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RAPID MICROBIOLOGICAL DETECTION

August 7, 1963

Prepared Under Bureau of Naval Weapons

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ABSTRACT

A new technique for the bioassay of ATP was developed in which the reaction is allowed to take place on filter paper. A major advantage of this technique is that it allows a rapid concentration of dilute ATP. A similar method which employs glass plates instead of filter paper is under development.

Studies were carried out with partially purified preparations of luciferase and luciferin in an effort to understand, and possibly eliminate the inherent light effect. While the mechanism of the inherent light effect is not yet defined, it appears that an initial "one step" treatment with calcium phosphate gel eliminates a major part of the inherent light without affecting the enzyme activity to any great extent.

The extraction of ATP from E. coli and S. cerevisiae using different solvents is now being studied. Using extraction with hot water as the basis of comparison, perchloric acid was not as effective while acetone when mixed with water at a ten to one ratio effected a significantly better extraction. Detection of 3,800 yeast cells has been realized. By using methanol extraction it has been possible to obtain a positive response from algae.

A number of chemical compounds were investigated for their effect on the bioluminescent reaction. The reaction rate was not accelerated by any of the compounds tested. The rate was inhibited by glycerol, methanol, and hydroxylamine.

Work will be continued along the lines of the studies described in this report. This includes development of more sensitive instrumentation, better extraction methods, and a rapid preliminary purification technique for the enzyme in order to eliminate inherent light.

RAPID MICROBIOLOGICAL DETECTION

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I. INTRODUCTION

A. Summary of Previous Work

The detection of microorganisms by the use of the bioluminescence reaction is based on the ATP* requirement of the reaction as it occurs in firefly lantern extracts. This method assumes, with a vast array of data in support, a ubiquitous distribution of ATP throughout the life spectrum. ATP thus emerges as a specific biological entity with its occurrence being concommitant with, and indicative of, the existence of life.

The general procedure which has been followed in the studies reported to date has consisted of dissolving lyophilized firefly lantern extract (Worthington Biochemical Corp.) in either distilled water or glycylglycine buffer (0.05 M-pH 7.8). The resultant solution contains, with one exception, all of the components necessary for the bioluminescent reaction (luciferase, luciferin, oxygen, and magnesium). The missing factor is ATP which, on being added, either as it exists in a microbial extract or as a standard solution, initiates the reaction below:

1) Luciferin + ATP $\xrightarrow{\text{Luciferase}}$ AMP-luciferin + pyrophosphate

2) AMP-luciferin $\xrightarrow{O_2}$ light + Oxyl AMP-luciferin

The above reaction yields one quantum of light for each molecule of ATP.[1]

Instrumentation used for quantitative light measurements consists of a reaction chamber in which a five mm Beckman cuvette containing either the ATP solution or the enzyme extract is placed, a photomultiplier tube (RCA 7265) before which the cuvette is positioned

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* Adenosine-5'-triphosphate

prior to start of reaction, and a Tektronix 502 oscilloscope with camera attachment. After the reaction is initiated by the injection of either ATP or enzyme-luciferin mixture,(depending on which has previously been placed in the cuvette), the resultant signal is followed on the oscilloscope.

By use of the above method, it has been possible to detect amounts of ATP in the order of 10^{-4} micrograms, and to detect ATP in about 16 different microorganisms including bacteria, yeast, and streptomycetes. Positive responses have also been obtained from lyophilized bacteria, dried yeast, fungal spores, and one soil extract. In the case of yeast, 40,000 to 50,000 cells could be readily detected. A ten fold improvement has been accomplished with the detection of 3,800 yeast cells during the current reporting period.

B. Goals of Current Studies

The study of bioluminescence as a microbial assay is now being directed toward the following goals:

(1) increased overall sensitivity with respect to the minimal amount of ATP necessary for a significant response, and

(2) the development of methods which will render the total ATP contained within microorganisms susceptible to reaction with the luciferase system of the firefly lantern extract.

II. SENSITIVITY STUDIES

In conducting studies designed to achieve maximal sensitivity in the assay of ATP utilizing the firefly bioluminescent reaction, there exists a variety of experimental approaches. The most promising include the following:

1. The addition of chemical compounds to the reaction mixture in order to achieve a maximal utilization of ATP for light production.

It has been observed by a number of investigators that when using a relatively crude preparation some of the ATP added to a system containing luciferase and luciferin does not give rise to a proportional amount of light.[2] This is due in part to the combination of ATP with biologically inactive oxyluciferin, and the inability of all of the luciferin molecules (active complex) to achieve the energy level necessary for light emission. In relatively crude extracts, there are, of course, other enzyme systems in competition for exogenous ATP.

2. The development of techniques whereby dilute solutions of ATP could be concentrated to a value sufficient for detection using the existing method, thereby increasing the overall sensitivity of the method.

3. A modification of the light measuring devices so as to obtain a greater percentage of the emitted light, a greater amplification of the signal produced at the first stage of the phototube, or an integration of the total current produced over a finite period of time.

4. Decreasing the reaction time by improved mixing of reagents.

A. Effects of Certain Compounds on Bioluminescent Reaction:

The effects of a number of compounds when added to the bioluminescent system were studied. The results are described in Table I. It had been hoped that ascorbic acid would reduce any inactive oxyluciferin, if present in the system, to the active luciferin. There was either no oxyluciferin present, or ascorbic acid does not possess sufficient negative potential to carry out the reduction. (There is no published value for the oxidation-reduction potential of oxyluciferin.) The rationale governing the use of glycerol, methanol, and methionine, is the stabilizing effect of these compounds (which has been observed by others) in a variety

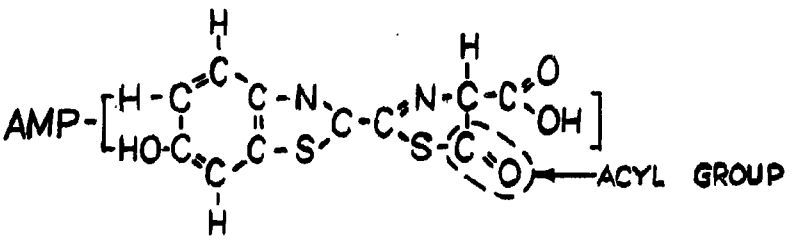
Table I

EFFECTS OF CERTAIN COMPOUNDS ON BIOLUMINESCENCE

Compound	Quantity	Response (millivolts)
None		300
Sodium ascorbate	4 μ moles	300
Glycerol	260 μ moles	175
Methanol	300 μ moles	100
Methionine	2 μ moles	285
Hydroxylamine HCl	5 μ moles	75
Coenzyme A	5 μ moles	310

Fifty mg of lyophilized extract were taken up in five ml of distilled H_2O. Standard ATP was prepared with a concentration of 1×10^{-2} gamma per 0.01 ml. Reaction mixture consisted of 0.2 ml of enzyme extract, 0.01 ml of ATP, and 0.1 ml of compound being tested.

of reactions in which free radicals and semiquinones are involved. The bioluminescent reaction would appear to proceed via one of these mechanisms. The glycerol and methanol were inhibitory whereas no measurable effect was observed with methionine. Hydroxylamine is extensively used for the binding of acyl groups, and was employed here to bind the acyl moiety of the luciferin - AMP complex (seen below)following light emission.



