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Title: EVALUATION OF A PHOTON-COUNTING TECHNIQUE FOR THE REACTION RATE DETERMINATION OF GLUCOSE

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A photon-counting technique which provides direct digital read-out of data has been developed and evaluated for the reaction rate determination of glucose. The enzyme catalyzed oxidation of glucose to gluconic acid and hydrogen peroxide with the subsequent reaction of hydrogen peroxide with excess iodide to produce tri-iodide is followed by the change in the absorbance of the solution. Equations are derived which relate the photometer response to the rate of change of the tri-iodide concentration in the solution. Optimum results for high or low glucose concentration ranges were obtained with a low or high enzyme concentration respectively. Methods for obtaining linear calibration curves with or without using a blank solution are discussed. Linear calibration curves were obtained for glucose concentrations from 10-110 ppm with a reproducibility of about 2 ppm.

Evaluation of a Photon-Counting Technique for the Reaction Rate Determination of Glucose

by

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INTRODUCTION

Reaction rate methods for analysis have become popular in both clinical and other analytical applications because they are rapid, sensitive, and in many cases selective. The general approach in analyses that use reaction rate measurements is to determine the initial rate of the reaction and relate it to the concentration of the species of interest in the sample. The species of interest may be one of the participating reactants in the reaction being monitored, or it may be a catalyst for the reaction being monitored. In following the rate of reaction, one may elect to follow either the appearance of one of the products of the reaction or the disappearance of one of the reactants involved in the reaction. Although the rate of the reaction may not necessarily be first order, one can usually arrange the conditions of the experiment so that the reaction initially follows pseudo-first order kinetics. The rate of reaction can then be linearly related to the concentration of the species of interest.

A variety of methods for determining the rates of reaction for analytical purposes have been reported. The majority of the systems have been applied to the determination of glucose by its oxidation in the presence of glucose oxidase to give gluconic acid and hydrogen

peroxide as products. Sunderman and Sunderman (27) have published a review of work done with this reaction and its application to glucose analyses. Some reaction rate methods have involved stopping the reaction after some specified time and measuring the amount of product formed or reactant lost. This can be time consuming. Other methods have simplified the analysis by making measurements of the reaction rate while the reaction is in progress.

The reaction rate method presented in this paper utilizes a photon-counting technique to photometrically determine the initial rate of reaction while the reaction is proceeding. The photometer gives a digital readout proportional to the total number of photons passing through a sample for a preset counting period. This number is proportional to the integral of the transmittance through the sample cell over the time period chosen. The integrated transmittance of a sample, R_c', is combined with the integrated transmittance of a blank, R_0', to give a value, $\log R_0'/R_c'$, which represents the integrated absorbance observed in the counting period. Under prescribed conditions, the integrated absorbance is shown to be proportional to the rate of reaction and to the concentration of glucose in the sample. The photon-counting method has the advantage of a relatively stable detection system, and of direct digital output which is suitable for computer coupling or for transmission from outlying field stations for remote monitoring systems.

HISTORY

The slope of a reaction curve is equal to the rate of the reaction involved. In order to determine the rate of a reaction, the detection system used must be able to determine the slope of the reaction curve either directly or indirectly. The slope of the reaction curve may be determined by following the rate of change of the concentration of one or more of the reactants or products in the reaction cell. Of the different methods available for following changes in concentration, the most commonly used reaction rate methods have employed potentiometric, amperometric, or photometric detection systems. Some of the different types of detection systems that have been reported are given below.

Two systems have been reported that use potentiometric measurements to determine the rate of reaction. Malmstadt and Pardue (14, 15) reported a potentiometric system for the detection of glucose in biological systems. The glucose oxidation reaction was coupled with the reaction of hydrogen peroxide with iodide in the presence of a molybdenum catalyst to give iodine. The iodine formed was used to follow the reaction potentiometrically. The time necessary for a predetermined change in the electromotive force of the sample cell was measured and related to the concentration of glucose in the sample. The reproducibility and accuracy of this system were given as

within 2% for glucose concentrations of 5-500 ppm. This method is rapid, sensitive, and gives an automatic readout of the data.

Pardue (18) reported a potentiometric system for measuring the slope of the reaction curve through the use of an electronic integrator that matched the slope of the reaction curve at near zero reaction time. The slope was related to the concentration of cystine in the reaction cell. A second application of this system was reported by Pardue (20) in which the concentration of glucose was determined. He used the same reactions that were used by Malmstadt and Pardue to determine glucose as mentioned previously. This method of electronic integration has the advantage of not requiring precisely linear reaction curves and is sensitive and gives automatic readout of the slope of the reaction curve.

Amperometric detection systems have also been used in determining glucose concentrations by reaction rate measurements. Pardue (19) reported an automatic reaction rate system in which the change in iodine concentration was followed by the change in current observed between a pair of polarized platinum electrodes. The concentration of glucose is related to the time necessary to observe a preselected change in current between the electrodes. A relative standard deviation of 2% for glucose concentrations of 50-200 mg/100 ml was given for this method. The method is sensitive and gives automatic readout of the data. Blaedel and Olson (2) have reported

an amperometric method for continuous reaction rate analyses. Their work was done with the glucose oxidation reaction. The hydrogen peroxide formed oxidized ferrocyanide to ferricyanide. The ferricyanide concentration was then determined with tubular platinum electrodes at two points in the flowing sample stream. The current at a fixed potential was measured simultaneously at both electrodes and the difference was related to the concentration of glucose in the sample stream. A relative standard deviation of 1% for glucose concentrations up to 100 ppm was reported for this system.

A different system for reaction rate measurements has been reported by Blaedel and Petitjean (3) for the determination of acetylacetone. They used a high frequency oscillator and measured the rate of change in conductivity of the sample. Accuracy of about 0. 3% was reported for this system. The measurement times are longer than with other detection systems.

A pH stat for use in reaction rate analyses was reported by Malmstadt and Piepmeier (16). Their method used a system that added small increments of a standard reagent to the sample cell to titrate an acid or base product, while keeping the pH of the solution at some preselected value. The number of equal increments of a standard reagent added over some period of time is proportional to the rate of reaction and can be read out on a recorder chart or reported digitally. Reproducibility and accuracy were reported as within 2%

for either glucose or urea determinations.

In addition to these electroanalytical techniques, several systems which use photometric measurement of reaction rates have been reported. Photometric methods for the determination of silver (28) and copper (8) using absorbance measurements have been published. Silver was determined from its catalysis of the persulfate oxidation of manganous ion to permanganate by following the change in absorbance of the solution with time. Copper was determined by its catalytic action on the autoxidation of resorcinol which results in the formation of a colored product. Accuracies of 5-10% were reported for sub-microgram quantities of these metals in samples.

An automatic system for the determination of the rate of formation of hydrogen peroxide in a primary reaction by its reaction with o-dianisidine in the presence of horseradish peroxidase to give a colored product has been reported by Malmstadt and coworkers (11, 13). The time necessary for a preset absorbance change was measured automatically and was related to the concentration of the reactants in the primary reaction. This system has been used to determine alpha-amino acids (11) and glucose (13). A standard deviation of 2% was given for the method. The method is sensitive and adapts to different determinations.

An adaptation of the method above was reported by Malmstadt and Hadjiioannou (9) for the determination of glucose in biological

samples. The difference between this method and that mentioned above was that the reaction of hydrogen peroxide with o-dianisidine to give a colored product was replaced by the reaction of hydrogen peroxide with iodide in the presence of a molybdenum catalyst to form iodine. In the presence of excess iodide, the iodine formed the triiodide complex which was detected photometrically. Relative errors within 2% were reported for this method and measurement times were given as 1-3 minutes. This method eliminated the need for horseradish peroxidase, but required larger samples than the previous method as well as longer measurement times. The same basic instrumentation was used for the determination of alcohol in blood (10), and of ultramicro amounts of iodine in biological samples (12). A further modification of this system was given by Hadjiioannou (6) for the determination of iodine in natural waters.

A continuous analysis system for glucose determination was reported by Blaedel and Hicks (1). The absorbance in the sample stream was measured at two points along the stream and the difference between the two readings was a measure of the rate of reaction since the two measurement points were fixed and the sample flow rate was constant. Concentrations up to 60 ppm of glucose were determined with a standard deviation of 1 ppm.

Pardue and Rodriguez (21) have reported a precision photometer which used optical feedback to control the light intensity and keep it

at a constant level. This system was reported to have a stability of 0. 01 % T over a one hour period. The use of this photometer for the determination of iodide by its catalysis of the cerium IV-arsenic III reaction was demonstrated. A relative standard deviation of 1. 3-2. 5% was reported for iodide concentrations of 0. 03-0. 003 μg/ml.

The detection systems mentioned above are not limited to use with one reaction, but may be modified for use with many reactions. The photon-counting photometer presented in this thesis is also capable of use with a variety of reactions with only minor modifications. The glucose oxidation reaction used by Malmstadt and Hadjiioannou (9) was chosen to demonstrate and evaluate the applicability of this photometer to reaction rate analyses. The hydrogen peroxide formed by the oxidation of glucose is reacted with iodide to form iodine which forms the colored tri-iodide ion with the excess iodide in solution. The rate of change of tri-iodide concentration was used to determine the rate of reaction. This reaction is of general use in clinical analyses of biological samples because the specificity of the enzyme for D-glucose reduces possible interference from other substances in the sample solutions.

THEORY

Reaction Rate Equations

The reaction rate determination of glucose is based on the oxidation of glucose in the presence of the specific enzyme glucose oxidase. A reaction mechanism for this oxidation has been given in King, Mason and Morrison (7) as:

$$E + S \underset{k_2}{\overset{k_1}{\rightleftharpoons}} E \bullet S \qquad (1)$$

$$E \bullet S \xrightarrow{k_3} EH_2 + \text{gluconolactone} \qquad (2)$$

$$EH_2 + O_2 \xrightarrow{k_{ox}} E + H_2O_2 \qquad (3)$$

where:

E = the oxidized form of the enzyme, glucose oxidase

S = the substrate (glucose)

$E \bullet S$ = the glucose-glucose oxidase complex intermediate

EH_2 = the reduced form of the enzyme

The hydrogen peroxide formed reacts with iodide in the presence of a molybdenum catalyst to form iodine which forms the colored tri-iodide complex with excess iodide in the solution. The reactions shown in Equations 4 and 5 are instantaneous relative to that

shown in Equation 3 (9).

$$H_2O_2 + 2I^- + 2H^+ \rightarrow I_2 + 2H_2O \quad (4)$$

$$I_2 + I^- \rightarrow I_3^- \quad (5)$$

Hydrogen peroxide can also react with any catalase present in the reaction cell. The glucose oxidase reagent contained 0. 5% catalase. Using a molecular weight of 250, 000 (26) for catalase, the catalase concentration in the reaction cell is found to be 2×10^{-8} M for the most concentrated enzyme solution. The iodide concentration in the reaction cell is about 0. 07 M. Although the reaction of hydrogen peroxide with catalase may be rapid, the reaction of hydrogen peroxide with catalase will be neglected due to the extreme excess of iodide. All of the hydrogen peroxide formed will be considered to react with iodide so that the rate of formation of tri-iodide will be equal to the rate of formation of hydrogen peroxide. From Equations 1, 2 and 3 the relationship between the total concentration of enzyme added, $[E_t]$, the concentration of free oxidized form of the enzyme, $[E]$, the concentration of enzyme tied up as the enzyme-substrate complex, $[E \bullet S]$, and the concentration of the reduced form of the enzyme, $[EH_2]$, is:

$$[E] = [E_t] - [E \bullet S] - [EH_2] = [E_t - E \bullet S - EH_2] \quad (6)$$

From Equations 1, 2, 3 and 6, the following expressions can be derived:

$$\frac{d[E \bullet S]}{dt} = k_1[E_t - E \bullet S - EH_2][S] - (k_2 + k_3)[E \bullet S] \quad (7)$$

$$-\frac{d[EH_2]}{dt} = k_{ox}[EH_2][O_2] - k_3[E \bullet S] \quad (8)$$

Assuming steady state conditions (5) for both $[E \bullet S]$ and $[EH_2]$, Equations 7 and 8 give:

$$[E \bullet S] = \frac{k_1[E_t][S] - k_1[EH_2][S]}{k_2 + k_3 + k_1[S]} \quad (9)$$

$$[EH_2] = \frac{k_3[E \bullet S]}{k_{ox}[O_2]} \quad (10)$$

Substituting Equation 9 into Equation 10 and rearranging yields:

$$[EH_2] = \frac{k_3[E_t][S]}{k_{ox}[O_2]K_m + k_{ox}[O_2][S] + k_3[S]} \quad (11)$$

where

$$K_m = \frac{k_2 + k_3}{k_1} .$$

From Equation 3 the following expression can be derived:

$$\frac{d[H_2O_2]}{dt} = k_{ox}[O_2][EH_2] \quad (12)$$

Then, substituting Equation 11 into Equation 12 and rearranging yields:

$$\frac{d[H_2O_2]}{dt} = \frac{k_3[E_t][S]}{K_m + [S][1 + \frac{k_3}{k_{ox}[O_2]}]} \qquad (13)$$

From the approximate values given (4) for the rate constants, k_1, k_2, k_3 and k_{ox}, and assuming $[O_2]$ remains constant at the saturation value of 2×10^{-4} M during the time of measurement, the value of $[S][1 + \frac{k_3}{k_{ox}[O_2]}]$ will be negligible with respect to K_m under the conditions set up for these experiments and the rate of appearance of hydrogen peroxide can be written as:

$$\frac{d[H_2O_2]}{dt} = \frac{k_3[E_t][S]}{K_m} \qquad (14)$$

Since $[E_t]$ is constant for any set of experimental runs, Equation 14 can be rewritten as:

$$\frac{d[H_2O_2]}{dt} = K'[S] \qquad (15)$$

where

$$K' = \frac{k_3[E_t]}{K_m}$$

Since the reactions shown in Equations 4 and 5 are instantaneous

relative to that in Equation 3, the following expression can be written:

$$\frac{d[H_2O_2]}{dt} = \frac{d[I_3^-]}{dt} \tag{16}$$

Since Beer's Law is obeyed for I_3^- using this photometer (23):

$$A = ab[I_3^-] \tag{17}$$

where:

A = the absorbance of the solution at any instant

a = the absorptivity of the species

b = the cell path length (1.4 cm)

Therefore, combining Equations 15, 16 and 17 yields:

$$\frac{dA}{dt} = ab\frac{d[I_3^-]}{dt} = ab\frac{d[H_2O_2]}{dt} = abK'[S] \tag{18}$$

Photometer Response

According to Equation 18, the initial rate of change of absorbance is proportional to the concentration of glucose in the sample if the initial rate of reaction is measured. Piepmeier, Braun and Rhodes (23) have shown that the absorbance measured with this photometer varies linearly with the concentration of tri-iodide in the cell. These absorbance measurements were made on a system in which the concentration of iodine was not changing. The total counts obtained in

a 10-sec counting period represent the integrated photon rate over the entire counting period. One tenth of the integrated counts would equal the photon rate in the first 1-sec interval of the 10-sec counting period. For the reaction rate work attempted here, the concentration of tri-iodide changed with time as shown in Equations 3, 4 and 5. Because the absorbance increased with time, the photon rate to the detector decreased with time. Therefore one tenth of the total accumulated counts in a 10-sec counting period would be less than the photon rate in the first 1-sec interval of the 10-sec counting period. It will now be shown that, under the reaction conditions established in the experiments described below, the logarithm of the integral of the photon rate obtained from the photometer over a 10-sec counting period is directly proportional to the rate of change of the tri-iodide concentration in the reaction cell.

