N71-26380
NASA CR-118659

AUTOMATED MICROBIAL METABOLISM LABORATORY

Contract No. NASW-1931

FINAL REPORT

1970

BIOSPHERICS INCORPORATED

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Report

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Prepared under
Contract No. NASW-1931

Prepared by:

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Prepared for:

National Aeronautics and Space Administration
Headquarters

28 April 1971

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ACKNOWLEDGEMENTS

The participation and assistance of the following Biospherics' personnel is gratefully acknowledged:

Mrs. Vivian Brooks

Mrs. Margaret Federline

Dr. Richard Fox

Mr. Richard Hughes

Mrs. Margaret Schiffman

Mrs. Susan Smith

Mr. Stanley Terry

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ABSTRACT

The aim of this program has been to solve some of the problems associated with life detection assays of the Automated Microbial Metabolism Laboratory (AMML) and to advance the state-of-the-art sufficiently for the assays to be incorporated into an automated breadboard instrument. During the year, significant advances have been made in the labeled carbon dioxide fixation-dark release and radioactive carbon and sulfur uptake experiments. In all cases, levels of the nonbiological signals were reduced and the overall precision of the experiments was increased by the incorporation of newly devised techniques into the assays. This greatly enhanced the sensitivity and reliability of these exobiology experiments over that attained in experiments prior to this program. Measurement of the kinetics of biological responses as well as the study of experimental variables increased the store of knowledge and experience relative to each of the assays.

During this program, the instrument components for the various experiments were designed, fabricated, integrated, and tested to demonstrate system feasibility. An integrated biology experiment was conducted using the instrument to perform the labeled release, labeled carbon and sulfur uptake, ATP production, and phosphate uptake experiments.

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I. SUMMARY

Attempts were made, during the biology-biochemistry research phase of this program, to upgrade laboratory procedures for the AMML life detection schemes. The nonbiological background from radioactive medium used in the labeled release experiment was studied. The kinetics and stoichiometry of $^{14}CO_2$ evolution from soils was measured as well as the effectiveness of Bard-Parker germicide as an inhibitor for this experiment.

The precision of the light fixation-dark release experiment was increased dramatically, due largely to the use of gas tight photosynthesis chambers designed specially for this purpose. The nonbiological background from soils was lowered considerably as a result of incorporation of a moist-$^{12}CO_2$ sweep into the experimental procedure. The combination of these two factors greatly increased the reliability and sensitivity of the assay.

The precision of the $^{14}C + ^{35}S$ uptake experiment was increased and its nonbiological background from soils greatly diminished through the use of a filter prewash and a soil rinse with medium containing unlabeled substrates. Here

again, the combination of these two factors increased the reliability of the assay and lowered the minimum detectable biological signal.

A study was made of the effects of varying concentrations and volume ratios of reagents upon the light output from the firefly bioluminescent reaction. The purpose of this experimentation was to determine satisfactory ranges for the volumes of enzyme and extractant to be used in the AMML instrument.

Very little success was achieved in attempting to remove substances from soil extracts which interfered in the colorimetric determinations of orthophosphate. These interferences were found to be organic as quantitative recoveries of phosphate were obtained after digestion of the soil extracts with perchloric acid. Significant biological phosphate uptake signals were obtained from soils and inocula from pure bacterial cultures after an overnight incubation period.

The engineering effort on this program was directed toward two goals: first, the biological testing of the AMML breadboard instrument fabricated during the previous year and second, the development of an advanced breadboard design of the Gulliver life detection instrument which could serve as

a backup instrument on an early Mars Lander.

The testing has been accomplished after expending considerable effort on modifications of the instrument to improve its operation and reliability.

An integrated biology experiment was conducted using the breadboard apparatus and the results clearly established the presence of biological activity in the test soil sample used. Also, this experiment demonstrated the feasibility of performing these assays automatically.

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II. INTRODUCTION

The goal of this program has been the functional integration of five independent, but reinforcing, experiments for the detection of extraterrestrial life into the AMML. The AMML represents an attempt to develop instrumentation for a second generation of exobiology experiments.

The AMML program, as presently conceived, consists of a number of distinct life detection experiments. One of these induces light-dependent and light-independent fixation of labeled carbon dioxide and subsequently measures the $^{14}CO_2$ reevolved during a dark incubation period. The remaining experiments are: uptake of radioactive carbon and sulfur substrates, detection of production of adenosine triphosphate (ATP), uptake of phosphate, and production of $^{14}CO_2$ from labeled organic substrates. These latter experiments did not originally include examination for a photosynthetic component. However, based upon recent laboratory findings that the effects of light upon the kinetics of these tests can be measured, all experiments will be monitored for photosynthesis, thereby adding four additional experiments to the complement.

Individually, each experiment will yield evidence relative to the presence of life, provide information on rates of metabolism, and determine the light dependency of the metabolism. However, as an integrated experiment, the total value should exceed the sum of its parts. The information obtained could indicate whether or not any life encountered is similar to or different from that on Earth. For example, the phosphate and sulfur tests might indicate the presence of life which, by yielding negative results in the ATP test, would be shown to follow an alien biochemical pathway. Carbon/phosphorus/sulfur utilization ratios might be obtained. ATP production might be related to carbohydrate utilization and phosphate uptake. Further, the system makes it possible to detect life which may be noncarbon based.

Experimental studies designed to investigate each of the life-detection assays will be described in the following sections, and significant improvements in the assays will be detailed. Integrated biology experiments conducted to test the AMML breadboard instrument will also be described.

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III. BIOLOGY-BIOCHEMISTRY RESEARCH PROGRAM

A. Labeled-Release Experiment

1. Nonbiological Background from Labeled Medium

Experiments were conducted in order to obtain estimates of the nonbiological signals from media containing four radioactive substrates, both individually and in combinations. The composition of basal M9 used to prepare these media is shown in Table 1. The labeled substrates studied are given in Table 2, along with their concentrations and the levels of radioactivity used.

a) Complete Labeled M9 Medium

Data pertaining to the level of the nonbiological signal released from the isotopically labeled M9 test medium were obtained from filter-sterilized preparations. Freshly prepared 0.4 ml aliquots of complete M9 medium (10 uCi/ml total activity) containing each of the labeled substrates (glucose, glycine, formate, and lactate) were transferred to each of two sets of sterile $1\frac{1}{4}$" diameter aluminum planchets. To each of one set of aliquots was added 0.1 ml of concentrated Bard-Parker germicide, and to each aliquot of the other set was added 0.1 ml of sterile distilled water. Each planchet was capped with an inverted planchet containing a filter pad moistened with 3 drops of a saturated

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Table 1

Composition of Basal M9 Medium

K_2HPO_4	1.0 g/l
NH_4NO_3	0.2 g/l
$MgSO_4 . 7H_2O$	0.2 g/l
NaCl	0.1 g/l
Soil extract *	100 ml/l
pH	7.0

* Soil extract was prepared by suspending 500 g of air-dried soil in 1300 ml of water. The mixture was then autoclaved for one-hour, filtered, and the liquid loss made up with sterile, distilled water to 1 liter.

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Table 2

^{14}C-Organic Substrates Incorporated
Into Basal M9 Medium

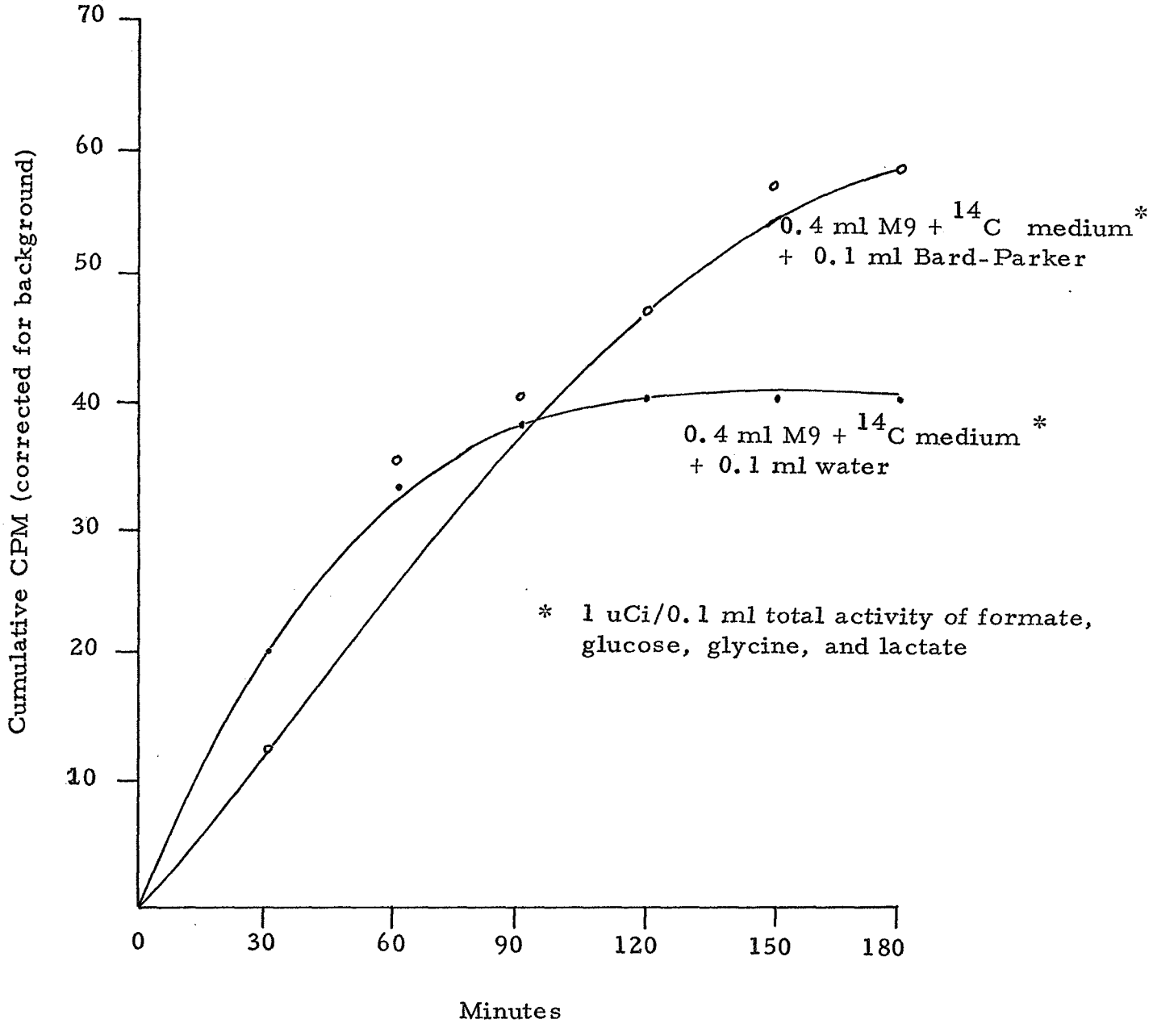
^{14}C-Substrate	uCi/ml	% w/v
Sodium Formate	6.5	0.002
D-Glucose (U)	1.3	0.005
Sodium DL-Lactate-1	1.3	0.002
Glycine-1	1.0	0.002

barium hydroxide solution to chemically collect the evolved, labeled carbon dioxide. Each collection planchet was replaced at regular intervals with a planchet containing a freshly moistened pad. From Figure 1 it is seen that the amount of the nonspecifically evolved $^{14}CO_2$ from the sample containing no Bard-Parker increased in a linear manner for 60 minutes to a level of about 40 cpm above background, then remained constant at this level for the remainder of the assay. The Bard-Parker-treated sample was not significantly affected by the presence of the inhibitor over the first 60 to 90 minutes. During the next 90 minutes, a slight increase in nonbiological response was observed. However, the total amount of nonbiological evolution was very small and the differences between the Bard-Parker and water experiments were hardly significant.

b) ^{14}C Components Individually in Basal M9

The nonbiological responses obtained from sterile solutions of each of the ^{14}C substrates, with and without the addition of Bard-Parker, are compared in Figure 2. In the absence of Bard-Parker, each constituent substrate had a total cumulative response after 4 hours of from 12 to 23 cpm above

Nonbiological Evolution of $^{14}CO_2$ from
Sterile M9 + ^{14}C Medium



BIOSPHERICS INCORPORATED	Fig. 1		
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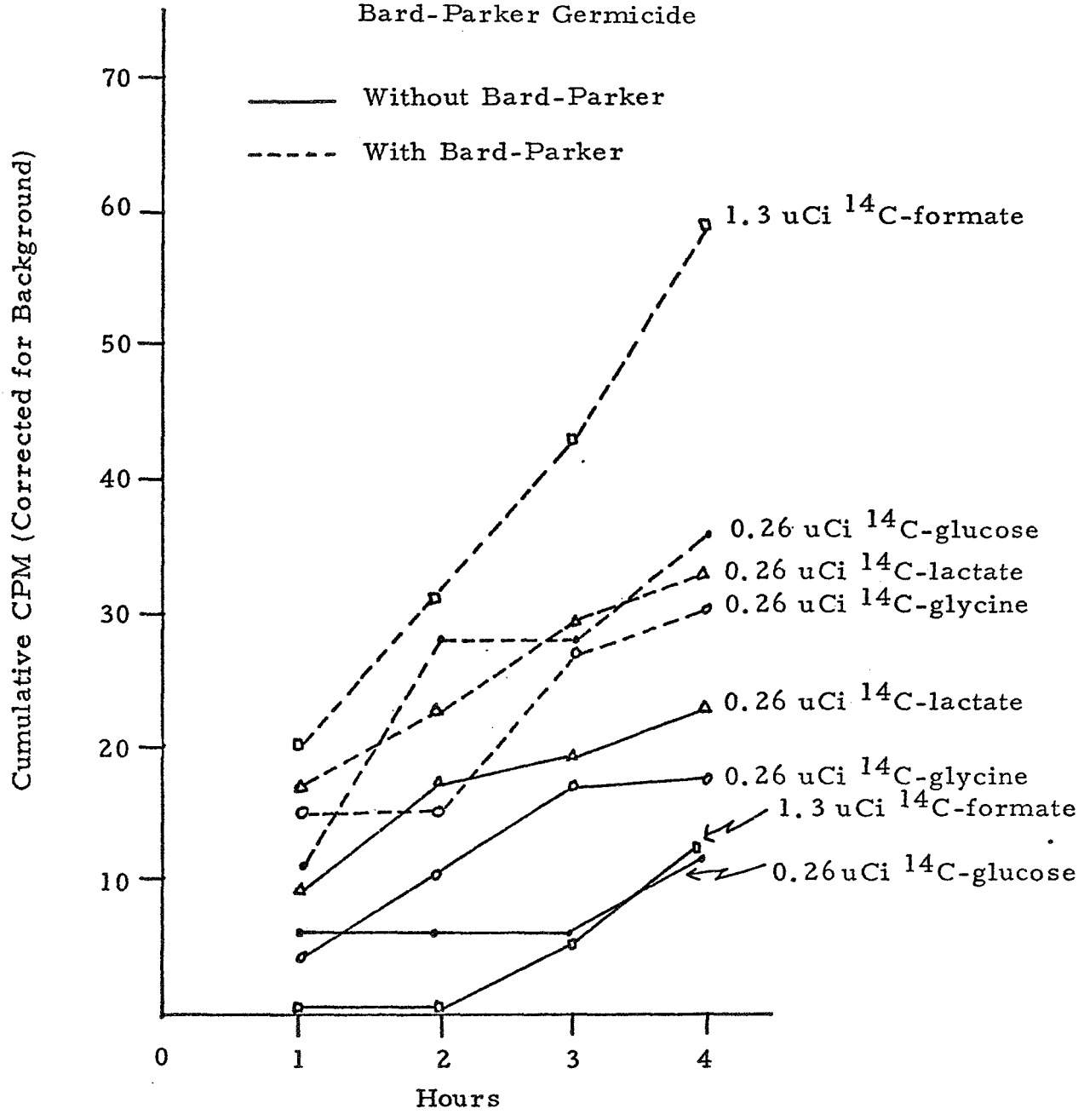
Nonbiological Background from Sterile M9 and ^{14}C-Organic Constituents With and Without Bard-Parker Germicide



BIOSPHERICS INCORPORATED Rockville, Maryland	Fig. 2			
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background. With the exception of labeled formate, the non-biological responses of the constituents increased approximately two fold when Bard-Parker was added. The nonbiological formate response was elevated five fold in the presence of the germicide. The labeled formate component of the total medium tends to be the least stable of the four organic substrates when associated with the inhibitor. Nevertheless, as will be shown later, the extent to which this molecule contributes to the overall response from viable soil is significant, and will furnish a strong argument against its elimination from the medium.

c) Effect of Bard-Parker on the Nonbiological Evolution from M9

The addition of Bard-Parker germicide to the total medium as a control increases the level of the non-biological background; however, its effect is modest. It is now believed that the effect of Bard-Parker on the nonbiological evolution will not seriously impair its usefulness as a control. A cumulative count of less than 100 cpm from evolved $^{14}CO_2$ collected over 4 to 5 hours has been demonstrated repeatedly when the medium was tested directly after preparation or at

various intervals over a five-week period during storage at 4°C.

2. Biological Response from Soil

a) Individual Labeled Substrates

Each of the labeled substrates was challenged against 100 mg of sifted local lawn soil. Equivalent soil aliquots were prepared and treated for 30 minutes with 0.2 ml of concentrated Bard-Parker before adding the appropriate labeled substrate. The results shown in Figure 3 demonstrate that the labeled formate component elicited the highest level of metabolically derived $^{14}CO_2$, followed respectively by labeled lactate, glucose, and glycine. It was also shown that the soil response from the ^{14}C-formate was extremely vigorous during the first hour, and exceeded the first-hour responses from labeled lactate and glucose by about a factor of 10, and from glycine, by a factor of 40. The rates of change of the individual responses were slight from the second to the fourth hour. Results from the untreated samples, with the exception of the labeled glycine-fertile soil combination, clearly indicate the biological nature of the assay, as evidenced by the large reduction in the amounts of evolved $^{14}CO_2$.

Labeled Release Experiment Comparing Medium Preparations of the Individual ^{14}C-Substrates - Each With 100 mg of Local Lawn Soil

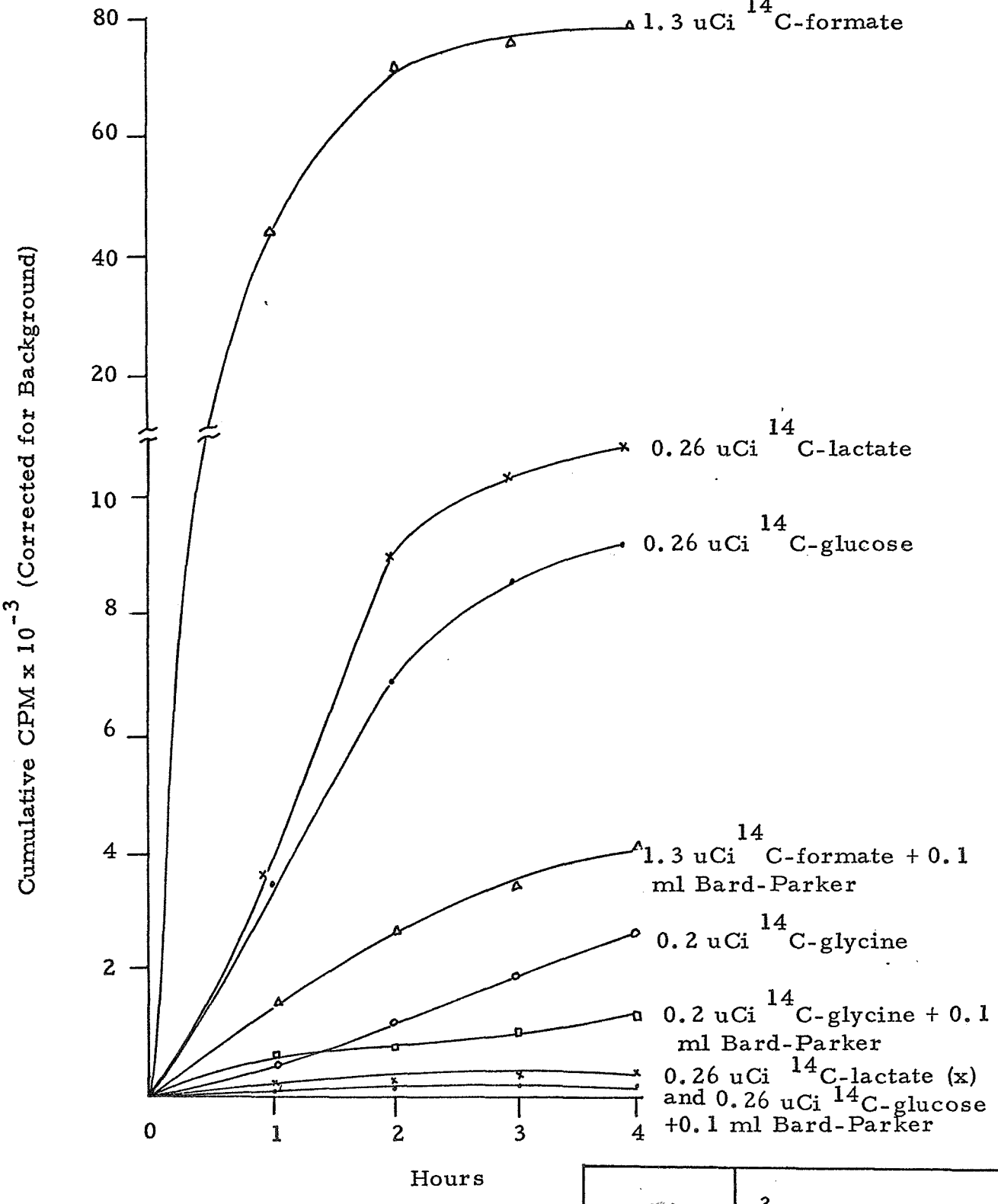

	Fig. 3			
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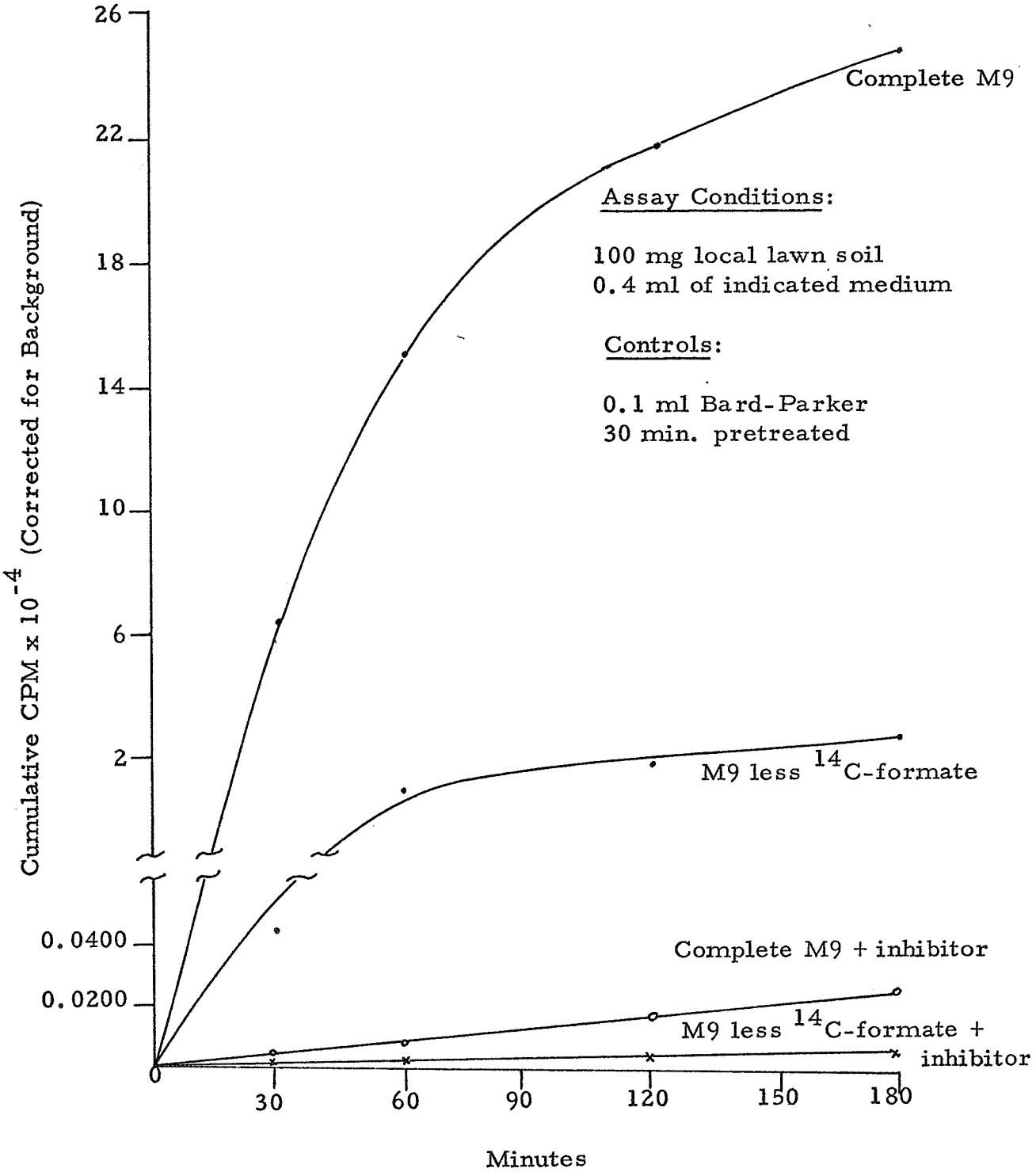
b) Labeled Release Responses from Total Labeled M9 and Labeled M9 Less ^{14}C-Formate Medium

In order to demonstrate further that the labeled formate component of the total medium contributes significantly to the total response from a fertile soil, comparisons were made of responses with the total labeled M9 and of those with a medium identical, except for the elimination of ^{14}C-formate. Controls for this experiment were prepared as usual. Figure 4 shows that including the labeled formate in the complete medium resulted in a signal which was an order of magnitude greater than one from the medium lacking the ^{14}C-formate substrate. The control soil samples for each medium preparation were drastically attenuated. These findings led to the interpretation that the responses derived from the uninhibited soils are real and of biological origin, and are not the result of uncharacterized soil chemical activity on the formate or on other components of the medium. Were this so, the total responses observed from the inhibited soil sample with the total labeled M9 medium would be expected to be orders of magnitude higher than those actually observed. The fact

Labeled Release Experiment Comparing Complete M9 + ^{14}C Medium with M9 + ^{14}C Medium Less Formate



BIOSPHERICS INCORPORATED Rockville, Maryland	Fig. 4			
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that the inhibited soil responses, with and without formate in the medium, were essentially at the same low level further demonstrates the attributes of this substrate as a component of the medium.

The data presented in Figures 3 and 4 indicate the initial preferential metabolic activity by a mixed soil flora on the ^{14}C-formate substrate. It now appears likely that the "early burst," typical of the labeled release experiment, is explained in large part by the rapid biochemical activity associated with the formate component.

c) Characteristic Responses from a Variety of Soils by the Labeled Release Experiment

Typical responses obtained from five different types of fertile soil by the labeled release assay are shown in Figure 5. The strength of the labeled release responses from each soil type represents a gradient of responses which would be anticipated for samples taken from different geographical and ecological locales. In Table 3 is a tabulation of test-to-control signal (T/C) ratios for these soil types. The cumulative, three-hour signals obtained from each soil which had been pretreated with inhibitor were equal to or less than 500 cpm, except for the botanical garden soil which was 1,124 cpm.

Labeled Release Experiment Comparing
Responses from Five Fertile Soil Types

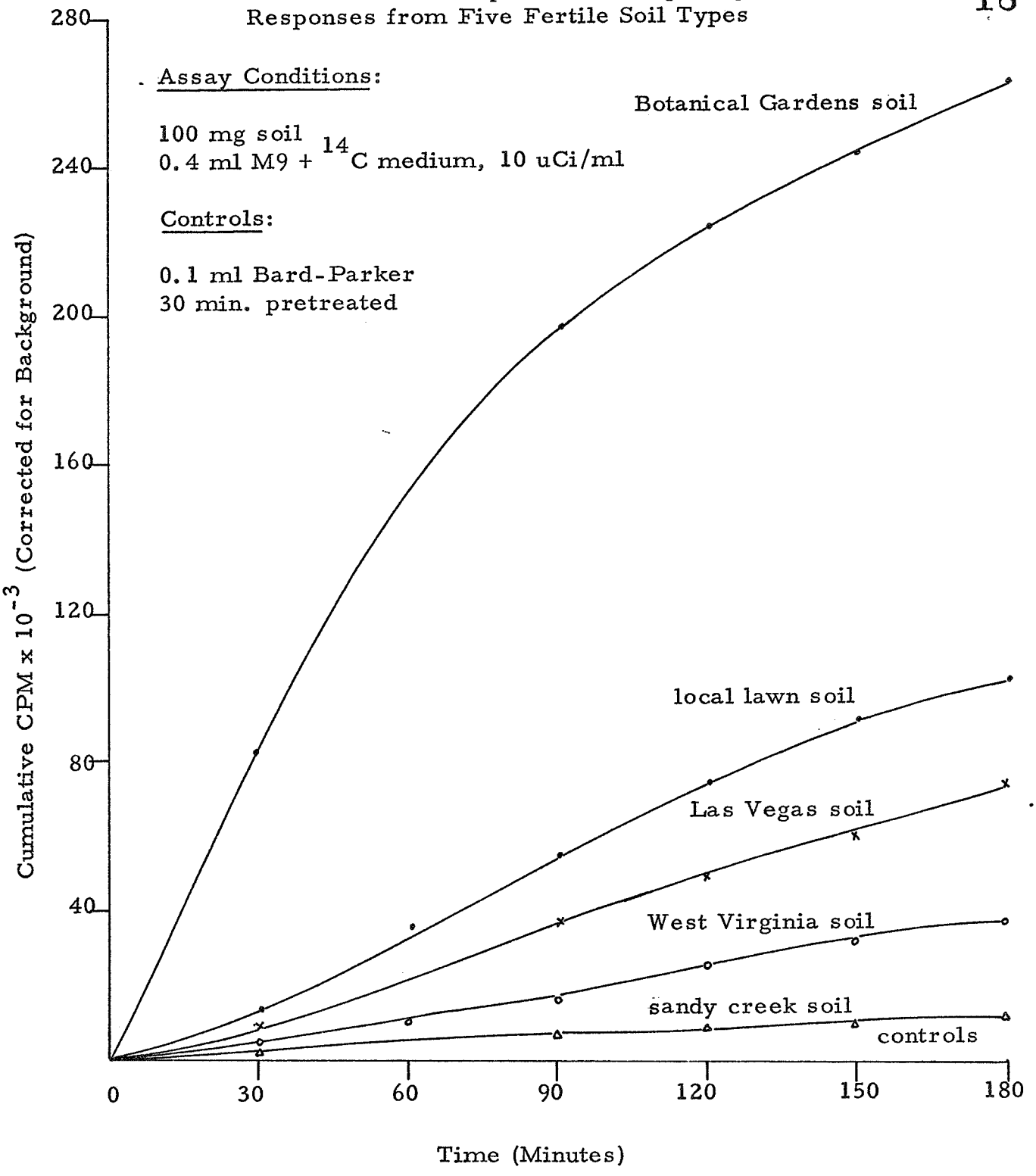

Fig. 5

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Table 3

T/C Ratios of Five Fertile Soils *

Soil Type	(T) Test Response (cpm)	(C) Control Response (cpm)	T/C
Botanical Gardens	263,122	1,124	234
Local lawn	103,000	500	206
Las Vegas	75,308	551	137
West Virginia	37,000	500	74
Sandy creek bottom	12,490	413	30

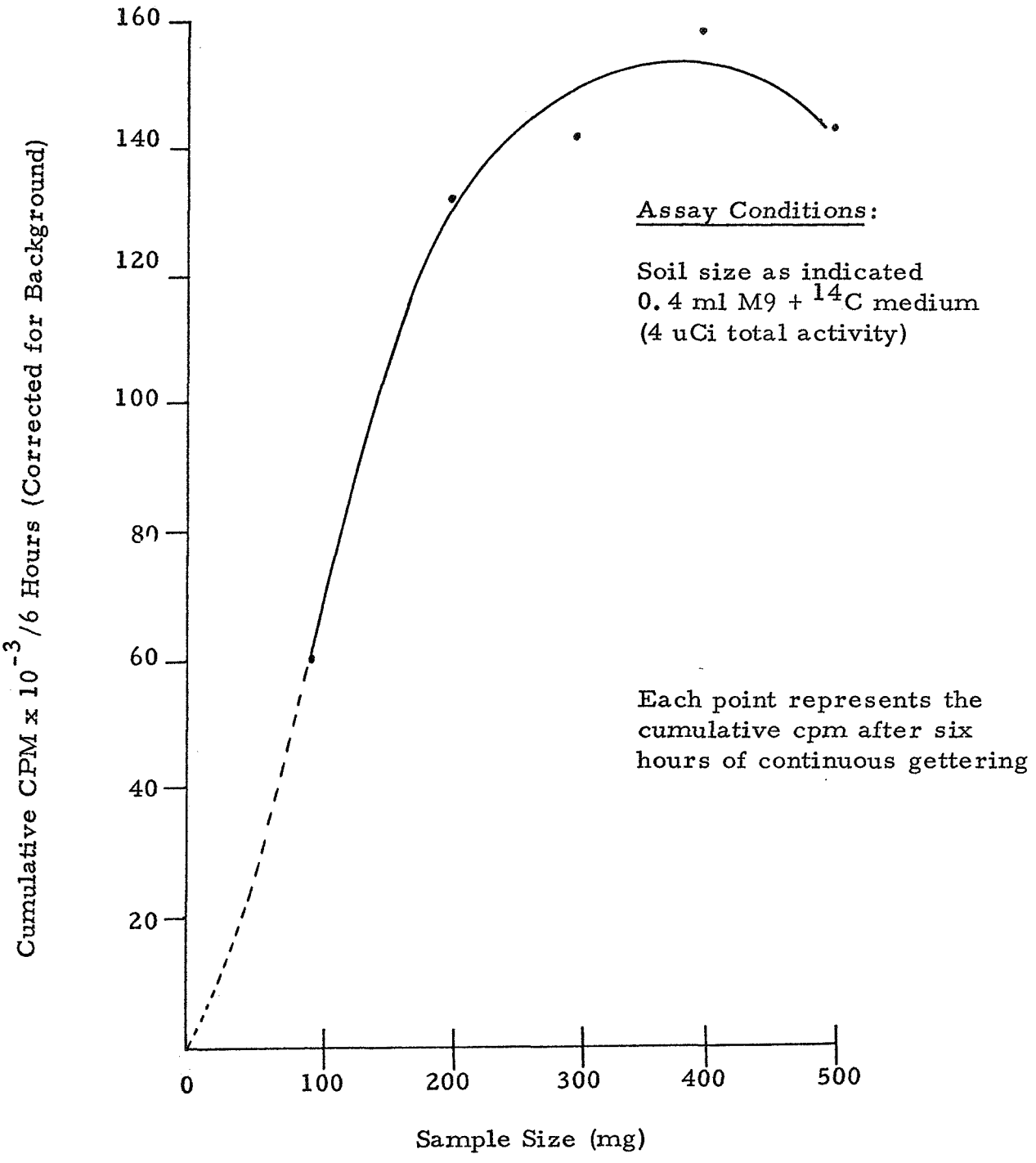
* T/C is the ratio of test-to-control cumulative radioactivity collected over a three-hour period.

3. Stoichiometry of the Labeled Release Assay

The stoichiometric relationship of the labeled release experiment was evaluated by employing decreasing ratios of medium (fixed at 0.4 ml)-to-soil (variable from 0 to 500 mg) as described in Figure 6. Upon extrapolating the line from zero, the responses were seen to be first order up to 200 mg of soil. When the weight of soil increased above this value, the response rate per mg of soil was markedly reduced. At a soil sample size of 500 mg, the metabolic response was attenuated to about the level of the 300 mg sample. The observed stoichiometry between soil inoculum size and signal may depend solely upon the uniform wetting of the contained soil sample with the fixed volume of medium. Soils which are naturally moist (about 5% aqueous content) would probably result in a more nearly uniform diffusion of medium throughout the sample with the fixed medium volume of 0.4 ml employed in this experiment. The moisture content of the test soil was not determined, but appeared to be relatively dry.

Although the soil surfaces of the samples greater than 200 mg tended to be homogeneously moistened with the medium, their percolation into the subsurface was perhaps incomplete.

Effect of Sample Size on the Labeled Release Response



BIOSPHERICS INCORPORATED Rockville, Maryland	Fig. 6			
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The chemical and physical parameters which influence the permeation of the medium into the soil need not be the only explanation available for the brief range of liquid/soil ratios over which the linear response was observed. The biological nature of the soil itself could be a factor. The problem of stoichiometry has been superficially studied during this program; therefore, definite conclusions relating to the problem are limited.

4. Effect of Moisture on the Labeled Release Assay

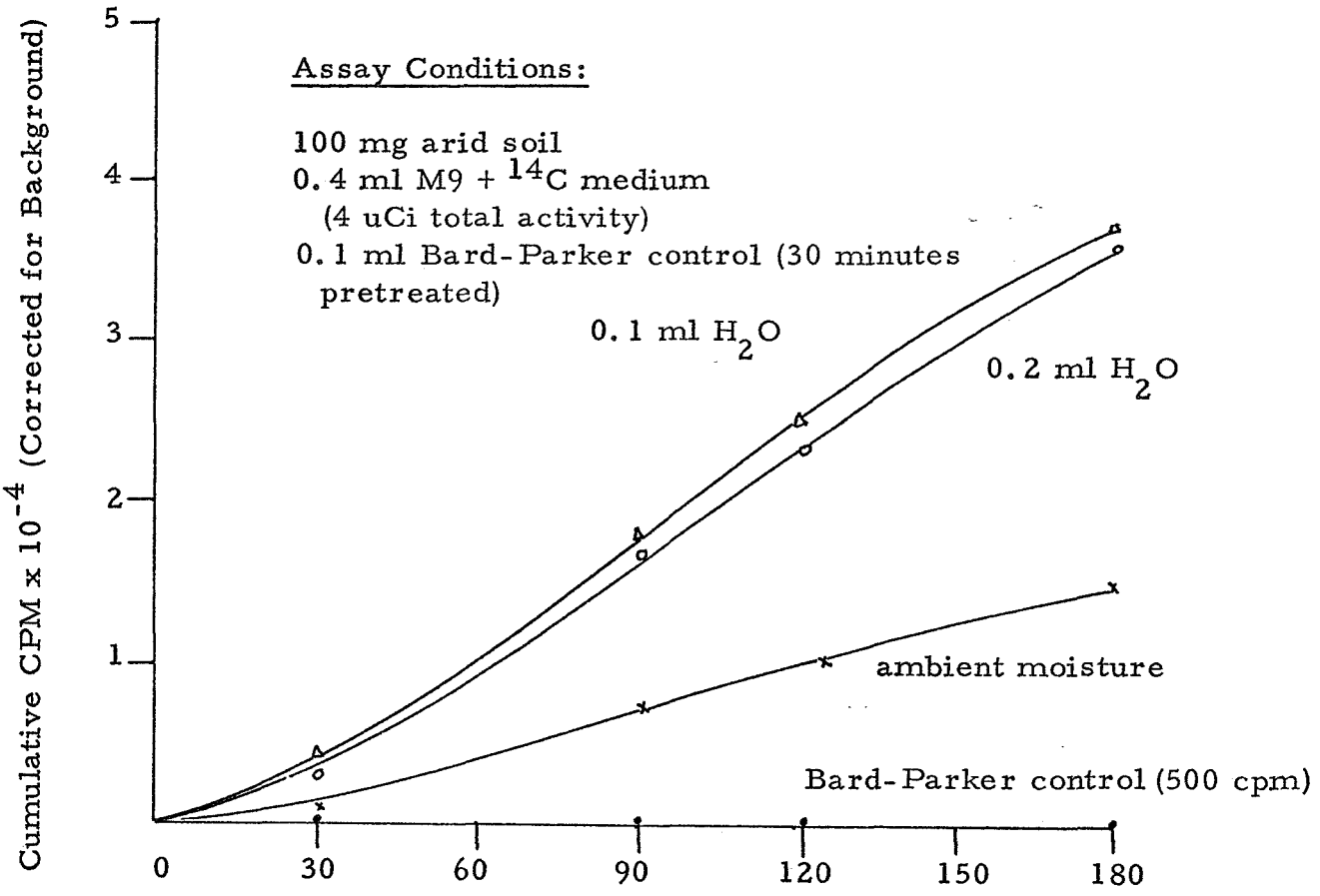
The addition of water to an arid soil has been shown to elevate the response observed in the labeled release assay. Figure 7 depicts the metabolic activity of an arid soil when challenged with the labeled M9 medium. The metabolic response from the same soil was more than doubled when the soil was moistened with 0.1 ml to 0.2 ml of water prior to the addition of the medium. The dramatic elevations of the labeled release response were not found in experiments conducted with soils having a higher initial moisture content.

5. Effect of Bard-Parker Germicide

a) Inhibitor Concentration

Bard-Parker germicide is an effective metabolic inhibitor when tested on pure cultures of microorganisms,

Effect of Added Moisture Upon the
Labeled Release Responses of Arid Soil



BIOSPHERICS INCORPORATED Rockville, Maryland	Fig. 7			
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and particularly when tested on soil inhabited by a mixed microbial flora. The parameters of inhibitor concentration, the size of soil sample or the duration of soil treatment with inhibitor influences the germicidal effectiveness of this substance. Data on the effect of inhibitor concentration upon response is described in Figure 8. In this experiment, cumulative responses on 100 mg portions of soil which had been pretreated with 0.1 ml portions of inhibitor diluted to contain from 0 to 100% Bard-Parker germicide were measured after five hours. It can be seen that the 10% inhibitor concentration reduced the biological response about sixfold, compared to the untreated control. By increasing the inhibitor concentration to 40%, a minimum metabolic response was reached, and increasing the concentration at 10% increments to 100% did not result in a further attenuation of the response.

b) Effect of Sample Contact Time and Sample Size with Bard-Parker

Increasing metabolic activity was observed when fixed volumes of inhibitor (0.1 ml, undiluted) and medium (0.4 ml) were applied to increasing soil sample sizes. Soils pretreated 30 minutes prior to the addition of medium resulted

Effect of Bard-Parker Concentration on the Evolution of $^{14}CO_2$ by the Labeled Release Experiment

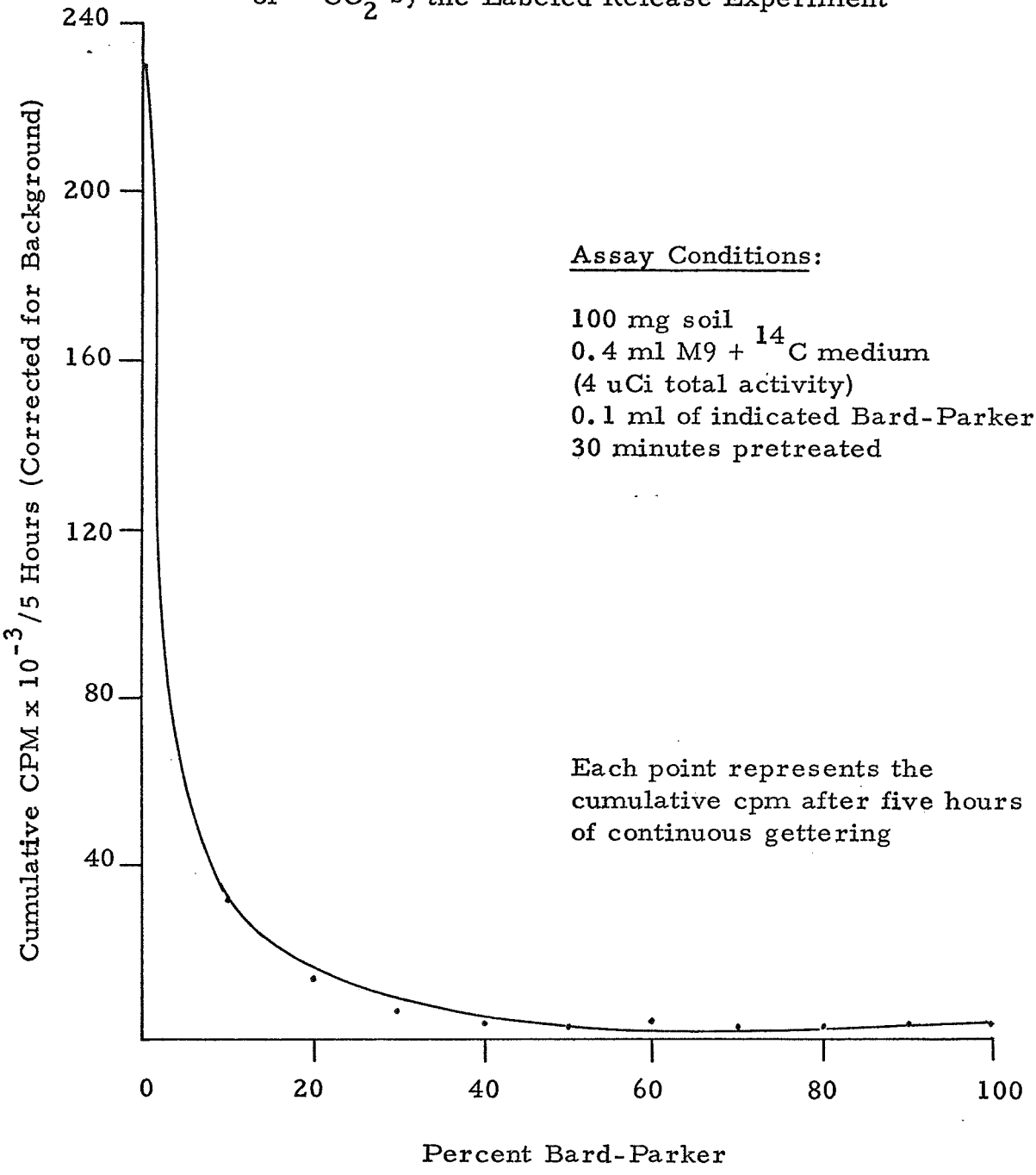

	Fig. 8		
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in lower responses than identical samples not subjected to the inhibitor pretreatment. This data is compared in Figure 9 where it is seen that the pretreated soil up to 300 mg evolved proportionately increasing amounts of metabolically labeled carbon dioxide. Inhibitor pretreatment of 400 mg and 500 mg samples resulted in a sharp increase in the rate of evolved $^{14}CO_2$, and approached the level of $^{14}CO_2$ outgassing of the non-pretreated samples.

