GasPak system in the culture of anaerobic bacteria. J. Appl. Bact. 35:71-75.

4. Dowell, V. R., Jr. and T. M. Hawkins. 1974. Laboratory methods in anaerobic bacteriology: CDC laboratory manual. DHEW Pub. No. (CDC) 74-8272. United States Government Printing Office, Washington, D. C.

5. Ferguson, I. R., K. D. Phillips, and P. V. Tearle. 1975. An evaluation of the carbon dioxide component in the GasPak anaerobic system. J. Appl. Bact. 39:167-173.

6. Finegold, S. M., W. E. Shepherd, and E. H. Spaulding. 1977. Practical anaerobic bacteriology. Cumitech 5. American Society for Microbiology. Washington, D. C.

7. Futter, B. V. and G. Richardson. 1971. Anaerobic jars in the quantitative recovery of clostridia, p. 81-91. In D. A. Shapton and R. G. Board (eds.), Isolation of anaerobes. Academic Press, Inc., New York.

8. Holdeman, L. V. and W. E. C. Moore (ed.). 1975. Anaerobic laboratory manual, 3rd ed. Virginia Polytechnic Institute and State University. Blacksburg, Virginia.

9. Rosenblatt, J. E., A. Fallon, and S. M. Finegold. 1973. Comparison of methods for isolation of anaerobic bacteria from clinical specimens. Appl. Microbiol. 25:77-85.

10. Rosenblatt, J. E. and P. R. Stewart. 1975. Anaerobic bag culture method. J. Clin. Microbiol. 1:527-530.

11. Tally, F. P., P. R. Stewart, V. L. Sutter, and J. E. Rosenblatt. 1975. Oxygen tolerance of fresh clinical anaerobic bacteria. J. Clin. Microbiol. 1:161-164.

