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Quarterly Progress Report No. 15

to

National Aeronautics and Space Administration
Radioisotopic Biochemical Probe

for

Extraterrestrial Life

Contract No. NASr-10

By

Hazleton Laboratories, Inc.
P.O. Box 30
Falls Church, Virginia

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I. SUMMARY

The testing of pure cultures of algae for radiological detection of photosynthetic activity was continued, and laboratory model photosynthesis chambers were completed. The algae tested for photosynthetic response were the green alga Scenedesmus quadricauda, the blue-green alga Nostoc commune, the red alga Porphyridium cruentum, and the marine diatom Nitzschia closterium. For S. quadricauda, light-induced $C^{14}O_2$ fixation as well as dark evolution of $C^{14}O_2$ were indicators of phototrophic activity. In testing N. commune, only net $C^{14}O_2$ fixation indicated photosynthetic activity; $C^{14}O_2$ evolution, although considerably greater than the amount fixed, was not markedly higher than that of controls. Striking photosynthetic responses were elicited from P. cruentum; both $C^{14}O_2$ evolution and $C^{14}O_2$ fixation were much greater than that of controls. In the study of the diatom N. closterium, relative net $C^{14}O_2$ fixation alone indicated phototrophic activity, whereas the amount of $C^{14}O_2$ evolution was not different from that of controls.

Results of previous experiments conducted in this program have indicated that pH of the test medium affected $C^{14}O_2$ exchange. Two algae, Euglena gracilis and Chlorella pyrenoidosa, were tested for photosynthetic response in systems buffered at acid, neutral, and alkaline pH levels. For both organisms, pH had no effect on net $C^{14}O_2$ fixation by live cells. In the case of Chlorella, the previously reported retention of $C^{14}O_2$ by killed cells in acidified aqueous solutions was confirmed, and this phenomenon has not been explained. There was no marked difference in $C^{14}O_2$ evolved by the organisms in acid or neutral systems, but there was a strikingly greater evolution of $C^{14}O_2$ in alkaline conditions. The non-metabolic absorption of $C^{14}O_2$ during the light phase and its subsequent

evolution from controls during darkness at alkaline pH's was as high as metabolic exchange of $C^{14}O_2$, amounting to a complete masking of metabolic activity.

Field and laboratory experiments have provided valuable information indicating the feasibility for the "in situ" Gulliver. Excellent positive responses were obtained within one minute and five minute incubation-collection periods directly from the soil surface with medium M9 containing (formate, glucose, lactate, glycine)-C^{14}.

Ammonium nitrate has been substituted for KNO_3 in M9, furnishing an additional nitrogen source as well as an ammonium salt to the medium.

Addition of 5% dimethyl sulfoxide did not appear to enhance the inhibitory effect of Bard Parker on Saccharomyces cerevisiae.

Seven new test soils and five soil isolates produced positive responses from basal C^{14} media.

A 50% final concentration of unlabeled L-glucose in a labeled inorganic salt medium did not inhibit the growth of Escherichia coli.

II. BIOLOGICAL INVESTIGATION

A. INVESTIGATIONS OF PHOTOSYNTHETIC RESPONSE

1. Introduction

Investigations conducted during the third quarter were concerned with further tests with a marine diatom and diverse phyletic color lines of algae for photosynthetic activity as measured by amount of light-induced $C^{14}O_2$ incorporation and subsequent $C^{14}O_2$ release during dark metabolism. The algae tested for relative responses and for effect of pH on $C^{14}O_2$ exchange were *Nostoc commune* (blue-green), *Scenedesmus quadricauda* and *Chlorella pyrenoidosa* (green), *Euglena gracilis* (protozoan-like euglenophyte), and *Porphyridium cruentum* (red). The marine diatom *Nitzschia closterium* was also tested.

2. Experimental

Method of $C^{14}O_2$ generation, light-dark exposure cycle, incubation of organisms during testing, $C^{14}O_2$ collection, and radioactivity determinations were as reported in previous quarterly reports.

a. Detection of Photosynthesis in the Green Alga *Scenedesmus quadricauda*

S. quadricauda was grown in the liquid medium previously defined for *Chlorella*. Although *S. quadricauda* is a green alga, it does not multiply as rapidly as *Chlorella*. It was therefore of interest to measure the magnitude of $C^{14}O_2$ exchange of a relatively slow-growing green alga. All treatments were triplicated, and cultures taken from a 27-day old stock contained 4.3×10^5 cells per flask. The results of this experiment are shown in Table II-1.

Table II-1

Photosynthesis of S. quadricauda

Thirty-Minute Exposure to $C^{14}O_2$ in Light Followed by 45 Minute Incubation in Dark

Treatments	Net Radioactivity - cpm							
	$C^{14}O_2$ Collected Final 15 Min. Dark Period				Net $C^{14}O_2$ Fixed			
	Replicate			Mean	Replicate			Mean
	1	2	3		1	2	3	
Cells	1964	2276	1800	2013	4759	5213	5630	5200
Water blank (pH 5.2)	661	610	486	585	13	21	24	19

The mean radioactivity in cellularly incorporated C^{14} was much greater than that retained by the blank. Although the anomalous evolution of $C^{14}O_2$ from controls of acid pH was again demonstrated, the average amount of $C^{14}O_2$ evolved from photosynthetic intermediates of S. quadricauda was considerably greater than that evolved from the blanks.

b. Detection of Photosynthesis in the Blue-Green Alga Nostoc commune

A 43 day-old culture of N. commune grown in soil extract medium (1) was split into two equal aliquots, one of which was heated at 80°C for one hour, while the other remained at room temperature. The pH of the heat-killed sample and the live culture was 5.7. Soil-extract medium served as a blank; its pH was 5.2. The live and killed cell treatments and the medium blank were exposed to $C^{14}O_2$, incubated and prepared for radioactivity determinations in the manner described in (a) above. Triplicate cultures each contained 3.1×10^5 cells. The results of this study are presented in Table II-2.