The superior inhibitor effectiveness achieved with the soil pretreatment (at least with samples as large as 300 mg) is simply a function of the contact time preceding inhibitor dilution upon the addition of the medium.

B. Light Fixation-Dark Release Assay

1. Introduction

Early in the study of the light fixation-dark release assay, experimentation was conducted in a variety of types of photosynthesis chambers varying in volumes from 10 to 20 ml. Encouraging preliminary results were obtained, although the chambers were not gas-tight. It was found with the soils used in these early trials that the nonbiological background was reduced to a satisfactory level by a brief pre-gettering period or by transfer of the samples to planchets for the dark release of labeled carbon dioxide.

Effect of Sample Size and Pretreatment Time Upon the Labeled Release Response from Bard-Parker Treated Soil

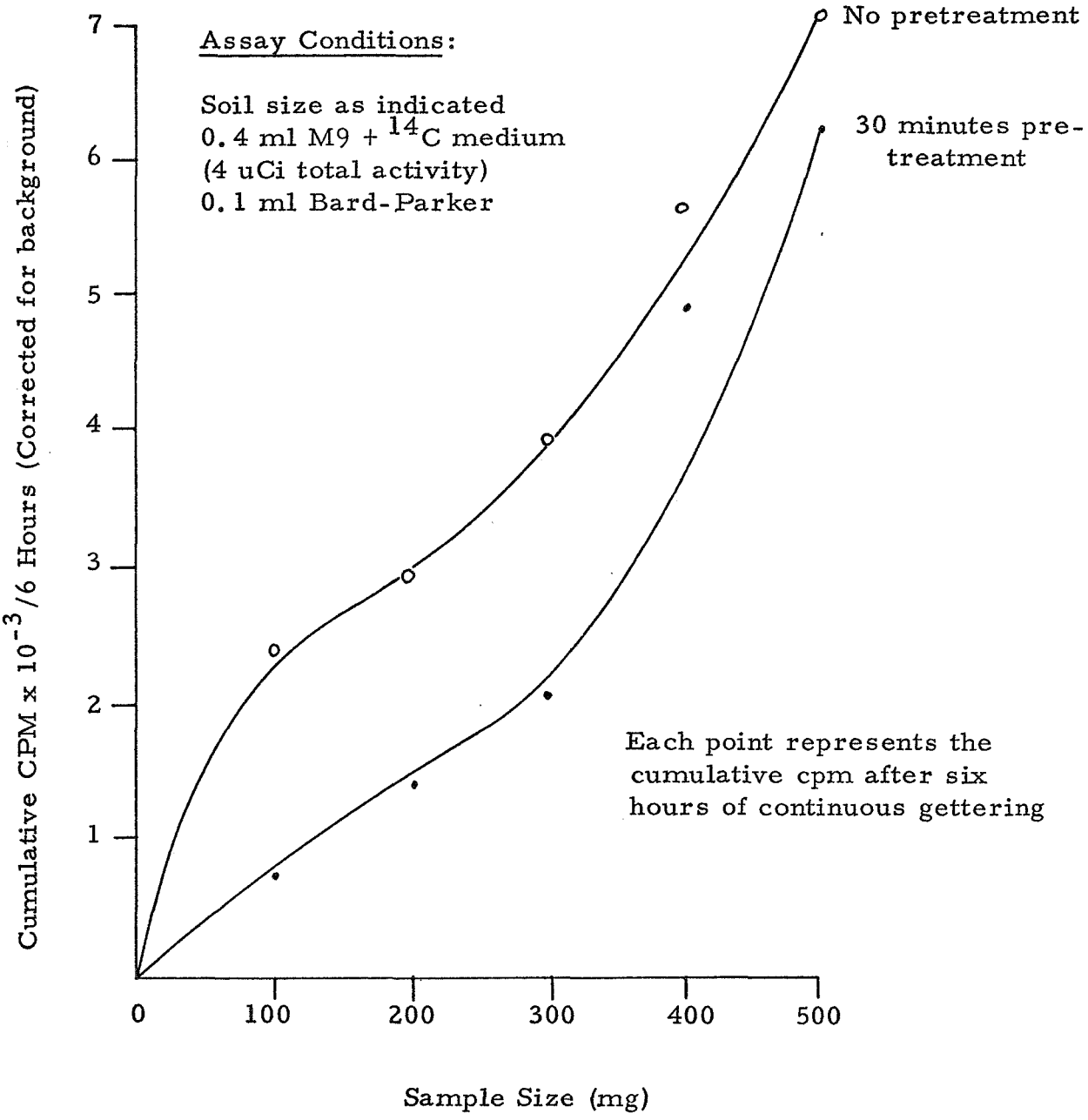

	Fig. 9			
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Several types of gas-tight photosynthesis chambers having volumes of about 10 ml were next tested in an attempt to increase the precision of the assay and to reduce the incidence of anomalous signals - that is, light signals less than dark or control signals and dark signals less than the controls. Anomalous results were still obtained with these gas-tight chambers, although their frequency was somewhat reduced. The precision of the test procedure was improved. However, several soils gave levels of nonbiological backgrounds high enough to render the assay insensitive in detecting photosynthetic activity. At this point, the 100 ml, gas-tight photosynthesis chamber was fabricated (Figure 10). This chamber was equipped with valves to permit a gas-flushing step, in addition to a removable bottom plug to allow for transfer of the soil sample after the light incubation period. A systematic investigation of some of the variables involved in the photosynthesis assay was initiated using this new experimental apparatus. The results are described in detail in this report.

2. Experimental Procedure

a) Precision of Light Fixation-Dark Release Experiment

A relative standard deviation of 40% to 90% was obtained in photosynthesis assays conducted with

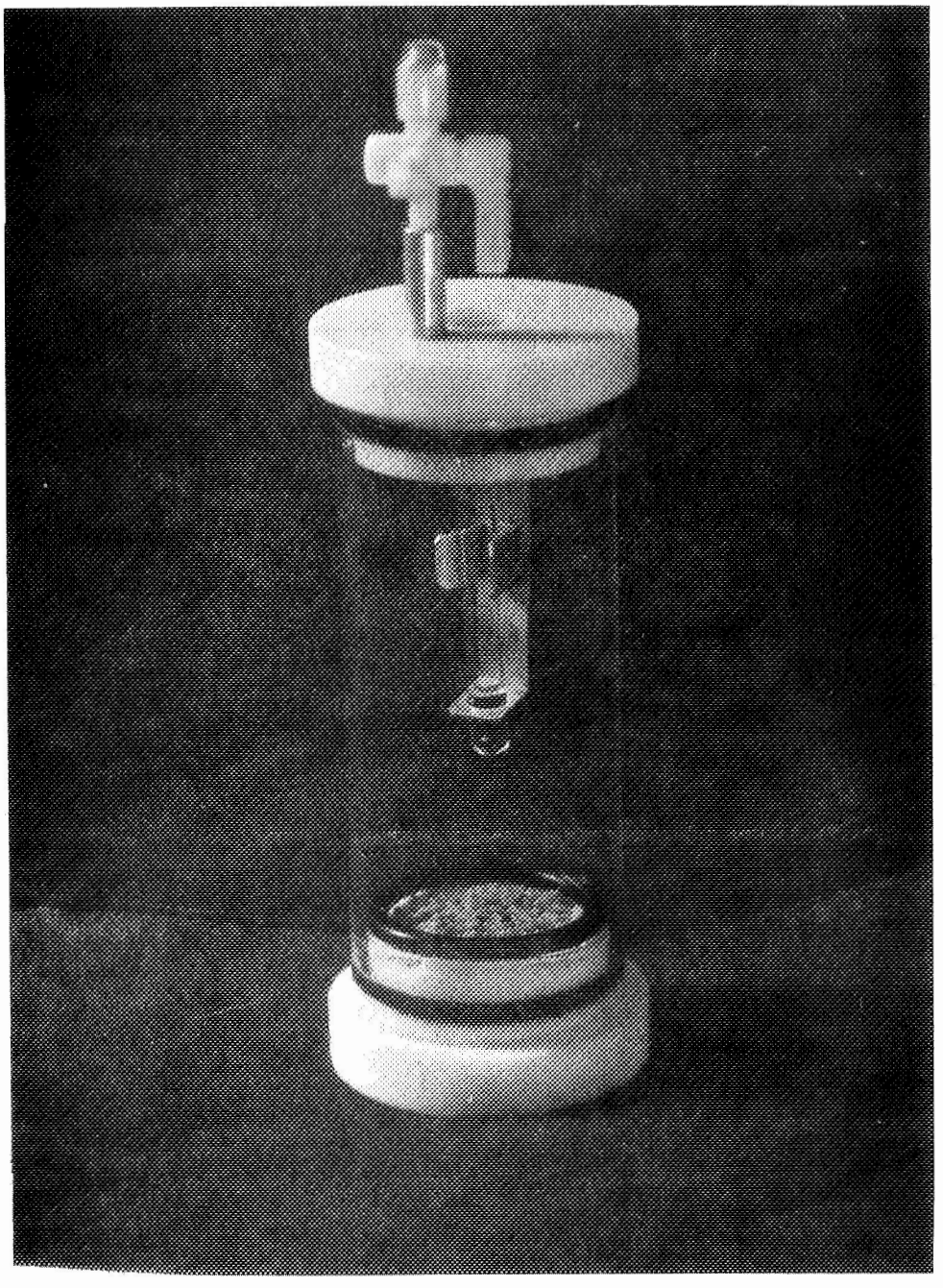

Figure 10

100 ml Gas-Tight Photosynthesis Chamber

various experimental chambers known not to be leak-tight. With the photosynthesis assays conducted in the gas-tight chambers having volumes of 10 to 100 ml, a standard deviation of 25% was routinely obtained. In addition to significantly improving the precision of the assay, the use of the gas-tight experimental chambers permitted exposure of the soil samples to a higher percentage of the labeled carbon dioxide during the incubation period.

b) Light Fixation-Dark Release Experiments

Table 4 summarizes the results of 59 experiments conducted with the gas-tight experimental apparatus; soils used were not flushed with gas. In these experiments, 1 gram soil samples were incubated in the light (500-foot candles) and dark for three hours in the presence of a 1 or 10 uCi/0.1 ml $NaH^{14}CO_3$ solution from which $^{14}CO_2$ had been generated by acidification and to which 0.1 ml of 18 $\underline{N}$ H_2SO_4 had been added to provide 0.01 or 0.1 uCi of $^{14}CO_2$/cc of headspace. Following the incubation, each soil sample was transferred to a planchet and gettered in the dark with $Ba(OH)_2$ wetted pads. Getter pads were changed at prescribed intervals. The first 30 to 60 minutes of gettering were considered the time sufficient to remove the nonbiological background. In 21 of 55

experiments, the signals from soil in the light chambers were greater than the signals from soil in the dark chambers. Seven of these 21 soil samples had been seeded with either algae cells or moss. The light signal was equal to the dark signal in 28 experiments, and the dark signal exceeded the light signal in six experiments. The controls were equal to or greater than the light signal in four experiments. The L/D ratios were greater than one in 14 experiments not supplemented with photosynthetic material and ranged from 1.6 to 5.2, with an average of 2.4. For the seven experiments with phototrophic-supplemented soil, the L/D ratios had a spread of 2.2 to 61, with an average value of 15. In six cases, the dark response was about twice that of the light response, with an average L/D ratio of 0.5. The L/C ratio was significantly less than one in four of 47 trials, and in five cases the control was greater than the dark response. The light response equaled the control in eight experiments and was significantly greater than the control in 35 out of 47 experiments. The range of L/C values greater than one for soil not supplemented with photosynthetic material was from 1.5 to 163, with a mean value of 18.

Since the relative standard deviation was found to be 25%, light responses equal to or greater than 150% of the dark response were considered indicative of photosynthetic activity at the 95% confidence level. In this manner, only 14 of the experiments performed on nonsupplemented natural soils and presented in Table 4 were indicative of photosynthetic activity. A number of experiments were performed on the photosynthetically nondefined soils by varying soil moisture content, duration of light intensity during incubation, and light composition and intensity. However, each of these variables was ineffective in eliciting a positive photosynthetic response from the soil samples, based on the latter statistical approach. It is probable that these soils contained a light-dependent autotrophic population below the threshold of detection for this assay. The onset of a seasonal, dormant period for microbial photosynthetic activity may have been a factor, in that these tests were conducted in the fall.

3. Investigation into the Mechanism of Anomalous Responses

The results presented in Table 4 show that responses from autoclaved controls were greater than the responses from light and/or dark chambers in five out of 47 experiments. These control soils were known to have been

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Table 4

Dark Release Experiments Conducted in Gas-Tight Chambers (No $^{12}CO_2$ Flush Used)

Order of Responses [1]	No. of Experiments
L > D > C	12 [2]
L = D > C	17
L > D = C	1
L = D = C	3
L = D	6
L > D	6 [3]
L > C	2
L = C	2
L > C > D	1
D > L > C	2
D > L = C	3
C > L > D [4]	1
C > L = D [4]	2
C = D > L [4]	1
	59

(1) Responses were different at the 95% or greater confidence level.

(2) Four of these 12 experiments were conducted on soils supplemented with photosynthetic organisms.

(3) Three of these six experiments were conducted on soils supplemented with photosynthetic organisms.

(4) The controls of these experiments were conducted with soils which had been autoclaved once. Most of the control soils were autoclaved two or more times.

autoclaved just once in four of the five instances. All control soils are now routinely autoclaved five hours per day for three days, then for 30 minutes just prior to their use in an experiment. A single autoclaving will not kill all spores, and, in fact, may even stimulate metabolic activity to a level higher than that in untreated soil.

The response from the dark chamber was higher than that from the light chamber in six of 55 experiments, as shown in Table 4. Although it is possible for light to exert an inhibitory effect upon the light-independent autotrophic organisms and, therefore, reduce the light signal, this was not felt to be the case in these experiments. After obtaining a number of L/D ratios significantly less than one, a study of this phenomenon was initiated. Two observations relative to this process were made. First, the temperature of the light incubation chambers was about 4°C higher than that of the dark chambers, due to heating effects during the exposure period. Secondly, considerable amounts of moisture were driven out of the soils and condensed on the walls of the light chambers during the incubation period. The temperature difference was eliminated either by placing the dark chambers in an incubator set at the proper temperature, or by wrapping them with aluminum foil and brown wrapping paper.

No condensation was noted on the walls of the dark chambers when the temperature was raised to that of the light chambers. The net result of this displacement of water from the soils in the light chambers probably was a lowered amount of nonbiologically fixed $^{14}CO_2$. Results presented later in this report show that increased moisture content in soils will increase physically-absorbed $^{14}CO_2$. To test this and other parameters which might affect the nonbiological responses from soil, the series of experiments to be described was investigated.

4. Studies on the Removal of Nonbiologically Fixed $^{14}CO_2$

One of the major complications of the photosynthetic assay had been the quantitative and qualitative discrimination between the biologically-derived and nonbiologically-derived $^{14}CO_2$ during the dark release portion of the experiment. Gettering the soil sample from one to two hours following the light incubation period had proven unsatisfactory for quantitatively removing $^{14}CO_2$ which was nonbiologically absorbed in the soil. The following experiments were designed to investigate the parameters of time, temperature, and moisture content as they affect $^{14}CO_2$ absorption and its release from soil. Figure 11 shows the arrangement of the experimental apparatus during light incubation.

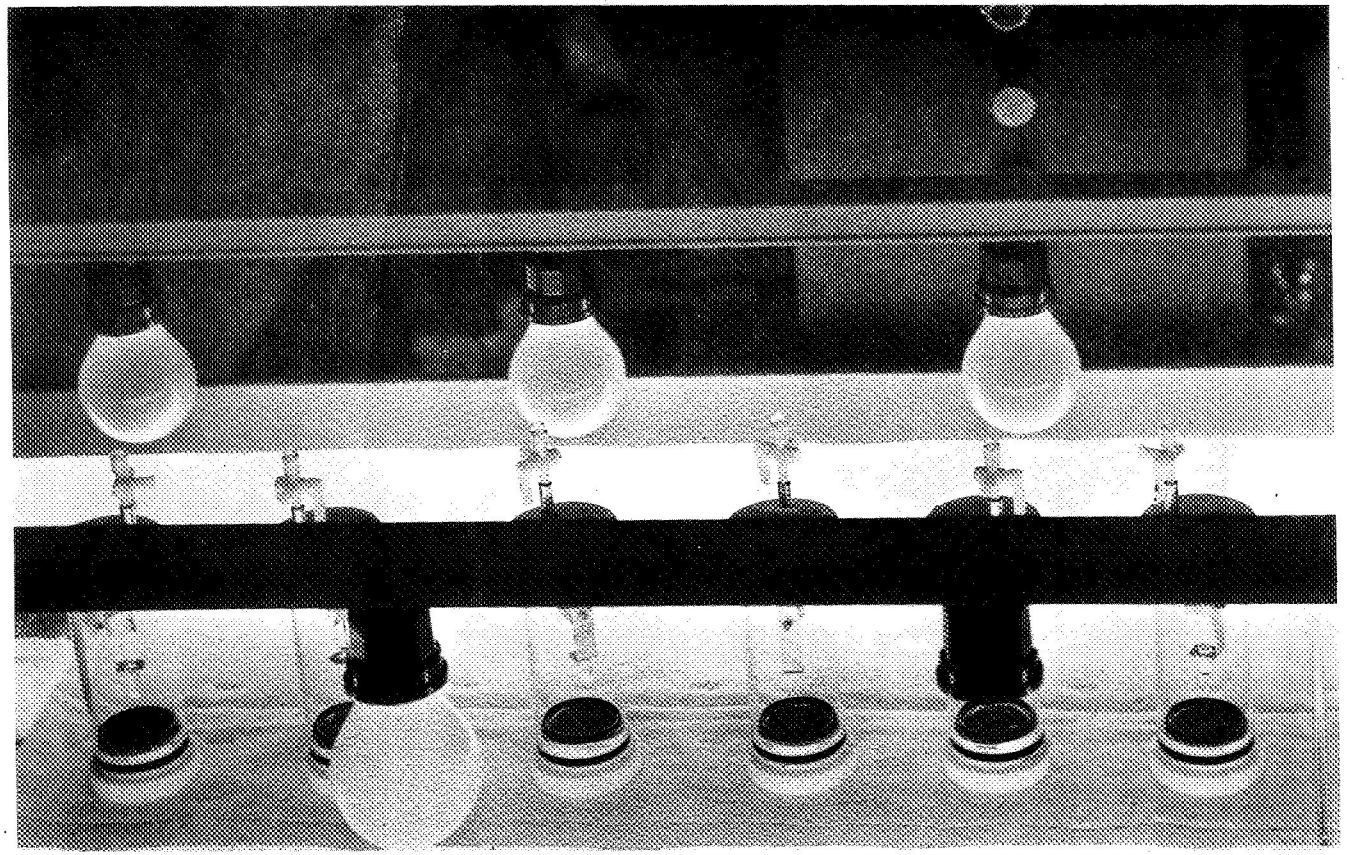

Figure 11

Arrangement of Photosynthesis Chambers During Light Incubation

a) Time and Temperature of Incubation with $^{14}CO_2$

The level of $^{14}CO_2$ nonbiologically fixed to a sterile soil was estimated by collecting the $^{14}CO_2$ desorbed over 20 hours during the dark release period, and was found to be independent of the light incubation temperature in the range from 20°C to 37°C.

Figure 12 shows the extent of the nonbiological fixation of $^{14}CO_2$ in two varieties of soil as a function of duration of light incubation, as measured by desorption in the dark during a 20-hour collection period. The amount of chemisorption of $^{14}CO_2$ in both soils was still proceeding at a significant rate after four hours of exposure to labeled carbon dioxide.

b) Effect of Added Moisture

Moisture has been found to stimulate both heterotrophic and photosynthetic activity when applied to most arid soils, but has little or no effect on soils that are naturally moist (i.e., contain more than approximately 5% moisture). The contribution of added moisture to the nonbiological fixation of $^{14}CO_2$ with two sterile soils is described in Figure 13. In both soils, the added moisture caused a significant increase in the amount of nonspecifically-adsorbed $^{14}CO_2$, as measured by

Nonbiological Fixation of $^{14}CO_2$ as a Function of Light Incubation Time

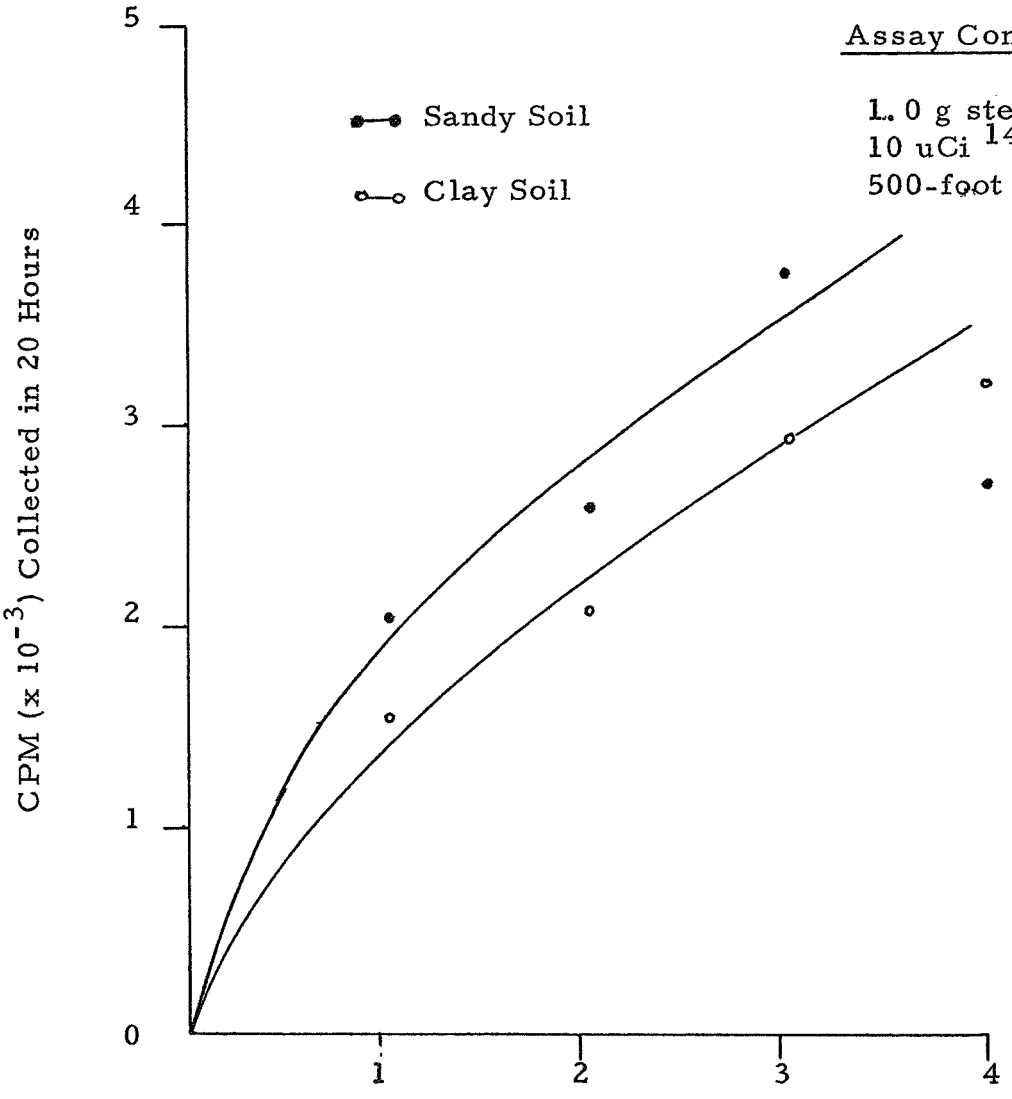

Fig. 12

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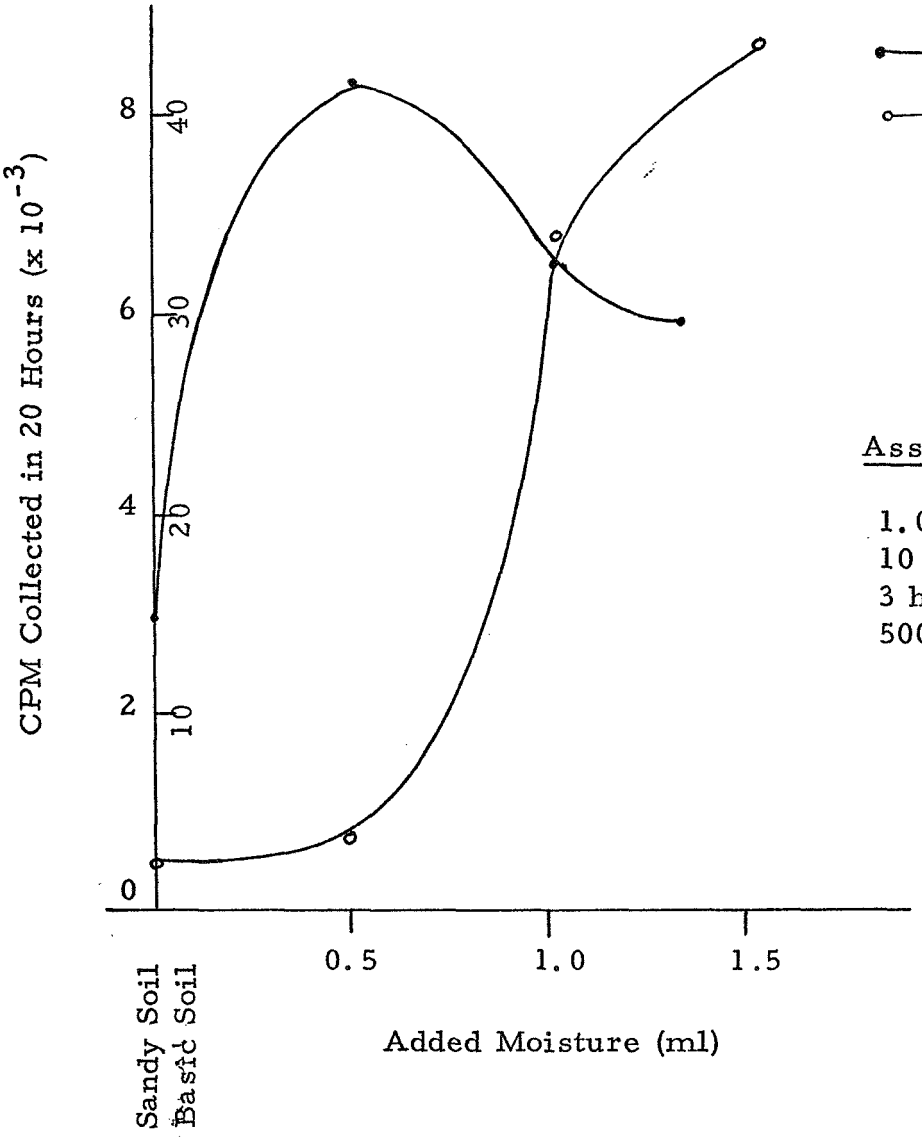
NASW-1931	AMML Final Report

Effect of Added Moisture Upon
$^{14}CO_2$ Evolution from Sterile Soil



Assay Conditions:

1.0 g sterile soil
10 uCi $^{14}CO_2$/100 ml
3 hours light incubation
500-foot candles

BIOSPHERICS INCORPORATED Rockville, Maryland	Fig. 13			
	Date: Apr'71	Init.	Scale	
NASW-1931 Cont. No.	AMML Final Report Report Title			

its desorption during the dark release period. Further, the amount of $^{14}CO_2$ adsorption and its rate of desorption appear to be a characteristic of the soil type.

In a separate experiment conducted with 1 gram of sterile, sandy soil at ambient moisture, a total of 600 cpm was collected during the dark release period, as compared with 11,000 cpm from 1 gram of the same soil moistened with 1 ml of water. Although these results argue against the addition of moisture to the soil, other studies to be described have indicated that water-augmented, nonbiologically fixed $^{14}CO_2$ can be minimized by $^{12}CO_2$ gas flushing. Thus, the addition of water to enhance metabolic activity of the soil remains feasible.

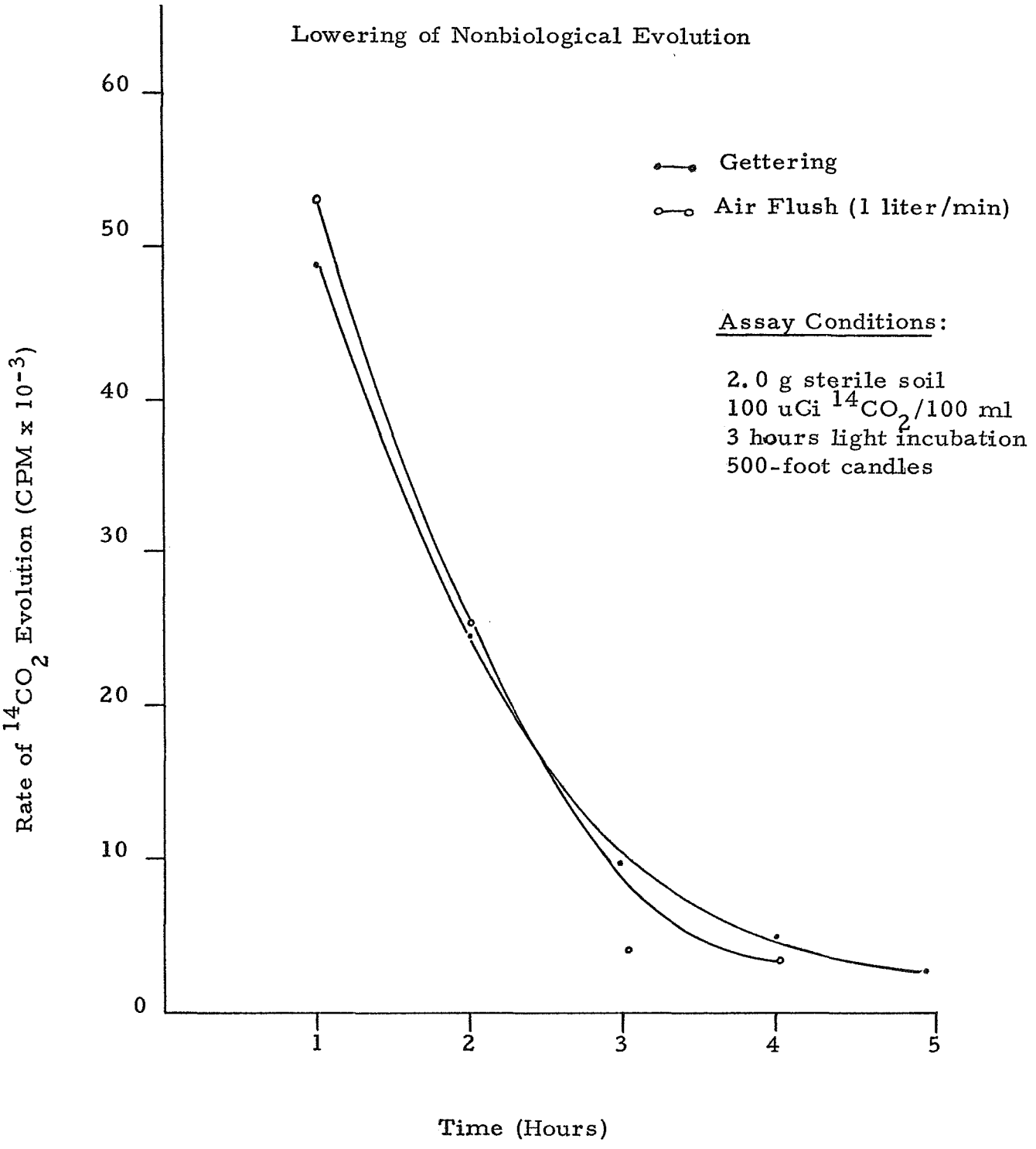
5. Gas-Flushing Techniques

The nonbiological responses from the majority of soils studied in early tests of the dark release experiments did not interfere with the detection of the biological signals. In most cases, the pregettering period of one to two hours lowered the adsorbed $^{14}CO_2$ to a satisfactory level. Later experiments with different soils and with higher specific activity of $^{14}CO_2$ demonstrated inadequacies in achieving low noise levels. Initial experiments with air and nitrogen gas flushes to remove chemisorbed

$^{14}CO_2$ indicated that this technique was not promising. This was verified by the experiment shown in Figure 14 which compared gettering without flushes to gettering preceded by a one-liter-per-minute air flush.

The use of a dry $^{12}CO_2$ sweep after the light and dark incubation periods with labeled carbon dioxide was studied in an attempt to develop a faster and more efficient method for the removal of nonbiologically fixed $^{14}CO_2$. This technique appeared very promising, since drastic reductions in the nonbiological evolution levels from soils were attained. The greatest reductions have been obtained on soils to which moisture had been added prior to the light incubation period. A tabulation of these experiments is presented in Table 5. A few biological tests were run using the dry $^{12}CO_2$ sweep to reduce chemisorbed $^{14}CO_2$. The initial results were encouraging and are described in Tables 6 and 7.

Later, several experiments were conducted employing a gas flush with $^{12}CO_2$ containing water vapor (Figure 15). The water vapor was added by passing the $^{12}CO_2$ through two water-filled impingers prior to using it for flushing. The results shown in Table 8 demonstrate the significant reduction in the non-biological noise of the California soil as a result of this new



BIOSPHERICS INCORPORATED Rockville, Maryland	Fig. 14		
	Apr'71 Date	Init.	Scale
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Table 5

Effect of $^{12}CO_2$ Flush upon Nonbiological Evolution in Dark Release Experiment (1)

Soil Description	$^{12}CO_2$ Flush	Added Moisture (ml)	Total $^{14}CO_2$ Released (cpm/20 hrs)
Botanical Gardens Soil, Wheaton Regional Park	1 hr-40 ml/min	0.0	79
Botanical Gardens Soil, Wheaton Regional Park	1 hr-40 ml/min	1.0	148
Botanical Gardens Soil, Wheaton Regional Park	none	0.0	217
Botanical Gardens Soil, Wheaton Regional Park	none	1.0	185
Sandy soil, Wheaton Regional Park	1 hr-40 ml/min	0.0	819
Sandy soil, Wheaton Regional Park	1 hr-40 ml/min	1.0	240
Sandy soil, Wheaton Regional Park	none	0.0	601
Sandy soil, Wheaton Regional Park	none	1.0	998/10
Creek bottom soil, Rock Creek Park	1 hr-40 ml/min	0.0	186
Creek bottom soil, Rock Creek Park	none	0.0	441
California soil -TRW	1 hr-40 ml/min	0.2	670
California soil -TRW	none	0.2	2085

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Table 5
(continued)

Soil Description	$^{12}CO_2$ Flush	Added Moisture (ml)	Total $^{14}CO_2$ Released (cpm/20 hrs)
Muddy soil, Rock Creek Park	0.5 hr-40 ml/min	0.0	400
Muddy soil, Rock Creek Park	none	0.0	1037

(1). A 1.0-g portion of sterile soil was illuminated under 500-foot candles of fluorescent light for three hours in the presence of 10 uCi of $^{14}CO_2$ in a 100-ml gas-tight chamber.

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Table 6

Summary of Light Fixation-Dark Release Experiments (1)
Using Sealed 100 ml Chambers and $^{12}CO_2$ Flushing

Order of Responses (2) (3)	
L > D > C	3
L = D > C	4

(1) A total of seven experiments was performed on four different soils.

(2) Responses are different at the 95 percent or greater confidence level.

(3) All control values were less than the corresponding dark and light values. All light values were equal to or greater than the dark values.

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Table 7

Light Fixation-Dark Release Experimental Data (1)

(Dry $^{12}CO_2$ Flush Used)

	Cumulative Responses (cpm)					
	Light		Dark		Control	
Soil Description	Indiv.	Avg.	Indiv.	Avg.	Indiv.	Avg.
Sandy Soil, Wheaton Regional Park	2206 2230	2218	1990 2403	2197	230 313	272
Terrarium soil (2)	1023 235	629	300 317	309	70 85	78
Terrarium soil (2)	899 551	725	301 328	315	54 4	29
Botanical Gardens Soil, Wheaton Regional Park	2774 2324	2549	1977 1883	1930	160 242	201
Botanical Gardens Soil, Wheaton Regional Park	2034 3547	2791	2222 2239	2231	109 28	69
California Soil - TRW	5034 6147	5591	7023 4877	5950	1177 1853	1515
Terrarium soil (2)	352 362	357	250	250	8 14	11

(1) Assay Conditions:
- 0.5 g soil, no added moisture;
- Control autoclaved three times in sealed canning jars containing a beaker of water;
- Incubation for three hours at 30°C under 500-foot candles fluorescent light (GE F48PG17CW Power Groove - Cool White);
- One-hour flush with $^{12}CO_2$ at 130-150 ml/min. through the soil;
- Dark release period - 18 to 24 hours.

(2) This soil was originally taken from a lawn. All higher plants including moss were removed and the soil was watered and stored in a closed terrarium under light for six weeks.

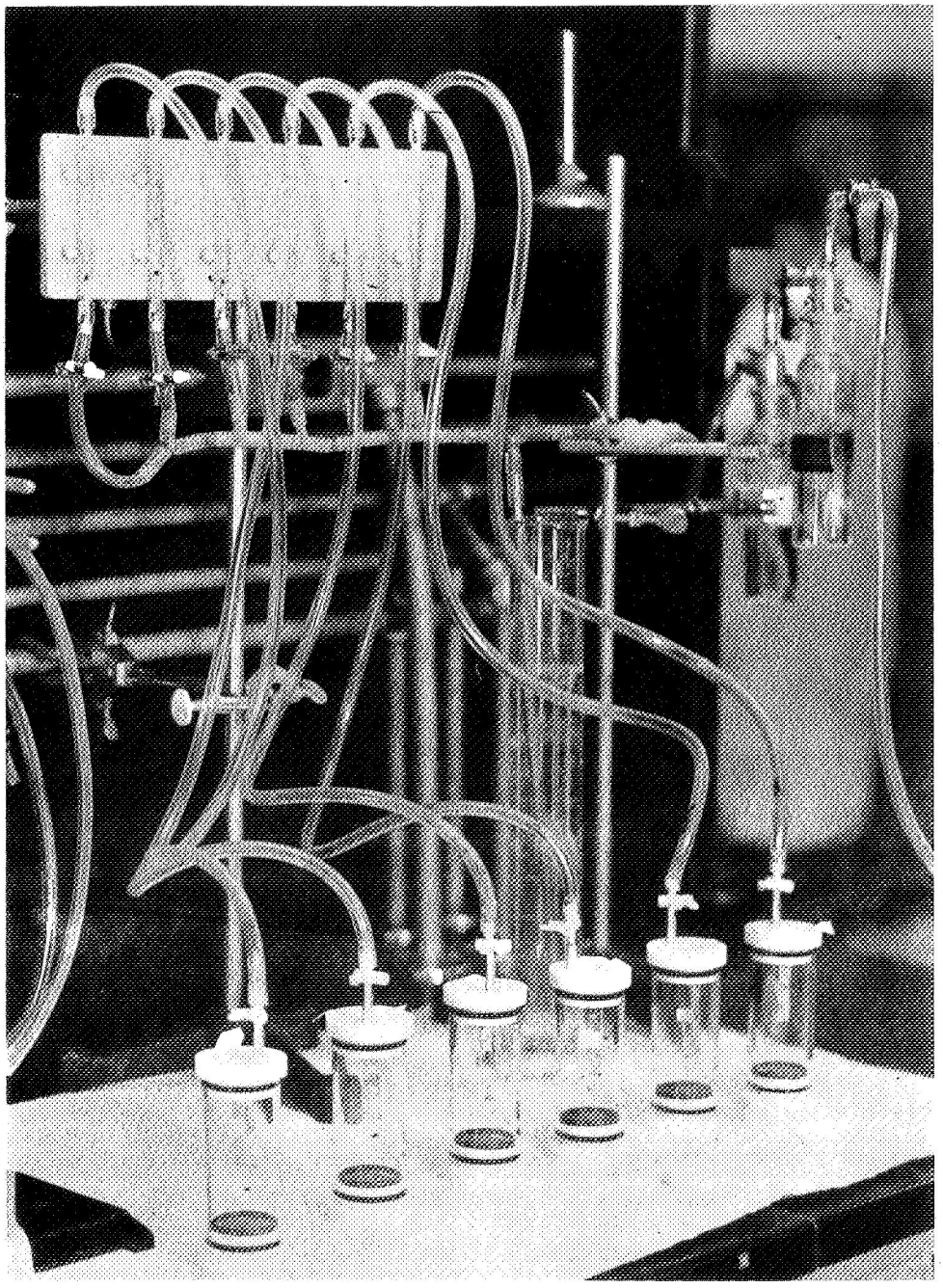

Figure 15

Moist $^{12}CO_2$ Flushing Apparatus and Arrangement

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Table 8

Improvement of the $^{12}CO_2$ Flushing Technique (1)
by the Addition of Water Vapor

Soil	Total $^{14}CO_2$ Released: Dry $^{12}CO_2$ Flush (cpm/20 hours)	Total $^{14}CO_2$ Released: Moist $^{12}CO_2$ Flush (cpm/20 hours)
Wooded area soil	26	44
Wheaton Regional Park	0	6
		18
		23
		49
		15
Sandy soil	272	339
Wheaton Regional Park		232
		78
Botanical Gardens,	49	39
Wheaton Regional Park	69	20
	201	100
California soil -TRW	2725	389
	1483	360
	1515	843
	2474	549
		634
		735
		475
		359
Terrarium soil (2)	11	21
	29	21
	78	46
	39	1

(1) A 1.0-g portion of sterile soil was illuminated under 500-foot candles of fluorescent light for three hours in the presence of 10 uCi of $^{14}CO_2$ in a 100-ml, gas-tight chamber. The soil was then flushed for one hour with dry or moist $^{12}CO_2$ at a rate of 130-150 ml/min.

(2) This soil was originally taken from a lawn. All higher plants, including moss, were removed and the soil was watered and stored in a closed terrarium under light for seven weeks.

flushing technique (less than 850 cpm compared to about 2,000 cpm after dry $^{12}CO_2$ flush - see Table 8). The $^{12}CO_2$ flush operates by the mass action effect, as it can exchange with $^{14}CO_2$. The addition of water vapor to the $^{12}CO_2$ resulted in an even more rapid displacement of $^{14}CO_2$, probably by de-activating polar sites in the soil which bind the $^{14}CO_2$.

