THE DESIGN AND FABRICATION OF AN INSTRUMENT FOR THE DETECTION OF ADENOSINETRIPHOSPHATE (ATP)

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NATIONAL AERONAUTICS AND SPACE ADMINISTRATION
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ABSTRACT

The development of a method for the detection of life in the upper atmosphere and outer space has been the goal of the investigations carried out in this laboratory during the past year and one-half.

The basis of the life detection system studied in this laboratory has been the selection of adenosinetriphosphate (ATP) as a monitor for the presence of life. The rationale of this selection stems from the universal occurrence of ATP in terrestrial organisms.

The method selected for the detection and quantitative assay of ATP exploits the bioluminescent reaction occurring in fireflies. In brief, the components of this reaction are an enzyme, luciferase, and two substrates, luciferin and ATP. With luciferase and luciferin held constant and in excess, the emission of light is theoretically proportional to the ATP added.

The work conducted during the tenure of this contract has proceeded along two lines. The first has consisted of experiments designed to establish the optimal conditions for the preparation of luciferase and luciferin, and also to establish the optimal reaction conditions for maximum assay sensitivity. The second phase of this work has been the design and fabrication of a highly sensitive and versatile instrument for the measurement of the light emitted during the bioluminescent reaction.

Column chromatography using Sephadex G-100 has been found to be the most effective means of obtaining luciferase in a partially purified state.

Because of difficulties in obtaining sufficient quantities of natural luciferin, this compound was synthesized from commercially available starting materials.

Reaction conditions, established as optimal for maximal sensitivity, included the following:

1. pH - 7
2. Temperature - $20^{\circ}C$
3. Buffer - Tris or Arsenate
4. Mg^{++} concentration - 1×10^{-2} M
5. Luciferin concentration - 1 mg/ml

Using optimal reaction conditions, the maximal sensitivity of the assay was determined. It was found that 1×10^{-8} γ of ATP could be detected with confidence levels ranging from 0.02% to 100%. The ATP responses were not linear at quantities lower than 10^{-7} γ.

An instrument for the monitoring of the light emitted during the bioluminescence reaction has been designed and fabricated in this laboratory. The design of this instrument was governed by two objectives. The first was to build a very sensitive unit, while the second objective was to have an instrument sufficiently versatile so as to allow the investigation of a variety of experimental variables.

A high signal-to-noise ratio was achieved by cooling the photomultiplier tube and the use of a discriminator circuit. The design of the photomultiplier circuit was such that the output of

the photomultiplier tube was a series of voltage pulses, thus allowing the efficient use of a discriminator circuit.

The system for the injection of ATP via a syringe was hydraulically controlled with provisions for continually varying the injection volume.

There was also incorporated into the instrument, circuitry for controlling and varying the light measuring period.

The reaction chamber was provided with a thermostatically controlled water bath.

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I. INTRODUCTION

Among the vast array of questions to which man is seeking answers in his investigations of the upper atmosphere and outer space, there is none more challenging and provocative than that pertaining to the existence of life outside terrestrial boundaries. A satisfactory answer to this question is the subject of intensive study by several investigators.

Basic to these studies is the establishment of the parameters which define life. A definition which is amenable to operational manipulation is one which characterizes life as the capacity of a molecular complex to perform certain functional activities including metabolism, growth, and reproduction. If the validity of the above definition is accepted, then it follows that a rational life detection system must be capable of measuring phenomena related to these criteria.

The detection of life in many instances is not a difficult matter, especially when there are visible manifestations such as characteristic form, movement, and reproduction. This, however, becomes increasingly difficult as the size of the organism becomes microscopic, and even more so as the number of organisms being sampled decreases. It is at this point that the classical life detection techniques begin to be dependent upon one of the manifestations mentioned above; namely, reproduction. Although adequate in many laboratory situations, techniques based on growth have the requirement of long time periods and rigorously controlled

conditions. Another factor which enters here is that for the detection of life beyond the surface of earth, techniques which are amenable to remote operation must be employed. The work to be described here grew out of the need for a rapid, sensitive technique for the detection of life.

All terrestrial life insofar as is presently known, is intimately associated with, and dependent upon, the nucleotide phosphate, adenosinetriphosphate (ATP) (1). The ubiquity of this compound in living organisms renders it an excellent indicator of the presence or absence of life. Furthermore, changes in the metabolic integrity of a living organism are frequently accompanied by variations in the steady state concentration of ATP in the organism. Thus, not only may ATP measurements provide the basis for the detection of life, but may also be exploited for the detection of metabolic changes, both normal and pathological.

In order to realize the full potential of ATP as a monitor for the presence of life in space, it was necessary to select and develop a method with which it could be assayed with the highest degree of sensitivity, accuracy, and rapidity.

There are two general techniques by which ATP may be measured. The first requires its isolation in the pure state after which it can be assayed by means of ultraviolet spectrophotometry. The second technique employs an enzyme system in which ATP is a substrate. The latter enjoys the advantage in that it does not

require that ATP be in a pure state. This gives it a simplicity and speed which cannot be approached by the first technique.

The choice of an enzyme system for ATP assay was dictated mainly by the degree of sensitivity of the assay methods for the products formed during the reaction between ATP and enzyme. In most of the enzymatic assays of ATP at very low concentrations, the primary enzyme is coupled with one in which a pyridine nucleotide is reduced at a rate proportional to the ATP concentration. Reduced pyridine nucleotide which has a very high extinction coefficient is then assayed at 340 mμ in the ultraviolet spectrophotometer. Another enzymatic reaction requiring ATP which appealed to us quite early was the bioluminescent reaction occurring in fireflies.

The components and mechanism of the bioluminescent reaction occurring in fireflies have been well established by the excellent work of McElroy and his associates (2-7).

In brief, the light emission in bioluminescence is the result of the reaction of oxygen with an oxidizable substrate (luciferin) catalyzed by an enzyme (luciferase). Luciferin must first react with ATP before it can be oxidized with light production. The first step of the reaction is this:

$$(1)\quad E + ATP + LH_2 \underset{}{\overset{Mg^{++}}{\rightleftharpoons}} E - LH - AMP + PP$$

The kinetics of the reaction indicate that ATP is not functioning here in an energy donor capacity but functions in an unknown catalytic fashion to change the electronic configuration of one or all of the energy states of luciferin (8). In the presence of oxygen, the light emission step takes place as follows:

(2) $E - LH_2 - AMP + O_2 \longrightarrow E - L - AMP + (H_2O_2)\ ? + \text{Light}$

E - L - AMP is a relatively stable complex and does not spontaneously disassociate to regenerate luciferase. The regeneration of the enzyme may be affected by either or both of the following reactions:

(3) $E - L - AMP + CoA \downarrow E + AMP + CoA - L$

(4) $E - L - AMP + PP \downarrow L + E + ATP$

Where:

E = Enzyme (luciferase)
LH - Luciferin
PP = Pyrophosphate
AMP = Adenylic acid
CoA = CoEnzyme A
L = Dehydroluciferin

On examination of reactions 1, 2, 3, and 4, it can be seen that the total light emitted during the course of the reaction

is a function of the concentration of luciferase, luciferin, ATP, CoA, O_2, and pyrophosphate. It has been shown, however (8), that the rate limiting step in the sequence is the reaction between ATP and luciferin. Therefore, in the presence of excess luciferase, the maximum intensity is a direct function of the concentration of luciferin and ATP. By keeping both luciferin and luciferase in excess, the maximum intensity of the emitted light is theoretically proportional to the ATP concentration.

The basic procedure consists of injecting either standard ATP solutions, or cellular extracts containing ATP, into a cuvette containing the enzyme system (luciferase, luciferin, and magnesium ion). The enzyme system is held at pH 7.4 with potassium arsenate buffer. The light, emitted as the result of the reaction between ATP and the enzyme system, strikes the surface of a photomultiplier tube giving rise to a current which can be measured by various means. A typical light response as measured with an oscilloscope is shown in Figure 1.

There are two ways in which the bioluminescent response with ATP, as portrayed in Figure 1, can be expressed. One is by measurement of the maximum intensity of the emitted light, which after reaching this maximum value, decays exponentially. With all other factors constant, the maximum intensity is directly proportional to the concentration of ATP. The alternative manner of expressing

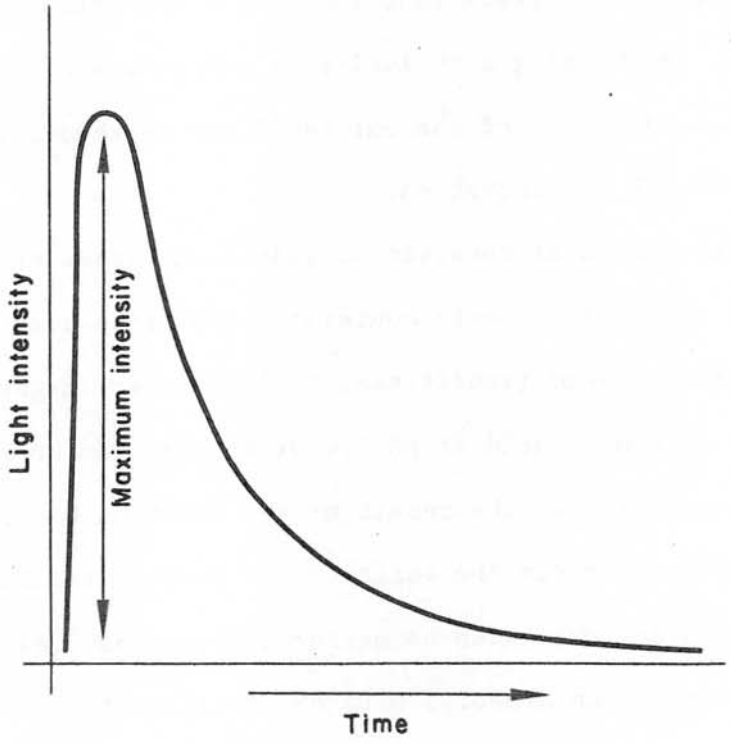


Figure No. I — A schematic representation of the bioluminescent reaction as recorded by an oscilloscope.

the response is by integration of either a part or all of the total amount of light emitted, i.e., area under the light intensity curve. Instrumentation currently available in this laboratory allows both types of measurements.

The advantages of the use of the bioluminescent assay for life detection in space may be enumerated as follows:

1. High degree of specificity
2. Assay requires very short time period
3. Instrumentation for light measurements are capable of great sensitivity and also may be easily miniaturized.

The specificity of the reaction for ATP and thus for life has, of course, only been established for terrestrial organisms. It is possible, however, that organisms which might be encountered in space will contain no ATP. It is also possible that nonbiological ATP may be found on other planets. This, in itself, would be of major biological significance indicating that prebiological evolution had developed to that point. The incorporation of an inhibited control and the monitoring for ATP production in the sample being tested could distinguish metabolic from nonmetabolic ATP.

Previous studies conducted under contract with the Naval Bureau of Weapons (9) established the feasibility of the bioluminescent assay for the rapid detection of small numbers of microorganisms. The studies to be described here represent improvements of this method, both in biochemistry and instrumentation.

II. RESEARCH GOALS

The goals of the studies carried out during the past 17 months were:

1. Biochemistry - The establishment of the optimal enzyme reaction conditions so as to achieve maximal measurable ATP response.

2. Instrumentation - The design and fabrication of an instrument for quantitative light (ATP bioluminescent) detection more sensitive than any previously available.

III. BIOCHEMISTRY

A. Introduction

The investigations to be described in this section were designed to accomplish the following:

1. Develop methods for the routine preparation of luciferase and luciferin.

2. Reduce the magnitude of the inherent light (light emitted by luciferase in the absence of added ATP) emitted by luciferase preparations.

3. Establish the reaction conditions for maximal light emission in response to ATP addition.

4. Determine the minimum quantity of ATP which can be measured using optimal reaction conditions and instrumentation.

B. Instrumentation

In the experiments to be described, light measurements were made with two instruments. The first was an instrument designed and fabricated by this laboratory (for the Naval Weapons Bureau) prior to the initiation of these studies. This instrument consists of a composite sensing and reaction chamber which contains an RCA 7265 photomultiplier tube and a rotating cylinder mounted in a block of aluminum in a manner which permits removal of the reaction chamber without exposing the phototube to extraneous light. As is shown in Figure 2, a section of the cylinder wall is cut out to accomodate the cuvette which is a 6 mm x 50 mm test tube. Immediately above the cuvette holder is a small injection port sealed with a replaceable light-tight rubber plug. The output of the photomultiplier is fed into a Tektronix Model 503 oscilloscope with a maximum sensitivity of 200 uv/cm. To observe and record the reaction, the cuvette containing the necessary reagents is positioned in the cuvette carrier without exposing the phototube. After the cuvette is rotated into position before the photomultiplier tube, the reaction is initiated by the injection of ATP via needle and syringe through the injection port. The light emission is measured on the oscilloscope in millivolts.

The second instrument used in these studies is one designed and built under this contract. This will be discussed in detail in Section IV. In brief, this unit differs from the first in the

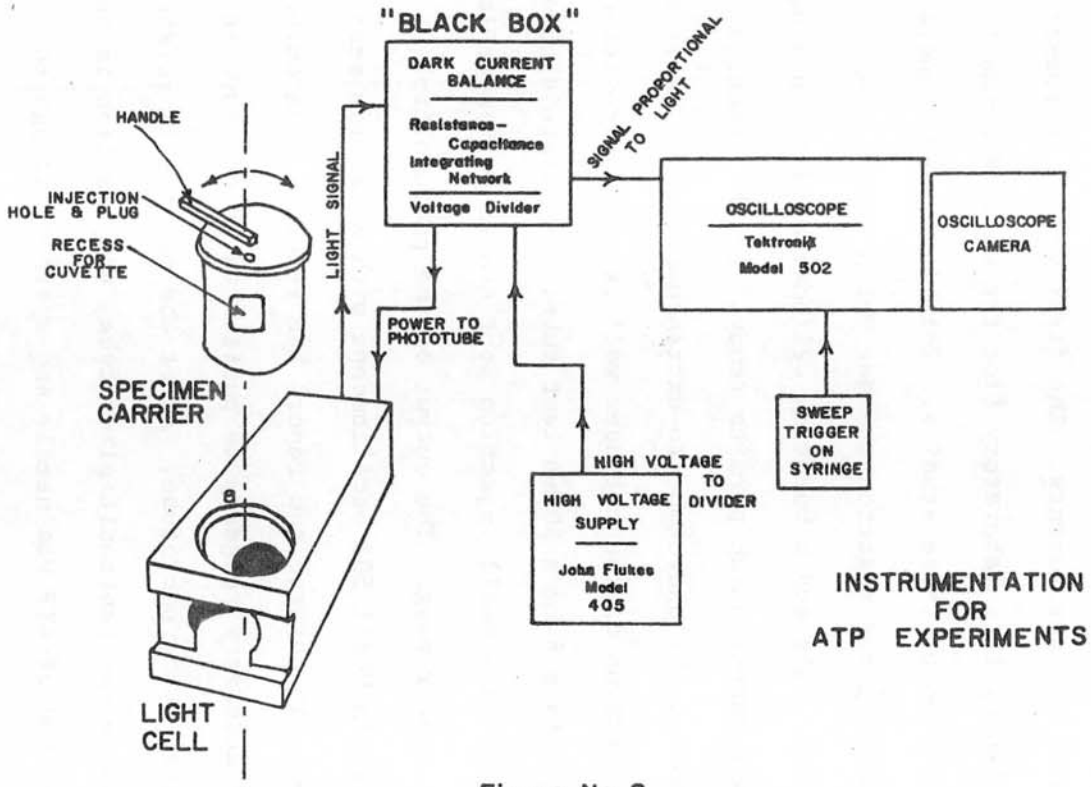

"BLACK BOX"
DARK CURRENT BALANCE
Resistance–Capacitance Integrating Network
Voltage Divider
SIGNAL PROPORTIONAL TO LIGHT
OSCILLOSCOPE
Tektronix Model 502
OSCILLOSCOPE CAMERA
HANDLE
INJECTION HOLE & PLUG
RECESS FOR CUVETTE
LIGHT SIGNAL
POWER TO PHOTOTUBE
SPECIMEN CARRIER
HIGH VOLTAGE TO DIVIDER
HIGH VOLTAGE SUPPLY
John Fluke Model 405
SWEEP TRIGGER ON SYRINGE
LIGHT CELL
INSTRUMENTATION FOR ATP EXPERIMENTS

Figure No. 2

following respects:

1. The output of the photomultiplier tube is a series of voltage pulses instead of an anode current as was the case with the previous unit. Thus, light emission is measured in pulses or counts per given time period.

2. The cathode of the photomultiplier tube is operated at -30°C. In addition, the use of a discriminator circuit permits a very low noise level to be attained.

3. The injection of ATP is mechanically controlled.

4. The counting time is selected and variable over a very wide range.

For purposes of identification throughout this report, the first instrument will be referred to as the "RCA Unit" while the second will be the "NASA Unit."

C. Preparation of Enzyme

1. Source

The starting material for the preparation of luciferase was desiccated firefly tail obtained commercially from both Sigma Chemical Company, St. Louis, and Worthington Biochemical Corporation, Freehold, New Jersey.

2. Preparation of Acetone Powder

It has been observed by Green and McElroy (10), and also

in this laboratory, that if the firefly tails are extracted directly with buffer, the extract contains an excess amount of lipid material which will interfere in subsequent purification procedures. Green and McElroy (10) found that this could be prevented by the preparation of acetone powders from the firefly tails as described below.

Desiccated firefly tails (ten grams) were ground to a fine powder with one gram of washed silica with a pre-chilled mortar and pestle. The powder was added to 600 ml of acetone which had been cooled to -20°C with dry ice. The suspension, after standing for five minutes, was filtered through a Buchner funnel under reduced pressure. The filter cake (residue) was washed with an additional 250 ml of cold (-20°C) acetone. Vacuum was maintained on the Buchner funnel until the filter cake was almost dry. The cake on its filter paper was taken to complete dryness under vacuum in a desiccator. The acetone powder when stored at -10°C in the presence of Drierite has been found to be stable for up to six months.

3. Extraction of Acetone Powder

For the extraction of luciferase activity from the acetone powder, various buffers have been compared. The solution initially used for the extraction of the acetone powder was Versene (0.01 M - pH 7.4). The extraction procedure was as follows:

The acetone powder (500 mg) was ground to a fine powder

with pre-chilled mortar and pestle. The powder was added to 7 ml of cold 1 x 10^{-3} M Versene at pH 7.4. The suspension was centrifuged at 1500 x G at 4°C for ten minutes. The supernatant solution was decanted and saved and the residue was re-extracted with two 3 ml volumes of cold water adjusted to pH 8 with 0.1 N NaOH. The three supernatant solutions were then pooled and centrifuged in the cold at 12,000 x G for thirty minutes. The supernatant (11.8 ml) containing the bioluminescent system was then assayed for activity.

The reaction mixture for the bioluminescence assay was as follows:

0.1 ml extract (1:1 dilution)
1.0 ml of 0.01 M $MgSO_4$ - 0.05 M Tris buffer - pH 7.4
0.1 ml of ATP solution (1γ)

The enzyme extract and the $MgSO_4$-Tris buffer were placed in a 5 mm Beckman cuvette. The cuvette was then positioned before the photomultiplier tube and the inherent light was measured. After biasing out the inherent light electronically, the bioluminescent reaction was initiated by the injection of ATP.

Typical assay:

1γ ATP response - 124,000 mv
Inherent light - 600 mv

An experiment in which the effectiveness of arsenate and Tris buffers are compared is described below.

Two 0.25 gram portions of acetone powder were extracted, one with 0.01 M Tris-10^{-3} M Versene, pH 7.4, and the other with 0.05 M Arsenate-10^{-3} M Versene, pH 7.4. The total volume for each extraction was 6.5 ml. The reaction mixture for the assay of activity was as follows:

0.3 ml extract
0.2 ml 0.01 M $MgSO_4$ - 0.05 M Tris, pH 7.4
0.1 ml ATP (0.01 %)

See Table 1 below for the results.

Table 1

Comparison of Tris and Arsenate as Extracting Agents

Extractant	ATP Response* (mv)	Inherent Light** (mv)
Tris	850	3700
Arsenate	2200	2600

*Instrument - RCA Unit

**Inherent light - Light emission by the enzyme mixture in absence of added ATP

Extraction of the acetone powder with arsenate buffer would appear to yield extracts with considerably more activity than those obtained with Tris Versene buffer.

Although the results of the above experiment would indicate that arsenate buffer is the more effective extracting agent, a

subsequent experiment in which the concentration of Tris buffer was increased to 0.05 M indicated that Tris was equally as effective as an extracting agent and, infact, even better if only the five-second response is measured. See Table 2 below.

Table 2

Effect of Assay Time on Responses of Enzyme Extracted with Tris and Arsenate Buffer

Extractant	ATP Response		
	RCA Unit (mv)	NASA Unit	
		c/30 sec	c/5 sec
Arsenate	1400	146,192	28,806
Tris	2850	153,722	40,420

Two 250 mg portions of acetone powder were extracted, one with 0.05 M Tris-10^{-3} M Versene, pH 7.4 and the other with 0.05 M arsenate-10^{-3} M Versene, pH 7.4. The total volume for each extraction was 6 ml. The reaction mixture for the RCA Unit consisted of 0.3 ml of extract, 0.2 ml of 0.01 M $MgSO_4$, and 0.1 ml injected ATP (1×10^{-3} δ). The reaction mixture for the NASA Unit consisted of 0.2 ml of extract, 0.1 ml of 0.01 M $MgSO_4$, and 0.01 ml of injected ATP (1×10^{-3} δ).

Visual inspection of the reaction course on the oscilloscope of the RCA Unit showed that the arsenate extract gave an ATP response, which while not having as high an initial intensity as the

Tris extract, did not decay as rapidly. This would explain the comparable responses of the two extracts when the reaction was measured over a thirty-second counting period. It was observed by Strehler and Totter (11) that arsenate ion prevented the rapid decay of bioluminescence.

The effectiveness of certain other buffers as extracting agents was studied. These buffers were sodium pyrophosphate, sodium tripolyphosphate, and potassium thiocyanate. Although these buffers have been widely used as extracting agents, two of them, namely, pyrophosphate and tripolyphosphate, would be expected to interfere in the bioluminescent reaction. They would, therefore, have to be removed prior to use of the enzyme for ATP assay. The comparison of the above described buffers was carried out as follows:

Four 250 mg quantities of an acetone powder of firefly lanterns were weighed out and extracted respectively with 0.05 M arsenate buffer, pH 7.4; 0.05 M tripolyphosphate, pH 7.4; 0.05 M pyrophosphate, pH 7.4. The extraction consisted of adding 0.5 ml of the buffer ($2^{\circ}C$) to the acetone powder after which the suspension was allowed to stand, with frequent stirring, in an ice bath for 15 minutes. At the end of this time, the suspension was centrifuged at 10,000 rpm for five minutes.

The supernatant was decanted off and placed in the ice bath while the residue was re-extracted as before. After centrifugation,

this supernatant was added to the first.

After assay the crude extracts were then placed on Sephadex G-25 columns* (10 mm x 150 mm) which had been equilibrated with 0.05 M arsenate buffer, pH 7.4. The columns were eluted with 0.05 M arsenate buffer at pH 7.4. This treatment would be expected to replace the extracting buffer with arsenate buffer. The luciferase emerging from the columns was assayed for ATP response. The results are given in Table 3 below.

Table 3

Effect of Various Buffers as Extractants of Acetone Powder

	Inherent Light c/30 sec	Net ATP Response c/30 sec
Arsenate	1,079,196	600,544
Arsenate after G-25	244,811	262,736
Tripolyphosphate	1,708,099	0
Tripolyphosphate after G-25	1,569,314	16,063
Pyrophosphate	1,050,155	26,683
Pyrophosphate after G-25	949,744	5,743
Thiocyanate	59,494	18,946
Thiocyanate after G-25	58,507	61,650

*This technique will be described in a later section.

Reaction Mixture

0.1 ml of extract
0.1 ml of luciferin* (0.5 mg/ml)
0.1 ml of $MgSO_4$ (0.02 M)
0.01 ml of ATP (1×10^{-3} M)

Counting period - 30 seconds
Instrument - NASA Unit

Luciferin is required here because the natural luciferin is separated from luciferase during passage through the Sephadex column.

The results described above are somewhat difficult to interpret, especially those after passage of the extracts through the Sephadex G-25 columns. In view of the work of McElroy, et al. (3), in which they reported that the base line level of bioluminescence was increased by the addition of pyrophosphate and tripolyphosphate, the high inherent light level of the non-chromatographed extracts prepared with the compounds is not surprising. The noteworthy observations here are: (1) the inherent light level of the extracts prepared using tripolyphosphate and pyrophosphate is not reduced by passage through Sephadex G-25, a procedure which effectively removes any low molecular weight compounds from the enzyme solution, and (2) the inhibition of light response with these two buffers on the

*The preparation of luciferin is described in Section III, D.

addition of ATP. The results seen with thiocyanate are difficult to explain but, in any event, the responses with it were too inhibited to justify further consideration of this extractant.

One possible reason for the continued high inherent light levels in the tripolyphosphate and pyrophosphate extracts after chromatography is that the buffer ions are so tightly bound to protein material that they are not removed. The inhibitory effects of pyrophosphate may be explained as being caused by pyrophosphate forcing the first reaction step to the left since pyrophosphate is a product of the first step. This would, however, not be the case with tripolyphosphate. This would indicate the possibility that although there is more active enzyme available for reaction, that there is a product released by the tripolyphosphate which competes with added ATP for luciferase. As will be discussed later, it is believed that this competing product is ATP which is in a state more favorable for reaction with the enzyme than exogenous ATP.

D. Preparation of Luciferin

One of the major limiting factors in the ultimate sensitivity of the bioluminescent reaction is inherent light. The assumption which has governed the experiments for its elimination has been that it is caused by the presence of bound endogenous ATP. Certain experiments which will be described later provide some

confirmation of this assumption.

The most obvious approach to the removal of inherent light, if our basic assumption was valid, was the removal of ATP and ATP precursors from the enzyme extract. As any purification of luciferase will result in the loss of luciferin, it was necessary to set up techniques for its preparation. These are described below.

It was first attempted to isolate luciferin from the crude enzyme extract described above. This was carried out using a method described by Bitler and McElroy (6). The procedure is given below.

Acetone powder (2.3 grams) in 23 ml of water was sonically disrupted for five minutes in an ice bath. The resulting slurry was then centrifuged at 10,000 rpm for ten minutes. The pH of the supernatant was adjusted to 3.5 with 0.5 N HCl, and the resulting precipitate was removed by centrifugation at 10,000 rpm for ten minutes. The supernatant (45 ml) was extracted four times with a total volume of 44.8 ml of ethyl acetate. The emulsion which formed on mixing was broken by centrifugation in the clinical centrifuge at 1700 x G for 15 minutes. The remaining interface solids were returned each time to the water phase.

The pooled ethyl acetate fraction was washed with 1.64 ml of water at pH 3.5 and the water removed by centrifugation in the clinical centrifuge at maximum speed for 15 minutes. The extract was then taken to dryness under vacuum at 25°C using a flash evaporator.

The dry residue was taken up in 11 ml of water at pH 8.3.

The crude luciferin prepared above was then purified by partition chromatography using a column described below.

A mixture of butanol (1240 ml), chloroform (138 ml) and water (459 ml) was prepared and allowed to equilibrate. The mixture was separated into two phases, a butanol chloroform phase, and a water phase (adjusted to pH 7 with 0.1 N NaOH).

Celite (120 grams) was washed with 500 ml of 6N HCl and then with sufficient water to remove excess acid. The celite was then oven dried at 130°C for three hours.

The dried celite was mixed with 60 ml of the water phase prepared above. This powdery mixture was then tightly packed by aliquots into a chromatography column (50 mm x 500 mm) using a close fitting tamper.

Luciferin (5 ml) after adjusting to pH 7.0 $\pm$ 0.1N HCl was mixed with 10 grams of dried celite. The mixture was placed on top of the celite column.

The column was then eluted with the butanol-chloroform mixture. The rate of elution was 150 ml/hour with 10 ml fractions being collected.

The fractions were assayed spectrophotometrically at 327 mμ (absorption maxima of luciferin). See Figure 3.

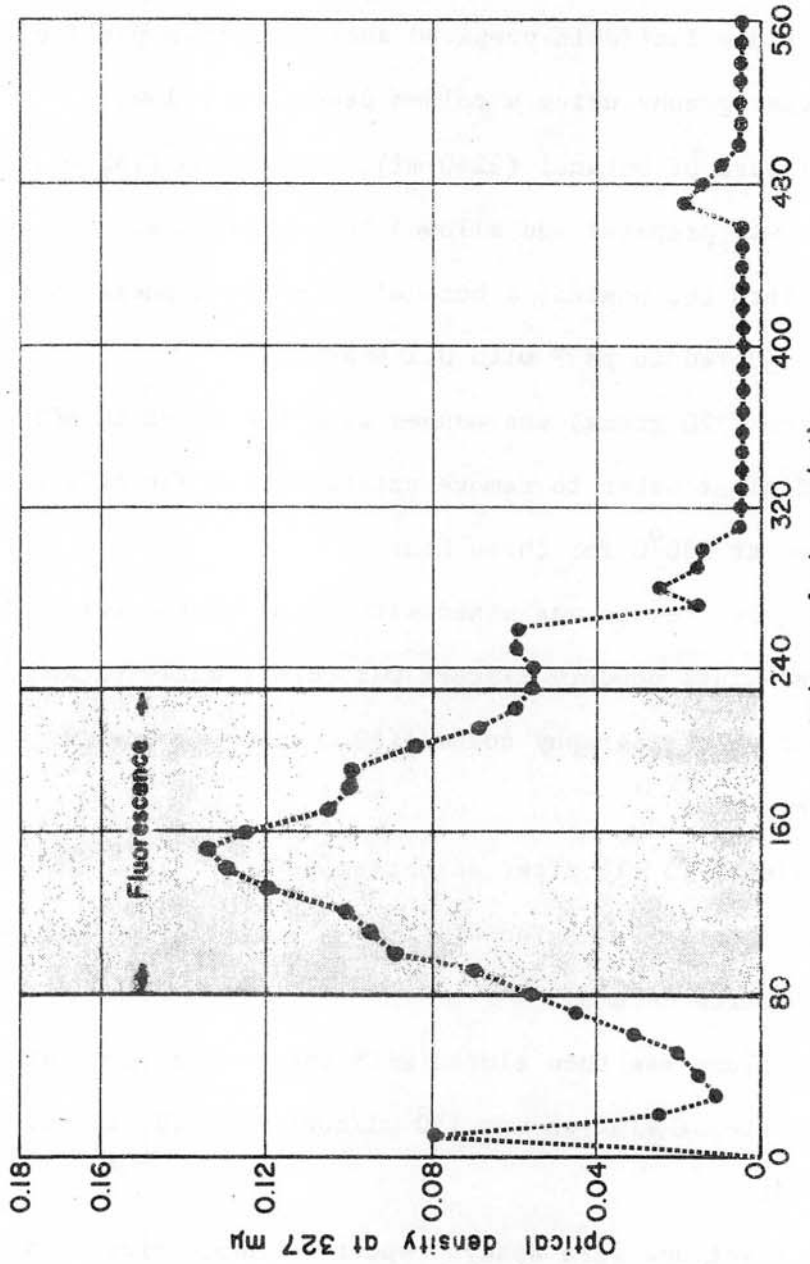


Figure No. 3 — Chromatography of natural luciferin.

The pooled fractions were extracted with 100 ml of water at pH 8.3. The water phase was shown to be active in the bioluminescent reaction.

It was recognized early that it would require an excessive amount of natural material to isolate the quantities of luciferin necessary for maximal light emission by given quantities of ATP. For this reason, it was decided to chemically synthesize luciferin. The procedure employed is one developed by White, et al. (12). It is described in detail in Appendix I. The determination of the optimal luciferin concentration will be described later.

E. Purification of Luciferase

1. Column Chromatography

a. Sephadex Molecular Sieve Gels

A technique which appeared promising as a means for removal of ATP was molecular-sieve chromatography. Separation by this type of chromatography is dependent on molecular dimension in contrast to ion-exchange chromatography which is a function of molecular charge effects. The most widely used stationary phase for molecular-sieve chromatography are artifically cross-linked polyglucose (dextran) chains. Standardized cross-linked dextrans are obtainable from Pharmacia Fine Chemicals under the trade name of Sephadex. The amount of cross-linking in this three dimensional network of dextran

chains determines the maximal size of a molecule which may penetrate into the interior of the network, i.e., the greater the cross-linkage, the smaller is the maximal size of the penetrating molecule. Because of its hydrophilic character, Sephadex swells considerably when placed in water, forming gel grains. The degree of swelling is determined by the degree of cross-linking.

When a solution containing a mixture of low and high molecular size species is introduced onto the surface of a column packed with a Sephadex gel, the small molecules enter the pores of the gel while the large molecules are confined to the liquid medium external to the gel proper. Elution with either water or a salt solution brings about separation of the two sizes of molecules. The small molecules by virtue of retardation within the gel are eluted after the larger molecules.

The following types of Sephadex molecular sieve gels have been used in these investigations.

Sephadex G-25 - Excludes molecules with a molecular weight greater than 5,000.

Sephadex G-50 - Excludes molecules with a molecular weight greater than 10,000.

Sephadex G-100 - Excludes molecules with a molecular weight greater than 100,000.

Sephadex G-200 - Excludes molecules with a molecular weight

greater than 200,000.

b. Preparation of Sephadex Gels

(1) Sephadex G-25

Sephadex G-25 (25 grams) was placed in an excess of 0.01 M Tris-10^{-3} M Versene-10^{-3} M $MgSO_4$, pH 7.4 and allowed to swell overnight.

A water jacketed column maintained at 5°C (500 mm x 25 mm) was packed with the gel to a height of 340 mm.

The volume of eluent necessary to elute an excluded molecule from the column (void volume) was determined by the use of hemoglobin. After placing hemoglobin on the surface of the column, Tris-Versene-$MgSO_4$ (as used in preparation of gel) was passed through until the red colored protein had left the column. The void volume was 82.5 ml.

(2) Sephadex G-50

Fifteen grams of Sephadex G-50 beads were allowed to swell overnight in 500 ml of 0.1 M Tris, pH 7.4 containing Versene at a concentration of 1×10^{-3} M. A chromatographic column (20 mm x 250 mm) was packed to a height of 175 mm with the Sephadex gel suspension. The column was water jacketed and kept at 5°C.

(3) Sephadex G-100

Sephadex G-100 (20 grams) was placed in an excess of 0.01 M

Tris-0.001 M $MgSO_4$-0.001 M Versene, pH 7.4 and allowed to swell for 48 hours.

A water jacketed column (100 cm x 25 mm) was packed to a height of 70 cm with the G-100 gel. An additional 10 cm of Sephadex G-25 was layered upon the surface of the G-100 gel. The column was then washed with 100 ml of buffer solution used in preparation of the gel.

(4) Sephadex G-200

The same procedure is followed as for the G-100.

(5) DEAE-Sephadex

DEAE-Sephadex is obtained by the introduction of diethylaminoethyl groups into Sephadex. These positively charged groups form ionic bonds with anions. The anions can, in turn, be displaced by anionic buffers with the ease of displacement being a specific property of the bound anion. Thus, a mixture of molecules bound to the DEAE-Sephadex may be separated on the basis of the rate of displacement by the eluent anionic buffer.

DEAE-Sephadex was prepared for use as follows:

Five grams of DEAE-Sephadex A-50 were stirred into 300 ml of distilled water. After one hour of standing, the excess water was decanted from the solution, and then the remaining solution was brought up to 300 ml.

The swollen gel was washed in a Buchner funnel with 500 ml of 0.5N HCl, followed by 500 ml of distilled water. It was then washed with 500 ml of 0.5N NaOH, followed by 500 ml of distilled water. The gel was neutralized to pH 7.4 with 0.5N HCl. It was finally washed with 500 ml of 0.01 M Tris buffer.

DEAE-Sephadex A-50 was packed into a column (20 mm x 250 mm) to a height of 180 mm.

c. Fractionation with Various Gels

Eluent fractions from the various columns used in these studies were collected using a Buchler automatic fraction collector. Provisions were made for continuous cooling of the fractions (5°C). Results obtained with the various gels are described below.

(1) Fractionation of Acetone Powder Extracts with Sephadex G-50

The purpose of these experiments was to determine the effectiveness of Sephadex G-50 in the separation of luciferase from luciferin, and also its effect on inherent light.

The gel was equilibrated with 0.1 M Tris (pH 7.4) - 0.001 M Versene. The column was packed to 175 mm and 15 ml of acetone powder extract was added to the column. The eluent was the same solution as that used for the equilibration.

Fractions were collected in 3 ml volumes and assayed for ATP activity. The fractions were also assayed spectrophotometrically

for protein using the Folin-Ciocalten method.

Forty-four 3 ml cuts of the fraction were collected during a four-hour period.

By U.V. detection of fluorescence, it was observed that tubes #23-44 contained the luciferin fraction.

All of the fractions were frozen and stored overnight. The following reaction mixture was used for the ATP assay:

0.1 ml of fraction
0.5 ml of luciferin (prepared from firefly)
0.4 ml of 0.05 M Tris-0.01 M $MgSO_4$ solution
0.1 ml of ATP (10%) injected

Instrument - RCA Unit

The results are described in Figure 4. It can be noted that the luciferase activity is associated with the fractions containing the highest quantity of protein.

(2) Fractionation of Acetone Powder Extract by Sephadex G-50 and DEAE-Sephadex A-50

DEAE-Sephadex was employed here in an attempt to separate luciferase from contaminating proteins.

Acetone powder extract (8 ml) was added to a Sephadex G-50 column and eluted with 0.01 M Tris-0.001 M Versene. Rate of flow of the column was 50 ml/hour.

The fractions were assayed for ATP activity.

The results are shown in Tables 4 and 5.

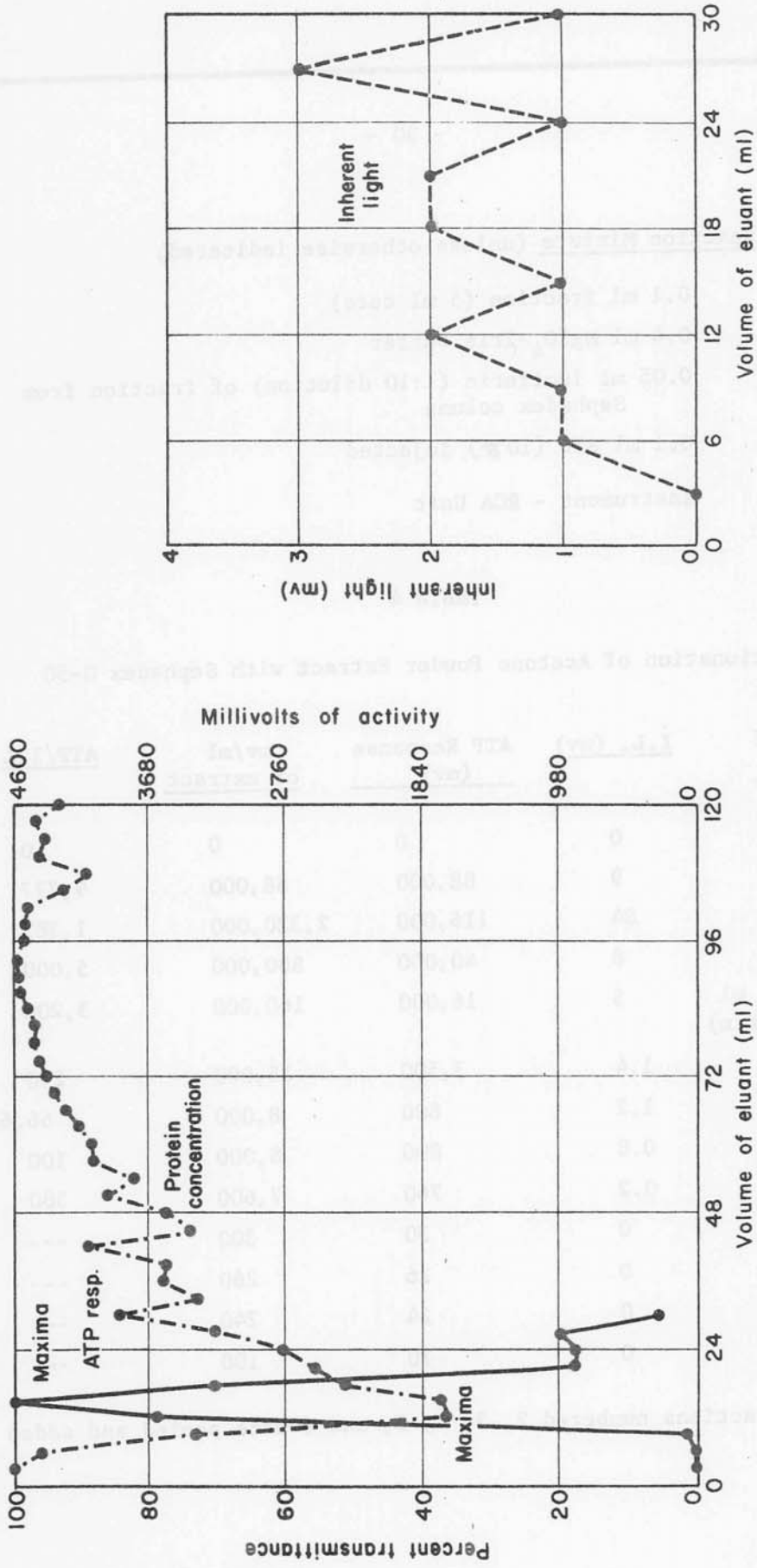


Figure No. 4 – The fractionation of a firefly extract with Sephadex G-50.

