Detection of Metabolically Produced Labeled Gas: The Viking Mars Lander

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Received May 5, 1971

A qualitative, nonspecific method will test for life on Mars in 1976 by supplying radioactive substrates to samples of the planetary surface material. If microorganisms are present, they may assimilate one or more of the simple labeled compounds and produce radioactive gas. The compounds have been selected on the basis of biological theory and terrestrial results. The measurement of radioactive gas evolved as a function of time constitutes evidence for life. A control performed on a duplicate, but heat sterilized, sample will confirm the biological nature of the results. The shape of the response curve obtained from the viable sample may provide information on the physiological state and generation period of the organisms. Data obtained from a wide variety of terrestrial soils demonstrate a rapid response and high sensitivity for the experiment. Its ability to make comparative studies of soil microorganisms is also demonstrated. Instruments have been developed to conduct the experiment automatically and a breadboard version of the instrument designed for the Viking mission is under construction. The Mars experiment is described and simulated return data are given.

I. INTRODUCTION

In this experiment, formerly called "Gulliver" (Levin *et al.*, 1962; Levin, 1963; Levin *et al.*, 1964; Levin, 1965; Levin and Heim, 1965; Levin, 1966; Levin and Perez, 1966; Levin, 1968; Levin, 1969; Levin *et al.*, 1970), radioactively tagged compounds are used to detect metabolic activity. The test is routinely performed in the laboratory by placing organisms or soil into a sterile planchet and adding sterile medium containing radioactive compounds. A similar planchet is fitted with a disk of filter paper moistened with a saturated solution of barium hydroxide. The second, or "getter," planchet is inverted over the first. Organisms assimilating the labeled substrates will evolve radioactive gas, principally $^{14}CO_2$ and, sometimes, including $H_2{}^{35}S$. The gas is gettered onto the surface of the filter disk. At intervals, the getter planchet is replaced. The exposed getter planchets are dried and then counted for radioactivity. When the evolved radioactivity is plotted as a function of time, metabolism and growth can be detected and the rates for each determined. Confirmation is obtained by running the identical experiment on a heat sterilized sample of the soil as a control. An idealized response is shown in Fig. 1, along with possible inferences.

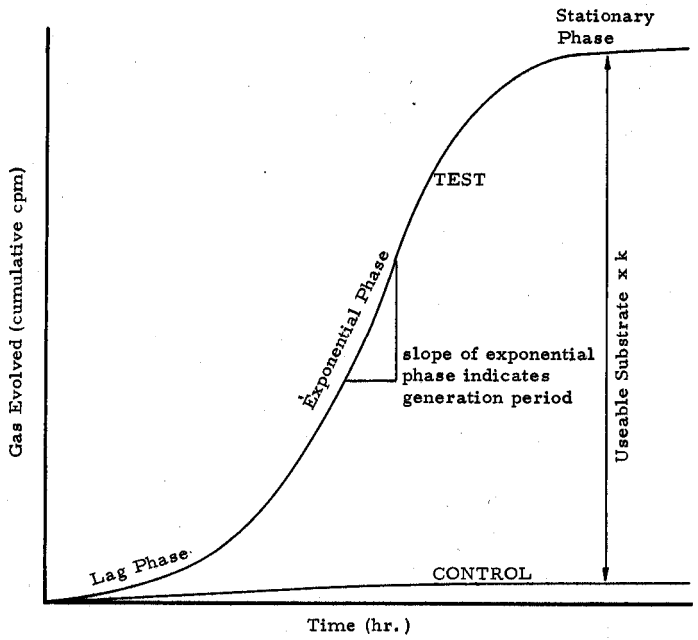

FIG. 1. Labeled release experiment idealized response.

The assumptions on which the experiment is based are: (a) although possible life on Mars may not be limited to microorganisms, the latter must be present to accomplish the biodegradation required for recycling of the organic matter; (b) the biochemical reactions at the cellular level are aqueous; (c) the organisms assimilate compounds from their environment, producing gas as an end product; (d) such compounds include relatively simple carbon molecules or ions and/or sulfate.

The use of isotopes lends speed and sensitivity to life detection. Results with test organisms are obtained within hours compared to the one or more days required by conventional microbiological methods which also measure evolved gas. A single cell should produce a response, just as in the case of the multiple tube dilution type determination. Actually, responses from cultures containing less than 25 cells (Levin *et al.*, 1962) and from soil samples containing approximately 200 cells (Levin and Perez, 1966) in 1 mg, the smallest amount of soil tested, have been obtained by the labeled release method. The sensitivity is also important from another standpoint. It permits the substrates to be offered in very dilute concentrations which minimize possible toxicity. Finally, the sensitivity increases the prospect for the detection of organisms with very low metabolic rates such as might be anticipated for Mars. A fortunate aspect of the method is that the radioactive gas evolved readily separates from the aqueous radioactive solution, eliminating an otherwise troublesome noise discrimination problem in detecting the product in the radioactive medium.

II. DEVELOPMENT AND TESTING

Under NASA sponsorship, considerable effort has been expended in formulation of suitable media, selection of radioactive substrates for the experiment, and in the development of automatic instrumentation to conduct the experiment on Mars. Two approaches were used to the basal media: a simple salts medium, and a complex medium with considerable organic

enrichment. The object of the former was to preclude introduction of compounds potentially inhibitory while the latter sought to satisfy possible requirements for complex molecules at concentrations dilute enough to minimize possible inhibition. These two media are given in Table I.

