

KINETIC STUDIES AND DEVELOPMENTS OF QUICK ASSAY FOR GLUCOSE ISOMERASE

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Abstract

Due to significant commercial importance, the production of invert sugar and fructose, using glucose isomerase from a suitable microorganism is currently studied. A relative simple extension of Michaelis-Menten kinetics with incorporation of enzyme inactivation is formulated. Determinations of reaction rate and its dependence on the stable variables, such as pH of solution, buffer solution, salt concentration, glucose quantity and isomerase quantity, are performed in this research.

I. INTRODUCTION

Fructose, which can be metabolized by diabetics without the aid of insulin, is used in dietetic foods. It is also used in the pharmaceutical industry. By now, extensive studies on making invert sugar and fructose from glucose have been made by Japanese industry. Many types⁽¹⁻⁸⁾ of the enzyme isolate from various microorganisms have been reported since the initial discovery by Marshall and Kooi⁽¹⁾ 1957. Substrate specificity of some of these enzyme for conversion to corresponding ketoses was restricted to D-glucose and D-xylose^(2, 9) and D-glucose, D-xylose and D-ribose⁽⁷⁻⁸⁾. Metal ions, such as magnesium ion^(1, 2, 9), cobalt ion^(7, 9), and manganese ion⁽⁸⁾, may be required for the enzyme activity to convert D-glucose to D-fructose. The enzyme is stable against heat up to 80°C particularly in the presence of magnesium or cobalt ions⁽⁹⁻¹⁰⁾. The pH dependency of enzyme activity varies with source i. e. the pH values at maximum activities are reported between 7 to 11⁽¹¹⁾ and 9 to 10⁽¹⁰⁾. An equilibrium-temperature relationship was reported of D-glucose to D-fructose employing the isomerizing enzyme for the temperature range of 25 to 70°C^(11,12).

Chemical reactor engineering principles have recently been used to investigate the industrial applications of enzyme technology. Most enzyme catalyzed reactions do not take place in a single step but proceed through a sequence of steps involving reactive substrate-enzyme complex. The basic mechanism proposed for enzyme reactions, known as the Michaelis-Menten mechanism, is useful for deriving a reaction rate expression involving only the concentrations of observable species, the concentrations of the complexes are eliminated by making the steady state approximation in which the net rates of formation of these complexes are set to zero.

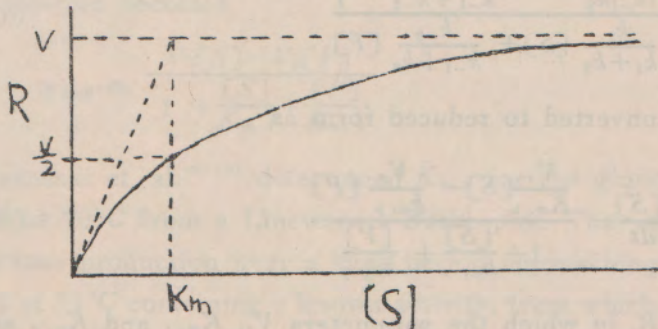
The simplest Michaelis-Menten mechanism can be expressed as



where enzyme is denoted by E , substrate by S , enzyme-substrate complex by ES , and the product by P . at high substrate concentration the enzyme sites are saturated, step 2 is rate determining, and the reaction is zero order in $[S]$; at low substrate concentration step 1 is rate determining, and the reaction is first order in $[S]$. It should be noted that enzymatic kinetics for high concentrations of substrate and product have not received much attention most probably due to their complexity. Single intermediate for steady state enzyme kinetic was formulated by Laidler⁽¹³⁾ in 1958. The solution of equation(1) for the steady state rate yields

$$R = -\frac{d[S]}{dt} = \frac{d[P]}{dt} = \frac{K_2[E]_T[S]}{K_m + [S]} = \frac{V[S]}{K_m + [S]} \quad (2)$$

where K_m , which gives the substrate concentration at which R has one-half constant k_2 is called the turnover number, and $[E]_T$ is total enzyme concentration. A typical characteristic of saturation in steady state enzyme reaction rate is illustrated in the following figure

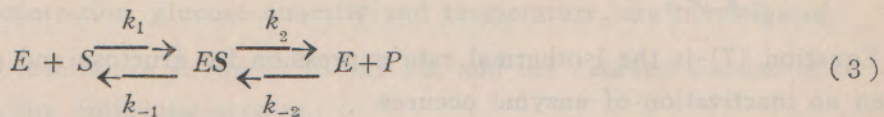


Sketch either graphs of R^{-1} versus $[S]^{-1}$, known as a line-weaver-Burke plot, or graphs of $\frac{[S]}{R}$ versus $[S]$ could be used to find V and K_m .