Reaction Mixture (unless otherwise indicated)

0.1 ml fraction (5 ml cuts)

0.4 ml $MgSO_4$-Tris buffer

0.05 ml luciferin (1:10 dilution) of fraction from Sephadex column

0.1 ml ATP (10 γ) injected

Instrument - RCA Unit

Table 4

Fractionation of Acetone Powder Extract with Sephadex G-50

	Volume of Enzyme	I.L. (mv)	ATP Response (mv)	mv/ml of extract	ATP/I.L.
1	0.1 ml	0	0	0	0
2	0.1 ml	9	88,000	88,000	9,777
3	0.05 ml	84	116,000	2,320,000	1,381
4	0.05 ml	8	40,000	800,000	5,000
5	(+ 0.06 ml luciferin)	5	16,000	160,000	3,200
6	0.1 ml	1.4	3,500	35,000	250
7	0.1 ml	1.2	800	8,000	66.6
8	0.1 ml	0.8	800	8,000	100
9	0.1 ml	0.2	760	7,600	380
10	0.1 ml	0	30	300	---
11	0.1 ml	0	26	260	---
12	0.1 ml	0	24	240	---
13	0.1 ml	0	10	100	---

Fractions numbered 2, 3, 4, 5, and 6 were pooled and added

to enough dry Sephadex G-50 to form a thick slurry. The slurry was allowed to stand for ten minutes and then was centrifuged at maximum speed for ten minutes. Volume of the supernatant was 4 ml.

The supernatant was assayed for ATP activity.

Reaction Mixture

Concentrated enzyme (as indicated)
0.05 ml luciferin (1:10)
0.04 ml $MgSO_4$-Tris buffer (0.05 M)
0.01 ml ATP (10 γ) injected

Table 5

Assay of Concentrated Active Fractions from Sephadex G-50

	Sample	I.L. (mv)	ATP (mv)	mv/ml of extract	ATP/I.L.
#1	0.05 ml concentrated extract	6.2	116,000	2,320,000	18709
#2	0.05 ml of 1:10 dilution of above	0.2	4,000	800,000	2000
#3	0.05 ml* re-extracted residue	12.0	50,000	1,000,000	4160

*Residue of the pooled extracts were re-extracted with 4 ml of 0.01 M Tris-0.001 M Versene and centrifuged at maximum speed for ten minutes.

The two supernatants from the concentration process were pooled, added to a DEAE-Sephadex A-50 column, and eluted with a gradient elution of 1 M Tris-0.001 M Versene (500 ml) into 0.01 M

Versene (500 ml).

The fractions were assayed for ATP activity.

Reaction Mixture

0.2 ml fraction (5 ml cuts)
0.005 ml luciferin (1:10)
0.3 ml $MgSO_4$-Tris buffer
0.1 ml ATP (10%) injected

Instrument - RCA Unit

See Figure 5 for results.

Although a rather sharp separation of the luciferase activity was achieved by the use of DEAE, the long period of time required for the fractionation (42 hours) makes it somewhat impractical for routine use.

(3) Sephadex G-25-G-200 Fractionation

Sephadex G-25-G-200 was used here for protein separation. Acetone powder (0.5 grams) was extracted with a total volume of 10 ml of 0.01 Tris-0.001 M Versene-0.001 M $MgSO_4$. The yield was 7.4 ml.

The 7.4 ml was placed on a Sephadex G-25-G-200 column and eluted with the Tris-Versene-$MgSO_4$ solutions. Flow rate of the column was 20 ml/hour.

The fractions were assayed for ATP activity and protein content. See Figure 6 for results.

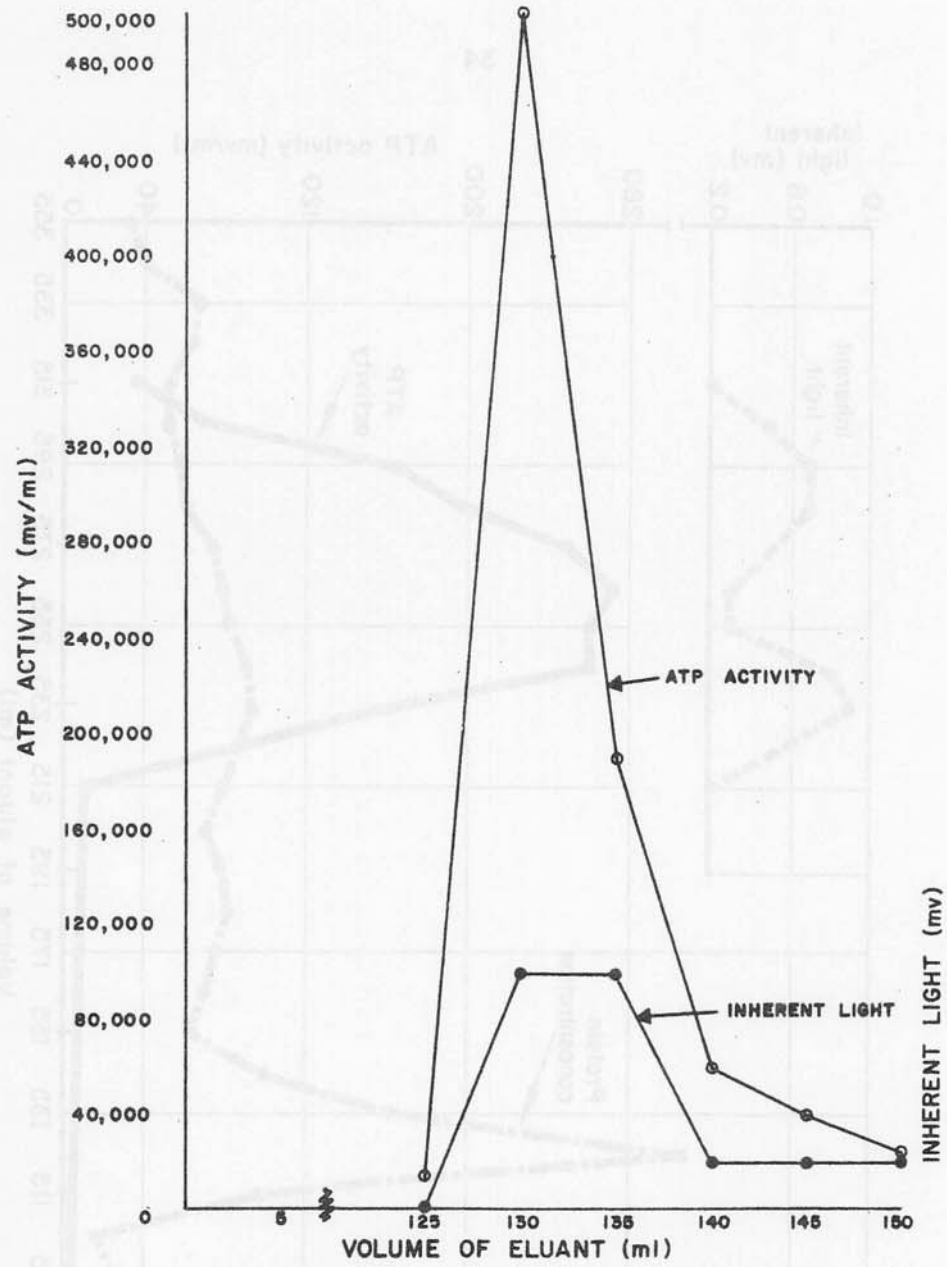


Fig. 5 – Fractionation of firefly extract with Sephadex G-50 and DEAE Sephadex A-50.

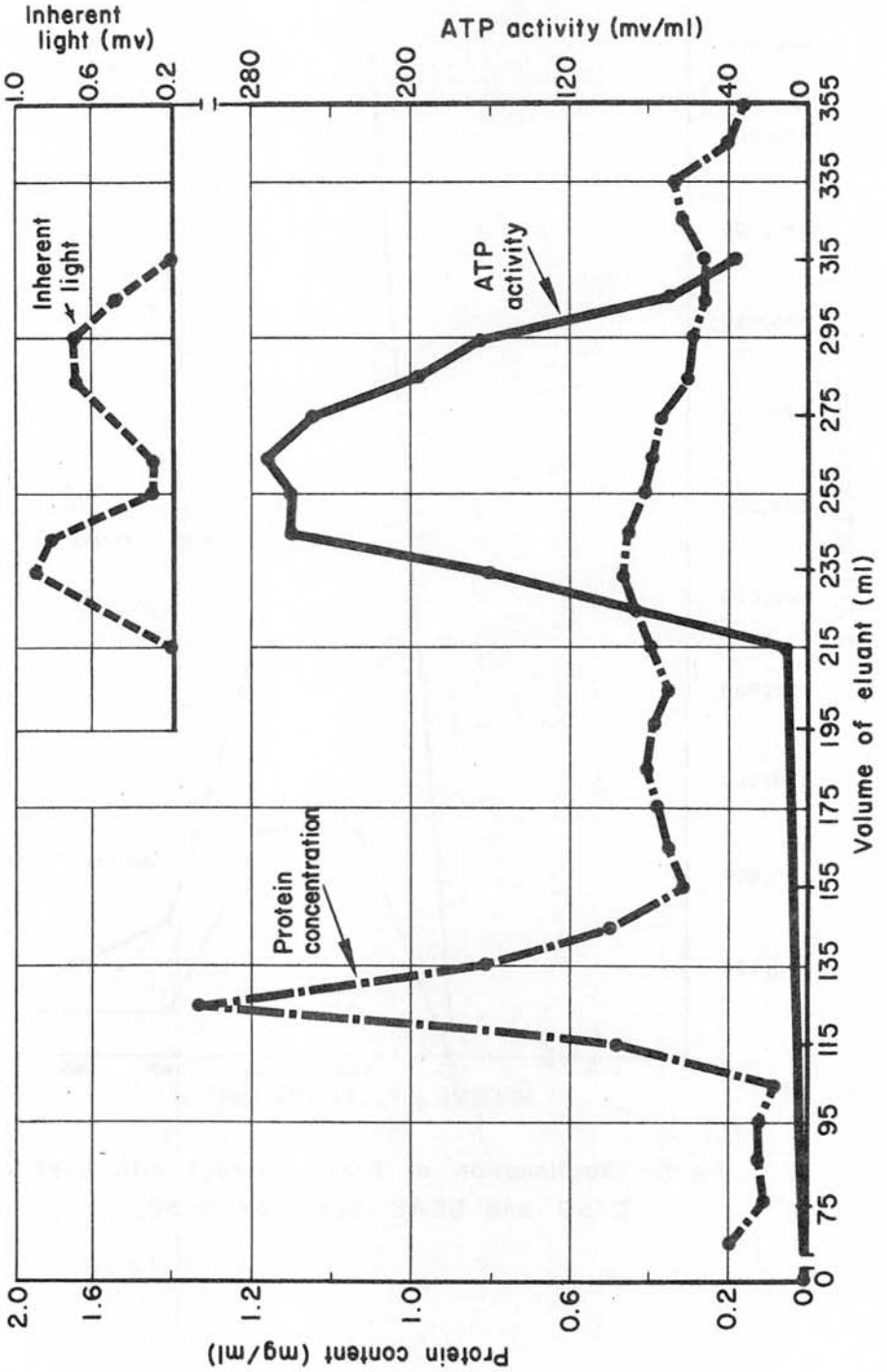


Figure No. 6 — Fractionation of firefly extract with Sephadex G -200.

A primary difference between the elution curve shown here and that seen using Sephadex G-50 is that the maximal luciferase activity is no longer associated with the maximum protein concentration. This in itself indicates a higher degree of purification. The curve for inherent light which shows a minima coinciding with the maximum ATP response should not be considered valid as subsequent fractionations repeatedly showed that the maximum inherent light coincided with the maximum ATP response.

Complete removal of luciferase from the Sephadex G-200 column required approximately 18 hours.

(4) Fractionation of Acetone Powder Extract with Sephadex G-100

An acetone powder extract (5 ml from 500 mg of acetone powder) was placed on the Sephadex G-100 column. The column was eluted with 0.05 M Tris-Versene-$MgSO_4$ which was previously described.

Reaction Mixture for Assay

0.1 ml of luciferin (1 mg/ml)
0.1 ml of $MgSO_4$ (0.01 M)
0.1 ml of enzyme extract
0.01 ml of ATP (1×10^{-3} γ)

The results are described in Figure 7. The assay was carried out with the NASA Unit. The elution of luciferase was complete in six hours. In view of the high activity of the fractions obtained using Sephadex G-100 and the short time period required,

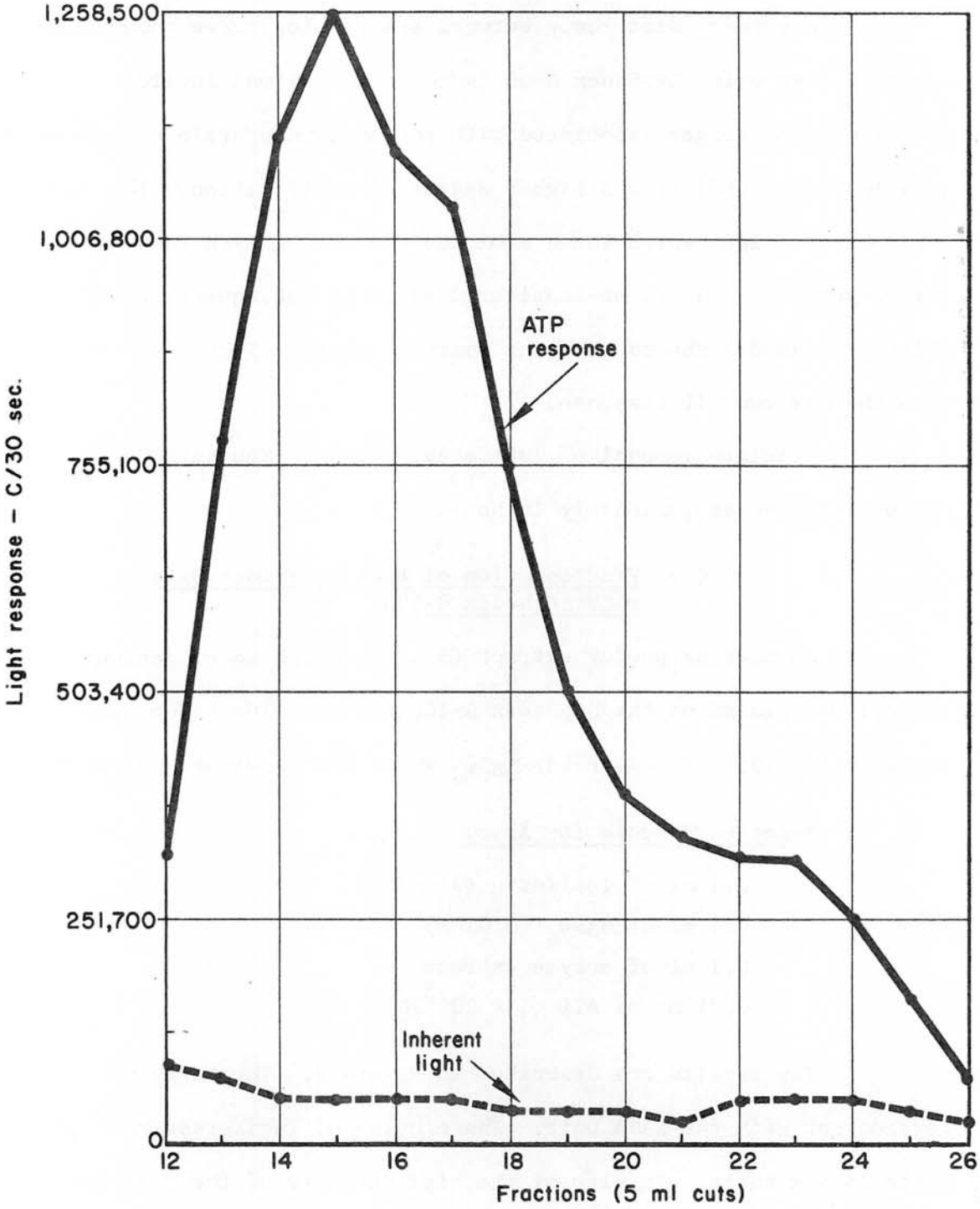


Figure No. 7 – Fractionation of firefly extract with Sephadex G-100

this gel is considered the best for routine use. However, if at a future date, it is decided to attempt a higher degree of luciferase purification, it may be necessary to employ gels such as Sephadex G-200 and DEAE Sephadex.

F. Removal of Inherent Light

As has been previously stated, it is believed by the writer that inherent light is caused by the presence of endogenous ATP. The observations that inherent light was not removed by Sephadex chromatography would indicate that the factors responsible for inherent light are bound to protein material in a tight fashion. With this as a rationale, a number of experiments was designed to attempt to remove contaminating ATP. They will be described below.

1. Crystallization of Luciferase

The effects of crystallization of luciferase upon the magnitude of inherent light were investigated here. The procedure described by Green and McElroy (10) was employed.

Acetone powder (5 grams) was extracted with 17 ml of 1×10^{-3} M Versene and 1.7 ml of 1N NaOH. The crude suspension after standing for 10 to 15 minutes was centrifuged at 3,000 rpm for ten minutes. The residue was re-extracted twice with 10 ml portions of 1×10^{-3} M Versene and 0.17 ml of 1N NaOH. The combined extracts were centrifuged at 13,000 rpm for 30 minutes. The volume

of the supernatant was 28 ml. The enzyme and the luciferin remained in the supernatant.

Calcium phosphate gel, at pH 7.5, was measured and centrifuged at 10,000 rpm for ten minutes, and the supernatant was discarded. Three parts of the crude enzyme extract (28 ml) were mixed with one part of prepared calcium phosphate gel (9.3 ml containing 16 to 17 mg of the gel/ml). The mixture was centrifuged at 10,000 rpm for ten minutes.

The supernatant (26 ml) from the first gel treatment was mixed, as described in the preceding paragraph, with 2 and 1/2 times the volume of calcium phosphate gel (10.4 ml). The luciferase was absorbed, and the luciferin remained in the supernatant. The supernatant was saved for luciferin recovery.

The luciferase was eluted from the gel with cold 20% saturated (0.82 M) $(NH_4)_2SO_4$ in 0.001 M Versene at pH 8.0. Three elutions of 17 ml each were made by centrifuging at 10,000 rpm for ten minutes. The combined eluent totaled 52 ml, and was adjusted to pH 8.0.

A 40% saturated fraction of $(NH_4)_2SO_4$ (12.64 grams) was made of the 52 ml of solution. The solution stood at room temperature for ten minutes, and was then centrifuged at 13,000 rpm for 15 minutes. The supernatant (56 ml) was poured off. The tubes were allowed to drain for five minutes, and then the precipitates were dissolved in a total of 5 ml of 0.001 M Versene - pH 8.0, and set

aside in ice.

The 56 ml of supernatant were taken up to 50% saturation with $(NH_4)_2SO_4$ (3.53 grams) and treated in the same manner as the 40% solution. Subsequent fractions of $(NH_4)_2SO_4$ at 60% (3.63 grams in 55 ml) and 70% (3.8 grams in 56 ml) were prepared and treated as the 40% and 50% concentrations.

The fractions were assayed for inherent light and ATP activity, and for protein content. Inherent light and ATP response were determined using the following reaction mixture:

0.01 ml of luciferin (purified)
0.1 ml of enzyme extract
0.9 ml of $MgSO_4$-Tris
0.1 ml of ATP (10 γ)

Protein content was determined spectrophotometrically at 280 mμ. See Figure 8 for results of the assays.

The next step in the procedure was the crystallization of the luciferase by dialysis. Although the 40% fraction exhibited the greatest activity, the 50% and 60% fractions were also selected for dialysis.

Each of the three fractions was placed in separate dialysis bags, and the bags suspended in a low salt solution of 0.001 M Versene, 0.01 M NaCl, and 0.002 M $NaHPO_4$ - pH 7.3. The fractions were dialyzed overnight. The contents of each bag were centrifuged for ten minutes at 10,000 rpm. The crystalline precipitate (present

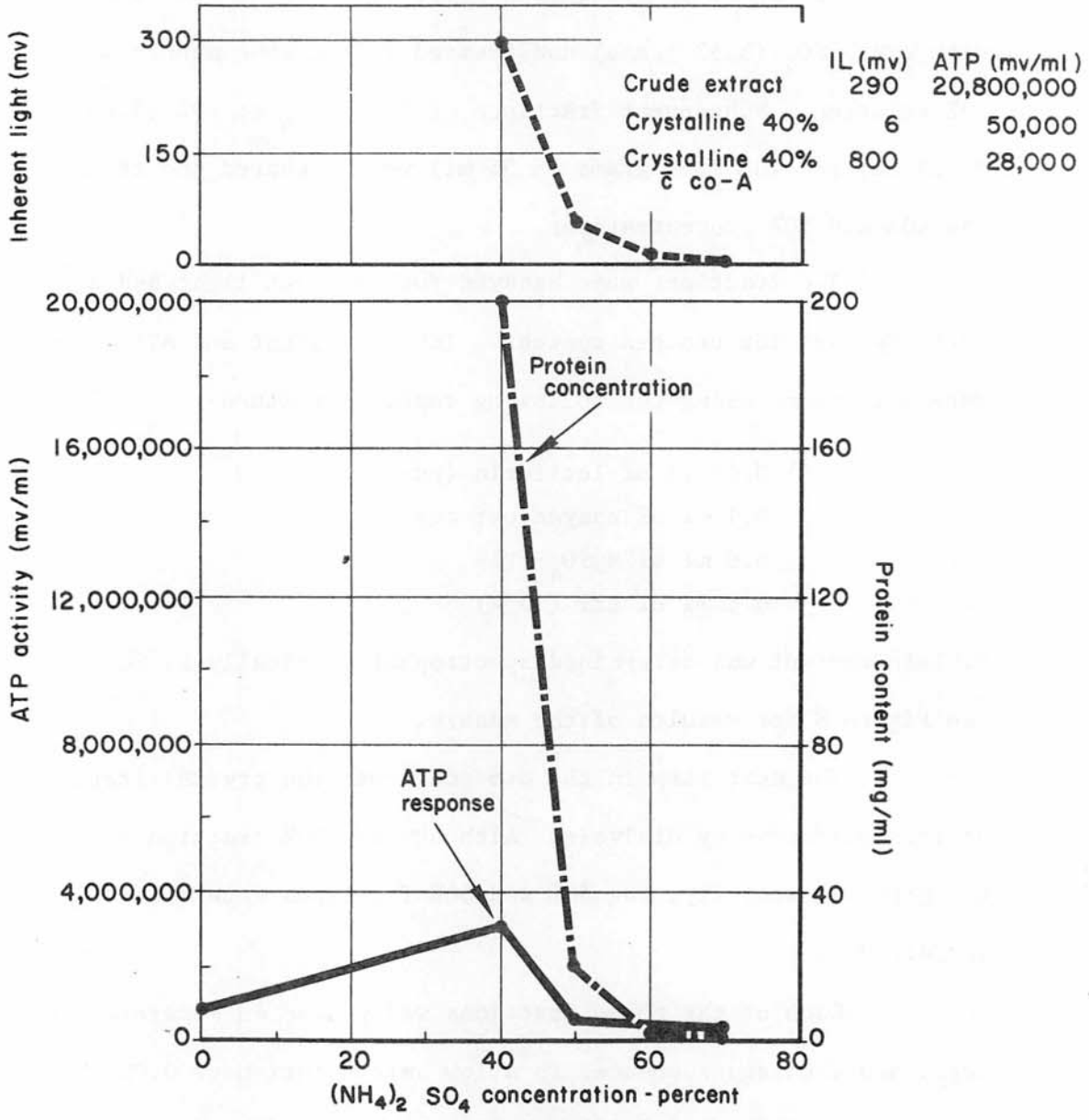


Figure No. 8 – Effect of luciferase crystallization on ATP response and inherent light.

in the 40% only) was taken up with 5 ml of a 0.001 M Versene - 0.4 M $(NH_4)_2SO_4$ solution - pH 8.0.

The 40% crystalline preparation was assayed for inherent light and ATP activity. The crystalline preparation was tested alone, and then tested with CoA. See Figure 8.

Although a considerable decrease in the inherent light is observed on the crystallization of luciferase, there is also a concomitant decrease in the ATP response. This renders an assessment of this technique difficult as the decrease in inherent light could be caused by either a decrease in inherent light per se or merely the decrease in the overall activity. The large amount of starting material required for crystallization made further studies along these lines impractical.

2. The Crystallization of Luciferase Fractions From the Sephadex G-50 Column

On the basis of luciferase being a euglobulin and thus insoluble in very dilute salt solutions, it was decided to attempt a selective crystallization of the luciferase present in the protein fraction from a Sephadex G-50 column.

Acetone powder extract (2.5 ml) was placed on a Sephadex G-50 column (pre-washed with 0.01 M Tris-0.001 M Versene) and eluted with the same Tris-Versene solution. The fractions were assayed for inherent light and ATP activity. See Figure 9.

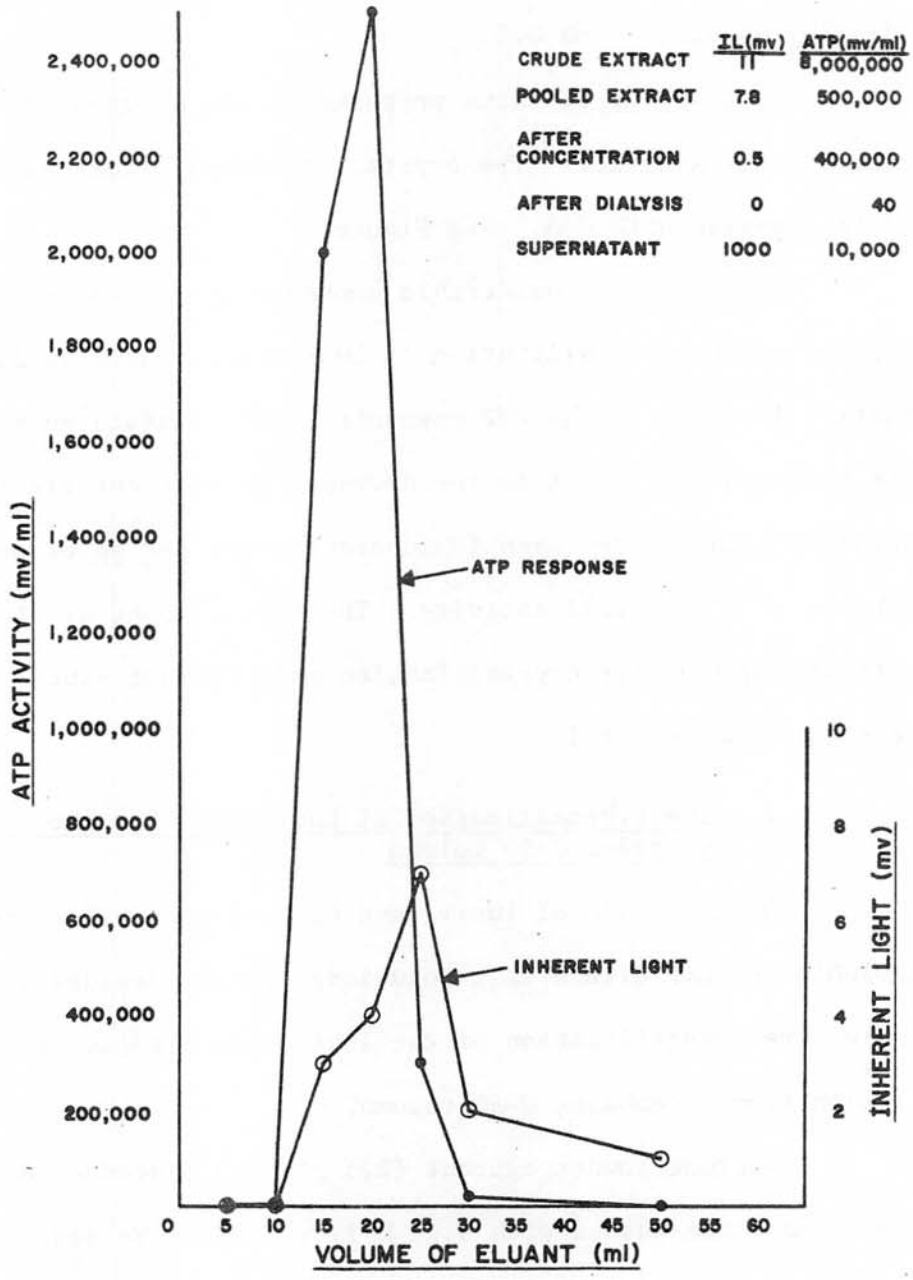


Figure No. 9—Crystallization of luciferase after purification on Sephadex G-50.

Eluent volumes 15, 20, and 25 milliliters were the most active fractions, so they were pooled for dialysis. The pooled extracts were centrifuged at 10,000 rpm for ten minutes; there was no precipitate. See Figure 9 for assay results. The solution was then concentrated down from 10.5 ml to 3 ml.

The concentration process was as follows:

Ficoll (5 grams) was placed in a dialysis bag which was placed in a 50 ml graduated cylinder. The luciferase fraction was poured onto the dialysis bag in the cylinder. The volume was reduced from 10.5 ml to 3 ml after two hours. See assay results in Figure 9.

The 3 ml of concentrated luciferase were placed in a dialysis bag and placed into a 0.001 M Versene bath. It was left to dialyze overnight. The contents of the dialysis bag were centrifuged at 10,000 rpm for ten minutes. The resulting crystalline precipitate was taken up with 5 ml of a 0.001 Versene - 0.4 M $(NH_4)_2SO_4$ solution - pH 8.0. The supernatant was also saved for analysis.

The reaction mixture for each ATP assay was:

0.01 ml of enzyme (except 0.005 ml crude extract)

0.5 ml of $MgSO_4$-Tris buffer

0.01 ml of luciferin

0.1 ml ATP (10 γ) injected

The results are described in Figure 9.

It is seen here that the precipitate after dialysis was devoid of activity. It was concluded that the protein concentration of the dialyzed solution was insufficient for crystallization.

3. Ammonium Sulfate Fractionation

In this experiment, luciferase was purified without going through the calcium phosphate gel treatment as was done previously. The dialysis treatment is also omitted.

One gram of acetone powder was extracted with a total volume of 10 ml of 0.01 M Tris-0.001 M Versene-0.001 M $MgSO_4$. There was a yield of 8.9 ml of extract.

To 8.4 ml of the extract, 3.9668 grams of solid $(NH_4)_2SO_4$ were added for a 70% saturation. This preparation stood at room temperature for ten minutes, and was then centrifuged at 10,000 rpm for ten minutes. There were 4 ml of supernatant.

The supernate from the 70% saturated solution of $(NH_4)_2SO_4$ (approximately 3.5 ml) was placed on a Sephadex G-50 column, and eluted with 0.01 M Tris-0.001 M Versene-0.001 M $MgSO_4$.

The crude extract, the supernatant, and the protein fractions were assayed for ATP activity.

Reaction Mixture

0.1 ml sample (0.1 ml of 1:10 crude extract and 0.05 ml of supernatant

0.1 ml luciferin

0.4 ml $MgSO_4$-Tris

0.1 ml ATP (1%)

See Figure 10 for results.

Fractions 2 through 9 (34 ml) were pooled and 5.984 grams of solid $(NH_4)_2SO_4$ added for 30% saturation. The solution was mixed and stood at room temperature for ten minutes. It was then centrifuged at 13,000 rpm for 15 minutes. There was no precipitate.

To this 30% saturated solution (37 ml), 2.294 grams of $(NH_4)_2SO_4$ were added to produce 40% saturation. This mixture was treated the same as was the 30% saturated solution. There was very little precipitate. The precipitate was drained on filter paper and was taken up with 5 ml of 0.4 M $(NH_4)_2SO_4$-0.001 M $MgSO_4$. This solution did not need centrifugation.

To the supernate (38 ml) of the 40% saturation, 2.394 grams of $(NH_4)_2SO_4$ were added to produce 50% saturation. This solution and the resulting precipitate were treated as those of the 40% saturation.

To the supernate (38 ml) of the 50% saturation, 2.508 grams of $(NH_4)_2SO_4$ were added to produce 60% saturation. It was treated as the preceding solutions and precipitates.

The solutions of the precipitates were assayed for ATP activity.

Reaction Mixture

0.1 ml sample	0.1 ml luciferin
0.4 ml $MgSO_4$-Tris	0.1 ml of ATP (1 γ) injected

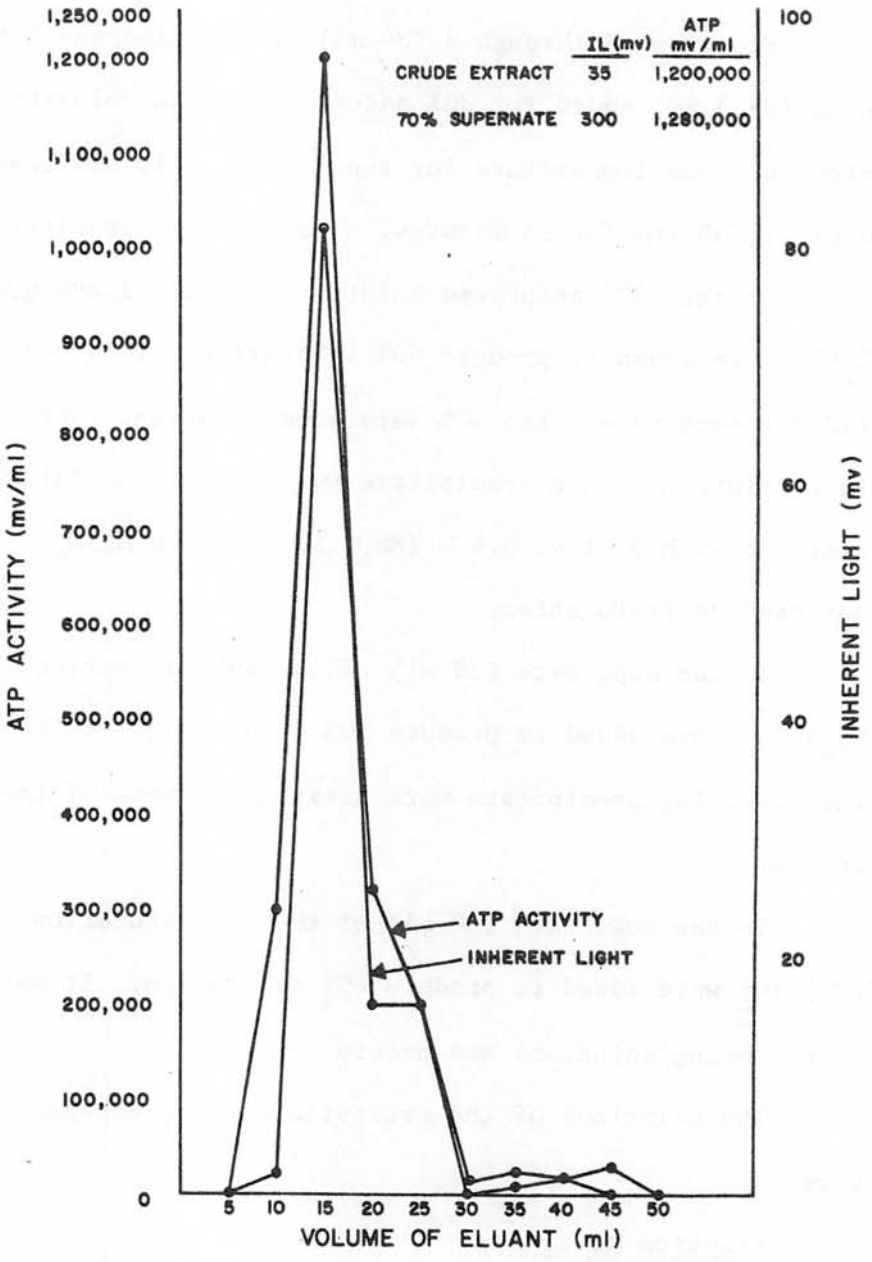


Figure No. 10 – Purification of luciferase with ammonium sulfate.

Table 6

Effect of Ammonium Sulfate Fractionation on Inherent Light and ATP Response

Sample	I.L. (mv)	ATP (mv)	mv/ml	ATP/I.L.
40%	0.1	500	5,000	5,000
50%	3.1	8,000	80,000	2,600
60%	0.5	5,000	50,000	10,000

The great loss in enzymatic activity during the final ammonium sulfate fractionation renders this procedure of little value.

4. Effect of Certain ATP Requiring Enzymes on Inherent Light

a. Potato Apyrase

This enzyme found in potatoes catalyzes the hydrolysis of ATP to give pyrophosphate and AMP (adenosine monophosphate). The preparation and results are described below.

About 250 grams of diced potatoes were homogenized for five minutes in a Waring Blendor with 250 ml of neutralized 0.04 M KCN, pressed through cheesecloth, and the extract centrifuged 4°C at 10,000 rpm for ten minutes. The enzyme was precipitated by the addition, with mechanical stirring, of 80.9 grams of powdered $(NH_4)_2SO_4$. The total volume of the mixture was 330 ml. The precipitate material was centrifuged at 10,000 rpm for ten minutes. The precipitate was allowed to drain for five minutes, and was then taken up with 5 ml

of 0.4 M $(NH_4)_2SO_4$-0.001 M Versene-0.001 M $MgSO_4$.

Reaction Mixture

0.2 ml of luciferase (1 mg/ml)
0.1 ml of $MgSO_4$-Tris
0.2 ml of extract from Sephadex G-25
0.1 ml of ATP (1×10^{-2} %)
0.1 ml of apyrase solution as prepared above

Table 7

Effect of Apyrase on Inherent Light and ATP Response

	Inherent Light (mv)	ATP Response (mv)	ATP/I.L.
Control (no apyrase)	250	20,000	80
Apyrase*	0	9,000	--

*Reaction mixture containing apyrase was incubated at room temperature for ten minutes, before initiation of the reaction.

It was conceivable, based on the results previously described, that apyrase could be employed as a means of eliminating inherent light. It is, of course, necessary to remove apyrase from luciferase prior to assay in order to obtain maximum ATP response. It was decided to attempt the separation using a Sephadex G-25-200 column.

