DETERMINATION OF BETA-AMYLASE BY OPTICAL ROTATORY CHANGES

by

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INTRODUCTION

Since the amylases were first discovered, numerous methods for their quantitative estimation have been developed. All of them are based on changes in physical or chemical properties of the solution undergoing enzymic reaction. With starch as substrate the changes in properties may be classified into five categories, viz., change of iodone staining property, decrease in viscosity, change of turbidity, increase in reducing power and the change of optical rotation of the solution.

All of them except the last have been widely employed for this purpose. While the first three were used for α-amylase, measurement of reducing sugars has been applied mainly in determination of β-amylase activity. This is because of the unique fashion in which β-amylase acts upon the substrate, producing maltose as the only reducing sugar.

These methods, however, are time consuming and sometimes rather empirical in nature. Furthermore they are not applicable to mixtures of α and β-amylase. In this work a simple and rapid method was developed for the quantitative evalution of β-amylase activity. The method makes use of the change in optical rotation during enzymic reaction, and the activity unit of β-amylase is defined in terms of change of optical rotation versus time. The method can be used for mixtures of α and β-amylase.

REVIEW OF THE LITERATURE

Enzymic Degradation of Starch by Amylases

Amylases are among the earliest know enzymes in biological systems. The existence of α and β-amylase was reported by Kuhn in 1924(20),.and further substantiated by Ohlsson(26) who fractionated malt diastase into two components, α and β-amylase.

α-amylases, which are found in a variety of living matter, are capable of catalyzing the hydrolysis of α-(1→4) D-glucosidic linkages in polysaccharides such as starch, glycogen or their degradation products. The enzymic action stops or slows down in the vicinity of a branching point, which contains the α-(1→6) D-glucosidic bond. The primary products are oligosaccharides and dextrins which are then broken down further to maltose, glucose, and oligosaccharides containing the α-(1→6) links.

α-amylase from various sources shows different action patterns. The α-amylase from human saliva, for example, hydrolyzes amylose to maltose, maltotriose, and maltotetraose as the main products near the achroic point(27). Sorghum and barley α-amylase, however, produce mainly maltohexaose, maltoheptaose, and maltooctaose(10,4). That of B. Substilis produces oligosaccharides of 5-7 glucose units(4). Fungal α-amylase yields products intermediate between those of salivary and B. Substilis amylases, namely, 3-5 glucose units(4). In addition, α-amylases, especially those of bacterial origin, also produce a slight amount of D-glucose as the initial reaction products.

The difference in action patterns of α-amylases can also be realized by comparing the ultimate limit dextrins obtainable after an extensive treatment of amylopectin or glycogen with α-amylases. In this way, the smallest α-dextrin obtained by malt α-amylase was found to be panose(3), that obtained by human saliva(25), pig pancreas and A. Oryzae α-amylase a tetrasaccharide, 6^3-α-glucosylmaltotriose(35), and by B. Subtilis a pentasaccharide, 6^2-α-maltosylmaltotriose(15).

Unlike α-amylases, β-amylases are found chiefly in higher plants such as barley, wheat, potato or soybean. During hydrolysis, β-amylase splits α-(1→4) glucosidic bonds forming maltose in the β-configuration(26) from the non-reducing end of the polysaccharide. The reaction proceeds continuously until a branching point is approached. This predicts a hundred percent conversion of amylose into maltose by β-amylase. However, generally only 70-85 per cent conversion of pure amylose is attained. Gilbert and Kenner postulated that(13,17) the glucosidic bond becomes sensitized to nonenzymic hydrolysis when a C-OH group is oxidized which is in the β position. Hydrolysis due to oxidation at C-6 and C-2positions produces two moles of amylose, one of them carrying an anomalous non-reducing group is resistent to enzymic hydrolysis. On the other hand, oxidation at C-3 position produces two moles of amylose, which all have normal (unmodified) non-reducing end groups and are hydrolyzed by β-amylase in the usual fashion.

Oligosaccharide of even numbers of glucose units is hydrolyzed completely to maltose(34). That with odd numbers

of glucose units produces maltose and maltotriose, the latter of which is hydrolyzed further to maltose and glucose at a much slower rate(33).

When amylopectin is treated with β-amylase and maltose is formed, there remain dextrins of high D. P. (degree of polymerization) value called β-amylase limit dextrin. It produces viscous solution and is colored purple by iodine.

Determination of β-amylase Activity

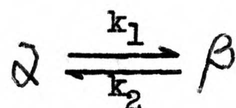
Although a large number of methods for the determination of β-amylases have been developed(36), those for β-amylase are universally based on the same principle. The basis of these methods is upon the regular increase in reducing power due to maltose production. Kneen and Sandstedt(10) defined their unit of β-amylase as the number of grams of starch converted to maltose by β-amylase in 1 hour at 30°C. Meyer et al(22) chose 1 unit of activity of -amylase as the number of mg of maltose pro produced when 1 ml enzyme was mixed with 1 ml of 1% starch in acetate buffer of pH 4.8 at 20°C for 3 minutes. Balls, Walden and Thompson(1) defined 1 unit of activity as the amount of enzyme which liberated reducing sugar equivalent to one milliequivalent of Fe(CN)3 in 10 minutes at 30°C in 30ml of 1% soluble starch in pH 4.8 buffer. Thoma and Koshland(31) defined one unit of activity of β-amylase as the amount of enzyme which produced 1 mg of maltose from 0.5% soluble in 30 minutes at pH 4.8 in 0.1M acetate buffer at 25 C.

All of these units are not different from each other in

any strict sense. They all were based on the amount of maltose produced (or starch converted into maltose) by the enzyme at a certain time period and temperature in acetate buffer of pH 4.8 which is near the optimum pH for most β-amylases(6).

Mutarotation of Maltose

Mutarotation was first discovered by Dubrunfaut(11) in 1846 who found that when glucose was dissolved in water, the optical rotation decreased until a final value was reached. For simple mutarotation, i.e., that during which only and forms exist



the reaction follows first order kinetics. The mutarotation coefficient $k_1 + k_2$ can be evaluated from the following equation(28)

$$k_1 + k_2 = (2.303/t)\ \log \frac{r_0 - r_\infty}{r - r_\infty}$$

where $k_1 + k_2$ is the mutarotation coefficient in $\min^{-1}$

t is the time for mutarotation in min

r_0 is the optical rotation in degrees before mutarotation

r is the optical rotation at time t

r_∞ is the optical rotation at equilibrium

Maltose like glucose, mannose, and lactose undergoes first order kinetics during mutarotation(16). The rate constant is governed

by a number of factors, namely, temperature, pH, solvent, and salt in solution. The effect of temperature upon the rate constant can be best recognized from the Arrhenius equation

$$\ln \frac{k}{k'} = (Q/R)(1/T_1 - 1/T_2)$$

where k and k' are rate constants at absolute temperature T_1 and T_2 respectively, Q is the heat of activation in cal, R is the gas constant. It is possible, therefore, to calibrate the rate constant to the proper temperature at which mutarotation takes place.

The presence of hydrogen and hydroxyl ions in solution also enter into catalysis of mutarotation. Since neither of them alone acts as effectively as the mixture, Hudson(28) proposed an equation to show the dependence of mutarotation coefficient upon the hydrogen and hydroxyl ions concentrations

$$k_1 + k_2 = A + B(H^+) + C(OH^-)$$

in which A, B, and C are constants. They can be determined empirically.

The effect of solvent on mutarotation of maltose and other sugars was studied by Grandchamp-chaudun in 1958(14). Using various alcohols and water mixtures, she noticed that the rate of mutarotation was lower in the mixture than in pure water.

Nicolle and Weisbuch(23, 24), who studied the effect of neutral salts upon the rate of mutarotation of maltose, found that sodium chloride or potassium chloride in water solution decreased mutarotation to a much lower value than in pure water.

Application of Optical Rotatory Methods to Amylolytic Studies

Enzymic degradation of starch has been followed extensively by optical rotatory methods by many workers. Among the early ones, Kuhn(20) found, from mutarotation data, that amylases of pancreas and Aspergillus oryzae yielded α-maltose as a primary fission product of starch, whereas malt amylase produced β-maltose and hence the names α and β-amylase were adopted. Later on, Ohlsson(27) found that malt amylose was a mixture of α and β-amylase. In 1936, Freeman and Hopkins(12) investigated the different behaviors of α and β-amylases using measurements of optical rotation. The concluded that mutarotation of the fission product was a characteristic of the enzyme itself and was independent of the substrate used. α and β-amylase can hydrolyze the same substrate but produce α and β-amylase respectively from it. Bird and Hopkins(1953), on mixing α or β-amylase with starch, reported that an initial increase in optical rotation was due to the formation of an enzyme-substrate complex(5). Thoma and Koshland (1960), in a study of the enzyme mechanism of β-amylase(31), proved with mutarotation datd that β-amylase hydrolyzed maltotetraose and amylodextrin to maltose with a quantitative inversion of configuration. Nowadays, changes in optical rotation have been useful in elucidation of stereochemical mechanism of enzymic reactions. Enzyme mechanisms so far developed involving catalysis at the optically active centers, e.g., single displacement, double displacement etc., are invariably dealt with through optical rotatory measurements.

MATERIALS AND METHODS

Preparation of Substrate

Starch solution. According to Meyer(21), hydrolysis by β-amylase is proportional to enzyme concentration over a wide range of substrate concentrations. The levels of 0.08% and 0.4% are well within this range. The starch solution was prepared by dissolving 8 or 40 grams of Merck's soluble starch in 1000 ml of 0.1 M sodium acetate buffer of pH 4.8. The buffer, as described by Colowick and Kaplan(8), was made by mixing 200 ml of 0.2 M acetic acid with 300 ml of 0.2 M sodium acetate solution and diluting to one liter with distilled water.

Preparation of Enzymes

Crystalline α and β-amylase. A commercial preparation of α and β-amylase obtained from Nutritional Biochemical Company was used in this work. The enzymes were adjusted to the strength desired by diluting with distilled water.

Malt Diastase Solution. Malt diastase was obtained from Nutritional Biochemical Company. 150 mg of malt diastase was suspended in 100 ml water to give a 0.15% enzyme solution.