It has been shown (23) that for this photometer:

$$A = ab[I_3^-] = \log \frac{I_0}{I_c} \qquad (19)$$

where:

A = absorbance of the solution

I_0 = the instantaneous photon flux through the blank

I_c = the instantaneous photon flux through the sample.

Therefore:

$$A = \log I_0 - \log I_c \tag{20}$$

Since the absorbance of the blank does not change with time, $\log I_0$ will be a constant so that:

$$A \propto \log I_c \tag{21}$$

Because $A \propto [I_3^-]$, and $d[I_3^-]/dt$ is constant,

$$\frac{dA}{dt} \propto \frac{d(\log I_c)}{dt} \propto \frac{d[I_3^-]}{dt} = \text{constant.} \tag{22}$$

Figure 1 shows the variation of $\log I_0$ and $\log I_c$ with time for a reaction of the type studied in this thesis. Since $\log I_c$ changes linearly with time, I_c will vary in a negative exponential fashion with time. The variation of I_0 and I_c with time is shown in Figure 2.

The integral under the $\log I_0$ curve in Figure 1 is represented by the total shaded area, R_0. The integral under the $\log I_c$ curve in Figure 1 is represented by the heavily shaded area, R_c. The equations for these areas are:

$$R_0 = \int_{t_1}^{t_2} \log I_0 dt \tag{24}$$

$$R_c = \int_{t_1}^{t_2} \log I_c dt \tag{25}$$

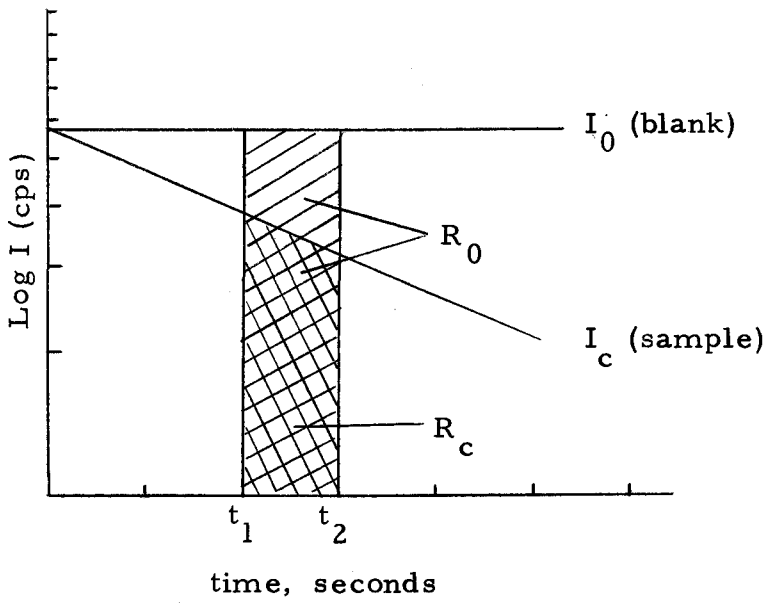

Figure 1. Relationship between log I and time.

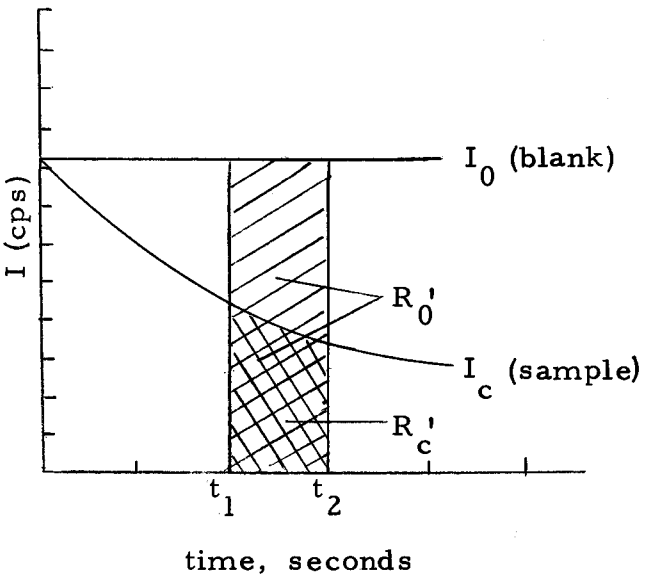

Figure 2. Relationship between I and time.

where t_1 and t_2 are the times when the counting interval of the photometer is started and stopped. The areas R_0' and R_c' are similarly defined for the I_0 and I_c curves in Figure 2, and represent the readout from the photometer. The equations for these areas are:

$$R_0' = \int_{t_1}^{t_2} I_0 dt \tag{26}$$

$$R_c' = \int_{t_1}^{t_2} I_c dt . \tag{27}$$

For any set of linear curves having the same ordinate intercept, such as those shown in Figure 1, it can easily be shown by shifting the origin to I_0 that the difference between the area under the I_0 curve and the area under any I_c curve is directly proportional to the negative slope of the I_c curve. Using simple geometric considerations, the areas under the I_0 and I_c curves in Figure 1 are found to be:

$$R_0 = \frac{1}{2}[(\log I_0)_1 + (\log I_0)_2]\Delta t \tag{28}$$

$$R_c = \frac{1}{2}[(\log I_c)_1 + (\log I_c)_2]\Delta t \tag{29}$$

where $\Delta t = t_2 - t_1$, and the subscripts 1 and 2 denote the points on the curves at times t_1 and t_2.

Subtracting Equation 29 from Equation 28 and rearranging, the

following expression for ΔR can be written:

$$\Delta R = \frac{\Delta t}{2}\left[(\log I_0)_1 - (\log I_c)_1 + (\log I_0)_2 - (\log I_c)_2\right] \tag{30}$$

where $R_0 - R_c = \Delta R$, which is the difference in the areas under the I_0 and I_c curves represented by the lightly shaded area in Figure 1. Because the slope of the blank curve is zero, $\log I_{0_1} = \log I_{0_2} = \log I_0$ and Equation 30 becomes:

$$\Delta R = \frac{\Delta t}{2}\left[(\log I_0 - \log I_{c_1}) + (\log I_0 - \log I_{c_2})\right] \tag{31}$$

Since the slope, b, of the I_c curve in Figure 1 can be expressed as:

$$b = \frac{\log I_0 - \log I_{c_1}}{\Delta t_1} = \frac{\log I_0 - \log I_{c_2}}{\Delta t_2} \tag{32}$$

where $\Delta t_1 = (t_1 - t_0)$ and $\Delta t_2 = (t_2 - t_0)$.

Equation 30 can be rewritten as:

$$\Delta R = \frac{\Delta t}{2}(\Delta t_1 + \Delta t_2)b \tag{33}$$

Therefore, from Equations 23 and 33, since t_1 and t_2 are constants:

$$\Delta R \propto b \propto \text{rate of reaction} \tag{34}$$

According to Equation 34, the change in ΔR will be proportional to the change in the slope of the reaction curves. Since the photometer readout is not R_0 or R_c but is R_0' or R_c' as defined in Equations 26 and 27, it will be shown that $\log R_c'$ is proportional to R_c.

The equation for any of the linear curves in Figure 1 can be written as:

$$\log I = k + bt \tag{35}$$

where:

k = the ordinate intercept of the log I vs. time curve

b = the slope of the log I vs. time curve

Using Equation 24 or 25, the general expression for the area under a log I vs. time curve can be written as:

$$R = \int_{t_1}^{t_2} \log I\,dt = \int_{t_1}^{t_2} (k+bt)dt = k(t_2-t_1) - \frac{1}{2}b(t_2^2-t_1^2) \tag{36}$$

Since (t_2-t_1), k, and $\frac{1}{2}(t_2^2-t_1^2)$ are constants for any particular set of runs, Equation 36 can be generalized as:

$$R = C + C'b \tag{37}$$

where:

$$C = k(t_2-t_1) \quad \text{and} \quad C' = -\frac{1}{2}(t_2^2-t_1^2)\,.$$

From Equation 31 the equation for any of the curves in Figure 2 can be written as:

$$I = e^{k+bt} \tag{38}$$

Using Equation 26 or 27, the general expression for the area under an I vs. time curve can be written as:

$$R' = \int_{t_1}^{t_2} I\,dt = \int_{t_1}^{t_2} e^{k+bt} = \left(\frac{e^k}{b}\right) e^{b(t_2-t_1)} . \tag{39}$$

Taking the logarithm of both sides of Equation 39:

$$\log R' = \log \frac{e^k}{b} + b(t_2-t_1) . \tag{40}$$

Since e^k/b is constant, Equation 40 can be generalized as:

$$\log R' = C'' + b\Delta t . \tag{41}$$

The difference in areas between any two curves having b equal to b_1 and b_2 respectively can be written as:

$$\Delta R = (C+C'b_1) - (C+C'b_2) = C'(b_1-b_2) \tag{42}$$

$$\Delta \log R' = (C''+b_1\Delta t) - (C''+b_2\Delta t) = \Delta t(b_1-b_2) \tag{43}$$

Taking the ratio of Equations 42 and 43:

$$\frac{\Delta R}{\Delta \log R'} = \frac{\Delta t(b_1 - b_2)}{C''(b_1 - b_2)} = \frac{\Delta t}{C''} = \text{constant} \tag{44}$$

Therefore:

$$\Delta \log R' \propto \Delta R \tag{45}$$

Combining the relationship:

$$\Delta \log R' = \log R_0' - \log R_c' = \log \frac{R_0'}{R_c'} \tag{46}$$

with Equation 34 and 45 yields the following expression:

$$\log \frac{R_0'}{R_c'} \propto \text{rate of reaction} \tag{47}$$

From Equations 19 and 47, it can be written that:

$$\log \frac{R_0'}{R_c'} \propto K'[S] \, . \tag{48}$$

Therefore, the logarithm of the ratio, R_0'/R_c', of the photometer readouts for the blank and sample is directly proportional to the glucose concentration in the sample if the reaction rate curves are linear and intersect at a point on the ordinate. The work presented in this thesis demonstrates that a linear calibration curve can be obtained for glucose concentrations between 10-110 ppm using this method.

EXPERIMENTAL

Instrumentation

The separate components of the photometer used in the experimental work of this thesis are shown in Figure 3.

Sample-Detector Housing

The light filters, reaction cell, photomultiplier tube, and linear pulse amplifiers were put in a 4" x 6" x 5" housing made from two 3" x 4" x 5" aluminum boxes. The light source was external to this unit which had a 6 mm x 0.1 mm slit fixed in one end to let in light at the desired level of intensity. A shutter was installed where the two boxes joined to facilitate sealing off the photomultiplier tube from the light source and from room light when the sample compartment was opened for sample changing. The inside of the unit was sprayed flat black and all joints were sealed to make the unit light tight. In addition, several layers of black cloth were wrapped around the unit to help eliminate room light. Figure 4 shows the physical arrangement of the housing unit and light source.

Light Source

The light source used in this work was a neon-filled calcium hollow-cathode lamp (Westinghouse No. 22610) which was run at

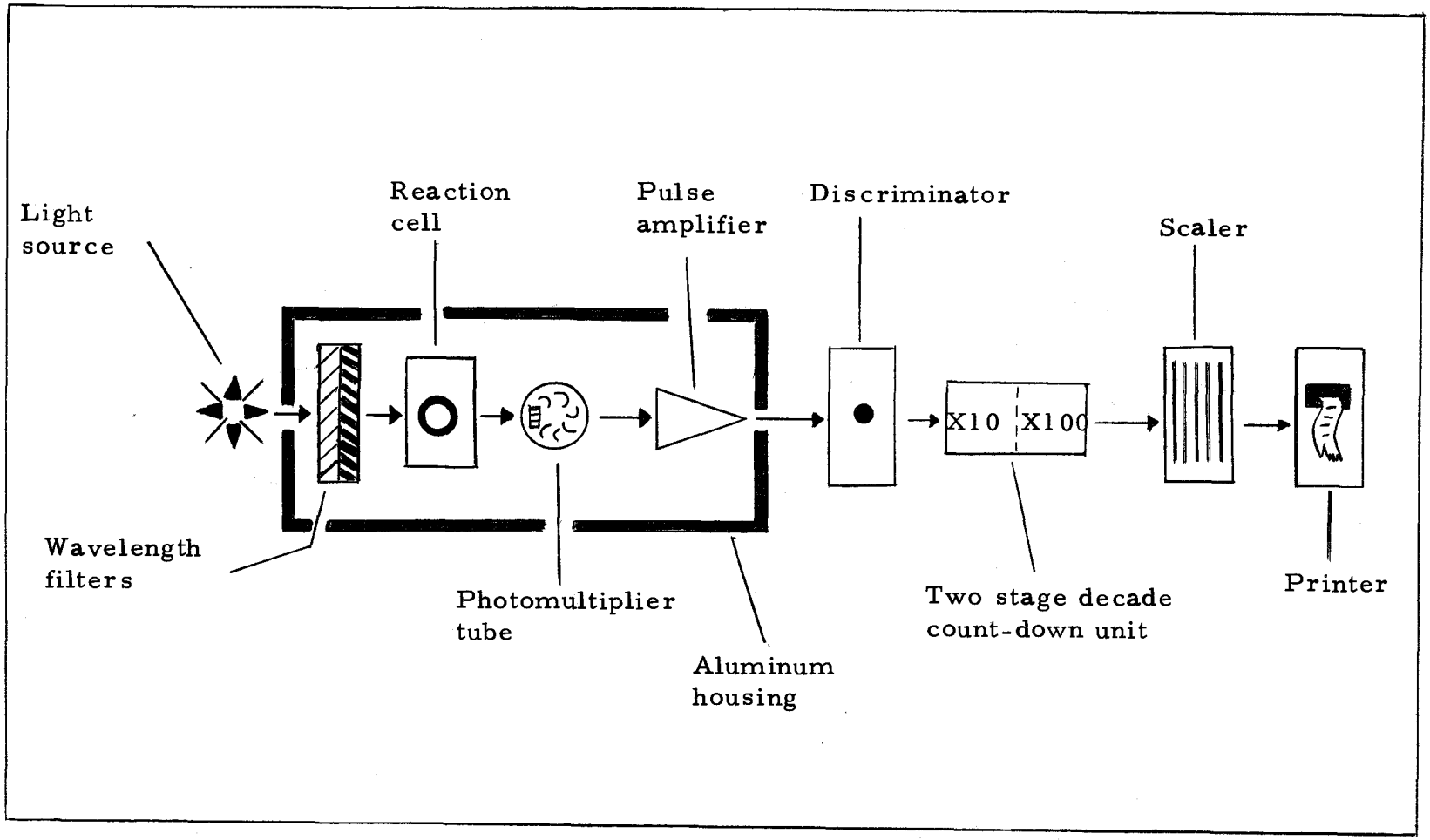

Figure 3. Component parts of the photometer for reaction rate studies.