FIG. 1

OXYGEN PROFILE OF GASPAK I VS GASPAK II IN A GASPAK 100 SYSTEM AT 24C

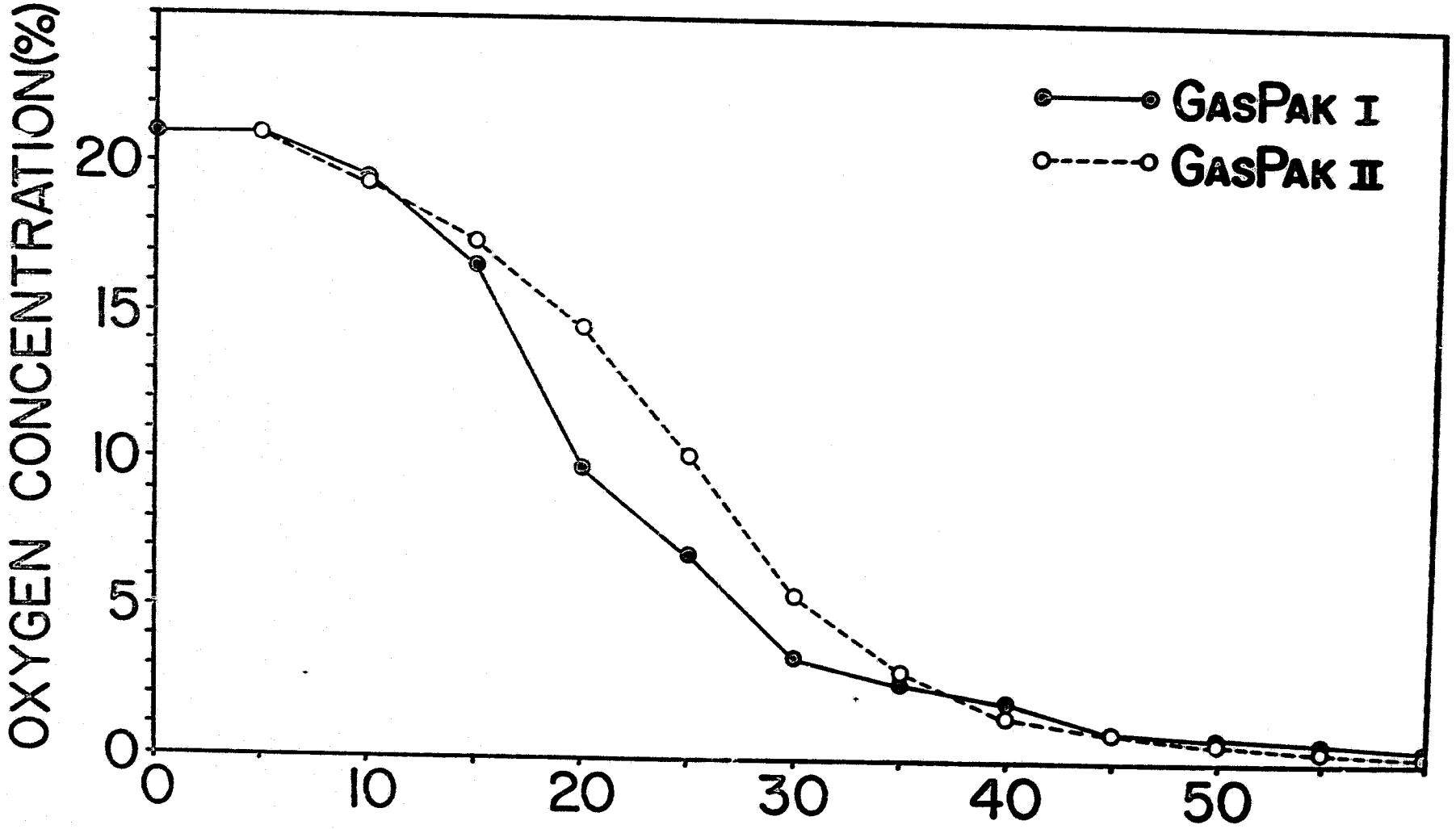


FIG. 2

