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BIOLOGICAL WATER QUALITY MONITORING USING CHEMILUMINESCENT AND BIOLUMINESCENT TECHNIQUES

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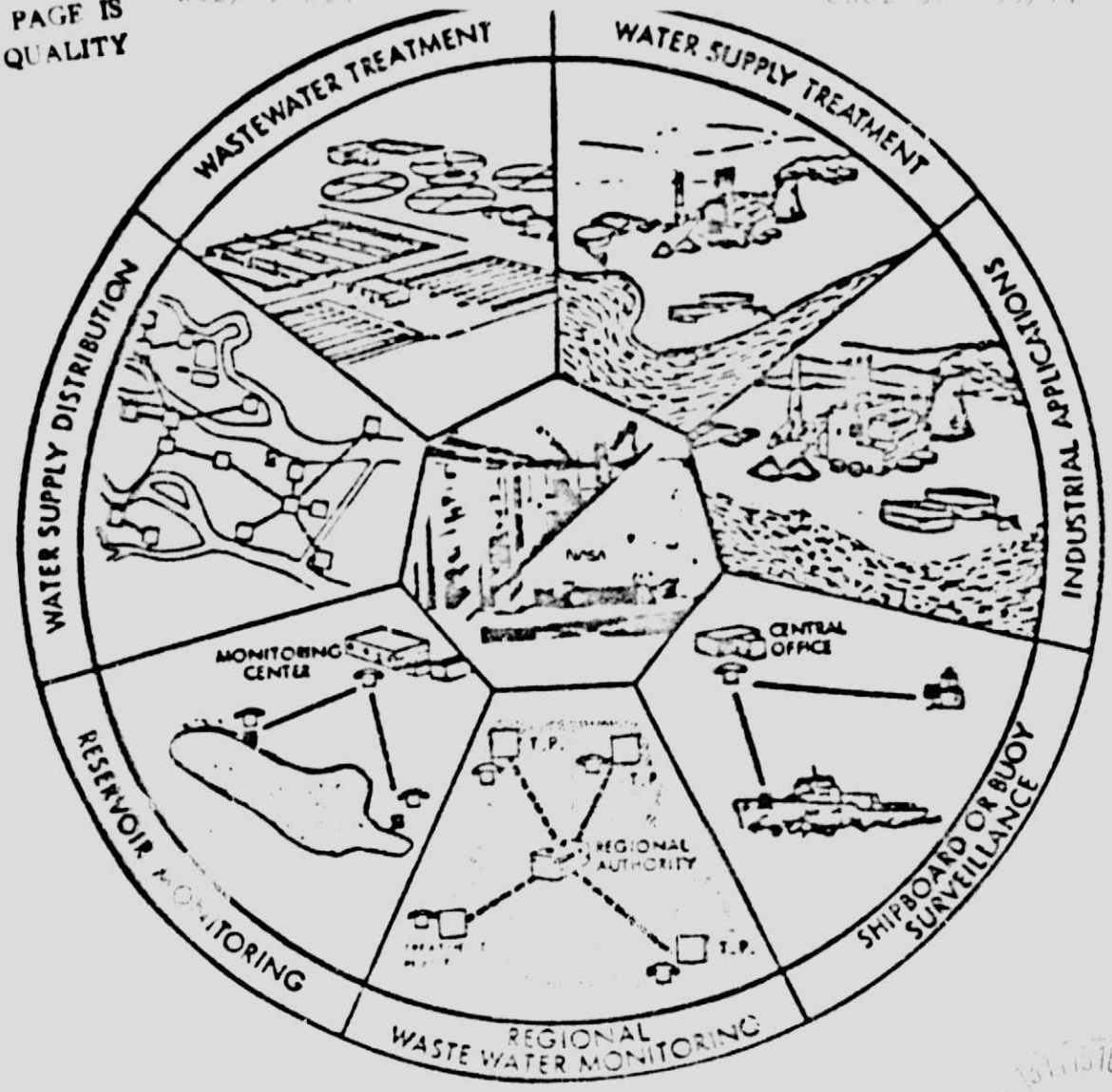

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Biological Water Quality Monitoring Using Chemiluminescent and Bioluminescent Techniques

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FINAL REPORT

Prepared for
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Abstract

Automated chemiluminescence and bioluminescence sensors have been developed for the continuous monitoring of microbial levels in water supplies. The optimal chemical procedures were determined for the chemiluminescence system to achieve maximum sensitivity. By using hydrogen peroxide, reaction rate differentiation, ethylene diamine tetraacetic acid (EDTA), and carbon monoxide pretreatments, factors which cause interference can be eliminated and specificity of the reaction for living and dead bacteria is greatly increased. By employing existing technology with some modifications, a sensitive and specific bioluminescent system was developed. Testing of the systems has shown that both systems are compatible with an automated system and will provide reproducible and reliable results.

INTRODUCTION

No continuous means of monitoring water quality with respect to microbial count is currently available to community wastewater treatment plants. Automated chemiluminescent and bioluminescent systems can accomplish this task in real-time with minimum operator assistance. A chemiluminescent system employing the reaction between luminol (5-amino-2, 3-dehydro-1,4 phthazinediane) and bacterial iron porphyrins and a bioluminescent system utilizing the reaction between firefly luciferase and bacterial adenosine triphosphate (ATP) have both been developed.

Previous to this work, the chemiluminescent system was plagued with the problem of non-specificity. Since other compounds besides iron porphyrins produce a chemiluminescant response, many compounds could interfere with the reaction and produce false signals. Agents such as metallic ions, chlorine, and extra-cellular iron porphyrins, all found in wastewater effluents, presented particular problems for the use of chemiluminescence as a bacteria monitor. Table 1 contains a list of some luminol activators and their relative luminol responses. Several methods had to be developed in order to eliminate these interferences if the system was to be used in contaminated wastewaters.

Much of the methodology for the bioluminescent system was already developed prior to the beginning of this project. The major effort was directed towards adopting the existing methodology to an automated system. Previous methodology involving manual sample analysis was converted to a flowing type of system involving very little operator manipulation, making the system much more compatible with an automated system.

CHEMILUMINESCENCE SYSTEM

Discussion and Results

The chemiluminescent system is based on the reaction between luminol, hydrogen peroxide and bacterial iron porphyrins such as those found in the electron transport chain. Since most aerobic bacteria contain iron porphyrins, a measurement of iron porphyrins in a sample can be used as an indication of the presence of bacteria. The chemiluminescence reaction between luminol and iron porphyrins is due to the oxidation of luminol according to the proposed reaction path given in Equation 1. (Ranhut, et al., 1966; Drew, 1939).