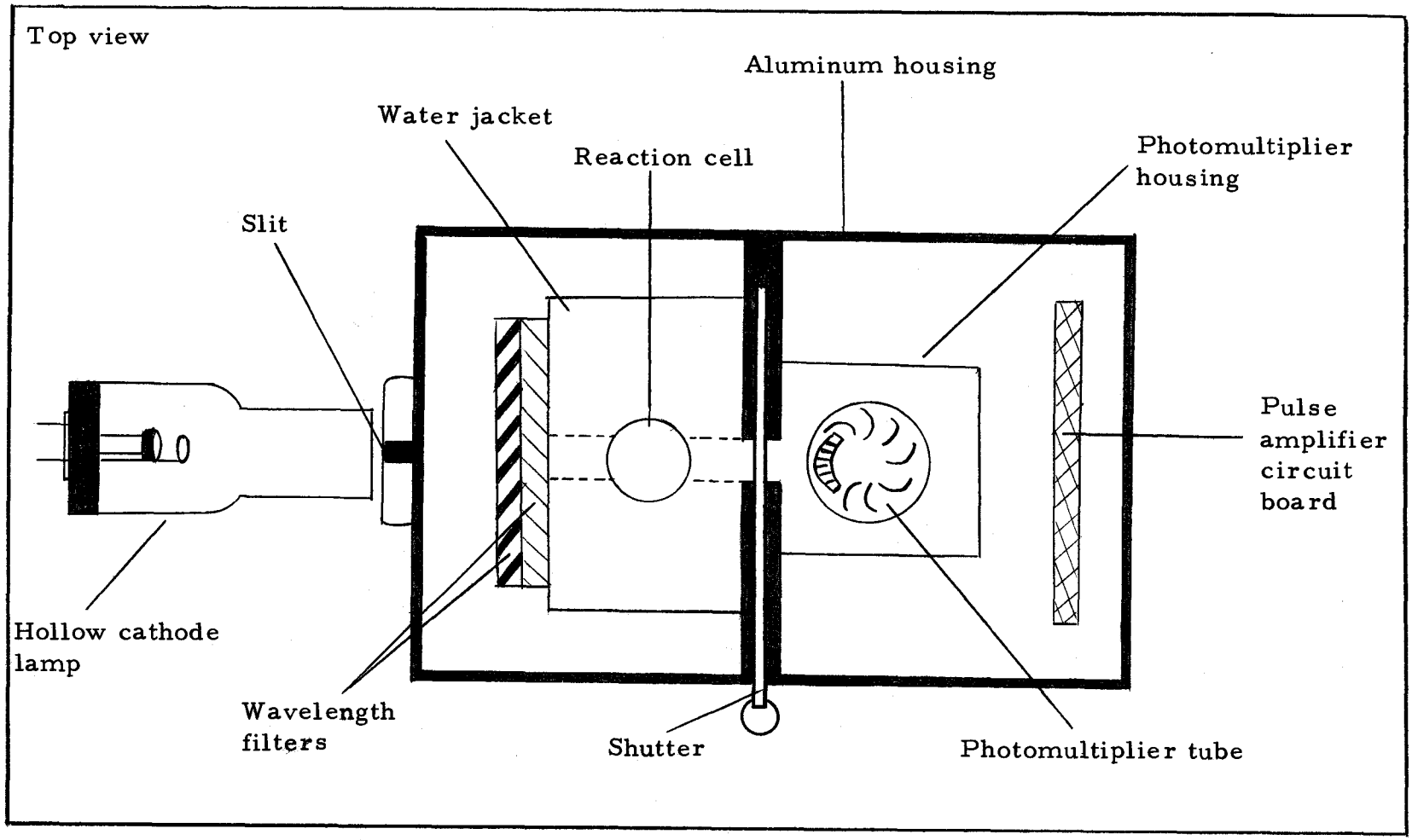

Figure 4. The housing unit and light source of the photometer.

either 0. 4 or 0. 6 ma. The power for the lamp was supplied by a Heath EUW-15 regulated power supply in series with four 100 K $\frac{1}{2}$-watt metal film resistors. The temperature coefficient of the resistors was less than 100 ppm/°C. Because of the low current used, the only lines observed from the hollow-cathode lamp were the neon lines from the filling gas and some lines from mercury present as an impurity. Two filters were used to isolate a portion of the spectral lines observed from the lamp. A 421 mμ interference filter with an 11 mμ band pass was used with an ultraviolet broad band filter which cut off above about 400 mμ to isolate a band of neon lines between 331 and 360 mμ (23).

Reaction Cell

The reaction cell was made from the bottom section of a 1. 4 I. D. soft glass test tube cut down to a height of 6. 5 cm. The cell was sealed into a lucite water jacket fashioned from 1/8" clear lucite which was subsequently sprayed flat black. The water jacket measured 4. 4 cm x 2. 7 cm x 6. 8 cm, and had a window 1. 2 cm x 3. 0 cm cut and sealed to the cell so the light beam would not pass through the circulating water. The water inlet on the jacket was at the lower edge of one side with the outlet located at the upper edge of the opposite side. The lucite-lucite seals were made with lucite dissolved in dichloroethane, and the glass-lucite seals were made with Clear Seal

(General Electric). The reaction cell with water jacket is shown in Figure 5.

Detection System

The light coming through the sample cell is detected with a HTV-R212 photomultiplier tube (Hamamatsu TV Co., LTD.). The pulses from the photomultiplier tube are amplified by a linear transistor amplifier having a gain of 1600. The pulses from the amplifier have a rise time of less than 50 nsec and a fall time of less than 0.3 μsec. The photomultiplier and amplifier circuits were built for previous work by Piepmeier and Cheung (24).

A single level discriminator made from a SN72710L differential comparator (Texas Instruments Incorporated) was installed following the pulse amplifier. The circuit for the discriminator is shown in Figure 6. The + 18 volts was supplied by a Heath IP-27 regulated low voltage power supply, and the -7.5 volts was supplied by a Heath EUW-17 transistorized power supply. The differential comparator has a 40 nsec response time. The level of the discriminator is set with the 10-turn 1K Helipot shown in Figure 6. The calibration of the discriminator was done with a square wave generator and was found to be linear within the 1% calibration of the generator. The discriminator level was chosen so that the majority of noise pulses were not counted but the larger signal pulses were passed through and counted.

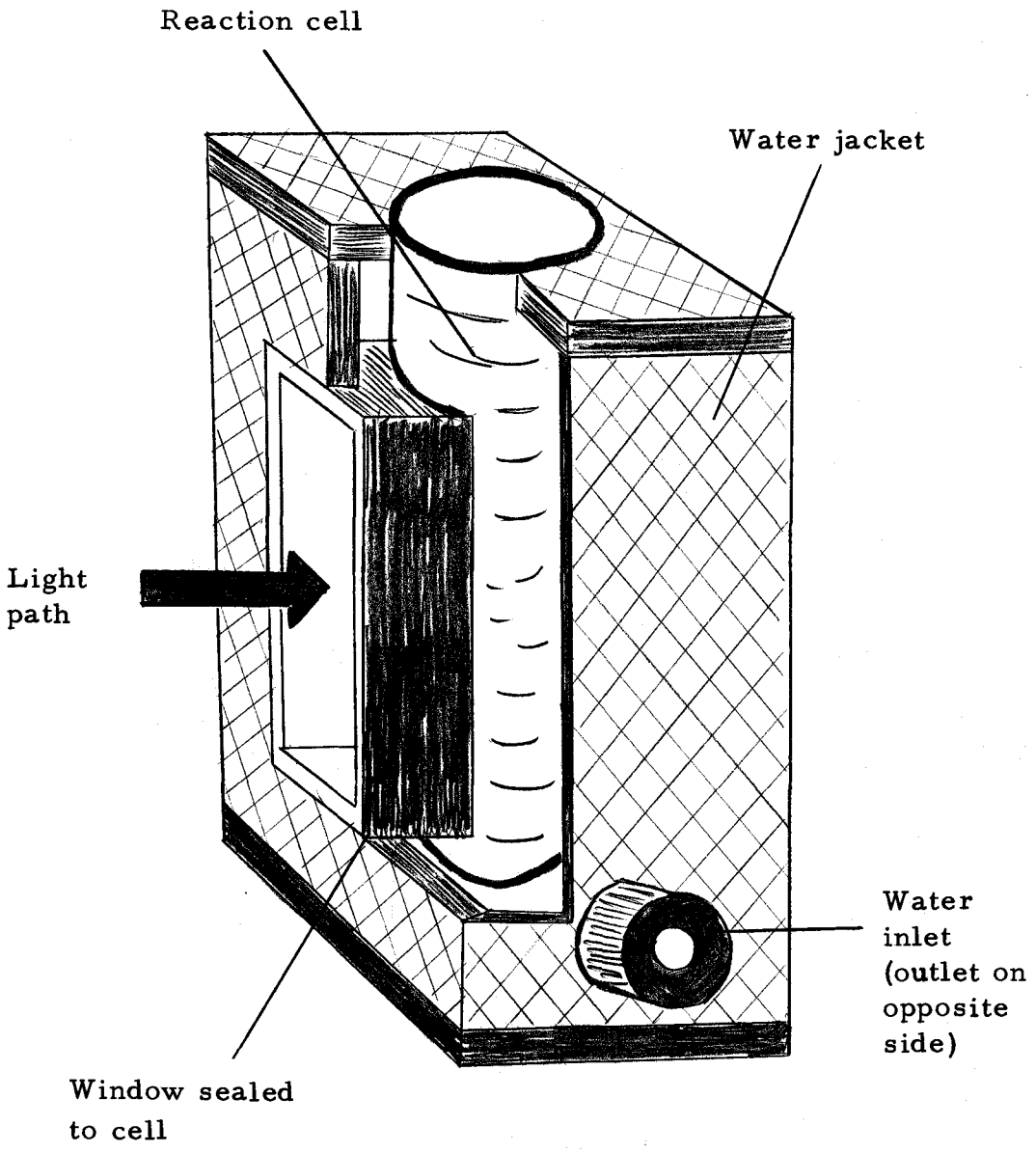

Figure 5. Cut-away view of the reaction cell and the water jacket.

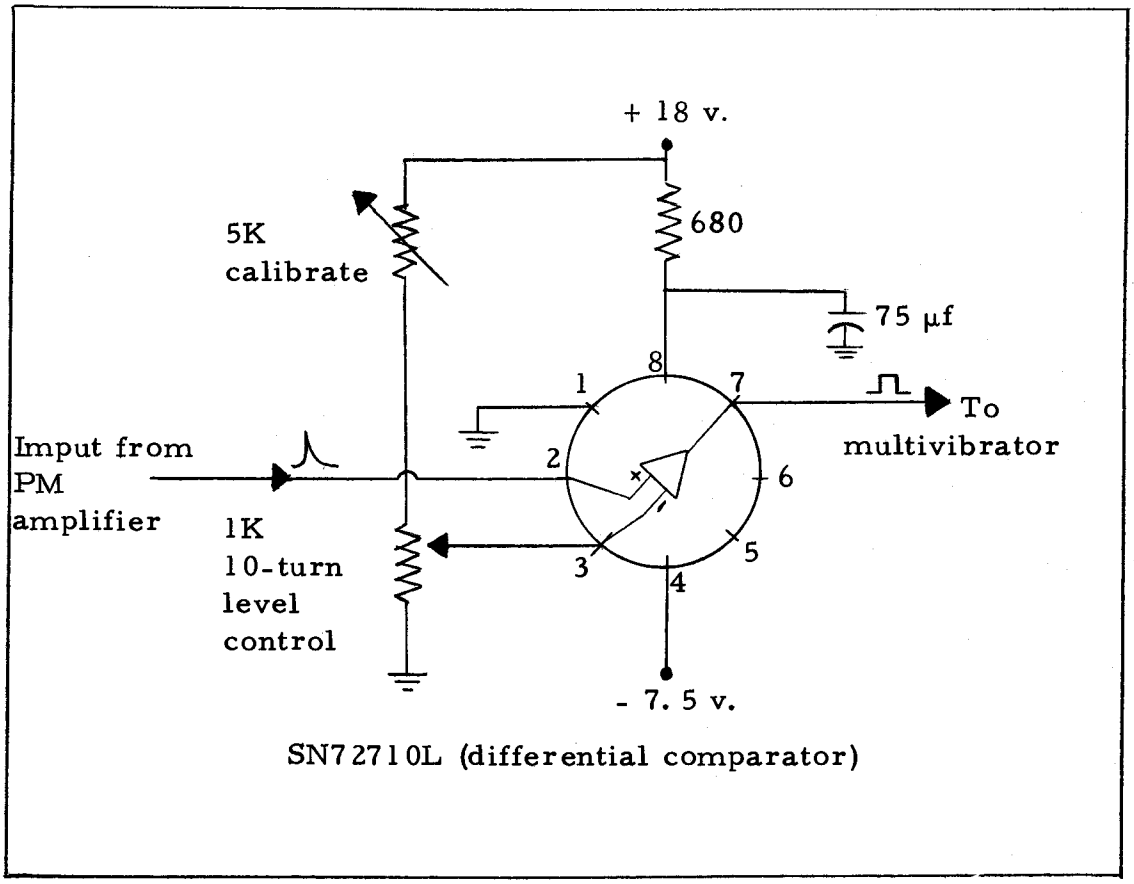

Figure 6. Single level discriminator circuit.

