GPO PRICE $ _____

CFSTI PRICE(S) $ _____

Hard copy (HC) _3.00_

Microfiche (MF) _.65_

ff 653 July 65
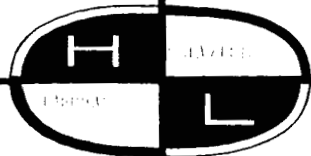
N67 16503

(ACCESSION NUMBER)

FACILITY FORM

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HAZLETON LABORATORIES

INCORPORATED • FALLS CHURCH, VIRGINIA

MONTHLY PROGRESS REPORT NO. 1

A STUDY TOWARD DEVELOPMENT OF AN AUTOMATED
MICROBIAL METABOLISM LABORATORY

Contract No. NASW-1507

Submitted to

National Aeronautics and Space Administration
Washington, D. C.

November 1, 1966

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I. INTRODUCTION AND SUMMARY

This is the first monthly progress report under Contract
No. NASW-1507 on "A Study Toward Development of an Automated Microbial
Metabolism Laboratory." The objectives of the current effort include the
development of life detection systems based on phosphate uptake and sulfur
uptake together with an examination of some of the engineering problems
associated with the instrumentation of these and related experiments.
The ultimate plan, not all of which is within the current program scope,
is the development of a "life detection subsystem" consisting of the
following metabolic experiments: 1. metabolism of radioactive sub-
strates, 2. heterotrophic photosynthesis, 3. autotrophic photosynthesis,
4. ATP production, 5. phosphate uptake, and 6. sulfate uptake. In
addition, various physical parameters will be measured on the Mars sur-
face such as light intensity, temperature, pH of soil, oxygen
concentration, background radioactivity and phosphate content of soil.
The final product will be a package of experiments integrated into a
single instrument which could serve as a "minimum biological payload"
or as the metabolic experiment subsystem of a "fully automated biological
laboratory."

Conventional phosphate assays have been studied this period as
well as an assay which measures phosphate levels in terms of counts
derived from ^{14}C-labeled triethylamine. In the former case, sensitivities
down to 3 µg/L of phosphate-P were observed. The ultimate sensitivity has
not yet been determined for the radioisotopic assay.

As in the Gulliver project, it is imperative that an almost universal growth medium be developed for this program. However, sensitivity in measurement of phosphate uptake will require a low phosphate medium which constitutes the background against which the measurement must be made. Attempts have been made to adapt the previous media, M9 and M11, by decreasing phosphate levels. In the initial phase of the work, cell cultures are being grown in more optimal media and then subcultured in the deficient media, after a period of conditioning in phosphate-free media.

When a high cell population is incubated in either M9 or M11 with low phosphate (KH_2PO_4 decreased from 1.0 g/L to 0.2 g/L), there is observed a dramatic decrease in the phosphate level of the extracellular solution within a few hours, indicating phosphate uptake by the cells.

The engineering effort this period has been concerned with the design of liquid processing systems, a study of the proposed single read-out system and a tentative investigation of photomultiplier tube selection.

II. PROGRESS DURING THE PERIOD

A. BIOCHEMISTRY

1. Metabolic Uptake of Phosphate

As discussed in the technical proposal, phosphorus is essential to the metabolism of all known forms of life. Every biological reaction is ultimately dependent upon phosphorus for energy conversion and transfer [1]. Furthermore, all known organisms are believed to be fastidious as to the form in which phosphorus may be accepted from the environment, as orthophosphate [2]. The chemical fact of the high-energy storage capacity of resonant bonds polymerizing phosphate ions makes phosphorus a strong candidate for a role in almost any conceivable form of extraterrestrial life.

In the thesis research of one of us [3], it had been shown that not only did microorganisms take up phosphate during growth phases, but they even take up phosphate in the absence of growth. Roberts, et al., [4] have also demonstrated rapid uptake of orthophosphate by Escherichia coli; significant amounts of ^{32}P-labeled orthophosphate were incorporated into the cells at 0° within three minutes.

On Earth orthophosphate is essential to both aerobic and anaerobic metabolism. It is reasonable to hypothesize that even in the low oxygen (or zero oxygen) environment on Mars, organisms will still utilize phosphate.

The experiment, then, would consist of supplying a suitable aqueous medium to a sample to be tested. An aliquot of the liquid phase would be removed by filtration and assayed for orthophosphate. This would establish the initial orthophosphate level contained in the medium and added by the introduction of the sample. At periodic intervals thereafter, aliquots would be similarly removed and assayed for orthophosphate. The uptake of orthophosphate by the test culture as opposed to no uptake or attenuated uptake demonstrated in a suitably inhibited control would be evidence for metabolism.

2. Orthophosphate Assay Procedures

Four types of analytical methods for determining the levels of orthophosphate have been under consideration: conventional methods (akin to the Fiske-Subbarow technique [5]), a triethylamine-phosphomolybdate method, a ^{14}C-triethylamine-phosphomolybdate method, and a method based on converting orthophosphate to adenosinetriphosphate (ATP) and then determining the ATP concentration by a bioluminescent technique.

3. Conventional Phosphate Assays

The conventional methods for the determination of phosphate suffer from at least one disadvantage as far as this program is concerned: the spectrophotometric read-out is not compatible with the other related experiments (e.g., Gulliver). However, the conventional

methods are essential in establishing the validity of novel methods of analysis and in preliminary experiments.

a. Ammonium Molybdate-Stannous Chloride Method

Based on the procedure in Standard Methods for the Examination of Water and Wastewater (6), the following ammonium molybdate-stannous chloride method has been developed:

Reagents: I. Ammonium molybdate - 10% aqueous ammonium molybdate $.4H_2O$ is diluted 1:3 with 50% H_2SO_4.

II. Stannous chloride - 400 mg. of $SnCl_2 \cdot 2H_2O$ is dissolved in 100 ml. of 10% HCl (prepared daily).

III. Phosphate standards - Stock solution (1000 mg. phosphate-P/L) contains 4.3916 g. of anhydrous KH_2PO_4 in 1000 ml. A few ml. of $CHCl_3$ are added as a preservative. Dilute solutions (down to 1 µg/ml) are stored in the refrigerator.

Procedure: I. Add 10 ml. of sample or standard to assay tube.

II. Add 0.1 ml. of ammonium molybdate reagent and swirl.

III. Immediately add 0.1 ml. of stannous chloride reagent and swirl.

IV. After 12 to 15 minutes at room temperature, read vs. reagent blank in Bausch and Lamb Spectronic 20 at 650 mμ in a 3/4 inch cell against a reagent blank.

Alternatives: When larger amounts of assay solution are desired, 100 ml. are placed in 250 ml. Erlenmeyer flasks and 1.0 ml. of each of subsequent reagents added. Originally, a water blank was used instead of a reagent blank, and a correction factor had to be applied to each reading.

Results: A typical response curve is given in Figure No. 1 and indicates reasonably good sensitivity down to about 25 μg. of phosphate-P per liter. Sensitivity could be extended by the use of a longer light path.

b. Ammonium Molybdate-Hydrazine Sulfate Method

Based on the procedure of Bruice, et al. (7), the following method has been tested:

Reagents: I. 5 N H_2SO_4

II. 0.15% hydrazine sulfate (w/v, distilled water)

III. 2.5% ammonium molybdate (.4 H_2O) (w/v, distilled water)

Procedure: I. To 5 ml. of sample in 10 ml. volumetric flask, add 2 ml. of 5 N H_2SO_4.

