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Labeled Release - An Experiment in Radiorespirometry

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Abstract. The Labeled Release extraterrestrial life detection experiment onboard the Viking spacecraft is described as it will be implemented on the surface of Mars in 1976. This experiment is designed to detect heterotrophic life by supplying a dilute solution of radioactive organic substrates to a sample of Martian soil and monitoring for evolution of radioactive gas. A significantly attenuated response by a heat-sterilized control sample of the same soil would confirm a positive metabolic response. Experimental assumptions as well as criteria for the selection of organic substrates are presented. The Labeled Release nutrient has been widely tested, is versatile in eliciting terrestrial metabolic responses, and is stable to heat sterilization and to the long-term storage required before its use on Mars. A testing program has been conducted with flight-like instruments to acquire science data relevant to the interpretation of the Mars experiment. Factors involved in the delineation of a positive result are presented and the significance of the possible results discussed.

1. Introduction

The announced goal for the NASA Viking Project is the exploration of Mars 'with special emphasis on the search for life' (Viking Project Office, undated pamphlet). The Viking Biology Instrument and its three life detection experiments (Levin, 1972; Horowitz *et al.,* 1972; Hubbard, 1976; Oyama, 1972; Oyama *et al.,* 1976) represent a direct attempt to detect Martian organisms and to begin to delineate the characteristics of any life found. The Labeled Release Experiment, which radiorespirometrically seeks heterotrophic metabolism, has undergone considerable development since its original conception in 1961 (Levin *et al.,* 1962). Since last described (Levin, 1972), the experiment has been refined to adapt it to flight hardware and to operational conditions. As the Viking spacecraft approach Mars, it seems timely to describe the experiment as it will be implemented on that planet beginning in the summer of 1976.

2. Experimental Technique, Sensitivity and Assumptions

Microbial radiorespirometry, a technique originally developed for the detection of specific organisms (Levin *et al.,* 1956; Levin, 1959), has evolved into the nonspecific microbial life detection test known as the Labeled Release experiment. In this experiment, a sample of Martian soil is provided to a culture chamber with Martian atmosphere in the headspace and is moistened with a dilute aqueous solution of selected ^{14}C-labeled organic substrates. The headspace is then periodically monitored for the evolution of radioactive gas as evidence for catabolism. The radioactive gas accumulates until a plateau is achieved, indicating exhaustion of the available substrate(s). Additional nutrient may then be applied and the kinetics of gas evolution again followed until a new plateau level is obtained. Upon completion of a test indicating a positive response, a control is conducted in which a duplicate portion of the same soil is heat-sterilized at 160 °C for three hours, cooled, and similarly tested in a fresh culture chamber. A significantly attenuated control response confirms the biological nature of the test result. The high sensitivity of the methodology to the presence of soil microorganisms is indicated in Figure 1 where as few as 50 colony-forming units are readily detected.

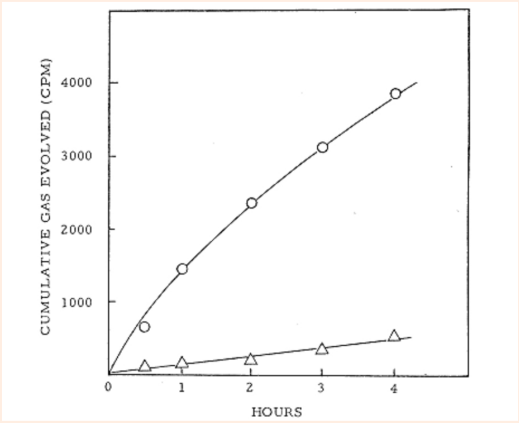

Figure 1. *Sensitivity of Labeled Release Technique*. To 0.5 grams of a soil from the Victoria Valley in the Antarctic was added 0.2 ml of a nutrient containing ^{14}C-labeled formate, glycine, glucose, and lactate (10μCi ml^{-1}) and ^{35}S-sulfate (10μCi ml^{-1}). The control soil was sterilized by heating for 30 min at 212 °C prior to nutrient addition. Radioactive gas evolved form each sample was then measured by the getter technique as a function of time. Separate experiments established that the soil contained 50 colony forming units.

The principal assumptions upon which all three Viking experiments are based are: (1) that microorganisms exist within the biotic system, (2) that metabolic rates are sufficient for detection during the elapsed time of the experiment, and (3) that the microorganisms are widely disseminated. Dissemination seems well justified (Hanel *et al.,* 1972; Straat *et al.,* 1974) in view of the high winds and associated dust storms (Leovy *et al.,* 1972) detected by Mariner 9. Specific assumptions for the Labeled Release Experiment are that life is carbon based, that one or more of the organic substrates selected for the heterotrophic experiment will be metabolized, and that one end product of metabolism will be a carbon-based gas. The experiment is conducted under predominantly moist conditions but provides a gradient from wet to 'dry' in the event that Mars organisms may be intolerant of large quantities of liquid water.

3. Labeled Release Nutrient

The organic substrates finally selected for the Labeled Release flight nutrient (VM1) are sodium formate, calcium glycolate, glycine, D-alanine, L-alanine, sodium D-lactate, and sodium L-lactate (Table I). Each substrate is present at 2 × 10^{-4} M concentration in unbuffered aqueous solution adjusted to pH 6-6.5. Substrate concentrations were kept low to avoid significant inhibition should one or more substrate be toxic. All carbon compounds are uniformly labeled at 2 μCi m^{-1} carbon and both optical isomers are offered for the enantiomorphic compounds.