REDOX POTENTIAL PROFILE OF GASPAK I Vs GASPAK II IN TRYPTICASE SOY BROTH AT 24C

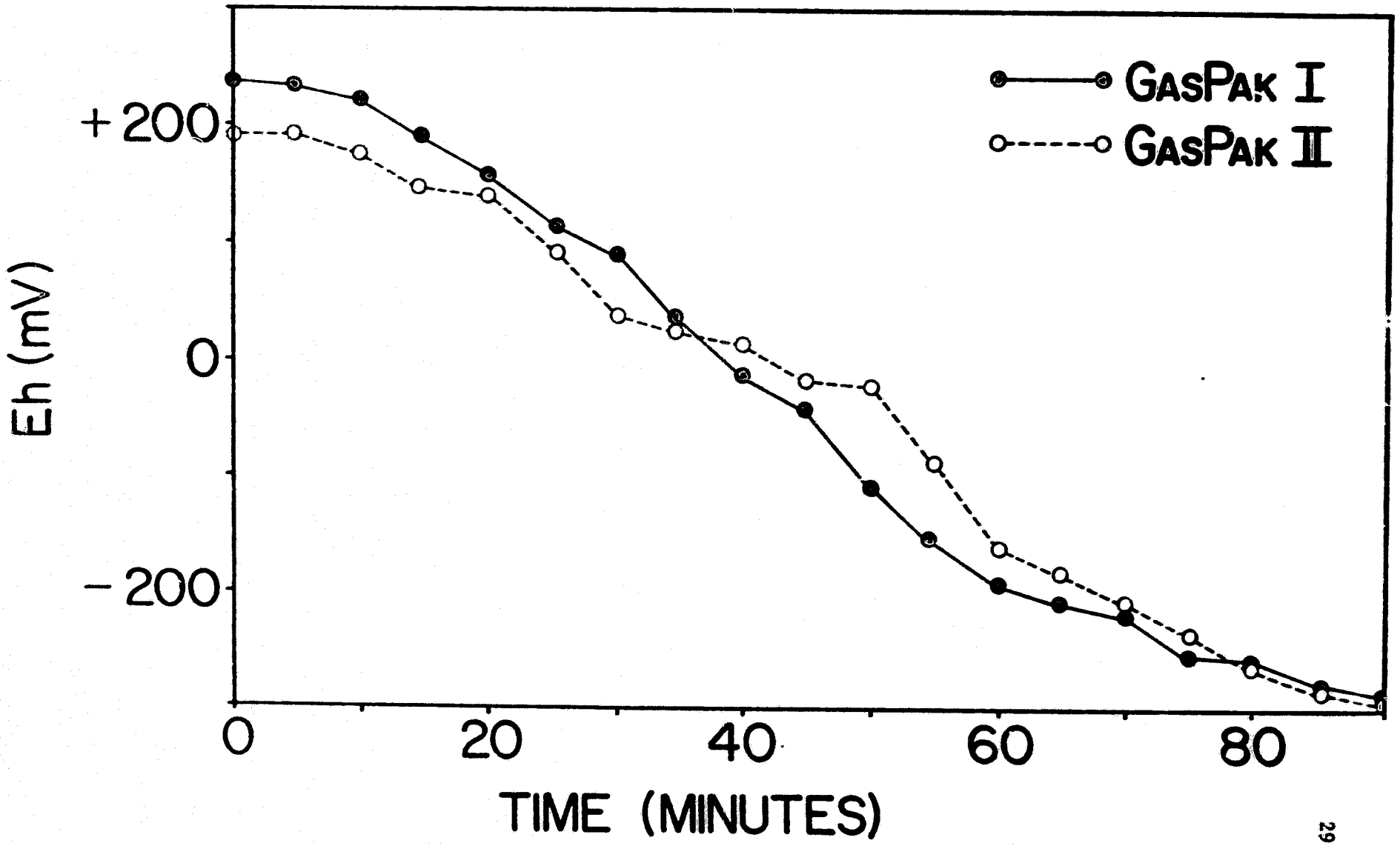
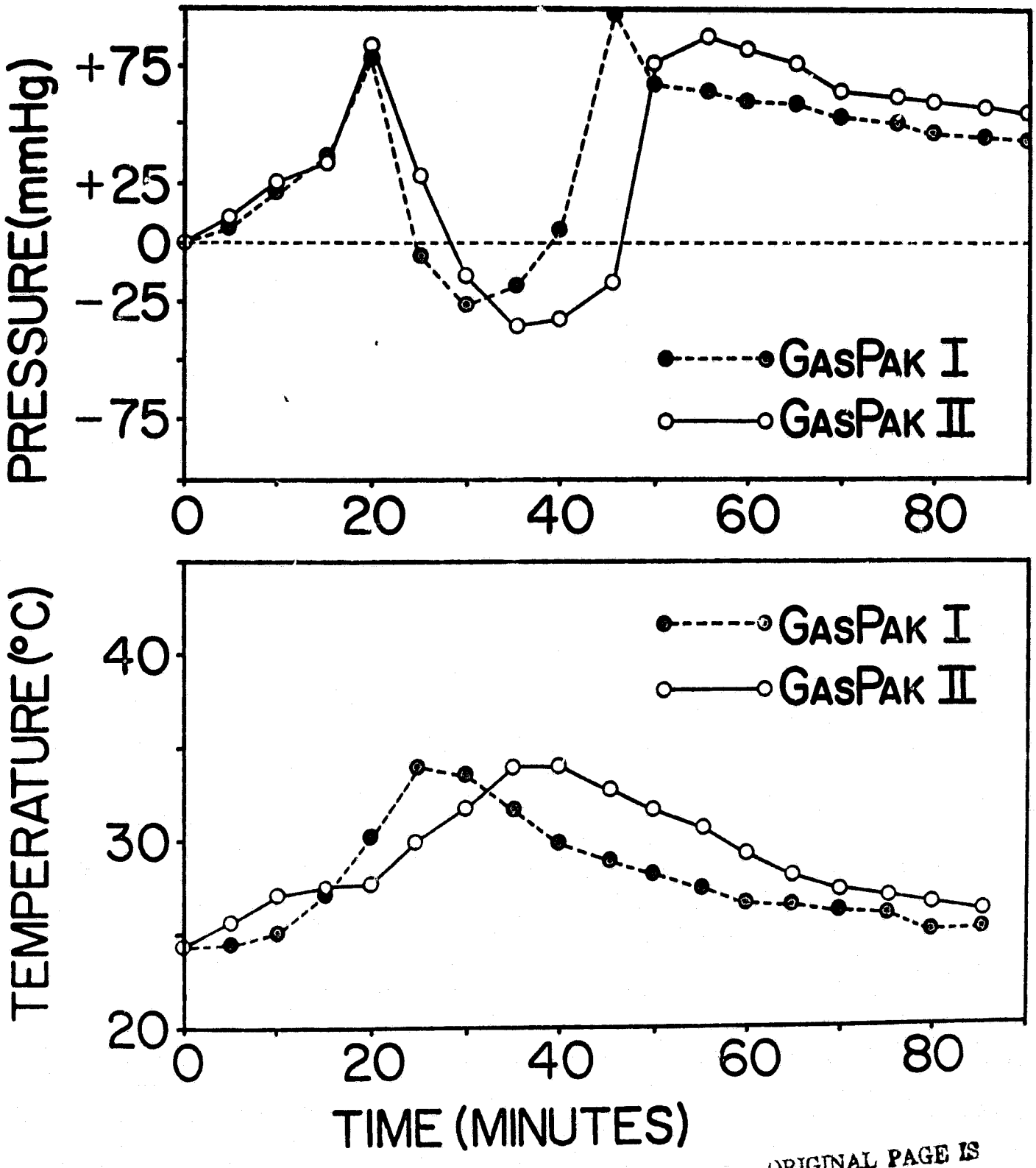