Table II-2

Photosynthesis of N. commune

Thirty Minute Exposure to $C^{14}O_2$ in Light Followed by 45 Minute Incubation in Dark

Treatments	Radioactivity - cpm							
	$C^{14}O_2$ Collected Final 15 Min. Dark Period				Net $C^{14}O_2$ Fixed			
	Replicate			Mean	Replicate			Mean
	1	2	3		1	2	3	
Live cells	2119	1723	1498	1780	334	378	668	463
Killed cells	1000	1648	1952	1533	23	10	28	20
Medium blank	1586	1896	1104	1528	30	26	16	24

Live cells of N. commune incorporated much more C^{14} than either the killed cells or the blank. Metabolic release of $C^{14}O_2$ in darkness could not be differentiated from non-metabolic release.

c. Detection of Photosynthesis in the Red Alga Porphyridium cruentum

Pure cultures of P. cruentum were grown in a sewage-sea water medium (2) sterilized by passage through a bacteriological filter.

A 25-day old culture of Porphyridium was split into two equal aliquots, one of which was heat-killed as previously described while the other remained at room temperature; both were tested for radiological detection of photosynthetic activity. Cell counts were rather low (1.3×10^4 cells per flask) and the medium was rather alkaline, pH 8.5. These factors may have been responsible for the lack of photosynthetic response.

A subsequent test of photosynthetic activity was conducted on a 38-day old culture of Porphyridium which appeared to be of about the

same gross cell density as the aforementioned culture. Culture density was increased by low-speed centrifugation and decantation of the supernatant. Triplicate cultures each contained 1.0×10^6 cells per incubation flask, and the pH of all treatments, which included a culture medium blank, was 6.0. Test conditions were as described above. Results of this experiment are shown in Table II-3.

Table II-3

Photosynthesis of P. cruentum

Thirty-Minute Exposure to $C^{14}O_2$ in Light Followed by 45 Minute Incubation in Dark

Treatments	Net Radioactivity - cpm							
	$C^{14}O_2$ Collected Final 15 Min. Dark Period				Net $C^{14}O_2$ Fixed			
	Replicate			Mean	Replicate			Mean
	1	2	3		1	2	3	
Live cells	1256	1922	2111	1763	27,890	35,934	58,497	40,773
Killed cells	114	147	175	145	27	19	53	33
Medium blank	46	-*	86	64	25	36	8	23

*Aberrant count

The above data show that a very high rate of photosynthetic fixation of $C^{14}O_2$ occurred, relative to that of the killed cells and the blank. The amount of $C^{14}O_2$ evolved from live cells in darkness was significant in relation to evolution from killed cells and the blank. Again, there was a considerable evolution of $C^{14}O_2$ from the acid medium containing killed cells.

d. Photosynthetic Response of the Marine Diatom *Nitzschia closterium*

A 40-day old culture of *N. closterium*, grown in a standard diatom medium (1), was centrifuged and most of the supernatant decanted. The cells were resuspended in the remaining volume of the supernatant to increase the culture density over that of the original culture. A portion of the concentrated culture was then heated as previously described to kill the cells and the remaining portion served as inoculum for the live cell treatment. Standard diatom medium was used as a blank. The pH of all preparations was 7.2. Test conditions were as previously described and triplicate cultures each contained 9.7×10^4 cells per flask. The results of this experiment are shown in Table II-4.

Table II-4

Photosynthesis of *N. closterium*

Thirty-Minute Exposure to $C^{14}O_2$ in Light Followed by 45 Minute Incubation in Dark

Treatments	Net Radioactivity - cpm							
	$C^{14}O_2$ Collected Final 15 Min. Dark Period				Net $C^{14}O_2$ Fixed			
	Replicate			Mean	Replicate			Mean
	1	2	3		1	2	3	
Live cells	119	212	123	151	4276	2338	1435	2683
Killed cells	247	126	122	165	1497	1607	1204	1436
Medium blank	182	111	101	131	1400	1787	1717	1635

The data show that $C^{14}O_2$ evolution from live cultures of *N. closterium* having a relatively low cell count was not detected.

Although a rather high amount of $C^{14}O_2$ was retained by killed cells and the blank, metabolic incorporation by live cells was indicated.

e. Effect of pH on $C^{14}O_2$ Solubility

The pH of the water blanks and of the algal medium in the test cultures greatly affects the amount of $C^{14}O_2$ leaving the air phase and entering the water phase of the exposure chamber. The $C^{14}O_2$ taken into solution, completely independent of any metabolic reaction, will come out of solution during the gas collection period and may completely mask the algal metabolism. To illustrate this, the following example is offered. Assume the volume of the air space is five times the volume of the liquid in the chamber in which the algal culture is exposed to $C^{14}O_2$ in the light. Then the distribution of the $C^{14}O_2$ between the air and water phases as a function of pH will be as follows at 25°C.

pH	Moles CO_2 in air/Moles CO_2 in water
2	64.0
3	64.0
4	64.0
5	61.5
6	44.7
7	12.1
8	1.4
9	0.14
10	0.01

It is thus seen that approximately five times as much $C^{14}O_2$ would go into solution at pH 7 as at pH 5. Accordingly, a correspondingly greater amount of $C^{14}O_2$ is available for collection from the pH 7 solution when the getter is introduced.

This problem may be resolved when algae are exposed to $C^{14}O_2$ in soil, rather than in water. Drying of the aqueous culture would

eliminate this solubility artifact from the C^{14} fixation determination.

Laboratory investigations of the pH problem as it affects $C^{14}O_2$ solubility and algal metabolism were undertaken.