OPTIMIZATION OF REACTION CONDITIONS

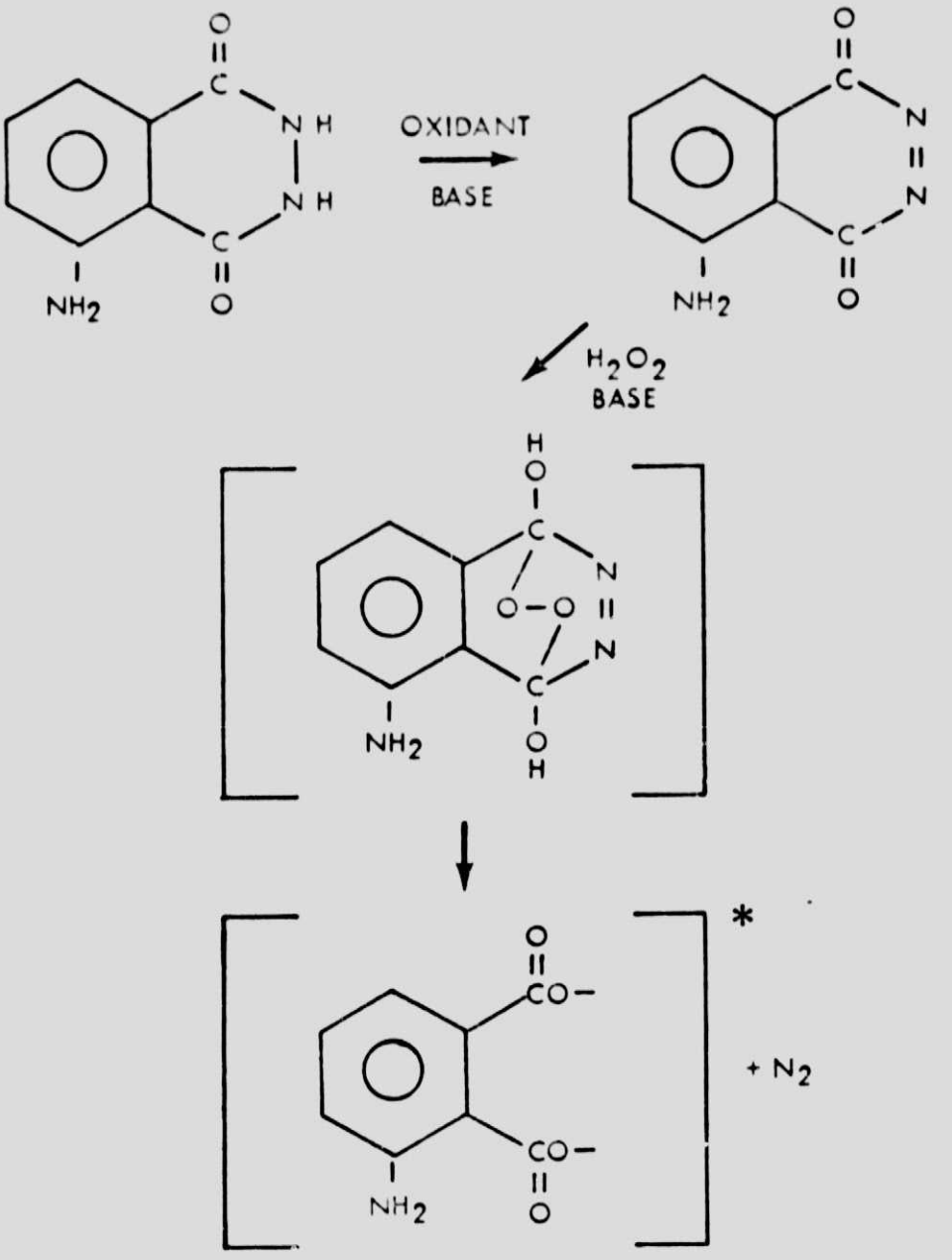
Purification of Luminol

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

TABLE 1

RELATIVE LUMINESCENCE RESPONSES OF LUMINOL ACTIVATORS

Compound	Concentration	Response	Fe Atoms/ Molecule
Catalase	1×10^{-7}M	9490	4
Hemoglobin	1×10^{-7}M	6500	4
Cytochrome c	1×10^{-7}M	25	1
Ferricyanide	1×10^{-7}M	5	1
Ferrous Sulfate	1×10^{-7}M	0.03	1
Cobaltous Chloride	1×10^{-7}M	2.6	0
Cl_2	1×10^{-7}M	0.03	0



EQUATION 1

recrystallizing luminol (J. T. Baker and Co.) twice in hydrogen bromide. The luminol · HBr 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.

Luminol was also purchased from several manufacturers to compare the quality of commercially available products with that produced in the laboratory. While luminol from several companies was considered inferior in quality, luminol from the Sigma Chemical Company approached the lab prepared luminol in terms of intensity of light response, linearity, and lower blank response. Although the lab prep luminol produced at least 25% more light for a given sample (See Figure 1), the Sigma luminol was selected for routine use because of its availability, coupled with the fact that 15% yields were achieved with laboratory preparations (10 grams required to produce 1.5 grams of purified luminol).

Luminol Concentration

The optimum luminol concentration was determined in terms of lowest blank and highest light response for a 4×10^7 Escherichia coli/ml sample. Figure 2 illustrates the sample response, blank response and endogenous light associated with increasing luminol concentrations.

A plot of the signal to noise ratio for the different luminol concentration can be found in Figure 3. Self-quenching of the reaction apparently becomes important when the luminol concentration is greater than 5×10^{-4}M. The optimum luminol concentration was thus determined to be 2.5×10^{-4}M

Hydrogen Peroxide

Two phenomenon are dependent on the hydrogen peroxide concentration; 1) peak light response, and 2) the related reaction rate curves. Figure 4 illustrates how the peak light response varies with different hydrogen peroxide concentrations in the luminol reagent for a bacteria sample. Figure 5 shows how the reaction rate curves change with those same hydrogen peroxide concentrations. 0.1% hydrogen peroxide was chosen as the optimum concentration due to the highest light output and yet sufficient reaction rate resolution of the interference signals from bacteria signals.

Sodium Hydroxide

The sodium hydroxide in the luminol reagent also serves two functions: 1) as an extractant to rupture the cells and release the iron porphyrins for the reaction, and 2) to provide an alkaline solution for the chemiluminescent reaction.

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).

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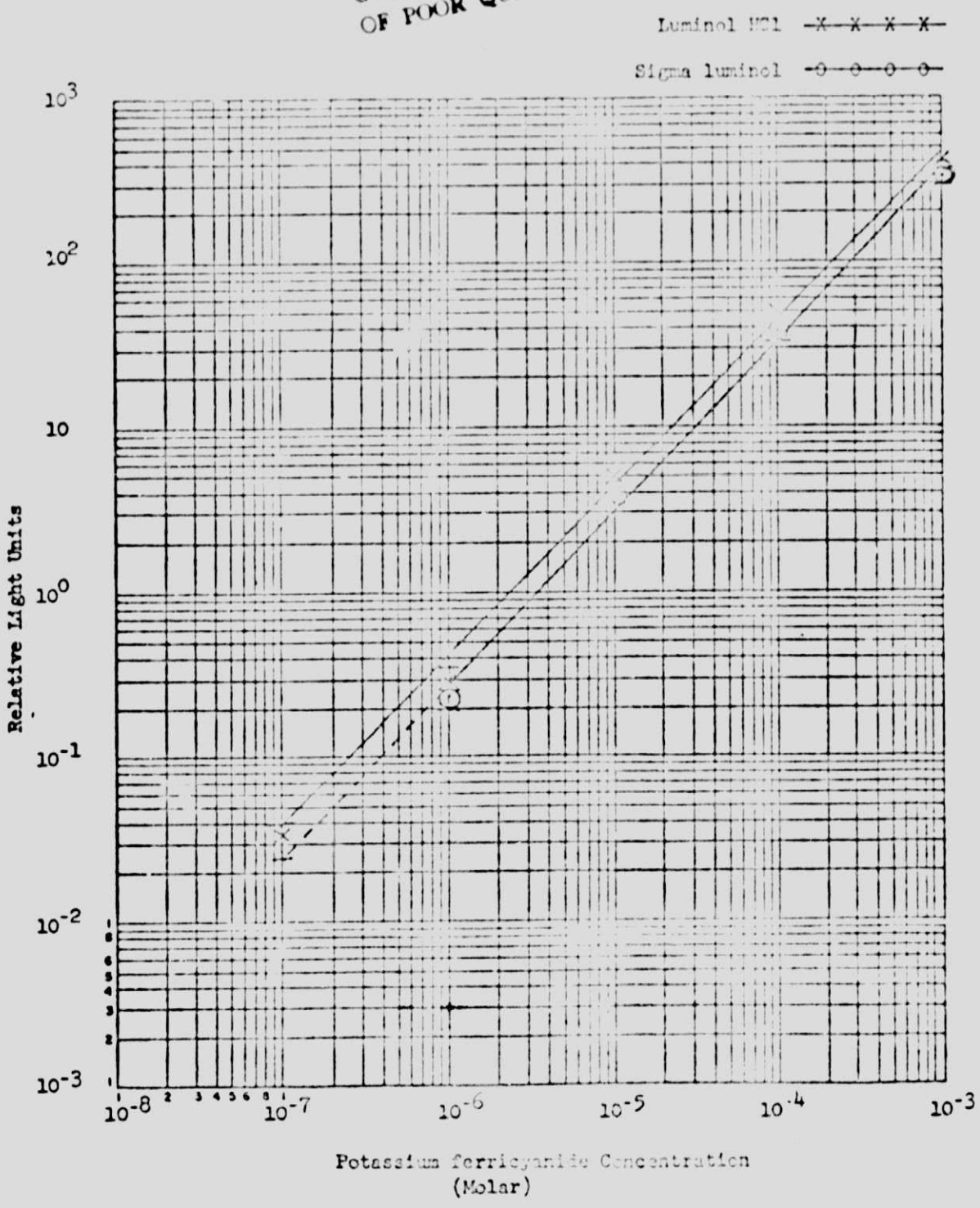


