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AUTOMATED MICROBIAL METABOLISM LABORATORY

Contract No. NASW-1731

FINAL REPORT

1969

BIOSPHERICS INCORPORATED

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Report

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by

BIOSPHERICS INCORPORATED
4928 Wyaconda Road
Rockville, Maryland 20853

for

NASA HEADQUARTERS

National Aeronautics and Space Administration

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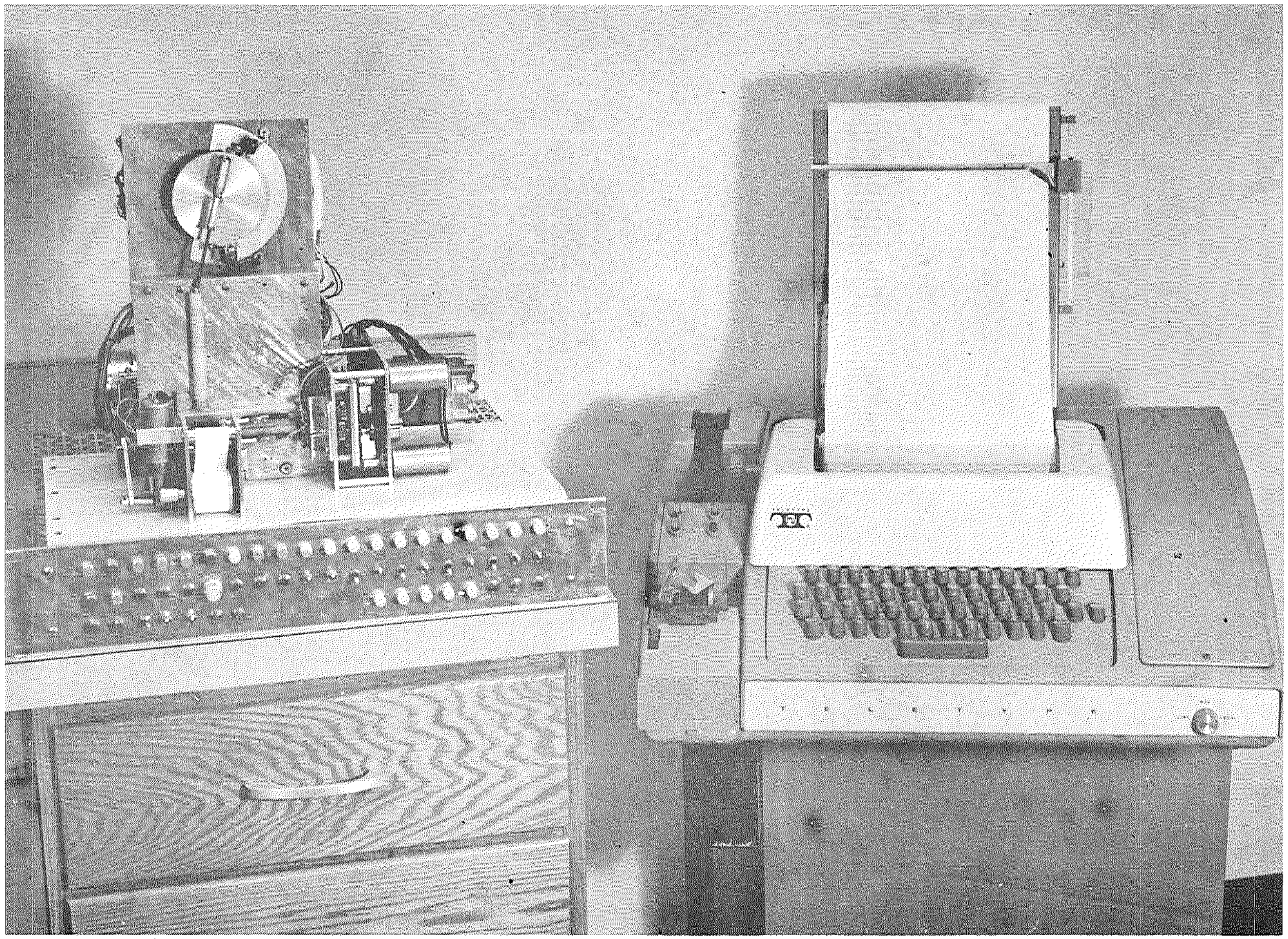

The Automated Microbial Metabolism Laboratory

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ABSTRACT

Several significant advances were made during the course of this developmental program on the Automated Microbial Metabolism Laboratory (AMML). The discovery of the photosynthesis activity index offered by phosphate uptake led to the recommendation that a photosynthetic component be examined for in all the experiments. In addition, a number of problem areas were uncovered. These were principally the non-biological signals from soils and substrates extracted from soils which interfere with the phosphate assay procedures.

The engineering effort on this program was directed toward the development and fabrication of an automated instrument that could be programmed to perform the various liquid biochemistry assays of the six AMML experiments. The instrument mechanism uses a 1 ml syringe-type transfer pump combined with a dual micro-slide-valve and roll-tape filter and is automatically controlled. The controller is a solid-state programmer which uses a Teletypewriter tape-reader as a stored program input.

Several detectors were also included in the instrument to monitor for ATP bioluminescence, ^{35}S and ^{14}C fixation and a ^{14}C-triethylamine phosphate determination.

I. Summary

The Automated Microbial Metabolism Laboratory (AMML) represents an attempt to develop moderately advanced instrumentation for extraterrestrial life detection missions in the mid or late 1970's. The program is aimed at simplifying and solving some of the complex instrumentation problems associated with multiple step assays, particularly those based on wet chemistry techniques.

One of the experiments supplies an aqueous solution of radioactive organic substrates to a sample of the planetary surface. The labels used are ^{14}C and ^{35}S. After application of the solution, the sample is monitored for the production of radioactive gas.

In an extension of this experiment, a light is introduced as a means for detecting photosynthesis. The light is turned on and off during the monitoring for radioactive gas production. Fluctuations in the rate of evolution of the radioactive gas corresponding to the light and dark periods are indicative of photosynthetic activity in the sample.

Another experiment seeks the detection of strict phototrophs. Radioactive carbon dioxide gas is supplied to a sample exposed to the light for a predetermined incubation

period. The $^{14}CO_2$ is then vented from the chamber. Light is excluded and the space above the sample is monitored for the dark evolution of $^{14}CO_2$ as an indication of endogenous respiration.

Another metabolic approach seeks to detect life by measuring the presence and increase of adenosine triphosphate (ATP) in a sample of the planetary material. In the experiment, lyophilized firefly luciferase is dissolved in aqueous buffer. In a separate chamber, a portion of the planetary material is chemically extracted to release any microbial ATP present. Aliquots of the enzyme preparation and the liquid extract are mixed in front of a photomultiplier tube. If any ATP is present, light is emitted by the reaction in proportion to the quantity of ATP present.

In the event that phosphate plays a role in the extraterrestrial life encountered, but that this vital nutrient does not participate as ATP, a phosphate uptake experiment was devised. This experiment seeks the incorporation of dissolved inorganic orthophosphate from an aqueous culture medium into which the sample is introduced. Uptake is detected by filtering and assaying aliquots of the phosphate-containing medium after inoculation. The orthophosphate assay is accomplished by first complexing the phosphate with molybdate and then precipitating the complex with ^{14}C-triethylamine. The precipitate is filtered and its radioactivity, which is directly proportional to the quantity of phosphate present, is measured on the dried filter tape.

The sixth experiment of the AMML is a labeled carbon and sulfate uptake test. A sample of the planetary material is introduced into an aqueous medium containing $^{35}SO_4$ and ^{14}C-organics. Aliquots of the suspension are periodically removed and filtered. The particulates retained by the membrane filter are dried and counted for radioactivity as an indication of uptake by microorganisms.

During the course of this year, the biology-biochemistry research phase of this program has produced several significant findings relative to the AMML array of experiments. The use of RM9 basal medium and labeled formate (developed under the Gulliver program) has enabled the detection of the heterotrophic respiration of as few as 10^3 algal cells within two hours. This represents an increase in sensitivity of three orders of magnitude for the detection of algae over previous results in this program.

The discovery that orthophosphate is taken up sufficiently rapid by several species of Chlorella to permit use of this ion as an indication of photosynthesis has added a new dimension to the AMML experiment. On the basis of this finding, it is recommended that the light-dependent nature of each of the life detection methods be measured by the introduction into the experiment of comparative light and dark incubation.

The addition to growth media of indoleacetic acid or low levels of the B vitamins which are carbon dioxide carriers was found to stimulate the growth of photosynthetic microorganisms. It was also found that phosphate uptake by photosynthetics was enhanced by the addition of 0.1% glucose to the medium. However, the small absolute amounts of phosphate taken up are best observed against a low phosphate background. The growth of microorganisms in low phosphate media was, therefore, studied and found to be satisfactory for the purposes of the AMML experiments. No morphological differences could be demonstrated between normal algal cells and phosphate starved organisms. Photosynthetic microorganisms grown under optimal physiological conditions maintained their capacity for rapid growth for up to five generations in low phosphate media.

Satisfactory laboratory tests were obtained on a semipermeable membrane material which could be used to retain moisture in the heterotrophic metabolism assay. This material will pass carbon dioxide freely while substantially reducing the rate of loss of water vapor.

The feasibility of conducting the light fixation-dark release test on soil samples was demonstrated. Satisfactory results were obtained on a number of soils. These tests indicated the presence of both photosynthetic and chemolithotrophic microorganisms in the soil samples.

The growth rate of microorganisms in the aqueous growth chamber constructed for the AMML was measured and found satisfactory indicating no serious inhibitors in the materials of construction.

A number of problem areas were uncovered during the course of these investigations. The nonbiological retention of labeled substrates by soils and filter materials was found to limit the sensitivity of the ^{14}C and ^{35}S uptake experiment. Further studies will be required to develop a wash procedure to lower the background level. Serious deficiencies were found in the colorimetric and radioisotopic phosphate assay procedures. Additional analytical method development will be required to apply these methods to soil extracts and to increase the sensitivity of the radioisotopic phosphate assay procedure. Nonspecific absorption of radioactivity by the filters from labeled triethylamine solutions is a factor in the latter.

The engineering program has applied the results from the biology-biochemistry research program toward the design and fabrication of a feasibility model of the AMML instrument. This instrument design centers about the use of a multi-port slide valve with a single syringe pump mechanism to provide a means to perform the aqueous wet chemistry assays. These assays are those that require metering, transferring, mixing and filtration and are performed automatically by the AMML instrument with the use of a programmer controller.

The automatic programmer controller consists of a teletypewriter tape-reader and logic circuitry which translates the program commands from a paper tape to control functions for the assay apparatus. This technique enables the operator to optimize the operating program easily by simply preparing a new program tape.

The AMML instrument incorporates several sensors that include an optical reaction cell coupled to the photocathode of a photomultiplier that is used to test for ATP specific bioluminescence. Solid-state and Geiger-Mueller beta detectors are used in the tests for ^{14}C and ^{35}S uptake and $^{14}CO_2$ evolution.

II. Introduction

The goal of this program has been the functional integration of six independent, but reinforcing, experiments into an Automated Microbial Metabolism Laboratory (AMML) for the detection of extraterrestrial life. In its completed form, the instrument should use the various subsystem components in multiple ways to minimize size, weight, complexity, and power requirements. The entire system, weighing perhaps 15 to 20 pounds, could serve as a biological payload for a small planetary lander or as a subsystem in a more elaborate extraterrestrial, biological, chemical, and physical exploration. This report describes the results of a one-year effort to refine the biological experiments and demonstrate their individual and collective feasibility. This program was aimed at simplifying and solving some of the complex instrumentation problems associated with multiple step assays, particularly those based upon wet chemistry techniques.

The AMML consists of the following specific biological experiments:

1. Radioisotopic biochemical probe (Gulliver, labeled release test)
2. Heterotrophic photosynthesis test
3. Autotrophic photosynthesis test

4. Adenosine triphosphate (ATP) measurement test
5. ^{14}C-organic and ^{35}S-sulfate uptake test
6. Orthophosphate uptake test

Although not studied as a part of this present program, the following nonbiological measurements should be made on the planetary surface by the lander instrument in order to complement the biological tests:

1. temperature
2. atmospheric oxygen
3. pH of the surface material
4. ambient light intensity
5. background radiation
6. soluble phosphate content of the surface material

This experiment array offers a broad spectrum of metabolic and biochemical windows through which extra-terrestrial life can be sought. The specific experiments listed above permit the AMML to monitor the interface between the biological system and the environment by examining processes involving the following important elements, compound and energy source:

1. The uptake and release of carbon heterotrophically from simple, dissolved substrates, and autotrophically

as carbon dioxide fixed photosynthetically,

2. The involvement of oxygen in the photosynthetic process,
3. The uptake of phosphorus in the simple, orthophosphate form,
4. The presence and production of adenosine triphosphate,
5. The uptake of sulfur in inorganic and organic form; and
6. The utilization of light as an energy source for metabolism.

Individually, each experiment might yield evidence on the presence of life and provide some information on metabolic rates. However, as an integrated experiment, the total value exceeds the sum of its parts. The information obtained could indicate whether or not any life encountered was similar to or very different from that on Earth. For example, the phosphate and sulfur tests might indicate the presence of life which, yielding negative results in the ATP test, would, thereby, 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.

III. Biology-Biochemistry Research Program

A. Supporting Research for Life Detection Experiments

1. Growth of Photosynthetic Microorganisms

a. Descriptions of Test Photosynthetic Microorganisms

Three strains of green algae were selected as sources of materials to be used in the laboratory photosynthetic studies. These strains, of the genus Chlorella, are described by Shihira and Krauss (1) as follows:

Chlorella sorokiniana (supplied by the University of Maryland, Department of Botany)

Cells spherical or ellipsoidal in inorganic liquid media, 3 x 2 u in small cells to 4.5 x 3.5 u in large cells, often becoming spherical, 4.5 to 5.5 u in diameter when grown on glucose. Chromatophore shallow, bowl-shaped; green but turning white in old inorganic cultures, and even more quickly on glucose media. Pyrenoid present.

Grows rapidly on agar without organic nutrients.

Good growth on inorganic liquid media. Glucose supports good growth in light and some growth in darkness. Galactose stimulates growth in light and weakly stimulates it in darkness. Mannose usually inhibits growth or may support little or no growth. Other sugars not effective. Acetate supports no growth in darkness.

Ammonia and nitrate utilized equally. Casein hydrolysate as a nitrogen source supports growth better than NH_4NO_3.

Yeast extract only slightly effective, thiamin not effective.

Maximum growth rate of 9.2 doublings per day at 39°C.

Chlorella vannielii

Cells always spherical, 3-13 u in diameter, nearer to 13 when grown on glucose media. Chromatophore bowl-shaped; always green. Pyrenoid present, but not always evident.

Good growth on agar or on any medium in light.

Grows well on inorganic media in light, glucose stimulatory in light, but weakly so in the dark. Galactose enhances growth in light but only weakly in the dark, mannose inhibits growth in light, other sugars ineffective. Acetate not stimulating in light and does not support dark growth.

Nitrate always supports better growth than ammonia as a nitrogen source. Casein hydrolysate and NH_4NO_3 serve equally as nitrogen sources.

Yeast extract and thiamin ineffective.

Dark growth always poor.

Chlorella vulgaris

Cell always spherical, 4-10 u in diameter. Chromatophore a reduced cup-shape; pea green. Pyrenoid present.

Not easily grown on agar, usually grows in a thin sheet. Slow growth rate.

Grows on inorganic media in light; glucose stimulatory in light and dark, although still with a slow growth rate. Galactose, mannose, and fructose strongly stimulate growth in light and support some growth in dark. Lactose, maltose, raffinose, and dextrin sometimes promote growth slightly in light. Acetate supports some growth in darkness.

Nitrate and NH_3 serve equally as nitrogen sources. Casein hydrolysate is generally more stimulatory than NH_4NO_3 as a nitrogen source.

Yeast extract enhances growth on glucose in either light or dark with NH_4NO_3 as a nitrogen source.

b. Illumination Chambers

Several artificial illumination sources were designed and constructed for use in the production of algae and for the laboratory studies of photosynthetic activity. The first consisted on a bank of four General Electric F20T12.CW, cool-white, 20-watt fluorescent bulbs. This was later replaced by four General Electric F48PG17.CW, cool-white, power-groove fluorescent bulbs. The cultures were placed at varying distances from the light bank in order to achieve the desired intensity of illumination. When solid cultures were used, inverted agar plates were placed on racks above the light source in such a manner that illumination was direct rather than through the translucent agar medium. The intensity of the light incident to the culture was monitored with a General Electric Type 213 light meter.

c. Compositions of Media Used for Study of Photosynthetic Microorganisms

Extensive research into the development of "universal" media was undertaken in earlier development of the AMML (2) and the labeled release (Gulliver) experiment (3). These media support, but do not necessarily optimize, growth of a broad spectrum of microorganisms. The media developed for AMML and investigated further during this program include the M9, RM9, and M11 media. The compositions of these media are shown in Table 1. Comparative studies of the growth of several photosynthetic organisms were undertaken with numerous media known to be optimal for these species in order to determine if it would be necessary or desirable to alter the present composition of the basal medium to accommodate the photosynthesis

Table 1

Composition of AMML Basal Media - M9, RM9, and M11

	M9	RM9	M11
K_2HPO_4	1.0 g/l	5.0 mg/l	1.0 g/l
$MgSO_4 \cdot 7H_20$	0.20 g/l	0.08 g/l	0.20 g/l
NH_4NO_3	0.20 g/l	0.20 g/l	0.19 g/l
NaCl	0.10 g/l	0.10 g/l	0.10 g/l
Soil Extract*	100 ml/l	100 ml/l	16.0 ml/l
pH	7.0 (HCl)	7.0 (HCl)	7.0 (HCl)
Tris	-	6.0 g/l	-
KNO_3	-	-	0.031 g/l
Malt Extract	-	-	0.19 g/l
Beef Extract	-	-	0.19 g/l
Yeast Extract	-	-	0.81 g/l
Ascorbic Acide	-	-	0.013 g/l
Bacto-Casamino Acid	-	-	0.25 g/l
Proteose Peptone #3	-	-	1.25 g/l

* 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.

experiments. A list of media for photosynthetics which were investigated during this project is provided in Table 2.

d. Growth and Assay Procedures

The procedure for the growth of cells of the three species of Chlorella studied during this program consisted of transferring an inoculum of the organism by a wire-loop from a parent culture to the appropriate medium followed by incubation until the desired yield was obtained. Routinely, a wire-loop inoculum of algae was transferred aseptically into 50 ml of medium and incubated either under continuous illumination or total darkness at the selected growth temperature to obtain an optical density, measured at 525 nm of about 0.5. Growth of the thermophile, Chlorella sorokiniana was achieved by the use of a water bath shaker thermostatically controlled at 39°C.

Growth yields of the algae suspensions were determined either by optical density measurements or by measuring the dry weight of cells. Aliquots of the cultures were sampled and desiccated to dryness in tared aluminum weighing vessels to determine the weight yield of cells. Alternately, the optical density was measured at 525 nm in a cuvet having a one centimeter light path and the weight yield determined from a standard curve. A standard curve for the growth assay of

Table 2

Chlorella Media Used for AMML Studies

1. Krauss Basal Inorganic Medium for Culture of *Chlorella* (1)

Compound	Concentration (g/l)
NH_4NO_3	1.0
K_2HPO_4	1.0
$MgSO_4 \cdot 7H_2O$	0.25
Na_2Mn EDTA	0.0071
Na_2Ca EDTA	0.0071
Na_2Co EDTA	0.0077
Na_2Cu EDTA	0.0093
Na_2Zn EDTA	0.0067
NaFe EDTA	0.038
MoO_3	0.001
H_3BO_3	0.010

2. Basal Inorganic Medium for Culture of *Chlorella* - University of Maryland

Compound	Concentration (g/l)
KNO_3	1.0
$MgSO_4 \cdot 7H_2O$	0.25
KH_2PO_4	0.80
K_2HPO_4	0.20
NaFe EDTA	0.005

Table 2 (continued)

Compound	Concentration (g/1)
Na_2Mn EDTA	0.001
Na_2Cu EDTA	0.001
Na_2Zn EDTA	0.001
Na_2Ca EDTA	0.001
Na_2Co EDTA	0.001

3. Proteose-Agar Medium for Culture of Chlorella

Compound	Concentration (g/1)
$NaNO_3$	0.10
$CaCl_2$	0.01
$MgSO_4 \cdot 7H_2O$	0.03
K_2HPO_4	0.03
KH_2PO_4	0.07
NaCl	0.01
Yeast Extract	0.01
Proteose	1.0
Agar	15
NaFe EDTA	0.005
Na_2Mn EDTA	0.001

Table 2 (continued)

Compound	Concentration (g/1)
Na_2Cu EDTA	0.001
Na_2Zn EDTA	0.001
Na_2Ca EDTA	0.001
Na_2Co EDTA	0.001
pH	6.2-6.5 (HCl)

4. Bristol's Proteose-Agar Medium for Cultures of Chlorella

Compound	Concentration (g/1)
$NaNO_3$	0.10
$CaCl_2$	0.01
$MgSO_4 \cdot 7H_2O$	0.03
K_2HPO_4	0.03
KH_2PO_4	0.07
NaCl	0.01
$FeCl_3$	0.0005
Agar	15
pH	6.5 (HCl)

5. $NaHCO_3$-Yeast Agar Preparation for Plates

Compound*	Concentration (g/1)
$NaHCO_3$	4.0

* These compounds are diluted up to one liter with Krauss basal inorganic medium for culture of Chlorella (1).

Table 2 (continued)

Compound*	Concentration (g/l)
Yeast Extract	0.10
Agar	15
pH	6.8

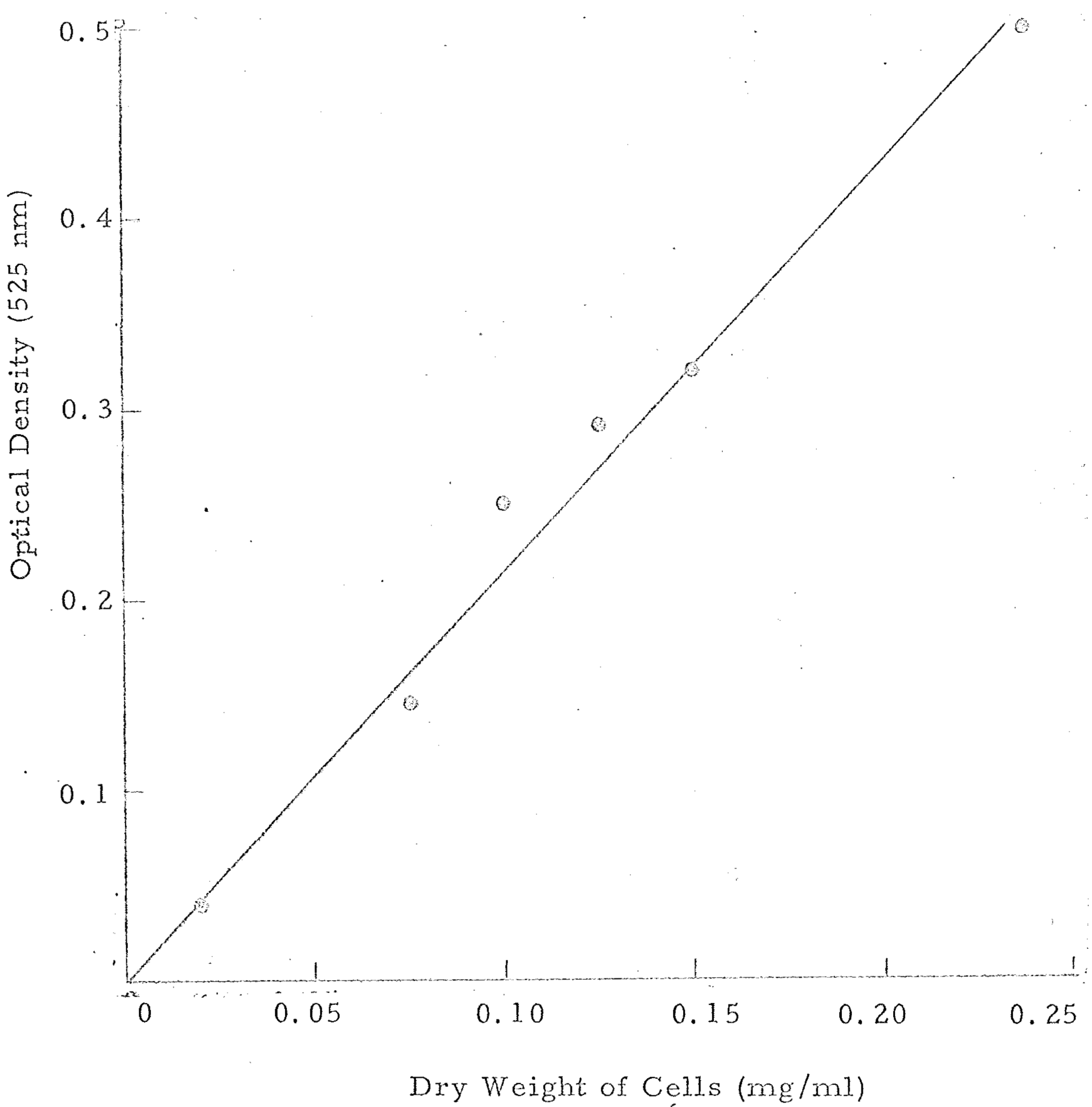
* These compounds are diluted up to one liter with Krauss basal inorganic medium for culture of Chlorella (1).

Chlorella sorokiniana by optical density measurements is shown in Figure 1. This figure shows that a virtually linear relationship exists between optical density and dry weight of cells per unit volume of cell suspension. A Bausch and Lomb "Spectronic 20" spectrophotometer was used to make routine assays of algal suspensions via optical density measurements at 525 nm.

High density cell populations were occasionally required for inocula and were obtained by centrifugation of a growing culture of cells. The pellet of viable cells was then resuspended in 0.1M Tris buffer using a Tenbroeck glass homogenizer. The centrifugal force required to yield the maximum recovery of viable cells was determined. The effect of centrifugal force upon the percentage of cells harvested is shown in Figure 2. All experimental results reported here on the growth of green algae were obtained using the above described procedures.

2. Development of Media for the AMML

Results presented in this section of the report describe the challenging of the AMML media with soil microorganisms as well as the results of attempts to increase the metabolic rates of photosynthetic organisms in these media through the use of various organic and inorganic additives.



Standard Curve for the Growth Assay of <u>Chlorella</u> <u>sorokiniana</u>

	Fig. 1		
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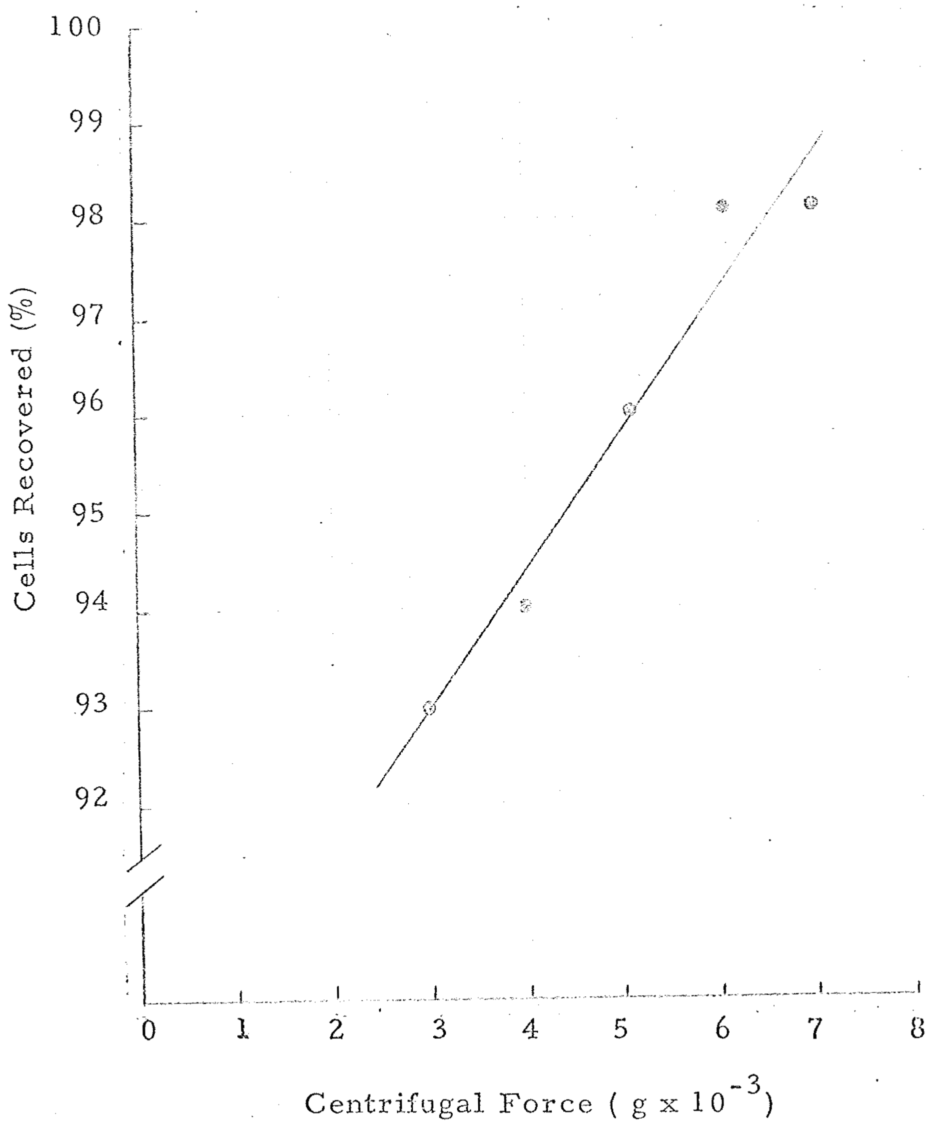

Effect of Centrifugal Force on the Harvest of Green Algae

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a. Soil Microbiology

1) Introduction

A study was conducted in which the levels of growth of soil microorganisms were compared on a standard bacteriological medium (trypticase soy agar) and on agar plates prepared with the AMML media M9, RM9, and M11. The purposes of this study were to demonstrate the ability of the AMML media to support the growth of soil microorganisms, to compare this growth supporting ability with a standardized bacteriological medium, and to provide a minimal estimate of the number of viable organisms in the test soil samples. It has been documented that the total number of viable organisms in soil may vary over many orders of magnitude depending upon the sample selected for investigation. It has also been documented that the number of organisms found using any one bacteriological medium may be several orders of magnitude lower than the actual numbers of organisms present in the soil sample.

2) Procedures

M9, RM9, M11 media and trypticase soy broth were solidified by the addition of 1.5% agar. The pH of each medium was adjusted to pH 7.0 prior to autoclaving. A thickness of approximately 3/8 inch of agar was provided by the use of 25 ml of media per plate. All plates were stored at 4^{o}C prior to inoculation in order to minimize drying.

All soils tested were treated in the following manner. A 1.0 g sample of the soil was suspended in 0.1M Tris buffer, pH 7.0. This suspension was shaken vigorously for one minute with a wrist action shaker. The shaking cycle was repeated after a 15 minute interval to permit solution of soluble particles. Dilutions ranging from 10^{-1} through 10^{-6} (or greater, if necessary), were prepared and 0.1 ml aliquots of each dilution were then plated in triplicate on each of the media under investigation. The plates were spread with a sterile glass rod. Following inoculation, the plates were sealed with plastic tape to retard loss of moisture, inverted, and stored at 30^{o}C. The plates were removed from the incubator and examined for growth after an incubation period of from 24 to 48 hours, depending upon the soil sample being tested. The plates were incubated for an additional 24 hours and then reassayed in the event that no growth was demonstrable after the initial incubation period.

3) Results and Discussion

The results of this study conducted on

six soils from the Eastern half of the United States are shown in Table 3. Although the AMML media generally, but not always, yielded lower numbers than the trypticase soy agar, it is significant to note that demonstrable growth was achieved with the solidified AMML media on each of the soils tested during the course of this phase of the program. Substrate concentrations in the AMML media have purposely been set at extremely low levels in order to minimize any possible inhibition of the metabolism of Martian organisms caused by an excess of organic compounds. Therefore, the AMML media were not expected to yield visible evidence of growth at the same levels as indicated by a standard bacteriological medium. It is significant to note that when organic substrate in the form of 1.0% glucose was added to M9, this medium then gave results equivalent to those obtained with trypticase soy agar.

b. Organic and Inorganic Additives to Promote the Growth of Photosynthetic Microorganisms

1) Introduction

The Biospherics Incorporated technical proposal for this program, "Automated Microbial Metabolism

Table 3

Growth of Soil Microorganisms on Trypticase Soy Agar and Solidified AMML Media

Soil Sampling Location	Medium	Bacteria/g Soil
Washington, D. C.	M9	$5x10^4$
	RM9	$9x10^3$
	M11	$2x10^6$
	TSB	$3x10^4$
Montgomery County, Indiana	M9	$1x10^6$
	RM9	$1x10^4$
	M11	$5x10^6$
	TSB	$5x10^7$
	M9+1. 0% glucose	$1x10^7$
Somerset County, Maryland	M9	$2x10^6$
	RM9	$2x10^6$
	M11	$3x10^6$
	TSB	$5x10^7$
	M9+1. 0% glucose	$6x10^7$

Table 3 (continued)

Soil Sampling Location	Medium	Bacteria/g Soil
Lenoir County, North Carolina	M9	$5x10^6$
	RM9	$2x10^5$
	M11	$3x10^6$
	TSB	$1x10^6$
Morgan County, West Virginia	M9	$5x10^4$
	RM9	$2x10^5$
	M11	$2x10^4$
	TSB	$4x10^6$
Wildwood, New Jersey	M9	$6x10^4$
	RM9	$1x10^5$
	M11	$1x10^7$
	TSB	$2x10^5$
	M9+1.0% glucose	$2x10^7$

Laboratory" (4), states that, "The medium previously used will be modified by the addition of various inorganic and organic constituents in an attempt to enhance the $^{14}CO_2$ production on a per cell basis. Vitamin B_{12}, thiamin, and niacin addition will be explored in axenic cultures on the basis of the possibility recently raised that these vitamins and co-factors may be essential to autotrophism."

The AMML as originally conceived had two photosynthetic components in its array of experiments; heterotrophic photosynthesis in which the rate of production of $^{14}CO_2$ was altered by light, and autotrophic photosynthesis in which labeled CO_2 was fixed in light and released during a dark incubation period. As will be described later, a significant advance achieved during this program resulted in the discovery of the light dependent nature of phosphate uptake by algae. This introduced a third photosynthetic experiment into the array of life detection schemes further emphasizing the importance of photosynthetic mechanisms. Laboratory research efforts to increase the metabolic activity of photosynthetics through the use of additives are described in this section. The effects of carbon dioxide, indoleacetic acid, and B vitamins upon the growth of algae were studied.

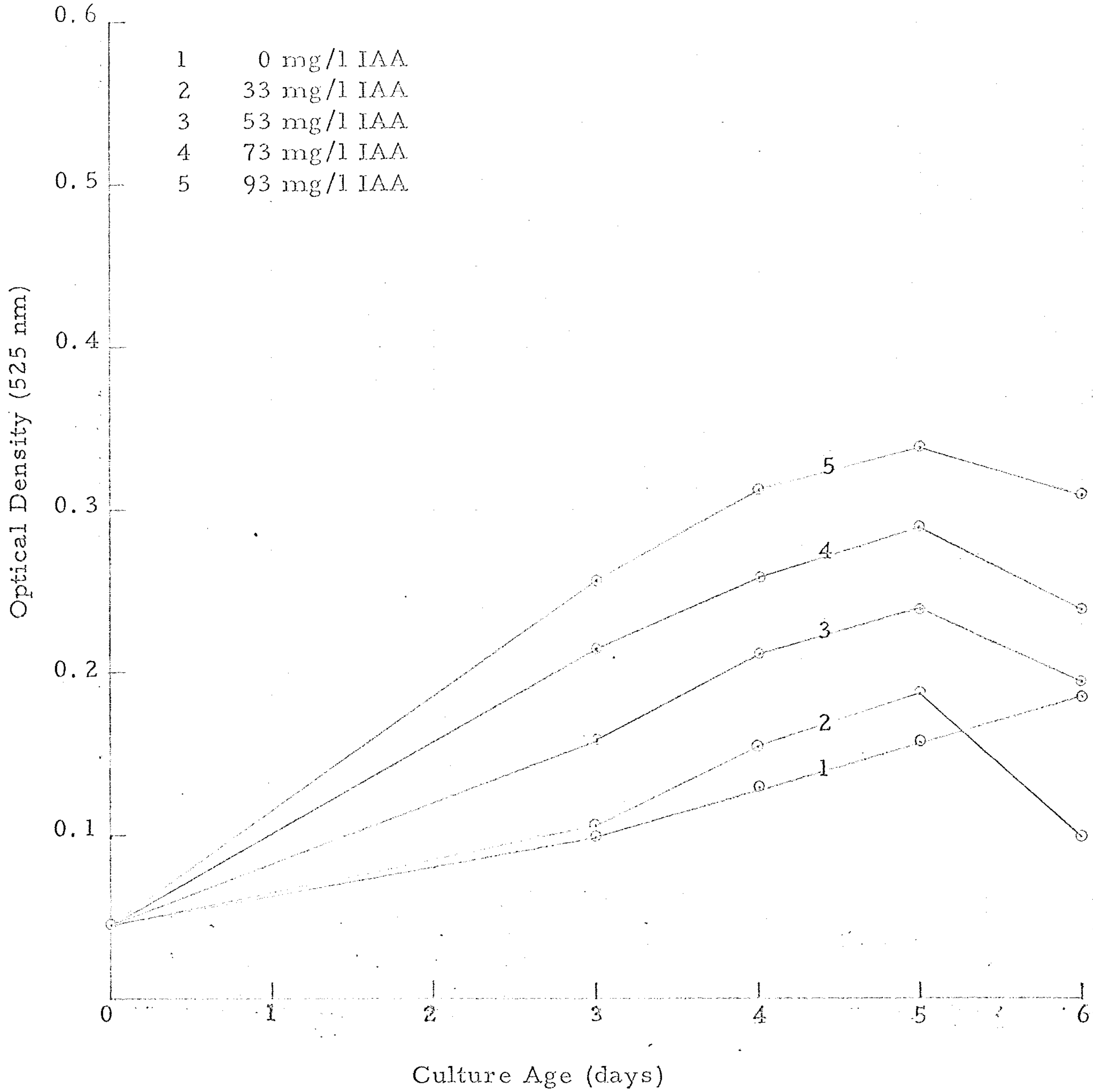
2) Effects of Indoleacetic Acid Upon Photosynthetic Growth

Indoleacetic acid (IAA) is known to enhance the photosynthetic activity of green plants. Therefore, this compound was examined for its ability to promote the growth of a photosynthetic microorganisms. The equipment and growth and assay procedures described earlier in this report were used to examine the effects of IAA additions to a basal *Chlorella* medium upon the growth of *Chlorella vulgaris.* Flasks of basal medium containing varying amounts of IAA were inoculated and examined for growth at 24 hour intervals by measuring the optical density at 525 nm.

The results of this experiment are shown in Figure 3. All levels of IAA studied showed some stimulation of growth. A three-fold increase in growth rate was attained with 93 mg/l IAA, the highest concentration studied. The addition of IAA will enable studies to be conducted on samples having marginal photosynthetic activity.

3) Study of B Vitamins to Enhance the Growth of *Chlorella*

As has been stated previously, this enhanced metabolic activity would increase the sensitivity of the various life detection methods. A likely possibility for stimulation of



Effect of Indoleacetic Acid (IAA) Upon the Growth of Chlorella vulgaris

	Fig. 3
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carbon dioxide production in photosynthetic as well as in non-photosynthetic microorganisms is the addition of B vitamins to the growth media. The possibility was recently voiced that there may be no strict phototrophs because all photosynthetic organisms may require vitamins (5). A study was undertaken of the effects of B vitamins upon the growth of photosynthetic organisms.

The list of B vitamins selected for their known abilities to act as growth co-factors is shown in Table 4. The growth of Chlorella sorokiniana in stationary, 50 ml cultures of RM9 medium under conditions of constant temperature and illumination was measured to study the effects of a variety of vitamin supplements. A scheme was devised which minimized the number of combinations to be tested in order to evaluate the antagonistic and/or synergistic effects of the vitamins as well as their individual effects upon growth. These combinations of vitamins along with the concentrations used are shown in Figure 4. Experiments, each consisting of 13 experimental flasks plus suitable controls, were run for two concentrations of each vitamin. Vitamin concentrations of 1 and 10 ug/ml of RM9 medium were selected for study.

The effects of two concentration levels of B vitamins upon the growth of Chlorella sorokiniana are summarized in

Table 4

Selected B Vitamins

Thiamine
Riboflavin
Vitamin B_6
Nicotinic Acid
Pantothenic Acid
Biotin
Vitamin B_{12} Group

Figure 4

Diagram of B Vitamin Combinations Studied

Biotin
Nicotinic Acid
Pantothenic Acid
Riboflavin
Thiamine
Vitamin B_6
Vitamin B_{12} Group

Nicotinic Acid
Riboflavin
Thiamine
Vitamin B_6

Biotin
Pantothenic Acid
Vitamin B_{12} Group

Riboflavin
Thiamine

Nicotinic Acid
Vitamin B_6

Biotin
Pantothenic Acid

Riboflavin

Thiamine

Nicotinic Acid

Vitamin B_6

Biotin

Pantothenic Acid

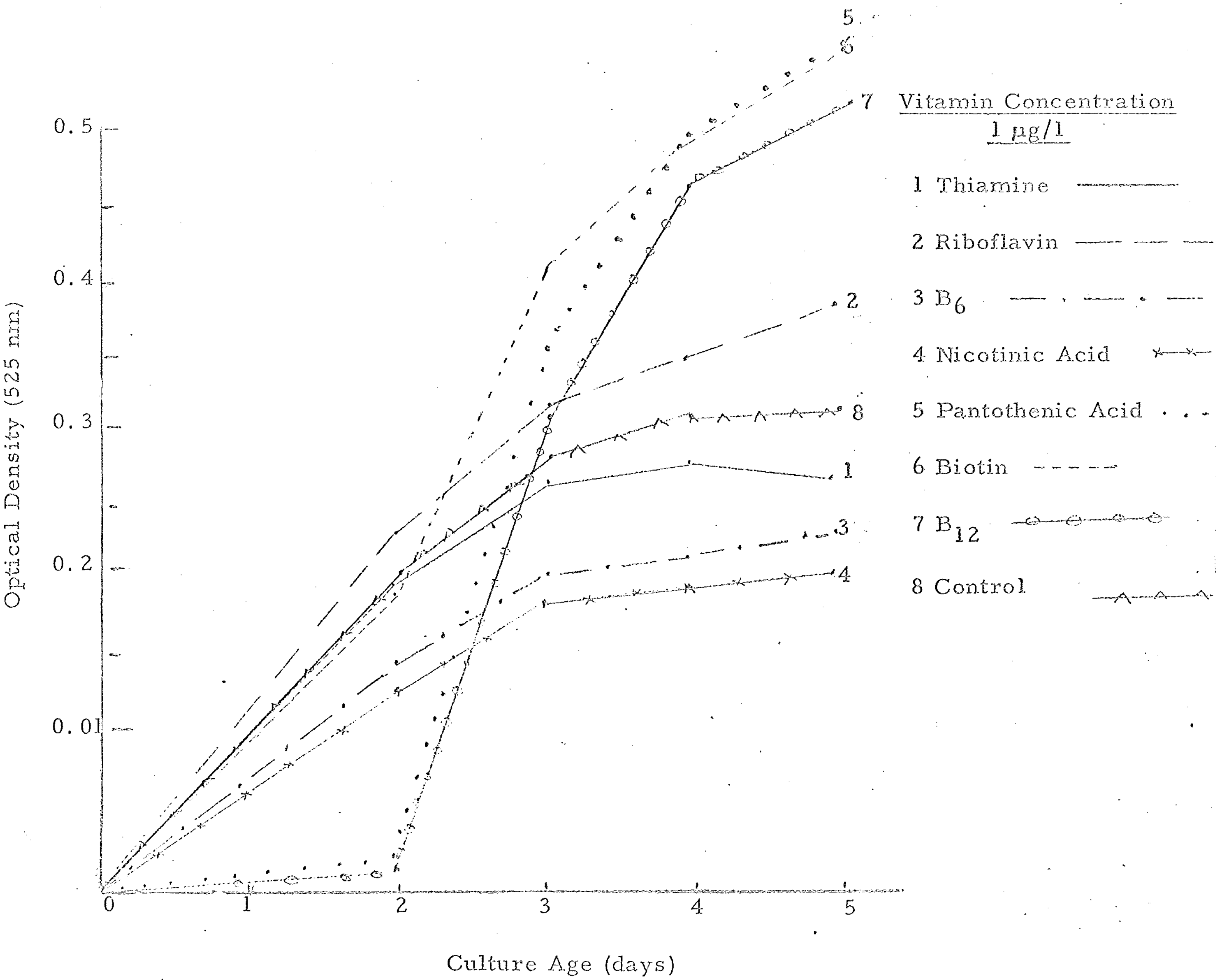
Vitamin B_{12} Group

vitamin concentrations of 1 and 10 ug/ml in RM9 were studied

Figures 5 and 6. A comparison of the levels of growth achieved after 120 hours of incubation showed that a definite stimulation of growth can be obtained by the B vitamins that are carbon dioxide carriers and that are active in the incorporation of one carbon compounds. Significant enhancement of growth was noted only at the 1 ug/ml concentration level. The addition of either biotin or pantothenic acid alone to the RM9 medium at a concentration of 1 ug/ml resulted in a growth enhancement of about 100%. The addition of the vitamin B_{12} group alone at this level resulted in an enhancement of about 85%. The biotin, pantothenic acid, vitamin B_{12} group combination at 1 ug/1 was the only combination studied which was particularly effective. This combination of vitamins resulted in an enhancement of about 100% in the growth of _Chlorella sorokiniana_ after 120 hours incubation.

