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Several Methods for Concentrating Bacteria in Fluid Samples

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16. Abstract

The sensitivities of the firefly luciferase - ATP flow system and luminol flow system have been established as 3 x 10^5 E. coli per milliliter and 1 x 10^4 E. coli per milliliter respectively. In order to achieve the detection limit of 1000 bacteria per milliliter previously established, a method of concentrating microorganisms is necessary. A Sartorius Membranfilter system is currently being investigated for concentrating bacteria. Catalase in 50% ethanol has been found to be a stable luminol standard and can be used up to 24 hours with only a 10% loss of activity. The luminol reagent is also stable over a 24 hour period. A method of preparing relatively inexpensive luciferase from dessicated firefly tails has been developed.

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INTRODUCTION

Quarterly Report #2 summarized the final optimal conditions for the firefly luciferase - ATP and luminol flow systems for detecting bacteria in wastewater effluent. The sensitivities of the ATP system and luminol system have been established as 3×10^5 bacteria per milliliter and 1×10^4 bacteria per milliliter respectively. In order to achieve the detection limit of 1000 bacteria per milliliter previously established, a method of concentrating microorganisms is necessary. Several techniques and apparatus have been studied with limited success. The Sartorius Membranfilter is currently being investigated for concentrating bacteria.

It is necessary that a standard be routinely used to check the activity of the luminol reagent for the luminol flow system. An aqueous catalase standard has previously been used; however, the solution is not very stable. Catalase in 50% ethanol is a stable luminol standard and can be used up to 24 hours with only a 10% loss of activity. The luminol reagent is also stable for 24 hours.

Experiments indicate that oxidized cytochrome-C is the species which reacts with luminol. Catalase, another luminol reactive porphyrin is also known to contain iron in the oxidized state. Since inorganic compounds react with luminol when they are in the reduced state (Fe^{2+}, Cl^-), a different reaction mechanism may be operating in the case of the oxidized iron porphyrins.

A method of preparing relatively inexpensive luciferase from dessicated firefly tails has been developed. This procedure results in an enzyme suitable for use with the firefly luciferase - ATP flow with about the same activity as the commercially available DuPont enzyme at one-tenth the cost.

I. Concentration of Bacteria Sample

The Sartorius Membranfilter described in Appendix A is currently being evaluated on its efficiency for concentrating a bacterial sample. The principle of the Sartorius ultrafiltration system is that of "tangential flow". Tangential flow should prevent bacteria as well as other particles from clogging and sticking to the filter surface by sweeping them across the filter surface.

The Sartorius Membranfilter has been tested in several configurations to determine the optimal operating conditions. 0.2 μ Nucleopore poly-carbonate filters have been used for most of these studies. Figure 1 is a schematic diagram of the recommended set-up for the Sartorius filtration system with tangential flow. Figure 2 is a simple straight through filtration system which requires a backwash step to remove the bacteria. Figure 3 illustrates the use of the tangential flow principle with a subsequent backwash step for removing the bacteria.

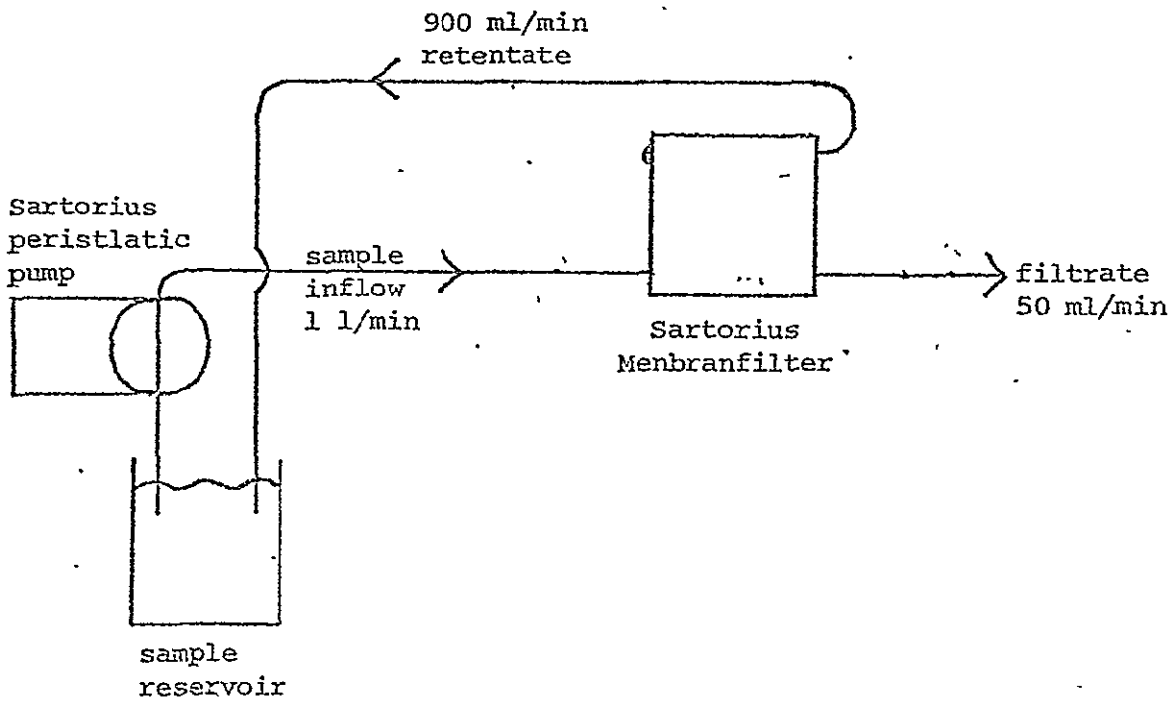


FIGURE 1. Schematic of Sartorius Membranfilter system using tangential flow.

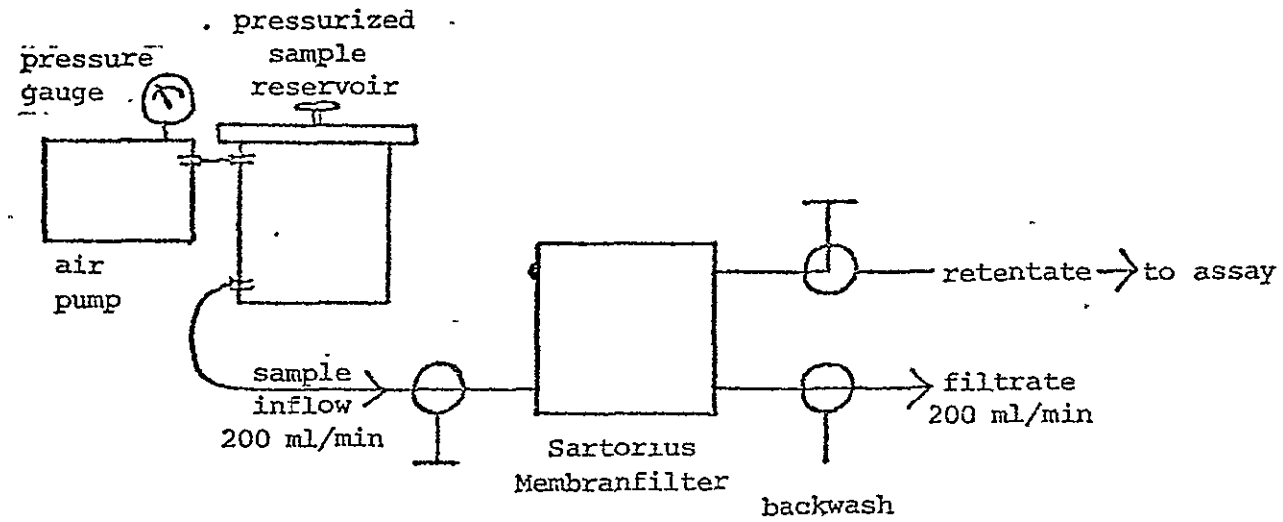


FIGURE 2. Schematic of Sartorius Membranfilter system using direct filtration and backwash. System is diagrammed in the concentrating mode; valves should be reversed for backwashing provided by a tap source of sterile, deionized water.

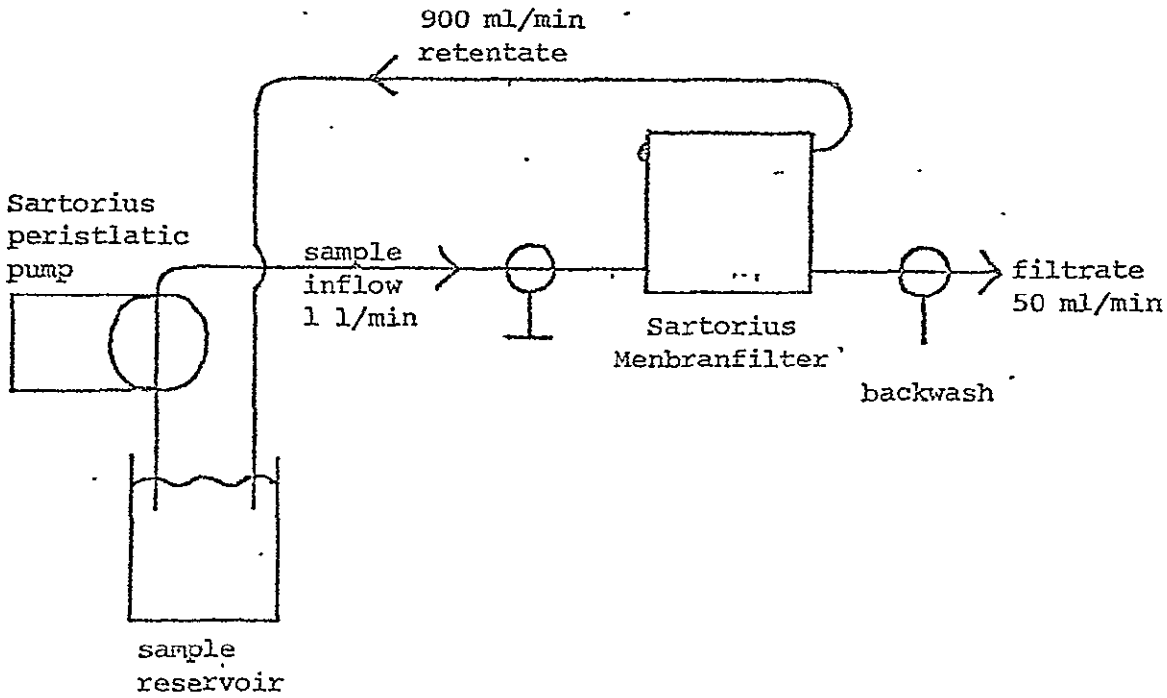


FIGURE 3. Schematic of Sartorius Membranfilter system using tangential flow and backwash. System is diagrammed in concentrating mode; valves should be reversed for backwashing provided by a tap source of sterile, deionized water.

The Sartorius Membranfilter system used as recommended with tangential flow has not proved to be an acceptable method of concentration. With each pass of the retentate over the filter surface bacteria are trapped and lost. While the bacteria may not be attached very tightly to the filter surface they nevertheless can not be retrieved and collected in the retentate as suggested by the manufacturer. 0.1% rhozyme and 0.001% Triton X-100 used as aids to prevent bacteria from sticking have proven unsuccessful.

The Sartorius Membranfilter has been used as a straight through filter with subsequent backwashing with limited success. A bacteria sample is simply pushed through the filter with 10 psi pressure to collect the bacteria on the poly-carbonate filter. The filter is then backwashed with portions of water to remove the bacteria from the filter surface. 60% recovery has been achieved with a 10 fold increase in concentration using 0.001% Triton X-100.

The third method for using the Sartorius Membranfilter system involves a combination of the above two procedures. The bacteria are "gently" concentrated on the filter using the tangential flow principle and once concentrated removed by backwashing. Preliminary results indicate that up to 90% recovery can be achieved with a 10 fold concentration using this method with 0.1% rhozyme or 0.001% Triton X-100.

Future work with filtration will include further development of the Sartorius Membranfilter system with the combination tangential flow and backwash operations. While 90% recovery is acceptable the consistency of those results must be proven. Improvements in concentration factor are also necessary. The ultimate goal of the system is to achieve 100% recovery with 1000 fold concentration.

II. Stable catalase standard for luminol reaction.

The catalase standard previously used for measuring the activity of the luminol reagent mixture consisted of freshly prepared catalase in deionized water. It has since been found that the catalase standard is not very stable once prepared and could lose as much as 50% activity within a 2 hour period. Some stabilization of the catalase standard can be achieved by addition of ethanol[1]/to the aqueous solution. 50% ethanol appears to be the optimal concentration for stabilization of the catalase standard with good luminol reactivity. Table 1 summarizes the results of the stability experiments.

TABLE 1. Percent loss of catalase activity after 19 hours.

1×10^{-5}M catalase in

H_2O	50% ethanol	100% ethanol
43%	10%	8%

As a result of this increased stability of catalase, the catalase standard in 50% ethanol should be reliable for up to 24 hours after preparation. It has also been found that the luminol reagent mixture is stable for over 24 hours and need only to be prepared once a day.

1/ J. E. Falk, Porphyrins and Metalloporphyrins, Elsevier Publishing Co., New York, 1964, p. 19.

III. Valence state of luminol reactants.

It was previously believed that the porphyrin reactants in the luminol reaction were in the (II) valence state. Recent studies with cytochrome-C indicate this hypothesis may be incorrect. Samples of cytochrome-C have been reduced with ascorbic acid with a corresponding reduction of signal from luminol indicating the reactive species is probably in the (III) state. Catalase, another luminol reactive porphyrin is known to contain iron in the III state. If it is true that inorganic iron in the (II) state is the luminol reactive form, then a different mechanism may be operating in the case of iron porphyrins (III).