The paper by Piepmeier, Braun and Rhodes (23) contains a discussion of the effect of photomultiplier voltage and discriminator setting on the signal-to-noise ratio and on the stability of the photometer. A monostable multivibrator was added following the discriminator to help eliminate noise from cable ringing and regeneration pulses from large pulses.

Following the multivibrator, two decade counting units were installed to divide the pulse frequency by 10 or 100 to increase the effective range of the five-column scaler from five to seven digits. The decade units were made using μL-923 and μL-914 (Fairchild) integrated circuits from a design described by Math (17). The scaler used with this photometer was a crystal controlled Hewlett-Packard Model 521C electronic counter with a Model 526A digital printer used for readout.

Constant Temperature System

The constant temperature bath used in this work consisted of a cylindrical glass container 12" across and 12" high, a thermoregulator, a centrifugal mixing pump, and a cell circulation pump which pumped at a rate of about 100 ml in 20 sec. A Merc-to-Merc Thermoregulator and "precision" electronic relay control box (Precision Scientific Company) were used to keep the temperature of the water bath at 30.5° C ± 0.2° C.

Reagents

All solutions were prepared with distilled water which had been passed through a mixed cation-anion resin bed. All reagents were Baker and Adamson reagent grade chemicals unless otherwise specified.

Buffer-Catalyst Solution

The buffer-catalyst solution was prepared by dissolving 42 grams of potassium monobasic phosphate, 82 grams of potassium dibasic phosphate, and 13 grams of ammonium molybdate $[(NH_4)_6Mo_7O_{24} \cdot 4H_2O]$ (Matheson Coleman and Bell, reagent grade) in enough water to make one liter of solution. The solution was stored in a polyethylene bottle. According to Malmstadt and Pardue (14) this solution is stable indefinitely.

Potassium Iodide Solution

A 4% solution of potassium iodide was prepared by dissolving 10 grams of the reagent in enough water to make 250 ml of solution. The solution was stored in a dark bottle away from direct light and was made fresh every 3-4 days.

Enzyme Solution

A solution prepared by dissolving 0.64 gram of glucose oxidase

(Sigma Chemical Company, type II, purified) in about 40 ml of water was filtered through Whatman Number 1 filter paper and diluted up to 100 ml in a volumetric flask. This solution was stored in a brown glass-stoppered bottle in the refrigerator for not longer than 2 days. Solutions containing 0.32 and 0.16 gram of enzyme in 100 ml of solution were prepared in the same manner. Based on a molecular weight of 149,000 for glucose oxidase (6), and assuming 20% purity, this would correspond to a concentration of 8.6×10^{-7} M for the 0.64 gram/100 ml solution, 4.3×10^{-7} M for the 0.32 gram/100 ml solution, and 2.15×10^{-7} M for the 0.16 gram/100 ml solution.

Glucose Standard Solutions

All standard glucose solutions were prepared by volumetric dilution of a 1000 ppm standard solution which was prepared by dissolving 1.000 gram of dextrose, anhydrous powder, in water and diluting to one liter. These standards were kept refrigerated when not in use.

Composite Reagent

One hundred and twenty-five milliliters of composite reagent were prepared by mixing 25 ml of the glucose oxidase solution, 50 ml of 4% potassium iodide solution, and 50 ml of buffer-catalyst solution together in a dark bottle. The solution, after mixing, was put into the constant temperature bath and used immediately after it reached

the temperature of the water bath. This reagent buffers at a pH of 6.5.

Procedure

Preparation of Equipment

All of the power supplies were usually left on standby to keep them warmed up. At least one hour of warm-up time was allowed if the power supplies had been shut off previously. The photomultiplier voltage and discriminator power were not left on, and were allowed 1-1$\frac{1}{2}$ hours to warm up before use. The water bath was turned on at least one-half hour prior to use to allow the water and reagent solutions to reach the appropriate temperature. During a run, all solutions used were kept in glass containers in the water bath to keep them at a constant temperature. The cell circulation pump was also started to bring the reaction cell up to temperature.

Measurement Step

The electronic counter was set on the automatic 10-sec counting period with a 1-sec display mode which delayed the start of the next counting period by 1 sec. Before making any experimental runs the counting period was checked by setting the input sensitivity dial on check. This gives a 10-kHz reference count rate to the counter and

should give a count of 100, 000 in 10 sec. After checking the counting period, the input sensitivity dial is turned full clockwise and left in this position.

The reaction cell was first rinsed a minimum of five times with deionized-distilled water to remove anything left from a previous run. The water is removed with an aspirator. A hypodermic syringe (Industrial and Scientific Instrument Company) with a 3" teflon needle (Hamilton) was rinsed with composite reagent before injecting 6. 4 ml of composite reagent into the reaction cell through an opening made in the housing directly above the cell. The stirring rod was inserted and 2. 0 ml of the glucose sample solution was injected at the same time the printer was turned on "record." The counter ran continuously, with the readout being controlled with the printer's record-on switch. After 11 sec from injection time, during which the sample was mixed by rotating the stirrer, the stirrer was withdrawn and the shutter was opened. The counter counted for 10 sec, printed out the accumulated counts, waited 1 sec, rezeroed, and began counting again. After about 100 sec the shutter was closed, the printer stopped, and the solution was removed with an aspirator. The cell was rinsed a minimum of five times with deionized-distilled water between runs. Usually three to five replicate runs were made with each concentration of glucose before the next concentration was run.

Because of the necessary operations after the injection time,

but prior to the opening of the shutter, the first two prints of the printer were not used in subsequent treatment of the data other than to obtain a measure of the dark current from the first print. The third count, which was the first useful one, began 23 sec after injection of the glucose sample into the reaction cell.

For the experimental runs made in this work, the blank solution contained 6. 4 ml of composite reagent and 2. 0 ml of deionized-distilled water. The sample solutions contained 6. 4 ml of composite reagent and 2. 0 ml of the appropriate standard glucose solution.

RESULTS

Treatment of Data

The numbers printed out by the photometer are the integrated areas under the I vs. time curves, Figure 2, for the blank and sample solutions. Each number obtained was first corrected for the dark counting rate by subtracting the dark current count from the gross accumulated counts to get the net accumulated counts for the particular solution in the reaction cell. The dark counting rate is the count obtained when the shutter is closed, and represents the noise pulses from the photomultiplier tube due to thermal noise and stray light. The net accumulated counts for the blank, R_0', and that for the sample solution, R_c', are combined according to the relationship $\log R_0'/R_c'$. This relationship was shown to be proportional to the rate of reaction, and to the glucose concentration in the sample. The $\log R_0'/R_c'$ values are plotted against their respective glucose concentrations to produce a calibration curve for determining glucose concentrations in unknown samples.

Although some variation was observed in R_0' between different sets of experimental runs, the value of R_0' would be expected to be constant for any one set of experimental runs. Because $\log R_0'/R_c' = \log R_0' - \log R_c'$, any error in R_0' will merely shift the calibration curve up or down but will not change the slope.

Therefore, for the calculations for each set of experimental runs a single blank was run and this value of R_0' was used for constructing the calibration curve for those runs.

Log I_c vs. Time Curves

One condition stipulated in the derivation of the photometer response equations was that the log I_c vs. time curves were linear, and a second condition was that the reaction curves had a common ordinate intercept. The latter condition is true only if the initial absorbance of all of the solutions is the same. Under the conditions used in this work, the concentration of tri-iodide in the samples at zero reaction time will be zero, and therefore if immediate mixing were possible and an immediate measurement of infinitely short duration could be made, the first count obtained for all samples and the blank should be the same. In an actual sample however, there might be an absorbance due to other species in the solution, which might differ from sample to sample depending upon the sources of the different samples. The blank solution would compensate for this extra absorbance if it were due to something in the reagents added to the reaction cell with both blank and samples. If the absorbance were due to something introduced into the reaction cell with the sample itself, an error would be introduced into the measurement which would cause that sample's reaction curve to be shifted relative to the reaction

curves of the other samples and of the standards. An error of 0. 01 units in the $\log R_0'/R_c'$ value could cause an error of up to 10 ppm in the reported glucose concentration. If an error of this type were suspected, one could run a standard of the glucose concentration obtained for the suspected sample and compare the reaction curves obtained, or use the method of standard additions. For the experiments run in this work, all of the solutions were from the same source so that any error due to unexpected absorbance would be the same in all samples.

For experiments run in this work, an immediate measurement was not possible. A waiting period of 23 sec was allowed for mixing and other necessary operations before any counts were taken for use in calculating the $\log R_0'/R_c'$ values for the calibration curve. In order to determine if the curves had a common ordinate intercept, the curves were graphically extrapolated to zero reaction time. The results for five samples run with an enzyme concentration of 0. 32 gram in 100 ml are shown in Figure 7. The photomultiplier voltage was 800 volts with the discriminator set at 0. 50 volt, and the light source was run at 0. 6 ma. The average ordinate intercept found for the curves was 50, 860 counts per second (cps), and a relative average deviation of less than 0. 5% was found for the ordinate intercept. Considering the uncertainties of the points on the curves and of the graphical extrapolation, these results show that the system fulfills

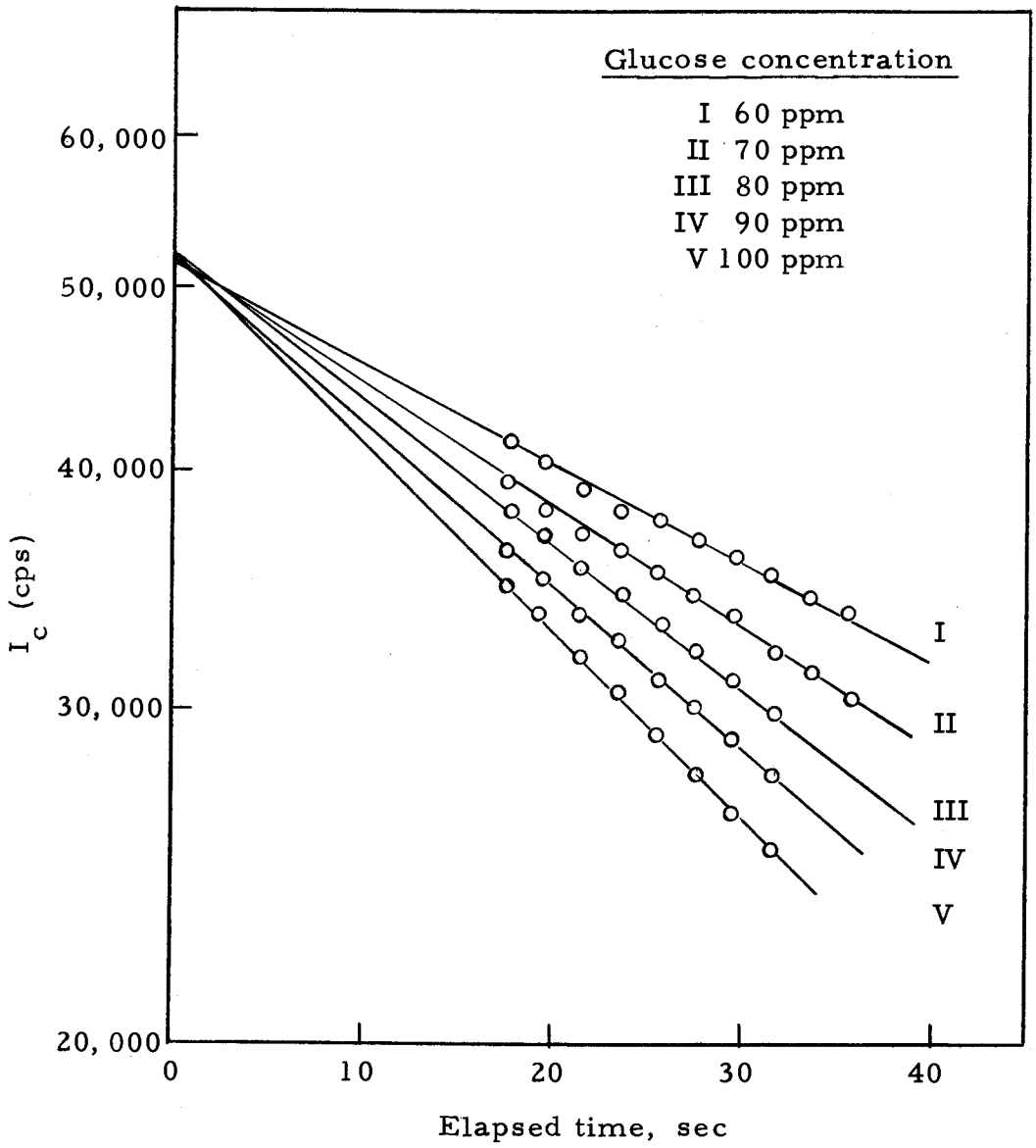

Figure 7. Graphical extrapolation of reaction curves to zero reaction time.

the conditions of linearity and of a common ordinate intercept that were stipulated in deriving the photometer response equations.

Calibration Curves

In order to be suitable for routine analyses, there should be a simple relationship between the data obtained from this photometer and the concentration of the species of interest in the solution. Equation 48, derived earlier in this paper, predicts that the log of the ratio of the photometer readout for the blank to that for the sample will be proportional to the concentration of glucose in the sample solution. Therefore, experiments were run to demonstrate that this photometer would provide linear calibration curves for the reaction rate determination of glucose in sample solutions.

