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A NEW, DIRECTLY COMPUTER-CONTROLLED PH STAT.(U)
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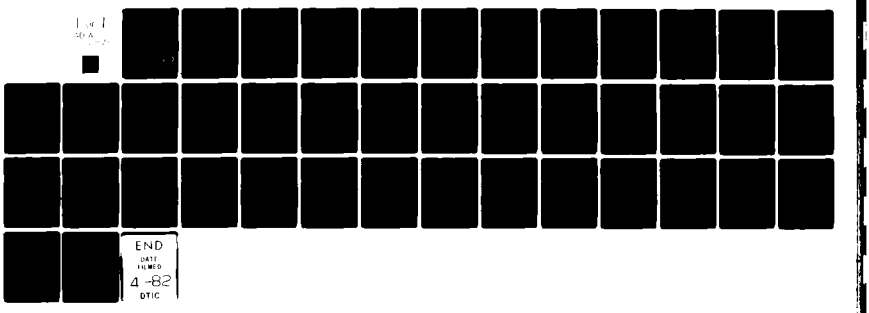

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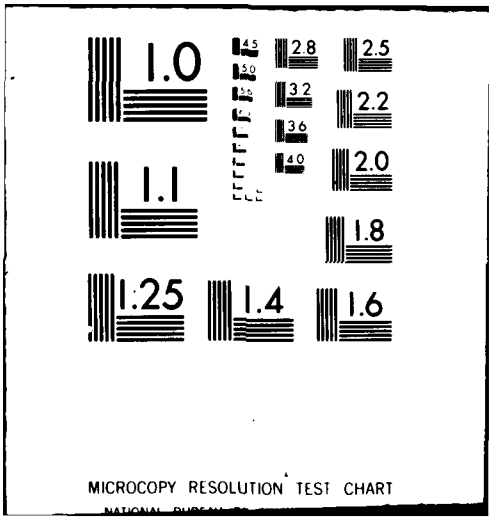
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The design and implementation of a computer-controlled pH-stat is presented. The titrant delivery system used in the design of the pH-stat is based on a computer-controlled droplet generator able to selectively add titrant aliquots of 0.06 μL. Precise titrant control enables the stationary control of pH to within 0.0008 pH units. Algorithms for program operation are structured as autonomous concurrent tasks and are written entirely in FORTRAN. The pH-stat is operated by means of simple directives entered into a terminal. During an experiment directives permit the graphic display of data and the

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alteration of experimental parameters. To evaluate the pH-stat, standard working curves are obtained from which the concentration of glucose in standard human blood serum is determined to within 3% of the value specified by the manufacturer. Two alternate methods of analysis - the fixed-time technique and the use of enzymatic induction times - are evaluated for the determination of glucose concentrations.

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A NEW, DIRECTLY COMPUTER-CONTROLLED pH STAT

by

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Prepared for Publication

in

ANALYTICA CHIMICA ACTA

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INTRODUCTION

Currently, potentiometric reaction-rate methods of analysis find a wide range of analytical application and a number of such procedures and corresponding instrumentation have been described [1-5]. Innovations in titrant delivery systems and computer automation, in particular, have enabled rapid, accurate, automated reaction-rate determinations; of special interest is the development of a directly digital computer-controlled titrant delivery system capable of producing microliter aliquots [6].

In the present study, reaction-rate methods are investigated by an instrumental pH-stat [7,8] placed under computer control. Real-time computer control processing not only eliminates tedious calculations but also enables real-time parameter modification according to experimental demands. Real-time programming enhances the flexibility of such an experiment in two ways:

(1) Feedback from the experiment enables accurate calculation of titrant rate, thus eliminating pH overshoot and undershoot by the pH-stat.

(2) Feedback from the experiment enables the dynamic alteration of sampling frequency, thus permitting rapid response in a dynamic system.

Real-time computer control and the precise nature of the directly digital computer-controlled titrant delivery system coupled with a highly sensitive electrometer allow the stationary control of pH to within 0.0008 pH units in the present experiment.

To illustrate the performance of the pH-stat, the familiar glucose-glucose oxidase reaction has been studied. The glucose-glucose oxidase reaction proceeds by a pseudo-first-order mechanism and has the following stoichiometry:

$$\text{glucose} + O_2 \xrightarrow{\text{glucose oxidase}} \text{gluconic acid} + H_2O_2$$

Glucose concentrations are determined by potentiometrically measuring the rate at which gluconic acid is formed. In accordance with first-order reaction-rate kinetics, the initial rate of reaction is proportional to the initial substrate concentration [9]. Because an enzymatic-catalyzed reaction rate is pH-dependent, pH control is necessary. In an effort to maintain a constant pH, the pH-stat adds alkaline titrant at a rate proportional to the formation of gluconic acid. Thus, the average titrant addition rate is proportional to the initial glucose concentration. A working curve is obtained, from which unknown sample concentrations can be determined. In this manner, determination of glucose concentrations as low as 10 μg/mL is possible.

THEORY

A rigorous treatment of reaction-rate theory is presented by Ingle and Crouch [10]. Only the formulation of the rate expression governing the studied reaction mechanism will be presented here. Reaction-rate methods of analysis form their basis in the calculability of an analyte concentration from the measurement of the initial rate of a reaction involving the analyte. In many clinical assays the analyte of interest is the substrate in an enzyme-catalyzed reaction of the form

$$S + E \underset{k_3}{\overset{k_1}{\rightleftharpoons}} [SE] \underset{k_4}{\overset{k_2}{\rightleftharpoons}} P + E \qquad (1)$$

where E is the enzyme, S the substrate, SE an enzyme-substrate complex, and P the product. The steady-state rate expression for the simple enzymatic reaction can be written as

$$-d[S]/dt = (k_2[S][E]) / ([S] + K_m) \qquad (2)$$

where K_m is the Michaelis constant. If the change in [S] is small compared to K_m over the observation period equation (2) simplifies to

$$-d[S]/dt = K'[S][E] \qquad (3)$$

And if the enzyme concentration remains constant for all analyses, equation (3) further simplifies to

$$-d[S]/dt = K''[S] \qquad (4)$$

The integration of equation (4) from t=0 (the reaction initiation time) to t=t (the time of observation) yields the relationship between the initial substrate concentration, $[S_0]$, and the substrate concentration, [S], at any time t

$$[S]_t = [S_0]\exp(-kt) \tag{5}$$

Substituting equation (5) into equation (4) yields the expression for the reaction-rate at any time t in terms of $[S_0]$

$$-(d[S]/dt)_t = K''[S_0]\exp(-kt) \tag{6}$$

By taking samples only during the initial part of the reaction (i.e., making t small) causes the exponential term in equation (6) to approach unity, and equation (6) simplifies to

$$-(d[S]/dt)_t = K'''[S_0] \tag{7}$$

Equation (7) forms the basis of reaction-rate techniques and states that the initial substrate concentration is proportional to the initial rate of reaction.

For many reactions, reaction-rate methods of analysis offer several advantages over alternative approaches.

Firstly, substrate concentrations can be determined shortly after mixing of reactants, so rate measurements for reactions with half-lives on the order of a few hours can be made in a few seconds or minutes.

Secondly, the determination can usually be completed before the occurence of possibly complex side reactions. In many cases, the initial-reaction-rate method can be of analytical use for reactions having complex rate mechanisms.

Thirdly, enzymes often possess a high degree of specificity for one substrate, enabling in situ analyses. This specificity also enables decisive determinations of closely related species that are difficult to physically separate but are differentiable by their kinetic properties.

Fourthly, because the pH-stat maintains the reaction at a constant pH, buffers are not necessary. This removal of buffers reduces the possibility of interfering side-reactions attributable to buffers, and also reduces the effect of pH on the rate of reaction.

Fifthly, reaction-rate methods require only a relative and not an absolute measurement. Therefore, factors which contribute to the error of an absolute measurement do not interfere with the reaction-rate measurement.

There are, however, many limitations to the reaction-rate methods which are often not present in equilibrium-based techniques.

The detection system can restrict the range of analyses performed by the reaction-rate method because the accuracy of the measured reaction-rate is dependent on the time response characteristics of the detection system. That is, the reaction-rate must not be so fast that the detection system does not faithfully and accurately register the rate. On the other hand, the reaction-rate must not be so slow that it is indistinguishable from drift in the detection system.

In order to achieve consistent results, experimental conditions must be carefully controlled in reaction-rate measurements. Factors which influence the reaction-rate are: pH, ionic strength, and temperature. The effect of pH and temperature on the reaction-rate for the glucose-glucose oxidase reaction has been studied by Malmstadt and Pardue [11].

Because reaction-rate methods measure only a small portion of the entire reaction, lower signal-to-noise ratios are often obtained than in equilibrium-based methods. This small measurement interval necessitates a more sensitive detection system for reaction-rate methods than the equilibrium-based approaches in order to achieve comparable detection limits.

EXPERIMENTAL

Instrumentation

As seen in Figure 1, the pH-stat is composed of four modular components:

(1) the microliter titrant addition system,

(2) pH detection system,

(3) reaction vessel,

(4) a mini-computer with graphics display and disk storage.