TABLE I

Labeled Release nutrient (VM1)

Substrate	Structure and label position (*)	Concentration	μCi ML^{-1}*	Specific Activity (Ci/Mole)
^{14}C-glycine	NH_3·*CH_2·*COOH	2.5 × 10^{-4}M	4	16
^{14}C-DL-alanine	*CH_3·*CH(NH_3)·*COOH	5.0 × 10^{-4}M	12	48
^{14}C-sodium formate	H*COONa	2.5 × 10^{-4}M	2	8
^{14}C-DL-sodium lactate	*CH_3·*CHOH·*COONa	5.0 × 10^{-4}M	12	48

^{14}C-DL-sodium lactate	$CH_3{}^*CHOHCOONa$	3.0×10^{-4}M	4	16
^{14}C-calcium glycolate	$(^*CH_2OH{\cdot}^*COO)_2Ca$	2.5×10^{-4}M		

*Total=34 (6.8×10^{-7} dpm ml^{-1})

Several guidelines governed the selection of these substrates. On the assumption that life on Mars may have evolved similarly to that on Earth, substrates were selected from among those which may, therefore, have been produced on Mars through prebiotic processes. These compounds would, then, have been available for chemical evolution of more complex life precursors and, finally, living systems. Thus, formate, glycine, alanine, lactate and glycolate are all simple Miller-Urey products (Miller and Urey, 1959; Buvet and Ponnamperuma, 1971), readily produced in a reducing atmosphere. Studies with a nonreducing simulated Mars atmosphere (Hubbard *et al.,* 1971) have demonstrated that, when exposed to ultraviolet light, a gaseous mixture of carbon dioxide, carbon monoxide, and trace amounts of water vapor produces acetaldehyde, formaldehyde, and glycolic acid. Also, the infall of carbonaceous chondrites may have contributed to the organics available on the surface of Mars for possible incorporation into the evolutionary process. Amino acids identified in the Murchison meteorite include both glycine and alanine (Kvenvolden *et al.,* 1970).

Another consideration in substrate selection was involvement in metabolic pathways of primitive terrestrial organisms. If the origin of life on Mars proceeded as on Earth, then early terrestrial life forms may be indicative of those anticipated on Mars (Straat *et al.,* 1974). Such organisms might include anaerobic fermenters, respirers, and phototrophs. Especially interesting are *Clostridia,* sulfate reducers, methane bacteria, and photosynthetic bacteria. *Clostridia,* classified extremely primitive by their ferredoxin structure (Hall, 1971), are known to ferment a wide variety of organics including amino acids. The early existence of sulfate reducers, which can utilize lactate, has been implied by enriched $^{32}S/^{34}S$ ratios in early rocks relative to those of juvenile sulfur found in meteorites and volcanoes (Peck, 1966-67). The primitive methane bacterium, *Methanobacterium formicicum,* requires formate as the sole carbon source (Stanier *et al.,* 1963) whereas photobacteria can utilize a variety of simple organics. *Bacillus oligocarbophilus,* while not considered primitive, presents interesting speculation for Martian biochemistry. This microorganism derives energy through the oxidation of CO to CO_2, a pathway postulated for Martian organisms (Wolfgang, 1970; Postgage, 1970) especially if coupled with sulfate reduction (Stephenson, 1949). Organic substrates utilized by these organisms include formic acid.

Other special considerations were involved in the selection of substrates. Thus, a significant nonbiological response should not be obtained from a particular substrate when tested with a wide variety of soils. Acetate, originally favored by other criteria, was rejected for this reason. For compounds with optically active centers, racemic mixtures were desirable to preclude the possibility of missing the Martian preference. For racemic alanine, however, special consideration was given to the inclusion of D-alanine, a known inhibitor of spore germination in *Bacillus* and *Clostridia.* In the original observations of Hills (Hills, 1949), DL-alanine at molar ratios of 0.03 (D/L = 1/30) inhibited spore germination by 50%. However, later reports by a variety of investigators (O'Connor and Halvorson, 1961; Krask, 1961; Uehara and Frank, 1964) indicate that molar ratios in excess of one or even two are generally required for significant inhibition. Schmidt (1958) reports that the initial inhibitory effect of DL-alanine on *B. subtillis* sporulation is overcome with time. In addition, enzymes are usually absolute in their specificity for a particular optical isomer and are not actively inhibited by the antipode (Dixon and Webb, 1958). Further, D-alanine is a main constituent of bacterial cell walls (Newton, 1970), an example of one of the rare utilizations of D-amino acids in nature. Therefore, alanine was included as a racemic mixture in that D-alanine would probably not prevent the detection of extraterrestrial life by this highly sensitive substrate-limited reaction and should, in fact, enhance prospects for that detection.

Versatility in the detection of a response was also considered in substrate selection. Each substrate has been shown individually to elicit a rapid response from a wide variety of soils. In addition, the VM1 nutrient has undergone extensive testing with a wide variety of pure cultures and soil types. Because of anticipated conditions on Mars, considerable emphasis was placed on anaerobic testing in a specially constructed anaerobic cabinet (Levin *et al.,* 1964). Specific pure cultures tested with VM1 include algae, fungi, actinomycetes, aerobic bacteria, strict and facultative anaerobic bacteria, halophiles, heterotrophs and autotrophs including phototrophs and sulfur bacteria. Laboratory results with a wide variety of soil types are shown in Table II. In addition, *in situ* field tests have been conducted seasonally with local soil and in the dry valleys of the Antarctic in collaboration with Dr Wolf Vishniac (Straat *et al.,* 1976). In all cases, the Labeled Release technique produced a positive response with the possible exception of two of the Antarctic soils which may, in fact, be naturally sterile soils. Responses obtained with VM1 compare favorably with those obtained with earlier nutrients (Levin, 1972) which were used extensively *in situ* to test many soil types, including field, sandy, highly mineralized, and fluvial soils. These tests included such extreme environments as Death Valley sand dunes, Salten Sea flats, the Antarctic, and areas above the timberline on White Mountain, California.

TABLE II
Labeled Release responses with various soils

Soil	Soil type	pH	Hour	Active*	Sterile*	S/N
North of Phoenix (Arizona)	Clay	7.0	2	242 936	1 490	163.0
			22	356 809	4 919	72.5
			46	382 688	8 072	47.4
			78	390 311	9 586	40.7
Dunes West of Yuma (California)	Dry drifting sand	7.3	2	3 075	1 600	1.9
			22	184 521	5 435	34.0
			46	238 910	8 968	26.6
			78	264 030	11 715	22.5
Red Rock (Arizona)	Red clay	7.8	2	39 769	1 645	24.2
			22	178 104	7 002	25.4
			46	229 277	11 132	20.6
			78	249 399	13 986	17.9
4 Mi. West of Yuma (Arizona)	Dry wash sediment	7.5	2	90 084	1 429	63.0
			22	353 064	6 674	53.9
			46	388 591	11 631	33.4
			78	401 177	15 879	25.3
El Centro (California)	Irrigation farmland	8.1	2	5 890	1 158	5.1
			22	170 905	3 596	47.5
			46	215 317	5 542	38.9
			78	233 593	8 153	28.7
Montezuma Well (Arizona)	White lime-stone clay	7.8	2	24 365	911	26.8
			22	181 790	3 129	58.1
			46	222 242	4 252	52.3
			78	236 259	5 422	43.6
Imperial (California)	Hydroscopic irrigation	6.9	2	872	1 004	0.9
			22	2 054	2 415	0.9
			46	39 211	4 516	8.7
			78	140 506	7 130	19.7
Sandy (Maryland)	Creek bed	7.5	2	1 781	516	3.5
			22	131 599	2 064	63.8
			46	173 321	3 723	46.6
			78	180 988	5 463	33.1
Woody	Humus	6.0	2	7 152	627	11.4
			5 1/2	27 684	1 292	21.4