An acetone powder extract (5 ml) was prepared. The extract was incubated with 1 ml of the apyrase prepared as described previously.

After incubation for one hour at room temperature, the inherent light was 0. The solution was then placed on the column and eluted with 0.05 M arsenate buffer, pH 7.4 (10 ml fractions). The results are given below.

Reaction Mixture

0.2 ml of luciferin (1 mg/ml)
0.1 ml of $MgSO_4$-Tris
0.4 ml of extract
0.1 ml of ATP (1×10^{-2} M)

Table 8

ATP Response and Inherent Light After Apyrase Removal

Fraction No.	Inherent Light (mv)	ATP (mv)	ATP/I.L.
#20	0	0	---
#21	5	600	140
#22	20	1000	50
#23	20	5000	250
#24	35	5000	140
#25	40	5000	125
#26	20	5000	250
#28	20	2000	1000
#30	5	800	160

The most obvious conclusion which may be derived from the studies reported here is that the factors responsible for inherent light are associated quite closely with the luciferase fraction of

the firefly lantern. This is based on the presence of inherent light in systems containing purified luciferase and synthetic luciferin. The experiments with apyrase indicate rather conclusively that the responsible factor for inherent light is ATP. The observation of a return of inherent light on removal of apyrase from the system points to a tight binding of the ATP to protein surface. It can be further hypothesized that there is a slow constant rate of dissocation of ATP from the protein surface. It is only the free ATP which gives rise to inherent light or is hydrolyzed by apyrase.

b. Myokinase

Myokinase catalyzes the reversible reaction between ATP and AMP to give two molecules of ADP. The results obtained by the use of this enzyme during the current report period were quite similar to those of the apyrase experiments. A representative experiment is as follows:

Reaction Mixture

0.1 ml of partially purified luciferase
0.1 ml of synthetic luciferin (0.2 mg/ml)
0.1 ml of $MgSO_4$ (1×10^{-2} M)
0.1 ml of myokinase (0.05 mg) - Sigma grade
0.1 ml of AMP (1 mg/ml)
10 λ of ATP injected (1×10^{-2} γ)

A control for the above reaction mixture was prepared by substituting 0.2 ml of water for the myokinase and AMP.

Table 9

Effect of Myokinase on Inherent Light

	Inherent Light (c/5 sec)	ATP Response (c/5 sec)
Myokinase treated mixture	10	697
Control	13,464	285,273

Additional confirmation of the role of ATP in the production of inherent light is found in the above data. We have not as yet separated the myokinase from luciferase to ascertain whether or not there is a return of inherent light. It is quite obvious, however, that although there is a drastic reduction of inherent light by myokinase, one cannot assay ATP in its presence.

c. 3-phosphoglyceric kinase

This is an enzyme which catalyzes a reversible transfer of phosphate between ATP and 3-phosphoglyceric acid. Using this enzyme, the following experiment was carried out.

3-phosphoglyceric acid (0.05 M) was prepared by dissolving 200 mg of the barium salt in 2 ml of 2N HCl and adding 2 ml of 2N H_2SO_4. The mixture was centrifuged at 10,000 rpm for ten minutes, after which the supernatant was adjusted to pH 7.4 and the volume brought up to 10 ml with water. An 0.2 mg/ml solution of phosphoglyceric kinase (Sigma) was prepared in arsenate buffer (0.05 M, pH 7.4).

Reaction Mixture

0.1 ml of luciferase from Sephadex G-200
0.2 ml of luciferin (0.5 mg/ml)
0.05 ml of 3-phosphoglyceric kinase
0.05 ml of phosphoglyceric acid
0.010 ml of ATP (1×10^{-3} M)

A control consisted of the above except that 0.1 ml of water was used instead of 3-phosphoglyceric kinase and phosphoglyceric acid.

Counting time was 30 seconds - NASA Unit.

Table 10

Effect of Phosphoglyceric Kinase on Inherent Light

	Inherent Light (c/30 sec)		ATP Response (c/30 sec) at 30 min
	0 time	30 min	
Kinase	2,026	727	197,858
Control	26,496	16,000	831,227

d. Pyruvate Kinase

Pyruvate kinase brings about the transfer of the terminal phosphate of ATP to pyruvic acid. A study of the ability of this enzyme to remove inherent light is described below.

Reaction Mixture

0.1 ml of luciferase from Sephadex G-200
0.1 ml of luciferin (0.5 mg/ml)
0.1 ml of pyruvate kinase (0.1 mg/ml)
0.1 ml of sodium pyruvate (0.1 M)
0.01 ml of ATP (1×10^{-3} γ)

Counting time was 30 seconds - NASA Unit

The control contained no pyruvate kinase and pyruvate.

Table 11

Effect of Pyruvate Kinase on Inherent Light

	Inherent Light After 15 min (c/30 sec)	ATP Response After 15 min (c/30 sec)
Pyruvate kinase	837	129,048
Control	17,256	950,000

The results obtained with both 3-phosphoglyceric kinase and pyruvate kinase are essentially the same as was obtained with apyrase and myokinase. Both enzymes drastically reduced the inherent light but competed with luciferase for injected ATP.

e. Hexokinase

Hexokinase is a trans-phosphorylase which catalyzes the

transfer of the terminal phosphate of ATP to glucose to give glucose-6-phosphate and ADP. Representative data obtained with the use of this enzyme are presented below.

Reaction Mixture

0.1 ml hexokinase (0.01 mg) Sigma
0.1 ml glucose (0.2 M)
0.1 ml $MgSO_4$ (0.01 M)
0.1 ml luciferin (0.2 mg/ml)
10 λ ATP (1×10^{-2} M)

Control for the above contained water instead of glucose and hexokinase.

Table 12

Effects of Hexokinase on Inherent Light

	Inherent Light (c/5 sec)	ATP Response (c/5 sec)
Control	3,415	126,302
Hexokinase	28	110,000

(15 minutes incubation)

As was found in the case of ATPase, the inherent light returned after separation of luciferase and hexokinase on a Sephadex column. As is seen, however, in the representative data given above and many other subsequent experiments, the ATP response is only reduced by a relatively small amount in the presence of hexokinase.

It has been found that the optimal concentration of hexokinase, i.e., maximal inherent light removal and minimal reduction of ATP response, is approximately 0.001 mg per reaction mixture. It thus appears that there is no necessity for the removal of hexokinase from luciferase prior to ATP assay.

A time course for the reduction of inherent light was run. The reaction mixture was the same as that described previously but with a different enzyme preparation. A control was run as before. The reaction mixtures were allowed to stand at room temperature in the reaction chamber and the inherent light measured at periodic intervals. The results are shown in Figure 11. It is seen that with hexokinase present, a minimal inherent level is attained after ten minutes.

A problem which has arisen with the use of hexokinase is that its effects differ depending on the commercial source. With certain commercial preparations from other sources the ATP response is inhibited drastically. It has been found that these preparations are contaminated with rather high levels of ATPase. Thus, it would appear that before hexokinase can be used in a routine fashion for the reduction of inherent light, it must be free of ATPase.

5. Removal of Inherent Light by Dialysis

If the binding of ATP ions to protein molecules is strictly an adsorption phenomenon, it should be possible to remove them by

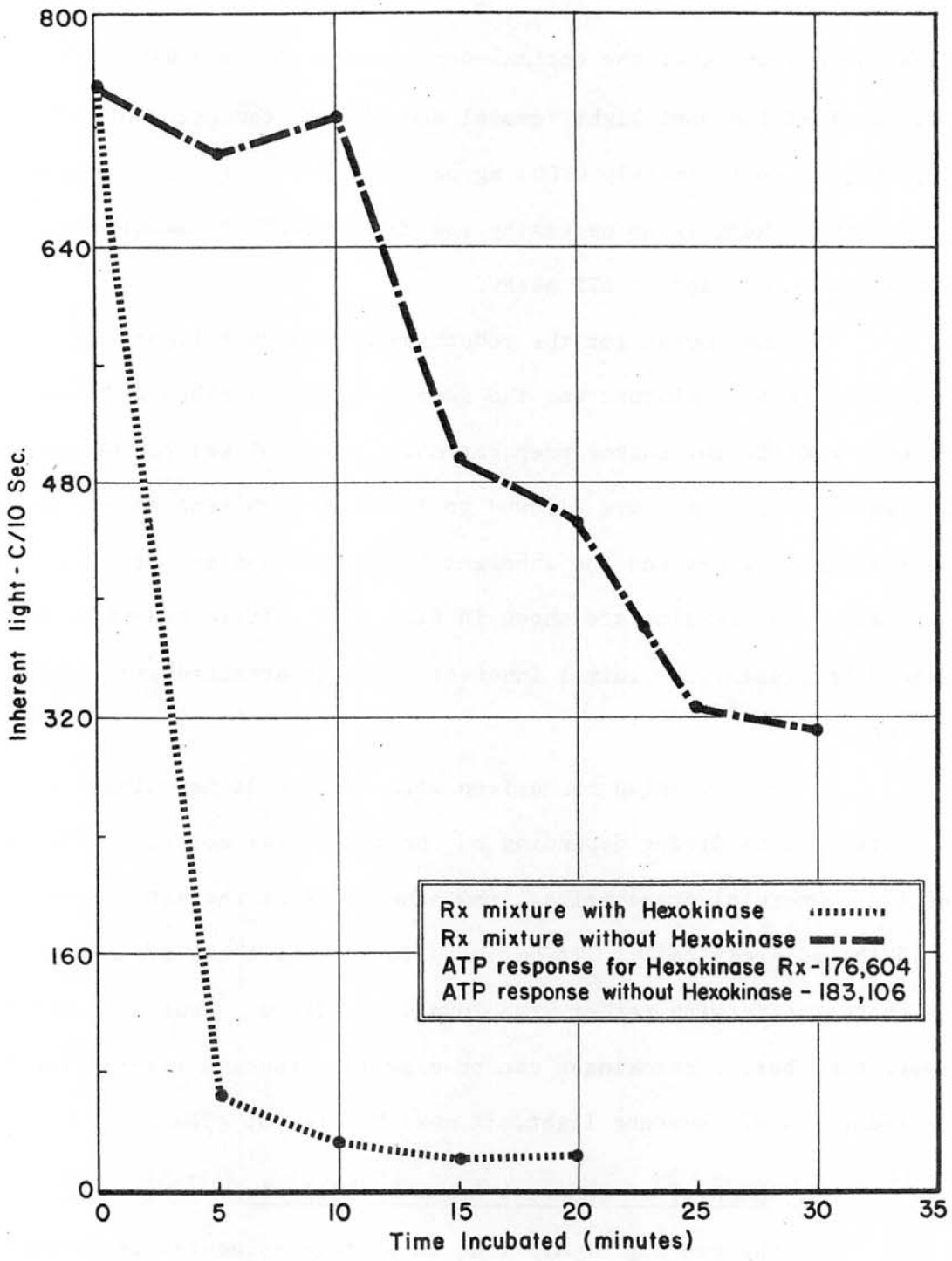


Figure No. 11 — Reduction of inherent light by Hexokinase.

exhaustive dialysis techniques. The results of experiments along these lines are described.

With dialysis equipment obtained from the National Instrument Laboratories, Rockville, Maryland, it was possible to carry out two types of dialysis. These were high pressure dialysis and low pressure dialysis.

High pressure dialysis involves the exertion of 150 pounds of pressure per square inch upon the surface of the contents of a dialysis bag, thereby increasing the rate of flow of the dialyzable components through the membrane and also reducing the back flow of ions. Thus, this procedure greatly shortens the time necessary for complete dialysis, and also reduces the volume of the solution within the dialysis bag.

Low pressure dialysis is the usual type except that low pressure (3 psi) is used to prevent wrinkles in the membrane surface. The National Instrument Laboratories instrument makes use of a unique agitation system for minimal equilibration time.

Results obtained with the above dialysis procedures were as follows.

a. <u>High Pressure Dialysis</u>

A partially purified luciferase fraction (20 ml) from a Sephadex G-200 column was dialyzed at $0^{\circ}C$ for six hours in the high pressure unit after which it was assayed for inherent light

and ATP (1×10^{-2} M) response.

Table 13

Effects of High Pressure Dialysis on Inherent Light

	Inherent Light (c/5 sec)*	ATP Response (c/5 sec)
Control (non-dialyzed)	4,950	165,000
Dialyzed sample	10,250	223,000

*Number of phototube output impulses during a five-second counting period.

It should be pointed out that the final volume of the luciferase fraction after dialysis was 7 ml. The results indicate that there was no loss in either inherent light or ATP response, but instead an increase in both due to the concentrating effect.

b. Low Pressure Dialysis

A partially purified luciferase fraction (5 ml) from a Sephadex G-200 column was dialyzed at 0°C for 21 hours. The contents of the dialysis bag were assayed for inherent light and ATP (1×10^{-2} M) response at designated intervals during this period.

Table 14

Effects of Low Pressure Dialysis on Inherent Light

Time (hrs)	Inherent Light (c/5 sec)	ATP Response (c/5 sec)
0	2386	196,999
2	4062	227,901
3	2187	188,305
4	3104	215,518
5	1615	191,136
21	199	487

Note: A non-dialyzed control which had stood at $0^{\circ}C$ for 21 hours had an inherent light count of 1877 and an ATP response of 183,375.

It was concluded on the basis of the above experiments that dialysis was not capable of removing the factors responsible for inherent light from the luciferase preparations.

6. Reduction of Inherent Light by Incubation of Luciferase-Luciferin at Elevated Temperatures

It has been frequently observed that the inherent light of luciferase-luciferin mixtures decreases on standing at room temperature. Experiments were carried out to determine the effect of temperature on the rate and extent of inherent light decrease.

Reaction Mixture

6 ml of luciferase from Sephadex G-200

6 ml of luciferin (0.5 mg/ml)

6 ml of $MgSO_4$ (0.01 M)

Aliquots (2 ml) of the above mixture were incubated at room temperature (23.5°), 30°C, and 37°C. Samples (0.3 ml) were removed at designated times and assayed for ATP response (1×10^{-3} M) and inherent light. (30-second counting period.)

Table 15

Effect of Incubation of Luciferase-Luciferin Mixtures at Various Temperatures on Inherent Light

Temp. °C	Time (min)	Inherent Light (c/30 sec)	ATP Response (c/30 sec)
23.5	0	26,000	1,011,000
" "	15	10,250	1,296,215
" "	30	4,900	1,210,340
" "	60	3,200	1,117,285
" "	120	2,100	1,115,176
30.0	0	28,750	1,319,164
" "	15	8,850	1,276,219
" "	30	4,296	1,118,617
" "	60	3,144	1,215,109
" "	120	1,221	1,019,656
37.0	0	29,756	923,041
" "	15	7,450	276,219
" "	60	2,034	169,895

On the basis of the data presented above, it appears that allowing the enzyme mixture to stand at 30°C for 120 minutes is a feasible technique for the reduction of inherent light. It is concluded that the reduction of inherent light on standing is caused by the depletion with time of the factors responsible for inherent light, i.e., endogenous ATP is used up.

7. Effect of Certain Environmental Conditions on Inherent Light Level

It has long been noted that the inherent light level of a luciferase-luciferin mixture drops on standing at room temperature. The decrease is rapid during the first few minutes after which the rate of decay is relatively slow. It has also been observed that reaction mixtures which have been allowed to stand until the inherent light level has undergone a considerable decrease, show an increase in the inherent light value on shaking. It was concluded that this was caused by the replenishment of the depleted oxygen content of the reaction mixture, and that at least part of the inherent light decrease was a reflection of the oxygen depletion. This would be compatible with the results reported in Table 15 in that the needed oxygen for the ATP response might have been present in the injected ATP solution. It was decided to determine the effect of continuously bubbling oxygen through a reaction mixture over a given time period in both light and darkness. The inherent light level and ATP response

would be compared with non-gased controls. The following reaction mixture was freshly prepared.

10 ml of luciferase from Sephadex G-100 column

10 ml of luciferin (0.5 mg/ml)

10 ml of 0.01 M $MgSO_4$

Four aliquots (5 ml each) were taken from the above freshly prepared mixture and treated in the following manner:

#1 Gased with compressed air in the dark

#2 Gased with compressed air in the light (100 W tungsten lamp at two feet)

#3 Allowed to stand in darkness

#4 Allowed to stand in light (tungsten lamp 100 W at two feet)

The samples were assayed with 10^{-3} δ of ATP at designated times. The results are presented in Table 16.

Table 16

Effect of Oxygen on ATP Response in Light and Dark

Time (min)	Gas-light		Gas-dark		Light		Dark	
	c/30 sec		c/30 sec		c/30 sec		c/30 sec	
	I.L.*	ATP	I.L.	ATP	I.L.	ATP	I.L.	ATP
0	168,291	715,512	168,291	715,512	168,291	715,512	168,291	715,512
15	3,120	868,222	4,445	957,134	2,620	845,933	3,343	866,388
45	1,830	870,302	2,753	821,893	1,510	825,063	1,458	824,780
115	1,114	843,205	1,554	806,420	1,033	835,635	996	850,000

*Inherent light

It is seen in the data above that the rate of decrease of inherent light is affected very little by the passage of oxygen through the reaction. Another observation, not shown above, was that if the samples which were gased were shaken, there was approximately a 50% increase in the inherent light level with a concomitant 10% decrease in the ATP response. This mixing phenomenon is unexplainable at this time. One can conclude, however, that reaction mixture is not oxygen limited after extended periods of incubation at room temperature. Hence, the removal of inherent light by incubation remains valid.

G. Optimal Reaction Conditions

1. Effect of pH

The optimal pH of the bioluminescence reaction was found by Green and McElroy to be 7.8 (10). However, with many enzyme systems the optimal pH will vary depending on conditions such as buffer type and the degree of enzyme purification. It was for this reason that the following experiment was carried out.

A partially purified luciferase fraction from a Sephadex G-100 column was passed through a Sephadex G-25 column with 0.005 M NaCl as the eluting agent. This removed arsenate buffer from the enzyme preparation. The following reaction mixtures were prepared using this enzyme.

0.1 ml of enzyme
0.05 ml of luciferin (1 mg/ml)
0.1 ml of 0.05 M arsenate at designated pH
0.05 ml of 0.01 M $MgSO_4$
0.01 ml of ATP (1×10^{-3} %)

The reaction mixtures were assayed at 0 time, 15 minutes, 30 minutes, 45 minutes, one hour, and one and one-half hours after standing at room temperature.

The results are presented in Figure 12.

The optimal pH of the reaction when measured at 0 time and at 15 minutes is 7.5. The reaction mixtures which were allowed to stand for longer periods of time at room temperature show an optimal pH of 7. This change in optimal pH is accompanied by a decrease in the activity of the pH 7.5 reaction mixture of approximately 30% compared to the pH 7.5 activity at 0 time. There was no decrease in the activity of the pH 7.0 reaction mixture which in fact showed a small increase over the first 45 minutes. It should further be noted that the inherent light level (see Figure 13) of the pH 7.0 reaction mixture was about one-half that of the pH 7.5 mixture throughout the entire time period. The inherent light of the pH 8.0 reaction mixture is about 30% greater than that seen at pH 7.5.

It would appear that while the actual enzymatic reaction proceeds at a maximal rate at pH 7.5, the enzyme itself is most

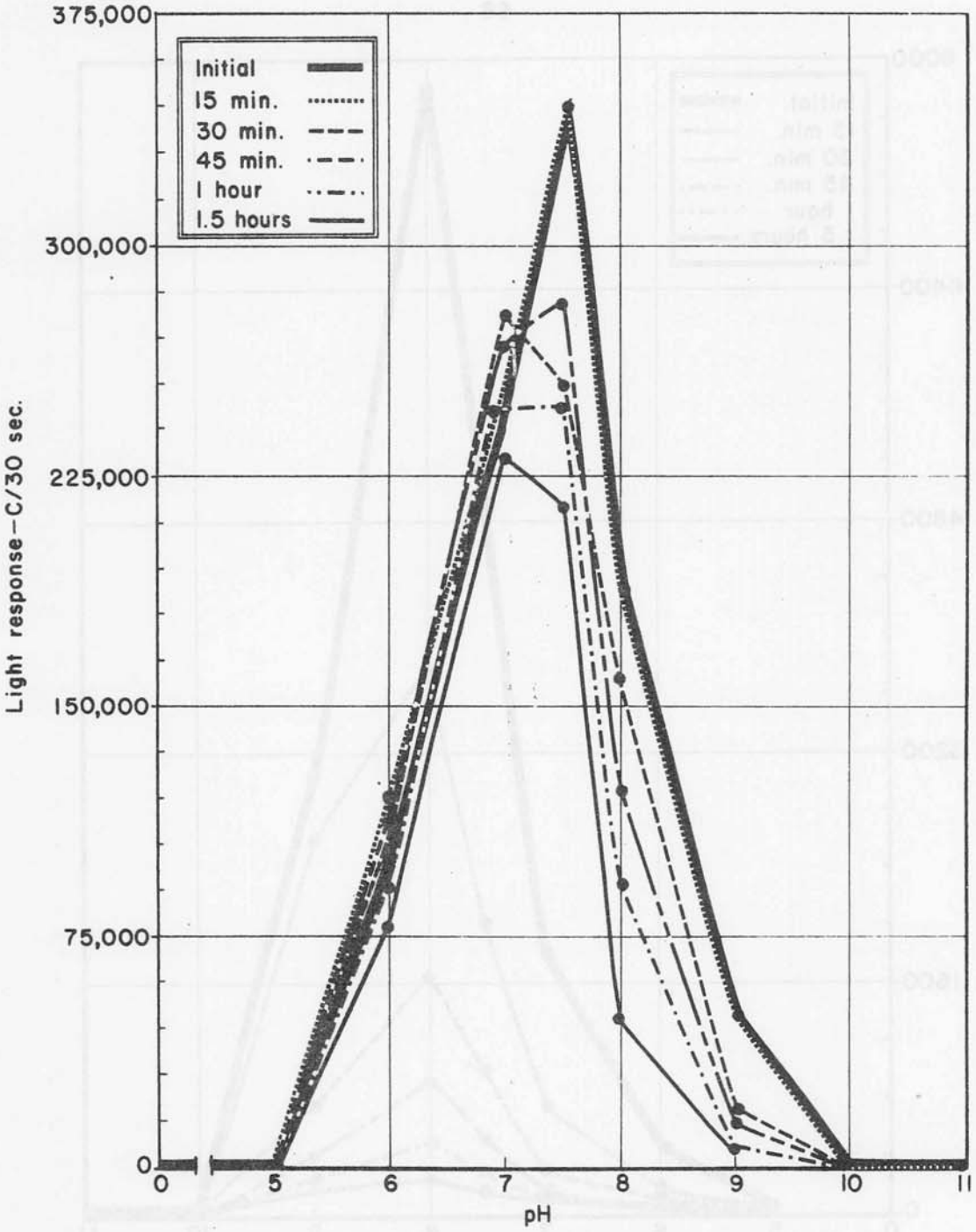


Figure No. 12 — Effect of pH on ATP response.

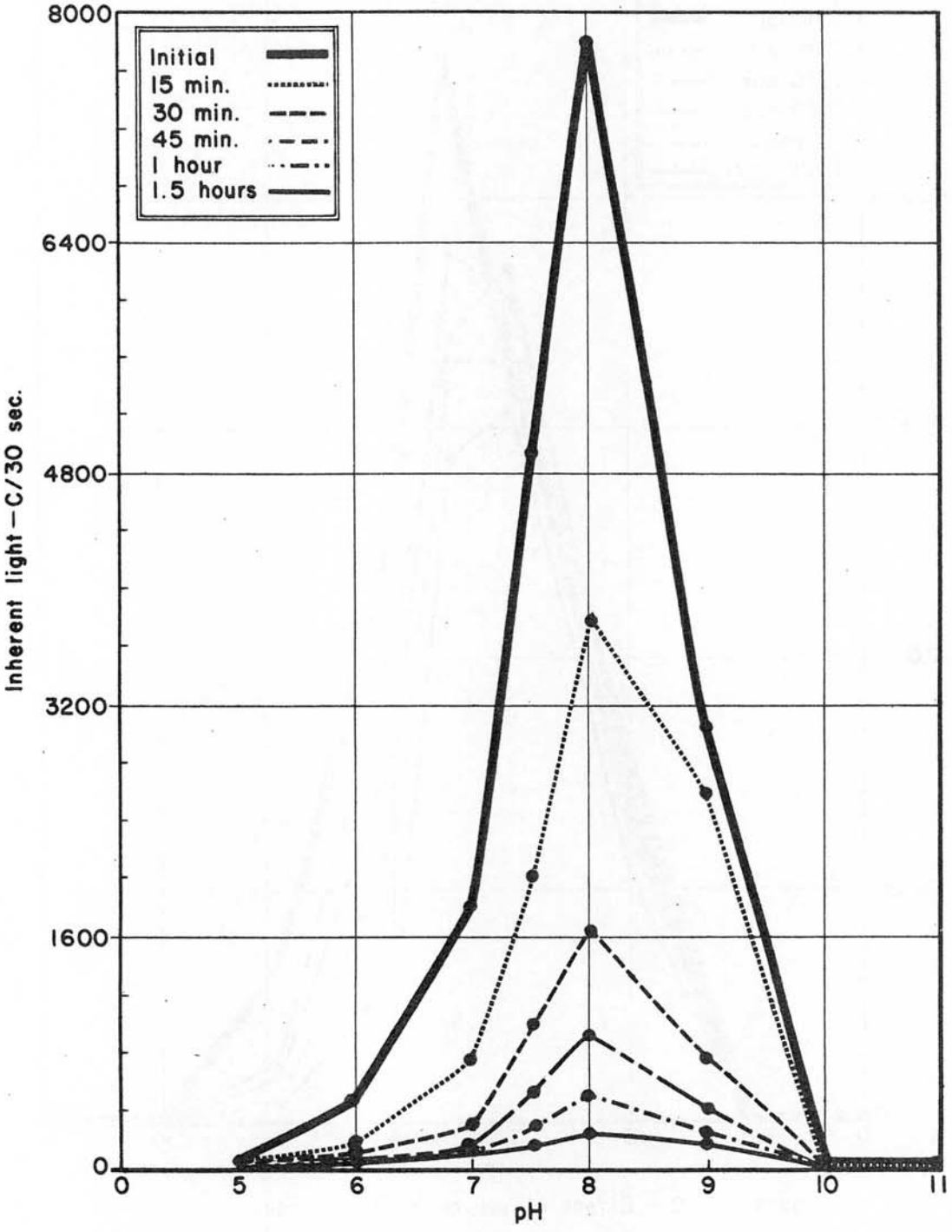


Figure No. 13 — Effect of pH on inherent light.

stable at pH 7.0. The increase in net response of the pH 7.0 reaction mixture is quite likely due to the increase in the inherent light level. This is a phenomenon which has frequently been observed here. The increase in inherent light with increase in pH is quite possibly a reflection of an increase in the rate of dissociation of protein bound ATP, and also that the rate of dissociation is sufficiently great as to offset the inhibition of ATP response seen at a basic pH. This might follow as the result of the anionic character of the phosphate functions of the ATP molecule.

2. Effect of Magnesium Concentration on Bioluminescent Assay

The experiment described below was carried out to determine the optimal magnesium concentration for the reaction condition currently being used.

Reaction Mixture

0.1 ml of partially purified luciferase
0.1 ml of luciferin (0.2 mg/ml)
0.1 ml of $MgSO_4$ at specified concentrations
10 λ ATP (1×10^{-2} γ)

30-second counting period

The results are described in Figure 14.

On the basis of the above data, the optimal $MgSO_4$ concentration is 1×10^{-2} M. Further studies would have to be made to establish whether this is true throughout a wide range of luciferin

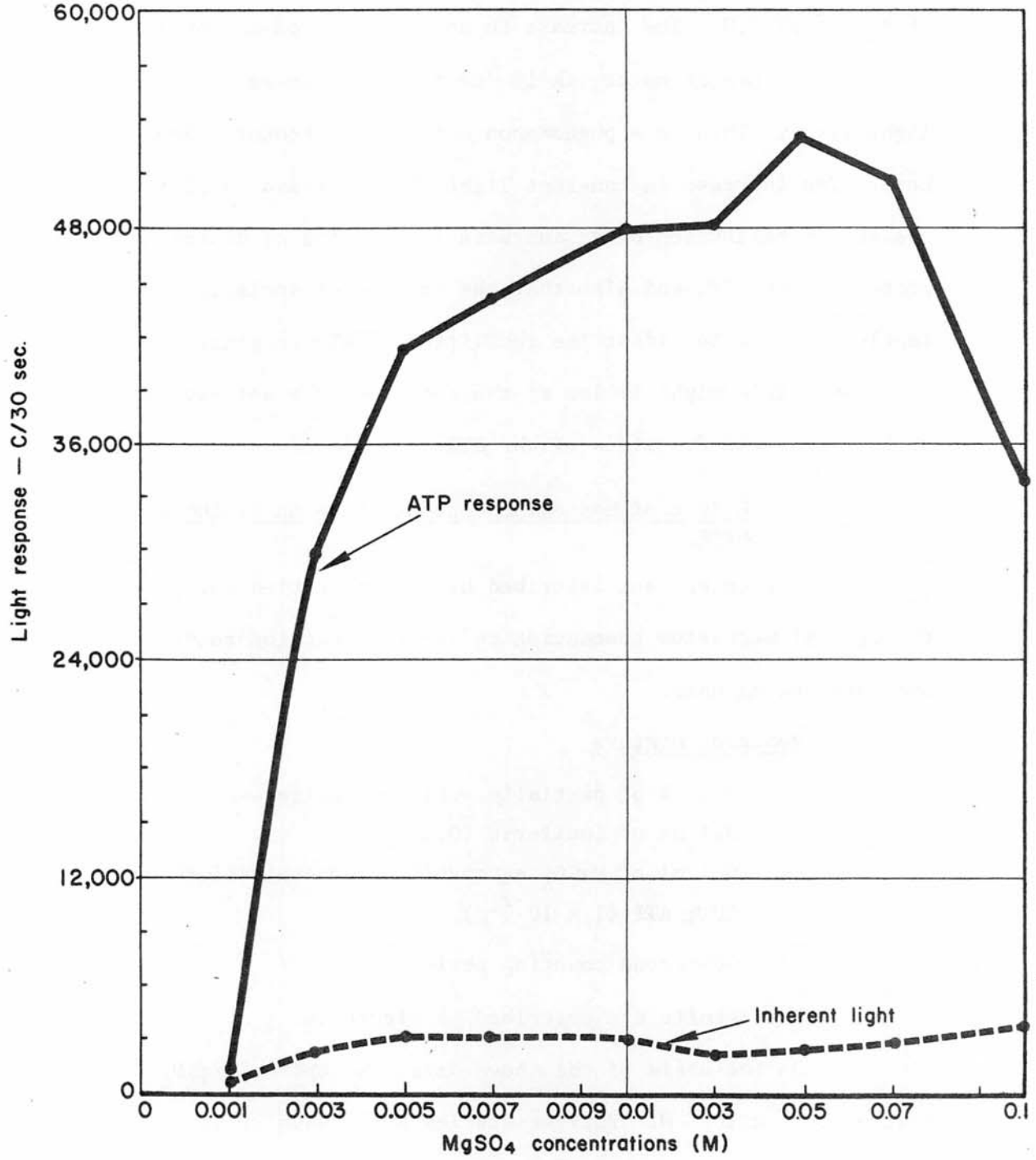


Figure No. 14 – Effects of magnesium concentration on ATP response.

and ATP concentrations.

3. Effect of Luciferin Concentration on Bioluminescent Assay

ATP response as a function of the luciferin was determined. The experimental procedure is described below.

Reaction Mixture

0.1 ml of partially purified luciferase
0.1 ml of luciferin at specified concentrations
0.1 ml of $MgSO_4$ (1×10^{-2} M)
10 λ ATP (1×10^{-3} M)

30-second counting period

The results are described in Figure 15.

It is noteworthy here that the ATP response begins to decrease at a luciferin concentration of 1 mg/ml while the inherent light level is continuing to rise. It is possible to speculate that the reason for the decrease in response at this higher luciferin concentration is caused by an increase in the concentration of contaminating oxyluciferin which would inhibit the reaction. No explanation can be brought forward at this time to explain the increase in inherent light at luciferin concentrations where the ATP response is inhibitied.

4. Effect of Type of Buffer

A comparison of the effects of different buffers on

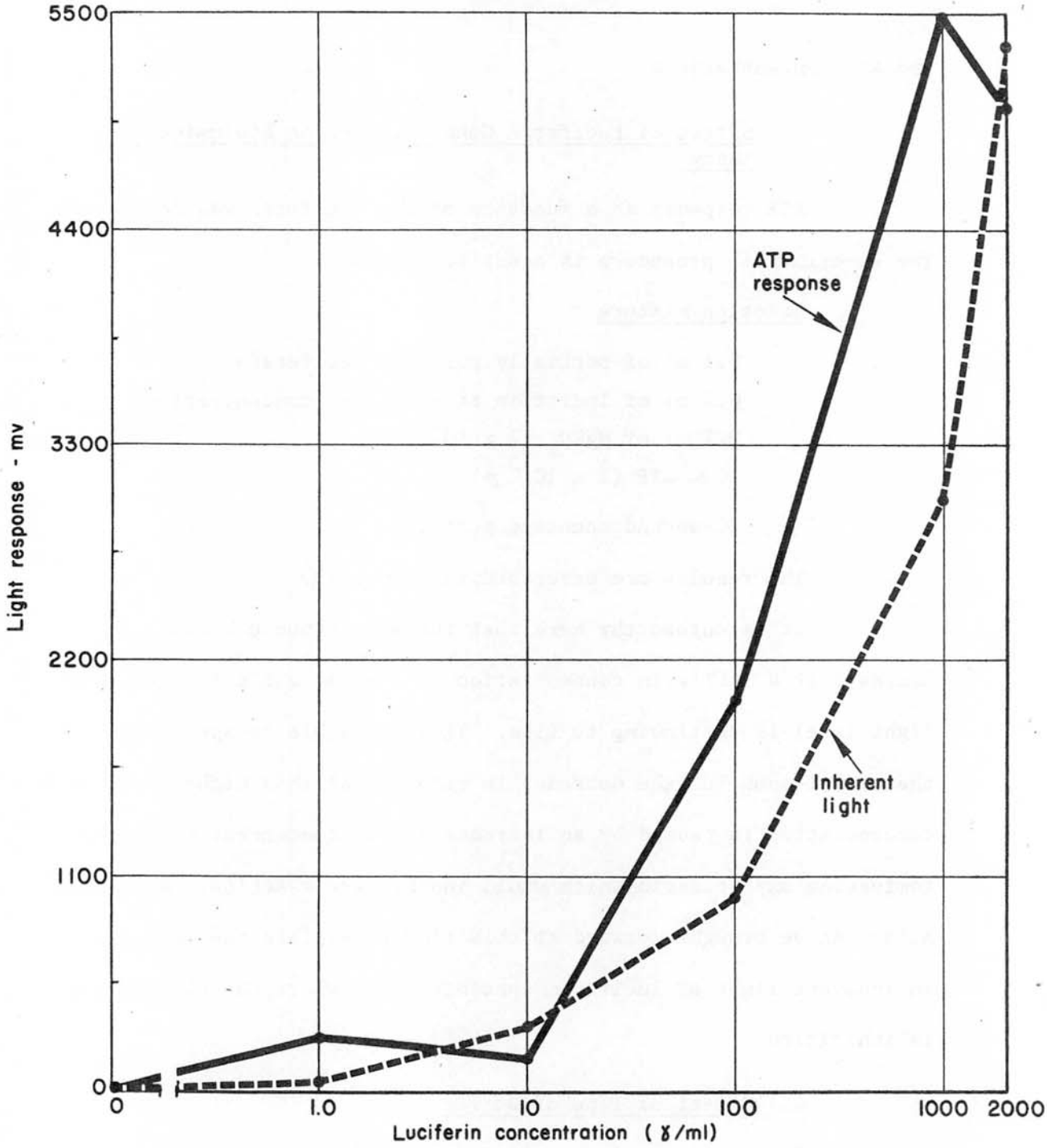


Figure No. 15 — Effect of luciferin concentration on ATP response.

luciferase activity has been made during this report period. The buffers studied were Tris, arsenate, phosphate, and glycyl-glycine. These studies are described below.

Four Sephadex G-25 columns (12" x 1/2") were prepared and equilibrated with 0.05 M glycyl-glycine, 0.05 M Tris, 0.05 M phosphate, and 0.05 M arsenate, respectively. All buffers were at pH 7.4. One ml aliquots of a partially purified luciferase fraction from a Sephadex G-200 column were placed on each of the Sephadex G-25 columns. The columns were eluted with the buffers with which they had been equilibrated. The breakthrough volume of each column was collected and assayed for activity. The results were as follows:

Reaction Mixture

0.1 ml luciferase
0.1 ml luciferin (0.2 mg/ml)
0.1 ml $MgSO_4$ (0.01 M)
10 λ ATP (1×10^{-1} M)

Table 17

Effect of Buffer Type on ATP Response

	Inherent Light (c/5 sec)	ATP Response (c/5 sec)
Phosphate buffer	268	170,686
Arsenate buffer	279	143,377
Tris buffer	288	197,302
Glycyl-glycine buffer	382	171,162

The data indicate that at least over a five-second counting period, there is little difference in the activity of luciferase when contained in the buffers studied. It is possible that if light production were followed over a longer period of time, greater differences might be seen.

5. Effect of Arsenate Buffer Concentration

It has been previously observed that the bioluminescent system is sensitive to high salt concentrations. A study to determine the optimal buffer concentration was carried out.

Arsenate buffer was removed from a partially purified luciferase preparation by passage through a Sephadex G-25 column with 0.005 M NaCl as the eluent. The reaction mixture for the experiment was as follows:

0.1 ml of enzyme
0.05 ml of luciferin (1 mg/ml)
0.05 ml of $MgSO_4$ (0.01 M)
0.1 ml of arsenate buffer (pH 7.5) at designated concentration

The assay was performed with the RCA photomultiplier unit. The results are given in Figure 16.

The inhibitory effect of a high salt concentration is quite apparent in the above data. The optimal buffer concentration is seen to be about 0.01 M.

