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Enclosure to 5-2510-HOU-2-1359

DEVELOPMENT OF A CHEMILUMINESCENT AND BIOLUMINESCENT SYSTEM FOR THE DETECTION OF BACTERIA IN WASTEWATER EFFLUENT

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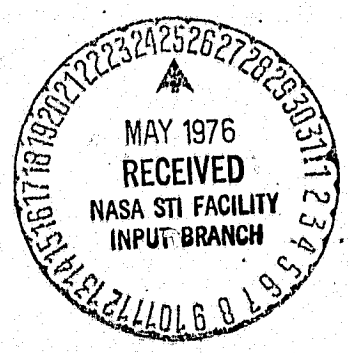
Quarterly Report for Period September - December 1975

(NASA-CR-144750) DEVELOPMENT OF A CHEMILUMINESCENT AND BIOLUMINESCENT SYSTEM FOR THE DETECTION OF BACTERIA IN WASTEWATER EFFLUENT Quarterly Report, Sep. - Dec. 1975 (Boeing Co., Houston, Tex.) 50 p HC $4.00 G3/51

N76-23824

Unclas
40543

Prepared for
Goddard Space Flight Center
Greenbelt, MD 20771



1. Report No.	2. Government Accession No.	3. Recipient's Catalog No.
1		

4. Title and Subtitle	5. Report Date
Development of a Chemiluminescent and Bioluminescent System for the Detection of Bacteria in Wastewater Effluent	6. Performing Organization Code
7. Author(s) Richard R. Thomas	8. Performing Organization Report No.
9. Performing Organization Name and Address The Boeing Company P. O. Box 58747 Houston, TX 77058	10. Work Unit No. 11. Contract or Grant No. NAS 5-22545
12. Sponsoring Agency Name and Address NASA/Goddard Space Flight Center Grace Lee Picciolo, Code 726 Greenbelt, MD 20771	13. Type of Report and Period Covered Type II September-December 75 14. Sponsoring Agency Code

15. Supplementary Notes

16. Abstract Automated chemiluminescent and bioluminescent sensors are being developed for continuous monitoring of microbial levels in wastewater effluent. Development of the chemiluminescent system has included optimization of reagent concentrations as well as two new techniques which will allow for increased sensitivity and specificity. The optimal reagent concentrations are 2.5×10^{-4} M luminol and 0.0125 M sodium perborate in 0.75N sodium hydroxide before addition of sample. The methods developed to increase specificity include 1) extraction of porphyrins from bacteria collected in a filter using 0.1N NaOH - 50% Ethanol and 2) use of the specific reaction rate characteristics for the different luminol catalysts. Since reaction times are different for each catalyst, the reaction can be made specific for bacteria by measuring only the light emission from the particular reaction time zone specific for bacteria. Developments of the bioluminescent firefly luciferase system have been in the area of flow system design.

17. Key Words (Selected by Author(s))	18. Distribution Statement
Chemiluminescent, Bioluminescent, Detection, Bacteria, Effluent	

19. Security Classif. (of this report)	20. Security Classif. (of this page)	21. No. of Pages	22. Price*
UNCLASSIFIED	UNCLASSIFIED		

*For sale by the National Technical Information Service, Springfield, Virginia 22151.

PREFACE

The purpose of this project is to develop automated chemiluminescent and bioluminescent sensors for the continuous monitoring of microbial levels in wastewater effluent in support of the Johnson Space Center Water Monitoring System. In particular the objectives include 1) the development of an optimal luminol reaction system and procedures for its use as a sensitive and specific means of microbial detection and 2) the necessary modifications of the GSFC bioluminescent firefly luciferase system.[1] The optimal reagent concentrations for the luminol system are 2.5×10^{-4} M luminol, and 0.0125 M sodium perborate in 0.75N sodium hydroxide before addition of sample. Two methods have been developed to increase the specificity of the luminol reaction; 1) extraction of porphyrins from bacteria collected on a filter using a 0.1N NaOH - 50% - EtOH solution, and 2) taking advantage of the differences in rates of reaction for various luminol catalysts. Since reaction times are different for each catalyst the reaction can be made specific for bacteria by measuring only the light emission from the particular reaction time zone specific for bacteria. At the present time, no modifications have been made on the bioluminescent firefly luciferase system except in the area of flow system design.

TABLES

ILLUSTRATIONS

INTRODUCTION

Community Wastewater Treatment Plants require continuous monitoring of the quality of the reclaimed water with respect to microbial count. Luminescence techniques can accomplish this in real time. A chemiluminescent system which can be used involves the reaction between luminol (5-amino-2, 3-dihydro-1, 4 phthalazedione) and bacterial porphyrins. The bioluminescent system which has been developed at GSFC is a result of the reaction between firefly luciferase and ATP (adenosine triphosphate). By measuring the amount of light emitted from either reaction the bacterial concentration can be determined.

The majority of water monitoring work at GSFC has been devoted to two basic problems which are inherent to the luminol system, 1) the reaction is not specific for bacteria (bacterial porphyrins) and 2) relatively large amounts of light can be generated even in the absence of any known reactant, i.e. high blank and endogenous light. Luminol has been used for the detection of hemoglobin, potassium ferricyanide, catalase, ferrous ions and cytochrome C as well as bacteria. In addition to the above catalysts, many other metallic ions and complexes initiate a light response from luminol. Because of this lack of specificity many interference problems could result when the luminol system is used in monitoring microbial levels in wastewater effluent. The second problem, that of high blank and endogenous light was also confronted. An effort was made to decrease the endogenous light and blank since these both limit the sensitivity of the luminol system.

The major thrust of the work with the bioluminescent system so far has been in the area of flow system design. The basic assay developed at GSFC has been used in the flow with emphasis on limiting the amount of firefly luciferase necessary for the assay in order to minimize cost. At the present time the system must involve discreet periodic assays of the flowing sample in order to be economically practical.

DISCUSSION AND RESULTS

Hydrogen Peroxide Concentration. The initial work involved optimization of hydrogen peroxide concentration which is necessary for the light response from a sample using the luminol reaction. This work involved the use of a discreet sampling method whereby a volume of sample was injected into the luminol reagent mixture. The Luminescence 760 Biometer, manufactured by E. I. Dupont de Nemours and Company, Inc., was the instrument used to measure the light emission from the luminol-peroxide reaction. Appendix A contains the report of all the work concerning this phase of the work. 1% hydrogen peroxide was determined to be the optimal concentration for the 5.67×10^{-7}M luminol solution resulting in a final reagent solution (before sample) of (0.5%) H_2O_2 and (2.84×10^{-7})M luminol in 0.75N NaOH. In addition to this work, it was shown that hemoglobin produced a linear response between the ranges of 10^{-8}M to 10^{-5}M, with the luminol-peroxide system. See Appendix A for complete information.

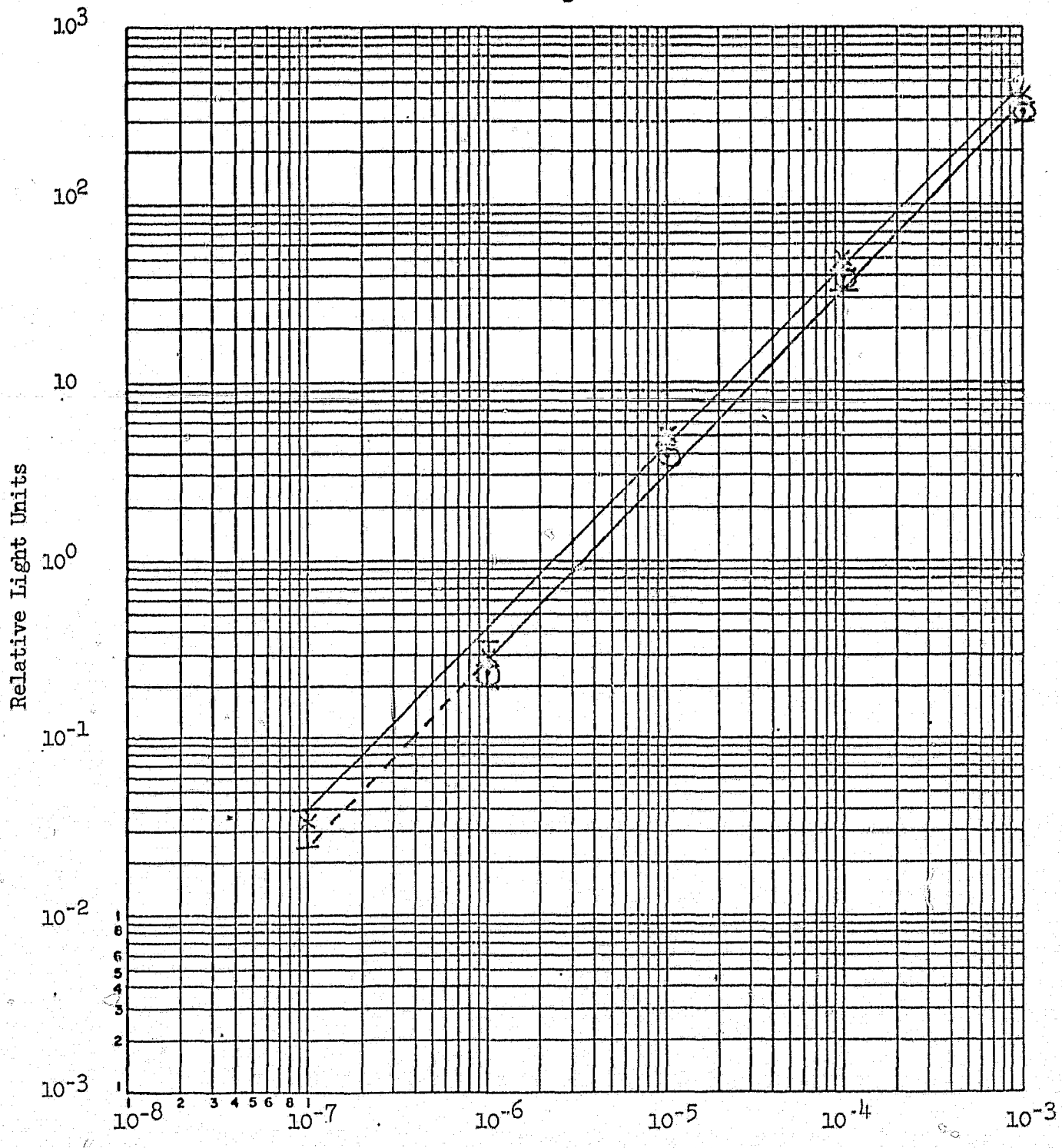
Purification of Luminol. In an effort to improve the linearity of light response from samples, increase the light emission, and lower the endogenous light and blank, a luminol purification scheme was undertaken. Luminol hydrochloride, determined to be the most pure by thin layer Chrotography, was produced by recrystallizing luminol (J. T. Baker and Co.) twice in hydrogen bromide. The luminol · HB~ was then dissolved in alkaline aqueous solution and precipitated out of solution by acidifying with hydrochloric acid. The resulting precipitate, luminol hydrochloride, produced the most linear light response for a hemoglobin sample, highest light emission for that sample and lowest blank using the discreet sampling method. The luminol solution before addition of sample contained equal volumes of (5.0×10^{-7})M luminol hydrochloride, in 1.5N NaOH and 1% hydrogen peroxide. Appendix B contains a report of all the work regarding the recrystallization of the luminol.

Luminol was later obtained from the Sigma Chemical Company and proved to be of much better quality than the original Baker luminol. the Sigma luminol was similar to the purified luminol in terms of light response and linearity for potassium ferricyanide (Figure 1). Considering the time required for recrystallization and experimental yields of 15%, the Sigma luminol is recommended for the remainder of the project.

Figure 1. Light response from luminol·HCl and Sigma luminol for potassium ferricyanide samples. The vertical bars represent standard deviation of the mean (n = 3-5).

Luminol·HCl -X-X-X-X-

Sigma luminol -O-O-O-O-



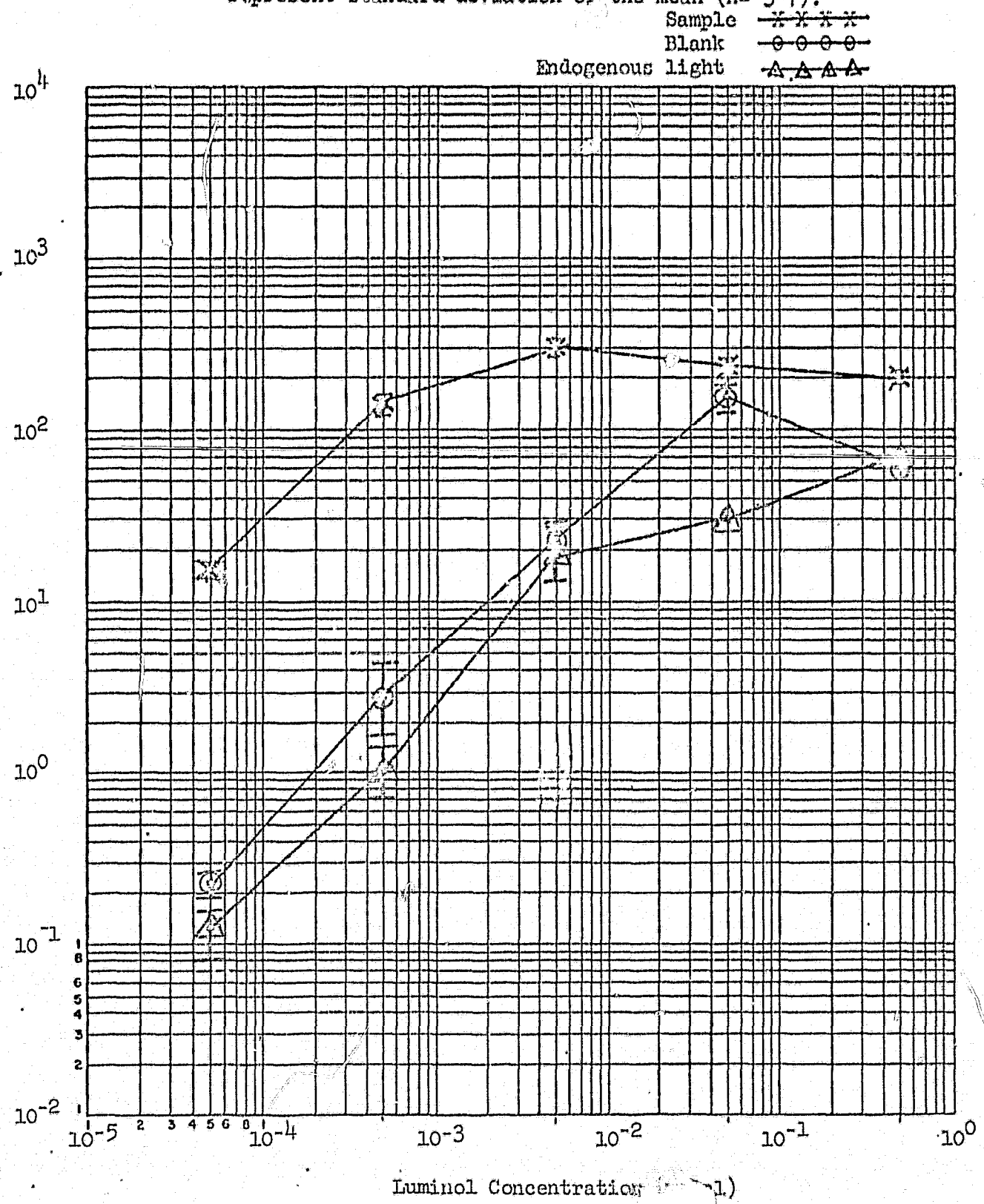
Optimal Luminol Concentration. The optimal luminol concentration was determined by consideration of the endogenous light, the blank, and highest light response for a sample. The determination was conducted using the discrete sampling method. The hydrogen peroxide concentration (1%) and the sample 1.0×10^{-7}M potassium ferricyanide were held constant while the concentration of luminol was varied. Figure 2 shows how all three parameters, endogenous light, blank, and sample response varied with luminol concentration. The optimal luminol concentration, highest light response for a sample with lowest blank (computed as activity (sample/blank)) was determined to be 5.0×10^{-4}M.