TABLE I

EXTENSIVELY TESTED LABELED RELEASE MEDIA

M9 (simple salts)		M11 (complex)			
K_2HPO_4	1.00g	K_2HPO_4	1.00g	Ascorbic acid	0.013g
$MgSO_4 \cdot 7H_2O$	0.20g	NH_4NO_3	0.19g	Bacto-casamino acid	0.25g
NH_4NO_3	0.20g	KNO_3	0.03g	Proteose peptone #3	1.25g
NaCl	0.10g	$MgSO_4 \cdot 7H_2O$	0.20g	Soil extract	16.0ml
Soil extract	100ml	NaCl	0.10g	Distilled H_2O	984ml
Distilled H_2O	900ml	Malt extract	0.19g	Medium adjusted to pH 7.0	
Medium adjusted to pH 7.0		Beef extract	0.19g		
		Yeast extract	0.81g		

Three considerations guided the choice of labeled substrates: (a) simple molecular or ionic species are more likely to be accepted than are complex ones; (b) test results on microorganisms and soils; and (c) the possible role of Miller-Urey type reaction products in the Mars evolutionary process. Racemic mixtures of those compounds possessing optically active centers are used to insure inclusion of the proper isomer for Martian organisms. The labeled substrates selected for use with the two basal media are shown with the concentrations and specific activities used to date in Table II. In tests on terrestrial soils, the labeled sulfate adds little to the magnitude of the response. However, sulfate is reduced by fairly primitive, anaerobic bacteria, such as the *Desulfovibrio* and, in the event the Mars microorganisms cannot utilize the labeled organics provided them, the sulfate might be reduced to $H_2{}^{35}S$ to yield a positive signal.

TABLE II

LABELED SUBSTRATES SELECTED FOR USE WITH M9 AND M11 MEDIA

Labeled substrate	Structure and position of label	μg/ml	nmoles/ml	μCi/ml	Specific activity (Ci/mole)
Formate	$H^{14}COONa$	20	194.1	6.5	22.1
Lactate	$CH_3CHOH^{14}COONa$	20	249.5	1.3	5.2
Glucose	$HO^{14}CH_2(^{14}CHOH)_4{}^{14}CHO$	50	277.5	1.3	4.7
Glycine	$H_2NCH_2{}^{14}COOH$	20	266.1	1.0	3.8
Sulfate	$Na_2{}^{35}SO_4$	207	860.6	10.0	11.6

Prior to the start of the Viking Project, four generations of feasibility instruments had been developed and successfully tested for the extraterrestrial experiment. These are shown in Fig. 2. “Sticky strings” were ejected to obtain soil samples in Models I, II, and III, while Model IV was designed to conduct an *in situ* experiment directly on the planetary surface. In each case, the medium was applied to the sample and the radioactive gas collected on a getter film deposited on the face of a radiation detector.

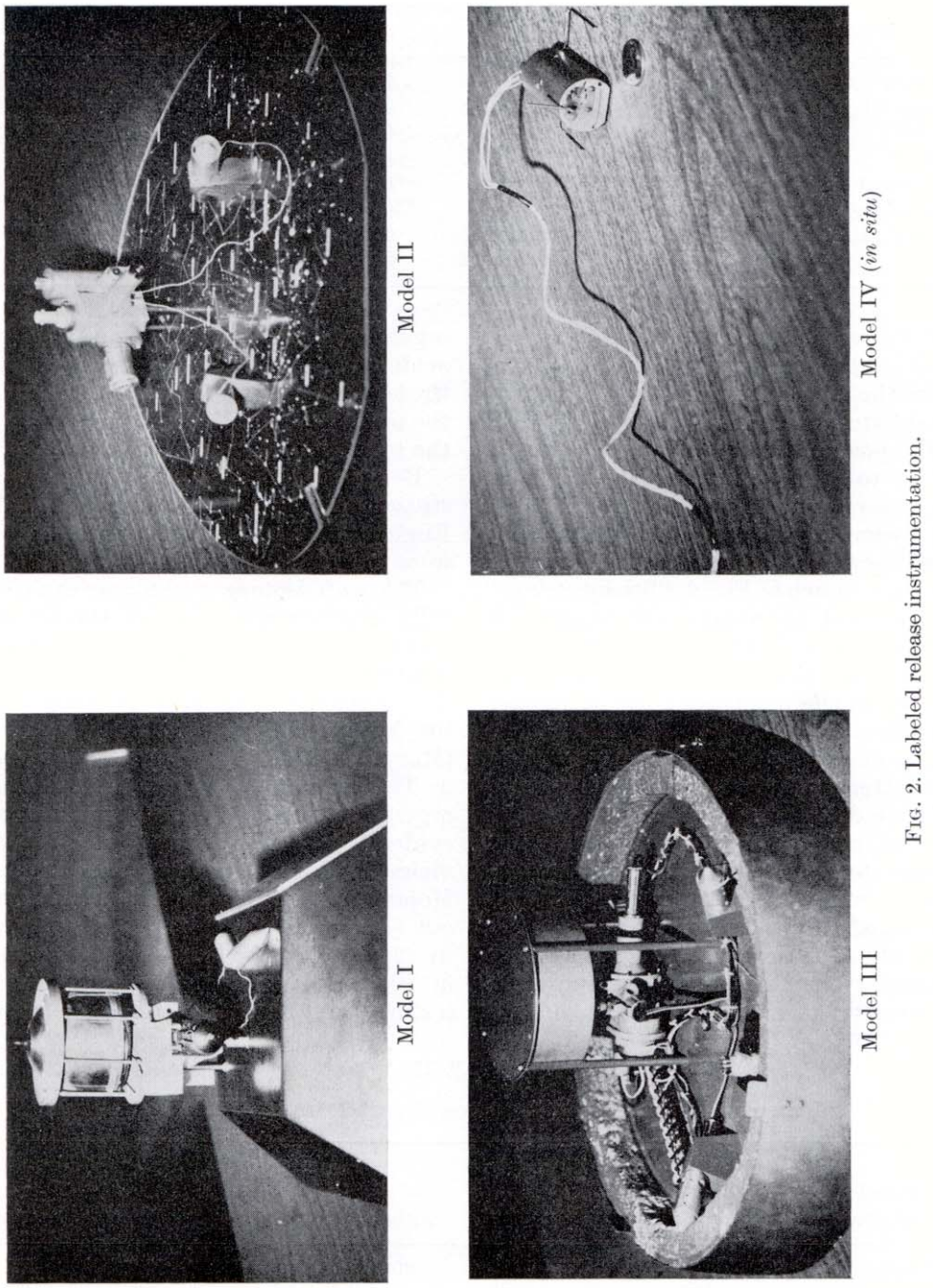

FIG. 2. Labeled release instrumentation.