4) Use of Carbon Dioxide to Promote the Growth of _Chlorella_

The use of added gaseous carbon dioxide to enhance the growth of photosynthetic microorganisms was examined. Surprisingly however, decreases in growth rates were observed when _Chlorella sorokiniana_, _Chlorella vannielii_, and _Chlorella vulgaris_ cultures were exposed to air enriched up to a level of 15% carbon dioxide. Experiments were performed in order to explain the decreased growth rates



Stimulation of Growth of Chlorella sorokiniana with Individual B Vitamin Supplements (1 ug/ml)

	Fig. 5-a		
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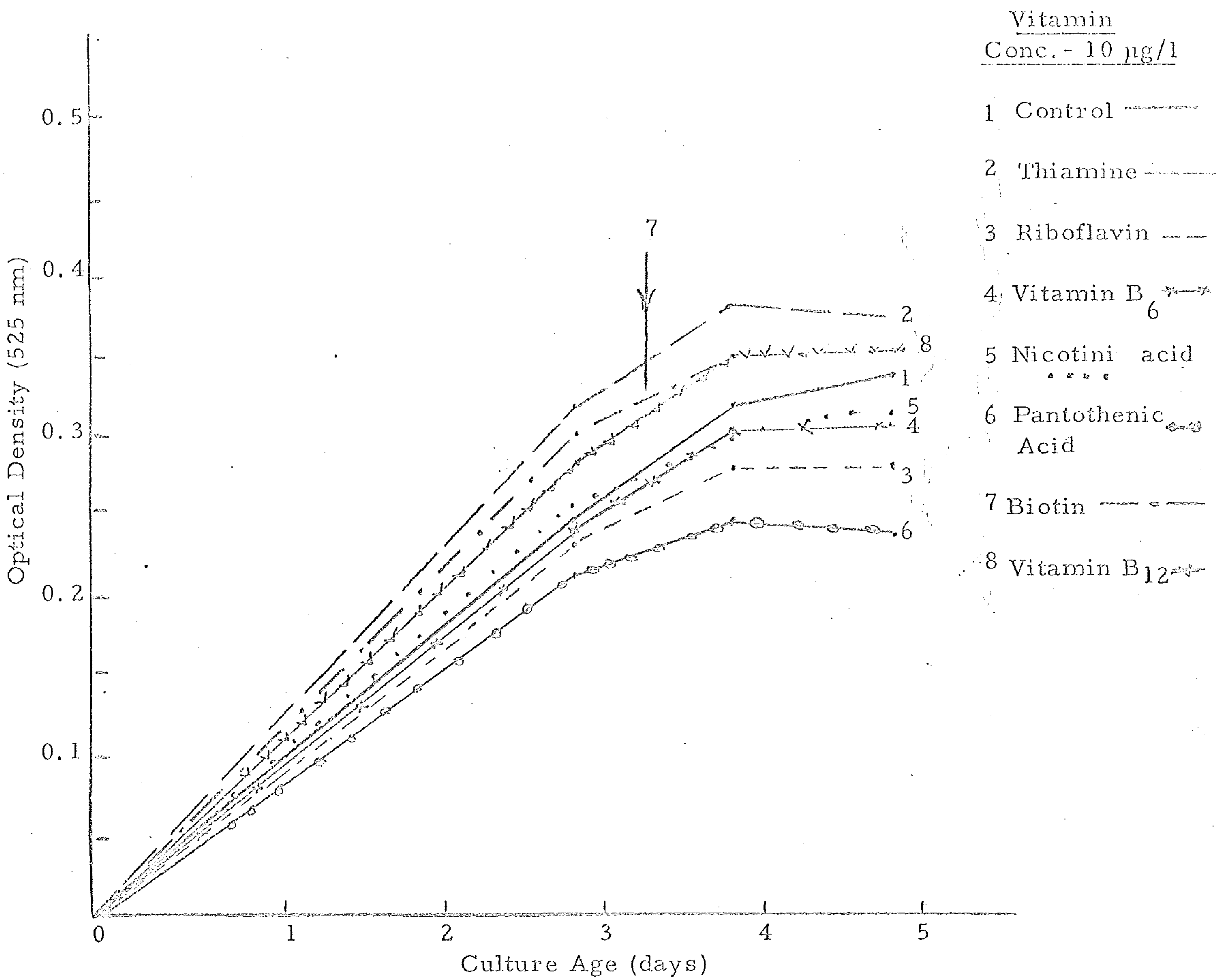

Stimulation of Growth of Chlorella sorokiniana with Individual B. Vitamin Supplements (10 ug/ml)

Fig. 5-b

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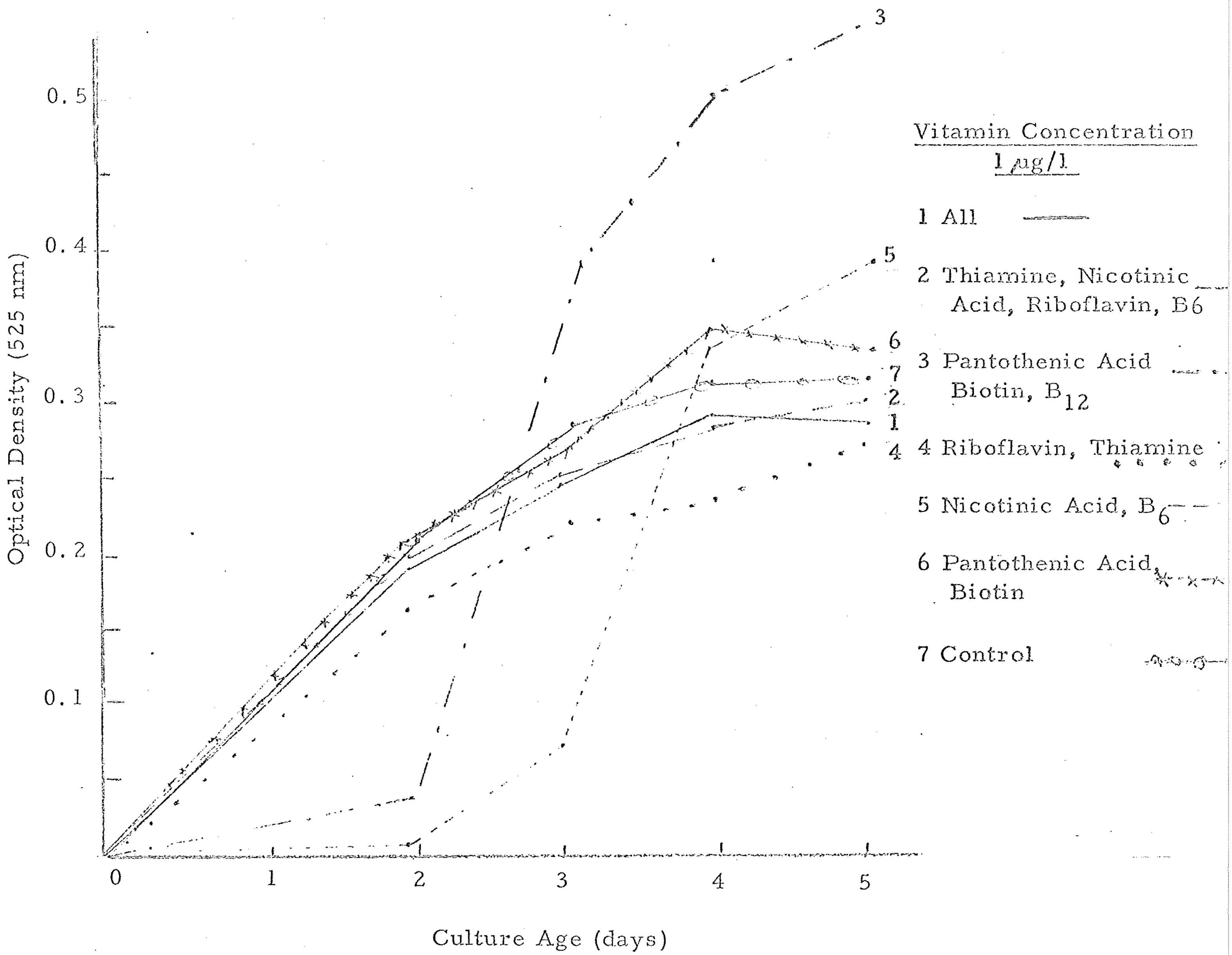

Stimulation of Growth of Chlorella sorokiniana with Combination B Vitamin Supplements (1 ug/ml)

BIOMETRICS INCORPORATED	Fig. 6-a		
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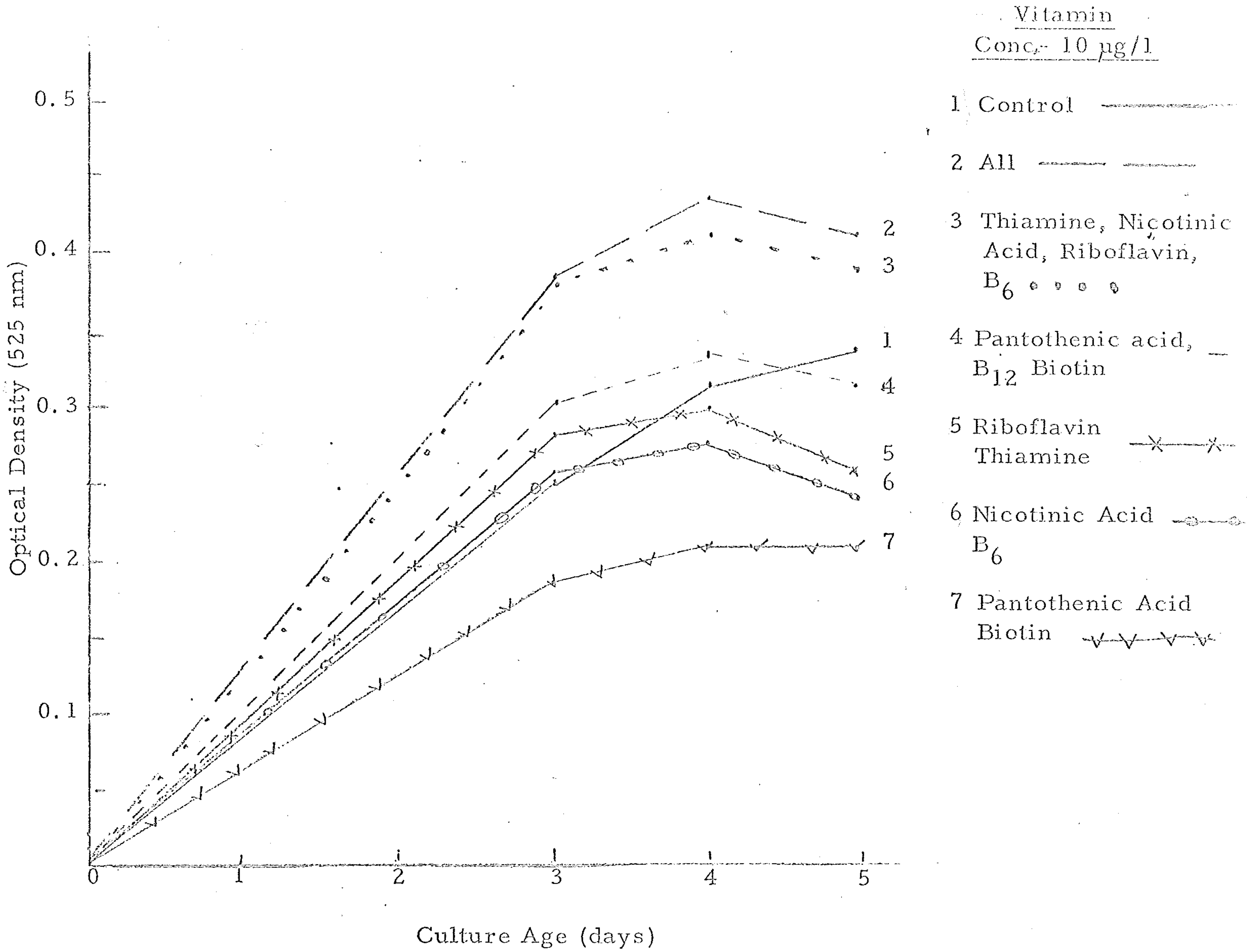

Stimulation of Growth of Chlorella sorokiniana with Combination B Vitamin Supplements (10 ug/ml)

BIOSPHERICS INCORPORATED	Fig. 6-b		
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on the basis of altered pH in the medium induced by gassing with carbon dioxide rich air.

The bicarbonate concentration required to maintain a pH of 7.0 in medium gassed with 15% carbon dioxide was determined. Media containing varying amounts of bicarbonate buffer at pH 7.0 were gassed at a constant rate with 15% carbon dioxide for the required length of time. Initial and final pH values were measured. The results of this experiment are shown in Table 5. A buffer concentration of at least 0.06 M sodium bicarbonate is required to maintain a pH of 7.0 in the medium.

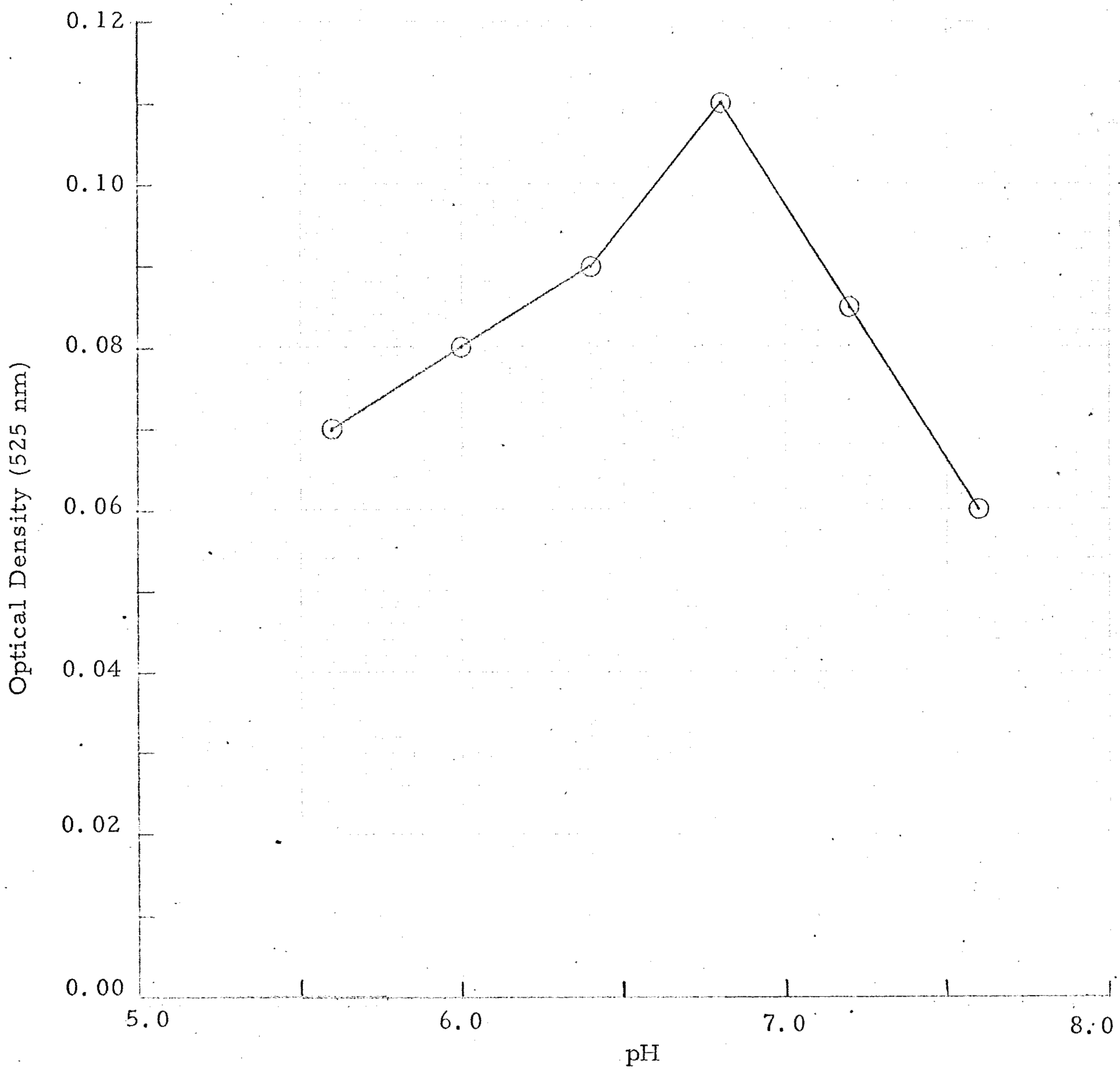
The nature of the pH dependence of *Chlorella sorokiniana* and *Chlorella vannielii* (the two most rapid growing strains investigated) was examined by preparing media identical in all respects but varying the pH through the additions of hydrochloric acid or sodium hydroxide. A pH range of from 6.0 to 7.6 was studied for effect on growth of *Chlorella* (6). The bicarbonate present proved sufficient to buffer the media adequately throughout the pH range chosen for study. The growth level achieved in stationary culture was examined after 72 hours in each of the flasks. Figures 7 and 8 show characteristic pH optima for the growth of each species. It is interesting to note that the slower growing species, *Chlorella*

Table 5

Effect of Bicarbonate Concentration Upon pH Stabilization in *Chlorella* Medium*

Sodium Bicarbonate Concentration		pH	
Molarity	mg/ml	Initial	Final
0.03	2.52	7.0	6.2
0.04	3.36	7.0	6.3
0.05	4.20	7.0	6.8
0.06	5.04	7.0	7.0
0.07	5.88	7.0	7.0

* The medium was gassed with 15% carbon dioxide in air



Effect of pH Upon Growth of Chlorella vannielii in Low Phosphate Medium

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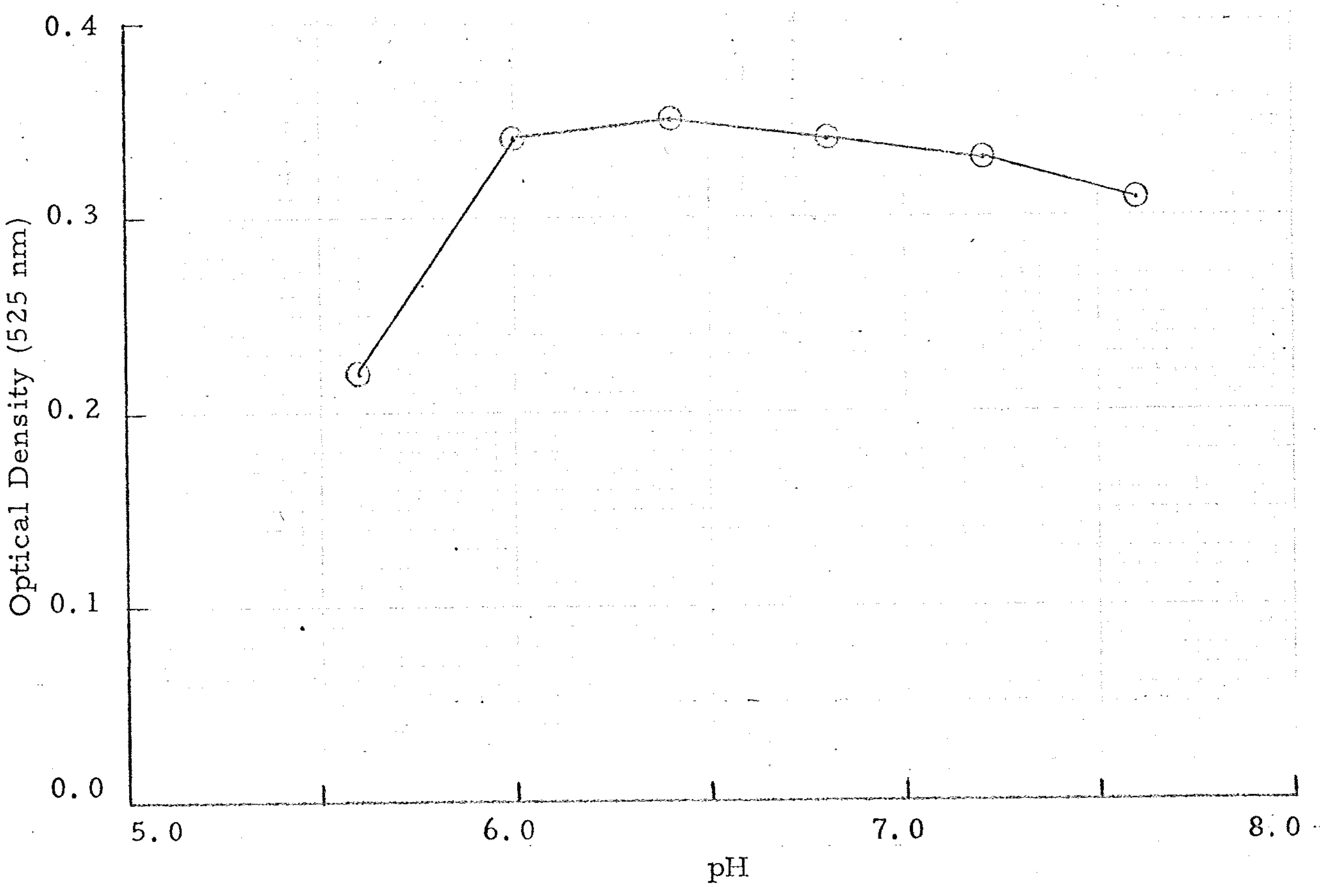

Effect of pH Upon Growth of Chlorella sorokiniana in Low Phosphate Medium

BIOSPHERICS INCORPORATED Rockville, Maryland	Fig. 8		
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vannielii, showed a very sharp maximum growth rate at pH 7.8 whereas Chlorella sorokiniana showed a broad optimal pH range from 6.0 to 7.2.

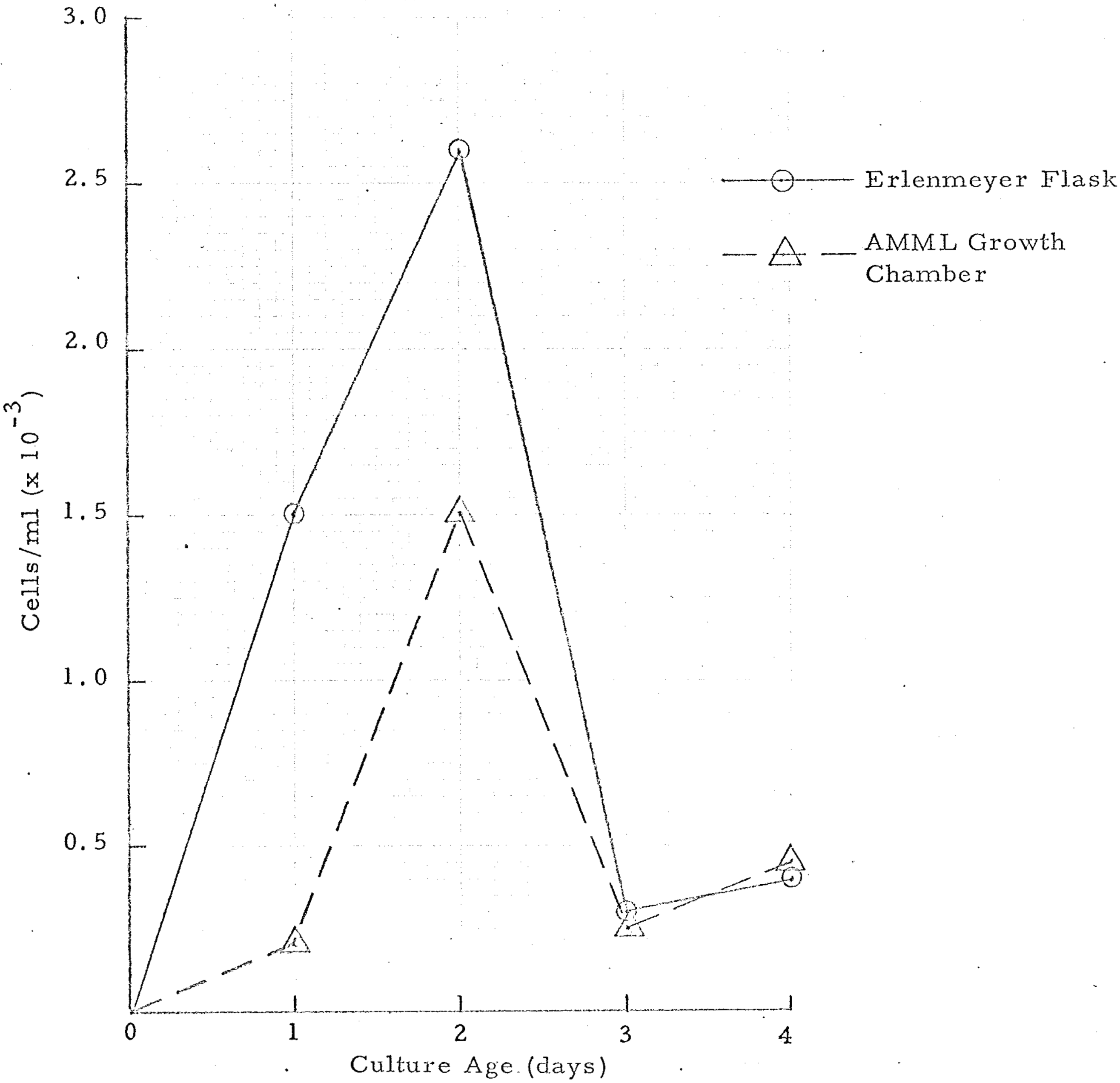
3. Test of the AMML Aqueous Growth Chamber

a. Introduction

Construction of the breadboard AMML will require materials not routinely used in studying microbial growth. The experimental growth chambers in which the soil inoculum will be incubated prior to each of the required assays has been tested for its ability to support the growth of microorganisms without inhibitory side effects. A comparison has been made of the growth obtained from parallel soil inocula in RM9 medium in the AMML aqueous growth chamber and in standard laboratory glassware. The aqueous growth chamber is shown in Figure 9.

b. Experimental

Aliquots of RM9 medium inoculated with 100 mg of viable soil were added to the aqueous growth chamber and to a 125 ml Erlenmeyer flask. Both systems were incubated at $25^{o}C \pm 2^{o}C$ under ambient light in the laboratory. At periodic intervals, samples from each system were taken, plated on bacteriological agar medium, and the



Comparison of Growth Obtained from Soil Samples Inoculated Into an Erlenmeyer Flask and the AMML Aqueous Growth Chambers

BIOSPHERICS INCORPORATED	Fig 9		
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extent of growth measured. The results are summarized below:

c. Results and Discussion

Figure 9 shows the growth obtained from identical soil samples under both sets of conditions. While a greater level of growth was achieved in the Erlenmeyer flask, a considerable level of growth was achieved in as little as 24 hours in the AMML aqueous growth chamber. Although differences in growth rate have been recognized, the materials used apparently do not inhibit growth from such soil inocula in a manner as to be prohibitive to the experiments. The slightly lower growth rate observed in the experimental chamber may not be ascribable to the construction material used. The positive results obtained with the materials selected indicate that the chamber is usable in the AMML.

B. Heterotrophic Metabolism

1. The Utilization of ^{14}C-Formate

A significant achievement of this program has been the development of a technique which will detect the heterotrophic metabolism of as few as 10^3 green algal cells in less than two hours. This represents an increase in sensitivity of three orders of magnitude over that last reported for algae. The addition of ^{14}C-formate to RM9 medium was responsible for the observed increase in sensitivity.

a. Experimental

Cells of log phase <u>Chlorella sorokiniana</u> were spun at 7000 X g for 15 minutes in a refrigerated centrifuge and then resuspended in basal RM9 medium. Appropriate dilution factors were determined from replicate direct counts in a Petroff-Hauser counting chamber. This technique had been checked by comparisons of visual counts with plate counts on algae which were incubated for several days. The purity of the cultures was determined by direct microscopic examination and cultures were discarded which had bacterial contamination levels exceeding approximately 1%. Phase-contrast, 1000X optics were used throughout. Aliquots of 0.1 ml of the appropriate dilution to yield 10^3 algal cells were transferred to 10 ml. Erlenmeyer flasks containing 0.3 ml of the AMML basal RM9 medium and 0.1 ml of radioactive substrate. The substrate chosen for investigation in these experiments was a ^{14}C-formate solution, 3.0 uCi/ml. The ^{14}C-formate had a specific activity of 0.454 mCi/mg. A series of 12 replicate test flasks were prepared and wrapped with aluminum foil to exclude light. Four sterile controls were also prepared and incubated at 39°C for two hours along with the test flasks. The Erlenmeyer flasks were then capped for 15 minutes with planchets containing filter pads moistened with saturated barium hydroxide solution. The planchets were then dried for five minutes under an infrared source and counted for radioactivity.

b. Results

The results of this experiment are shown in Table 6. The average response from the test flasks was 567 cpm, seven times that from the sterile controls. This experiment demonstrates the ability to detect the dark respiration of as few as 10^3 algal cells in less than two hours.

2. Heterotrophic Photosynthesis

Several experiments were conducted in order to examine the effect of the duration of the light exposure period upon the differential signal, dark minus light, obtained in the heterotrophic photosynthesis life detection test. Contrary to anticipated findings, a decrease in the differential response was noted when the incubation period was extended from two to five hours.

Table 6

Heterotrophic Metabolism of Chlorella sorokiniana

Responses (cpm)		
Test	Control	
470	82	
454	82	
720	60	
326	99	
418		
709		
673		
418		
709		
637		
418		
688		
567 ± 153	81 ± 10	average ± standard deviation

Assay Conditions:

temperature	$39^{o}C$
illumination	none
medium	0.3 ml RM9
^{14}C-formate	0.1 ml at 3.0 uCi/ml, 0.45 mCi/mg
inoculum	0.1 ml containing 10^3 algal cells
control	sterile
time	2 hours

3. Silastic Membrane

The use of a semi-permeable membrane designed to pass carbon dioxide and retard water vapor was explored for incorporation into the AMML instrument. The rate of loss of water vapor from aqueous solutions exposed to the Martian atmosphere will be very high due to the extant low pressure and extremely low relative humidity. An evaluation was conducted of a "Silastic" membrane material, manufactured by Dow Corning, for the purpose of reducing the rate of loss of water vapor in the heterotrophic metabolism experiment.

a. Experimental

The ability of the semi-permeable "Silastic" membrane to pass $^{14}CO_2$ was measured by performing the labeled release experiment under ambient terrestrial conditions in aluminum planchets covered with the "Silastic" membrane. A 0.3 ml portion of AMML basal RM9 medium containing ^{14}C-formate was added to eight planchets and an inoculum of 1.4×10^4 microorganisms added. Bard-Parker germicide solution was added to four of the planchets. Two of these control planchets and two of the remaining four test planchets were sealed with "Silastic" using an adhesive applied to the rims of the planchets. All eight experimental planchets were then covered with inverted, blank planchets except for the last five minute interval of the incubation periods shown in Table 7. During this interval, the planchets were covered with filter pads moistened with a saturated solution of barium hydroxide in order to collect evolved $^{14}CO_2$. The results of this experiment are presented in Table 7.

A separate experiment was conducted in order to measure the retardation of evaporation under ambient conditions. In this experiment, 0.3 ml portions of distilled water were added to several replicate planchets. These planchets were then sealed with the "Silastic" membrane using adhesive as described above. The planchets were then weighed periodically in order to compare their rates of water loss with open planchets which initially contained 0.3 ml of distilled water. The results of this experiment are shown in Figure 10.

An experiment, similar to the one described above, was conducted at 10 millibars pressure in order to examine the rate of water loss through "Silastic" membrane under Martian atmospheric pressures. This experiment was only semi-quantitative in nature due to the difficulty in performing manipulations in the vacuum chamber. For this same reason no attempts were made to examine the transport of $^{14}CO_2$ through "Silastic" under reduced pressures.

Table 7. Labeled Release Experiment Conducted with a Semi-Permeable Membrane *

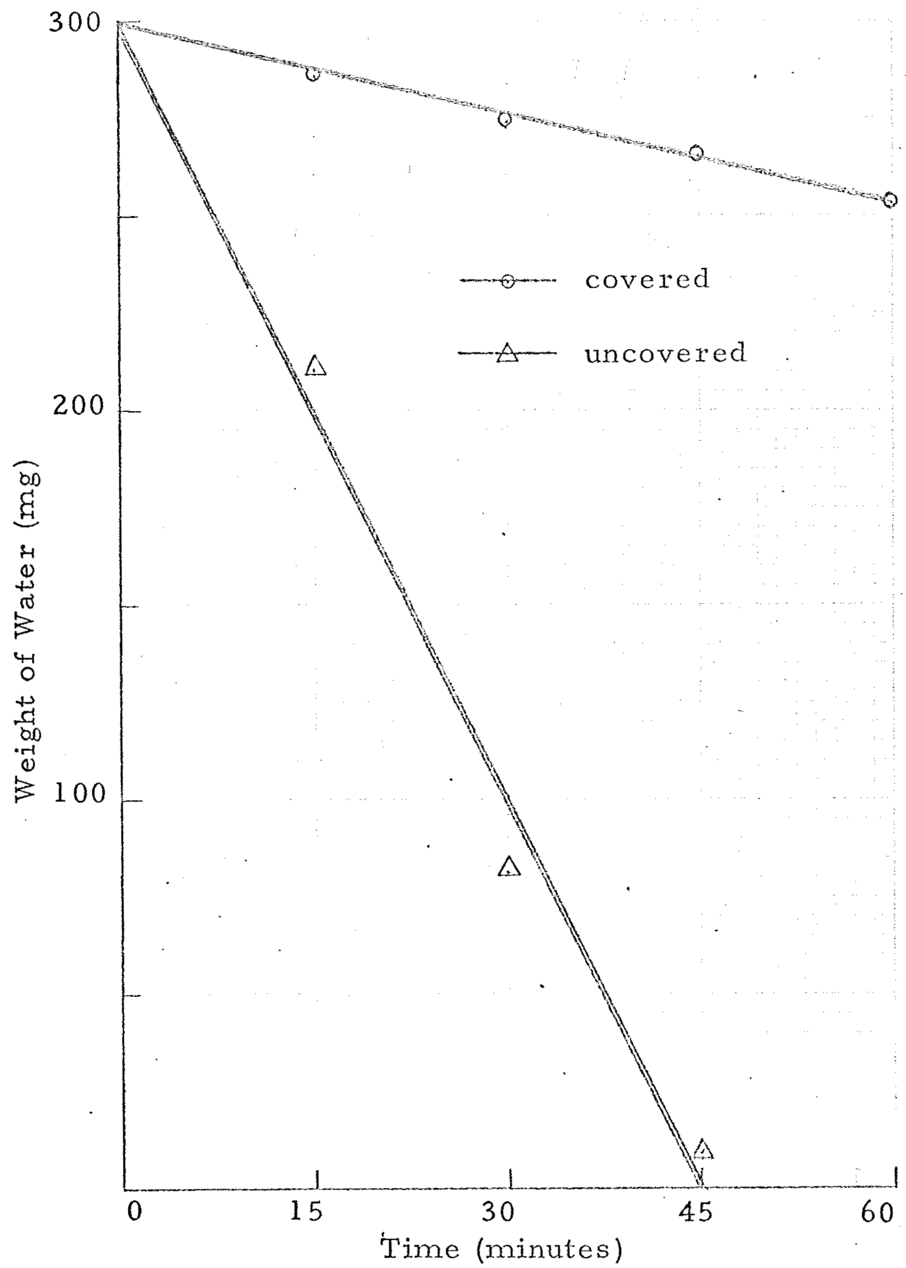
Incubation Time (min)	Responses (cpm) **			
	Membrane	No Membrane	Membrane Bard-Parker Control	No Membrane Bard-Parker Control
30	152	194	67	55
60	144	205	55	52
90	210	233	43	56
120	163	182	69	39

* The semi-permeable material used in this experiment was a silicone "Silastic" membrane manufactured by Dow Corning. The planchets were sealed with the membrane using rubber cement.

** Each of the values reported is the average of duplicate determinations.

Assay Conditions:

inoculum	1.4×10^4 soil bacteria/0.1 ml
substrate	0.3 ml of ^{14}C-formate in basal RM9, 9 uCi/ml
controls	contained 0.1 ml of 0.8% Bard-Parker solution
planchets	were gettered for five minutes at the end of each incubation period.



Retardation of Water Loss by Silastic Membrane

Assay Conditions:

Pressure - Ambient
Temperature - $25^{o} \pm 2\ ^{o}C$
Sample - 300 mg distilled water
Covered planchets sealed with "Silastic" membrane using rubber cement

Fig. 10

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b. Results and Discussion

Table 7 shows the results of the labeled release experiment conducted under ambient terrestrial conditions. Only about a 10% loss in signal was noted with the "Silastic" membrane after the initial one hour period of incubation and a total of only 18% loss was observed over the total two hour course of the experiment. The results presented in Figure 10, similarly, are very encouraging. Only very slight loss of water was observed with the "Silastic" covered planchets in three-quarters of an hour while complete loss occurred in the open planchets during this same period. The experiment conducted under Martian pressures again demonstrated the ability of the semi-permeable membrane to substantially reduce the rate of evaporative loss of water. Although this experiment was only semi-quantitative in nature, due to the difficulties involved with performing manipulations under vacuum, the rate of loss of water through "Silastic" membrane did not appear to be substantially greater under a pressure of only 10 millibars.

These experimental results are very encouraging and indicate the applicability of "Silastic" membrane to the AMML. This membrane may be used to prevent excessive losses of water in experiments requiring the collection and measurement of evolved $^{14}CO_2$.

C. Light Fixation-Dark Release Experiment

1. Introduction

In the light fixation-dark release experiment (LF-DR), soil samples are incubated in the light and in the dark with labeled carbon dioxide. The soils are then placed in the dark at the end of the incubation period and the release of $^{14}CO_2$ measured. The difference in $^{14}CO_2$ produced by the light and dark incubated soils during the dark release period is a measure of the photosynthetic activity of the sample. The amount of $^{14}CO_2$ evolved by the dark incubated soil above that evolved by a sterile, control soil serves as a measure of the light independent fixation of $^{14}CO_2$ by soil microorganisms.

This life detection scheme has an inherent advantage in that the introduction of potentially alien or inhibitory chemical compounds to the sample is not required. The test may be conducted under ambient conditions with no added moisture. The use of $^{14}CO_2$ will not stress the organisms as the Martian atmosphere is composed primarily of carbon dioxide. In addition, it is highly probable that, if life exists on Mars, the organisms will fix carbon dioxide from the atmosphere.

2. Experimental Results

Most of the early LF-DR tests were conducted in 10 ml Erlenmeyer flasks which had been modified by the addition of a 0.5 ml center well. The soil samples were introduced into the main compartments of the flasks and 1.0 uCi of $NaH^{14}CO_3$ in 0.25 ml of solution added to the center wells. Labeled carbon dioxide was then generated by the addition of 0.1 ml of 12N hydrochloric acid to the center wells. The flasks were stopped immediately and exposed to the selected light conditions for the desired incubation period. Earlier work with sterile soils had indicated that non-biological fixation of $^{14}CO_2$ could sometimes contribute to a high background and mask the biological signals. In order to reduce the magnitude of the nonbiological background, a 15 minute air flush of the flasks was used followed by a one hour period in which the flasks were capped with barium hydroxide saturated pads contained in inverted planchets. All flasks were then placed in the dark and gettered for the prescribed periods with basic pads.

Results obtained on a natural soil using the modified 10 ml Erlenmeyer flask are shown in Table 8. The total dark release signal collected in three hours from the light

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Table 8

Light Fixation-Dark Release Experiment Conducted in Modified 10 ml Erlenmeyer Flasks

Dark Release Period (hours)	Responses (cpm) Light	Dark	Autoclaved Control	Empty Flask
0-1	443	295	46	52
1-2	603	420	86	94
2-3	2414	633	144	145

Assay Conditions:

sample	1.0 natural soil
temperature	$25^{o}C$
illumination	300 foot candles for four hours
isotope	0.28 uCi $^{14}CO_2$
flush	15 minutes with air followed by one hour of gettering

incubated soil was about three times that of the dark incubated soil, indicating a significant level of photosynthetic activity. The total signal from the dark incubated soil was about four times that of the autoclaved control soil. It was found that marginal photosynthetic signals could be obtained within a three hour dark release period from a variety of soils.

After this early success, a number of soil samples were examined which gave significantly larger nonbiological background values using the procedure and apparatus described above. After some experimentation, a second exposure chamber was devised which allowed for a transfer of the soil sample following the incubation period with $^{14}CO_2$. The new chambers consisted of 3/4 inch OD glass tubing, two inches in height. The bottoms of the chambers were sealed with aluminum foil covered rubber stoppers. The tops of the light chambers were sealed with glass cover slips lubricated with silicone stopcock grease and the dark chambers were sealed with inverted planchets. Disposable cuvets, cut to fit the chambers, were used to hold the radioisotope solution and for the generation of $^{14}CO_2$ with 18N sulfuric acid. After the light exposure period, the soils were transferred to aluminum planchets for the dark release portion of the experiment. The soil planchets were gettered by covering them with inverted planchets containing filter pads impregnated with saturated barium hydroxide solution.

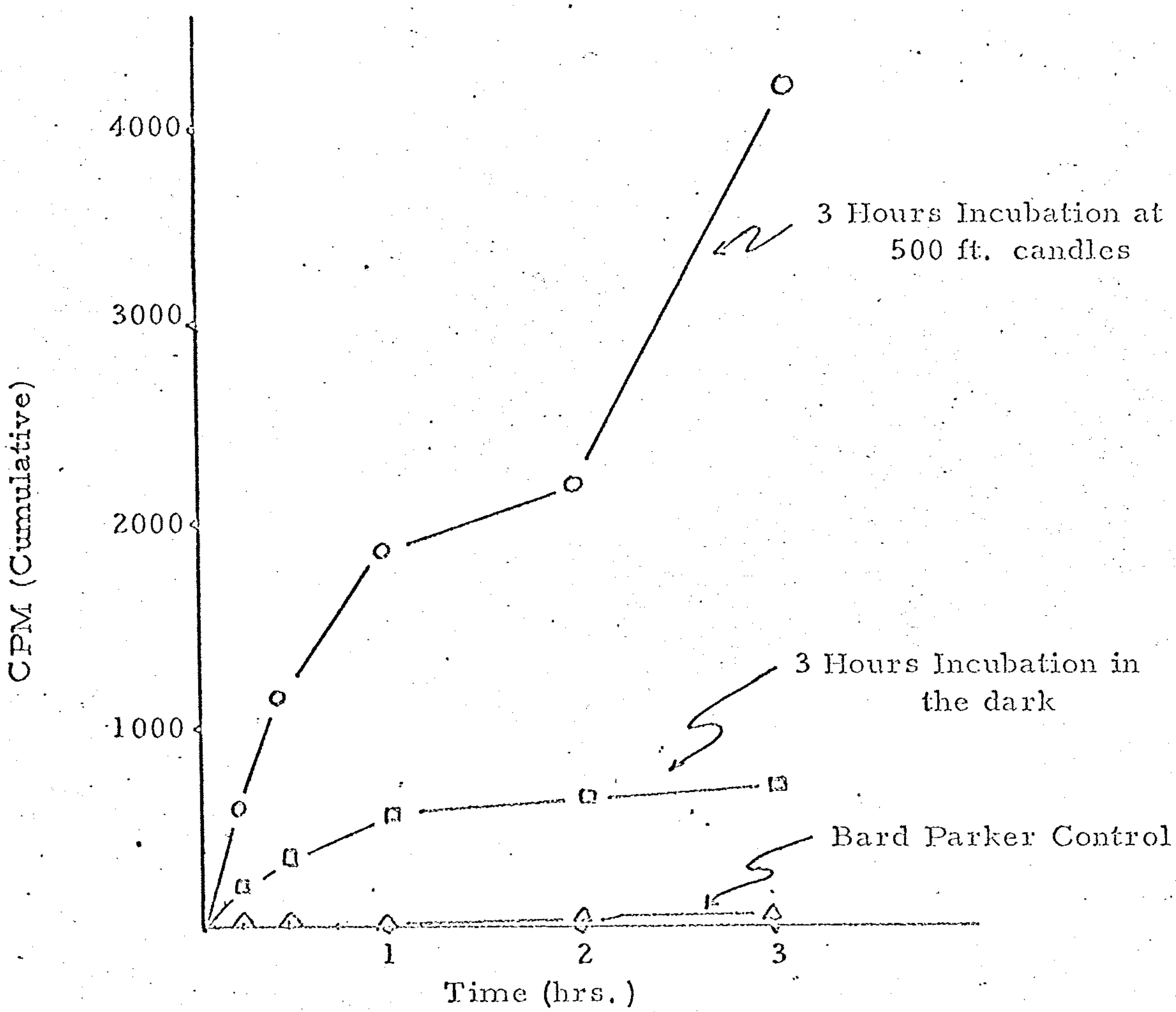
Examples of experimental results obtained using the soil transfer technique are shown in Figures 11 and 12. Figure 11 shows the assay of a local garden soil while Figure 12 demonstrates the effect of added water upon the photosynthetic response from an arid soil. A number of soils were assayed in this manner and produced positive photosynthesis responses.

D. Detection of Phosphate Metabolism

1. Introduction

As proposed for this program, the depletion of phosphate-phosphorus (PO_4-P) from liquid growth medium by "simulated Martian microorganisms" has been investigated as a life detection method. This method entails periodic phosphate measurements on aliquots of the AMML medium to follow the uptake by the extraterrestrial organisms. Experience with terrestrial microbial populations has led to a desired minimum detectable PO_4-P uptake of about 0.5 ppm. The low level of uptake postulated places severe restrictions on the phosphate analytical method and upon the

Demonstration of Metabolic Response from Garden Soil in Photosynthetic Fixation and Release Test



Assay Conditions:

Radioisotope - 1.0 µCi $NaH^{14}CO_3$/10 cc gas volume

Soil Type - 0.5 gm Garden Soil

Illumination - 500 Foot Candles; G.E. Power Groove Fluorescent

Fig. 11		
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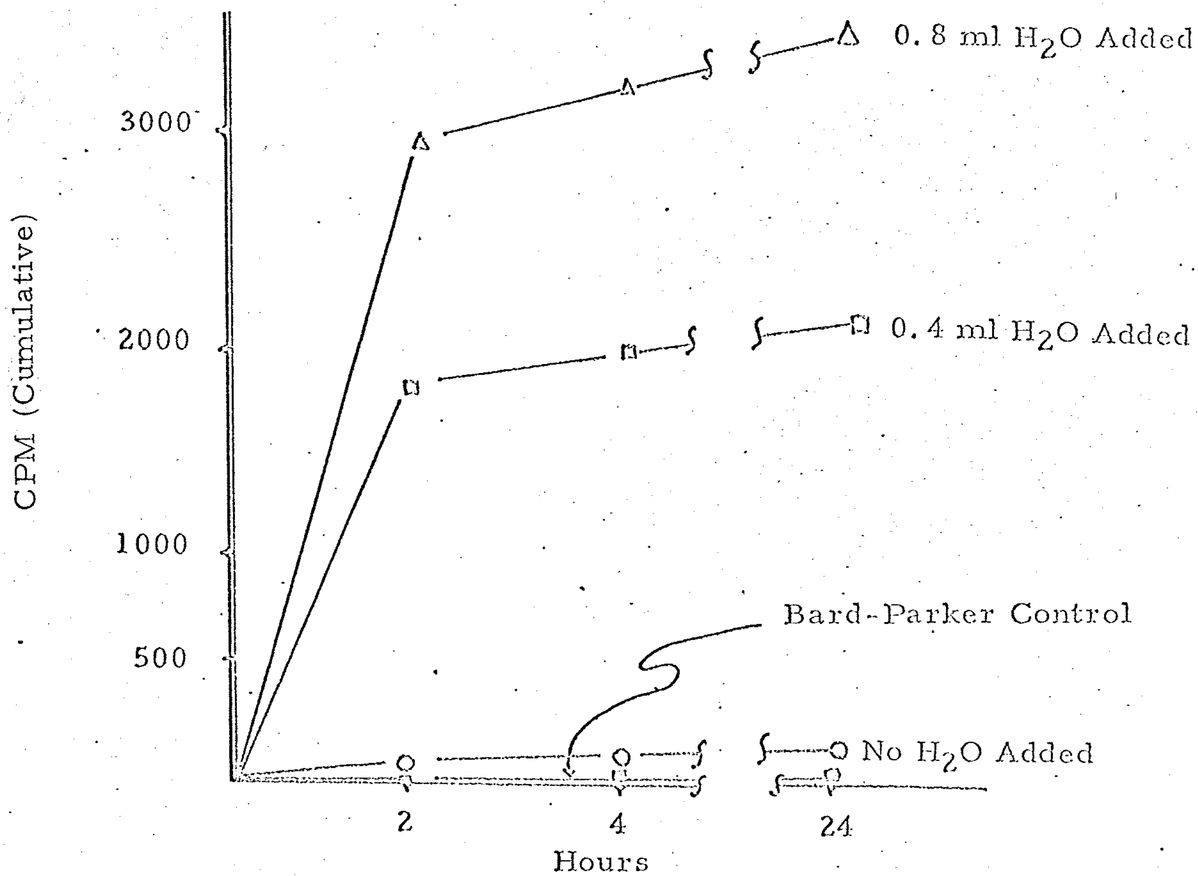
Effect of Supplemental Moisture on $^{14}CO_2$ Fixation and Release from an Arid Soil



Assay Conditions

Illumination - 500 Foot Candles for 3 Hours
Isotope - 1 μCi $NaH^{14}CO_3$
Soil - 0.5 gm Air Dried
Gas Volume - 10 ml

	Fig. 12
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phosphate concentration of the medium in order that a favorable signal-to-noise ratio (S/N) can be obtained from this experiment. The signal in this case refers to the difference between the phosphate level of the medium containing the sterile control soil and the medium containing the test soil. The noise then refers to the phosphate level remaining in the medium containing the test soil. In order to measure an uptake of 0.5 ppm PO_4-P, an analytical method is required which has a sensitivity of at least 0.2 to 0.3 ppm. The S/N will be undesirably low at initial PO_4-P concentrations of above 3 ppm in the AMML medium, assuming the minimum uptake of 0.5 ppm. An initial PO_4-P concentration of 1 ppm was selected for the medium as this will give a S/N of 1, again assuming the minimum uptake. This requirement is complicated by the possibility that the soil inoculum may contain large amounts of soluble phosphate and therefore place the life detection scheme into a relatively insensitive area of operation. Should this occur, alternate procedures such as dilution of the suspension, reinoculation of fresh medium with a smaller amount of soil, or filtration to remove soluble phosphate followed by resuspension of the soil in fresh medium would be required.

