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STUDIES RELATED TO THE DEVELOPMENT OF THE VIKING 1975 LABELED RELEASE EXPERIMENT

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16 Abstract

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Results indicate (1) Label is released by sterilization-treated soil (2) Substantial quantities of label are retained in solution under basic conditions (3) The substrate used as well as position of label in the molecule, affect release of label (4) Label release is depressed by radiolytic decomposition of substrates (5) About 10^5 organisms are required to produce a detectable response*

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SUMMARY

The labeled release life detection experiment on the Viking 1975 Mars mission is based on the concept that microorganisms will metabolize radioactive organic substrates in a nutrient medium and release radioactive carbon dioxide. During the time that operational procedures were being defined and hardware assembled, several experiments, using laboratory equipment, were carried out to evaluate various aspects of the concept

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These results, which affected experiment design and operation, suggest additional areas for testing, add to the data base for interpretation of flight results, and have significance for broader application of this technique for assessing microbial activity

INTRODUCTION

The ability of microorganisms to degrade organic compounds is commonly used to indicate microbial activity in biological samples Release of carbon dioxide (CO_2) is one of several techniques for measuring organic substrate decomposition (ref 1)

Release of CO_2 from a suitable medium by a soil sample forms the basis for the labeled release experiment (refs 2 through 5), one of three life detection experiments on the Viking 1975 mission to Mars. A nutrient medium containing radioactive (C^{14}) substrate is incubated with a soil sample If microorganisms are present, one or more of the labeled compounds may be metabolized, and the products released as gases, principally $C^{14}O_2$ The detection of labeled gas released from the substrates constitutes evidence for the presence of biological processes in the soil Sterilized soil is used as a control

Experiments reported here were designed to investigate certain aspects of the concept during the time that the labeled release experiment was being defined and hardware assembled The experiments describe label release by sterilization-treated soil, effect of soil pH, substrate utilization and stability, and sensitivity of the method The equipment, soils, and substrates used for these studies were those specified and available at the time and were not necessarily the same as

ultimately selected for flight For example, an active $C^{14}O_2$ getter was used here, whereas, in the Viking flight experiment, $C^{14}O_2$ accumulates in the headspace above the soil and is periodically monitored

These studies contributed to the final design and operation of the labeled release experiment, and suggested a number of specific areas to be explored during the ongoing testing phase using the flight-like test standard module instrument These experiments also add to the data base that will be used in interpreting data returned from the Martian surface Finally, the experimental results described below are of significance to the broader use of this technique for the detection of microbial species in terrestrial biological samples

MATERIALS AND METHODS

Substrates

Several radioactive substrate mixtures were prepared by adding labeled organic substrates to a basal medium (see table 1) The substrate mixtures were sterilized by filtration through 0 45-μm membrane filters, aerated by shaking at room temperature for a few hours, sealed in sterile glass vials, and stored at 4° C Some aliquots of substrate 3 were sealed under nitrogen (N_2) Sterility was demonstrated by lack of colony growth on plates streaked with the substrate mixtures and incubated under various conditions The pH (paper) was 7 2

Soil

Several test soils were used in the experiments described here Their properties are shown in table 2 Soil a was air dried, sieved, and the 35–80 mesh fraction used. The remaining soils were used as supplied.

Experimental Chamber

Two 10- by 20-mm glass culture tubes were attached together with a rubber tubing sleeve (see fig 1(a)) The bottom half contained soil and substrate (200 mg and 200 μl, respectively, unless otherwise noted) while the upper half (getter assembly) contained a 12- by 40-mm rolled filter paper moistened with saturated barium hydroxide ($Ba(OH)_2$) After incubating for an interval at room temperature, the getter assembly was removed (a new getter assembly was immediately added to the bottom half), placed in a vial, and counted in a scintillation counter using a scintillation fluid containing 4 g PPO,[1] 0 1 g POPOP,[2] 700 ml toluene, 300 ml ethanol Total accumulated C^{14} counts/min (cpm) were calculated and plotted against time of incubation, as shown in figure 1(b) The overall efficiency of C^{14} collection and liquid scintillation counting was about 62 percent of calculated dis/min.

[1] PPO = 2, 5-Diphenyloxazol.

[2] POPOP = 1, 4-bis-(2-(4-Methyl-5-Phenyloxazolyl)) – Benzene, scintillation grade

TABLE 1 – SUBSTRATE MIXTURES

Substrate number	Basal medium	Organic substrates	Final concentration (mM)	Final activity (μCi/ml)
1	M-9[a]	1-C^{14} glycine	0 01	0 2
		U-C^{14} glycine	0.09	1.0
		U-C^{14} D-glucose	0.5	1 5
		1-C^{14} DL-sodium lactate	0.06	1 5
2	M-9[a]	1-C^{14} glycine	0 08	1 8
		2-C^{14} glycine	0 1	1 8
		U-C^{14} D-glucose	0 1	1 8
		1-C^{14} DL-sodium lactate	0 4	2 0
		3-C^{14} L-sodium lactate	0 1	0 4
3	RM-9[b]	C^{14} formate	0 1	6 5
		1-C^{14} DL-sodium lactate	0 03	1 3
		1-C^{14} glycine	0 07	1 0
		U-C^{14} D-glucose	0 3	1 3

[a]M-9 (ref 5) K_2HPO_4 (1 00 g), $MgSO_4 \cdot 7H_2O$ (0 20 g), NH_4NO_3 (0 20 g), NaCl (0 10 g), soil extract (100 ml), distilled H_2O (900 ml), pH 7 0

[b]RM-9 (ref 6) K_2HPO_4 (5 0 mg), $MgSO_4 \cdot 7H_2O$ (80 mg), NH_4NO_3 (0 20 g), NaCl (0 10 g), soil extract (100 ml), tris-HCl (6.0 gm), distilled H_2O (900 ml), pH 7 0