The first experiments were run with the same enzyme concentration as used by others with this reaction (9, 14). A solution containing 0. 64 gram of glucose oxidase in 100 ml was used in making up the composite reagent used in the experiment. The photomultiplier voltage was 850 volts with the discriminator set at 0. 25 volt. The light source was run at 0. 6 ma for the reaction curves shown in Figure 8 and at 0. 4 ma for the reaction curves shown in Figure 9. The reaction curves obtained with the 60 and 80 ppm glucose standards, as shown in Figure 9, began to curve away from the time axis almost immediately after the 10-sec counting periods began. With the lower

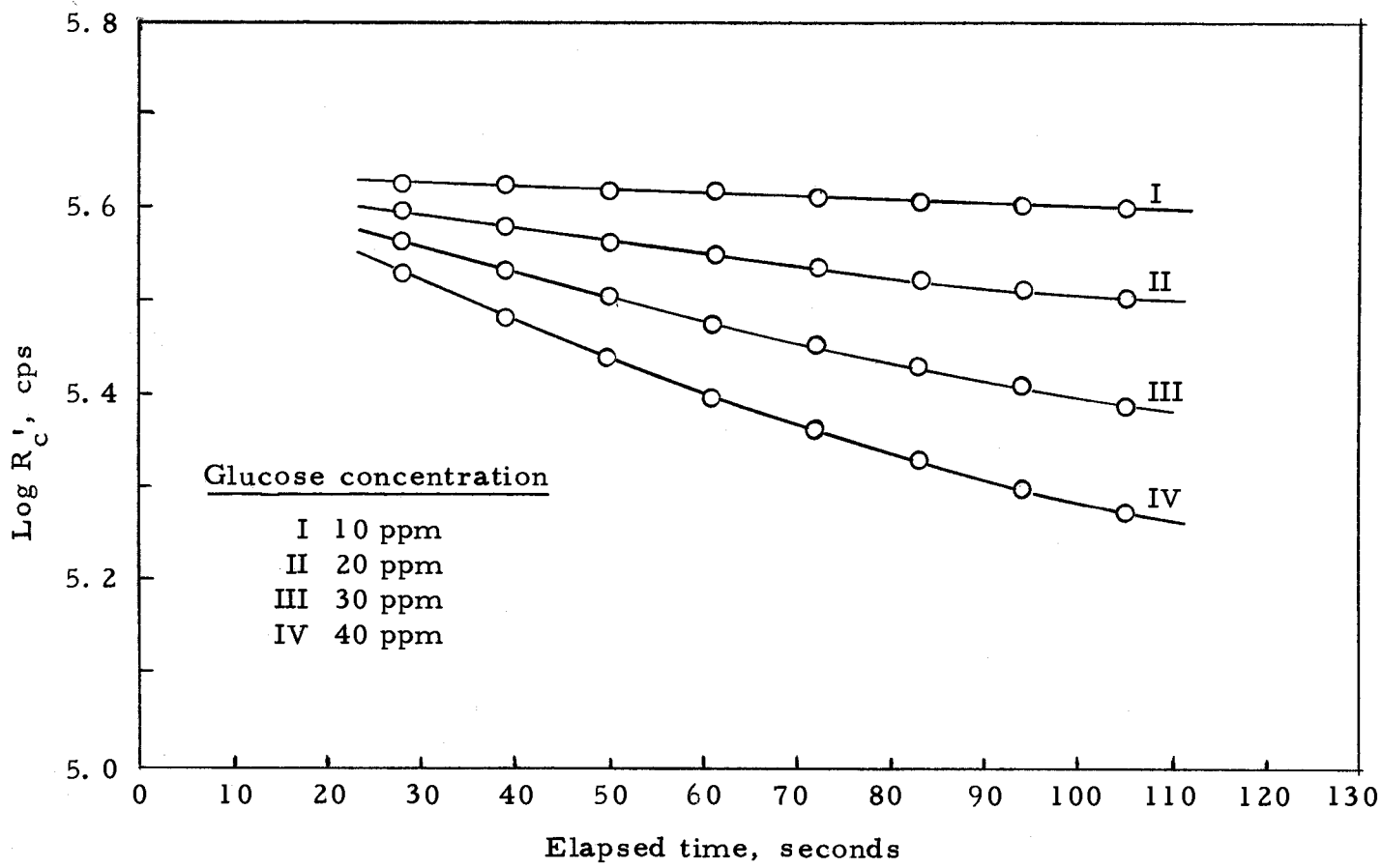

Figure 8. Reaction curves for an enzyme concentration of 0.64 gram/100 ml.

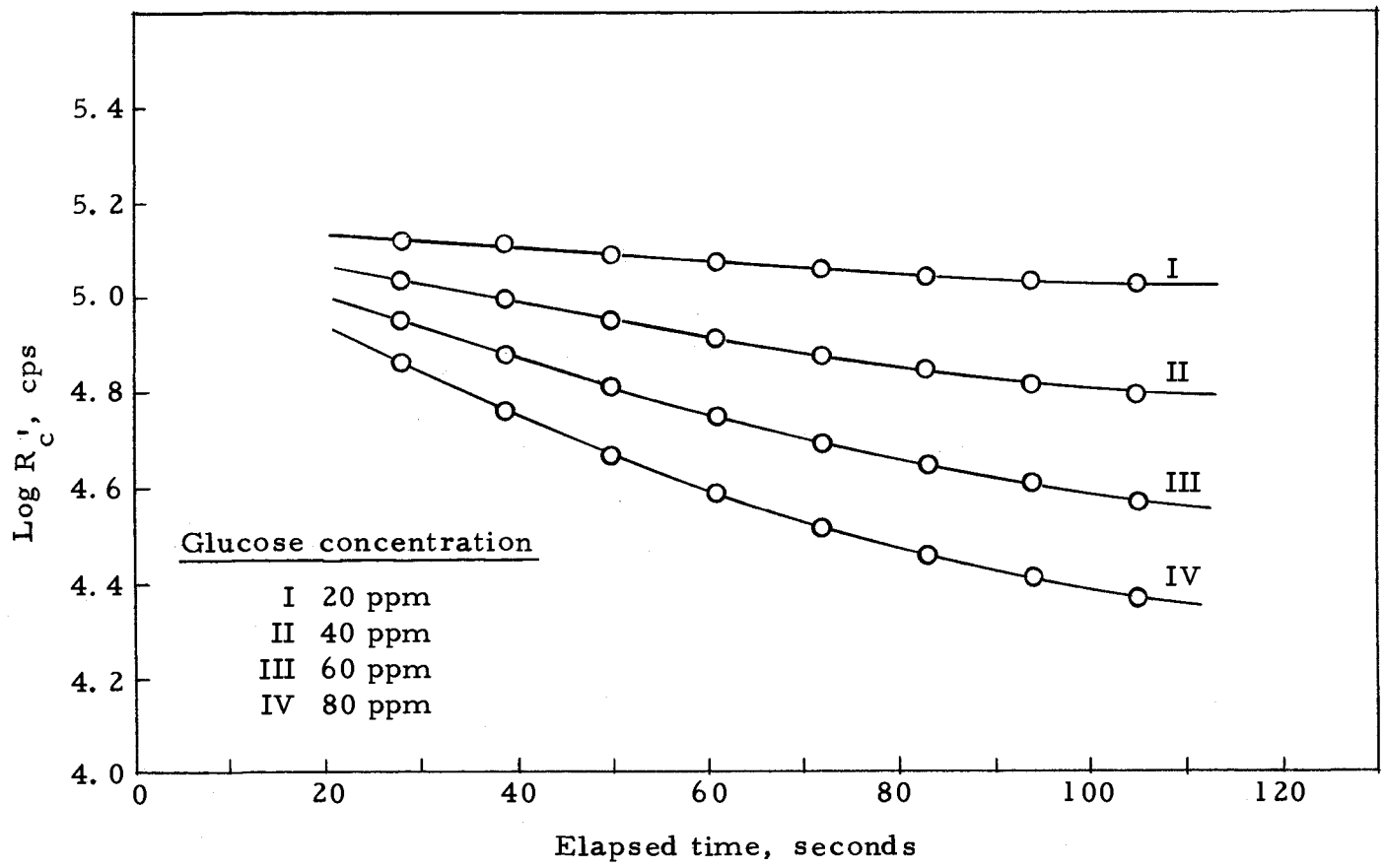

Figure 9. Reaction curves for an enzyme concentration of 0.64 gram/100 ml.

glucose concentrations the curvature did not begin as soon, but was still present in the later portions of the curves. There was no observed induction period with the 10-80 ppm standard solutions used in these experiments. In this thesis the term, induction period, is defined as that time period between the beginning of the reaction and the time when the reaction curve becomes linear. The appearance of an induction period may indicate that the reaction is not following first order kinetics in the initial period of reaction.

The first of the 10-sec counts shown in the reaction curves in Figures 8 and 9 was used to calculate the $\log R_0'/R_c'$ values used for each of the glucose concentrations shown in the calibration curves in Figures 10 and 11. As predicted by Malmstadt and Hadjiioannou (9), the calibration curves do not pass through the origin because some finite amount of glucose is used up during the measurement time. This would tend to shift the calibration curve so that it intercepts the concentration axis rather than passing through the origin. The data for Figure 10 are shown in Table 1 with the experimental deviations observed for the R_c' values.

The calibration curve shown in Figure 11 shows that, at a glucose concentration of 80 ppm, the $\log R_0'/R_c'$ value was higher than predicted for a straight line. This would be expected if the reaction curve for this glucose concentration were non-linear over the 10-sec counting period in such a way as to give an R_c' value smaller than

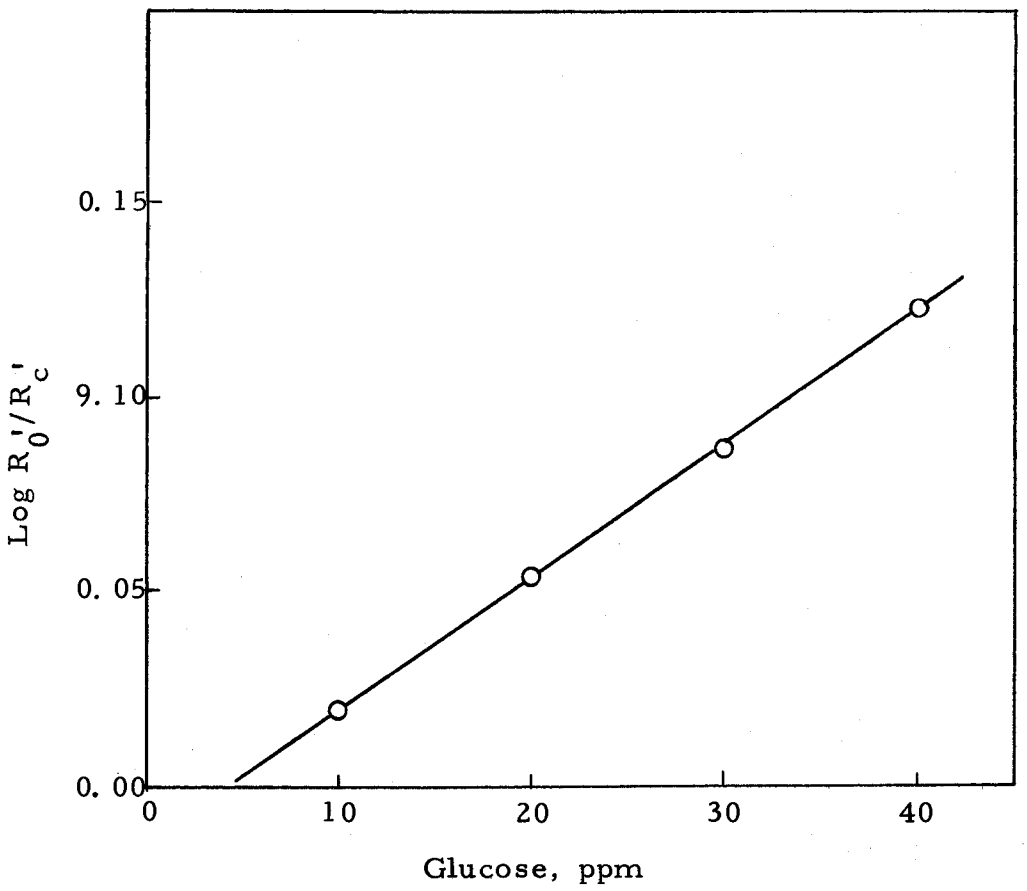

Figure 10. Calibration curve for an enzyme concentration of 0. 64 gram/100 ml.