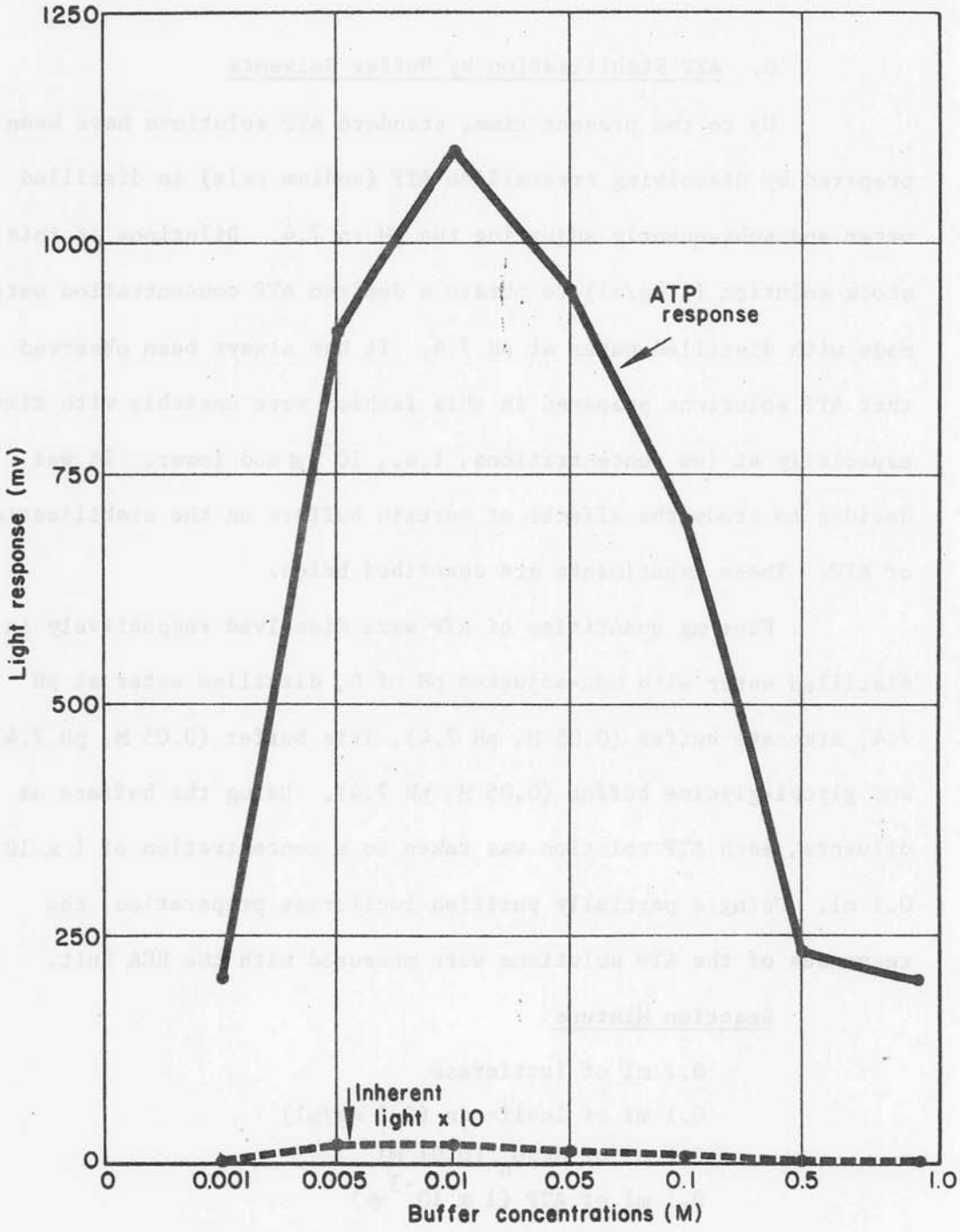


Figure No. 16 — Effect of arsenate buffer concentration on ATP response.

6. ATP Stabilization by Buffer Solvents

Up to the present time, standard ATP solutions have been prepared by dissolving crystalline ATP (sodium salt) in distilled water and subsequently adjusting the pH to 7.4. Dilutions of this stock solution (1 mg/ml) to obtain a desired ATP concentration were made with distilled water at pH 7.4. It has always been observed that ATP solutions prepared in this fashion were unstable with time, especially at low concentrations, i.e., 10^{-3} γ and lower. It was decided to study the effects of certain buffers on the stabilization of ATP. These experiments are described below.

Five mg quantities of ATP were dissolved respectively in distilled water with non-adjusted pH of 6, distilled water at pH 7.4, arsenate buffer (0.05 M, pH 7.4), Tris buffer (0.05 M, pH 7.4), and glycyl-glycine buffer (0.05 M, pH 7.4). Using the buffers as diluents, each ATP solution was taken to a concentration of 1×10^{-3} γ / 0.1 ml. Using a partially purified luciferase preparation, the responses of the ATP solutions were measured with the RCA Unit.

Reaction Mixture

0.1 ml of luciferase
0.1 ml of luciferin (0.5 mg/ml)
0.1 ml of $MgSO_4$ (0.01 M)
0.1 ml of ATP (1×10^{-3} γ)

Table 18

Effect of Buffer on ATP Stability

Buffer	ATP Response (millivolts)				
	0	15 min	1 hr	2 hrs	6 hrs
Arsenate	1400	1350	1300	1350	1350
Tris	1450	1400	1400	1375	1400
Glycyl-glycine	1400	1250	1300	1400	1350
H_2O - pH 6	550	360	120	40	10
H_2O - pH 7.4	500	280	70	14	8

The results indicate that arsenate, Tris, and glycyl-glycine serve equally well as buffers. Since the luciferase is routinely prepared in arsenate buffer, it was decided to use arsenate buffer for the ATP preparations as well.

In the above experiment the ATP solutions were standing at room temperature over the indicated time period. As a further precaution in selecting arsenate, the ATP in arsenate buffer was kept at $0^{\circ}C$ for an additional four days and then assayed. There was no decrease in response. It has also been observed that the arsenate ATP solutions exhibit no decrease in light response after a 15-minute boiling period.

7. Effect of Counting Time on ATP Response

The ideal counting period would be one terminating concomitantly with cessation of the statistically significant light

emission due to the injection of ATP. It has been observed, however, that the period of light emission is subject to wide variations dependent to a great extent on the past history of the luciferase preparation. An example of this is shown in the experiment described below.

A partially purified luciferase preparation (100 ml) was subjected to $(NH_4)_2SO_4$ fractionation. Three fractions (40%, 50%, and 60%) were obtained. These fractions (10 ml each) were assayed in the following fashion.

Reaction Mixture

0.1 ml of fraction
0.1 ml of luciferin (0.2 mg/ml)
0.1 ml of $MgSO_4$ (1×10^{-2} M)
10 λ ATP (1×10^{-3} γ)

Table 19

Effect of Counting Time on ATP Response

	c/5 sec		c/30 sec	
	Inherent Light	ATP	Inherent Light	ATP
Extract before $(NH_4)_2SO_4$	29,200	80,650	161,081	328,953
40% fraction	3,128	15,505	16,646	66,595
50% fraction	6,615	26,068	35,697	358,446
60% fraction	10,944	24,830	92,435	149,849

On the basis of the responses shown during the five-second counting period, it would be concluded that the most active preparation was the luciferase prior to $(NH_4)_2SO_4$ fractionation, with the 50% and 60% fractions being of almost equal potency. A very different order of activity is observed after a 30-second counting period. Here, the 50% fraction is the most active. The high activity shown by the 50% fraction is most likely due to a decrease in pyrophosphate relative to luciferase resulting in the emission of light at a high level over a longer period of time.

In order to determine whether the optimal counting period varied with the ATP concentration, the experiment described below was carried out.

Reaction Mixture

0.1 ml of luciferase from Sephadex G-200
0.1 ml of luciferin (0.5 mg/ml)
0.1 ml of $MgSO_4$ (0.01 M)
0.1 ml of ATP at designated concentrations

The results are seen in Table 20.

Table 20

Optimal Counting Period as a Function of ATP Concentration

ATP Concentration	Counting Time (sec)	Inherent Light	ATP Response	Net ATP Response	Net ATP/ Inherent Light
1×10^{-3} γ	2	763	34,956	34,193	44.8
" "	5	1,952	104,556	102,604	52.5
" "	10	3,179	189,113	185,934	58.5
" "	30	9,342	465,131	456,089	48.8
" "	60	20,121	730,017	709,896	35.3
1×10^{-4} γ	2	622	5,767	5,145	8.3
" "	5	1,749	15,379	13,630	7.8
" "	10	3,302	29,050	25,748	7.8
" "	30	9,624	69,520	59,896	6.2
" "	60	21,182	98,183	77,001	3.6
1×10^{-5} γ	2	696	1,107	411	0.6
" "	5	1,878	3,245	1,375	0.7
" "	10	3,942	6,787	2,845	0.7
" "	30	10,710	16,671	5,961	0.6
" "	60	19,598	27,632	8,034	0.4
1×10^{-6} γ	2	734	748	14	0.02
" "	5	1,572	1,720	148	0.1
" "	10	3,202	3,426	224	0.1
" "	30	10,056	10,235	179	0.02
" "	60	19,354	19,554	200	0.01

It is evident from the data presented above that for quantities of ATP down to the level of 1×10^{-5} γ, there is still measurable light emission even after 60 seconds. On looking at the

ATP/inherent light ratio, it is seen that for 1 x 10^{-3} and 1 x 10^{-4} of ATP, the most favorable ratio (highest) is realized at ten seconds, while for the 1 x 10^{-5} and 1 x 10^{-6}, it is at five seconds. There are two parameters to be considered in the selection of optimum counting time. The first is the fact that the higher the ATP/inherent light ratio (signal/noise), the greater is the statistical validity of the measured net response. The second is that maximum sensitivity is obtained by the measurement of total light emission. As is seen in the above data, the time period necessary for the achievement of the two conditions does not necessarily coincide. It would thus appear that the optimal counting period will be a compromise between maximal sensitivity and maximal statistical validity of the responses. This compromise value will be a function of the ATP concentration, activity and purity of the luciferase preparation, and the inherent light level.

8. Effect of Temperature on ATP Response

The reaction chamber of the NASA Unit is constructed so that water at any desired temperature can be circulated around the reaction cuvettes. This provision allowed the determination of the optimal reaction temperature and also of temperature on inherent light. A representative experiment along these lines is described below.

The experiment was carried out using a partially purified luciferase preparation from a Sephadex G-100 column. The following reaction mixture was used:

0.1 ml of enzyme
0.1 ml of luciferin (1 mg/ml)
0.1 ml of $MgSO_4$ (0.01 M)
0.01 ml of ATP (1×10^{-3} M)

Counting time - 30 seconds

Each cuvette containing the above reaction mixture was allowed to stand at the specified temperature for five minutes prior to the injection of ATP.

The results of this experiment are shown in Figure 17.

A rather sharp optimal reaction temperature is seen at 20°C. This is at variance with that observed by Green and McElroy (10) who reported an optimal temperature of 23.5°C. It is impossible at this time to explain the discrepancy. It may possibly be caused by differences in the techniques of obtaining and maintaining the various temperatures.

An observation of great interest here is that the optimal reaction temperature is different from the temperature at which maximal inherent light is observed. At present, there are two possible explanations that can be put forward. The first would be that the increased inherent light at elevated temperatures is a reflection of an increased rate of dissociation of ATP from the

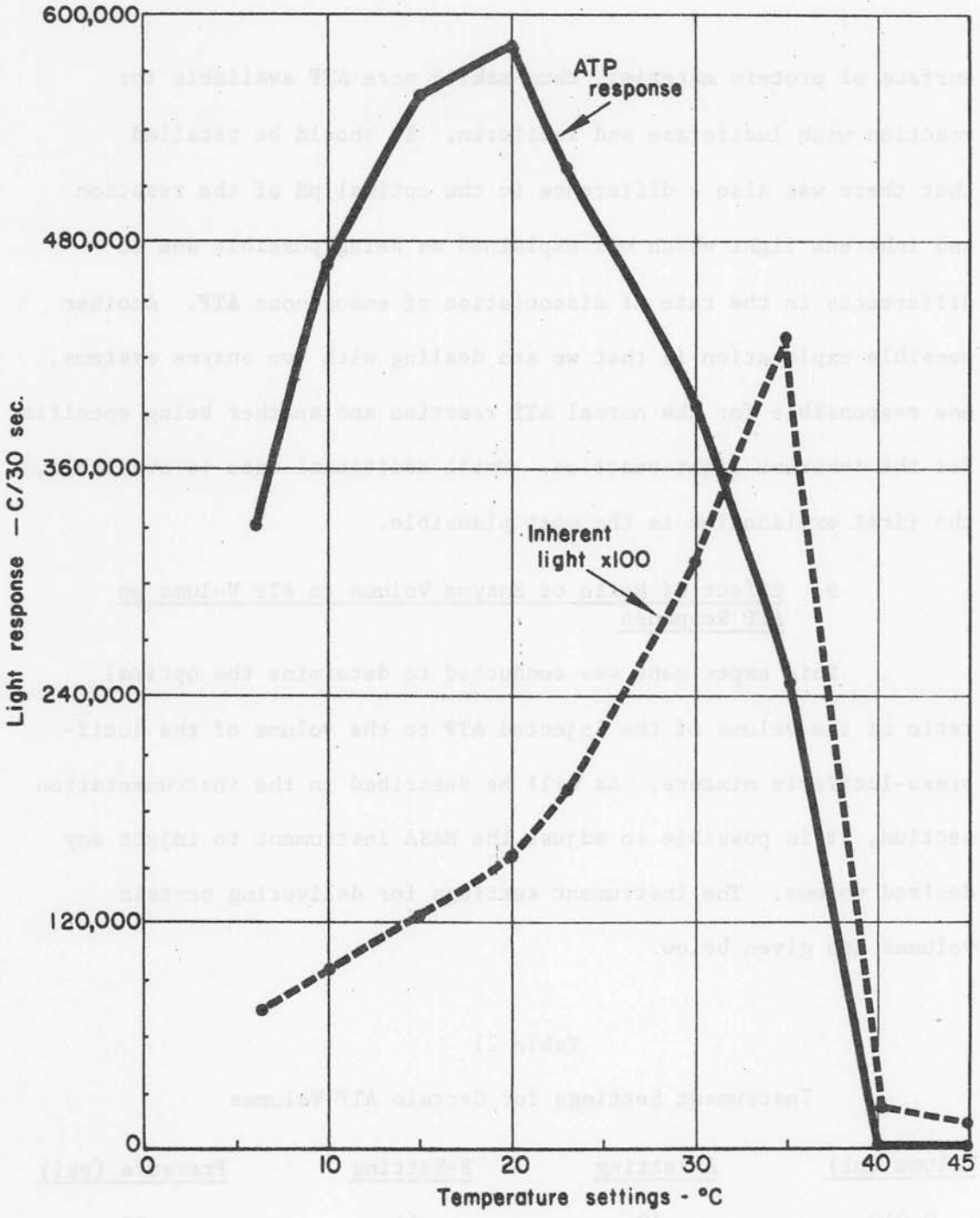


Figure No. 17 — Effect of temperature on ATP response.

surface of protein material, thus making more ATP available for reaction with luciferase and luciferin. It should be recalled that there was also a difference in the optimal pH of the reaction and inherent light which was explained as being possible due to differences in the rate of dissociation of endogenous ATP. Another possible explanation is that we are dealing with two enzyme systems, one responsible for the normal ATP reaction and another being specific for the inherent light reaction. Until additional data is available, the first explanation is the most plausible.

9. Effect of Ratio of Enzyme Volume to ATP Volume on ATP Response

This experiment was conducted to determine the optimal ratio of the volume of the injected ATP to the volume of the luciferase-luciferin mixture. As will be described in the instrumentation section, it is possible to adjust the NASA instrument to inject any desired volume. The instrument settings for delivering certain volumes are given below.

Table 21

Instrument Settings for Certain ATP Volumes

Volume (ml)	A-Setting	B-Setting	Pressure (psi)
0.010	10	11	50
0.025	10	13	50
0.050	10	15	50
0.075	10	15	65
0.100	10	18	50
0.150	10	18	81
0.200	10	20	81

A lyophilized reaction mixture containing luciferase and luciferin was used in this study (preparation will be described in a later section). The lyophilized mixture was prepared so that the quantity of enzyme placed in the cuvette was the same regardless of the volume. The standard ATP was prepared so that there was always 1×10^{-3} γ injected independent of the volume. The total volume of the reaction solution after ATP injection was 0.3 ml in all cases. Experimental results are described in Table 22.

Table 22

Effect of Ratio of Enzyme Volume to ATP Volume

Enzyme/ATP	Inherent Light c/30 sec	ATP Response c/30 sec
29	444	126,091
11	463	124,009
5	625	96,286
3	390	69,404
2	490	46,100
1	412	37,396
0.5	494	24,158

It is seen that the optimal ratio of enzyme to ATP is approached at approximately 30:1. Because of the disposable syringe currently being used, it was impossible to exceed this ratio. It is

believed that the differences seen in ATP response due to changes in ratio of enzyme to ATP is the result of differences in mixing times.

10. Light Emission by Water and Buffers

A phenomenon which has been frequently observed during the course of these studies is the emission of light as the result of the injection of solutions which presumably are free of ATP. A comparison of the magnitude of this effect in water and various buffers was made. This was done as described below.

Using sterile deionized water, the following solutions were prepared:

1. H_2O - pH adjusted to 7.4
2. Tris buffer - 0.05 M, pH 7.4
3. Arsenate buffer - 0.05 M, pH 7.4
4. Glycyl-glycine buffer - 0.05 M, pH 7.4

The above solutions (10 lambdas) were injected into a standard reaction mixture consisting of:

0.1 ml of partially purified luciferase in arsenate
0.1 ml of luciferin (1 mg/ml)
0.1 ml of $MgSO_4$ (0.01 M)

The results are given in Table 23.

Table 23

ATP Response Due to Injection of Water and Buffers

Solution	Inherent Light c/30 sec	ATP Response c/30 sec
Water	65	68
Tris	68	642
Arsenate	72	692
Glycyl-glycine	75	1016

The only immediate explanation of the above effects is that the buffer components are contaminated with ATP. This phenomenon is of great importance as it will give rise to increasing interference at low ATP concentrations. It warrants further investigation.

11. Effect of Mixing Time and Oxygen on ATP Response

It has long been felt, in an intuitive fashion, that the maximum initial ATP response was related to the speed of ATP injection. It was observed, however, that on varying the injection speed of the NASA Unit from 1500 m seconds to 600 m seconds, there was no significant change in the peak light output. In order to examine the effects of an even faster injection speed, arrangements were made with Dr. Quentin Gibson of the Johnson Research Foundation, University of Pennsylvania, to use his rapid mixing equipment. By

the use of this equipment, it was possible to obtain complete mixing of ATP with the luciferase-luciferin in 3 m seconds.

Although the experiments were carried out in a rather qualitative fashion, the results indicated once again that the speed of mixing was not critical.

A possibly important difference in Gibson's procedure is that he uses two syringes, one containing the luciferase-luciferin mixture and the other containing ATP. Both syringes were injected by the application of hydraulic pressure into a cuvette facing the photon, whereas in the studies conducted in this laboratory, the ATP is injected into a static enzyme mixture.

An experiment to determine addition to the reaction mixture was also carried out in Dr. Gibson's laboratory. This was done by first removing oxygen from both the luciferase-luciferin system and the ATP solution by exhaustive evacuation. The two solutions were mixed in a cuvette positioned before a photomultiplier tube. The reaction was initiated by the addition of oxygen. The increase in ATP response as the result of this procedure over that where oxygen is present at the beginning was about 10%. It was concluded that the small gain seen here did not justify the fabrication of the accessories necessary for this technique.

12. Optimal Enzyme Concentration

Due to the variability among luciferase preparations and their lack of purity with respect to protein, it is impossible to establish a definite optimal concentration. This would require the use of a crystalline luciferase preparation. It was of interest, however, to determine the effects of changes in the concentration of both the crude luciferase preparations and the partially purified preparations on the ATP response. Representative experiments will be presented here. It should be stressed, however, that the values obtained will vary from one preparation to the next.

a. Crude Luciferase

A crude lyophilized firefly lantern extract (Sigma grade) was dissolved in distilled water at various concentrations and assayed for activity using the RCA Unit.

Reaction Mixture

0.3 ml of extract
0.1 ml of ATP (1×10^{-2} M) injected

The results are shown in Figure 18.

The inhibition observed at concentrations above 50 mg/ml is quite striking. The factors responsible are as yet unknown. It is also of interest and possibly of significance that the inherent light continues to rise even after the ATP reaction is inhibited. It may be that the increased endogenous ATP as the result of the

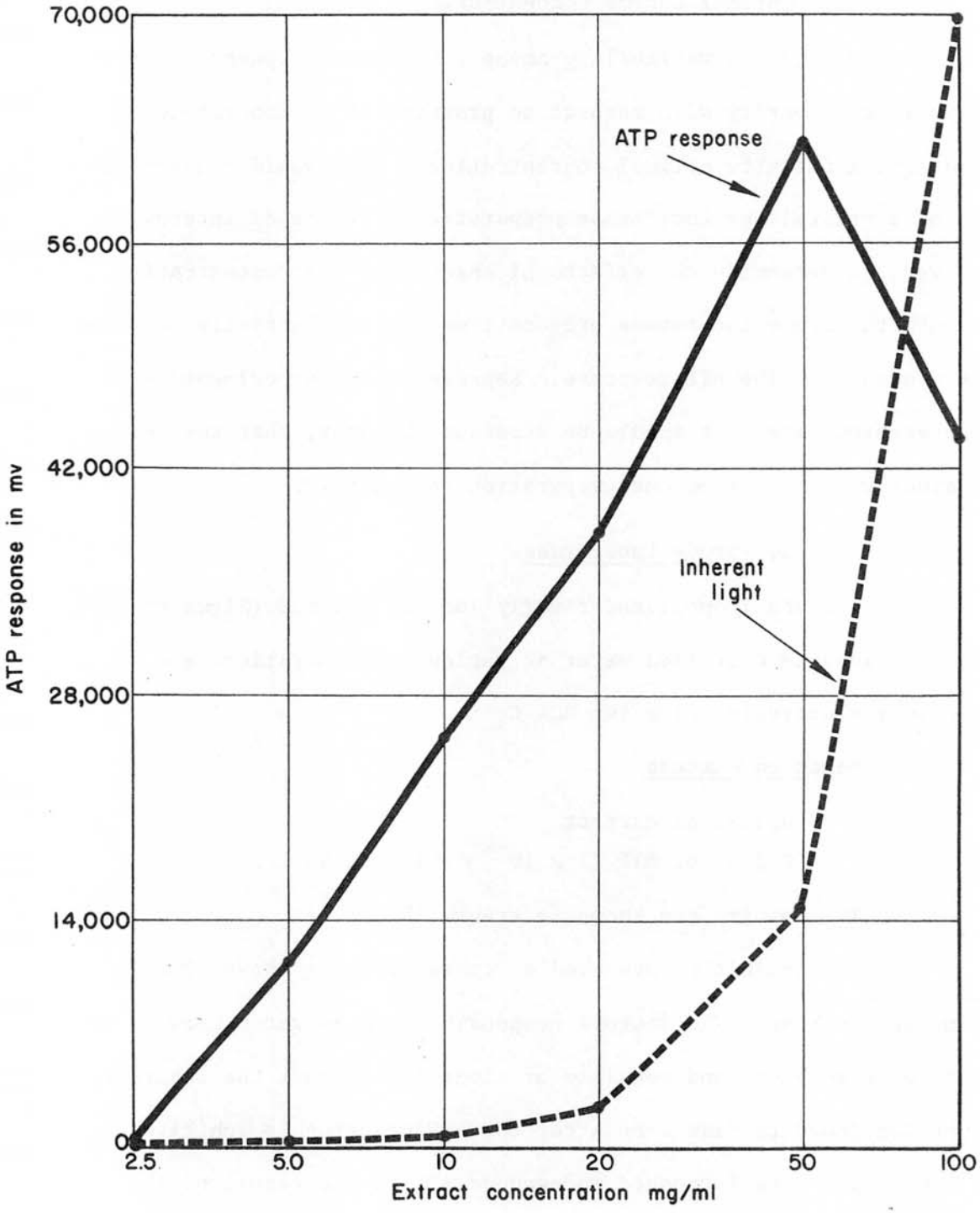


Figure No. 18 — ATP response as a function of lyophilized crude extract concentration.

higher protein concentration more than offsets the inhibition effect.

b. Partially Purified Luciferase

Using a partially purified luciferase preparation from a Sephadex G-100 column, the following experiment was carried out.

Various dilutions in 0.05 M arsenate of the enzyme as it emerged from the column were prepared and assayed for activity.

Reaction Mixture

0.1 ml extract at specified dilution
0.1 ml of luciferin (1 mg/ml)
0.1 ml of $MgSO_4$ (0.01 M)
0.01 ml of ATP (1×10^{-3} M)
Counting time - 30 seconds; NASA Unit

This experiment is described in Table 24.

Table 24

ATP Response As a Function of Luciferase Dilution

Dilution	Inherent Light c/30 sec	ATP Response c/30 sec
Stock	221,695	1,735,847
1:1	172,740	1,016,907
1:2	115,938	968,120
1:3	70,647	762,254
1:4	58,209	736,324
1:5	42,861	594,349
1:10	18,813	328,999

The above experiment is of value from the standpoint of enzyme conservation. It is evident that this particular preparation could be easily diluted 1:2 without an intolerable drop in ATP response. It is recommended that each enzyme preparation be assayed in the above manner to determine the maximal dilution of the enzyme.

H. Enzyme Storage

A prime requirement for the luciferase system if it is to be used as a life detection system is stability over extended periods of time. Luciferase being an enzyme, as is true for most enzymes, is stable only under rigorously controlled conditions. These conditions include temperature, moisture, pH, and gaseous environment. The studies to be described represent attempts to elucidate some of the conditions necessary for the stabilization of the luciferase-luciferin system.

1. Storage in Solution

It was noted very early during these studies that luciferase when purified on Sephadex columns lost most of its activity on freezing and thawing. This is a phenomenon which is rather common among enzymes. Freezing of the enzyme in solution was not considered any further.

It was decided to exploit another characteristic of enzymes, their increased stability in the presence of their substrates.

The following experiment was carried out.

A 10 ml partially purified luciferase fraction was mixed with 10 ml of luciferin (1 mg/ml) and 10 ml of $MgSO_4$ (0.01 M). The reaction mixture was allowed to stand in a cold bath at $4^{o}C$ with aliquots assayed periodically for ATP response. See Table 25 for results.

Reaction Mixture

0.3 ml of enzyme - luciferin
0.01 ml of ATP (1×10^{-3} %)

Counting time - 30 seconds; NASA Unit

Table 25

Stability of Luciferase-Luciferin Solution in Cold

Time (Days)	Inherent Light c/30 sec	ATP Response c/30 sec
0	83,306	1,121,528
1	323	1,032,734
2	234	1,005,745
8	215	1,003,625
30	300	626,333
60	105	425,243

It is evident that luciferase when stored under the above conditions exhibits a rather high degree of stability. It would be of interest to observe the stability of the system in a

nitrogen atmosphere under the same conditions of temperature and time.

2. Lyophilization

Lyophilization is a classical technique for the storage of labile biological intermediates. The storage of a compound in a dry state has rather obvious advantages over storage in a liquid state. It was therefore decided to evaluate this technique. A representative lyophilization is described below.

A reaction mixture containing 20 ml of a partially purified luciferase preparation from a Sephadex G-100 column, 20 ml of luciferin (1 mg/ml), 20 ml of $MgSO_4$ (0.1 M), and 200 mg of bovine serum albumin was prepared and assayed.

Reaction Mixture

0.3 ml of enzyme
0.01 ml of ATP (1×10^{-3} γ)

Inherent light - 3250 c/30 sec
ATP response - 1,070,300 c/30 sec

The mixture was then lyophilized overnight. A dry powder weighing 750 mg was obtained. Various concentrations of the reaction mixture were dissolved in distilled water and assayed to determine the optimal concentration. This is shown in Figure 19.

Reaction Mixture

0.3 ml of preparation at specified concentration
0.01 ml of ATP (1×10^{-3} γ)

Counting time - 30 seconds; NASA Unit

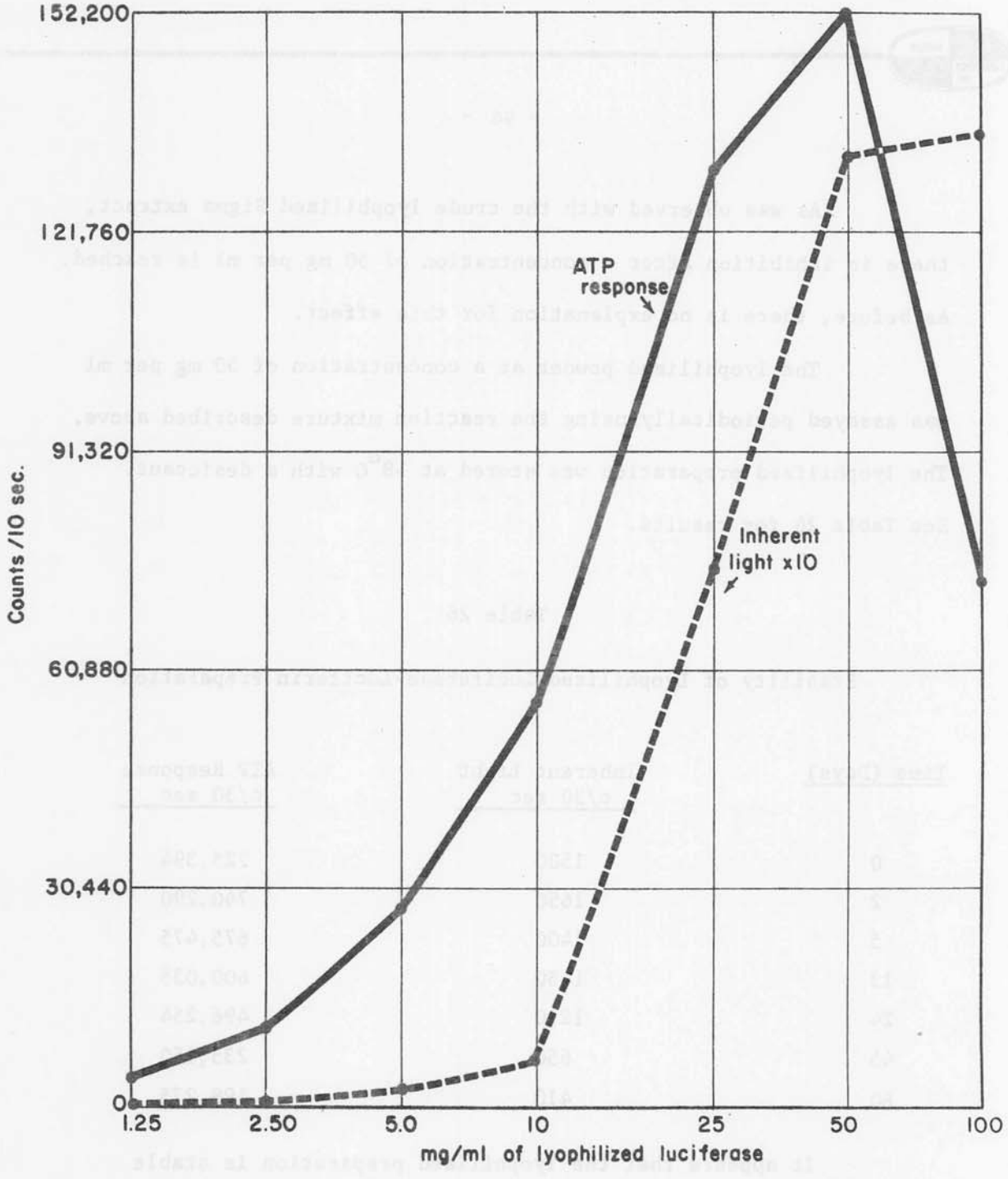


Figure No. 19 — ATP response as a function of lyophilized purified enzyme concentration.

As was observed with the crude lyophilized Sigma extract, there is inhibition after a concentration of 50 mg per ml is reached. As before, there is no explanation for this effect.

The lyophilized powder at a concentration of 50 mg per ml was assayed periodically using the reaction mixture described above. The lyophilized preparation was stored at -8°C with a desiccant. See Table 26 for results.

Table 26

Stability of Lyophilized Luciferase-Luciferin Preparation

Time (Days)	Inherent Light c/30 sec	ATP Response c/30 sec
0	1500	725,394
2	1650	740,290
5	1400	675,475
13	1450	600,035
24	1210	496,254
45	650	235,150
60	410	198,275

It appears that the lyophilized preparation is stable for approximately two weeks and then begins to become inactive. It has been suggested that the lyophilized preparations be stored at lower temperatures under nitrogen to possibly prevent this.

I. Sensitivity and Linearity of ATP Assay

A series of experiments were conducted to determine the maximum sensitivity of the bioluminescent ATP assay under defined conditions and also to determine the linearity of response over a range of ATP concentrations.

All of the studies to be described were carried out using luciferase preparations from Sephadex G-100 columns. Although there was variation in activity between the various preparations, the only differences in the experimental conditions were the buffer solvent for ATP and the counting time. The inherent light level was reduced by standing at room temperature for two hours.

The results of these experiments are described in Tables 27 through 34 and in Figures 20 through 26.

Table 27

Light Response as a Function of ATP Concentration

Experiment #1

Quantity of ATP Injected (ml)	Number of Replicates	Mean Net* Response (c/5 sec)	Standard Deviation (c/5 sec)
0 (water injected)	16	-250	84
10^{-2}	8	195,671	1,652
10^{-3}	8	85,419	10,298
10^{-4}	8	9,635	1,534
10^{-5}	8	1,539	212
10^{-6}	8	443	167
10^{-7}	16	89	89

Reaction Conditions

0.1 ml of luciferase from Sephadex G-100 column

0.1 ml of luciferin (0.5 mg/ml)

0.1 ml of $MgSO_4$ (0.01 M)

0.01 ml of ATP dissolved and diluted in distilled water

Temperature of reaction mixture - 24°C

Instrument - NASA Unit

Inherent light - three measurements for each ATP replicate

Mean inherent light - 877-1798 c/5 sec

Standard deviation of inherent light - 117-290 c/5 sec

*ATP response after subtraction of inherent light and net water response

Table 28

Light Response as a Function of ATP Concentration

Experiment #2

Quantity of ATP Injected (g)	Number of Replicates	Mean Net* Response (c/10 sec)	Standard Deviation (c/10 sec)
0 (water injected)	5	43	23
10^{-3}	4	371,468	6,457
10^{-4}	3	66,631	1,053
10^{-5}	4	6,579	842
10^{-6}	5	822	67
10^{-7}	5	27	26
10^{-8}	5	31	20
10^{-9}	5	-13	22

Reaction Conditions

0.1 ml of luciferase from Sephadex G-100 column

0.1 ml of luciferin (0.5 mg/ml)

0.1 ml of $MgSO_4$ (0.01 M)

0.01 ml of ATP dissolved and diluted in distilled water

Temperature of reaction mixture - 24°C

Inherent light - three measurements for each ATP replicate

Mean inherent light - 254-292 c/10 sec

Standard deviation - 14-22 c/10 sec

*ATP response after subtraction of inherent light and net water response

Table 29

Light Response as a Function of ATP Concentration

Experiment #3

Quantity of ATP Injected (g)	Number of Replicates	Mean Net* Response (c/10 sec)	Standard Deviation (c/10 sec)
0 (Tris injected)	5	82	24
10^{-3}	5	412,822	10,757
10^{-4}	5	73,545	15,497
10^{-5}	5	8,067	1,185
10^{-6}	5	1,047	61
10^{-7}	5	88	18
10^{-8}	5	2	19

Reaction Conditions

0.1 ml of luciferase from Sephadex G-100 column

0.1 ml of luciferin (1 mg/ml)

0.1 ml of $MgSO_4$ (0.01 M)

0.01 ml of ATP dissolved and diluted in 0.05 M Tris

Temperature of reaction mixture - $15^{o}C$

Inherent light - three measurements for each replicate

Mean inherent light - 208-232 c/10 sec

Standard deviation of inherent light - 14-19 c/10 sec

*ATP response after subtraction of inherent light and net Tris response

Table 30

Light Response as a Function of ATP Concentration

Experiment #4

Quantity of ATP Injected (γ)	Number of Replicates	Mean Net* Response (c/10 sec)	Standard Deviation (c/10 sec)
0 (Tris injected)	3	355	-22
10^{-3}	3	342,743	15,583
10^{-4}	3	63,786	6,477
10^{-5}	3	8,268	645
10^{-6}	3	626	65
10^{-7}	3	-48	42
10^{-8}	3	-119	33
10^{-9}	7	38	251

Reaction Conditions

0.1 ml of luciferase from Sephadex G-100 column

0.1 ml of luciferin (1 mg/ml)

0.1 ml of $MgSO_4$ (0.01 M)

0.01 ml of ATP dissolved and diluted in 0.05 M Tris - pH 7.4

Temperature of reaction mixture - 15°C

Instrument - NASA Unit

Inherent light - three measurements for each ATP replicate

Mean inherent light - 228-355 c/10 sec

Standard deviation of inherent light - 11-14 c/10 sec

*ATP response after subtraction of inherent light and net Tris

Table 31

Light Response as a Function of ATP Concentration

Experiment #5

Quantity of ATP Injected (g)	Number of Replicates	Mean Net* Response (c/10 sec)	Standard Deviation (c/10 sec)
0 (Arsenate injected)	3	73	21
10^{-3}	3	320,932	28,994
10^{-4}	3	43,975	10,068
10^{-5}	3	6,439	607
10^{-6}	3	975	132
10^{-7}	3	121	10
10^{-8}	5	60	46
10^{-9}	5	119	15

Reaction Conditions

0.1 ml of luciferase from Sephadex G-100 column

0.1 ml of luciferin (1.0 mg/ml)

0.1 ml of $MgSO_4$ (0.01 M)

0.01 ml of ATP dissolved and diluted in arsenate buffer

Temperature of reaction mixture - 24°C

Inherent light - three measurements for each ATP replicate

Mean inherent light - 66-121 c/10 sec

Standard deviation of inherent light - 6-22 c/10 sec

*ATP response after subtraction of inherent light and net arsenate response

Table 32

Light Response as a Function of ATP Concentration

Experiment #6

Quantity of ATP Injected (ϑ)	Number of Replicates	Mean Net* Response (c/10 sec)	Standard Deviation (c/10 sec)
0 (Arsenate injected)	7	1,757	256
10^{-3}	3	242,466	16,364
10^{-4}	3	43,178	925
10^{-5}	3	4,423	1,074
10^{-6}	3	1,665	19
10^{-7}	3	498	398
10^{-8}	3	436	81
10^{-9}	7	261	333

Reaction Conditions

0.1 ml of luciferase from Sephadex G-100 column

0.1 ml of luciferin (1.0 mg/ml)

0.1 ml of $MgSO_4$ (0.01 M)

0.01 ml of ATP dissolved and diluted in arsenate buffer

Temperature of reaction mixture - 15°C

Inherent light - three measurements for each ATP replicate

Mean inherent light - 124-228 c/10 sec

Standard deviation of inherent light - 12-33 c/10 sec

*ATP response after subtraction of inherent light and net arsenate response

Table 33

Light Response as a Function of ATP Concentration

Experiment #7

Quantity of ATP Injected (g)	Number of Replicates	Mean Net* Response (c/10 sec)	Standard Deviation (c/10 sec)
0 (Arsenate injected)	3	150	22
10^{-8}	7	84	52
10^{-11}	2	19	--

Reaction Conditions

0.1 ml of luciferase from Sephadex G-100 column

0.1 ml of luciferin (1 mg/ml)

0.1 ml of $MgSO_4$ (0.01 M)

0.01 ml of ATP dissolved and diluted in 0.05 M arsenate buffer - pH 7.4

Temperature of reaction mixture - $15^{\circ}C$

Instrument - NASA Unit

Inherent light - three measurements for each ATP replicate

Mean inherent light - 1045-1492 c/10 sec

Standard deviation of inherent light - 389 c/10 sec

*ATP response after subtraction of inherent light and net arsenate response

Table 34

Estimates of Levels of Confidence for the Detection of Low Concentrations of ATP

Expt. #	Quantity of ATP (g)	Mean ATP Response (c/10 sec)	Lower 95% Confidence Limit (c/10 sec)*	Confidence Level For a Positive Response (%)
#1	10^{-7}	89	-89	84
#2	10^{-7}	27	-25	85
#2	10^{-8}	31	-9	94
#2	10^{-9}	-13	-57	28
#3	10^{-7}	88	52	100
#3	10^{-8}	2	-36	54
#4	10^{-7}	-48	-132	13
#4	10^{-8}	-119	-185	0.02
#4	10^{-9}	38	-464	56
#5	10^{-7}	121	101	100
#5	10^{-8}	60	-32	90
#5	10^{-9}	119	89	100
#6	10^{-7}	498	-298	89
#6	10^{-8}	436	274	100
#6	10^{-9}	261	-405	78
#7	10^{-8}	84	-20	95