Chlorella pyrenoidosa. A culture of a high-temperature strain (7-11-05) of this green alga was centrifuged and the cells resuspended in 0.06 M phosphate buffer. This preparation was split into two equal aliquots, one of which was heated as previously described to kill the cells; the other aliquot served as the live cell treatment. This procedure was used for systems buffered at pH 4.5, 7.0, and 8.7. For pH 4.5, 0.06 M KH_2PO_4 was used; equal amounts of 0.06 M KH_2PO_4 and Na_2HPO_4 were used for pH 7.0; and 0.06 M Na_2HPO_4 was used for pH 8.7. Respective cell counts at the three pH levels were 3.1×10^7, 3.0×10^7, and 2.9×10^7 cells per incubation flask. Test conditions were as described previously and treatments were duplicated. The results of this experiment are shown in Table II-5.

Table II-5

Effect of pH on Detection of Photosynthesis of C. pyrenoidosa

Thirty-Minute Exposure to $C^{14}O_2$ in Light Followed by 45 Minute Incubation in Dark

Treatments	Net Radioactivity - cpm					
	$C^{14}O_2$ Collected Final 15 Min. Dark Period			Net $C^{14}O_2$ Fixed		
Cells	Replicate 1	Replicate 2	Mean	Replicate 1	Replicate 2	Mean
Live, pH 4.5	638	1,134	886	20,903	42,699	31,801
Killed, pH 4.5	92	90	91	2,524	4,101	3,312
Live, pH 7.0	1,007	1,140	1,073	36,951	56,951	46,663
Killed, pH 7.0	15	24	20	26	40	33
Live, pH 8.7	5,801	21,037	13,419	37,417	41,130	39,273
Killed, pH 8.7	19,343	9,118	14,230	137	78	107

Although there was considerable variation between replicates of certain treatments, significant results were obtained. Over the range of pH imposed, there was no definite effect of pH on the metabolic activity of C. pyrenoidosa to fix $C^{14}O_2$. Again, it was demonstrated that killed cells at an acid pH retained much $C^{14}O_2$; there was slight retention by killed cells at neutrality; and, there was moderate retention at pH 8.7. At pH 4.5 and 7.0, the metabolic evolution of $C^{14}O_2$ from photosynthetic compounds could be detected, but at pH 8.7, the metabolic release of $C^{14}O_2$ was masked by dissolution of $C^{14}O_2$ from the water.

Euglena gracilis. The Vischer Strain of this euglenophyte was used in this study of pH effects. Cells were centrifuged and resuspended to increase culture density as described above. In this experiment killed cell controls were not prepared; instead, replication was increased. Live cells were suspended in 0.06 M phosphate buffer systems at pH 4.5, 7.0, and 8.6; calculated cell counts at the three pH levels were, respectively, 8.2×10^4, 10.5×10^4, and 12.7×10^4 cells per incubation flask. Treatments were triplicated and test conditions were as previously described. The results of this experiment are shown in Table II-6.

Table II-6

Effect of pH on Detection of Photosynthesis by E. gracilis

Thirty-Minute Exposure to $C^{14}O_2$ in Light Followed by 45-Minute Incubation in Dark

Treatments	Net Radioactivity - cpm							
	$C^{14}O_2$ Collected Final 15 Min. Dark Period				Net $C^{14}O_2$ Fixed			
	Replicate			Mean	Replicate			Mean
	1	2	3		1	2	3	
pH 4.5	425	285	533	414	673	805	652	711
pH 7.0	455	377	-*	416	610	643	704	652
pH 8.6	13,132	10,276	7,069	10,159	643	789	486	639

*Aberrant count

As shown for C. pyrenoidosa, pH had no effect on the ability of cells of E. gracilis to fix $C^{14}O_2$ photosynthetically. There was no difference in $C^{14}O_2$ evolution in darkness at pH 4.5 and 7.0, but there was a striking increase in $C^{14}O_2$ evolution at 8.7, amounting to a roughly 25-fold response over those observed at the two lower pH's.

3. Discussion and Conclusions

Although a relatively low concentration of cells of the green alga, Scenedesmus quadricauda was tested, the results shown in Table II-1 indicate that light-induced cellular incorporation of $C^{14}O_2$ and its subsequent metabolic release in darkness could be detected by the radiological method. Scenedesmus cells divide at a slower rate than cells of Chlorella pyrenoidosa; moreover, few cells of Scenedesmus when tested, were actively dividing. It has therefore been shown that, although growth, in terms of cell multiplication, was occurring at a slow rate, photosynthetic metabolism was sufficiently active to permit distinct radiological detection of phototrophic activity.

Detectable light-induced $C^{14}O_2$ incorporation by the blue-green alga Nostoc commune was demonstrated (Table II-2), but metabolic release of $C^{14}O_2$ by living cells during darkness could not be differentiated from that of killed cells or the blank. Cell concentration in the experimentals was relatively low (3.1×10^5 cells per flask), and it is probable that the number of cells was insufficient to allow discrimination between metabolic release and non-metabolic release of $C^{14}O_2$ in darkness during a single light-dark regime.

Results presented in Table II-3 show that photosynthetic metabolism of the red alga Porphyridium cruentum in concentrations of 1.0×10^6 cells per flask can be easily detected radiologically in acid

conditions; however, photosynthetic responses could not be detected for 1.3×10^4 cells in alkaline conditions.

Photosynthetic fixation of $C^{14}O_2$ was detected in the marine diatom Nitzschia closterium at pH 7.2, although metabolic evolution of $C^{14}O_2$ could not be differentiated from non-metabolic release during incubation in darkness (Table II-4). Here again, it is probable that the cell concentration was not sufficiently high to discriminate between metabolic and non-metabolic release in alkaline conditions. Considerable retention of $C^{14}O_2$ occurred in the killed cell treatment and in the blank; here, therefore, retention of $C^{14}O_2$ by killed cell treatments under alkaline conditions was detected.

It has been shown in previous quarterly reports that certain control treatments at acid pH, containing heat-killed cells or lacking them, absorbed appreciable amounts of $C^{14}O_2$ during the light phase and released it during the subsequent dark phase. Results presented in this report (Tables II-1, II-2, and II-3) are further demonstrations of this phenomenon.