IV. Luciferase Preparation.

DuPont purified luciferin-luciferase has been utilized in the firefly luciferase-ATP flow system. In an attempt to defray operating expenses a less expensive supply of luciferase was sought. Luciferase has been extracted from dessicated firefly lanterns. With appropriate purification steps and addition of synthetic luciferin the resulting enzyme costs only one-tenth that of DuPont with better activity.

The procedure used by Margaret A. McGarry and Emmett W. Chappelle for obtaining highly purified, less expensive luciferase involves preparation from dessicated firefly tails as outlined below:

A. Preparation of acetone powder

1. Grind firefly lanterns (Sigma Chemical Company, Worthington, Calbiochem - $20 per gram) and small amount of sand with mortar and pestle, keeping the mixture cold with liquid nitrogen or acetone-dry ice mixture.

2. Add cold acetone (4°C) (at least 100 ml per 5 grams of tails) and wait 10 minutes to dissolve lipids.

3. Filter solution through a Buchner funnel and wash with cold acetone.

4. The powder should be completely dried and stored at -20°C.

B. Preparation of crude luciferase-luciferin extract

1. Add 10 ml cold 0.05M Tris, pH 7.75 with 1×10^{-3}M Clelands reagent per gram of acetone powder. Mix gently at 10°C for 30 minutes.

2. Centrifuge the solution at 10,000 RCFXG for 10 minutes and collect the supernate, discard precipitate.

C. Luciferase purification

1. Bring above supernate to 30% $(NH_4)_2SO_4$ at room temperature and discard precipate.

2. Then, bring solution to 70% $(NH_4)_2SO_4$ and collect precipitate-luciferase.

3. Wash precipitate in 70% $(NH_4)_2SO_4$ pH 7.75.

4. Dissolve washed pellet in Tris buffer: 0.05M, pH 7.75, 1×10^{-3}M Clelands' reagent, 1×10^{-2}M $MgSO_4$ (minimum volume 2.5 ml per gram acetone powder). Centrifuge to clarify. Apply enzyme to Sephadex G-200 column and elute with same Tris buffer. Assay fractions by mixing small portion 1:1 with luciferin in Tris buffer.

5. Pool fractions with high activity and low inherent light. (1 gram acetone powder should produce about 20 ml enzyme with activity equal to DuPont product). Dilute pool with Tris buffer to desired activity level. Add 0.1 mg luciferin per ml diluted fraction pool. Aliquot and lyophilize enzyme. Store dessicated at -20°C.

6. Rehydrate in H_2O for 0.05M Tris pH 7.75 or 0.20M Tris pH 8.2 for 0.25M Tris pH 8.2.

V. ASM and AWWA Presentations.

In December, 1975, a paper entitled "Chemiluminescent vs. Bioluminescent Methods for Detection of Bacteria in Wastewater Effluent" was presented at the 1975 Water Quality Technology Conference Workshop. The topics covered in the workshop included New Techniques in Microbiological Instrumentation. A copy of the presentation can be found in Appendix B. Another paper was presented at the American Society for Microbiology Convention in May 1976. This paper can be found in Appendix C.

Ultrafiltration System

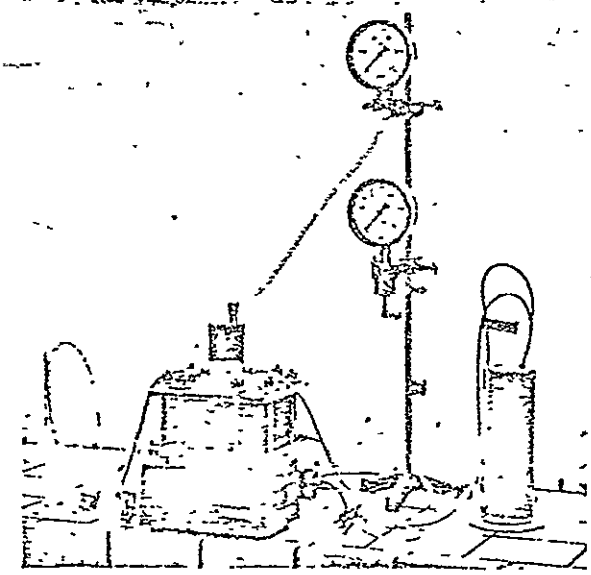
Efficient ultrafiltration of high molecular weight substances such as proteins, enzymes, and viruses has been extremely difficult, mainly because these substances effectively block the pores of an ultrafilter by forming a film of increasing thickness on the filter surface.

The use of laboratory shakers, vibrators and magnetic stirrers has up to now provided relief to a limited degree by agitating the proteins away from the filter surface. These traditional methods have now become obsolete with the introduction of a "tangential flow" principle suggested by Strohmaier in 1964

"Tangential flow" prevents blocking of the pores by sweeping the residues off the filter surface and thus opens the way to efficient and economical concentration, separation and purification of high molecular weight substances, for large or small volumes

The new Sartorius ultrafiltration system is based on Strohmaier's "tangential flow" principle

It is autoclavable with filters in situ and can be used with filtration areas of 170 cm² to 2550 cm², in steps of 170 cm².



Description

The Sartorius ultrafiltration system consists of the following parts:

Cat. No SM 16525 ultrafiltration cell and pressure valve
Cat. No SM 16896 membrane pump, 220 V, or
Cat. No SM 16915 membrane pump, 110 V.

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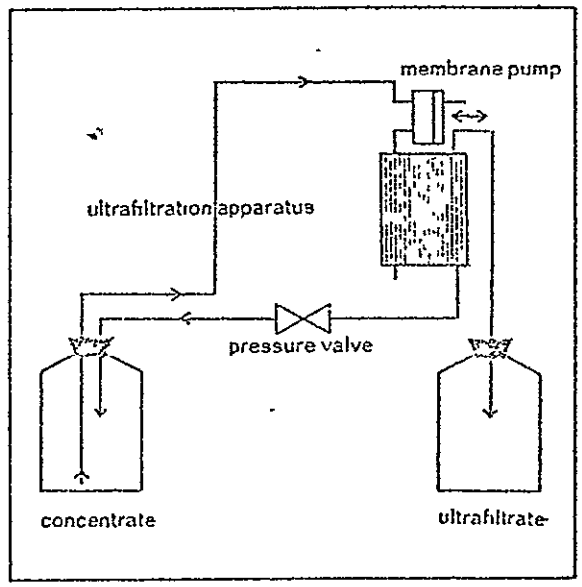
Ultrafiltration Apparatus
Cat. No. SM 16525

Technical data	
Plates made of	polycarbonate. Base and cover plate as well as the locking bolts and nuts are made of stainless steel
Size of filters	160×160 mm
Filtration area	170 cm² per plate
Max. filtration area	2550 cm² using all 15 plates
Max. pressure for the ultrafiltration apparatus	~10 bar (140 psi)
Connection	connecting nipple with securing nut for tubing of 4 mm ID, 6 mm OD
Weight	7.7 kg
Dimensions	190×190×140 mm
Pressure valve made of	stainless steel, membrane made of neoprene
Max. permissible pressure	~4 bar (60 psi)
Adjusted pressure on delivery	~2 bar (20 psi) (for membrane pump SM 16896 or SM 16915)

Any number of the filter-supporting plates of the ultrafiltration cell, up to the maximum of 15, can be used for a particular experiment. Each plate is separated by an ultrafilter. The plates have V-shaped grooves 0.7 mm wide running parallel to each other, which ensures a completely even flow over the filter. The liquid to be concentrated is first pumped into the distributing channel of the inlet plate. From there it flows into the grooves and across the membrane into the collecting channel and is then fed back into the storage container. The ultrafiltrate which has passed through the memorane flows through the channel on the outlet plate into the receiver vessel.

The number of plates chosen depends on the volume of the liquid to be filtered

Flow during the filtration of high molecular substances using the ultrafiltration system



Membrane Pump

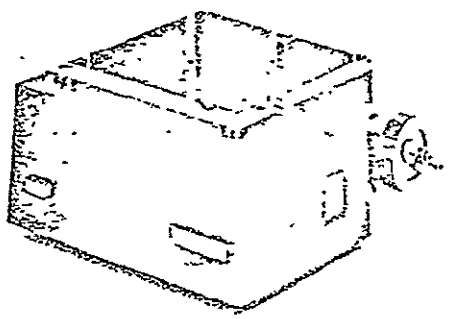
Cat. No. SM 168 96 and SM 169 15

Technical data

Membrane	Neoprene
Output (water)	500 ml/min
Max. permissible pressure	~2 bar (30 psi)
Weight	4.6 kg
Dimensions	200×200×100 mm
Connection	Connecting nipples (outlet with securing nut) for tubing of 4 mm ID, 6 mm OD

Cat. No. SM 168 96 Line voltage 220 V, 50 Hz
Cat. No SM 169 15 Line voltage 110 V, 60 Hz

The membrane pump SM 168 96 is designed specially for the Sartorius ultrafiltration apparatus. The output of 500 ml/min is sufficient for the tangential flow over the membrane. A pressure of 2 bar builds up when the membrane pump is used in combination with the pressure valve The suction head of the membrane pump can be taken off and is autoclavable
When concentrating solutions with a high solid content or when two ore more ultrafiltration apparatuses are connected parallel to each other the output must be increased (0 5 to 0 8 l/min per instrument).



A higher flow rate (proportional to the pressure difference) can be achieved by evacuating the receiver vessel Use of the laboratory pump Cat No. SM 166 12 is recommended

Tubing

Cat. No SM 166 52
PVC (Standard) 4 mm ID, 6 mm OD, 2 m long, not autoclavable

Cat No SM 166 54
Polyamide (optional) 4 mm ID, 6 mm OD, 2 m long, autoclavable

Application

The Sartorius ultrafiltration system is suitable for the concentration and separation of colloids and biopolymeric substances such as proteins, viruses and enzymes in pharmacy, microbiology, medicine and biochemistry; furthermore for other polymeric substances, with due regard to the chemical resistance of the polycarbonate.
The instrument has also been used successfully for the ultra cleaning of these substances by dialysis.

Sterilization

The complete system, with ultrafilters SM 121 33, 34 or 36 in position, can be autoclaved, together with the removable suction head of the membrane pump (the remainder of the membrane pump is not autoclavable) Thus all parts of the system which come in contact with the solution may be sterilized.

Procedure	Fill the entire system with distilled water prior to autoclaving
Autoclave time	45 min
Autoclave temperature	121 °C
Autoclave pressure	1 bar (15 psi)

The ultrafiltration apparatus, without filters, can also be sterilized with steam The filters must then be sterilized chemically by immersing them in a 3 % formalin solution.

For molecular weight cut-off of Sartorius ultrafilters see Filter Catalog SM 200.

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APPENDIX B

ABSTRACT

Chemiluminescent vs. Bioluminescent Methods for Detection of Bacteria in Wastewater Effluent

To be presented at the 1975 Water Quality Technology Conference Workshop - New Techniques in Microbiological Instrumentation, by Grace Lee Picciolo, Emmett W. Chappelle, Richard R. Thomas.

A chemiluminescent system and bioluminescent system are presently under development for the rapid detection of bacteria in water samples. The chemiluminescent system involves the reaction between luminol (5-amino-2, 3-dihydro-1, 4 phthalazinedione) and bacterial porphyrins while the bioluminescent system is a result of the reaction between firefly luciferase and ATP. By measuring the amount of light emitted from either reaction the bacterial concentration can be determined. Both systems display good linearity and a sensitivity limit of approximately 10^5 E. coli per milliliter of seeded wastewater. The bioluminescent system is a more specific reaction, however, the higher cost of reagents limit its use to discreet sampling. The lower cost of reagents for the chemiluminescent system make that assay more suited for continuous monitoring of bacteria levels in wastewater. The combination of both systems in one package allows for continuous monitoring of bacteria levels in wastewater with provisions for periodic back-up tests specific for bacteria.

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CHEMILUMINESCENT VS. BIOLUMINESCENT METHODS FOR DETECTION OF BACTERIA IN WATER SAMPLES

INTRODUCTION

Two rapid methods for the detection of total bacteria in water samples are being developed by the laboratory at Goddard, Greenbelt, Maryland. Both methods employ the principle of luminescence. There are three primary sources of light: thermal, chemical, and biological (slide 1). Thermal light is the result of radiation emitted from molecules which have been excited by high temperatures. Chemiluminescence, commonly called cold light, is light emitted from molecules which have been excited by a chemical reaction and bioluminescence is merely chemiluminescence which is taking place in a biological system.

Luminescence under controlled conditions can be used as an analytical tool (slide 2). The characteristics of a luminescent system include the relative ease of light measurements through appropriate instrumentation, the potential for automation and flexibility - there exist a number of reactions providing a variety of assays. The most important feature of a luminescent system is that it provides immediate results. The systems are sensitive, specific (within certain limitations) and reproducible.

A chemiluminescent system and bioluminescent system have been developed for the rapid detection of bacteria in water samples. The water samples we have been concerned with are wastewater effluent

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samples however this system should be able to be used for potable water as well. The chemiluminescent system involves the reaction between luminol and bacterial porphyrins while the bioluminescent system is a result of the reaction between firefly luciferase and ATP. By measuring the amount of light emitted from either reaction the bacterial concentration can be determined (slide 3).

Bioluminescence is light production by living organisms. This is the phenomenon where the exitation of the light emitter to the excited state is mediated by an enzyme. This is seen in many members of the plant and animal kingdoms with practically every group of lower organisms containing some members that have the ability to produce light. The most common organism which displays this phenomenon is the firefly (slide 4).