Based on theory alone, it is not yet possible to calculate the rate accurately enough to design a chemical reactor. Thus, successful design of a chemical reactor requires accurate knowledge of the experimental reaction rate. With incorporation of enzyme inactivation, our kinetic model is a simple extension of the Michaelis-Menten kinetics.

II. SIMPLE EXTENSION OF THE MICHAELIS-MENTEN KINETICS

The simple Michaelis-Menten mechanism does not suffice since the reaction is reversible with an equilibrium constant near unity. The kinetic model for this case gives



Use the steady state approximation on the enzyme-substrate complex, we have

$$[ES] = \frac{k_1[E][S] + k_{-2}[E][P]}{k_{-1} + k_2} \quad (4)$$

Applying a balance on the total enzyme concentration $[E]_T = [E] + [ES]$, we obtain

$$[E] = \frac{[E]_T}{1 + \frac{k_1[S] + k_{-2}[P]}{k_{-1} + k_2}} \quad (5)$$

The rate law for P is

$$\frac{d[P]}{dt} = k_2[ES] - k_{-2}[E][P]$$

Then

$$\frac{d[P]}{dt} = \frac{[E]_T \left\{ \frac{k_2 k_1}{k_{-1} k_2} [S] - \frac{k_{-1} k_{-2}}{k_{-1} + k_{-2}} [P] \right\}}{1 + \frac{k_1}{k_1 + k_2} [S] + \frac{k_{-2}}{k_{-1} + k_2} [P]} \quad (6)$$

Equation (6) can be converted to reduced form as

$$R = \frac{d[P]}{dt} = -\frac{d[S]}{dt} = \frac{V_s}{K_{m,s}} [S] - \frac{V_p}{K_{m,p}} [P] \quad (7)$$

$$1 + \frac{[S]}{K_{m,s}} + \frac{[P]}{K_{m,p}}$$

for the reaction rate, R , in which the parameters V_s , $K_{m,s}$ and $K_{m,p}$ are given by

$$V_s = k_2[E]_T \quad (8)$$

$$K_{m,s} = \frac{k_{-1} + k_2}{k_1} \quad (9)$$

$$K_{m,p} = \frac{k_{-1} + k_2}{k_{-2}} \quad (10)$$

and the one missing parameter V_p may be obtained by a Haldane relationship as

$$V_p = -\frac{V_s K_{m,p}}{K_{eq} \cdot K_{m,s}} = k_{-1}[E]_T$$

The equilibrium constant K_{eq} is given by (11)

$$K_{eq} = \frac{k_1 k_2}{k_{-1} k_{-2}} \quad (12)$$

Equation (7) is the isothermal rate expression for fructose and glucose when no inactivation of enzyme occurs.

Incorporation of enzyme inactivation, first order inactivation⁽¹⁴⁻¹⁵⁾ is assumed for which

$$[E]_T = [E]_0 e^{-\alpha t} \quad (13)$$

where $[E]_T$ is the total enzyme concentration at any time and is a steady decreasing quantity due to inactivation. α is the inactivation parameter. If we let

$$V_s' = [E]_0 k_2 \quad (14)$$

$$V_p' = [E]_0 k_{-1} \quad (15)$$

and

$$V_s = \frac{[E]_0 k_2}{K_{m,s}} \quad (16)$$

$$V_p = \frac{[E]_0 k_{-1}}{K_{m,p}} \quad (17)$$

Rate equation becomes

$$R = e^{-\alpha t} \frac{V_s[S] - V_p[P]}{1 + \frac{[S]}{K_{m,s}} + \frac{[P]}{K_{m,p}}} \quad (18)$$

Takasaki et, al.^(9, 16) determined $K_{m,s}$ for the glucose conversion to be 0.16 M at 70°C from a Lineweaver-Burke plot. They also presented a plot of fructose production over a time period approaching equilibrium for cell extract at 70°C containing a known activity, from which the remaining parameters V_s and $K_{m,p}$ can be estimated. We have calculated these to be

$$K_{m,p} = 0.081 \text{ M}$$

and

$$\frac{V_s}{[E]_r} = k_2 = 0.21 \text{ mg fructose}/(\text{min} \cdot \text{mg enzyme}).$$

III. QUICK ASSAY FOR GLUCOSE ISOMERASE

A incubation recipe is composed of 0.5ml of 1M acetate (pH 6.5), 0.02ml of salts (0.5M $MgCl_2$ and 0.05M $CoCl_2$) sample, and filled with 0.2M fructose to make the total volume to 1 ml. The glucose isomerase is incubated with fructose for 10 minutes. The glucose formed is analyzed by the quick procedures (as shown in Appendix) with glucostate, where non-enzymatic glucose generation, calibration with enzyme, kinetic constants, and effects of buffer, salt concentration, glucose quantity and temperature, are investigated.