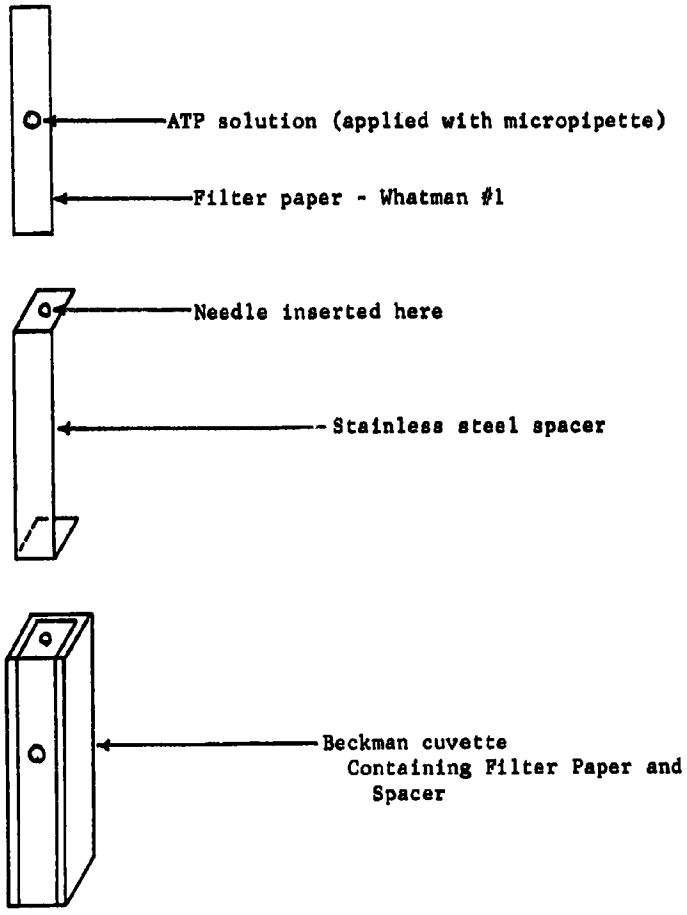
This would conceivably displace the reaction to a greater degree in the direction of light emission. Once again, however, there was marked inhibition of the reaction. Coenzyme A is a normal component of the reaction cycle, being responsible for the splitting of the product-inhibited luciferase to regenerate the active enzyme.[3] The results described in Table I would indicate either a saturating concentration of endogenous coenzyme A, or the initial absence of any product-inhibited luciferase. The concentration of the compounds used in the above experiment are equivalent to those used routinely in enzyme studies.

B. Bioluminescent Reaction on Filter Paper

A method which is undergoing development at present involves the use of cellulose chromatography paper (filter paper) as the site of the bioluminescent reaction. The relatively simple design of the reaction cell is shown in Figure 1. The procedure consists of pipetting an aliquot of the ATP solution (not more than 10 lambda)

Figure 1

FILTER PAPER TECHNIQUE FOR DETECTING ATP



on to the surface of the filter paper strip and allowing it to dry. The drying may be accomplished(two to three minutes) by allowing it to stand in air at ambient temperatures, or more rapidly, by the use of a small warm air blower. The strip is then placed in a ten mm Beckman cuvette and secured against one face by means of a metal spacer.

A three inch spinal needle with a two ml syringe containing the enzyme extract is inserted into the hole at top of metal spacer and allowed to rest on bottom of cuvette. After the cell is positioned in front of the photomultiplier tube, the enzyme extract is introduced into the cell. The enzyme extract moves up the filter paper by capillary action and on reaching the ATP reacts to give a bioluminescent response. The linearity of the reaction is described in Figure 2. The emitted light is measured as a function of ATP concentration. Figure 2 also shows the response of the reaction when carried out in the usual liquid phase. The greater initial intensity of the latter is immediately evident. However, on comparing the shapes of the curves (Figure 3) encompassing the total light emitted, it is seen that the total light emitted by the filter paper reaction is as great and possibly greater than the liquid phase reaction. The reason for the difference in curve shape remains to be explained.

The method described above, while not increasing sensitivity over the technique employing an aqueous environment, does have one great advantage. It allows the concentration of ATP from a dilute solution by the simple process of alternately spotting and drying successive aliquots of a solution, thereby, in effect, giving greater sensitivity from the point of view of the entire system, as is shown in Figure 4. It also appears that the magnitude of the inherent light is reduced.