ATP (slide 5) is a biochemical compound that is unique to all living organisms, its purpose in the cell to store energy for use in all other cellular reactions. By making use of the light producing substances from fireflies, which are commercially available from many companies, a sensitive assay for ATP can be developed (slide 6). Under ideal reaction conditions, the amount of light emitted from the reaction is proportional to the amount of ATP. If the amount of ATP per cell is known, then the number of cells present can be determined from the light emitted.

The procedure for any ATP assay is relatively simple and quick. Apyrase is added to the bacterial sample. This apyrase destroys any non bacterial ATP which might interfere with the results. Nitric

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acid is then added to the sample to break open the bacterial cells and at the same time release the ATP for subsequent assay. The solution is then diluted in half with distilled water and the prepared sample is then injected into luciferase (firefly extract). Total time for sample preparation - 20 minutes.

Slide 7 shows the rate of the reaction when ATP is injected into the luciferase. The height of the curve is proportional to the amount of ATP in the sample. Slide 8 is a graph showing the relationship between the amount of purified ATP injected into the system and the corresponding light emission. Slide 9 shows actual bacterial samples with the amount of light produced from each. Measurements were made using three different light measuring instruments.

Since the amount of ATP is fairly constant per cell for many species of bacteria (slide 10), the light emitted from a sample should represent the total number of bacteria. Slide 11 shows how the light response varies with E. coli concentration in wastewater effluent. The curve is linear over a wide range of bacteria concentrations with a limit sensitivity of 5×10^5 bacteria/ml. Note the luminol curve, the system which I will now describe.

Luminol

The principle of the luminol chemiluminescence method for detecting bacteria is based on the reaction shown in slide 12. Irons porphyrins (complex molecules with an iron group) from

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bacteria act as a "catalyst" for the reaction of luminol and peroxide (later perborate) in the presence of sodium hydroxide. When all the reagents are present in excess, the amount of light emitted from the reaction (λmax. 425 nm) is proportional to the concentration of bacterial porphyrins. This number can then be related to the number of bacteria present in the sample.

The procedure for the luminol system is very quick and simple. The bacterial sample is directly pumped into an area where the luminol solution is mixed and then through a coiled glass tube which is adjacent to a photomultiplier tube. (Slide 10). The slide shows the luminol response to various concentrations of E. coli in wastewater effluent.

Instrumentation

Several photometers are commercially available with various price ranges (slide 13). The Aminco Chem-Glow Photometer is used in many of the studies in our lab. Slide 14 shows the photometer with an automatic injection system which has been used for the bioluminescent assay. Also pictured is the optional integrator which indicates the total light response from the system.

For the luminol work, a Buchler peristaltic pump was employed for the circulation of the sample and reagents. Shown is the flow head which allows for the light measurement for the flow system (slide 15).

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Discussion

Two luminescent systems have been presented for the rapid detection of bacteria in wastewater samples - the luminol chemiluminescent assay and the ATP luciferase assay. The characteristics of the ATP assay are:

1) specificity - the system is specific for ATP, the light response represents the total number of bacteria present in the sample;
2) sensitivity - the sensitivity limit is on the order of 1×10^5 bacteria per ml. The sensitivity can of course be improved by methods of concentration which I will explain in a minute;
3) the assay is rapid;
4) and has the potential for automation;
5) the system produces reliable and reproducible results.

The characteristics of the luminol system are similar except for #1 - specificity. In addition to iron porphyrins catalyzing the reaction, metallic ions can also produce a luminol response. The levels of iron in the water of course determine the magnitude of the problem.

Sensitivity

Let me go back to the matter of sensitivity. The sensitivity must necessarily be improved to detect the lower levels of bacteria found in potable water. Sensitivity can be improved by concentrating the number of bacteria in the sample. Two methods can be used:

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1) centrifugation and 2) filtration. Centrifugation has been used with success however this method eliminates much of the potential for an automated system. Filtration is the alternative to centrifugation. A potential problem exists in the fact that once bacteria are absorbed on to a filter, it is difficult to quantitatively remove them. A technique has been developed in which the active factor for each system can be selectively extracted, in the case of ATP, nitric acid will break open the cells on the filter and release the ATP. Sodium hydroxide and ethanol mixture will release the porphyrin for the luminol reaction. In each case the filtrate is assayed.

Another very real problem with filtration, especially in trying to filter wastewater effluent, involves the actual physical problem of trying to filter this liquid since it can contain much particulate matter. This may or may not be a problem for potable water.

Depending on the use of the assay there may be some drawbacks in the area of cost. The luciferase - ATP assay costs approximately $0.60 per injection. This most likely will limit the use of the assay if a continuous monitoring of bacteria is needed. If discreet periodic sampling is desired, the ATP system is quite adequate. The chemiluminescent system on the other hand costs less than one cent per assay. In this case cost should not restrict the continuous monitoring of bacteria levels.

We propose that the most efficient rapid bacteria detection system would incorporate both the chemiluminescent and the bio-

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luminescent systems. The chemiluminescent system would permit continuous monitoring of bacterial levels. A high light response from the luminol system would indicate the possibility of dangerous bacteria levels. The bioluminescent system would then be called upon to verify the presence of high levels of bacteria.

A very real potential exists for a system such as this. If indeed there is a real need for such a system in the realm of potable water such a system can and should be developed.

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1. THERMAL

2. CHEMICAL

3. BIOLOGICAL

SLIDE 1. THREE PRIMARY SOURCES OF LIGHT.

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SLIDE 2.

LUMINESCENCE AS AN ANALYTICAL TOOL

CHARACTERISTICS:

- LIGHT MEASUREMENT
  - AUTOMATABLE
  - FLEXIBLE - VARIETY OF ASSAYS
  - IMMEDIATE RESULTS
  - SENSITIVE
  - SPECIFIC
  - REPRODUCIBLE

SLIDE 5.

ADENOSINE TRIPHOSPHATE (ATP)

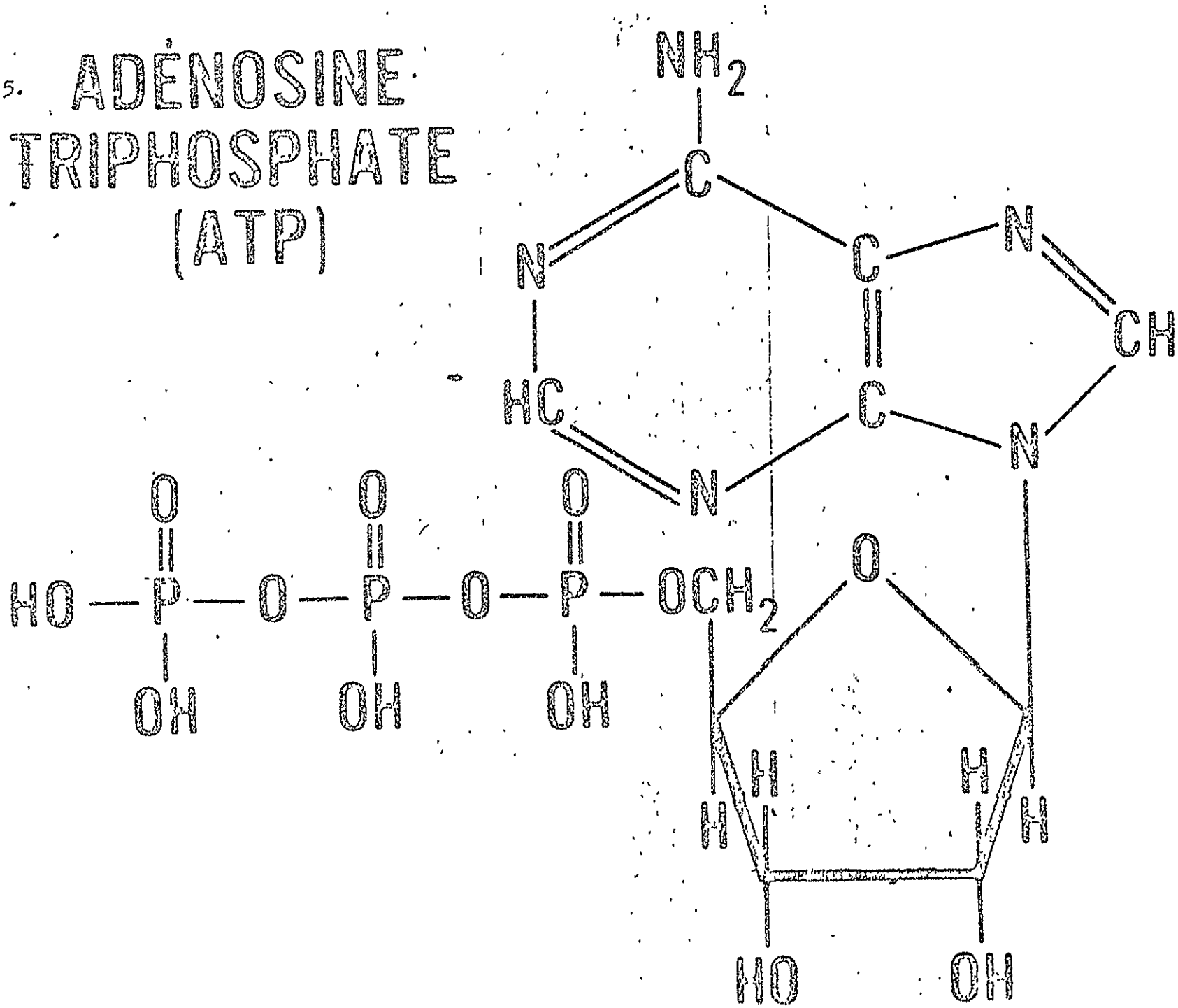

FIGURE 6.

FIREFLY LIGHT REACTION MECHANISM (PLANT, 1968)

$$E + LH_2 + ATP \underset{}{\overset{Mg^{++}}{\rightleftharpoons}} E \cdot LH_2 + AMP + PP$$

$$E \cdot LH_2 + AMP + O_2 \longrightarrow E + AMP + CO_2 + h\nu + T$$

WHERE:

