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Report

AUTOMATED MICROBIAL METABOLISM LABORATORY

Contract No. NASW-1931

Annual Report

1971

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Prepared for:

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Headquarters
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19 October 1971

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ACKNOWLEDGEMENTS

The participation and assistance of the following Biospherics' personnel is gratefully acknowledged:

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ABSTRACT

This program explored the effect of several environmental parameters on previously developed life detection systems. Initial attempts were made to conduct all the experiments in a "moist" mode (high soil volume to water volume ratio). However, only labeled release and measurement of ATP were found to be feasible under conditions of low moisture. Therefore, these two life detection experiments were used for most of the environmental effects studies. Three soils, Mojave (California desert), Wyaconda (Maryland, sandy loam) and Victoria Valley (Antarctic desert) were generally used throughout this study. The environmental conditions studied included: incubation temperature $3^{o}C$ - $80^{o}C$, ultraviolet irradiation of soils, variations in soil/liquid ratio, specific atmospheric gases, various antimetabolites, specific substrates, and variation in pH. An experiment designed to monitor nitrogen metabolism was also investigated.

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I. SUMMARY

During the past year, initial work on the Automated Microbial Metabolism Laboratory (AMML) was directed toward an attempt to adapt various assay procedures which had previously been tested on "wet" soil culture systems (1 g soil/30 ml medium) to "moist" soil culture systems (1:1 or less volume ratio of liquid to soil). This study was initiated as a result of increasing evidence for the scarcity of liquid water on Mars, which raised the possibility of adverse effects of large quantities of water on Mars organisms. Hence, it seemed prudent to explore means for conducting the experiments with minimal water addition. It was also desired to study the effects of moisture level and specific atmospheric gases on the life detection tests of the AMML.

Although ATP assays were successfully performed, phosphate uptake in "moist" experiments was not detectable. This was caused partially by the interference of leaching and absorption of phosphate in soils and by the large dilution factor required to provide an adequate assay volume with its dilution error. ^{14}C and ^{35}S uptake experiments also presented large, variable background problems in the moist

were also elevated by the higher temperatures, but remained below the levels of the test responses.

Analysis of data based upon the kinetics of labeled release showed that the differences between viable and sterile soils release rates were greater than when analysis was based upon cumulative evolution of radioactivity after plateauing.

All soils showed a greater relative difference between rate of response from the viable and control portions at the lower temperature, but this difference appeared to be significant only for the Victoria Valley soil.

Ultraviolet Irradiation Studies: Experiments were conducted in which 0.5 g of soil (depth approximately 1 mm) were irradiated with 91 milliwatts/cm^2 of 2537 Angstrom radiation for periods of time up to 10^6 seconds. Soil so treated was then tested for activity by the labeled release experiment.

Mojave soil was unaffected by UV exposures of 10^5 seconds and suffered only a 40% decrease in activity from 10^6 seconds (11.5 days) exposure. Wyaconda and Victoria Valley soil, on the other hand, showed decreased activity after 10^3 seconds exposure and both suffered a ten fold decrease in activity after 10^6 seconds exposure.

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Moisture Studies: Labeled release and ATP analyses were performed on soil cultures which ranged from 0.3 g soil sample immersed into 10 ml of RM9 medium (high liquid/soil ratio), to five microliters added to 1 g of air dried soil. The initial rate of evolution of radioactivity from viable soils was found to be much lower for high liquid/soil ratios. The maximum initial rate of evolution occurred at liquid/soil ratios of 0.1 ml/g to 0.01 ml/g. The nonbiological evolution of radioactivity did not appear to be affected by the liquid/soil ratio, and varied in proportion to the radioactivity added. At high ratios there was little or no difference between the initial rate of response from viable and sterile soils. However, extended incubation of high ratio soil cultures resulted in a significant difference between control and viable responses.

ATP assays performed on the Mojave soil cultures supported the labeled release data. A liquid/soil ratio of 0.005 showed 100 fold more production of ATP per ml of medium added than a liquid/soil ratio of 0.4 ml/g.

Phosphate uptake and ^{14}C and ^{35}S uptake was measured in "wet" experiments. Cultures were assayed in the lag, logarithmic and stationary phases of growth.

Uptake of ^{14}C and ^{35}S was observed in all soils during logarithmic and stationary growth. The uptake by viable soils exceeded sterile soils by approximately twenty to eighty fold. Phosphate uptake of 0.1 mg/l was observed with Wyaconda and Mojave soil, however, Victoria Valley soil showed no uptake.

Atmosphere Studies: Several experiments were conducted in various selected gaseous environments. It was found that a ^{14}C and ^{35}S gettering experiment could not be conducted in a gas mixture of $N_2/H_2/CO_2$ (90:10:10). Presumably, the CO_2 in the atmosphere saturated the $Ba(OH)_2$ getter rendering it ineffective for collection of evolved $^{14}CO_2$.

Successful labeled release gettering experiments were conducted in atmospheres of N_2/H_2 (90:10), N_2, N_2/H_2 CO (90:9:1) and N_2/O_2 (99:1). Anaerobic evolution of labeled gas was approximately 50% of that obtained aerobically. Anaerobic production of ATP was also reduced 50% from that obtained aerobically. The rate of labeled release in a low oxygen mixture N_2/O_2 (99:1) fell between the aerobic and anaerobic response.

Antimetabolite Studies: A number of antimetabolites were tested for effect on the labeled release experiment.

Concentrations which had been found effective in earlier studies, or which are recommended for disinfection, were tried. Their effects on the inhibition of Mojave and Wyaconda soil were compared. The effects of these agents on nonbiological evolution of radioactivity were also compared. Treatments with mercuric chloride, Bard-Parker germicide, sodium hypochlorite and ethylene oxide were found to be ineffective in severely inhibiting the evolution of radioactivity from Mojave soil, but did reduce the activity of Wyaconda soil by at least an order of magnitude. Potassium cyanide (0.05M) was effective in the inhibition of both Wyaconda and Mojave soils. Viable soils showed no more evolution of radioactivity than heat sterilized controls when KCN was added to both. The antibiotics tested, penicillin and chloramphenicol, had little or no effect on the evolution of radioactive gases.

Substrates Studies: Labeled release experiments with Wyaconda and Mojave soil were conducted using media containing a single labeled substrate. These substrates were tested alone in the medium, and in the presence of other nonlabeled substrates. The substrates studied

were: ^{14}C-lactate (both DL and L), ^{14}C-D-glucose, ^{14}C-glycine, ^{14}C-formate, ^{14}C-glycolate, ^{14}C-acetate and ^{14}C-L-alanine. Glucose and lactate produced approximately twofold more evolved radioactive gas than formate, glycine, acetate or glycolate. Efficiencies in terms of radioactivity evolved versus radioactivity added were determined for the substrates studied. Formate produced the highest efficiency of response and glucose produced the lowest. However, both of these substrates produced a higher initial rate of ^{14}C-gas evolution than the other substrates tested.

Medium containing $^{35}SO_4^{=}$ and unlabeled organic substrates was tested with an organism which carries out anaerobic respiratory sulfate reduction. A 100 fold viable/nonviable response ratio was obtained in a 90% N_2, 10% H_2 atmosphere.

pH Studies: Experiments were conducted to determine the effect of a buffered versus a nonbuffered medium. The effect appeared to be soil dependent. However, in one case, buffering resulted in increased labeled release, ATP production and ^{14}C and ^{35}S uptakes.

Nitrogen Studies: The possibility for development of a life detection monitor based upon nitrogen metabolism was

approached. The nitrogen fixing complex (nitrogenase) converts acetylene to ethylene, which can be detected by vapor phase chromatography. Preliminary testing of this system on soils has been highly encouraging.

II. INTRODUCTION

The Automated Microbial Metabolism Laboratory (AMML) as conceived and developed is composed of functionally independent, but complementary, second-generation exobiology experiments. These experiments are designed to show confirmation of life detection on the basis of a spectrum of metabolic activities and provide a measure of comparative data. As previously described (1), the AMML includes: light-dependent and light-independent fixation of labeled carbon dioxide and subsequent evolution of $^{14}CO_2$ during dark incubation, uptake of radioactive ^{14}C-organic substrates and $^{35}SO_4^{=}$, production of adenosine triphosphate (ATP), uptake of phosphate, and production of radioactive gas from labeled organic substrates. These life detection systems which were developed and validated on test soils, were in the past subjected to a rather specific set of test conditions. Recent data have supplied more precise information on Martian conditions. It was, therefore, proposed that the effect of these more vigorous conditions on the characteristics of response from certain life detection systems should be studied on soils including those from extreme terrestrial soils.

In view of the dearth of water on Mars, attempts were made to modify the assay systems to accommodate low water/soil cultures. Under these conditions, the detection systems which appeared most viable received the greatest emphasis during this phase of work.

Also, mindful that the AMML test array, and other life detection tests as well, lack experiments based on nitrogen metabolism, an attempt was made to devise one.

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III. EXPERIMENTAL BIOLOGY

A. Attempts to Adapt the AMML Assay Procedures to "Moist" Culture Conditions

Increased evidence for the scarcity of liquid water on Mars prompted attempts to adapt the various assay procedures of the AMML, which had previously been tested on high liquid/soil ratio culture systems (1), to low liquid/soil ratio, or "moist" experiments. This system was also required in order to investigate the effect of moisture and atmospheric gases more Mars-like than previously studied.

Details of the individual assays are presented in the Appendix. However, the generalized procedure was as follows:

> Labeled release of the soil culture was conducted for a designated time. The entire culture was then transferred to a membrane filter apparatus, filtered, and washed. The filter residue was counted for uptake of ^{14}C and ^{35}S, and the filtrate assayed for PO_4-P.

Other cultures were extracted and assayed for ATP. In order to design a workable culture system, it was necessary to determine the following:

- Amount of medium necessary to produce a measurable response.
- Amount of soil which could be effectively washed and counted for retained radioactivity in the $^{14}C + ^{35}S$ uptake experiment.
- Wash regimen necessary.
- Influence of soil leaching on the ATP and phosphate assay.
- Concentration of PO_4-P in the medium which is required to show uptake yet provide a measurable concentration in the wash filtrate.
- Treatment of controls best suited for all assays.

A series of experiments was conducted to determine if a single culture system having a low liquid/soil ratio could be used for all the above mentioned assays. The design was as follows:

- Experiment Nos. 1 and 2 - The soil sample was 0.025 g and the volume of medium was 0.4 ml of complete RM9 with a concentration of added phosphate of 60 mg/l K_2HPO_4 (11 mg/l PO_4-P).

- Experiment No. 3 - The soil sample was 0.1 g and the volume of medium was 0.2 ml of complete RM9 with a concentration of added phosphate of 5 mg/l K_2HPO_4 (0.9 mg/l PO_4-P).
- Experiment No. 4 - The soil sample was 0.5 g and the volume of medium was 0.1 ml of complete RM9 with a concentration of added phosphate of 250 mg/l K_2HPO_4 (46 mg/l PO_4-P). This high concentration of phosphate was used to accommodate a trial modification of the phosphate and ^{14}C and ^{35}S uptake assays. The planchet contents were emptied into 10 ml of RM9 medium without added phosphate and without labeled organics. A 1 ml aliquot of this 10 ml, stirred volume was then filtered, washed, and assayed for PO_4-P, and ^{14}C and ^{35}S uptake.

The procedure used was as follows: The indicated quantity of soil sample was weighed into aluminum planchets. 0.2 ml of Bard-Parker germicide was added to controls and other soil samples received 0.2 ml of sterile,

distilled water. Medium was added to each planchet and gettering was conducted as described in the Appendix, Section 2-A.

Duplicate planchets were harvested at the beginning of the incubation period and assayed for ATP, PO_4-P, and ^{14}C and ^{35}S uptake. The growth phases of the cultures were followed by observation of the labeled release experiment. PO_4-P and ^{14}C and ^{35}S uptake were again measured during the logarithmic phase, and finally ATP, PO_4-P, and ^{14}C and ^{35}S uptake were measured when the culture had reached the stationary growth phase.

The representative data presented in Table 1 were obtained in Experiment No. 2. As shown, no significant biological signal in terms of ^{14}C and ^{35}S uptake, was observed. PO_4-P uptake was variable. However, a definite increase in ATP was observed. Table 2 shows that a medium addition of only 0.2 ml RM9 to 0.5 g soil caused a twofold increase in the ATP level. Although there was a significant increase in ATP in all experiments, it was noted that a greater actual increase was observed when the liquid/soil ratio was low. In Table 1, an ATP increase of approximately 4×10^{-3} μg/0.4 ml was observed; however, as shown in Table 2, 7×10^{-3} μg/0.1 ml were observed. The low liquid/soil ratio appeared to enhance ATP production.

This effect of liquid/soil ratio is discussed in greater detail later in this report.

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TABLE 1

Results of ATP, Phosphate, and ^{14}C and ^{35}S Uptake in System Containing 0.025 g Soil*, 0.4 ml RM9 Medium**

Assay	0 Incubation Time	Logarithmic Growth Phase 27 hrs.	Stationary Growth Phase 49 hrs.	Bard-Parker Control 49 hrs.
ATP	1.2×10^{-3}	-	5.5×10^{-3}	3.2×10^{-4}
(µg/sample)	9.5×10^{-4}	-	5.0×10^{-3}	3.5×10^{-4}
PO_4-P	4.12	0.75	0.75	4.37
(µg/sample)	4.37	1.0	11.2	4.37
^{14}C & ^{35}S	3,289	85,722	77,553	100,889
(cpm/sample)	5,058	75,116	116,441	163,580

* Soil was collected at Sterling Park, in Northern Virginia.

** RM9 medium (containing 50 mg/l K_2HPO_4) was prepared as given in Appendix - Section 1-A.

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TABLE 2

ATP Production by 0.5 g Soil* and 0.1 ml RM9** Medium

	0 Incubation Time	Stationary Growth Phase 28 hrs.	Bard-Parker Control 28 hrs.
ATP μg/sample	1.35×10^{-2}	2.23×10^{-2}	6.8×10^{-4}
	1.57×10^{-2}	2.16×10^{-2}	5.4×10^{-3}

* Soil was collected at Sterling Park in Northern Virginia.

** RM9 medium (containing 250 mg/l K_2HPO_4) was prepared as given in Appendix -Section 1-A.

Additional attempts to measure a biological uptake of ^{14}C and ^{35}S and PO_4-P in "moist" cultures were made. These attempts which were presented and discussed in detail in the First Quarterly Progress Report (2) were generally unsuccessful. It was, therefore, decided that these assays should be performed in "wet" culture as had been used successfully in early studies.

Treatment of soil for use as a nonviable control was studied to determine a procedure which would produce a low nonbiological response. Bard-Parker germicide had been used most successfully in earlier work. However, the possible interference of this agent in the uptake experiments prompted a comparison of Bard-Parker treated soils with heat treated soils. Heat treated soils were found to produce less background.

Figure 1 shows a comparison of labeled release from soil which was subjected to various Bard-Parker and heat treatments. Heat treated soils produced a lower level of nonbiological response than Bard-Parker treated soils. Dry heat sterilization was selected as the method of choice since it was rapid and effective. The 180°C, 15-minute treatment was subsequently changed to 212°C, 30 minutes after lack of sterility was detected in several controls.

B. Soil Selection

Three soil types were selected for comparative study in the present program. They were selected on the basis of ecological

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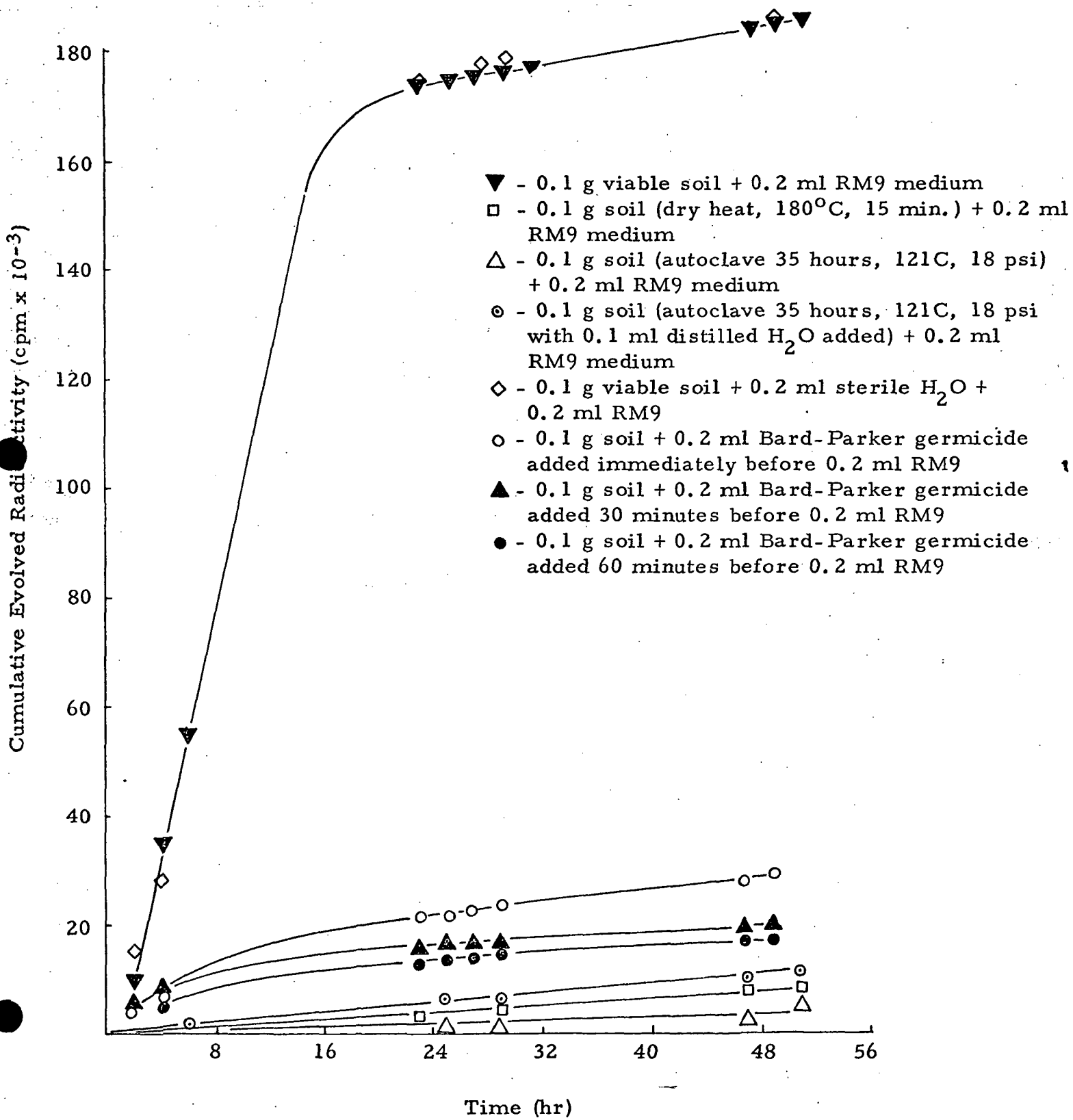
FIGURE 1

Results of Labeled Release Experiment
Comparing Methods of Control Preparation



and geographical distribution, differences in plated numbers of bacteria, and differences in the observed kinetics of the labeled release experiment. The three soils under study were:

Mojave #75 - This soil was obtained from the desert soil collection at the JPL Soil Science Laboratory and is described in the NASA Technical Report No. 32-977, "Desert Soil Collection at the JPL Soil Science Laboratory," Jet Propulsion Laboratory, California Institute of Technology, Pasadena, California, 15 October 1966. The sample is a coarse, sandy desert soil collected aseptically from the surface to a depth of $\frac{1}{4}$ inch on 14 November 1961. It was stored in a plastic whirl-pack in a 500 g aliquot as collected until received by Biospherics. It was then aseptically sifted with a number 14 mesh screen and stored in a sterile screw top Nalgene bottle. Data supplied by JPL indicated that it contained 2 ppm inorganic phosphate, 60 ppm sulfate, 0 ppm nitrate, and had a pH of 7.0. Aerobic plate counts on Czapek Dox agar revealed the presence of 6×10^6 organisms per gram of soil*. Colonies were visible after 24 hours of incubation at 25°C.

Wyaconda - This soil was obtained near Biospherics in Rockville, Maryland on 7 October 1970. The collection was at the site of construction where vegetation had been cleared some months earlier. Soil from the surface to a depth of 2 inches was collected, dried, and sifted with a number 14 mesh screen and stored in a sterile screw top Nalgene bottle. The soil was a sandy clay containing small particles of organic material. It was of finer particle size than the Mojave soil. Aerobic plate counts on nutrient agar revealed the presence of 1×10^7 organisms per gram of soil*. Colonies were visible after 24 hours of incubation at 25°C.

Victoria Valley #538 - This soil from Antaractic was also obtained from the Desert Soil Collection at JPL. It was not described in the NASA Technical Report. However, information supplied indicated that it was collected at a depth of 1 to 6 inches and chemical analysis was as follows: 0.3 ppm inorganic phosphate, 22 ppm sulfate, 6 ppm nitrate, pH 6.9. Growth under CO_2 at room temperature on nutrient agar revealed the presence of 10^2 organisms/gram of soil*. Colonies were visible after 13 days incubation at 25°C. The

soil was aseptically sifted with a number 14 mesh screen at Biospherics and stored at room temperature in sterile Nalgene screw top bottles. Texture of the soil was coarse granular similar to the Mojave soil in particle size.

Preliminary testing of the three soils demonstrated that known quantities of ATP added to the Mojave soil could be 90% recovered. Only relatively small quantities of added ATP could be recovered from the other two soils. Possibly there were inhibitory substances in the soil which extracted along with the ATP and caused adverse effects to the firefly light producing reaction. Alternatively, the ATP could have been biologically or chemically degraded. Several extraction techniques were used to no avail. For purposes of the current study, the decision was made to measure ATP changes in only the Mojave soil.

* All soils were plated on nutrient agar, trypticase soy agar (TSA) and Czapek Dox agar. Incubation was conducted aerobically at 25°C, and 3°C, anaerobically (H_2) at 25°C and in candle jars (5% CO_2) at 25°C and 3°C. Counts presented in the text were for the medium and incubation conditions which yielded the greatest number of counts.

A preliminary investigation of organism types in the Mojave and Wyaconda soil revealed that nearly 80% of all colonies obtained from Mojave soil were spore forming rods. In the Wyaconda soil, there were less than 8% spore forming rods and 50% of all colonies were due to organisms which formed mycelial growth.

C. Temperature Studies

1. General

The labeled release experiment was performed on the three test soils at four different temperatures, 3°C, 25°C, 35°C, and 80°C. The effects of temperature on the kinetics of $^{14}CO_2$ evolution from viable as well as heat sterilized control soils were measured. Production rates of ATP in soil samples were also measured under conditions of the various incubation temperatures. In each of these experiments the soil sample was 0.5 g and the medium 0.2 ml of RM9 medium (Appendix - Section 1-A) containing ^{14}C-formate, ^{14}C-lactate, ^{14}C-glucose, ^{14}C-glycine and $^{35}SO_4$ (total activity 20 μCi/ml).

2. Effect of Incubation Temperature of Mojave Soil

The effect of incubation temperature on the labeled release experiment which was performed on the viable and heat sterilized Mojave soil is shown in Figures 2 and 3, respectively. At all four incubation temperatures tested, the cumulative count viable responses exceeded the nonviable responses by at least 200 fold. The viable response at 80°C was always extremely rapid. However, in similar experiments the cumulative counts did not always reach the high level seen for 80°C in Figure 2. This was probably because the organisms sometimes died at this high temperature prior to the onset of the limiting conditions which arrest growth at lower temperatures.

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FIGURE 2

Effect of Incubation Temperature on the Biological Evolution of Radioactivity from Mojave Desert Soil

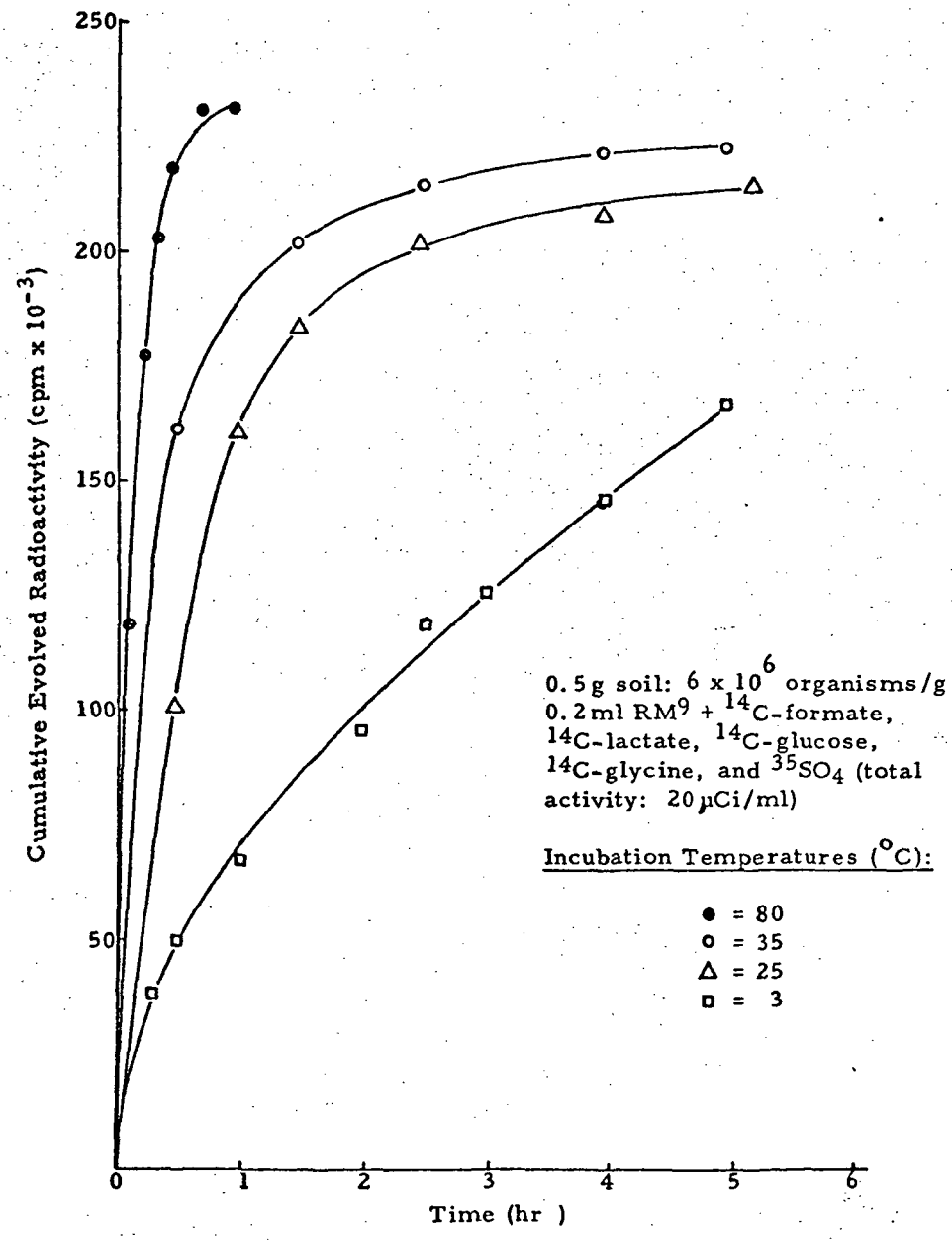

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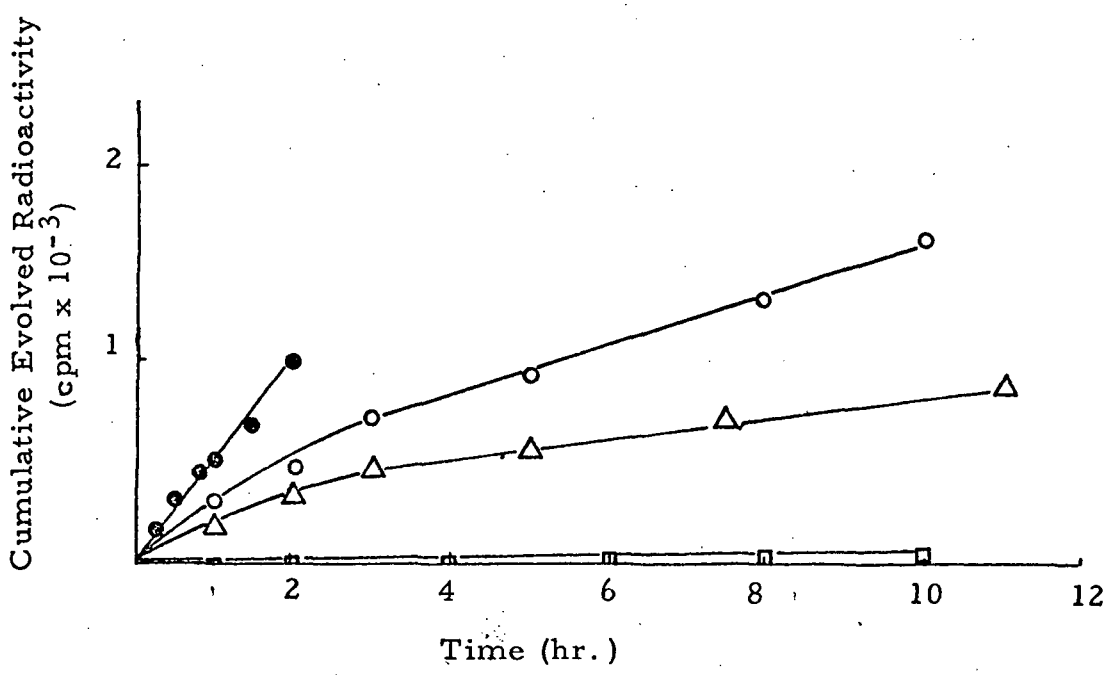
FIGURE 3

Effect of Incubation Temperature on the Nonbiological Evolution of Radioactivity from Heat Sterilized Mojave Desert Soil

0.5 g soil: dry heat sterilized 30 minutes at 212°C.
0.2 ml RM9 + ^{14}C-formate, ^{14}C-lactate, ^{14}C-glucose, ^{14}C-glycine and $^{35}SO_4$ (total activity: 20 μCi/ml)



Support for the theory of cellular death has been provided by results of ATP assays conducted in conjunction with the labeled release experiments.

ATP measurements were made on sterile and viable soil at the end of each temperature experiment. Results are presented in Table 3. At 3°C, 25°C, and 35°C, an increase in viable soil ATP of five to eight fold was observed during the course of the experiment. The final difference between sterilized and viable soils was two orders of magnitude. Results of the 80°C experiment were different. The viable soils decreased in ATP and showed a final value similar to that for the heat sterilized control. This decrease in ATP had occurred despite a measured high evolution of $^{14}CO_2$. Thus, the organisms may have metabolized for only a portion of the test and then died. To test for the possibility of ATP production followed by degradation, an experiment was conducted at 80°C in which replicate planchets were assayed for ATP at intervals during incubation. Results of this experiment are presented in Figure 4. As shown, the ATP concentration was found to rise initially and then fall sharply after ten minutes incubation, validating the hypothesis.

To explore the possibility of ATP losses at lower temperatures, a similar experiment was conducted on Mojave soil at 3°C. The results of this experiment are shown in Figure 5. No loss in ATP was observed.

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TABLE 3

Effect of Temperature on the Production of ATP in Mojave Soil

0.2 ml of RM9 medium containing ^{14}C-formate, ^{14}C-lactate, ^{14}C-glucose, ^{14}C-glycine, and $^{35}SO_4$ (total activity 20 μc/ml) were added to 0.5 g of soil. ATP measurements in duplicate was made after the labeled release indicated attainment of stationary growth.

	Concentration of ATP(μg/0.5 g soil) Prior to Incubation	
	Viable	Sterile
	.0325	.0023
	.0290	.0018

Incubation Temperature	Concentration of ATP μg/0.5 g soil After Incubation*	
	Viable	Sterile
3°C	.2544	.0025
	.1543	-
25°C	.1805	.0050
	.1947	.0093
35°C	.1758	.0080
	.1598	.0059
80°C	.0045	.0029
	.0038	.0025

*Incubation period varied according to the time required for a plateau in labeled release.