The above anomalies of $C^{14}O_2$ exchange under acid and basic conditions led to studies of pH effects in buffered systems. The pH studies (Tables II-5 and II-6) clearly show the effect of concentration of hydrogen and hydroxyl ions on $C^{14}O_2$ exchange. In both cases, $C^{14}O_2$ evolution by live cells was comparable in neutral or acid conditions; in alkaline conditions, however, evolution of $C^{14}O_2$ was greatly stimulated. The dark evolution of $C^{14}O_2$ in alkaline conditions followed the extensive non-metabolic absorption of the labeled gas during exposure in the light. The importance of the effect of pH on CO_2 solubility has been discussed earlier, as have possible methods for controlling this problem.

In the Chlorella experiment (Table II-5) both the live- and killed-cell treatments evolved comparable high counts in $C^{14}O_2$ at pH 8.7, whereas $C^{14}O_2$ fixation data for this pH clearly show the function of living cells in $C^{14}O_2$ uptake. Net $C^{14}O_2$ fixation by live cells was comparable, regardless of pH, in the Chlorella experiment and in the Euglena study.

The photosynthesis studies made in this program show that $C^{14}O_2$ was readily incorporated by living cells when they were placed in a fiberglass matrix through which the labeled gas was passed. As reported earlier (Quarterly Report No. 13), photosynthetic uptake was not elicited when $C^{14}O_2$ was passed over cell suspensions in the center well of Warburg vessels. Carbon dioxide administered to decidely acid systems would not be very soluble therein, and would be rather unavailable for cellular uptake. However, cells suspended in acid buffers or media and placed on fiberglass pads photosynthetically fixed $C^{14}O_2$. Such systems might be viewed as semi-solid in nature, containing cells having moistened cell walls exposed to the porous gas passages. Therefore, low solubility of $C^{14}O_2$ in acid conditions did not prevent sufficient exposure of the gas to cell membranes for metabolic incorporation. Based on this premise, it is conceivable that in cases where killed cells retained radioactivity, the heat-killing process created CO_2-bonding sites on cell walls or cell membranes, or released CO_2-bonding agents to the ambient fluid. In analysing the retention of radioactivity by killed cells at low pH (eg. pH 4.5, Table II-5), it may be assumed that a complexing or chelating mechanism was operative. Such a system might consist of carbon-containing substituent groups whose dissociation activity would not follow the Henderson-Hasselbach relationship for pK, pH, bicarbonate ion, and CO_2 concentration, and the quantitative loss of CO_2 in darkness would not occur.

4. Tasks for the Fourth Quarter

The program designed to screen pure cultures of algae for photosynthetic response is considered complete. The yellow-green alga, *Botrydiopsis arhiza* has not been successfully grown to a useful culture density; this organism, therefore, will not be further studied.

Laboratory model photosynthesis chambers are complete. Upon completion of preliminary studies of labeled gas manipulation and recorder calibration, natural soil samples and sterile soils inoculated with pure cultures of phototrophic algae and bacteria will be tested for photosynthetic response.

B. IN SITU DETERMINATIONS

Biological support for the "in situ" Gulliver has commenced. Planchet and in situ experiments were conducted in the field and in the laboratory to compare (1) responses directly from soil surfaces, from 1/4 inch soil cores placed in planchets, from mixed soil-horizon samples packed in planchets, and from 100 mg of mixed soil-horizon samples in planchets; and (2) incubation and collection response periods.

The field portion of the experiment was conducted on garden soil in the Washington, D. C. area. A site approximately one foot square was selected. Each phase of the experiment was performed in triplicate, but due to the very nature of soil even this limited area would contain a large variety of mixed populations. As a result, the replicates are to be considered as individual responses. Medium M9 containing C^{14} formate, glucose, lactate, glycine, Table II-7, II-8, was used throughout. Three soil determinations were performed.

(1) In situ - 0.04 ml of C^{14} medium was placed directly on the soil surface. Wet $Ba(OH)_2$ collection pads in planchets were immediately placed over the sites. One set was allowed to remain for one hour. A second set of inoculations was covered for 15 minutes; collecting planchets were removed and replaced with fresh $Ba(OH)_2$ pads and a second 15 minute incubation-collection made; a third and fourth 15 minute incubation-collection period followed.

(2) Soil core, planchet determination - soil cores (approximately 1/4 inch profile) from the same site were carefully and aseptically removed and placed within planchets, soil surface upward; 0.04 ml of C^{14} media were placed on the soils; incubation-collection procedures were the same as in (1) above.

(3) Mixed soil-horizon sample, planchet determination - the

procedure was identical to (2) above with the exception of thoroughly mixing the soil profile sample before placement in the planchets.

(4) Soil, laboratory, planchet determination - soil from the experimental site was removed to the laboratory, portioned out in 100 mg quantities, placed in planchets containing the same C^{14} medium (0.5 ml) and incubated and collected for the same time intervals. This enabled comparison of the routine laboratory method and results to those obtained in the field.

The responses are presented in Table II-9. The in situ results were very encouraging, particularly those obtained during the first fifteen minute periods.

In view of these excellent early responses, a second field-laboratory experiment was conducted. The same general procedure was followed, only the incubation and collection periods were altered. Four completely separate (non-consecutive) incubation-collection periods were made, consisting of one minute, five minute, twenty minute and sixty minute periods each. The responses were again excellent, Table II-10. The one minute and five minute responses from the organisms present on the soil surface (in situ) were very high in themselves and also in comparison to those obtained in the laboratory at comparable periods. These experiments have provided valuable information indicating the feasibility of the in situ method of detecting metabolic activity directly from the soil surface.