Malt α-amylase. Malt α-amylase is generally prepared by heating malt extracts to a temperature at which the β-amylase is inactivated. The temperature is not the same for all malt extracts In most cases, heating at 70°C for 15 min will be adequate. Other methods of separating these enzymes were reviewed by Bamann and

Myrbäck(2). In the present work, 100 ml of 0.15% diastase solution was heated in a water bath at 70°c for fifteen minutes(29). After cooling immediately to room temperature, all β-amylase was precipitated out by centrifugation at 15,000 rpm (73,000xg) for 30 minutes in a Servall Superspeed Centrifuge. The supernatant was adjusted to 50% saturation with ammonium sulfate at 5°c. After standing for two hours, most of the α-amylase was precipitated. It was dissolved in 11 ml of 0.2% calcium acetate solution(9) to give an α-amylase solution of the desired strength. The percentage recovery was found to be 50%.

Determination of β-amylase Activity

Soluble starch of two concentrations, i.e., 0.08% and 0.4%, were used as substrate. β-amylase of different starting concentrations (the starting concentration of β-amylase was so chosen as to reach the achroic point in 15-20 minutes for 1 ml enzyme reacting with 9 ml substrate at 30°c.) were diluted to various concentrations for the two substrates. To 9 ml of substrate was added 1 ml of enzyme. The mixture was incubated in a water bath at 30°c, and the stop watch was immediately started. In the mean time, a portion of the hydrolyzing mixture was withdrawn and transferred to the polarimeter cell and optical rotation measured at 30°c.

Determination of β-amylase in the presence of malt α-amylase was carried out in the same manner except that the enzymes made by mixing various volumes of α and β-amylase to give mixture containing different proportions of amylases. The substrate

used was 0.08% soluble starch.

Determination of Maltose

Maltose produced during β-amylolysis was determined by the Somogyi method(30). This was also performed at two substrate levels, namely, 0.08 and 0.4% soluble starch. For the lower substrate level, 0.08%, 1 ml Somogyi reagent was used, and for the higher substrate level, 0.4%, 5 ml Somogyi reagent was used. The procedure was as follows.

To 9 ml of starch solution was added 1 ml of β-amylase solution and the mixture was incubated in a water bath at 30°c. The mixture was divided into two parts. For one part, the optical rotation was read continuously in the polarimeter. From the second part, 1 ml of solution was withdrawn succesively at one minute intervals and Somogyi reagent added, which stopped the enzymic reaction immediately. (β-amylase was inactivated by cupric sulfate in the reagent.) After boiling for 30 minutes and cooling to room temperature, the solution was titrated with 0.005 N sodium thiosulfate.

Chromatography

<u>Hydrolysis of soluble starch by malt diastase</u>. A chromatographic analysis of the hydrolysates was performed as follows. To 9 ml of 0.08% starch solution (at pH 4.8) was added 1 ml of malt diastase solution (0.15% diastase in solution), and the hydrolysis carried out in a water bath at 30°c Portions of the hydrolyzing mixture were withdrawn at 0.5, 5, 10, 15, and 20

minutes and boiled for 2 minutes to stop the enzyme reaction. Then 30 of each of the hydrolysates were applied on Whatman No. 1 paper. Chromatography was carried out in a glass chamber w with butanol-pyridinewater as developing reagent(9). After running for six ascents, the reducing sugars were detected by silver nitrate in alkaline solution(9).

Chromatography of the hydrolysates preparedly by -amylase and commercial α-amylase were carried out in the same way except for commercial α-amylase the substrate used was 0.4% soluble starch in 0.1 M sodium phosphate buffer at pH 6.8(8).

Polarimeter

The polarimeter used in this work is a type 143A ETL-NPL Automatic Polarimeter (see the photograph below). It is a photoelectric polarimeter which employs the Faraday Magneto -optic effect to measure the optical rotation. The yellow filter (wavelength =-5893A) was used throughout this work. There are two scales, $\pm 0.5^{\circ}$ and $\pm 0.1^{\circ}$, on the polarimeter, the latter of which gives fives times expanded values. The polarimeter cell used in the experiment is a specially designed one. It is made of a 0.5" x 2" glass cylinder covered with a piece of round glass on each ends. The outside of the cell is a condenser-like water jacket, through which water circulates to control the temperature. Solutions to be measured were injected, with a syringe, into the cell through a ting side arm which goes upward out of the water jacket. A photograph of the cell is shown below.

Type 143A ETL-NPL Automatic Polarimeter.

0.5" x 2" Polarimeter Cell.

RESULTS AND DISCUSSION

It is the purpose of the present work to develop a new method for the determination of β-amylase activity, based on the quantitative production of maltose from starch by the enzyme. Evaluation of β-amylase activity on this basis has been very common. While a variety of activity units have been proposed(1,31,21), the measurements of maltose have been by chemical methods. In this work, however, the optical rotation is followed as a parameter in estimating maltose production, which in turn measures the enzyme activity quantitatively. The possibility of employing optical rotatory changes as a measure of maltose production can be recognized from the appreciable difference in the specific optical rotations between β-amylase, +111.7°(32), limit dextrins, +200°(18), and starch, +200°. Of the reaction products, only β-maltose has a low specific optical rotation. Decrease in the total optical rotation during hydrolysis of starch by β-amylase is mainly due to maltose production. The feasibility of this method is predetermined by two factors, both of which should be experimentally proved. Firstly, in order to interpret maltose production exactly, there should exist a linear relationship between the rate of change of optical rotation and the velocity of maltose production. Secondly, a proportionality should exist between the rate of change of optical rotation and the amount of enzyme used.

Optical Rotation and Maltose Production.

1). Theoretical consideration: By definition, the observed optical rotation of sugars in solution is related to their concentrations in the following manner:

$$[\alpha]_D = \frac{\alpha}{l \times c} \qquad (A)$$

where $[\alpha]_D$ is the specific optical rotation of the sugar in circular degrees

α is the observed optical rotation of the sugar

l is the length of the polarimeter cell in dm (or the effective length of the cell giving the same readings as those obtained from a standardized polarimeter)

c is concentration of sugar in grams per ml

From equation (A), it is evident that the observed optical rotation of a specific sugar is directly proportional to its concentration in solution. To evaluate the proportionality constant, equation (A) may be rewritten as:

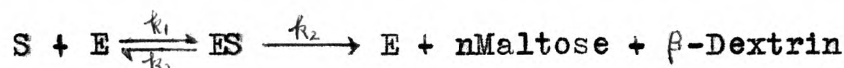
$$\alpha = k \times c$$

where $k = l \times [\alpha]_D$

In the present work, the effective length was measured with standard solutions of sucrose, glucose, and maltose (dried at 55°c under vacuum). With 0.1% solutions the observed optical rotation at 30°c for sucrose was 0.0432, for glucose 0.034, and for maltose hydrate 0.084. When these values were put into equation (A) together with corresponding specific

rotations, i.e., +66.5 for sucrose, +52.7 for glucose, and +130.4 for maltose hydrate(28), the effective lengths calculated were respectively o.650, 0.645, and 0.642 dcm. The averagevalue of 0.645 was adopted in this work.

In cosidering the hydrolysis of starch by β-amylase:



$$S + E \underset{k_2}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_2} E + n\text{Maltose} + \beta\text{-Dextrin}$$

and assuming the optical rotation to be additive, the observed rotation of the entire solution would then be the sum of those of its constituents. Neglecting the contributions of optical rotation by free enzyme and the effect of mutarotation (see proof later) then:

$$\alpha_{total} = \alpha_{[S]} + \alpha_{[ES]} + \alpha_{[M]} + \alpha_{[D]} \qquad (C)$$

where $[M]$ is mole concentration of maltose and $[D]$ is mole concentration of β-Dextrin. From equation (B), with the introduction of proportional constants:

$$\alpha_{total} = k_{[S]}[S] + k_{[ES]}'[ES] + k_{[M]}'[M] + k_{[D]}'[D] \qquad (D)$$

Upon differentiation with respect to time:

$$\frac{d\alpha_{total}}{dt} = k_{[S]}\frac{d[S]}{dt} + k_{[ES]}\frac{d[ES]}{dt} + k_{[M]}\frac{d[M]}{dt} + k_{[D]}\frac{d[D]}{dt} \qquad (E)$$

all the terms in the right hand side of equation (E) can be shown to be proportional to $[ES]$ in the following way:

$$[S] = [S_{total}] - [ES] - \frac{[M]}{n}$$

$$\frac{d[S]}{dt} = -\frac{d[ES]}{dt} - \frac{1}{n}\frac{d[M]}{n}$$

for steady state(7): $\frac{d[ES]}{dt} = 0$

Thus $\frac{d[S]}{dt} = -\frac{d[M]}{n\,dt}$

$\frac{d[M]}{dt}$ is the velocity of enzymic reaction which is proportional to $[ES]$

$\frac{d[S]}{dt}$ is therefore proportional to [ES]. The last term in equation (E), $k_{[D]}\frac{d[D]}{dt}$, is the rate of formation of β-Dextrins. Its value is proportional to [ES]. All the terms in the right hand side of equation (E) now can be conbined and expressed as k'[ES] or rather $k\frac{d[M]}{dt}$ (because $\frac{d[M]}{dt}$ is proportional to [ES]). Equation (E) might assume another form:

$$\frac{d\alpha_{total}}{dt} = k\frac{d[M]}{dt} = kv \qquad \text{(F-1)}$$

or $$\alpha_{total} = k[M] + \text{Constant} \qquad \text{(F-2)}$$

Equation (F-1) gives the linear relationship between the reaction velocity and the change in optical rotation per unit time. This makes it possible to express the velocity of β-amylolysis in terms of change of optical rotation per unit time, which in turn can be employed as a quantitative unit for the estimation of β-amylase activity.