In operation, the pH-meter continuously monitors the pH of the reaction solution. At a selected frequency the computer reads the output of the pH-meter, compares it with a stored reference potential, and determines whether a change in pH has occurred. If one has, the computer calculates the addition rate of titrant that is necessary to return the pH of the reaction solution to its command value. The titrant addition

rate is entered into the microliter titrant addition system and titrant is added to the reaction vessel at the specified rate. The average addition rate for several time intervals is directly proportional to the intial rate of reaction.

The following sections discuss the components of the pH-stat.

Microliter Titrant Addition System. Enabling the pH-stat to precisely control pH is the microliter titrant addition system (MTAS), shown in Figure 2. The MTAS can on command add increments of titrant having a volume of approximately 0.06 μL. The design of the titrant delivery system is based not on a conventional syringe mechanism but rather on the generation and selection of charged droplets by an electric field. The titrant delivery system is designed with no moving parts, no burets that require refilling, and a fast response time to a request for the addition of titrant. An extensive description of the operation and design of the MTAS has already been presented [6,12,13]. Only those features unique to the present device will be described here.

The MTAS operates on the ability to selectively separate submicroliter droplets from a flowing droplet stream. Those droplets separated from the droplet stream are directed into the reaction cell. The droplet stream is generated by a technique first described by Lord Rayleigh [14] and later refined by Schneider and Lindblad [15,16]. In this approach, an intense periodic perturbation of a liquid jet forced through a small orifice produces uniform-sized droplets. In the present apparatus, droplets are generated by a vibrating 27-gauge metal capillary. The capillary is mounted with epoxy cement perpendicularly to one end of a piezoelectric ceramic bimorph (PZT-5H, Clevite Corp., Bed-

ford, OH) through a 1/32-inch hole drilled into the bimorph. To drive the bimorph, an oscillator (Audio Oscillator, Model 202D, Hewlett-Packard Company, Palo Alto, CA) is used to generate a 1KHz, 100 volt P-P sine wave.

The separation of individual droplets from the droplet stream is based on the attraction of an electrically charged droplet to a charged surface of opposite polarity. Individual droplets are charged by the application of an electric field immediately prior to droplet formation. Under these conditions, charges of the same polarity in the liquid jet are repelled toward the grounded capillary. Upon droplet formation a net charge of opposite polarity as the charging electrode is retained. In this system, a charging electrode is placed immediately beyond (0.5 cm) the tip of the capillary. Charging of a droplets is accomplished by energizing this electrode with an electrical pulse of appropriate amplitude and width. By proper application of this pulse, individual droplets can be charged in a selective manner.

Separation of charged and uncharged droplets occurs as the droplet stream passes through an electric field. In this system, two planar electrodes located 2 cm apart and 0.5 cm below the charging electrode are held at a differential potential of 1000 volts by a high voltage power supply (244 High Voltage Power Supply, Keithly Instruments, Cleveland, OH).

Titrant is supplied to the MTAS via a 1.0 liter reservoir pressurized with air to 6 psig. Air pressure is controlled with a single-stage tank regulator (Model 02-2600, Air Products, Allentown, PA).

To evaluate the precision with which the titrant can be delivered to the reaction vessel, the variations in titrant rate were determined.

The droplet volumes are determined by instructing the MTAS to deliver titrant at its maximum rate for a specific time period. The titrant is collected in a weighing bottle after which it is immediately capped and weighed. The droplet volume can be calculated from the weight of collected titrant and the number of droplets added to the weighing bottle. With this technique, the average maximum titrant delivery rate is 0.056 mL/sec, and the average droplet size is 0.056 µL with a relative standard deviation of 0.06 percent. These results are obtained using a 27-guage capillary with a bimorph driving frequency of 1KHz.

Electrodes and Reaction Vessel. Changes in pH are measured potentiometrically with a combination Ag/AgCl ceramic junction electrode (No. 6023-03, 3.0mm diameter, Ingold Electrodes, Inc., Lexington, MA), and electrometer (model 602 Solid State Electrometer, Keithley Instruments, Cleveland, OH), whose analog output (0-1V) feeds the laboratory computer (PDP-11/34, Digital Equipment Corp., Maynard, MA). A sensitive electrometer is required because the total change in pH is less than 0.001 pH units over a typical observation period for a glucose concentration of 10 µg/mL.

During a determination and between analyses, the pH electrode was maintained in a solution of equal pH and ionic strength as the reaction solution in order to minimize any non-equilibrium drift [17]. The addition of enzyme to the reaction cell and the rinsing of electrodes after a determination are two procedural steps in which the pH and ionic strength of each solution must be carefully matched. Also, because reaction products can change the ionic strength of the reaction solution, an electrolyte (0.01M KCl) was added to mask any changes in ionic strength.

The 5.0 mL reaction cell is temperature-regulated at 30° C by water circulated from a thermostated bath through a jacket surrounding the vessel. Titrant and titrate are vigorously mixed using a 1.0 cm Teflon magnetic stirring bar driven by a flowing-water-driven stirrer. To minimize electrode response time, the titrant entry point was placed as near as possible to the pH electrode in the reaction vessel. The proximity of titrant and electrode creates a region of high transient titrant concentration, giving the pH electrode an initial "kick".

Computer Interface. The pH-stat is able to add titrant at computer-selectable rates, measured in terms of the number of droplets charged per second. The charging of droplets is accomplished by the computer interface shown in Figure 3. The interface consists of three main units:

(1) a computer-controlled frequency divider.

(2) pulse-delay and pulse-width adjustment circuitry.

(3) high-voltage transistor switch.

The rate at which droplets are to be charged and therefore deflected into the sample vessel is loaded by the computer onto its common instrument bus (designed in this laboratory). From this bus the rate is latched into the frequency divider of Figure 3 (74197 binary presettable counters/latches). The common instrument bus communicates with negative logic. Therefore, the rate which the frequency divider "sees" is the one's complement of the rate which the computer supplied. The bimorph driving signal, which is reduced to a TTL-compatible voltage with a voltage divider and shaped with a Schmitt trigger (7414 hex Schmitt-trigger inverters), clocks the frequency divider. When the frequency divider is enabled, the one's complement of the rate is latched into the counters which then count in a positive direction, toward zero. The

transition of the counters from one to zero causes a negative transition in the sign bit, which triggers the pulse-generating circuitry and also triggers monostable M3 that generates a 100 nanosecond pulse to reload the frequency divider.

The pulse-generating circuitry consists of two monostables (74123 dual retriggerable monostable multivibrators with clear), M1 and M2, respectively. When triggered by the frequency divider, M1 generates a positive pulse, termed the delay pulse. When triggered by the negative transition of the delay pulse, M2 generates a positive pulse, termed the charging pulse, which directly feeds the amplifier circuitry connected to the charging ring.

The width and temporal position of the charging pulse is crucial because it provides synchronization between droplet formation and droplet charging. Because the width and delay are empirically determined, an error checking circuit is provided in case the duration of either the pulse delay or width is too great. The error checking is accomplished with the use of two J-K flip-flops (74109 dual J-K positive-edge-triggered flip-flops with preset and clear) in conjunction with the sign bit of the frequency divider and the outputs of M1 and M2. The outputs of M1 and M2 are connected to the J-K inputs of flip-flops F1 and F2, respectively; the sign bit is connected to the clock input of both flip-flops. The outputs of M1 and M2 should be low (pulses ended) at the transition of the sign bit; if not, the pulse delay or pulse width is too great. The flip-flops detect this condition because the negative triggering of the sign bit latches the J-K inputs to the Q outputs of the flip-flops. Thus a high at the Q output of the flip-flop indicates an error.

The charging pulse [C] is amplified by the simple high-voltage amplifier circuit shown in Figure 4. The TTL charging pulse drives transistor T1 which fires the high-voltage transistor T2. The output of T2 feeds the charging ring of the droplet generator.

Computer Program

The computer program that controls the pH-stat is written entirely in FORTRAN IV and operates under the PDP-11 RSX-11M V3.1 real-time operating system. This operating system supports many utility programs for the performance of concurrent tasks. Two important utilities in the present study are:

(1) the resume-suspend constructs,

(2) event flags.

The resume-suspend constructs provide a means for tasks to gain partial control over task scheduling. A task can relinquish control of the processor by the *suspend command*. The processor, however, continues execution of other tasks. The suspended task cannot regain control of the processor until resumed by the *resume command*.

Event flags are a means to accomplish task synchronization. Synchronization is accomplished when a task suspends execution until an event flag is activated. When the event flag is activated, the task resumes execution.

Modularity in program design and efficient use of the CPU are achieved by subdividing the program into individual concurrent tasks. Communication between tasks is accomplished using a shared data region; synchronization, when necessary, is accomplished with the use of event flags and suspend-resume constructs. All tasks are linked to a shara-

ble, memory-resident FORTRAN library thereby eliminating any unnecessary duplication of code.

There are eight concurrent tasks involved in controlling the pH-stat:

(1) the monitor,
(2) the data display,
(3) the parameter change,
(4) the data acquisition,
(5) the decision maker,
(6) the data store,
(7) the calibrator,
(8) and the results.

The function of each of these tasks will be discussed separately.