Table II-7

COMPOSITION OF BASAL MEDIA-UNLABELED

Medium M9

	per liter
K_2HPO_4	1.0 g
NH_4NO_3	0.2
$MgSO_4 \cdot 7H_2O$	0.2
NaCl	0.1
Soil extract*	100.0 ml
	pH 7.0

Medium M8

	per liter
K_2HPO_4	1.0 g
KNO_3	0.5
$MgSO_4 \cdot 7H_2O$	0.2
NaCl	0.1
	pH 7.0

Medium M5

	per liter
K_2HPO_4	1.0 g
KNO_3	0.5
$MgSO_4 \cdot 7H_2O$	0.2
NaCl	0.1
Malt extract	3.0
Beef extract	3.0
Yeast extract	13.0
Ascorbic acid	0.2
L-cystine	0.7
Bacto casamino acid	4.0
Proteose peptone #3	20.0
Soil extract*	250.0 ml
	pH 7.2

Medium 1/5 M5 - Medium M5 diluted 5-fold with distilled water

Medium 1/16 M5 - Medium M5 diluted 16-fold with distilled water

*Soil extract prepared by suspending 500 g of air dried soil in 1300 ml H_2O containing 0.1% Na_2CO_3. The mixture is autoclaved for one hour, filtered, and liquid loss made up to 1000 ml with water.

Table II-8

BASAL MEDIA-C^{14} SUBSTRATES

Formate-Glucose-Lactate-Glycine

C^{14} Substrates	Sp. Act. (mc/millimole)	mM	uc/ml	% (w/v)
Sodium formate	25.0	0.26	6.5	0.002
D-glucose-U.L.	3.0	0.42	1.3	0.007
DL-sodium lactate-1	5.0	0.26	1.3	0.002
Glycine-1	4.4	0.22	1.0	0.002

Formate-Glucose-Lactate

C^{14} Substrates	Sp. Act. (mc/millimole)	mM	uc/ml	% (w/v)
Sodium formate	25.0	0.26	6.5	0.002
D-glucose-U.L.	3.0	0.42	1.3	0.007
DL-sodium lactate-1	5.0	0.26	1.3	0.002

Table II-9

IN SITU AND PLANCHET DETERMINATIONS - I

Inoculum	Radioactivity - cpm Incubation-Collection Periods (min)				
Soil Organisms All Taken Within One Square Foot Plot	0-15	15-30	30-45	45-60	0-60
In Situ					
Field	57,754	6,311	4,069	2,462	35,710
0.04 ml medium	54,457	5,151	3,939	2,270	27,937
	---	---	2,900	2,305	---
Planchet					
Soil Core	29,711	12,163	7,273	4,032	92,354
Field	30,662	11,118	5,625	3,894	81,337
0.04 ml medium	33,820	2,498	5,747	3,322	71,997
Planchet					
Mixed soil-horizon sample	80,069	61,687	10,688	7,243	4,589
Field	82,858	55,997	5,299	4,617	2,739
0.04 ml medium	52,330	12,877	8,312	4,957	---
Planchet					
100 mg mixed soil-horizon sample	97,621	97,423	110,609	79,093	89,969
Laboratory	115,775	106,870	140,335	113,739	145,111
0.5 ml medium					
Media Controls					
Laboratory	33	11	20	22	1,149
0.5 ml medium					

Medium M9-(formate, glucose, lactate, glycine)-C^{14}

Table II-10

IN SITU AND PLANCHET DETERMINATIONS - II

Inoculum Soil Organisms All Taken Within One Square Foot Plot	Radioactivity - cpm Total Incubation-Collection Periods From Individual Samples (minutes)			
	1	5	20	60
In Situ				
Field	21,970	51,134	36,142	43,392
0.04 ml medium	11,435	54,849	46,624	35,843
	25,285	42,964	55,205	25,710
Planchet				
Soil Core	---	21,428	28,089	57,091
Field	---	57,180	36,145	49,111
0.04 ml medium	---	50,381	37,888	53,169
Planchet				
Mixed soil-horizon sample	---	53,224	48,317	84,700
Field	---	77,463	44,176	54,438
0.04 ml medium	---	26,947	42,404	49,144
Planchet				
100 mg mixed soil-horizon sample	923	9,961	72,275	133,571
Laboratory	1,125	13,053	71,613	129,070
0.5 ml medium	1,620	6,132	73,170	124,796
Media Controls				
Laboratory	32	35	59	112
0.5 ml medium	34	41	63	120
	24	43	56	110

Medium M9-(formate, glucose, lactate, glycine)-C^{14}

C. MEDIUM DEVELOPMENT

1. Basal Media Substrates - Unlabeled

The sole source of nitrogen in the unlabeled, basal M9 medium is potassium nitrate. Ammonium nitrate might be substituted and provide ammonium nitrogen as well as nitrate nitrogen.

Previous determinations (Quarterly Report No. 14) showed that concentrations of 0.01, 0.1, 1.0, 1.5, and 3.0 grams per liter of ammonium nitrate in M9-C^{14} media produced responses from various soil inocula that were similar to those obtained from standard M9-C^{14}. The study was continued, comparing ammonium nitrate to potassium nitrate on a nitrogen weight per weight basis. Details follow:

Method ----- Planchet determination

Media ----- 0.5 ml/test

(1) Standard M9-C^{14}

a. 0.5 g/l KNO_3 13.86% N = 69 mg/l N

(2) Modified M9-C^{14}

a. KNO_3 - deleted

b. 0.198 g/l NH_4NO_3 35.00% N = 69 mg/l N

Inocula -----

(1)	*E. coli* 0.1 ml	6×10^5 cells/test
(2)	Alaska, Fairbanks	100 mg soil
(3)	Alaska, Palmer	100 mg soil
(4)	Florida, Daytona Beach	100 mg soil
(5)	Virginia, Orange	100 mg soil

Successive Incubation Periods ----- 1, 3, 5, 24 hours

$C^{14}O_2$ Collection Time ----- final 15 minutes of each incubation period

Results ----- Table II-11. The responses from both media were, again, basically similar. Although there appeared to be no strikingly obvious advantage of the ammonium nitrate, it was, nevertheless, deemed advisable

to substitute the ammonium nitrate (0.2 g/l) for the potassium nitrate in the M9 basal medium since it increased the potential response spectrum of the medium.

