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The Case for Extant Life on Mars and its Possible Detection by the Viking Labeled Release Experiment

Gilbert V. Levin[1]

and

Patricia Ann Straat[2]

[1]Arizona State University, Tempe, Arizona

[2]National Institutes of Health (Retired)

Both authors were affiliated with Biospherics Incorporated, Rockville, Maryland, throughout the development and execution of the Viking Labeled Release Experiment.

Address Correspondence to: Gilbert V. Levin

10709 Blossom Dr., Goodyear, AZ 85338, 443-695-0061, gilvlevin@gmail.com

ABSTRACT

The 1976 Viking Labeled Release (LR) experiment was positive for extant microbial life on the surface of Mars. Experiments on both Viking landers, 4,000 miles apart, yielded similar, repeatable, positive responses. While the authors eventually concluded that the experiment detected martian life, this was and remains a highly controversial conclusion. Many believe that the Mars environment is inimical to life and the LR responses were non-biological, attributed to an as-yet-unidentified oxidant (or oxidants) in the martian soil. Unfortunately, no further metabolic experiments have been conducted on Mars. Instead, follow-on missions have sought to define the martian environment, mostly searching for signs of water. These missions have collected considerable data regarding Mars as a habitat, both past and present. The purpose of this paper is to consider recent findings about martian water, methane, and organics that impact the case for extant life on Mars. Further, the biological explanation of the LR and recent non-biological hypotheses are evaluated. It is concluded that extant life is a strong possibility, that abiotic interpretations of the LR data are not conclusive, and that, even setting our conclusion aside, biology should still be considered as an explanation for the LR experiment. Because of possible contamination of Mars by terrestrial microbes after Viking, we note that the LR data are the only data we will ever have on biologically pristine martian samples.

Key Words: Extant life on Mars—Viking Labeled Release experiment—Astrobiology—Extraterrestrial life—Mars.

1. Introduction

The Viking Labeled Release (LR) Experiment (Levin, 1972; Levin and Straat, 1976a, 1976b) was an experiment in radiorespirometry whereby 14C-labeled organics were injected onto a soil sample in a test chamber and continuously monitored for the subsequent evolution of radioactive gas. The experiment was designed to test for the presence of life on the surface of Mars by monitoring for metabolism. The positive results obtained (Levin and Straat, 1976a, 1976b) are consistent with biology, but have been challenged by a variety of non-biological active agents, and by doubts that putative martian organisms could exist in the harsh environmental conditions. However, recent findings of water availability, complex organic molecules possibly of biological significance, and the periodic appearance of methane warrant a re-evaluation of the potential for life on Mars whether or not the LR experiment detected it.

2. The Viking Labeled Release Experiment

The Viking Labeled Release (LR) experiment (Levin, 1972; Levin and Straat, 1976a, 1976b) was one of three life detection metabolic tests on the 1976 Viking Mission to Mars. The question is whether the positive results of this experiment detected extant life or some chemical agent in the regolith. The experiment was designed to look for metabolism whereby one or more simple organic compounds are consumed and one or more carbon-based gases are evolved. The other two life detection experiments were the Gas Exchange (GEx) experiment (Oyama, 1972; Klein *et al*., 1976; Oyama and Berdahl, 1977), which tested for microbial life exposed first to humidity only, then to water, and then to immersion in a complex "chicken soup" of organic nutrients and supplements, and the Pyrolytic Release (PR) experiment (Horowitz *et al*., 1972, 1977; Klein *et al*., 1976), which was designed to test for microorganisms that photosynthetically incorporated $^{14}CO_2$ and/or ^{14}CO in a simulated Mars atmosphere and environment with and without the addition of water. A fourth Viking experiment, the GCMS, was included to analyze organics expected to be widely present on the surface of Mars (Biemann *et. al*., 1977). While not a life detection experiment *per se*, it was believed that, if life existed on the planet, organics would be detected by the GCMS and serve as confirmation of life.

The LR experiment was based on the belief that early Mars and Earth possessed similar primordial environments, each of which produced Miller-Urey type organic compounds that were then available for the genesis of life and its subsequent metabolism and evolution. In the LR experiment, a nutrient solution consisting of Miller-Urey compounds tagged with ^{14}C was added to a sample of martian soil, and the mixture was continuously monitored for evolution of radioactive gas. Early laboratory experiments showed the technology to be very rapid and extremely sensitive, detecting as few as 30 cells in a sample (Levin *et. al*., 1956). The sensitivity of the method reflects the fact that this is a metabolic experiment that utilizes radioactive isotopes and does not depend on culturing, at least initially, thereby eliminating the lag typically observed in classical microbiological methods. The method can thus detect microbial

metabolism even for those soil microorganisms that defy culturing, which may explain why soils found "sterile" by classical microbiological methods tested positive by the LR method. The sensitivity is further enhanced by the radioisotopic methodology, which permits the use of low substrate concentrations and thereby reduces potential toxicity to any alien organisms.

The LR nutrient substrates were sodium formate, sodium lactate, glycine, alanine, and calcium glycolate (Levin and Straat, 1976a), all Miller-Urey compounds. Both left-handed and right-handed isomers of alanine and of lactate were included to provide for a possible different chirality with martian life. Concentrations of the selected compounds were only 2.5×10^{-4} molar each to minimize toxicity, with each carbon uniformly labeled with ^{14}C at 2 μCi/m. The experimental procedure consisted of adding 0.115 ml of nutrient solution to 0.5 cc soil sample in a 3.5 cc cylindrical test chamber that had a 2 cm diameter. This provided a moisture gradient starting with the injected liquid at the center of the sample and progressing to minimal moisture at the periphery of the sample. This "moist" mode greatly decreased the response time because no water reservoir had to be saturated with gas before the gas could evolve from the liquid. Other environmental conditions chosen for the experiment were a temperature of 10^{o} C ± 2^{o} C to guarantee liquidity of the nutrient, and martian atmosphere with a helium overpressure of 85 mbar to assure liquidity in the event the martian atmosphere was below the triple point. A major advantage of this simple experiment is that the radioactive end-product is gaseous, easily separating itself from the radioactive liquid nutrient solution on the soil and thereby eliminating any interfering noise from the injected nutrient.

Terrestrial microbial utilization of the substrates in the LR nutrient involves mostly decarboxylation, although other carbon atoms can be metabolized as well. Because metabolic pathways on Mars are unknown, each carbon in each LR substrate was tagged with ^{14}C, and the specific radioactivity was the same for each carbon. This permitted quantification of the amount of carbon gas released, regardless of the carbon atom from which it was derived. There were seven substrates in the LR nutrient for a total of 17 carbons, all present at the same concentration and the same specific radioactivity.

The Labeled Release technique was first developed in 1956 for the rapid detection of microorganisms (Levin *et al.*, 1956) before its selection in 1970 as one of the Viking life detection experiments. Over the entire 20-year development period for this experiment, thousands of LR tests were performed on a wide variety of soils, many of which were provided by NASA as bonded samples from harsh environments around the world (Levin and Straat, 1976a). Field tests were also made in which working models of the instrument were taken to extreme environments, for example, Antarctica, White Mountain above the timber line, Salton Sea flats, Death Valley sands, all of which responded positively. With the exception of three naturally sterile soils, discussed below, an active response was obtained from every soil tested, which was then eliminated in a duplicate sample by application of a control regime to destroy any life present.

This control regime was an essential part of the experiment to confirm that the initial response was biological, not chemical. Extensive laboratory experiments established that the most effective yet practical sterilization regime was heating the soil sample at 160ºC for three hours, followed by cooling before testing with the ^{14}C nutrient. It was thought that this treatment would be sufficient to kill any martian microbial life and thus confirm that an active first response was evidence for life. If an active response were caused by chemistry instead of biology, the heat control response would be expected to be virtually the same as the active response; *by definition*, then, a positive response is one in which an active response is verified by a negative control. Three naturally sterile soils (Moon, Surtsey, and one Antarctic sample) that tested negative, (i.e., the active and control sequences were essentially the same) showed the validity of the LR in not giving false positives (Levin and Straat, 1976b).

