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State of aggregation of amylose in aqueous solutions and its relationship to iodine complex formation

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NOTE TO USERS

STATE OF AGGREGATION OF AMYLOSE IN AQUEOUS SOLUTIONS AND ITS RELATIONSHIP TO IODINE COMPLEX FORMATION

by

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I. INTRODUCTION

At present two somewhat extreme theories have been proposed to explain the interaction of amylose and iodine. Some investigators, notably K. H. Meyer, are of the opinion that the formation of the amylose-iodine complex is an adsorption phenomenon, the iodine being adsorbed in the interstices of an aggregate of amylose. Others adhere to a helical theory which stipulates that the amylose-iodine complex is in the form of a helix with the iodine arranged linearly within the coil, held in place by dipole-induced dipole interaction. An important aspect of the helical theory concerns the behavior of iodine toward amylose molecules of different lengths. Experiments by F. L. Bates have indicated that longer amylose particles complex at a lower free iodine concentration than shorter molecules. This implies that the sigmoidal character of the potentiometric iodine titration curve represents heterogeneity within the amylose.

Recent investigations have indicated that the linear fraction of starch is heterogeneous with respect to molecular weight. This is particularly true of corn amylose for which variations of twenty fold have been reported for some fractions.

This investigation was begun with the view of determining a molecular weight distribution from the iodine titration curves. However, before much progress could be made in this direction, it was necessary to establish equilibrium between iodine and amylose during the titration. These conditions are not attained in the performance of a normal titration.

With this problem in mind, many varied experiments were performed in establishing some of the factors which influence the ability of amylose to bind iodine. As these experiments progressed, it became increasingly evident that aggregates of amylose were responsible for some of the effects observed.

Carried on concurrently with these experiments was a spectrophotometric investigation of the amylose-iodine complex with particular emphasis upon the behavior of the wave length of maximum absorption as a function of temperature. A critical investigation by R. R. Baldwin of the absorption spectra of the amylose-iodine complex had indicated that the wave length of maximum absorption was related to the molecular weight of amylose. Furthermore, evidence had accumulated to show that larger molecules were capable of forming a more stable complex and consequently should resist disassociation at elevated temperatures more than short molecules. This suggested that a determination of the wave

length of maximum absorption at various temperatures should indicate a distribution of molecular weights. After experiments of this nature were performed on several amyloses, evidence was secured which also indicated that aggregates were responsible for the results obtained.

The problem of determining a molecular weight distribution was hence abandoned in favor of an investigation to confirm the presence of amylose aggregates by the light scattering technique. Not only was it necessary to relate these findings with the preceding potentiometric iodine titrations and spectrophotometric experiments, but also it was of interest to attempt a correlation of these results with the existing theories concerning the nature of the amylose-iodine complex.

II. HISTORICAL

A. Events Leading to the Present Concept of Starch

Developments in the general field of starch chemistry in the past decade have served to define more clearly the nature of the chemical valences of starch. However, the physical forces which are operative within the granule are not so clearly understood. The presence of crystalline regions has been established by X-ray diffraction studies and also from the optical properties. Much effort has been expended in constructing models of the starch granule. Most of this work has been recently reviewed (43, p. 157-178, 53, 29).

No attempt will be made to review all of the literature leading to the present concept of the chemical bonds in starch. A comprehensive literature survey has recently been made by Kerr (43).

It has been well established that starch consists of two major components: a linear and a branched fraction. Maquenne and Roux (48) were the first to introduce the names amylose and amylopectin for the linear and branched component respectively. This nomenclature was later adopted by Meyer (53), who was probably responsible for its widespread use.

Methylation studies in the late 1920's on starch illustrated a predominance of 2,3,6-trimethyl glucose indicating either an α-1:4 or an α-1:5-glucosidic linkage. Haworth and workers (34) and also Irvine and Macdonald (40), by repeated treatment with dimethyl sulfate and alkali, methylated all of the hydroxyl groups to get an almost quantitative yield of 2,3,6-trimethyl glucose. Also it was observed that upon enzymatic hydrolysis of starch, a high yield of maltose resulted, which indicated that most of the glucose units were united by α-1:4-glucosidic linkages. Freudenberg and Friedrich (26) succeeded in isolating from methylated starch a small amount of 2,3,4,6-tetramethyl glucose in addition to 2,3,6-trimethyl glucose.

During this time evidence was accumulating that a linear component could not account for all of the properties exhibited by starch, since a linear structure should behave in a manner similar to cellulose.

Staudinger and Eilers (82) concluded that large units of starch consisted of definite molecular entities rather than indefinite micelles composed of many small molecules held together by secondary valences. This conclusion was based upon the results of viscosity studies on starch and its methylated derivatives. The measurement of molecular sizes in several solvents gave comparable values for the original, the acetylated and the regenerated starch. Later

Staudinger (83) suggested a branched structure to explain the discrepancies between physical measurements and end group assay.

Freudenberg and Boppel (28) supplied additional evidence that a branched structure might exist by isolating a small amount of 2,3-dimethyl glucose from fully methylated starch. The indications are that, in addition to α-1:4-glucosidic linkages, α-1:6-glucosidic linkages are also involved. These results were obtained with whole starch and did not indicate which fraction contained the branched structure, or indeed did not show that two components were involved.

Recently Halsall and coworkers (32) have criticized the work of Freudenberg concerning the existence of the α-1:6-glucosidic linkage. Their argument arises from the experimental difficulties encountered in the complete methylation of amylopectin and the fact that trimethyl glucose tends to undergo demethylation. The above workers attempted to minimize these difficulties by use of disaggregated, low-molecular weight starch. However, 2,3-dimethyl glucose again predominated.

A direct proof for the existence of α-1:6-glucosidic linkages may be furnished by Montgomery, Weakly and Hilbert (56). They reported the isolation of crystalline derivatives of 6-α[-D glucopyranosyl]β-D-glucose after hydrolyzing waxy cornstarch with purified amylolytic enzyme.

Pascu and Hiller (60, 61) reported a new concept for the structure of starch in which small molecules are incorporated into the starch chain. The simple sugars are visualized as existing in their hydrated, open chain form to interrupt the normal sequence of α-1:4-glucosidic bonds. These small molecules supposedly hold the long chain molecules together by acetal or hemiacetal bonds. Presumably these bonds are very easily split by hydronium ions even at a pH of 5.9 to 6.3 during autoclaving.

This concept has been criticized by Halsall, Hirst and Jones (31) on the basis that more than one mole of formic acid would be obtained per end group. Periodic acid is specific for contiguous hydroxyl groups producing formic acid in the case of three or more contiguous groups and yields a dialdehyde for the two adjacent hydroxy groups (15, 42). Bourne and Peat (14) objected to Pascus's formulation on the grounds that the semiacetal link would be resistant to acid hydrolysis and that the amylose separated from potato starch under alkaline conditions by the use of aluminum hydroxide (12) could not have originated in the partial hydrolysis of amylopectin.

In connection with the above arguments concerning Pascu's concept of starch it should be pointed out that both Pascu (60) and Bourne (14) were able to isolate a fraction from a waxy maize sample which behaved like amylose. Pascu

was of the opinion that this amylose resulted from the splitting of hemiacetal linkages, whereas Bourne and Peat considered amylose to be initially present.

