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Report

Annual Report
1973

"Automated Microbial Metabolism Laboratory"

Contract No. NASW-2280

Prepared for:

National Aeronautics and Space Administration
Headquarters
Washington, D. C. 20546

Prepared by:

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Rockville, Maryland 20852

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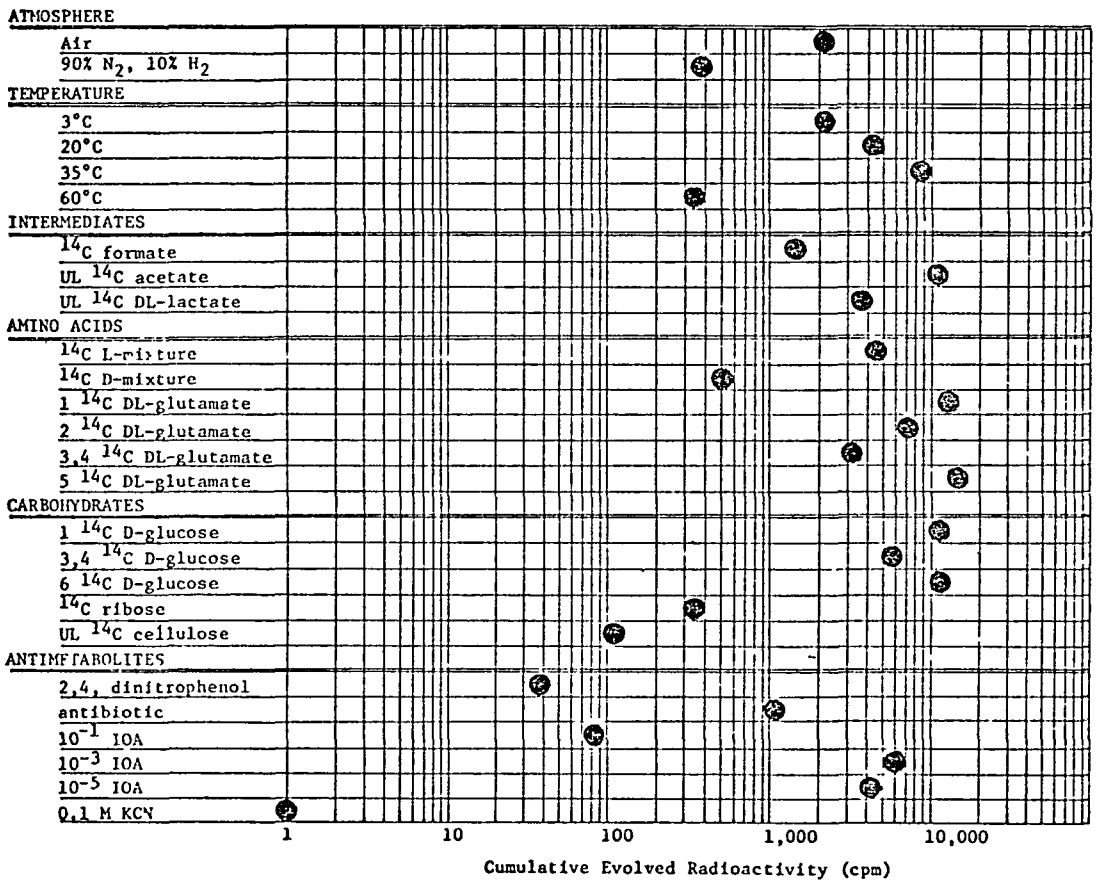
Soil or Organism Phoenix Soil Isolate #1 Incubation Time 2 Hours

Standard Medium FLGG



Frontispiece

Soil Isolate Library Profile

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ABSTRACT

Development of the Automated Microbial Metabolism Laboratory (AMML) concept was continued. The focus of effort was upon the advanced labeled release experiment. Specifically, labeled substrates, inhibitors, and temperatures were investigated to establish a comparative biochemical profile. A library of profiles on soil and pure cultures of bacteria isolated from soil was begun.

A separate task, to develop strategy for the return of a soil sample from Mars, was also undertaken.

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SUMMARY

Laboratory experimentation directed toward the development of methods for detecting and elucidating extraterrestrial metabolism has been performed. Work cented upon the use of the basic Viking labeled release experiment (detection of radioactive gas evolved from ^{14}C labeled substrates by soil microorganisms) using a series of specifically labeled substrates, various environmental conditions, and chemical inhibitors. Selection and testing of these parameters constituted the main effort. Orientation was toward establishing methods for obtaining data for comparison with terrestrial data and toward methods which might be used to assess potential biohazards of alien life forms.

A series of test parameters, listed in Table 1, was investigated. Work was concentrated on specific aspects of the entire series, such as glutamate metabolism, temperature tolerance, and the multiple substrate addition mode for conducting tests. In addition, a soil and pure culture test profile library involving all parameters was begun. An example of a characteristics profile which results from the test series thus far developed is shown in the Frontispiece. In order to provide a most complete library, profiles at three time intervals, T_2, T_6, and T_{22} (the subscript refers to hours of incubation), were prepared. The T_2 profile in the Frontis Piece presents initial responses. However, comparison of T_2 with T_6 or T_{22} provides further information concerning enzyme

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Table 1

Candidate Test Parameters for the Advanced Labeled Release Experiment

ATMOSPHERE ABOVE SOIL

1. Air (aerobic)
2. 90% N_2, 10% H_2 (anaerobic)

TEMPERATURE

3. $3^{o}C$
4. $20^{o}C$
5. $35^{o}C$
6. $60^{o}C$

SUBSTRATES

Intermediate Metabolism

7. ^{14}C formate
8. ^{14}C acetate
9. ^{14}C lactate

Amino Acids - Protein Metabolism

10. ^{14}C L-amino acid mixture
11. ^{14}C D-amino acid mixture
12. 1 ^{14}C DL-glutamate
13. 2 ^{14}C DL-glutamate
14. 3, 4 ^{14}C DL-glutamate
15. 5 ^{14}C DL-glutamate

Carbohydrate Metabolism

16. 1 ^{14}C D-glucose
17. 3, 4 ^{14}C D-glucose
18. 6 ^{14}C D-glucose
19. ^{14}C ribose
20. 1 ^{14}C cellulose

Antimetabolites

21. 2, 4 dinitrophenol
22. antibiotic antimicotic mixture
23. 10^{-1}M IOA
24. 10^{-3}M IOA
25. 10^{-5}M IOA
26. KCN

Note: Media and antimetabolite solutions and procedures are given in Appendix I.

induction, metabolic rate, inhibition, and the like. For example, the information on Phoenix Soil Isolate No. 1, shown in the Frontispiece, allows prediction that this organism is facultatively anaerobic but more active aerobically and obtains energy chiefly by oxidative phosphorylation; has a temperature optimum at or above 35°C, but is killed by 60°C; metabolizes lactate, acetate, and formate but shows greater response to acetate and lactate than formate; prefers L-amino acids over D-amino acids; exhibits citric acid cycle metabolism of glutamate, metabolizes glucose via a combination of pathways most likely including the glucuronic acid pathway; does not rapidly degrade the biopolymer cellulose; is not susceptible to penicillin, strytomycin and amphotericin B; is inhibited by 10^{-1} Molar but not by 10^{-3} Molar iodoacetic acid; and is completely deactivated by 0.1 M KCN.

Four natural soils were investigated using the labeled release experiment at temperatures ranging from 0°C to 80°C. Data were analyzed for peaks in the rate of respiration as well as total radioactivity evolved. Data on these soils which contained natural mixed populations appeared to indicate optima at 20°C, 35°C and 60°C.

Glutamate metabolism utilizing specifically labeled DL-glutamate was studied. Natural soils and some pure culture soil isolates showed the following pattern of utilization: $1\ ^{14}C > 2\ ^{14}C \cong 5\ ^{14}C \gg 3\text{-}4\ ^{14}C$. Such a $^{14}CO_2$ yield pattern may be explained by citric acid

cycle metabolism. Other pure culture results showed the following pattern: 1 $^{14}C \geq 2\ ^{14}C > 3\text{-}4\ ^{14}C \gg 5\ ^{14}C$. These latter data do not appear to conform with the citric acid cycle pathway of glutamate dissimilation. The study demonstrates the utility of the method for investigating pathways of metabolism.

Also investigated in conjunction with glutamate metabolism was the multiple addition mode for sequentially adding substrates to a single growth chamber. The feasibility of performing the glutamate metabolism experiment in this manner has been demonstrated. Yields of CO_2 from specifically labeled glutamate are unaffected by the order of addition or by preceding additions of ^{14}C glucose, ^{14}C formate and ^{14}C lactate. The possibility of forming a large portion of the tests given in Table 1 in a single growth chamber is indicated.

The effect of several inhibitors on test soils and pure cultures isolated from those soils was investigated. Iodoacetic acid was used as a model in a procedure designed to determine inhibitory levels. Results indicate that the in vitro sensitivity level of inhibitors may be determined by the labeled release technique.

Another task was directed toward development of a strategy for the return of a soil sample from Mars. The history of planetary quarantine is reviewed. The rationale for returning a sample from Mars is considered. An initial assessment of the potential biological hazards involved in returning a sample is discussed.

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A canvas of leading scientists familiar with the space program was conducted for opinions regarding value and justification, hazards, and quarantine recommendations for return Mars samples. From the diverse opinions collected, a series of questions has been formulated outlining those issues required for full assessment of a return Mars sample.

This background information will serve as foundation for the ongoing considerations of a return Mars sample program.

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I. ADVANCED LABELED RELEASE

A. Introduction

Research has been continued as part of the AMML concept to develop a series of labeled release experiments designed to provide comparative biology as well as life detection capabilities. Efforts described in this report are directed toward: (1) the selection of various parameters which may provide a metabolic fingerprint of pure cultures and soil populations, (2) advancement of state-of-the-art techniques for conducting the experiments, and (3) establishment of a soil/pure culture data bank to provide a library of labeled release response profiles for comparison with each other and with possible future extraterrestrial responses.

A tentative list of distinguishing parameters including substrates, atmospheres, antimetabolites, and temperature has been established. The effects of temperature and the metabolism of ^{14}C glutamate have been investigated most extensively; however, all parameters listed in Table 1 were explored in labeled release experiments on a variety of soils and on pure cultures isolated from those soils.

B. Temperature Studies

Four test soils were charged with a medium composed of ^{14}C glucose (5 μCi/ml, 10^{-3}M) and ^{14}C glutamate (5 μCi/ml, 10^{-3}M), and incubated at various temperatures ranging from $0^{o}C$ to $80^{o}C$.

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Evolved radioactivity was collected at intervals with $Ba(OH)_2$ wetted filter pads and counted with a Nuclear Chicago gas flow counter. Evolved radioactivity by the four soils at the various incubation temperatures is plotted cumulatively in Figures 1 through 4.

All test soils were found to be biologically active over the entire 0° - $80^{\circ}C$ range of incubation temperatures which were studied. However, both the rate of $^{14}CO_2$ evolution and the total cumulative evolved $^{14}CO_2$ were temperature dependent. Twenty-four hour cumulative evolved radioactivity (cpm) for various incubation temperatures is shown in Figure 5. Wyaconda and Meadow soil showed the greatest production of $^{14}CO_2$ at temperatures of $60^{\circ}C$ and $70^{\circ}C$. The Yuma and Phoenix soils, on the other hand, showed maximum production of $^{14}CO_2$ at $40^{\circ}C$ and $50^{\circ}C$, and total production was considerably reduced by incubation temperatures of $70^{\circ}C$. All soils showed a lower cumulative evolution of $^{14}CO_2$ at $80^{\circ}C$ than was found at lower temperatures, thus indicating that this temperature is at or near the upper limit of tolerance for the organisms present in these soils. Figure 2 best demonstrates the relationship between temperature and cumulative $^{14}CO_2$ evolution since most of the soil responses had plateaued by 24 hours. Production of CO_2 generally appears to be much greater at higher temperatures than at lower temperatures. Therefore, proportionately, a greater amount of nutrient must be cycled through energy-producing, CO_2 generating

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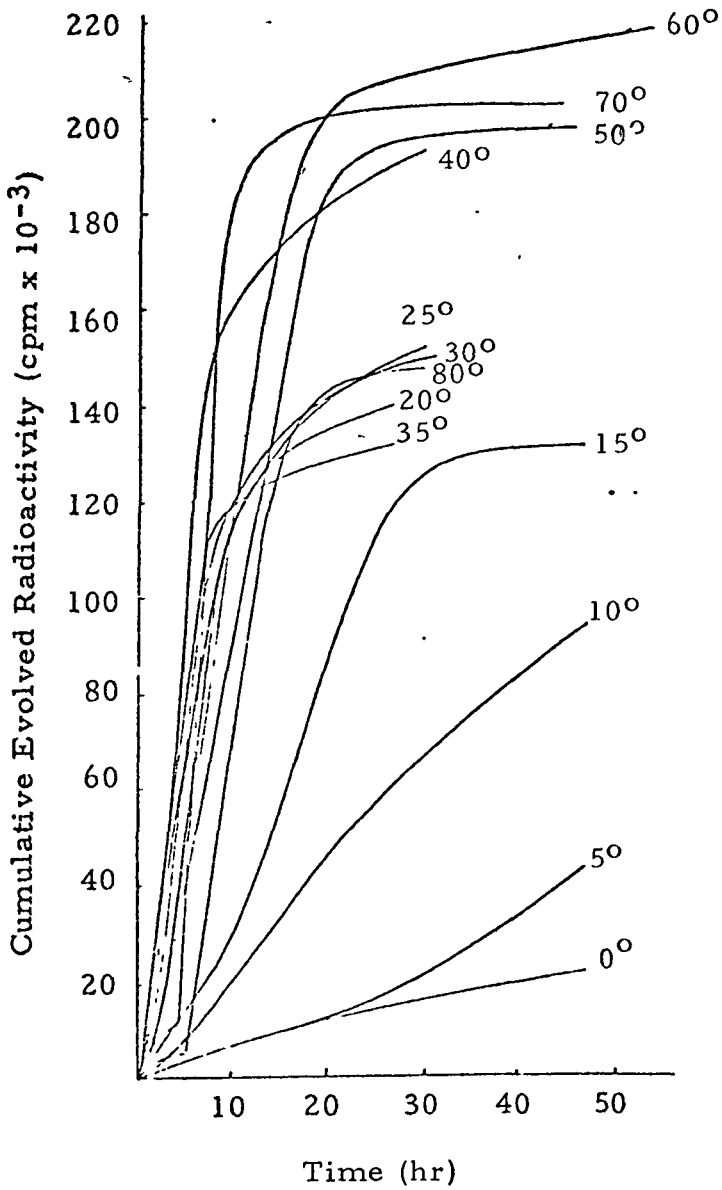

Figure 1

Effect of Temperature on the Radiorespiration of Wyaconda Soil

Experimental Design: 0.2 cc soil, 0.1 ml H_2O containing 10^{-3} M UL ^{14}C D-glucose and UL ^{14}C L-glutamate. Curves were established on the basis of duplicate points taken at 1, 3, 5, 7, 23, 27, 31 and 46 hrs. Sterile controls run at each temperature were less than 1% of the viable response except at the highest temperatures where the viable response was depressed.

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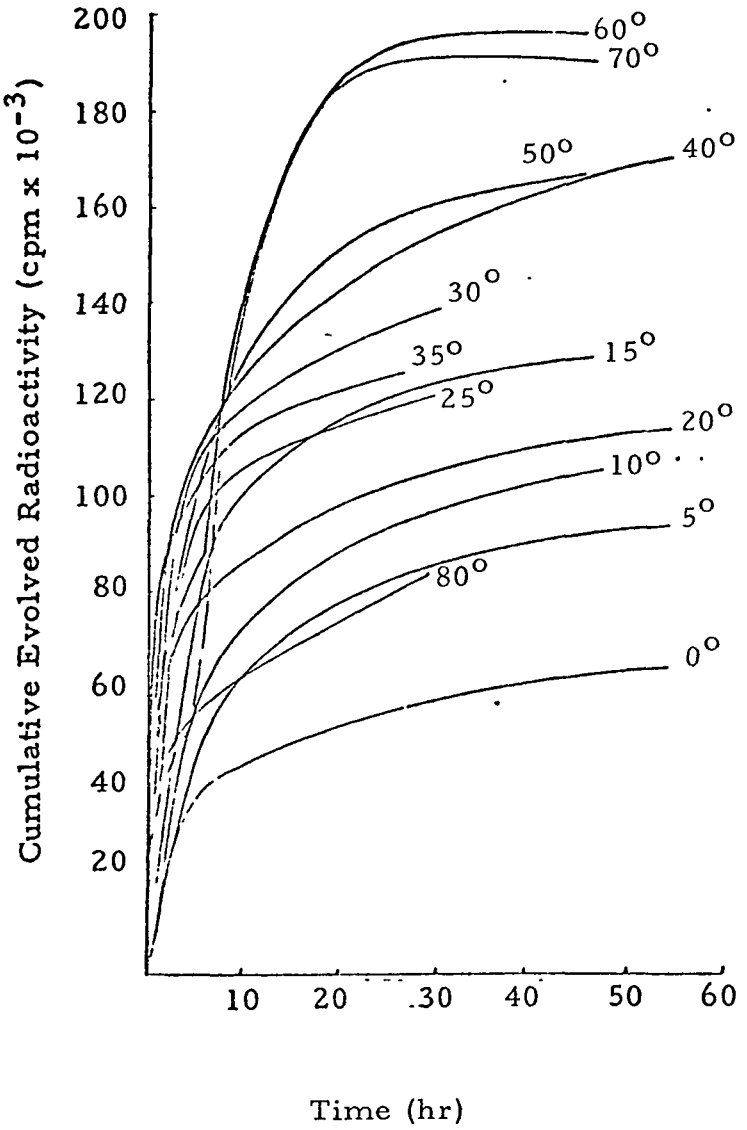

Figure 2

Effect of Temperature on the Radiorespiration of Meadow Soil

Experimental Design: 0.2 cc soil, 0.1 ml H_2O containing 10^{-3} M UL ^{14}C D-glucose and UL ^{14}C L-glutamate. Curves were established on the basis of duplicate points taken at 1, 3, 5, 7, 23, 27, 31 and 46 hrs. Sterile controls run at each temperature were less than 1% of the viable response except at the highest temperatures where the viable response was depressed.

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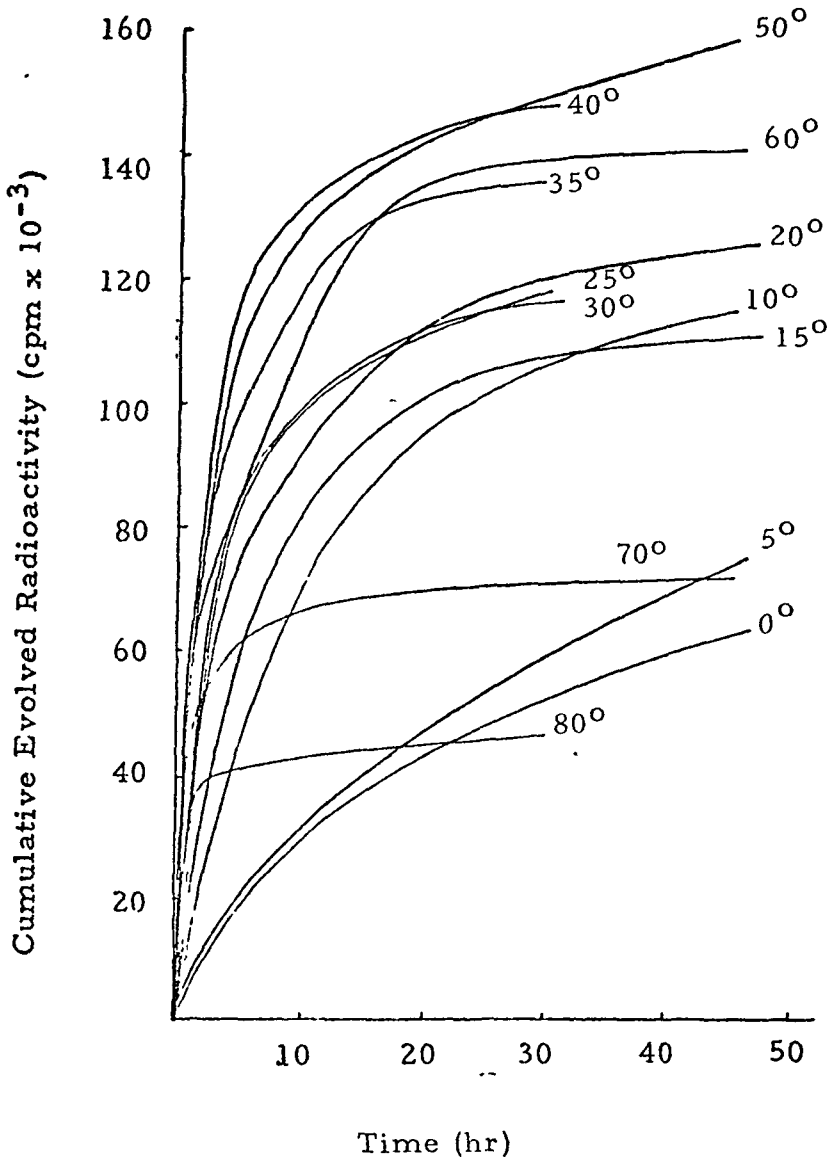

Figure 3

Effect of Temperature on the Radiorespiration of Phoenix Soil

Experimental Design: 0.2 cc soil, 0.1 ml H_2O containing 10^{-3} M UL ^{14}C D-glucose and UL ^{14}C L-glutamate. Curves were established on the basis of duplicate points taken at 1, 3, 5, 7, 23, 27, 31 and 46 hrs. Sterile controls run at each temperature were less than 1% of the viable response except at the highest temperatures where the viable response was depressed.

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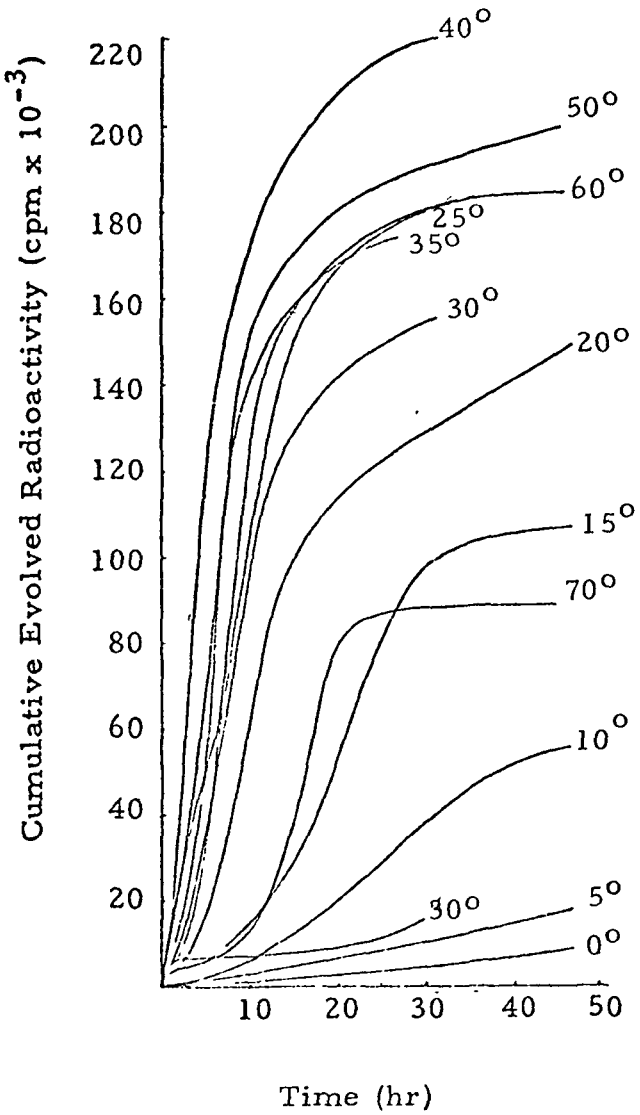

Figure 4

Effect of Temperature on the Radiorespiration of Yuma Soil

Experimental Design: 0.2 cc soil, 0.1 ml H_2O containing 10^{-3} M UL ^{14}C D-glucose and UL ^{14}C L-glutamate. Curves were established on the basis of duplicate points taken at 1, 3, 5, 7, 23, 27, 31 and 46 hrs. Sterile controls run at each temperature were less than 1% of the viable response except at the highest temperatures where the viable response was depressed.

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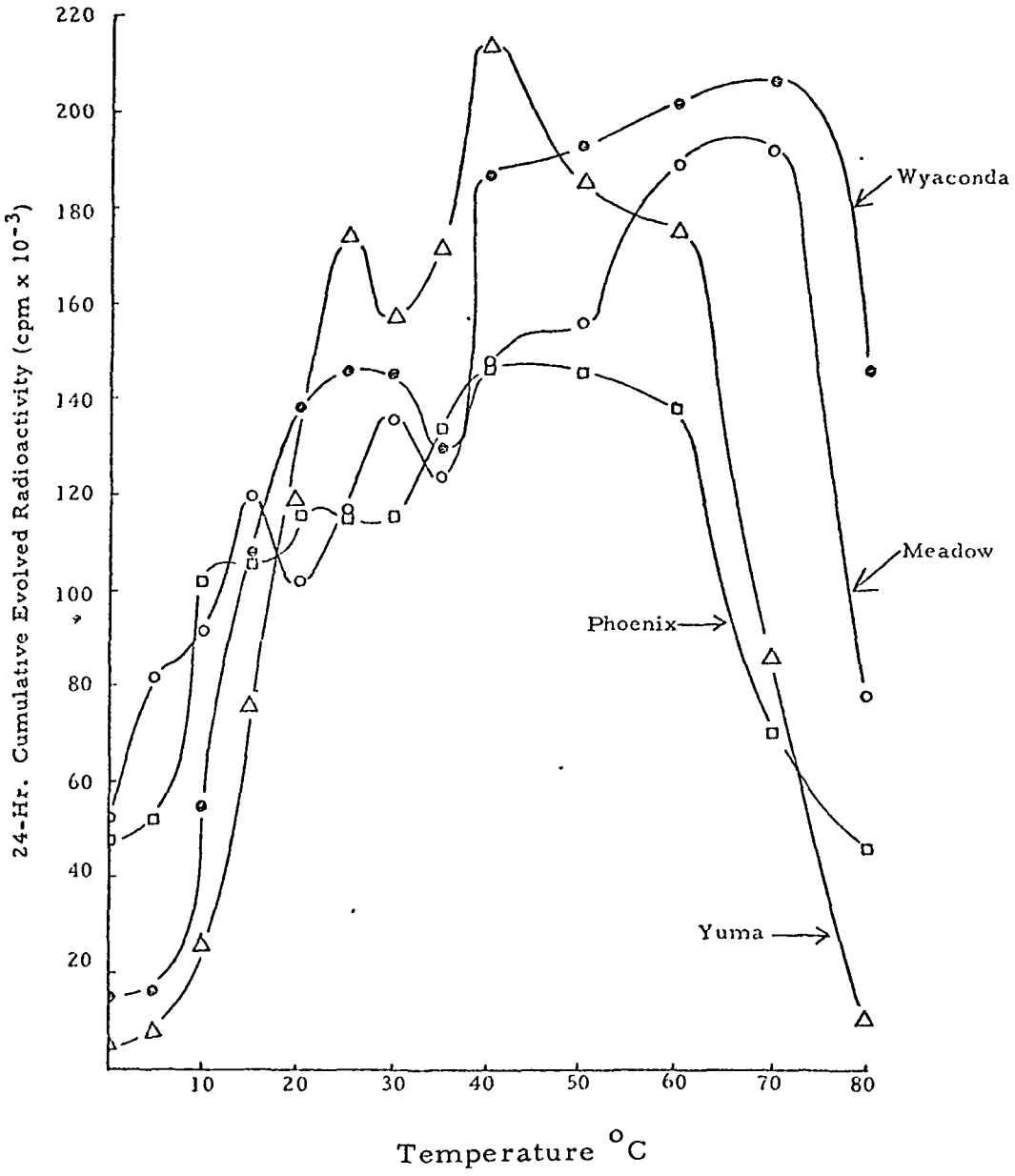

Figure 5

Twenty-Four Hour Cumulative Evolutions of Radioactivity of Test Soils as a Function of Temperature

Experimental Design: 0.2 cc soil, 0.1 ml H_2O containing 10^{-3}M UL ^{14}C D-glucose and UL ^{14}C L-glutamate. Sterile controls were less than 1% of viable response.

metabolism at the higher temperatures. Increased temperature most likely imposes a greater energy of maintenance requirement on cells, which must be compensated for by reduced growth and increased endogenous metabolism. Indirect support for this explanation rests in the well known fact that greater mass yields are obtained at temperatures below those which produce maximum growth rates (1). The unexpected finding that the Meadow and Wyaconda soils from Maryland showed CO_2 evolution maxima at higher temperatures than the CO_2 evolution maxima for the two western soils was very interesting. Dilution plate counts incubated at 65°C showed that Meadow and Yuma soils contained more thermophyles than the other test soils.

The kinetics of $^{14}CO_2$ evolution at 60°C, 70°C and 80°C with Wyaconda soil and Meadow soil were also interesting (see Figures 1 and 2). Evolution of radioactivity at these elevated temperatures began at a relatively low level and continued at a decreasing rate for a few hours. Suddenly an extremely rapid evolution occurred which continued until plateau. These results are in contrast with the usual results at room temperatures in which the addition of medium gives rise to an immediate and rapid evolution of CO_2. Many times the first hour rate of evolution is the greatest. A possible explanation for this difference may be that the higher temperatures inactivated those organisms which produce the immediate response at lower temperatures, but at the same

time activated a thermophilic population, which after a lag of several hours began a phase of rapid respiration. Germination of spores, for example, would require a lag of several hours. On the other hand, the effect may have been caused by shock on the organisms which took several hours to adjust to the new environment.

The initial rate of $^{14}CO_2$ evolution (cpm/1st. hour) for each soil was plotted vs the incubation temperature. As shown in Figure 6, the Meadow and Phoenix soils produced a much greater initial rate of $^{14}CO_2$ production than the Wyaconda and Yuma soils. This may be caused in part by the total numbers of organisms present. Plate counts for these soils showed 4×10^7, 2×10^7, 8×10^6 and 4×10^6, respectively (see Table 1, Appendix). All four soils showed the greatest rate of respiration at $35^\circ - 40^\circ C$; however, there appeared to be additional peaks at $20^\circ C$ (except for Wyaconda) and $60^\circ C$.

Psychophiles sometimes show their greatest growth rate at $20^\circ - 30^\circ C$ and may be responsible for the peaks at $20^\circ C$. Many saprophytic species of mesophiles also have a temperature optimum at $20^\circ C$ (2).

Mesophiles which inhabit homiothermic animals are found in soil and generally display temperature optima in the range of $35^\circ - 40^\circ C$. This relatively large group of organisms may be responsible for the peaks between $35^\circ C$ and $40^\circ C$ which occurred in all soils.

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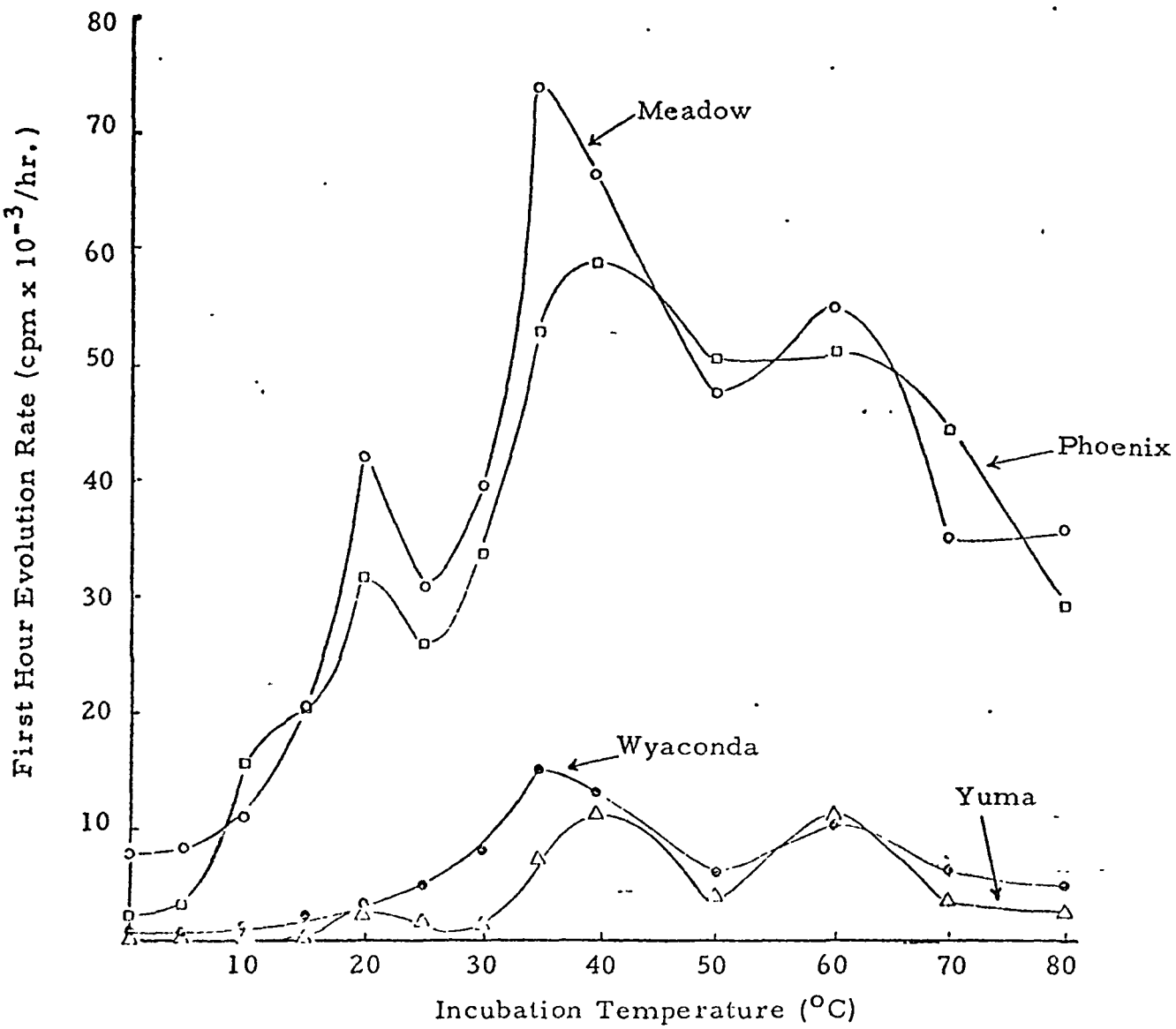

Figure 6

First Hour Rate of Radiorespiration by
Soil Incubated at Various Temperatures

Experimental Design: 0.2 cc soil, 0.1 ml H_2O containing 10^{-3} M UL ^{14}C D-glucose and UL ^{14}C L-glutamate. Sterile controls were less than 1% of viable response.

Thermophiles grow best between 55°C and 60°C and, therefore, may be responsible for the peaks at the initial respiration rate shown by all soils at 60°C.

It appears, on the basis of this study, that the radioisotopic procedure may be used to determine the temperature range and optima for biological respiration of species in natural soil. In addition, it may be possible to grossly estimate numbers of microbes from the initial rate of respiration at a given temperature.

The three temperatures which showed apparent optima 20°C, 35°C, and 60°C were selected for inclusion in the list of candidate parameters. In addition, a 3°C incubation temperature was included since nonbiological evolution is greatly reduced at that temperature.

C. Glutamate Studies

1. Soil Assays

Experiments were performed in which 0.1 ml of medium containing DL-glutamate specifically labeled in the 1, 2, 3-4 or 5 carbon atom was individually added to soil. All substrates were made up to concentrations of 10^{-3} M in distilled water. Evolved radioactivity was collected at 15-minute intervals over a period of several hours, and the results plotted as shown in Figures 7 and 8.