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FIGURE 4

ATP Concentration and Labeled Release of
Mojave Desert Soil Incubated at 80°C

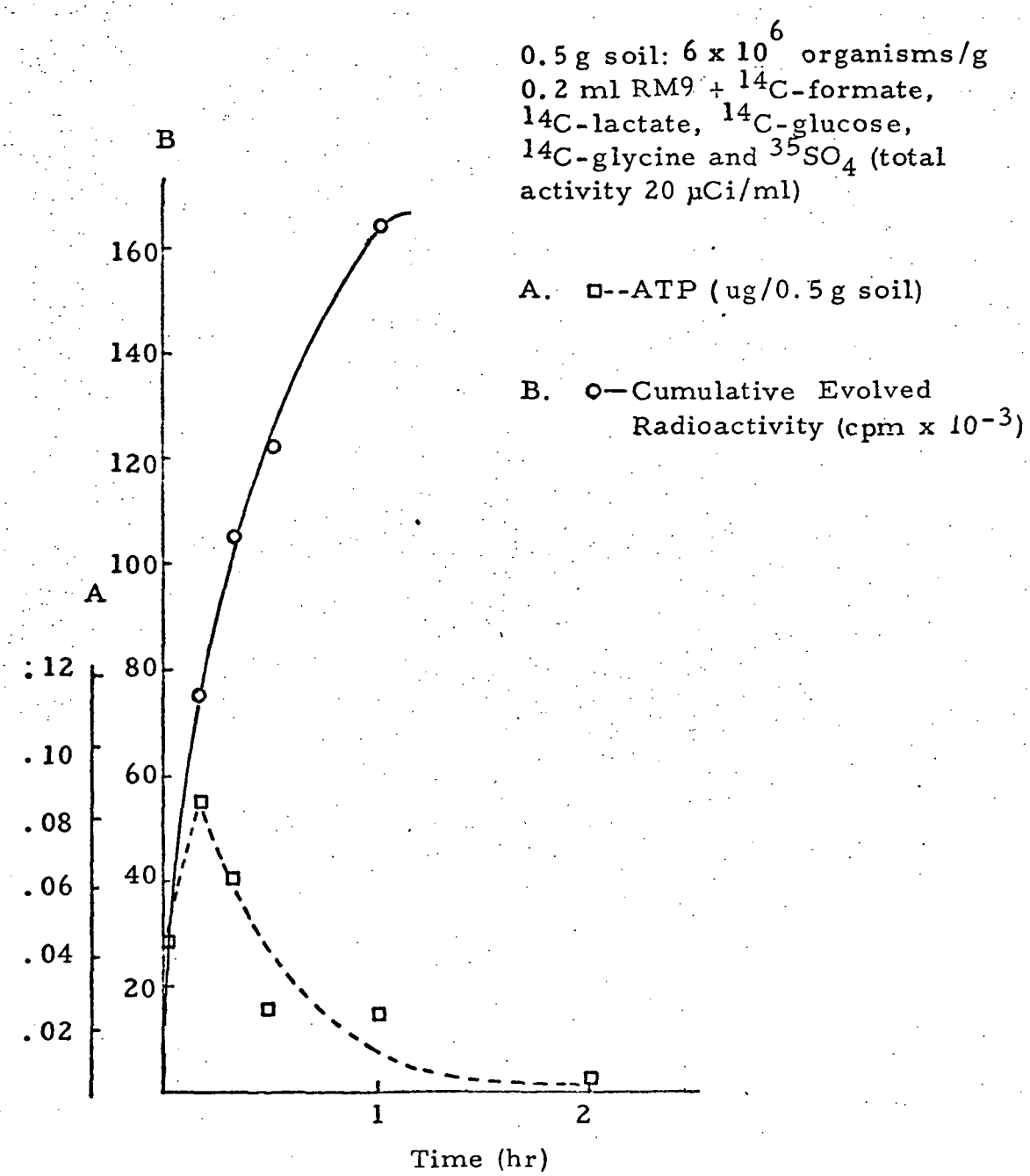

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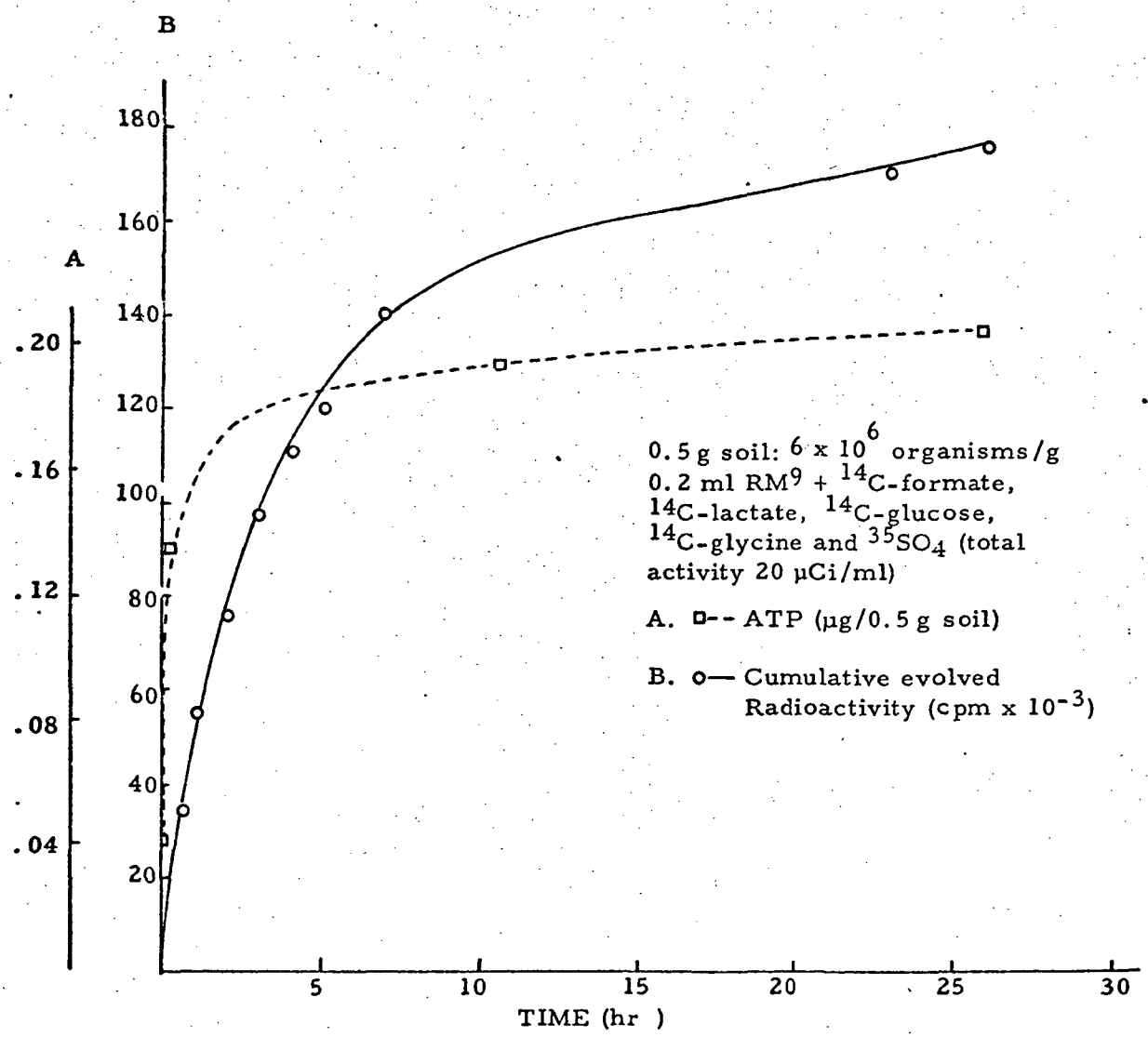
FIGURE 5

ATP Concentration and Labeled Release
Mojave Desert Soil Incubated at 3°C



Of additional interest is the fact that at least half the ATP production occurred during the first 15 minutes of the experiment.

A Q_{10} calculation for the effect of temperature on the rate of response of both viable and sterilized soils was made. As has been shown for other biological processes, the Q_{10} was found to decrease as the temperature increased. The influence of temperature on the kinetics of radioactive gas evolution is shown in Figure 6. The rates were determined graphically from Figures 2 and 3 at approximately half maximum cumulative evolution. Figure 6 shows that the rate of viable and control responses from Mojave soil at any one temperature are separated by at least three orders of magnitude.

3. Effect of Incubation Temperature on Wyaconda Soil

The effect of incubation temperature on the labeled release experiment which was performed on both viable and heat sterilized soils is shown in Figures 7 and 8. At the 3°C, 25°C, and 35°C incubation temperatures the viable response (cumulative counts) exceeded the sterile response by a minimum of 40 fold. The 80°C incubation resulted in an initial 40 fold difference between viable and control cumulative counts. However, the organisms were apparently killed very rapidly. The viable response at 25°C was similar to that at 35°C, but 3°C incubation very strongly reduced the rate of evolution. The effect of temperature on the rate of evolution of radioactivity is

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FIGURE 6

Effect of Incubation Temperature on the Rate of Evolution of Radioactivity from Mojave Desert Soil

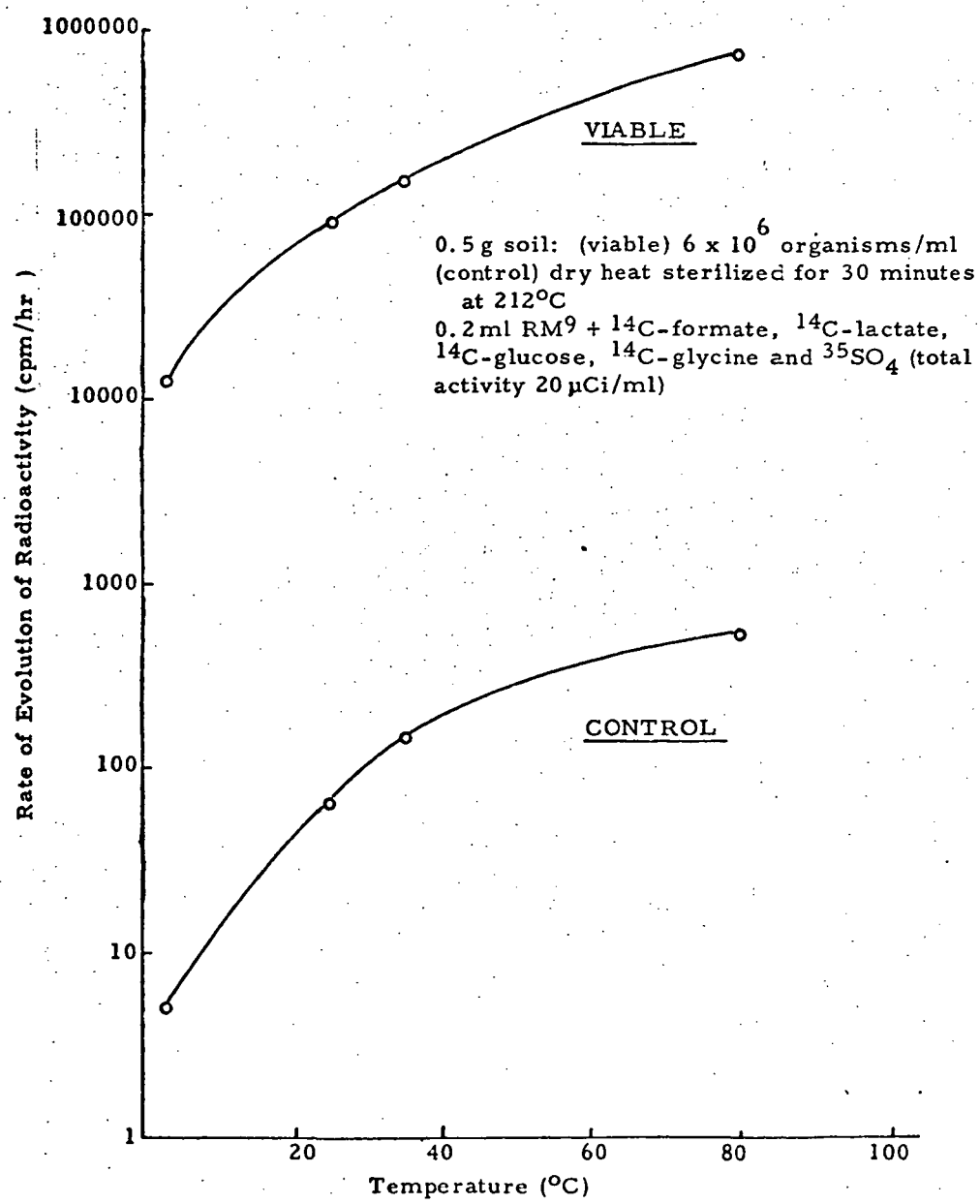

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FIGURE 7

Effect of Incubation Temperature on the Biological Evolution of Radioactivity from Wyaconda (Maryland) Soil

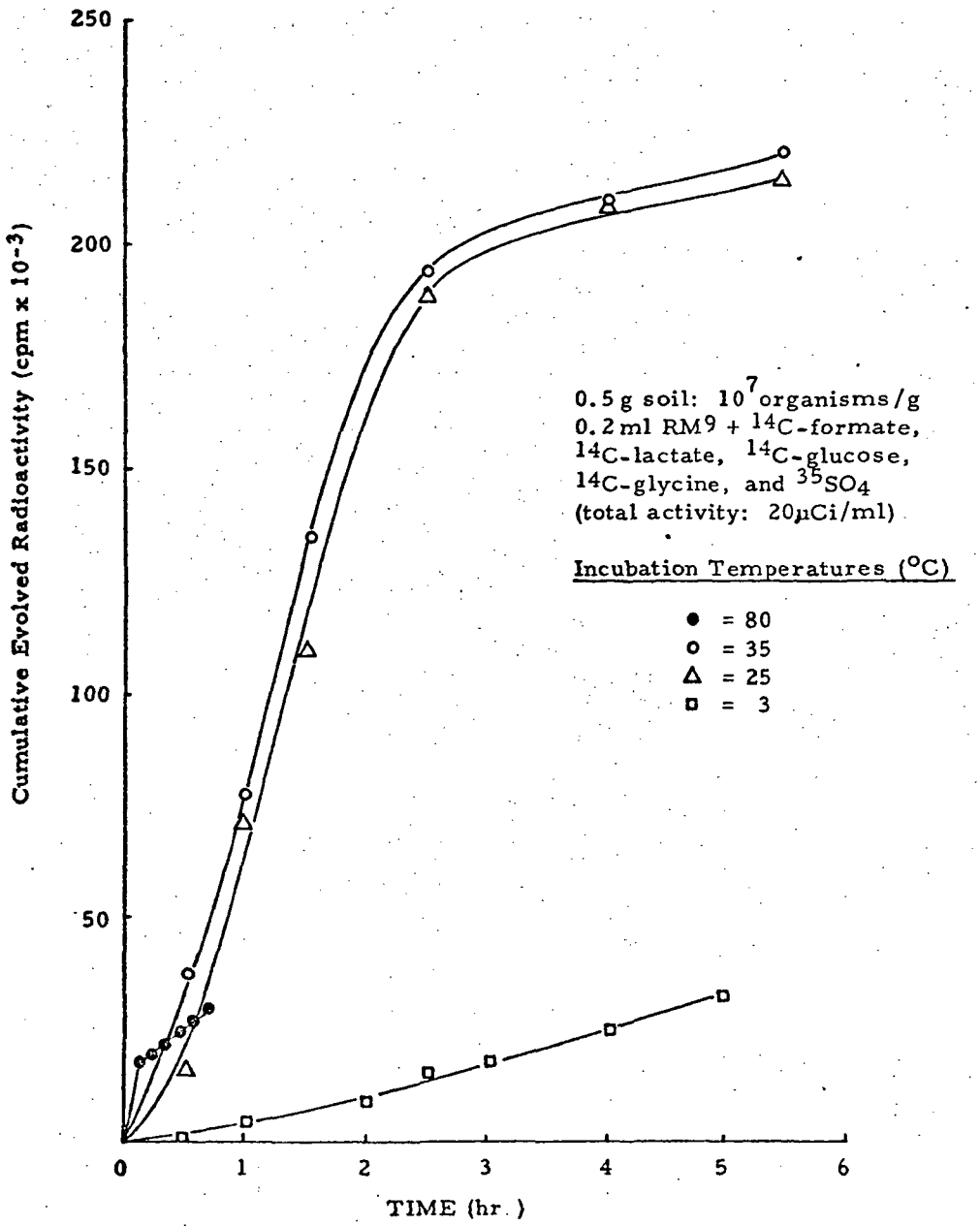

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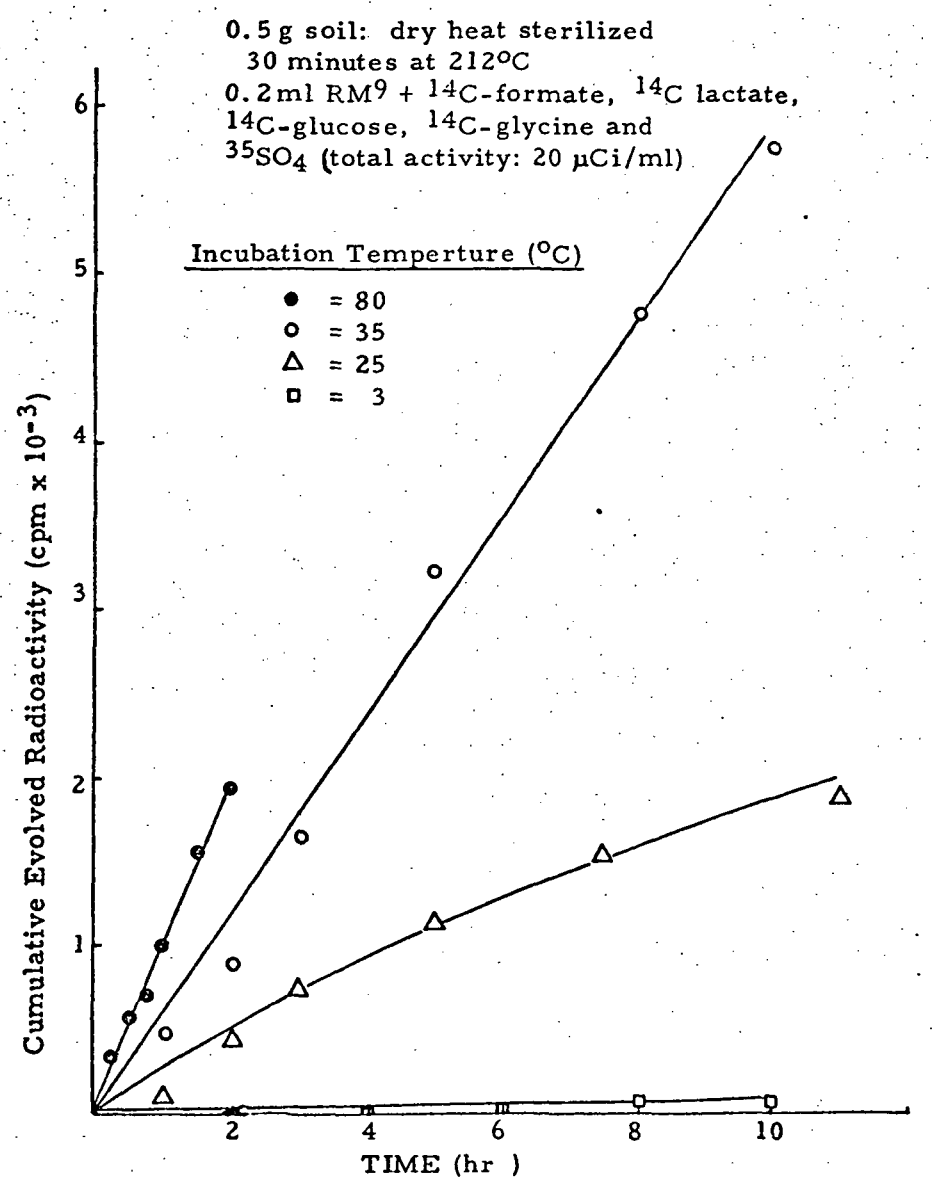
FIGURE 8

Effect of Incubation Temperature on the Nonbiological Evolution of Radioactivity from Heat Sterilized Wyaconda (Maryland) Soil



shown in Figure 9. The rate of response of the viable soil at any one temperature exceeded the rate of response from the sterile soil by two orders of magnitude.

4. Effect of Incubation Temperature on Victoria Valley Soil

The effect of incubation temperature on the labeled release experiment performed on both viable and heat sterilized controls is shown in Figures 10 and 11. The magnitude of response from this soil was much lower than the other soils tested. However, cumulative evolution of radioactivity from viable soils that was three fold greater than the nonviable evolution was obtained within a few hours. Soil platings required two weeks of incubation before organisms were detected. Figure 12 shows the effect of temperature on the rate of response from both viable and sterile soils. The greatest difference between signal and noise occurred at 3°C where nearly two orders of magnitude separated the viable and sterile responses. The sterilized soils produced the same shaped rate-versus-temperature curve as had been produced by the other sterile soils. However, the viable soil rate-versus-temperature curve was somewhat different for Victoria Valley soil. Incubation temperatures of 3°C or 25°C produced similar responses and the 35°C and 80°C incubation produced similar, but greater responses.

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FIGURE 9

Effect of Incubation Temperature on the Rate of Evolution of Radioactivity by Wyaconda (Maryland) Soil

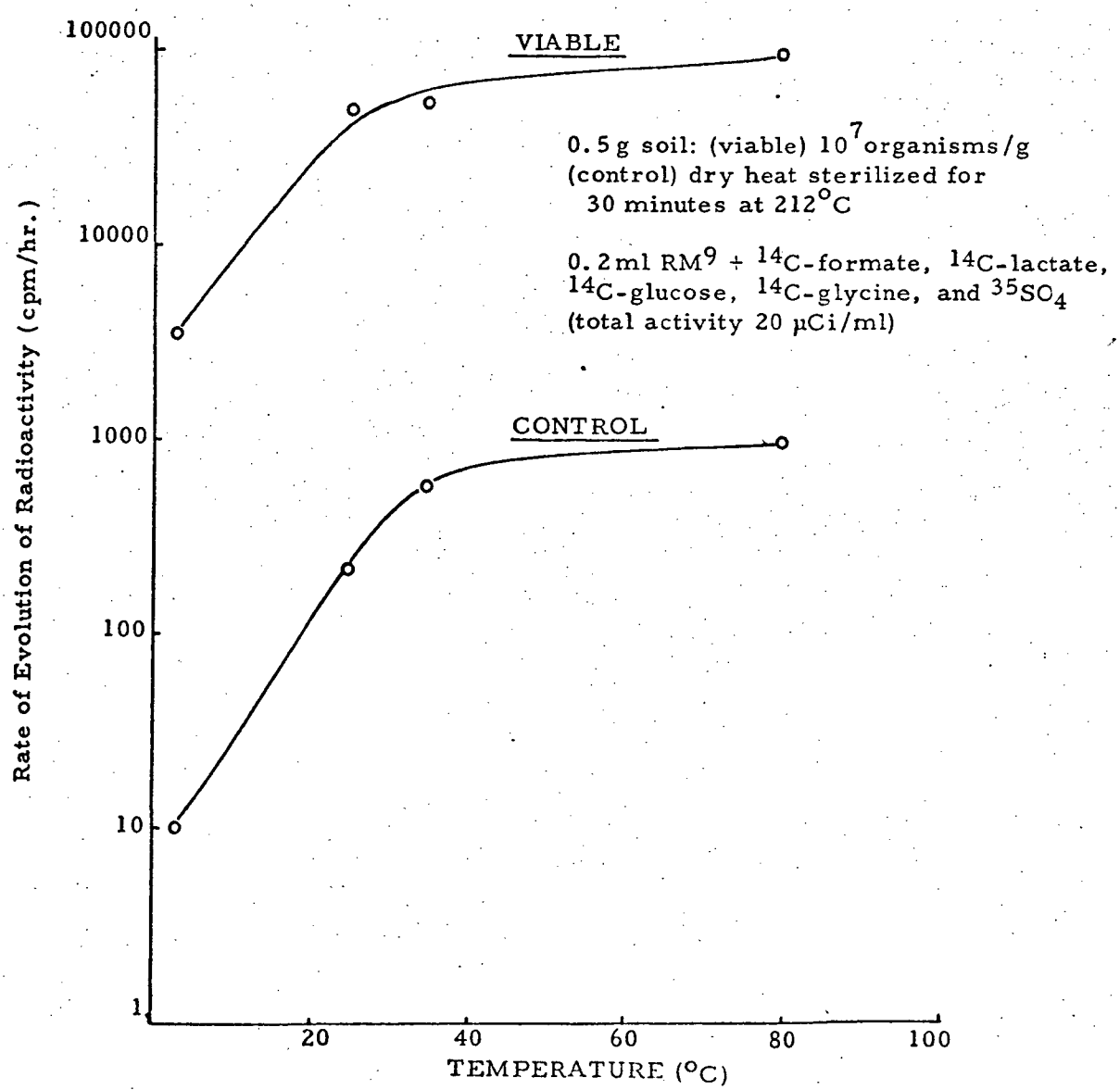

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FIGURE 10

Effect of Incubation Temperature on the Biological Evolution of Radioactivity from Victoria Valley (Antarctica) Soil

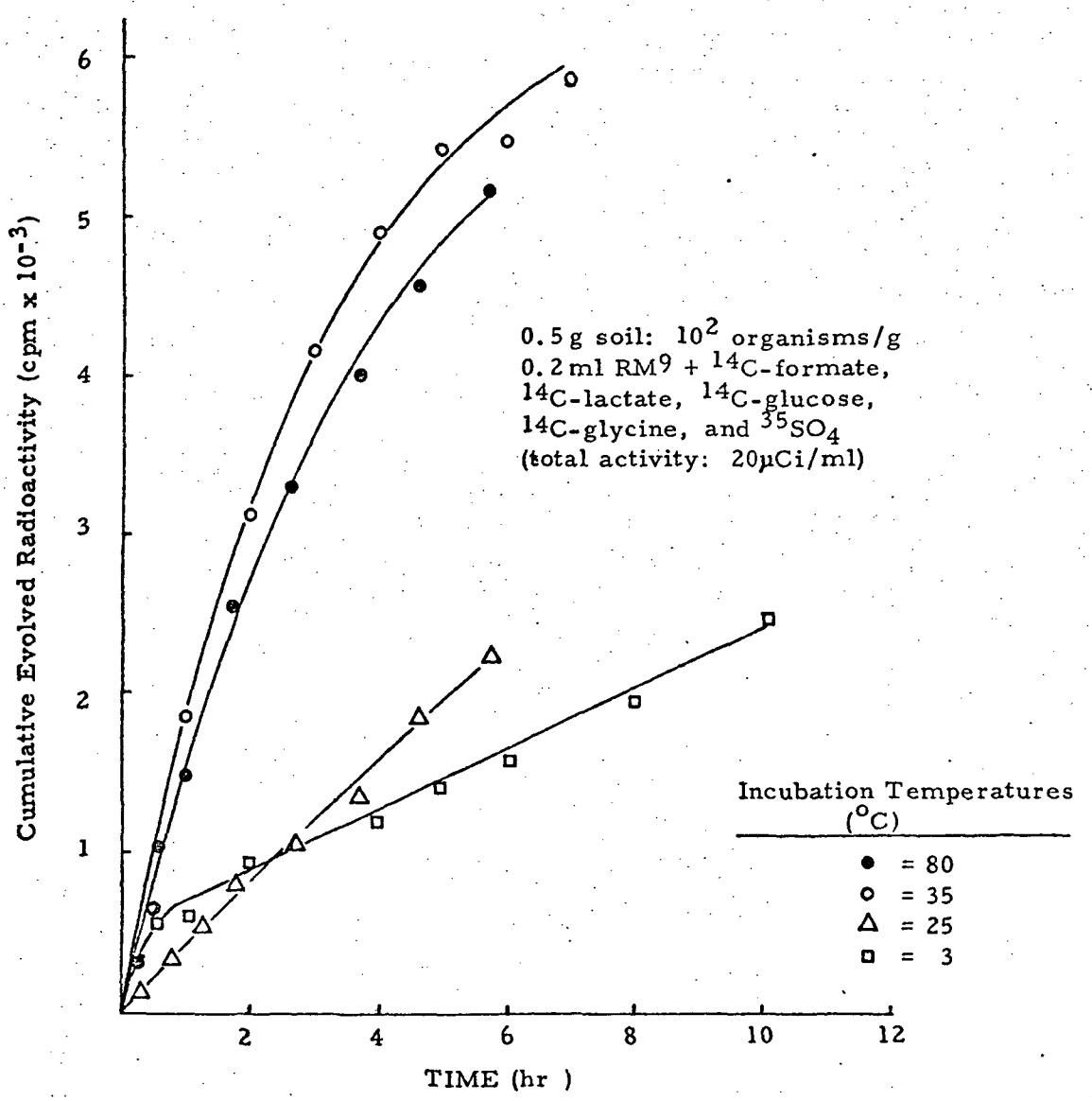

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FIGURE 11

Effect of Incubation Temperature on the Nonbiological Evolution of Radioactivity from Heat Sterilized Victoria Valley (Antarctica) Soil

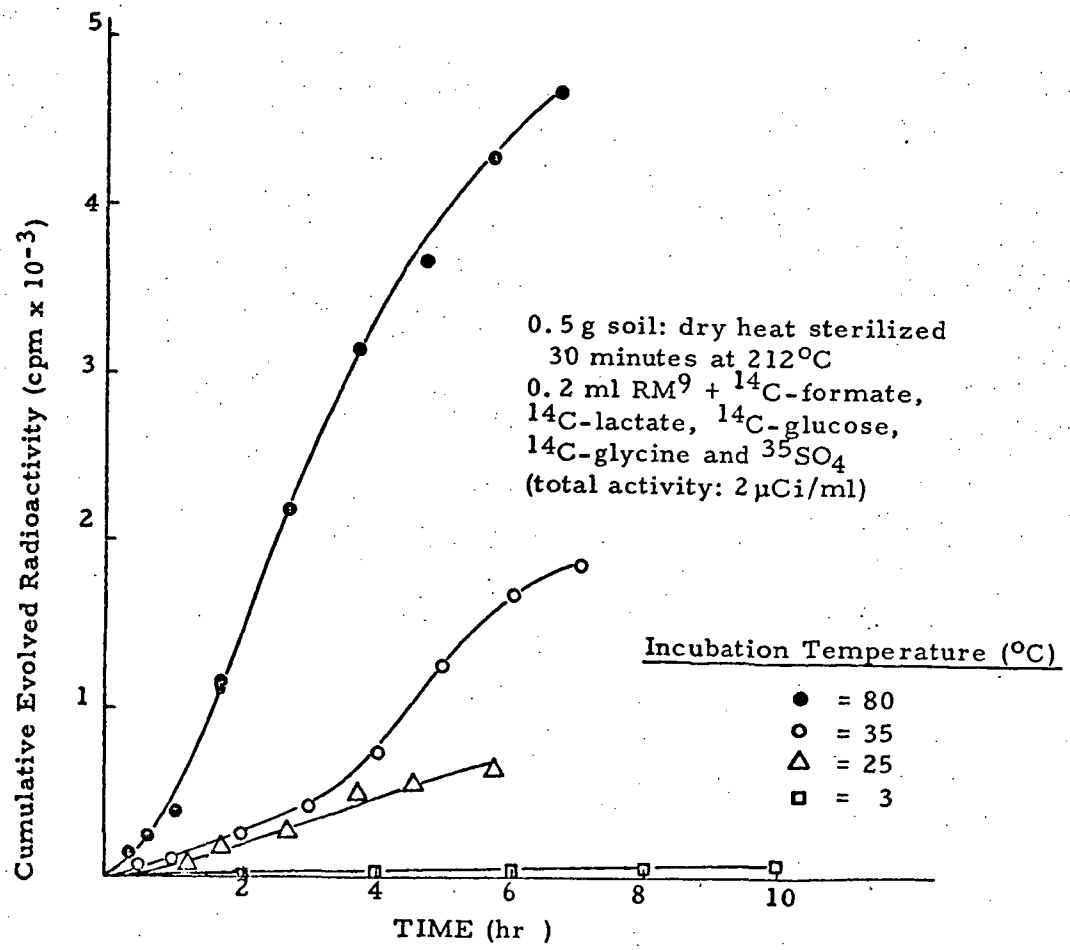

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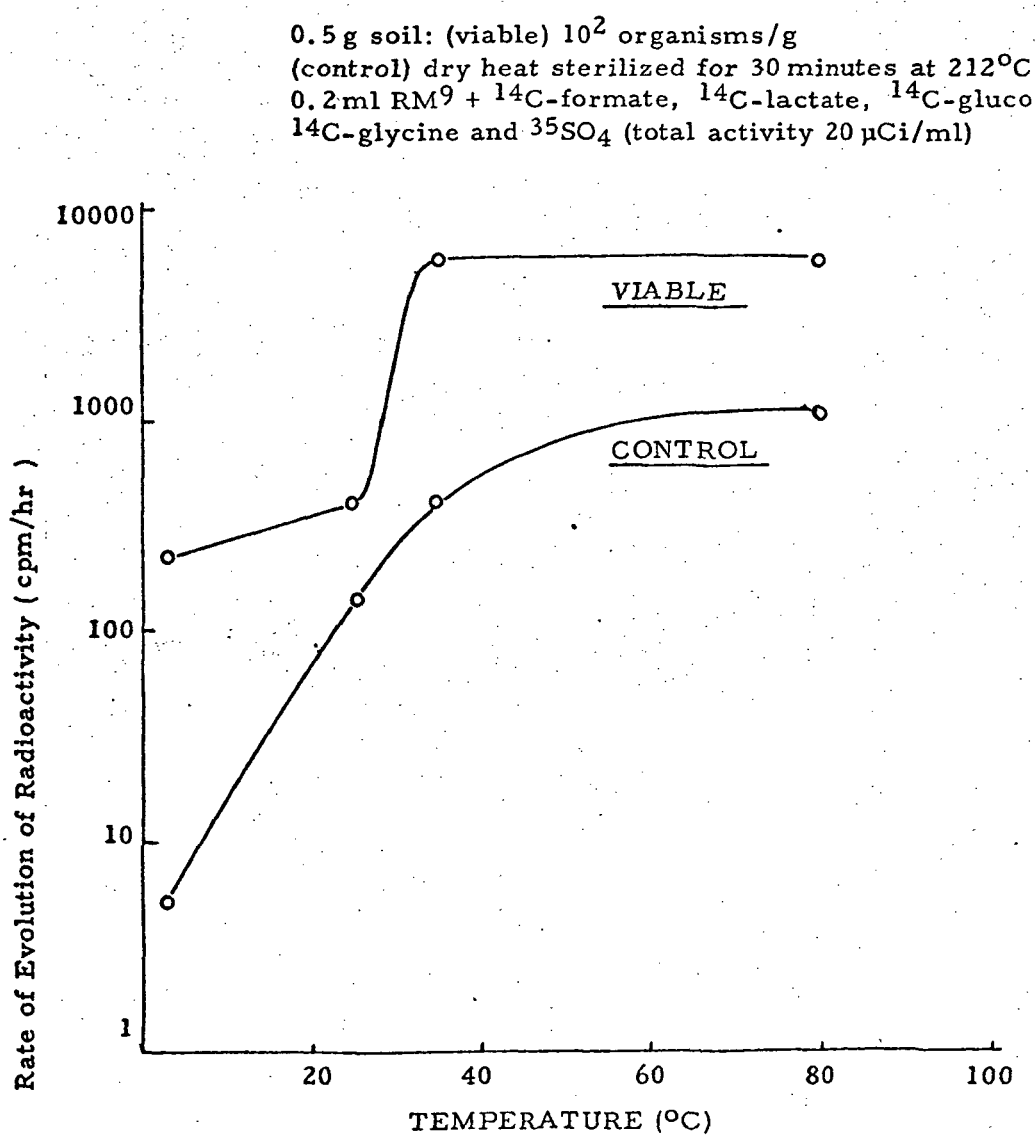
FIGURE 12

Effect of Incubation Temperature on the Rate of Evolution of Radioactivity by Victoria Valley (Antarctica) Soil



All soils produced a lower rate of nonbiological response at the lower incubation temperature and thereby showed a maximum difference between biological and nonbiological responses at that temperature. This indicates a favorable development if extrapolated to Mars.

