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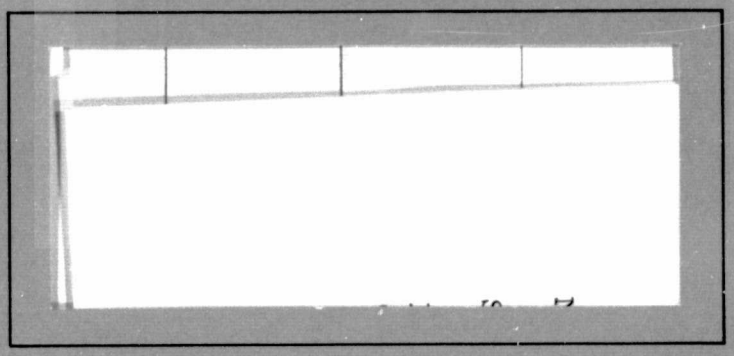

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Report

Annual Report
1974

"Automated Microbial
Metabolism Laboratory"
Contract No. NASW-2280

Prepared for:

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Headquarters
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Prepared by:

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SUMMARY

The Labeled Release concept has been advanced to accommodate a post-Viking mission designed to extend the search, to confirm the presence of, and to characterize any Martian life found. In addition, it is to obtain preliminary information on control of the life detected. The Advanced Labeled Release concept utilizes four test chambers, each of which contains either an active or heat sterilized sample of the Martian soil. A variety of ^{14}C labeled organic substrates can be added sequentially to each soil sample and the resulting evolved radioactive gas monitored. The concept can also be expanded to test effects of various inhibitors and environmental parameters on the experimental response. The current Viking '75 Labeled Release hardware is readily adaptable to the Advanced Labeled Release concept.

Theoretical considerations are given to the choice of parameters most appropriate for delineation of Martian life by this approach. Substrate selection is based on those organics which may be present on Mars, organics which are utilized by primitive terrestrial organisms, substrates which are key intermediates in terrestrial metabolism, and substrate stability. Of those compounds selected, DL isomerism and label positions are

rotated as appropriate. Similarly, inhibitor selection is directed toward further delineation of biochemical mechanisms as well as possible control. Environmental parameters are also varied to determine both optimal conditions and ranges of tolerance.

From the list of substrates, inhibitors and environmental parameters generated from theoretical considerations, those appropriate for a demonstration of the concept with terrestrial organisms have been utilized. The experimental results confirm the feasibility of the chamber design and experimental sequence. Using candidate substrates, experiments are reported which demonstrate the success of the multiple addition mode. Further, substrates in which DL isomerization and label positions are rotated confirm known terrestrial pathways. The effects of atmospheric composition, temperature and inhibitors are also readily demonstrated.

Although the Advanced Labeled Release concept is highly versatile as a second order life detection and characterization test, difficulties encountered in automating biochemical experiments have prompted the consideration of returning a sample of the Martian soil to Earth where it can be examined under the protection of a quarantine facility. Such a direct examination would permit

maximum scientific value to be realized. In anticipation of a Mars return sample mission, we have examined its advisability from the viewpoints of mission justifications, hazards, and risks. Anticipated engineering problems as well as problems associated with obtaining unanimous approval from the scientific and lay communities are discussed.

Strong precautions are recommended for early return of a Mars sample. Ideally, the Martian soil should be fully characterized prior to a return. For an early return, precautionary measures could include return of either soil sterilization or a viable sample with full capability of assessing the hazard and sterilizing if necessary. In either case, the sample should be under rigorous quarantine control.

Preparatory to the return of a sterile sample, means should be developed for the extraction of biologically significant information from such sterile soils. As one approach, experiments are reported in which enzymatic activity is sought following soil sterilization by heat. The preliminary results indicate that soil does not afford protection to enzymatic activity during minimal heat sterilization. This conclusion is in general agreement with the results of other investigators who have reported varying degrees of destruction of biological and chemical information even with mild sterilization procedures.

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I. INTRODUCTION

A follow-on Viking mission to Mars should provide not only a first order life detection test but also a second order experiment by delineating some of the characteristics of that life. One of our approaches to this problem is to search for a carbon-based Martian biochemistry by providing a series of ^{14}C labeled substrates and monitoring the subsequent evolution of radioactive gas. By appropriate choice of substrates one should be able to do preliminary interplanetary comparative biochemistry. Inhibitors can also be utilized in conjunction with substrates for further comparative research and to provide baseline information regarding control of a future return Mars sample.

The concept proposed to accomplish this consists of multiple additions of substrates to the same soil sample. A major advantage of this approach is that it makes maximum use of existing technology by expanding the Labeled Release Experiment of the Viking '75 mission to Mars. In the current program, we envision expansion of the multiple addition concept by performing many sequential additions to the same soil sample, thus introducing the capability for rapid sequential comparison of the effectiveness of a broad spectrum of single substrates or inhibitors. This test is considerably more flexible than the Viking '75 experiment which provides the soil sample with only one mixture of several substrates.

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To obtain maximum information utilizing a minimum of tests with the Advanced Labeled Release concept, it is necessary to make a judicious selection of substrates and inhibitors. This report discusses the theoretical basis for the selection of substrates, inhibitors, and environmental conditions for a future Martian experiment. However, these conditions are not entirely appropriate for a feasibility demonstration using terrestrial organisms. Thus, modification of the Martian test was made to permit a laboratory demonstration of the concept. The results of this demonstration are presented in the second section of this report.

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II. ADVANCED LABELED RELEASE

A. Theoretical Grounds for Selection of Substrates, Inhibitors, and Environmental Conditions

1. Rationale for Substrate Selection

Candidate substrates for an Advanced Labeled Release Experiment include organics possibly present on the surface of Mars and key organic compounds serving as substrates for primitive terrestrial organisms, particularly those at intersections or branching points of biochemical pathways. Selected substrates offer additional flexibility by rotation of the label position, isomeric configuration, or order of addition. Some limitations are imposed on the selection, however, by stability requirements to terminal heat sterilization and long-term storage. Further, contact of selected substrates with sterile soils must not produce interfering levels of noise. Based on these arguments, a recommended list of candidates is developed.

With regard to the organics possibly present on Mars, the "General Theory of Biology" is the biologists explanation of the phenomenon of life. The theory establishes a logical continuum of increasingly complex development from elements, to simple compounds, to life precursors, to single-celled living organisms, and finally to differentiated organisms. Such a continuum avoids the necessity for explaining

the existence of life through some unique event which would constitute a sharp discontinuity in our developing understanding of the universe. The direct consequence of a General Theory of Biology is that life will evolve independently on countless planets which lie in "life zones" with respect to their stars and which experience the appropriate environmental history. If the theory is correct, then it is logical that organics present on the surface of Mars may well be life precursors for Martian life, whether or not it has already developed. If it has developed, such organics may serve as substrates for its continued existence. This concept, then, provides one basis for selection of substrates with which to challenge Martian soil. Information relevant to those substrates possibly present on the Martian surface is derived from the following sources:

a. Chemical Evolution Studies in Reducing Atmospheres

Chemical synthesis of biologically relevant organic molecules has been achieved (1) and verified in many laboratories by subjecting appropriate gaseous mixtures to electric discharges, ultraviolet radiation and even shock waves (for a comprehensive review, see ref. 2). Reacting gases have included various mixtures of methane, ammonia, hydrogen, water vapor, carbon monoxide, and carbon dioxide. The early experiments of Miller showed that

high energy radiation of a mixture of CH_4, NH_3, H_2O and H_2 produced glycine, alanine, aspartic acid and alpha-amino-n-butyric acid. Other similar mixtures have also produced additional amino acids, including serine and glutamic acid as well as formaldehyde, formate, and the purine adenine. The addition of H_2S to such a gaseous mixture results in the production of cysteic acid. Hydrocarbons result from irradiation of methane. Since Mars in its early stages following accretion may well have had a reducing atmosphere, the products of such reactions are candidate substrates for Martian organisms.

b. Chemical Evolution Studies in Simulated Mars Atmospheres

Studies as early as 1897 indicated that irradiation of mixtures of carbon dioxide and water resulted in the production of formaldehyde. More recently (3), experiments were performed under a nonreducing gaseous mixture more closely simulating that of the present Mars atmosphere. Thus, when exposed to ultraviolet light, a gaseous mixture consisting of carbon dioxide, carbon monoxide, and trace amounts of water vapor produced acetaldehyde, formaldehyde, and glycolic acid. These organics, then, may be present on the Martian surface and available as substrates for Martian organisms.

c. Influx of Meteorites and Carbonaceous Chondrites

Organic precursors formed on the surface of Mars are undoubtedly supplemented by the infall of carbonaceous chondrites. These are known to bombard the surface of Mars with greater frequency than on Earth and to contain a variety of organic compounds. The amino acids identified in the Murchinsen meteorite, for example, are glycine, alanine, valine, proline, glutamic acid, and aspartic acid as well as several not found in proteins, such as sarcosine, 2-methyl alanine, and alpha-amino-n-butyric acid. A similar content has been identified in the Murray meteorite. Both D and L isomers have been identified for many of the amino acids. In addition to amino acids, other organic compounds identified in meteoritic material include a variety of hydrocarbons, especially saturated alkanes. It is of interest to note that most of the chondritic organic matter can be accounted for by Fischer-Tropsch type reactions in the solar nebula (4), a finding which supports an early atmosphere consisting minimally of hydrogen, carbon monoxide, and carbon dioxide.

d. Viking '75 Organic Analyses Results

Viking '75 results from mass spectrometric analysis of organics present on the surface of Mars will provide information most appropriate for the selection of substrates for an Advanced

Labeled Release Experiment. Sufficient latitude should exist in any follow-on Viking mission to encompass these findings. From these considerations, then, possible substrates which may be or have been available on the Martian surface for utilization by Martian organisms are the amino acids, glycine, alanine, valine, proline, aspartate, and glutamate, and, also, formaldehyde, acetaldehyde, glycolic acid, formic acid, adenine, and a variety of hydrocarbons. These, then, become candidates for a second order Labeled Release Experiment on Mars.

e. Organic Substrates for Primitive Terrestrial Organisms

If the origin of life on Mars proceeded under atmospheric conditions similar to those thought to have occurred on Earth, then it is not unreasonable that primeval Martian life may have biochemically resembled some primeval terrestrial organisms. However, environmental differences now existing between the two planets suggest that advanced Martian forms may have evolved in a different direction. While possibly true, we wish to point out that advanced terrestrial life forms have not evolved to the exclusion of primitive life forms. Well-adapted primitive bacterial types have persisted. Thus, it is reasonable to assume that certain primitive Martian types may

still co-exist with advanced life forms. That such primitive types may indeed have adapted to current Martian conditions is suggested by the fact that bacterial adaptations to hostile terrestrial environments are often accomplished not only by advanced organisms but also by primitive sulfate reducers, methane bacteria, and blue-green algae. Thus, since primitive terrestrial bacteria are possible models for primitive Martian types, their metabolic requirements should be taken into account in substrate selection for Advanced Labeled Release.

On primeval Earth, the principle mode of metabolism of the first life forms is thought to have been anaerobic fermentation of abiogenically formed organic compounds. Current theory holds that as these heterotrophs gradually consumed the available organic energy sources, autotrophs appeared. The most likely primitive terrestrial organisms are the anaerobic fermenters, respirers, and photosynthesizers, none of which evolve or utilize oxygen. Clostridia, sulfate reducers, methane bacteria, and photosynthetic bacteria are especially interesting. Oxygen-evolving blue-green algae are also considered primitive, although they probably appeared somewhat later. The particular bacteria of interest are described below along with some of the evidence classifying them as primitive. One other terrestrial

bacteria with a biochemistry perhaps suited to present Martian atmospheric conditions is also listed although it is considered somewhat more advanced. Because of the possible role of such organisms in the development of Martian life, their organic substrates are noted as candidates for Advanced Labeled Release.

1). Clostridia - This genus was classified primitive by a recent structural study (5) of bacterial ferredoxins. Ferredoxins are primitive metalloproteins, believed to have played a significant role in the development of fermentative bacteria. Clostridia contain the simpliest known ferredoxin with a molecular weight of about 6,000. The simplicity of structure is compatible with spontaneous formation under primitive conditions. Spontaneous polymerization of activated amino acids using a montmorillonite clay catalyst has produced polypeptides up to 4,000 in molecular weight (6). The ferredoxin active site is thought to contain only iron and sulfur, both of which spontaneously add to the polypeptide chain under anaerobic conditions. The six amino acids found in the Murchison meteorite constitute 64 percent of the amino acid content of ferredoxin. Such evidence dates Clostridia early in the evolutionary scheme.

Clostridia anaerobically ferment a wide variety of organic substrates including soluble sugars, amino acids, glutamic acid, ethanol, acetic acid, starch, pectin, cellulose, and protein. This anaerobic respirer has, however, been reported (7) to function as an autotroph, deriving energy by the oxidation of molecular hydrogen utilizing carbon dioxide as sole carbon source according to the following oxidative scheme:

$$H_2 + CO_2 \longrightarrow CH_3COOH$$

This suggests to us that the earliest autotrophs may have been early heterotrophs which simply evolved alternate biochemical pathways to obtain energy and carbon. This hypothesis provides a logical continuum between heterotrophs and autotrophs.

2). Sulfate Reducers - Evidence for the early existence of Desulfovibrio, an anaerobic sulfate-reducing heterotroph, has been obtained from isotopic ratios of sulfur (8). Desulfovibrio has been reported to fractionate ^{32}S and ^{34}S during sulfate reduction, preferentially utilizing ^{32}S. Since rocks formed 2 - 3.5 billion years ago are enriched in their ^{32}S content over that in juvenile sulfur found in meteorites and volcanoes, the early existence of sulfate reducers is implied. Three and one-half billion years is

the age given the oldest sediments and the approximate appearance of life forms (9 , 10). Sulfate reducers utilize lactic acid as an organic substrate although, like Clostridia, they have been reported to function autotrophically by deriving energy through the following schematic oxidation of molecular hydrogen (7):

$$H_2 + SO_4 \longrightarrow H_2S$$

3). Methane Bacteria - Methane-evolving bacteria are similarly classified primitive because they anaerobically, like Clostridia and Desulfovibrio, derive energy by the oxidation of molecular hydrogen using carbon dioxide as sole carbon source:

$$H_2 + CO_2 \longrightarrow CH_4$$

As heterotrophs, they utilize simple substrates, such as short-chain fatty acids (formate, acetate, propionate, butyrate, valerate, and caproate) and some alcohols (methanol, ethanol, primary and secondary alcohols). These substrates closely resemble those compounds formed by abiogenic processes as well as those found in carbonaceous chondrites. Their association with petroleum in deep oil wells implies their early ability to have utilized hydrocarbons (11). It has been postulated (11) that the primordial soup resembled a fuel, consisting primarily of hydrocarbons.

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4). Photosynthetic Bacteria - Terrestrial anaerobic autotrophs which do not evolve oxygen are the photosynthetic purple sulfur bacteria and green bacteria. Their biochemical requirements suggest their adaptability to primeval conditions. On the basis of structural relationships of ferredoxins (5), it has been proposed that evolution proceeded as follows: anaerobic fermentative bacteria $\longrightarrow$ green photosynthetic bacteria $\longrightarrow$ red photosynthetic bacteria $\longrightarrow$ algae $\longrightarrow$ higher plants. Other evidence that bacterial photosynthesis is primitive and preceeded algal and plant oxygen-evolving photosynthesis is derived from a recent study (12) of Precambrian stromatolites in Yellowstone National Park. These early stromatolites appear to result from photosynthetic bacteria, implying that bacterial photosynthesis preceeded algal oxygen-evolving photosynthesis and that oxygen may not have appeared until later than previously supposed. Purple sulfur bacteria capture the sun's energy and utilize H_2S as an electron donor according to the following equation:

$$CO_2 + H_2S + H_2O \longrightarrow (CH_2O) + H_2SO_4$$

This reaction is directly analogous to the reaction conducted by green plants. As phototrophs, these bacteria predominantly utilize inorganic substrates although they are known to anaerobically

decompose proteins and to metabolize simple organic substrates in the dark.

5). Blue-Green Algae - While these algae are considered the most primitive of oxygen-evolving photosynthetic organisms, oxygen evolution appears to be a later evolutionary development. Thus, these bacteria are more advanced than those discussed above, a conclusion supported by structural ferredoxin studies (5). The prime reaction of these phototrophic organisms is with inorganic substrates. However, they are capable of utilizing simple organic substrates in dark reactions and are thus detectable by a Labeled Release Experiment.

6). Bacillus oligocarbophilus - These and similar terrestrial autotrophic microorganisms make interesting speculations for Martian biochemistry since they derive energy through the oxidation of CO to CO_2. It has been postulated (13, 14) that such a pathway is not impossible for chemosynthetic Martian organisms and could be coupled to sulfur reduction as has been reported to occur in cell-free extracts of Desulfovibrio (15). Organic substrates utilized by these organisms are formic acid, formaldehyde, acetone, and butyric acid.

In summary, if primitive terrestrial bacteria are representative of primitive Martian microorganisms, then anaerobic

fermenters, respirers, and photosynthesizers derserve special attention as candidate Martian prototypes. In these categories, Clostridia, sulfate reducers, and methane bacteria are primitive heterotrophs readily detected by labeled release-type experiments. Photosynthetic bacteria and blue-green algae, while detectable by labeled release techniques, are of lesser interest. Bacillus oligocarbophilus, although not as primitive, has an attractive biochemistry for Martian atmospheric conditions.

The substrates of interest for these four bacterial types, then, are sugars, amino acids, formate, acetate, butyrate, lactate, glutamate, formaldehyde, acetone and ethanol. Other possible substrates include higher molecular weight compounds such as starch, pectin, cellulose, and proteins. These, then, are prime candidate substrates for Advanced Labeled Release.

f. Key Intermediates and Complexity Levels in Terrestrial Metabolism

Another approach for the selection of substrates for an Advanced Labeled Release Experiment is to select key intermediates in terrestrial metabolism. By appropriate selection of such intermediates, it should be possible to determine whether or not similar metabolic pathways are extant in Martian organisms.

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To select such substrates, one can consider various metabolic pathways of terrestrial metabolism:

1). Degradation of High Molecular Weight Molecules

The breakdown of carbohydrates to simple sugars, proteins to amino acids, fats to glycerol and fatty acids, and nucleic acids to purines and pyrimidines occurs in all living systems. Although not accompanied by the evolution of CO_2, subsequent metabolism of these monomer products results in the evolution of CO_2.

2). Breakdown of Simple Sugars

The Embden-Meyerhoff Pathway is the main pathway for the anaerobic breakdown of hexoses to pyruvate via a series of phosphorylated 3-carbon intermediates. Two alternate pathways for the conversion of glucose to pyruvate are also known. One, the Hexose Monophosphate Shunt, proceeds via a series of 3- to 7-carbon phosphorylated sugars with the ultimate complete oxidation of glucose to CO_2. The other, the Entner-Doudoroff Pathway, proceeds via gluconic acid. Glyceraldehyde-3-phosphate is an intermediate in all three pathways.

3). Subsequent Metabolism of Pyruvate

Pyruvate occupies a key position in terrestrial

metabolism, participating in several subsequent pathways. Pyruvate is converted to ethanol via acetaldehyde in alcoholic fermentation and to lactic acid via mammalian glycolysis. Lactic acid is also produced in lactic acid bacteria by a sequence of reactions identical to those observed in muscle. Pyruvic acid may also undergo reversible reduction to lactic acid, be converted back to glucose, reversibly form oxaloacetic or malic acids, or be reversibly transaminated to alanine. Still other organisms convert pyruvic acid to acetoacetic acid via acetaldehyde and acetic acid. Subsequent pathways of acetoacetic acid result in the formation either of butyric acid or isopropyl alcohol and CO_2. However, the major fate of pyruvate in most mammalian cells is its oxidation to CO_2 and acetyl coenzyme A.

4). Aerobic Breakdown of Carbohydrates

In the presence of oxygen, carbohydrates are converted to pyruvate and subsequently to CO_2 and H_2O via Krebbs tricarboxylic acid or citric acid cycle. Here, pyruvate is first converted to acetyl CoA which combines with oxaloacetate to form citrate. Citrate is then in turn converted to cis-aconitate, isocitrate, α-ketoglutarate, succinate, fumarate,

malate, and finally again to oxaloacetate, thereby completing the cycle in a series of energy producing reactions. Each turn of the cycle results in the loss of 2 CO_2 molecules. In addition to pyruvate and acetyl CoA, oxaloacetic acid and alpha ketoglutaric acid are in key positions since they are also formed from several side reactions. Acetyl CoA is derived from fatty acids, leucine, tyrosine, and phenylalanine. Transamination of alanine, glutamic acid and aspartic acid results in production of pyruvate, α-keto-glutarate, and oxaloacetate, respectively.

5). Other Metabolic Conversions of Acids of the Citric Acid Cycle

Several other pathways exist for metabolic conversions of citric acid cycle intermediates. Of these, the most important is the glyoxylic acid cycle found in plants and microorganisms. Here,acetate, presumably via acetyl CoA, condenses with glyoxylic acid to form malic acid. This reaction is analogous to the condensation of acetyl CoA with oxaloacetic acid to form citric acid. The glyoxylic acid cycle then involves conversion of malate to oxaloacetic acid, citrate, and isocitrate which then converts to succinate plus glyoxylate. Glyoxylate recycles whereas moles of succinate are subsequently oxidized to glucose=6-phosphate and 2 moles of CO_2. Glyoxylic acid is formed

either from glycine or glycolic acid and, in some organisms, is broken down first to oxylic acid and then to formic acid.

6). Purines and Pyrimidines

Purines may be oxidized to uric acid and either excreted or further broken down to glyoxylic acid and urea. In some microorganisms, the glyoxylic acid is converted to oxalic acid whereas urea is hydrolyzed to CO_2 and NH_3. Some anaerobic Clostridia use purines as sole carbon and nitrogen source, deriving energy from their fermentation to NH_3, CO_2, formate, acetate, and glycine. Pyrimidines break down to their respective beta amino acids which are in turn further degraded to CO_2 and other products.

From this discussion, several compounds become immediately apparent as occupying key positions in terrestrial metabolism, namely, pyruvate, acetyl CoA, oxaloacetic acid, and α-ketoglutarate. These then, as well as the compounds from which they are derived, become prime substrate candidates for Advanced Labeled Release. In addition, for more specialized microbial metabolism, acetate, glyoxylic acid and their precursors, as well as several purines and pyrimidines are of interest. The candidates derived from this type of analysis, then, are summarized below:

Pyruvate (hexose, alanine, glycine, lactate, glycolic, acid)

Acetyl CoA (pyruvate, fatty acids, acetate, leucine, tyrosine, phenylalanine)

Oxaloacetate (aspartate, tyrosine)

α-Ketoglutarate (glutamic acid, arginine)

Acetate (pyruvate, acetaldehyde)

Glyoxylic acid (glycine, glycolic acid)

Uric acid (xanthine, urea)

Uracil (thymine, orotic acid)

Comparison of gas evolution responses from a wide variety of these compounds should establish the degree of similarity of Martian to terrestrial metabolism. In addition, utilization of high molecular weight proteins, carbohydrates, fatty acids,or nucleic acids with subsequent gas evolution could serve to indicate further similarities and differences in ability to degrade such substances. This in turn implies whether or not such substances are present in Martian organisms since, in all terrestrial organisms, enzyme systems exist for continual turnover (synthesis and degradation) of most high molecular weight material.

g. Additional Considerations

Once a substrate has been selected for study, it is important to consider internal variations of structure to obtain maximum information. Two possibilities are immediately apparent:

rotation of the position of the radioactive carbon and utilization of both D and L isomers for optically active compounds. The types of information to be attained from each approach is outlined below:

1). Rotation of Radioactive Label - If a Labeled Release Experiment is to be conducted with only one substrate or group of substrates, then each carbon should be uniformly labeled to optimize chances of detecting metabolism. However, if a series of substrates can be added sequentially, then the opportunity exists to rotate that label to specific positions. This becomes a powerful tool for pathway delineation and comparison to terrestrial metabolism. For example, specific labels have successfully been utilized to distinguish pathways for glucose utilization. Thus, the carboxyl group of pyruvate, which is subsequently lost upon conversion to acetyl CoA, is derived from carbons #3 and #4 of glucose in the glycolytic pathway,but from carbons #1 and #4 in the Entner-Doudoroff Pathway. Similar examples can be cited for other candidate substrates showing selective preference for a particular carbon in a metabolic scheme.

2.) Comparison of D and L Isomers - Terrestrial systems have developed a stereospecificity for L-amino acids and D-sugars to be selectively incorporated into high molecular weight proteins and carbohydrates. However, although their occurrence is

rare, D-amino acids and L-sugars are found in nature. D-amino acids are found in peptide links in antibiotics of some microorganisms. Bacillis brevis and Bacillis polymyxa, for example, respectively produce gramicidin and polymyxin, both of which contain D-leucine, L-galactose has been isolated from plants and from the molluscan polysaccharide galactogen. In addition to the rare incorporation into higher molecular weight material, D-amino acids and L-sugars are also catabolized by many organisms which possess isomerases capable of interconversion of L and D forms. Thus, both isomeric forms of sugars or amino acids can result in the subsequent evolution of CO_2. Terrestrially, then, a stereospecificity may only be visible upon hydrolysis of high molecular weight material rather than in catabolism although it is possible that different utilization rates exist for the two isomeric forms. L-glucose in man, for example, is catabolized to the exclusion of D-glucose. If differences in rate of utilization of isomers occurs in Martian organisms, then an Advanced Labeled Release Experiment could determine the stereospecificity of Martian organisms by comparing D and L isomers sequentially. However, equal utilization does not mean that stereospecificity does not exist at the polymer level.

In summary, each substrate selected for study offers the opportunity for examining: a) mechanistic pathway by rotation

of label position, and b) Martian stereospecificity by comparison of optical isomers.

3). Stability to Heat Sterilization and Long-Term Storage

One prime difficulty in selection of organic substrates is the spacecraft quarantine requirement. Thus, the Viking '75 Labeled Release nutrient will be exposed to at least three heat sterilization treatments, each of which consists of a 40-hour exposure to 113°C. Not all organic compounds can withstand such severe treatment although stability is considerably enhanced by anaerobic conditions during heating. Compounds such as glucose and hexoses carmelize whereas aldehydes lacking an alpha hydrogen (formaldehyde) permute to their corresponding acid plus alcohol in air or anaerobically. Formaldehyde and acetaldehyde furthermore readily form low molecular weight material (dimers, trimers, etc. on heating). This eliminates these compounds as candidates for Advanced Labeled Release unless alternate sterilization techniques can be utilized.

Following heat sterilization, substrates must survive long-term storage during transit to Mars plus additional storage prior to launch, a total storage time of 12 to 18 months before use. Assuming heat stability, the major factor affecting long-term stability is beta disintegration of ^{14}C labeled compounds with subsequent radiolysis of water molecules to produce reactive free hydroxyl radicals.

These, in turn, cause substrate decomposition by decarboxylation or other mechanisms. However, provided the radioactive concentration is maintained sufficiently low (less than 30 μCi/ml), no more than 10 - 15% decomposition is anticipated per 24 months.

In summary, before final selection of substrates for an Advanced Labeled Release Experiment, it is imperative that each be completely tested for stability to heat and long-term storage.

4). Nonbiological Reactions with Sterile Soils

Organic substrates added to heat sterilized soil frequently produce nonbiological evolution of radioactive gas. This response is generally minimal, totaling only slightly more than occurs in the absence of soil and usually totaling no more than 1% of the biological response obtained from the corresponding active soil. However, certain substrates react in the presence of soil to produce a high nonbiological response. Acetate and formate, for example, have been observed to react with heat sterilized soil of low pH (less than pH 5.0). Neither reaction occurs with neutral or basic soil. Pyruvate reacts strongly with all sterile soils independent of soil pH. Although the mechanisms of these responses have not been thoroughly investigated, the data emphasize the

importance of examining all selected substrates for the absence of such nonbiological responses that could mask biological responses. If such responses are severe, then the substrate must either be eliminated or appropriate conditions provided (i.e., buffering capacity, anaerobiosis, or new soil sterilization method) to eliminate the response.

5.) Miscellaneous Considerations

Several additional minor factors must be considered in the final selection of candidate substrates for an Advanced Labeled Release Experiment. For example, water solubility is a criterion that eliminates hydrocarbons and fatty acids of molecular weight higher than butyric. Conditions for optimal stability also involve selection of appropriate pH. Carboxylic acids are generally more stable as the salt than as the free acid. Oxalic acid, for example, readily decomposes at pH 4.5 whereas lactic acid forms dimers at low pH. Finally, in selecting a substrate series, the series must be sufficiently broad in scope, preferentially utilizing several classes of substrates, including at least one sugar or sugar derivative, one fatty acid, and both isomers of one amino acid. These in turn should span the different types of rationale justifying substrate selection. Further, any effects of the order of addition must be fully understood.

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2. Prime Substrates for an Advanced Labeled Release Experiment on Mars

From the preceeding discussion, it is obvious that many factors must be considered before a particular substrate can qualify as a candidate for the Advanced Labeled Release Experiment. Table 1 summarizes those substrates selected on the basis of potential presence on Mars, utilization by primitive terrestrial organisms, or key role in terrestrial metabolism. As indicated, several substrates satisfy two or more of these categories, making them prime substrate candidates. Priority numbers are thus assigned to these substrates depending on the number of categories satisfied by the particular substrate.

Following the priority assignment for selected substrates, the suitability of the substrate is examined from the viewpoints of heat stability, nonbiological noise with sterile soils, water solubility and high vapor pressure. If any of these criteria are known to be problems with a particular substrate, that substrate is eliminated, as indicated. It should be noted, however, that not all of the remaining substrates have been tested for heat stability or nonbiological noise. Thus, acceptance and recommendation of a particular substrate must be considered tentative, pending future examination of suitability.

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Table 1

Selection of Candidate Substrates for an Advanced Labeled Release Martian Test

Compound	Basis for Compound Selection						Basis for Compound Elimination			Priority Assignment*
	Could be Present on Mars			Substrate for Primitive Bacteria	Key Terrestrial Role		Unstable to Heat or Storage	Non-Biol Noise	Not Water Soluble or High Vapor Pressure	
	Product of Reduced Gases	Product of Current Mars Gases	Found in Carbonaceous Chondrites		Key Role	Precursor for key				
Amino Acids										
Glycine	x		x	x		x				1
Alanine	x		x	x		x				1
Aspartic	x		x	x		x				1
Glutamic	x		x	x		x				1
Valine			x							3
Serine	x									3
Proline			x							3
Leucine						x				3
Tyrosine						x				3
Phenylalanine						x				3
Arginine						x				3
α-Amino Butyric	x									3
Cysteine	x									3
Aldehydes, Ketones, and Alcohols										
Acetone				x					x	E
Acetaldehyde		x				x	x		x	E
Formaldehyde	x	x		x			x		x	E
Methanol				x					x	E
Ethanol				x					x	E

*Lowest Number = highest priority

E = eliminated

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Table 1 (continued)

Selection of Candidate Substrates for an Advanced Labeled Release Martian Test

Compound	Basis for Compound Selection						Basis for Compound Elimination			Priority * Assignment
	Could be Present on Mars			Substrate for Primitive Bacteria	Key Terrestrial Role		Unstable to Heat or Storage	Non-Biol Noise	Not Water Soluble or High Vapor Pressure	
	Product of Reduced Gases	Product of Current Mars Gases	Found in Carbonaceous Chondrites		Key Role	Precursor for key				
Fatty Acids										
Formate	x			x				?		2
Acetate				x	x	x		?		2
Acetyl CoA					x	x	x			E
Propionate				x						3
Butyrate				x					x	E
Valerate				x					x	E
Caproate				x					x	E
Other Acids										
Pyruvate					x	x		x		E
Lactate				x		x				2
Gluconic						x				3
Glycolic		x				x				2
Glyoxalic					x					3
Oxaloacetate					x					3
-Ketoglutarate					x					3
Purines, Pyrimidines										
Adenine	x					x				2
Uric Acid				x		x				2
Xanthine				x		x				
Uracil						x				3
Thymine						x				3
Orotic Acid						x				3

*Lowest Number = highest priority

E = eliminated

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Table 1 (continued)

Selection of Candidate Substrates for an Advanced Labeled Release Martian Test

Compound	Basis for Compound Selection						Basis for Compound Elimination			Priority* Assignment
	Could be Present on Mars			Substrate for Primitive Bacteria	Key Terrestrial Role		Unstable to Heat or Storage	Non-Biol Noise	Not Water Soluble or High Vapor Pressure	
	Product of Reduced Gases	Product of Current Mars Gases	Found in Carbonaceous Chondrites		Key Role	Precursor for key				
Polymers										
Starch				x		x	x			4
Protein				x		x	x			4
Cellulose				x		x	x			4
Miscellaneous										
Glucose						x	x			E
Hydrocarbons	x		x	x					x	E
Urea				x		x				2

*Lowest Number = highest priority
E = eliminated

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From the final list of candidates in Table 1, those with the highest priority assignment (priority #1) are the amino acids glycine, alanine, aspartate, and glutamate. Other compounds also highly favored (priority #2) are acetate, formate, lactate, glycolic acid, adenine, uric acid, and xanthine. The remaining compounds (priority #3) are a variety of additional amino acids, urea, propionate, gluconic acid, glyoxalic acid, oxaloacetate, α-ketoglutarate, uracil, thymine, and orotic acid. Several of these compounds are optically active and can be used for determination of stereoisomeric specificity. All (except formate and urea) can be utilized for label rotation studies although the amino acids, acetate, lactate, oxaloacetate and α-ketoglutarate are most highly recommended for such a study. Final selection of candidates, however, depends on suitability studies.

Finally, polymeric material such as starch, cellulose, and protein could be utilized in an Advanced Labeled Release Experiment. While it is recognized that these polymers are heat sensitive, it should be noted that heating destroys polymeric function by disrupting tertiary structure. However, it may not affect ability of microorganisms to decompose the primary structure of these polymers. Thus, while not considered prime candidates, these polymers could make interesting substrates, especially if alternates to heat sterilization are devised.

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3. Rationale for Inhibitor Selection

Once a labeled release response has been obtained by utilization of a particular substrate or group of substrates, additional information can be gained by examining the sensitivity of that response to inhibitors added prior to or simultaneous with the substrate. This type of study can also provide important preliminary information regarding future control of a return Mars sample.

With pure enzyme systems, inhibitors are a classical means of revealing mechanisms, structure, and biochemical pathways. However, some of the disadvantages of working with soils are the heterogeneity of their constituent microbial populations and the soil nonbiological constituents which may react with the inhibitors. With a Mars soil sample, there is the additional complication of the unknown basic nature of the biochemical components. These problems minimize the diagnostic value of a particular inhibitor.

Difficulties also exist with selection of specific inhibitors to determine mechanisms and structure. For example, competitive inhibitors are often so highly specific that many may have to be tested before achieving inhibition of an unknown multicomponent system. Alternatively, other inhibitors have multiple action, thereby requiring supplementary studies with several other inhibitors

to ascertain the particular mechanism. For example, several inhibitors would be required to determine whether the mechanism of cyanide inhibition was its action as a metal chelator or as a sulfhydryl agent. Although such correlative studies would be quite informative in a pure system, their value is limited here because it would not be known whether sequential inhibitors were operating at the same site. Finally, even within a particular class of inhibitors, as, for example metal chelators, some may inhibit and others may not, depending both on specificity and on affinity for the catalytic site.

These problems make the selection of a limited number of inhibitors difficult. In order to minimize the number of inhibitors to be studied, we recommend grouping inhibitors of similar function and testing a variety of group types collectively. While the information obtained in this fashion will be less specific, the approach both minimizes the number of required tests and maximizes the probability of obtaining an inhibitor. Thus, it appears to be the most practical means of obtaining preliminary information. Following is a discussion of the various functional inhibitor categories and those inhibitors recommended.

a. Respiratory Poisons and Metal Chelators

Cyanide, azide, hydrogen sulfide and carbon monoxide inactivate heavy metal catalysts by forming highly stable

complexes with the metal. They inhibit many, although not all, enzymes which contain iron or copper as part of their active site and are classical inhibitors of respiratory enzymes such as cytochrome oxidase. However, they are not particularly specific since they inhibit a large number of enzymes which do not involve metal catalysis. Cyanide and versene (EDTA = ethylenediaminetetra-acetate) have been considered the standard inhibitors for detecting metal catalysis in general. Other less frequently studied chelators are α, α'-dipyridyl, o-phenanthroline, salicylaldoxime, 8-hydroxyquinoline, and diethyldithiocarbamate. Many other compounds also have chelation properties, including buffers such as pyrophosphate, glycylglycine, and TRIS and even such compounds as ATP, citrate, and oxalate. A combination of cyanide, azide, and versene (which are the most powerful, stable and water soluble chelators in this group) appears most suitable for a Labeled Release inhibitor study. Hydrogen cyanide and carbon monoxide, being gases, are not readily adaptable, although carbon monoxide is recommended for atmospheric studies (see next Section).

One difficulty with studying inhibition by metal chelation is the interaction of the chelator with nonbiological soil metals, effectively removing the inhibitor from interaction with the biological system. For this reason, metal chelators should be

added to soils in concentrations higher than normally utilized for pure cultures.

b. Heavy Metals

Salts of heavy metals, such as silver, copper, mercury, lead, cadmium, and zinc inactivate most enzymes in high concentrations. In fact, these agents act as general protein precipitants. The mechanism of inactivation is unknown although it may involve interaction with thiol or carboxyl groups. Usually the inhibition is reversed by addition of metal-complexing agents such as cyanide and versene (EDTA). These six heavy metals can readily be combined to their solubility limit to form an effective biological inhibitor group for an Advanced Labeled Release Experiment.

c. Reagents for Thiol Groups

Three classes of agents are known to react with thiol groups of enzymes depending on the location of the group and whether it is a free sulfhydryl or a disulfide group. In the first class are the alkylating reagents such as iodoacetate (IAA), ethyl iodoacetate and iodoacetamide which irreversibly alkylate unmasked sulfhydryl groups. The second category contains organic compounds of mercury or arsenic which reversibly form mercaptides with thiol groups of both masked and unmasked sulfhydryls.

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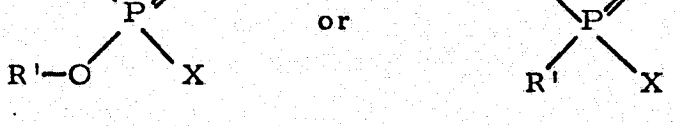
Most widely used of these compounds is para-chloromercuribenzoate (PCMB). The final class includes thioloxidizing groups such as oxidized glutathione, which reversibly convert thiol groups into disulfides. Reduced glutathione, mercaptoethanol and cysteine, will perform the reverse reaction, namely the conversion of disulfide groups into thiol groups. Ideally, one inhibitor from each of these classes should be combined into one inhibitor package. However, since agents of the first two categories interact with agents of the third category, it is recommended that two inhibitor packages be prepared from this group, one containing PCMB, IAA, and ethyl IAA and the other containing reduced glutathione, mercaptoethanol, and cysteine. Of these two groups, the one containing PCMB, IAA and ethyl IAA should be the more potent inhibitor.

d. Irreversible Organophosphorus Inhibitors

These inhibitors, collectively known as "nerve gases," are highly toxic to the central nervous system and are of the general formula:



Where R and R' are alkyl groups and X is F, CN, or O $C_6H_4NO_2$

They are highly specific inhibitors of enzymes which possess esterase activity, especially cholinesterase and acetylcholinesterase. They also are known to inhibit trypsin, chymotrypsin, thrombin, acetylesterase, and some lipases, all of which possess some esterase activity. A combination of the stronger of these compounds, including diisopropylphosphorofluoridate (DFP) should be a most effective inhibitor should Martian organisms possess enzymes with esterase activity.

e. Competitive Inhibitors

Many competitive inhibitors exist, most of which are limited in their action to one enzyme. Although studying each individually is impractical, a group of such inhibitors designed to compete with several enzymes of a particular pathway would be quite interesting. Krebbs Cycle intermediates are particularly favored for such a study because a specific example exists for most of these enzymes. Malonate, for example, is a classical and powerful competitive inhibitor of succinate dehydrogenase. Fumarase is inhibited by the salts of about a dozen di- and tricarboxylic acids but not by monocarboxylic acids. Fluorocitrate

specifically inhibits aconitase. Thus, a group of such competitive acids could be prepared which would compete with reactions within the Krebbs Cycle or the glyoxylic acid cycle.

f. Other Inhibitors for Distinguishing Carbohydrate Pathways

Classically, certain inhibitors have been used to distinguish pathways of glucose metabolism. The Hexose Monophosphate Shunt, for example, is not blocked by IAA, fluoride, or arsenite whereas glycolysis is sensitive to these inhibitors. Thus, with pure systems or microbial populations, inhibitor sensitivity can establish the particular pathway. However, since we are dealing with an unknown and possibly heterogeneous population and since most of these agents are included in other inhibitor packages, we do not recommend repackaging groups of inhibitors for this type of study.

g. Enzymes as Inhibitors

Enzymes which are capable of reducing macromolecules to monomer units or otherwise destroying their structure are inhibitors which are quite interesting and revealing as a group. These include RNase, DNase, and various snake venoms which destroy nucleic acid structure, and proteinases which hydrolyse

proteins. Lysozyme, which destroys bacterial cell walls, could also be included in this category. Whether or not these enzymes inhibit terrestrial organisms will in part depend on permeability. For Martian organisms, both permeability and macromolecular structure are factors. Although of considerable interest, this is a difficult package to prepare because of group incompatibility (proteinases may destroy other enzyme categories),and instability to heat sterilization,and long-term storage. Special means would have to be examined to overcome these problems.

h. Antibiotics

Inhibition of a Martian response by a group of antibiotics would be a highly significant finding. A grouping of such agents should encompass as broad a spectrum as possible, including bactericidal agents for both gram positive and gram negative organisms as well as fungicidal agents. One recommended grouping contains a mixture of penicillin for gram positive bacteria, streptomycin for both gram positive and gram negative organisms, and the general fungicide "Fungizone" containing amphopericin B.

i. Antibodies

The most highly specific inhibitors known are antibodies prepared against specific enzymes. If antibodies could

be prepared against Martian organisms or macromolecules, they would also be expected to be highly effective. While of intense interest for future experiments, the preparation of such antibodies requires preliminary experiments with Martian soil before its use in an Advanced Labeled Release Experiment. However, this type of experiment should be borne in mind should its performance become practical.

In summary, the inhibitors selected for study in an Advanced Labeled Release Experiment are groups containing functionally similar inhibitors. The designated categories are metal chelators (cyanide, azide, versene), heavy metals (silver, copper, mercury, lead, cadmium, zinc), thiol reagents (PCMB, IAA, ethyl IAA), organophosphorus inhibitors (DFP), competitive inhibitors (Krebbs Cycle intermediates), and antibiotics (penicillin, streptomycin, Fungizone). In addition, if stability and compatibility problems can be solved, an enzyme package (RNase, DNase, snake venoms, proteinases, lysozyme) could provide interesting information. With each inhibitor package, preliminary insights are obtained into the nature of Martian catalytic units and potential control mechanisms of a future return Mars sample. As with substrates, however, final selection of the inhibitors would depend on their stability

to heat sterilization as well as absence of interaction with substrates. For competitive inhibitors, the final selection is also dependent on the final substrate selection.

4. Selection of Environmental Conditions

In selecting environmental conditions to examine for effect on Martian metabolism, two objectives should be considered. First, environmental conditions should be determined to optimize Martian activity. Second, environmental conditions should be determined which may be inhibitory to Martian life. Such information supplements that obtained with water soluble inhibitors and is important in the event that a Mars sample is returned to Earth in a future mission. As a basis for selecting those conditions best suited to meet these objectives, a review of the Martian environment and possible adaptations by Martian organisms was made.

Mariner 9 results (16, 17) have shown Martian surface temperatures ranging from 145°K to about 280°K with diurnal fluctuations of as much as 90°K. Surface pressures in equatorial zones range from about 3 to 8 mb, averaging about 5 mb. The atmosphere consists almost entirely of CO_2, although trace amounts of CO, O_2, O_3, water vapor, and silicon dust have been detected.

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Intense ultraviolet radiation directly strikes the surface of the planet, requiring some protective mechanism on the part of Martian organisms, perhaps analogous to terrestrial enzymatic repair mechanisms. Mariner 9 results (18) have shown that the surface of Mars is covered by sand and dust. Strong winds (19), which continually distribute this material, insure the widespread distribution of microbial heterotrophs and nonphotosynthetic autotrophs. Microbial phototrophs, however, may be absent or restricted to protected environments where the sun's energy is not excluded by the shifting dust. Liquid water has not been detected on Mars although it may exist transitorily whenever a source is available, the temperature is above 273°K, and the surface atmospheric pressure exceeds 6.1 mb. Thus, should Martian life exist, it must have adapted to extreme aridity or become restricted to limited areas of low altitude where liquid water may be transitorily available. Possible adaptive mechanisms may include the ability to increase the internal water concentration over that of the environment, analogous to the "bioaccumulation" phenomenon whereby terrestrial organisms concentrate metal ions (20, 21).