- 6 -
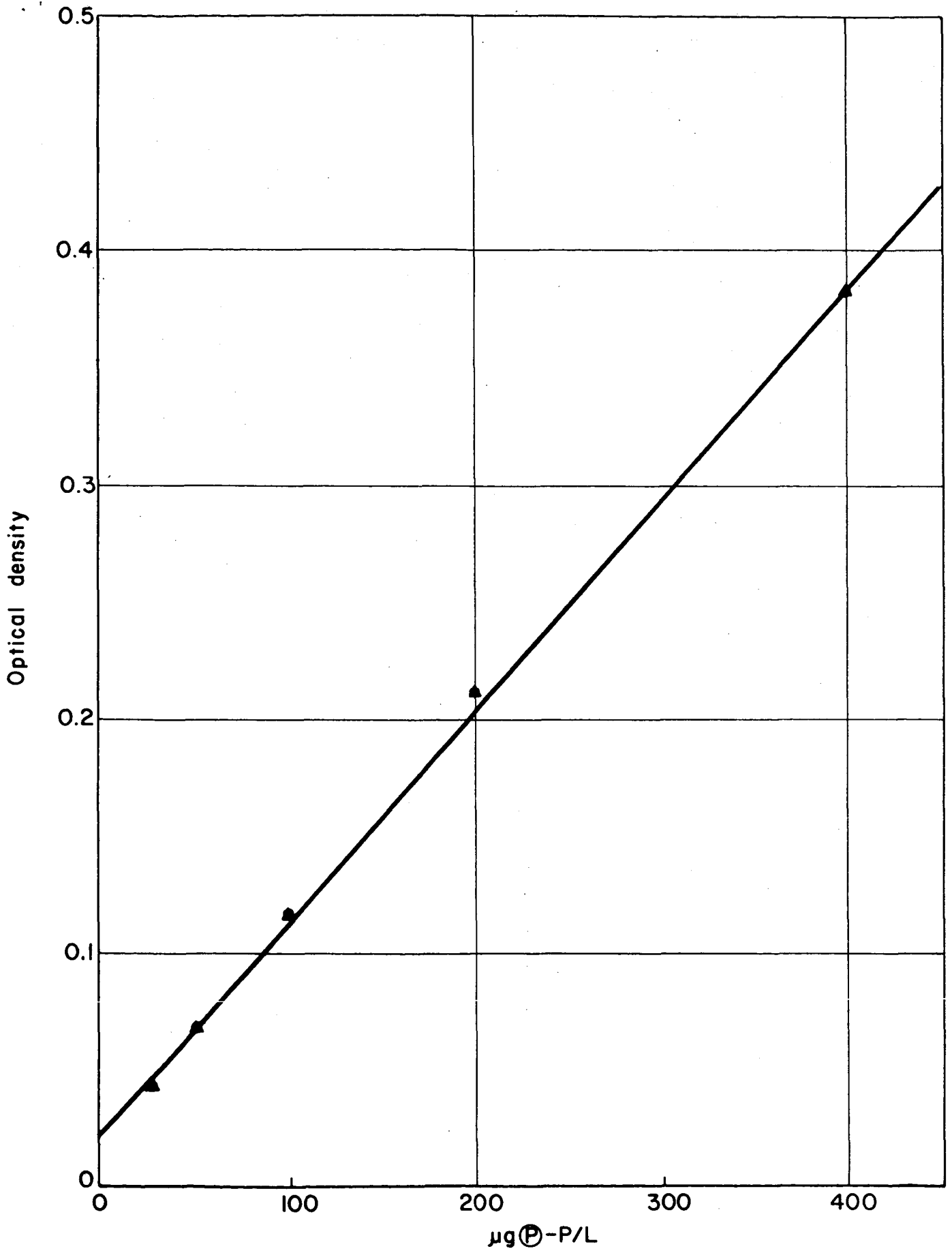
Figure No. I — Phosphate -P standard curve, ammonium molybdate-
stannous chloride method.

II. Add 0.5 ml. of hydrazine sulfate reagent

and 1.0 ml. of the ammonium molybdate

reagent.

III. After filling to volume with distilled water and

mixing, transfer to tube. Boil for 10

minutes, cool and read in 4 cm. cell in

Beckman DB at 730 mμ against a reagent blank.

Results: A typical curve is included in Figure No. 2 and

indicates reasonably good sensitivity even at

5 μg. phosphate-P per liter.

c. Ammonium Molybdate-Aminonaphthol Sulfonic Acid Method

Based on the procedure of Sunderman and Sunderman (8), the

following method has been tested:

Reagents: I. 5 N H_2SO_4

II. 2.5% ammonium molybdate .4 H_2O (w/v) in

distilled water

III. Aminonaphthol sulfonic acid reagent:

To 19.5 ml. of 15% sodium bisulfite (w/v)

in a glass-stoppered cylinder are added

0.5 g. of 1,2,4-aminonaphthol sulfonic acid.

After adding 5 ml. of 20% sodium sulfite

(w/v), the flask is shaken until the sul-

fonic acid goes into solution. (The reagent

is stable for one month in the refrigerator.)

1.0

o·········o Ammonium molybdate-hydrazine
sulphate method

▲————▲ Ammonium molybdate - aminonapthal
sulfonic acid method

0.5

0.6

Optical density

0.4

0.2

0

0   10   20   30   40

μg℗-P/L

Figure No. 2 — Phosphate—P standard curves by two
conventional methods.

Procedure: I.   To 15 ml. of sample or standard in a 25-ml.
                  volumetric flask are added successively
                  2.5 ml. of 5 N H_2SO_4, 2.5 ml. of ammonium
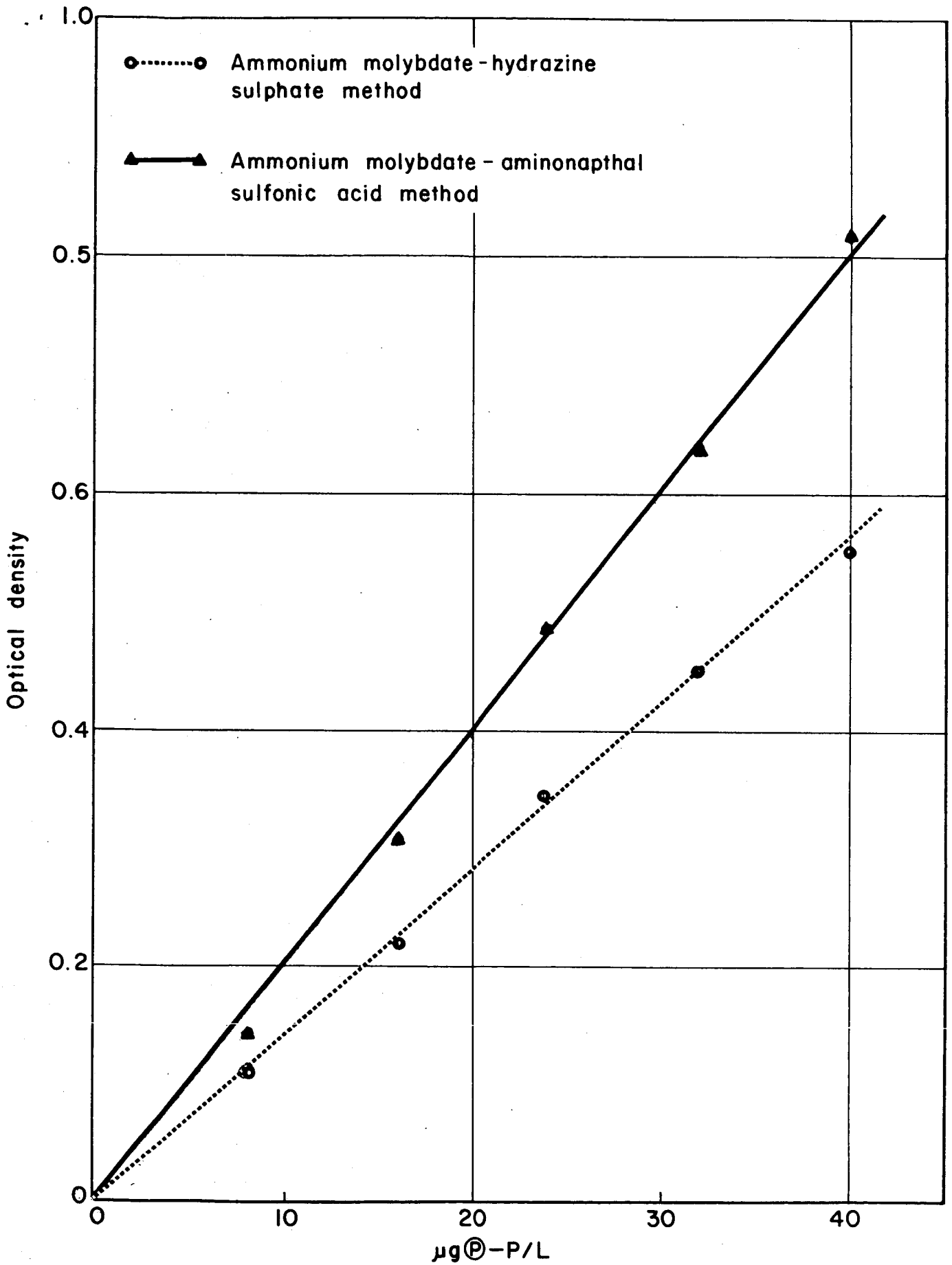   molybdate solution and 1.0 ml. of the aminon-
                  aphthol sulfonic acid reagent.

            II.   After filling to volume with distilled water,
                  the mixture is allowed to stand for 5 minutes
                  at room temperature and then read, against a
                  reagent blank, in a 4 cm. cell in the Beckman
                  DB spectrophotometer at 700 mμ.