Pure cultures tested during the development of the LR experiment (Straat and Levin, 1972) included not only heterotrophs (algae, fungi, actinomycetes, aerobic bacteria, strict and facultative anaerobic bacteria), but also phototrophs and autotrophs. Heterotrophs included *Mucor rouxii, Fusarium oxysporum, Candida albicans, Waksmania rosea, Mycobacterium smegmatis, Chlamydomonas reinhardi, Halobacter halobium, Desulfovibrio desulfuricans,* and *Streptosporangia rosea*. Active responses were obtained from these heterotrophs as confirmed by their controls. Phototrophs included *Chlorella vulgaris, Oscillatoria sp.,* and *Chromatium sp.,* all producing positive responses. The results of these studies with pure cultures serve to emphasize the versatility of the LR in detecting microbial metabolism (Straat and Levin, 1972).

The LR flight instrument has been described elsewhere (Levin and Straat, 1976a).

Essentially, it contained four incubation cells in a carousel that rotated such that each cell could be positioned to receive a soil sample delivered from the lander sample distribution box. Once soil was received, that test cell was rotated under a head-end and sealed. Any evolved gas following nutrient injection passed through a 13 inch "swan neck" tube (to preclude carry-over of radioactive aerosol or dust particles) to a detector chamber where radioactivity measurements were made. The head–end of the test cell contained two heaters and a temperature sensor for controlling and recording temperature, both during the test runs and during heat sterilization of the control soil samples. The second heater boosted the temperature of the soil sample to the desired 160ºC.

A Test Standards Module (TSM) of the instrument, duplicating all critical flight instrument parameters and experimental conditions, was constructed and tested at TRW, Inc., at Redondo Beach, California, and later operated at the NASA Ames Research Center. This instrument was used to test the various developmental stages of the experiment and create a library of responses. In addition, an actual flight instrument, called SN103, was used to conduct a final pre-flight test of the LR experiment on a California soil ("Aiken" soil) under Mars conditions (Figure 1). The soil, which contained approximately 10^5 aerobic microorganisms per gram, was maintained for

three days under these harsh conditions before being injected with the nutrient. Despite this severe pre-treatment, the soil remained viable, as shown by the strong active response following both first and second injection, confirmed by the negative response of the heated control.

3. The Viking LR Data

Vikings 1 and 2 were launched August 20, and September 9, 1975, and landed safely on Mars July 20 and September 3, 1976, respectively. On July 30, 1976, sol 10, the first LR run was conducted on a martian soil sample (Viking 1, cycle 1; i.e., VL1-1). The soil sample had been collected on sol eight by the sampling arm, which dredged to a depth of about four cm. The soil was stored in the sample distribution box at approximately 10^{o} C for the two intervening sols. As shown in Figure 2, upon nutrient injection, the LR response was immediate and strongly active, within the lower portion of the range of responses (Figure 3) from terrestrial viable soils (Levin and Straat, 1976a; Levin, 2006). After the run was complete, the critical 160^{o}C control was performed on a duplicate sample of the same soil (VL1-2). The response was negative (Figure 2). Taken together, the results from VL1-1 and VL1-2 are consistent with a life response (Levin and Straat, 1976b).

Gas evolution in VL1-1 began to level off after around two sols, at a level corresponding either to total utilization of only one carbon in the nutrient, or partial utilization of several or all of the carbon substrates, and had nearly plateaued seven sols after the first nutrient injection. This plateau could indicate either running out of substrate or "death" of the active agent, or both. An additional injection of nutrient on Sol 7 failed to produce additional gas evolution, indicating that the plateau resulted from lack of active agent rather than lack of substrate. Instead, there was an immediate depletion in the headspace gas, attributed to re-absorption of CO_2, by the wetted alkaline soil (Levin and Straat, 1976b).

Most terrestrial soils responded positively to a second injection of nutrient, as demonstrated in Figure 1. The failure of the Mars sample to respond positively to the addition of more substrate indicated that the active agent was no longer active. Reabsorption of gas following second injection was therefore attributed to a physico-chemical reaction involving a carbon dioxide-bicarbonate-water-soil equilibrium in the test cell. In support of this conclusion, laboratory studies in the TSM demonstrated $^{14}CO_2$ uptake following water injections in the presence of two Mars analog soils as well as in the absence of soil (Levin and Straat, 1979b).

As seen in Figure 4, Antarctic soil #664 acted similarly in the TSM when a second injection of the LR nutrient was made, although the percentage drop was not nearly as great as in the Mars samples (Levin and Straat, 1986). This was a NASA-bonded test soil with a pH of 8.1. Several other Antarctic soils, one naturally sterile and the others with a sparse microbial population, were also examined in our TSM test program. Soil #664 was the only one that showed re-absorption

following 2nd injection, and the only one at high pH; this re-absorption is most likely reflecting a physico-chemical reaction, as proposed for the martian sample.

Scientific protocol dictated that the third run, VL1-3, verify or contest the positive result of VL1-1. A fresh sample of the soil was taken from the same area. The response was again positive (Figure 5), validating the positive response from the first cycle. Second and third injections produced the same re-absorption of gas seen with VL1 cycle 1, following by a slow linear re-evolution of radioactive gas.

The fourth and final LR run at Viking site 1 (VL1-4) was on a sample collected from the same area that had produced the positive response in VL1-1. However, at the time of the injection for this run, the soil had been stored in the sample distribution box, in the dark, open to the martian atmosphere, but maintained at temperatures ranging between 10° C and 26° C for 141 sols. Two nutrient injections were made about three hours apart, which allowed time to observe a response from the first injection before administering the second. Each injection resulted in a null response (Figure 6). Apparently, long-term storage of an active soil at 10° – 26°C inactivates an initially active sample. This, again, is consistent with biology. We would not expect to see such losses in terrestrial analog soils because these temperature are within normal range for most terrestrial soils, even over long periods of time. On Mars, however, although soil may temporarily see 10°C or even higher temperatures during a normal diurnal cycle, at least during the warmer times of the martian year, the *daily* exposure to such elevated temperature would be brief; Martian organisms would therefore be expected to be much more likely to succumb to long term storage at 10°C – 26°C than would terrestrial microorganisms. Thus, possibly stressed by long term isolation from their natural environment at elevated temperatures, the putative martian organisms may have died.

All Viking site 1 test and control responses for first injections for the first 7sols are shown in Figure 6 (Levin and Straat, 1979a). The LR first injection results at Viking site 2, some 4,000 miles distant, are shown in Figure 7 (Levin and Straat, 1979a). The first sample on the second lander (VL2-1) produced a positive response, essentially duplicating the positive responses of VL1 cycles 1 and 3. During the mission, because some scientists questioned the 160° C control as evidence of life, the Viking Biology Team unanimously agreed that pre-heating a sample to 50° C for three hours was more likely to distinguish between chemistry and biology; few oxidizing chemicals are affected by such a low temperature, whereas martian organisms, acclimated to extremely low ambient temperatures, might be adversely affected. Calculations confirmed that activation of only the booster heater in the head-end should result in soil temperatures around 50°C, although this had never been attempted in advance of the mission. Thus, for VL2-2, a fresh sample was acquired from the same location that supplied the positive sample and heated by activating only the booster heater. The soil temperature achieved during this "low-temperature sterilization" was determined by extrapolation of the thermal signal to be 51°C. As shown in Figure 8, the initial response following first injection was greatly reduced,

but not eliminated (Levin and Straat, 1977a). A series of small peaks, not seen in any previous or subsequent runs in either the flight instrument or the TSM, was evolved. The peculiar kinetics suggested a possible instrument anomaly, but, on careful investigation, the engineers could find no problem with the instrument, nor was any detected over the remaining course of the mission over which it functioned properly. Comparing only the first peak to the amount of gas evolved in VL2-1 during an equivalent time period showed that only about 15% as much gas was evolved; in total, significantly less gas was produced over the remaining sols than seen in VL2-1.

