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Title: Design of a Chemiluminescence Photometer and its Application to Trace Chromium (III) Analysis

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The design and construction of a chemiluminescence photometer is described. The design is based on a discrete sample system which has a number of advantages over the previously described flow cell system and centrifugal analyzer. The advantages include simple and inexpensive equipment, small sample size, versatile electronic readout system, and temperature controlled cell holder. The system may be used for the determination of Cr(III) in natural samples.

Digestion procedures are developed for measuring Cr(III) in natural samples since Cr(III) can be determined by chemiluminescence analysis only if it is present in solution as free Cr(III). The Cr(III) determination is made using the luminol and hydrogen peroxide system.

Reagent concentrations and reaction conditions are optimized and a calibration curve is obtained. The detection limit is 4×10^{-10} M or about 5 ppt. The dyna-

mic range is 10^{-9} M to 10^{-5} M and the relative standard deviation is about four percent. A digestion procedure for biological samples was developed and tested on an NBS Orchard leaf standard. The Cr levels were found to be 2.2 $\pm$ 0.4 ppm.

Design of a Chemiluminescence Photometer and its Application to Trace Chromium (III) Analysis

by

Steve Dow Hoyt

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TABLE OF CONTENTS

List of Illustrations

List of Tables

Design of a Chemiluminescence Photometer and Its Application to Trace Chromium (III) Analysis

I. INTRODUCTION

Chemiluminescence is the emission of light from an excited intermediate or a product produced in a chemical reaction. The emission process falls in the category of luminescence because the excited electronic state is produced by a non-thermal process. Any type of luminescence is a potential tool for analysis since the number of photons emitted due to the luminescence is related to the concentrations of the luminescing species and of other species which affect the rate of luminescence. In the case of chemiluminescence analysis, the luminescence signal produced by mixing certain reactants with the analyte is related directly to the concentration of the analyte.

Chemiluminescence analysis is an attractive technique and the recent reviews (1,2,3) attest to its growing popularity. The instrumentation is simple and easily made portable for possible field use. The detection limits are low for many metals and analysis can be carried out over a wide range of concentrations. In addition, the technique is specific for certain oxidation states.

Recently (4,5) chemiluminescence instrumentation has been described and used for ppb and sub-ppb determinations

of Cr(III) which is of great importance in biological and medical investigations. Determination of Cr(III) at these levels is difficult since few analytical techniques are available to the analyst, and those which are available can be expensive and require specialized equipment.

This investigation is concerned with the design and construction of a simple chemiluminescence photometer and its application to the determination of trace amounts of Cr(III). The instrument uses a discrete sampling system in which the reagents and analyte solutions are mixed in a standard spectrophotometer cell for each determination. The resultant radiation peak is detected by a photomultiplier tube and the peak height and peak area are recorded.

The instrumentation is applied to the chemiluminescence reaction between basic luminol and hydrogen peroxide which is greatly enhanced by Cr(III). Reagent concentrations and instrumental parameters are varied to obtain optimal conditions for analysis of Cr(III). A simple analysis procedure for Cr(III) is developed and applied to biological and water samples after they were digested. The feasibility of the digestion and chemiluminescence analysis procedures is checked with a NBS standard reference material.

II. HISTORY

A. Introduction

The emission of light by certain biological organisms has been observed for many years and is called bioluminescence (BL). It was not until the latter part of the 19th century that a chemical (lophine) was isolated that produced chemiluminescence (CL). BL is actually a specific type of CL which involves an enzyme reaction. Since the discovery of CL, many molecules have been found that can react to produce light. Usually CL and BL reactions are oxidations of the general form:

$$A + B + C \rightarrow L^* \rightarrow P + h\nu$$

where A represents an oxidizing agent or coenzyme; B represents an activator, inhibitor, or enzyme; C is a molecule which undergoes oxidation to produce a CL species; L* is an excited product or intermediate; P is the product of the reaction; and $h\nu$ represents a photon. The quantum efficiency of a CL reaction is defined as,

$$\phi = \frac{\text{number of photons emitted}}{\text{number of molecules reacting}}$$

In general, the highest efficiencies are associated with BL reactions.

The most important application of BL to chemical analysis is the determination of ATP (adenosine triphosphate) based on the luciferin-luciferase system. The reaction

provides a detection limit of 0.1 picomoles, and the BL signal is linear with respect to ATP concentration over 5 orders of magnitude (1). If it is assumed that the percentage of ATP in living matter is constant, the determination of ATP concentration provides a direct measure of the biomass. There are other bioluminescence systems used for analysis such as the bacterial system for FMN (flavine mononucleotide) determinations and the Aequorea system for determination of the cations Ca(II), Co(III), Pb(II), and Yb(III)(6).

CL analysis has not received much attention until recently because the quantum efficiencies are much lower than with BL. The smaller CL signal was difficult to measure with the photographic film used by early researchers. The use of the photomultiplier tube to measure low light levels has led to the development of CL analytical techniques. CL methods of analysis can be divided into gas phase analysis and solution analysis. Gas phase analyzers are commercially available and are used primarily for measuring atmospheric pollutants such as S, SO_2, P, O_3, NO, NO_2, CO, and BO_2 (7,8,9).

For analysis in solutions, the two common organic CL substances used are luminol (5-amino-2,3-dihydro-1,4-phthalazendione) and lucigenin (N,N'-dimethyldiacridinium dinitrate). Analysis is based on the activation and deactivation by certain metals of luminol or lucigenin CL in basic

solutions with hydrogen peroxide. Analytical procedures have been developed for Cu(II) (10), Mn(II) (11,12), Co(II) (13), V(III) (14), Fe(II) and Fe(III) (15), Cr(III) (4).

The following sections will provide background information on the mechanism of the luminol-peroxide reaction, chromium chemistry and analysis, hydrogen peroxide chemistry, instrumentation for CL, CL-Cr(III) analysis, and digestions of biological samples.

B. Mechanism of the Aqueous Luminol Reaction

The chemiluminescent properties of luminol were first discovered by Albrecht in 1928 (16) who proposed a mechanism for its reaction with oxygen. Since that time there have been over 200 papers and some excellent review articles (17,18) dealing with the mechanism and reaction of luminol. A complete mechanism is still not known because the reaction of luminol in aqueous solutions requires a strong oxidizing agent such as hydrogen peroxide, and many of the reaction products are destroyed by the peroxide.

One of the earliest products of the luminol reaction to be identified was nitrogen gas (18). Several of the earlier workers (19,20) postulated that the light was given off by an excited state of luminol decomposing to the azo form or by an excited azo form of luminol produced from a biradical intermediate. There was also some speculation

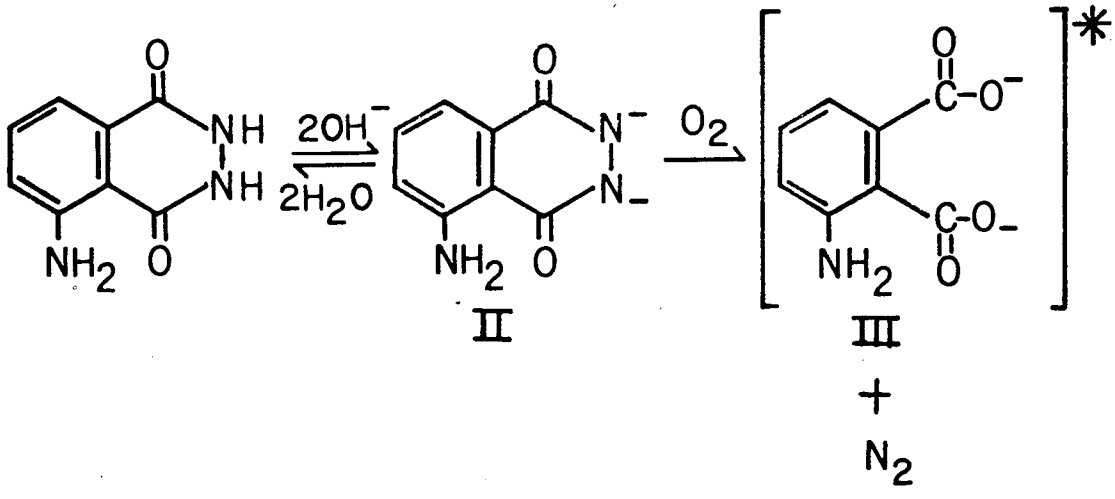
about a transannular peroxide formed in the reaction of luminol with peroxide (21).

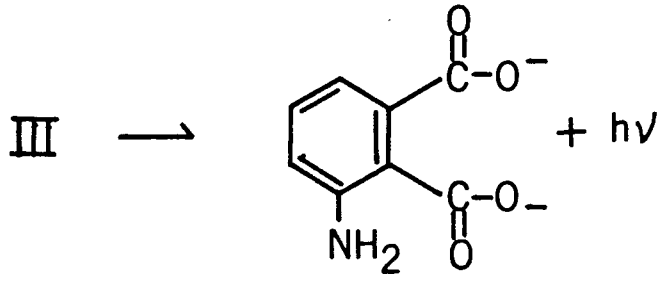
The following facts are now accepted (22): 1) Oxygen is required for the reaction to take place. 2) In basic solutions the anion of luminol is the reactant. 3) Free radicals are involved since the addition of radical chain stoppers inhibits CL. 4) Nitrogen is a product of the reaction.

More recently White (23) has done a considerable amount of work on the reactions of luminol in aqueous and non-aqueous solvents. The reaction conditions in non-aqueous basic solutions are much less severe because only O_2 and luminol are required and hence some of the products can be identified. White's work has led to the formulation of a more complete mechanism in aqueous solutions which is shown in figure 1. The products of the reaction with oxygen in dimethyl sulfoxide have led to the identification of the 3-aminophthalate dianion (species III in figure 1) as the light emitting species (24).

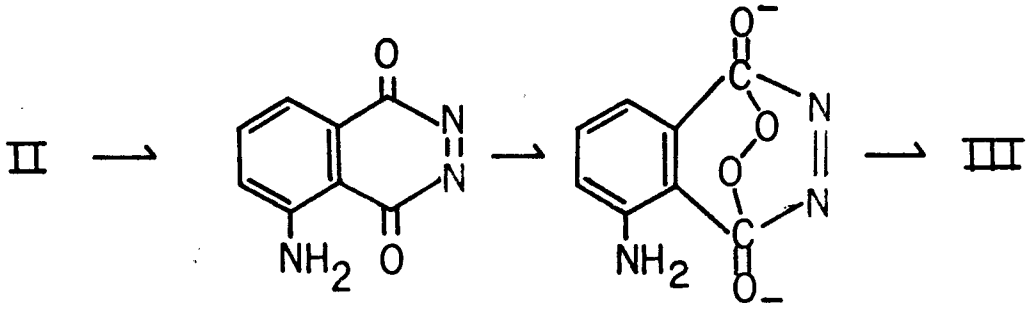
White confirmed that the two oxygens that added to luminol came from the oxygen gas and not from hydroxide (24), and that in the absence of oxygen, basic solutions of luminol would be stable indefinitely. The first and second dissociation constants for luminol are 10^{-6} and 10^{-13} respectively, so that in basic solution (pH = 12) the luminol exists primarily as the mono anion form (II) with only

Figure 1. Proposed luminol reaction pathway and intermediates.





PROPOSED INTERMEDIATES



a small amount of the dianion present.

Much of the early controversy about the reaction mechanism centered around the interpretation of the chemiluminescence and fluorescence spectra. Figure 2 shows the CL spectrum for basic luminol, the fluorescence spectrum for acidic (pH 4.0) luminol, and the absorption spectrum of basic luminol (25). Since the differences in the luminescence spectra are slight, early workers concluded that the neutral luminol molecule was responsible for chemiluminescence. Subsequent work (24) has shown that there are differences in the spectra, and that the fluorescence quantum yield for basic luminol is zero, hence some product of the luminol reaction is responsible for the chemiluminescence. The dependence of the fluorescence quantum yield of luminol and 3-aminophthalic acid on pH is shown in figure 3. The 3-aminophthalic acid was postulated to be the species responsible for chemiluminescence since the luminol CL and the 3-aminophthalic acid fluorescence are similar in terms of spectra and dependence of quantum yield of pH.

C. Chromium Chemistry

Chromium can exist in many different oxidation states ranging from Cr(II) to Cr(VI). The more important stable oxidation states are Cr(II), Cr(III), and Cr(VI) (26). Of these the most stable form is Cr(III) which is of primary

Figure 2. Absorption, chemiluminescence, and fluorescence spectra of luminol.

a) Absorption spectrum

b) Chemiluminescence spectrum

c) Fluorescence spectrum

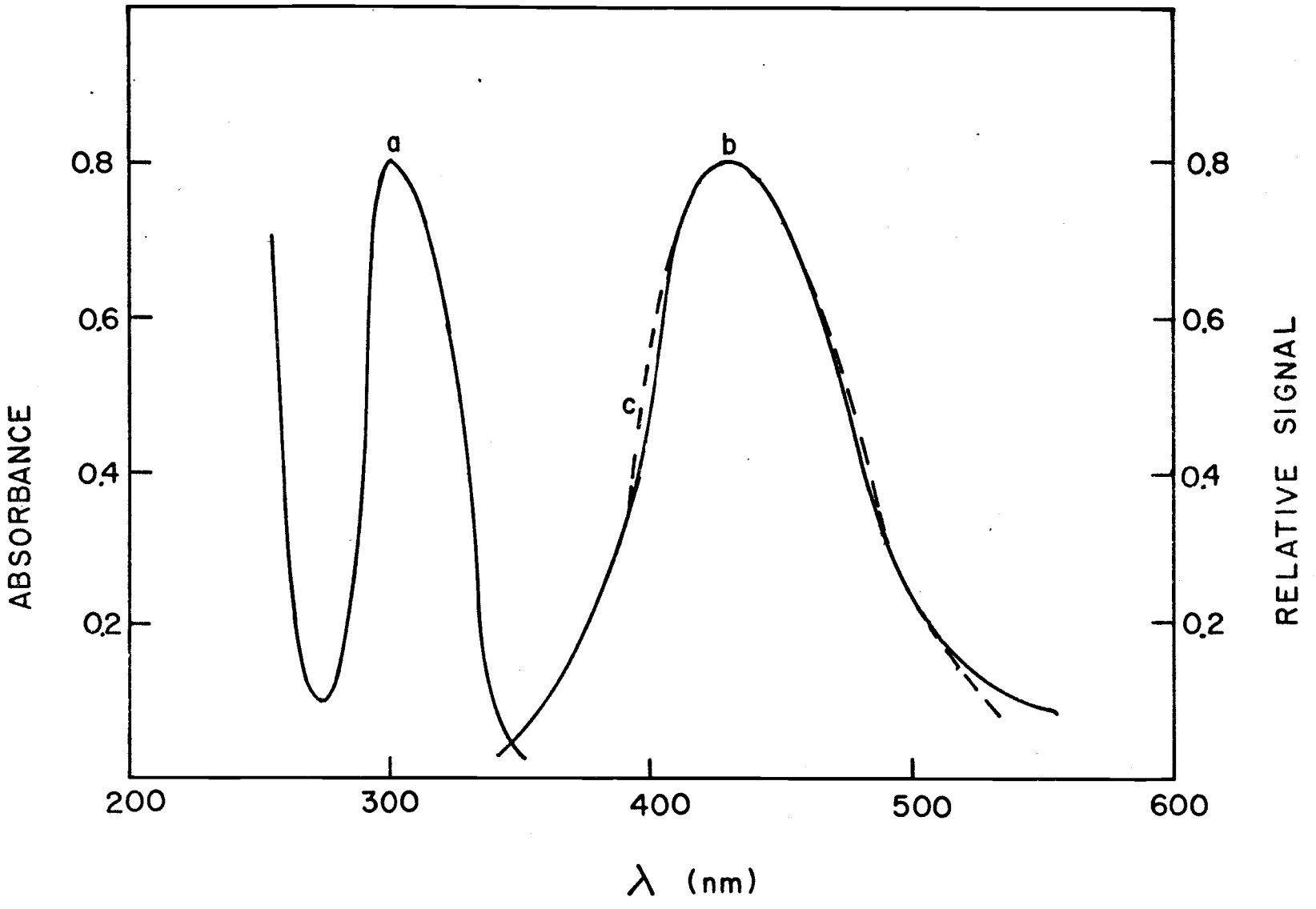
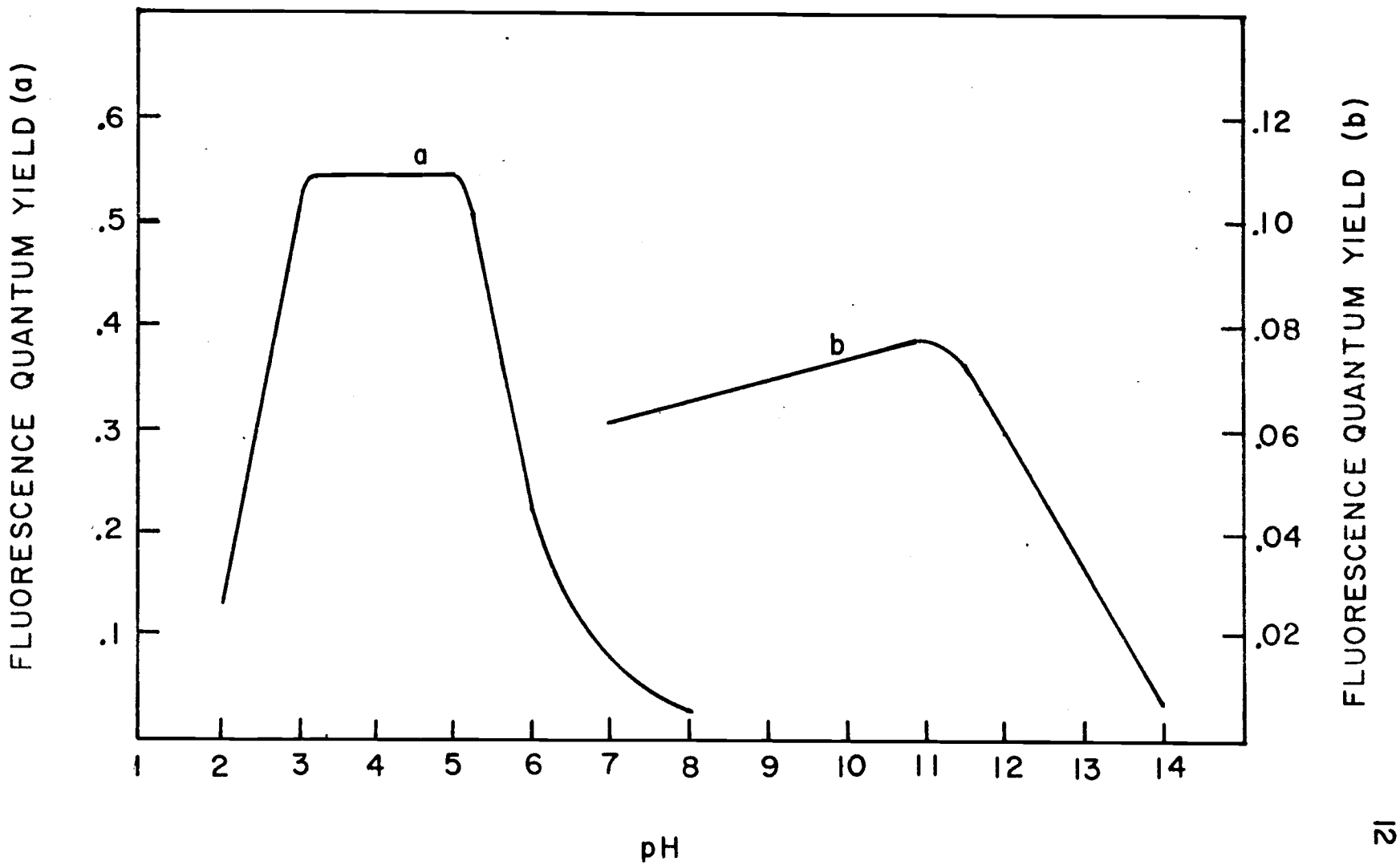

a
b
c
0.8
0.6
0.4
0.2
ABSORBANCE
RELATIVE SIGNAL
200
300
400
500
600
λ (nm)