The constant k can be calculated theoretically as follows: Owing to the relatively low concentration of enzyme, it is reasonable to assume that the contributions to observed optical rotation of the solution are due to maltose, β-Dextrins, free starch molecules, and enzyme substrate complex. If α_t is the total observed optical rotation of the solution and $\alpha_{[M]}$, $\alpha_{[D]}$, $\alpha_{[S]}$, $\alpha_{[ES]}$, are the optical rotation due to maltose, β-dextrins, free starch molecules, and enzyme-substrate complex respectively, then

$$\alpha_t = \alpha_{[M]} + \alpha_{[D]} + \alpha_{[S]} + \alpha_{[ES]} \qquad \text{(G)}$$

or, to express in terms of concentrations $[S_t]$, $[D]$, $[S]$, $[S_{ES}]$

$$\alpha_t = k_{[M]}[M] + k_{[D]}[D] + k_{[S]}[S] + \{k_{[S]}[S_{ES}] + a[S_{ES}]\} \qquad \text{(H)}$$

where $[S_{ES}]$ is the concentration of starch forming enzyme-substrate complex. $\alpha_{[ES]}$ is expressed as the observed optical rotation due to $[S_{ES}]$ plus an increasement by enzyme-substrate complex formation, a S_{ES} . Since specific rotation of β-dextrin is very close to that of starch(18), and $[D] + [S] + [S_{ES}] = [S_t] - [M]$ (it should be remembered that the concentrations used here are in gm per ml instead of a molar basis as used before), thus if $k_{[D]}$ is replaced by $k_{[S]}$ and $[S_{ES}]+[S]+[D]$ by $[S_t]-[M]$ equation (H) becomes

$$\alpha_t = k_{[M]}\cdot[M] + k_{[S]}\{[S_t]-[M]\} + a[S_{ES}]$$

rearrange:

$$\alpha_t = \{k_{[M]} - k_{[S]}\}[M] + k_{[S]}\cdot[S_t] + a[S_{ES}] \quad \text{(I)}$$

The last terms in equation (I) is observed rotation before hydrolysis. If it is denoted by α_{t_0}, then

$$\alpha_{t_0} - \alpha_t = \{k_{[S]} - k_{[M]}\}[M]$$

divide both side by Δt

$$\frac{\Delta\alpha}{\Delta t} = \{k_{[S]} - k_{[M]}\}\frac{\Delta M}{\Delta t}$$

or

$$\frac{d\alpha}{dt} = k\,\frac{d[M]}{dt} \quad \text{(F-1)}$$

where $k = k_{[S]} - k_{[M]}$

let l = 0.645 dm(page 16), $[\alpha]_{D,\,maltose} = +111.7^\circ$(32), $[\alpha]_{D,\,starch} = +197^\circ$ (measured at 0.08% starch concentration in 0.1M sodium acetate buffer of pH 4.8 at 30°c.), the k value calculated is 0.55 deg ml g^{-1} or 0.055 deg-ml mg^{-1}, which is the change of observed optical rotation due to the production of 1 mg of maltose monohydrate in 1 ml solution.

2). Experimental proof. Toverify the linearity between the reaction velocity and the rate of change of optical rotation

a simultaneous determination of $d[M]/dt$ and $d\alpha/dt$ was carried out. Maltose production was determined by the Somogyi method(30), while at the same time a continuous recording of optical rotation was made in the polarimeter. Data for maltose produced during hydrolysis are listed in Table IIIb and Table IVb, where the amount of maltose was obtained by dividing through the volume of 0.005 N sodium thiosulfate used for titration by the equivalent volume, which is the volume of thiosulfate equivalent to 1 mg of maltose. For one assay, where the substrate used was 0.08% soluble starch, this was found to be 8.75 ml/mg maltose. For the other one, where 0.4% soluble starch was used, this was 4.5 ml/mg maltose. Optical rotation data are presented in Table IIIa and Table IVa. Both maltose and optical rotation are plotted versus time in Fig. IIIa, where optical rotation can be read directly for the corresponding values of maltose in mg. T There are listed in Table IIIc and Table IVc. When the amount of maltose produced at each time is plotted against the observed optical rotation of the solution in Fig. IIIb, straight lines are obtained as expected from the theoretical point of view, the slopes of which are about 0.050, a value close to that calculated theoretically.

<u>Optical Rotation and Enzyme Concentration</u>. In this work the linearity between amount of enzyme used and the rate of change of optical rotation within the optimum substrate level was established. The optical rotation of the reaction mixture at each level of enzyme concentration was recorded respectively.

The data are summarized in Table I and Table II and plotted in Fig. Ia and Fig. IIa. The initial slope of each curve is measured and plotted against the enzyme concentrations in Fig.Ib and Fig.IIb which all give straight lines.

The initial slope taken corresponds to the steady state of enzymic reactions. This steady state is characterized by the constant concentration level of enzyme-substrate compound(5), or constant reaction velocity. The existence of the steady state gives a constant initial slope of optical rotation--time curve, which makes it measurable within a certain period of time. The length of this period depends upon the concentration of substrate and enzyme. Meyer and Press(21) in their study of starch degradation by β-amylase have given a list of data for the relationship of $V = v/(E)$ (the ratio of reaction velocity to enzyme concentration) to substrate concentration, which also shows the dependence of steady state upon concentration of substrate and enzyme. Under high concentration of substrate and low concentration of enzyme, there was a longer period of time within which the reaction velocity was constant, or in other words the steady state existed, whereas under opposite condition the time for steady state was shorter. Therefore at a certain substrate level, as the concentration of enzyme goes higher, the linear portion of the optical rotation--time curve will tend to be shorter, and a poor measurable slope results. This could cause trouble in the method. However, it can be easily handled by measuring the diluted enzyme solution and multiplying the result properly to give

the correct value for the original enzyme solution.

Activity Unit. The activity unit of β-amylase in this work was so defined as to represent the amount of enzyme capable of producing maltose hydrate at a velocity of 1 $mg.ml^{-1}min.$, when 1 ml of enzyme was added to 9 ml of 0.08% (or 0.4%) soluble starch in 0.1 M sodium acetate buffer of pH 4.8 at 30°c. Since the proportionality constant was calculated to be 0.055, the rate of production of maltose hydrate at 1 mg ml^{-1} min. will correspond to the decrease of optical rotation at a rate of 0.055 deg./min.. Hence, the amount of enzyme that can give a change of optical rotation of 0.055 deg./min. under above conditions was defined as 1 unit of activity of β-amylase at the substrate level of 0.08% or 0.4% soluble starch. As a matter of fact, 1 unit activity of β-amylase defined at 0.08% substrate level is not equ equivalent to that at 0.4% level. Moreover, the activity unit defined as degrees per minute corresponding to 1 mg ml^{-1} min. of maltose production is also subject to change with different cell length. Nevertheless, the unit itself, as interpreted as numbers of miligrams of maltose produced under a certain condition, is actually the same as those reported by most workers in the field.

Mutarotation Considerations. The quantitative liberation of maltose in β-configuration has been proved by Thoma and Koshland (31) in a kinetic study of the mutarotation of maltose released from amylodextrin and maltotetraose. This indicates that during the early stage of hydrolysis no α-maltose is produced. The effect of mutarotation of β-maltose can be evaluated as follows:

Applying the equation of mutarotation to β-maltose

$$k_1 + k_2 = (1/t)(2.303)\log \frac{r_0 - r_\infty}{r - r_\infty}$$

Replacing the optical rotation by concentrations

$$k_1 + k_2 = (1/t)(2.303)\log \frac{C_0 - C_\infty}{C - C_\infty} \qquad (A)$$

where C_0 is the concentration of β-maltose before mutarotation

C is the concentration of β-maltose at any time

C_∞ is the concentration of β-maltose at equilibrium

The mutarotation coefficient $k_1 + k_2$ for β-maltose hydrate in the absence of buffer is 0.0053 min. (16)

for t = 10 min., equation (A) becomes

$$\frac{0.0053 \times 10}{2.303} = \log \frac{c_0 - c_\infty}{c - c_\infty}$$

$$1.06c = c_0 + 0.06c_\infty$$

$$C = 0.94\, C_0 + 0.057C_\infty$$

$$C_0 - C = 0.06\, C - 0.057C_\infty$$

The maximum difference of $C_0 - C$ can be obtained by assuming that all β-maltose mutarotate to α-maltose. Then, $C_\infty = 0$ and $C_0 = 0.048$ $= 0.08 \times 60\% = 0.048$ (assure 60% hydrolysis of 0.08% soluble starch) which gives a value of 0.0029 for $C_0 - C$. This difference in concentration corresponds to a change in optical rotation of 0.002°:

$$\alpha = \frac{(111.7)(0.62)(0.0029)}{(100)} = 0.002^\circ$$

This value should be even smaller in the presence of buffer. It is negligible during optical rotatory measurements. In this work, therefore, it was not taken into considerations.

Applications. An important application of this method is determination of β-amylase in the presence of α-amylase. As is usually the case α-amylases from plant sources are contaminated with β-amylase, thus it has been essential to test the homogeneity of the enzyme after purification. So far, there has not been any satisfactory method for this purpose. The method developed here is applicable to those α-amylases which produce oligosaccharides of more than five glucose units from starch together with very small amounts of glucose, maltose or some maltotriose. An examination of Table VI shows that oligosaccharides with more than five glucose units have relatively high specific rotations, e.g., $[\alpha]_D^{25}$ of maltopentaose is 178.3°, that of maltohexaose is 179.9°, maltoheptaose 182.9°, maltooctaose 182.2°, and maltononaose 186.1°. All these values are the equilibrium rotations. They are lower than the rotations of pure α forms that are first released from starch molecules by β-amylase. Production of oligosaccharides of more than five glucose units therefore bring about no significant change to the total optical rotation of the reaction mixture. As shown in Plate I, degradation products of starch by malt α-amylase contain mostly G_6 -G_8 and maltose with only small quantity of glucose, and maltotriose. In this experiment, a mixture of malt β-amylase and α-amylase was used as enzymes containing various content of β-amylase were incubated with 0.08% starch, the optical rotation was recorded continuously with respect to time. A list of data is summarized in Table V and plotted in Fig. IVa. The initial

slope, or $d\alpha/dt$, of each curve in fig. IVa are plotted against β-amylase concentrations in Fig IVb which results in a straight line. This proves that it is possible to detect the contamination of β-amylase in malt α-amylase by optical changes during hydrolysis of the starch to G_6, G_7, G_8, as main products, it seems that this method might as well be applicable to sorghum amylases.

In Plate II, where the chromatographic analysis made for bacterial α-amylase, the degradation products of starch at the early stage of hydrolysis contain more glucose, maltose and some maltotriose which produce remarkable decrease in the total optical rotation of the reaction mixture (see Table VII) and makes the method inapplicable to this enzyme. As for human s salivary α-amylase, although it produces G_2, G_3 and G_4 as the main products from starch, the absence of glucose makes the method still applicable to the enzyme. The production of glucose appears to be a criterion for the applicability of this method.

The detectable activity of β-amylase by this method has been found to be 0.0062 units which corresponds to a change of optical rotation of 0.0003 degrees per minute.

Table I. Hydrolysis of 0.08% soluble starch by β-amylase in 0.1M sodium acetate buffer of pH 4.8 at 30°C. Optical rotation was taken at ±0.1° scale*.