The third cycle at VL2 was a positive run to ensure the integrity of the instrument. At this point, a new possibility was raised: the LR positive responses could be attributed to "activation" of the soil by the intense flux of UV light impacting the martian surface. Activated soil components, it was contended, could react non-biologically with the nutrients to evolve radioactive gas. To test the UV activation theory, the soil sample for VL2-3 was collected just at dawn by moving a rock (Notch Rock) with the sampling arm, and obtaining a sample that had been shielded from UV light by the rock for eons. After two days in the test cell, the soil was injected with the nutrient solution. The strong positive VL2-3 response (Figure 7) dispelled the UV theory and confirmed instrument integrity.

An attempt was then made to clarify the singular results of VL2-2 by repeating that experiment in VL2-4. A fresh soil sample, taken from the same location as the fresh samples for VL2 cycles 1 and 2, was pre-heated, aiming for 50^{o} C as before. This time, however, the temperature attained in the soil was extrapolated to be 46^{o} C. The LR VL2 -4 response (Figure 7) produced essentially the same kinetics as the positive VL2 -1 and VL2-3 samples, but with only about 30 percent of the amplitude. Clearly the low-temperature treatment had reduced the magnitude of the active response, a result strongly in support of biology; few chemical oxidants would be expected to show such temperature sensitivity. Although the unusual kinetics of VL2-2 are unexplained, the one clear conclusion that can safely be made is that the magnitude of the initial several hours of reaction was greatly reduced by the low-temperature sterilization, in agreement with VL2-4. As for the effect of the unintended 5^{o} temperature difference between the two low-temperature sterilizations, while it hints of biology in that small temperature differences could affect microbial survival, no conclusion can be made in the absence of further data.

VL2- 4 used the last of the four LR test chambers. Since nutrient solution was still available, an additional run was improvised whereby stored soil collected for VL2-4 was added on top of a previously tested soil sample. This VL2-5 soil was taken from the same area as that for VL2 -4, but the soil had been held in the sample distribution box for 84 sols at approximately 10^{o} C before the onset of VL2-5. As seen in Figure 7, long-term storage of the sample used for VL2-5 totally inactivated the soil, producing a negative response**.**

In summary of the Viking LR data, characteristics determined for the active agent are listed in Table 1. Each of these characteristics is reminiscent of responses by a compendium of terrestrial

microorganism species, including the initial positive responses, the 160ºC and 50ºC heat controls, the re-absorption of evolved gas upon second injection of nutrient, and death from isolated long-term storage. Further, the Viking LR data show similarity to the SN103 data (Levin and Straat, 1977b) obtained with California soil held under martian conditions for three days prior to testing (Figure 1), and are within the lower range of responses from other viable terrestrial soils both in amplitude and kinetics (Figure 3), including those from Barrow and Palmer, Alaska, and Antarctic soil 726. All in all, the results of the Viking LR experiment are consistent with a biological explanation.

4. Non-Biological Explanations for the LR Data

Despite the positive LR results, the scientific community has remained skeptical of the biological interpretation. Doubt increased greatly when the GCMS reported failure to detect organic matter in the martian soil or atmosphere (Biemann *et al*., 1977). In the years since Viking, many attempts have been made to explain the LR results non-biologically, but, so far, no non-biological explanation has met all the criteria. Zent and McKay (1994) reviewed most of the earlier oxidant theories, finding them unconvincing, and offered their theory that atmospheric photochemically produced oxidants react with the Mars regolith to create complexes that caused the Viking Biology results. However, none of the theoretical oxidants satisfy the Viking LR thermal control data.

One study frequently cited in explaining the Viking LR data is that of Navarro- González *et al*. (2003) who conducted LR-type reactions *in situ* in the extremely arid Atacama Desert. They claimed that the soil tested contained only trace organics and extremely low levels of culturable bacteria. LR-type reactions were conducted with ^{13}C-labeled alanine and glucose enantiomers. When results from a mixture of L-alanine and D-glucose were essentially the same as those from a mixture of D-alanine and L-glucose, the soil sample was considered abiotic. Experiments were also conducted with ^{13}C- labeled formate; active responses resulting from the addition of formate to Atacama soil was attributed to an unidentified non-biological oxidant rather than biology because the chiral experiments with alanine and glucose had indicated that the soil was sterile. While these authors do not specifically claim to have resolved the Viking LR results, the implication is that the Viking results are most likely caused by a non-biological soil oxidant.

While those Atacama results superficially resemble the Viking LR results, the conditions of the two studies are drastically different. The most critical part of the LR experiment is verifying a positive response by conducting an identical experiment on a 160ºC heat-treated sample of the same soil. This would have been especially important in the Atacama study with formate. Formate is known (Straat and Levin, 1971) to react non-biologically with certain terrestrial soils, particularly those of low pH; pre-sterilization of the soil with heat at 160º C does not eliminate this reaction, showing that the LR technology readily distinguished this chemical reaction from a life response. Had a sterilization control been conducted at the Atacama site, the reaction with

formate may have been the same as with the non-sterilized reaction, unlike the Mars result. Another consideration is that the formate concentration in the Atacama studies was 2000 times greater than that used on Mars, and 10 times greater than the concentrations of the alanine and glucose isomers used in the Atacama experiments. Thus, while the Atacama studies may provide an important model for testing experiments designed for future Mars missions, they cannot, as these authors themselves concluded, be used to interpret the Viking LR results.

The discovery during the Phoenix mission (Hecht *et al.*, 2009) that perchlorates were present on Mars at approximately 0.5 wt % levels provided yet another candidate for the oxidation of the LR nutrients. Perchlorates have since been found at several Curiosity sites by Sample Analysis at Mars (SAM) (Glavin *et al.,* 2013), implying that perchlorates may be widespread on the surface of Mars. However, perchlorates cannot account for the Viking LR data because perchlorates are stable under all thermal conditions of the Viking LR experiment, including the 160^{o}C control (Quinn *et al*., 2013).

Quinn *et al*. (2013) proposed that products from perchlorate exposed to ionizing radiation could account for the LR positive results. They showed that perchlorate exposed to ^{60}Co in a carbon dioxide environment produces hypochlorite, oxygen, and chlorine dioxide. Hypochlorite added to ^{13}C-labeled LR nutrients produced $^{13}CO_2$. Based on their results, they claimed that galactic cosmic rays and radiation particles from the sun can produce hypochlorite from perchlorate, which can then interact with ^{14}C-labeled amino acids, especially alanine (present in the Viking LR nutrient solution), and release radioactive gas. They further showed that hypochlorite is destroyed by heating for three hours at 160^{o} C, meeting that requirement of the Viking LR control cycle.

While tantalizing as a non-biological explanation for the Viking LR results, there are some problems with this theory. Whether perchlorate, or its irradiation products, were present at the VL1 and VL2 sites has not been established, although Navarro-González et al. (2010) conducted studies that led them to imply perchlorate may have been present. The Quinn data also do not address two critical aspects of the LR experiment: First, the active LR agent on Mars is adversely affected by heating at approximately 50o C, and, second, is destroyed by long-term storage in the dark at 10o C, both of which are strong plusses for biology; these critical controls have yet to be performed with irradiation products of perchlorate. Nonetheless, despite deficiencies, this hypothesis remains perhaps the most attractive of the non-biological explanations because of the likely widespread distribution of perchlorate and, especially, because of the sensitivity of hypochlorite to 160oC.

5. Discussion and Conclusions

The general consensus at the time of Viking was that the LR positive response was non-biologically caused. However, since then, information from later missions to Mars has called the

basis of that consensus into question, and it is timely to reconsider a biological explanation for the LR results. On the side of biology, the LR active results were strongly positive and obliterated by heat treatment at 160°C. Perhaps the strongest experimental evidence for biology is that pre-treatment of the soil around 50°C partially destroyed the active agent, and that long-term storage around 10°C completely destroyed it. In our pre-mission testing program, we showed (Straat and Levin, 1970) that a 4-hour, 90°C pre-heat treatment significantly reduced terrestrial soil activity; Mars microbes, acclimated to lower environmental temperatures, would be expected to be even more temperature sensitive. Few chemical agents that would oxidize any of the LR nutrients are affected by such low temperatures, which is why it is so important that candidate non-biological agents be subjected to those temperature regimes as a control prior to testing for activity. The Mars active result was also similar in magnitude and kinetics to pre-mission tests of a California soil in a flight instrument (Figure 1) and to results from a variety of terrestrial soils tested either in the field or in the TSM (Figure 3). Against the biological hypothesis are the candidate non-biological hypotheses, some of which come tantalizingly close to approximating the LR results (Navarro- González *et al*., 2003; Quinn *et al*., 2013). However, none as yet have completely replicated the LR thermal data, mainly because the critical 50°C and 10°C controls are lacking.