As of this writing, several thousand separate tests have been run on pure cultures of many different species of microorganisms, mixed cultures, and a wide array of soils. Positive test results were obtained from all samples otherwise demonstrated to contain viable microorganisms. In the case of several highly acidic soils, some chemical degradation of the labeled substrates produced more than the usual trace of $^{14}CO_2$ in the control, but the test results were significantly higher.

Because oxygen is, at best, a trace constituent of the Mars atmosphere (Mars Engineering Model, 1970) anaerobic tests were included as shown in Fig. 3.

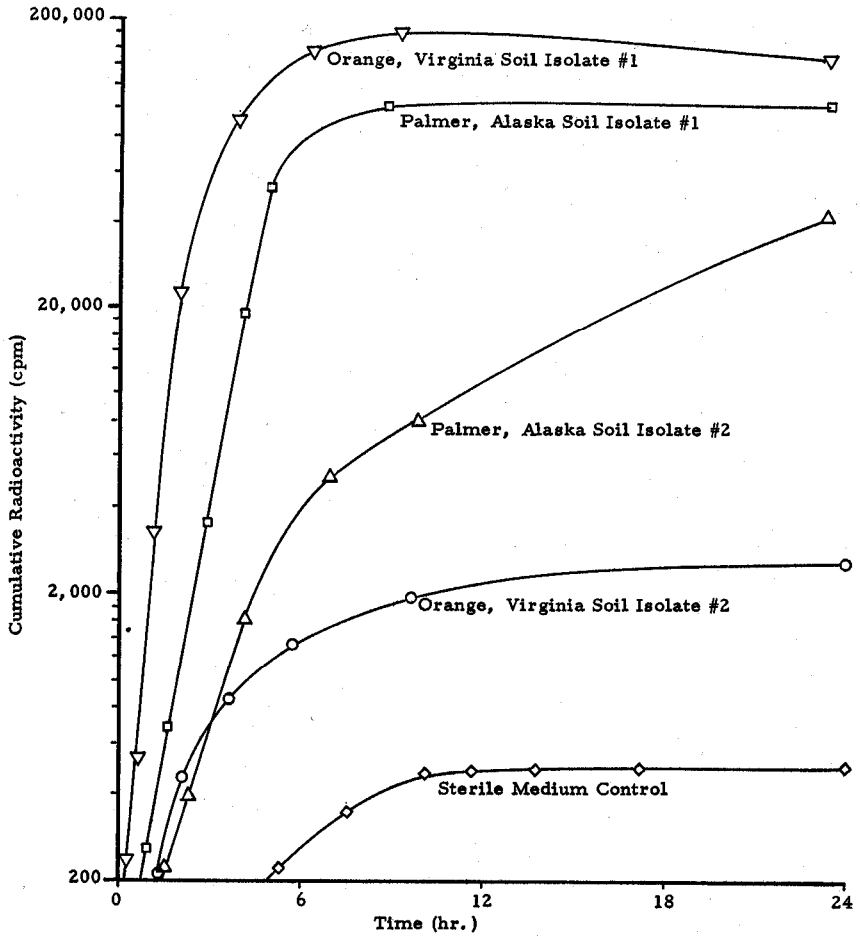

FIG. 3. Response of anaerobic soil isolates to medium M9–C^{14}. Anaerobic cabinet-recorder determination.

The radioisotope technique can faithfully monitor the effect of the environment on metabolism. It also permits comparative studies of microorganisms. The difference in response to temperature by Mojave Desert soil and Wyaconda (Maryland) field soil is shown in Figs. 4 and 5. The greater adaptation of the desert organisms to wide temperature swings is evident. Similarly, the effects of ultraviolet light on both soils were determined. Mojave and Wyaconda soils were spread out (<1 mm thick) and exposed to 91 mW uv (2537 Å) for the time periods indicated in Figs. 6 and 7 immediately prior to testing. The responses seen in the same figures again show the superiority of the desert soil to withstand environmental rigors. Both of these tests indicate the way Mars organisms may have evolved to cope with their inhospitable (by terrestrial standards) environment.

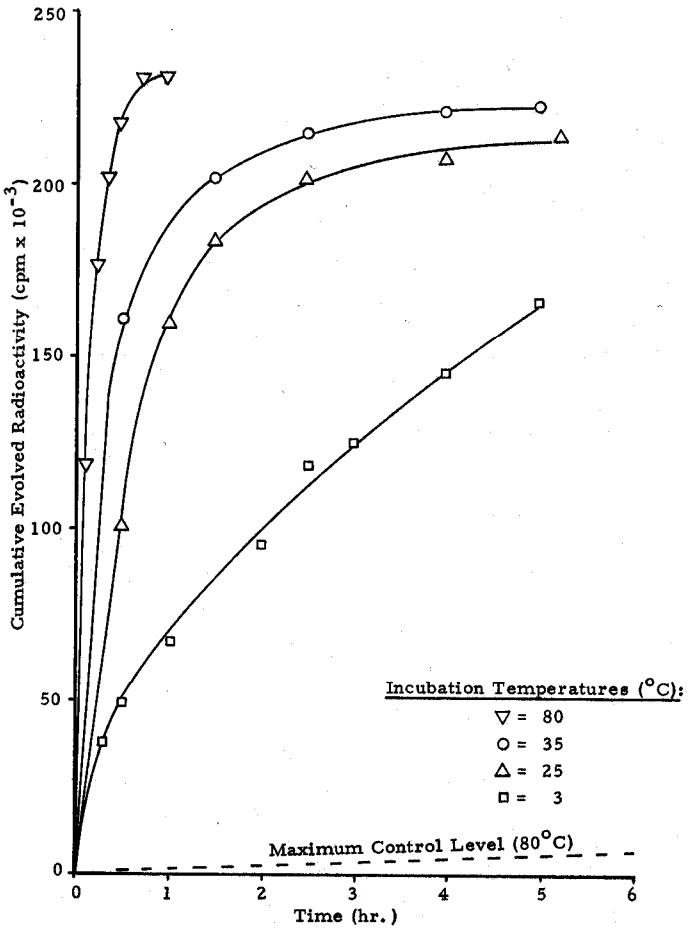

FIG. 4. Effect of incubation temperature on Mojave Desert soil labeled release. 0.5g soil: 5×10^5 organisms/g. 0.2ml RM9 + ^{14}C-formate, ^{14}C-lactate, ^{14}C-glucose, ^{14}C-glycine, and $^{35}SO_4$ (total activity: 20μCi/ml).