Concentration	Time	Optical rotation	Achroic point	Initial slope(deg/min)
	1'30"	0.400		
	2'45"	0.395		
	5'00"	0.390		
	6'50"	0.385		
0.10	9'00"	0.380	82'00"	0.0028
	10'45"	0.375		
	15'00"	0.370		
	18'45"	0.365		
	23'00"	0.361		
	1'30"	0.387		
	2'45"	0.380		
	4'00"	0.370		
	4'40"	0.365		
0.25	6'00"	0.360	30'30"	0.0069
	8'10"	0.355		
	10'45"	0.350		
	16'00"	0.345		
	20'00"	0.343		
	1'30"	0.380		
	1'45"	0.375		
	2'15"	0.370		
	2'35"	0.365		
0.50	3'15"	0.360	15'45"	0.0141
	4'00"	0.355		
	5'15"	0.350		
	11'30"	0.341		
	15'00"	0.340		
	1'15"	0.365		
	1'29"	0.360		
	1'43"	0.355		
	2'08"	0.350		
0.75	2'28"	0.345	8'15"	0.0210
	4'05"	0.330		
	5'25"	0.325		
	8'00"	0.320		
	11'00"	0.315		

* See " Materials and Methods " (Polarimeter)

Table II. Hydrolysis of 0.4% soluble starch by β-amylase in 0.1M sodium acetate buffer of pH 4.8 at 30°C. Optical rotation was taken at -0.5° scale.

Concentration	Time	Optical rotation	Achroic point	Initial slope
0.125	1'30"	0.375	54'00"	0.0024
	3'10"	0.370		
	5'30"	0.365		
	8'05"	0.360		
	12'35"	0.353		
	13'30"	0.350		
	18'20"	0.344		
	22'25"	0.340		
0.250	1'30"	0.372	27'45"	0.0053
	2'20"	0.370		
	3'00"	0.365		
	3'55"	0.360		
	4'35"	0.355		
	5'35"	0.350		
	6'25"	0.345		
	7'40"	0.340		
	8'50"	0.335		
	10'45"	0.330		
	13'00"	0.325		
	17'35"	0.320		
0.500	1'10"	0.375	14'00"	0.0102
	1'40"	0.370		
	2'10"	0.365		
	2'40"	0.360		
	3'05"	0.355		
	3'37"	0.350		
	4'05"	0.345		
	4'43"	0.340		
	5'15"	0.335		
	6'05"	0.330		
	6'55"	0.325		
	9'00"	0.320		
	14'30"	0.315		
1.00	2'00"	0.365	6'30"	0.0200
	2'16"	0.360		
	2'30"	0.355		
	2'45"	0.350		
	3'10"	0.345		
	3'40"	0.340		
	4'50"	0.335		
	6'15"	0.330		
	8'45"	0.325		
	13'00"	0.325		

Table IIIa. Optical rotation during β-amylolysis of 0.08% soluble starch in 0.1M sodium acetate buffer of pH 4.8 at 30°C. Readings were taken at ±0.1°scale.

Time in min	Optocal rotation in degrees
1'30"	0.385
1'45"	0.382
1'55"	0.380
2'15"	0.375
2"35"	0.370
3'00"	0.365
3'30"	0.360
4'30"	0.355
7'30"	0.350
9'00"	0.349

Table IIIb. Maltose production during β-amylolysis of 0.08% soluble starch in 0.1M sodium acetate buffer of pH 4.8 at 30°C.

Time(min)	Thiosulfate used in ml	Net volume(ml)	mg maltose(Dividing by 8.75 ml)
0	27.38	0	0
1	26.88	0.50	0.057
2	26.05	1.33	0.152
3	25.86	1.52	0.174
4	25.40	1.98	0.226
5	24.99	2.39	0.274
6	24.97	2.41	0.276
7	24.98	2.40	0.274

Table IIIc. Optical rotation versus maltose production during β-amylolysis of 0.08% soluble starch in 0.1M sodium acetate buffer of pH 4.8 at 30°C.

Time in min	mg of maltose	Optical rotation at ±0.1° scale
1'30"	0.08	0.385
2'00"	0.10	0.379
2'30"	0.13	0.372
3'24"	0.18	0.360

Table IVa. Optical rotation during β-amylolysis of 0.4% soluble starch in 0.1M sodium acetate buffer of pH 4.8 at 30°C. Readings were taken at ±0.5° scale.

Time in min	Optical rotation in degrees
2'00"	0.370
2'20"	0.365
2'45"	0.360
3'05"	0.355
3'25"	0.350
3'50"	0.345
4'20"	0.340
4'50"	0.335
5'45"	0.330
6'50"	0.325
10'40"	0.320

Table IVb. Maltose production during β-amylolysis of 0.4% soluble starch in 0.1M sodium acetate buffer of pH 4.8 at 30°C.

Time(min)	Thiosulfate used in ml	Net volume in ml	mg maltose(dividing net vol. by 4.5 ml)
0	24.43	0	0
1	23.10	1.33	0.295
2	22.12	2.31	0.512
3	20.58	3.85	0.885
4	19.09	5.34	1.185
5	18.16	6.27	1.390
6	17.80	6.63	1.470
7	17.73	6.70	1.490
8	16.98	7.45	1.660

Table IVc. Optical rotation uersus maltose production during β-amylolysis of 0.4% soluble starch in 0.1M sodium acetate buffer of pH 4.8 at 30°C.

Time in min	mg of maltose	Optical rotation
2'00"	0.58	0.370
2'35"	0.76	0.362
3'00"	0.88	0.356
3'30"	1.02	0.349

Table V. Hydrolysis of 0.08% soluble starch by mixture of β and malt α-amylase in 0.1M sodium acetate Buffer of pH 4.8 at 30°C. Optical rotation was taken at ±0.1° scale.

Concentration of -amylase	time	optical rotation	achroic point	initial slope(deg/min)
0.05	2'00"	0.390	16'15"	0.0012
	8'15"	0.390		
	11'45"	0.385		
	17'00"	0.380		
	20'30"	0.375		
0.10	2'10"	0.388	12'30"	0.0018
	3'30"	0.384		
	6'00"	0.380		
	8'10"	0.375		
	11'45"	0.370		
	13'30"	0.365		
	18'20"	0.355		
	22'20"	0.350		
0.25	2'00"	0.369	12'30"	0.0048
	3'35"	0.360		
	5'10"	0.355		
	5'50"	0.350		
	6'35"	0.345		
	8'00"	0.340		
	8'55"	0.335		
	10'30"	0.330		
	12'50"	0.321		
	15'00"	0.320		
0.50	1'30"	0.372	12'10"	0.0092
	2'00"	0.370		
	2'20"	0.365		
	2'50"	0.360		
	3'40"	0.355		
	4'40"	0.350		
	5'10"	0.345		
	6'00"	0.340		
	7'25"	0.335		
	8'40"	0.330		
	10'00"	0.325		
	13'45"	0.320		
0.75	1'25"	0.370	15'00"	0.0140
	1'30"	0.365		
	2'00"	0.360		
	2'35"	0.360		
	2'55"	0.350		
	3'20"	0.345		
	3'58"	0.340		
	4'30"	0.335		
	6'15"	0.330		
	7'45"	0.325		
	12'00"	0.321		
	15'00"	0.320		

Table VI. Specific optical rotations of some linear starch oligosaccharides(32)

Saccharide	Symbol	Specific optical rotation $[\alpha]_D^{20}$		
α-Glucose	Ø	Amorphous	+112 → +52.7	(C, 4; water)
β-Glucose	Ø	Amorphous	+18.7 → +52.7	(C, 4; water)
α-Maltose	0-Ø		+173	(calculated value)
β-Maltose	0-Ø	Crystalline hydrate	+111.7	(C, 4; water)
Maltotriose	0-0-Ø	Amorphous	+159.4	(C, 1.06;water)
Maltotetraose	$0-(0)_2-Ø$	Amorphous	+163 +177	(C, 0.885; water) (water; 15°C)
Maltopentaose	$0-(0)_3-Ø$	Amorphous	+178.3 +180.3	(C, 0.85;water) (water; 15°C)
Maltohexaose	$0-(0)_4-Ø$	Amorphous	+179.7 +184	(C, 0.93;water) (water; 15°C)
Maltoheptaose	$0-(0)_5-Ø$	Amorpuous	+182.9 +186.4	(C, 0.86;water) (water; 15°C)
Maltooctaose	$0-(0)_6-Ø$	Amorphous	+182.2	(C, 0.685;water)
Maltononaose	$0-(0)_7-Ø$	Amorphous	+186.1	(C, 0.855;water)
Maltodecaose	$0-(0)_8-Ø$	Amorphous	+167.3	(C, 0.925;water)
Starch			+200	(water)

Ø represents reducing glucose residue.

Table VII. Observed optical rotation during hydrolysis of soluble starch by some α-amylases.

Enzymes	Time	Optical rotation	Achroic point	Conditions
Malt α-amylase	1'30"	0.390	17'30"	1 ml enzyme reacting with 9ml 0.4% soluble starch in 0.1 M sodium acetate buffer of pH 4.8 at 30°C. ±0.5° scale.
	2'30"	0.389		
	3'00"	0.390		
	4'30"	0.390		
	6'30"	0.390		
	9'00"	0.390		
	12'30"	0.390		
	15'00"	0.390		
	18'00"	0.389		
Bacterial α-amylase	1'45"	0.370	10'00"	1 ml enzyme reacting with 9 ml 0.08% soluble starch in 0.1 M sodium phosphate buffer of pH 6.9 at 30°C. ±0.1° scale.
	2'30"	0.365		
	3'05"	0.360		
	3'50"	0.355		
	4'30"	0.350		
	5'20"	0.345		
	6'15"	0.340		
	7'50"	0.335		
	10'00"	0.330		
	13'30"	0.324		
	24'15"	0.320		
	42'00"	0.320		
Human salivary α-amylase	1'45"	0.365	45'00"	0.8 ml enzyme reacting with 8 ml 0.08% soluble starch in 0.01 M sodium phosphate buffer of pH 6.8 at 25°C. ±0.1° scale.
	4'15"	0.370		
	5'00"	0.368		
	11'00"	0.368		
	13'15"	0.368		
	16'30"	0.365		
	19'15"	0.363		
	30'00"	0.360		
	45'00"	0.360		
	60'00"	0.355		