Figure 3. Fluorescence quantum yield as a function of pH.

a) Luminol

b) Aminophthalic acid


FLUORESCENCE QUANTUM YIELD (a)
.6
.5
.4
.3
.2
.1
a
b
.12
.10
.08
.06
.04
.02
FLUORESCENCE QUANTUM YIELD (b)
1
2
3
4
5
6
7
8
9
10
11
12
13
14
pH

interest here as an activator for the CL reaction of luminol and hydrogen peroxide.

There are a number of Cr(II) compounds known, and all of them are strong reducing agents. Solutions of Cr(II) tend to be readily converted to Cr(III) by molecular oxygen. Almost all of the Cr(VI) compounds formed are oxides which act as good oxidizing agents. Of the oxides the best known are chromate and dichromate which exist in equilibrum. Dichromate ($Cr_2O_7^{-2}$), the stronger oxidizing agent, predominates in acid solution and chromate (CrO_4^{-2}) exists in basic solution.

Cr(III) primarily forms octahedral complexes which are relatively inert because the displacement reactions are very slow. The most common oxide is chromic hydroxide $Cr_2O_3 \cdot nH_2O$ with variable waters of hydration. It is formed on addition of hydroxide ion to chromium salts. In acid solutions the oxide and Cr(III) salts form the water complex $Cr(H_2O)_6^{+3}$. Important processes in Cr(III) chemistry is olation and oxolation. Olation is the linking of chromium complexes with hydroxide bridge groups. If the ligands are aquo or hydroxo groups, heating in basic solution will cause polymerization which is called oxolation.

In biological samples the Cr(III) form appears to be predominant, and there is no good evidence for the existence of Cr(VI)(27). To keep the Cr(III) in solution at physiological pH's and prevent it from undergoing olation, the

Cr(III) must be in a complex which is more stable than the hydroxide complex. Any attempt to measure free Cr(III) concentrations woule require this complex to be destroyed.

Recent studies of the physiological effects of Cr(III) show that chromium levels are high in patients with tumors, and Cr(III) can be responsible for impaired glucose tolerance factor (28).

The common trace analysis techniques for Cr are shown in table 1 along with information about the detection, limit, sample size, and the valence of Cr. Only X-Ray fluorescence with preconcentration is able to measure Cr(III) at sub-ppb levels often found in biological systems. Of course other criteria such as required sample size, and speed of analysis must be considered.

D. Hydrogen Peroxide.

Hydrogen peroxide (H_2O_2) is an essential reactant for the CL determination of Cr(III) where a metal ion-peroxide complex is thought to oxidize the luminol (37). H_2O_2 is more acidic than water (pK_a of 11.85) and acts as a strong oxidizing agent at all pH's although in basic solutions the oxidation takes place much faster (38).

The decomposition of H_2O_2 solutions depend on many factors and follows the basic reaction shown below (39).

$$H_2O_2\ (l) = H_2O\ (l) + \tfrac{1}{2}O_2\ (g)$$

TABLE 1. METHODS FOR CHROMIUM ANALYSIS

Method	detection limit (ppb)	sample size	Cr form	Reference
Coulometric Titration	15	6 ml	VI	29
Inductively Coupled Plasma Emission	1	--	any	30
Mass Spectrophotometric with Chelates	1	1 µg	III	31
Spectrophotometric Diphenylcarbazide	10	3 ml	III	32
Neutron Activation Analysis	450	10 g	any	33
X-Ray Fluorescence Chromium Oxalate	0.16	--	III	34
Flame Atomic Absorbtion	5	--	VI	35
Gas Chromatography of Chelates	3	1 µl	III	36
Carbon Rod Atomic Absorption	1	5 µl	any	54

Trace metals, radiation in the 200 - 400 nm region, and higher temperatures increase the rate of decomposition (39). The decomposition of 99% H_2O_2 increases with pH and is minimal in the pH range of 4.5 $\pm$ 0.5. For a given pH, more concentrated solutions were more stable than the dilute ones. At room temperature H_2O_2 can be stored in polyethylene bottles with a minimum of decomposition (39).

E. Instrumentation for Chemiluminescence

The simplicity of CL and BL instrumentation is illustrated by Figure 4 which shows the different configurations used for measurement of the luminescence signal. All that is needed is a light-tight sample module which contains a cell in which the sample and reagents are mixed and a means of measuring the radiant power emitted during the reaction. Either the instantaneous intensity, peak height, the integrated intensity, or the integral between selected times is measured. Babko (10,12,13,14) and other early workers (11) used the photographic detection system (figure 4a) and hence measured the total integrated CL by densitometer measurement of film or by weighing the silver deposits. More recently phototubes (PT) or photomultiplier tubes (PMT) (figure 4b) have been used in place of the film. This improves the limit of detection for most systems and increases the speed of analysis. The signal modifier consists of a current-to-voltage converter and, in more

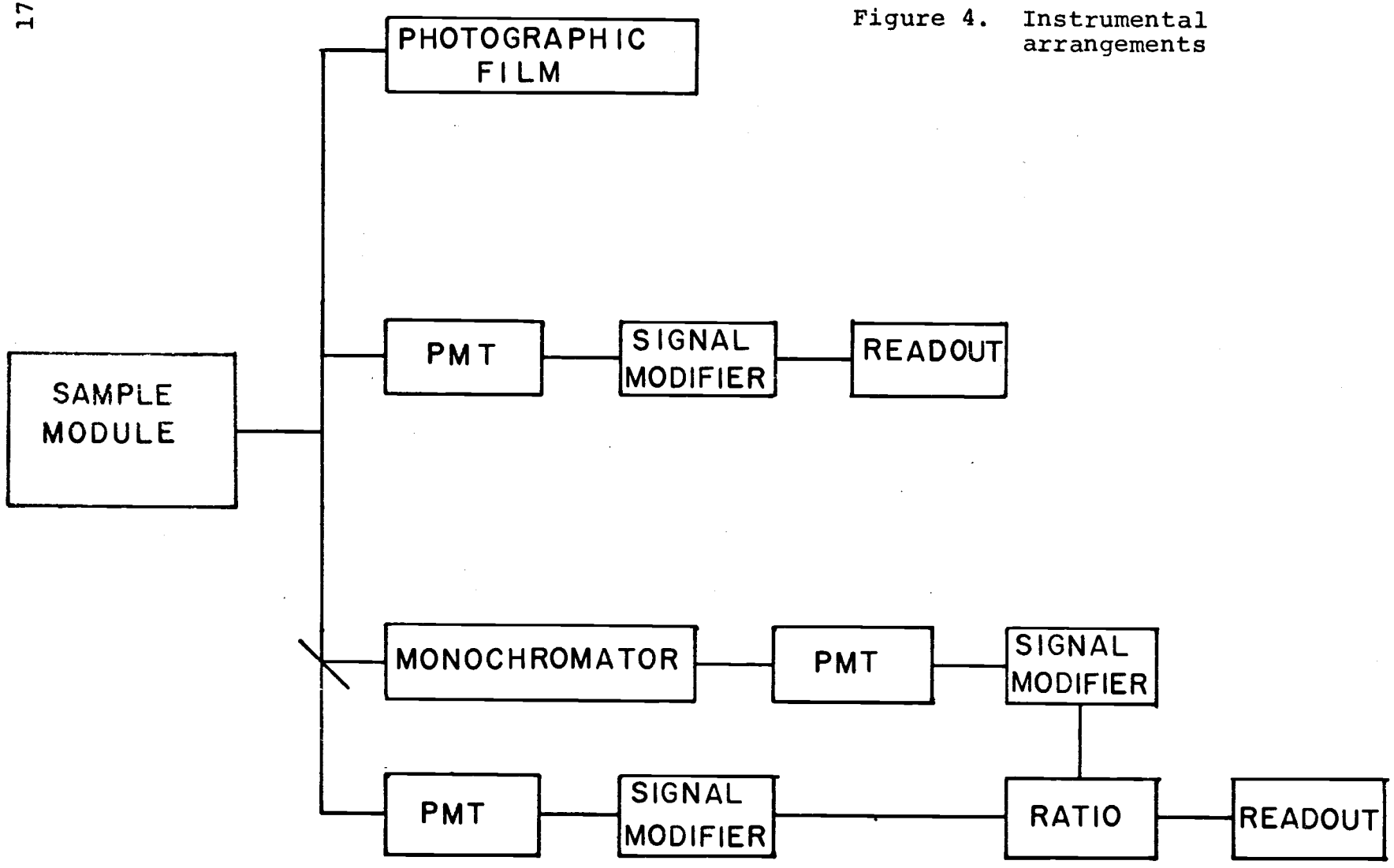


Figure 4. Instrumental arrangements

sophisticated systems, a peak detector or integrator is also incorporated.

Three types of sample modules have been developed a discrete sampling system (40,41), a flow system (14), and a centrifugal analyzer (5,42). With the discrete sampling system all reagents except the analyte are placed in a reaction cell in front of a PMT. A lid is placed over the reaction cell, the analyte solution is injected into the reaction cell with a syringe, and the photocurrent output is measured as peak height or peak area. In the flow system the reagents are continuously pumped through a reaction chamber in which case the integral of the light intensity for the time spent in the reaction chamber is measured (14). The centrifugal analyzer uses a spinning action to mix the samples, and then measures them as they pass a PMT. The advantages and limitations of each system are listed in Table 2.

The instrumentation shown in figure 1c utilizes a monochromator so that a chemiluminescence spectrum may be obtained by running samples at a number of different wavelengths and making a composite spectrum. The double beam feature is needed to correct for differences in the total CL for each sample run for the spectrum.

F. Chemiluminescence Chromium Analysis

A method for chemiluminescence Cr(III) analysis was

TABLE II. COMPARISON OF CL SAMPLING METHODS

Discrete System	Flow System	Centrifugal Analyzer
versatile readout system	peak area measurement	versatile readout system
small sample	large sample	small sample
external magnetic stirring	internal stirring by gas bubbles	external stirring by rotor
temperature controlled cell holder	difficult to use temperature controlled holder	difficult to use temperature controlled cell holder
more complicated electronics (if peak detector or integrator are used)	simple electronics	computor electronics readout required
No multi-sample capability	simple to make repetitive measurements on one sample	multi-sample analysis capability (up to number of cells in rotor)
simple instrumentation that can be made portable	complicated flow cell and syringe system that cannot be easily made portable	extremely complicated instrument that could not be easily made portable
low cost	moderate cost	high cost
sample cell easily modified for samples requiring O_2	can be used with O_2	cannot be used for samples requiring O_2

first developed by Seitz, et.al. (4) in 1972. The technique is based on Cr(III) acting as an activator for the reaction between basic luminol and H_2O_2. Since other metals also serve as activators some masking is required. Addition of EDTA to the solutions was found to immediately complex most of the interfering ions. Since the Cr(III)-EDTA complex is formed slowly at ambient temperatures, the Cr can be determined by making measurements soon after the EDTA is added.

The instrumentation is based on a flow cell system (15) in which the reactants (luminol, H_2O_2) and the samples are pumped by a motor driven syringe system into a 1 by 2 cm glass cell. After the reactants enter the cell they are mixed by nitrogen gas pumped into the cell. The light emission is measured by a PMT and recorded on a chart recorder. The output of the PMT represents the integration of the light intensity between the time the reactants enter the cell and when they leave the cell.

The results of the optimization study with the above system are shown in table 3 where the concentrations of the reactants are the initial concentrations. A plot of log peak intensity vs. log Cr(III) concentration gave a line that was linear with a slope of 1 between 3×10^{-9} and 10^{-6} M Cr(III). The detection limit, defined as the concentration producing a signal twice the noise level, was 5×10^{-10} M Cr(III). Even with EDTA added to the samples they

TABLE III. OPTIMUM CONDITIONS FOR Cr(III) ANALYSIS

Reagent	flow cell conc.	centrifugal analyzer conc.
luminol	4×10^{-4} M	10^{-3} M
H_2O_2	2×10^{-2} M	0.2 M
pH	10.8	10.3 *
flow rate	4.41 ml/min	----
Cr(III)	5×10^{-9}--10^{-6} M	0.1 --- 0.7 ppm
H_3BO_3 buffer	0.1 M *	0.12 M *
EDTA	10^{-2} M *	10^{-2} M *

* not optimized

found a significant interference from 10^{-5} M Fe(II) and Fe(III), and 10^{-7} M Co(III). Their system was used to measure free Cr(III) in the ppb range in natural water samples.

A subsequent paper by Li and Hercules (43) described the use of the apparatus for the measurement of Cr in biological samples (43). The samples were digested using a 3:1:1 nitric- perchloric-sulfuric acid mixture. Standard additions were used to measure the Cr(III) levels in orchard leaves (2.4 ppm), bovine liver (0.87 ppm), and human blood (0-200 ppb). The interferences from iron and cobalt were minimized by heating the samples just below boiling for 2 minutes after the initial measurement with EDTA. The heating complexed the Cr(III) with the EDTA so when the samples were measured again the signal would be due only to the iron and cobalt. By subtracting the two readings, the Cr(III) concentration was obtained. One problem that resulted from using real samples was the sample loop used to inject samples in the flow system clogged after 3 or 4 analyses and had to be removed and boiled in nitric acid.

A recent paper by Bowling, et. al. (5) describes a CL method for the determination of Cr(III) using a centrifugal fast analyzer (42). The analyzer is basically a rotor with 15 cuvettes on the outside and two sample chambers connected to each cuvette. The Cr(III) samples or standards and

the H_2O_2 is placed in one sample chamber and the luminol-EDTA solution is placed in the second chamber. Rotation causes the reagents and samples to mix and the signal from each cuvette is observed when it passes across the photomultiplier tube face. A PDP 8/I controls the data collection. The rotor's speed is 1000 rpm and the data is collected on the second revolution and every other revolution after that until 21 readings have been taken. This allows both peak height and peak area measurements to be taken. The optimum reagent concentrations are also shown in table 3. No detection limit was reported but the calibration curve went down to 50 ppb. The system was used to measure both the Cr(III) and the Cr(VI) in drinking water samples. The Cr(VI) was measured by reducing a 15 ml Cr(VI) sample with 0.1 ml of 30% H_2O_2 and 0.05 ml of 1 M HCl to Cr(III). The solution was heated to near boiling for 30 minutes to remove the excess peroxide. The Cr(III) levels were measured and equated with the Cr(VI) concentration. The Cr(VI) levels in natural water were found to be between 50 and 600 ppb and no Cr(III) was observed.

G. Digestions of Biological Materials

The two methods commonly used for the destruction of organic matter in biological samples for analysis are acid digestions with one or more mineral acids and dry ashing in a high temperature muffle furnace. Both methods have been

reviewed in detail (44,45,46).

The most popular acid digestion method for biological samples involves heating a 3:1:1 mixture of nitric, perchloric, and sulfuric acid to 210° C in an open container with the sample. Perchloric acid can be used alone but explosions can result if it comes in contact with easily oxidized organic materials or a dehydrating agent. The sulfuric acid provides increased oxidizing power but can be left out if the sulfate interferes (44,47). Another common digestion procedure involves placing the sample and nitric acid in a Teflon-lined acid bomb and heating for a few hours. The acid bomb reduces volatilization and speeds up the digestion process (48). The advantages of the acid digestions are the speed with which they can be done, the low temperature, and the ease of transferring the sample from the container. The disadvantages are contamination from the acids and the difficulty of subsequent pH adjustment.