(Maryland)	Humus	6.0	25	98 049	2 590	37.9
			56	207 011	15 312	13.5
Aiken	Clay	5.9	1	7 444	322	23.1
			5 1/2	28 525	1 297	22.0
			25	62 415	4 124	15.1
			46	103 470	6 771	15.3
Sterling Park (Virginia)	Clay	6.5	1	9 039	385	23.5
			5 1/2	45 288	1 312	34.5
			25	276 108	4 817	57.3
			46	308 174	7 737	39.8
Creek Bed (Maryland)	Sandy	7.4	1	667	152	4.4
			5 1/2	3 903	737	5.3
			25	203 878	2 230	91.4
			46	270 755	3 918	69.1
Thermal California (California)	Sandy	7.7	1	20 422	260	78.6
			5 1/2	49 920	758	65.9
			25	219 135	3 683	59.5
			56	309 395	6 370	48.6
Death Valley (E. Calif. and S. Nevada)	Sandy	8.3	1	14 413	344	41.9
			5 1/2	47 818	1 598	29.9
			25	103 103	4 203	24.5
			46	175 049	5 986	29.2
Pine Tree (Maryland)	Humus	4.2	1	53 190	698	76.2
			6	178 079	3 614	49.3
			25	311 565	10 651	29.3
			46	338 963	16 360	20.7

*Average cumulative gas evolved (CPM)

Active and heat sterilized (30 min at 200 °C) samples of 0.5 cc of each soil were assayed in duplicate for radioactivity evolved after the addition of 0.125 ml of VM1 - (getter technique).

A severe constraint in the selection of substrates was stability. Scheduling and flight requirements imposed a delay of approximately two years between nutrient preparation and use on Mars. During the interim, the glass ampoules containing the nutrient were loaded into the Biology Instruments which was sterilized for 54 h at 120 °C. Prior to launch, the entire lander was sterilized for 20 h at 110 °C, exclusive of approximately 45 h of temperature ramp-up and ramp-down time. Because of these temperature constraints, glucose, used in earlier work (Levin, 1972), was eliminated and glycolate substituted. To enhance stability during heating, the inorganic medium previously utilized (Levin, 1972) was also eliminated and distilled water chosen as solute. Separate experiments indicated little effect on biological responses in the absence of supplied organics, probably because essential nutrients were leached from the soil particles by the thin film of water provided. Each selected substrate was individually placed in distilled water and tested anaerobically for stability to heat sterilization.

Once defined, the nutrient was prepared under stringent quality control (Straat, 1972a, 1972b) which ensured 99% chemical and radio-chemical purity and specific activities within 10% of the desired value for each compound. An initial preparation was used for a 30-month stability test which incorporated a heat regime consisting of two cycles at 113 °C, one 40 h and the other 54 h long, each separated by 18 months to correspond to the flight regime anticipated at the time. The results established the stability of the nutrient as adequate for the extent of the mission (Straat, 1975). The most significant long-term effect was the gradual accumulation of $^{14}CO_2$ in the nutrient, resulting from beta-induced generation of free hydroxyl molecules followed by substrate decarboxylation. However, this decomposition is less than 1% and the accumulated $^{14}CO_2$ is removed by flushing with helium shortly after landing on Mars, thereby reducing it to a level which prevents significant interference with the sensitive response of the experiment.

4. The Instrument

The Viking Biology Instrument, described elsewhere (Klein, 1974, 1976; Klein *et al.,* 1976) consists of four modules, one for each of the three Viking life detection experiments and a fourth containing common support services. The Labeled Release module consists of four incubation test cells mounted in a carrousel. Each cell can be moved and sealed beneath a head end assembly containing a heater and plumbing terminals for nutrient delivery and for gas removal and delivery. Eight miniaturized solenoid valves operate the plumbing system. The headspace in the sealed test cell is connected to two solid state beta detectors, also equipped with a heater, through a 13-in long, 0.105 in i.d. tube which is bent at several points, to prevent contamination of the detector with radioactive particulates from the test cell. The total volume of this assembly is approximately 8.5 cc and gas equilibrium between the test cell and the detectors is established within thirty minutes. All materials which contact soil or nutrient were tested for toxicity and chemical compatibility prior to selection.

A schematic of the Labeled Release Module is shown in Figure 2. The nutrient is contained in a sealed glass ampoule within a reservoir. Early in the experimental sequence after landing, the ampoule is broken by a mechanical striker driven under the force of high pressure helium (135 psi) from the common support module. Low pressure helium (18 psi) is then bubbled through the nutrient for five hours via valves S/61, S/59 and S/47 in a nutrient degassing operation. To deliver VM1 to a test cell, high pressure helium is used to drive the nutrient into the metered volume contained within the plumbing bounded by valves S/59, S/61, S/44, and S/45. This VM1 volume is then released through S/45 to the sealed test cell containing the soil sample under Martian atmosphere plus a slight overpressure of helium to prevent violent boiling of the added nutrient. The total pressure at the onset of the experiment is 69 torr including the vapor pressure of water. Following injection, the radioactive gas evolved is counted at four-minute intervals for the first two hours and at 16-minute intervals thereafter. At the end of the experiment, the radioactive gas is removed by purging and the soil is dried by brief heating to prevent explosive evaporation upon opening the cell. A fresh test cell is then rotated beneath the head end assembly and a three-hour cleanup is accomplished by heating the head end and detectors. After cooling, nutrient trapped, and thus heated in the metered volume between S/59, S/61, S/44, and S/45, is wasted through S/44. The system is then ready to accept another soil sample into a clean test cell. For a control cycle, the soil sample is heated for three hours at 160 °C in the test cell prior to the addition of nutrient.