Experimental temperatures of 80°C, the highest temperature tested, produced the highest nonbiological evolution of radioactivity. Planchets containing only sterile medium also evolved considerable radioactivity at 80°C, and the rate of evolution appeared to correlate with the degree of drying which occurred. To test the physical effects of soil particles on nonbiological evolution at 80°C, the following experiment was conducted: Duplicate 0.5 g samples of soil and glass powder (200 mesh) were weighed into sterile planchets. These and two additional planchets, for medium alone, were then placed in petri dishes and heated at 215°C for 30 minutes. After sterilization, the petri dishes with planchets were cooled and placed in an 80°C incubator. After temperature equilibration 0.2 ml of sterile RM9 medium containing ^{14}C-lactate, ^{14}C-glucose, ^{14}C-formate, ^{14}C0glycine and $^{35}SO_4$ (total activity 20 μCi/ml) were added. Temperature-equilibrated getters were changed at one-hour intervals. Throughout the experiment, small containers of water were maintained in the petri dishes. Results are shown in Figure 13. Nonbiological evolution of radioactivity from medium was significantly reduced by addition of powdered glass. The

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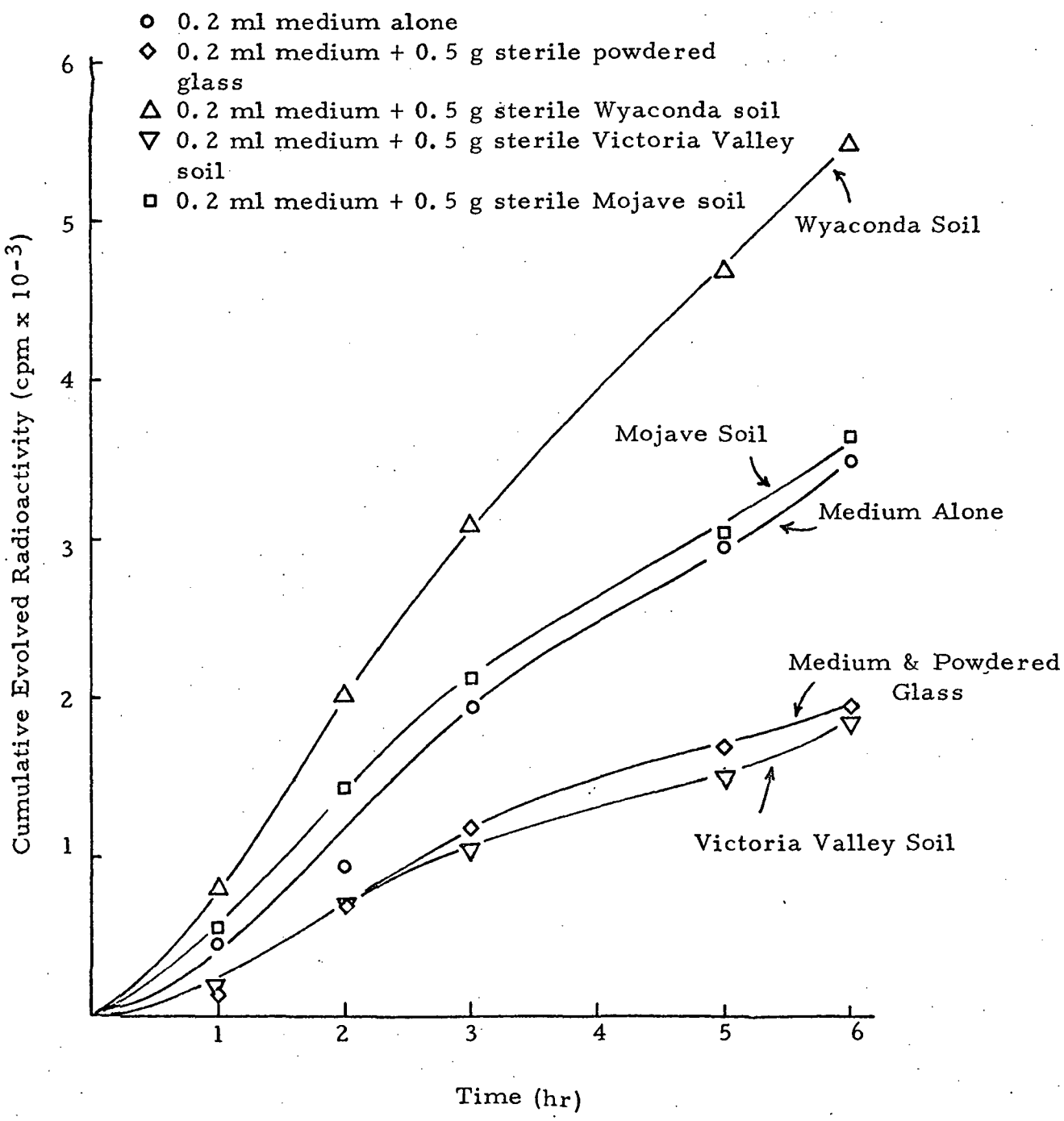
FIGURE 13

Effect of Soil or Powdered Glass on the Nonbiological Evolution of Radioactivity at 80°C



Victoria Valley soil produced an effect similar to the glass powder. Wyaconda soil increased the evolution and Mojave soil produced background similar to the planchet with medium alone.

D. Ultraviolet Irradiation Studies

Soils were exposed to UV irradiation (intensity 90 milliwatts per cm^2, 2537 Angstroms) for periods up to 10^6 seconds, and subsequently tested for labeled release. Results are shown in Figures 14 through 17.

The activity of Mojave soil was reduced 40% by the 10^6 seconds exposure. Wyaconda and Victoria Valley soils on the other hand, showed a decreased response of 30% after 10^4 seconds of exposure. An exposure of 10^6 seconds on these latter two soils resulted in a ten fold decrease in response rate.

Control soils (heat sterilized prior to UV exposure) were unaffected by UV exposure.

E. Moisture Studies

Findings concerning the low concentration of water on Mars spurred the attempts described earlier in this report to adapt life detection assays to "moist" rather than "wet" experiments. It was known from earlier work (3) that a high soil-to-liquid ratio produced a very rapid "early burst" evolution of radioactivity. However, a detailed study of soil to liquid ratios had not been performed. The following experiments were conducted to show the effect of soil/liquid

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FIGURE 14

Effect of Ultraviolet Light on Mojave Desert Soil as Determined by Labeled Release Experiment

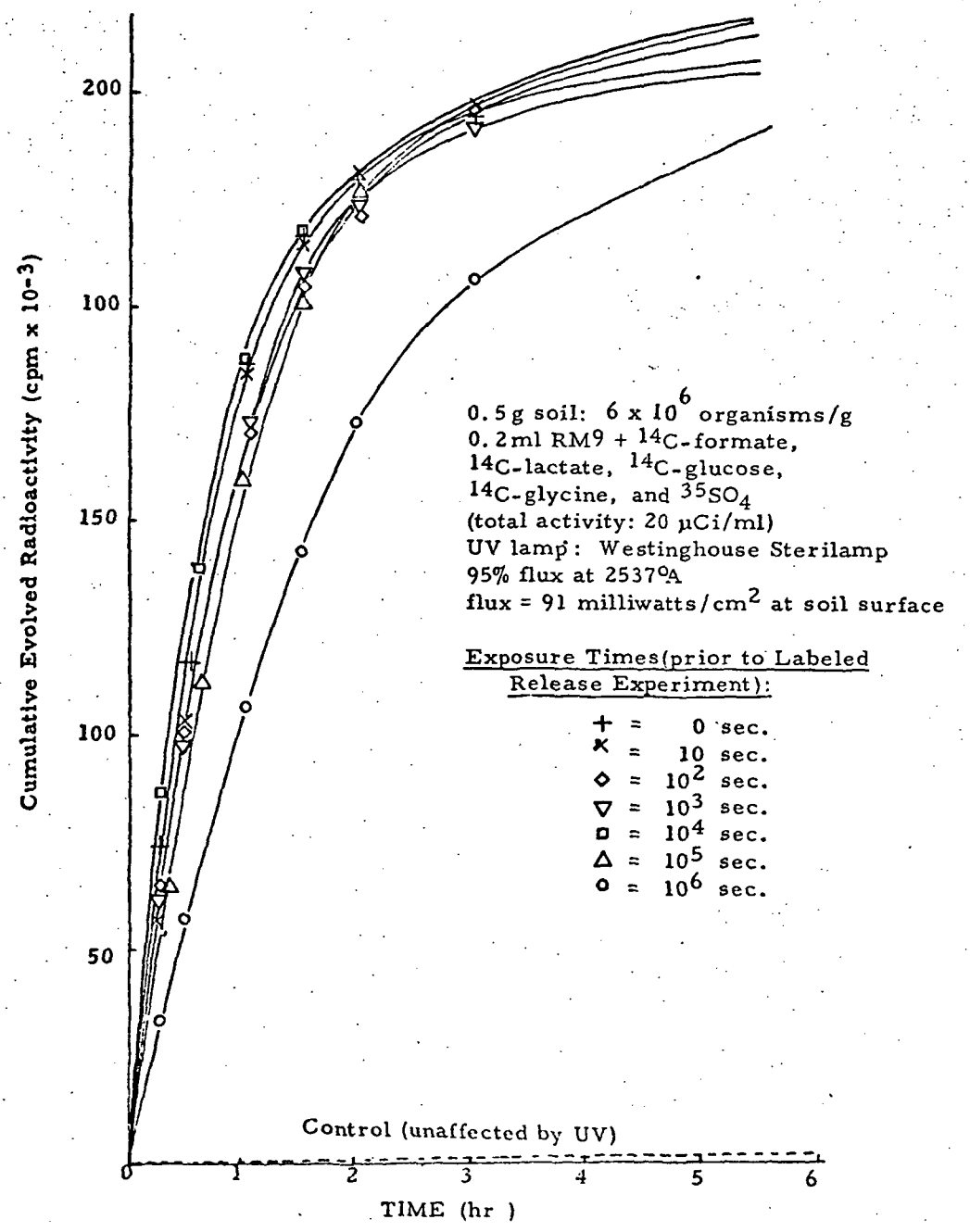

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FIGURE 15

Effect of Ultraviolet Light on Wyaconda (Maryland) Soil as Determined by Labeled Release Experiment

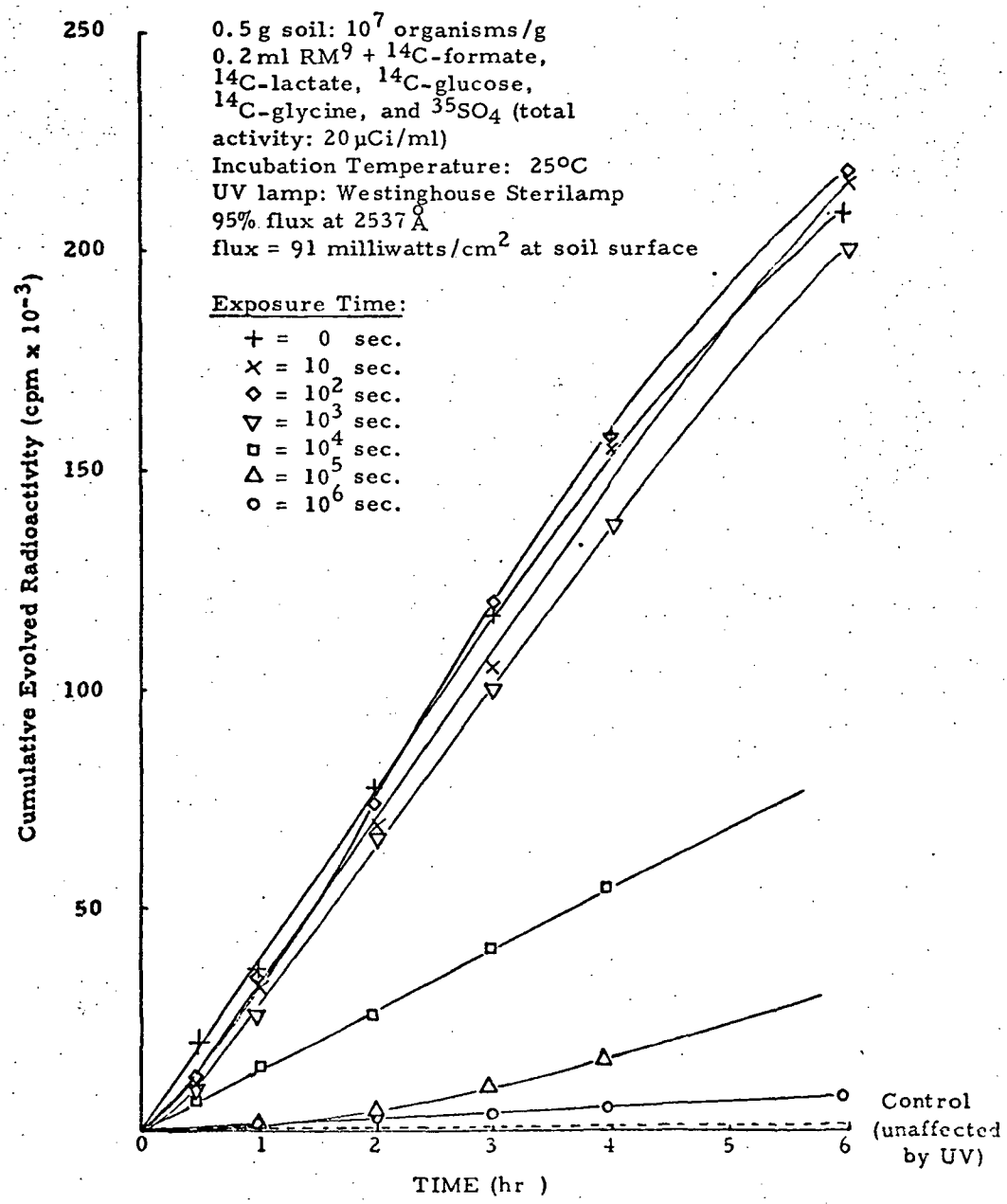

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FIGURE 16

Effect of Ultraviolet Light on Victoria Valley (Antarctica) as Determined by Labeled Release Experiment

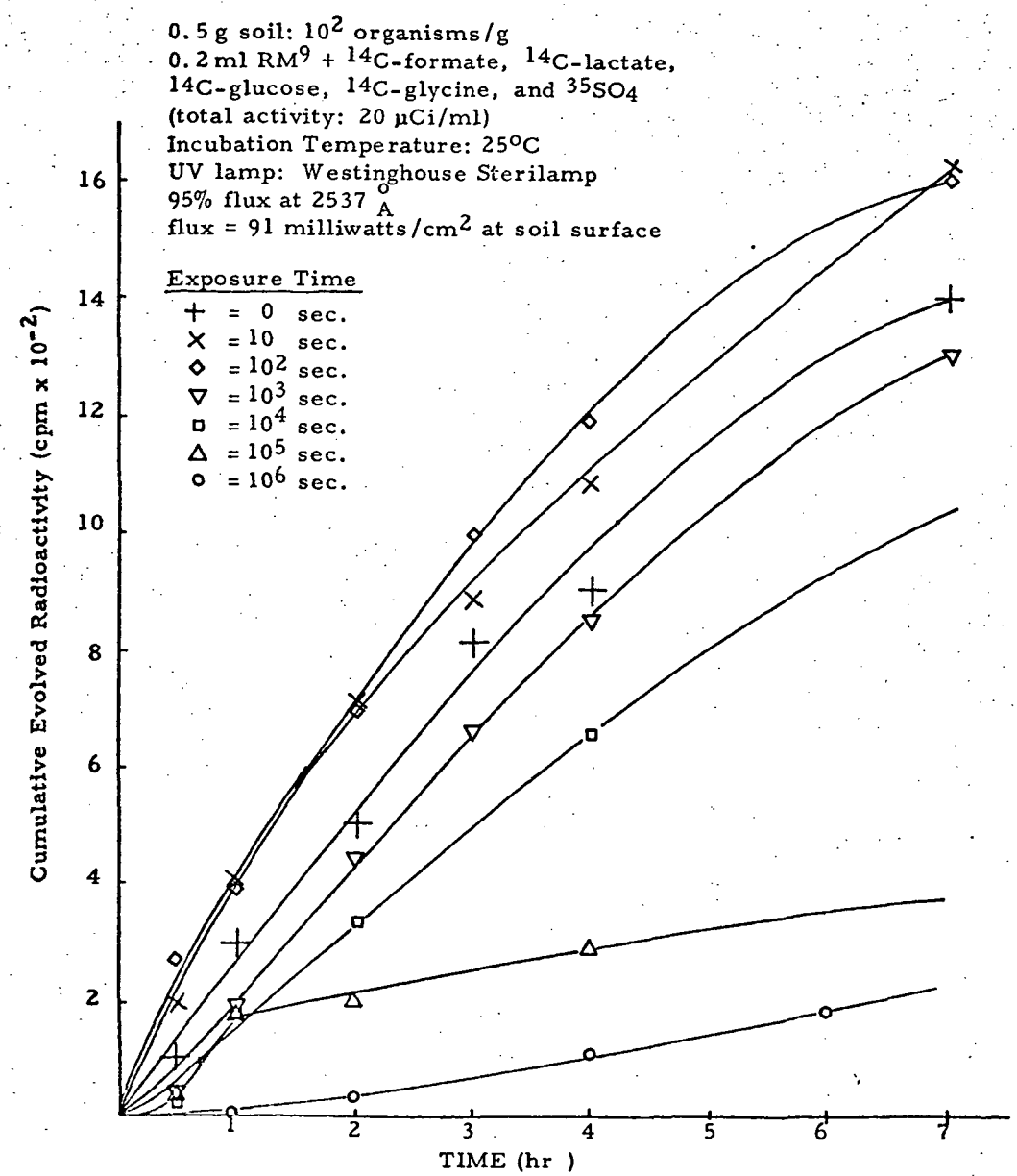

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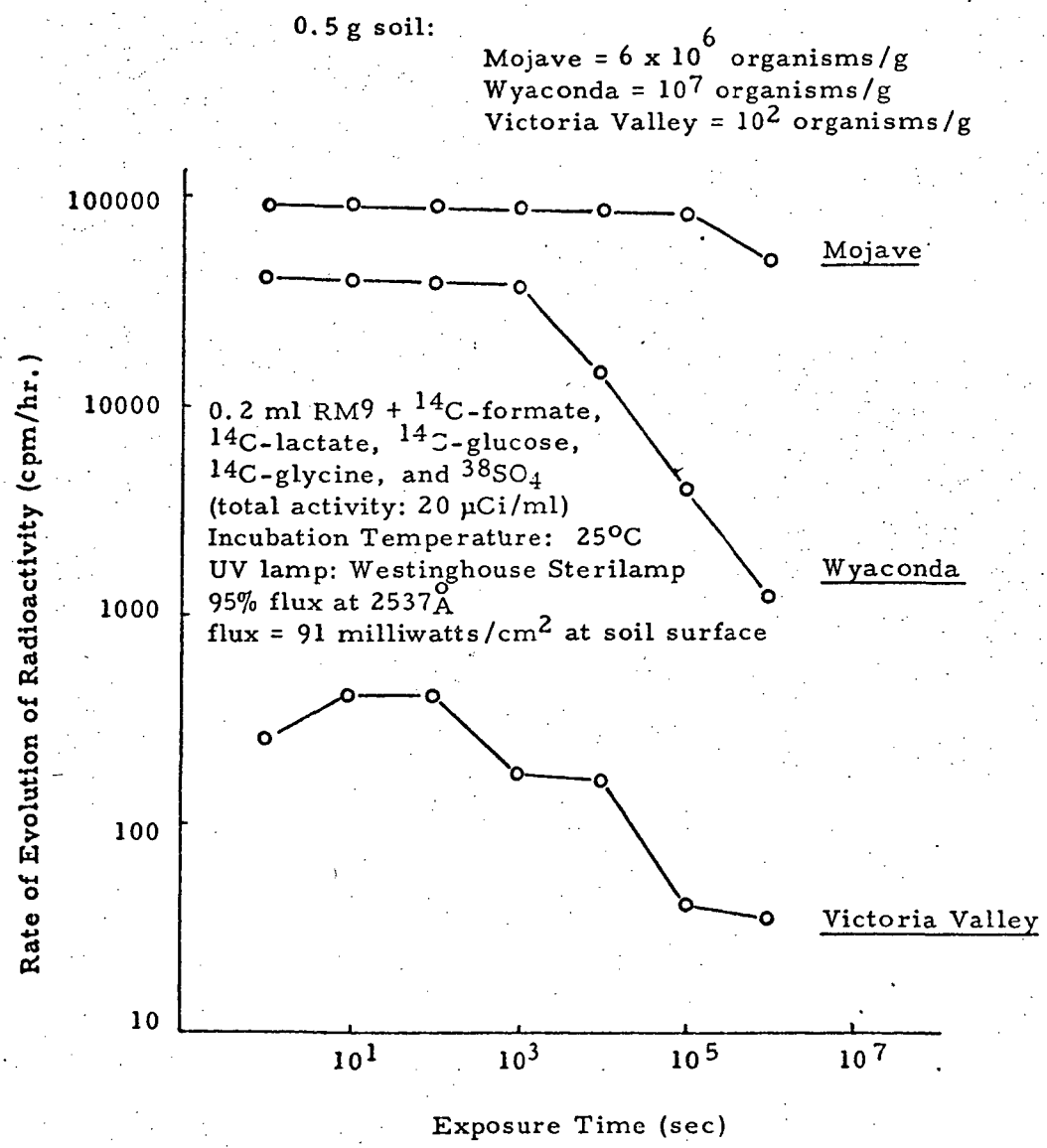
FIGURE 17

Effect of Ultraviolet Light on the Rate of Evolution of Radioactivity from Mojave Desert, Wyaconda (Maryland), and Victoria Valley (Antarctica) Soil



ratios and to determine the ratio which would produce a maximum response.

Weighed quantities of soil and measured amounts of RM9 medium were placed into planchets in combinations which made up a broad range of soil/medium ratios. The "wettest" of these ratios was conducted in magnetically stirred glass tubes which were adapted and capped with getters as shown in Figure 18. Soil and medium quantities which were used to produce the range of ratios studied are given in Table 4. Labeled release was followed in the usual manner. Mojave soil was assayed for the production of ATP. Phosphate uptake and ^{14}C and ^{35}S uptake were measured in the "wet" (33 ml/g) experiments on all three soils.

1. Results of Labeled Release

The overall effect of the medium/soil ratio on the evolution of radioactivity from Wyaconda soil is shown in Figure 19. The specific activity of the medium was the same in all but one case (Ratio 33). Therefore, the total radioactivity added was determined by the volume of medium used. This resulted in the differences in plateaus of cumulative evolved radioactivity. The higher medium/soil ratios do appear to show a lag in the evolution of radioactivity which is not present in low medium/soil ratios.

It has been suggested that this observed lag may be caused by the entrappment of metabolically produced $^{14}CO_2$ by the water phase (4).

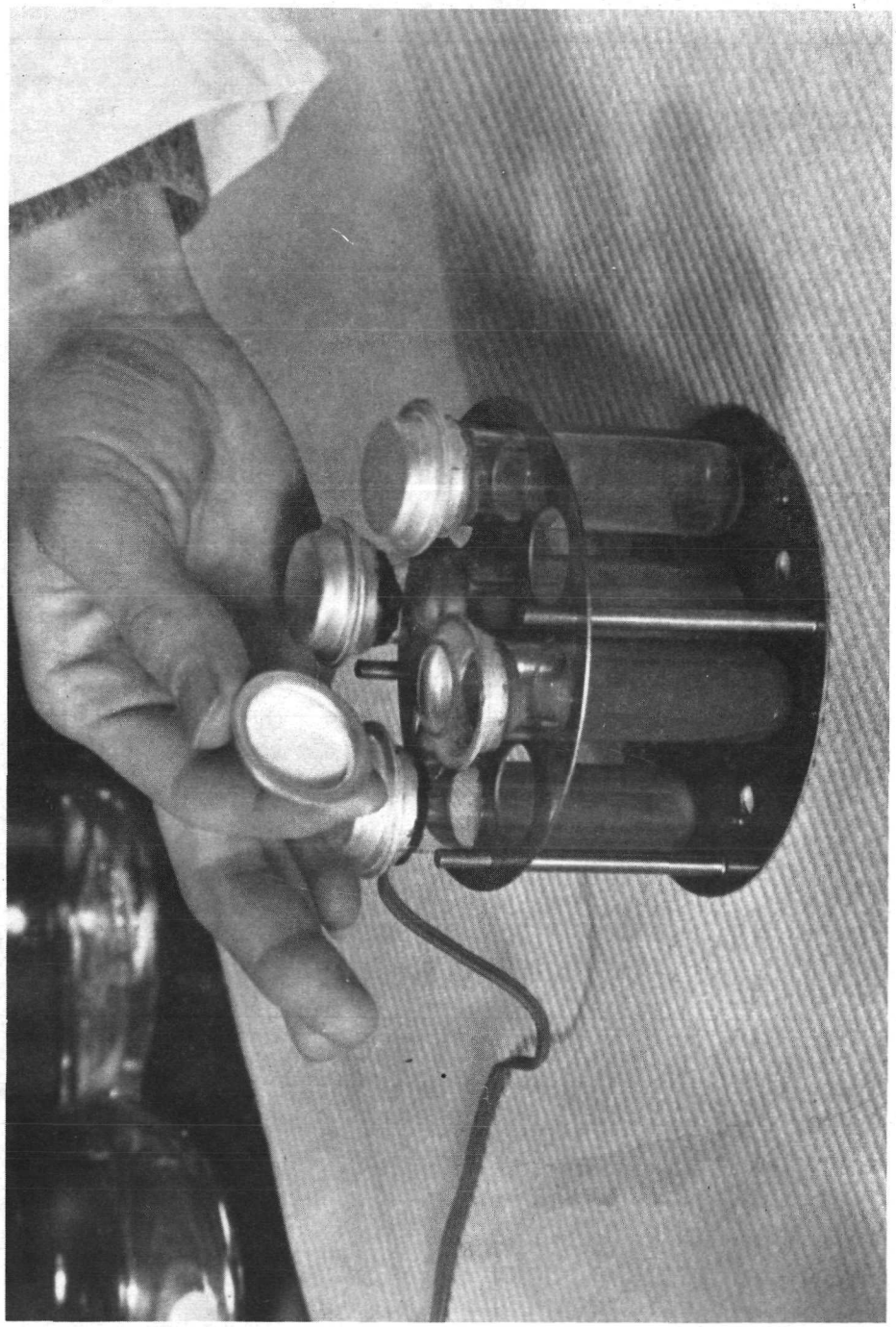


FIGURE 18

Stirred Tubes Used for Gettering 10 ml Culture System

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TABLE 4

Quantities of Soil and Medium Which Were Used to Produce the Range of Liquid/Soil Ratios Studied

RM9 Medium (ml)***	Soil (g)	Ratio (ml/g)
10.0**	0.3	33
0.5	0.05	10
0.2	0.10	2.0
0.2	0.5	0.4
0.1	1.0	0.1
0.05	1.0	0.05
0.02*	1.0	0.02
0.005*	1.0	0.005

* Small volumes were measured using a Hamilton syringe.

** Total activity 2 μCi/ml.

*** Medium was RM9 containing ^{14}C-lactate, ^{14}C-formate, ^{14}C-glucose, ^{14}C-glycine and $^{35}SO_4$ (total activity 20 μCi/ml).