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Investigation of growth responses from inoculated M9 (formate, glucose, lactate, glycine)-C^{14} has continued with the addition of various vitamins to the medium. The nine vitamins most commonly used as supplements were reviewed for C^{14} medium compatability and for sterilization stability. Seven were selected and incorporated into the M9-C^{14} at effective but low levels for the first study.

Routine planchet determinations were performed. The vitamins tested were nicotinic acid (niacin, B_3), riboflavin (B_2), thiamine hydrochloride (B_1), biotin (H), cyanocobalamin (B_{12}), folic acid (B_c, M), and paraaminobenzoic acid (H^1). The vitamin supplementation did not, generally, improve responses.

Table II-11

RESPONSES TO MEDIA M9-C^{14}, M9-C^{14} CONTAINING NH_4NO_3 IN PLACE OF KNO_3

Planchet Determination

Inoculum	Medium (0.5 ml)	Net Radioactivity - cpm Successive Incubation Periods* (hrs)			
		1	3	5	24
E. coli	KNO_3-M9-C^{14}	1305	3005	6479	6462
6×10^5 cells	NH_4NO_3-M9-C^{14}	1515	3487	7655	1893
Alaska, Fairbanks	KNO_3-M9-C^{14}	4141	4746	8833	1784
soil 100 mg	NH_4NO_3-M9-C^{14}	3972	4596	9475	1396
Alaska, Palmer	KNO_3-M9-C^{14}	7891	8278	14482	1213
soil 100 mg	NH_4NO_3-M9-C^{14}	7792	7947	14585	1233
Florida, Daytona Beach	KNO_3-M9-C^{14}	26	62	276	14869
soil 100 mg	NH_4NO_3-M9-C^{14}	63	74	228	12043
Virginia, Orange	KNO_3-M9-C^{14}	31207	29992	34871	934
soil 100 mg	NH_4NO_3-M9-C^{14}	34357	31191	32457	855
Sterile Media	KNO_3-M9-C^{14}	12	5	3	1
Controls	NH_4NO_3-M9-C^{14}	11	11	11	18

*$C^{14}O_2$ collection time - final 15 minutes of each incubation period

D. ANTIMETABOLITES

Bard Parker, a commercial germicide, continues to be used successfully. Recent reports citing increased cell permeability induced by the chemical compound dimethyl sulfoxide (DMSO) led to the possibility of its use in enhancing the inhibitory action of the Bard Parker antimetabolite.

Studies were undertaken with Saccharomyces cerevisiae to determine the effect of DMSO on the pure culture as well as compatability with the C^{14} medium. Determinations were performed on S. cerevisiae correlating decreasing concentrations of Bard Parker with DMSO at a level not in itself inhibitory or stimulating. This was done to determine a possible synergistic effect of the DMSO on inhibition by the Bard Parker by virtue of the supposed increased permeability. The procedures were as follows:

Effect of DMSO on S. cerevisiae, C^{14} Medium

Method ----- Planchet determination

Medium ----- 0.5 ml Sabouraud broth, 2 uc/ml glucose-C^{14}

DMSO Concentrations ----- 1, 2, 5, 10%

Inocula ----- 0.1 ml S. cerevisiae 5×10^5 cells/test

Successive Incubation Periods ----- 1, 3, 5, 24 hours

$C^{14}O_2$ Collection Time ----- final 15 minutes of each incubation period

Results ----- Table II-12. The 5% DMSO concentration was selected on the basis that, of itself, it neither inhibited nor promoted growth but was probably sufficiently high to indicate the sought synergistic effect.

Effect of 5% DMSO + Bard Parker on Inhibition

Method, Medium-C^{14}, Inoculum, Incubation-Collection Period - as above.

Bard Parker Concentrations ----- 20.0, 10.0, 5.0, 2.5, 1.3, 0.6, 0.3%.

Results ----- Table II-13. Results showed no advantage for including the DMSO, but confirmed the excellent performance of the Bard Parker.

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'Brook Aloetex Concentrate,' a commercial iodine-detergent complex, was screened for use as a possible antimetabolite. The active ingredients in the product are nonylphenoxy-polyethoxy-ethanol-iodine complex, ethylenediaminetetraacetic acid (EDTA), and diglycolic acid. A planchet determination was first performed to establish the compatability of the compound with the four radioactive substrates of the M9 medium. Since excessive degradation of the sterile C^{14} medium occurred, further testing was precluded.

Table II-12

EFFECT OF DMSO ON GROWTH RESPONSE OF S. CEREVISIAE IN C^{14} MEDIUM

Planchet Determination*

DMSO Concentration	Net Radioactivity - cpm			
	Successive Incubation Periods** (hrs)			
	1	3	5	24
S. cerevisiae	1518	3111	5745	177
S. cerevisiae + 1%	1403	3255	5924	197
S. cerevisiae + 2%	1420	2788	5353	221
S. cerevisiae + 5%	1570	2820	5301	164
S. cerevisiae + 10%	1141	1624	2500	97

*Medium - 0.5 ml Sabourauds Broth containing 2 uc/ml glucose-C^{14}
Inoculum - 0.1 ml *S. cerevisiae* 5×10^5 cells/test