FIGURE 2

CHEMILUMINESCENT RESPONSE TO *E. COLI* WITH VARYING LUMINOL CONCENTRATIONS

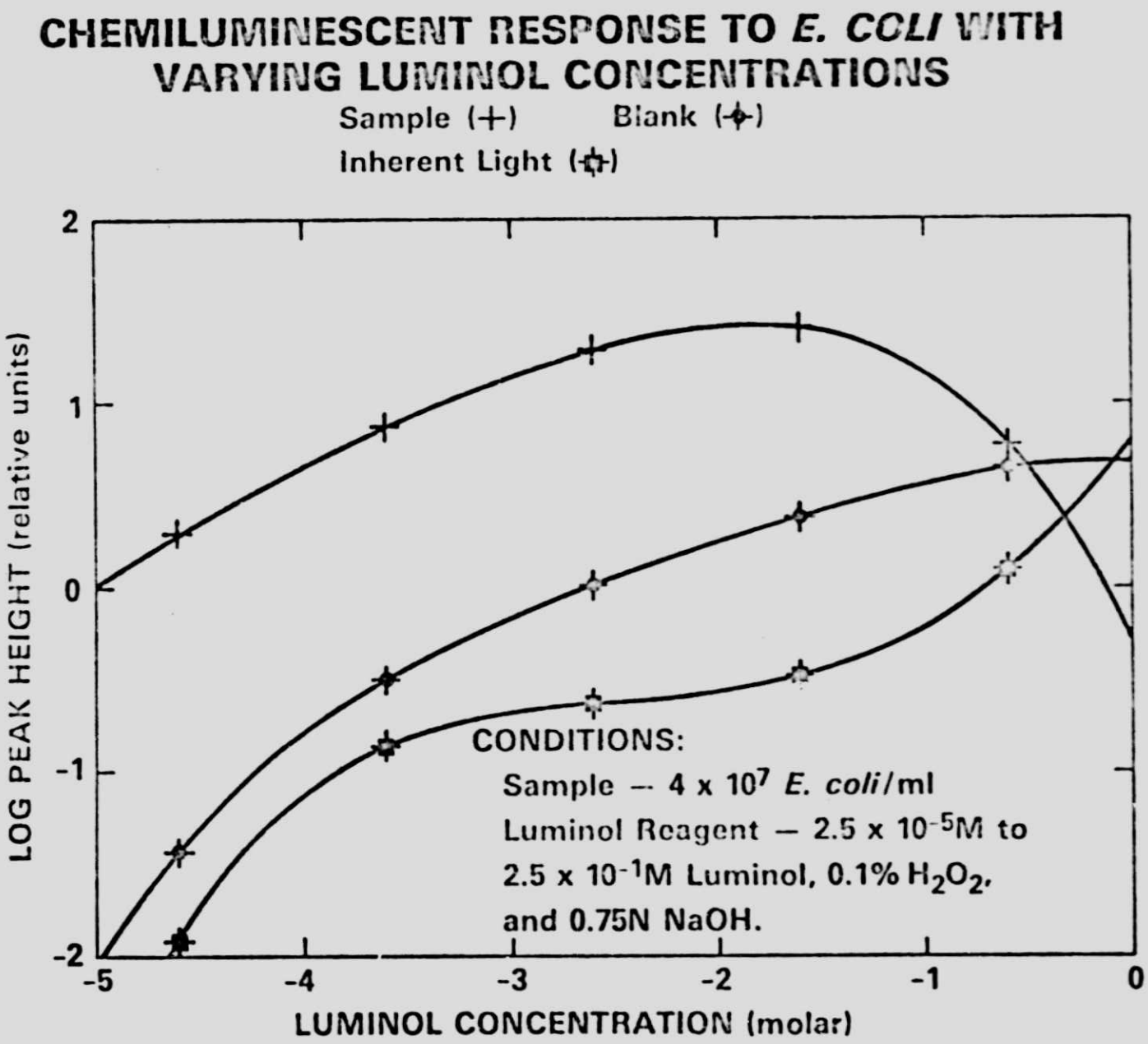


FIGURE 3

SIGNAL TO NOISE RATIO FOR VARIOUS LUMINOL CONCENTRATIONS

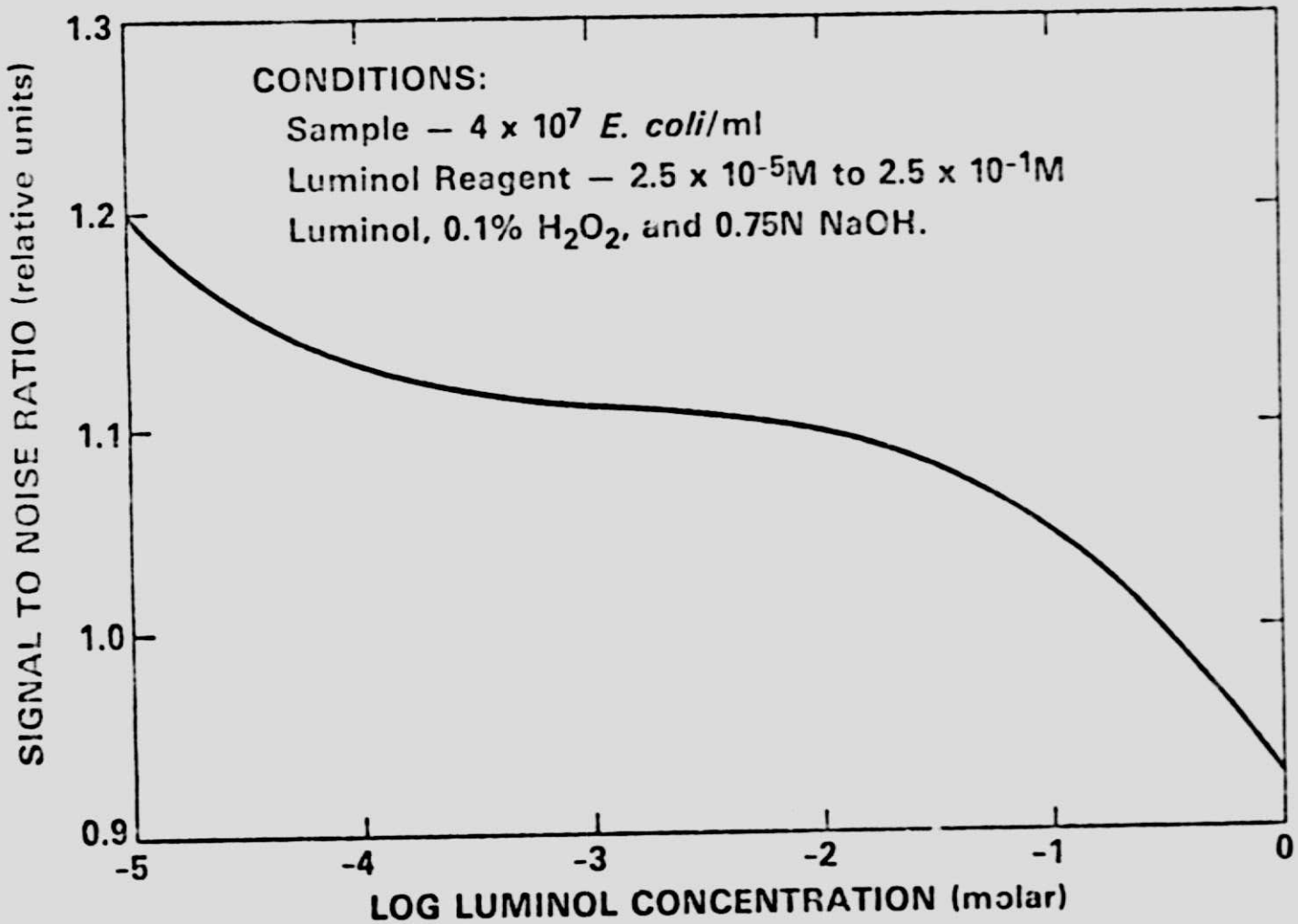


FIGURE 4

CHEMILUMINESCENT RESPONSE OF *E. COLI* WITH VARYING HYDROGEN PEROXIDE CONCENTRATIONS

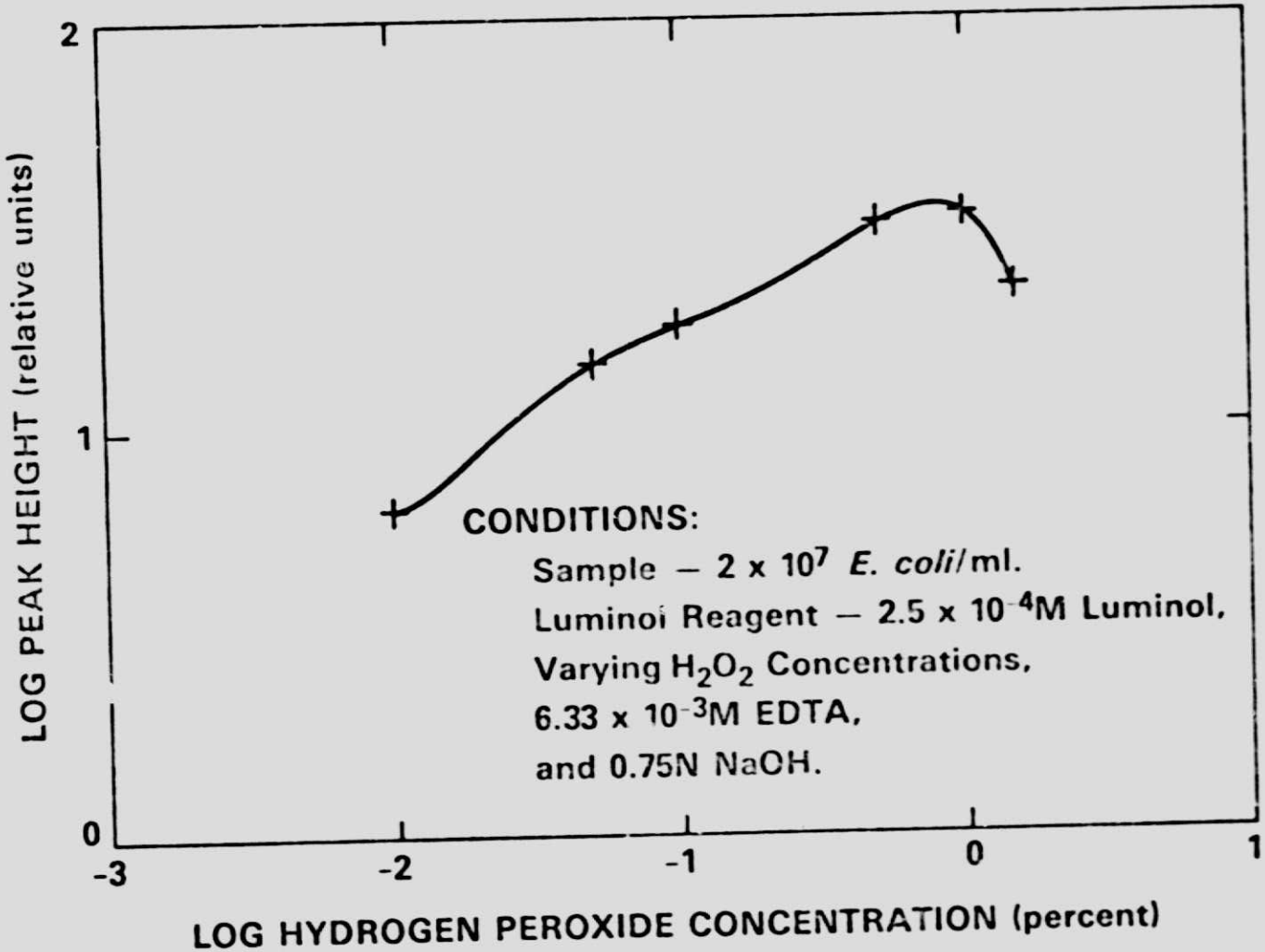
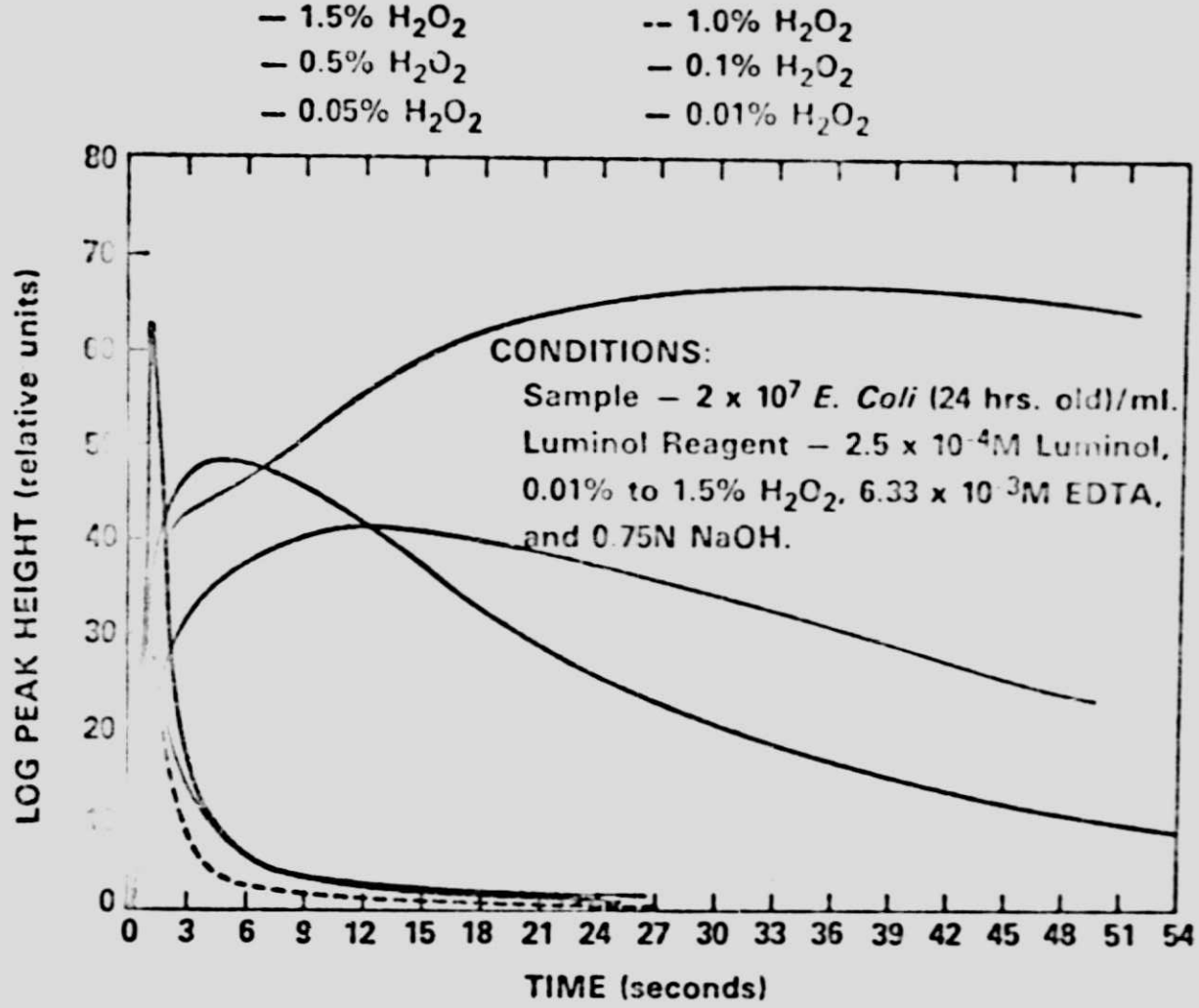


FIGURE 5

REACTION RATE CURVES FOR *E. COLI* WITH HYDROGEN PEROXIDE CONCENTRATIONS



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The optimum pH was the criteria for selecting the sodium hydroxide concentration. Luminol chemiluminescence with transition metals such as iron and cobalt (II) show a maximum around pH 10.9 while the maximum chemiluminescence for E. coli is exhibited around pH 12.4. 0.75N sodium hydroxide which has a pH of 12.4 is therefore used as the basis for the luminol reagent.