The monitor interfaces the operator to the pH-stat. It interprets the directives given by the operator and, depending upon the directive, either sets an event flag, resumes a task, or aborts a task.

The display directive enables the operator to view parameters at any time throughout an experiment. The display task is concurrent with data acquisition, permitting the operator to observe dynamic changes of the experimental parameters. Pertinent displayed parameters are: elapsed experimental time, average titrant rate, average change in titrant rate, sampling frequency and, if desired, all data points collected thus far.

The change directive enables the operator to alter program parameters from their default values. The change task is also concurrent with data acquisition, enabling parameter alteration as the determination progresses. Affectable parameters are:

(1) sample type and concentration. The sample can be either a standard or unknown. If the sample is a standard, its concentration must be declared.

(2) minimum experiment duration. The minimum duration of the determination after which the pH-stat can terminate the run if the data are satisfactory.

(3) maximum experiment duration. The maximum duration of a determination when the pH-stat has not automatically terminated the run.

(4) initial sampling frequency. The frequency at which the acquisition task records data. This frequency is dynamically modified to meet environmental conditions.

(5) data base identification. All data are stored in a data base bearing this name.

The data acquisition directive initiates an analysis by the simultaneous activation of the acquisition task and the decision maker. The combined efforts of these two tasks accomplish the data acquisition, the determination of starting and ending times, and the sampling frequency.

A flow diagram of the acquisition task is shown in Figure 5. Upon inception, the task immediately suspends itself until resumed by the monitor after receipt of the data acquisition directive. Once the task is resumed by the monitor, it records and saves the initial command pH. The following cycle is then initiated:

> The sampling frequency is loaded into the computer and task execution is suspended until the clock interrupts. With the reactivation of the task by the clock interrupt, the pH and time are recorded, a new titrant rate is calculated based on the difference between the current pH and its command value.

This cycle continues until instructed to stop by either the decision maker upon the determination of the end of analysis, or by the monitor upon an operator directive.

The algorithm for calculating a new titrant rate is critical in the new pH stat. This algorithm does not calculate an absolute titrant rate from an observed changed in pH, but rather determines the necessary incremental change in titrant from the current titrant rate. The algorithm considers two factors in the calculation of the change in titrant rate:

(1) the difference between the current pH and the control pH,

(2) the difference between the pH at the last sampling and the current pH.

The first factor insures that the reaction will be held at the control pH. The second factor determines the direction and potential in which the pH is currently changing. Equation (8) represents this algorithm mathematically.

$$Rate = Average°Rate - (f1*(pHcontrol - pH) + f2*(pHlast - pH))/(2*f3) \quad (8)$$

where Rate is the new titrant rate, Average°Rate the average rate, pHcontrol the control pH, pH the current pH, pHlast the pH at the last sampling, f1 and f2 weighting factors, and f3 is the conversion from change in pH to change in titrant rate.

The data collected by the acquisition task are viewed by the decision maker to determine the following conditions:

(1) The start of the analysis. The analysis is not considered to begin until after a significant rate has been noted. This delay encompasses any enzymatic induction period.

(2) The end of the analysis. The operator sets a maximum analysis duration which the pH-stat does not exceed. Even if the data obtained prior to this duration limit have an average change in rate equal to zero (within selected tolerence limits) and has a low variance, the analysis is ended.

(3) The sample frequency. Fast reactions have the potential of "getting out of hand" and are sampled at a higher frequency than slower reactions. The sampling frequency varies in proportion to the reaction-rate.

A flow diagram of the decision maker is shown in Figure 6. When activated, the decision maker pauses as the acquisition task collects the first few data points. This pause ensures that any unstable recordings caused by the initial mixing of enzyme and substrate solutions are ignored, after which the decision maker determines if the current titrant rate is significant by comparing its magnitude to a minimum rate value; if the rate is greater, the beginning of the analysis is declared. Any changes to be made in sampling frequency are determined by comparing the change in titrant rate to zero; if the rate is changing (within tolerence limits), the sampling frequency is increased. The end of the determination is triggered by comparing the variation in titrant rate with a selected value; if they are equal and the minimum analysis duration has been exceeded, the analysis is ended. The analysis is also ended if the selected maximum analysis duration has been exceeded.

After an analysis, the operator can elect to permanently retain the data by issuing a store directive. This directive enters all parameters and data including the current time and date into the data base.

The calibration directive determines a factor used by the acquisition task to convert the change in pH to the change in titrant rate. This factor is obtained by recording the change in pH when a constant rate of titrant is introduced into a blank reaction cell.

The results directive presents in tabular form both a summary of the standard substrate analyses used to obtain a working curve and the unknown sample data.

It should be noted that there are several advantages to the multi-tasking approach for the design of real-time systems. Firstly, because the acquisition task and the decision maker operate concurrently, the computation time for the decision maker need not be limited to the sampling interval of the acquistion task, thus permitting the use of complex algorithms in a real-time environment. Secondly, other computer-controlled experiments and program development can be performed concurrently with a pH-stat determination. Thirdly, tasks such as the display, change, and store are loaded into memory only when they are requested, after which they are removed. This dynamic loading of tasks permits the overall program size to be very large while necessitating only a small portion of memory-resident code.

Reagents

To ensure that all solutions have nearly the same ionic strength and pH, they are made up in a 0.01N KCl stock solution adjusted to pH 6.5 with NaOH. Because Chloride ion (0.2M) competitively inhibits the studied reaction [18,19] the concentration of KCl in the stock solution is kept low.

Glucose standards are prepared whose concentration range encompass the unknown concentration. Typical working curve ranges are 10-250 and 250-1000 μg/mL.

A glucose oxidase solution is prepared by diluting 0.2 grams of glucose oxidase (E.C. No 1.1.3.4, No 6-6125, Sigma Chemical Company) to 250.0 ml and storing under refrigeration.

Standardized human blood serum (MONI-TROL I, Lot Number LTD-165, DADE Reagents Inc., Miami, Fla 33152) is deproteinized by the method described by Malmstadt and Pardue [20]. Because the blood sera are

contained in 5 mL vials, the deproteinization procedure is slightly modified from that used for the 0.02 mL aliquots used in the previous work [20]. For deproteinization, a 5 mL aliquot of serum is mixed with 20 mL of a solution of 1.8% Barium Hydroxide and 2.0% Zinc Sulfate Septihydrate. This mixture is filtered, the filtrate diluted to a total volume of 50 mL (1:10 dilution), and adjusted to pH 6.5 with dilute HCl. For analysis, a 2.0 mL aliquot of the deproteinized serum is used.

A solution of 0.0005N NaOH serves as a titrant.

Procedure. A 2.0 mL glucose sample is introduced into the reaction vessel immediately followed by a 1.0 mL aliquot of the glucose oxidase reagent. The computer acquisition routine is activated and the analysis proceeds as outlined in the previous section. After the analysis, the reaction vessel is thoroughly rinsed with stock solution.

RESULTS AND DISCUSSION

Determination of Attainable pH Control

The required sensitivity of the pH detection system can be appreciated from the observed change in pH over a 60 second observation period for various glucose concentrations (Table 1). For example, a glucose concentration of 20 μg/mL produces a total change in pH of only 0.008 pH units or, equivalently, a change in electrode potential of 0.3 mV. The precision with which the pH-stat can maintain a constant pH was determined by measuring the noise at the output of the pH meter by means of a strip-chart recorder. With this technique, the noise is determined to be approximately 0.0008 pH units, or a change in electrode potential of 0.05 mV.

Glucose Standards. To evaluate the performance of the pH-stat system, a series of standard glucose samples was examined. The concentration range of standards chosen was between 10-1000 µg/mL. Typically, six measurements were recorded for each standard concentration in order to evaluate the system's precision.

Shown in Figure 7 is the resulting linear relationship between reaction-rate and glucose concentration over the range 10-1000 µg/mL. The relative standard deviations at glucose concentrations of 10, 100, and 1000 µg/mL were 20%, 5%, and 2%, respectively.

Determination of Glucose in Human Blood Serum. To validate the performance of the pH-stat, the determination of glucose in a "real" sample (human blood serum) was undertaken.

Glucose standards were prepared at concentrations of 50, 100 and 150 µg/mL and a linear working curve (cf. Fig. 7) was obtained based on the measured reaction-rate of each standard. From this working curve and the measurement of the sample reaction rate, the serum glucose concentration was directly printed out in terms of milligrams per deciliter.

The average measured concentration for six serum samples was 90 mg/dL with a relative standard deviation of 7%. This measured glucose concentration is in agreement with the value specified by the manufacturer (92 ± 3.6 mg/dL obtained by a Beckman Glucose Analyzer).

Glucose Determinations by Enzymatic Induction Times

The glucose-glucose oxidase reaction does not immediately follow first-order kinetics. Only after an initial delay period, termed the induction time, does the reaction proceed by first-order kinetics. The induction time

can limit the utility of this reaction for time-critical analyses. However, the induction period is known to be inversely proportional to substrate concentration [21]. Therefore, this time-to-concentration relationship provides an alternative method of analysis.

To evaluate this alternative method, a series of standard glucose concentrations between 20-1000 µg/mL was examined. All glucose standards were tested at two enzyme concentrations in order to determine the effect of enzyme activity on induction time. Ordinarily, six trials were performed at each standard concentration to verify precision. The induction time was determined from the first continuous deviation from a constant pH.