Summaries of the photosynthesis assays using the moist $^{12}CO_2$ flush are presented in Tables 9, 10, 11, and 12. The precision was acceptable, and no anomalous values were obtained. This technique showed promise in alleviating the nonbiological noise problem and has demonstrated capability to detect metabolism in soils. Photosynthesis has been demonstrated also, but not in all of the samples tested. The data presented in Table 12 for samples assayed in the spring did not show any clear increase in photosynthesis activity over that of the samples examined during the previous fall. However, the fresh, sandy soil acquired in the spring showed the largest photosynthetic signal observed by us to date in a natural soil sample.

6. Use of Bard-Parker Germicide for Control Soils

The control responses presented thus far in the light fixation-dark release data have been obtained from soils sterilized by repeated autoclaving. A study was made of the use

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Table 9

Summary of Light Fixation-Dark Release Experiments Using Sealed 100 ml Chambers and Moist - $^{12}CO_2$ Flushing

November 1969 Experiments (1)

Order of Response (2) (3)	
L > D > C	4
L = D > C	9

(1) A total of 13 experiments was performed on six different soils.

(2) Responses are different at the 95 percent or greater confidence level.

(3) All control values were less than the corresponding dark and light values. All light values were equal to or greater than the dark values.

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Table 10

Light Fixation-Dark Release Experimental Data (1)

(Moist $^{12}CO_2$ Flush Used)

November 1969 Experiments

Soil Description	Date	Cumulative Responses (cpm) Light Indiv.	Light Avg.	Dark Indiv.	Dark Avg.	Control Indiv.	Control Avg.
Sandy soil, Wheaton Regional Park	11/17/69	1398	1755	1396	1584	128	182
		2112		1771		236	
" "	11/22/69	2820	2613*	1649	1531	206	339
		2406		1412		472	
Botanical Gardens soil, Wheaton Regional Park	11/17/69	730	813	453	658	27	20
		895		862		12	
" "	11/19/69	1870	1668	1689	1689	39	39
		1466		----		---	
Wooded area soil, Randolph Road	11/18/69	658	763	594	664	30	23
		867		734		16	
" "	11/19/69	1721	1673*	415	414	18	18
		1624		413		---	
" "	11/20/69	692	754*	485	501	0	6
		815		516		12	
" "	11/24/69	1133	1016	816	816	44	44
		899		----		---	

*Significant for photosynthesis at the 95 percent or greater confidence level.

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Table 10
(continued)

Light Fixation-Dark Release Experimental Data (1)

November 1969 Experiments

Soil Description	Date	Cumulative Responses (cpm) Light Indiv.	Light Avg.	Dark Indiv.	Dark Avg.	Control Indiv.	Control Avg.
Terrarium soil (2)	11/18/69	1332	925*	228	247	14	21
		518		265		27	
"	11/21/69	199	285	195	213	23	23
		371		231			
Clay soil, Wyaconda Road	11/25/69	4192	5556	5663	5281	165	165
		6919		4898			
" "	11/26/69	5676	5141	3865	3991	134	127
		4605		4116		120	
Wooded area soil, Georgia Avenue	11/26/69	1851	1773	1725	1648	110	126
		1695		1570		142	

(1) Assay Conditions:
- 1.0 g soil, no added moisture;
- Controls autoclaved three times in sealed canning jars containing a beaker of water;
- Incubation for three hours at 30°C under 500-foot candles fluorescent light (GE F48PG17CW Power Groove - Cool White). 0.1 uCi/cc $^{14}CO_2$.
- One-hour flush with $^{12}CO_2$ at 130-150 ml/min. through the soil. $^{12}CO_2$ passed through two water-filled impingers to raise humidity prior to contact with soil.
- Dark release period - 18 to 24 hours.

(2) This soil was originally taken from a lawn. All higher plants, including moss, were removed and the soil was watered and stored in a closed terrarium under light for seven weeks.

* Significant for photosynthesis at the 95 percent or greater confidence level.

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Table 11

Summary of Light Fixation-Dark Release Experiments
Using Sealed 100 ml Chambers and Moist-$^{12}CO_2$ Flushing
April-May 1970 Experiments(1)

Order of Response(2)(3)	
L > D > C	5
L = D > C	9

(1) A total of 14 experiments was performed on nine different soils.

(2) Responses are different at the 95 percent or greater confidence level.

(3) All central values were less than the corresponding dark and light values. All light values were equal to or greater than the dark values.

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Table 12

Light Fixation-Dark Release Experimental Data (1)(2)

(Moist $^{12}CO_2$ Flush Used)

April-May 1970 Experiments

Soil Description	Date	Cumulative Responses (cpm) Light Indiv.	Light Avg.	Dark Indiv.	Dark Avg.	Control Indiv.	Control Avg.
Sandy soil, Wheaton Regional Park	4/21/70	695 417	556	562 438	500	101 52	77
Fresh sandy soil, Wheaton Regional Park	5/8/70	30649 26918	28784*	3734	3734	375 897	636
Fresh Botanical Garden soil, Wheaton Regional Park	5/1/70	4999 5066	5033	6351 6458	6405	73 127	100
Wood area soil, Randolph Road	4/22/70	683 812	748	585 587	586	9 26	18
Fresh wooded area soil, Randolph Road	5/4/70	1708 1864	1786	1721 2149	1935	87 14	51
Terrarium soil (3)	5/8/70	297 321	309*	130 143	137	30 12	21
Terrarium soil (3) containing moss	5/5/70	7518 13877	10698*	1567 1528	1548	15 0	8
California soil -TRW	4/20/70	2236	2236	2197 1628	1913	487 60	274
" "	4/24/70	4488 4191	4340*	2656 2656	2656	697	697
" "	4/28/70	2223 2365	2294*	1002 1159	1081	691 775	733

* Significant for photosynthesis at the 95 percent or greater confidence level.

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Table 12
(continued)

Light Fixation-Dark Release Experimental Data(1)(2)
April-May 1970 Experiments

Soil Description	Date	Cumulative Responses (cpm) Light Indiv.	Light Avg.	Dark Indiv.	Dark Avg.	Control Indiv.	Control Avg.
California soil-TRW	4/29/70	1726	1574	1297	1448	888	892
		1422		1599		896	
" "	4/30/70	2570	2728	3105	2470	496	548
		2886		1835		600	
California soil, Torrance	4/27/70	581	538	545	563	108	140
		495		581		171	
Fresh creek bank soil, Wheaton Regional Park	5/5/70	4161	3229	2614	2514	162	154
		2296		2413		146	

(1) Assay Conditions:
1.0 g soil, no added moisture.
Controls autoclaved three times in sealed canning jars containing a beaker of water.
Incubation for three hours at 30°C under 500-foot candles fluorescent light (GE F48PG17CW Power Groove - Cool White). 0.1 uCi/cc $^{14}CO_2$.
One-hour flush with $^{12}CO_2$ at 130-150 ml/min. through the soil. $^{12}CO_2$ passed through two water-filled impingers to raise humidity prior to contact with soil.
Dark release period - 18 to 24 hours.

(2) All soils, except the fresh soils, were stored in the laboratory over the winter. The fresh soils were obtained at locations near the original sampling sites.

(3) This soil was originally taken from a lawn. All higher plants, including moss, were removed and the soil was watered periodically in a closed terrarium.

of Bard-Parker germicide for reducing the metabolic activity of soils in this assay and the responses from treated soils were compared with those from autoclaved soils. These data are presented in Table 13. The responses from the Bard-Parker treated soils were all considerably higher than those from the autoclaved soils and in several cases, approached the magnitude of the dark responses. It was concluded, therefore, that pre-treatment with Bard-Parker germicide for ten to 20 minutes prior to $^{14}CO_2$ exposure was not adequate to reduce the response from control soils to a satisfactory level.

C. ^{14}C and ^{35}S Uptake Experiment

1. Introduction

Development of the ^{14}C and ^{35}S uptake life detection experiment has been described in the final reports from two previous programs sponsored by the National Aeronautics and Space Administration (NASA) - (1, 2) and in the Third Quarterly Progress Report of the current program, Contract No. NASW-1931 (3). Briefly, this experiment calls for passing a 1 ml portion of the soil-labeled medium suspension through a membrane filter, washing the filter to remove radio-activity retained by nonbiological processes, and then drying

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Table 13

Use of Bard-Parker Germicide for Control Soils

Soil Description	Cumulative Responses (cpm)(1) Bard-Parker	Autoclaved
California soil, TRW	1991	274
" " "	2537	697
Sandy soil, Wheaton Regional Park	244	77
Wooded area soil, Randolph Road	103	18
California soil, Torrance	569	140

(1) Assay Conditions:
1.0 g soil, no added moisture.
Bard-Parker germicide control soils pretreated with 1.0 ml for 10 to 20 minutes.
Autoclaved control soils heated at least three times for 4 hours in sealed canning jars containing a beaker of water.
Incubation for three hours at 30°C under 500-foot candles fluorescent light (GE F48PG17CW Power Groove - Cool White). 0.1 uCi/cc $^{14}CO_2$.
One-hour flush with $^{12}CO_2$ at 130-150 ml/min. through the soil. $^{12}CO_2$ passed through two water-filled impingers to raise humidity prior to contact with soil.
Dark release period - 18 to 24 hours.

(2) Responses reported are averages of duplicate determinations.

and counting the filter. During the course of this research, estimates have been obtained of the magnitude of biological signals to be expected from pure cultures of microorganisms and from natural soil samples. The effects of mechanical agitation and of additions of glucose and thioglycollic acid upon the biological signals have been studied. A satisfactory medium has been developed for the experiment, as well as an efficient antimetabolite for the control. A number of wash regimens and filter types have been studied in order to reduce the nonbiological background to a minimum and to increase the precision of the method. Estimates of nonbiological noise have been obtained from a wide variety of sterile and antimetabolite-treated soils.

Much of the experimentation described in the Third Quarterly Progress Report of Contract No. NASW-1931 will not be included here, as a significant improvement in the method was achieved during the final quarter of the program. Many of the experiments presented in the Third Quarterly Report were repeated, using the improved techniques, and are presented in the following description of the experimental work.

2. Nonbiological Background of the ^{14}C and ^{35}S Uptake Experiment

a) Summary of Results Prior to Use of Filter Prewash

During the latter portion of the program, it was discovered that prewashing the membrane filters with a solution of unlabeled substrates considerably reduced the level of nonbiological retention of labeled substrates. A good deal of preliminary experimentation had been completed up to this time, and the results are summarized here.

Basal medium containing the unlabeled substrates was selected for use as a filter wash solution on the basis of experimental results presented in Table 14. The use of a wash regimen of 2-2 ml filter rinses or from 2 to 4-1 ml filter rinses reduced the nonbiological retention of several filter types to a level of 0.1 to 0.3% of the applied radioactivity which could be measured with a coefficient of variation ranging from 40% to 80%. Gelman Acropor AN-450, Gelman Type E Glass Fiber, and S&S Bac-T-Flex membrane filters gave the lowest nonbiological retention levels of all the filters studied. Experiments conducted to measure nonbiological retention by soils demonstrated that retention by the soil-filter combination was not significantly higher than retention by the filters alone.

b) Nonbiological Background From Filters and Soils

Six separate experiments were conducted

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Table 14

Removal of Adsorbed Radioactivity from
Acropor AN-450 Membrane Filters by Wash Solutions

Wash Regimen		Basal Medium	% of Applied
Solution	Volume (ml)		Radioactivity Retained
Water	5 x 1	M9	0.14
"	5 x 1	M9	0.01
"	5 x 1	M9	0.15
"	5 x 1	M9	0.09
"	5 x 1	M9	0.38
Basal M9	5 x 1	M9	0.02
"	5 x 1	M9	0.08
"	5 x 1	M9	0.08
"	5 x 1	M9	0.04
0.15M NaCl	5 x 1	M9	0.01
0.01N H_2SO_4	5 x 1	M9	0.01
"	5 x 1	M9	0.01
"	5 x 1	M9	0.07
0.15M NaCl,	5 x 1	M9	0.03
0.015M $Na_3C_6H_5O$	5 x 1	M9	0.09
Water	4 x 1	RM9	0.53
0.01N H_2SO_4	4 x 1	RM9	0.18
Basal RM9	4 x 1	RM9	0.36
RM9 + ^{12}C + ^{32}S	4 x 1	RM9	0.07
"	2 x 2	RM9	0.16
"	2 x 2	RM9	0.07
"	2 x 2	RM9	0.19

Assay Conditions: One ml of the ^{14}C and ^{35}S solutions was passed through Gelman Acropor AN-450 membrane filters and washed as indicated. The filters were then dried and counted. The applied radioactivity was estimated by evaporating and counting aliquots of the solution in planchets. The percentages reported are averages of two or more replicate determinations. The radioactivity level of the M9 medium was 20 uCi/ml and of the RM9 medium, 2.0 uCi/ml.

during the latter portion of this program to measure the effects of a filter prewash upon the nonbiological retention of radioisotopes by filters. In these experiments, replicate 1 ml portions of RM9 + ^{14}C + ^{35}S medium were passed through microbial membrane filters. The medium composition is given in Table 15. Half of each set of filters was prewashed, just prior to filtration of the labeled medium, with 1 ml portions of RM9 containing only unlabeled substrates. Filter prewash solutions containing the usual and ten times the usual concentrations of unlabeled substrates were studied. The filters were then washed with 2-2 ml portions of RM9 containing the unlabeled substrates, dried, and monitored for retained radioactivity. Results of the experiments are shown in Table 16. These experiments demonstrated that the prewash technique described here reduced the nonbiological retention of isotopes by membrane filters by a factor of about five with no attendant loss, and perhaps a slight improvement, in precision. The use of a prerinse solution having a higher concentration of unlabeled substrates failed to improve the results. This constituted a significant advance in the development of the ^{14}C and ^{35}S uptake assay, and the filter prewashing technique was used throughout the remainder of the program in performing the experiments described in this section.

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Table 15

Composition of RM9 + ^{14}C + ^{35}S Medium

K_2HPO_4	5.0 mg/l
$MgCl_2 . 6H_2O$	166 mg/l
NH_4NO_3	200 mg/l
NaCl	100 mg/l
Soil extract*	100 ml/l
pH	7.0 (HCl)
Tris	6.0 g/l
^{14}C-Sodium Formate**	20 mg/l
^{14}C-D-Glucose (u.l.)**	50 mg/l
^{14}C-DL-1-Sodium Lactate**	20 mg/l
^{14}C-1-Glycine**	20 mg/l
^{35}S-Sodium Sulfate**	3.4 mg/l
Total radioactivity**	2.0 uCi/ml

* Soil extract was prepared by suspending 500 g of air-dried soil in 1300 ml of water. The mixture was then autoclaved for one hour, filtered, and made up to one liter with sterile, distilled water.

** The medium has been used at several levels of total radioactivity at ratios of 6.5 formate:1.3 glucose:1.3 lactate: 1.0 glycine:10.0 sulfate.

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Table 16

Effect of Filter Prewashing Upon Nonbiological Retention of Radioactivity by Membrane Filters

Filter Type	No. of Replicates	Applied Radioactivity Retained ± Coefficient of Variation (%) No. Prewash	Prewash	Prewash (10x)
S&S, Bac-T-Flex, C-5, 0.45 u	8	0.26 ± 75	0.06 ± 21	----------
"	4	0.44 ± 14	0.06 ± 70	0.06 ± 24
"	8	0.12 ± 65	0.03 ± 27	----------
Gelman, Acropor, AN-450, 0.45 u	8	0.13 ± 74	0.07 ± 79	----------
Millipore, HAWP, 0.45 u	8	0.31 ± 40	0.04 ± 40	----------
"	4	0.40 ± 13	0.04 ± 50	0.07 ± 35

Membrane filters, having a diameter of one inch, were compared for retention of applied radioactivity from 1-ml portions of RM9 + ^{14}C + ^{35}S medium having a total activity of 2 uCi/ml. Replicate filters from each experiment were prewashed with RM9 containing unlabeled substrates in the concentrations specified in Table 15. In some cases, a prewash solution containing ten times the specified concentrations was used. Retention by these filters was then compared with that of filters which had not been prewashed. All filters were washed, after exposure to the radioactive medium, with two 2-ml portions of RM9 containing the unlabeled substrates, dried, and then counted.

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Additional experiments were performed in an attempt to improve the filter washing procedure. These combined the filter prewetting technique with dilution of the labeled medium just prior to filtration. Each replicate S&S Bac-T-Flex Type C-5, 0.45 u, membrane filter was prewashed with a 1 ml portion of RM9 + ^{12}C + ^{32}S medium. Filtration of 1 ml portions of RM9 + ^{14}C + ^{35}S medium through half of each set of filters followed. Next, 1 ml portions of labeled medium, which had been diluted with 4 ml of cold medium, were passed through every remaining filter. All filters were washed three times with 1 ml portions of cold medium, then dried and counted. The results of this study, shown in Table 17, indicated that predilution was somewhat effective in lowering the percentage of applied radioactivity retained. Although these results appeared promising, the effects of medium predilution upon the experiment were not studied further. In order to economize on time and minimize the manipulations required to perform the assay in the laboratory, this technique was not incorporated into the biology experiments discussed later.

The possibility of reducing the percentage retention by the filters of applied radioactivity by increasing the volume of medium filtered was next studied. The biological signal, as

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Table 17

Effect of Predilution of Labeled Medium Upon the
Nonbiological Retention of Radioactivity by Membrane Filters

Filter Type	No. of Replicates	Applied Radioactivity Retained ± Coefficient of Variation (%)	
		No. Predilution	Predilution
S&S, Bac-T-Flex, C-5, 0.45 u	8	0.05 ± 33	0.01 ± 25
S&S, Bac-T-Flex, C-5, 0.45 u	8	0.03 ± 20	0.02 ± 44

Each membrane filter was prewashed with a 1-ml portion of RM9 + ^{12}C + ^{32}S and filtration of 1-ml portions of RM9 + ^{14}C + ^{35}S medium through each of one half of the filters followed. Then, 1-ml portions of labeled medium, which had been diluted with 4 ml of cold medium, were passed through each remaining filter. All filters were washed with three 1-ml portions of cold medium, dried, and monitored for radioactivity.

well as the nonbiological retention of radioisotope by soil, will be directly proportional to the volume of soil-medium suspension filtered. A higher signal-to-noise ratio will result in the event that filter retention does not increase in direct ratio with the volume of medium applied. Three separate experiments were performed in which the retention of radioisotope by membrane filters was measured as a function of volume of labeled medium filtered. Replicate sets of microbial filters were prewashed with 1 ml portions of RM9 containing the unlabeled substrates and used to filter volumes of 1 to 4 ml of RM9 + $^{14}C + ^{35}S$ containing 2 uCi/ml. The filters were then washed with cold RM9, dried, and counted. The results of these experiments are shown in Table 18. In general, the percentage of nonbiological retention of radioisotopes by the filters decreased as the applied radioactivity increased. This means that the signal-to-noise ratio can be increased by increasing the size of the aliquot filtered in the $^{14}C + ^{35}S$ uptake experiment. It may be desirable to take advantage of this phenomenon in cases where marginal biological signals are being measured.

Experimentation was conducted to determine the number

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Table 18

Retention of Radioisotopes by Membrane Filters as a Function of Applied Radioactivity*

Filter Type	No. of Replicates	Vol. of Medium Filtered (ml)	Retention ± Coefficient of Variation (%)
S&S, Bac-T-Flex, C-5, 0.45 u	4	1	0.021 ± 31
	4	2	0.022 ± 37
	4	3	0.019 ± 43
Millipore, HAWP, 0.45 u	4	1	0.044 ± 5
	4	2	0.022 ± 25
	4	3	0.023 ± 39
S&S, Bac-T-Flex, C-5, 0.45 u	3	1	0.028 ± 9
	3	2	0.027 ± 73
	2	3	0.018 ± 16
	3	4	0.015 ± 31

*Each filter was prewet with a 1-ml portion of RM9 containing unlabeled substrates and then used to filter varying volumes of RM9 + ^{14}C + ^{35}S containing 2 uCi/ml. The filters from the top two experiments listed were washed with two 2-ml portions of cold RM9. Filters from the last experiment listed were washed with four 1-ml portions of cold RM9. All filters were then dried and counted.

of 1 ml filter washes required to reduce to a minimum the non-biological retention of radioisotopes by membrane filters and by soils. These experiments were all conducted in a similar fashion. Each replicate set of filters was prewashed with a 1 ml portion of RM9 containing unlabeled substrates and used to filter 1.0 ml aliquots of RM9 + ^{14}C + ^{35}S medium or aliquots of the labeled medium-sterile soil suspension. The suspensions contained 1.0 g of sterile soil in 50 ml of RM9 + ^{14}C + ^{35}S medium, 2 uCi/ml, which was stirred magnetically for one-hour prior to use in an experiment. From each experiment, duplicate filters were taken with no washing, after washing with 1-1 ml portion of cold RM9, and so on until a complete set of duplicate filters had been washed with from 0 to 5-1 ml portions of RM9 containing unlabeled substrates. All of the filters were then dried and monitored for radioactivity. The results obtained with microbial filters alone are shown in Table 19. The non-biological backgrounds of the filter-sterile soil combinations are shown in Table 20.

c) Summary

The use of a filter prewash solution containing cold formate, lactate, glycine, glucose, and sulfate in

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Table 19

Effect of Wash Regimen Upon Nonbiological Retention of Radioisotope by S&S Bac-T-Flex, C-5, 0.45 u Filters*

No. 1-ml Washes	% of Applied Radioactivity Retained		
	Expt. 1	Expt. 2	Expt. 3
0	1.12	----	1.26
1	0.06	----	0.06
2	0.08	0.06	0.04
3	0.03	0.04	0.04
4	0.04	0.04	0.03
5	0.03	0.04	0.04

*A set of 12 Bac-T-Flex filters was prewashed for each experiment by filtering 1 ml of RM9 containing cold substrates. A 1-ml portion of RM9 + ^{14}C + ^{35}S, 2 uCi/ml, was then passed through each of the 12 filters. Duplicate filters were rinsed with from zero to five 1-ml portions of cold RM9. The filters were then dried and counted to monitor the radioactivity retained. The retention values reported are the averages of duplicates.

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Table 20

Effect of Wash Regimen Upon Nonbiological Retention of Radioisotope by Sterile Soil-Membrane Filter Combinations*

Soil Description	No. of 1-ml washes	% of Applied Radioactivity Retained	
Wooded area soil, Wheaton Regional Park	0	1.15	1.22
	1	0.13	0.11
	2	0.13	0.05
	3	0.08	0.04
	4	0.05	0.03
	5	0.03	0.04
Botanical Garden soil, Wheaton Regional Park	0	1.96	
	1	0.17	
	2	0.09	
	3	0.09	
	4	0.07	
	5	0.05	
Sandy creek bottom soil	0	0.80	
	1	0.10	
	2	0.04	
	3	0.05	
	4	0.03	
	5	0.03	

*A 1.0-g portion of sterile soil was stirred magnetically for one hour in 50 ml of RM9 + ^{14}C + ^{35}S medium, 2 uCi/ml prior to use. A set of 12 S&S, Bac-T-Flex, C-5, 0.45 u filters was prewashed for each experiment by filtering 1-ml portions of RM9 containing unlabeled substrates. A 1-ml portion of labeled medium-soil suspension was then passed through each of the 12 filters. Duplicate filters were rinsed with from zero to five 1-ml portions of cold RM9. The filters were then dried and counted to monitor the retention of radioactivity. The retention values reported are the averages of duplicate determinations.

the basal medium has resulted in a considerable reduction in the nonbiological retention of radioisotopes by membrane filters. The retention by filters, which had been in the range from 0.1 to 0.3% without the prewash, has been lowered to 0.02 to 0.04% with the revised technique. Furthermore, the precision, which had ranged from a coefficient of variation of 40 to 80% without a filter prewash, has been improved slightly by the prewash to a level of 20 to 70%.

A minimum of 3-1 ml washes with RM9 containing unlabeled substrates was required to reduce the nonbiological retention of radioisotopes by S&S Bac-T-Flex, C-5, 0.45 u membrane filters to 0.03 to 0.04% of the applied radioactivity. The addition of soil to the experiment increased nonbiological retention slightly. However, the results obtained with a limited number of soils indicated that 5-1 ml washes were required to reduce the nonbiological background of soil-filter combinations to 0.03 to 0.05%.

A slight, further reduction in the percentage of nonbiological retention by filters was possible by increasing the applied radioactivity. This was done by increasing the volume of labeled medium filtered for the ^{14}C and ^{35}S uptake experiment.

Reductions to a level of 0.02% of the applied radioactivity were achieved by taking a 3 rather than a 1 ml aliquot for filtration.

3. Biological Investigations of the ^{14}C and ^{35}S Uptake Experiment

Biological experimentation was conducted with known inocula of pure cultures to determine the number of microorganisms and the time required to yield a detectable signal. Studies were conducted with a number of viable soils to become more familiar with their properties in this life detection method. Examination for a photosynthetic component of the experiment was made by incubating cultures in the presence and absence of light. Bard-Parker germicide-treated and sterile controls were carried through the assay procedure, along with the viable samples.

a) Biological Studies with Pure Cultures

Biological studies of ^{14}C and ^{35}S uptake were performed using pure cultures of Streptococcus faecalis, Escherichia coli, and Alternaria solani. Uptake by Chlorella sorokiniana was also measured to investigate the photosynthetic nature of the experiment.

These experiments were conducted by inoculating 30 ml of RM9 + ^{14}C + ^{35}S medium, 2 uCi/ml, with a known number of

cells in a 1 ml volume. A separate, identical, flask was set up if photosynthesis was to be studied. In this case, one flask was incubated on a light box under approximately 500-foot candles of fluorescent light. The second flask was wrapped with aluminum foil to exclude light and incubated on the same light box. A control flask was prepared in a similar fashion by adding 1.0 ml of concentrated Bard-Parker germicide to the inoculated medium. All flasks were stirred magnetically throughout the incubation period. Duplicate 1 ml aliquots were removed periodically (2 ml aliquots were taken if phosphate uptake was to be measured simultaneously) to follow the uptake of ^{14}C and ^{35}S. The aliquots were passed through S&S Bac-T-Flex, Type C-5, 0.45 u membrane filters which had been prewet with 1 ml of RM9 containing the unlabeled substrates. The filters were then rinsed four times with 1 ml portions of the cold RM9, dried, and counted.

The results of uptake studies conducted with pure cultures are shown in Table 21. These experiments demonstrate a strong positive response in all cases, as well as the effectiveness of Bard-Parker germicide. The two experiments shown in Table 22 and Figure 16 were conducted with Chlorella sorokiniana to demonstrate the photosynthetic nature of the ^{14}C and ^{35}S

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Table 21

Uptake of ^{14}C and ^{35}S by Pure Cultures*

Microorganism	Incubation Time (hrs.)	% of Applied Radioactivity Retained			
		100 Cell Inoculum		1000 Cell Inoculum	
		Test	Control	Test	Control
E. coli	0.2	0.01	0.02	0.02	0.02
	2	0.02	0.02	0.02	0.02
	4	0.02	0.02	0.02	0.02
	22	1.81	0.03	2.34	0.02
S. faecalis	0.05	0.04	0.02	0.04	0.03
	2	0.06	0.03	0.06	0.03
	4	0.03	0.03	0.03	0.05
	21	15.0	0.03	12.0	0.05
A. solani	0	----	----	0.03	0.04
	1	----	----	0.05	0.02
	3	----	----	0.04	0.03
	5	----	----	0.04	0.03
	24	----	----	6.64	0.04

*30-ml portions of complete RM9, 2 uCi/ml, were inoculated with 1-ml aliquots of cell suspension. Cell densities of the inocula were determined by microscopic chamber counts. Control flasks were prepared by adding 1-ml of concentrated Bard-Parker germicide. All flasks were stirred magnetically and aliquots removed periodically for the assay of ^{14}C and ^{35}S uptake. The procedure for filtration and assay of the aliquots is described in the text. The values reported are the averages of duplicate determinations.

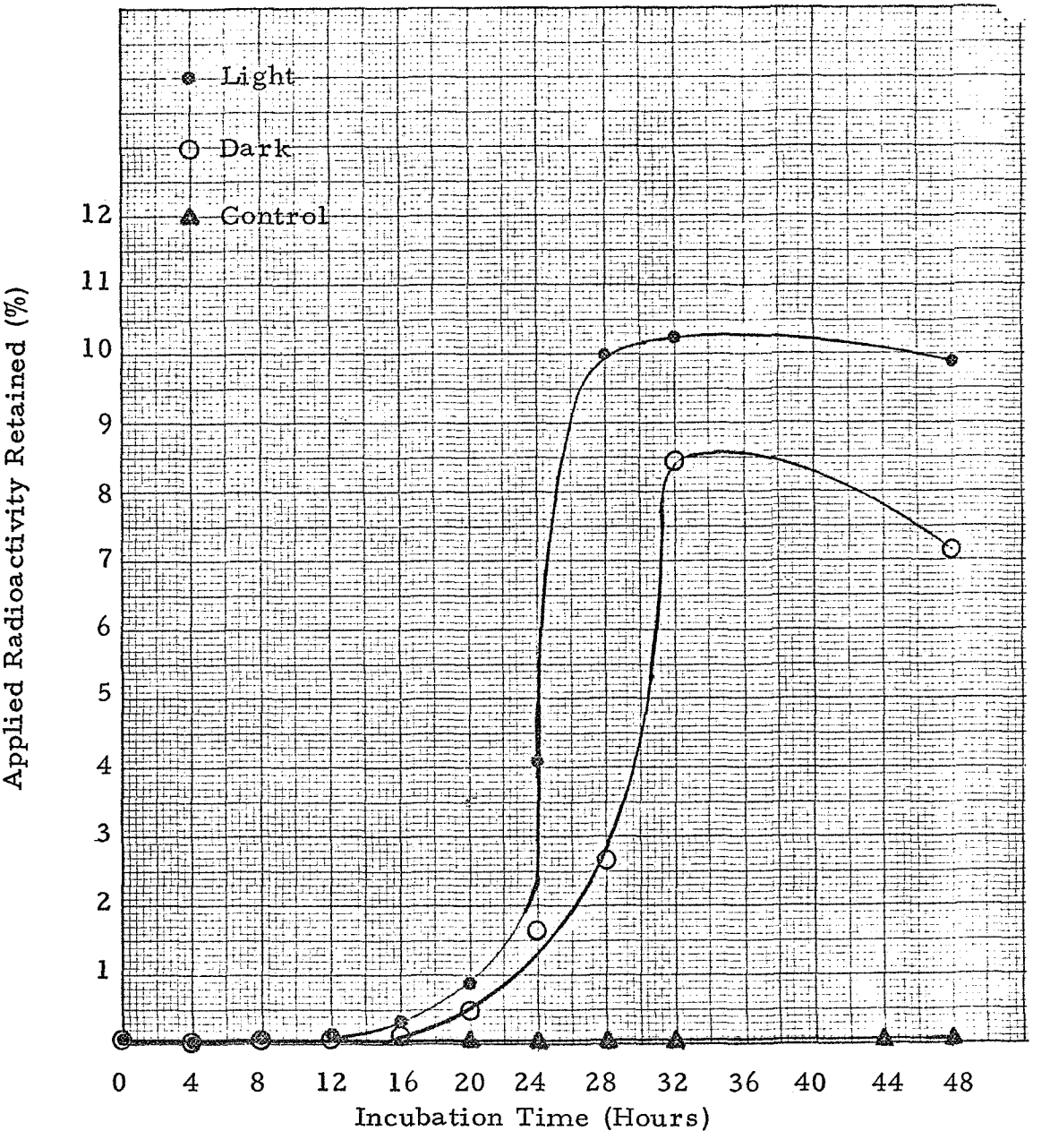
Table 22

Uptake of ^{14}C and ^{35}S by Chlorella sorokiniana*

Incubation Time (hrs.)	% of Applied Radioactivity Retained		
	Light	Dark	Control
0.1	0.04	0.03	0.02
1	0.03	0.03	0.03
2	0.05	0.04	0.03
3	0.04	0.05	0.04
4	0.04	0.03	0.03
5	0.07	0.07	0.05
23	11.7	7.95	0.05

*30-ml portions of complete RM9, 2 uCi/ml, were added to three flasks. The flasks were each inoculated with 1 ml of a suspension of C. sorokiniana containing 2×10^7 cells/ml. One flask was wrapped with aluminum foil to exclude light, and a 1-ml portion of concentrated Bard-Parker was added to a second flask to serve as a control. All three flasks were stirred magnetically on a light box under 500-ft. candles of fluorescent light. Duplicate aliquots were removed periodically from each flask for assay following the procedure described in the text. Values reported are averages of the duplicate determinations.

Kinetics of ^{14}C and ^{35}S Uptake by C. sorokiniana *



* 75 ml portions of complete RM9, 2 uCi/ml, were added to three flasks. The flasks were each inoculated with 1 ml of a suspension of C. sorokiniana containing 2×10^7 cells/ml. One flask was wrapped with aluminum foil to exclude light and a 1 ml portion of concentrated Bard-Parker was added to a second flask to serve as a control. All three flasks were stirred magnetically on a light box under 500-foot candles of fluorescent light. Duplicate aliquots were removed periodically from each flask for assay following the procedure described in the text. Values reported are averages of the duplicate determinations. The applied radioactivity was 8.75×10^5 cpm/ml.

Fig. 16

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uptake experiment. Here again, high positive signals were obtained after an overnight period. The differential between signals from the light and dark incubated flasks clearly served to illustrate photosynthesis.

b) ^{14}C and ^{35}S Uptake by Soils

A number of ^{14}C and ^{35}S uptake experiments were conducted with soils in laboratory experiments simulating volumes and procedures of the AMML instrument. Results of the biological experimentation with soils are shown in Table 23. In these experiments, 30 ml portions of complete RM9, 2 uCi/ml, were inoculated with 0.6 g of the soil sample. The light, dark, and control flasks were treated and assayed in a manner identical to that used for the uptake experiments conducted with C. sorokiniana. In several cases, small biological signals were observed before the overnight period. Generally speaking, however, an overnight incubation period was required to elicit a substantial signal from the viable soils. Here again, as in the case of studies with pure cultures, the Bard-Parker germicide was effective in maintaining the control signals at a constant level which was small, relative to the biological signals obtained after overnight incubation periods. Dark signals higher than the signals from light-

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Table 23

^{14}C and ^{35}S Uptake by Soils*

Soil Description	Incubation Time (hrs.)	% of Applied Radioactivity Retained		
		Light	Dark	Control
Wheaton, Maryland soil**	0.2	0.05	----	0.03
	2	0.14	----	0.07
	4	0.26	----	0.06
	23	1.30	----	0.12
	29	1.11	----	0.06
Sandy creek bottom soil	0.2	0.03	0.03	0.07
	2	0.15	0.03	0.05
	5	0.03	0.05	0.02
	24	1.35	2.56	0.02
	47	1.33	1.45	0.03
Terrarium soil***	0.2	0.08	0.05	0.05
	2.5	0.09	0.03	0.05
	4	0.07	0.04	0.05
	6	0.06	0.06	0.05
	24	2.25	1.80	0.09
Wooded area soil, Wheaton Regional Park	0.2	0.07	0.05	0.05
	2.5	0.11	0.10	0.08
	68	2.10	2.15	0.11
Sandy creek bottom soil	0.2	0.03	0.03	0.05
	2	0.07	0.04	0.04
	20	2.36	3.37	0.05
	26	3.07	4.14	0.04
California soil, TRW	0.1	0.03	0.06	0.03
	3	0.07	0.06	0.05
	5	0.09	0.12	0.06
	23	2.54	3.37	0.06
Wooded area soil, Wheaton Regional Park	0.2	0.11	0.06	0.09
	2	0.26	0.28	0.11
	4	0.32	0.31	0.11
	23	3.76	3.25	0.17

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Table 23
(continued)

^{14}C and ^{35}S Uptake by Soils*

Soil Description	Incubation Time (hrs.)	% of Applied Radioactivity Retained		
		Light	Dark	Control
Wooded area soil, Wheaton Regional Park	0.2	0.09	0.08	0.04
	3	0.37	0.35	0.04
	5	0.50	0.40	0.04
	23	2.72	2.73	0.07
	27	2.63	3.02	0.07
	49	2.73	3.14	0.06
Sandy creek bottom soil	0.1	0.04	0.05	0.05
	2	0.08	0.08	0.09
	4	0.15	0.07	0.08
	22	2.98	3.24	0.11
	28	3.81	3.47	0.10
	45	2.37	2.61	0.11
Terrarium soil***	0.1	0.05	0.04	0.06
	2	0.06	0.07	0.06
	20	2.28	3.21	0.07
	26	2.93	4.23	0.06

*Three flasks containing 30-ml portions of complete RM9, 2 uCi/ml, were inoculated with 0.6 g of soil. One flask was wrapped with aluminum foil to exclude light, and a 1-ml portion of Bard-Parker germicide was added to a second flask to serve as a control. All three flasks were stirred magnetically under 500-ft. candles of fluorescent light on a light box. Duplicate aliquots were removed periodically from each of the flasks. The aliquots were assayed for incorporated radioactivity as described in the text. Values reported are averages of duplicate determinations.

**The light flask in this experiment was incubated at 50- foot candles. No dark flask was run.

***This soil was stored in the laboratory and watered periodically.

incubated flasks were frequently obtained, and photosynthesis in soil samples could not be detected in a reliable fashion. Further studies are required to investigate this phenomenon which may be related to nonhomogeneous samples, temperature effects, or other unknown causes.

c) Precision of the ^{14}C and ^{35}S Uptake Experiment

As mentioned previously, the precision of the ^{14}C and ^{35}S uptake experiment at low signal levels near the nonbiological background of 0.03 to 0.05% is about 20 to 70% (coefficient of variation). An experiment conducted to measure the precision at higher signal levels is shown in Table 24. This experiment was conducted without the filter prewash technique. However, the biological signals measured were high enough to render the nonbiological background level insignificant. The experiment was conducted by filtering replicate aliquots of a cell suspension which had taken up considerable quantities of radioactive carbon and sulfur. The overall coefficient of variation was 5% at this uptake level.

D. Firefly Bioluminescent Assay

1. Introduction

A study of the effects of changing concentrations and volume ratios of reagents upon the light output from the

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Table 24

Precision of ^{14}C and ^{35}S Uptake
Experiment at Higher Signal Levels

	^{14}C and ^{35}S Uptake (cpm)			
	22 Hours	23 Hours	24 Hours	26 Hours
	21371	23456	26281	30375
	22062	24308	27869	31127
	23278	25720	28198	32467
	20918	24696	28375	33305
	19381	24956	28448	30871
	23307	23146	28179	32485
$\bar{x}$ =	21720	24380	27892	31772

Assay conditions: 30 ml of RM9 medium containing 2 uCi/ml of ^{14}C and ^{35}S substrates was inoculated with E. coli and allowed to incubate, with stirring, at 23°C. Six replicate 1-ml aliquots were removed and filtered through Acropor AN-450 filters at the indicated intervals. Filters were washed twice with 2-ml portions of unlabeled RM9, dried, and then counted. The overall coefficient of variation was found to be five percent.

firefly bioluminescent reaction has been completed during this contract period. Some of the data presented in this section were obtained under Contract No. NAS5-10464 with the Goddard Space Flight Center of the National Aeronautics and Space Administration (1). The purpose of this experimentation was to determine satisfactory ranges for the volumes of enzyme and extractant to be used in the AMML instrument and to investigate some unusually rapid bioluminescent reactions occasionally observed in past studies.

2. Discussion

The first case to be considered is that of the well-known "standard curve" for ATP. In this instance, fixed volumes of ATP solutions of varying concentrations are injected into fixed volumes of enzyme. For each particular volume ratio (volume ratio is defined as the volume of enzyme solution divided by the volume of ATP solution injected), a straight-line relationship exists between response and ATP concentration. This linear relationship holds over many orders of magnitude from the lower limit of detection to the saturation level of the photomultiplier tube, or to the point at which enzyme concentration starts to become limiting.

The second case to be considered is one in which the volume ratio and concentrations of reagents remain constant while the total volumes of enzyme and ATP solutions are varied. An idealized plot of such an experiment is shown in Figure 17. This experiment, performed under Contract No. NAS5-10464, shows a linear increase in output as the volumes are increased. This relationship will hold until the geometry of the system causes a variation in the percentage of emitted light reaching the photomultiplier tube.

A third situation is one in which a fixed weight of ATP is injected at a fixed volume ratio into varying volumes of enzyme solution; that is, the concentration of ATP solution decreases as the volumes of ATP solution and enzyme solution increase. A plot of this type of experiment is shown in Figure 18. It shows a gradual increase in response as the volumes increase. This increased response is due to an increasing percentage excess of enzyme as the volumes grow larger.