INTERFERENCE REMOVAL TECHNIQUES

Hydrogen Peroxide Pretreatment

Hydrogen peroxide pretreatment can be used to eliminate extra-bacterial iron porphyrins which would otherwise produce false chemiluminescent signals. In hydrogen peroxide oxidation as in natural degradation of iron porphyrins, a bridge-carbon atom is eliminated from the porphyrin nucleus. Very little chemiluminescent response is observed from the resulting dissociated iron atom. Figure 6 shows the effect of hydrogen peroxide pretreatment concentration with time on a 10^{-7}M catalose sample. At all concentrations, the greatest reduction of chemiluminescent signal occurs in the first five minutes. Figure 7 illustrates the effect of hydrogen peroxide pretreatment concentration on a bacteria sample, E. coli. At final pretreatment concentrations less than 1.5% H_2O_2, no significant loss of chemiluminescent signal was observed. A final concentration of 0.5% hydrogen peroxide for a 2-minute period was selected as optimum for effective reduction of non-cellular iron porphyrins and little loss of signal from the bacteria sample. Table 2 shows the effect of 0.5% H_2O_2 pretreatment on a number of luminol activating agents.

Reaction Rate Differentiation

Hydrogen peroxide does little to eliminate false signals from inorganics such as the transition metals, ferricyanide, or chlorine. By utilizing the different reaction rates characteristic of the various luminol activating agents, these interfering materials can be eliminated. Figure 8 illustrates how the reaction rates of ferricyanide and ferrous sulfate differ from iron porphyrins such as catalose and those found in the bacterium, E. coli. If the light measurement is recorded at a point 5 seconds after the initial mixing of sample and luminol reagent, only the catalose and E. coli response would be observed.

EDTA

Some wastewater effluents contain some materials which appear to inhibit luminol chemiluminescence. It is known, for example, that some amino acids, in particular amino acids containing sulfhydryl groups such as cysteine and other compounds such as thiourea inhibit the luminol reaction. Table 3 is an example of the effect of 6.33×10^{-3}M EDTA on the inhibitors found in secondary effluent. 63% of the response from bacteria is inhibited in effluent without EDTA while less than 10% inhibition is observed with EDTA. It is speculated in these cases that EDTA may be chelating some metallic ion; however, the actual source and site of the inhibition is not yet known.

FIGURE 6

CHEMILUMINESCENT RESPONSE OF HYDROGEN PEROXIDE PRETREATED CATALASE

(✧) 0.05% H_2O_2 (✧) 0.5% H_2O_2

(✧) 0.25% H_2O_2 (+) 1.5% H_2O_2

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FIGURE 7

CHEMILUMINESCENT RESPONSE OF HYDROGEN PEROXIDE PRETREATED *E. COLI*

(✦) 0.25% H_2O_2 (✧) 5.0% H_2O_2

(✧) 0.05% H_2O_2 (+) 1.5% H_2O_2

(✡) 0.5% H_2O_2

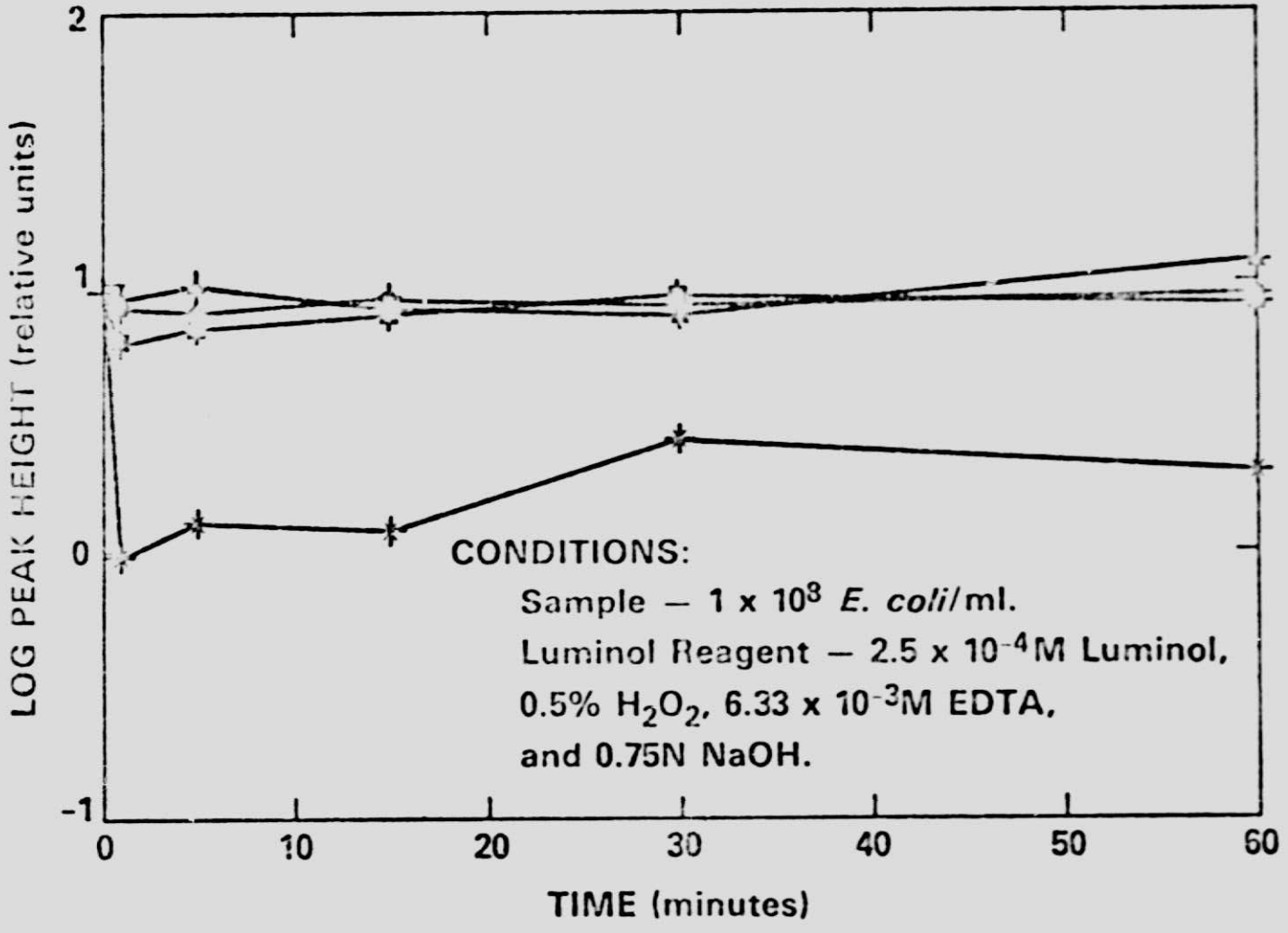
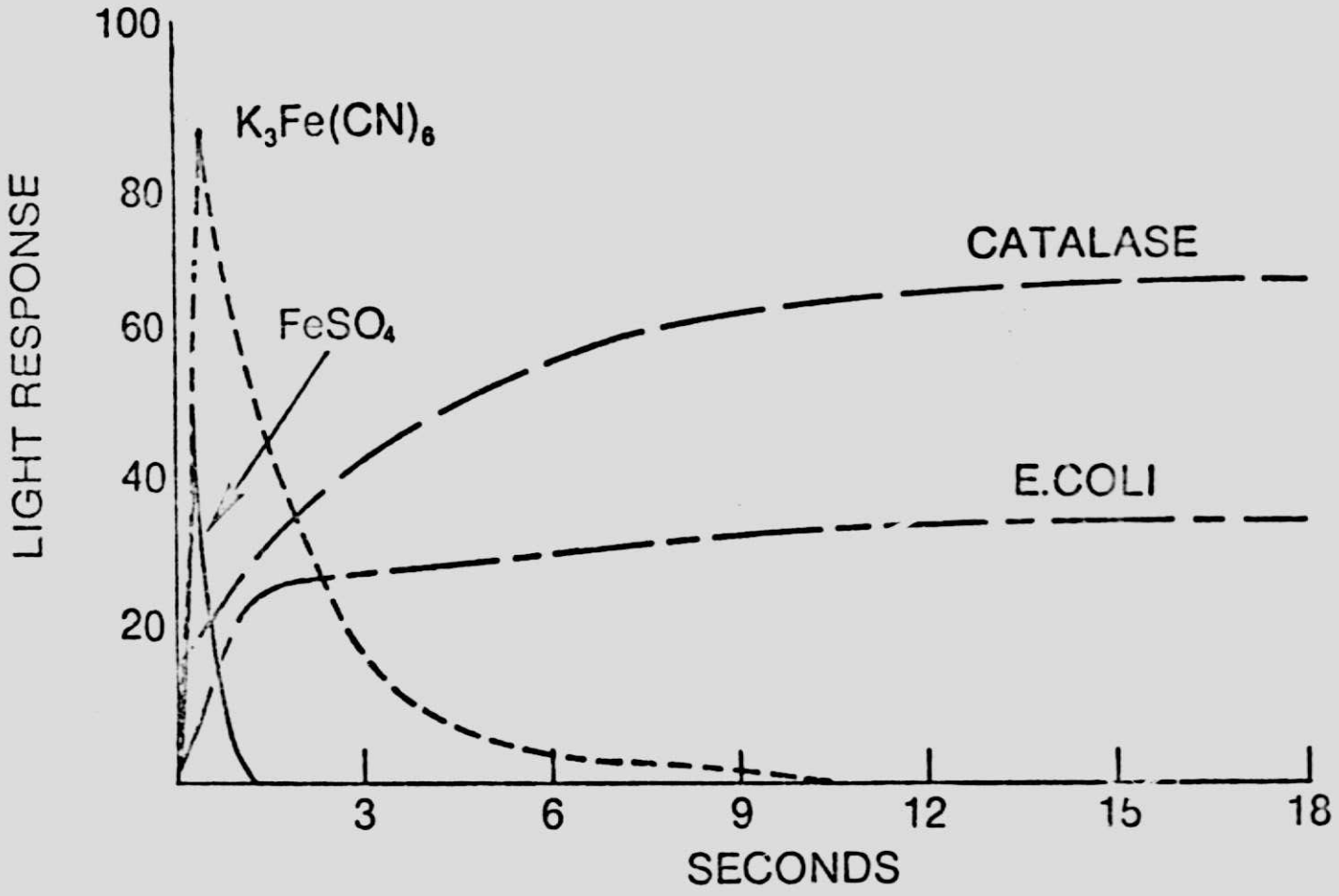