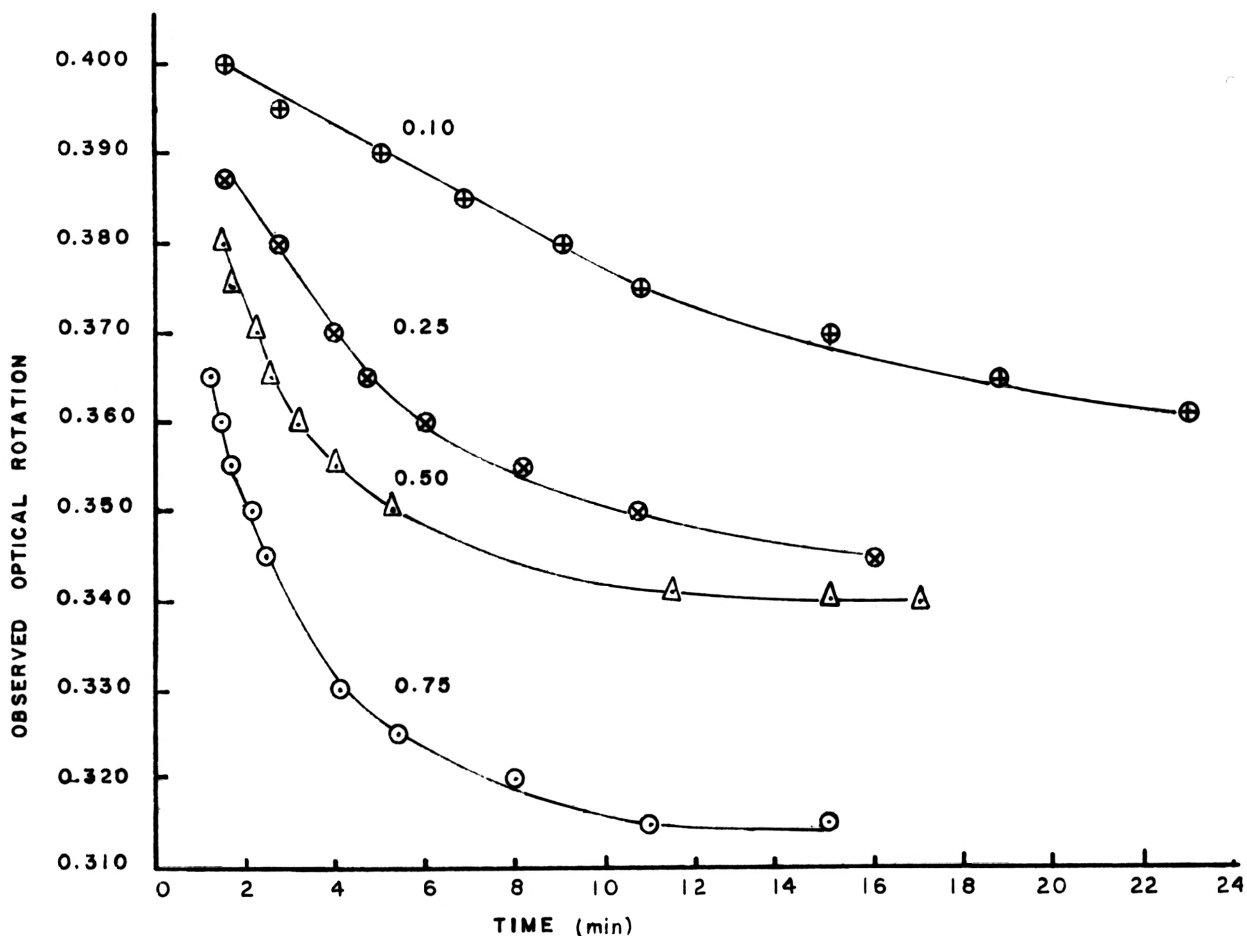


Fig. Ia. Optical rotatory changes during hydrolysis of 0.08% soluble starch by β-amylase in 0.1 M sodium acetate buffer of pH 4.8 at 30°C. Dilutions of β-amylase stock solution were 0.1, 0.25, 0.50, and 0.75.

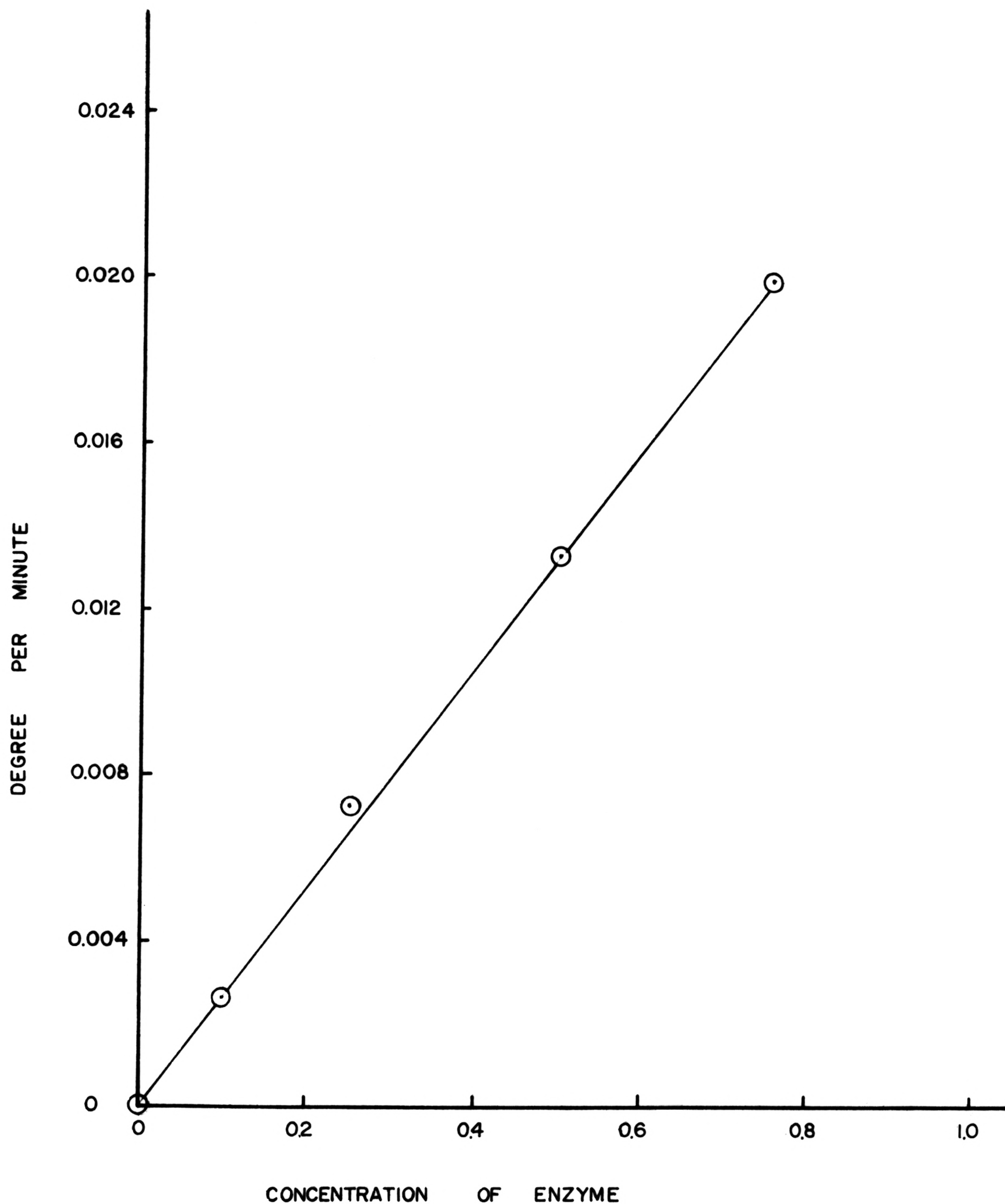


Fig. Ib. Initial slopes of Fig. Ia. versus β-amylase concentrations.

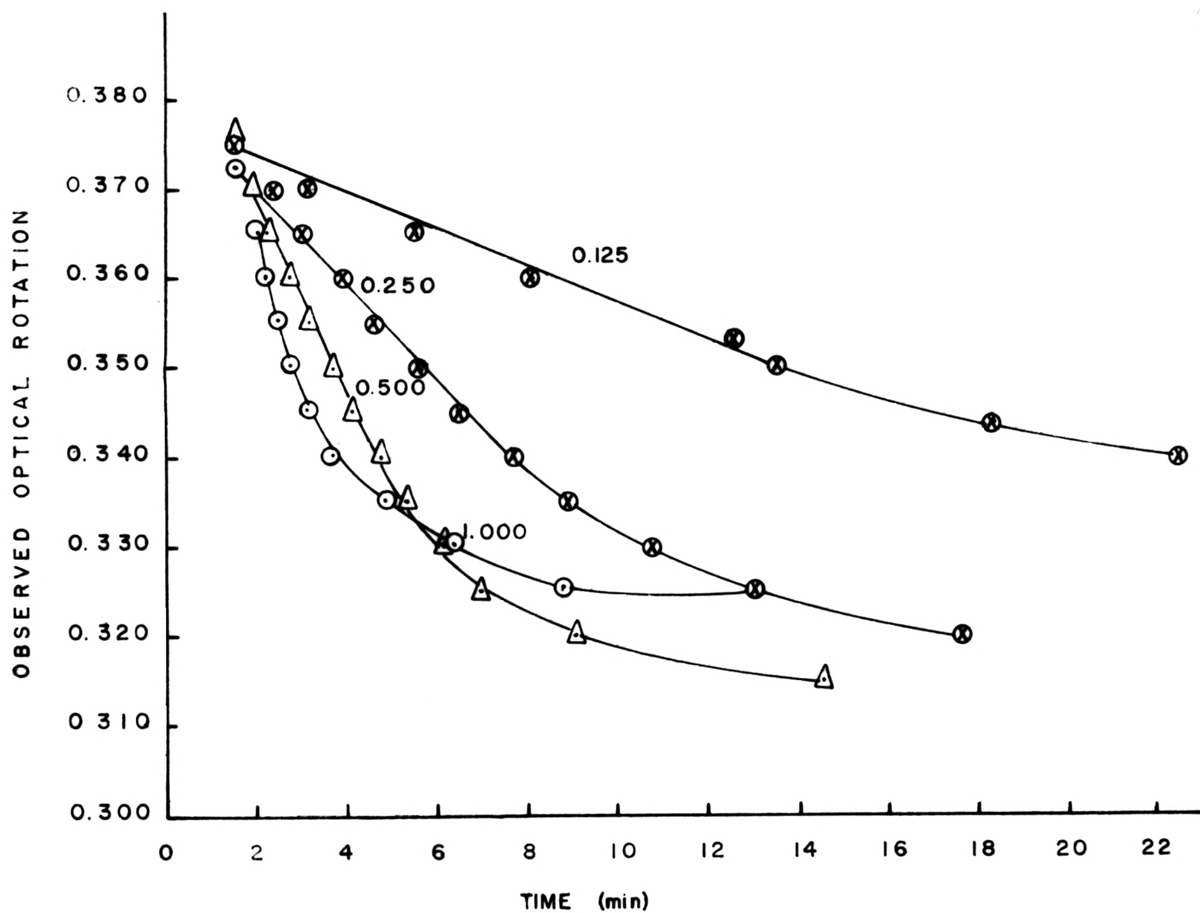


Fig. IIa. Optical rotatory changes during hydrolysis of 0.4% soluble starch by β-amylase in 0.1 M sodium acetate buffer of pH 4.8 at 30°C. Dilutions of β-amylase stock solution were 0.125, 0.250, 0.500, and 1.000.