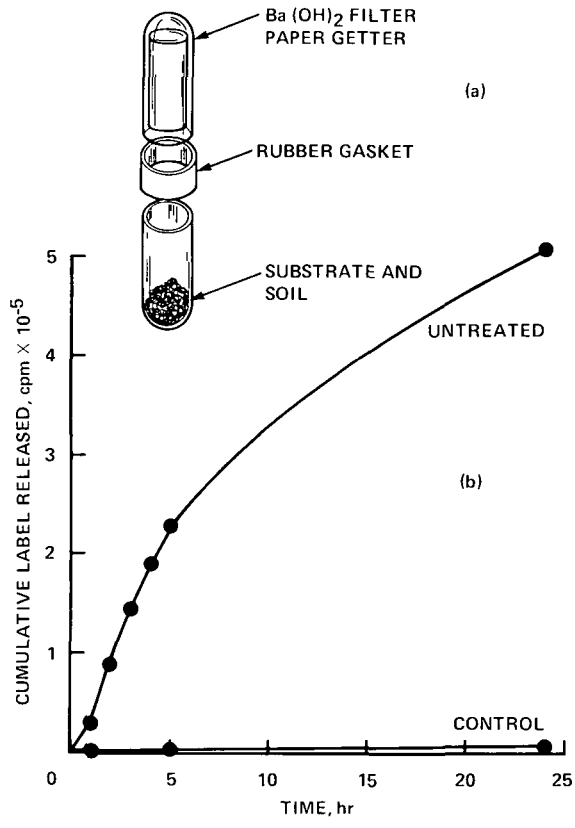
TABLE 2.– TEST SOILS

Soil sample[a]	Description	Paste pH[b]	Bacterial content[c] (aerobes/gm)
a	San Francisco, clay loam	—	$1X10^7$
b	Siskiyou, sandy loam	6 3	$5X10^6$
c	Waukena, sandy loam	10 3	$5X10^6$
d	Bowers Clay, clay	8 1	$5X10^7$
e	Death Valley	8 6	$1\ 4X10^3$
f	Aiken, clay loam	5 6	$1\ 6X10^7$
g	Creek Bed, coarse sand	—	—

[a]Soils a – f were available at Ames Research Center Soil g was supplied by Dr G V Levin, Biospherics, Inc , Rockville, MD

[b]Soil-water slurry.

[c]1 percent tryptic soy agar



(a) Experimental chamber
(b) Label release from substrate 1 by untreated and control (germicide-treated) samples of soil a

Figure 1.– Laboratory apparatus for, and results of, a typical label release experiment

To determine whether $C^{14}O_2$ leaked from the system, a known amount of sodium carbonate ($Na_2C^{14}O_3$) was placed in the chamber, sulfuric acid (H_2SO_4) added, and the $C^{14}O_2$ content of the getter and solution measured All of the starting radioactivity was accounted for

Replicate samples (two to four) were run in all experiments, the data shown represent average values.

Substrate Pretreatment

For testing stability of substrate compounds, 1-ml aliquots of substrate 3 were sealed in 5-ml glass vials under air or N_2 The vials were subjected to one or more of the following treatments heat (40 hr at 125° C in a laboratory oven), gamma radiation (6 hr at room temperature in a cobalt-60 gamma ($Co^{60}\gamma$) cell delivering a total of 1.008 Mrad), and X-ray radiation (10 hr at room temperature in a GE Maxitron 300 delivering a total of 1 Mrad at 55 keV) Prior to use in an experiment, the vials were broken open and flushed aseptically for 20 to 30 min with a mixture of 5 percent CO_2 and 95 percent N_2, at a flow rate of 10 to 15 cc/min, to remove volatile reaction products

Controls

Four methods of soil sterilization were employed autoclaving (2 hr, 121° C, 15 psi (1 05 kg/cm^2) pressure, followed by dry heat for 3 hr at 165° C), dry heat (3 hr at 160° C), germicide (30 min before substrate addition, 0 1 ml Bard-Parker germicide[3] was added to soil), and irradiation (21 or 40 Mrad in a $Co^{60}\gamma$ cell) In addition, label release from substrate was routinely measured in the absence of added soils

[3] Formaldehyde germicide, Bard-Parker, Division of Becton, Dickinson and Co , Rutherford, N J

EXPERIMENTAL RESULTS

Effect of Soil Sterilization on Label Release

Samples of soil a were irradiated, treated with germicide, autoclaved, or heated as described in the previous section. Incubations were carried out with substrate 1 for 121 hr Label released from an untreated soil and from substrate alone are included as controls The results are shown in table 3.

TABLE 3 – EFFECT OF METHOD OF SOIL STERILIZATION ON SUBSTRATE LABEL RELEASE

Soil treatment	Total label released (cpm)
None (active)	715,000
Irradiated	19,500
Germicide	17,500
Autoclaved	5,240
Dry heat	5,100
Substrate alone (no soil)	2,840

Significant label was released from substrate incubated with the treated soil The amount depended upon the method of sterilization Germicide-treated or irradiated soils catalyzed substantial release of label (about 2 5 percent of that released by untreated soil), while autoclaved or dry-heated soils released intermediate amounts (about 0.7 percent) Substrate alone released some label (0 4 percent) However, it was always less than when soil was present

Unless otherwise noted, germicide-treated soil was used as a control because of ease of handling

Label Release as a Function of Soil pH

Soils b, c, and d, with different chemical and physical properties but similar bacterial counts, were incubated for 5 hr with substrate 2. Soils used as controls were pretreated with germicide The results are shown in figure 2.

The three soils, all with bacterial numbers in the range of 10^6 to 10^7/g showed label release responses which could be correlated with the soil paste pH. At the end of 5 hr, the Siskiyou (paste pH 6 3), Bowers Clay (paste pH 8 1), and Waukena (paste pH 10 3) soils yielded recoveries of 8, 0 8, and 0 09 percent (percent of label initially present which was released and trapped on the getter), respectively Label released by all three controls was approximately 0 02 percent