Results: A typical curve is included in Figure No. 2 and shows
           sensitivity of about 3 μg. phosphate-P per liter.

It should be noted that the first described method was measured
in 1 cm. cells, whereas the latter two methods were measure in 4 cm. cells.
Nevertheless, the third method is more sensitive than the second which is
more sensitive than the first, even after multiplying the O.D. readings
of the first method by 4.

4.   Specific Precipitation of Orthophosphate

        Sugino and Miyoshi (9) have described a variation on the
ammonium molybdate method of determining the concentration of ortho-
phosphate which depends on the formation of a phosphate-triethylamine-
molybdate precipitate rather than the formation of a colored complex,
as in the above more conventional methods. Not only is this method

not sufficiently sensitive (it is good to 5×10^{-6} M phosphate), but it would not be practicable in our program. However, there is the possibility that substitution of ^{14}C-labeled triethylamine for the unlabeled material may yield a technique which is sufficiently sensitive to measure phosphate uptake by a few microorganisms and which would have a compatible read-out with the other experiments in the program.

Reagents: I. 4 N perchloric acid

II. 0.08 M ammonium molybdate

III. 0.8 M triethylamine hydrochloride

Procedure: I. To 1.0 ml. of sample or standard, add successively 0.05 ml. of 4 N perchloric acid, 0.25 ml. of 0.08 M ammonium molybdate and 0.05 ml. of triethylamine hydrochloride.

II. After a few minutes at room temperature, the precipitate is removed by centrifuging at 1500 g. for 5 minutes.

III. The concentration of phosphate in the supernatant is determined by one of the conventional methods; the total amount of phosphate in the precipitate is determined by dissolving the precipitate in 2 ml. of 1 N ammonia, then diluting 1:500 with water and using a conventional assay to determine phosphate recovery.

Results: Typical results are summarized in Table No. 1.

Table No. 1 - Phosphate recovery in precipitation
method

CONC. OF PHOSPHATE IN STARTING SOLN.	RECOVERY OF PHOSPHATE IN PPT
0.10 mg/L	0.08
0.20	0.21
0.40	0.42
0.60	0.60
0.80	0.76

The concentration of phosphate in the supernatant was too low to be detected by the ammonium molybdate-stannous chloride method.

5. Radioisotopic Assay for Orthophosphate

The obvious methods for radioactive assay of orthophosphate use the isotope ^{32}P. However, the relatively shoft half-life (14.3 days) precludes the use of this isotope in a Mars flight. However, it should be possible to utilize the long-lived isotope, ^{14}C, by quantitatively precipitating phosphate with ^{14}C-triethylamine hydrochloride in the Sugino-Miyoshi procedure. High specific activity triethylamine should lead to the most sensitive results, but so far only low activity has been available.

Reagents: I.   4 N perchloric acid

   II. 0.08 M ammonium molybdate

   III. ^{14}C-triethylamine

As received, the triethylamine had a specific activity of 1.1 millicuries per millimole. There were 7.8 millicuries per ml. and 7.1 millimoles per ml. The contents were diluted 1:100 with water to elute them. This solution was stored in the refrigerator; it contained 78 μC/ml or 1.7×10^8 DPM.

Procedure: I.   The ^{14}C-trimethylamine solution was further diluted 1:10 with 0.8 M triethylamine hydrochloride (unlabeled). To 1.0 ml. of varying

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concentrations of phosphate were added 0.05 ml. of 4 N perchloric acid, 0.25 ml. of ammonium molybdate and 0.05 ml. of the labeled tri- ethylamine hydrochloride.

II. After a few minutes at room temperature, the tube was centrifuged at 1500 g. for five minutes.

III. The supernatant was placed in a planchet and 1 ml. of solution containing ammonium molybdate and perchloric acid was added to the centrifuge tube.

IV. After resuspending the precipitate, it was centrifuged again. The supernatant was added to the original planchet and the precipitate to a second planchet. After evaporating the solutions in the planchets to dryness, they were counted in a Widebeta gas proportional counter (with an efficiency of approximately 30%).

Results: Typical results for the precipitate are shown in Figure No. 3. The limits of sensitivity are difficult to determine on this curve, and the experiment will be repeated with longer counting times. The supernatant had counts of about 4,000 CPM, indicating that there
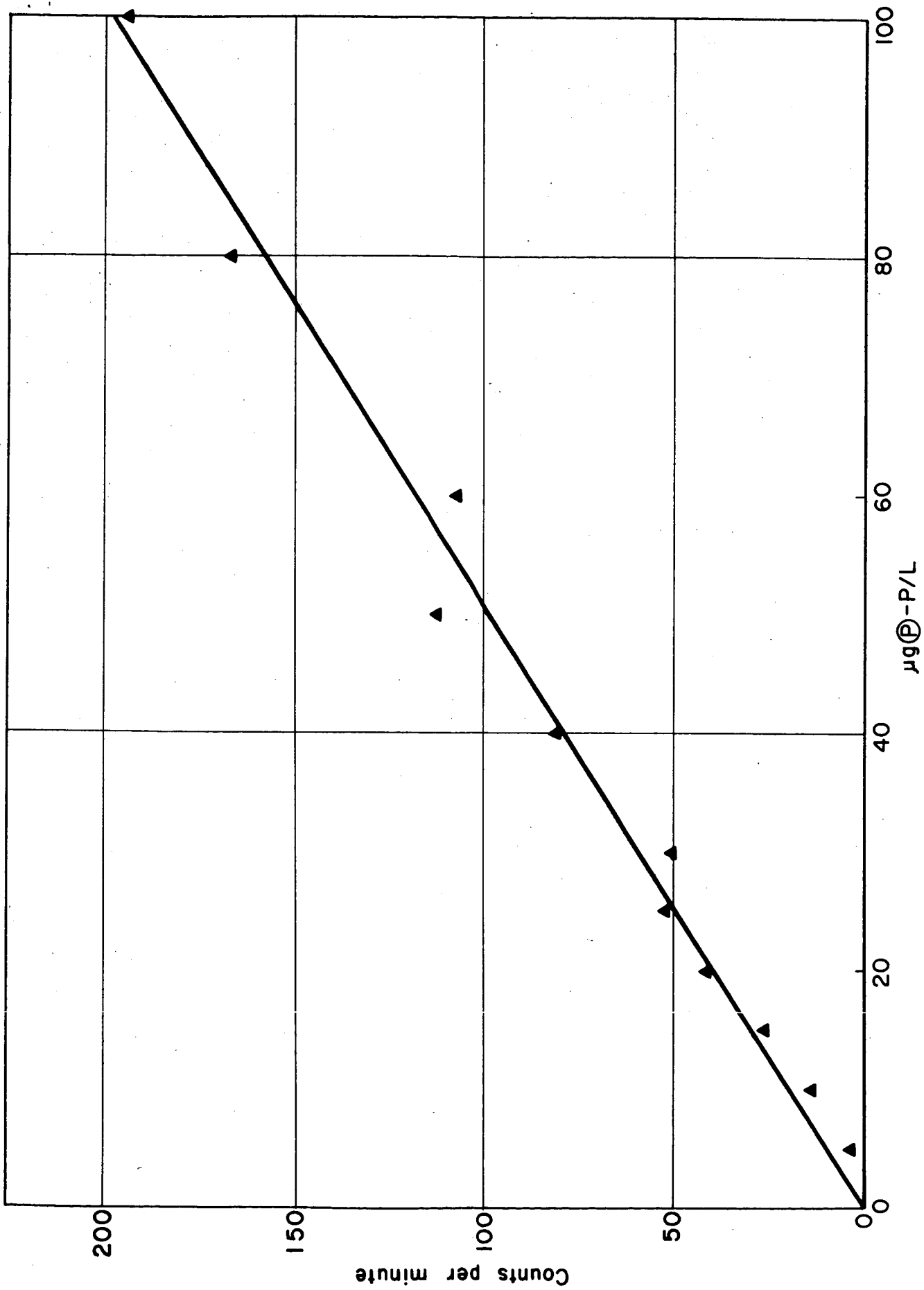
Figure No. 3 – Phosphate – P standard curve using ^{14}C – triethylamine

may have been too great an excess of triethylamine
hydrochloride for these low levels of phosphate.