B. Amylose-Iodine Complex

1. Theories concerning its constitution

Since the discovery of the blue starch-iodine complex by deClaubry in 1814 (20), a controversy has existed concerning the interaction of iodine and starch. Barger (3) and Radley (67) have presented a historical account of much of the early work done in attempting to characterize the starch-iodine complex. The debate has been concerned with whether an adsorption complex of variable composition was formed between starch and iodine, or whether a true stoichiometric relationship existed. At least fourteen different compounds have been suggested as representing the composition of the starch-iodine complex. Rouvier (70) was of the opinion that compounds of the general formula $(C_6H_{10}O_5)_{16}I_n$, where n equals 2, 3, 4 and 5, are formed as the amount of iodine is increased. Lottermoser (47) considered the reaction between iodine and starch as an adsorption phenomenon and obtained typical adsorption curves supposedly showing this behavior.

The features of the starch molecules which give rise to

the blue color have been the cause of much speculation. Some investigators have considered the bridge oxygen atoms in the pyranose ring of glucose to be centers of adsorption for iodine in the formation of the complex (8, 9). Their reasoning was based upon the observation that many other compounds which contain an internal oxygen bridge were found to produce a blue-black color with iodine.

In this connection Jackson and Hudson (41, 42) disrupted the pyranose ring between positions 2 and 3 by means of periodic acid to yield a product which does not show an iodine reaction. Furthermore, it has been shown that free hydroxyl groups are not essential to the formation of the blue complex since fully methylated starch is able to exhibit the blue color (55).

A new idea concerning the configuration of starch chains in aqueous solutions was introduced by Hanes (33) to explain the appearance of dextrine of six glucose units when starch was treated with α-amylase. Hanes suggested that the starch chain might be in the form of a spiral in which each turn contained six glucose units.

These ideas were expanded by Freudenberg (27) to account for the blue complex formed between starch and iodine. His models showed that it was possible to have helices with a hydrocarbon lining and the hydroxyl groups protruding outward.

An extensive investigation of this concept was carried out by Bear and coworkers. This work has been recently reviewed by Rundle and Hixon (74) and by Kerr (43, p. 157-178). The essential features of the model as verified by these workers are that the helix is 12.97 angstroms in diameter and the length of a turn in the helix corresponds to 7.91 angstroms (73).

The long axis of the iodine molecule was found to be parallel to the long axis of the starch iodine complex (71).

Later it was postulated that the interaction between starch and iodine in the amylose-iodine complex was dipolar and that additional stabilization may occur as a result of the formation of resonating polyiodine chains (85).

A controversial feature of the helical concept concerns the role of the individual amylose molecules. According to the proponents of this concept, the individual molecules are coiled around a linear array of iodine molecules and do not necessarily depend upon an aggregate for complex formation. It has been demonstrated that the larger molecules have a greater tendency to bind iodine. Also if half the theoretical amount of iodine is added to a heterogeneous amylose solution, the iodine will not be distributed between all of the molecules but will be complexed with half of the amylose (72).

In spite of the strong arguments presented in favor of a helical mechanism, not all workers agree with the concept. Meyer (53) explained the reaction between amylose and iodine as an adsorption phenomenon in which iodine is deposited in the interstices of colloidal particles. Some of his arguments were based on the blue color produced by colloidal or poorly crystallized materials and oxygen bridged, cyclic compounds.

Bernfeld and Meyer (10) investigated the equilibrium involved in the formation of the starch-iodine complex. Their conclusion was that several molecules of starch reacted with several molecules of iodine to form a complex expressed by the equilibrium $K\,[\text{starch-iodine}] = [\text{starch}]^n(I_2)^m$ where $n, m > 2$. The implication of this equilibrium is that one molecule of amylose does not react with several moles of iodine, as advanced by the helical theory, but that an adsorption of iodine by an amylose aggregate is occurring.

Also Meyer (53) believes that it is not coincidental that linear molecules have a greater ability both to aggregate and also to complex. Furthermore, the longer linear molecules have a still greater tendency to aggregate and a correspondingly greater ability to complex.

2. Potentiometric iodine titration

The introduction of the potentiometric iodine titration (5) to the study of starch and its fractions has helped to place the two component concepts of starch on a sound basis. Previous to the adoption of the titration procedure a good criterion for determining the purity of amylose fractions was lacking. This method, besides being convenient for the determination of the quantity of iodine bound by amylose, offered a means of following the formation of the complex throughout the titration. According to the helical theory, a sigmoidal type of titration curve indicates that solutions of amylose are heterogeneous with respect to chain length. This implies that a preferential absorption of iodine occurs during the titration. Substantiating this implication was the discovery that the free iodine concentration during the formation of the complex is characteristic of the amylose preparation (5, 72). This behavior was attributed to the difference in chain length of the various amyloses. Viscosity and osmotic pressure measurements apparently confirmed this view (24, 25).

Recent use has been made of the potentiometric method as an analytical instrument in the determination of the amylopectin impurity in starch and impure amylose. It was found to be more accurate for this purpose than spectrophotometric measurements on the complex (39).

Some variations have been made in the experimental procedure by the use of two iodine electrodes instead of the single electrode in combination with a calomel reference electrode (30, 79). It was claimed that better results were obtainable in regions of high free iodine concentration which is necessary in the titration of amylopectin.

The potentiometric iodine titration has been used in determining the influence of the iodide ion on the composition of the amylose-iodine complex. Bates, French and Rundle (5) found that the amount of iodine bound by the amylose component of starch varied inversely with the iodide concentration. A later investigation by Baldwin, Bear and Rundle (2) disclosed that a decrease in the iodine content of the helices from one molecule per six glucose units to about one per eight occurred when the concentration of the iodine was increased through a wide range. These authors concluded that a partial replacement of iodine by tri-iodide ions had taken place.

Gilbert and Marriott (30) found that during the early stages of the titration when the iodine concentration was low, the amylose complex was associated with a molecular ion of composition (I_8^-). For a given iodide concentration the number of iodine molecules in the complex increases steadily with an increase in iodine activity.

3. Absorption spectrum of the iodine complex

A method somewhat similar to the potentiometric iodine titration in its applicability to the study of starch and starch fractions is based on the absorption spectrum of the iodine complex. This method, unlike the potentiometric iodine titration, has been in use for many years.

Recently Baldwin (1) has made an extensive investigation of the starch-iodine complex and its relation to the structure and configuration of starch. A correlation was made between the molecular size (25) of the various types of amylose and the absorption maximum, molecular extinction coefficient, and characteristic potential (4). These results indicated that the absorption maximum and molecular extinction coefficients increase with the molecular weight of the amylose. The characteristic potential, which is a measure of the free iodine concentration when fifty per cent of the iodine is bound, decreases as the molecular weight increases.

An important factor influencing the formation of the starch-iodine complex is temperature, recognized early by Lassaigne (46). When heated to 80° or 90° C. the complex is destroyed. It reforms upon cooling, but not to the original intensity.