Figures 8 and 9 show the relationship between induction time and glucose concentration for enzyme concentrations of 0.28 g/L and 0.8 g/L, respectively. The relative standard deviation for the induction time of each standard concentration was between 5-10%. The relative difference between the observed average induction time and the predicted value (by least squares fit) was 1-3%.

Understandably, induction times were found to be dependent on enzyme concentration (cf. Figures 8 and 9). An increase in enzyme concentration from 0.28 g/L to 0.8 g/L produced a corresponding decrease in induction time by a factor of approximately three. This dependency provides a convenient method for adjusting induction time so determinations can be performed in a reasonable time period.

From these results, it is evident that induction times can be used for the precise determination of glucose concentrations. There are two apparent advantages to this method:

(1) instrumentation is simple and inexpensive: the only requirements are a sensitive pH-meter and a timer,

(2) pH effects are eliminated since the pH remains constant during the induction time.

A pH-stat is obviously not needed for this method because the measured induction time occurs prior to reaction commencement.

Glucose Determinations by Fixed-Time Reaction-Rate Methods. Because pH affects reaction-rate, fixed-time techniques have not been widely utilized in potentiometric reaction-rate analyses [22]. However, if the measured change in pH is kept small (0.001-0.1 pH units), the effects of pH will not significantly affect the reaction-rate measurement. To evaluate the practical utility of fixed-time techniques in such a situation, the reaction-rate of several glucose standards was measured by monitoring small changes in pH. In this experiment, the change in pH was recorded over the initial 30 seconds of the reaction.

Figure 10 shows the relationship between fixed-time-measured reaction rate and glucose concentration over the range 20-1000 µg/L. The resulting straight line, with a slope of $4.3 \pm 0.1 \times 10^{-3}$ pH units/second, is applicable over a broad range of concentrations. Because the effects of pH are specific for each enzymatic reaction, the linearity of reaction-rate with substrate concentration must be determined for each reaction.

CONCLUSION

The directly computer-controlled pH-stat has been shown to accurately determine glucose concentrations in human blood serum. The microliter titrant addition system (MTAS) was determined not to be the precision-limiting component of the pH-stat. The MTAS has no moving parts, except for a vibrating bimorph, and is constructed from simple, inexpensive components [23].

The programs for controlling the pH-stat were structured as autonomous concurrent tasks, permitting the direct translation of simultaneous, real-time activities into structured tasks. Operator directives were accepted at any time throughout an experiment, permitting the real-time display of data and experimental parameters.

Under certain conditions two alternative methods for the analysis of glucose can be used. The fixed-time reaction-rate method of analysis can yield quantitative results when the measured change in pH is small. However, because the effects of pH are specific for each enzymatic reaction, the fixed-time technique must be evaluated for each reaction. The determination of glucose concentration by enzymatic induction time can also yield quantitative results. Again, however, induction times are specific for each reaction and this technique must therefore be evaluated for each reaction.

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Table 1

Effect of Glucose Concentration on Observed Change in pH Over 60-second Period

Glucose Concentration (ug/mL)	Average Change in pH over a 60 second Observation Period
1000	0.260
750	0.195
500	0.148
250	0.058
150	0.039
100	0.030
50	0.018
20	0.008

Figure Legends

Figure 1. Functional Organization of a pH-Stat. The pH-stat consists of four modular components: (1) a minicomputer with graphic display and disk storage, (2) the microliter titrant addition system (MTAS), (3) a sensitive pH-meter, and (4) a reaction cell.

Figure 2. Schematic Diagram of the Microliter Titrant Addition System.

Figure 3. Schematic Logic Circuit Diagram of the MTAS Computer Interface. The MTAS computer interface receives from the computer the titrant rate expressed as a binary number and produces a pulse train at an appropriate frequency and duty cycle. See text for discussion.

Figure 4. Schematic Circuit Diagram of the High-Voltage Switch. The high-voltage switch receives a TTL-compatible pulse as input and produces a high voltage pulse.

Figure 5. Flow Diagram of the Acquisition Task. The acquisition task provides minimum control for the pH-stat. The objectives of the acquisition task are to record the pH at a selected frequency and determine the corresponding change in titrant rate.

Figure 6. Flow Diagram of the Decision Maker. The decision maker performs complex decisions in the control of the pH-stat. The objectives of the decision maker are to determine the start and end of the experiment and the sampling frequency.

Figure 7. Working curve obtained with New pH-stat;

Intercept = -0.02 ± 0.04

Slope = $5.4 \pm 0.1 \times 10^{-3}$

$S_{y \cdot x} = 0.087$

$S_{y \cdot x}$ = Standard error of estimate.

Figure 8. Effect of Glucose Concentration on Observed Enzymatic Induction Time;

[Enzyme] = 0.28 g/L.

Intercept = $-5.1 \pm 7 \times 10^{-4}$

Slope = $4.4 \pm 0.1 \times 10^{-5}$

$S_{y \cdot x} = 1.0 \times 10^{-3}$

$S_{y \cdot x}$ = Standard error of estimate.

Figure 9. Effect of Glucose Concentration on Enzymatic Induction Time;

[Enzyme] = 0.8 g/L.

Intercept = $-0.9 \pm 4 \times 10^{-4}$

Slope = 1.5 0.1 $\times 10^{-4}$

$S_{y \cdot x} = 4.8 \times 10^{-4}$

$S_{y \cdot x}$ = Standard error of estimate.

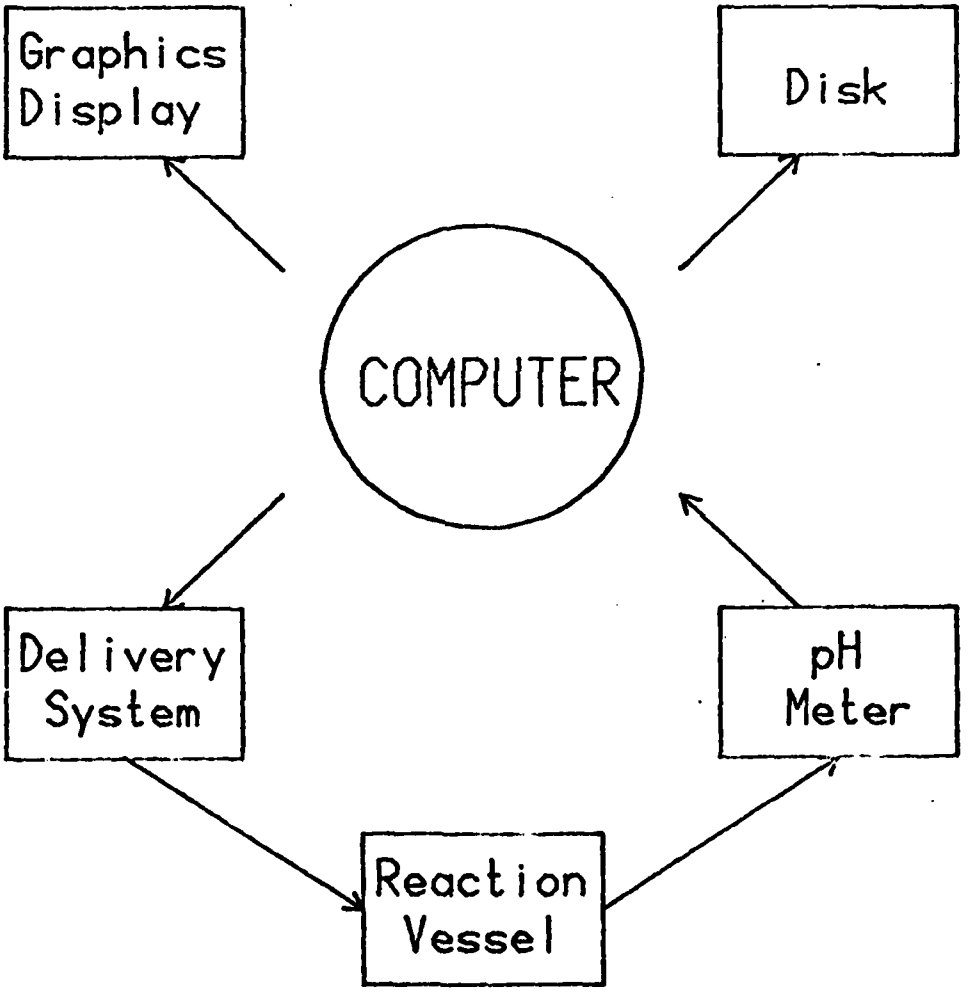
Figure 10. Observed Reaction-Rate vs Glucose Concentration using Fixed-Time Measurements.