6. Growth Medium

As in the Gulliver project, it is necessary to use an almost
"universal" growth medium, i.e., a medium in which almost all micro-
organisms will grow to some extent. Obviously, this "universal" growth
medium will not be optimal for all, or most, or even possibly any of
the organiams. As a starting point, some experiments were done using
media M9 and M11 developed previously (see Tables No. 2 and No. 3).
These media are not well suited for phosphate uptake experiments since
they each contain high amounts of phosphate (1.0 g/L). It is a well-
established principle that a small difference between two large numbers is
more difficult to detect than that same difference between two small num-
bers. Thus, to detect the phosphate uptake of a few microorganisms, it
will be necessary to have a medium with a low phosphate concentration.
It will, of course, have to be demonstrated that the low phosphate medium
will sustain metabolism sufficiently for the experiment. As a first step,
media M9 and M11 were used, merely decreasing the concentrations of phos-
phate and adding glucose. The latter was added as a carbohydrate source
to promote additional phosphate uptake through substrate phosphorylation.
Since growth of E. coli was limited on such a medium, later experiments
have depended on the technique of growing the organisms on a more optimal
medium (Difco Lactose Broth), depleting phosphate reserves by placing the
cells in a phosphate-free solution, and then transferring the cells to

Table No. 2 – Composition of M9

K$_2$HPO$_4$ 1.0 g/L

NH$_4$NO$_3$ 0.2 g/L

MgSO$_4 \cdot$7H$_2$O   0.2 g/L

NaCL   0.1 g/L

*Soil Extract   100.0 ml.

pH 7.0

*Soil extract prepared by suspending 500 g. of
air-dried soil in 1300 ml. H$_2$0. Mixture is
autoclaved for one hour, filtered, and liquid
loss made up to 1000 ml. with distilled
sterile water.

Table No. 3 - Composition of M11

K_2HPO_4	1.0 g/L
KNO_3	0.031 g/L
$MgSO_4 \cdot 7H_2O$	0.2 g/L
NaCl	0.1 g/L
Malt Extract	0.19 g/L
Beef Extract	0.19 g/L
Yeast Extract	0.81 g/L
Ascorbic Acid	0.013 g/L
L-cystine	0.044 g/L
Bacto-casamino Acid	0.25 g/L
Proteose peptone #3	1.25 g/L
Soil Extract[*]	16.0 ml/L
NH_4NO_3	0.19 g/L

*Same treatment as for M9

phosphate-low M11 or M9 for the uptake experiments. Obviously, it is not proposed to use this system in the final process, but it is convenient in the preliminary experiments which are required in order to establish the most sensitive assay system.

7. Phosphate Uptake by E. Coli

In a typical experiment, an inoculum of E. coli cells is added to sterile medium. While incubating at 37°, aliquots are removed at various time periods, filtered through a Gelman GA-6 Metricel filter (0.45 micron) to remove bacteria and the phosphate level determined on the filtrate after suitable dilution. The zero time readings have represented approximately 10 minute readings, but in the more recent work, the zero time aliquots have been removed in less than one minute. The phosphate assays have been by conventional methods (by the ammonium molybdate-stannous chloride method unless otherwise indicated). Almost all of the experiments have involved duplicate flasks incubated at 37° with shaking and duplicate flasks incubated at 37° without shaking. The inoculum for the shaking flasks was taken from a culture which had been shaken and unshaken cultures were used to supply inoculum for the non-shaking.

As a check on the reliability of methods, M9 medium with a 0.5% glucose supplement was inoculated with E. coli and incubated for 24 hours at 37° with and without shaking. Filtered aliquots were assayed for phosphate-P at zero time and after 24 hours, both by the ammonium molybdate-stannous chloride method and by the triethylamine precipitation

method. In the latter case, both the precipitate and the supernatant remaining after precipitation were assayed for phosphate level (by the ammonium molybdate-stannous chloride method). The results are summarized in Table No. 4. The completeness of precipitation in the triethylamine-ammonium molybdate procedure can be seen here.

A more extensive series was run, using both M9 and M11 media, supplementing the media with 0.2% glucose in each case. These results are given in Table No. 5. Again, the completeness of precipitation can be seen. The M9 zero time readings with the colorimetric method seem dubious. In all other cases, there was a good phosphate uptake during the 24-hour incubation.

To increase the sensitivity of measuring phosphate uptake, the phosphate content of the media was decreased by decreasing the amount of K_2HPO_4 from 1.0 g/L to 0.2 g/L (this does not decrease the total phosphate to 1/5 the original amount since the phosphate contributed by the extracts remains constant). As a check, phosphate analyses were made using both colorimetric and precipitation methods (Table No. 6). These results would indicate that the phosphate contribution from extracts is considerably higher in the M11 medium than in the M9.

Using this decreased concentration of phosphate (0.2 mg. KH_2PO_4 per liter of medium), the concentration of phosphate not assimilated in E. coli cells was measured by the ammonium molybdate-stannous chloride procedure for several time periods of incubation. The results are summarized in Table No. 7. In all cases, there is a dramatic decrease in

Table No. 4 - Phosphate assay by colorimetric and
precipitation methods[*]

	COLORIMETRIC METHOD	PRECIPITATION METHOD[**]	
		PRECIPITATE	SUPERNATANT
Sterile Control	217.5 mg/L	220.6 mg/L	0.6 mg/L
E. coli, shaken, 37°, 24 hours	207.5	249.9	0.4
E. coli, stationary, 37°, 24 hours	232.5[***]	238.1	0.5

[*]Medium is M9 with a 0.5% glucose supplement.

[**]The precipitation method results are expressed in terms of original solution -
   i.e., the precipitate is dissolved in aqueous ammonia and then brought back
   to the original volume.

[***]This value must represent phosphate contained in the inoculum which was
   released during incubation. Phosphate uptake which may have occurred in
   either or both cultures was probably observed by the end of 24 hours (see
   results in Table No. 7).

Table No. 5 - Phosphate assay by two methods, using
both M9 and M11 media

	COLORIMETRIC METHOD	PRECIPITATION METHOD	
		PRECIPITATE	SUPERNATANT
Zero Time			
M9, Sterile Control	215.0 mg/L	240.5 mg/L	0.5 mg/L
M11, Sterile Control	210.0	245.5	0.5
M9, Shaking	143.5	226.1	1.1
M11, Shaking	212.0	255.4	0.4
M9, Stationary	180.0	263.9	0.9
M11, Stationary	225.0	265.2	0.2
After 24 Hours Incubation at 37°			
M9, Shaking	215.0 mg/L	200.8 mg/L	0.8 mg/L
M11, Shaking	200.0	182.8	0.1
M9, Stationary	210.0	190.1	0.8
M11, Stationary	200.0	175.1	0.1

N.B.: Both M9 and M11 were supplemented with 0.2% glucose.
(See last two footnotes in Table No. 4.)

Table No. 6 - Phosphate assays of media with
decreased KH_2PO_4

	COLORIMETRIC METHOD	PRECIPITATION METHOD	
		PRECIPITATE	SUPERNATANT
M9 (0.2 g. KH_2PO_4/L + 0.2% glucose)	40 mg/L	45 mg/L	0.2 mg/L
M11 (0.2 g. KH_2PO_4/L + 0.2% glucose)	75 mg/L	80 mg/L	0.4 mg/L

Table No. 7 - Phosphate uptake by <u>E. coli</u> for
various periods of incubation at 37°

MEDIUM* AND MODE	HOURS OF INCUBATION			
	0	3	5	24
M9, shaking	50 mg/L	40 mg/L	25 mg/L	40 mg/L
M9, stationary	40	5	40	44
M11, shaking	60	15	47	57
M11, stationary	90	10	50	75

*Note: Both media had decreased KH_2PO_4 (0.2 g/L instead of 1.0 g/L) and
had 0.2% glucose added as supplement.

/

the concentration of phosphate, indicating uptake by the cells, followed

by the unexplained return towards the original phosphate levels noted

in Reference 3.

B. ENGINEERING

Work in the first period was confined to supporting the biochemistry
effort and beginning a study of scintillation detectors best adapted to the
needs of a package as described in the proposal.

Conceptual design work has started in the areas of liquid
transport and pumping. Inasmuch as there are many areas where biochemical
development must be performed before instrumentation can be designed, this
first phase effort was concentrated in outlining possible solutions to
areas semi-defined that will eventually be part of the overall program.

For this purpose the PO_4 uptake experiment was taken as the
basis for our discussions, bearing in mind that it is a representative
model for the ATP and SO_4 uptake subsystems of the ultimate package.

Sample Delivery

The selection and development of a sample collection system for
the integrated life detection package is not within the scope of this
program. Inasmuch as the parameters of a lander capsule are unknown at
this time, it will be assumed that a nominal amount of sample will be
delivered to the instrument. The sample probably will consist of dust
with different particle sizes and it is possible that some large lumps may
be present at the delivery point.