The results are shown in Figures 1-8, and the detailed discussion is given in the following section.

IV. RESULTS AND DISCUSSION

Although the Michaelis-Menten type of reaction has been treated by Chu and Hougen⁽¹⁷⁾, the applicability of their results are limited, because of lack of consideration of the general modulus. A relative simple extension of Michaelis-Menten kinetics with incorporation of enzyme inactivation is formulated, and the general rate equation is given by Equation (18).

The developments of quick assay for glucose isomerase are shown in Figures 1-8. Figure 1 shows that the amount of non-enzymatic glucose generated during the 10-minutes incubation of a blank. This is important at pH 8, and at pH 9, it swamps the determination. Clearly, it is worth-while to stay at pH 7 or below, if possible. Figure 2 shows four possibilities about the effect of buffer. Phosphate buffer precipitates the cobalt and magnesium and gives unreproducible results. Phosphate without buffer is not very active and some other choice is necessary. Ammonium chloride, used by Smiley and Strandberg, shows the largest activity, but has no buffering capacity in that range. Acetate buffer appears to be the best bet; pH 6.5 was chosen instead of 7 to give more buffering capacity. Figure 3 shows the results of the effect of salt concentration.

Several runs were made using the recommended ratio of cobalt to magnesium, but varying the total amount. The concentrations recommended to us appear to be about the best, just reaching the plateau.

About the calibration with enzyme, several runs were made with varying amounts of isomerase. Each of the rates of change of absorbance was converted to glucose by means of Figures 4 and 5, and the units of activity was calculated from that using the definition of international unit, one micromole substrate converted per minute. This corresponds to 0.18 mg glucose per minute. The result is shown in Figure 6. It is nicely linear up to 0.2 units isomerase, but deviates above that for unknown reasons. Figures 5 and 6 also show the rate of change of absorbance as a function of the quantity of glucose present. The line through the points is a Michaelis-Menten equation,

$$\text{rate} = \frac{0.833 G}{G + 1.450}$$

The plot is linear up to about 0.03 mg glucose, which is as much as we can expect with 1 ml of glucostat. The non-linear portions are usable, though, by correcting later data to the same rate as given here for the standard, 0.0180.

Figure 7 shows Lineweaver-Burke plots with and without salts. Both kinetic constants are different, both tending to make lower rates without salts, so that a simple explanation of the effect of salts is not indicated.

The effects of temperature have shown in Figure 8, which containing Arrhenius plots for soluble enzyme and whole cells. The activation energy is $19,000 \pm 1,000$ for soluble enzyme and 15,000 for whole cells, suggesting some diffusional limitation for the whole cells. The value of 19,000 is different from the value obtained at the glucose end of the concentration range, suggesting the kinetics are different.

The Michaelis constant with salts is only 16% of the value obtained at the glucose end of the concentration range, suggesting that fructose is a better substrat than glucose.

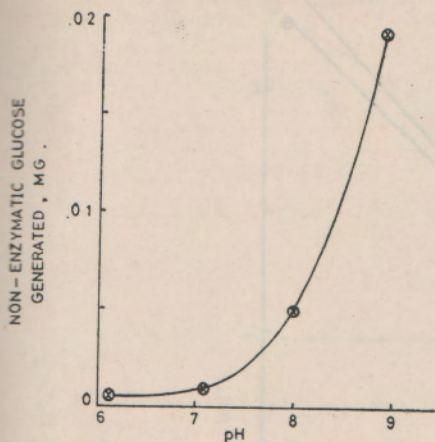


Fig 1. Effect of pH on non-enzymatic glucose generation. Phosphate buffer, no salts.