Another attempt to decrease blank and endogenous light - Ammonium hydroxide vs. sodium hydroxide. In an effort to decrease the endogenous light and eliminate the high luminol blank, ammonium hydroxide was tried as an alternative to sodium hydroxide as the base. If the extranneous light from the luminol reaction was due to impurities in the sodium hydroxide it was expected that the endogenous light would decrease since ammonium hydroxide can be obtained in a purer form. Table I contains the results of the experiment. These results show that the use of ammonium hydroxide produced the same endogenous light from luminol as sodium hydroxide. Although a lower blank was found using the ammonium hydroxide, a lower response was also found from the sample. Ammonium hydroxide appears to quench the luminol reaction resulting in very little light emitted from the luminol system even with high sample concentrations. Because of this, sodium hydroxide will be used as the base for the remainder of the project.

TABLE I. Sodium Hydroxide vs. Ammonium Hydroxide as a Base, including Blank, Endogenous Light, and Luminol Response to a 1×10^{-7}M $K_3Fe(CN)_6$ Sample.

	Blank	Endogenous Light	Sample 1×10^{-7}M $K_3Fe(CN)_6$
	(relative light units)		
5×10^{-3}M luminol in 1.5N NaOH	6.2	7.2	6.9×10^2
5×10^{-3}M luminol in 1.5M NH_4OH	2.6	7.4	0.5

Figure 2. Light response from various luminol concentrations for 1×10^{-7}M potassium ferricyanide samples including blank and endogenous light values. The vertical bars represent standard deviation of the mean (n= 3-7).



Optimal Oxidant. Hydrogen peroxide, dissolved O_2 in ethanol, and sodium perborate. Hydrogen peroxide has been the most common oxidant for the luminol reaction. Another oxidant which has been used is bubbled oxygen in the luminol solution.[2] It was hypothesized that the high amount of oxygen dissolved in ethanol (5 x that of water) would be sufficient for the luminol reaction but not too strong an oxidant to produce the high blanks and endogenous light which have so far been observed. Table II shows the results of experiments using ethanol and a hemoglobin sample. The ethanol system had very low blanks and endogenous light but only because the sensitivity was greatly diminished. The ethanol system is therefore not recommended at this time.

TABLE II Dissolved Oxygen vs. Hydrogen Peroxide as the Source of Oxygen for the Luminol Reaction. (5.0×10^{-3}M Luminol).

	Blank	Endogenous Light	Samples	
			1.47×10^{-10}M Hemoglobin	1.47×10^{-9}M Hemoglobin
		(relative light units)		
1% H_2O_2	2.2×10^{2}	3.1×10	3.3×10^{2}	4.3×10^{3}
Oxygen dissolved in 25% ethanol	7.5×10^{-1}	3.4×10^{-1}	6.5×10^{-1}	4.5×10^{-1}
Oxygen dissolved in 50% ethanol	8.4×10^{-1}	7.6×10^{-1}	7.7×10^{-1}	7.0×10^{-1}

Sodium perborate was explored as an alternative to hydrogen peroxide. While keeping the luminol and sample concentrations constant, concentrations of 2.5×10^{-4}M to 2.5×10^{-2}M sodium perborate were examined to determine what concentration produced the maximum luminol response from a hemoglobin sample. Table III contains the experimental results. Using the discreet sampling system, a 60% increase in light response was produced with 0.025M sodium perborate compared to the 1% hydrogen peroxide. Considering the increased light response and greater stability of sodium perborate, 0.025M (0.0125M in luminol solution) is recommended and will be used for the remainder of the project.

TABLE III Hydrogen Peroxide vs. Sodium Perborate.
(5×10^{-5}M Luminol in 1.5N NaOH)

	Endogenous Light	Blank	Sample 1.47×10^{-9}M Hemoglobin
	(relative light units)		
1% H_2O_2	9.0×10^{-1}	1.6×10	2.0×10^{2}
2.5×10^{-2}M sodium perborate	4.1×10^{-1}	1.7×10	3.4×10^{2}
2.5×10^{-3}M sodium perborate	1.6×10^{-1}	3.2	5.1×10
2.5×10^{-4}M sodium perborate	6.6×10^{-2}	6.6×10^{-1}	6.2

Determination of lysing ability of nitric acid, sodium hydroxide and the luminol system for E. coli. The luminol solution before addition of sample consists of 0.75N sodium hydroxide. This concentration has been assumed to be sufficient for extraction of the bacterial porphyrins for subsequent assay by the luminol solution. A comparison was made between 0.1N nitric acid extraction, extraction with 0.5N sodium hydroxide, and the usual extraction by the luminol system. Table IV contains the experimental results.

Results with E. coli show that the luminol mixture is an effective extractant and its use can and should be continued.

TABLE IV Comparison of Three Methods of Extraction - 0.1N Nitric Acid, 0.5N Sodium Hydroxide and the Luminol System.

	Extractant		
	0.1N Nitric Acid	0.5N Sodium Hydroxide	Luminol System
	(relative light units)		
Luminol Response from E. coli sample	4.0×10^{2}	2.9×10^{2}	4.0×10^{2}

Flow System. The initial luminol work was conducted using the discreet sampling method as described in Appendix B. This injection type system was later converted to a constant flow system. Figure 3 is a diagram of the specially designed flow head which was attached to the Aminco Chem-Glow Photometer. A Buchler peristaltic pump was used to force the reagents and sample to a coiled tube where the solutions are mixed and light detected by the photomultiplier tube. Good linear results from bacteria samples can be obtained as is shown by the E. coli curve in Figure 4.

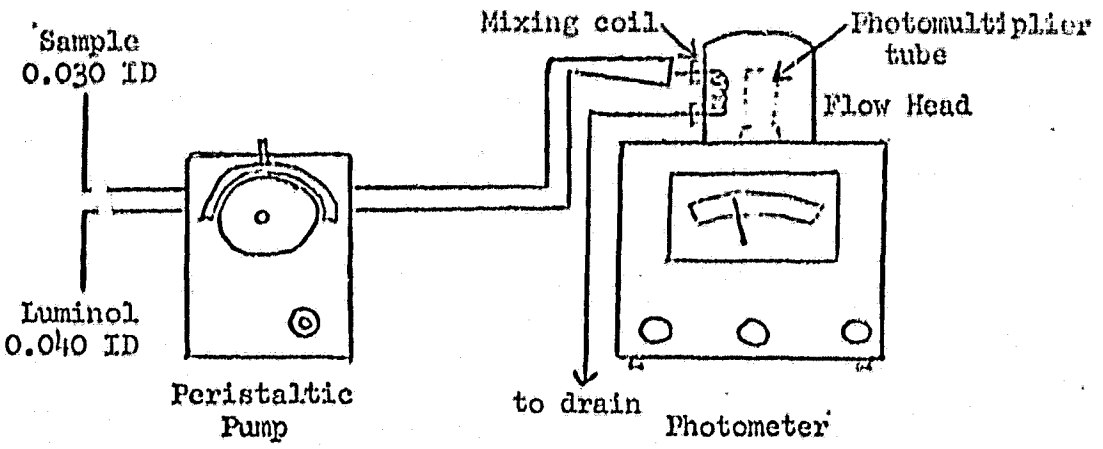


Figure 3

The effect of chlorine on the luminol system and its elimination. Using the luminol flow system several concentrations of chlorine were assayed. The chlorine samples were prepared by dilutions of chlorox bleach in distilled water, Cl_2 concentrations determined by the ortho-tolidine method. 2.5×10^{-4}M luminol and 0.0125M $NaBO_3$ in 0.75N NaOH were used throughout the study. The results of three Cl_2 concentrations can be found in Figure 5. Untreated chlorine reacts linearly like many other luminol catalysts and as such can present a problem for bacterial detection in chlorinated samples.

By using sodium thiosulfate much of the interference caused by chlorine in samples can be eliminated. Table V shows the effect of sodium thiosulfate on chlorine concentration used in this experiment, a very high 10 ppm. Sodium thiosulfate effectively eliminates 98% of the interference while lowering the bacterial response 20%. Sodium thiosulfate is necessary if there is chlorine present in the bacterial samples. (If the residual chlorine is less than 10 ppm as it should be, less sodium thiosulfate should be needed and there should be less effect on the bacterial sample.)

TABLE V The Effect of Sodium Thiosulfate on Chlorine and Chlorinated Bacteria Samples. (5×10^{-4}M Luminol in 1.5N NaOH plus 0.02M Sodium Perborate)

No Sodium Thiosulfate

	E. coli in H_2O	10 mg Cl_2/liter	E. coli in 10.1 mg Cl_2/liter
	(relative light units)		
Luminol Response	5.0×10^2	3.0×10^3	7.8×10^2
With 500 mg Sodium Thiosulfate/liter			
Luminol Response	3.8×10^2	2.6×10	5.0×10^2

Figure 4. Light response from various concentrations of E. coli using the luminol-perborate flow system.

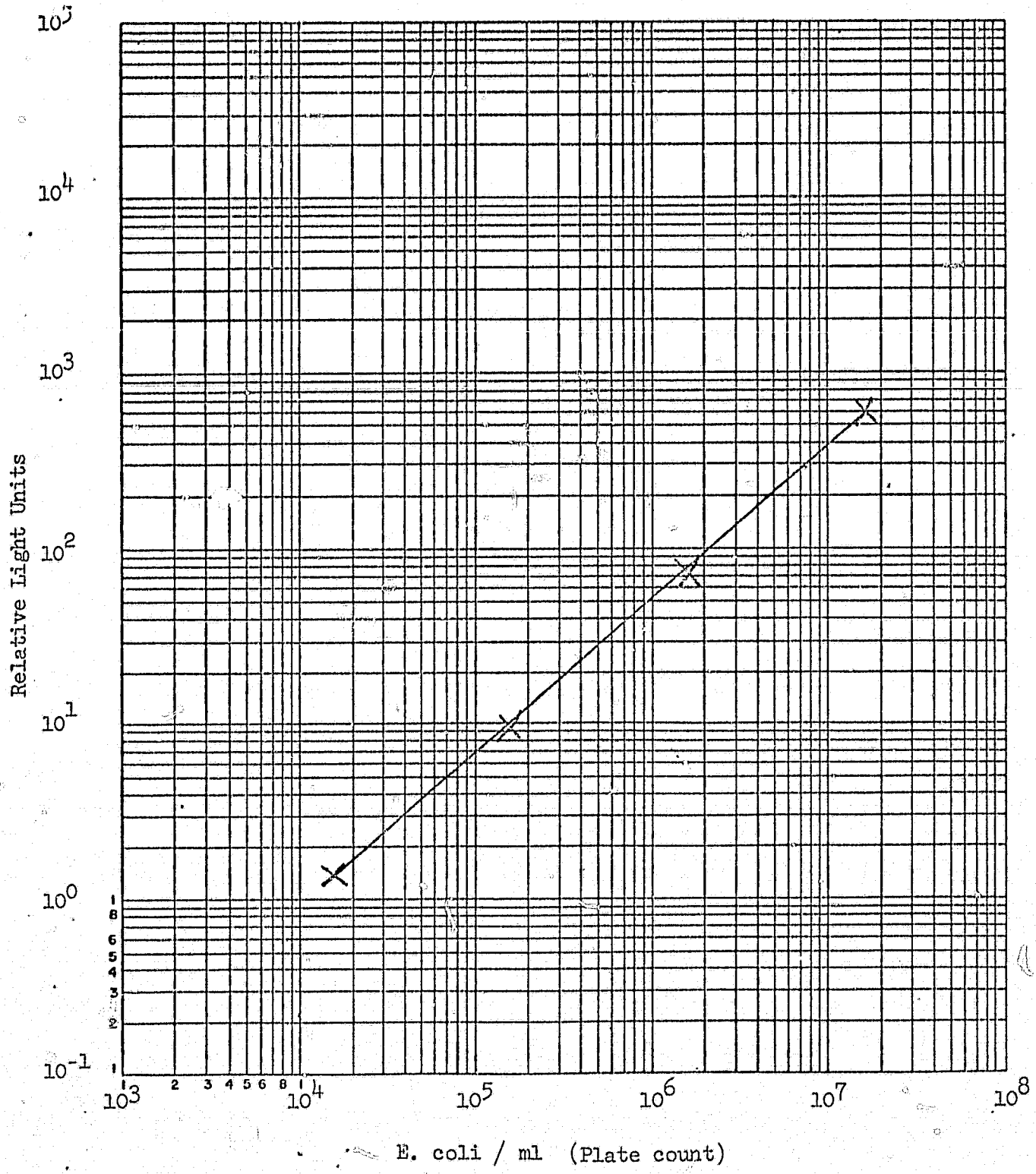
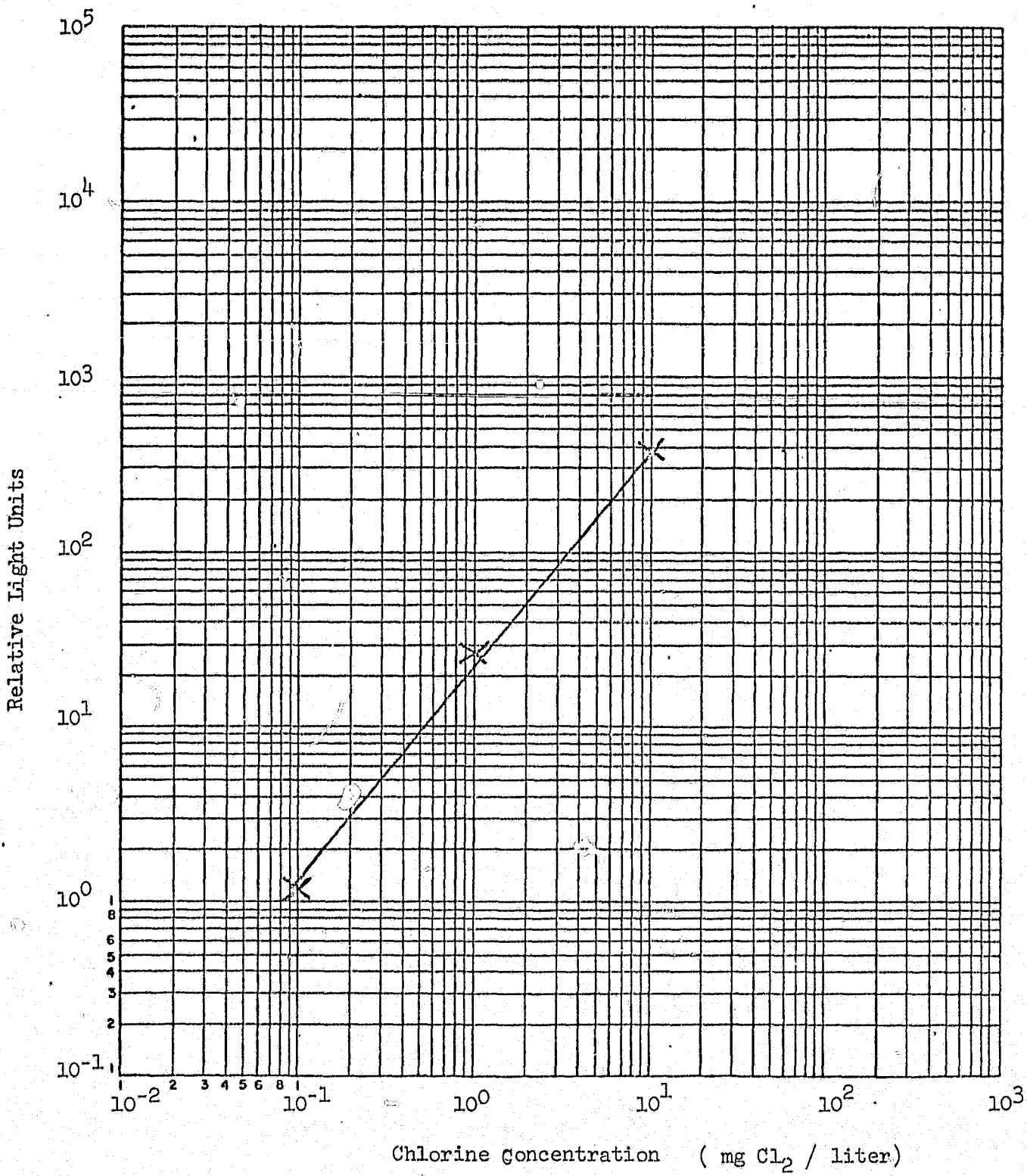