*Counting time in Experiment #1 was five seconds

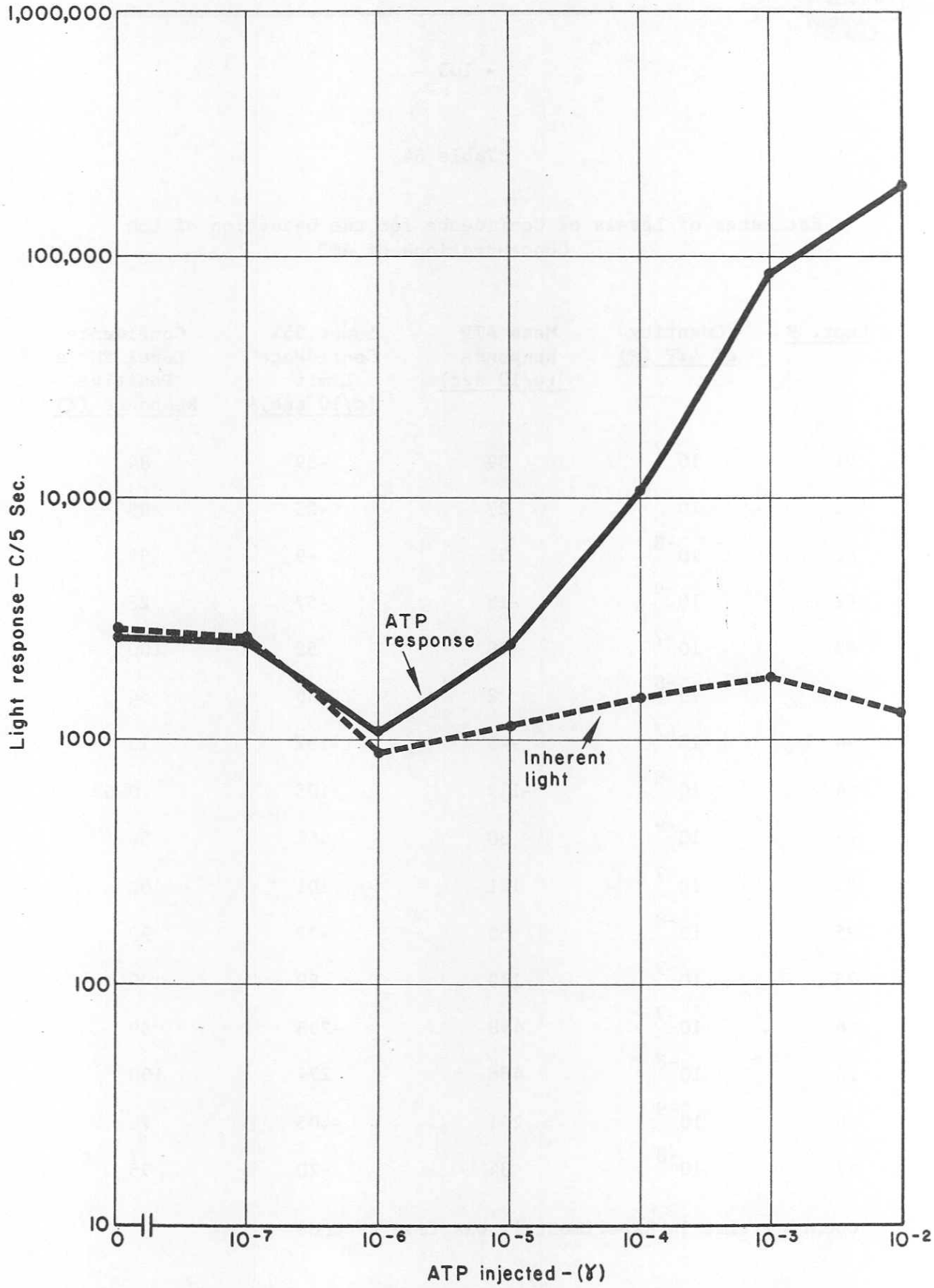


Figure No. 20 – Light response as a function of ATP concentration – Experiment No. 1

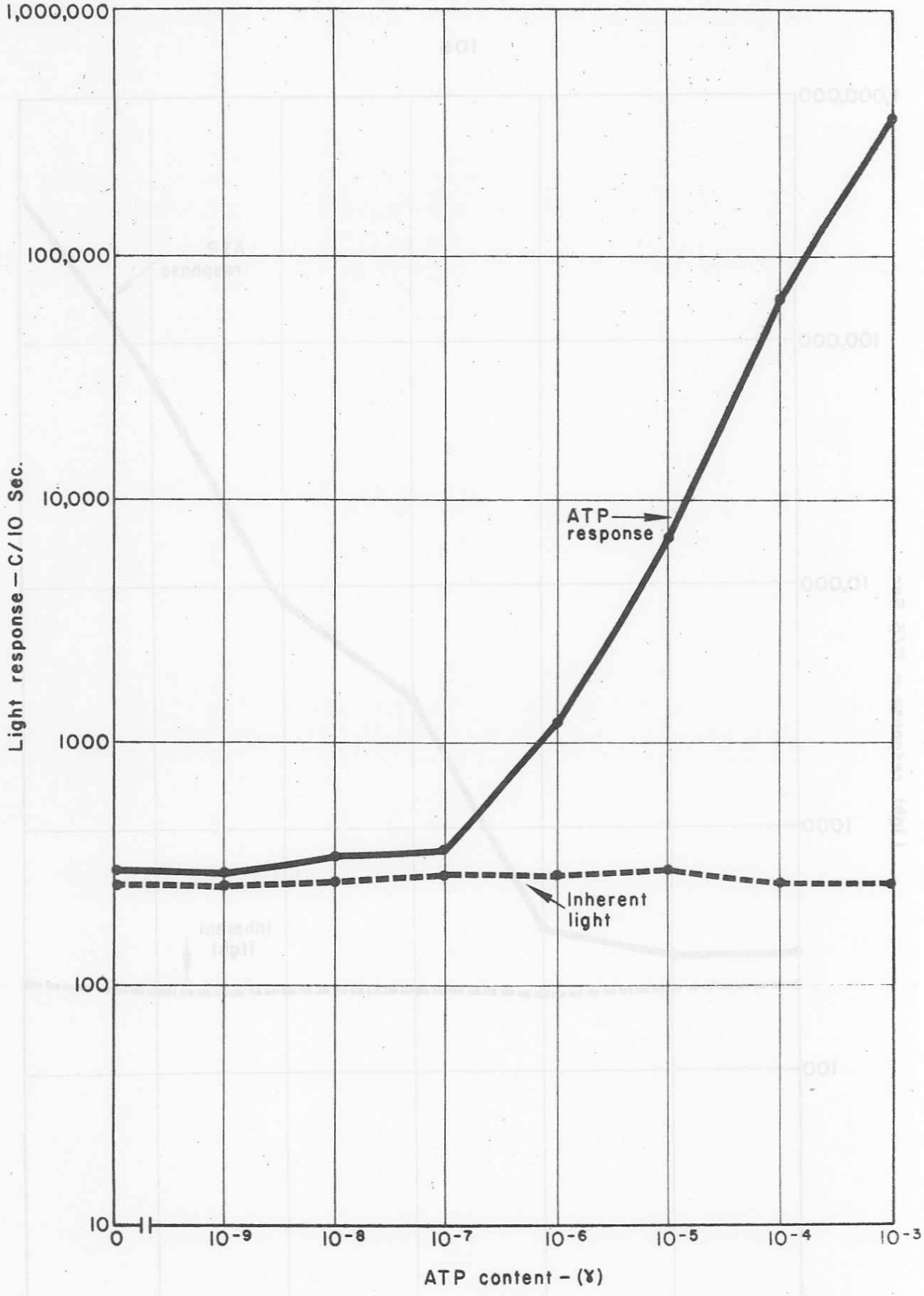


Figure No. 21 – Light response as a function of ATP concentration – Experiment No. 2

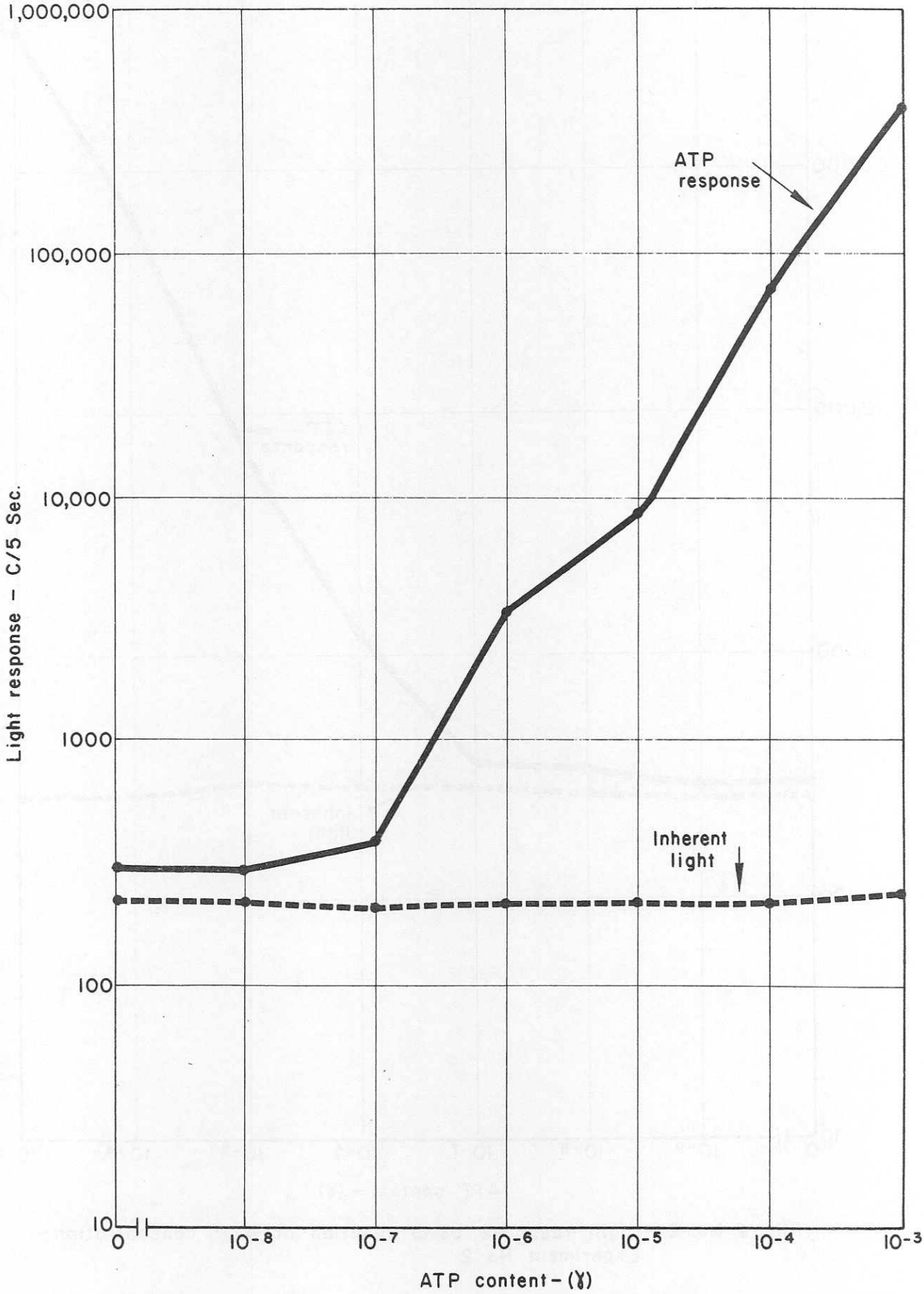


Figure No. 22 - Light response as a function of ATP concentration - Experiment No. 3

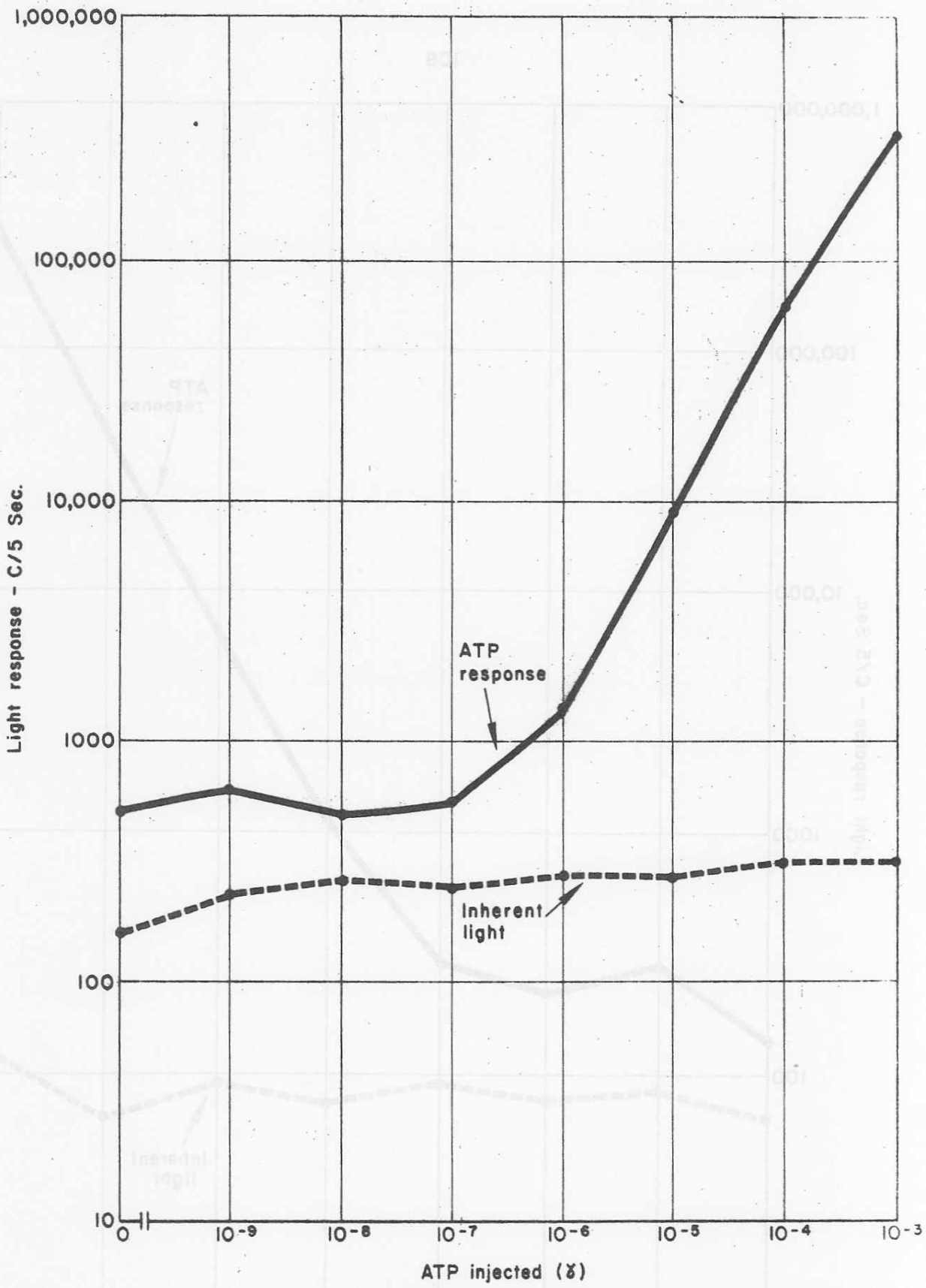


Figure No. 23 – Light response as a function of ATP concentration – Experiment No. 4.

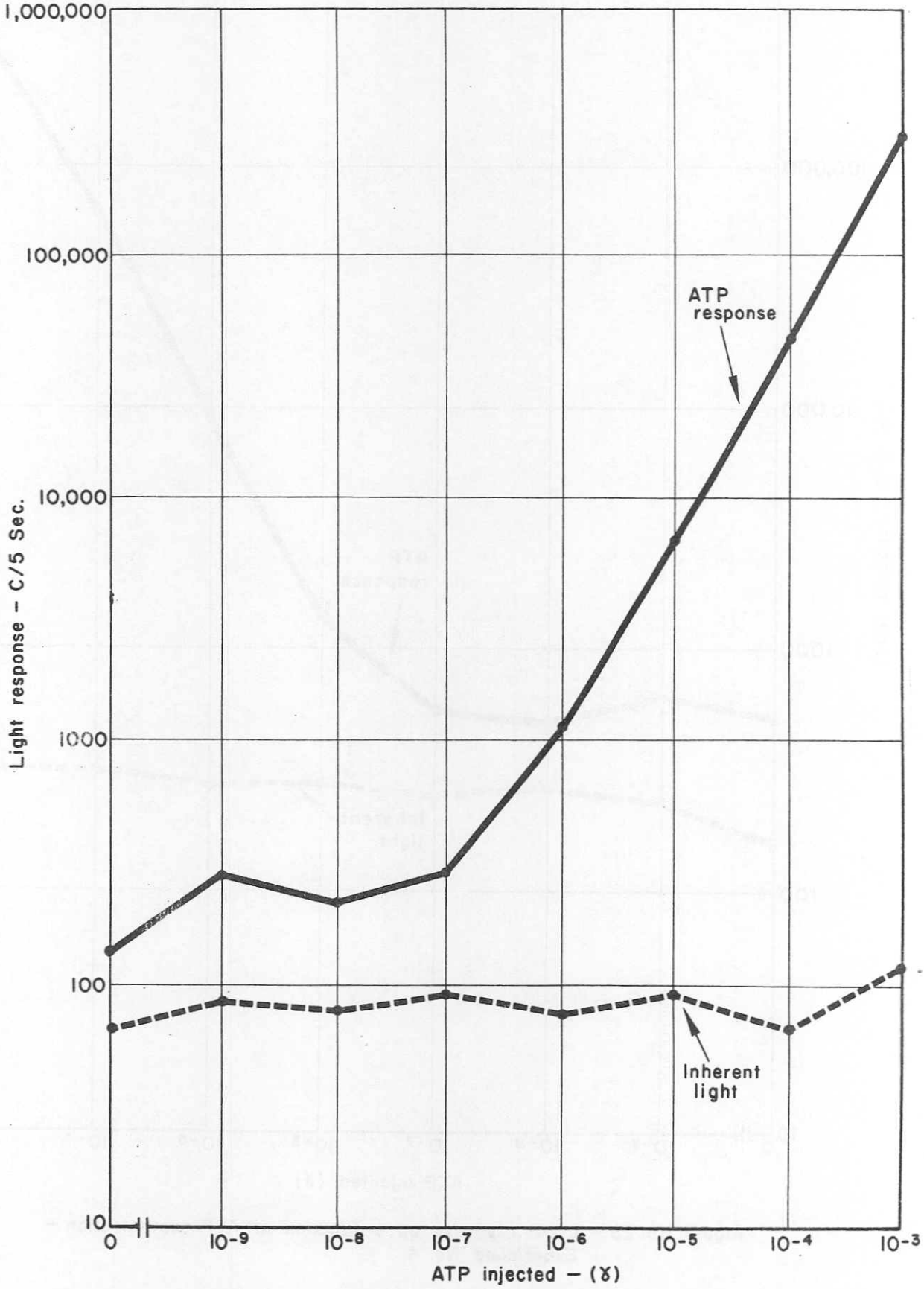


Figure No. 24 — Light response as a function of ATP concentration — Experiment No. 5

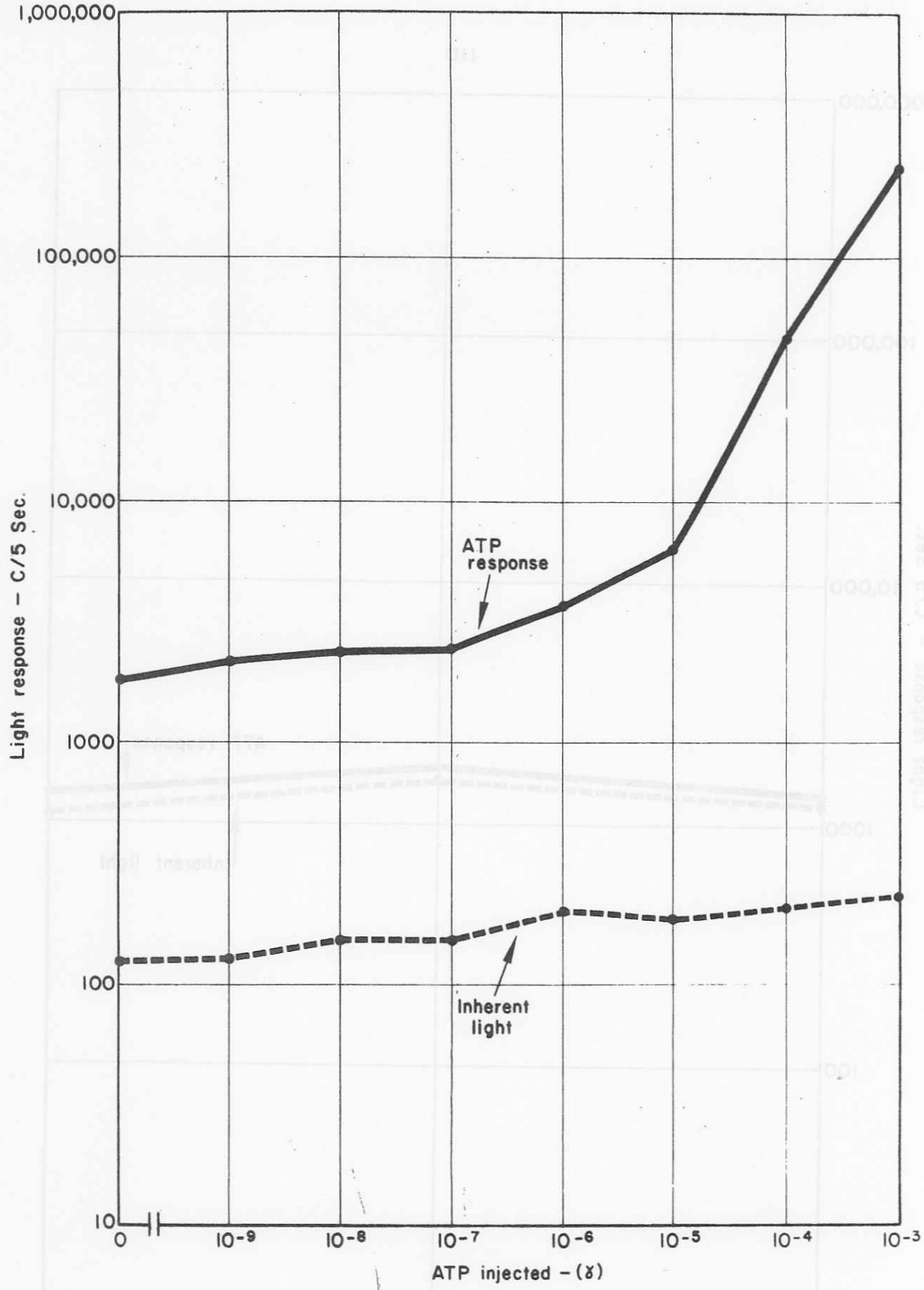


Figure No. 25 – Light response as a function of ATP concentration – Experiment No. 6

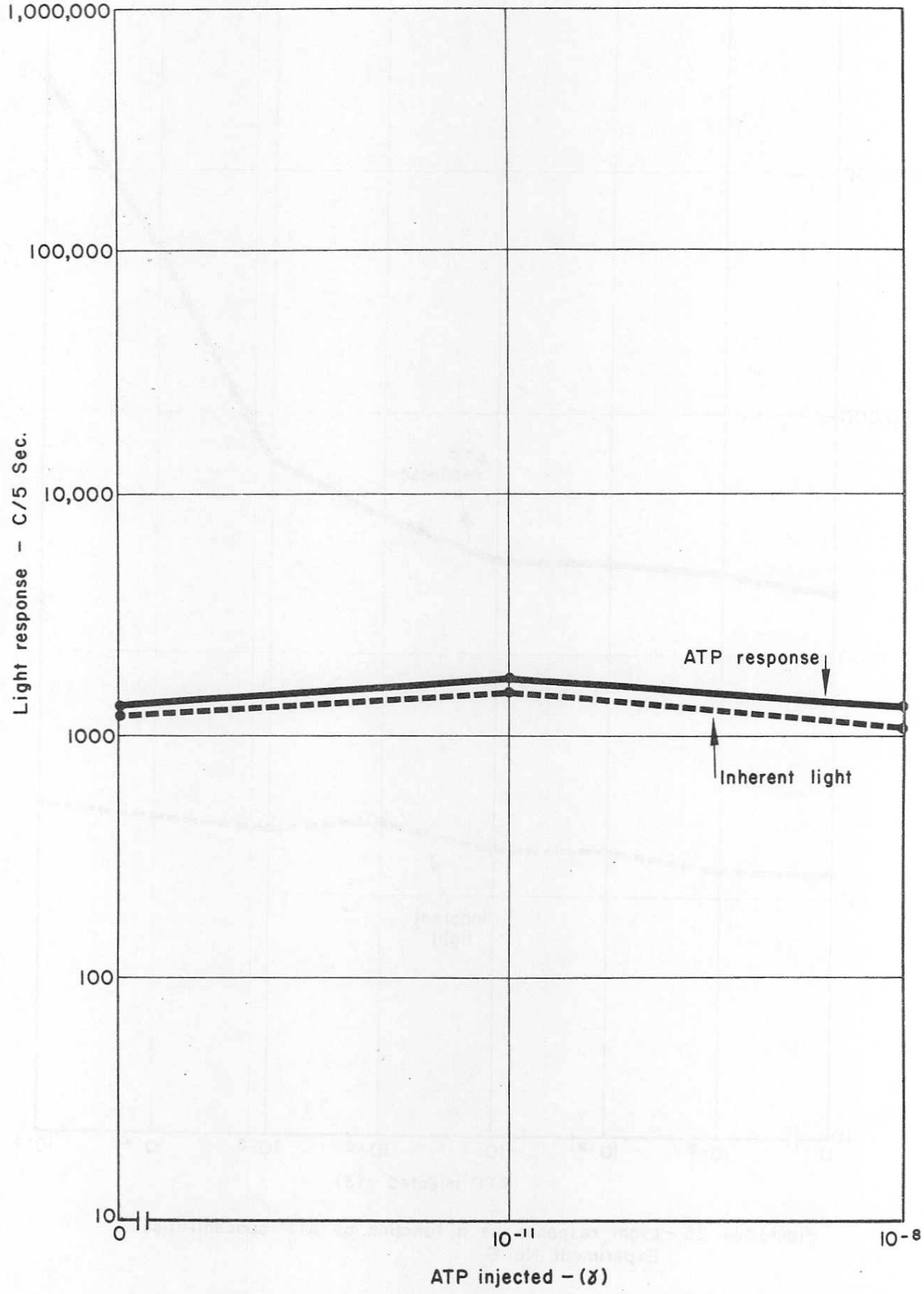


Figure No. 26 – Light response as a function of ATP concentration – Experiment No. 7.

It is seen in the data presented above that ATP can be detected down to a level of 10^{-8} γ with levels of confidence ranging from 0.02% to 100%, and indeed, a positive response is elicited at 10^{-9} γ with confidence limits of 28% to 78%.

On analysis of the data presented above, two problems become quite apparent. The first is the variability in the levels of confidence with which low ATP concentrations can be measured. The second problem is the lack of linearity of light response to ATP at concentrations less than 10^{-7} γ.

There are many factors which might possibly cause the variations seen in the light response at low concentrations. Some of these factors are:

(1) Lack of reproducibility in the volume of injected ATP.

(2) Non-uniformity in cuvette size; this would cause variations in the length of the light path to the photomultiplier tube.

(3) Inherent problems in pulse counting photometers; it has been observed that the response of the NASA Unit to a standard light source with constant light emission is subject to variation. This is due to the random emission of the voltage pulses elicited by light incident to the photomultiplier tube.

It is obvious that additional study is necessary to conclusively establish the nature of the factors responsible for the variations in ATP response.

Nonlinearity in ATP response at low concentration must also be the subject of further investigations. It is impossible to decide at this time whether this nonlinearity is due to instrumentation or if it is biochemical in origin, i.e., ATPase contamination, binding of ATP to container walls, etc.

IV. INSTRUMENTATION

A. Rationale of Instrument Design

The objectives which governed the design of the instrumentation for bioluminescent measurements were as follows:

1. High sensitivity
2. Flexibility to allow the evaluation of a variety of variables
3. Facilities for controlled environment.

B. Instrument Design and Operation

The theory of operation, instrument design, and actual operation are described in Appendix II - Instruction Manual for ATP Detection Instrument.

C. Instrument Calibration

A set of sixty measurements was recorded for each of three light levels obtained using a masked standard light source. There are data reproduced here (See Tables 35, 36 and 37) showing the readings obtained by counting phototube output pulses for a

Table 35

RESPONSE OF ATP DETECTION INSTRUMENT TO STANDARD LIGHT SOURCE

U.S. Radium Corp., G150 Serial 52522

Date of Test: Dec. 21, 1964

Source Temperature: 24°C Photocathode temperature: -25°C

Area of Source Exposed: 0.012 in^2

Discriminator Settings: 500

Pulse Counting Interval: 5 seconds

Counts recorded from exposed source

Light flux from exposed, filtered source: 1.16×10^{-9} lumens

1741	1744	1802	1780
1751	1738	1753	1722
1716	1750	1731	1761
1727	1734	1744	1739
1747	1753	1772	1747
1757	1747	1753	1732
1788	1746	1721	1729
1726	1743	1717	1749
1723	1734	1772	1766
1742	1746	1731	1749
1735	1738	1718	1772
1734	1744	1734	1736
1739	1731	1755	1721
1748	1748	1729	1754
1736	1729	1744	1769

Average count/interval	1744
Variance	305
Standard Deviation	18

Table 36

RESPONSE OF ATP DETECTION INSTRUMENT
TO STANDARD LIGHT SOURCE

U.S. Radium Corp., G150 Serial 52522

Date of Test: Dec. 21, 1964

Source Temperature: 24°C Photocathode temperature: -25°C

Area of Source Exposed: 0.012 in^2

Discriminator Settings: 500

Pulse Counting Interval: 5 seconds

Counts recorded from exposed source through a 12.5% transmission factor filter

Light flux from exposed, filtered source: 1.45×10^{-10} lumens

339	350	312	329
344	339	323	344
334	319	357	364
305	346	309	352
343	326	329	312
319	312	312	319
363	341	322	334
307	322	347	347
311	360	311	323
338	344	334	309
316	312	326	327
339	331	331	350
342	329	358	318
324	341	317	322
348	326	320	340

Average count/interval	331
Variance	232
Standard Deviation	16

Table 37

RESPONSE OF ATP DETECTION INSTRUMENT TO STANDARD LIGHT SOURCE

U.S. Radium Corp., G150 Serial 52522

Date of Test: Dec. 21, 1964

Source of Temperature: 24°C Photocathode temperature: -25°C

Area of Source Exposed: 0.012 in^2

Discriminator Settings: 500

Pulse Counting Interval: 5 seconds

Counts recorded from exposed source through a 0.0125% transmission factor filter

Light flux from exposed, filtered source: 1.45×10^{-13} lumens

14	12	15	21
19	19	15	19
19	13	17	16
19	17	12	12
14	16	18	13
13	18	12	15
16	17	19	12
16	18	14	17
21	14	14	17
11	16	17	16
15	21	19	18
17	12	16	9
17	14	14	21
18	13	13	19
15	14	13	16

Average count/interval	16
Variance	8
Standard Deviation	3

precise five-second period as registered on the read-out panel on the instrument. Also shown are the average counts, the computed variance and standard deviation of the set of measurements. The standard deviation has not been corrected for any error of the calculated mean.

A set of fifty measurements was taken with the dark slide closed but with all other factors the same as those above. These data are reproduced here (See Table 38) together with the computations necessary to determine the mean and the standard deviation of the set of measurements.

The conclusion that can be drawn from these data is that the lowest light level tested (1.45×10^{-13} lumens) can be distinguished from the phototube dark count with a confidence limit of ninety-nine percent when a five-second reading of each are made and compared. For light levels significantly lower than this (by at least one order of magnitude) the ability to confidently discriminate between light and dark counts obtained rapidly diminishes. It is important to notice that the number of counts recorded is not a linear function of the light intensity but a much slower decreasing one. This characteristic makes it difficult to differentiate between two levels of approximately the same intensity. Figure 27 is a plot of the present data showing the average counts

Table 38

ATP DETECTION INSTRUMENT
DARK COUNT MEASUREMENT

Date of Test: Dec. 22, 1964

Photocathode Temperature: -25°C

Discriminator Setting: 500

Pulse Counting Interval: 5 seconds

6	7	9	9	10
9	11	9	13	12
4	8	9	11	7
6	5	11	10	5
7	10	5	8	8
10	6	12	7	6
7	6	8	4	12
4	10	7	8	3
8	13	6	12	6
10	11	6	7	13

Average count/interval	8
Variance	8
Standard Deviation	3

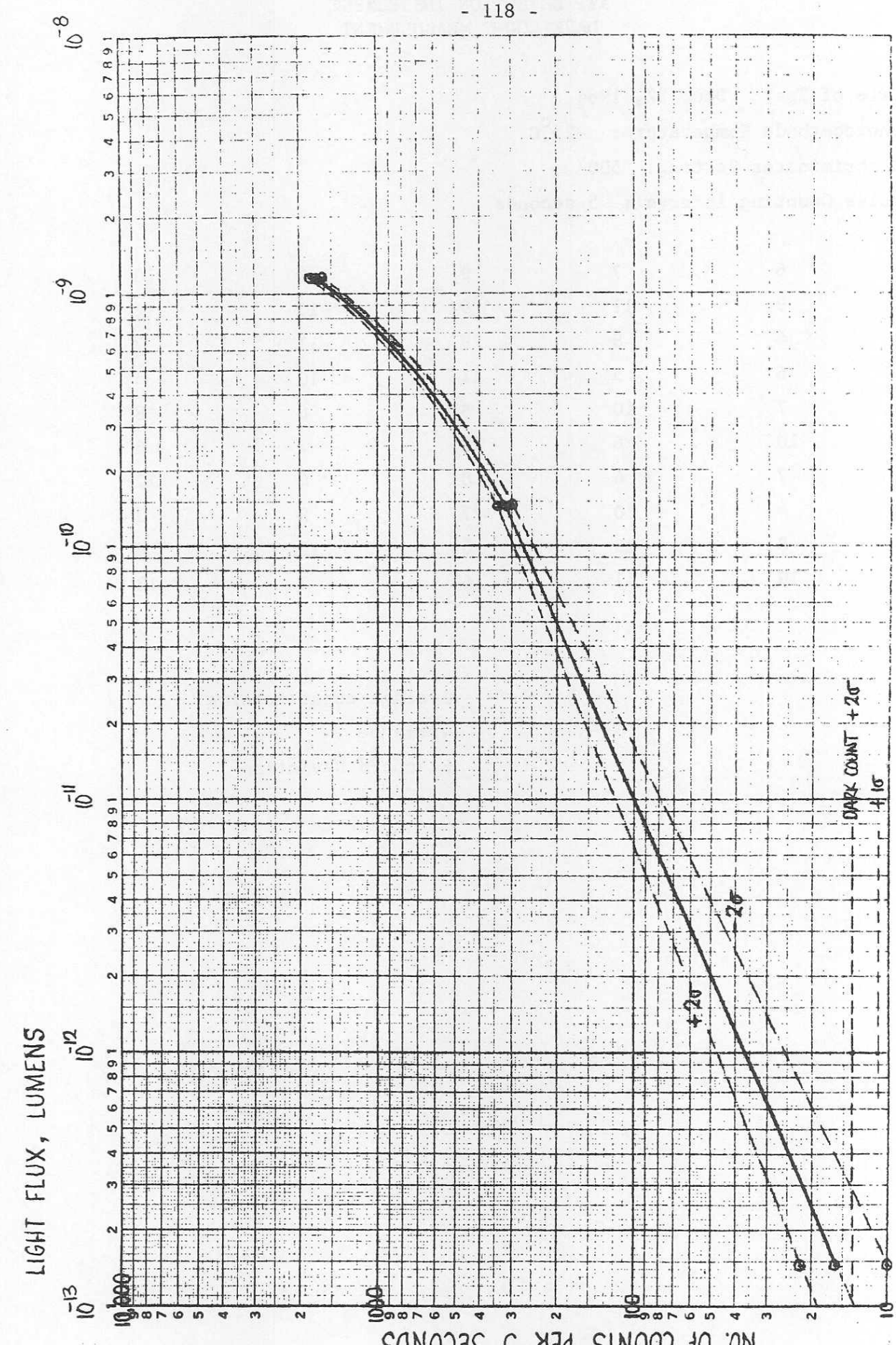


FIG 27 ATP DETECTION INSTRUMENT
LIGHT FLUX DETECTION SENSITIVITY

and the standard deviation observed for the three light levels analyzed. At the threshold of reliable detection, the minimum change in intensity that can be detected with a ninety-five percent confidence level in a single measurement is practically one order of magnitude.

Two factors can be used to improve the confidence level. One is to increase the time of measurement; the other is to take multiple samples (another version of increasing the observation time). One reading for a longer time must be treated differently from separate readings when analyzing the data. The present five-second measurement period was established empirically for the instrument calibration and may not be an optimum interval. The change in observation period is the reason for the slight difference in minimum detectable light level derived from the data presented here and data previously reported.

As a result of experiments conducted to establish an instrument calibration curve, it was determined that for the present enzyme system, an injection volume of ATP of 0.01 ml was desirable. The original syringe selected for the injection does not possess this capacity so a disposable type 1 ml syringe was substituted. Due to the difference in dimensions of the two syringes, modification of the instrument syringe holder is required. The modification has been designed and will be fabricated. In the interim, temporary

measures have been taken to allow the use of the new syringe.

The substitution of the larger capacity syringe allows a simplification of the sterilization procedure since these syringes are available in a clean, sterile condition designed for one-time use. It is more feasible at this time to use the disposable design than to pursue the sterilization procedure. As the experiment is refined, the injected sample size may be reduced and the original syringe re-employed. As reported previously, the larger volume of injected sample appears to result in a more rapid mixing of the components.

A calibration curve was established for the instrument using an enzyme preparation described in the Biochemistry Section.

For the calibration of the instrument, glass cuvettes were used to contain the enzyme-luciferin mixture. A volume of 0.3 ml of enzyme mixture was employed, and the loaded cuvette was allowed to stand at room temperature for at least ten minutes before being placed in the reaction chamber. This was done in an attempt to stabilize the inherent light level. At one point in the calibration procedure, fresh enzyme mixture was prepared from the stock solution in order to ascertain whether or not the activity of the enzyme had changed significantly during the calibration run (some four hours duration). The results obtained showed no change

in the enzyme activity.

The ATP samples were prepared by dilution of a stock solution so that the requisite quantity of ATP was contained in each 0.01 ml volume to be injected. During the measurement of the response to gamma quantities of ATP, fresh ATP solutions of the same concentration were prepared and used to determine whether there was any substantial hydrolysis of the ATP with time in the very dilute solution. There was no significant difference in the measured response between the fresh and the aged ATP samples.

The instrument was prepared for calibration by allowing it to stand ready for a period of two hours. The only parameter which might require this long for stabilization is the temperature of the phototube front face. The thermo-electric cooler temperature will stabilize in less than twenty minutes but the thermal path to the photocathode consists of a heat sink compound in contact with the glass neck of the phototube and a longer period of cooling was allowed to bring the photocathode to the thermo-electric cooler temperature. Ten readings (five seconds each) were taken of the phototube dark current to confirm the anticipated value, and the calibration procedure was begun.

The first measurements were made on the effect of introducing a water sample of 0.01 ml into the 0.3 ml enzyme

mixture. Sixteen separate reactions were initiated using sterile, deionized water. In each case five separate measurements were taken of the inherent light level and one reading of the sample light level after injection. The response count began with the initiation of the injection. The injection of the 0.01 ml water sample was completed in 25 milliseconds.

The data show that, in each case, the light emitted by the reaction as measured by the instrument decreased. This is due to the water diluting the enzyme mixture that is within the field of view of the phototube. The light emitted decreased by approximately ten percent. The average loss for the sixteen samples and the standard deviation of the values were computed for the purpose of correcting subsequent data for the change in inherent light level produced by dilution of the enzyme mixture by the volume of the injected sample. This change in the inherent light level due to dilution has a substantial effect on the ability to detect small quantities of ATP as will become apparent in the discussion of the calibration data to follow.