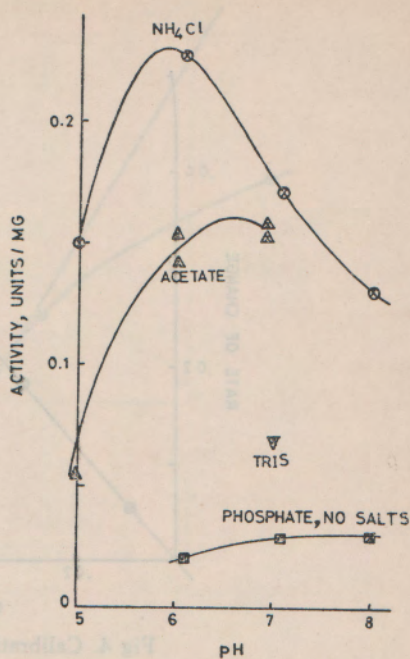


Fig 2. Isomerase assay, pH effect

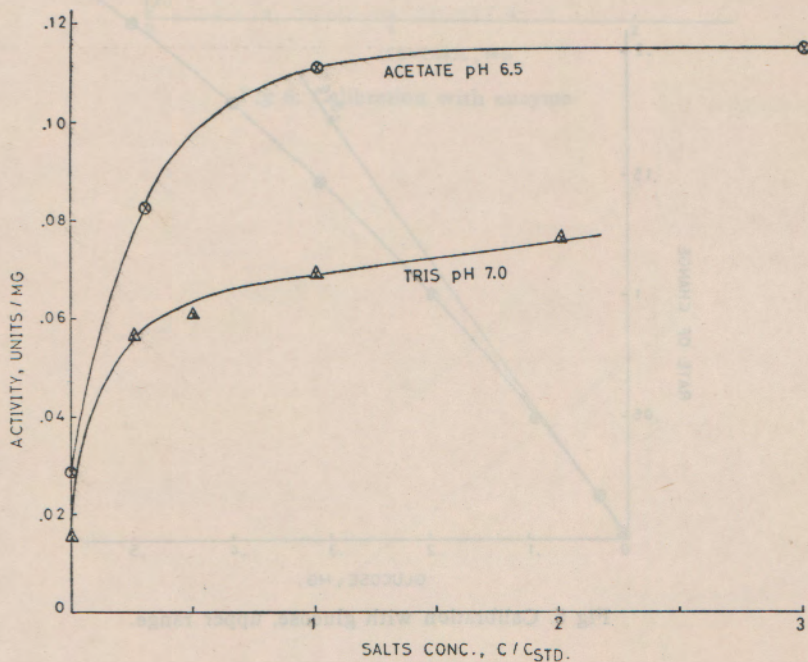


Fig 3. Effect of salts (0.2M fructose)

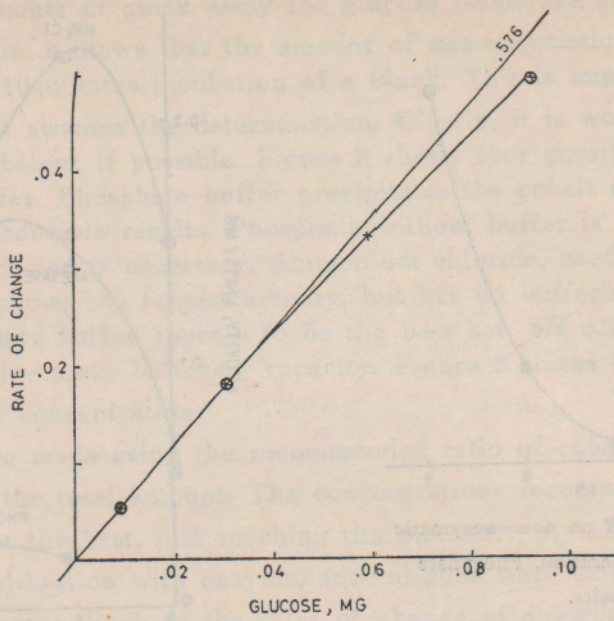


Fig 4. Calibration with glucose; for a rate of Change of .0180 for .0311 mg glucose.