III. DESIGN FOR VIKING

As new data on Mars are brought in by spacecraft and ground observation, theoretical and practical aspects of the experiment are updated. In addition, the development of detailed plans for the Viking Mission has had an important impact on the manner in which the experiment can be conducted.

The discovery that the atmospheric pressure on Mars is between 0.5 to 2.5% that of the pressure on Earth suggests that there is little likelihood of liquid water surviving in free form for appreciable periods of time. Hence, the liquid medium in the experiment requires protection from the Mars atmospheric pressure. On the other hand, the positive detection of water vapor on Mars justifies the important experimental assumption of an aqueous biochemistry. The amount of water detected in the Mars atmosphere is only approximately 0.1% that of the Earth. The temperature and pressure range make it likely that water is not usually in liquid form on or near the surface of Mars. The question of whether organisms evolved under these conditions could survive complete immersion in water is thus raised. Inasmuch as the light scattering experiment discussed in this issue already poses this question, it was decided to convert the “labeled release”

experiment from an “immersed” to a “moist” mode. In earlier stages of the program, the experiment and instrumentation were designed to immerse 100 mg or less of soil sample into several ml of labeled medium. Now, the effort is directed toward applying a fraction of a ml of aqueous solution to several grams of soil. In this manner, most of the sample is subjected to only a thin film of water. This change may also make it unnecessary to include inorganic constituents or soil extracts in the “medium.” These should be leached out of the Martian sample in relatively high concentration by intimate cont act with the thin film of water applied. Similarly, the pH of the solution will be determined by the soil and, hence, should be suitable for any indigenous organisms. A comparison of results achieved by the “moist” and “wet” labeled release experiments is shown in Fig. 8. The rapid initial response, apparently devoid of any lag, is characteristic of the “moist” experiment.

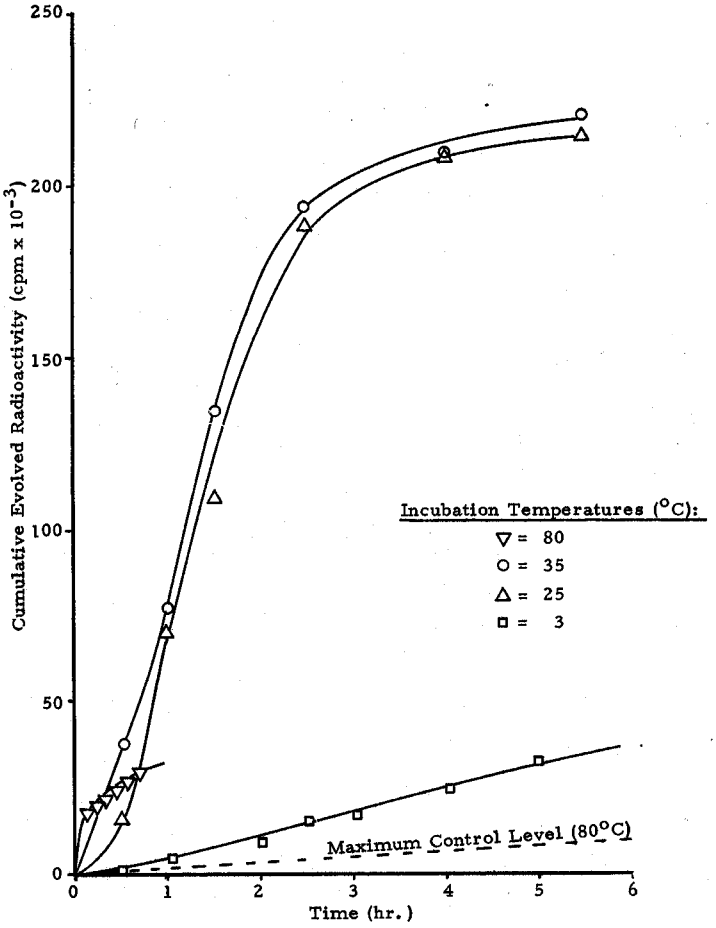

FIG. 5. Effect of incubation temperature on Wyaconda (Maryland) soil labeled release. 0.5g soil: 10^7 organisms/g, 0.2ml RM9 + ^{14}C-formate, ^{14}C-lactate, ^{14}C-glucose, ^{14}C-gylcine, and $^{35}SO_4$ (total activity: 20 μCi/ml).

The discovery that the Mars atmosphere is composed almost wholly of carbon dioxide maintains the credibility of two of the other important assumptions underlying the experiment: the carbon basis of life, and the metabolic production of gas. Recent findings by Hubbard, Hardy, and Horowitz (1970), working on the Viking biology experiment, that the one-carbon compound, formaldehyde, may be formed on the surface of Mars, further strengthens these

assumptions and the selection of formate as one of the labeled substrates.