Provisions to separate or grind large pieces of the soil sample
should be made. Vibratory coarse primary filters with sieves of a size
compatible with the sample preparation system must be used. After the

sample is prepared for each specific experiment, a secondary filter in
the micron range will have to be used to eliminate any particles that
may endanger the proper operation of the mechanical parts of the instru-
ment. As long as processing samples for the different experiments
involves only the addition of chemicals in liquid form to the soil sample,
it is very unlikely that mechanical operations will take place before the
fine secondary filter is reached; consequently, the primary filter can
be a coarse sieve.

Sample Distribution

        The soil sample delivered to the package must be divided and
introduced to both the unit that performs the basic experiments and
the control unit. Dividing an amount of coarse, filtered soil does not
seem to present a serious problem. A simple rotary distributor with a
metering cavity can be used for this purpose (Figure No. 4). The
metering cavity can be made small so several cycles will be required
to distribute the sample into the two subsystems contemplated. The
purpose of this scheme is to assure that the two subsystems receive
an equal amount of sample before the supply is exhausted.

        Programming the operation of the fractioning drum and the
distribution disc is simple. A maximum predetermined number of cycles
will be permitted to occur to introduce the maximum amount of sample
desired, even if an excess exists at the receiver. By this arrangement
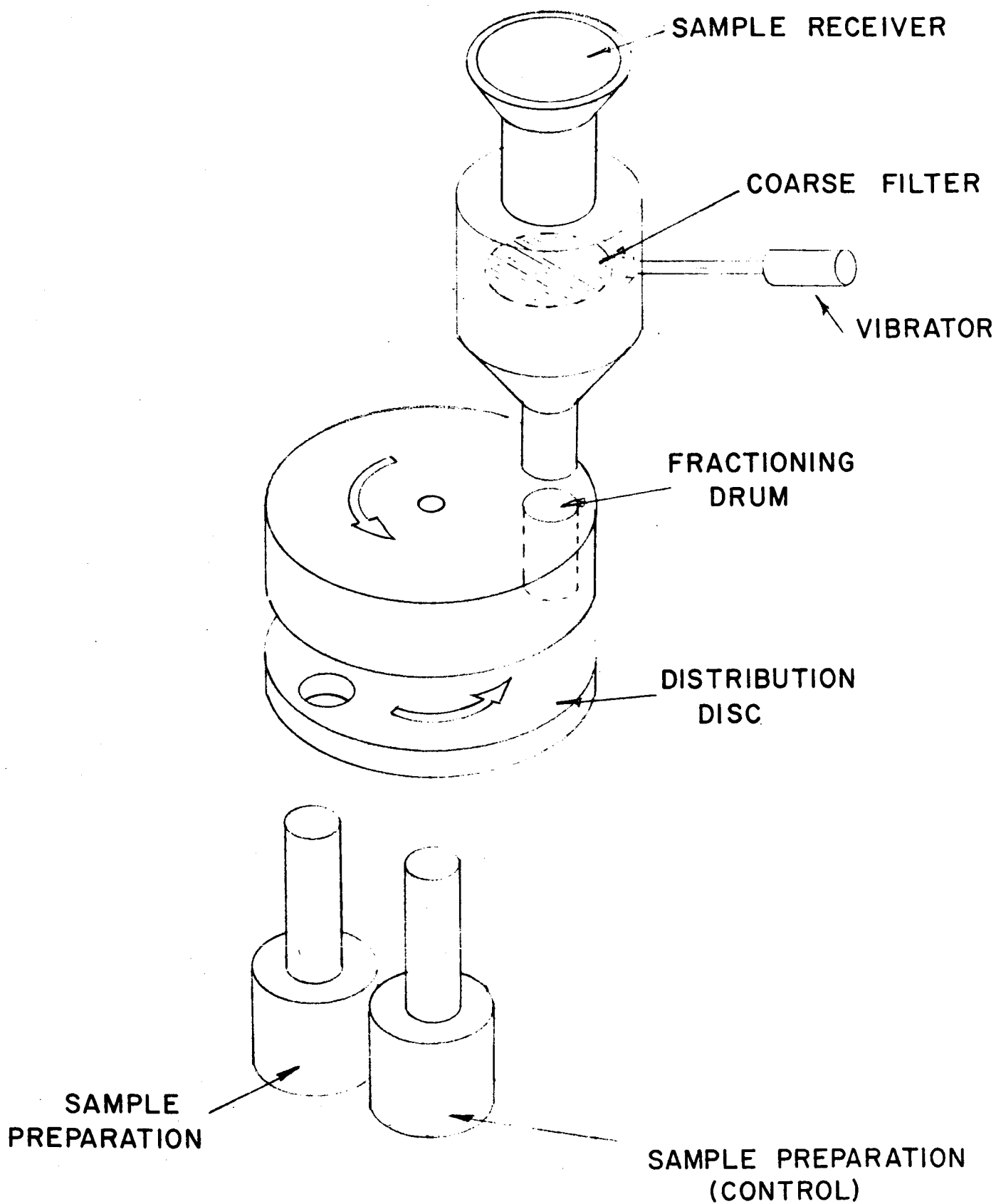it is possible to predict that any quantity of sample in the receiver,

SAMPLE RECEIVER

COARSE FILTER

VIBRATOR

FRACTIONING DRUM

DISTRIBUTION DISC

SAMPLE PREPARATION

SAMPLE PREPARATION (CONTROL)

Figure No. 4 — Sample distribution

short of the maximum required will be equally divided among the two subsystems.

Sample Preparation, PO$_4$ Uptake

The preparation chamber is, in effect, an incubation chamber from which, at predetermined intervals, aliquots will be extracted for further processing.

The medium will be transported in sealed glass ampuls to prevent its evaporation during the trip to Mars. It is assumed at this point of the program that the capsule, when it lands on Mars, will have a selected orientation. Some of the solutions proposed for the different problems of processing samples and transporting liquids are based on this assumption.

A scheme for delivering the liquids transported in ampuls has been tested (Figure No. 5). Ampuls are located in housing that protects them from shock and vibration during land transportion and the launch phase of the instrument. Upon landing and acquisition of the sample by the capsule, a programmer will fire the hermetic squib that breaks the ampul. In tests performed, a piston actuator, Atlas 1 MT 114 has been used to propel the one-gram steel dart that breaks the ampul. Different sizes of ampuls were tested; the largest one, with a volume of 10 cc., shattered into a large number of fragments. There was no liquid entrapment and the glass particles were contained by the coarse filter in the bottom of the housing. Gravitational flow of the liquids in discharge