Much of the literature concerning the behavior of the starch-iodine complex upon heating has been reviewed by

Radley (67). Some of the reasons given for the change in color of the complex upon heating include the greater affinity of hot water for starch particles (64), the volatilization of the iodine upon heating with a concurrent formation of a colorless compound between starch and residual iodine (63), and the expansion of starch aggregates upon heating with a reaggregation upon cooling to cause the blue color to reappear (62).

It was also found that the blue color of the complex may persist at elevated temperatures provided a large excess of iodine was present (6). Another interesting observation was made showing that the addition of hydrochloric acid to the starch-iodine complex permitted the color to remain even at boiling temperatures (81).

C. Recent Advances in Starch Fractionation

1. Methods of effecting starch dispersion

Most of the methods for separating the two major components of starch depend upon the progressive swelling of the starch granule until a homogeneous sol or paste is formed. The most widely used method of accomplishing dispersion involves use of the autoclave at fifteen to twenty pounds pressure. Much criticism has arisen concerning the damage of starch fractions at these temperatures.

Higgenbotham and Morrison (39) have studied the effect of time, temperature and rate of stirring on the dispersal of various types of starch pastes in aqueous solution. To detect chemical modification viscosity measurements were carried out on the triacetates. They concluded that the degree of modification during autoclaving was dependent upon the type of starch used as well as time, temperature and rate of stirring. The yield of amylose was found to be practically the same whether or not complete dispersion was attained; however, the amylose obtained with incomplete dispersion was believed to be contaminated with amylopectin.

Meyer and coworkers (51) found that, in addition to a decrease in viscosity, a small increase in the copper number was observed when starch was heated at a pH of 6 for two hours at 126° C.

It is possible to circumvent the modification of starch fractions due to autoclaving by other fractionation procedures. Some workers have prepared amylose by leaching the swollen starch granules with hot water at temperatures near 70° (54). However, it is evident that complete separation of amylose is not accomplished. Although a product is obtained which is modified to a minor extent if at all, the yield of amylose is poor and contaminated with some branched material. The purity, however, may be improved by recrystallization with butyl alcohol (44).

Bois and Vallieres (11) obtained crystalline amylose by dispersing starch in .5 N. sodium hydroxide (freed from oxygen) at 37° for several days until a clear solution was obtained. The amylose was crystallized with butyl alcohol after dialysis of the basic solution against distilled water. These authors claimed to have obtained a product which was not degraded.

Meyer and workers (52) have studied the solubility in a large variety of solvents and found that starch could be dispersed in 33 per cent aqueous chloral hydrate, hydrazine hydrate, ethylenediamine hydrate, lithium bromide and zinc chloride.

In the performance of experiments of a fundamental nature, such as determining the chain length distribution of amylose in a particular variety of starch, it is necessary to have all of the amylose present in a form which is not degraded. This may be impossible since the branched and unbranched fractions may be associated in such a manner that conditions which will disperse them will cause some degradation.

The use of aqueous solutions of pyridine in dispersing starch has been known for some time. Hess and workers (37, 38) used various mixtures of pyridine and water in the acetylation of starch. In fact one of the oldest methods for the separation of amylose from starch makes use of the

fact that amylose precipitates when a dispersion of starch in hot aqueous pyridine is cooled (69).

Mullen and Pascu (57) have reviewed numerous methods of preparing starch esters and concluded that their method of dispersing starch in an azeotropic mixture of pyridine and water (60 per cent pyridine) with subsequent esterification gave an ester which was degraded very little. They also concluded that all other methods given in the literature for the preparation of starch esters lead to degradation. These authors studied the gelatinization temperature of starch as a function of pyridine concentration and concluded that the lowest gelatinization temperature occurred with 30 per cent pyridine.

The investigation of Higginbotham and Morrison (39) disclosed that hot aqueous solutions of pyridine were better solvents for starch than aqueous solutions which were autoclaved.

It can be gathered from the above discussion that aqueous solutions of pyridine may serve in two capacities: at elevated temperatures as a dispersing agent and at low temperatures as a complexing agent.

2. Various fractionating agents used

Most of the older fractionation methods have been adequately reviewed by Schoch (76) and Kerr (43, p. 157-178).

It is sufficient to say that before 1940 fractionations in general gave only a partial separation of the components. The characterization of the components was hampered because of a lack of methods which since have been devised. Briefly, the older methods include aqueous leaching of gelatinized starch granules, electrosedimentation, selective adsorption, retrogradation and use of enzymes.

Recent improvements have made it possible to crystallize amylose in a much purer form. Schoch (77) used butyl alcohol to precipitate the amylose component after removing fatty material and autoclaving for two or three hours at 18 to 20 pounds pressure. Later Schoch (76) used pentasol as the preferred method which gave a greater yield of amylose but possibly included some material originally classified as amylopectin. Haworth, Peat and Sagrott (36) and also Bourne and coworkers (13) introduced the use of thymol as a precipitating agent and claimed a sharper separation of the starch components. The starch paste was prepared by dispersing starch in boiling water for half an hour and cooling before the addition of thymol. Later Haworth and Peat (35) expanded the number of complexing agents to include cresol, carvacrol, chloro- and bromo-nitrophenol. The same method for preparation of starch paste was used. Schoch and Williams (78) used fatty acids to separate the amylose component of starch. A yield of 29 per cent of the original

defatted cornstarch was obtained. Whistler and Hilbert (87) found that many different classes of organic compounds, such as esters, ketones, mercaptans, carboxylic acids and nitroparaffins, which were capable of forming hydrogen bonds with amylose, form complexes with amylose. In particular they studied the formation of crystalline amylose-nitroparaffin complexes from nitroethane, 1-nitropropane and 2-nitropropane. The latter two complexing agents compared favorably with butyl alcohol. The disperson of the starch was accomplished by autoclaving under steam pressure.

Bourne and coworkers (12) introduced a method by which the order of complexing was reversed. By the use of aluminum hydroxide they were able to absorb the amylopectin from the solution and leave an amylose fraction which was purer than a thymol precipitated amylose although a lower yield of 13 per cent maximum was obtained.

D. Molecular Weight of Starch Fractions

There is at present no adequate information concerning the molecular weight of amylose and amylopectin. The older molecular weights were too low and those obtained more recently from starch fractions, which presumably have a greater purity, vary considerably among themselves. Actually variations of 50 to 100 fold have been encountered in some instances for both the amylose and amylopectin

fraction. It is difficult to ascribe some of these large variations to the heterogeneity of the sample.

Both physical and chemical procedures have been used in attempting to arrive at satisfactory values for the molecular weights. Physical methods have in general been limited to those carbohydrate solutions which do not undergo an appreciable change with time. Neutral aqueous solutions of amylose have been avoided in most cases because of their tendency to retrograde.

Recently Potter and Hassid (65) have carried out molecular weight determinations on a variety of amyloses by osmotic pressure measurements on the acetylated derivatives. According to them, degradation was kept at a minimum by carrying out the reaction at room temperatures by using formamide as a solvent and acetylating with a mixture of acetic anhydride and pyridine. From osmotic pressure measurement of the acetylated amyloses in chloroform they obtained molecular weights for amylose ranging from 100,000 to 210,000 and for amylopectin from 1 to 6,000,000 when corrected to the weight of the deacetylated product. These values were compared with those found earlier (66) by a periodate oxidation method in which oxidation was performed at 2° C. with sodium periodate instead of periodic acid which has been used by other workers for an end group assay (15, 42). The molecular weights for the various amyloses

previously mentioned was 67,000 to 157,000. Potter and Hassid considered the discrepancies due to a slight branching in the amylose.