**$C^{14}O_2$ collection time - final 15 minutes of each incubation period

Table II-13

EFFECT OF 5% DMSO + BARD PARKER ON INHIBITION OF S. CEREVISIAE IN C^{14} MEDIUM*

Planchet Determination

Inoculum	Concentration		Net Radioactivity - cpm			
	DMSO	Bard Parker	Successive Incubation Periods** (hrs)			
			1	3	5	24
Uninhibited S. cerevisiae	--	--	284	520	615	1664
	5%	--	174	310	349	1345
S. cerevisiae	--	20%	2	0	1	0
	5%	20%	0	3	0	5
S. cerevisiae	--	10%	0	3	0	0
	5%	10%	0	0	0	0
S. cerevisiae	--	5%	0	0	0	2
	5%	5%	0	0	0	0
S. cerevisiae	--	2.5%	6	8	0	0
	5%	2.5%	8	6	2	0
- - - - -						
Uninhibited S. cerevisiae	--	--	1336	3219	5709	98
	5%	--	1155	2437	4323	150
S. cerevisiae	--	5%	6	2	0	2
	5%	5%	5	2	1	1
S. cerevisiae	--	2.5%	22	6	5	0
	5%	2.5%	15	3	6	0
S. cerevisiae	--	1.25%	28	11	0	0
	5%	1.25%	26	17	8	0
S. cerevisiae	--	0.62%	94	31	16	8
	5%	0.62%	62	23	12	4
S. cerevisiae	--	0.31%	185	133	108	4
	5%	0.31%	159	105	51	11

*Medium - 0.5 ml Sabourauds Broth containing 2 uc/ml glucose-C^{14}

Inoculum - 0.1 ml S. cerevisiae 5×10^5 cells/test

**$C^{14}O_2$ collection time - final 15 minutes of each incubation period

E. TEST ORGANISMS AND RESPONSES

1. Soils

Seven additional soils from the test collection have been processed. General information pertaining to the sites from which the soils were collected is given in Table II-14. Cell numbers were obtained, Table II-15; and routine planchet determinations performed with M9 medium containing formate, glucose, lactate, and glycine C^{14} substrates. All seven test soils produced positive responses, Table II-16.

This group completes the soil screening phase of the program. In all, 24 new soils were tested. A complete summary will be presented in the forthcoming Annual Report.

2. Soil Isolates

The evaluation of the possiblity that general soil responses are largely attributable to one or several common species continued. Determinations were performed on the five soil isolates described in Table II-17, in the following manner.

Method ----- Planchet determination

Medium ----- 0.5 ml M8-C^{14} plus 100 mg sterile soil per test

C^{14} Substrates -----	Sp. Act. (mc/millimole)	mM	uc/ml	% (w/v)
Sodium formate	25	0.26	6.5	0.002
D-glucose-U.L.	3	0.42	1.3	0.007
DL sodium lactate-1	5	0.26	1.3	0.002

Inocula ----- 0.1 ml soil isolate suspension

(1) 2D Rocky Mt., Colorado
(2) 4C Metuchen, New Jersey
(3) 5AA Orange, Virginia
(4) 5B Orange, Virginia
(5) 5T Orange, Virginia

Successive Incubation Periods ----- 45, 90, 160 minutes

$C^{14}O_2$ Collection Time ----- final 15 minutes of each incubation period

Results ----- Table II-18. The responses obtained from all five isolates were positive.

Screening of the final group of soil isolates is now in process. After this is completed, an analysis of the data will be made.

Table II-14

NEW TEST SOILS - SOIL SITE INFORMATION

Soil Site	Soil Type	Temperature Range (°C)	Average Precipitation (inches/year)
Arizona, Tuscon	desert	-6 to 49	7
California, Sawyers Bar	mountain	-34 to 42	10
Minnesota, St. Paul	field	-37 to 42	25
Missouri, Bolivar	field	-6 to 46	30
South Dakota, Rapid City	field	-42 to 44	17
Utah, Salt Lake City	field	-34 to 42	14
Washington, Sequin	field	-6 to 42	30

Table II-15

NUMBERS OF MICROORGANISMS ISOLATED FROM TEST SOILS

Soil	Organisms/100 mg Soil	
	Tryptone-glucose Agar	Sabouraud Agar
Arizona, Tuscon	280,000	500
California, Sawyers Bar	170,000	250
Minnesota, St. Paul	130,000	900
Missouri, Bolivar	78,000	1750
South Dakota, Rapid City	57,500	1450
Utah, Salt Lake City	165,000	450
Washington, Sequin		
Additional Soil Samples*		
California, Needles	245,000	15,000
Colorado, Derby	150,000	950
Idaho, Boise	150,000	350
Maine, Orono	77,000	350
Montana, Bozeman	140,000	150
Oklahoma, Chandler	59,500	1,900
Texas, Ferris	160,000	2,100
Virginia, Blacksburg	34,000	150

*Soil responses and characteristics reported in Quarterly Progress Report No. 14

Table II-16

RESPONSES FROM NEW SOILS OF TEST COLLECTION

Planchet Determination*

Soil	Net Radioactivity - cpm Successive Incubation Periods** (hrs)				
	0.75	1.5	3	5	24
Arizona, Tuscon	2354	3591	4807	7949	975
California, Sawyers Bar	1130	1403	1934	5679	966
Minnesota, St. Paul	2527	4598	5925	7382	1113
Missouri, Bolivar	3277	4579	5186	6174	797
South Dakota, Rapid City	11089	16671	17568	18119	591
Utah, Salt Lake City	2762	4838	7066	9489	1316
Washington, Sequin	1659	2034	2708	6510	1015

*100 mg soil/0.5 ml medium M9 (formate, glucose, lactate, glycine)-C^{14}

**$C^{14}O_2$ collection time - final 15 minutes of each incubation period

Table II-17

GENERAL CHARACTERISTICS OF TESTED SOIL ISOLATES

Soil Source	Soil Isolate Code	Cell Characteristics	Colony Description*
Colorado, Rocky Mt.	2D	Gram-variable rod	Round, irregular edges, yellow-white, flat, rough, dry, opaque, 1/4-3/8 inch diameter
New Jersey, Metuchen	4C	Gram-positive rod	White, feathery, outgrowths, spreading, wooly subsurface growth
Virginia, Orange	5AA	Gram-negative rod	Round, yellow, shiny, raised, 1/4 inch diameter
Virginia, Orange	5B	Gram-positive filament	Streptomycete, round, white, dull, raised, sporulating, 1/8 inch diameter
Virginia, Orange	5T	Gram-negative rod	Round, white, transparent, yellow-raised opaque center, 1/8 inch diameter