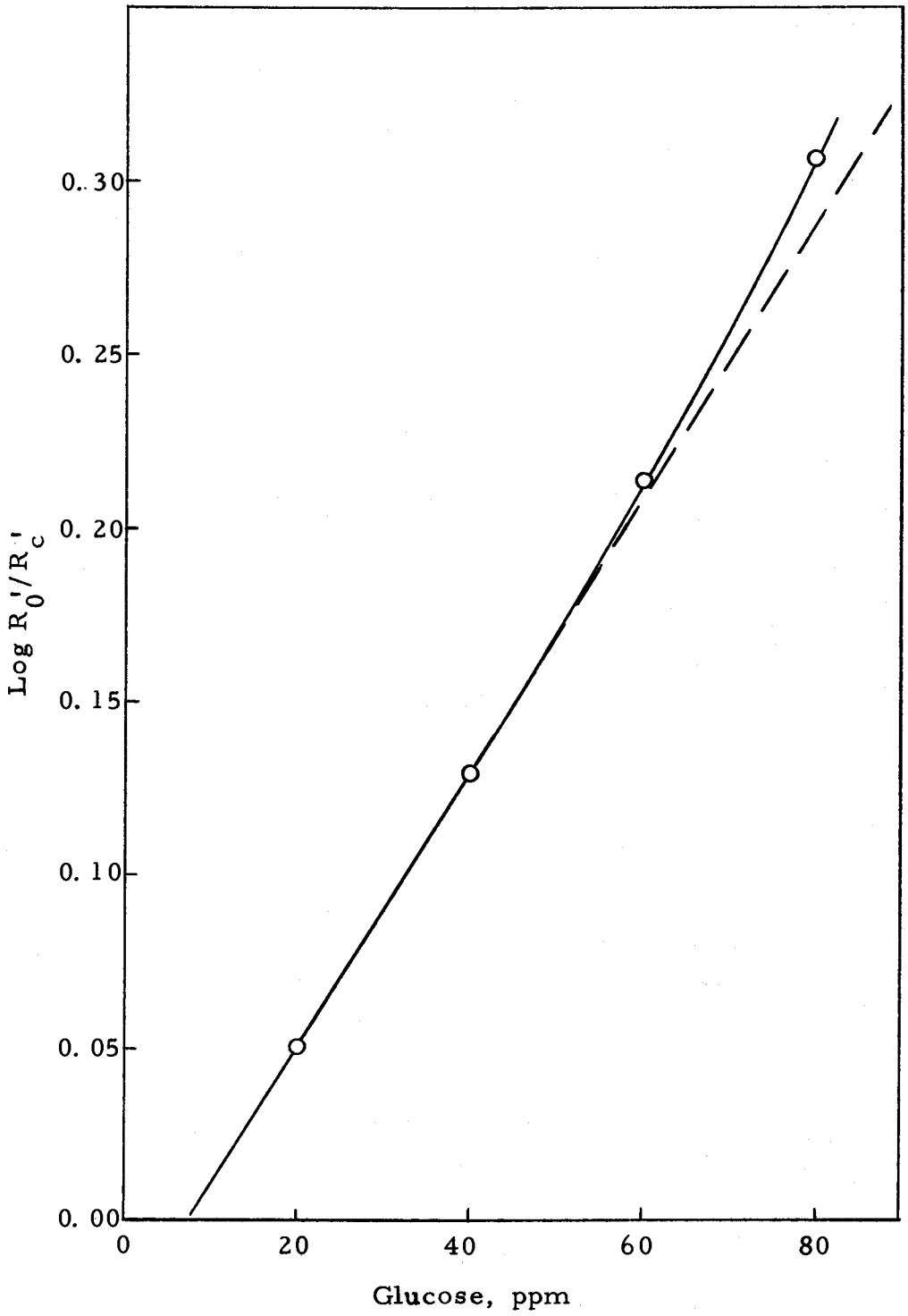

Figure 11. Calibration curve for an enzyme concentration of 0. 64 gram/ 100 ml.

would be obtained if the curve were linear. From the reaction curve for the 80 ppm glucose standard in Figure 9, it is evident that the curve is not linear and that the R_c' value for the reaction curve will be less than if the curve were linear. The 60 ppm reaction curve in Figure 9 also is not precisely linear, and therefore the slightly high value of $\log R_0'/R_c'$ for this concentration in the calibration curve in Figure 11 is not surprising. The data for Figure 11 are shown in Table 2 with the experimental deviations for the R_c' values.

The curvature of the reaction curves in Figures 8 and 9 was due to the fact that as the reaction proceeded the glucose concentration was depleted until the conditions for a pseudo-first order reaction were no longer true and the rate of reaction was no longer constant. Because the problem of curvature of the reaction curves is related to the rate of the reaction, it was proposed that if the reaction were slowed the reaction curves would stay linear for a longer period of time. In Equation 14 earlier in this thesis, the rate of reaction was shown to be directly proportional to the concentration of the enzyme and glucose in the cell. Accordingly it was decided that a reduction in the enzyme concentration might help provide more linear reaction curves for the higher glucose concentrations, thereby extending the upper limit of the linear portion of the calibration curve.

The enzyme concentration was decreased to 0.32 gram in 100 ml for the enzyme solution used in making up the composite reagent.

Table 1. Photometer response for low glucose and high enzyme concentrations.
Enzyme = 0.64 gram/100 ml

ppm glucose	R_c'	$R_c+\Delta$ *	$\log \frac{R_0'}{R_c'}$ **	$\log \frac{R_0'}{R_c'+\Delta}$	% error	Absolute error ppm glucose
10	429653	433509	0.0187	0.0149	20.3	2.0
20	396133	399339	0.0538	0.0504	6.3	1.3
30	366453	373351	0.0871	0.0795	8.7	2.6
40	337933	340935	0.1232	0.1193	3.2	1.3

* $\Delta = \left[\frac{\sum_{i=1}^{N}(m-x_i)^2}{N-1}\right]^{1/2}$ N = number of trials, m = average value and x_i = individual values.

**R_0' = 448510 and is considered constant.

Table 2. Photometer response for high glucose and high enzyme concentrations.
Enzyme = 0.64 gram/100 ml

ppm glucose	R_c'	$R_c+\Delta$ *	$\log \frac{R_0'}{R_c'}$ **	$\log \frac{R_0'}{R_c'+\Delta}$	% error	Absolute error ppm glucose
20	130800	133086	0.0504	0.0430	14.7	2.9
40	109200	111503	0.1291	0.1216	5.8	2.3
60	89900	90379	0.2133	0.2111	1.0	0.6
80	72500	73542	0.3068	0.3006	2.0	1.6

* Δ see Table 1 above.
** R_0' = 146933 and is considered constant.

For the experiments run with this enzyme concentration, the photomultiplier voltage was 800 volts with the discriminator set at 0.50 volt and the light source was run at 0.6 ma. The reaction curves obtained under these conditions are shown in Figures 12 and 13. There was a noticeable increase in the linearity of the reaction curves for the higher glucose concentrations. Unfortunately an induction period was observed for the 20 and 10 ppm glucose standards in Figure 12 and 13 respectively making the initial portions of these reaction curves remain almost parallel to the time axis.

The calibration curves corresponding to the reaction curves in Figures 12 and 13 are shown in Figures 14 and 15. The first of the 10-sec counts in the reaction curves was used to calculate the $\log R_0'/R_c'$ values for the calibration curves in Figures 14 and 15. The calibration curve shown in Figure 14 is linear over the 20-50 ppm range shown. The data for this calibration curve and the experimental deviations observed for the R_c' values are shown in Table 3.

Concentrations up to 110 ppm were used in order to extend the linear range of the calibration curve, and the results are shown in the calibration curve in Figure 15. The data for Figure 15 and the experimental deviations for the R_c' values are given in Table 4. The $\log R_0'/R_c'$ values for the higher glucose concentrations were found to lie on the linear calibration curve with the exception of the 110 ppm glucose standard which was slightly high.

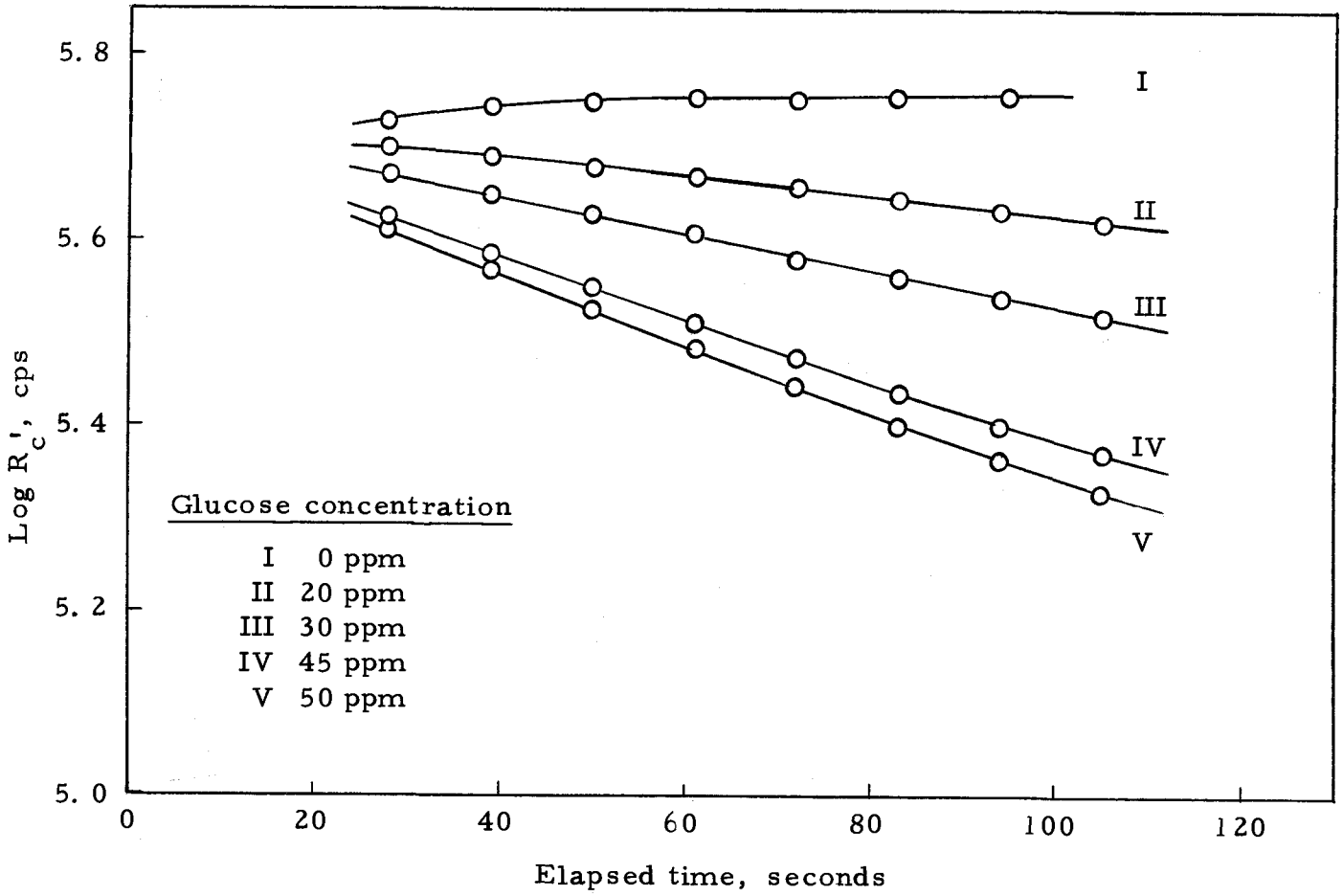

Figure 12. Reaction curves for an enzyme concentration of 0. 32 gram/100 ml.

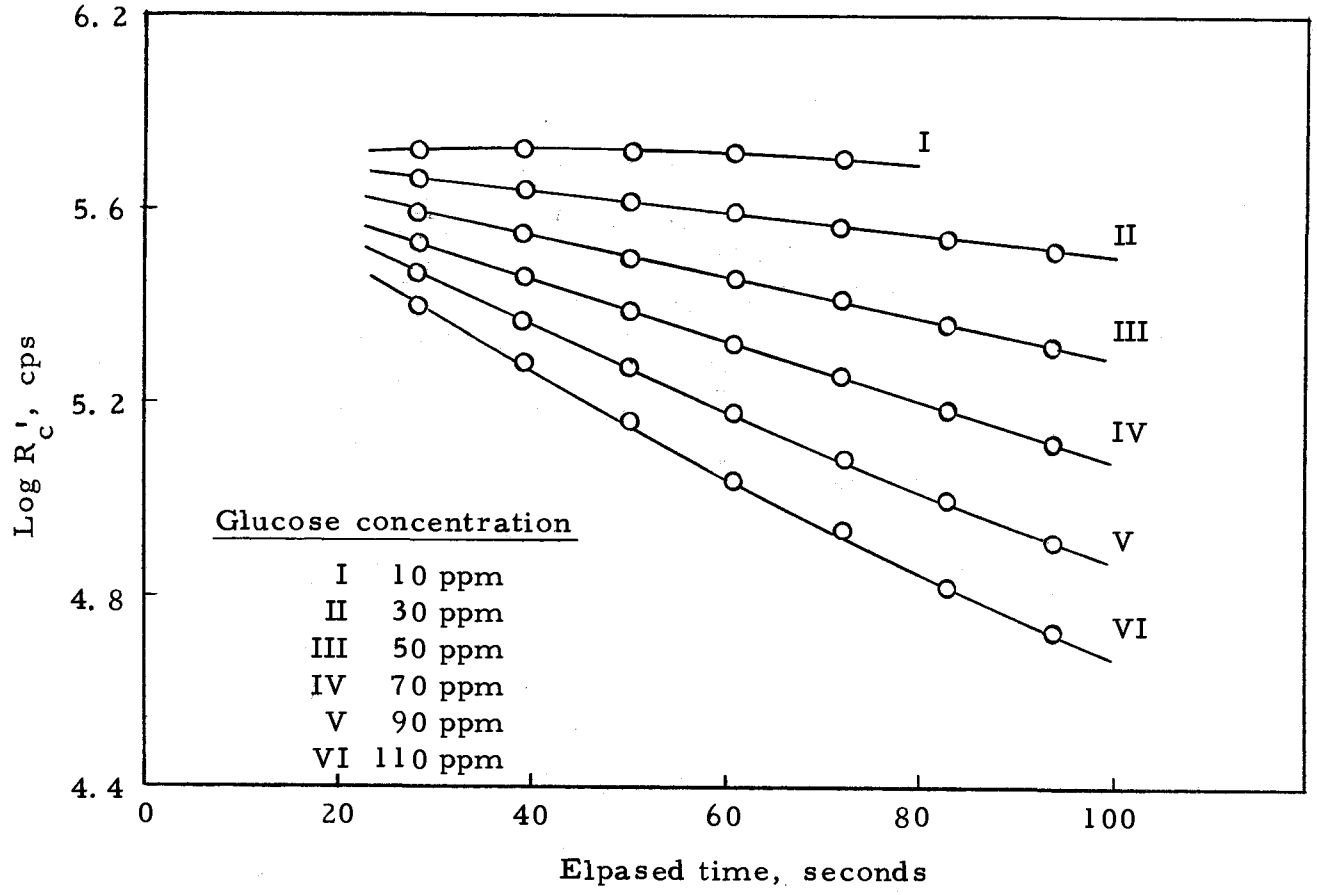

Figure 13. Reaction curves for an enzyme concentration of 0. 32 gram/100 ml.