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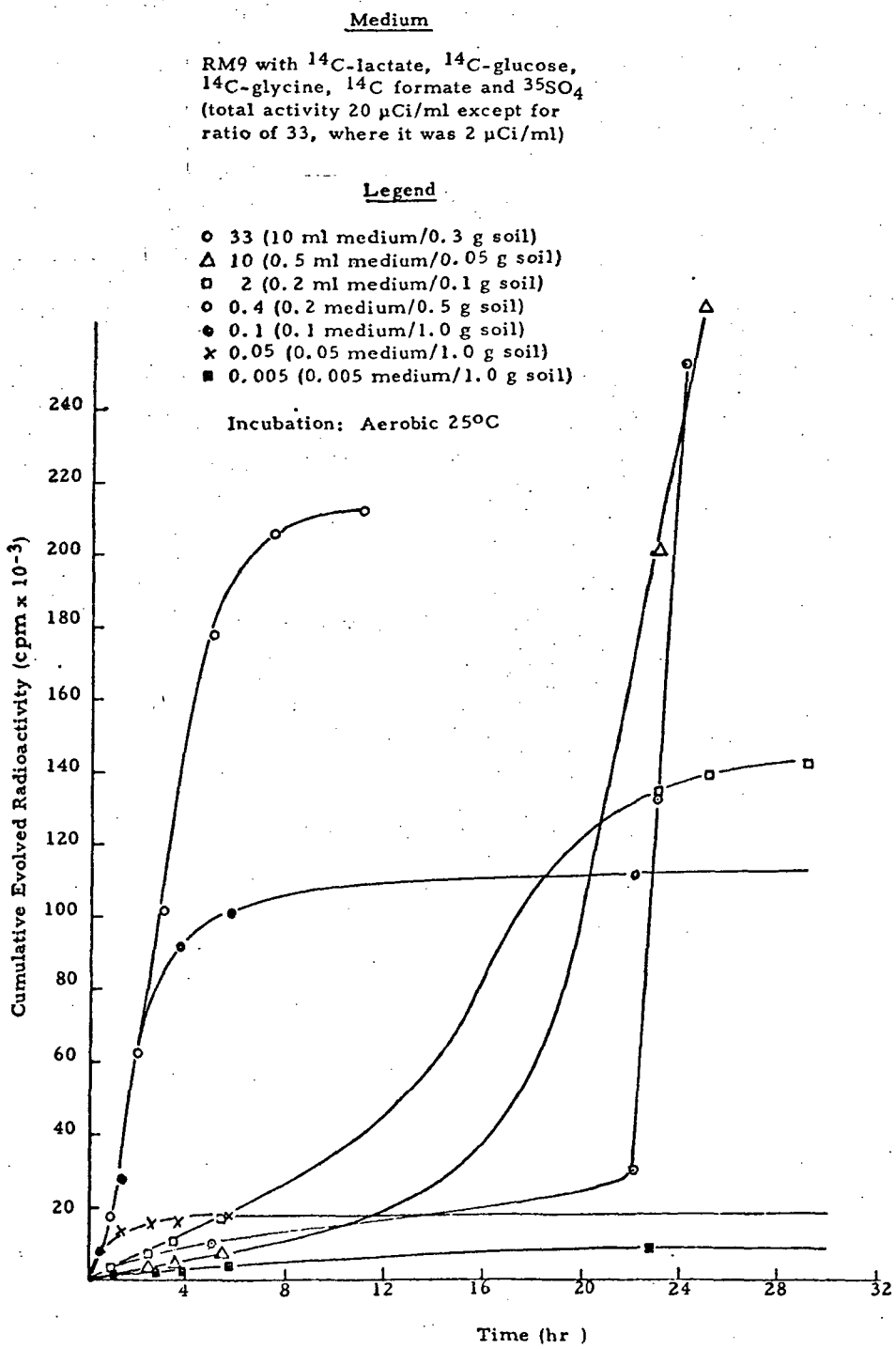
FIGURE 19

Effect of Medium/Soil Ratio on the Evolution of Radioactivity from Wyaconda Soil



A separate labeled release experiment (Ratio 0.4) was halted at four hours by the addition of trichloracetic acid (TCA) and the $^{14}CO_2$ subsequently released from the medium collected and measured. The results of this experiment are shown in Table 5. Since 6,736 cpm of radioactivity was trapped by 0.4 ml of medium, the entrappment of approximately 17,000 cpm might be expected per ml of liquid present. There is a possibility that some of the released $^{14}CO_2$ was contained in cells which were ruptured by the TCA treatment. However, this experiment seems to confirm the fact that some metabolically produced $^{14}CO_2$ is retained by the medium. The quantity retained would be influenced by several factors such as pH, temperature, stirring, chemical and physical influence of soil and the rate of $^{14}CO_2$ production.

In order to interpret the labeled release results of the various medium/soil ratios and to compare the rates with which radioactive gas was metabolically produced, the ratio of viable/sterile evolution was calculated. Data presented in Figure 19 were divided by the cumulative evolved radioactivity from corresponding sterile controls. Figure 20 shows the ratio of viable/sterile response that was obtained from the various ratios of medium/soil. It is immediately apparent that the three highest medium/soil ratios required several hours until a ten fold difference between viable and sterile response was obtained. On the other hand, the low medium/soil ratios produced a 100 - 1000 fold difference between viable and sterile response within

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TABLE 5

Entrapment of $^{14}CO_2$ by Liquid

Culture System	0.4 ml RM9 medium (20 μCi/ml total radioactivity) 0.2 g Wyaconda soil Ratio 2 ml/g	
	Viable	Sterile
four-hour cumulative evolved radioactivity (cpm)	20,186	498
	22,188	648
Evolved radioactivity (cpm) after addition of 0.25 ml of 10% TCA	10,021	4,858
	11,910	3,602
Biologically produced $^{14}CO_2$ which was released by TCA (Viable - Sterile)*	6,736	

* Other studies have shown that acid produces a nonbiological release.

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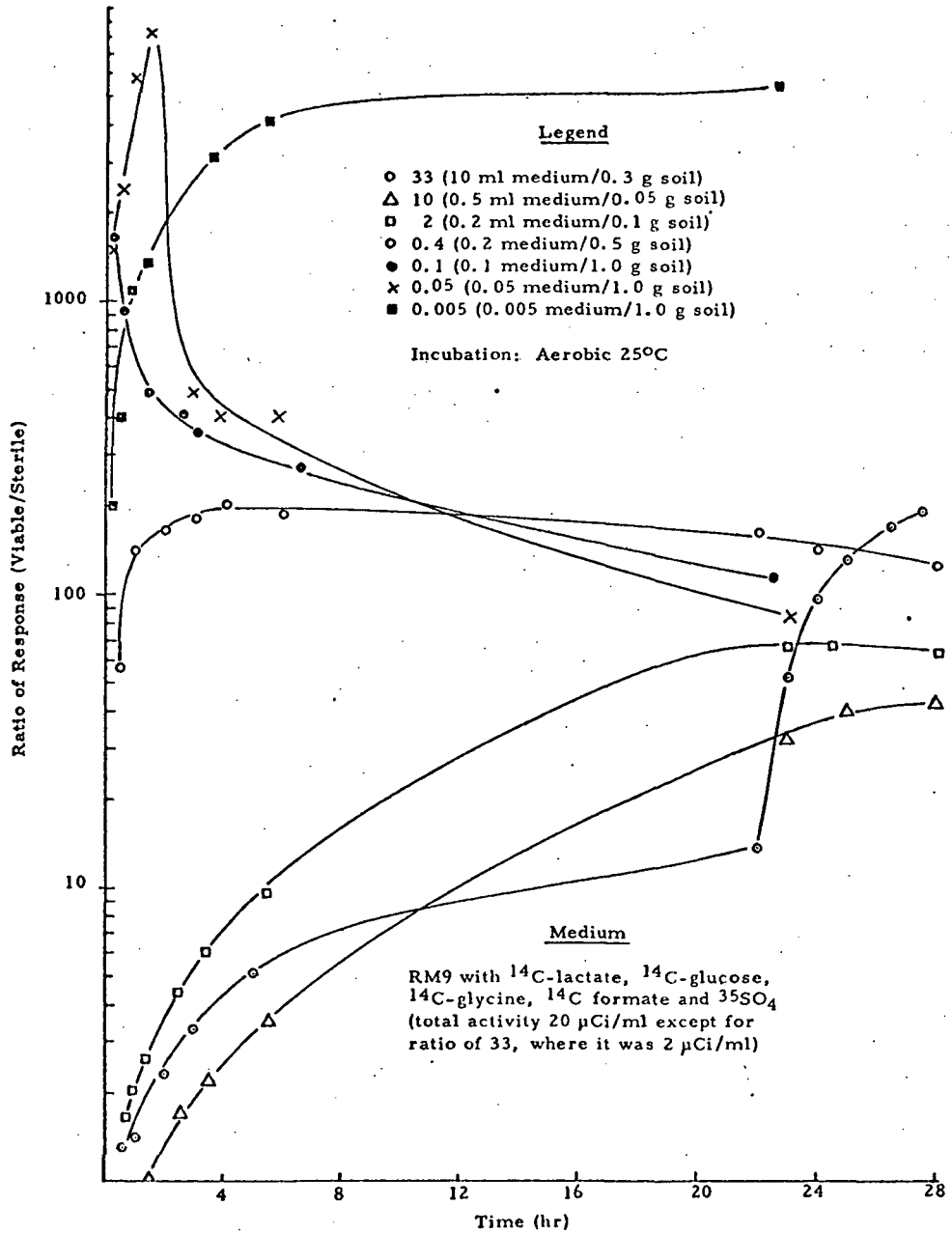
FIGURE 20

Effect of Medium/Soil Ratio on the Ratio of Viable/Sterile Labeled Release



the first hour. Since the viable and sterile systems were identical except for the heat treatment of the sterilized soil, retention of $^{14}CO_2$ by medium would be expected to be similar. The difference in viable and sterile response should occur after CO_2 saturation of the viable medium. Viable systems containing large volumes of medium would in theory require a longer period of metabolism before the medium became saturated with CO_2 and evolution of $^{14}CO_2$ from its surface became rapid. However, sterile systems would be expected to evolve radioactivity at a low rate and therefore require a very long incubation before evolution would occur. This was not the case. The response from both viable and sterile high medium/soil ratio systems was of similar magnitude during the initial hours of incubation, and it was relatively proportional to the volume of medium used.

It appears that the initial metabolic activity is actually greater in the low medium/soil ratios. An explanation may be simply the large relative inoculum size (naturally contained in the soil) which would result in low medium/soil ratios. Also, it may be theorized that larger quantities of water dilute the naturally existing environment to the point where cells must undergo a period of readjustment before growth and/or metabolism occur. Small quantities of water, on the other hand, probably form a thin-film or monolayer which does not move native inorganics, growth factors, and organisms from their natural juxtaposition.

The avoidance of a lag in response may be of utmost importance. The delayed response which occurs in the wetter soil cultures is dependent upon adaptive survival and proliferation of at least a fraction of the microbial flora.

Soils which contain relatively few organisms of selected genera may not produce survivors after a period of suboptimal conditions such as those which a high liquid/soil ratio experiment may impose. The conditions which favor the measurement of an initial burst of radioactivity would be highly advantageous.

Similar experiments were performed on Mojave and Victoria Valley soils, with essentially the same results as those presented for the Wyaconda soil.

Of the ratios tested, those between 0.4 - 0.005 ml/g gave a high initial biological response. However, the smaller ratios require either a very small medium addition and corresponding low total addition of radioactivity of a large quantity of soil. A soil sample of 1 g to which is added 0.1 ml of medium (ratio 0.1 ml/g) is the most likely candidate for future experimental testing.

2. ATP Production

Experiments were performed to test the effect of the medium/soil ratio on the production of ATP. RM9 medium and Mojave soil were added to planchets in ratios as shown in Table 6.

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TABLE 6

Medium to Soil Ratios Used for ATP Studies

Soil (g) *	Medium (ml) **	Ratio ml/g
1.0	0.005	0.005
1.0	0.05	0.05
1.0	0.1	0.1
0.5	0.2	0.4

* Mojave soil

** RM9 medium containing ^{14}C-formate, ^{14}C-lactate, ^{14}C-glucose, ^{14}C-glycine and $^{35}SO_4$ (total activity 20 μCi/ml).

After addition of medium to the soil, gettering was conducted to follow the evolution of radioactivity. After one hour, the cultures containing 0.005, 0.05 and 0.1 ml of medium had reached a plateau in ^{14}C evolution. At that time, they were extracted and assayed for ATP. Heat sterilized controls and several 1 g samples of viable soil, without medium, were also assayed. The results of this experiment are presented in Figure 21. The concentration of ATP (0.037 μg/g of soil) which was measured in Mojave soil before growth, gives further indication concerning the number of bacteria present in that soil. Several workers (5, 6, 7) have determined that the concentration of ATP per bacterial cell is in the neighborhood of 5×10^{-10} μg.

Therefore:

$$\frac{0.037\ \mu g\ ATP/g\ soil}{5 \times 10^{-10}\ \mu g\ ATP/cell} = 7 \times 10^{7}\ cells/g\ soil$$

This value is somewhat higher than the (6×10^{6}) cells/g soil) value that was obtained in a plate count of Mojave soil. However, commonly recognized difficulties of soil plating could easily explain the difference.

The concentration of ATP in heat sterilized soil plus medium (Figure 21) was considerably lower than viable soil without medium. This would be expected since cellular death is associated with a disappearance of ATP. The curve also showed a slight decrease at the lower medium/soil ratios.

Viable soil and medium after growth showed concentrations of ATP that were eight fold to ten fold higher than those found in sterile

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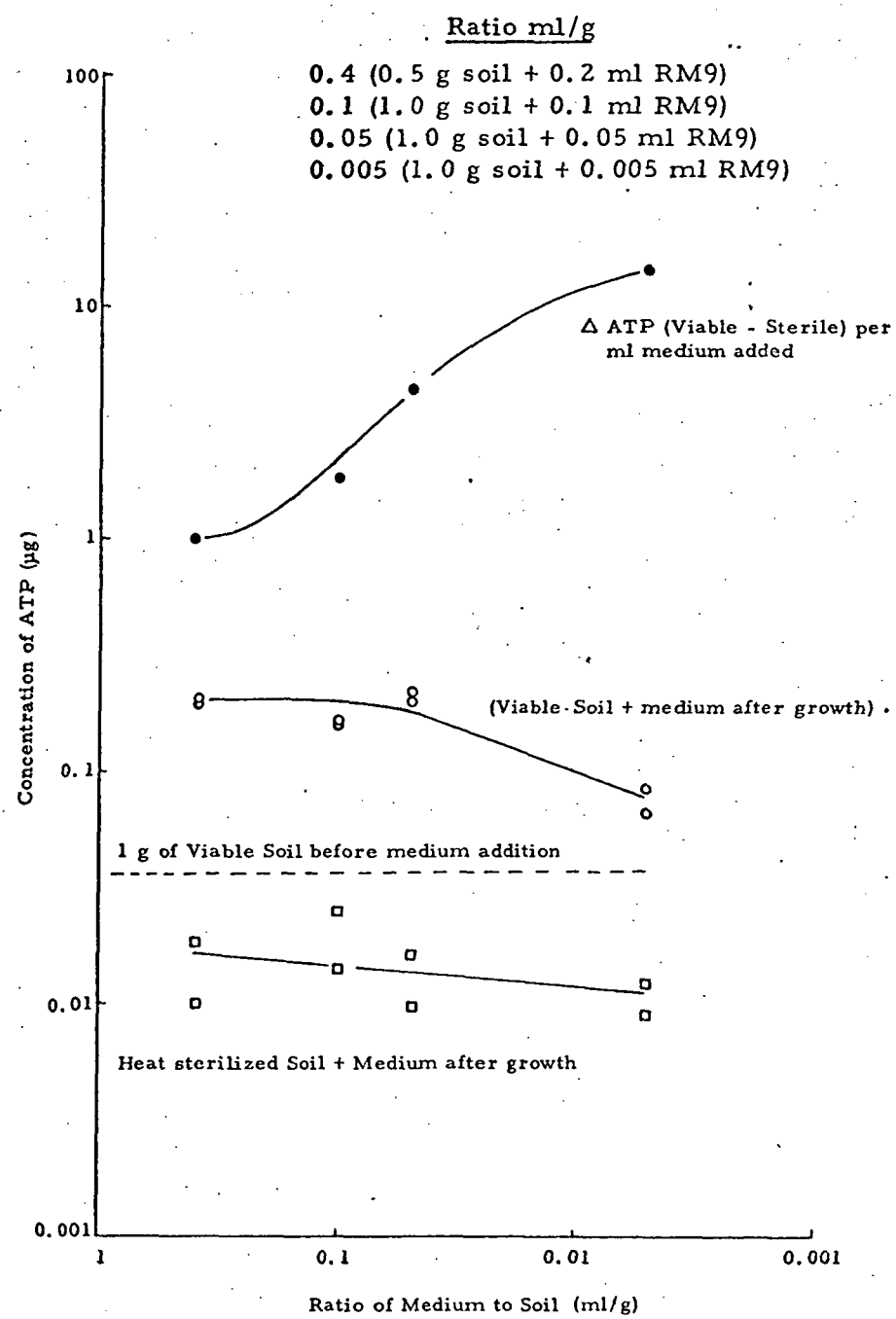
FIGURE 21

Effect of Medium/Soil Ratio on ATP Production by Mojave Soil



controls, depending upon the quantity of medium added. Since the affecting agent in ATP production was the medium, a plot was made of the difference between ATP concentrations of viable and sterile cultures per ml of medium added versus the medium/soil ratio. This plot, also shown in Figure 21, dramatically showed that the ATP produced per ml of medium added was greatly enhanced by a low/medium soil ratio.

3. Phosphate Uptake and ^{14}C and ^{35}S Uptake

Phosphate utilization and ^{14}C and ^{35}S uptake in "wet" cultures of Mojave, Wyaconda and Victoria Valley soils were conducted using 0.3 g of soil in 10 ml of RM9 medium containing ^{14}C-formate, ^{14}C-lactate, ^{14}C-glucose, ^{14}C-glycine, and $^{35}SO_4$, (total activity 2 μCi/ml). The cultures were incubated at 25°C in the stirred tubes shown in Figure 18. Direct gettering of the tubes resulted in the curves shown in Figures 22 through 24. At times indicated on these graphs, sample portions were withdrawn and assayed for PO_4-P and ^{14}C and ^{35}S uptake. The results of these assays are given in Tables 7 through 9. All three soils tested showed a ^{14}C and ^{35}S uptake by viable soils which exceeded sterile soil uptake by 20 to 60 fold. Most of the uptake occurred early in the logarithmic phase of growth, but the incorporated ^{14}C and ^{35}S remained measurable at any time thereafter. A decrease of PO_4-P concentration concomitant with the labeled release

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FIGURE 22

Labeled Release of "Wet" Mojave Soil Culture
(Sampling Points for ^{14}C and ^{35}S Uptake,
and PO_4-P Assay are Indicated)

○ 0.3 g viable soil
● 0.3 g heat sterilized soil
10 ml RM9 medium containing ^{14}C-formate, ^{14}C-lactate, ^{14}C-glucose, ^{14}C-glycine and $^{35}SO_4$ (total activity 2 μCi/ml)

Incubation at 25°C.

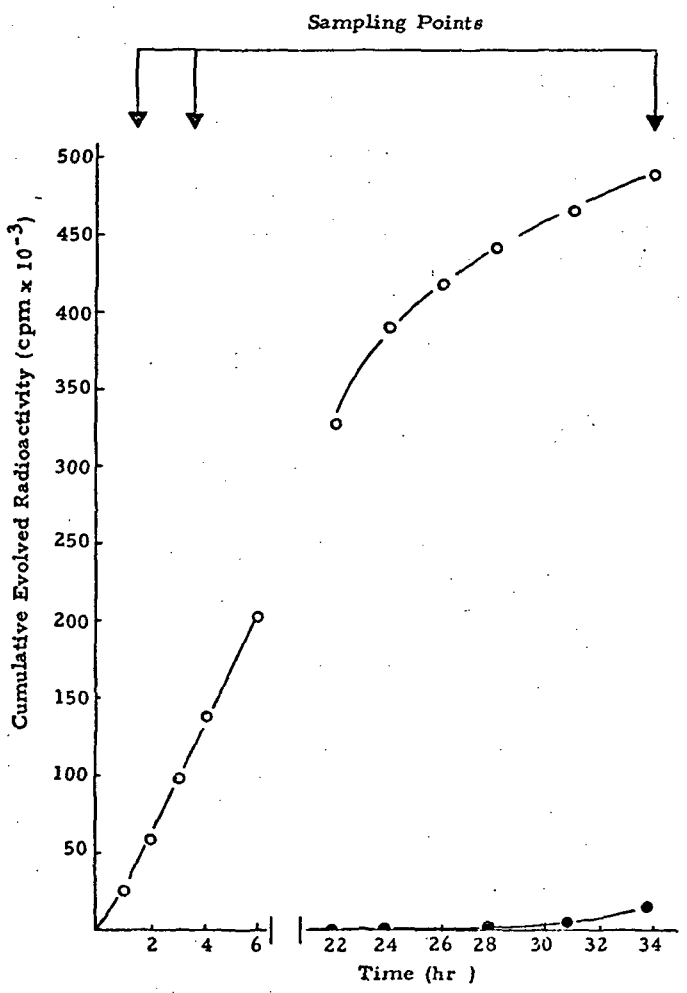

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FIGURE 23

Labeled Release of "Wet" Wyaconda Soil Culture
(Sampling Points for ^{14}C and ^{35}S Uptake
and PO_4-P Assay are Indicated)

○ 0.3 g viable soil
● 0.3 g heat sterilized soil
10 ml RM9 medium containing ^{14}C-formate, ^{14}C-lactate, ^{14}C-glucose, ^{14}C-glycine and $^{35}SO_4$ (total activity 2 μCi/ml)

Incubation at 25°C.

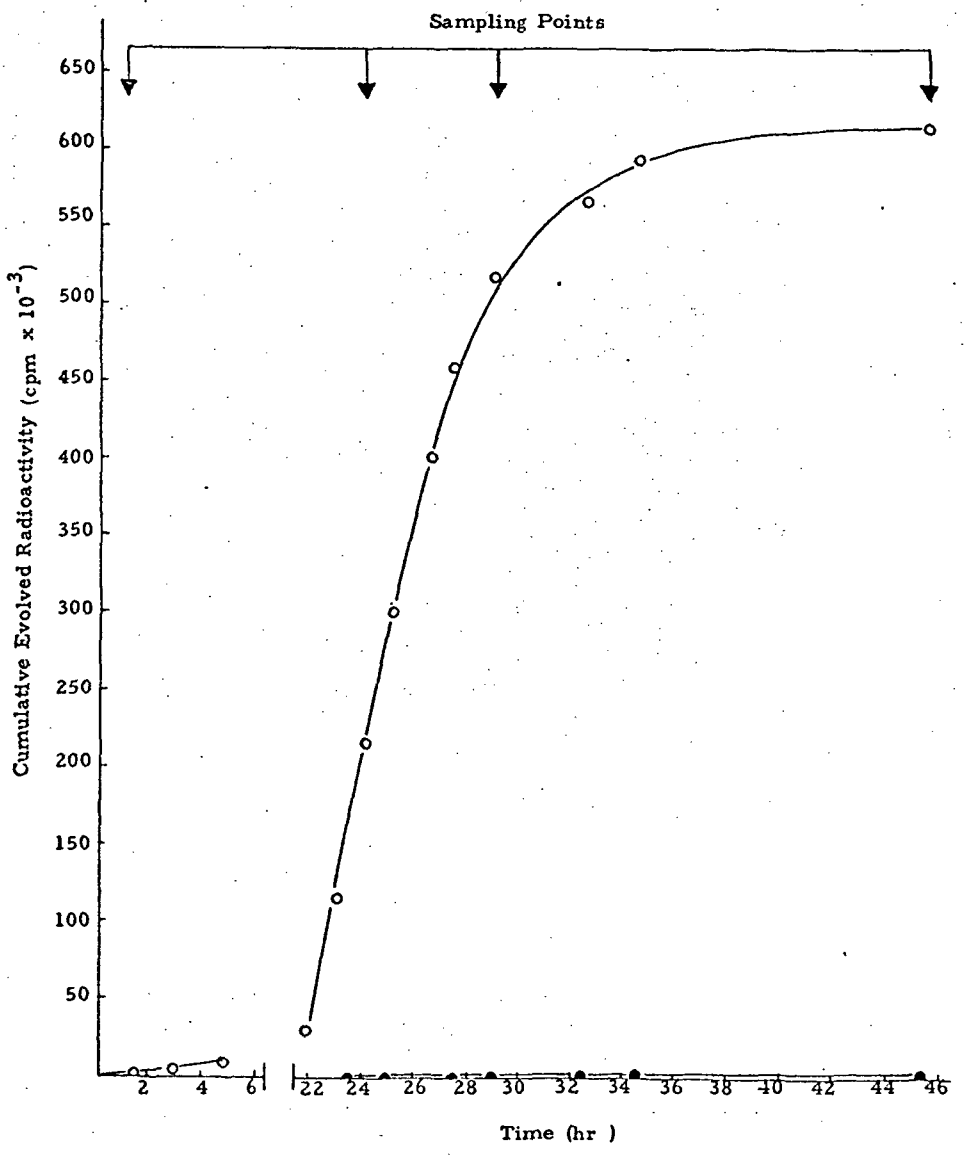

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FIGURE 24

Labeled Release of "Wet" Victoria Valley Soil Culture (Sampling Points for ^{14}C and ^{35}S Uptake, and PO_4-P Assay are Indicated)

○ 0.3 g viable soil
● 0.3 g heat sterilized soil
10 ml RM9 medium containing ^{14}C-formate, ^{14}C-lactate, ^{14}C-glucose, ^{14}C-glycine and $^{35}SO_4$ (total activity 2 μCi/ml)

Incubation at 25°C.

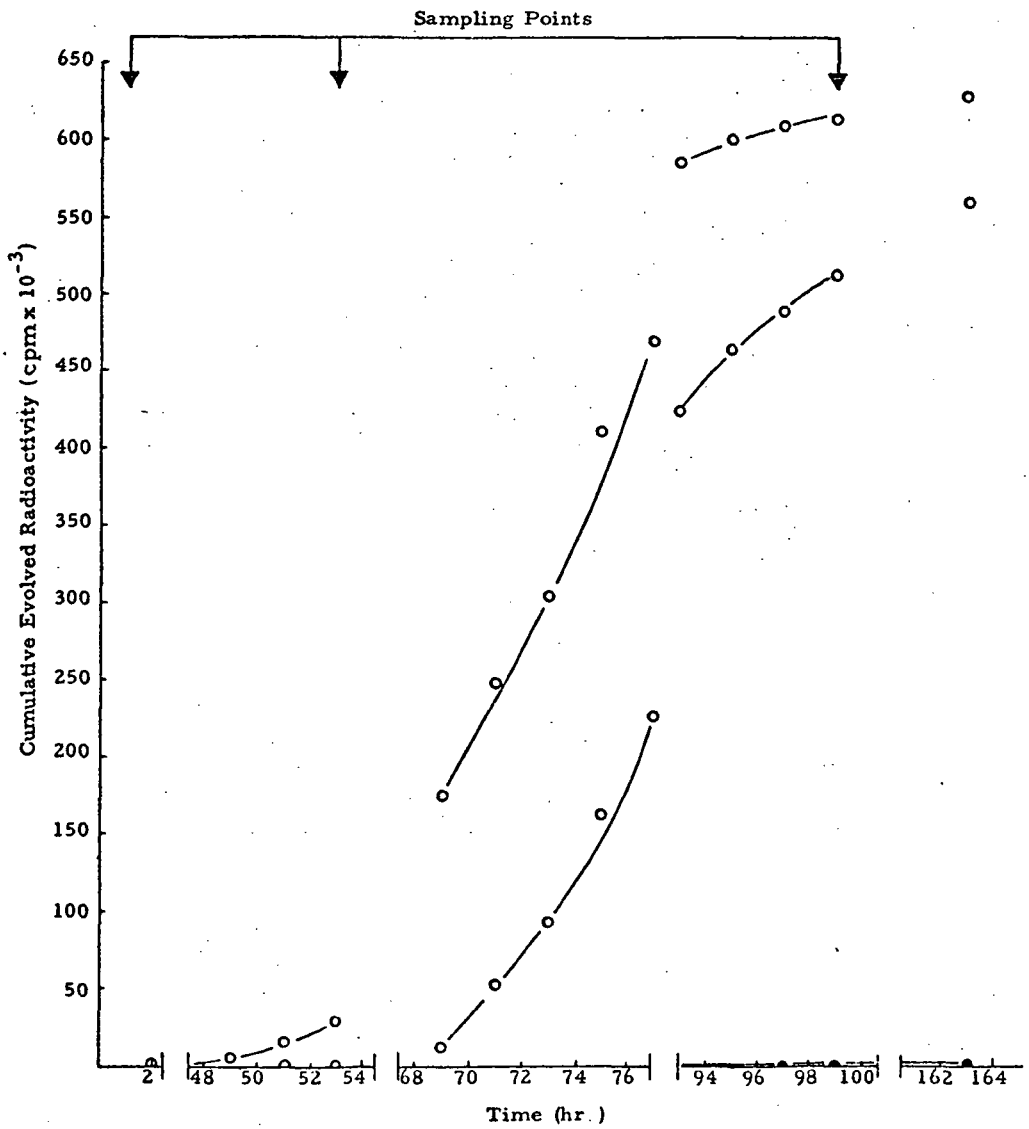

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TABLE 7

Results of ^{14}C and ^{35}S Uptake and PO_4-P Assay of the "Wet" Mojave Soil Culture Shown in Figure 22

^{14}C and ^{35}S Uptake CPM

Time	Viable Soil		Sterile Soil	
$1\frac{1}{2}$ hours	20,418	20,161	795	815
$3\frac{1}{2}$ hours	30,923	28,970	1,206	923
34 hours	34,740	32,529	1,230	852

PO_4-P (mg/l)

Time	Viable Soil		Sterile Soil	
$1\frac{1}{2}$ hours	0.42	0.45	0.49	0.50
$3\frac{1}{2}$ hours	—	0.45	0.45	—
34 hours	0.34	0.32	0.49	0.49

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TABLE 8

Results of ^{14}C and ^{35}S Uptake and PO_4-P Assay of "Wet" Wyaconda Soil Culture Shown in Figure 23

^{14}C and ^{35}S Uptake CPM

Time	Viable Soil		Sterile Soil	
1 hour	871	930	960	1,176
24 hours	27,374	28,343	932	1,200
29 hours	29,553	27,923	1,207	1,491
45½ hours	21,261	26,277	1,110	982

PO_4-P (mg/l)

Time	Viable Soil		Sterile Soil	
1 hour	0.16	0.16	0.16	0.16
24 hours	0.06	0.06	0.12	0.12
29 hours	0.16	0.15	0.23	0.24
45 hours	0.19	0.19	0.25	0.33

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TABLE 9

Results of ^{14}C and ^{35}S Uptake and PO_4-P Assay of "Wet" Victoria Valley Soil Culture Shown in Figure 24

^{14}C and ^{35}S Uptake CPM

Time	Viable Soil		Sterilized Soil	
45 minutes	366	648	700	1,135
53 hours	19,168	1,814	639	1,407
99 hours	28,005	26,100	541	381

PO_4-P (mg/l)

Time	Viable Soil		Sterilized Soil	
45 minutes	0.17	0.17	0.18	0.18
53 hours	0.11	0.14	0.13	0.14
99 hours	0.13	0.12	0.12	0.13

curve was also observed. Mojave and Wyaconda soils showed a PO_4-P decrease of approximately 0.1 mg/l. Victoria Valley showed no PO_4-P uptake.

F. Atmosphere Studies

A series of labeled release and ATP experiments were conducted in the Biospherics' environmental chambers in the presence of various gas mixtures. The purpose of these experiments was to determine the effect of an anaerobic, microaerophylic, CO_2 and CO atmosphere on the test procedure and level of response as compared with that obtained in an aerobic environment.

1. Procedure

Prenumbered getters and planchets containing sterile soils were loaded into an environmental chamber (see Figure 25). The chamber with attached glass wool filters was then placed in the autoclave and sterilized at 18 psi, 120°C for 45 minutes. It was then removed from the autoclave, cooled and planchets containing viable soil aseptically added. Test gas was flushed through the system and medium to displace air. A flush of the test gas mixture was also maintained throughout the test period. The chamber was not opened to the air during manipulations required for medium addition and getter changes.