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The environmental parameters readily condusive to study by the labeled release technique are atmospheric composition, temperature, pressure, water, and possibly ultraviolet radiation. Recommendations for variation of each of these parameters is discussed below to obtain maximum information regarding reaction optima and environmental inhibitors of Martian organisms.

a. Atmospheric Composition

Having obtained a response in a Martian atmosphere consisting mainly of CO_2, examination of other atmospheric compositions could reveal essential components of the reaction as well as atmospheric inhibitors. Five gases of high interest are pure CO_2, CO, H_2, O_2, and air. Failure to produce a response with pure CO_2 could indicate that some atmospheric trace constituent in the Martian atmosphere is important for the Martian reaction. Utilization of pure CO, a trace constituent in the Martian atmosphere, could indicate the existence of autotrophic pathways which oxidize CO to CO_2. Since primitive terrestrial organisms such as Clostridia, methane bacteria, and sulfate reducers are capable of utilizing H_2, this gas is also of high interest for a Martian study. In addition, should a response be obtained in the presence of O_2, this could indicate the onset of more highly evolved

forms. If, on the other hand, O_2 is inhibitory to a labeled release response, it indicates a possible form of control for a return Mars sample. Similar conclusions can be deduced using air as the reaction atmosphere although the specific gas(es) responsible for a particular response or lack of response may not be known. These then, become the key gases for study. Additional gases that could be considered are "inert" gases such as N_2 and helium. These could determine whether any gas is a necessity in the reaction and, if so, serve as a control for gas flushing.

b. Temperature

On Viking '75, responses will be obtained from reaction conditions approximating Mars ambient conditions, allowing partial diurnal temperature fluctuations. After this initial study, several isothermal temperatures could be examined by the labeled release technique to determine optimal temperature, thermal death point, and the necessity for diurnal fluctuations. Terrestrially, optimal reaction temperatures are usually in the range of 20° - 37°C, depending on the particular reaction under investigation, although certain thermophiles and psychrophiles are adapted to temperatures well above and below this range. Above the temperature optimum, the reaction rate diminishes as the temperature increases until no

further reaction occurs. Death at high temperatures is associated with protein and nucleic acid denaturation which usually occurs above 50°C. On Mars, native organisms would be adapted to colder temperatures than terrestrial organisms and one would anticipate that both the temperature optima and the thermal death point may be lower than on Earth. If so, this would reinforce the hypothesis that ambient terrestrial conditions would be lethal to Martian organisms. With these considerations in mind, a broad preliminary temperature curve could be selected to include points such as -10°C, +5°C, +20°C, +35°C, and +60°C. These five points would readily establish the general shape of the Martian temperature curve, showing whether the reaction is faster at freezing temperatures and whether the optima and death points occur significantly below terrestrial values.

c. Pressure

With regard to pressure, all Labeled Release experiments should be conducted as close as possible to ambient conditions, to which Martian organisms are undoubtedly best adapted. However, one interesting experiment could readily be performed aimed strictly at determining whether a large pressure increase acts as an inhibitory control mechanism for a return Mars sample. Thus, the pressure of a test cell could be raised to simulate

terrestrial pressures, preferably using air as test gas. On the Viking '75 hardware, for example, opening the test cell for one minute exposure to the pressurized helium source results in a pressure rise from 5 to 250 torr. Thus, this important experiment is readily adaptable to existing hardware.

d. Water

Whereas water is considered a necessity for life processes, as previously discussed, one would expect Martian organisms to be well-adapted to the arid Martian conditions. In fact, it is possible that Martian organisms would "drown" if exposed to vast amounts of liquid water. Not knowing the sensitivity of Martian organisms to water, the Viking '75 experiment has been designed to provide soil with a moisture gradient from wet to dry. The possibility that a wet condition may be lethal to Martian organisms has led to the suggestion that a return Mars sample be returned to an oceanic island or a ship. The Advanced Labeled Release Experiment offers the potential of determining the validity of this hypothesis by varying the amount of liquid substrate placed on the soil. As with pressure, however, one need study only two extremes, one in which a small amount of liquid is in contact with a dry soil, thereby providing a moisture gradient, and one in

which the soil is completely wetted or immersed in the liquid substrate.

e. Ultraviolet Radiation

Labeled Release Experiments are conducted in a test cell wherein organisms are protected from the intense ambient UV radiation. If a test cell could be adapted to optionally receive such radiation, ultraviolet sensitivity could be determined. It would be of considerable interest to determine whether protection of Martian organisms is afforded by external (protected niche, atmospheric dust, etc.) or internal (protective shield, repair enzymes, etc.) means. As with pressure and water, only two points need be considered - namely, the responses in both the presence and absence of ambient ultraviolet radiation.

In summary, variation of environmental parameters can provide considerable information relative to interplanetary comparative biochemistry as well as control of a return Mars sample. Variations of atmospheric composition, temperature, pressure, water, and possibly ultraviolet radiation are readily adaptable to the Labeled Release technique. Whereas atmospheric composition and temperature studies would ideally require about five points each,

the pressure, water, and ultraviolet studies would require only one experiment each beyond the control to establish extreme effects.

5. Selection of Approach, Substrates, Inhibitors, and Conditions for Terrestrial Study

It is obvious from the foregoing discussions that a large number of test cells would be required if each recommended substrate, inhibitor, and environmental parameter were to be tested with a fresh soil sample in a fresh test cell. Consequently, in this program we have examined the feasibility of multiple liquid additions to the same soil contained within the same test cell. During our breadboard experience with the Viking '75 hardware, we demonstrated that sequential liquid plus soil additions into the same test cell did not provide adequate data. However, when a sequential liquid addition alone was made to the same soil, as in a commandable injection, the results were quite adequate. In the current program, we have expanded the concept by showing that many additions can be made to the same soil with quite satisfactory results. This now permits the sequential addition of many different single substrates to one soil sample in one test cell, thereby reducing the number of necessary samples and test cells. In fact, in these experiments, only four test cells and samples were required

to examine 27 different test conditions and to compare both active and control responses.

To demonstrate the feasibility of the multiple addition concept for Advanced Labeled Release, we have selected substrates, inhibitors, and environmental conditions from our list of prime candidates discussed in previous sections. Wherever possible, our selection reflected the most favored candidates for a Martian test. However, our emphasis here was not the delineation of the final Martian experiment, but the demonstration of the principle and approach using terrestrial soils. Consequently, the choice of parameters was also influenced by terrestrial metabolism and substrate availability.

The single substrates chosen for this study were acetate, formate, glutamate, DL-lactate, and DL-phenylalanine. All substrates appearing on the list of candidates (Table 1) and acetate, formate, glutamate and lactate are prime candidates for a Martian test. Formate, acetate, and lactate are also substrates for primitive anaerobic organisms whereas both formate and glutamate have been formed by nonbiological primitive conditions. Acetate is a key intermediate in terrestrial metabolism and, along with phenylalanine, a precursor for acetyl CoA. Glutamate and lactate are precursors for oxaloacetate and pyruvate, respectively, both of which are key compounds in Krebbs Cycle. Pyruvate, a

highly favored candidate substrate, was eliminated early because of nonbiological gas evolution when placed in contact with sterile soils. Phenylalanine was selected because preliminary experiments comparing D and L isomers showed a difference in rate between these two isomers. Thus, these five substrates cover the broad spectrum of rationale for substrate selection . To complete the spectrum, experiments involving rotation of label position were performed with acetate, lactate, and glutamate. Finally, in addition to single substrates, VM1 (the Viking '75 Labeled Release nutrient containing uniformly labeled formate, glycine, DL-alanine, DL-lactate, and glycolic acid) was also studied. This provided some correlation with individual substrates as well as continuity with the Viking '75 program. The mixed nutrient was used to demonstrate effects of inhibitors, atmospheric composition, temperature, and moisture content.

Regarding inhibitors, three classes were chosen: the metal chelator cyanide, the heavy metal package consisting of silver, lead, mercury, cadmium, zinc, and copper, and the antibiotic package consisting of penicillin, streptomycin and Fungizone. These represent three of the most important inhibitor packages recommended for a Martian test and are also well-suited for a terrestrial demonstration.

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Three environmental parameters were examined. The choice of atmospheres was air and an anaerobic mixture of nitrogen and hydrogen gases. This choice was directed strictly toward a terrestrial demonstration of the principle by the getter technique and,hence,could not encompass those gases most desired for a Martian experiment. Temperature curves were performed using 3^{o}, 20^{o}, 35^{o}, and 60^{o}C, temperatures approximately coincident with the ideal Martian temperature curve except for the point below freezing where terrestrial metabolism proceeds slowly. Finally, the "damp" versus "wet" conditions for moisture content were compared to complete the spectrum for environmental parameter variation. Variations of ultraviolet radiation and pressure were not investigated in the current work because they are not readily adaptable to a demonstration by the sequential getter technique. However, the sensitivity of Labeled Release responses from terrestrial soils pre-treated with ultraviolet radiation has been reported in earlier investigations. Some effects of pressure extremes have also been examined in the Viking hardware under separate contract. Although not repeated here, both parameters are nonetheless recommended for an Advanced Labeled Release Experiment on Mars using Viking-type hardware.

The results of our study using these substrates, inhibitors, and environmental parameters are presented in the following section. They readily demonstrate the versatility of the Advanced Labeled Release multiple addition concept in accommodating a large number of reaction conditions to maximize information regarding Martian life with a minimum of hardware changes from the Viking '75 hardware.

B. Experimental Design of Test Chamber - Medium Addition Sequence

The design and rationale of an Advanced Labeled Release Experiment based on single addition of soil and multiple sequential additions of media into each of four test chambers has been discussed in the preceeding section of this report. The feasibility of multiple addition testing has been extensively investigated. The proposed methodology utilizes a four chamber battery of tests, including:

Chamber 1 - Determination of the effect of various atmospheric gases and selection of that gas which produces an optimum response.

Chamber 2 - Determination of the effect of incubation temperature and selection of the optimum temperature for performing Martian biochemical tests.

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Chamber 3 - Nonbiological control for the other three test chambers. Sterile soil is dosed with a battery of ^{14}C labeled substrates and subjected to the experimental temperature range.

Chamber 4 - Determination of the possible inhibitory effects of water on Martian organisms is performed initially by dosing with 0.01 ml and 0.5 ml of medium, respectively. A series of specifically labeled substrates are then added to obtain patterns in metabolic $^{14}CO_2$ evolution, which might provide evidence for the pathways of metabolism. The substrate series is followed by an antimetabolite series designed to provide methods for microorganism control. (The temperature and atmospheric gas series should also provide information concerning methods of control).

This battery of tests was designed to challenge the multiple addition mode and provide criteria of feasibility based upon terrestrial soil results. Wherever possible, substrates and/or conditions paralleled Martian rationale; however, aerobic, 25°C studies on terrestrial soil were used predominately in these model tests.

1. Chamber 1 - Containing viable soil allows for performance of the Labeled Release Experiment under various atmospheric conditions. A proposed sequence of atmospheric conditions and medium additions is shown in Figure 1. Results of this series should provide information on which atmosphere

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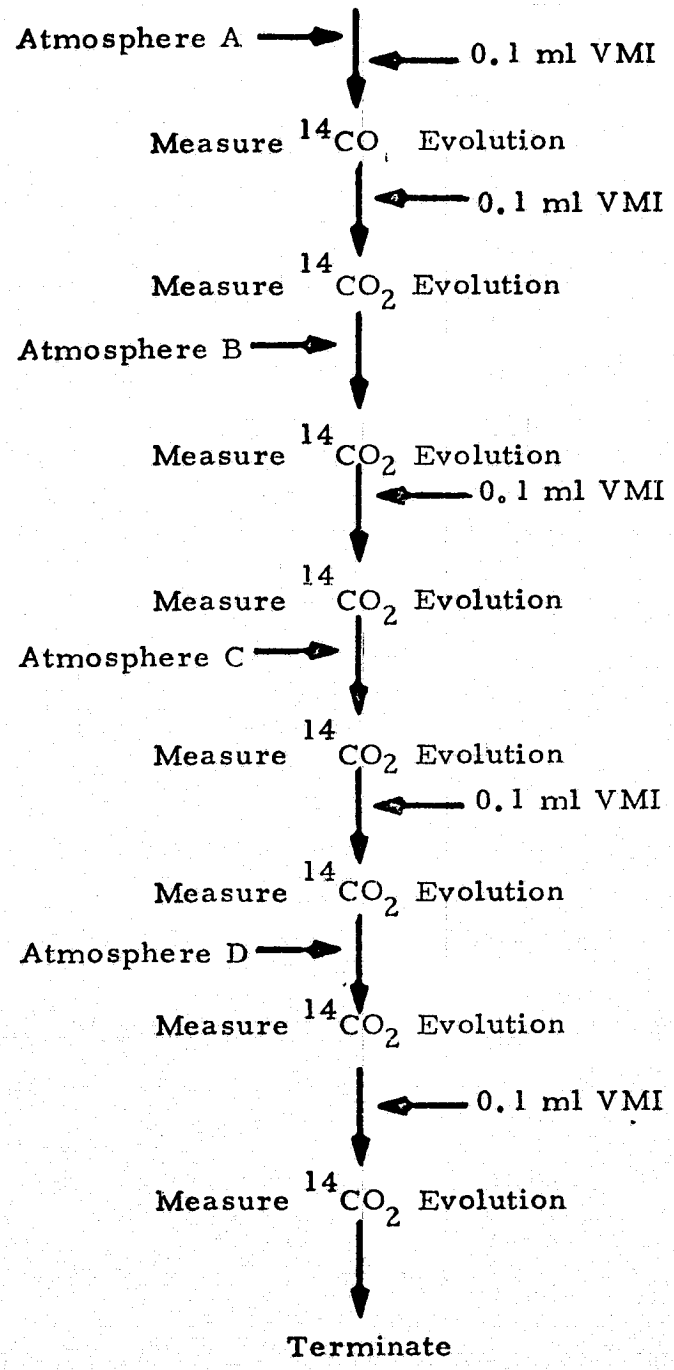
Figure 1

Chamber 1
Addition Sequence
Atmosphere Test

Ambient Temperature, Viable Soil (0.21 cc)



gives the optimum response and which might serve to control growth and therefore dictates the atmospheric conditions which should be used for the other three test chambers. The ^{14}C labeled medium to be used in this series is VM1, which has undergone rigorous testing as a part of the current Viking development. The gaseous atmospheres, which might be studied in this series are CO_2, CO, H_2, N_2, and 20 percent O_2 (air). Current experimental laboratory methods for conducting the Labeled Release involves CO_2 collection with $Ba(OH)_2$; therefore, testing under an atmosphere of CO_2 or CO are not feasible. An alternative detection system (ionization chamber or solid state) would be required to perform tests under conditions of a CO_2 or CO atmosphere.

Demonstration of the methodology has been performed using atmospheres of air and N_2:H_2 (90 percent: 10 percent). The rationale for determining the sequence of gases was to test for medium induced biological activity. The difference in kinetics between a first and second addition is primarily a biological phenomenon and, therefore, provides valuable information. That a first medium addition increased cell numbers or induced degradation systems would be a highly interesting finding.

2. Chamber 2 - Containing viable soil was used to establish the temperature optimum and range of activity of organisms in a test soil. As shown in Figure 2, the addition sequence for Chamber 2, VM1 medium is added sequentially to test soil and each addition is preceded by an increase in the incubation temperature. Temperatures to be used under simulated Martian conditions may or may not coincide with the demonstration procedure temperatures of 3°C, 20°C, 35°C and 60°C, which have been studied. The 3°C temperature is within the range of Martian ambient temperature, yet above freezing for the medium. Terrestrial soil produces a signal at this temperature and some psychrophiles might show an optimum. The 20°C temperature is approximately the upper limit for the Martian ambient temperature (although 30°C may be experienced in the hot model of the planet). Many soil organisms are active at this temperature. The 35°C temperature is optimum for many terrestrial organisms. Most organisms which inhabit warmblooded animals have their optimum at this temperature and many of these organisms are found in soil.

Thermophiles are active at 60°C. To date, soils thus far tested at 60°C showed decreased activity. Such a temperature would be expected to inhibit Martian organisms; therefore, the test,

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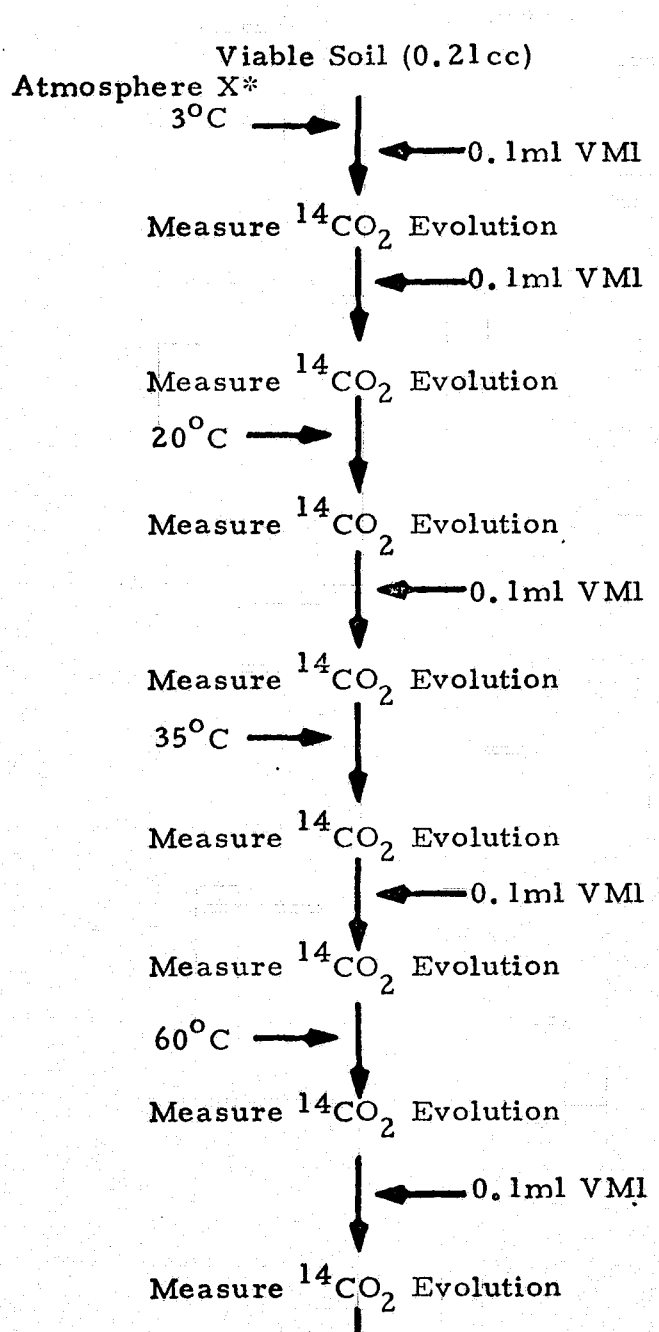
Figure 2

Chamber 2
Addition Sequence
Temperature Test



*Atmosphere selected on basis of Chamber 1 results

if used on Mars, should provide evidence for inhibition or cidal effects of heat.

As with Chamber 1, two successive media additions are made at the first temperature. As described for Chamber 1, these additions are necessary and constitute a repeat of results obtained for Chamber 1 (additions 1 and 2) at a different temperature.

The temperature optimum (that which produced the highest rate and cumulative evolution) will be selected for further tests with Chambers 3 and 4.

3. Chamber 3 - (Figure 3) will be charged with soil which has been presterilized. A series of medium additions and incubation conditions will be imposed. The results of Chamber 3 will serve as a nonbiological control for interpretation of results from Chambers 1, 2 and 4. The sterile medium series is tested prior to the viable medium series in order to provide baseline information as an aid to decision making concerning the timing of subsequent additions.

No control series is scheduled to parallel the Chamber 1 tests since the various gases themselves may impose a control-like situation and only one atmospheric gas will be tested with

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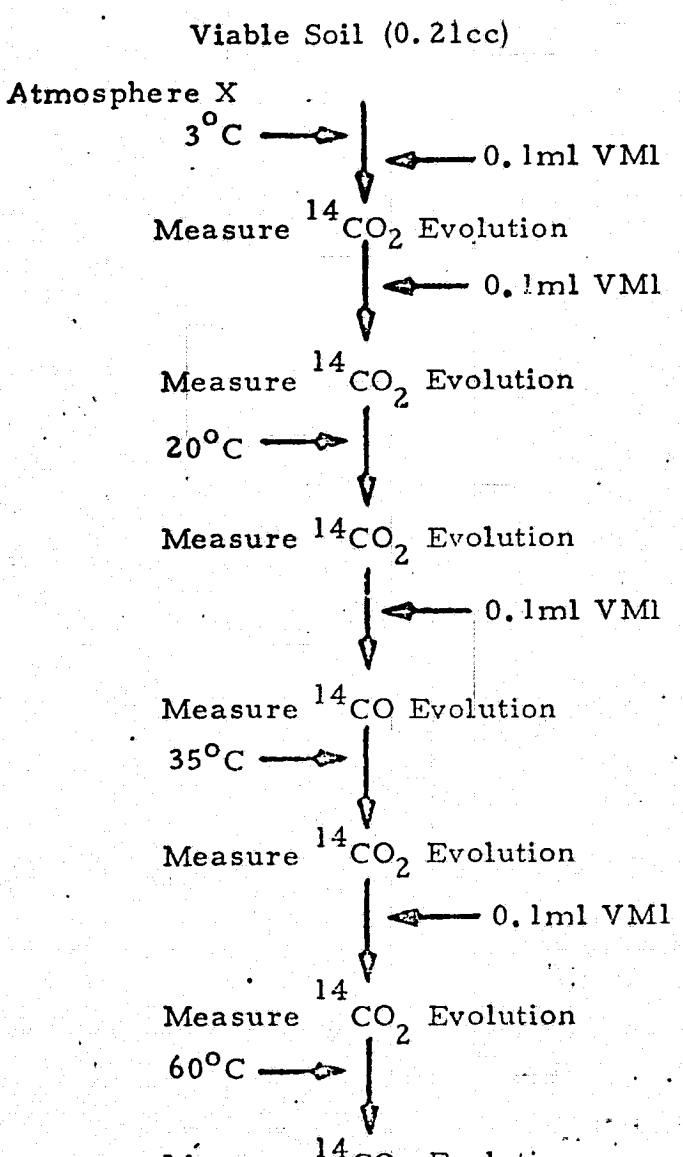
Figure 3

Chamber 3
Addition Sequence
Sterile Control



(continued)

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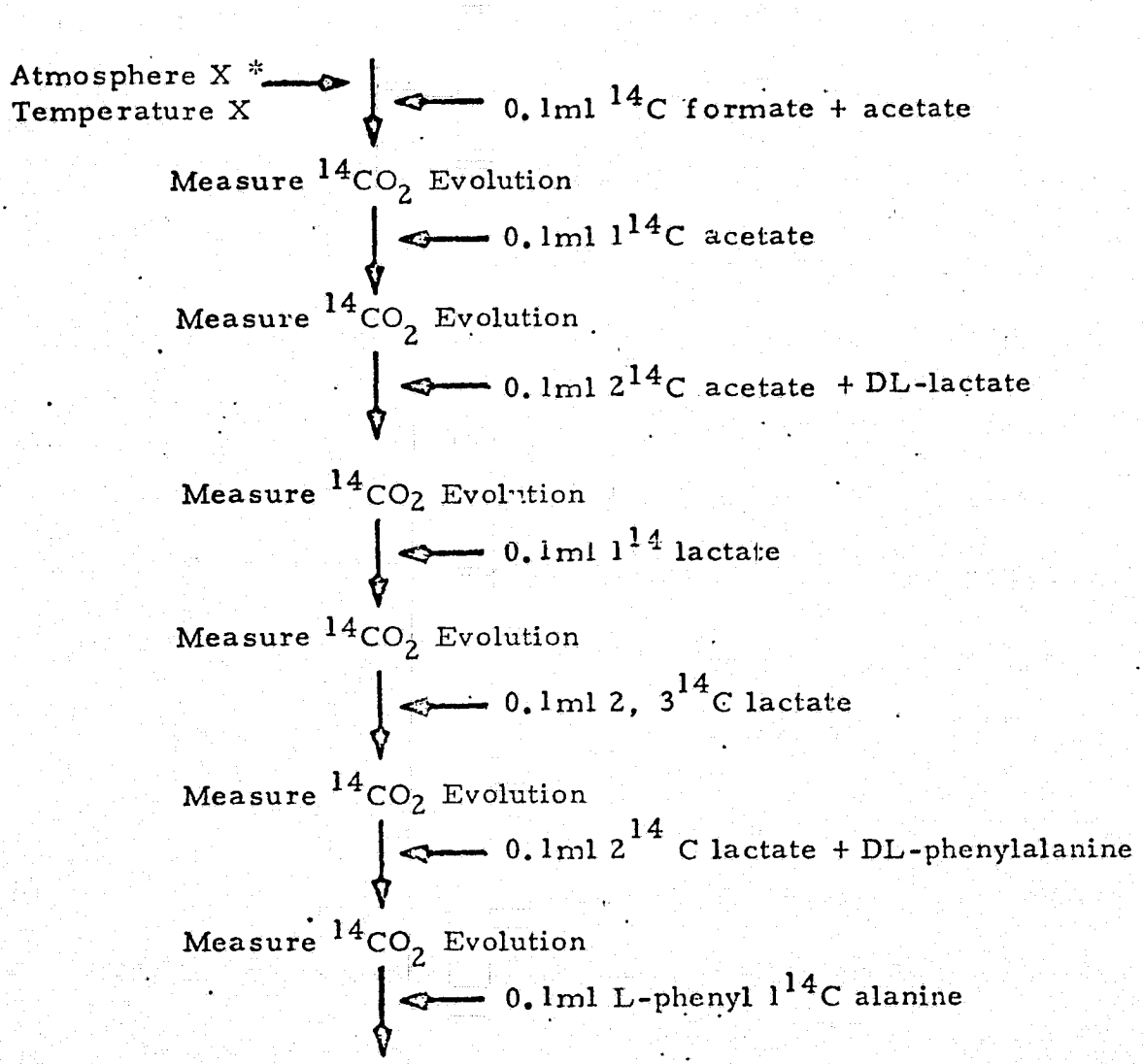
Figure 3 (continued)

Chamber 3
Addition Sequence
Sterile Control



(continued)

* Atmosphere and Temperature selected on the basis of results from Chambers 1 and 2

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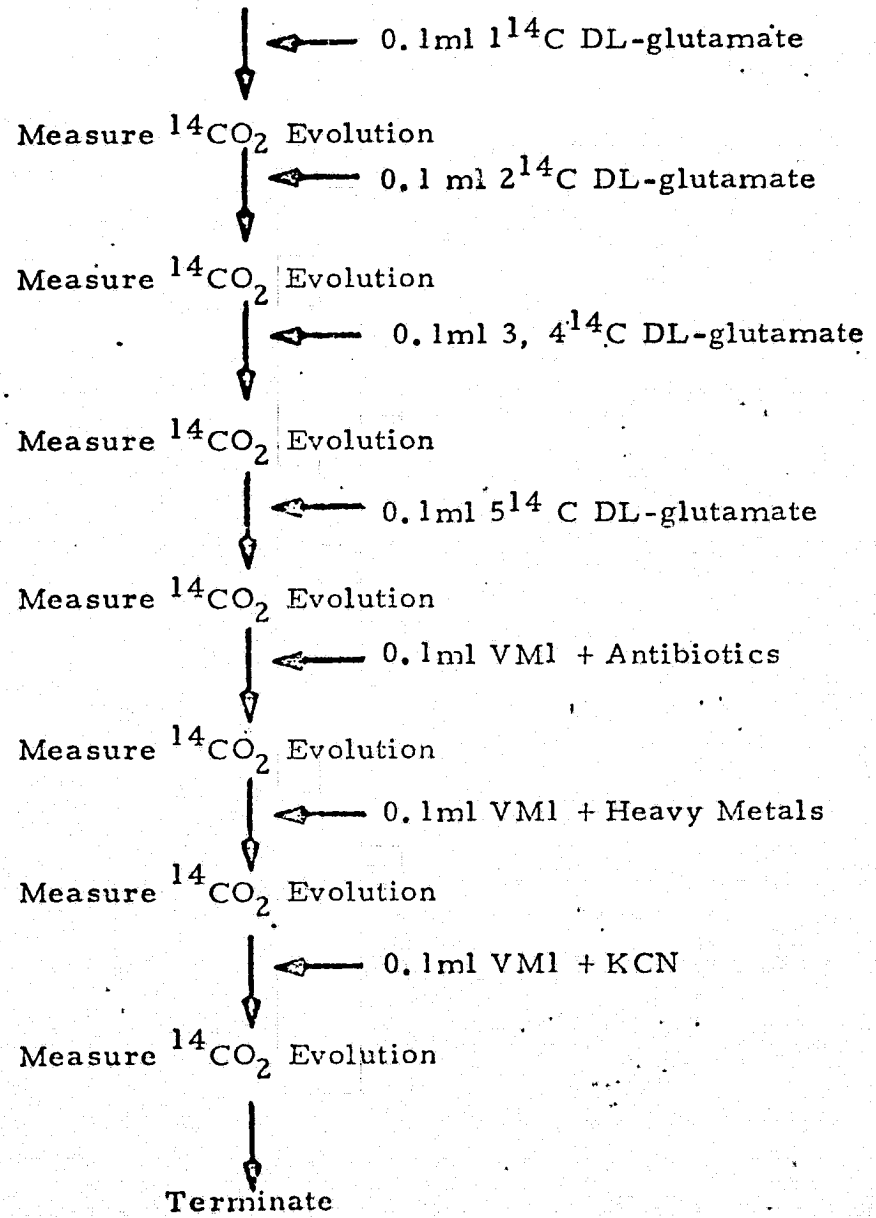
Figure 3 (continued)

Chamber 3

Addition Sequence
Sterile Control



the substrate series. A control for the selected atmosphere will result from the temperature control series.

The same protocol used for Chamber 2 will be followed with Chamber 3. Results obtained in the sterile vs. viable system will be analyzed to determine the noise-to-response ratio which produces the most clearly defined data. This, in conjunction with the characteristics of the viable response, will provide additional criteria for selection of a temperature optimum for further tests. Employing the selected temperature, the media, as shown in Figure 3, are added sequentially in the order given. The kinetics of nonbiological evolution may be established and, the nonbiological baseline at any point in the sequence may be established.

4. Chamber 4 - (Figure 4) constitutes the main comparative biochemistry test series. Experiments to determine the effect of water and antimetabolites are included. As shown in the addition sequence for Chamber 4, the initial addition of medium to viable soil, 0.01 ml/0.21cc, will result in a partially moistened system, which will give results of the labeled release under conditions of minimum water. The second medium addition to

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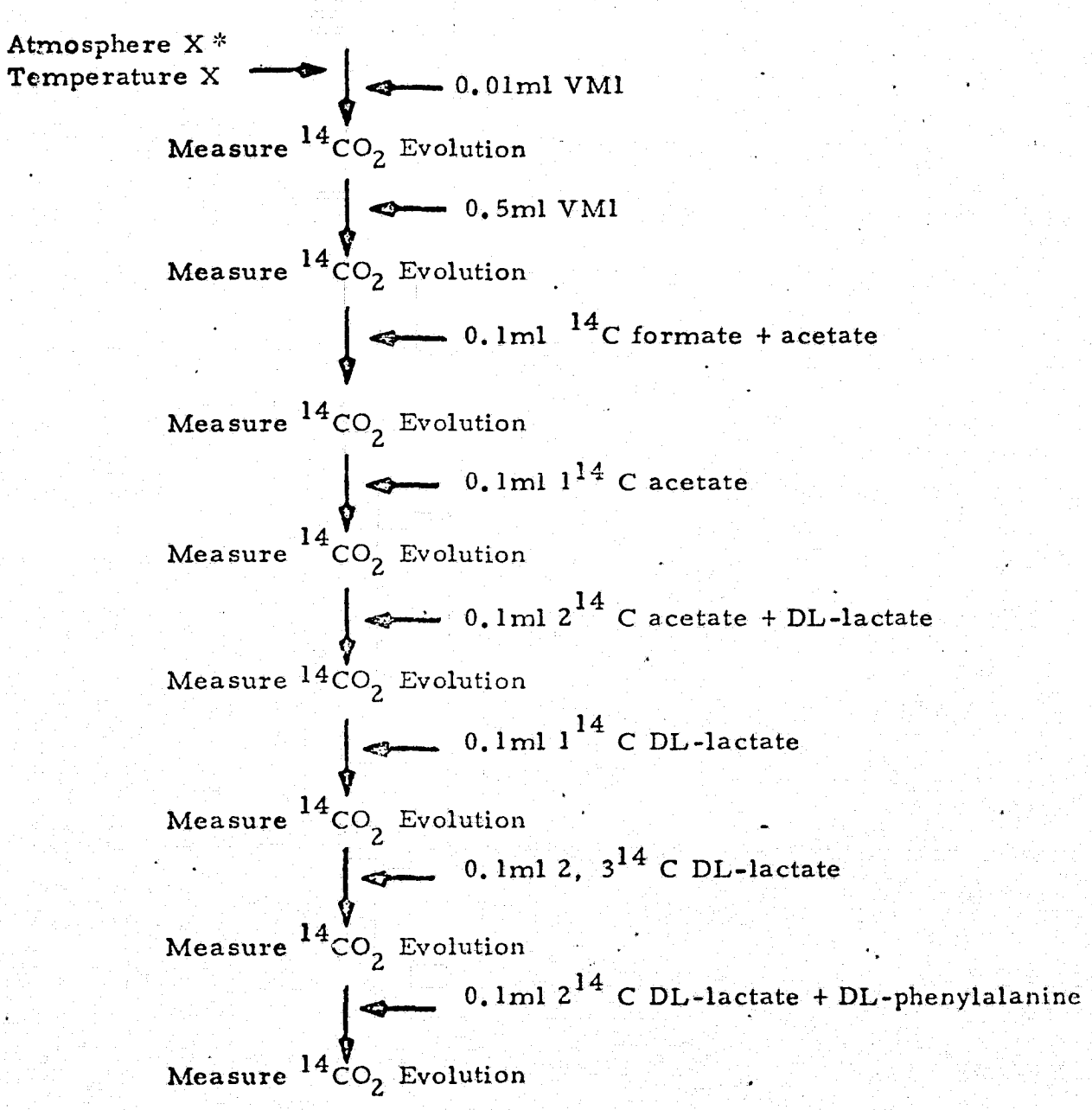
Figure 4

Chamber 4
Addition Sequence
Water, Metabolism and Antimetabolite Tests



(continued)

* Atmosphere and Temperature selected on the basis of results from Chambers 1 and 2

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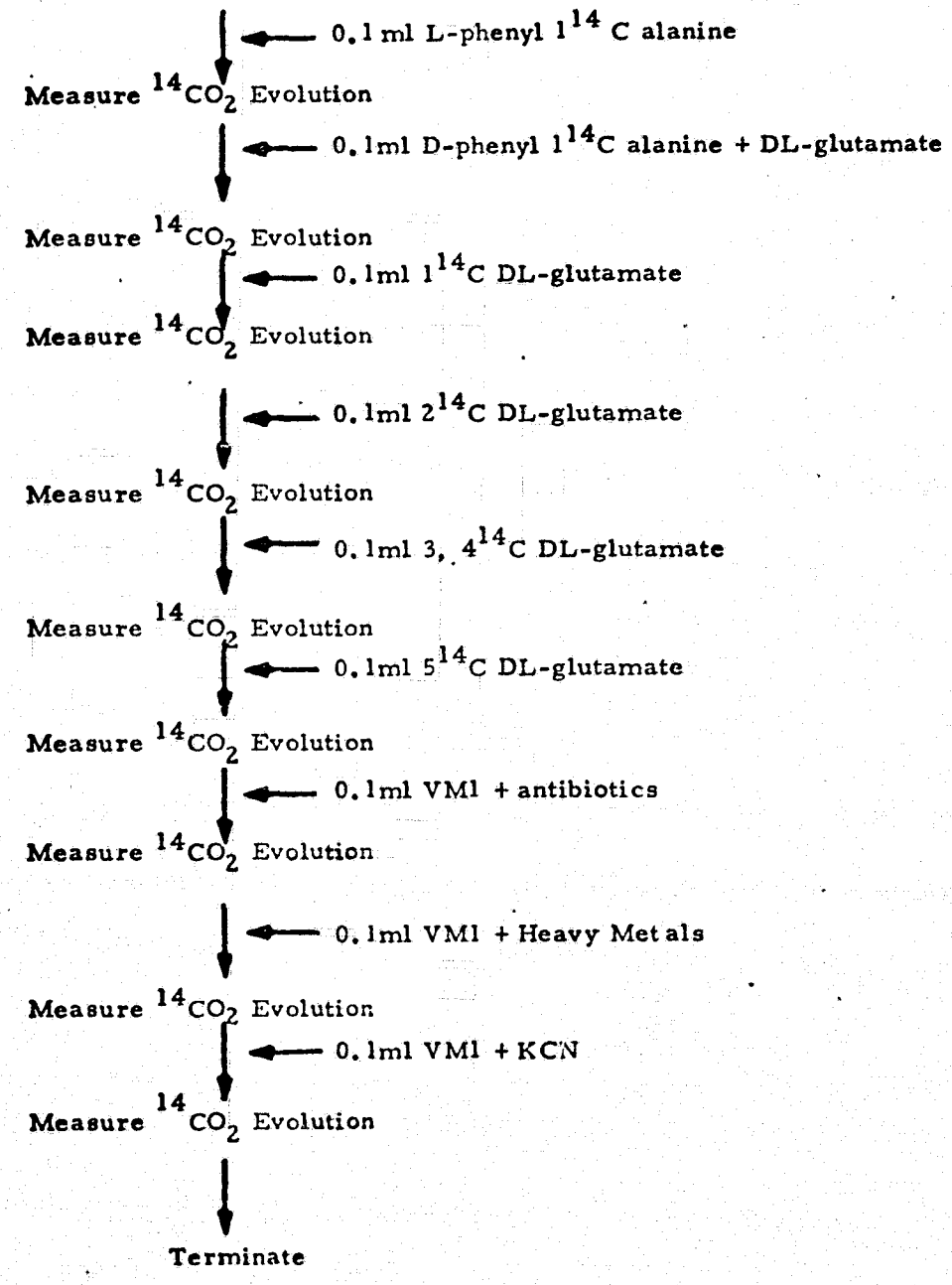
Figure 4 (continued)

Chamber 4

Addition Sequence
Water, Metabolism and Antimetabolite Tests



Chamber 4, 0.5 ml VM1 will provide a test for the possibility of inhibition by water. This second addition will result in a thoroughly wetted sample, and also bring the volume of added medium in the test series in line with the volume added in the control series (control 0.5 ml, test 0.51 ml).

The selection of a series of specifically labeled substrates has been directed by practical as well as by theoretical considerations, which have already been discussed.

Chemical stability during storage and sterilization as well as experimental results of the labeled release obtained with selected substrates moderated the original selection. Although the substrate series presented here is tentative, it has been tested and found to provide reproducible results.

Formate, acetate and lactate are simple carboxylic acids, which occupy key metabolic positions. Acetate is activated to acetyl CoA and lactate oxidized to pyruvate by most biological systems. These two compounds, acetyl CoA and pyruvate, are perhaps the most important biosynthetic and degradative intermediate compounds in terrestrial metabolism. The use of specifically labeled acetate and lactate can provide much data for predicting the pathways of metabolism.

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Glutamate, although structurally more complex than the former substrates, is important for amino acid synthesis and is generally metabolized via the citric acid cycle. Use of specifically labeled glutamate has produced a pattern in $^{14}CO_2$ evolution which is easily explained by the citric acid cycle.

D & L phenylalanine have been tentatively chosen to provide a test for specificity of the two forms. Although a specificity for the D or L form may be demonstrated, phenylalanine is viewed principally as a demonstration substrate rather than a candidate for Martian testing. Other ^{14}C labeled substrates with D and L forms which might demonstrate specificity with terrestrial soils and are theoretically desirable are not readily available and will probably need to be specially prepared.

As shown in the addition sequences for Chambers 3 and 4, a series of antimetabolites is scheduled to follow the substrate series, and is designed to provide much needed information concerning the control of Martian organisms. The order of addition is in accordance with expected levels of inhibition, i. e., the first addition should be least inhibitory and the final addition most inhibitory. Agents currently scheduled for this series are antibiotics (mixture of penicillin, streptomycin and Fungizone),

heavy metals (a mixture containing Hg, Pb, Ag, Cu and Cd is currently being tested) and KCN (found to be strongly inhibitory in earlier studies).

C. Preliminary Experimentation

Many preliminary experiments were conducted to establish the feasibility of the multiple addition methodology. Findings of these tests have been incorporated into the four chamber, sequential media addition series.

1. Order of Temperature Incubation

To determine if the order of temperature incubation might influence radiorespiration, a series of experiments was conducted in which incubation temperature was in the order of $3^{\circ}C \rightarrow 20^{\circ}C \rightarrow 35^{\circ}C \rightarrow 60^{\circ}C$ as well as in the order $60^{\circ}C \rightarrow 60^{\circ}C \rightarrow 35^{\circ}C \rightarrow 20^{\circ}C \rightarrow 3^{\circ}C$. Successive medium additions were made at each incubation temperature.

Results of the above series of medium additions and incubation temperatures are shown in Figures 5 and 6, respectively. Incrementally evolved radioactivity (cpm/hr) was plotted vs. incubation time. The percent recovery of added radioactivity collected at a given temperature was calculated as follows:

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Figure 5

Evolution of $^{14}CO_2$ by Meadow Soil
at Various Temperatures

Multiple Addition Mode, Low Temperature to High Temperature

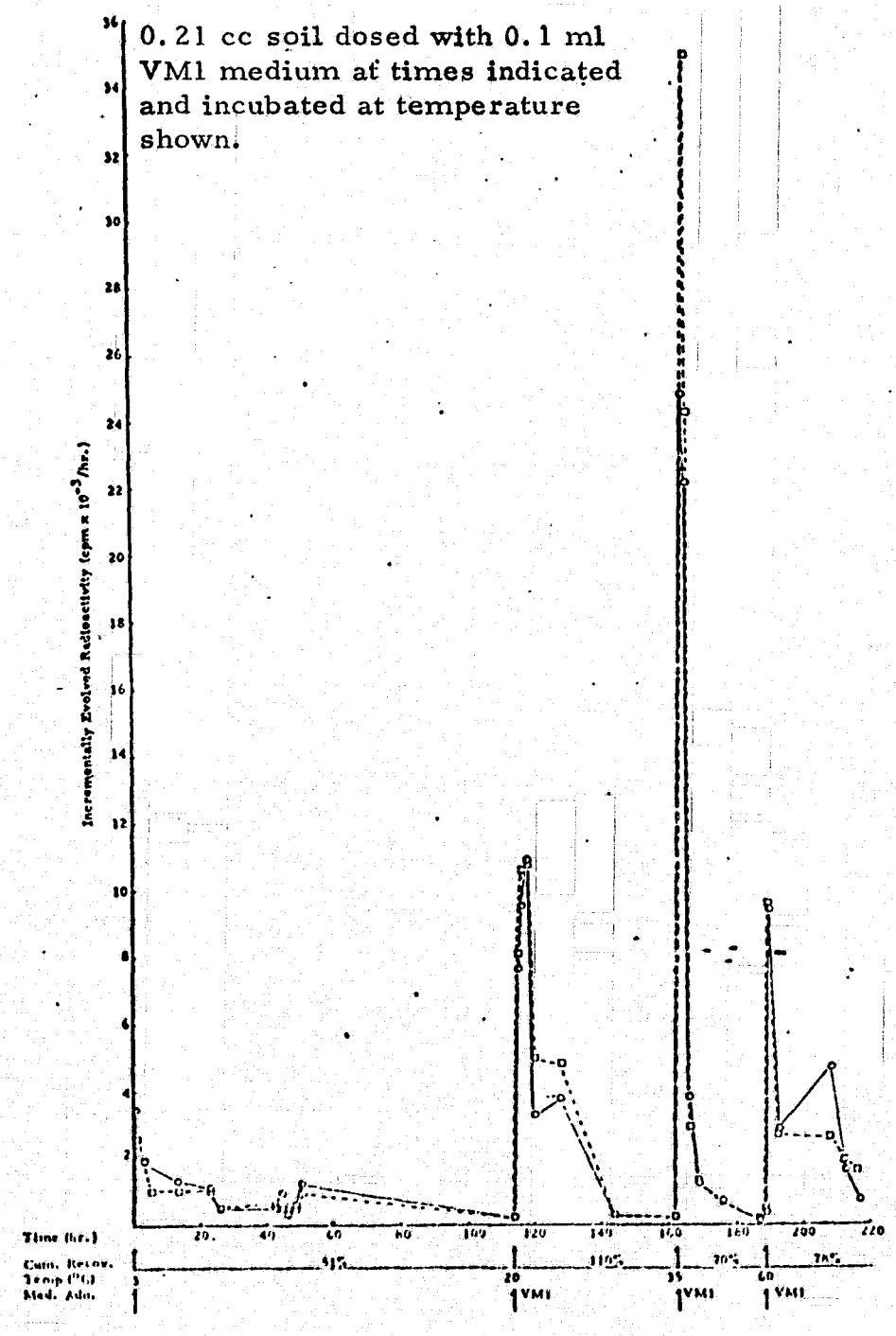

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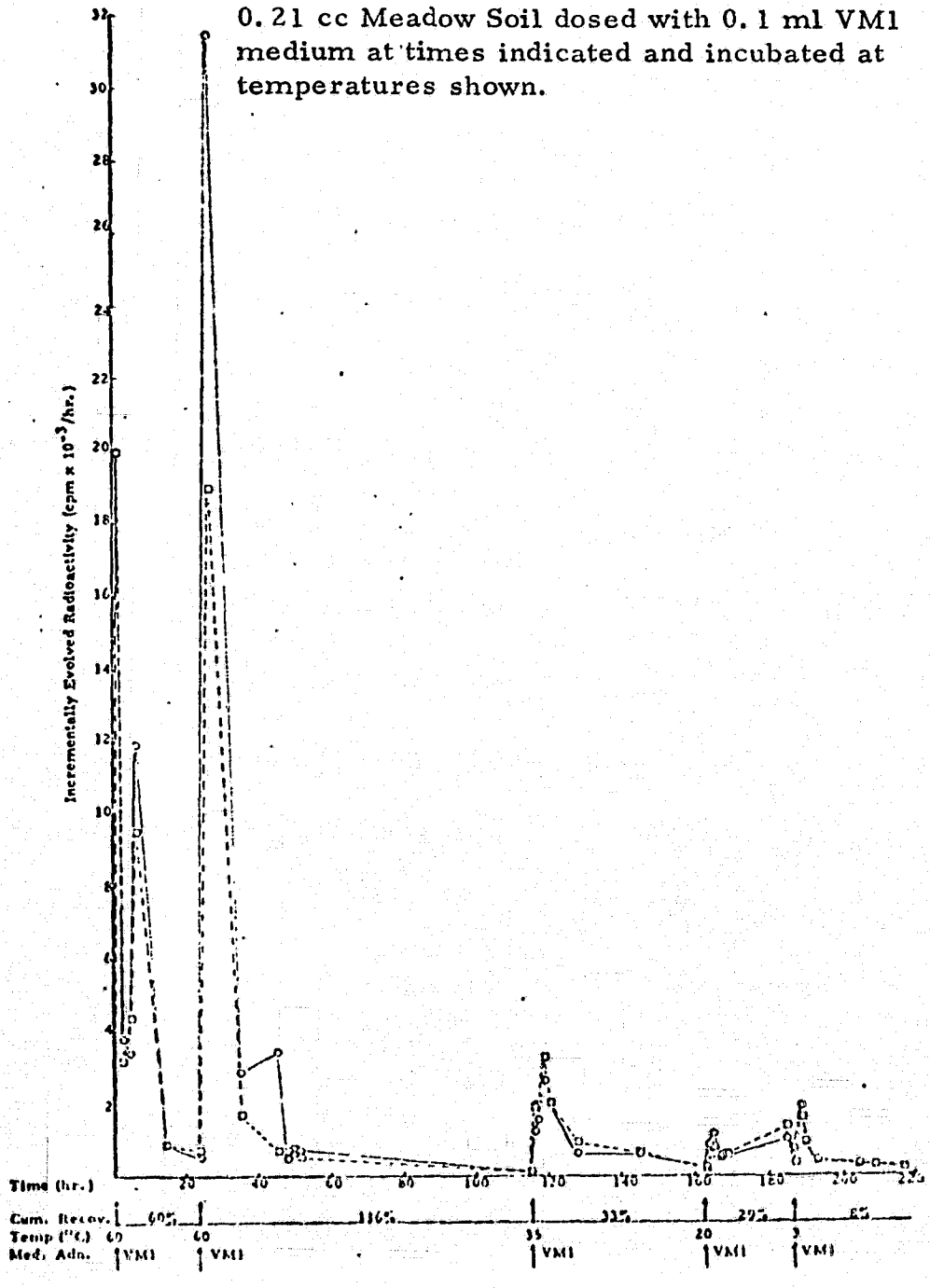
Figure 6

Evolution of $^{14}CO_2$ by Meadow Soil
at Various Temperatures

Multiple Addition Mode, High Temperature to Low Temperature



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0.1 ml added x 3.4 μCi/ml = 0.34 μCi added

0.34 μCi added x 2.22×10^6 dpm/μCi = 7.55×10^5 dpm added

7.55×10^5 dpm added x 13.4 ± 2.02% (collection and counting efficiency) = $1.01 \times 10^5 \pm 1.52 \times 10^4$ cpm added

$$\frac{\text{cpm recovered} \times 100}{1.01 \times 10^5 \text{ cpm added}} = \% \text{ recovery}$$

As seen in Figure 5, natural Meadow soil dosed with ^{14}C labeled medium and incubated at 3°C produced an initial small peak followed by continued low level activity (500 - 1000 cpm/hr) for more than 50 hours. Peaks in activity occurred immediately following subsequent shifts to higher temperatures and accompanying medium additions. The greatest rate of radiorespiration occurred at 35°C at which nearly 50% of the added radioactivity was evolved during the first two hours of incubation at that temperature. Total radioactivity recovered at the 3°C, 20°C, 35°C and 60°C incubation temperatures was 51%, 110%, 70% and 78%, respectively. The initial incubation temperature resulted in the lowest recovery; however, it appeared that some of the unrecovered radioactivity from the 3° incubation was evolved during the subsequent 20° incubation.