Figure 5. Response from various chlorine concentrations using the luminol-perborate flow system.



Extraction on a filter surface. A method has been developed which should increase both the sensitivity and specificity of the luminol reaction. This is accomplished by collecting bacteria from a sample on to a Gelman 0.45 μ acropor filter and then washing the interferring materials off with a solvent (0.1N NaOH, saline, or deionized water). The bacteria can then be ruptured with 0.1N NaOH - 50% EtOH to selectively wash through the bacterial porphyrins. This portion is then assayed with the luminol system.

The optimal filtration and extraction procedure is as follows:

(1) A known volume of sample is collected on a Gelman 0.45 μ acropor filter.

(2) The material on the filter is then washed with 10 ml of 0.1N sodium hydroxide.

(3) The bacteria are then ruptured using 1 ml of 0.1N sodium hydroxide with 50% ethanol. The filtrate which contains the soluble porphyrins is then assayed using the luminol flow system.

This method has been shown to work in a flow system. A Swinnex-13 (Millipore) 13 mm diameter filter holder was positioned in the sample line between the peristaltic pump and the photometer. See Figure 6. A Gelman acropor 13 mm diameter filter AN-450, 0.45 μ pore size was used and was replaced before each new sample.

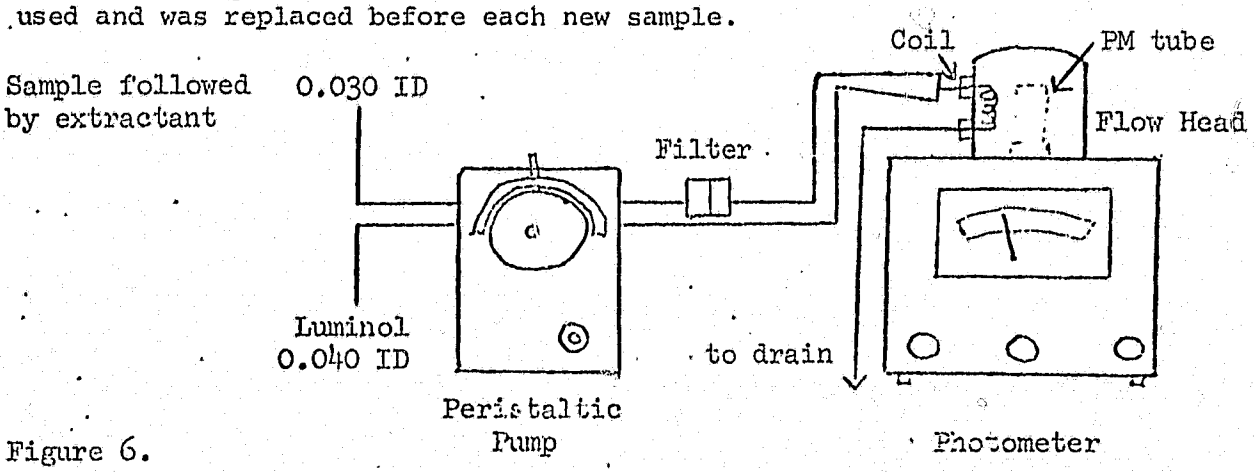
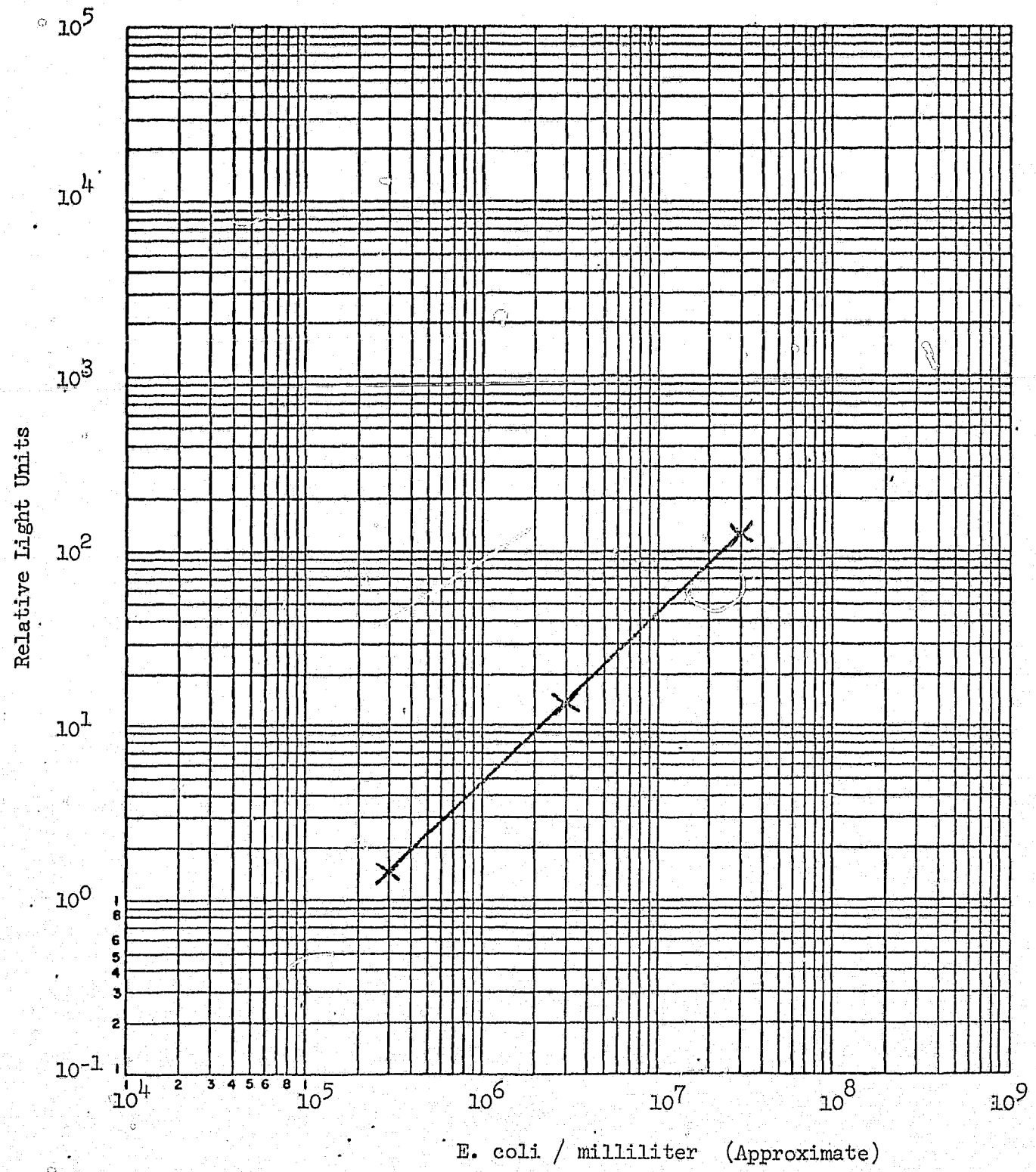


Figure 6.

Two milliliters of various concentrations of E. coli in saline were pulled through the in-line filter by the peristaltic pump. Five ml of saline were then pulled through to wash the cells on the filter. The collected bacteria were then extracted with 1 ml of 1.5N sodium hydroxide-50% Ethanol (Not Optimal) and that extract assayed as it passed through the reaction cell with the luminol mixture. Figure 7 is a graph of the experimental results. For a pure sample of E. coli, the light response is linear for changing concentrations in the in-line filter flow system. The porphyrins component from the bacteria appears to be released instantaneously on contact with the extractant.

Figure 7. Luminol light response to various concentrations of E. coli extracted off an in-line filter and assayed using the luminol-perborate flow system.



Acceptable washes were determined by washing an E. coli sample collected on a filter with 5 ml of wash. The bacteria were then extracted with 1.5N NaOH - 50% EtOH and the extract assayed with the luminol system to determine any loss of signal response due to washing. Table VI contains a list of the washes and concentrations used with the corresponding luminol response after extraction. It appears that except for ethanol any of those washes mentioned can be used without any harmful effects to the bacteria.

TABLE VI Results of Bacteria Washes

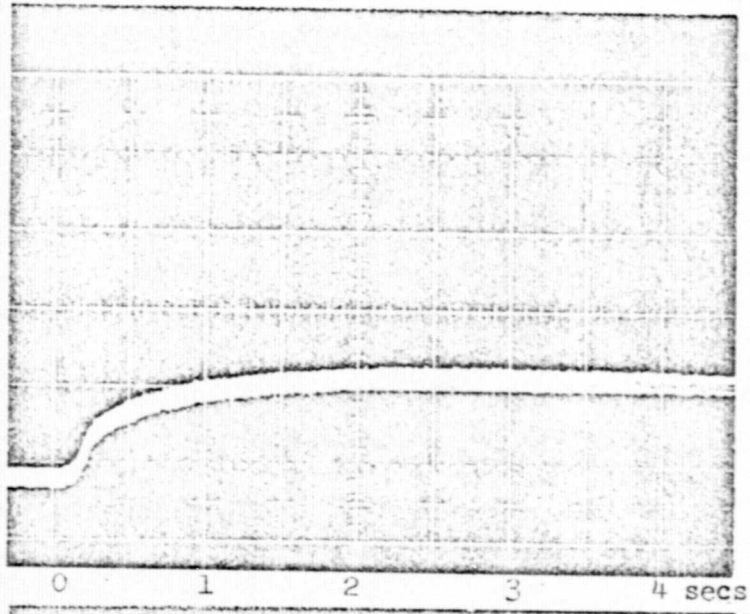
Washes	Mean Response from 10^7 E. coli extracted off filter
Sucrose	5.48×10^7 mv
0.3N NaOH	5.82×10^7 mv
0.75N NaOH	4.99×10^7 mv
1.0N NaOH	4.19×10^7 mv
1.5N NaOH	5.36×10^7 mv
3.0N NaOH	4.28×10^7 mv
Ethanol	2.69×10^7 mv
Blank from 1.5N NaOH washed filter	3.59×10^6

Time Rate of Various Catalysts for the Luminol Reaction.