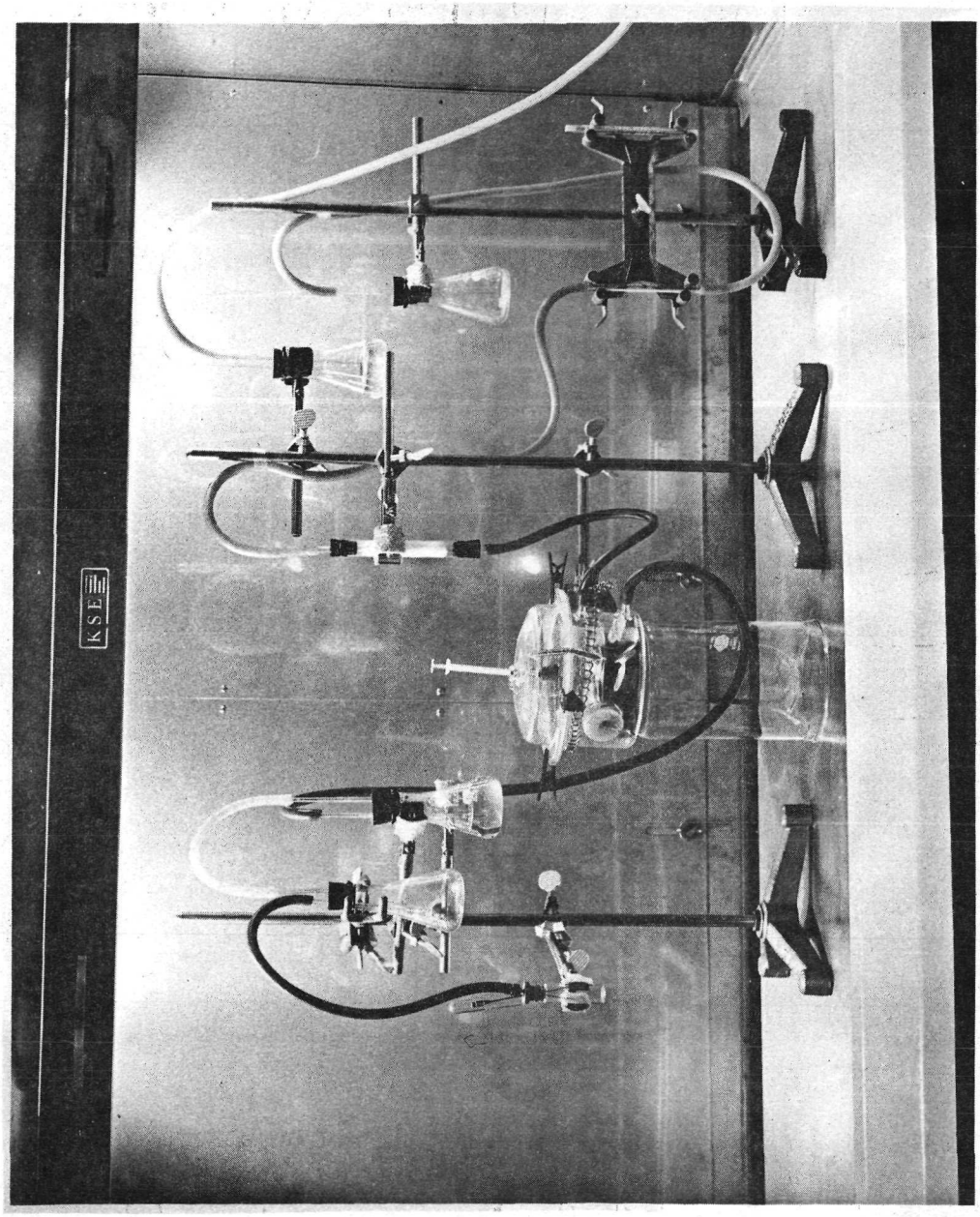

FIGURE 25

Environmental Chamber Designed by Biospherics for Incubation of Soil Samples Under Various Gases

2. $N_2/H_2/CO_2$ Atmosphere

In view of the high CO_2 and low O_2 concentration of the Martian atmosphere, a gas mixture containing 80% N_2, 10% H_2, and 10% CO_2 was tested using Mojave soil. Cumulative evolved radioactivity from viable and sterile soils after 46 hours of incubation was 1,800 and 800 cpm, respectively. This was in such sharp contrast to aerobic culture results, which had always produced over 100,000 cpm after a few hours, that interference of the CO_2 atmosphere with the test procedure was suspected.

A second experiment was conducted in which $Ba(OH)_2$ impregnated getters were placed over planchets containing water. These planchets were subjected to the $H_2/N_2/CO_2$ atmosphere, removed from the chamber, and then used to getter an aerobic Wyaconda soil culture. Results of this experiment, shown in Table 10, indicate that the Ba(OH) getters are rendered incapable of trapping evolved radioactivity by exposures of one hour to a 10% CO_2 atmosphere. Gettering experiments in a CO_2 atmosphere were concluded to be infeasible. Further studies excluded the use of CO_2.

3. Effect of Various Gaseous Atmospheres on Mojave, Wyaconda and Victoria Valley Soil

Labeled release experiments on Mojave, Wyaconda, and Victoria Valley soil were conducted in the environmental chambers under the following gas mixtures:

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TABLE 10

Interference of a 10% CO_2 Environment on $Ba(OH)_2$ Gettering of $^{14}CO_2$ Evolved Radioactivity

Treatment of Getters In $N_2/H_2/CO_2$ Atmosphere	Getter Period 0.2 ml RM9 * 0.5 g Wyaconda Soil	Cumulative Counts Collected CPM
20 hours	3 hours	1,249 1,248
No Treatment	3 hours	Approx.100,000
1 hour	1 hour	85 345
No Treatment	1 hour	5,035 6,556

* RM9 contained ^{14}C-formate, ^{14}C-lactate, ^{14}C-glucose, ^{14}C-glycine and $^{35}SO_4$ (total activity 20 μCi/ml)

100% N_2

90% N_2, 10% H_2

90% N_2, 9% H_2, 1% CO

99% N_2, 1% O_2

The results obtained in these studies were compared to results obtained in air and are presented in Figures 26 through 31. All three soils produced a lesser response under anaerobic conditions than that obtained aerobically. However, in all cases, this lowered response was significantly higher than that produced by sterile controls. There appeared to be no difference in the response obtained with 90% N_2. 10% H_2, 90% N_2/9% H_2/1% CO and 100% N_2. But, the 99% N_2, 1% O_2 mixture produced a rate of evolution intermediate between the aerobic and anaerobic response. Also noted was the fact that anaerobic culturing greatly reduced the plateau of cumulative evolved radioactivity. This might be explained in terms of the incomplete substrate utilization, which would occur when fermentative pathways of metabolism were being utilized.

Nonbiological response obtained from Wyaconda and Mojave soils (Figures 29 and 30) were not affected by the gas mixture used. On the other hand, a comparison of many sterile controls from several experiments tends to indicate that the Victoria Valley nonbiological response may be higher in air than under anaerobic conditions (see Figure 31).

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FIGURE 26

Effect of Various Gas Mixtures on the Labeled Release from Viable Mojave Soil

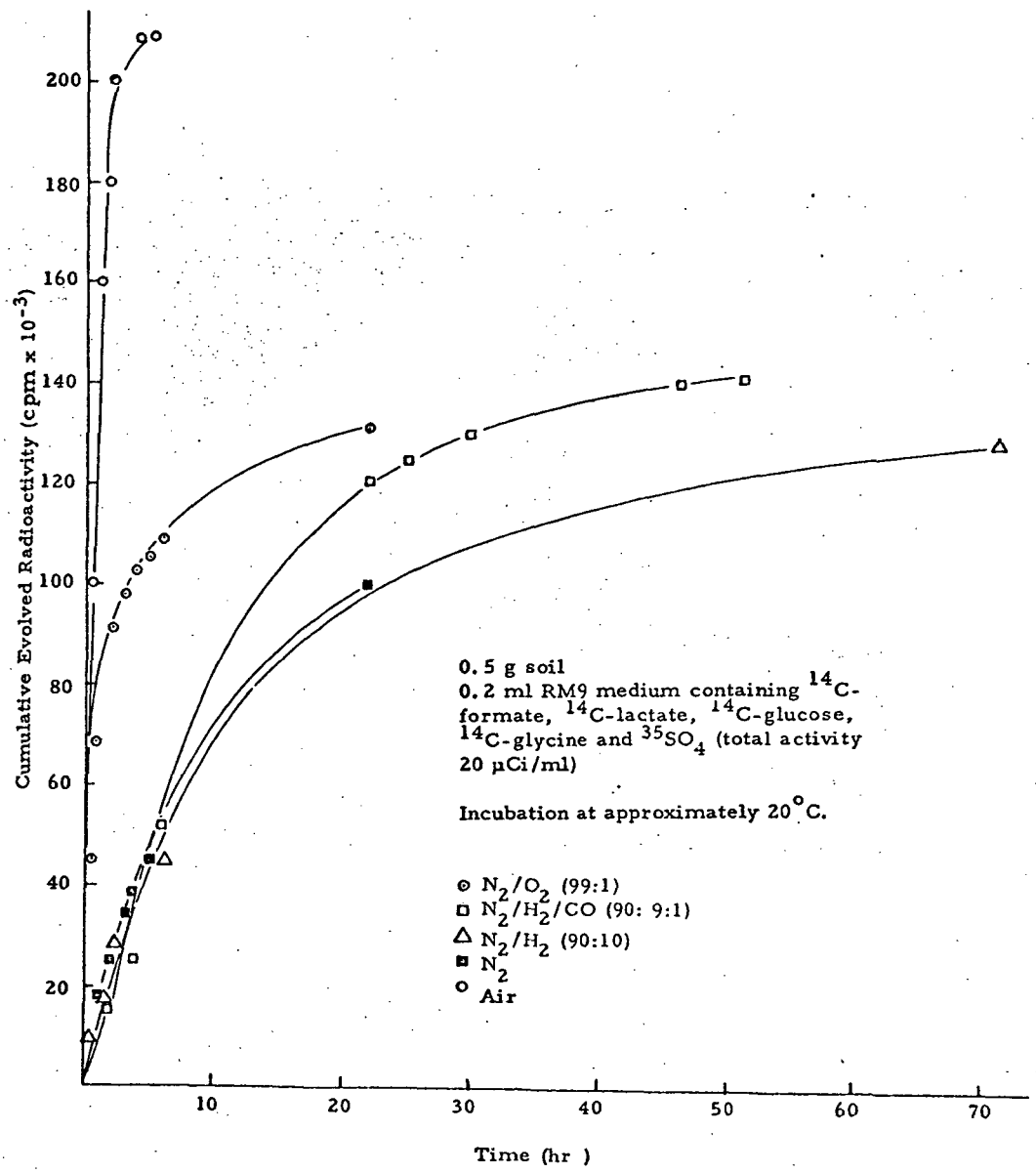

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FIGURE 27

The Effect of Various Gas Mixtures on the Labeled Release from Wyaconda Soil

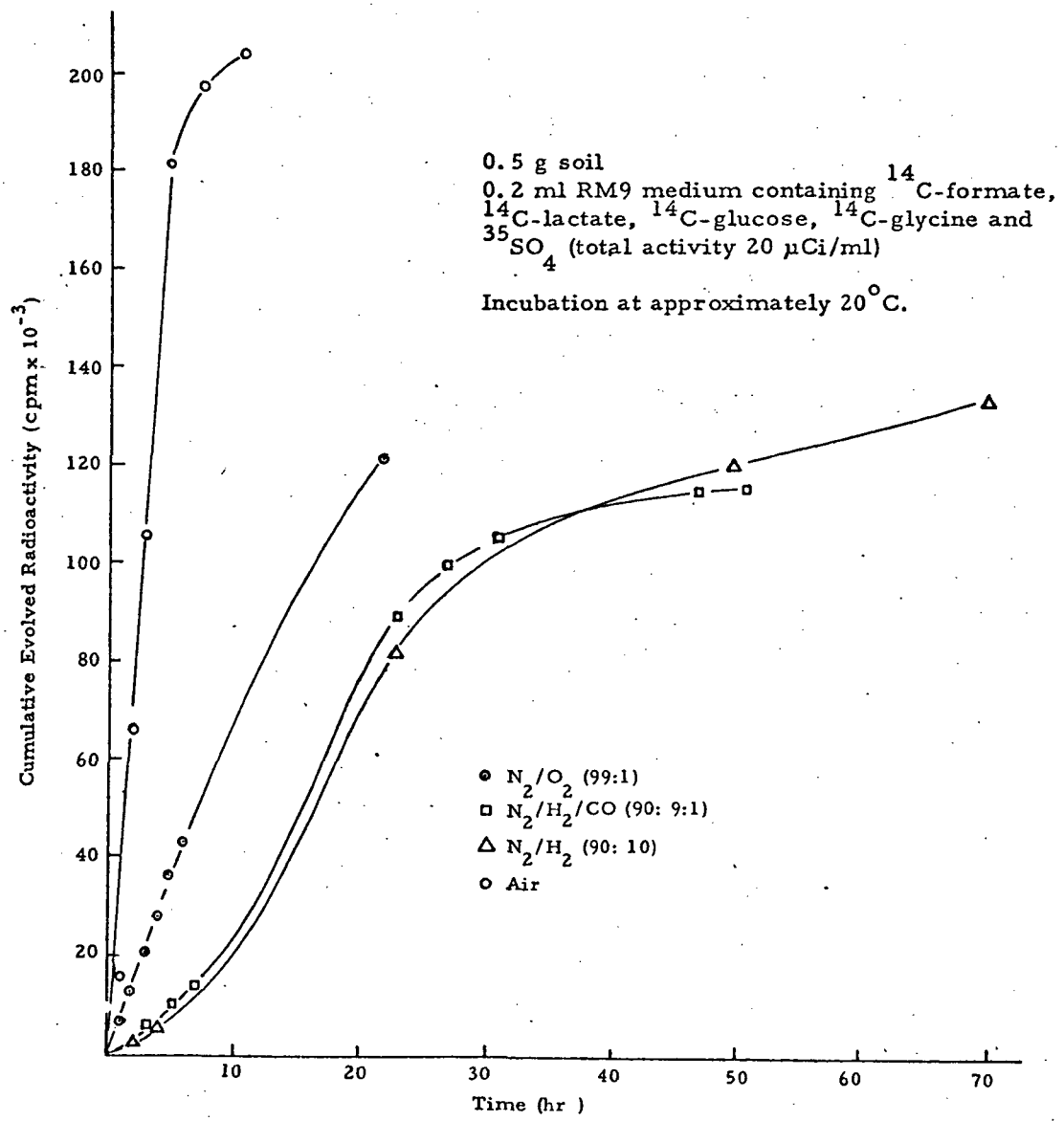

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FIGURE 28

The Effect of Various Gas Mixtures on the Labeled Release from Viable Victoria Valley Soil

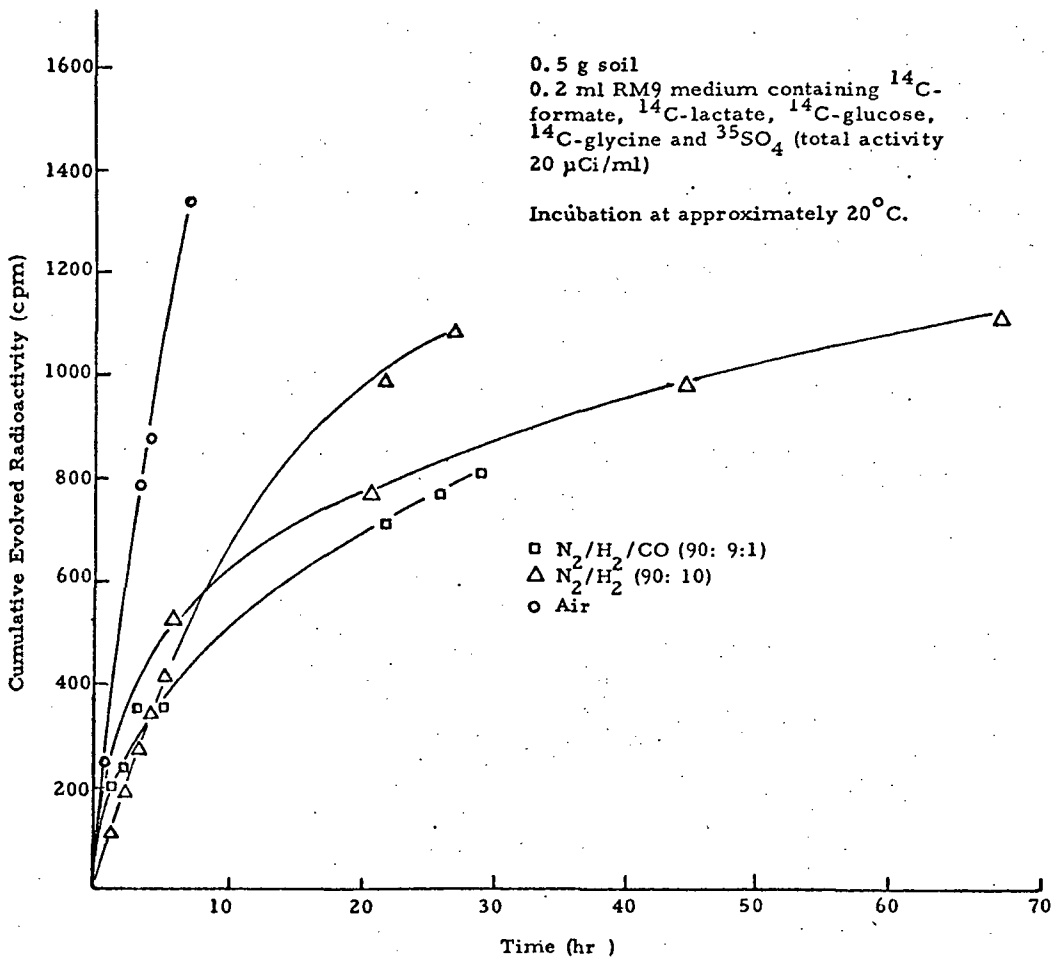

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FIGURE 29

Effect of Various Gas Mixtures on the Nonbiological Evolution of Radioactivity by Heat Sterilized (30 min. 212°C) Mojave Soil

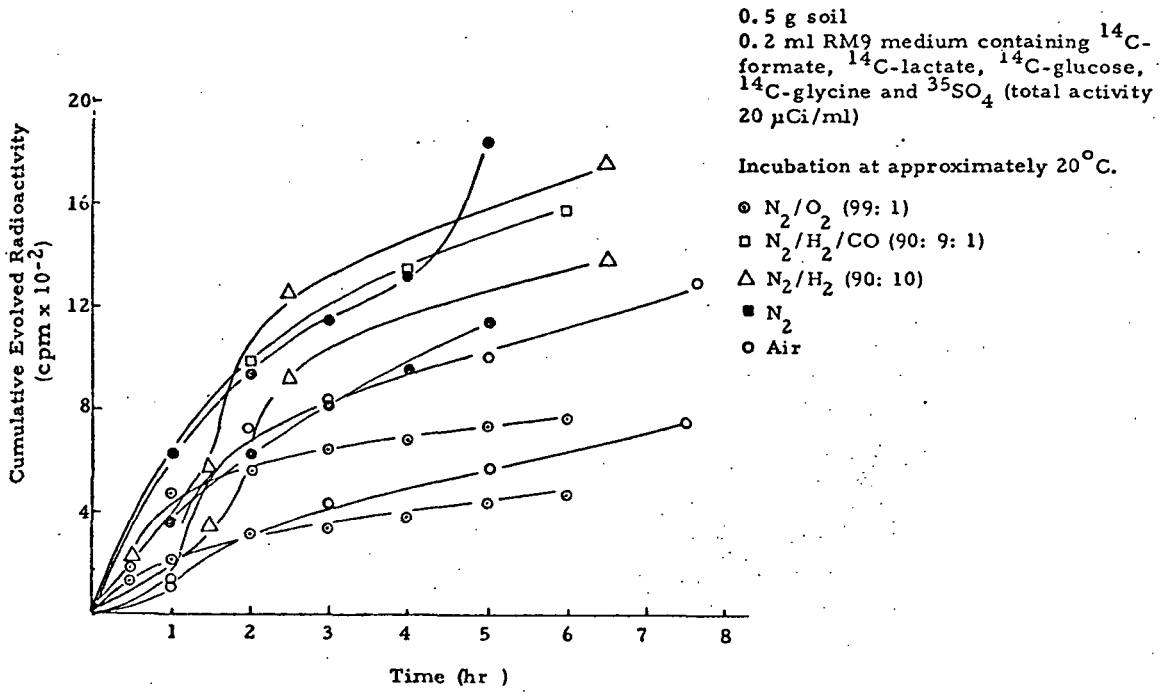

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FIGURE 30

Effect of Various Gas Mixtures on the Nonbiological Evolution of Radioactivity by Heat Sterilized (30 min. 212°C) Wyaconda Soil

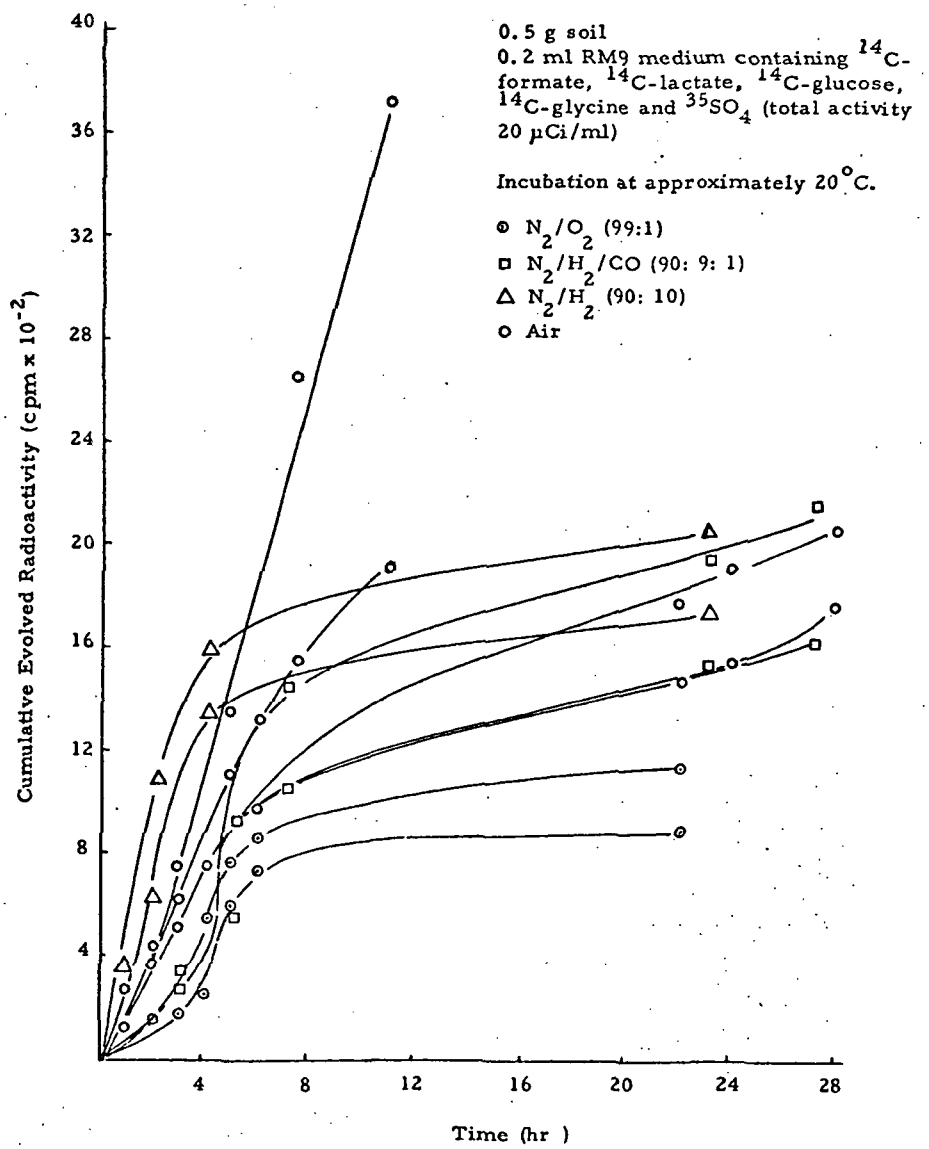

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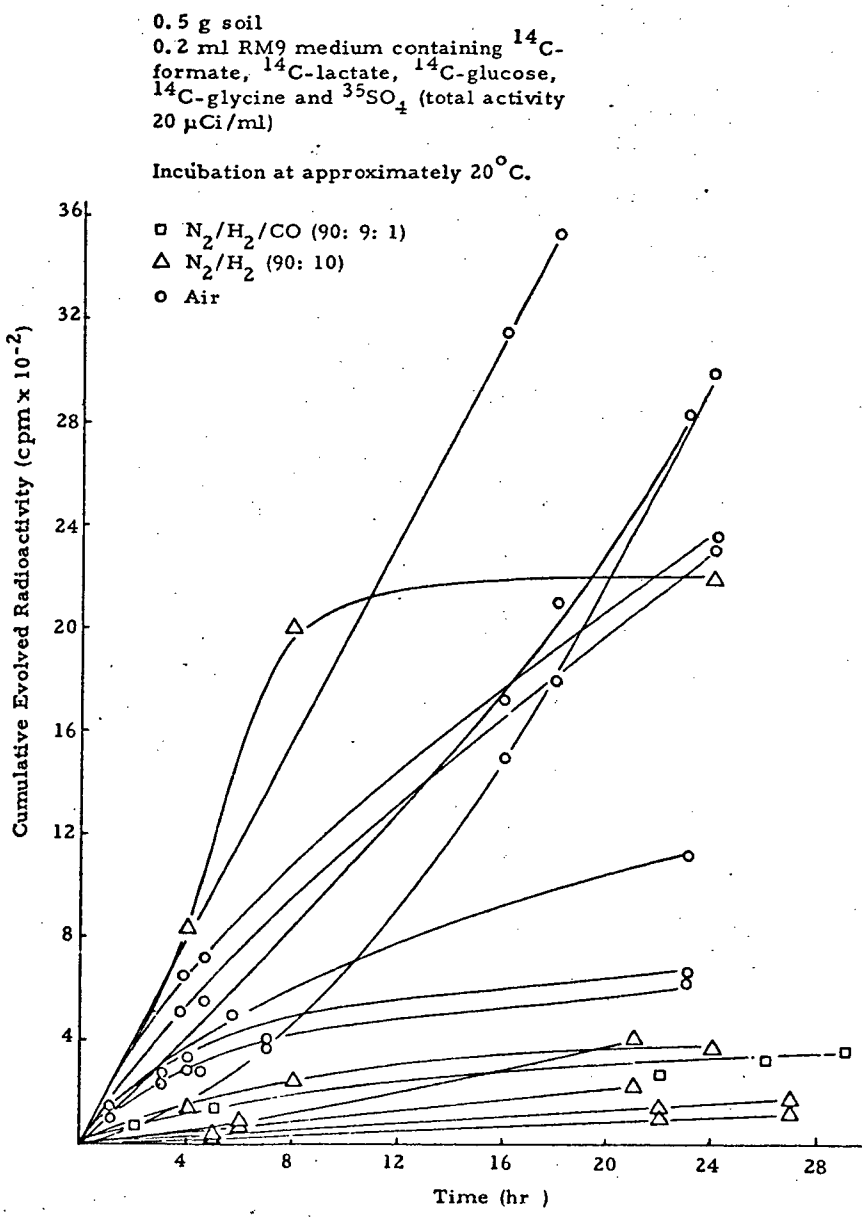
FIGURE 31

Effect of Various Gas Mixtures on the Nonbiological Evolution of Radioactivity by Heat Sterilized (30 min. 212°C) Victoria Valley Soil



4. Anaerobic Production of ATP

The production of ATP in Mojave soil (see Table 11) was less during anaerobic incubation than during aerobic incubation. Nevertheless, the N_2/H_2 culture resulted in viable soil ATP levels that were 20 fold higher than sterile controls.

The anaerobic ATP levels shown in Table 11 are approximately one-half the values obtained aerobically. This value supports the labeled release data shown in Figure 26. Anaerobic culture produced a cumulative evolved radioactivity with plateaued at approximately 60% of the cumulative evolved radioactivity observed during aerobic incubation.

G. Antimetabolite Studies

The use of antimetabolites for treatment of control soils has been studied in the past. The possibility of their use in Martian experiments has three obvious advantages. First, a technique for cold sterilization would simplify engineering design and allow for a broader range of material and test equipment. Second, the susceptibility or resistance of Martian organisms to selected antimetabolites would reveal important facts concerning their metabolism or structure. Third, important preliminary information concerning organism control and decontamination (a prerequisite for manned flights or sample return) would be obtained.

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TABLE 11

ATP Production Under Aerobic and Anaerobic Conditions

Atmosphere	ATP μg/g Soil After Growth		Average Difference Between Viable & Sterile Soils
	Viable	Sterile	
Air	.1805 .1947	.0093 .0050	.185
90%N_2, 10%H_2	.0979 .0996	.0025 .0050	.095

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The approach in this study was to test a broad range of agents which have differing modes of action upon cells. Concentrations which have been shown to be effective in other applications were used. The following antimetabolites have been tested on Wyaconda and Mojave soil:

- Mercuric Chloride - a heavy metal which poisons enzymes by formation of mercaptides with sulfhydryl groups.
- Potassium Cyanide - chiefly inhibits cytochrome oxidase.
- Sodium Hypochlorite - nonspecific oxidizing agent widely used for disinfection.
- Bard-Parker Germicide - was used effectively in the past to treat soils used for controls. It is a commercial preparation of 65.26% Isopropanol, 2.75% Methanol, 8.00% Formaldehyde, and 0.5% Hexachlorophene. Its action is chiefly upon the cell wall.
- Ethylene Oxide - chemically active gas used in cold sterilization.
- Antibiotics -

  Chloramphenicol antibiotic which is effective against both gram positive and gram negative bacteria.

Penicillin antibiotic which is effective against gram positive bacteria.

The procedure used in all these experiments was to add 0.3 ml of a solution containing 0.2 ml RM9 medium and 0.1 ml of antimetabolite, to 0.5 g of soil. Viable control soils were treated with 0.3 ml of a solution containing 0.2 ml RM9 medium and 0.1 ml of sterile distilled water. Incubation was at 25°C on a laminar flow bench. Gettering of labeled release was conducted in the usual fashion.

1. Mercuric Chloride

The effect of 1000 ppm mercuric chloride ($HgCl_2$) on Mojave and Wyaconda soil is given in Figures 32 and 33. Very little inhibition of the Mojave soil was observed; however, a 60 fold decrease in activity of the Wyaconda soil occurred.

2. Potassium Cyanide

Studies done earlier with other soil types had shown that a concentration of 0.05M KCN was an effective inhibitor (8). Potassium cyanide (KCN, 0.05M) strongly inhibited both Mojave and Wyaconda soil. (See Figures 34 and 35). The response of viable soil plus KCN was the same as that obtained with heat sterilized soil and KCN.