The procedure for data correction was to determine the least number of counts lost due to dilution alone, and to add this number to the response obtained from a reaction due to the injection of ATP in the same volume of water. Since the inherent light

level is variable from sample to sample, a net change in light level is taken to represent the response to the injected ATP. If the inherent light level was constant from test to test, the procedure would be somewhat simpler.

The data for the water reaction is included in this report together with the calculation of the standard deviation of the set of sixteen measurements*.

The first calibration set was obtained with eight samples by injecting 1×10^{-2} gamma of ATP in a 0.01 ml water solution. The data for these samples are included in this report together with the calculation of the standard deviation of the set of measurements. Subsequent sets of data, consisting of eight samples each, were obtained for injections of quantities of 10^{-3}, 10^{-4}, 10^{-5}, and 10^{-6} gammas of ATP. The data for each of these sets are attached together with the calculation of the mean response and the standard deviation for each set of measurements.**

For the determination of the response due to the injection of a 10^{-7} gamma quantity of ATP, sixteen measurements were made. These data are attached to this report. Upon examination, the data are inconsistent, since after correcting for the least expected dilution loss there is a net loss after the injection of ATP. It is unlikely that the injection of ATP results in no emission of

*See Table 39
**See Table 40-45

Table 39

RESPONSE OF ATP DETECTION INSTRUMENT
TO INJECTION OF WATER INTO ENZYME MIXTURE

Date of Test: Dec. 22, 1964

Volume of Enzyme Mixture: 0.3 ml Injected H_2O Volume: 10 lambda

Reaction Temperature: 24°C Photocathode Temperature: -25°C

Discriminator Setting: 500 Glass cuvette

Pulse Counting Interval: 5 seconds Injection Time: 0.025 seconds

Sample No.	Inherent Light (average)	Response
1	3212	2811
2	3195	2888
3	3209	2889
4	2965	2629
5	3098	2851
6	3007	2675
7	3114	2813
8	2216	2121
9	2734	2436
10	2744	2516
11	2846	2638
12	2786	2658
13	2857	2676
14	2849	2608
15	2699	2484
16	2783	2620

Average count/interval	-250
Variance	6997
Standard Deviation	84

Table 40

RESPONSE OF ATP DETECTION INSTRUMENT
TO ATP SAMPLE INJECTION

Date of Test: Dec. 22, 1964

Volume of Enzyme Mixture: 0.3 ml Injected H_2O volume: 10 lambda

Reaction Temperature: 24°C Photocathode Temperature: -25°C

Discriminator Setting: 500 Glass Cuvette

Pulse Counting Interval: 5 seconds Injection Time: 0.025 seconds

Sample Content: 10^{-2} gamma ATP

Sample No.	Inherent Light (average)	Response
1	937	193,985
2	1282	195,445
3	1798	199,292
4	1265	195,425
5	1198	196,990
6	1148	196,878
7	1084	196,257
8	1593	199,403

Average count/interval	195,522
Variance	2.7×10^6
Standard Deviation	1650

Table 41

RESPONSE OF ATP DETECTION INSTRUMENT
TO ATP SAMPLE INJECTION

Date of Test: Dec. 22, 1964

Volume of Enzyme Mixture: 0.3 ml

Reaction Temperature: 24°C

Discriminator Setting: 500 Glass Cuvette

Pulse Counting Interval: 5 seconds Injection Time: 0.025 seconds

Sample Content: 10^{-3} gamma ATP

Sample No.	Inherent Light (average)	Response
1	1947	70,181
2	1964	93,441
3	1923	100,401
4	1880	85,050
5	1847	85,012
6	1993	83,354
7	1176	98,981
8	1656	79,316

Average count/interval	87,068
Variance	7.249×10^8
Standard Deviation	10,180

Table 42

RESPONSE OF ATP DETECTION INSTRUMENT
TO ATP SAMPLE INJECTION

Date of Test: Dec. 22, 1964

Volume of Enzyme Mixture: 0.3 ml

Reaction Temperature: 24°C

Discriminator Setting: 500 Glass Cuvette

Pulse Counting Interval: 5 seconds Injection Time: 0.025 seconds

Sample Content: 10^{-4} gamma ATP

Sample No.	Inherent Light (average)	Response
1	1602	9233
2	1713	11,531
3	1747	12,331
4	1565	12,286
5	1550	11,975
6	822	11,636
7	1461	8826
8	1570	9289

Average count/interval	9485
Variance	2.40×10^6
Standard Deviation	1550

Table 43

RESPONSE OF ATP DETECTION INSTRUMENT
TO ATP SAMPLE INJECTION

Date of Test: Dec. 22, 1964
Volume of Enzyme Mixture: 0.3 ml
Reaction Temperature: 24°C
Discriminator Setting: 500 Glass Cuvette
Pulse Counting Interval: 5 seconds Injection Time: 0.025 seconds

Sample Content: 10^{-5} gamma ATP

Sample No.	Inherent Light (average)	Response
1	1075	2378
2	1279	2317
3	1072	2564
4	1295	2925
5	1087	2296
6	941	2170
7	1181	2577
8	1110	2128

Average count/interval	1465
Variance	79,732
Standard Deviation	282

Table 44

RESPONSE OF ATP DETECTION INSTRUMENT
TO ATP SAMPLE INJECTION

Date of Test: Dec. 22, 1964
Volume of Enzyme Mixture: 0.3 ml
Reaction Temperature: 24°C
Discriminator Setting: 500 Glass Cuvette
Pulse Counting Interval: 5 seconds Injection Time: 0.025 seconds

Sample Content: 10^{-6} gamma ATP

Sample No.	Inherent Light (average)	Response
1	989	1237
2	1229	1203
3	959	1481
4	873	1108
5	874	997
6	802	849
7	679	813
8	612	870

Average count/interval	305
Variance	9598
Standard Deviation	98

Table 45

RESPONSE OF ATP DETECTION INSTRUMENT
TO ATP SAMPLE INJECTION

Date of Test: Dec. 22, 1964
Volume of Enzyme Mixture: 0.3 ml
Reaction Temperature: 24°C
Discriminator Setting: 500 Glass Cuvette
Pulse Counting Interval: 5 seconds Injection Time: 0.025 seconds

Sample Content: 10^{-7} gamma ATP

Sample No.	Inherent Light (average)	Response
1	3292	3133
2	2665	2390
3	2739	2592
4	2788	2472
5	2757	2573
6	2901	2655
7	2670	2585
8	2517	2463
9	2760	2526
10	2801	2629
11	2666	2430
12	2400	2172
13	2460	2429
14	2288	2256
15	2359	2270
16	2631	2529

Average count/interval	-49
Variance	11,380
Standard Deviation	107

additional light. The most logical explanation for these results is that the correction of the data for the least reduction in inherent light level is not adequate to reveal the average difference, and the variance of the results is the product of the large standard deviation of the dilution loss and a large standard deviation in the actual response of this particular enzyme preparation to a quantity of 10^{-7} gammas of ATP.

Figure 28 is a graph of the net response due to the injection of a quantity of 10^{-4}, 10^{-5}, and 10^{-6} gammas of ATP drawn in order to show the resolution available at the limit of reliable detection. From the results obtained from these calibration data, it is possible to resolve the difference in the response due to the injection of one microgamma of ATP and four microgammas of ATP with at least a 95% confidence level. At a slightly lower confidence level, it is possible to detect the difference between one and 2.5 microgammas of ATP. Extrapolation of the data to the water response level shows that it should be possible to detect the response due to 5×10^{-7} gammas of ATP using this particular enzyme substrate.

A qualitative review of the calibration data, the design of the test and the characteristics of the biochemical experiment lead to several conclusions which should be considered

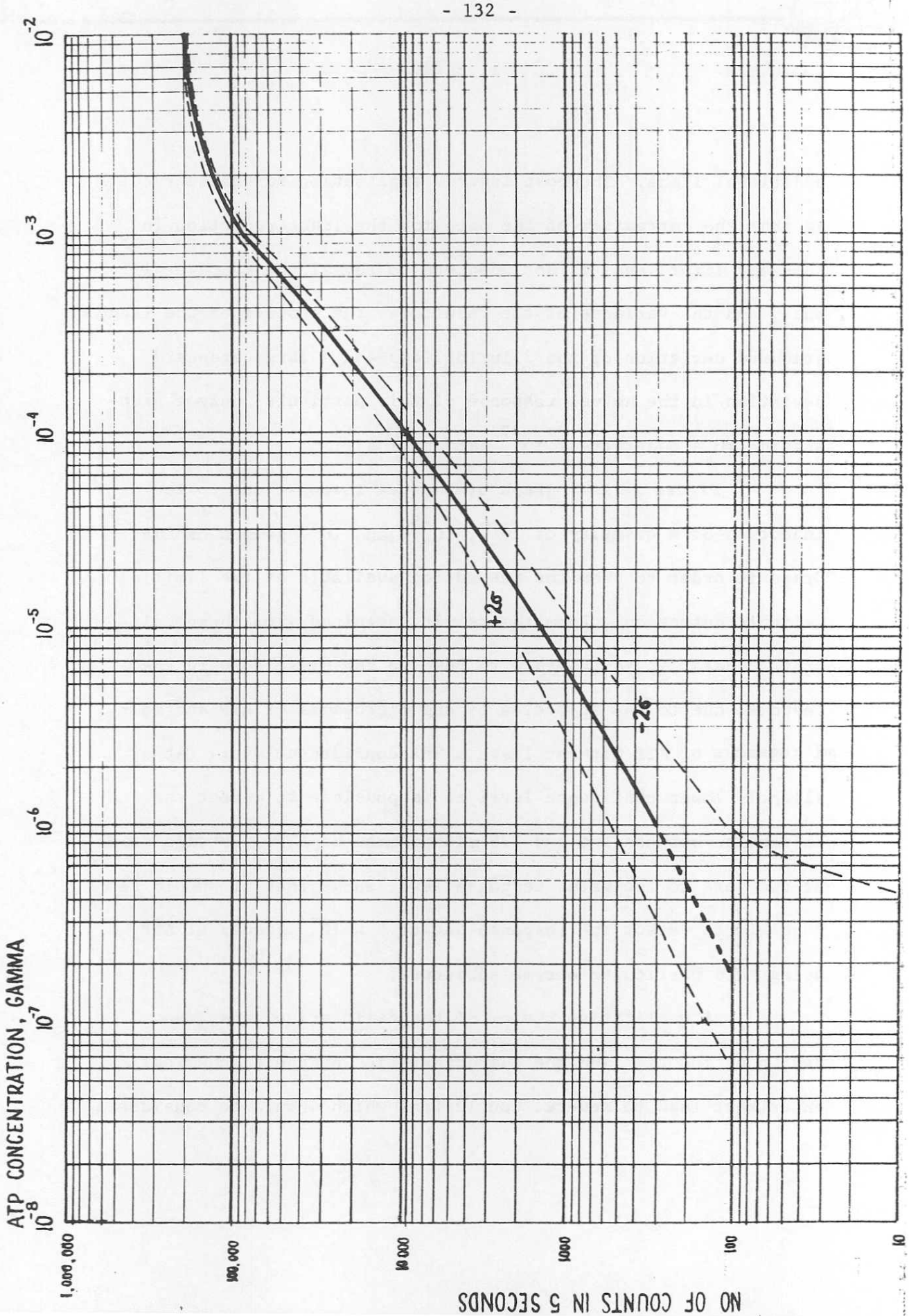


FIG 28 ATP DETECTION SENSITIVITY

in evaluating the significance of the calibration. This calibration is valid only for the specific enzyme preparation employed. There appear to be several important factors connected with the enzyme preparation which vitally affect the detection threshold available with the experiment. Both the activity and the inherent light level must be investigated more extensively before the ultimate sensitivity can be determined. In the design of the test, it would appear that several factors could be manipulated to improve the sensitivity of the experiment. First, the enzyme volume could be reduced to reduce the inherent light, and also to keep the entire quantity within the field of view of the phototube. This alteration would eliminate the necessity for correction due to dilution. Second, accurate measurement of the enzyme volume would reduce variations in inherent light caused by different quantities of material being presented to the field of view of the phototube when samples smaller than the total field of view are used. Third, the counting time could be increased. The five second period was established in order to count only during the portion of the reaction where the signal-to-background ratio was increasing. Extending this time will result in significantly higher net counts since it is observed that the signal-to-background ratio does not decrease rapidly with time. From the data accumulated to date, the count period could be safely extended

by a factor of five. Fourth, the injection volume could be reduced. This would reduce any effects caused by dilution.

V. DISCUSSION AND CONCLUSIONS

As was pointed out at the beginning of this report, the investigations carried out during the tenure of this contract had as their goals the establishment of methods for the routine preparation of luciferase and luciferin, the establishment of optimal reaction conditions, the reduction of inherent light, and the determination of the maximum sensitivity of the assay technique. It is believed that these goals have been attained. The preparation of luciferase using Sephadex chromatography can be carried out in a relatively simple reproducible fashion. The activity of the enzyme is consistently high.

The advantages of the use of synthetic luciferin at relatively high concentrations have been well established during the course of these studies.

The reaction conditions for maximum ATP response have now been established.

The reduction of inherent light has continued to be a somewhat baffling problem. Although the data presented in this report indicate that the ultimate factor responsible for inherent light is ATP, the mechanisms responsible for its presence are still

unknown. The observation that the optimal pH and temperature of inherent light is different from those determined for maximal ATP response is subject to a variety of interpretations. A rather intriguing possibility is that another enzyme is involved in the production of inherent light. The proof of this must, of course, await further investigation. The present status of inherent light is that while it may be reduced by certain of the expediencies described in this report, it remains a problem to be solved.

The finding that quantities of ATP as low as 10^{-8} g can be detected is extremely encouraging. However, the variability and lack of linearity at low ATP concentrations are problems which will require additional study.

VI. RECOMMENDATIONS

Based on the results of the studies described in this report, it is recommended that the following areas be investigated.

A. Further improvement in the sensitivity of the bioluminescent ATP assay method.

B. Modification of the ATP assay for flight conditions.

C. Investigation of other complementary life detection methods.

A. Improvement of Sensitivity of ATP Assay

1. Removal of Inherent Light

A major factor which continues to limit the ultimate sensitivity of the bioluminescent ATP assay is that of inherent light.

The problem of complete elimination of inherent light has not yet been solved. Various methods of minimizing it have been developed. These include the use of hexokinase, aging, and fractionation by Sephadex. It has been indicated by several of the studies carried out thus far that the source of inherent light is protein bound ATP and that the level of the inherent light is a function of the rate of dissociation of the protein-ATP complex. It can be assumed that a certain amount of the bound ATP is not bound to luciferase but to other inert proteins. It would thus follow that the higher the specific activity of a protein fraction, the lower would be the inherent light for a given amount of activity. This condition could be approached by further purification of luciferase, possibly to crystallization. Large scale crystallization has never been previously attempted here because of a shortage of firefly tails.

With the availability of increased quantities of fireflies, it is proposed to investigate the effects of further purification of luciferase upon the magnitude of inherent light. The methods of purification will include column chromatography with ion exchange resins and synthetic molecular sieve materials, and also ammonium

sulfate fractionations.

Studies in this report have indicated that there is a difference in the optimal temperature and pH of inherent light and ATP response. It has been recently observed by another investigator that there is a certain amount of inherent light reduction following ultrafiltration of the luciferase extract. This would indicate that at least some of the inherent light is associated with particulate material. With this in mind, it is proposed to examine both the effects of ultrafiltration and ultracentrifugation upon the magnitude of the inherent light. The optimal temperature for exogenous ATP response was found to be 20°C, while that of the inherent light was between 30°C and 35°C. The pH of maximal ATP response was approximately 7.4 while that of the inherent light was approximately 8.3. It is also indicated that the reaction responsible for inherent light is inhibitory toward the exogenous ATP reaction. The above findings might conceivably be suggestive of separate enzymatic mechanisms being responsible for inherent light. A substantiation of this would, however, require a great deal of additional study as there are other possible explanations of the above data. In any event, however, it may be possible to exploit the above differences in the optimal conditions for inherent light versus ATP response as a possible means of reducing inherent light.

2. Increase in Rate of Reaction

Under present reaction conditions, the kinetics are such that upon the addition of ATP to the enzyme, there is a rapid initial rise in the intensity of the emitted light followed by a slow exponential decay. It is obvious that the signal-to-noise ratio during the measurement of the emitted light would be much more favorable if the light of the exponential phase was incorporated into the rapid initial phase.

The interpretation of the exponential phase as given by McElroy, et al., is that it is due to product inhibition of luciferase. It would thus follow that there are two possible means of reducing the magnitude of the exponential phase; namely, removal of the product from the enzyme surface, and by increasing the initial enzyme concentration. Both of these have been attempted.

Studies by McElroy, et al. (14), have shown that the product of the bioluminescent reaction, dehydroluciferylandenylic acid, can be split off from luciferase by either pyrophosphate or coenzyme A. The use of pyrophosphate for this purpose is complicated by virtue of pyrophosphate being a product of the first step in the sequence of reactions, and thus would poise the reaction to the left if present at the time of ATP addition. This was manifested in our experiments by a reduction in ATP response when the reaction was carried out in the presence of pyrophosphate. It was also observed

that there was a high concomitant rise in the inherent light level.

The results obtained when the reaction was performed in the presence of coenzyme A were equally as unrewarding. The ATP response was reduced while at the same time there was a rise in the level of inherent light to a value equivalent to several gammas of ATP.

It is possible that if the factors responsible for inherent light were removed from the enzyme system, the results obtained with both pyrophosphate and coenzyme A would be quite different.

On attempting to decrease the duration of the exponential phase of the reaction by the use of higher concentrations of partially purified luciferase, it was found that above a certain concentration (this varied from preparation to preparation), there was no further increase in reaction rate, but instead a decrease accompanied by a rise in the level of inherent light. As was previously mentioned, it is believed that a certain amount of the endogenous ATP responsible for inherent light is bound to inert protein; thus it may possibly be concluded that the above observation is a reflection of the inhibition caused by the high concentration of endogenous ATP.

As all of the observations described above appear to be influenced by the inherent light phenomenon, the effects of pyrophosphate, coenzyme A, and high luciferase concentrations using luciferase at a higher state of purity should be re-investigated.

B. ATP Assay Under Flight Conditions

A major problem which remains to be studied is that of rendering the ATP assay method compatible with rocket flight conditions. The optimal reaction conditions at this time have been established only for laboratory instrumentation and it is quite likely that it will not be compatible to duplicate these conditions in a flight system. The goals of these studies to be proposed should be:

(1) Determine the effects of environmental flight conditions upon the bioluminescent reaction.

(2) Modify the reaction conditions within biochemical limits to ensure compatability with anticipated instrumentation.

1. Environmental Flight Conditions

Having established the optimal reaction conditions in the laboratory, it remains to be determined how well these conditions can be maintained during rocket flight. The abnormal conditions which may be encountered must also be defined.

2. Effect of Environmental Flight Conditions Upon the Bioluminescent Reaction

The effects of those flight conditions, which are at variance with laboratory conditions, upon the bioluminescence reaction should be studied. The particular conditions to be studied will depend upon the guidelines established above.

C. Compatability of Flight Instrumentation and Reaction Conditions

It will be necessary to assess the compatability of anticipated flight instrumentation with the previously established reaction conditions. It is almost certain that the unique nature of the instrumentation for flight conditions will necessitate a number of changes in the reaction conditions.

The ATP assay method as it will be used under flight conditions may be divided into the following sequence of steps:

(1) Collection of sample
(2) Extraction of ATP
(3) Presentation of ATP to enzyme system
(4) Bioluminescent reaction
(5) Measurement of light intensity

While sample collection is purely instrumental, it will cast a certain influence upon the most feasible extraction procedure. For this reason, a number of extraction techniques would be examined, both chemical and physical. These would include extraction by chemicals such as trichloroacetic acid, perchloric acid, and dimethyl sulfoxide. The major physical method to be studied would be disruption by ultrasonic vibration. From the above techniques and possibly others, one may be selected which is most compatible with the collection procedure and also with subsequent steps.

The form and fashion in which the ATP and enzyme will be introduced to each other would be objects of extensive investigation.

The state of the enzyme system during flight will be dependent upon factors such as duration of flight and the degree of temperature control. It has been established here that the enzyme system when lyophilized under certain conditions, may be stored in the dry cold state for short periods of time with little loss in activity, whereas in solution, it is stable for relatively long periods in the cold.

Our present procedure for mixing ATP with enzyme involves the injection via syringe of the ATP into the enzyme mixture. Other mixing procedures should be evaluated.

D. Other Complementary Life Detection Methods

1. Rationale

The ideal condition for life detection during space flight would be one where a number of biological criteria could be assayed simultaneously, i.e., "a shotgun approach." This would reduce one of the risks inherent in the use of only one life detection method, this being the possibility of encountering an organism which would lack the factor necessary for a positive response. The "shotgun" approach is, however, limited by instrumentation problems. The instrumentation necessary for the measurement of a variety of biological intermediates could become so involved and cumbersome as to be impractial. This type of approach would be most feasible with selected compounds which could be assayed with the same instrumentation and possibly the same reaction mixture cuvette.

It is believed that compounds participating in bioluminescent reactions are ideally suited for this type of approach.

The use of other bioluminescent systems as life detection techniques to complement the bioluminescent ATP assay should thereby be investigated. The systems which appear to offer promise are described below.

2. Bacterial Luminescence

A cell free extract of luminous bacteria (Photobacterium fischeri) capable of exhibiting luminescence was first prepared by Shoup and Strehler in 1951. As the result of studies conducted by a number of investigators over the next few years (these studies are described in the excellent review by Cormier and Totter [15]), the following reaction mechanism has been established.

$$(1)\quad DPNH + H^{+} + FMN \underset{}{\overset{\text{DPNH oxidase}}{\rightleftharpoons}} FMNH_2 + DPN$$

$$(2)\quad FMNH_2 + RCHO + O_2 \xrightarrow{\text{luciferase}} \text{light (490 m}\mu\text{)}$$

DPNH - reduced pyridine nucleotide
FMN - flavin mononucleotide
RCHO - long chain aldehyde

Reaction No. 1 is not a true component of the bioluminescent system but merely serves to provide reduced FMN. This is a function which a number of other enzyme systems could provide. Thus, the

true components of the system are $FMNH_2$, a long chain aldehyde, oxygen, and luciferase. The component of primary interest in this system is $FMNH_2$ which is probably as widespread throughout living organisms as is ATP. It appears that under properly selected conditions, the bacterial bioluminescence could be exploited for use as a sensitive quantitative method for the assay of FMN.

3. Bioluminescent System in Sea Pansy

The bioluminescent system found in the sea pansy (Renilla reniformis) which has been extensively studied by Cormier (16) appears to offer promise as the basis of a life detection method.

The mechanism for the reaction is briefly as follows:

(1) Luciferin + DPA $\xrightarrow{\text{luciferase}}$ activated luciferin

(2) Activated luciferin + O_2 $\xrightarrow{\text{luciferase}}$ light + Products

DPA - 3', 5'-diphosphoadenosine

As DPA plays a prominent role in sulfate transfer reactions, it might be expected to be rather widespread in its occurrence in living organisms. A difficulty to be encountered with DPA is that it appears to function as a cofactor and not as a substrate. It has been shown that in the presence of excess luciferin, wide variations in the DPA concentration do not change the total light emitted. It is possible, however, that initial light intensities would show a linear response to changes in DPA concentration.

The feasibility of the above system for possible use as a life detection method is worthy of study.

4. Chemical Luminescence Assay

In 1962, Seliger and McElroy (17) demonstrated a chemiluminescence of firefly luciferin in the absence of luciferase. This was done by condensing adenosine monophosphate (AMP) with luciferin in dimethyl sulfoxide as the reaction medium. This reaction also requires dicyclohexylcarbodiimide. Luminescence occurred on the addition of NaOH pellets to the condensation product. It is believed that a system of this kind holds possible promise for use as a life detection method.

VII. APPENDIX I - SYNTHESIS OF FIREFLY LUCIFERIN
[2-(6-hydroxy-2-benzothiazolyl)-Δ^2-thiazoline-4-carboxylic acid

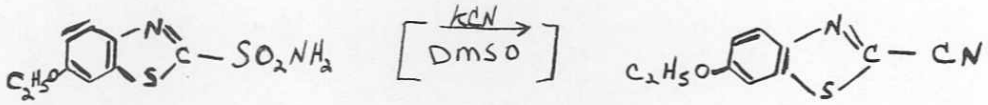
The synthesis of firefly luciferin was performed following the original procedure described in 1963 by E. H. White. As we were able to obtain a considerable amount of 6-ethoxybenzothiazole-2-sulfonamide as a gift from the Upjohn Company, the whole procedure consisted of only three steps:

1. Conversion of 6-ethyoxybenzothiazole-2-sulfonamide into 2-cyano-6-ethoxybenzothiazole.

2. Conversion of 2-cyano-6-ethoxybenzothiazole into 2-cyano-6-hydroxybenzothiazole.

3. Reduction of D-cystine into D-cysteine and condensation of the freshly prepared D-cysteine with 2-cyano-6-hydrobenzothiazole into D-luciferin.

A. Conversion of 6-ethoxybenzothiazole-2-sulfonamide into 2-cyano-6-ethoxybenzothiazole



$C_9H_{10}O_3N_2S_2$
258.318

$C_{10}H_8ON_2S$
204.246

1. Material

a. 10 grams of 6-ethoxybenzothiazole-2-sulfonamide (0.38 mole).

b. five grams potassium cyanide (0.077 mole)

c. 1000 ml of dimethyl sulfoxide (dry) and 525 ml chloroform.

d. 485 grams activated alumina (Merck acid-washed)

e. 2.5 liter petroleum ether (b.p. 30-60°C)

f. 1.8 liter ether

g. Sodium sulfate anhydrous

2. Equipment

a. 2 liter three-necked round bottomed flask with standard joints.

b. Mechanical stirrer

c. Drying tube

d. Oil bath

e. Thermometer

f. 3-4 one beaker

g. 3-4 one suction filtration flask

h. Buchner funnel about 12 cm

i. One liter separating funnel

j. Chromatographic tube

k. 500 ml flasks to collect fractions

3. Procedure

(a) and (b) under the Material section are combined in 1000 ml of DMSO in a two-liter round bottomed flask equipped with

a mechanical stirrer and a drying tube, and heated under continuous stirring two and one-half hours on an oil bath (t = 125°C). The peak at 320 mu in the ultraviolet spectrum of the product is used to monitor the reaction.* The reaction mixture is then cooled and diluted with ice and water (1000 ml). A precipitate is formed which is separated by suction filtration, thoroughly washed with water and dried on air. The yellowish precipitate is dissolved in 450 ml of chloroform. This solution is then dried over sodium sulfate and evaporated. The residue (5.5 grams) is dissolved in 75 ml of dry chloroform and 85 grams of Merck acid-washed alumina added. Then this mixture was evaporated to dryness. The resulting mixture of compound and alumina is placed on the top of a column made up of 400 grams of alumina in petroleum ether. The product is then eluted with a mixture of ether and petroleum ether (1:1). A typical chromatography is summarized in the following Table 1.

*0.1 ml of the reaction mixture is diluted to 10 ml with absolute methanol and of this solution 0.2 ml are made up to 2.5 ml with the same solvent. D_{320} after 2.5 hours of heating reaches 0.4-0.45. Further heating of the reaction mixture does not increase this value.

Table 1

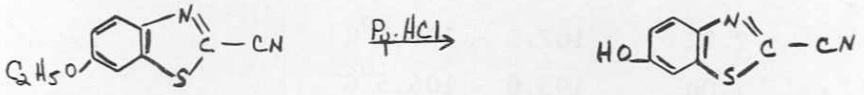
Fraction	Ml	g	M.P.	Remarks
1	350	0.23		Needs further purification
2	" "			
3	" "	2.22	107.5 - 108.5°C	
4	" "	1.00	105.0 - 106.5°C	
5	" "	0.41	104.0 - 105.0°C	
6	" "	0.09		Needs further purification
7	" "	0.01		Discarded
8	" "			
9	" "			

The products of fraction 3 and 4 are usually pure enough to be used for preparation of 2-cyano-6-hydroxybenzothiazole without further purification. The rest of the material should be combined with corresponding fractions from other experiments and rechromatographed over Merck acid-washed alumina. 2-cyano-6-ethoxybenzothiazole can be further purified by crystallization from isooctane (one gram from approximately 25 ml) - m.p. 197.5 - 18.5°C (Lit. 108-108.2°C).

4. Yield

3.22 grams (fraction 3 and 4) (40.76%) + 0.73 grams (fraction 1, 2, 5, and 6) (9.24%)

B. Conversion of 2-cyano-6-ethoxybenzothiazole into 2-cyano-hydroxybenzothiazole



$C_{10}H_8ON_2S$
204.246

$C_8H_4ON_2S$
176.194

1. Material

a. 4 grams of 2-cyano-6-ethoxybenzothiazole (0.0196 mole).

b. Pyridine hydrochloride prepared from 42.8 grams (0.54 mole) of dry hydrogen chloride.

c. 32 grams celite

d. 4 grams activated charcoal

e. 700 ml absolute ethanol

f. 100 ml sulfuric acid

2. Equipment

a. 250 ml three-necked round bottomed flask with standard joints.

b. Drying tube

c. Tube for introducing hydrogen chloride

d. Three gas washing bottles

e. T piece

f. Oil bath capable of being heated to 200°C

g. Thermometer

h. 100-200 ml beaker

i. 125 ml suction filtration flask

j. Buchner funnel - about 2 cm

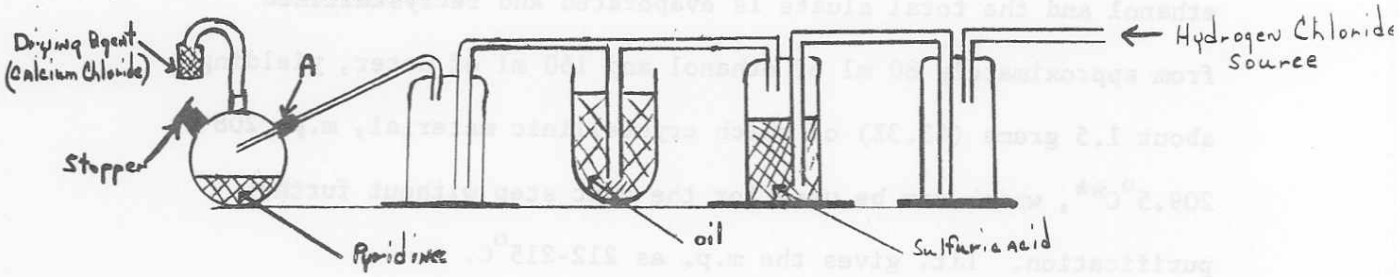
k. Chromatographic tube

l. Flasks for collecting fractions

3. Procedure

Pyridine hydrochloride is prepared by introducing dry hydrogen chloride into dry pyridine until the weight of the product reaches or slightly exceeds the theoretical amount. The arrangement used for this procedure is depicted in the following scheme.

Scheme 1 -



After the entire amount of pyridine has been converted into the hydrochloride, the hydrogen chloride introduction is interrupted (at Point A, see the Scheme 1), and the gas introducing tube is replaced by a stopper. The reaction flask is then heated on an oil bath (F = 190°), and 2-cyano-6-ethoxybenzothiazole is added. Heating is continued under occasional shaking for about 100'. The progress of the reaction is followed by taking samples periodically

and using the shift in the ultraviolet spectrum in alkali to determine the extent of dealkalization.*

After approximately 100 minutes, the reaction mixture is cooled to room temperature and diluted with 200 ml of water. The precipitate which is formed is filtered, washed with about 40 ml of cold water and dried on the air. Yield: 2.5874 grams. This material is dissolved in about 100 ml of absolute ethanol and run through a column containing 4 grams of activated charcoal and 32 grams of celite. The column is washed with 600 ml of absolute ethanol and the total eluate is evaporated and recrystallized from approximately 80 ml of ethanol and 160 ml of water, yielding about 1.5 grams (43.3%) of which crystallinic material, m.p. 208 F, 209.5°C**, which can be used for the next step without further purification. Lit. gives the m.p. as 212-215°C.

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*2-cyano-6-ethoxybenzothiazole has a maximum at 320 mu and 2-cyano-6-hydroxybenzothiazole at 322 mu. Whereas the maximum of the ethoxy- compound does of course not change in alkaline medium, the peak of the OH group in 2-cyano-6-hydroxybenzothiazole shifts under the same conditions (in 0.025 N ethanolic NaOH) from 320 to 390. Typical values are listed in Table 2 on the next page.

**m.p. is measured in a capillary and the capillary is introduced into the apparatus when temperature reaches 185°C

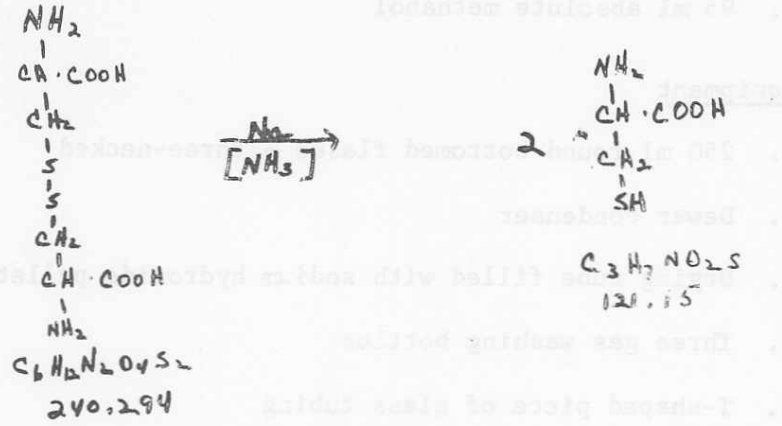
Table 2

Time	D_{320}	D_{390}	$\frac{D_{320}}{D_{390}}$
15'	0.585	0.126	4.7
30'	1.45	0.98	1.47
70'	0.078	0.368	0.21
90'	0.05	0.345	0.144

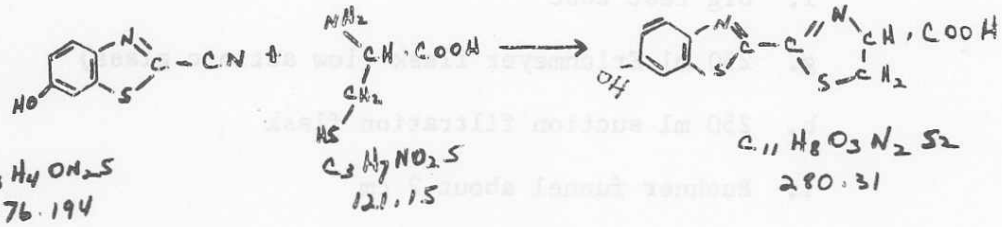
The decrease in the ratio $\frac{D_{320}}{D_{390}}$ indicates the progress of the reaction. This value reaches approximately 0.2 eventually.

C. 2-(6-hydroxy-2-benzothiazolyl)-Δ^2-~~tenazoline~~-4-carboxylic acid (D-luciferin)

(I)



(II)



1. Material

   a. 364 mg (1.51 mmoles) D-cystine

   b. 503 mg (2.88 mmoles) 2-cyano-6-hydroxybenzothiazole

   c. Liquid ammonia (about 150 ml)

   d. Sodium metal

   e. Ammonium chloride

   f. Nitrogen

   g. N sodium hydroxide solution

   h. N hydrochloric acid

   i. 95 ml absolute methanol

2. Equipment

   a. 250 ml round bottomed flasks - three-necked

   b. Dewar condenser

   c. Drying tube filled with sodium hydroxide pellets

   d. Three gas washing bottles

   e. T-shaped piece of glass tubing

   f. Big test tube

   g. 250 ml Erlenmeyer flask (low actinic glass)

   h. 250 ml suction filtration flask

   i. Buchner funnel about 2 cm

3. Procedure (Consists of three parts)

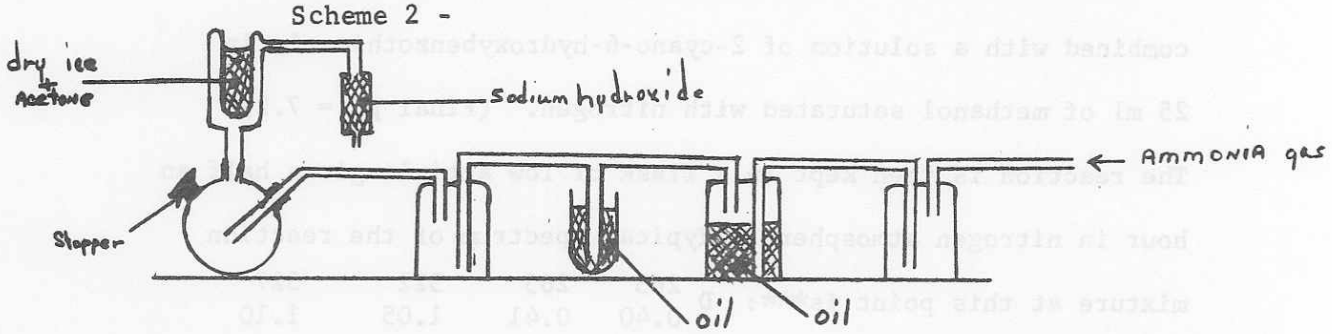
   a. Preparation of liquid ammonia

b. Reduction of D-cystine to D-cysteine

c. The actual condensation of D-cysteine with 2-cyano-6-hydroxybenzothiazole.

a. Dry liquid ammonia is prepared by introducing ammonium gas into a reaction flask fitted with a Dewar condenser in a set-up indicated in the following Scheme 2.

Scheme 2 -



b. After enough ammonia has been condensed, the tube introducing it into the reaction flask is replaced by a stopper. D-cystine is dissolved in the ammonia, and small pieces of sodium metal are added until the blue color of the reaction mixture persists for ten minutes.* The excess is then destroyed with the necessary amount of ammonium chloride and ammonia is then allowed to evaporate in a stream of nitrogen.

*During this entire procedure, it is <u>essential</u> to exclude moisture completely.

c. The white crystalline residue is dissolved in 70 ml of water saturated with nitrogen and pH of the solution is adjusted to 8.0 using 1N solution of hydrochloric acid (8.7 ml)** and N sodium hydroxide (0.5 ml)** respectively. Then 70 ml of methanol saturated with nitrogen are added and this mixture is combined with a solution of 2-cyano-6-hydroxybenzothiazole in 25 ml of methanol saturated with nitrogen. (Final pH = 7.2). The reaction is then kept in a flask of low actinic glass half an hour in nitrogen atmosphere. Typical spectrum of the reaction mixture at this point is***:

D	263	265	322	327
	0.40	0.41	1.05	1.10

The solution is then acidified with 7 ml of N hydrochloric acid (pH = 1.5) and kept refrigerated overnight. Crystals separated by suction filtration under nitrogen, washed with about 20 ml of water-methanol mixture (1:1) and dried in nitrogen atmosphere. Yield: 0.6176 grams (78%).

** These volumes vary and depend on the excess of sodium used in the reduction of cystine.

*** 0.01 ml of the reaction mixture in 25 ml of absolute methanol

Appendix II

INSTRUCTION MANUAL FOR ATP DETECTION INSTRUMENT

TABLE OF CONTENTS

TABLE OF CONTENTS (Continued)

FIGURES

FORWARD

It is the purpose of this Manual to present the theory of operation, and the design details of the ATP Detection Instrument. In addition, this Manual provides the information required to place the Instrument into operation, check its proper operation, calibrate it, and interpret the data derived from the experiment.