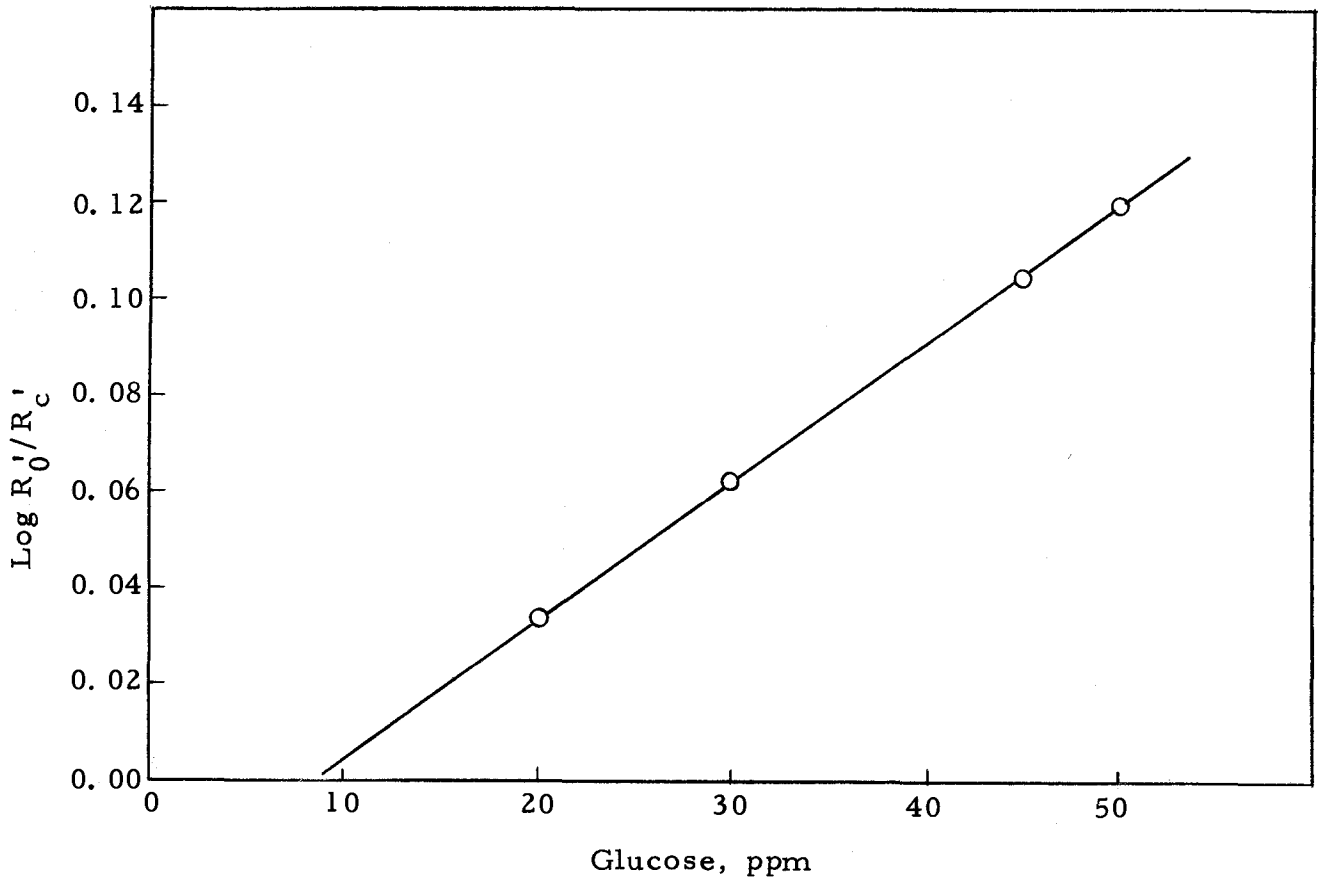

Figure 14. Calibration curve for an enzyme concentration of 0. 32 gram/100 ml.

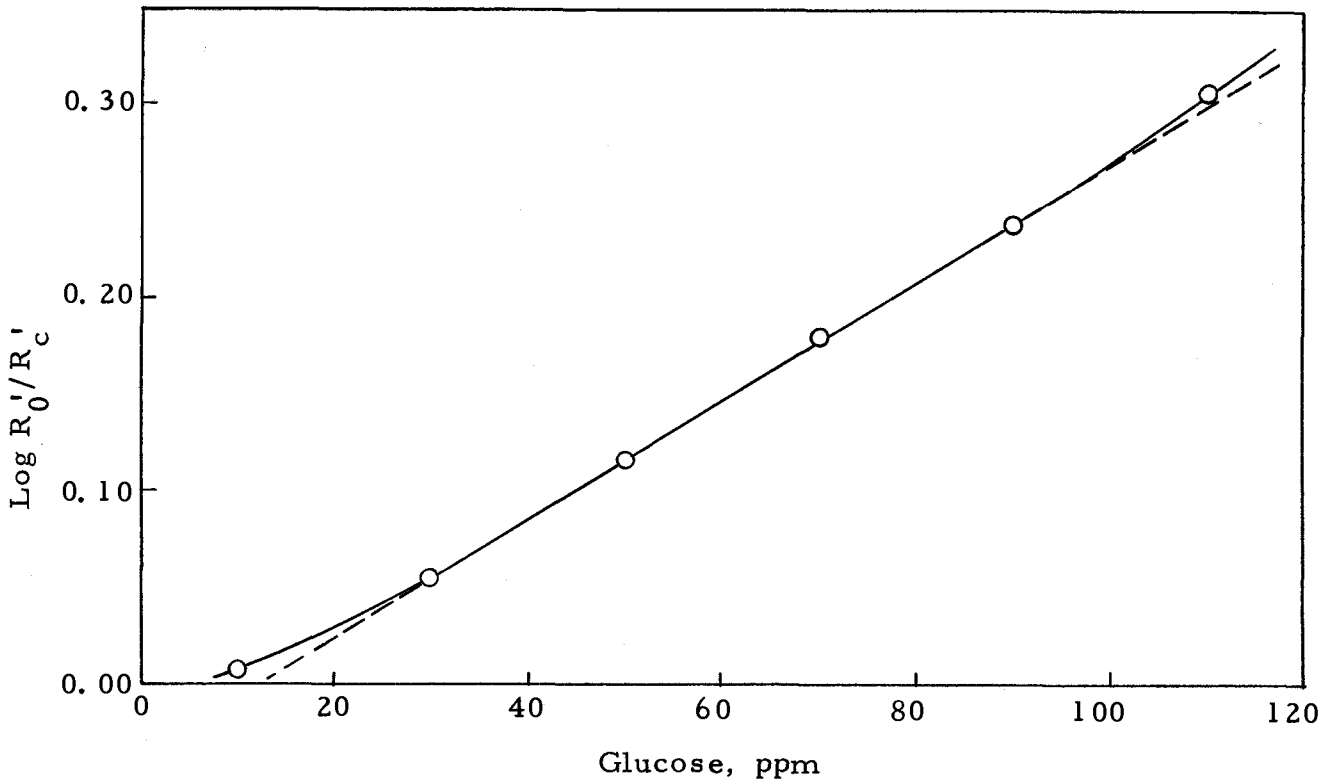

Figure 15. Calibration curve for an enzyme concentration of 0. 32 gram/100 ml.

Table 3. Photometer response for low glucose and medium enzyme concentrations. Enzyme = 0.32 gram/100 ml

ppm glucose	R_c'	$R_c+\Delta$*	$\log \frac{R_0'}{R_c'}$ **	$\log \frac{R_0'}{R_c'+\Delta}$	% error	Absolute error ppm glucose
20	502318	504432	0.0342	0.0322	5.8	1.2
30	470852	474090	0.0622	0.0592	4.8	1.4
45	427310	431994	0.1045	0.0997	4.6	1.8
50	413095	414587	0.1193	0.1176	1.4	0.5

* $\Delta = \left[\frac{\sum_{i=1}^{N}(m-x_i)^2}{N-1}\right]^{1/2}$ N = number of trials, m = average value and x_i = individual values.

** R_0' = 543433 and is considered constant.

Table 4. Photometer response for high glucose and medium enzyme concentrations. Enzyme = 0.32 gram/100 ml

ppm glucose	R_c'	$R_c+\Delta$*	$\log \frac{R_0'}{R_c'}$ **	$\log \frac{R_0'}{R_c'+\Delta}$	% error	Absolute error ppm glucose
10	525797	527575	0.0069	0.0056	18.8	1.9
30	469800	471796	0.0558	0.0538	3.6	1.1
50	407480	409993	0.1176	0.1149	2.3	1.2
70	352983	356600	0.1801	0.1755	2.6	1.8
90	307480	309517	0.2398	0.2370	1.2	1.1
110	263365	264758	0.3071	0.3049	0.7	0.8

* Δ see Table 3 above.
** R_0' = 534260 and is considered constant.

The $\log R_0'/R_c'$ value for the 10 ppm standard does not lie on the linear calibration curve. A possible reason for the deviation of the 10 ppm standard is that the 10-sec count used in calculating the $\log R_0'/R_c'$ value for this standard was taken long before the induction period for this standard was over. The induction period caused the straight line portion of the reaction curve to intercept the ordinate at a point not common to the other reaction curves as stipulated in the theoretical treatment of photometer response. Therefore the calibration curve is no longer linear in this region. If a time is chosen when the induction period for the 10 ppm standard is over, the reaction curves for the higher glucose concentrations have become non-linear. The linear range of the calibration curve appears to be from 20-100 ppm glucose.

It is worthwhile to note that the relative change in R_c' during the induction period for the 10 ppm glucose sample is 4.5% and that the instrument is capable of detecting changes in R_c' of 0.2%. The detectable change in glucose concentration during any one run is therefore 0.03 ppm from one 10-sec counting period to the next. Therefore the induction period is well resolved above the noise level of the photometer.

In order to test this apparent trend of increasing linearity for higher glucose concentration reaction curves and increased induction period for lower glucose concentrations with decreasing enzyme

concentrations, a further reduction in the enzyme concentration was made. The reaction curves obtained with a composite solution made up with an enzyme solution containing 0. 16 gram in 100 ml are shown in Figure 16. The photomultiplier voltage was 800 volts with the discriminator set at 0. 50 volt and the light source was run at 0. 6 ma. Some increase in linearity for the higher glucose concentrations was noted. The 110 ppm standard still had some curvature in the latter portion of the reaction curve. An induction period was noted with the 10, 30, and 50 ppm glucose standards.

The first 10-sec count for the reaction curves in Figure 16 was used for the calculation of $\log R_0'/R_c'$ for the calibration curve in Figure 17. Little improvement in the agreement of the high glucose concentration values with a linear line was observed for this enzyme concentration relative to that obtained with the 0. 32 gram/100 ml solution used previously. Although the 30 and 50 ppm standards showed induction periods in their reaction curves, their $\log R_0'/R_c'$ values lie on the linear calibration curve. The 10 ppm value again is not on the linear line. The data for Figure 17 with the experimental deviations for the R_c' values are given in Table 5.

Because the linear range of the calibration curve was not increased significantly with the 0. 16 gram/100 ml enzyme concentration over that for the 0. 32 gram/100 ml concentration, it seemed evident that a further decrease in the enzyme concentration would give

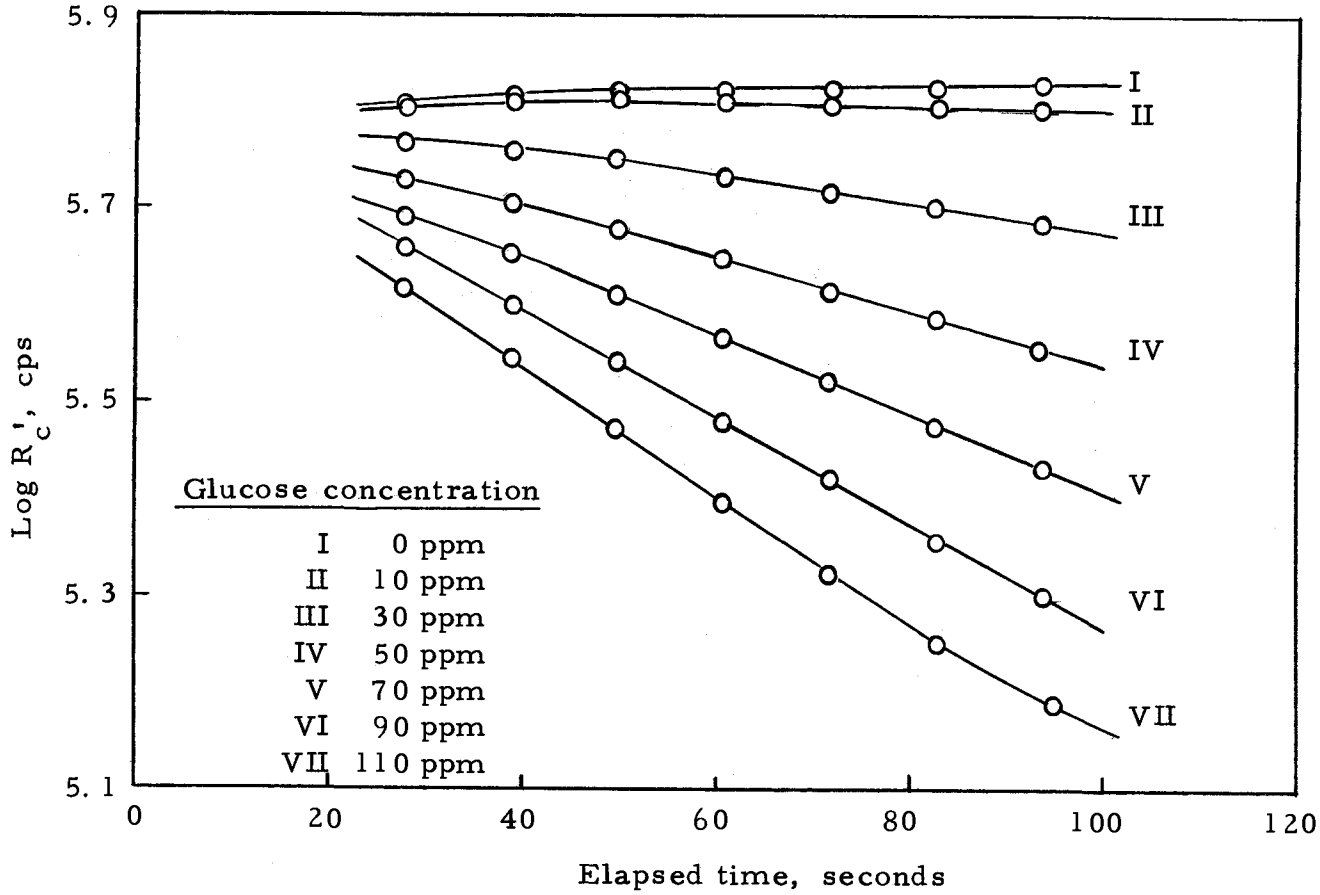

Figure 16. Reaction curves for an enzyme concentration of 0.16 gram/100 ml.