To dry ash a sample in a muffle furnace,the sample must first be dried and then placed in a crucible. The crucibles are transferred to a muffle furnace and heated to between 500° C and 600° C for 4 to 5 hours (47,45,46). After cooling, the samples must be put into solution which often takes a small amount of acid. Problems with dry ashing are volatilization of some elements, difficulty in removing the ashed sample from the crucible walls, contam-

ination from the crucible, and the dissolution of the compounds formed.

A new method for dry ashing employs a low temperature oxygen plasma. An RF field is used to generate a plasma in a partially evacuated quartz cylinder through which oxygen is flowing. The oxygen interacts with the RF field to form an active oxygen plasma which reacts readily with organic matter to form CO_2 and H_2O (49). The volatilization from the containers is reduced. However the ashing time is usually 2 to 4 hours.

III. INSTRUMENTATION

A. Introduction

As discussed in the background section, the three basic CL sample modules are the discrete sampling system, the flow cell system, and the centrifugal analyzer. The discrete sampling system approach was chosen because we desired to build a simple, rugged instrument that could be operated in the field. This approach has been used by others (40,41), and is the basis for the bioluminescence instruments built by DuPont, JRB, and Aminco.

The advantages of the discrete system over the flow system are that it eliminates the use of N_2 gas for reagent mixing; it requires a smaller sample volume; it is possible to use a more versatile electronics system which measures the instantaneous signal, the peak height, and the peak area; and it eliminates changing the sample loop after every three measurements for real samples. Some of the advantages our system has over previous and commercial discrete sampling units include temperature control for the sample cell, a large light tight compartment to allow room for modifications to the cell holder, compatability with a monochromator, a magnetic stirrer, a cooled PMT housing to reduce dark current noise, and a versatile electronics system. The construction of the sample compartment, the cooled PMT housing, and the electronics are covered in the

rest of the chapter.

B. Sample Compartment

A sample compartment designed to meet the specifications outlined in the previous section is illustrated in figures 5 and 6, and the specific components are identified in table 4. The three essential parts of the sample compartment are the housing, the sample cell holder, and the stirring mechanism.

The housing was constructed from milled aluminum plates in the box form shown in figure 5 and was held together with 6-32 machine screws. The inside edges of the box were sealed with a black rubber compound to prevent light leaks. The sample compartment is divided into an upper (main) compartment and a lower (motor) compartment. The photomultiplier opening is designed to be compatible with a Heath model EU-700 monochromator so a CL spectrum could be obtained. Water and gas (if needed) enter the motor compartment through bulkhead swagelock fittings (j) attached to the back plate and flow through ¼" copper tubing to a second set of bulkhead fittings when entering the main sample compartment. Access to the sample compartment is through adjustment ports (f) and (h) which have screw plate covers with felt gaskets.

The temperature controlled sample cell holder was machined from brass and provides temperature control by

TABLE IV. IDENTIFICATION OF COMPONENTS OF SAMPLE MODULE

Letter	Description	Material
a	stirring motor	
b	carriage supports	steel
c	stirring magnet	ALNICO
d	sample cell holder	brass
e	swagelock injection port	brass
f	access port & cover	aluminum
g	adjustable feet	steel
h	access port & cover	aluminum
i	sample cell	glass
j	bulkhead swagelocks	brass
k	sample module lid	aluminum
l	PMT window	
m	movable cell block	brass
n	water circulator holes	
o	adjustment screws 8-32	steel
p	stirring motor shaft	steel
q	movable carriage	aluminum

Figure 5. Front view of sample module

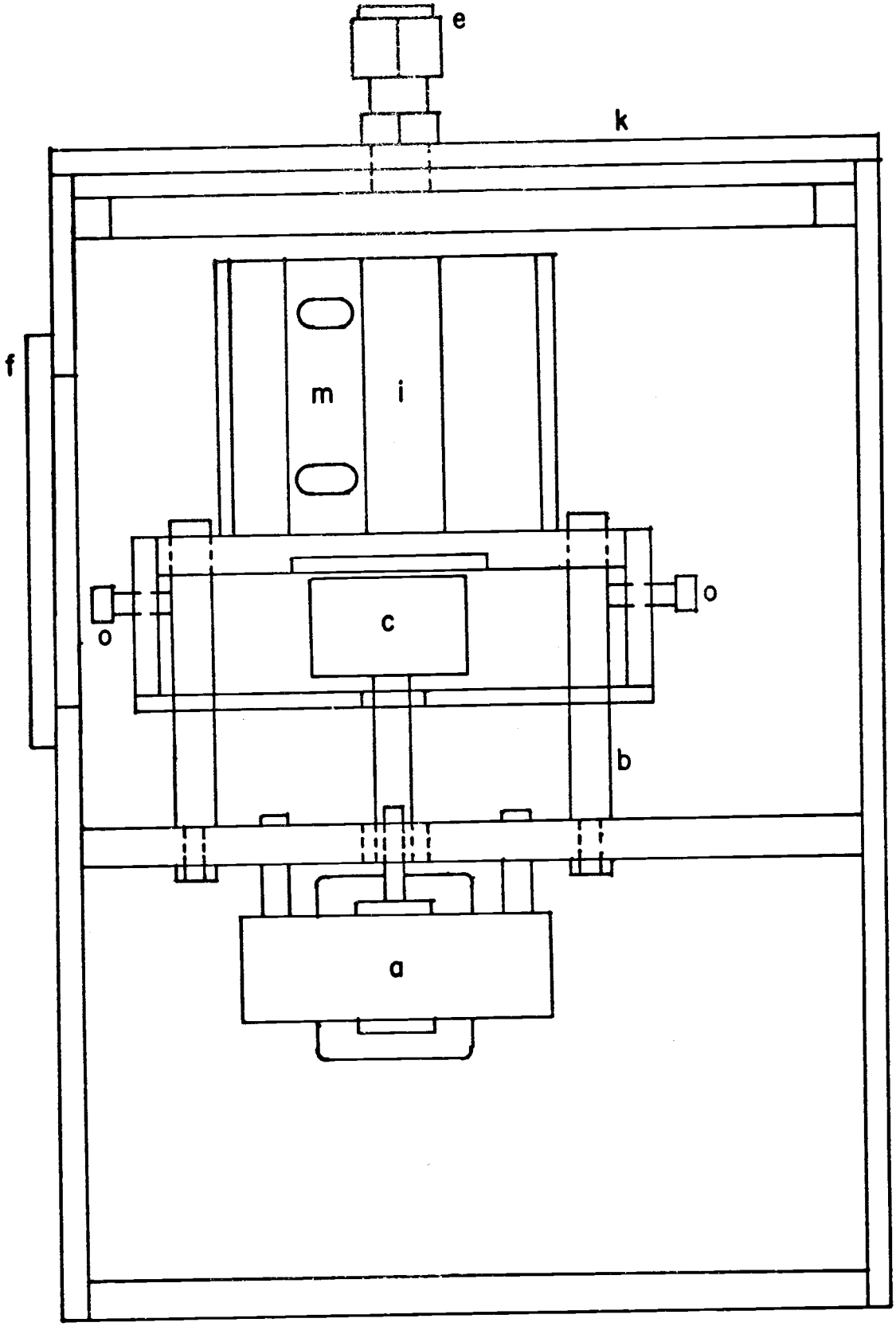
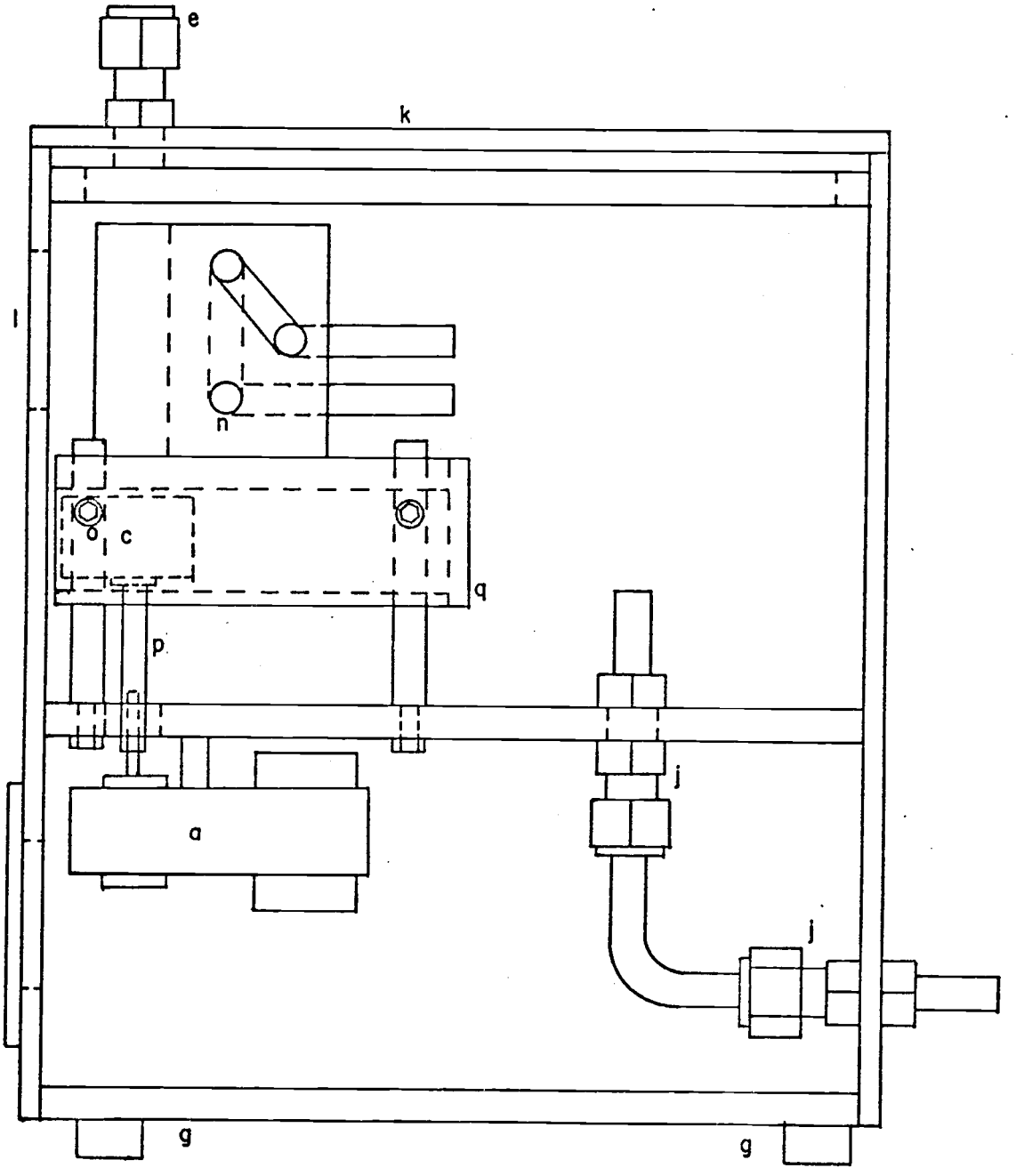

e
k
f
m
i
o
o
c
b
a

FIGURE 6 Side view of sample module

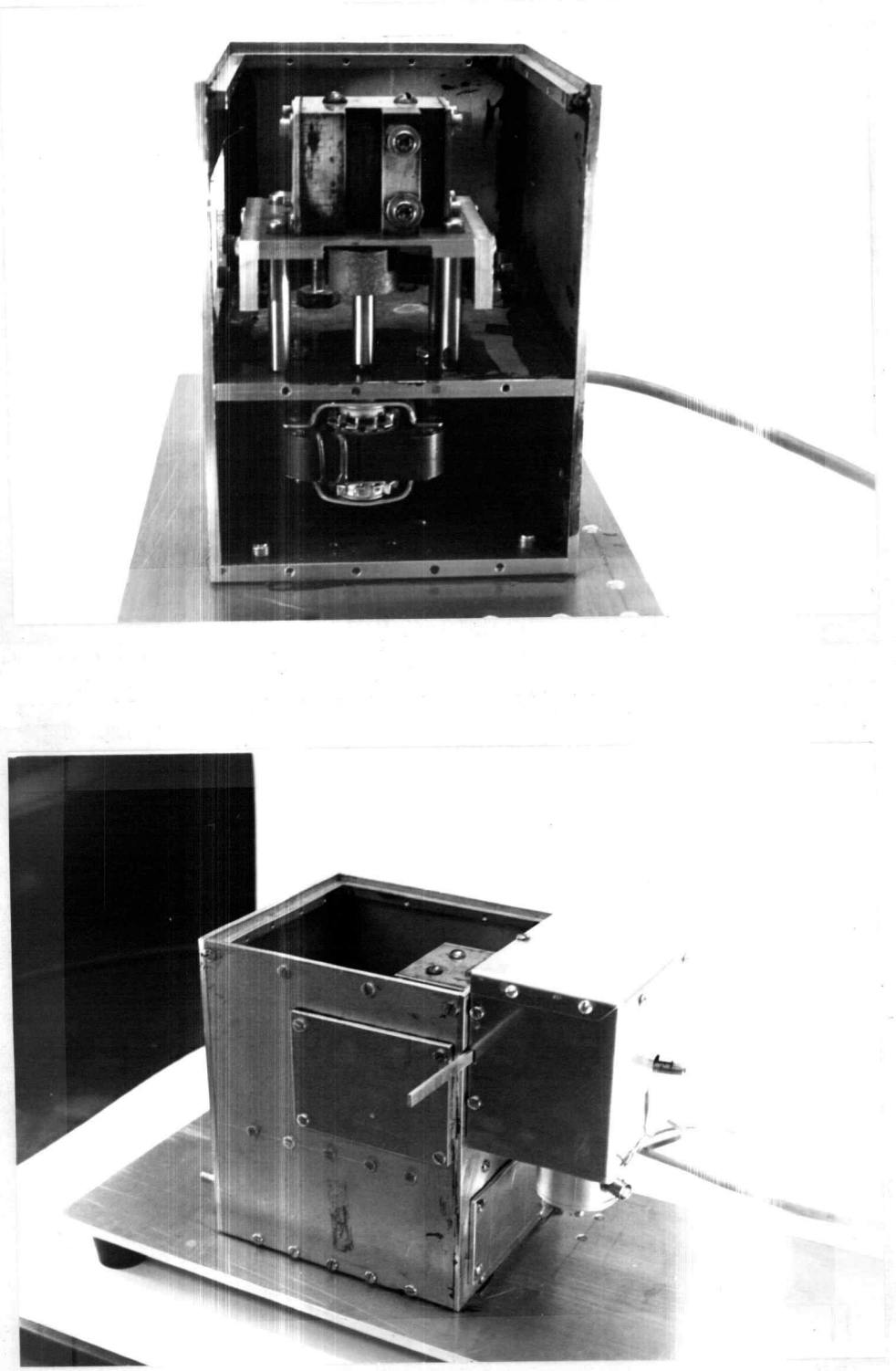


water circulating through holes (n) drilled in the block. Copper tubing was expoxied into the holes and is connected to the bulkhead swagelock connectors by Tygon tubing. Temperature controlled water is circulated by a Haake model FJ temperature bath and pump. This cell holder is unusual because the cell is surrounded on three sides by the cell holder since there is no need for an excitation window. The sample cell is a 1 cm square glass cell and is held in position (i) of the sample cell holder by the movable block (m) which keeps the cell in thermal contact with the holder.

The cell holder is attached to a movable carriage (q) which provides a 3/4" adjustment in height and can accommodate different sample cell holders and cells. Four set screws (o) hold the carriage in position. To reach the screw, adjustment plate (f) must be removed. The sample cell holder is held to the carriage by two brass screws on the top of the cell holder which allows it to be removed for cleaning.

Stirring magnet (c) is connected to a motor with a brass shaft. The shaft is hollow and is held in position with a set screw so it can adjust with the height of the carriage. The speed is controlled by a Variac outside the motor compartment. For stirring a special teflon coated magnetic stirring bar designed for the 1 cm square cell (Bel-Art F-37150) was driven by a small induction motor. Photographs of the completed housing are shown in Figure 7.

Figure 7. Photographs of sample module housing.



C. Cooled Photomultiplier Housing

Cooled photomultiplier housings are used to reduce the dark current noise caused by thermionic emission. For small light levels, the reduction in dark current noise will improve the signal-to-noise ratio (S/N) if dark current noise is significant. Minimization of dark current noise is especially critical for tubes that have extended response in the infrared (e.g. S-1,S-20) since they are more susceptable to thermionic emission.

The design and construction of the cooled photomultiplier housing used with this CL appratus was discussed in detail in a recent paper (51). The housing consists of a light-tight compartment in which nitrogen gas cooled by liquid nitrogen is circulated to cool the PMT, a PMT socket and appropriate dynode resistors and electrical connectors, and a shutter mechanism. Since the PMT was never operated under low temperature conditions for CL measurements because dark current noise was not limiting, the construction and operation will only be briefly reviewed.

The housing was designed so that the side-on PMT photocathode is close (1½") to the CL reaction cell in order to insure efficient light collection. Other important characteristics of the housing include continuous temperature control and temperature measurement between 20 and -60°C, a shutter mechanism, quartz windows for UV res-

ponse, simplicity of construction, simple replacement of PMT, and small size.

For all CL measurements, an RCA C-37025C PMT which has a high cathode spectral responsivity from 200-900 nm was used. Cooling this tube, biased at -1200 V, to -60°C reduced the dark current and dark current noise by factors of about 30 and 3, respectively, compared to the room temperature values.