The harsh environment of Mars has also been cited as unfavorable to a biological interpretation, particularly because of the lack of liquid water. Yet findings from recent missions provide strong evidence that liquid water is available, albeit in low amounts, either in pure or absorbed form (Levin 1997; Rennó *et al*., 2009; Ming *et al*., 2014), as frost (such as seen at the Viking 2 landing site, Figure 9) or as briny saltwater (Martin-Torres *et al*., 2015; Ojha *et al*., 2015). The temperature of the atmosphere immediately above the surface of large regions of Mars frequently reaches the 20°C range (Gomez-Elvira *et al*., 2014, Mischna *et al*., 2014), which is sufficient to provide liquid water from near-surface ice since atmospheric pressure has never been recorded below the triple point, except at high elevations. Considering the ability of terrestrial extremophiles to adapt to harsh arid environments, it would seem that, if life were ever present on Mars, it could have adapted and evolved as Mars transformed to cold conditions where water was scarce. Life may therefore still exist, if only in a cryptobiotic state, subject to resuscitation whenever water becomes available. Overall, the studies of water on Mars do not preclude extant life, whether or not the LR detected it. While the availability of water at the Viking sites was unknown at the time the LR was performed, the availability of water on Mars generally favors a biological interpretation of the LR.

Lack of biologically relevant organic molecules on the surface of Mars has also been considered a major detriment to extant life. Recent reports of complex organics, possibly of biological importance, are encouraging (Freissinet *et al*., 2015), although analyses are ongoing and details are not yet available. Simple organic substrates, such as amino acids or other organics present in the LR nutrient, have not yet been detected on Mars, although the putative martian microbes that produced the LR reaction were demonstrating heterotrophic metabolism by utilizing such

organics as substrates. While autotrophs seem a more likely possibility for martian microbial life because of the apparent scarcity of simple organics, it's possible that martian autotrophs would be facultative and able to utilize simple organics whenever they are available, such as through meteoritic fallout or other non-biological processes.

The sporadic appearance of methane (Webster *et al*., 2015; Mumma *et al*., 2009) suggests methanogens as possible martian life forms. Mumma *et al*. (2009) stated that methane may be biologically accumulated underground where water may be available, and that geological activity, such as seasonal fissuring, might cause periodic release. Because Mumma *et al*. also proposed that a sink was needed to explain the overly rapid disappearance of released methane, Levin and Straat (2009) postulated an anaerobic methanogen-methanotroph ecology on the martian surface where methane-consuming methanotrophs could provide such a sink. Halophiles have also been suggested as candidates for martian life forms (Leuko *et al*., 2010). In addition to their ability to tolerate salinity extremes, many terrestrial halophiles withstand damaging radiation, temperature extremes, and low oxygen atmosphere (DasSarma, 2006). Some terrestrial halophiles are also methanogenic (Ollivier *et al*., 1994; Oren, 2002). However, Martin-Torres *et al*. (2015) and Ojha *et al*. (2015) indicated that water activity in the postulated perchlorate salt brines would be too low for habitability by terrestrial microbes, although this would not preclude halophilic activity at lower salt concentrations. Perchlorates, first discovered on Mars in 2009 (Hecht *et al*., 2009) and apparently widespread on the surface (Glavin *et al.*, 2013), are toxic to many terrestrial flora and fauna, although several perchlorate-utilizing terrestrial microorganisms have been isolated and described (Coates and Achenbach, 2004; Waller *et al*., 2004; Bardiya and Bae, 2011). Other extant chemoautotrophic life forms may yet be defined as candidates for extant martian life.

The LR experiment was designed to monitor microbial metabolism whereby simple organic molecules are consumed to yield carbon dioxide or other carbon gases derived from the LR substrates. The flight instrument was not capable of identifying the labeled gas (or gases) evolved. Laboratory simulations have shown that the primary gaseous end-product of the LR reaction was carbon dioxide (Levin and Straat, 1979b), although other carbon-based gas would also have been detected. Several methanogenic species are known (Miyamoto, 1997; Chaban *et al*., 2006) that can utilize formate, one of the LR substrates, or other organics in the generation of methane. Thus, while methanogens could not be responsible for the entire active LR response, the LR soil sample could have included methanogens, and the evolved gas could have included methane as well as carbon dioxide. Also, as previously discussed, the LR experiment dropped a small amount of liquid nutrient on a relatively large soil sample, creating a water gradient from wet, where the nutrient landed, to dry or almost dry at the periphery of the soil sample. It is entirely possible that the liquid added to the LR soil sample re-activated cryptobiotic martian microorganisms.

We have long proposed a chiral metabolic experiment as a follow-on to the Viking LR experiment (e.g., Levin, 1998). Chemical reactions cannot distinguish between stereoisomers, whereas all known terrestrial life forms preferentially utilize L-amino acids and D-carbohydrates. Preferential utilization of one or the other such isomer on Mars would be indicative of a life response. However, some biological utilization of D-amino acids and L-carbohydrates does occur in terrestrial microorganisms. For example, D-alanine is a known constituent of bacterial cell walls (Newton, 1970; Lam *et al*., 2009), and the toxicity of a Chinese mushroom has been attributed to two novel unsaturated D-amino acids (Stone, 2012). Martinez-Rodriguez *et al*. (2010) reported incorporation of D-amino acids into peptide antibiotics. Moazeni *et al*. (2010) reported utilization of both D-lactate and L-lactate by microbial communities, although L-lactate is consumed only after a lag. Zhang and Sun (2014) provided evidence that bacteria use D-amino acids as a source of nitrogen by running enzymatic racemization in reverse. While such reactions may occur in nature, they are rare and do not negate the concept of using chiral compounds to differentiate chemical from metabolic reactions, because it would be expected that the rates of biological utilization of the two isomers would differ, whereas kinetics for chemical reactions of both should be the same. But, in any event, the cogent use of controls could readily distinguish between a chemical and a biological response.

In summary, in the absence of a non-biological agent that satisfies all Viking findings, and in view of environmental evidence that Mars may well be able to support extant life, it seems prudent that the scientific community maintain biology as a viable explanation of the LR experimental results. It seems inevitable that astronauts will eventually explore Mars. In the interest of their health and safety, biology should be held in the forefront of possible explanations for the LR results. Plans for any return Mars sample mission should also take into account that such a sample may contain viable, even if dormant, alien life. Protected "Special Regions" on Mars, deemed possibly habitable by terrestrial hitch-hikers (Rummel *et al*., 2014), may even need to be redefined, especially based on the liquid water findings by Martin-Torres *et al*. (2015) and by Ojha *et al*. (2015). It is noted, however, that Mars may already have been infected by the many spacecraft that have landed there; although Viking was heat-treatedto reduce microbial counts, no other spacecraft have been similarly treated. The Viking LR experiment is thus in the unique position of being the only pristine life detection experiment that we will ever have. We strongly recommend that life-seeking experiments be considered for future missions. These should include the continued search for organic molecules of biological importance (e.g., amino acids, simple carbohydrates, lipids, DNA, protein); the conduct of further metabolic experiments, including a search for chiral preference in metabolism; the close examination of any tantalizing surface features; and perhaps even microscopic examination of martian soil with and without the addition of water or water vapor, reminiscent of the experiments of Anthony van Leeuwenhock who discovered the phenomenon of cryptobiosis approximately 300 years ago (Clegg, 2001).