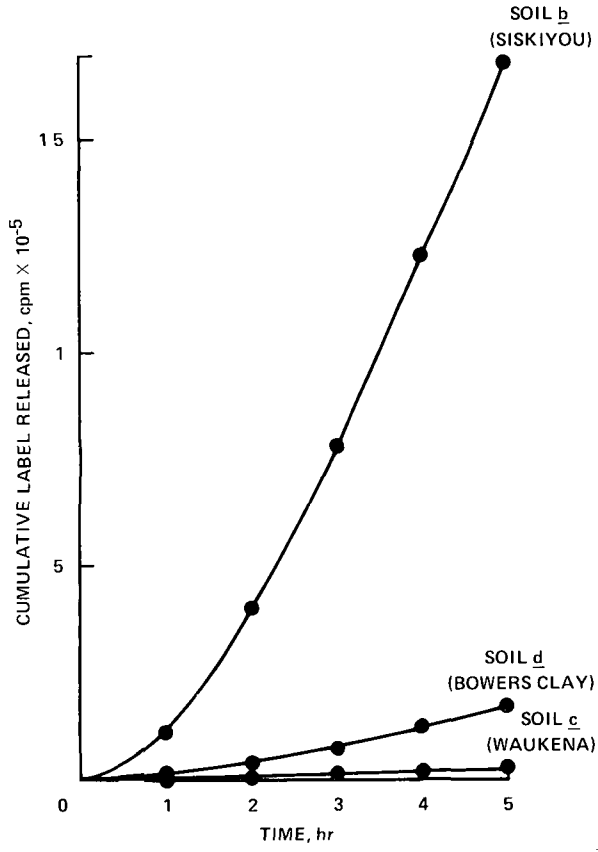


Figure 2.– Label release from substrate 2 added to three soils with different properties

Retention of $C^{14}O_2$ in Solution

Four experimental chambers were assembled, two containing soil a and two with soil a pretreated with germicide. At the end of a 2-hr incubation with substrate 2, all getters were removed and counted, replaced with new ones, and 0.25 ml of 10 percent trichloroacetic acid (TCA) was injected into one untreated and one germicide-treated reaction mixture. After 20 min, all getters were again removed and counted. The results are shown in table 4

In the incubations with untreated soil, large quantities of $C^{14}O_2$ were released from the acidified sample, as compared to that released from the unacidified sample In the germicide controls, the TCA-mediated $C^{14}O_2$ release was consistent with the low level of activity in these samples This shows that interactions between the TCA and substrate were not responsible for the release of the additional $C^{14}O_2$ observed with the untreated soil.

TABLE 4.– EFFECT OF TRICHLOROACETIC ACID ON $C^{14}O_2$ RELEASE

	$C^{14}O_2$ released (cpm)			
	Untreated soils		Germicide controls	
Initial incubation 2 hr	71,000	58,800	133	108
Treatment after initial incubation	None	TCA added	None	TCA added
Subsequent incubation 20 min	16,600	81,100	47	132

Substrate Utilization

Figure 1(b) shows label release by soil from a medium containing a mixture of radioactive substrate components. To test for differential utilization, the release of label from each radioactive substrate component was examined individually Also, individual substrates, with different carbon atoms labeled, were tested to see if some atoms were more readily released than others

To each of five aliquots of M-9 basal medium, one substrate compound, labeled as shown, was added to the indicated final concentration and final activity (a) 1-C^{14} glycine (0 07 mM, 1.7 μCi/ml), (b) 2-C^{14} glycine (0 09 mM, 1 7 μCi/ml), (c) U-C^{14} D-glucose (0.1 mM, 1.7 μCi/ml), (d) 1-C^{14} DL-sodium lactate (0 06 mM, 0 3 μCi/ml), and (e) 3-C^{14} L-sodium lactate (0 1 mM,

0 3 μCi/ml) Soil a was incubated with each of the five substrates Cumulative label released was tabulated and percent recovery of label calculated as a function of incubation time Germicide-treated soil was used as a control for each substrate

Figure 3 indicates that the percent of label released from the starting material was dependent on both the nature of the substrate and the position of the label in the molecule Within a given molecule (lactate or glycine), the carboxyl carbon was released more effectively than the interior carbon atoms Label was released more readily from the carboxyl positions of lactate and glycine than from the uniformly labeled glucose

The controls released label ranging from 2 5 percent for 1-C^{14} glycine to 0 08 percent for U-C^{14} D-glucose

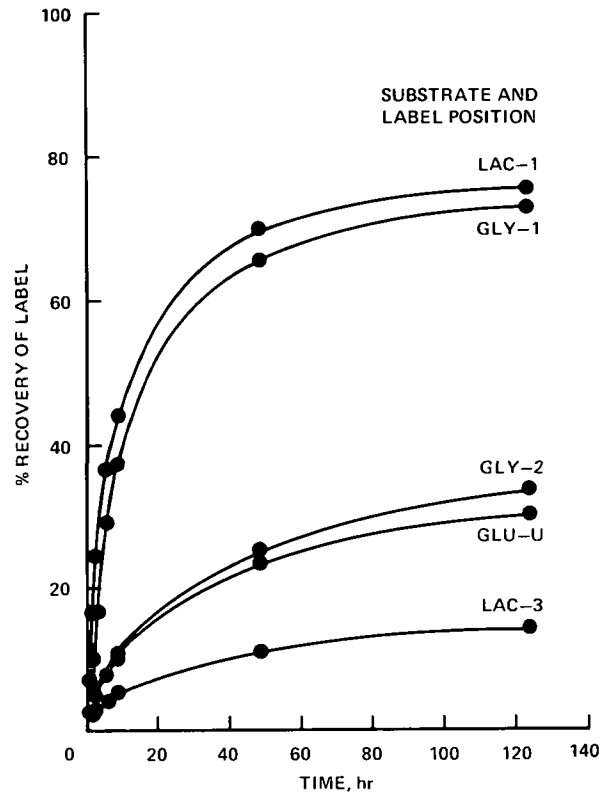


Figure 3.– Percent recovery of label released by soil a from various substrates 1-C^{14} glycine (GLY-1), 2-C^{14} glycine (GLY-2), U-C^{14} D-glucose (GLU-U), 1-C^{14} DL-sodium lactate (LAC-1), and 3-C^{14} L-sodium lactate (LAC-3)

Radiation-Induced Substrate Decomposition

Substrate 3, sealed either under air or N_2, was subjected to heat and/or gamma radiation, or X-ray radiation as noted in Materials and Methods Following these treatments, but prior to use, the substrate mixture was flushed with CO_2 and N_2 and counted to determine how much, if any, labeled substrate was converted to volatile decomposition products Table 5 shows the percentage of label remaining in the treated solutions In each case, the percentages refer to the untreated control Even in the worst case, no more than 14 percent of the initial label was volatilized by the heat and/or radiation treatment

Soils e, f, and g (250 mg each) were then incubated for 24 hr with 50 μl of each of the treated and flushed labeled substrate mixtures Figure 4 shows the label released in the first 8 hr by each soil from substrate irradiated under air but not heated A control was run with unirradiated substrate, and a dry heat sterilized soil control was run with both irradiated and unirradiated substrate Of the sterilized soil controls, only the one run with unirradiated substrate is shown, since irradiated substrate yielded similar or lower counts.