Intercept = 0.05 ± 0.07

Slope = $4.3 \pm 0.1 \times 10^{-3}$

$S_{y \cdot x} = 0.13$

$S_{y \cdot x}$ = Standard Error of Estimate.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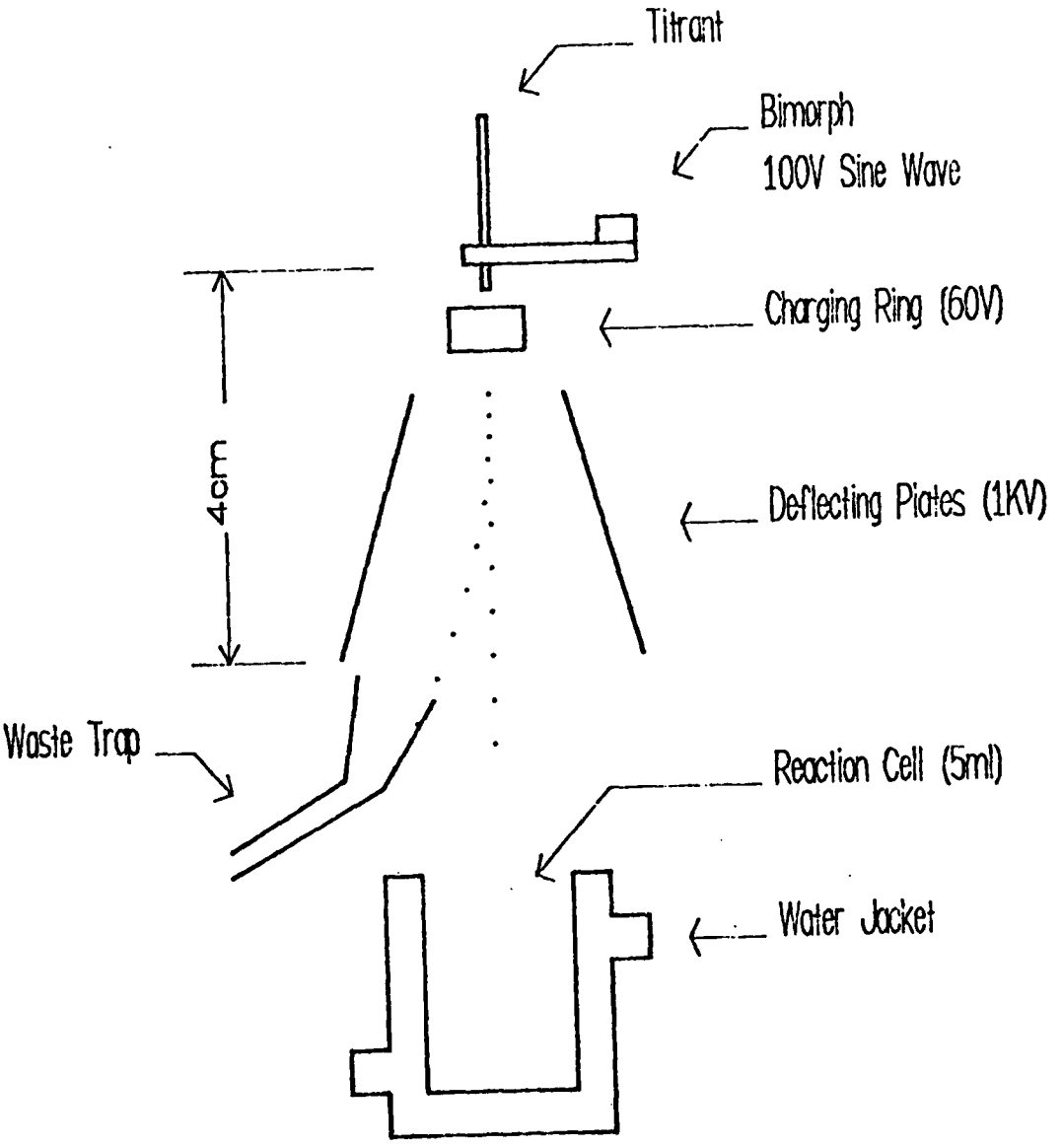
pH Meter
Reaction Vessel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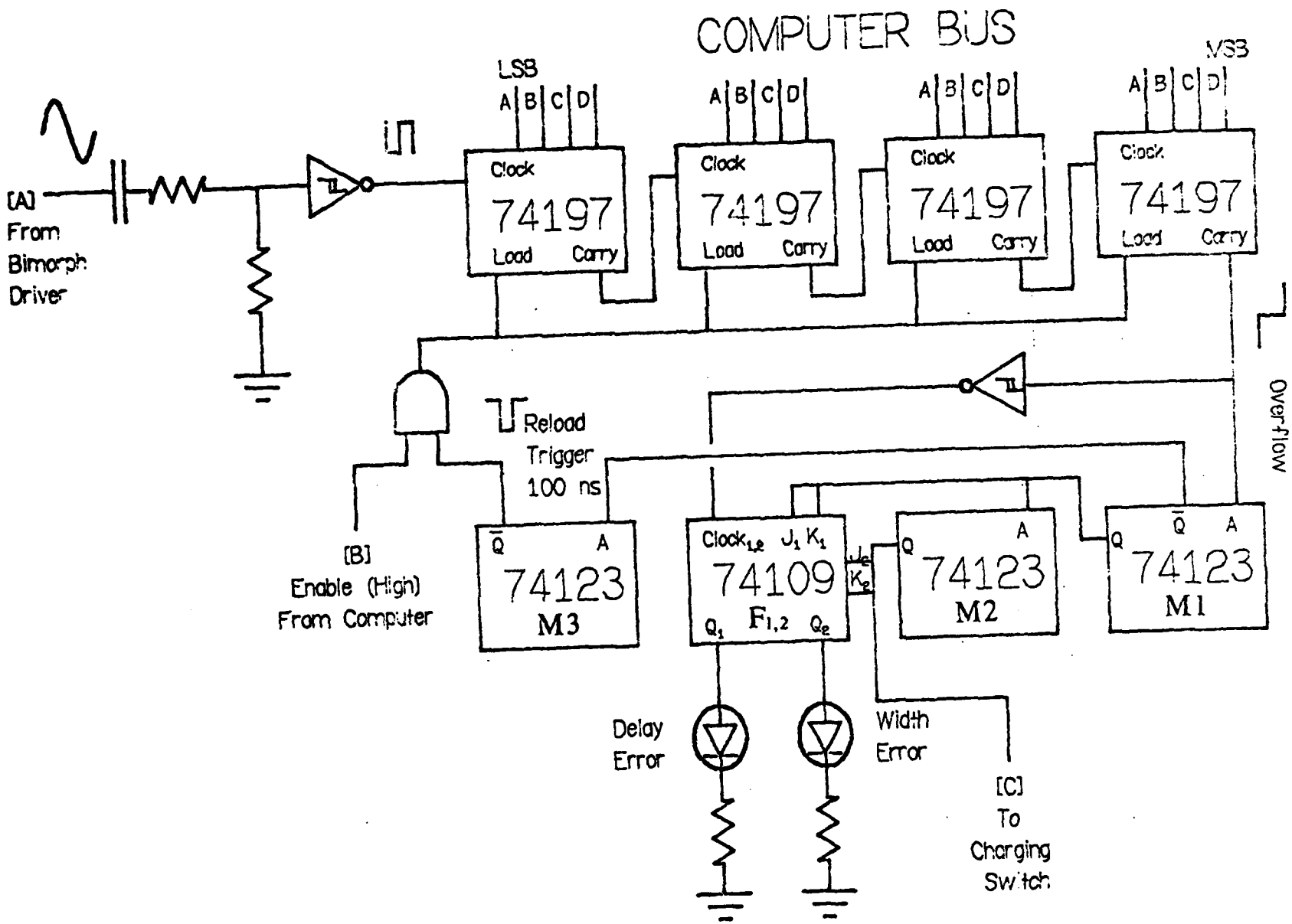
Waste Trap
Reaction Cell (5ml)
Water Jacket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MSB
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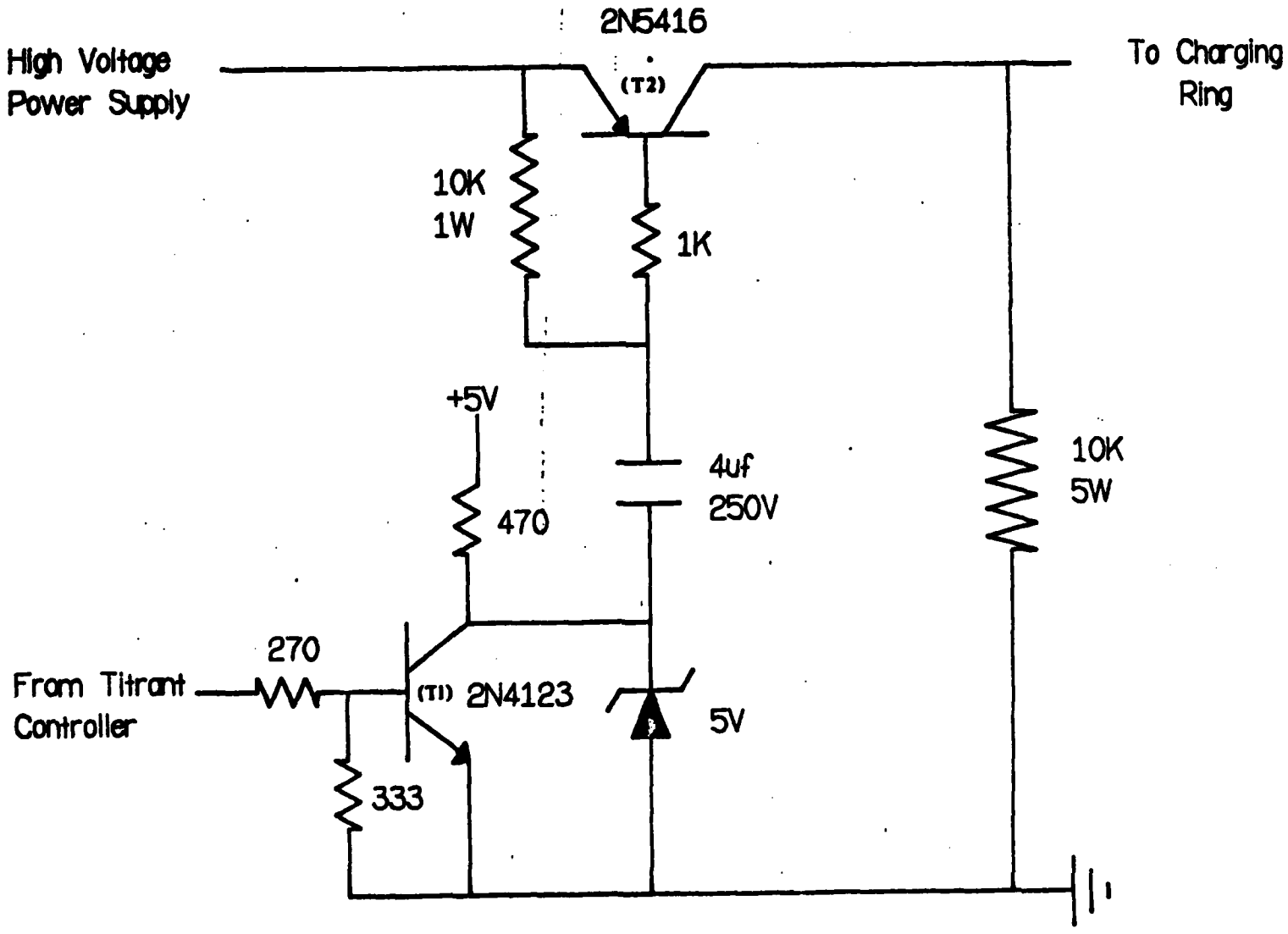
Load
Carry
[A]
From
Bimorph
Driver
Reload
Trigger
100 ns
Over-flow
[B]
Enable (High)
From Computer
Q
A
74123
M3
Clock1,2 J1 K1
74109
Q1 F1,2 Q2
J2
K2
74123
M2
74123
M1
Delay
Error
Width
Error
[C]
To
Charging
Switch