The kinetics of evolution for the differently tagged molecule varied somewhat among experiments. As typified by Figure 7, except

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Soil:	0.21 cc Meadow soil not pretreated
Incubation:	Room temperature, in scintillation vials flushed with air (40 cc/min.)
Gas Collection:	Hyamine used to trap $^{14}CO_2$
Medium:	0.1 ml, 10^{-3}M, 5 µCi/ml ● 1 ^{14}C DL-glutamate ○ 2 ^{14}C DL-glutamate □ 3-4 ^{14}C DL-glutamate ■ 5 ^{14}C DL-glutamate
Controls:	<50 cpm (not shown) Results are divided by 5.6 for comparison with $Ba(OH)_2$ results

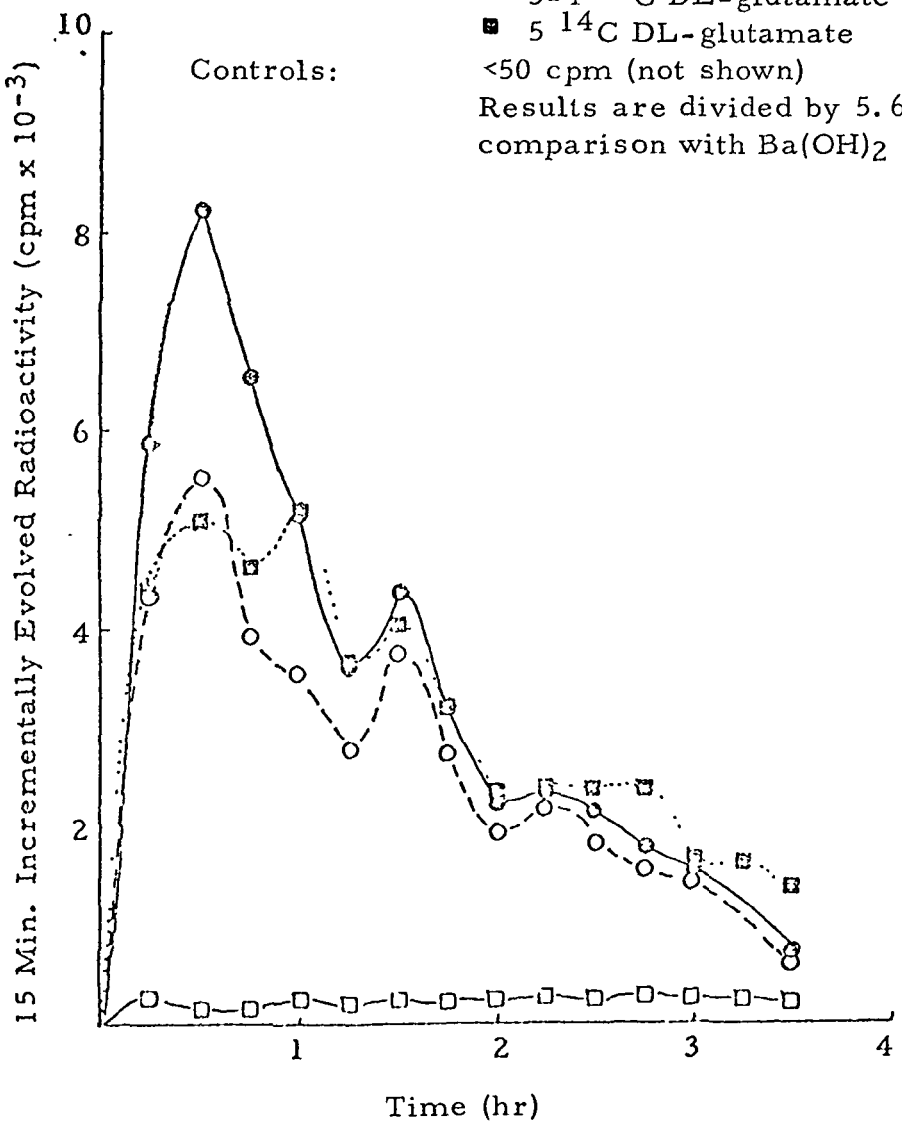

Figure 7

Evolution of $^{14}CO_2$ from 1, 2, 3-4 and 5 ^{14}C DL-glutamate by Meadow Soil

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Soil:	0.21 cc Meadow soil not pretreated
Incubation:	Room temperature, aerobic
Gas Collection:	$Ba(OH)_2$ used to trap CO_2
Medium:	0.1 ml, 10^{-3} M, 5 μCi/ml
	● 1 ^{14}C DL-glutamate
	○ 2 ^{14}C DL-glutamate
	□ 3-4 ^{14}C DL-glutamate
	▣ 5 ^{14}C DL-glutamate
Controls:	<50 cpm (not shown)

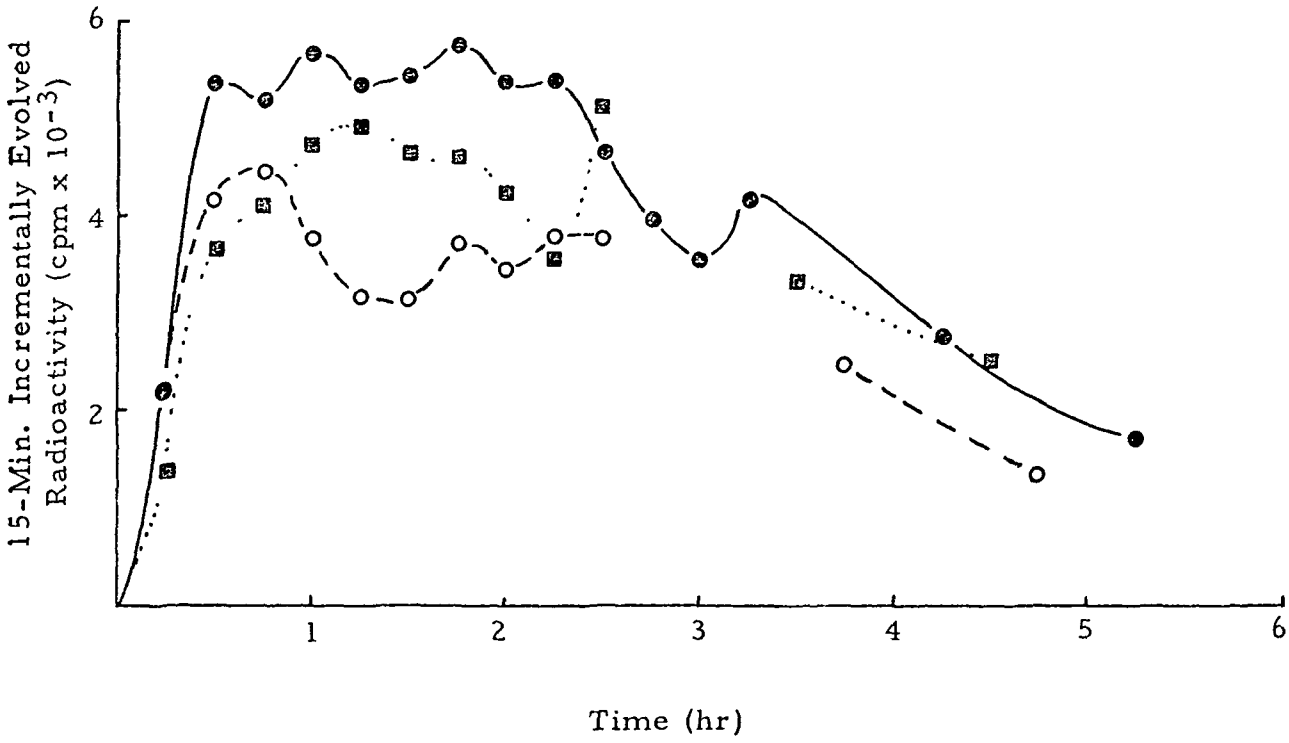

Figure 8

Evolution of $^{14}CO_2$ from 1, 2, 3-4 and 5 ^{14}C DL-glutamate by Meadow Soil

for the 3-4 tagged glutamate, evolved radioactivity sometimes peaked within the first hour of incubation and then fell rapidly thereafter. On other occasions, as shown in Figure 8, evolved radioactivity reached a maximum within the first hour and then remained at that level for two or more hours before falling off slowly. The phenomenon of an early peak was characteristically associated with soils having larger numbers of microorganisms and the results showing no peak resulted from soils having fewer numbers of microorganisms. However, as exemplified by Figures 7 and 8, a single soil sometimes gave rise to both types of response. Lack of hogeneity of soil samples may account for some of this difference. In spite of these differences in kinetics, the overall interpretation of results are unaltered. In both experiments shown in Figures 7 and 8, the pattern of $^{14}CO_2$ yield from specifically labeled glutamate was: 1 ^{14}C > 2 ^{14}C ≅ 5 ^{14}C >> 3-4 ^{14}C. A comparison of two-hour cumulative evolution, which was a time point used to establish the library profiles, is shown in Table 2. No difference in the two-hour cumulative $^{14}CO_2$ evolution exists.

It was found that the magnitude of response could be enhanced by treating a soil sample with nonlabeled glutamate 18 to 24 hours prior to the addition of the specifically labeled glutamate. Typical results of a pretreated soil are shown in Figure 9.

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Table 2

Two-Hour Cumulative Evolved
Radioactivity in Two Experiments
Shown in Figures 7 and 8

Substrate	Two-Hrs. Cumulative Evolved $^{14}CO_2$ (cpm)	
	Exp. Fig. 7	Exp. Fig. 8
1 ^{14}C DL-glutamate	39,300	40,250
2 ^{14}C DL-glutamate	29,000	31,450
5 ^{14}C DL-glutamate	32,750	32,200
3-4 ^{14}C DL-glutamate	1,300	1,600

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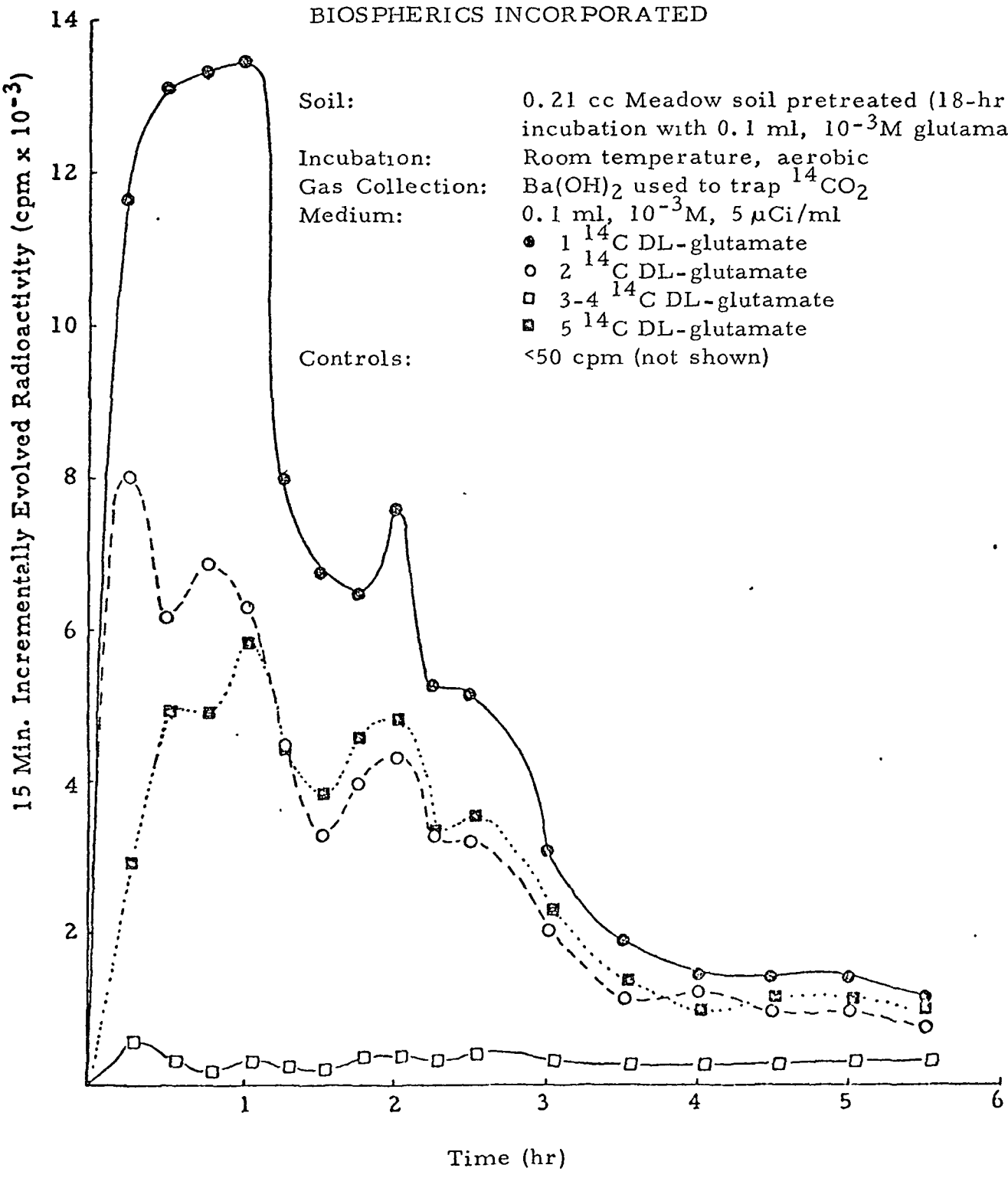

Figure 9

Evolution of $^{14}CO_2$ from 1, 2, 3-4 and 5 ^{14}C DL-Glutamate by Pretreated Meadow Soil

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In all three of these experiments (Figures 7, 8 and 9), 1 ^{14}C glutamate produced the greatest evolution of $^{14}CO_2$. A lesser, but significant, amount of $^{14}CO_2$ resulted from 2 ^{14}C glutamate and 5 ^{14}C glutamate. The 3-4 ^{14}C glutamate produced only very low levels of evolved radioactivity. This pattern in labeled release from glutamate may be readily explained by catabolism via a deamination to alpha-ketoglutaric acid which is then cycled through the citric acid cycle. As shown in Figure 10, alpha-ketoglutaric acid undergoes a decarboxylation of the one carbon atom as an initial step. The resulting succinic acid is symmetrical; therefore, the 2 and 5 carbon atoms would be indistinguishable beyond that step. Cycling of labeled compound, assuming condensation of labeled oxaloacetic acid with unlabeled acetyl-CoA through citric acid, etc., would result in a decarboxylation of oxaloacetic acid, which corresponds to the 2 and 5 carbon atoms of the original labeled glutamate molecule. Decarboxylation of the resulting alpha-ketoglutarate would then liberate the remaining 2, 5 glutamate carbon atoms; however, these atom positions continue to be actively decarboxylated with each successive cycle. The 3-4 carbon atoms of glutamate more slowly move to positions on the citric acid cycle intermediates which undergo decarboxylation. The condensing reaction moves the 3-4 carbon atoms of the original glutamate molecule to the 2 and 3 carbons of isocitric acid (positions comparable to the 2, 3 carbon position of the original glutamate molecule).

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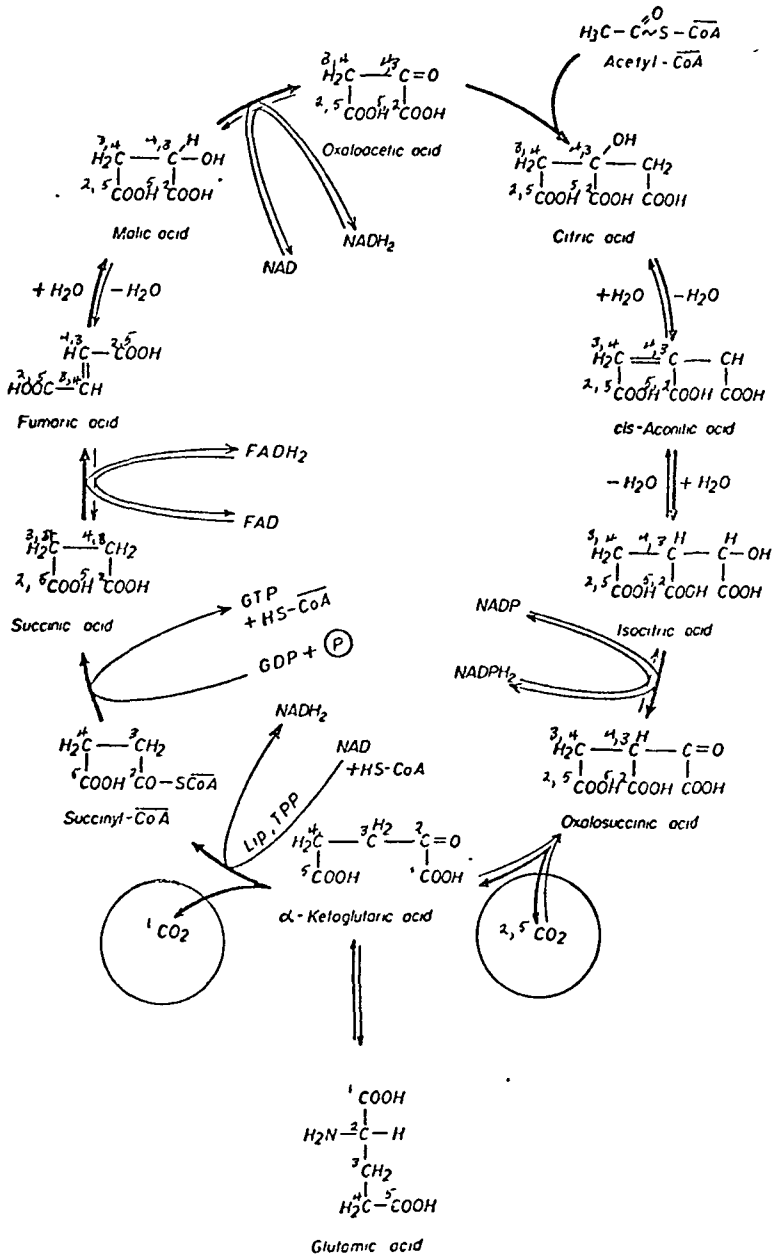

Figure 10

Evolution of $^{14}CO_2$ From ^{14}C Glutamate Via Citric Acid Cycle*

*Modified from Karlson (3).

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Two complete cycles of the TCA cycle are necessary to move one-fourth of the 3-4 labeled glutamate carbon atoms to positions eligible for decarboxylation. Therefore, the $^{14}CO_2$ release from 3-4 ^{14}C glutamate must occur slowly from the TCA cycle and from side reactions outside the cycle, which may function less in energy production and to a greater extent in synthetic processes. The resultant low yield of $^{14}CO_2$ from 3-4 ^{14}C glutamate would be expected.

Yields of $^{14}CO_2$ from glutamate labeled in the 1, 2, 3-4 and 5 carbon positions are thus readily explained on the basis of citric acid cycle metabolism.

Wang (4) has demonstrated a similar pattern of $^{14}CO_2$ evolution using pure cultures of Brevibacterium. To provide more definitive evidence that glutamate was degraded via the citric acid cycle pathway, a series of experiments was conducted using specific inhibitors of the citric acid cycle. If, in the presence of the inhibitor, the pattern of $^{14}CO_2$ evolution from 1, 2, 3-4 and 5 ^{14}C DL-glutamate was significantly changed, this would provide strong evidence for the operation of that cycle in organisms contained in the test soil sample.

Inhibitors tested include the following:

- o <u>Malonic Acid</u> - substrate analog of succinic acid which competes with succinic acid for enzyme active site and thereby prevents the conversion of succinic acid to fumaric acid (5).

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- Trans-aconitic Acid - antimetabolite which interferes with the metabolism of cis-aconitic acid (6).

- Fluoroacetate - reported to substitute for acetate and undergo activation and condensation with oxaloacetic acid to form fluorocitrate. Fluorocitrate is unable to undergo the aconitase reaction, thereby blocking the cycle (7, 8).

- Fluorocitrate - is unable to undergo the aconitase reaction, thereby blocking the cycle (7, 8).

- 2, 4 Dinitrophenol - uncouples oxidative phosphorylation (9). Since the citric acid cycle generates reduced co-enzymes, which produce energy through oxidative phosphorylation, then a blockage of this energy scheme might be expected to result in a shift in the energy yielding pathways.

Each of the above inhibitors was tested with the labeled release technique at various concentrating using soil and ^{14}C DL-glutamate. The concentration selected for tests with 1, 2, 3-4, and 5 ^{14}C glutamate was in each case that which decreased the $^{14}CO_2$ evolution slightly as determined in preliminary runs. In all cases this concentration of inhibitor was at least ten fold greater than the concentration of the test substrate glutamate.

None of the inhibitors listed above changed the pattern of $^{14}CO_2$ evolution. There are several possible explanations which include: permeability and organism specificity to inhibitors, operation of the alternate non-citric acid cycle pathways, and partial operation of the citric acid cycle which has been blocked.

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Experiments involving 1, 2, 3-4 and 5 ^{14}C glutamate performed under conditions of anaerobiosis showed the most dramatic effect. Soil was flushed with 90% N_2 and 10% H_2 for approximately one hour prior to the introduction of labeled medium. Incubation was then continued in the presence of a continuous flush with this gas. Comparison of results shown in Figure 11 with those shown in Figure 7 (the aerobic control) demonstrate that anaerobiosis greatly reduces the $^{14}CO_2$ evolution from all labeled positions. The importance of aerobic mechanisms for degradation of glutamate by organisms in this soil are indicated.

2. Pure Culture Assays

The predominating organisms in Meadow soil were isolated on TSA plates. Eighteen-hour growth on these plates was removed with a cotton swab and suspended in 0.85% saline at a concentration of approximately 10^9 colony forming units (CFU). A 0.1 ml aliquot of this suspension was dosed with 0.1 ml of 1 ^{14}C, 2 ^{14}C, 3-4 ^{14}C and 5 ^{14}C DL-glutamate. Results from Meadow soil and three organisms isolated from that soil are given in Figures 12 to 15. Data given in Table 3, which was obtained in a separate series of experiments performed to produce the library profiles, also supports the data shown in Figures 12 to 15.

Although the Meadow soil produced the typical citric acid cycle pattern of evolution, the pattern of evolution from all three pure culture

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Soil:	0.21 cc Meadow soil not pretreated
Incubation:	Room temperature in scintillation vials flushed with 90% N_2, 10% H_2 (40 cc/min)
Gas Collection:	Hyamine used to trap $^{14}CO_2$
Medium:	0.1 ml, 10^{-3}M, 5 uCi/ml ● 1 ^{14}C DL-glutamate ○ 2 ^{14}C DL-glutamate □ 3-4 ^{14}C DL-glutamate ■ 5 ^{14}C DL-glutamate
Controls:	< 50 cpm (not shown) Results are divided by 5.6 for comparison with $Ba(OH)_2$ results.

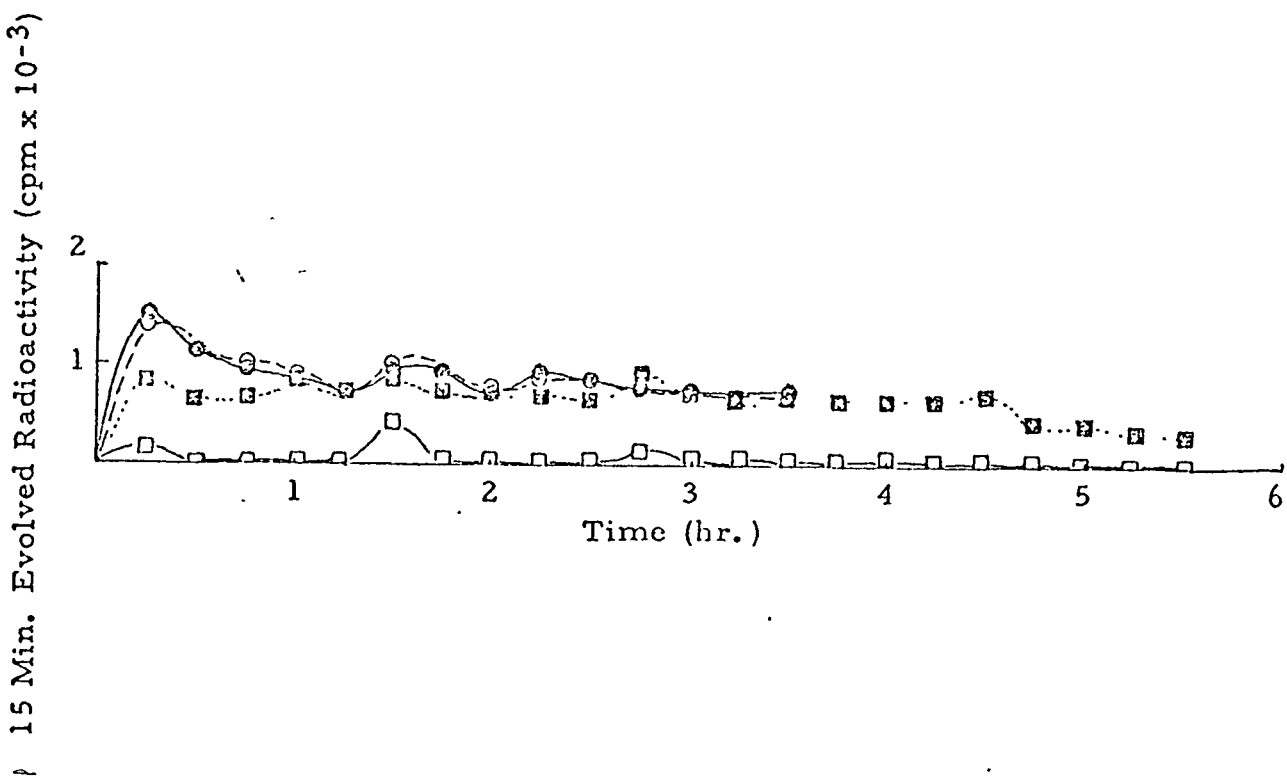

Figure 11

Evolution of $^{14}CO_2$ from 1, 2, 3-4 and 5 ^{14}C DL-glutamate by Meadow Soil Under Conditions of Anaerobiosis

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Soil:	0.21 cc Meadow soil
Incubation:	Room temperature, aerobic
Gas Collection:	$Ba(OH)_2$ getters used to trap $^{14}CO_2$
Medium:	0.1 ml, 10^{-3} M, 5 μCi/ml
	● 1 ^{14}C DL-glutamate
	○ 2 ^{14}C DL-glutamate
	△ 3-4 ^{14}C DL-glutamate
	□ 5 ^{14}C DL-glutamate
Controls:	<50 cpm (not shown)

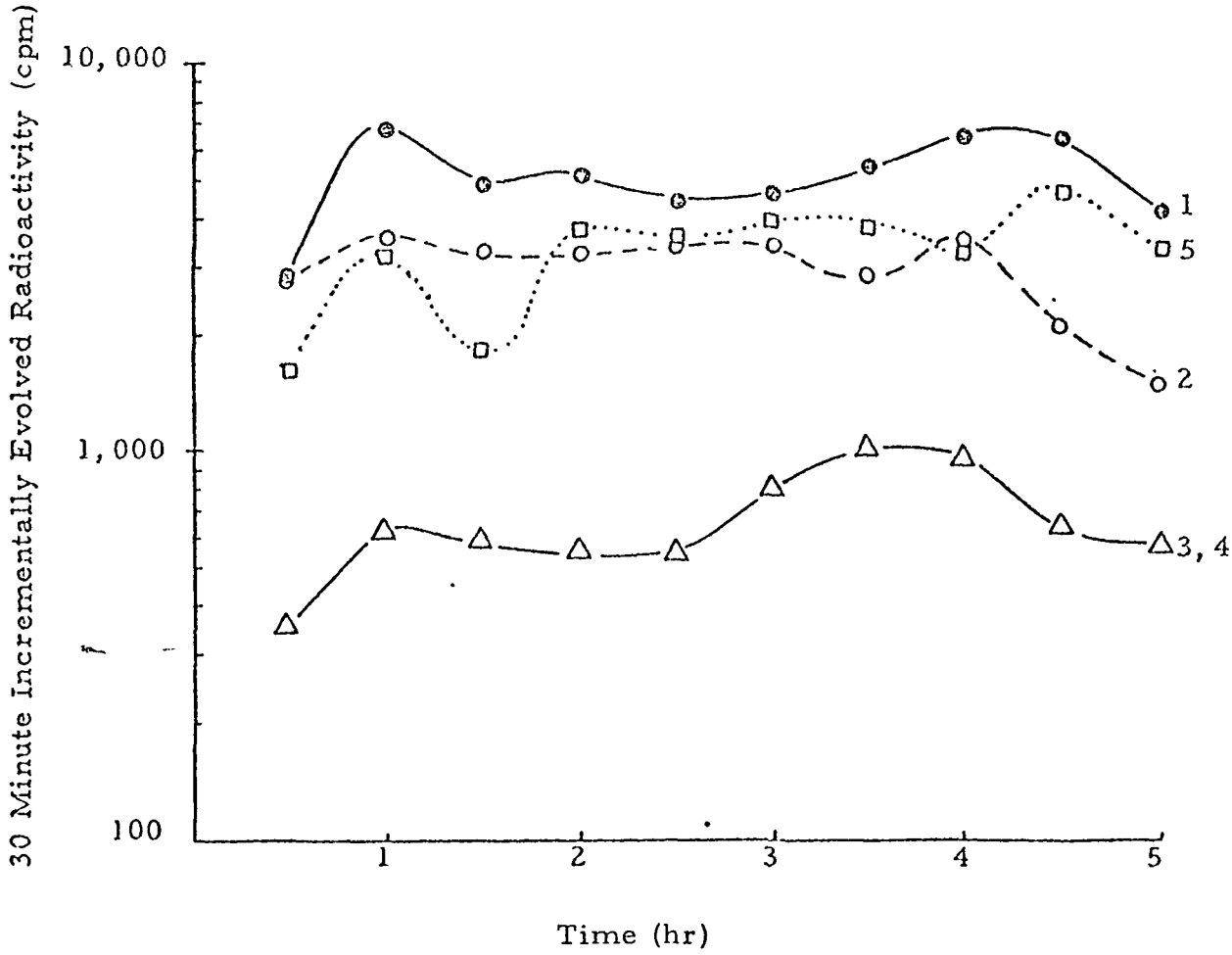

Figure 12

Evolution of $^{14}CO_2$ from 1 ^{14}C, 2 ^{14}C, 3-4 ^{14}C and 5 ^{14}C DL-glutamate by Meadow Soil

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Inoculum:	0.1 ml (10^9 CFU/ml) Meadow Soil Isolate No. 1
Incubation:	Room temperature, aerobic
Gas Collection:	$Ba(OH)_2$ getters used to collect $^{14}CO_2$
Medium:	0.1 ml, 10^{-3}M 5 uCi/ml
	● 1 ^{14}C DL-glutamate
	○ 2 ^{14}C DL-glutamate
	△ 3-4 ^{14}C DL-glutamate
	□ 5 ^{14}C DL-glutamate
Controls:	<50 cpm (not shown)

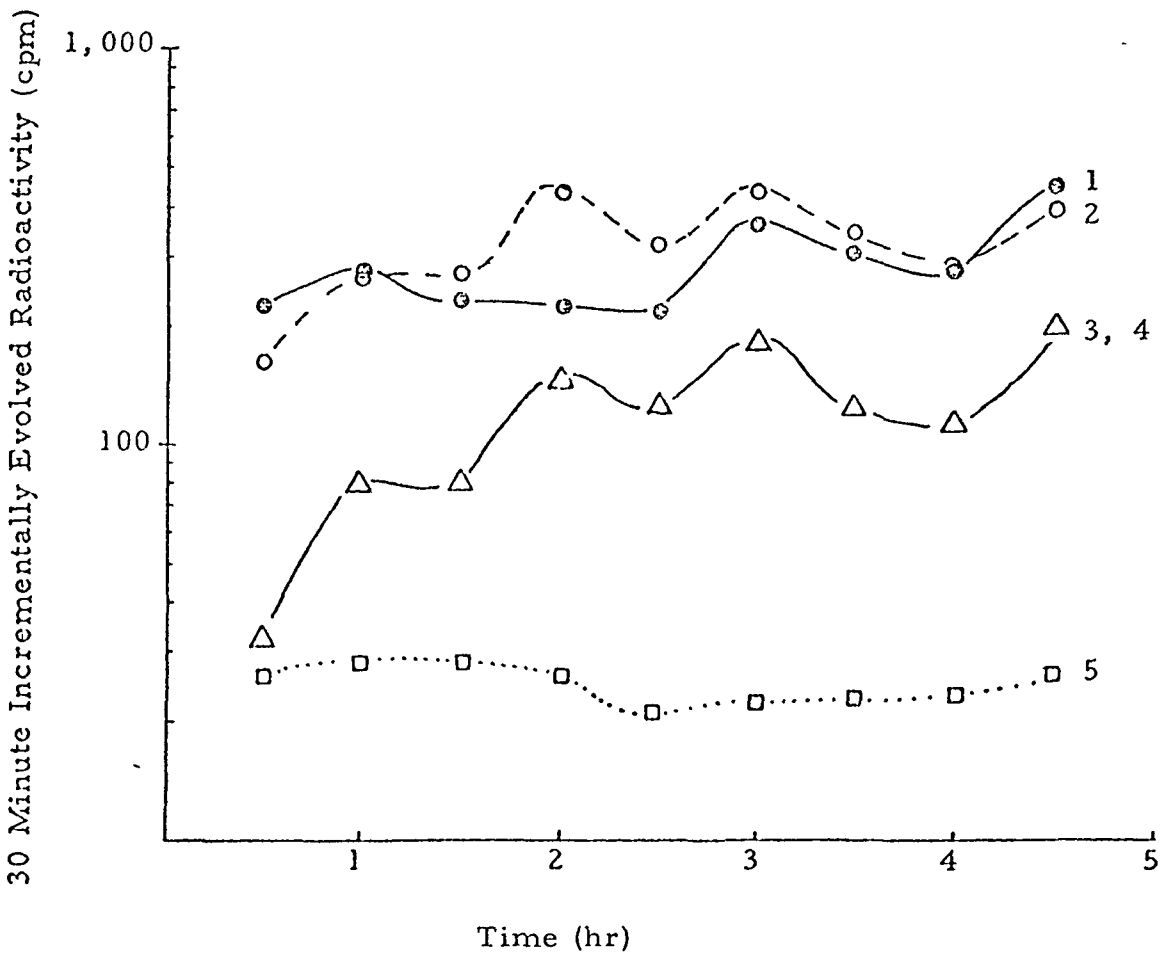

Figure 13

Evolution of $^{14}CO_2$ from 1 ^{14}C, 2 ^{14}C, 3-4 ^{14}C and 5 ^{14}C DL-glutamate by Soil Isolate No. 1

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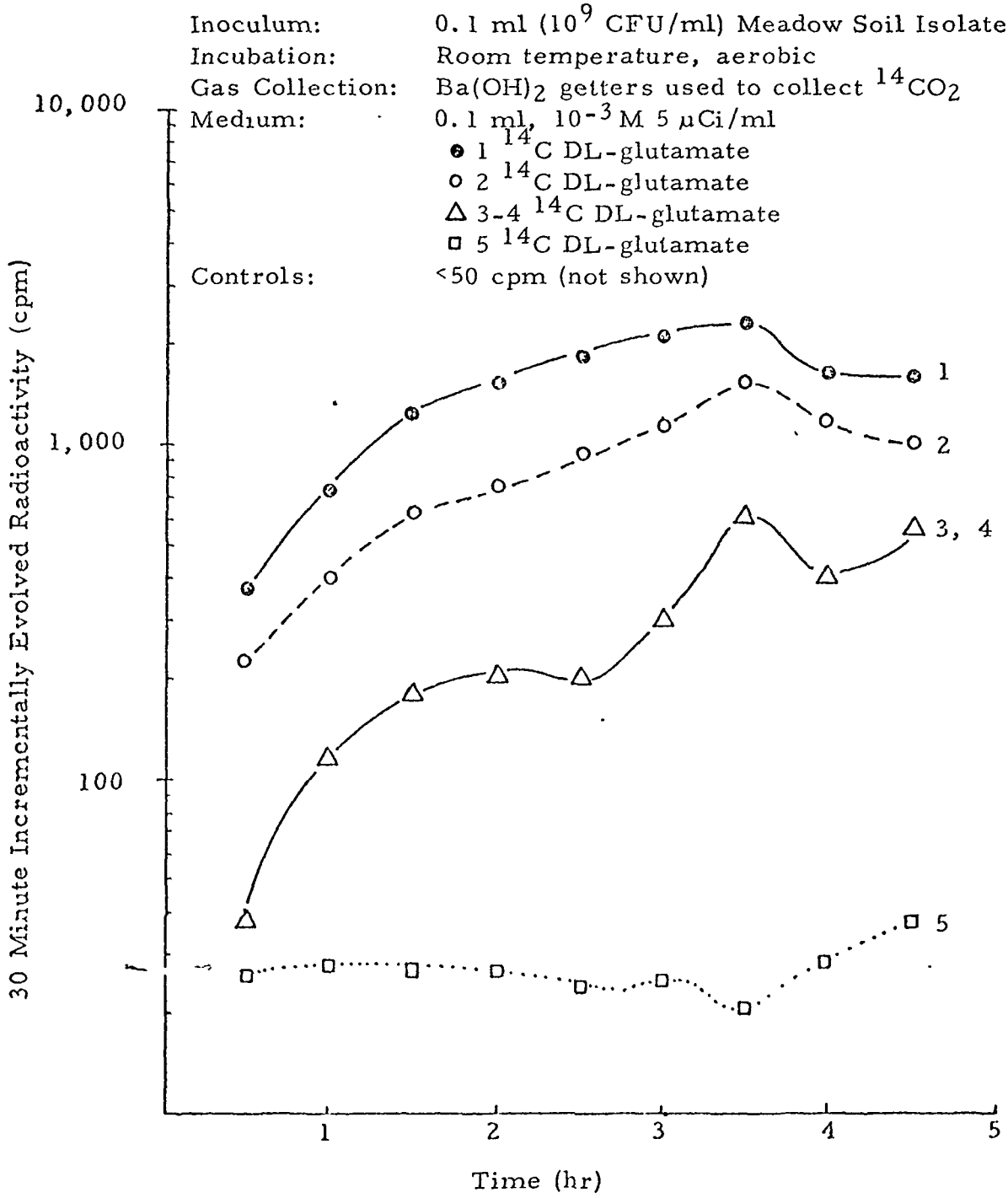

Figure 14

Evolution of $^{14}CO_2$ from 1 ^{14}C, 2 ^{14}C, 3-4 ^{14}C and 5 ^{14}C DL-glutamate by Soil Isolate No. 2

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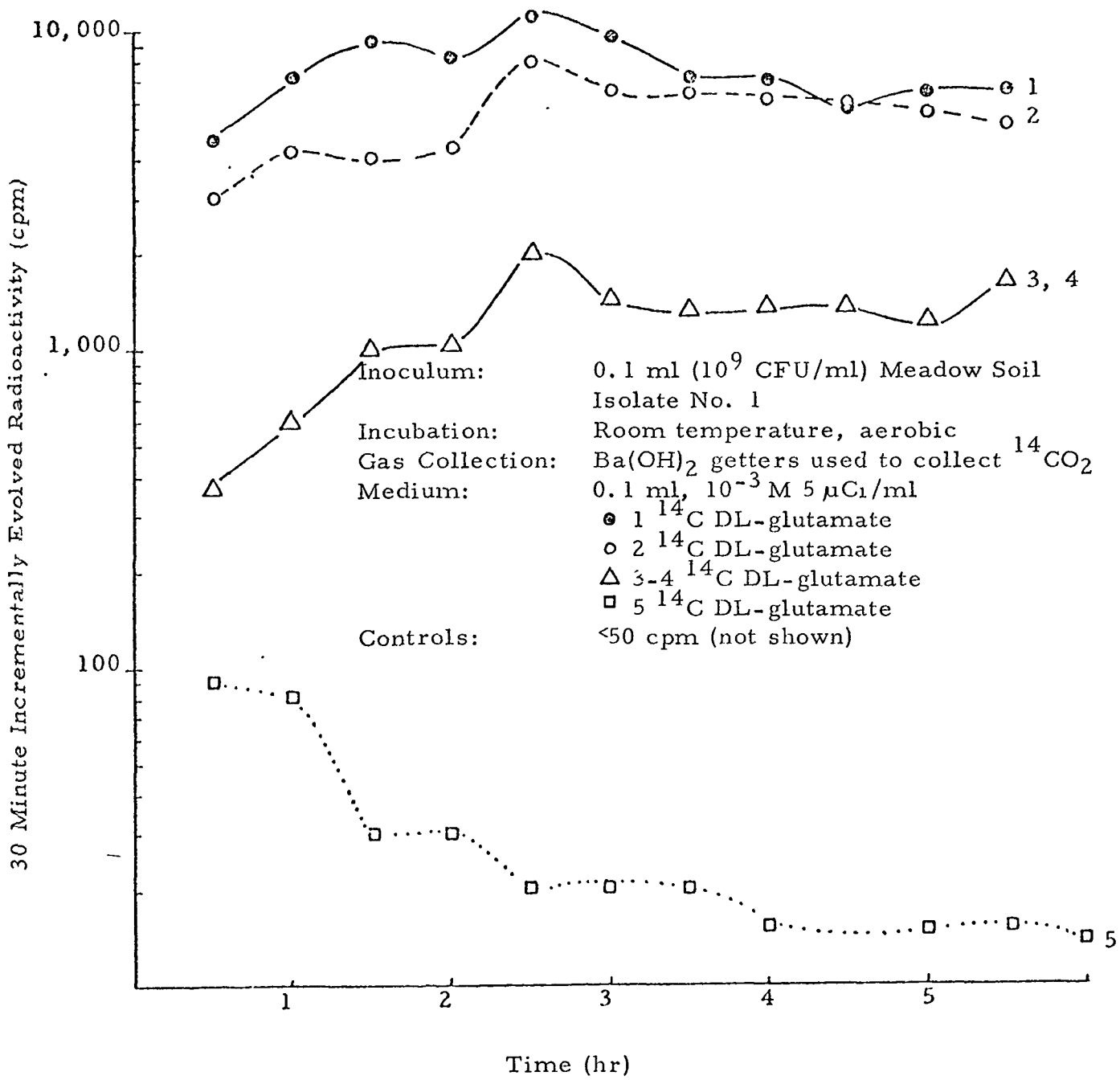

Figure 15

Evolution of $^{14}CO_2$ from 1 ^{14}C, 2 ^{14}C, 3-4 ^{14}C and 5 ^{14}C DL-glutamate by Soil Isolate No. 3

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Table 3

Twenty-Two Hour Cumulative Evolved Radioactivity
from Specifically Labeled Glutamate

Substrate	CPM/μCi of Labeled Carbon			
	Natural Soil	Soil Isolate No. 1	Soil Isolate No. 2	Soil Isolate No. 3
1 ^{14}C DL-glutamate	28,000	1,100	31,000	22,000
2 ^{14}C DL-glutamate	17,000	1,500	16,000	20,000
3-4 ^{14}C DL-glutamate	8,800	900	28,000	13,000
5 ^{14}C DL-glutamate	16,000	45	4,000	700

isolates was different; i.e. 1 $^{14}C \geq 2\ ^{14}C > 3\text{-}4\ ^{14}C \gg 5\ ^{14}C$. This pattern of evolution cannot be explained by the citric acid cycle since the decarboxylation of alpha-ketoglutarate results in succinate--a symmetrical compound in which the original 2 and 5 carbons of glutamate are indistinguishable from each other. The pathway involved excludes the decarboxylation of the 5 carbon of glutamate.