Figure 5 gives the results of a similar experiment using substrate heated and irradiated under air The substrate control in this case was heated but not irradiated. As in the previous experiment, sterilized soil controls were run with each treated substrate

TABLE 5 – EFFECT OF SUBSTRATE TREATMENT ON LABEL VOLATILIZATION

Substrate treatment	Atmosphere	Label left in solution[a] (%)
Untreated	Air or N_2	100
Heated 40 hr, 125° C	Air	97
X-ray, 1 Mrad		88
X-ray, heated		86
$Co^{60}\gamma$, 1 Mrad		93
$Co^{60}\gamma$, heated		86
Heated 40 hr, 125° C	N_2	100
$Co^{60}\gamma$, 1 Mrad		91

[a]After flushing with CO_2 and N_2 as described in Materials and Methods.

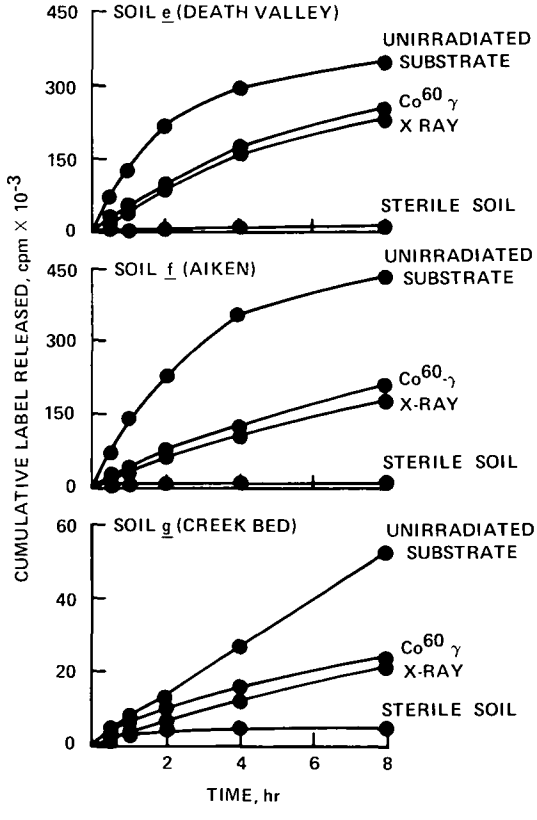


Figure 4.– Label release by three different soils from substrate 3 irradiated under air

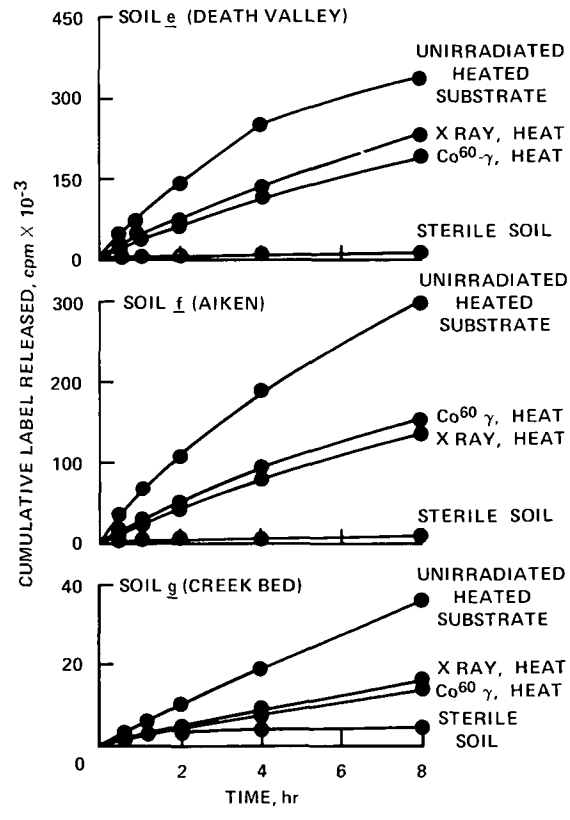


Figure 5.– Label release by three different soils from substrate 3 heated and irradiated under air

Figure 6 shows label released in the first 8 hr by the three test soils from substrate heated or irradiated under N_2 All other conditions were the same as in the preceding experiments

In each case, the scale on which the results for Creek Bed soil (soil g) was plotted was reduced by a factor of five relative to the other soils, since Creek Bed soil was less active than the others during the first 8 hr. Irradiation of substrate with either $Co^{60}\gamma$ or X-ray results in a depression of label release which is approximately the same for either form of radiation. The depression is noted with all three soils and occurs whether the substrate was heated or not heated (compare figs 4 and 5) and whether it was treated under air or under N_2 (compare figs 4 and 5 with 6).

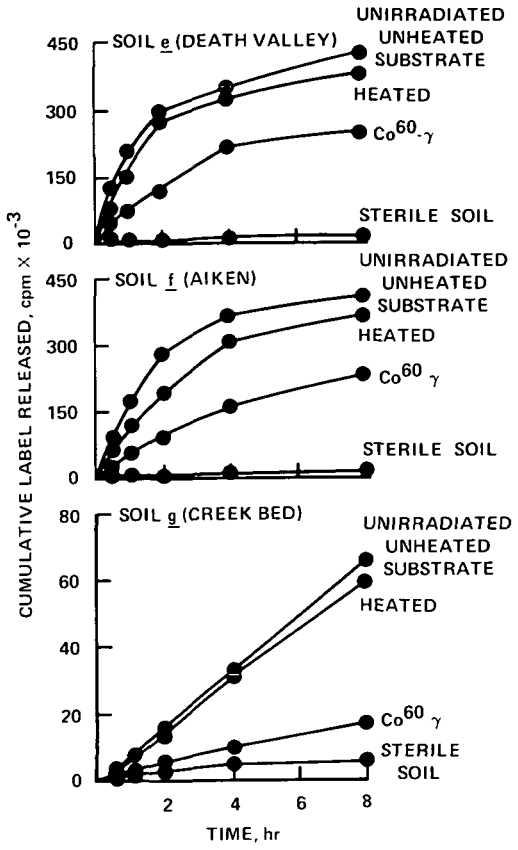


Figure 6.– Label release by three different soils from substrate 3 heated or irradiated under N_2