An idealized curve of the next case to be discussed is shown in Figure 19. This experiment was performed under Contract No. NAS5-10464 and involved varying the volume ratio by adding a fixed weight of ATP, in varying volumes, to a fixed

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Relationship of Volume of Reactants to Response from Firefly Bioluminescent Reaction

$$\text{Volume Ratio} = \frac{\text{Volume Enzyme Solution}}{\text{Volume ATP Injected}} = 10$$

Conc. ATP Solution = 10^{-1} ug ATP/ml

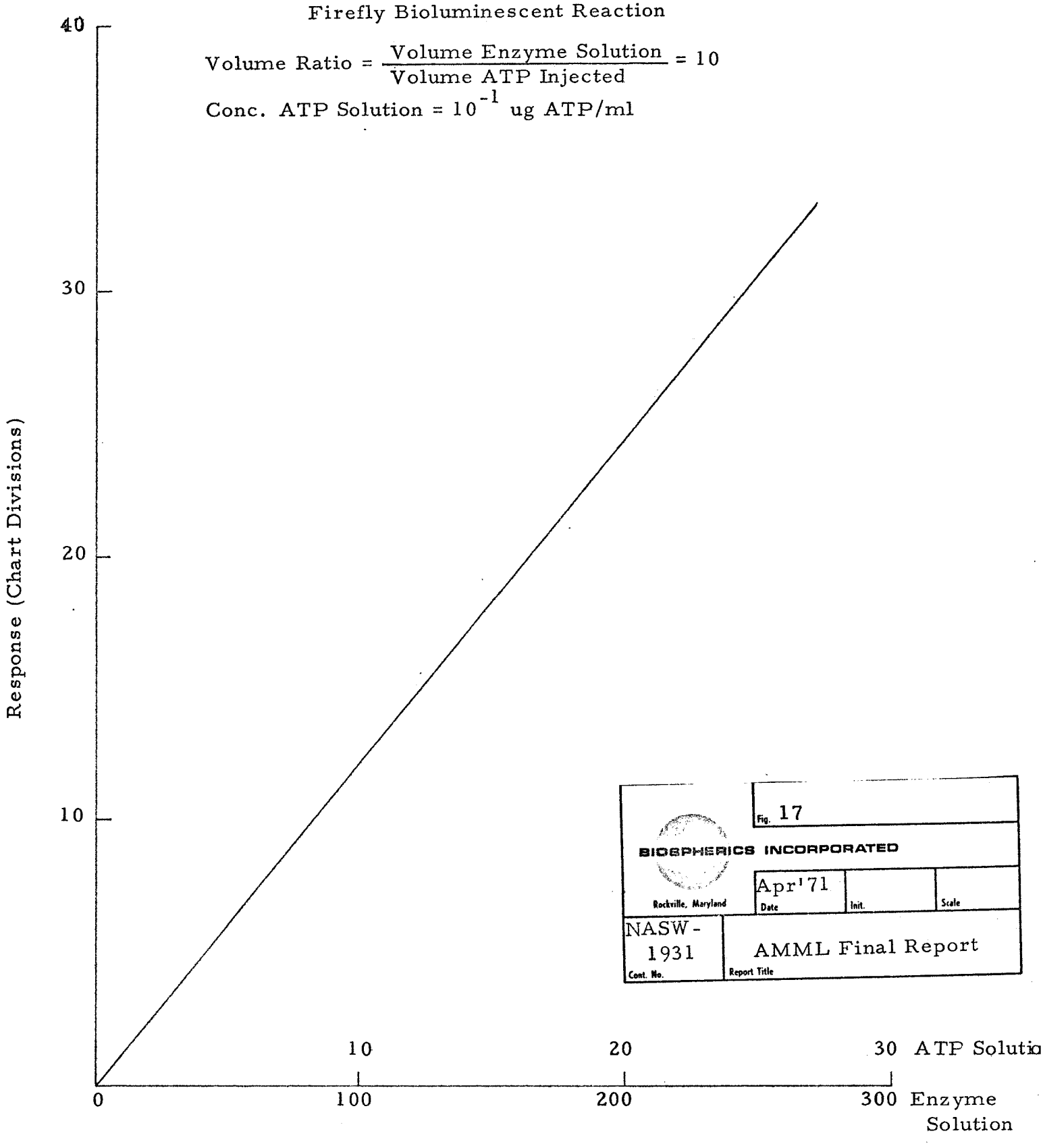

Fig. 17

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NASW-1931 (Cont. No.) | AMML Final Report (Report Title)

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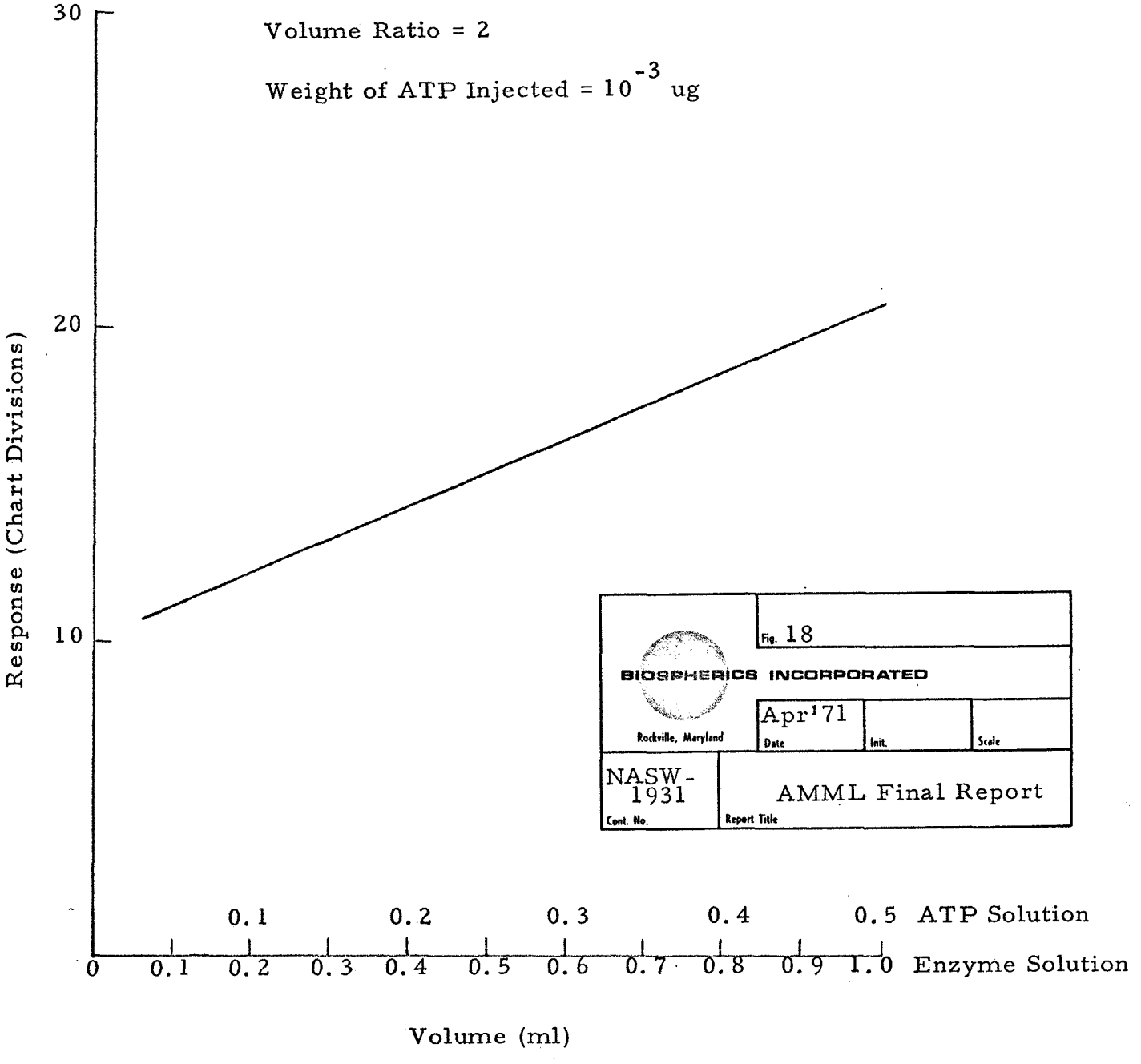
Relationship of Response from Fixed Weight of ATP to Volume of Reactants



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Relationship of Volume Ratio to Response from Fixed Weight of ATP

Enzyme Solution Volume = 0.10 ml

Weight ATP Injected = 10^{-3} ug

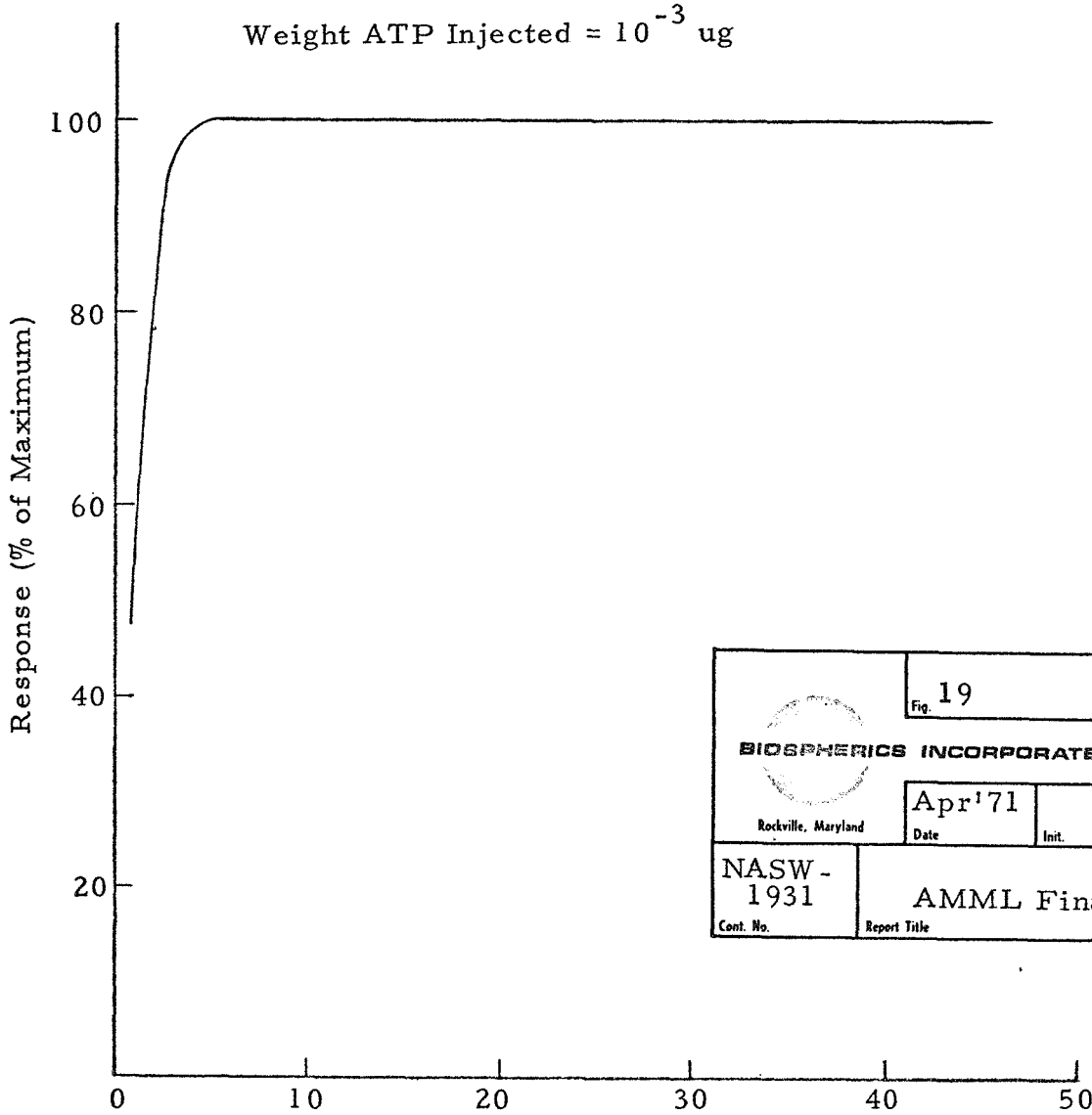

Fig. 19

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volume of enzyme solution. The figure showed that the response for a given weight of ATP was constant down to a volume ratio of about three, and then decreased rapidly below this point. The volume ratio which has been selected for use in the AMML is two and, as is evident from Figure 19, it is extremely important that lower ratios not be used.

The last case to be considered is one in which increasing volumes of a fixed concentration of ATP solution were added to a fixed volume of enzyme. The results of several of these experiments, performed under the present program, are shown in Figures 20, 21, and 22. The figures show a slight negative curvature as the amount of added ATP is increased. This bending reflects the negative effects of a decreased volume ratio upon response. Several of these experiments were conducted at higher ATP volumes and concentrations and lower enzyme concentrations than those normally employed in the laboratory. The purpose for this was to examine for unusually rapid decline in the bioluminescent decay curves under these exaggerated conditions. Extremely rapid responses followed by almost equally rapid decay had been observed from time to time in the past during operation of the automated instruments. This

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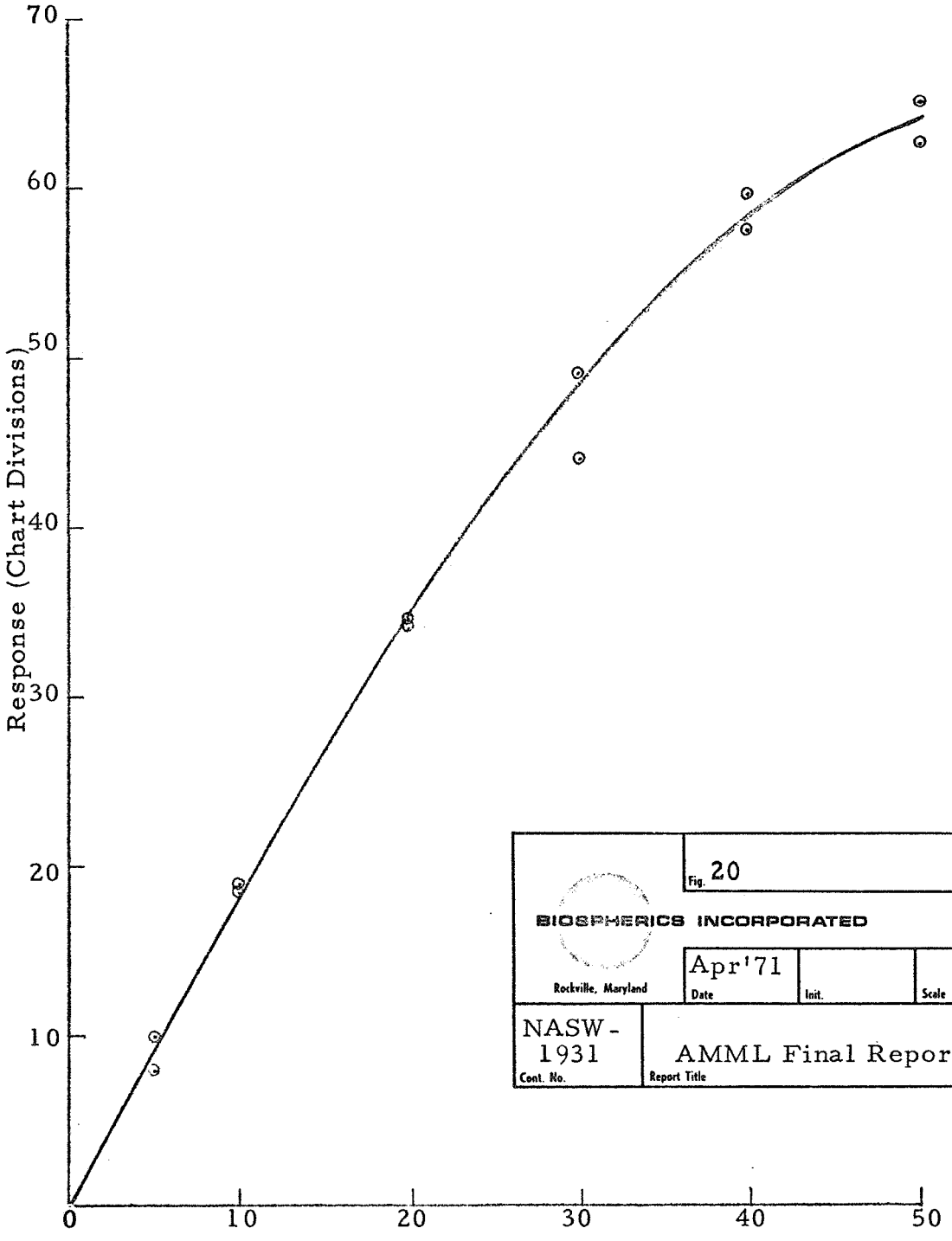
Relationship of Response to Volume of 0.10 ug ATP/ml Solution Injected

Volume of Enzyme Solution = 0.10 ml



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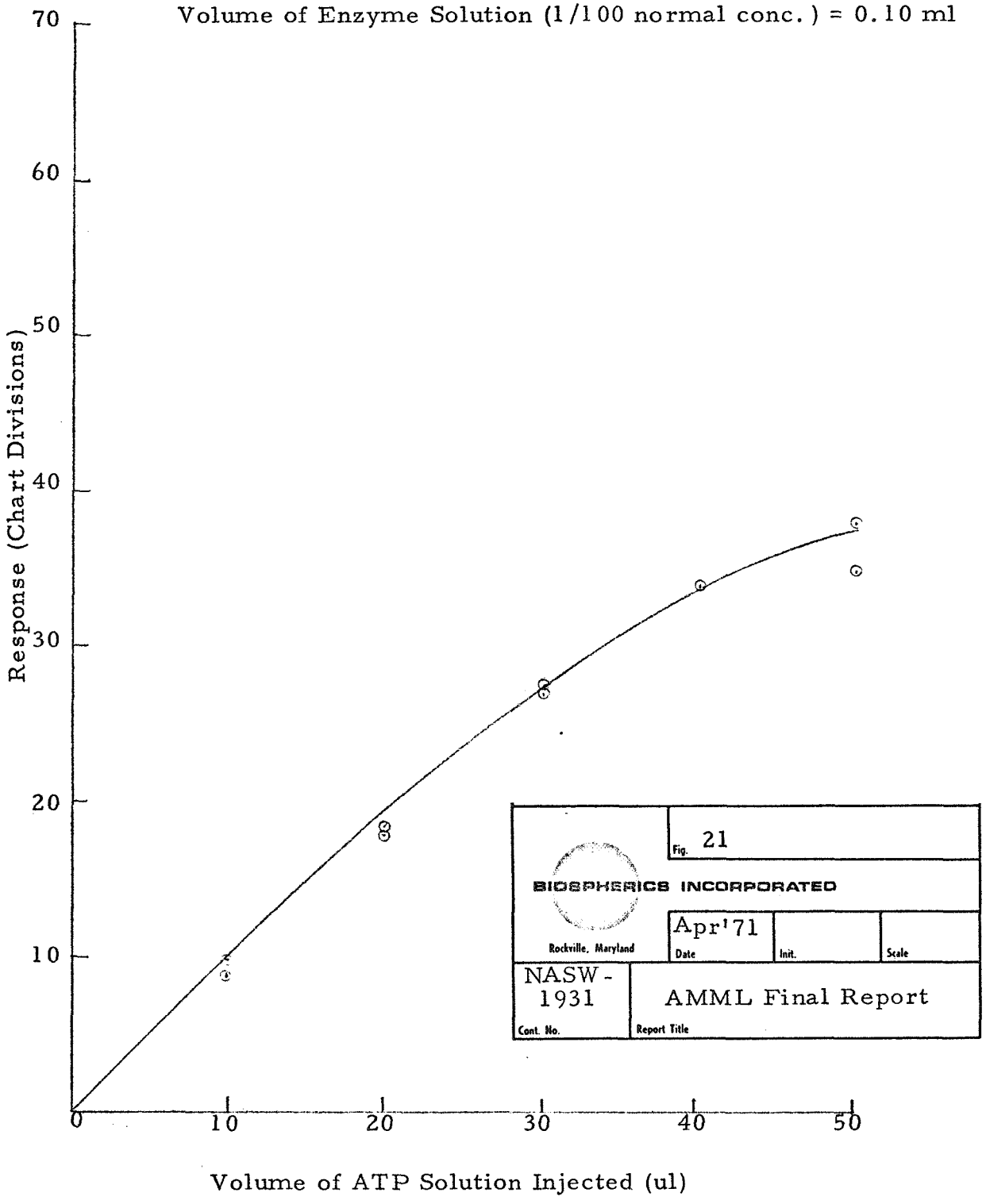
Relationship of Response to Volume of 10 ug ATP/ml Solution Injected



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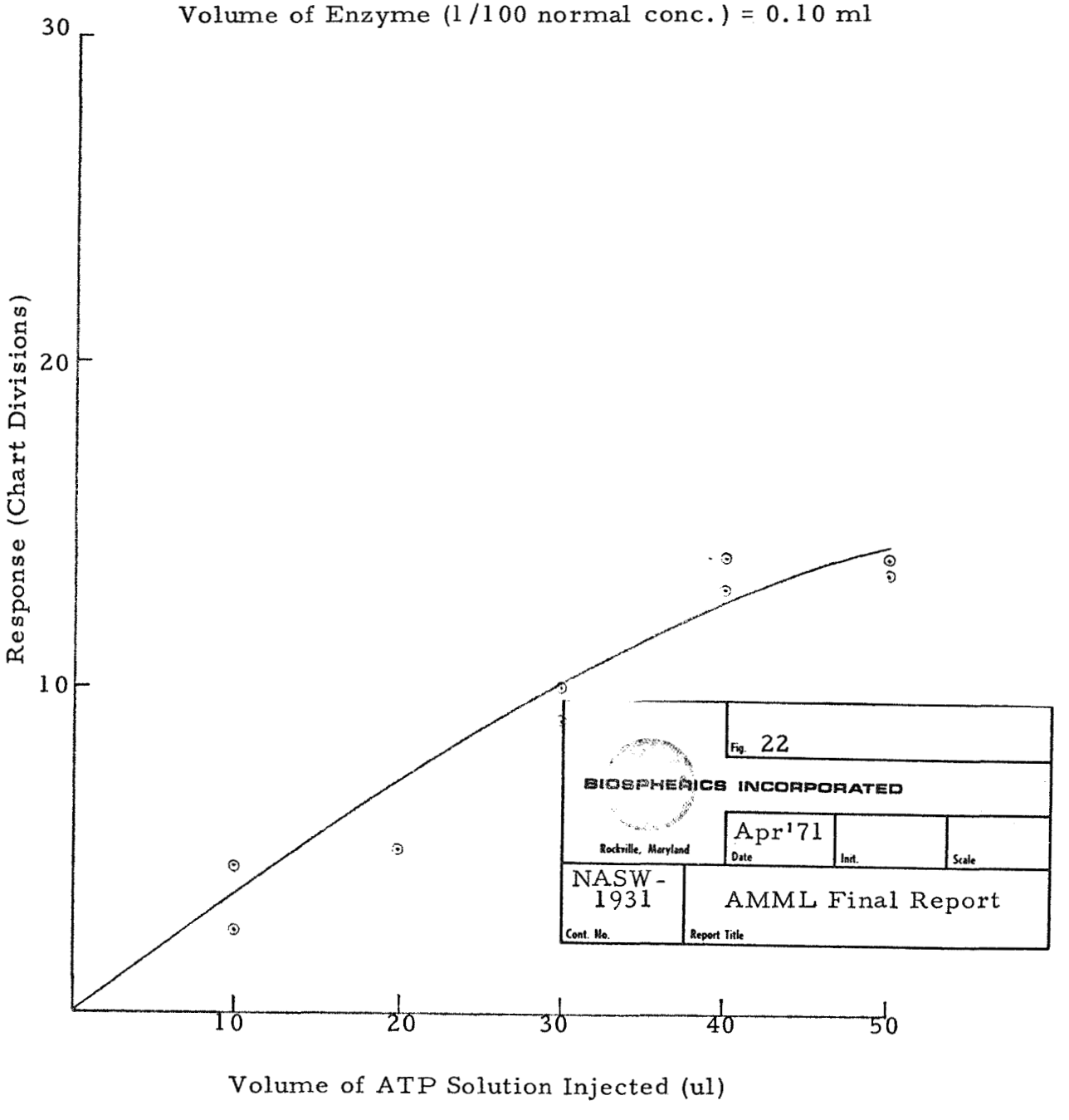
Relationship of Response to Volume of 1.0 ug ATP/ml
Solution Injected into Diluted Enzyme



phenomenon has been observed on the AMML instrument and on the instrument built for the Goddard Space Flight Center under Contract No. NAS5-10464. Examination of the rates of response and decay from the individual injections performed in the laboratory revealed no evident alterations in the slopes of curves obtained. Explanations involving properties of the different photomultipliers used or response time of the individual recorders have been ruled out as sources of these unusually shaped response curves obtained with the automated instruments. No satisfactory explanation has been found for the unusually rapid reaction occasionally observed. Present thinking is that the phenomenon may be related to the viscosity of the enzyme solution.

E. Phosphate Uptake Assay

1. Colorimetric Determination of Orthophosphate

a) Introduction

Research conducted under the previous program, NASW-1731, has shown serious positive interferences from soil to the determination of phosphate using the ^{14}C-radioisotopic modification of the procedure of Sugino and Miyoshi (4). The molybdenum blue colorimetric procedure (5) was found to give recoveries of 90% or better on soil extracts up to

a concentration of 0.3 mg/l PO_4-P. Recoveries fell off rather sharply, in some cases, at higher PO_4-P levels. Since some measure of success had been attained with the colorimetric procedure, further research was initiated under the present program in attempts to apply this technique to soil extracts.

The phosphate uptake life detection test does not require high analytical accuracy, as the method is based upon the measure of concentration differences. Precision, however, is very important, as differences in phosphate levels as low as 0.2 mg/l must be detectable. This means that recovery levels as low as 80% to 90% would be acceptable if the precision of the measurements were satisfactory. For this reason, attempts to improve recoveries in the analytical method by addition of steps which would greatly increase the complexity of automation were not made at this point in the program. For example, addition of bromine to remove interferences by organic materials extracted from soil and solvent extraction of molybdophosphoric acid to separate interferences were not studied initially. Rather, the addition of a cationic ion exchange resin into the analytical method was investigated, as this step can be introduced into the automated instrument with little complexity.

b) Determination of Phosphate in RM9 and in Soil Extracts

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In preparation for the analysis of soil extracts from phosphate uptake experiments, a colorimetric procedure was set up which required only 5 ml of sample. The procedure was performed as follows:

Reagents:

1. Ammonium Molybdate Solution - a 1% aqueous solution of $(NH_4)_6Mo_7O_{24}\cdot 4H_2O$ is diluted with three volumes of 5% sulfuric acid.
2. Stannous Chloride Solution - 40 mg of $SnCl_2\cdot 2H_2O$ is dissolved in 100 ml of 1% hydrochloric acid (prepared fresh daily).

Procedure:

1. Add 5.0 ml of sample or standard to a 10 ml Erlenmeyer flask.
2. Add 1.0 ml of ammonium molybdate reagent and mix thoroughly.
3. Immediately add 1.0 ml of stannous chloride reagent and mix.
4. After 12 to 15 minutes at room temperature, read at 690 nm in a 13 mm cell against distilled water.

Several typical standard curves are shown in Figure 23. The

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Standard Curves for the Colorimetric Determination of PO_4-P

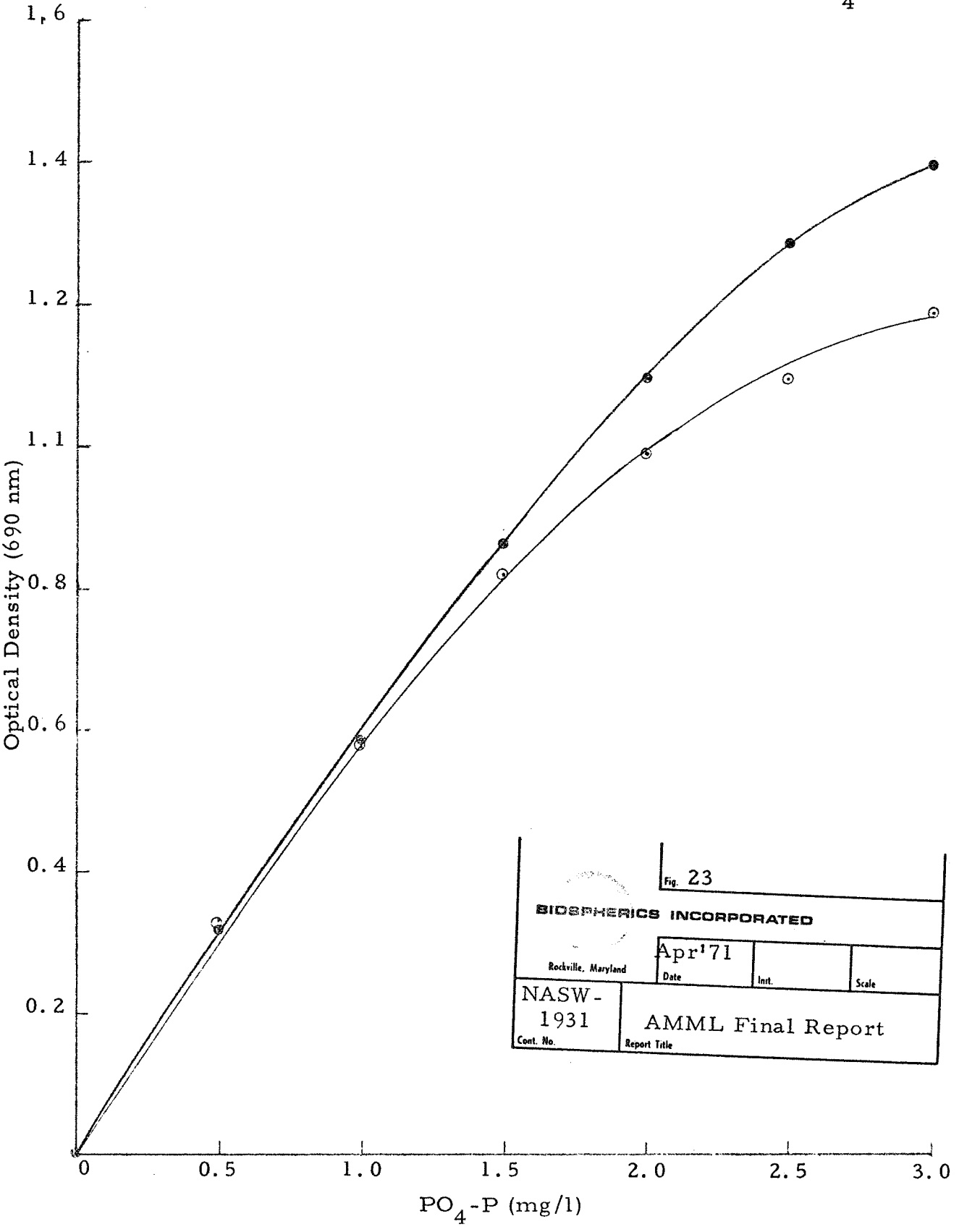

Fig. 23

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slope of the curve begins to drop off markedly at higher concentrations, but was found to be satisfactory in the range of interest from 0 to about 1.5 mg/l PO_4-P.

The initial experiments on this phase of the program consisted of recovery studies performed in RM9 medium. RM9 medium is prepared with 100 ml of soil extract per liter (see Table 15), and was examined to see just how well the procedure described above could handle medium alone. Complete RM9 medium was prepared from three different soil extract solutions. The three media were prepared to contain only unlabeled substrates. Increasing dilutions of each of the media were assayed for orthophosphate; the results were plotted as shown in Figure 24 and extrapolated to infinite dilution; and recoveries were calculated, based upon the "Y" intercept. In addition to recoveries of 79% and 79% obtained on the first extract mentioned in Figure 24, recoveries of 92% and 91% were obtained on RM9 containing two other soil extracts. These results demonstrate that RM9 medium alone can be assayed fairly well at levels up to 1 mg/l PO_4-P with no further modifications in the colorimetric procedure.

Additional recovery checks were made on test extracts prepared by inoculation of 30 ml of RM9 medium with 1.0 g

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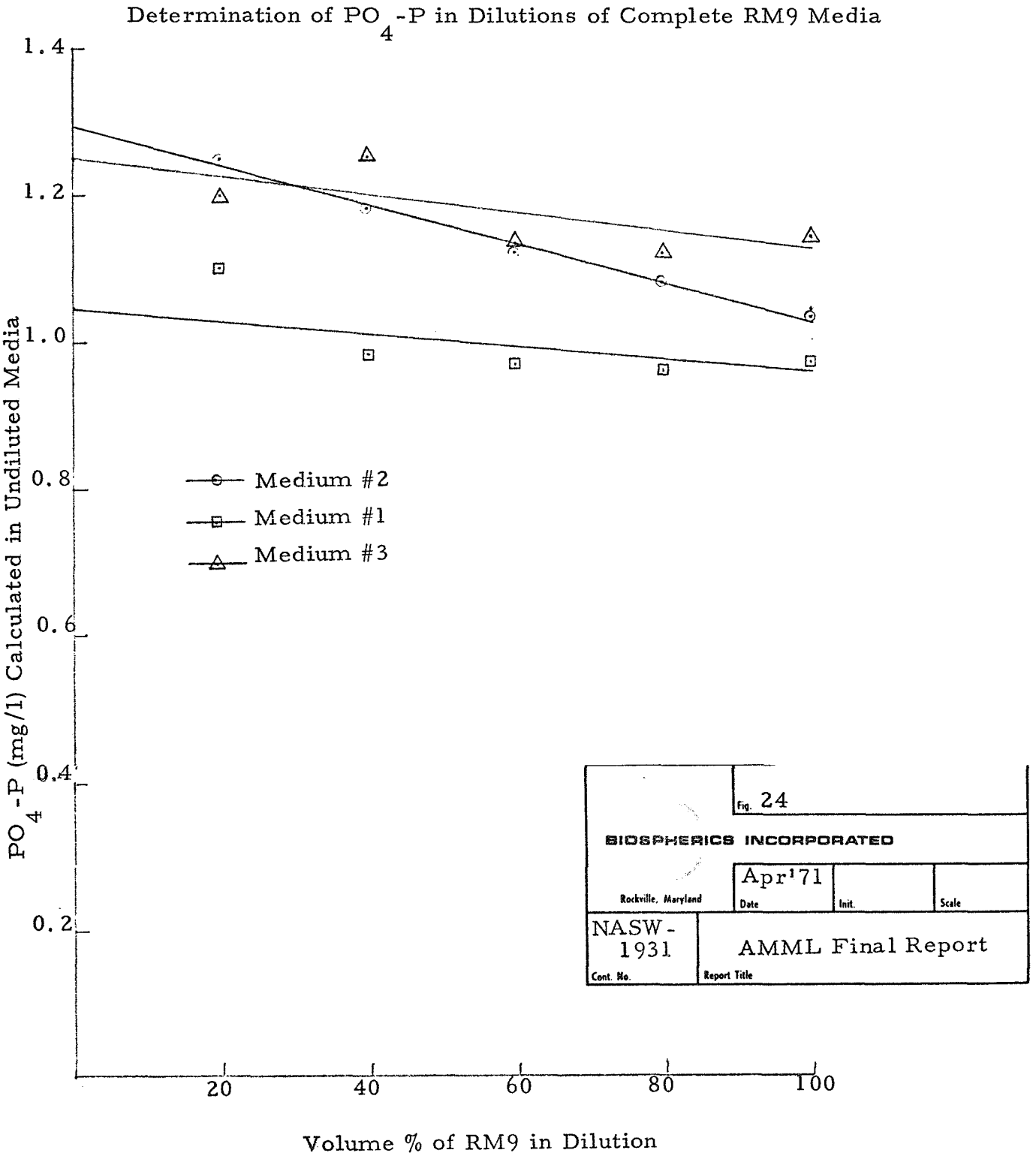

portions of various autoclaved soils. The suspensions were allowed to stir for 24 hours, filtered through 0.45 u membrane filters, and then assayed for added phosphate. Recoveries obtained on these test extracts are shown in Table 25. Although the majority of recoveries from the test soil extracts were satisfactory, there are a few instances in which poor results were obtained. As noted in previous studies, recoveries tend to drop off with increasing phosphate concentration. Recoveries lower than 80% were obtained at phosphate concentrations greater than 0.6 mg/l with several test soil extracts.

c) Analysis of Soil Extracts Using Ion Exchange Resins

Two cationic ion exchange resins were studied for their ability to remove interferences to the molybdenum blue colorimetric procedure for phosphate. The first of these was Amberlite IR-120, a strongly acidic, sulfonated polystyrene-type resin. The other resin studied was Chelex 100, a styrene-divinylbenzene copolymer matrix having iminodiacetic acid exchange groups. Chelex 100 is a chelating ion exchange resin which shows high preferences for heavy metals over such cations as sodium, potassium, and calcium.

Table 25

Recoveries of Added PO_4-P from Test Soil Extracts

Soil	PO_4-P Added (mg/l)	PO_4-P Found (mg/l)	Recovery* (%)
California soil, TRW	0.0	0.75	---
	0.2	0.88	93
	0.4	1.21	105
	0.6	1.33	99
	0.8	1.43	92
	1.0	1.53	88
	0.0	1.17	---
	1.0	1.79	83
	0.0	1.20	---
	1.0	1.82	83
	0.0	0.62	---
	0.96	1.17	74
Wheaton, Maryland soil	0.0	0.23	---
	0.2	0.42	98
	0.6	0.82	99
	1.0	1.21	99
Florida soil	0.0	0.72	---
	0.2	0.91	99
	0.6	1.23	93
	1.0	1.53	89
Terrarium soil	0.0	0.13	---
	0.2	0.32	97
	0.4	0.51	96
	0.6	0.71	97
	0.8	0.92	99
	1.0	1.04	92

Table 25
(continued)

Recoveries of Added PO_4-P from Test Soil Extracts

Soil	PO_4-P Added (mg/l)	PO_4-P Found (mg/l)	Recovery* (%)
Botanical Garden soil	0.0	1.37	---
Wheaton Regional Park	0.2	1.43	91
	0.6	1.53	78
	1.0	1.67	71
Wooded area soil,	0.0	0.14	---
Wheaton Regional Park	0.2	0.29	85
	0.6	0.56	76
	1.0	0.77	68

Assay Conditions: Complete RM9 medium containing unlabeled substrates was inoculated with autoclaved soils, 1 g of soil per 30 ml of medium, and allowed to stir overnight. Autoclaved soils were not sterile in every case and the RM9 medium was prepared with no added phosphate in some experiments. Soil suspensions were centrifuged and then filtered through 0.45 u membrane filters. Soil extracts were spiked by adding 1-ml portions of phosphate standards to 25 ml of extract. These solutions were then analyzed by the colorimetric procedure described previously.

*Recoveries are based on the total amount of phosphate present; that is, the amount added plus the amount found in the unspiked extract.

The initial ion exchange experiments were conducted by mixing Amberlite IR-120, in the hydrogen form, with phosphate standard solutions and comparing the resultant optical densities with untreated standards after adding the colorimetric reagents. Optical densities considerably lower than the untreated phosphate standards were obtained upon treatment of 10 ml of standard solution with 2 g and 4 g of ion exchange resin. An acid wash, followed by repeated water washes of the Amberlite failed to raise the optical densities to an acceptable level. The Amberlite was converted to the sodium form by being washed four times with a saturated sodium chloride solution. The resin was then rinsed several times with distilled water, and a phosphate standard curve was prepared from untreated standards, standards treated with 4 g/10 ml of Amberlite IR-120 (Na), and standards treated with 4 g/10 ml of Chelex 100 (Na). The results of this experiment are shown in Figure 25. Recoveries were run at the same time on RM9 medium containing known levels of phosphate. Recoveries from the RM9 were calculated from the individual standard curves shown in Figure 25 for each treatment. The results are presented in Table 26. Although the standard curve prepared with Amberlite was closer to the curve from the untreated samples, Chelex 100 gave considerably higher recoveries.

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Effect of Ion Exchange Resins upon Optical Densities from Phosphate Standards

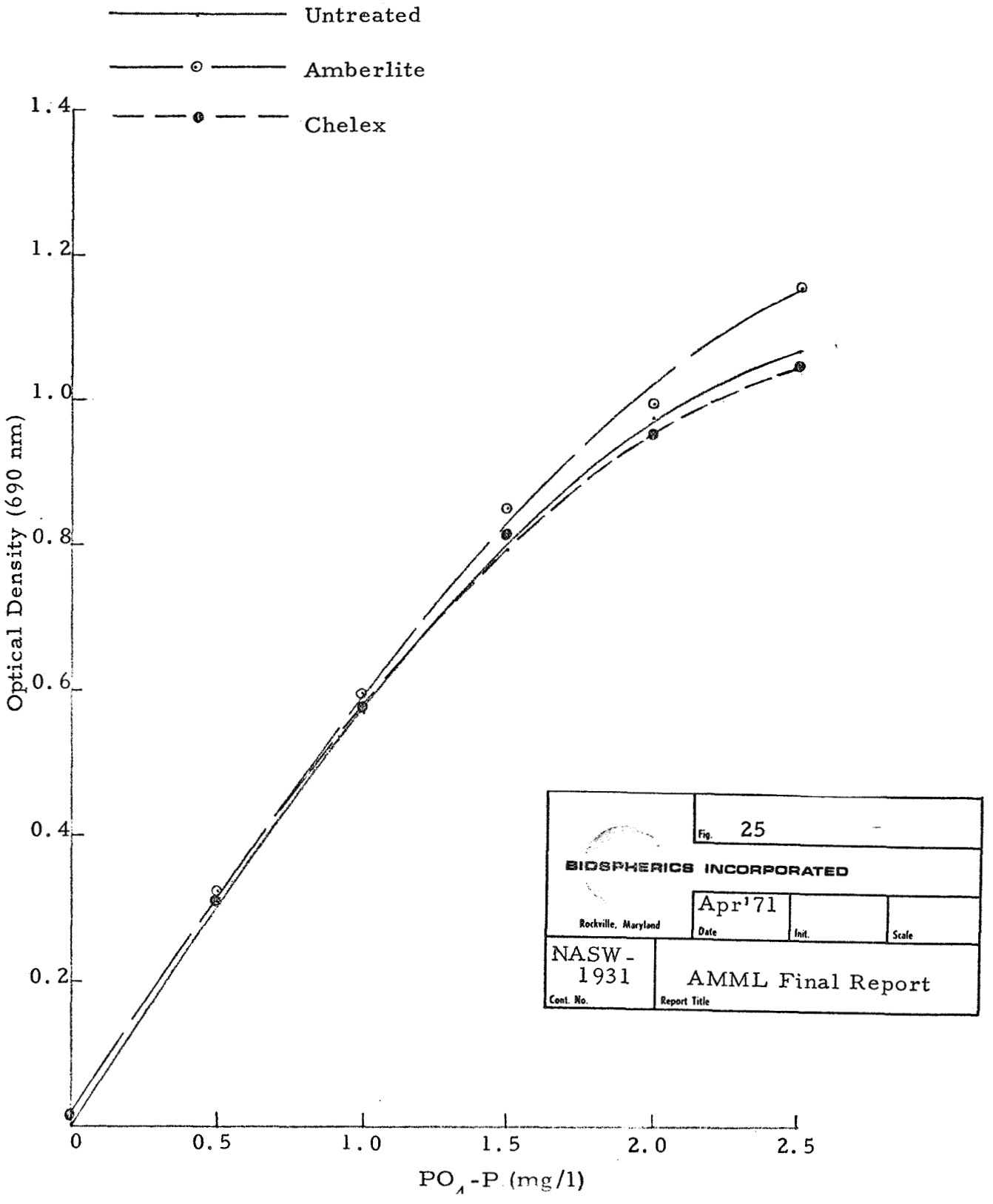

Fig. 25	
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Table 26

Phosphate Recoveries from RM9 Treated with Ion Exchange Resins *

PO_4-P Added (mg/l)	No Treatment		Amberlite (Na)		Chelex (Na)	
	Conc. (mg/l)	% **	Conc. (mg/l)	% **	Conc. (mg/l)	% **
0.0	0.13	---	0.14	---	0.13	----
0.5	0.64	102	0.60	94	0.64	101
1.0	1.11	98	1.02	89	1.16	103
1.5	1.64	101	1.32	81	1.75	107

Assay Conditions: Concentrated phosphate standard solutions were added to RM9 at the indicated levels and assayed colorimetrically in the usual manner, and after treatment with 4 g of Amberlite IR-120 (Na) or Chelex 100 (Na) per 10 ml of standard. The standards were stirred for 15 minutes with the resins prior to colorimetric assay of the supernatants.

*The pH values of the sample solutions in equilibrium with the ion exchange resins were unknown in this experiment.

**Recoveries are based upon the total amount of phosphate present; that is, the amount added plus the amount found in the unspiked extract.

Next, six groups of three recovery experiments were conducted using treatment with 0, 2, and 4 g of Chelex 100 (Na) per 10 ml of test soil extract. Recoveries in the 1.3 to 2.0 mg/l PO_4-P range were from 74% to 82% for no treatment, 80% to 104% for treatment with 2 g/10 ml, and from 84% to 108% for treatment with 4 g/10 ml.

These initial results looked very promising; however, some discrepancies appeared in subsequent experimentation. A few recovery values were obtained which were significantly higher than 100%. In addition, considerable scatter in the standard curves was noted from time to time with the ion exchange resin (Na)-treated solutions. This effect seemed to be more pronounced at higher phosphate levels and in the standards treated with 4 g/10 ml. The experiment described in Table 26 was repeated using Chelex 100 (Na) at a level of 2 g of resin per 10 ml of phosphate solution. Similarly, satisfactory results were again observed with recoveries from RM9 ranging from 94% to 99% and a standard curve very close to the curve from untreated standards. In an attempt to identify the cause of these erratic results, standard curves were constructed in the range from 0 to 2.0 mg/l PO_4-P with untreated standards and with standards

treated with Chelex 100 (Na) at levels of 2 and 4 g/10 ml. The Chelex treated solutions this time gave significantly higher optical densities than untreated solutions and, in addition, gave a greater number of spurious points. The pH's of the treated solutions were measured and found to be about 11. Thus, the cause for the erratic results was due to variations in sample pH. A standard curve constructed from an acid-washed Chelex 100, which yielded solutions having pH's around 3, gave significantly lower optical densities than the untreated standards. Both the Chelex 100 and the Amberlite IR-120 resins were converted to the sodium form by washing them with several portions of a saturated sodium chloride solution. This was followed by several distilled water rinses and adjustment of the supernatant with 0.5 N HCl to a pH of 6 to 7 in equilibrium with the resins. These resins then gave standard curves close to the untreated standards when tested at a level of 2 g or 4 g/10 ml of phosphate standard. In addition, no spurious points were obtained using the pH adjusted resins.

Recovery experiments were performed using these ion exchange resins on test extracts prepared by inoculation of 30 ml of RM9 medium with 1.0 g portions of various autoclaved

soils. As described previously, the suspensions were stirred for 24 hours and then filtered through 0.45 u membrane filters. Results of the experiments are presented in Table 27. It is readily apparent from these recovery values that the addition of either cationic ion exchange resin into the analytical procedure fails to significantly improve the colorimetric assay. It was concluded from these studies that the metallic ions were not responsible for low orthophosphate recoveries from test soil extracts in RM9.

Phosphate recovery checks were conducted on several test soil extracts which had been digested with $HClO_4$ to destroy all organic matter. Essentially, quantitative phosphate recoveries were obtained. This demonstrated conclusively that positive ions were not the principal cause for low recoveries, and suggested that organics in the test extracts may be responsible for the interference.

Introduction of activated charcoal into the assay to remove organic compounds from the test soil extracts failed to solve the problem of low recoveries. Low, erratic results were obtained with phosphate standards and test soil extracts treated with 2 g of charcoal/10 ml of solution.

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Table 27

Phosphate Recoveries from RM9 Soil
Extracts Treated with Ion Exchange Resins*

Soil	Ion Exchange Resin	PO_4-P Added (mg/l)	PO_4-P Recovered** (%)
Combined soil #1	None	0.97	100
	Chelex, 2g/10ml	1.01	92
California soil, TRW	None	0.95	95
	Amberlite, 2g/10ml	1.02	97
Wooded area soil, Wheaton Regional Park	None	0.95	87
	Amberlite, 2g/10ml	1.02	87
Wooded area soil, Wheaton Regional Park	None	1.05	81
	Chelex, 0.5g/10ml	1.05	71
	Amberlite, 0.5g/10ml	1.05	71
Combined soil #2	None	0.99	55
	Chelex, 2g/10ml	0.99	52
	Amberlite, 2g/10ml	0.99	55
California Soil	None	1.02	31
	Amberlite, 2g/10ml	1.02	33

Assay Conditions: Complete RM9 medium containing unlabeled substrates was inoculated with autoclaved soils, 1 g of soil per 30 ml of medium, and allowed to stir overnight. Soil suspensions were centrifuged and then filtered through 0.45 u membrane filters. Soil extracts were spiked by adding 1-ml portions of phosphate standards to 25 ml of extract. These solutions were then analyzed by the colorimetric procedure described previously.