FIG. 3
PRESSURE AND TEMPERATURE PROFILES OF GASPAK I Vs GASPAK II AT 24C



Since GasPak II became generally available, BBL has provided samples of numerous different lots to our laboratory for evaluation. Results of experiments evaluating several hundred GasPak IIs are on file and resulted in further improvement. For example, in experiments on some of the first new GasPaks it was observed that the front paper surface often charred. We determined that the catalyst was placed too high on the pak. Being above the water level, the catalyst generated sufficient heat to char the paper. BBL began lowering the catalyst on the pak and the problem was resolved.

In other experiments, it was observed that the fine-particle catalyst often flew off of the pak and actually sparked inside the jar. These "fire-flies" are an obvious hazard in this environment with H_2 in the jar although they did not occur until late in the reaction after the catalyst heated up. At this time, there is insufficient O_2 for a flame, and no explosion, flash, or flame was observed. The problem was produced by application of excessive palladium on alumina catalyst. This produced a multiple layering of the catalyst which "popped off" upon heating. Reduction of the amount of catalyst application has resolved this problem.

In running the Brewer anaerobe jar with a GasPak, there are certain key factors to observe in evaluating how well it is operating. The following is a general summary of evaluating almost 1,000 of the new GasPak IIs:

Average Time To Formation Of Condensation
(From The Reaction Of $H_2 + O_2$ H_2O)
Minimum=25 Minutes-----Maximum=42 Minutes