After 24 hr, the accumulated counts were compared to starting activity and percent recoveries calculated. These recoveries (table 6) were based on label present in substrate after treatment and flushing and, therefore, were not affected by losses due to radiolytically produced volatiles The depression of label released by soils from irradiated and heat/irradiated substrates observed at 8 hr still holds after 24 hr, with losses ranging up to 90 percent compared to untreated substrate. Substrate heated under air yielded less label than substrate heated under N_2

After 24 hr, the amount of label released from untreated substrate by Creek Bed soil was the same as for the other test soils, indicating that the kinetics of release between 8 and 24 hr were very different for this soil However, the depression of label released from treated substrates by this soil was much more pronounced after 24 hr than at 8 hr, and much greater than occurred in the other two soils. Finally, Creek Bed soil was less able than the other soils to release label from substrate subjected to combined heat and irradiation.

Correlation of Response with Microbial Content

Estimation of the minimum number of organisms required to release label significantly greater than control values was determined by two methods dilution of soils of known bacterial content with sterilized soil, and using known numbers of cells from pure cultures in place of soil or together with sterilized soil.

TABLE 6 – LABEL RELEASE FROM TREATED SUBSTRATES BY SOILS

Substrate treatment	Atmosphere	Label released in 24 hr (%)		
		Aiken	Death Valley	Creek Bed
Untreated	Air or N_2	52	52	54
Heated 40 hr, 125° C	Air	45	50	42
X-ray, 1 Mrad	↓	42	42	33
X-ray, heated	↓	37	41	10
$Co^{60}\gamma$, 1 Mrad	↓	41	40	24
$Co^{60}\gamma$, heated	↓	38	40	10
Heated 40 hr, 125° C	N_2	49	53	56
$Co^{60}\gamma$, 1 Mrad	↓	39	37	5

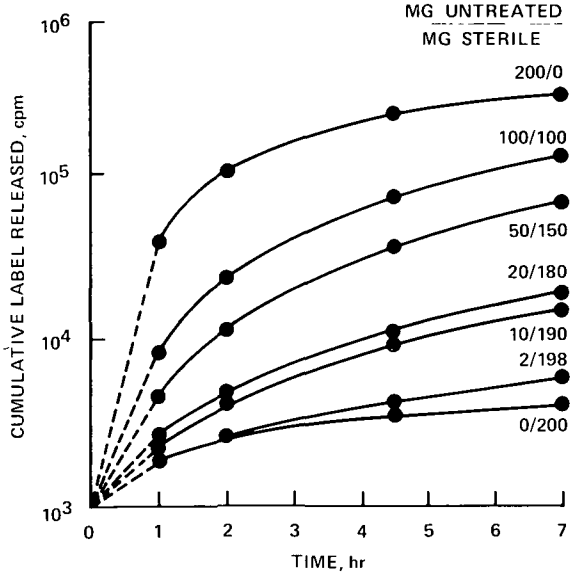


Figure 7 – Effect of dilution of untreated with sterilized soil a on label release from substrate 1

Soil dilution.– Soil a, mixed (diluted) with autoclaved soil a in the proportions shown in figure 7, was incubated with substrate 1 Figure 7 shows the first 7 hr of a 76-hr incubation

Amounts of label significantly greater than the sterilized soil control were released from all soil mixtures, except the 1 100 dilution, during the first hour. After 4 hr, even the 1 100 sample started to release detectable quantities Since the unsterilized soil had 10^7 bacteria/g, these results indicated that 10^5 organisms (in 200 mg of soil) were needed before label release occurred at a rate greater than the control There was some proportionality between the quantity of unsterilized soil present (and therefore cell number) and total net label release during the 7-hr incubation period

After 76 hr, all soil samples (except the autoclaved control) had released greater than 550,000 cpm (data not shown) This indicated that samples containing initially low numbers of cells ultimately released label as completely as those samples with higher initial cell counts

Another set of soil dilution experiments was carried out with soils b and c. These mixtures were prepared by mixing appropriate quantities of untreated soil (of known bacterial content) with corresponding autoclaved soil (three cycles of 1-hr autoclaving, 24 hr apart) In soil c the dilutions yielded samples containing 0, 10^2, 10^3, 10^4, and 10^5 bacteria (aerobic) in 200 mg Substrate 2 was added to each and incubated for 55 hr Another sample of the soil containing 10^5 bacteria was treated with germicide and used as the control The first 6 hr of this incubation are shown in

figure 8 The sample containing 10^5 bacteria at the start was the only one that gave a positive response in the first 6 hr

After 55 hr, all untreated samples had released more than 460,000 cpm The germicide control and the sample containing only autoclaved soil both released about 5,000 cpm during the same time period (data not shown).

A similar set of experiments was conducted with soil b (data not shown) The dilutions yielded samples containing 0, 10, 10^2, 10^3, and 10^4 bacteria (aerobic) in 200 mg After 6 hr incubation, there was no significant difference between the label released by any of the samples and the germicide control. However, after 55 hr, all samples had liberated nearly 900,000 cpm, except the germicide and autoclaved controls

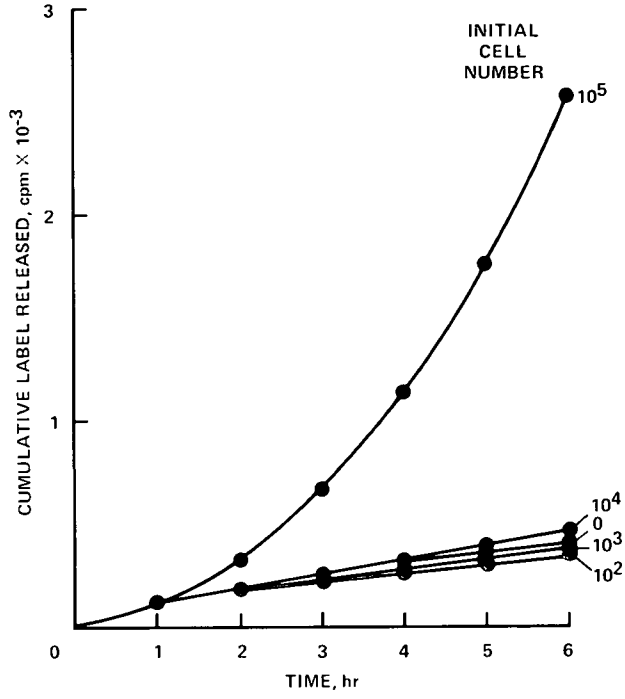


Figure 8.– Label release from substrate 2 by soil c The bacterial content was adjusted by diluting untreated with sterilized soil