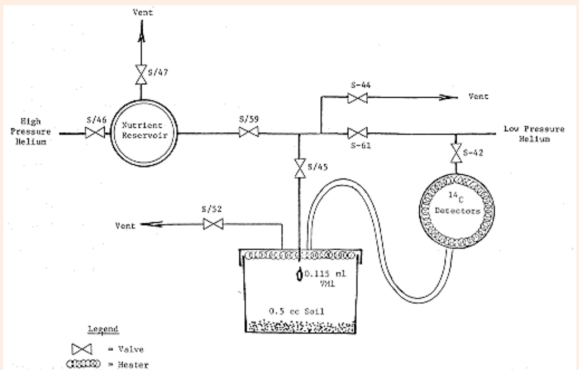

Fig. 2. *Schematic of the Labeled Release Experiment.*

All consumables (helium, nutrient, electrical power) are sufficient to support four complete soil cycles, each with two injections of nutrient during the cycle. The experiment is conducted in ambient Martian atmosphere modified by the overpressure of helium as discussed above. The internal test cell temperature undergoes a diurnal cycle governed by the Martian Sol (approximately 24 h and 40 min). However, because of the necessity to maintain the instrument above freezing, the test cell temperature will not be allowed to fall below 9-10 °C. During the warmest part of the Martian Sol, the test cell could rise as high as 27 °C, a temperature near the upper limit of that attained on the surface of Mars.

Significant events for the Labeled Release experiment are ampoule breakage and degassing three Sols after landing, initial soil delivery on Sol 8, nutrient injection on Sol 10, commandable nutrient injection on Sol 17, and termination and clean-up initiating on Sol 19. The second soil cycle is scheduled to begin on Sol 27 with nutrient injection on Sol 29. Program variables include the ability to perform commandable injection, selection of test or control sequences, selection of fresh soil sample, and the choice of initiating or terminating an individual sequence. Sequences have also been developed to permit single channel counting as a contingency against failure of one detector channel. However, all decisions must be made at least four days in advance of their execution on Mars because of time restrictions governing the communication of commands to the spacecraft.

5. The Science Program

Active research in adapting the radiorespirometry technique to the detection of extraterrestrial life began in 1961 and has been continuously maintained (Levin, 1963, 1966, 1968, 1972; Levin *et al.,* 1962, 1970; Levin and Carriker, 1962; Levin and Perez, 1967). Since 1971, this effort has been largely directed toward modification of the experiment for Viking hardware compatibility, providing assistance to the NASA contractors in hardware design and materials selection, verifying the capabilities of the Labeled Release Module, designing experimental flight sequences and strategies, and acquiring relevant test data necessary for interpretation of flight data. Part of the latter effort was expended on improving the scientific aspects of the experiment and on obtaining data with a 'Test Standards Module' (TSM). The TSM (Figure 3) instrument is flight-like in configuration with respect to test cells, head end assembly, detector assembly, and nutrient reservoir with these subsystems arranged similarly to those in the flight package. These components are contained under a bell jar in which a simulated Martian atmosphere of carbon dioxide at a pressure of 5 torr is established. As in the flight instrument, both high and low pressure helium sources are available. The TSM heaters and flight-type solonoid valves may be manually operated to perform an entire experimental sequence. In addition, the incubation test cell is equipped with a temperature cycler which can provide a diurnal temperature regime closely matching that anticipated during mission operations.

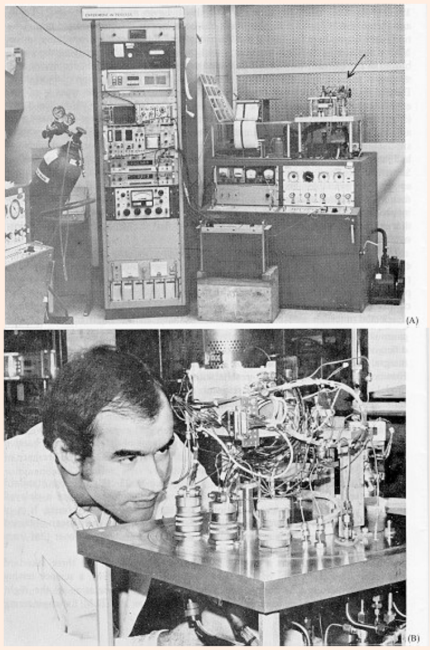

Fig. 3. *Labeled Release Test Standards Module*. The Test Standards Module (A) contains Labeled Release test cells, detectors, nutrient reservoir, valves, and heaters which are essentially identical to flight components. The instrument may be operated by manual manipulation of the valves and heaters to perform an entire flight sequence. The location of the flight components is indicated by arrow. These components, enlarged in (B), are covered during an experiment with a bell jar which can be evacuated to contain a Martian atmosphere. The temperature of the test cell may be regulated to obtain isothermal or diurnal temperature patterns, as desired.

The TSM has been an invaluable aid in bridging the gap from the laboratory equipment to the flight instrument. Most early experimental data (Levin, 1972) relied upon a 'getter' technique whereby evolved $^{14}CO_2$ was collected on a getter pad moistened with a solution of saturated barium hydroxide and placed over the culture chamber containing soil and radioactive nutrient. These pads were replaced at selected intervals and counted for radioactivity. In the flight instrument, the getter system has been replaced with a detector assembly which continuously counts gaseous radioactivity as it is evolved. Because of these and configurational differences, the possibility existed that the kinetics of gas evolution, considered an integral part of the Labeled Release experiment, might differ in the two systems. However, comparison of getter results with those obtained from an earlier TSM model shows similar kinetics and response magnitudes under terrestrial conditions for each of three standard Viking test soils selected by the NASA Viking Project Office (Figure 4). Thus, it was demonstrated that past getter experience could be used in the development and interpretation of the flight experiment. However, the TSM and flight instruments have the added advantages of detection of radioactive carbon-based gases other than $^{14}CO_2$ and the detection of $^{14}CO_2$ in the presence of quantities of $^{12}CO_2$ that might saturate the getter.