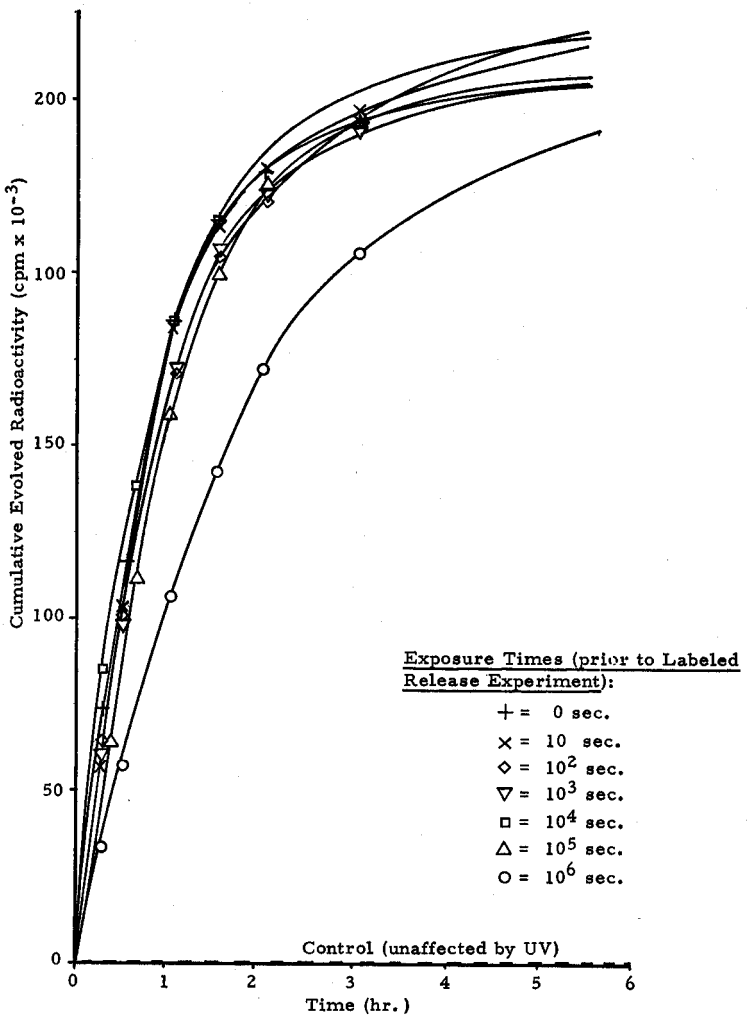

FIG. 6. Effect of ultraviolet light on Mojave Desert soil as determined by labeled release experiment. 0.5g soil: 5×10^5 organisms/g, 0.2ml RM9 + ^{14}C-formate, ^{14}C-lactate, ^{14}C-glucose, ^{14}C-glycine, and $^{35}SO_4$ (total activity: 20μCi/ml). UV lamp: Westinghouse sterilamp; 95% flux at 2537Å; flux = 91mW/cm^2 at soil surface.

Strictures imposed on the Mission design have resulted in special problems. Power for the landed science capsule will be provided by radioisotope thermal generators (RTGs). This energy source will permit the biology experiments to operate 90 days, much longer than believed possible during the formative stage of this experiment. But the labeled release experiment must pay a price for this advantage. The RTGs will impose a constant background radiation signal of approximately 2500 counts/mm on the beta detector. Counting statistics thus become very important for the detection of a faint signal. In order to obtain three sigma confidence for a 150 dpm signal, background will have to be monitored for 24 hr and the signal will have to be counted for 4 hr in the instrument, currently under flight hardware development by TRW, Inc.

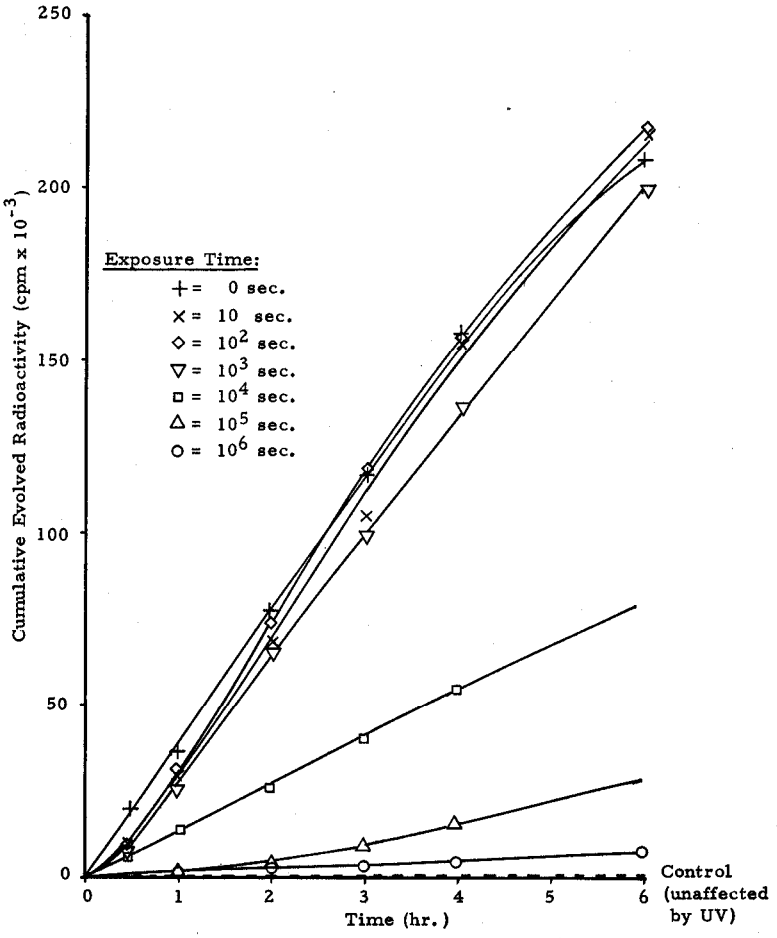

FIG. 7. Effect of ultraviolet light on Wyaconda (Maryland) soil as determined by labeled release experiment. 0.5g soil: 10^7 organisms/g, 0.2ml RM9 + ^{14}C-formate, ^{14}C-lactate, ^{14}C-glucose, ^{14}C-glycine, and $^{35}SO_4$ (total activity: 20 μCi/ml). Incubation temperature: 25°C; uv lamp: Westinghouse sterilamp; 95% flux at 2537 Å; flux = 91 mW/cm^2 at soil surface.