E = FIREFLY LUCIFERASE

LH_2 = REDUCED LUCIFERIN

ATP = ADENOSINE TRIPHOSPHATE

AMP = ADENOSINE MONOPHOSPHATE

PP = PYROPHOSPHATE

T = THIAZOLINONE

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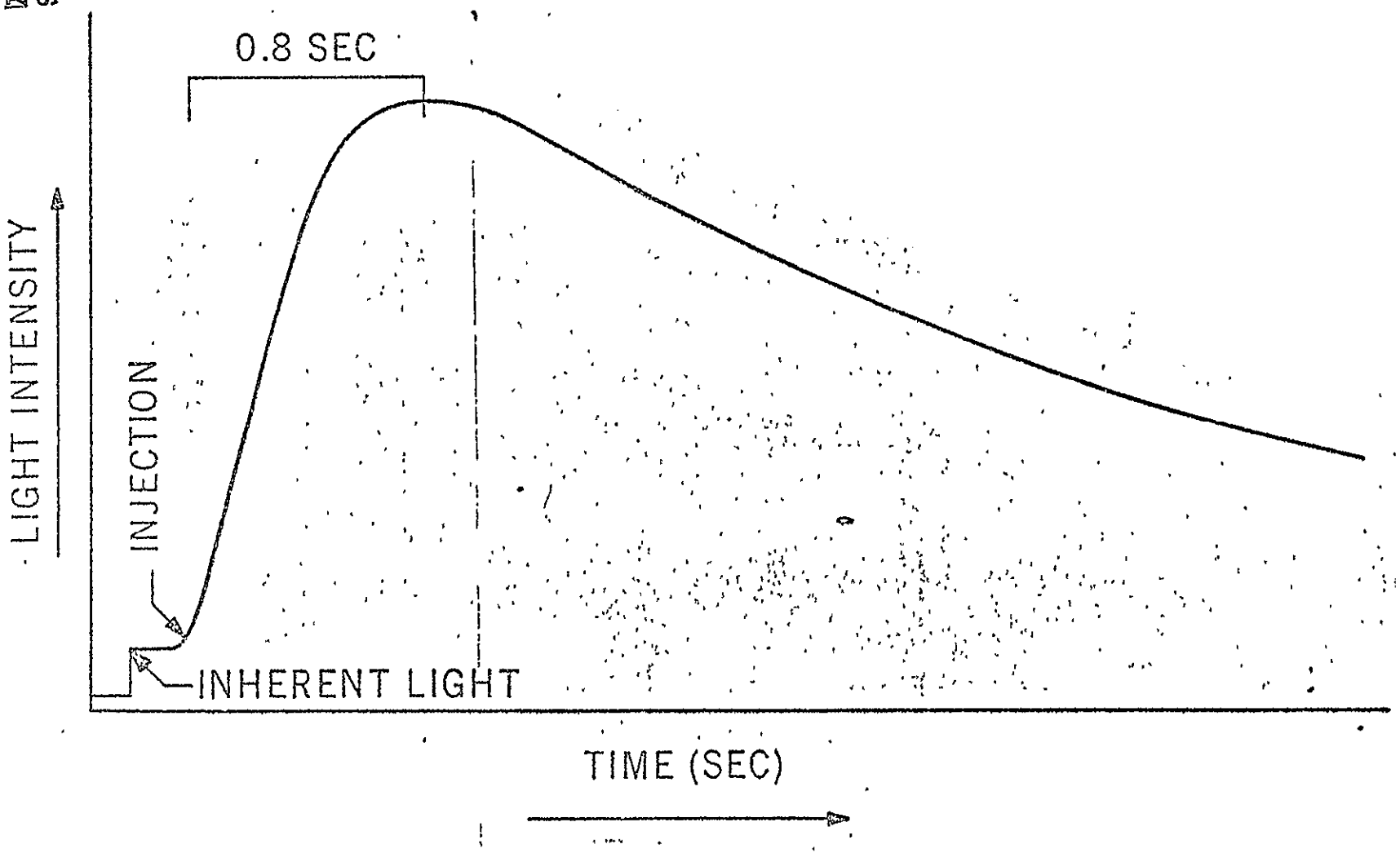
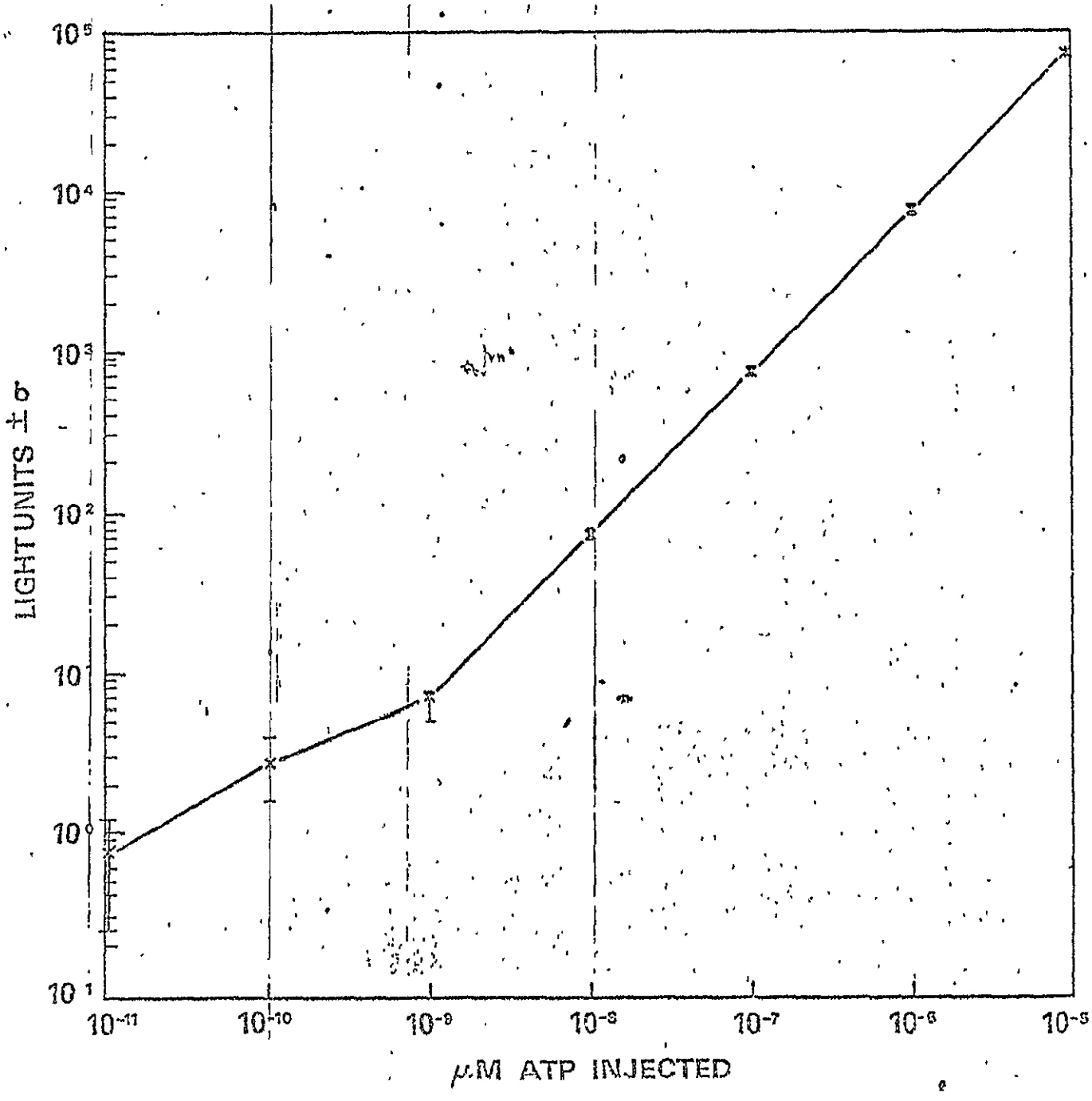


SLIDE 7. FIREFLY LUCIFERASE - ATP REACTION RATE.



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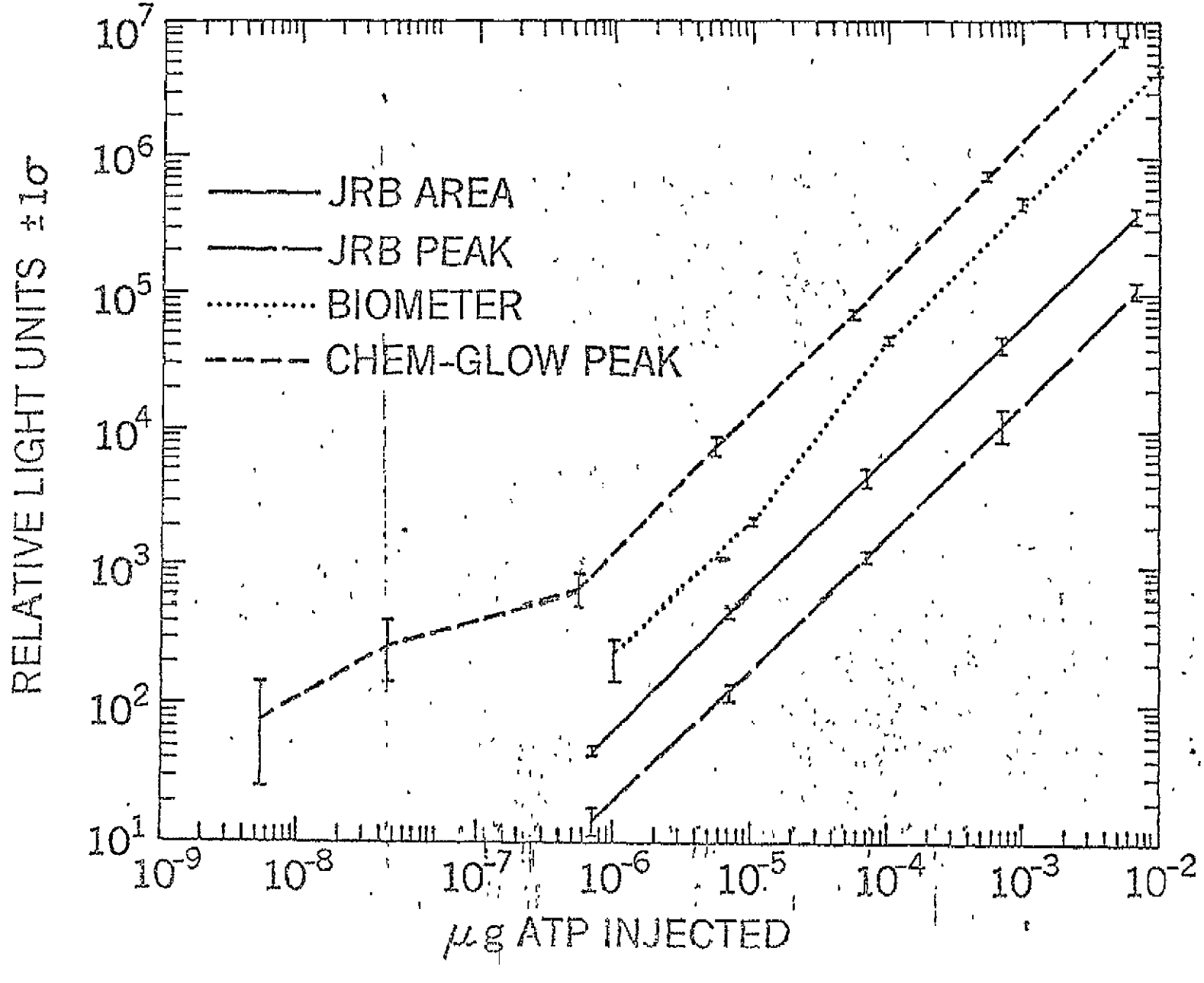
SLIDE 9.

ATP CONCENTRATION CURVE WITHOUT NITRIC ACID COMPARING THREE PHOTOMETERS



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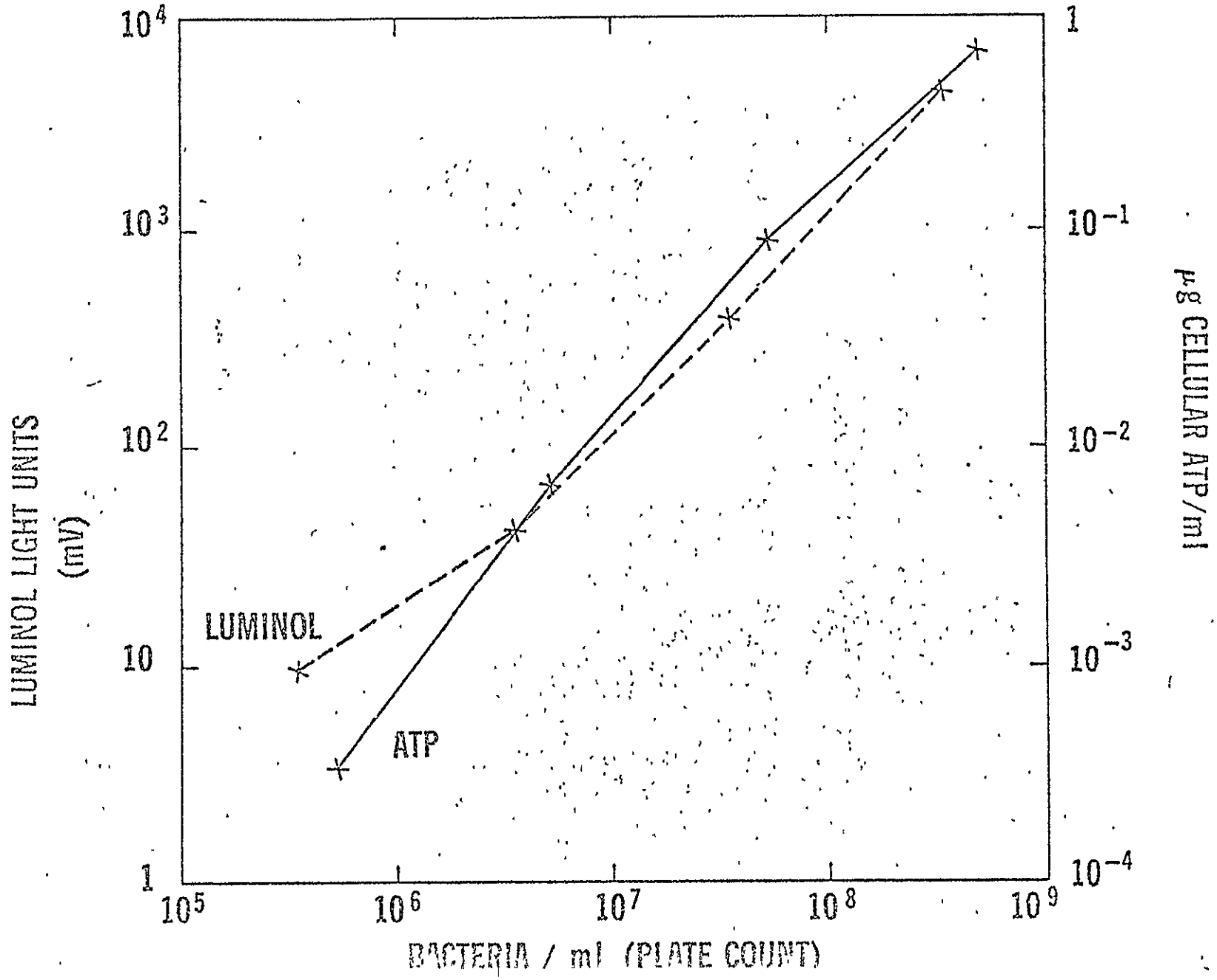
Species	μgm ATP per cell ($\times 10^{-10}$)
Escherichia coli	1.2
Streptococcus faecalis	1.1
Staphylococcus aureus	1.8
Staphylococcus epidermidis	8.2
Proteus mirabilis	4.3
Klebsiella	2.8
Pseudomonas	0.5
Proteus rettgeri	1.4
β streptococcus	1.5

SLIDE 10. ATP CONCENTRATION PER CELL FOR VARIOUS SPECIES OF BACTERIA.

SLIDE 11.