% O_2 At Time Of Condensation
Average=15.9%

Jar Temperature At Time Of Condensation
Average=27°C

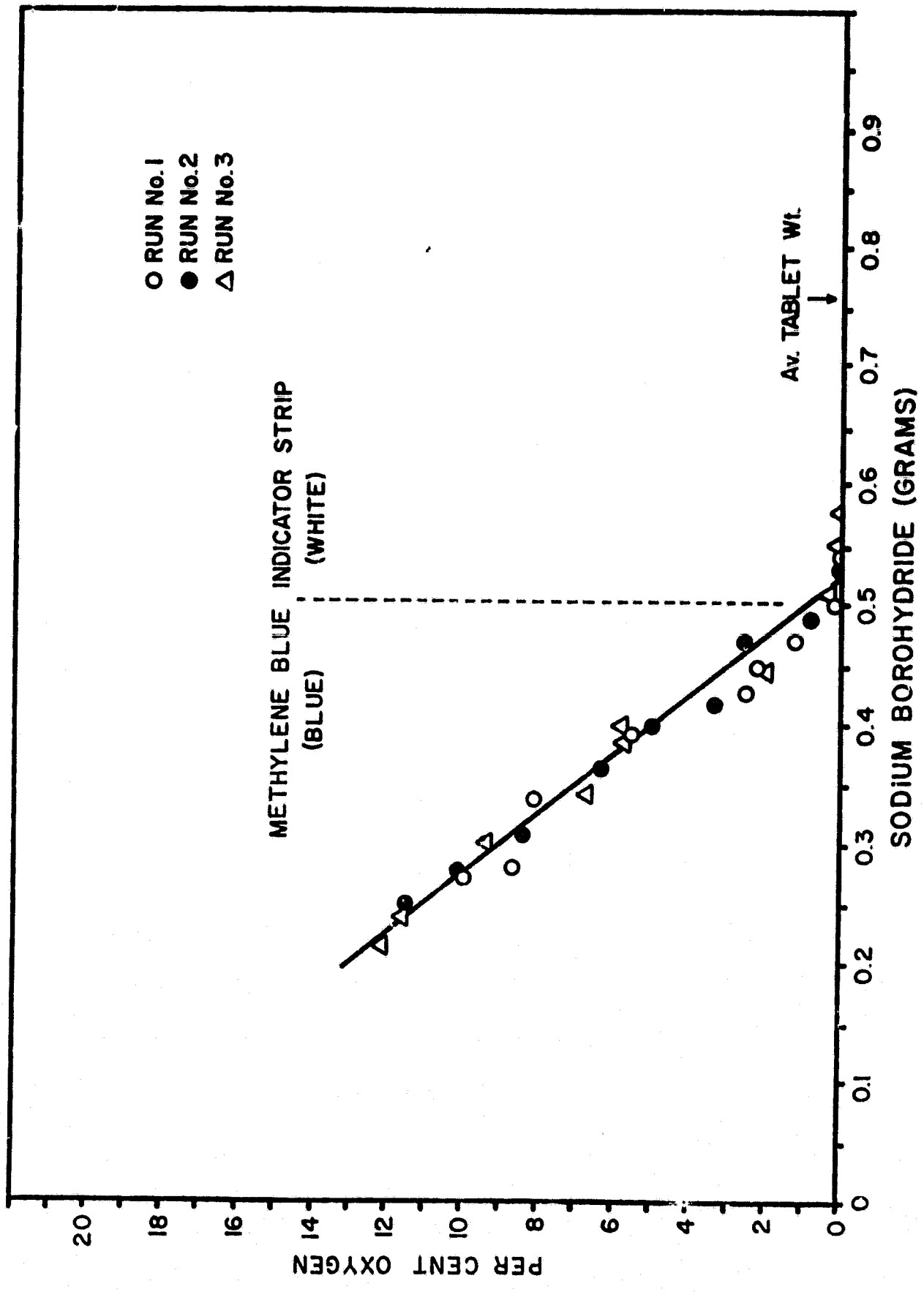
Jar Temperature At Time Of 1% O_2
Average=31.6°C

Jar Temperature At Time Of Reduction Of Indicator
Average=26.2°C

Time To Achieve 1% O_2
Average=40 Minutes

The GasPak II is designed to yield a final atmosphere with a slight excess of H_2. The average $NaBH_4$ tablet weighs 0.76 grams and routinely generates atmospheres of 0.01% O_2. A series of experiments determined that approximately 0.55 grams $NaBH_4$ is sufficient to generate an anaerobic environment. These experiments were run in empty jars. If the jars contained petri dishes or tubes of media, the amount of excess H_2 increases accordingly. This information underscores the need for observing precuations when working with anaerobe jars, even when opening them at the termination of incubation. It also provides information which is useful when making calculations to use the GasPaks to establish anaerobic atmospheres in containers other than the GasPak anaerobe jars, or to establish microaerophilic environments.

The new GasPak IIs are sufficiently versatile to allow establishment of anaerobic environments in containers other than anaerobe jars. This has potential application for storage of spacecraft components of unusual size or configuration in a non-oxidizing atmosphere. Their unique advantages make them much more reliable and our laboratory recommends their routine use for NASA's anaerobic microbiology.


O RUN No.1
● RUN No.2
Δ RUN No.3
METHYLENE BLUE INDICATOR STRIP
(BLUE) (WHITE)
Av. TABLET Wt.
PER CENT OXYGEN
SODIUM BOROHYDRIDE (GRAMS)
0
2
4
6
8
10
12
14
16
18
20
0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9

NEW ANAEROBIC PLATE

In Report No. 10, we described attempts to develop a new anaerobic plate capable of being opened and resealed at least three times and continue to establish anaerobic atmospheres. This work has not progressed further because of delays in receiving specially designed plates. These investigations will be continued at a later time.