*Grown on tryptone-glucose agar

Table II-18

RESPONSES OF SOIL ISOLATES IN M8-C^{14} MEDIUM

Planchet Determination*

Soil Source	Soil Isolate	Net Radioactivity - cpm			Cells/test
		Successive Incubation Periods**			
		45 min	90 min	180 min	
Colorado, Rocky Mt.	2D	16,236	22,367	42,295	3×10^6
New Jersey, Metuchen	4C	28,164	31,268	28,308	2×10^6
Virginia, Orange	5AA	36,815	47,885	58,901	123×10^6
Virginia, Orange	5B	7,775	8,479	9,987	4×10^6
Virginia, Orange	5T	72,533	91,636	114,234	547×10^6

*Medium - 0.5 ml M8 containing (formate, glucose, lactate)-C^{14}; 100 mg sterile soil
Inoculum - 0.1 ml soil isolate suspension

**$C^{14}O_2$ collection time - final 15 minutes of each incubation period

F. STERIOISOMERS

Experimentation has continued in determining the effects of L-glucose on test organisms. Previous studies (Quarterly Progress Report No. 14) indicated that L-glucose, when incorporated in concentrations up to 56% of the total glucose in labeled M9 medium, did not inhibit the growth of Serratia marcescens or microorganisms present in four test soils. The responses of Escherichia coli, however, were doubtful as stated in the aforementioned report.

As a result, further study with E. coli was undertaken. An inorganic salts medium was prepared (Table II-19), permitting glucose to be the sole carbon source.

The experiment was performed as follows:

Method ----- Planchet determination

Medium ----- 0.5 ml, C^{14} labeled inorganic salts medium

Glucose Concentrations

	D-glucose-C^{14} mM	%	D-glucose mM	%	L-glucose mM	%	Total Glucose mM
Medium (A)	0.36	100%	---		---		0.36
Medium (B)	0.36	50%	0.36	50%	---		0.72
Medium (C)	0.36	50%	---		0.36	50%	0.72

Inoculum ----- 0.1 ml E. coli 6.4×10^6 cells/test

Successive Incubation Periods ----- 1, 3, 5, 24 hours

$C^{14}O_2$ Collection Time ----- final 15 minutes of each incubation period

Results ----- Table II-19. The following observations were made from the responses obtained:

(1) Medium A containing 0.36 mM of D-glucose-C^{14} produced greater responses from the E. coli during the three and five hour

incubation-collection periods than medium B containing 0.72 mM D-glucose of which 0.36 mM (50%) was labeled. This indicated that in the range of glucose concentration used, increased concentration did not result in increased $C^{14}O_2$ metabolism but showed merely the relation to concentration of the labeled component.

(2) The inoculated medium (C) with 50% (0.36 mM) of D-glucose-C^{14} and 50% (0.36 mM) of L-glucose produced greater responses during the three to five hour period than the inoculated medium (B) containing the same amount of labeled glucose but with 50% (0.36 mM) of unlabeled D-glucose. This would indicate that the L-glucose was not interfering in the metabolism of E. coli and also that the label-concentration effect was present.

(3) Responses from the inoculated medium (C) containing the 50% (0.36 mM) L-glucose and 50% (0.36 mM) D-glucose-C^{14} were surprisingly greater than those from the inoculated medium (A) containing 100% (0.36 mM) D-glucose-C^{14} indicating that L-glucose did not interfere in the metabolism and also that it might have, in some unknown manner, promoted the utilization of the D form. Racemases may also be present in the E. coli organisms which could possibly convert the L form of glucose into the D form, but this would have resulted in an increase of unlabeled D, thus yielding, at the most, an equal response and not one which is greater.

In summation, the presence of 50% L-glucose in the labeled inorganic salts medium did not inhibit the growth of E. coli. Turbidimetric studies are in process with the medium containing glucose in the L form only. This is encouraging in its indication that the incorporation of racemic mixtures of carbohydrates and, perhaps, other organic substrates into the medium may not be inhibitory.

Table II-19

RESPONSES FROM E. COLI IN C^{14} MEDIA* CONTAINING D-GLUCOSE, L-GLUCOSE

Planchet Determination

Inoculum	Successive Incubation Periods (Hours)**	Net Radioactivity - cpm C^{14} Media - Glucose Content		
		A	B	C
		D-glucose-C^{14} 0.36 mM 100%	D-glucose-C^{14} 0.36 mM 50%	D-glucose-C^{14} 0.36 mM 50%
			D-glucose 0.36 mM 50%	L-glucose 0.36 mM 50%
		Total mM 0.36	Total mM 0.72	Total mM 0.72
E. coli	1	23	15	37
6×10^5 cells/test	3	147	63	228
(0.1 ml)	5	484	211	640
	24	65	111	95

*Inorganic Salts Medium 0.5 ml/test D-glucose-U.L.-C^{14} 2 uc/ml

NH_4Cl	1.0 g
Na_2HPO_4	6.0
KH_2PO_4	3.0
NaCl	5.0
$MgSO_4$	0.1

per liter

**$C^{14}O_2$ collection - final 15 minutes of each incubation period

G. MEETINGS, PERSONNEL

Meetings

The Fifteenth Annual AIBS Meetings of the Biological Sciences, in Boulder, Colorado, August 23-28, 1964, were attended by Drs. Gilbert V. Levin and Allen H. Heim. A paper entitled "Significance and Status of Exobiology" was given by Dr. Levin.

Staff members were in attendance at a symposium on Recent Developments in Research Methods and Instrumentation held at the National Institutes of Health, Bethesda, Maryland, on October 5-8, 1964. Dr. Levin participated in the Exobiology Session, presenting "Gulliver - An Instrumented Radioisotopic Experiment to Detect Life on Mars."

Personnel

Paul E. Wilkins, Engineering Physicist, has become associated with the Radioisotopic Biochemical Probe for Extraterrestrial Life.

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QUARTERLY PROGRESS REPORT NO. 15

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Respectfully submitted,

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