*Amberlite IR-120 and Chelex 100 cationic ion exchange resins were converted to the sodium form by washing several times with a saturated sodium chloride solution. The resins were then rinsed twice with distilled water. The pH of the supernatant in equilibrium with the resin was adjusted to from 6 to 7 with 0.5 N HCl.

**Recoveries are based upon the amount of phosphate added.

d) Miscellaneous Experiments

Retention by filter materials is known to be a problem when solutions of low phosphate concentrations are passed through paper. The retention of phosphate by five different filters has been shown to be negligible, and these materials are satisfactory for use in the phosphate uptake life detection test. The filters studied were Acropor AN-450, Metricel VF-6, Bac-T-Flex C-5, Millipore HAWP, and Gelman Type E Glass Fiber.

Both Bard-Parker and Cidex germicide solutions were found to interfere with the colorimetric assay for phosphate. The effects of the germicide solutions were studied at a concentration of 1 ml per 30 ml of solution, which is about the level proposed for the AMML experiments. Bard-Parker solution caused extremely low recoveries, and Cidex solutions analyzed as if they contained several hundred ppm of PO_4-P. The addition of an ion exchange resin to the solutions failed to improve the analyses.

Optical densities as high as 0.05 were obtained on blank solutions treated with Chelex 100 and Amberlite IR-120. This blank value was lowered to about 0.01, using several different

resin regeneration and wash solutions. However, it has not been possible to completely eliminate this blank value. The blank absorbancy was studied as a function of the amount of Chelex used, but did not increase significantly as the weight of resin used was increased.

e) Summary

Test soil extracts prepared in RM9 and containing 1 mg/l PO_4-P could not be analyzed successfully by the molybdenum blue colorimetric procedure. Low recoveries were obtained, probably due to the presence of interferring organic matter in solution. Positive ions were not responsible for the low recoveries, and introduction of cationic ion exchange resins into the assay did not significantly improve the assay. Attempts to improve phosphate recoveries through the use of activated charcoal were not successful. The colorimetric procedure can be used for the biological experiments; however, sensitivity will suffer, due to the low recoveries frequently obtained. Quantitative phosphate recoveries were obtained from test soil extracts following digestion with $HClO_4$. The assay cannot be conducted successfully in the presence of either Cidex or Bard-Parker germicide solutions.

2. Biological Uptake of Orthophosphate

Phosphate uptake studies were conducted with bacteria and with soil microorganisms. Examination for a photosynthetic component of the phosphate uptake was made in a number of test soil samples. The molybdenum blue colorimetric procedure for orthophosphate was used to follow the biological uptake, despite the analytical limitations described in the previous section of this report.

a) Experimental

Uptake of phosphate from RM9 by Escherichia coli was studied. One ml portions of inocula containing 100 and 1000 cells were added to separate 30 ml portions of RM9 in covered 50 ml Erlenmeyer flasks. Chamber counts were used to determine dilution factors for the inocula. The RM9 was stirred magnetically and taken for filtration and assay at selected intervals throughout the course of the experiment. Two ml aliquots of the RM9 suspension were filtered through 0.45 u membrane filters and one ml of the filtrates was assayed for phosphate by the molybdenum blue colorimetric procedure. The results of this experiment are shown in Table 28. Considerable phosphate uptake was obtained from both flasks after the overnight incubation period.

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Table 28

Phosphate Uptake by Escherichia coli

Incubation Period (Hrs.)	Dissolved PO_4-P (mg/l)	
	100 Cell Inoculum	1000 Cell Inoculum
0.2	3.4	4.1
2	3.4	4.1
4	3.5	4.2
24	1.8	2.3

Assay Conditions: Separate 30-ml portions of RM9 in 50-ml flasks were inoculated with 100 or 1000 cells. The flasks were covered and stirred magnetically throughout the incubation period. Periodically, 2-ml portions of the suspensions were removed and filtered through 0.45 u membrane filters. One-ml aliquots of the filtrates were analyzed for phosphate by the molybdenum blue colorimetric method.

Biological experimentation was conducted with soils in much the same manner. One gram portions of the soils were used to inoculate 30 ml portions of RM9, and soils which had been autoclaved repeatedly were used as controls. A series of experiments was performed in which the photosynthetic nature of phosphate uptake by soils was examined. In these experiments, one flask was incubated in a light box at about 500-foot candles illumination, another flask was wrapped with aluminum foil to exclude light during the incubation, and a third flask was inoculated with soil which had been autoclaved repeatedly to serve as a sterile control. The results of the photosynthetic experiments with soils are shown in Table 29. Other uptake experiments with soils are shown in Table 30.

b) Summary

Significant phosphate uptake signals were obtained from microorganisms in RM9 after an overnight incubation period. Detectable signals were obtained with inocula of 100 cells and 1000 cells, and 1 g of various viable soils. The results in Table 29 showed that photosynthetic activity was not readily detectable in soils, even in the case with terrarium soil containing visible amounts of photosynthetic

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Table 29

Photosynthetic Uptake of Phosphate
by Soil Microorganisms

Soil	Incubation Period (Hrs.)	Dissolved PO_4-P (mg/l) Light	Dark	Control
California soil, TRW	0.2	1.2	1.2	0.9
	2	1.2	1.3	0.9
	4.5	1.3	1.2	0.9
	16	1.4	1.2	1.0
	24	1.4	0.6	1.1
	30	0.9	0.5	1.2
	48	0.6	0.3	1.3
Sandy creek bottom soil	0.2	1.0	0.8	0.9
	2	1.1	1.0	0.8
	4	0.5	1.1	0.9
	73	0.5	0.4	---
	96	0.6	0.4	1.0
Botanical Garden soil, Wheaton Regional Park	0.2	1.5	1.8	---
	2	2.2	2.1	---
	21	0.7	0.9	---
	27	0.6	0.4	---
California soil, TRW	0.1	1.4	1.8	1.4
	3	2.1	2.1	1.7
	5	2.0	2.2	1.8
	23	0.4	0.4	---
Wooded area soil, Wheaton Regional Park	0.2	1.1	1.1	---
	3	1.0	1.1	---
	5	0.8	0.9	---
	23	0.2	0.2	---
	27	0.3	0.2	---
	49	0.3	0.1	---

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Table 29
(continued)

Photosynthetic Uptake of Phosphate
by Soil Microorganisms

Soil	Incubation Period (Hrs.)	Dissolved PO_4-P (mg/l) Light	Dark	Control
Terrarium Soil*	0.1	2.2	---	
	2	1.5	0.9	
	20	0.1	0.1	
	26	0.2	0.2	

Assay Conditions: Separate 30-ml portions of RM9 in 50-ml flasks were inoculated with 1 g of soil. The control flasks were inoculated with 1 g of soil which had been autoclaved repeatedly. The flasks were covered and stirred magnetically throughout the incubation period. The light flasks were incubated on a light box under 500-foot candles of fluorescent illumination. The dark flasks were wrapped with aluminum foil to exclude light and incubated on the light box along with the control and light flasks. Portions were removed periodically for filtration and analysis of the filtrate for phosphate.

*The terrarium soil was maintained under artificial light and watered periodically in the laboratory. The soil contained visible amounts of moss at the time of this experiment.

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Table 30

Uptake of Phosphate by Soil Microorganisms

Soil	Incubation Period (Hrs.)	Dissolved PO_4-P (mg/l) Test	Control
Wheaton, Maryland soil	0.2	1.2	1.0
	2	1.2	1.1
	4	1.8	1.2
	21	0.8	1.1
	25	0.4	1.2
Wheaton, Maryland soil	0.2	1.5	1.4
	2	1.4	1.4
	4	1.7	1.5
	23	0.1	1.4
	29	0.2	1.1
Sandy creek bottom soil	0.2	0.6	0.8
	2	1.5	1.7
	5	1.3	---
	24	0.1	---
	47	0.3	1.8

Assay Conditions: Separate 30-ml portions of RM9 in 50-ml flasks were inoculated with 1 g of soil. Control flasks were inoculated with 1 g of soil which had been autoclaved repeatedly. The test and control flasks were covered and stirred magnetically throughout the incubation period. Two-ml portions of the suspensions were removed periodically for filtration and assay of the filtrates for phosphate.

materials. The signals from dark-incubated flasks were significantly higher than the light signals in the majority of these experiments. This may indicate that light is inhibiting uptake by the nonphotosynthetic microorganisms, resulting in a lower total signal. Despite shortcomings in the colorimetric phosphate analytical procedure, the technique was very satisfactory for these experiments.

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IV. ENGINEERING SUPPORT PROGRAM

A. Introduction

The primary goal for the engineering effort on this program has been the performance of biological testing of the AMML breadboard instrument which was fabricated during the previous year under contract No. NASW-1731. This goal was accomplished after expending considerable effort on the refinement and simplification of the instrument to improve its operation and reliability. Among these refinements was the development of apparatus for conducting the light fixation-dark release experiment with automated techniques.

During the first quarter of this program, engineering effort was directed toward an alternate task - the development of an advanced breadboard design of the Gulliver life detection instrument which could serve as a configuration for a backup instrument on an early Mars Lander.

B. Gulliver V Development

In May 1969, at the suggestion of NASA, a program to design and fabricate an advanced model of the Gulliver instrument was commenced. This design was envisioned as having the capability of performing the labeled release experiment on as many as six soil samples sequentially. At this time in

the history of the Viking Program (for 1973), the necessity for a backup biology instrument was considered vital in the event the hybrid instrument being planned for the Mission was not developed on schedule.

The advanced Gulliver (Mark V) design is an "updated" version of the Mark III Gulliver (6, 7). In operation, soil samples are brought into this instrument one at a time through a gas-sealed inlet. Once the soil is within the incubation chamber, labeled nutrient is added and metabolically produced $^{14}CO_2$ is monitored with a gettered-type solid-state detector. When another soil sample is available at the inlet, it is brought into the incubation chamber with the previous sample, and is then processed in parallel. This operation is repeated until a total of six samples has been added to the chamber.

In August 1969, the details of the backup design were documented and they are included in this report as Appendix I.

C. AMML Test Program Development

1. Instrument Concept

The current AMML breadboard instrument is designed to implement the five aqueous biochemical experimental assays that have been designated for the program and described previously (2). Basically, the instrument is a liquid media

transport system which can dispense and mix liquids from six reservoirs, perform filtration of, and elution through solids suspended upon the filtration media, and transfer the liquids to six other receptacles. A syringe-type pump, multiple inlet valve, filter tape system, reagent vessels, and an array of sensors which comprise the instrument are controlled with a programmer which directs the performance of the biological assays.

In addition to the breadboard instrument, apparatus was designed and fabricated which allowed the conduct of the "dry" biochemical assay for the light fixation-dark release experiment. One design was used in performing the experiment repeatedly in laboratory testing and another demonstrated the conceptual design for the automated unit which will be incorporated into the breadboard instrument system.

The operational parameters for the breadboard apparatus include:

1. Dispensing volume: fixed by the 1 milliliter syringe pump
2. Number of dispensations: limited only by the reagent reservoir volumes

3. Reagent solutions: nonprecipitating liquids which are not solvents for Teflon or PVC, nor corrosive to stainless steel
4. Program delay: adjustable to five minutes
5. Number of program steps: no limit
6. Number of various filtered transfers: six maximum
7. Number of various unfiltered transfers: three maximum
8. Number of mixed transfers: six maximum
9. Programming: sequential steps punched on Teletype tape; nonprogramming narrative can be included to describe the operation

While the present design of the AMML has been developed to monitor for metabolic activity using the five specific experimental assays, the mechanical and electrical capabilities of the instrument are adaptable to any other biochemical or chemical processes which meet the above listed parameters.

2. Programming the Instrument for Tests

A comprehensive description of the method used to program the AMML was presented in Part IV -C of the Final

Report for the Contract No. NASW-1731 (3). Table 31 is the control format which was developed for programming the instrument together with the control functions listed in Table 32.

A program for controlling the AMML consists of a number of lines of programming information. Each line is a single step in a sequence of operations which combine to perform an experimental assay. An understanding of the programming technique, along with a functional knowledge of the operations of the instrument (see Figure 26) is important for the successful development of programs. However, an understanding of electronic and logic circuitry is not required.

Test programs can be developed by following four simple steps:

First: Outline the program in detail and sequentially, as the assay must be performed by the instrument

Second: Assign valve inlets for the various reagent reservoirs, growth chamber(s), and sensors

Third: Develop the details of the programming sub-routines; i.e., load or unload the syringe, advance filter tape, move from position to position of the valves, etc.

Fourth: Convert the programming details to the proper codes and prepare the control tape.

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Table 31

Formating Details for a Programming Step

Step 1:	"BELL" (Control + G)
Step 2:	"CR" (Carriage Return)
Step 3:	"LF" (Line Feed)
Step 4:	Item Number (X)
Step 5:	"SPACE"
Step 6:	"SYNC" (Control + V)
Step 7:	Direction (< or >)
Step 8:	N (Number of position change, minus 1)
Step 9:	Letter designation of device (A, B, S, etc)
Step 10:	"SPACE"
Step 11:	Written description of control command
Step 12:	Repeat sequence starting with Step 1 for new command

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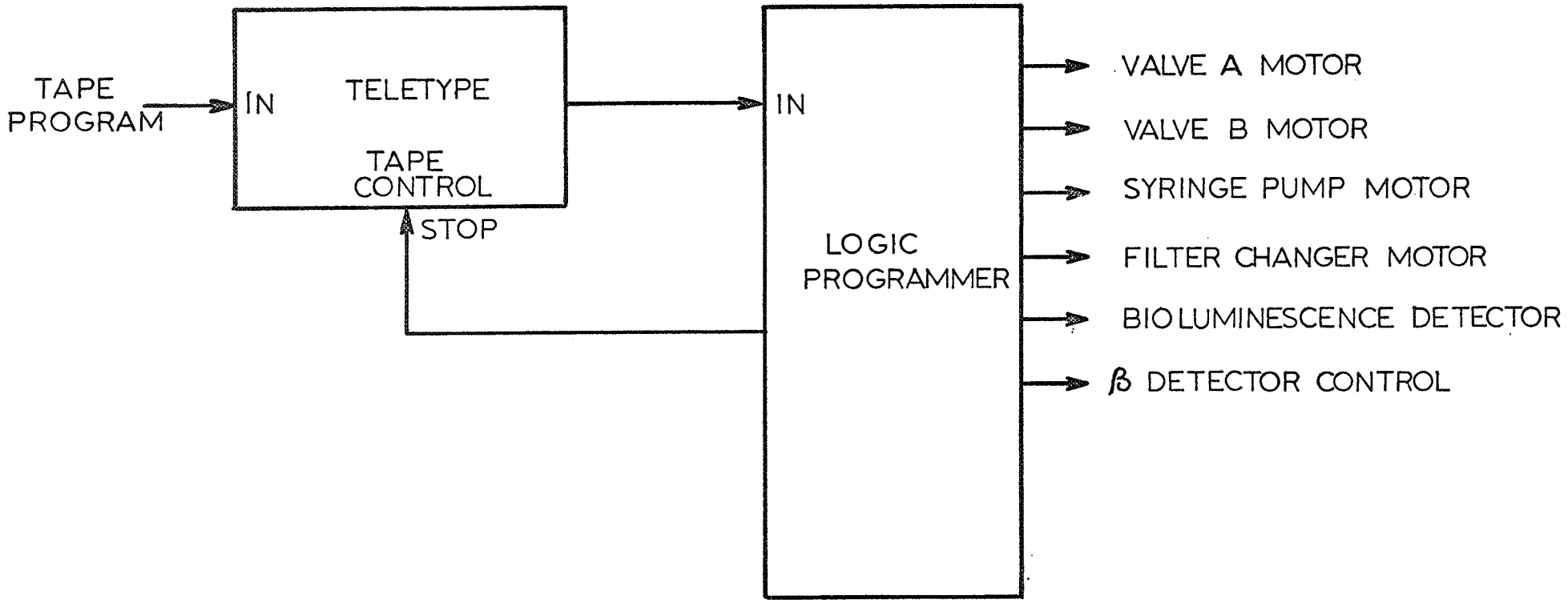
Table 32

Operational Codes for AMML Program

Operation Code	Control Function
A	Valve A Motor Drive
B	Valve B Motor Drive
D	Program Delay (1 min.)
F	Filter Changing Cycle
P	Bioluminescence Detector
R	Beta Detector
S	Syringe Motor Drive
>	UP, ON, or RIGHT
<	DOWN, OFF, or LEFT
0 thru 5	Move One thru Six Valve Positions (Operation code is (N-1) valve positions)
"BELL"	Holds Programmer if in $\overline{EOC}$
"SYNC"	Provides Method for Synchronizing Character Detection



BLOCK DIAGRAM - AMML PROGRAMMER

BCS INCORPORATED Rockville, Maryland	Fig. 26		
	Date	Init.	Scale
NASW 1731 Cont No	AMML Final Report Report Title		

3. Experimental Programs Developed

a) ATP Assay

The operational program which has been developed for the performance of the ATP assay by the AMML is shown in Figure 27. In operation, the instrument acquires a sample from the aqueous growth chamber, deposits the suspended material (including the microorganisms) onto the filter tape, elutes the material on the filter tape with an extractant for ATP, and combines this with the luciferase complex while it is viewed by the luminescence sensor. An additional program is performed in this assay, in that the filtrate from the filtration is collected in a separate holding chamber for further processing by the phosphate assay. Subsequent to the previous operations, all chambers, valves, and lines used in the assay are washed so that the next assay may be performed in them.

b) ^{14}C and ^{35}S Uptake

The operational program which is designed to assay the suspended material in the growth chambers for uptake of ^{14}C and ^{35}S labeled substrates is detailed in Figure 28. As in the previous program, an aliquot of sample from the growth

```
#1. >0A POS 3 D.W. WASH
#2. >0S SYRINGE FILL
#3. <0A POS 4 BLOCK
#4. <0B POS 5 DUMP
#5. <0S SYRINGE EMPTY
#6. <1A POS 6 ENZ.
#7. >0B POS 4 BLOCK
#8. >0S SYE FILL
#9. >1A POS 4 BLOCK
#10. >0B POS 3 RX CHAMBER
#11. <0S SYRINGE EMPTY
#11A. <1A POS 6 ENZ.
#11B. <0B POS 4 BLOCK
#11C. >0S SYRINGE FILL
#11D. >1A POS 4 BLOCK
#11E. >0B POS 3 RX CHAMBER
#11F. <0S SYRINGE EMPTY
#12. >0A POS 3 D.W. WASH
#13. <0B POS 4 BLOCK
#14. >0S SYRINGE FILL
#15. <0B POS 5 DUMP
#16. <0A POS 4 BLOCK
#17. <0S SYRINGE EMPTY

#18. <0F FILTER CHANGE
#19. >0A POS 3 D.W. WASH
#20. >0B POS 4 BLOCK
#21. >0S SYRINGE FILL
#22. <0B POS 5 DUMP
#23. <0A POS 4 BL><LOCK
#24. >2A POS 1 SAMPLE
#25. >0B POS 4 BLOCK
#26. >0S SYRINGE FILL
#27. <2A POS 4 BLOCK
#28. <0B POS 5 DUMP
#29. <0S SYRINGE EMPTY
#30. >0F FILTER CHANGE
#31. >1A POS 2 EXTRACT
#32. >0B POS 4 BLOCK
#33. >0S SYRINGE FILL
#34. <1A POS 4 BLOCK
#35. <0B POS 5 DUMP
#36. <0S SYRINGE EMPTY
#37. >2A POS 1 SAMPLE
#38. >0B POS 4 BLOCK
#39. >0S SYRINGE FILL
#40. <2A POS 4 BLOCK >
#41. >0B POS 3 R.X. CHAMBER
#42. <0S SYRINGE EMPTY
#43. >1A POS 2 EXTRACT <
#44. <0B POS 4 BLOCK
#45. >0S SYRINGE FILL
#46. <1A POS 4 BLOCK
#47. >0B POS 3 R.X. CHAMBER
#48. <0S SYRINGE EMPTY
#49. >0D DELAY RECORD REEACTION
#37. >0S SYRINGE FILL
```

```
#38. <1B POS 5 DUMP
#39. <0S SYRINGE EMPTY
#40. >1B POS 3 RX CHAMBER
#41. >0S SYRINGE FILL
#42. <1B POS 5 DUMP
#43. <0S SYRINGE EMPTY
#44. >1B POS 3 RX CHAMBER
#45. >0S SYRINGE FILL
#46. <1B POS 5 DUMP
#47. <0S SYRINGE EMPTY
#48. >0F FILTER CHANGE
#49. >0A POS 3 D.W. WASH
#50. >0B POS 4 BLOCK
#51. >0S SYRINGE FILL
#51A. <0A POS 4 BLOCK
#52. >0B POS 3 RX CHAMBER
#53. <0S SYRINGE EMPTY
#54. >0A POS 3 D.W. WASH
#55. <0B POS 4 BLOCK
#56. >0S SYRINGE FILL
#57. <0A POS 4 BLOCK
#58. >0B POS 3 RX CHAMBER
#59. <0S SYRINGE EMPTY
#60. >0A POS 3 D.W. WASH
#61. <0B POS 4 BLOCK
#62. >0S SYRINGE FILL
#63. <0A POS 4 BLOCK
#64. >0B POS 3 RX CHAMBER
#65. <0S SYRINGE EMPTY
#66. >0A POS 3 D.W. WASH
#67. <0B POS 4 BLOCK
#68. >0S SYRINGE FILL
#69. <0A POS 4 BLOCK
#70. >0B POS 3 RX CHAMBER
#71. <0S SYRINGE EMPTY
#72. >0S SYRINGE FILL
#73<<<<>FILL
#73. <1B POS 5 DUMP
#73A. <0S SYRINGE EMPTY
#74. >1B POS 3 RX CHAMBER
#75. >0S SYRINGE FILL
#76. <1B POS 5 DUMP
#77. <0S SYRINGE EMPTY
#78. >1B POS 3 RX CHAMBER
#79. >0S SYRINGE FILL
#80. <1B POS 5 DUMP
#81. <0S SYRINGE EMPTY
#82. >1B POS 3 RX CHAMBER
#83. >0S SYRINGE FILL
#84. <1B POS 5 DUMP
#85. <0S SYRINGE EMPTY
#86. >1B POS 3 RX CHAMBER
#87. >0S SYRINGE FILL
#88. <1B POS 5 DUMP
#89. <0S SYRINGE EMPTY
#90. >0F FILTER CHANGE
#91. <1A POS 6 ENZ. >>
```

Figure 27

ATP Assay Program

```
1. >0A POS 3 COLD RM 9
2. >0S SYRINGE FILL
3. <0A POS 4 BLOCK
4. <0B POS 5 DUMP
5. <0S SYRINGE EMPTY

1. >0A POS 3 COLD RM 9
1A. >0B POS 4 BLOCK
2. >0S SYRINGE FILL
3. <0A POS 4 BLOCK
4. <0B POS 5 DUMP
5. <0S SYRINGE EMPTY
6. <0A POS 5 HOR RM 9
7. >0B POS 4 BLOCK
8. >0S SYRINGE FILL
9. >0A P04 BLOCK
10. <0B POS 5 DUMP
11. <0S SYRINGE EMPTY
12. >0A POS 3 COLD RM 9
13. >0B POS 4 BLOCK
14. >0S SYRINGE FILL
15. <0A POS 4 BLOCK
16. <0B  POS 5 DUMP
17. <0S SYRINGE EMPTY
18. >0A POS 3 COLD RM 9
19. >0B POS 4 BLOCK
20. >0S SYRINGE FILL
21. <0A POS 4 BLOCK
22. <0B POS 5 DUMP
23. <0S SYRINGE EMPTY
24. >0A POS 3 COLD RM 9
25. >0B POS 4 BLOCK
26. >0S SYRINGE FILL
27. <0A POS 4 BLOCK
28. <0B POS 5 DUMP
29. <0S SYRINGE EMPTY
30. >0A POS 3 COLD RM 9
31. >0B POS 4 BLOCK
32. >0S SYRINGE FILL
33. <0A POS 4 BLOCK
34. <0B POS 5 DUMP
35. <0S SYRINGE EMPTY
36. <0F FILTER CHANGE  DRY
```

Figure 28

^{14}C ^{35}S Assay Program

chambers is acquired and deposited on the filter tape. After rinsing the filter with nonlabeled medium several times, the tape is advanced, dried, and then monitored for ^{14}C and ^{35}S.

c) Labeled Release (Heterotrophic)

The growth chamber(s) are monitored periodically for $^{14}CO_2$ which may be released from the aqueous medium as the result of heterotrophic fixation and respiration. No special programming tape was developed for this test program because the operation requires only a single command to activate the growth chamber detector(s).

d) Labeled Release (Photosynthesis)

This assay is an extension of the previous assay with only the addition of a command to turn on and off the incubation chamber illumination source.

e) Light Fixation-Dark Release

The mechanism which was designed to perform the light fixation-dark release experiment automatically has been assembled in its simplest form to allow the demonstration of the concept. In the further development program, where the complete system will be demonstrated, the full automation of this experiment will be implemented and a control program will be developed to perform the assay.

f) Phosphate Uptake

The AMML breadboard instrument had been designed to perform a phosphate assay using the ^{14}C-triethylamine precipitation method; however, this assay failed to yield sensitivities which were suitable for the levels of phosphate concentration desired for the experiment. A colorimetric assay has now been developed which has the potential for meeting the experiment requirements. Again, if further evaluation of this technique proves successful, it, too, will be implemented in the automated system and a control program will be developed.

4. Instrument Calibration

Two major areas are of importance in the calibration of the instrument. One involves the radiation detectors and the system to verify the conversion of labeled substrates to $^{14}CO_2$. The other concerns the standardization of ATP reagents and the sensor system.

a) Radiation Standardization

The method selected to calibrate the radiation sensors has involved the use of standard radiation sources. The source employed for the AMML test program has been a ^{14}C source which has been coated on a plastic disk.

b) ATP Standards

Calibration of the ATP assay system for the

various levels of sensitivity expected was accomplished by the use of standards in combination with distilled water. These standards are tested by use of an automated control system which has been preset to prime the reagents into the proper vessels and to use the standard in place of the extraction process. The response to this was used as a reference to calibrate the instrument. Blanks consisting of distilled water provide a means to calibrate the instrument response to background levels.

D. AMML Instrumentation Program

1. Design and Modification of the Breadboard

Figure 29 shows the current breadboard apparatus which was used in performing the biological testing, and includes the accessories fabricated to support this. As a result of the testing program for the AMML breadboard, the instrument which was fabricated during the previous program was found to require modifications.

a) Valve Mechanism

The valve assembly for the instrument shown in Figure 30 is the heart of the instrument. The body and slider are fabricated from Teflon because it resists corrosion, is nontoxic, and possesses a low coefficient of friction which permits the sliding members to operate

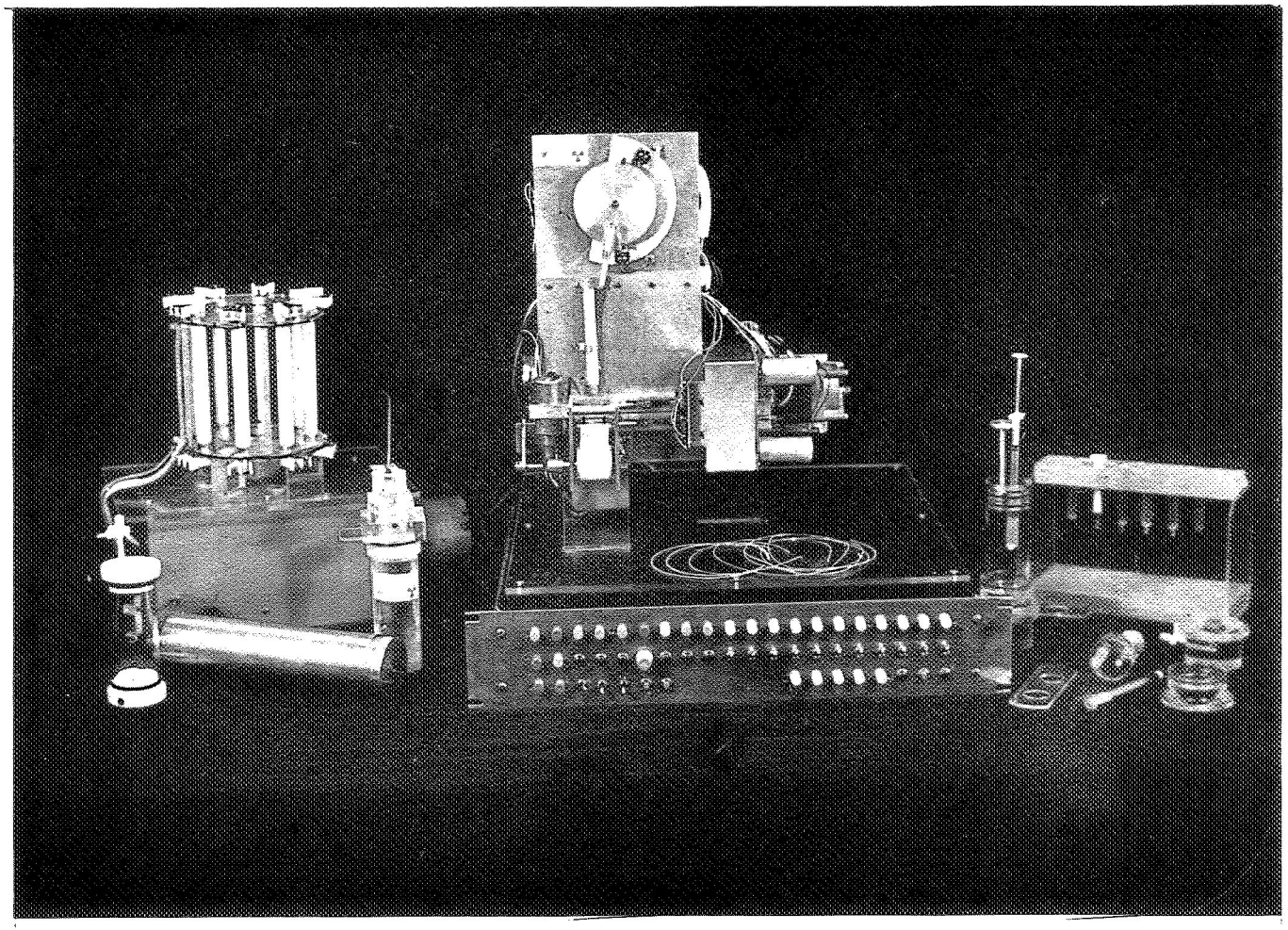


Figure 29

AMML Breadboard Apparatus

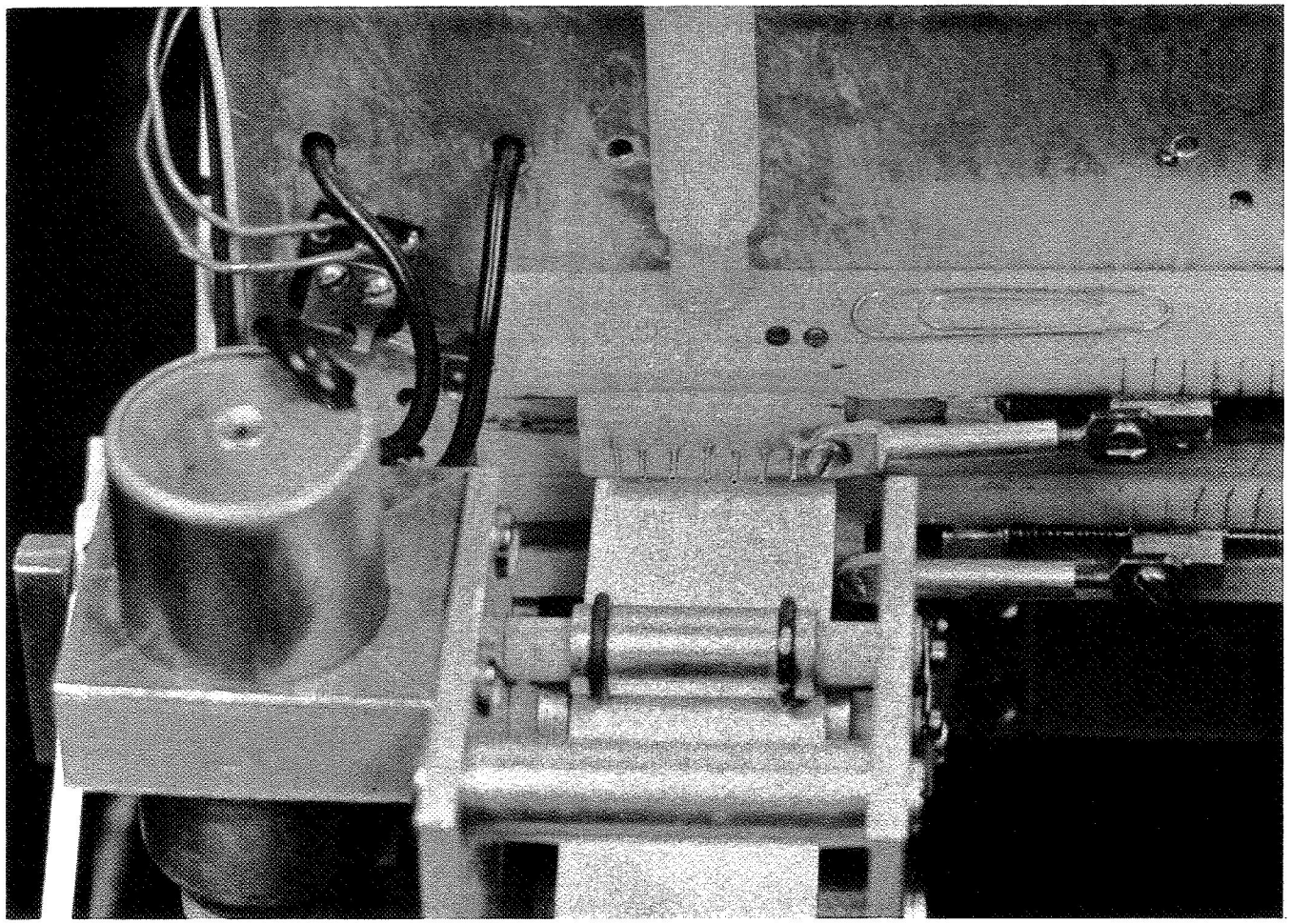


Figure 30

Valve Mechanism

smoothly. Because this material is flexible, minor misalignments tend to distort the assembly and result in leakages. Correction of the leakages necessitated a lengthy realignment process, including the addition of shims to provide the required O-ring pressure. When future versions of this valve are contemplated, it is recommended that another material be selected. Non-corrosive stainless steel or titanium is recommended.

b) Valve Drive Unit

The slider of the valve mechanism is actuated by a lead-screw arrangement powered through a clutch, coupled to a reversible motor. During the course of testing the breadboard, the modifications made to this unit entailed redesigning the double yoke coupling device, the reduction gear bearing plate, and the clutch mechanism which engages either the upper or lower valve to the motor driver.

c) O-rings

The seven inlets for each valve (upper and lower) are sealed with miniature O-rings which are fitted around the hypodermic tubing. These O-rings and the inlet were shown in Figure 30 and are the primary cause of leakages during the testing. Leakage was caused by the fact that the O-ring material (Buna-S) tends to set after being clamped for an extended period

of time. Buna-S was selected because it appeared to have the best properties of those materials which were available in the O-ring size (size 2-1) required for this application. This problem will be solved in future models of the valve by providing a flexible pressure plate that will accommodate the effect of setting, and by obtaining O-rings specially fabricated from a more suitable material.

d) Radiation Detectors

Two radiation detectors to monitor for ^{14}C were employed in the breadboard apparatus. One is used for the the uptake measurements on the filter tape and the other, for the evolution of $^{14}CO_2$ over the aqueous growth chamber. Original plans called for the use of solid-state detectors that had been optimized for the detection of ^{14}C. These proved to be light-sensitive and caused a problem during initial testing. An Amperex-Type 18515 GM detector was selected for a detector; however, a problem developed in the monitor for the aqueous growth chamber which is discussed in a subsequent section (2 a). A type 100-300 surface barrier solid-state detector manufactured by Ortec was used to monitor the $^{14}CO_2$ over the aqueous growth chamber.

e) Controller/Mechanism Integration

The most serious problem encountered during

the integration of the system was noise pulses which caused the programmer to generate false commands. This problem is typical for systems in which low level solid-state electronics are used in combination with AC-operated relays and motors. The decision to use AC motors for this application had been made originally because of their cost, availability, and simplicity in design; however, future versions of the instrument will employ DC motors. (In another development program performed concurrently with this program, DC motors were successfully employed with a similar programmer/controller). Figure 31 shows the typical modifications which were necessary to overcome the noise pulses problem.

2. Accessory Development and Integration

a) Aqueous Growth Chambers

The aqueous growth chambers which were designed during the previous program are shown in Figure 32. These were made to contain an aliquot of medium which is inoculated with a soil sample and then allowed to incubate and dispense samples of aqueous suspensions through a 200 mesh filter.

During testing with soils bearing a high silt content, several problems were encountered, including the clogging of

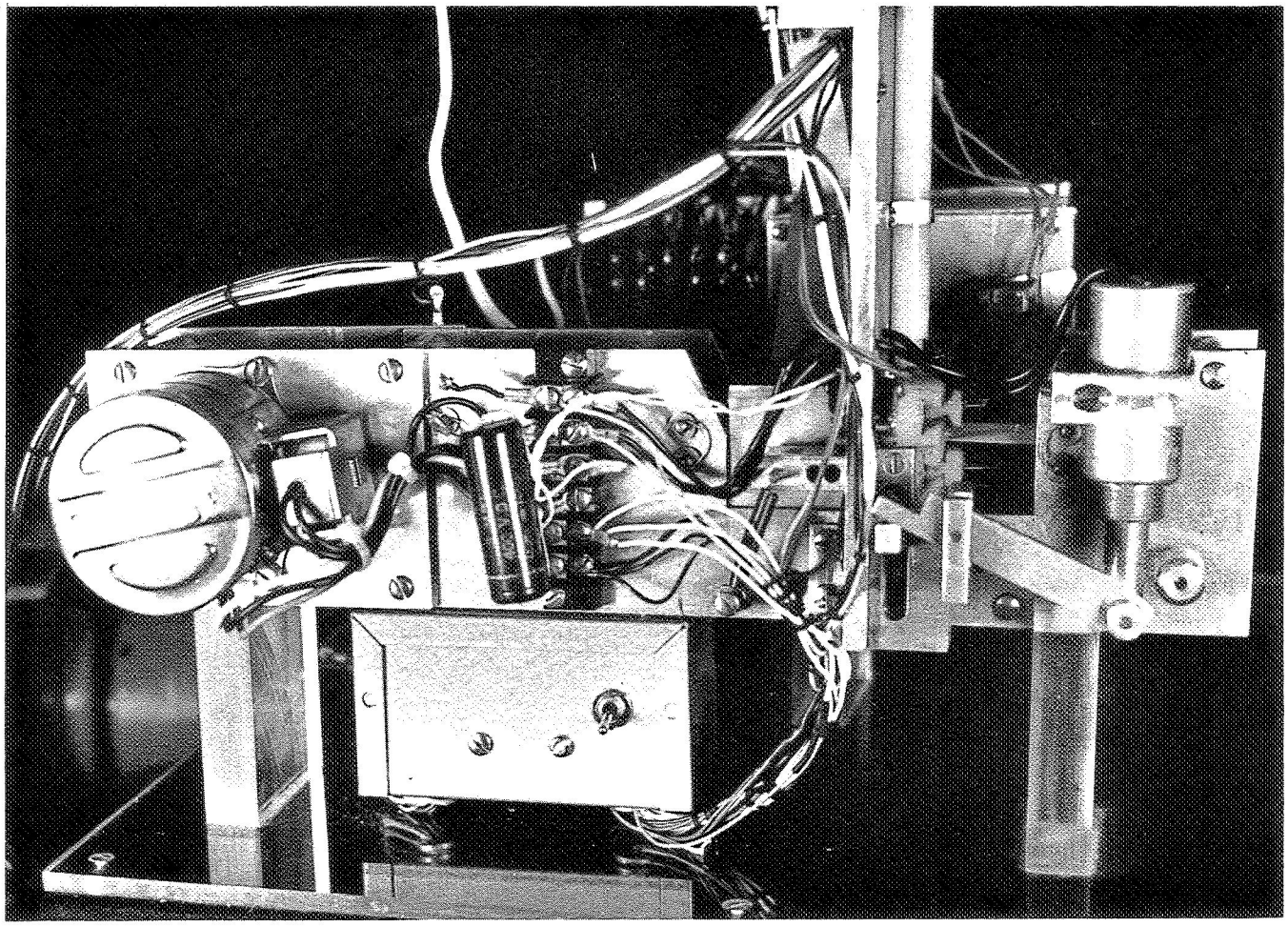


Figure 31

Filter Tape Drive System

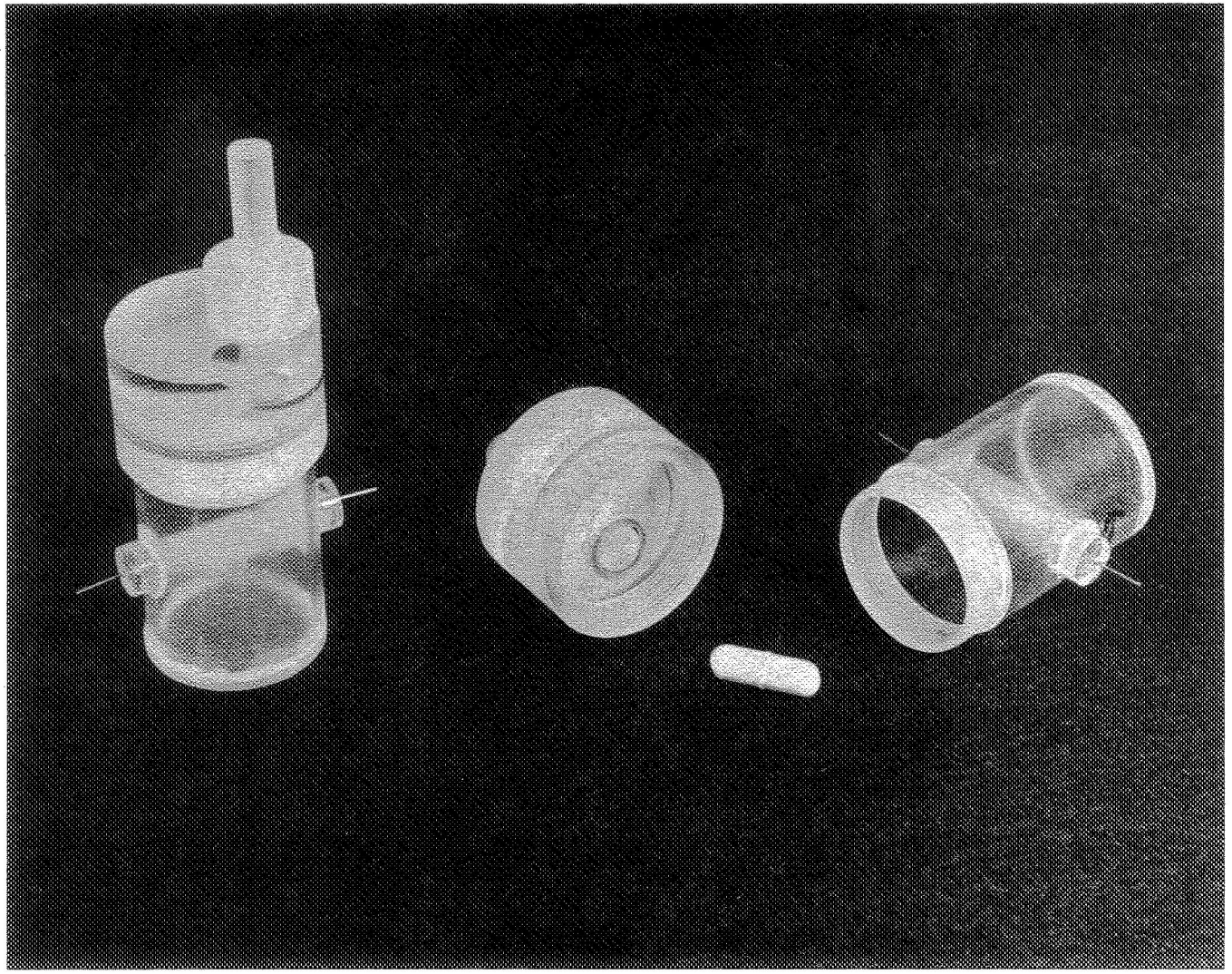


Figure 32

Original Aqueous Growth Chambers

the 200 mesh screen, clogging of the filter tape, and stratification of the medium if it was not stirred during incubation. Several procedures were tried and proved unsuccessful. Among these was the attempt to select particle sizes and dilution.