Pure cultures – An organism (small, motile rod) was isolated from soil a cultured in Brain Heart Infusion (BHI, Difco) broth at room temperature. A stationary culture was harvested, resuspended in BHI, and counted in an electronic particle counter (numbers were verified by plate counts) It was then diluted in BHI until 0 1 ml samples contained 10^2, 10^4, 10^6, and 10^8 cells. One-tenth ml of each dilution was placed in an experimental chamber (0.1 ml sterile water served as a control) together with 200 mg of soil a which had been sterilized by irradiation (21 Mrad) The reaction was initiated by addition of substrate 1, and the results are shown in figure 9

This experiment indicated that 10^4 to 10^6 cells were needed to produce significant label release within the first few hours However, even 10^2 cells were detected after 30 hr of incubation

E coli K12 was grown aerobically at 30° C in 2 percent glucose, 1 percent yeast extract, 2 percent peptone medium Cells were harvested in stationary phase (15 hr), washed, resuspended in growth medium, and counted in an electronic particle counter Dilutions in growth medium were made such that 10^3, 10^4, 10^5, 10^6, 10^7, and 10^9 cells were contained in 0 1 ml. One-tenth ml of fresh growth medium was used in place of irradiated soil To this was added 0 1 ml of the respective cell dilutions (0 1 ml of sterile water served as control), and substrate 1. Label release was followed for 24 hr, with the first 7 hr shown in figure 10.

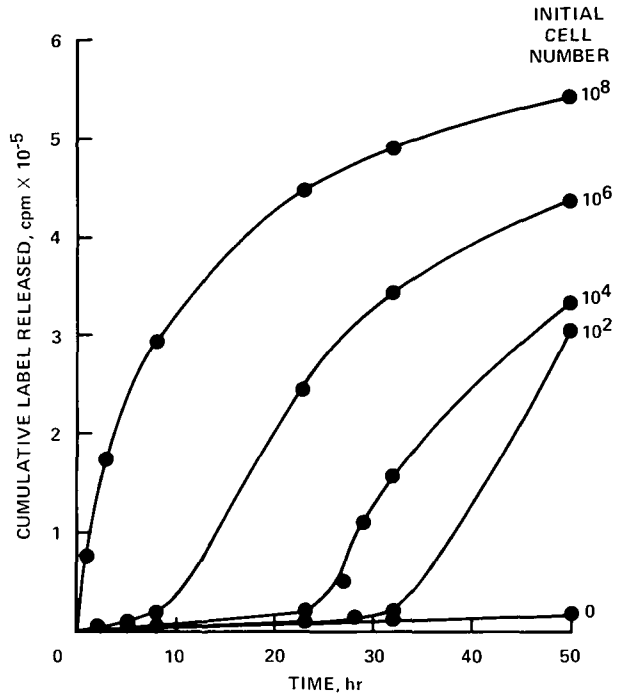


Figure 9 – Label release from substrate 1 by a soil microorganism as a function of initial cell number

An initial concentration of 10^6 cells gave a significant early response The curves for 10^3, 10^4, and 10^5 cells were not significantly different from the control during the experimental time interval shown After 24 hr, however, these samples had released 14,700, 44,100, and 87,400 cpm, respectively, compared to 1,400 cpm for the control.

S cerevisiae, strain LK2G12, was grown as described for *E coli* and harvested in log phase (14 hr) Experimental procedures were as above, except that initial cell numbers in 0 1 ml were 10^2, 10^4, 10^6, and 10^8, and incubation was for 7 hr The data (fig. 11) indicated that a minimum of between 10^4 and 10^6 cells were needed to produce a response in the early hours of incubation.

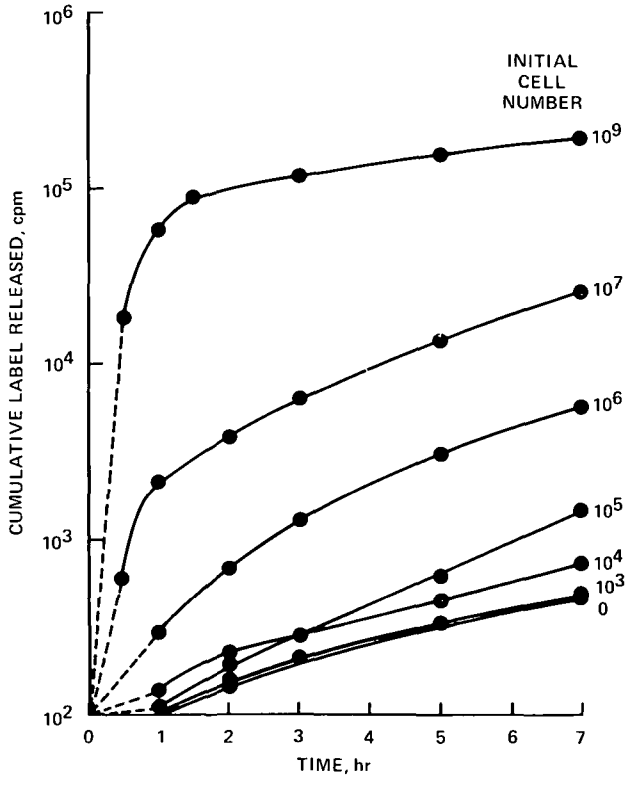


Figure 10.– Label release from substrate 1 by *E coli* as a function of initial cell number