TABLE 2

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

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

FIGURE 8



REACTION RATE CURVES FOR VARIOUS LUNINOL OXIDIZING AGENTS (ARBITRARY CONCENTRATIONS)

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SAMPLE	RESPONSE (RELATIVE LIGHT UNITS) NO EDTA	6.33×10^{-3}M EDTA
1×10^{7} E.coli/ml deionized water	5.2	5.2
1×10^{7} E.coli/ml 0.45μ filtered secondary treated effluent	1.9	4.7

TABLE 3 EFFECTS OF 6.33×10^{-3}M EDTA ON THE INHIBITORS IN SECONDARY EFFLUENT

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Carbon Monoxide

Although the luminol system described up to this point is specific for bacteria, the system cannot differentiate live from dead cells. A new technique involving bubbling carbon monoxide (CO) through a bacterial suspension results in reduced CO-complexed iron porphyrins in live bacteria which do not chemiluminescently react with luminol. Using the luminol-CO method, the difference between the response from an untreated sample (measurement of live and dead bacteria) and a CO treated sample (measurement of dead bacteria only) is directly related to the concentration of live bacteria in the sample. The percent reduction of luminol response can also be directly related to the percent living bacteria in a sample as illustrated in Figure 9.

Incorporation of Methodology Into an Automated Flow System

Figure 10 is a schematic of the luminol biosensor incorporating EDTA for eliminating inhibitors, 6 second delay for reaction rate resolution to eliminate metallic ions and chlorine, and carbon monoxide to differentiate live from dead bacteria. Note that the hydrogen peroxide is not included in this system. Figure 11 shows a comparison of the luminol response without CO versus the total bacteria present determined with a Coulter electronic particle counter. Figure 12 shows results of a correlation between the luminol biosensor for living bacteria and the firefly luciferase-ATP analysis. These analysis in tap water and wastewater effluent demonstrate good correlation ($\sim$ 2=0.96) for total bacteria and living bacteria in waters which would most likely contain interferences.

BIOLUMINESCENT SYSTEM

Discussion and Results

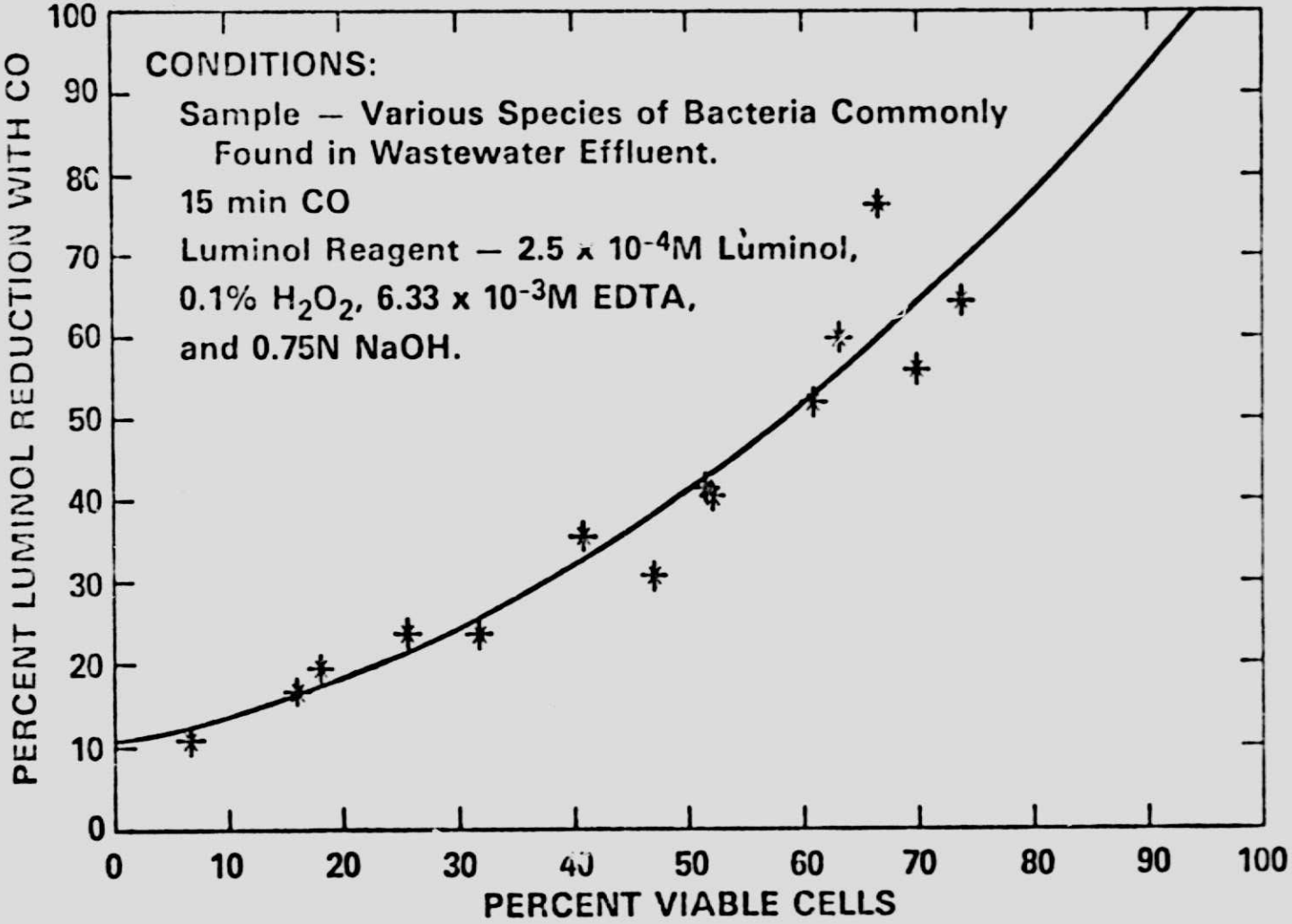
Employing the existing methodology, Figure 13 illustrates how the firefly luciferase-ATP assay has been adapted to a flowing automated system. The system consists of a 2-minute nitric acid extraction step to rupture and release bacterial ATP followed by dilution with deionized water. The prepared sample then mixes with buffered luciferase in front of the photomultiplier on the bioluminescence measured. ATP standards and deionized water blanks must be periodically assayed and thus, by knowing the concentration of ATP per cell (3.0×10^{-10}mg ATP/bacteria), the bioluminescence can be related to the concentration of bacteria.