Figure 2

COMPARISON OF BIOLUMINESCENT RESPONSE AS OCCURRING ON FILTER PAPER AND IN LIQUID PHASE

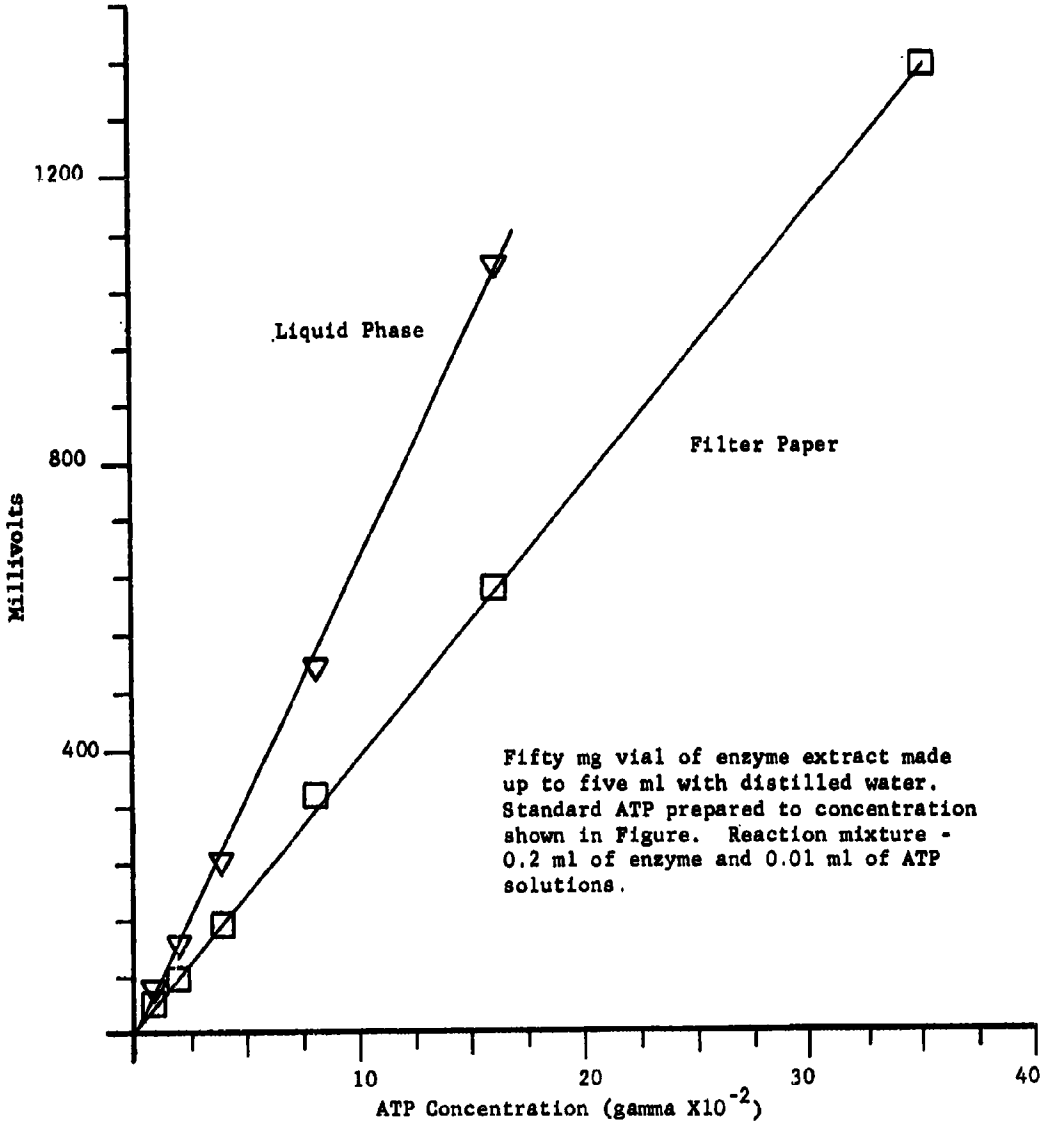
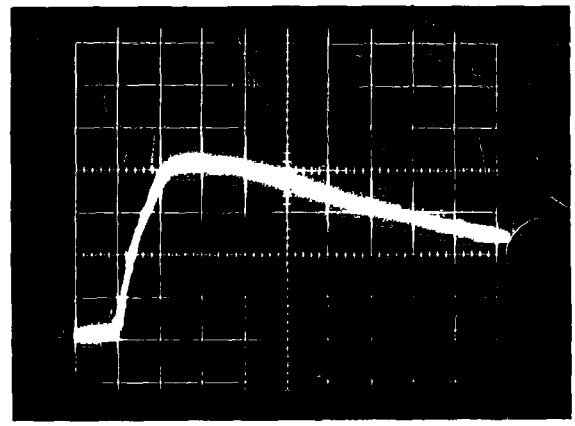
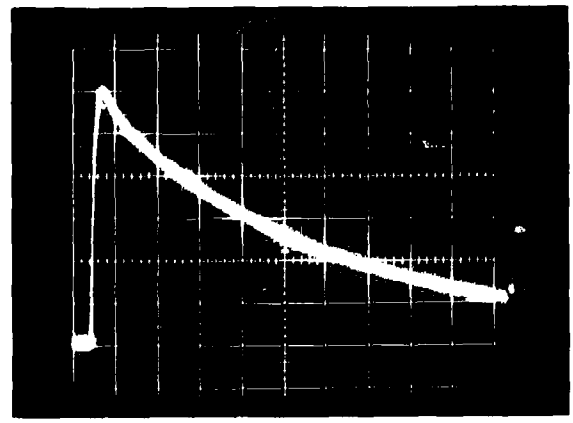