2N5416
(T2)
High Voltage
Power Supply
To Charging
Ring
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1W
1K
+5V
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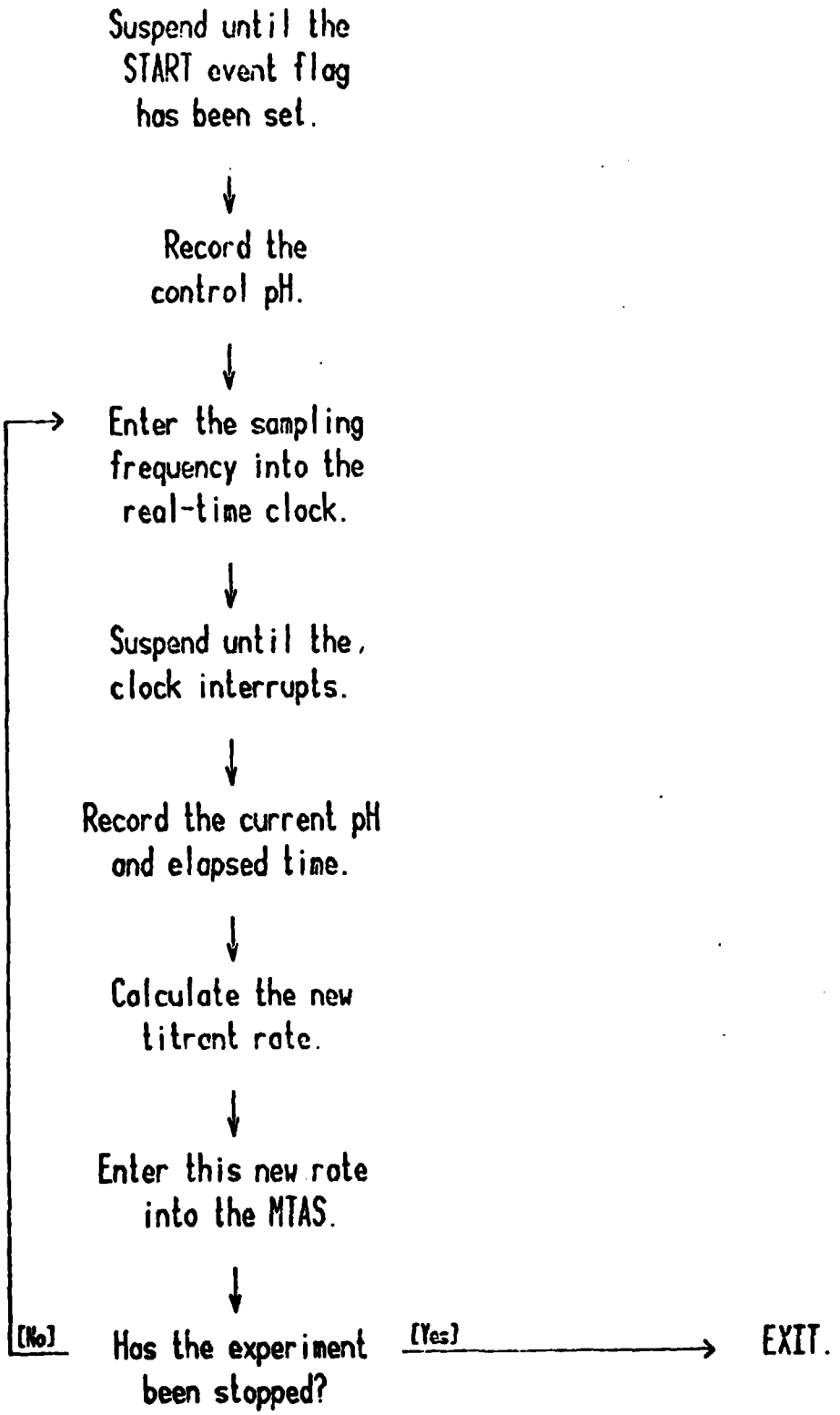