When the isolates were added back to sterile soil, $^{14}CO_2$ was rapidly evolved from 5 ^{14}C glutamate (see Figure 16). These findings indicate that some factor in the soil stimulates citric acid cycle catabolism of glutamate in cells which, when grown on TSA, fail to show that pattern of catabolism. As shown in Figure 17(a), a filter-sterilized extract prepared by mixing 0.5 g Meadow soil with 2.0 ml of H_2O had the same stimulatory effect on $^{14}CO_2$ evolution from 5 ^{14}C DL-glutamate by TSA grown cells. It was also found that cell suspensions in 85% saline regain the ability to produce $^{14}CO_2$ from 5 ^{14}C DL-glutamate without soil or soil extract (Figure 17(b)), when the saline suspension is left standing 24 hours or longer. The soil extract has little additional effect on these old cell suspensions.

A pathway of glutamate dissimilation which excludes the production of CO_2 from the 5 carbon atom of glutamate has not previously been described. Insufficient information is available from this study to propose a breakdown scheme. However, several conclusions may be

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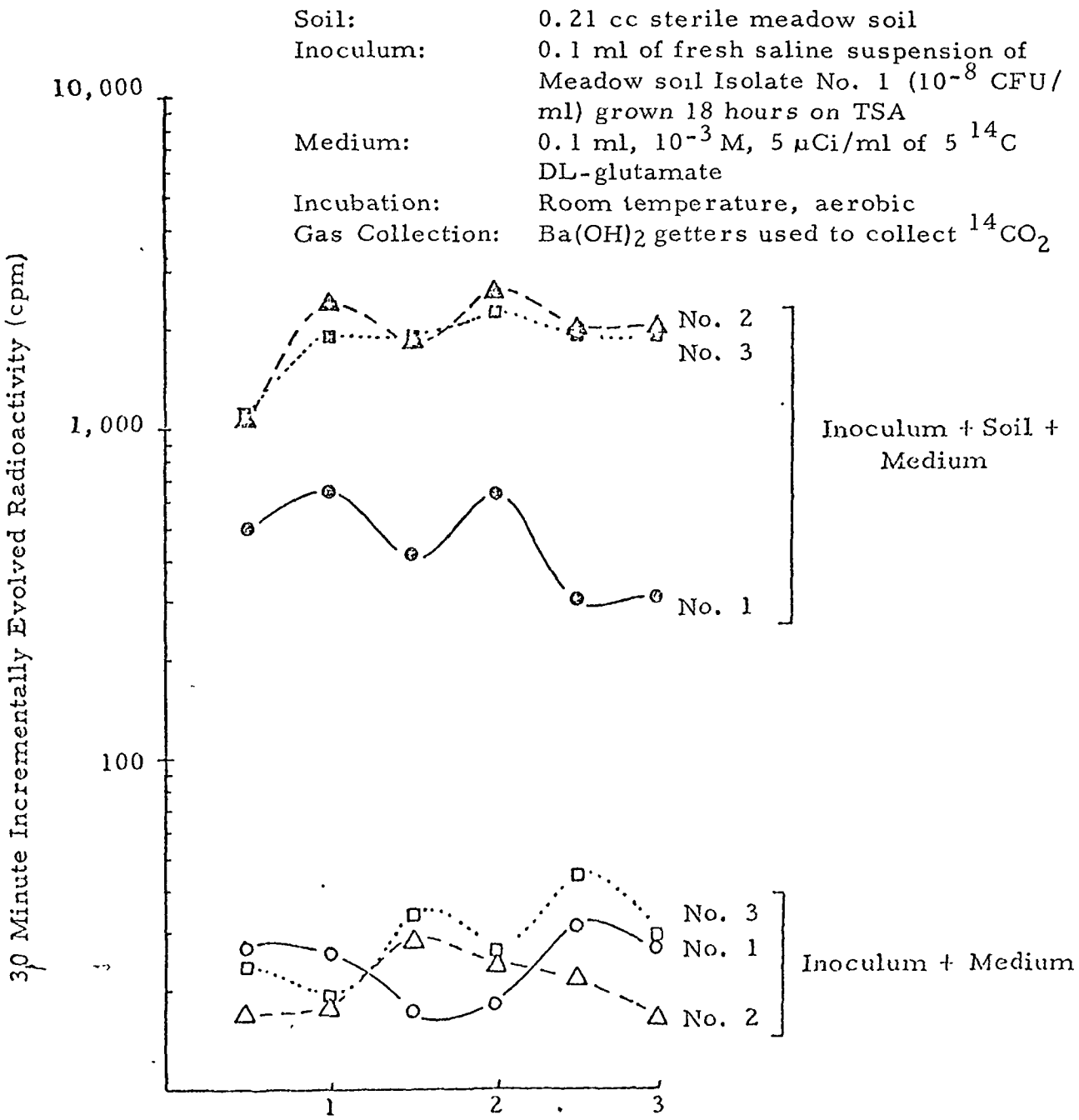

Figure 16

Influence of Sterile Soil on the Evolution of $^{14}CO_2$ from 5 ^{14}C DL-glutamate by Soil Isolates

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Soil Extract:	0.1 ml prepared by mixing 0.5 g Meadow soil with 2.0 ml of H_2O and filter sterilizing
Medium:	0.1 ml, 10^{-3}M, 5 μCi/ml, 5 ^{14}C DL-glutamate
Inoculum:	Bacteria were grown 18 hours on TSA, then suspended in 0.85% saline at a concentration of approximately 10^8 CFU/ml
Gas Collection:	$Ba(OH)_2$ was used to collect $^{14}CO_2$

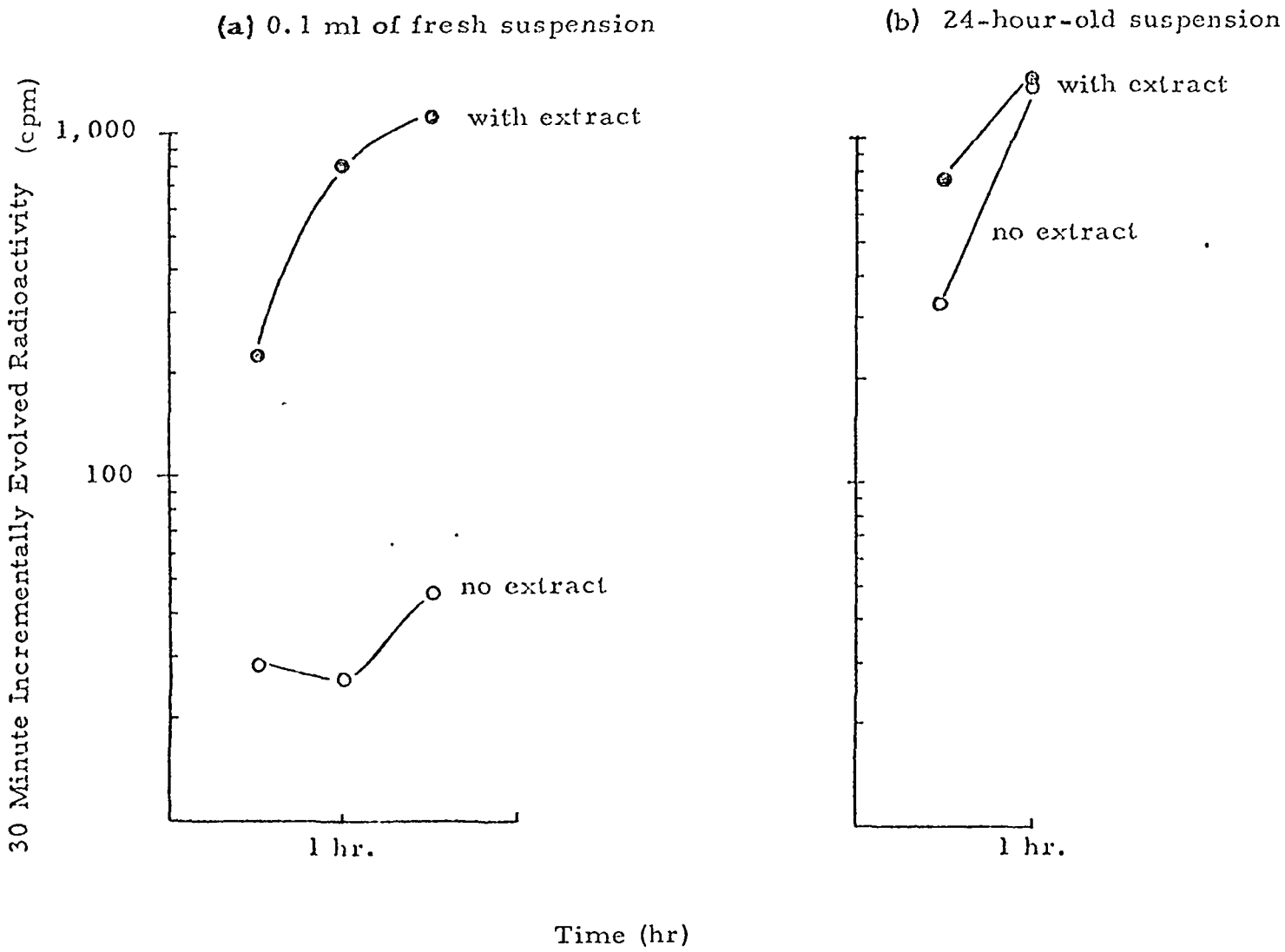

Figure 17

Effect of Soil Extract on $^{14}CO_2$ Evolution from 5 ^{14}C-glutamate

made: (1) Organisms in the soils tested predominately utilize the citric acid cycle for breakdown of glutamate; (2) These same organisms are inhibited from utilizing the citric acid cycle for glutamate breakdown when they are isolated from soil grown on TSA and suspended in saline; (3) These pure cultures do rapidly dissimilate glutamate, but do so via some unknown pathway; (4) Inhibition of the citric acid cycle dissimilation of glutamate may be overcome rapidly by adding sterile soil or water extract of the soil to the pure culture or slowly by allowing the saline cell suspension to stand until the inhibiting levels of organic nutrients accumulated from the TSA are metabolized or until required factors are elaborated by the cells.

These findings demonstrate the utility of the technique for distinguishing pathways of metabolism. The importance of conducting tests on organisms in their natural state; i.e. in soil, is also exemplified.

3. Multiple Addition Mode

A series of experiments was conducted to determine if media containing 1 ^{14}C, 2 ^{14}C, 3-4 ^{14}C and 5 ^{14}C DL-glutamate, respectively, could be added sequentially to a single soil sample and yield results similar to those obtained when those labeled media were added to separate soil samples. Additions were made on consecutive days after $^{14}CO_2$ evolution from the previous addition had fallen to a low baseline level. Figure 18 shows results of a series of sequential

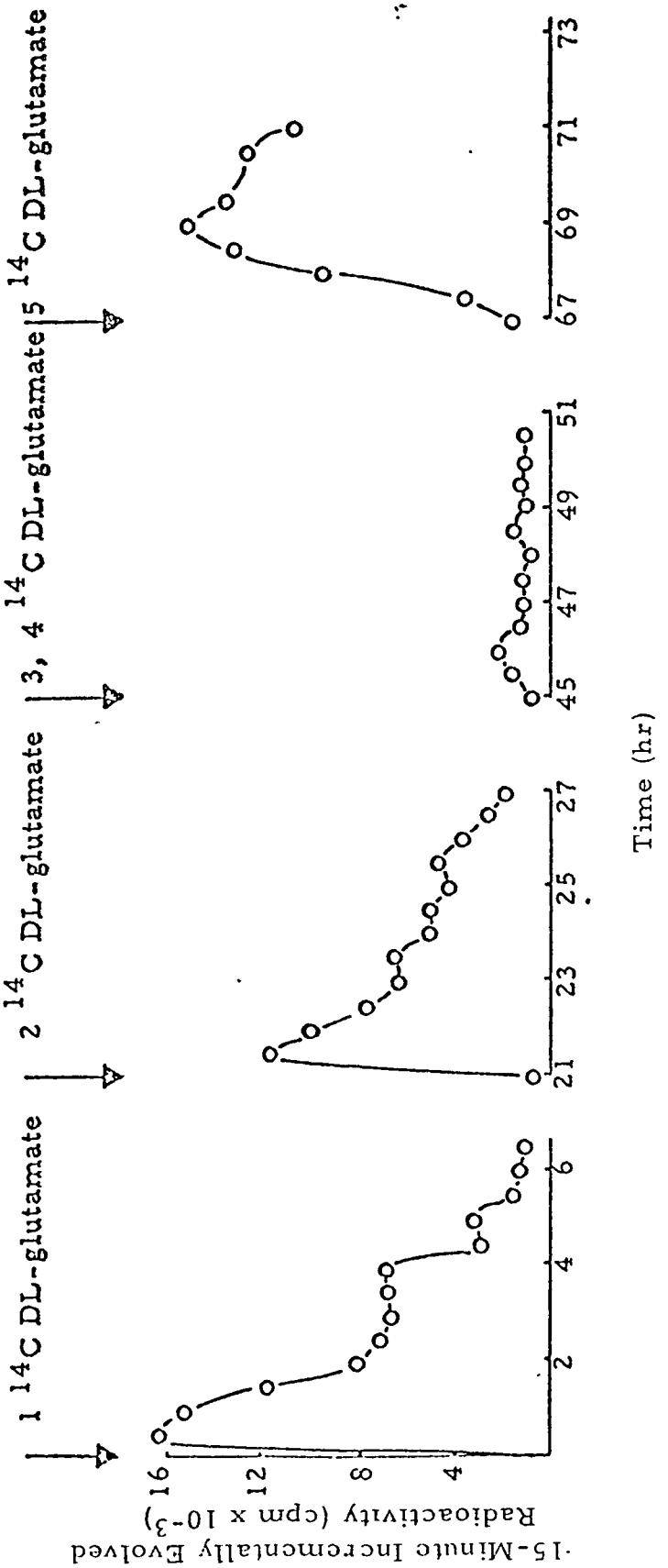

Figure 18

Sequential Addition of 1, 2, 3-4 and 5 ^{14}C DL-glutamate to Meadow Soil

0.21 cc of viable meadow soil was dosed with 0.1 ml of DL-glutamate 24 hours prior to the addition of 1 ^{14}C DL-glutamate. Substrates (no soil) were added at time shown. Incubation was in $Ba(OH)_2$ capped vials at room temperature.

additions of glutamate which correspond quite well with results of the single addition mode (compare with Figures 7 through 9).

To determine if the order of addition of specifically labeled glutamate or the prior addition of some ^{14}C labeled substrate other than glutamate might significantly affect subsequent additions, a series of sequential additions was made involving 1 ^{14}C, 2 ^{14}C, 3-4 ^{14}C and 5 ^{14}C DL-glutamate, 1 ^{14}C D-glucose, ^{14}C formate and 1 ^{14}C lactate. The chronology of additions varied as shown in Figures 19 to 22. Replications of sequential additions of a given substrate, even when preceded by different substrate additions, agreed well. A summary of glutamate results showing the mean and range of all values obtained from the multiple addition experiment shown in Figures 19 - 22 is given in Figure 23. Mean values showed that 1 ^{14}C > 2 ^{14}C $\cong$ 5 ^{14}C >> 3-4 ^{14}C; however, there was considerable overlap in the range of values obtained with the 1 ^{14}C, 2 ^{14}C and 5 ^{14}C labeled glutamate. Considerably less $^{14}CO_2$ was evolved from the 3-4 ^{14}C labeled positions so that even the highest values obtained with the specifically labeled glutamate did not approach the lowest values obtained with the 1 ^{14}C, 2 ^{14}C and 5 ^{14}C labeled glutamate. These data show that consistent results may be obtained for sequential additions of 1 ^{14}C, 2 ^{14}C, 3-4 ^{14}C and 5 ^{14}C DL-glutamate using the multiple addition mode regardless of the order of addition.

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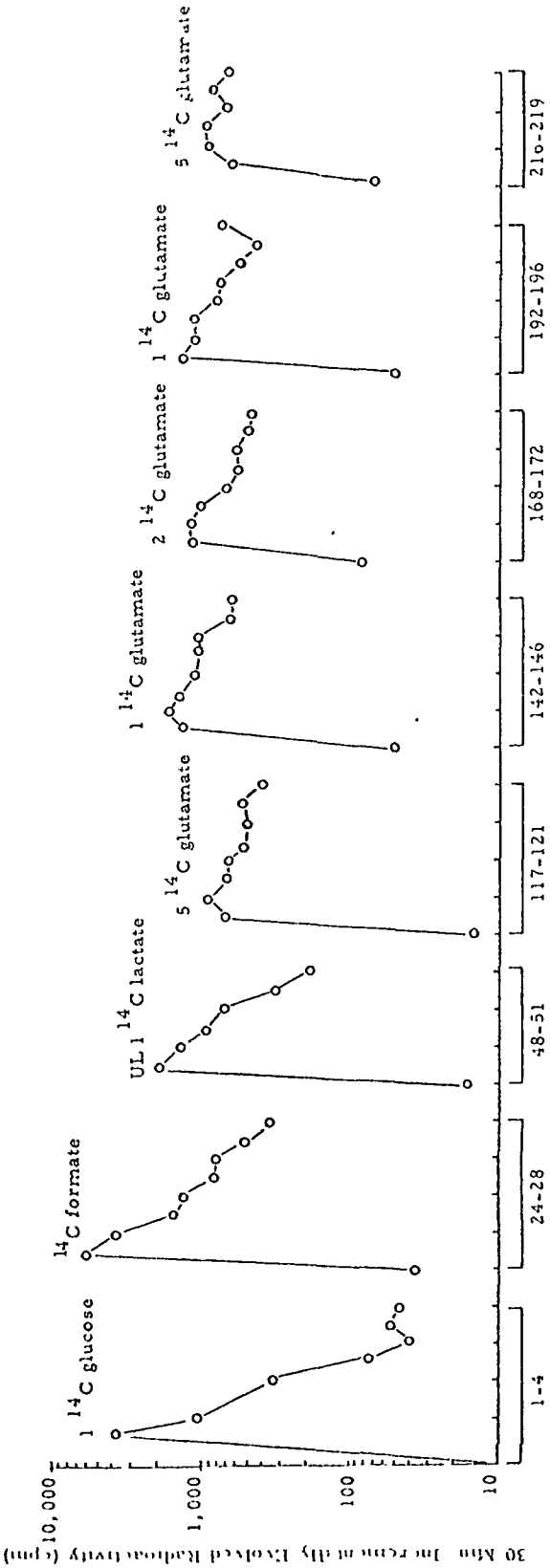

Figure 19

Multiple Addition of Labeled Substrates to Meadow Soil

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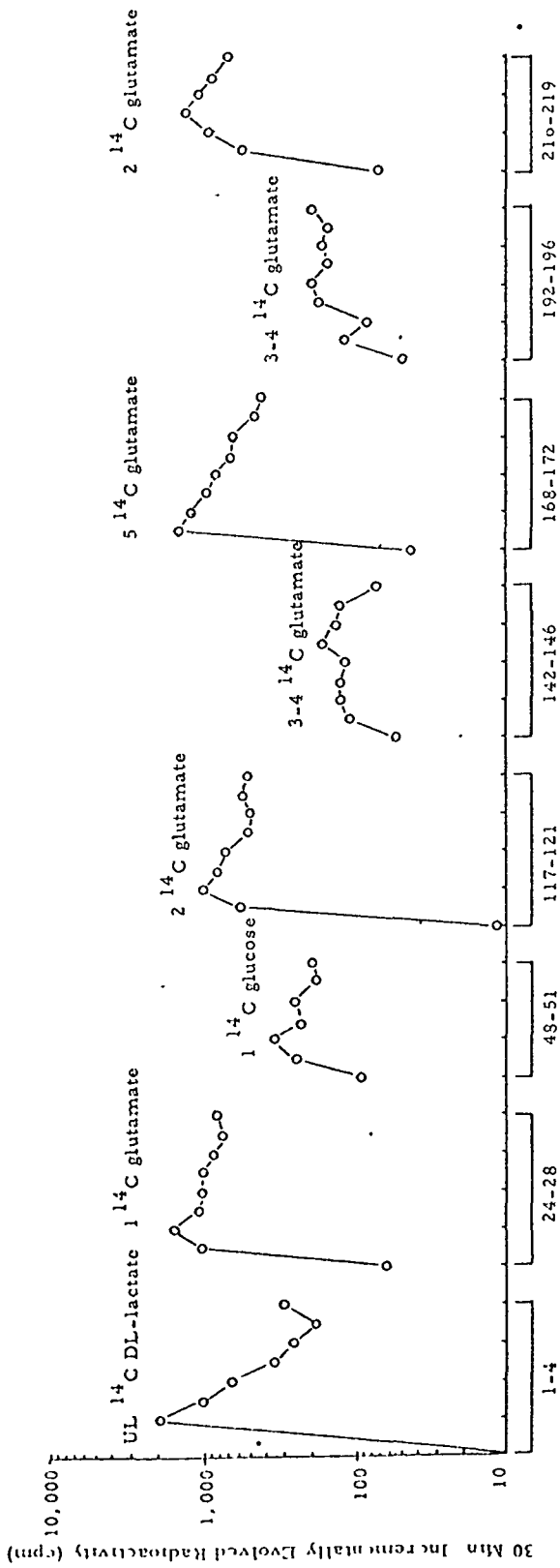

Figure 20

Multiple Addition of Labeled Substrates to Meadow Soil

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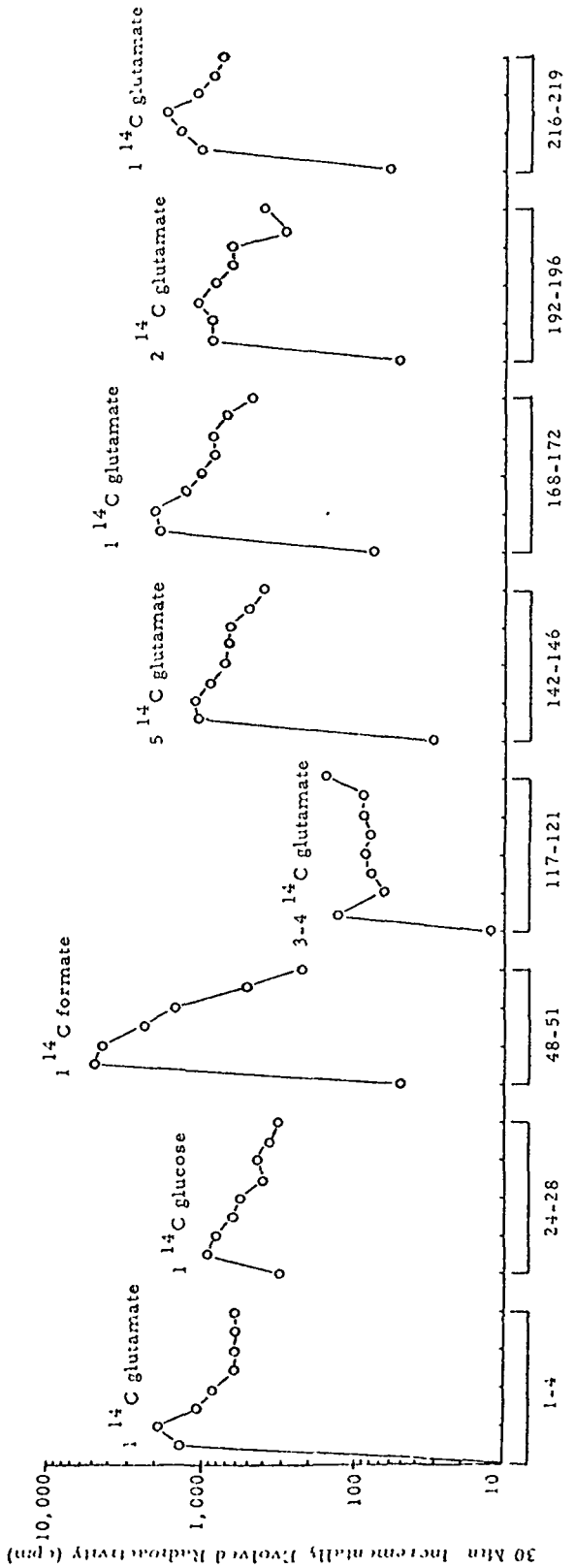

Figure 21

Multiple Addition of Labeled Substrates to Meadow Soil

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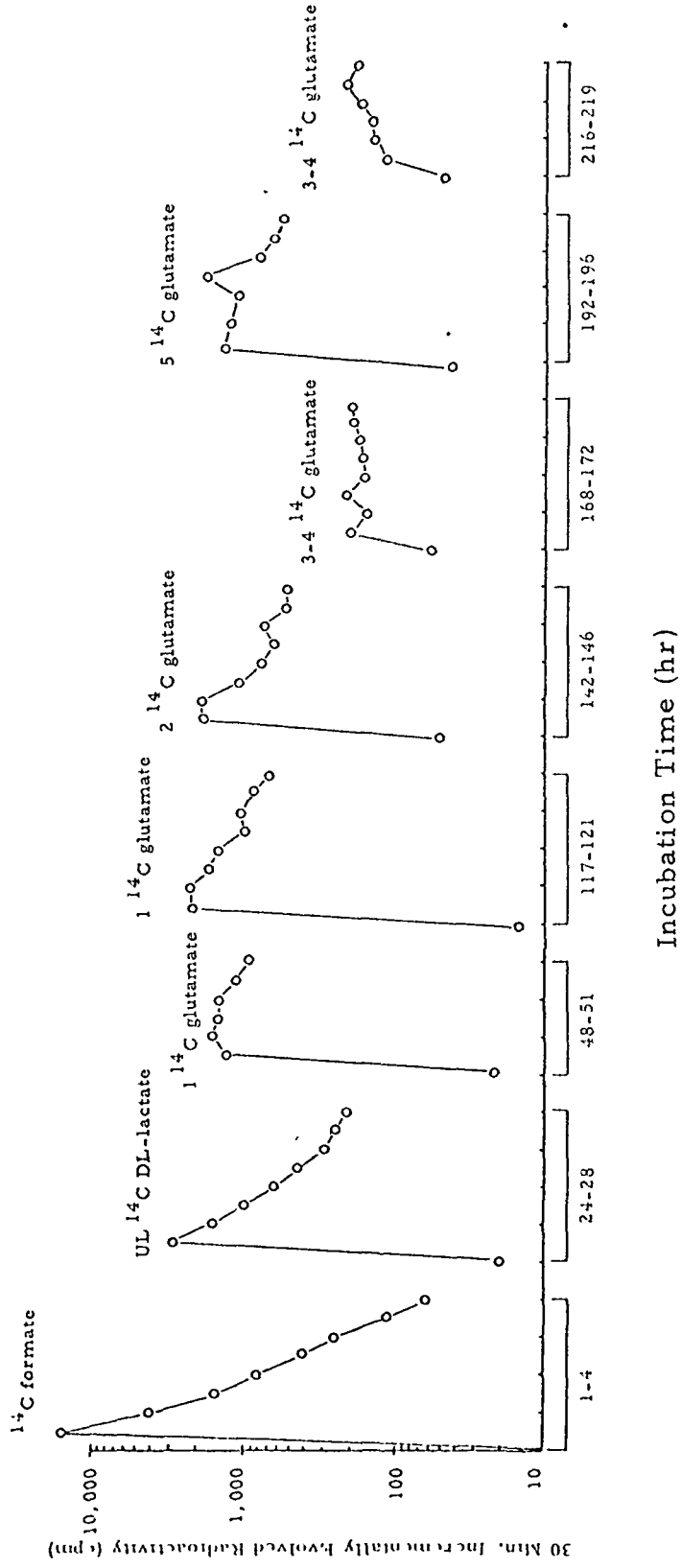

Figure 22

Multiple Addition of Labeled Substrates to Meadow Soil

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Soil: One addition of 0.21 cc of Meadow soil was used
Incubation: Room temperature, aerobic
Gas Collection: $Ba(OH)_2$ used to collect $^{14}CO_2$
Medium: 0.1 ml of 10^{-3} M, ^{14}C specifically labeled DL-glutamate
- ● 1 ^{14}C DL-glutamate
- ○ 2 ^{14}C DL-glutamate
- □ 3-4 ^{14}C DL-glutamate
- ▣ 5 ^{14}C DL-glutamate

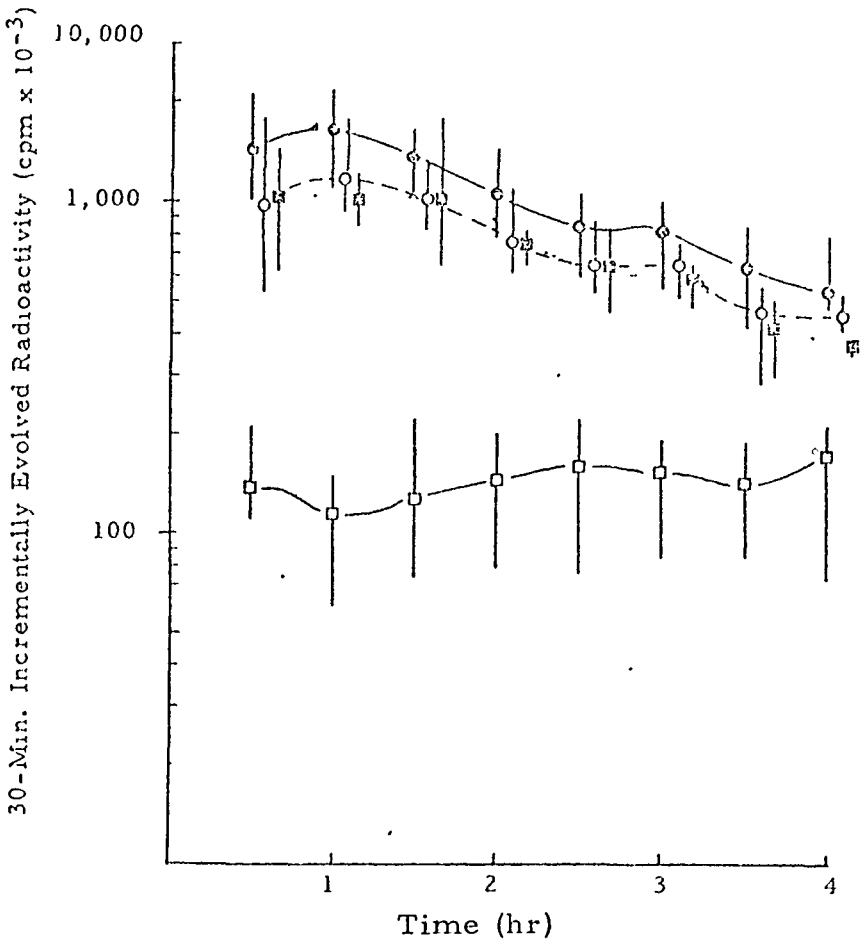

Figure 23

Metabolism of 1 ^{14}C, 2 ^{14}C, 3-4 ^{14}C and 5 ^{14}C DL-glutamate by Meadow Soil

Substrates were added sequentially as shown in Figures 19 to 22.

The basic feasibility of multiple addition for at least specifically labeled glutamate is thus firmly established.

D. Library Soil and Pure Culture Profiles

1. Introduction

The candidate substrates and conditions listed in Table 1 were tested against several soils and pure cultures of organisms isolated from those soils. This was performed to verify the various candidate test parameters and to initiate the establishment of a library of profiles corresponding to various soil types and presumably to various microbial species. It was also questioned whether the pattern of response from pure cultures isolated from the soil would be similar to the pattern of response obtained from the natural soil from which they were isolated. Two or three of the predominating microbial species obtained from dilution plate counts on TSA were isolated from each soil and tested individually.

Kinetic data were collected for each parameter tested, and profiles corresponding to two hours, six hours and 22 hours of incubation were constructed. These three time points appeared to be the minimum needed to show rate differences and to detect inhibition which may occur early during incubation but may be overcome later.

Soils and pure cultures tested in this phase of study included: Meadow soil and three pure culture isolates of Meadow soil, Phoenix

soil and two pure culture isolates of Phoenix soil, and Aiken soil and one pure culture isolate from Aiken soil. Results are presented in Appendix II, "Soil and Soil Isolate Library of Profiles."

2. Atmosphere Effects

All soils and soil isolates tested showed greater production of $^{14}CO_2$ when incubated under aerobic conditions as opposed to anaerobic conditions. Meadow and Phoenix soils showed at least five fold differences between aerobic and anaerobic incubation after two hours; however, after 22 hours of incubation, the anaerobic evolution was only slightly less than aerobic evolution. Cultures isolated from these soils showed much greater differences in anaerobic and aerobic incubation than did the soils, even after 22 hours of incubation. Since only aerobic isolation procedures were conducted, these results are not surprising. Results from these two soils indicate that anaerobic and/or facultatively anaerobic organisms are also present and actively metabolize the substrates added.

Aiken soil showed a wide difference in the anaerobic and aerobic response, and this remained after 22-hour incubation. It would appear that anaerobic organisms play a lesser role in the population structure of the Aiken soil than in the other two test soils.

3. Temperature

The four temperatures selected produced relatively clear-cut profiles identifying the temperature which produced maximum

$^{14}CO_2$ evolution as well as providing information on the kinetics of that evolution. Meadow soil showed a peak in evolution which occurred at 35°C; all three isolates from this soil also showed temperature peaks at 35°C.

Phoenix soil produced maximum $^{14}CO_2$ at 60°C; however, the two isolates from this soil showed maxima in the 20° - 35° range. It is presumed that thermophylic organisms not isolated and studied in significant numbers must also be present in Phoenix soil.

It is possible that a considerable portion of the 60° evolution is brought about by organisms whose tolerance limit is considerably below that temperature. Note that all soils and isolates produced significant quantities of $^{14}CO_2$ at 60° even though they showed maxima in evolution at lower temperatures. It was also generally found that most of the $^{14}CO_2$ evolved over a 22-hour incubation period occurred during the first two hours of incubation. An interesting protocol might be to make a second medium addition at the elevated temperature. Enhanced response would indicate growth at that temperature; however, a decreased response may mean that the incubation temperature kills vegetative metabolizing cells.

4. Metabolism of Formate, Acetate and Lactate

All three intermediate substrates, ^{14}C formate, UL ^{14}C DL-lactate and UL ^{14}C acetate at the same specific activity gave rise to significant levels of evolved $^{14}CO_2$ from all soils and pure

culture isolates tested. However, the pattern of evolution from these substrates differed depending upon the soil and pure culture. All three soils produced the greatest amount of $^{14}CO_2$ from ^{14}C formate, and several cultures gave similar results. However, Meadow Soil Isolates No. 1 and 3 and the Aiken soil isolate evolved considerably more $^{14}CO_2$ from ^{14}C lactate than from ^{14}C formate. Phoenix Soil Isolate No. 1 showed the greatest evolution of $^{14}CO_2$ from ^{14}C acetate.

Interpretation of these data is linked to other biochemical tests. At the very least, however, the metabolism of one, two and three carbon compounds is demonstrated.