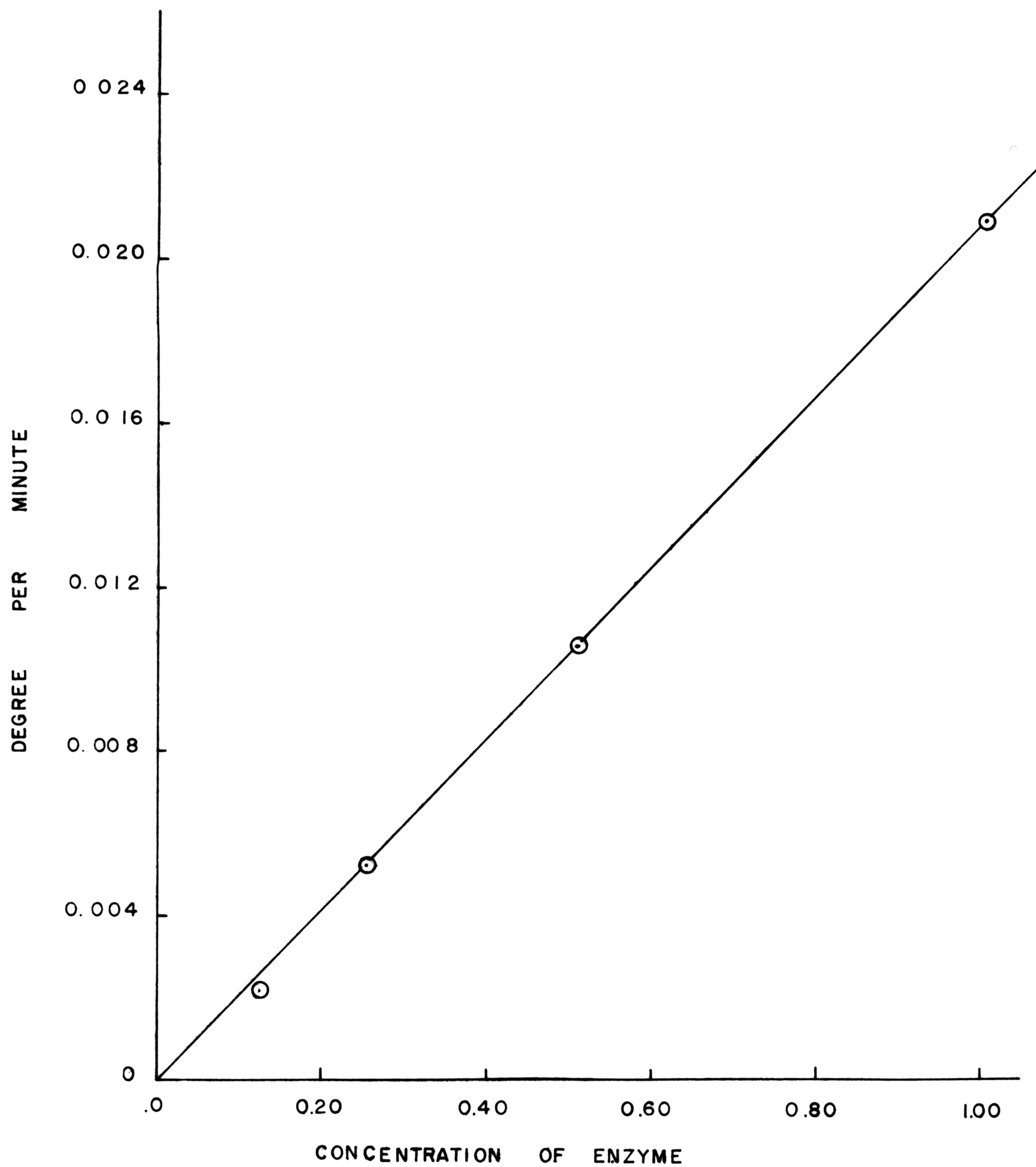


Fig. IIb. Initial slopes of Fig IIa. versus β-amylase concentrations.

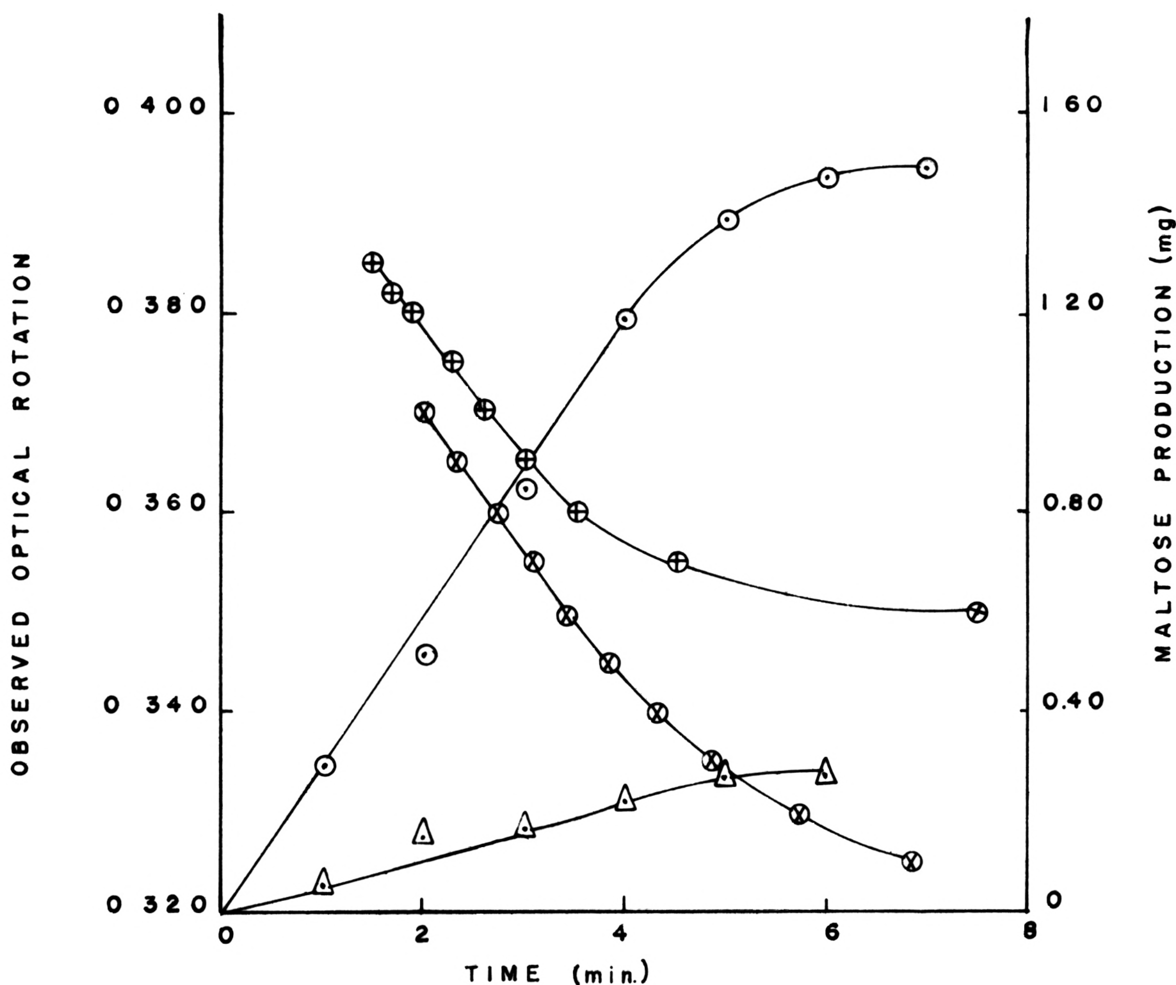


Fig. IIIa. Optical rotation and maltose production during hydrolysis of starch by β-amylase in 0.1M acetate buffer of pH 4.8 at 30°C.