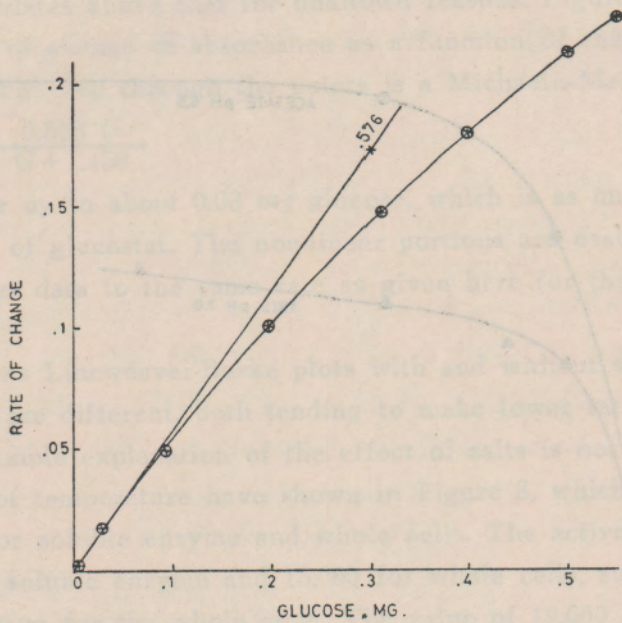


Fig 5. Calibration with glucose, upper range.

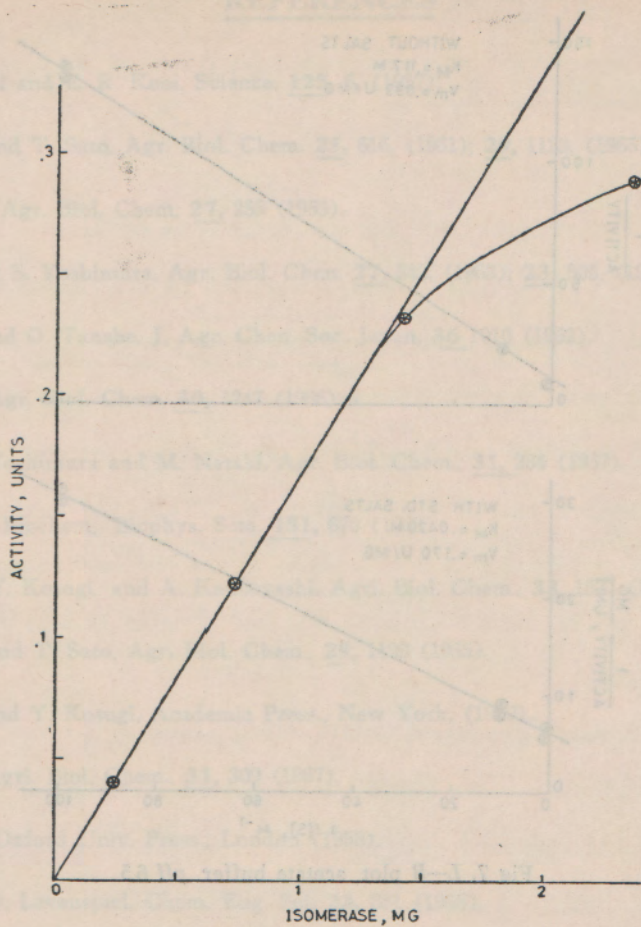
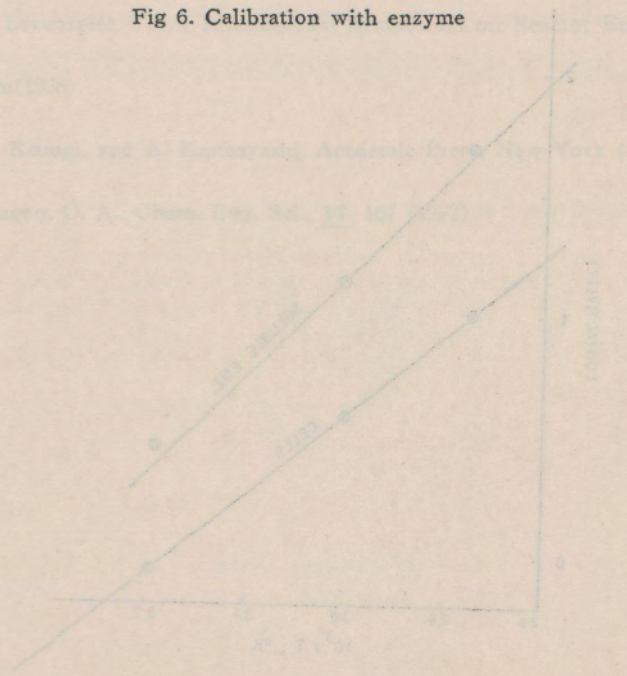