5. Amino Acid Metabolism

After 22-hour incubation, the three soils showed similar or slightly greater $^{14}CO_2$ production from L-amino acids than from D-amino acids. Isolated cultures also tended to show this pattern. Two of the pure cultures, Aiken Soil Isolate and Meadow Soil Isolate No. 1, however, did show more $^{14}CO_2$ evolution from D-amino acids than from L-amino acids. Although the hydrolysis of most protein yields exclusively L-amino acids (10, 11), the once held theory that D-amino acids were biologically inactive has been largely disapproved. Some naturally occurring plant and microorganism materials have been found to contain D-forms and the ability to degrade D-amino acids via specific D-amino acid oxidases, transaminases, or racimases to the L forms have been

widely found (11, 12). Some members of the D-series are actually degraded more rapidly than the corresponding L-enantiomorphs. The L-amino acid mixture tested in these experiments contained 3 ^{14}C L-serine, 1 ^{14}C L-leucine and UL ^{14}C L-alanine. The D-amino acid mixture contained 3 ^{14}C D-serine, 1 ^{14}C D-leucine and UL ^{14}C D-alanine. A more complete list of labeled amino acids should be tested, and those members selected which show the greatest degree of isomeric preference in test soils.

Several distinct patterns in the evolution of $^{14}CO_2$ from 1 ^{14}C, 2 ^{14}C, 3-4 ^{14}C and 5 ^{14}C DL-glutamate by soil and soil isolates were observed. Typical citric acid cycle metabolism gave rise to the pattern: 1 ^{14}C > 2 ^{14}C ≅ 5 ^{14}C >> 3-4 ^{14}C. Meadow soil, Phoenix soil, Aiken soil and Aiken isolate all showed this basic pattern of evolution and presumably the mixed flora predominantly utilizes that pathway for dissimilation of glutamate.

Another pattern of $^{14}CO_2$ evolution which has been discussed in Section I. C. 2 of this report was observed primarily in the isolates of Meadow soil. Results were typically as follows: 1 $^{14}C \geq$ 2 ^{14}C > 3-4 ^{14}C >> 5 ^{14}C.

A pathway which would give rise to such a labeling pattern has not been described. However, these isolates also possess the typical citric acid cycle pathway since addition of the organisms to sterile soil produces the typical citric acid cycle pattern.

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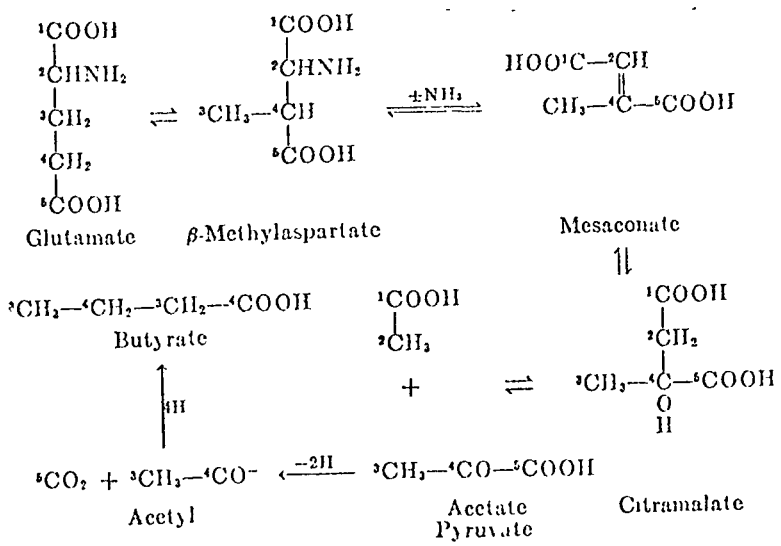
The following pattern was observed in Phoenix Soil Isolate No. 1: 1 $^{14}C \cong 5\ ^{14}C > 2\ ^{14}C > 3\text{-}4\ ^{14}C$.

Unequal yields from the 5 carbon and 2 carbon positions indicate at least some metabolic involvement other than via the citric acid cycle. The higher rate of evolution from the 5 position might be explained by a utilization of the pathway which has been described for Clostridium tetanomorphum (13), which is shown below:



Further breakdown of acetate and pyruvate, as shown by the results for ^{14}C acetate, ^{14}C lactate and ^{14}C formate, occur rapidly.

However, these intermediates were uniformly labeled and, therefore, cannot provide support for the supposed pathway of acetate and pyruvate dissimilation. The usefulness of specifically labeled acetate and lactate in place of the uniformly labeled compounds are thus demonstrated and will be considered for future studies to refine the technique.

6. Carbohydrate Metabolism

The involvement of various possible pathways for the dissimilation of specifically labeled glucose may be predicted from radiorespirometric experiments. Metabolism by unknown organisms and natural soil populations probably occurs via several pathways simultaneously thereby precluding quantitative estimates of pathway utilization such as those made by Wang (4). However, some individual pathways, if predominantly active for a particular organism or soil population, show differential and characteristic patterns in $^{14}CO_2$ evolution. Generally the Embden-Meyerhof-Parnas (EMP) pathway results in a predominance of $^{14}CO_2$ from the 3 and 4 carbon positions of glucose. On the other hand, the hexose monophosphate pathway (HMP) and the Entner-Doudoroff (ED) pathway result in a predominance of $^{14}CO_2$ from the 1, and 4 carbon positions of glucose.

A high yield of $^{14}CO_2$ from 6 ^{14}C glucose would be indicative of the glucuronic acid pathway (GA).

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Although the present study was limited to 1 ^{14}C, 3-4 ^{14}C and 6 ^{14}C D-glucose, definite indications of the several possible pathways were observed. All three soils showed a pattern of 1 ^{14}C > 3-4 ^{14}C > 6 ^{14}C, thereby indicating that the ED and/or HMP pathways predominate in these soils. Pure culture isolates, on the other hand, showed a pattern of 3-4 ^{14}C > 1 ^{14}C ≅ 6 ^{14}C or 3-4 ^{14}C ≅ 1 ^{14}C > 6 C, thus indicating a greater involvement of the EMP pathway than was found in the natural soils.

Phoenix Soil Isolate No. 1 showed a pattern in which 1 ^{14}C > 6 ^{14}C > 3-4 ^{14}C. The fact that the sixth carbon atom was more rapidly decarboxylated than the third or fourth carbon atoms may indicate involvement of the GA pathway.

Ribose utilization provides additional evidence for the presence of the HMP and GA pathways.

Surprisingly, cellulose, thought to be slowly utilized, was metabolized to a high degree by all soils and soil isolates except Phoenix Soil Isolate No. 1. The capability of degrading this biopolymer indicates a high level of development. Enzymes for breakdown would have been dependent upon the presence of cellulose at some stage in evolution. Thus, a positive response from an unknown soil charged with labeled cellulose would provide two-fold information: (1) that organisms present possess enzymes capable of degrading cellulose and (2) that cellulose probably exists or once existed in the evolutionary environment.

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7. Antimetabolite Studies

Experiments were conducted by mixing various antimetabolites with ^{14}C labeled medium and dosing soil or pure culture suspensions in the normal fashion. Medium and conditions were otherwise the same as in the aerobic experiments. Therefore, this latter experiment serves as an uninhibited control.

The results show that 2, 4 dinitrophenol, which uncouples oxidative phosphorylation, was strongly inhibitory to all soil isolates, but only slightly inhibitory towards the soils. The 0.1 M KCN was similarly effective in inhibiting pure culture suspensions, but much less effective against natural soils. The soils may offer protection for some organisms and may cause chemical deactivation of the 2, 4 dinitrophenol and KCN.

The antibiotic mixture which contained penicillin 5,000 U, Streptomycin 5,000 μg and Fungazone* 12.5 μg per milliliter was administered in two doses, the second following the first after 24 hours. Results presented in Appendix II are for the second addition. Pure cultures of soil isolates were largely sensitive to the mixture, Phoenix Soil Isolate No. 1 being an exception. Meadow and Phoenix soils were not inhibited by the antibiotic mixture; however, the Aiken soil was inhibited by approximately an order of magnitude.

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*Fungazone is an E. R. Squibb & Sons trademark for Amphotericin B.

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The effect 10^{-1} M, 10^{-3} M and 10^{-5} M concentrations of iodoacetic acid (IAA) was used to demonstrate the method for determining inhibitory concentrations. Soils were generally much less inhibited than pure cultures by all three concentrations of IAA; however, a definite inhibition pattern was found for both pure cultures and soils. In most cases, the inhibition pattern was 10^{-5} M $\cong$ 10^{-3} M $<<$ 10^{-1} M. This means that the critical inhibiting concentration of IAA was between 10^{-3} M and 10^{-1} M.

In overview of all parameters, it is obvious that Meadow and Phoenix soils produced a vigorous response in the first two hours. Aiken soil showed a response in the first two hours which was approximately one order of magnitude lower. This fact seems to correlate well with the total numbers of organisms grown from these soils (see Table 2, Appendix I). Continued studies may provide sufficient data to approximate the number of heterotrophic organisms present in unknown soil.

It was generally observed that natural soils produced a much less distinctive pattern of results than pure culture isolates from that soil; i.e. all substrates and conditions produced a near maximum response with soils. Aiken soil, having the fewest organisms, showed the most distinctive pattern.

Natural populations of organisms in soil are comparised of many different species, all displaying overlapping metabolic capabilities

which provide for community utilization of almost any natural substrate and survival under a broad range of conditions. Therefore, characteristic patterns of metabolism from natural soil would be expected only when the majority of species present function optimally within narrow limits. Several parameters of the profile such as atmosphere, temperature, glutamate metabolism, and inhibition by KCN appeared to demonstrate these general population characteristics. However, the organism specific effects of some substrates and inhibitors are completely overridden by overall capabilities of the whole microbial community.

Soil may also provide a physical or chemical protection to its inhabitants and, thereby, strongly buffer the action of adding antimetabolites.

The fact that the specific action of a substrate or inhibitor cannot be demonstrated on natural soils should not be grounds for excluding it from planetary tests if the compounds influence key pathways or steps. Extraterrestrial soils, if similar to Earth soils and if containing mixed flora, may also display a rather nonspecific pattern of metabolism. However, the possibility should not be overlooked that foreign soil populations may lack some of the metabolic capabilities displayed by Earth soil microbial communities. In that case, definite patterns similar to those found with pure Earth cultures might be obtained. It is the profile of Martian metabolism and possible holes in biochemical capabilities with respect to Earth capabilities which are specifically sought. If, on the

other hand, these do not exist, very valuable information on an important similarity between Earth and the extraterrestrial soil populations will have been found.

The profile library of soils and soil isolates which has been initiated in this study will be used as a working base for further studies. A careful reevaluation of each substrate and set of conditions is planned before expansion of the library is continued.

Attempts will be made to utilize the multiple addition mode for producing library results and the list of parameters as developed may require modification in order to do so. Examination of the substrates within the light of stability and compatability with the Viking test system must also guide future thinking.

In conclusion, it appears that a test system similar to the current Viking labeled release experiment, with the added capability for repeated additions of individual media and modifications in incubation temperature or atmosphere, could determine a broad array of biochemical and physiological characteristics of any organisms found. These characteristics would provide at least some tolerance limits for growth and control of the organisms--minimum requirements for any return of alien soil to Earth. A workable basis for assessment of the potential biohazard from organisms encountered might be provided by such a series of characteristics.

If, as planned, soil and pure culture libraries are established for a broad array of organisms and soil types, it is foreseeable that the data

bank will be voluminous, and retrieval may become difficult. The possibility of developing a card reader, computer stored data, or other type of automated sorting device should be seriously considered for future library storage.

II. RETURN MARS SAMPLE

A. Background

In the spring of 1973, astronaut geologist, Dr. Jack Schmitt, told the United Press that man would be exploring Mars in the not-too-distant future. "Now I don't know what the reasons will be that we will head for Mars, but the goals will be even more exciting than anything we were able to imagine on the Moon," he said.

Undoubtedly, the capability for sending men to Mars lies within our grasp and man's inquisitiveness will not be denied. However, before such trips can be reasonably condoned, we must first determine the hazards entailed. The physical hazards may be fairly well anticipated and, in any event, would be confined to the astronaut crews. Tragedies of the sort that besets explorers might occur, but no catastrophe to the general population could ensue. The biological hazards of infectious diseases or ecological displacements, on the other hand, cannot presently be anticipated nor delimited. Their impact may go far beyond the space crew upon its return. Infection and rampant, invasive growths by alien organisms could change the Earth in a fearful manner. Additional information about Mars must be obtained before an intelligent estimate of the problem can be made. Then a careful plan must be evolved and put into practice to protect the Earth against back contamination from the red planet before orderly exploration by man or the return of Mars samples to our world can safely commence.

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The discipline of planetary quarantine probably began in 1957 when the National Academy of Sciences expressed concern over the possible biological contamination of planets we intended to investigate within our impending space program. Two reasons for this concern were cited: (a) the possibility of influencing the evolution or biological status of the target planet, and (b) the prospect that contamination of the planet with terrestrial organisms might preclude the historic scientific opportunity to detect indigenous life on another planet.

In 1958, the International Council of Scientific Unions (ICSU), through its ad hoc Committee on Contamination by Extraterrestrial Exploration (CETEX) recommended a code to prevent lunar and planetary contamination. In 1959 and 1960, NASA promulgated an official policy statement to the effect that it was essential that no act be performed that would irretrievably preclude the use of a celestial body as a base for scientific investigations (14). Since 1959, the Committee on Space Research (COSPAR) of the ICSU has attempted to bring about international cooperation on planetary quarantine policy and technology. The initial concern of the planetary quarantine program was the accidental impact of unsterilized, nonlanding vehicles onto the surface of the planets (15).

This concern was soon broadened to include all possible modes of planetary contamination by nonlanders and landers.

NASA mounted a significant research and development effort in planetary quarantine with early and heavy emphasis on preventing contamination of the planets. The primary thrust, however, remained on prevention of contamination of planetary targets with terrestrial organisms. The consequences of which were carefully assessed (16).

The Soviet Union expressed support for the concept of contamination control and cited three principal reasons: (2) the loss of certainty in identifying indigenous organic matter on the target planet, (b) the, perhaps, irrevocable loss of determining the presence of extraterrestrial life and (c), the possibility of changing the evolutionary history of the target planet (17).

With the approach of manned lunar flight capability, the problem of dangerous back contamination of the Earth came to the fore of planetary quarantine considerations. In 1964, the National Academy of Sciences convened a "Conference on the Potential Hazard of Back Contamination from the Planets," (18). The study formed much of the basis for the subsequent lunar quarantine procedures.

An examination of Federal regulations at that time showed that the U.S. Public Health Service, the Department of Agriculture and the Department of the Interior were responsible for various aspects of biological contamination control (19). None of these regulations, of course, had been directed at planetary quarantine. Accordingly, an interagency agreement among the NASA, Department of Agriculture, Department of Health, Education and Welfare (containing the Public Health Service), the Department of the Interior and the National Academy of Sciences was drafted for the "Protection of the Earth's Biosphere from Lunar Sources of Contamination" on August 24, 1967 (20). The specific dual rationale cited was the necessity to protect the Earth's ecology and the scientific need to preserve integrity of samples returned from the Moon. An extensive protocol for examinations of the returning astronauts and the lunar samples was devised along with an elaborate Lunar Receiving Laboratory (LRL) specifically designed for the conduct of the program. The men and samples were to be transferred to and maintained behind biological barriers where the examinations were to be performed. Criteria were established for the quarantine and release of the astronauts and samples.

In the ensuing years, an overall planetary quarantine program evolved with the following objectives:

1. The protection of planetary quarantine targets of exploration from terrestrial contamination which could interfere with, confuse or render ambiguous the scientific investigation of the planet's biological status.

2. The preclusion of influencing the biological evolution of target planets through introduction of terrestrial organisms or biochemicals.

3. The preservation of the integrity of planetary samples returned to Earth for scientific examination.

4. Protection of the health of astronauts on planetary missions and return flights.

5. Protection of the general population from pathogenic extraterrestrial organisms or mutated terrestrial organisms returned with the astronauts, spacecraft or samples.

6. Protection of the biosphere against any untoward, adverse consequence of the introduction of extraterrestrial agents.

The LRL and quarantine facility were built and the programs carried out on the returning astronauts and samples essentially as planned. However, the fact that no untoward events occurred is attributed to the sterility of the Moon and not to the efficacy of the quarantine program. Numerous contaminations of the terrestrial biosphere with lunar material occurred. Terrestrial exposure to lunar material began with the reentry of the Apollo spacecraft into the Earth's atmosphere when lunar particles were undoubtedly abraded from the spacecraft and disseminated into the atmosphere. Upon impact

with the ocean, particles clinging to the spacecraft became water-borne. There were additional opportunities for the escape of lunar material during the transfer of the spacecraft and astronauts to the receiving aircraft carrier and quarantine station. Numerous other exposures (21) occurred after the astronauts and samples had been installed in the LRL and while the quarantine program was in progress (22).

The lunar quarantine program might best be considered a dress rehearsal for a Mars quarantine program. The lunar program was warranted because of the severity of the catastrophe that could be inflicted on Earth by foreign organisms, but most scientists agreed, based on our pre-Apollo knowledge of the Moon, that the likelihood of indigenous organisms on the Moon was highly remote. The lack of an atmosphere, the lack of solar shielding and the lack of biologically available water at the Moon's surface had been well established and strongly argued against the likelihood that the Moon's surface harbored living organisms. With the completion of the lunar quarantine program, NASA sponsored an extensive study (23) to determine the possible role of the LRL in the post-Apollo period with respect to the containment and examination of extraterrestrial samples. Dr. Gilbert V. Levin participated in this study and subsequently visited the LRL

specifically to consider its usefulness in a return Mars sample program. He strongly agrees with the study statement: "It was generally agreed that current LRL quarantine protocol is by no means adequate to protect the Earth from contamination by injurious agents of extraterrestrial origin." At best, it was felt that the LRL might be used for "the development of techniques and protocols for back contamination testing of a returned Mars sample" and for the "resolution of planetary quarantine problems associated with returning extraterrestrial samples to Earth." Thus, many scientists use a completely different frame of reference when assessing the potential hazard of returning samples to Earth from Mars than they did in assessing that hazard with respect to the Moon. The prospect for indigenous life on Mars is ranked several orders of magnitude higher than that for the Moon even by those scientists generally conservative in estimating the prospect for life on Mars.

B. Rationale for Return Mars Sample

There is virtually no disagreement among scientists that the examination of surface samples of Mars conducted in terrestrial laboratories by competent investigators would produce information of great value to many aspects of planetary science. No matter how sophisticated instruments become for automated

analysis and experimentation on planetary surfaces, the depth of understanding obtained in this manner cannot be as great as that which would be obtained by highly qualified scientists working with Mars samples in well-equipped, modern laboratories. Many physical and chemical examinations of the samples could be performed with instruments and techniques not amenable to remote automation. Moreover, scientists examining the specimen would direct the examination in an evolving manner in accordance with results obtained which no spacecraft computer or ground link could approach. In no field could direct examination by scientists be more advantageous than in biology. As of this writing, the most pressing scientific question with regard to Mars is whether or not life exist on that planet. The principal objective of the Viking Mission is the resolution of that problem.

In developing a rationale for a Mars return sample program, the prospects for life on Mars must be somehow assessed as that probability will greatly influence the type of plan required. One approach to lend perspective to the possibility of Martian life is to examine the Martian environment to determine whether it could support life as we know it on Earth. This eliminates the need for initial speculation on exotic life systems. Our current

information on Mars indicates that in all known respects with one possible exception, the Mars environment is capable of supporting anaerobic microorganisms. That exception is water. The problem with water on Mars with respect to life is three fold: (2) the abundance of water, (b) the availability of water to potential ecological niches and (c), the availability of water in liquid phase. Mariner 9 has determined that the quanity of precipitable water vapor in the Mars atmosphere is approximately two orders of magnitude below that in the Earth's atmosphere. Nonetheless, large portions of Mars are covered with an atmosphere at or approaching water vapor saturation on a seasonal basis. The absolute quantity of water on Mars is unknown. The principal water reservoirs are probably the polar caps, permafrost, and connate water released through volcanic outgassing, the extent of each being unknown at this time. The temperature and pressure of Mars, as currently known, are such that no definitive statement is possible concerning the existence of water in liquid phase. The data at hand would indicate, that except, possibly, for extremely low elevations on the planet's surface, water would exist only as ice or vapor. However, various theories have been proposed to suggest that biologically significant quantities of liquid water might be made available

diurnally or seasonally at or near the surface of the planet. Should this be the case, there seems little doubt that terrestrial organisms could survive and probably grow on Mars. If, on the other hand, liquid water is not available, and one wishes to constrain his speculation to extraterrestrial life forms functioning through an aqueous biochemistry, some mechanism would have to be hypothesized whereby the organisms can extract water from the atmosphere or from ice to maintain an internal aqueous environment. This would not seem to be an overly formidable evolutionary step. This approach would indicate that the probability for Martian life is significant.

It must be cautioned, however, that even if the present environment on Mars were a duplicate of that on Earth, this would not guarantee the existence of life on Mars. If a general theory of biology prevails, the disconcernment of which is the true investigatory goal of exobiology, planetary conditions other than those prevailing would have been required for the evolution of indigenous Mars life. Too little is known about the planet at this time to determine such a history.

If life exists on Mars, it is not unlikely that it could survive in the Earth's environment. Hence, the dilema is set concerning the return of Mars samples to Earth: we want to obtain Mars samples because a significant probability exists that they would contain extra-

terrestrial life, but the probability is also significantly high(er) that such organisms could proliferate on Earth with unknown consequences to our biosphere.

What is the cost-benefit ratio of returning a Mars sample to Earth? As opposed to a civil works project, the sample return project does not lend itself to a dollar evaluation. It is not possible to place monetary value on the knowledge to be gained nor on possible direct application of that Knowledge. Nonetheless, most scientists agree that the value added to our knowledge would be very large.

A brief survey of these benefits is possible through considering possible scenerios confronting a return Mars sample mission:

1. The Viking Mission Has Detected Life Through Its Automated Lander

A return sample would offer the best means to confirm the results and to determine the various forms of life, its biochemistry, the environmental responses, interactions with Earth forms, evolutionary relationships among forms found and comparison of terrestrial forms.

2. Life Exists on Mars But Has Not Been Detected by the Experiments on Viking

Return samples can be exhaustively examined by the entire arsenal of techniques available to the modern organic chemistry, biochemistry, microbiological and biological laboratories. Life forms

incapable of responding to Viking life detection tests or other life detection tests presently planned would be found and the result might be a large savings in the expenditures for future, otherwise fruitless, planetary missions.

3. Life Does Not Exist on Mars and Viking Has Returned a False/Positive Signal

Examination of return samples, including one from the site of the Viking false/positive, would reveal the true situation.

4. Life Does Not Exist on Mars and Viking Has Returned a Negative Signal

Viking and other automated landers will find it more difficult to prove that life does not exist than to establish the presence of life on Mars. The absence of life can only be proven by repeated samplings and exhaustive tests. In answering the biological question with respect to Mars, the samples would be carefully searched for biological and biochemical fossils. Any evidence of past life forms or biological precursors would be a prime objective of such an evaluation. In testing the validity of a general theory of biology, all disciplines would be brought to bear upon the samples to determine environmental factors and epochs could be established. Evidence for chemical, organic chemical, biochemical and life precursor evolution would be the key objective.

5. Manned Exploration of Mars is Proposed

Prior to the exposure of astronauts to the Mars environment, and, subsequently the astronauts to the Earth, an exhaustive analysis of possible hazards to the spacecraft crew must be made. The examination of return Mars samples offers the best method of assessing these hazards.

The cost of an automated return sample mission to Mars would probably be several billion dollars - several times the cost of the 1975 Viking Mission. It has been estimated that a manned return sample mission to Mars would cost approximately $40 billion (24).

On the cost side of the ledger, the principal unknown is the hazard that the return of Mars samples would pose to terrestrial biology.

What is that hazard? Two fairly extreme perspectives delineate the problem. One can make the comparison between the introduction of Mars material to Earth and the initial contact of Europeans with the Western Hemisphere. The prospect that the Europeans would contract some deadly infectious disease were very high. This was because the immunological pool of the Europeans might be expected to be devoid of defenses against infectious agents which had had time for isolated development in the New World. Since the infectious agents would be competing for an ecological niche among life forms and environments

similar to those in which they had developed, the prospects for a successful invasion of the new host were high. The situation might be said to have been far more serious than that posed by the Mars return sample. Yet the historic confrontation of agents and hosts was not catastrophic to life on Earth (although the resulting spread of infectious disease among the Europeans was, and continues to be very serious in accumulated deaths and illnesses caused by the introduction of syphillis and tobacco). By comparison, the probabilities of the existence of Martian organisms, their survival in our environment, and their interaction with our life forms (the two biochemistries and genetic machinery perhaps differing fundamentally), are all very low and, when multiplied together to obtain the total probability, become vanishingly small.

Those at the other end of the hazards spectrum fear the possibility of an impact so severe as to threaten the existence of many or all biological species on Earth. This could result from the modification of some fundamental ecological system, the introduction of particularly virulent pathogens or from direct toxic or poisonous effects (21). Perhaps the most insidious and difficult to detect danger would be the modification of a terrestrial ecological system. A hypothetical example might be the blocking of nitrogen fixation by an organism invading this relatively small, but vital ecological niche.

Exotic species performing in this performing in this manner are not likely to be checked by defense mechanisms evolved on Earth (25). And, the fact that alien organisms may have arisen from an entirely different biochemical sequence than terrestrial life does not assure that they are innocuous to terrestrial life (26). We have examples of organisms which can metabolize or transform alien molecules such as DDT and other synthetic materials.

Despite the lunar quarantine program, there are those who contend that the serious issue of back contamination of Earth has received essentially no consideration (26), that neither research nor significant discussion has as yet been undertaken and that essentially no effort has been devoted to develop new quarantine technologies. The only absolutely certain approach available today would be to expose the entire ark of living things to a wide variety of Martian samples--obviously infeasible. However, no alternative has been developed (26). Even given such an impossible tour de force, what would be a reasonable incubation period? Terrestrial examples, such as scrappie with its 11 year incubation period and the 30 year latent period between the ingestion of an asbestos fiber and the production of a malignant tumor, confound the quarantine officer. In view of such facts, some have contended (21) that we have no basis for limiting quarantine to any workable period of time.

There are secondary hazard ramifications to be considered among the costs. Mutations occurring naturally in terrestrial organisms have been the cause of serious epidemics such as Asian flu. Terrestrial organisms are constantly exposed to mutagenic agents including, recently, man-made ones. The possibility exists that Martian organisms which are either not detected or which pass quarantine may subsequently mutate on Earth and produce major repercussions.

Thus, while the benefit from a return Mars sample may be immense, the cost could be infinite. Scientists might be willing to take a risk of, say, 10^{-6} that a catastrophic event will occur from the introduction of Mars material to Earth, but they do so realizing the great opportunity to answer scientific questions that the sample will provide. The lay public, on the other hand, not seeing any direct benefit from the sample, but fearing the hazard, may be reluctant to accept even that degree of risk (26).

C. Canvas of Scientific Opinion on Return Mars Sample Benefit and Risk

In an attempt to obtain current thinking from members of the biological community active in planetary work, Dr. Patricia A. Straat of Biospherics interviewed several prominent investigators concerned with space exploration and extraterrestrial life detection. Views regarding the value and justification for a return Mars sample,

the potential hazards of back contamination, and quarantine recommendations were solicited. The ideas expressed in these interviews are summarized below:

Joshua Lederberg, Stanford Medical School, has in the past been opposed to returning a Mars sample. However, he now recommends a consideration of what information is necessary to enable such a decision to be made. Although he feels the decision is premature if made now, the coming Viking Mission will contribute important information to the issue by delineating Mars as a habitat. Since Martian conditions differ considerably from terrestrial conditions, Dr. Lederberg strongly recommends that experiments designed to test the effects of terrestrial environments on Martian organisms be conducted on Mars prior to returning a sample to Earth. Studies such as these would afford the potential of destroying a return sample in the event of a mishap. The effects of liquid water are of special interest since the water content of Mars suggests the speculation that liquid water at elevated temperatures may destroy Martian life. However, if terrestrial conditions are not inhibitory, a decision becomes more difficult for a risk versus value assessment. Or, if life is not detected by Viking missions, a great deal of contingency information regarding the habitat is important in assessing the risk.

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Consideration of returning a sample from Mars poses a dilemma. Before bringing it back, it is necessary to know a great deal about Martian biochemistry; however, it may be necessary to bring back the sample first in order to obtain this knowledge in sufficient detail. While automated instruments can accomplish a great deal, large investments will be required to develop automated experiments having the flexibility of detailed control by human intelligence from Earth.

Bringing back a Mars sample is a prerequisite for manned missions since it is impossible to return the man without Martian soil on him. On the other hand, it may not be necessary to do these experiments in series; a man could be sent to Mars and, if he survives, utilized to return the Mars sample to Earth with the concommittant risk reduction. As of now, however, Dr. Lederberg does not feel sufficient information is available to make a decision. A great deal will depend on the results of future Viking missions after which factors such as risk, value, and cost can be considered as major trade-offs.

With regard to quarantine of a return Mars sample, Dr. Lederberg strongly advocates the use of a space station where the chances of control or sample destruction are maximized. Other specific quarantine recommendations would be containment of the sample in a quarantine laboratory located either on an oceanic island or possibly on a ship

(which could be incinerated) using liquid water as a final barrier in the event it is shown to be inhibitory to Martian life. However, as illustrated by the Moon return samples, he feels that because of human errors there will always be breaks, leaks, and accidents within any quarantine system. He recommends minimization of this risk by utilizing some already operational quarantine facility, such as Plum Island, and utilizing personnel with long practice in handling deadly pathogens in that particular laboratory. Such a procedure also eliminates the necessity of training new personnel to handle return samples. However, for absolute safety, it is probably best to conduct all experimentation on Mars with automated instruments.

Harold P. Klein, Ames Research Center, does not feel that it is justified to return a sample of Mars to Earth until it is better established that there will be no subsequent hazards to terrestrial life. He feels that the probability of life on Mars has been enhanced by the recent Mariner 9 data, increasing the concern over a potential hazard involved in returning a Mars sample. If the forthcoming Viking biology experiments are positive, the pressure for immediate return of a sample for study will be high. In this case, Dr. Klein is strongly against returning a sample and feels that there will be a need for experimentation to be continued on Mars rather than on Earth for some period of time. Such experimentation could include automated

biochemical and metabolic studies and possibly the exposure of a variety of terrestrial organisms or even delicately balanced ecosystems to Martian soil. Such experiments are considered necessary to provide the confidence that when a sample is returned, it is not dangerous either because it has no deleterious effect or because it can be controlled. Finally, only after such preliminary study would he recommend sending a man to Mars. However, even with this approach, the risk reduction is not complete since not all terrestrial organisms can be tested. Consequently, a return sample must still be fully isolated in a quarantine barrier.

In the event that Viking life detection experiments are negative, Dr. Klein would, nonetheless, recommend similar precautions and repeated missions before returning a sample. The reason for this is that negative results would be obtained by Viking experiments either because they are of relatively low sensitivity or because the specific sampling sites were inappropriate. In addition, if no organics are found, he feels an explanation is in order since they are anticipated. If organic material is present in the soil, Dr. Klein would be suspicious of soil biogenicity. Only after repeated missions with negative life detection results and after the composition and distribution of the organic material is thoroughly understood should a sample be returned for analysis, and then only under full quarantine procedures. Some

consideration should be given to bringing back a sterile sample, but Klein considers this of little biological interest.

Leonard Zil, Ames Research Center, expressed the opinion that returning a Mars sample is justified because more sensitive measurements, such as analysis for traces of organic carbon, are possible here and can be made by trained researchers. Further, direct comparisons can be made with Lunar and Earth samples. Mars may also be the only planet from which we can return a sample since Venus and Jupiter may be out of the question. Such comparative studies are deemed quite important to the space program and the understanding of the evolution of the solar system, galaxy and universe. However, in view of the potential hazard of a return Mars sample, Dr. Zil felt that information from Viking is necessary before a decision can be made as to whether to return a sample. If Viking Biology Experiments are negative, then there probably is no life on Mars and a sample can probably be safely returned. However, if Viking is positive, much more information is required before we can safely return a sample. The difficulty expressed is that we cannot assess the hazard without sending up terrestrial life to Mars but even here there is a problem of host specificity.

With regards to quarantine precautions, Dr. Zil felt that the handling of the Moon samples was inadequate, but that many of the

problems were unavoidable. Thus, he suggested that perhaps the samples should be returned to a sky lab equipped with minimum personnel and with the capability to incinerate if necessary. The system recommended was to contain the samples in a sealed container which could be sterilized by heat or some other mechanism prior to the return if biological experiments are not planned. This container should be placed in a Class 3 barrier which is a glove box with an internal negative pressure, sterilized with ethylene oxide and used for work with deadly pathogens. This barrier could then contained in a laminar flow room within the orbiting laboratory.

Vance Oyama from Ames Research Center has conducted extensive life detection studies with Moon soil and is familiar with the effectiveness of the quarantine barrier utilized to prevent back contamination from the Moon. In his interview he expressed the opinion that a Return Mars Sample is necessary for an "in-depth" study of the properties of Martian soil because of the availability of various techniques and facilities which allow handling of large numbers of samples treated in a variety of conditions. To conduct such a study on a return sample is well justified to understand the place of Mars in the history of the solar system, to answer questions on the origin of life, and to gain insight into the possible uniqueness of Earth as a biological habitat. He feels that much can be derived

from a return Mars sample to answer questions regarding the geological, chemical or pre-biological, and biological evolution of Mars and of Earth. However, these return samples should be collected from as many diverse sites as feasible.

Regarding potential hazards, Oyama strongly felt that whether or not a Mars sample is returned to Earth should depend on the results from the Viking '75 Mission and on several additional Post Viking Missions. If these life detection tests provide negative results, then it would be plausible to return a sample for extensive analytical tests, including additional life detection tests. In this instance, the hazard to man would probably be considered minimal since infectivity requires a certain compatibility between pathogen and host. However, if ambiguous or positive results were obtained by Viking Missions, Oyama would be against returning a sample to Earth because of the large potential hazard of contamination.

For quarantine recommendations, Oyama favored returning the sample either to a Moon station or to an artificial satellite. Here the returning spacecraft could be "swallowed" and later sent to outer space if necessary. Within the way station, work could be done either with a robot or by manned operation using barriers and rubber gloves. Here the major problem is the interface between man and the sample. Differential pressure barriers pose problems because to protect man,

the lower pressure must be with the sample. Operationally, however, this makes it difficult to use the gloves which are extended into the vacuum and have no tactile surface. If, on the other hand, the sample is protected, man is at a lower pressure in a clean room with a secondary curtain of air protection. The aternative favored by Oyama is to operate remotely with a robot, protecting man by separation from the sample with a pressure wall between the man and sample chambers. Mr. Oyama did not feel that the sample should be brought back to Earth either directly or via a relay station because he felt that no safe mechanism exists for completely preventing contamination. He cited the handling of Moon samples as a case in point.

Edward Merek, Ames Research Center and co-worker with Vance Oyama, feels that the return of a Martian soil sample is not justified for biological experimentation because such studies could more effectively be performed on the surface of Mars. Thus, the survival of Martian organisms both in transit and within the terrestrial environment may preclude valid data from being obtained in biological experiments performed on a return sample. Further, conducting studies on Earth would be difficult because of constraints necessarily imposed by a quarantine barrier. Return samples will also be limited in size and may represent only one or a few geographical locations. On Mars, the sampling potential is considerably higher and many

experiments could be conducted at various times of day and at numerous geographical locations with the added advantage of utilizing the Martian habitat and viable organisms.

Although Dr. Merek feels that biological experimentation is best conducted on the surface of Mars, a return sample nonetheless has value for geological and chemical soil analyses. Such detailed analyses could, in fact, provide the basis for additional biological experiments to be performed on the surface of Mars. To avoid possible hazards of back contamination, the return sample would best be returned sterilized although there is a fundamental difficulty in sterilizing soil of totally unknown properties.

In addition to these interviews where individual points of view have been expressed, Dr. Straat also held a brief conference on March 30, 1973 with several Ames personnel interested in the issue of a return Mars sample. Those present included Drs. Richard Johnson, Larry Hochstein, Leonard Zil, Harold Klein, James Lanyi, and Bob McElroy. Although this group had no specific recommendations for quarantine other than can be found in classical textbooks for the handling of deadly pathogens, considerable time was devoted to a discussion of the justification, importance, and hazards of returning a Mars sample. The concensus of the group was that the decision must be considered from two viewpoints:

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1. On scientific grounds, a Martian sample should be returned to Earth. Thus, if proper samples can be obtained by a rover from a variety of areas in sufficient quantities, the types of studies that can be done on Earth are broader in scope than those which can be automated. These include geological dating, vast numbers of growth conditions to study living systems, and any type of experiment that requires logic feedback from scientists of a type that cannot be anticipated years in advance of the experiments.

2. On logical grounds, no Martian sample should be returned to Earth. Since the risk in returning a sample cannot be identified, it is simply not logical to return it. The major issue is infectivity and the mechanism by which Martian organisms would multiply. If the infecting mechanism and the terrestrial hosts could be known, a more precise assessment of the risk could be made. However, even then, ecology is not sufficiently advanced as a science to assess the implications of contamination by even one seemingly insignificant organism. Also,

assuming that very sophisticated experiments can be conducted with remote, automated instruments, the incremental information to be gained from a return sample may not be worth the risk.

Norman Horowitz, California Institute of Technology, favors returning a sample from Mars provided it is an active or fertile sample and feels that this is ultimately necessary for biological and geological investigations. The strongest argument for a return Mars sample would be an unambiguous positive response from Viking '75 which, especially if repeated in a follow-on Viking Mission, would justify returning a sample from the same region. If, however, Viking is negative, a return sample may not justify for biological reasons until we learn where to obtain an active sample. Dr. Horowitz does not feel that Martian life poses a threat to life on Earth and is far more concerned about keeping the Martian sample alive under terrestrial conditions than with infecting man. He feels that it is sufficient to handle a return Mars sample in the Lunar Return Sample Laboratory. Only if an orbiting laboratory is pre-existing and available should the return sample be quarantined there. Further, he feels that a return sample mission should precede a manned mission only if it is cheaper and easier. If both cost the same, then a man should be used to collect the return Mars sample. Dr. Horowitz stated that

some scientists are opposed to returning a viable sample to Earth but that most seem to be agreed that a sterilized sample can be safely returned. In this case, he recommends a study of different techniques for sterilizing and containing the sample for maximum preservation of biological, chemical, and morphological features.