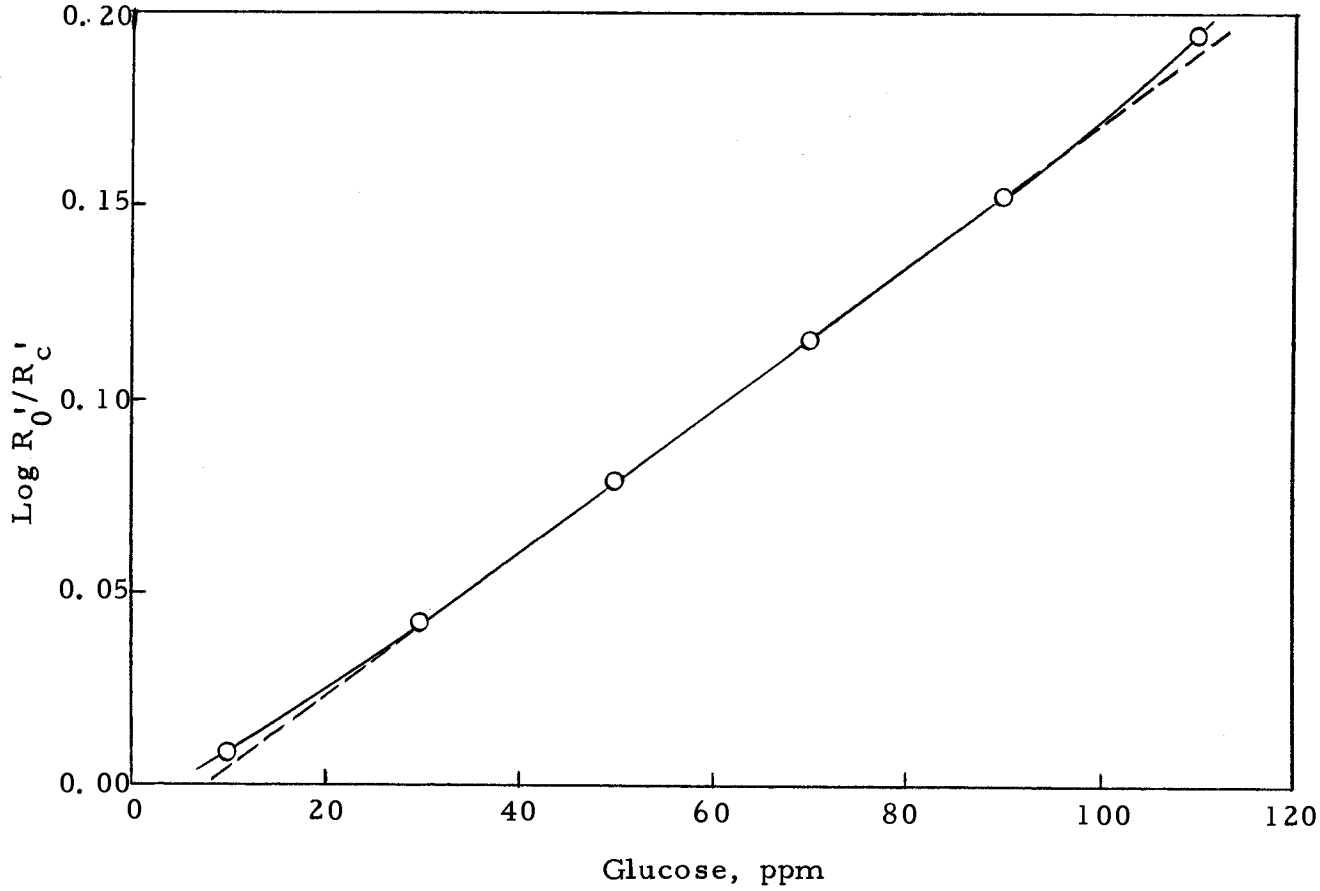

Figure 17. Calibration curve for an enzyme concentration of 0. 16 gram/100 ml.

Table 5. Photometer response for high glucose and low enzyme concentrations. Enzyme = 0.16 gram/100 ml

ppm glucose	R_c'	$R_c+\Delta$ *	$\log \frac{R_0'}{R_c'}$ **	$\log \frac{R_0'}{R_c'+\Delta}$	% error	Absolute error ppm glucose
10	633350	636715	0.0082	0.0060	26.8	2.7
30	585483	586490	0.0422	0.0418	0.9	0.3
50	537687	539572	0.0795	0.0781	1.8	0.9
70	494360	494572	0.1159	0.1156	0.3	0.2
90	454565	455732	0.1523	0.1514	0.6	0.5
110	416310	417130	0.1906	0.1898	0.4	0.4

* $\Delta = \left[\frac{\sum_{i=1}^{N} (m-x_i)^2}{N-1} \right]^{1/2}$ N = number of trials, m = average value and x_i = individual values.

** R_0' = 645600 and is considered constant.

no significant improvement. This work indicates that for any particular glucose concentration between 10-110 ppm there is an optimum enzyme concentration to use with this photometric technique. In the 10-50 ppm glucose range, the higher enzyme concentration would be the best choice. For an intermediate range of 30-80 ppm glucose, the second enzyme concentration would be a wise choice. For glucose concentrations above 80 ppm, the best choice would be the lower enzyme concentration. Some overlap of these ranges is noted, and for an unknown in which there is no previous indication of the possible glucose concentration several trials might be necessary to obtain optimum results. For routine analyses in which the glucose concentrations are frequently known fairly closely, the most convenient conditions could be chosen more easily, or dilutions could be made to bring the glucose concentration into the desired range. If high glucose concentrations were being run, dilution to a lower range would decrease the amount of original sample needed per determination.

The experimental deviation for the photometric technique presented in this thesis is shown in Table 6 for some representative glucose concentrations. The experimental error decreased as the enzyme concentration decreased. The maximum error predicted by the counting statistics for R_c' is given in Table 7 for those experimental runs given in Table 6. The error due to counting statistics is calculated as the square root of the total accumulated counts for a run.

The predicted statistical error did not decrease significantly as the enzyme concentration decreased. Because the maximum statistical error is less than the observed experimental error, and because the observed error decreased with decreasing enzyme concentration and the statistical error did not, the major source of error would seem to be due to the experimental procedure rather than to the counting statistics. The most likely source of error in the experimental procedure would be in injecting the solutions into the reaction cell. The error in the volume of solution injected into the reaction cell was determined to be less than 2% which is approximately the same order of magnitude as the observed error in most cases. A more accurate injection system would help increase the precision of this technique to near the level of error predicted by the counting statistics.

Table 6. Experimental uncertainties in glucose determinations.

Enzyme concentration gram/100 ml	Glucose ppm	Percent error	Absolute error ppm glucose
0.64	20	14.7	2.9
	40	5.8	2.3
	60	1.0	0.6
	80	2.0	1.6
0.32	10	18.8	1.9
	50	2.3	1.2
	90	1.2	1.1
	110	0.7	0.8
0.16	10	26.8	2.7
	50	1.8	0.9
	90	0.6	0.5
	110	0.4	0.4

Table 7. Statistical errors in glucose determinations.

Enzyme concentration gram/100 ml	Glucose ppm	$\sqrt{R_c'}$	Absolute error ppm glucose
0.64	20	362	0.2
	40	330	0.3
	60	298	0.4
	80	269	0.3
0.32	10	725	0.7
	50	638	0.3
	90	554	0.3
	110	514	0.3
0.16	10	795	0.6
	50	733	0.3
	90	674	0.3
	110	645	0.3

DISCUSSION

A limitation of this photometer for reaction rate analyses, as suggested by Pardue and Rodriguez (21), is the need to accumulate sufficient counts to have reasonable counting statistics. This was the major reason for discarding the use of the 1-sec counting periods for the glucose determinations. With 10-sec counting periods the relative error due to counting statistics was only a few tenths of a percent. For the 1-sec counts the error was up to about one percent.

One method for solving the problem of low accumulated counts with 1-sec counts would be to increase the light intensity. This can be done up to about 50, 000 cps, but pulse pile-up begins to interfere if the count rate is increased much above this number (23). Therefore, the problem of low numbers of counts would best be alleviated by increasing the count rate acceptance of the detector unit being used, so an increase in light intensity could be tolerated. The 50, 000 cps limit only corresponds to the amount of light reaching the detector, not that amount starting out from the light source. With highly absorbing solutions the light intensity can be increased over that used for less absorbing solutions without exceeding the 50, 000 cps limit.

Another aspect of the count accumulation problem stems from the fact that the solutions being observed are becoming more absorbing as time passes. Therefore, as the concentration of glucose

increases, the rate of reaction increases and the count rate to the detector will decrease more rapidly so fewer counts will be collected in the specified counting period. This would result in a larger statistical counting error for the fast reaction than for the slow reaction as was observed in this work. One way to counteract this statistical counting error would be to modify the photometer so that it would precisely determine the time necessary to obtain a specified number of counts. For a system of this type the accumulated counts would be the fixed quantity and the counting time would be the variable quantity. The inverse of the time would be related to the rate of reaction and to the glucose concentration. The measurement of time for a predetermined change in absorbance was used by Malmstadt and coworkers in their detection systems. Because the number of counts could be selected to give the desired statistical error, this would be the first step in freeing this type of photometer from the limitation of statistical counting error.

A different type of problem was more troublesome than the counting statistics. The photometer did not give the same total count for the same glucose solution on different days. This makes it necessary to run a calibration curve for each day's runs. Although an attempt was made to normallize the values obtained for R_c' on various days by the ratio of the R_0' values, it was not successful. The reason for this was probably the result of differences in the enzyme

solutions used on the various days. Each time the enzyme solutions were prepared in exactly the same manner, but because of the filtration step, the final concentration of the enzyme may have varied slightly between solutions. The age of the enzyme solution definitely has an influence on the results obtained and enzyme solutions were not used if they were over two days old. The most likely cause for the differences observed between different enzyme solutions was the turbidity of the enzyme solution which probably increased the variation observed between runs made on different days.

The induction period observed with the low glucose concentrations can be explained in part on the basis of the behavior of the blank solution. The transmittance of the blank increased non-linearly with time and leveled off as shown in Figures 12 and 16. The reason for the increase is not known, but the increase became more pronounced as the enzyme concentration was decreased. Because there was no glucose added to the blank, the behavior observed may have been caused by some reaction between the reagents in the composite reagent or by a gradual clearing of the turbid composite reagent due to the dilution with deionized-distilled water. If the same reaction were occurring in the sample runs, the net effect might be to cause the reaction curves to appear more linear than they would be if the blank behavior were not typical of all experimental runs. If the blank behavior were due to a reaction that is inhibited when the glucose

reaction occurs, then the blank behavior would not be typical of all runs and would have little effect on the reaction curves of those samples which have sufficient glucose to make the glucose reaction the only one of significance.

The change in the slope and shape of the calibration curve when a correction for the blank behavior is made was found to be negligible for the lowest enzyme concentration where the largest effect would be expected. Therefore no correction was made for the calibration curves with the other two enzyme concentrations.

The correction for the blank behavior did not make the 10 ppm standard lie closer to the linear calibration curve, and did not help to make the calibration curve pass through the origin. The latter problem can be explained, but only in part, by the fact that some substrate was tied up as the $E \cdot S$ intermediate. The theoretical expression for the reaction rate, Equation 13, expresses the rate in terms of the free substrate concentration. If a significant amount of substrate is initially tied up as $E \cdot S$, the free substrate concentration and therefore the reaction rate would be lower than expected, particularly for low initial substrate concentrations. This would help to cause the curvature near the origin of the calibration curve. However, the lowest ratio of substrate to enzyme concentration at which a deviation from a linear calibration curve was observed was 30:1. If all of the enzyme were complexed as the intermediate, the actual substrate

concentration would only be 3. 3% lower than assumed. This is approaching the precision of the method and cannot account for the large curvature of the calibration curve near the origin.

The dark current is not an extreme problem with this photometer because a correction can be made for the dark current since the first reading obtained for each run is a measure of the dark current. The dark current is mostly due to thermal noise in the photomultiplier and could be reduced if the photomultiplier tube were cooled.

Some of the advantages of this photometer for reaction rate analyses result from its unique detection system. The readout obtained is digital without the need of an analog to digital converter. The readout is proportional to the total number of photons passing through the sample cell during the counting period and the log of the ratio of the readout for the blank to that for a sample has been shown to be proportional to the rate of reaction and to the glucose concentration in the sample.

The system used in this work for glucose determinations can be easily adapted for use in other systems. The determination of iodide with the cerium IV-arsenic III reaction can be done with this system with a change in the light filters used. Other systems may require the use of a different light source as well as different filters. A highly stable light source as suggested by Pardue and Rodriguez (21) could be used in this system.

The light intensity required for this photometer is small and makes the use of a radioisotopic light source like that of Ross (25) possible. The power consumption is small enough (23) that a portable system could be made for field work.

The measurement time for a determination is less than one minute, which makes the system practical for routine analyses. If the system were modified so that a higher light intensity could be used, the time per measurement could be cut down through the use of 1-sec counts.

SUMMARY

The photon-counting technique presented in this thesis has been shown to be suitable for the routine reaction rate determination of glucose. Equations have been derived relating the photometer read-out, R_c', to the changing absorbance of the solution in the reaction cell. Proportionality between $\log R_c'$ and glucose concentration was predicted by the equations and the experimental results support the prediction.

Linear calibration curves have been obtained for glucose concentrations in the range of 10-110 ppm. The reproducibility of the points on the calibration curves was found to be about 2 ppm over the entire range. It was found that there was an optimum enzyme concentration for any particular range of glucose concentrations. Low glucose concentration determinations gave more precise results with a high enzyme concentration, but high glucose concentration determinations were better with a low enzyme concentration.

The major source of error in this technique was found to be the experimental procedure rather than the counting statistics for the values obtained for R_c'. The most probable cause of experimental error was variation in the volume of composite reagent and sample solution injected into the reaction cell.

The use of a standard addition method to establish a calibration

curve without using a blank solution was discussed. This method is necessary when the sample background absorbance is significantly different from that of the standard glucose solutions.

Due to the unique detection system used, the photometric technique presented in this thesis has the advantages of digital readout, low power consumption, easy adaptation to different determinations, and short measurement times per sample. The photometer itself is small enough to be made portable, however the need of a constant temperature bath for kinetic work would make the construction of a portable model of this photometer for reaction rate studies more difficult.

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