SEALED GLASS
AMPUL

STEEL
DART

AMPUL HOUSING

HERMETIC PISTON
ACTUATOR ATLAS
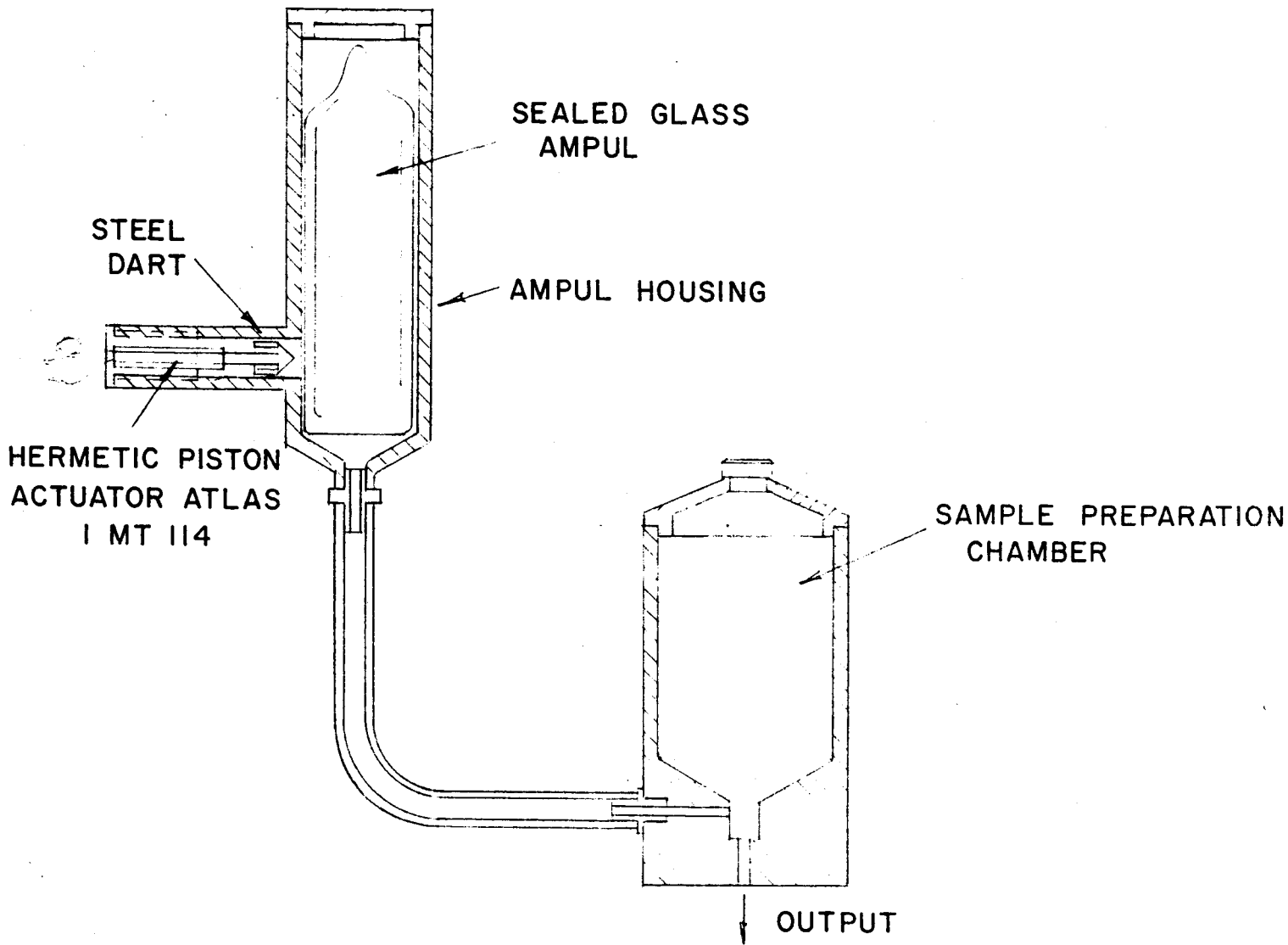I MT II4

SAMPLE PREPARATION
CHAMBER

OUTPUT

Figure No. 5 — Liquid storage and delivery

pipes as small as 1/16 of an inch internal diameter was excellent. It is proposed to use this system for liquid handling in the preparation chambers of all the experiments involved in this program. It eliminates the use of pumps and valves, and its reliability can be enhanced by using double squibs for each ampul. Its low electrical power requirements and easy programming are further reasons for recommending its use.

Aliquot Extraction and Filtering

At predetermined intervals, aliquots of the culture must be taken and pumped to individual chambers where a reagent for the phosphate analysis is added. Single use chambers are used to prevent contamination between aliquots taken at different time intervals.

The extraction of aliquots can be performed by means of a piston pump built in the preparation chamber (Figure No. 6). A gear motor attached to the cover of the preparation chamber turns a lead screw that drives a piston into a cavity in the bottom of the housing. The motor can be programmed to drive the piston the full length of the cavity or, if a volume of liquid smaller than the volume of the cavity is desired, it can be made to stop before the end of the cavity. Conversely, if a volume of liquid larger than the cavity is desired, the pump can be recycled. The output of the pump is directed to a distribution rotor. The rotor has passages that connect with the input of a fine filter located immediately ahead of the aliquot chamber. A check valve in the rotor passages prevents liquid returning to the pump in its return stroke.

SAMPLE FROM
SAMPLE DISTRIBUTION

GEAR
MOTOR

PREPARATION
CHAMBER

DRIVE SCREW

PISTON

DISTRIBUTION ROTOR
CHECK VALVE
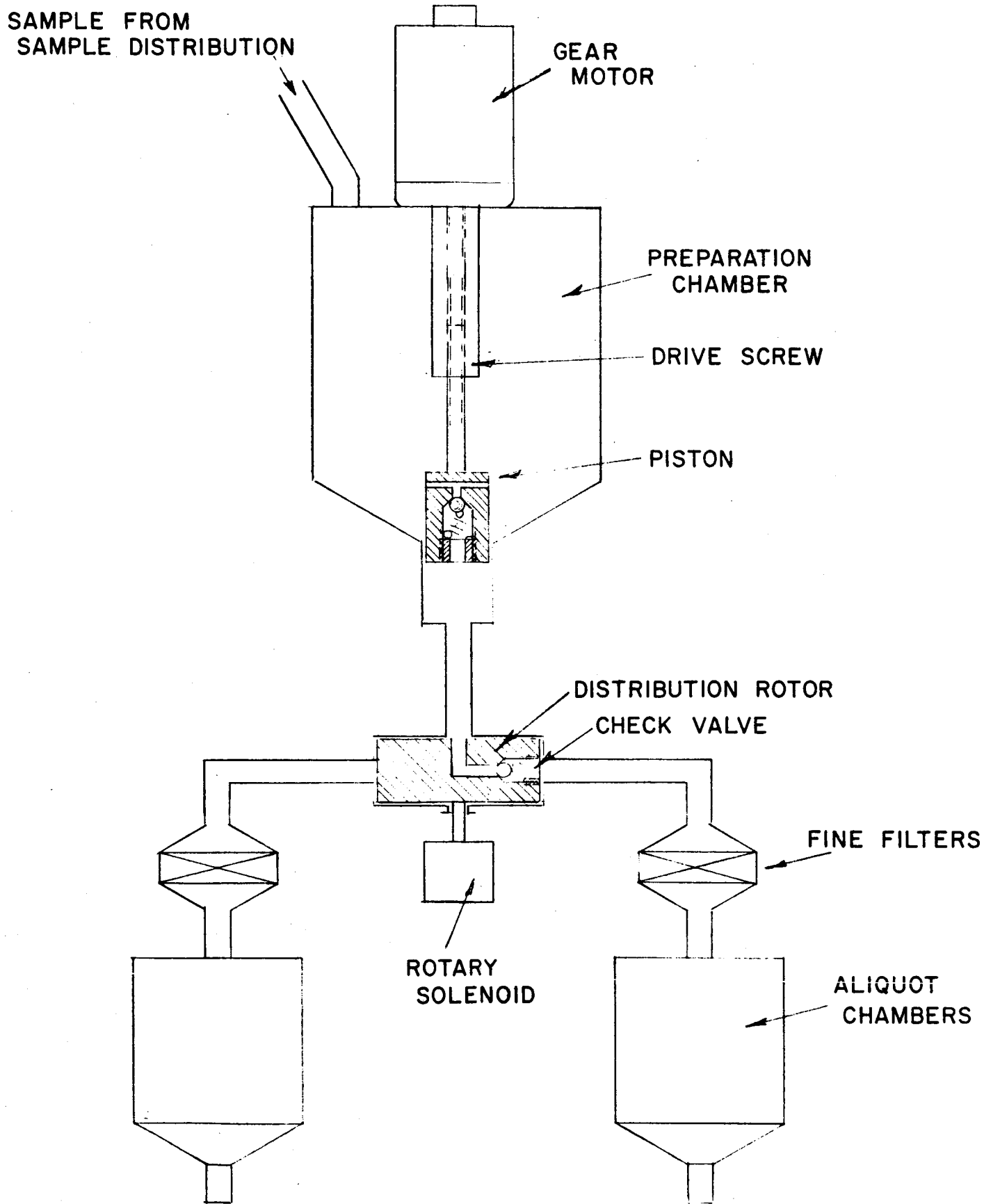
FINE FILTERS

ROTARY
SOLENOID

ALIQUOT
CHAMBERS

Figure No. 6 — Aliquot extraction and filtering

As soon as the injection to one of the aliquot chambers ceases, a rotary solenoid indexes the rotor to the next aliquot chamber. The pump gear motor is reversed and reloading for the next injection phase takes place.

Millipore filters, 0.45 micron, are used to filter the aliquots before they are introduced in the chambers.

Aliquot Chambers

Each aliquot is prepared by adding triethylamine or an ATP precursor mixture (whichever analytical method is selected) before it is injected into the reaction chamber.

Experiments with the ATP precursor mixture will dictate if it can be stored in a single container and later distributed to the aliquot chambers, or if it has to be stored in individual containers to be opened at the time each reaction is to take place. In any event, it is very likely that the mixture will be carried in sealed containers to protect it from the space environment.

A device similar to the aliquot extractor can be used to pump and fraction the triethylamine or the ATP precursor mixture if it is carried as a single batch. On the other hand, if individual portions of the reagents are desired for each aliquot, a number of ampuls may be used.