HISTORY AND SIGNIFICANT CONTRIBUTIONS OF NASA GRANT NGR 44-095-001 SUPPORTED BY NASA'S PLANETARY QUARANTINE AND PLANETARY PROTECTION FROM SEPTEMBER, 1972 - DECEMBER, 1980

In 1972, the applicant submitted a proposal entitled "A Study of Psychrophilic Organisms Isolated from the Manufacture and Assembly Areas of the Spacecraft to be Used in the Viking Missions" to the National Aeronautics and Space Administration. This proposal was approved and NASA's Planetary Quarantine Program began funding this project in September, 1972, under grant number NGR 44-095-001. From that time, new topics were investigated and NASA continued funding this research until December, 1980. Because of the expanded scope of this project, NASA approved a change of title of this grant in 1974, and it was continued under the new title with the original grant number and funding from the Office of Planetary Biology and Bioscience.

The results of investigations under this grant were published in ten semi-annual reports, a number of scientific papers, two book chapters, and data has been presented at various symposia, seminars, and conferences. Included in this report are two manuscripts which have been submitted for publication.

A summary of the major accomplishments of previous research and its relevance to NASA's planetary exploration is given below:

Due to the possibility of the Viking landers transmitting terrestrial organisms to the surface of Mars and because of the low temperature of the planet, the objective of our first study was to investigate the presence of psychrophilic organisms from areas associated with the Viking spacecraft. After demonstrating

the presence of obligate psychrophiles from soil samples from Cape Canaveral, Florida and Denver, Colorado (manufacture site of the Viking spacecraft) (3), we later demonstrated that most of these would grow in a simulated Martian environment if water was present (4). We then demonstrated that these psychrophilic sporeformers were susceptible to the dry-heat cycle used for decontamination of the Viking spacecraft (6).

Using various sampling procedures, the JPL Planetary Quarantine Laboratory at Cape Canaveral isolated a significant level of airborne, sporeforming bacteria from spacecraft facilities which could withstand the Viking dry-heat cycle. These isolates ("hardy" organisms) were sent to the Science Research Center for confirmation of identification and partial physiological characterization including limits of growth based on temperature, pH, oxygen requirement, salt concentration, and others. Because of the significance of the "hardy" organisms, we continued investigations and demonstrated that these reproduced in the simulated Martian environment if sufficient moisture was present. In another study, we demonstrated that these organisms could be isolated from a sample after exposure to spacecraft conditions, deep space conditions, and the simulated Martian environment.

While performing microbial assays of soil samples from Cape Canaveral, we isolated numerous sporeforming baccili that grew over an unusually broad temperature range (0°-55°C). We determined the incidence of these in Cape Canaveral soils, identified most of them, investigated their physiological requirements, and determined that several of them survive the Viking dry-heat

cycle (1). Pyrochromatograms of several of these isolates demonstrated the presence of several peaks which appear to be temperature dependent. When the organisms were grown at different temperatures, some of the peaks diminished while others increased.

The protocol for microbial assay of the Viking spacecraft resulted in 2.0 ml of heat-shocked sample which was to be discarded. We were asked to develop a protocol for use of this 2.0 ml in assaying for psychrophilic and mesophilic obligate anaerobes. Working in cooperation with the JPL PQ Lab, which provided space for this work, we assayed 3,470 samples and isolated no obligate anaerobes. All of the 17 colonies which were isolated proved to be facultative anaerobes.

Because Jupiter is anaerobic and phosphorus does not exist as phosphate, we designed experiments and demonstrated anaerobic utilization of phosphite and hypophosphite (2,5). The isolates were from Cape Canaveral soil samples, and results indicated that they could serve as potential terrestrial contaminants of Jupiter. Of the numerous investigations to demonstrate anaerobic utilization of phosphine, none have resulted in positive data. The assay of these isolates indicates that none can utilize phosphine anaerobically as a source of phosphorus but that phosphine is not toxic to the microorganisms.