D. Electronics

The photocurrent from the PMT is amplified and converted to a voltage by a Keithley model 124 current amplifier. The signal from the amplifier is monitored and modified by different types of circuitry and readout devices as shown in figure 8 in order to ascertain the optimal arrangement. The specific commercial components are listed in table 5.

The integrator was constructed from an operational amplifier wired in the standard configuration shown in figure 9; (52). The various capacitors in the feedback circuit provide different values for the transfer function governed by equation 1.

$$e_{out} = \frac{1}{RC} \int e_{in}\, dt$$

Relay RY2 shorts the integrator capacitor (reset) and relay RY1 disconnects the input from the integrator (hold). The operation of the relays is controlled by switches on the front panel or alternately by 5 VDC logic pulses from an

TABLE V. ELECTRONIC COMPONENTS

Component	Manufacturer	Model
PMT	RCA	C-31025C
Current Amplifier	Keithley	124
PMT Power Supply	Keithley	244
Storage Scope	Tektronix	564B
Chart Recorder	Heath	SR-255B
Digital Voltmeter	Fluke	8000A

Figure 8. Block diagram of electronics arrangements tested.

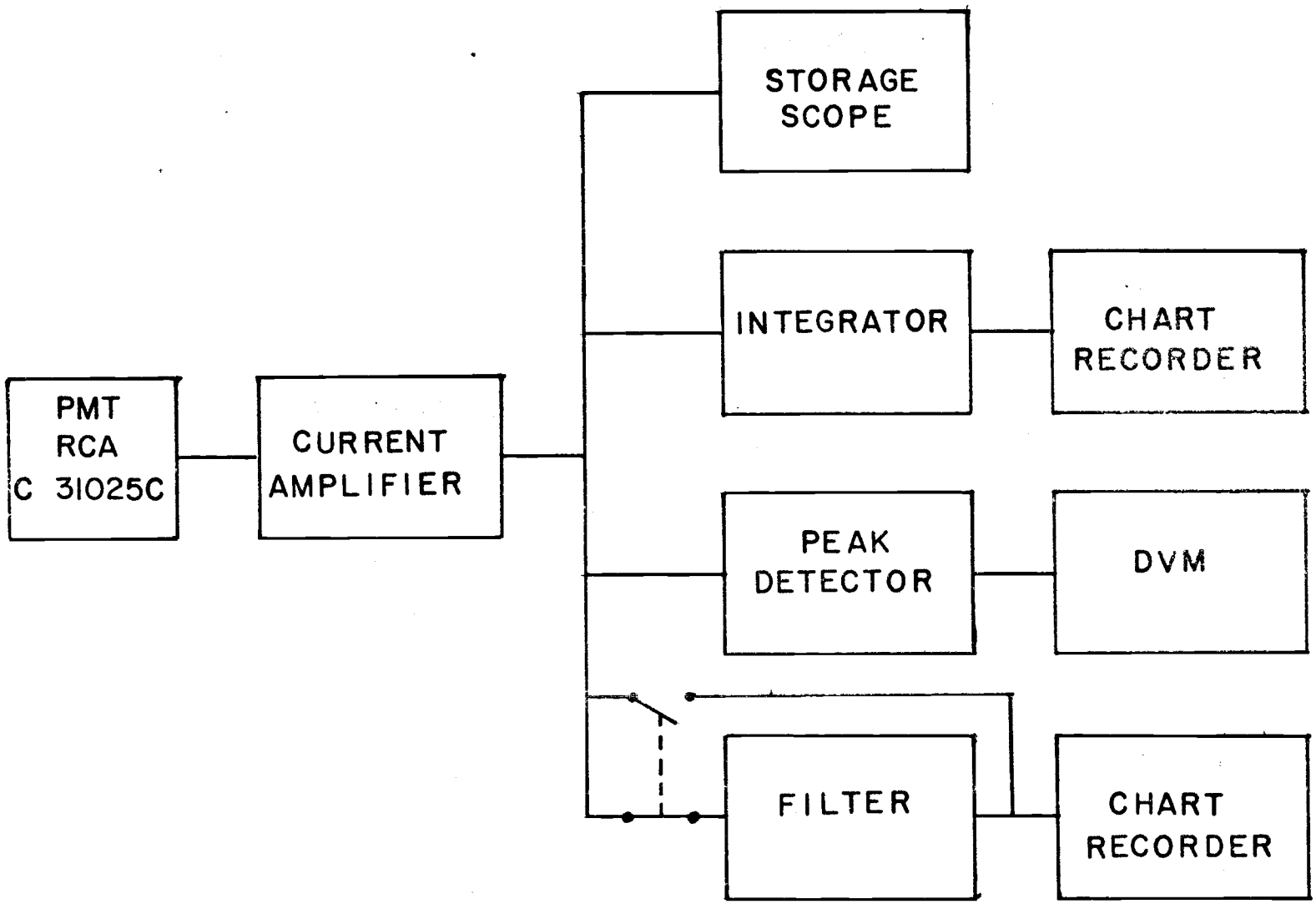

PMT
RCA
C 31025C
CURRENT
AMPLIFIER
STORAGE
SCOPE
INTEGRATOR
CHART
RECORDER
PEAK
DETECTOR
DVM
FILTER
CHART
RECORDER

Figure 9. Integrator circuit diagram.

a) Digital control circuits

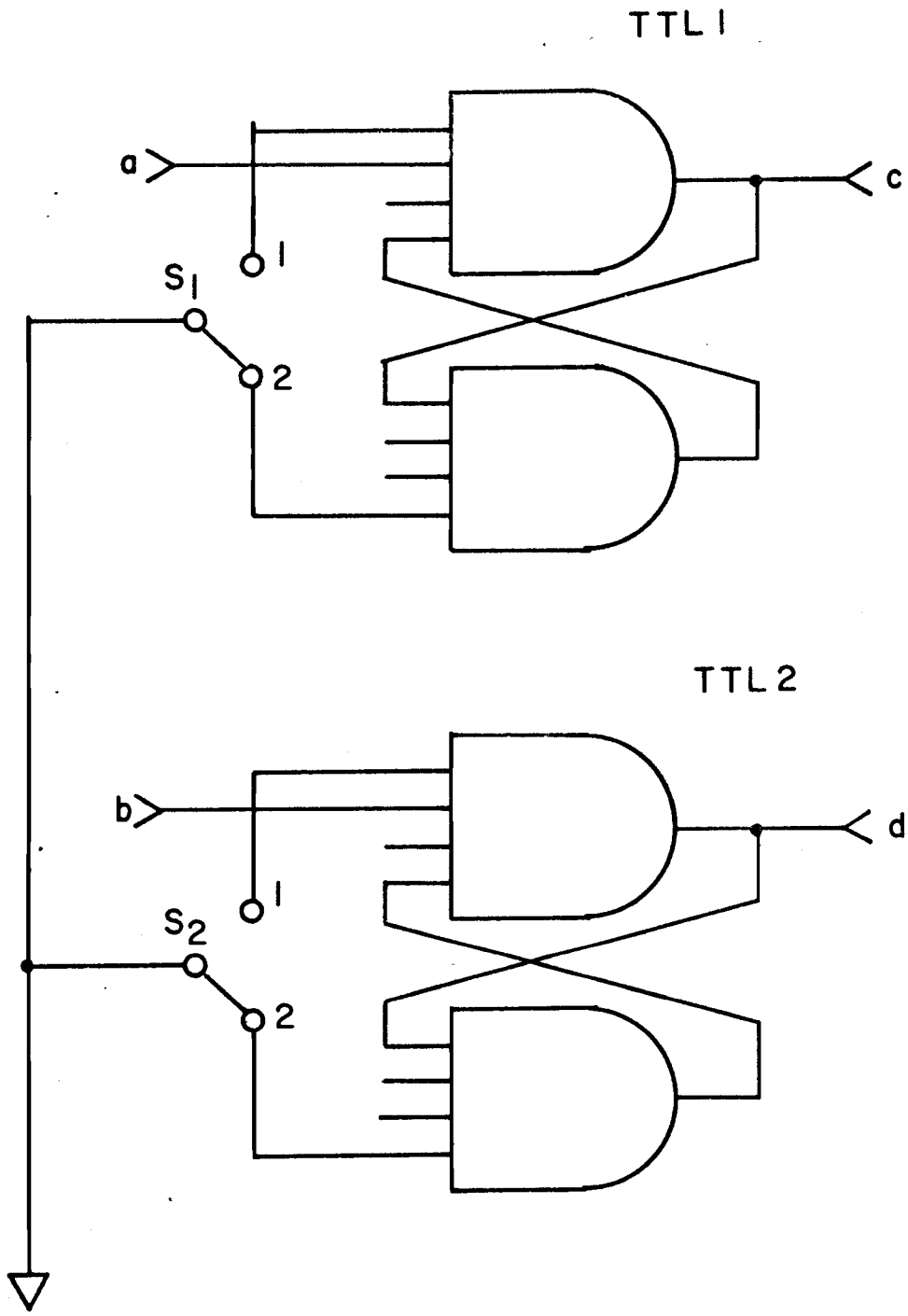
a & b are external control inputs, c & d are outputs to the control relays in figure 10b. S1 in position 1 corresponds to "hold", and S2 in position 1 corresponds to "reset".

S1	SPST
S2	SPST
TTL1	SN 7420N
TTL2	SN 7420N

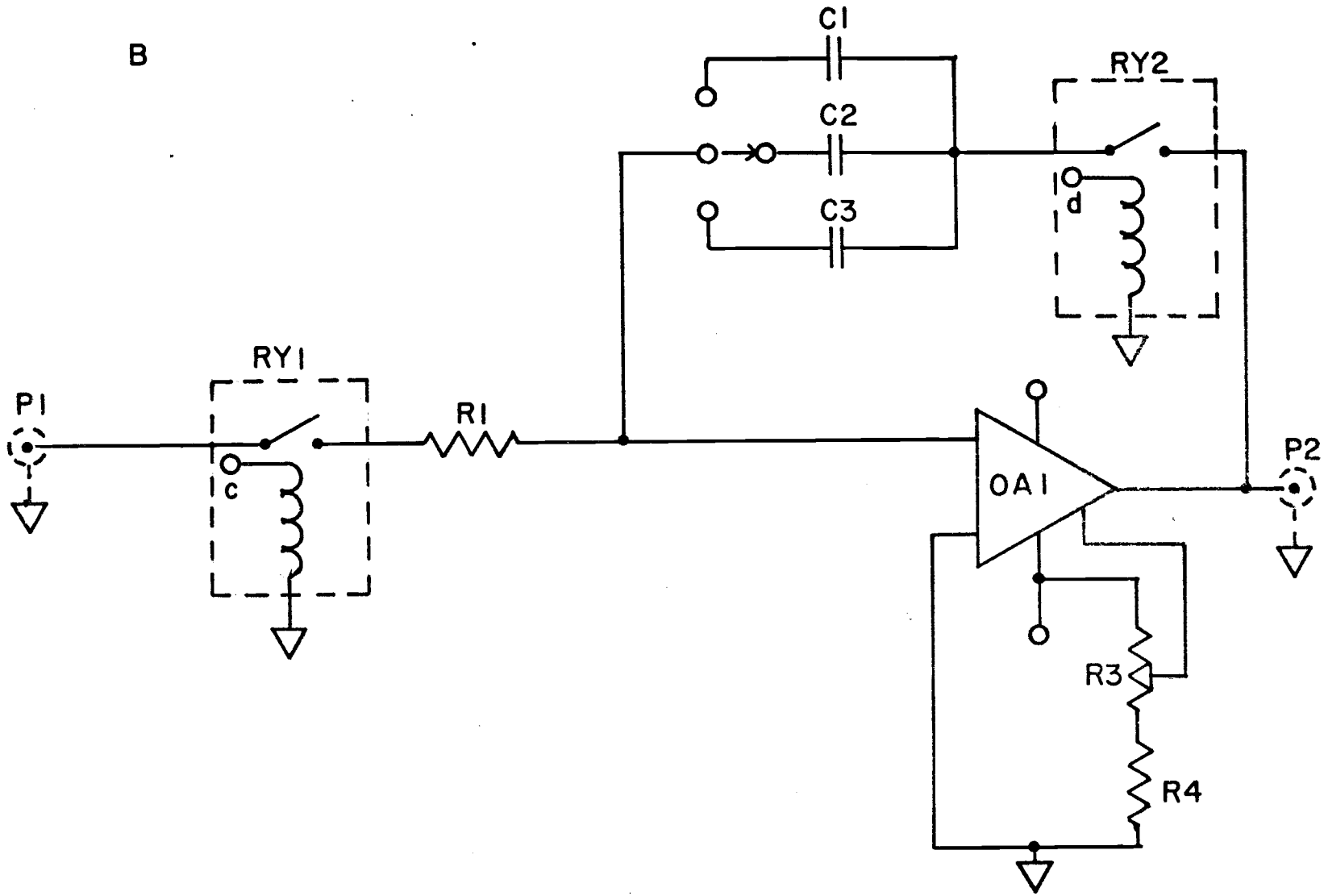
b) Operational amplifier integrator

C1	5 uF
C2	1 uF
C3	.1 uF
OA1	Function Modules 380J
P1	BNC input
P2	BNC output
R1	100K
R2	56K
R3	50K
RY1	mercury wetted relay
RY2	mercury wetted relay

A



B



external timing circuit connected to BNC connectors at the back of the chassis.

The performance of the integrator was tested by measuring the output voltage of the integrator using a Heath Voltage Reference Source model EU-80A to input a known voltage for a known period of time controlled by an external timing circuit. The results show that the measured output was within 3% of the calculated output, and the relative standard deviation of the runs was within 0.25%.

Comparison of the instantaneous CL signal recorded on a storage scope to that recorded on the chart recorder indicate that the chart recorder response was slow enough to attenuate the signal by up to 25% and that the attenuation depended on the signal level. The input filter circuit shown in figure 10a was used to provide time constants of 1 and $\frac{1}{2}$s and to see if the chart recorder could be used directly if the filter's time constant was greater than the recorders. Even with a 1s time constant, the response of the recorder was still not constant across its scale, and the recorder cannot be used for accurate peak height measurements, but it can be used for optimization studies where it is desired to monitor only changes in the peak height.

A peak detector based on a design by Piepmeier (53) was constructed to provide a means of accurately measuring the peak height without the use of a storage scope. The

Figure 10. Peak detector and filter circuit diagram.

a) Filter circuit

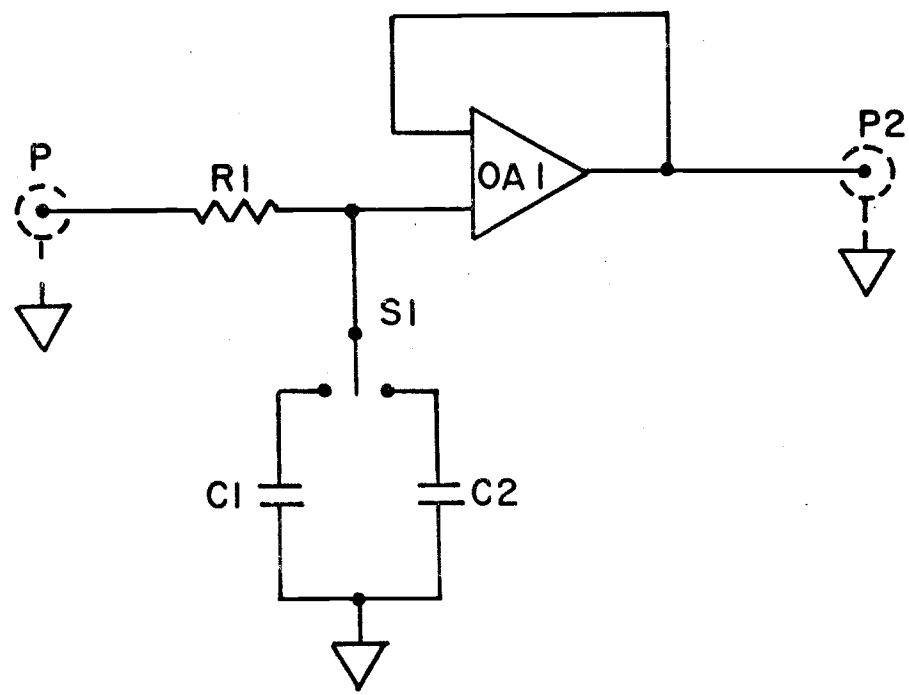
C1	1.0 uF
C2	0.5 uF
OA1	A540J Anolog Devices
P1	BNC filter input
P2	BNC filter output
R1	1 M
S1	SPDT toggle switch

b) Peak detector circuit

C3	1.0 uF
D1, D2	1N457
OA2, OA3	A504J Analog Devices
P3	BNC input peak detector
P4	BNC output peak detector
R2,R3	1 K
R4	10 K
S2	SPST peak detector shorting switch

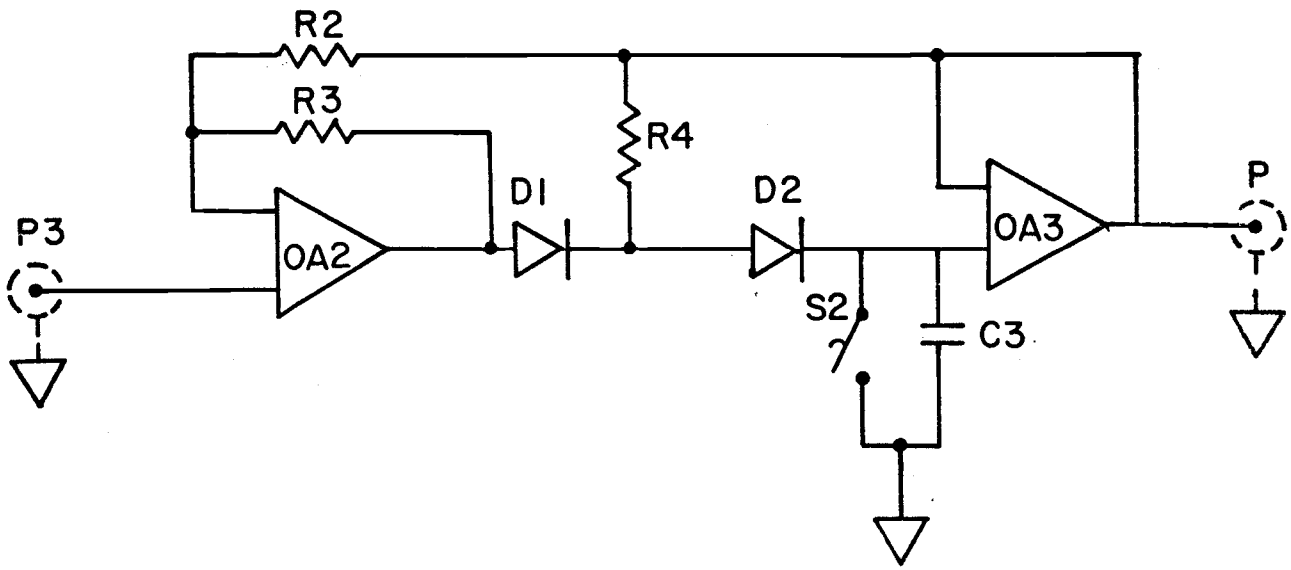
FILTER

A



B

PEAK DETECTOR



schematic is shown in figure 10b. The peak detector circuit is comprised of a simple peak detector consisting of OA3,C3, and D2. The peak voltage is passed through D2, stored on C3, and then measured through OA3 which acts as a follower. In order to improve stability, D1 and R4 are included to reduce the leakage from C3 through D2. The charging time of C3 is very slow; to increase it, OA2 is used as a follower with variable gain. When there is no charge on C3, OA2 has a high gain, but as the charge increases the gain decreases until it reaches unity when C3 is equal to the input voltage. Actual Cr chemiluminescence peaks were simultaneously recorded on the storage scope and peak detector. The storage scope could only be read to 5% and since most of the peak detector readings were within this range, the peak detector was judged to work satisfactorily.