Meadow soil, dosed with ^{14}C labeled medium and incubated at 60° C as shown in Figure 6, produced an immediate

double peak which accounted for approximately 60% of the added radioactivity within 24 hours. A second addition of labeled medium produced a single large peak in activity which resulted in 116% of the second medium addition. Apparently some of the radioactivity not released during the first 24-hour incubation period was evolved after the second medium addition. Subsequent medium additions at 35°C, 20°C and 3°C showed much less activity than had been found when an increasing order of temperature incubation was studied. The initial rate of respiration and the total recovery of added radioactivity were both depressed. Incubation at 60°C apparently killed or deactivated most organisms which are active at the lower temperatures. Reduction in numbers of bacteria active at the lower temperatures would explain the decreased reaction rate, but not the low overall recovery.

Phoenix soil was subjected to the same experimental procedures as the Meadow soil. As shown in Figures 7 and 8, very similar results were obtained with the two soils. The Phoenix soil was somewhat more active at 60°C than the Meadow soil. When the initial incubation was at 60°C, the Phoenix soil showed one peak as opposed to two for the Meadow soil. Also, the

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Figure 7

Evolution of $^{14}CO_2$ by Phoenix Soil
at Various Temperatures

Multiple Addition Mode, Low Temperature to High Temperature

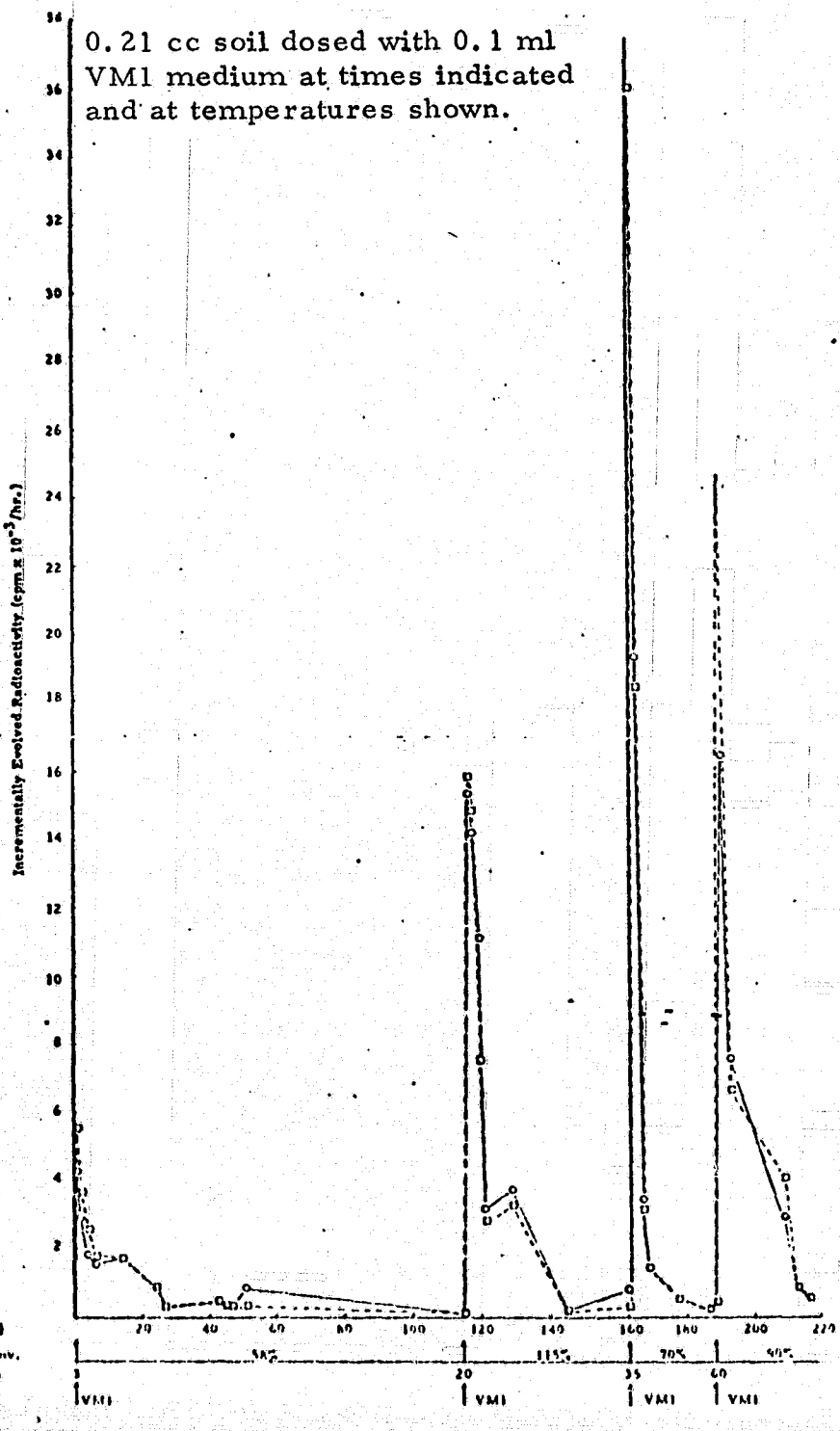

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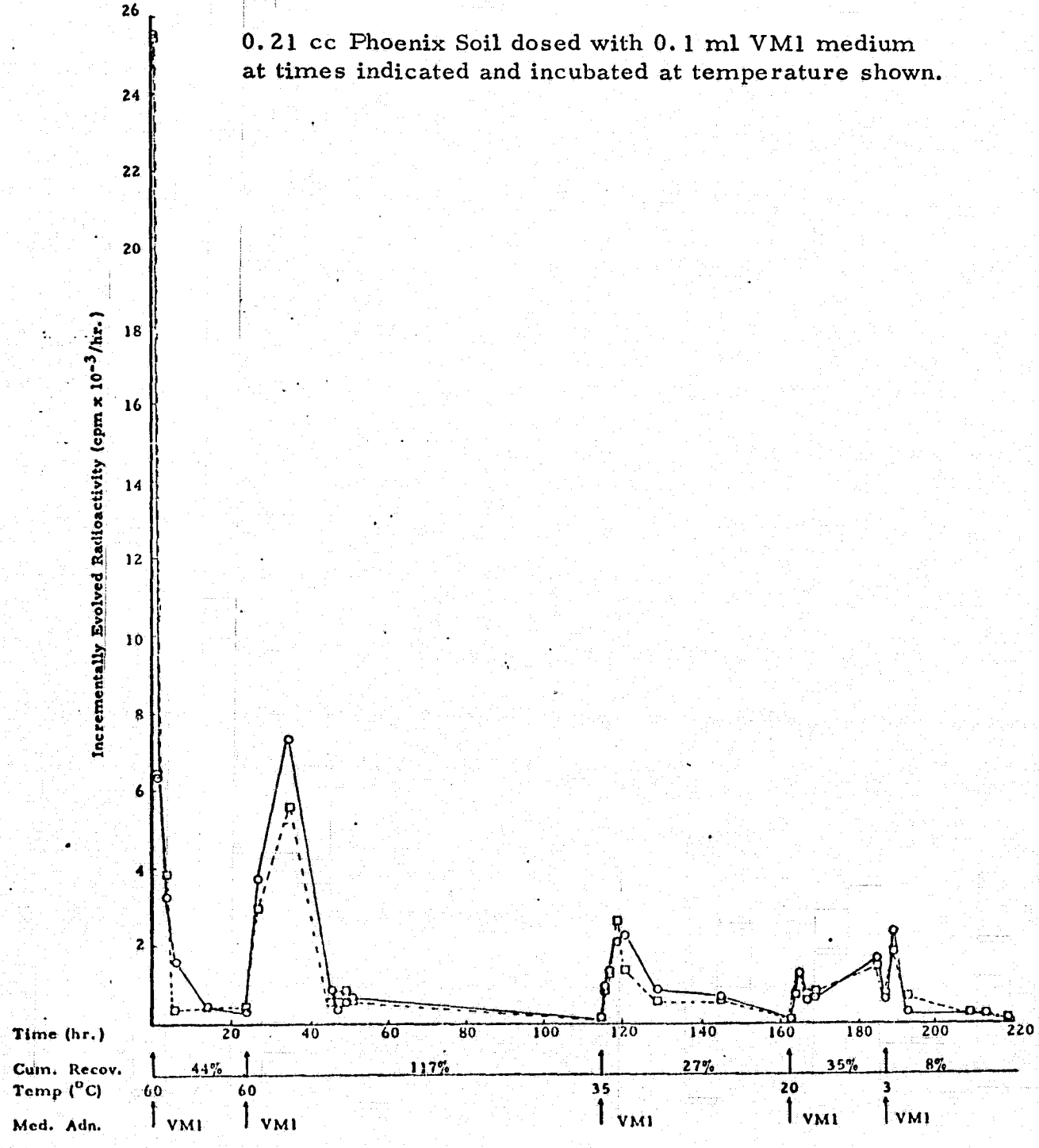
Figure 8

Evolution of $^{14}CO_2$ by Phoenix Soil
at Various Temperatures

Multiple Addition Mode, High Temperature to Low Temperature



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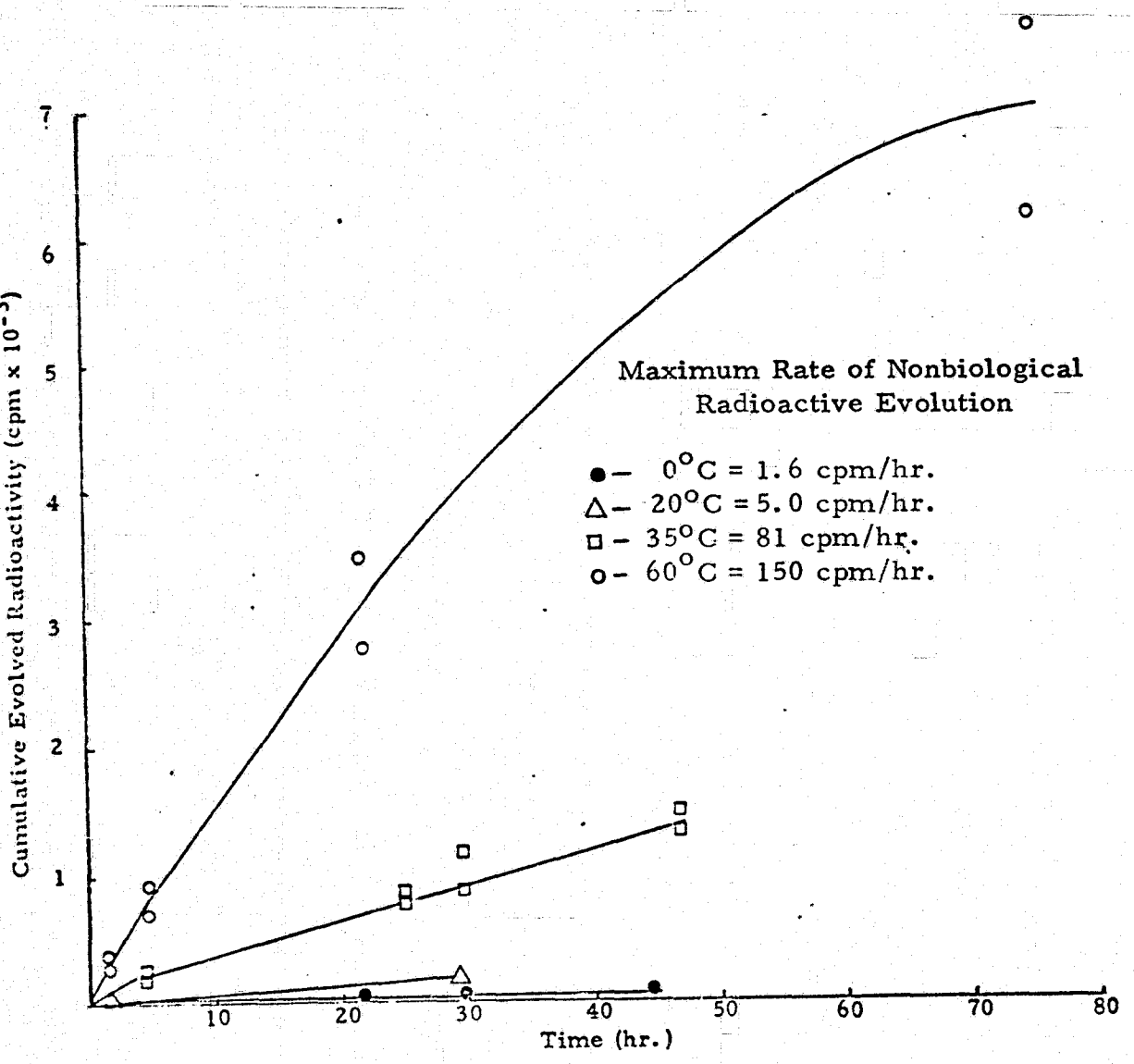
Phoenix soil, after a regimen of increasing temperatures, showed a greater peak at 60°C than had been obtained with the Meadow soil.

2. Nonbiological Evolution of Radioactivity

A series of heat sterilized control soils (312°C, 30 min.) were dosed and incubated sequentially at the four incubation temperatures. Results are shown in Figure 9. Nonbiological evolution of radioactivity was found to be temperature dependent; however, only at the 60° incubation temperature was the amount of evolution significant. But even the 150 cpm/hour nonbiological rate obtained at 60° would not affect the plots as shown in Figures 4-8. However, the cumulative amount of this radioactivity evolved over a long incubation time could be considerable. Nonbiological evolution would, however, have little affect on the interpretation of results from soils such as Phoenix and Meadow. Consider,for example, the 60° incubation conducted in Figure 8. Nonbiological evolution would not affect either peak and at most could be responsible for no more than 2,000 cpm of the 45,618 cumulative cpm evolved after the first medium addition and no more than 10,000 cpm of the 119,823 cumulative cpm evolved after the second medium addition. Nonbiological affects are less than 10% at 60° and, therefore, fall within the limits of reproducibility of the method. Soils

Figure 9

Nonbiological Evolution of Radioactivity at Various Incubation Temperatures



may be encountered, however, which show little biological activity at 60°C; and, under these circumstances, affects of nonbiological release would require greater consideration. In such cases, heat sterilized controls become increasingly important in distinguishing biological responses from nonbiological noise.

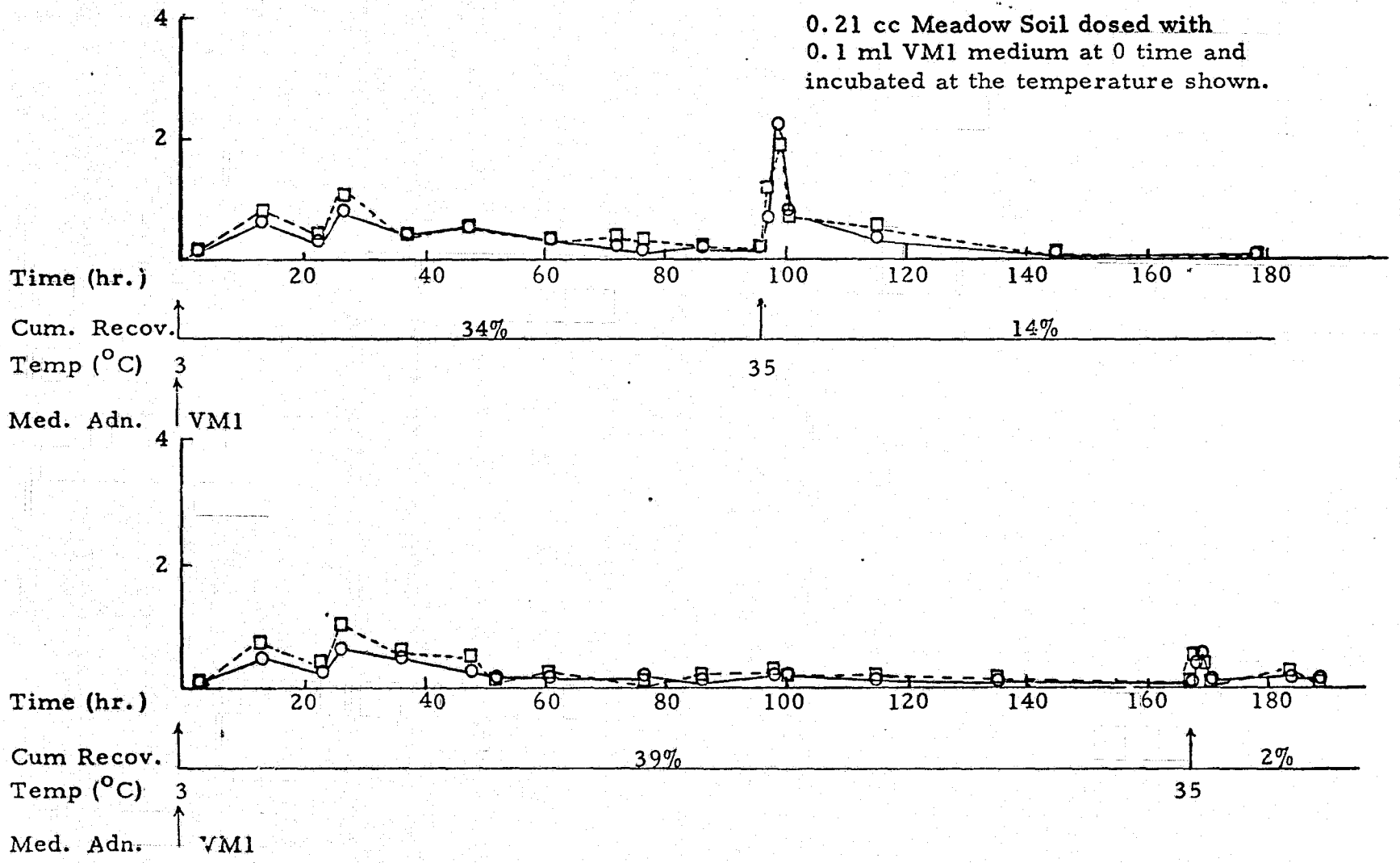
Of additional importance in separating biological and nonbiological responses is the fact that,in the control series presented in Figure 9, an imposed temperature shift did not produce any peak in nonbiological evolution.

3. Effect of Temperature Shift on the Biological Evolution of Radioactivity

Experiments have shown that a soil which is dosed with ^{14}C labeled medium evolves radioactivity rapidly at first but then evolves only very low amounts of radioactivity when incubated at that temperature over an extended period of time. When the same soil is again dosed with medium and incubated at a higher temperature, an immediate high rate of $^{14}CO_2$ evolution results. Experiments have been performed in which the temperature shift was made without adding additional ^{14}C labeled medium. As shown in Figure 10, a temperature shift alone may induce a peak in activity. The magnitude of this temperature-induced peak appeared to be determined by the amount of unevolved radioactivity

Figure 10

Effect of Temperature Shift on the Evolution of Radioactivity



from a previous addition since a shift to 35°C made after 100-hour incubation at 3°C resulted in a greater peak than a shift to 35°C made after 170 hours. The temperature induced evolution could considerably influence results in which a shift to a higher temperature is accompanied by a ^{14}C medium addition. Figure 11 shows the results of an experiment in which soil was dosed with labeled medium, incubated at 3°C for 95 hours, shifted to 35°C for 22 hours and then dosed with additional labeled medium. Approximately 40% of the added radioactivity was recovered during the 3°C incubation period; however, an additional 20% was recovered during the 35°C incubation period prior to the second medium addition. These data confirm the explanation of data shown in Figures 5-8 that some unevolved radioactivity from the first medium addition was evolved during the second medium addition in response to the increased temperature. Curiously, however, the pattern of the "early burst" still appears.

4. Successive Medium Additions at Constant Temperature

Soil samples were placed at a given temperature for one hour, then dosed with VM1 medium and the evolved radioactivity collected. When evolution of radioactivity had dropped to low levels, a second medium addition was made at that temperature.

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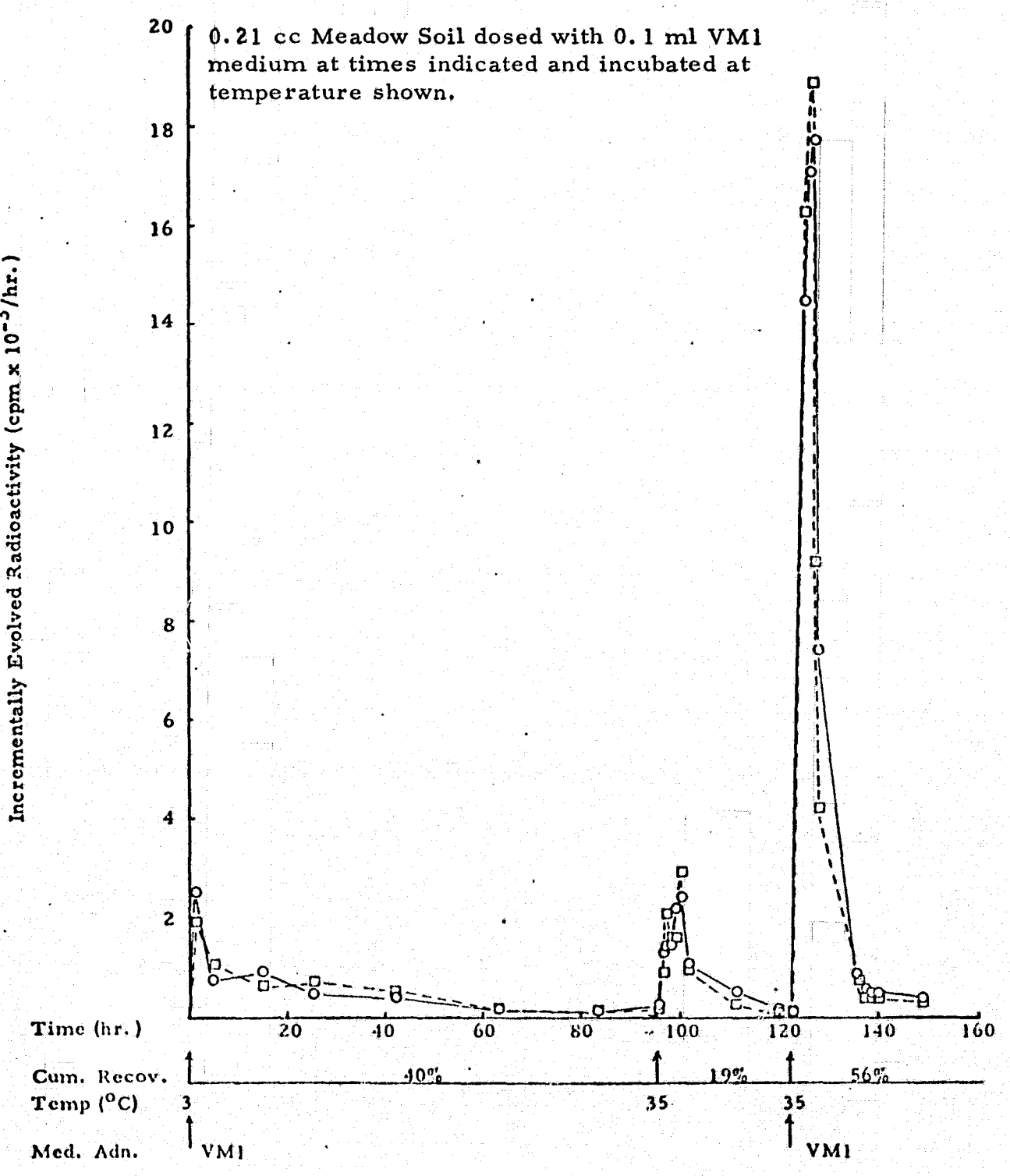
Figure 11

Effect of Temperature Shift from 3°C on the Evolution of $^{14}CO_2$ by Soil



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Results obtained at the four experimental temperatures 3°C, 20°C, 35°C and 60°C are shown in Figures 12-15. In every case the second medium addition produced a greater peak in activity than occurred during the first addition. At 3°C incubation (Figure 12) the second addition produced a greater recovery of radioactivity than the first addition. At other incubation temperatures the recovery was similar for first, second, and, in the case of 20°C incubation, third additions.

Figure 15 shows the double peaks which occurred following the first medium addition. This phenomenon was also observed in Figure 6. It was noted, however, that a second medium addition at 60°C failed to show double peaks, probably indicative of adaptation of the organisms which were active at this elevated temperature.

5. Temperature Adaptation by Soil Organisms

An experiment was performed to test the effect a 60°C temperature adaptation period may have on $^{14}CO_2$ evolution. Four replicate Meadow soil samples were dosed with VM1 medium and incubated at 3°C. After 95-hour incubation, two of the soil cultures were moved to 60°C incubation. At 123 hours the other two soil cultures were moved to 60°C incubation

Figure 12

Evolution of $^{14}CO_2$ by Meadow Soil
Sequential Addition at 3°C

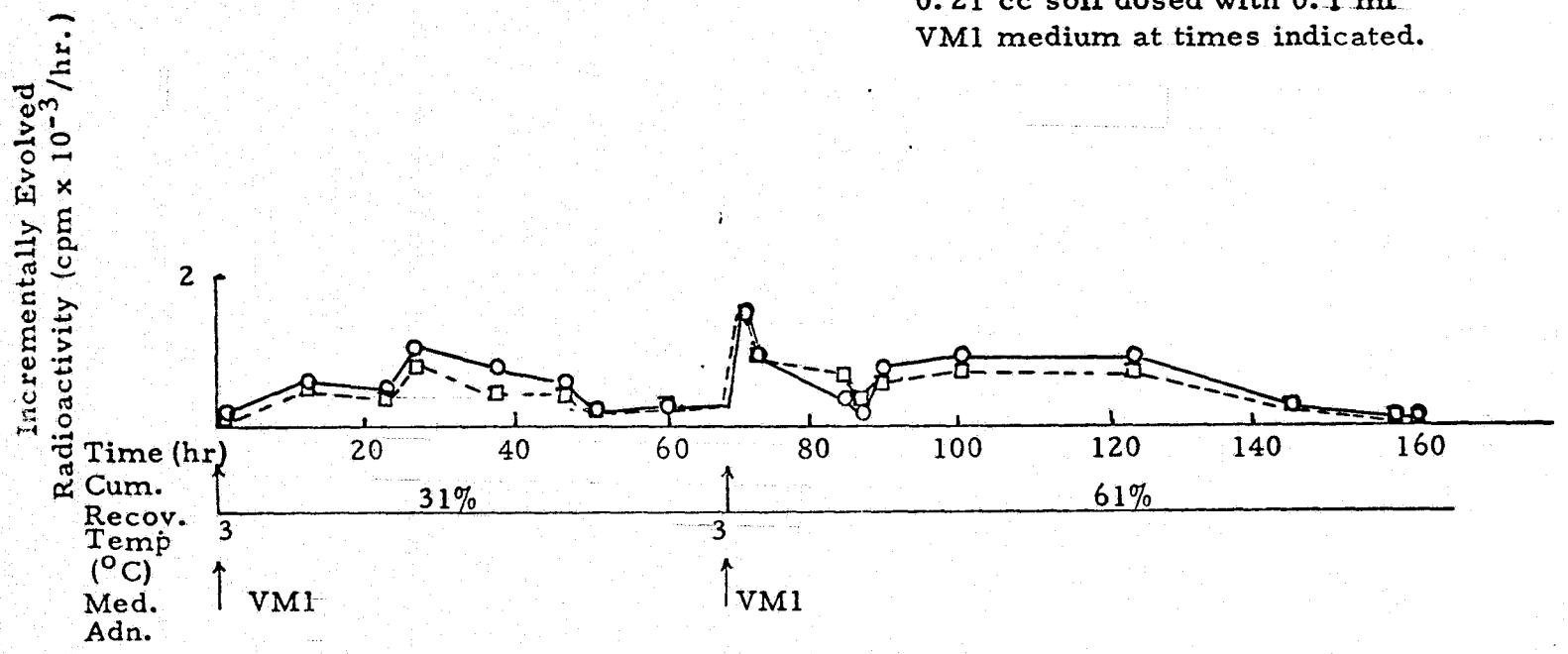

Figure 13

Evolution of $^{14}CO_2$ by Meadow Soil
Sequential Addition at 20°C

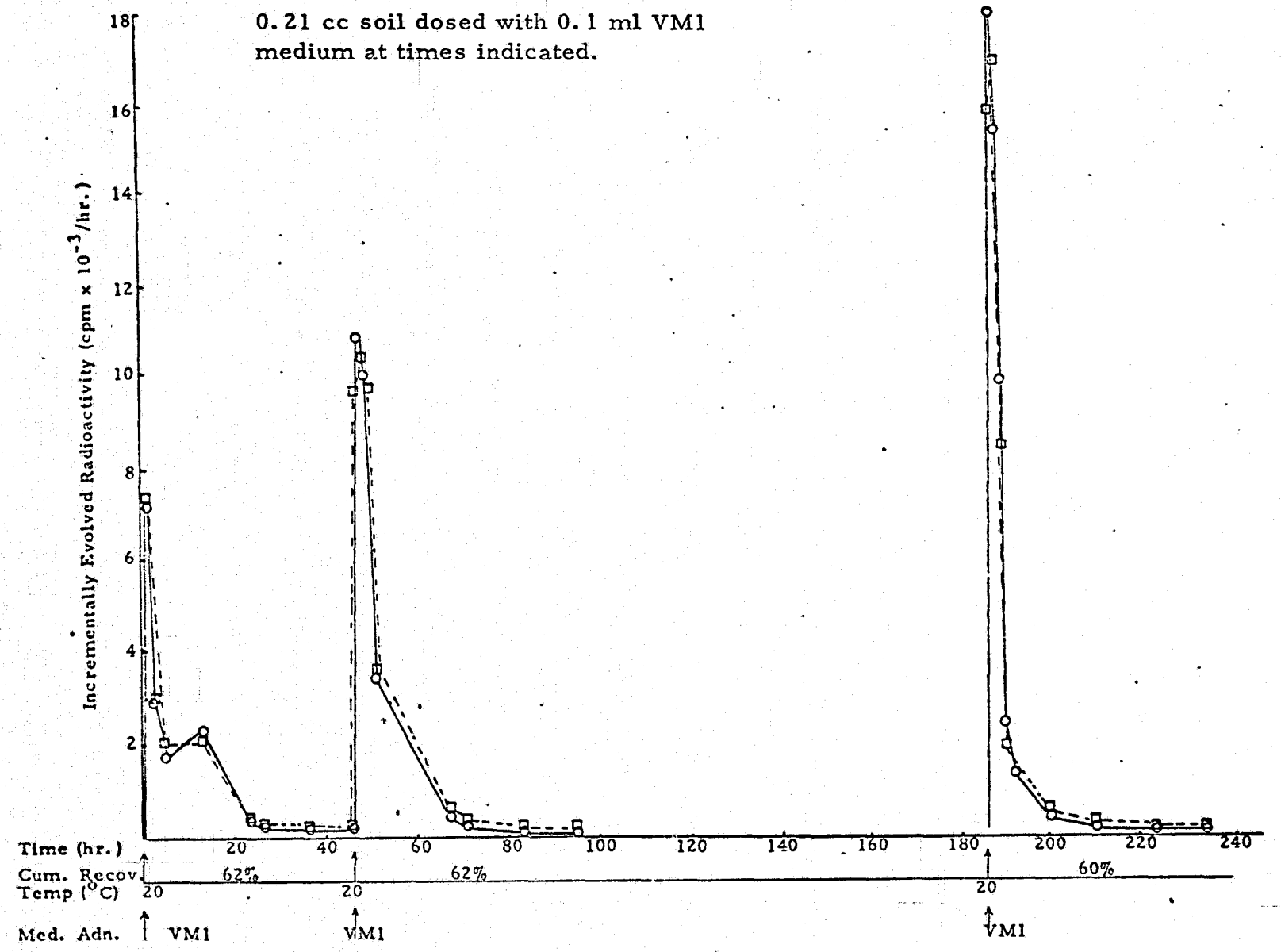

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Figure 14

Evolution of $^{14}CO_2$ by Meadow Soil
Sequential Addition at 35°C

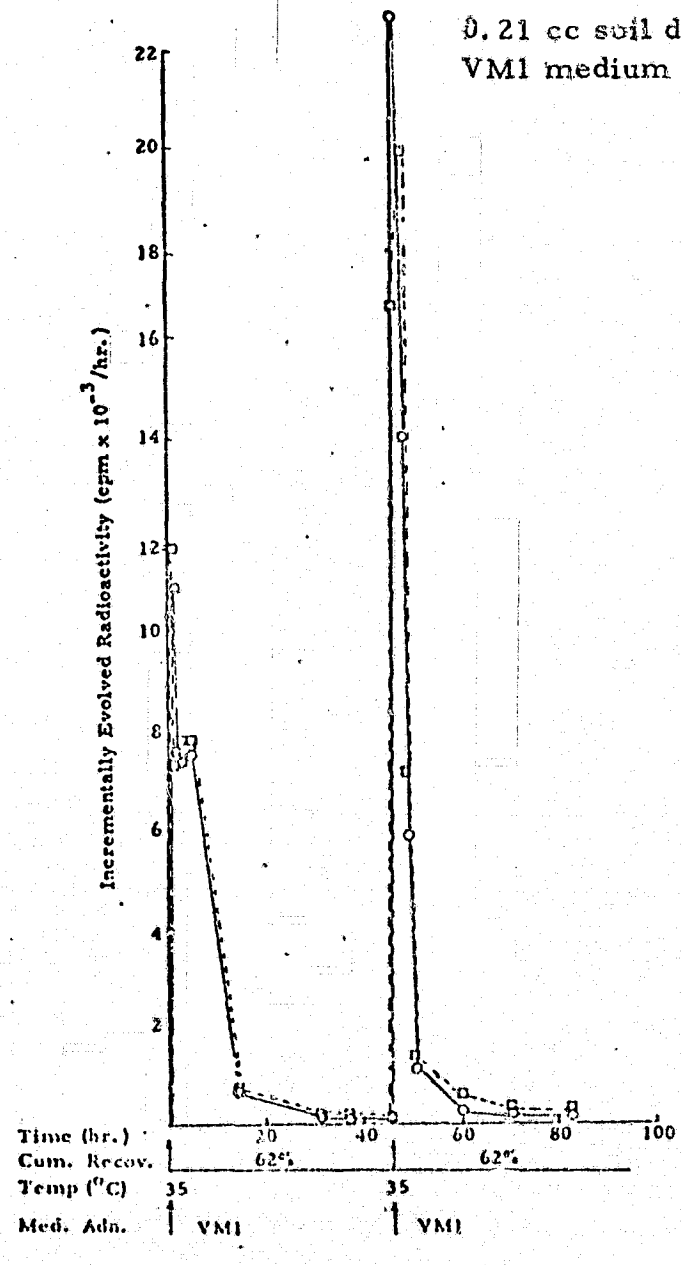

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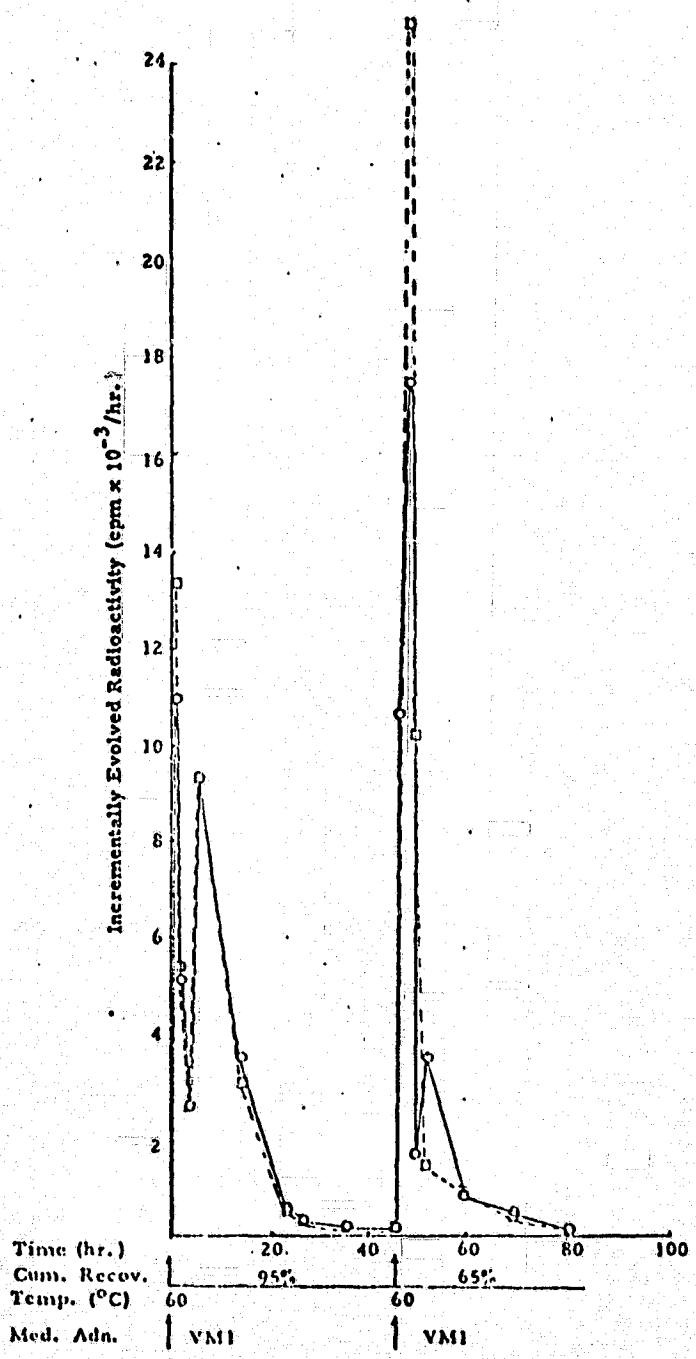
Figure 15

Evolution of $^{14}CO_2$ by Meadow Soil
Sequential Addition at 60°C



C-2

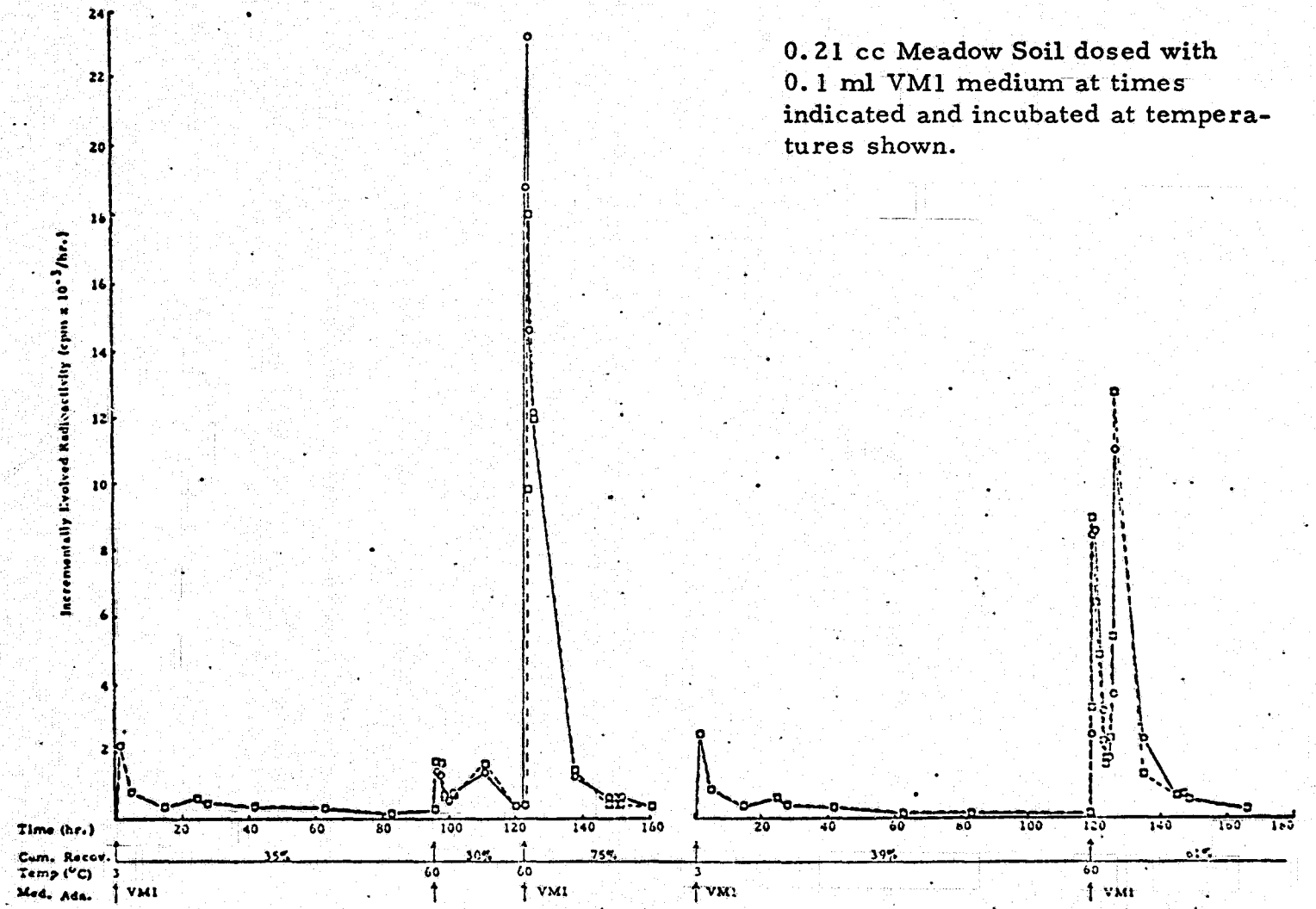
and both sets of soil samples were dosed with VM1 medium. Evolved radioactivity was collected throughout and results are shown in Figure 16. The soil which was incubated at 60°C for 28 hours prior to the second addition of VM1 medium produced a high single peak in activity immediately following the medium addition. The soil which had not been shifted to 60°C for 28 hours prior to medium addition produced two peaks in activity similar to those observed in the earlier experiment.

When the temperature was shifted to 60°C without medium addition, two small peaks in activity occurred which correspond quite well to the two peaks of evolution which resulted when a medium addition was made along with a shift to 60°C incubation. The reason for the observed phenomenon is not known; however, it is apparent that a shift in temperature from 3°C to 60°C induces metabolic activity which did not occur at the lower temperature. Except for quantitative differences, the peaks in this metabolism appear to be similar regardless of whether they arise from non-metabolized substrates remaining from the earlier addition or from the addition accompanying the temperature shift.

This phenomenon of two peaks is definite and reproducible. A possible explanation is that the two peaks represent two individual

Figure 16

Effect of Temperature Acclimatization on $^{14}CO_2$ Evolution by Soil



0.21 cc Meadow Soil dosed with 0.1 ml VM1 medium at times indicated and incubated at temperatures shown.

segments of the microbial population. Perhaps the first peak is caused by organisms most functional at the low temperature which are induced to metabolize rapidly at the increased temperature until death results. The second peak might then be due to thermophilic organisms which are induced to function after a lag of several hours at the higher temperature.

On the other hand, a reverse order of activity by temperature dependent segments of the population might be theorized. The first peak after a temperature shift might result from thermophiles poised for metabolism at the higher temperature, and the second peak could result from facultative thermophiles which metabolize at the lower temperature but which require a period of adaptation before becoming active at the higher temperature.

In any case, it is obvious that after a period of incubation at 60°C, a medium addition gives rise to an immediate rapid response corresponding to the first peak. The phenomenon of double peaks does not occur after a soil population has "adapted" to an increased temperature.

6. Retention of Viability in Long-Term Experiments

The effect of prolonged incubation of a soil culture which had reached plateau after a previous medium addition was

tested. In the multiple addition system, a plateau of evolution of radioactivity is comparable to exhaustion of the added medium and organism inactivity - perhaps death. However, it is important that subsequent doses of media be made to cultures which have not lost viability. Since it is difficult to determine when $^{14}CO_2$ evolution has reached completion, a culture may remain in a nutrient-deprived state for an indefinite period before another medium addition is made.