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Abbreviations

GCMS, Gas Chromatograph Mass Spectrometer; GEx, Gas Exchange; LR, Labeled Release; PR, Pyrolytic Release; SAM, Sample Analysis at Mars; NASA, National Aeronautics and Space Administration; TSM, Test Standards Module; SN103, a flight instrument used for tests prior to the mission; VL1, Viking Lander 1; VL2, Viking Lander 2; VL1-1, Viking Lander 1 cycle 1

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TABLE 1

CHARACTERISTICS OF ACTIVE AGENT DETECTED BY VIKING LR EXPERIMENT

1. Produced positive response when injected with nutrient solution, similar in kinetics and amplitude to responses produced by LR test of a number of terrestrial viable soils.
2. Inactivated by pre-heating to 160oC for three h, similar to terrestrial tests of active soils.
3. Significantly reduced when pre-heated to approximately 50o for three h.
4. Inactivated when stored approximately two months in the dark in the soil distribution box at approximately 10oC.
5. Inactivation of soil not caused by UV exposure
6. A second injection of nutrient solution to positively responding soil caused approximately 25% of gas already evolved to disappear from detector cell (probably reabsorbed into soil), gradually to re-evolve. Active agent no longer available at time of second injection.
7. Widespread distribution of active agent (Viking sites 1 and 2 are 4,000 miles apart.

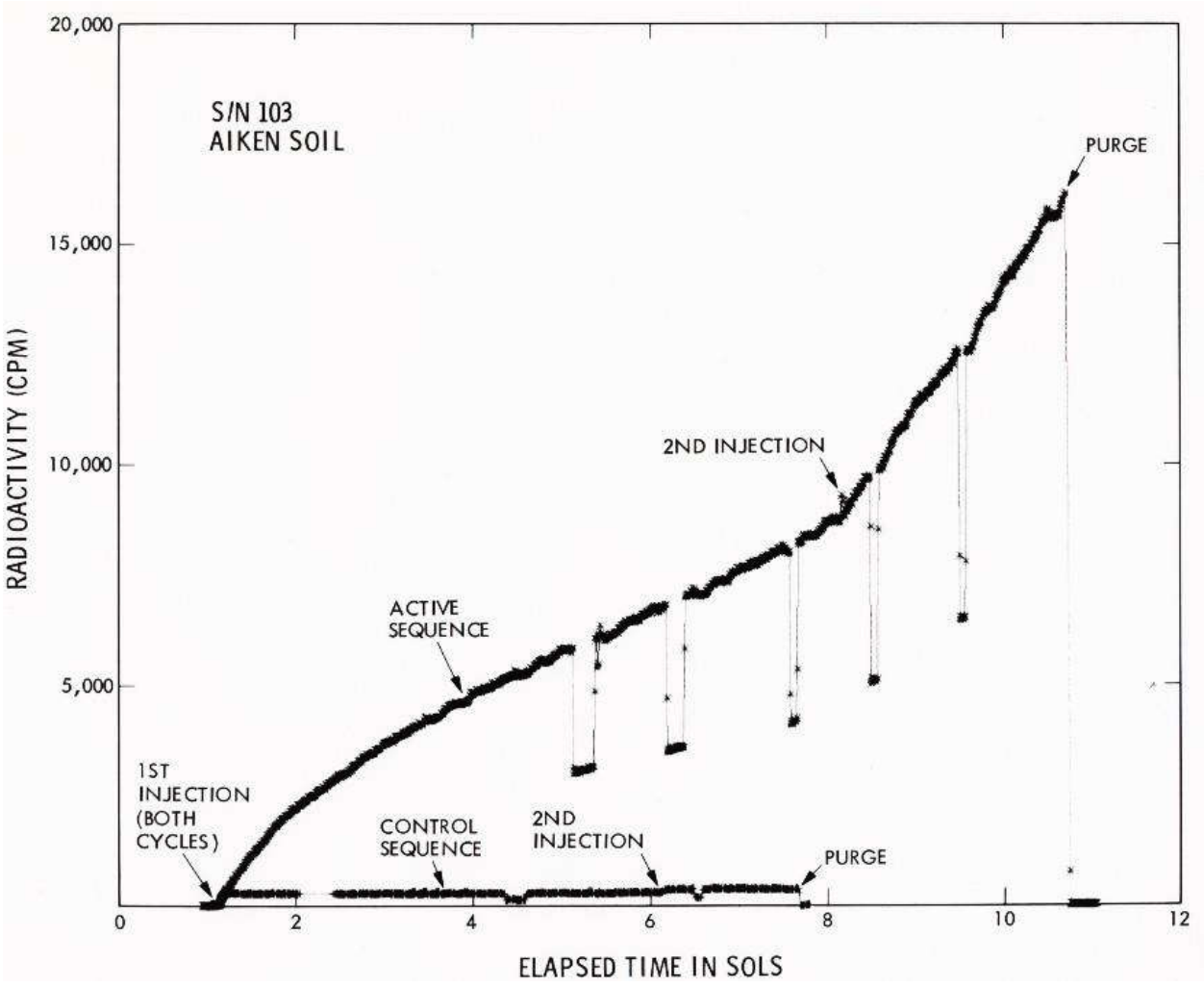

Figure 1. LR results from California ("Aiken") soil under martian conditions in the SN103 flight instrument. Nutrient was added either to untreated soil (active sequence) or to soil that had been preheated for 3 h at 160°C (control sequence). A second nutrient injection was added to the active and control samples approximately 7 and 5 sols, respectively, after first injections. Brief intervals where counts are reduced by approximately half reflect times when only one of the two beta detectors were utilized (single channel counting mode). [Reprinted from Levin, G.V. and Straat, P.A. (1977b) Life on Mars? The Viking Labeled Release Experiment. *Biosystems*, Vol 9/2-3, pp165-174, September 1977; with permission from Elsevier.]

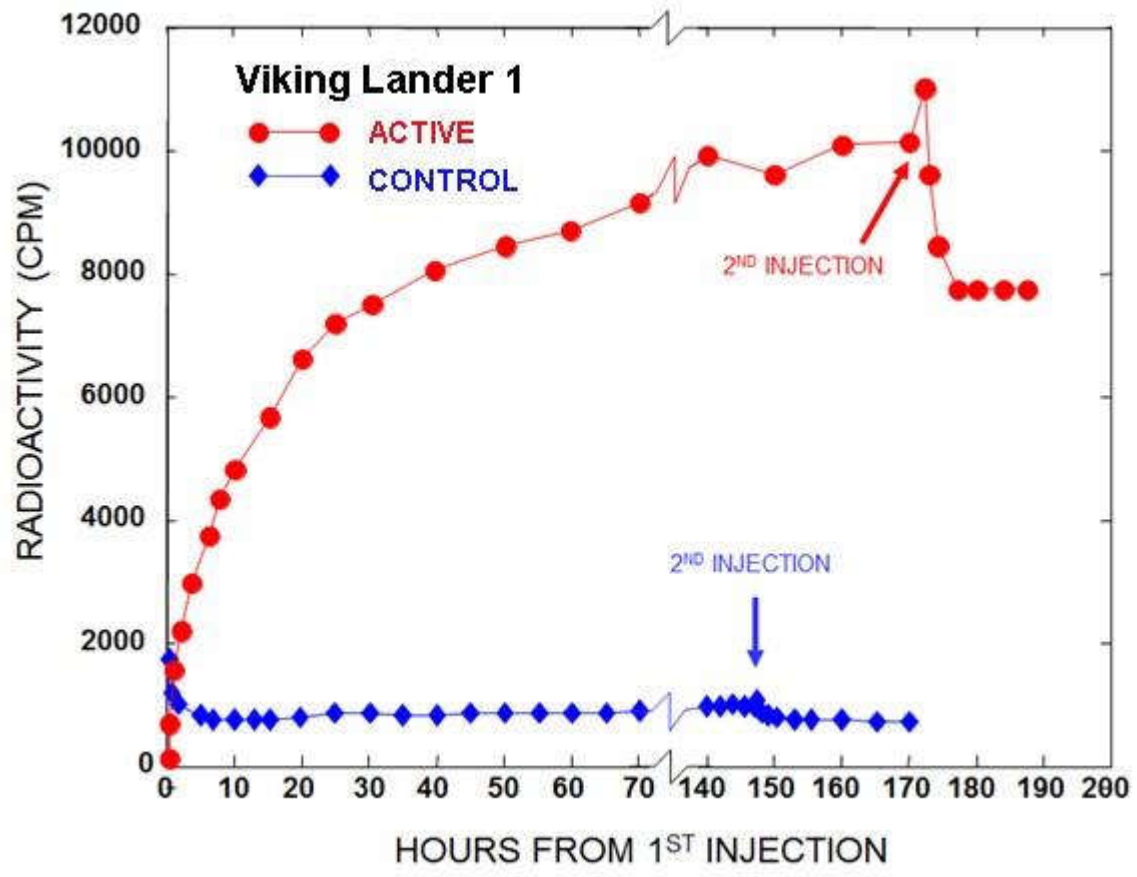

Figure 2. LR response to first and second nutrient injection in VL1 cycle 1 (active) and VL1 cycle 2 (160oC control). [Adapted from Levin and Straat, 1976b]