FIREFLY LUCIFERASE AND LUMINOL RESPONSE FROM E COLI SEEDED WASTEWATER EFFLUENT



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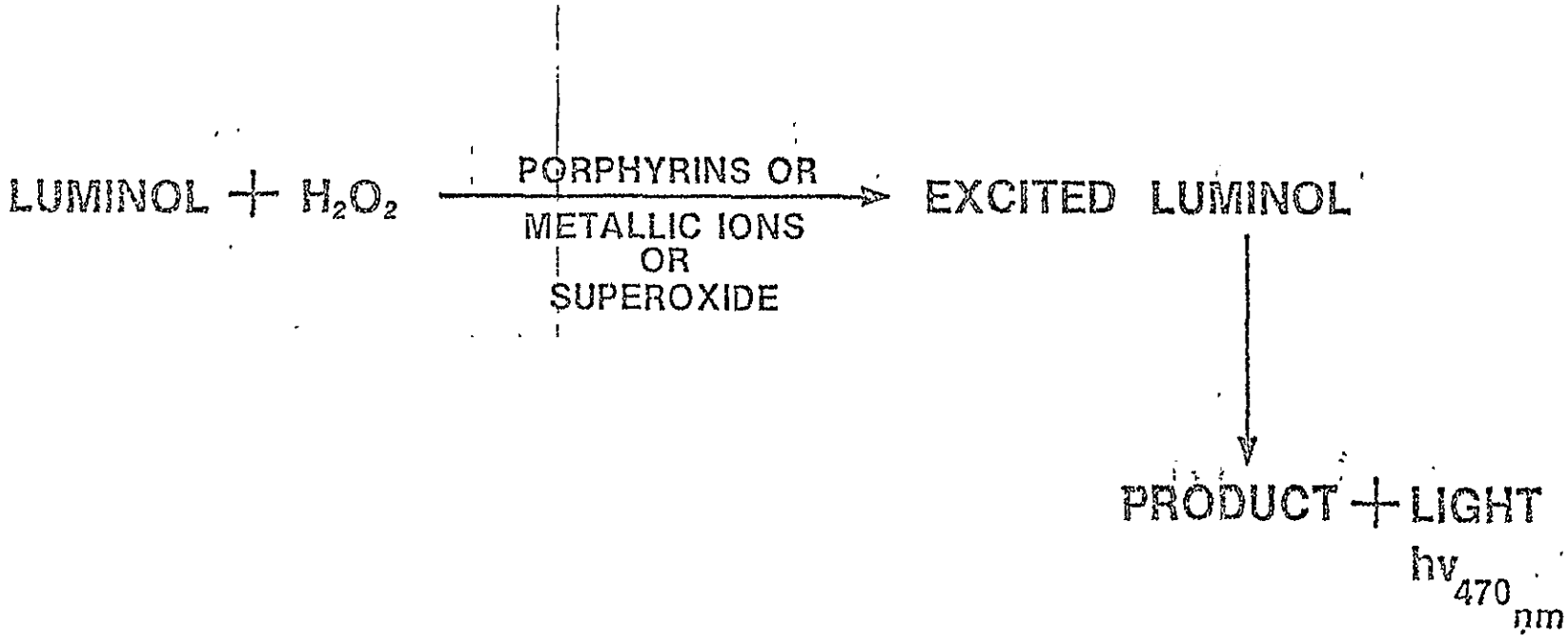
SLIDE 12.

LUMINOL LIGHT REACTION



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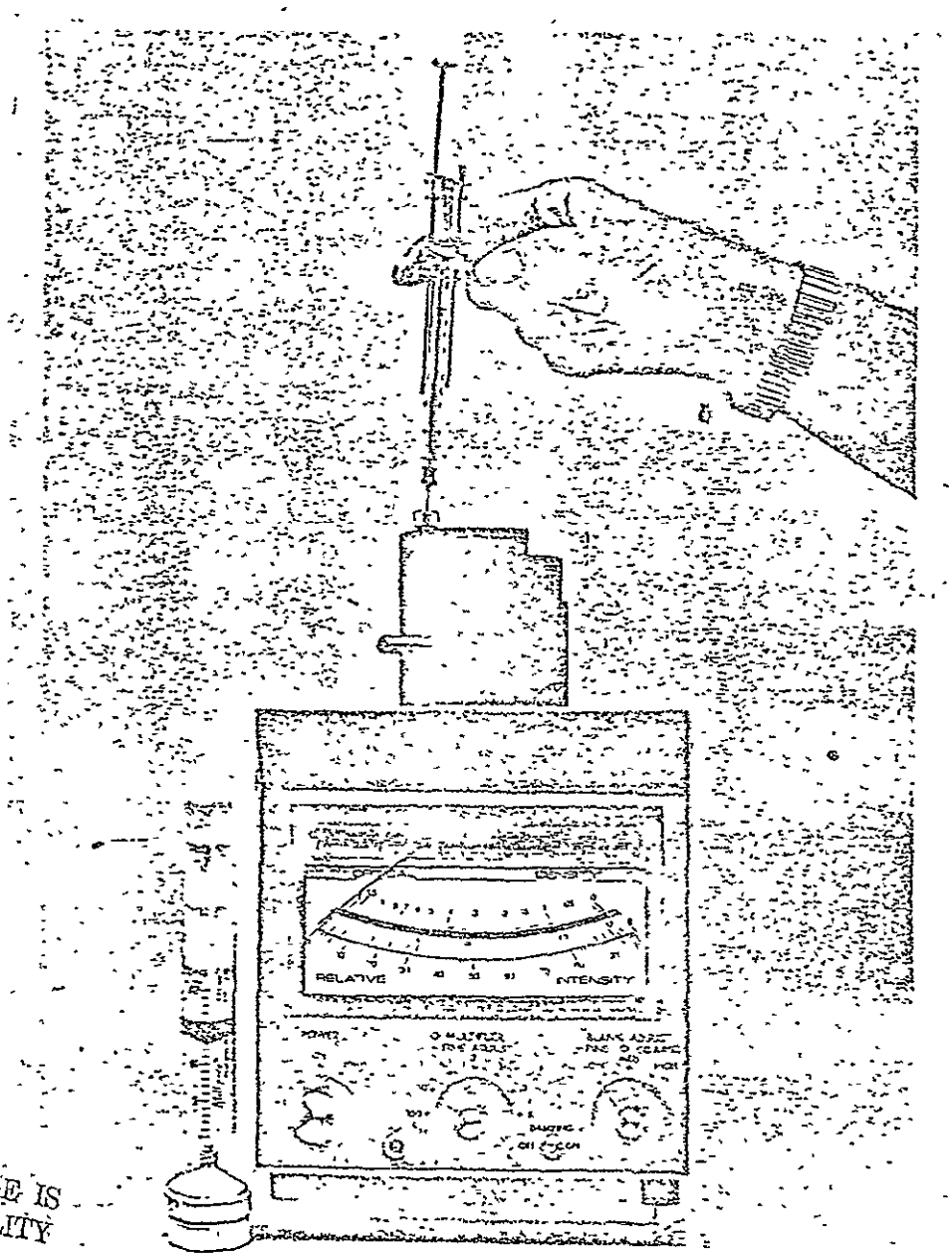
INSTRUMENTATION

COMPANY	PHOTOMETER	PRICE
AMINCO	CHEM GLOW	~ $ 800
	INTEGRATOR	~ $1,800
DUPONT	BIOMETER	~ $6,000
SAI (JRB)	ATP PHOTOMETER	~ $5,000

SLIDE 13: SEVERAL COMMERCIALLY AVAILABLE PHOTOMETERS.



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SLIDE 14. THE AMINCO CHEM-GLOW PHOTOMETER WITH AUTOMATIC INJECTION APPARATUS.

SLIDE 15. LUMINOL FLOW SYSTEM WITH FLOW HEAD ATTACHMENT.

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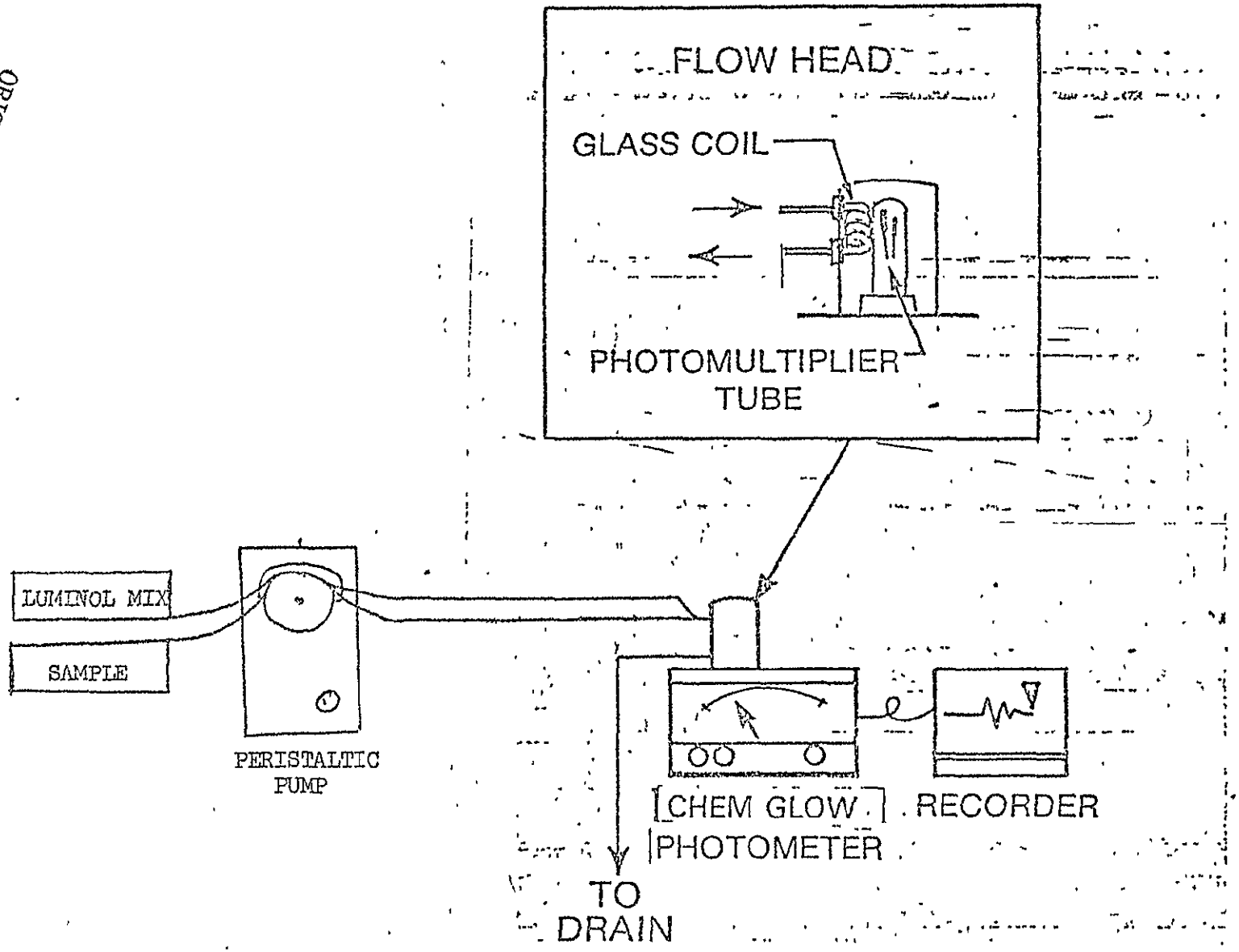

APPENDIX C

Presented at the American Society for Microbiology Convention,

Atlantic City, New Jersey May 1976

Chemiluminescent vs. Bioluminescent Methods for Detection of Bacteria in Wastewater Effluent.

R. R. THOMAS, ~~J. W. DEMING~~, D. A. HIBLEY, R. TAYLOR. Boeing Co., Houston, Tx., Hahnemann Med. Col. & Hosp., Philadelphia, Pa., NASA Johnson Space Flight Ctr., Houston, Tx.

A chmiluminescent system and a bioluminescent system are presently under development for the rapid detection of bacteria in water samples. The chemiluminescent system involves the reaction between luminol (5-amino-2, 3-dihydro-1,4 phthalazinedione) and bacterial porphyrins while the bioluminescent system is a result of the reaction between firefly luciferase and adenosine triphosphate (ATP). By measuring the amount of light emitted from either reaction the bacterial concentration can be determined. Both systems display good linearity and a sensitivity limit of approximately 10^5 E. coli per milliliter of seeded wastewater. Improvement in the functional sensitivity can be made by processing the sample by concentration, i.e., centrifugation or filtration. In waste water effluent, this may be difficult due to the presence of particulates. The luciferase ATP assay when combined with an ATPase for extra-bacterial ATP hydrolysis is a specific reaction. The lower cost of reagents for the chemiluminescent system make that assay suited for continuous monitoring of bacterial levels. Complementary use of both systems allows for continuous luminol monitoring of wastewater with periodic back-up luciferase tests specific for bacteria.

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CHEMILUMINESCENT VS. BIOLUMINESCENT METHODS FOR DETECTION OF BACTERIA IN WASTEWATER EFFLUENT

Automated chemiluminescent and bioluminescent systems are under development for the rapid detection of bacteria in water samples. While these systems were originally developed for the purpose of continously monitoring microbial levels in wastewater effluent (in conjunction with the Johnson Space Center Water Monitoring System) these systems should easily be adaptable to most any fluid sample including drinking drinking water, etc.

The chemiluminescent system used for monitoring microbial levels involves the reaction between luminol (5-amino-2,3 dihydro-1,4 phthalazinedione) and bacterial porphyrins. The bioluminescent system which has already been described is a result of the reaction between firefly luciferase and adenosine triphosphate (ATP). By measuring the amount of light emitted from either reaction the bacterial concentration can be determined.

The principle of the luminol chemiluminescence method for detecting bacteria is based on the reaction shown in Figure 1. In this method, a base such as sodium hydroxide, hydrogen peroxide, and a luminol stimulating factor are required for a luminescnt response. A number of compounds produce a luminescent response: ferricyanide and hypochlorite or a number of chelated transition metals such as ferrous and cobaltousions with hydrogen peroxide. In the case of the luminol bacteria detecting system the most important compounds are probably iron containing porphyrins such as catalase. When all the necessary luminol reagents are

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present in excess, the amount of light emitted from the luminol reaction (λ_{max}=425 nm) is proportional to the concentration of porphyrins present. If only bacterial iron porphyrins are being measured, the light response can then be related to the number of bacteria present in the sample.