The need for terminal heat sterilization of the entire spacecraft prior to launch (to preclude the possibility of contaminating Mars and to protect the integrity of the life detection experiments) imposes another problem. The medium and substrates will be subjected to 125°C for a total of 118 hr according to current plans. Recent laboratory tests determined the deterioration of the labeled, sterile substrates when sealed in glass vials and subjected to the sterilization regimen in air and in nitrogen. Increasing the initial concentrations of three substrates can compensate for the moderate losses found. However, no trace of the fourth organic substrate, glucose, remained. Studies are now underway to replace glucose. The radioactive sulfate suffered essentially no loss. The effect of the terminal heat sterilization on the ability of the substrates to support growth was then examined. Aliquots of 0.2 ml of the heat treated substrates were added to 500 mg portions of a viable soil. The cultures were incubated at room temperature for 4 hr during which the evolved gas was collected for each. Figure 9 presents these results and also shows the amounts of activity released by each substrate as a sterile control after permitting passive gas exchange.

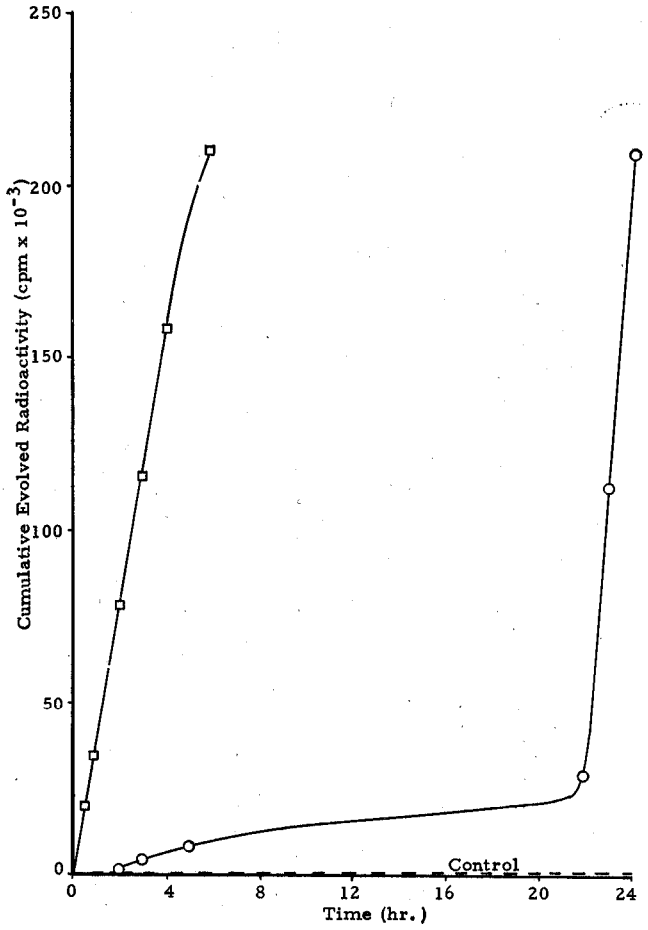

FIG. 8. Labeled release experiment "moist" vs. "wet" mode. □ = Moist: 0.5 g Wyaconda (Md.) soil: 10^7 organisms/g, 0.2 ml RM9 + ^{14}C-formate, ^{14}C-lactate, ^{14}C-glucose, ^{14}C-glycine, and $^{35}SO_4$ (total activity: 20 μCi/ml). ○ = Wet: 0.3 g Wyaconda (Md.) soil: 10 ml RM9 + ^{14}C-formate, ^{14}C-lactate, ^{14}C-glucose, ^{14}C-glycine, and $^{35}SO_4$ (total activity: 2 μCi/ml). Incubation temperature = 25°C.

Radioactive carbon dioxide is a principal product of the heat degradation of the labeled substrates. Additional radioactive gas will be released by the medium as a result of the self-degradation of the labeled compounds during the two or more years which will elapse between sealing the medium in the instrument and using it on Mars. This period includes approximately one year required for the trip. Realtime and accelerated storage tests of this effect are underway.

In order to offset the loss in sensitivity caused by partial degradation of the labeled substrates, initial concentrations and specific activities will be adjusted. Immediately prior to the start of the experiment on Mars, radioactive gases liberated by the above mechanisms will be purged from the medium by the use of a carrier gas or the Mars atmosphere.

This long storage time creates a particular problem with the radioactive sulfate. Because of the short half-life of ^{35}S, approximately 0.4% of this label will reach the surface of Mars, assuming 24 months storage. Accordingly, this substrate must be placed in the medium in relatively high specific activity. It must now be determined whether the disintegrations from the ^{35}S will be overly destructive of the other compounds in the medium and whether the

decomposition products may be toxic. If so, and no suitable solutions are achieved, the use of sulfate will be abandoned.