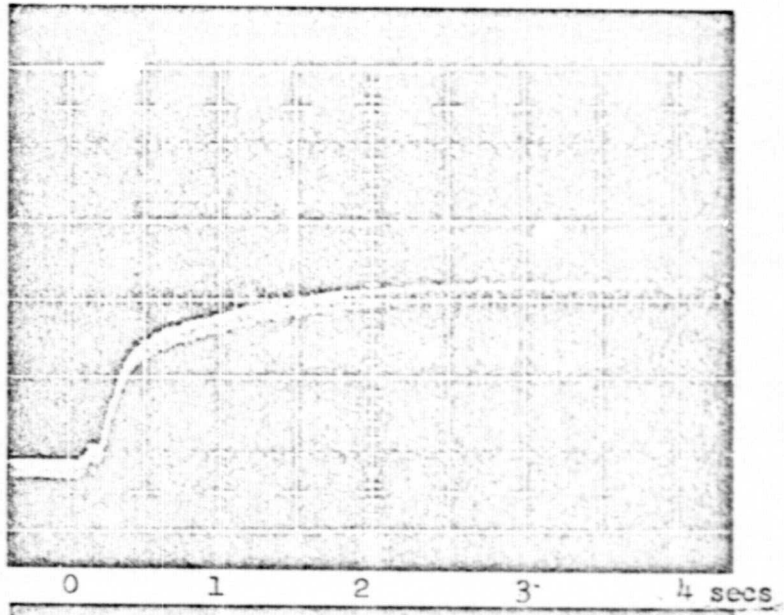
It has been determined that luminol reaction rates vary depending on the particular catalyst present. From the differences in reaction rates, the various luminol catalysts can be differentiated. Figures 8 & 9 show the response curves of various catalysts as a function of time. Hemoglobin, catalase, potassium ferricyanide, ferric chloride, and cytochrome C were all tested. The porphyrin molecules have a longer reaction time than do the inorganic molecules. Figure 10 illustrates how these characteristic curves can be used to differentiate between catalysts. The response from the potassium ferricyanide is quite rapid, and is essentially zero after 3 secs, while the response from hemoglobin remains high even after 4 seconds. The light response from hemoglobin can be differentiated from the ferricyanide in a mixture since at four seconds into the reaction the ferricyanide response is zero. At this point only hemoglobin is still reacting with the luminol. Whole bacteria have an even longer reaction period

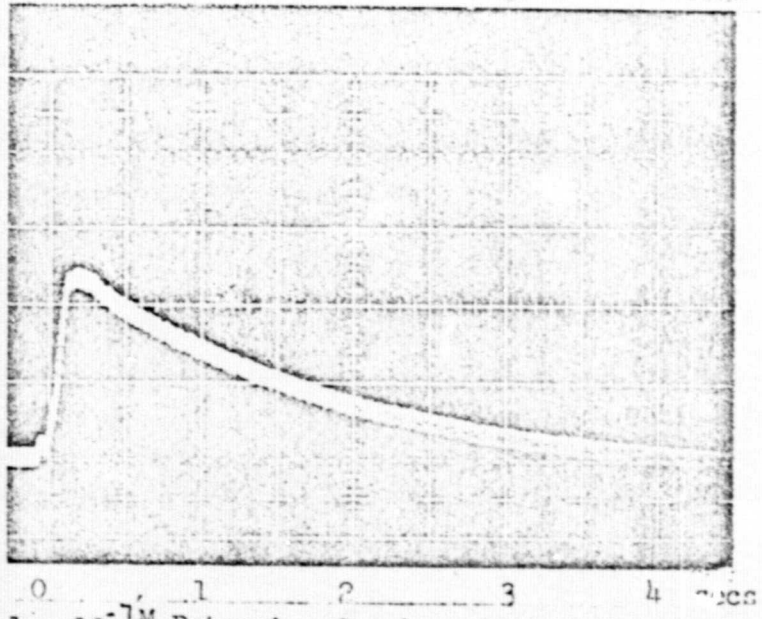
Figure 8. Luminol response to various catalysts as a function of time.

1×10^{-9} M Hemoglobin
Amplitude = 0.1 v Chem Glow x 30

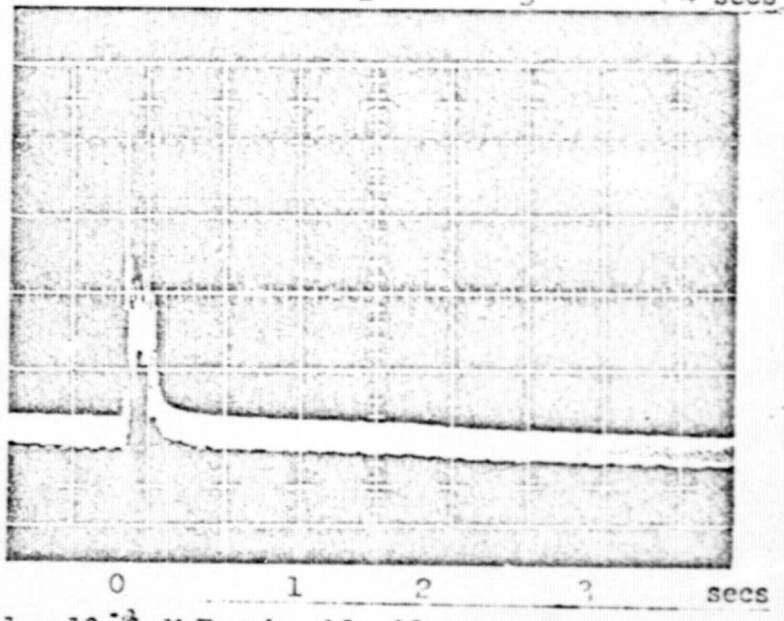


1×10^{-8} M Catalase
Amplitude = 0.1 v Chem Glow x 30





1×10^{-7} M Potassium ferricyanide
Amplitude = 0.1 v Chem Glow x 10

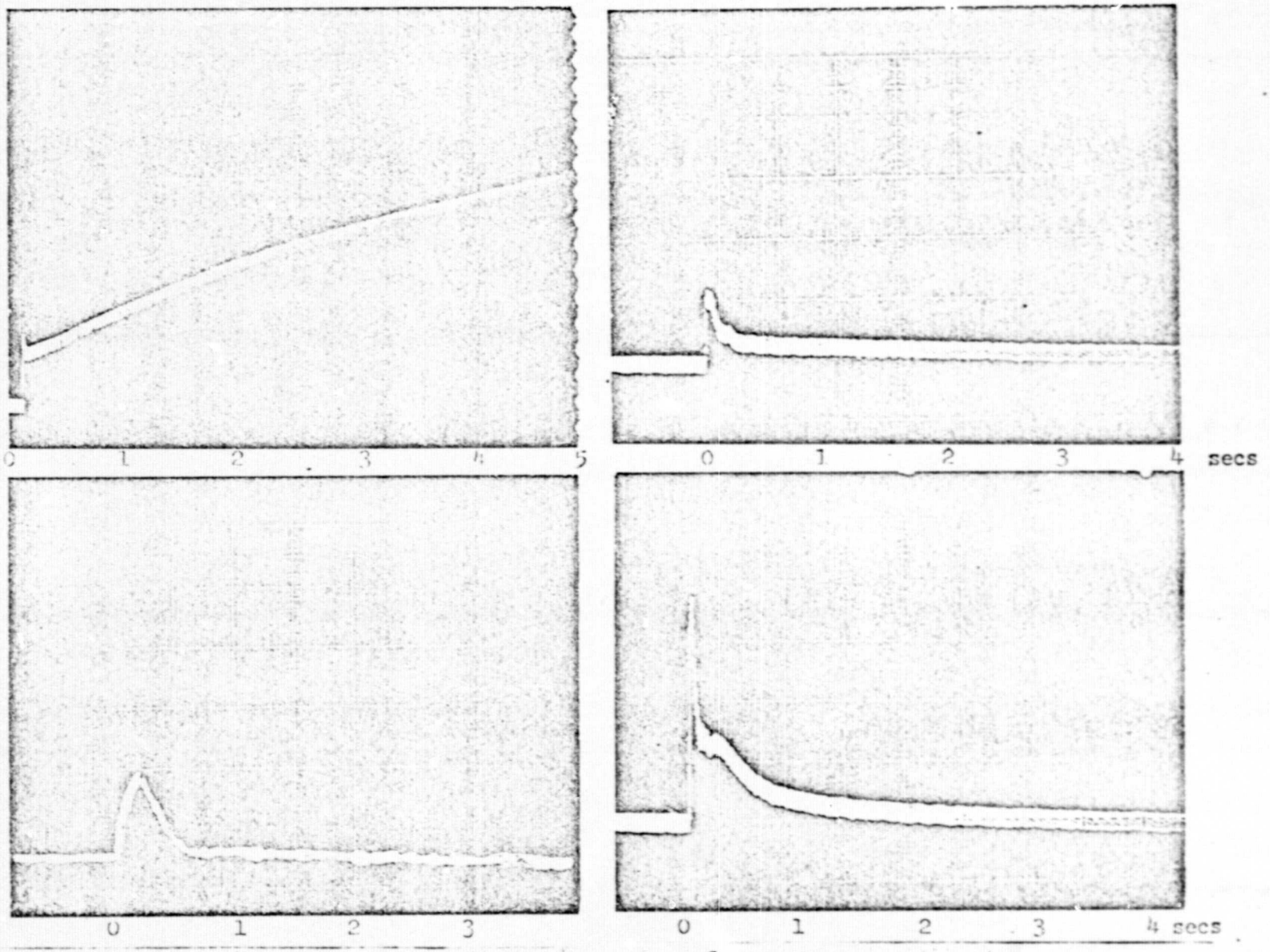


1×10^{-3} M Ferric chloride
Amplitude = 0.1 v Chem Glow x 100

Figure 9. Luminol response to various catalysts as a function of time.

1×10^{-7} M Cytochrome c
Amplitude = 0.1 v Chem Glow x 10

Deionized water
Amplitude = 0.1 v Chem Glow x 3

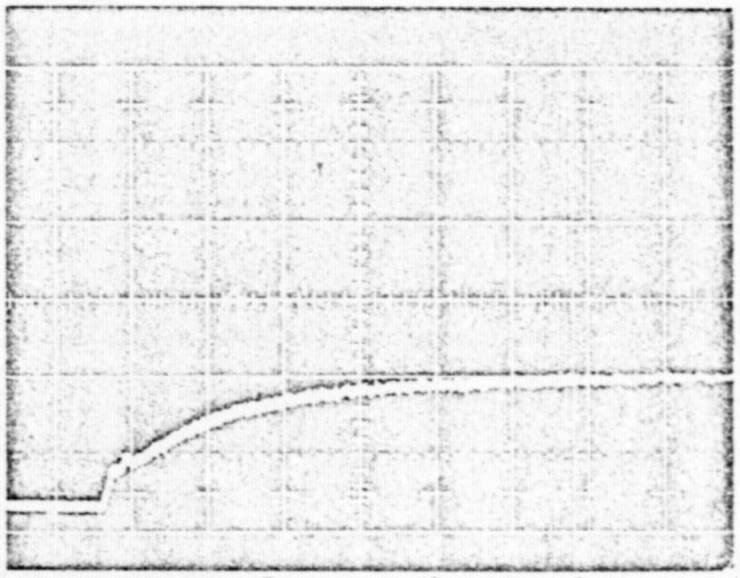


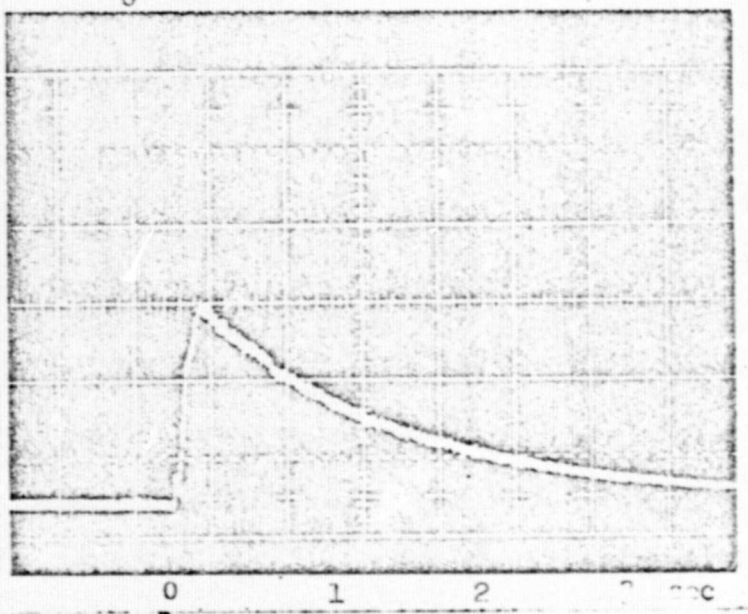
1×10^{-4} M Chlorophyll a in 90% acetone
Amplitude = 0.1 v Chem Glow x 3

1×10^{-2} M Magnesium sulfate
Amplitude = 0.1 Chem Glow x 3

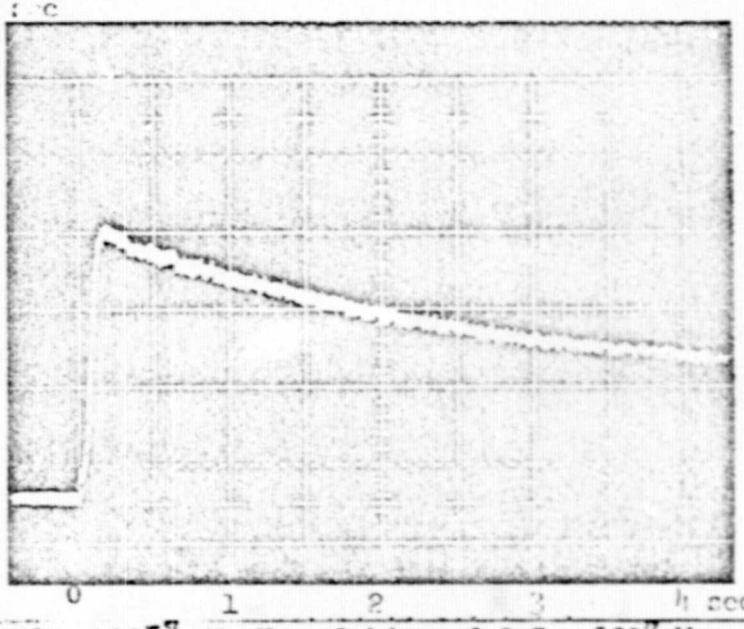
Figure 10. Use of reaction rate characteristics to differentiate between catalysts.