The experiment shown in Figure 13 shows the results of a medium addition made more than 100 hours after a preceding medium addition had reached plateau. These results and similar ones obtained at 35°C and 60°C verified that the Meadow soil population remains viable for a considerable time after being dosed with VM1 medium. Successive medium additions and incubation at various temperatures were conducted for as long as 520 hours. Soil so treated showed typical evolution patterns.

7. Multiple Addition vs. Single Addition: Temperature Effects

Experiments were performed to compare the $^{14}CO_2$ evolution from a single medium addition with that from multiple addition. Triplicate vials containing 0.21 cc of soil were dosed

with 0.1 ml of VM1 medium and incubated sequentially at 3°C, 20°C, 35°C and 60°C. Nine vials, each containing 0.21cc of soil, were also dosed with 0.1 ml of VM1 medium and incubated at 20°C, 35°C and 60°C, in triplicate. These single addition vials were incubated for one hour at the experimental temperature prior to addition of the ^{14}C labeled medium. Results are shown in Figures 17-19. At all three temperatures, the single addition mode produced lower peaks in activity and the time required for $^{14}CO_2$ evolution to drop to baseline values was longer. The single addition produced two peaks in activity, the second peak being the greater. This two peak phenomenon was most pronounced at the 60°C incubation temperature. The multiple addition mode, on the other hand, produced a high single peak.

Both procedures, however, demonstrated that the Meadow soil was more active at 35°C and 60°C than at 20°C. The experiment shows that either the single addition or the multiple addition modes may be used to show the effect of temperature. However, comparisons between specific substrates or incubation conditions should exclude the comparison of first addition response to a subsequent addition response. This in no way invalidates the

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Figure 17

Comparison of Multiple Addition
and Single Addition Responses at 20°C

0.21cc Meadow Soil
0.1ml VM1 medium (3.4μCi/ml)
Data represents triplicate results

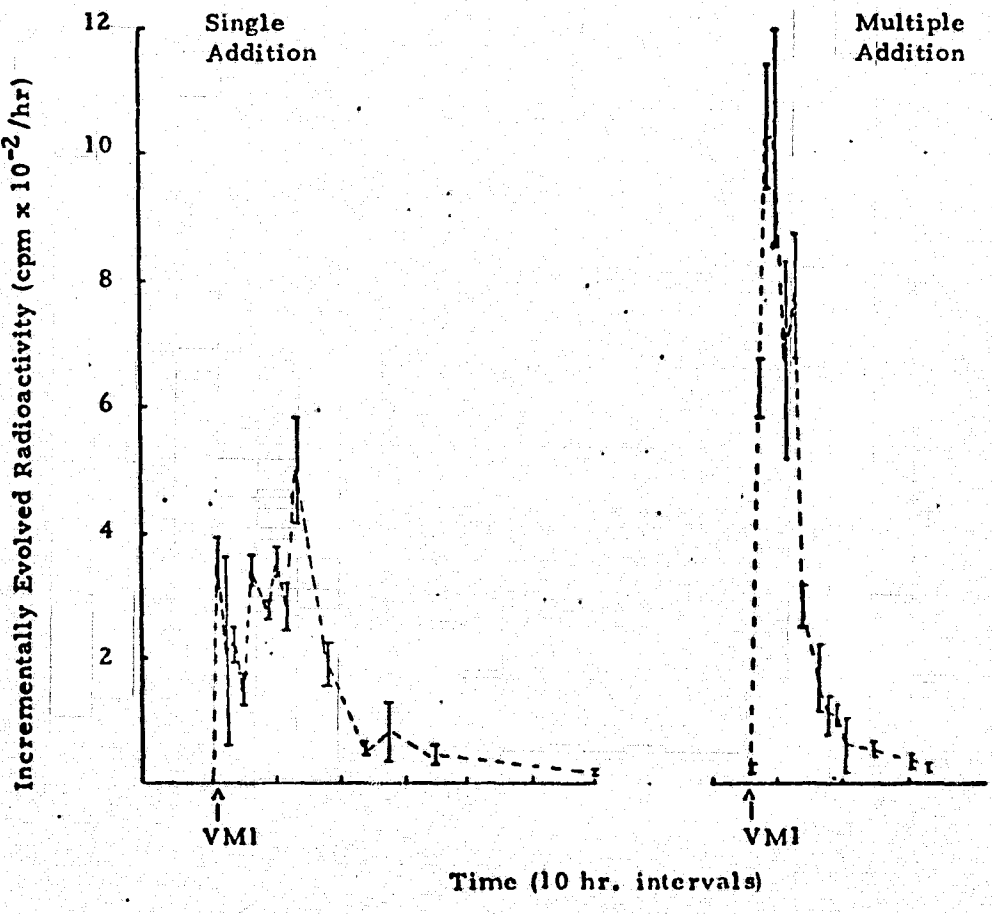

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Figure 18

Comparison of Multiple Addition and Single Addition Responses at 35°C

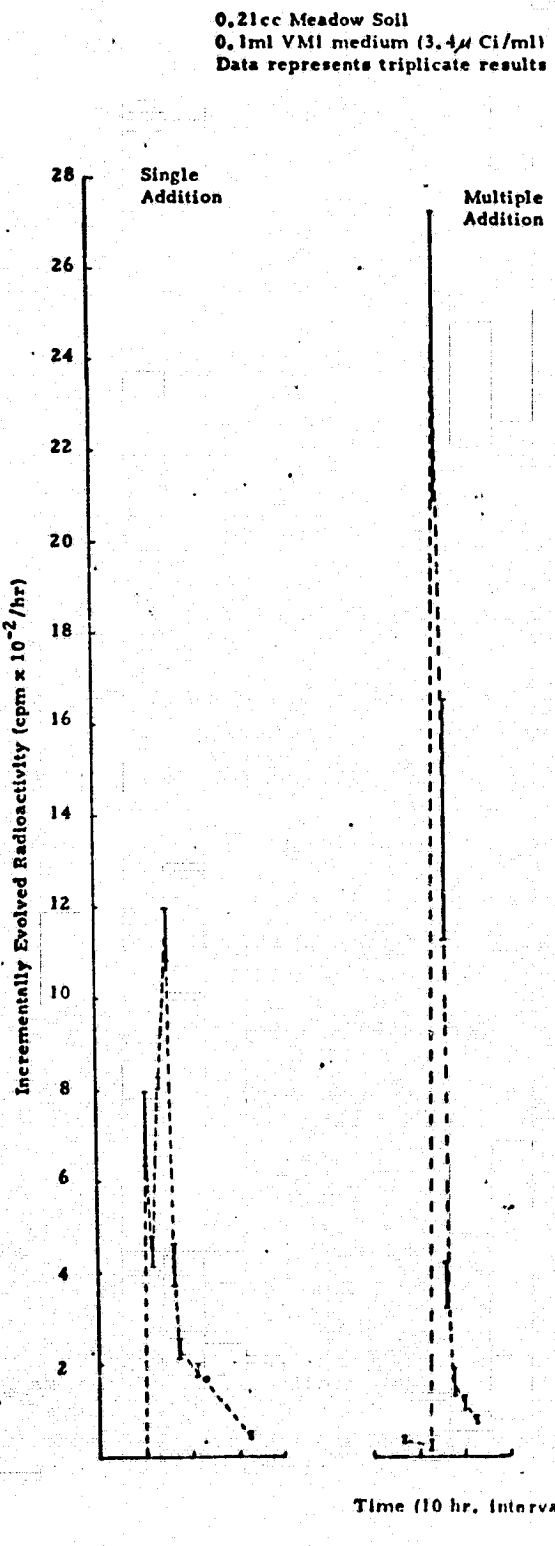

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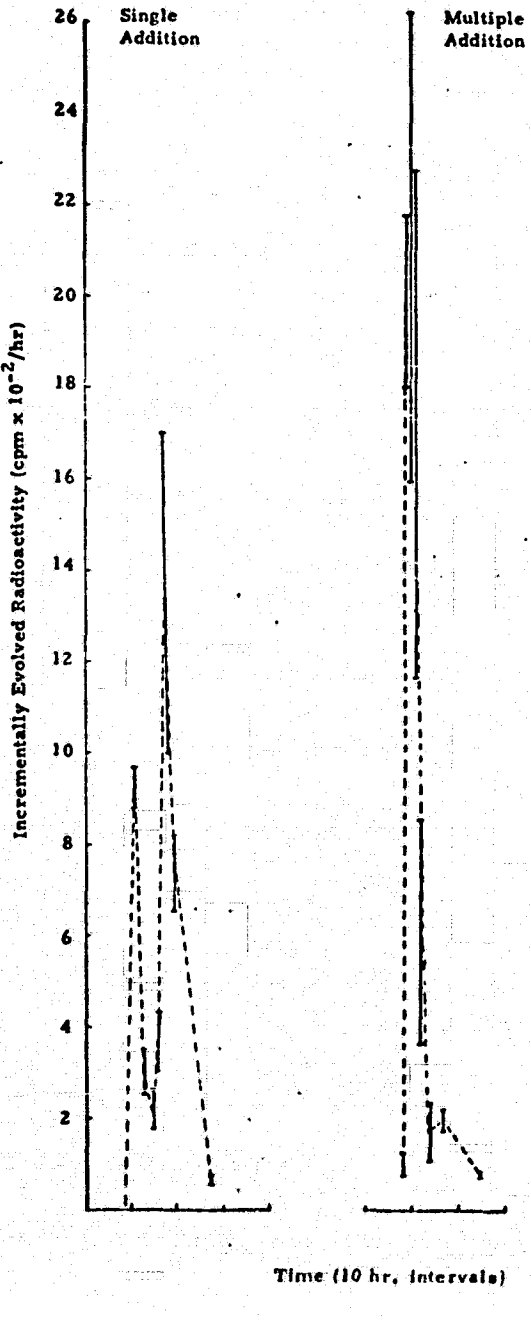
Figure 19

Comparison of Multiple Addition
and Single Addition Responses at 60°C

0.2 cc Meadow Soil
0.1ml VM1 medium (3.4 μCi/ml)
Data represents triplicate results



multiple addition mode of experimentation; but does require that comparisons of a series of additions exclude the first such addition.

8. Specifically Labeled Lactate and Acetate: Single Addition

The possibility of using specifically labeled lactate and acetate to obtain comparative biochemical information was investigated experimentally. It had been found in earlier experiments that the patterns of $^{14}CO_2$ evolution from ^{14}C formate and from uniformly labeled lactate and acetate varied somewhat depending upon the soil. Lactate and acetate are closely related to pyruvate and acetyl CoA, two key intermediates in terrestrial metabolism. Therefore, the kinetics of CO_2 generation from the individual carbon atoms of these compounds might provide evidence for the operation of various pathways in unknown organisms. Preliminary experiments were conducted in which additions of $1^{14}C$ lactate, $2^{14}C$ lactate, $1^{14}C$ acetate and $2^{14}C$ acetate were made to Mojave, Phoenix, and Meadow soils using the single addition mode. Results are shown in Figures 20-22. Although the magnitude of response from each substrate was soil dependent, the greatest amount of $^{14}CO_2$ evolution for each soil was from $1^{14}C$ lactate. The second most active substrate

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Figure 20

Evolution of $^{14}CO_2$ from Specifically Labeled Lactate and Acetate by Mojave Soil

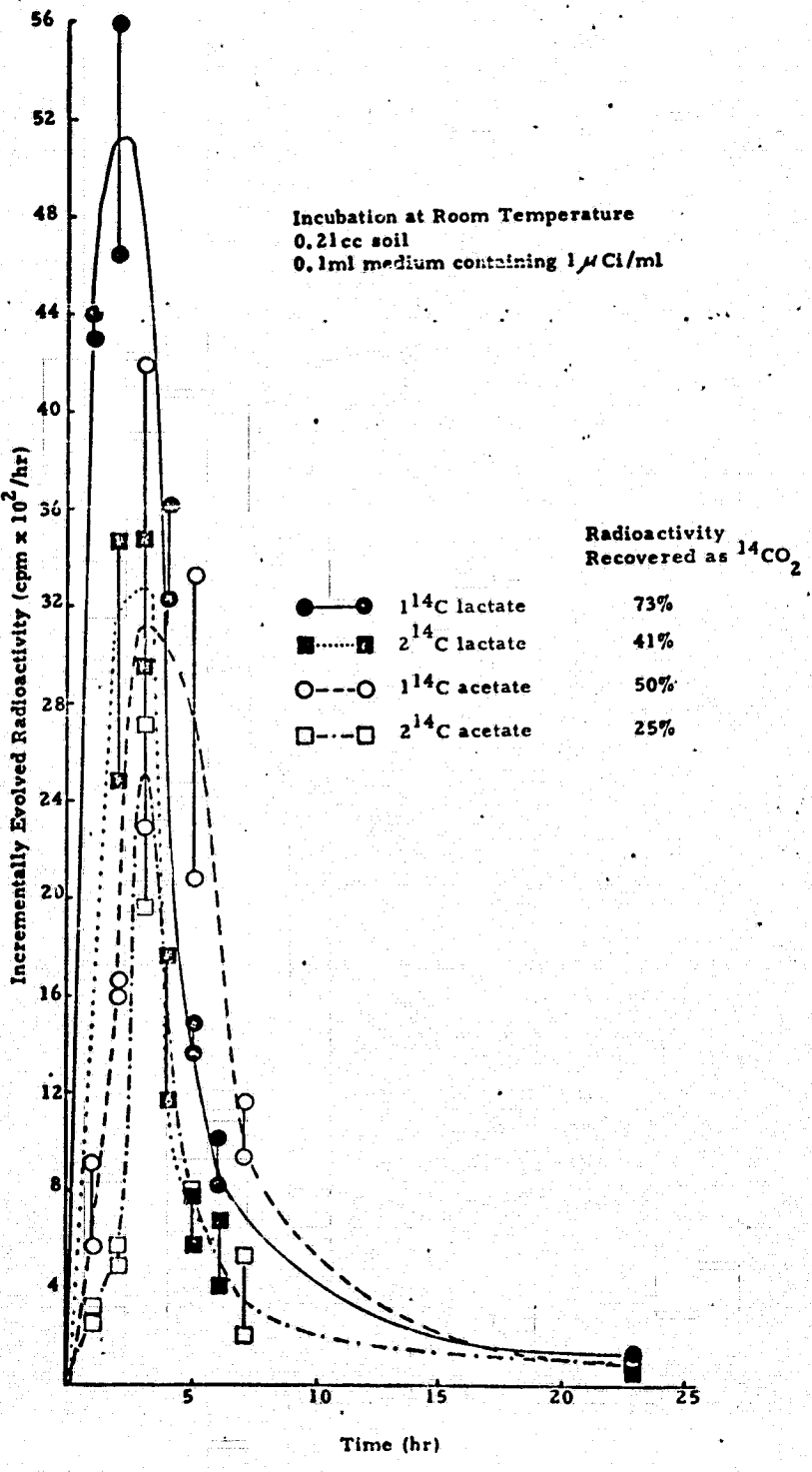

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Figure 21

Evolution of $^{14}CO_2$ from Specifically Labeled Lactate and Acetate by Phoenix Soil

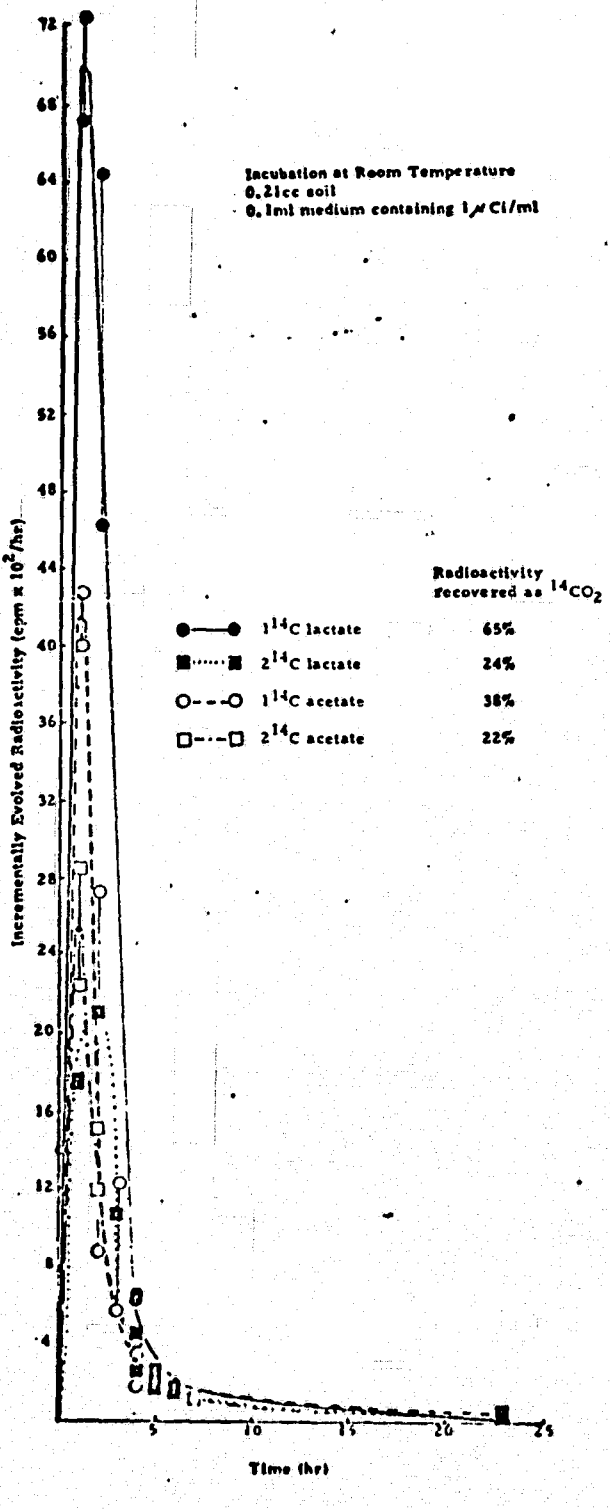

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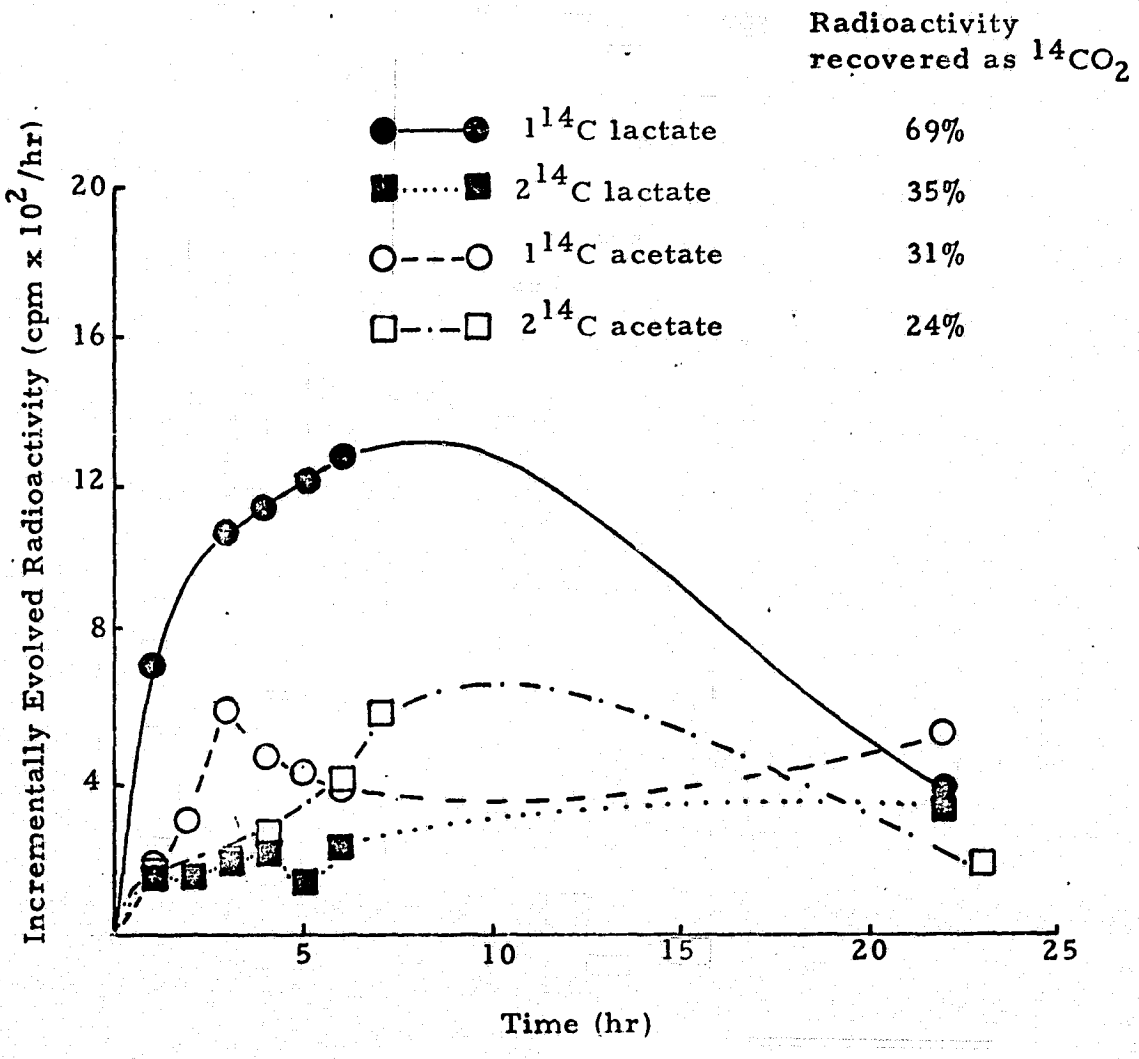
Figure 22

Evolution of $^{14}CO_2$ from Specifically Labeled Lactate and Acetate by Meadow Soil



Incubation at Room Temperature
0.21cc soil
0.1ml medium containing 1μ Ci/ml

was $1^{14}C$ acetate. The results demonstrate the facility with which decarboxylation occurs and that these carbon atoms are primarily expelled as the result of degradation. The fact that acetate produced lesser amounts of $^{14}CO_2$ may be due to a greater involvement of this compound in synthetic processes. With the Mojave soil, it is interesting that the $2^{14}C$ lactate produced a peak and evolution of $^{14}CO_2$, which was similar to that obtained with $1^{14}C$ acetate, possibly indicating that the degradation product of lactate decarboxylation primarily follows a pathway similar to that of acetate. Meadow soil showed similar recoveries of $^{14}CO_2$ from $1^{14}C$ acetate and $2^{14}C$ lactate. However, the kinetics of evolution from these compounds are dissimilar. The $2^{14}C$ lactate was evolved at a much slower rate.

The recovery of $^{14}CO_2$ from ^{14}C acetate with Phoenix soil was greater than from $2^{14}C$ lactate, possibly indicating that these two substrates are involved in different pathways of metabolism in this soil. The acetate may have become involved in energy yielding pathways, perhaps the citric acid cycle, whereas the two carbon fragment of lactate degradation may have been shunted to synthetic systems.

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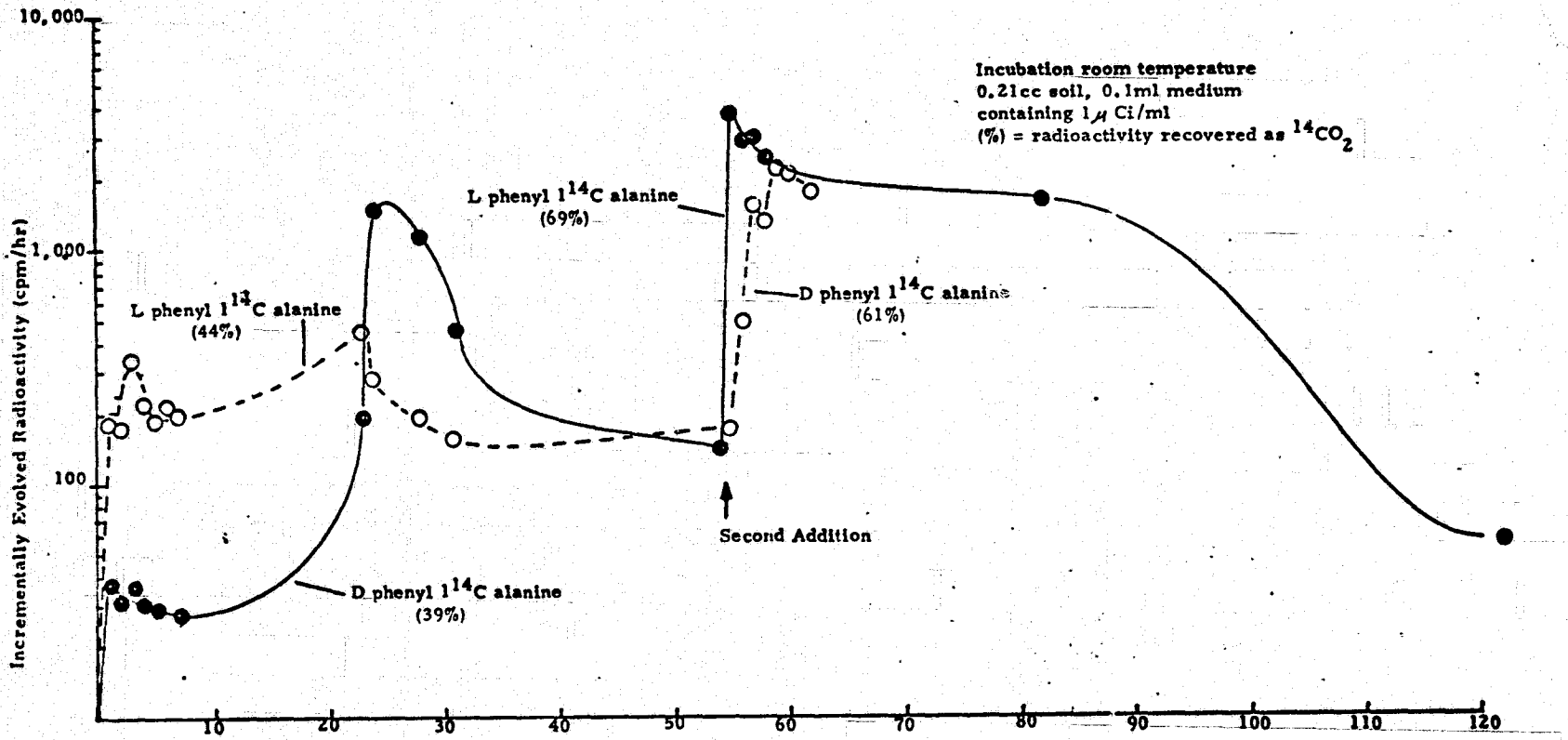
Further discussion of the degradation pathways of lactate and acetate will be presented in conjunction with the interpretation of the results of the multiple addition tests.

9. Specificity for D vs. L Substrate Configuration

Media to demonstrate a specificity for D vs. L substrate configuration were incorporated into the addition series early in the study. A large difference in the $^{14}CO_2$ evolution from D vs. L glucose was obtained. However, requirements for heat sterilization of the media destroyed these substrates, making them unsuitable for use in planetary landers. A medium containing UL ^{14}C D alanine, 1 ^{14}C D leucine and 3 ^{14}C D serine was compared with a medium containing UL ^{14}C L alanine, 1 ^{14}C L leucine and 3 ^{14}C L serine. Differences in the $^{14}CO_2$ evolved from these media were not great. Another possible candidate which was tested is phenylalanine. D phenyl 1 ^{14}C alanine and L phenyl 1 ^{14}C alanine were dosed onto separate samples of Meadow soil. Radioactivity was collected over a period of 54 hours and then a second dose of media was added. Soil initially dosed with D phenyl 1 ^{14}C alanine was dosed the second time with L phenyl 1 ^{14}C alanine, and vice versa. Results, as shown in Figure 23, demonstrate that the L form produced a more rapid response than the D form. It was also observed, however, that the second addition response was

Figure 23

Evolution of $^{14}CO_2$ from D and L Phenyl 1 ^{14}C Alanine by Meadow Soil



greater than the first addition response, independent of the D or L form. The magnitude of response from a first addition of L phenylalanine was less than a second addition of D phenylalanine. It was therefore obvious that a first addition of one substrate could not be compared with subsequent additions of another substrate.

An experiment was performed to determine if a prior addition of phenylalanine affects a subsequent response from an addition of phenylalanine. Results shown in Figure 23 indicated a definite influence. However, it was not possible to conclude that phenylalanine specifically enhanced the second addition, since the initial addition of medium to that soil was phenylalanine and therefore the response enhancement might be nonspecific. (See discussion in Section II-C-7 of this report). Would a first addition of a substrate other than phenylalanine also show the same effect? To answer this question, an experiment was conducted in which media containing 2 ^{14}C lactate and 2 ^{14}C lactate plus unlabeled DL phenylalanine were dosed onto soil incubated 24 hours and then subsequently dosed with D or L phenyl 1 ^{14}C alanine. The ^{14}C lactate was used since it is proposed to precede phenylalanine in the multiple addition sequence. (See Chamber Addition Sequence, Figure 4).

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As shown in Figure 24, preincubation of the soil in the presence of DL-phenylalanine enhanced the rate of $^{14}CO_2$ evolution from both D and L-phenyl 1 ^{14}C alanine added subsequently. However, the L form showed a greater rate and a higher peak than the D form. The yield of $^{14}CO_2$ from both the D and L forms was the same with and without a preincubation in DL-phenylalanine. These data show that both forms are utilized. However, the rate of degradation of the D form is much slower and may involve a rate limiting racemase.

It should also be noted from Figure 24 that the presence of DL-phenylalanine did not effect the evolution of $^{14}CO_2$ from 2 ^{14}C lactate. These findings suggested that ^{14}C labeled substrate experiments performed in the multiple addition mode should be preceded by a preincubation in that substrate. This could be done by adding unlabeled substrate to the media preceding that ^{14}C labeled substrate.

10. Total Colony Counts of Multiple Addition Experiments

It has been shown that a first addition of ^{14}C labeled medium to soil produces a rate of $^{14}CO_2$ evolution less than that from a subsequent addition. This phenomenon is not substrate specific and occurs when the initial substrate differs from the

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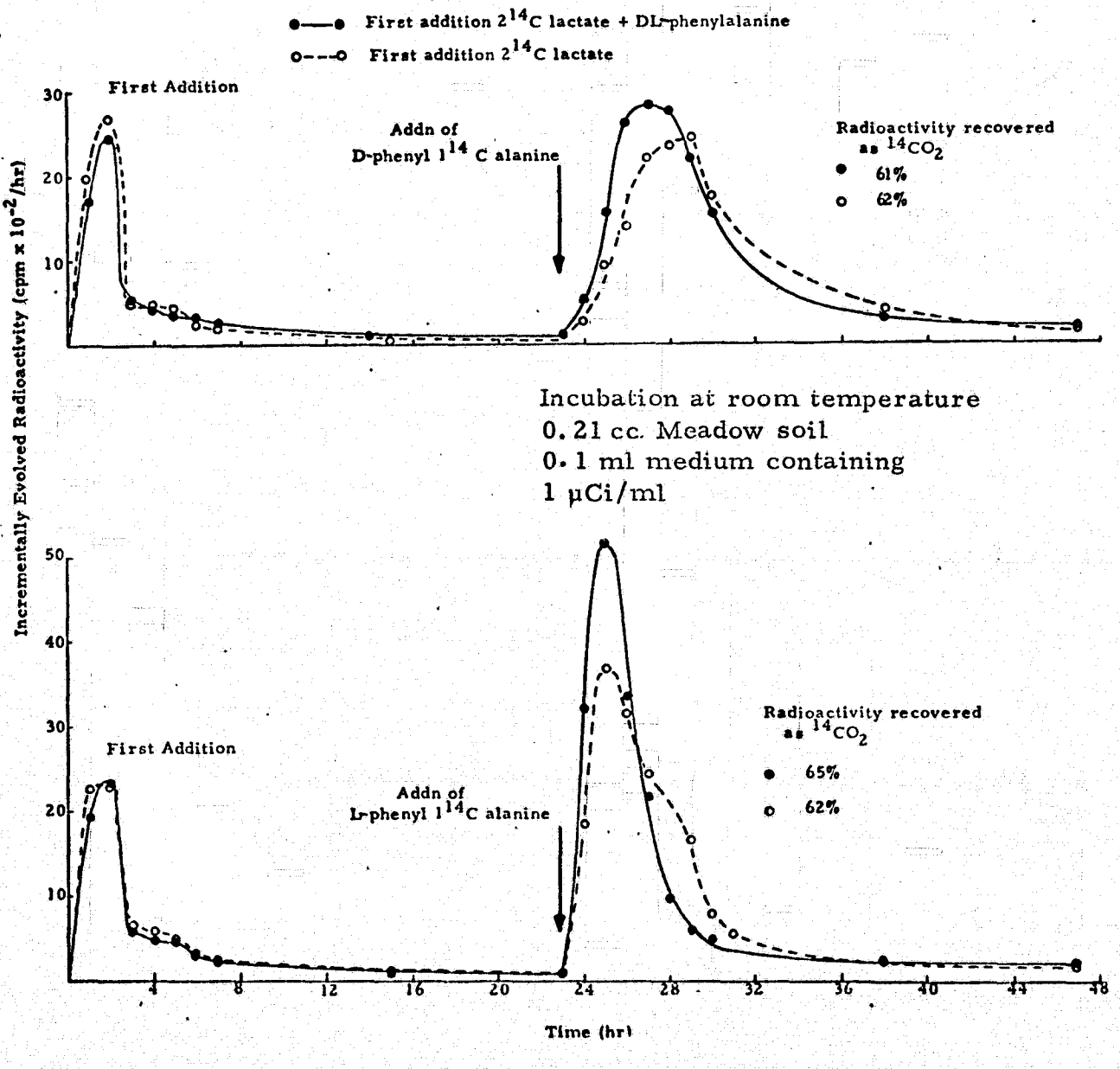
Figure 24

Evolution of $^{14}CO_2$ from D and L-Phenyl $1^{14}C$ Alanine with and without Preincubation in Phenylalanine



subsequent addition. A possible explanation is that the microorganisms reproduce after the first medium addition and, therefore, subsequent additions of medium involve larger numbers of organisms.

Total plate counts were conducted on a series of soil samples which had been subjected to multiple additions of media. Soil cultures which had received one or more sequential doses of VM1 medium were brought to 2 ml with distilled water, and sonicated to loosen microorganisms from soil particles and to break up clumps. Aliquots of this suspension were diluted and pour plates of nutrient agar prepared. Plates were incubated for six days at room temperature and the total number of colonies counted. Results obtained over a period of 118 hours,which included three medium additions, are shown in Figure 25. No significant increase in the number of colonies occurred during the first 22 hours; however, a two to five fold increase was found after 24 hours of incubation. The number of colonies found after subsequent medium additions did not appear to change significantly and ranged from 6×10^7 to 3×10^8 colonies per cc of soil added. Plate counts obtained on a long-term (440 hours) multiple addition experiment which involved the addition of 13 individual substrates (a total of 1.3 ml H_2O) showed 2×10^8 to 5×10^8 colonies/-cc soil.

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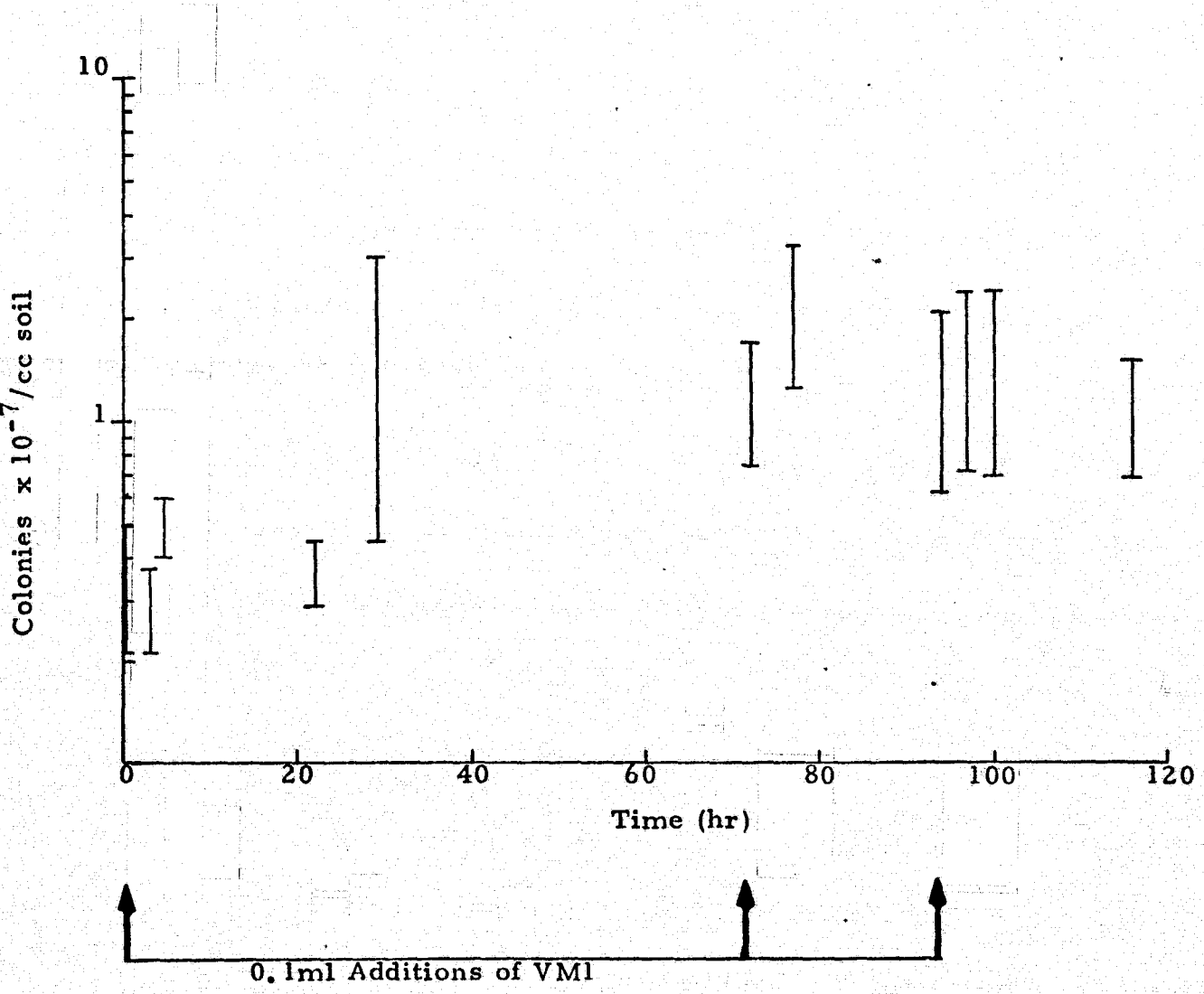
Figure 25

Number of Colonies Plated from Meadow Soil During Multiple Addition of Media

Medium for Plate counts was Nutrient Agar. Plates were incubated at room temperature for six days. 0.21 cc Meadow soil was dosed with 0.1ml VM1 and incubated at room temperature. Three soil replicates and four plate count replicates (12 values) were used to establish each bar.



As shown in Figure 25, the addition of a substrate appeared to increase cell numbers. However, the number of bacteria as determined by colony counts remained relatively constant after the second and third medium additions. It was also noted that the colonies obtained after a long-term multiple addition experiment were morphologically similar to those obtained with fresh soil. Furthermore, the pour plates of these soil suspensions showed that the relative abundance of the various morphologically distinguishable species remained unchanged.

These data suggest that the first substrate addition produces an increase in microorganisms; however, continued incubation and additional substrate additions produce only minor fluctuations in the number of microorganisms. The small increase in numbers of organisms may explain the greater rate of substrate degradation, by subsequent additions beyond the first. Further, it indicates that the first addition is sufficient to permit growth of the organism to the maximum number. The ecological system will support third and later additions and show no further increase in the rate of $^{14}CO_2$ evolution. At present, there is no explanation for the corresponding increase in the yield of $^{14}CO_2$ from additions subsequent to the first.

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11. Stimulation of $^{14}CO_2$ Evolution by Subsequent Substrate Additions

The position of the ^{14}C carbon atom in the labeled substrate affects not only the rate at which $^{14}CO_2$ is produced, but also the total yield of $^{14}CO_2$ which occurs. It is suspected that an addition of another substrate may induce the further degradation of a substrate remaining from a previous addition. To test this possibility, a series of ^{14}C labeled substrates was added to soil, and the evolution of $^{14}CO_2$ monitored. Following the peak in $^{14}CO_2$ evolution, a second addition of an unlabeled substrate was made. As shown in Figures 26-28, the addition of unlabeled substrate in cases where evolution of radioactivity from the initial medium addition had dropped to a low level resulted in a slight but detectable peak in evolution.

In cases where the radioactive evolution from a first addition of labeled substrate had not yet returned to a low level, a following addition of nonlabeled substrate produced a greater peak in activity. However, this peak was nowhere near the order of magnitude obtained by additions of labeled substrate.

These experiments provided assurance that, in the multiple addition mode, $^{14}CO_2$ evolution which occurs after an addition of ^{14}C labeled substrate, arises from that substrate and not from

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Figure 26

Effect of an Addition of Formate on the Evolution of $^{14}CO_2$ from ^{14}C Labeled VM1 Medium

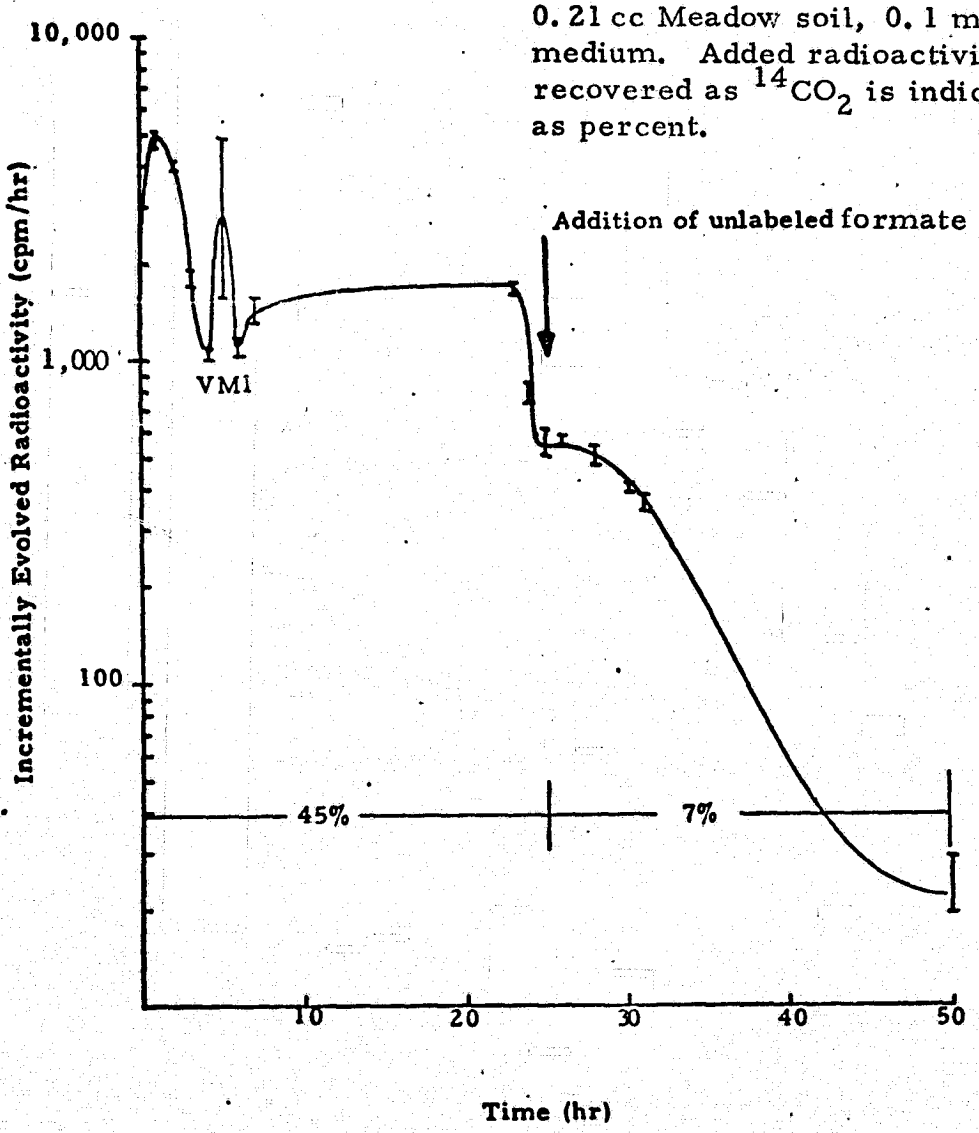

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Figure 27.