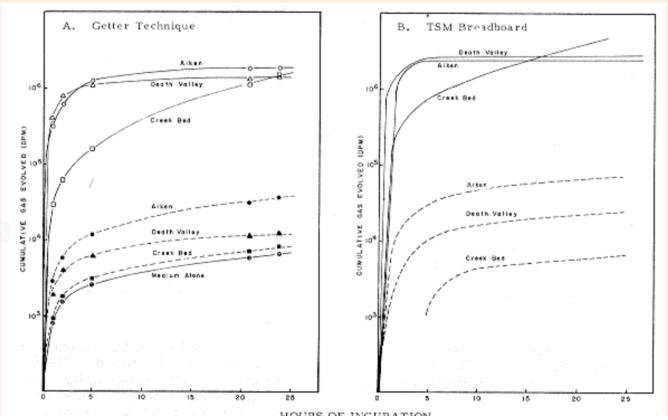

Fig. 4. *Comparison of Labeled Release getter data with TSM data*. Viable (———) and heat sterilized (------) samples of the three standard soils, as indicated, were examined for labeled release activity by the getter technique (A) or by utilizing an early model of the TSM (B). Each assay was conducted with 0.5 cc of soil under terrestrial conditions. Most soils received 0.125 ml of a nutrient containing ^{14}C formate, glycine, glucose, and lactate (10 μCi ml^{-1} and ^{35}S-sulfate (10 μCi ml^{-1}). For active Creek Bed soil in the TSM Breadboard, however, 0.25 ml nutrient was used. Data was converted to DPMs using a predetermined counting efficiency of 3% for the TSM and 10% for the getter technique.

When subjected in the TSM to Mars atmosphere at 23 °C, the three standard test soils with VM1 yield the kinetics shown in Figure 5. As shown, a delayed response is obtained with gas evolution occurring in two distinct phases. It is of considerable interest that even under these Martian conditions, the plateau obtained from each terrestrial soil is approximately two orders of magnitude over that from the corresponding heat sterilized control.

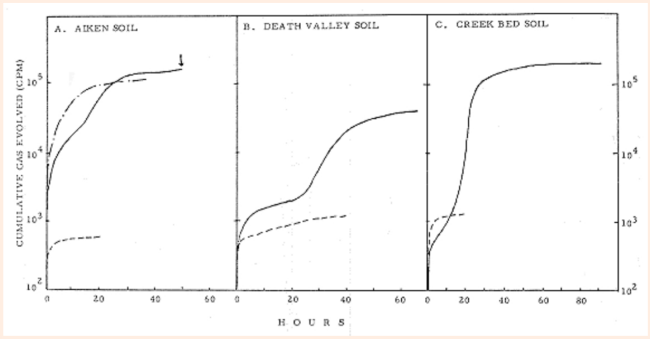

Fig. 5. *Activity from Three Standard Soils Under Martian Atmosphere in TSM*. A 0.5 cc sample of Aiken, Death Valley, or Creek Bed soil, as indicated, was added to the TSM and subjected to Martian atmospheric conditions. Radioactivity evolved after the addition of 0.18 ml VM1 to each active (———) and control (------) soil is shown as a function of time. For active Aiken, a second commandable injection (—— · ——) was performed at the indicated position. The kinetics for the commandable injection are shown after subtraction of the plateau from the first injection. Control samples of each soil were heat sterilized in the TSM at 160 °C for three hours.

Having established that a response could be elicited from the three standard test soils under the harsh Martian conditions used in the TSM, a science testing program was evolved to obtain data relevant to the interpretation of the flight experiment. However, because of program demands on the TSM for engineering purposes, not as much science data has been collected as had been planned. Early phases of this program were aimed at nutrient studies and at responses elicited from active soils. Active soil experiments were mainly concerned with the establishment of proper operation of the flight hardware and the development of flight sequences. As a result of many TSM tests, the necessity for design changes in the flight hardware and sequences became apparent. Additional experiments provided support data for tests conducted with flight prototype modules. Later phases of the science testing program were directed at sterile soils and at the development of criteria to distinguish a positive response. Throughout the program, the impact of potential malfunctions due to faulty nutrient degassing or delivery, failure to deliver soil, noisy detectors, or test cell gas leakage has also been examined. Because an entire flight sequence requires a minimum of two weeks to execute, experiments had to be carefully selected and many were of necessity abbreviated, being stopped as soon as a plateau was obtained.

Since the plateau level attained by a particular soil is directly proportional to the amount of nutrient injected, experiments with the VM1 nutrient were conducted early to determine the volume injected and the reproducibility of that injection. Injections made into liquid scintillation vials established that volume at 0.115 cc ± 8%. Additional nutrient tests using a glass test cell established the overpressure required to prevent explosive boiling upon injection of the liquid nutrient into the test cell. Other experiments determined the number of 'bleeds' necessary to remove heat damaged nutrient from the metered injection cavity following head end heating. The degassing procedure was established, tested, and shown to be sufficient to remove most of the nonbiological $^{14}CO_2$ gas accumulated during the two-year storage prior to the experiments on Mars. However, that not all the gas is completely removed by the procedure is evidenced by the small outgassing seen with sterilized soils (Figures 4, 5).

In conjunction with providing support data for flight instrument tests, TSM temperature studies have yielded relevant science data showing the effects of isothermal temperature on responses from Creek Bed (Figure 6) and from Aiken (Figure 7) soils. As seen, both active soils respond similarly although the temperature effect is far more pronounced with Creek Bed soil. In each case, decreased temperature causes a delay in the onset of the second phase of gas evolution although ultimately the same plateau level is attained regardless of temperature. The 10 °C regime, in fact, represents that anticipated in the test cell for the 'Cold Case' environmental model of the landing site. Figure 8 shows the effect of diurnal temperature cycling typical of a 'Hot Case' landing site in which the test cell temperature fluctuated between 10 °C and 27 °C. As shown, the Aiken active response resembles that obtained at 10 °C although pronounced increases in evolved gas correlate with diurnal temperature rises. Data obtained for the Warm Case, in which the test cell temperature fluctuated between 10 °C and 18 °C, are intermediate in response. Later isothermal studies at 10°, 15°, and 20 °C under Mars atmospheric conditions showed no significant temperature effect on either the kinetics or the magnitude (approximately 500 cpm) of the sterilized Aiken soil response.

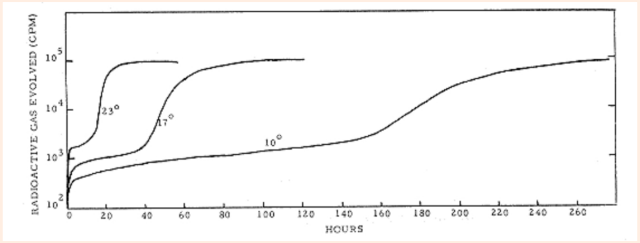

Fig. 6. *Effect of Temperature on Labeled Release from Creek Bed Soil*. Labeled Release data for isothermal tests are shown for active Creek Bed soil at 10°, 17°, and 23 °C, as indicated. For each run, 0.5 cc soil was placed in the TSM test cell and equilibrated under Martian atmospheric conditions for two hours at the desired temperature before the addition of 0.115 ml VM1 nutrient. The resulting radioactive gas evolved was monitored continuously as a function of time.