0.5×10^{-9} M Hemoglobin
Amplitude = 0.05 Chem Glow x 10



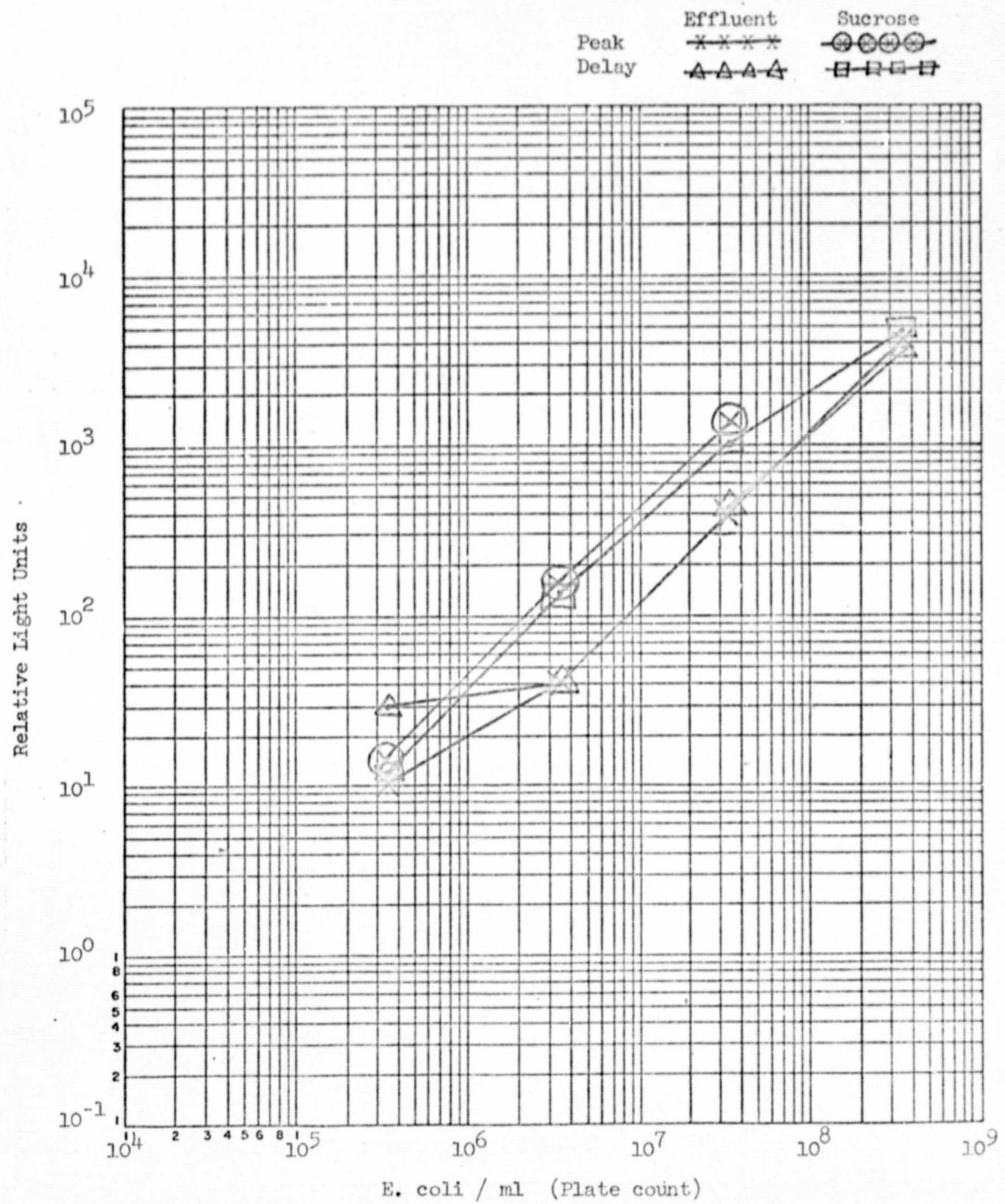


0.5×10^{-7} M Potassium ferricyanide
Amplitude = 0.05 v Chem Glow x 10



0.5×10^{-7} M Hemoglobin and 0.5×10^{-7} M Potassium ferricyanide
Amplitude = 0.05 C.Glow x10

Figure 11. Luminol assay of E. coli seeded wastewater effluent and isotonic sucrose (with and without time-delay response).

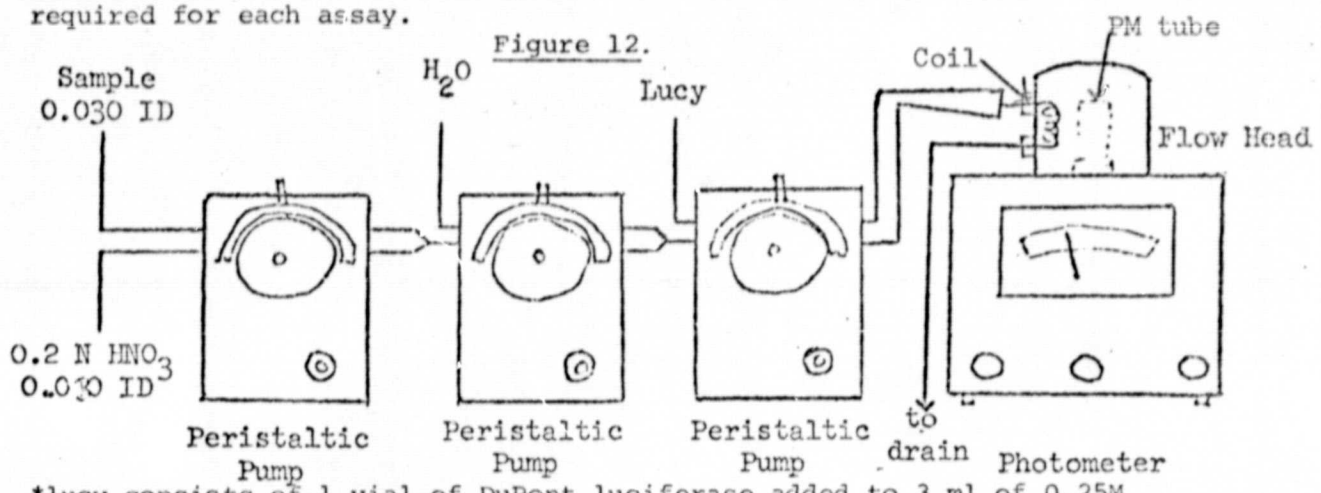


as long as 15 seconds for E. coli which should allow for differentiation between soluble porphyrins and whole bacteria.

Luminol assay of E. coli seeded wastewater effluent. Wastewater effluent was obtained from a local sewage treatment plant. Wastewater effluent and isotonic sucrose (9.25%) were seeded with E. coli. The luminol flow system was used for assaying the samples. Figure 11 shows the results of that assay. The response from the E. coli in isotonic sucrose was approximately 30% higher than that from the effluent. The time rate method (delay response) was used in order to eliminate any interference however according to the results no interference was present.

The basic bioluminescent system developed by GSFC is continuing to be used for the detection of bacteria in wastewater samples. This system involves the reaction between adenosine triphosphate (ATP) and firefly luciferase. The work so far has involved the incorporation of the assay in a flow system design. Figure 12 is a diagram of the ATP flow system. 0.3 ml of luciferase are required for each assay.

Figure 12.



*lucy consists of 1 vial of DuPont luciferase added to 3 ml of 0.25M Tris, 0.01M $MgCl_2$, 10^{-3}M Cleland's reagent, pH 8.2.

Work in the future will include further development of the extraction of the bacteria on the filter. In particular, experiments will be conducted to determine the efficiencies of the washes for eliminating interference of the luminol system. Further work may be necessary for the time rate method for elimination of luminol interferences.

Further development of the bioluminescent system will concern the areas of sample preparation. The sample should be assayed in its most concentrated form for optimal sensitivity. This will include optimal sample to nitric acid ratios. Correct pH and optimal mixing point for maximum light detection will be determined.

Appendix A.

EFFECTS OF HYDROGEN PEROXIDE CONCENTRATIONS AND ORDER OF ADDITION OF REACTANTS ON THE LIGHT RESPONSE OF CRUDE LUMINOL TO HEMOGLOBIN USING A DUPONT BIOMETER

January 30, 1975

by

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This report is the result of a one month study during a mini-mester cooperative program between GSFC and Loyola and Goucher

Time is an essential factor in the detection and treatment of bacterial infections. Present methods of detection, however, necessitate an incubation period of 24 to 48 hours. The development of a rapid automatable technique for the detection of infection in biological fluids has been undertaken at Goddard Space Flight Center. One such study deals with the use of the luminol chemiluminescence method for rapid detection of bacteria.

The principle of the luminol chemiluminescence method for detecting bacteria is based on microbial activation of the oxidation of the luminol monoanion by hydrogen peroxide. See figure 1 for the general reacti n mechanism. In an aqueous alkaline solution luminol (5-amino-2,3-dihydro-1,4-phthalzinedione) is reported to be in the form of the monoanion which can be rapidly and energetically oxidized by hydrogen peroxide in the presence of iron porphyrins contained in microorganisms[1]. The intermediate products of oxidation could include free radicals such as hydroxyl, luminol, O_2^-, and luminol endoperoxide[2]. A final reaction product, the aminophthalate dianion, is formed in an electronically excited state which decomposes to the ground electronic state with loss of the excess energy as blue light (230 nm). When the reagents are in excess, the intensity of the light has been shown to be proportional to the concentration of bacterial porphyrins. Interference by free Fe^{++} has been minimized through use of ethylene diamine tetraacetate (EDTA), a chelating agent which binds any free Fe^{++}.

This chemiluminescence method has advantages over even a bio-luminescence method. Reagents used in this method are inexpensive

and remain stable at room temperature. Preparation of the reagent and the analytical technique also do not require the degree of care and manipulation that is needed in the bioluminescence method. Since cells are lysed upon introduction to the alkaline luminol solution, separate steps for lysing, and thus time are not necessary. Complications due to deterioration of porphyrins in the presence of lysed cells are also avoided. Overall, this chemiluminescence method demonstrates great adaptability to field applications in which adequate laboratory facilities are not present.

This paper reports the results of various investigations into the different parameters affecting the luminol reaction. Factors investigated included the response to a particular porphyrin, hemoglobin; the effect of various hydrogen peroxide concentrations; as well as the order of the addition of reactants.

Experimental

Apparatus. The Luminescence 760 Biometer manufactured by E. I. Dupont de Nemours and Co., Inc. was the instrument used to measure the light emission from the luminol-peroxide reaction. A schematic diagram of the system can be found in Figure 2. An information bulletin for the instrument is reproduced in Appendix A.

The Biometer is a photometer specifically designed for measurement of luminescent reactions. As can be seen in Figure 2, the system consists of a rotary reaction chamber coupled to a photomultiplier tube. A section of the rotary chamber is cut out to accomodate a 6-by 50- mm glass cuvette. Immediately above the cuvette holder is a small injection port through which the sample is injected via

needle and syringe. An analog signal from the photomultiplier tube is amplified and utilized to charge a "memory" capacitor. This design allows the peak to be accumulated for three seconds and exhibited in the form of a digital display. The instrument also provides automatic range change over five decades to accomodate a wide range of light intensities. Figure 3 shows a typical response curve. Data are presented in terms of peak height (maximum light intensity).

Reagents. The stock luminol solution contained 5.65×10^{-7} M luminol, 1.5 N NaOH, and 4.46×10^{-6} M EDTA. Hemoglobin samples were prepared from crystalized hemoglobin. Hydrogen peroxide solutions were prepared from 30% reagent grade hydrogen peroxide. All reagents were prepared using distilled water.

Procedure. A 0.3 ml sample volume was used in this study. 0.1 ml of the stock luminol mixture and 0.1 ml of the hemoglobin sample were pipetted into the reaction cuvette. 0.1 ml of the hydrogen peroxide was then injected into the cuvette via tuberculin syringe. The sample was then assayed with the Biometer.

Results and Discussion

Response to Hemoglobin Concentration. Figure 4 is a plot of light intensity catalyzed by hemoglobin as a function of concentration. from 1.47×10^{-7} M to 1.47×10^{-5} M hemoglobin. The response is linear

throughout this range.

Hemoglobin concentrations greater than 1.47×10^{-5} M could not be investigated due to the limited range of the Fiometer. 10^{-8} M hemoglobin proved to be the detection limit.

Effect of Hydrogen Peroxide Concentration. 5.67×10^{-7} M luminol and an intermediate hemoglobin concentration of 1.47×10^{-8} M were tested with varying H_2O_2 concentrations. The concentrations tested ranged from .01% to 5%. Figure 5 illustrates the light emissions obtained with the respective H_2O_2 concentrations. A plateau was reached with values from 0.1% to 1%, with a decline in light emission prior to and following these points. H_2O_2 concentrations from 1% to 5% were then tested with 5.67×10^{-7} M luminol and a high hemoglobin concentration of 1.47×10^{-6} M. Again, as illustrated by Figure 6, maximum light emission was obtained with 1% H_2O_2. Adaptability o 1% H_2O_2 to yield high light emission in .he presence of both high and intermediate hemoglobin concentrations was demonstrated.

Effect of Sequence of Addition of Reactants. Three possibilities regarding the order of addition of reactants to the luminol system were investigated: 1) hydrogen peroxide added to luminol and hemoglobin, 2) hemoglobin added to luminol and hydrogen peroxide, and 3) luminol added to hemoglobin and hydrogen peroxide. Figure 7 demonstrates that maximum light emission occurs when hemoglobin is

injected to the luminol and hydrogen peroxide solution. Minimum light emission occurs when hydrogen peroxide is injected into the hemoglobin and luminol mixture. A possible explanation for these results is based on the fact that oxygen rapidly oxidizes Fe^{++} to ferric hydroxide ($Fe(OH)_3$). Although the mechanism of aqueous luminol is not known, it is likely that an intermediate produced in the oxidation of Fe^{++} by dissolved oxygen is the species reacting with luminol[3]. From this assumption, it can be concluded that some of the luminol had already been oxidized prior to injection of hydrogen peroxide. High residual light readings before injection of hydrogen peroxide correlate with these conclusions. No light producing reaction takes place between luminol and peroxide, or between the hemoglobin and the peroxide. Thus, maximum light emission is obtained with injection of hemoglobin and luminol, respectively.

Effect of 50% Ethanol as Oxidizing Agent. In this experiment, 50% ethanol was used as the source of the oxidizing agent rather than hydrogen peroxide which had formerly been used. The reason for this change of procedure is due to the high oxidation potential of hydrogen peroxide which caused a high light emission from the blank. It is believed that by taking advantage of the higher solubility of oxygen in an organic solvent such as ethanol,

the dissolved oxygen will be sufficient to oxidize the Fe^{++} catalyzed luminol reaction while at the same time keeping the blank at a relatively low light level.

Figure 8. shows the luminol reaction in the 50% ethanol solvent to exhibit a linear response to the hemoglobin concentration within 10^{-8} to 10^{-6} M. In the ethanol solvent the detection limit of the hemoglobin concentration was increased to 1.47×10^{-9}M. This is a ten-fold improvement over the peroxide system. The maximum limit of detectability wason the order of 10^{-6} M, after this point, linearity greatly decreased.