Effect of a Lactate Addition on the Evolution of $^{14}CO_2$ from ^{14}C Formate

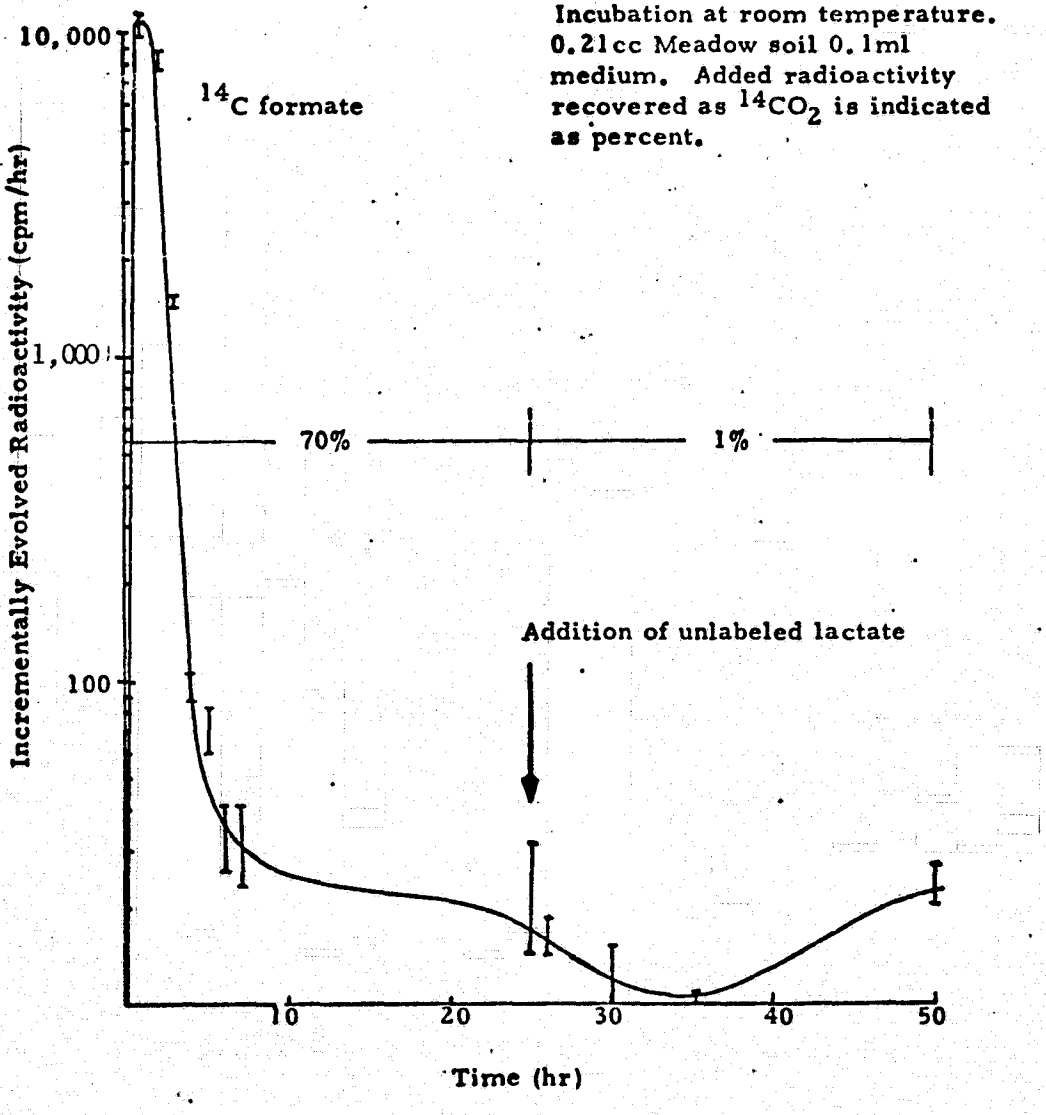

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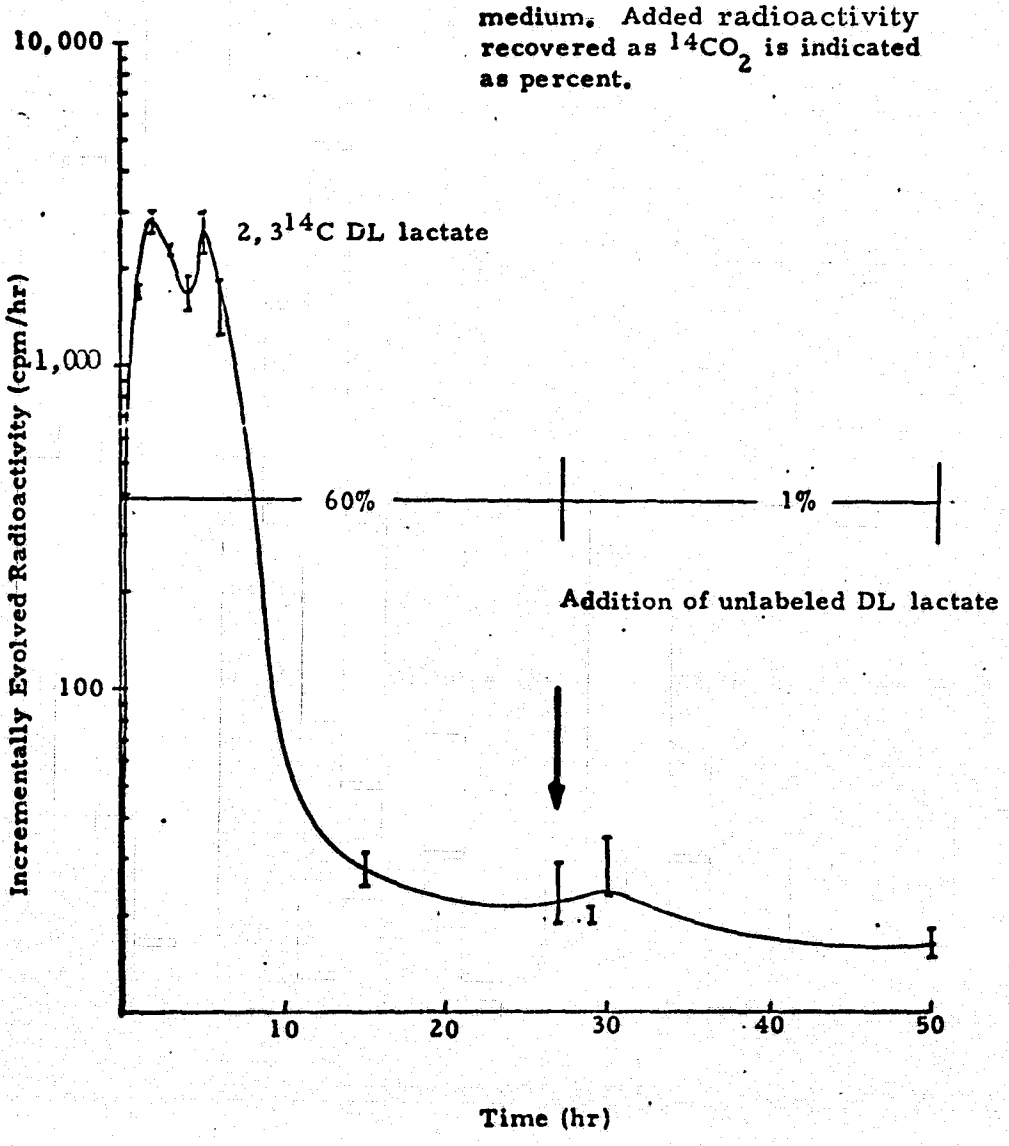
Figure 28

Effect of a Second DL-Lactate Addition on the Evolution of $^{14}CO_2$ from 2, $3^{14}C$ DL Lactate



the metabolism of residual ^{14}C labeled substrates or intermediates remaining from earlier additions.

12. Effect of Nonlabeled Substrates in the ^{14}C Labeled Medium

A multiple addition series which included ^{14}C labeled VM1, $1^{14}C$ formate, $1^{14}C$ acetate, $2^{14}C$ acetate, $1^{14}C$ lactate, $2\text{-}3^{14}C$ lactate, $2^{14}C$ lactate, L-phenyl $1^{14}C$ alanine, D-phenyl $1^{14}C$ alanine, $1^{14}C$ glutamate, $2^{14}C$ glutamate, $3\text{-}4^{14}C$ glutamate, and $5^{14}C$ glutamate was conducted. Media containing these specifically labeled substrates were sequentially added to a single soil sample. Two independent series of media were investigated, both of which included the same ^{14}C labeled substrates. Series (A) was composed of media which contained one ^{14}C labeled substrate and no unlabeled substrates. Series (B) was composed of media which contained one ^{14}C labeled substrate and an unlabeled substrate which was supplied to induce activity for the labeled substrate scheduled for the next medium addition. The media used in the two test series are listed in Table 2 in the order in which they were added to the soil.

By using the medium Series B, a soil culture was not dosed with a ^{14}C labeled substrate with which it was unfamiliar.

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Table 2

Media* Used for Multiple Addition Tests
Listed in Order of Addition

Code	Series (A)	Series (B)
VMI	VMI **	VMI *
F	1^{14}C formate	1^{14}C formate + acetate
A_1	1^{14}C acetate	1^{14}C lactate
A_2	2^{14}C acetate	2^{14}C acetate + DL-lactate
L_1	1^{14}C L-lactate	1^{14}C L-lactate
L_3	2-3^{14}C L-lactate ***	2-3^{14}C L-lactate
L_2	2^{14}C L-lactate	2^{14}C L-lactate + DL-phenylalanine
PA_L	L-phenyl 1^{14}C alanine	L-phenyl 1^{14}C alanine
PA_D	D-phenyl 1^{14}C alanine	D-phenyl 1^{14}C alanine
G_1	1^{14}C DL-glutamate	1^{14}C DL-glutamate
G_2	2^{14}C DL-glutamate	2^{14}C DL-glutamate
$G_{3,4}$	3-4^{14}C DL-glutamate	3-4^{14}C DL-glutamate
G_5	5^{14}C DL-glutamate	5^{14}C DL-glutamate

* All substrates at a concentration of 2.5×10^{-4}M.

** VMI, 1 μCi/ml composed of UL^{14}C L-alanine, UL^{14}C D-alanine, UL^{14}C glycine, ^{14}C formate, UL^{14}C DL-lactate, UL^{14}C glycolic acid. (Total radioactivity 3.4 μCi/ml).

*** Data are obtained for 3^{14}C lactate by multiplying cpm for 2-3^{14}C lactate (1 μCi/ml) x 2 and subtracting cpm for 2^{14}C lactate (1 μCi/ml).

3^{14}C lactate is not commercially available except as a special item.

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The induction of enzymes no longer was involved in the response from a given substrate, and differences in the rate and yield of $^{14}CO_2$ were interpreted more easily in terms of metabolic pathways.

Results of multiple addition tests on Meadow soil utilizing the media additions listed in Series A and B were very similar. The presence of unlabeled substrates did not affect the kinetics or total cumulative amount of $^{14}CO_2$ evolved from the substrate with which they were mixed. However, the fact that little or no effect was induced by the unlabeled substrates is not considered to be justification for excluding them. The possibility of either enhancement or inhibition exists. The probability of the former would seem to be greater. Although not observed, it is possible that dilution of the radioactivity by the unlabeled substrate could decrease the rate of $^{14}CO_2$ evolution from the labeled substrate.

D. Long-Term Multiple Addition Tests with Viable Soil

A series of multiple additions of media containing ^{14}C labeled substrates were made to eight replicate samples of Meadow soil. The media and times of addition were as shown in Table 3. Radioactive gas was collected, measured, and expressed as the

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Table 3

Sequence of Multiple Addition to Meadow Soil

Time of Addition (hr.)	Code	Substrate
0	VM1	VM1
26	F	^{14}C formate
49	A_1	1 ^{14}C acetate
112	A_2	2 ^{14}C acetate
137	L_1	1 ^{14}C lactate
158	L_3	3 ^{14}C lactate
180	L_2	2-3 ^{14}C lactate
282	PA_2	L-phenyl 1 ^{14}C alanine
308	PA_D	D-phenyl 1 ^{14}C alanine
330	G_1	1 ^{14}C glutamate
355	G_2	2 ^{14}C glutamate
379	G_{3-4}	3-4 ^{14}C glutamate
409	G_5	5 ^{14}C glutamate

amount evolved per hour. Results of this series of substrate additions are shown in Figure 29. The error bars on the graph indicate the range in values obtained for the eight replicates. These data demonstrate the reproducability of peaks obtained with various substrates and show that after reaching a peak, the evolution in radioactivity drops to a level of 100-250 cpm after approximately 24 hours.

Results obtained in a similar experiment involving viable Phoenix soil (4 replicates) are shown in Figure 30. This latter series included an initial test for the effect of high and low moisture using two additions of VM1 (0.01 ml and 0.5 ml, respectively), and a final series of additions which included antimetabolites. The recovery of added radioactivity for the two soils is shown in Table 4.

The small volume of medium (0.01 ml) added to Phoenix soil produced a response rate of 1,000 cpm/hr vs. 5,000 cpm/hr for the larger volume of medium (0.5 ml). Since the larger volume of medium contained 50 times more radioactivity, the dose adjusted rate of response from the smaller volume was ten-fold more rapid than from the larger volume. The recovery of added radioactivity is also greater for the 0.01 ml addition than for the 0.5 ml addition. It was found in preliminary studies that the rate of evolution and

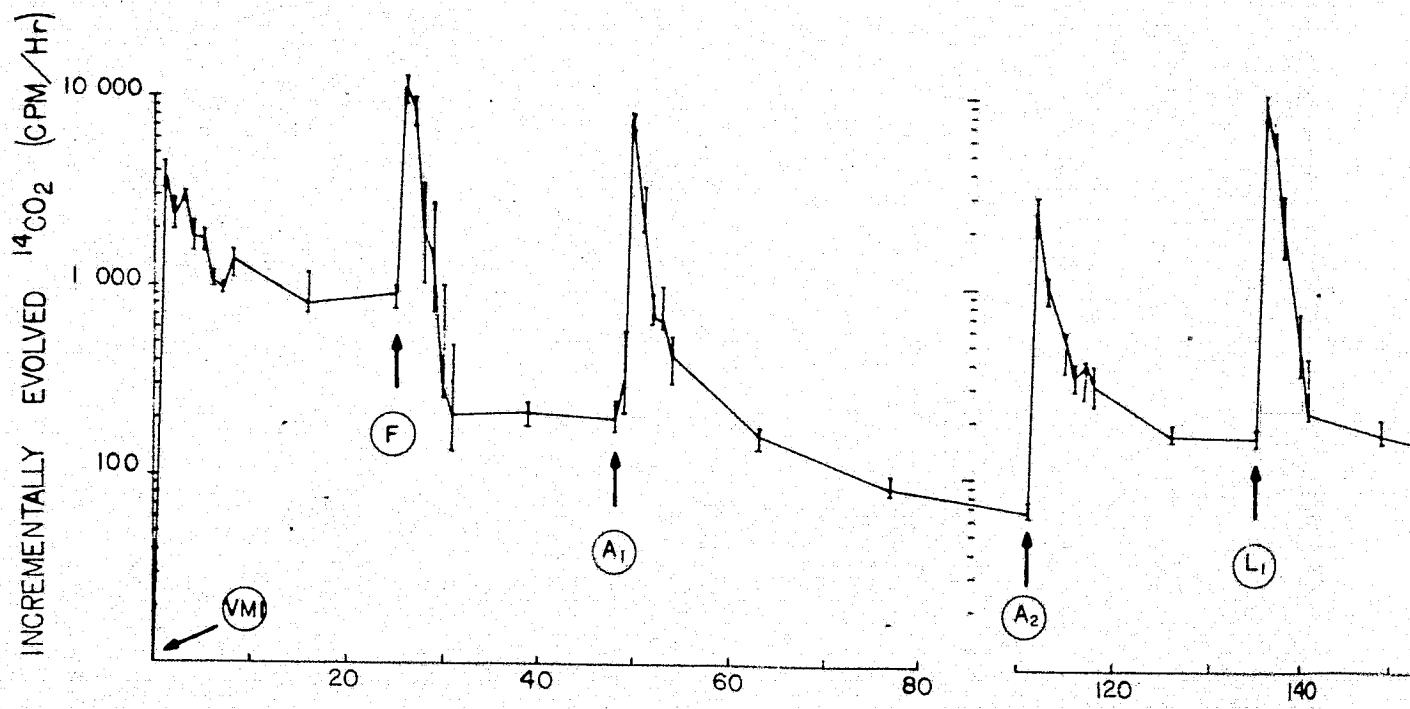
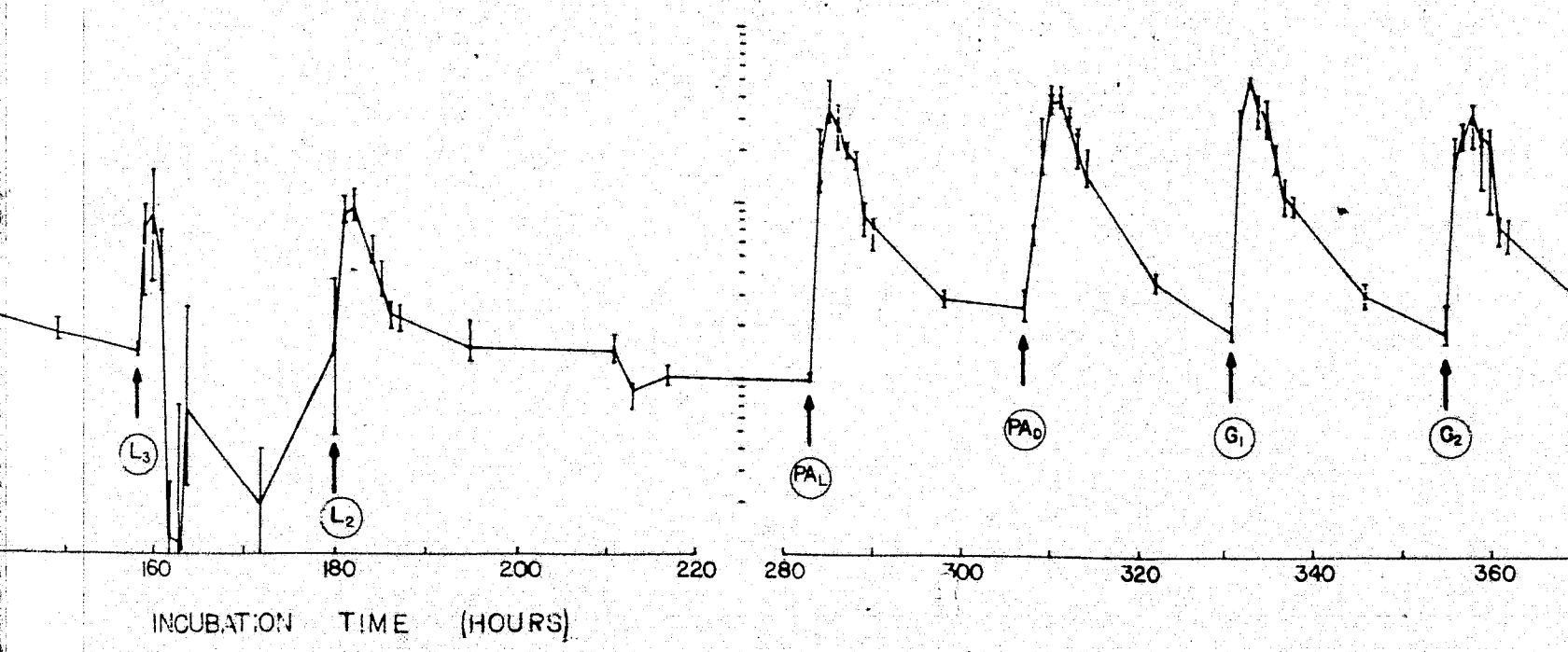


FIGURE 29

MULTIPLE ADDITION SUBSTRATE SERIES

MEADOW SOIL TEST



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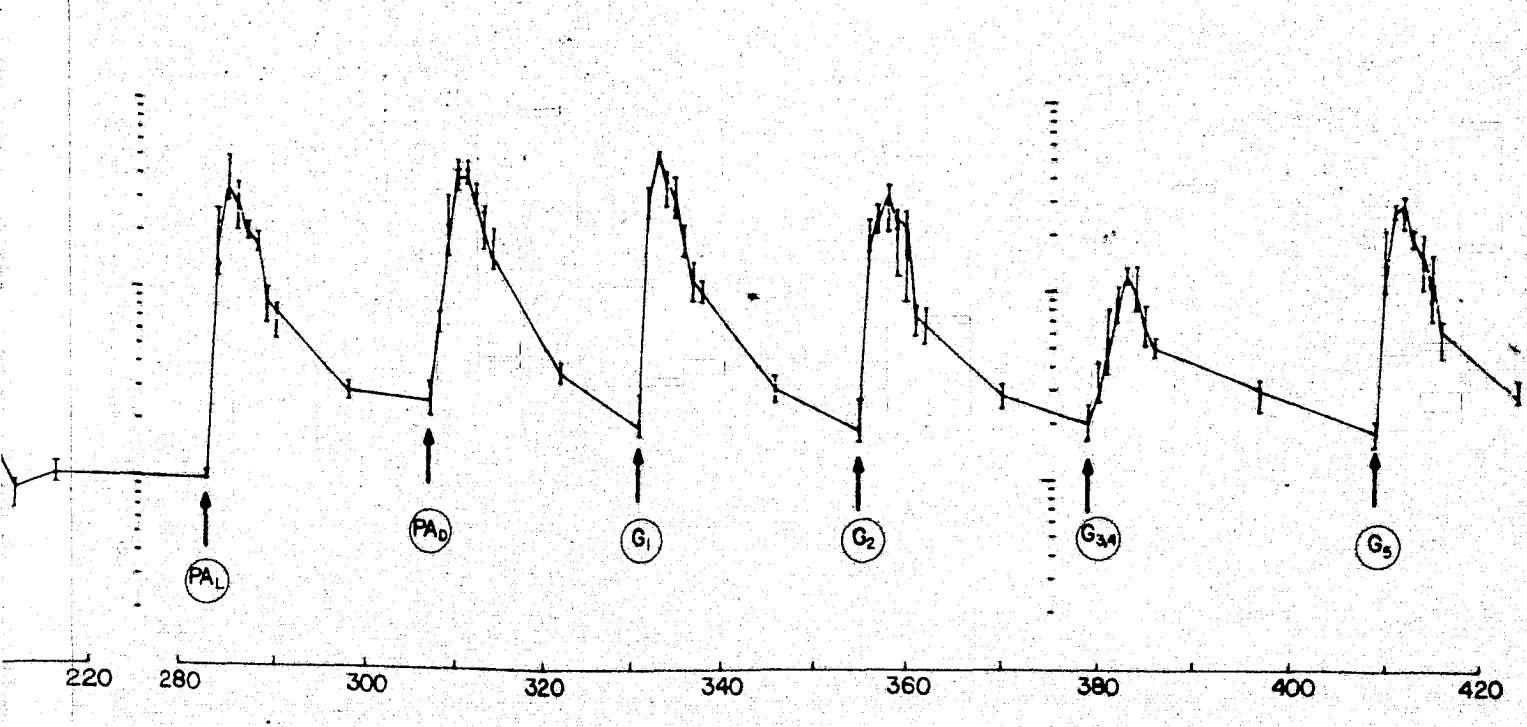
RATE SERIES



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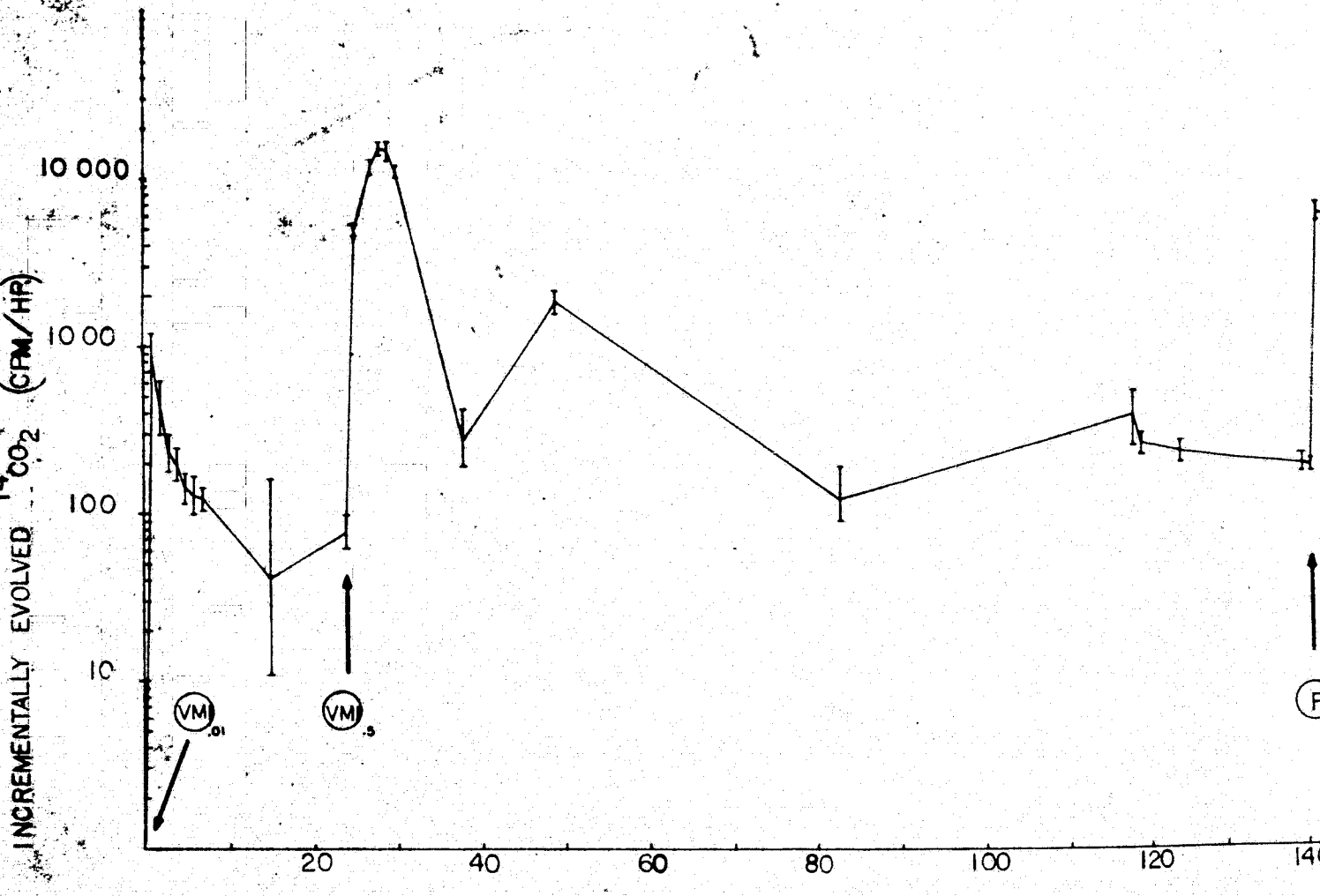

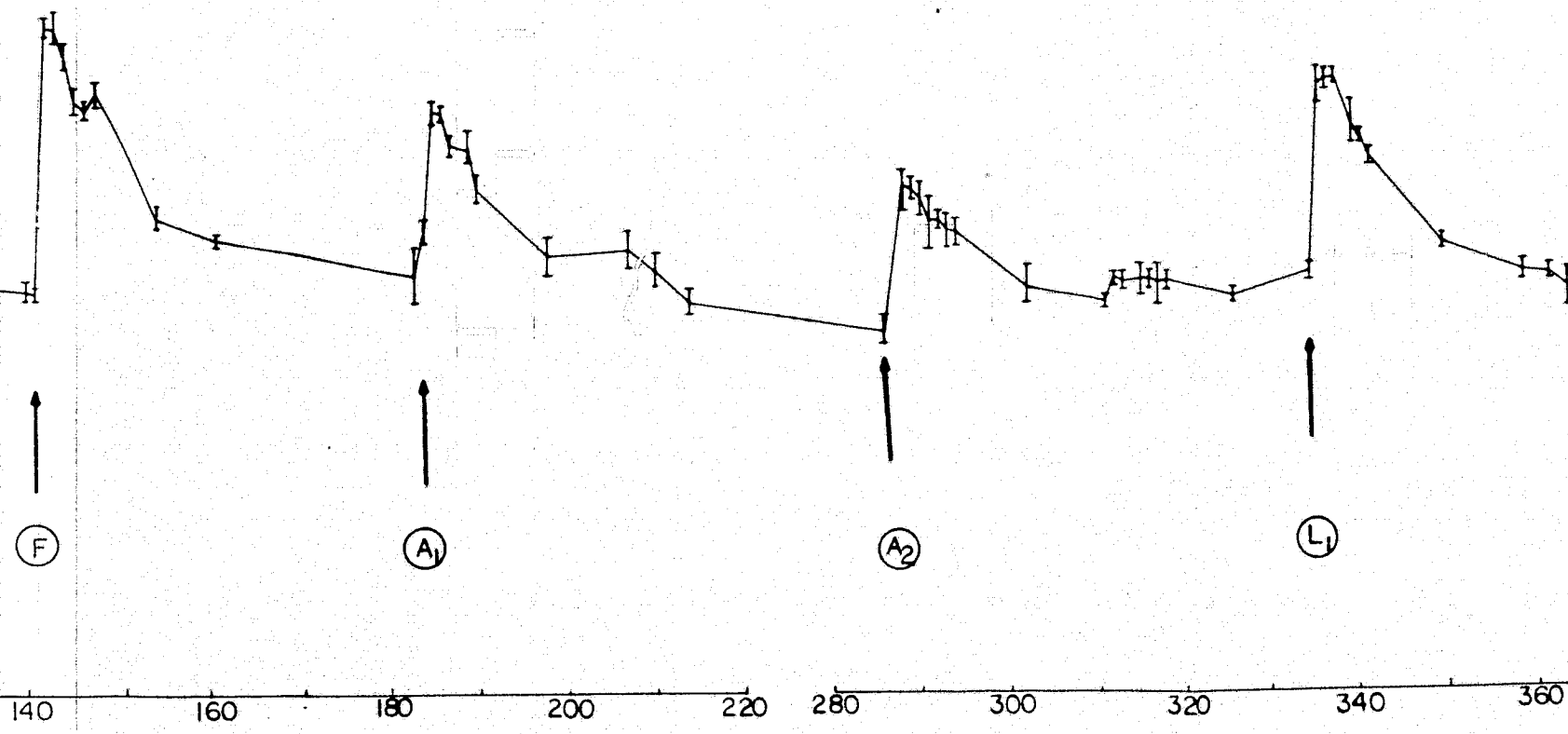

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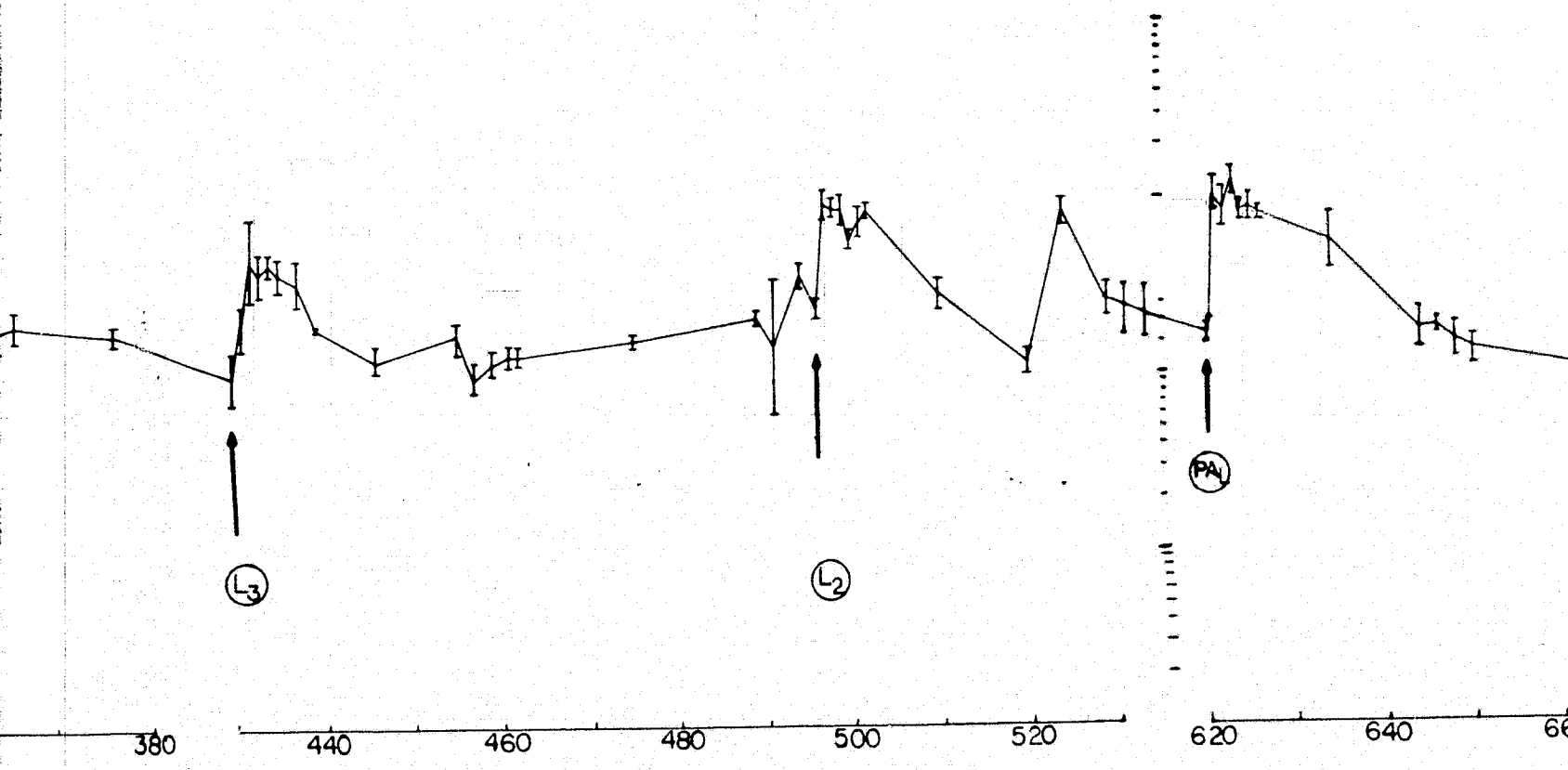

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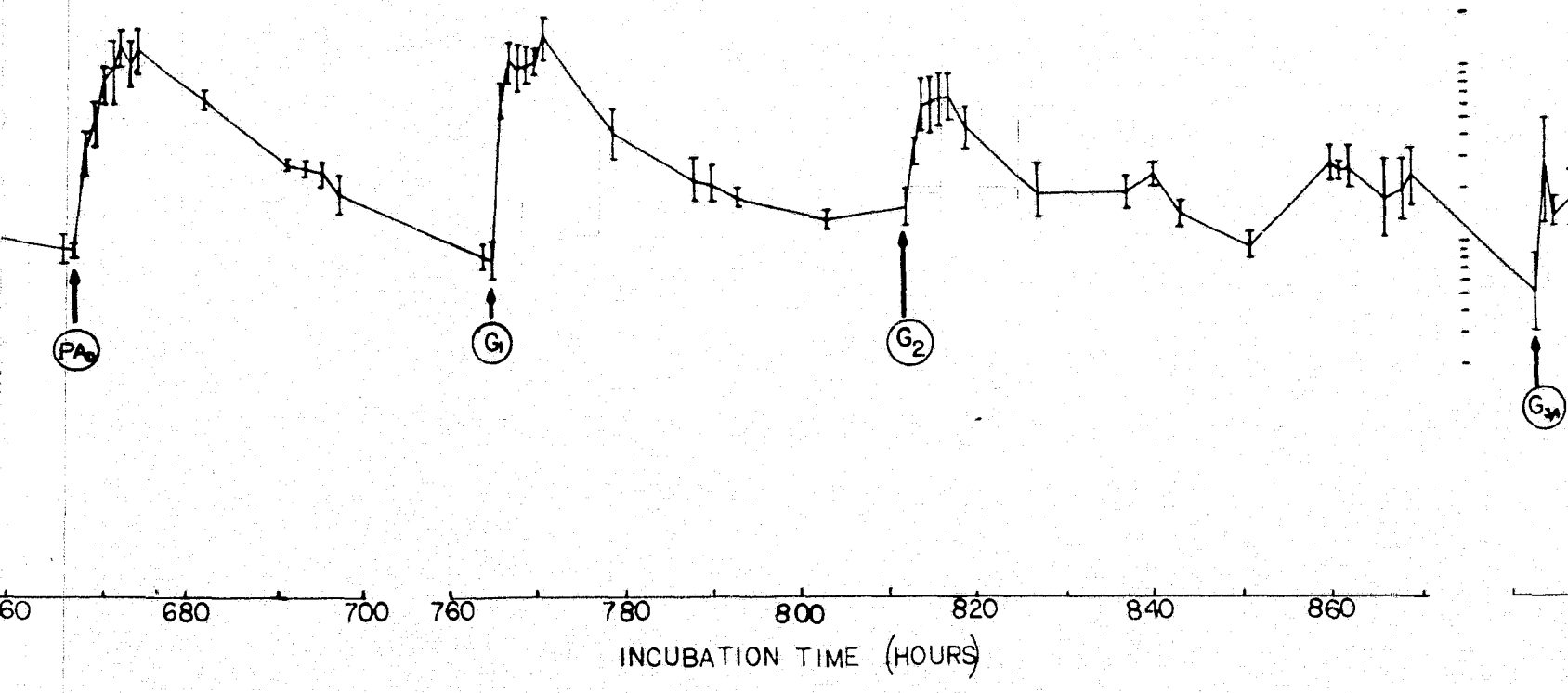
FIGURE 30

MULTIPLE ADDITION

CHAMBER 4 ADDITION SEQUENCE

WATER, METABOLISM, AND ANTIMETABOLITES

PHOENIX SOIL



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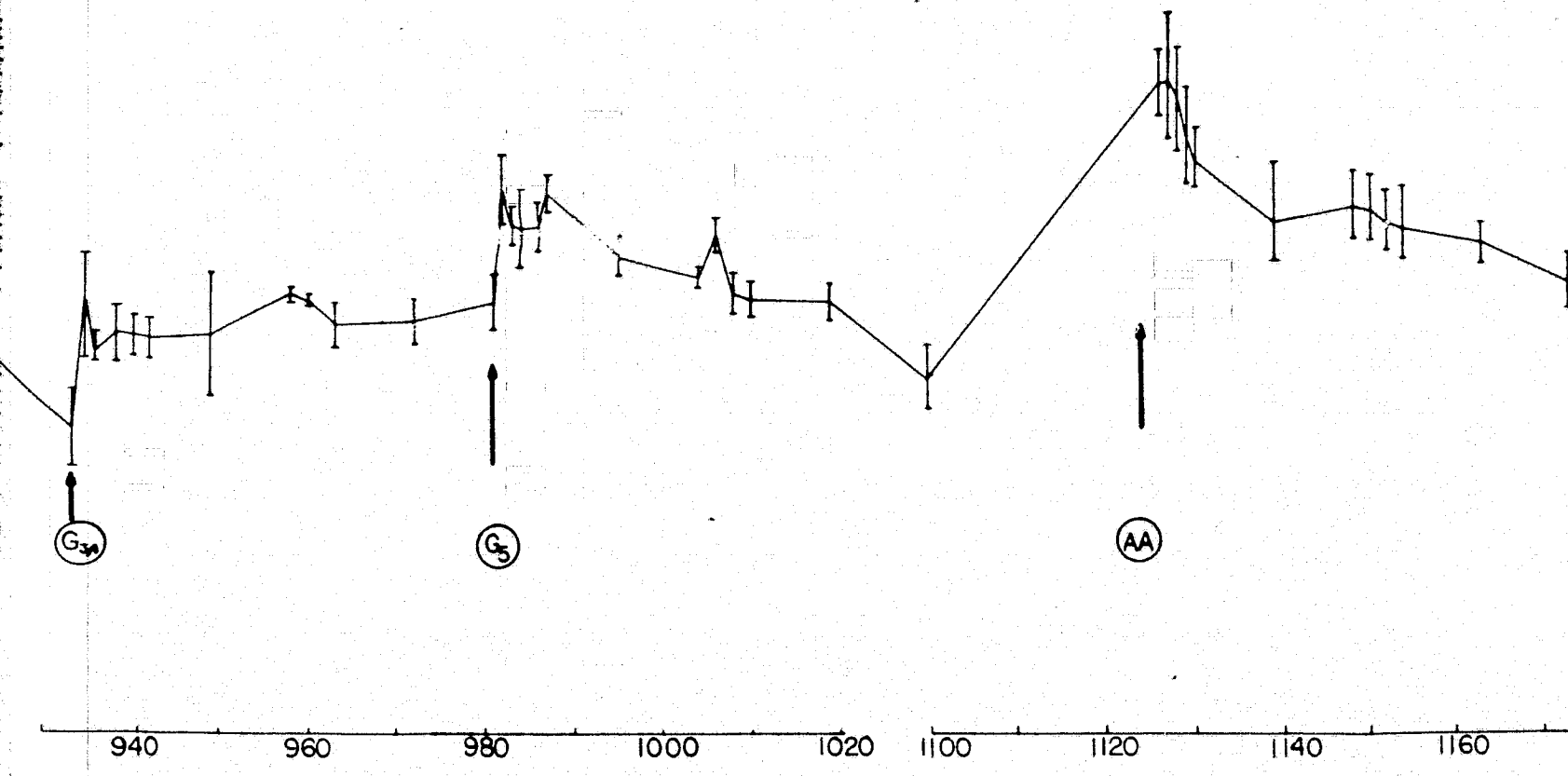

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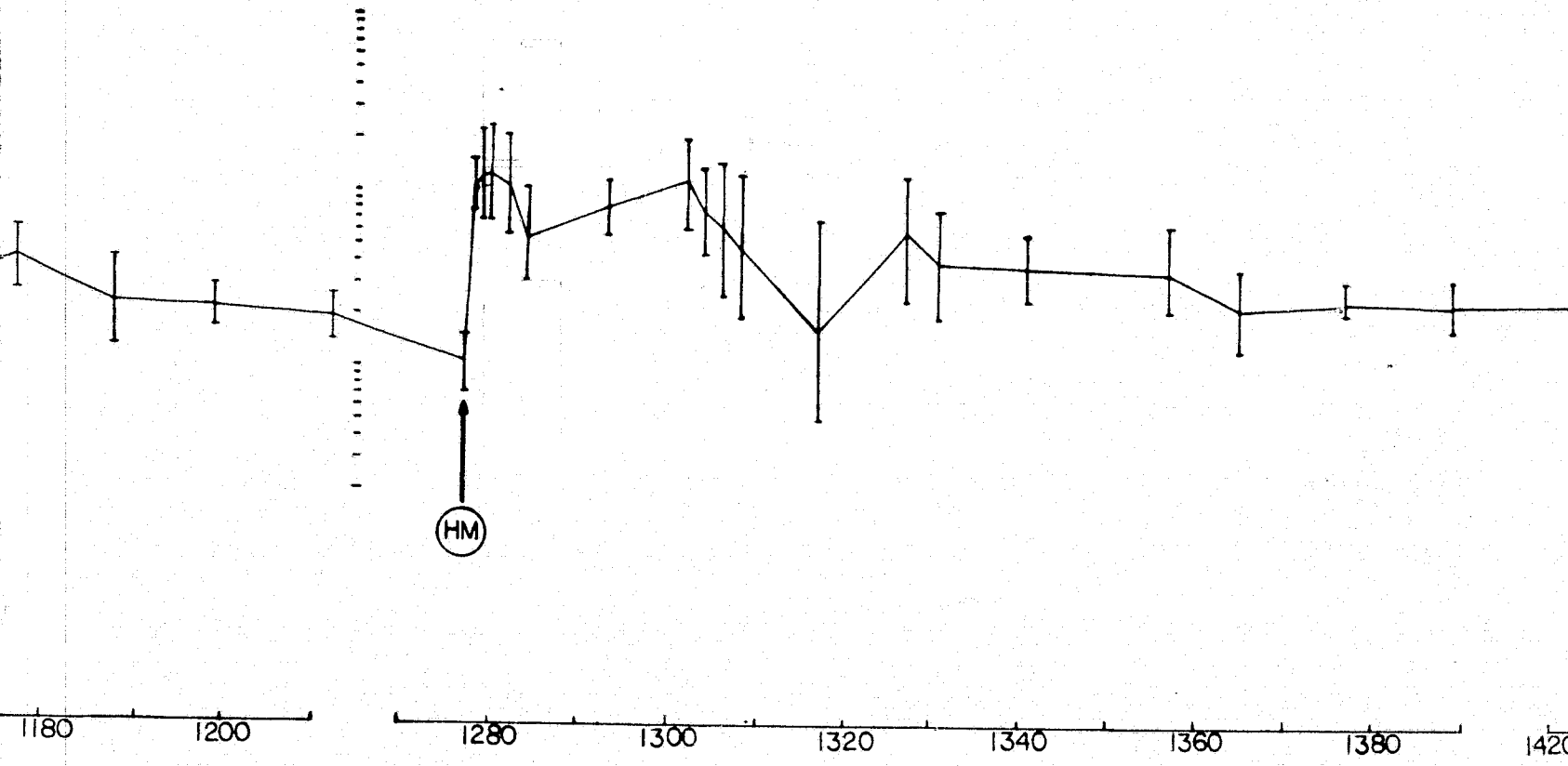

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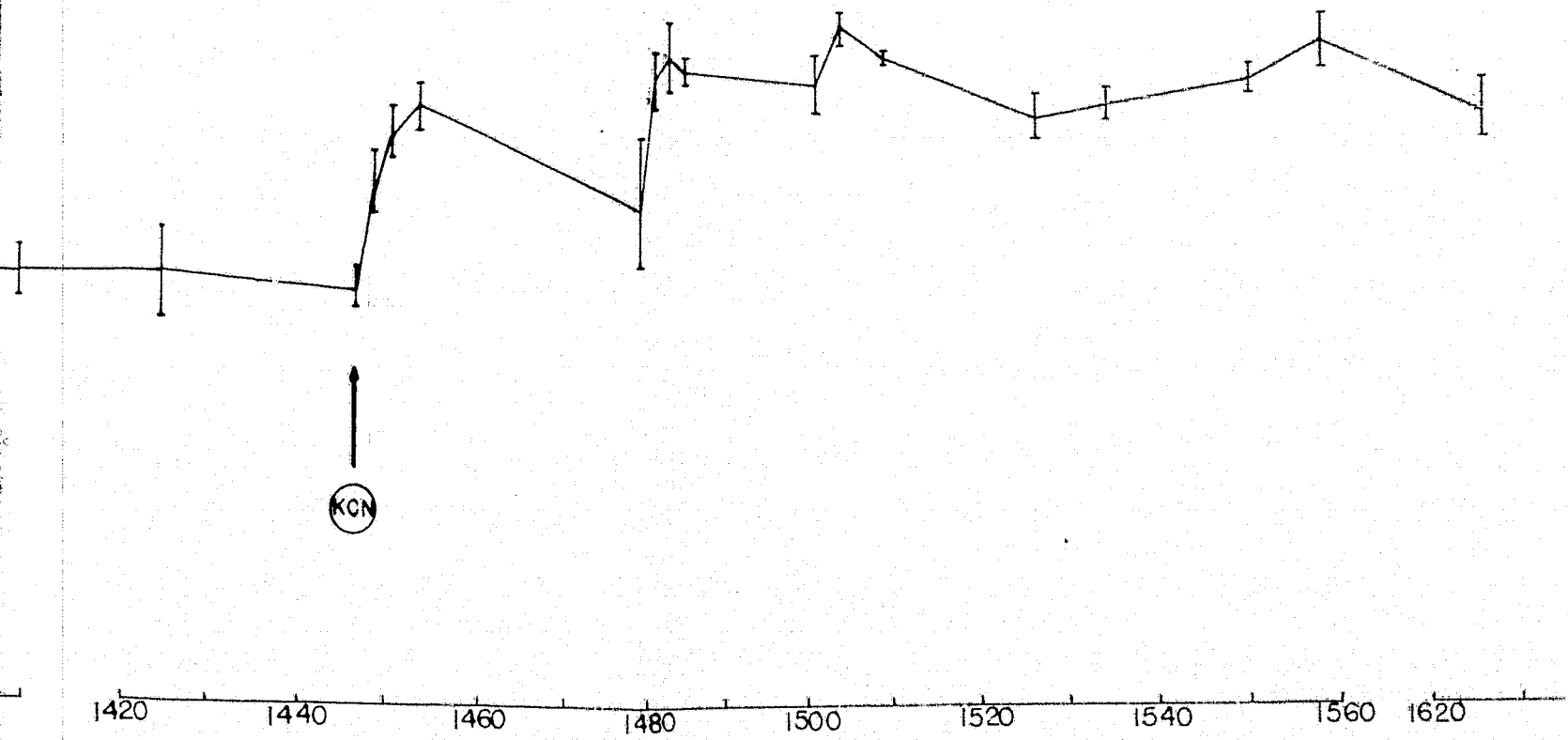

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Table 4

Recovery* of Added Radioactivity
from Viable Soil

Substrate Code	% Recovery	
	Meadow Soil	Phoenix Soil
VM1 0.01	NA	32
VM1 0.5	NA	18
VM1 0.1	30	NA
F	92	127
A_1	71	81
A_2	37	37
L_1	101	101
L_3	7	0
L_2	22	50
PA_L	62	51
PA_D	80	87
G_1	80	61
G_2	52	55
G_{3-4}	17	17
G_5	58	93
$VM1_{AA}$	NA	77
$VM1_{HM}$	NA	70
$VM1_{KCN}$	NA	40

* Includes all evolved radioactivity trapped with $Ba(OH)_2$ until the time of the next ^{14}C labeled substrate addition.