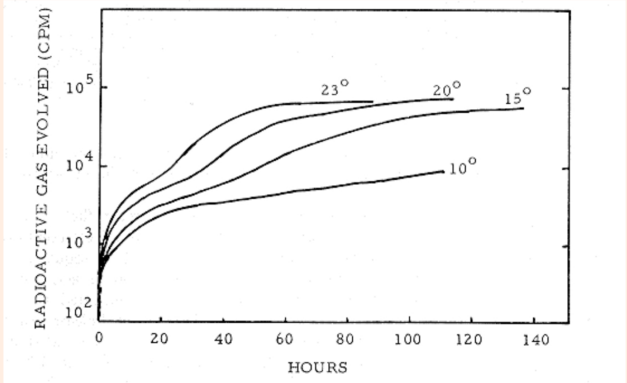

Fig. 7. *Effect of Temperature on Labeled Release from Aiken Soil*. Labeled Release data for isothermal tests are shown for active Aiken soil at 10 °C, 15 °C, 20 °C, and 23 °C, as

indicated. For each run, 0.5 cc soil was placed in the TSM test cell and equilibrated under Martian atmospheric conditions for two hours at the desired temperature before the addition of 0.115 ml VM1 nutrient. The resulting radioactive gas evolved was monitored continuously as a function of time.

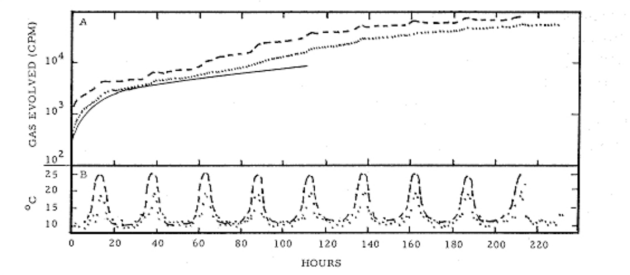

Fig. 8. *Effect of Diurnal Temperature Cycling on Labeled Release from Aiken Soil.* (A) Active Aiken soil Labeled Release data obtained with Hot Case (— — —) and with Warm Case (· · · · · ·) temperature patterns are compared to those obtained at constant 10 °C test cell temperature (———). For each run, 0.5 cc soil was placed in the TSM test cell and equilibrated under Martian atmospheric conditions for two hours at the desired temperature before the addition of 0.115 ml VM1 nutrient. Injection in the diurnal tests occurred at 10 °C, at the same point in the cycle programmed for flight. The resulting evolved radioactive gas was monitored continuously as a function of time. (B) Temperature (°C) at the bottom of the test cell as recorded during Hot Case (— —) and Warm Case (· · · · · ·) tests.

Figure 9 represents data obtained from a Labeled Release flight module contained within a Viking Biology Instrument (Flight S/N 103). Test conditions were Warm Case diurnal under Mars atmosphere. An entire flight sequence was performed from ampoule breakage and nutrient degassing through an active Aiken cycle followed by a control Aiken cycle. Temperature data indicated that the Warm Case condition will maintain temperatures in the Labeled Release test cell between 10 and 14.5 °C, somewhat below those previously anticipated and studied in the TSM. However, when corrected for temperature differences, the S/N 103 results match the TSM data in both kinetics and magnitude of response. The data from active Aiken were intermediate between a 10 °C isothermal curve (Figure 7) and a Warm Case curve ranging from 10° to 18 °C (Figure 8). Little or no effect of diurnal cycling was observed on the sterile response. The data ensure that the flight instrument is capable of conducting a Labeled Release experiment and that the TSM data are relevant for interpretation of flight data. Apparent 'notches' in the gas evolution (Figure 9) reflect single channel counting, which, during the mission, will be used only in the event of a faulty detector channel.

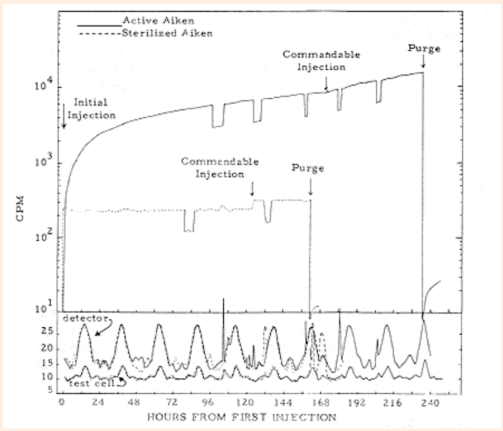

Fig. 9. *Labeled Release activity with Aiken Soil in a Flight Instrument.* Labeled Release data with Aiken soil was obtained from an LR module contained within a flight instrument (S/N 103). A complete flight sequence was used consisting of ampoule breakage, nutrient degassing, active Aiken cycle, and sterilized (three hours at 160 °C in the test cell) Aiken cycle. Each soil sample (0.5 cc) was subjected to Martian atmospheric conditions for approximately 58 hours prior to the addition of 0.115 ml VM1. The temperature regime of each test was Warm Case Diurnal. Evolved radioactivity, head end temperature of the test cell, and detector temperature were monitored as a function of time. For each test, commandable injection occurred as indicated. 'Notches' in the evolved counts result from brief periods in which only one detector channel was used for counting as a system check.

Other TSM experiments have determined variability of sterile replicates and the anticipated magnitude of the sterile response. In a series of four sequential experiments with sterilized Aiken soil, the response plateau from the first run was higher than those obtained from subsequent runs (Table III). This phenomenon has been observed on several occasions and may result from additional nutrient degassing during heat cleanup between the first and second run. Additional experiments have examined the plateau magnitude of sterilized soils as a function of soil pH (Table IV). As anticipated from the $C0_2$-carbonate equilibrium, the plateau increased from 150 cpm to 660 cpm as the soil pH decreased from 8.3 to 4.6. The concern in each case is prevention of a false positive result. However, the responses are low level compared to any positive responses yet encountered from terrestrial organisms. Nonetheless, these results will be factored into significance levels established for data obtained on Mars. Additional experiments with Antarctic soils of low microbial population, as well as with sterilized soils of varying inorganic content, including lunar soil, will be conducted prior to touchdown to continue building a 'reference library' for use in interpreting the Labeled Release data returned from Mars.