A colorimetric and a radioisotopic phosphate analytical method were studied and research performed to modify these procedures for application in the AMML instrument. Comparative studies were made of the results obtained on soil extract by these two methods. Biological experimentation with soils simulating the instrument operation was conducted in the laboratory using both phosphate assay methods. Phosphate uptake experiments were performed with algae in low phosphate medium and the photosynthetic nature of this uptake was discovered as is described subsequently. It is on the basis of this discovery that we now recommend examination for a photosynthetic component in the ^{14}C and ^{35}S uptake, ATP production, and PO_4-P uptake life detection schemes. If conducted in this manner, the AMML instrument will determine the light dependent properties of each of the five proposed experiments.

2. Chemistry

a. Colorimetric Determination of Orthophosphate as Molybdenum Blue

The molybdenum blue colorimetric method (7) for the determination of orthophosphate was selected for study here as it is one of the most thoroughly investigated and widely used methods available today. In addition, this method is relatively sensitive and generally free from serious interferences.

The method is based upon formation of moylbdenum blue by reduction of the phosphomolybdic acid complex. The analytical procedure developed for use in the laboratory is described below:

Reagents:

1. Ammonium Molybdate Solution - a 10% aqueous solution of $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$ is diluted with three volumes of 50% sulfuric acid.

2. Stannous Chloride Solution - 400 mg of $SnCl_2 \cdot 2H_2O$ is dissolved in 100 ml of 10% hydrochloric acid (prepared fresh daily).

3. Phosphate Standards - A stock solution containing 4.3916 g/l (1000 ppm PO_4-P) is prepared and a few ml of chloroform are added as a preservative. Dilutions down to 1 ppm may be stored in a refrigerator.

Procedure:

1. Add 10 ml of sample or standard to the assay tube.

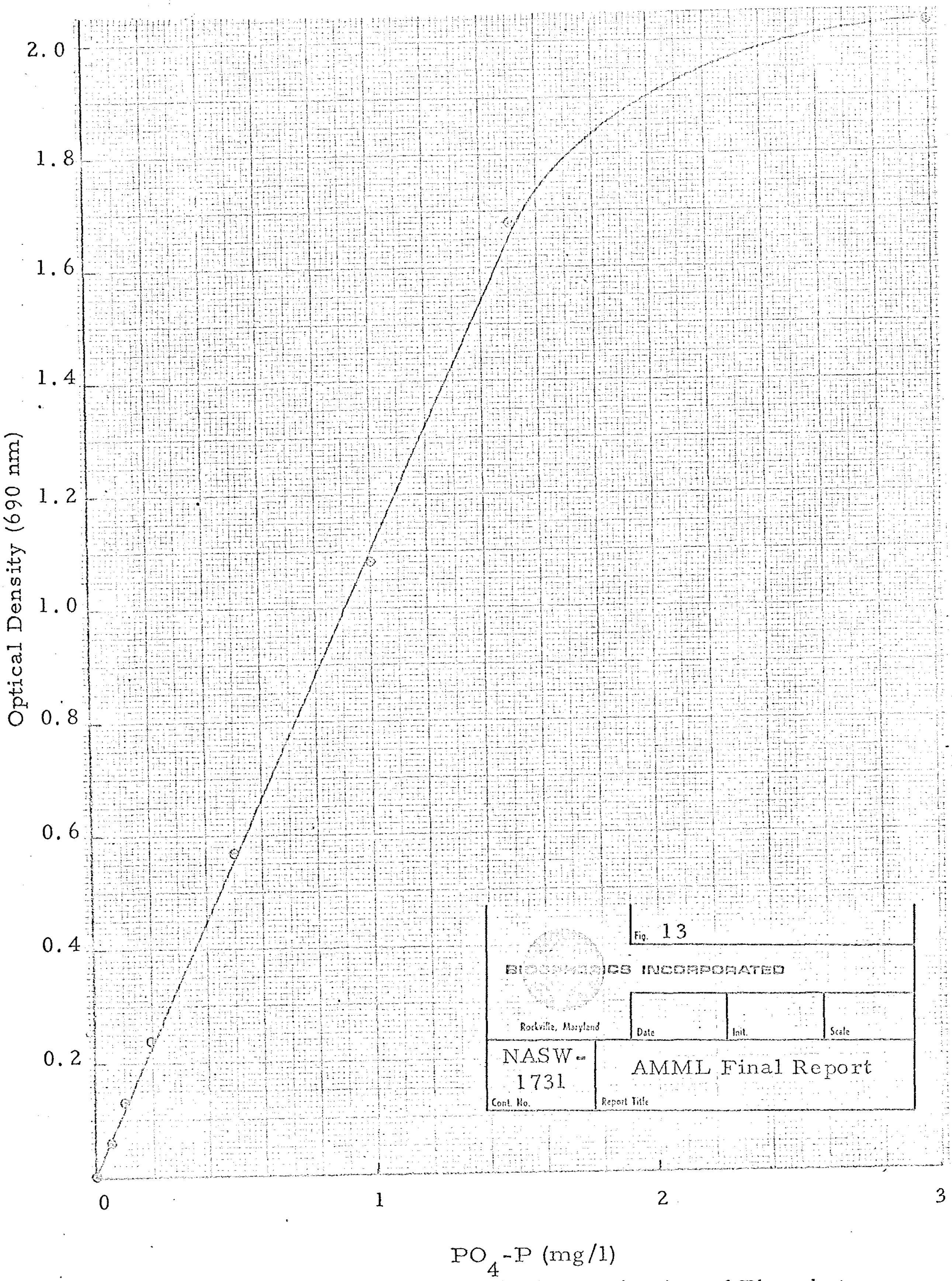
2. Add 0.2 ml of ammonium molybdate reagent and mix thoroughly.
3. Immediately add 0.2 ml of stannous chloride reagent and mix thoroughly.
4. After 12 to 15 minutes at room temperature, read at 690 nm in a 13 mm cell against a reagent blank.

A typical standard curve is shown in Figure 13. The limit of sensitivity of this method is about 0.05 ppm PO_4-P. The sensitivity may be increased by the use of a longer light path. The standard curve begins to deviate from linearity at about 2 ppm PO_4-P.

1) Interference of EDTA with the Colorimetric Phosphate Assay

Initial studies on the uptake of phosphate by green algae grown in defined algal medium showed anomalous results. An investigation of the cause of the discrepancies noted was undertaken and it was found that the chelating agent used in the Chlorella medium interfered with the formation of the phosphomolybdate complex in the assay. A study was made to determine the magnitude of this problem in light of the possibility that chelated metals may be indicated for the AMML medium finally selected.

Phosphate standards were prepared at levels up to



Standard Curve - Colorimetric Determination of Phosphate

0.3 ppm PO_4-P in distilled water and in 10^{-3}, 10^{-4}, and 10^{-5} M ethylenediaminetetraacetic acid (EDTA). These solutions were then analyzed by the molybdenum blue colorimetric method.

The results of this experiment are presented in Table 9. These results show that, at the phosphate levels studied here, interferences by the EDTA just begin to become noticeable at a concentration of about 10^{-4} M. EDTA levels above this concentration will interfere more severely. However, a quantitative relationship exists between phosphate and the optical density at 690 nm even in the presence of interfering amounts of EDTA. It is possible to circumvent this interference, at least in the presence of moderate amounts of EDTA, by preparing the phosphate analytical standards in medium rather than in distilled water. Krauss medium, optimal for several photosynthetic organisms, has an EDTA concentration of only 1.8×10^{-4} M and will present no analytical problem if this technique is employed.

2) Colorimetric Phosphate Analyses of Soil Extracts

The AMML media (Table 1) contain as much as 100 ml of a soil extract per liter of medium. These soil extracts were prepared by autoclaving a soil-water suspension. The extracts were then filtered prior to addition

Table 9

Interference of Ethylenediaminetetraacetic Acid in the Molybdenum Blue Colorimetric Assay for Orthophosphate

PO_4-P Added (mg/l)	EDTA (M)	PO_4-P Recovered (mg/l)	(%)
0.1	10^{-5}	0.100	100
0.2	10^{-5}	0.200	100
0.3	10^{-5}	0.300	100
0.1	10^{-4}	0.100	100
0.2	10^{-4}	0.198	99
0.3	10^{-4}	0.292	97
0.1	10^{-3}	0.079	79
0.2	10^{-3}	-	-
0.3	10^{-3}	0.238	79

to the medium. Several soil extracts were prepared and phosphate standards made up in solutions containing a final concentration of 10% extract. These phosphate solutions were then assayed by the molybdenum blue colorimetric method in order to test for interferences in the AMML medium as a result of the addition of soil extract.

The results of this experiment are shown in Table 10. The analyses were satisfactory up to a level of about 0.3 ppm PO_4-P and deteriorated rapidly above this concentration. As a result of this study, all phosphate solutions containing soil extract were diluted below 0.3 ppm PO_4-P prior to colorimetric assay. Additional research, perhaps the introduction of an ion exchange resin prior to color development or a higher concentration of color reagents, is required to expand the useful range of this assay on solutions containing soil extracts. Although it is anticipated that further experimentation will resolve these analytical problems, a working system involving automated dilutions could be devised for the AMML instrument which would circumvent the interferences described here.

b. Radioisotopic Assay of Orthophosphate

The radioisotopic modification of the procedure of Sugino and Miyoshi (8) was selected for study

Table 10

Interference of Soil Extract with the Molybdenum Blue Colorimetric Assay for Orthophosphate

	PO_4-P Found			
	Extract 1		Extract 2	
Sample	mg/l	% Recovery	mg/l	% Recovery
10% Soil Extract	0.054	-	0.040	-
10% Soil Extract + 0.1 mg P/l	0.144	90	0.141	101
10% Soil Extract + 0.2 mg P/l	0.239	93	0.217	89
10% Soil Extract + 0.3 mg P/l	0.335	90	0.313	91
10% Soil Extract + 0.4 mg P/l	0.358	76	0.420	95
10% Soil Extract + 0.5 mg P/l	0.441	77	-	-

as this method is especially compatible with the AMML instrument. In addition, the method appeared to offer the advantages of simplicity and adequate sensitivity. The method is based upon formation of the ^{14}C-triethylamine analog of the well known ammonium phosphomolybdate precipitate which has been widely used for the gravimetric determination of phosphorus. The radioactive precipitate is then filtered, washed, dried, and counted. These four unit operations are integral parts of the AMML system already in use in other life detection schemes.

The procedure used in laboratory experiments and proposed for the AMML instrument was as follows:

> To 1 ml of sample solution is added 0.05 ml of 4N perchloric acid, 0.25 ml of 0.08 M ammonium molybdate($(NH_4)_6Mo_7O_{24}\cdot 4H_2O$), and 0.05 ml of 0.8 M ^{14}C-triethylamine hydrochloride (TEA.HCl), 5 uCi/ml.

This suspension is mixed and allowed to stand for two minutes prior to filtration through a 0.45 u microbial filter. The precipitate is rinsed with 1 ml of the wash solution described below:

> 10 ml distilled water
>
> 0.5 ml 4N $HClO_4$
>
> 2.5 ml 0.08 M $(NH_4)_6Mo_7O_{24}\cdot 4H_2O$
>
> 0.5 ml 0.8 M TEA.HCl (unlabeled)

The filter is then dried and counted. Results are read off of a standard curve prepared in a similar manner. A typical standard curve prepared early in the program is shown in Figure 14. The curve appeared to be linear in the range from 0 to 60 ppm PO_4-P, but the sensitivity (approximately 150 cpm/ppm PO_4-P) was not adequate. Curves of this type pointed up a number of problem areas and further experimentation was undertaken to improve the assay.

1) Analytical Method Development for the Radioisotopic Phosphate Assay

Several techniques were studied in an attempt to improve the radioisotopic assay method for orthophosphate. A number of filter types were studied to test their ability to retain quantitatively the labeled precipitate and for their ability to pass the dissolved ^{14}C-TEA so as to have as low a background as possible. A significant improvement in the filterability of the labeled precipitate resulted from incorporation of a two minute aging period between final mixing of the sample plus reagents and the filtration step.

The results of an early experiment conducted to study the retentive ability of glass fiber filter mats and 0.45 u Gelman cellulose acetate membrane filters are shown in

Table 11. In this experiment, the phosphate was precipitated with unlabeled reagents. The precipitates were then filtered on the selected filter materials and then washed in the manner prescribed by the radioisotopic method. The triethylammonium phosphomolybdate precipitates were dissolved off of the filters with dilute ammonia and the recovered phosphate measured using the molybdenum blue colorimetric procedure. These results demonstrated that both types of filters have adequate retentive properties. However, this experiment did not measure the amounts of radioactivity retained by the filters which was not associated with phosphate. Later experiments proved the glass fiber filter mats to be unsatisfactory as a lower sensitivity was obtained when labeled precipitate was counted on this material. This was probably caused by penetration of the labeled precipitate below the surface of the filter.

Several different filter types were studied for their ability to pass the soluble radioactive reagents not associated with phosphate. The results of this experiment are shown in Table 12. The low background levels obtained with the Teflon filters were very encouraging. However, later experiments of the filter. The results shown in Table 13 demonstrate that this manual technique was effective both in quantitative recovery of the precipitate and in lowering the background level. However, the manual procedure was abandoned after a number of tests were run as the method could not readily be incorporated into the automated instrument. Later advances described in the next paragraph compensated for abandoning the manual procedure.

Several wash procedures have been evaluated for the removal of non-specifically bound isotopic label. While all procedures tested tend to lower background activity somewhat, no single method is particularly effective. Attempts at presaturating binding sites on the filters with unlabeled TEA have been made and these results are shown in Table 14. An improvement of about 50% resulted when the membrane filter was prewet with 0.04 M ^{12}C-TEA. Table 15 shows the effects of prefiltration of the reagents through a 5.0 u membrane filter prior to use in the radioisotopic assay. The first filtration reduced the background by 50 to 60% while the second filtration was essentially without effect.

The two minute aging period for formation of the labeled precipitate, prefiltration of the reagents, and prewetting of the 0.45 u Millipore cellulose acetate membrane with 0.04 M ^{12}C-TEA filter have been incorporated into the final procedure

Table 11

Retention of Triethylammonium Phosphomolybdate Precipitate by Glass Fiber Filter Mats and by Gelman Cellulose Acetate Microbial Filters

PO_4-P Standard (mg/l)	Volume Filtered (ml)	Filter Type	Recovery (%)
0	1.0	Glass Fiber	-
0	1.0	Microbial	-
5	1.0	Glass Fiber	89
5	0.5	Glass Fiber	98
10	1.0	Microbial	105
50	1.2	Glass Fiber	99
50	1.0	Microbial	105
100	1.0	Glass Fiber	98
100	1.0	Microbial	103

Table 12

Retention of Radioactivity by Candidate Filter Materials for the Radioisotopic Phosphate Assay

Filter	Activity Retained $\pm \sigma$* (cpm)	Retention (%)
0.45 u cellulose acetate	157 $\pm$ 19	0.14
5.0 u polyvinyl chloride	200 $\pm$ 105	0.19
Teflon - 500 P	78 $\pm$ 34	0.08
Teflon - 500 PA	44 $\pm$ 6	0.05

* The values reported are averages of three replicate determinations corrected for background.

Assay Conditions:

1.0 ml	distilled water
0.05 ml	4N $HClO_4$
0.25 ml	0.08 M $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$
0.05 ml	0.8 M ^{14}C-TEA, 5 uCi/ml

showed that both the 500 P and 500 PA filters did not quantitatively retain the labeled precipitate. The range of pore sizes of the Teflon filters was very wide and ranged from 10 to 50 u. Unfortunately, Teflon filters with the smaller pore sizes required to retain the precipitate are not presently available for study, but it appears that they might resolve the high background problem. Several brands of 0.45 u cellulose acetate membrane filters were studied and appeared to be roughly equivalent in their performance. Stainless steel filters were also studied, but did not perform as well as the cellulose acetate filters.

In using the membrane filter holder assembly in the usual manner, the edges of the filter were held between the sintered glass filter support on the bottom and the rim of the glass funnel on top. It was felt that perhaps radioactive material in the solution was wetting this protected area and was not subsequently removed during washing of the radioactive precipitate. An experiment was conducted in which the funnel was removed and not used during the filtration or the subsequent wash step. The suspension to be filtered was carefully placed onto the filter so as to effect a quantitative transfer. Similarly, the wash solution was carefully applied so as to minimize losses yet wash the entire surface

Table 13

Effect of Filtration Method on Background Radioactivity in Radioisotopic Phosphate Assay

Phosphate Level	Cellulose Acetate Filter Pore Size	Radioactivity Retained $\pm\sigma$ (cpm)*	
(mg PO_4-P/l)	(u)	Standard Filtration	Manual Filtration
0.0	0.45	162 ± 25	71 ± 2
0.5	0.45	302 ± 30	206 ± 20
0.0	5.0	132 ± 17	88 ± 21
0.5	5.0	275 ± 34	248 ± 32

* The values reported are the averages of six determinations corrected for background

Table 14

Effect of Prewetting Filter * with Unlabeled Reagents
Upon the Non-Specifically Bound Radioactivity

Prewetting Solution	Activity Retained $\pm \sigma$ ** (cpm)	Retention (%)
None	157 $\pm$ 19	0.14
Water	229 $\pm$ 75	0.20
0.04 M ^{12}C-TEA	84 $\pm$ 9	0.08
0.8 M ^{12}C-TEA	103 $\pm$ 45	0.10

* Millipore, cellulose acetate, 0.45 u membrane filters were used in this experiment.

** The values reported are averages of five replicate determinations corrected for background.

Table 15

Reduction of Background by Filtration of Reagents Prior to the Radioisotopic Assay*

Phosphate Level (mg PO_4-P/l)	Radioactivity (cpm)** No Filtration	1st Filtration	2nd Filtration
0.0	207	92	89
0.2	286	176	185
0.5	422	306	293

* Each of the required reagents were filtered through 5.0 u membrane filters as indicated.

** The values reported are the averages of triplicate determinations.

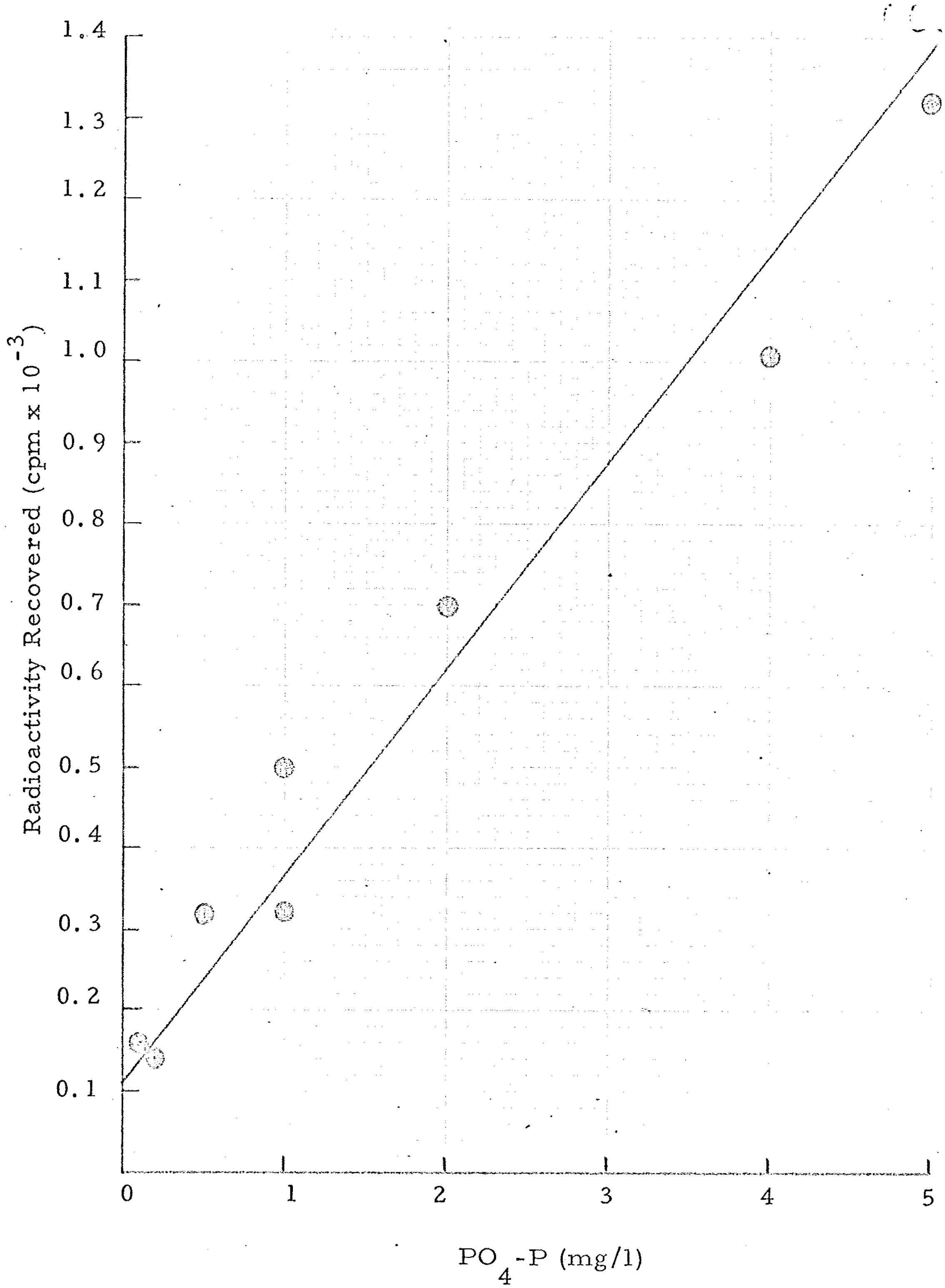
for the radioisotopic measurement of orthophosphate. Standard curves were prepared using the finalized procedure and are shown in Figures 15 and 16. Figure 15 demonstrates a limit of sensitivity about 0.3 ppm PO_4-P, however, the precision shown here is not altogether satisfactory and further study is needed to improve this method.

2) Simplification of the Radioisotopic Assay Method

Experimentation was conducted in order to simplify the radioisotopic phosphate assay procedure for incorporation into the AMML instrument. A new procedure was developed which incorporates the precipitation reagents into a single solution prepared as described below:

1.0 ml	4N $HClO_4$
5.0 ml	0.08 M $(NH_4)_6Mo_7O_{24}\cdot 4H_2O$
0.8 ml	0.8 M TEA.HCl
3.2 ml	distilled water

A slight precipitate forms immediately upon mixing these solutions. This may be removed by filtration or by centrifugation at 13,000XG for five minutes. A second precipitate will form in the combined reagent after storage for several days. The reagent should be filtered through a 0.45 u or 5.0 u membrane filter just prior to use in the assay. The employment of a single combined reagent



Standard Curve - Radioisotopic Assay of Phosphate, 0-5 mg/l

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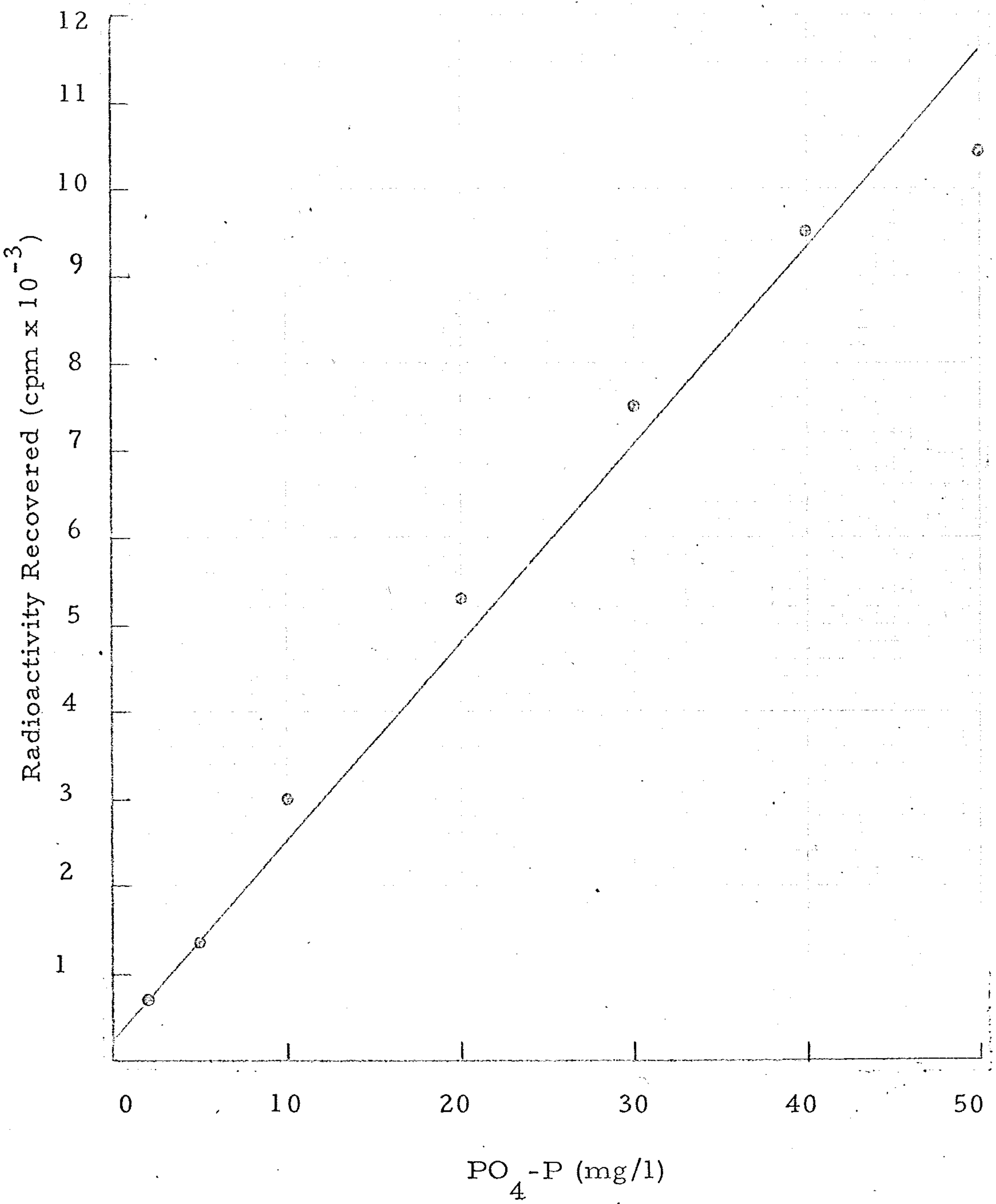

Standard Curve - Radioisotopic Assay of Phosphate, 0-50 mg/l

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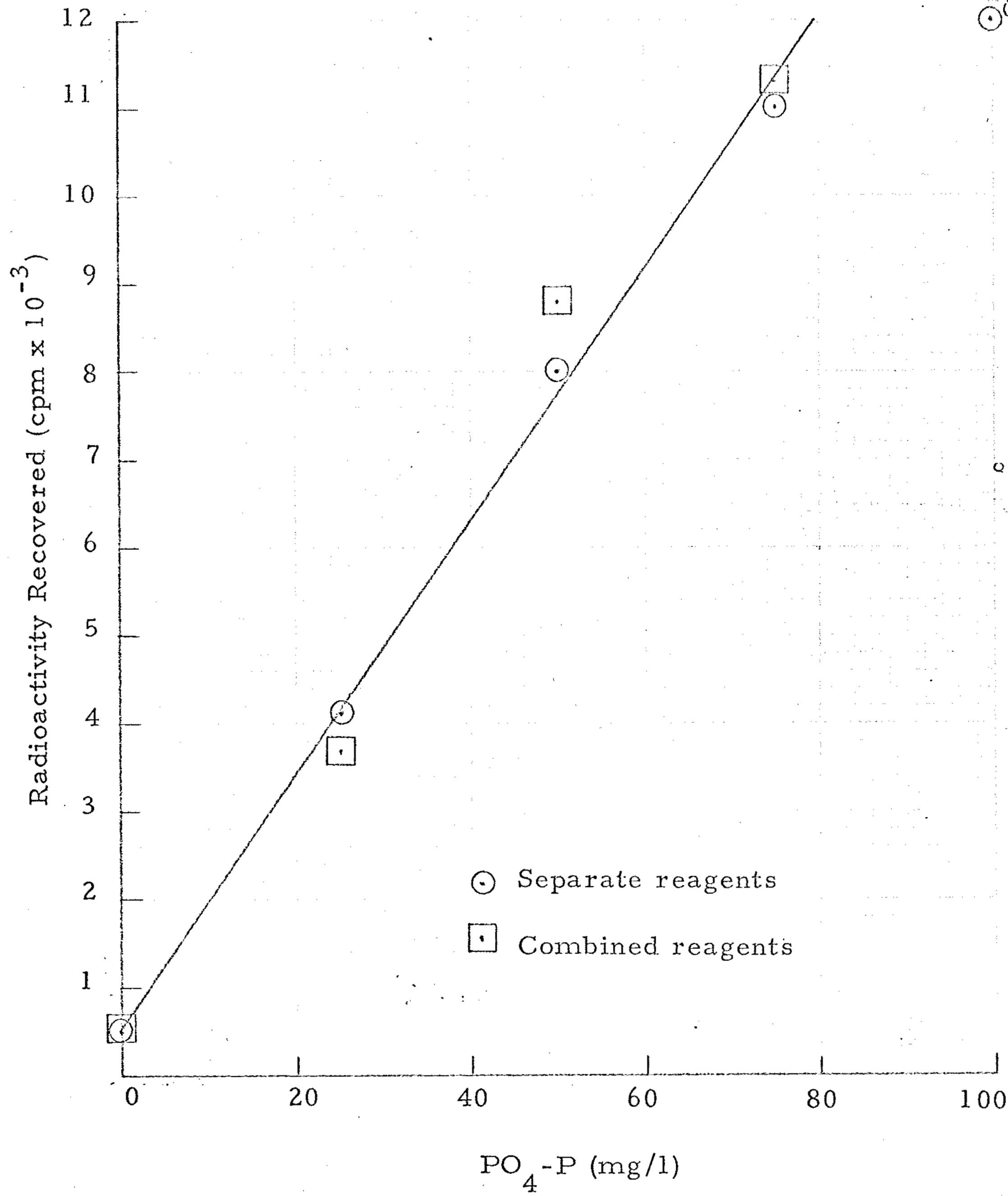
in the instrument would considerably simplify the operation over that required to add three separate solutions to the phosphate sample.

The precipitation reaction was conducted using the combined reagent in a syringe comparable to the mechanism in the current AMML design. A sample of 0.5 ml of phosphate standard and 0.5 ml of combined reagent was mixed in a 2.5 cc disposable syringe and the resulting suspension was injected through a 0.45 u Gelman membrane filter. The syringe was then filled with one ml of wash solution to clean the syringe and rinse the precipitate and filter pad. The filters were then assayed for radioactivity. The experiment presented in Figure 17 shows a comparison of results obtained using the combined reagent and the usual laboratory procedure involving the preparation and addition of three separate reagents. Comparable results were obtained using these two techniques for the radioisotopic measurement of phosphate.

3) Radioisotopic Analyses of Soil Extracts for Phosphate

An extensive investigation was conducted to determine the applicability of the radioisotopic phosphate assay to the AMML instrument. RM9 and water were inoculated



Radioisotopic Phosphate Assay - Use of a Combined Reagent for the Precipitation of Phosphate

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with soils and allowed to stand for 24 hours, approximating the aqueous growth chamber of the instrument. Aliquots of the aqueous supernatant were then filtered and analyzed using the radioisotopic method. Colorimetric assays were performed simultaneously to provide a standard value for comparison. As previously recommended, the aliquots for colorimetric analyses were diluted to give an optical density below 0.3 in order to ensure accurate measurements. Some of the analytical values obtained are reported in Table 16.

The results in Table 16 show that the radioisotopic phosphate assay method is not generally applicable to the analyses of soil extracts. The radioisotopic method gave results considerably higher than the colorimetric procedure in most cases. The agreement between the two methods was very good with several of the soils. In general, the agreement between the methods was better for the water than for the RM9 medium. This indicated that the soil extract in the medium was contributing a positive interference to the radioisotopic method.

Considerable research is required to adapt the radioisotopic method for use in the AMML. In addition to the problems discussed previously for marginal sensitivity and precision and a high blank level, there is a serious positive

Table 16

Analyses of Soil Extracts for Phosphate *

Soil	g Soil/ 50 ml	Medium	PO_4-P Found (ppm) Radioisotopic	Colorimetric
Death Valley	0.5	RM9	1.5	0.1
" "	0.5	H_2O	0.5	0.1
JPL #100	0.5	RM9	2.2	0.9
" "	0.5	H_2O	0.6	0.7
JPL #120	0.5	RM9	2.2	0.9
" "	0.5	H_2O	0.6	0.5
JPL #121	0.5	RM9	3.1	1.2
" "	0.5	H_2O	1.2	0.9
North Carolina	0.5	RM9	0.2	0.1
" "	0.5	H_2O	0.5	0.5
" "	2.0	RM9	0.5	0.2
" "	2.0	H_2O	1.0	1.0
Chilean #259	0.5	RM9	3.0	1.0
	0.5	H_2O	0.5	0.2
Chilean #271	0.5	RM9	1.7	1.0
	0.5	H_2O	0.6	0.2
Indiana	0.5	RM9	0.8	0.6
	0.5	H_2O	0.6	0.6

Table 16 (continued)

Soil	g Soil/ 50 ml	Medium	PO_4-P Found (ppm) Radioisotopic	PO_4-P Found (ppm) Colorimetric
Indiana	2.0	RM9	2.7	2.4
	2.0	H_2O	4.2	4.2
Indiana (Auto-claved)	0.5	RM9	1.7	1.4
	0.5	H_2O	1.5	1.5
Taylor Street	0.5	RM9	0.1	0.0
	0.5	H_2O	0.6	0.7
Taylor Street	1.0	RM9	0.1	0.1
	1.0	H_2O	0.9	0.9

* Indicated weight of soil was placed in 50 ml of RM9 or water and allowed to extract for 24 hours. Phosphate assays were performed after the extraction period.

interference to the method extracted from soils by aqueous media. The use of a cationic exchange resin to remove interfering positive ions is one possible approach to this problem.

3. Biology

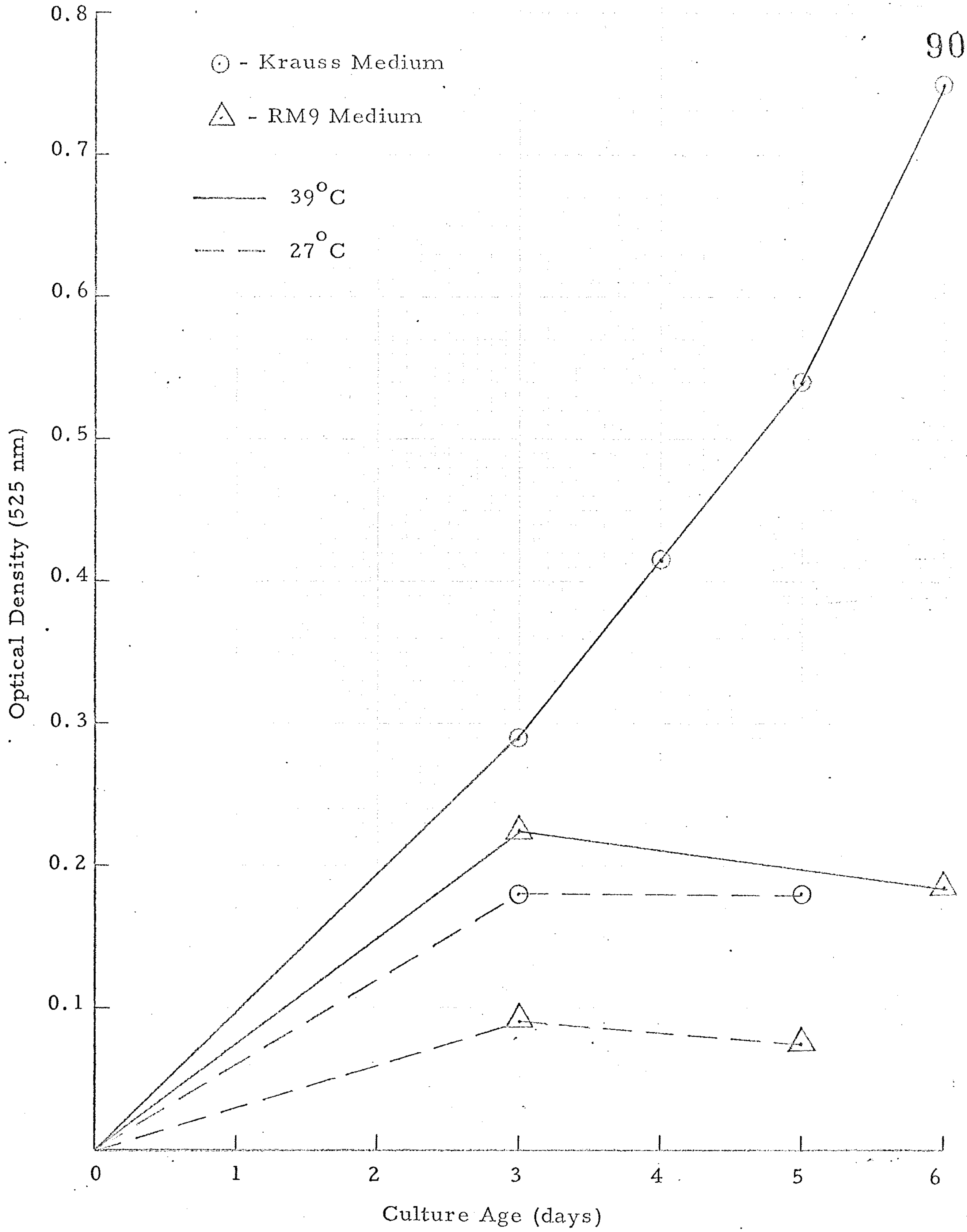
a. Introduction

As discussed earlier in this section, the phosphate uptake life detection scheme requires that the extraterrestrial microorganisms be placed in a low phosphate medium in order that a limited uptake can be measured with some degree of sensitivity. The biological investigations conducted for this phase of the program consisted of experiments designed to study the effects of low phosphate media upon the growth of algae, the uptake of phosphate by algae, and the uptake of phosphate by soil microorganisms.

b. Effects of Low Phosphate Media Upon the Growth of Algae

1) Experimental Results

The growth of *Chlorella sorokiniana*, *Chlorella vannielii*, and *Chlorella vulgaris* was compared at 27° and 39°C in a low phosphate AMML, RM9, and in Krauss *Chlorella* medium. The results are shown in Figures 18, 19, and 20, respectively. The figures show that, generally, the growth rate of *Chlorella* levels off sometime after 72 hours in the low phosphate



Growth of Chlorella sorokiniana at 27° and 39°C in Krauss and Low Phosphate Media

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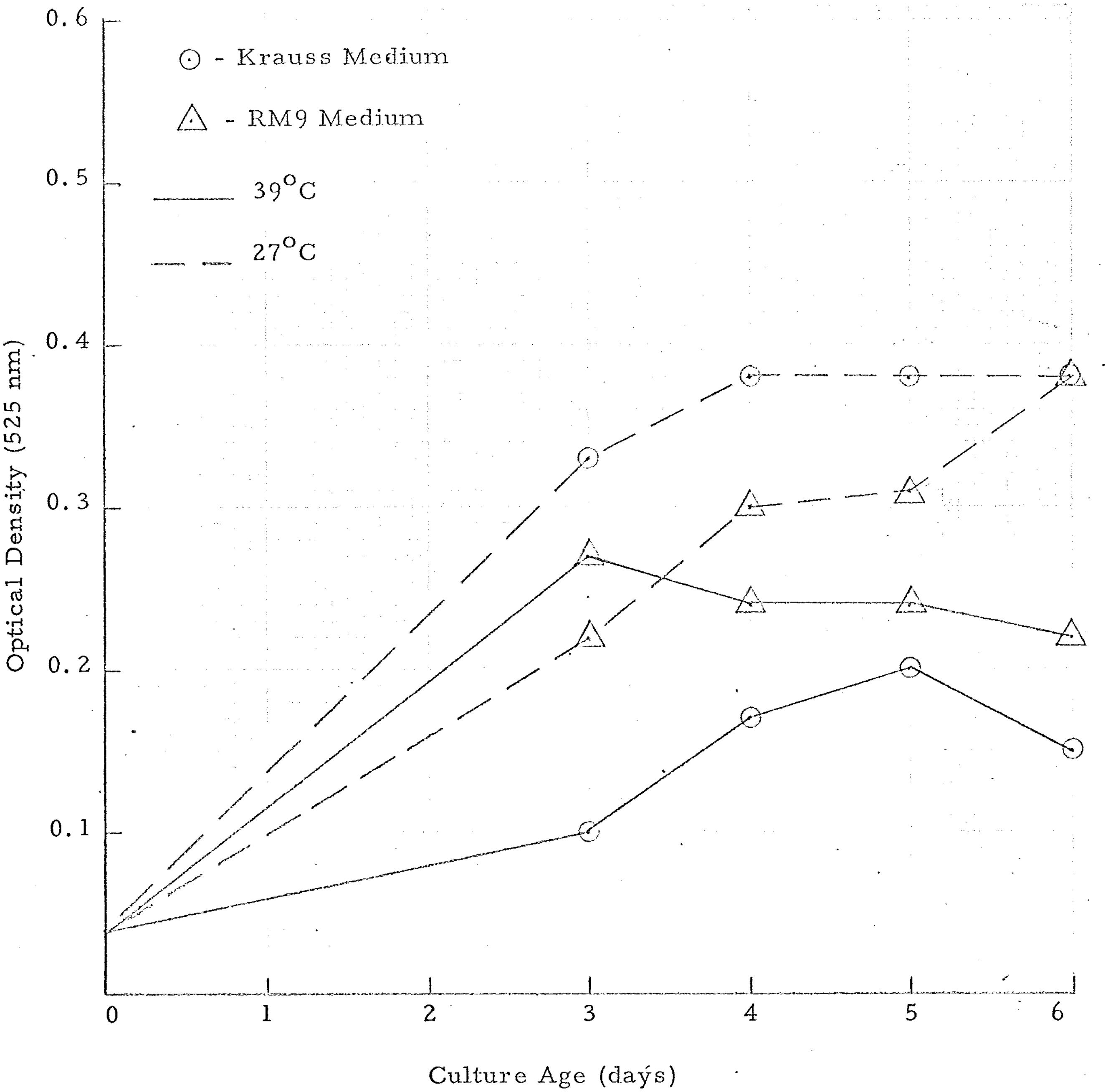

Growth of Chlorella vannielii at 27° and 39°C in Krauss and Low Phosphate Media

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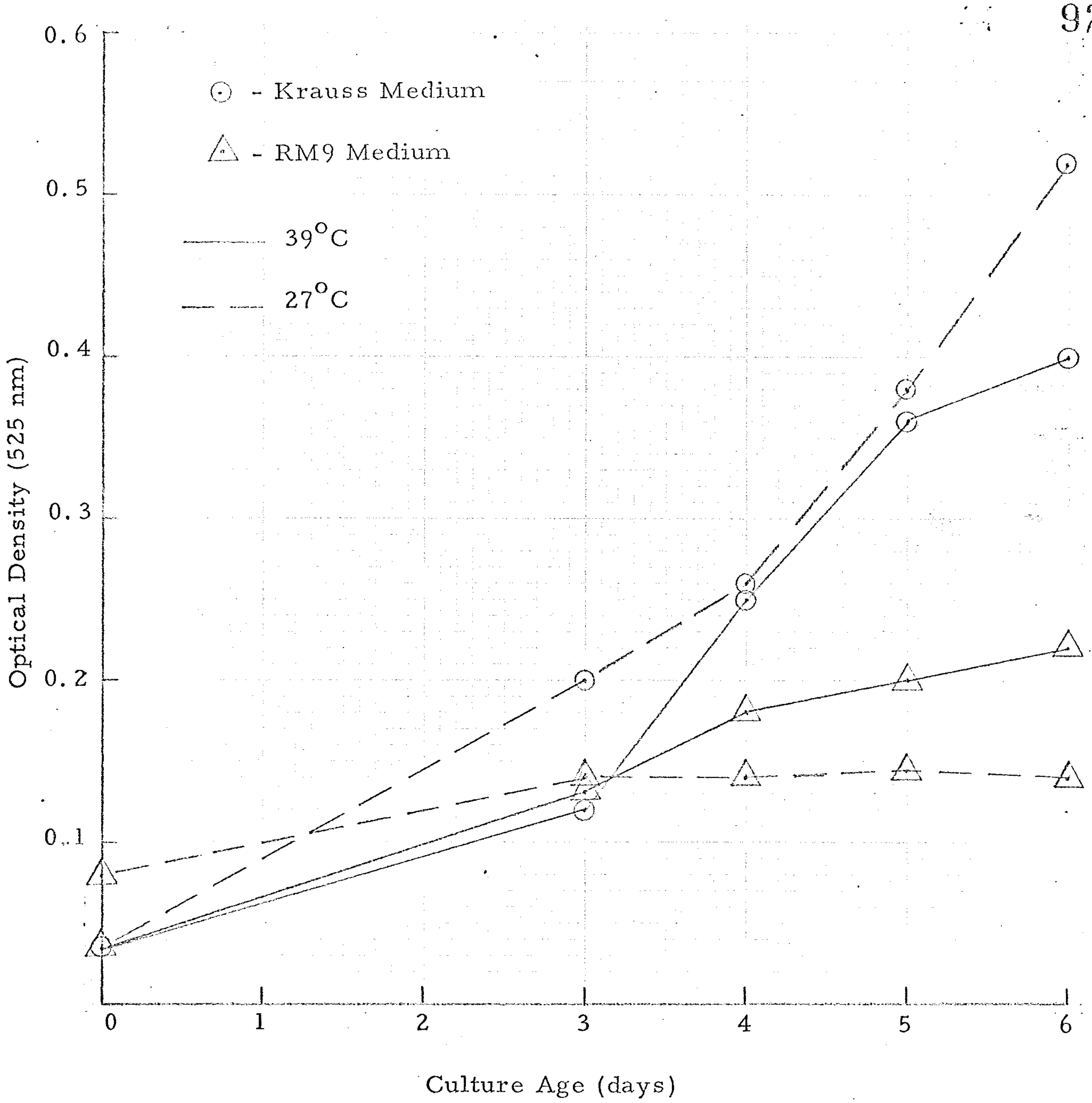

Growth of Chlorella vulgaris at 27° and 39°C in Krauss and Low Phosphate Media

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medium. However, until at least the 72nd hour, growth in the low phosphate medium was always at greater than 50% of that in Krauss' medium. This result is highly encouraging from the standpoint of suitability of RM9 for the aqueous growth chamber in that it is unlikely that the AMML will be designed to obtain data for more than 72 hours unless more medium is added.