NA No Addition

recovery of added radioactivity is less from an initial medium addition than from a subsequent addition; therefore, the true differences in responses produced by 0.01 and 0.5 ml additions are greater than those demonstrated.

A possible explanation is that the microorganism-to-substrate ratio is greater for the smaller volume,and this results in the more rapid metabolism of available substrate. Also, the small volume of medium may primarily satisfy maintenance energy requirements and be channeled through energy yielding steps rather than anabolic processes. Substrate in the larger volume, on the other hand, may saturate energy requirements and also serve as a carbon source for growth. A lower recovery of $^{14}CO_2$ from added radioactivity would be expected to accompany growth.

The carboxyl labeled atoms of ^{14}C formate, $1^{14}C$ acetate, and $1^{14}C$ lactate all produced a rapid high yield of $^{14}CO_2$, with the highest yield of CO_2 coming from the formate and lactate. The rapidity with which formate is degraded is probably due to the action of dehydrogenase,which produces reduced cofactor according to the equation:

$$HCOOH \rightleftharpoons CO_2 + 2H^+ + 2\,e^-$$

The $1^{14}C$ lactate is most likely degraded via oxidation to pyruvate followed by decarboxylation of pyruvate to yield CO_2 and acetyl CoA.

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Several pathways for utilization or further degradation of the acetyl CoA might be considered. Foremost is the citric acid cycle. Condensation of acetyl CoA with oxaloacetate would cause the carbon atoms of the acetyl CoA to be liberated beginning with the second turn of the cycle. Both carbon atoms would rotate to positions for decarboxylation at the same time due to passage through succinate, a symetrical compound (22). Therefore, if degradation of acetyl CoA proceeded primarily via the citric acid cycle, equal yields of $^{14}CO_2$ from 2 and 3 ^{14}C lactate would be expected. Data represented graphically in Figures 29 and 30 and recoveries given in Table 4 do not support this route of metabolism. With Meadow soil,approximately three-fold more $^{14}CO_2$ was recovered from the $2^{14}C$ lactate than from the $3^{14}C$ lactate. The Phoenix soil showed a 50% recovery of ^{14}C as CO_2 from $2^{14}C$ lactate but none from $3^{14}C$ lactate. Following the rapid and complete degradation of $1^{14}C$ lactate to $^{14}CO_2$, a small fraction of the remaining two carbon fragments,most likely acetyl CoA, may enter the citric acid cycle, but the bulk of this compound must be involved in synthetic pathways (amino acid or lipid metabolism) or other catabolic processes. The accumulation of a two or one carbon byproduct is unlikely in the presence of the

highly versatile soil population, and data obtained on formate and acetate degradation also argue against this possibility. Results obtained with $1^{14}C$ acetate and $2^{14}C$ acetate support the explanation that the citric acid cycle is not the exclusive pathway for the utilization of acetyl CoA. Similar results for labeled acetate were obtained with both test soils, and showed that approximately two-fold more $1^{14}C$ acetate than $2^{14}C$ acetate is converted to $^{14}CO_2$.

The degradation of acetate requires activation to acetyl CoA. This activation requires energy and may proceed via the following:

Acetokinase

$$\text{Acetate} + \text{ATP} \rightleftharpoons \text{Acetyl Phosphate} + \text{ADP}$$

$$\text{Acetyl Phosphate} + \text{Coenzyme A} \longrightarrow \text{Acetyl CoA} + \text{Phosphate}$$

or

Aceto CoA-Kinase

$$\text{Acetate} + \text{Coenzyme A} + \text{ATP} \longrightarrow \text{Acetyl CoA} + \text{AMP+PP}$$

Since the degradation of lactate and acetate probably involves a common intermediate, acetyl CoA, the degradation rate and yield of $^{14}CO_2$ from $2^{14}C$ lactate and $3^{14}C$ lactate might be expected to compare with $1^{14}C$ acetate and $2^{14}C$ acetate, respectively. However, the yield of CO_2 from acetate was much greater than from the two and three carbon atoms of lactate. This would

suggest that a greater percentage of acetyl CoA from acetate had been used for energy-yielding metabolism, perhaps via the citric acid cycle, than had been used from the acetyl CoA derived from lactate.

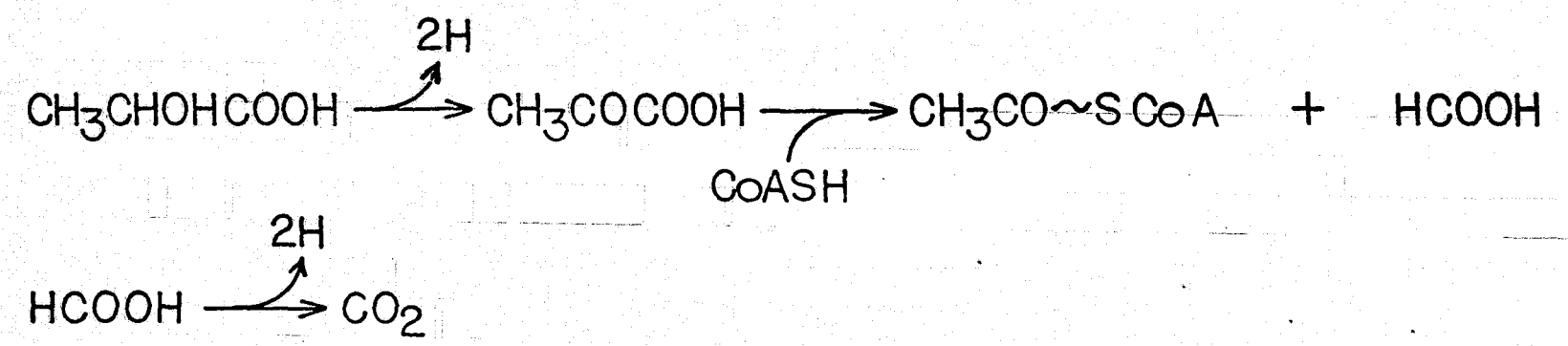
Energy considerations of lactate vs. acetate metabolism may provide an explanation. The most likely schemes for production of acetyl CoA from the two compounds is as shown in Figure 31. Lactate dehydrogenation results in the production of reduced cofactor. A clastic split of pyruvate to acetyl CoA would produce formate, an additional source of reduced cofactor. In an aerobic system, these two reduced cofactors might produce a net gain of six ATP moles per mole of acetyl CoA produced. Activation of acetate on the other hand, leads to a net loss of one mole of ATP per mole of acetyl CoA produced.

It might easily be reasoned, therefore, that the same population of organisms would process acetyl CoA from lactate differently than acetyl CoA from acetate. In the former, a net gain in six ATP would allow for a high percentage of the acetyl CoA to be directed toward cellular carbon needs, i. e., protein and lipid synthesis. The energy requiring activation of acetate would cause a greater percentage of that acetyl CoA to be

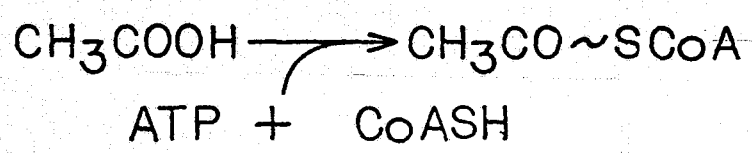
Figure 31

Schemes for Production of Acetyl CoA
from Lactate and Acetate

LACTATE



ACETATE



directed through energy producing pathways which ultimately lead to the generation of CO_2.

Both soils showed a greater rate of evolution of CO_2 from L-phenylalanine than from the D-form, thus demonstrating specificity of the mixed soil flora for the L-isomer.

The pattern in $^{14}CO_2$ evolution from $1^{14}C$, $2^{14}C$, $3\text{-}4^{14}C$, and $5^{14}C$ glutamate by Meadow soil is consistent with citric acid cycle metabolism.

Following deamination of the glutamate molecule and its incorporation into the cycle as a ketaglutarate, decarboxylation of the 1 carbon atom would result in succinate. Succinate, a symetrical compound, would be cycled and the two carboxyl carbon atoms arising from the 2 and 5 carbon atoms of the original glutamate would be decarboxylated at an equal rate. The 3 and 4 carbon atoms of the original glutamate would gradually rotate to positions on the citric acid cycle intermediates which may be decarboxylated. However, several cycles are required,and the intermediates containing these labeled atoms may be involved in synthetic processes,thereby stabilizing these 3 and 4 carbon atoms in cell constituents. The rate of $^{14}CO_2$ evolution from labeled glutamate degraded via the citric acid cycle would be expected to follow the pattern:

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Rate

$1^{14}C > 2^{14}C = 5^{14}C >> 3\text{-}4^{14}C$

Meadow soil shows this pattern both in rate and yield of CO_2. The percent recovery of CO_2 from glutamate was:

Yield

$1^{14}C = 80\%$

$2^{14}C = 52\%$

$5^{14}C = 58\%$

$3\text{-}4^{14}C = 17\%$

Phoenix soil showed a pattern, which in the rate and yield of evolution of CO_2, corresponded to:

Rate

$5^{14}C > 1^{14}C \cong 2^{14}C >> 3\text{-}4$

Yield

93% 61% 55% 17%

These results are consistent with the profiles obtained for Phoenix soil in earlier studies using the single addition mode (23). The high rate of evolution and yield of CO_2 from the 5 carbon position of glutamate might be explained by a pathway which has been described for Clostridium tetanomorphum as shown below:

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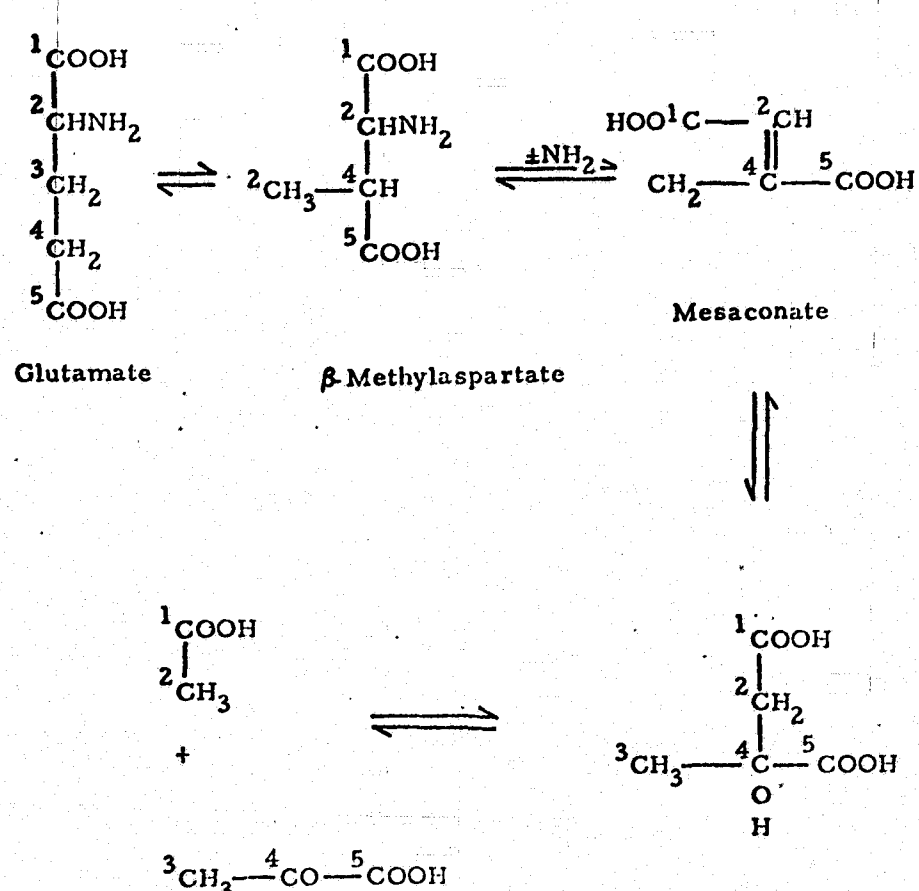

This pathway results in the production of acetate and pyruvate. The carboxyl group of the pyruvate originated from the 5 carbon atom of glutamate and thus would be decarboxylated at a high yield like the 1 carbon of lactate which has been demonstrated. The series of antimetabolite additions included:

AA (Antibiotic, Antimyiotic) + VM1

HM (Heavy Metals) + VM1

KCN (Potassium Cyanide) + VM1

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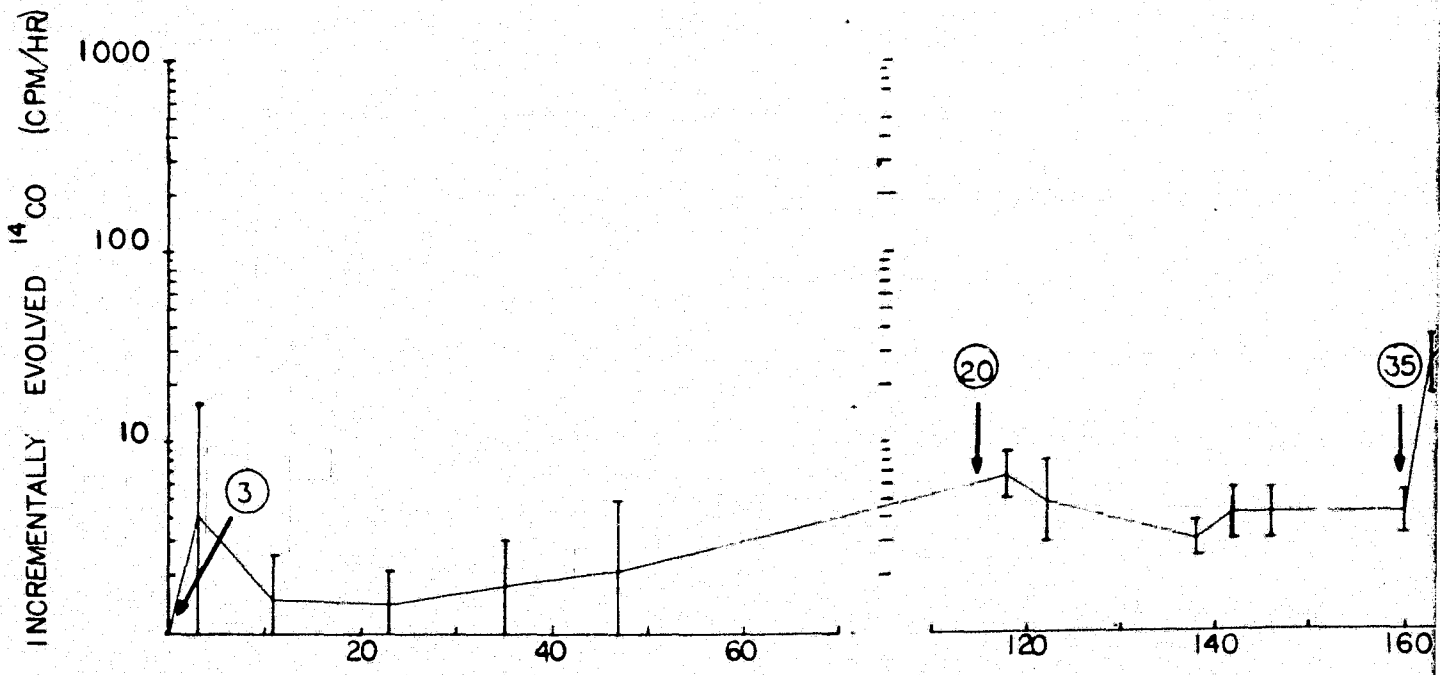
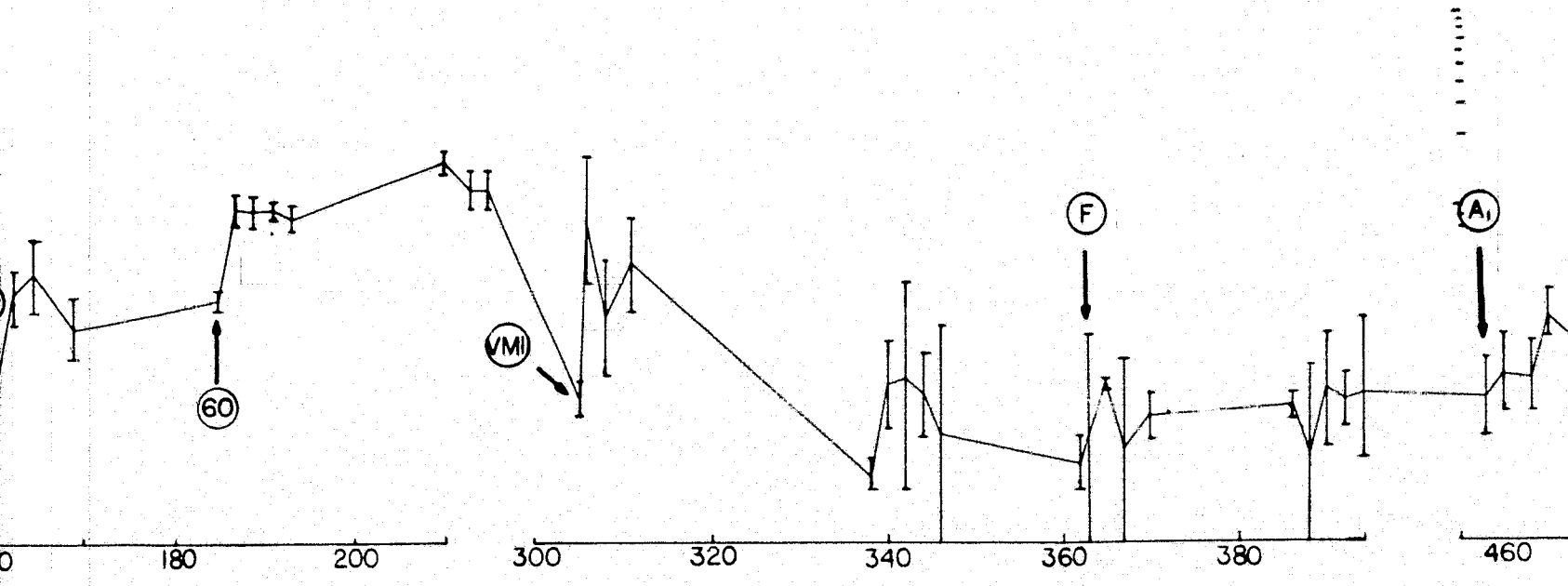
None of these agents totally inhibited the radiorespirometric response. However, heavy metals and KCN depressed the initial rate of CO_2 evolution. All three agents produced a nontypical prolonged moderate level of evolution.

E. Long-Term Multiple Addition Tests with Sterile Soil

Four replicate aliquots of each test soil (0.21 cc of Phoenix and Meadow soils) were placed in 20 ml glass vials and placed in the dry air oven at 210°C for 60 minutes. The soils were cooled and then dosed sequentially and incubated as indicated for the Chamber 3 sequence (Figure 3). Filter pads wetted with saturated $Ba(OH)_2$ and stuck to the inside of the caps with stopcock grease were used to collect evolved radioactive gas. These pads were changed several times per day and the radioactivity on each determined. Figures 32 and 33 are continuous plots of the incrementally evolved radioactivity (cpm/hr incubation period) obtained for the two soils throughout the entire series of sequential additions. The percent recovery of added radioactivity for each medium addition is given in Table 5.

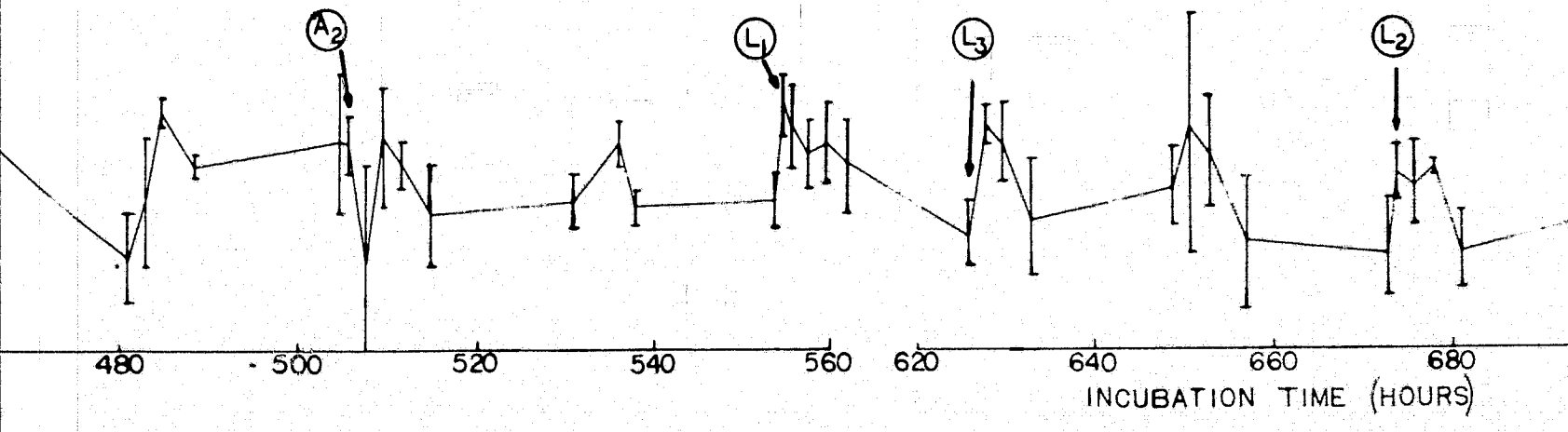
The initial series of additions involved 3°C, 20°C, 35°C and 60°C incubation temperatures. As shown, nonbiological

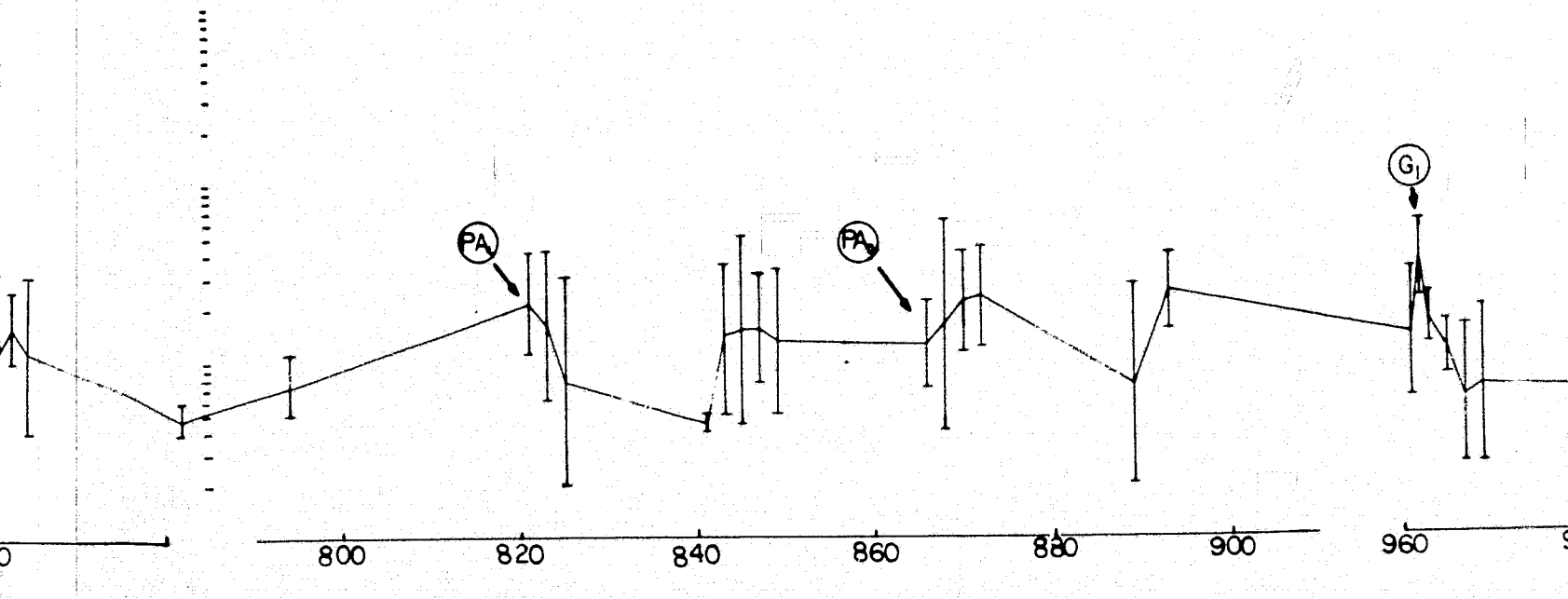




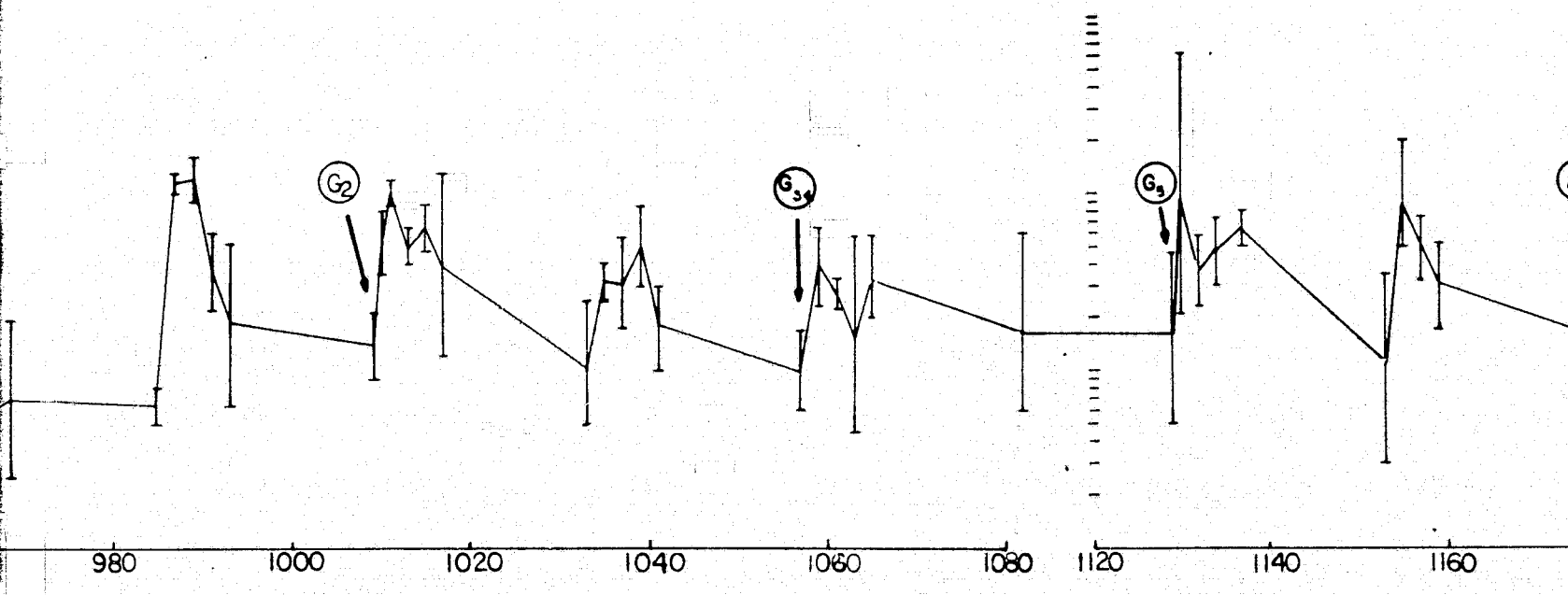
FOLDOUT FRAME 2

FIGURE 32
MULTIPLE ADDITION
CHAMBER 3 ADDITION SEQUENCE
STERILE CONTROL
MEADOW SOIL





FOLDOUT FRAME 4



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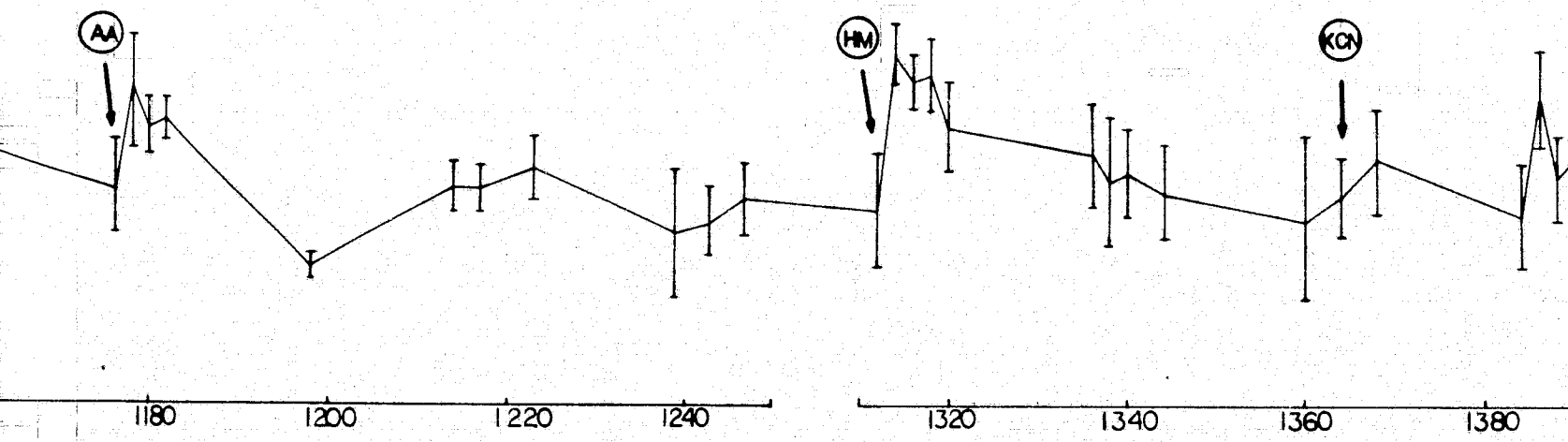
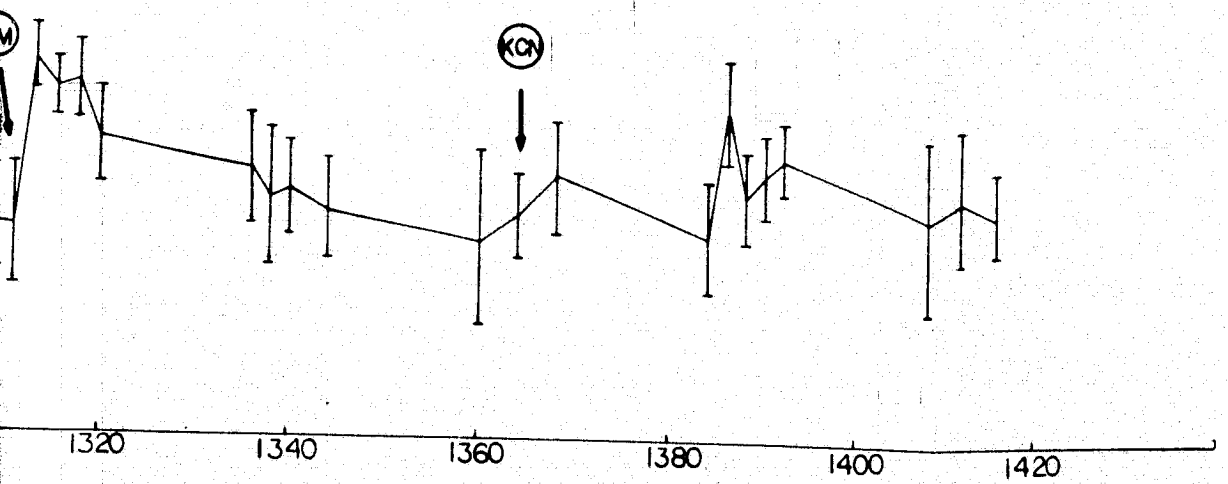
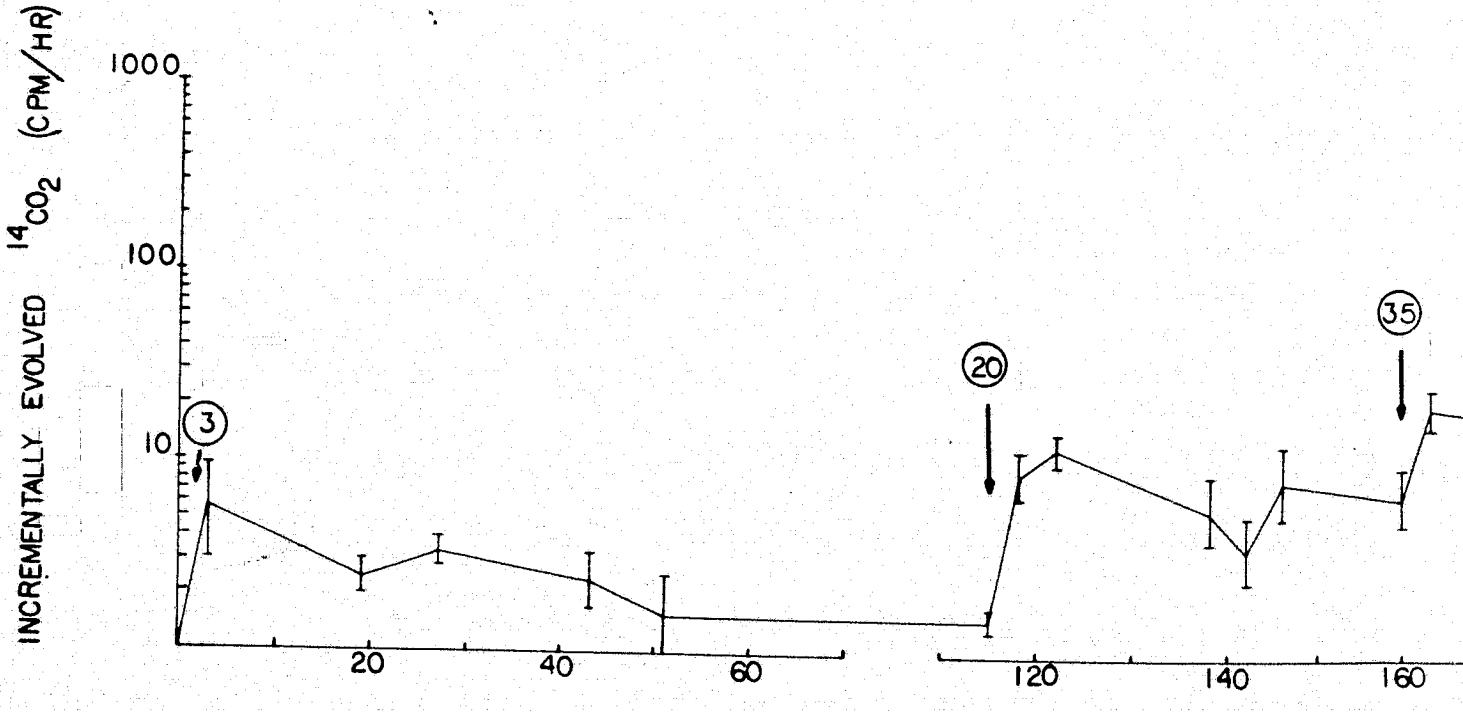
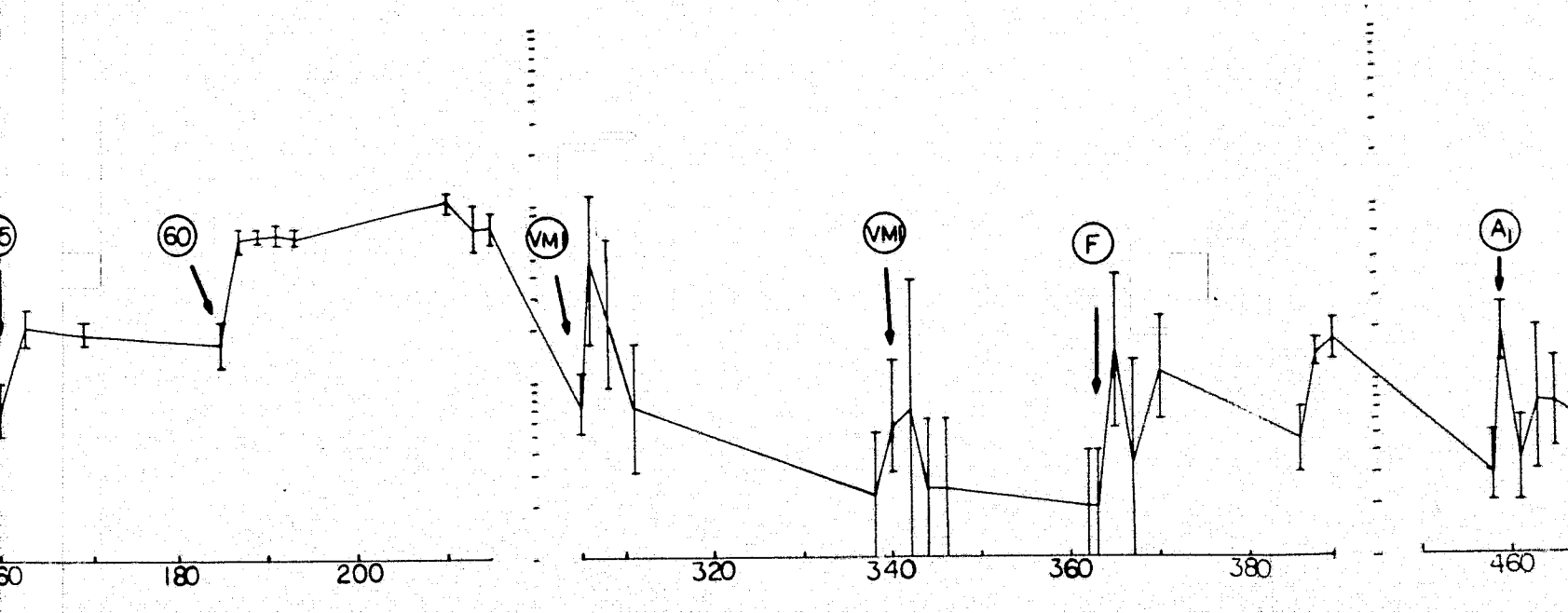


FOLDOUT FRAME 6


M
KCN
1320
1340
1360
1380
1400
1420





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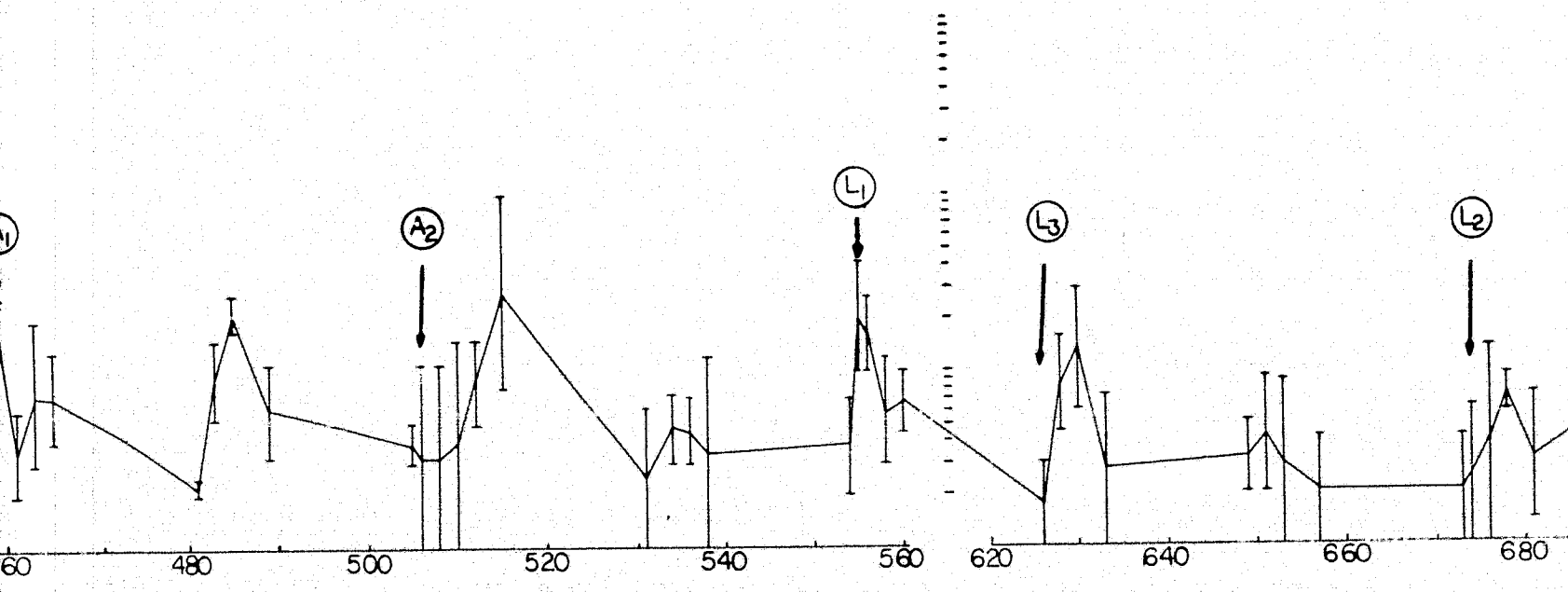
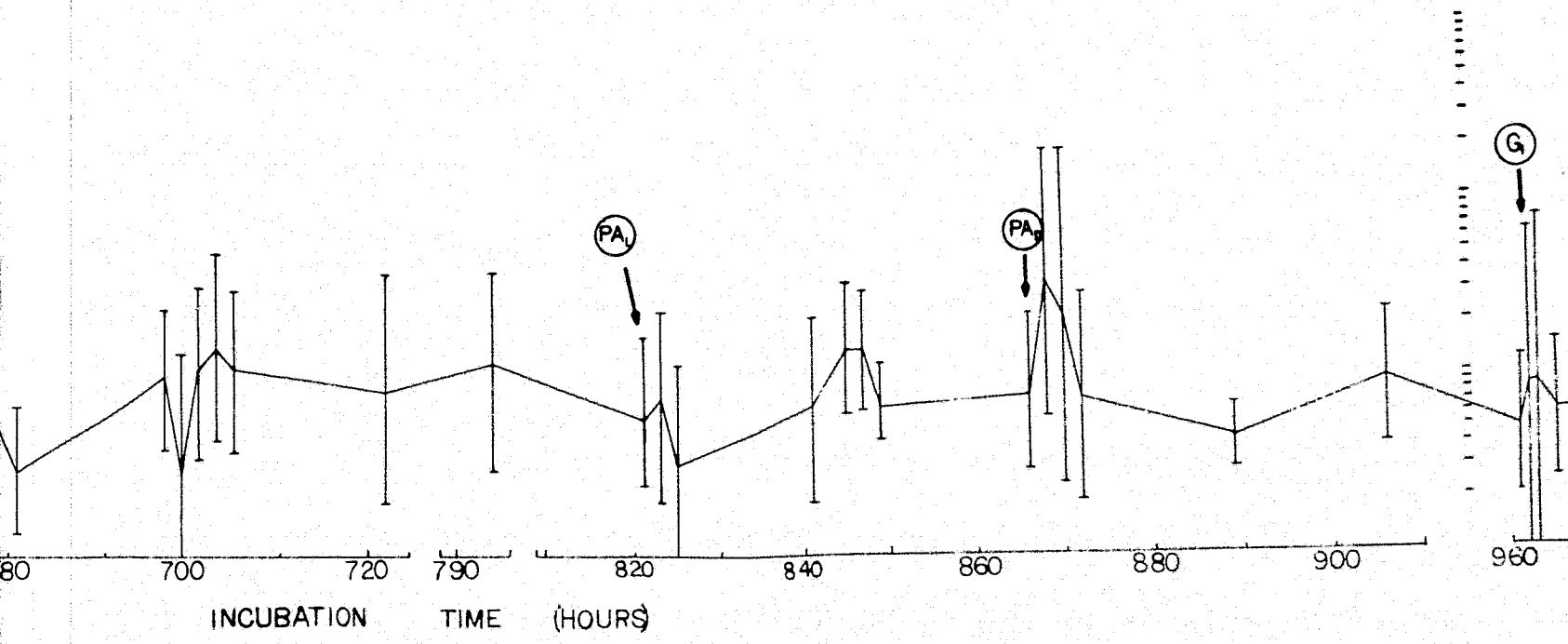