CONCLUSION

The chemiluminescent luminol-iron porphyrin reaction and the bioluminescent luciferase-ATP reaction have been adapted to an automated system for monitoring bacteria in water and wastewater. As a result of newly-developed techniques for eliminating interference and improving specificity with the luminol system, both assays are rapid and specific for live bacteria. Because the luminol system produces similar results as the ATP system and is less expensive to operate, the chemiluminescent system is the assay of choice. In addition, the chemiluminescent reagents are more stable, eliminating the need for frequent reagent preparation. Because of all the advantages of the chemiluminescent system, the assay has unlimited applications in the field of continuous real-time bacteria water monitoring.

FIGURE 9

PERCENT LUMINOL REDUCTION WITH CARBON MONOXIDE VS. PERCENT VIABLE CELLS



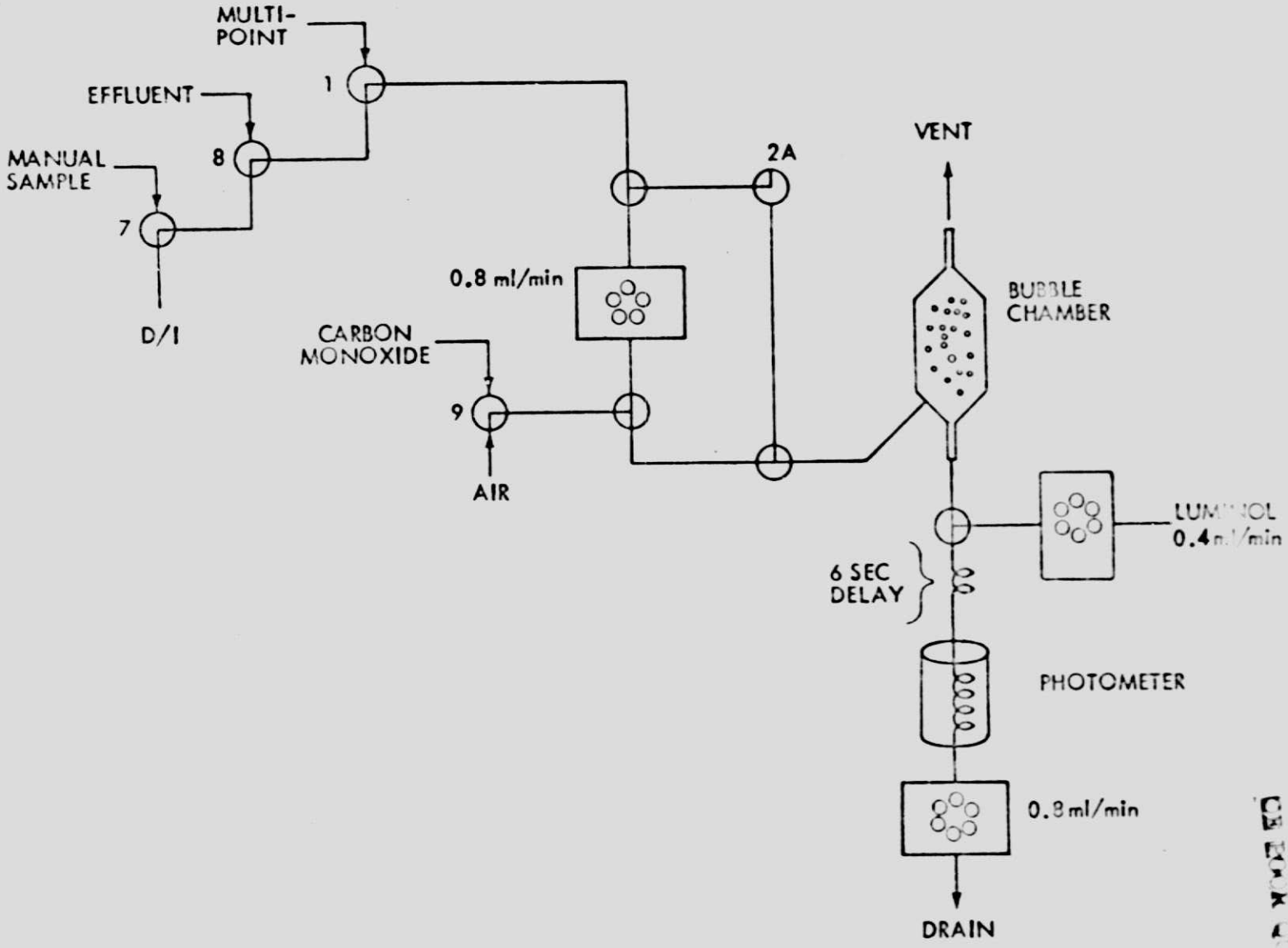


FIGURE 10. SCHEMATIC OF LUMINOL-CARBON MONOXIDE BIOSENSOR

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FIGURE 11

LUMINOL RESPONSE OF *E. COLI* IN TAP WATER VS. TOTAL CELL COUNT (COULTER PARTICLE COUNT)

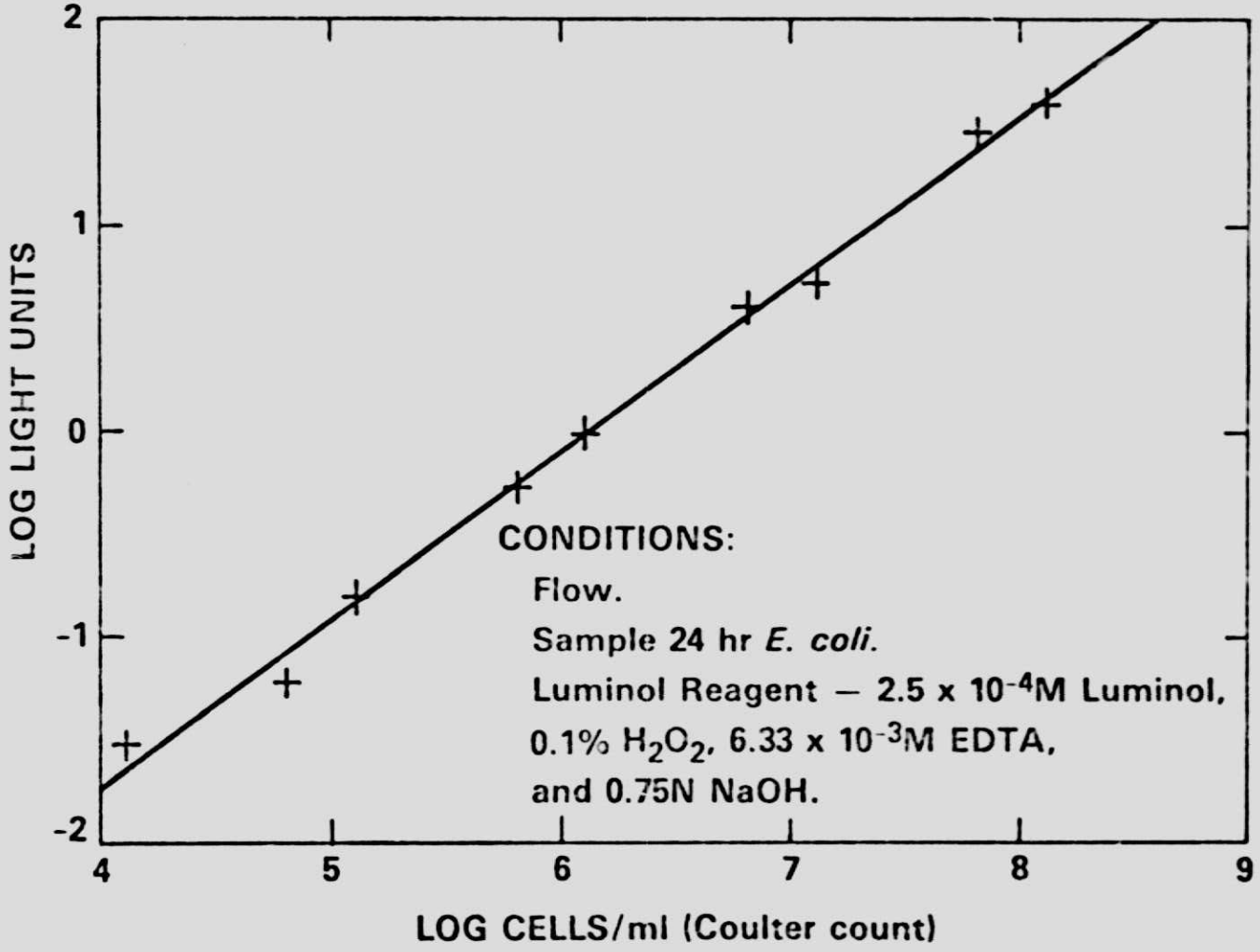

FIGURE 12

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LUMINOL - CO MEASUREMENT OF LIVING BACTERIA (IN PHOTOMETER UNITS, % FULL SCALE) VS. LIVING CELLS PER MILLILITER DETERMINED BY FIREFLY LUCIFERASE ATP ASSAY.