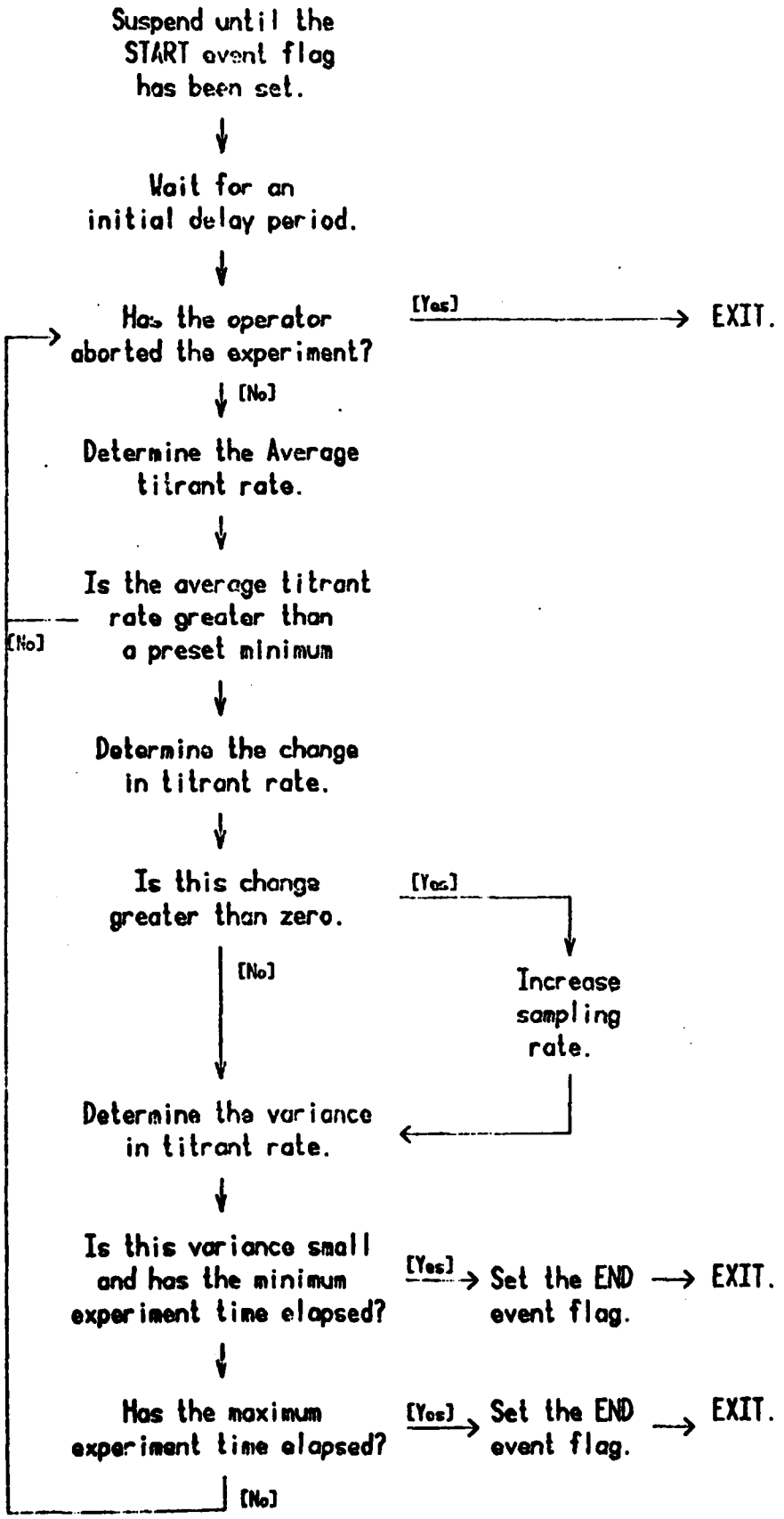
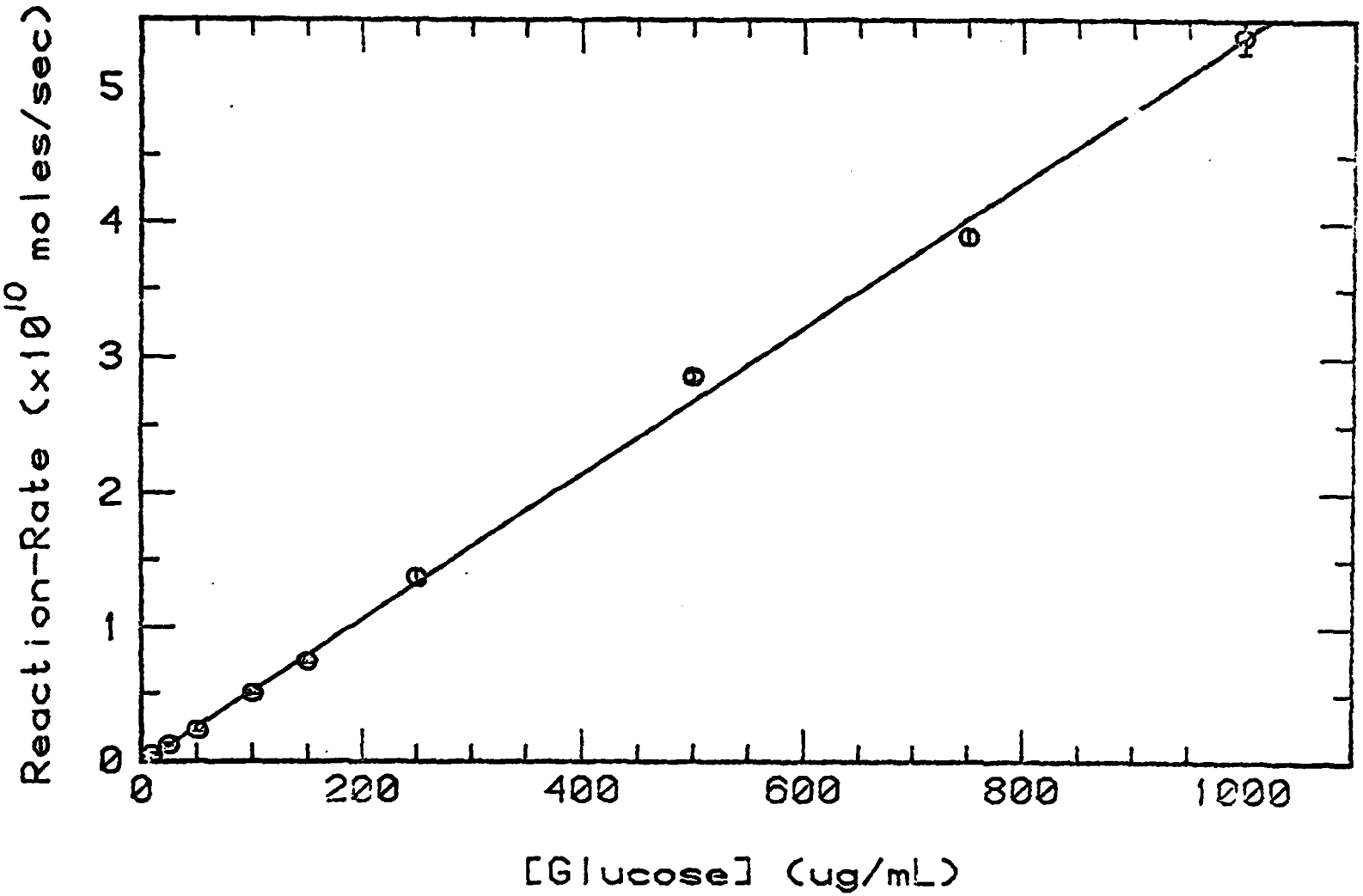
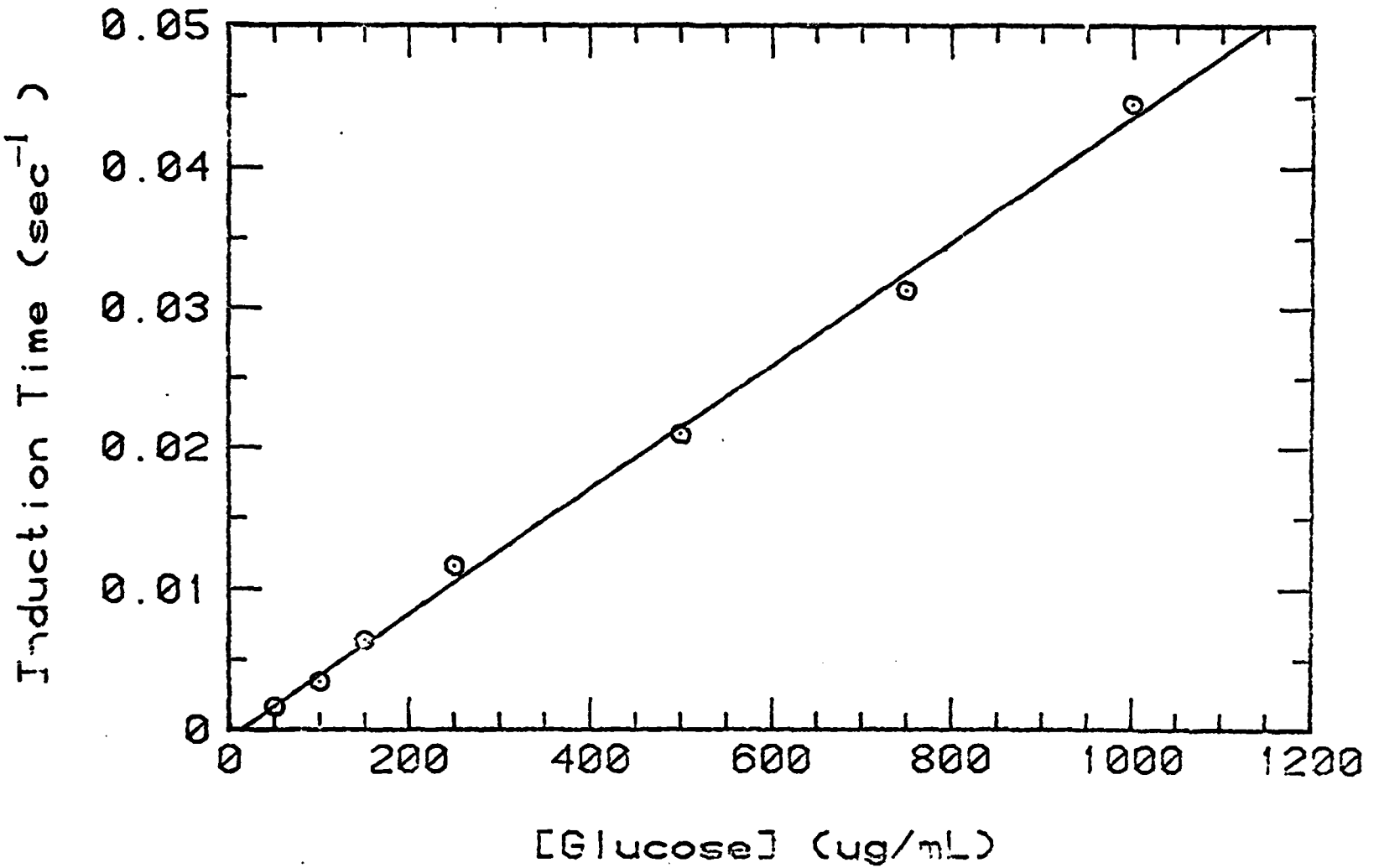
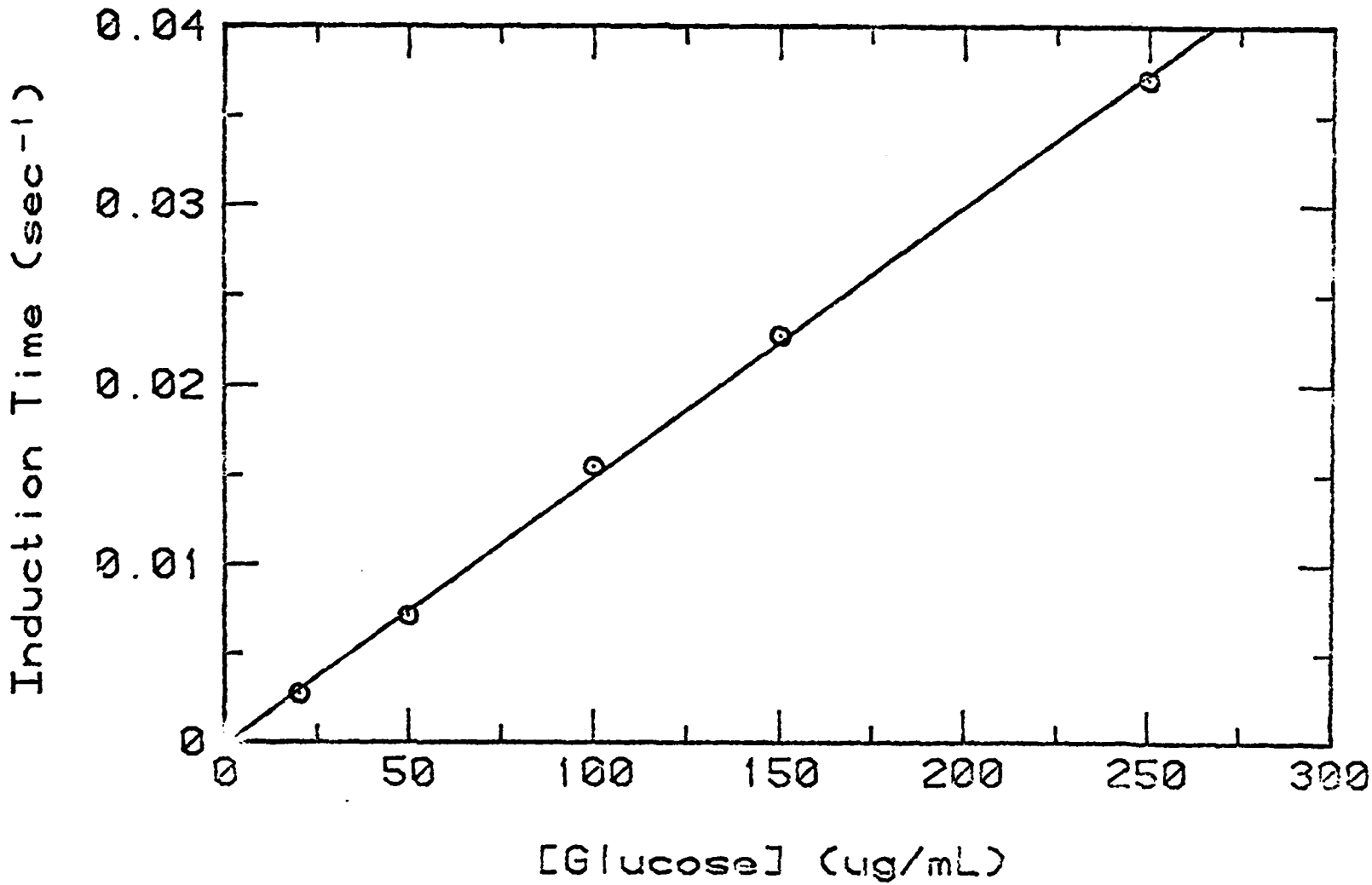
270
From Titrant
Controller
(T1) 2N4123
5V
333