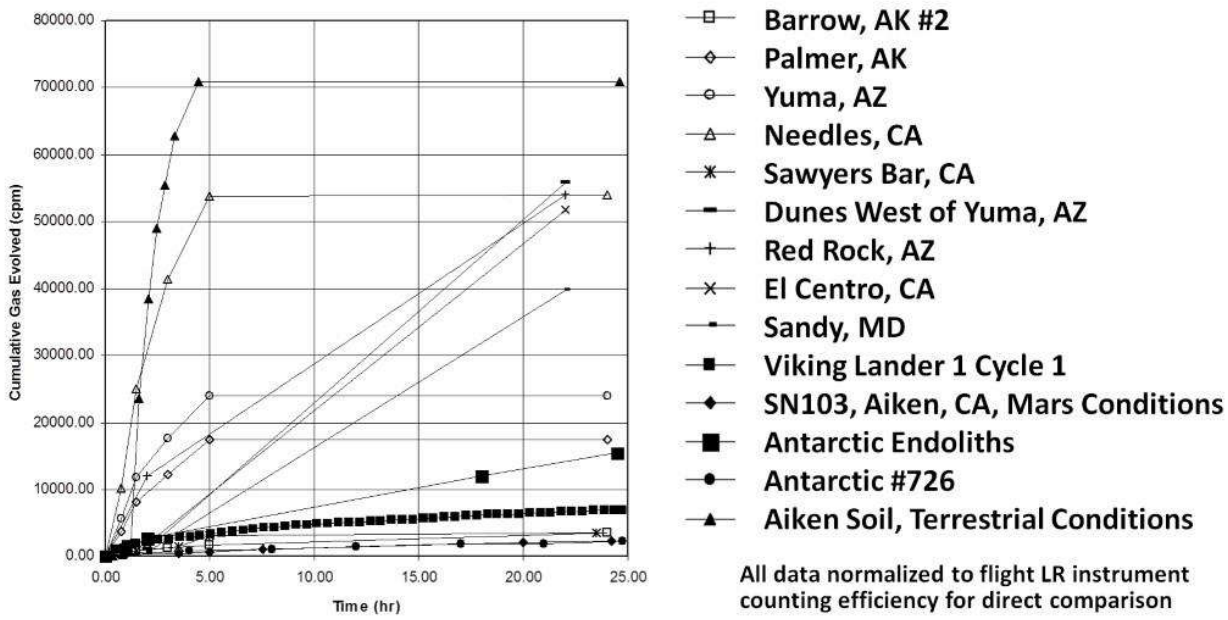

Figure 3. Comparison of Viking LR response with those from several viable terrestrial soil samples. Experiments with terrestrial soils were conducted by the "getter" technique, except for the SN103 data, which were obtained with a flight instrument. All data have been normalized to flight LR instrument counting efficiency for direct comparison. The Viking response (VL1-1) is shown by heavy black squares with frequent time intervals toward the bottom of the figure. [Reprinted from Levin, G.V. Modern myths of Mars. *Proc. Instruments, Methods, and Missions for Astrobiology, Proc. of SPIE 6309*, 1-15, 2006; with permission from SPIE Proceedings; figure compiled from Levin and Strat 1976a, 1976b.]

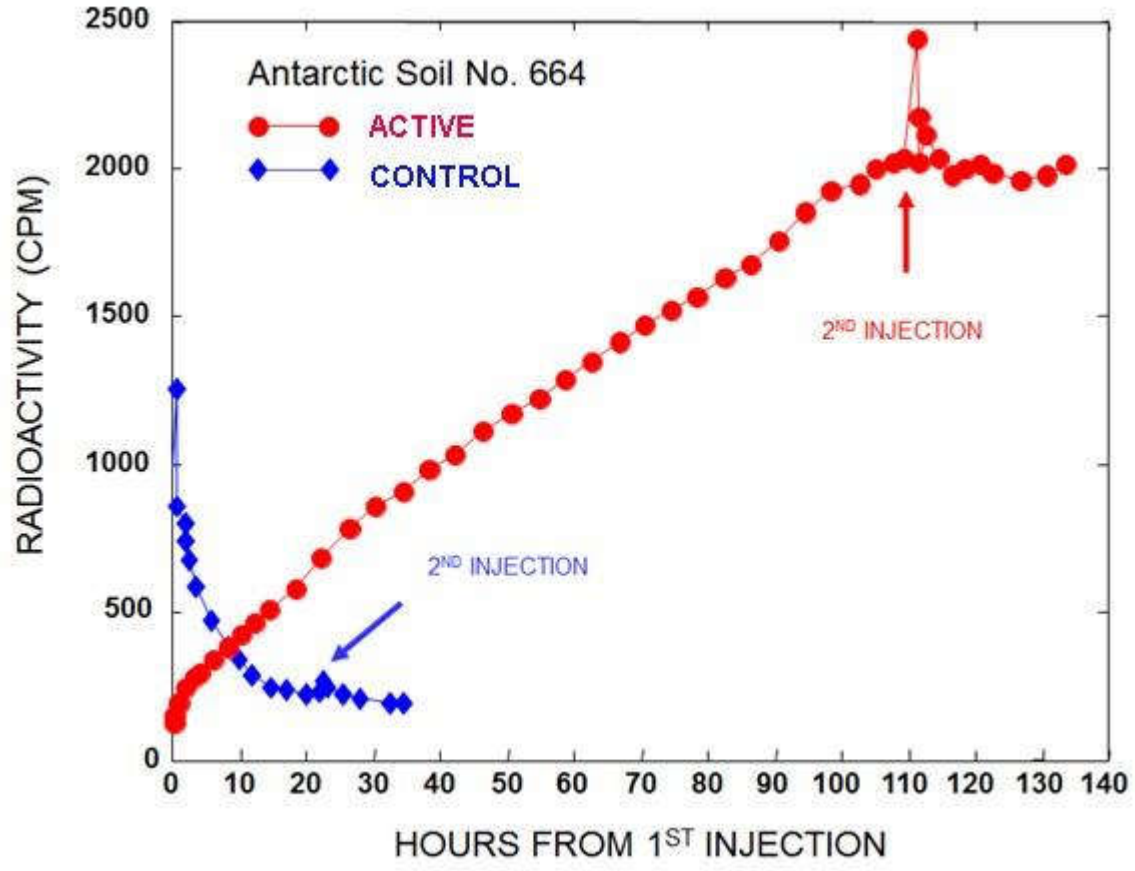

Figure 4. LR response to first and second nutrient injection added to Antarctic soil #664 in the TSM (active). A duplicate run was conducted on soil pre-sterilized at 160ºC (control). [Reprinted from Levin, G.V. and Straat, P.A. (1986) A reappraisal of life on Mars. Figure presented at the NASA Mars Conference in conjunction with the American Astronautical Society (AAS) held July 21-23, 1986, Washington, D.C., U.S.A. Published in *The NASA Mars Conference*, edited D.B. Reiber, *AAS Science and Technology Series*, © 1988, v.71, pp 187-207 © 1988 with permission from Univelt, Inc.]