IV. PROCEDURE

A. Analysis Procedure

The basic injection procedures used for the optimization studies and analysis are outlined below. With the shutter closed and the lid off the sample module, luminol solution is put into the reaction cell with a 1 ml Eppendorf pipet followed by the hydrogen peroxide added with a 1/2 ml Eppendorf pipet. The lid is put in place, the shutter is opened, and the integrator or peak detector is set to the run position. One-half milliliter of the Cr(III) solution is injected rapidly in a reproducible manner with a ½ ml Hamilton gas tight syringe (a platinum needle should be used at low concentrations). One ml instead of ½ ml of Cr(III) was used for the luminol and H_2O_2 optimization studies discussed in Chapter V. The signals are recorded and then the shutter is closed. The lid of the sample compartment is removed and the solution is removed by suction with a disposable pipet connected to a vacuum aspirator bottle. Three milliliters of double distilled water in a polyethylene (poly) wash bottle are used to rinse the cell twice before another sample is injected. For samples that might contain interfering ions, 100 µl of 10^{-1} M EDTA should be added with a syringe to the sample in a test tube and allowed to stand two minutes before injecting into the cell.

For optimization studies peak heights were measured on

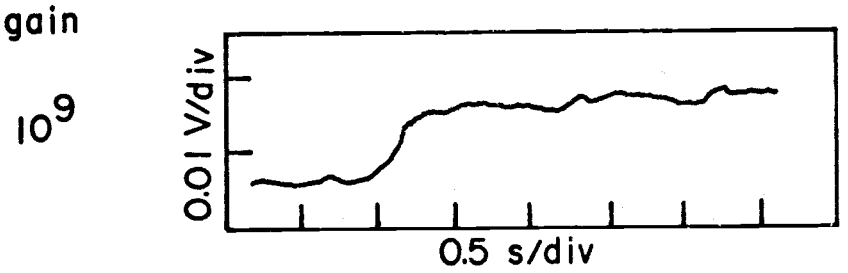
a chart recorder connected directly to the current amplifier while the final calibration curve and analysis measurement readings were taken from a digital voltmeter connected to the peak detector.

Peak areas were measured off recorder tracings of the output of the integrator. Photographs of storage scope tracings in figure 11 show the difference between the background, blank, and analyte signals at lower concentrations. The Cr and blank peak appear on top of the background reaction. At concentrations below 10^{-7} M, the blank signal must be subtracted from the total signal to obtain the signal due just to Cr(III) in the sample.

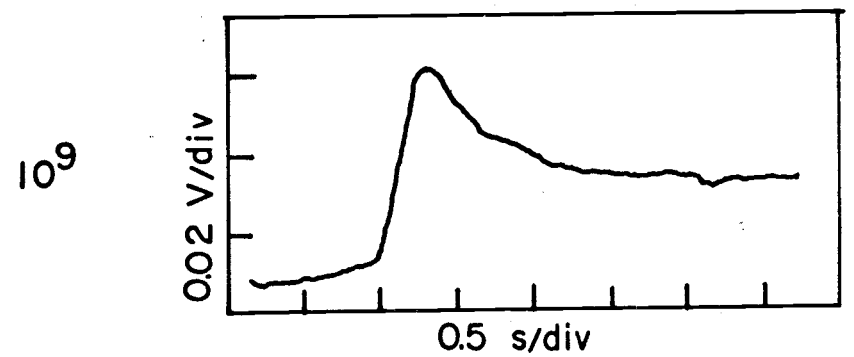
B. Solution Preparation

All of the solutions were made with double distilled water from a Corning Water still (model AG-3), attached to the house distilled water. All chemicals used were analytical reagent grade or better as specified. For solutions where the concentration was not critical, calibrated poly bottles were used. The final pH of all solutions was adjusted by adding either 0.5 N NaOH or 1 N HCl with a buret while monitoring with a micro-pH electrode and Chemtrix digital pH meter (model 60). Standard buffer solutions of pH 4.00, 7.00, and 10.00 were used to calibrate the meter. The optimum reagent concentrations are listed in table 6.

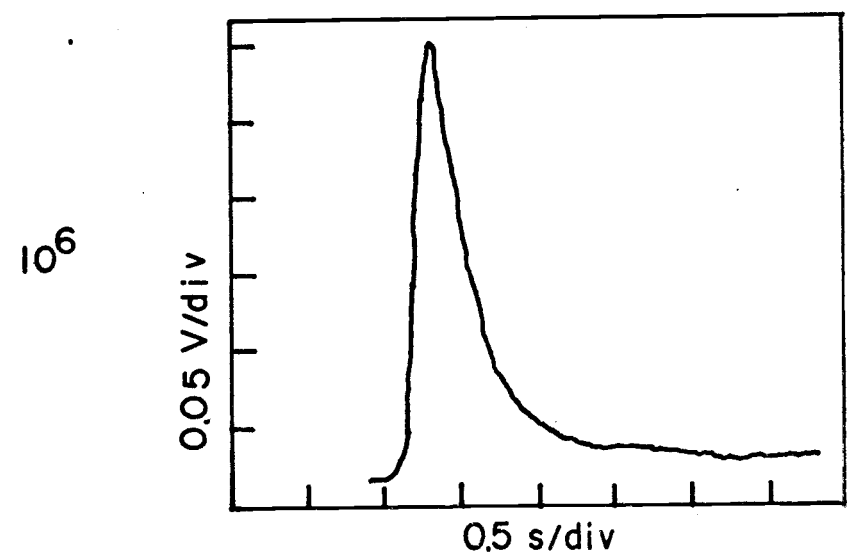
Figure 11. Scope tracings for CL reaction.



Background (H_2O_2 + luminol)



Blank (H_2O_2 + luminol + distilled water)



Analyte (H_2O_2 + luminol + 10^{-6} M Cr.)

TABLE VI. OPTIMUM CONDITIONS

Reagent	peak height	peak area
luminol [a]	8×10^{-5} M	8×10^{-5} M
hydrogen peroxide [a]	10^{-1} M	10^{-2} M
luminol pH	12.0	11.0
cell temperature	as high as possible	24° C
$NaHCO_3$ buffer	0.05 M	0.05 M
EDTA	10^{-3} M	10^{-3} M
Cr(III)	10^{-8} -- 10^{-5} M	10^{-7} -- 10^{-5} M

a- final cell concentration

A 0.1 M luminol stock solution was made by diluting 3.544 grams of Eastman luminol crystals (5-amino-2,3-dihydro 1,4-phthalazinedione) with distilled water in a 200 ml volumetric flask. A recrystalization was not carried out and no problems with impurities were discovered. The luminol solutions used for optimization were made by addition of the appropriate volume of luminol stock solution to a 1 liter volumetric flask followed by the addition of 100 mls of 10^{-1} M EDTA solution to complex with interfering metal ions, and 500 mls of 0.100 M $NaHCO_3$ (8.4 g of $NaHCO_3$ in 1 liter) to buffer the solution. The pH was adjusted by adding about 5 mls of 0.5 N NaOH and the solution was diluted to volume with distilled water.

For optimization, the hydrogen peroxide stock solution of 7.5×10^{-1} M was made by weighing 85.5 grams of Mallinckrodt Analytical Reagent grade 30% hydrogen peroxide and diluting it to 1 liter. The solutions used in the optimization were made in 100 ml volumetric flasks from dilutions of the stock solution with the addition of 10 mls of EDTA and adjustment of the pH to 3.5 with about 1 ml of 1.0 N HCl before final dilution. The pH of the hydrogen peroxide solution should be below pH 4.5 in order to minimize decomposition (27).

A 10^{-3} M Cr(III) stock solution was prepared by diluting 0.4001 grams of Baker and Adamson reagent grade chromium nitrate to 1 liter. Standard Cr(III) solutions were made

from dilutions of the stock solution in 100 ml volumetrics. The pH was adjusted to 4.0 with about 1 ml of 1 N HCl to keep the formation of $Cr(OH)_3$ to a minimum.

The 8×10^{-5} M luminol solution used for actual analysis was made in a 1 l flask from 16 mls of 0.001 M luminol stock solution with 100 mls of 10^{-2} M EDTA, 500 mls of 0.10 M $NaHCO_3$ buffer, and about 5 mls of 0.5 N NaOH to adjust the pH to 11.0. The 3×10^{-2} M H_2O_2 solution used for analysis was made directly from 13.60 grams of 30% hydrogen peroxide and 100 mls of 10^{-2} M EDTA diluted to 1 l after adjusting the pH to 4.0 by adding about 1 ml of 1 N NaOH.

V. OPTIMIZATION

A. Introduction

To take full advantage of the CL analysis procedure, the reagents concentrations, reaction conditions, and instrumental variables need to be optimized. The criterion for optimization for both the peak height and peak area studies was to adjust conditions to give the maximum signal to background ratio. Since the baseline noise is proportional to the background signal, this should optimize the S/N for small Cr(III) concentrations. The optimum concentrations found by Seitz, et. al. (4) were used until a new optimum value was found. Three measurements were made for each condition or concentration and the mean value was calculated. Five were taken if the standard deviation was high.

B. Luminol

For the luminol optimization, 10 solutions ranging from 10^{-5} to 10^{-2} M luminol at pH 11.0 were used. For 10^{-6} M Cr(III) there is no significant blank peak so the peak heights were measured from the base of the peak to the top. To measure the peak area the integrator was run until the output reached a constant value indicating the reaction was complete. At the lowest concentration luminol solution, the reaction continued for over one minute and the output

of the integrator after one minute was taken as the integrator signal.

Figure 12 shows optimization curves for the peak height and peak area and background reaction signal versus luminol concentration. The concentration axis is labeled in final cell luminol concentration which is 2/5 of the initial concentration. The bending off of both curves above 10^{-4} M appears to coincide with the Cr(III)-luminol complex formation postulated by Seitz and Hercules. Below 10^{-4} M luminol, the integrated signal is fairly constant which indicates that the total CL intensity is independent of luminol concentration. The peak signal drops off at lower concentrations because the reaction is slower giving a broader peak so that the peak height is not proportional to the peak area.

The data on the luminol-background reaction signal appears to follow the luminol-Cr curve closely. The optimum final cell concentration for both peak height and peak area measurements is 8×10^{-5} M luminol. For the peak area measurements, lower luminol concentrations are not advantageous because the analysis time is increased.

C. Hydrogen Peroxide

The optimum luminol concentration at pH 11.0 determined in the previous section was used for the hydrogen peroxide optimization. Six hydrogen peroxide solutions ranging

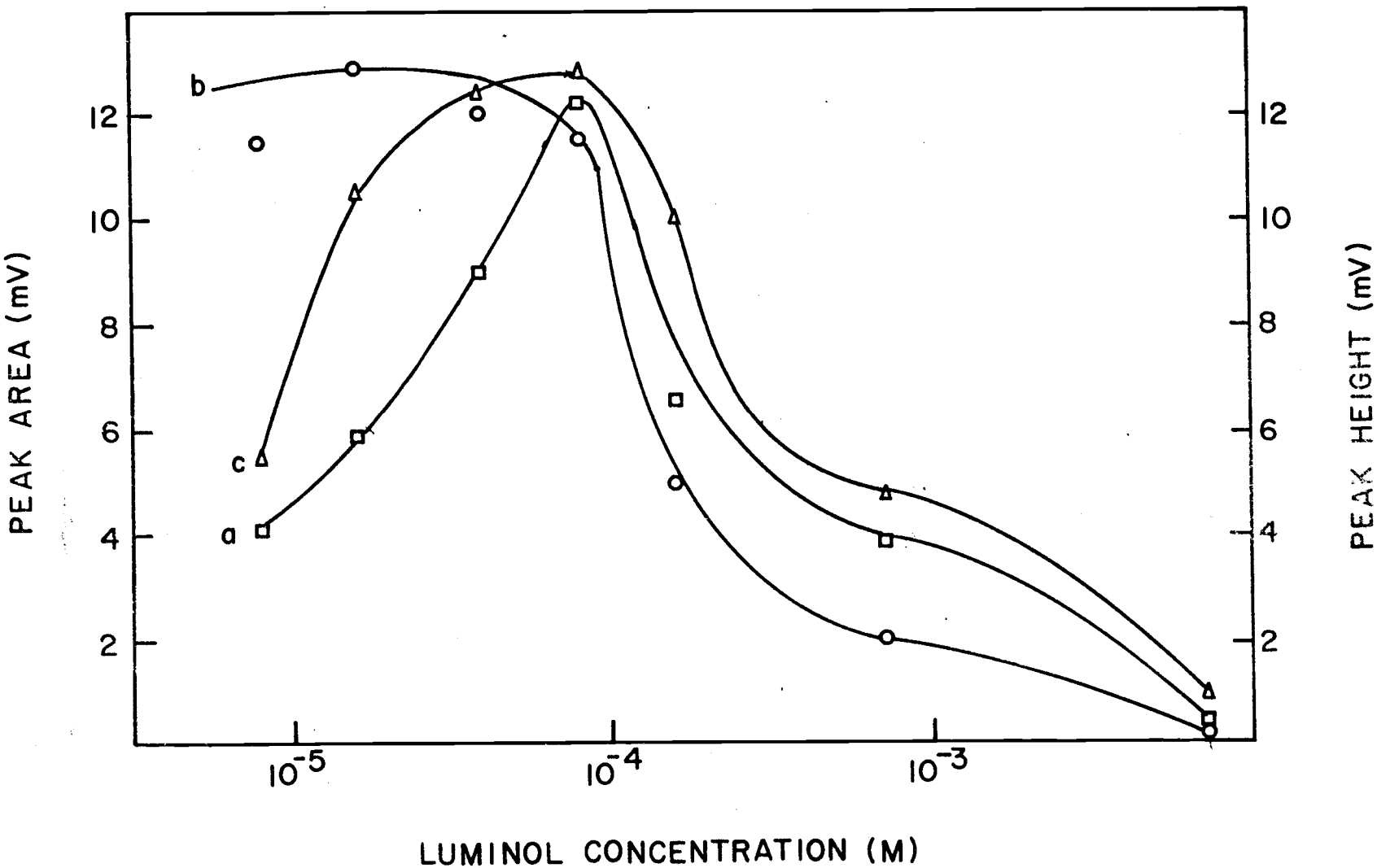
Figure 12. Luminol concentration optimization

a) Peak height

b) Peak area

c) Background

Conditions: 1 ml luminol (variable conc.) with 10^{-2} M EDTA, 0.05 M $NaHCO_3$ buffer, and pH 11.0; ½ ml 2 x 10^{-3} M H_2O_2 (final cell conc.) with 10^{-2} M EDTA, and pH 4.0; 1 ml 5 x 10^{-7} M Cr(III) (final cell conc.), pH 3.5; gain 10^6 V/A; integrator 5 μF. Background studies done with ½ ml H_2O_2 injected into 1 ml luminol; gain 10^8 V/A.


PEAK AREA (mV)
PEAK HEIGHT (mV)
LUMINOL CONCENTRATION (M)
12
10
8
6
4
2
10^{-5}
10^{-4}
10^{-3}
a
b
c

from 7.5×10^{-4} M to 2.5×10^{-1} M were used. The optimization curves for Cr and the background are shown in figure 13. The concentrations reported are final cell concentrations which are 1/5 the initial concentration. The optimum value for peak area is 10^{-2} M and for peak height is 10^{-1} M (final cell concentrations). A concentration of 3×10^{-2} M was chosen as a compromise optimum value for peak height and peak area measurements because at higher concentrations the background signal increases significantly.