In a continuation of this research, a second experiment was conducted in order to study the effects of a low phosphate medium upon the growth rates of photosynthetic microorganisms. For several weeks prior to the experiment, cultures of *Chlorella sorokiniana*, *Chlorella vannielii*, and *Chlorella vulgaris* were maintained under normal growth conditions. Transfers were made to fresh *Chlorella* medium every 72 hours. At the initiation of the experiment, 5 ml inocula of these cultures were transferred to 50 ml portions of fresh, sterile, *Chlorella* medium prepared with the omission of phosphate. The composition of this medium is shown in Table 17. The growth of the organisms was followed by measuring the increases in turbidity. At 72 hour intervals following the initial inoculations, 5 ml aliquots of each culture were

Table 17

Composition of Basal *Chlorella* Medium-Phosphate Depleted

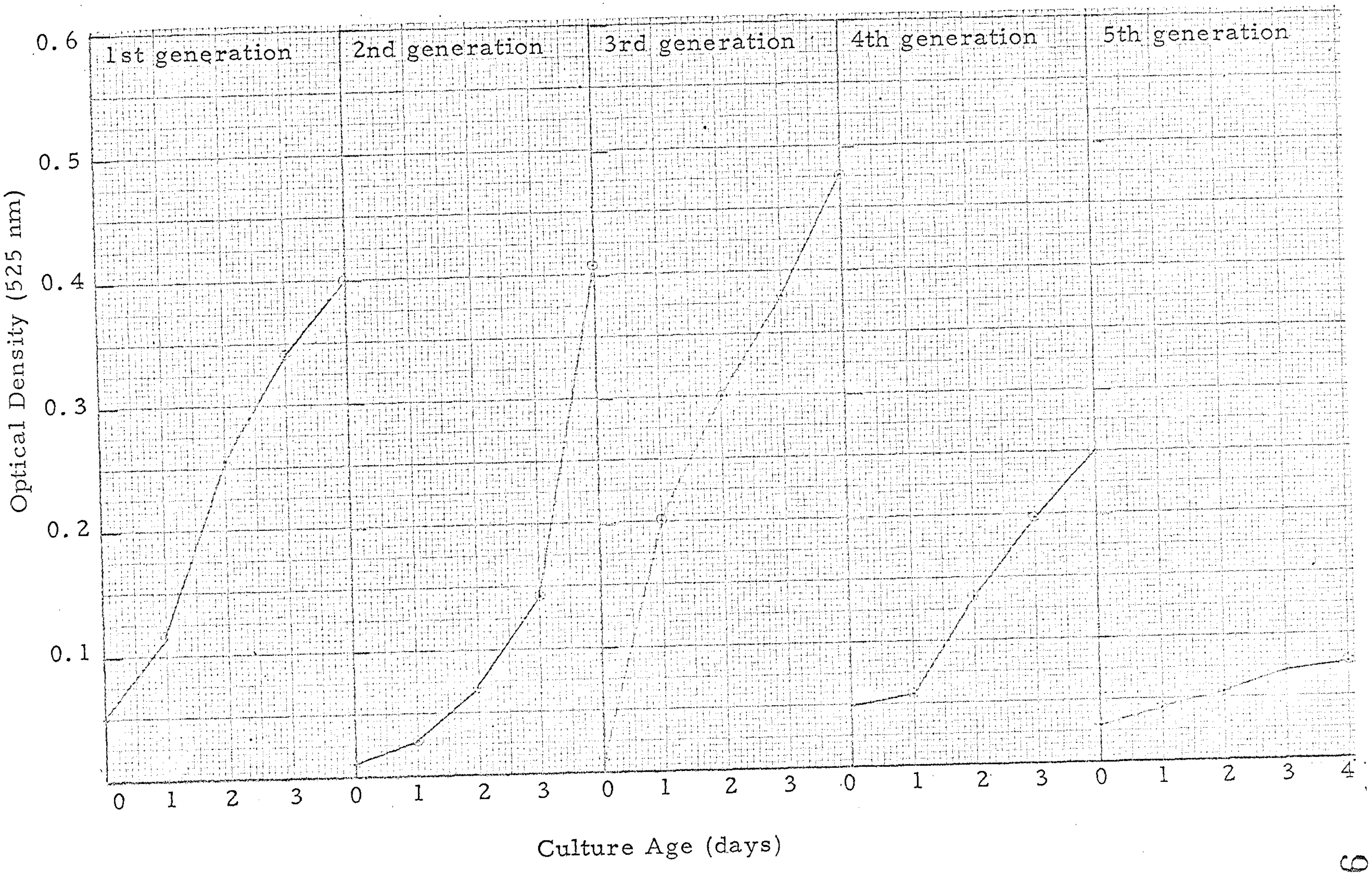
Compound	Concentration
KNO_3	1000
$Mg\ SO_4 \cdot 7H_2O$	250
Na Fe EDTA	5
Na_2 Mn EDTA	1
Na_2 Co EDTA	1
Na_2 Zn EDTA	1
Na_2 Ca EDTA	1
Na_2 Cu EDTA	1

transferred to fresh, sterile, phosphate-free medium. The transfers were continued at 72 hour intervals until no further growth was obtained. The results of these experiments are shown in Figures 21, 22, and 23. In each case, a decrease was noted in growth rate of the algae with each succeeding generation. However, it is most significant to note that a high rate of growth was maintained for several generations in a medium of drastically reduced phosphate concentration. Microscopic comparisons of the normal stock algal cells with the phosphate-starved organisms showed no strikingly apparent differences in morphology. The microscopic examinations were made under 400X phase contrast and 1000X oil-immersion phase contrast.

A similar experiment was conducted with Chlorella sorokiniana grown in RM9 medium containing about 1 ppm PO_4-P. These results indicate that photosynthetic microorganisms grown under normal physiological conditions maintain the capacity for rapid growth for up to five generations in a low phosphate medium.

2) Discussion

The results of the biological experimentation conducted on the growth of photosynthetic microorganisms in



Decrease in Growth Rate of <u>Chlorella sorokiniana</u> in PO_4-Depleted Medium

Fig. 21

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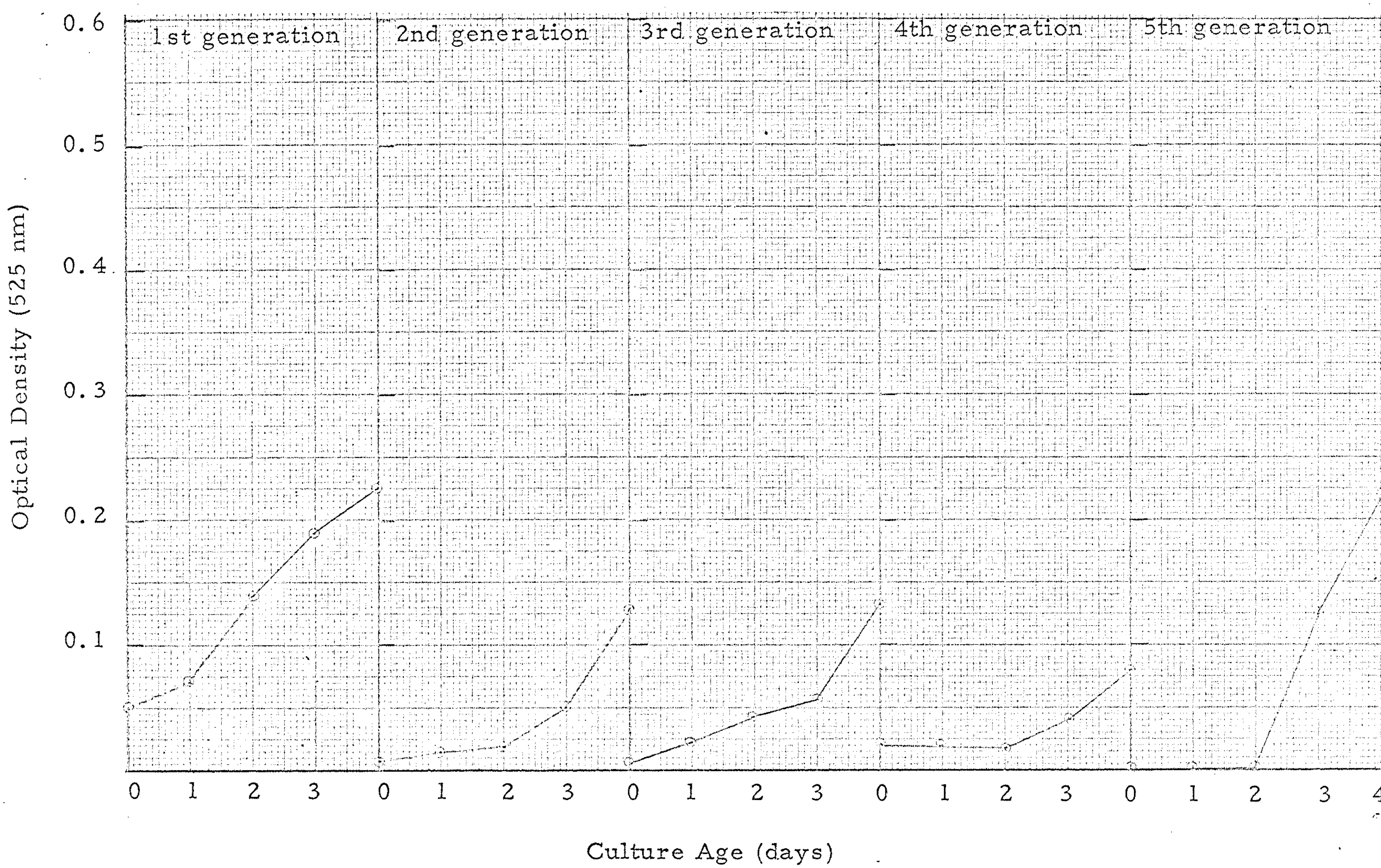

Decrease in Growth Rate of Chlorella vannielii in PO_4-Depleted Medium

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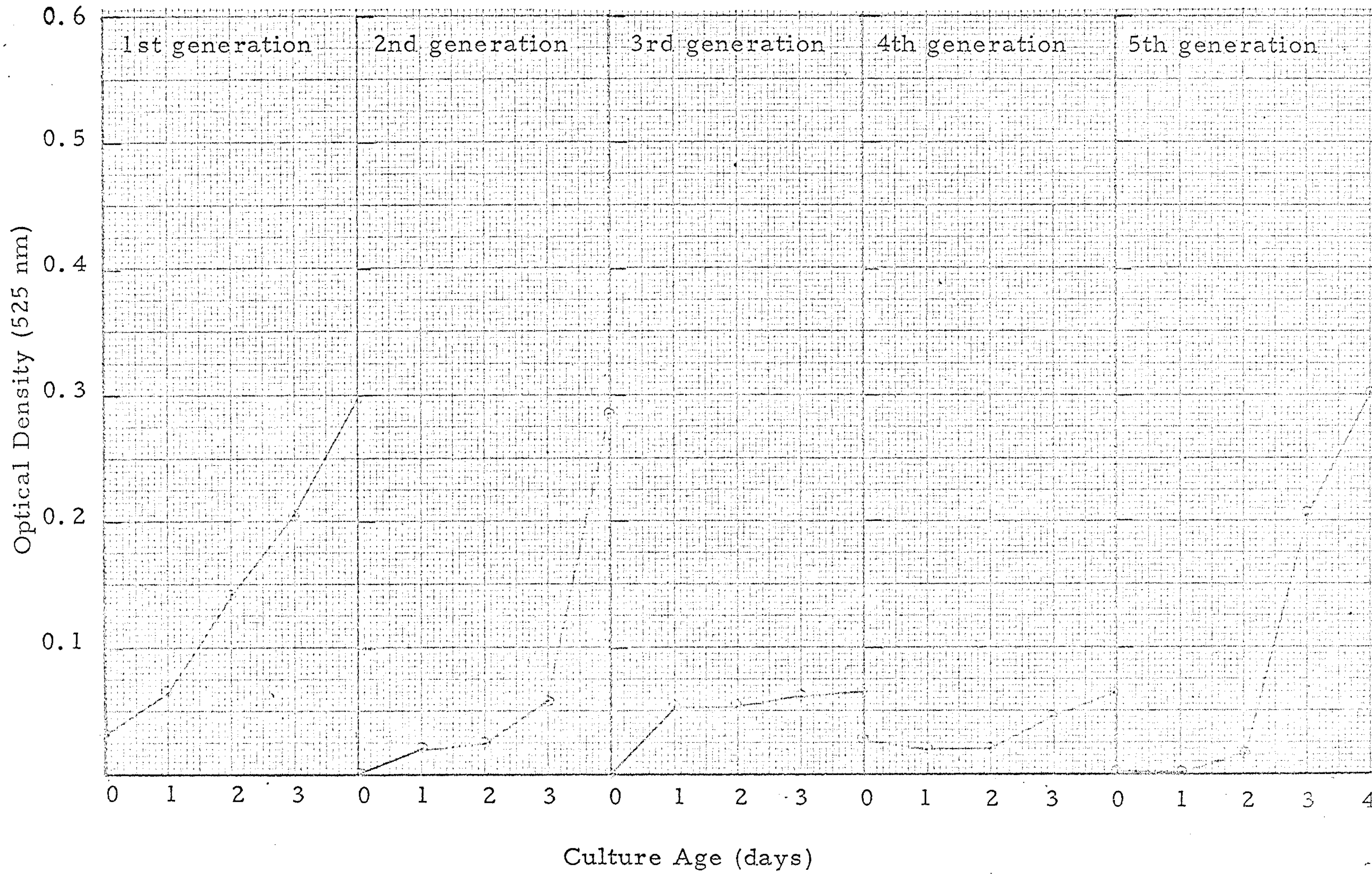

Decrease in Growth Rate of Chlorella vulgaris in PO_4-Depleted Medium

Fig. 23

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low phosphate media have been extremely encouraging. The organisms studied maintained their ability to grow at satisfactory rates for many generations in low phosphate medium. Even in essentially phosphate-free medium, the algae were able to grow at rapid rates for several generations. No morphological differences could be demonstrated between normal algal cells and phosphate starved organisms.

As a result of these studies, it is believed that a growth medium of very limited inorganic phosphate content will be acceptable for the aqueous growth chamber of the AMML.

c. Phosphate Uptake by Algae

1) Introduction

The rates of growth and simultaneous uptake of phosphate by pure cultures of <u>Chlorella sorokiniana</u> and <u>Chlorella vannielii</u> were measured in low phosphate medium. The influence of light upon the rates of growth and phosphate uptake were also examined in these experiments.

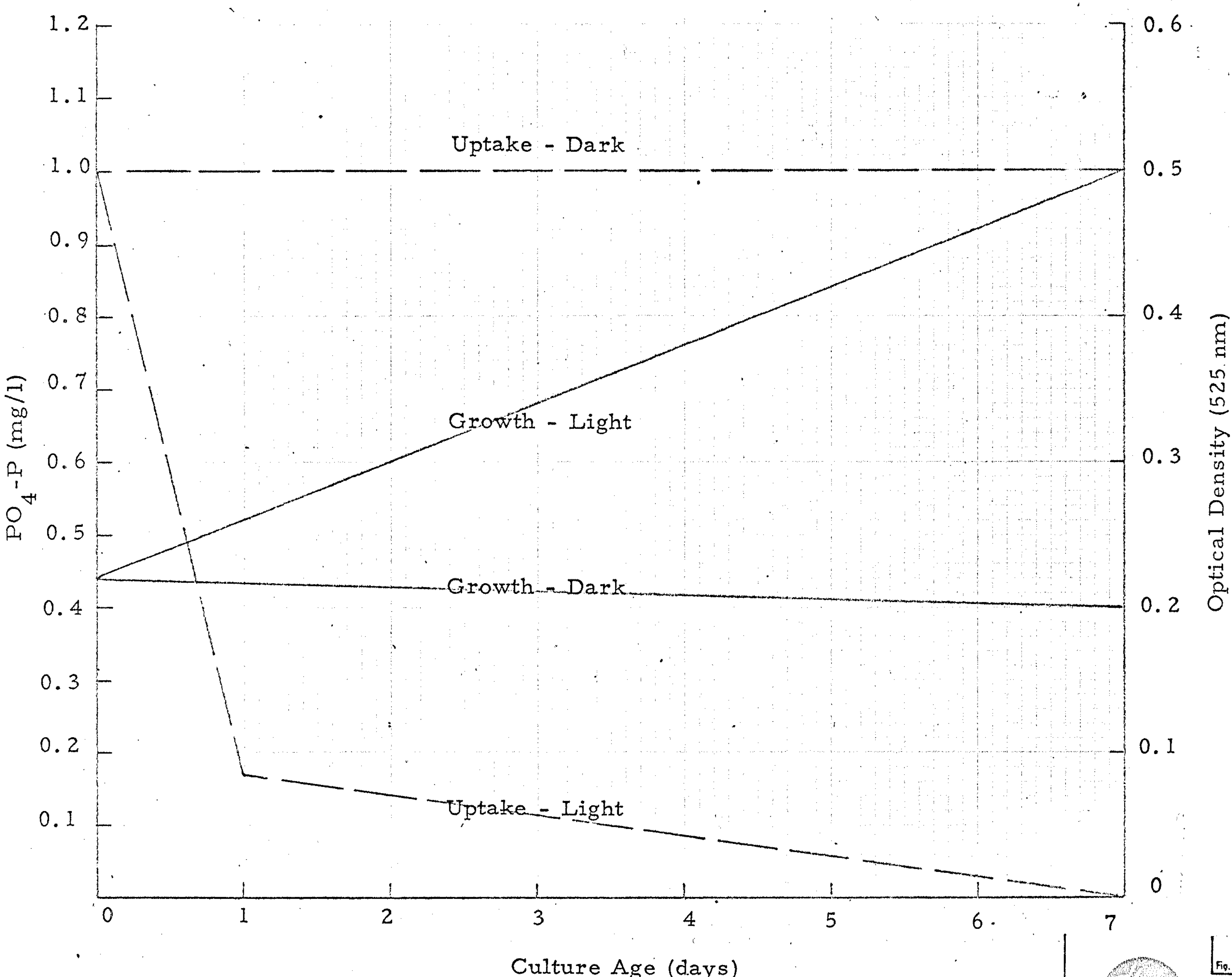
2) Experimental

Algal cells were grown as described earlier in this report in any one of several growth media. Cells were harvested by centrifugation at 5000Xg for five minutes and the pellet thus formed was washed three times with sterile, 0.1 M Tris buffer, pH 7.0. Following the three wash and centrifugation cycles, the cells were suspended in 300 ml of the experimental low phosphate medium and treated as per the experimental protocol. RM9 medium containing about 1 mg/l PO_4-P was used in these experiments. The cell suspensions were subdivided into the required number of light and dark incubated flasks and suitable sterile controls provided, if required. The flasks to be dark incubated were covered with aluminum foil to exclude all light and placed in the light exposure chamber so as to be at the same temperature as the light flasks.

The growth rates of the *Chlorella* were followed using optical density measurements as described previously. Phosphate determinations were made on filtered aliquots of the test cultures using the molybdenum blue colorimetric procedure. Generally, soluble phosphate and cell population were measured simultaneously at the beginning, at the end, and at selected intervals during the course of the experiments. Phosphate uptake experiments were performed with *Chlorella sorokiniana* and *Chlorella vannielii* in low phosphate medium.

3) Results and Discussion

The results of the experiment conducted with *Chlorella sorokiniana* are shown in Figure 24. The growth



Uptake of Phosphate by *Chlorella sorokiniana* in Basal RM9 Medium

Fig. 24

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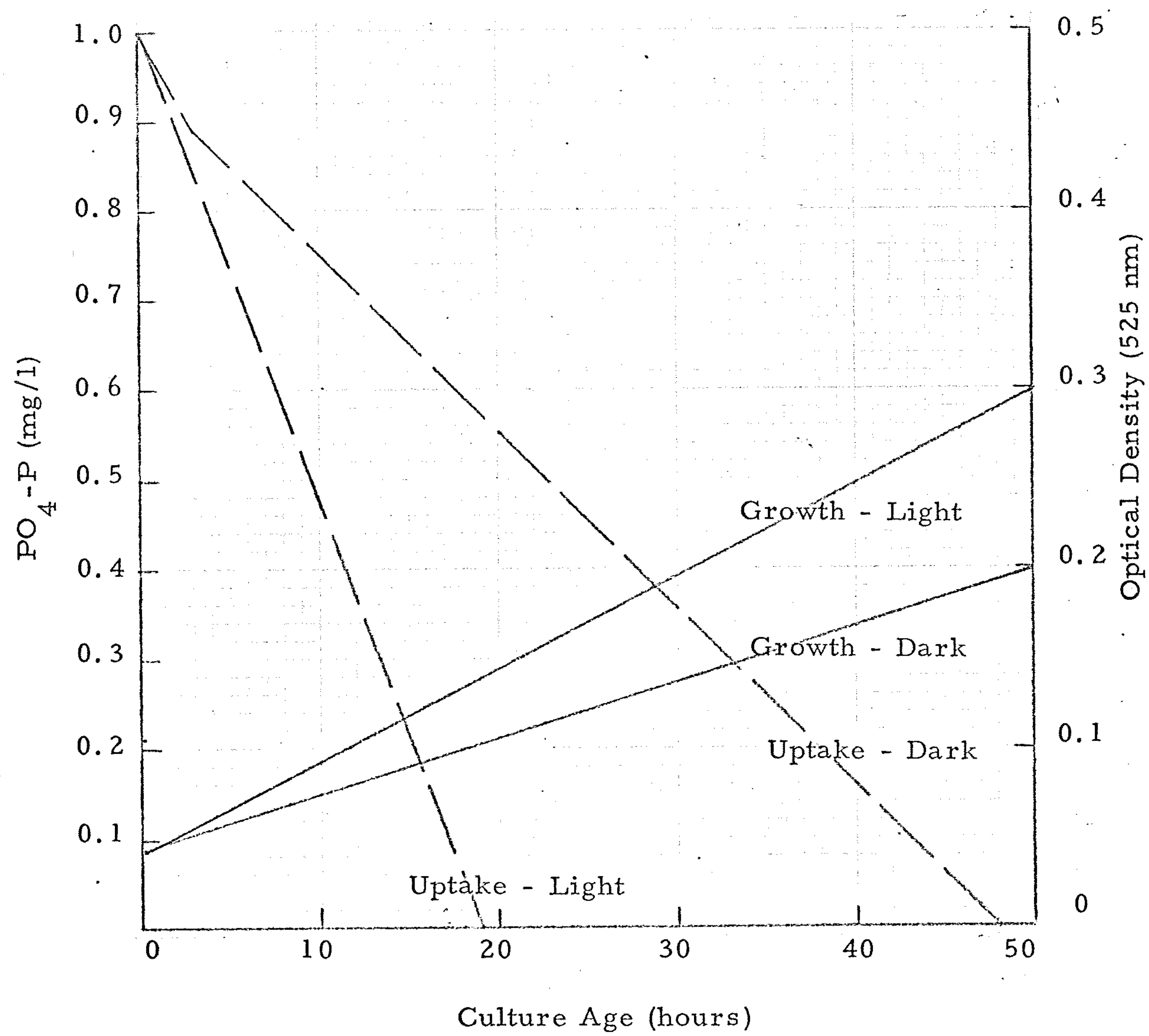
of this organism is completely light dependent in the medium provided. Similarly, the uptake of phosphate by the organisms was limited only to growing cells and occurred at a very high rate during the first 24 hours. This initial sharp drop in the phosphate concentration of the medium demonstrates an advantage for this life detection technique from the standpoint of short-duration experiments. Thus the change in phosphate concentration at 24 hours was approximately 75% compared to change of approximately 25% in optical density over the same period. The results of the experiment conducted with Chlorella vannielii are presented in Figure 25. This experiment was of much shorter duration but was sufficient to reduce the phosphate level of the medium to practically zero in one day in the light incubated culture, and in two days in the dark incubated culture.

The growth rates of both species in RM9 was lower than that observed when optimal Krauss medium was provided. However, these experiments have demonstrated that the low phosphate RM9 medium employed here may be used to provide a significant level of growth for photosynthetic microorganisms in the AMML. Since the results obtained with Chlorella vannielii grown on low phosphate media support



Uptake of Phosphate by Chlorella vannielii in Basal RM9 Medium

BIOSPHERICS INCORPORATED Rockville, Maryland	Fig. 25		
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the investigation conducted with Chlorella sorokiniana, we are most encouraged to note the striking difference in the rate of phosphate uptake by photosynthetic cells when grown in the light over that which occurs in the dark. This result is especially interesting when the history of the cells is considered. In both cases, the cell material used for the assay was collected from laboratory cultures of the strains under standard growing conditions. One might thus safely predict that the cells were in a physiologically active state, containing a level of endogenous phosphate sufficient to satisfy growth requirements. Earlier work on the uptake of phosphate by bacteria (9) has indicated that cells grown in phosphate-free media will tend to take up the phosphate very rapidly when released from the starvation condition. However, in the cases demonstrated above, the rapid phosphate uptake by cells under conditions where photosynthesis was occurring, indicating a high state of physiological activity, leads us to believe that the measurement of phosphate uptake could be used to demonstrate photosynthetic activity in the AMML. In the light of this study, the recommendation has been made that all of the metabolic processes measured by the AMML apparatus be examined under conditions of illumination and non-illumination.

d. Algal Phosphate Uptake in the Presence of Organic Substrate

1) Introduction

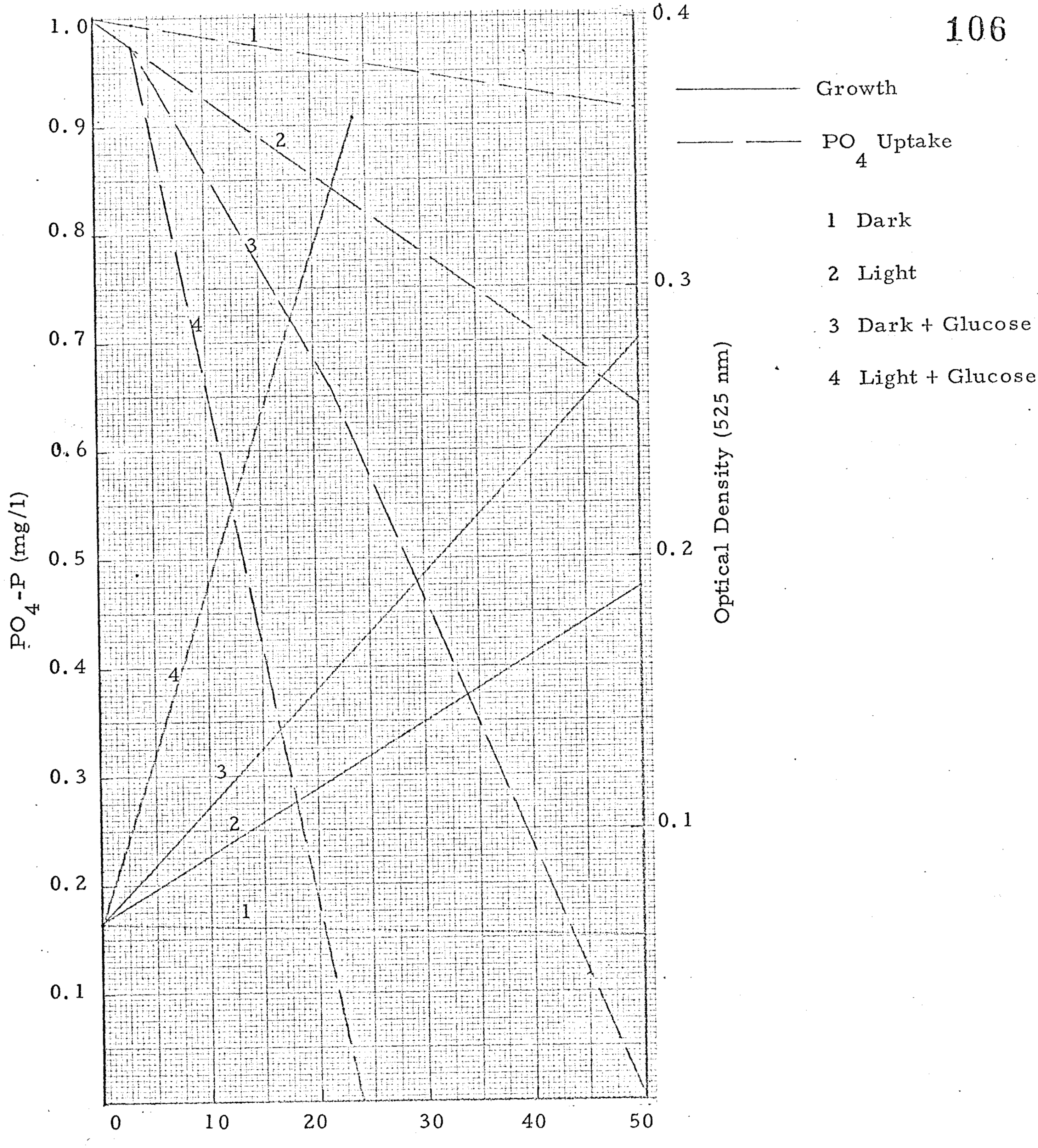
Due to the facultative nature of most autotrophic organisms, the study of their growth characteristics is affected by the presence of organic compounds. With this in mind, the following determinations of phosphate utilization in the presence and absence of organic substrates, in the light as well as under no illumination, was investigated.

2) Experimental

A suspension of rapidly growing Chlorella vannielii was utilized as the test organism. From healthy culture of cells in log phase, aliquots were harvested and divided into experimental and control aliquots. Growth cultures were prepared in RM9 medium as described in the preceding section with 0.1% glucose added as a carbon source. The basal inorganic medium utilized was that described by Krauss (see Table 2) with carbonate as the carbon source and without the chelated metal components.

3) Results and Discussion

The results are summarized in Figure 26. It is observed that whereas the inorganic dark control showed no uptake of orthophosphate over a period of 48 hours, the



Heterotrophic and Autotrophic Uptake of Phosphate by *Chlorella vannielii*

Fig. 26

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Rockville, Maryland

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addition of glucose to the non-illuminated medium caused a slow, but continuous, uptake of orthophosphate by C. vannielii. Heterotrophic growth of this organism on glucose has been well documented by a number of authors (1). In the presence of light, the RM9 low phosphate mineral salts medium showed a very low level of phosphate uptake. However, in the presence of an organic substrate, a very high level of activity was observed, with a complete disappearance of the available phosphate within 24 hours.

e. Phosphate Uptake by Soil Microorganisms

1) Introduction

Having investigated the usefulness of phosphate uptake as a life detection test for photosynthetic microorganisms, application of this life detection scheme to soil microorganisms was explored next. The limitations placed upon the assay by introduction of soils having a high soluble orthophosphate content and by interfering materials extracted from the soils into the growth medium have already been discussed. The problems caused by the slow rate of leaching of phosphate from the soil into the medium are discussed in this section.

2) Experimental

The general procedure employed for conducting this test in the laboratory was to inoculate 50 ml of RM9 medium or distilled water with amounts of soil varying from 0.5 to 2.0 g. This suspension was stirred for about 15 minutes and an aliquot taken for an initial soluble phosphate assay. The suspension was then incubated and aliquots taken for assay at the prescribed time intervals. The aliquots were filtered through 0.45 u Millipore cellulose acetate filters prior to being assayed for phosphate by the molybdenum blue colorimetric procedure. The filtrates were diluted, if required, to obtain an optical density no higher than 0.3. This precaution was required to minimize interferences with the colorimetric assay procedure.

3) Results and Discussion

The study of the leaching of phosphate from sterile control soils is presented in Table 18. These results show that a significant amount of solubilization occurs in 24 hours and thus it would be possible to have a substantial biological uptake of phosphate with no net decrease, or perhaps, even a substantial increase, in the dissolved phosphate level of the medium. In order to circumvent this problem, a sterile control soil will be required for the AMML and phosphate uptake can only be detected by comparing the dissolved orthophosphate levels from separate media inoculated simultaneously with sterile and viable soil after identical incubation periods.

Table 18

Leaching of Phosphate from Control Soils *

Soil Inoculum	Weight (g)	Medium	Dissolved Orthophosphate (ppm PO_4-P) Initial	24 Hours
Indiana Soil, Autoclaved	0.5	RM9	1.0	1.4
" " "	0.5	H_2O	0.3	1.4
" " "	1.0	RM9	1.4	2.4
" " "	1.0	H_2O	0.6	2.2
" " "	2.0	RM9	1.5	3.3
" " "	2.0	H_2O	1.0	3.2
Taylor Street, Autoclaved	1.0	RM9	1.0	1.3
" "	1.0	H_2O	0.4	0.8
Washington, D. C. Soil, Autoclaved	1.0	RM9	0.9	1.1
" " " Bard-Parker	1.0	RM9	1.3	3.8

* This experiment was conducted by inoculating 50 ml of the specified medium with the indicated weights of soil. Soils indicated as autoclaved were heated for 15 minutes at 15 psig. One soil was treated with 0.75 ml of Bard-Parker germicide. Dissolved orthophosphate measurements were made on filtered aliquots of the media at the specified time intervals. The PO_4-P concentration of the RM9 medium was approximately 1 ppm.

The results of biological phosphate uptake studies conducted on a number of soil samples in RM9 medium are shown in Table 19. Phosphate uptake was detected in a total of eight out of 20 soils tested on the basis of a decrease of at least 0.2 ppm in the soluble PO_4-P level. This may very well have been due to leaching of phosphate counterbalancing microbial uptake in some cases. The introduction of a phosphate analysis after four hours of incubation produced some interesting results, especially in the case of the RM9 medium which was inoculated with 1.0 g of Indiana soil. Phosphate uptake would have gone undetected in this case were it not for this analysis at an intermediate time period. Another important piece of information which may be gathered from this experiment pertains to the size of soil inoculum used. Results with the North Carolina, Indiana, and Taylor Street soils show greatest phosphate uptake at an inoculum size of 0.5 g, the lowest level studied.

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

1. Analysis

The basis of this life detection scheme is the biological uptake of labeled carbon and sulfur compounds by extraterrestrial soil microorganisms. Current recommendations call for inoculation of RM9 + ^{14}C + ^{35}S substrates with

Table 19

Phosphate Uptake by Soil Microorganisms in RM9 Medium

Soil Inoculum	Weight	Dissolved Orthophosphate ($ppm\ PO_4$-P) Initial	4 Hours	24 Hours
Chilean Soil #259	0.5	1.0	-	1.0
" " #285	0.5	1.0	-	1.3
" " #271	0.5	1.0	-	1.0
" " #266	0.5	1.0	-	0.8
" " #268	0.5	1.1	-	1.2
Death Valley Soil	0.5	0.9	0.9	0.1
JPL #100	0.5	1.0	0.9	0.9
JPL #101	0.5	0.9	0.8	0.6
JPL #110	0.5	0.9	-	1.0
JPL #113	0.5	0.9	-	0.9
JPL #114	0.5	1.0	-	0.9
JPL #115	0.5	0.9	-	0.9
JPL #118	0.5	1.0	-	0.9
JPL #120	0.5	0.9	-	0.9
JPL #121	0.5	1.0	-	1.2
JPL #123	0.5	0.9	-	0.5
North Carolina Soil	0.5	0.9	0.8	0.1
" " "	1.0	0.9	0.6	0.1

Table 19 (continued)

Soil Inoculum	Weight	Dissolved Orthophosphate ($ppm\ PO_4$-P) Initial	4 Hours	24 Hours
North Carolina Soil	2.0	0.8	0.7	0.2
Washington D. C. Soil	1.0	0.9	-	0.4
Indiana Soil	0.5	1.4	1.8	0.6
" "	1.0	1.6	2.6	1.5
" "	2.0	1.9	2.6	2.4
Taylor Street Soil	0.5	1.0	-	0.0
" " "	1.0	1.0	-	0.0
" " "	2.0	1.0	-	0.1

soil in the aqueous growth chamber of the AMML. Periodically, aliquots of this suspension will be removed and filtered. The filters will then be washed, dried, and counted in order to monitor the biological incorporation of radioactivity. If the magnitude of the biological signal - that is, the combined net uptake of ^{14}C and ^{35}S by the soil microorganisms - is low compared to the magnitude of the nonbiological signal - that is, radioactivity retained by the filters and the soil particles themselves - then the sensitivity of the assay will suffer. Accordingly, experiments were designed to estimate the magnitudes of the biological and the nonbiological signals.

2. Retention of Radioactivity by Microbial Filters

a. Introduction

A number of different types of membrane filters of pore sizes appropriate for the retention of microorganisms in the ^{14}C and ^{35}S uptake experiments were examined. Membrane filters manufactured by Schleicher and Scheull, Gelman Corporation, and Millipore Corporation were studied.

b. Experimental

Three standard radioactive solutions were prepared in RM9 medium, each containing a single labeled substrate at the concentration normally used in the uptake experiments. A solution containing 10 uCi/ml of ^{35}S and

3.4 mg/l sodium sulfate, a solution containing 1.3 uCi/ml of ^{14}C-D-glucose (U) and 50 mg/l of D-glucose, and a solution containing 6.5 uCi/ml of ^{14}C-formate and 20 mg/l of sodium formate were prepared for this study. Eighteen filters were set up for each type of filter investigated. One ml aliquots of the ^{14}C-glucose solution were passed through six of the filters, 1 ml aliquots of the ^{14}C-formate solution were passed through a second set of six filters, and 1 ml aliquots of the ^{35}S-sulfate solution passed through the remaining set of six. Two filters from each of the three sets of six were then dried and counted for radioactivity in the usual manner. A second pair of filters from each of the three sets of six was rinsed with 0.5 ml of RM9 medium containing no radioactive substrates. The final pair of filters from each of the three sets of six filters was rinsed with 5.0 ml of the unlabeled medium. All filters were then dried and counted. Bactiflex membrane filters from Schleicher and Scheull, Metricel filters from Gelman Corporation, and HAWP filters from Millipore Corporation were studied in this experiment.

c. Results and Discussion

The results of this experiment are shown in Table 20. The Gelman Corporation Metricel membrane

Table 20

Retention of Radioactivity by Membrane Filters

Vol. of RM9 Wash (ml)	Radioactivity Retained (%)*,** Bactiflex	Metricel	HAWP
^{14}C-D-Glucose (U) Solution (480, 000 cpm/ml) - 1 ml applied			
0	1.20	1.67	2.53
0.5	0.14	0.34	0.33
5.0	0.29	0.19	0.24
^{14}C-Formate Solution (701, 900 cpm/ml) - 1 ml applied			
0	0.79	0.63	2.19
0.5	0.50	0.27	0.80
5.0	0.34	0.13	0.57
^{35}S-Sulfate Solution (1, 986, 000 cpm/ml) - 1 ml applied			
0	2.03	1.60	2.09
0.5	0.55	0.70	0.24
5.0	0.14	0.16	0.27

* The applied radioactivity was estimated by evaporating to dryness and counting replicate aliquots of each solution.

** Each of the values reported is the average of duplicate determinations.

filters appeared to retain a little less radioactivity than the other two filter types studied here. In addition, the 5.0 ml wash was shown to reduce the radioactivity retained to a level below that achieved with the 0.5 ml wash.

3. Retention of Radioactivity by Autoclaved Soils

a. Introduction

An experiment parallel to the one described previously, was conducted to measure the nonbiological retention of radioactive substrates by soils. Three soils were studied in order to develop a washing procedure that would result in a low nonbiological background. The Gelman microbial filter was selected, on the basis of the previous experimental results, for use in this study.

b. Experimental

Soils from Indiana, West Virginia, and New Jersey were investigated in this experiment. Each of the soils were passed through a 200 mesh sieve and the absorptive properties of the sieved soils compared with those of the unsieved soils. The soils were autoclaved, prior to use in the experiment, at 15 psig for 20 minutes in order to remove vegetative cells. The three radioisotopic solutions of ^{14}C-glucose, ^{14}C-formate and ^{35}S-sulfate made up for the previous experiment, were diluted 1 to 4 with RM9 and used here. Cold substrates were added to keep each concentration constant despite the dilution. Gelman Corporation Metricel membrane filters of 1 inch diameter and 0.45 u pore size were used for this experiment.

The sieved and unsieved soil samples from each of the three soils were tested individually, making a total of six samples tested. The general procedure followed is described below. Three sterile test tubes were set up for each soil sampled studied. Ten ml of ^{14}C-glucose solution was added to the first of these three tubes, 10 ml of ^{14}C-formate solution was added to the second, and 10 ml of ^{35}S-sulfate solution added to the third tube. Each of these three tubes was then inoculated with 100 mg portions of the soil sample. A total of 15 membrane filters were set up for each soil sample; 1 ml portions of the ^{14}C-glucose suspension were passed through each of five filters, 1 ml portions of the ^{14}C-formate suspension were passed through a second five filters, and 1 ml portions of the ^{35}S-sulfate suspension were passed through the remaining five filters. Each of the five filters, from the three sets of filters, was washed with a specified volume of unlabeled RM9 medium. The wash volumes chosen for study here were 0, 0.5, 1.0, 2.0, and 5.0 ml.

c. Results and Discussion

The results of this experiment are presented in Table 21. A wash volume of 1.0 ml of unlabeled RM9 medium served to reduce nonbiological background to the lowest levels attained here. Larger volumes of wash solution did not appreciably lower this background. The nonbiological background from sieved soils was about the same as the unsieved soils after washing with volumes of 1 ml or greater. In general, the formate is more difficult to remove than the glucose, which is somewhat harder to wash off than the sulfate. Of course, this order of retention might be altered on other types of soils. It should be pointed out that the figures for retention of radioactivity listed in Table 21 include the retention by the Metricel membrane filter. The filter retains approximately 0.2% of the applied radioisotope for each of the labeled compounds studied here.

On the basis of this experiment, two separate rinses of the filter with 1 or 2 ml volume have been recommended for incorporation in the AMML.

4. ^{14}C and ^{35}S Biological Uptake Experiment

A limited number of biological tests were initiated having obtained an estimate of the total nonbiological background in the ^{14}C and ^{35}S uptake experiment. The tests were con-

Table 21

Retention of Radioactivity by Autoclaved Soils *

Soil Type	Sieved	Vol. of RM9 Wash (ml)	Radioactivity Retained (%) **		
			^{14}C-Formate	^{14}C-Glucose	^{35}S-Sulfate
Indiana	-	0	1.11	1.21	0.92
"	+	0	1.60	1.01	0.43
"	-	0.5	0.62	0.64	0.52
"	+	0.5	1.41	0.50	0.22
"	-	1.0	0.69	0.35	0.21
"	+	1.0	0.70	0.43	0.37
"	-	2.0	0.86	0.41	0.42
"	+	2.0	0.56	0.34	0.20
"	-	5.0	0.62	0.57	0.23
"	+	5.0	0.82	0.35	0.15
West Virginia	-	0	0.93	0.85	0.95
" "	+	0	1.52	0.82	0.74
" "	-	0.5	0.35	0.30	0.35
" "	+	0.5	0.68	0.60	0.35

Table 21 (continued)

Soil Type	Sieved	Vol. of RM9 Wash (ml)	Radioactivity Retained (%)** ^{14}C-Formate	^{14}C-Glucose	^{35}S-Sulfate
West Virginia	-	1.0	0.36	0.28	0.48
" "	+	1.0	0.55	0.47	0.17
" "	-	2.0	0.35	0.48	0.51
" "	+	2.0	0.52	0.47	0.21
" "	-	5.0	0.46	0.26	0.37
" "	+	5.0	0.62	0.40	0.23
New Jersey	-	0	1.39	0.86	0.83
" "	+	0	2.32	0.48	1.16
" "	-	0.5	0.62	0.53	0.56
" "	+	0.5	0.51	0.40	0.47
" "	-	1.0	0.55	0.34	0.32
" "	+	1.0	0.55	0.31	0.29

Table 21 (continued)

Soil Type	Sieved	Vol. of RM9 Wash (ml)	Radioactivity Retained (%)** ^{14}C-Formate	^{14}C-Glucose	^{35}S-Sulfate
New Jersey	-	2.0	0.77	0.30	0.27
" "	+	2.0	0.47	0.35	0.37
" "	-	5.0	0.64	0.28	0.55
" "	+	5.0	0.31	0.27	0.50

* The results reported here are actually the total retention by 0.45 u Metricel filters (Gelman Corporation) and the soil particles.

** The applied radioactivity was estimated by evaporating to dryness and counting replicate aliquots of each solution.

ducted using a soil obtained locally. Soil treated with Bard-Parker germicide was used for the controls. Three media prepared in basal RM9 medium were used in these tests, one medium contained the four ^{14}C-organic substrates, one contained the ^{35}S-sulfate as substrate and the third contained both ^{14}C and ^{35}S labeled compounds. Two separate 10 ml portions of each of the three media were inoculated. To one portion of each medium was added 100 mg of viable soil and to the other was added an equal amount of soil containing 0.3 ml of Bard-Parker germicide. The suspensions were mixed thoroughly and incubated on a shaker for five hours. The entire 10 ml of suspension was then filtered, washed several times with unlabeled RM9 basal medium, dried, and then monitored for radioactivity.

The results of this experiment are shown in Table 22. Although the test signals are significantly higher than the controls, even for the short incubation period of five hours studied here, the lower limits of sensitivity were approached in these assays. A microbial population one order of magnitude lower than that in soil studied would not have yielded a detectable signal. Additional research will be required to optimize the biological signal and to reduce the nonbiological background so that smaller microbial populations can be detected.