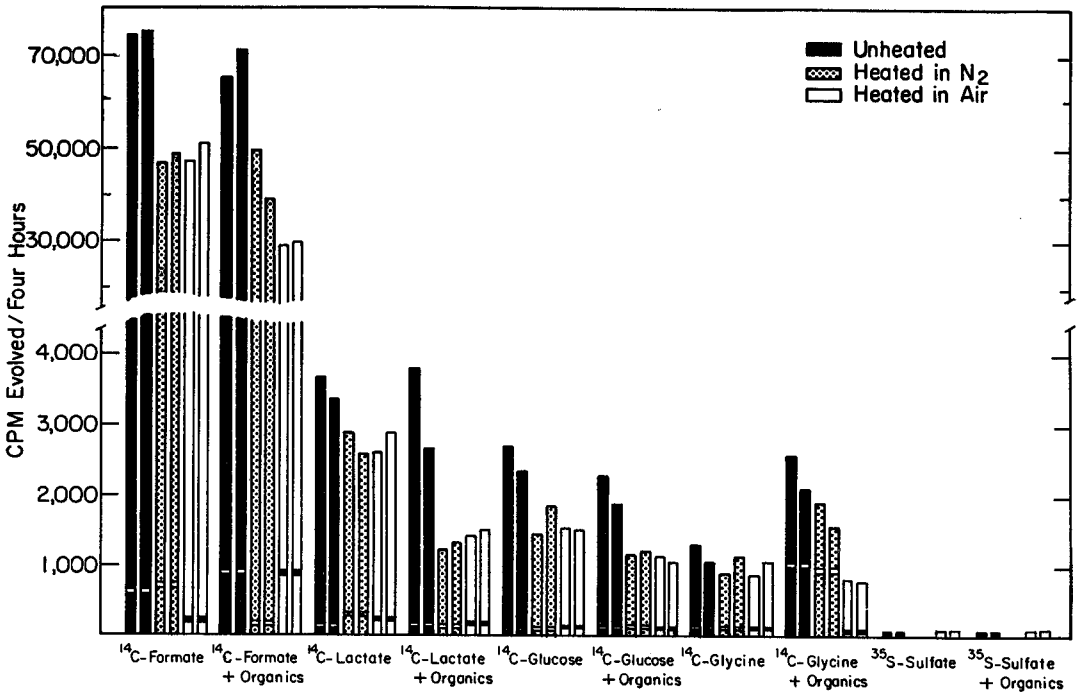

FIG. 9. Effect of terminal heat sterilization on labeled gas release in the presence of sandy soil.

One final problem, common to all four biological experiments, is being resolved. This is the treatment of the control sample. The tentatively selected 160°C for 3 hr does not offer sufficient assurance that the organisms will be inactivated. As seen in Fig. 10, randomly selected terrestrial soils have shown microbial survival after exposure to 160° for 2 hr. Higher temperatures may have adverse effects on chamber sealing materials. A time-temperature regimen sufficiently severe to destroy any life present but not damage the instrument must he determined.

The status of the flight instrument design permits the following account of how the labeled release experiment will operate on Mars. One cubic centimeter of soil will be delivered to one of the two chambers of the labeled release portion of the biology instrument. The chamber will also accommodate at least 5 cm^3 of the Mars atmosphere. This will reduce the possibility that some vital trace gas in the Martian atmosphere will be consumed, causing an early termination of the response. The sealed medium will then be released and purged to remove radioactive gases accumulated in it. Back-pressure will be maintained to preclude excessive loss of water to the outside atmosphere. Martian atmosphere at a slight over-pressure will be established in the test chamber. Two-tenths of a ml of the purged medium will be released onto the soil which will be maintained at 10 ± 5°C. The radiation detector, consisting of an array of solid state devices designed to maximize signal to noise in the ^{14}C-^{35}S segment of the energy spectrum, will take cumulative readings every 32 min. Technical difficulties preclude the use of a getter, but the evolved radioactive gases will diffuse to the detector space.

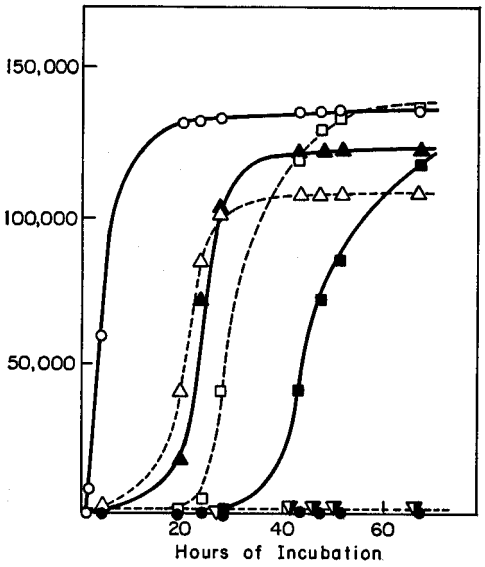

FIG. 10. Kinetics of gas release from labeled medium in the presence of soil. Samples of sandy soil were heated at 160°C for 10 min (open triangle, vertex up), 30 min (filled triangle, vertex up), 1 hr (open square), 2 hr (filled square), 3 hr (open triangle, vertex down), or 4 hr (filled triangle, vertex down) before testing for biological activity in the labeled medium. Gas released by unheated soil samples (open circle) and by medium in the absence of soil (filled circle) is also shown.

The experiment can be continued for a period of 15 days. If the response is negative, a fresh soil sample taken from a different site and/or after some interesting event, such as passage of the wave of darkening, will be added to the test chamber, along with a fresh supply of medium. Demonstration that the chamber can be reused after a negative response is given in Fig. 11. Here, radioactive medium was added to sterilized, local field soil. After 24 hr produced no response, a viable sample of the same soil and fresh medium were added. A classic growth curve resulted. A total of three experiments can be accommodated in the test chamber. Should a positive response be detected, a portion of the same sample, saved for this purpose in the sampling system, will be added to the second, or control, chamber.

This duplicate soil aliquot will be heated to kill the organisms. The labeled release experiment will then be performed on this control precisely as it was on the test. A total of three test and one control assays will be possible within the 90-day lifetime of the capsule. A nominal sequence will be programmed into the instrument, but override commands from Earth will be able to alter it. Data acquisition, telemetry, and Mission control procedures, however, will impose a delay of approximately two and one-half days between an event warranting an override command and enactment of the command by the instrument on Mars.