⊕-⊕ : optical rotation of 0.08% starch

△-△ : maltose production in 0.08% starch

⊗-⊗ : optical rotation of 0.4% starch

⊙-⊙ : maltose production in 0.4% starch

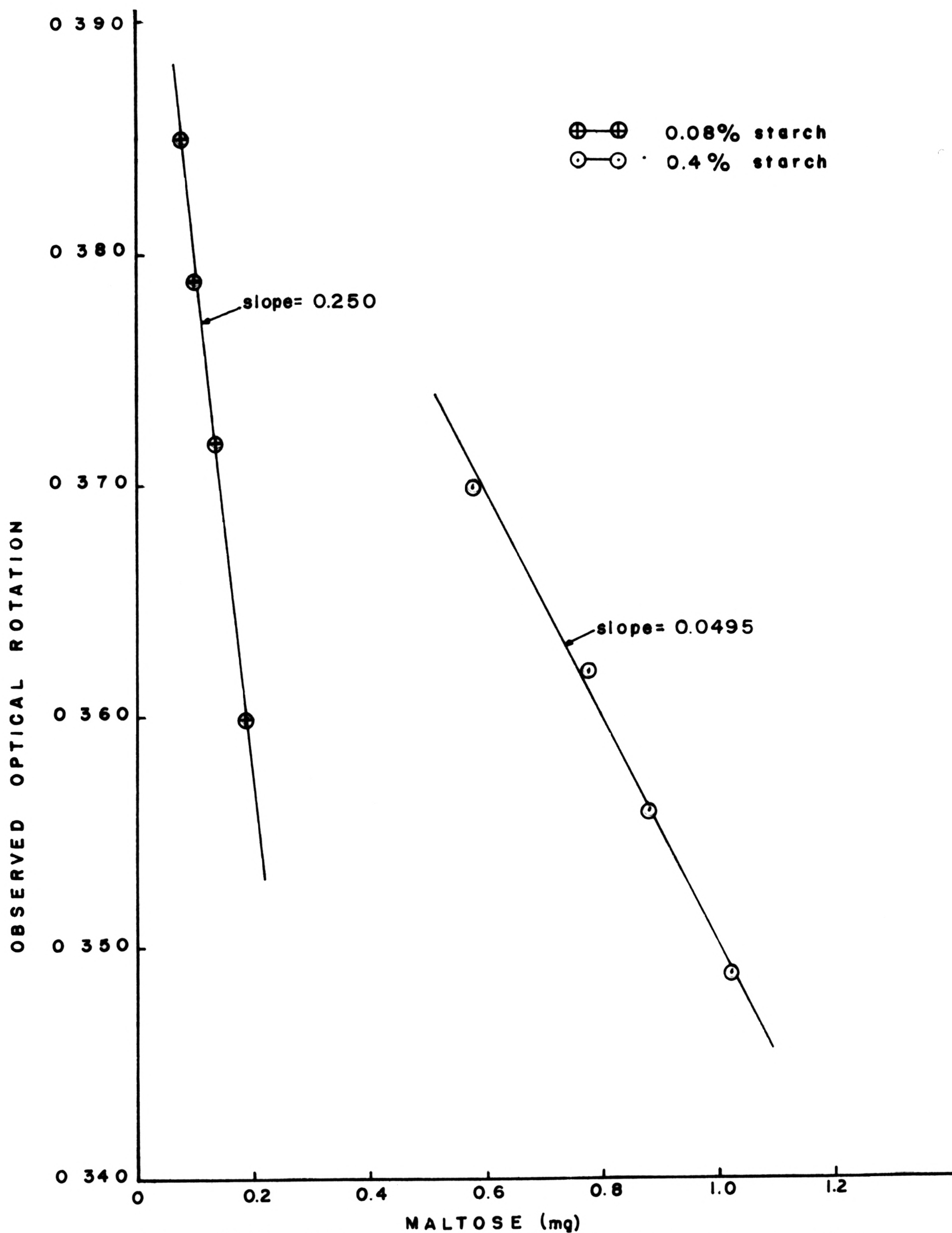


Fig. IIIb. Observed optical rotation versus maltose production during hydrolysis of soluble starch by β-amylase in 0.1M sodium acetate buffer of pH4.8 at 30°C.

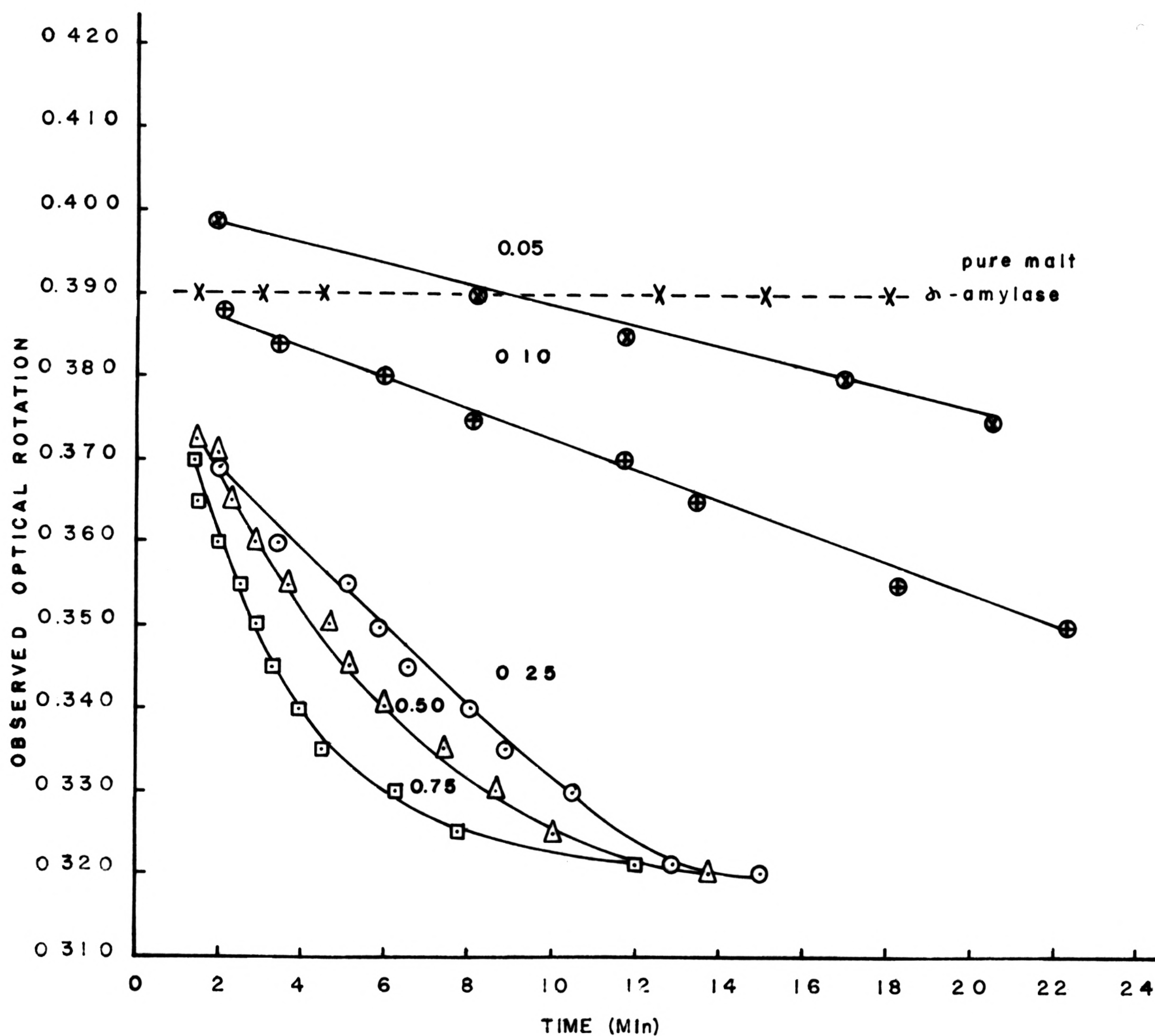


Fig. IVa. Optical rotatory changes during hydrolysis of 0.08% soluble starch by mixture of β and malt α-amylase in 0.1 M sodium acetate buffer of pH 4.8 at 30°C. Concentrations of enzyme were expressed in terms of β-amylase content.

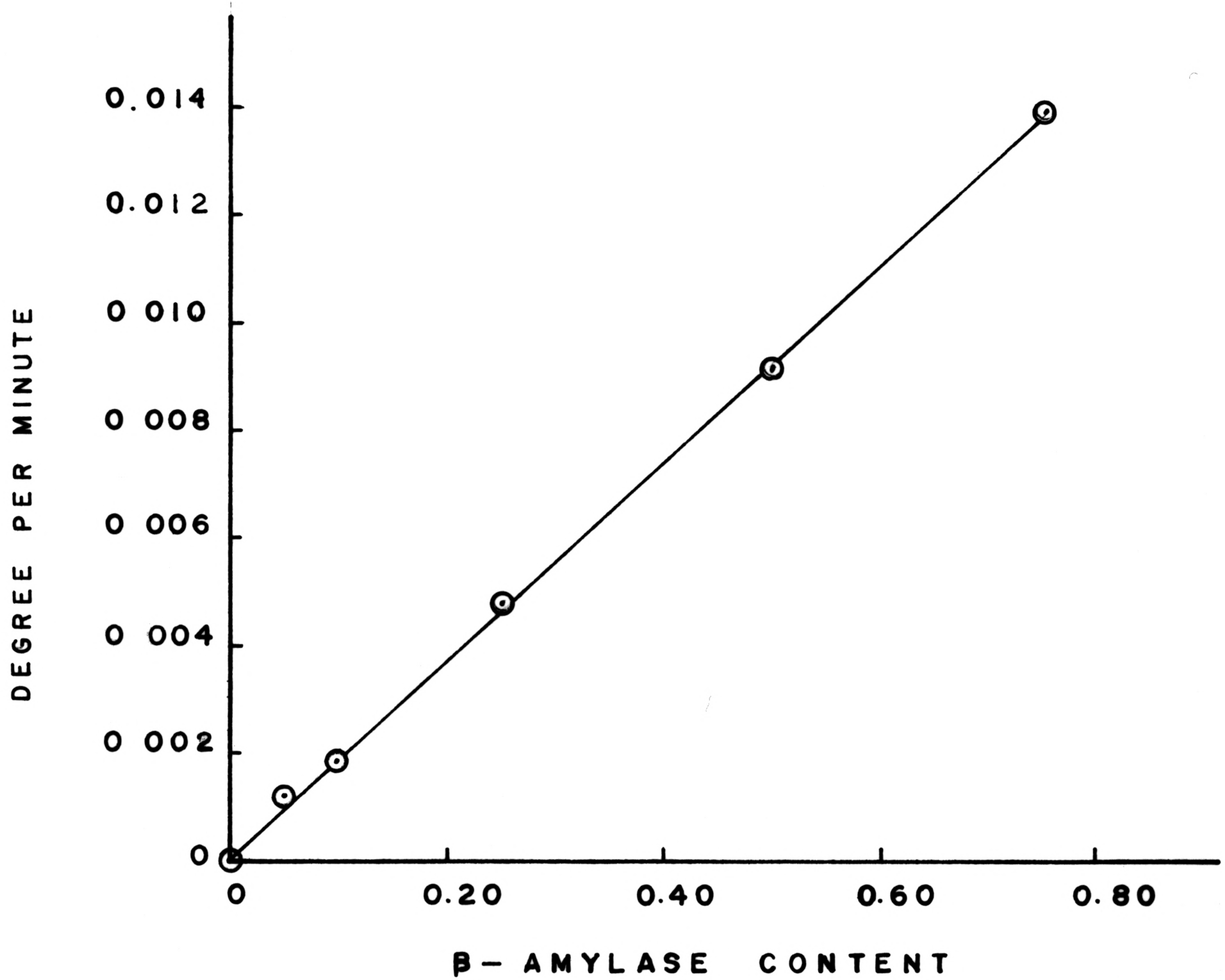


Fig. IVb. Initial slope taken from Fig. IVa versus β-amylase concentrations in the enzyme mixture.