Table 22

The Incorporation of ^{14}C and ^{35}S Substrates by Soil Microorganisms

Medium	Incorporated Activity (cpm)*	
	Test	Control
^{14}C-Organics in RM9 (0.3 uCi/ml)	362	296
^{35}S-Sulfate in RM9 (0.2 uCi/ml)	266	92
^{14}C + ^{35}S in RM9 (0.3 uCi/ml ^{14}C + 0.2 uCi/ml ^{35}S)	649	386

Assay Conditions:

Temperature	25°C
Basal Medium	RM9
Substrates	sodium formate - 20 ppm
	D-glucose - 50 ppm
	DL-sodium lactate - 20 ppm
	glycine - 20 ppm
	sulfate - 31 ppm
Inoculum	100 mg soil in 10 ml medium
Activity	0.3 uCi/ml total ^{14}C-organics
	0.2 uCi/ml ^{35}S-sulfate
Control	0.3% Bard-Parker Germicide
Incubation Period	5 hours

* These results are the averages of triplicate determinations.

F. Adenosine triphosphate (ATP) Bioluminescence Assay

1. Introduction

An experiment was performed to determine if reaction products of the firefly bioluminescence reaction remaining in the reaction chamber would interfere with the subsequent assay for ATP. This experiment will give an estimate of the effects of inadequate rinsing of the AMML reaction chamber between ATP assays.

2. Experimental

The bioluminescence assay was conducted in a laboratory instrument in 50 mm x 6 mm OD reaction cuvets. A 0.1 ml portion of the ATP solution was injected into a 0.2 ml of reaction mixture in a cuvet positioned in front of a photomultiplier tube. The experiment was conducted as follows:

1. A 10^{-2} ug/ml standard solution of ATP was assayed in a clean, dry cuvet.
2. This same cuvet was emptied without rinsing and a second assay performed with fresh reagents.
3. This cuvet was emptied, rinsed with 0.3 ml of distilled water, emptied, and

a third assay performed with fresh reagents.

4. This procedure was repeated two additional times starting with a clean, dry cuvet each time.

5. This same experimental protocol was followed to give triplicate assays with 10^{-1} ug/ml standard ATP.

3. Results and Discussion

The results of this experiment are recorded in Table 23. The responses recorded after emptying the cuvets and after rinsing with distilled water were approximately equal. This indicates that the reaction products of the ATP bioluminescence reaction will not interfere with the subsequent assay. Both of these responses, however, were only 70% to 80% of the values obtained initially in clean, dry cuvets. This was caused by dilution of the reaction mixture by liquid incompletely emptied from the cuvets. A prerinse of the reaction chamber will be required in the instrument in order to dilute out the first assay to the same level as in subsequent tests. This prerinse might not be required if the liquid hang-up in the AMML is not significant.

Table 23

ATP Bioluminescence Assay - Interference of Reaction Products

ATP Standard (ug/ml)	Responses (nanoamperes)		
	initial	after emptying	after rinsing & emptying
10^{-1}	18.5	14.5	15.0
	20.0	16.0	15.0
	18.5	14.5	13.5
	$\bar{x}$ = 19.0	$\bar{x}$ = 15.0	$\bar{x}$ = 14.5
10^{-2}	1.9	1.2	1.6
	2.1	1.5	1.3
	1.4	1.3	1.1
	$\bar{x}$ = 1.8	$\bar{x}$ = 1.3	$\bar{x}$ = 1.3

IV. Engineering Development

A. Instrument Design Considerations

The engineering program was concerned with the functional integration of the six biological experimental measurements into a hybrid system. The program called for the development of engineering feasibility apparatus which would enable laboratory verification that the measurements could be combined into a single system.

As mentioned previously, the six experimental measurements are:

1. Sulfate uptake by the microorganisms in the growth medium.
2. Phosphate taken up by these microorganisms in the growth medium.
3. Assay for ATP.

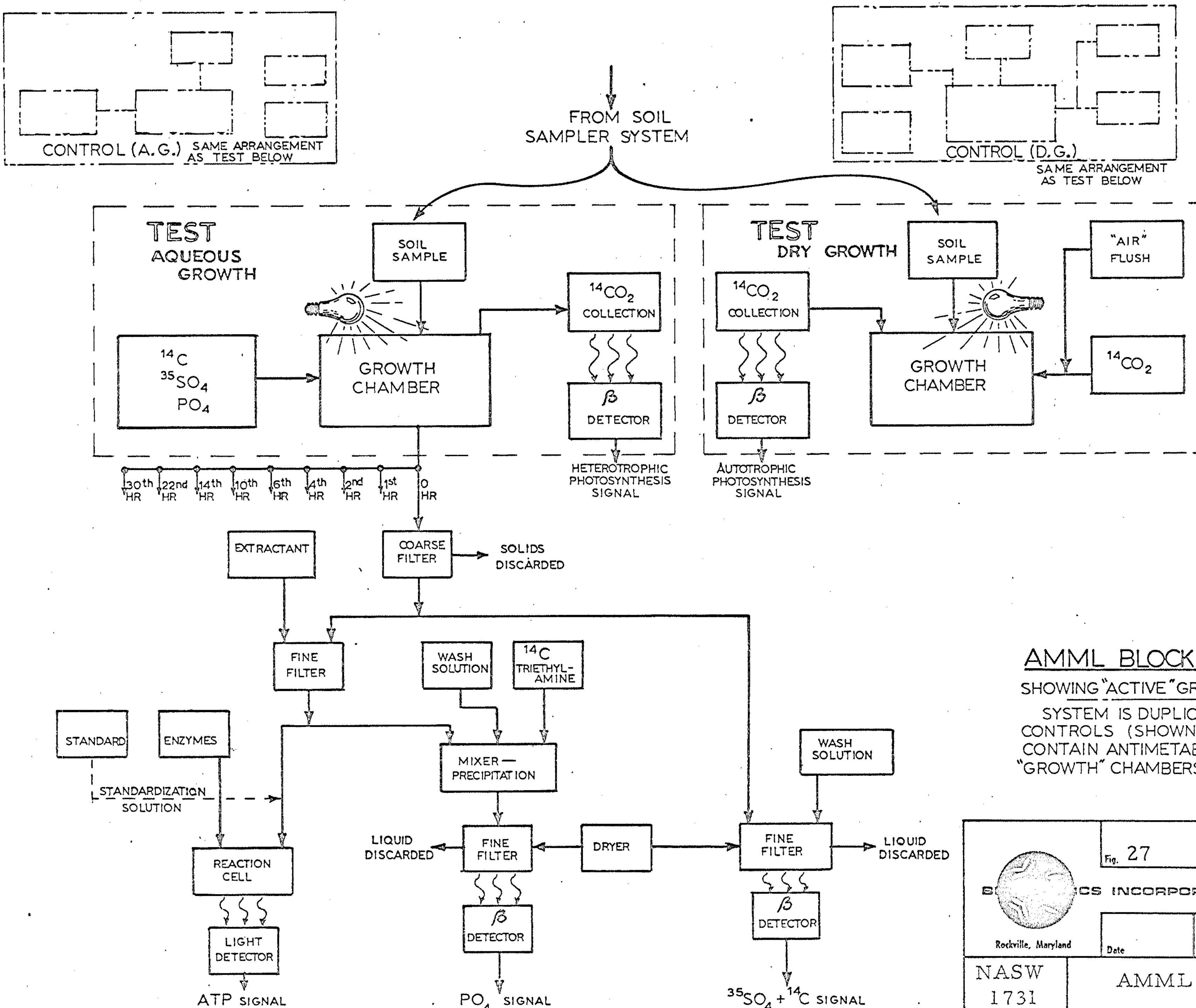
4., 5. The heterotrophic assimilation of ^{14}C from labeled substrates with the production of $^{14}CO_2$ and the modulation of $^{14}CO_2$ evolved in the light and dark as evidence for photosynthetic activity.

6. The phototrophic assimilation of $^{14}CO_2$ by microorganisms in the light and subsequent release in the dark.

The concept for the demonstration apparatus that combines these measurements is diagramed in Figure 27. The first five measurements are performed in the aqueous growth chambers that contain about one gram of the unknown "soil" sample and about 30 ml of nutrients that are labeled with various ^{14}C and ^{35}S compounds. A trace quantity of phosphate is also included so that its consumption by the microorganisms may be assayed periodically by a ^{14}C triethylamine precipitation technique. The initial and subsequent increases of adenosine triphosphate (ATP) in the microorganisms are assayed with the firefly bioluminescence technique.

The sixth experimental measurement examines a "soil" sample for the evolution (in the dark) of $^{14}CO_2$ that had been previously photosynthetically fixed. This measurement requires dry growth chambers.

The entire instrument system is operated automatically with a program controller/teletypewriter tape-reader that allows the flexible programming of the detailed operation of the experimental measurements.



AMML BLOCK DIAGRAM

SHOWING "ACTIVE" GROWTH CHAMBERS.

SYSTEM IS DUPLICATED FOR CONTROLS (SHOWN AT TOP) WHICH CONTAIN ANTIMETABOLITE IN "GROWTH" CHAMBERS.

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B. Hardware Development and Integration

The AMML experimental program involves two basic types of measurements: the aqueous wet-chemistry measurements, and the gaseous evolution assays. The function of the AMML is shown in the diagram of the overall instrument (Figure 28) that consists of five basic components: the growth chambers, reagent storage reservoirs, liquid transfer and filtering mechanism, the detectors, and the automated programming controller. The effort during this year has been primarily directed toward solving those problems which involve the integration of hardware for the three aqueous wet-chemistry analyses and the development of a control system to automatically perform this analysis. The hardware which has been constructed and tested during this program is shown in Figure 29 and described in the following:

1. The Growth Chambers

Two sets of growth chambers are used in the AMML instrument. These sets consist of pairs of chambers - one for the test measurements and the other for the controls. The control chambers are configured identically to the test chambers and are loaded with the same ingredients except that the control chambers have an antimetabolite added also.

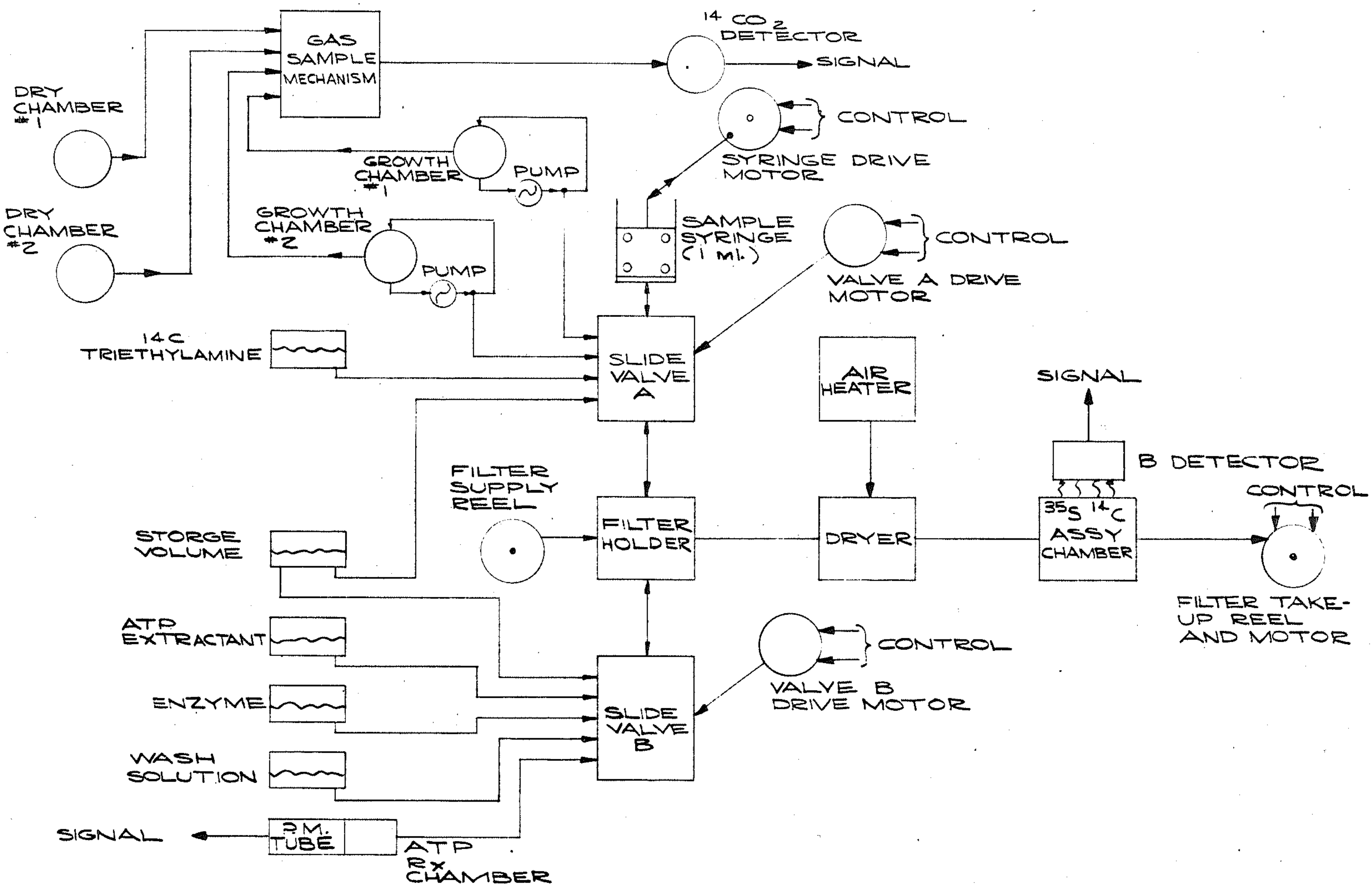

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	Fig. 28
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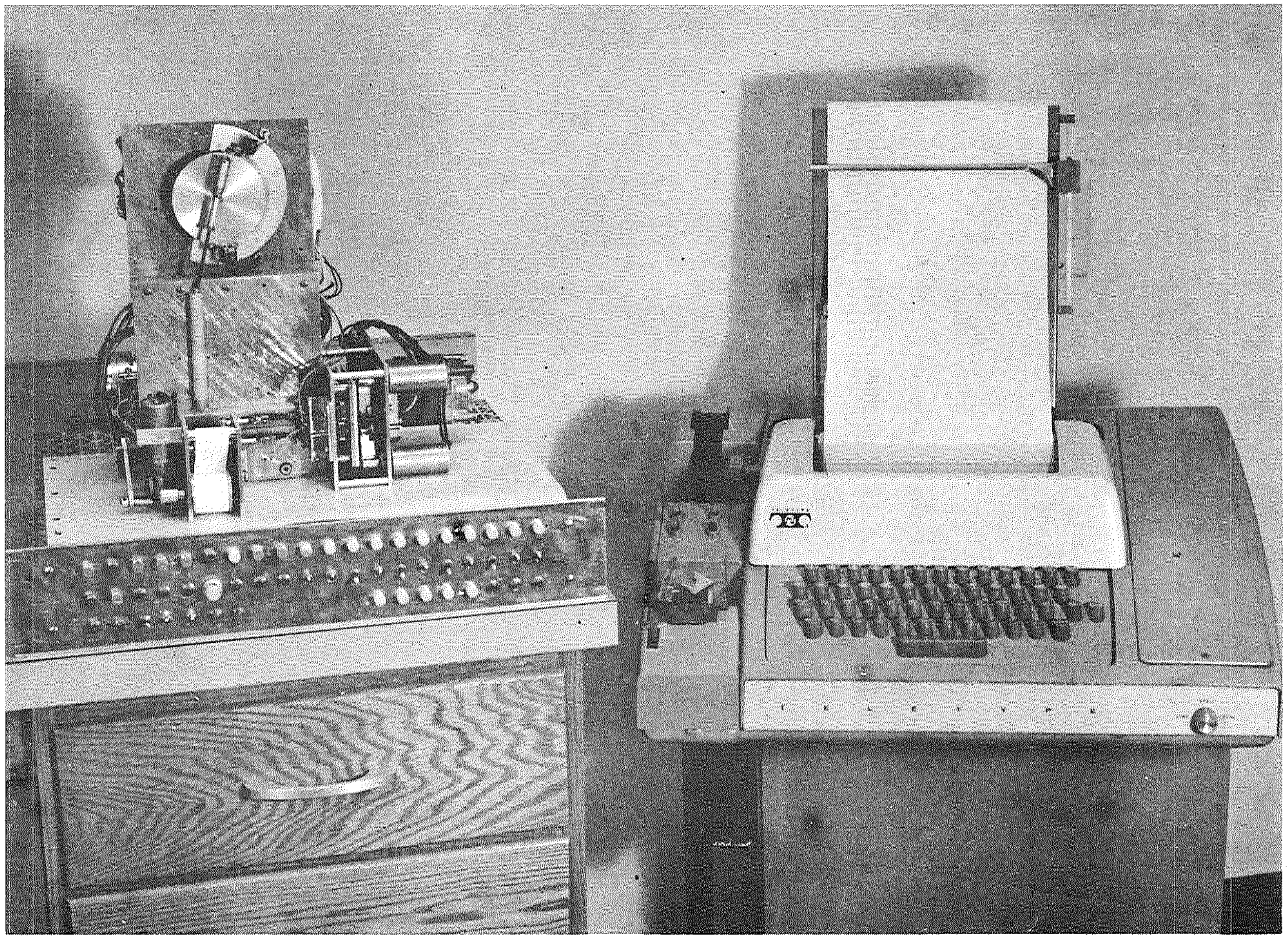


Figure 29

The AMML Hardware & Programming Controller

The final design of the dry growth chambers has not been established as yet.

The aqueous media growth chambers shown in the photograph (Figure 30) contain 30 to 40 ml of a solution of the labeled nutrient that is inoculated with 1 gram of "soil" samples. Upon inoculation, the "soil" inlet is closed so as to seal the chambers from further exchange or loss to the atmosphere as shown in Figure 31. $^{14}CO_2$ evolution by the microorganisms in the media is monitored by use of getter-type detectors so that both the heterotrophic and phototrophic labeled carbon experiments can be performed.

Photosynthetic fixation is obtained by programming a light source to illuminate the growth chambers with approximately 300 lumens. The program of light and dark cycles by the present breadboard instrument is manual, but will ultimately be controlled by an automated system. The program calls for the first hour (0-1) to be a light cycle, the second (1-2) a dark cycle. Hours 2-4, 6-10 and 14-22 are further light cycles and 4-6, 10-14, and 14-22 are dark cycles. Gas analysis for $^{14}CO_2$ that is evolved is assayed at the end of each light and dark cycle.

A magnetic stirrer and a Model RD074 circulation pump manufactured by Extracorporeal Medical Specialties continuously mixes and re-circulates the aqueous solution in each of

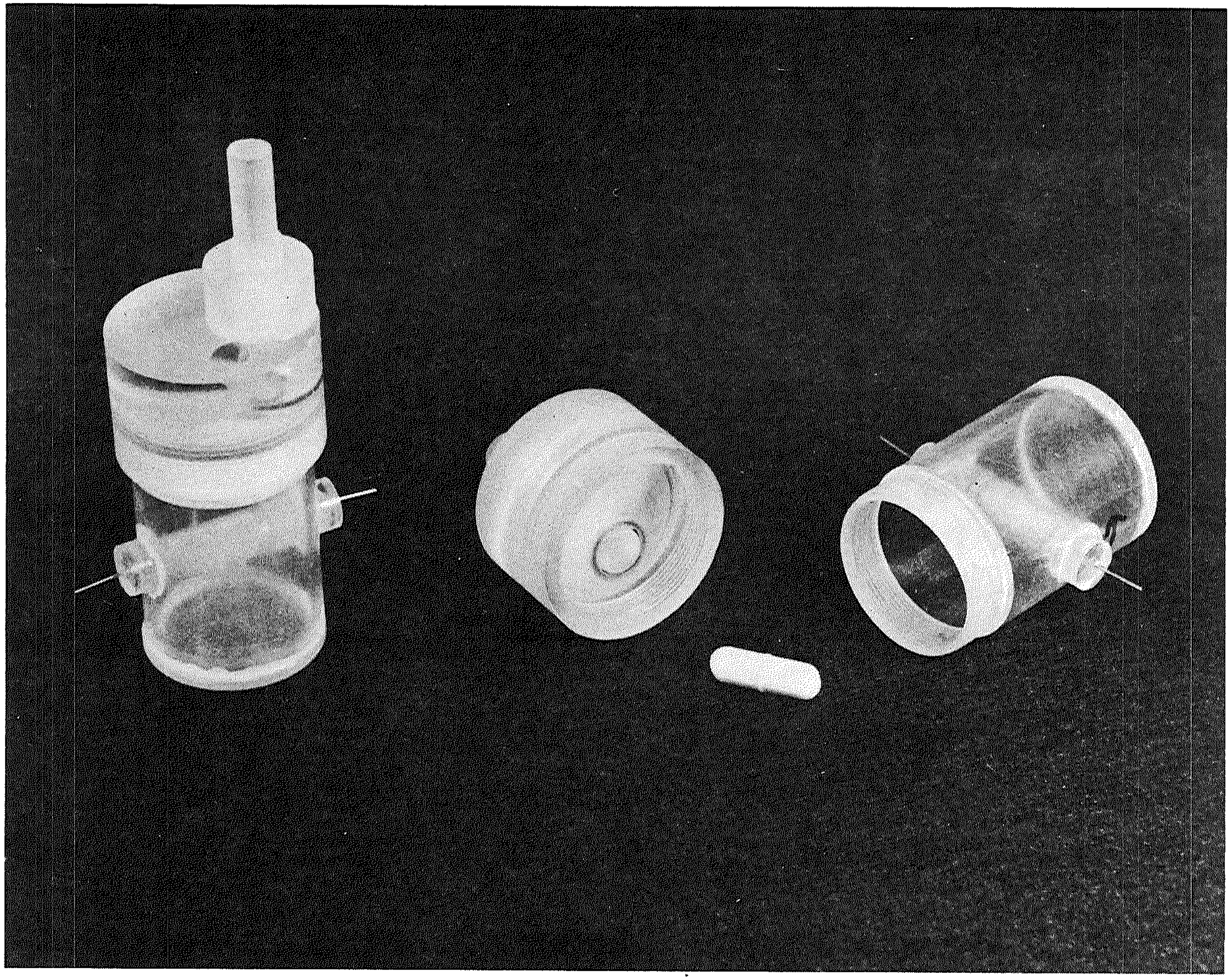
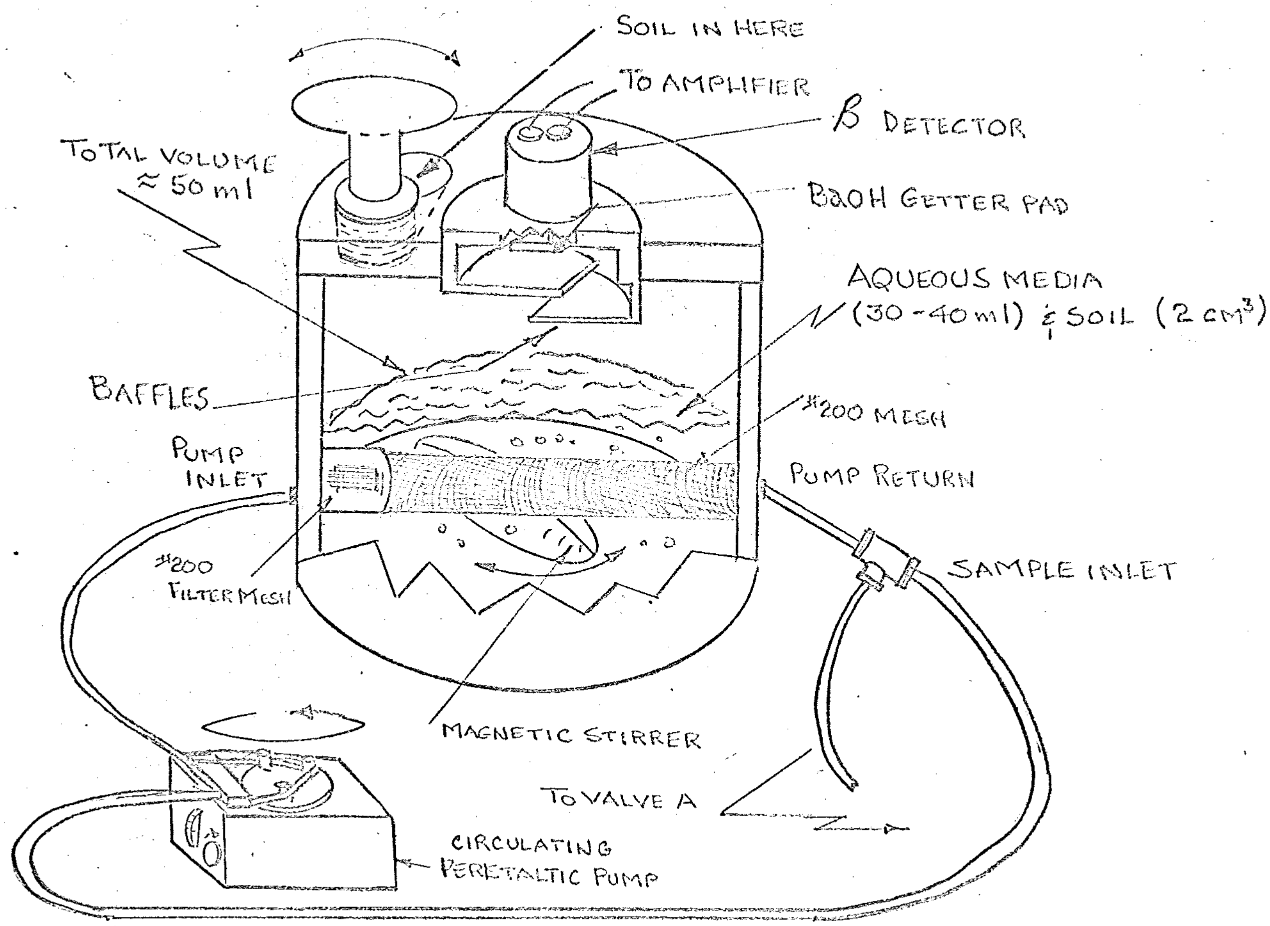


Figure 30

Aqueous Media Growth Chambers



Title: Aqueous Media Growth Chamber	
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the growth chambers to encourage uniform dispersion of any microorganisms. Outlets are provided in the re-circulating lines so that samples for the wet-chemistry analysis may be obtained periodically. The inlets to these lines are covered with a coarse filter (approximately 100 micron) and located near the middle of the fluid level of the chambers to prevent silting and clogging. Such a filter is required because of the small diameter tubing which is used in micro liquid handling system.

2. Liquid Transfer and Filtering Mechanism

A syringe-type liquid transfer pump provides the basic mechanism for transferring the various aliquots of reagents and aqueous solution from the growth chambers and other solutions that are required for the operation of the AMML instrument. This mechanism is shown in the photograph in Figure 32. The pump is designed to transfer a one milliliter aliquot of liquid from one reservoir to another or to one of the experimental reaction chambers with minimal retention of the transferred liquid. The pump is combined with two multiple inlet micro valves and a tape-filter mechanism and is detailed in Figure 33.

The two micro-valve manifolds are detailed in Figure 34 and the photograph in Figure 35, and are designated Valve A and Valve B. Valve A is coupled directly to the syringe pump and gives access to samples from the growth chambers

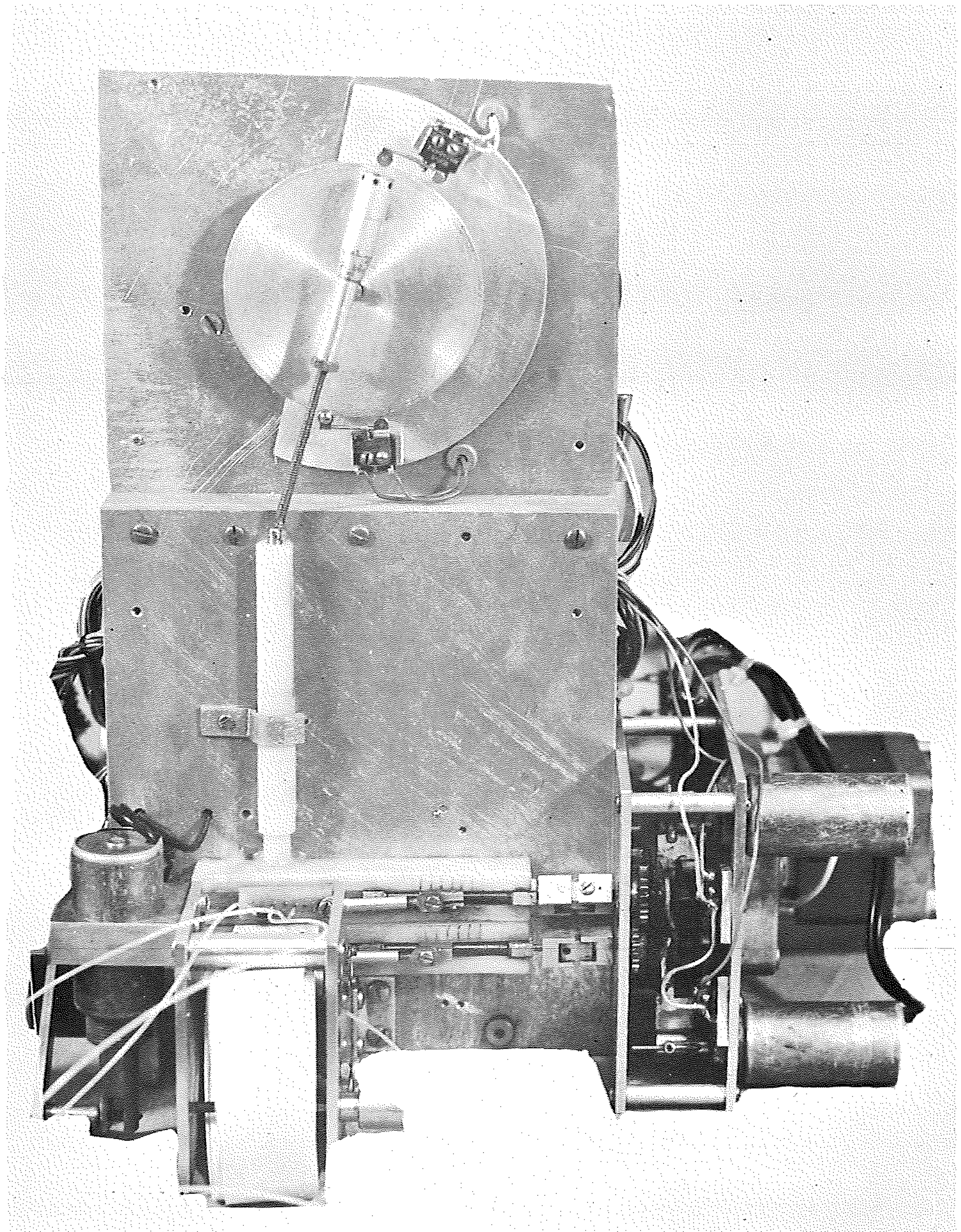
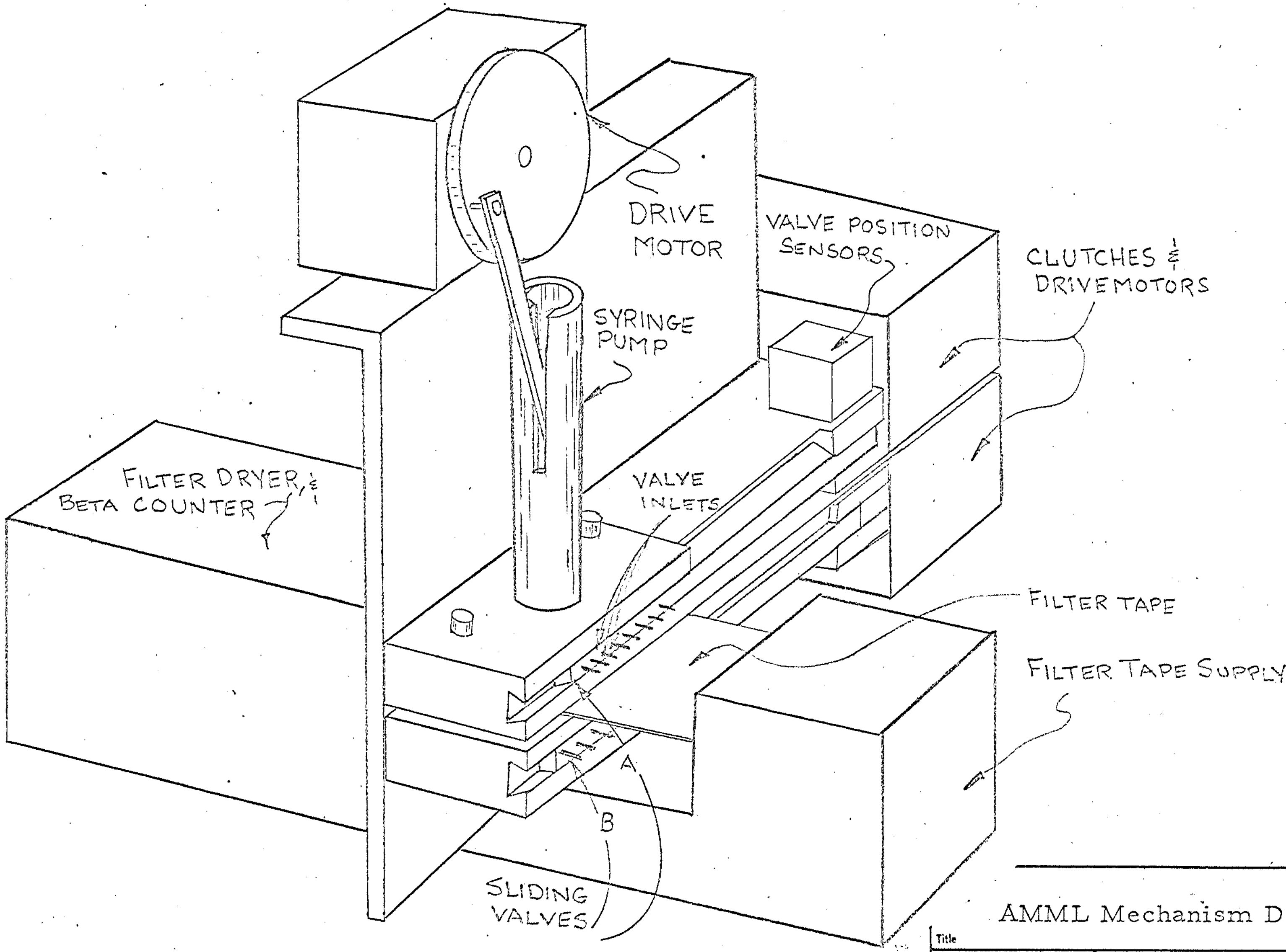


Figure 32

Liquid Transfer and Filtering Mechanism



Title: AMML Mechanism Details			
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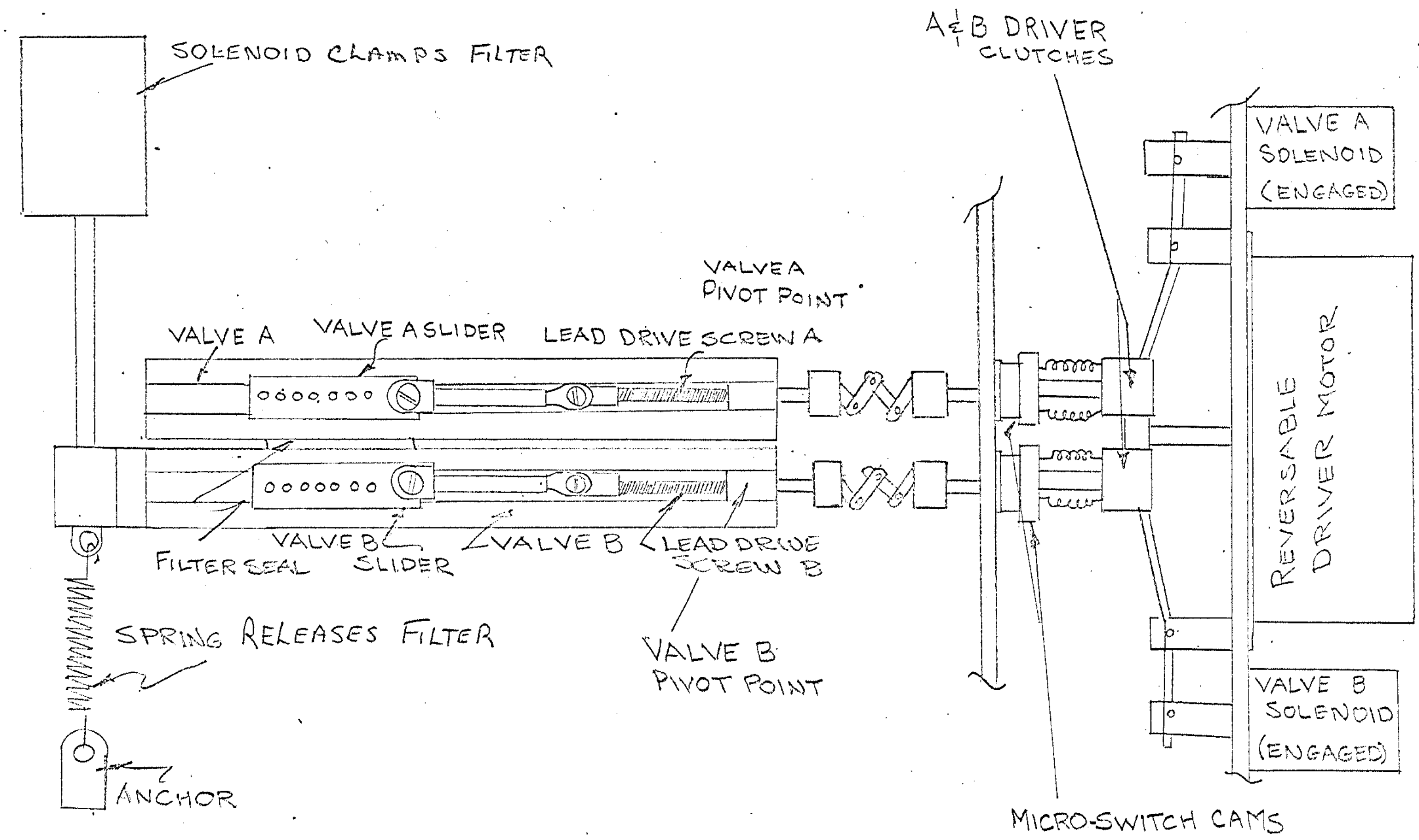


Title: Slide Valves and Drive Mechanism	
B[...]CS INCORPORATED, Rockville, Maryland	Fig. 34
	Date / Init. / Scale
NASW 1731 (Cont. No.)	AMML Final Report (Report Title)

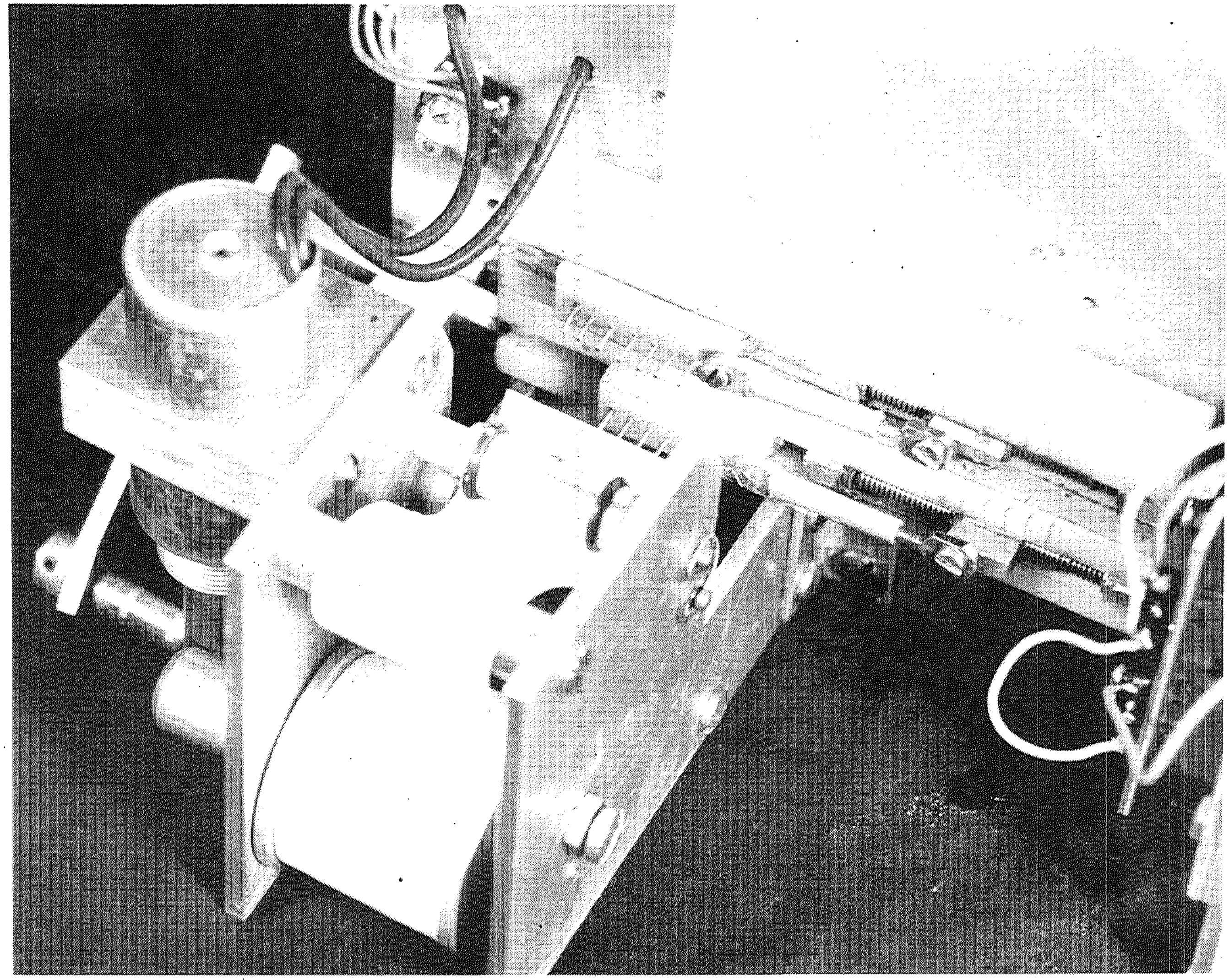


Figure 35

Slide Valve Mechanism Details

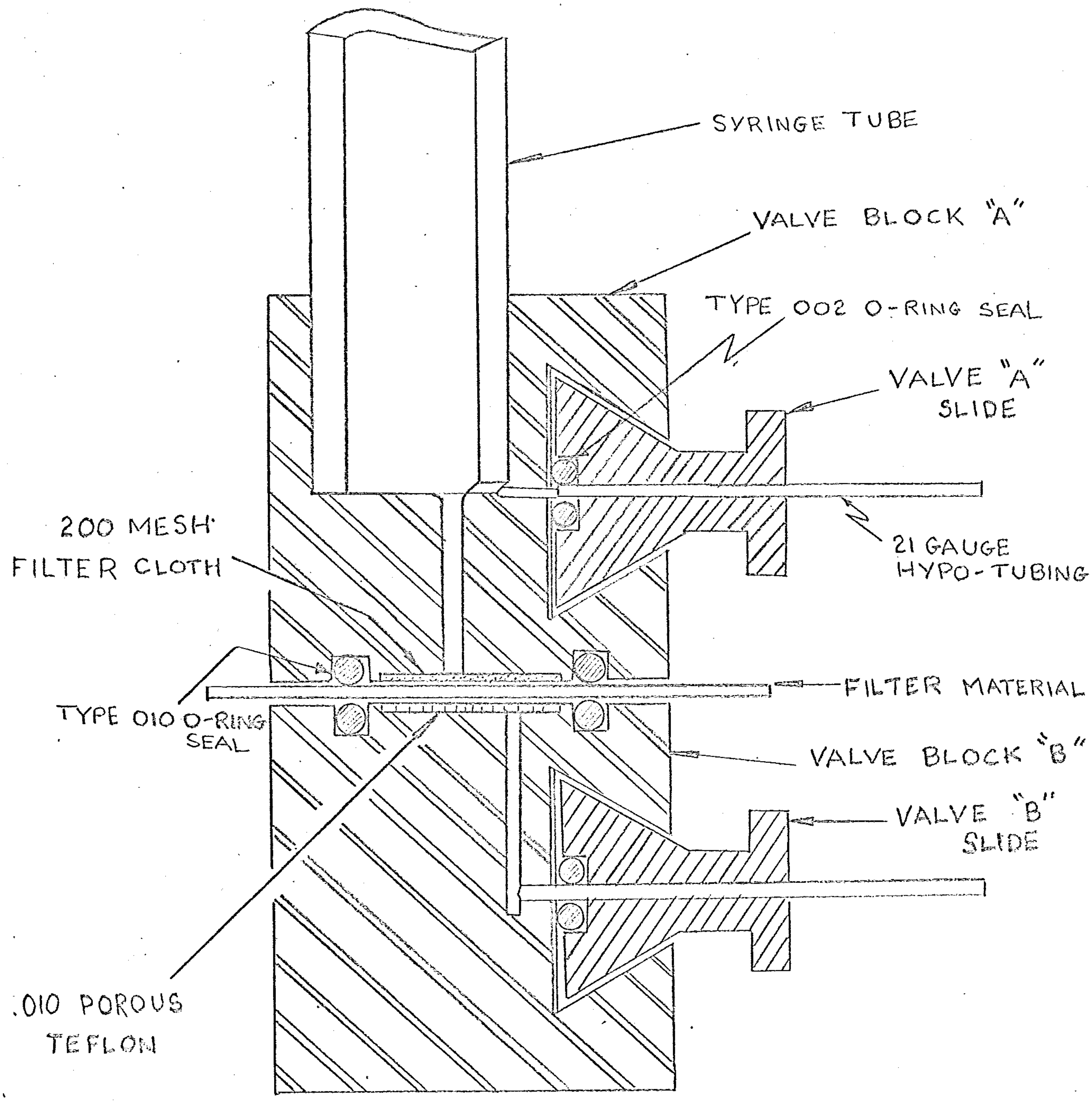
and to those liquids and reagents that must be brought into the syringe pump before filtering.

A changeable filter assembly is shown in Figures 36 and 37 below the Valve A block. This mechanism is designed with particular attention toward reducing the liquid retention or hang-up that is accomplished by the use of a thin, porous filter support under the filter (a 0.010 inch thick porous teflon which has 50% voids, supplied by Technical Fluorocarbons Engineering, Inc.), a small active surface area and head space over the filter which is filled with a disk of 200 mesh stainless steel filter cloth (0.005 inches thick).