Because most of the planets have little or no free oxygen, a major emphasis of our recent research efforts dealt with techniques of anaerobic microbiology. We have evaluated several procedures for use in space-related anaerobic micro-

biology and have concentrated on new or improved procedures. In this report, we described investigations of new anaerobic procedures utilizing equipment furnished by BBL Microbiology Systems, Cockeysville, Maryland, and employing several new ideas of Dr. John H. Brewer who developed much of the anaerobic equipment now in use (anaerobe jars, GasPak anaerobe systems, thioglycollate medium, anaerobic agar, anaerobic petri dishes, and others). One of these investigations has led to in-depth evaluation of the new hydrogen generator produced by BBL (GasPak II).

Many laboratories do not properly monitor their anaerobic equipment, and the catalyst becomes poisoned or they fail to use appropriate anaerobic indicators. The result is that many times the jar is placed in the incubator, and it may take hours for the indicator to change so that one has no knowledge of how long it took for anaerobic conditions to actually exist. A new GasPak H_2/CO_2 generating system (BBL) with the catalyst applied directly onto the aluminum foil envelope has been developed so that a new catalyst is available each time the jar is run. Also, in the GasPak, the final reaction of the H_2/CO_2 generator was often alkaline, and much of the CO_2 was reabsorbed. A new formulation of the CO_2 tablet has been incorporated into the GasPak II envelope to assure 9-10% CO_2.

The new GasPak II offers distinct advantages over the former GasPak system, especially in space-related anaerobic microbiology. Anaerobic sampling will be more consistent because there is no danger of trying to reuse a poisoned catalyst. It will also be possible to use any air-tight, sealable container to establish anaerobic conditions. The need for a specifically designed anaerobe jar is negated by the presence of the catalyst on the disposable

pak. This characteristic alone should be of value to the space program because it offers the advantage of anaerobically storing or transporting items of unusual shapes and sizes, provided that a sufficient number of GasPaks are used.

A major task of our research was to determine microbial response to various pressures of H_2, CH_4, NH_3, and H_2S. In these experiments, specific soil isolates from Cape Kennedy and soil samples were subjected, in nutrient solutions, to pure gases and mixtures from standard pressure to 1500 psi. Samples were obtained and enumerated periodically to examine population changes. Results of these studies were presented at the National Meeting of the American Society for Microbiology in May, 1980, and generally indicate population increases of the major types of bacteria in CH_4, H_2S, and H_2. However, all vegetative cells were killed quickly in NH_3. Spores were capable of survival, but not germination in NH_3. Mixtures of the four gases were also toxic, presumably due to the presence of NH_3.

This research has been concerned with investigations of selected populations of microorganisms from spacecraft environments and their potential role in terrestrial contamination of planets, especially Mars and Jupiter. The first objective has been met with our descriptions of psychrophiles, omnitherms, "hardy" organisms, phosphite utilizers, and anaerobes. The second objective has been met with our work on the simulated Martian environments, effects of various gases at various pressures, anaerobic utilization of hypophosphite, and others.

A review of our proposals, reports, and publications reveals that the Hardin-Simmons University Science Research Center has fulfilled its objectives described in the various proposals and completed special studies often requested by NASA's division of Planetary Protection. It is our hope that much of this research has generated base-line data which will also be useful to NASA in programs other than the ones for which it was originally intended.

LITERATURE CITED

1. Brewer, J. H. and T. L. Foster. 1977. Isolation and characterization of omnitherms and facultative anaerobes from Cape Canaveral soil samples. Life Sciences and Space Research. 15:53-58. Pergamon Press, New York.

2. Foster, T. L. and L. Winans, Jr. 1977. Anaerobic utilization of phosphite/phosphine as a sole source of phosphorus. Life Sciences and Space Research. 15:81-86. Pergamon Press, New York.

3. Foster, T. L. and L. Winans, Jr. 1975. Psychrophilic microorganisms from areas associated with the Viking spacecraft. Applied Microbiology. 30:546-550.

4. Foster, T. L., L. Winans, Jr., R. C. Casey, and L. E. Kirschner. 1978. Response of terrestrial microorganisms to a simulated Martian environment. Applied and Environmental Microbiology. 35:750-757.

5. Foster, T. L., L. Winans, Jr., and S. J. S. Helms. 1978. Anaerobic utilization of phosphite and hypophosphite by Bacillus sp. Applied and Environmental Microbiology. 35:937-944.