Plate I. Hydrolysis products of 0.08% soluble starch by malt α-amylase to the achroic stage in 0.1 M sodium acetate buffer of pH 4.8 at 30°C.

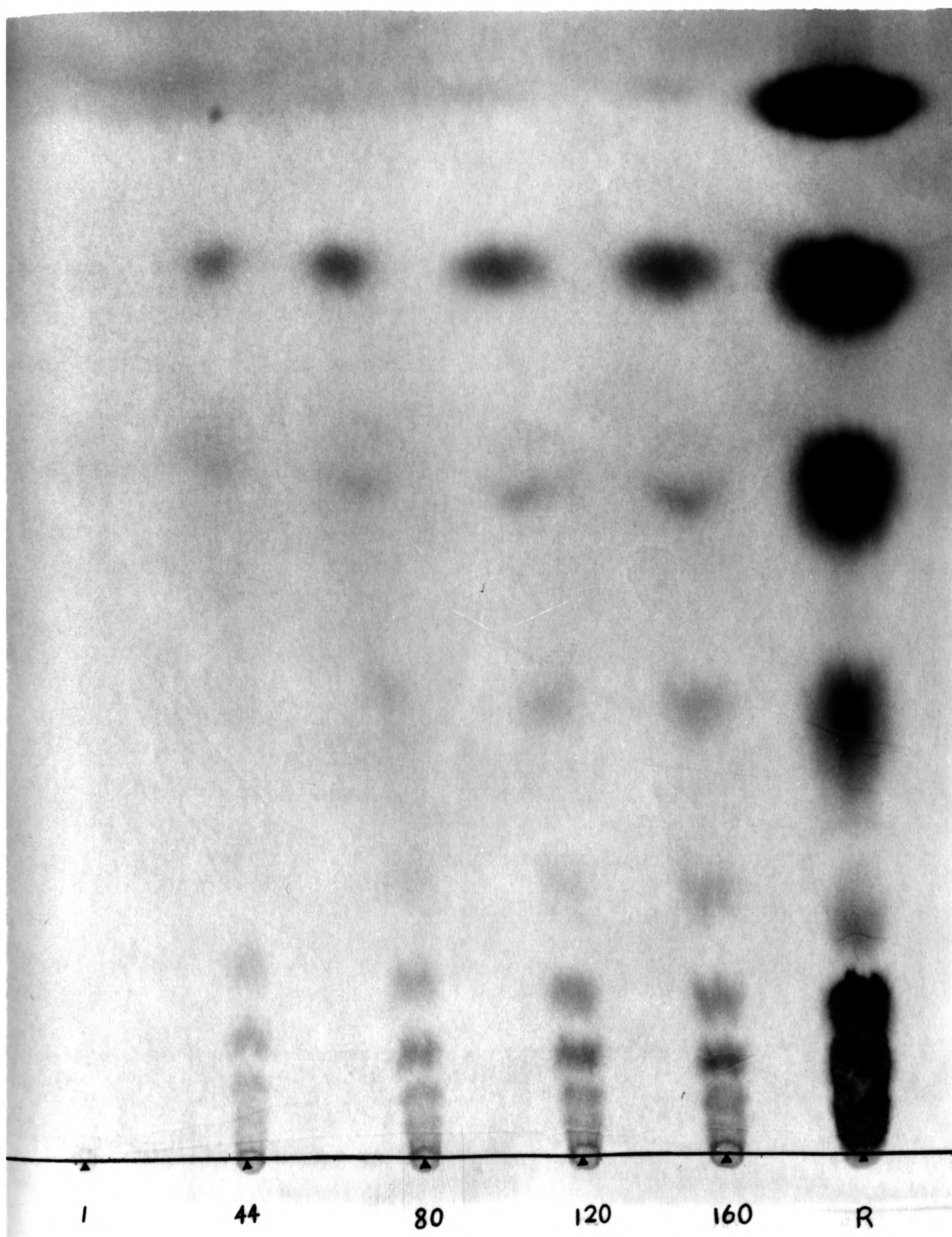
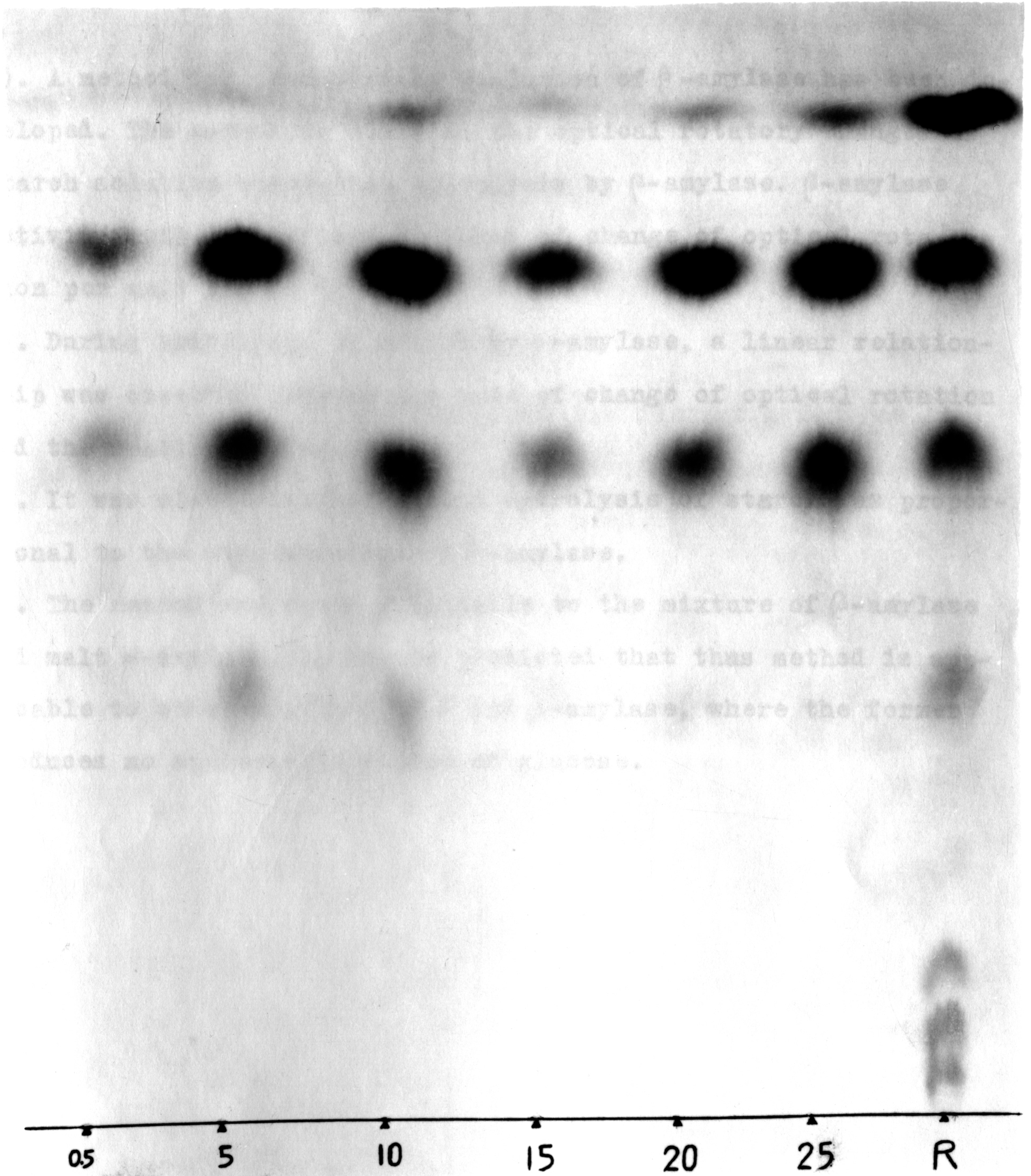

1
44
80
120
160
R

Plate II. Hydrolyzed products of 0.1% soluble starch by bacterial α-amylase to the achroic stage in 0.01 M sodium phosphate buffer of pH 6.9 at 30°C.



05
5
10
15
20
25
R

SUMMARY

1). A method for quantitative evalution of β-amylase has been developed. The method is based on the optical rotatory changes of starch solution undergoing hydrolysis by β-amylase. β-amylase activity unit was defined in terms of change of optical rotation per unit time.

2). During hydrolysis of starch by β-amylase, a linear relationship was observed between the rate of change of optical rotation and the reaction velocity.

3). It was also established that hydrolysis of starch was proportional to the concentration of β-amylase.

4). The method was found applicable to the mixture of β-amylase and malt α-amylase. It may be predicted that thus method is applicable to other mixtures of α and β-amylase, where the former produces no appreciable amount of glucose.

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DETERMINATION OF BETA-AMYLASE BY OPTICAL ROTATORY CHANGES

by

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The methods that have been most commonly used for determination of β-amylase are based on evaluation of maltose produced from starch. This is usually done by determining as reducing sugar and the activity unit expressed as the amount of enzyme required to produce a certain amount of maltose within a certain period of time under given conditions. These methods, however, are not applicable to the mixtures of α and β-amylase, because α-amylase also produces reducing sugars. It has been a problem to detect the contamination of β-amylase during the purification of some α-amylases from plant sources. The method developed here determines maltose by measuring the decrease in optical rotation due to production of maltose during hydrolysis of starch. The activity unit is defined as that amount of enzyme which will produce a change in optical rotation corresponding to the production of β-maltose hydrate at a rate of 1 mg ml^{-1}min.. The instrument used in this work was a type 143A ETL-NPL Automatic Polarimeter. It is an electronically controlled self-balancing photoelectric polarimeter. To be a quantatative method, the linear relationship between maltose production and change of optical rotation of the hydrolyzing mixture was confirmed. Maltose was determined by the Somogyi method(30) and the optical rotations were taken with the polarimeter. Secondly, the rate of change of optical rotation was proved to be proportional to the amount of enzyme used at each time. When this method was applied to the mixture of malt α and β-amylase and hydrolysis of soluble starch carried out at 30°C, the same proportional relationship was found between the rate of change of optical rotation and β-amylase contents in malt α-amylase. This included

the zero value in optical rotatory changes for pure malt α-amylase. A chromatographic analysis of the degradation products of soluble starch by malt α-amylase revealed that the relatively constant reading in optical rotation during hydrolysis might be due to the formation of maltose, maltohexaose, maltoheptaose, and maltooctaose as the main products at the early stage of hudrolysis. It was postulated that the method may be applicable to those α-amylases that produce no significant amount of glucose.