FOLDOUT FRAME 3

FIGURE 33
MULTIPLE ADDITION
CHAMBER 3 ADDITION SEQENCE
STERILE CONTROL
PHOENIX SOIL



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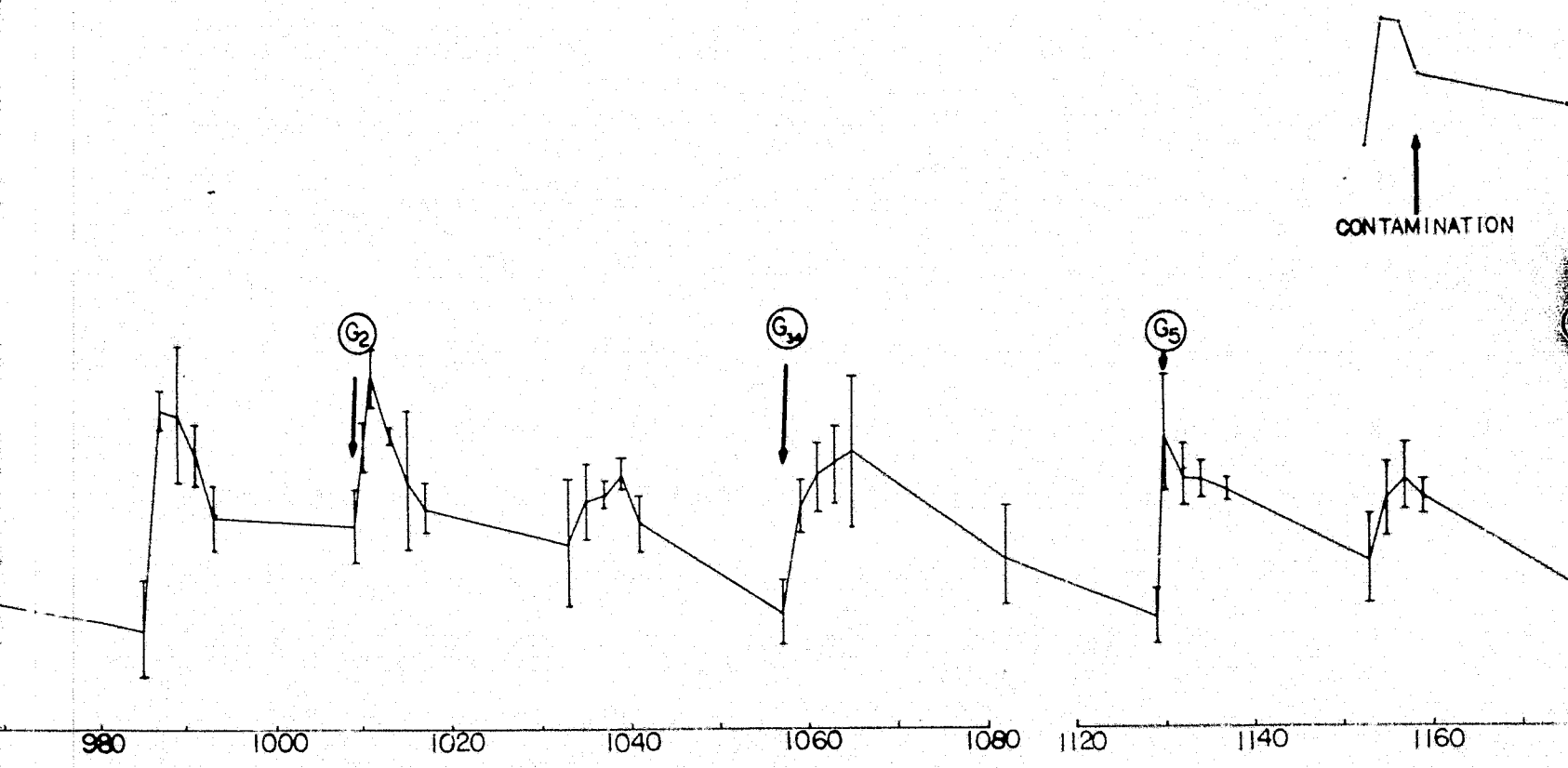

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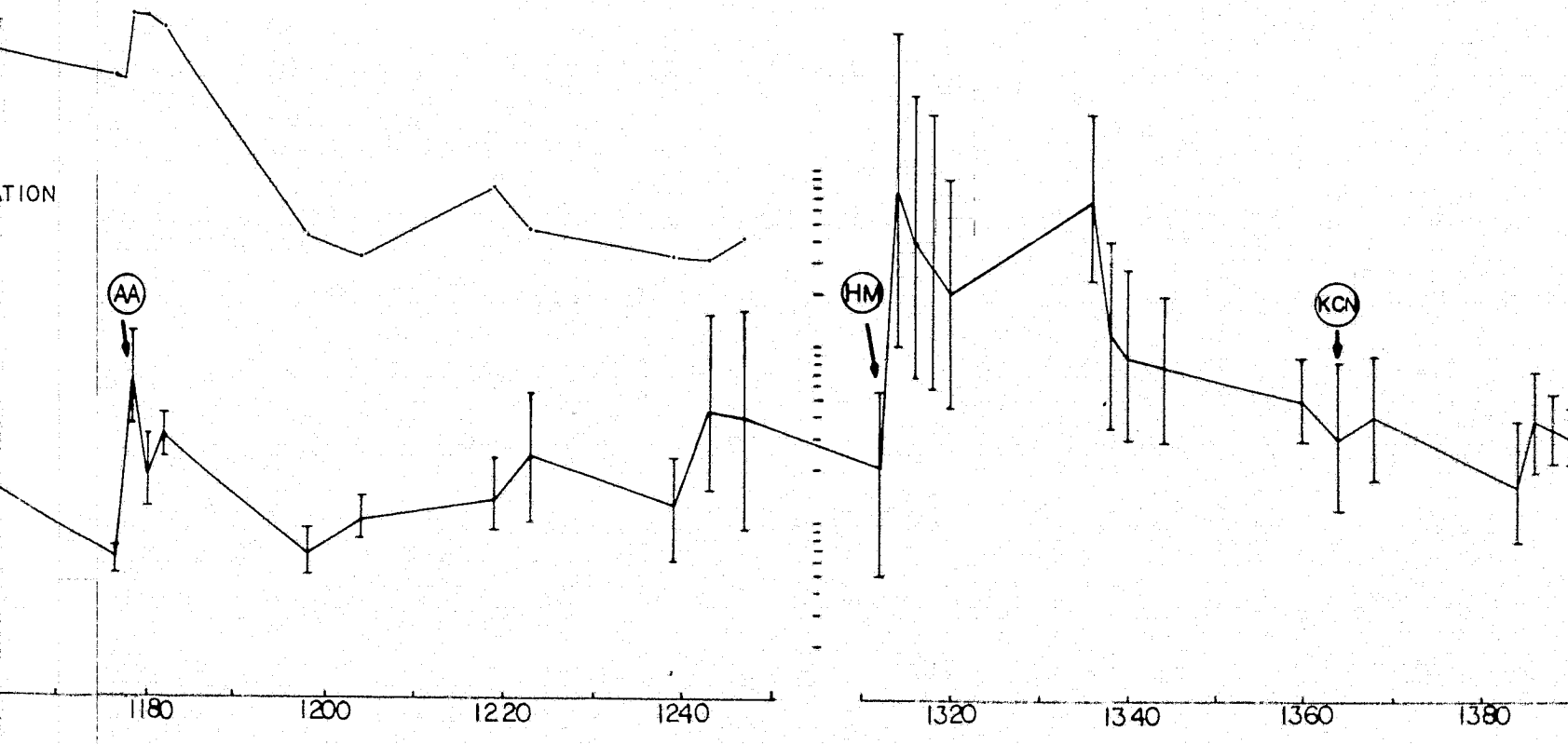

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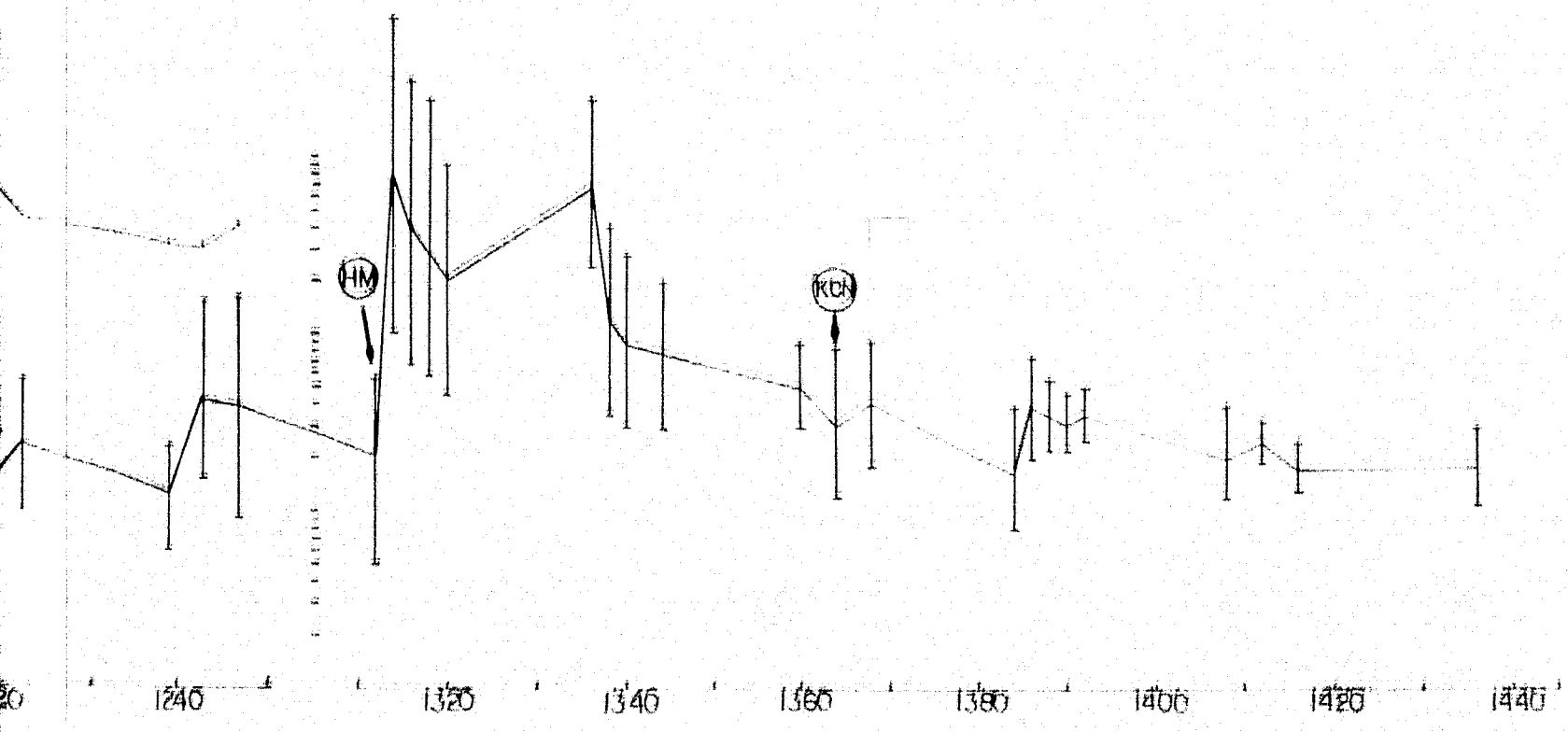

1240 1320 1340 1385 1380 1400 1420 1440

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Table 5

Recovery of Added Radioactivity from
Sterile Phoenix and Meadow Soils

Substrate Addition *	Percent Recovery (%)	
	Phoenix Sterile	Meadow Sterile
$VM1_3$	0.3	0.2
$VM1_{20}$	0.3	0.2
$VM1_{35}$	0.5	0.7
$VM1_{60}$	2.8	4.5
VM1	0.8	0.2
F	1.4	2.0
A_1	0.8	1.7
A_2	1.0	1.3
L_1	0.6	1.3
L_3	0	0.7
L_2	4.9	2.9
PA_L	1.4	1.7
PA_D	2.8	4.0
G_1	2.9	3.5
G_2	2.9	3.6
G_{3-4}	1.1	3.3
G_5	1.9	4.2
$VM1_{AA}$	5.5	2.2
$VM1_{HM}$	14.9	1.5
$VM1_{KCN}$	1.7	2.9

* See Glossary for medium codes. (Appendix II)

evolution from both soils was temperature-dependent. When the four incubation temperatures of 3°C, 20°C, 35°C and 60°C were used, the average nonbiological evolution of radioactivity accompanying each respective temperature was approximately 3 cpm/hr, 8 cpm/hr, 20 cpm/hr and 80 cpm/hr. When the incubation temperature was reduced from 60°C to 23°C for the remainder of that test, the nonbiological response fell to a level of approximately 10 cpm/hr. Throughout the remainder of the medium addition series, the nonbiological evolution appeared to increase very slowly and reached approximately 100 cpm/hr during the addition of ^{14}C labeled glutamate. These highest levels of nonbiological evolution are well below the peak response levels obtained with viable soil. The baseline level of radioactive evolution from viable soil (that occurring between sequential medium additions) was approximately 100 - 200 cpm/hr.

One of the sterile vials (Phoenix soil) became contaminated and exhibited a burst in evolved radioactivity after 1, 150 hours. Readings were continued on this vial, and it is interesting to note that evolution of radioactivity decreased during the addition of the antimicrobials (antibiotic antimycotic, heavy metals mixture, KCN). The heavy metals mixture induced an undesirable high nonbiological

peak in all vials, however, this evolution fell again and thereafter the evolution of $^{14}CO_2$ from the contaminated vial was within the range of evolution seen in the sterile vials. These results suggest that the antimicrobial series effectively inhibited the "drop-in" contaminate, however, with viable soils, these same antimicrobials were largely ineffective.

F. Modification of Current Viking Hardware to Accommodate Advanced Labeled Release

The multiple addition Advanced Labeled Release Experiment could be adapted to the current Viking '75 Labeled Release hardware with relatively little modification. As discussed earlier, only four test cells are required to perform the large number of test conditions described. The current VLR hardware has four test cells available, each of sufficient size for the more sophisticated experiment. The most significant hardware adaptation is the accommodation of a battery of different liquid reservoirs to host the various substrates and inhibitors since the current VLR hardware has only one such reservoir.

Figure 34 diagrams the LR module for Viking '75. Nutrient is contained within a sealed ampoule inside the reservoir. The ampoule is broken upon introduction of high pressure helium through valve S/46 to the reservoir pistons at each end of the

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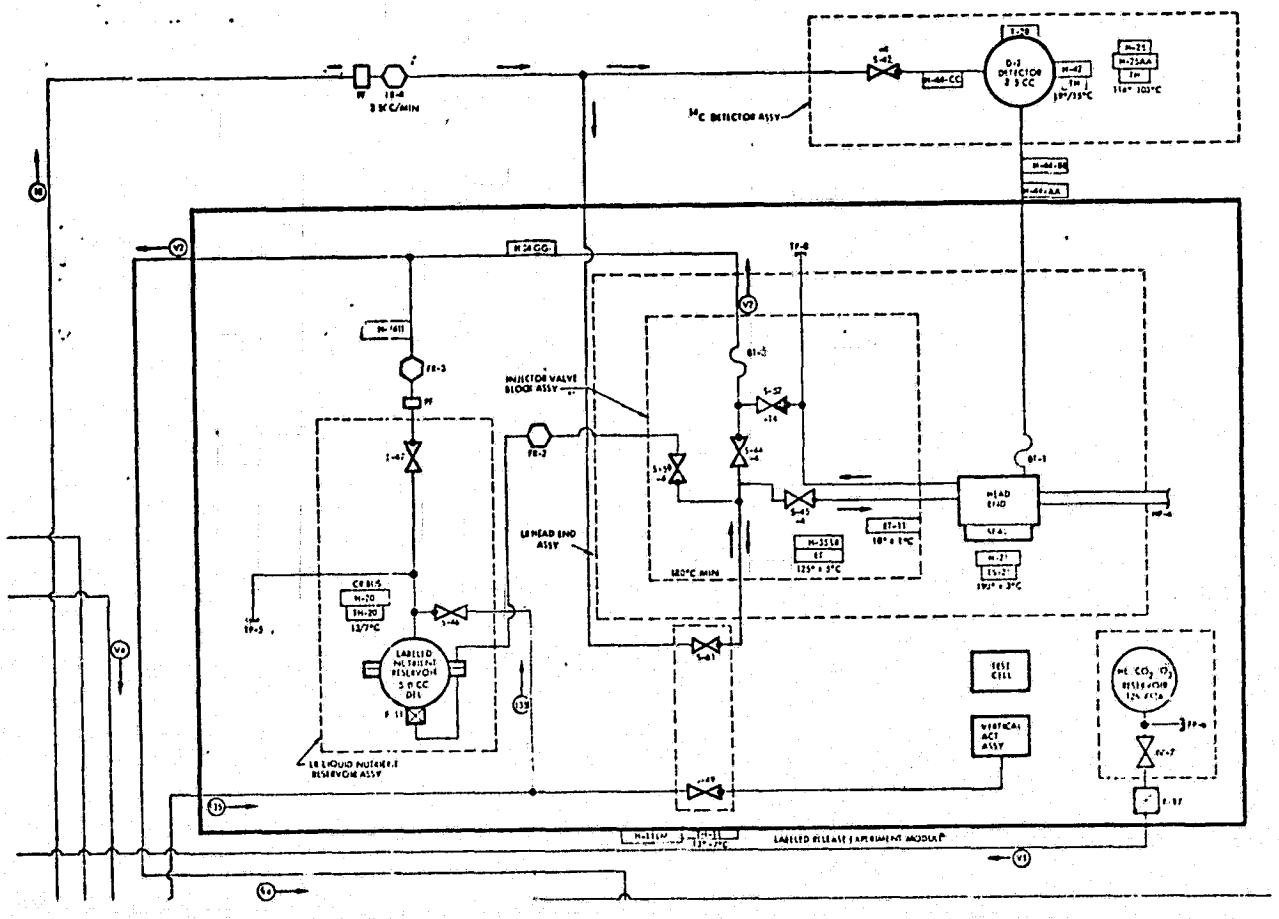
Figure 34

Diagram of LR Module for Viking '75



ampoule. The nutrient is then degassed inside the reservoir by bubbling low pressure helium which flows through S/61, then through S/59 to the reservoir and out S/47. Finally, nutrient is injected into the test cell by first utilizing high pressure helium through S/46 to fill a metered cavity bounded by S/59, S/61, S/44 and S/45. The contents of the cavity are then emptied into the test cell by opening S/45.

One suggested means of accommodating a battery of liquid reservoirs is shown in Figure 35. As shown, the single reservoir is replaced by a series of reservoirs, only 5 of which are shown in Figure 35. Each is isolated by locating a valve at the top and bottom of the reservoir. With this arrangement, the existing hardware can be utilized as is for all operations utilizing only sequence changes. Thus, to selectively break the ampoule in Reservoir #1 which is isolated by the hypothetical valves S/91 and S/92, S/46 and S/91 are opened to the high pressure helium source to permit selective breakage of Reservoir #1. The contained liquid is then degassed by passing low pressure helium through S/61, through S/59 and S/92, through Reservoir #1 and out S/91 and S/47. The liquid in Reservoir #1 is selectively used to fill the metered cavity by opening S/92 only and forcing the liquid through S/59 under high pressure helium. Similarly, the liquid from any other selected

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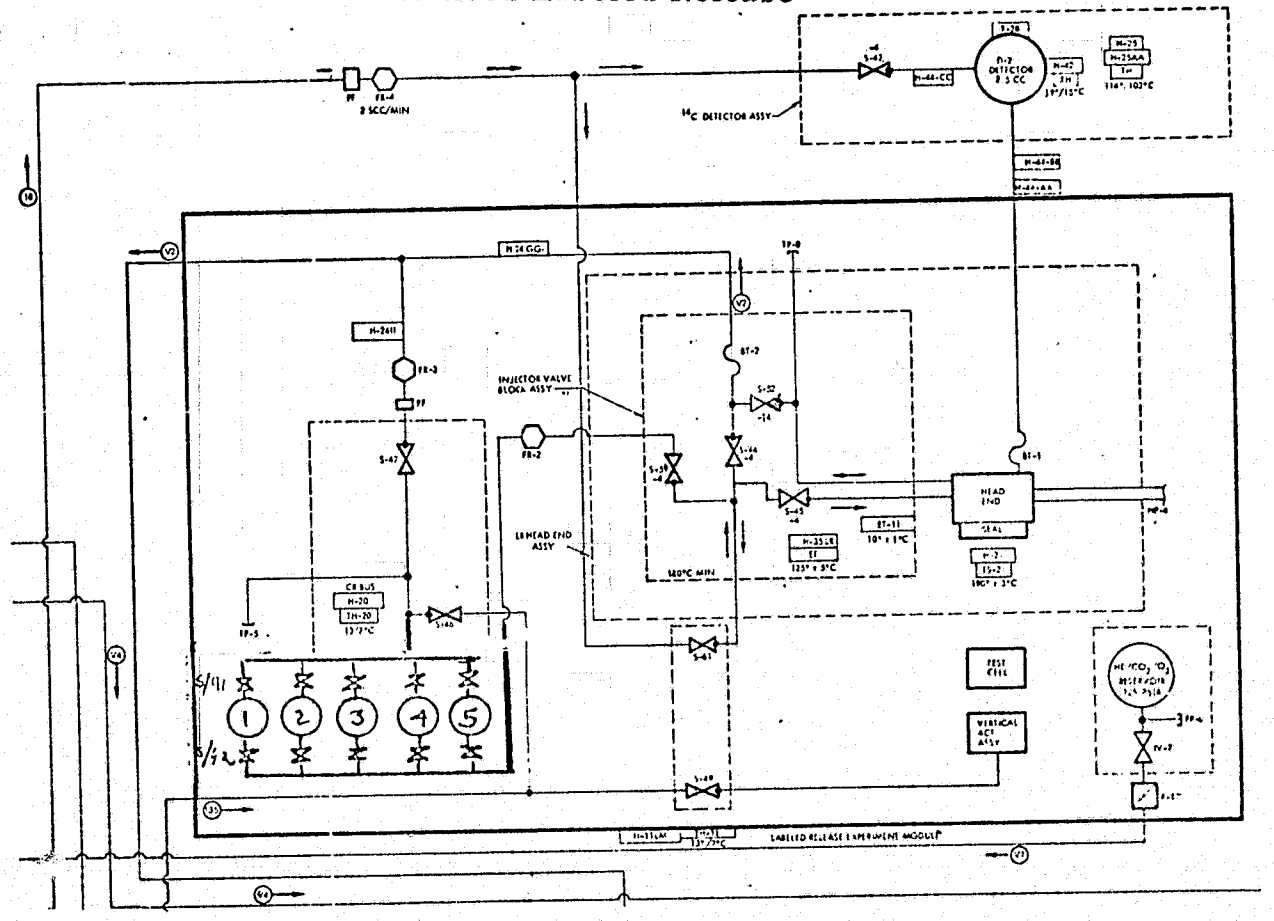
Figure 35

Diagram of LR Module Showing Modifications
for Advanced Labeled Release



reservoir can be used to inject a given test cell. Cross contamination can be minimized between injections from different reservoirs by a flushing procedure. Thus, after using the contents of Reservoir #1, in preparation for an injection from the contents of Reservoir #2, the entire nutrient line downstream of valve S/92 is cleared by opening both S/59 and S/44 and exposing the line to Martian pressures. The line is then flushed by opening the valve to Reservoir #2 and filling both the line and the metered cavity with the contents of Reservoir #2. The contents of the metered cavity is then discarded through S/44 and evaporated by exposure to Martian pressures. This flushing procedure is repeated at least once more before filling the metered cavity for injection into the test cell through S/45. It is estimated that cross contamination by this procedure would be less than 1%.

The most difficult adaptation to the current VLR hardware is the location of the required battery of reservoirs which replace a single reservoir. However, it should be noted that only one or two injections are required for each liquid in the Advanced Labeled Release Experiment. Thus, each individual reservoir can be considerably smaller than the current Viking '75 LR reservoir which must accommodate approximately 9 ml of nutrient for all operations.

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G. Discussion of Results

These long-term multiple addition experiments demonstrate the feasibility of conducting a series of Labeled Release experiments in a single test chamber. Provided proper attention is given to sequence, timing and other details of medium addition, the single chamber may be used repeatedly without inbetween sterilization cycles. The influence of earlier experiments appears to be a minimal difficulty and, in some instances, may provide an advantage in the form of an adapted or increased soil population.

A number of factors served as input for the development of the experimental design which was undertaken in these long-term multiple addition studies. Theoretical considerations, which ranged from theories of organic evolution to current data on the physiochemical environment of Mars, were integrated with basic modes of terrestrial metabolism. Key intermediate compounds, which by the nature of their reactivity or pattern of decomposition might provide comparative biochemical information, were especially sought. Microorganisms of particular interest were those having a primitive metabolism or possessing characteristics which would allow for survival in a theorized premordial environment. The series of antimetabolites, like the series of substrates,

possessed the potential for providing biochemical information, but were also designed as empirical tests to find means to control the organisms. A number of practical considerations also heavily influenced the experimental design. Experimental results with various substrates eliminated them from use. For example, pyruvate, a theoretically very important intermediate, was not used since a high level of nonbiological evolution was found to occur. Glucose was eliminated due to the fact that it decomposes during heat sterilization. Conditions of the test were geared to a terrestrial environment for purposes of demonstration. However, those are the optimum conditions for terrestrial microorganism - the test situation. The selection of a four chambered system was definitely influenced by the existing engineering designs. The possibility that significant comparative biochemical data could be obtained with relatively modest changes in current hardware makes the proposed experimental approach especially attractive.

Basically, the four chamber testing schedule involves:

a) Chamber 1. An investigation of the effect of the atmosphere and selection of that one which provides the greatest reactivity.

b) Chamber 2. The use of the most optimum atmosphere and an investigation of the most optimum incubation temperature. This series will enable selection of the optimum temperature.

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c) Chamber 3. Employing a heat sterilized soil inoculum and optimum conditions of atmosphere the entire temperature, substrate and antimetabolite series will be tested to provide control values.

d) Chamber 4. The ^{14}C substrate and antimetabolite series is tested using viable soil. Specific results obtained with the test soils have been discussed in the text. However, the results of this series interpreted in the light of Chamber 3 results should provide much information on the route of metabolism of the selected substrates and possible means of control.

Before the procedural sequence and selection of substrates had been tested in the long-term experiments, numerous individual tests were performed using the single addition mode. After tentative selection of the substrates, numerous tests were made to explore the various aspects of the multiple addition methodology.

The order in which temperature tests were performed was found to be important. If the temperature progressed stepwise from 3^{o} to 25^{o} to 35^{o} to 60^{o}, results were similar to those obtained in single addition experiments. The soils tested showed the greatest activity in the 25^{o} and 35^{o} range. If, however, the temperature progressed stepwise from 60^{o} to 35^{o} to 25^{o} to 3^{o}, then results were significantly different. Relatively little activity was obtained at the 35^{o}, 25^{o} and 3^{o} temperatures. These data indicate that initial high incubation temperatures kill the major portion of the soil

population which is active at lower temperatures. However, lower temperatures are not harmful to the thermophilic organisms. A "lower-to-higher" temperature sequence was therefore indicated. Nonbiological evolution of radioactivity was found to be temperature dependent - the higher temperature produced significantly higher rates of chemical evolution. In most soils possessing a large population of organisms, the biological response is clearly defined from the nonbiological effect. In soils with low microbial populations, and therefore a low radiorespirometric response, the nonbiological evolution caused by increased temperature could introduce problems of interpretation.

An interesting effect, not understood but well documented, is the burst of radioactivity which occurs when a multiple addition system is shifted to a higher temperature. The phenomenon is strictly biological since control soils show no such burst of $^{14}CO_2$ evolution. For some reason, soil cultures previously dosed with ^{14}C labeled media and incubated until plateau are induced to rapidly evolve some of the remaining ^{14}C compounds when the temperature of incubation is raised. Extending the time of incubation after plateau tends to reduce the magnitude of

evolution. This finding dictated that a temperature shift and medium addition should not be made simultaneously; otherwise, the evolution of radioactivity resulting from an earlier medium addition, but induced by the temperature shift, would be mixed with that resulting from the freshly added medium.

Successive media additions utilizing the same medium and temperature produce relatively similar results, save the initial addition. Soils dosed initially evolve radioactivity more slowly and over a longer period of time than soils dosed a second, third, or nth time. Enumeration studies of the viable bacteria in soils indicate that a distinct increase of two-six fold occurs after the first medium addition, but then the viable population remains relatively constant. An especially important finding was that the period of time between media additions did not affect the radiorespirometric response. It was feared that an enhanced population of organisms which were fed during a particular medium dosing might die or at least become inactive if not dosed again after a reasonably short period of time. However, experimental results showed a high level of activity after a period of inactivity of more than 100 hours.

Unless information on the enhancement of a population or the effect of water volume is specifically sought, a first

addition result should never be compared with the results of a subsequent addition. A soil should always be primed with a medium addition before comparisons are made.

The use of specifically labeled acetate, lactate and glutamate have provided considerable evidence for the operation of various pathways of metabolism. Glutamate especially provides evidence for the operation of the citric acid cycle. The yields of CO_2 produced from the individual carbon atoms of acetate and lactate indicated that these two compounds are metabolized in quite different fashion by the soils studied. The carboxyl group of the lactate is rapidly liberated and recovered in high yield as CO_2. The most likely intermediate product of the lactate decarboxylation is acetyl CoA. The first step in acetate utilization or degradation by most organisms is activation to acetyl CoA. We would therefore expect the yields of CO_2 from the second carbon atom of lactate and the first carbon atom of acetate to be similar, and the third carbon atom of lactate and the second carbon atom of acetate to be similar. Instead,it was found that much more radioactivity resulted from the acetate - especially from the first carbon atom. The most likely explanation is that the first carbon atom of lactate was utilized

for energy production, thereby producing an excess which was coupled to synthetic incorporation of the resulting C_2 fragment. On the other hand, the activation of acetate to acetyl CoA requires energy, thereby creating an ATP deficit which must be satisfied by utilization of the acetyl CoA in energy yielding mechanisms.

Irrespective of the interpretations and results obtained on these individual soils, these data point to the kinds of information which might be extracted from the results of a Martian soil test. Similar results on more and varied soil samples and/or pure cultures would support interpretation.

Phenylalanine may not be a theoretically good choice of substrate for which to demonstrate specificity for the D vs. L form. This compound was selected empirically and, for the purposes of demonstration, worked well. The L form was utilized more rapidly than the D form.

The long-term multiple addition tests provided no really new information on the soils tested, but they stand as the culmination of a long and tedious development. They firmly establish the feasibility of performing many sequential additions of varied ^{14}C labeled compounds, under selected conditions, to a single culture vessel. The evolved radioactivity from each addition stands alone as a discrete profile of information which may be

interpreted in the light of other equally discrete profiles. The wealth of information which might be extracted from a successful multiple addition experiment dwarfs the objectives of the current Viking mission, yet the engineering requirements are only moderate modifications of existing state of the art.

Current thinking with regard to post Viking missions is shifting in the direction of a return Mars sample. However, a positive life signal from Viking in 1976 would most likely strengthen and unify current opposition sufficiently to postpone, or even prevent a return sample mission as the next step. The multiple addition Advanced Labeled Release Experiment stands as a candidate alternative.

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III. RETURN MARS SAMPLE

A. Introduction

Although automated spacecraft, in theory, can obtain considerable information regarding biological and chemical properties of Mars, experience with the Viking '76 mission has emphasized the complexity of automating even simple biochemical experiments. To obtain the sophisticated types of information required by scientists for detailed comparative planetology, it may be necessary to return a sample of the Martian soil to Earth where equipment and personnel are readily available for detailed analyses. However, in view of the potential risk of exposing the terrestrial ecosystems to Martian samples, considerable effort is required to establish whether such a mission can be conducted in a safe manner. It has been the purpose of the current investigations to examine critical questions related to these issues. Specifically, our efforts have been directed toward outlining justifications for and potential hazards of returning a Mars sample to Earth, delineating types of information necessary to control, contain, or totally destroy Martian life and the means for acquisition of this knowledge, and identification of proper quarantine procedures and related engineering problems. Plans have also been considered

for educating the scientific and lay communities regarding the benefits, risks and associated safeguard measures for a Mars Sample Return Mission.

Our approach to these issues, discussed in the following sections, has encompassed both theoretical and laboratory efforts. The theoretical sections have incorporated viewpoints from: 1) several leading scientists involved with extraterrestrial life studies, 2) two NASA-sponsored Return Mars Sample Symposia (November 1973 at JPL and June 1974 at NASA Headquarters) and, 3) Interactions between Biospherics and the Center for Theoretical Biology at the University of New York at Buffalo.

Associated with the theoretical effort has been a laboratory study centering on means of controlling, inhibiting and sterilizing a Mars soil sample. Control and inhibition have been discussed in the foregoing section where the AMML concept, presented as a modification of the current Viking concept, is programmed to determine effects of inhibitors on Martian metabolism. Selected inhibitors have either known effects on terrestrial metabolism or are environmental factors potentially inhibitory to Martian organisms (oxygen, liquid water). Laboratory studies demonstrating the feasibility of this concept have been presented. In addition, a gentle sterilization procedure has been sought which would preserve

key biological and chemical features in the killed organisms. While other investigators (24) have sought preservation of morphological or molecular structural features, we have sought to detect the survival of cell-free enzymes. The rationale is that these enzymes, possibly present as exozymes, may be bound to soil particles, and examples have been reported of bound enzymes surviving sterilization temperatures (25). Thus, monitoring for sterility by plating techniques, an enzyme survival was sought by radiorespirometry. The results, reported in the following Sections, indicate that enzymatic activity does not survive even minimal heat sterilization procedures.

B. Theoretical Considerations

A mission designed to return a sample of the Martian soil to a terrestrial laboratory must answer two complicated questions:

1. Is there sufficient justification to return a sample to Earth?

2. If so, how should sample return be accomplished? Several return modes are possible which can be summarized as follows: $\pm$ man $\pm$ sterilization $\pm$ space station.

Our examination of these questions has been conducted mainly from a scientific rather than a technical viewpoint since it is assumed that sufficient technical expertise is available to develop all

necessary hardware. To initiate our scientific evaluation, we interviewed several prominent scientists involved in extraterrestrial life detection and space research (26). Based on the varied opinions expressed and on the resulting internal discussions, we formulated the following major scientific areas of concern regarding a Mars Sample Return Mission:

- Scientific values from automated landers vs. return samples.
- Scientific knowledge necessary prior to returning a sample.
- Sample selection
- Health hazards and pathogenicity
- The role of man before, during or after returning a sample.
- Value of returning a sterile sample and method of sterilization.
- Location of a quarantine laboratory
- Maintenance of quarantine
- Types of analyses
- Termination of study and disposal of sample

The potential risks that exist within each of these ten categories serve as guidelines to the discussions that follow.

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1. Justifications for the Return of a Mars Sample

Full justification for the return of a Mars sample can only be formulated after assessment of all risk factors in each of the ten categories above. Since this depends not only on theoretical discussions, but also on data to be obtained from forthcoming lander missions and on hardware assessment as it is developed, any justifications for sample return must be considered preliminary. The major justifications at this time for preparing for such a mission may be summarized as follows:

- The biological values obtained from study of a return sample far exceed those to be gained from automated landers within any given period of time. Only by extended and repeated missions can automated landers yield the information to be gained by one or possibly two return sample missions.
- NASA has shown an interest and political pressure for a return is anticipated.
- Should Martian life exist, a study of that life under controlled laboratory conditions would be scientifically imperative.
- Potential difficulties in developing an adequate operational quarantine facility will require an extensive testing program to determine the effectiveness of the constructed facility. If a return mission is anticipated anytime within the next ten years, it is strongly recommended that engineering of this facility be immediately initiated. No justification is complete without a risk assessment of the operational facility.

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2. Health Hazards Imposed by a Return Mars Sample

The probability of an independent life on Mars, while remote, is nonetheless finite. That possibility greatly exceeds the pre-Apollo probability that life existed on the moon. Nonetheless, that probability was believed to justify the extensive precautions taken at the Lunar Receiving Laboratory. The results of the recent Mariner 9 mission indicate past and present environmental conditions whereby the origin and evolution of life on Mars can be imagined. Adaptations of that life to the current Martian environment are not beyond serious speculation. Should Martian life exist and be transported intact to Earth, it may be pathogenic to man either directly or indirectly through other susceptible members of the ecosystem. Arguments advanced against this potential hazard are that diseases are mediated through genetic mechanisms and that Martian life may not be of sufficient genetic similarity to be pathogenic to terrestrial organisms. Further, survival of Martian life in the terrestrial environment is unlikely. Atmospheric oxygen, highly toxic in the terrestrial primeval environment, high surface temperatures and pressures, and high water activity all threaten the survival of Martian organisms.

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3. Risk Assessment and Information Required for Analysis of Hazard Potential

Based on current information, the pathogenicity issue cannot be resolved. Diverse opinions exist from individual risk assessments of the above considerations. In view of this dilemma, we have made an analysis of relative risks depending on available information. Further, we have considered types of information desired in order to reduce this risk and the probability of obtaining such information.

Information regarding life on Mars will be obtained from the Viking '75 mission. Although the USSR landers were to have provided biologically related data in the spring of '74, these missions failed. If Viking '75 is successful, this mission will provide information on the similarity and possible compatibility of Martian biochemistry with that of the Earth, the effect of heat sterilization on biochemical responses, soil organics and inorganics, water content and trace atmospheric constituents. Assuming proper functioning of the Viking '75 landers, the following result combinations are possible:

- Positive biology, positive organic analysis (+, +)
- Positive biology, negative organic analysis (+, -)

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- Negative biology, positive organic analysis (-, +)
- Negative biology, negative organic analysis (-, -)
- Contradictory or ambiguous information (?, ?). This possibility will be ignored in this analysis since its treatment would be highly data dependent.

The potential risk is not alleviated by any of these results. Thus, a positive biology signal indicates the presence of a Martian life which is potentially quite hazardous because of possible biochemical compatibility with terrestrial life. The susceptibility of this signal to control by mild heat sterilization can only reduce but not eliminate the potential biohazard. A negative signal only somewhat reduces the risk of returning a sample from Mars. Martian life similar to terrestrial life might exist but not be present at our sampling site. Alternatively, Martian life may be present but undetected because it is not sufficiently similar to terrestrial life to respond to our tests. Positive organic analysis is within the realm of prediction regardless of the presence of life, because the presence of organic material is anticipated by general theories of abiotic organic compound formation. However, the remaining possibilities, namely, negative organic analysis with or without positive biology, are without a

terrestrial counterpart and, therefore, warrant cautious procedure.

Taking these considerations as a whole, then, two risks are apparent for a return sample: a biohazard risk and a sample selection risk. The assessment of these risks could be as follows:

Viking '75			Relative Biohazard Risk			Relative Selection Risk	
Biol.	Org.	Heat Effect	Very High	High	Mild	High	Mild
+	+	None	x				x
+	+	Inhibits		x			x
+	-	None	x			x	
+	-	Inhibits		x		x	
-	+	-			x	x	
-	-	-			x	x	

On this basis, a decision for the return of a sample is still difficult except perhaps where positive biology is obtained which is not affected by heat. (Sterilization of Mars soil on Viking '75 is performed by heating at 160°C for three hours). In this case, a sample should not be returned without further study on Mars. Where the biohazard is somewhat reduced, either by heat control or by negative biology results, the risk of selecting a representative sample is increased. This is because negative biology or organic results may have been site specific. Positive results, on the other hand, suggest widespread

distribution. In all cases, then, further study is either highly desirable or necessary (very high biorisk) before a sample return can be considered.

If data from the forthcoming Viking '75 mission is insufficient for satisfactory risk assessment, consideration must be given to additional information which should be obtained on Mars before returning a sample to Earth. Five areas where information is desired are immediately apparent:

- Inhibitors for control of a possible pathogen, especially those found in the terrestrial environment not common to Mars (oxygen, water).
- Substrate specificities to determine similarity of biochemical pathways.
- Long-term preservation of sample within enclosed container (this gives us knowledge of whether attempts to return a live sample will be fruitful).
- Life detection tests and organic analyses at a variety of sites to determine universality of responses.
- Effect of Martian life on selected terrestrial organisms or ecosystems.

The importance of answering all questions for safe sample return cannot be overstated. However, it should be noted that the order of importance varies according to the initial observations. Thus, where negative biological or organic results are obtained, tests at additional sites may be most important. Where positive

biology is obtained which is not susceptible to nominal heat control, inhibitors probably are the most important. On the basis of such data, it would seem that the Return Sample Mission should be abandoned only if Martian life definitely cannot be controlled or inhibited. However, the difficulty of establishing that all Martian life forms can be controlled or inhibited is immense.

The question of the effect of Martian life on terrestrial life, while extremely important, is unfortunately not a question readily answered either a priori or by automated landers prior to returning a sample. In a delicately balanced ecosystem, it is impossible to determine which species are important for testing; it would be necessary to test all Earth forms on Mars. Even if this were feasible, a negative effect on Mars does not necessarily guarantee a negative effect on Earth. A pathogenic effect may not appear until certain synergistic conditions are met or may occur only after a long incubation period. Initially harmless Martian organisms may mutate into pathogens when placed in terrestrial conditions. Thus, while it may be interesting to test one or two selected terrestrial species on Mars, it is important to realize the limitations of these tests.

From the above considerations, it becomes apparent that one cannot design arguments or experiments which can lead to the

logical conclusion that it is completely safe to return a sample from Mars. One can only reduce the unknown risk by appropriate choices in sample handling. One can, however, reach the logical conclusion that it is unsafe to return a sample from Mars. Such would be the case if Martian life showed terrestrial biochemical pathways or was insensitive to various inhibitors. But in the event that it is deemed advisable to return a Mars sample, sample acquisition and handling must be considered from the standpoints of site selection, health hazards, manned mission, sterile sample, and location of quarantine laboratory. A summary of the problems in each category is as follows:

- Site Selection: The major issue involved in sample selection is to select one that contains as much biological information as possible. If Viking missions detect biology, the return sample should be selected to contain living organisms. If Viking missions fail to detect biology, the sample should contain as much organic information as possible. Perhaps Viking-type experiments can be conducted on the sample to ensure the initial presence of anticipated biology and organics. If Martian responses are highly heterogeneous, it may be necessary to provide a rover for adequate sampling.

- Health Hazard and Pathogenicity: This is the major issue in returning a sample from Mars. The scientific community appears divided on it. There are those who contend that disease mechanisms depend upon similarity

and compatibility in biochemical pathways. Thus, only if Martian organisms are remarkably similar to terrestrial organisms could they pose a potential pathogenic threat. Others contend that Martian organisms may have a completely unanticipated and harmful effect. Still others feel that even if the initial effect is harmless, a pathogenic effect may appear at a later date due to mutations or long incubation periods. One additional view is that Martian life would be extremely difficult to maintain under terrestrial conditions and probably could not live long enough to be pathogenic. The division of the scientific community can probably only be resolved by extensive experimentation on (return) samples. Probably the only indication of the potential hazard is data on whether or not positive responses are obtained by Viking-type landers, preferably with rovers, whether inhibitors are found which can control this life, and whether or not an effective quarantine can be maintained.

- Manned or Unmanned Mission: The arguments in favor of sending a man to Mars to obtain the return sample are the increased aid in sample selection and the in situ test for pathogenicity. Thus, one possible scenario is that if the man survives, he can be returned to Earth with reduced risk possibilities. However, a returned man always concommittantly returns some sample, even inadvertently (perhaps within him). Thus, a return manned mission should also be considered a return sample mission. In an unmanned mission, quarantine is more easily maintained although the advance knowledge of short-term effects on man in situ is not obtained.

- Sterilization of Return Sample: The major reason for returning a sample is for biological purposes. Ideally, a viable sample

would be returned. However, for maximum safety, it would be better to sterilize the sample. A sterile sample, although theoretically devoid of metabolic activity, can still yield morphological data as well as organic and inorganic data if the sterilization technique is sufficiently mild. A major problem is in the determination of a sterilization technique effective on unknown organisms that still preserves as much information as possible. The assessed risk must be a major factor in determining the severity of the sterilization procedure.

- Location of the Quarantine Laboratory: Possible locations suggested for a quarantine laboratory are in space (Mars or Earth orbit, moon) or on Earth (preexisting inland facility, de novo facility, or oceanic island). The choice of the type of facility depends upon three different risk assessments: the potential biohazard of the return sample, the probability of the return spacecraft crashing on Earth, and the probability of maintaining an effective quarantine. The laboratory can either be completely automated and remotely manipulated or manned.

Examples of selection of handling modes based on biohazard assessments are as follows:

Highest Risk (+ biology, + organics, no inhibition, life -- widespread)

Unmanned mission
Severely sterilized (if return at all)
Space station

High Risk (+ biology, + organics, inhibition, life widespread)

Unmanned mission
Sterilized
Space station

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Moderate Risk (+ biology, + organics, inhibition), (life not widespread)

Unmanned mission
Mild sterilization, perhaps by several different techniques
Space station or isolated Earth station

Mild or Low Risk (-biology, ± organics)

Unmanned or manned
Unsterile
Isolated terrestrial station

Additional factors to be considered are those concerned with the probability of spacecraft crash impact on Earth, probability of quarantine maintenance and termination of the study in the event that the sample poses a larger threat than anticipated. Also to be considered are the relative costs of various choices, although generally the higher costs will be associated with the greatest precautions. Each one of these factors can influence the mode of sample handling relative to the potential biohazard either by increasing or decreasing the degree of precaution to be taken.

4. Quarantine and Hazard Control

It is apparent at this time that a decision to return a Mars sample cannot be made on logical grounds. The strong concern for possible contamination of Earth expressed by a number of scientists precludes consideration of an early return of a viable sample. Even though a larger number of scientists

may express confidence that such hazard is negligible or can be satisfactorily contained, they cannot meet the burden of proof. As a possible transcending public health concern, the threat must be the controlling factor until set to rest. Proving that no adverse environmental impact can result from a Mars sample returned to Earth is impossible at this time. The myriad of potential hosts and ecological niches and the very long incubation times that would have to be accommodated for adequate testing render an empirical approach extraordinarily difficult.

It is our conclusion, therefore, that the return Mars sample planning should be constrained to considerations of the return to Earth and the study of sterilized samples of Mars soil.