Read-out System

An investigation has been made of a number of factors which are critical in the design of an automated device that provides for moving various light sources into a viewing position in front of an electron multiplier tube. Since the light output from any of the experiments may be of very low intensity, perhaps as low as to require single photon counting, the optical sensitivity and the system noise level will be of great importance.

At this time, actual volumes and cross-sectional areas of the possible light sources are not known but they are not expected to differ enough to invalidate any of the conclusions drawn about optimum systems. For purpose of design, a source one inch in diameter was selected with uniform brightness across the surface, an index of refraction at the interface of 1.485, a variable viewing time period, and no polarization or collimation of the light energy emitted from the surface.

There are two mechanisms envisioned for the production of light. One is the product of a bioluminescent reaction and the other is a scintillation in an optically clear material produced by the deceleration of a radioactive particle, in this case a beta particle. Both of these sources may produce small amounts of light (5×10^{-13} lumens) as the conclusion of a successful experiment so that, in general, no sacrifice in optical efficiency should be made with present-day photodetector sensitivities. The light output of the bioluminescent reaction and from the scintillator material being considered has energy in the 400 to 550 mμ region of the spectrum

and this range has been used when considering indices of refraction or transmission of optical elements. A cursory examination of the light output mechanisms of these two sources shows that, for scintillators activated by low energy beta particles, the light output pulse from the second reaction within the scintillator material (the one producing 405 mμ wavelength energy in Pilot B, for instance) has a considerably greater length than the light output pulse obtained from a single molecular source in the bioluminescent reaction. This difference offers the possibility of operating both experiments by selection of the pulse width. Pulse-width discriminators have been in use for a number of years and a relatively unsophisticated circuitry will differentiate between these two with less than one percent error. The wavelength of both sources, being similar, will produce the same quantum yield in a detector so that discrimination between the two on the basis of pulse amplitude is not possible. The rate at which pulses appear may be completely arbitrary, i.e., either the fastest rate may appear or the rates may be equal. This permits two choices: use entirely separate sources produced by the different mechanisms, or attempt to view the two mechanisms simultaneously and differentiate on the basis of pulse width. The latter approach seems to offer additional advantages and is chosen to pursue for a more complete evaluation of its feasibility.

The bioluminescent experiment, as far as the end-point emission of light is concerned, is relatively short-lived. It will be a one-per-source, and then the source will have to be replaced. This replacement operation will be mechanical and is one of the limitations placed on the

design of the optical system coupling. The scintillation experiment, on the other hand, is a relatively long-lived one which will operate over a period of hours and can be viewed at any convenient time without decoupling the optical system. This optimum coupling of the optics for the scintillator is critical since scintillator efficiencies for beta particles are intrinsically low and, in this case, the radioactive source producing the scintillation is distributed over as large an area as possible.

Photomultiplier Tube Selection

The basic photomultiplier tube can be of the Cesium-Antimony type exhibiting a wavelength of maximum response around 407 mµ. A photocathode and multiplier sensitivity of at least 100 microamperes per lumen is required for the low level expected from the light sources. Tubes of this type are available at 1.25 and 1.5 inches in diameter with metal-ceramic structure and flat faces. These tubes are mechanically rugged and will withstand 20 g. acceleration throughout the frequency range 20 to 3000 cycles and shocks of 11 milliseconds duration at a 50 g. level. The leakage noise, vacuum integrity, etc., are suitable for space use.

One parameter which will be compromised in the dual experiment is the selection of the larger photocathode effective area than is desirable. Previous development on an optical system for the detection of bioluminescent reactions (10) has employed a photomultiplier tube with a small (0.1 inch) effective photocathode area with good results. It

appears to be more efficient to channel light to a small low noise
photocathode area than to eliminate the optical parts by employing a
large photocathode. The large photocathode results in considerably
more noise signal.

The lowest accelerating voltage possible will be determined
experimentally. At the present time, the photomultiplier gain for
commonly employed multiplier electron optical systems is designed for
200 to 300 volts per dynode, and for a 10-stage tube the overall supply
voltage is 3 kv. Regulation of this voltage is essential for making
valid comparisons between readings made at different times. Experience
has shown that primary low voltage regulation is adequate if it can be
controlled to 0.25%. Conventional electrostatic and magnetic shielding
will be required for the photomultiplier.

The phototube output will be taken in the form of pulses.
Two amplifier chains will be needed, one for slow scintillator pulses
and one for fast bioluminescence created pulses. Both of these chains
can be constructed from integrated circuit components. Time discrimina-
tion for slow pulses will be obtained by inductive changing of a reactor
which will be the largest component of the amplifier system. Initial
development will consider the dual amplifier with the provision that one
amplifier may be eliminated if it is possible to read a bioluminescent
light reaction through a steady background of scintillator pulses. The
scintillator pulses alone can be read while bioluminescent sources are
being changed or before they are activated, but since the scintillator in

the test assembly to be proposed cannot be decoupled it will not be possible to read bioluminescent light alone unless the scintillator experiment is delayed until all of the bioluminescent samples have been examined.

Scintillator Material and Mechanical Design

The various scintillator materials available have been examined and two selected for test. There are two mechanical designs proposed as well. The basic selection is made on materials that will produce a high light output and not possess any subsidiary mechanical properties that would render them inapplicable to the experiment. Calcium iodide (Eu) has one of the highest light outputs but is so deliquescent that it should not be employed. Sodium iodide (Tl) has a high light output but is subject to surface effects. It has been reported as having poor decay characteristics, making it undesirable in pulse width discrimination applications. Anthracine is acceptable mechanically and physically as is Pilot B with the light output for Pilot B somewhat less than for anthracine.
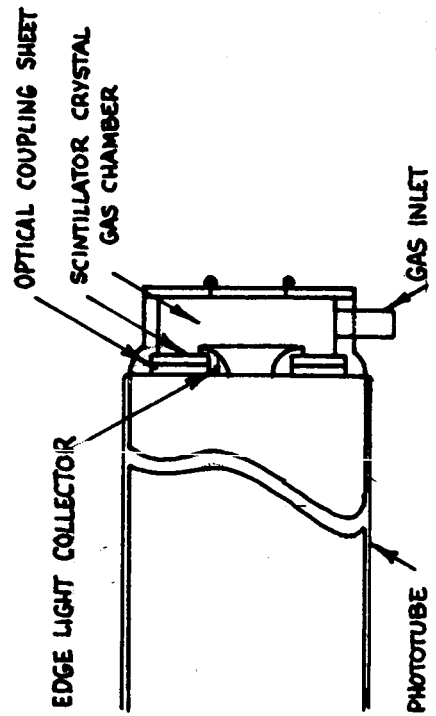
For experimental data it is proposed to test an annular ring of both anthracine and Pilot B as a scintillator by optically coupling the material to the flat face of the photomultiplier with a sheet of LTV-602 (GE). This material has an index of refraction of 1.406 in the wavelength range of interest and shows a 93 percent transmission factor. A very thin sheet (0.02 inch) will be used and mechanical pressure will be used as a sealant. The compression will be vacuum exposed and readjusted so no entrained air bubbles are retained. An alternate coupler could be a
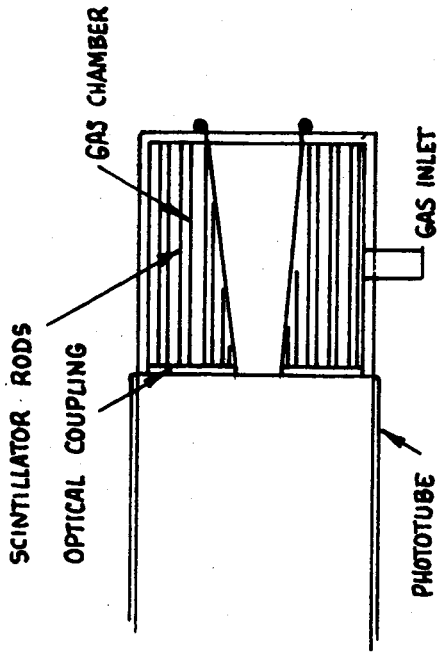
silicone grease (Dow Corning #QC-2-0057) but this is considered less satisfactory than the sheet material. A captive ring of Lucite will be made to capture edge light from the scintillator material and direct it onto the photomultiplier face. Figure No. 7A is a sketch of the assembly.