Martin Alexander, Cornell University, is against returning a sample from Mars. The risk involved is considered high and the willingness to accept the risk depends on the relative vested interest of the investigator. Since some scientists have a vested interest in a return sample, he is inclined not to rely on these individuals for decisions on potential risks from back contamination. He feels that if life is found on Mars, automated landers cannot perform enough tests to determine whether a sample can be safely returned without endangering any important terrestrial species. He feels that automated landers can learn a great deal about Mars, although they are very limited in their ability to define potential hazards to the terrestrial biosphere. However, he feels that a decision by nonscientists in favor of a return sample is likely. Consequently, he stresses the urgency for research to plan for an effective quanrantine assuming a high risk. He feels that there is no current effective quarantine and that it would be a mistake to assume that one could be developed in two or three years. He feels that the Lunar Receiving Laboratory was a farce and that

quarantine laboratories should be required to maintain the rigorous standards exhibited by laboratories of the Communicable Disease Center in Atlanta or the Plum Island facility, both of which handle virulent organisms. Research areas recommended for study to prepare for a return sample are:

1. Means of detecting disease carriers and carrier state in man and animals.
2. Ability to detect pre-clinical expressions of disease in man, animals and plants.
3. A sensible definition of species essential to the biosphere and a careful selection from this list of those which should be included in the quarantine.

Carl Sagan, Cornell University, is completely against returning any Mars sample which has not been sterilized at 500°C for two days. He feels that the probability of life on Mars is sufficiently high to warrant extreme caution and the risk too great to allow the return of any organic or biological material to Earth, to Earth orbit or to a Lunar base. Since not much science can be done with a sterile sample, he sees no reason to return a sample. His feelings are sufficiently strong, that should it be decided to return a sample in the near future, he would make a widespread public appeal to prevent the action. He also feels that use of a satellite for a return sample

quarantine station is not sufficiently viable to sell to Congress. He is against manned missions, even one way manned missions. A manned mission would cost several hundreds of billion dollars and he feels that the other things are needed more urgently. Since the exploration of Mars by automated landers is far less costly, he feels this is the way to proceed and that all the information that is necessary can eventually be obtained by automated landers. When asked what information he would require before agreement to return a sample, he commented that we should know the results of Viking 1976 and of many follow-on Viking missions which have studied a wide variety of geographical locations. Further, the amount and type of organic matter present on Mars should be fully known. Only after as thorough and scrupulously careful program of unmanned missions should a sample be returned to an isolated laboratory, not earlier than 2000 A. D., and maintained in quarantine for an extensive period--leprosy, he notes, has an incubation period of about one decade. However, he feels that it is too early to construct a logic tree of when and how to return a sample.

Wolf Vishniac, University of Rochester, favors returning a sample from Mars to Earth provided the results of Viking '76 are negative. The risk of returning a sample is a function both of the probability of life on Mars and the probability of infection. If Viking does not detect life, the probability of infection is small, either

because Mars does not have Earth-like organisms, a requirement for pathogenicity, or because Martian life differs from terrestrial life and consequently would not have an infective mechanism. Although the probability of life on Mars is greater than that of life on the Moon, he feels that we are fully committed to the space program and it is justified on scientific grounds to return a sample for close examination under controlled laboratory conditions. In order to avoid some of the problems encountered with the Lunar sample, quarantine measures should be in the domain of a supervisory committee consisting entirely of scientists. He feels that if traffic between the two planets develops, back contamination cannot be prevented and the purpose of the quarantine should be to prevent massive spread. However, he is convinced that, even if the return sample were pathogenic, terrestrial life is hardy and catastrophic consequences are unlikely. To ensure maximum safety, the sample should be returned by an unmanned mission because more confidence exists for maintaining quarantine. Cost, however, may be a more important factor than risk in deciding upon a particular course of action. Since an unmanned return sample mission may require the same size spacecraft as a manned mission, it may be difficult to justify an unmanned mission. Use of an orbiting quarantine laboratory is difficult to justify financially. Further, unless maintained by one-way personnel, a disease could still be transmitted if someone became

sick and recovered. It is also easier to protect scientists on Earth because many protective devices depend on gravity. Although, as a scientist, he would like to see a sample returned from Mars, he commented that, if absolute safety is the primary consideration, then, intellectually, a sample should not be brought back.

Paul Lowman, Goddard Space Flight Center, outlined the following advantages of returning a Mars sample for geological analysis:

1. No time lag in feedback for performing an operation as would exist for remote manipulation.
2. Better integration of analyses since many investigators can simultaneously work on the same sample.
3. Preservation of a sample portion for future work with improved techniques.
4. Not necessary to design and build new instruments. No restraints on weight, size, power, or durability of analytical instruments.
5. Higher resolution of the optical microscope or scanning electron microscope relative to space instruments.
6. No data rate problem. For the large amount of information required, the data transmission band width would have to be extreme to accommodate the information load.

As an example, the return Lunar sample was cited where 10 mg was used to perform a more accurate, complete, and detailed study than could have been accomplished in situ.

The information desired by geologists are the major, minor, trace, and rare earth elements, as well as the various isotopes, present in the surface. Exposure to cosmic rays can also reveal the turn-over rate of surface materials whereas the scanning electron microscope can obtain the crater impact history of the micro-level. Relative dating is obtained from photo interpretation of superposition and cross cutting relationships whereas radiometric data provides a measure of absolute age. Igneous rock petrology and metamorphism studies are also desired. Much of this information can theoretically be obtained on Mars in situ, although, perhaps, not with the degree of sensitivity as on Earth. Major elemental analyses can be obtained in situ by X-ray fluroscence, alpha scattering, or neutron capture experiments. Minor elemental analyses can be performed by the ion microprobe mass analyzer (IMMA). Major minerals are obtained by x-ray diffraction and television microscopy, the latter of which also reveals particle size distribution. The major limitation of in situ measurements is radiometric dating which can theoretically be obtained by the IMMA. However, considering cost, size, and

reliability of instruments available on Earth, Dr. Lowman is skeptical about the reliability and accuracy of the IMMA probe on Mars.

Although, as a geologist, he favors a return Mars sample, Dr. Lowman points out that for the same money, many orbital surveys and much in situ work could be done on Mars. This could include not only geology, but several studies which cannot be accomplished by a return sample, such as seismology, planetology, atmospheric analyses, magnetic fields, heat flow and radiation environment. As a scientist, however, he is concerned about back contamination and consequently is against a return sample until it is well-established that it is non-pathogenic.

Clearly, then, following this brief survey of ideas expressed by prominent space scientists, the question of a return Mars sample is quite complex. While all recognize the potential hazard, undoubtedly man's curiosity to understand his place in the cosmos will place high pressure on returning a sample for detailed study by competent scientists in advanced laboratory conditions. While differences of opinion exist on whether or not a sample should be returned, many differences undoubtedly arise from a lack of defined circumstances under which the sample should be returned and how it should be handled. Given a set of conditions and vital information from future Viking missions,

the questions of risk, value, and cost can be more clearly, although perhaps not adequately, evaluated. Questions to be analyzed to establish common ground for discussions among scientists are:

1. How much can we learn from automated landers versus a return Mars sample?
2. How much knowledge is necessary before returning a sample?
   a. If life is detected by automated landers.
   b. If life is not detected by automated landers.
3. In what order should we proceed?
   a. Man before return sample.
   b. Return sample before man.
   c. Man simultaneous with return sample.
4. Where should a quarantine laboratory be established?
5. How should the sample be transferred to and contained within the quarantine facility?
6. What are the hazards within the quarantine laboratory?
   a. Those originating from psychological errors.
   b. Those originating from design and construction errors.
   c. Those originating from scientific errors and lack of techniques.

7. What new techniques need development?

8. How do we terminate the study?

   a. At what point do we relax the quarantine?

   b. How do we destroy the sample if necessary?

D. The Basis for a Return Mars Sample Program

There seems but little doubt that manned flight to Mars will occur provided earlier recognizance does not reveal the planet to be inimmicably hostile to him. The potential hazard on Mars greatly exceeds that which existed concerning the Moon in the pre-Apollo era. Major and detailed planning will be required to design an appropriate program to satisfy the scientific community that sufficient safeguards have been taken and to inform the public adequately so it accepts the assurances of the scientists. The program will entail numerous and various life detection and analysis experiments on a variety of Martian sites. But this phase of the program can only assure us of danger; it cannot guarantee safety. The next required phase will be the examination of a variety of samples of Mars material by competent biological scientists. One of the key determinations of the program will be the decision as to where this examination will take place. It is initially to take place on Earth, a suitable and detailed protocol will have to be worked out for containing the sample, transporting it, transferring it, examining it and disposing of it with complete safety. Variations of

this plan may permit early or total examination of the sample in isolated laboratories on Mars, on one of the Martian Moons, in Mars orbit, on the Earth's moon, in Earth orbit, or during transit from Mars to Earth. All of these possibilities, however, will require the same absolute isolation of the sample from the men as would be required in an Earth-based Mars return sample laboratory. Only two alternatives to the above plan exist: (1) permit less stringent barriers between the examining scientists and the samples with the acknowledged understanding by all parties concerned that developments may make it necessary, however unlikely the probability might be, that the men would have to be sacrificed, (2) avoid any Earthward contact between Mars and Earth. The latter demands a very high price from man but might be preferable to accepting a high risk to the biological organisms or ecological balance on the Earth.

E. Future Program

The future effort on this return Mars sample task will be directed at elaborating Section D - The Basis for a Return Mars Sample Program. Emphasis will be on scientific considerations augmented with some engineering concepts should these prove waranted.

Approved by:

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APPENDIX I

METHODS

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I. MEDIA & ANTIMETABOLITES

Different media which contained the substrates and/or antimetabolites given are listed by number. Total radioactivity is given for media containing more than one labeled substrate.

A. Media Composition

1.	UL ^{14}C D-glucose	5 μCi/ml	1×10^{-3}M
2.	UL ^{14}C L-glutamate	5 μCi/ml	1×10^{-3}M
3.	UL ^{14}C pyruvate	3 μCi/ml	5×10^{-5}M
4.	UL ^{14}C D-glucose	5 μCi/ml	1×10^{-3}M
	UL ^{14}C L-glutamate	5 μCi/ml	1×10^{-3}M
	Total	10 μCi/ml	
5.	1 ^{14}C DL-glutamate	5 μCi/ml	1×10^{-3}M
6.	2 ^{14}C DL-glutamate	5 μCi/ml	1×10^{-3}M
7.	3,4 ^{14}C DL-glutamate	5 μCi/ml	1×10^{-3}M
8.	5 ^{14}C DL-glutamate	5 μCi/ml	1×10^{-3}M
9.	VM-I		
	UL ^{14}C L-alanine	.6 μCi/ml	2.5×10^{-4}M
	UL ^{14}C D-alanine	.6 μCi/ml	2.5×10^{-4}M
	UL ^{14}C glycine	.4 μCi/ml	2.5×10^{-4}M
	^{14}C formate	.2 μCi/ml	2.5×10^{-4}M
	UL ^{14}C DL-lactate	1.2 μCi/ml	2.5×10^{-4}M
	UL ^{14}C glycolic acid	.4 μCi/ml	2.5×10^{-4}M
	Total	3.4 μCi/ml	
10.	FLGG		
	^{14}C formate	1 μCi/ml	1×10^{-3}M
	UL ^{14}C DL-lactate	1 μCi/ml	1×10^{-3}M
	UL ^{14}C glycine	1 μCi/ml	1×10^{-3}M
	UL ^{14}C L-glutamate	1 μCi/ml	1×10^{-3}M
	Total	4 μCi/ml	

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11.	1 ^{14}C DL-glutamate	1 μCi/ml	2×10^{-4}M
12.	2 ^{14}C DL-glutamate	1 μCi/ml	2×10^{-4}M
13.	3,4 ^{14}C DL-glutamate	1 μCi/ml	2×10^{-4}M
14.	5 ^{14}C DL-glutamate	1 μCi/ml	2×10^{-4}M
15.	L-amino acids mixture		
	3 ^{14}C L-serine	1 μCi/ml	3.33×10^{-4}M
	1 ^{14}C L-leucine	1 μCi/ml	3.33×10^{-4}M
	UL ^{14}C L-alanine	1 μCi/ml	3.33×10^{-4}M
	Total	3 μCi/ml	
16.	D-amino acids mixture		
	3 ^{14}C D-serine	1 μCi/ml	3.33×10^{-4}M
	1 ^{14}C D-leucine	1 μCi/ml	3.33×10^{-4}M
	UL ^{14}C D-alanine	1 μCi/ml	3.33×10^{-4}M
	Total	3 μCi/ml	
17.	1 ^{14}C D-glucose	1 μCi/ml	2×10^{-4}M
18.	3,4 ^{14}C D-glucose	1 μCi/ml	2×10^{-4}M
19.	6 ^{14}C D-glucose	1 μCi/ml	2×10^{-4}M
20.	UL ^{14}C D-ribose	1 μCi/ml	2×10^{-4}M
21.	Cellulose	5 μCi/mg	
22.	UL ^{14}C acetate	1 μCi/ml	2×10^{-4}M
23.	^{14}C formate	1 μCi/ml	2×10^{-4}M
24.	UL ^{14}C DL-lactate	1 μCi/ml	2×10^{-4}M

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B. Antimetabolite Composition

	Concentration at Preparation	Concentration of Antimetabolite in Medium
Trans-aconitic Acid	1×10^{-2}M	5×10^{-3}M
Monofluroacetic Acid	1×10^{-2}M	5×10^{-3}M
	1×10^{-3}M	5×10^{-4}M
	1×10^{-4}M	5×10^{-5}M
2, 4 Dinitrophenol	Saturated	
	5×10^{-3}M	2.5×10^{-3}M
	1×10^{-3}M	5×10^{-4}M
	5×10^{-4}M	2.5×10^{-4}M
Malonic Acid	1×10^{-2}M	5×10^{-3}M
KCN	1×10^{-1}M	5×10^{-2}M
Iodoacetic Acid	1×10^{-1}M	5×10^{-2}M
	1×10^{-3}M	5×10^{-4}M
	1×10^{-5}M	5×10^{-6}M
Antibiotic:	Penicillin 10,000 u/ml	500 u Penicillin
Antimycotic	Fungizone 25 ug/ml	(1.25 ug Fungizone)
	Streptomycin 10,000 ug/ml	(500 u Streptomycin)

C. Preparation of Media

1. Labeled substrate or substrates were added to a sterile vial to provide sufficient radioactivity for the final volume. The prescribed concentration was achieved by adding unlabeled substrate. This combination was brought to the correct volume with distilled H_2O which had been previously autoclaved for 20 minutes at 15 psi,

121°C, and cooled. The medium was then sterilized by two passages through 0.22 μ pore size Millipore membrane filters.

2. Freshly prepared medium generally exhibited a higher than background count when 0.1 ml of the sterile medium was gettered for one hour. This nonbiological evolution was reduced by shaking the sterile medium on a Magni-Whirl reciprocating shaker. Medium was shaken until 0.1 ml evolved less than 100 cpm of radioactivity during a one-hour gettering period.

3. Radioactivity was measured at the time of medium preparation and at the time of each subsequent experiment using the following procedure:

   a. 0.01 and 0.02 mls of radioactive medium were pipetted in duplicate into scintillation vials, each containing 10 ml dioxane cocktail.

   b. Vials were counted for one minute each in an LS-230 Beckman scintillation counting system.

4. Media were routinely stored at 3°C. As a precaution, media which were stored for several weeks were refilter-sterilized before use.

D. Preparation of Antimetabolites

1. Antimetabolites were prepared at a concentration practical for accurate weighings and diluted with

distilled H_2O to the desired experimental concentration.

2. Iodoacetic acid was recrystallized prior to preparation and maintained in the dark following hydration to prohibit deterioration.

3. Antibiotic solution was stored at -10°C. All other antimetabolites were stored at room temperature.

4. Media was premixed with antimetabolites prior to each experiment in a 1 : 1 proportion and dispensed to soil or pure culture samples.

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II. SOILS

A. Determination of Sample Size

Earlier experiments had shown that a soil/liquid ratio of 0.5 gram soil to 0.1 ml medium was optimum for Wyaconda and Mojave soils. Due to the difference in soil density, however, other soils exhibited a varied degree of wetting at this soil/liquid ratio. It was, therefore, decided that sample size should be based on volume rather than weight and should be correlated with the volume of Wyaconda soil which had produced good results in earlier studies. This decision was substantiated in that the future sampling on Mars will also be based on volume.

Weight/volume measurements were made on each soil by weighing 1.0 gram of soil into a calibrated vial and then filling the vial to a predetermined volume mark with a known volume of water.

$$\begin{array}{r} \text{Volume of Vial Empty} \\ -\ \underline{\text{Volume of Water Added}} \\ \text{Volume of 1 Gram Soil} \end{array}$$

A volume of 0.21 cc with correlated with 0.5 gram Wyaconda soil was selected for experimental sample size as supported by earlier test data. Several precision scoops were made to measure each soil at a volume of 0.21 cc. The repeatability of this method is shown in Table 1.

B. Handling & Characterization of Soils

All test soils collected by Biospherics were air dried in a laminar flow bench, sieved aseptically with a #18 mesh sieve (1 mm

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Table 1

Soil Measurement with Volumetric 0.21 cc Scoops

Repetition	Weight of Soil
1	0.251
2	0.232
3	0.235
4	0.240
5	0.241
6	0.233
7	0.220
8	0.241
9	0.252
10	0.241

Avg. $\pm$ s.d. $.238 \pm .009$

opening), and stored in sterile polypropylene bottles at air-conditioned room temperature (approximately 23°C). Aiken soil is a Project Viking test soil characterized by Ames Research Center and was used as supplied.

Table 2 lists the test soils and summarizes the results of descriptive determinations which characterize each soil. Methods for these determinations follow:

C. Methods of Soil Analysis

1. pH

One gram of soil was mixed with one ml distilled water and stirred on a magnetic stirrer for 15 min, Sample containers were then covered with parafilm and pH determinations made at the times noted in Table 2. Measurement of the slurry was made with a Corning Model 10 pH meter using a combination electrode. As indicated, the pH of heat sterilized soil was also measured.

2. Wt./Vol.

The weight of 0.21 cc of soil as measured with the scoops was determined.

3. Plate Counts

Soil was placed in sterile H_2O (1 g/10 ml) and sonicated for one minute with a Heat Systems - Ultrasonic, Inc. Sonifier Model W185D using a cup horn. Soil suspensions were then diluted, plated on nutrient agar, Tripticase Soy Agar and Czapek Dox Agar and incubated

Table 2

Test Soil Characteristics

Soil	pH 0 Time 6 05	Wt./Vol.	Plate Counts (CFU)	Texture	% Moisture	% Volatile Solids
Wyaconda Rockville, Md. Collected 9/1/72 Soil bared by construction several years earlier. Sparce Weeds	Natural 90 min. 6.95 3 hr. 6.93 20 hr. 6.96 Heat Treated 0 time 6.03 90 min. 6.45 20 hr 6.57	0.50 g/.22 cc	23°C N.A. 8.25×10^6 TSA 4.7×10^6/gm Czapek Dox 1.86×10^6/gm 65°C TSA < 10^3	Sandy Loam 52% Sand 37% Silt 11% Clay	1.1	3.7
Meadow Gaithersburg, Md. Collected 9/1/72 Planted several years before - now in meadow	Natural 0 time 6.04 90 min. 6.75 20 hr. 6.99 Heat Treated 0 time 6 03 90 min. 6.45 20 hr 6 57	0.17 gm/.22 cc	23°C N A. 4.1×10^7/gm TSA 2.95×10^7/gm Czapek Dox 3.8×10^7/gm 65°C TSA 3.3×10^4	Loam 38% Sand 47% Silt 15% Clay	9.5	7.4
Yuma 4 mi west of Yuma, Ariz. Collected 3/9/72 Dry wash sediment, mesquite brush, sparce grass	Natural 90 min. 6.90 7.82 Heat Treated 20 hr. 7.62	0.34 g/.22 cc	23°C N A. 4.48×10^6/gm TSA 2.58×10^6/gm Czapek Dox 1.7×10^6/gm 65°C TSA 4×10^3	Loam 42% Sand 47% Silt 21% Clay	5.1	6.5
Phoenix North of Phoenix, Ariz. on Mesa Collected 3/11/72 Sparce grass, prickly pear & yucca	Natural 90 min. 6.70 20 hr. 7.25 Heat Treated 20 hr. 6 66	0.32 g/.22 cc	23°C 2.15×10^7/gm N.A. TSA 1.25×10^7/gm Czapek Dox 1.05×10^7/gm 65°C TSA < 10^{-3}	Clay 18% Sand 36% Silt 46% Clay	4.3	5.4
Arlington - Project Vixing Soil	Natural 0 time 5.55 90 min. 5.35	0.19 gm/.22 cc	25°C TSA spread plates 7.6×10^5/gm Czapek Dox 5.6×10^5/gm		2.2	12.6

* Methods of analysis are given in Section II. C. - Appendix I.

at room temperature for three days. Plates prepared in the same manner with TSA were also incubated at 63°C for 16 hours, to estimate the number of thermophyls.

4. Percent Moisture

Sample dried at 104°C (2).

5. Percent Volatile Solids

Sample fired at 550°C (2).

6. Texture

Determination of texture were conducted by the Agronomy Department, University of Maryland. Procedure for mechanical analysis by hydrometer method was as follows:

a. Weigh out 100 g of air-dry sandy soil (light textured) of 50 g of clay or silt loam soil (medium to heavy texture). Transfer to a 250 ml beaker. Cover with water. Add 5 ml of 10% Calgon and allow to stand overnight.

b. Transfer to a metal dispersion cup and fill about 2/3 full with H_2O.

c. Place dispersion cup on mixer and stir for five minutes.

d. Transfer contents from the dispersion cup to a Boyoucos Cylinder.

e. Place the hydrometer in the suspension very gently and bring to volume with distilled water. If 50 g of soil is used, bring the suspension to the lower mark (1130 ml). If 100 g is used, bring the suspension to the upper mark (1205 ml).

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f. Carefully remove the hydrometer and shake the cylinder throughly by placing a large stopper over mouth of cylinder and inverting several times to obtain a uniform suspension.

g. Place cylinder on a table and note the time. Carefully but quickly place the hydrometer in the suspension. At the end of 40 seconds take the hydrometer reading.

h. Remove the hydrometer and take the temperature of the suspension being careful not to disturb the suspension.

i. Take the second hydrometer and temperature readings at the end of two hours.

Calculations

At the end of 40 seconds, the sand fraction has settled (0.05 mm and larger), but the silt plus clay is still in suspension. One hour reading would indicate the $-_5$ micron material in suspension. A two-hour reading would indicate the $-_2$ micron material (clay). For every degree F above 67°, 0.2 of a hydrometer graduation must be added to the hydrometer reading. For each degree below 67°F, subtract 0.2 of a graduation.

(1.) *40 Sec. Reading*

$$\frac{\text{40 second hydrometer reading}}{\text{weight of sample}} \times 100 = \%\ \text{Sand}$$

(2.) Two-Hour Reading

$$\frac{\text{2 hour hydrometer reading}}{\text{weight of sample}} \times 100 = \%\ \text{Clay}$$

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(3.) 100 - (% Sand & % Clay) = % of Silt

(4.) Temperature Correction

C - 0.1 (T-67)
(C is the hydrometer correction. Round off to nearest whole number. T is the temperature of suspension in ^{o}F).

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III. SOIL ORGANISM ISOLATES

A. Method of Isolation and Description

Serial dilutions of suspensions of each of the test soils were plated on Trypticase Soy Agar (TSA). Colony forming units per gram were determined to be as follows:

Meadow	2.95×10^7 CFU/gm
Phoenix	1.25×10^7 CFU/gm
Aiken	7.60×10^5 CFU/gm

Colonies of the most predominating types were also picked and streaked onto TSA plates. Table 3 summarizes significant characteristics determined by staining and microscopic examination.

B. Maintenance

Cell isolates were maintained on TSA without glucose at room temperature and transferred biweekly. Attempts to keep cultures for a two-week period at 3°C resulted in the loss of Phoenix Soil Isolate No. 2.

C. Preparation of Cell Suspension for Assay

Eighteen-hour cultures which had been streaked on TSA plates and incubated at 35°C were used for each experiment. Growth on the plates was removed using a sterile cotton swab and suspended in 0.85% saline to an optical density of approximately one at 420 nm (Bausch and Lomb Spectronic 20). Dilution spread plates were prepared routinely

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Table 3

Characteristics of Soil Isolates

Soil & Cell Isolate	Colony Morphology	Gram Stain	Cell Morphology
Meadow Soil Isolate #1	Large Spreading Grayish White.	Gram Positive	Spore forming rod. ≅ 3 μ in Length. Appeared Singly & in chains
Meadow Soil Isolate #2	Small Granular Yellowish Gray Colonies.	Gram Negative	Rods in short chains. (Pleomorphic) 4 5 μ - 7.54 μ in Length Granular
Meadow Soil Isolate #3	Raised, Mucoid Grayish White Colonies. Opaque. Evenly Circular.	Gram Negative to Gram Variable	Long, Thin Rods, 7 μ and Longer. Extensive Chain Formation.
Phoenix Soil Isolate #1	Raised, Mucoid Cream Colonies. Evenly Circular.	Gram Negative	Short Rods, ≅ 2 μ in Length Form Short Chains
Phoenix Soil Isolate #2	Irregular Dry Colonies.	Gram Negative	Short Rods, ≅ 1 μ in Length Form Short Chains
Aiken Soil Isolate #1	Raised, Granular, Mucoid, Grayish White Colonies.	Gram Positive	Long Rods, ≅ 6 μ in Length. Terminal Spores, Granular Protoplasm, Form Short Chains.

accompanying each assay. Most cell suspensions yielded approximately 10^8 CFU/ml.

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IV. SELECTION OF EXPERIMENTAL CONFIGURATION

A. Screw-Cap Vial Test System

In the study of temperature ranges and optima for biological activity in soils, it was necessary to establish a labeled release system which was applicable to both high and low temperature studies.

Earlier work had shown that drying, which occurred rapidly at higher temperatures, strongly influenced results. The difficulties had been partially overcome by humidification of the incubator. Since the effects of small temperature increments were to be studied, it was apparent that differences in humidity and water loss must be controlled. The test system devised was comprised of a 25 ml glass scintillation vial with a 20 mm Schleicher and Schuell #470 Nutropad inserted in the screw cap.

Test runs in comparison with the planchet system using ^{14}C glutamate and ^{14}C glucose are shown in Figures 1 and 2. These experiments demonstrated that considerable evolved radioactivity may be lost from the planchet system, especially during long collection periods. The screw capped vial showed a 2-3 fold increase in gettered radioactivity over the planchets. The vials also showed a slightly lower control level than was obtained with planchets.

In addition to preventing the escape of radioactivity, there appeared to be other advantages of screw-capped vials. Vials were

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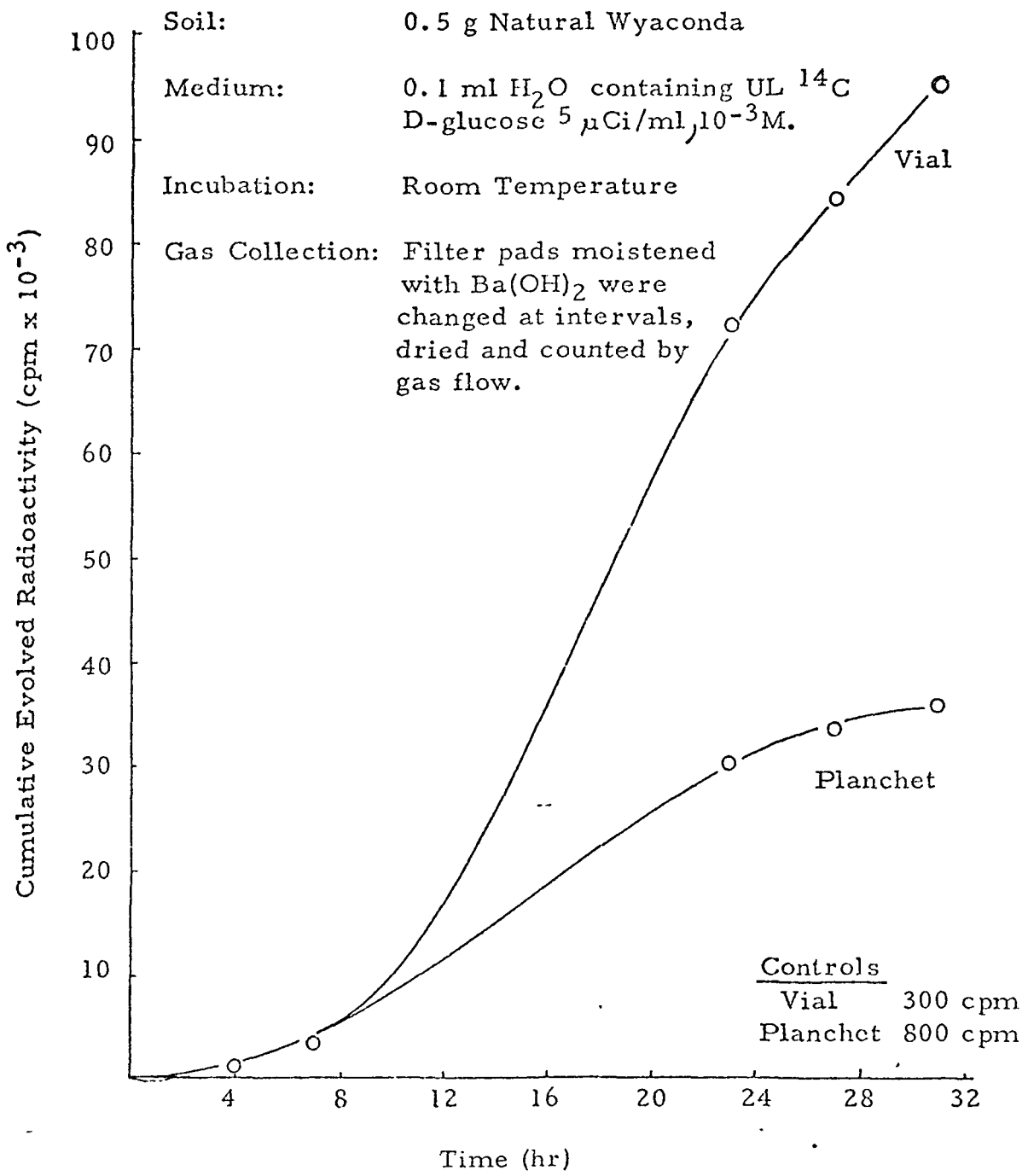

Figure 1

Comparison of the Closed Vial vs. Planchet for Conducting Labeled Release Using ^{14}C Glucose

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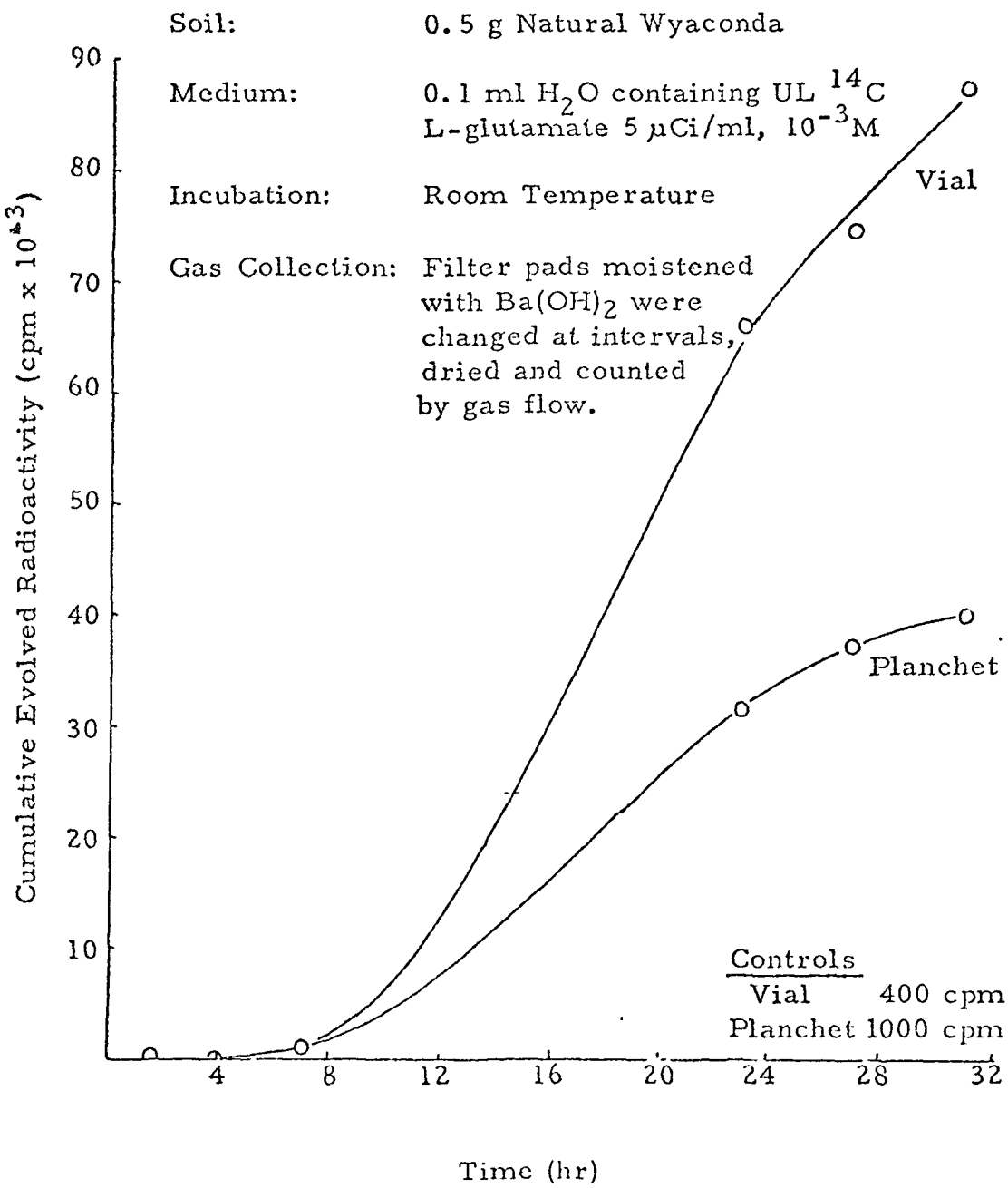

Figure 2

Comparison of the Closed Vial vs. Planchets for Conducting Labeled Release Using ^{14}C Glutamate

nearly as simple as planchets for conducting the assay. Getter pads were changed with sterile forceps and the inexpensive caps were discarded at the end of an experiment. The tight fitting screw cap prevented the escape of moisture from the system; therefore, humidity was maintained at 100% regardless of the incubation temperature. The vials could be immersed in a water bath for careful temperature control. The 20 ml volume presumably provided adequate O_2 for aerobic organisms.

The quantitative nature of the experiments involving radiorespiration of specifically labeled compounds to demonstrate the presence of predominating metabolic pathways indicated the desirability of the vial labeled release method. High efficiency of collection as well as prevention of evaporation was necessary in an attempt to quantitate small metabolic differences between predominating metabolic pathways of natural populations of soil microorganisms.

B. Methods for CO_2 Collection

Two methods of $^{14}CO_2$ collection were used. The first employed the use of a filter pad moistened with saturated $Ba(OH)_2$ or 20% KOH which was placed in the lid of the vial. The second method (Figure 3) involved passing a sterile humidified gas through the growth chamber and trapping CO_2 from the exit gas in one milliliter of hyamine hydroxide in a second scintillation vial. The latter method provided a

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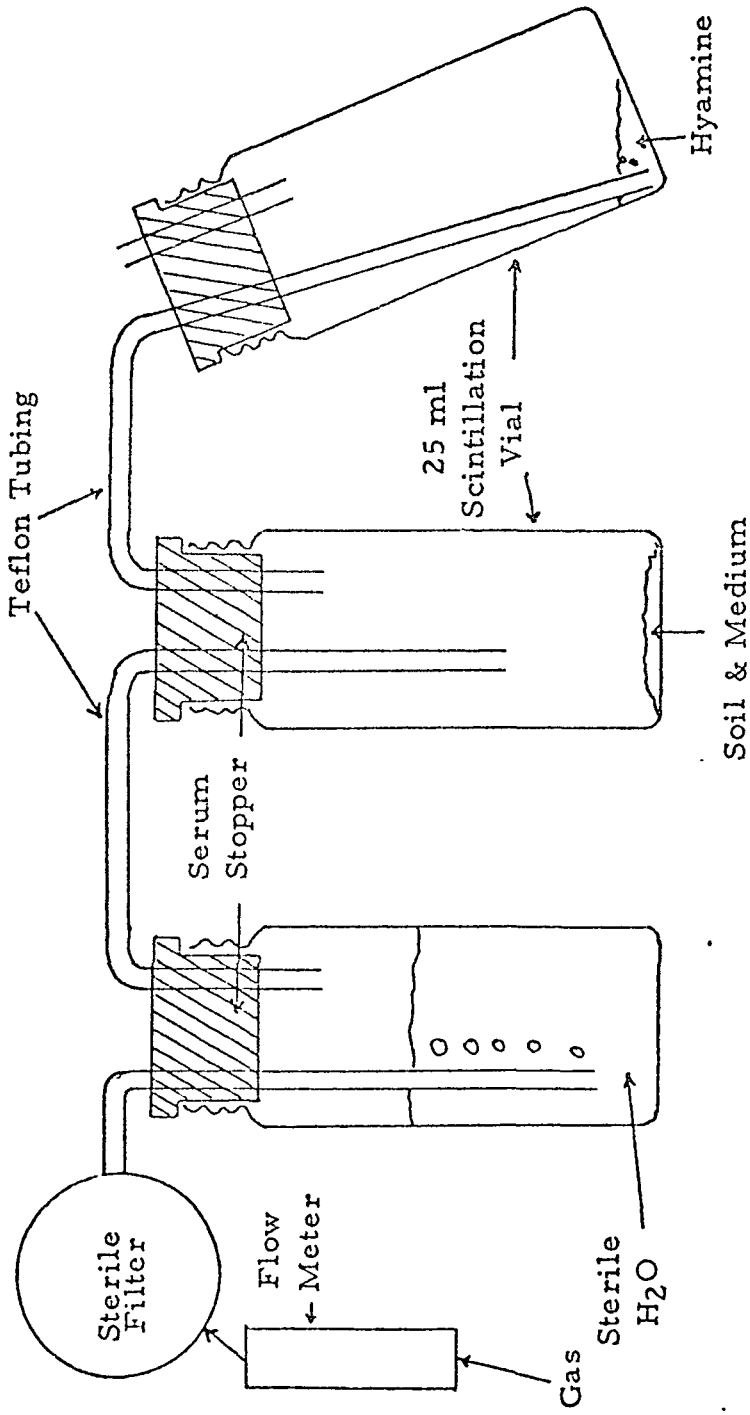

Figure 3

Apparatus Used for Flush Experiments

means for conducting anaerobic experiments. Anaerobiosis was established by passing 90% N_2 : 10% H_2 through the growth chamber (40 cc/min.) for approximately one hour prior to the introduction of labeled medium. This medium was introduced by injection with a syringe and needle through the serum stopper. No attempt was made to degas the medium prior to injection.