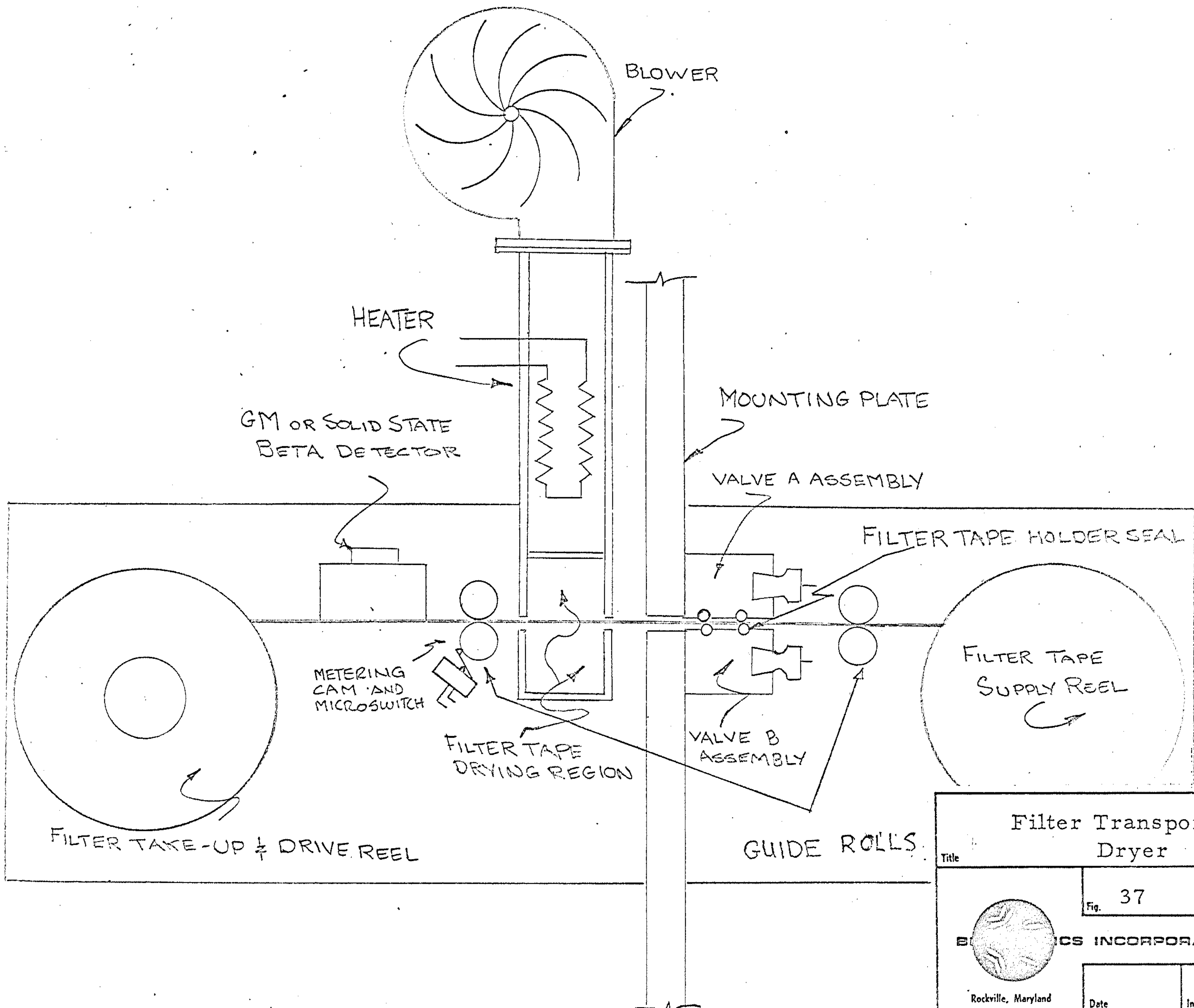
Valve B and its manifold couples to the channel below the filter assembly. This valve gives access to a second set of liquid reservoirs and reaction volumes. All interconnecting orifices, void volumes, and liquids channels in the valves, filter mechanisms, and syringe are designed for minimal liquid retention. The small retention volumes coupled with an operational program scheme help to assure that the subsequent liquid transfers are not affected by the previous liquid which was in the system.

a. The Syringe Pump

The syringe pump shown in the previous figures, is an all-nylon device which is capable of reproducibly transferring one-milliliter aliquots. The plunger of the syringe uses



Title: Details of Valve and Filter Seals			
B[illegible]ICS INCORPORATED, Rockville, Maryland	Fig. 36		
	Date	Init.	Scale
Cont. No. NASW 1731	Report Title: AMML Final Report		



Title: Filter Transport and Dryer	
B[illegible]ICS INCORPORATED, Rockville, Maryland	Fig. 37
	Date / Init. / Scale
Cont. No. NASW 1731	Report Title: AMML Final Report

a double o-ring seal to assure a low leakage when transferring high viscosity fluids.

The pump is accuated with a 10 rpm drive motor. A single detent cam is provided with two microswitches that identify the top and bottom positions of the plunger.

b. Slide Valve Mechanism

The slide valves which control the distribution of the liquid reagents require a unique positioning mechanism to control their movement in the automated program. This mechanism which employs a single drive motor and clutching arrangement is shown in Figure 38. The motor powers the clutches and only one is engaged at a time so as to couple the lead screw of the sliding valves. Solenoids are connected through a yoke that retains these clutches in their rest positions until a command is received.

A new valve position is set through the use of a cam and microswitch arrangement that generates a series of four pulses for each position change. These pulses are sent to the programmer circuit where they are accumulated in a binary register that is preset to overflow when it reaches the desired new position and then signals the controller to discontinue its operation. The slide valve design minimizes

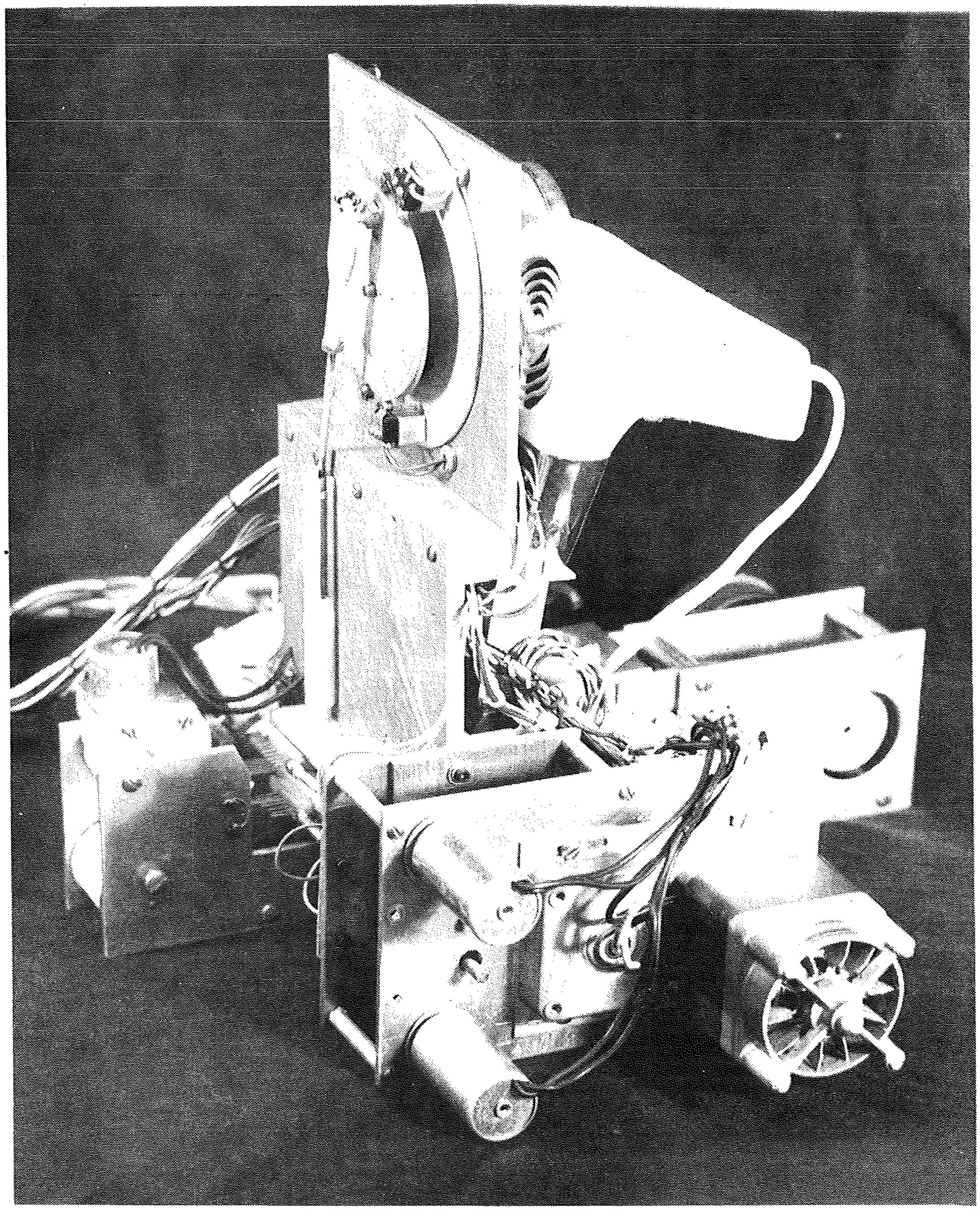


Figure 38

View of Valve Positioning Drive System

positioning errors caused by overshoot due to motor overtravel and mechanical backlash in the system, and it has been possible to position each valve orifice to within .005 inch (the valve orifice has an I.D. of .017 inch). Details of the o-ring sliding valve seal design have been shown in Figure 34. The valves are made of hypodermic tubing that is sealed to the face of the housing with pressure from the dovetailed construction of the slider. Only one outlet is connected at a time, and it is sealed to the wall of the body of the valve with use of miniature o-rings.

c. Filter Transport/Dryer Mechanism

The filter that is placed between Valve A and Valve B (Figure 39) is changed periodically as required in the operation of the measurement routine. A mechanism to perform this changing operation has been constructed and is shown in detail in Figure 37. This mechanism uses a nylon-web/filter-tape made of membrane or other material that is stored on a supply reel and moved through the mechanism on guide rollers to a take-up reel by a motor drive. The guide rollers provide the necessary tensioning and positioning action to keep the tape accurately located in its movement through the mechanism and also provide the means to meter the length of tape being transported in each filter-change cycle. Metering is accomplished with the use of a cam and microswitch that

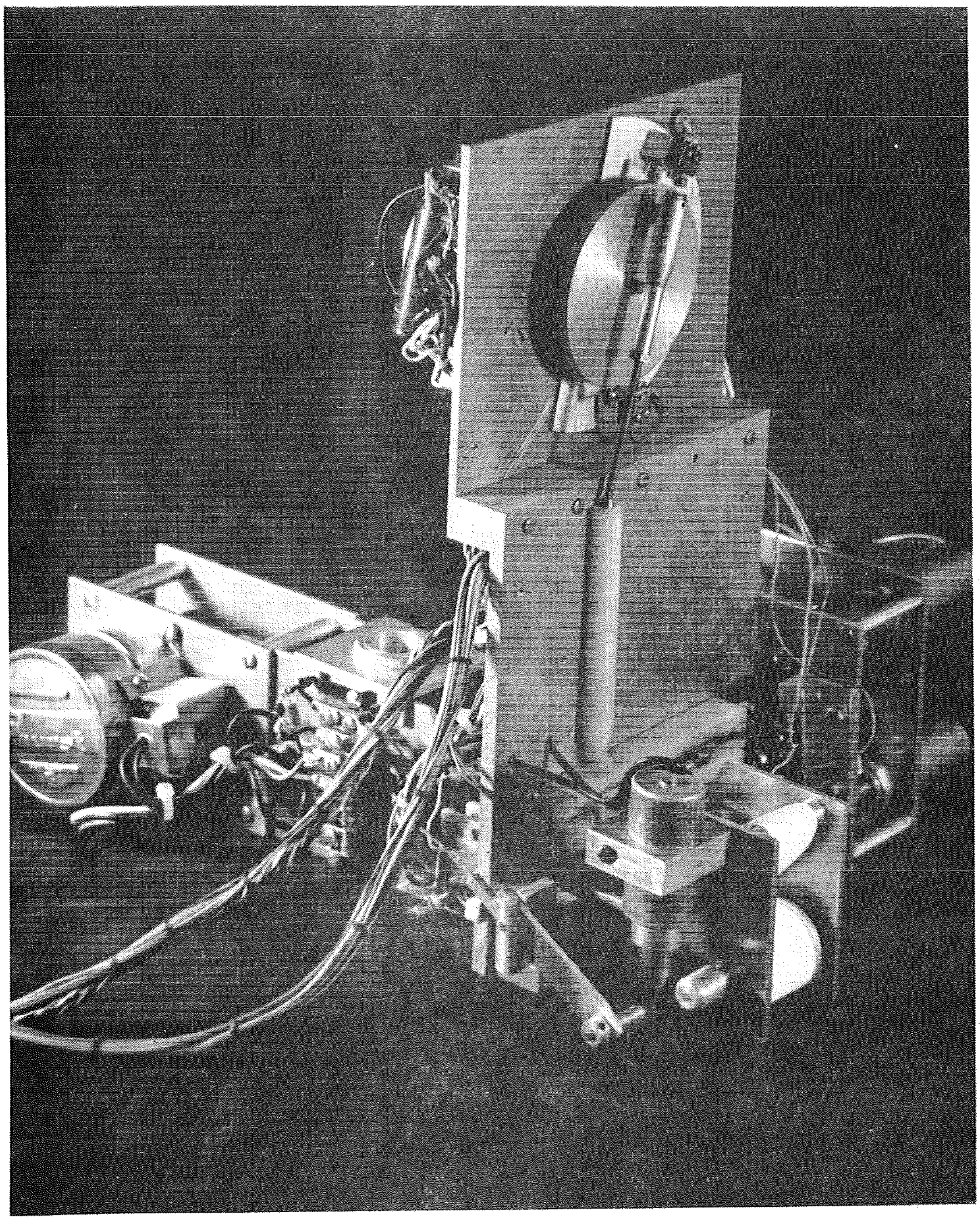


Figure 39

View of Filtering Mechanism

is attached to one of the guide rollers.

During the liquid transfer, the filter tape is clamped between Valves A and B by a solenoid that holds the two valve assemblies together so that they seal the tape through pressure on a pair of o-rings on each side of the tape as detailed in Figure 34. During a filter-change cycle, the solenoid is de-energized, and a spring releases the clamping action to allow the tape to move freely between the valves.

After a tape section has been used for filtering, it is transferred to the drying region. Here, hot air is forced across the filter to drive out the moisture. The operation of the dryer is controlled to apply sufficient heat to dry the filter without overheating it.

The third position for the filter is a cooling region where it is allowed to return to room temperature.

The last active position before the filter tape is stored on the take-up reel is the beta radiation detector region. In this region, a beta-sensitive detector is located in close proximity to the upper surface of the filter tape to assay the beta radiation from the surface of the filter tape.

The filter-change cycle is accomplished by a command that is received from the automatic program controller. The mechanism is designed to operate automatically through its change cycle from this command by first deactivating the clamping solenoid, secondly, activating the take-up reel motor, thirdly, deactivating the motor drive upon receipt of a signal from the metering cam-switch, and fourthly, reactivating the solenoid clamp and generating a cycle-complete signal to the controller.

3. Reagent and Solution Storage

The problem of storing the various reagents and solutions that are required for the operation of the AMML breadboard has been met by use of a very simple scheme. Various sizes of standard disposable hypodermic syringe barrels are coupled to the valve assemblies through 24-gauge teflon tubing and standard disposable hypodermic needles. This scheme also provides a means to monitor the status of the liquid transfers directly as the program proceeds. It is not necessary to store large quantities of reagents and solutions for the operation of the breadboard instrument, since these are prepared by manual techniques directly before the instrument is tested.

In the case of a prototype AMML, it may be necessary to provide pressurized storage reservoirs that are made from materials that will stand the long term storage, sterilization cycle, high pressures, and chemicals without degradation. Materials for these reservoirs have been considered in this research program, and preliminary tests have been conducted on a material similar to that used for the storage of blood in blood packs. Table 24 summarizes the requirements for the various reagent and solution storage volumes.

C. Automatic Programmer Controller

1. Design Details

The complexity of the experimental apparatus described above, coupled with a requirement for automated operation of the various functions required in this sytem, creates a demand for a fairly intricate programming mechanism. A spacecraft version of the programmer will no doubt take full advantage of the advanced techniques that are presently available with large-scale, integrated circuits (LSI) for programming the AMML through the complex functions. Such a programmer was far beyond the economic feasibility for the scope of this Contract, so an alternate scheme was developed for the demonstration apparatus.

A search of the various multipoint switches and programming apparatus for this type of applicator resulted in arriving at a conclusion that these were inadequate. As a result of this search, coupled with recent experience by one of us (W. A. L.), a new approach was suggested which involved the use of punched

Total Volume of Aqueous growth chamber	50 cm^3
Volume of Aqueous medium	30-40 ml*
Number of assays from test Aqueous growth chamber made at: 0, 1, 2, 4, 6, 10, 14, 22 and 30 hours	9*
ATP ASSAY:	
Enzyme	36 ml*
ATP Extractant	9 ml*
PO_4 ASSAY:	
Triethylamine Reagent	9 ml*
^{35}S + ^{14}C ASSAY:	
No special reagents required	
Wash solution	108 ml*

* Note: If assays are made so as to control chamber, this amount or number must be doubled.

Table 24

Summary List of AMML Liquid Reservoir Requirements tape to store the program for the operation of the AMML. An additional reason for using this rather than a "wired programmer," was the flexibility it offered the experimenter as he determined the optimal experimental program. A new program may be obtained with this system by simply writing a new tape that incorporates the changes.

An inquiry to several manufacturers of tape-readers resulted in the selection of a unit manufactured by the Teletype Corporation. Since a tape-punch was also required for the preparation of program tapes, it was decided to purchase a complete Model TC33 Teletypewriter. This is described in Table 25.

A program was initiated to develop a compatible logic package to translate the teletypewriter's output data into control functions for the instrument's operation. A simplified block diagram of the teletypewriter/logic programmer is shown in Figure 40.

Details of a step-by-step program for a typical experimental cycle is shown in Figure 41 and Table 26. This shows the conditions of each valve, the syringe, and the filter changes. In each step of the operation, only one mechanism is controlled at a time. Its operation must be completed and a verification received before the programmer can proceed to the

Description

Teletype Corporation Model 33TC Standard Duty Send-Receive Page Printer Set with Tape Perforator and Reader (Private Line Version) with these features:

Set Features (Less Punch and Reader)

Eight-level, 11.0 unit code (1.0 unit start and 2.0 unit stop pulse)

8-1/2" friction feed platen capable of handling one or two ply paper (maximum roll diameter 5")

Data communications type wheel arrangement with associated four-row keyboard layout (American Standard Code for Information Interchange)

115 V AC 60 cycle synchronous motor unit

Gears for 100 wpm operation (110.0 Bauds)

Adjusted for 72 character line

Horizontal spacing 10 character per inch

Vertical spacing 3 or 6 lines per inch

Selector magnet driver can accept .020 or .060 amperes with proper wiring (strapping)

Two-tone cover: Ivory and Greige

Standard Teletype nameplate

Copyholder

Answer-back mechanism (21 character) actuated by the "here-is" key

Answer-back drum to be coded by the customer

Function box "on-line" operation of line feed, carriage return, and bell

Space suppression at end-of-line

Control key (functions)

Shift key (locks out keys without shift case)

Break key and "Here-is" key

Power supply transformer

Convenience outlet

3-way switch (off, on-line and local)

Wiring provisions for optional full duplex operation

Sheet metal stand with greige color finish

Tape Reader Features

3-way operating switch for start, stop and free position

"End-of-Tape" mechanism to shut down reader

Feed sensing electromagnet is pulsed by the distributor

Power pack assembly

Parallel wire output to distributor

Operating sequence; reads tape and feeds

Capable of reading fully perforated tape

(continued)

Table 25
Teletypewriter
Equipment Features and Components

Tape Punch Features

Four operating buttons, "OFF", "ON", "BACKSPACE", and "RELEASE"

1" Core for tape supply

Fully perforated 8-level code holes and feed hole

"V" shaped tape tearoff

Operating sequence; feed and punch

Removable chad box (Designed for attaching to stand)

General Information

Interoperates with existing teletypewriters having corresponding features
Fewer options possible compared to Model 35 Heavy-Duty Equipment
Actual and schematic wiring diagrams packaged with set
Set shipped completely assembled less stand and chad box
For applications requiring a broader range of features, the Teletype Model 35 line of equipment is recommended.
The above equipment requires a channel capable of handling 110.0 Bauds (Bits per second) for operation at 100 wpm. If for use on Dataphone Service with "Bell" Data Subset or its equivalent, we can furnish the necessary interface coupler under Part No. 198420, at $78.20 net each plus Part No. 186145 Cord with Connector at $5.60 net each. (P/N 186136 - voltage) coupler.
One copy each of Bulletins 273B Vol. 1, 310B Vol. 2, and 1184B furnished with each set

Dimensions

Complete set less stand:

Width	22"
Depth	18-1/2"
Height	8-3/8"
Weight	44 lbs.

Stand: Supports Teletypewriter

Width	17-3/4"
Depth (at Top)	8"
Depth (at Bottom)	6-1/2"
Height	24-1/2"
Weight	12 lbs.

Estimated Service Life

7500 Hours at 60 or 66 wpm
4500 Hours at 100 wpm

Lubrication Intervals

Printer, Tape Punch and Reader

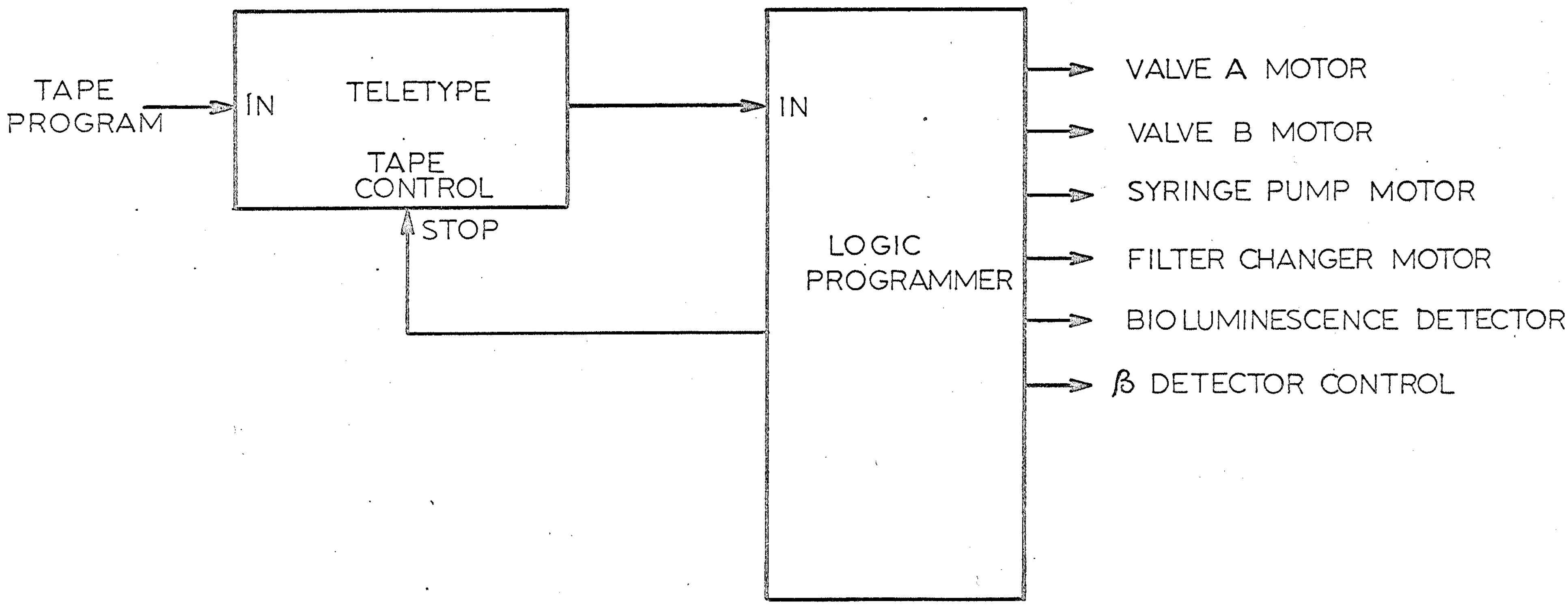
100 WPM	500 Hours or 6 months, whichever is first
60 or 66 WPM	1000 Hours or 1 year, whichever is first

Preventive Maintenance and Overhaul

At 60 or 66 WPM	At 100 WPM
2500 and 5000 hours	1500 and 3000 hours

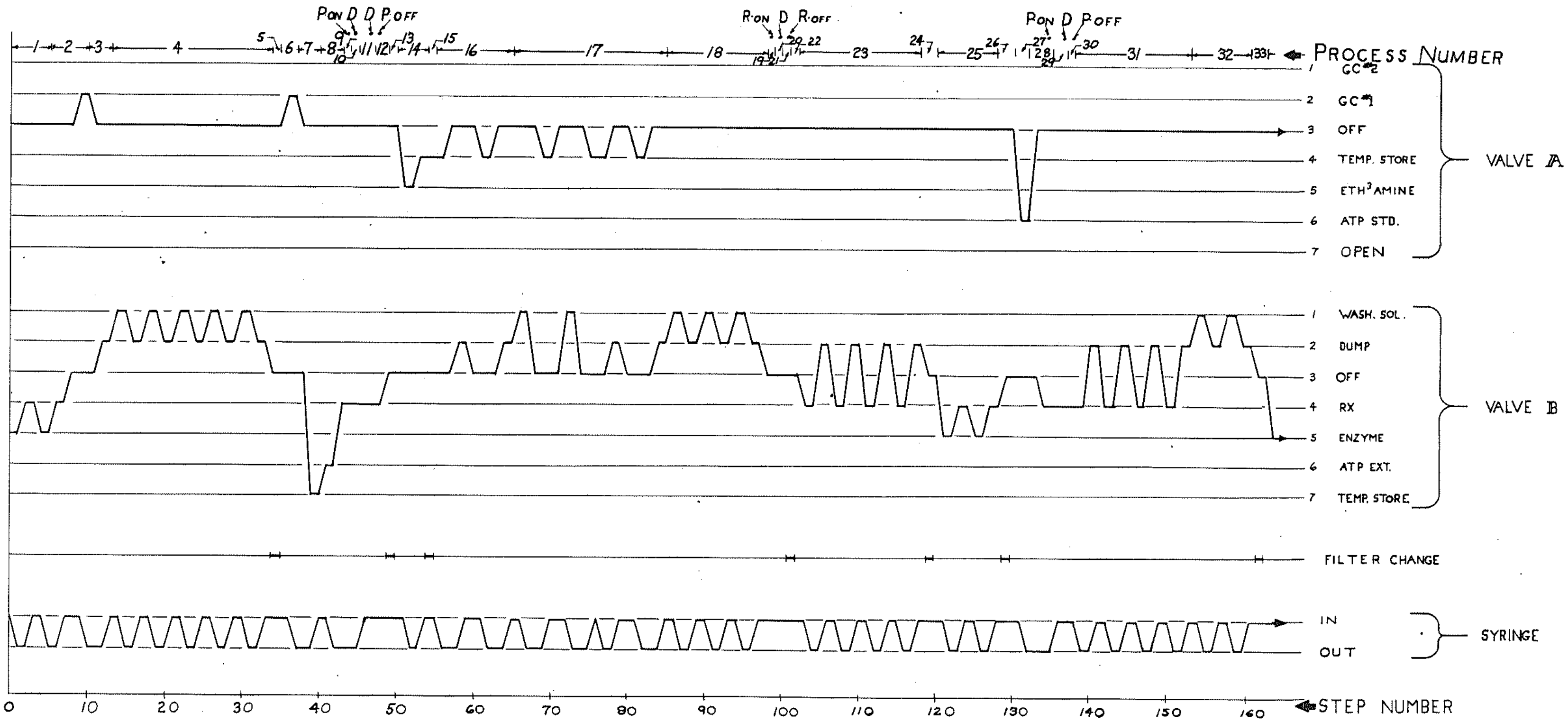
Note: Hours indicates actual operating time.

(Table 25 continued)



BLOCK DIAGRAM - AMML PROGRAMMER

B...CS INCORPORATED, Rockville, Maryland	Fig. 40		
	Date	Init.	Scale
Cont. No. NASW 1731	Report Title: AMML Final Report		



Note:
Process Numbers are described in the following Table 26.

Title: Typical AMML Experimental Test Cycle			
BIOSPHERICS INCORPORATED Rockville, Maryland	Fig. 41		
	Date	Init.	Scale
Cont. No. NASW 1731	Report Title: AMML Final Report		

Process Number	Operation	Process Number	Operation
1	Fill ATP R_x with 2 ml Enzyme	19	Turn ON R, Radiation Detector
2	Acquire first 1 ml sample from GC#1	20	Delay program one minute
3	Filter sample and dump liquid	21	Turn OFF R, Radiation Detector
4	Wash Filter 5X with 1 ml	22	Advance Filter to Dryer
5	Advance Filter to Dryer	23	Remove liquid from ATP R_x (4x1 ml)
6	Acquire second 1 ml sample from GC#1	24	Advance Filter
7	Filter sample and store liquid	25	Fill ATP R_x with 2 ml Enzyme
8	Wash 1 ml ATP Extractant through Filter	26	Advance Filter
9	Turn ON P, Bioluminescence Detector	27	Fill with 1 ml ATP standard solution
10	Delay program one minute	28	Turn ON P, Bioluminescence Detector
11	Inject Syringe into ATP R_x and observe bioluminescence for one minute	29	Inject Syringe into ATP R_x and observe bioluminescence for one minute
12	Turn OFF P, Bioluminescence Detector	30	Turn OFF P, Bioluminescence Detector
13	Advance Filter to Dryer	31	Remove liquid from ATP R_x (4x1 ml)
14	Fill with 1 ml triethylamine and add this to temporary storage	32	Advance Filter
15	Advance Filter	33	Wash Syringe with 2X 1 ml wash solution
16	Remove the 2 ml from temporary storage, Filter this and dump liquid		
17	Wash temporary storage with 2 ml wash solution, remove thru Filter and dump liquid		
18	Wash Filter with an additional 3X 1 ml wash solution and dump liquid		

Table 26

Details of Program in Figure 41

next command. If this verification is not received for some reason, the program will be held in this step. In this step-by-step operation, the teletypewriter reads the program code from the tape and transmits this signal to the logic package. While the AMML mechanism is operating, the teletypewriter may be programmed to print a written description of the operation. An example of printout is shown in Table 27.

The standard Model TC33 teletypewriter requires only minor modifications for use in this application. Since it operates synchronously with its tape-reader during the normal printout mode, the AMML programmer must provide a program-interrupt to stop the tape-reader whenever an operation requires more time than it takes to print the written description of the operation. The program-interrupt is accomplished by using the coincidence of a "cycle not complete" code ("EOC") and a "BELL" non-printing code that is included on the program tape at the end of each completed instruction and message. The program-interrupt disengages the teletypewriter's tape-reader whenever these conditions are met. An END OF CYCLE command is generated whenever: 1) A valve being controlled reaches the required new position, 2) The syringe pump completes a transfer cycle, 3) A filter change is completed, 4) A thirty-second WAIT has been completed, or 5) The beta

```
AT START VALVE A IS AT POSITION 3 (OFF); VALVE B IS AT POSITION
5 (ENZYME); SYRINGE IS EMPTY; UNUSED FILTER IS IN PLACE.

#1. >0S SYRINGE - FILL (ENZYME )
#2. <1B VALVE B LEFT TO POSITION 4 (R-X)
#3. <0S SYRINGE - EMPTY
#4. >1B VALVE B RIGHT TO POSITION 5 (ENZYME)
#5. >0S SYRINGE - FILL
#6. <1B VALVE B LEFT TO POSITION 4 (R-X)
#7. <0S SYRINGE - EMPTY
#8. <1B VALVE B LEFT TO POSITION 3 (OFF)
#9. <0F CHANGE FILTER
#10. <1A VALVE A LEFT TO POSITION 2 (G.C.#1.)
#11. >0S SYRINGE - FILL
#12. >1A VALVE A RIGHT TO POSITION 3 (OFF)
#13. <1B VALVE B LEFT TO POSITION 2 (DUMP)
#14. <0S SYRINGE - EMPTY
#15. <1B VALVE B LEFT TO POSITION 1 (WASH SOL.)
#16. >0S SYRINGE - FILL
#17. >1B VALVE B RIGHT TO POSITION 2 (DUMP)
#18. <0S SYRINGE - EMPTY
#19. <1B VALVE B LEFT TO POSITION 1 (WASH SOL.)
#20. >0S SYRINGE - FILL
#21. >1B VALVE B RIGHT TO POSITION 2 (DUMP)
#22. <0S SYRINGE - EMPTY
#23. <1B VALVE B LEFT TO POSITION 1 (WASH SOL.)
#24. >0S SYRINGE - FILL
#25. >1B VALVE B RIGHT TO POSITION 2 (DUMP)
#26. <0S SYRINGE - EMPTY
#27. <1B VALVE B LEFT TO POSITION 1 (WASH SOL.)
#28. >0S SYRINGE - FILL
#29. >1B VALVE B RIGHT TO POSITION 2 (DUMP)
#30. <0S SYRINGE - EMPTY
#31. <1B VALVE B LEFT TO POSITION 1 (WASH SOL.)
#32. >0S SYRINGE - FILL
#33. >1B VALVE B RIGHT TO POSITION 2 (DUMP)
#34. <0S SYRINGE - EMPTY
#35. >1B VALVE B RIGHT TO POSITION 3 (OFF)
#36. >0F CHANGE FILTER
#37. <1A VALVE A LEFT TO POSITION 2 (G.C.#1)
```

Table 27

AMML Teletypewriter Printout of Typical Control Program and bioluminescence detectors acknowledge that they have been turned ON or OFF.

The block diagram and explanatory legend of the AMML programmer is shown in Figure 42 and Table 28 and a front panel layout in Figure 43 . The programmer consists of a shift-register which takes the serial signal from the teletypewriter's commutated signal and provides a means for parallel readout and detection of a single character. These characters are made up of an 11-BIT code based upon the ASCII* code and are detailed in Figure 44 . The 11-BIT code always begins with a "Start" signal, represented by a "0" followed by 7-BITS of character identification, "1's" and "0's," a parity BIT (always a "1" in this case), and last two STOP code BITS, "1's."

Character detection is accomplished by gates which examine the shift-register output for the desired combination of "1's" and "0's." When the required combination occurs in coincidence with a clock pulse (CL), an output signal is generated which is used to initiate an operation or is stored in a binary memory element for delayed usage. The binaries remain set in this condition until they are RESET at the end of the cycle.

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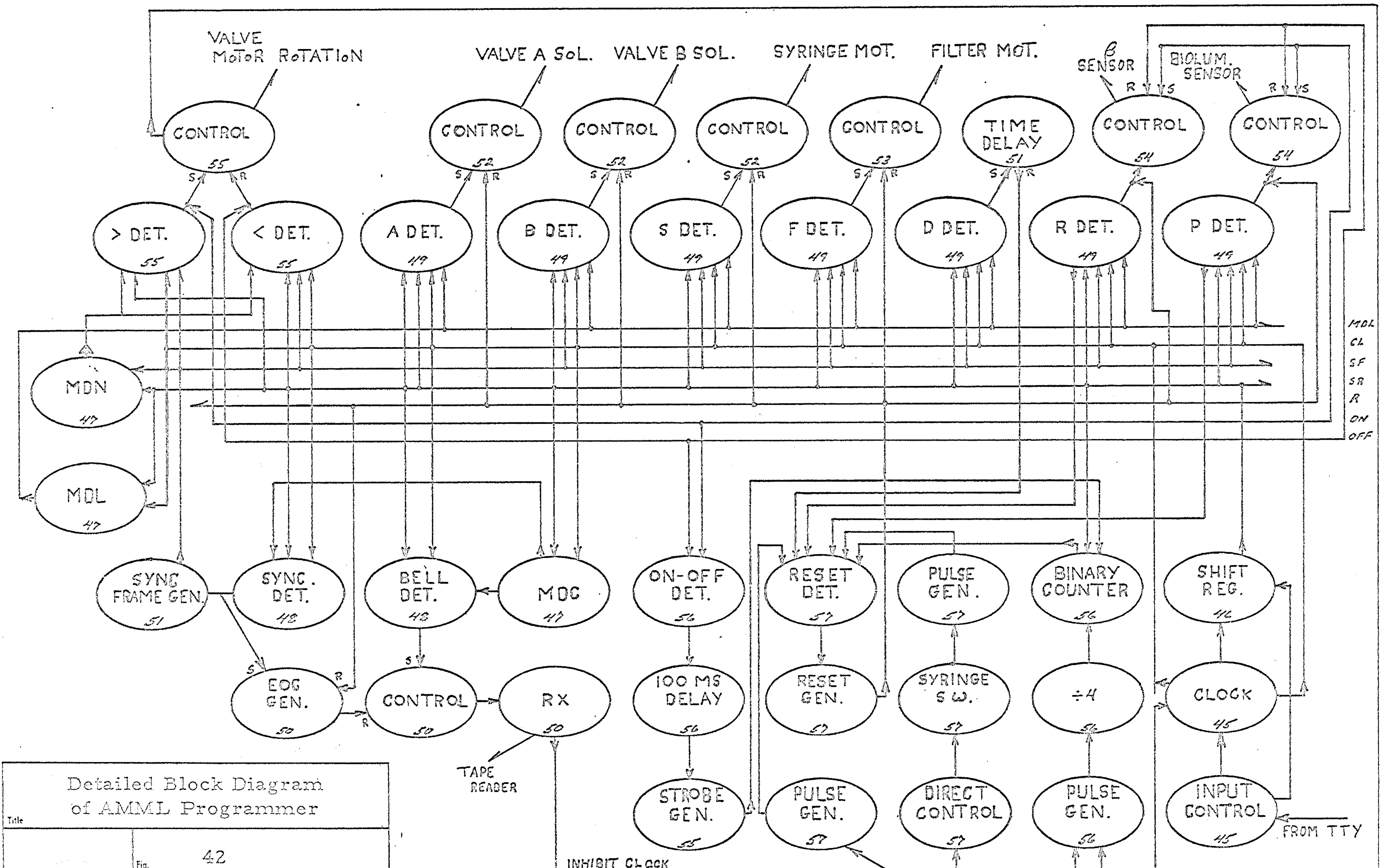
* American Standard Communication Information Interchange.



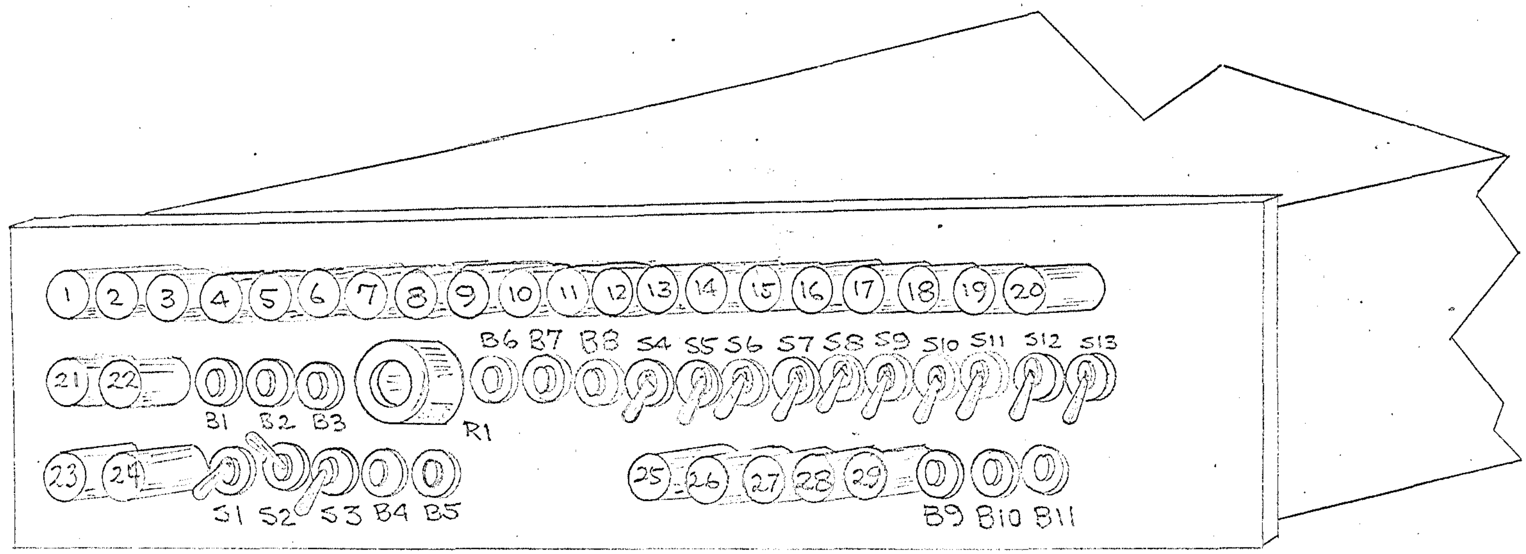
NOTE: NUMBERS ARE FIGURE NUMBERS WHICH SHOW EACH CIRCUIT.

Title: Detailed Block Diagram of AMML Programmer			
	Fig. 42		
BIOSPHERICS INCORPORATED			
Rockville, Maryland	Date	Init.	Scale
Cont. No. NASW 1731	Report Title: AMML Final Report		

BELL =	Conditional Hold
SYNC =	Synchronizing Pulse to Open Data Gate
CL =	Clock Pulse Bus
R =	Reset Bus
EOC =	End of Cycle Command Level
SR =	Shift Register Signals
ON =	"ON" Level from Direction Detector
OFF =	"OFF" Level from Direction Detector
STROBE =	Timing Pulse when TTY is Synchronized for Number Entry
SF =	SYNC Frame - Gate is Open During 32 Second Data Entry Period
Rx =	Relay
TTY =	Teletypewriter
MDC =	Master Detector, Control
MDL =	Master Detector, Letter
MDN =	Master Detector, Number

Table 28

Explanatory Legend for Figure 42



SWITCHES

B1	SYRINGE OPERATE
B2	FILTER OPERATE
B3	RAD. & BIOLUM. DETECTORS ON
B4	PROGRAM RESET
B5	TAPE READER RESET
B6	RAD. & BIOLUM DETECTORS OFF
B7	VALVE A OPERATE
B8	VALVE B OPERATE
B9	BINARY COUNTER STROBE (SET)
B10	SR. & BINARY (RESET)
B11	SR. DATA ENTRY (SET)
S1	A.C. POWER
S2	TAPE READER CONTROL
S3	CLOCK (SR) CONTROL
S4	ELEVEN DATA BITS FOR
↓	MANUAL ENTRY INTO
S13	SHIFT REGISTER
	POTENTIOMETER
R1	DELAY TIME CONTROL
	(10 – 100 seconds)

INDICATORS

1	EOC – PROGRAM RUNNING (GREEN)
2	$\overline{\text{EOC}}$ PROGRAM WAITING (RED)
3	SYRINGE (S)
4	FILTER (F)
5	RADIATION DETECTOR (R)
6	DELAY PROGRAM (D)
7	BIOLUMINESCENCE DETECTOR (B)
8	VALVE A (A)
9	VALVE B (B)
10	SHIFT REGISTER BIT 11
11	" " " 10
12	" " " 9
13	" " " 8
14	" " " 7
15	" " " 6
16	" " " 5
17	" " " 4
18	" " " 3
19	" " " 2
20	" " " 1

21	DIRECTION < LEFT (RED)
22	DIRECTION > RIGHT (GREEN)
23	TAPE READER
24	A.C. POWER
25	BINARY COUNTER BIT 32
26	" " " 16
27	" " " 8
28	" " " 4
29	" " " 2

Title: Programmer Front Panel

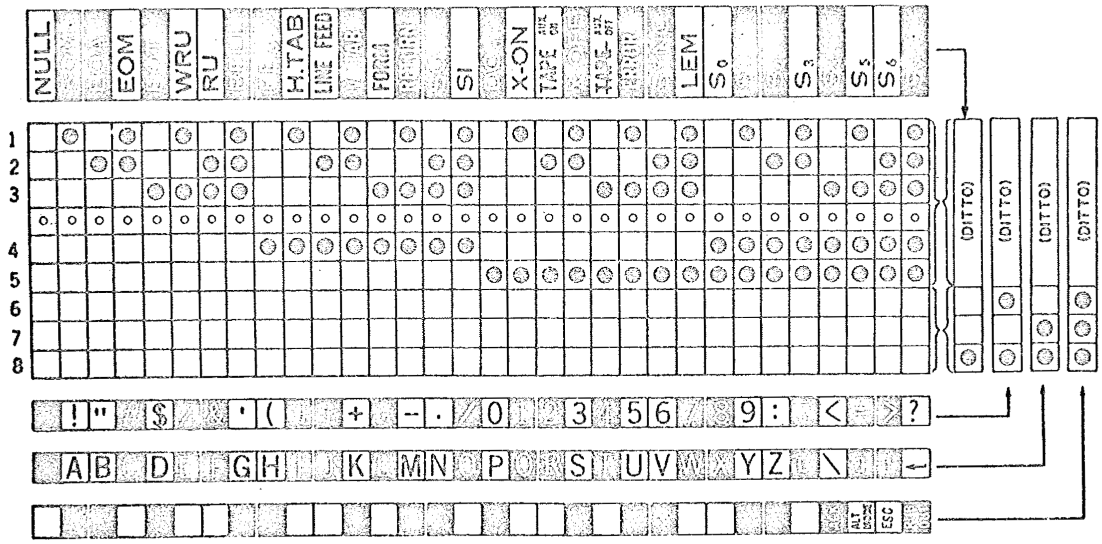
Fig. 43

B[illegible]CS INCORPORATED
Rockville, Maryland

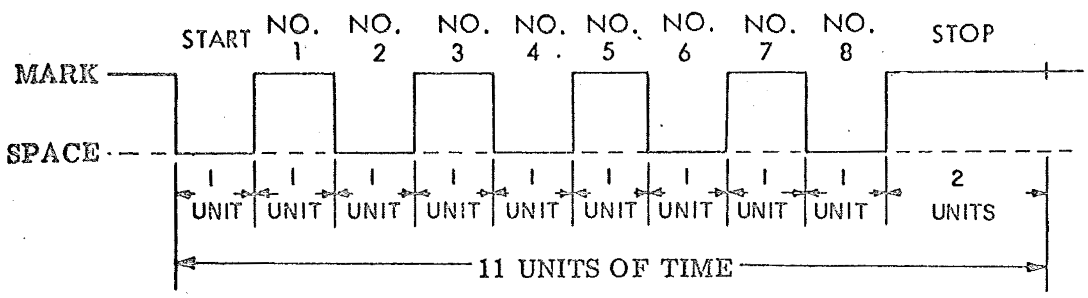
Date | Init. | Scale

Cont. No.: NASW 1731

Report Title: AMML Final Report



CHARACTER ARRANGEMENT (ASCII CODE)



CURRENT WAVE FORM FOR LETTER "U" (WITH "EVEN PARITY")

Title: ASCII and Teletypewriter Code	
	Fig. 44
D[illegible]CS INCORPORATED, Rockville, Maryland	Date / Init. / Scale
Cont. No. NASW 1731	Report Title: AMML Final Report

The operational codes which are combined with control characters are shown in Table 29. A typical program code consists of four characters. The first character of the program code is a "SYNC" code that initiates a 0.3 second gate that is used to activate the detectors for the three characters which follow. The second character is a ">" or "<" which is used to program the RIGHT or LEFT, UP or DOWN, or ON or OFF. The fourth character is a device designation character which is also described in Table 29. The third character is used to preset a step control register which is used to control the number of valve positions which must be moved.