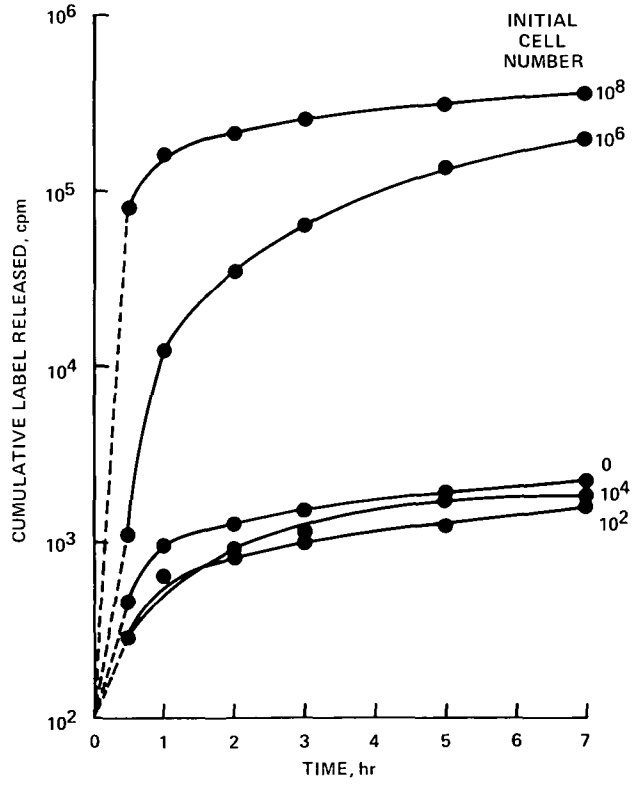


Figure 11.– Label release from substrate 1 by *S cerevisiae* as a function of initial cell number.

DISCUSSION

Release of label from radioactive organic compounds is a valuable technique for exploring the biological activity of natural environments. However, this method requires careful preparation of controls to discriminate between what may be nonbiological release and that definitely attributable to biological activity. In the present study, four methods, including one used on the Viking 1975 mission (dry heat, 3 hr, 160° C), were used to sterilize soil samples used for controls The data in table 3 show that the technique of soil sterilization had a marked influence on the extent to which label release by control samples approached zero Even without added soil, sterile substrate released some label. This probably resulted from natural decomposition of the labeled compounds and must be taken into account in interpreting experiments.

Soil subjected to sterilization treatment, regardless of method, always increased the rate of label release from substrate, indicating that soil had a sterilization-resistant capacity for catalyzing substrate decomposition This activity could have resulted from sterilization-resistant organisms (ref 7, dry-heat-resistant organisms), a product of prior biological activity (sterilization-resistant enzymes), or some chemical or physical property of the soil samples (microenvironments within the soil which protect enzymes and/or organisms). The release of label from sterile substrate by sterilization-treated soil (regardless of its cause) is important when judging the biological competency of soils only marginally populated with active organisms, and may restrict the sensitivity in these cases

Figure 1(b) shows the results of a typical label release experiment using a biologically rich soil. After an interval of rapid label release, the reaction slowed, as indicated by the gradual flattening of the curve of total label release In the best cases, only about 75 percent of the label was recovered after 120 hr of incubation This means that a large fraction of the initial radioactivity was not released and/or trapped by the getters

There are a number of possible explanations for such an occurrence First, the unrecovered label may have leaked from the system (this was unlikely, however, as noted in Materials and Methods) Second, there may have been preferential utilization of substrates, with one or more of the labeled substrates not being utilized as efficiently as the others Third, the substrates may have been metabolized to $C^{14}O_2$, but a sizeable fraction of this $C^{14}O_2$ was trapped in the reaction mixture by adsorption, solution, etc Fourth, a fraction of the substrates may have been metabolized to nonvolatile components or bound to or incorporated into structural components of the cells. Fifth, substrates may have been converted to volatile products, other than $C^{14}O_2$, which were not trapped by the $Ba(OH)_2$ getters.

The experiments reported here provide evidence that at least two of these possibilities may have contributed to this effect For instance, figure 3 shows preferential substrate (and specific molecular structure) utilization with percent recoveries in 120 hr varying from 14 percent (3-C^{14} L-sodium lactate) to 75 percent (1-C^{14} glycine and 1-C^{14} DL-sodium lactate) It is noted that carboxyl carbons are readily converted to gaseous products, whereas interior carbons are not released as rapidly under these experimental conditions. Many possible explanations exist for this result However, no experimental attempt was made to trace the metabolism of individual carbon atoms

Experiments carried out with soil b (Siskiyou), soil c (Waukena) and soil d (Bowers clay) indicated that with roughly similar numbers of organisms there was a dramatic difference in label release (fig 2) Although this may have been due, in part, to metabolic differences of the organisms contained in each of the three soils, the properties of the soils themselves could have been responsible Both Bowers clay and Waukena soils are considerably more basic than Siskiyou soil and might have retained $C^{14}O_2$ in solution to a greater extent than Siskiyou, since the solubility of CO_2 increases markedly with increasing pH

Further support for this possibility was obtained in the experiment that involved adding TCA to a reaction mixture that had been actively releasing label for 2 hr (see table 4) Acidification resulted in release, during the next half hour, of more label than had already been released up to that point and five times the amount of label expected to be released during this interval if there was no addition of TCA. These results showed that considerable amounts of $C^{14}O_2$ were trapped in solution, at least during short incubations, even in the presence of an active getter Therefore in high-pH environments, the technique may require that pH be lowered after incubation to free released label from solution, and thereby make it available for trapping and subsequent counting

Determination of the sensitivity of the label release technique, in terms of the minimum number of organisms required to detect a valid response, is complicated by possible increase in cell numbers during incubation From a knowledge of the kinetics of both growth and CO_2 production, it should be possible to establish a relationship between the number of cells and the amount of CO_2 produced In practice, however, the necessary kinetic data are not generally obtainable, particularly for the mixed populations found in soil It should also be noted that the rate of CO_2 production in a population is not a simple function of the numbers of cells, but also reflects their physiological condition which governs their metabolic activity Nevertheless, under the specific experimental conditions used here, a lower limit of detectability was estimated to be about 10^5 organisms. This limit was based on the release of label by the test population during a time interval short enough to minimize growth (the first 1 to 2 hr of incubation) Figures 7 and 8 show that, in the soil dilution experiments, 10^5 cells gave a positive response in the first 2 hr, whereas 10^4 cells did not. In the three pure culture experiments (figs 9, 10, and 11), 10^6 cells gave a positive response, but 10^4 did not This led to the conclusion that, under these experimental conditions, about 10^5 organisms, or more, were required to detect a label release response significantly greater than the control in the first 2 hr of incubation, independent of the soil or culture type. However, greater sensitivity was observed during experiments carried out for other purposes (figs. 4, 5, and 6) In these cases, with Death Valley soil, 10^3 cells produced a significant response in 1 to 2 hr However, the experimental conditions differed in that a different substrate mixture was used and the ratio of soil to nutrient volume was larger