In order to bring the radioactive source to the scintillator, a chemical getter will be deposited by evaporation on the exposed scintillator face. Previous experiments attempting to evaporate lithium hydroxide layers onto a clean surface have not been completely satisfactory due to the decomposition of the compound at the temperature at which evaporation is achieved. An alternative to the evaporation of the hydroxide is the deposition of a thin layer of lithium and then converting it to the hydroxide chemically. The lithium hydroxide is applied to act as a getter material for the carbon dioxide which contains C^{14}, a beta emitter. The average beta energy is 50 kev so that minimum thickness of getter can be applied without incurring appreciable absorption of the beta particles in the getter material itself. The penetration of the beta particles into the scintillator will be less than 10^{-5} cm. so that scintillator thickness is minimal. The thinner the scintillator disc the less conversion efficiency it will have for ambient gamma radiation or background particle capture. The front surface of the scintillator will be deep etched chemically to increase the available surface area for deposit of the getter material.

An alternate design for the scintillator is the application of rod-shaped material of scintillator quality optically coupled to the photocathode and coated with a getter material. Such a design can increase

A. FLAT SCINTILLATOR DESIGN

Labels: EDGE LIGHT COLLECTOR, OPTICAL COUPLING SHEET, SCINTILLATOR CRYSTAL, GAS CHAMBER, GAS INLET, PHOTOTUBE

B. ROD SCINTILLATOR DESIGN

Labels: GAS CHAMBER, SCINTILLATOR RODS, OPTICAL COUPLING, GAS INLET, PHOTOTUBE
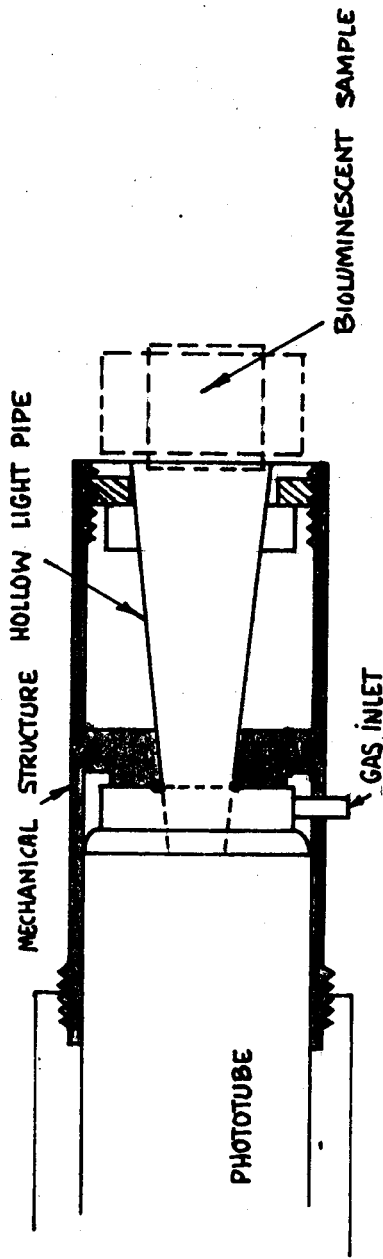
C. COMBINED OPTICAL SYSTEM

Labels: BIOLUMINESCENT SAMPLE, MECHANICAL STRUCTURE, HOLLOW LIGHT PIPE, GAS INLET, PHOTOTUBE

FIG. 7 OPTICAL SYSTEM COMPONENTS

the area of the getter which is an important factor if the device were to be applied in a carbon dioxide-rich atmosphere. The same considerations appear in the deposit of getter, but the overall efficiency would be somewhat lower since the production of a scintillation event in a rod results in light moving in both directions, one of which is away from the light detector. By incorporating white ends, however, approximately 65 percent of the reverse travel light can be directed toward the photodetector.

A serious consideration was given to fiber optical components as light collecting agents. The transmission efficiency of non-image forming bundles, in most of the visible spectrum, is typically 18 percent for non-parallel light. To increase this figure, it is required to use coated fibers and restrict any dimensional changes to less than 20 times the rod diameter. For the present, the overall efficiency of fiber optics simply for conducting light is not particularly attractive, nor required. The use of the scintillator material, PVT or Pilot B in rod form, 0.062 inch in diameter will adequately perform its own light conduction to the detector surface. The rods will be attached to a light diffuser ring in the form of an annulus coupled to the photomultiplier tube face with a Lucite guard ring to capture edge light. Figure No. 7B shows a sketch of the experimental assembly. This design anticipates the use of a conical light pipe for the collection of light from the bioluminescent sources. In order to collect light from a one-inch source and concentrate it properly at the center of the phototube on a 0.2 inch diameter spot, the light pipe would need to be of appreciable length.

Light Collector System for Bioluminescent Source

The bioluminescent sources are assumed to have a one-inch diameter aperture and the recoverable light will be emitted uncollimated, unpolarized and uniformly over the aperture. In fact, the aperture may be somewhat smaller than the projected one-inch since it will be a function of the reacting chemical interface between two liquids being rapidly mixed. In any event, if light can be efficiently collected from the complete aperture it will be collected from fractions as well. The optimum aperture will be determined by the final design of the liquid mixing system and the optical field of view should eventually be tailored to match the source area since the bioluminescent materials may emit a continuous background. Since this is a noise signal and it is uniformly distributed across the source area, no more of this area should be viewed than is absolutely necessary.

The light collector proposed is a hollow reflective cone. The use of a filler material would necessitate two additional optical interfaces at which losses could occur. The inlet diameter is as large as can be accommodated by the mechanical design of the source translator mechanism. By making the inlet aperture 20 percent greater than the source and coupling it close mechanically, the field of view of the photomultiplier can be increased to a solid angle of at least 160°. It is envisioned that the bioluminescent reaction chamber will be double ended and that an identical optical system will be mounted on the other side. Previous experiments using a reflector in the back of the bioluminescent reaction chamber have not materially increased the radiation in the forward direction

so that viewing the reaction chamber with identical optical systems on either side should result in nearly identical observations by both systems.

The light collector cone will concentrate the light energy on the center of the photomultiplier tube face in the center of the annulus formed by either of the scintillator arrangements. Figure No. 7C shows a sketch of the complete light assembly.

Signal Conditioning

The conduct of the biological experiments may be programmed to coincide with telemetry transmission so that rates, intensities, etc., may be transmitted directly in real time. However, the real-time requirement may impose restrictions on the conduct of experiments that would result in a degradation of the experiment results. If the outcome of each experiment can be stored for arbitrary reading after a fixed time period, then data transmission can be simplified considerably. The bioluminescent experiments are sequences which last fractions of a minute and all data must be gathered within this time period. The collection of radioactive gas products of a reaction will take a minimum of 15 minutes and, depending on the information desired, can take up to several hours or even days.

The basic information derived from the proposed experimental techniques is whether a signal exists during the test interval that is greater than the background. To gain confidence in the validity of the measurement, a statistical measure must be applied to the background, and to the signal, and a threshold value assigned to the signal. If the

signal exceeds the predicted threshold during the test interval, then the answer from the experiment is positive. These decisions can all be made electrically within the experiment; the logic and the threshold ratios must be made in designing the apparatus.

The proposed approach in designing the prototype instrument is to determine the background electrically, assuming an initial integration time of five seconds. The integrated background is used as a reference signal by holding a charge on a capacitor. The experiment read-out is then made, integrated with the same time constant, and compared electrically with the reference signal. A bias voltage, representing the required threshold to determine a confidence level, will be applied to the signal channel. If the signal exceeds the threshold, then a positive result will be indicated by the change of state of a switch. It is proposed that the various switch outputs be scanned at the start of the experiment, and then after the sequence of experiments is completed. More than one threshold can be incorporated so that, in addition to a yes-no determination, intensity information can be measured. Tests can be compared with controls to provide a total yes-no determination.

Of course, if analog data transmission facilities are available, the signals derived from an experiment are not limited to the yes-no type.

III. PLANS FOR NEXT PERIOD

The most sensitive ^{14}C-triethylamine phosphate assay will be developed. This may require obtaining amine with higher specific activity. Attempts will be made to determine the minimum number of micro-organisms this assay will detect, both under conditions of all phosphate-depletion and under conditions without such depletion. Not only will the experiments be conducted using E. coli, many of the microorganisms which have been tested in the Gulliver program will be tested here. The objectives will be both to be sure that these organisms can grow in the new recommended medium and to determine minimum numbers of cells required to detect phosphate uptake.

Another biochemical program which will be worked on during the second period is the quantitation assay of inorganic phosphate by con-version to ATP. The sensitive assay of ATP is not considered a problem as this is done routinely in these laboratories. However, the reaction of ADP with inorganic phosphate seems replete with difficulties.

The final biochemical program for the second period will involve systems for measuring sulfate uptake.

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