The bioluminescent system- the firefly luciferase assay for detecting bacterial ATP involves extracting the ATP from the bacteria with nitric acid with subsequent dilution and assay. The amount of light emitted from the reaction if proportional to the amount of ATP which should be directly related to the bacteria concentration.

Since many conpounds besides bacterial porphyrins produce a light response, common interfering agents including metallic ions, chlorine, and extra cellular porphyrins, it is necessary to differentiate these "interferences" from the bacterial porphyrins. Several techniques have been developed to accomplish this:

1) Sodium thiosulfate can be used to reduce chlorine to the non-reactive chloride. 98% reduction of signal from a sample containing 10 ppm chlorine was achieved with 500mg sodium thiosulfate per liter. No interaction between the sodium thiosulfate and iron porphyrins have been observed.

2) A technique has been developed for eliminating luminol interference caused by extra cellular porphyrins such as catalase and hemoglobin. This method involves a pre-incubation of the sample with a dilute concentration of hydrogen peroxide which destroys the tetrapyrole structure of the interfering soluble porphyrins and thus inactivates them.[1]

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The porphyrins within the bacteria remain intact and will only react with the luminol reagent after the cells have been ruptured by the sodium hydroxide in the luminol reagent.

Figure 2 shows the effect of hydrogen peroxide concentration with time on a catalase sample. The greatest reduction of signal takes place within the first five minutes of the incubation. Figure 3 shows the effect of hydrogen peroxide concentration and time on a sample of stationary phase E. coli. At concentrations less than 1.5%, no significant reduction of response from E. coli was observed. From these experiments, 0.5% hydrogen peroxide for a two minute period was determined to be the optimal pretreatment condition for the luminol sample. Figure 4 shows the effect of this pretreatment on three species of bacteria, E. coli, Bacillus subtilis, and Pseudomonas aeruginosa. The growth stage of some species of bacteria does influence the susceptability of the bacteria to the pretreatment. It is therefore necessary that the growth phase of the bacteria in a sample be known or at least be constant.

While some loss of signal is observed with pretreated bacteria, the loss is not significant (at least for E. coli) compared to the loss of signal from other pretreated materials. Figure 5 shows the effect of 0.5% hydrogen peroxide pretreatment on a number of compounds capable of stimulating a luminol light response. Over 90% of the interference due to porphyrin material can be eliminated using this technique.

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3) Not all the interference can be eliminated using the hydrogen peroxide pretreatment. To eliminate the effects of inorganic interference we have taken advantage of the different reaction rates of the various luminol reactants. Figure 6 shows the reaction rate curves of E. coli, catalase, ferricyanide, and ferrous sulfate. If the light measurement was taken at a point ten seconds after mixing the sample with the luminol, only the catalase and E. coli response would be observed. When used in conjunction with the hydrogen peroxide pretreatment, the reaction rate resolution method should make the luminol system specific for bacteria.

Since there was a need for an automated continuous method for monitoring microbial levels in fluid samoples, a flow type system was in order. A schematic diagram of the luminol flow system is shown in Figure 7. The system incorporates the two methods for eliminating interference problems. The hydrogen peroxide pretreatment allows the sample to pre-react with the hydrogen peroxide at a concentration of 0.4% for a two minute period. The sample then reacts with the luminol reagent (2.5×10^{-4} M luminol, 0.1% H_2O_2 and 0.75 N NaOH) for a period of ten seconds before reaching the photomultiplier tube. This step, rate resolution, eliminates the interference from inorganic materials. Results from a tap water sample show that 98% of the interference can be eliminated.

The peak light produced from the luminol system is measured to determine the bacteria conentration. Figure 8 shows the luminol flow system response to washed E. coli. The functional sensitivity of the system is about 1×10^4 E. coli per millilter.

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A comparison of the chemiluminescnt and the bioluminescent system can be broken down as follows:

1) Looking at the reactions at face value, the firefly luciferase reaction is a much more specific reaction compared to the luminol reaction with the multitude of factors capable of stimulating a light response. However when one considers the interference elimination techniques described, the specificity of the luminol system can greatly be improved. Preliminary tests with wastewater effluent indicate that any interference present will not affect either system.

2) The sensitivity of the flow systems has been mentioned as 1×10^4 E. coli per milliliter for the luminol system and 1×10^5 E. coli per milliliter for the firefly luciferase ATP system. Figure 11 shows the use of the two systems for detecting E. coli in seeded wastewater effluent. With no interference elimination techniques employed, a sensitivity of 5×10^5 E. coli per milliliter was achieved. With the interference elimination techniques included in the systems, the sensitivities further improve.

A method for concentrating bacteria is currently under development and should improve the sensitivities much more.

3) The final area for comparison is in the area of cost. The use of the firefly luciferase ATP system is limited to discreet assays due to the cost factor, each assay, 0.2 milliliters of luciferase costing approximately $0.60.

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If an assay needed to be performed periodically the ATP system should be quite adequate. For a continuous monitoring of a fluid sample, as in the case of wastewater effluent, the luminol flow system is ideal. The reagents necessary for the luminol system are inexpensive and cost should not restrict the continuous monitoring of bacteria levels.

References

1. Falk, J.E., "Porphyrins and Metalloporphyrins, New York, Elsevier Publishing Co., 1964, p. 21.

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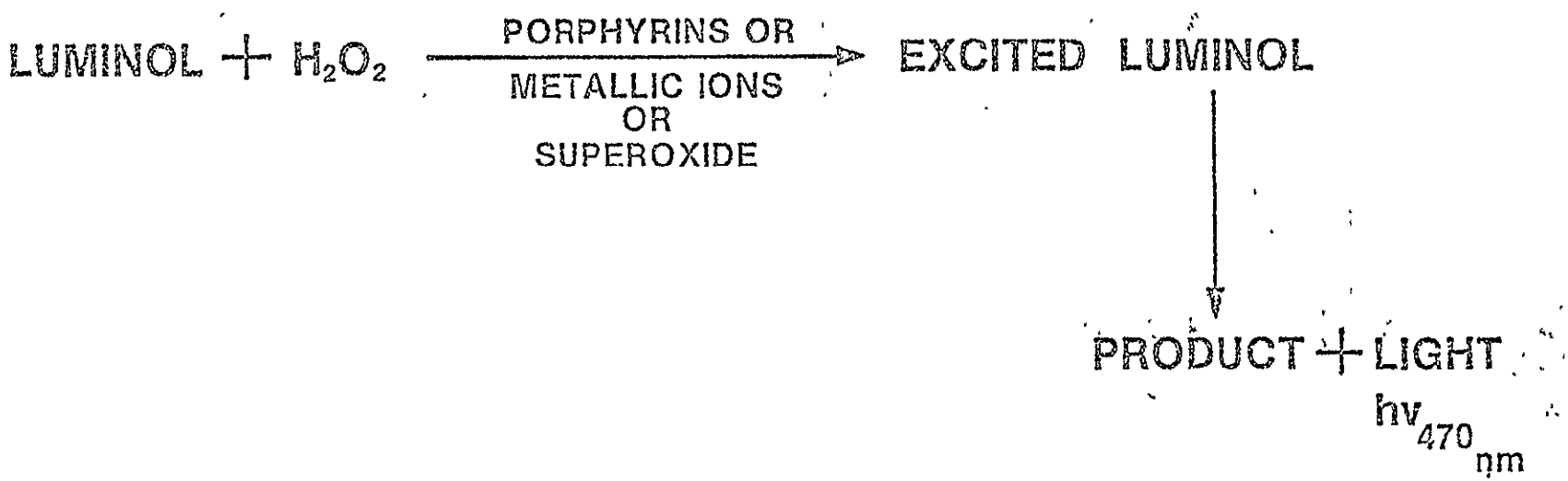
FIGURE 1. LUMINOL LIGHT REACTION



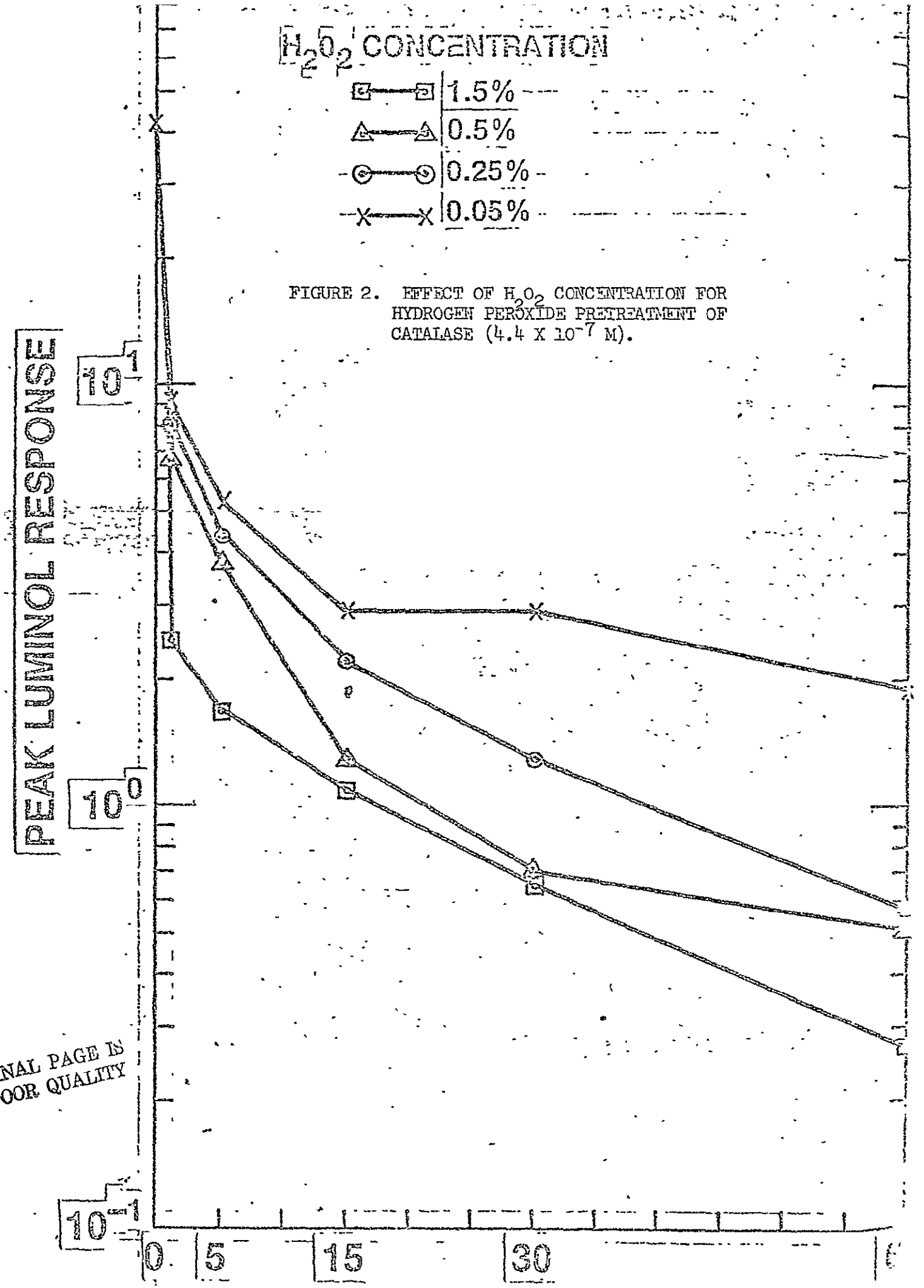


FIGURE 2. EFFECT OF H_2O_2 CONCENTRATION FOR HYDROGEN PEROXIDE PRETREATMENT OF CATALASE (4.4×10^{-7} M).