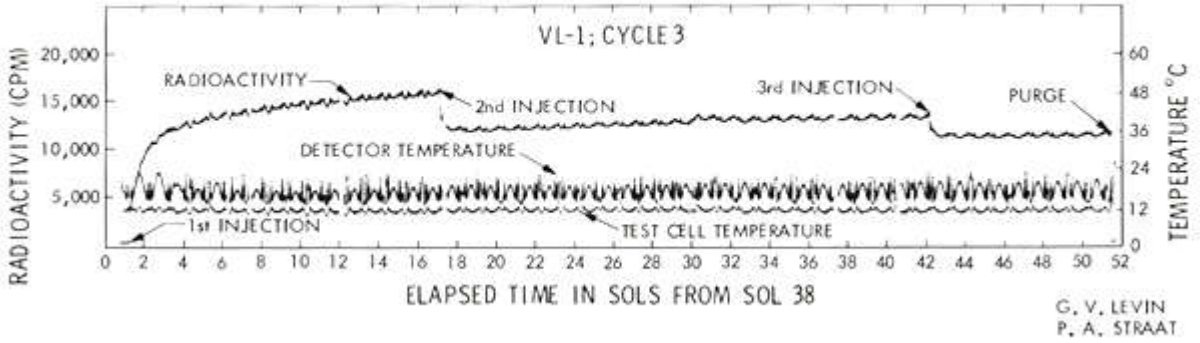

Figure 5. Complete VL1 cycle 3. A fresh sample was used in this active cycle. Data shows times of first, second, and third injections. [Reprinted from Levin, G.V. and Straat, P.A. (1977b) Life on Mar? The Viking Labeled Release Experiment. *Biosystems*, Vol. 9/2-3, pp165-174; with permission from Elsevier.]

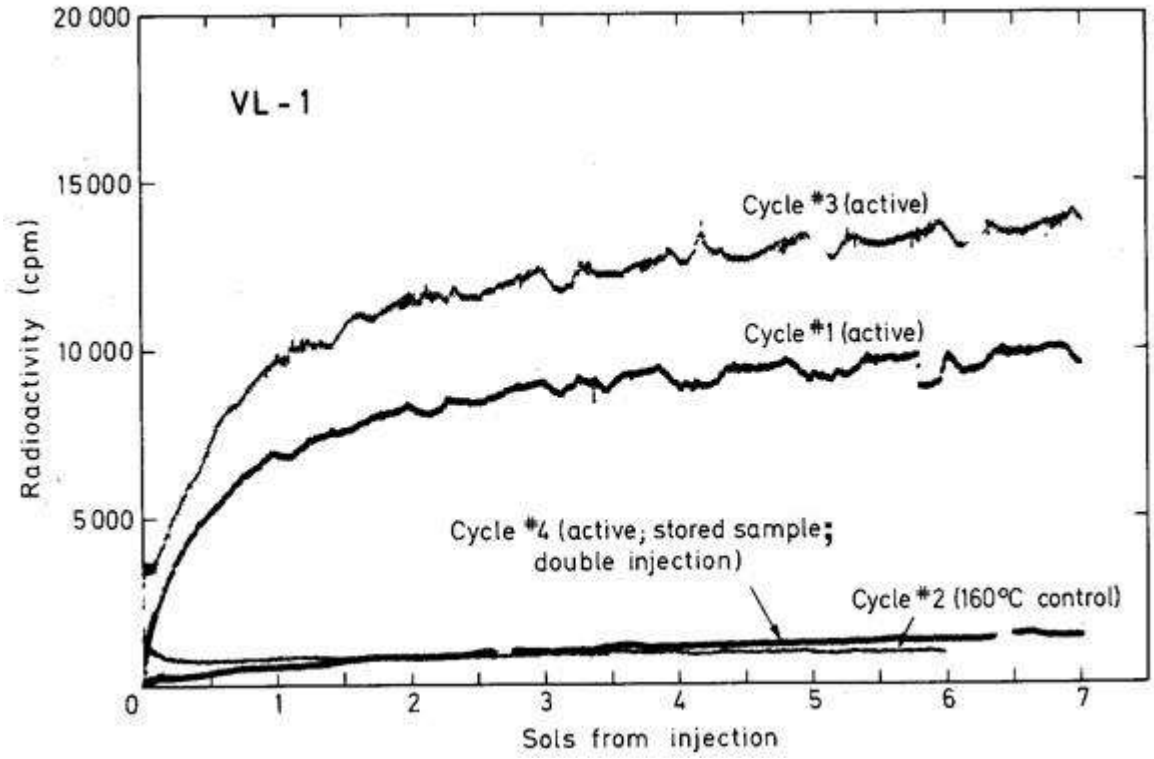

Figure 6. All first injection cycles of VL1. A fresh sample was used for the active sequence of cycles 1 and 3, whereas the sample used for active cycle 4 was stored for approximately 141 sols at 10-26°C prior to use. For cycle 2, a stored portion of the same sample used for cycle 1 was heated for 3 h at 160°C prior to nutrient injection. All data have been corrected for background counts observed prior to nutrient injection. [Reprinted from Levin, G.V. and Straat, P.A. (1979a) Comparison of the Viking Labeled Release Experiment on Mars. *J. Mol Evol* 14:167-183, 1979; Journal Molecular Evolution ©by Springer-Verlag, 1979; with permission of Springer]

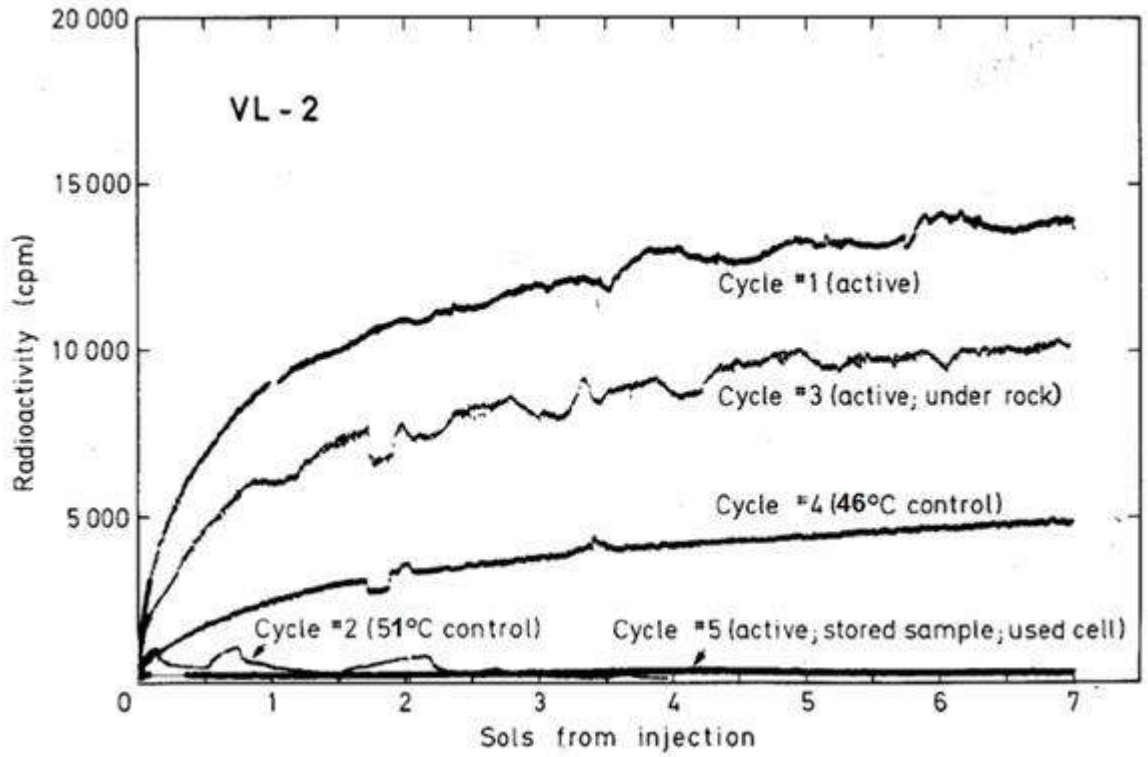

Figure 7. All first injection cycles of VL2. A fresh sample was used for the sequences except cycle 5, which used the same sample as for cycle 4 but was stored for approximately 84 sols before nutrient injection. All soil samples were taken from the same site except the sample for cycle 3, which was taken from under Notch Rock. All data have been corrected for background counts observed prior to nutrient injection. [Reprinted from Levin, G.V. and Straat, P.A. (1979a) Comparison of the Viking Labeled Release Experiment on Mars. *J. Mol Evol* 14:167-183, 1979; Journal Molecular Evolution ©by Springer-Verlag, 1979; with permission of Springer.]