As had been shown in the example in Table 29, the program code word is followed with a description of the operation being performed.

2. Program Preparation

Programming tapes are prepared with the use of the tape perforator on the Model TC33 teletypewriter. In preparing a program tape, the exact format is dictated by the actual function that must be performed by the programmer.

The first operation is to push in the tape punch to "ON" and the teletypewriter power switch to the "LOCAL" position. A few inches of leader tape is advanced through the tape punch by use of the "BREAK" key.

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 $\overline{\text{EOC}}$
"SYNC"	Provides Method for Synchronizing Character Detection

These Operation codes are used to control the AMML programmer. As an example, the word format would be: "SYNC" $\underline{<}$ $\underline{1}$ A. A typewriter message should follow; "valve a Left to positions 2 (GC. #1)"

Table 29

Description of Operation Codes

Initial written instructions for the operator should be printed first. These should include set-up details such as any manual settings he should make before the automatic program commences. These instructions should be followed by 1) "SYNC" (V plus CONTROL), 2) "BELL" (G plus CONTROL), 3) Carriage Return, and 4) Line Feed, so as to make sure all program resets have been made. This control format for the AMML is shown in Table 30 . The printout format which follows had been shown in Table 27 starts with an item number, followed by a period, space, and the control character "SYNC" (which is written as V plus CONTROL). The "SYNC" character is followed by a "greater than" or "less than" symbol which indicates an up-down, on-off, or a right-left operation, a number (N-1) indicating the number of steps (N), if any, which must be detected in the operation command, and last a device designation character which is a letter of the alphabet.

Following the four-character control word is a written description of the operation being performed that does not have control capability. This provides the user a means to visually check the steps of the program as it progresses.

A "BELL" CONTROL follows which is used to stop the program operation in the event that the control sequence has

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

Table 30

Control Format

not been completed. The incomplete sequence is indicated on the RED "$\overline{\text{EOC}}$" lamp. Upon completion of the sequence, the GREEN "EOC" goes ON, and the program automatically re-starts. A "CARRIAGE RETURN" and "LINE FEED" are the next characters, followed by the next item number which repeats the above sequence.

After a complete sequence of events is recorded on the punch tape, additional operator instructions may also be printed. The tape is then advanced for a few inches through the punch with the use of the "BREAK" key on the keyboard of the teletypewriter and removed from the punch. The pre-pared tape is then wound, keeping the beginning of the tape at the outside of the coil and stored until its use is required in performing a programming function.

3. Program Tape Use

The teletype controls should initially be in the following positions:

a) The main switch is in the "LOCAL" position.

b) The tape punch is in the "OFF" position.

c) The tape-reader is in the "FREE" position.

All connections from the tape-reader to the program con-troller must be in place, and the system turned ON and com-pletely reset (depress all red buttons on the programmer front panel). The start of the program tape is placed in the tape-reader with the leader in the reading area and the tape-reader latched closed. The system is then ready to start an automatic program.

The automatic program is initiated by moving the switch on the tape-reader to the "READ" position. The first operation of the teletypewriter will be the printing out of the necessary initial conditions that must be manually set-up in the AMML. When these are printed, the reader will stop until the operator actuates the master "RESET" push button on the AMML programmer to acknowledge that the initial conditions have been met. From this point on, the entire sequence of events occurs automatically, and the operator need only to monitor visually the progress of the program to assure its error-free operation. At the end of the automatic sequence, the teletypewriter will print additional instructions to the operator and cease operation. Depending upon the exact character of the program which is stored on the punch tape, the end of tape can signify several conditions: this could be the end of the complete program, an intermediate pause between programs, or an alternate program selection point. This selection must be made by the operator.

4. Operational Details

The output signal from the teletypewriter is a series of sequentially coded pulses. During an idle condition, the signal is a D. C. level ("1's"). Data signals are pulses ("0's") in groups of 11-BIT characters that are coded in a very particular manner consisting of first a START which is a "0" followed by an 8-BIT code to conform to the American Standard Communication Information Interchange (ASCII), followed by 2 STOP codes "1's" which complete the character code. The ASCII code describes all letters of the alphabet, numbers, punctuation, descriptive characters, and some non-printing control functions, as described previously in Table 29. Each character is transmitted by the teletypewriter during a 0.1 second period followed by the next character sequentially as long as data is being transmitted by the teletypewriter. The individual BITS of data for each of the characters transmitted at a rate of 110 Hz, occupy a time frame of 9.1 ms.

The automatic program controller accepts the signal from the teletypewriter through an input conditioner that converts the signal level from the teletypewriter to a standardized 5 volt logic level pulse. Each transition of the input signal from "1" to "0" creates a synchronizing pulse which is used to keep the internal 110 Hz clock oscillator in synchrony with the teletype data frame.

The clock oscillator (CL) has two functions. One is to command the Shift Register (SR) to shift the data BITS to the adjacent storage element, and the other is to provide a master strobe command to interrogate data at the proper time as it passes through the Shift Register.

The Shift Register is an eleven binary storage element connected in such a manner that when commanded to shift, the first binary transfers its data BIT (either a "0" or a "1") to the second binary, the second to the third, etc., through the entire Shift Register. The shift command in this case is a 110 Hz clock pulse corresponding to the rate of data as it is received from the teletypewriter. In operation, the Shift Register temporarily stores the serially-coded information from the teletypewriter so that each complete character is stored in the Shift Register once during the readout cycle.

As these characters pass through this Shift Register, some are used to create command in the logic system. A "BELL" command is used to interrogate the programmer to determine if it is still in operation from the previous command by detecting the presence ("1") in the END-OF-CYCLE (EOC). If the EOC is "0", the programmer continues to accept the next character that is transmitted. If the EOC is "1," ($\overline{EOC}$), the programmer signals the tape-reader to STOP. The transmission of data by the teletypewriter ceases until the EOC again is "0." The EOC is controlled by the Reset Generator that receives its signal from the completion of the device operations.

When the SYNC character is received, a 0.32 second SYNC FRAME (SF) is generated so that the next three characters can be identified in the logic programmer. The next character in the command is either a " >" or a "< ," and is used to give a direction to the command, as described previously in Table 29 . The third programming character is used for setting the valve positions and is a number 0-5 indicating the number of steps, if any, which must be counted during a particular sequence. (A "0" is used for the other controls.) These numbers are preset into a binary counter that has 2^8 BITS of counting capacity. This counter uses the first two binaries to pre-divide the signals from the valve cam switches that are located on the slide valve drive system. (Each valve position change requires four complete revolutions of the drive motor.) The last character to be received is a letter code used to identify the actual mechanism which is being programmed during this cycle. A description of the letter code designations used by the AMML have been shown in Table 29 , and may be expanded to include additional letters of the alphabet as they may be required.

This design has applications which could extend beyond that of the AMML program, and it is possible to make simple modifications to the characteristics of the command word so that more flexibility can easily be obtained. One such modification is the incorporation of several decade counters that can be preset with more digits that are added to the command word. Such applications as digital-linear position indicators and digital-angular indicators that can have a 10^6 resolution would require a simple modification requiring additional decades to be added. Additionally, for high-precision, complex applications, a digital comparator can also be incorporated so that the programmer would then be capable of controlling multiple drive high-resolution transducer-inductors.

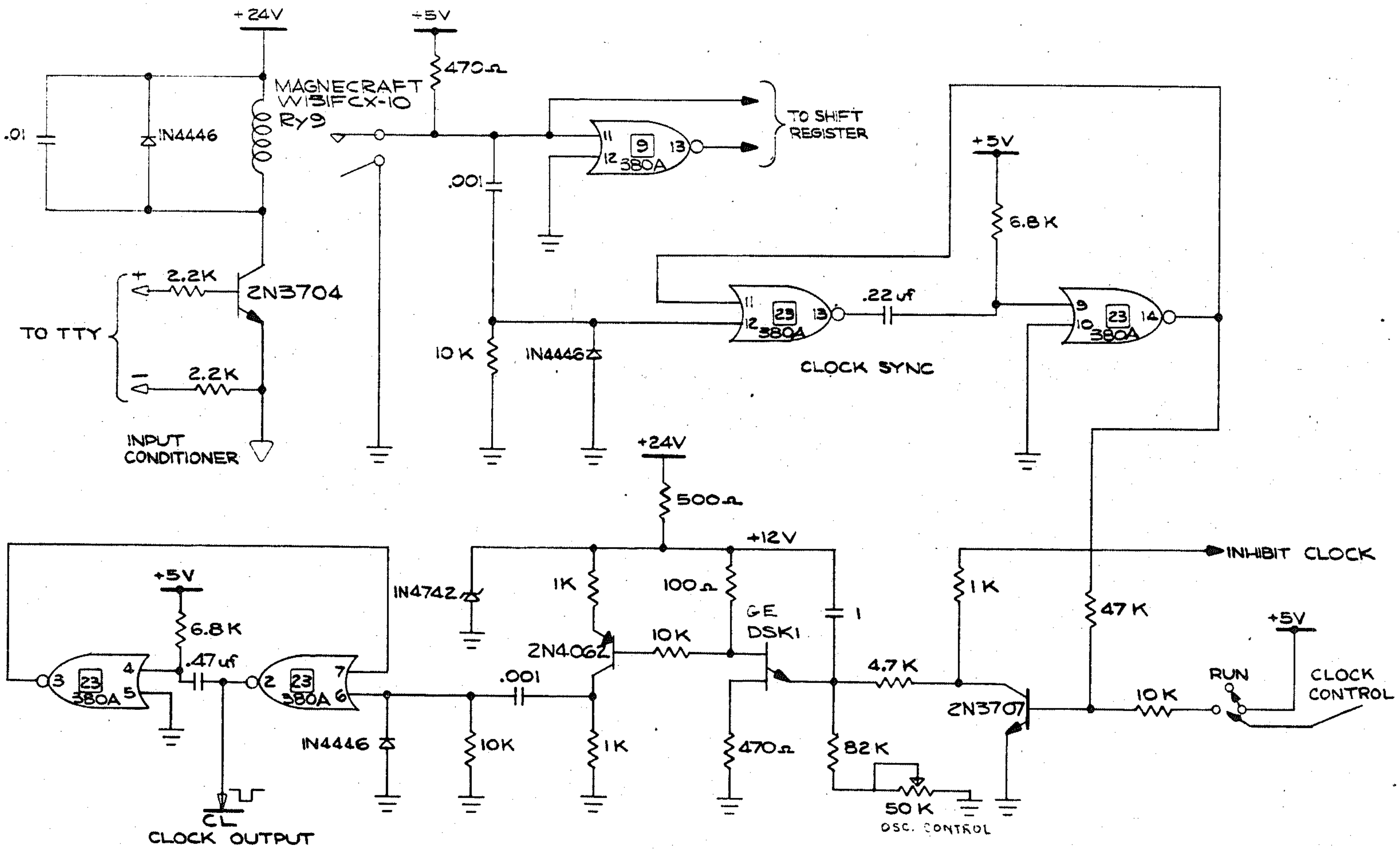
The AMML programmer design uses integrated circuits manufactured by Signetics which have been chosen for their simplicity of assembly, economy, high noise immunity, and ready availability. The most important design consideration in using these integrated circuits is the strict observation of the input and output loading rules particularly, in the case of the output from the Shift Register binaries where additional drivers have been added to prevent overloading. In addition, output drive capability is conserved by grouping the redundant portions of the character identification together. This is accomplished by the use of three 4-BIT detectors for the identification of 1) the nonprinting control characters (MDC), 2) the letters (MDL), and 3) the numbers (MDN). These three detectors are also synchronized further with a clock pulse signal (CL).

5. Logic Circuit

The functional group of logic circuits which are used to perform the details of the operation described above are covered in the following description. The logic circuit diagrams use symbols that are described in Table 28 .

a. Input Conditioner

The output signal from the teletypewriter is obtained from the magnet drive circuit through an ungrounded source of 5 volt pulses. These are used to drive a transistor amplifier that actuates a mercury-wetted-contact relay which in turn normalizes the signal to the logic pulse levels as shown in Figure 45 . This type of relay was selected because of its reliability, fast response and freedom from contact bounce. The input conditioner operates so that the teletypewriter's "Marking" transmission ("1's") produces a "1" signal to the logic circuits, and the "Space" transmission ("0's") produces a "0" signal. This logic circuitry defines a "1" as a signal



INPUT CONDITIONER AND CLOCK GENERATOR

Title: Input Conditioner and Clock Generator

Fig. 45

BIOSPHERICS INCORPORATED
Rockville, Maryland

Date | Init. | Scale

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between +3.8 and +5.0 volts and a "0" between 0.0 and +0.6 volts.

The relay drives two circuits. One, the synchronizing circuit for the Clock Oscillator, forces a resetting of the unijunction oscillator every time that the input signal has a "0" to "1" transition. This scheme assures that the 110 Hz free-running clock Oscillator is always in phase lock with the teletypewriter commutator. The Shift Register also uses the relay signal along with an inverter circuit which provides the complementary signal to the second input to the Shift Register.

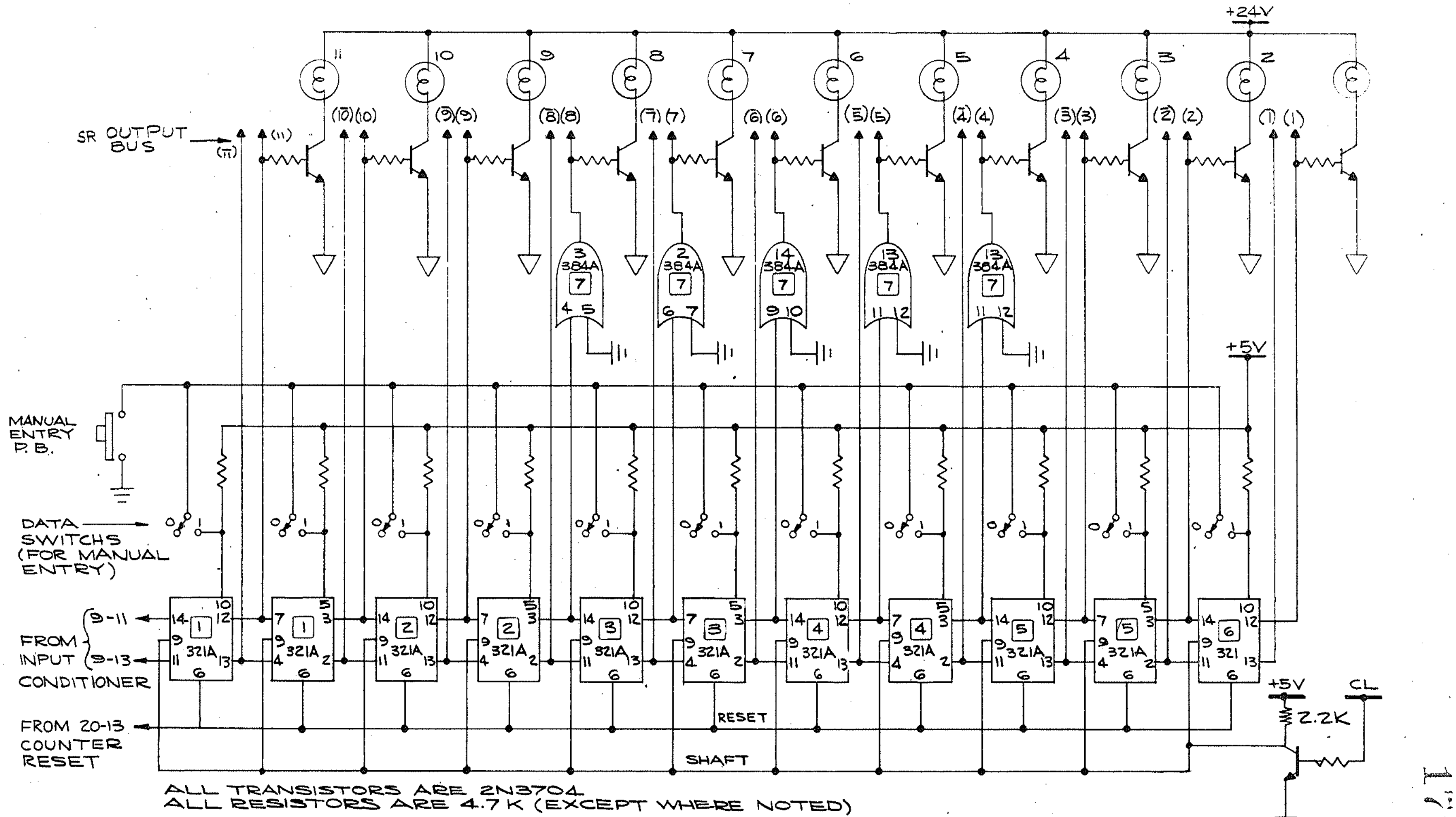
The unijunction oscillator uses a G. E. Type 5DK1 complementary unijunction transistor which has improved stability characteristics over the standard unijunction transistor. In this circuit, the emitter is connected to the RC timing circuit that has a normal period of 9.2 milliseconds. The synchronizing circuit is also connected to the emitter through a smaller (4.7K) resistor and briefly (for about 1 millisecond) applies this resistance across the timing resistor causing the circuit to reset at the synchronizing time. At other times, this synchronizing circuit appears as a very high impedance and has no effect upon the timing circuit. An additional input is included with the timing circuit which is used as an

Inhibit Clock to inhibit the operation of the oscillator during the intervals that the Shift Register must remain static.

The output of the unijunction oscillator is obtained from base #1 which is a negative going pulse that has an amplitude of 5 volts and a width of about 0.1 milliseconds. This signal is coupled to the Clock Generator through a PNP transistor. The Clock Generator (CL) output is a negative going pulse with a 2 millisecond width and is used to time all the programmer's logic detection and data transfer so that these functions will occur in synchrony and in their proper sequence. In operation, the detectors strobe the data from the Shift Register while (CL) is at "0," while the transition of "1" causes the Shift Register to accept a new data BIT.

b. Shift Register

The Shift Register (SR) circuit is detailed in Figure 46 and consists of 11 binary storage elements. Each binary element consists of a slave and master memory element. Data can be entered into the register either directly into the master memory through the direct set S_D, or reset R_D inputs or through the slave memory upon command (through the J and K inputs that are enabled with the Clock input). In each case, the $\bar{Q}$ and Q assume the logic of the S_D and R_D or J and K inputs, respectively.



Title: Shift Register			
BIOSPHERICS INCORPORATED, Rockville, Maryland	Fig. 46		
	Date	Init.	Scale
Cont. No. NASW 1731	Report Title: AMML Final Report		

In operation as a Shift Register, the J and K inputs of the binary element are connected to the previous Q and $\bar{Q}$ or the input conditioner. A shift command on the Clock input (CL) operates by disconnecting the slave from the master during the "1" signal when the slave also assumes the condition of the input. The state of the slave is transferred to the master during the "0" signal.

The program controller is designed so that data characters may be manually entered into the Shift Register for testing purposes through the use of preselected toggle switches and the manual entry push button on the front panel. This enables the user to trouble-shoot the circuitry in the event of operational difficulty. A manual reset push button is also provided to clear any data in the register.

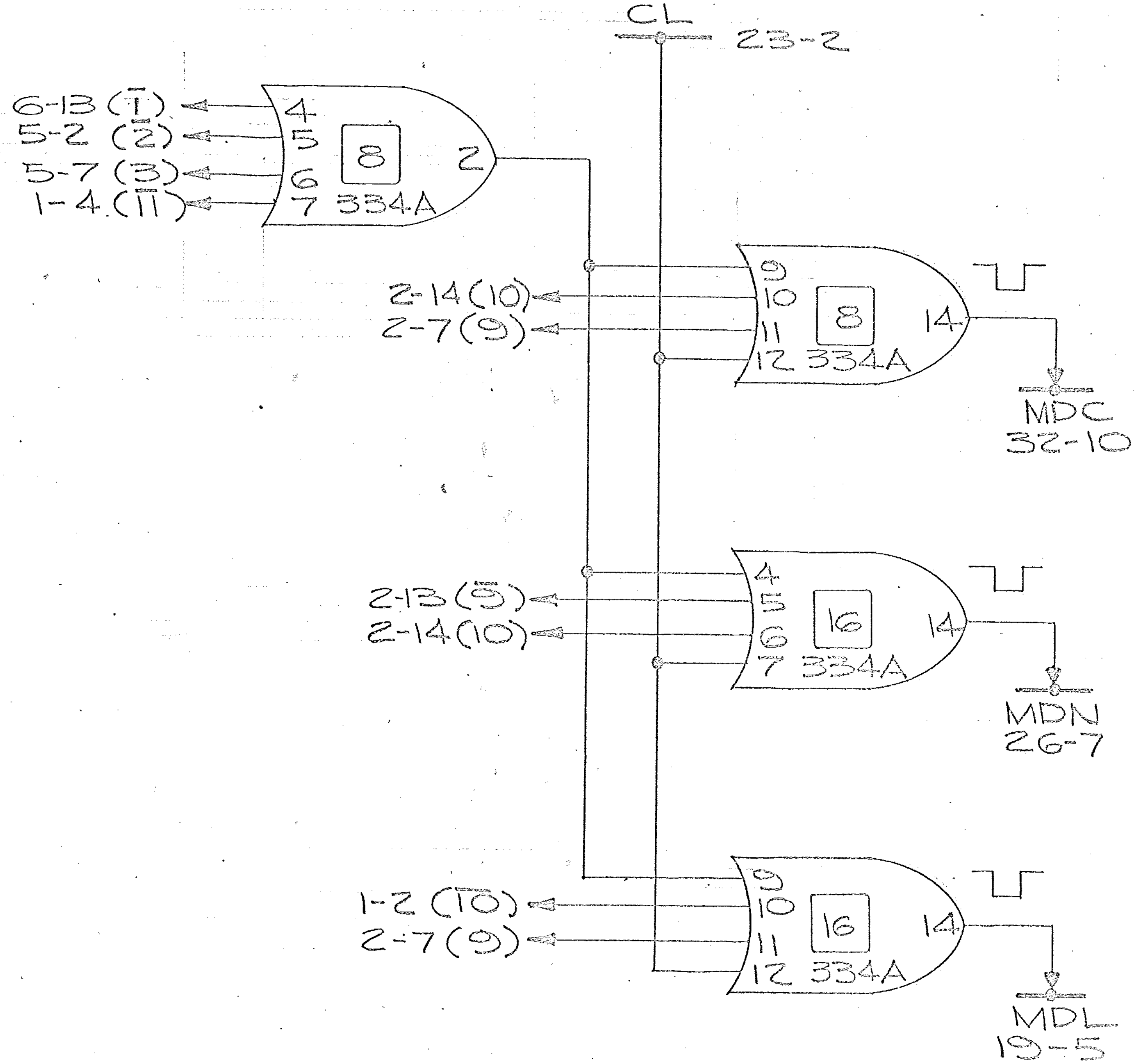
The twenty-two output lines from the Shift Register provide signals to the various detector circuits and to drivers for the front panel indicator lamps. The lines represent both the Q and $\bar{Q}$ of all the Shift Register elements and are indicated 1 and $\bar{1}$ for the output of SR #1; 2 and $\bar{2}$ for SR#2; etc. Because five of these lines are used beyond the capacity of the output of binary, additional drive current is provided with the use of a type 384A OR Gate.

c. Data Detectors

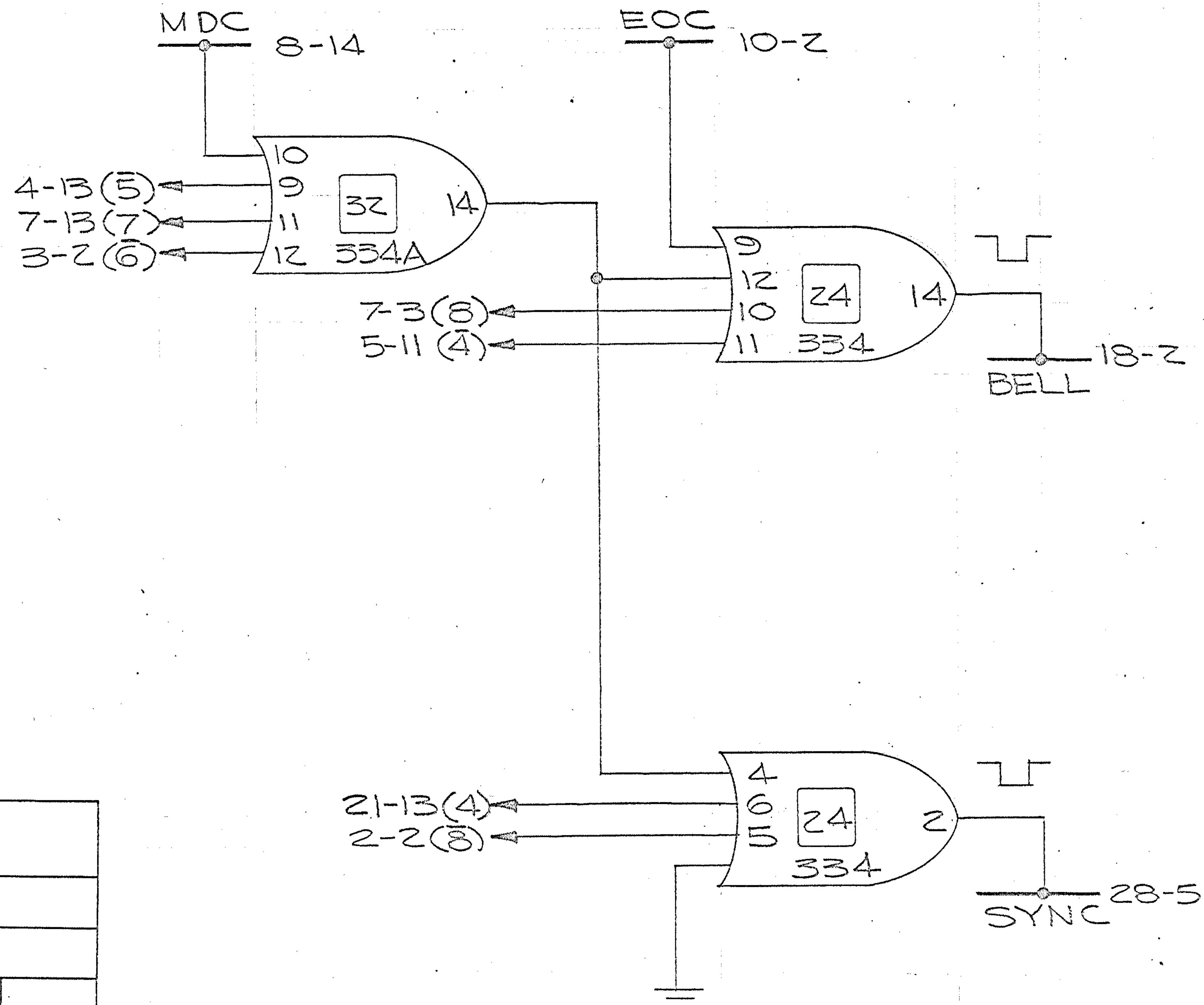
Several data detectors are included in the circuitry for the program controller. Included in these are groups of master detectors as shown in Figure 47 , the command detectors in Figure 48 , and device designation detectors in Figure 49. Each of these detectors consists of groups of OR Gates that are used in combination so as to detect the presence of a condition in which all inputs are "0."

The master detectors (MDC, MDL and MDN) examine the contents of Shift Registers 1, 2, 3, 9, 10, and 11 as well as the presence of a CL pulse. A pulse is produced at the output of these detectors whenever a control (MDC), letter (MDL), or number (MDN) symbol is present in the Shift Register.

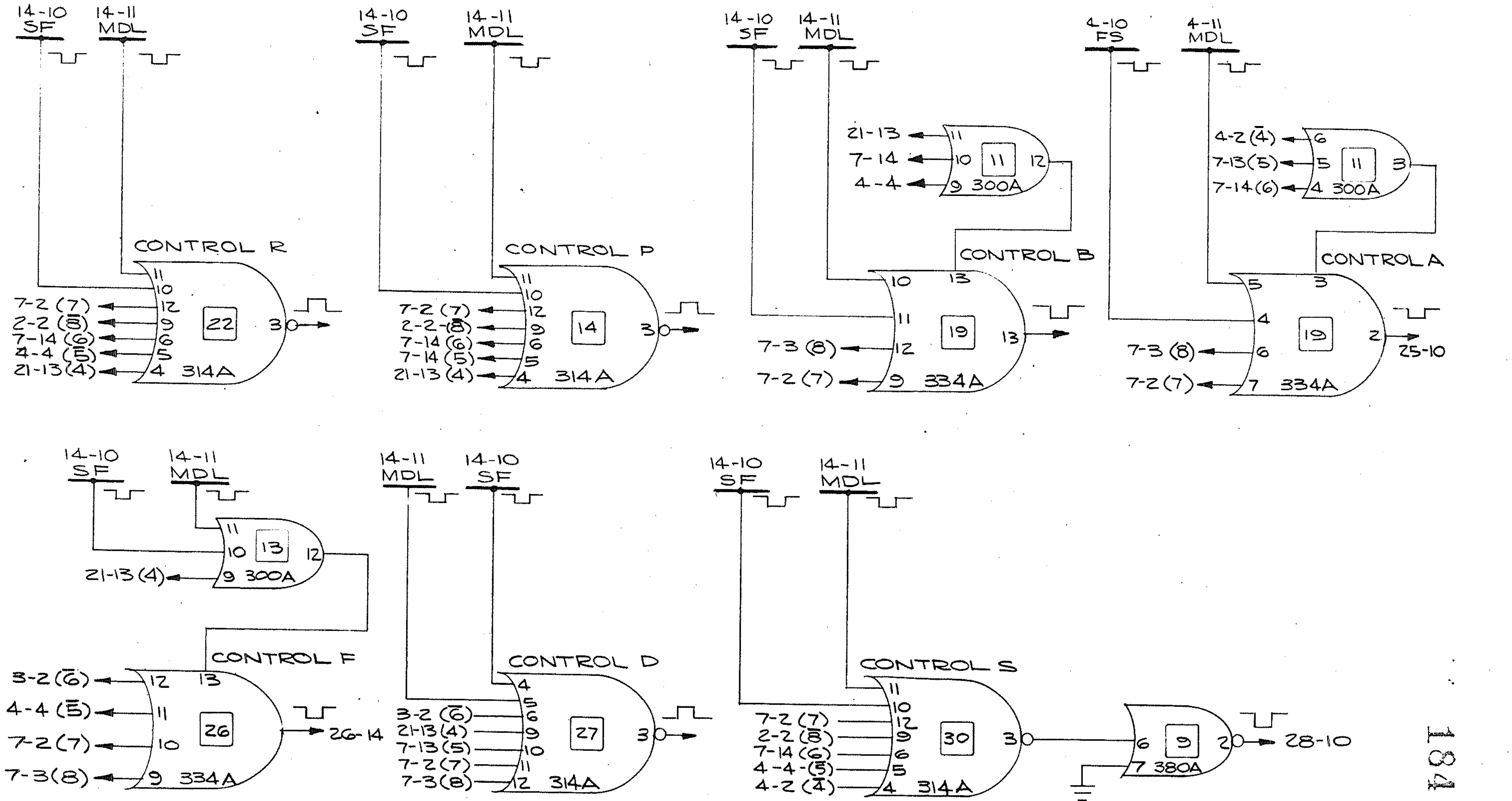
The two control detectors are used for the BELL character and SYNC character. The BELL character interrogates the status of the program controller so that if the programmer has not been reset by the termination of the previous control sequence, the tape-reader in the teletypewriter is disabled, and the readout sequence is held at this point. In the case where the program controller has been reset before the BELL character is received, the teletypewriter's tape-reader will continue without pausing. This circuit is detailed in Figure 50 .



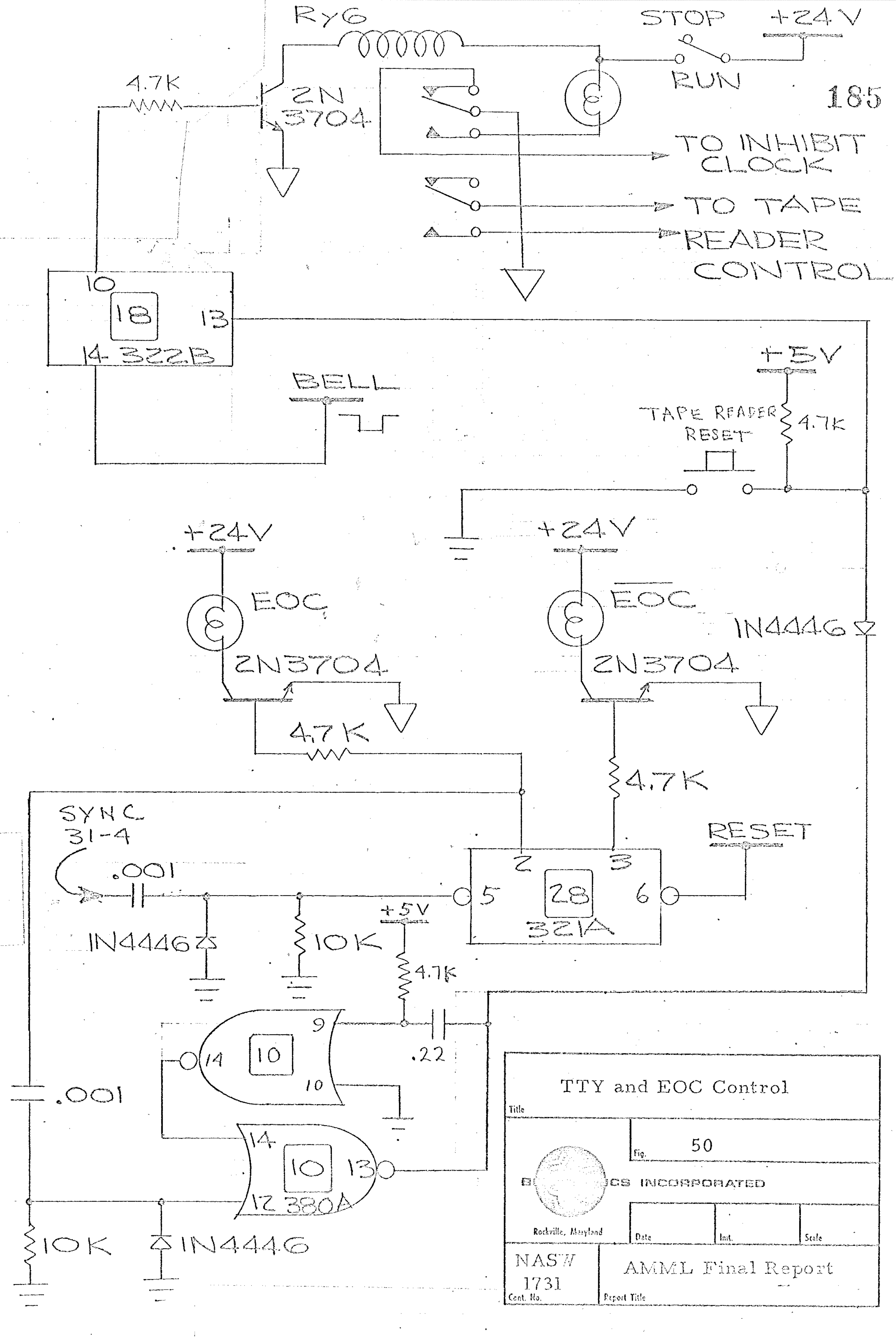
Master Detectors Title			
	47 Fig.		
B… ICS INCORPORATED			
Rockville, Maryland	Date	Init.	Scale
NASW Cont. No. 1731	AMML Final Report Report Title		



Title: Command Detectors	
Fig. 48	
BIOSPHERICS INCORPORATED, Rockville, Maryland	Date / Init. / Scale
Cont. No. NASW 1731	Report Title: AMML Final Report



Title	Device Designation Detectors
Fig.	49
BIOPHYSICS INCORPORATED, Rockville, Maryland	Date / Init. / Scale
Cont. No. NASW 1731	Report Title: AMML Final Report

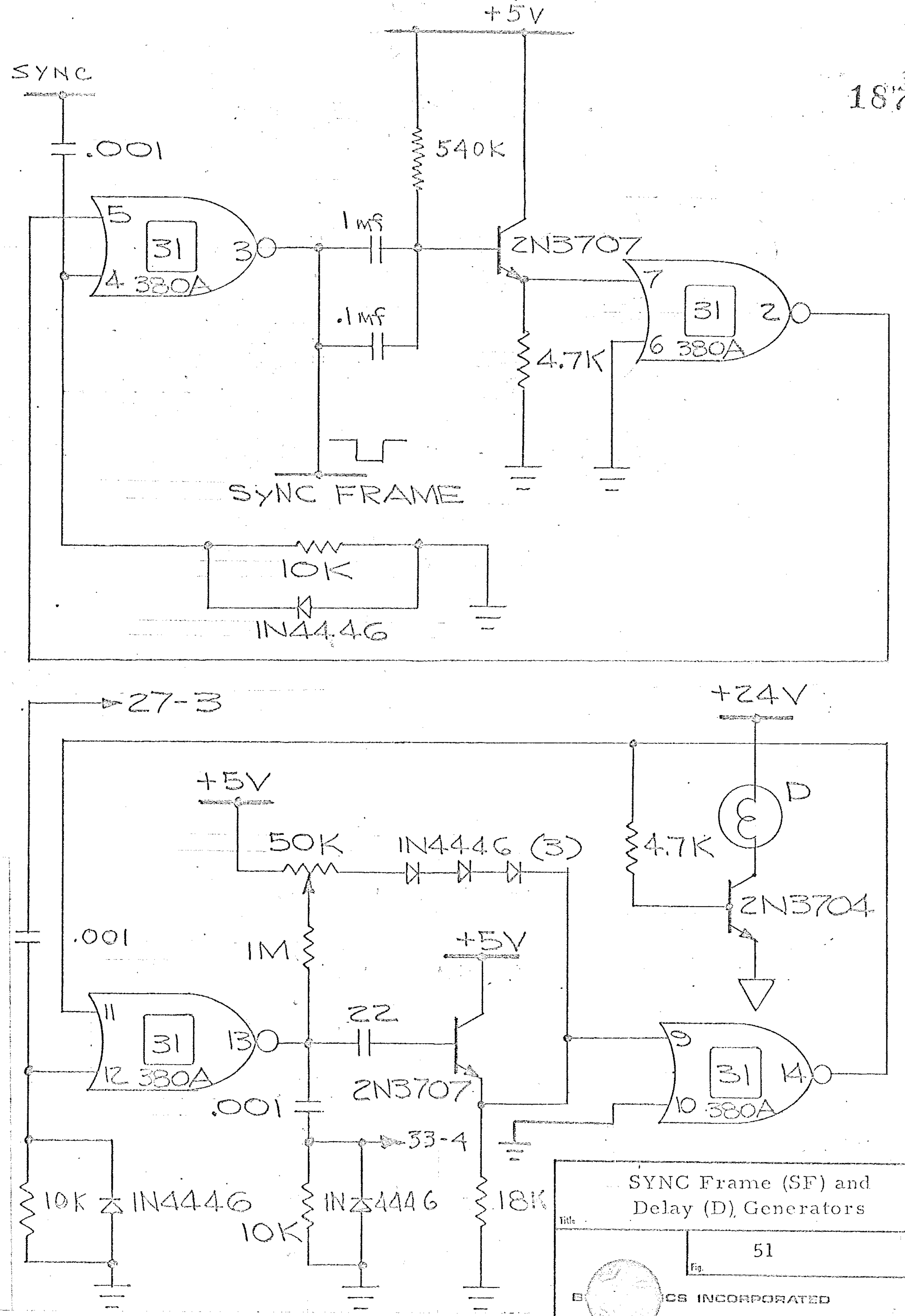


TTY and EOC Control	Title
Fig. 50	
B[illegible]CS INCORPORATED, Rockville, Maryland	Date / Inst. / Scale
NASW 1731 (Cont. No.)	AMML Final Report (Report Title)

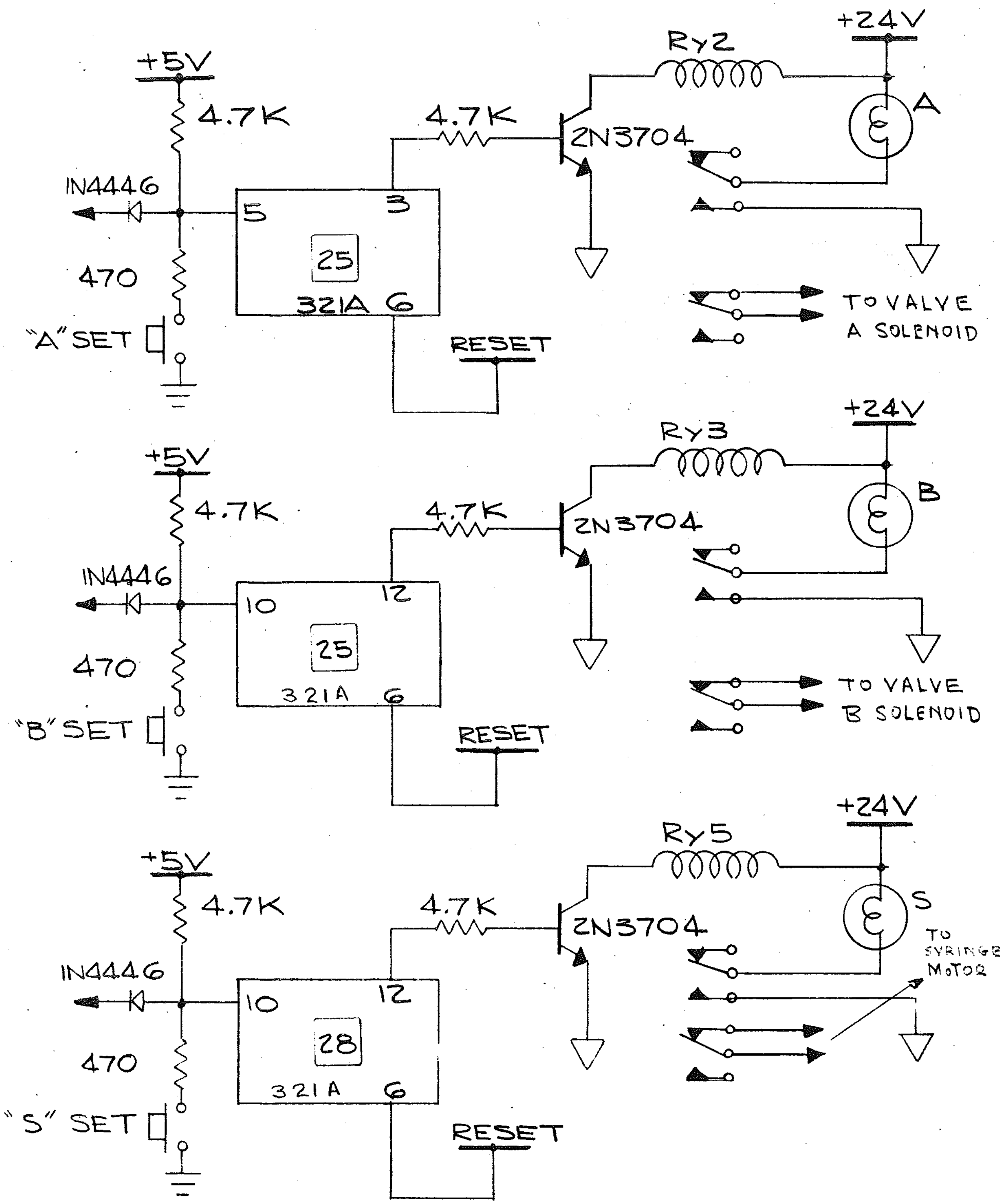
The SYNC character opens the detection gate (SYNC Frame Generator SF in Figure 51) for 0.32 seconds and enables the detection of the next three characters received. These characters are used in controlling the various devices of the AMML. The SYNC character may also hold the program by commanding the End-of-Control (EOC) element to "0." The EOC will remain in this condition until the device being programmed has completed its operating sequence. This character is used to control one of the various devices on the AMML breadboard.

In the case of the Valve A and B and the Syringe, the receipt of an "A," "B," or "S" is used to set the Direct Set (S_D) input of the associated memory element as shown in Figure 52 . The filter command from the letter "F" is coupled to a pulse generator which initiates the action of a short-time delay relay that is held in briefly while the filter mechanism is activated. A switch then holds the relay until the operation is completed. This circuit is detailed in Figure 53 . Memory elements which control relays for the radiation detector R and bioluminescence detector P can be turned ON or OFF by use of the direction control in conjunction with the P or R. This circuit is detailed in Figure 54 .