Figure 3

RESPONSE FROM 10^{-1} GAMMA ATP AT SENSITIVITY OF 200 MILLIVOLTS



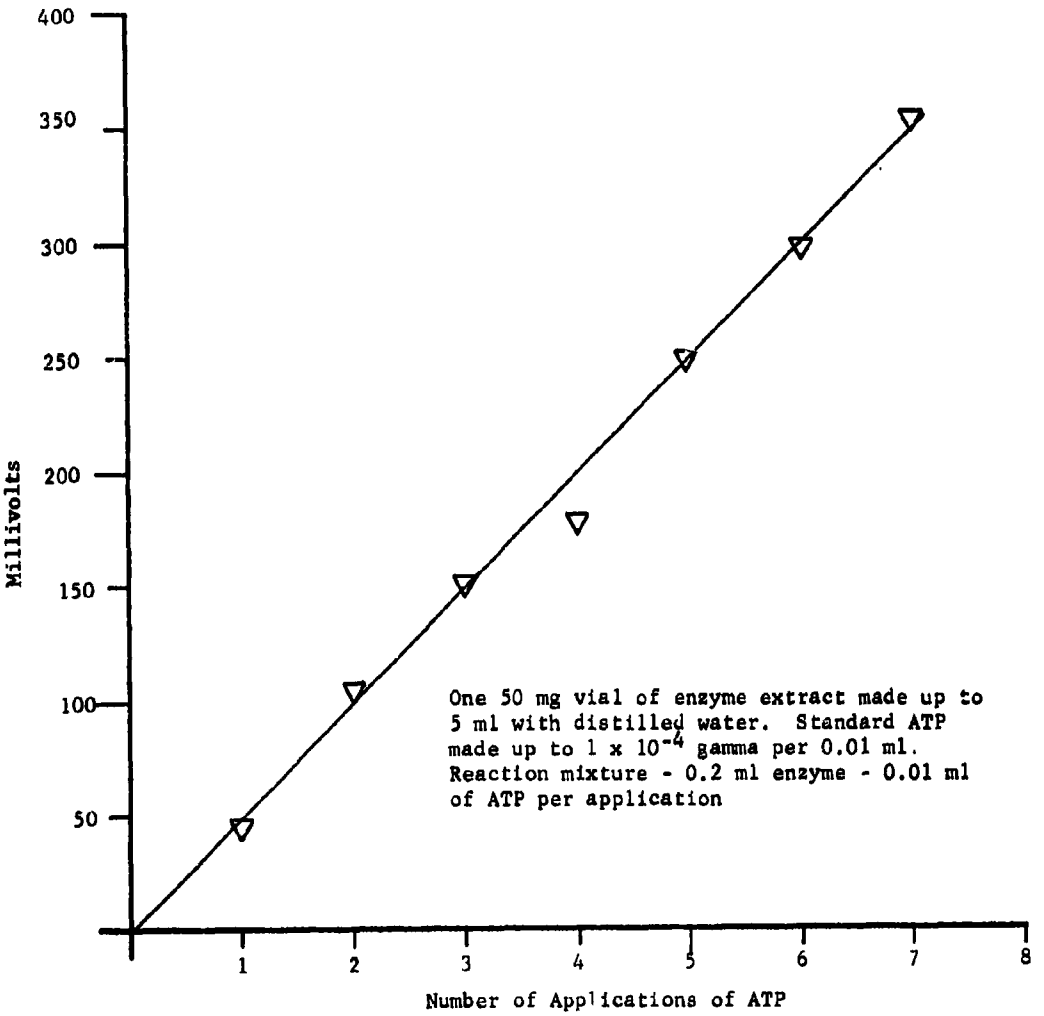
a. Filter Paper



b. Liquid Phase

Figure 4

BIOLUMINESCENT RESPONSE AS RESULT OF REPEATED APPLICATIONS OF ATP ON FILTER PAPER



Another approach under investigation at this time, which in principle is quite similar to the use of filter paper, is one utilizing glass plates instead of paper. A small volume of solution containing ATP is placed on a three inch by one inch glass plate and dried for one minute, after which a second plate of the same dimensions but with a mirrored surface, is placed in exact coincidence with the spotted plate. After the pair of plates is positioned before the phototube, the reaction is initiated by depressing the plates into a small trough containing the enzyme extract which contacts the ATP by moving up between the plates by capillarity. This system offers the advantages of the filter paper technique, and allows an approximation of 4π geometry from the standpoint of light recovery. Preliminary determinations indicate a linearity of response similar to that of liquid phase reactions. A working prototype, which is shown in Figure 5,is now under construction.

Another means of increasing the overall sensitivity is being considered. As envisioned now, the reaction would be carried out on the positive side of a parabolic mirror with the reactants deposited on its surface as aerosols. With the reactants present as almost monomolecular layers, the mixing time would be very rapid and self absorption would be minimal. The mirrored surface would insure that all of the emitted light struck the phototube.

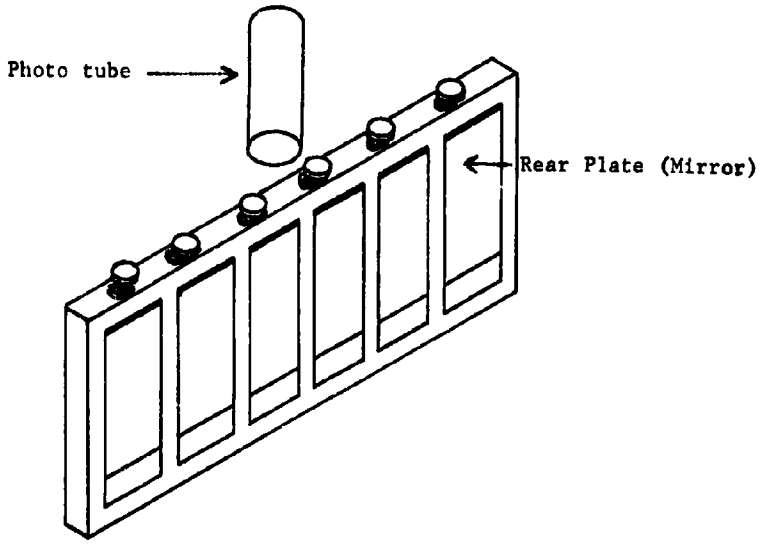
III. STUDIES WITH PARTIALLY PURIFIED LUCIFERASE AND LUCIFERIN

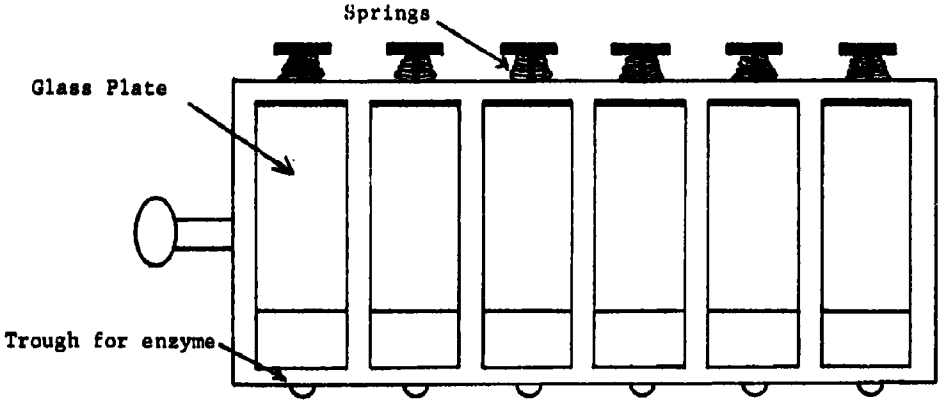
A. Fractionation of Luciferase and Luciferin

The commercial preparations of lyophilized extract (luciferase and luciferin) exhibit considerable variation in activity. Also these relatively crued preparations emit a low level of inherent light which is independent of added ATP. Although crystalline luciferin and

Figure 5

GLASS SLIDE HOLDER AND DARK CHAMBER FOR BIOLUMINESCENT REACTION





luciferase can be prepared, the excessive cost of such preparations seemed unwarranted at this phase of the program. It was thought, however, that a better understanding of both the extract variability and the light effect could be obtained through the use of partially purified luciferase and luciferin.

The separation and partial purification of luciferase and luciferin was carried out according to methods described by McElroy *et al.*[4, 5] outlined as follows:

Five grams of the Worthington crude firefly extract were combined and suspended in 100 ml of deionized water. The pH of the resultant solution was 6.4. It was adjusted to 8 with 1N NaOH. The resulting suspension was centrifuged for 15 minutes and the supernatant was then mixed with calcium phosphate gel which had been prepared as follows: 2.0 g of calcium phosphate gel (14.3 percent, aged) was centrifuged 15 minutes and the supernatant discarded. After being stirred 15 minutes at 10-15°C the mixture, which attained a pH of 7.9, was centrifuged. The gel (I) was discarded and the supernatant (II) was treated with 10.52 g of calcium phosphate gel prepared as before. After mixing the gel with supernatant II and adjusting the pH to 8 (1N NaOH), the mixture was stirred 15 minutes at 10-15°C and centrifuged 15 minutes. After this procedure, essentially all of the luciferin remains in the supernatant (III) whereas the enzyme is absorbed on the gel (II). From this point the procedure diverges into two techniques:

1) isolation of luciferin
2) isolation of luciferase

B. Isolation Of Partially Purified Luciferin

The crude supernatant (III) was acidified with two ml of 20 percent sulfuric acid to pH 3.5 with a final volume of 96 ml. It was then extracted three times with a total volume of 200 ml of ethylacetate.