The effect of the organic solvent on the molecular weight of the acetates of corn amylose was studied by Cleveland and Kerr (17). They found that out of several selected solvents used, chloroform gave 70,000, the lowest molecular weight encountered. The conclusion was drawn that starch acetates tend to become insoluble in organic solvents when the molecular weight exceeds that of corn amylose. The above workers obtained a molecular weight of 73,000 for a pentasol fractionated, corn amylose sample when recrystallized with butanol. A pentasol fractionated sample not recrystallized with butyl alcohol gave a value of 80,000 (16). These fractions were obtained from autoclaved cornstarch sols.

Kerr (43, p. 194-196) was able to obtain molecular weights of less than 200,000 for corn amylopectin subfractions by using formamide as a solvent and acetylating with acetic anhydride and a pyridine catalyst.

Much lower values for the molecular weight of corn amylose have been reported by Meyer and coworkers. However, Meyer (52) obtained the low values by working with amylose extracted from starch with hot water at 70°. Fractions were obtained with molecular weights varying from 10,000 to

50,000. More recently, Meyer and coworkers (50) have obtained corn amylose fractions by a combination of two procedures. The fraction soluble in water at 70° was removed following his previous method, and the remainder of the amylose removed according to Schoch's method. The water soluble fraction had a molecular weight of 40,000 and the fraction of amylose remaining a weight of 340,000. The average molecular weight of both fractions was 72,000, which is in agreement with the results of Kerr. Meyer obtained his results by use of a method which involves a reduction of dinitrosalicylic acid by the aldehyde groups of amylose. This method was developed by Sumner (86) for the determination of glucose.

Lamm (45) determined the molecular weight of starch and amylose in aqueous solutions by use of ultracentrifugal measurements. His solutions were prepared by dispersing starch in concentrated aqueous zinc chloride, diluting with water and removing the zinc chloride by dialysis. Lamm found starch particles having molecular weights ranging from 100,000 to 1,000,000. With amylose, particles were detected having molecular weights of 300,000 to 1,800,000.

Samec (75), using aqueous solutions of amylose, found molecular weights of 200,000 from osmotic pressure measurement.

Much lower values were obtained by Beckmann and Landis

(7). They determined the molecular weight range of corn amylose prepared by dry grinding before dispersal in water and found a molecular weight range of from 17,000 to 225,000.

Foster and Hixon (25) determined the molecular weights of corn and tapioca amylose from osmotic pressure and viscosity measurements. They found the molecular weights to be 47,000 and 75,000 for corn and tapioca amylose respectively. Their measurements were carried out on the acetylated amyloses.

E. Light Scattering

The use of light scattering in the determination of size and shape of large molecules has advanced rapidly in the past few years. The theoretical aspects leading up to its present application to molecular weights of solutions of polymers has followed three distinct stages.

First, Lord Raleigh (68) treated the simplest case of an ideal gas at low density and derived an expression relating turbidity, τ, to the polarizability, α, of the gas:

$$\tau = \frac{8}{3}\pi \frac{(2\pi)^4}{\lambda} \quad n\alpha^2 \qquad (1)$$

where λ is the wave length of the light and n is the number of molecules per cubic centimeter. In this

treatment the particles are considered to be in a random position with respect to each other.

During the second stage, it became apparent that molecules in liquids, however, do not scatter light independently of each other. The total intensity is not a summation of individual particles as with ideal solutions of ideal gas. Smoluchowsky (80) considered the turbidity as a function of local fluctuations in density of particles which will give local inhomogeneities in the index of refraction and will scatter light. Einstein (23) extended Smoluchowski's theory for liquid mixtures in which a fluctuation in concentration causes further inhomogeneities in refractive index which causes the light to be scattered. The final equation as given by Einstein for liquid mixtures is:

$$\tau = \frac{8\pi^3}{3\lambda^4} \frac{RT\bar{V}_o}{N_o(-\partial\mu_o/\partial c)} (\partial\epsilon/\partial c)^2 \qquad (2)$$

where $\bar{V}_o$ is partial molal volume of solvent, N_o is Avagadro's number, μ_o is Gibbs chemical potential of solvent and ϵ is the optical dielectric constant.

The third stage was the modification of Einstein's equation by Debye (18, 19) to eliminate the chemical potential and optical dielectric constant term in equation (2) and introduce the measurable terms osmotic pressure and optical refractive index. This leads to the equation

$$\tau = \frac{32\pi^3}{3} \frac{n_o^2}{N_o \lambda^4} \frac{(c\,\partial n/\partial c)^2}{c\,\partial(P/RT)/\partial c} \qquad (3)$$

where n_o and n are the refractive index of solvent and solution respectively. Furthermore for dilute solutions the osmotic pressure may be written in the form

$$P = \frac{c}{M} RT + Bc^2 \qquad (4)$$

where B is the interaction constant. This leads to the final equation

$$\frac{Hc}{\tau} = \frac{1}{M} + \frac{2Bc}{RT} \quad \text{where } H = \frac{8\pi^3 n_o^2}{3N_o \lambda^4} (\partial n/\partial c)^2 \qquad (5)$$

The molecular weight is a weight average molecular weight (88). In practice the second term on the right is eliminated for molecular weight determinations by plotting $\frac{Hc}{\tau}$ versus c for a series of concentrations in which the slope determines the value of B. This value according to Oster (59) can be related to the size and shape of the solute molecules and to their interactions with each other and with the solvent. Furthermore the greater the solubility the more the particles deviate from spherical shape and thus, greater is the slope.

If the solute molecules are isotropic and small with respect to the wave length of light (less than one-tenth to one-twentieth), equation (5) may be applied without any correction. With particles of linear dimension greater than

one-twentieth the wave length of light, the particles no longer act as point scatterers, and for exact molecular weights a correction must be applied. This is generally referred to as a dissymmetry correction.

For particles which are small and isotropic, the scattered light is plane polarized and vibrates in a direction parallel to the electric vector of the incident light. Anisotropic particles do not behave in this manner. The oscillations induced by the incident light are in the direction of the greatest polarizibility of the particle. These oscillations have an oblique orientation which gives rise to a vertical and horizontal component and thus causes an increase in intensity of light scattered at 90°.

The depolarization correction has been found to reduce the molecular weight by 2 to 10 per cent (49).

Doty and Kaufmann (22) have studied the correction to be applied to the molecular weight of cellulose acetate and found the correction to be dependent upon the molecular weight with the depolarization value decreasing with molecular weight. Since on the basis of size alone the effect should be in the opposite direction, they conclude that anisotropy must be a sensitive function of molecular weight. Something may be found out about the size range and anisotropy of the particles by measuring three polarization values using polarized and unpolarized incident

light (59, 22).