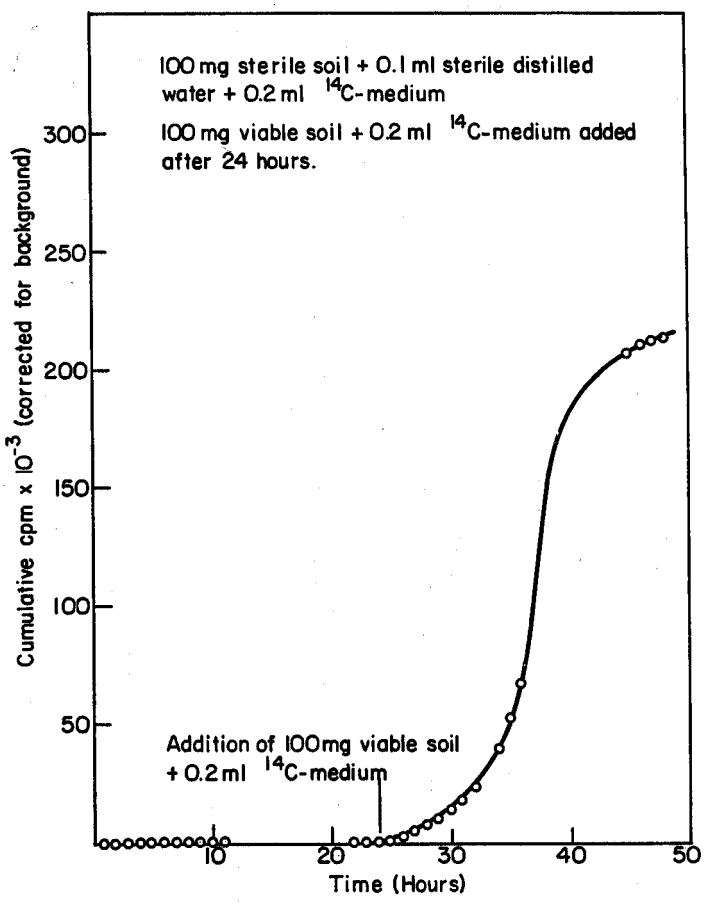

FIG. 11. Reuse of chamber for positive response following initial negative test.

The exploration of Mars is a highly complex mission and searching for life there is clearly the most difficult task assigned to Viking. The odds are long, but the unprecedented, potential reward warrants the effort. If the paths of evolution, science, and engineering intersect in July 1976, curves such as those shown in Fig. 12 (labeled release test and control data from Wyaconda soil degraded for anticipated error and background levels) may be received from Mars.

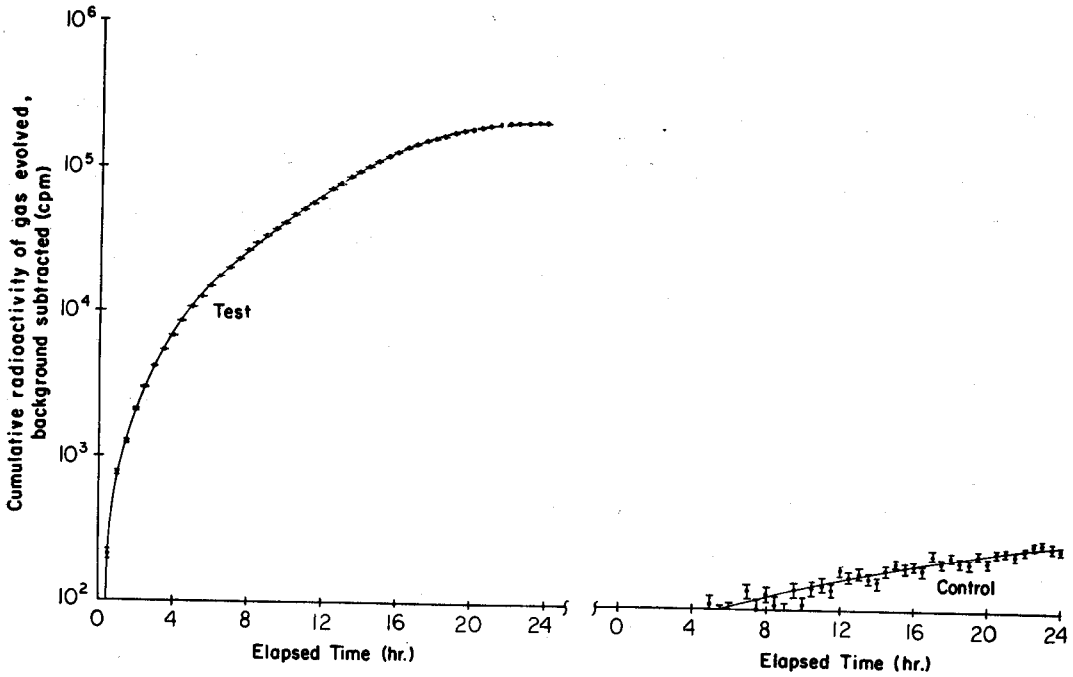

FIG. 12. Typical labeled release test results plotted to simulate Viking lander data. Active sample: 200 mg field soil from Wyaconda (Maryland). Control sample: 200 mg field soil from Rockville, Md., heat sterilized. Medium: RM9 (M9 + Tris). Labeled substrates: ^{14}C-D-glucose (u.l.), ^{14}C-l-glycine, ^{14}C-sodium formate, ^{14}C-DL-l-sodium lactate. Medium plus substrates volume: 0.2 ml. Radioactivity of medium plus substrates: 10 μCi/ml. Note: actual test data have been artificially degraded by randomizing process using an equivalent RTG background. Vertical bars show ± 1 standard deviation.

ACKNOWLEDGMENTS

The author wishes to thank the National Aeronautics and Space Administration for its support of the entire developmental program described herein. He also wishes to acknowledge the extensive contributions of his colleagues at Biospherics: Mr. William A. Lindgren, Mr. Donald G. Shaheen, Dr. Patricia Ann Straat, and Dr. J. Rudolph Schrot, along with the technical assistance of Mrs. Margaret Federline, Mrs. Margaret Schiffman, and Mrs. Susan Smith, and the help of earlier co-workers identified in the referenced works.

The contribution of Dr. Adrian Dahl, Martin Marietta Corporation, and Mr. Mac Chapman, TRW, Incorporated, in degrading the data shown in Fig. 12 to simulate its return from a labeled release experiment conducted on Mars is acknowledged.

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