D. pH

The luminol and H_2O_2 concentrations determined in the previous section were used for the pH optimization. Nine luminol solutions were made with initial pH's in the range of 10.0 to 12.1.

Figure 14 shows the peak height and peak area optimization data. Since the background values remained constant above pH 10.5 the optimum pH values for peak height is 12.0 and for peak area is 11.0. For subsequent measurements, a pH of 11.0 was used unless only peak height measurements were made in which case the pH was changed to 11.2.

E. Cell Temperature

The temperature dependence of the CL signal was studied by varying the cell temperature by changing the temperature of the circulating water from the bath. For

Figure 13. Hydrogen peroxide optimization.

a) Peak height

b) Peak area

c) Background

Conditions: 1 ml 8 x 10^{-5} M luminol (final cell conc.) with 10^{-2} M EDTA, o.05 M $NaHCO_3$ buffer, and pH 11.0; ½ ml H_2O_2 (variable conc.) with 10^{-2} M EDTA and pH 4.0; 1 ml 5 x 10^{-7} M Cr (III) (final cell conc.), pH 3.5; gain 10^6 V/A; integrator 5 µF. Background studies done with ½ ml of H_2O_2 injected into 1 ml of luminol; gain 10^8 V/A.

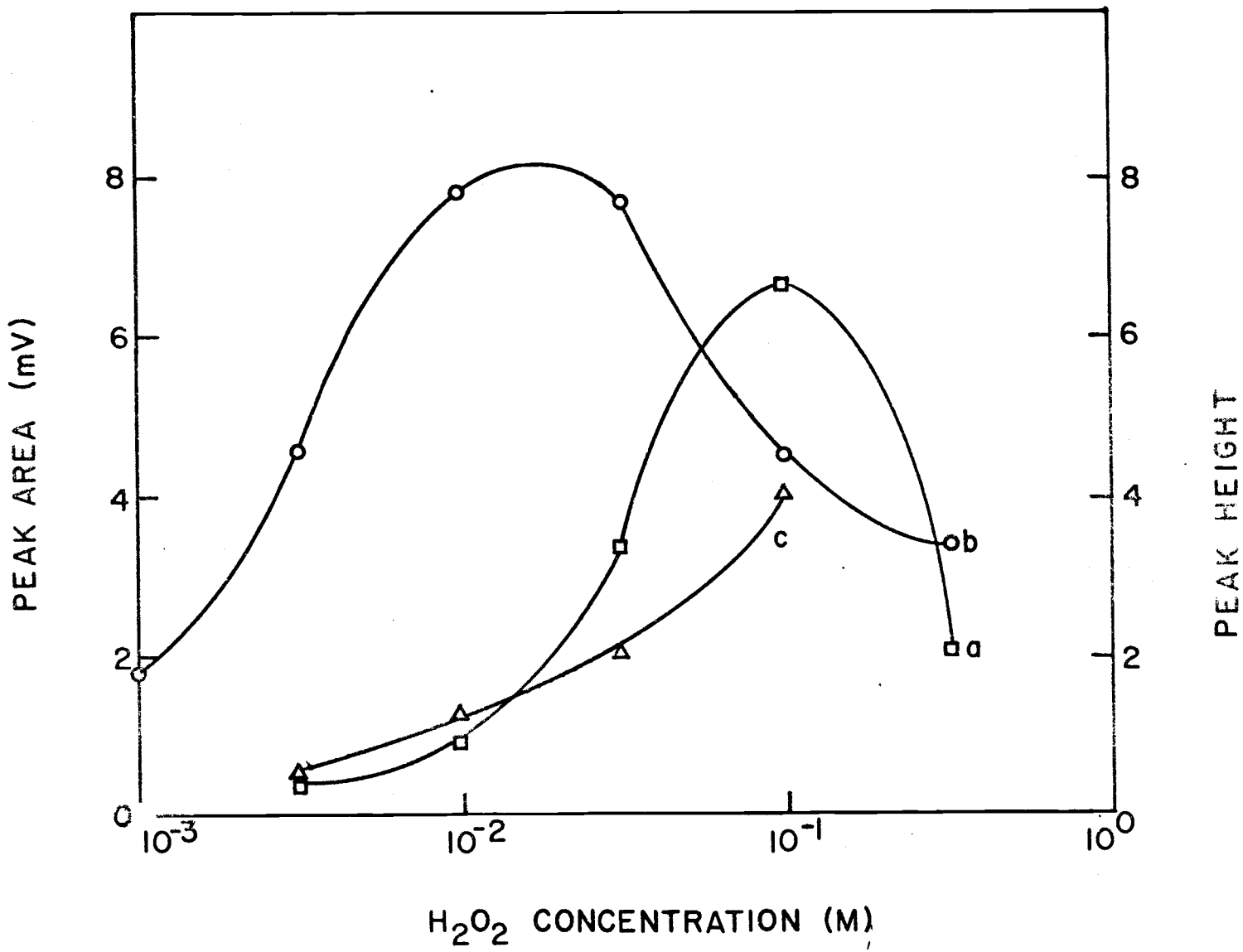
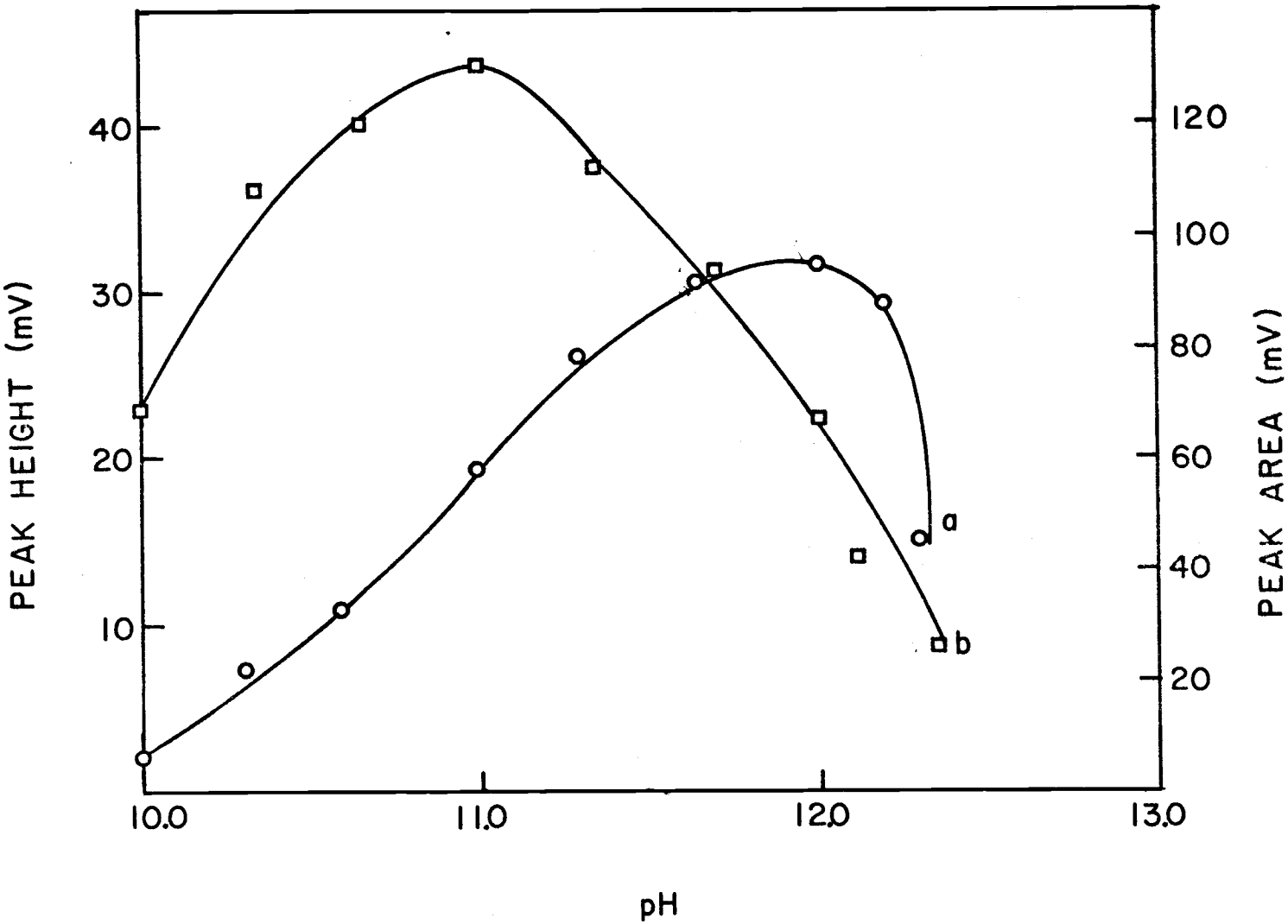

PEAK AREA (mV)
PEAK HEIGHT
8
6
4
2
0
10^{-3}
10^{-2}
10^{-1}
10^{0}
a
b
c
H_2O_2 CONCENTRATION (M)

Figure 14. pH optimization

a) Peak height

b) Peak area

Conditions: 1 ml 8 x 10^{-5} M (final cell conc.) with 10^{-2} M EDTA, 0.05 M $NaHCO_3$ buffer, pH 11.0; ½ ml 3 x 10^{-2} M H_2O_2 (final cell conc.) with 10^{-2} M EDTA and pH 4.0; ½ ml 5 x 10^{-7} M Cr(III) (final cell conc.) and pH 3.5; gain 10^6 V/A; integrator 5 μF.


PEAK HEIGHT (mV)
40
30
20
10
120
100
80
60
40
20
PEAK AREA (mV)
10.0
11.0
12.0
13.0
pH
a
b

low temperatures, a coil of copper tubing inserted in an ice bath was connected between the cell holder and temperature bath. The temperature in the cell was measured by placing a thermometer in the cell, which was filled with water, and allowing it to equilibrate before taking the reading.

Several points were taken for each temperature recorded. The Cr(III) solutions were pre-cooled in a water bath for the low temperature measurements and the luminol and H_2O_2 solutions were allowed to equilibrate in the cell before the measurement.

Figure 15 shows the results for the peak height and peak area optimization. The optimum temperature for peak area measurements is about room temperature (24° C) while for the peak height measurements the signal continually increases with temperature over the measurement region. For convenience a temperature of 25° C was selected for analysis. The improvement of the signal with higher temperatures is caused by the increased rate of the CL reaction. The leveling off with the peak area measurements is probably caused by a decrease in the quantum efficiency.

F. Buffer Solutions

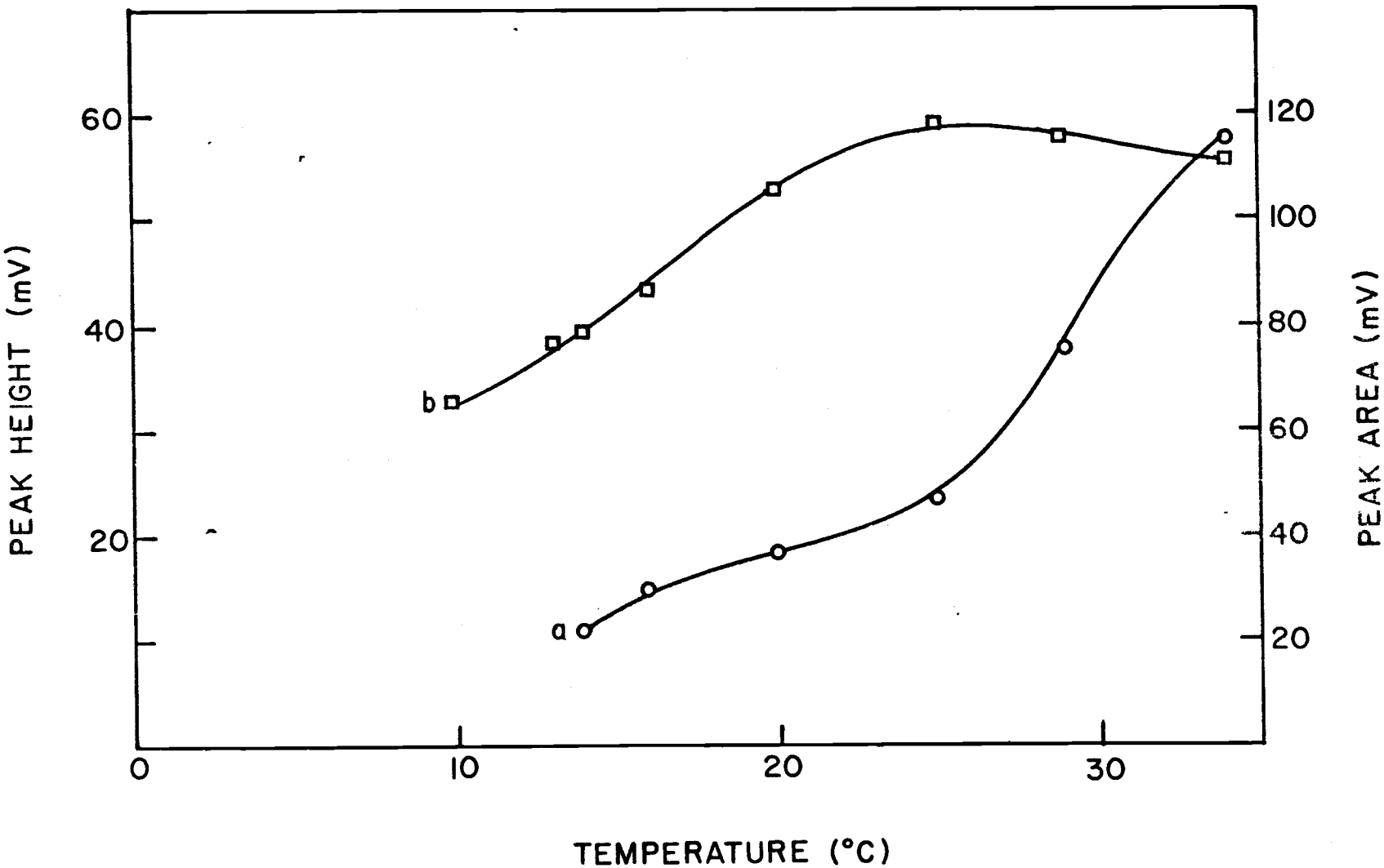
A test of three buffers commonly used in the pH range of 10 to 11 was made in order to select the buffer that gave the largest signal. The 0.05 M buffers chosen were

Figure 15. Cell temperature optimization

a) Peak height

b) Peak area

Conditions: 1 ml 8×10^{-5} M (final cell conc.) with 10^{-2} M EDTA, 0.05 M $NaHCO_3$ buffer, pH 11.0; ½ ml 3×10^{-2} M H_2O_2 (final cell conc.) with 10^{-2} M EDTA and pH 4.0; ½ ml 5×10^{-7} M Cr(III) (final cell conc.) and pH 3.5; gain 10^6 V/A; integrator 5 μF.


PEAK HEIGHT (mV)
60
40
20
0
10
20
30
TEMPERATURE (°C)
120
100
80
60
40
20
PEAK AREA (mV)
b
a

from sodium bicarbonate (pK_2 = 10.33), boric acid (pK = 9.23), and sodium dihydrogen phosphate (pK_2 = 7.21, pK_3 = 12.32). Seitz, et. al. (4) chose boric acid even though it is not an optimum buffer for the pH 11.0 region. The buffers were compared at the pH values of 10.0 and 11.0.

TABLE VII. BUFFER OPTIMIZATION

Buffer	initial pH	final pH	Average Peak Area	Average Peak Height
$NaHCO_3$	10.2	10.1	111.3	19.7
$NaHCO_3$	11.0	10.3	129.2	42.5
H_3BO_3	10.2	9.8	72.4	32.1
H_3BO_3	11.0	10.5	77.0	41.8
Na_2HPO_4	11.0	9.2	35.5	4.1

Table VII lists the initial pH of the 8×10^{-5} M luminol-buffer solution and final pH measured after H_2O_2 and Cr(III) solutions are injected into the cell. A pH 11.0 $NaHCO_3$ buffer was finally chosen because it gave the largest signal. The data indicate a substantial decrease in pH which is due to the hydrogen peroxide acting as a weak acid. This was verified by substitution of distilled water adjusted to pH 4.0 for the peroxide solution in the standard injection procedure and the pH remained unchanged at 11.0. The pH change was reduced from 0.55 pH units to 0.24 pH units when a 0.5 M $NaHCO_3$ pH 11.0 buffer was used.

G. Decomposition of the Luminol and Peroxide Solutions

The effect of storage on the luminol and hydrogen peroxide solutions was studied by monitoring the concentration of freshly prepared solutions for several days until a change in composition was noticed. The decomposition of the peroxide solution was checked with a standard procedure (39) which involves titration with potassium permanganate and is based on the reaction:

$$5\ H_2O_2 + 2MnO_4^- + 6H^+ \rightarrow 5O_2 + 2Mn^{+2} + 8H_2O$$

The initial H_2O_2 analytical concentration of 0.2515 N was calculated from the titration data and compared to the expected value of 0.2400 N. The titration had to be done without EDTA in the solution since its presence produced high, unreproducible results because the EDTA complexed with the Mn(II) produced by the reduction of permanganate. The absence of M_n(II) in the solution obscures the end point change. The results showed that there was no significant change in the peroxide concentration for five days and then it fell off gradually at a rare of about 1% per day. Hence the H_2O_2 solutions were stable for about one week.

The decomposition of the luminol solution was checked by monitoring the peak and integrated chemiluminescence intensity over five days. The results show that the intensity increased about 19% for the first day when leveled off

and remained constant for five days at which time the experiment was ended. The concentrations of Cr(III) and H_2O_2 were assumed not to vary over this time, which was checked for the H_2O_2 with the permanganate titration.