INSTRUCTION MANUAL

ATP DETECTION INSTRUMENT

I. Purpose of the ATP Detection Instrument

This Instrument has been designed and constructed as a laboratory tool which is to be utilized in the study of the parameters which influence the detection of sub-gamma amounts of ATP (adenosine-triphosphate) through the use of the ATP-luciferin-luciferase bio-chemical reaction.

The substance ATP is a biochemical constituent of all known living cells. The detection of this substance is evidence of the presence of cellular products, hence life processes as far as they are known. The ATP Detection Instrument has been designed to achieve a high detection sensitivity in order to reveal extremely small amounts of ATP, such as may be present in a single microorganism.

The development of this Instrument was initiated as a part of a NASA program to improve the sensitivity and methodology of the firefly-lantern enzyme biochemical system as a detector of ATP.

II. Theory of Operation

A. Biochemical System

1. A number of biochemical reactions which result in bioluminescence are known to exist. One of the best known and studied is the biochemical reaction used by the firefly.

2. The basic firefly lantern-enzyme reaction takes place in the following steps:

$$\text{(a)}\quad LH_2 + (ATP) + E \underset{}{\overset{Mg^{++}}{\rightleftharpoons}} E \cdot LH_2 - AMP + PP$$

$$\text{(b)}\quad E \cdot LH_2 - AMP + O_2 \longrightarrow E \cdot L - AMP + h\nu \text{ (light photon)}$$

where: LH_2 = luciferin
E = enzyme (luciferase)
PP = pyrophosphate
L = dehydroluciferin
AMP = adenylic acid

3. If all of the components of this reaction, with the exception of ATP, are present in excess, the emission of light is quantitatively specific for the amount of ATP introduced.

B. Instrument

1. The ATP Detection Instrument is designed to be used as a flexible laboratory tool in the study of the parameters which influence the progress of the firefly-lantern enzyme reaction upon the introduction of ATP and the subsequent emission of light. The Instrument provides for the introduction of a quantity of biochemical components, known as the enzyme mixture, which contains the components of equation A 2(a) with the exception of ATP. The enzyme-mixture is

contained in a cuvette which is placed in a temperature controlled bath and positioned in front of a light collecting optical system. The cuvette assembly is contained in a light-tight reaction chamber which also includes a precision syringe that is filled with a sample of ATP dissolved in water. The syringe is positioned directly over the enzyme mixture cuvette and is displaced by a hydraulic drive to discharge a controlled quantity of ATP sample into the enzyme-mixture.

2. The injection of a quantity of ATP into the enzyme-mixture results in the emission of light quanta which are collected by the optical system and directed onto the photocathode of a photomultiplier vacuum tube. The photomultiplier tube is operated to obtain a high signal-to-noise ratio at low light levels. The photocathode is cooled to reduce the thermal noise and pulse amplitude discrimination is employed to reduce the effects of photomultiplier noise.

3. The emission of light resulting from the injection of ATP into the enzyme-mixture is not instantaneous. The light output vs. time history of a reaction shows that, in general, there is an initial rapid rise in the emission of light to a peak value, followed by a decay in the rate at which light is emitted with the passage of time. The ATP Detection Instrument is designed to be a flexible tool for the study of the biochemical reaction. The Instrument allows exp'oration of the variables of injection time and volume, temperature, sample sizes and preparation procedures, monitoring schedules and data collection.

4. Figure 1 is a block diagram of the ATP Detection Instrument which shows all of the individual operational segments and the signal and control flow lines.

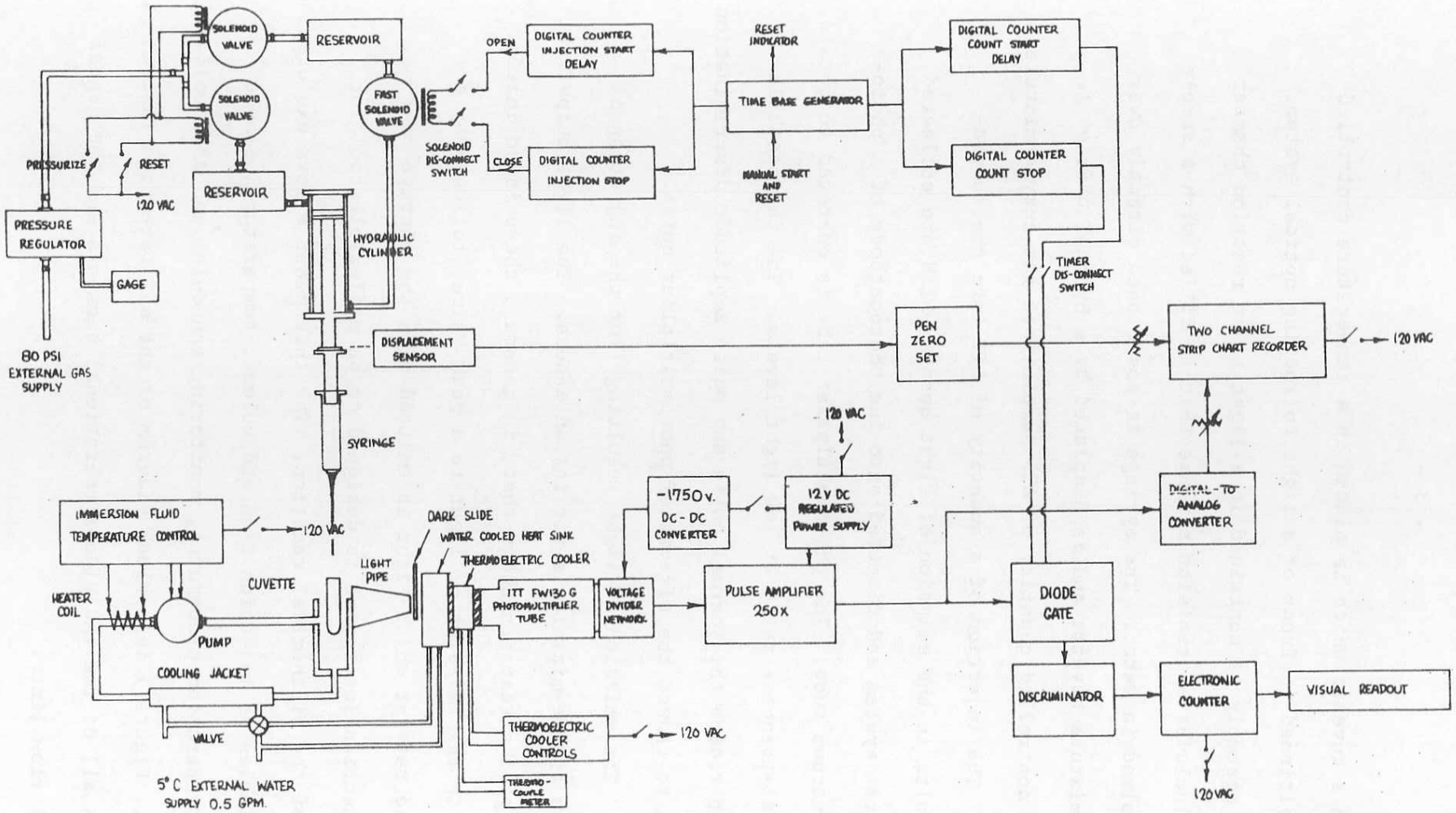


FIG. 1 BLOCK DIAGRAM ATP DETECTION INSTRUMENT

HAZLETON LABORATORIES, INC.

III. Design of the ATP Detection Instrument

A. Console

1. The Instrument is designed to be housed in a console which contains all of the required components. The console does require external electric power (120 v, 4.5 A), a source of compressed air (80 psi) and an external water supply (0.5 gal/min @ 5°C temperature).

The controls are grouped according to function on the panels of the console and all required adjustments in controls can be made by an operator seated in front of the console. The major components of the Instrument are installed as subassemblies and are interconnected with electrical cables or fluid couplings.

2. Figure 2 is a block diagram of the subassemblies contained in the Instrument and their interconnections.

3. Figure 3 is a schematic diagram of the console identifying the front panel controls and the location of the major subassemblies contained in the Instrument.

B. Reaction Chamber (see Figure 4 for schematic diagram keyed to text)

1. Physical Description

The reaction chamber is an inverted T-shaped stainless steel housing mounted on the right side of the Instrument cabinet. An access door which is hinged at the bottom and lowers into the open position makes a light tight closure when secured shut with the four thumb latches. The interior of the chamber is painted dead black to eliminate internal light reflections.

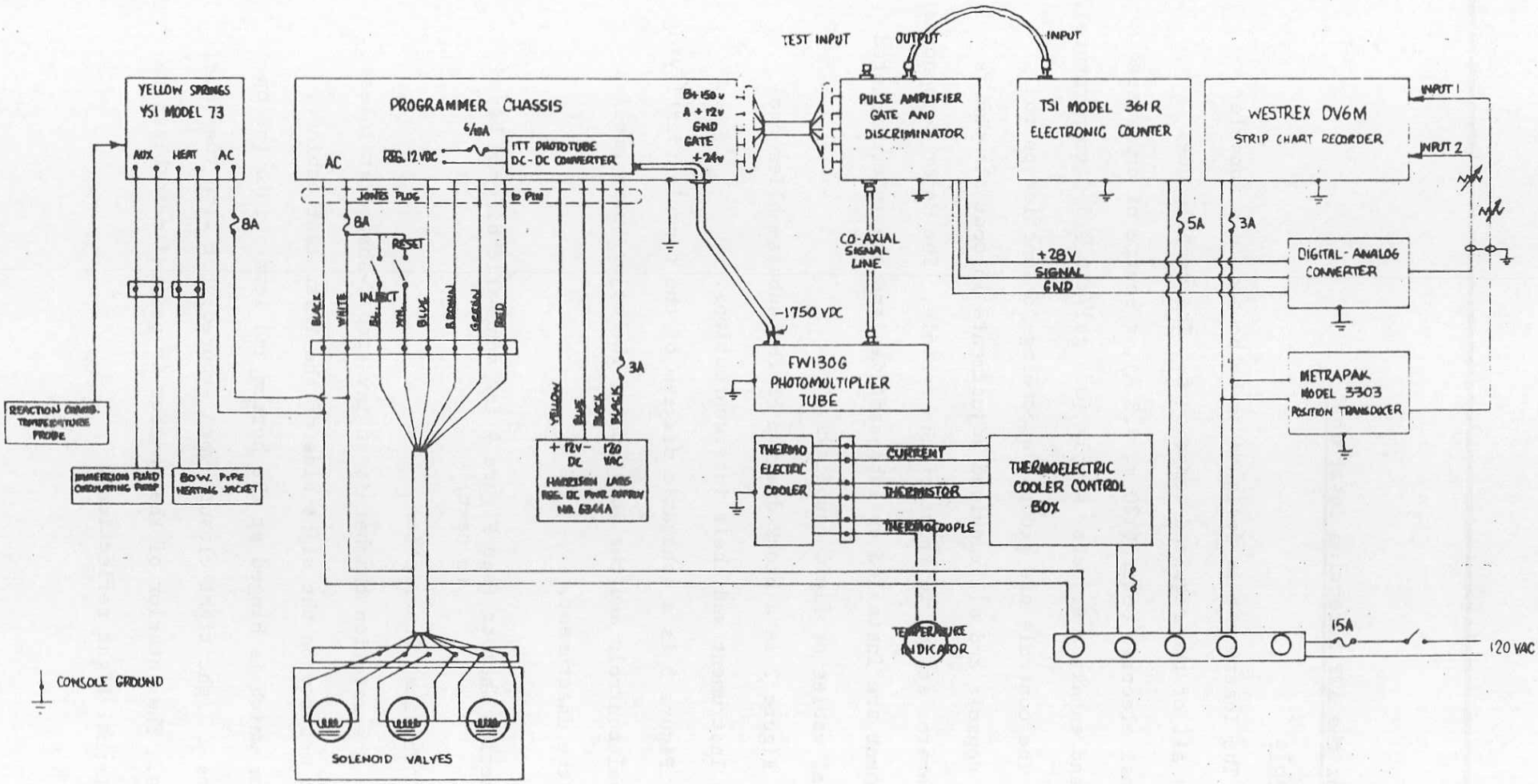
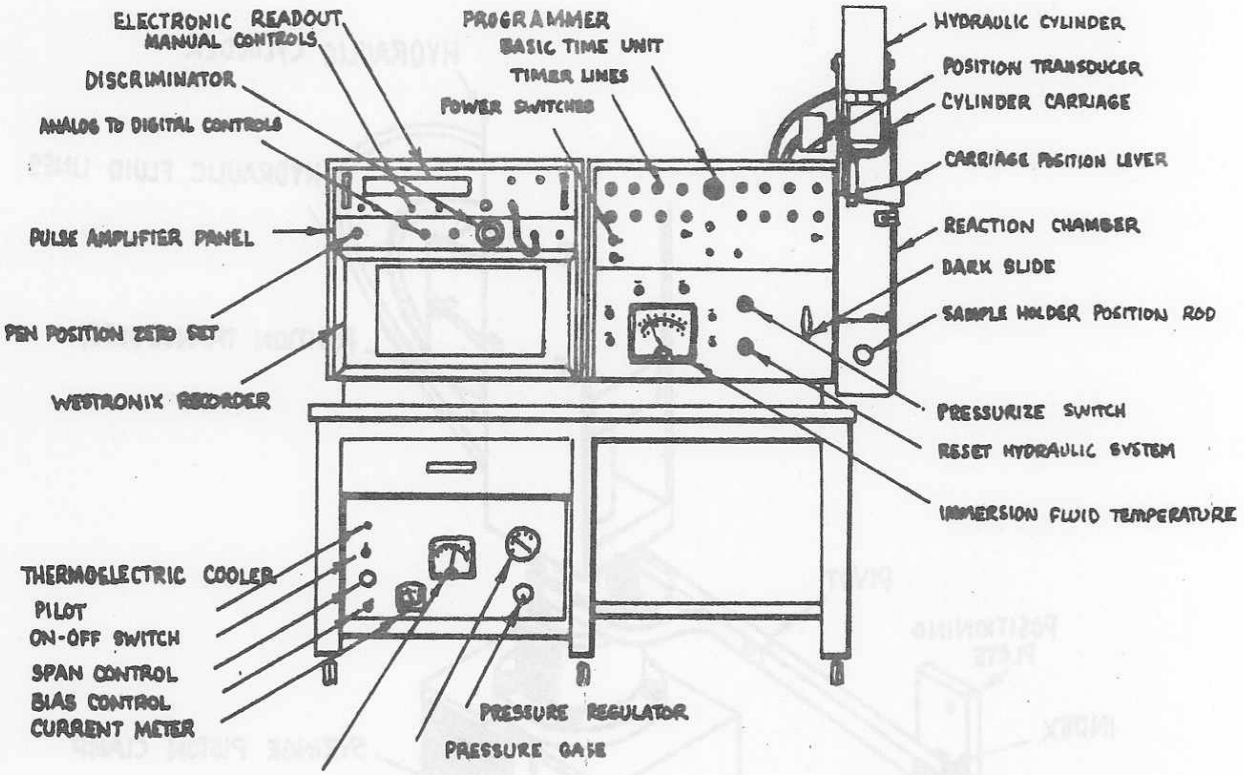


FIG. 2 SCHEMATIC BLOCK DIAGRAM
ATP DETECTION INSTRUMENT
HAZLETON LABORATORIES, INC.



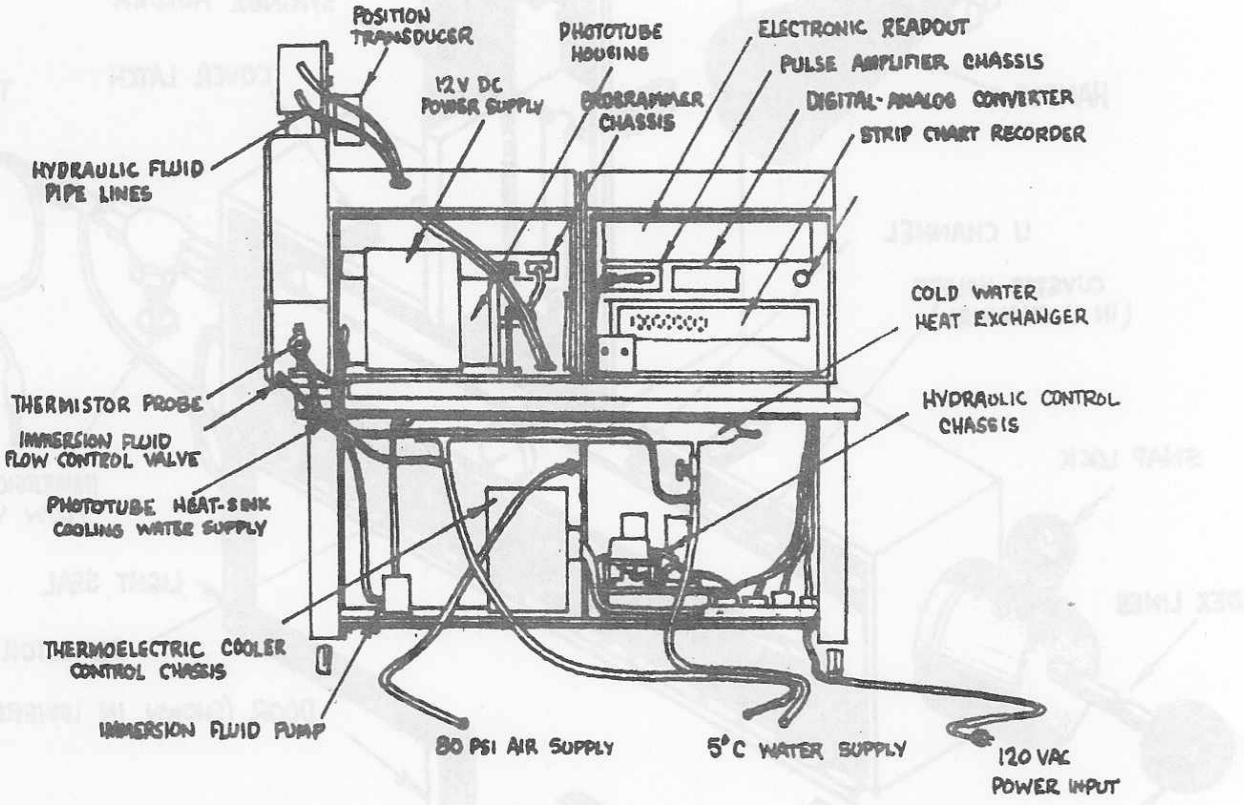


FIG. 3 CONSOLE LAYOUT FRONT & REAR VIEWS

HAZLETON LABORATORIES, INC.

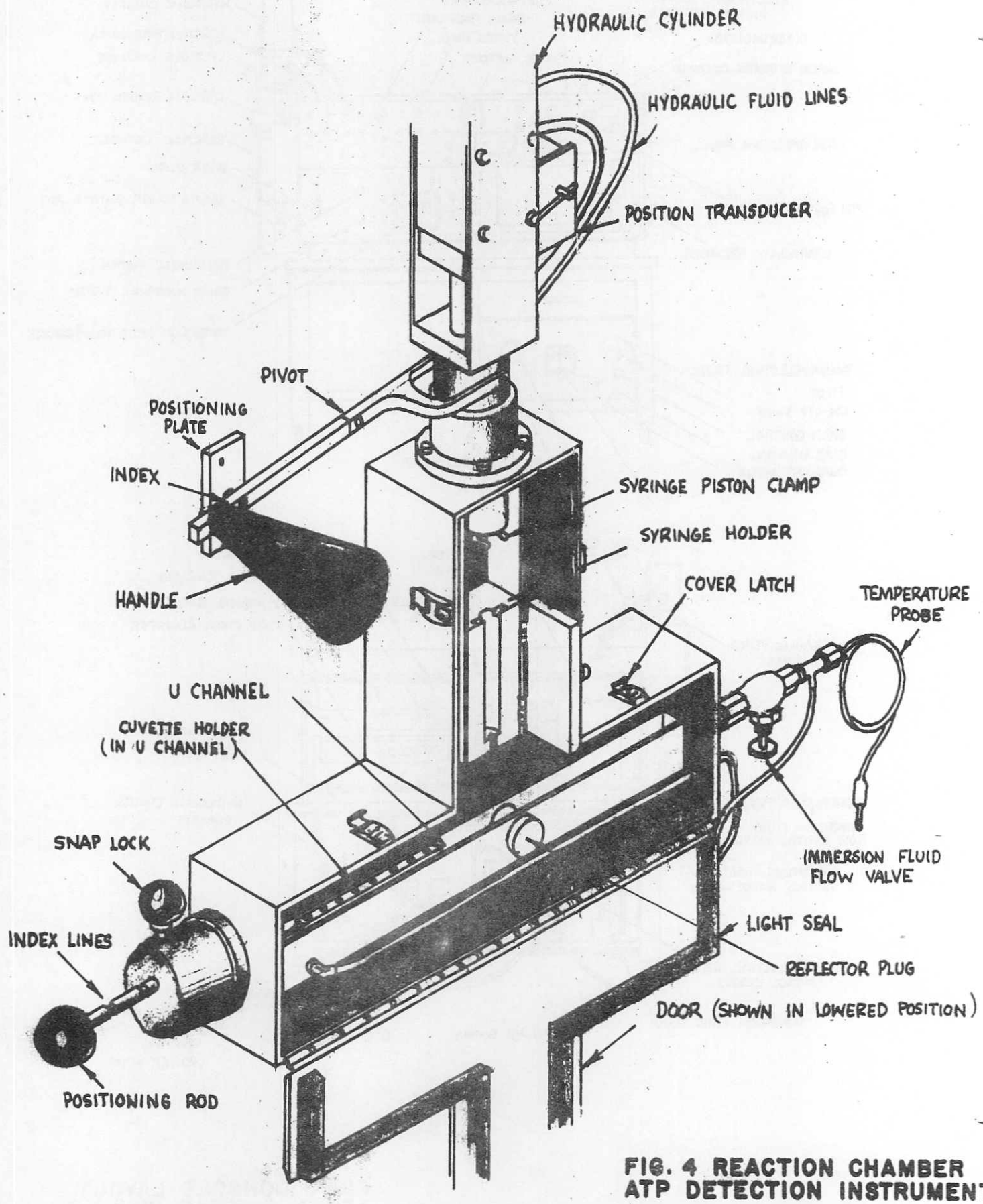


FIG. 4 REACTION CHAMBER ATP DETECTION INSTRUMENT

HAZLETON LABORATORIES, INC.

2. Environmental Control of the Enzyme Mixture

Within the reaction chamber is a U-channel, running the full width of the T, which is filled with the immersion fluid that is used to control the temperature of the enzyme mixture cuvette. This fluid is pumped through the channel from the back of the Instrument to the front. The temperature is sensed by a thermocouple immersion thermometer inserted into the fluid inlet piping fixture at the rear of the chamber. The fluid in the immersion bath is in a closed system and is circulated by a small vane pump located on the bottom shelf board in the rear of the console. The fluid is pumped through copper tubing and there are two heat exchangers attached to sections of this tubing. One consists of an electrical resistance element which is used to introduce heat to the fluid; the other is a cold water jacket which removes heat from the immersion fluid when external cooler water is circulated through the jacket. A flow of one-half gallon per minute of $5^{\circ}C$ temperature water is adequate for proper operation of the cooling jacket. The temperature of the immersion fluid is controlled by a Yellow Springs Instrument Co. YSI Thermistemp Model 73 indicating controller. This controller is used to control the immersion fluid temperature with an accuracy of $\pm 1^{\circ}$ within the range of $5^{\circ}C$ to $50^{\circ}C$. The temperature of the fluid is set and read on the lower right panel of the console and indicator lights are provided to indicate whether a heating or cooling cycle is in progress. Turning the temperature controller on automatically starts the circulating pump.

The temperature controller is turned on and the thermometer calibrated by pressing the "calibrate" button. The temperature indicator needle should move to the red line on the left side of the meter scale. If it does not come to rest on the red line, a screwdriver adjustment of the screw head located above the calibrate button will bring the needle to the proper position. With the thermometer calibrated the temperature indicated on the thermometer is the reaction chamber temperature. To set the controller to a desired operating point, push the "temperature set" button and adjust the screw head above it until the thermometer reads the desired temperature. The difference between the "set" temperature and the desired temperature determines whether heating or cooling is required and the "heat" and "cool" pilot lights above the thermometer indicate which cycle is in progress.

3. Cuvette Holder

The U-channel in the reaction chamber is made to accept the cuvette holder. There are two cuvette holder designs, one, a holder for glass, test tube-like cuvettes, the other a metal frame with cuvettes drilled into the body of the frame. Both holders are of the same size and provide eight cuvettes in each holder. The holder fits inside of the U-channel and rides on a ridge at the bottom of the U. One end of the holder has a hook mechanism attached that is designed to fit over the end of a rod which enters the front of the reaction chamber and is used to displace the cuvette holder horizontally within the U-channel when the reaction chamber is closed. The positioning rod is indexed so that it can be snapped into position in any one of eight

positions. Each index line places one cuvette into position in front of a light collecting system within the chamber. The positioning rod can be rotated about its axis for minor changes in positioning. When one cuvette is properly centered, all cuvettes will be properly centered by the index positions on the positioning rod.

4. Cuvette Design

A cuvette is the volume in which the biochemical reaction takes place. Glass cuvettes will hold 0.3 ml enzyme mixture, while the metal cuvette will hold 0.15 ml. For routine work, disposable glass cuvettes are preferred because they are easily sterilized and prepared. The glass cuvette holder has spaces for eight small-bore glass test tubes. When glass test tubes are used, a reflector is placed behind the test tube to direct the light produced by the reaction into the optical system. This reflector is in the form of a plug inserted into a hole bored into the center of the U-channel. This plug must be in place when an immersion fluid is used, but is removed when it is desired to calibrate the Instrument with a standard light source. (The immersion fluid must be drained from the channel before removing the plug.) The metal cuvettes are nickel plated and act as their own reflectors. The metal cuvette is sealed on the front by a microscope slide cover-glass that is replaced each time the cuvette is prepared for a reaction experiment.

5. Syringe and Syringe Mounting

The injected component of the reaction is placed in a syringe which is positioned in a holder directly over the center of the light collecting system and the center of the cuvette positioned

for injection within the reaction chamber. Two syringe holders have been provided, one for use with a disposable 1 cc capacity syringe (Yale, sterile, 20G 1 1/2 and a 22G, 1 1/2 sterile disposable needle) and the other for a Hamilton microliter 701-N precision syringe. The disposable syringe is used for routine experiments since it does not require cleaning and sterilization before using. The syringe body is supported in the holder and the extended piston is engaged by a spring clamp at the top of the reaction chamber. The spring clamp is attached to the piston of a hydraulic cylinder which extends into the interior of the reaction chamber. The cylinder itself is external to the reaction chamber and is mounted on a moveable platform. The hydraulic fluid is piped to the cylinder through flexible plastic tubing. Movement of the hydraulic cylinder piston causes the syringe plunger to be pushed into the syringe barrel. By controlling the hydraulic cylinder displacement, the volume of sample injected into the enzyme mixture cuvette can be metered.

6. Syringe Position Indicator

The displacement of the hydraulic cylinder piston is measured by a position transducer, a Metrapak Model 3303 (Brush Instruments, Inc.). This sensor produces an electrical output which is a function of the angular position of the shaft of the sensor which is mechanically coupled to the hydraulic cylinder piston with a bar link. The output of the position sensor is connected to the left-hand channel of the strip chart recorder so that syringe plunger travel can be recorded.

7. Hydraulic Drive System

The hydraulic fluid is pressurized by an external gas

supply of 80 psi. This pressure is regulated by a flow valve located on the lower left hand drawer panel of the console. Immediately above the flow valve is a pressure gage which indicates pressure on the hydraulic fluid. The external gas is connected to the hydraulic system through a fixture located in the center of the lower rear compartment. The application of pressure to the hydraulic fluid is controlled by fast acting electrical solenoid valves. The valves, pressure regulator, exhaust and fluid reservoirs are mounted on a sub-chassis plate in the compartment behind the lower left hand drawer panel. The solenoid valves are operated by 120 v A.C. Two control the direction of fluid flow and are operated by momentary contact push button switches, one, "pressurize", and the other, "reset" which are located beside the immersion bath temperature control on the lower right hand panel on top of the console. When the piston of the hydraulic cylinder is extended (syringe plunger depressed) the cylinder may be returned to the starting position by momentarily pushing the "reset" button. This retracts the piston back into the cylinder. To allow the piston to push the syringe, the pressure on the fluid must be reversed, and this is accomplished by momentarily pushing the "pressurize" button. The pressurizing action causes an ejection of a small quantity of air from the exhaust which will produce a sound similar to uncapping a bottle of soda. The hydraulic system must be pressurized after being reset before the control solenoid will function.

The displacement of the hydraulic cylinder is controlled by electrically opening a valve to start the movement, and closing it

to stop the flow. The open and close signals are generated by an electronic timer. This timer is adjustable so that the length of time the pressure is applied to the cylinder is under the control of the operator. The time and the pressure variables combine to establish a range of available injection cycle times and volumes. The electronic timer is described more fully under the section of this Manual dealing with pulse counting (Section C6).

8. Injection System Positioning

The entire hydraulic cylinder-syringe combination is moveable in the vertical direction. The movement is controlled by a lever fitted with a handle which is within reach of the operator. This assembly is made moveable because the syringe must be immersed in the enzyme mixture when an injection takes place, but must be lifted clear of the cuvette when the cuvette holder is moved to place a different cuvette in position for a reaction. Failure to raise the syringe assembly before moving the cuvette holder will result in a bent syringe needle which will not accurately enter subsequent cuvettes placed in position for a reaction. In the lowered position, the tip of the syringe needle is positioned at the center of the light collection system and on the center line of the cuvette. In the raised position a ball lock in the handle base snaps into a depression in the face plate under the lever on the console side. Tension of the ball spring is regulated by a set screw at the base of the handle.

9. Light Collection System

The light generated as a result of the injection of a

sample into an enzyme-mixture is radiated from the cuvette. A plexiglass light pipe, which is a reflectorized conically-shaped piece is mounted in the back wall of the reaction chamber at a level even with the cuvette in position under the syringe. The large end of the light collector is visible when the reflector plug is removed from its normal position in the side of the U-channel in the reaction chamber. The light collector extends through the end panel of the console in order to conduct the light into the phototube chamber. The diameter of the light pipe at its smaller end in the phototube chamber is 0.1 inch.

10. Dark Slide

A sliding wedge of blackened brass sheet is positioned at the small end of the light pipe so that it can be interposed between the end of the light pipe and the phototube. This element is called a dark slide and is used to shield the phototube from room light when the reaction chamber door is opened. Exposure of the phototube to high light levels desensitizes the phototube and raises the noise level for several hours. The position of the dark slide is controlled from the lower right hand panel on the top of the console by rotating a control knob. This knob may be turned 90° in either direction from the "open" position to close the dark slide. The rotation is translated by a mechanical linkage into a vertical motion which raises or lowers the slide in front of the phototube. The dark slide is intended to be kept closed except when actually making a reaction reading.

C. Light Intensity Monitoring

1. Photomultiplier Tube

The light emitted by the ATP-enzyme mixture is collected by the light collection system and is directed through the dark slide assembly to the photocathode of the photomultiplier tube. The tube employed is an International Telephone and Telegraph Co. (ITT) model FW 130G. This tube is a sixteen dynode photomultiplier tube which has an S11 phosphor photocathode which is 0.1 inch in diameter. (The base of the light pipe is also 0.1 inch in diameter.)

The potential supplied to the cathode of the photomultiplier is -1750 volts developed by an ITT solid state DC to DC converter mounted under the electronic timer chassis. The dynode potential divider is constructed according to ITT recommendation and consists of 1% tolerance deposited film resistors.

The phototube is mounted in a magnetic shield inserted into a phenolic tube inside of a six-inch diameter aluminum tube attached to the reaction chamber end of the console behind the right hand console panels. The dark slide control on the lower right hand console panel rotates a shaft which enters the phototube housing and drives the dark slide mechanism through a cam arrangement.

The phototube chamber can be removed by disengaging the dark slide control and removing the compression thumb-screw. The phototube proper is housed inside the phenolic tube which is attached to the cooling assembly.

2. Phototube Cooler

In order to reduce the thermal emission of electrons from the photocathode, the temperature of this cathode surface is reduced. This cooling is accomplished by an International Energy Conversion thermoelectric (solid-state) cooler. This assembly consists of a series of thermoelectric junction elements mounted on one side to a heat-sink plate, and on the other to a copper ring which is fashioned to fit loosely around the photocathode end of the phototube. The inside of this ring is coated with a silicone heat-sink compound (General Electric, Insulgrease G-641) to obtain a good thermal connection between the phototube and the inside surface of the ring.

The phototube cooling controls are located on the left side of the lower left hand drawer panel of the console. These controls consist of an on-off switch, pilot light, temperature set knob (blue), bias current control, cooler current meter and a thermometer. To adjust the cooler, rotate the blue knob and the bias control completely clockwise, and turn the switch "on". When the cooler is turned "on" the pilot lights, a bias current of 7.5 amperes flows and the temperature will begin to decrease within 30 seconds. When the temperature reaches the desired level, the blue knob is rotated clockwise until the current meter reading drops to zero. This sets the temperature control point and the cooler will automatically regulate at this temperature. It is recommended by the manufacturer that when the temperature controller is cycling, the bias control be advanced to supply a bias current (read on the current meter) approximately equal to the current required to

maintain the set temperature. Since the cooler is intended to operate at the lowest temperature obtainable (at least -20°C) this bias control can be rotated fully clockwise and left in this position. With the design heat-sink conditions temperatures of -35°C to -40°C are obtained.

The heat removed from the phototube assembly is conducted away by a circulating water bath flowing through a channel in the end plate of the phototube chamber. Water is supplied to this heat sink from the same source of water used in the reaction chamber environment control (see 3 (b) 2). Copper tubing is used to pipe the water to the heat sink. The thermoelectric cooler will cool the phototube to at least 40° below the heat sink temperature.

The phototube requires approximately one hour to achieve operating temperature. The thermometer indicates the temperature determined by a thermocouple mounted in the ring around the phototube; there is a lag in the cooling of the photocathode itself due to the resistance of the thermal path in the mounting.

The electronic control for the thermoelectric cooler is boxed in a subassembly placed on the lower shelf in the back of the Instrument. Electrical cables connect this unit to the cooler and the front panel controls.

3. Pulse Amplification

Each time a light quantum interacts with the photocathode of the photomultiplier tube, electrons are emitted from the photocathode material. The number of electrons emitted is a function of the energy

given up by the interacting quantum and the efficiency of the photoelectric process. In the spectral range over which the particular photocathode material is sensitive, there are from one to six electrons emitted per quantum absorbed.

The electrons are guided through an electron-optical system within the tube and accelerated by a potential difference of several hundred volts. After acceleration, the electrons impact on a collector electrode coated with a material that produces additional electrons through the process of secondary emission. These new electrons are directed and accelerated toward a second secondary emitter electrode and similarly for fourteen more stages. This multiplication process produces a charge of electricity which is collected by an anode electrode within the tube. If this anode is connected through a resistance to the cathode, the charge flows in the form of an electric current and a potential drop appears across the resistance.

Since the emission of electrons from the photocathode is the result of the interaction of a single quantum, a finite electric charge is delivered to the anode and a "pulse" of voltage appears across the anode-cathode resistance for each photon absorbed. The duration of the pulse is on the order of 10^{-7} seconds so that, at least for a small number of interacting quanta, there is a one-to-one correspondence between interacting quanta and voltage pulses appearing across the anode resistor.

The magnitude of the voltage of a pulse is a function of the number of electrons ejected by the interacting quantum and the

multiplication obtained through secondary emission. For the phototube employed in the ATP Detection Instrument, pulses due to electrons emitted by the photocathode, have a voltage amplitude of 0.5 to 6 millivolts. This amplitude is directly dependent on the phototube supply voltage since this potential controls the secondary emission constant for the multiplier stages of the phototube. The phototube power supply is regulated to maintain the potential constant to 0.1%. Deviations greater than this produce significant differences in the amplitude of the voltage pulses appearing at the anode.

The pulse amplitudes at the phototube are not adequate to drive a counter, so they are amplified in a three stage vacuum-tube amplifier which has a cathode follower output stage. The gain of this amplifier is 250 at a frequency of 100 kc, and the bandwidth of the amplifier is such that the gain at 1 mc is 60. This bandwidth permits the amplification of the leading edge of the pulses which are used to actuate an electronic counter.

4. Pulse Amplitude Discrimination

The phototube pulse output amplitude spectrum contains a large number of discreet pulse amplitudes. Pulses originate at electrodes other than the photocathode, and secondary emission coefficients of the many multiplier stages are not a constant. However, pulses originating at the photocathode are the largest in amplitude (since they have received the greatest amplification in the phototube) and are the only pulses of interest in the Instrument.

In order to discriminate against low voltage pulses, an amplitude control is inserted in the output circuit of the pulse amplifier. The electronic counter (see Section 5) requires a threshold voltage to register a count, and the amplitude control regulates the number of pulses from the pulse amplifier which will trip the counter mechanism. It is a ten-turn potentiometer which is controlled from the front panel of the console.

The pulse amplifier is located on the left top of the console, just above the dual channel recorder, on a narrow panel. There are input and output coaxial connectors which allow a quick check of the amplifier characteristics of accessability to the phototube output and amplifier output from the front panel. The discriminator control is a ten-turn dial with zero reading being zero volts, the complete discrimination level. The discriminator characteristic is linear with dial readings of 0 to 1000. The power required by the pulse amplifier is supplied from a power supply located in the timer chassis and connected by means of a multiconductor cable. The output is normally taken from the front panel, and supplied to the input connector of the electronic counter.

5. Electronic Counter

Pulses appearing at the output of the amplifier-discriminator which exceed 18 millivolts peak are accepted by the electronic counter and registered on the numeric readout contained in the counter assembly. The electronic counter is a Transistor Specialties model 361R, and is a complete subassembly installed in the upper left hand panel position on the top of the console. The counter may be operated

as an event counter using its own internal timer or as an event counter which registers for externally determined time periods. This latter mode of operation is designed to be employed in the ATP Detection Instrument. To achieve this kind of operation, the counter is turned 'on', allowed to stabilize for fifteen minutes with the time interval selector in the Manual position. The reset and ready functions must be operated manually by pushing the reset button, followed by pushing the ready button. Both of these are momentary closure switches and do not require a long hold-down time.

The trigger level of the counter is adjustable by two controls on the counter panel. One of these is a decade range switch which should be in the X1 position (fully clockwise). The other control knob has been removed and replaced with a potentiometer lock-nut since this control adjusts the triggering sensitivity and polarity of the input signal.

The counter has a self-checking capability by switching the function switch to Period A, and the Interval switch to 10^{-3}. Under these conditions the counter will register 1000. Other settings of the Interval switch will result in other count totals (see the counter instruction manual).

The counter, as employed in the ATP Detection Instrument, is operated in the manual mode; that is, it will register the total of all the pulses appearing at the input that are of sufficient voltage level. The source of pulses is controlled by the Programmer (see Section 6) so that a connection between the pulse amplifier and the

counter is made only during a desired time interval. This coupling between the amplifier and the counter is made through a diode which is normally biased so that it is not conducting and hence an open circuit. When pulses are to be counted, the bias voltage is removed by the programmer so that the diode will conduct pulses from the amplifier to the counter. This diode gate circuit is built into the amplifier unit. Once the gate circuit is turned off, the total count remains on the readout until the manual "reset" button is pressed. After resetting the readout to zero, the counter must be set for another count cycle by pressing the "manual timing" button.

6. Programmer

The programmer in the ATP Detection Instrument is built on the chassis behind the upper right hand panel on the top of the console. It contains a time base generator and four sets of counting circuitry with switching and power supplies.

The time-base generator uses the 60 cycle line frequency to produce a unit time interval of 25, 50, or 100 milliseconds. The choice of this interval is made with the selector switch in the center of the panel. An indicator light (green) in the center of the panel lights when the counters are set to zero. To reset the counter, the "reset" button is depressed. This will cause the green light to come on and indicates the counters are set to zero. To start the timer, the start button is depressed. This connects the time-base generator to the counters.