TABLE III

Series of sterile aiken soil runs performed under warm case diurnal conditions (each separated by heat clean-up)

Order performed	First injection*	Commandable injection*
1	550	330
2	275	225
3	320	-
4	325	-

*Gas evolved at plateau (CPM)

(TSM Data, Martian conditions)

TABLE IV

Effect of soil pH on plateau of control soil

Soil	Soil pH	Gas evolved at plateau (CPM)

Death Valley	8.3	150
Creek Bed	7.6	530
Aiken	6.0	545
Aiken	6.0	415
Pine Tree	4.6	660

(TSM Data, Martian conditions)

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6. Prospects for Life on Mars, Its Detection by the Labeled Release Experiment, and Significance of Results

Prospects for life on Mars revolve principally about two central and related issues. The first is whether the theory of evolution can be extended into a general law of biology under which - given circumstances, environmental and elemental distributions similar to those on Earth - other planets must necessarily generate living systems. Second, does Mars now have, or has it had, sufficient liquid water to sustain the development and continuance of life? Available information indicates that, in all other respects, with the possible exception of available nitrogen, Mars could sustain many types of terrestrial organisms.

Water vapor, seasonally approaching saturation levels, has been detected over large regions at levels of 20-30 precipitable microns (Conrath *et al.,* 1973) and speculation exists that liquid water may be transitorily available on the planet's surface. Adaptations to extreme aridity are known to occur on Earth in the cold, dry, saline deserts of Antarctica where the moisture content is 1.4%, or less, by weight. Terrestrial microbes present rapidly multiply upon the artificial addition of liquid water (Cameron and Mereck, 1971) although *in situ* reproduction has been questioned (Horowitz *et al.,* 1972). Certain bacteria and invertebrates survive extreme drying for as long as 40 y in a state of 'cryptobiosis,' reviving upon the addition of water (Crowe and Cooper, 1971). Martian organisms may similarly 'hibernate' during dry seasons, or eras, and flourish during wet periods (Straat and Levin, 1971; Sagan *et al.,* 1973).

'Water pumps', such as proposed by Robinson (1954, 1956) could accomplish an active transport of water for internal concentration by Martian organisms. Alternatively, active transport of water could occur by the 'protein flexure' theory (Goldacre, 1952) in which protein configuration operates as a pump to regulate cellular water content. A recent report (Ling and Walton, 1976) proposes that ATP absorbed on specific protein sites is the factor which, in turn, controls this protein configuration. Further, the data show that the cellular water exists in a physical state different from that of a dilute aqueous solution. In this newly described state, it is retained against external forces, even in the absence of intact membranes. Based on these theories, one might postulate that Martian organisms internally 'fix' atmospheric water vapor to provide the form and concentration required for metabolic reactions.

The nominal mission will direct two Viking landers to widely different regions where each Labeled Release experiment will perform three soil cycles, each with a duration of 12 days. Planning is presently underway by NASA to extend the Viking Mission thereby permitting incubations to continue for several months and to encompass different seasons. Few environmental insults are imposed by the Labeled Release experiment on the Martian sample. These are limited to a small overpressure of helium and prevention of freezing during the cold part of the diurnal temperature cycle. In addition, although liquid water is present in a wet to 'dry' gradient, water vapor exists in the test cell at greater than ambient. The sensitivity of the instrument, the basis for labeled substrate selection, and the extensive, successful experience with terrestrial organisms strongly indicate a good potential for the Labeled Release Experiment to detect Martian microorganisms should they be present.

Because of the impact of a positive result, the conclusion requires a high level of certainty. The kinetics of gas evolution, the absolute magnitude of the response at plateau, and a comparison of the test and control results must all be evaluated in detail. Despite the limitations imposed on the TSM program, considerable data relevant to each issue have been accumulated. Most important has been the compilation of a 'reference library' of sterile soil kinetics and response magnitudes. Factors influencing variations in this response have also been cataloged and will be used to evaluate the significance of differences between active and control cycles. Such factors include variability in the amount of nutrient and soil delivered, possible effects of heat sterilization, temperature differences between active and control cycles, changes with time in the nonbiological gas level of the nutrient, counting statistics, and effects of electronic or mechanical malfunctions. By appropriate application of TSM data, it will be possible to construct error bars around each response. In addition, the wide experience with terrestrial microorganisms may offer useful comparisons. Most important, however, will be the sterilized control data which are essential to avoid a false positive interpretation. Present criteria delineate a positive response by a three sigma level difference between test and control.

The Viking life detection mission constitutes the first direct field test of what some call the General Theory of Biology. Results from all three life detection experiments will be supported by the determination of surface organic material (Anderson *et al.,* 1972). The scientific significance of a positive result is obvious. Biologists, and the world at large, would have two planets upon which to base evolutionary theories of the universe. However, a negative result from all four experiments would also be highly significant. Should no evidence be found either of past or present life forms, or of organic molecules of sufficient complexity to indicate some degree of prebiotic evolution, a significant reappraisal of the biological theory will be indicated.

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References

Anderson. D. M., Biemann, K., Orgel, L. E., Oro, J., Owen, T., Schulman, G. P., Toulmin, P., III, and Urey, H. C.: 1971, *Icarus* **16,** 111.

Buvet, R. and Ponnamperuma, C. (eds.): 1971, *Chemical Evolution and the Origin of Life,* NorthHolland Publishing Co., Amsterdam.

Cameron, R. E. and Merek, E. L.: 1971, Technical Report 32-1522, National Aeronautics and Space Administration.

Conrath, B., Curran, R., Hanel R., Kunde, V., Maguire, W., Pearl, J., Pirraglia, J., and Welker, J.: 1973, *J. Geophys. Res.* **78,** 4267.

Crowe, J. H. and Cooper, A. F.: 1971, *Sci.* Amer. **225** (6), 30.

Dixon, M. and Webb, E. C.: 1958, *Enzymes,* Academic Press, New York.

Goldacre, R.: 1952, *Internat. Rev. Cyt. **1,*** 135.

Hall, D. O., Cammack, R., and Rao, K. K.: 1971, *Nature* **233,** 136.