After each extraction an emulsion phase was formed which was then broken by centrifugation. The ethylacetate fractions were combined, filtered, and then evaporated at 30 mm Hg in a nitrogen atmosphere at 25-35°C. The residue was dissolved in 10 ml of deionized water. This solution will be subsequently referred to as "luciferin I." The flask was then washed with an additional 10 ml of deionized water becoming "luciferin II". Neither luciferin I nor luciferin II exhibited any inherent light or any light emission with ATP, but when mixed with the crude luciferase solution (as prepared below), both gave a definite bioluminescent reaction with 10^{-1} gamma of ATP.

C. Isolation of Partially Purified Luciferase

The gel (II) was then washed twice with a total volume of 80 ml of cold dilute base (pH-8). The supernatant was discarded and the gel washed with 20 ml of cold two percent ammonium sulfate solution (pH-8). The supernatant was discarded and the gel extracted twice with a total volume of 95 ml of cold seven percent ammonium sulfate solution (pH-8). From the supernatant after centrifugation, luciferase was precipitated and fractionated by stepwise partial saturation of the solution with ammonium sulfate. The following fractions were collected:

up to 36% saturation
36-50% "
50-55% "
55-60% "
60-65% "
65-70% "

The precipitation was carried out with constant stirring at 15°C. A pH of 8 was maintained throughout the entire procedure by adding 1N sodium hydroxide when necessary. After each precipitation, the reaction mixture was allowed to settle 3-20 hours and then centrifuged

for 15 minutes. Each precipitate was dissolved in five ml of 0.001 M ethylenediaminetetraacetate solution, pH = 7.9. Each fraction was devoid of inherent light at an oscilloscope sensitivity of two mv/cm and gave no light reaction with 10^{-1} gamma ATP. When mixed with luciferin I or II all of the fractions gave a definite reaction with 10^{-1} gamma ATP with no inherent light. The results are summarized in Table II. It should be noted that the response curve (intensity vs. time) differs greatly from those seen with crude extracts. See Figure 6.

D. Inherent Light and Dilution Effect

Although partial purification completely removes the inherent light, it also results in a considerable decrease in total activity. It was, therefore, decided to determine the activity of the extract after subjecting it only to the first calcium phosphate gel treatment. Two vials of crude Worthington firefly extract were combined and suspended in two ml of 0.05 M glycylglycine buffer (pH = 7.8). The mixture was centrifuged ten minutes, and an aliquot of 0.04 ml of the supernatant brought up to 0.2 ml with glycylglycine buffer. The inherent light in this solution was measured and after biasing out the signal, 10^{-1} gamma ATP in 0.1 ml of water was injected and the response recorded. The remainder of the supernatant was mixed with calcium phosphate gel (prepared by centrifuging 60 mg of the aged gel and discarding the supernatant) and shaken vigorously for 15 minutes. After centrifugation for ten minutes, 0.04 ml of the resulting supernatant was brought up to 0.2 ml with the standard glycylglycine buffer. The inherent light was measured, biased out, and 10^{-1} gamma ATP in 0.1 ml water was injected using the standard technique.

The results are summarized in Table III.

Table II

BIOLUMINESCENT RESPONSE BY AMMONIUM SULFATE FRACTIONS OF LUCIFERASE

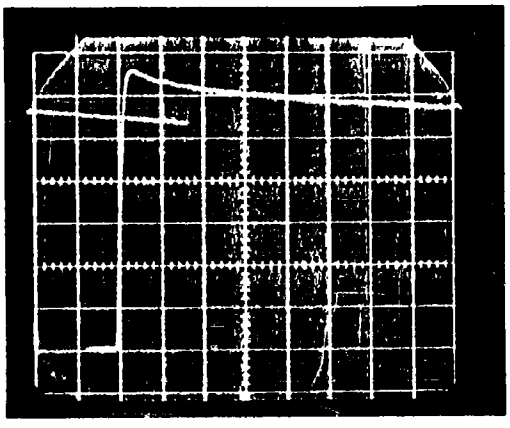
Luciferase Fraction	Response	Total Protein Conc. (mg/ml)*
up to 36% sat.	25 mv	1.324
36 - 50%	230 mv	0.818
50 - 55%	190 mv	0.428
55 - 60%	290 mv	0.506
60 - 65%	90 mv	0.378
65 - 70%	110 mv	0.305

* Protein concentration was calculated from UV absorption according to Methods in Enzymology; vol. III, p. 451

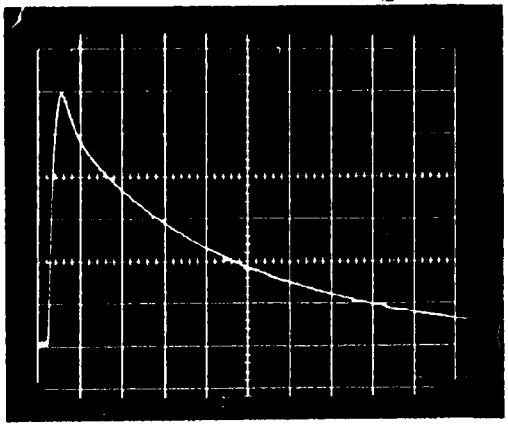
The responses listed in this table were obtained by mixing 0.1 ml of the respective luciferase fraction with 0.1 ml of luciferin II solution, 0.1 ml of 0.02 M $MgSO_4$ and 0.5 ml of glycylglycine buffer pH = 7.8 and injecting 0.1 ml of solution containing 10^{-1} gamma ATP. All the preparations were kept refrigerated, but not frozen.

Figure 6

BIOLUMINESCENT RESPONSE FROM ATP USING PARTIALLY PURIFIED LUCIFERIN AND LUCIFERASE OR CRUDE EXTRACT



a. $1X10^{-1}$ gamma ATP, 0.5 ml of glycylglycine buffer (pH 7.8), 0.1 ml of luciferase (55-60% fraction), 0.1 ml of luciferin, and 0.1 ml of $2X10^{-2}$M $MgSO_4$



b. $1X10^{-1}$ gamma ATP and 0.2 ml of crude extract (50 mg in 5 ml of glycylglycine buffer)

Table III

EFFECT OF SINGLE TREATMENT WITH CALCIUM PHOSPHATE GEL, ON ACTIVITY OF BIOLUMINESCENT SYSTEM

Before treatment with gel		After treatment with gel	
Inherent Light	Response*	Inherent Light	Response*
120 mv	2000 mv	40 mv	1800 mv
75 mv	1800 mv	26 mv	1600 mv
20 mv	1600 mv	15 mv	1650 mv

* $1X10^{-1}$ gamma ATP

These results show that in extracts with high inherent light, the calcium phosphate gel treatment removes over 60 percent of the inherent light while decreasing the response only about ten percent. Further study involving variation in the concentration of the extract as well as in the amount of the applied calcium phosphate gel is in progress.