3. Bard-Parker Germicide

Bard-Parker germicide (Figures 36 and 37) was only slightly effective at both 5% and 33% concentration for inhibition of

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FIGURE 32

Effect of $HgCl_2$ (1000 ppm) on the Evolution of Radioactivity from Wyaconda Soil

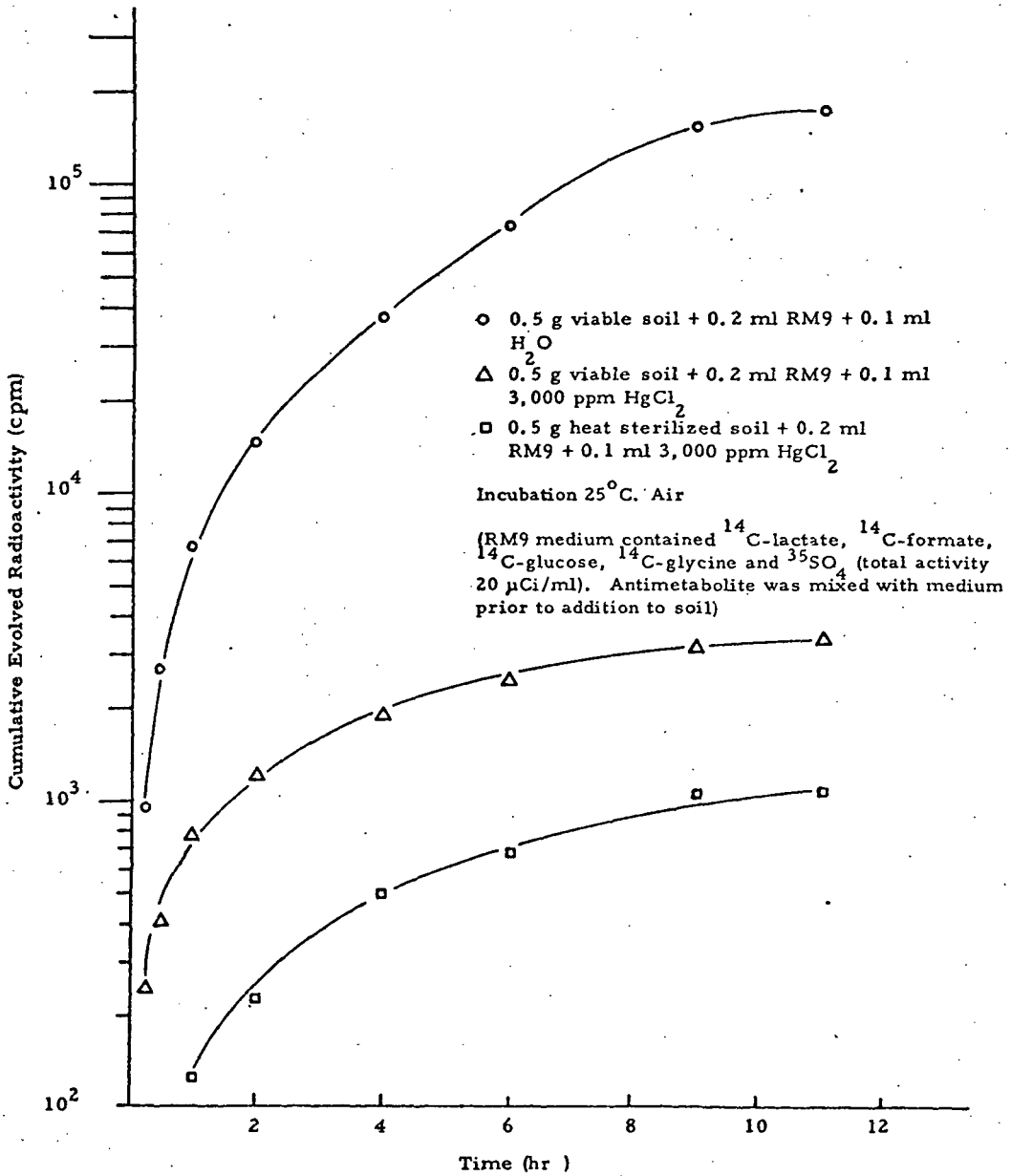

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FIGURE 33

Effect of $HgCl_2$ on the Evolution of Radioactivity from Mojave Soil

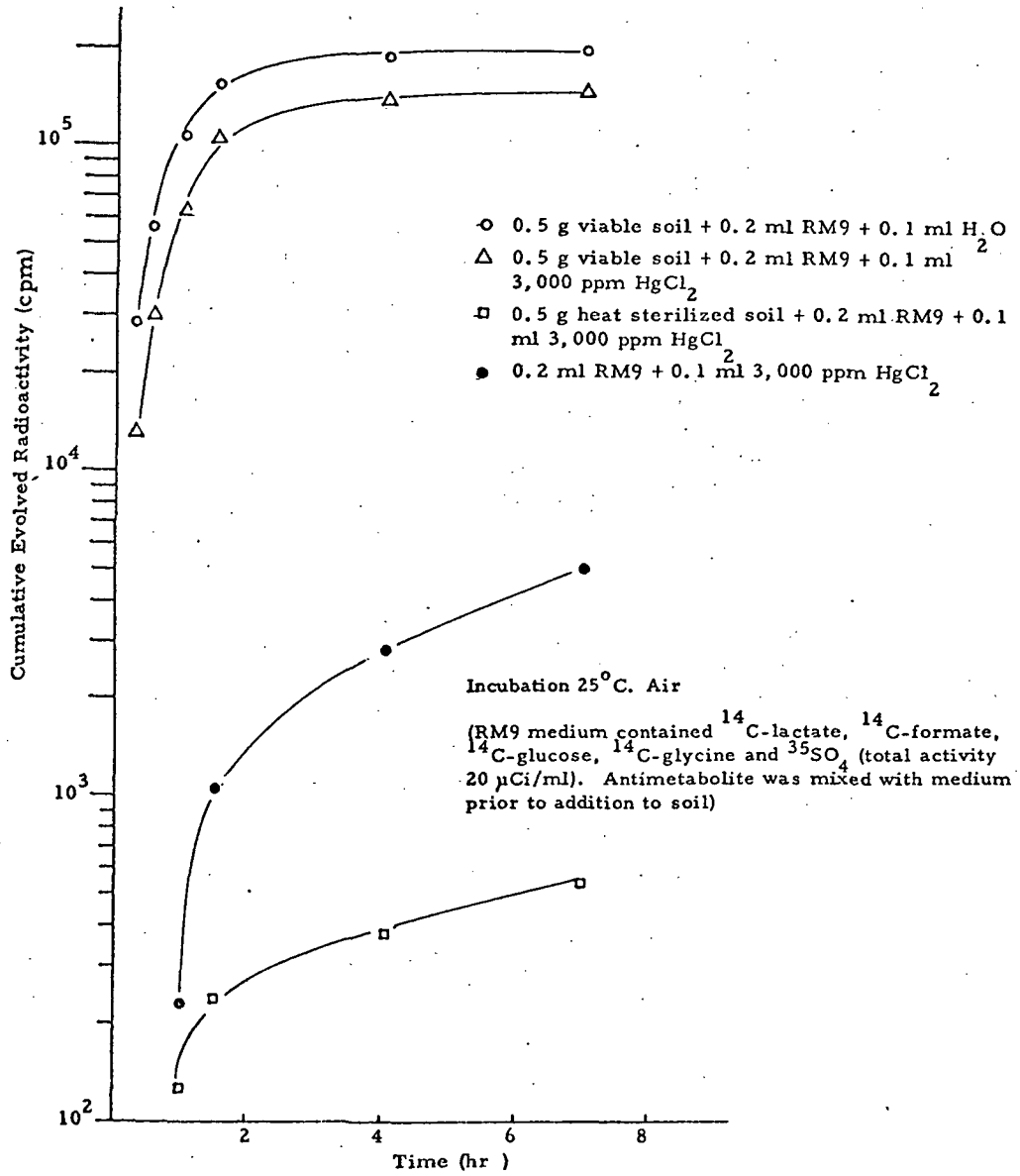

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FIGURE 34

Effect of KCN (0.05M) on the Evolution of Radioactivity from Wyaconda Soil

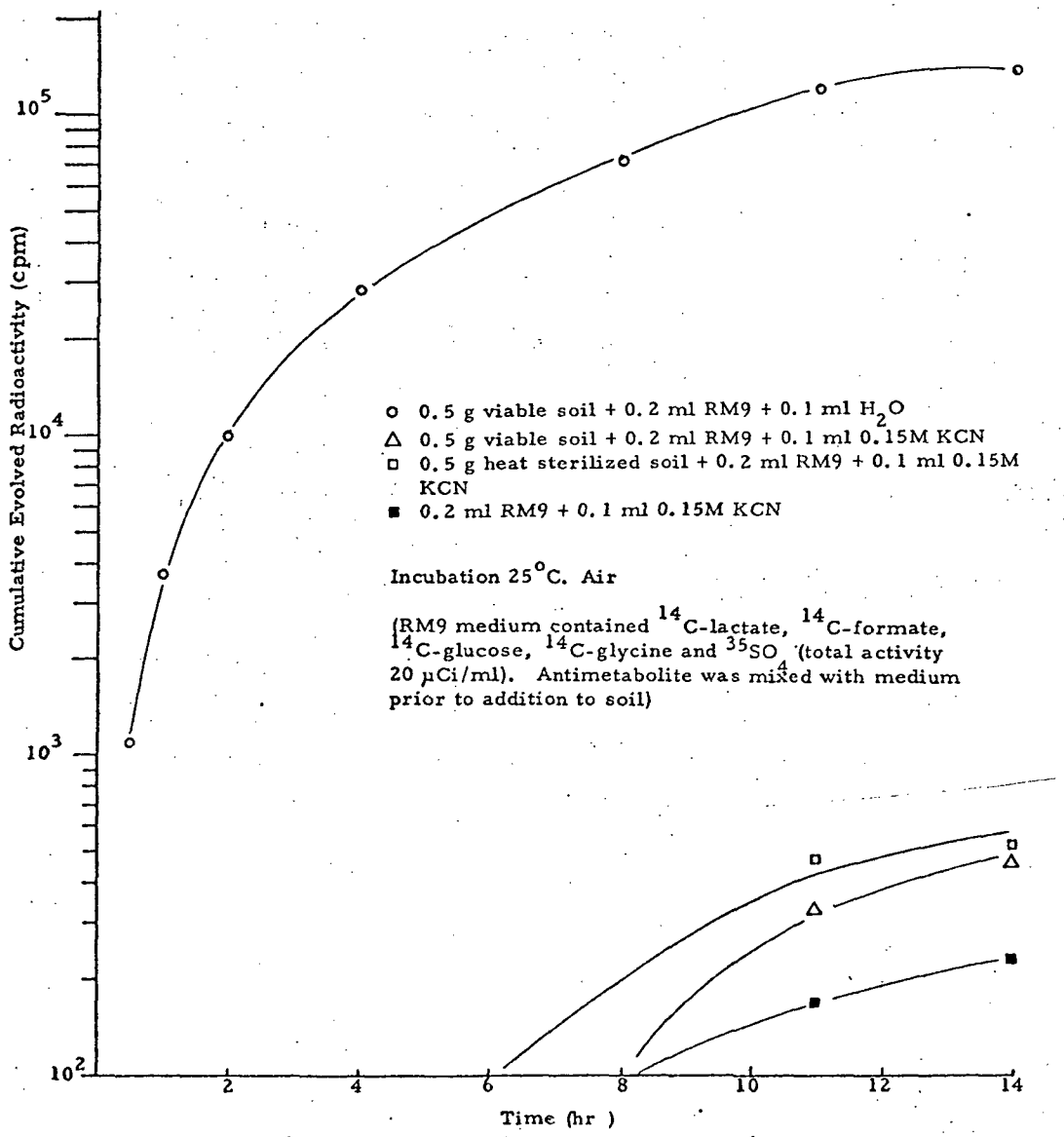

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FIGURE 35

Effect of KCN (0.05M) on the Evolution of Radioactivity from Mojave Soil

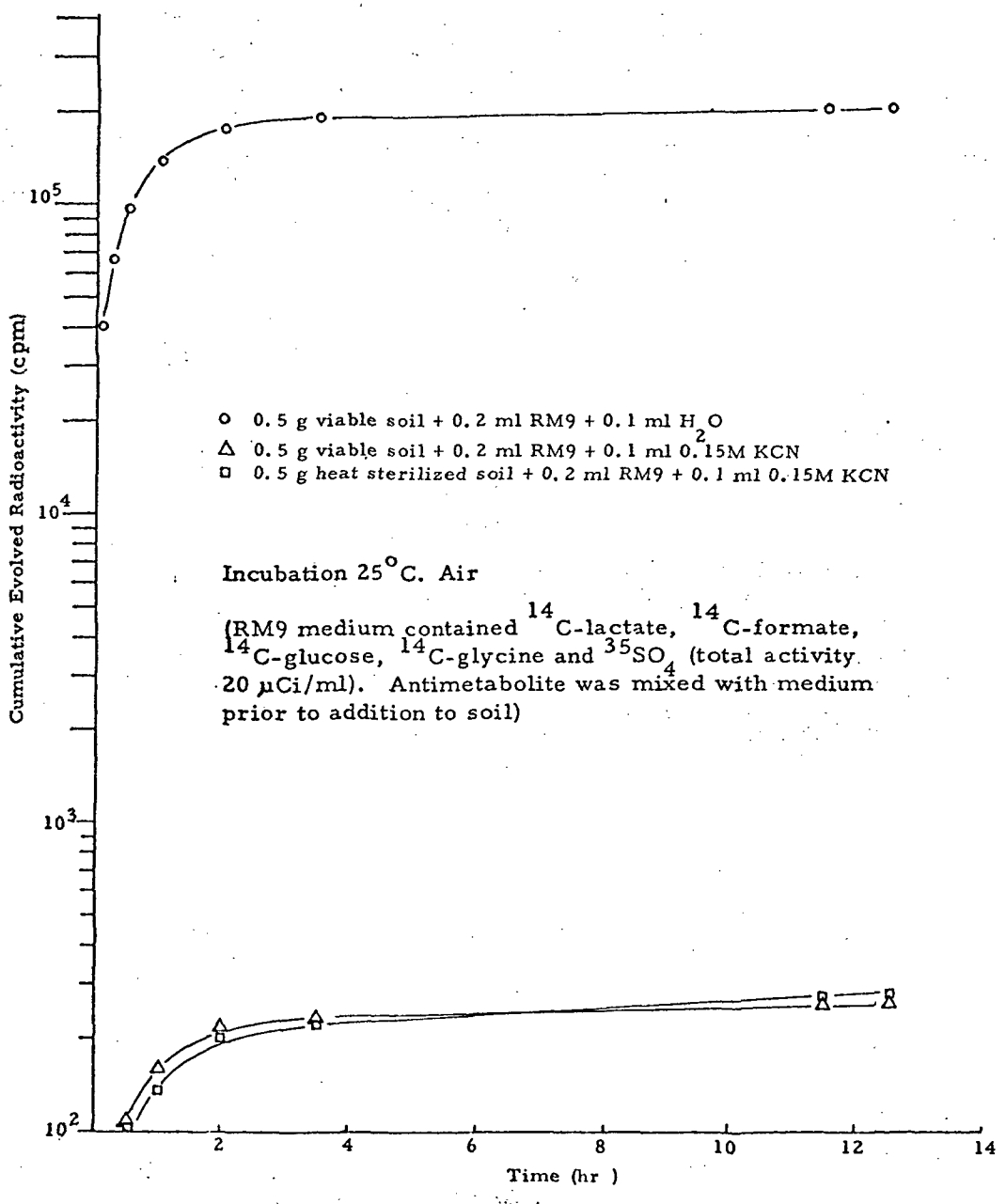

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FIGURE 36

Effect of Bard-Parker Germicide (BP) on the Evolution of Radioactivity from Wyaconda Soil

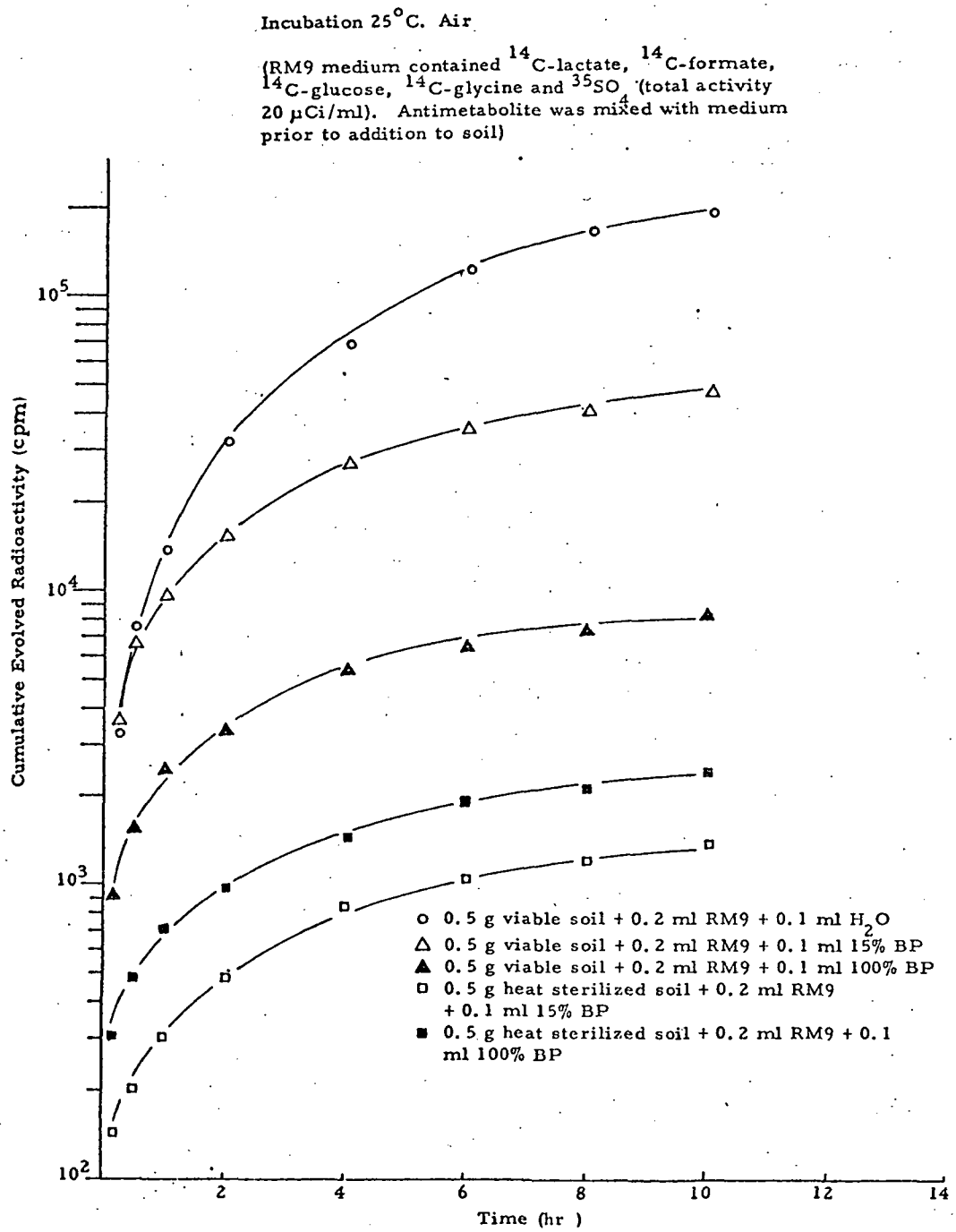

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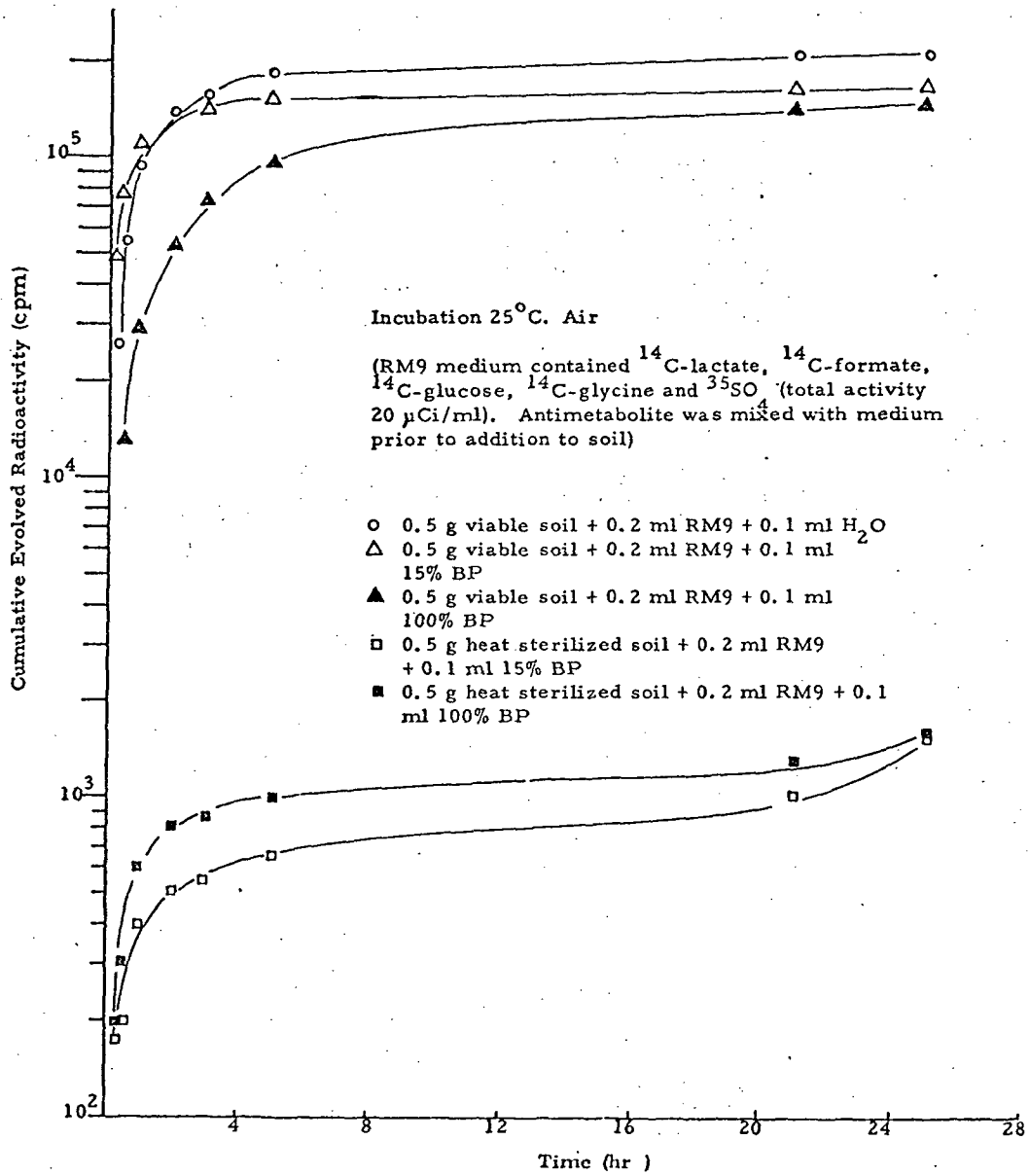
FIGURE 37

Effect of Bard-Parker Germicide (BP) on the Evolution of Radioactivity from Mojave Soil



Mojave soil. It was somewhat more effective against Wyaconda soil, and at the 33% concentration reduced the cumulative evolved radioactivity after ten hours by 20 fold.

Figure 38 shows that an adverse nonbiological evolution of radioactivity occurred when Bard-Parker germicide was added to the medium. This effect was reduced considerably, however, in the presence of sterile soil (see Figures 36 and 37). Since these findings conflicted with earlier program studies (10) in which Bard-Parker was used effectively as a control inhibitor, the experiment was repeated using a "wetter" liquid/soil ratio as had been employed before. The results of this experiment are shown in Figure 39. A liquid/soil ratio of 0.3 ml/0.1 g rather than 0.3 ml/0.5 g caused the Bard-Parker germicide to be approximately 30 fold more effective at the same concentration. The cumulative labeled release from viable soil and Bard-Parker germicide was only slightly higher than the response obtained from the heat sterilized soil plus Bard-Parker.

That the Bard-Parker germicide was effective under "wet" conditions, but not under "moist" conditions is most interesting. An explanation is not obvious at this time, and further work is clearly needed. Physical separation or dilution of Bard-Parker from the medium by soil particles, or biological factors may be responsible. However, the fact that "wetter" cultures may undergo a lag in growth not seen in "dryer" experiments, indicates a difference in metabolism

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FIGURE 38

Effect of Bard-Parker Germicide (BP) on the Nonbiological Evolution of Radioactivity from RM9 Medium

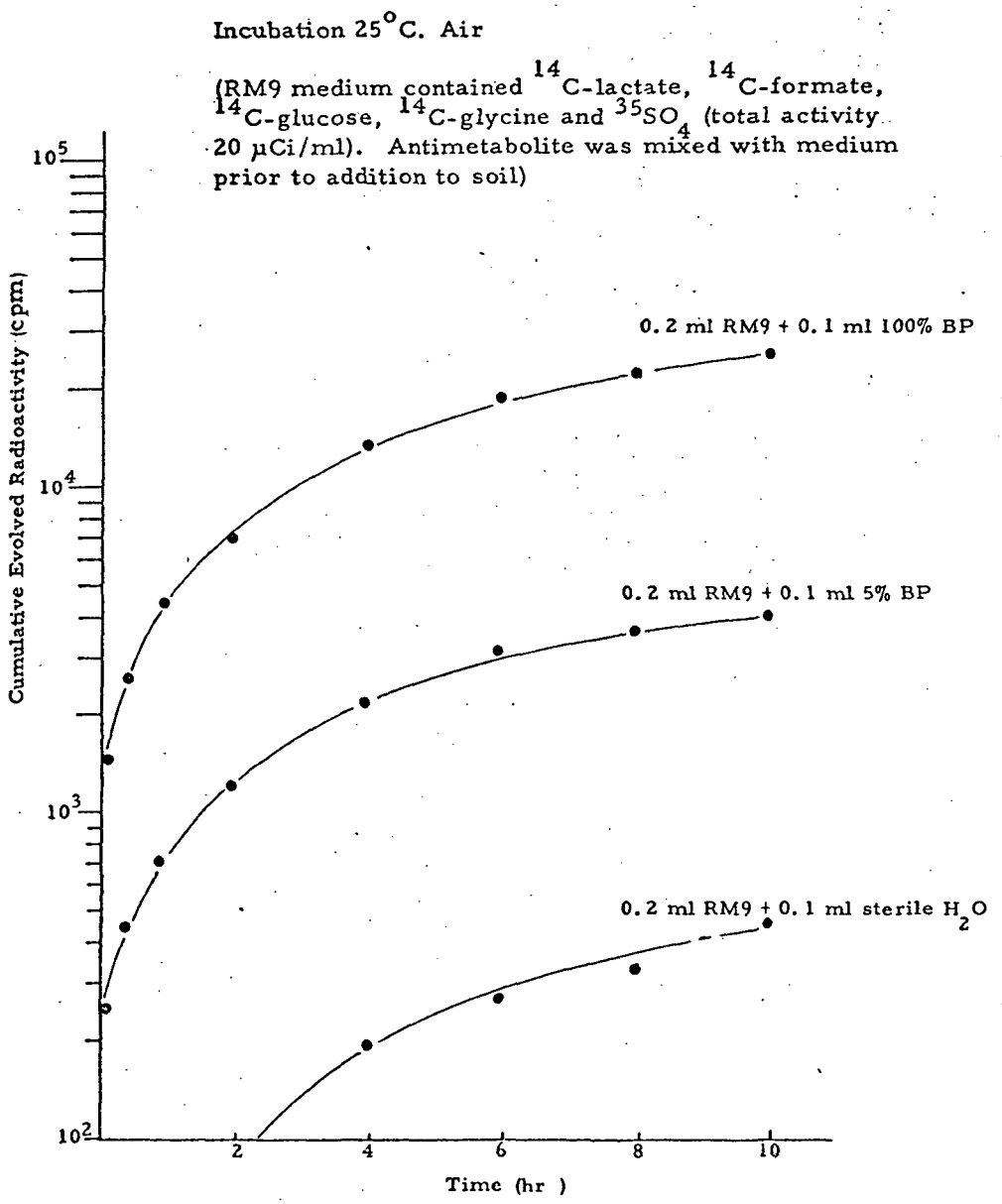

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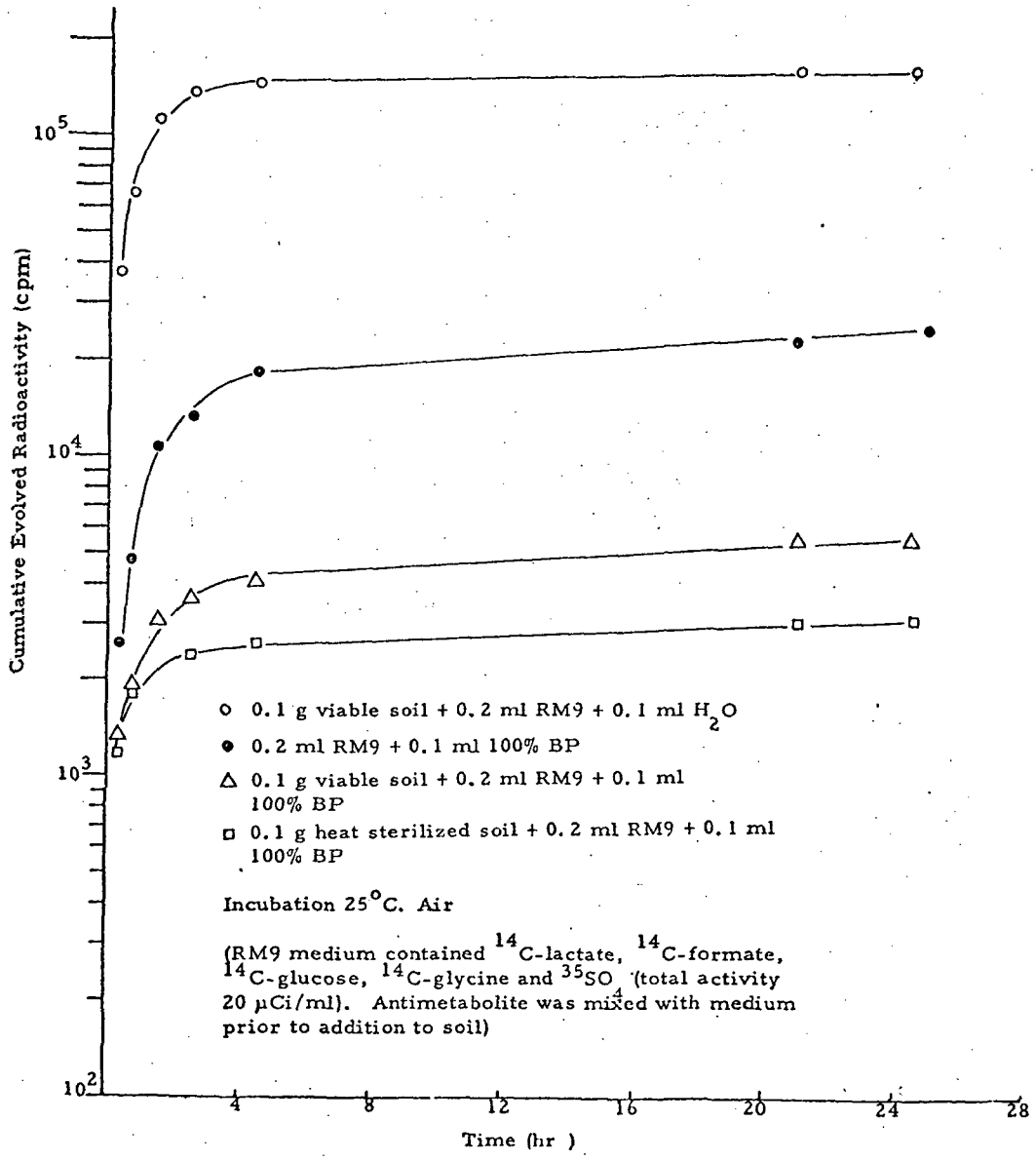
FIGURE 39

Effect of Bard-Parker Germicide (BP) on the Evolution of Radioactivity from Mojave Soil (high medium/soil ratio)



or viability. Cells in lag phase growth may be more susceptible to the effects of the Bard-Parker germicide.

4. Sodium Hypochlorite

Concentrations of 5 and 50 ppm had no detectable effect on the evolution of radioactivity by viable soils (not shown). A concentration of 500 ppm as shown in Figures 40 and 41 decreased the response of Wyaconda soil by seven fold, but had little effect on the Mojave soil.

5. *Ethylene Oxide*

Ethylene oxide sterilization was conducted on 0.5 g soil samples contained in 22 mm planchets. Treatment was with 56 mg/l $(CH_2)_2O$ at a relative humidity of 52% and temperature of 54°C for six hours. As shown in Figures 42 and 43, the treatment was only slightly effective in inhibiting the evolution of radioactivity from Mojave soil. The cumulative response from Wyaconda soil was reduced approximately 20 fold by the ethylene oxide treatment. The experiment was repeated with soil samples which were thinly spread on filter paper during $(CH_2)_2O$ exposure. The results of this experiment were no different than those presented.