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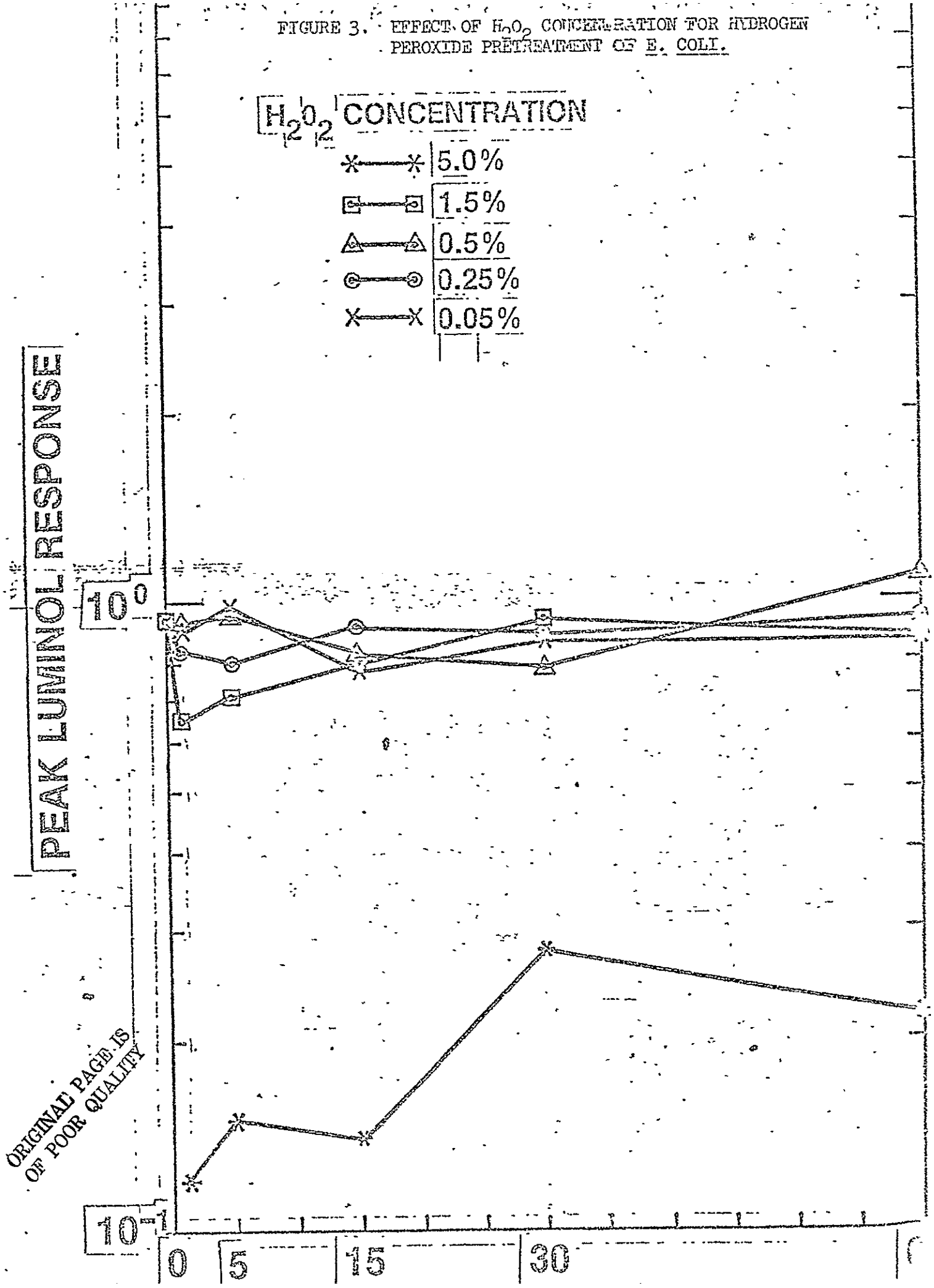
FIGURE 3. EFFECT OF H_2O_2 CONCENTRATION FOR HYDROGEN PEROXIDE PRETREATMENT OF E. COLI.



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SPECIES	REDUCTION OF SIGNAL	
	GROWTH PHASE	
	STATIONARY	LOGARITHMIC
ESCHERICHIA COLI	8%	24%
BACILLUS SUBTILIS	16%	36%
PSEUDOMONAS AERUGINOSA	43%	40%

FIGURE 4. EFFECT OF 0.5% HYDROGEN PEROXIDE PRETREATMENT ON BACTERIA

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OXIDIZING AGENT	REDUCTION OF SIGNAL
CATALASE	94%
HEMOGLOBIN	95%
EXTRACTED BACTERIAL PORPHYRINS (NaOH - EtOH)	97%
POTASSIUM FERRICYANIDE	50%
COBALT(OUS) CHLORIDE	20%
FERROUS SULFATE	0

FIGURE 5. EFFECT OF 0.5% HYDROGEN PEROXIDE PRETREATMENT ON SOME LUMINOL OXIDIZING AGENTS

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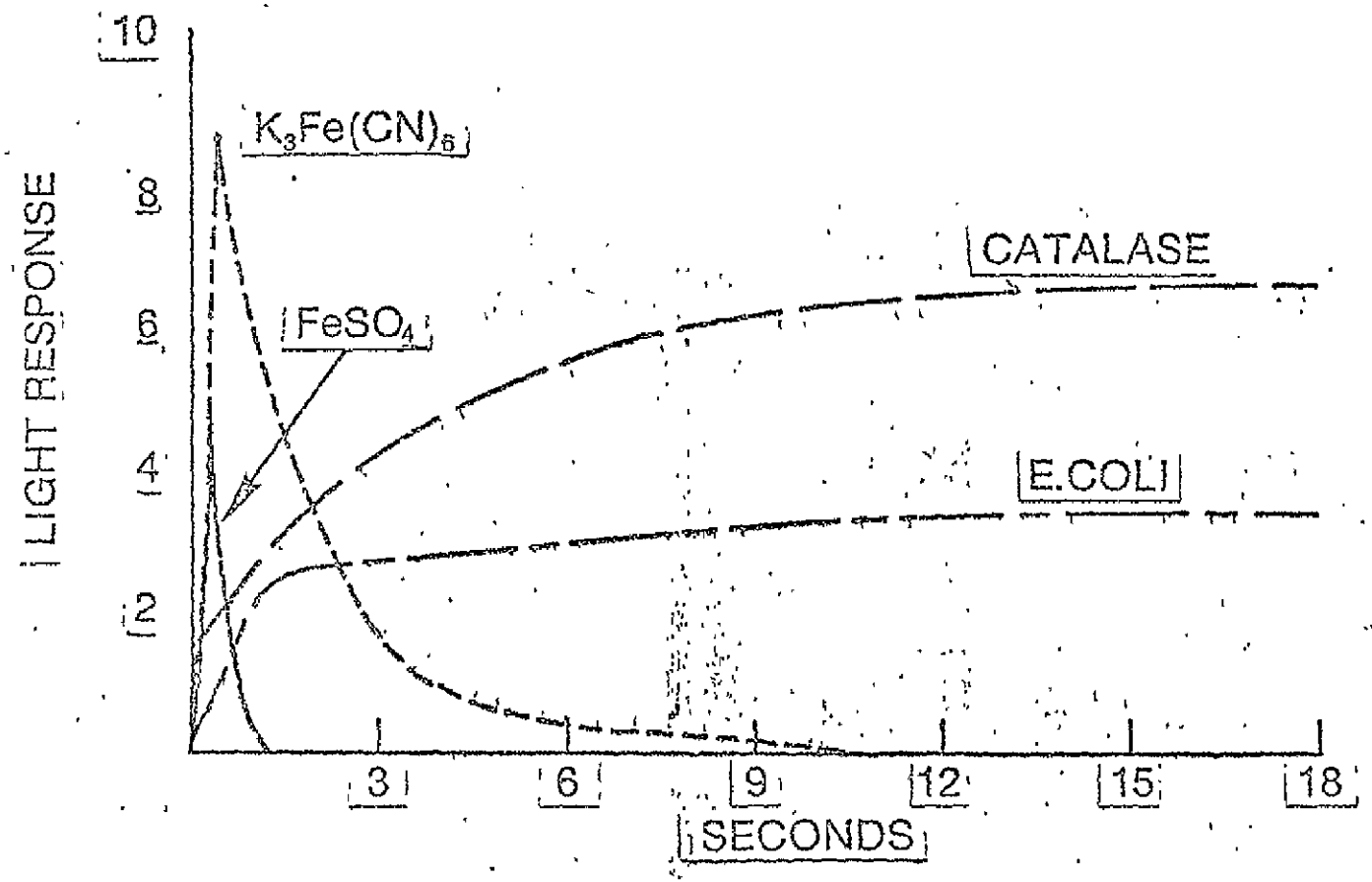

FIGURE 6. REACTION RATE CURVES FOR VARIOUS LUMINOL OXIDIZING AGENTS (ARBITRARY CONCENTRATIONS).

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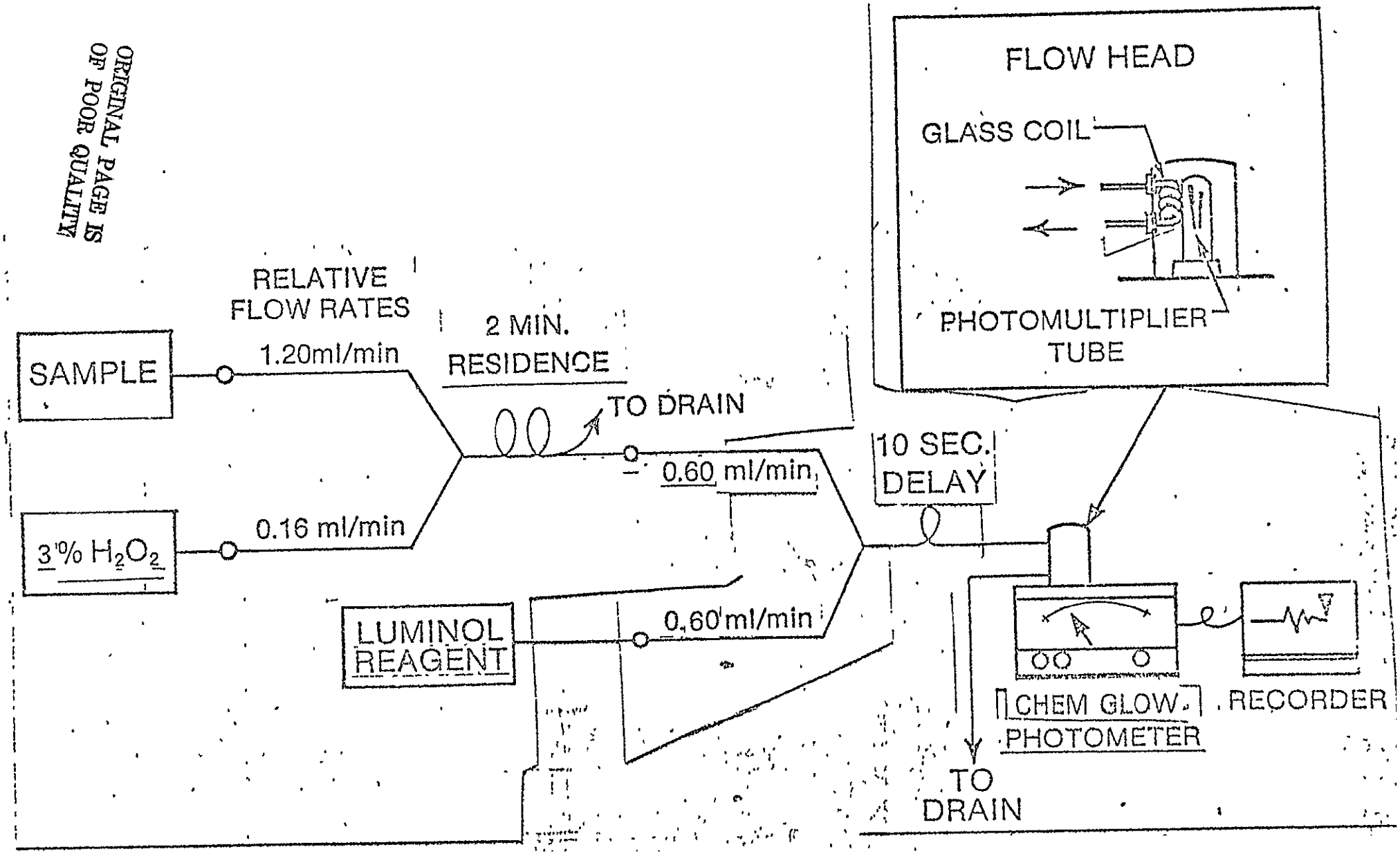
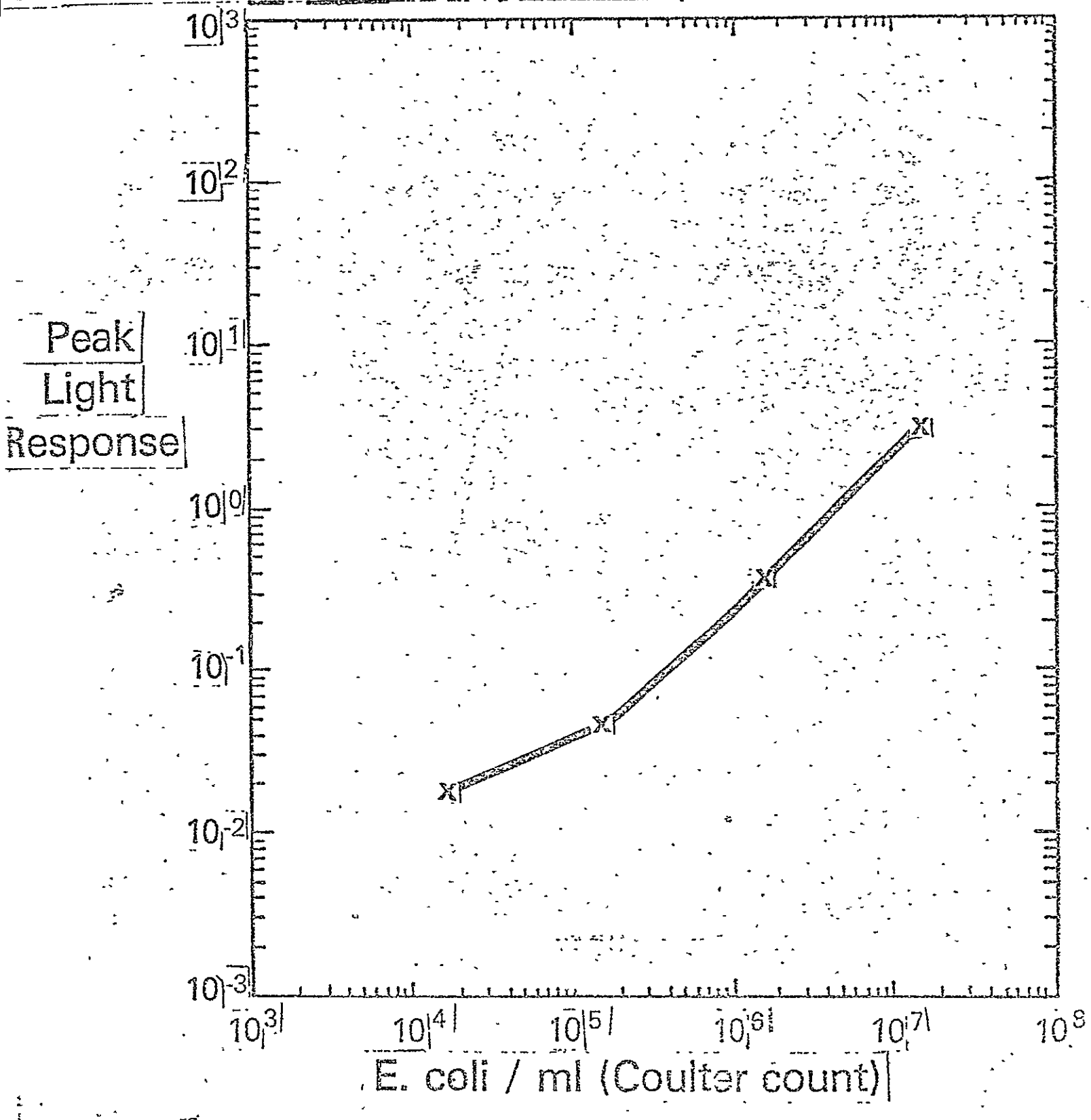


FIGURE 7 AUTOMATED LUMINOL FLOW SYSTEM INCORPORATING HYDROGEN PEROXIDE PRETREATMENT AND REACTION RATE RESOLUTION FOR ELIMINATING INTERFERENCE.