In three other experiments (data not shown), 10 bacteria or less at the start gave strongly positive responses, but only after 35 to 90 hr of incubation, such an interval being sufficient for substantial growth Data shown here (fig 9) indicated that, starting with 100 cells in broth culture, significant label was released, but only after 30 hr of incubation, during which an increase in turbidity of the incubation medium was also observed.

Therefore, under the experimental conditions employed here, at least 10^5 cells were needed to detect an immediate response This number could be lower and may be dependent on the experimental conditions (soil-to-nutrient volume ratio, active getter, etc) These data suggest that care is required in interpretation of responses from nonreproducing slowly-metabolizing organisms

Radiation-induced decomposition of the labeled organic substrate molecules could limit the versatility of the label release technique If volatiles are produced by this process, they can be effectively removed by thorough aeration of the substrate mixture prior to use. However, radiolytic decomposition of the organics can also result in the production of nonvolatile inhibitory compounds or compounds not readily utilized by the test organisms, and the effect on experimental results may be significant

Radiolytic decomposition can increase significantly under various conditions. For example, storage of the radioactive substrate mixture for a long period of time, as is required on the Viking flight to Mars (ref. 8), may amplify this effect Similarly, increasing the specific radioactivity of the mixture, which would have resulted from the proposed addition of sodium sulfate ($Na_2S^{35}O_4$) to the Viking labeled release nutrient (ref. 5), can also promote increased degradation Therefore, in an attempt to quantitate the probable effects of radiolytic decomposition, accelerated radiation treatment of substrate 3 was carried out The radiation doses defined in Materials and Methods were calculated to approximate levels expected to be generated in a substrate mixture if it contained amounts of $Na_2S^{35}O_4$ proposed for inclusion (10 μCi/ml at Mars) and if it were to be stored for the proposed flight time to Mars (24 mo, including preflight storage time)

The data in table 5 verify the assumption that breakdown of substrate by radiolytic processes results in the production of some volatiles However, the quantities of volatiles produced were sufficiently small that they impacted the experiment only minimally. A more serious problem, as revealed by examination of table 6 and figures 4, 5, and 6, is caused by either inhibitors or refractory products formed in solution The data show that soil catalyzed a lower percentage of label release from treated substrates than from untreated substrates. Although the bulk of the label remained in solution following treatment, it was apparently no longer in a form that could be converted to volatile products by the active species. In the most extreme cases, label recovered in 24 hr, and corrected for loss of radiolytically formed volatiles, was reduced to 1/10 that of an untreated substrate control.

Irradiation in the presence of oxygen increased substrate degradation, as measured by label release by Creek Bed soil but not by the other two soils. This suggests that Creek Bed soil is a more sensitive indicator of substrate damage and that the biological component of Aiken and Death Valley soils is more tolerant of at least some of the damaged materials produced in irradiated substrate It should also be noted that the presence of oxygen tended to increase damage caused by heating alone In this case, both the Aiken and Creek Bed soils were useful in indicating substrate damage.

Heat treatment was compared with radiation-induced damage, and synergism between the two stress factors was examined Although in ordinary applications heating could be avoided, spacecraft sterilization constraints for flight applications (ref. 5) require that substrate be subjected to heating comparable to that employed here. Heating was found to increase degradation in radiation-treated substrate samples, as measured by label release by Creek Bed soil

Creek Bed soil, which was unusual in that it responded much more slowly than the other soils to all substrate additions, was more severely affected by all substrate treatments (table 6 and figs. 4, 5, and 6). This suggests that using the results obtained from one soil to predict the behavior of other soils must be approached with care The general observation, however, that radiation treatment

causes changes to the substrate molecules such that they are less efficiently metabolized by soil organisms is supported by measurements with all three test soils

These experiments indicate that radiation-induced substrate decomposition can have a significant effect on the results of label release experiments However, the effect can probably be markedly lessened by selection of organic substrate molecules which are more stable to radiolytic decomposition and by using the lowest specific radioactivities possible under the given experimental conditions

Results reported here aided in the final design and operation of certain aspects of the Viking labeled release experiment; for example, establishment of the control sterilization regime at 160° C dry heat for 3 hr, selection of specific nutrient substrate components, and elimination of $Na_2S^{35}O_4$ from the nutrient medium. Additional experiments to be carried out during the current testing phase, as suggested by this report, include responses from soils with differing microbial populations and chemical and physical properties, effect of soil pH on $C^{14}O_2$ release, and label release by sterilized soil

CONCLUSIONS

This study was made to evaluate various aspects of the concept on which the labeled release life detection experiment on the Viking 1975 Mars mission is based. Measurement of release of radioactive carbon dioxide from radioactive organic substrate by soils was accomplished using routine laboratory equipment. The major conclusions are

1 Label is released from substrate by sterilized soil and is dependent on the method of sterilization This may be an important factor when judging the biological competency of soils marginally populated with active organisms.

2 Quantity of label released from substrate by soils depends on the specific substrates used as well as on the position of the label within the molecule

3. Since the solubility of carbon dioxide increases markedly with increasing pH, basic soils may cause significant amounts of label to be retained in solution.

4 Although sensitivity of the method depends on factors such as the physiological condition and nature of the organisms and the experimental and environmental conditions, small numbers of cells can be detected given enough time for sufficient growth or metabolic activity.

5 Detection of an early response requires approximately 10^5 organisms.

6 Radiolytic decomposition of substrates may result in the production of nonvolatile inhibitory compounds or compounds not readily utilized by soil microorganisms

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Moffett Field, Calif 94035, January 9, 1976

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