Fig 6. Calibration with enzyme



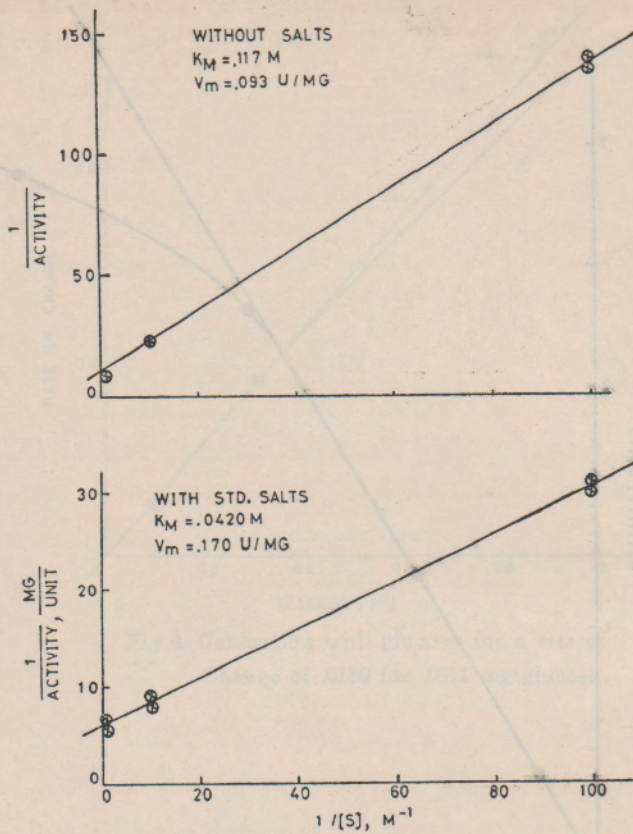


Fig 7. L-B plot, acetate buffer, pH 6.5.

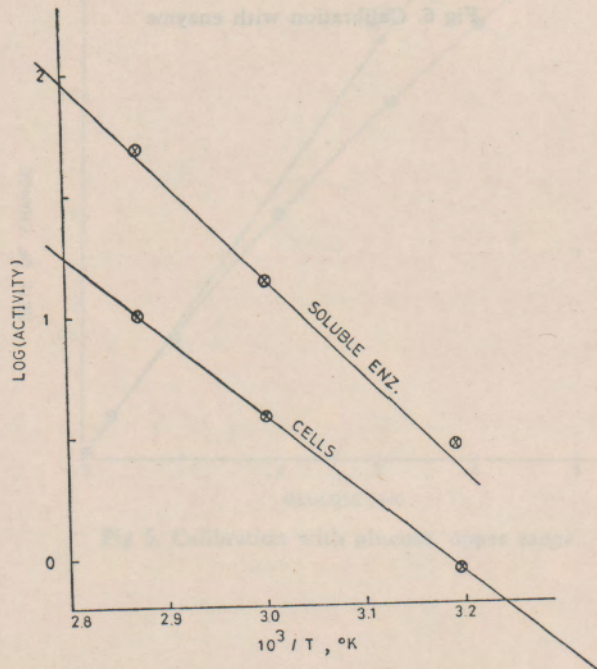


Fig 8. Arrhenius plot.

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APPENDIX

A. Procedure for soluble enzyme:

1. Keep the glucostat at 25°C .
2. Incubate the recipe above for 10 minutes at 60°C .
3. Cool to 25°C ; this takes about 3 minutes in a 25°C bath.
4. Add 1 *ml* glucostat, immediately place in cuvette in the spec, and plot absorbance against time at 430 *nm*.
5. Disregard the first minute, during which time dust and dianisidine fibers are settling and give an erratic line. Take the next minute or two for the slope; in the case of blanks, the slope is constant for about 3-4 minutes, but at higher glucose, the rate begins to change with time.
6. Run a blank.
7. Run a standard, our usual 0.0311 *mg* glucose.
8. Correct the observed rate of change of the sample by the ratio of 0.0180 to the standard rate (from item 6). Find the quantity of glucose from Figures 4 and 5. Calculate the units of activity by dividing this by 1.8. If desired, divide by the sample weight to give units per *mg*. if desired, calculate activity at saturation using the Michaelis constant of 0.0420 *M*.

B. Procedure for whole cells

Double the incubation recipe to give 2 *ml* total. After cooling, centrifuge for ten minutes. Pipette off 1 *ml* of the clear fluid, bring to 25°C , add 1 *ml* glucostat, and proceed as above.