FIGURE 8.

PEAK LIGHT RESPONSE FROM VARIOUS CONCENTRATIO
OF WASHED E. COLI USING THE LUMINOL FLOW SYSTE



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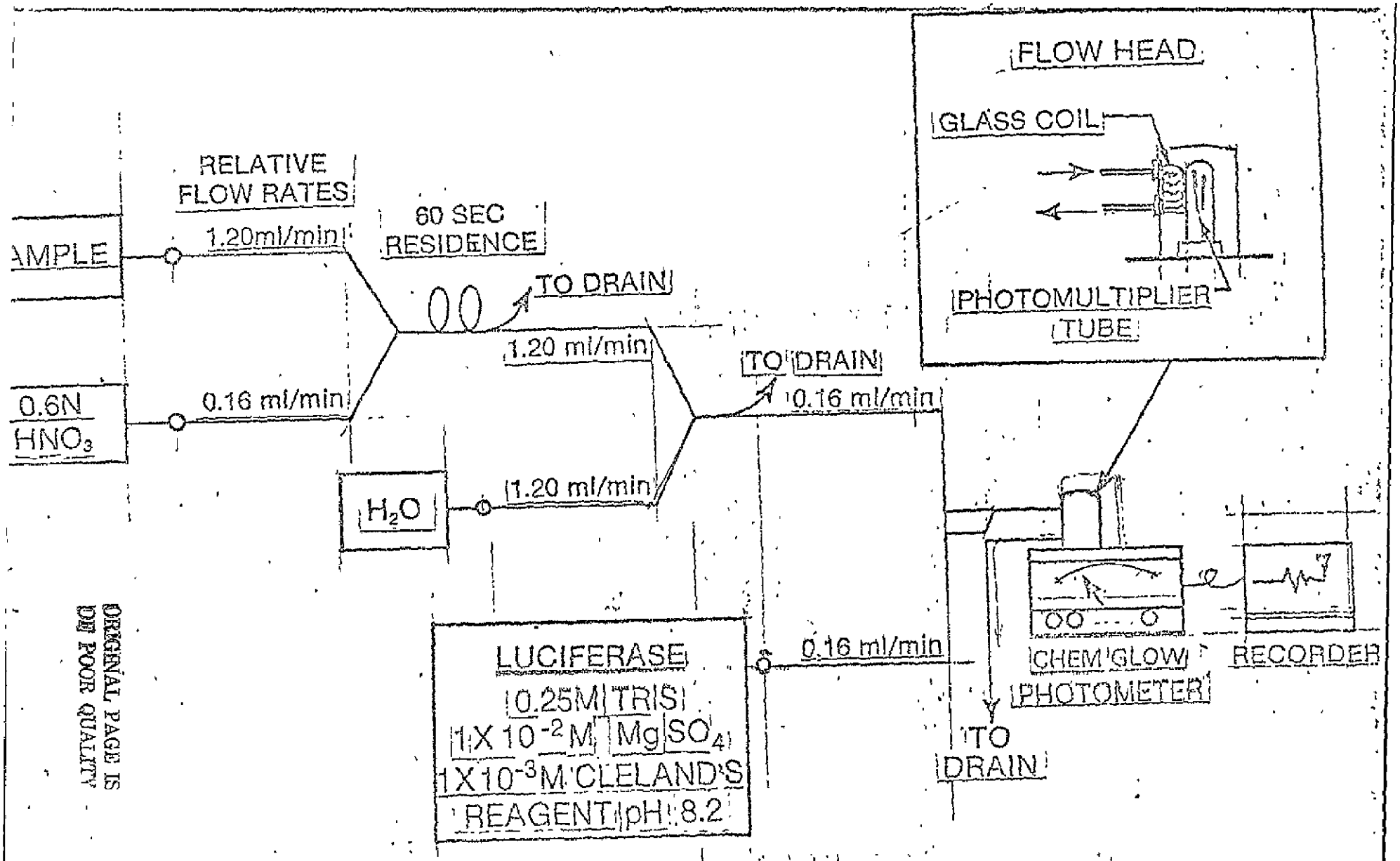

FIGURE 9. AUTOMATED FIREFLY LUCIFERASE FLOW SYSTEM FOR DETECTING BACTERIAL ATP INCLUDING NITRIC ACID EXTRACTION AND SUBSEQUENT DILUTION.

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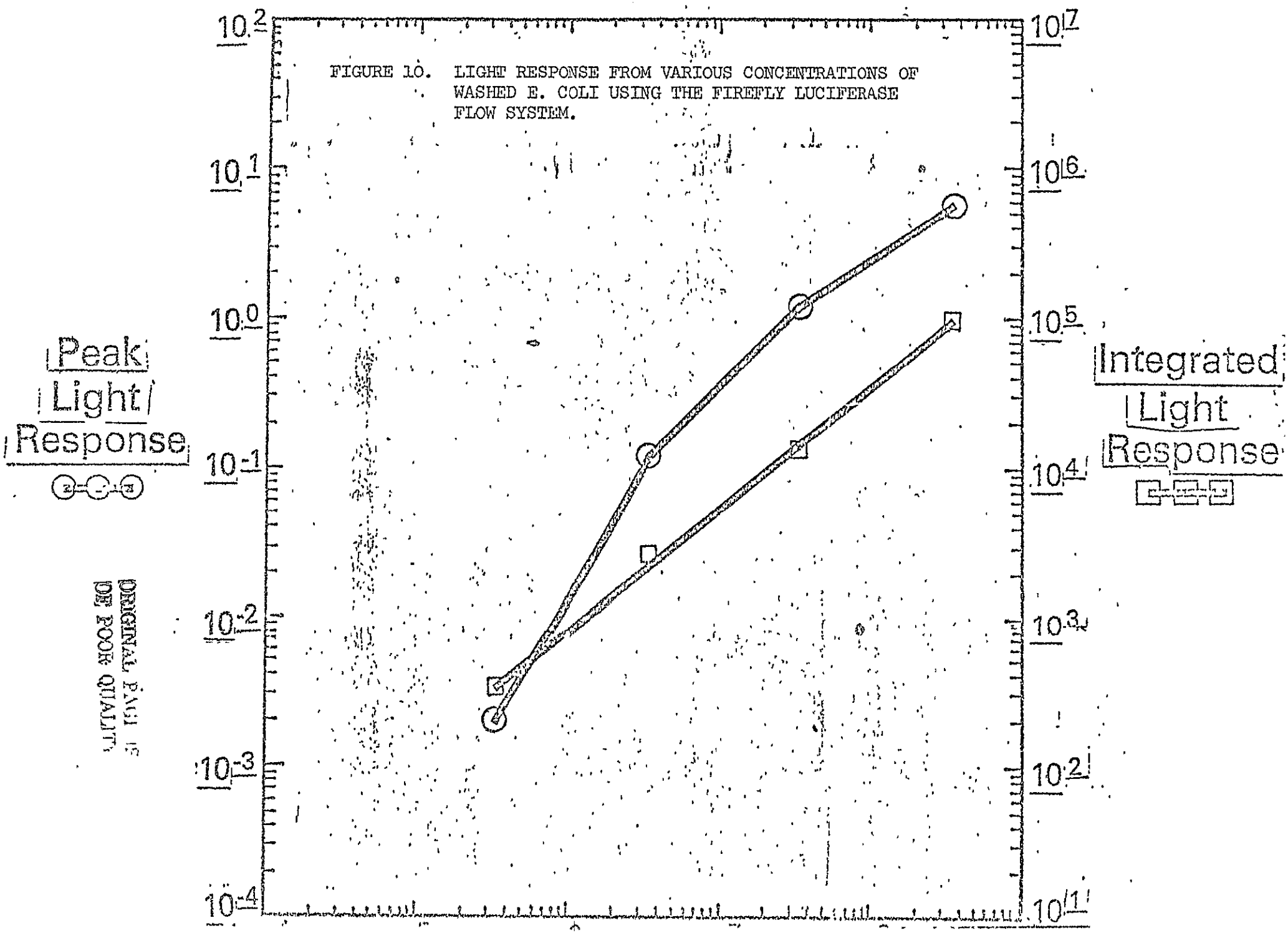

FIGURE 10. LIGHT RESPONSE FROM VARIOUS CONCENTRATIONS OF WASHED E. COLI USING THE FIREFLY LUCIFERASE FLOW SYSTEM.

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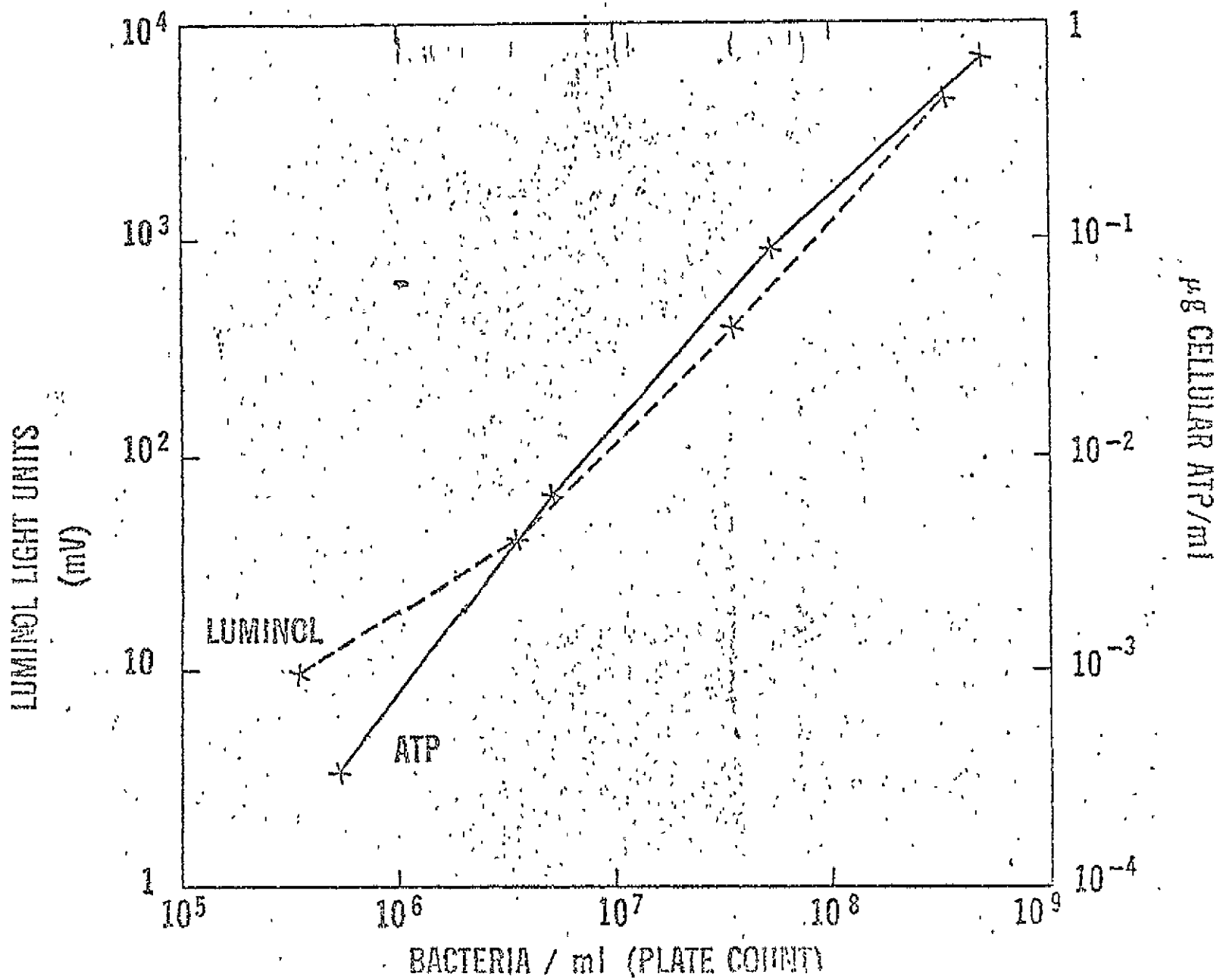
FIGURE 11. FIREFLY LUCIFERASE AND LUMINOL RESPONSE FROM E COLI SEEDED WASTEWATER EFFLUENT



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