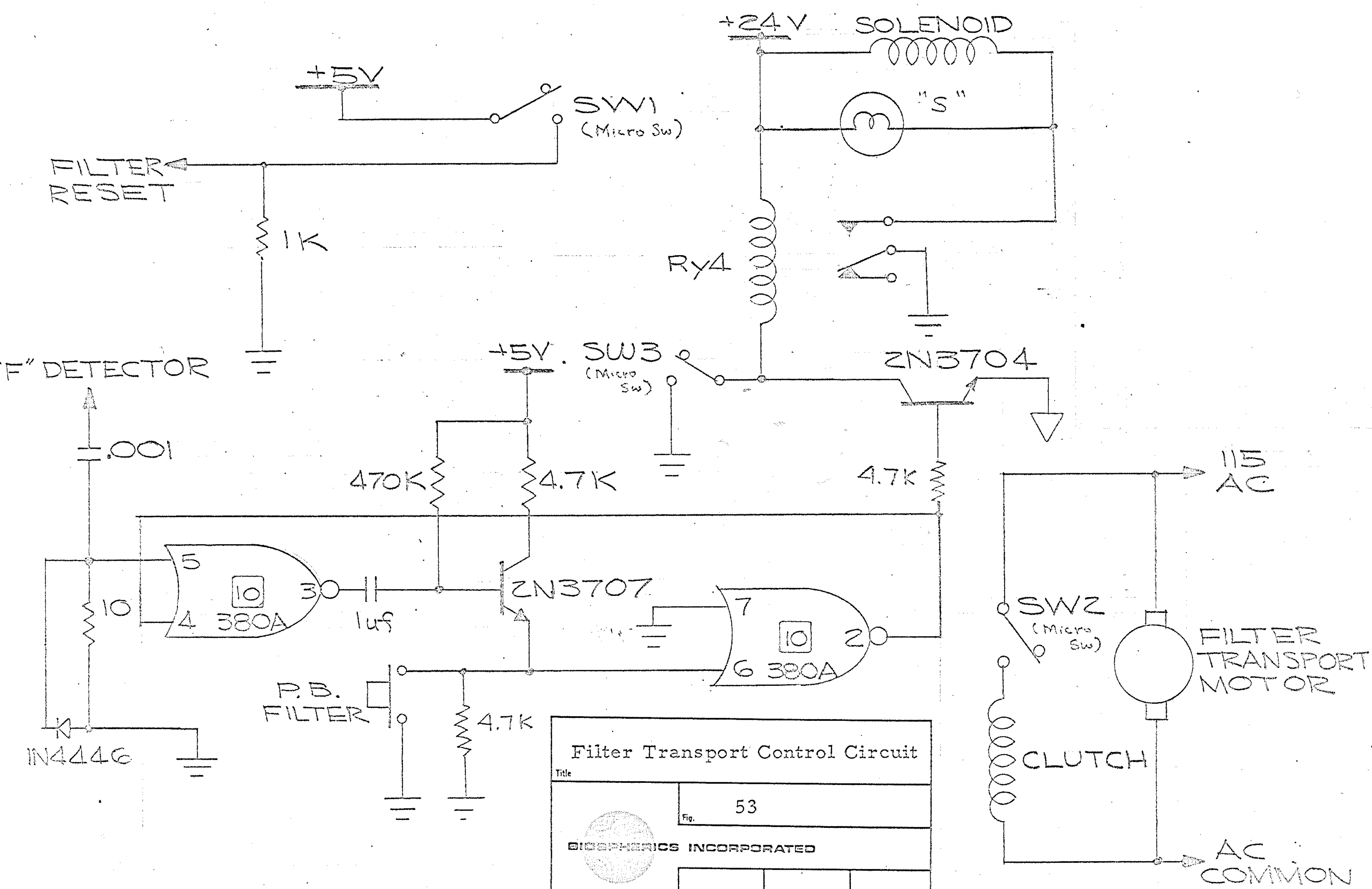
Another device character is the letter D which activates



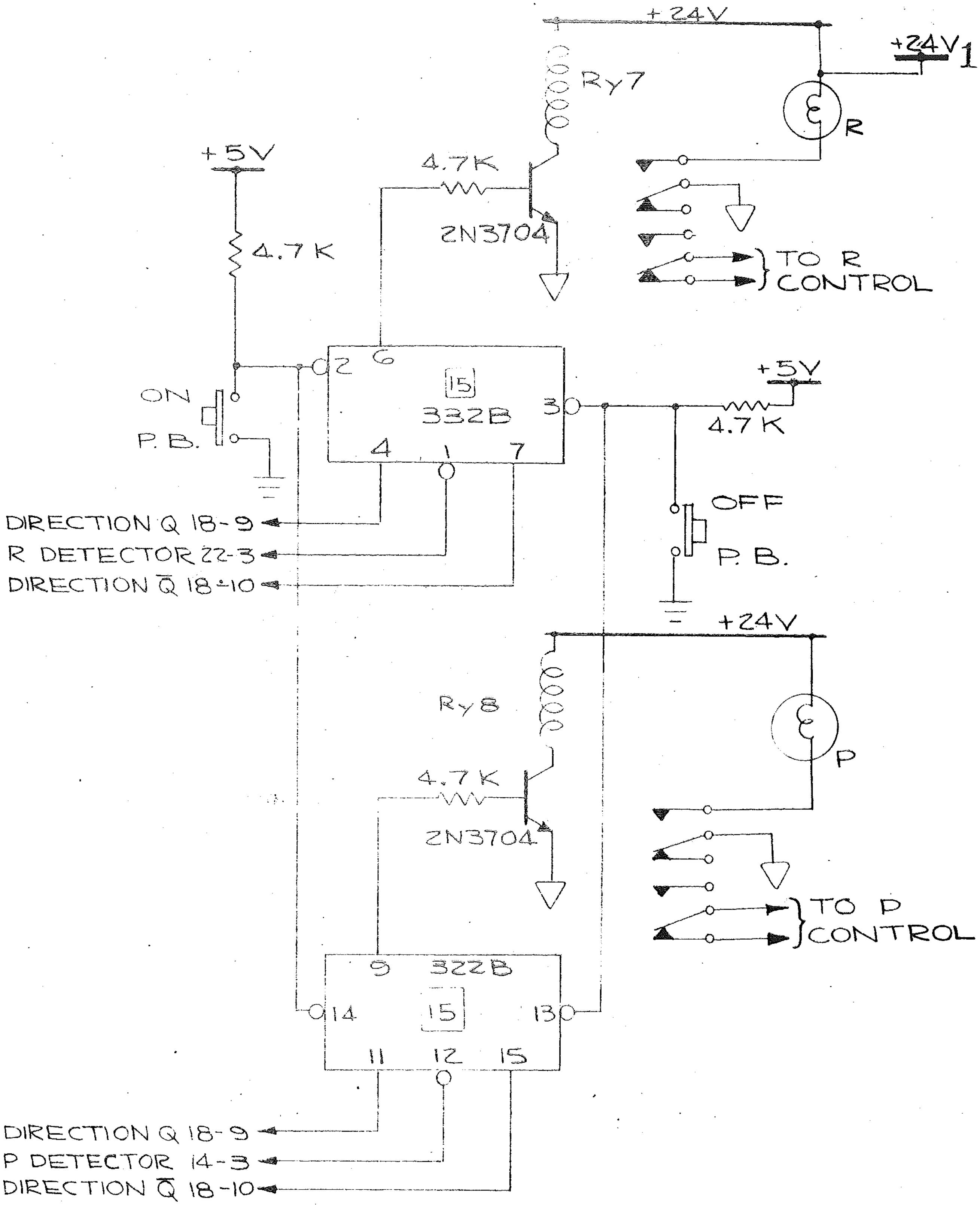
Title	SYNC Frame (SF) and Delay (D) Generators		
B[illegible]ICS INCORPORATED Rockville, Maryland	Fig. 51		
	Date	Init.	Scale
NASW Cont. No. 1731	Report Title: AMML Final Report		



Title: A, B, & S Memories and Controls			
	Fig. 52		
BIOSPHERICS INCORPORATED, Rockville, Maryland	Date	Init.	Scale
NASW 1731 (Cont. No.)	Report Title: AMML Final Report		



Title: Filter Transport Control Circuit			
BIOSPHERICS INCORPORATED, Rockville, Maryland	Fig. 53		
	Date	Init.	Scale
Cont. No.: NASW 1731	Report Title: AMML Final Report		



Title: Bioluminescence and Radiation Detector Control Circuits			
BIOPHYSICS INCORPORATED Rockville, Maryland	Fig. 54		
	Date	Init.	Scale
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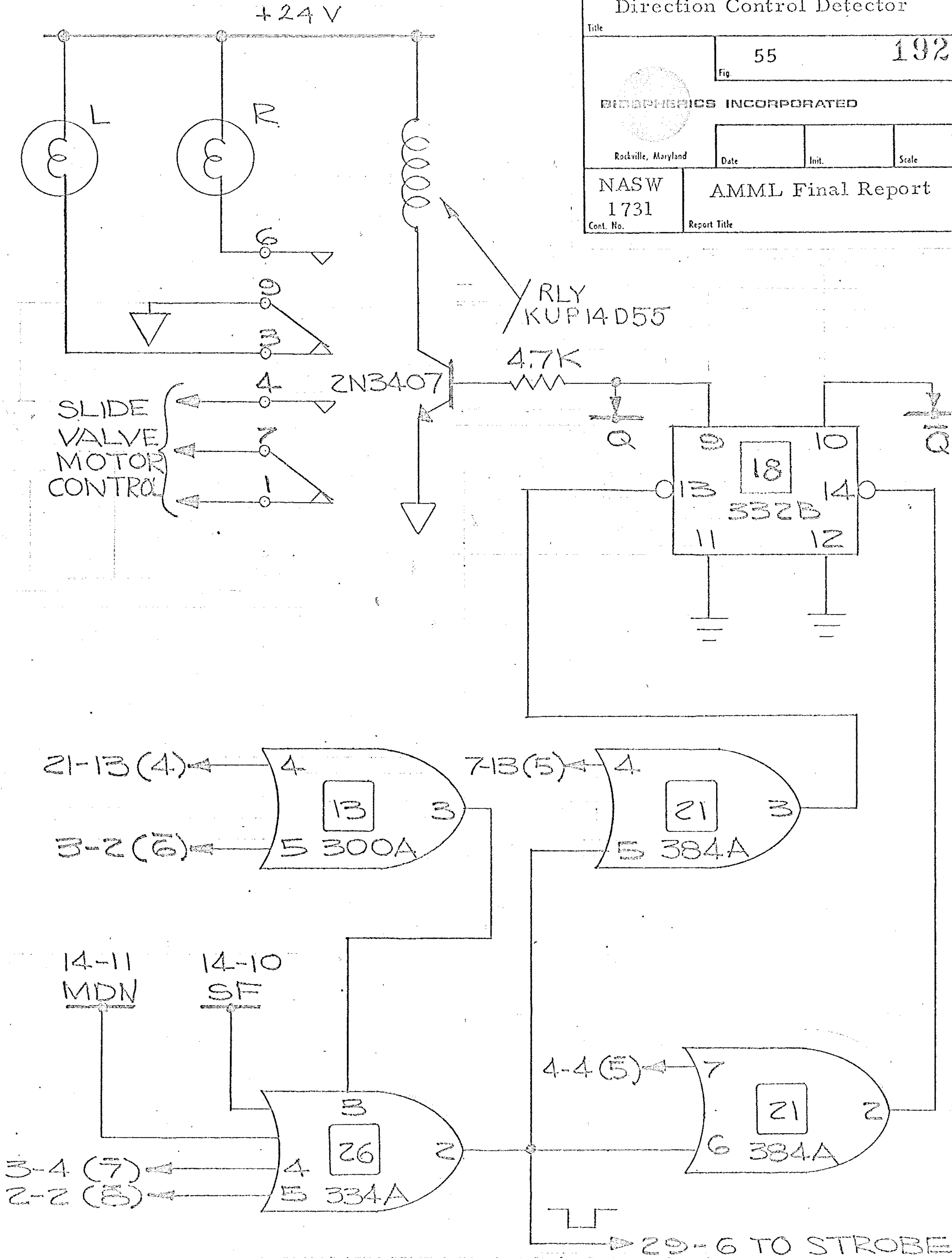
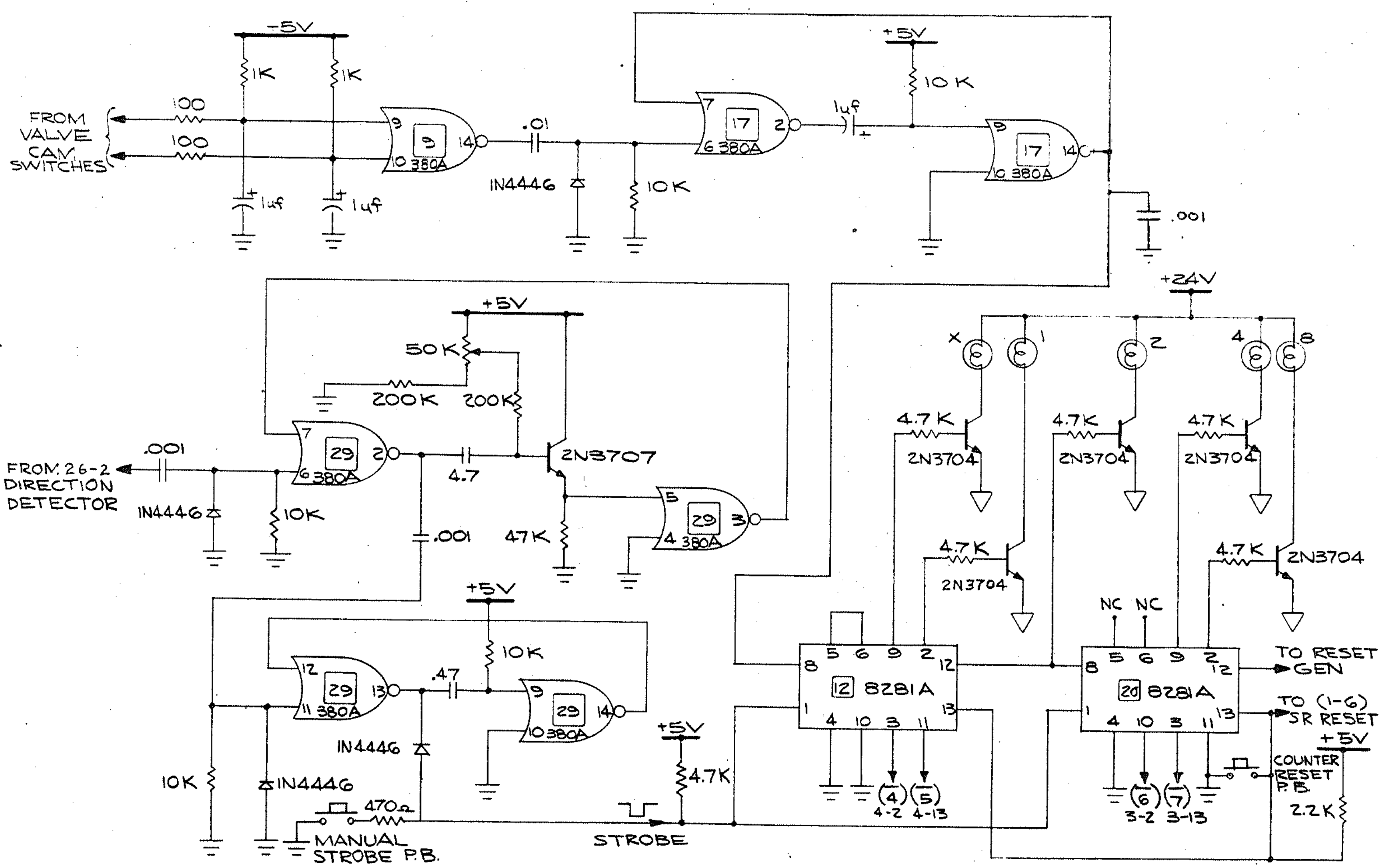
a delay in the program (shown previously in Figure 51). This delay can be adjusted over a period from 20 seconds to one minute, thereby holding the program off during this period. At the end of the delay period, a reset command is generated, and the system then proceeds on to the next program command. This delay is useful whenever a pause in the program is required for chemical reactions, mixing, or during the readout of detectors as desired.

The direction control characters (> or <) are grouped with the number symbols. These are used to command either a RIGHT or LEFT, UP or DOWN, and ON or OFF control in the program. The direction control detector is shown in Figure 55 and consists of an OR Gate array which examines the contents of Shift Registers 4, 5, 6, 7, and 8. The output of the detector is coupled to both the Direct Set (S_D) or the Direct Reset (R_D) input of a memory element that drives a control relay. The relay operates the Right and Left lamps on the front panel and also the reversible motor that actuates the slide-valve mechanism. The logical Q and $\overline{Q}$ are used as inputs to other detectors.

The number characters that follow the direction character are used to set the binary register that is shown in Figure 56 . This register is used to count the steps during the operation



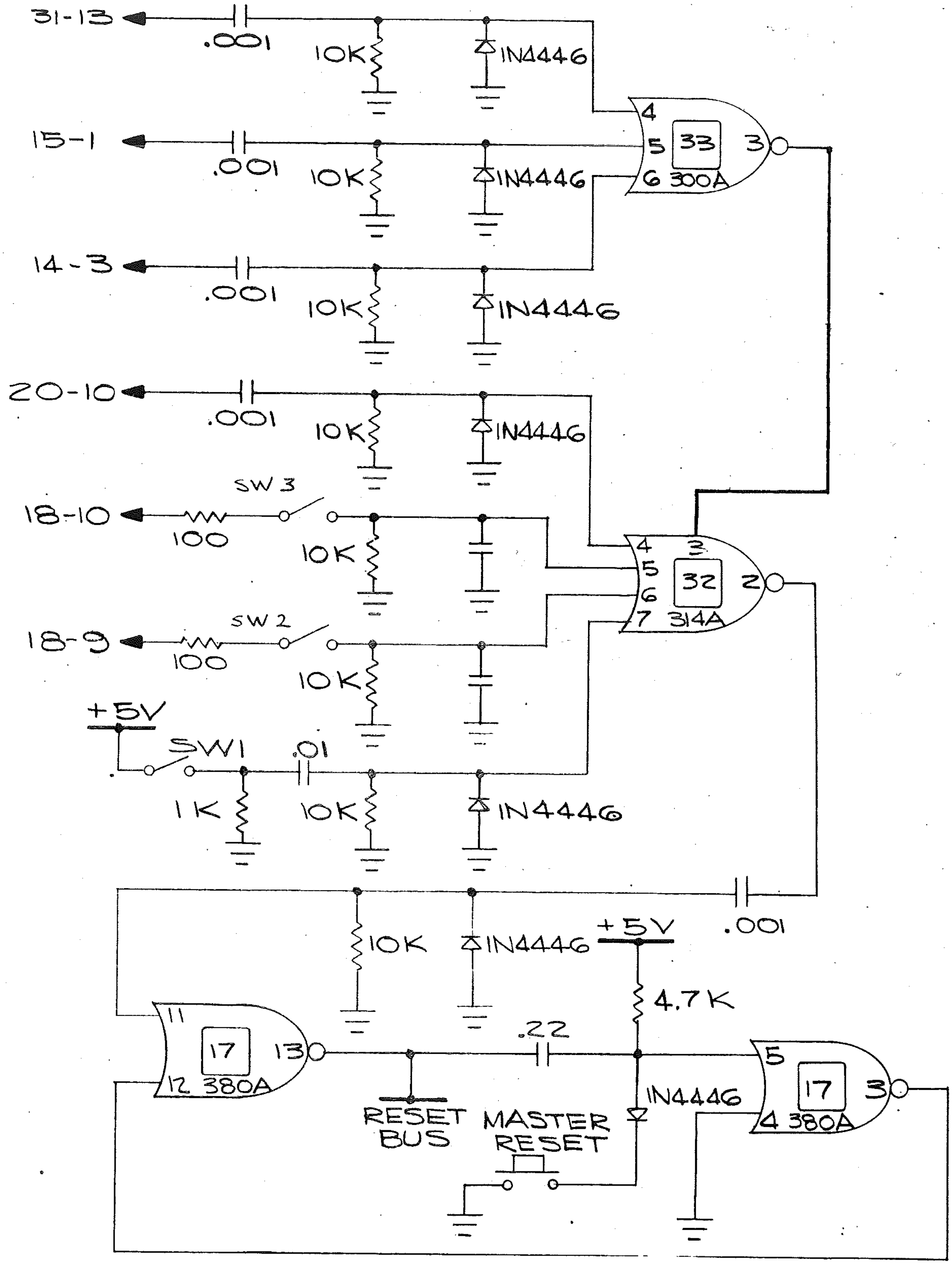
Title	Direction Control Detector		
BIOPHYSICS INCORPORATED, Rockville, Maryland	Fig. 55		192
	Date	Init.	Scale
Cont. No. NASW 1731	Report Title: AMML Final Report		



Binary Counter for Valve Control Circuit Title	
BIOSPHERICS INCORPORATED Rockville, Maryland	Fig. 56
	Date · Init. · Scale
NASW-1731 Cont. No.	AMML Final Report Report Title

of the slide-valve controls from the AMML apparatus. The first two stages of the counter predivide the input pulses that are generated by the cam microswitches on the valve controls. The next four binaries are preset from Shift Registers $\overline{4}, \overline{5}, \overline{6}$, and $\overline{7}$ during the transmission of the number character. The seventh binary is always preset to "0" and is used as an overflow register so that when the preceding binaries accumulate the required number of pulses upon overflow, this register goes to "1," and its output signals the end of the sequence and operates the reset generator. The binary counter is preset by use of the binary strobe command that is generated upon receipt of either the $>$ or $<$ character. The strobe pulse follows the direction character after a 0.1 second delay. After receipt of the strobe command, the binary counters operate in a normal fashion and accumulate the input pulses in a binary sequence.

The central control for the program completion is the generation of a reset pulse. This reset is used to deactivate all the devices that are being controlled. As shown in Figure 57, this circuit is controlled by the presence of any of seven signals: Delay termination, R control, P control, Binary Counter overflow, UP Syringe microswitch, DOWN Syringe microswitch, and the Filter transport cycle completion.



Title: Reset Pulse Generator

Fig. 57

BIOSPHERICS INCORPORATED

Rockville, Maryland

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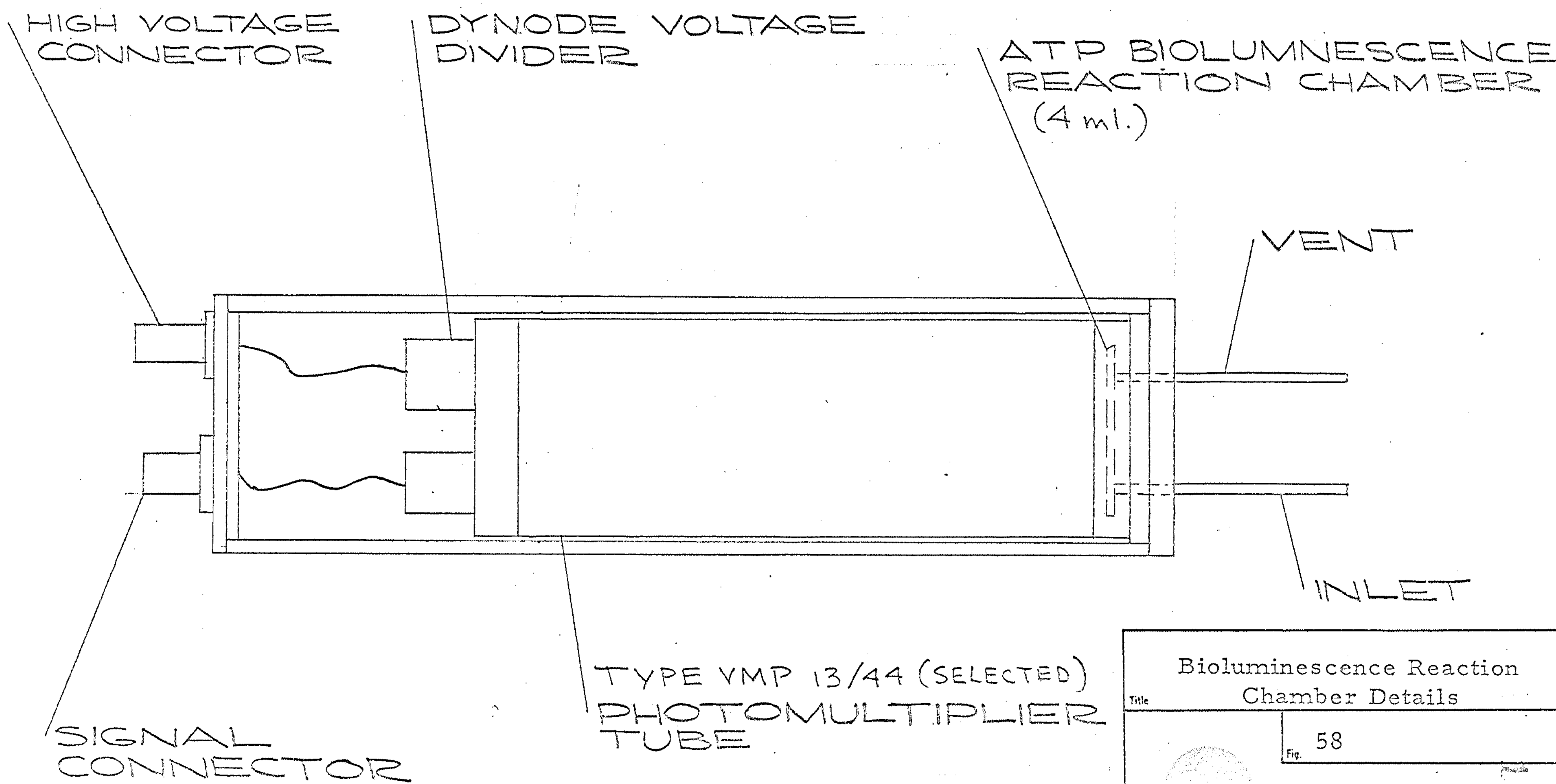
D. Sensors and Measurement Circuits

1. ATP Measurements

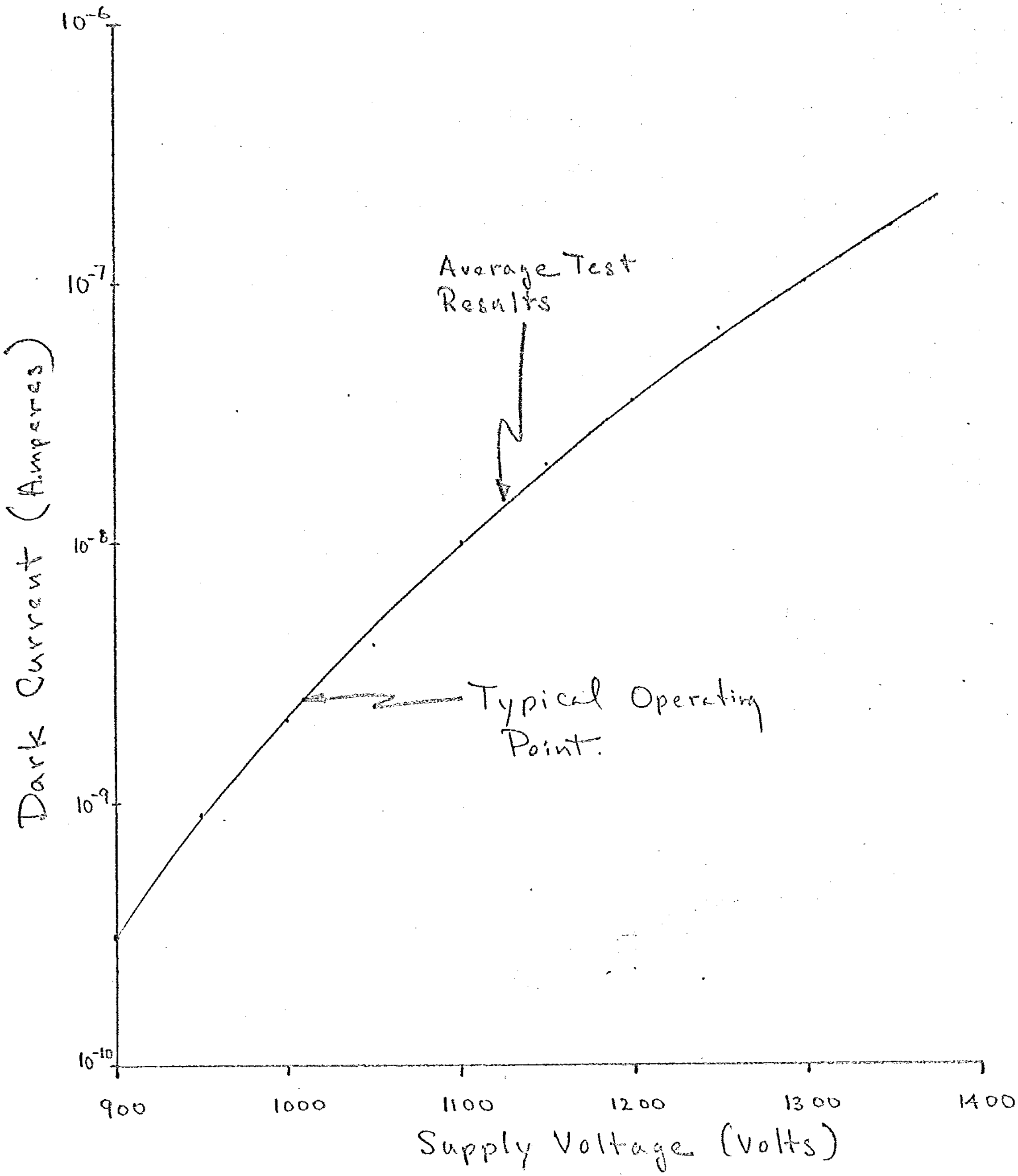
The tests for adenoisine triphosphate-specific bioluminescence are carried on in an optical reaction cell which is coupled to the Valve B manifold through small diameter teflon tubing. The chamber is coupled optically to the photocathode of the photomultiplier as shown in Figure 58. An integral reflector surrounds the reaction chamber to increase the light collection efficiency from the bioluminescence reaction on the photocathode. The chamber is fabricated so that there will be minimal liquid retention or hang-up.

In operation, the ATP reaction chamber is first filled with the enzyme in its buffer solution, and then the unknown ATP sample in its extraction solution is quickly injected into the volume. The resultant bioluminescence emission is measured by the photomultiplier and its associated electronic circuitry.

The photomultiplier used in this assembly is the Centronix type VMP 13/44K, a 13-dynode tube which has a sensitivity of 200 AMP/lumen when operated at 1330 volts. The dark current is specified as 1.5 nanoampers while operated with this sensitivity. Figure 59 shows the relationship of dark current from this photomultiplier as a function of supply voltage.



Title: Bioluminescence Reaction Chamber Details			
BIOSPHERICS INCORPORATED, Rockville, Maryland	Fig. 58		
	Date	Init.	Scale
Cont. No.: NASW 1731	Report Title: AMML Final Report		



Title: Photomultiplier Dark Current vs. High Voltage Supply			
BIOSPHERICS INCORPORATED, Rockville, Maryland	Fig. 59		
	Date	Init.	Scale
Cont. No.: NASW 1731	Report Title: AMML Final Report		

The read-out circuitry for this sensor is shown in Figure 60 and consists of several commercially available instruments that are used as a matter of convenience in this breadboard demonstration project.

2. $^{14}CO_2$ Measurements

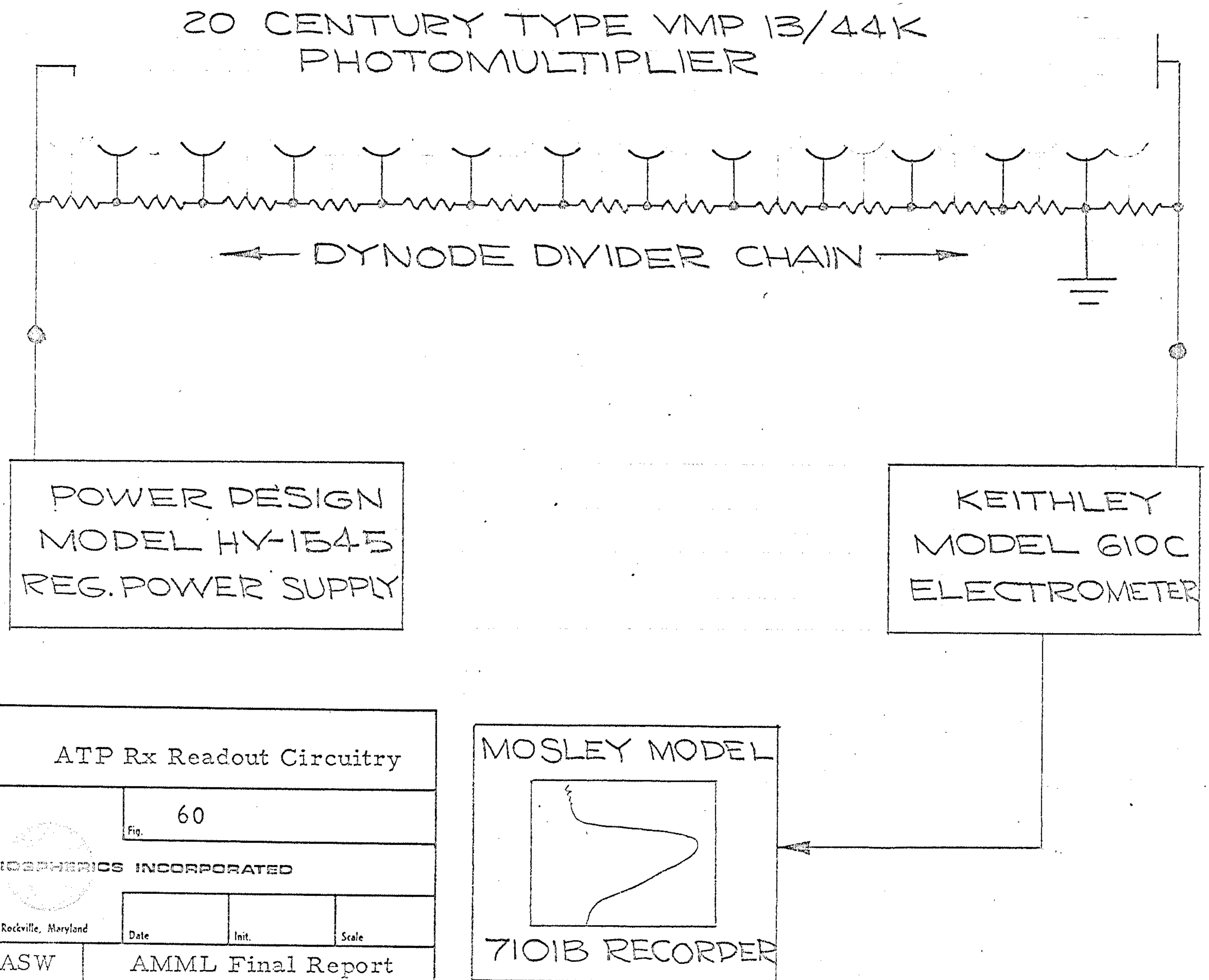
Several new techniques were investigated during this research program to develop improved schemes to measure $^{14}CO_2$. One technique consisted of a scheme to acquire and compress a sample of gas containing $^{14}CO_2$ into a small detection volume containing a solid-state detector as shown in Figure 61.

A specially sealed detector was obtained from ORTEC of Oak Ridge, Tennessee. The detector had been selected for a noise resolution of less than 9 kev. The preliminary testing of this system showed that although the scheme was feasible, it was not adaptable to the breadboard instrument.

In addition to this approach, the breadboard apparatus can also accommodate the standard Gulliver type GM-getter $^{14}CO_2$ detectors that have been used successfully in previous development programs.

3. ^{14}C and ^{35}S Measurements

The apparatus to assay beta radiation from the filter of the residual ^{14}C and ^{35}S will use either a solid-state detector such as the ORTEC type BA-020-300-100 surface



Title	ATP Rx Readout Circuitry
Fig.	60
BIOSPHERICS INCORPORATED, Rockville, Maryland	Date / Init. / Scale
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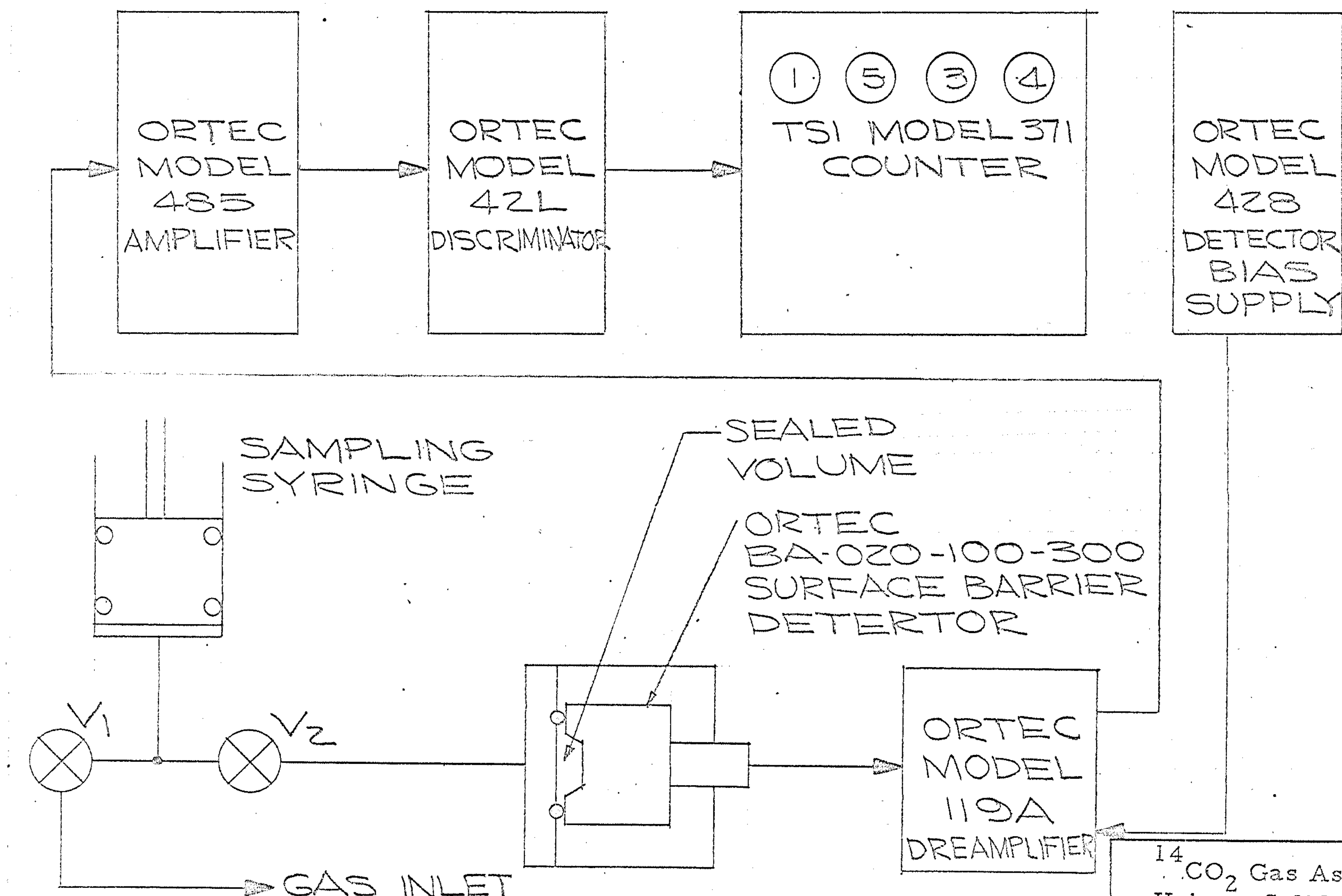

Title: $^{14}CO_2$ Gas Assay Technique Using a Solid-State Detector			
CS INCORPORATED, Rockville, Maryland	Fig. 61		
	Date	Init.	Scale
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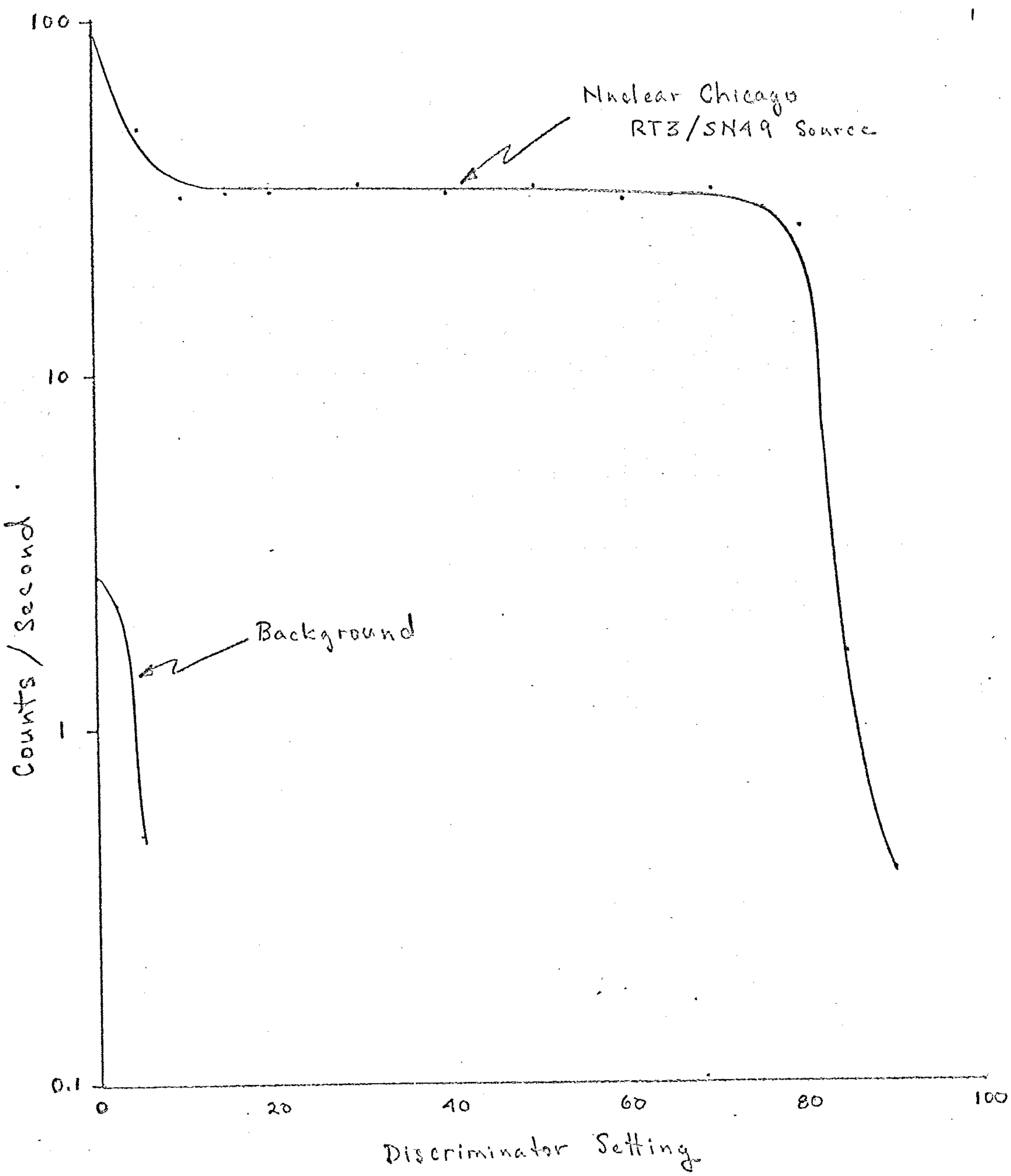
barrier detector or a GM counter. Preliminary tests of the operating characteristics of the ORTEC detector have been performed with test equipment previously shown in Figure 61 and preliminary tests are shown in Figure 62 . Additional results from this development indicate that this system can be operated with a discriminator setting of 26 kev, with about 15% detection efficiency of the ^{14}C activity from the filter tape with a background rate less than 1 count per second.



Title: Tests of the ORTEC Surface Barrier Detector	
BIOSPHERICS INCORPORATED, Rockville, Maryland	Fig. 62
	Date / Init. / Scale
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V. The AMML Testing Program

Upon completion of the AMML instrument, the apparatus was tested with several sample programs that reflected the best programming information available at this time. A set of instructions has been developed for each assay that will provide the basic operational program. As biological and operational data is obtained in future programs, these instructions must be modified to reflect these changes. The present operational instructions are described in the following:

A. The ATP Assay

1. Prepare the program tape to provide for the necessary steps of adding enzyme, buffer, extractant (or ATP standard) to the optical reaction chamber, as well as the cycle to remove the fluids from the chamber and rinse it. This is similar to the program described previously in Figure 41.

2. Test the operation of the breadboard apparatus with the program tape to verify its operation and that the sequence is written correctly.

3. Use hand-operated hypodermic syringes to test the photomultiplier/optical reaction chamber combination with small quantities of enzyme and an ATP standard to

obtain a rough calibration of the sensitivity of the detection system. After initial responses are obtained, increase the quantities of the reagents to the amount used in the instrument so as to verify the operational sensitivity of the breadboard apparatus in the test cycle.

4. Integrate the optical reaction chamber to the breadboard apparatus and perform tests to provide a standard curve to the limit of sensitivity with standard ATP samples at various concentrations (i.e., 10^{-2}, 10^{-3}, 10^{-4}, etc. gamma per 0.01 ml).

5. Using a known culture which has been assayed for ATP concentration, use the extraction cycle from the test program to remove the ATP from the organisms that are deposited on the filter in the AMML breadboard.

6. Repeat #5, except substitute cultures taken from the AMML growth chambers that have previously been inoculated with known test soil samples.

B. The ^{35}S and ^{14}C Uptake Measurements

1. Prepare a program tape similar to the above which will prepare a filter with microorganisms from a growth medium that have been cultured with the type of nutrients required for the AMML operation. The program should include a filtration cycle, a drying cycle, and a counting cycle.

2. Test the program and breadboard apparatus in a simple operation that consists of filtering organisms on standard millipore filter disks. Manually remove these from the apparatus for drying and counting by the standard laboratory instruments.

3. Using sections of filter tape, repeat the filtering cycle above to deposit the microorganisms on the filter tape. Allow the program to transfer these into the drying apparatus of the AMML breadboard, and then into the beta detection chamber for assay of ^{14}C and ^{35}S.

4. Repeat step 3, except use microorganisms that are taken from the AMML growth chambers that have been inoculated with known soil samples.

C. The Phosphate Uptake Determination

1. Prepare the program for the operation of the AMML breadboard apparatus for the mixing of the triethylamine (T. E. A.) and aliquots of the test sample; perform the precipitation to dry and count the filter and to wash the apparatus.

2. With distilled water substituting for the reagents, verify the operation of the apparatus.

3. Verify the mixing of the T. E. A. and subsequent precipitation of standard phosphate solutions.

4. Combine step 3 with a filtration cycle, using standard millipore filters.

5. Repeat the cycle of step 4, except use labeled T. E. A. and add a filter change cycle to remove the filter. Then transfer it manually to the laboratory dryer and counter for verification of the precipitation procedure.

6. Substitute aliquots of liquid from the AMML growth chambers for the phosphate standards and repeat step 5.

7. Test step 5 with short sections of filtering tape and add operation of the AMML dryer and ^{14}C assay cycle to the program.

8. Combine step 6 with step 7.

D. Integrated System Testing

Prepare a program tape which combines the three tests that are described in A, B, C above. The system should then be tested using this program to verify the operation of the complete breadboard apparatus for all of the assays.

VI. Recommended Future Program

The Automated Microbial Metabolism Laboratory research and development program has uncovered a number of problem areas that need further studies. The suggested future research program that is described below will require the use of the AMML feasibility instrument. As these studies continue, the instrument will be refined further so as to incorporate improvements and modifications that result from the program.

A. Light Fixation-Dark Release Test

Further refinements should be made in the apparatus and techniques required for this assay in order to increase the precision and obtain the maximum in sensitivity. It is recommended that a leakproof exposure chamber be designed and fabricated for laboratory studies. The design should permit the use of various gas flushes to investigate the removal of nonbiologically fixed $^{14}CO_2$.

Additional biological studies should be conducted on a variety of soils once satisfactory precision and background levels have been achieved. Determination should be made on the sensitivity, effects of added moisture and the effects of type and duration of light exposure. The optimum sample size as well as amount and specific activity of added carbon dioxide should be determined in order to obtain a favorable signal-to-noise ratio. Bard-Parker germicide should be tested further to ensure that it will act as a satisfactory antimetabolite in this assay.

B. ^{14}C and ^{35}S Uptake Test

Nonbiological retention of labeled substrates by the filter materials and soil particles has resulted in a high background level and had an unfavorable effect upon the sensitivity of this test. Further studies are required to select a filter material and to develop a wash regimen which will lower the nonspecific absorption of the isotopes to a more desirable level.

Biological studies of the ^{14}C and ^{35}S uptake tests should be conducted to measure the number of cells which can be detected by this test and determine the photosynthetic component of the uptake. Further experimentation will be required to determine the applicability of Bard-Parker germicide and to gain experience in the assay of different soil types with this test.

C. Phosphate Uptake Test

Serious inadequancies in the application of the colorimetric and radioisotopic phosphate assay procedures have been found in the current laboratory research program. Further analytical method development will be required to apply these techniques to soil extracts. In addition, alternate analytical methods should be examined as candidates for the phosphate assay.

Additional biological studies of soils and the photosynthetic nature of their phosphate uptake are required. Further testing of Bard-Parker germicide for this assay would be desirable.

D. Firefly Bioluminescent ATP Assay

The relationships of response to volume ratio of reaction mixture to extractant, and to total volume should be studied in greater detail. Such studies are a requisite for determining the optimal volumes to be used in the AMML.

VII. LITERATURE CITED

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