Hanel, R., Conrath, B., Hovis, W., Kunde, V., Lowman, P., Maguire, W., Pearl, J., Pirraglia, J., Prabhakara, C., Schlachman, B., Levin, G., Straat, P., and Burke, T.: 1972, *Icarus* **17,** 423.

Hills, G. M.: 11949, *Biochem. J.* **45,** 363.

Horowitz, N. H., Cameron, R. E., and Hubbard, J. S.: 1972, *Science* **176,** 242.

Horowitz, N. H., Hubbard, J. S., and Hobby, G. L.: 1972, *Icarus* **16,** 147.

Hubbard, J. S.: 1976, this issue, p. 281.

Hubbard, J. S., Hardy, J. P., and Horowitz, N. H.: 1971, *Proc. Nat. Acad. Sci., U.S.* **68,** 574.

Klein, H. P.: 1974, *Origins of Life* **5,** 43 1.

Klein, H. P.: 1976, this issue, p. 273.

Klein, H. P., Lederberg, J., Rich, A., Horowitz, N. H., Oyama, V. I. and Levin, G. V.: 1976, *Nature,* in press.

Krask, B. J.: 1961, in Halvorson, H. O. (ed.), *Spores II*, Burgess Publ. Co., Minnesota, p. 89.

Kvenvoiden, K., Lawless, J., Pering, K., Peterson, E., Flores, J., Ponnamperuma, C., Kaplan, I. R., and Moore, C.: 1970, *Nature* **228,** 923.

Leovy, C. B., Briggs, G. A., Young, A. T., Smith, B. A., Pollack, J. B., Shipley, E. N., and Wildey, R. L.: 1972, *Icarus* **17**, 373.

Levin, G. V.: 1959, *Method of Making Bacteriological Determinations,* U.S. Patent No. 2,914,447.

Levin, G. V.: 1963, *Adv. App. Microbiol.* **5,** Academic Press, Inc., New York, p. 95.

Levin, G. V.: 1966, in Dempsey, J. C. and Polishuk, P. (eds.), *Radioisotopes for Aerospace, Part II: Systems and Applications*, Plenum Press, New York, p. 347.

Levin, G. V.: 1968, *Adv. App. Microbiol.* **10**, Academic Press, Inc., New York, p. 55.

Levin, G. V.: 1972, *Icarus* **16,** *153.*

Levin, G. V. and Carriker, A. W.: 1962, *Nucleonics* **20,** 7 1.

Levin, G. V. and Perez, G. R.: 1967, *Search for Extraterrestrial Life* **22,** Advances in Astronautical Science Series, American Astronautical Society, Publications Office, Tarzana, California.

Levin, G. V., Harrison, V. R., and Hess, W. C.: 1956, *J. Am. Water Works Assoc.* **48,** 75.

Levin, G. V., Heim, A. H., Clendenning, J. R., and Thompson, M. F.: 1962, *Science* **138,** 114.

Levin, G. V., Horowitz, N. H., Heim, A. H., and Thompson, M. J.: 1964, *Radioisotopic Biochemical Probe for Extraterrestrial Life,* Third Annual Progress Report, Contract No. NASr-10, Resources Research, Inc., Washington.

Levin, G. V., Shaheen, D. G., Lindgren, W. A., and Rich, E.: 1970, *The Detection of Metabolism,* Presented at Intern. Congr. Microbiology., Mexico City, August 1970.

Ling, R. N. and Walton, C. L.: 1976, *Science* **191,** 293.

Miller, S. L. and Urey, H. C.: *1959, Science **130**,245.*

Newton, J. W.: 1970, *Nature* **228**, 1100.

O'Connor, R. J. and Halvorson, H. O.: 1961, *J. Bacteriology* **82,** 706.

Oyama, V. I.: 1972, *Icarus* **16,** 167.

Oyama, V. I., Berdahl, B. J., Carle, G. C., Lehwalt, M. E., and Ginoza, H. S.: 1976, this issue, p. 313.

Peck, H. D., Jr.: 1966-67, *Some Evolutionary Aspects of Inorganic Sulfur Metabolism,* Lecture on Theoretical and Applied Aspects of Modern Microbiology, University of Maryland.

Postgate, J.: 1970, *Nature* **226,** 978.

Robinson, J. R.: 1954, *Symp. Soc. Exp. Biol.* VIII, Academic Press, New York, p. 42.

Robinson, J. R.: 1956, *J. Physiol.* **134,** 1.

Sagan, C., Toon, O. B., and Gierasch, P. J.: 1973, *Science* **181,** 1045.

Schmidt, C. F.: 1958, in Halvorson, H. O. *(ed.), Spores,* Amer. Inst. Biol. Sci., Washington, p. 56.

Stanier, R. Y., Doudoroff, M., and Adelberg, E. A.: 1963, *The Microbial World,* Second Edition, Prentice-Hall Inc., Englewood Cliffs.

Stephenson, M.: 1949, *Bacterial Metabolism,* Third Edition, Longmans, Green and Co., New York.

Straat, P. A.: 1972, *Methods Manual for VM1 Preparation,* Contract No. GD2-010009 (Martin-Marietta), Biospherics, Inc., Rockville, Maryland.

Straat, P. A.: 1972, *VM1 Preparation Manual,* Contract No. GD2-010009 (Martin-Marietta), Biospherics, Inc., Rockville, Maryland.

Straat, P. A.: 1975, *L. R. Nutrient Stability Testing,* Final Report, Contract No. GD2-01009 (Martin-Marietta), Biospherics, Inc., Rockville, Maryland.

Straat, P. A. and Levin, G. V.: 1971, *IRIS Biological Experiments,* November Progress Report, Contract No. NAS5-11294, Biospherics Inc., Rockville, Maryland.

Straat, P. A., Levin, G. V., and Lowman, P. D., Jr.: 1974, *Mariner 9: Prelude to First Field Test of a General Theory of Biology?,* X-922-74-310, Goddard Space Flight Center, Greenbelt, Maryland.

Straat, P. A., Levin, G. V., and Vishniac, W.: 1976, manuscript in preparation.

Uehara, M. and Frank, H. A.: 1964, in Campbell, L. L. and Halvorson, H. O. (eds.), *Spores III,* Burgess Publishing Co., Minnesota, p. 38.

Viking Project Mission to Mars: undated, Viking Project Office, NASA Langley Research Center, Hampton, Virginia.

Wolfgang, R.: 1970, *Nature* **225,** 876.