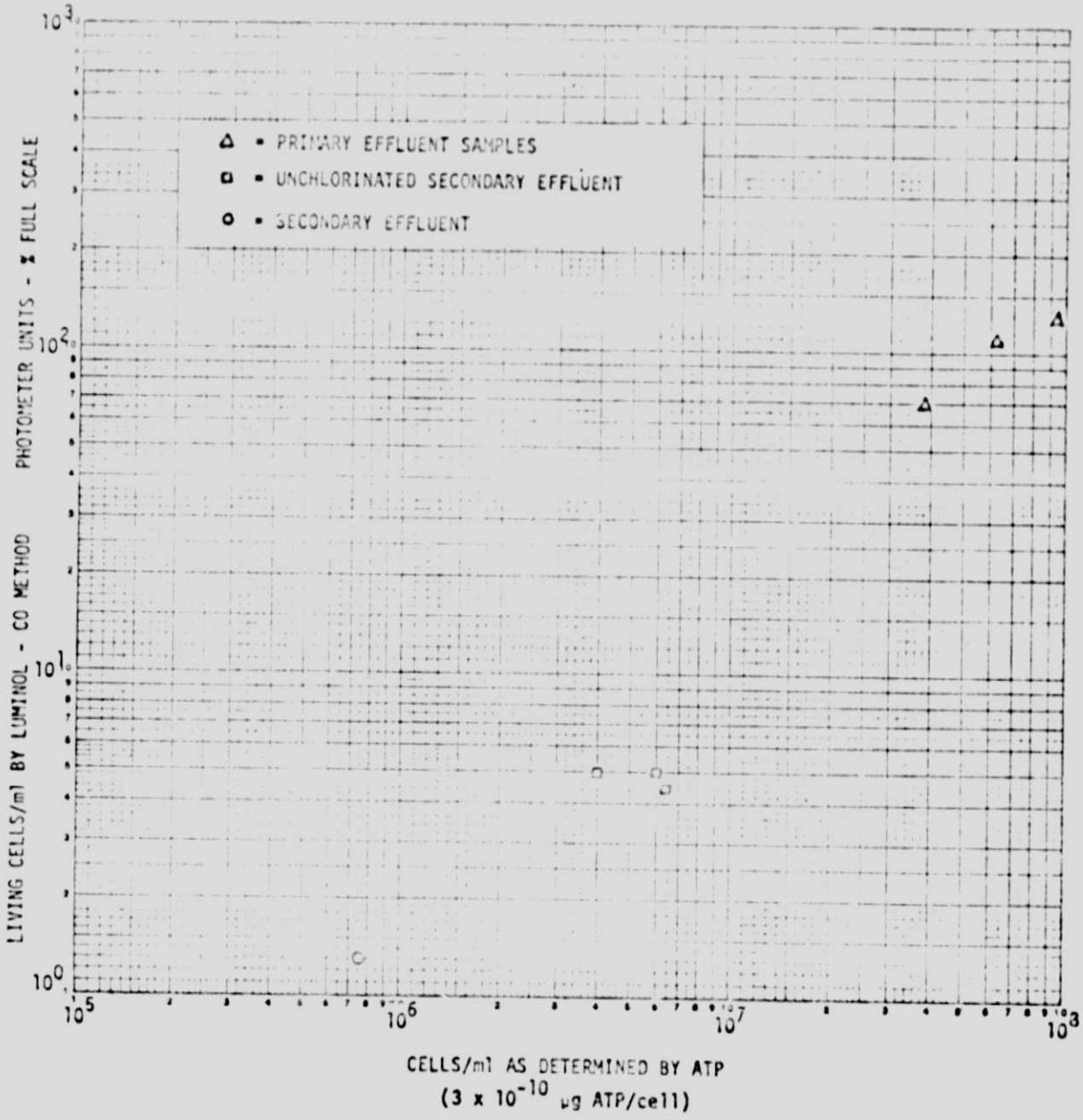
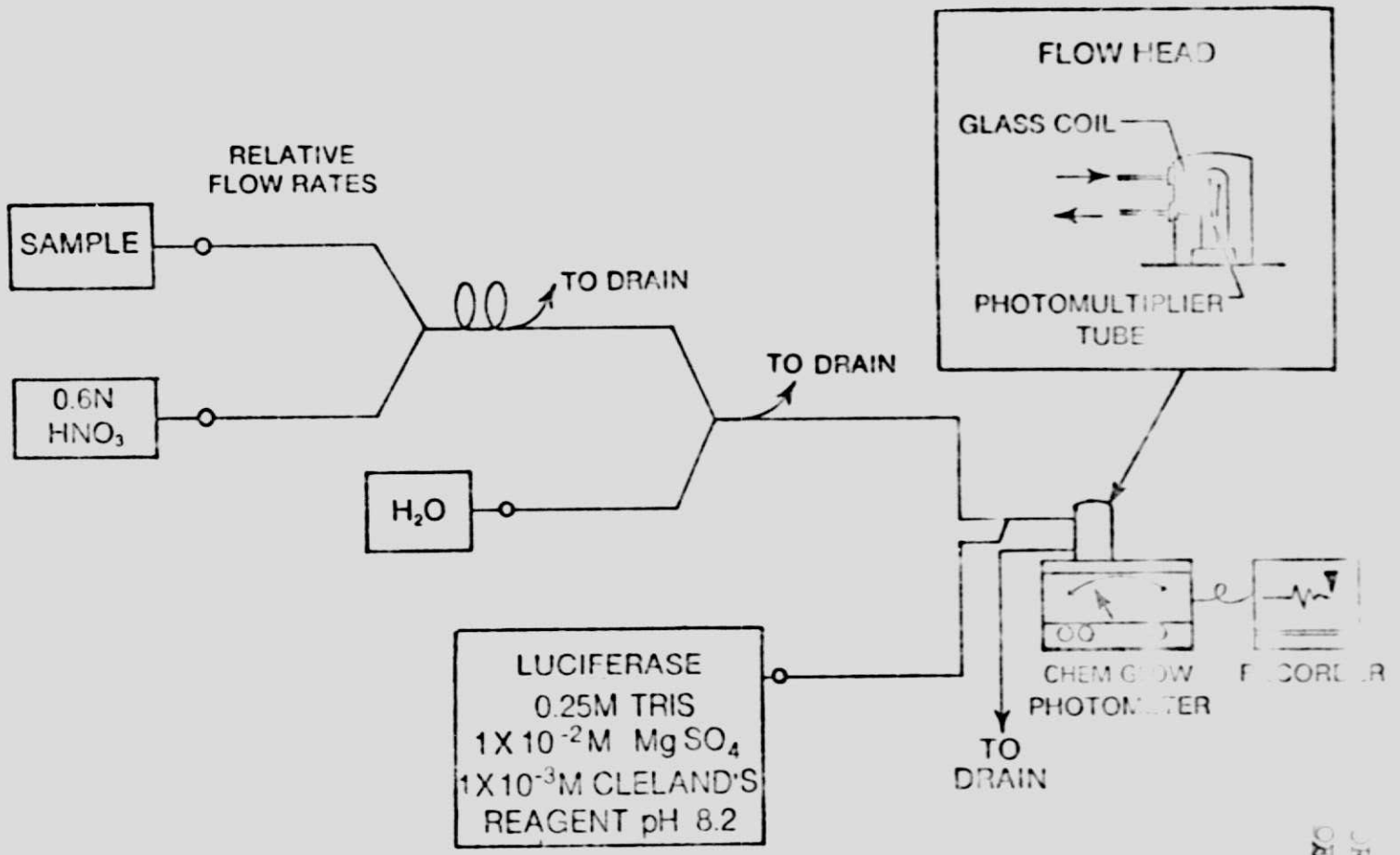


FIGURE 13



AUTOMATED FIREFLY LUCIFERASE FLOW SYSTEM FOR DETECTING BACTERIAL ATP INCLUDING NITRIC EXTRACTION AND SUBSUQUENT DILUTION

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