A solution to the problem was found to consist of the following:

> The soil sample is preprocessed by adding it to an aliquot of medium which is then incubated for two hours.
>
> A supernatant of this aqueous suspension is withdrawn, added to the aqueous growth chamber, and then processed by the standard AMML program.

Although this technique greatly dilutes the inoculum, satisfactory metabolic response characteristics have been obtained using the process.

Despite the previously mentioned advantage for the GM counter, problems were encountered in using it for the detection of evolved $^{14}CO_2$ over the aqueous growth chamber. The mica window of the GM counter, because of the cleavages in the mica's crystal structure, tends to absorb $^{14}CO_2$ in a way that makes it difficult to remove. This self-gettering by a GM

counter may be advantageous in a system where the detector will be used for only one test; however, in the AMML breadboard testing, the system must be reusable for many tests. The surface barrier detectors were reevaluated for this application, and it was found that they could be cleaned when their surfaces became contaminated. A scheme was subsequently devised wherein the problem of light sensitivity was eliminated when the detector was monitored only in the dark.

Another problem was encountered. During long incubation periods, the detector's surface tends to be cooler than the surrounding chamber and moisture condenses on the surface, thus degrading the signal from the ^{14}C. This condensation was avoided by using a Silastic membrane, .001 inches thick, to isolate the detector and prevent a moisture buildup at the surface. Figure 33 shows the final design of this sytem.

b) Dark Release Chambers for Laboratory Experiments

Figure 10 showed the test cells which were developed during the previous program to laboratory test the dark release assay. The design worked well during this experimentation, but it is not adaptable for conversion to an automatic system. Problems with the absorption of $^{14}CO_2$

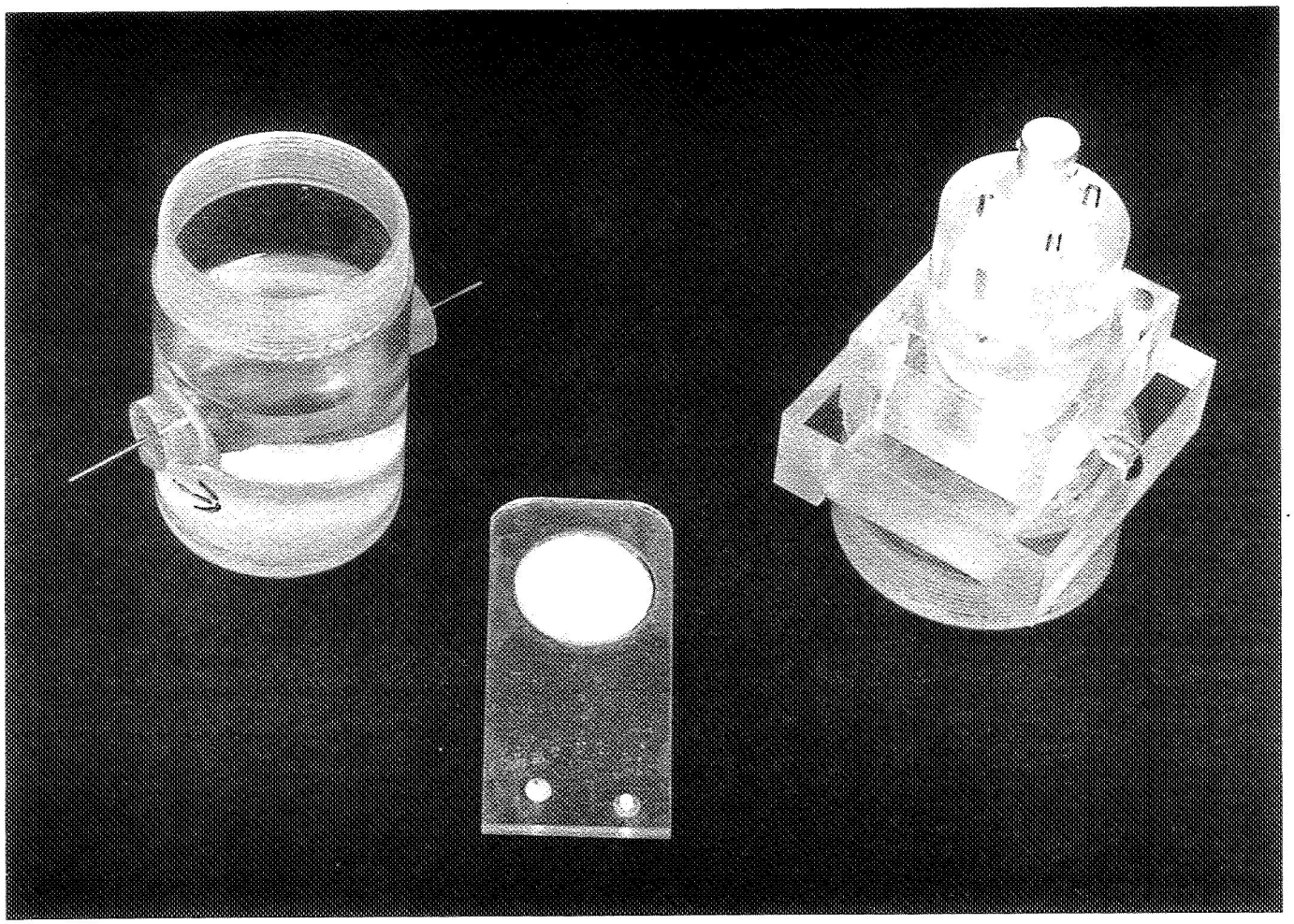


Figure 33

Modified Aqueous Growth Chamber

by the O-rings, discussed elsewhere, were encountered but can be solved by using other materials which do not absorb CO_2.

c) AMML Dark Release Chamber

Figure 34 shows the proposed configuration of a dark release chamber which is adaptable to automated assay techniques. It consists of a series of plungers and syringes which may be actuated by motor-driven lead-screw controlled devices. The system allows for inoculation with $^{14}CO_2$ gas, photosynthetic incubation, flushing, and the subsequent monitoring for $^{14}CO_2$ which is released by the sample during dark incubation. The syringe at the top of the chamber serves a dual purpose in that it is a $^{14}CO_2$ gas generator and a flush-valve outlet. The bottom portion of the unit consists of a screen to hold the soil sample and a device to position a getter in the system for monitoring the dark released $^{14}CO_2$.

d) Reagent Storage

Several systems have been examined for reagent storage for the AMML breadboard. Of these, the best appears to be a floating piston reagent chamber, which is depicted in Figure 35. In terrestrial applications, under atmospheric conditions, the system does not require pressurization of the upper volume; however, for extraterrestrial applications,

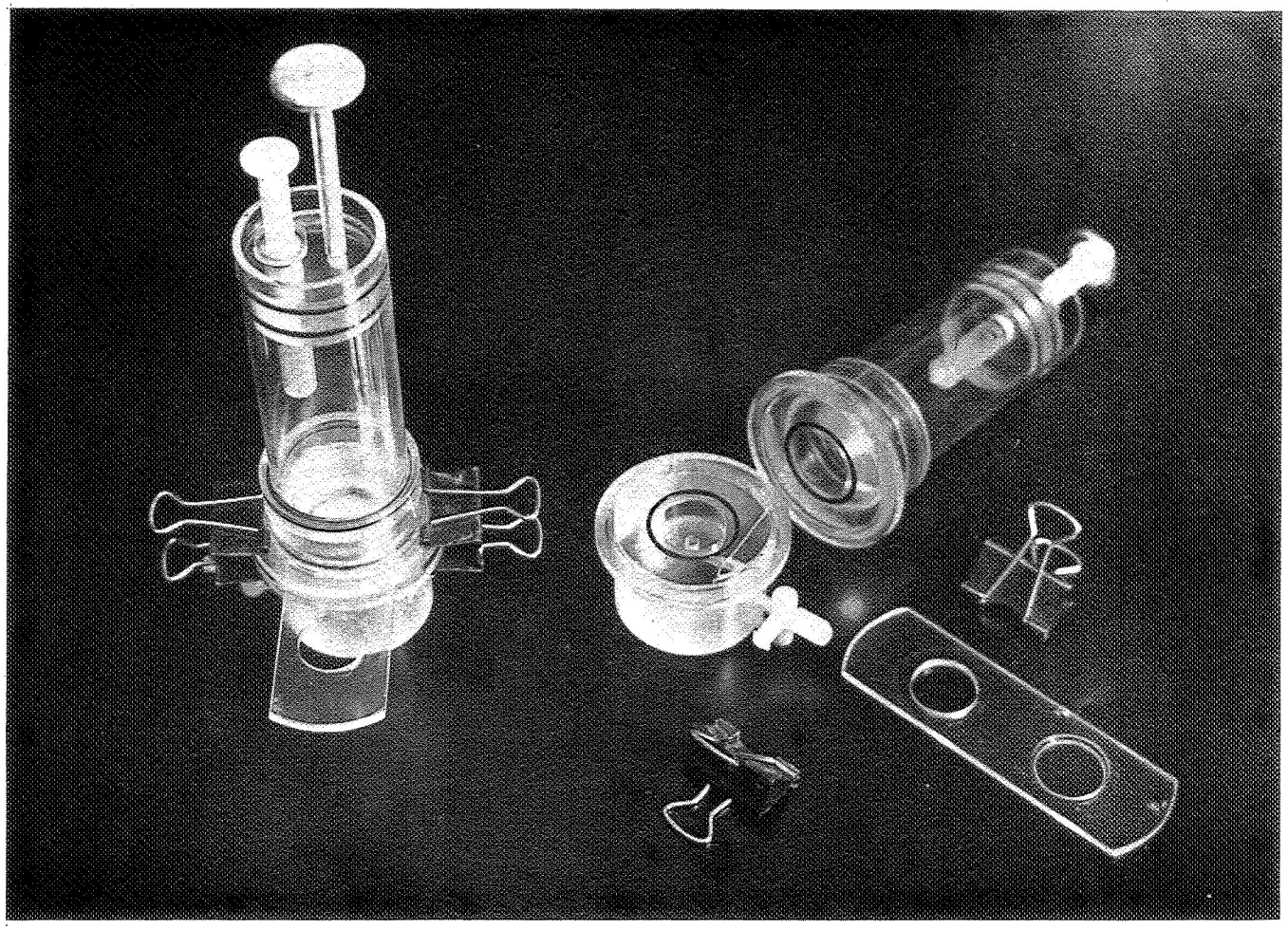


Figure 34

Dark-Release Chamber for AMML Concept

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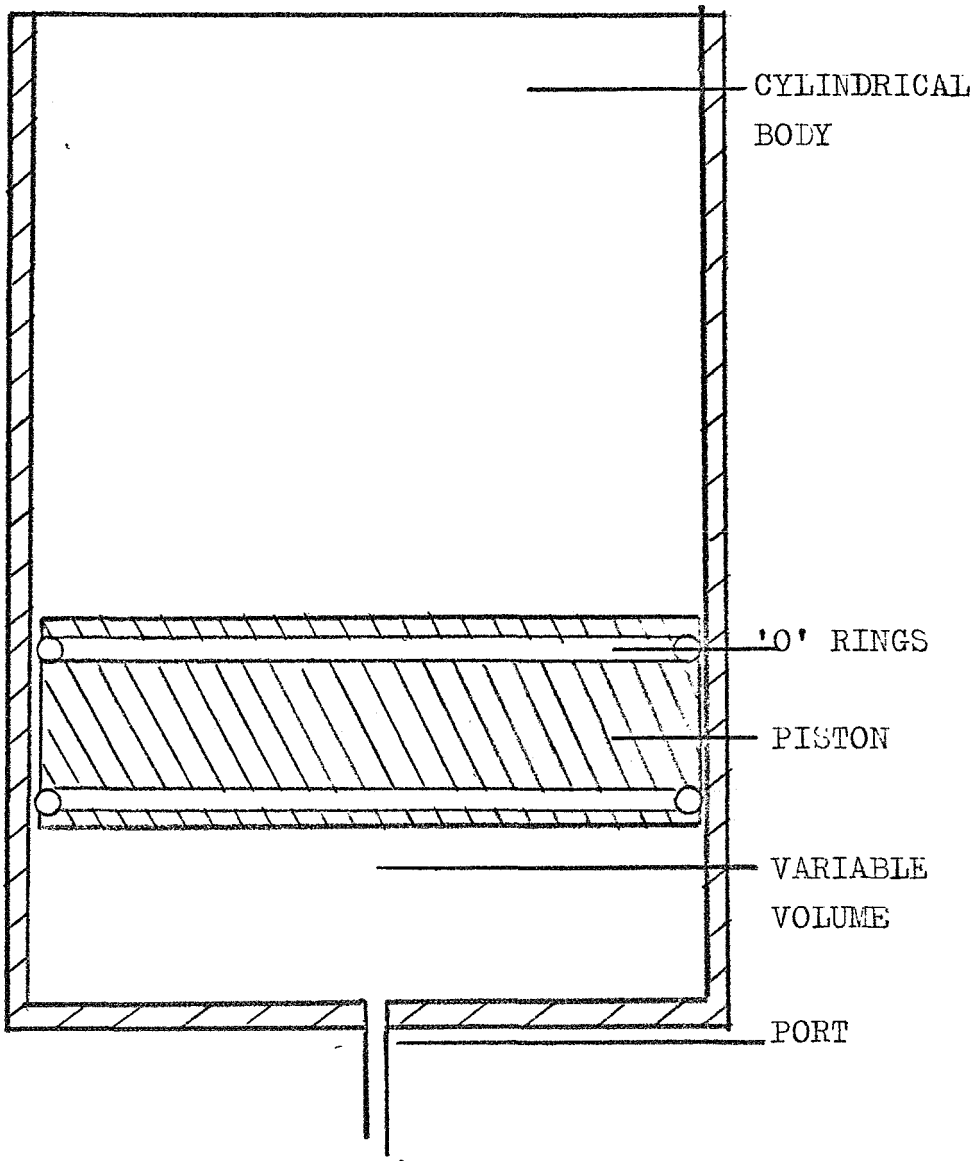

Title: Reagent Storage Concept for AMML	
	Fig. 35
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Rockville, Maryland	Date: Apr'71 / Init. / Scale
Cont. No.: NASW-1931	Report Title: AMML Final Report

this volume must be pressurized with a gas supply. This system will perform in a zero gravity environment and it can be made to withstand the forces which develop during sterilization.

e) Filter Tape

Originally, the filter tape selected for use with the breadboard apparatus had a .45 u pore size, which has a high retention efficiency for mixed species of bacteria. During the testing, however, extremely fine silt particles caused the system to clogg, so an alternate pore size (1.2 u), which alleviated the clogging problem and still maintained the retention efficiency at a relatively high level (better than 99%), was selected.

Since the breadboard apparatus requires a tape-type filter media with a fairly high tensile strength, a nylon-backed filter material was used. Preliminary tests were conducted using the Millipore material. This proved suitable from the filtration aspects; however, the filtration media on the surface was easily damaged, so an alternate source of material was investigated. A material manufactured by the Gelman Company, Acropor-Type AN-1200, was found to overcome the problem and also proved suitable for this application.

f) Incubation Light Source

The incubation light source shown in Figure 36 was developed to provide the necessary illumination intensity for the aqueous growth chamber experiments. The system provides a uniform illumination of 400-foot candles for the aqueous growth chamber. Forced air-cooling of this unit helps limit the temperature rise of the sample during incubation.

E. Biology Experimentation with the AMML Breadboard Instrument

The light fixation-dark release chambers designed for the AMML breadboard instrument (Figure 34), were tested manually and their performance compared with the apparatus normally used in the laboratory (Figure 10). Duplicate light and sterile control chambers were set up with the laboratory apparatus and one light, and one control chamber was set up with the AMML equipment. A one-gram portion of sandy creek bottom soil was placed in each chamber and exposed to 10 uCi of $^{14}CO_2$, which corresponded to 0.1 uCi of labeled gas-per-ml of head space in both types of chambers. Soil which had been sterilized by repeated autoclaving was used in the control vessels. The chambers were

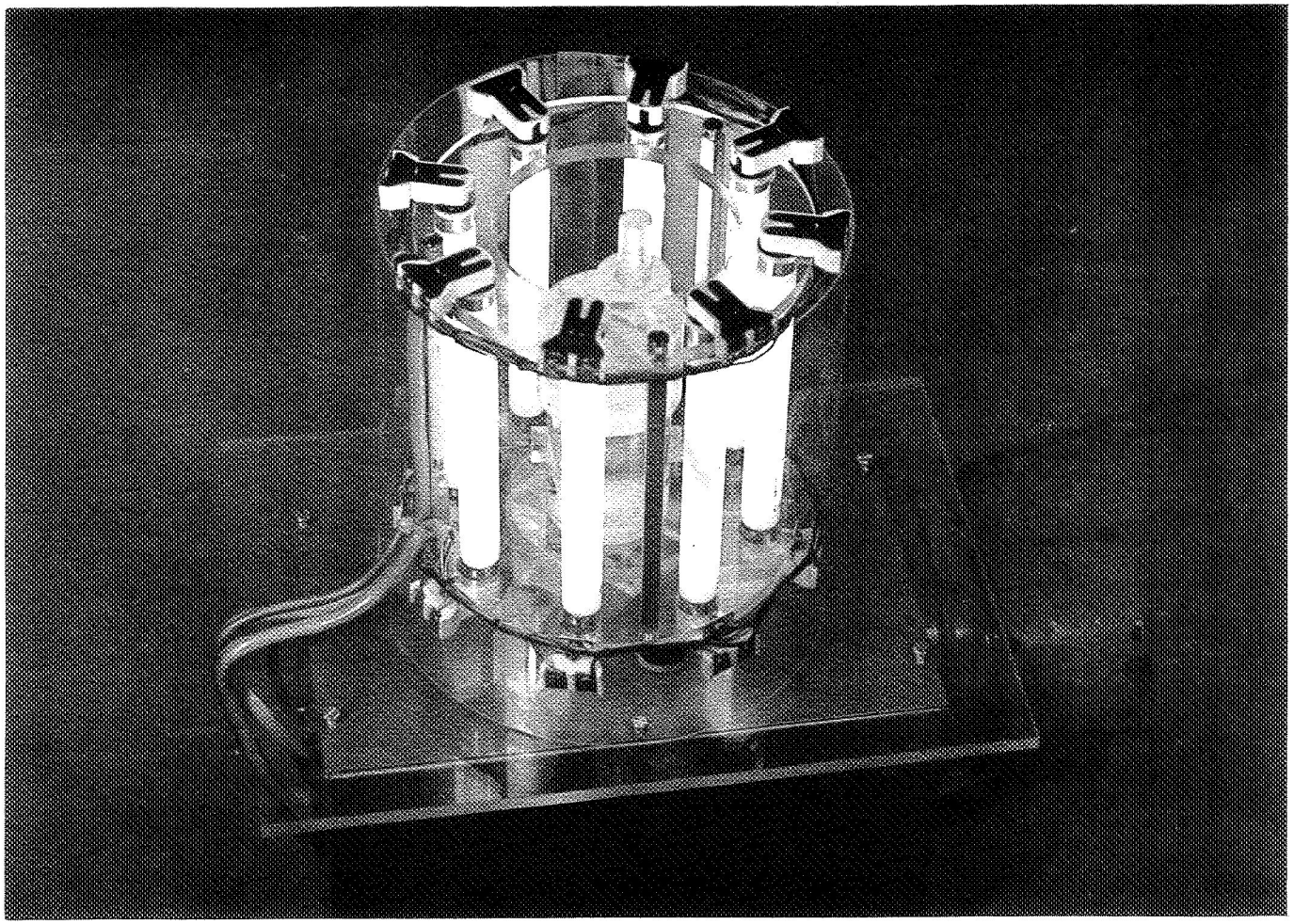

Figure 36

AMML Aqueous Growth Chamber Incubation Light Source

incubated for three hours under illumination of 400-foot candles and flushed with moist carbon dioxide for one-hour at a flow rate of 200 ml/minute. The soil in the laboratory chambers was then transferred, as usual, to planchets and the $^{14}CO_2$ evolved was gettered by moist filter pads impregnated with a saturated $Ba(OH)_2$ solution. The soil contained in the chambers designed for the AMML bread-board instrument was gettered in place with the base-impregnated moist filter pads. The inverted planchets containing the getter pads were changed at preselected intervals, in order to follow the kinetics of $^{14}CO_2$ evolution. The results of this comparative test are shown in Table 33. The cumulative signals from the AMML light and control chambers were about three and nine times higher, respectively, than those from the light and control chambers of the laboratory apparatus. This was later found to be caused by high nonbiological backgrounds from the Buna N O-rings used to seal the AMML chambers. These O-rings absorbed relatively large amounts of labeled carbon dioxide during the incubation period, which was then partially evolved during the dark release portion of the assay. Buna N was found to retain substantial amounts of radioactivity absorbed

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Table 33

Light Fixation-Dark Release Assay

Test of Incubation Chambers for the
AMML Breadboard Instrument

Gettering Interval (Hours)	Cumulative Radioactivity (cpm)*			
	Laboratory Chambers**		AMML Chambers	
	Light	Sterile Control	Light	Sterile Control
0-1.5	627	264	24	0
1.5-17.5	1067	432	514	277
17.5-32	1238	477	3879	4165

Assay Conditions:

Each chamber contained a 1 g portion of sandy creek bottom soil and was incubated on a light box for three hours at 400-foot candles. A level of 10 uCi of $^{14}CO_2$ was generated in each 100 ml chamber. The control soil was sterilized by repeated autoclaving. At the end of the incubation period, all chambers were flushed for one-hour with moist carbon dioxide at a flow rate of 200 ml/minute. The soil in the AMML chambers was then gettered with $Ba(OH)_2$ impregnated pads. The soil in the laboratory chambers was transferred to planchets for gettering.

* These values have been corrected for background.

** The averages of duplicate determinations are reported.

in this fashion for several days or more unless baked-out in an oven at 120°C for several hours.

Operation of the reaction chamber and photomultiplier of the AMML breadboard instrument was checked out by running a standard curve prior to conducting an integrated biology experiment with the instrument. This test was performed manually by injecting 1 ml portions of various ATP solutions from 10^{-5} to 10^{-2} ug ATP/ml into 2 ml of reaction mixture contained in the reaction chamber. Reaction mixture obtained from the Instrument and Equipment Division of du Pont was used for this experiment. The ATP standard solutions were prepared in 0.01 M arsenate, 0.01 M EDTA, 0.01 M Tris, and pH 7.4 buffer saturated with n-butanol. The standard curve generated is shown in Figure 37.

An integrated biology experiment was conducted with an inoculation of a mixed culture of soil microorganisms. The inoculum was prepared by incubating a 1 g portion of a local soil for an overnight period in about 50 ml of RM9 containing unlabeled substrates. The soil suspension was allowed to settle for 15 minutes and 1 ml of this inoculum was added to 50 ml of RM9, 2 uCi/ml, in the growth chamber of the AMML breadboard instrument. The results of the

ATP Standard Curve for AMML Breadboard Instrument

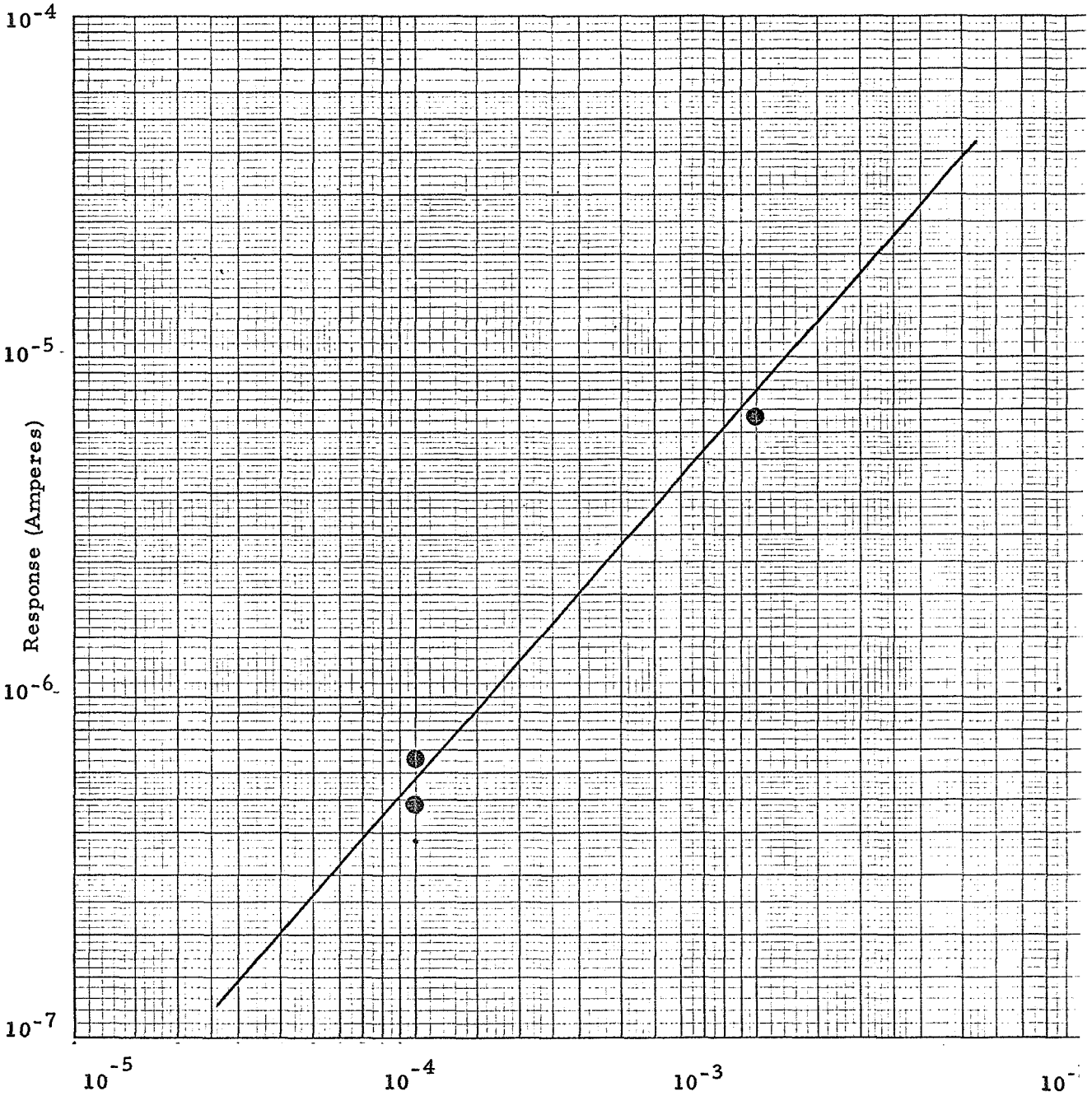

Fig. 37

BIOSPHERICS INCORPORATED
Rockville, Maryland

Date	Int.	Scale
Apr'71		

Cont. No.	Report Title
NASW-1931	AMML Final Report

integrated biology experiment are shown in Figure 38. All results shown were obtained by the instrument, with the exception of the colorimetric phosphate assay, which has not been satisfactorily instrumented. Filtrates obtained automatically by the instrument were assayed manually using the molybdenum blue laboratory procedures for orthophosphate. A preliminary ATP production experiment was manually performed prior to the integrated AMML breadboard test to predetermine the optimum sensitivity settings for measuring the bioluminescent responses during the course of the incubation period. Response curves comparable to those obtained in laboratory experiments were obtained from the AMML breadboard instrument. The results clearly establish the presence of biological activity in the soil and give insight into the biochemistry encountered. Such a response from an extraterrestrial soil would constitute a highly successful and valuable experiment.

Integrated Biology Experiment with AMML Breadboard Instrument

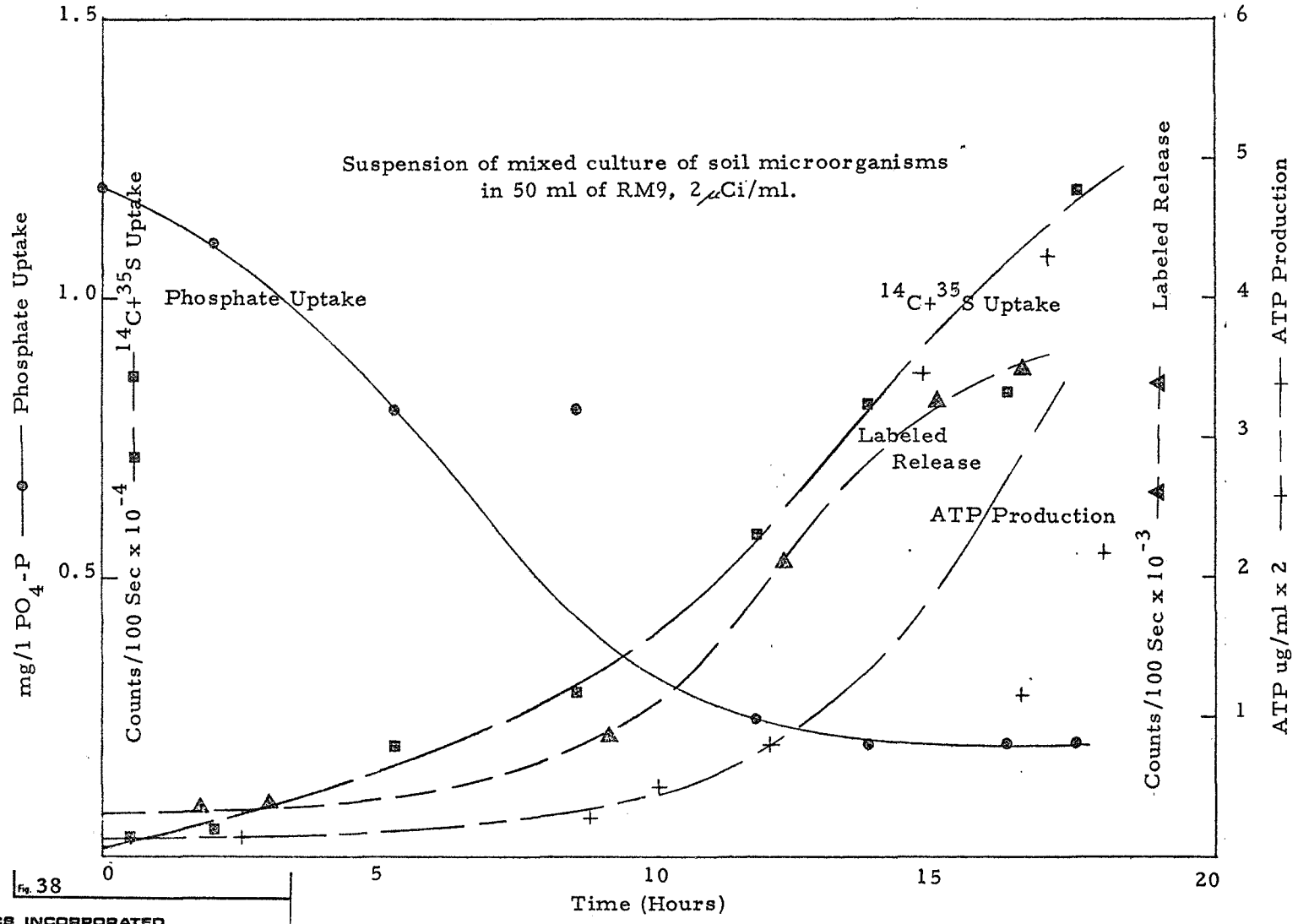

Fig. 38

BIOSPHERICS INCORPORATED
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Date	Init.	Scale
Apr'71		

NASW-1931 AMML Final Report

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V. DISCUSSION

A. Program Evolution

1. NASW-1731

This contract permitted the development of an instrumentation concept for an automated metabolic laboratory. Emphasis was placed on automation of wet chemistry techniques, suitable for second generation life detection experiments and comparative biochemical studies of life found. The product was a breadboard apparatus capable of conducting a number of microbial metabolic assays. Limitations were established and a number of problems defined.

2. NASW-1931

Additional effort was contracted for the purpose of quantifying the biology performed by the AMML and integrating and testing the various experiments. Minor modifications were achieved to improve operation and efficiency.

B. Status

Methods and programs have been developed for fairly complex operations involving the metering, pumping, transferring, filtering and drying of liquids and suspensions. The required sensor and support system have also been developed and the overall feasibility demonstrated. The automated conduct of all of the experiments with the exception of

the assay for PO_4 is efficient, practical and reliable. The AMML, while only an engineering model, has performed thousands of programmed experiment operations with a record of few failures. The assay for phosphate, wherein a precipitate is formed and transported, is not feasible. Excessive mechanical binding is caused by the precipitate which is insoluble.

The AMML mechanical and electronic components have provided the degree of control and flexibility required for a remote laboratory. Sensor sensitivity has been adequate for the levels of test. More sensitive detection devices are available for spaceflight configurations as required.

VI. RECOMMENDATIONS

A. Engineering

The engineering model of the AMML has demonstrated the feasibility of conducting integrated metabolic experiments automatically. It has, in the course of design, suggested a number of improved approaches and design concept. One approach for an advanced mechanical configuration with simplified electronics will permit numerous organic and inorganic experiments other than those that might result in the formation of insoluble products on multiple use components.

An advanced design for the AMML has been proposed (8, 9) and would provide additional valves, a turret distribution system, plastic bag reagent chambers and a practical limit of perhaps 15 reagents, and a four-thousand step program. Common laboratory functions such as boiling, freezing, filtering, and centrifuging would be possible in the same volume occupied by the present model. The many aspects of potential improvements in technology are represented in the following recommendations:

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1. Advanced AMML Prototype Model. It is recommended that an advanced phase of AMML be given serious consideration. This phase would be for a versatile assay laboratory, capable of performing organic and inorganic chemistry and biochemistry experiments and assays. It would include selected laboratory functions, such as boiling, heating, freezing, filtering, and continuous flow centrifugation, exchangable as modules for other applications. Thus, an instrument would be developed with the capability of being programmed for a wide variety of experiments as conceived by individual experimentors. Although experiments have been proposed in association with the AMML, the instrument is not constrained to these. Unit processes not currently part of the instrumental complement could be incorporated as modules where needed.

2. It is recommended that the potential spinoff applications for hospital laboratory specimen analysis be examined. Public health facilities,

clinics, and military and civilian hospitals could benefit from the automated laboratory processes evolved from the AMML.

B. Biology-Biochemistry

1. Labeled Release Experiment

The labeled release experiment is capable of producing very reliable indications of microbial life from all types of soils studied to date. The sensitivity and precision of this assay are eminently suited to life detection experiments. However, additional studies are recommended in the following cases:

a) Effect of moisture and sterilization temperature upon the nonbiological background from soils.

b) Selection of labeled substrates for incorporation into the AMML medium.

c) Effect of environmental conditions; i.e., temperature, moisture, atmosphere, etc., upon the biological signals.

d) Photosynthetic nature of $^{14}CO_2$ production from labeled organics.

2. Light Fixation-Dark Release Experiment

The light fixation-dark release experiment has been developed as a laboratory assay, largely as a result of research conducted under the subject program. An enhanced light signal is readily detectable from local soil samples laden with photosynthetic organisms. A dark signal significantly higher than that of autoclaved controls is obtained from viable soils; however, in looking at a variety of soil types, it has proven extremely difficult to consistently detect photosynthesis. This probably reflects the actual state of the samples investigated, as evidenced by the difficulties noted in observing soil photosynthetic activity, with the ^{14}C and ^{35}S uptake experiment.

Additional experimentation using this assay on fresh soils from more diverse locations of the continental United States is recommended. More research is required to develop a suitable antimetabolite for this assay. A study of the effects of environmental conditions upon the biological responses would be desirable.

3. ^{14}C and ^{35}S Uptake Experiment

The ^{14}C and ^{35}S uptake experiment has been improved considerably during the subject program, and a very good life detection assay has resulted. Several areas

are recommended for additional study. As with the light fixation-dark release experiment, photosynthetic activity was not detected from the soil samples studied. It is recommended that the ^{14}C and ^{35}S uptake experiment be conducted in parallel with the light fixation-dark release assay on fresh soils from across the country.

4. ATP Production

The ATP production life detection assay is in a fairly advanced state of development, although the precision and quantitation of the assay can be further improved.

5. PO_4-P Uptake Experiment

The limiting factor, at present, for the PO_4-P uptake experiment is the analytic method for orthophosphate. The labeled triethylamine method is inadequate for the determination of soluble orthophosphate in RM9-containing materials extracted from soils. The colorimetric method may be suitable, but additional research is required to solve this problem. It is recommended that information be obtained on the rates of leaching of orthophosphate from soils, and on the rates of conversion of polyphosphate in soil extracts to orthophosphate.

6. Nitrogen-Based Life Detection Experiment

The absence of a nitrogen-based life detection

assay is conspicious in the AMML array of life detection experiments. It is recommended that one be devised and the feasibility examined in some future program.

7. General Comments

At present, the labeled release experiment and the light fixation-dark release experiment are the only ones which have been conducted in the "moist" rather than the "immersed" condition. It is recommended that further developmental work be performed on the other assays to determine the feasibility of conducting these tests with the addition of a minimum of liquid medium.

It is further recommended that additional integrated biological experiments be conducted to determine the synchronization of the responses. The integrated experiments should include examination for photosynthetic activity.

VII. LITERATURE CITED

1. A Study Toward Development of an Automated Microbial Metabolism Laboratory, final report, NASA Contract No. NASW-1507, Hazleton Laboratories, Inc., Falls Church, Va., 1 Oct. 1966-31 Aug. 1967.

2. Automated Microbial Metabolism Laboratory, final report, NASA Contract No. NASW-1731, Biospherics Inc., Rockville, Md., 1 Mar. 1970.

3. Automated Microbial Metabolism Laboratory, Third Quarterly Progress Report, NASA Contract No. NASW-1931, Biospherics Inc., Rockville, Md., 15 Mar. 1970.

4. Sugino, Y. and Miyoshi, Y., J. Biol. Chem., 239, 2360, 1964.

5. "Standard Methods for the Examination of Water and Wastewater," American Public Health Association, Inc., New York, Twelfth Edition, 1965.

6. Radioisotopic Biochemical Probe for Extraterrestrial Life, Second Annual Progress Report, NASA Contract No. NASr-10, Hazleton Laboratories, Inc., Falls Church, Va., March 1963.

7. Radioisotopic Biochemical Probe for Extraterrestrial Life, Third Annual Progress Report, NASA Contract No. NASr-10, Hazleton Laboratories, Inc., Falls Church, Va., March 1964.

8. Advanced Automated Microbial Metabolism Laboratory, Technical Proposal, Ames Research Center, National Aeronautics and Space Administration, Moffett Field, California, 3 April 1970.

9. "The Detection of Metabolism," G. V. Levin, D. G. Shaheen, W. A. Lindgren and E. Rich, Presented at International Congress for Microbiology, Mexico City, Mexico, August 1970.

APPENDIX I

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APPENDIX I

DETAILS OF GULLIVER V DEVELOPMENT
(Excerpts from the following report)

AMML
GULLIVER V
ADVANCED BREADBOARD
DEVELOPMENT PROGRAM

First Quarterly Progress Report
(May 6 to August 31, 1969)
Contract Number NASW-1931

Prepared by

BIOSPHERICS INCORPORATED
4928 Wyaconda Road
Rockville, Maryland 20853

for

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

September 15, 1969

B. Mechanical Design

The design for the Gulliver V is shown in Figure 9. It consists of an inlet for the soil sample (assumed to be from the Viking experiment auger distribution system); 5 cm^3 sample chambers (unused chambers are stored collapsed); a gas-seal inlet trap; an incubation chamber volume; a $^{14}CO_2$ getter and detector; a CO_2 absorber (scrubber); nutrient storage and dispensing mechanism and nutrient processing provisions (filtering and pre-gettering). Detailed drawings of the present design are shown in Appendix C.

As operation commences, chamber #1 is in position ready to receive the first soil sample. This chamber is filled by gravity from the experiment auger distribution system within the Viking lander. After the chamber is filled, the drive motor, coupled through the actuator cable, moves this chamber through the gas-seal inlet area to its first incubation position. Here, nutrient is added to the soil, and the initial incubation period is commenced.

While chamber #1 is located in the first incubation position, chamber #2 is open (from its collapsed stored position) ready to receive a soil sample upon command. Upon receipt of this command, the sample is added to chamber #2 which, when filled, is moved through the gas-seal area to the first incubation position where nutrient is added. Chamber #1 is now in the second incubation position, and monitoring of sample continues along with the second sample.

As each sample is brought into the incubation chamber, a scrubber-getter is opened for a few minutes so as to remove much of the atmospheric CO_2 brought in with the soil. This helps to prevent this atmospheric CO_2 from saturating the detector's getter.

The nutrient reservoir holds 16 ml of liquid nutrient that is applied upon command in increments of 2 ml. The nutrient injection actuator and valve mechanism operate in a sequence which seals the reservoir at all times except during an injection cycle.

The nutrient must be processed to remove $^{14}CO_2$ that is generated during the sterilization and subsequent storage cycle. For this, a pre-getter cartridge is added to the tube which interconnects the nutrient reservoir to the incubation chamber (Figure 10).

Mechanical components that resulted from the design task of this program have been detailed in the figures that are included in Appendix C. These drawings are in sufficient detal for fabrication.

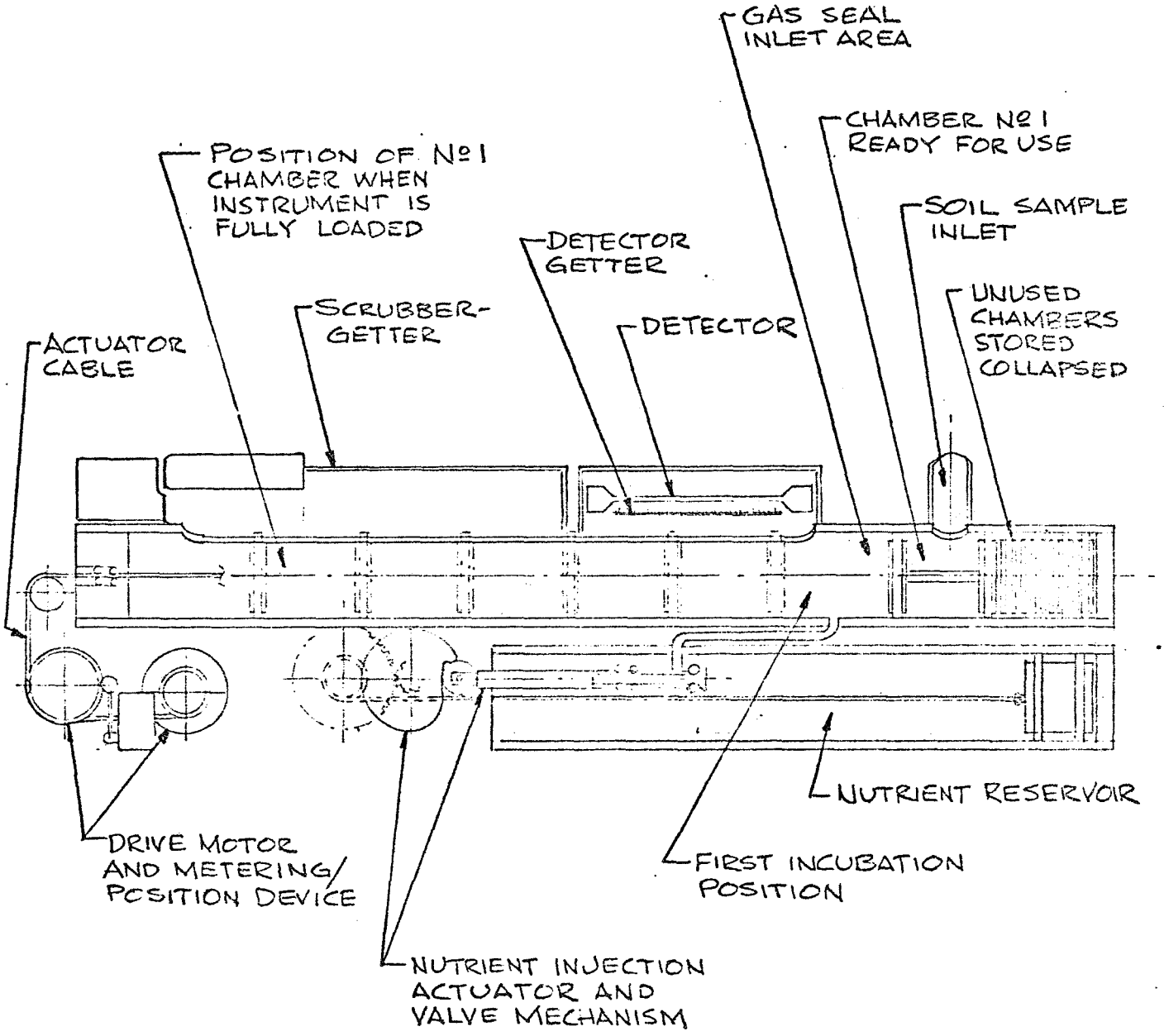

Figure 9 - Mechanical Design of Gulliver V

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Rockville, Maryland

E. Current Status of Development Program

During a meeting of the Viking Direct Biology Instrument Team early in August, a decision was reached by the members that the group would not recommend continuing the development of any back-up instrumentation for the 1973 Viking mission. This recommendation was transmitted to NASA Headquarters, and during a subsequent meeting with Biospherics, it was decided to discontinue the development program on the Gulliver V instrument. With this decision, effort on this research program was evaluated, and an attempt was made to bring the program to a point that would be most beneficial to NASA.