As has been previously mentioned, there is a measurable quantity of light emitted by the firefly extract in the absence of exogenous ATP. The mechanism of this inherent light reaction is as yet undefined. Among the experiments carried out toward an elucidation of this effect has been one in which the inherent light has been measured as a function of the dilution of the extract. This is described in Figure 7. It is seen here that while the inherent light falls off with increased dilution, there is a concomittant and proportional decrease in response to a constant amount of ATP. It was speculated that this decrease in activity on dilution might be due to limiting concentrations of luciferin. An experiment was done to check this possibility. A luciferin preparation was used which had been purified to the extent that there was no luciferase present. The results are shown in Figure 8.

The change in reaction rate is measured as a function of enzyme dilution with and without purified luciferin. It is seen that, even at dilutions where apparently the enzyme is not limiting, there is little, if any, effect on adding additional luciferin. This lack of luciferin effect is also shown in Figure 9 where the initial light intensity is plotted as a function of extract dilution. It would thus appear that the effect of dilution upon the reaction is not due to decreased luciferin, and further, as is shown by the lack of change in rate in the region where the initial intensity is changing, is not due to a limiting luciferase

Figure 7

EFFECT OF ENZYME EXTRACT DILUTION

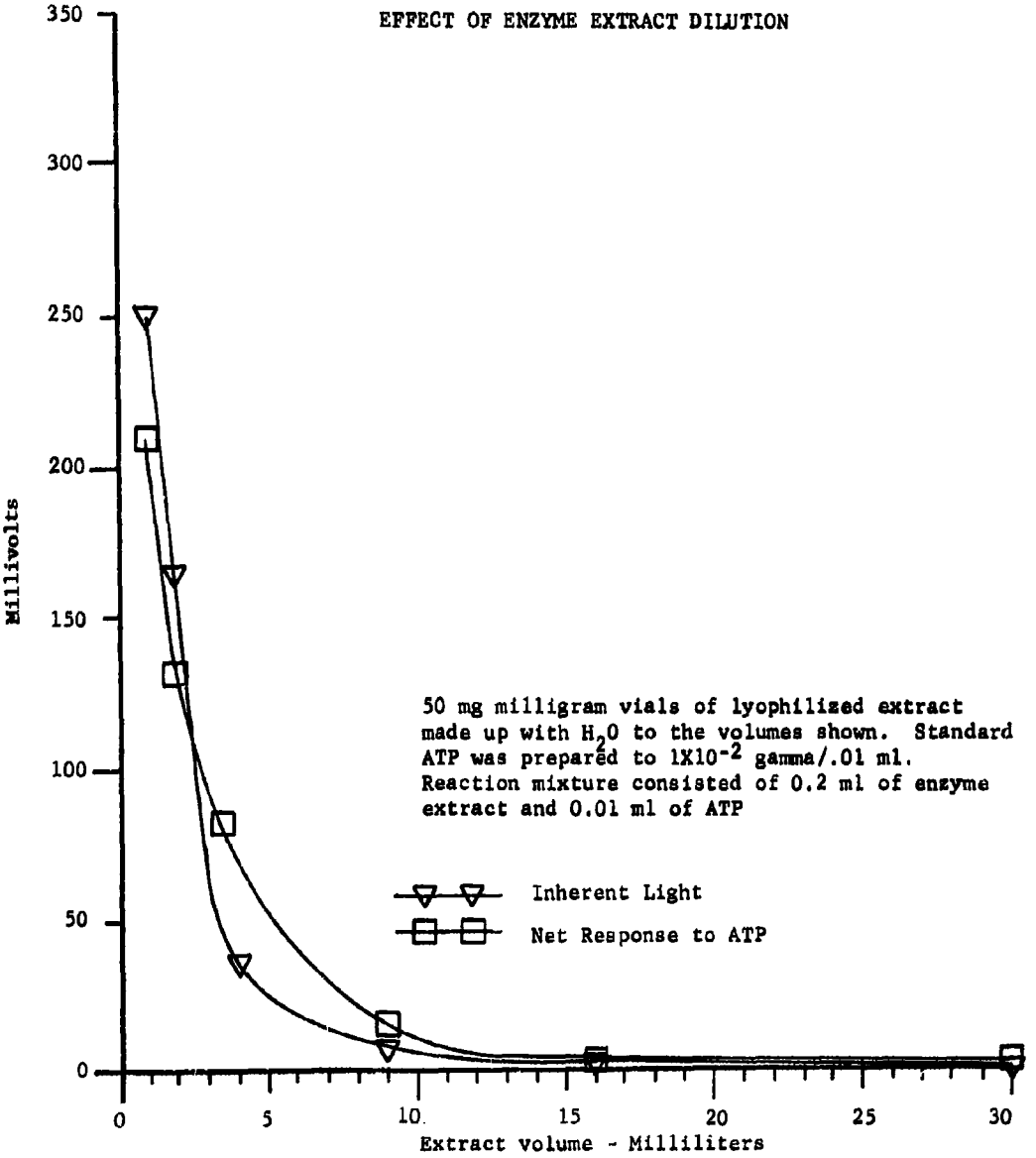
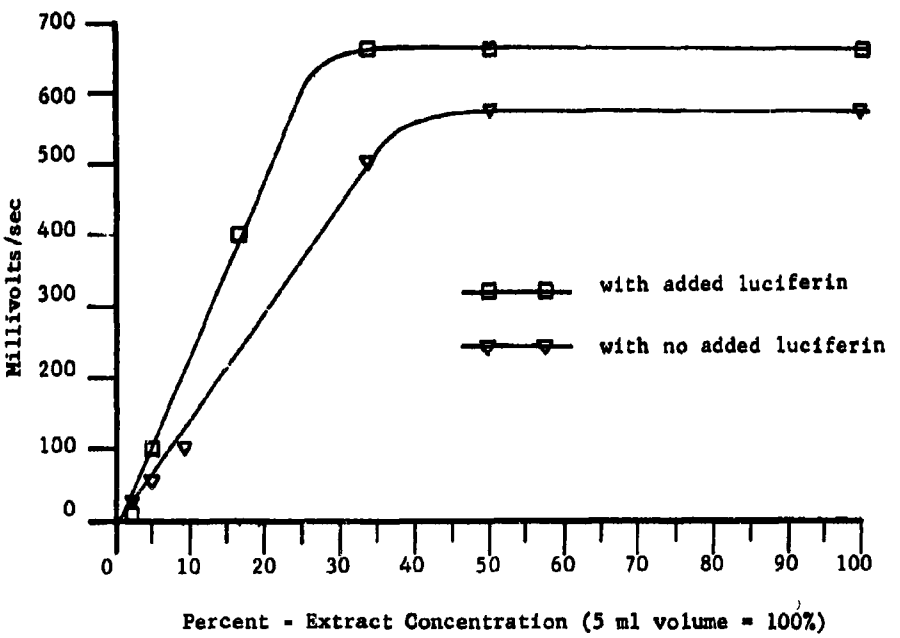