Radioactivity trapped with hyamine was determined in a Beckman LS-230 liquid scintillation counter (LSC). The counting cocktail was composed of the following components added in this order:

500 ml Toluene
2 g PPO
0.025 gm POPOP (add while stirring)
500 ml methanol

Counting efficiency of the cocktail, as determined with benzoic acid standard, was found to be 77% of calculated dpm. Addition of 1 ml Hyamind reduced the efficiency to 62%. Flushing the cocktail with N_2 for one minute prior to counting increased efficiency by approximately 5%. The efficiency of $^{14}CO_2$ collection of 1 ml Hyamine at a flush rate of 40 cc/min. was found to be 95%; therefore, the overall efficiency of collection and liquid scintillation counting was approximately 59%. It was also noted that a 1 : 3 mixture of Hyamine in methanol exhibited no loss in gettering efficiency and greatly lengthened the gettering interval. (Evaporation limited gettering with a 1 ml vol. to approximately 30 mins.)

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The feasibility of using KOH to trap $^{14}CO_2$ was also tested. Collection efficiency of 0.2 ml, 20% KOH in a 25 ml vial receiving flush gas at the rate of 40 cc/min. was much poorer than with Hyamine. Bubbling of flush gas through KOH was not tested. It was found that up to 0.3 ml of aqueous KOH, with or without added carbonate, did not affect the accounting efficiency of the cocktail.

Efficiency of the first method of gas collection using a filter pad with $Ba(OH)_2$ and counting by gas flow was also compared to filter pad collection with 20% KOH and counting by liquid scintillation (pad was placed in cocktail). The method was tested using a system as shown in Figure 4, wherein a known amount of NaH $^{14}CO_3$ was acidified and the evolved radioactivity collected. Results and calculated relative efficiencies (added radioactivity/recovered radioactivity) of the two methods are shown in Appendix I, Tables 4 and 5. Since the counting efficiency of the toluene methanol cocktail is approximately 77%, the following total efficiencies are obtained for the three collection and counting methods used:

1. $Ba(OH)_2$ pad collection and quantitation by gas flow - 10.4% efficiency.

2. KOH pad collection and quantitation by liquid scintillation - 57% efficiency.

3. Hyamine flush collection and quantitation by liquid scintillation - 59% efficiency.

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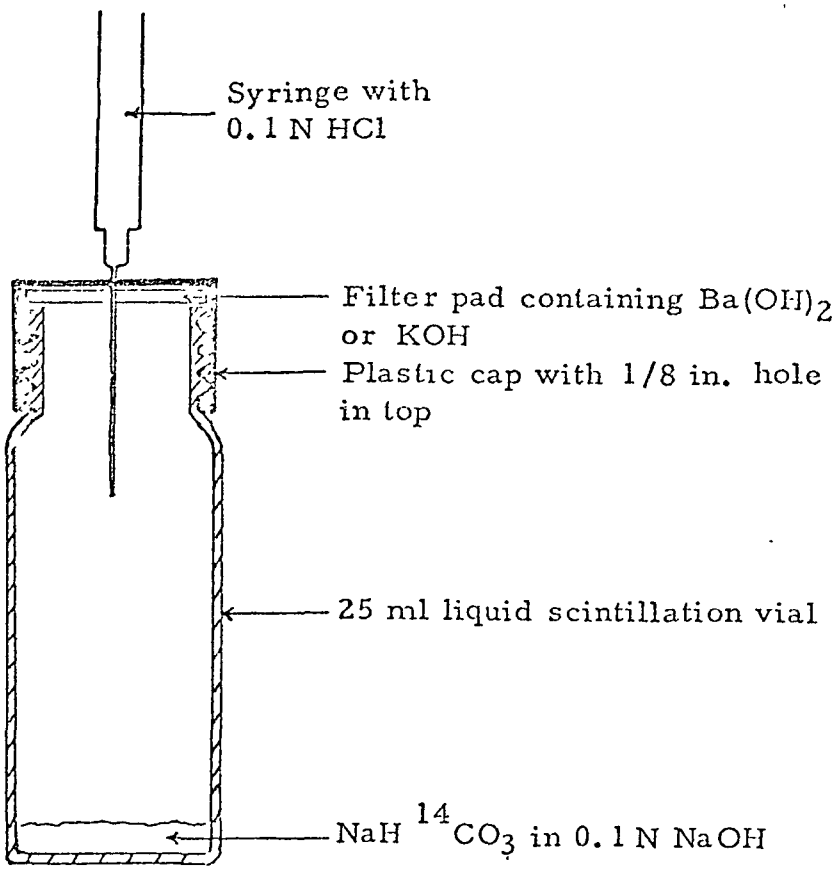

Figure 4

Apparatus Used for Determining the $^{14}CO_2$ Collection Efficiency of $Ba(OH)_2$ and KOH

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Table 4

Efficiency of KOH Pad Collection
and Liquid Scintillation
Counting of $^{14}CO_2$ with Toluene Methanol

Added Radioactivity Measured by Liquid Scintillation	9,740	9,740	19,450	19,450	97,350	97,350
Radioactivity Collected with KOH & Counted by Liquid Scintillation	7,480	7,420	14,958	14,600	67,000	71,200
Efficiency of KOH Collection & Liquid Scintillation Counting	77	76	77	75	69	73

$$\text{Avg. relative efficiency} \left(\frac{\text{collected radioactivity}}{\text{added radioactivity measured}} \right) = 74\%$$

Since:

$$\frac{\text{cpm collected} \times 100}{\text{cpm added}} = 74\% \text{ efficiency}$$

and:

$$\text{dpm added} = \text{cpm added} \times \frac{1}{0.77} \text{ (counting efficiency)}$$

then:

$$\frac{\text{cpm collected} \times 100}{\text{dpm added}} = 74\% \times 0.77 = 57\% \text{ overall efficiency}$$

Table 5

Efficiency of $Ba(OH)_2$ Pad Collection and Gas Flow Counting of $^{14}CO_2$

Added Radioactivity Measured by Liquid Scintillation	6,260	12,500	25,000	75,100	125,200	399,400	798,840	1,597,685
Radioactivity Collected with $Ba(OH)_2$ and Counted by Gas Flow (avg. of five replicates)	847	1,628	3,730	11,630	16,800	29,200	102,800	198,800
Efficiency of $Ba(OH)_2$ Collection & Gas Flow Counting	13.5 s.d. ± 1.5	13.0 s.d. ± 0.5	14.9 s.d. ± 1.3	15.5 s.d. ± 2.6	13.5 s.d. ± 2.9	12.2 s.d. ± 1.4	12.8 s.d. ± 2.8	12.4 s.d. ± 2.3

$$\text{Avg. relative efficiency} \left(\frac{\text{collected radioactivity}}{\text{added radioactivity measured}} \right) = 13.5\%$$

Since:

$$\frac{\text{cpm collected} \times 100}{\text{cpm added}} = 13.5\%$$

and:

$$\text{dpm added} = \text{cpm} \times \frac{1}{0.77} \text{ (counting efficiency)}$$

then:

$$\frac{\text{cpm collected} \times 100}{\text{dpm added}} = 13.5\% \times 0.77 = 10.4\% \text{ overall efficiency}$$

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The calculated difference in the efficiencies of Method No. 1 and Method No. 2 is 5.6 fold. Actual measurements of radioactivity collected and counted by the individual methods, when compared by the method of least squares showed a ratio of 4.1. Data in Appendix II, for atmosphere, was obtained by the Hyamine and LSC method and has been reduced by a factor of 1/4.1 to make it comparable to other data.

It should be noted in Appendix Table 5 that increased quantities of $^{14}CO_2$ did not appreciably reduce the efficiency of collection and counting.

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V. PROCEDURE FOR CONDUCTING THE LABELED RELEASE EXPERIMENT

Step 1. Glass scintillation vials were sonication cleaned in a 10% Radiac detergent solution, rinsed thoroughly with tap and distilled H_2O and dried. Vials with disposable caps were autoclaved at 15 psi, 121°C, for 20 minutes.

Step 2. Sifted soil samples were placed into the sterile vials using the sterile scoop. 0.1 ml liquid cell suspension was placed in each vial for pure culture assay.

Step 3. Any necessary pretreatment was made. Temperature assays were preincubated at test temperature for one hour. Hyamine collection samples were preflushed with 90% H_2 - 10% H_2 for anaerobic samples and air for aerobic samples. Any pre-enhancement with cold substrate occurred as early as 16 hours prior to initiation of the experiment.

Step 4. Schleicher & Schuell No. 470 pads were moistened with saturated $Ba(OH)_2$ solution and placed aseptically in the lids of the vials employing the $Ba(OH)_2$ collection system. One ml of a 1 : 5 hyamine hydroxide in methanol solution was placed in the last vial in the flushing-labeled release apparatus.

Step 5. The labeled medium was introduced to the $Ba(OH)_2$ test system using a sterile pipette and to the hyamine test system using a sterile syringe and needle to inject through the serum stopper. The

latter procedure was to minimize oxygen reaching anaerobic samples. The antibiotics, antimetabolites, and inhibitors employed in the $Ba(OH)_2$ test system were premixed with the labeled medium prior to introduction to the soil or cell suspension.

Step 6. After a timed interval, the gettering pads in the $Ba(OH)_2$ test system were replaced in the lids with freshly moistened $Ba(OH)_2$ pads. In the hyamine flushing system, a vial with fresh hyamine : methanol replaced the previous vial which was removed at the same intervals. This regimen was repeated throughout the experiment.

Step 7. The getter pads were dired under an infrared lamp for about 20 minutes. Radioactivity retained on each pad was determined in a Nuclear Chicago D-47 Gas Flow Counting Apparatus flushed with Q-gas. Correspondingly, 10 ml of toluene methanol cocktail (Section IV.-B.) was added to each vial containing hyamine-methanol which had been used as a $^{14}CO_2$ collection agent in the flush test system. These vials were then counted in the Beckman LS-230 scintillation counting system for a measure of $^{14}CO_2$ entrapment.

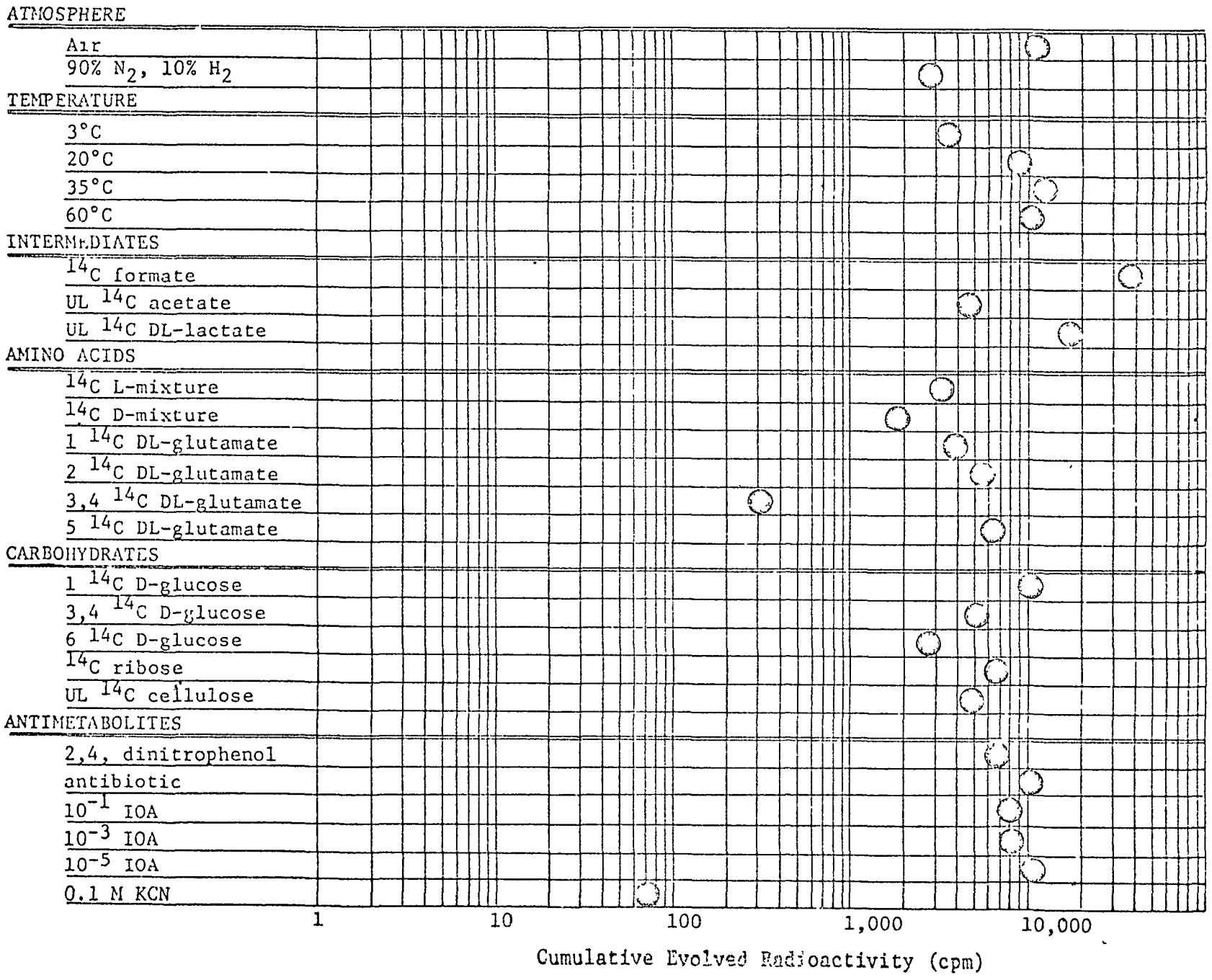
APPENDIX II

SOIL AND SOIL ISOLATE LIBRARY OF PROFILES

Soil or Organism Meadow Soil

Incubation Time 2 Hours

Standard Medium FLGG



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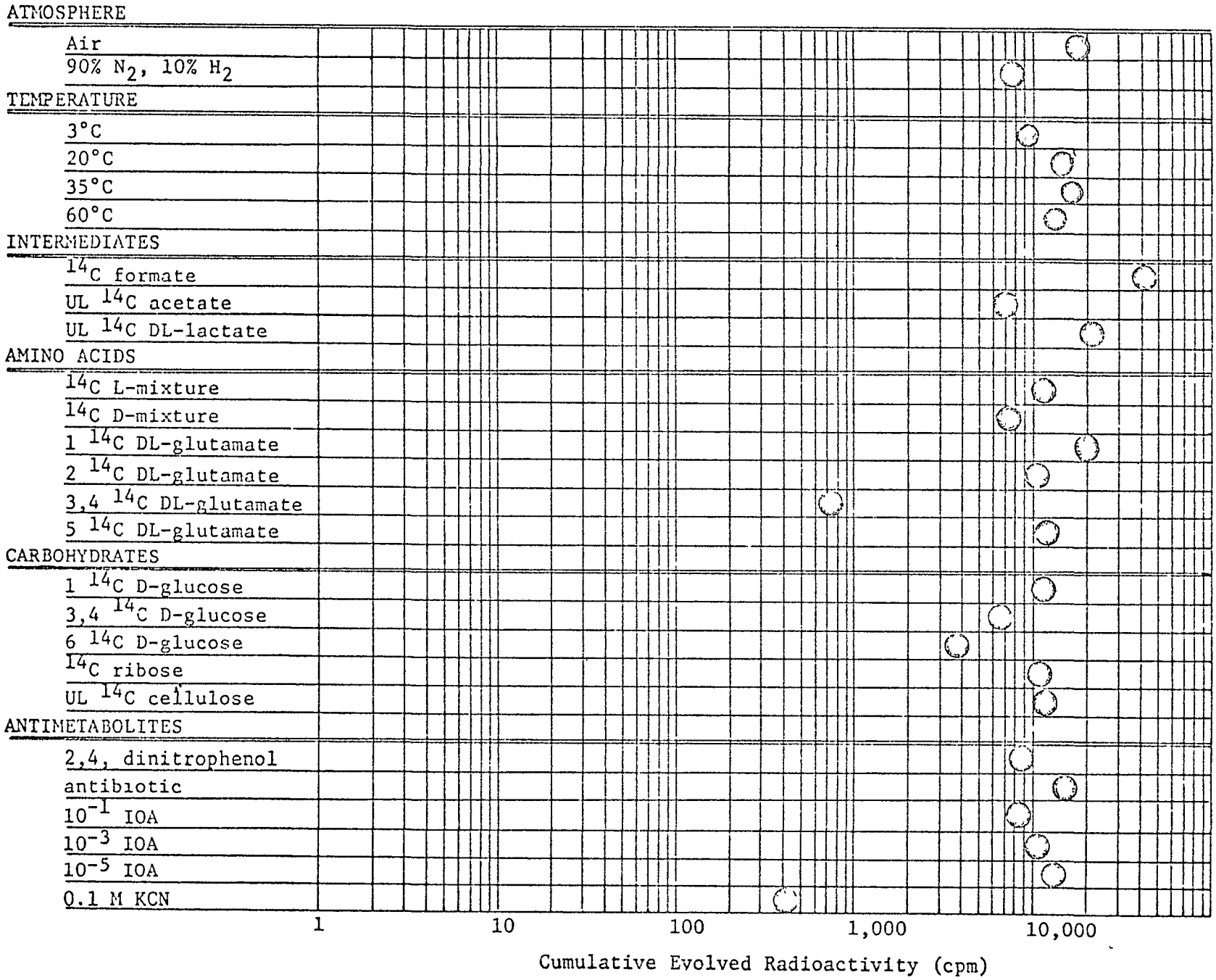
Contract No. NASW-2280
Apendix II

Soil or Organism Meadow Soil

Incubation Time 6 Hours

Standard Medium FLGG



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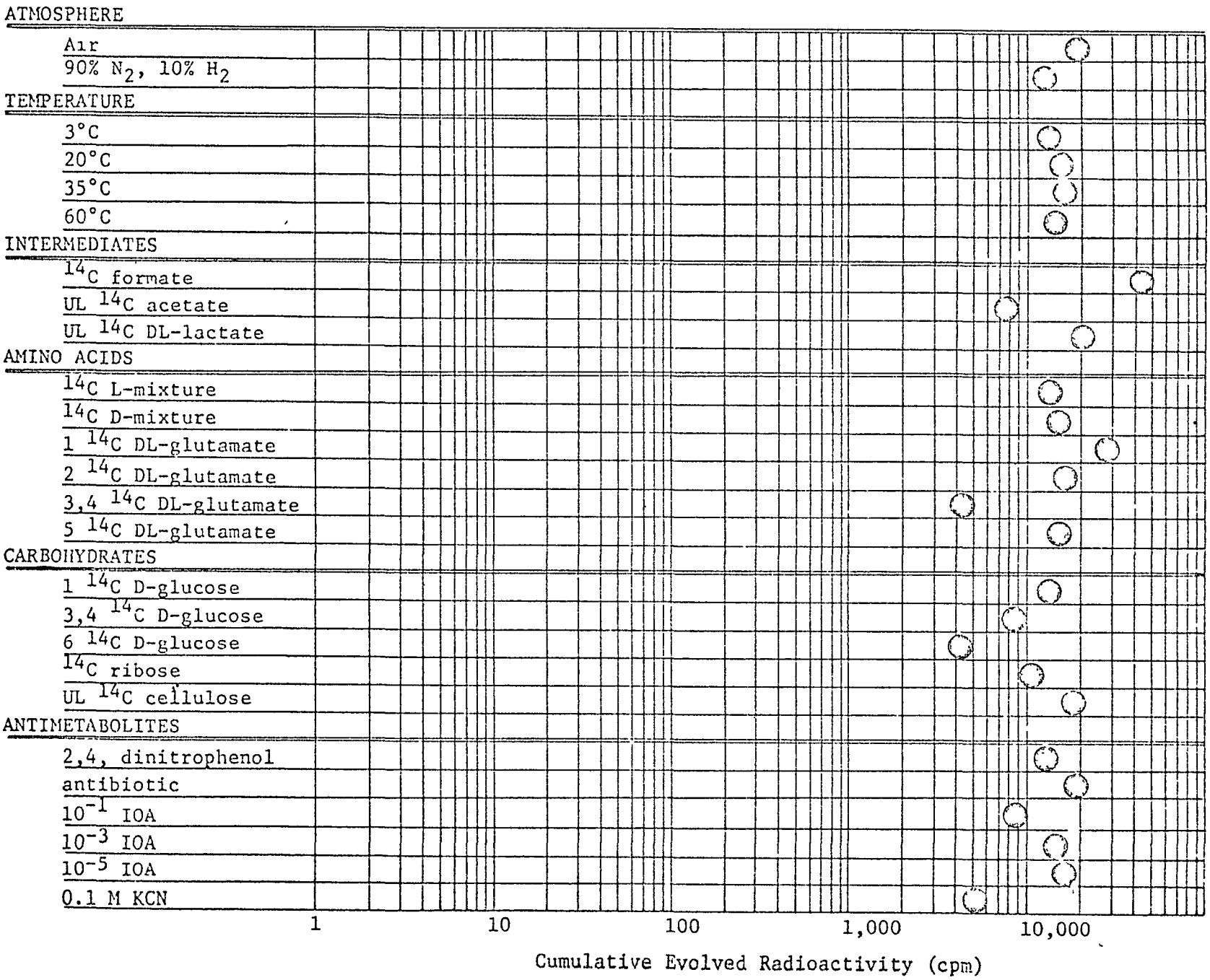
Soil or Organism Meadow Soil

Incubation Time 22 Hours

Standard Medium FLGG



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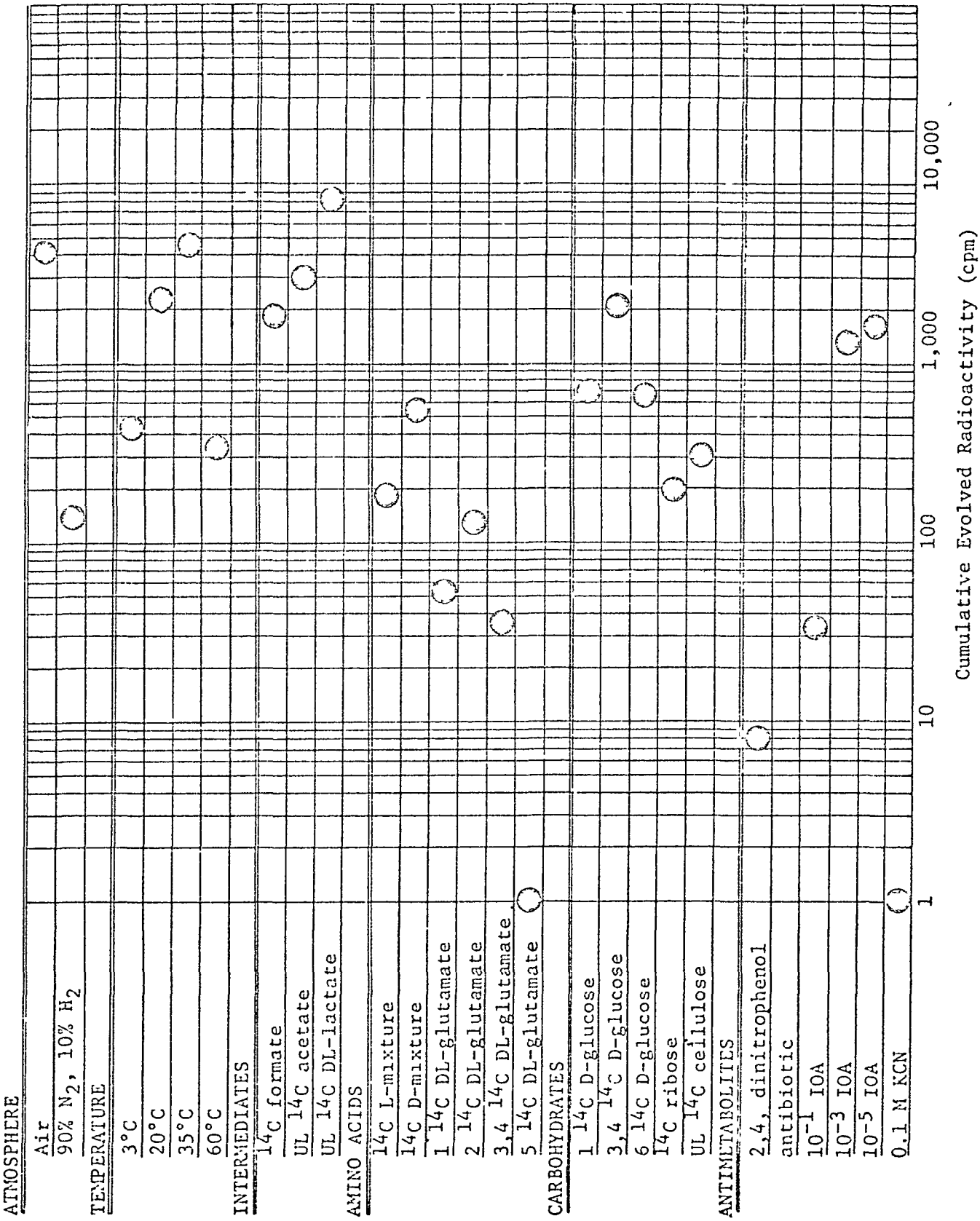
Soil or Organism Meadow Soil Isolate #1 Incubation Time 2 Hours

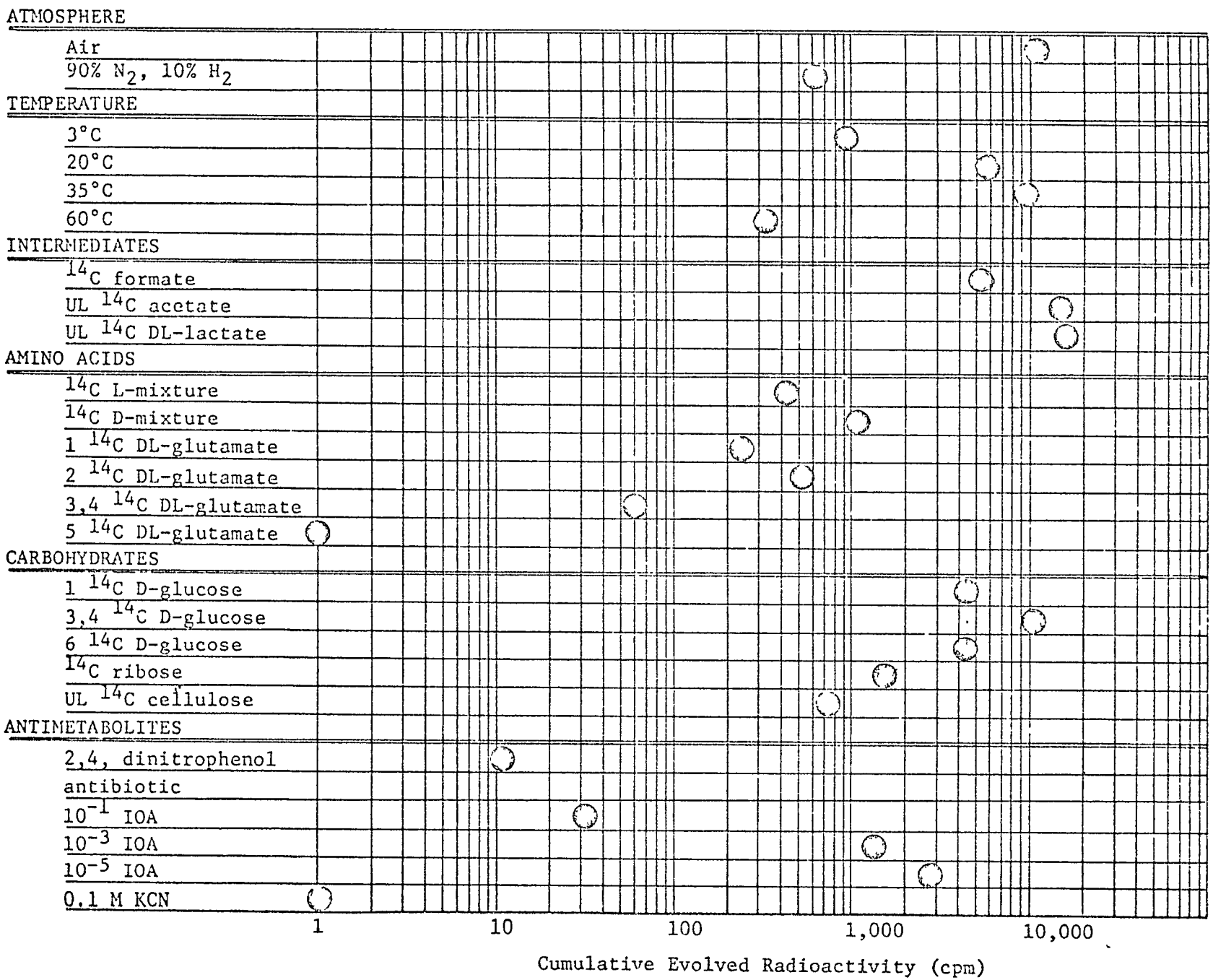
Standard Medium FLGG



Soil or Organism Meadow Soil Isolate #1

Incubation Time 6 Hours

Standard Medium FLGG



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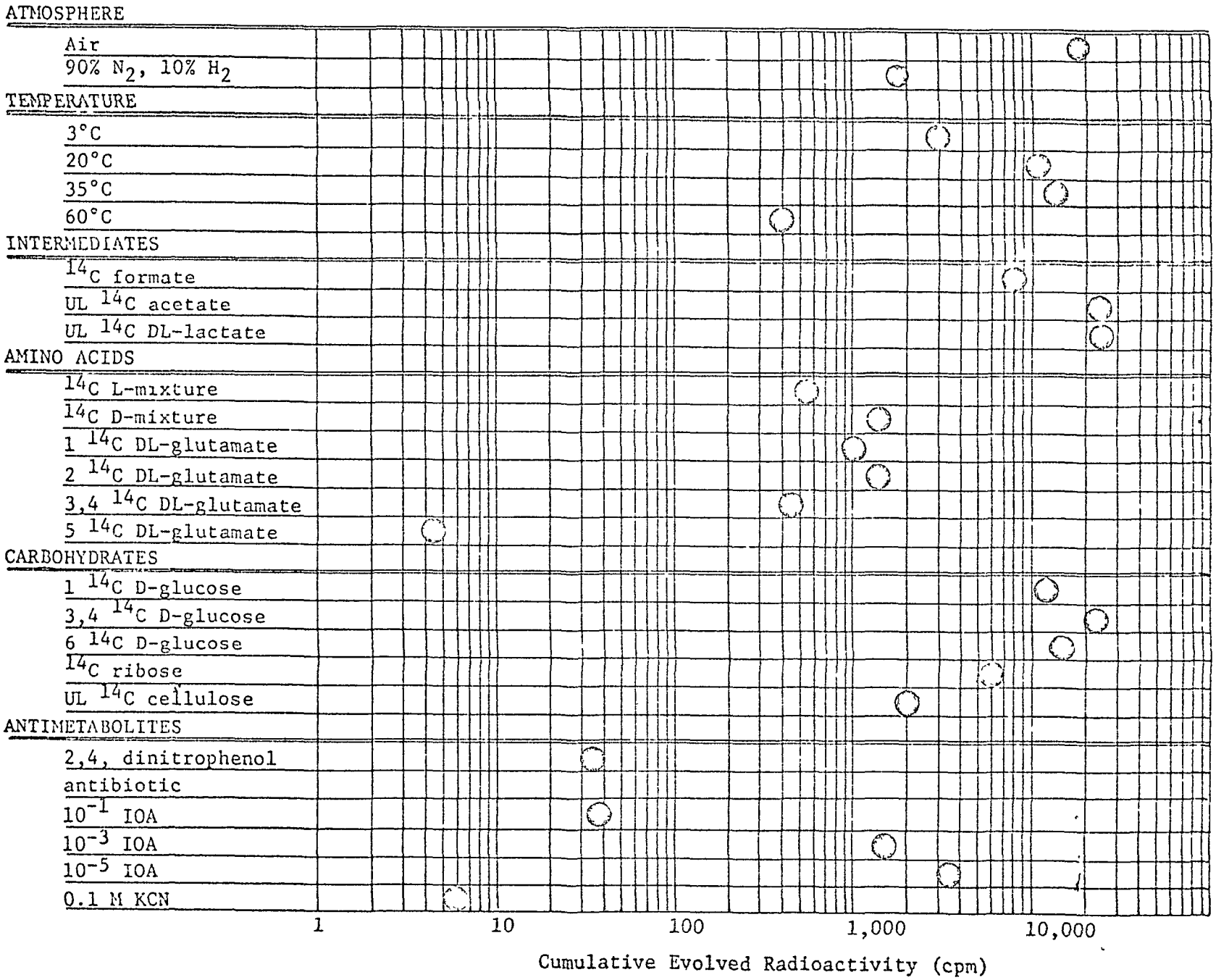
Soil or Organism Meadow Soil Isolate #1

Incubation Time 22 Hours

Standard Medium FLGG



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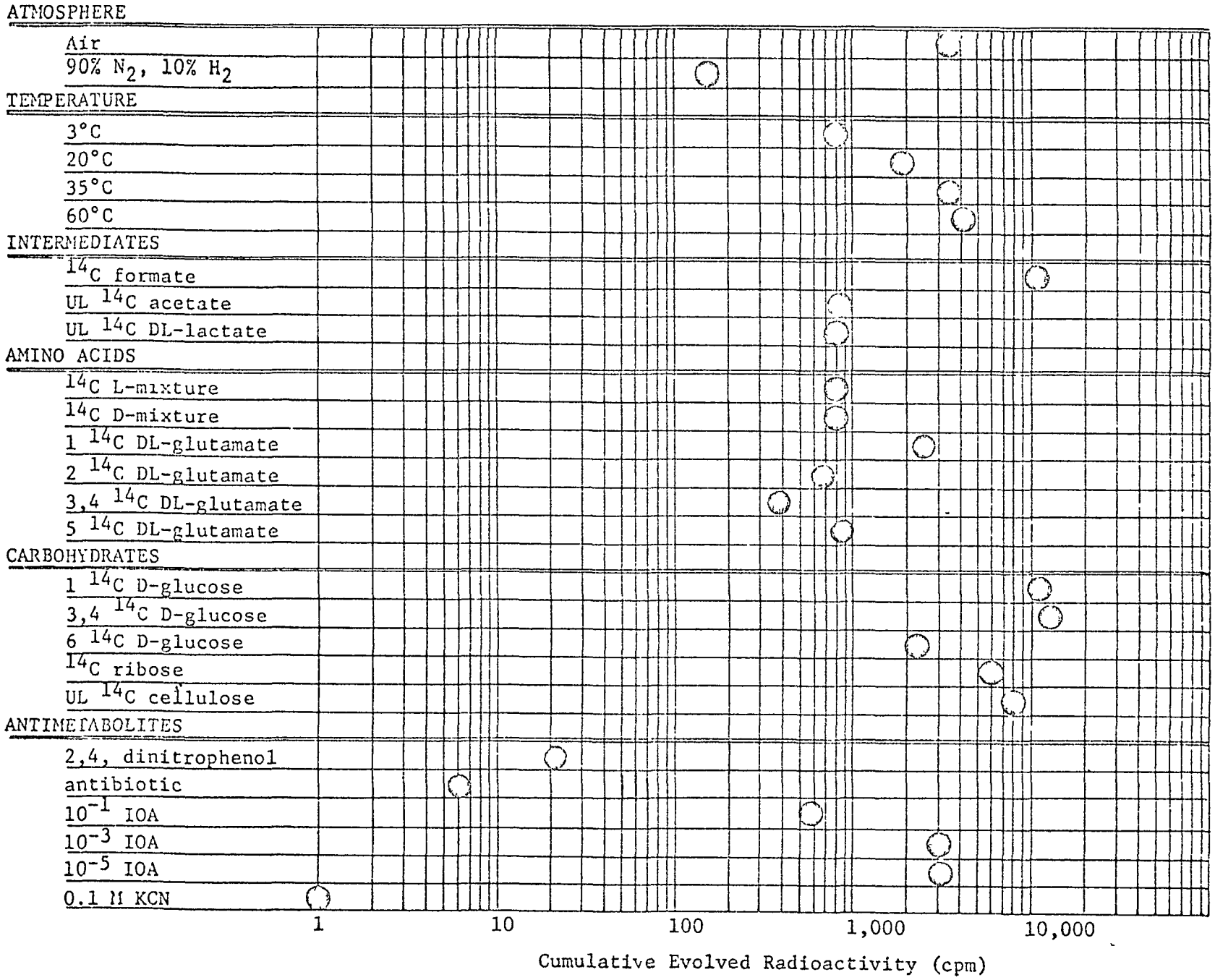
Soil or Organism Meadow Soil Isolate #2