Particles which have a linear dimension approaching the wave length of light will show dissymmetries of varying values. The dissymmetry is a ratio of light scattered at two angles equally spaced on either side of 90°, usually at 45° and 135°. This behavior arises from the fact that particles longer than about one-fifteenth the wave length of light do not behave as point scatterers but instead radiate light from all component dipoles. A phase interference results which depends on the distance the ray must traverse in getting to a particular segment of the particle and from there to the observer. The difference in total distance the two rays must travel is greater in the backward direction than the forward which causes a phase lag and destructive interference. Very little interference occurs in the forward direction. Consequently light which is scattered at 90° will show some decrease in intensity depending on the length of the particle, causing a decrease in the observed molecular weight. It is recalled that depolarization tends to increase the observed molecular weight.

Methods for making dissymmetry corrections have been described by Stein and Doty (84). The method requires that a model representing either a rod, coil or sphere be chosen

to represent the particle in question. From intrinsic dissymmetry measurements (dissymmetries extrapolated to zero concentration which is dependent only on a parameter which refers to the ratio between characteristic dimensions of the polymer and wave length) one is able to obtain a ratio of a characteristic dimension of the molecule to the wave length of incident light used. Graphs have been theoretically derived to give this ratio (89, 21, 58, 49). This ratio can now be introduced into one of three equations which relates angular intensity distribution to the angle of scattering and ratios of characteristic dimension to the wave length. Equations have been derived to give this relation for a rod, a sphere and a coil (58, 84). Since the ratio is now known and is a constant, a plot of intensity, I, versus the angle, θ , and extrapolated to zero° will give a maximum intensity which corresponds to forward scattering and is very little influenced by phase interference. Consequently the ratio I at 0° to I at 90° gives the desired correction factor. A characteristic dimension of the particle has also been obtained.

III. SOME FACTORS WHICH INFLUENCE THE WAVE LENGTH OF MAXIMUM ABSORPTION OF THE AMYLOSE-IODINE COMPLEX

A. Influence of Temperature on Wave Length of Maximum Absorption[1]

It is well known that the blue color of the amylose-iodine complex fades at elevated temperatures and reappears upon cooling. This behavior implies that the stability of the complex is greatest at low temperatures and decreases as the temperature rises. Near 80° the blue color disappears.

Baldwin (1) has shown that a correlation exists between λ_{max} for the blue complex and the iodine binding affinity as determined by Bates (4). Amyloses which possess a low binding affinity exhibit high values for λ_{max}. Evidently the same factors which are involved in the formation of the iodine complex and are responsible for low binding affinities are also responsible for the increased λ_{max}. The investigations of both Baldwin and Bates may be interpreted to mean that the stability of the amylose-iodine complex at a constant temperature is a function of the chain length of the amylose with the longer chains possessing the greater

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[1] Hereafter in this section the wave length of maximum absorption will be known as λ_{max}.

stability. Perhaps the longer chains also have a greater stability at elevated temperatures. If this is correct, it should be possible by simply heating to preferentially dissociate the complex formed by shorter molecules. Conversely, if an excess of iodine is added to hot solutions of amylose which are then allowed to cool slowly, the longer amylose molecules should perhaps complex first. Thus, it should be possible to observe this behavior by following λ_{max} as the complex is slowly formed or destroyed.

Experiments were performed with several different amyloses. First, λ_{max} was followed at various temperatures as the complex was heated. Next, the complex was gradually reformed by cooling slowly, and λ_{max} determined at the same temperatures.

The amylose solutions were prepared by dispersing the required amount of amylose in KOH to give a concentration of 0.267 per cent after neutralization with hydrochloric acid. Aliquots of these solutions were diluted to a final concentration of 0.002 per cent. The iodine concentration used for complex formation was 1.6×10^{-4} N. and the potassium iodide and potassium chloride concentrations were 5×10^{-3} N. and 3×10^{-2} N. respectively.

To those solutions which were heated a layer of mineral oil was added to help prevent the loss of iodine.

The instrument used to obtain the absorption data was a Model 14 Coleman Universal spectrophotometer. The solutions were contained in 19 x 105 millimeter, round cuvettes.

A surprising variation was observed between the maximum found at room temperature and that found in hot solutions of the complex. A higher value by between 35 and 40 millimicrons was found in the former case. This is about three times greater than the difference noted between a potato and a corn amylose preparation on which intrinsic viscosity measurements indicated a considerable difference in molecular weight. These low λ_{max} values obtained above 40° correspond to those produced by an acid modified, corn amylose preparation.

The behavior of λ_{max} was followed as the amylose-iodine complex was slowly heated. At each temperature selected for a measurement ten to fifteen minutes were allowed for the complex to attain equilibrium. In addition λ_{max} was followed in a similar manner on the same solution as the solution cooled.

The interesting observation was made that most of the decrease in λ_{max} occurred while the temperature was rising from 25° to 45°. Between 45° and 80° very little additional change was observed. Furthermore, upon cooling this solution and reforming the complex, the maximum followed about the same course with one exception. When the solution

was cooled from 45° to room temperature, a value was obtained which was about 20 millimicrons lower than that obtained before heating. Thus it appears that the overall effect of heating an amylose-iodine solution is to reduce λ_{max} by about 20 millimicrons. A temperature below 45° is capable of causing the major portion of the decrease. This behavior is shown in Figure 1 for a tapioca and corn amylose preparation.

A similar behavior in λ_{max} was observed by adding the iodine to a hot amylose solution and cooling. This was expected since most of the decrease in λ_{max} was observed previously to have occurred below 45°.

In this connection it was of interest to study the behavior of λ_{max} for amylose-iodine solutions after heating at 80° for various time intervals. One might expect an additional change to occur upon heating for longer periods of time if oxidative degradation or cleavage of the α-1:4 or α-1:6-glucosidic linkages occurred. The behavior of λ_{max} for a corn and tapioca amylose is shown in Table I. The experiment was performed by adding an excess of iodine to several amylose solutions heated to 80°. The solutions were held at 80° for zero, two, and four hours and then quickly cooled and λ_{max} determined. The corn amylose solution exhibited only a slight further decrease after