Figure 8

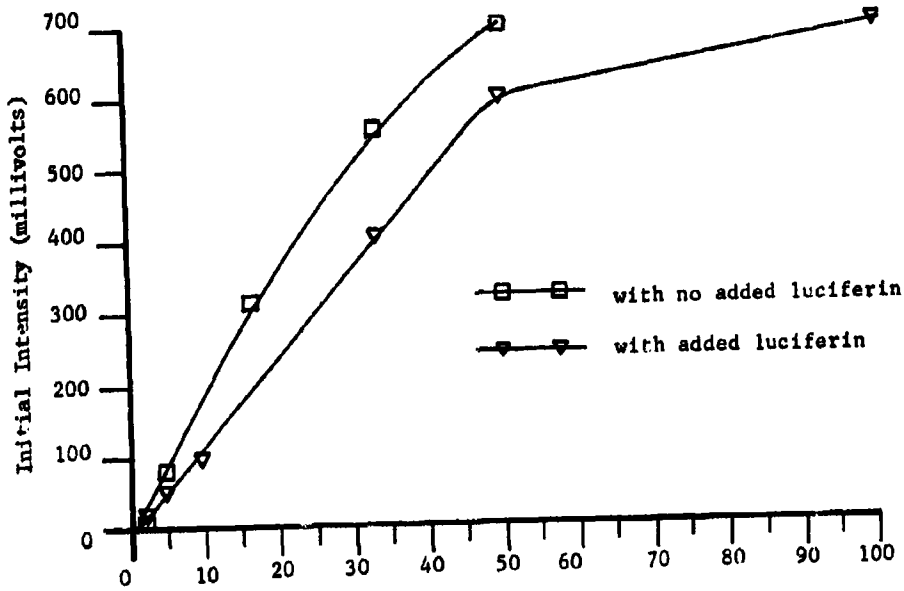
RATE OF BIOLUMINESCENT REACTION AS
FUNCTION OF EXTRACT DILUTION



Standard ATP prepared to 10^{-1} gamma/0.01 ml, enzyme extract prepared to volumes described. Reaction mixture - 0.2 ml enzyme extract and 0.01 ml ATP. Liquid phase conditions

Figure 9

INITIAL INTENSITY OF BIOLUMINESCENT REACTION AS FUNCTION OF EXTRACT DILUTION



Standard ATP prepared to 10^{-1} gamma/0.01 ml, enzyme extract prepared to volumes described. Reaction mixture - 0.2 ml enzyme extract and 0.01 ml ATP. Liquid phase conditions

concentration. Thus, there is at present, no satisfactory explanation for the dilution effect or the inherent light. Figures 8 and 9 show also the limit of dilution of the lyophilized enzyme extract for the measurement of ATP at concentrations up to 5×10^{-2} gamma. This is approximately 50 mg in five ml of solution.

IV. EXTRACTION OF ATP FROM MICROORGANISMS

One of the most critical factors to be dealt with during the course of these studies is a means by which the ATP within microbial cells can be quantitatively and rapidly made available for reaction with luciferase. The procedure need not accomplish extraction per se if the cells are sufficiently disrupted to allow free access to luciferin and luciferase. The procedure should also result in minimal destruction of ATP.

The microorganisms investigated, with the exception of a spore suspension, were 24 hour broth cultures which were washed once with equal volumes of ATP-free broth, and then resuspended in the same volumes of ATP-free broth. Aliquots of the resulting cell suspensions or dilutions prepared with corresponding sterile broth were employed directly in all tests. Difco Sabouraud broth was employed in the culturing and preparation of washed cell suspensions of Saccharomyces cerevisiae, while Difco Nutrient broth was used for Escherichia coli and Serratia marcescens. The Bacillus subtilis var. globigii spore suspension was prepared with distilled water.

The firefly extract was prepared daily by reconstituting 50 mg of firefly lantern extract with five ml of deionized water and centrifuging for ten minutes at 1700 G. The supernatant was then immediately cooled in an ice bath. Two-tenths ml aliquots

contained in a five mm cuvette were employed in each determination. One-tenth ml aliquots of the microbial ATP preparations were injected into the extract via syringe.

The relative efficiency of other procedures was compared with the hot water extraction procedure.

A. Perchloric Acid Extraction

Perchloric acid (5%) has been employed as a standard reagent for extracting ATP from tissues.[6]

Preliminary investigations of this extraction procedure were undertaken with three organisms: Escherichia coli, Saccharomyces cerevisiae, and a Bacillus subtilis spore suspension.

The responses obtained from suspensions of Escherichia coli and Saccharomyces cerevisiae are presented in Table IV. Using KOH for neutralization, perchloric acid extraction appears to be somewhat more efficient than hot water extraction. The Bacillus subtilis spore suspension failed to respond when tested under the conditions described. In the case of spores, the question of whether the extraction technique is inadequate, or whether the level of ATP during this life phase is insufficient for detection is unresolved. It may become necessary to induce sporulation prior to ATP assay if other measures fail. An interesting observation during these experiments, which may have important ramifications, was that NaOH when used for neutralization of perchloric acid causes a drastic decrease in the ATP response. Studies are contemplated in which it will be determined whether the site of inhibition is on the ATP or the enzyme system.

B. Acetone Treatment of Cells

Acetone has been employed for the disruption of a wide variety of cells. It seemed feasible to examine this procedure as a possible method of liberating microbial ATP for this system. A preliminary

Table IV

PERCHLORIC ACID EXTRACTION COMPARED TO HOT WATER EXTRACTION

Organism	Extraction Time (min)	Peak Deflection (mv) Hot Water Extr.	Peak Deflection (mv) $HClO_4$ Extr.
A.			
E. coli	0.5	480	640
E. coli	1.0	-	640
E. coli	1.0	-	600
S. cerevisiae	1.0	1,300	1,800
S. cerevisiae	2.0	1,400	1,600
B.			
S. cerevisiae	1.0	2,200	50
S. cerevisiae	2.0	1,000	50
S. cerevisiae	3.0	1,400	0

A. pH adjusted with KOH

B. pH adjusted with NaOH

\- Determinations not made

One tenth ml of 60% perchloric acid (Baker and Adamson, Reagent Grade) was added to one ml of cell suspension resulting in a final perchloric acid concentration of about 5.4%. At the conclusion of the desired extraction period the preparation was adjusted to pH 7.8 - 8.0

investigation employing acetone is described in Progress Report No. 1. The procedure had failed to elicit a response when evaluated visually under conditions of dark adaptation. However, it was decided to reinvestigate this system in greater detail utilizing instrumented rather than visual detection. Preliminary studies were conducted during the present quarter and indicated that at least a ten-to-one acetone to cell suspension volume ratio is required. Accordingly, one ml of washed cell suspension was added to ten ml of acetone. A one ml aliquot of the resultant suspension was taken to dryness under air and the residue reconstituted with deionized water to one ml.