6. Winans, L., Jr., I. J. Pflug, and T. L. Foster. 1977. Dry-heat inactivation of psychrophilic spores. Applied and Environmental Microbiology. 34:150-154.

BIOGRAPHICAL SKETCH OF PRINCIPAL INVESTIGATOR

Terry L. Foster, Ph.D.

1.	Born	April 4, 1943 Mt. Pleasant, Texas
2.	Marital Status	Married Two Children
3.	Home Address	3493 Santa Monica Abilene, Texas 79605 915-698-3617
4.	Education	Mt. Pleasant High School, 1961 Baylor University, 1961-62 North Texas State University, 1962-65 (Bachelor of Arts-Biology) North Texas State University, 1965-67 (Master of Arts-Microbiology) Texas A & M University, 1971-73 (Doctor of Philosophy-Microbiology)
5.	Experience	Instructor, Hardin Simmons University, 1967-70 Assistant Professor, HSU, 1970-73 Assistant Professor, HSU, 1973-78 Research Professor, HSU, 1979-present Principal Investigator, 1972-present (HSU Science Research Center)
6.	Honors and Memberships	American Society for Microbiology American Society for Microbiology, Texas Branch Outstanding Young Men of America, 1972 and 1975 Outstanding Young Educators of America, 1974 Who's Who in Texas, 1975
7.	Presentations	Invited to present a seminar on simulated Martian environments at Ames Research Center, September, 1974 Presented paper at the "Working Conference on the Significance of Heat-Resistant Organisms to the Viking Biopackage" at Ames Research Center, November, 1974 Presented one paper and co-authored another presented at the 19th COSPAR meeting in Philadelphia, 1976 Presented papers at the following AIBS/NASA Spacecraft Sterilization Technology Seminars or Planetary Quarantine Panel Meetings: (continued on next page)

7. Presentations (continued)

New Orleans, Louisiana in January, 1972
Denver, Colorado in July, 1973
San Francisco, California in February, 1974
Denver, Colorado in July, 1974
Cape Canaveral, Florida in December, 1974
Cape Canaveral, Florida in August, 1975

Presented one paper and co-authored another presented at the National ASM Meeting in New Orleans, Louisiana, May, 1977

Presented one paper at the National ASM Meeting in Los Angeles, California, May, 1979

Presented one paper at the National ASM Meeting in Miami Beach, Florida, May, 1980

8. Publications

Foster, T. L. and L. Winans, "Psychrophilic Microorganisms from Areas Associated with the Viking Spacecraft", Applied Microbiology 30:546-550 (1975).

Winans, L., I. J. Pflug, and T. L. Foster, "Dry-Heat Inactivation of Psychrophilic Spores", Applied and Environmental Microbiology", 34:150-154 (1976).

Foster, T. L. and L. Winans, "Anaerobic Utilization of Phosphite/Phosphine as a Sole Source of Phosphorus", Life Sciences and Space Research 15:81-86 (1977).

Brewer, J. H. and T. L. Foster, "Isolation and Characterization of Omnitherms and Facultative Anaerobes from Cape Canaveral Soil Samples", Life Sciences and Space Research 15:53-58 (1977).

Foster, T. L., L. Winans, R. C. Casey, and L. E. Kirschner, "Response of Terrestrial Microorganisms to a Simulated Martian Environment", Applied and Environmental Microbiology 35:730-737 (1978).

Foster, T. L., L. Winans, and S. J. S. Helms, "Anaerobic Utilization of Phosphite and Hypophosphite by Bacillus sp.", Applied and Environmental Microbiology 35:937-944 (1978).

Foster, T. L., L. Winans, and J. H. Brewer, "Evaluation of a New Microquantity Blood Collector", Journal of Clinical Microbiology 7:305-306 (1978).

8. Publications (continued)

Foster, T. L., "Psychrophilic and Omnithermal Organisms in the NASA Interplanetary Program", In Advances in Decontamination and Sterilization: A Survey. NASA Publication, May, 1977.

Brewer, J. H. and L. Winans, "Chemical Sterilization", In Advances in Decontamination and Sterilization: A Survey. NASA Publication, May, 1977.

Brewer, J. H. and T. L. Foster. Served on Task Force for the Revision of NASA Standard NHB 5340.1B, 1978.