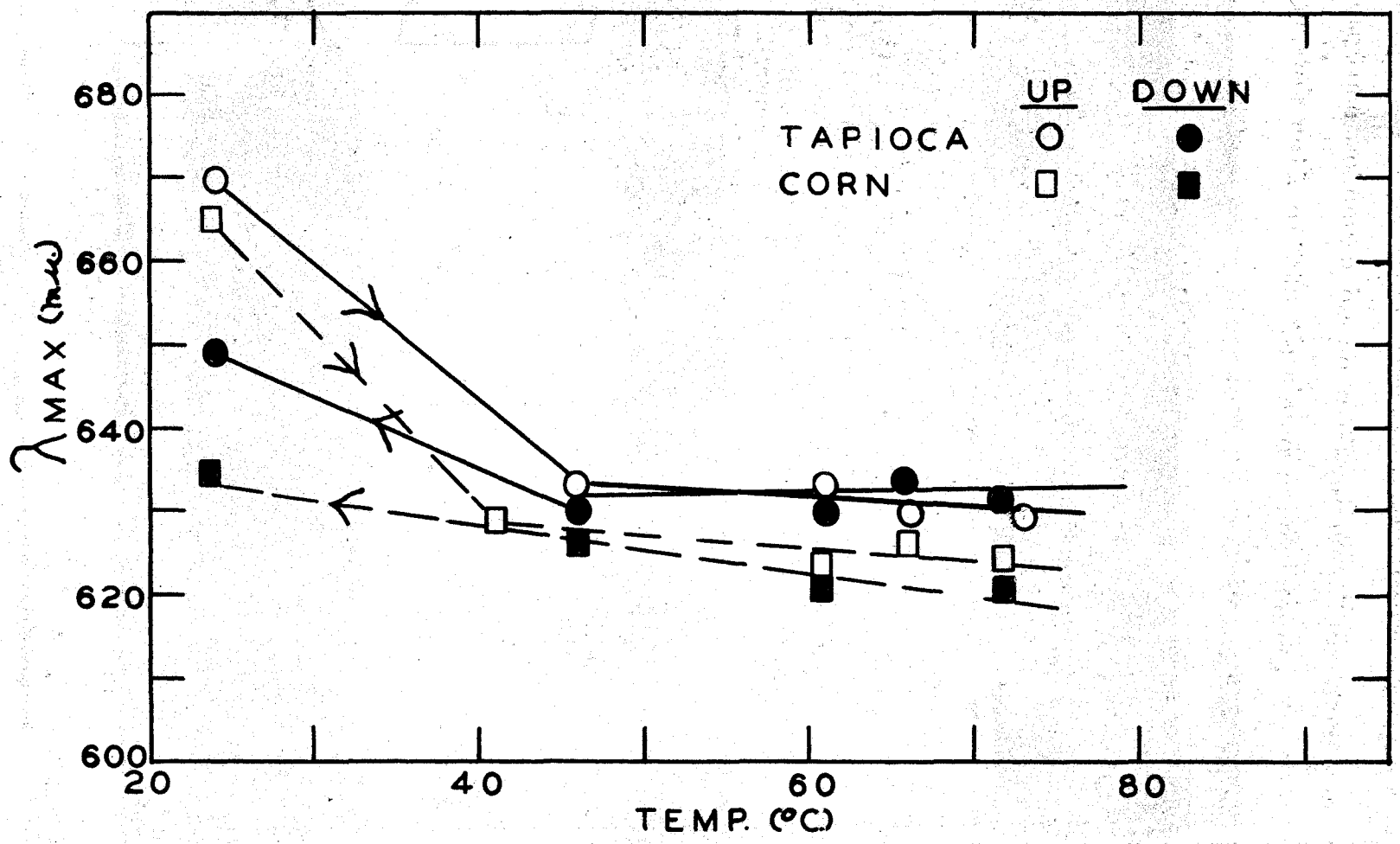

Figure 1. Influence of temperature on the wave length of maximum absorption of iodine complexes formed from tapioca and corn amylose.

Table I

Variation in Wave Length of Maximum Absorption Produced By Heating Amylose-Iodine Complex

Hours Heated	Temp.	λ_{max} (mu)
Corn Amylose		
0	80°	620
2	80°	615
4	80°	615
(Control) Not heated		665
Amylose heated for ½ hr. at 25° and cooled before complexing	80°	665
Tapioca Amylose		
0	80°	640
2	80°	625
4	80°	625
(Control) Not heated		670

heating for two or even four hours.

A control was run on a corn amylose solution which was held at 80° for thirty minutes in the absence of iodine. After this amylose was complexed at room temperature, the value of λ_{max} was found to have remained unchanged.

The tapioca amylose showed a slight shift to a lower maximum upon heating for two hours but evidenced no further decrease after four hours.

Samples of amylose were heated under nitrogen at 80° for various time intervals. This procedure gave the same λ_{max} as was obtained in similar experiments without nitrogen.

Another observation which may have a bearing upon the explanation of these results is that the unheated complexes gradually settle out after standing three or four days. The heated complexes may be kept for several weeks with no precipitation. Perhaps the ability of an amylose-iodine solution to form large aggregates is a factor which influences the value of λ_{max}.

Several experiments were carried out with mixtures of corn and potato amylose to determine the behavior of λ_{max} as the complex was formed by slowly cooling the solutions which had been heated to 80°. This was to observe whether or not selective complex formation would occur. It was thought that this might be evident even though a major

shift in the maxima had already taken place at the elevated temperature. However, there was not sufficient variation to warrant a definite conclusion.

Since the change in λ_{max} before and after heating an amylose-iodine complex does not appear to be temperature reversible, it is assumed that some irreversible process has occurred in the physical state of the amylose. The fact that most of this change takes place at temperatures below 45° would tend to eliminate the possibility of a chemical change due to oxidative degradation. This is further substantiated by the observation that about the same decrease is observed whether the complex is kept under oxygen or nitrogen at an elevated temperature. Also, continued heating at 80° has little additional effect.

From these results it is suggested that two types of aggregates are instrumental in causing the observed behavior. First, it is suspected that aggregates of amylose are initially present which cause the observed high λ_{max}. The dispersal of these aggregates under the influence of iodine at elevated temperatures is believed to be the cause of the observed shift.

The second type of aggregate which might be involved is that produced from amylose under the influence of salt and the complexing agent. This aggregate is loosely constructed and very easily dispersed. An aggregate of this nature may

be responsible for the increased maximum observed upon cooling from 45° to room temperature. Most of the subsequent experiments will be devoted to the problem of amylose aggregates and their influence on the iodine binding affinity of amylose.

B. Influence of Time of Standing in Alkaline Solution on Wave Length of Maximum Absorption

The influence of KOH on amylose is of major importance since this medium has been used not only as a solvent in viscosity work but also, in many instances, as a dispersing agent for amylose. The belief is widespread that molecular dispersion is attained rather rapidly in KOH. Experiments were performed to observe the behavior of λ_{max} as amylose stood in KOH.

A corn amylose preparation was allowed to stand in KOH for varying lengths of time. At intervals of fifteen minutes, three hours, and two weeks samples were withdrawn and neutralized. The λ_{max} was determined and the results shown in Table II. A decrease in the maximum of 35 millimicrons is observed after two weeks, whereas no appreciable change occurs in three hours. No precautions were taken to exclude oxygen from the basic solution. In view of the possibility of oxidative degradation it would perhaps have

Table II

Influence of Time in KOH on Corn Amylose As Shown by Wave Length of Maximum Absorption of the Amylose Iodine Complex

Time in KOH	λ_{max} (mu.)
15 minutes	655
3 hours	655
2 weeks	625
2 weeks and regenerated	625

been wise to keep the basic solution under nitrogen and determine the maxima at more frequent time intervals. This procedure has since been used in preparing amylose solutions for potentiometric iodine titrations and light scattering experiments. These will be discussed in detail in subsequent sections.

It may be significant that this λ_{max} obtained after two weeks in KOH corresponds to that obtained upon heating an amylose iodine complex at 80° for a short time. The change in maximum produced by the KOH treatment is not reversible upon regeneration. This is attested by the fact that a sample regenerated after two weeks in KOH will not show the original value but instead will remain at the value evident before regeneration.