Due to the greater sensitivity and lower blank value, the ethanol system warrants further investigation.

FOOTNOTES

[1]Henry Linschitz in "Light and Life," W.D. Mc Elroy and B. Glass, Ed., Johns Hopkins Press, Baltimore, Maryland, 1961, 173.

[2]Emil H. White, ibid., 189.

[3]W. Rudolf Seitz and David M. Hercules, "Determination of Trace Amounts of Iron(II) Using Chemiluminescence Analysis," Analytical Chemistry, 44 No. 13, 2145, (1972).

FIGURE 1

DETECTION OF BACTERIA *via* CHEMILUMINESCENCE OF LUMINOL

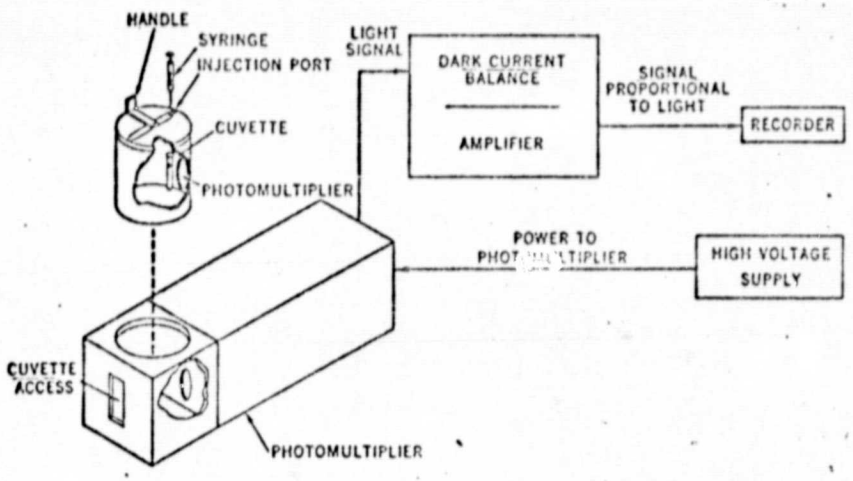


Figure 2. Light measuring instrumentation.

Figure 3. Typical response curve for luminol - peroxide reaction. Recorded using a Chem-Glow Photometer, Aminco, Inc., and X - Y Recorder.

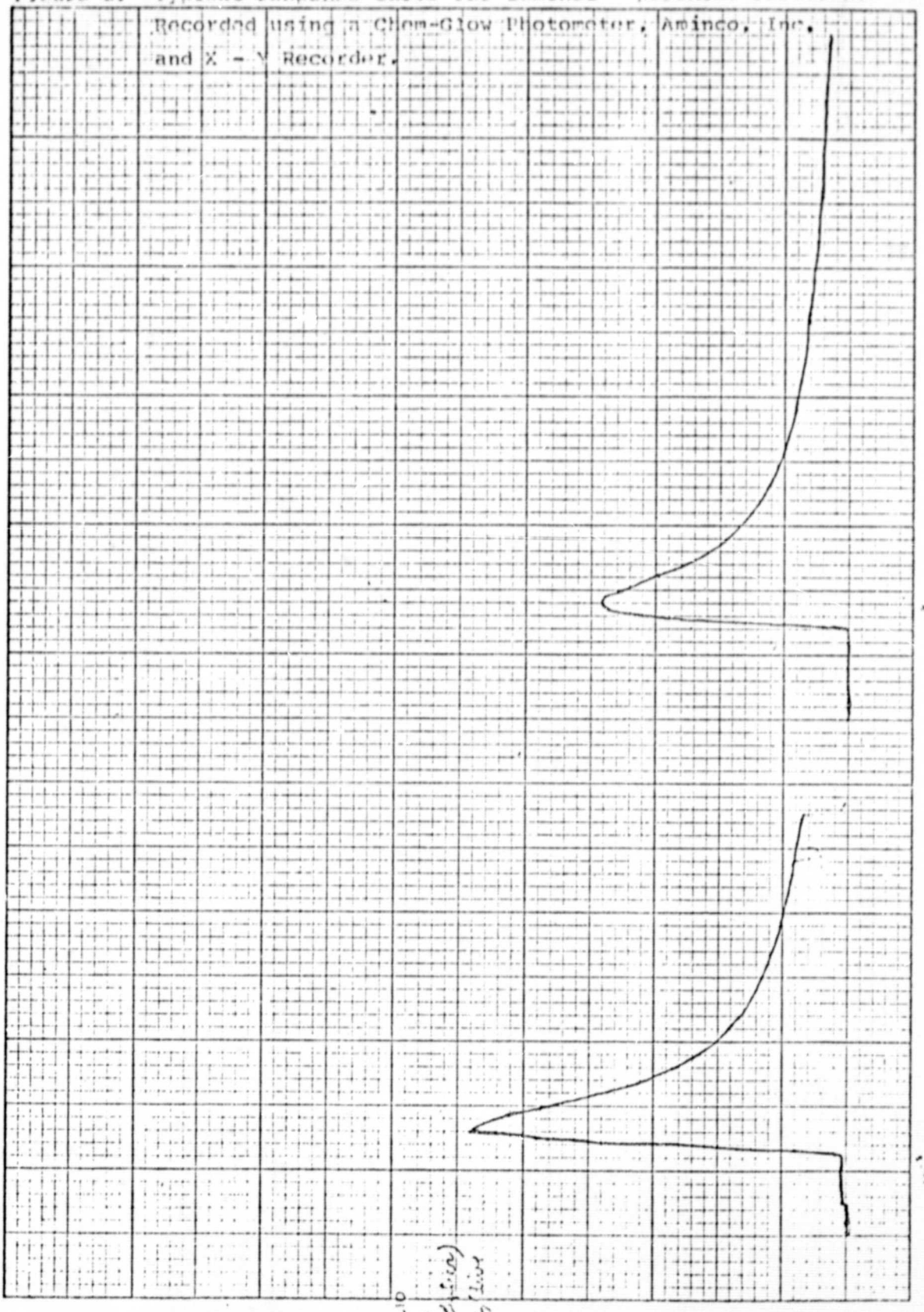


Figure 4

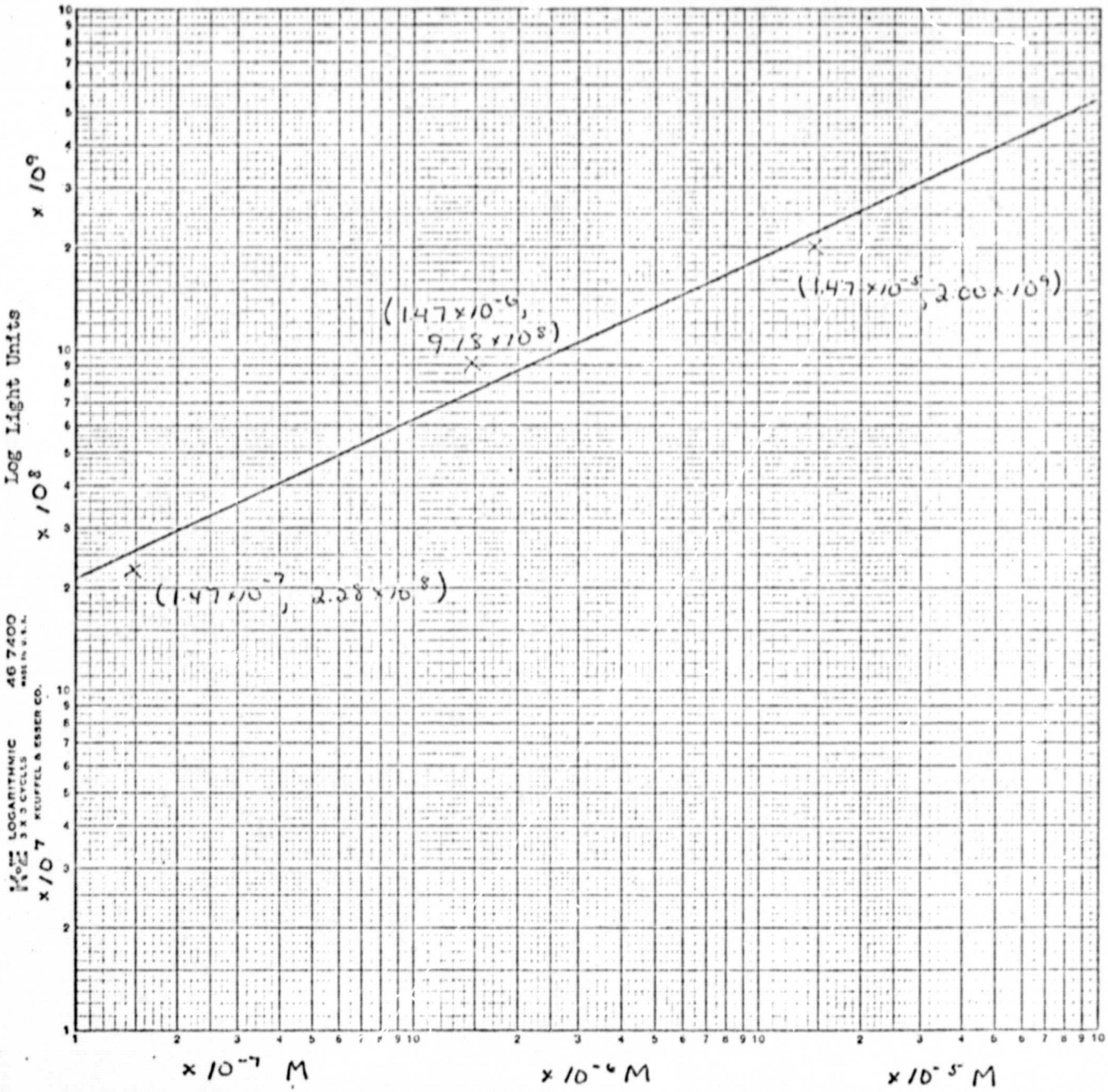

Log Light Units
x 10^9
x 10^8
x 10^7
(1.47 x 10^-6, 9.13 x 10^8)
(1.47 x 10^-5, 2.00 x 10^9)
(1.47 x 10^-7, 2.28 x 10^8)
x 10^-7 M
x 10^-6 M
x 10^-5 M
Log of Hemoglobin Concentration (Molar)