A hot water extraction was also performed along with each acetone study. One ml of the same washed cell suspension was added to ten ml of deionized water. One ml was extracted in a boiling water bath for the same time as the corresponding acetone preparation. At the termination of the extraction period the preparation was rapidly chilled, brought to one ml with deionized water, and tested.

Representative responses with S. cerevisiae are presented in Table V.

The present data indicate an approximate three fold increase in efficiency over hot water extraction using Saccharomyces cerevisiae as the test organism. The same increase in efficiency has been observed with yeast cells taken directly from a stock slant into broth.

Higher ratios of acetone to cell suspension have not yet been explored. The response with standard ATP when treated with acetone and hot water are shown in Table VI.

The inverse effect of acetone on standard ATP solution as compared to microbial suspensions may be due to a number of factors, either acting alone or in combination. Acetone may not

Table V

RELATIVE EFFICIENCY OF HOT WATER EXTRACTION
AND
ACETONE TREATMENT OF SACCHAROMYCES CEREVISIAE*

Extraction Time (min)	Peak Deflection (mv) Hot Water Extr.	Acetone Treatment
1	70	260
1	50	120
5	60	460
5	150	360
5	70	200
5	60	140
5	100	250
10	70	250

* Cells were from a 24 hour Sabouraud broth culture

Table VI

EFFECT OF ACETONE ON STANDARD ATP

Treatment	Treatment Time (min)	Peak Deflection (mv)
None*	0	110
Hot water*	1	120
Acetone*	1	30
Hot water*	5	120
Acetone*	5	40
Hot water*	10	110
Acetone*	10	30
None**	0	700
Acetone**	1	520

* Treatment and assay were performed under the same conditions as those employed with microbial suspensions. One gamma of standard ATP in one ml of deionized water was treated in each case. The quantity of ATP used was 10^{-2} gamma per test.

** One ml aliquots of standard ATP were taken to dryness and again treated in the same manner employed with microbial suspensions. The quantity of ATP used was 10^{-1} gamma per test.

act as a true extracting agent, but may disrupt the cellular structure to the extent that the water which is added in the final stage of the procedure functions as the extractant. Other factors such as the formation of an acetone-ATP complex and the degree of solubility of ATP in acetone may also be involved. The difference in response as shown by standard ATP and that present in microorganisms may be due to differences in the chemical state of ATP in the two systems.

The sensitivity of the acetone procedure in terms of actual cell numbers is presented in Table VII. In this case, two types of organisms were tested: Saccharomyces cerevisiae and Serratia marcescens. In the case of Saccharomyces cerevisiae there appears to be no difficulty in detecting approximately 4,000 cells (Figures 10, 11). A greater number of Serratia marcescens cells are required at this stage of the development.

If initial deflection is used as a criterion of response magnitude, the response depicted in Figure 10 is approximately twice that of Figure 11. The response depicted in Figure 11 is distinct from the spurious responses previously reported with water and sterile broth. The latter responses consist of deflections with no area.

On the basis of this examination of a Serratia marcescens cell suspension, the acetone procedure again appears to be more efficient than hot water extraction. Further investigation is required to determine the sensitivity of the method in terms of actual cell numbers.

For the first time, algae have been studied with regard to both the concentration and ease of extraction of ATP within them. By extraction with 70 percent methanol, it has been possible to obtain a significant ATP response (250 mv) from 50,000 Chlorella pyrenoidosa cells.

Table VII

SENSITIVITY OF THE ACETONE PROCEDURE

Cell Treatment	Treatment Time (min)	No. of Cells Per Test	Peak Deflection (mv)
Saccharomyces cerevisiae			
None	0	37,000	0
Hot water	1	37,000	70
Acetone	1	37,000	260
None	0	38,000	0
Hot water	1	38,000	50
Acetone	1	38,000	120
Hot water	1	3,800	10
Acetone	1	3,800	18
Serratia marcescens			
None	0	1,310,000	0
Hot water	1	1,310,000	10
Acetone	1	1,310,000	50
Hot water	5	1,310,000	10
Acetone	5	1,310,000	50

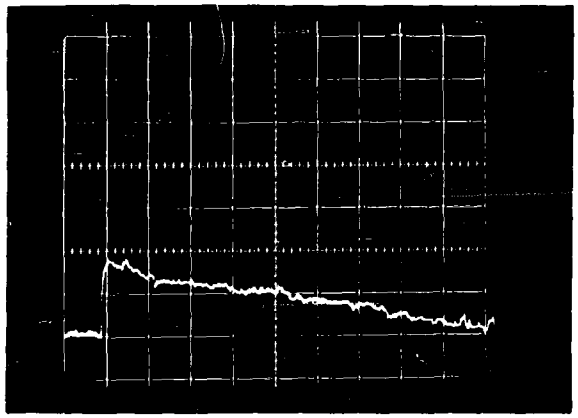


Figure 10. Thirty-eight hundred S. cerevisiae cells - acetone extraction. Oscilloscope sensitivity - 10 mv/cm

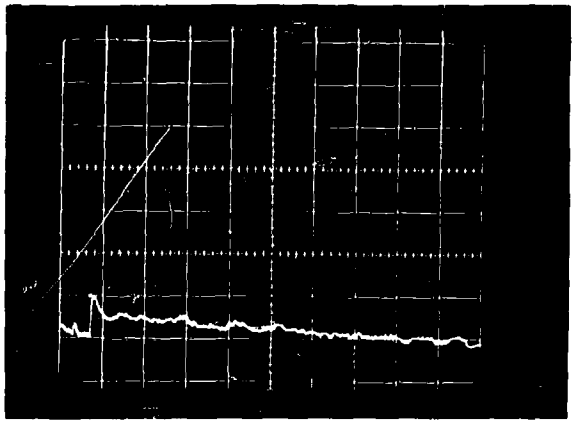


Figure 11. Thirty-eight hundred S. cerevisiae cells - hot water extraction. Oscilloscope sensitivity 10 mv/cm

Preliminary investigations have been undertaken with propylene oxide and dimethyl sulfoxide. Although the present data preclude definitive conclusions, further investigations of these reagents as extractants appear warranted.

The decrease in overall sensitivity which is observed here and elsewhere in this report with standard ATP is a reflection of a recent malfunctioning of the photomultiplier tube.

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