Incubation Time 2 Hours

Standard Medium FLGG



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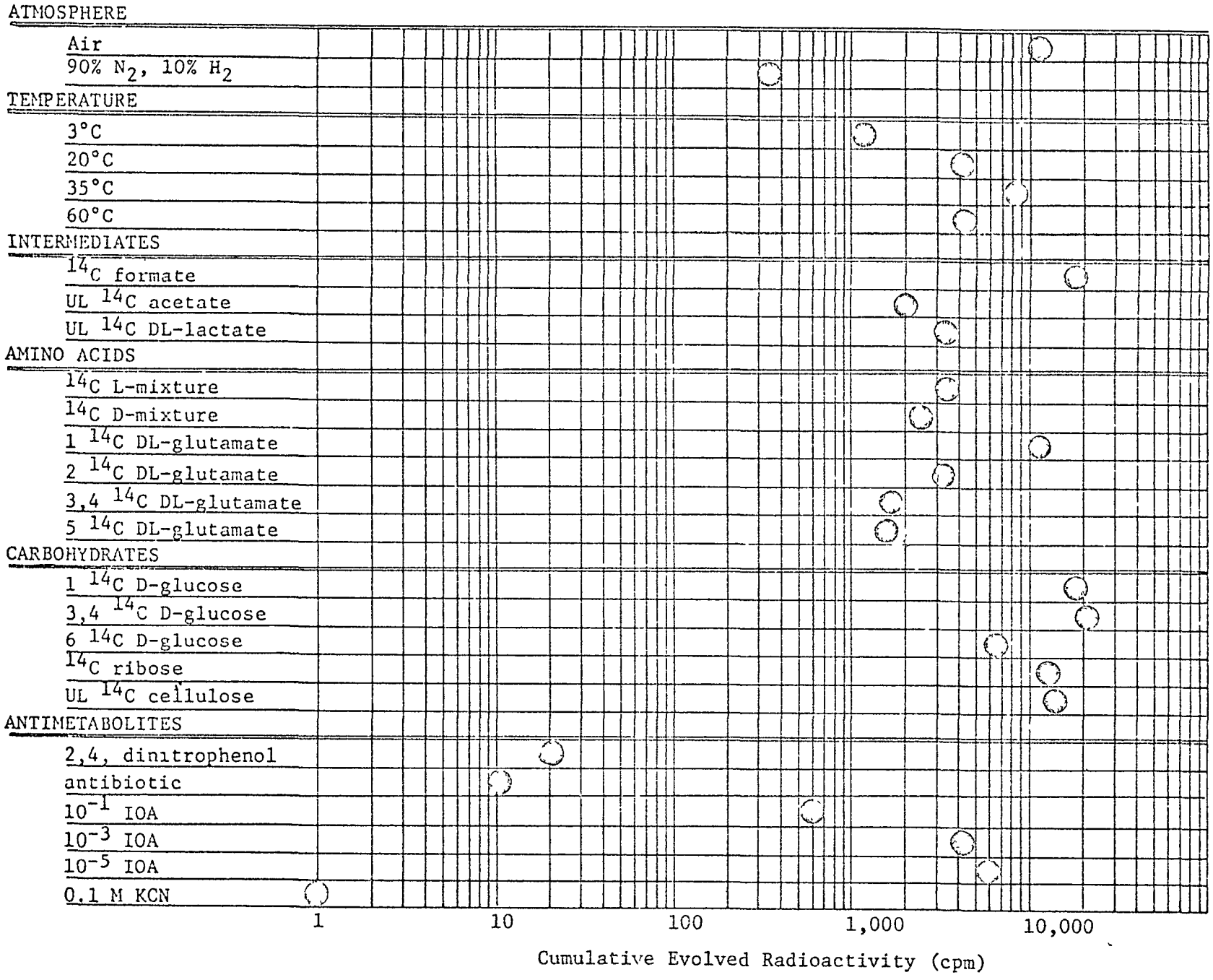
Soil or Organism Meadow Soil Isolate #2

Incubation Time 6 Hours

Standard Medium FLGG



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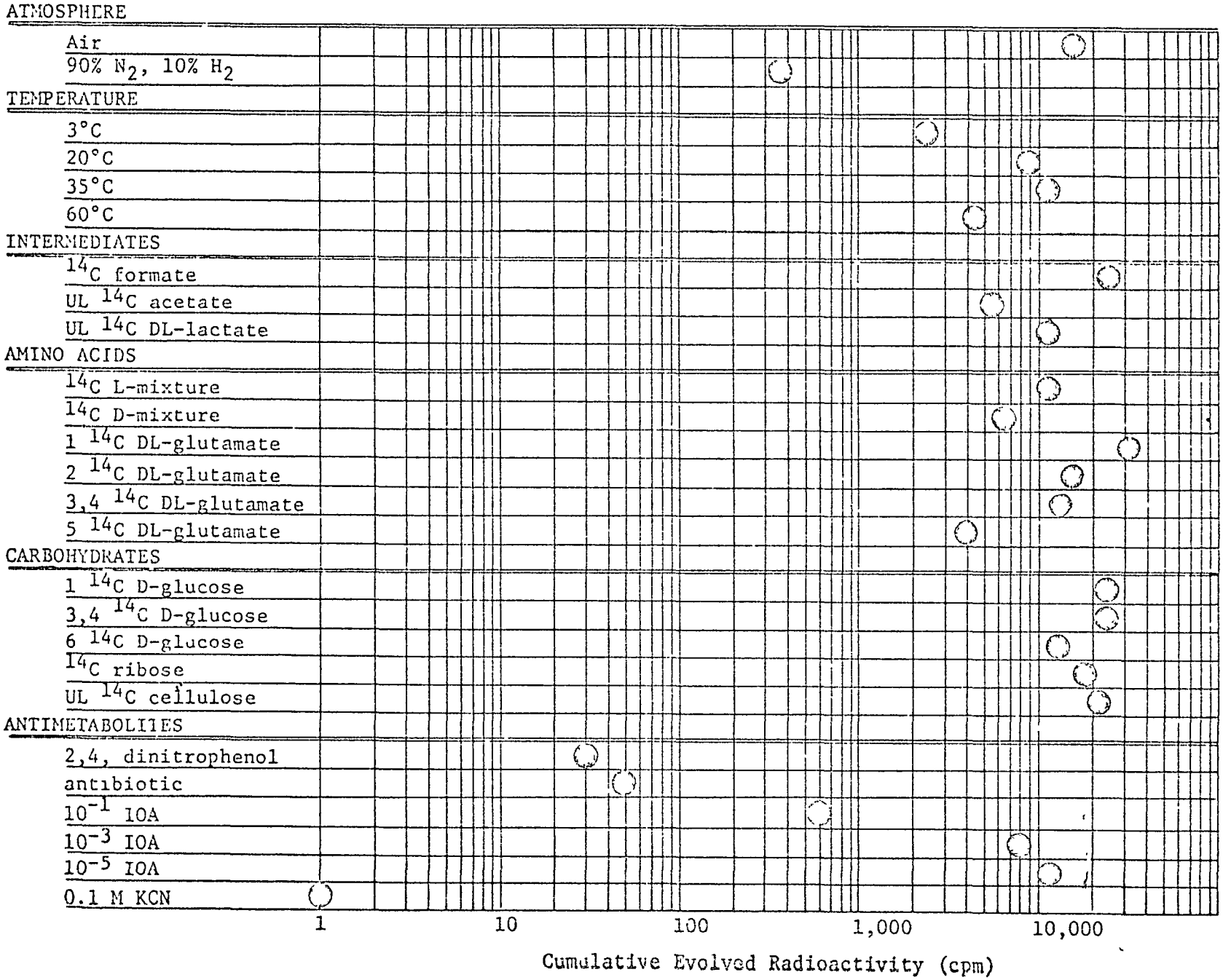
Soil or Organism Meadow Soil Isolate #2

Incubation Time 22 Hours

Standard Medium FLGG



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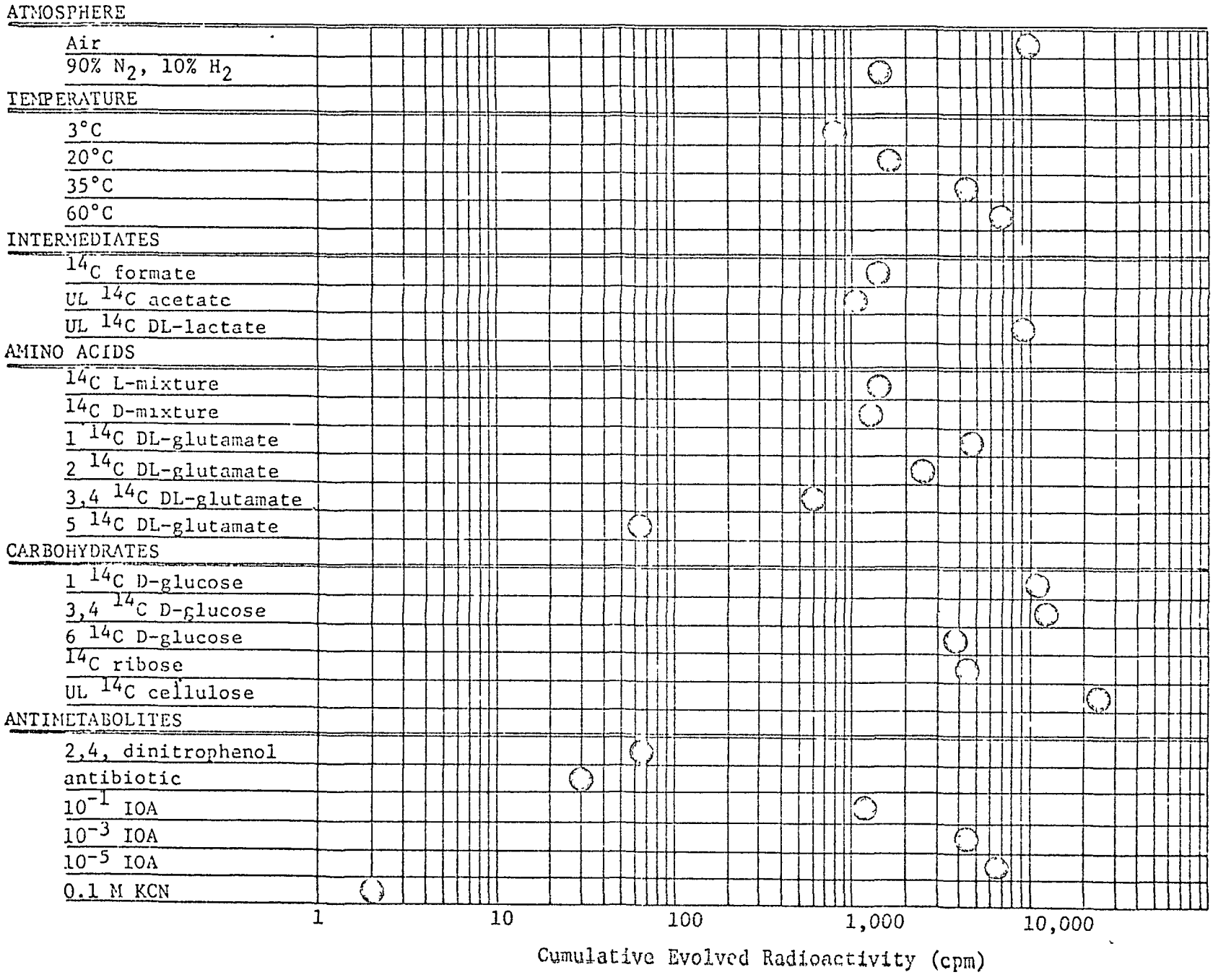
Soil or Organism Meadow Soil Isolate #3

Incubation Time 2 Hours

Standard Medium FLGG



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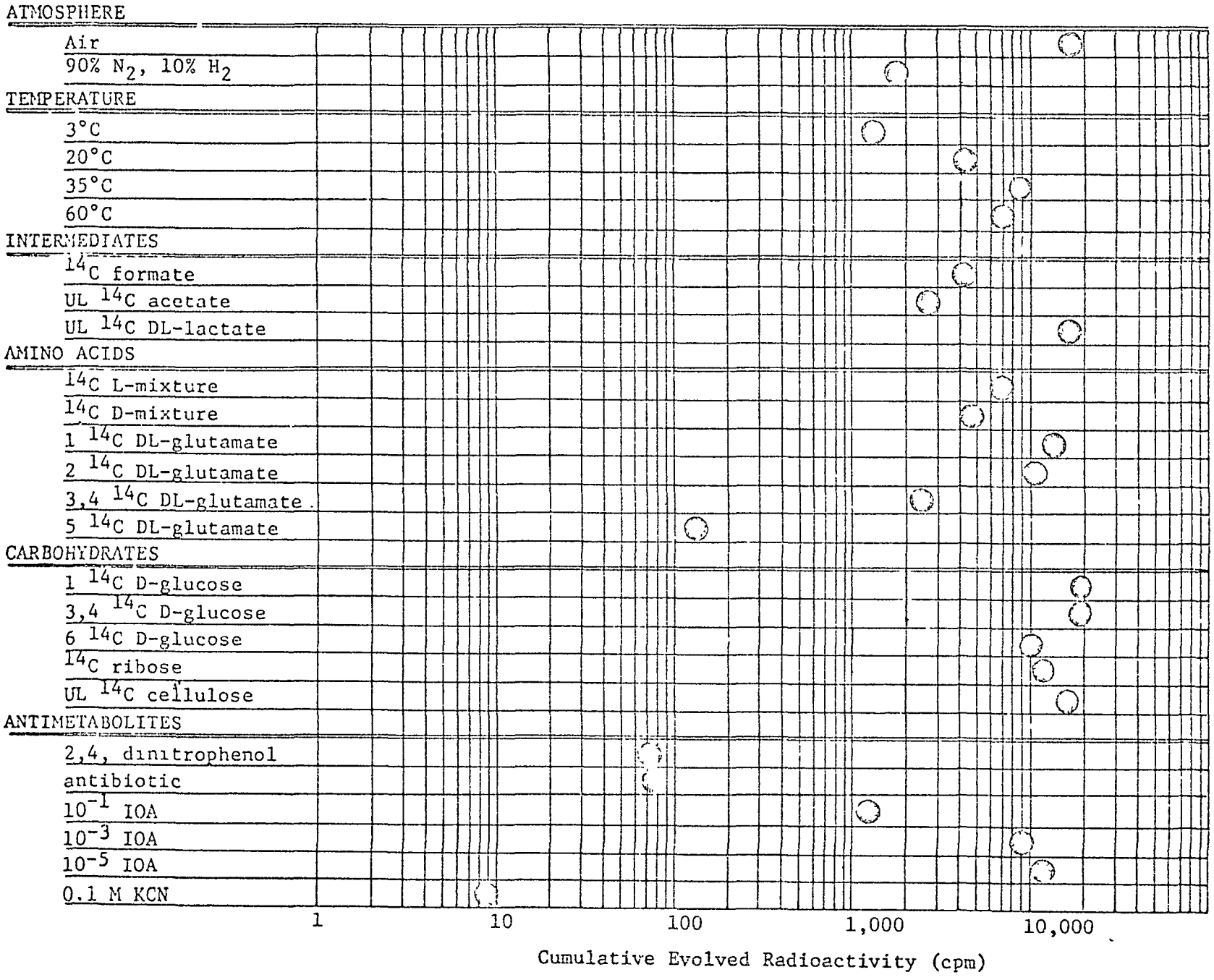
Soil or Organism Meadow Soil Isolate #3

Incubation Time 6 Hours

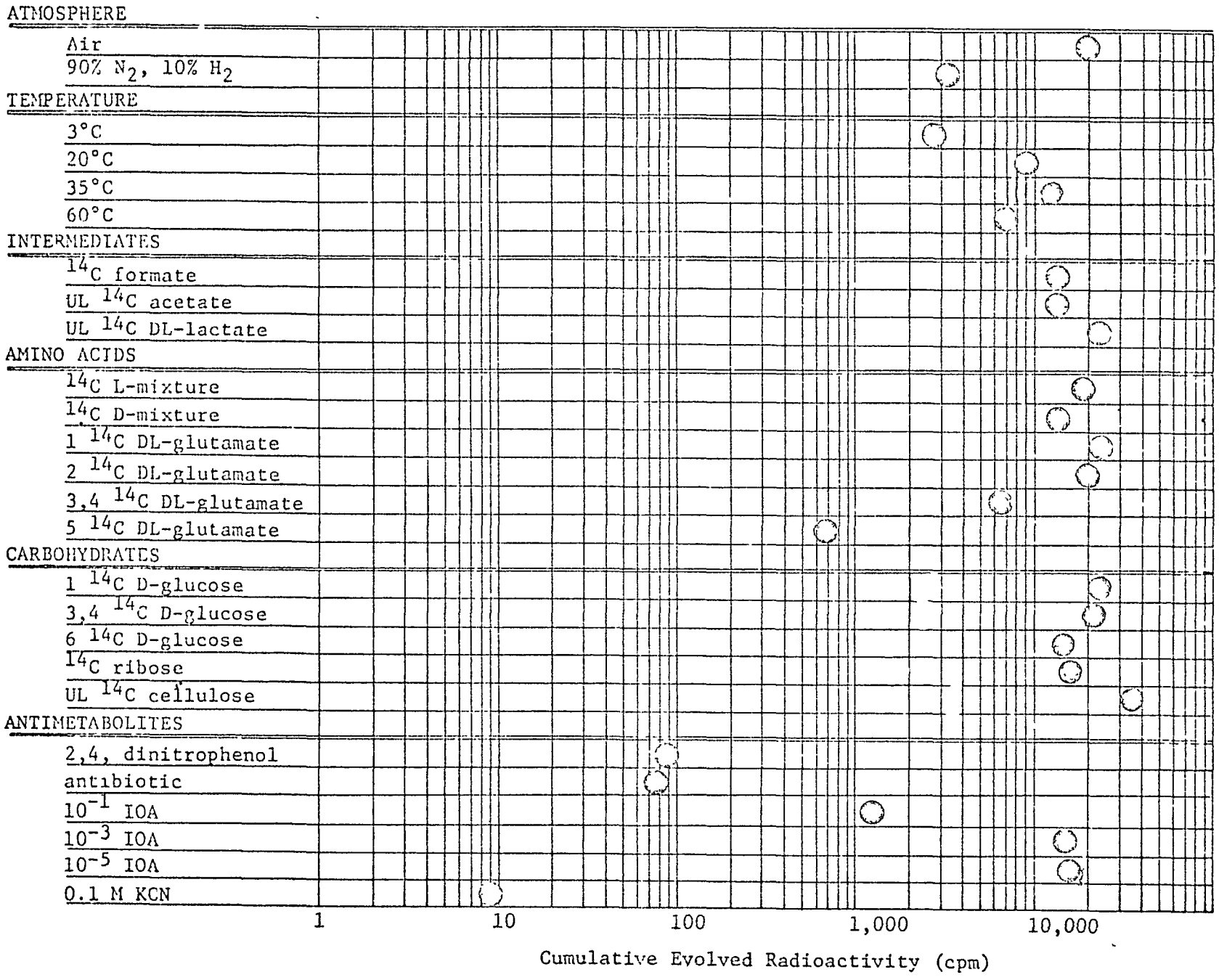
Standard Medium FLGG



Soil or Organism Meadow Soil Isolate #3

Incubation Time 22 Hours

Standard Medium FLGG



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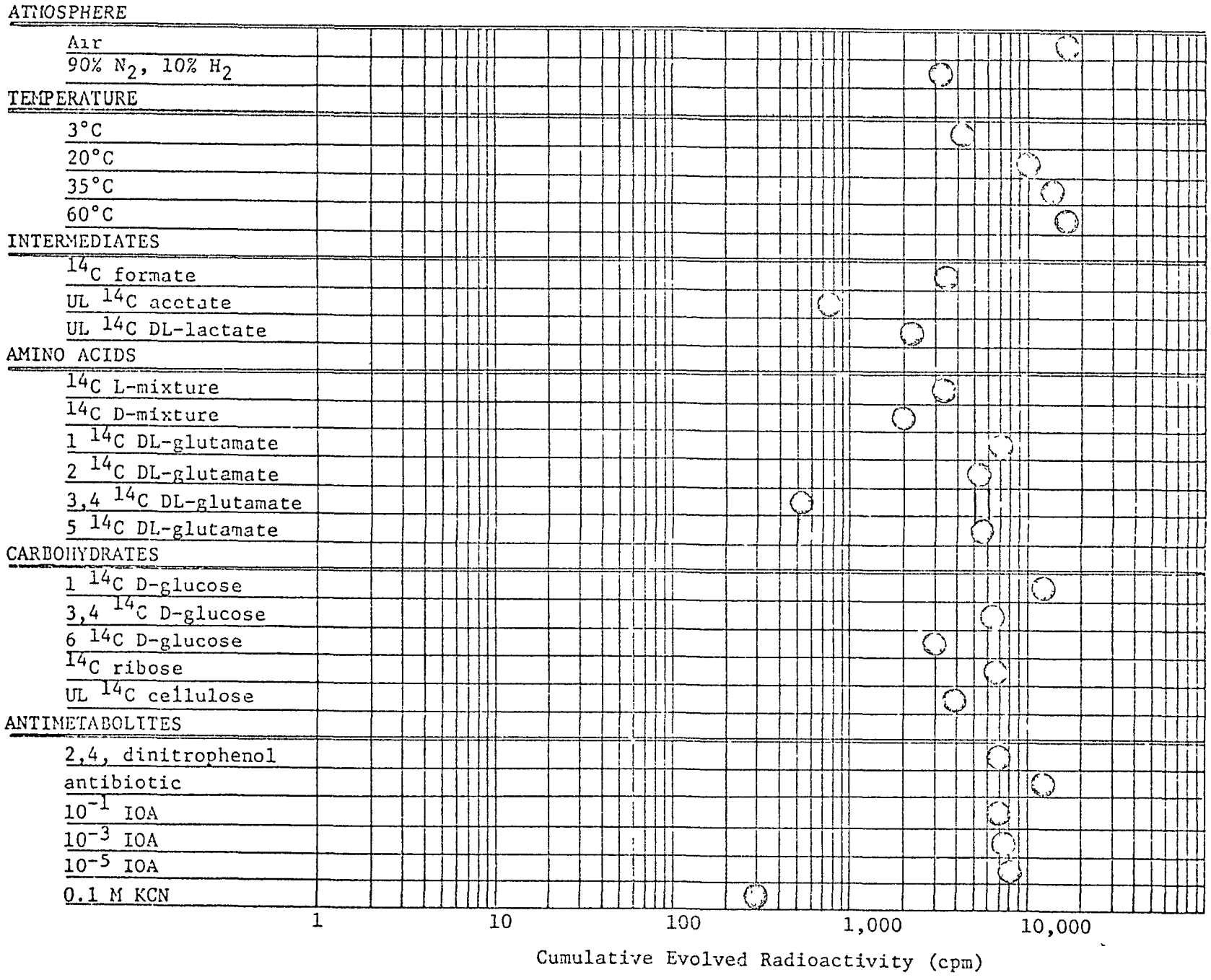
Soil or Organism Phoenix Soil

Incubation Time 2 Hours

Standard Medium FLGG



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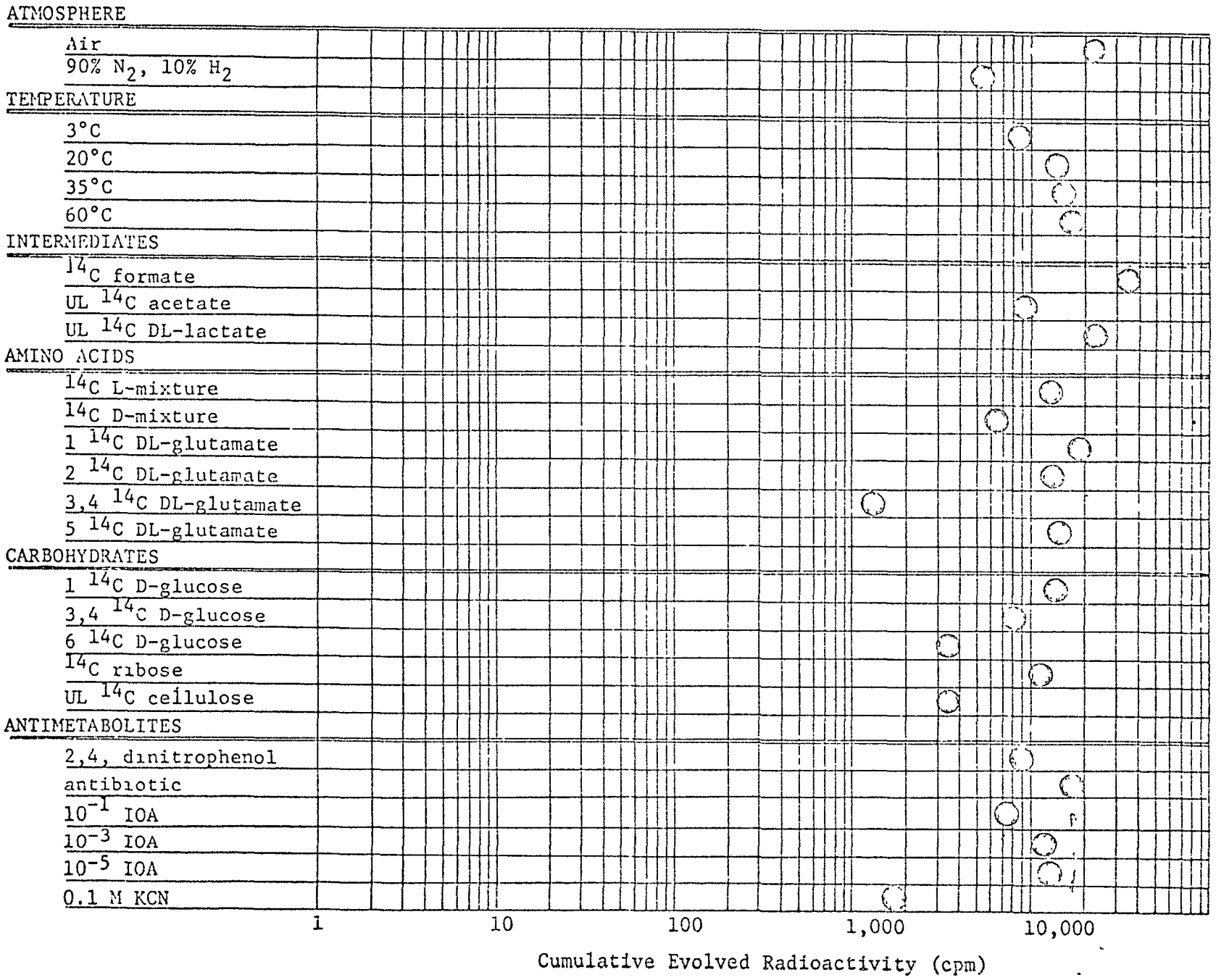
Soil or Organism Phoenix Soil

Incubation Time 6 Hours

Standard Medium FLGG



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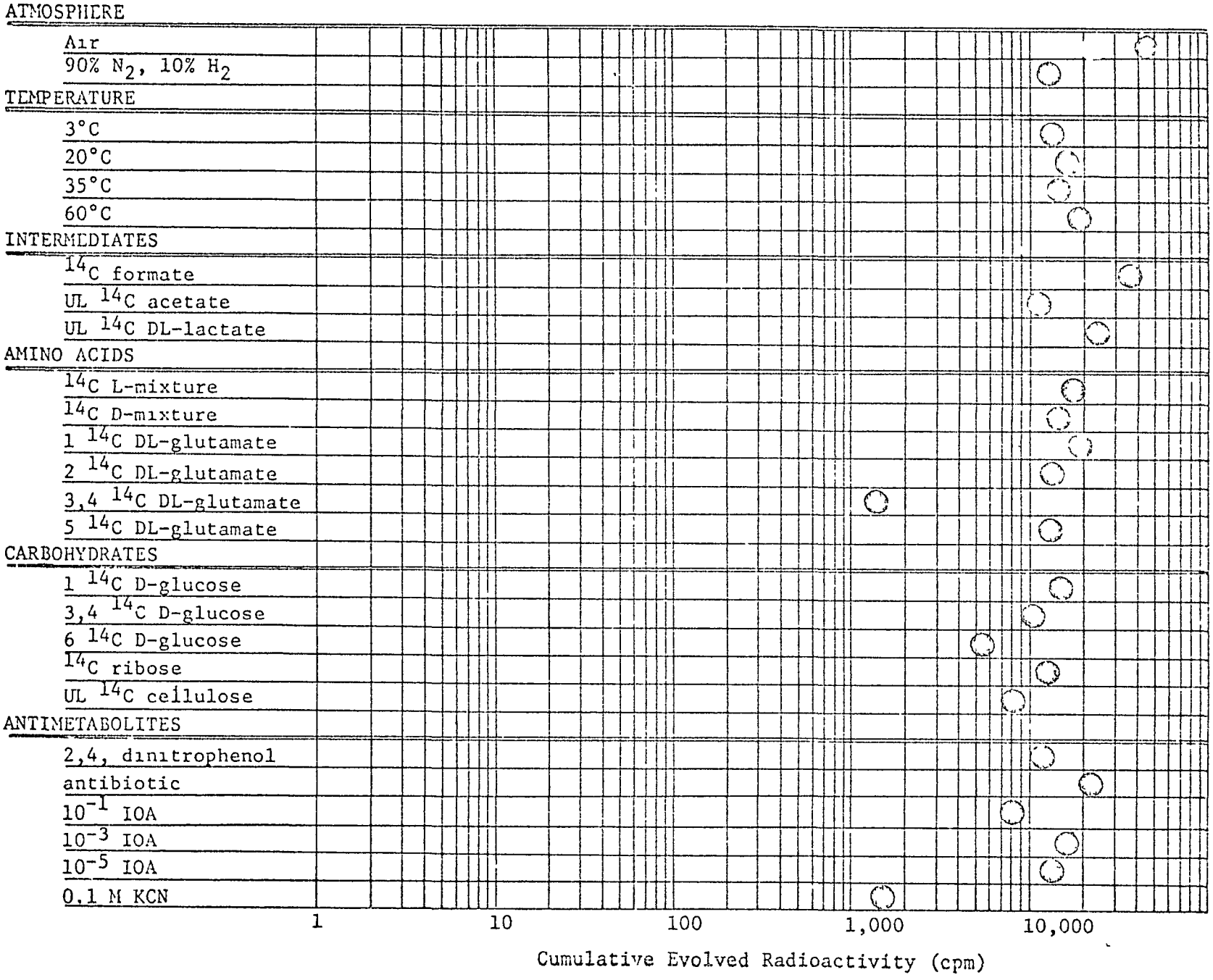
Soil or Organism Phoenix Soil

Incubation Time 22 Hours

Standard Medium FLGG



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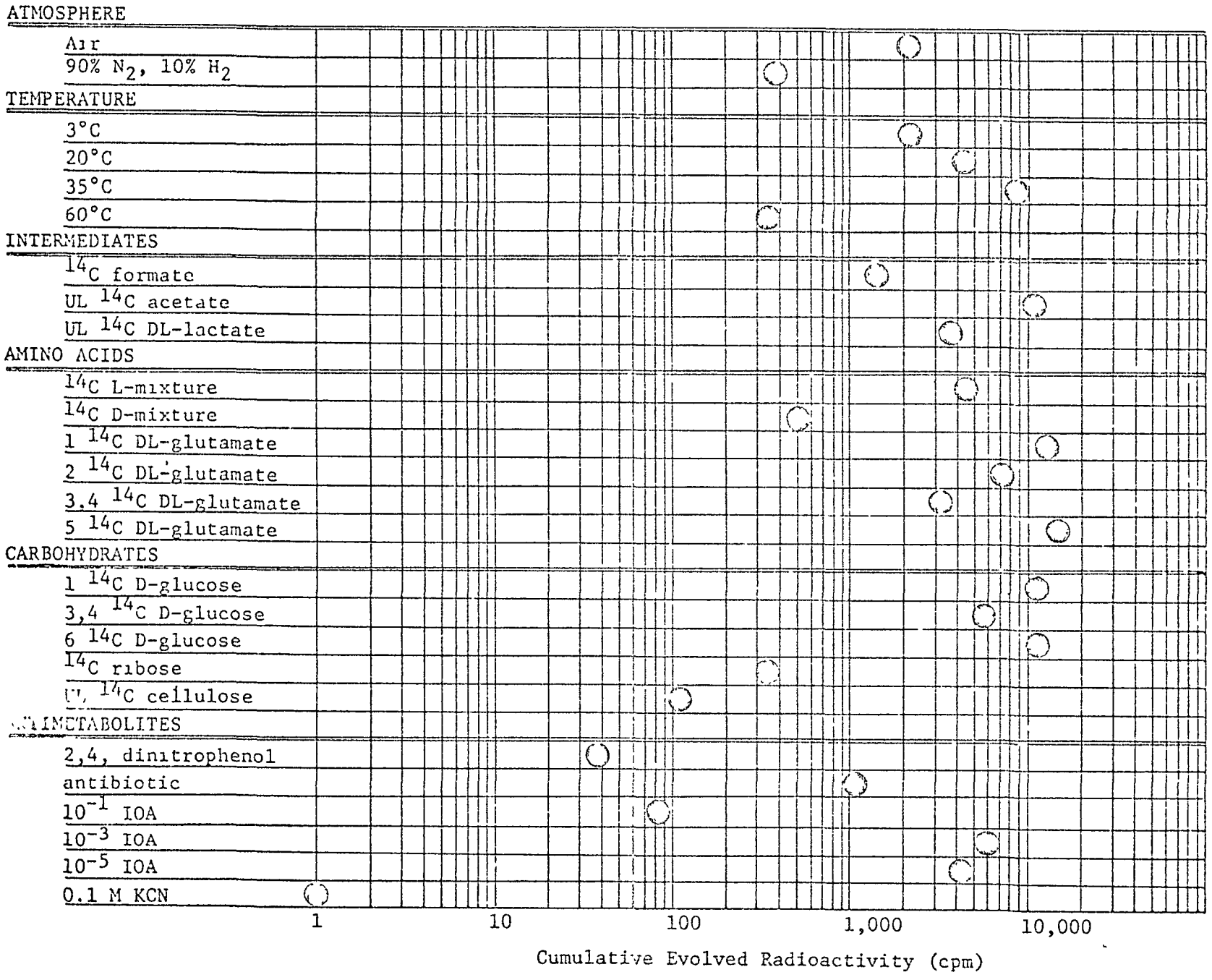
Soil or Organism Phoenix Soil Isolate #1

Incubation Time 2 Hours

Standard Medium FLGG



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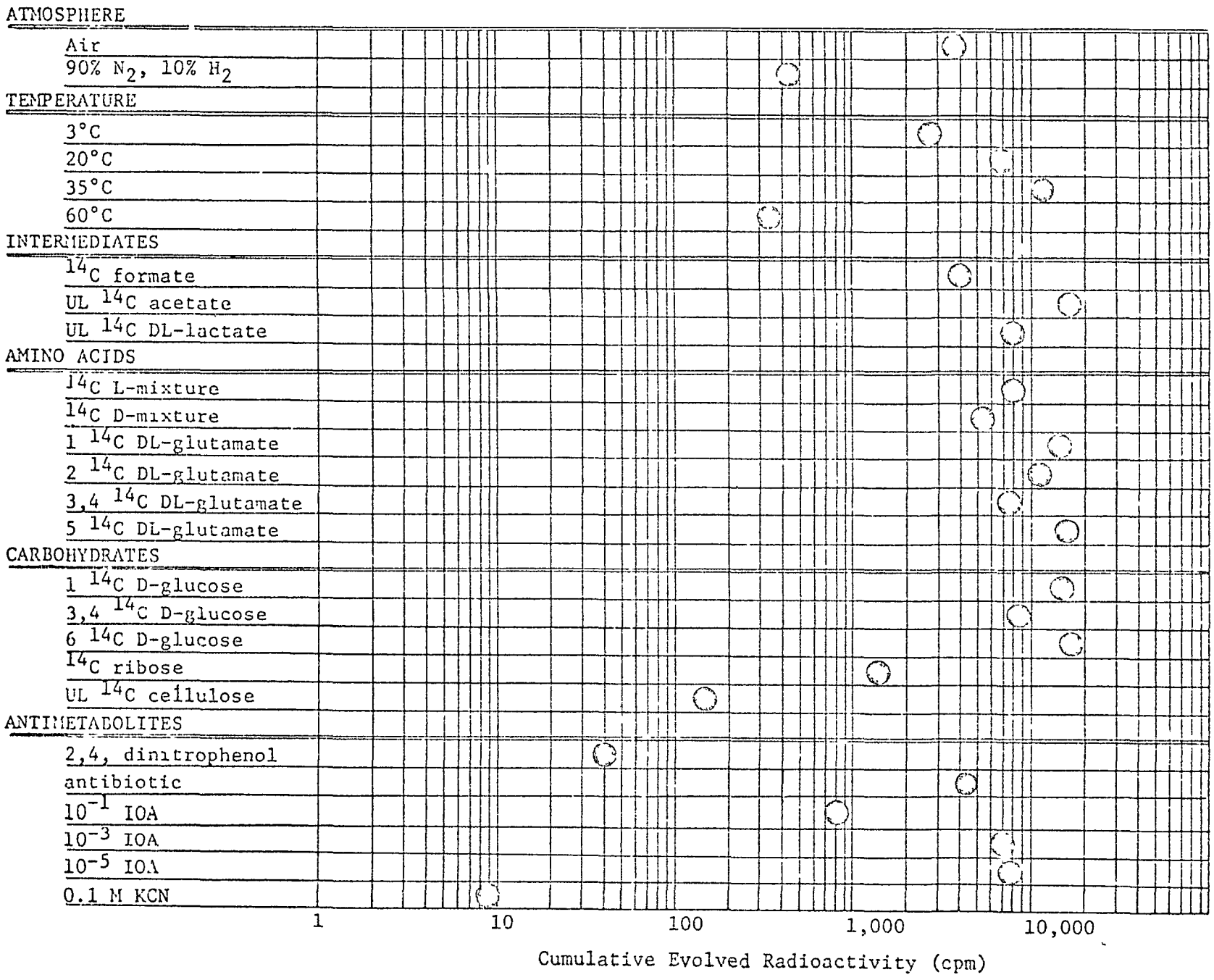
Soil or Organism Phoenix Soil Isolate #1