There are four counters which are identical. Each one has a set of four dials, each numbered from 0 to 9. The dials are arranged in horizontal rows. The right hand knob is the units dial, and tens, hundreds, and thousands decades are located proceeding from right to left from the units dial. Setting these dials adjusts the counter to count from the start instant to the number of units indicated on the dials. To determine the time interval, the dial settings are multiplied by the basic time interval selected.

Timer number one, referred to as Timer line A, controls the initiation of the injection cycle. A setting on this timer is in effect a time delay between pushing the start button and the start of the injection cycle. When the timer has counted the set number of pulses indicated on the dials, it operates a switching circuit that operates a relay to open a hydraulic solenoid to start the injection cycle.

Timer number two, referred to as Timer line B, which is directly under Timer line A, controls a relay which closes the hydraulic solenoid to stop the injection cycle. The length of the injection cycle is the difference in time setting between Timer line B and Timer line A. To obtain the real time, the two sets of dial readings are subtracted; the result is multiplied by the basic time interval chosen with the center selector dial. The minimum injection time interval available is one unit of 25 milliseconds duration.

A timer override switch is located below the two rows of dials which make up Timer lines A and B. In the "on" position, the

timer will operate the solenoid valves in the hydraulic system. In the "off" position the solenoids do not operate. This switch is included so that the other pair of timers can be operated independently of the injection system.

Timer number three, referred to as Timer line C, controls the start of the pulse counting interval by removing the bias voltage from the gate circuit (see Section 5). The start of the counting period may preceed, coincide with, or follow the start of the injection cycle depending on the relation of the dial settings of line C to the dial settings on line A.

Timer number four, referred to as Timer line D, controls the end of the pulse counting interval. The count interval is the difference between the dial settings on lines D and C multiplied by the basic time interval.

A timer override switch is located below the two rows of dials which make up Timer lines C and D. In the 'on' position the timer controls the count interval. In the 'off' position the pulse amplifier is connected to the counter continuously.

The programmer has a power switch and a phototube power supply on-off switch. The pulse amplifier power supply is also located in the programmer chassis. A multi-conductor cable connects the amplifier to the power supply. A second cable is used to interconnect the programmer and the solenoid valves. These cables thread through holes in the console base to their respective terminations.

7. Recorder

A Westrex model DV-GM dual channel strip chart recorder is an integral part of the ATP Detection Instrument. When recordings are to be made, the recorder is operated by its own power switches to turn it on and to start the chart drive. There are two input circuits to the recorder:

(a) The left hand channel is connected to the syringe position transducer (see Section 3 (b) 6). Since this transducer has an arbitrary direct current output for the rest position (depending on the mounting of the transducer with respect to the connection to the piston on the hydraulic cylinder), a zero position control is provided to accurately set the pen indicator to zero. This control is located immediately above the left end of the recorder. The output voltage range of the transducer exceeds the span of the recorder so a voltage divider is used to adjust the magnitude of the transducer voltage to register full scale when the syringe piston is at its lowest level. This adjustment is a semi-fixed one and is made by a potentiometer mounted on a bracket behind the recorder. Unless the position transducer mounting or coupling linkage is changed this adjustment is not required to be altered after being set initially.

(b) The right hand channel is connected to a digital-to-analog converter circuit which is built into a chassis mounted on the pulse amplifier panel. Pulses are conducted to an integrator circuit which produces a direct current output that is linearly related to the number of pulses per second. By recording this signal, the pen

deflection is a measure of the pulse rate, and thus the light intensity. Since the actual photomultiplier pulses are of such short duration, a pulse regeneration circuit is included in the converter. Each short pulse from the amplifier triggers a one-shot multivibrator that generates a longer duration pulse. These longer pulses are integrated to produce a greater voltage level output than would be possible from integrating the original short pulses. A choice of regenerated pulse lengths is provided by a front panel selector switch above the recorder on the amplifier panel. For a small number of pulses, long time duration is desirable; for increased pulse rates the duration of the regenerated pulses must be reduced so that incoming pulses do not occur while the previous regenerated pulse is being formed. If this happens, the output will remain constant even though the number of input pulses increases. A potentiometer is located next to the pulse width selector for the purpose of setting the deflection amplitude to a convenient value. It is possible to calibrate the pen deflection for pulse rate by using a signal generator to provide known pulse rates connected to the pulse amplifier input connector. This is not normally required since the chart is used to record a qualitative measure of the light output vs. time of the reaction being monitored. The recorder input operates continuously and is not gated on or off by the count timer; thus a record of light output vs. time will be recorded. The pulse width and the signal amplitude are determined empirically for the light level being produced by the reactions being monitored.

IV. Operating Procedure

A. Instrument Set-Up

1. With the Instrument connected to a source of electrical power, cold water and gas pressure, the programmer, phototube power supply, electronic counter and thermoelectric cooler are turned on with their respective power switches. Pilot lights indicate the on condition of each of these units with the exception of the phototube power supply. A period of one hour should be allowed for complete cooling of the phototube from room temperature even though the thermometer indicates the desired temperature. The cooler may be left on indefinitely if desired so that the warm-up period can be reduced to fifteen minutes.

2. If sample environmental control is to be used, check the filling of the sample holder channel with distilled water to within one/quarter of an inch of the top without the sample holding rack in the channel. Turn on the temperature controller, calibrate the thermometer and set the desired temperature. If recordings are to be made, turn on the recorder.

3. After fifteen minutes of warm up: (a) press the "reset" button on the programmer to insure that the ready light (green) is on, (b) adjust the hydraulic pressure to 60 lbs. with the pressure regulator valve under the pressure gage, (c) press the hydraulic system "reset" button and wait until the hydraulic cyclinder retracts to its topmost position. After the cylinder is in position, press the "pressurize" button to charge the hydraulic system. This action is accompanied by an exhaust cycle and a slight downward motion of

the cylinder piston. During this setting, the timers must be reset properly or when the "pressurize" button is depressed the hydraulic cylinder will travel its full stroke downward. For this reason it is desirable to delay loading a syringe into the reaction chamber until the "pressurize" setting is made. The system cannot be pressurized if the injection control timers (timer lines A and B) are all set to zero. Use the units dial set to 1 on both lines or any other convenient settings.

B. Sample Preparation

1. Serilization

The purpose of sterilization and cleaning is to insure that no trace of microorganisms or other source of ATP is present in, or can inadvertently be admitted to the enzyme mixture or the sample to be analyzed for the presence of ATP. The enzyme mixture is contained in cuvettes supported in a holding rack and the sample to be analyzed is confined to a syringe. These must be sterile and chemically clean, not only of ATP but any other contaminants which might interfere with the desired biochemical reaction.

The cuvette designs are such that the metal type may be disassembled and the rack with the cover glasses removed, washed in 0.1 Normal HCl, rinsed in deionized, distilled water, and autoclaved at 121°C for 20 minutes. The alternate cuvette rack, designed for routine work, which uses disposable miniature test tubes need not be cleaned as long as the test tubes themselves are sterile and clean before the introduction of the enzyme mixture. The glass tube cuvettes

are cleaned in 0.1 Normal HCl by boiling for ten minutes. Following the boiling, the tubes must be rinsed thoroughly in sterile deionized water for ten cycles, protected from the entry of particulates and oven dried.

The disposable syringe is supplied in a sterile clean condition and need not be treated further provided care is exercised in not letting the tip touch anything and restricting its exposure to room air. The precision syringe is treated the same as the glass cuvettes; it is disassembled, boiled in 0.1 N HCl for ten minutes, rinsed and dried.

The reaction chamber should be dust free to prevent the entry or release of particulates into the cuvettes. It should be periodically wiped clean with a sterile soft cloth moistened with disinfectant. The reaction chamber should be kept closed except during loading and unloading.

Cuvettes should not be allowed to stand open exposed to room air without a cover. Aluminum foil or thin mylar film which is dust free can be placed over the cuvettes during storage, standby, or Instrument loading.

C. Enzyme Mixture and Sample Injection

There are a variety of preparation schedules for enzyme mixtures and injection samples. The quantity of enzyme placed in the glass cuvette is at least 0.15 ml and no more than 0.3 ml. The filling criteria are basically whether the needle of the injection syringe is below the liquid level in the cuvette when the syringe carriage is lowered into position for an injection (topmost position of the carriage control lever), and the volume of the injection to be made and its effect on dilution of the enzyme mixture.

The metal cuvettes will hold 0.15 ml enzyme sample and are designed for use with the Hamilton syringe. Using this syringe, when the carriage is in position for injection, the tip of the needle is in the center of the cuvette.

The syringes hold either lcc (the disposable type) or ten lambda (Hamilton) and can be partially or completely filled depending on the total volume required for a set of injections.

Specific preparation schedules for the enzyme mixtures and injection samples will be assembled as an appendix to this Manual after the completion of experimental work programs to determine the most suitable combination of ingredients and processing.

D. Loading the Samples in the Instrument

The reaction chamber door is opened by releasing the four spring clips and lowering the door into the drop position. The dark slide on the phototube must be closed when the reaction chamber is opened. Failure to close the dark slide first may result in sensitizing the photocathode for a period of several hours, especially if it is exposed to the light from fluorescent lamps. The reaction chamber door is heavy and has sharp edges so that care is required not to drop it accidentally causing bodily injury or a severe impact against the Instrument cabinet. A strip of rubber cemented to the Instrument side is to be used as a bumper for the door top in the drop position.

The loaded cuvette racks are placed into the U-shaped trough in the reaction chamber so that the end of the rack with the fork is at the front of the Instrument (left side facing the reaction

chamber). The hydraulic cylinder carriage is raised to its topmost position for this operation. It is easier to load a sample holding rack without a syringe installed, but with care it can be accomplished. The fork on the sample rack fits over a boss on the holding rack positioning rod. The sample rack may be installed with the position rod indexed into any of the eight positions. However, it is easier to do if the position rod is fully extended from the front of the reaction chamber so that the boss is at the extreme left side of the channel.

A syringe is loaded and the body placed in the holder provided. The lip on the syringe piston is engaged in the spring clip at the top of the syringe holding assembly. As explained in Section IV A, the hydraulic system should be pressurized before loading the syringe.

A check should be performed on the syringe needle alignment by lowering the carriage with the lever and observing whether the needle will enter the cuvette. The needles are quite thin and subject to bending during handling (especially the sterilization of the Hamilton syringe). Minor changes in cuvette position are made by rotating the position rod to the right or the left to center the cuvette entry port under the needle. In extreme cases, the needle may have to be straightened.

When needle alignment is accurate for one cuvette, it is accurate for all eight, so the reaction chamber may be closed and secured. The door is secured to compress the light gasket around the

door to prevent the entry of ambient room light. A pinhole light leak will completely blind the instrument to accurate reaction monitoring.

E. Pre-Injection Monitoring

Two pieces of information are derived from pre-injection monitoring: (1) a phototube "dark count" which is a measure of the thermal and background noise generated by the phototube, and (2) an inherent light count which is a measure of the inherent light radiated by the enzyme mixture in the cuvette positioned in front of the light collector. In order to make these measurements:

1. Disable the solenoid timer by turning the switch located below Timer lines A and B to the off position.

2. Set the desired counting period by selecting a basic time interval and set Timer line C to at least one unit. Set Timer line D to the number of time units desired plus one (to compensate for the setting on line C). Recommended monitoring periods for initial use of the Instrument are either 5 or 10 seconds. (Settings on Timer line C, 0001 and 0201 on Timer line D with a basic time unit of 25 milliseconds will generate a five second interval.)

3. Depress the programmer reset button along with the Manual reset and Manual start buttons on the electronic counter to set the readout to zeros and ready the timer.

4. Set the discriminator to the desired reading (see Section V, F).

5. Make certain the dark slide is in the closed position.

6. Press the start button on the programmer. This will cause the green light to go out and the amplifier gate will be

automatically closed at the end of the first time unit. The gate will open automatically at the end of the time interval, but the green light will remain out until the reset button on the programmer is depressed.

7. The count registered on the readout on the counter is the dark count. Ordinarily five such readings are taken by repeating steps 3 and 6 to obtain five values of dark count. Experience will tell (see Section V, B) whether the dark count is in the right range for the temperature, discriminator, and time interval chosen for the measurement.

8. The inherent light reading is taken on the cuvette in position in front of the light system. The cuvette number is obtained by counting the grooves in the cuvette positioning rod that are outside the reaction chamber. To take this count, open the dark slide, and repeat steps 3 and 6. Ordinarily five such inherent light readings are taken so that they can later be averaged (see Section V, D). The inherent light level decreases with time so the readings should be taken in quick succession and the injection made as soon as the inherent light counts are complete.

F. Injection Cycle

The completion of the dark count readings and the inherent light readings completes the pre-injection monitoring. The preparation for an injection cycle is made in the following steps:

1. The hydraulic system should have been pressurized.

2. The desired volume of sample to be used is computed in terms of length of cylinder stroke to deliver. For the two syringes

supplied, a calibration chart of time settings and hydraulic pressure for specific sample volumes is presented in Figure 5.

3. The desired pressure is set by adjusting the pressure valve to give the desired gage pressure.

4. The injection start time is set into Timer line A and stop time into line B in multiples of the basic time interval. In general, injection and count starting times are the same so Timer line A and line C should have the same reading.

5. Press the programmer reset button to make sure the green light is on; press the two set buttons on the electronic counter.

6. Lower the syringe carriage by raising the lever to the top of its travel to place the tip of the syringe needle into the cuvette inside the reaction chamber.

7. Turn the solenoid control switch to the on position.

8. Open the dark slide.

9. Press the programmer start button.

10. Record the total count shown on the counter read-out.

11. Turn the solenoid control switch to off.

12. Raise the syringe carriage by drawing the control lever to the lowest position.

13. Place a new cuvette into position by translating the rod to the next index position.

G. Data Recording

The data acquired from a particular experiment may be recorded on a data form such as is shown in Figure 6. This form makes provisions

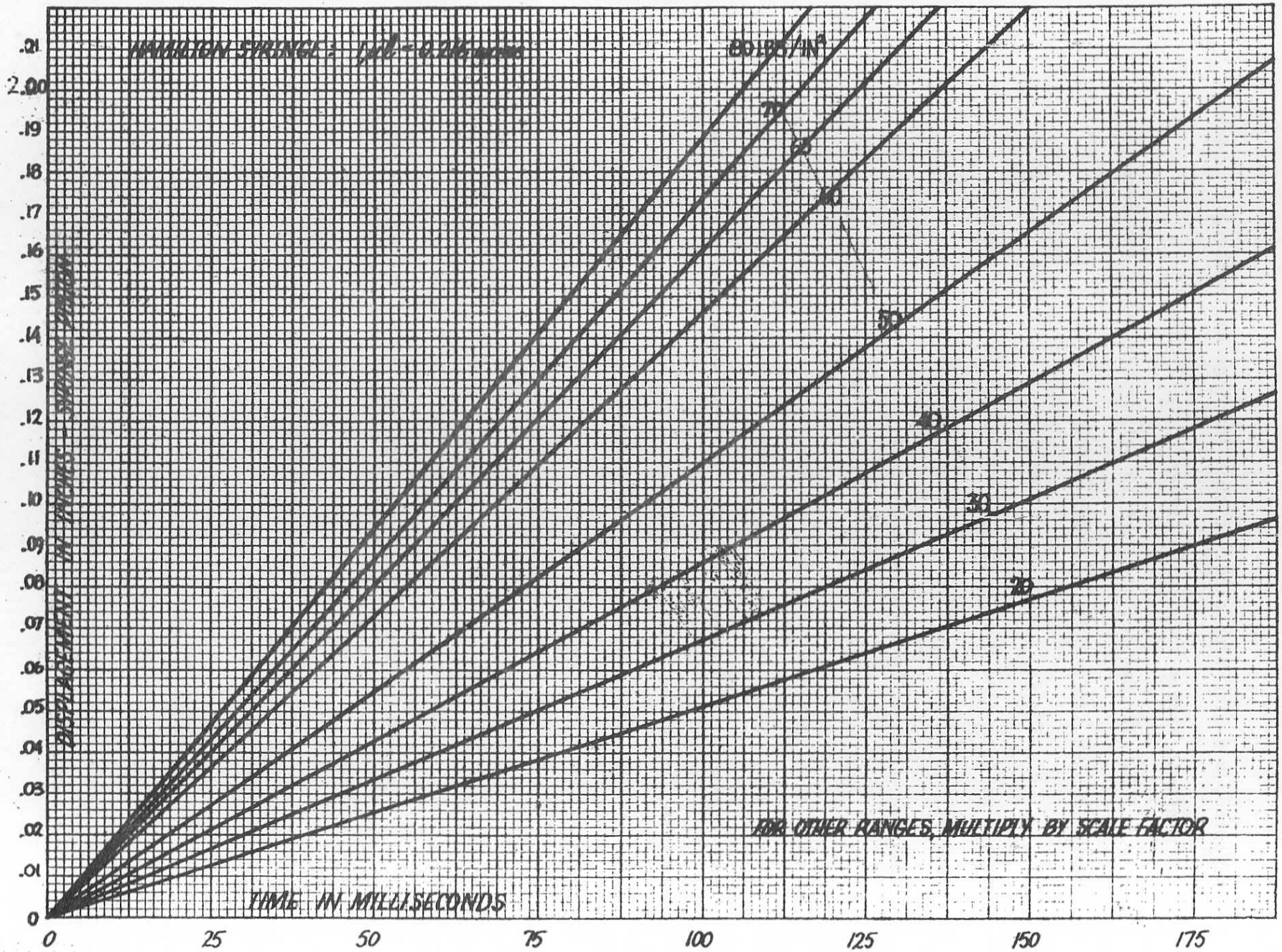


FIG. 5 ATP DETECTION INSTRUMENT
SYRINGE DISPLACEMENT VS TIME FOR SPECIFIC HYDRAULIC PRESSURES

ATP DETECTION INSTRUMENT DATA SHEET

TIME INTERVAL _____ MS.

ATE: _____

TIMER A _ _ _ _ _ TIMER C _ _ _ _ _

PURPOSE OF TEST:

TIMER B _ _ _ _ _ TIMER D _ _ _ _ _

Volume: _____ ATP sample, concentration __/__ Temperature _____ reaction,

_____ enzyme _____ photocathode

Count Period _____ seconds, starting _____. Cuvette _____

Injection time _____ seconds Dark Count _____

Injection pressure _____ lbs/in^2

Enzyme Preparation:

Discriminator Setting _____

No.	Inherent Light					Response

Sample No.	Inherent Light					Response

FIG. 6 SAMPLE DATA SHEET
ATP DETECTION INSTRUMENT
HAZLETON LABORATORIES, INC.

for keeping a record of all of the significant parameters. It may be completed before or after the injection or used as the statement of the experimental design.

V. Calibration Procedure

A. Purpose

Calibration is effected for two reasons: one, to measure the basic light sensitivity of the Instrument; and two, to measure the light emitted from the reaction of know quantities of ATP. The calibration is used to determine average response and the standard deviation of measurement. Data analysis will provide the resolution of the experiment.

B. Instrument Set-Up for Calibration

The instrument is prepared for calibration in the same manner as it is prepared for an experimental reading of dark count or an inherent light measurement. The use of a standard light source calibration run requires that the immersion fluid be drained from the sample rack and the center plug (reflector surface) removed for the insertion of the standard light source. When standard sample concentrations of ATP are used to determine Instrument response, the Instrument is set up for operation just as for any injection experiment.

C. Using the Standard Light Source

A standard light source (U. S. Radium Corp., G-150 phosphor serial no. 52522) is supplied with the Instrument. This source is a

fluorescent phosphor excited by C^{14} which produces a surface brightness of .015 microlamberts when it has not been exposed to any light whatsoever for a period of 24 hours. The exposure of the active surface of the light source, even to dark-room light, will cause a change in its light output. It is therefore very important to handle the light source carefully.

The intensity of light from the source is relatively high for testing the instrument, even though it is barely discernable to the human eye in total darkenss. To reduce the surface area and hence the total light output, a mask has been designed which has two holes in its front surface. One of these is 0.125 inches in diameter; the other is .0465 inches in diameter. A cover can be placed over either of the holes to obtain two different source areas. In addition to the mask, a set of three neutral density filters is supplied which provide transmission factors of 12.5%, 1.25%, and .0125%. These are used by placing them in the U-channel between the standard light source face and the end of the light-pipe on the rear lip of the channel.

A time interval of five seconds is used to accumulate a count. Since the light output from the source is continuous, it is possible to take a large number of readings. Sixty readings have been used in determining the average response and the standard deviation of the measurements. The data required in a standard light source measurement is shown in Figure 7. The ordinate is the light level in lumens; the abscissa is the number of counts recorded. The raw data was reduced by the method outlined in Section V, E.

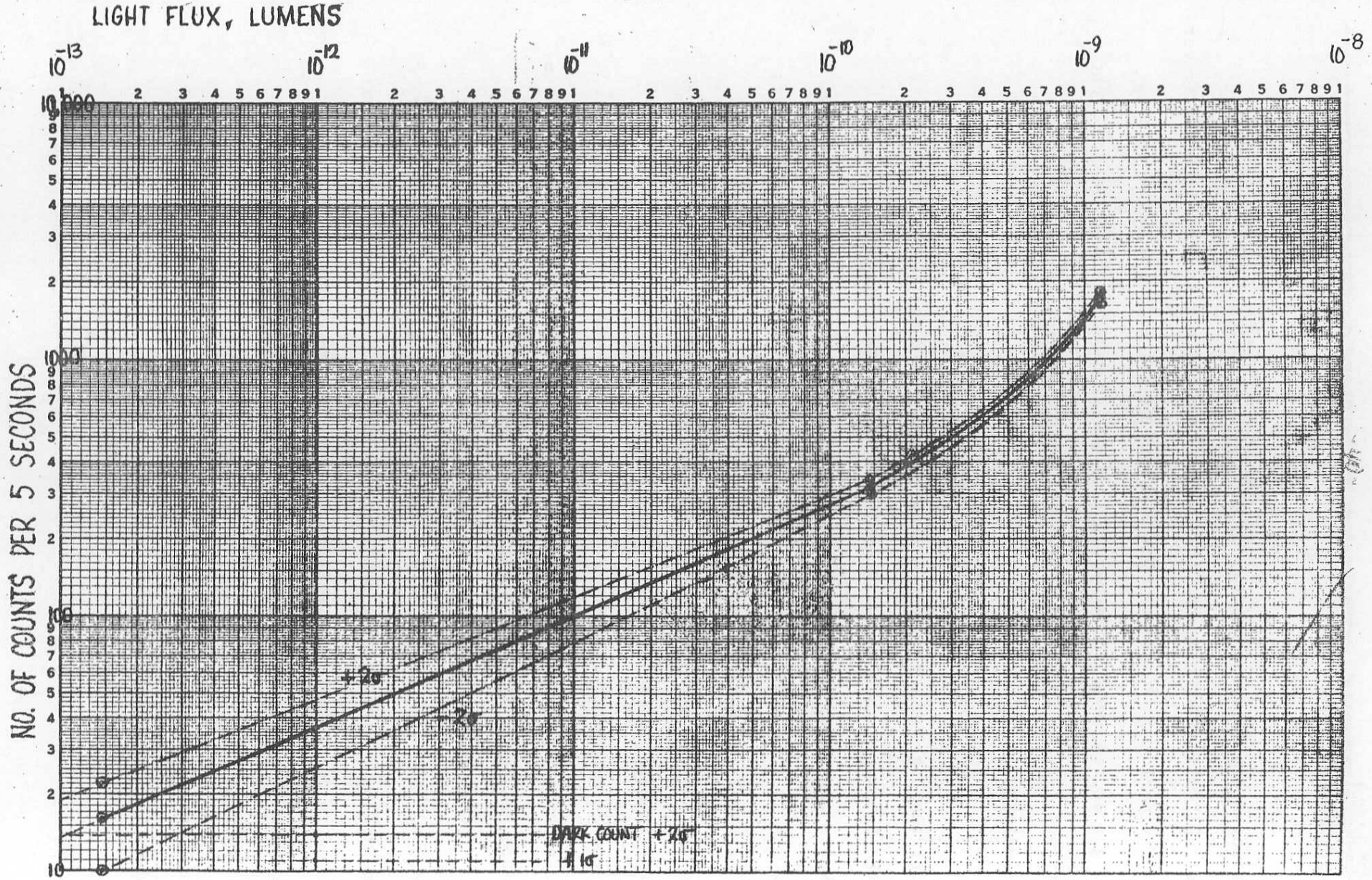


FIG. 7 ATP DETECTION INSTRUMENT
LIGHT FLUX DETECTION SENSITIVITY

The following calculations are required to interpret the results of a standard light source test:

1. Since the source brightness is .015 microlamberts and a lambert is a lumen per square centimeter, the total light flux radiated normal to the source surface is the brightness divided by the source area:

(a) $.015 \times 10^{-6}$ lamberts = $\frac{\text{lumens}}{2.857 \text{ cm}^2}$ = 4.29×10^{-8} lumens

2. The mask exposes a fixed percentage of the surface area. Thus a .125 inch diameter hole has an area of .0123 in^2 which is 2.7% of the source area. Therefore, the light flux emitted is equal to:

(a) $.027 \times 4.29 \times 10^{-8}$ lumens or 1.15×10^{-9} lumens.

3. The use of a neutral density filter between the source and the light collecting system reduces the light intensity by the transmission factor. Since three such filters are available, the light levels produced are:

(a) 1.15×10^{-9} lumens x .125 or 1.43×10^{-10} lumens

(b) 1.15×10^{-9} lumens x .0125 or 1.43×10^{-11} lumens

(c) 1.15×10^{-9} lumens x .000125 or 1.43×10^{-13} lumens

The use of the source, the mask and the filters provide twelve separate light levels for calibration purposes. The calibration is carried out in lumens since this is a standard method of rating phototube sensitivity and light intensities. Changing source areas reveals differences in optical efficiency and the use of stacked plates in the neutral density filters results in additional losses due to internal reflections, so

the absolute accuracy and the repeatability of some points is poorer than others.

It should be noted that the light intensities calculated are for the source and not at the photocathode surface. In order to relate true light level comparisons between the standard source and a cuvette, the distance from the photocathode of each source must be accounted for. The ratio of two light intensities varies as the ratio of the squares of the distance to the source. In the ATP Detection Instrument the standard light source in position is located 3.8 inches from the photocathode, while the center of the cuvette is 2.73 inches from the photocathode. The ratio of the square of these dimensions is 0.49. Thus, approximately, a reaction emitting half the light intensity of the standard source will produce the same number of counts as are obtained from the standard light source.

No attempt has been made to match or determine the spectral characteristics of the standard light source vs. the light spectrum of either inherent light or the light emitted by an ATP-enzyme reaction.

D. Use of Standard ATP Concentrations

The measurement of the Instrument response to known concentrations of ATP is carried out using the procedure outlined in Section IV. In order to determine the dispersion of the experiment, replicate experiments are conducted by running a series of identical injections of ATP into a set of enzyme mixtures. Since a sample rack will hold eight cuvettes, a minimum of eight samples are run under the same

conditions. As the variance of the individual tries increases, a larger number of replicates are taken.

Inherent light readings are taken on each cuvette to establish an average value; one response reading is taken, and the two subtracted to obtain a net response. The average response and the standard deviation are computed for the set of measurements.

The reaction response is a function of the particular enzyme mixture used, the concentration of ATP in the injected sample, the sample volume and the resultant speed of mixing. For large sample volumes, there is a decrease in the inherent light level due to dilution of the enzyme mixture which is determined by injecting sterile, deionized water into the enzyme. This decrease in background is subtracted from the inherent light readings obtained.

Figure 8 is a plot of the number of counts recorded in a five-second period, beginning with the start of the injection, for a range of ATP concentrations in a constant volume of sample. This curve represents the response of a particular enzyme mixture. By suitably processing this mixture, reduced inherent light and a greater activity may be achieved. The limiting factor in the Instrument sensitivity in the detection of small quantities of ATP is, at present, the inherent light level. If the inherent light were not present, the limiting factor would be the phototube dark current.

E. Data Analysis

The data acquired from standard light source tests and injections of known amounts of ATP into enzyme mixtures is analyzed

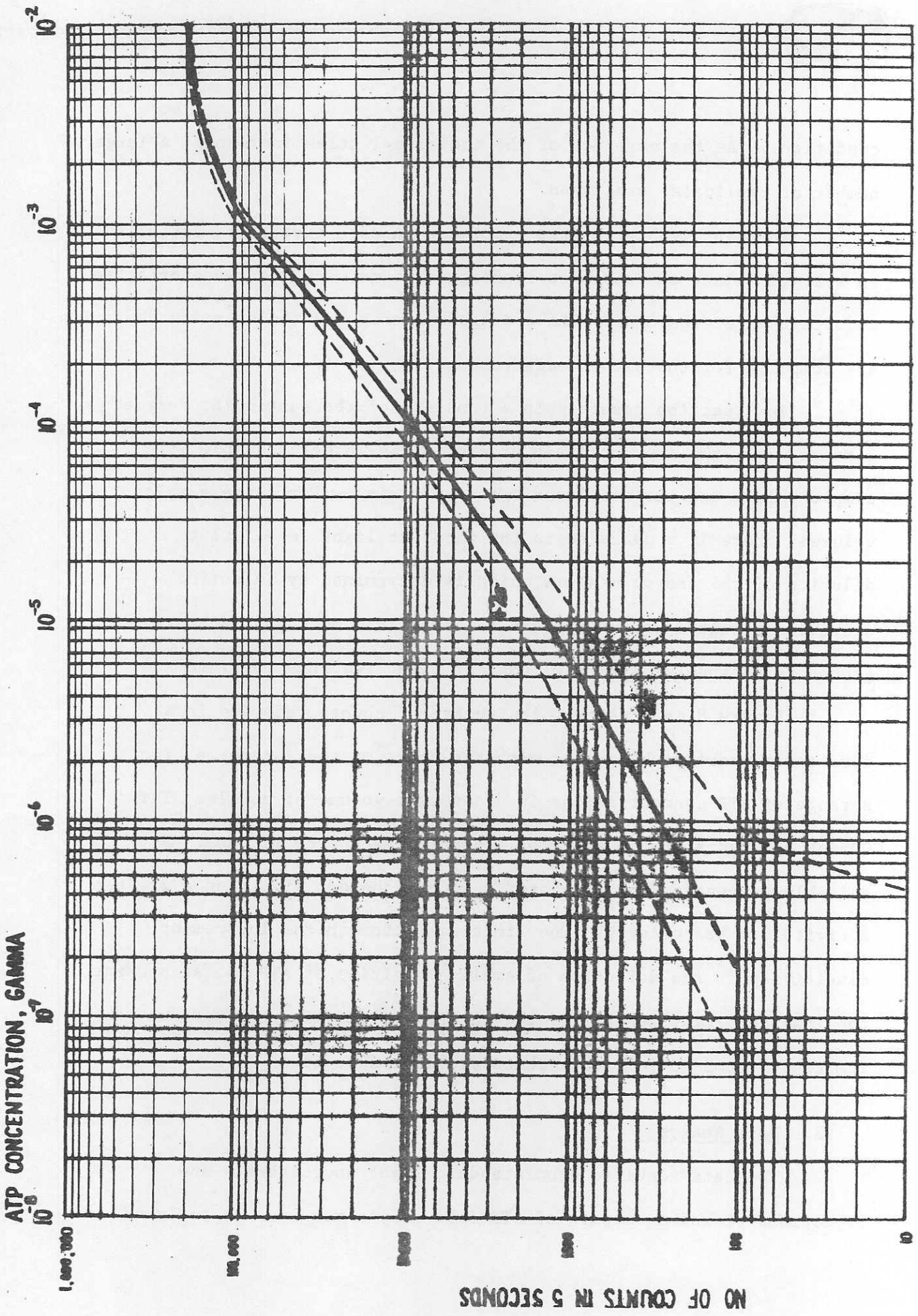


FIG. 8 ATP DETECTION SENSITIVITY

to obtain the best estimates of the average and the standard deviation. To do this, replicate readings of the response of the instrument are averaged; the deviation of each reading from the average, and its square computed, and the variance computed by summing the squares and dividing by a number one less than the number of readings. The standard deviation is the square root of the variance.

In order to make the best decision on the probable utility of a single determination and to determine the minimum statistically significant interval that can be resolved, a confidence level of 95% is adopted. Two standard deviations below one reading that produces the same number of counts as two standard deviations above another, represents the minimum reliably distinguishable difference in ATP concentration when using a single reading. When more than one reading can be used, either the confidence level is raised or the concentration difference interval can be decreased keeping the confidence level at 95%.

Examination of the curves (Figure 7 and 8) shows that the percentage variation in readings increases at the lowest light levels to the point when the uncertainty in the reading includes the level which is the dark count. This is the limiting sensitivity as determined by these tests. It should be noted that there is no attempt to separate Instrument, experimental or reaction variables. An estimate of the Instrument error can be made by comparing the standard deviation of Figure 7 and 8. In the former, the variance is less by a considerable factor even though the standard light source is fundamentally a scintillation counter of C^{14} disintegrations which is a statistical process and must demonstrate some variance.

The ATP detection sensitivity and resolution exhibited in Figure 8 is not the ultimate sensitivity of the Instrument, simply the data acquired during a specific calibration run. A separate run with a different enzyme should produce the same curve displaced upward or downward depending on the activity of the enzyme. A lower inherent light level will reduce the variance and increase the resolution.

VI. Manuals and Instructions Included

The following is a list of the commercial equipment manuals supplied for components used in the ATP Detection Instrument.

1. Instruction Manual, Model 371R Apti-meter Transistor Specialties, Inc., Plainview, L. I., N. Y.
2. Instruction Manual, Model DV-GM Strip Chart Recorder Westronics, Inc., Fort Worth, Texas
3. Instructions, YSI Thermistemp Model 73 Indicating Controller, Yellow Springs Instrument Company, Yellow Springs, Ohio
4. Instructions, Metripak, Position Sensing Transducer, Brush Instruments, Cleveland 14, Ohio
5. Instruction and Operating Manual, Model 6344A Power Supply, Harrison Laboratories, Berkley Heights, New Jersey
6. Schematic, parts list, correspondence, on Thermoelectric cooler, International Energy Conversion, Inc., Garland, Texas
7. U. S. R. C. "Isolite" Standard Source, U. S. Radium Corporation, Morristown, New Jersey
8. Data Sheet, FWG-130G Phototube, International Telephone and Telegraph, Fort Wayne, Indiana

The wiring diagrams for the Programmer, the pulse amplifier, and the digital-to-analog converter are included in a folder supplied with the equipment manuals for the commerical sub-assemblies. These diagrams have color codes and voltage measurements for maintenance and trouble shooting purposes. A parts list and circuit board layout are included for parts location use.

The mechanical drawings showing all of the fabricated parts, and assembly drawings, are listed on the drawing list. The drawing set is included as part of this manual but for convenience is packaged separately.

VI. Drawing List

Top Assemblies

Drawing No.	Description
D 1002	Reaction Chamber Weldment
D 1069	Cuvette Reaction Chamber Assembly
D 1070	Injection System Assembly
D 1071	Phototube Housing Assembly

Sub-Assemblies

C 1008	Cuvette Assembly
C 1013	Reaction Chamber Door
C 1020	Cuvette Holder Assembly

Detail Drawings

B 1001	Thermoelectric Cooler
C 1003	Left Chassis Side
C 1004	Right Chassis Side
B 1005	Weld Angle

B 1006	End Gusset
C 1007	Chassis Bottom
B 1009	Mounting Strip
B 1010	Mounting Strip
B 1011	Window Chamber
B 1012	Cuvette Chamber
C 1013	Reaction Chamber Door
B 1014	Top Plate
B 1015	Reflector
B 1016	Reflector
B 1017	Reflector
B 1018	Dark Slide Disc
B 1019	Bushing
C 1020	Cuvette Holder
B 1021	Mounting Ring
B 1022	Track Slide
B 1023	Track Slide
B 1024	End Plate
B 1025	Mounting Ring
B 1026	Piston Chamber
C 1027	Mounting Arm
B 1028	Sliding Shaft
B 1029	Syringe Holder Bracket
B 1030	Mounting Bracket
B 1031	Reaction Tube
C 1032	Syringe Holder
D 1033	Reaction Chamber Mounting Plate
C 1034	Phototube End Plate
C 1035	Phototube Front Plate
B 1036	Phototube Mounting Bracket
B 1037	Rod Mounting Ring
B 1038	Pull-Rod
B 1039	Detent Pin

B 1040	Tube Holder
B 1041	Retainer Pin
B 1042	Dark Slide Shaft
B 1043	Slider Block
B 1044	Swivel Shaft
B 1045	Sliding Shaft
B 1046	Pivot
B 1047	Pivot Shaft
B 1048	Dark Slide
B 1049	Bushing
B 1050	Shaft Coupling
B 1051	Shaft
B 1052	Slide Positioner
B 1053	Washer
B 1054	Lift Bar
B 1055	Positioner Bracket
B 1056	Swivel Pin
B 1057	Pivot Block
B 1058	Spring Pin
B 1059	Retaining Block
B 1060	Swivel Piston
B 1061	Phototube Housing Details
B 1062	Sliding Piston
B 1063	Sleeve
B 1064	Mounting Ring
B 1065	Mounting Ring
C 1066	Phototube Housing
B 1067	Base Plate

Routine Procedure for ATP Assay

Desiccated firefly tails (5 grams) are ground to a fine powder with 250 mg of washed silica with a pre-chilled mortar and pestle. The powder is then added to 600 ml of acetone which has been cooled to -20°C with dry ice. The suspension, after being allowed to stand for five minutes, is filtered on a Buchner funnel under reduced pressure. The filter cake (residue) is washed with an additional 250 ml of cold (-20°C) acetone. Vacuum is maintained on the Buchner funnel until the filter cake is almost dry. The cake is then placed on filter paper and taken to complete dryness under vacuum in a desiccator. This preparation will be referred to as an acetone powder.

The acetone powder (500 mg) is then extracted with 7 ml of cold arsenate buffer (0.05 M - pH 7.4). The buffer also contains Versene at a concentration of 1×10^{-3} M. The suspension is centrifuged at 1500 x G at 4°C for ten minutes. The supernatant solution is decanted and saved and the residue is re-extracted with two 3 ml volumes of cold water adjusted to pH 8. The three supernatant solutions are then pooled and centrifuged in the cold at 12000 x G for 30 minutes. The supernatant (11.8 ml) containing the bioluminescent system is then assayed for activity.

Partially purified luciferase is prepared by passage of the crude extract through a Sephadex G-100 column prepared as follows:

Sephadex G-100 (20 grams) is placed in an excess of 0.05 M arsenate (pH 7.4) - 0.001 M Versene, pH 7.4 and allowed to swell for 48 hours.

A water jacketed column (100 cm x 25 mm) is packed to a height of 70 cm with the G-100 gel. An additional 10 cm of Sephadex G-25 is layered upon the surface of the G-100 gel. The column is then washed with 200 ml of buffer solution used in preparation of the gel.

Eight ml of the acetone powder extract is placed on the Sephadex column. The column is then eluted with 0.05 M arsenate, pH 7.4, containing Versene at a concentration of 1×10^{-3} M. The 10 ml fractions which are collected are then assayed for activity using the following reaction mixture:

0.1 ml of fraction
0.1 ml of luciferin* prepared by dissolving crystalline luciferin in 0.05 M arsenate - pH 7.4 (1 mg/ml)
0.1 ml of $MgSO_4$ (1×10^{-2} M
0.01 ml of ATP (1×10^{-3} M) is injected

The most active fractions are then pooled and luciferin and $MgSO_4$ are added to the total volume in 1:1:1 ratio. The mixture is then allowed to stand in the cold (5°C) for at least two hours to reduce the inherent light level when stored at 5°C, the above reaction mixture is stable up to one month.

*Synthesis of luciferin is described in Appendix I.

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