6. Antibiotics

Two antibiotics, Chloramphenicol (100 μg/ml) and Penicillin-G (10 μg/ml) were tested for effect on the evolution of radioactivity from soils. As shown in Figures 44 and 45, the

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FIGURE 40

Effect of Sodium Hypochlorite (500 ppm NaClO) on the Evolution of Radioactivity from Wyaconda Soil

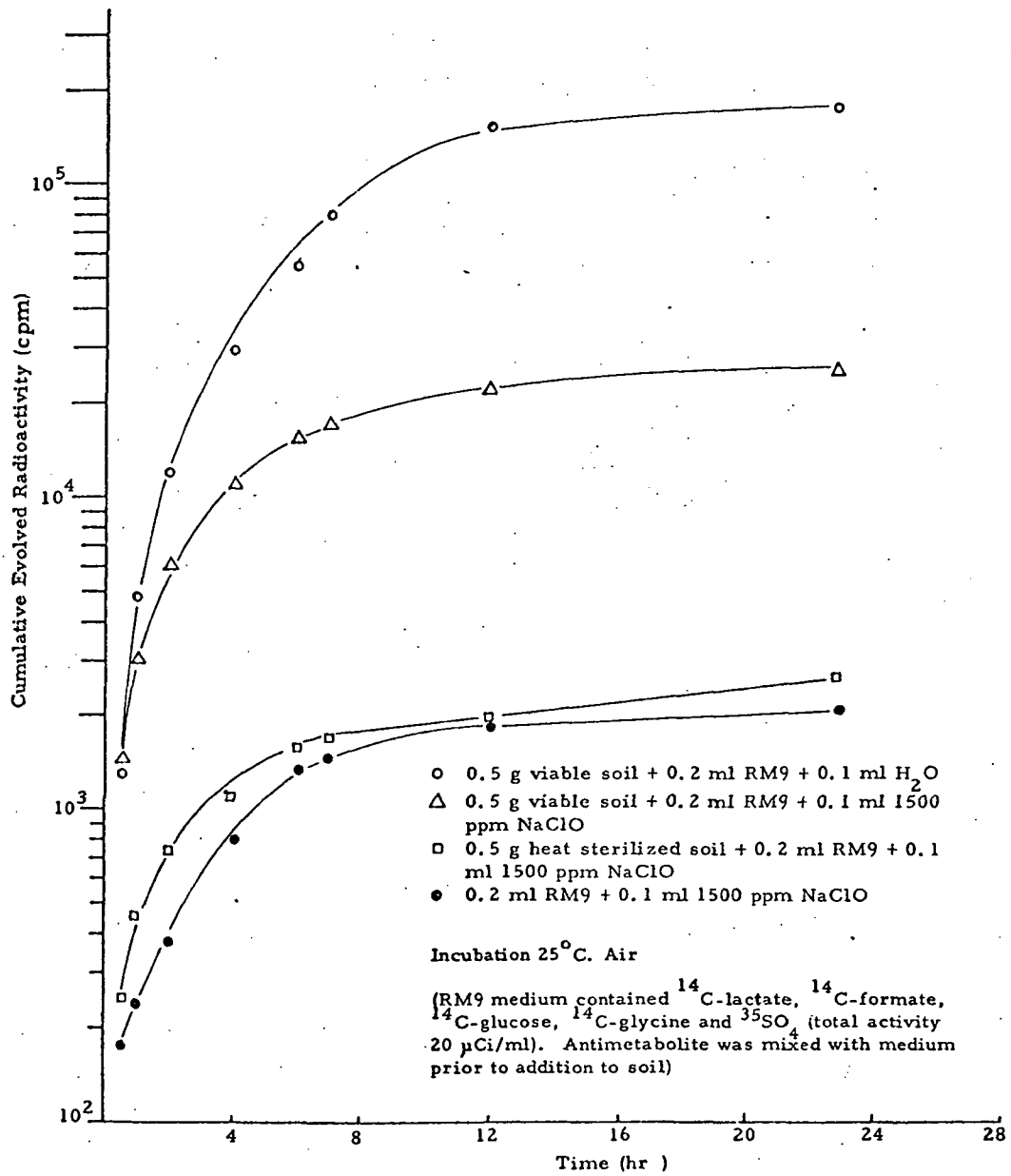

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FIGURE 41

Effect of Sodium Hypochlorite (500 ppm NaClO) on the Evolution of Radioactivity from Mojave Soil

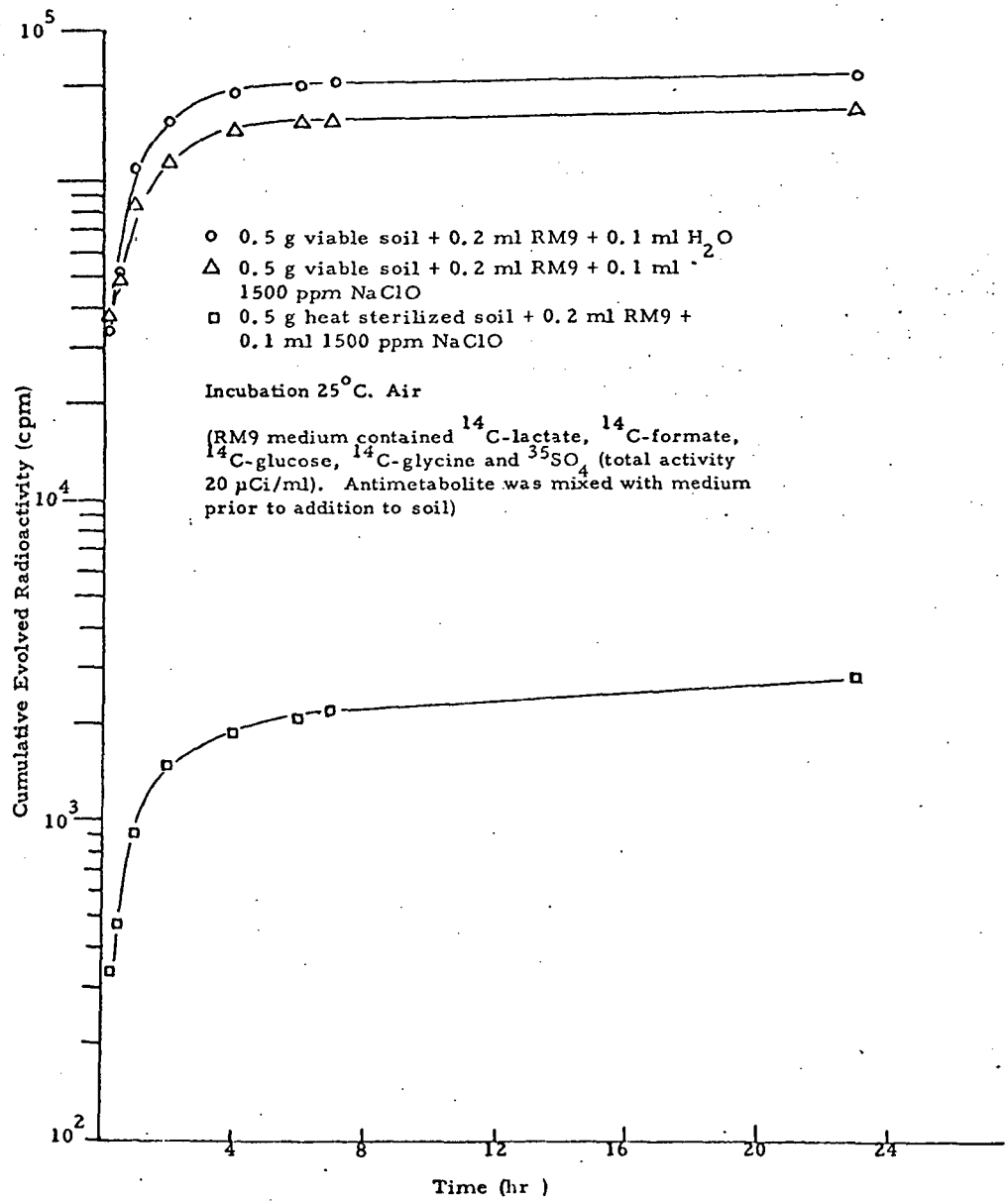

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FIGURE 42

Effect of Ethylene Oxide on the Evolution of Radioactivity from Wyaconda Soil

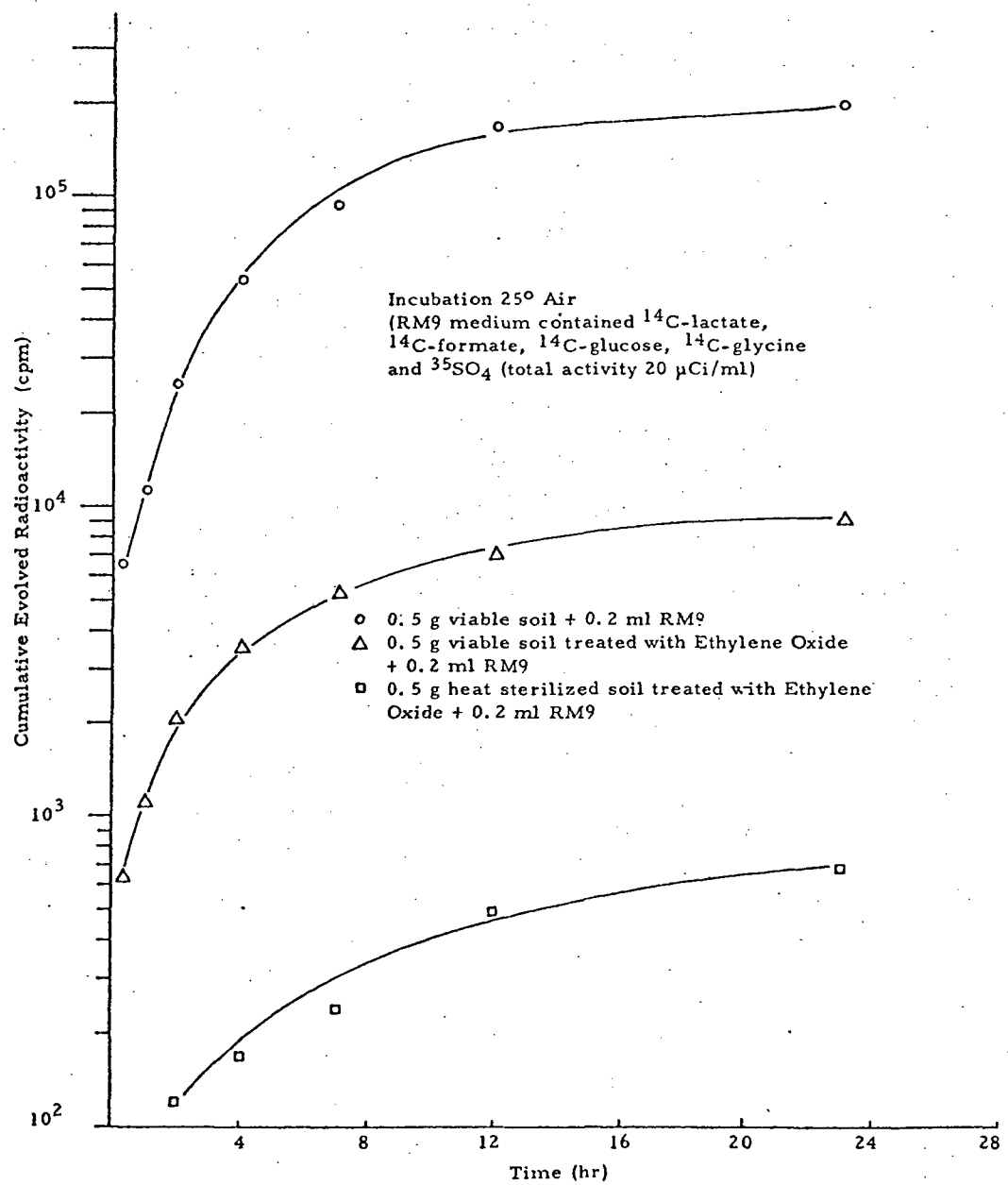

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FIGURE 43

Effect of Ethylene Oxide on the Evolution of Radioactivity from Mojave Soil

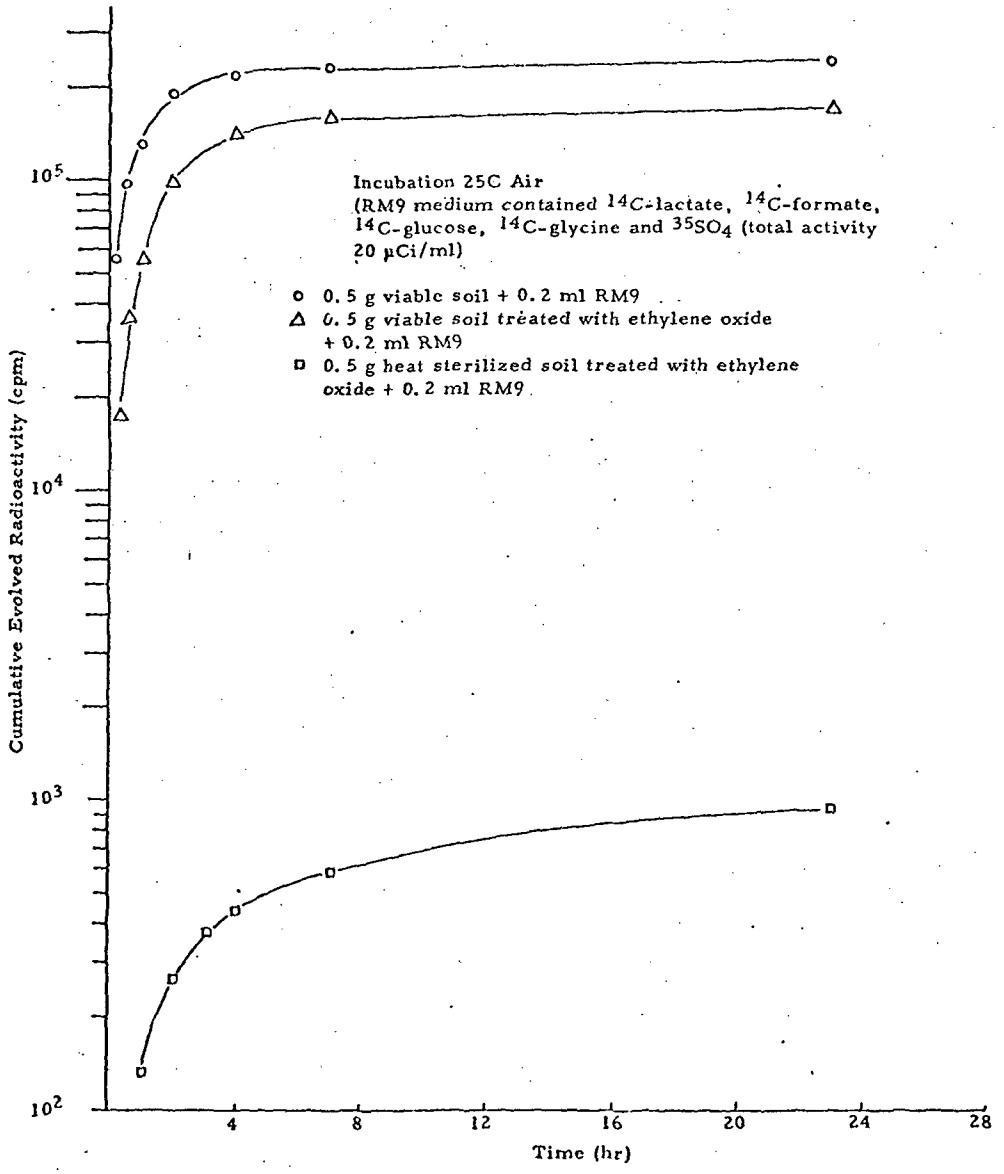

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FIGURE 44

Effect of Chloramphenicol (100 μg/ml) and Penicillin-G (10 μg/ml) on the Evolution of Radioactivity from Wyaconda Soil

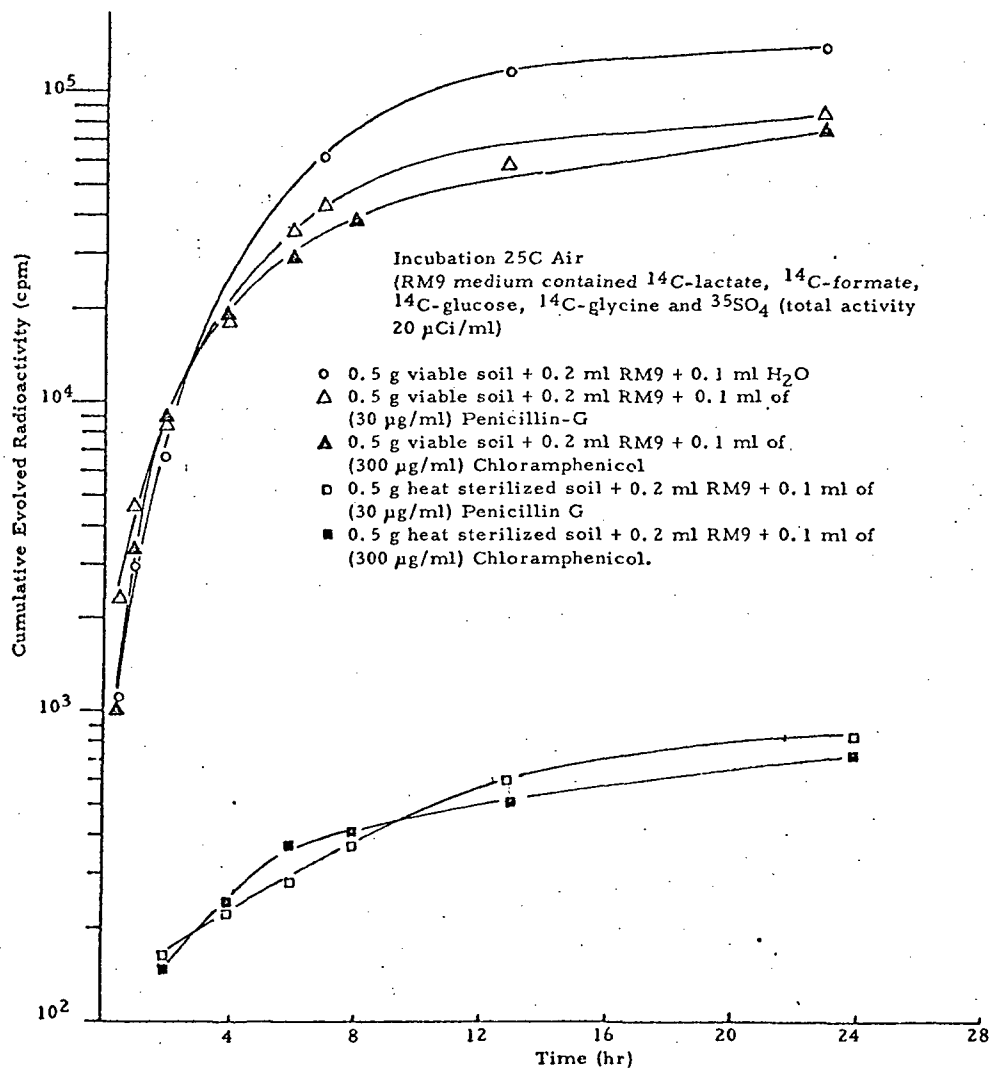

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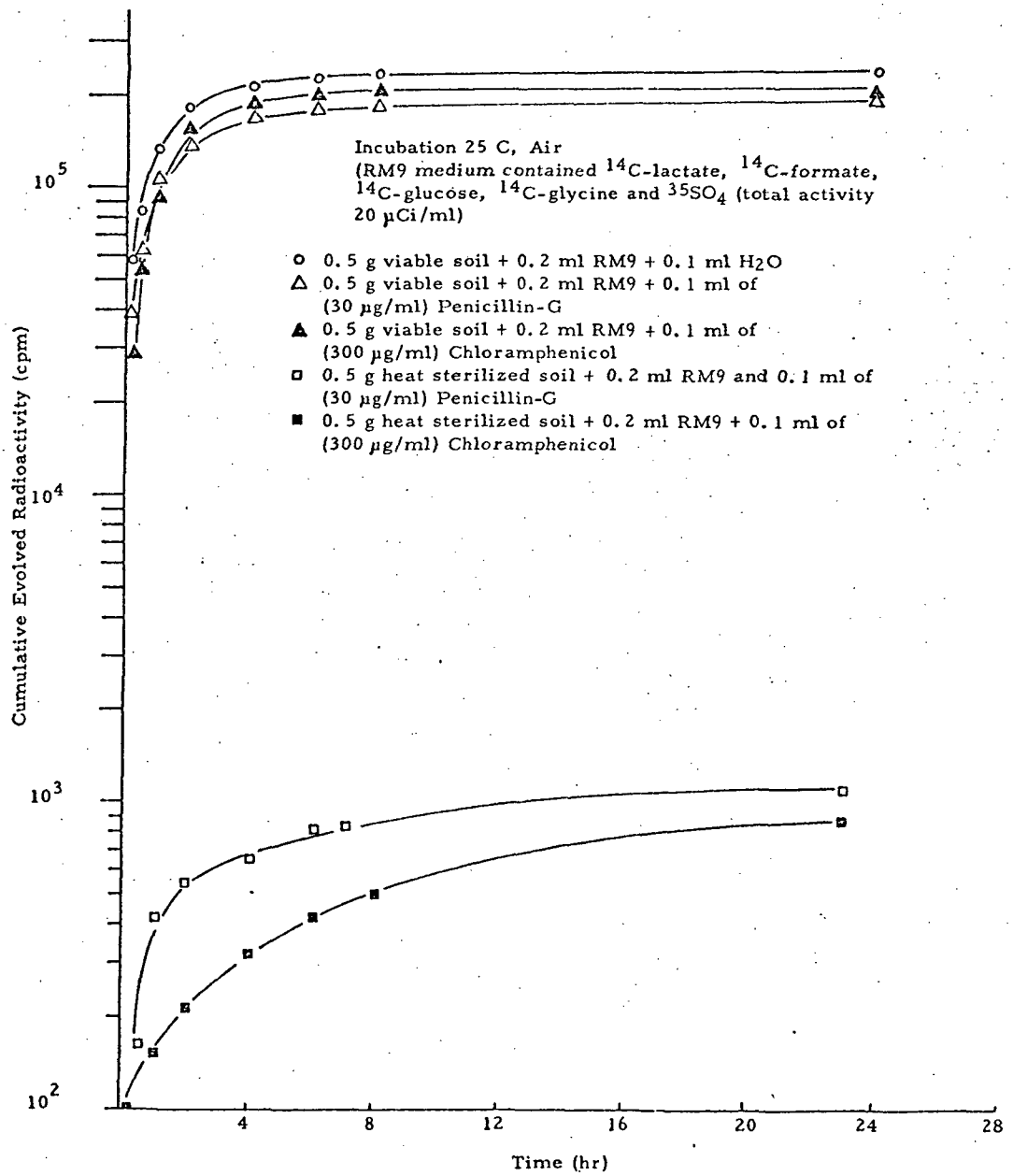
FIGURE 45

Effect of Chloramphenicol (100 μg/ml) and Penicillin-G (10 μg/ml) on the Evolution of Radioactivity from Mojave Soil



concentrations of antibiotics tested were only slightly effective in inhibiting the evolution of radioactivity from Wyaconda soil and had no effect on the Mojave soil.

H. Substrate Studies

Various labeled substrates were individually tested by the labeled release to determine their contribution to the overall response from the complete RM9 medium. In addition to ^{14}C-formate, ^{14}C-lactate, ^{14}C-glucose and ^{14}C-glycine, other candidate substrates which have been recommended (9) were also tested. All substrate concentrations were made to 2.5×10^{-4} M and the radioactivity of the substrates adjusted to 1 μCi/ml per substrate carbon atom. For example, RM9 with ^{14}C-formate was prepared to contain a total activity of 1 μCi/ml, whereas RM9 with ^{14}C-glucose was prepared to contain 6 μCi/ml. Medium was prepared in which ^{14}C-formate, ^{14}C-DL-lactate, ^{14}C-glucose and ^{14}C-glycine were present, both singly and in combination with the other unlabeled substrates.

Labeled release was conducted using Mojave, Wyaconda and Victoria Valley soil.

There was no difference in the kinetics of ^{14}C evolution, regardless of whether the labeled substrate was alone or in conjunction with other unlabeled substrates. The kinetics of ^{14}C evolution from medium containing individually labeled substrates in the presence of Mojave soil, are shown in Figure 46. There appeared

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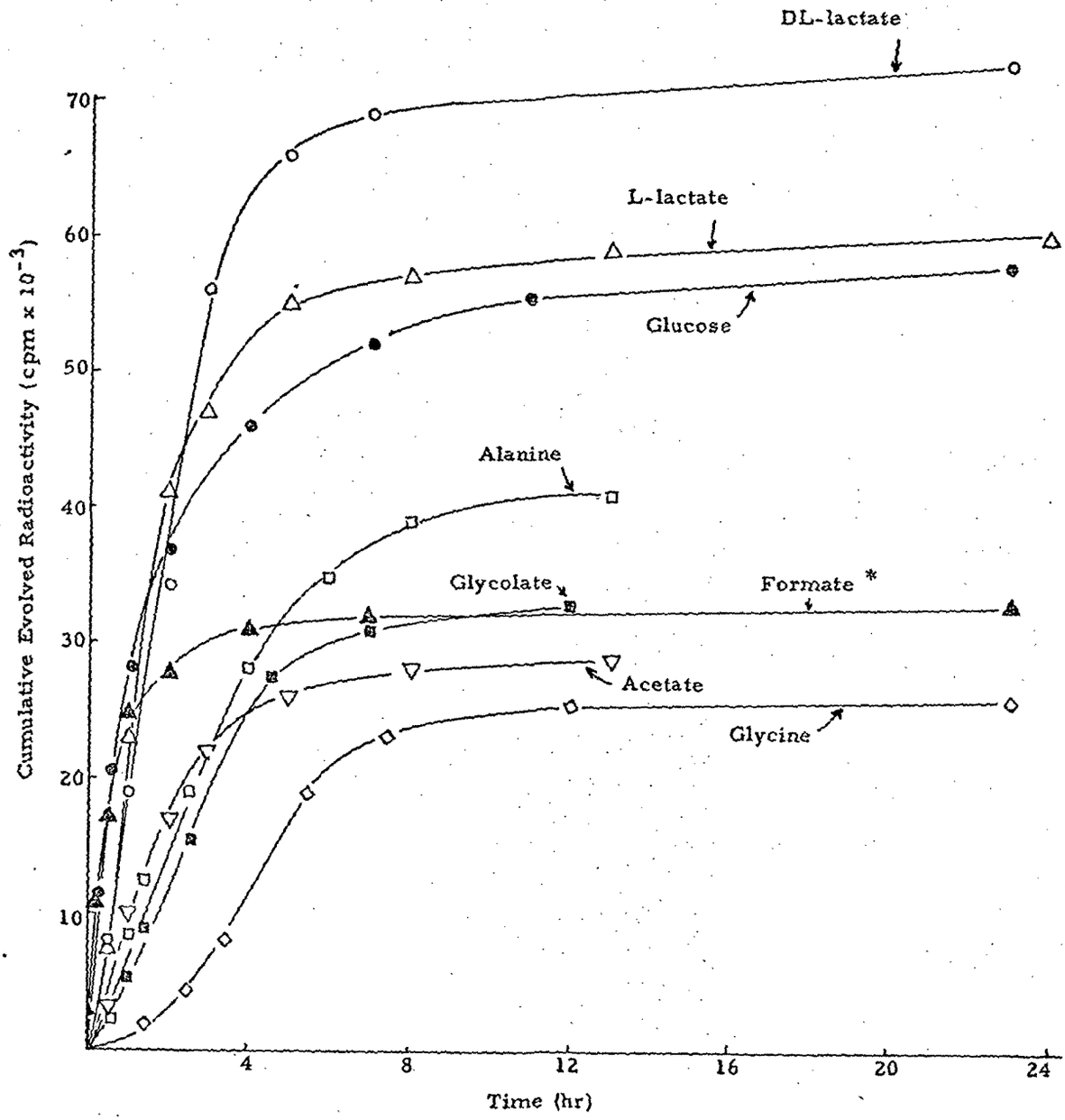
FIGURE 46

Evolution of Radioactivity from RM9 Medium Containing Various Labeled Single Substrates, by Mojave Soil



* The measured amount of radioactivity in ^{14}C-formate medium was 3.73 times lower than the average of all other substrates. Values of ^{14}C evolution as presented have been multiplied by 3.73.

to be two distinct families of curves. Media containing ^{14}C-glucose, ^{14}C-DL-lactate, and ^{14}C-L-lactate produced a cumulative ^{14}C evolution that was approximately two-fold higher than that produced from medium containing ^{14}C-formate, ^{14}C-glycine, ^{14}C-acetate or ^{14}C-glycolate. Medium containing ^{14}C-alanine produced a cumulative evolution of radioactivity that appeared intermediate between these two groups.

The substrates ^{14}C-glycine, ^{14}C-glycolate and ^{14}C-acetate are universally labeled and are at twice the specific activity of ^{14}C-formate. Since all four of these substrates evolved a similar amount of radioactivity, it would appear that an equivalent of one carbon from each was being utilized, perhaps by a similar series of reactions.

Lactate and glucose both produced a similar level of evolved radioactivity. Since that level was approximately twice the amount produced from the one and two carbon substrates, it appears that an equivalent of two carbons are liberated from both of these substrates. The data tend to show that the glucose is not metabolized via a three carbon split, or if so, only one of the three-carbon fragments is metabolized in a scheme similar to lactate utilization.

Alanine produced a level of evolved radioactivity, which was equivalent to the release of slightly less than 1.5 carbon atoms. However,

if an efficiency is defined as the radioactivity evolved per total radioactivity added, then alanine would have approximately the same efficiency as the two-carbon substrates. See Table 12.

Formate produced the highest efficiency of ^{14}C turnover and glucose produced the lowest.

It was also noted that the initial rate of evolution of radioactivity was substrate dependent. Formate and glucose produced the highest initial rate of ^{14}C evolution. Of all substrates tested, glycine stood out as producing the longest lag in evolution of radioactivity.

Results of medium testing with Wyaconda (Figure 47) soil were similar to those obtained with Mojave soil. Two families of curves were again obtained. However, the production of labeled gas was generally not as great.

With the exception of glucose, all substrates showed a lag in radioactive evolution--glycolate produced the longest lag. Evolution of ^{14}C from glucose occurred at a lower rate than in the presence of the Mojave soil. However, the initial rate with Wyaconda soil greatly exceeded that produced from all other substrates.

Responses obtained from DL-lactate and L-lactate were approximately equal in terms of total radioactivity, thus indicating that both enantiomorphs are used with similar efficiency. D and L lactate are commonly metabolized by two independent and specific

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TABLE 12

Radioactivity Evolved from Various Labeled Substrates by Wyaconda Soil

Substrate	Added Radioactivity (cpm)*	Radioactivity Evolved (cpm)	Ratio+
Formate	2,500	8,000	3.20
Lactate	27,000	51,000	1.90
Alanine	26,000	40,000	1.54
Glycolate	19,000	28,000	1.47
Acetate	19,000	27,000	1.42
Glycine	20,000	28,000	1.40
Glucose	55,000	47,000	0.85

* Measured by $Ba(OH)_2$ method described in Appendix (Section 1-C, Step 4)

\+ Evolved radioactivity (cpm) divided by added radioactivity (cpm) by BaOH method.