H. Study of Blank Reaction

When the chromium blank (distilled water) is injected, a signal is observed above the background. Some of the postulated problems include: metal ions in the double distilled water, metal ions leaching out of the poly bottles or syringes, and variations in the amounts of dissolved O_2 in the solutions.

Peak height measurements were made for the following three solutions to test the effect of dissolved O_2; bubble distilled water, double distilled water with O_2 bubbled through it for 10 minutes (to saturate the solution with O_2), and double distilled water with N_2 bubbled through it for 10 minutes (to remove O_2). The slight decrease (5%) in the peak heights observed when either gas was bubbled in solution indicates that the O_2 levels are not critical.

The blank peak heights obtained from distilled water stored in polyetheylene, teflon and glass containers for two days and injected into 8×10^{-5} M luminol, 10^{-3} M EDTA and 3×10^{-2} M H_2O_2 were 21.4, 26.0 and 27.3, respectively. Polyethylene was chosen because it gave the lowest value. A test conducted on the cleaning procedures for poly bottles

showed that bottles acid washed with a 50% nitric acid solution and thoroughly rinsed with distilled water gave an identical signal to new poly bottles with no treatment. Poly bottles used for the standards and samples were rinsed with double distilled water before use to remove any particles.

House distilled water gave a blank signal over twice as large as that from the double distilled water. After an initial measurement of untreated distilled water, EDTA was added as described in the procedure section to complex with any metal ion interferents. Figure 16 shows how the signal changed with time upon addition of EDTA. The points at zero hours are the blanks without EDTA. The blank peak produced by the double distilled water is soon eliminated by the EDTA additions which suggests that the blank signal was due to trace contaminants in the distilled water. The improvement in the house distilled water is not as good, perhaps due to higher levels of impurities not complexed by the EDTA (see interference section).

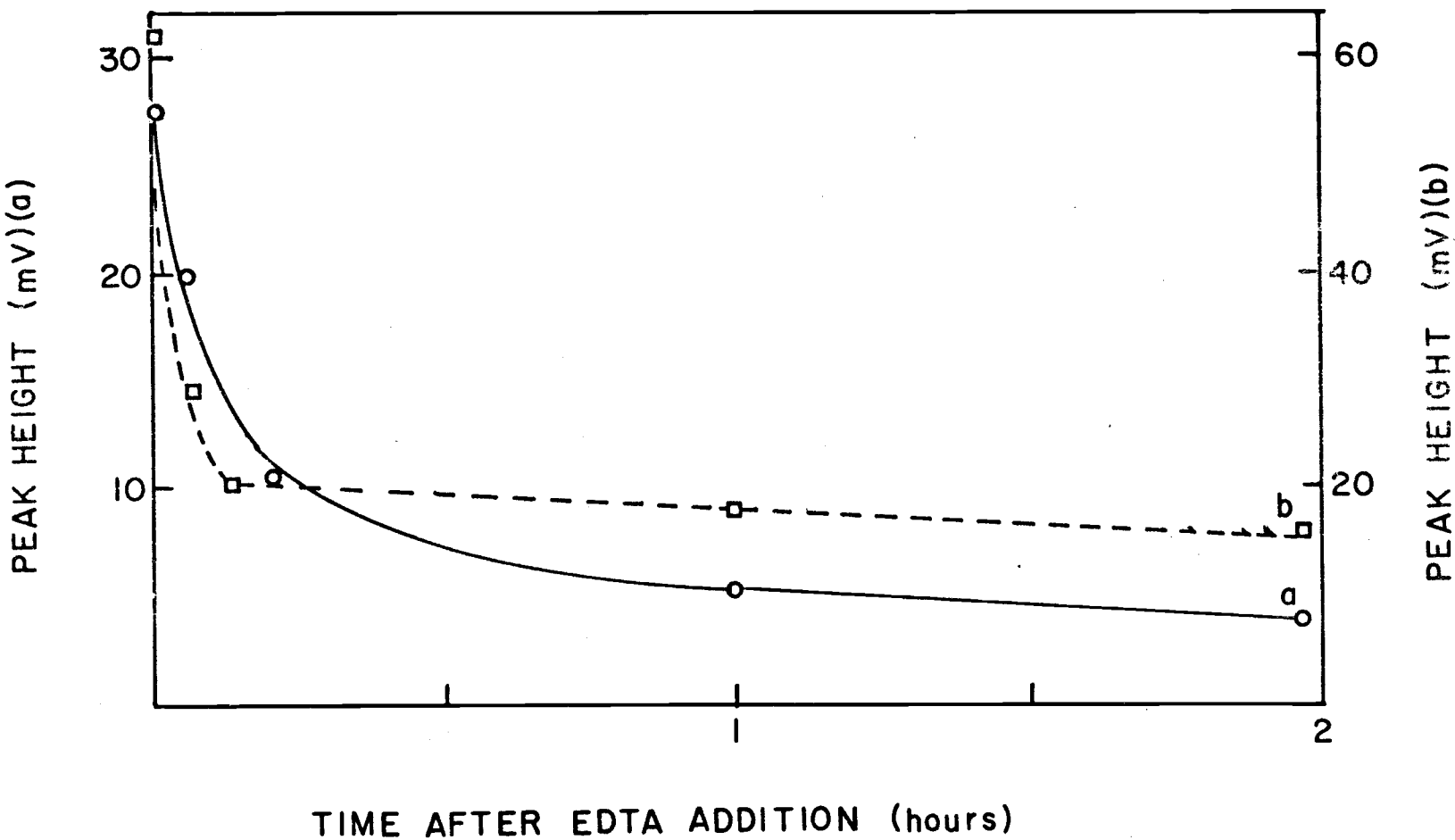
One fact discovered by accident is that H_2 bubbled in the solution results in an increase in signal by about 200 times. Also if the teflon stirring bar is placed in concentrated HNO_3, a large peak signal is observed and over 40 rinsings are required to reduce the signal to the original level.

Figure 16. Change in blank signal with EDTA addition.

a) Double distilled water.

b) House distilled water.

Conditions: 1 ml 8 x 10^{-5} M (final cell conc.) with 10^{-2} M EDTA, 0.05 M $NaHCO_3$ buffer, pH 11.0; ½ ml 3 x 10^{-2} M H_2O_2 (final cell conc.) with 10^{-2} M EDTA and pH 4.0; ½ ml 5 x 10^{-7} M Cr(III) (final cell conc.) and pH 3.5; gain 10^6 V/A; integrator 5 μF.


30
20
10
60
40
20
1
2
a
b
PEAK HEIGHT (mV)(a)
PEAK HEIGHT (mV)(b)
TIME AFTER EDTA ADDITION (hours)

V. EDTA

As discussed in the history section, other researchers have added EDTA to complex potential interfering ions. Although the Cr-EDTA complex forms much more slowly than other metal-EDTA complexes, analysis must be run before any significant complex formation.

To find the optimum EDTA concentration, the following procedure was employed. Ten ml aliquots of 10^{-4} M and 3×10^{-8} M Cr(III) standard solutions were added to 100 ml volumetric flasks. One milliliter of the appropriate EDTA solution was added to give final EDTA concentrations of 10^{-2} M, 10^{-3} M, and 10^{-4} M after dilution. The time dependence of the CL signal is shown in figures 17 and 18. The value at zero time is the value obtained if no EDTA is added. For 10^{-3} M and 10^{-4} M EDTA, at both Cr concentrations, there is an initial increase in signal when EDTA was added, and then the curve levels off. The results for 10^{-5} M Cr and 10^{-2} M EDTA show that the peak area and peak height signals decrease rapidly with time. For the 3×10^{-9} M Cr(III) solution, the signal levels off at a lower concentration after the initial peak rise in the signal. The EDTA concentration chosen for analysis is 10^{-3} M which gives a stable signal and provides more EDTA for complexing than 10^{-4} M. Clearly the 10^{-2} M EDTA concentration recommended by Seitz, et.al.(4) is not optimal.

Figure 17. EDTA reaction with 10^{-5} M Cr(III).

a) 10^{-2} M EDTA Δ

b) 10^{-3} M EDTA □

c) 10^{-4} M EDTA ○

Conditions: 1 ml 8 x 10^{-5} M (final cell conc.) with 10^{-2} M EDTA, 0.05 M $NaHCO_3$ buffer, pH 11.0; 1/2 ml 3 x 10^{-2} M H_2O_2 (final cell conc.) with 10^{-2} M EDTA and pH 4.0; 1/2 ml 5 x 10^{-7} M Cr(III) (final cell conc.) and pH 3.5; gain 10^{-6} V/A; integrater 5 μF.

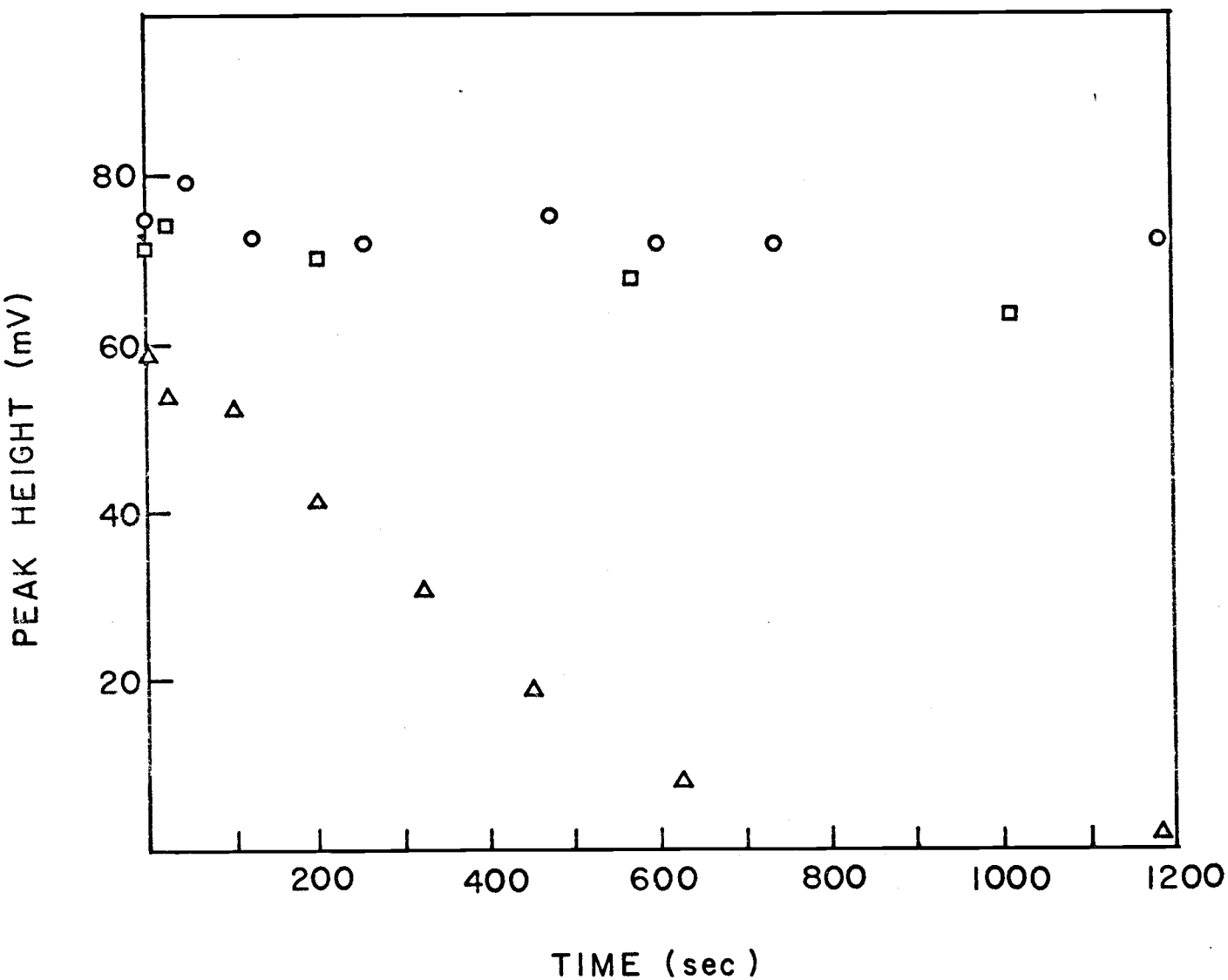

80
60
40
20
PEAK HEIGHT (mV)
200
400
600
800
1000
1200
TIME (sec)

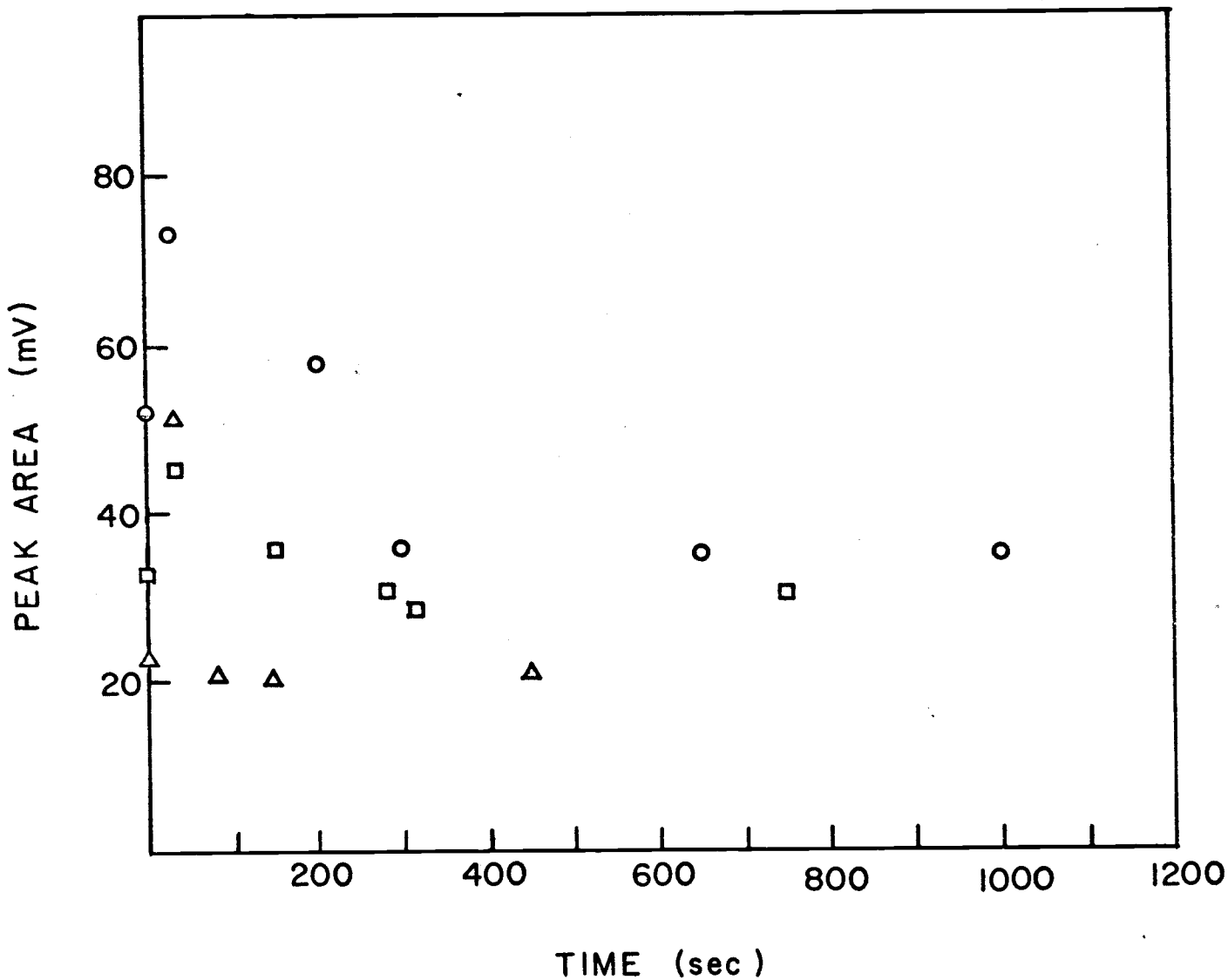
Figure 18. EDTA reaction with 3×10^{-9} M Cr(III) solution.

a) 10^{-2} M EDTA △

b) 10^{-3} M EDTA □

c) 10^{-4} M EDTA ○

Conditions: 1 ml 8×10^{-5} M (final cell Conc.) with 10^{-2} M EDTA, 0.05 M $NaHCO_3$ buffer, pH 11.0; ½ ml 3×10^{-2} M H_2O_2 (final cell conc.) with 10^{-2} M EDTA and pH 4.0; ½ ml 5×10^{-7} M Cr(III) (final cell Conc.) and pH 3.5; gain 10^8 V/A; integrator 5 uF.


80
60
40
20
PEAK AREA (mV)
200
400
600
800
1000
1200
TIME (sec)

J. pH of Cr(III) Solutions

3×10^{-6} M Cr(III) standard solutions adjusted to different pH's in the range of 2.0 to 5.0 were run in order to find the optimum sample pH. The results indicate that the pH of the samples used for analysis should be adjusted to be in between 2.0 and 4.0.

VI. RESULTS

A. Chromium (III) Calibration

Solutions with Cr(III) concentrations from 10^{-4} M to 10^{-10} M were run to determine the dynamic range of analysis and the shape of the calibration curve. Measurements were made for peak height and peak area without EDTA, and for peak height with solutions containing EDTA. The results shown in figures 19 and 20 illustrate the remarkable dynamic range of the technique.

To test the reproducibility of the calibration curve the same points were run one day later. The points run on the second day are 24% lower, but the shape of the curve is identical. The plot with EDTA added (see figure 19) falls 42% lower than that for solutions without EDTA, but the shape of the calibration curve remains the same. The lower signal is caused by some Cr(III)-EDTA complex formation.