The hardware development program now has progressed to a point where components could be manufactured from the detailed design drawings. The design provides for bringing the soil sample into the incubation chambers through a gas-seal that can survive the abrasion caused by soil particles that interfere with the gaskets. This problem has been solved by the use of a "Quad-Kup" seal that is described in Appendix C. This type of seal provides a "knife edge" that scrapes and wipes the surface to keep the soil particles enclosed within each chamber.

A problem of storing the unused soil sample chambers in a minimum volume has been solved by use of a triple chain system to interconnect the sealing discs that form each sample chamber.

Atmospheric CO_2 that is brought in with the soil sample to the incubation chamber might saturate the detector's getter. This problem has

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been solved by use of a "scrubber" getter that will have excess gettering capacity combined with a mechanism to open this "scrubber" for a few minutes after each new soil sample has been brought into the incubation chamber.

The nutrient storage system requires a means to provide a seal that will contain the fluid during the sterilization cycle and still allow metering of the nutrient to the soil sample in the incubation chamber. This sealing has been accomplished by the cam-operated valve that is combined with the nutrient injection drive system.

Portions of the hardware for the Gulliver V have been constructed so as to mock-up the design concepts considered critical. Effort on the program was terminated just at the point that the final components were to be fabricated for use in the demonstration instrument. The mock-up components demonstrated the operational details satisfactorily. The materials (plastic rather than stainless steel) that had been used in fabricating the chamber housing and coupling mechanism exhibited a minor problem relating to non-uniform diameters which would be corrected in the final construction. The O-rings that were used rather than the special "Quad-Kup" rings now proposed did not provide the required scraping and wiping action.

Tests of the solid-state detector to be used to monitor the beta particles from ^{14}C showed that these devices can be used to detect 65% of the spectrum of beta particles that impinge its surface, with a resultant noise background of less than 10 counts per minute.

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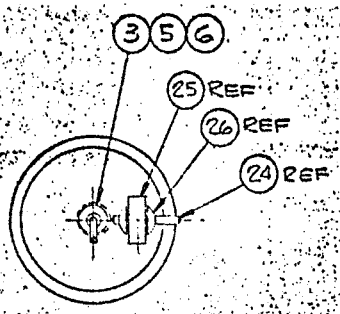
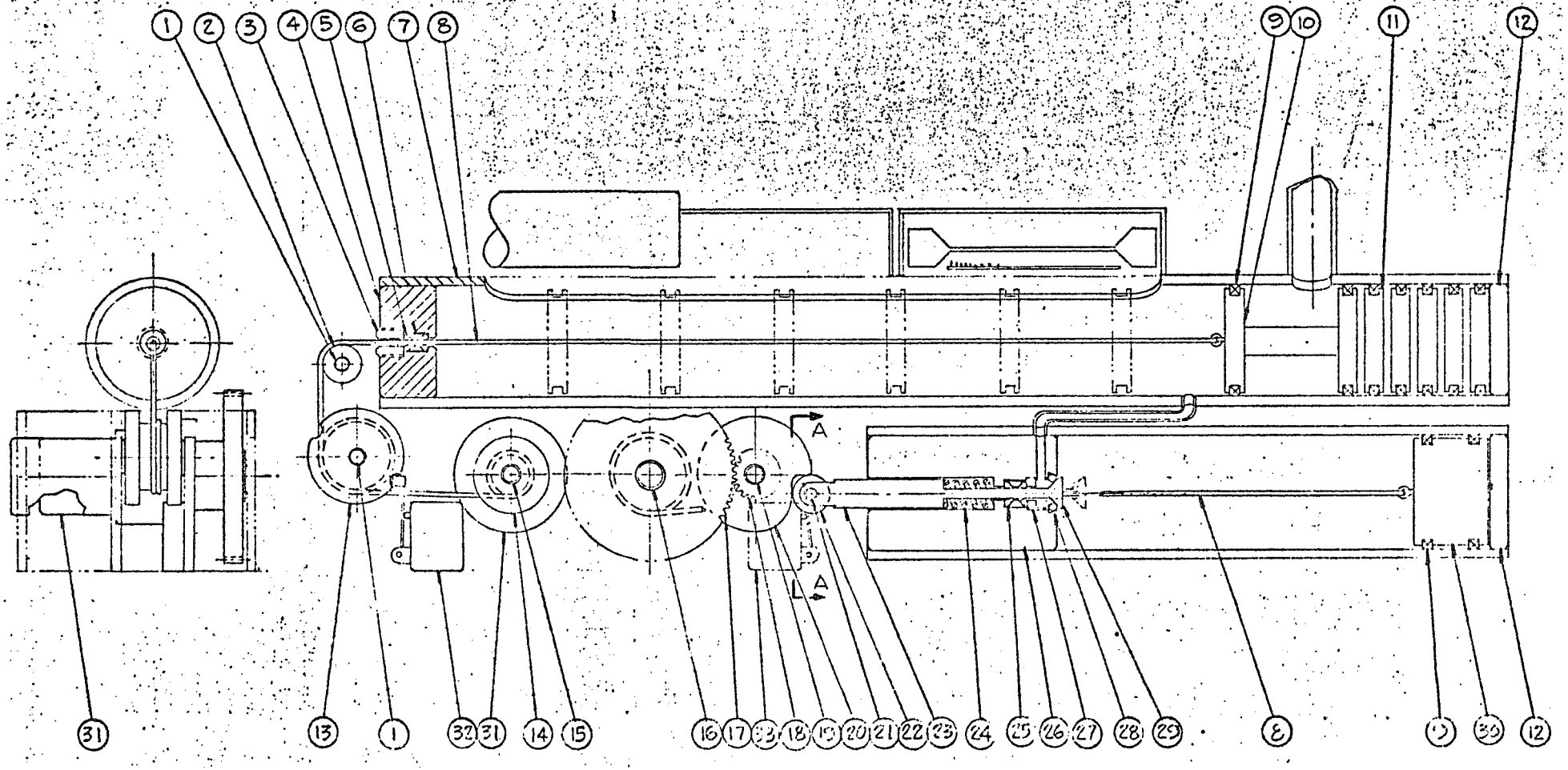
Title	Assembly Details for Gulliver V
Fig.	1
BIOSPHERICS INCORPORATED	
Rockville, Maryland	Sept '69 (Date) / (Init.) / (Scale)
Cont. No. NASW-1931	Report Title: First Quarterly Progress Report



VIEW A-A



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Appendix C

Detailed Drawings

of

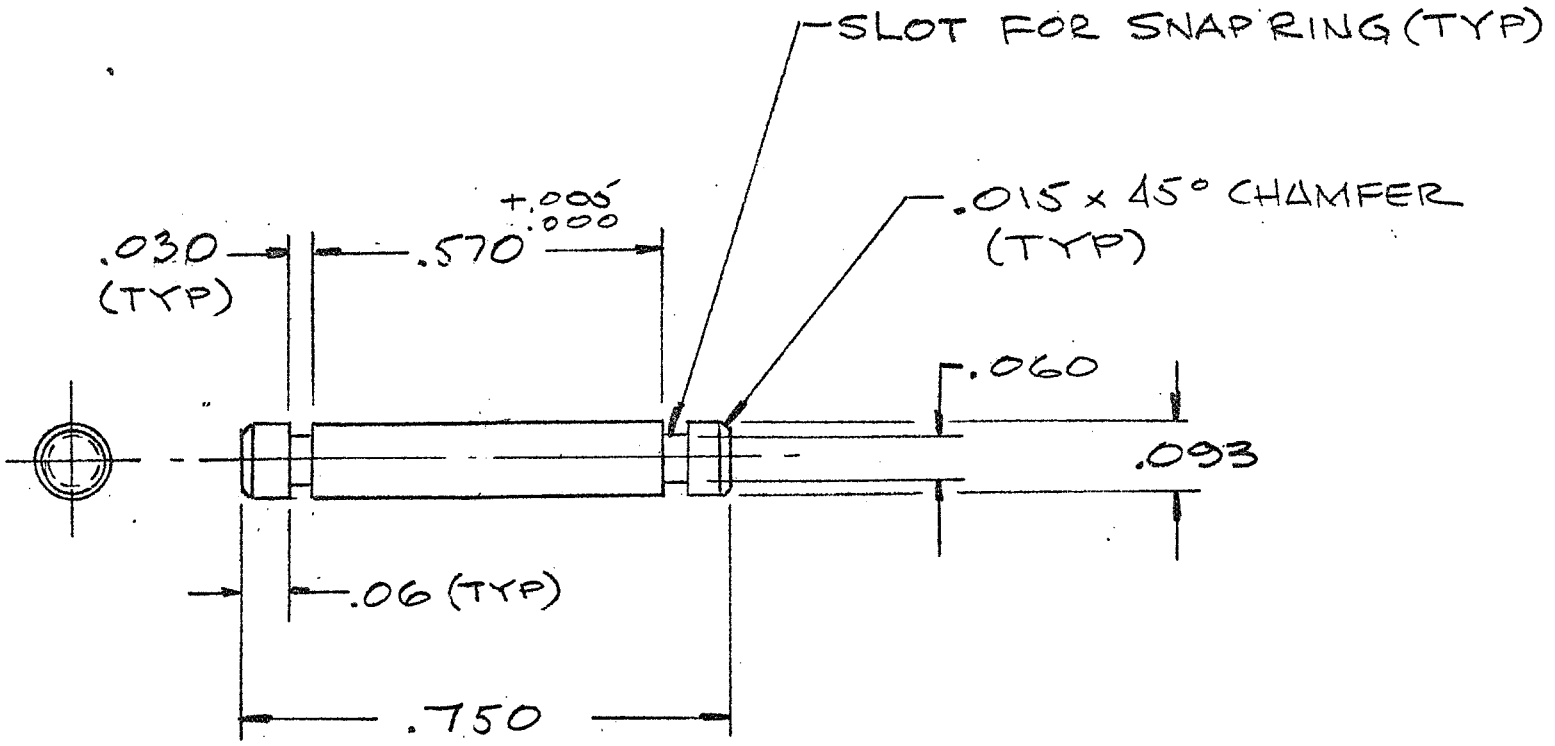
Gulliver V

Guide for Mechanical Parts Gulliver V Assembly Drawing

Item No.	Description
1	Shaft, Metering Drum and Idler
2	Idler
3	Seal Retainer
4	Insert, Front
5	O-Ring, Parker P/N 2-001
6	Spacer, O-Ring. Beveled
7.	Testing Chamber
8	Teflon Coated Copper Wire 24 AWG
9	Quad-Kup Seal Minnesota Rubber P/N - PD-692513
10	Double Disc
11	Disc
12	Plug, Rear
13	Metering Drum and Cam
14	Drive Drum
15	Shaft, Drive Drum Testing Chamber
16	Shaft, Drive Drum, Syringe
17	Spur Gear Boston Gear P/N 17AN80
18	Spur Gear Boston Gear P/N 175N20
19	Shaft Drive Drum Syringe
20	Cam Valve Plunger

Guide for Mechanical Parts Gulliver V Assembly Drawing Continued

Item No.	Description
21	Stem Cam Roller
22	Cam Roller
23	Plunger Guide
24	Syringe, Valve Plunger
25	O-Ring Parker P/N 2-003
26	Plug Front
27	Retainer O-Ring
28	O-Ring Parker P/N 2-006
29	Plunger
30	Piston



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BIOSPHERICS INC.
ROCKVILLE, MD.

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SHAFT, METERING DRUM AND IDLER
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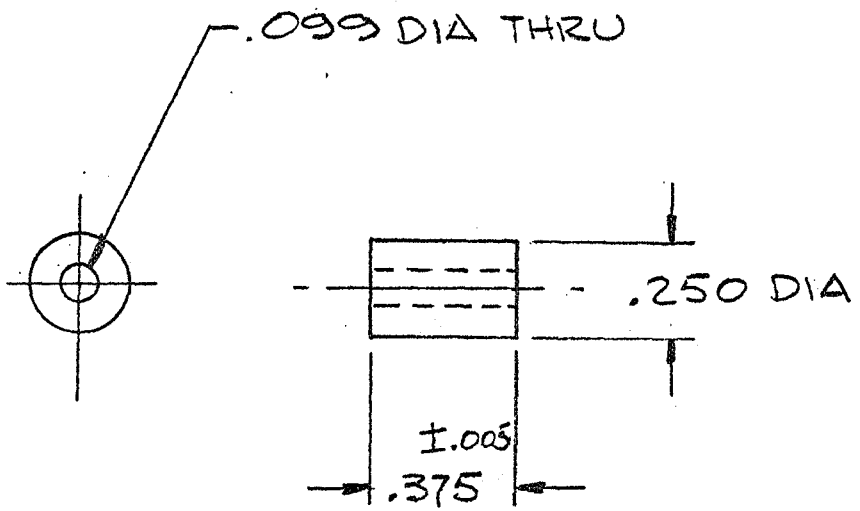
GULIVER V
CONT. NO. NASW-1931



BIOSPHERICS INC.
ROCKVILLE, MD.

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FIN; CHROMATE

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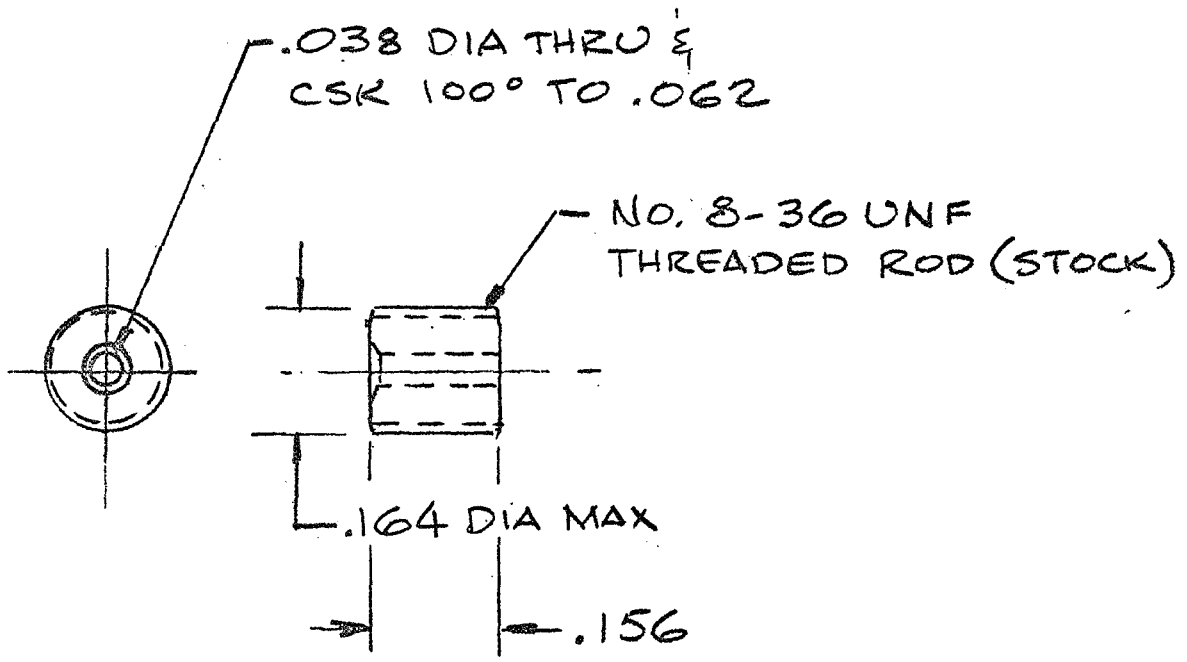
IDLER
TESTING CHAMBER
GULIVER V
CONT. NO. NASW-1931



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BIOSPHERICS INC.
ROCKVILLE, MD.

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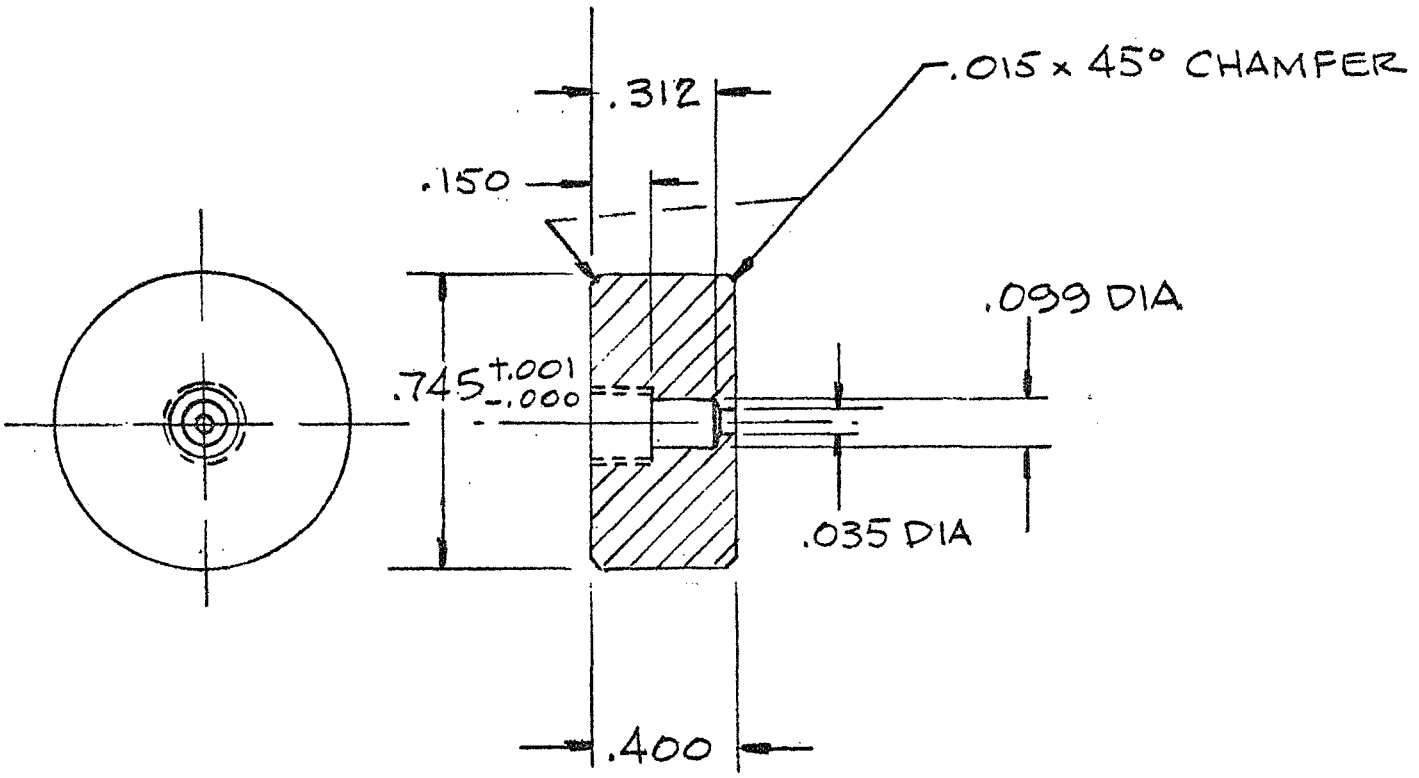
SEAL RETAINER
TESTING CHAMBER
GULLIVER V
CONT. NO. NASW-1931



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ROCKVILLE, MD.

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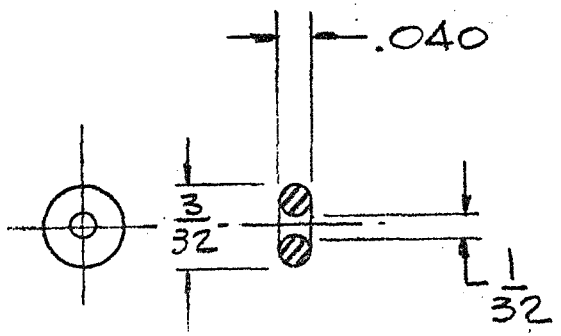
GULIVER V
CONT. NO. NASW-1931



O-RING P/N 2-001
PARKER SEAL CO.

BIOSPHERICS INC.
ROCKVILLE, MD.

SCALE: 4/1

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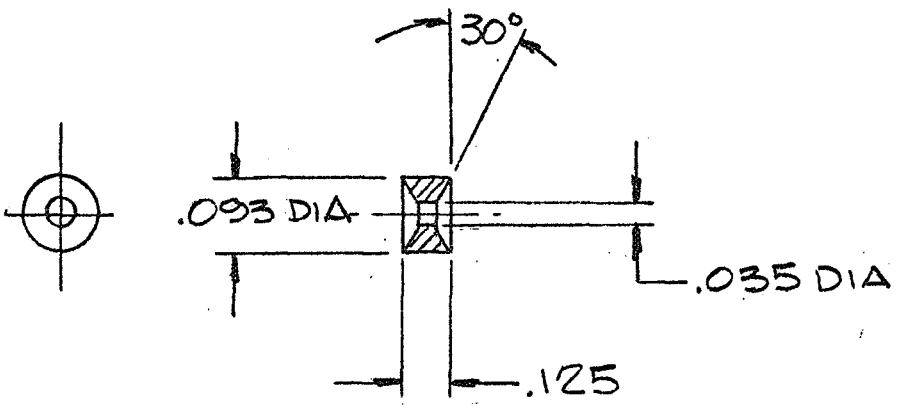
TESTING CHAMBER
GULIVER V
CONT. NO. NASW-1931



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MTL; AL ALY, 6061-T6
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DWG No 6
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GULIVER V
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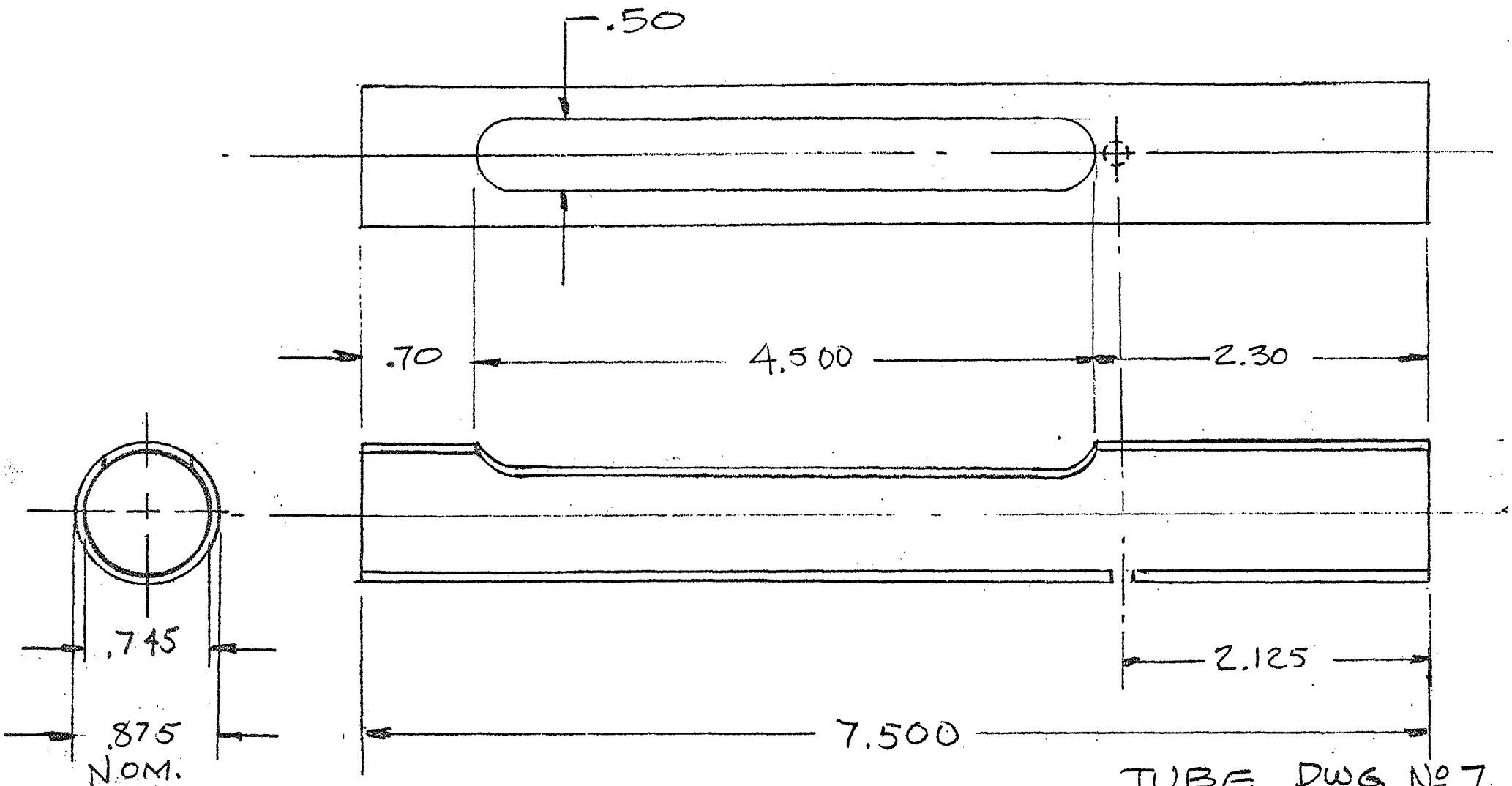

TUBE DWG Nº 7

SCALE: 1/1

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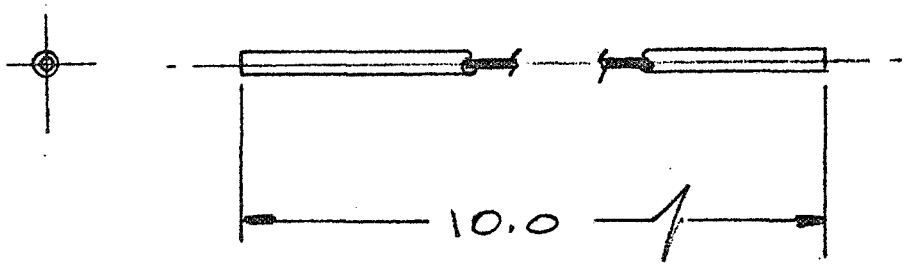
GULIVER V
CONT. NO. NASW-1931



TEFLON COATED COPPER WIRE
24 AWG

BIOSPHERICS INC.
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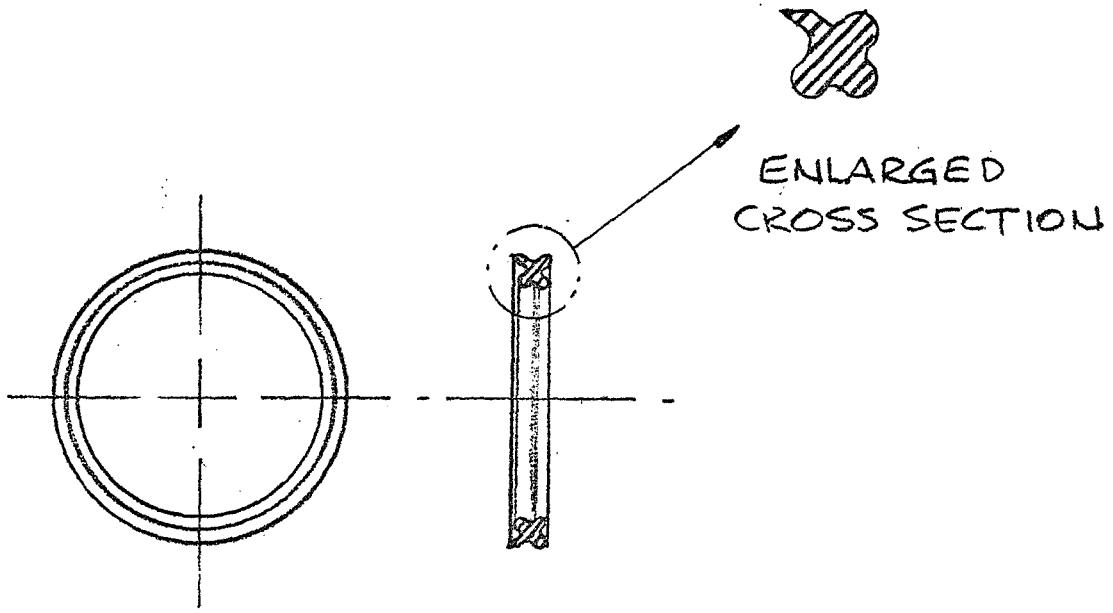
GULIVER V
CONT. No. NASW-1931



QUAD-KUP® SEAL

FOR .745 ID CYLINDER
MTL: VITON
MINNESOTA RUBBER CO.

BIOSPHERICS INC.
ROCKVILLE, MD.

SCALE: 2/1

DWG № 9
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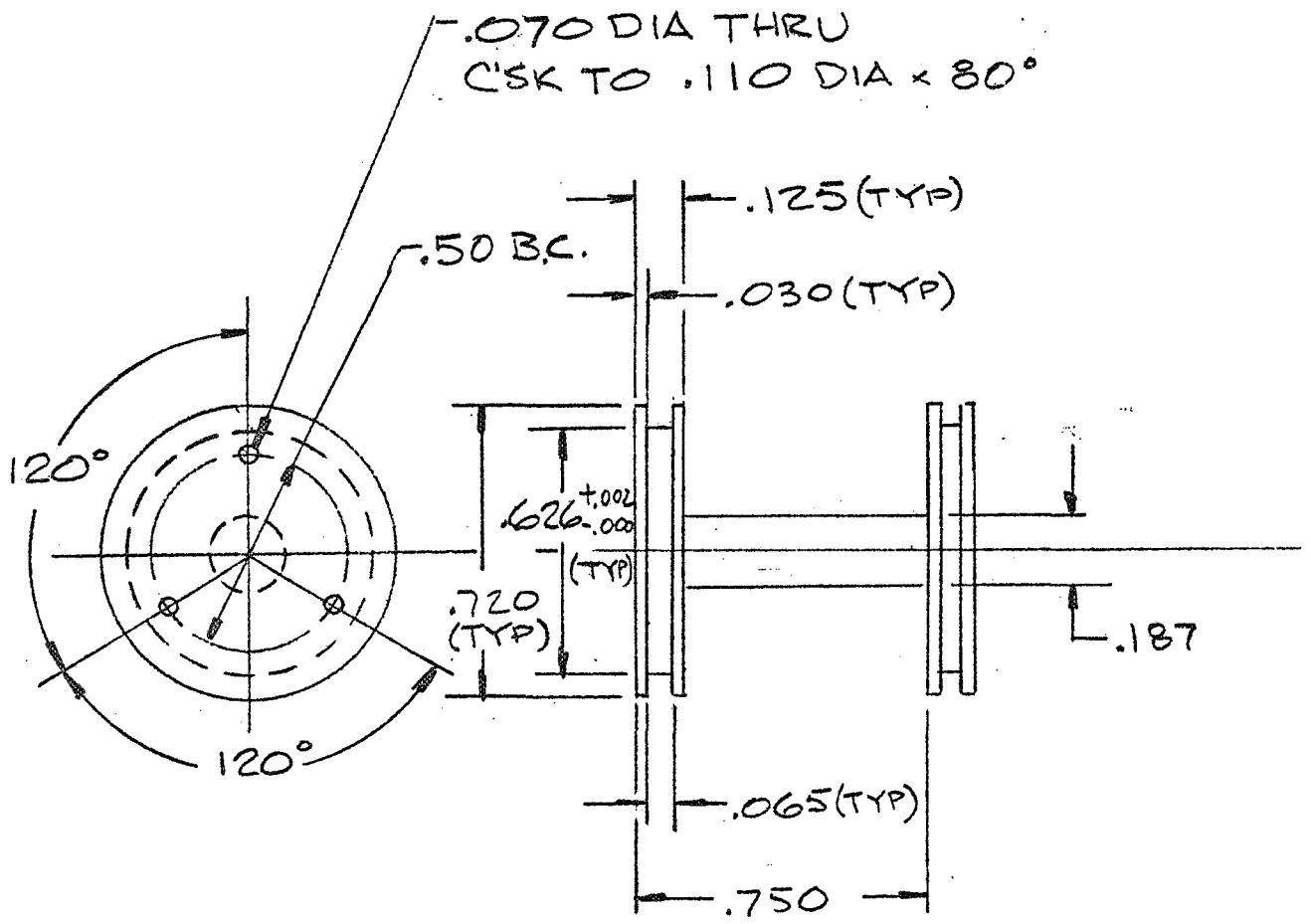
TESTING CHAMBER
GULIVER V
CONT. No. NASW-1931



BIOSPHERICS INC.
ROCKVILLE, MD.

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FINISH: CHROMATE

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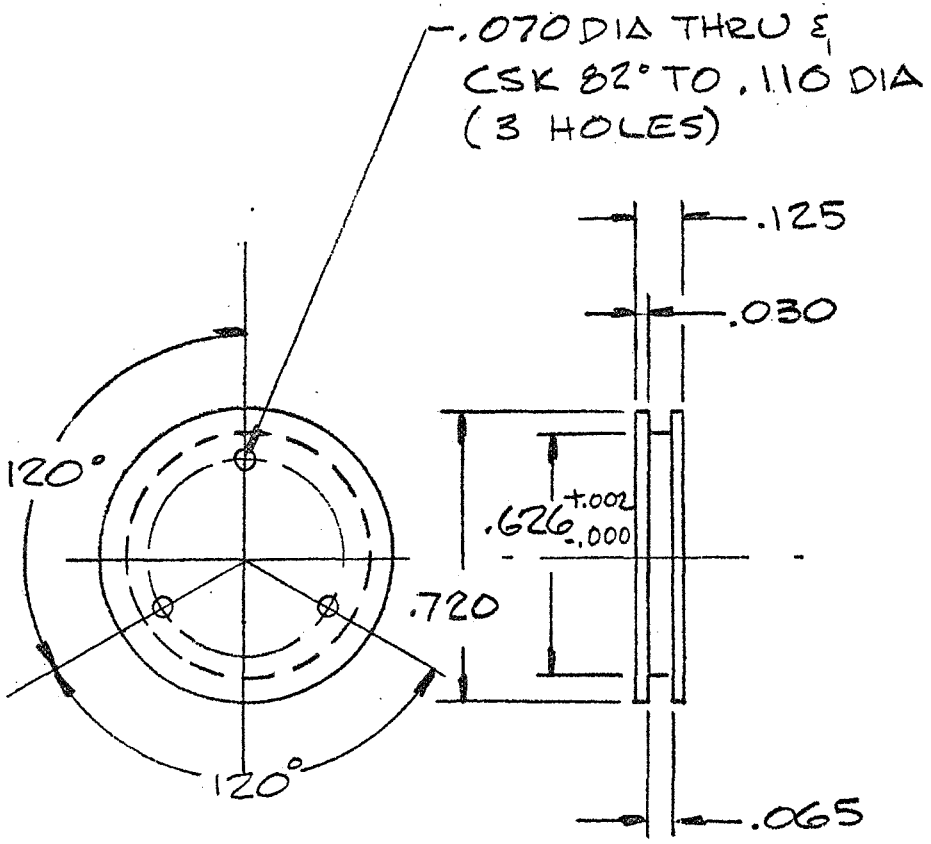
DOUBLE DISC
TESTING CHAMBER
GULIVER V
CONT. NO. NASW-1931



BIOSPHERICS INC.
ROCKVILLE, MD.

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DISC
TESTING CHAMBER
GULIVER V
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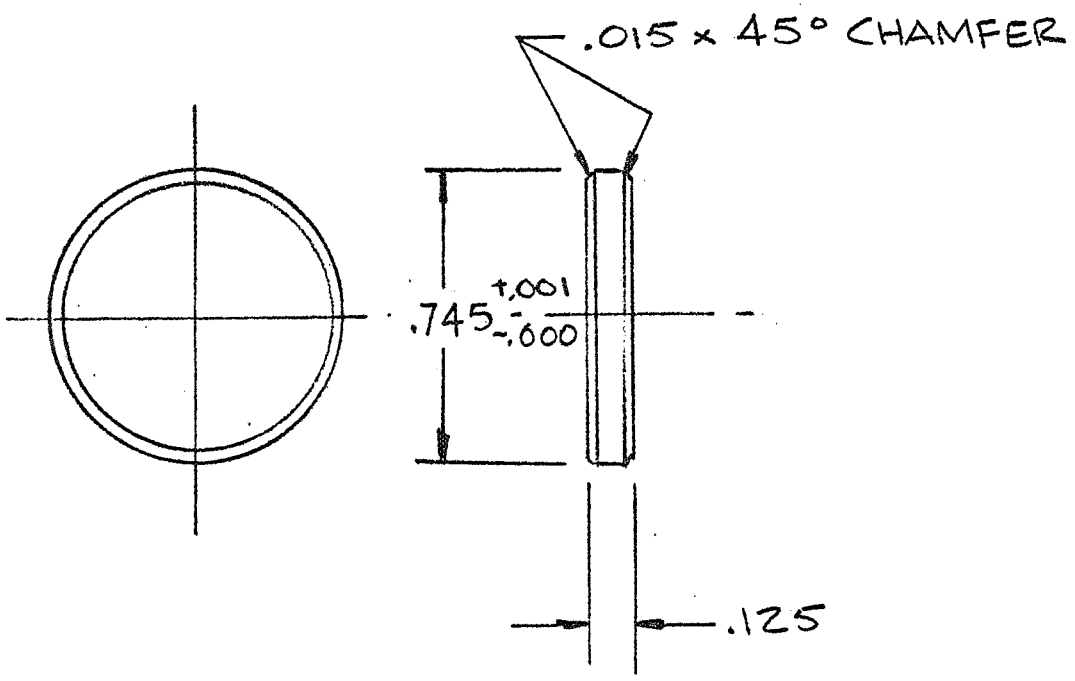

BIOSPHERICS INC.
ROCKVILLE, MD.

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MTL: AL ALY, 6061-T6
FINISH: CHROMATE

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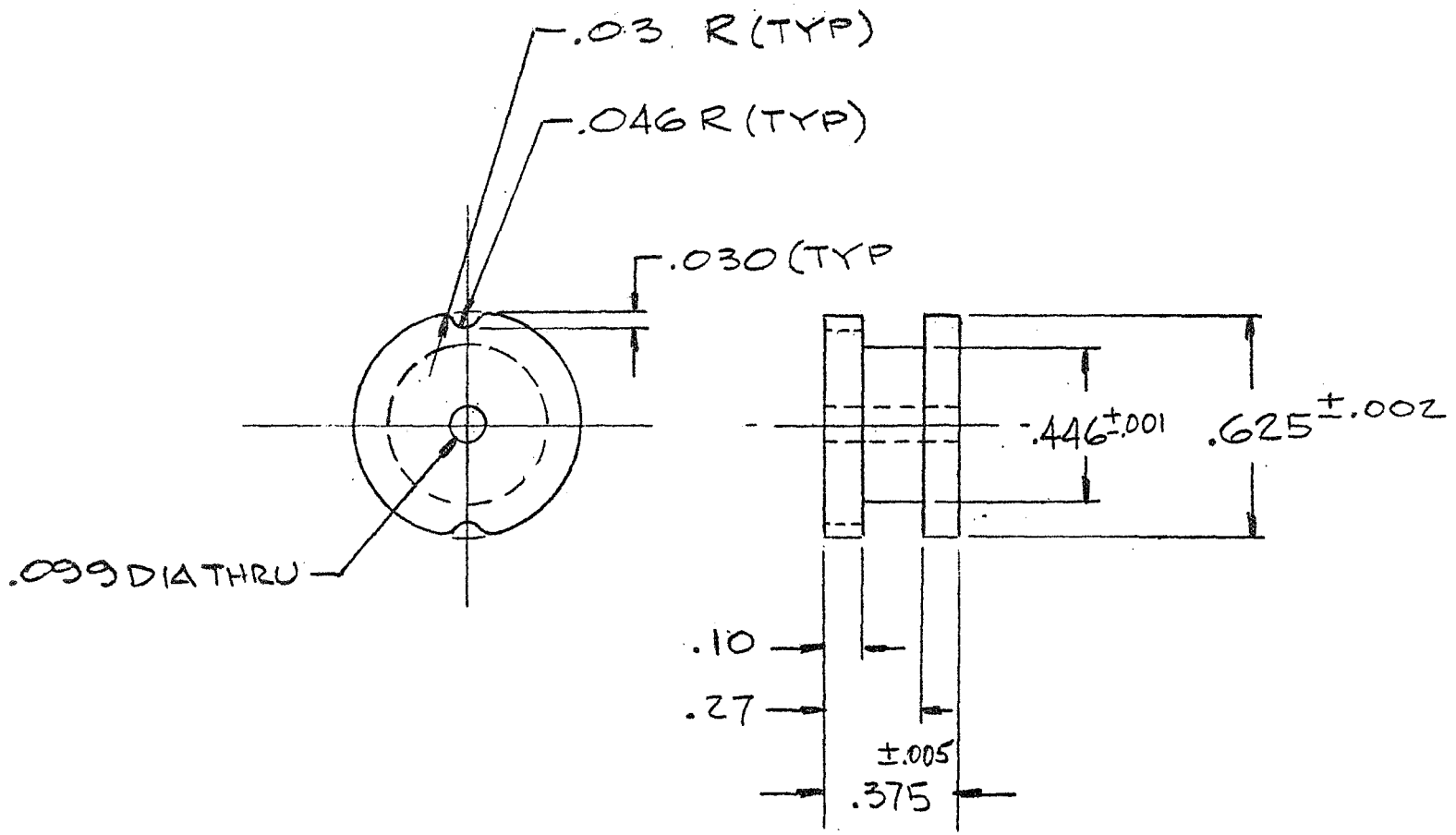
TESTING CHAMBER
GULIVER V
CONT. NO. NASW-1931



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ROCKVILLE, MD.