Figure 5

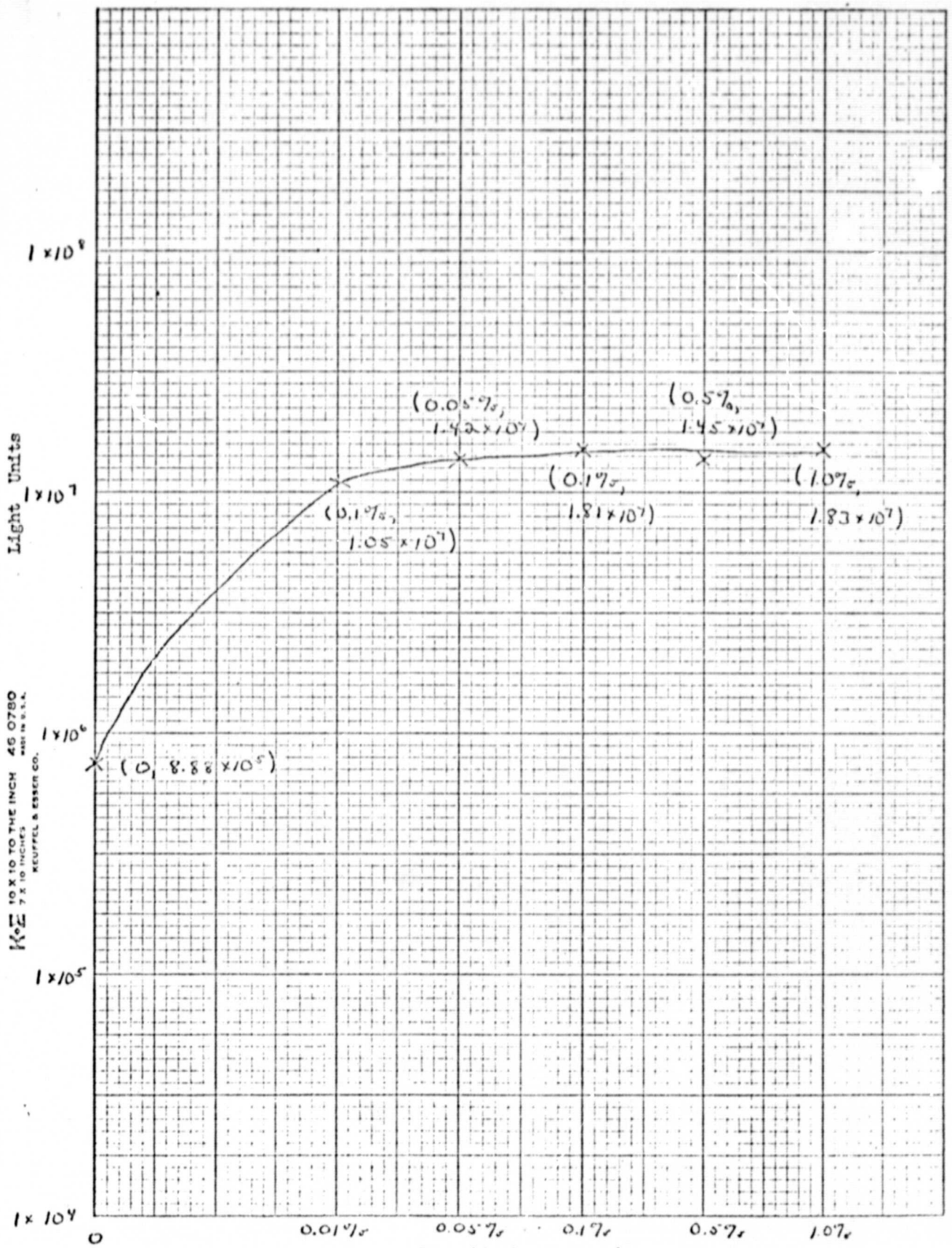


Figure 6

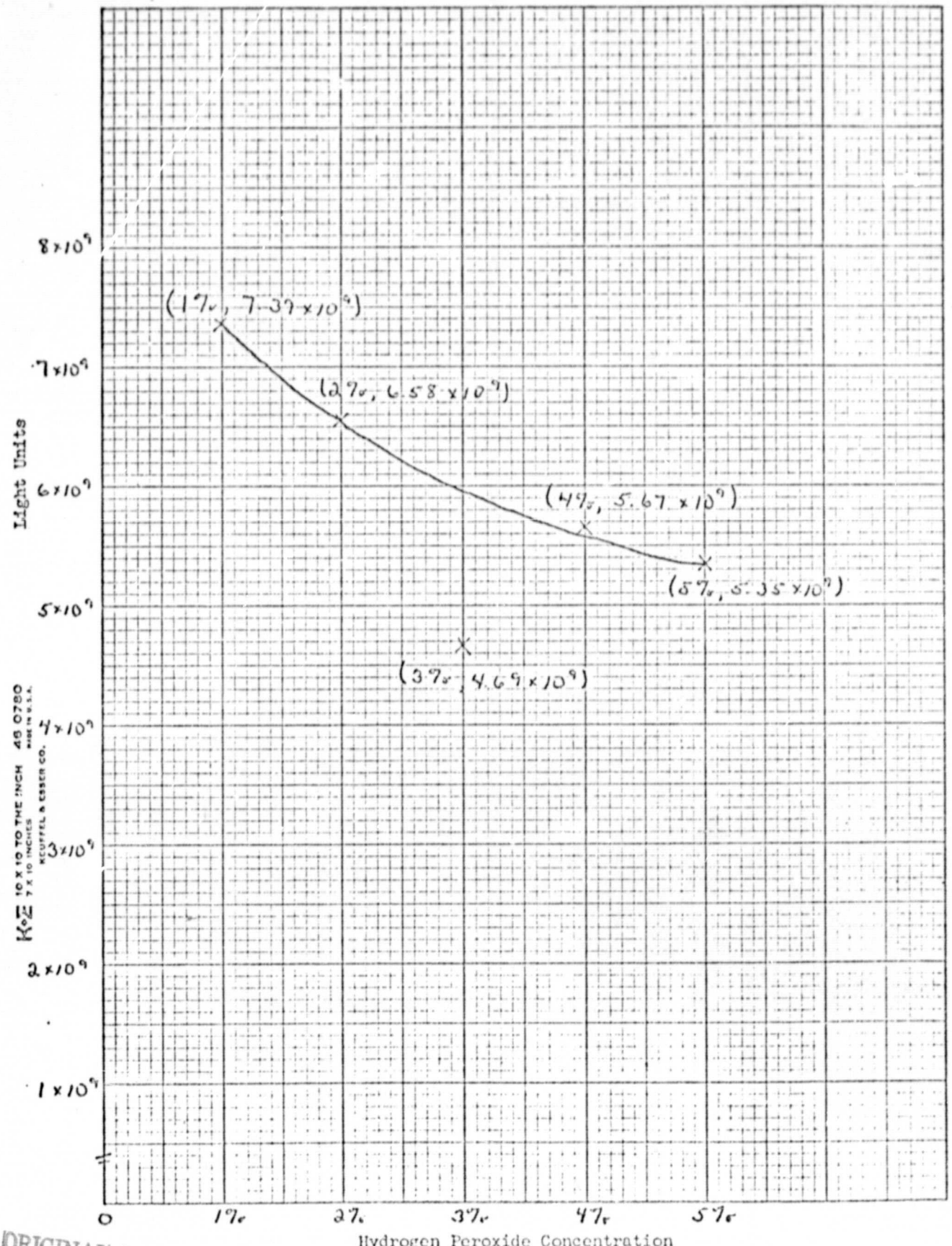
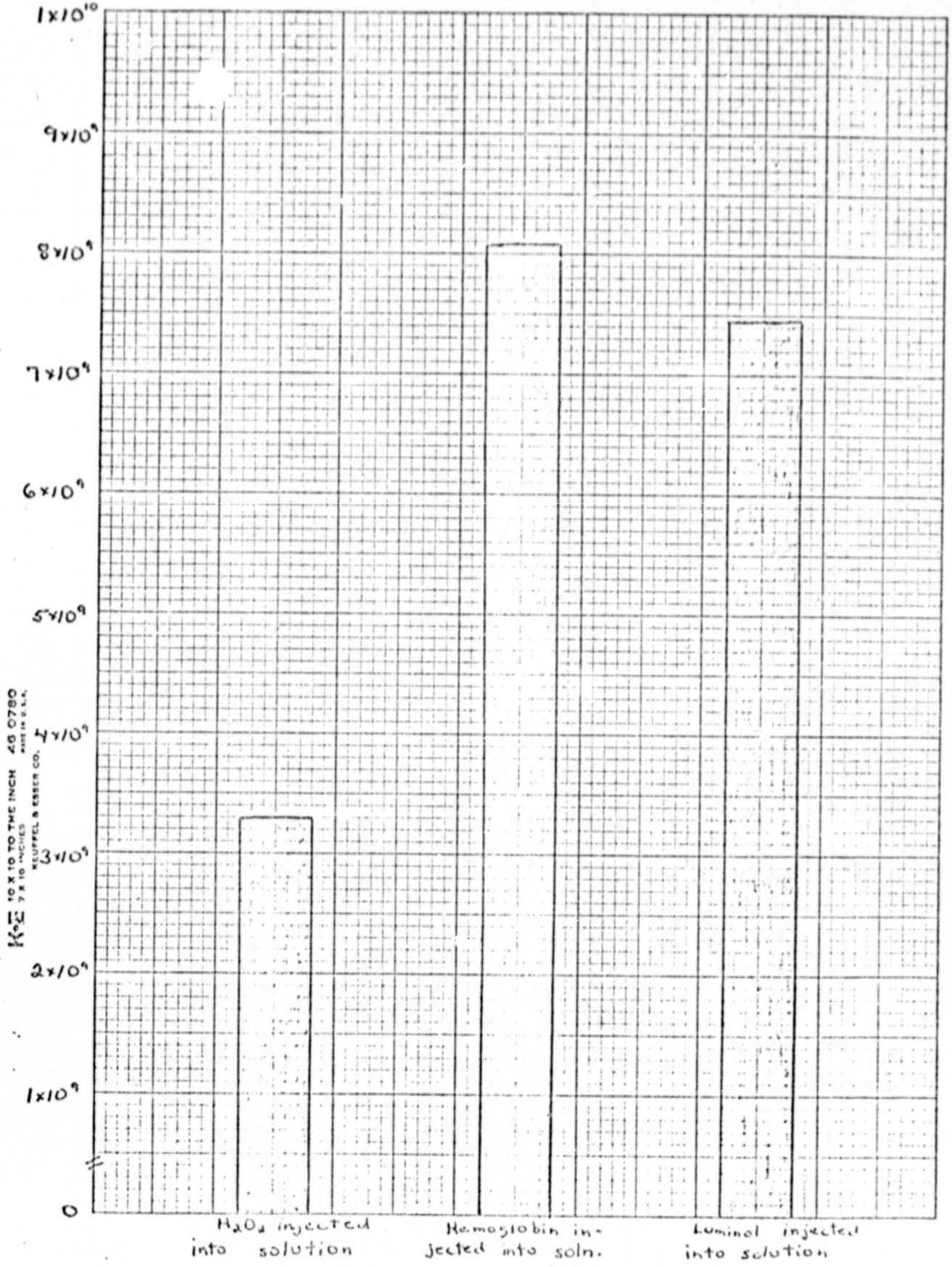


Figure 7


1x10^10
9x10^9
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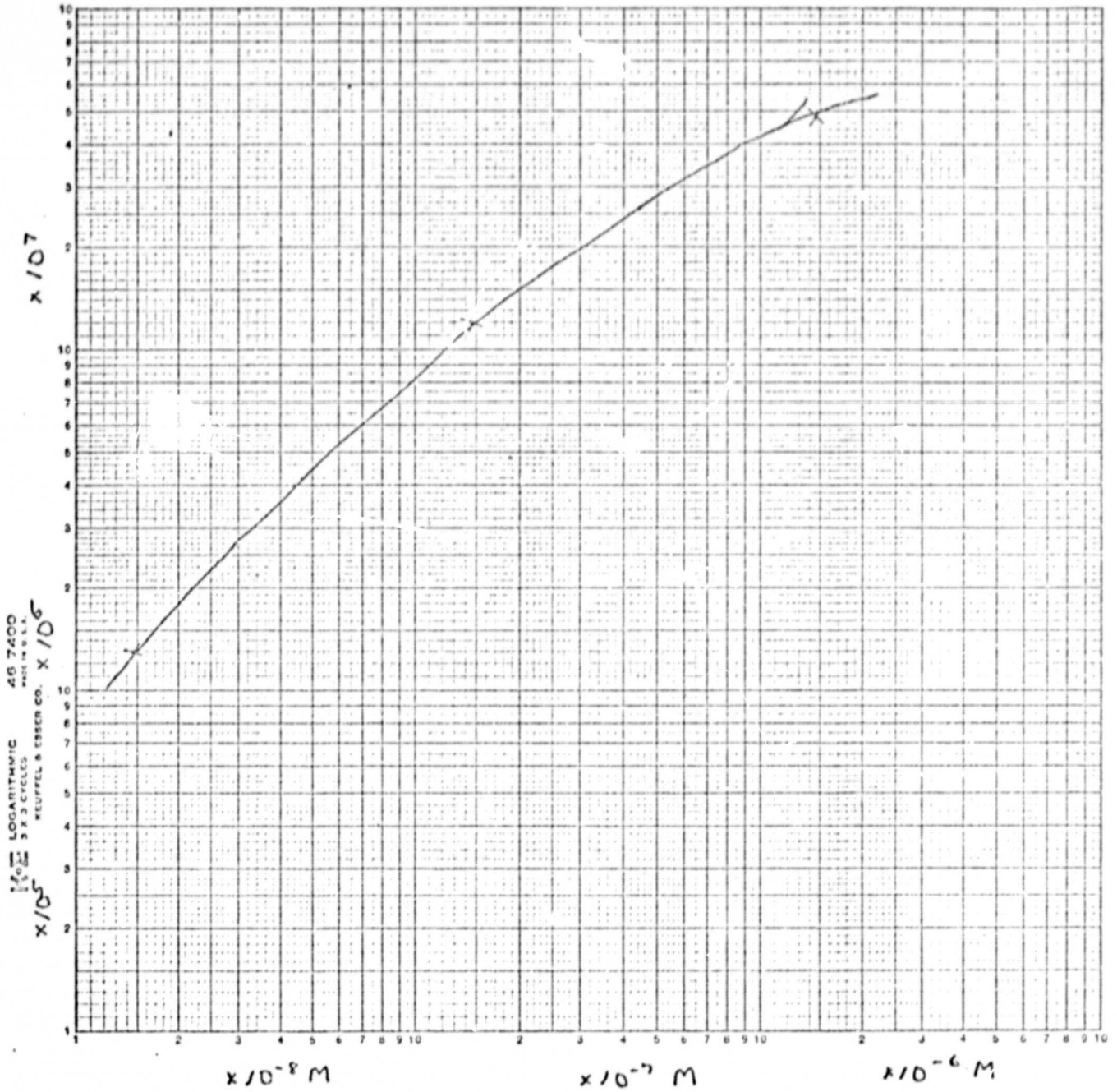
0
KoE 10 X 10 TO THE INCH 46 0780
7 X 10 INCHES KEUFFEL & ESSER CO.
H2O2 injected into solution
Hemoglobin injected into soln.
Luminol injected into solution

Figure 8.


x 10⁷
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LOGARITHMIC
3 X 3 CYCLES
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x 10⁻⁸ M
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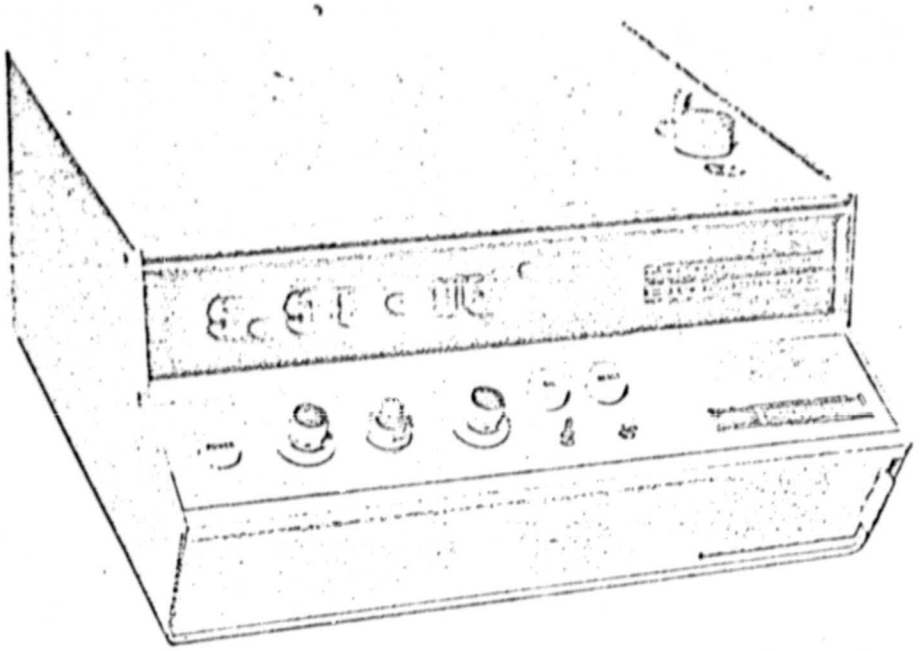
Appendix A.

luminescence biometer



The Du Pont Luminescence Biometer[1] is a semi-automatic photometer for measurement of bioluminescent and chemiluminescent reactions. Using the firefly luciferase-luciferin reaction for rapid assay of adenosine triphosphate (ATP), it is sensitive to 10^{-13} grams.

Studies by Du Pont and Hazleton Laboratories, Inc.[2] have shown that the quantity of cellular ATP extracted from bacterial cells is proportional to the total number of cells. A study of thirteen bacteria species[3] showed a range from 2.2 to 10.3 x 10^{-10} µg ATP/cell, and indicated that a mean value of 5 x 10^{-10} µg ATP/cell could be used for determining total bacteria counts.

The Du Pont system can be applied to the measurement of viable bacterial populations in urine, blood, food, dairy products, water and other research and industrial applications.

FEATURES

- Rapid test procedure: bacterial count in 10 minutes; ATP measurement in 10 seconds.
- Digital readout direct in ATP or bacteria
- Sensitive to 10^{-13} grams of ATP or 1000 bacterial cells
- Sample size: normally 10 microliters
- Parallel strip chart record optional
- Automatic compensation for dark current and inherent light
- Automatic ranging of readout over five decades
- Biometer performance checked by standard light source
- Sample preparation kits available for a variety of applications

Bibliography

Cormier, M.J., D.M. Hercules, and J. Lee. Chemiluminescence and Bioluminescence. New York: Plenum Press, 1973.