The peak area data for Cr(III) without EDTA addition is shown in figure 20. The curve has about the same shape as the peak height curve but deviates at the low end due to the difficulty in measuring the peak area on top of a large background signal at low concentrations.

The curves for the peak height measurements are linear from 10^{-5} to 10^{-8} M and have a slope of 1.33 on a log-log plot. The average relative standard deviation for peak height measurements without EDTA is 4.4%, and with EDTA it

Figure 19. Peak height calibration curve.

a) Without EDTA

b) With EDTA, 10^{-3} M

Conditions: 1 ml 8 x 10^{-5} M luminol (final cell conc.) with 0.05 M $NaHCO_3$ buffer, pH 11.0, ½ ml 3 x 10^{-2} M H_2O_2 (final cell conc.), pH 4.0; ½ ml Cr(III) variable conc., pH 3.5; gain variable.

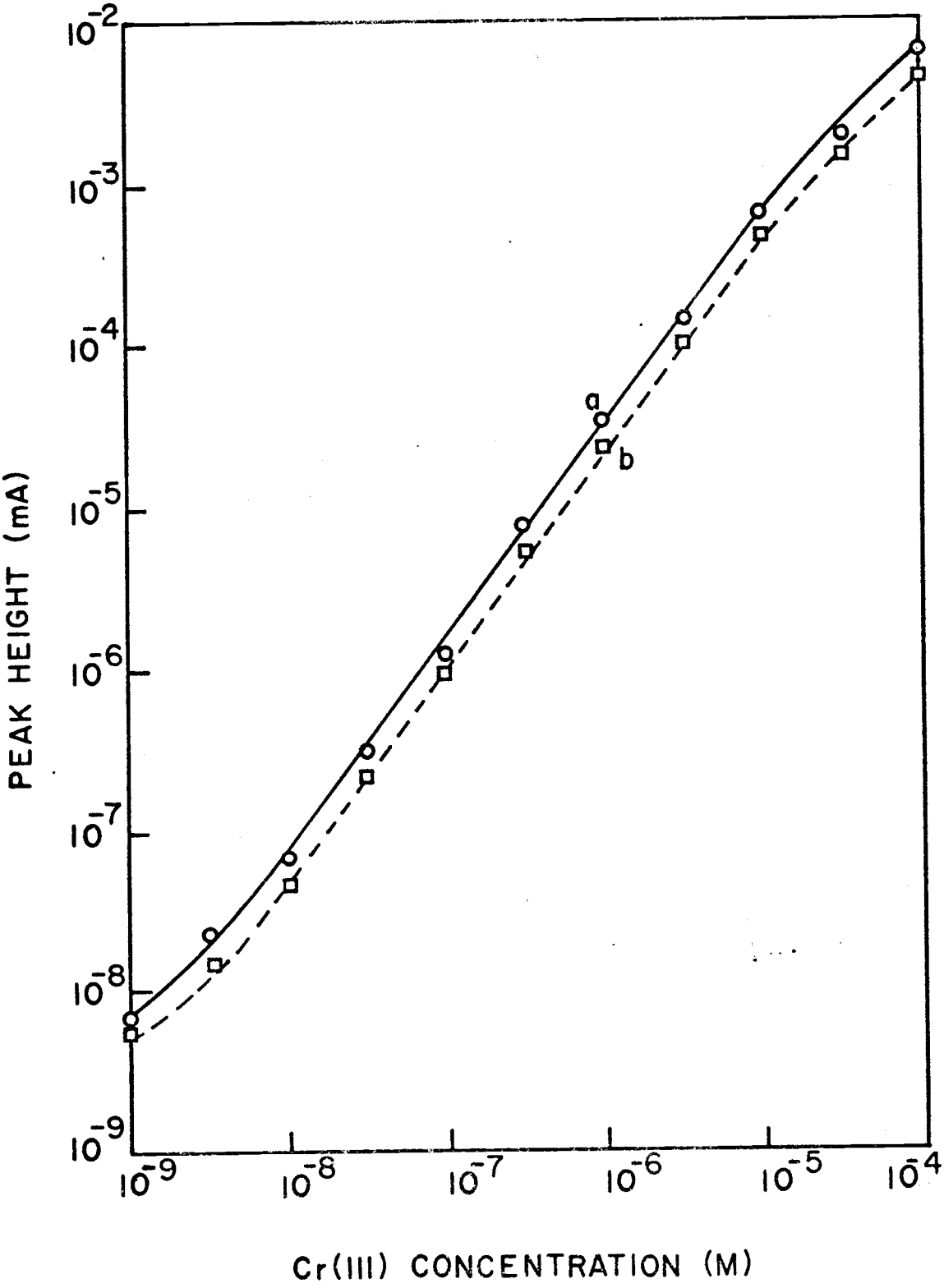
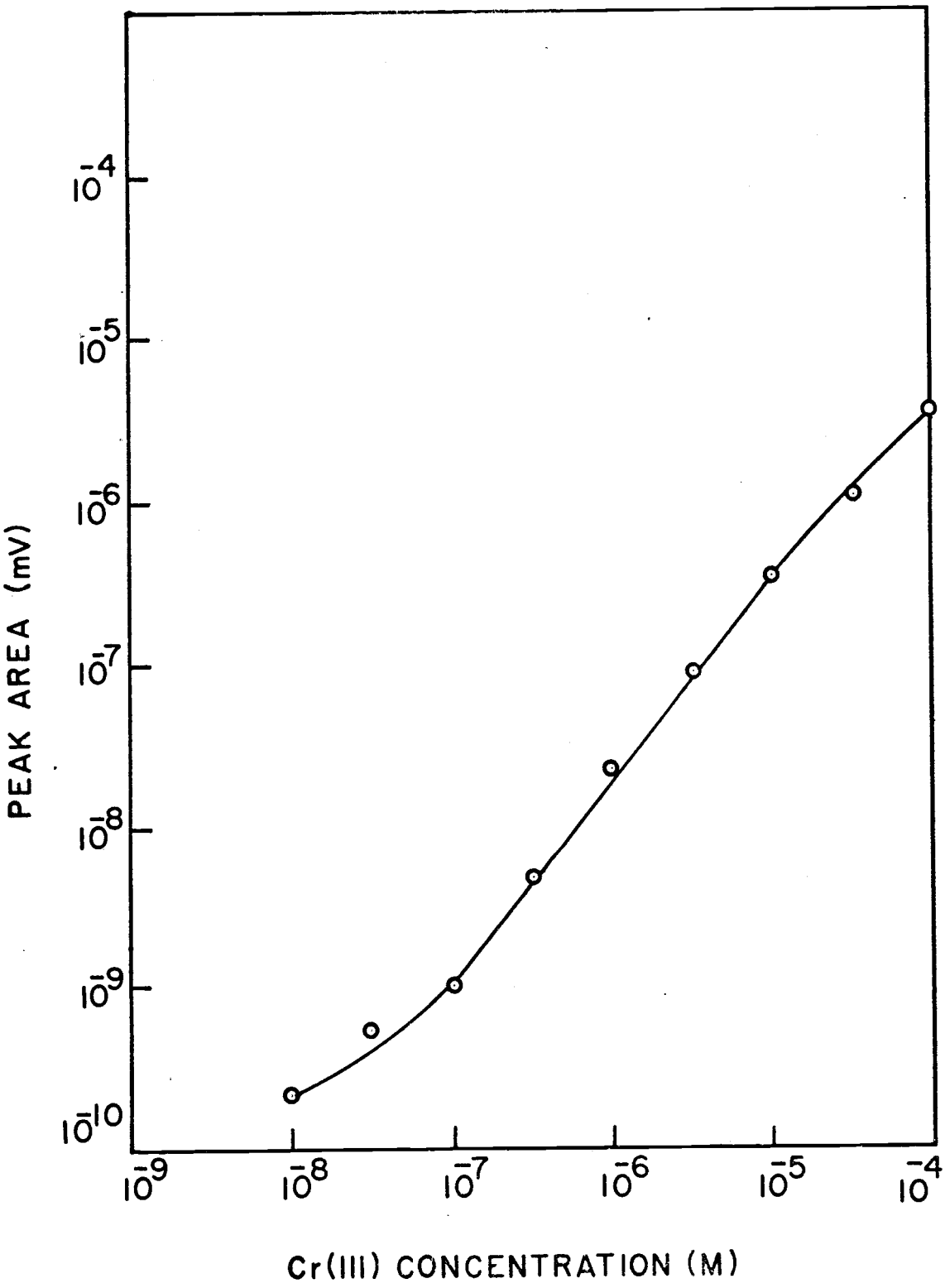

PEAK HEIGHT (mA)
10^-2
10^-3
10^-4
10^-5
10^-6
10^-7
10^-8
10^-9
a
b
10^-9
10^-8
10^-7
10^-6
10^-5
10^-4
Cr(III) CONCENTRATION (M)

Figure 20. Peak area calibration curve.

Conditions: 1 ml 8 x 10^{-5} M luminol (final cell conc.) with 0.05 M $NaHCO_3$ buffer, pH 11.0 ½ ml 3 x 10^{-2} M H_2O_2 (final cell conc.), pH 4.0; ½ ml Cr(III) variable conc., pH 3.5; gain variable; integrator 5 μF.


PEAK AREA (mV)
10⁻⁴
10⁻⁵
10⁻⁶
10⁻⁷
10⁻⁸
10⁻⁹
10⁻¹⁰
10⁻⁹
10⁻⁸
10⁻⁷
10⁻⁶
10⁻⁵
10⁻⁴
Cr(III) CONCENTRATION (M)

is 3.1%. For the peak area calibration curve the linear region is 10^{-7} to 10^{-5} M, the slope is 1.17, and the average relative standard deviation is 6.0% for all points but is 2.8% for points above 3×10^{-7} M. The non-linearity at higher concentrations is due to the precipitation of $Cr(OH)_3$ which could be observed visually when a 3×10^{-3} M Cr(III) solution was injected. The detection limit, defined as the concentration producing a signal twice the background signal RMS noise, is 4×10^{-10} M, or about 0.05 ppb. In actual practice concentrations lower than 10^{-9} M could not be measured because lower concentrations gave about the same signal as the 10^{-9} M solution.

B. Interferences

EDTA is added to the Cr(III) solutions just before analysis to prevent metal ion interferences. In order to test the effectiveness of the EDTA, 10^{-6} M solutions of common metal ions which catalyze the luminol-peroxide reaction were made and tested with 3×10^{-7} M Cr(III) solution. The results are shown in table VIII.

Of the solutions tested, Fe(III), Fe(II), and Co(II) all interfere even with the addition of EDTA, while the Cu(II) interference is eliminated with EDTA. These results are reasonable since it is known that iron and cobolt complexes are very labile and that basic peroxide solution

will destroy the complexing agent (52). In addition to the metal ion interferences, other ions which interfere at high levels are SO_4^{-2}, NH_4^+, SO_3^{-2}, and NO_2^- all of which cause a decrease in the signal. The sulfite and nitrite decrease the chemiluminescence by causing the destruction of the hydrogen peroxide. Any other chemicals which cause the decomposition of hydrogen peroxide will also interfere.

TABLE VIII. INTERFERENCES

Cation + EDTA + Cr(III)	Signal Relative to Cr(III) + EDTA
Fe(III)	1.21
Fe(II)	1.25
Co(II)	1.40
Cu(II)	1.03

Cation + Cr(III)	Signal Relative to Cr(III)
Cu(II)	1.23
Co(II)	1.34

Conditions: 8×10^{-5} M luminol (final concentration_ with 10^{-3} M EDTA, 3×10^{-2} M H_2O_2, (final concentration), pH 11.2, 10^{-3} M EDTA (when used), 3×10^{-7} M Cr(III), 10^{-6} M for each interfering ion.

VII. ANALYSIS

A. Digestion Procedures

The chemiluminescence analysis technique measures only the free Cr(III) in the samples. If the Cr is complexed, as it might be in water samples containing organic matter or in biological samples, the Cr complexes must be destroyed by dry ashing or acid digestion before measurement. All of the procedures tried have some shortcomings which are listed in table 9. In general the acid digestions suffer from metal ion contamination in the acids and pH adjustment problems described in the next section. The dry ashing takes longer and it is difficult to remove all the sample from the ashing containers. Digestions using sulfuric acid were especially bad since the sulfate resulted in a reading much lower than expected due to sulfate interference in the reaction. From the information in table 9, the best digestion procedures are the perchloric acid digestion with nitric acid using ultra pure acids and the nitric acid--hydrogen peroxide digestion where the hydrogen peroxide is heated so it decomposes.

B. pH Adjustment

After acid digestion of samples the pH must be adjusted to between 2.0 and 4.0. Sodium hydroxide was finally

TABLE IX. DIGESTION PROCEDURES

Procedure	acid ratio	time heated	Difficulties	pH adjustment
HNO_3:$HClO_4$; H_2SO_4	3:1:1	3 hrs	Interference from sulfate. Metal ion contaminants.	M
HNO_3:$HClO_4$	3:1	2 hrs	Metal ion contamination	H
HNO_3 acid bomb	--	1 hr	Need one acid bomb per sample. Metal ion contamination	H
HNO_3:H_2SO_4	3:1	3 hrs	Interference from sulfate Metal ion contamination	M
HNO_3:H_2O_2	5:1	4 hrs	Metal ion contamination Interference from H_2O_2	E
Plasma Asher	--	4 hrs	Time required Incomplete Ashing Incomplete sample transfer	E
Plasma Asher (rinse with HNO_3)	--	4 hrs	Time required Contamination from acid Not all samples dissolve	H
Muffle Furnace	--	5 hrs	Time required. Incomplete sample transfer	E

E easy

H hard

M moderately hard

chosen as the neutralization base. Ammonium hydroxide was first used because it has lower levels of trace metal contamination than any other strong base. It was found that the ammonium hydroxide at high levels totally inhibits the CL reaction and could not be used. Potassium hydroxide has the disadvantage that the potassium forms a precipitate with perchloric acid which had to be filtered.

The pH adjustment was done using sodium hydroxide at two concentrations, 6 N and 1 N. The sample was placed in a beaker on a magnetic stirrer to mix the solution. 6 N NaOH was added dropwise from a buret until the pH was about 2.0, then the 1 N NaOH was added dropwise to get the pH close to 3.5.

Samples that were run without acid digestion were adjusted to pH 3.5 by adding 1 N NaOH from a buret. Real samples could be run without pH adjustment if they fell in the range of 2.0 and 4.0.

C. Analysis of Natural Water Samples

To measure the uncomplexed Cr(III) in natural water samples it is not necessary to destroy the organic material in the sample. Four Willamette River water samples were gathered at the locations listed in table 10. The pH of the river water was found to be about 7 so it was adjusted to pH 3.5 with a small amount of HCl. The samples were first run directly after addition of EDTA. Next the

samples were heated for 4 minutes to complex the Cr(III) and were measured again. The readings were subtracted to give the uncomplexed Cr(III) signal.

A determination was also made on a tap water sample from the lab. It was treated like the samples above and found to give a signal below the lowest standard (1 ppb). The concentration was estimated to be 0.6 ppb.

TABLE X. Cr CONCENTRATIONS IN NATURAL WATER SAMPLES

Sample Location	Concentration (ppb)
Willamette Park	1.1
Peoria Boat Launch	1.3
Entrance of Mary's River	1.3
Riverview Marina	1.1
Tap Water	0.6

D. Determination of Biological Samples

For determination of ppb concentrations of Cr(III) in biological samples an acid digestion is used. Since contamination from the acid was a problem "Superpure" acids are used which have very low metal concentrations

A 5:2 nitric-perchloric acid digestion is used. The sample and nitric acid mixture is refluxed in test tubes on a Technican block digester for about 1 1/2 hours. The perchloric acid is added and heated at 230° C until the perchloric acid is fuming. The samples are removed, cooled, and the pH is adjusted to about 3.0 as described earlier.

To test the digestion procedure and the analysis technique, NBS standard reference orchard leaves were digested using the above procedure. The Cr(III) concentration was found to be 2.2 ppm which compares favorably with the accepted value of 2.3 ppm. The relative standard deviation is 20%.

The large relative standard deviation probably resulted from the digestion procedure. Seitz et. al. (43) postulated that the holes in the block digester must be within 2° C in order to get a low standard deviation. On the Technicon block digester there is a variation larger then 2° C between holes.

VIII. CONCLUSIONS

The design and construction of a simple CL photometer has been described in detail. This instrument should serve as a useful prototype upon which to base a portable instrument applicable to field use. Some of the important characteristics of the photometer include temperature controlled cell holder, magnetic stirring, cooled PMT, high collection efficiency, and a versatile electronics readout system.

The CL photometer was applied to the ppb and sub-ppb determination of Cr(III) with luminol and hydrogen peroxide in basic solutions. Reagent concentrations were optimized to achieve the best signal-to-background ratio. The potential of the technique for determining Cr(III) was demonstrated by a calibration curve from 10^{-9} to 10^{-4} M and a detection limit of 5×10^{-10} M.

Co(II), Fe(II), Fe(III), and Cu(II) interfere by activating the same CL reaction as Cr(III). Addition of 10^{-3} M EDTA eliminates the interference from Co(II) and allows an indirect determination of Cr(III) in the presence of Co(II), Fe(II), and Fe(III).

Samples of Willamette river water were tested and found to contain 1.0 to 1.6 ppb uncomplexed Cr(III).

The potential of CL analysis has not been fully explored. The photometer described is a versatile tool useful for further studies with other metals and other

CL reagents. A more complete understanding of the mechanism involved is required to adjust solution conditions to eliminate interferences.

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