While the examination of sterilized return Mars samples seems the only feasible approach, this rationale suffers from a serious problem. Even though the intent is to sterilize the sample, our present state of knowledge cannot assure us that any sterilization technique which will preserve biological information will, indeed, kill the alien organisms. Hence, the "sterilized" return sample must be received as if it contained viable organisms, thereby completing a sorites to the first, unacceptable, position of introducing Martian organisms to Earth. All of the biological containment techniques and facilities required for a "live" sample would also be

required for a minimally "sterilized" one. The only way to avoid this problem is to treat the sample in a manner yielding a probability of essentially one, that it has been sterilized. Whether or not such a minimum severity regimen will allow significant biological information to survive may be an area worth investigating.

Some efforts attempting to minimize sterilization to preserve biological and chemical features have been reported at the NASA Symposia in November 1973 and June 1974. These investigations analyzed the effects of heat and chemical sterilization on preservation of cellular morphology and structure of significant biological macromolecules, organic compounds, and inorganic compounds. No sterilization procedure has yet been identified which is not severely detrimental to the scientific worth of the sample. Although the degree of destruction varies with the procedure, biological properties and organic compounds are clearly most susceptible to soil sterilization. Our own laboratory effort, reported in later Sections, supports this conclusion by showing loss of soil enzymatic activity, presumably from cell-free exoenzymes, with minimal heat sterilization. An additional approach has been suggested by Danielli's group

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at the Center for Theoretical Biology, State University of New York at Buffalo. This group has proposed the use of immunological techniques to obtain biological information from sterilized Martian samples on Earth and from nonsterilized samples on Mars. The appended Minutes (Appendix I) of the group's meeting summarize the approach.

Although it appears extremely difficult to define an appropriate minimum sterilization procedure for terrestrial soils, the difficulties are compounded in defining an appropriate minimal procedure for Martian soils. The following problems are immediately apparent:

- Mars organisms may be less susceptible to a particular sterilization technique than terrestrial organisms. A candidate qualifying for this category is sterilization by UV radiation. Since Martian organisms are exposed to more UV radiation than terrestrial organisms, they may have protective mechanisms to minimize damage from this source.

- Mars organisms may be more susceptible to a particular technique and thereby be completely destroyed by it. A candidate qualifying for this category is sterilization by heat. Since Martian organisms are not exposed to as high temperatures in their habitat as terrestrial organisms, heat sterilization may be far more destructive.

- Sterilization techniques which are dependent on particular chemical reactions may be too geocentric for application to Martian organisms.

Assuming, however, that these problems can be solved, the crux of the problem remains to be addressed: "What is the minimum severity of treatment which will satisfy the scientific community that P = 1 for sterilization?" Only upon resolution of this problem can such a sterilized sample be returned to Earth without safety precautions. It is unlikely, however, that cognizant scientists, health and environmental authorities could agree on the required level of treatment of the sample. If there is to be a return Mars sample project, it will, most likely, involve some level of destructive treatment of the sample plus biological containment quarantine and examination.

5. Containment, Sterilization and Destruction of a Return Mars Sample

Regardless of whether a Mars return sample is returned sterilized or viable, that sample must be regarded as a viable sample, and appropriate containment precautions taken. The following means for containment might be explored:

a) The original sample container might be potted in epoxy, in clay which might then be vitrified, or in solid glass poured or formed around the container. The container might then be stored in concentrated sulfuric acid or a material similarily destructive of organic compounds but not damaging to the encasing material.

b) The sample container might be buried in a hermetically sealed vault. The vault

might be sealed by the same techniques mentioned in a) above. This method would provide easier access to the sample at some time in the future should it be desired to exhume it for experimentation or examination.

c) The sample container might be placed in orbit for storage.

d) The sample container might be sent to the moon for storage.

e) The sample container might be returned to Mars for storage.

Paragraphs c), d) and e) above assume that the sample had initially been returned to Earth. There is an element of risk inherent in the Earth landing and subsequent re-launching of the sample container. The sample container would have to be designed as, or placed in, a primary shield to prevent dissemination of the sample in the event of an accident. The container should incorporate devices for emitting light, sonic and radio signals to aid in its recovery in the event of accidental loss over land or in water.

Provision should exist for determining whether the risk of returning that sample to Earth and opening the container is permissible both before and after sterilization. Two unacceptable risks seem apparent. First, some evidence, obtained perhaps through hermetically sealed probe inserts discussed in earlier

reports, may indicate that the enclosed sample contains living organisms which survived sterilization attempts (or inhibition attempts for a viable sample). The second unacceptable risk would be information similarly obtained indicating the presence of macromolecules in the sterilized sample. Were this the case, the possibility of releasing genetic material hazardous to terrestrial biota could not be eliminated.

In either such event, the refractory nature of the sample might be the basis for a decision to ensure its containment indefinitely or until the hazards associated with the sample were understood to the point of their virtual elimination.

In the event that the preliminary examination of the sample indicates a degree of hazard sufficient to warrant destruction of the sample, suitable methods and facilities should be immediately available. Such indications may be high levels of apparent metabolic activity or reproduction of the sample despite attempts at sterilization; or the indication of large amounts of varieties of macromolecules which also are refractory to destruction when put through the sterilization procedures. In such an event, the following methods for destroying the sample might be considered:

a) Construct the sample container of a material that can retain its integrity when subjected to very high temperatures. The sample,

still within its original container, then might be repeatedly heated to temperatures, to be determined, which will yield sufficient assurance of sample destruction as evidenced by monitoring.

b) The sample and container might be melted in some molten material at a sufficiently high temperature to be determined.

c) The sample in its container might be subjected to intensive ionizing radiations at elevated temperatures for time-temperature dosages and energy spectra to be determined.

d) The sample container and sample might be incinerated at a temperature to be determined. The incinerator would have to be designed to preclude the emission of particulates.

e) The sample in its original container might be diverted or launched toward the Sun for gravity capture and destruction. In this event, similar precautions with respect to launch and flight accidents as those described for c) and d) above would be required.

Should the preliminary investigation establish the absence of hazard, the sample could be returned to the Earth quarantine facility and removed from its container for intensive studies. However, were an unexpected hazard to develop during this phase of the investigation, means would have to exist for containing and destroying the entire sample. This strongly indicates that, even if the early tests find no hazard, the next, or intermediate, phase of the investigation should be carried out by automated

instruments within an absolute biological and chemical barrier. This entire "laboratory" should thus be designed for transport, storage or destruction by the means described above for the sample in its original container.

6. Anticipated Engineering Problems

Some of the difficult engineering problems associated with a safe and productive Mars Sample Return program are apparent from the preliminary considerations afforded by this study.

a. Sample Acquisition

Site selection may hold the key to success for any Mars Sample Return program. It will be extremely important, therefore, to provide a sampling system that can determine and provide access to the desirable sample sites. The sampler must also be able to obtain the sample from a depth predetermined or determined on the basis of lander-gathered information.

The sampler must be free of chemical and biological contamination on all surfaces which contact the sample. These surfaces must also be free of potentially toxic or inhibitory material.

Once the sample is obtained, it must be sequestered in a manner which effectively provides a hermetic seal from the environment. The container must prevent any escape of Martian

material and also prevent the intrusion of external material into the sample.

Perhaps the major problem associated with sample acquisition and containment is that of preventing sample material from remaining on the outside of the sample container where it can become a potential contaminant. An alternate approach might be to assure the removal of any such material from the exterior surfaces of the sample container. In any event, great difficulty is foreseen in developing a method which will absolutely prevent the deposition of Mars surface material on the sample container where it could contaminate spacecraft system components and serve as a source of pervasive contamination spreading through the return vehicle, receiving laboratory,and terrestrial biosphere.

b. Prevention of Unintentional Sample Return

The Mars sample return vehicle will be subject to contamination by particulates upon relaunch from the surface of Mars and in passing through the dust supported by the Mars winds. Providing a suitable bioshield or other means for removing such contamination will be a difficult problem to solve. For example, when the Apollo command modules splashed down in the ocean, they undoubtedly innoculated the ocean with moon material. Similarly, moon material escaped in the transfer of men and

equipment from the command module to the receiving laboratory.

c. Handling

Sample handling will have to be performed in a manner which prevents external contamination of the sample container and which maintains the integrity of the sample.

d. Transport and Storage

A key problem in the transport and storage of the sample is that of maintenance of environmental conditions similar to those from which the sample was extracted. Care must be taken not to violate the ambient conditions of Mars in a manner which would do damage to organisms or biochemicals. The potential importance of diurnal temperature cycling should be considered. However, the principal environment problem within the sample container may be that of providing the necessary gaseous constituents of the atmosphere. Were any Martian life present in the sample, it is likely to require gases from the atmosphere and release other gases. Lack of the former over the long flight storage period could result in loss of viability, Similarly, the accumulation of expired gases may be toxic. It may be necessary to analyze the head space composition of the sample container shortly after sampling and to provide a means

for maintaining that composition during transport and storage. A combination of gas chromatography for atmospheric analysis and a feedback activated gas supply system and scrubbing system might be required.

e. Sample Examination

Methods for preliminary examination of the sample to assess the biological hazard will be required on Mars after the sample is secured and during transport to Earth. Should it be decided either to permit examination of the sample directly by scientists or to return the sample to Earth for examination, absolute bio-barriers will be required. Methods for direct or remote sample examination without violation of bio-barriers need to be developed. The bio-barrier system used in the Lunar Receiving Laboratory, adaptated from biological warfare facilities, are inadequate to this task. That type of barrier suffered from the dual liability of barrier failure and severe limitation of manipulative freedom for sample examination.

f. Distribution of Samples

After the samples have been examined in the quarantine facility and there is sufficient evidence to permit release of samples to outside laboratories, suitable methods are required for distribution to external laboratories. Even though all evidence

indicates no hazard to the biosphere, great care will have to be exercised in the continued study of the samples. The same precautions concerning containment, transportation, storage,and examination as discussed for the original sample will have to be taken. Possible consequences of the remote possibility that such samples may actually contain a time or condition dependent hazard necessitate such care. Complete records of sample distribution and experimentation will be required. Finally, a method of surveillance of each sample by the cognizant board or authority will be required.

g. Final Disposal of Samples

After completion of each sample study at its ultimate laboratory, means for final disposal will have to be implemented. These could consist of onsite disposal or transportation of samples to a central location for controlled disposal. Disposal may be by total destruction or prominent, absolute containment unless the experimentation conclusively demonstrates the inoculous nature of the sample. A complete record and control system for disposal will be required under the authority of the cognizant board.

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Should living organisms be detected in the samples, an exhaustive research program to assess all hazards and potential impact to the terrestrial biosphere will have to be undertaken before the life forms can be permitted to be introduced into the natural environment for economic or other purposes.

7. Approach for and Problems Related to Educating the Lay and Scientific Communities

The scientific community will approve the return of a Mars sample only if assurance is given that the mission can be accomplished without risk to the terrestrial biosphere. The facility design and operational problems of the Lunar Receiving Laboratory permitted the escape of lunar sample material. Had the moon contained life harmful to terrestrial life, the Earth would have been in grave trouble. Thus, a Mars Sample Return Mission will find considerable difficulty in convincing the scientific community of adequate containment. Following is a discussion of four steps deemed necessary to ensure mission safety from the viewpoint of back contamination and to convince the scientists and lay public that all precautions have been properly executed.

a) A receiving quarantine laboratory must be constructed well in advance of the mission, staffed with personnel thoroughly trained in quarantine procedure, and a sufficient number

of "dry run" demonstrations performed to ensure smooth functioning throughout the entire operation. This must include transfer of the sample from the returned lander to the quarantine facility, sample handling within the facility, and sample destruction or disposition at the end of the mission. Each scientist who is to perform experiments with the Martian soil within the quarantine laboratory must also be thoroughly rehearsed in these handling techniques.

b) Mission safety should, at least in part, be directly under the auspices of some non-NASA governing board composed of members of the Public Health Service, the Environmental Protection Agency, the Department of Agriculture, the Department of Defense, and the National Academies of Sciences and Engineering. Approval of both the design and operation of the facility should fall within the jurisdiction of this body. Security and Technical Officers, responsible to the governing board, should be appointed to ensure proper operation during the mission and coordinate with their NASA operational counterparts.

c) Since little knowledge regarding the potential hazard of the sample will be available prior to a return, a major factor ensuring safety would be enroute assessment of the potential hazard of the sample. Methods of performing such analyses

have been mentioned in previous sections but minimally should include several life detection schemes coupled with possible means of controlling that life. For example, were the Labeled Release Experiment to serve as such a probe, the metabolic response could be monitored before and after the application of a particular sterilization technique. Based on these criteria, should the sample prove more hazardous than previously assessed, provision should be made for total sterilization or spacecraft deflection.

d) The final critical aspect of a program to ensure safety is a complete listing of potential emergencies and contingency plans for handling them. Emergencies emanating from human sources (accident or illness), hardware failures, or a soil sample exceeding an arbitrary hazard level must all be considered. Questions of philosophy, such as emergency treatment or disposition of infected personnel, must also be satisfactorily resolved well in advance.

While fulfilling these objectives may satisfy some segment of the scientific community that the potential risk has been minimized, a major difficulty with this plan is that not all scientists will agree that adequate precautions have been taken and that the

risk reduction is reasonable. As long as discussion exists within the scientific community, it is unlikely that the lay public will accept the risk imposed by a Mars Sample Return Mission. Furthermore, the writings of popular scientists have had and will have a major influence on the public. For example, novels such as Andromeda Strain have already educated the lay public to the fact that hazards will exist despite extreme precautionary quarantine procedures. Since public safety is an issue, it is quite likely that this may become the most publicized of all NASA ventures. In order to maintain a viable mission, it is strongly recommended that the safety aspects of the mission be thoroughly planned well ahead of time and that a strong science and public information program be established. Differences among scientists should be resolved to their minima before presenting the planned mission to the lay public. Should opposition to the mission be so great from either the scientific or lay communities as to preclude it, then this should be known early before vast sums of money are spent.

If mission acceptance depended only on the assurance of safety, unaminity of opinion would be difficult enough. However, assuming complete safety assurance, the next major stopping point is the high cost of the mission which must be weighed against the

potential scientific benefits. This trade-off is highly subjective and extreme viewpoints can be identified. For the scientist, many questions of scientific benefit are readily envisioned - questions which challenge fundamental biological and geological theories. For the layman, however, the mission may not appear quite as beneficial since there is little immediate benefit. Past adventures undertaken have involved the opening of frontiers where there was potential economic reward for the participants or the society. Any economic rewards resulting from the proposed Martian exploration probably lie beyond the life span of the threatened public. This fact increases the layman's susceptibility to any dissension among scientists regarding mission safety.

8. Concluding Remarks

At the June 1974 NASA-sponsored Symposium, a study group recommended to NASA that a viable soil sample be returned to Earth. The major factors behind this recommendation were:

- The implication that a Return Sample Mission would be forthcoming.
- The probability expressed by NASA that the space program may sponsor only one more mission to Mars within the next decade or two, namely, the Return Sample Mission.

- The extremely high expense of the mission.
- The evidence that sterilization is severely detrimental to the subsequent study of biological and organic chemical properties.
- The purpose of the entire Mars endeavor to determine whether or not indigenous life exists on Mars.

It should be noted that these justifications are not based on strictly scientific considerations but, rather, on a political situation dictating that this may be the one and only opportunity for this generation to examine Martian life. The scientific approach would be to proceed more cautiously with a logical progression of missions. However, the actual data available prior to a return Mars mission will be only those obtained from the Viking '75 mission. At best, the data will be preliminary, although they may allow some estimation of the potential risk as discussed in a preceeding section. In any event, the potential risk involved in a return sample mission is sufficiently high to warrant extensive precaution. Thus, the only acceptable conditions under which a Martian soil sample should be returned to Earth are with demonstration of thoroughly adequate containment and quarantine measures. As discussed in a previous Section, this includes the construction of a quarantine facility, thorough training of all personnel, complete contingency planning

in the event of all conceivable accidents, and thorough integration of all scientific investigations with quarantine measures. Most important, adequate "dry run" demonstrations of the effectiveness of this quarantine facility and transfer of the sample into it are absolutely necessary to instill confidence in the scientific and lay communities. No sample should be allowed on Earth without these demonstrations, even if it means spacecraft deflection at the last moment. Coupled with the Earth quarantine procedure, it is imperative that provision be made onboard the return spacecraft for an enroute assessment of the potential hazard. Finally, provision must be made for diversion, total sterilization,or destruction enroute to Earth or sterilization after entry in the event the hazard assessment exceeds anticipated levels. Unless these conditions are met in full, it is considered unlikely that the scientific community will be unanimous in approving the return of a viable sample. The lay public and the Congress, in turn, would not be likely to approve the program.

C. Experimental Results

Our laboratory effort associated with the Mars Return Sample has sought a "mild" means of soil sterilization which preserves biologically significant information. Other investigators have

established that certain bound proteins survive heat sterilization. Luciferase, for example, immobilized on Sephadex is reported to survive 135°C for 36 hours, retaining 40% of the original enzymatic activity (27). Since extracellular enzymes may be bound to soil particles which could afford similar protection, we have sought a sterilization procedure which would destroy viability while retaining enzymatic activity. However, our preliminary results, reported below, indicate that little, if any, enzymatic activity in soil survives even a minimal heat treatment required for sterilization. No attempts were made to protect or immobilize enzymes in soil prior to sterilization.

As a preliminary effort, typical garden soil (WCH) was selected as a model soil. Prerequisite to an examination of enzymatic activity in this soil, heat sterilization experiments were performed to determine the minimum treatment necessary to eliminate all bacterial growth. This was accomplished by adding trypticase soy broth (TSB) or thyoglycolate broth to the soil after each heat regime, periodically removing aliquots of the broth, and plating the aliquots on agar. Soil broth cultures and agar plates were incubated for at least four days at room temperature to determine if viable bacteria were present. The results of these experiments (Table 6) show that with WCH soil, heat treatment at 175°C for one

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Table 6

Soil Cultures

Test Soil	Heat Treatment	Nonsterile Vials Found/Vials Tested
Garden Soil (WCH)	145°C - 1 hr	4/4
Garden Soil (WCH)	165°C - 1 hr	4/23
Garden Soil (WCH)	175°C - .5 hr	0/2
Garden Soil (WCH)	175°C - 1 hr	1/33
Wyaconda Soil (Fresh)	175°C - 1 hr	0/3
Wyaconda Soil (Old)	175°C - 1 hr	1/3
Pine Soil	175°C - 1 hr	0/3
Woody Soil	175°C - 1 hr	1/3

hour generally produced sterility, whereas treatment for one hour at either 145°C or 165°C generally failed to produce sterility.

To ensure that the minimal heat treatment did not hydrolyze the protein present in the soil, we examined soil extracts for tyrosine content before and after protein precipitation with trichloroacetic acid. Soils were extracted before and after heat treatment (175°C for one hour) by mixing 30 g soil with 60 ml 0.2 NaOH, sonicating one minute, and centrifuging. The supernatant was then analyzed for tyrosine by the Folin method of Lowry (27). As shown in Table 7, soil extracts contain both acid soluble amino acids and acid precipitable protein, as previously reported (28). Heat treatment appears to cause a reduction in the tyrosine content of the soil. However, it is seen that (Table 7) this reduction results from losses in the free amino acid fraction rather than from the acid insoluble protein fraction. Thus, it may be concluded that the soil conditions are not sufficiently acidic to cause a heat-catalyzed protein hydrolysis.

To determine whether or not enzymatic activity can survive the minimal heat treatment, a preliminary examination was made for phosphatase activity. Activity was sought by incubating 5 g soil with 50 mg glycerol phosphate (prepared by mixing 500 mg/100 ml water and adjusting to pH 9.5) for one hour at 37°C. At the end

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Table 7

Tyrosine Content in Soil Extracts

Sample	Tyrosine (mg/g Soil)		
		TCA	
	Total Extract	Soluble	Insoluble
Soil MF No Heat	0.20	0.046	0.13
Soil H 175°C - 1 hr	0.35	0.14	0.20
Soil H No Heat	0.52	0.26	0.21
Soil H 175°C - 1 hr	0.37	0.19	0.20
Soil H No Heat	0.62	0.35	0.26

of the incubation period, the slurry was centrifuged and the clear supernatant was analyzed for orthophosphate according to the vanadomolybdophosphoric acid colorimetric method (29). This method will detect only orthophosphate and is insensitive to organic phosphate present in glycerol phosphate. Thus, only if phosphatase activity converted glycerol phosphate to orthophosphate would a positive reaction be obtained over and above normal soil levels. The results show that WCH soil contains a significant amount of orthophosphate after incubation with glycerol phosphate (approximately 0.5 O.D. units corresponding to 0.6 ug phosphate per gram soil). However, with WCH soil heated for one hour at 175°C, less than 0.01 µg orthophosphate was detected after incubation with glycerol phosphate. Because no evidence of phosphatase activity was detected, after the minimal heat treatment, this approach was abandoned.

A second approach for determining survival of enzymatic activity after minimal soil sterilization entailed Labeled Release Experiments performed with a variety of ^{14}C labeled organic substrates before and after various heat treatments of WCH soil. Activity was sought by mixing 0.21 cc of soil with 0.02 ml of TSB or thioglycolate broth and the ^{14}C labeled substrate. Subsequently evolved radioactivity was collected by $Ba(OH)_2$ soaked getter pads

and counted in a Nuclear-Chicago gas flow counter. Typical results (Figures 36-39) appear to contradict those obtained by plating techniques (Table 6). Thus, heat treatments of 165°C for one hour and 145°C for one hour are not sufficient to sterilize the soil (Table 6). However, these treatments arrest the evolution of radioactivity from ^{14}C-urea and l-^{14}C-serine as substrates (Figures 36, 38). On the other hand, the evolution of radioactivity from ^{14}C-glucose was initially depressed for approximately 24 hours but then rose during extended incubation (Figure 37). In two instances, (Figures 37, 39), heat treatments at 145°C or 165°C for one hour were more effective in arresting metabolism than 175°C for 0.5 hour.

The finding that enzymatic activity is depressed in soils under heat conditions in which bacteria survive is not encouraging to our objective. Further, the varying degrees of inhibition depending upon the type of ^{14}C labeled substrate suggests that certain microorganisms in the population are killed by the heat treatment while others are allowed to survive and that these survivors are characterized on the basis of their reactivity to assorted substrates.

Since mixed populations in soils may contain a few heat resistant strains which may prevent detection of enzymatic activity

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Figure 36

Evolution of Radioactivity from Heat
Treated WCH Soils Dosed with ^{14}C Urea

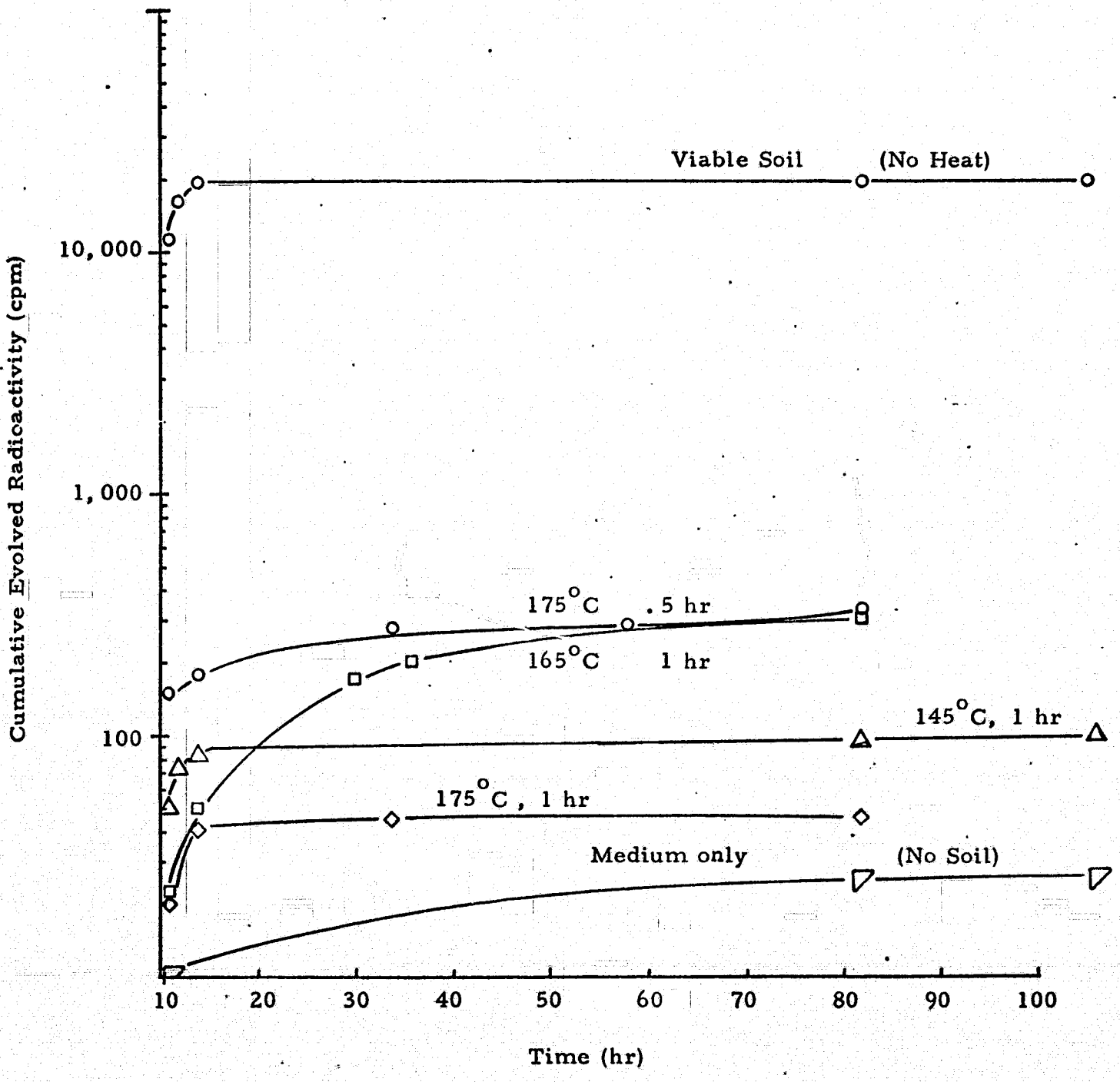

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Figure 37

Evolution of Radioactivity from Heat
Treated WCH Soils Dosed with UL ^{14}C Glucose

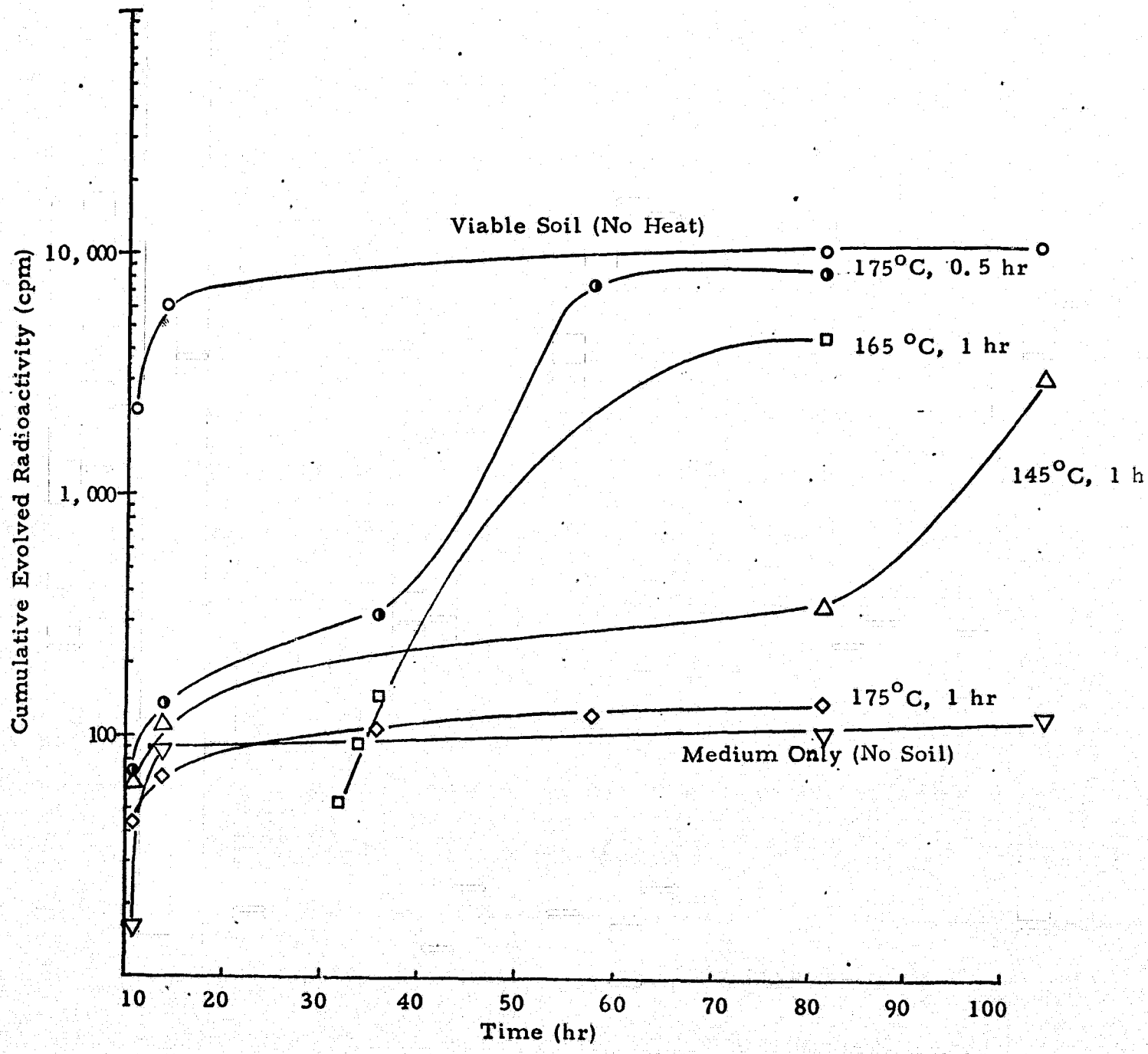

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Figure 38

Evolution of Radioactivity from Heat Treated WCH Soils Dosed with $1^{14}C$ Serine

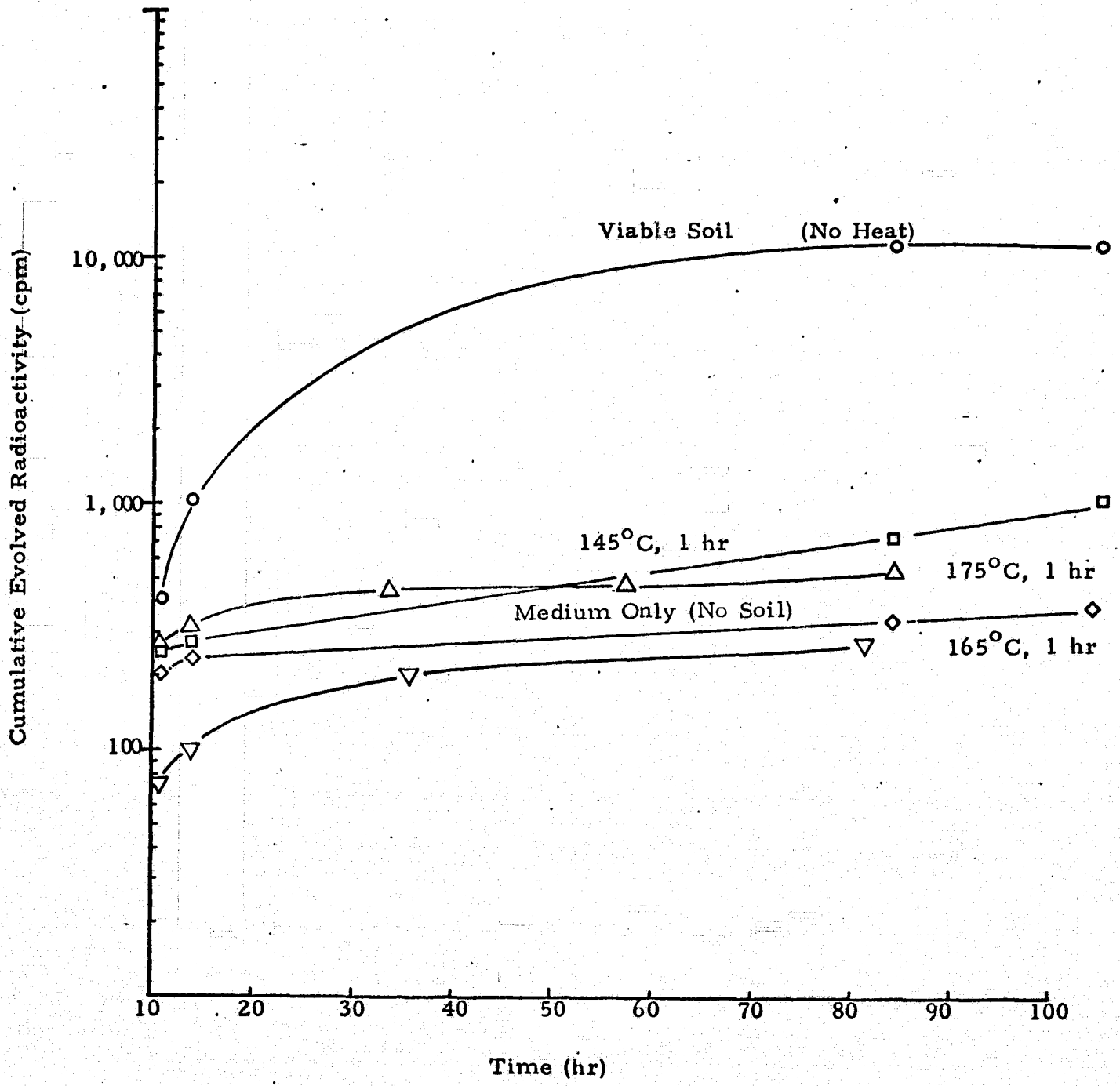

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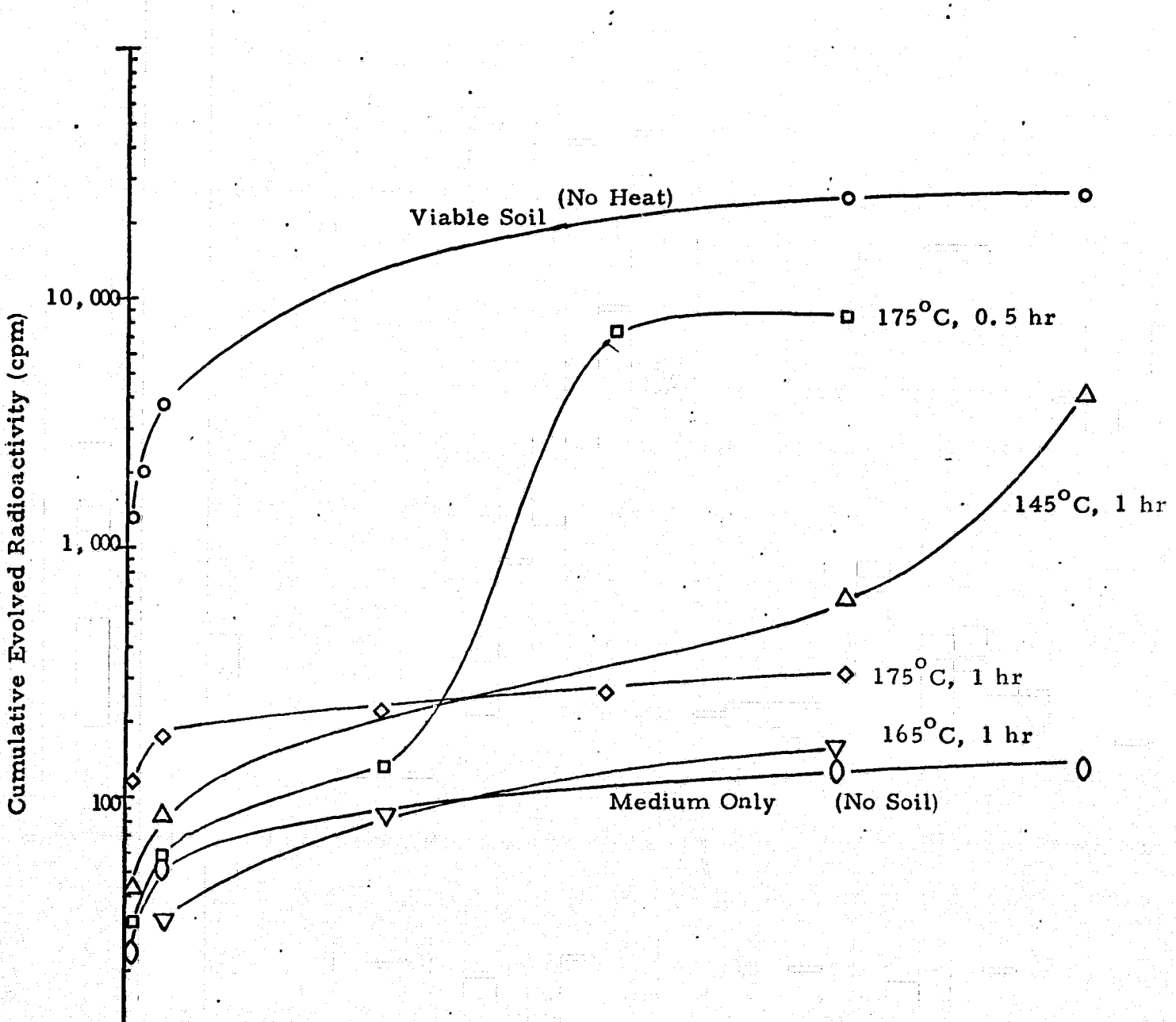
Figure 39

Evolution of Radioactivity from Heat
Treated WCH Soils Dosed with VM1 Medium



where sterility has been achieved, experiments have been initiated with pure cultures in an attempt to detect differential heat sensitivity of bacteria and enzymes. The method chosen was to examine the effect of heat on extracellular enzymes. To avoid difficulties potentially encountered in defining sterility, attempts have been made to isolate cell-free enzymes from Staphylococcus epidermidis after cell disruption by sonication. Typical results are shown in Tables 8 and 9. Sonicated cells, although reduced in viable cell number by three orders of magnitude, showed greater activity with all ^{14}C labeled substrates, possibly because cell disruption enhanced activity by removing permeability barriers (Table 8). However, most of that activity was removed by filtration or centrifugation indicating that the responsible enzymes are either within the remaining viable bacteria or bound to membrane fragments. Similar results were obtained when these cells were disrupted by a French Press (Table 9). However, since neither technique resulted in significant extraction of cell-free enzymatic activity from whole cells and cell fragments, this approach for differentiating heat sensitivity of enzymes and bacteria was abandoned. Future experiments will attempt to differentiate heat sensitivity on the sonicate with its enhanced enzymatic activity.

Table 8

Evolution of Radioactivity by Sonicated
Staphylococcus epidermidis

20 Minutes cpm of Evolved Radioactivity

Substrates	Whole [1] Cell Suspension	Sonicated [2] Cell Suspension	Supernatant [3] from Sonicate	Filtrate [4] from Sonicate	Control Substrate Alone
^{14}C UL Urea	1,183	11,352	220	229	21
^{14}C UL Glucose	307	2,300	74	35	28
UL ^{14}C Sucrose	970	1,803	54	41	16
UL ^{14}C Trehalose	26	220	21	31	19

0.02 ml of substrate (in distilled water) containing 0.045 μCi was mixed with 0.02 ml of the suspensions tested.

1. Cells suspended in phosphate buffer (1.2 x 10^{11} cells/ml plate count)

2. Cells suspended in phosphate buffer and sonicated (1.8 x 10^{8} cells/ml - plate count)

3. Sonicated mixture was centrifuged at 13,000 x g for ten minutes. Supernatant was tested.

4. Sonicated mixture was filtered through a Millipore 0.22 μ pore sized membrane filter. Filtrate was tested.

N.B. All test solutions and suspensions had been frozen overnight and were tested simultaneously.

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Table 9

Evolution of Radioactivity by French Press Treated Staphylococcus epidermidis

20 Minutes cpm of Evolved Radioactivity

Substrates	Extract of [1] French Press	Filtrate [2] from Extract	Control Substrate Alone
^{14}C Urea	4,562	516	21
UL ^{14}C Glucose	514	17	23
^{14}C Formate	113	44	25
1 ^{14}C DL Lactate	2,441	109	34
1 ^{14}C D Gluconate	1,562	40	26
1 ^{14}C Mannose	90	28	23
1 ^{14}C Serine	607	35	11

0.02 ml of substrate (in distilled water) containing 0.045 µCi was mixed with 0.02 ml of the solution tested.

1. An 18-hour culture of Staphylococcus epidermidis grown on TSA, was suspended in C_1 8% NaCl for extraction with a French Press.

2. The extract prepared as in 1 above, was filtered through a Millipore 0.22 µ pore size membrane filter.

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From this preliminary survey, it is concluded that techniques for demonstrating survival of enzymatic activity following minimal heat treatment for sterility are not readily available. Further, our evidence suggests that enzymatic activity is destroyed along with viability. This conclusion is in general agreement with those of other investigators (24) who have shown considerable destruction of biologically important molecules even with the most gentle sterilization procedures. However, because of the high desirability of delineating means of obtaining biological information from sterile soils, some further experimentation is warranted. Additional soils can be examined for a variety of enzymatic activities which by virtue of binding to soil particles may acquire heat resistance. Clay soils may be especially interesting. Also interesting for future efforts are possible means of protecting soil enzymes during heat treatment.

Respectfully submitted,

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APPENDIX I -
Minutes

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APPENDIX

Mars Working Party Minutes of Immunological Studies Subgroup, Held Friday, 30 November 1973

Present: P. Bigazzi, S. Cohen, J.F. Danielli, T. Yoshida

Absent: C. Van Oss

1. The group as a whole was of the view that immunological methods are highly suitable for identifying organic macromolecules, either before or after sterilization. Since immunological methods are competent to examine macromolecule fragments of relatively small size, loss of information by sterilization will be very much less important than would be the case, for example, if enzymic activity was studied. Thus the immunological approach, whilst not able to establish definitively that living organisms are present or have been present in a specimen, can provide very substantial evidence bearing on the probability of the existence of organisms, and the strength of this evidence mounts as the number of identifiable antigens increases.

2. Although immunological methods can be used optimally where the organisms to be studied can be cultured, a great deal of information can become available without culturing, provided macromolecules or fragments of macromolecules

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can be brought into aqueous solution or suspension.

3. In connection with the study of Mars, three distinct approaches must be considered:

(a) the innoculation of whole animals with the objective of producing antibody;

(b) the innoculation of cell cultures with the objective of producing antibody, or otherwise identifying antigens;

(c) interaction of antigens with prepackaged antibody.

Procedure (a) cannot at the present time be envisaged as appropriate, except in a manned space laboratory or in a terrestrial laboratory. Thus at the present time these procedures can probably be used only with sterile samples from Mars.

Procedures involving cell cultures: at present there are no adequate procedures, but very substantial progress has been made in this field of immunology, and if progress continues at the same rate, it may well be that ten years from now appropriate cell culture systems could be dispatched for use in Mars landers. In the development of such packages, study of sterilized Martian cell samples would be of great value.

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(continued)

The use of prepackaged antibody: such antibodies can be used in any proposed site of investigation, provided adequate temperature control, etc., is available. In general, use of prepackaged antibody presupposes that at least sterile Martian samples have been available for use as antigens. There may, however, in view of the cross reactions which exist with antibodies of different species, be some value in observing the interaction of Martian antigens with antisera prepared to a selected range of terrestrial species, since this would provide some information about the degree of relationship between Martian macromolecules and terrestrial macromolecules.

4. The recent development of the use of bacterial endotoxins, e.g., lipopolysaccharides, etc., to trigger cell multiplication in the immune response is extending the sensitivity of immunological studies so that in the future many studies will be possible with nanogram amounts of antigen, as contrasted with the milligram amounts necessary with classical techniques. This development greatly extends the value of immunological studies of Mars samples.

5. The logical sequence of studies would be as follows:

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(continued)

(a) terrestrial soils

(b) sterilized terrestrial soils

(c) Martian sterilized soils

(d) preparation of cell and/or antibody packages for use on Mars.

Almost everything which is required for steps (a) and (b) is readily available.

6. An appropriate division of responsibility between Biospherics and the Center for Theoretical Biology would be:

(a) joint selection of appropriate terrestrial soil samples;

(b) specimen handling and sterilization by Biospherics;

(c) preparation of water soluble antigen specimens and characterization of these specimens by Biospherics;

(d) identification of soil organism macromolecules and fragments of macromolecules by the Center for Theoretical Biology.

7. In order to establish how much information is lost by sterilization and other steps in specimen preparation, a

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number of known pure antigens should be taken through all the steps of all the procedures.

J. F. Danielli

APPENDIX II -

Medium Addition Sequence and Code

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Media Used for Multiple Addition Tests

CODE	MEDIA
3 20 35 60	Incubation Temperature (^{o}C) 0.1 ml VM1 medium
VM1	VM1 *
F	1^{14}C formate + acetate
A_1	1^{14}C acetate
A_2	2^{14}C acetate + DL-lactate
L_1	1^{14}C L-lactate
L_3	2-3^{14}C L-lactate **
L_2	2^{14}C L-lactate + DL-phenylalanine
PA_L	L-phenyl 1^{14}C alanine
PA_D	D-phenyl 1^{14}C alanine + DL-glutamate
G_1	1^{14}C DL-glutamate
G_2	2^{14}C DL-glutamate
G_{3-4}	3-4^{14}C DL-glutamate
G_5	5^{14}C DL-glutamate
AA	VM1 and 0.1 ml antibiotic antimycotic mixture (Penicillin 10,000 µ/ml, Streptomycin 10,000 mcg/ml, Amphotericin B 25 mcg/ml).
HM	VM1 and 0.1 ml heavy metal mixture containing 1 mg/ml of $Hg(NO_3)_2$, $Zn(NO_3)_2$, $Cu(NO_3)_2$, $Ag(NO_3)_2$, $Cd(NO_3)_2$, $Pd(NO_3)_2$ Note: Not all components were completely soluble.
KCN	VM1 and 0.1 ml of 0.1 M KCN

* VM1, 1 µCi/ml composed of UL^{14}C L-alanine, UL^{14}C D-alanine, UL^{14}C glycine, ^{14}C formate, UL^{14}C DL-lactate, UL^{14}C glycolic acid. (Total radioactivity 3.4 µCi/ml).

** Data are obtained for 3^{14}C lactate by multiplying cpm for 2-3^{14}C lactate (1 µCi/ml) x 2 and subtracting cpm for 2^{14}C lactate (1 µCi/ml).

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