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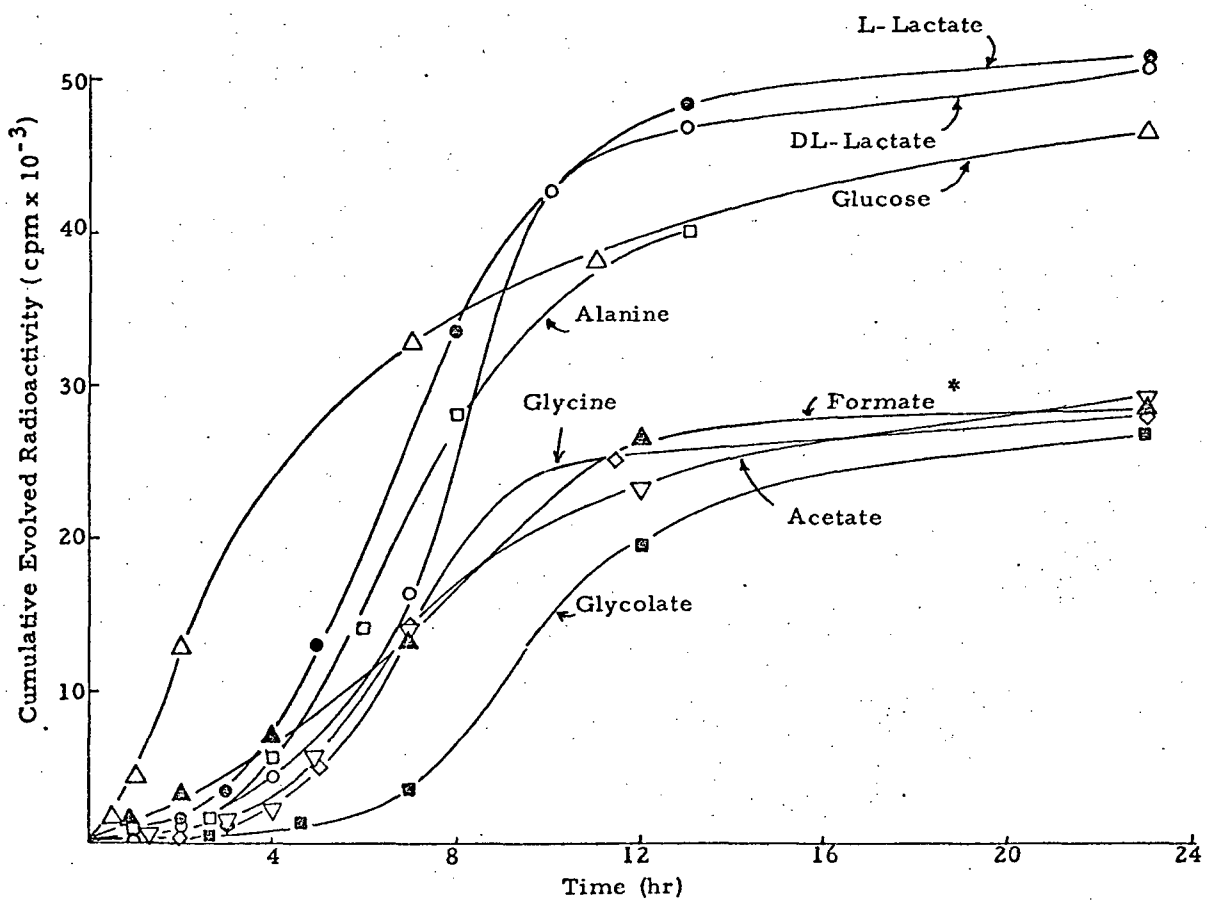
FIGURE 47

Evolution of Radioactivity from RM9 Medium Containing Various Labeled Single Substrates by Wyaconda Soil



* The measured amount of radioactivity in ^{14}C-formate medium was 3.73 times lower than the average of all other substrates. Values of ^{14}C evolution as presented have been multiplied by 3.73.

enzymes or by racemization by special racemase enzymes. Nonbiological evolution of radioactivity from individual substrates amounted to no more than 3% of the biological responses, except in the initial stages of glycine and acetate utilization. The ratio of viable to nonviable response was as great as 10,000:1 in some cases.

A test of the signal obtained from medium containing nonlabeled organics and $^{35}SO_4$ was conducted anaerobically in the environmental chambers, using a culture of Desulfovibrio desulfuricans cholinicas. A 0.1 ml sample of a day-old D. desulfuricans culture was added to planchets which were then placed in the chamber and flushed with N_2/H_2 (90:10). To this, was added 0.2 ml of RM9 containing 3.4 mg/l of $^{35}SO_4$ (10 uCi/ml) and 2.5×10^{-4} M glucose, lactate, formate, glycine. Evolved radioactivity was trapped with $Ba(OH)_2$ in the normal fashion. Results were as shown in Figure 48. The response was not as great as with labeled carbon; however, a 100-fold ratio between viable and control responses was obtained. D. desulfuricans is a strict anaerobe, unable to grow at oxidation potentials above 200 mv. The conditions provided by this experiment were probably less than optimum; however, a significant biological signal was observed. Nonbiological evolution of radioactivity by a nonviable culture was essentially at background level.

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FIGURE 48

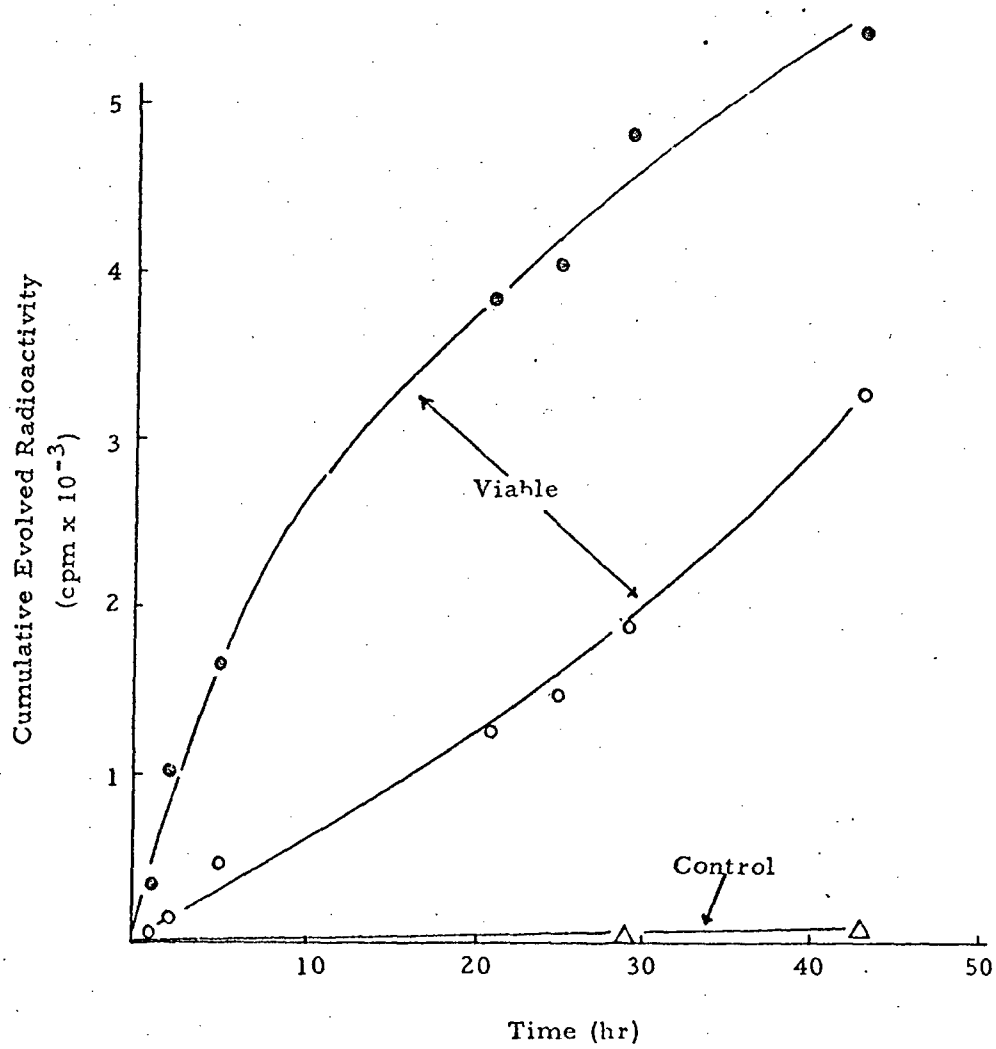
Evolution of Radioactivity from RM9 Medium Containing $^{35}SO_4$ by *Desulfovibrio desulfuricans cholinecus*

0.2 mg of RM9 containing 3.4 mg/l of $^{35}SO_4$ = (10 uCi/ml) and 0.25 M in glucose, lactate, formate and glycine

Incubation was under 10% H_2, 90% H_2 at 25°C

Viable contained 0.2 ml of 24-hr. culture of *D. desulfuricans*

Control was both medium alone and very old *D. desulfuricans* culture



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I. pH Studies

Experiments were conducted to determine if the response from soil was affected by buffering the soil/medium culture at a pH which was different from the pH achieved when that soil was immersed in unbuffered medium. 0.3 g of soil were immersed into 10 ml of RM9 medium containing 2.4×10^{-4} M ^{14}C-lactate, ^{14}C-glucose, ^{14}C-glycine, ^{14}C-formate and $^{35}SO_4$ (total radioactivity 2 μCi/ml). Duplicate cultures in which the medium contained 0.05 M Tris buffer were compared with cultures having no Tris buffer. The results of an experiment employing Mojave soil are shown in Figures 49. The buffered medium which resulted in a culture with a lower pH showed increased labeled release, ATP production and ^{14}C uptake, above that obtained for the unbuffered system.

A similar experiment which was performed with woody soil (unbuffered pH 5.7, buffered pH 6.7) showed no difference in labeled release, ATP and ^{14}C uptake due to buffering.

Although Tris buffer caused enhancement of response from the Mojave soil, the effect would appear to be soil dependent, and in theory might be inhibitory in some cases. The pH of low liquid/soil ratio was not measured, but it is likely that the 0.05 M Tris would not appreciably change the pH.

The concentrations of buffer that would be required to stabilize the pH in these systems would likely be very high, and might cause

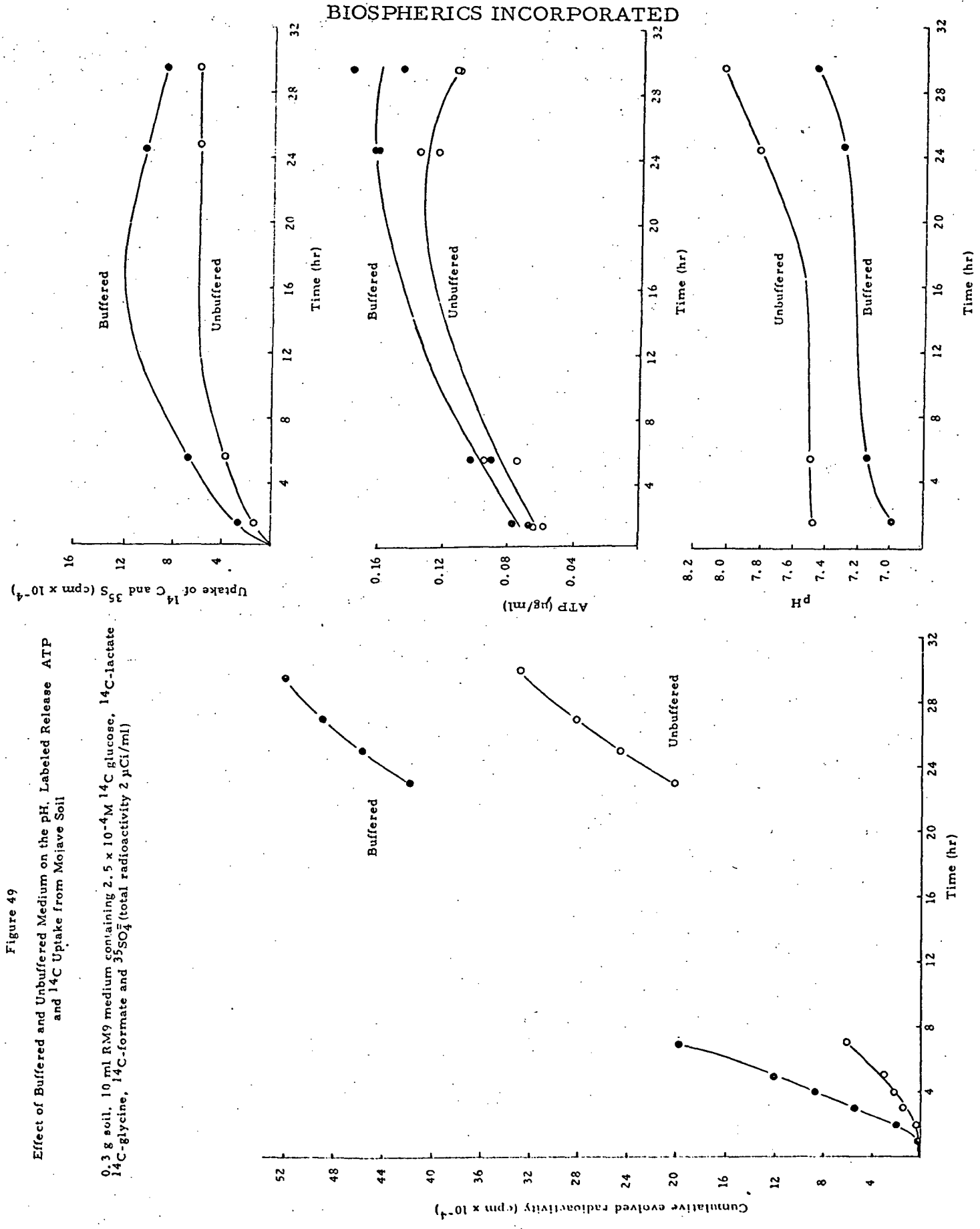

Figure 49

Effect of Buffered and Unbuffered Medium on the pH, Labeled Release ATP and ^{14}C Uptake from Mojave Soil

0.3 g soil, 10 ml RM9 medium containing 2.5×10^{-4}M ^{14}C glucose, ^{14}C-lactate ^{14}C-glycine, ^{14}C-formate and $^{35}SO_4^{=}$ (total radioactivity 2 µCi/ml)

inhibition of some organisms. Suggestions concerning media for the "Viking 75" mission have made the inclusion of Tris or another strong buffer less attractive (9).

J. Nitrogen Studies

The development of a technique for monitoring microbial nitrogen metabolism was approached in a preliminary way in this program. Methods for detecting life as measured by changes in the concentration of nitrogen compounds were viewed for possible inclusion in the AMML. From the aspect of theoretical importance, sensitivity, and compatibility with other life detection schemes planned, a measurement of N_2 - fixation by the technique of acetylene reduction appeared most feasible.

It has been shown that the nitrogen-fixing complex (nitrogenase) also reduces acetylene to ethylene (11). On the basis of this reaction, Stewart et al (12) have described a method for in situ measurement of N_2-fixation rates in root nodules and algae. With a few modifications, the experiment described by these authors was tested on soil.

The results thus far are highly encouraging. It appears that in addition to monitoring nitrogen metabolism, the method may also detect the presence of phototrophic activity.

IV. ENGINEERING

The engineering effort on this program has been directed toward a software rather than hardware development. As the various new and revised metabolic assays were developed in the laboratory, these were reviewed with respect to their application in the AMML breadboard instrument.

The initial concept for the basic AMML processing system is still adequate for the aqueous portion of the assays. The incorporation of a "moist" soil culture system, however, will require additional hardware and new techniques must be developed to perform this type of assay. Several concepts of suitable techniques have been reviewed and one of these shows considerable promise in meeting the requirements for the system. This concept entails the use of separate test chambers for each assay sample. The samples are incubated "moist," then eluted individually so that an aqueous extract may be removed for the assay. (ATP, ^{14}C and ^{35}S uptake, phosphate uptake, or others which may be included in the future).

The automated programmer concept which was developed for the AMML breadboard uses a punched program tape and numerical control technique is directly compatible with the current assay requirement.

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APPENDIX I

METHODS AND TECHNIQUES

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APPENDIX I - METHODS AND TECHNIQUES

Section 1 - Media

A. The composition of RM9 medium which was used throughout the program, with the exception of the section on, "Substrate Studies," is shown in Table 1.

TABLE 1

Composition of RM9 Medium

Basal:	K_2HPO_4	25 mg/l	
	$MgCl_2 \cdot 6H_2O$	66 mg/l	
	NH_4NO_3	200 mg/l	
	NaCl	100 mg/l	
	Soil Extract*	100 mg/l	
	Tris	6 g/l	
	Adjusted to pH 7.0 with HCl		
Radioisotopes:	(^{14}C) Sodium Formate	6.5 mCi/l	20 mg/l
	D-(UL-^{14}C) Glucose	1.3 mCi/l	50 mg/l
	DL-(1-^{14}C) Sodium Lactate	1.3 mCi/l	20 mg/l
	(UL-^{14}C) Glycine	1.0 mCi/l	20 mg/l
	Sodium Sulfate (^{35}S)	10 mCi/l	3.4 mg/l

*Soil extract was prepared by suspending 500 g of air-dried soil in 1,300 ml of water. The mixture was then autoclaved for one hour, filtered, and made up to one liter with sterile, distilled water.

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B. The ^{14}C organic substrate concentrations were adjusted during the phase of study on substrates. Media used contained one or more labeled or unlabeled substrates in concentrations and specific activities as shown.

TABLE 2

Composition of RM9 Medium Used in Substrate Studies

Basal:	Same as Table 1		
Radioisotopes:	(^{14}C) Sodium Formate	1 μCi/ml	2.5×10^{-4} M
	D(UL-^{14}C) Glucose	6 μCi/ml	"
	D(UL-^{14}C) Sodium Lactate	3 μCi/ml	"
	L(UL-^{14}C) Sodium Lactate	3 μCi/ml	"
	(UL-^{14}C) Glycine	2 μCi/ml	"
	L(UL-^{14}C) Alanine	3 μCi/ml	"
	(UL-^{14}C) Sodium Acetate	2 μCi/ml	"
	(UL-^{14}C) Sodium Glycolate	2 μCi/ml	"
	(^{35}S) Sodium Sulphate	10 μCi/ml	3.5 mg/l

C. Preparation of RM9 Medium

Step 1. Basal medium is made up in 1 liter batches, placed in 100 ml aliquots and autoclaved for 20 minutes, 18 psi, 120°C.

Step 2. Labeled substrate is added to obtain the required radioactivity level. The concentration is then brought to the prescribed level with unlabeled substrate.

After addition of labeled substrate(s) the solution is filter sterilized through a 0.22 μ pore-size membrane filter.

Step 3. Freshly prepared medium generally shows a higher than background count when the sterile medium is gettered. This nonbiological emission can be reduced by shaking the sterile medium in a horizontal water bath shaker at 35°C. Medium is shaken until 0.2 ml shows a count of 100 cpm or less for a one-hour gettering period.

Step 4. Radioactivity is measured before and after each experiment using the following procedure:

a. Three drops of saturated $Ba(OH)_2$ are placed in duplicate stainless steel concentric planchets

b. To each of these planchets, 0.02 ml of medium and 0.4 ml of distilled water are added.

c. The planchets are dried under an infrared light for approximately 30 minutes.

d. Planchets are counted for one minute in a Nuclear Chicago D-47 Gas Flow Counting Apparatus.

Step 5. Media is stored at 3°C until used. It is filter sterilized again before use and a measurement of radioactivity (as in Step 4) is performed prior to each experiment.

Section 2

A. Procedure for Conducting the Labeled Release Experiment:

Step 1. Sifted soil samples (14 mesh screen), ranging in size from 25 mg to 100 mg, are weighed into sterile, one-inch aluminum planchets. Planchets are precleaned by boiling in Sparkleen detergent until water wets the aluminum evenly. They are then thoroughly rinsed with tap and distilled water.

Step 2. Control soils (unless otherwise stated) are prepared by dry heat sterilization. Planchets containing a weighed quantity of soil are placed in glass petri dishes and placed in a hot air oven at 212°C for 30 minutes.

Step 3. Planchets are placed in a holding rack or in sterile petri dishes and set on a laminar flow bench. An experiment is initiated by the addition of labeled medium to soil. Immediately upon addition of the labeled medium, each soil containing planchet is

capped with an inverted planchet lined with a filter pad moistened with three drops of saturated $Ba(OH)_2$ (see Figure 1).

Step 4. After a timed interval, the gettering pads were replaced with planchets lined with freshly moistened $Ba(OH)_2$ pads. This regimen was followed throughout the entire experiment.

Step 5. The getter pads were dried under an infrared lamp for about 15 to 20 minutes and transferred to clean planchets. Radioactivity retained on each pad was determined in a Nuclear Chicago D-47 Gas Flow Counting Apparatus flushed with Q-gas.

B. Loss of radioactive gas due to leakage of planchets was checked in the following manner:

Four planchets containing viable soil and labeled medium were capped with getters and placed in a 14 cm diameter glass petri dish containing five planchets having exposed getter pads saturated with $Ba(OH)_2$. Placement of getters and viable cultures was as shown in Figure 2. During a 24-hour incubation period, the getters on the viable cultures were changed after 2, 4, 6, 8, and 24 hours. The open getters were changed after 6 and 24 hours. Results were as shown in Table II. Although it is likely that some radioactive gas escaped completely, the

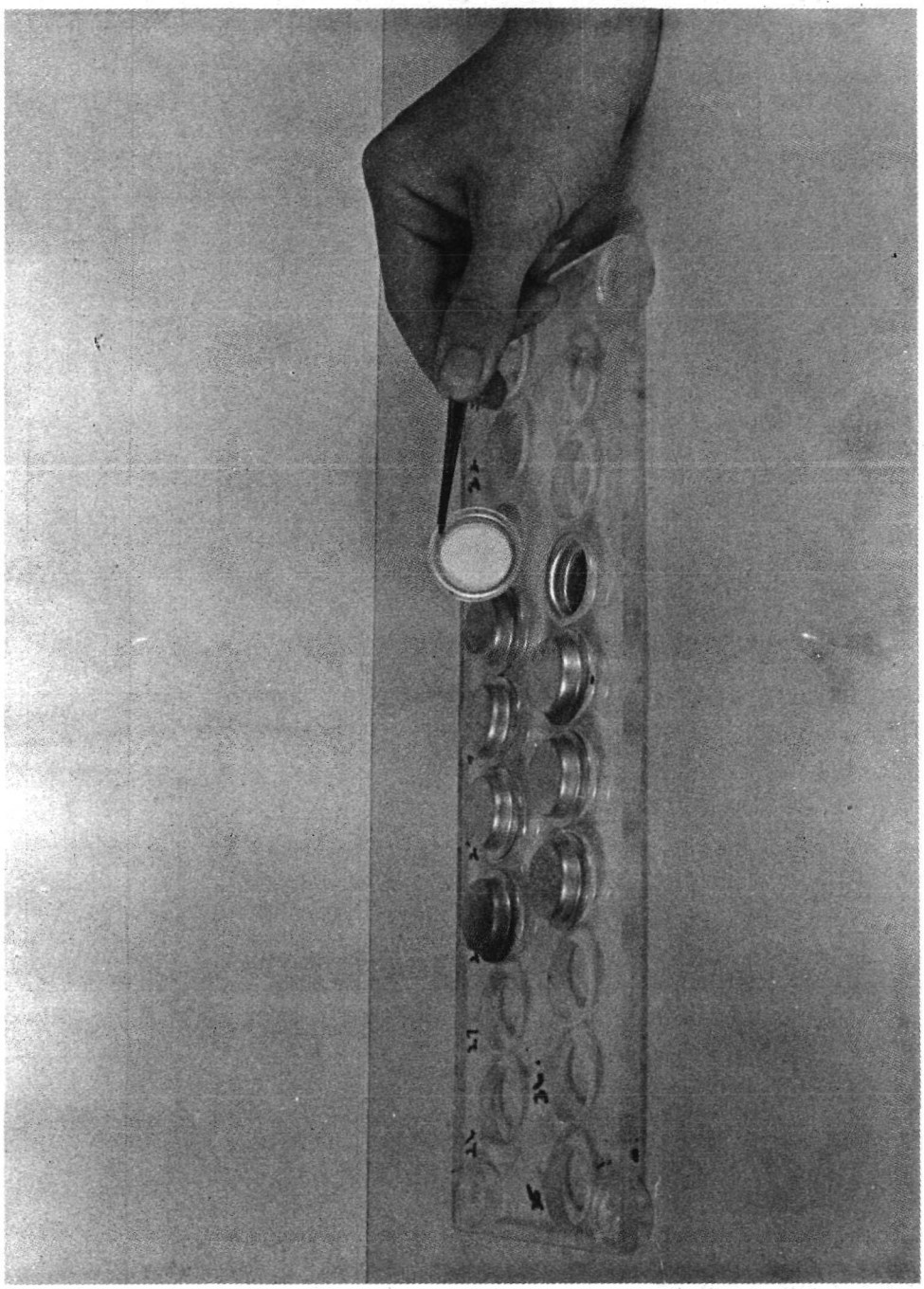

FIGURE 1

Planchets Used for Labeled Release Gettering Experiment

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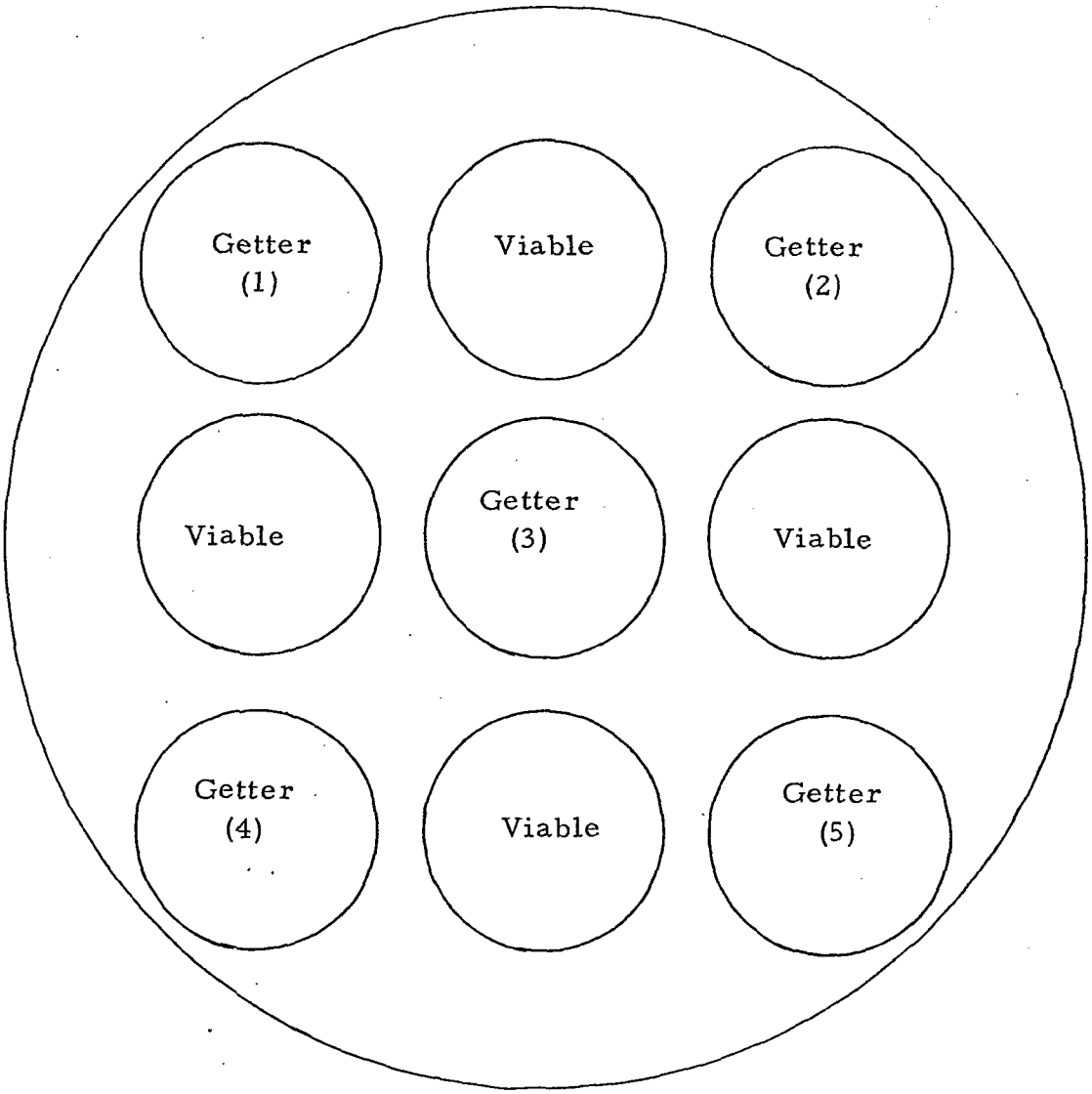
FIGURE 2

Placement of Getters and Viable Planchets for Gas Leakage Experiment



Actual Size

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TABLE 3

Leakage of Radioactivity from Planchets

Time (hr)	Radioactivity Trapped Above Viable Cultures (cpm)			
0 - 2	21,754	21,832	22,507	19,083
2 - 4	24,382	20,182	29,908	26,392
4 - 6	25,674	20,651	31,332	29,482
6 - 8	27,926	27,761	27,246	36,652
8 - 24	96,100	102,417	92,137	126,051

Total: 829,500

Time (hr)	Radioactivity Trapped by Open Getters				
	(1)	(2)	(3)	(4)	(5)
0 - 6	826	688	660	413	133
6 - 24	3273	3280	5024	2750	1240

Total: 18,280

crossover contamination could not exceed this "worst case" situation. Crossover contamination which resulted was approximately 2%.

Section 3

A. Procedure for Performing the ATP Assay
Butanol Sonication Extraction:

The following procedure was used during attempts to adapt the AMML assay procedures to "moist" culture conditions:

Step 1. Soil/medium sample was rinsed quantitatively into a centrifuge tube with 5 ml of 0.02 M, pH 7.5 arsenate buffer.

Step 2. Five ml of n-butanol was added and the mixture was sonicated for two minutes at 65 watts, 20 kc/sec., in an ice bath.

Step 3. The mixture was centrifuged at 6,000 x g for five minutes to separate the butanol and water. The water phase was removed and assayed by the firefly bioluminescence technique.

B. Boiling Tris buffer extraction: This procedure was used except when indicated above.

Step 1. Soil/medium sample and planchet (or 1 ml of liquid culture) is dropped into a beaker containing approximately 20 ml of boiling Tris buffer.

Step 2. The mixture is cooled on ice, rinsed into a 25 ml volumetric flask, and brought to volume.

Step 3. A portion of the mixture is filtered and assayed by the firefly bioluminescence technique.

C. Firefly Bioluminescence ATP Assay:

A 10 µl quantity of sample is measured into a cuvette using a 50 ul Hamilton syringe having a 10 cm piece of 26 gauge teflon spaghetti tubing attached to the needle. The cuvette is placed in the luminescence measuring device and a 50 µl quantity of enzyme reaction mixture (duPont) injected. The millivolt response which results is quantitated on the basis of known standards.

Section 4

A. Procedure for Performing Phosphate Uptake Experiments on "Moist" Culture Systems

Step 1. The soil/medium sample was quantitatively transferred to a 25 mm diameter millipore filtering apparatus which contained a 0.45 µ pore-size membrane filter which had been previously wet with 0.1 ml of unlabeled RM9 medium without added phosphate. Unlabeled RM9 medium without added phosphate was used to transfer and wash the soil. The volume used was varied from 2 - 5 ml as designated in the text.

Step 2. The filtrate was caught in a clean test tube, two drops of chloroform were added as a preservative, and the tube was stoppered and refrigerated until phosphate assay.

B. Procedure for Performing Phosphate Uptake Experiments on "Wet" Culture Systems

Step 1. A one (1) ml sample of stirred liquid culture was quantitatively removed and transferred to a 25 mm diameter millipore filtering apparatus which contained a 0.45 μ pore-size membrane filter which had been previously wet with 0.1 ml of unlabeled RM9 medium without added phosphate. Four (4) one-ml portions of RM9 medium without phosphate were used to wash the filter.

Step 2. The filtrate was caught in a clean test tube, two drops of chloroform were added as a preservative, and the tube was stoppered and refrigerated until phosphate assay.

C. Procedure for Colorimetric Phosphate Assay

Reagents:

1. Ammonium Molybdate Solution - a one percent aqueous solution of $(NH_4)_6 Mo_7O_{24} \cdot 4H_2O$ is diluted with three volumes of five percent sulfuric acid.

2. Stannous Chloride Solution - 40 mg of $SnCl_2 \cdot 2H_2O$ is dissolved in 100 ml of one percent hydrochloric acid (prepared fresh daily).

Procedure:

1. Add 5.0 ml of sample or standard to a 10 ml Erlenmeyer flask.
2. Add 1.0 ml of ammonium molybdate reagent and mix thoroughly.
3. Immediately add 1.0 ml of stannous chloride reagent and mix.
4. After 12 to 15 minutes at room temperature, read at 690 nm in a 13 mm cell against distilled water.

Section 5

Procedure for performing ^{14}C and ^{35}S uptake:

Membrane filters resulting from Section 4-A, Step 1, were dried under infrared light and transferred to clean planchets. Radioactivity retained on each pad was determined in a Nuclear Chicago D-47 Gas Flow Counting Apparatus, flushed with Q-gas.