It was suggested previously that aggregates of amylose perhaps were responsible for the high value of λ_{max}. It was of interest to see if the micelles produced in the initial stages of retrogradation after a long KOH treatment would show a higher maximum. For this purpose a sample of corn amylose which had stood in KOH for several weeks was used. The basic solution was neutralized and allowed to stand for various lengths of time. Although the intensity of the light transmitted decreased as the solution slowly retrograded, there was no change in the value of λ_{max}.

These results imply that KOH causes a slow dispersion of the suggested amylose aggregates. These aggregates appear to be a special type in that they cause a high value of λ_{max}, whereas those formed during the initial stages of retrogradation (after standing two weeks in KOH) do not experience an increase in value.

IV. PREPARATION OF CORN AMYLOSE SUBFRACTIONS WITH 15 PER CENT AQUEOUS PYRIDINE

Aqueous solutions of pyridine have been found to be not only excellent dispersing agents for starch but also good complexing agents for amylose. This dual nature may be controlled by varying the temperature. From preliminary experiments it has been observed that a temperature of approximately 50° may be critical. Below this temperature amylose will gradually precipitate, and above this dispersion will occur. The lowest temperature for maximum gelatinization has been obtained with 30 per cent pyridine (57); however, both dispersion and crystallization will occur with 15 per cent pyridine with proper control of the temperature.

The slow rate of formation of the amylose-pyridine complex suggested a possible means for the selective crystallization of molecules of varying affinities for pyridine. If the same factors are involved as in the formation of the amylose-iodine complex, then one would expect from the helical theory that the long molecules have a greater affinity for pyridine and crystallize first. This suggests two methods for the subfractionation of an amylose: first, controlling the temperature within such

narrow limits that only fractions with a certain range of chain length will crystallize, and second, controlling a temperature so that the rate of crystallization is slowed to such an extent that cuts of decreasing chain length may be taken at various time intervals. The second method was in general followed, partly because of the difficulty of maintaining the narrow temperature range required in the first method and partly because of the long initiation period required for crystallization at the temperatures which permitted only the longer particles to complex. An interesting variation of the first method which may circumvent the difficulty of the latter would involve first the formation of the complex at room temperature followed by gradual destruction of the complex by raising the temperature. At various time intervals during the temperature rise the material could be centrifuged and the supernatant analyzed. The short material presumably would be obtained in the initial cuts.

An indispensable preliminary to a good subfractionation is the elimination of amylopectin. It is difficult to detect trace amounts of this branched material and also difficult to differentiate between short and slightly branched material. Consequently many of the inconsistencies in physical and chemical properties of various amyloses may

be ascribed to unknown quantities of amylopectin, slightly branched material, short material or some combination of these. The problem of eliminating amylopectin and subsequent subfractionation of the linear component was attacked by using 15 per cent aqueous pyridine solution at 45° temperature.

A. Preliminary Experiments With 15 Per Cent Pyridine as a Complexing Agent for Amylose

A study was made of the influence of amylopectin on the fractions obtained as crystallization proceeded with a mixture of equal parts of potato amylose and potato amylopectin at room temperature. Potentiometric iodine titrations were used as a criterion for amylose content and relative chain length. A plot of per cent iodine bound versus log of the free iodine concentration was used to obtain this information. The per cent of iodine bound was found from the intersection of straight lines connecting the horizontal and vertical points of the plot. The binding affinity is defined as the free iodine concentration when 50 per cent of the iodine required to completely complex the amylose is bound.

Figure 2 shows the progress of the subfractionation of the mixture. Cuts 1, 2 and 3 represent fractions which

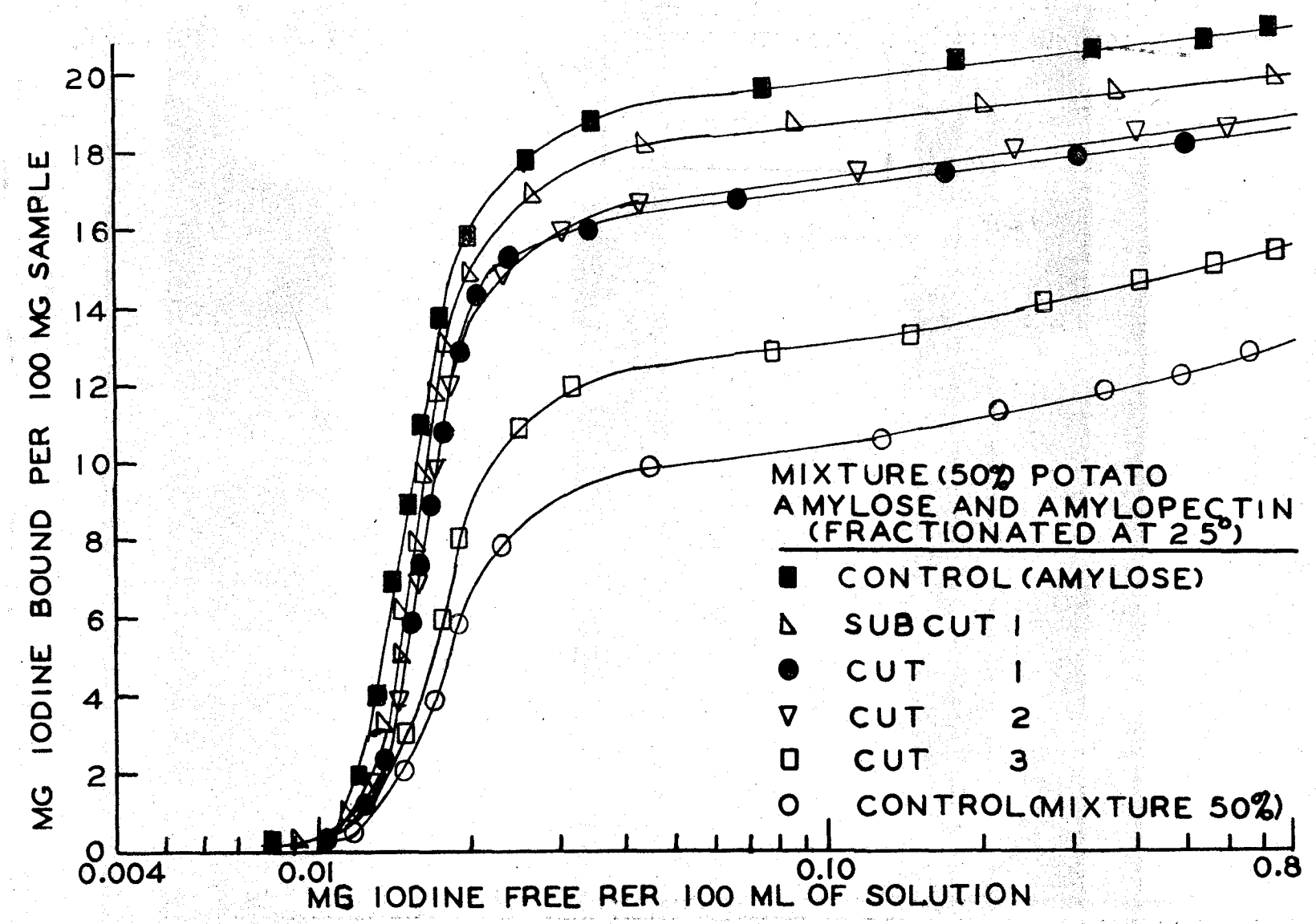

Figure 2. Potentiometric iodine titration curves representing the fractionation of a 50 per cent mixture of potato amylose and amylopectin with 15 per cent pyridine at room temperature.

crystallized upon standing at room temperature. Cut 1 was further fractionated to give sub-cut 1. From an examination of this data it appears that the subfractions are contaminated with amylopectin, with the greatest contamination occurring in Cut 3. Sub-cut 1, obtained by a fractionation of Cut 1, was apparently much freer of amylopectin and closely approached the original potato amylose in per cent iodine bound and in binding affinity.