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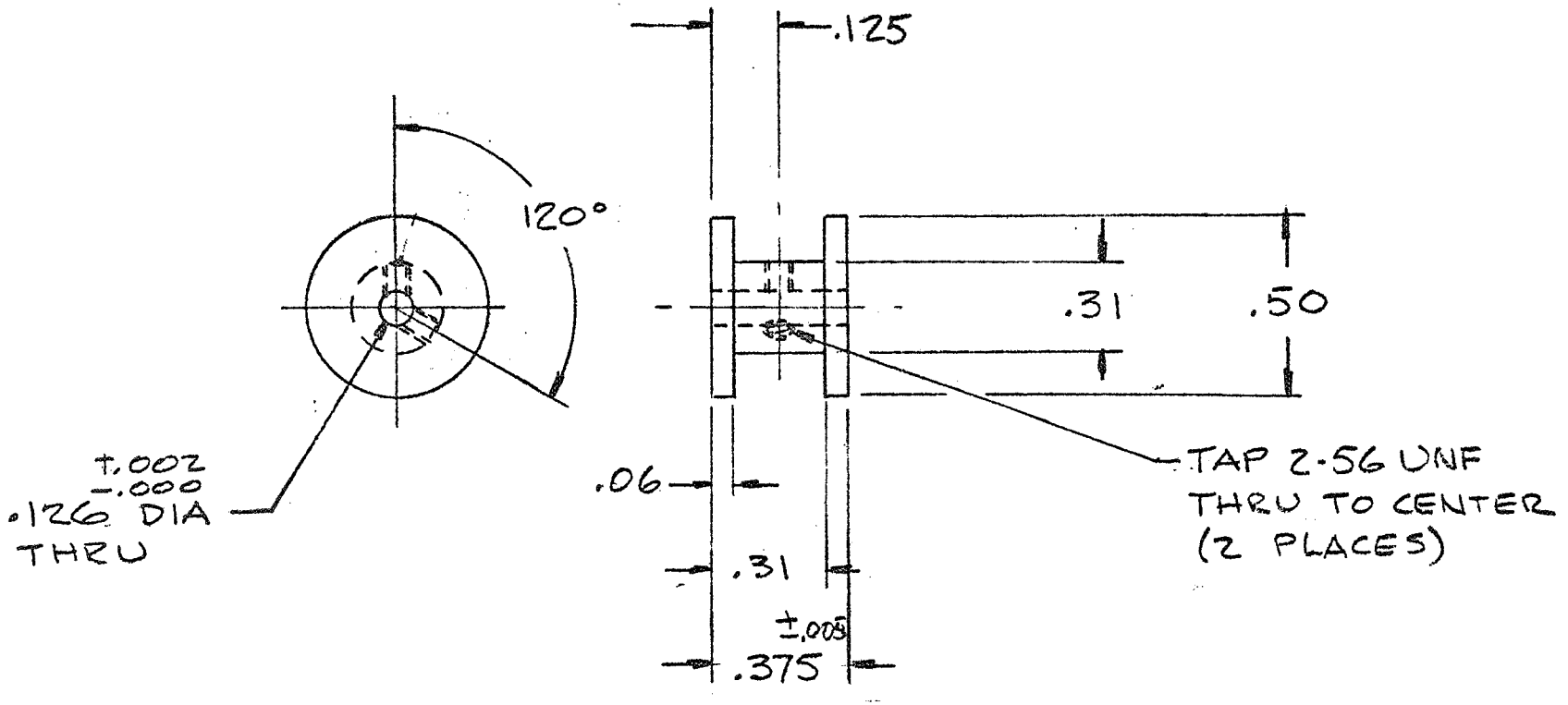
TESTING CHAMBER
GULIVER V
CONT. NO. NASW-1931



BIOSPHERICS INC.
ROCKVILLE, MD.

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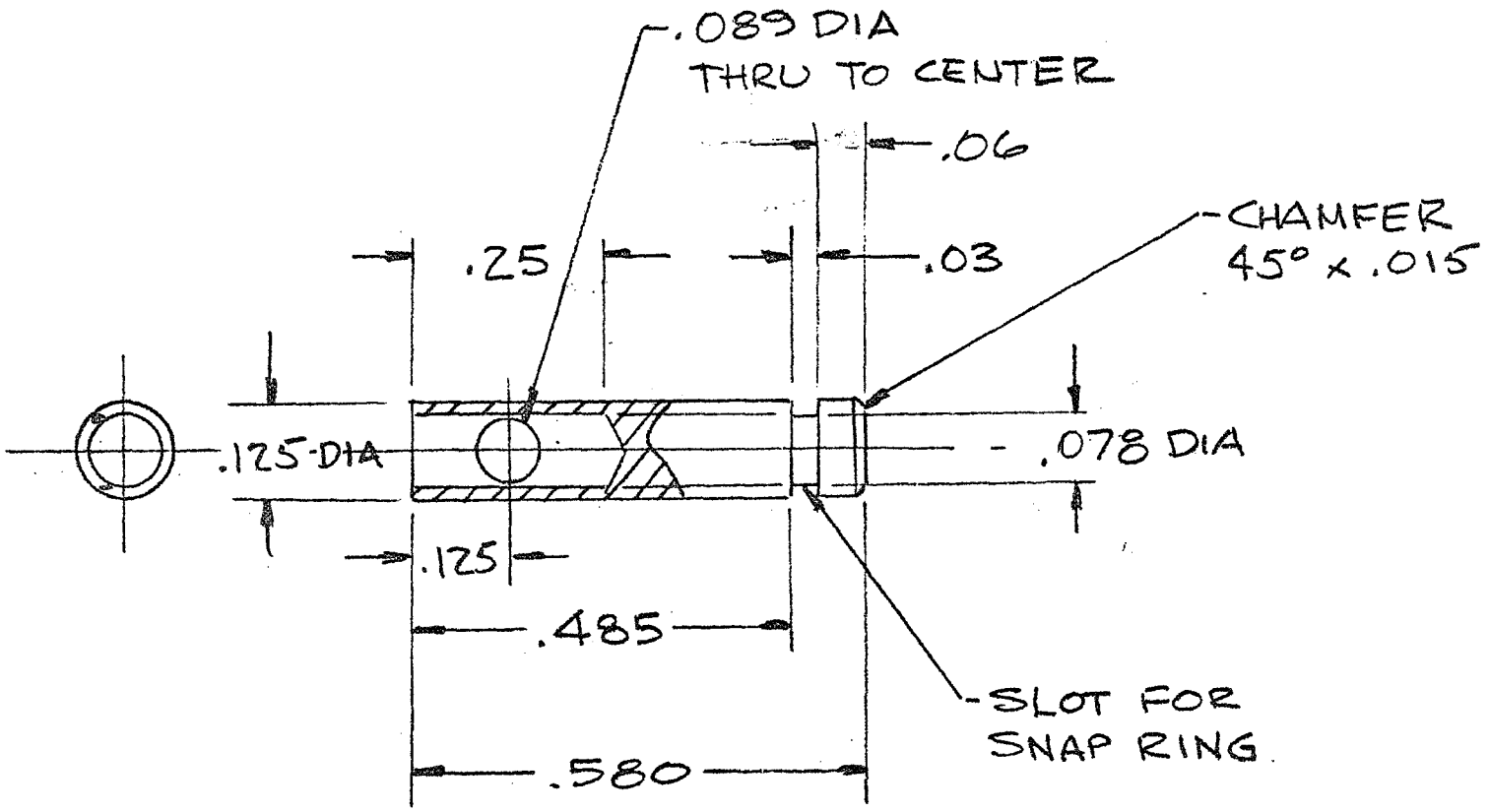
FIN: CHROMATE

DWG NO 14
DRIVE DRUM
TESTING CHAMBER
GULIVER V
CONT. NO. NASW-1931



BIOSPHERICS INC.
ROCKVILLE, MD.

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MTL: STL

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SHAFT, DRIVE DRUM
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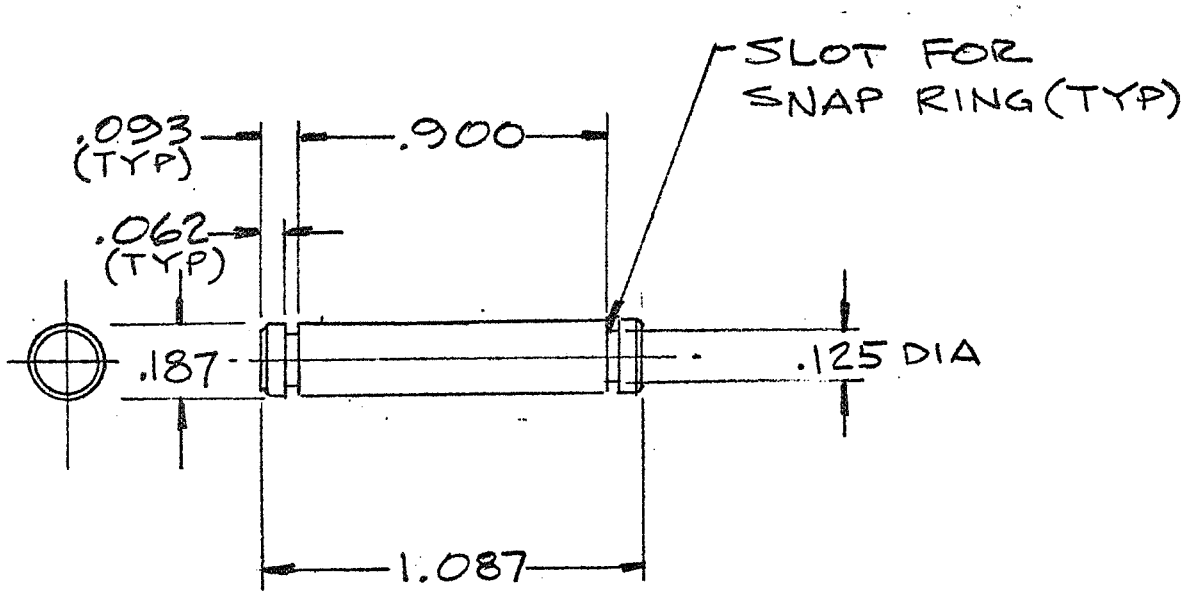
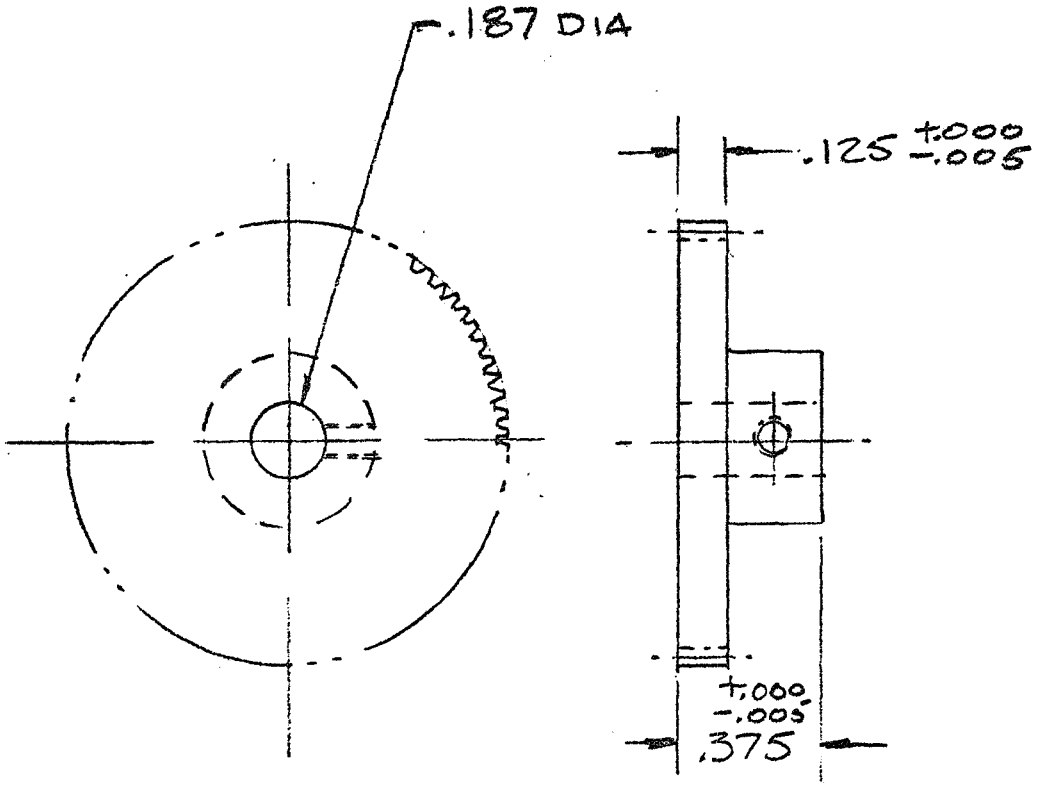
GULLIVER V
CONT. NO. NASW-1931



BIOSPHERICS INC.
ROCKVILLE, MD.

SCALE: 2/1
MTL: STL

DWG No 16
SHAFT, DRIVE DRUM
SYRINGE
GULLIVER V
CONT. No. NASW-1931

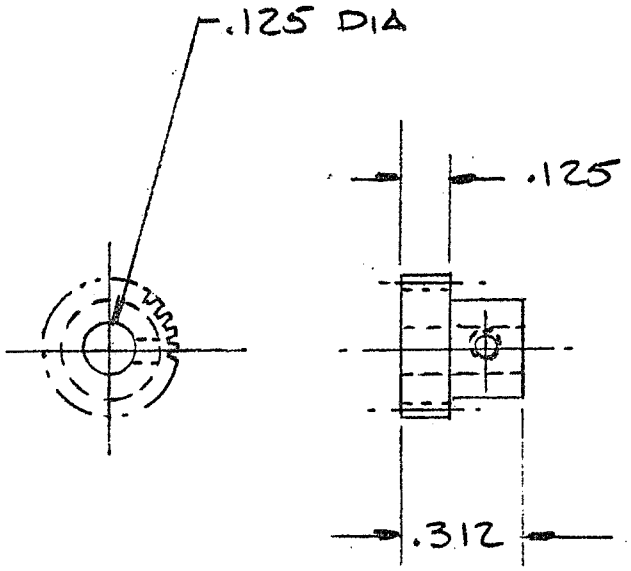


SPUR GEAR
ALUMINUM, TYPE 2024
72 PITCH
80 TEETH
PITCH DIA 1.111
P/N 17AN80
BOSTON GEAR

BIOSPHERICS INC.
ROCKVILLE, MD.

SCALE: 2/1

DWG NO 17
CONTROL DWG
SYRINGE
GULLIVER V
CONT. NO NASW-1931

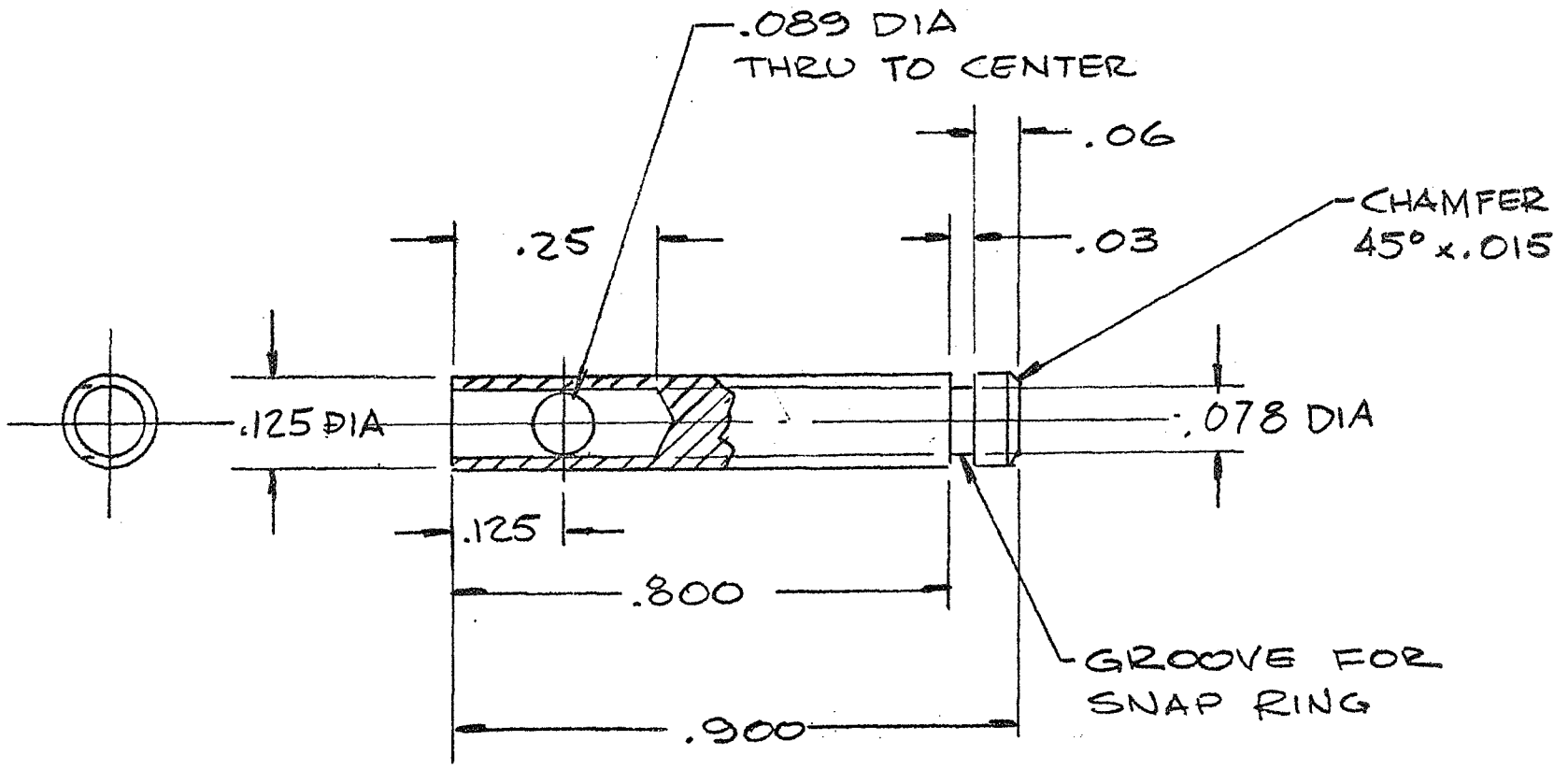


SPUR GEAR
ALUMINUM
72 PITCH
20 TEETH
PITCH DIA .2778
P/N 17SN20
BOSTON GEAR

BIOSPHERICS INC.
ROCKVILLE, MD.

SCALE: 2/1

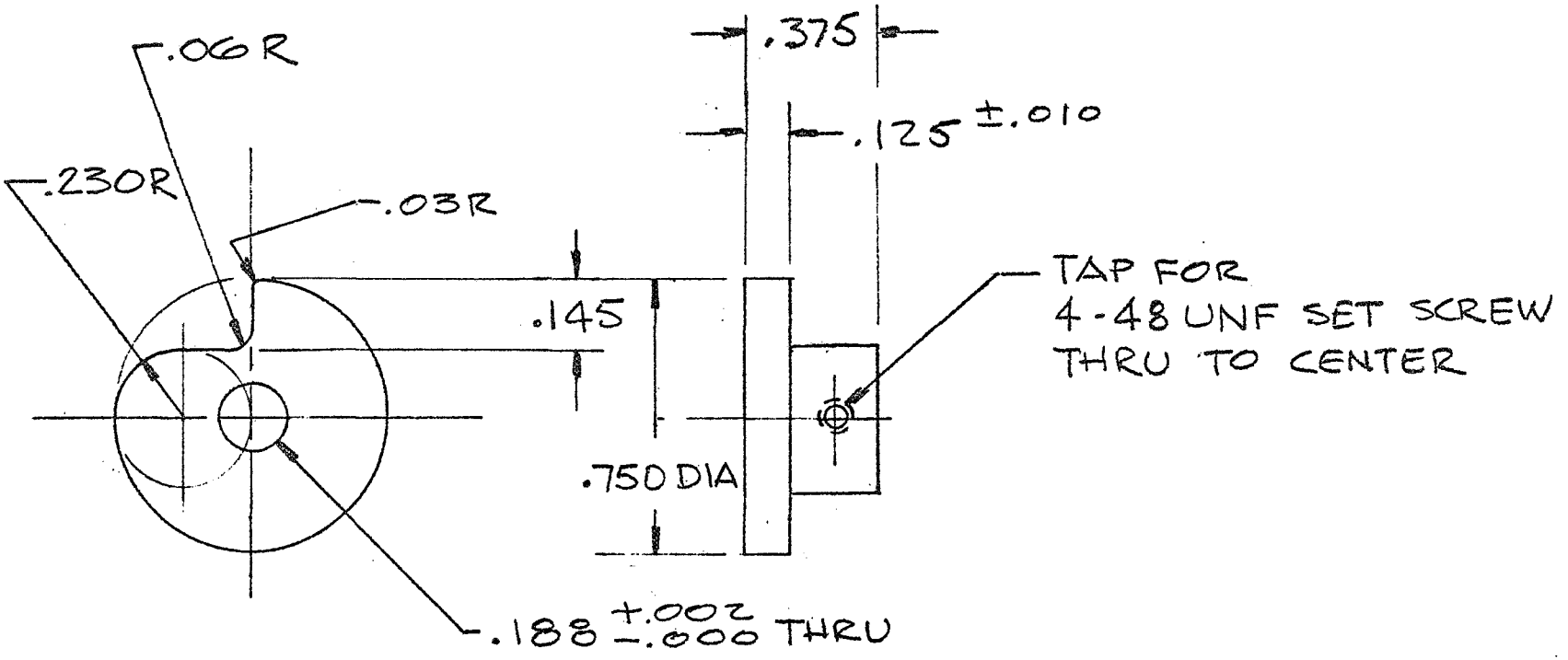
DWG NO 18
CONTROL DWG
SYRINGE
GULLIVER V
CONT. NO. NASW-1931



BIOSPHERICS INC.
ROCKVILLE, MD.

SCALE : 4/1
MTL : STL

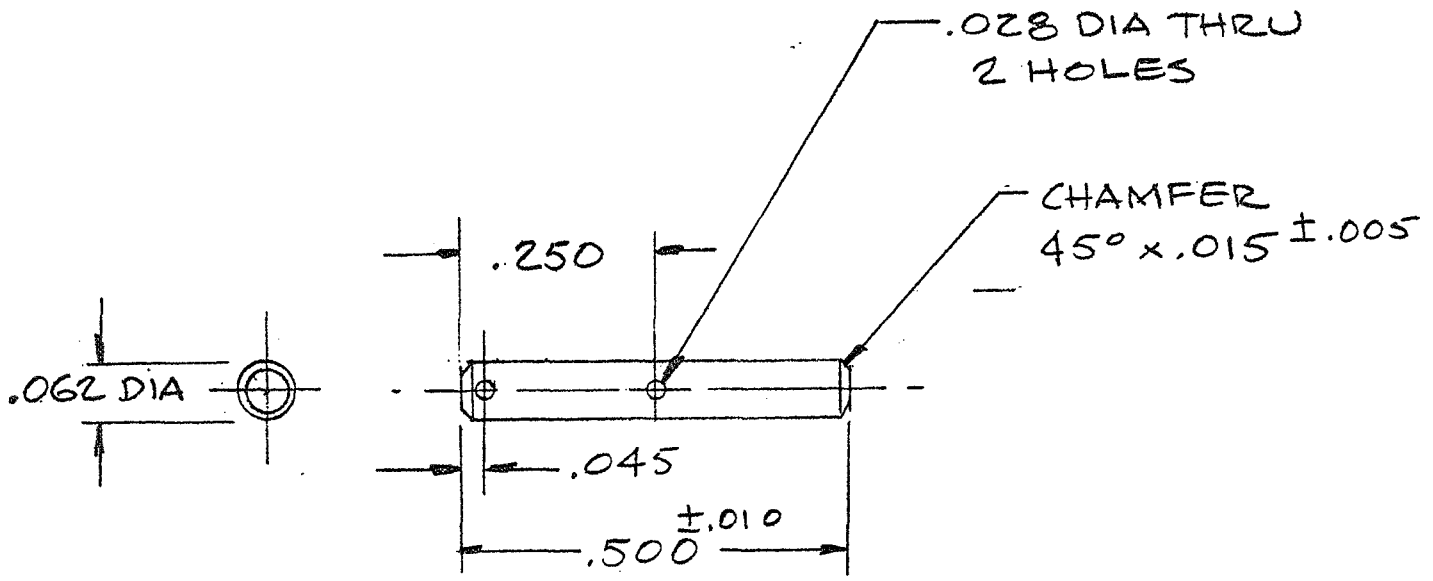
DWG NO 19
SHAFT, DRIVE DRUM
SYRINGE
GULLIVER V
CONT. NO. NASW-1931



BIOSPHERICS INC.
ROCKVILLE, MD.

SCALE: 2/1
MTL: AL ALY, 6061-T6
FINISH: CHROMATE

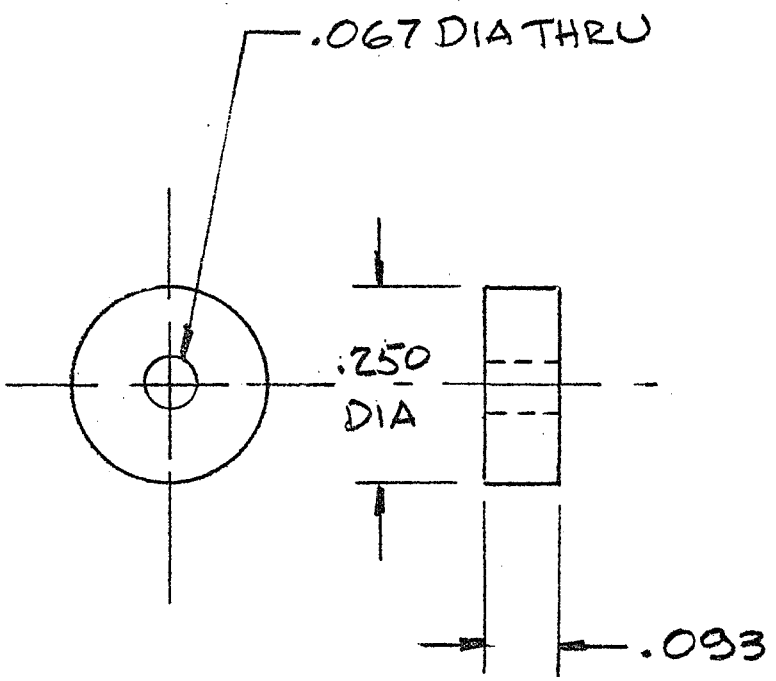
DWG Nº 20
CAM, VALVE PLUNGER
SYRINGE
GULIVER V
CONT. No. NASW-1931



BIOSPHERICS INC.
ROCKVILLE, MD.

SCALE: 4/1
MTL: STL

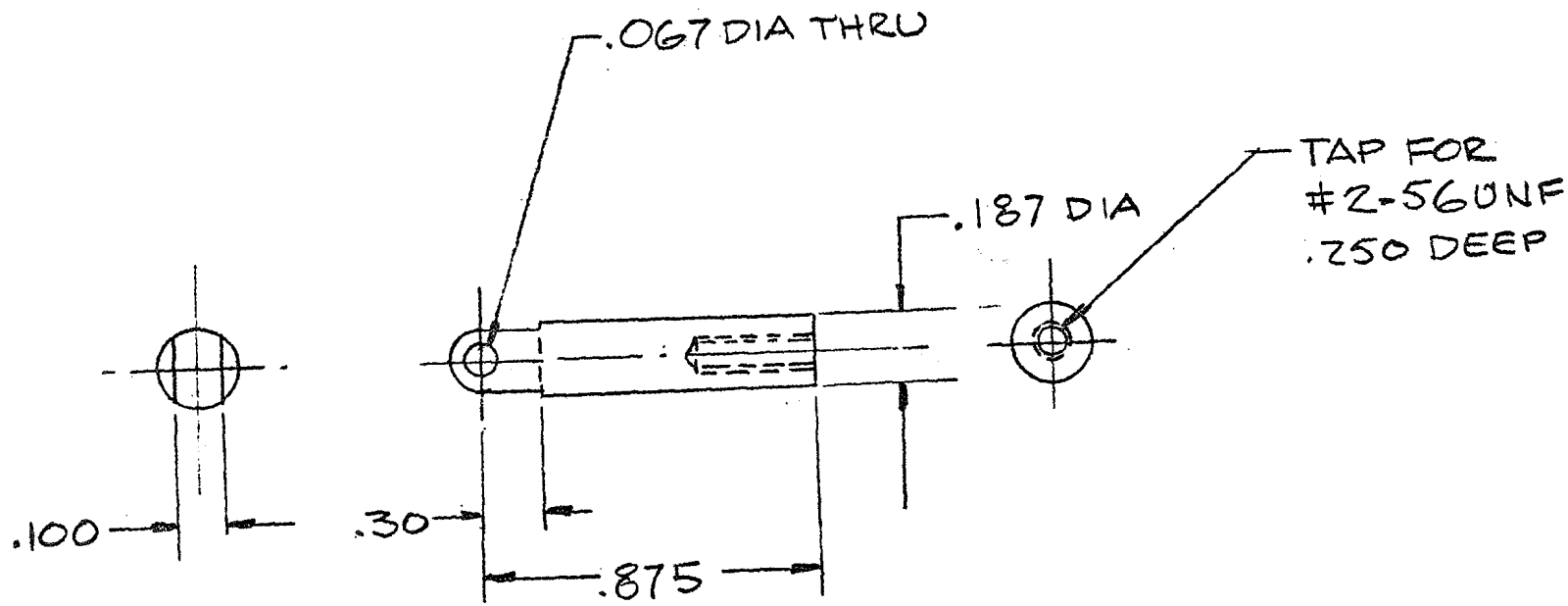
DWG NO 21
STEM, CAM ROLLER
SYRINGE
GULLIVER V
CONT. NO. NASW-1931



BIOSPHERICS INC.
ROCKVILLE, MD.

SCALE: 4/1
MTL: AL ALY, 6061-T6
FIN: CHROMATE

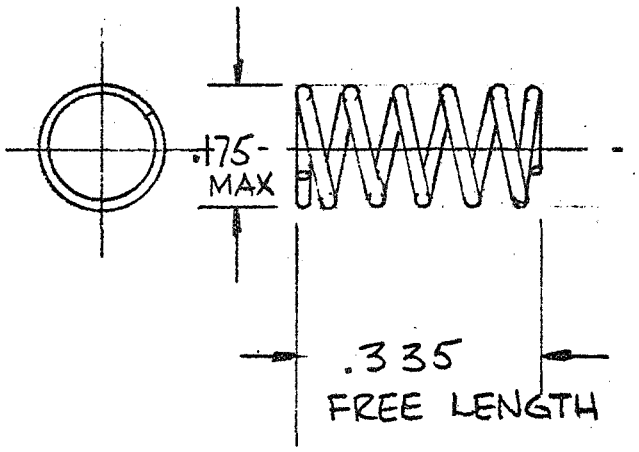
DWG Nº 22
CAM ROLLER
SYRINGE
GULLIVER V
CONT. No. NASW-1931



BIOSPHERICS INC.
ROCKVILLE, MD.

SCALE: 2/1
MTL: AL ALY, 6061-T6
FIN: CHROMATE

DWG No 23
PLUNGER GUIDE
SYRINGE
GULIVER V
CONT. NO. NASW-1931

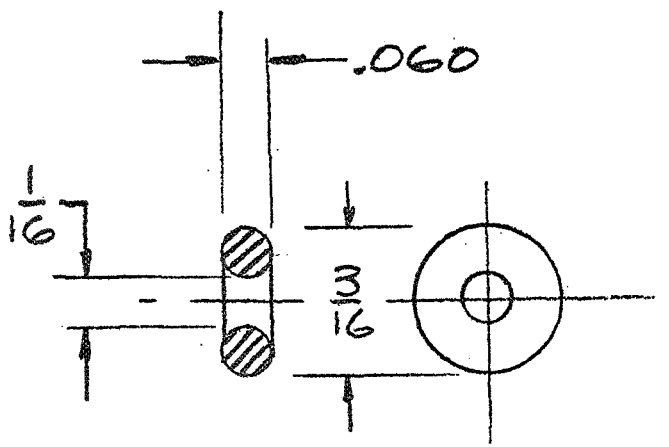


5 lb RATING

BIOSPHERICS INC.
ROCKVILLE, MD.

SCALE: 4/1
MTL: STL

DWG NO 24
SPRING, VALVE PLUNGER
SYRINGE
GULLIVER V
CONT. NO. NASW-1931

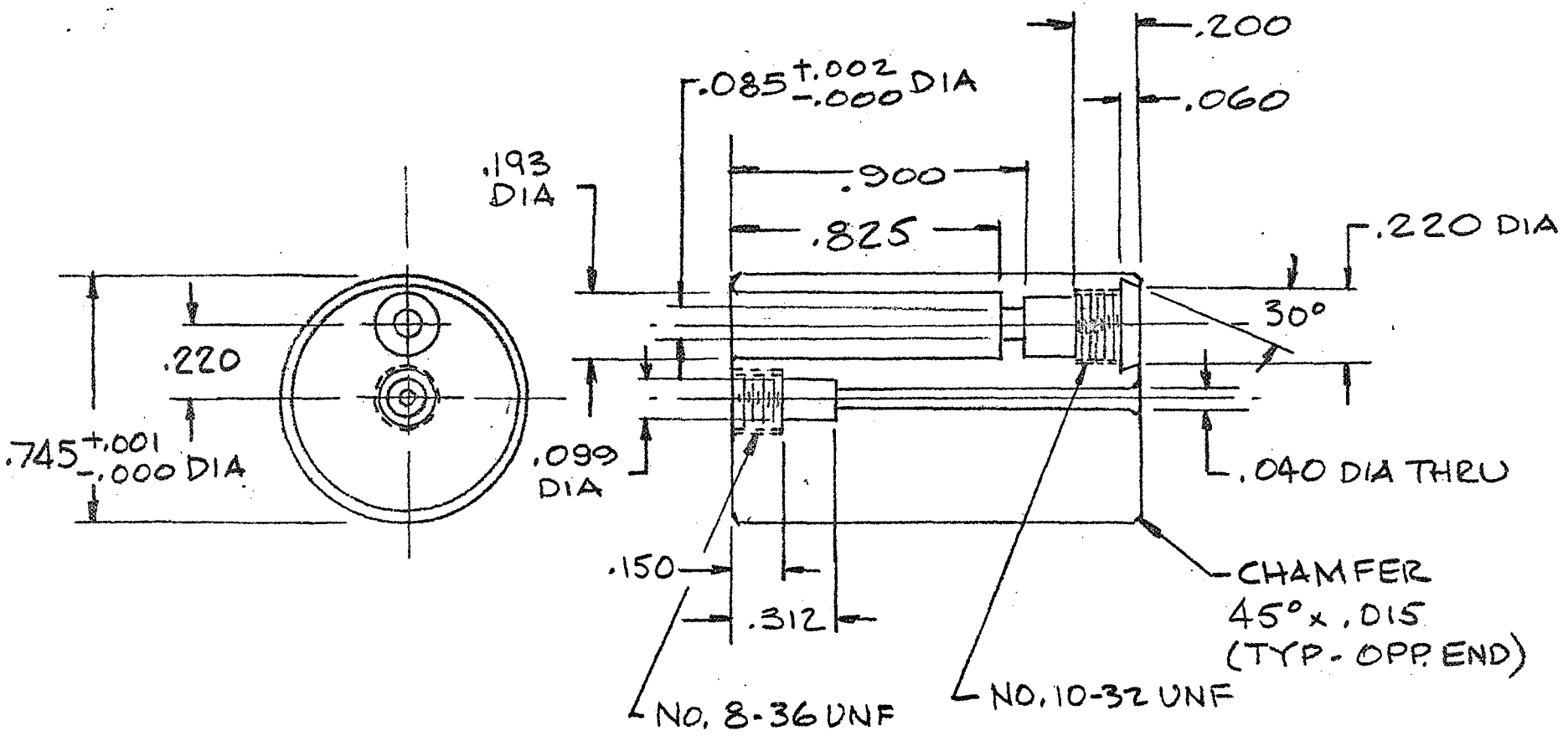


O-RING P/N 2-003
PARKER SEAL CO.

BIOSPHERICS INC.
ROCKVILLE, MD.

SCALE: 4/1

DWG Nº 25
CONTRA DWG
SYRINGE
GULLIVER V
CONT. NO. NASW-1931

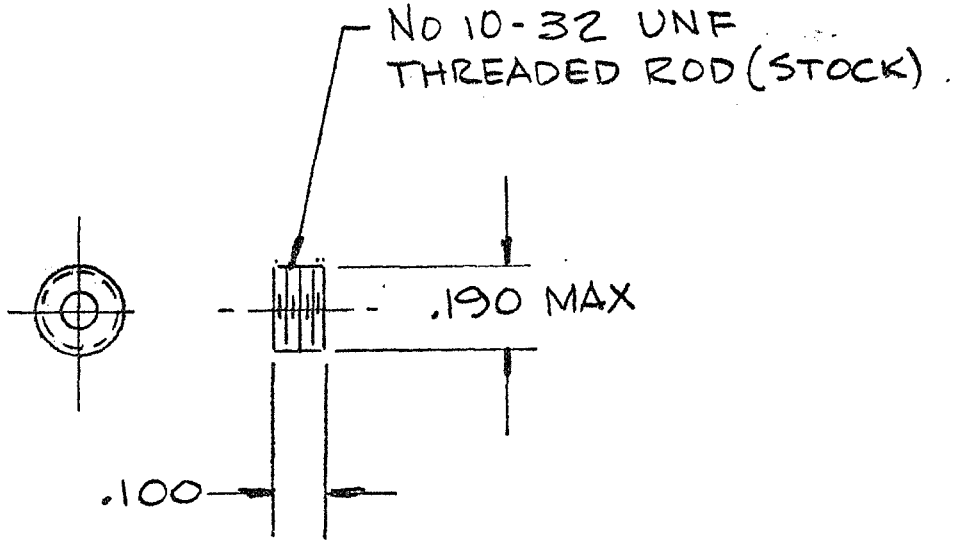


DWG No. 26

BIOSPHERICS INC
ROCKVILLE, MD.

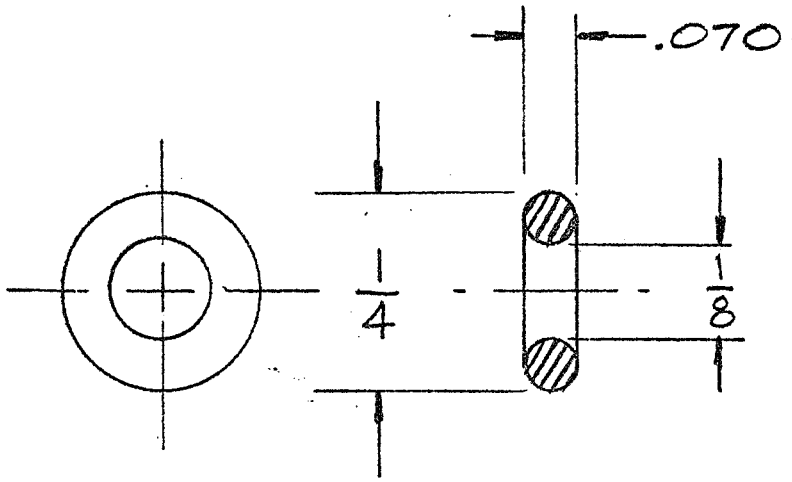
SCALE: 2/1

PLUG, FRONT
SYRINGE
GULLIVER V
CONT. No. NASW-1931



BIOSPHERICS INC.
ROCKVILLE, MD.

DWG No 27
RETAINER, O-RING
SYRINGE
GULLIVER V
CONT. No. NASW-1931

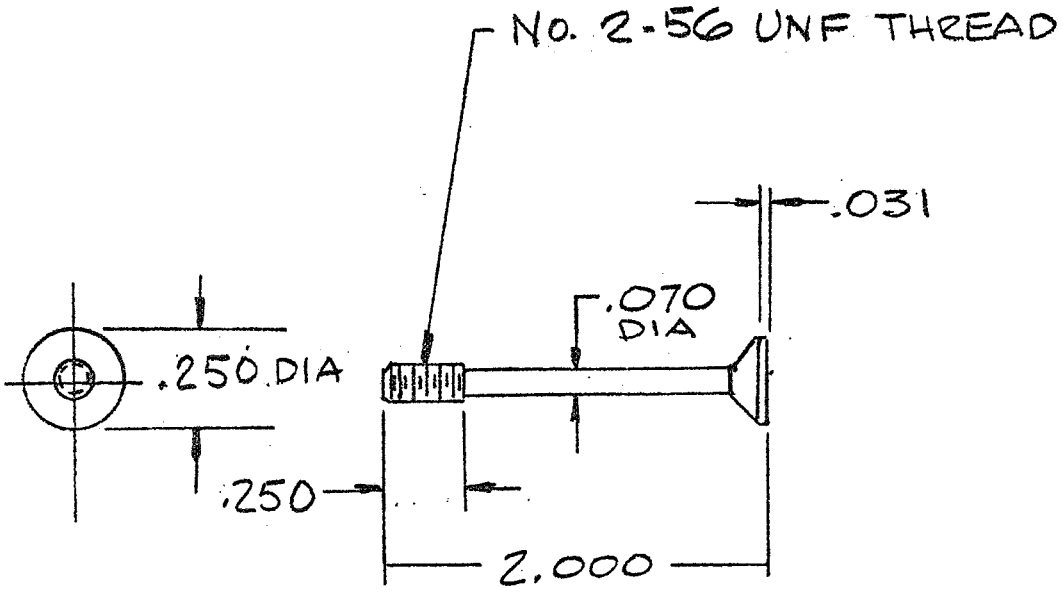


O-RING P/N 2-006
PARKER SEAL CO.

BIOSPHERICS INC.
ROCKVILLE, MD.

SCALE: 4/1

DWG NO 28
CONTROL DWG
SYRINGE
GULLIVER V
CONT. NO. NASW-1931

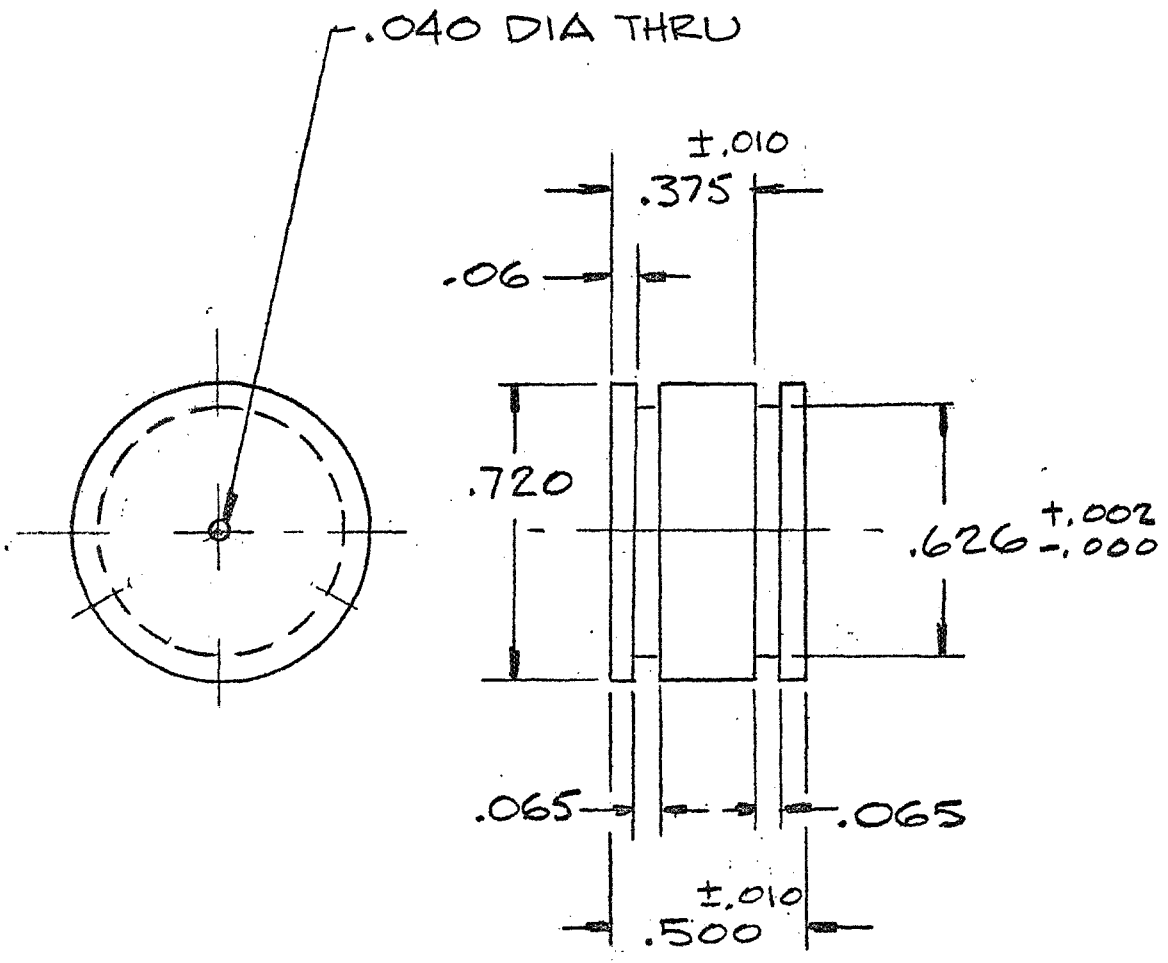


BIOSPHERICS INC.
ROCKVILLE, MD

SCALE: 2/1
MTL: AL ALY, 6061-T6
FIN: CHROMATE

DWG Nº 29

PLUNGER
SYRINGE
GULLIVER V
CONT. No. NASW-1931



BIOSPHERICS INC.
ROCKVILLE, MD.

DWG Nº 30
PISTON
SYRINGE
GULIVER V
CONT. NO. NASW-1931