Incubation Time 6 Hours

Standard Medium FLGG



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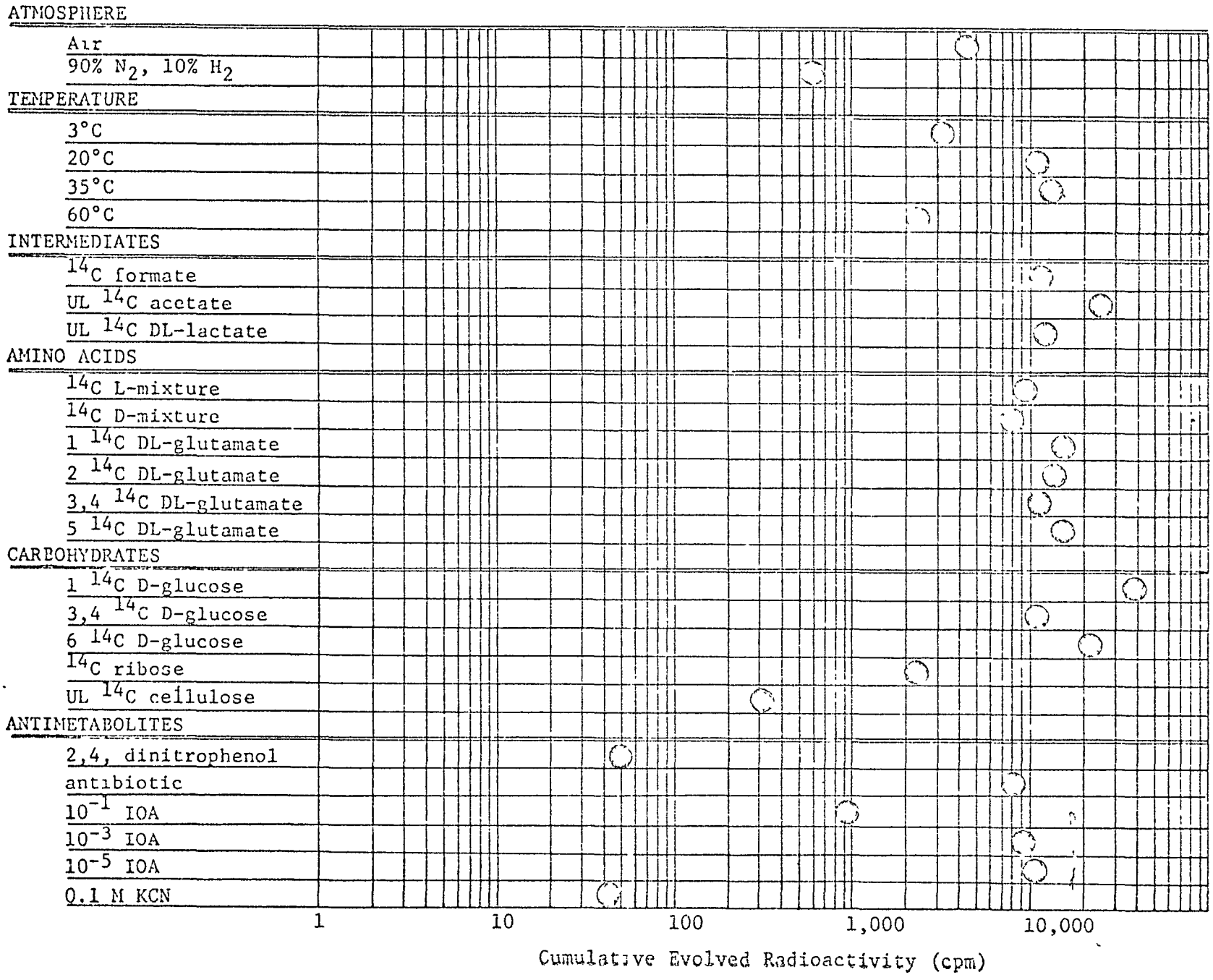
Soil or Organism Phoenix Soil Isolate #1

Incubation Time 22 Hours

Standard Medium FLGG



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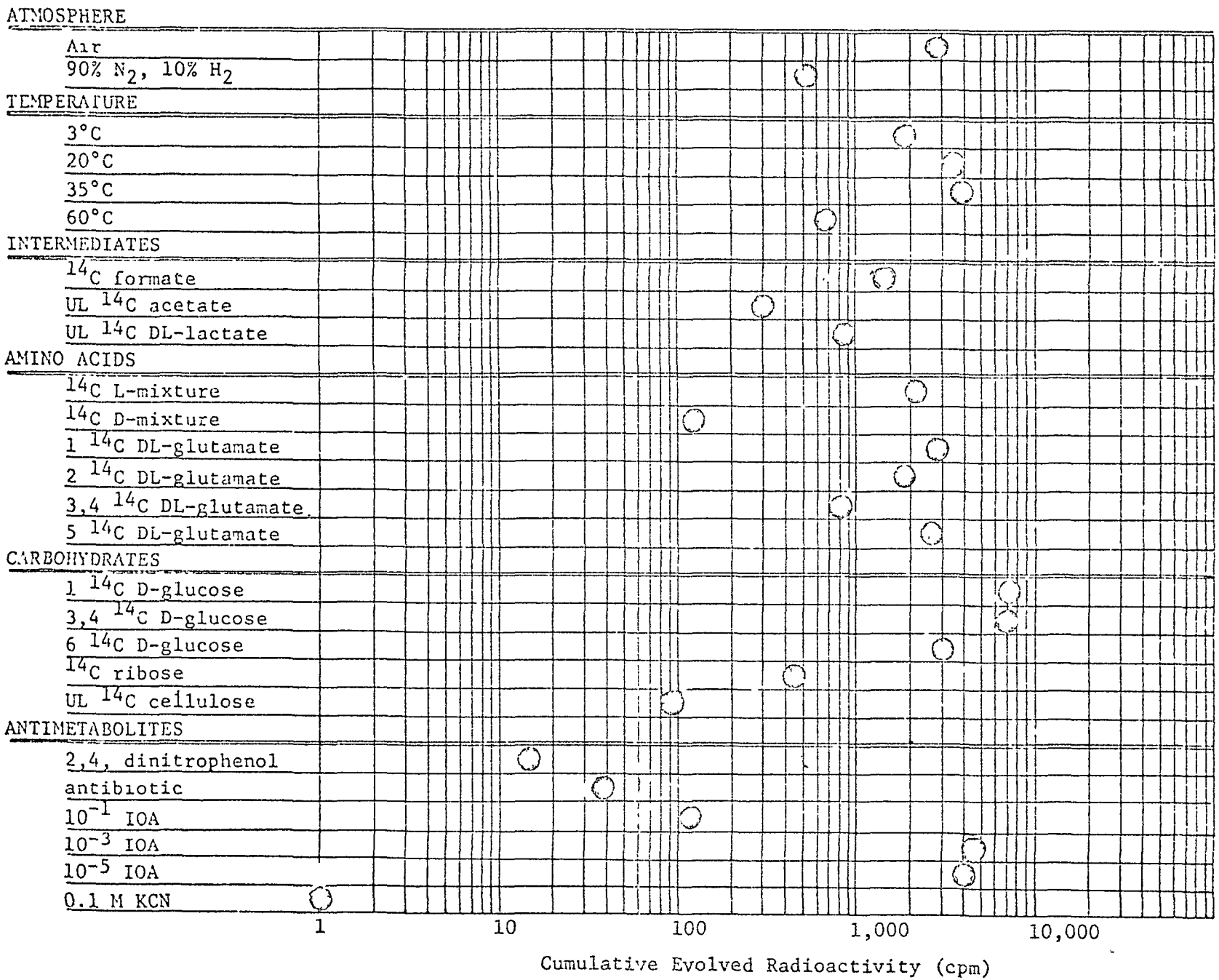
Soil or Organism Phoenix Soil Isolate #2

Incubation Time 2 Hours

Standard Medium FLGG

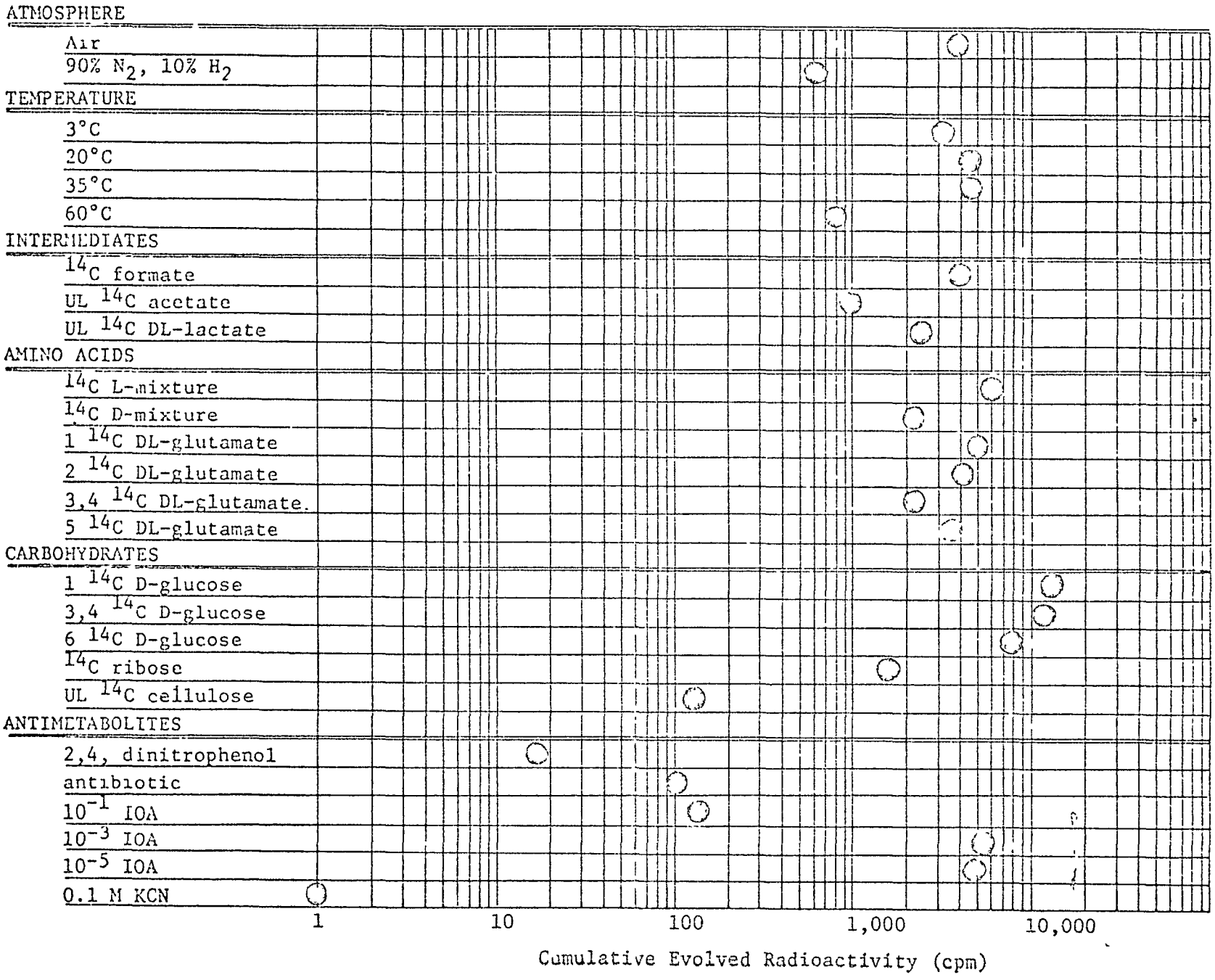


BIOSPHERICS INCORPORATED

Soil or Organism Phoenix Soil Isolate #2

Incubation Time 6 Hours

Standard Medium FLGG

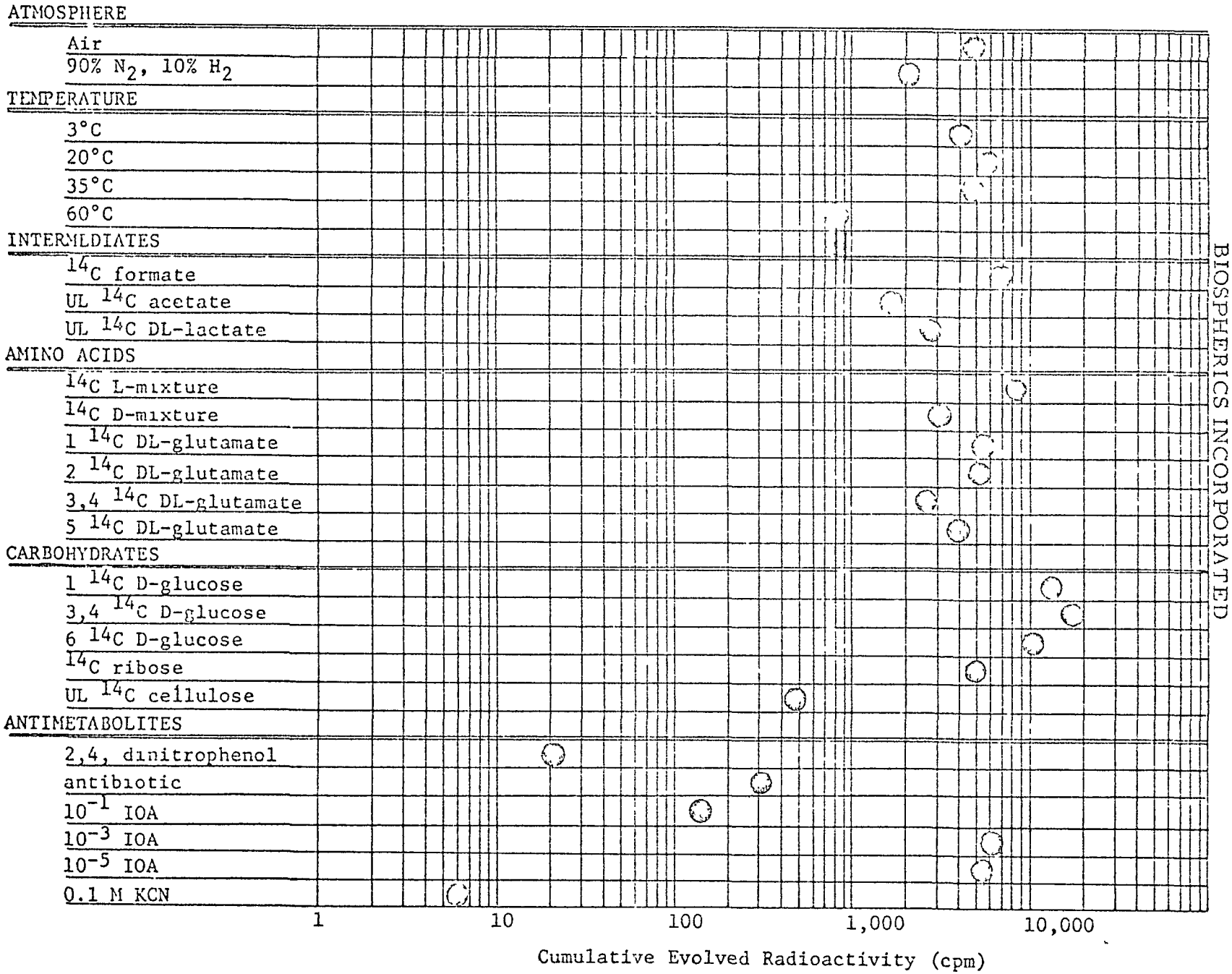


BIOSPHERICS INCORPORATED

Soil or Organism Phoenix Soil Isolate #2

Incubation Time 22 Hours

Standard Medium FLGG

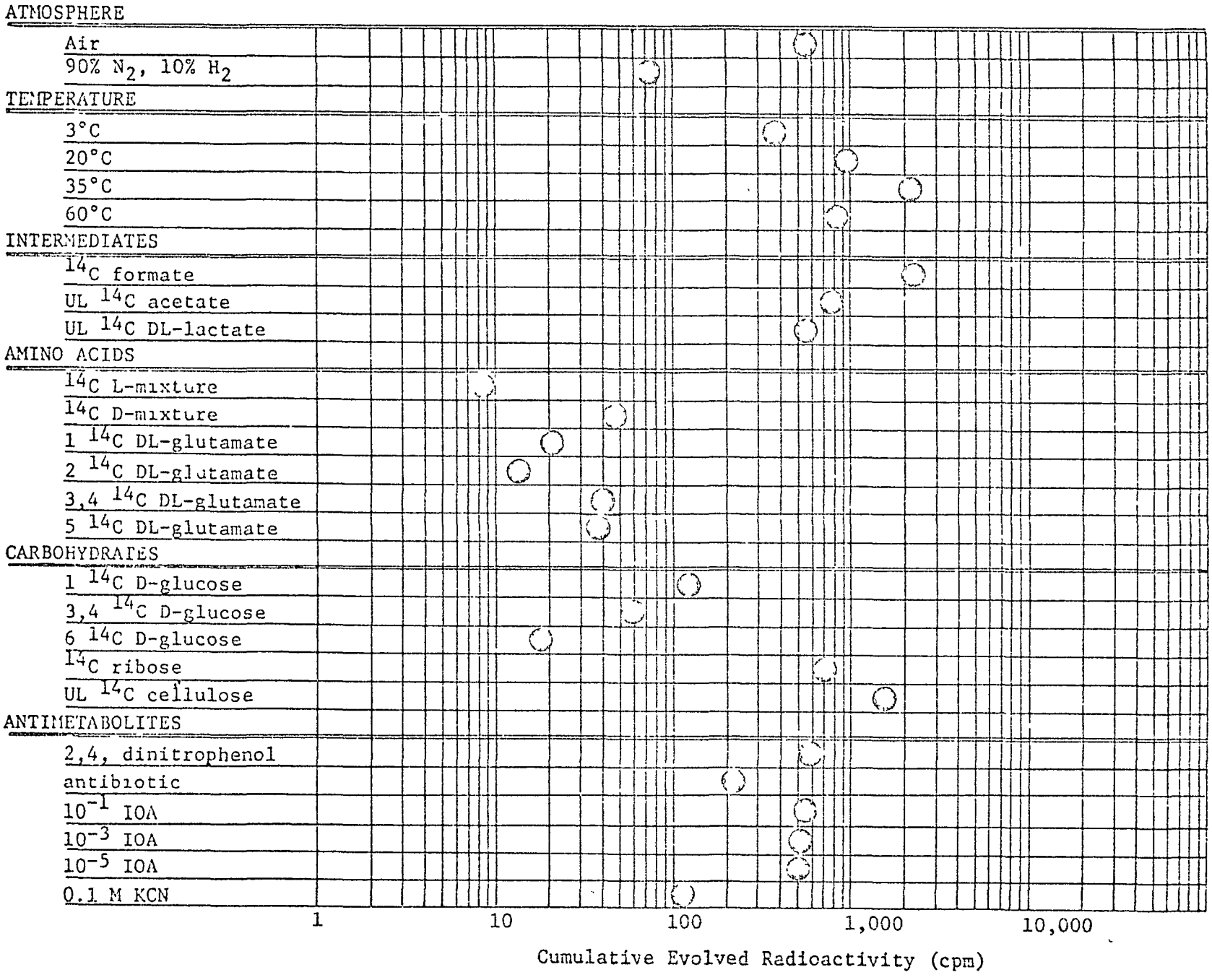


BIOSPHERICS INCORPORATED

Soil or Organism Aiken Soil

Incubation Time 2 Hours

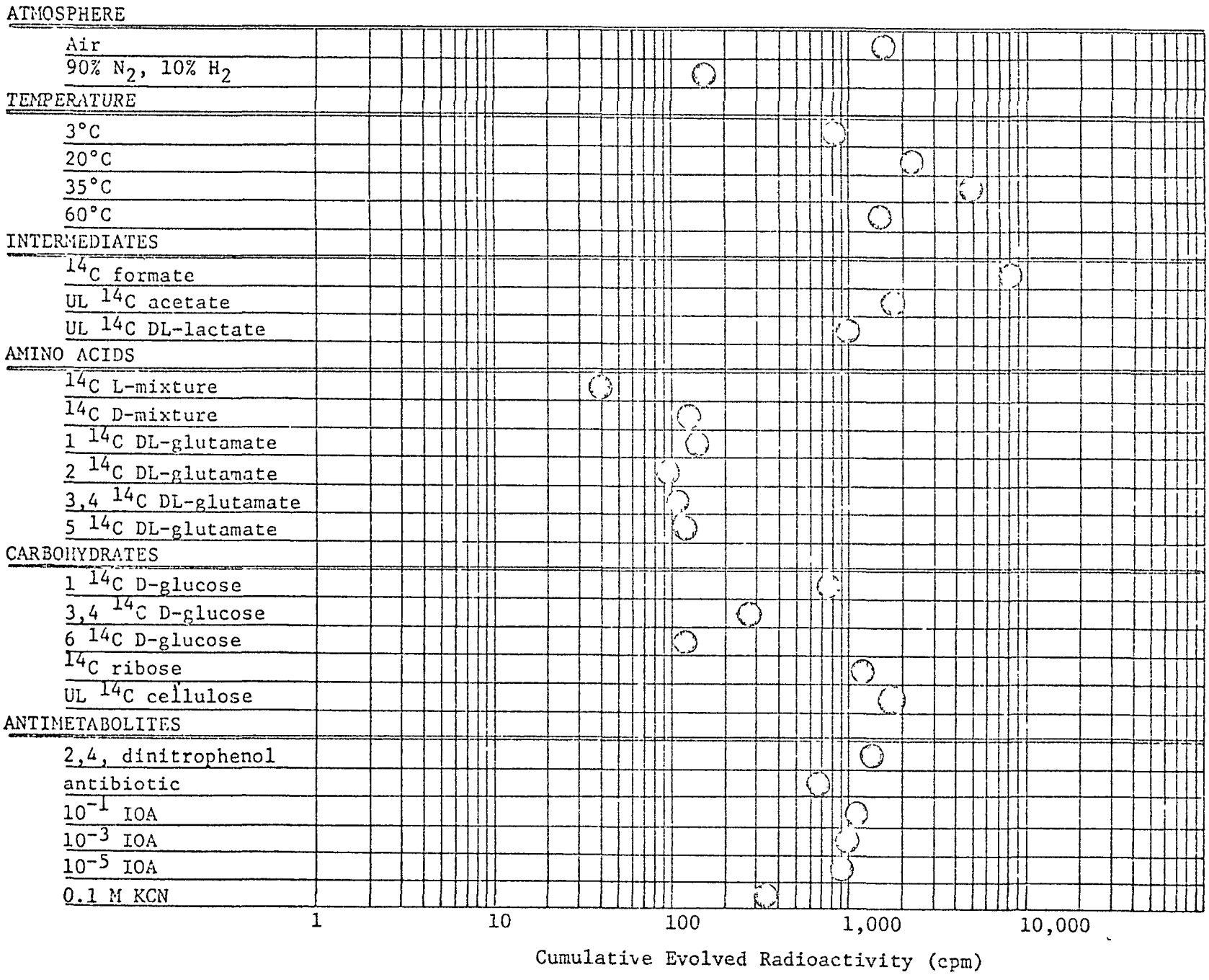
Standard Medium VM-I 3.4 uCi/ml



Soil or Organism Aiken Soil

Incubation Time 6 Hours

Standard Medium VM-I 3.4 uCi/ml

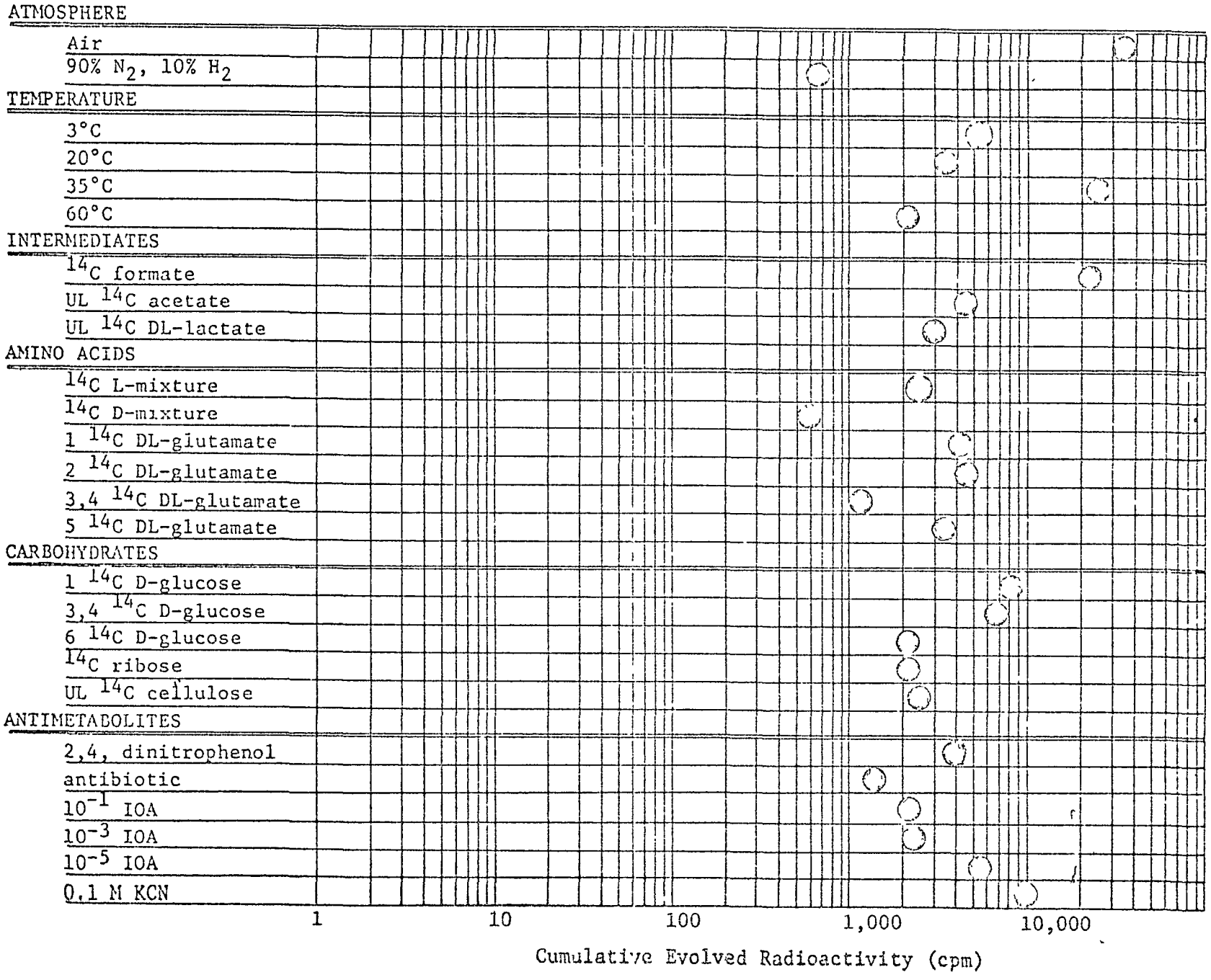


BIOSPHERICS INCORPORATED

Soil or Organism Aiken Soil

Incubation Time 22 Hours

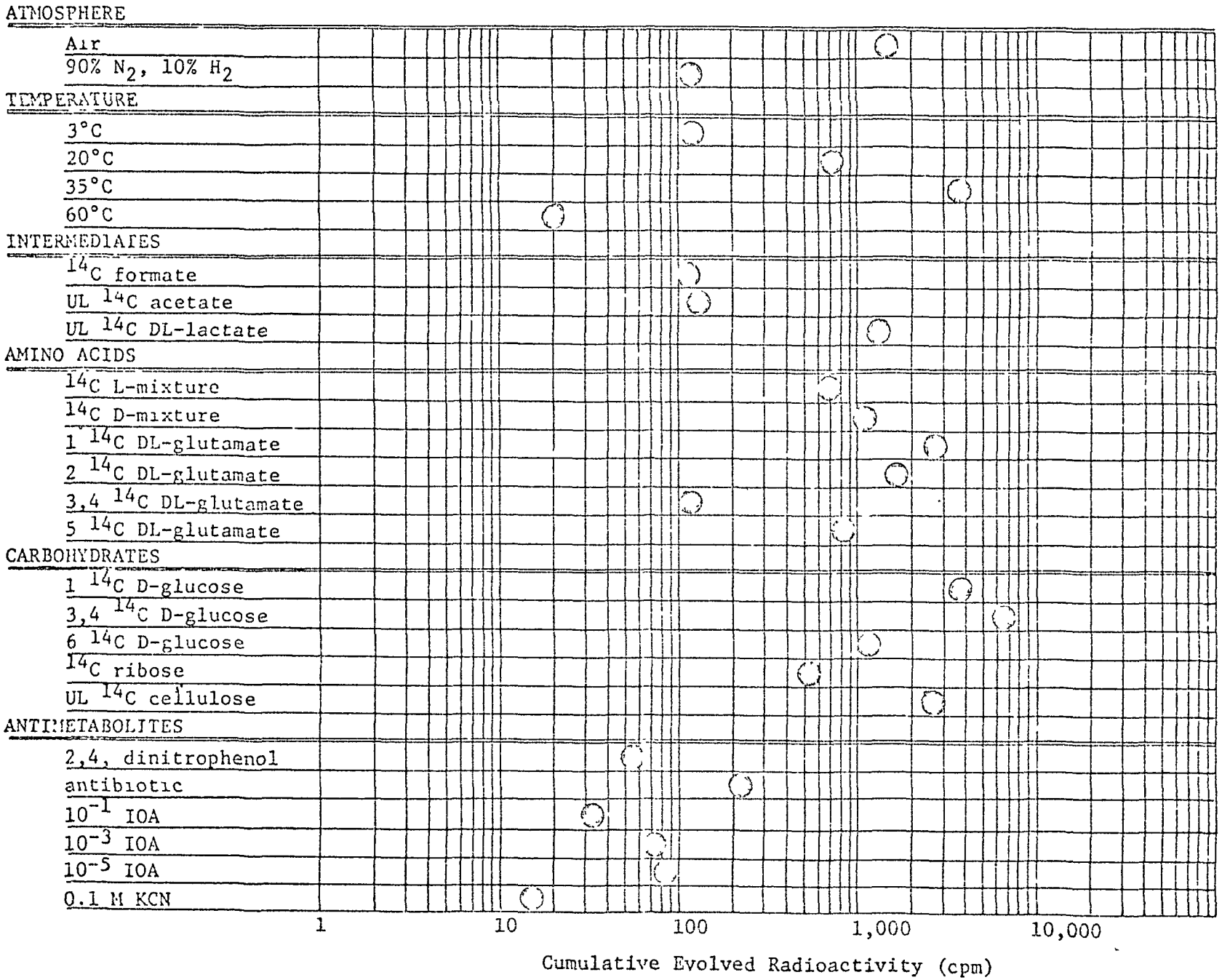
Standard Medium VM-I 3.4 uCi/ml



Soil or Organism Aiken Soil Isolate #1

Incubation Time 2 Hours

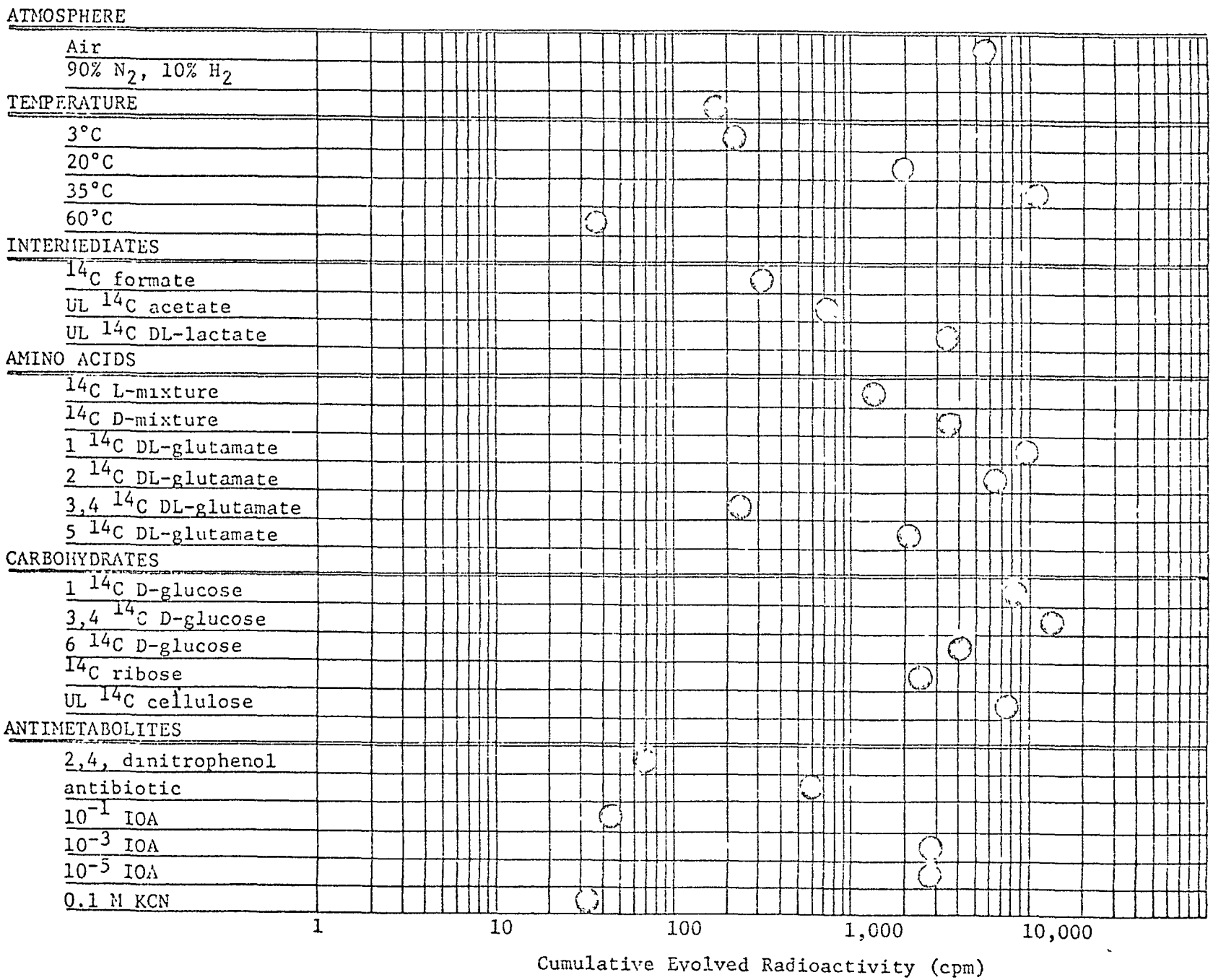
Standard Medium VM-I 3.4 uCi/ml



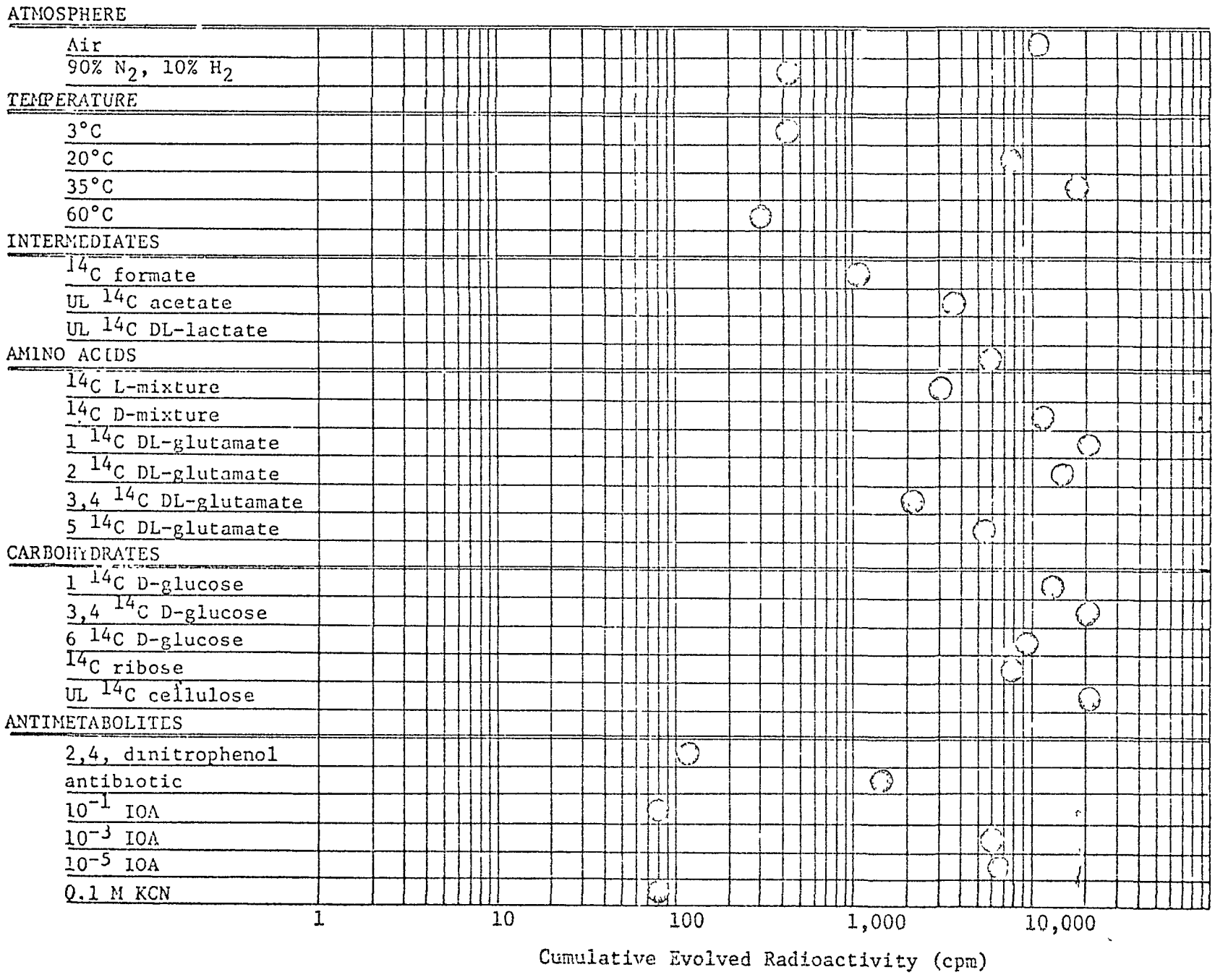
Soil or Organism Aiken Soil Isolate #1

Incubation Time 6 Hours

Standard Medium VM-I 3.4 uCi/ml



BIOSPHERICS INCORPORATED

Soil or Organism Aiken Soil Isolate #1 Incubation Time 22 Hours

Standard Medium VM-I 3.4 uCi/ml