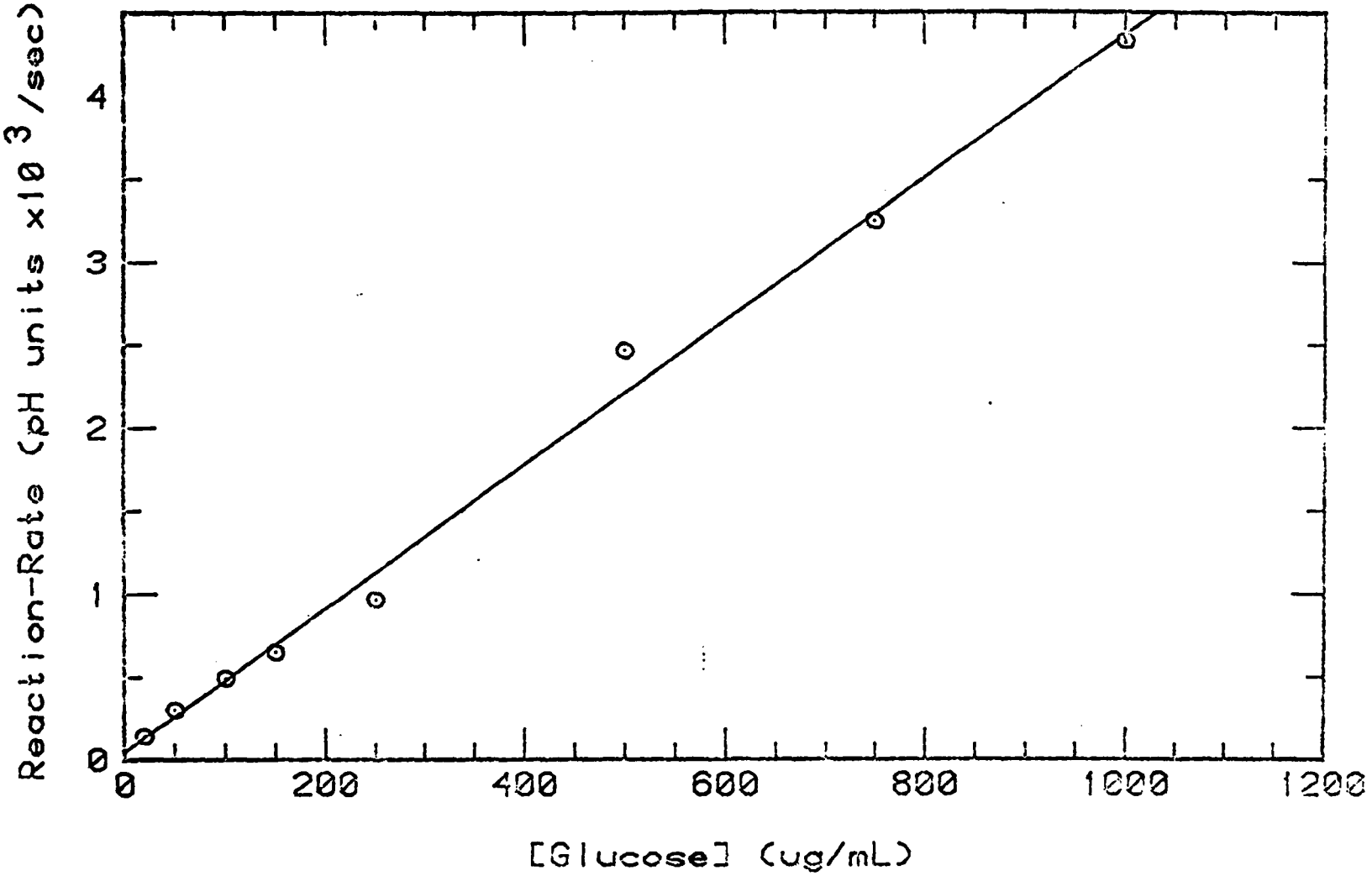

Suspend until the START event flag has been set.
Record the control pH.
Enter the sampling frequency into the real-time clock.
Suspend until the clock interrupts.
Record the current pH and elapsed time.
Calculate the new titrant rate.
Enter this new rate into the MTAS.
Has the experiment been stopped?
[No]
[Yes]
EXIT.


Suspend until the START event flag has been set.
Wait for an initial delay period.
Has the operator aborted the experiment?
[Yes]
EXIT.
[No]
Determine the Average titrant rate.
Is the average titrant rate greater than a preset minimum
[No]
Determine the change in titrant rate.
Is this change greater than zero.
[Yes]
Increase sampling rate.
[No]
Determine the variance in titrant rate.
Is this variance small and has the minimum experiment time elapsed?
[Yes]
Set the END event flag.
EXIT.
Has the maximum experiment time elapsed?
[Yes]
Set the END event flag.
EXIT.
[No]


Reaction-Rate (x10^10 moles/sec)
0
1
2
3
4
5
0
200
400
600
800
1000
[Glucose] (ug/mL)


0.05
0.04
0.03
0.02
0.01
0
Induction Time (sec^{-1})
0
200
400
600
800
1000
1200
[Glucose] (ug/mL)


0.04
0.03
0.02
0.01
0
Induction Time (sec-1)
0
50
100
150
200
250
300
[Glucose] (ug/mL)


Reaction-Rate (pH units x10 3 /sec)
0
1
2
3
4
0
200
400
600
800
1000
1200
[Glucose] (ug/mL)

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