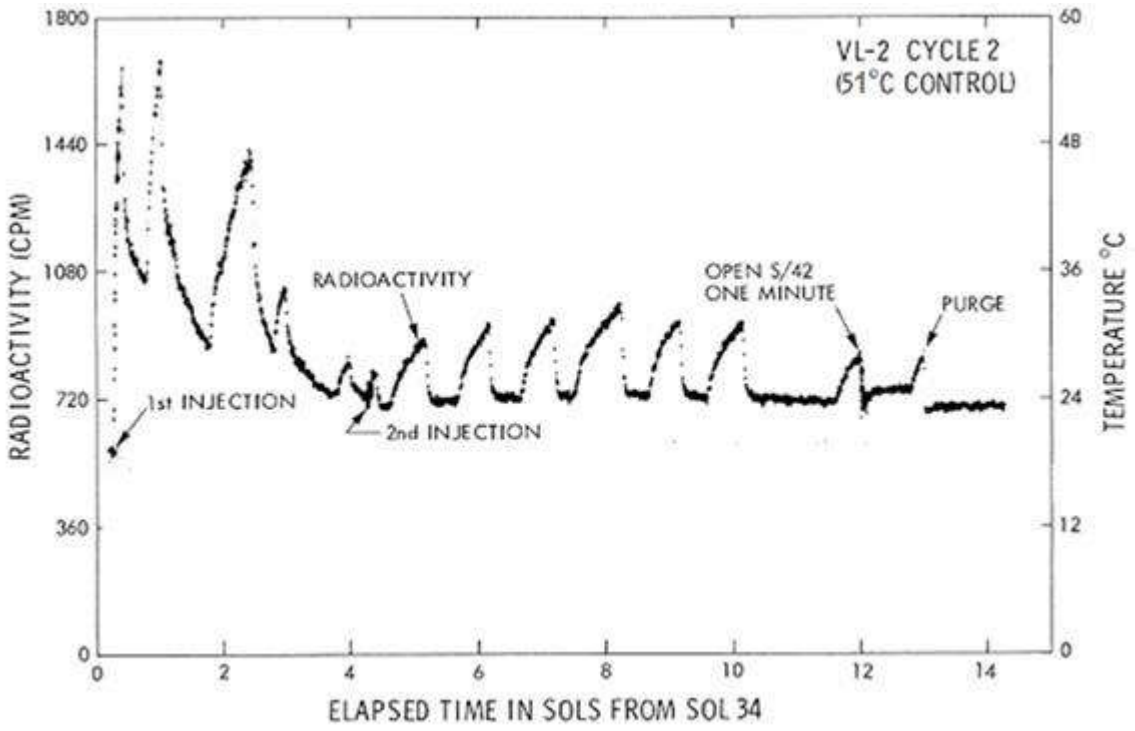

Figure 8. VL2 cycle 2 LR responses to first and second injections. After correcting for background, the cumulative radioactivity evolved in the first peak following first nutrient injection was approximately 15% of that evolved during VL2 cycle 1 in the same time period; little else evolved over the subsequent sols. Following second injection, the peaks represent diurnal movement of gas back and forth from the test cell chamber to the detector chamber as determined by turning off the thermal electric coolers for one sol between Sols 10 and 12, which eliminated the fluctuation. [Reprinted from Levin, G.V. and Straat, P.A. (1977a) Recent Results from the Viking Labeled Release Experiment on Mars. *J Geophys Res* 82(28):4663-4667.]

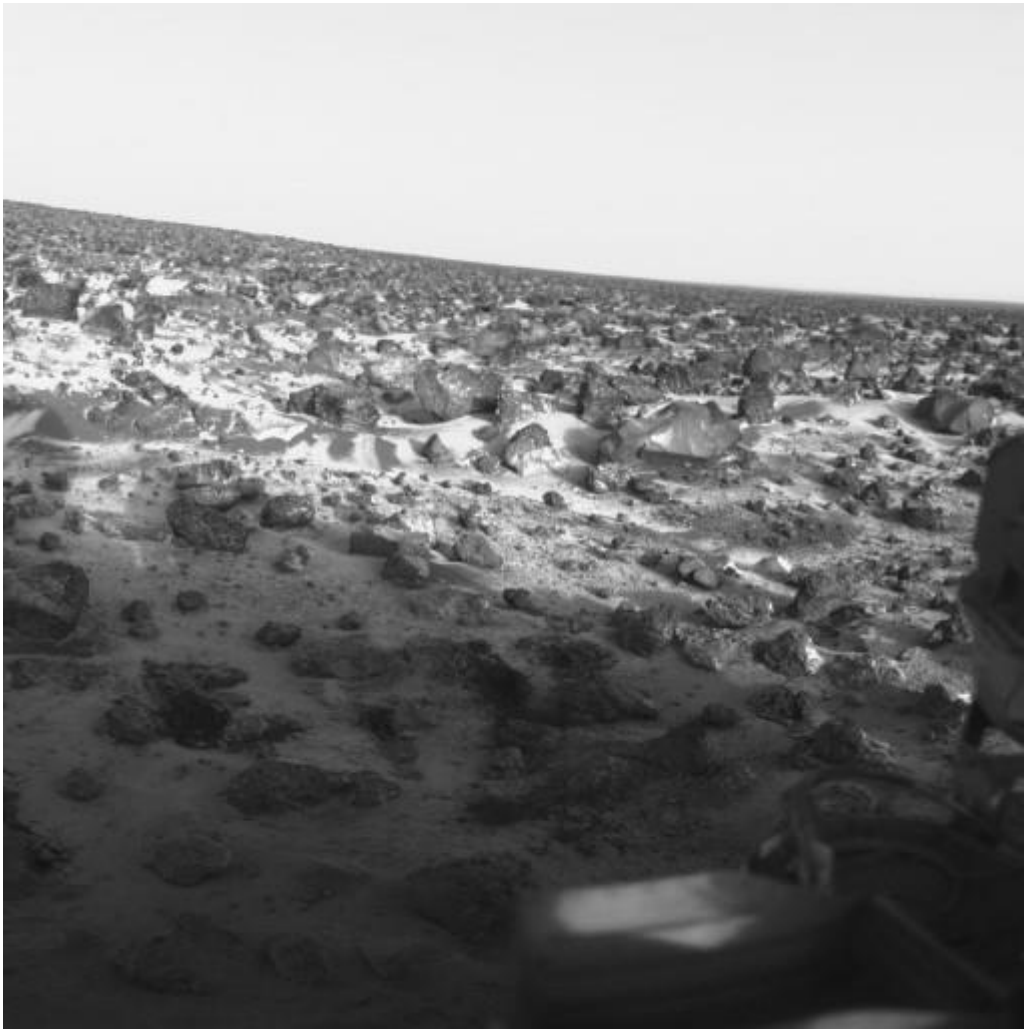

Figure 9. Frost at the Viking 2 Lander site. Image acquired May 18, 1979, almost one martian year after the Viking Lander 2 first detected frost at this site. [Image credit: NASA; Viking 2 Lander image P-21873 (other ID 21I093/960) available online at http://nssdc.gsfc.nasa.gov/imgcat/html/object_page/v12_p21873.html.]