Johnson, Frank H. and Haneda, Yata. Bioluminescence in Progress. Princeton: Princeton University Press, 1966.

Mc Elroy, W.D. and Glass, B. Light and Life. Baltimore: Johns Hopkins Press, 1961.

Oleniacz, Walter S.; Pisano, Michael A.; Rosenfield, Martin H.; and Elgart, Robert L. "Chemiluminescent Method for Detecting Microorganisms in Water," Environmental Science and Technology

Seitz, Rudolf W. and Hercules, David H. "Determination of Trace Amounts of Iron(II) Using Chemiluminescence Analysis" Analytical Chemistry 44 No. 13 (Nov. 1972): 2143-2149.

Appendix B.

A FURTHER STUDY OF THE LUMINOL REACTION SYSTEM

Ch 420 L Chemistry Research

Loyola College

Baltimore, Maryland

Richard R. Thomas

20 May 1975

Luminol hydrochloride from three recrystallizations of reagent grade luminol produces a linear light response for various hemoglobin concentrations as well as the highest light emission for a given hemoglobin sample. The detection limit for hemoglobin is 1.47×10^{-11} M with the response linear up to at least 1.47×10^{-8} M. Blank levels have been decreased to acceptable levels and reproducability improved to within 5% variation. Luminol chemiluminescent detection of bacteria was achieved within a range of about 10^{6} bacteria per milliliter to the detection limit of about 10^{5} bacteria per milliliter which is in agreement with the literature findings.[1]

Certain basic procedures and operating conditions were reported in "A Preliminary Study of the Luminol Reaction".[2] Further refinements of the system were necessary and several problems needed to be solved before the system could be put into operation as a bacterial detection system.

The areas investigated and described in this paper include the determination of the optimal luminol compound in terms of purity, linear light response, and maximum light emission. Basic procedural modifications were necessary to eliminate unexplainably high blank levels and unacceptable variations in repeated sample assays. Finally an attempt was made to test the system as a bacterial detection tool.

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[1] N.D. Searle, "Applications of Chemiluminescence to Bacterial Analysis," presented at the 2nd Annual Meeting of the American Society for Photobiology, July 22-26, 1974, University of British Columbia, Vancouver, B.C.

[2] R.R. Thomas, "A Preliminary Study of the Luminol Reaction," NASA / Goddard Space Flight Center, 30 Jan 1975.

Apparatus

The Aminco Chem-Glow Photometer, Model J4-7441, shown in Figure 1, was used throughout this study. The photometer consists of a reaction chamber mounted on top of a photomultiplier microphotometer. When the sample is injected into the reaction chamber, the emitted light from the luminescent reaction is reflected to the photomultiplier tube which converts the light to corresponding electrical signals. The signals were then recorded using strip chart recorders. Two recorders were used during the course of the investigation, a Sargent recorder and a Houston Instruments integrating recorder.

Procedure

The basic procedure for the assay of a sample included pipetting 0.1 ml of 5.0×10^{-7} M luminol compound in 1.5 N NaOH and 0.1 ml of 1% hydrogen peroxide into a reaction cuvette. 0.1 ml of the sample was injected into the reaction cuvette via tuberculin syringe and the light emission of the reaction was measured by the Chem-Glow Photometer and recorded on the strip chart recorder.

Results and Discussion

I. Determination of Optimal Luminol Compound

Six luminol compounds were studied: (1) reagent grade luminol, J.T. Baker Chem. Co.; (2) luminol hydrobromide #1, prepared by recrystallization of the reagent grade luminol in concentrated hydrobromic acid; (3) luminol·HBr #2, prepared by two recrystallizations of the reagent grade luminol; (4) luminol hydrochloride, prepared by acidifying (with hydrochloric acid) an alkaline solution of luminol·HBr #2 in water and acetone; (5) the sodium salt of luminol; and (6) sodium lumniol prepared by M.M. Rauhut.

As shown in Figure 2, the luminol·HCl exhibited the best linear response to hemoglobin samples within a range of 1.47×10^{-9} M to 1.47×10^{-11} M. Overall, the maximum light response was also exhibited by this compound. It should be noticed that a new minimum limit of detection for a hemoglobin sample was established by the luminol·HCl. The new detection limit is 1.47×10^{-11} M hemoglobin compared to the detection limit of 1.47×10^{-8} M previously established in "A Preliminary Study of the Luminol Reaction."

II. Procedural Modifications

In an effort to decrease blank levels, it was observed that impurities contained in the injection syringe contributed to high light levels of blanks. By rinsing the syringe in 0.1 M EDTA, the blank levels can be significantly decreased.

Reproducability does not seem to be significantly affected by the measurement of total light emission as opposed to the peak light emission of the luminol reaction. Mixing the reaction solution, luminol solution and hydrogen peroxide, does however greatly affect the reproducability of the light emission for a particular sample. A homogeneous mixture of the luminol and peroxide solutions improves the reproducability to within a 5% variation.

III. Applications to Bacterial Detection

A sample containing approximately 10^6 bacteria per ml[3] (E. Coli B) of nutrient broth was detected using the luminol reaction system. The detection limit for the system was found to be approximately 10^5 bacteria per ml of nutrient broth. This is the approximate range and

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[3] Determined by visual inspection of turbidity by Rev. James T. Maier, S.J., Assistant Professor of Biology, Loyola College.

detection limit found by N.D. Searle.

Much work still needs to be done in this area of the application of the luminol system to the detection of bacteria. In the future, most of the investigations will be in this direction.

Acknowledgement

The research described here was carried out in the laboratories of the Department of Chemistry of Loyola College, Baltimore Maryland. The author wishes to thank NASA / Goddard Space Flight Center for the use of their equipment and special thanks to Dr. David F. Roswell for his advice and assistance.

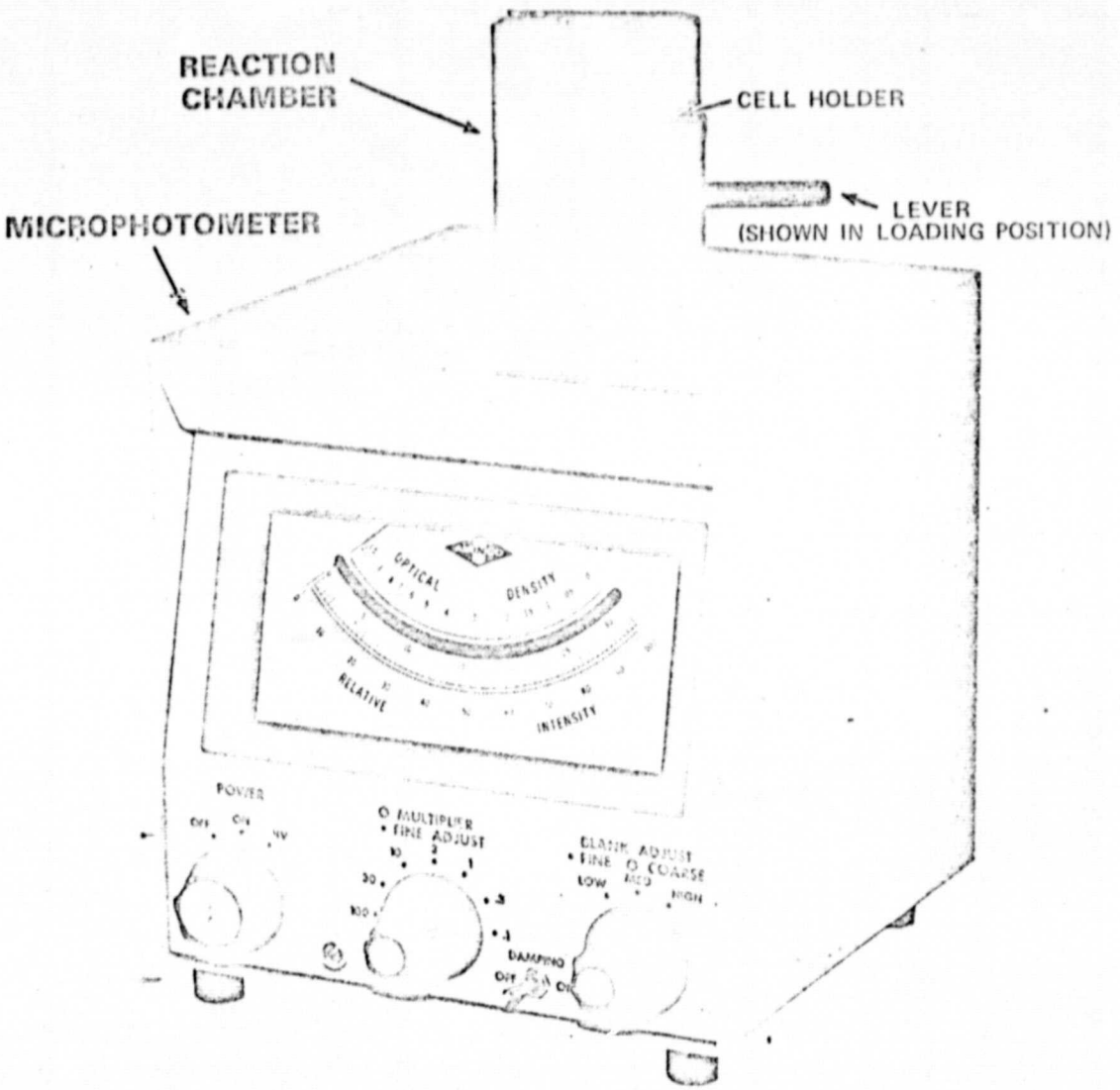
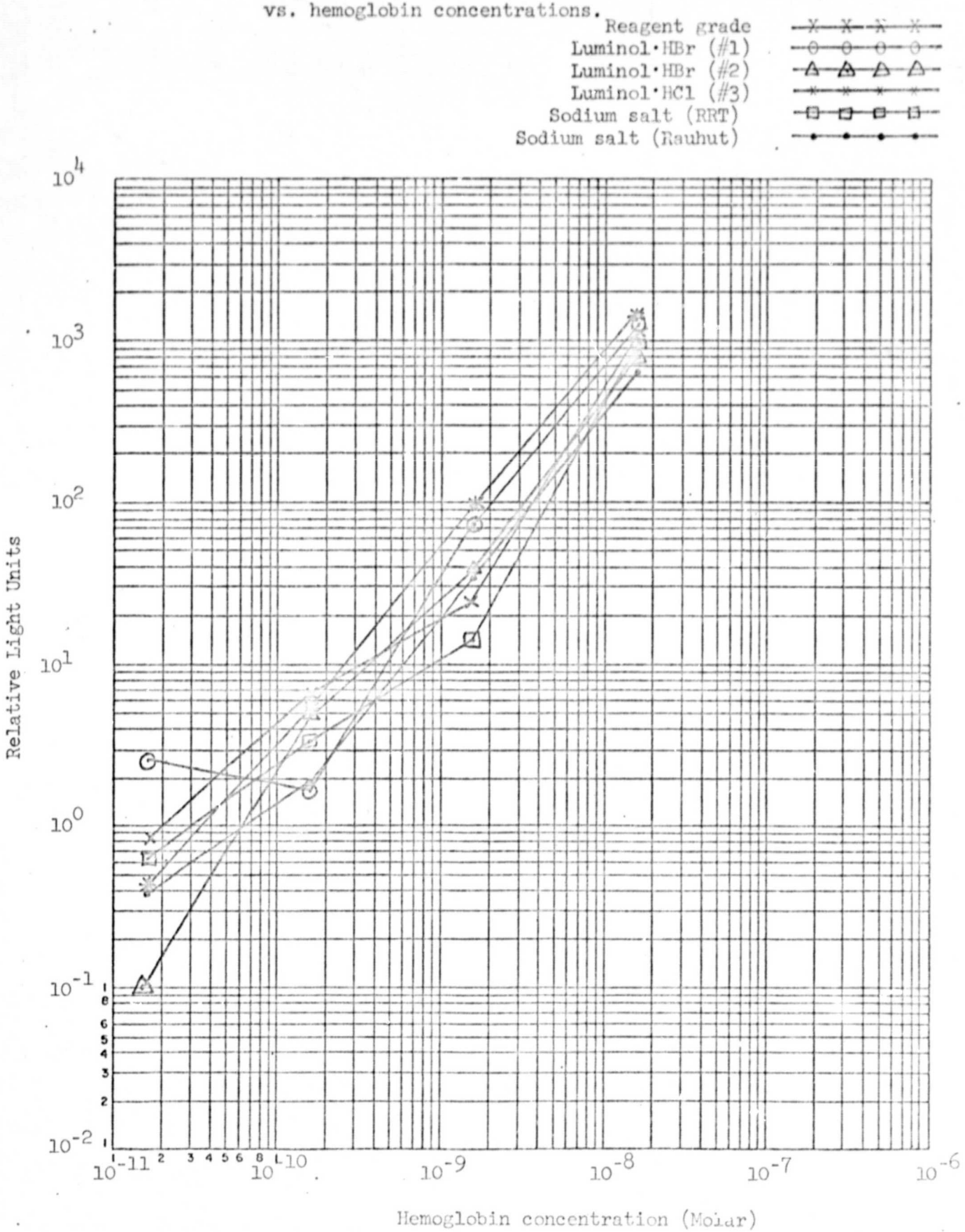


Figure 1. CHEM–GLOW Photometer.

Figure 2. Graph of light emissions from various luminol compounds vs. hemoglobin concentrations.



References

1. E. W. Chappelle and G. L. Picciolo. "Laboratory Procedures Manual for the Firefly Luciferase Assay for Adenosine Triphosphate (ATP)." NASA X-726-75-1. January 1975.

2. W. R. Seitz and D. M. Hercules. "Determination of Trace Amounts of Iron (II) Using Chemiluminescence Analysis". Analytical Chemistry. 44, 13, November 1972, p. 2143.

Bibliography

Ewetz, L. and Strangert, K. A Simple Method for Detection of Bacteriuria with an Automated Chemiluminescence Technique. ACTA Path. Microbiol. Scand. Section B. 82:375-381,1974.

Olenïacz, W. S., Pissno, M. A., Rosenfeld, M. H. and Elgart, R. L. Chemiluminescent Method for Detecting Microorganisms in Water. Environmental Science and Technology. 2:1030-1033, 1968.

Searle, Norma D. Applications of Chemiluminescence to Bacterial Analysis. Analytical Applications of Bioluminescence and Chemiluminescence. E. W. Chappelle and G. L. Picciolo, eds., NASA SP-388, 1975.