An experiment similar in nature to the above was carried out at room temperature with a pentasol fractionated corn amylose sample fractionated by Shoch's method. Amyloses prepared by the use of pentasol as the complexing agent contain some material which is either slightly branched or very short in length which is not precipitated by butyl alcohol. This sample appears to be ideal for subfractionation since it reportedly contains all the amylose in the original starch preparation (76).

Figure 3 shows the results of potentiometric iodine titrations on the cuts obtained as crystallization proceeded. Included also are curves representing the original material as well as a subfraction of Cut 1. Table III gives the per cent of iodine bound, the binding affinity and the weight of each cut. The control is observed to represent predicted results if subfractionation were accomplished. As with the mixture of potato amylose and

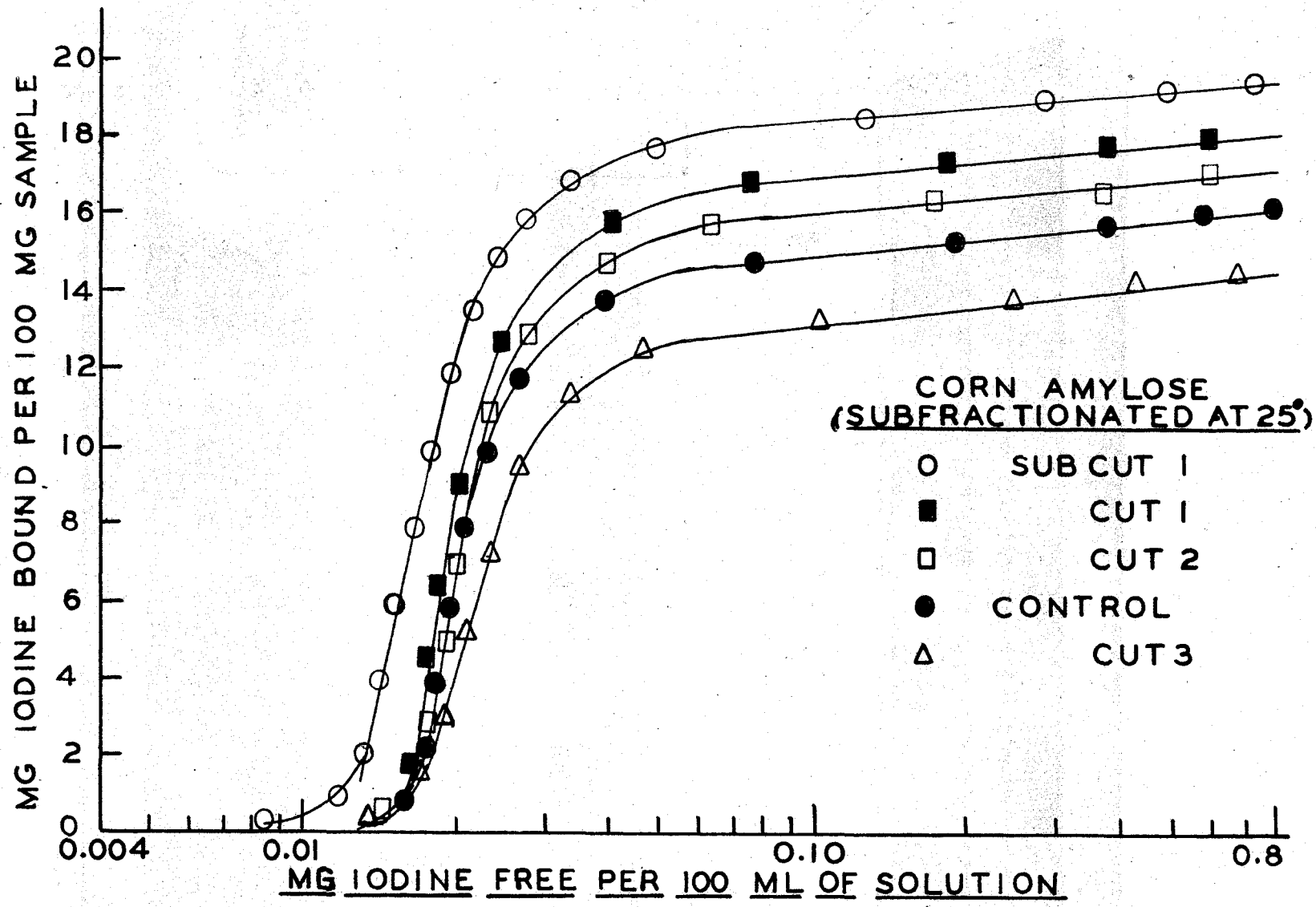

Figure 3. Potentiometric iodine titration curves representing the subfractionation of impure pentanol fractionated corn amylose at 25°.

Table III

Subfractionation of Corn Amylose (Pentasol) With 15 Per Cent Pyridine

At 25° Before Elimination of Amylopectin	Cut 1	Cut 2	Cut 3	Sub-Cut 1
Wt. of Recovered Cuts (Grams)	0.3164	0.1808	0.0381	0.081
% Iodine Bound	16.2	14.1	12.4	17.6
Binding Affinity Mg I_2 free/100 ml Soln.	0.0197	0.0203	0.0240	0.0173

At 45° and Room Temperature After Elimination of Amylopectin	Cut 1	Cut 2	Cut 3	Cut 4
Wt. of Recovered Cuts (Grams)	0.7755	4.0759	0.8183	0.7916
Temp. at Which Cuts Removed	45°	Room	Room	Room
% Iodine Bound	17.2	17.2	15.3	10.2
Binding Affinity Mg I_2 free/100 ml Soln.	0.016	0.0173	0.0180	0.0230

amylopectin previously discussed, a subfraction of Cut 1 showed a marked increase in the per cent of iodine bound as well as in the binding affinity. It is reasonable to suspect that any amylopectin present in the original corn amylose would affect the various cuts in a manner similar to that found in the previous experiment with potato amylose and amylopectin. The necessity of removing amylopectin prior to subfractionation is apparent.

Since an aqueous pyridine solution, as previously mentioned, may serve either as a complexing agent or a dispersing agent depending upon the temperature, it is feasible that a temperature just below that at which amylose fails to precipitate would tend to cause a maximum solubility in the branched fraction while allowing the linear fraction to precipitate. Thus a more complete separation of the two components would be effected. Preliminary experiments with amylopectin in 15 per cent pyridine at 45° showed no tendency for this fraction to precipitate upon standing for several days at this temperature. The amylose fraction, on the other hand, crystallized in the usual manner after an induction period of eighteen to twenty hours. The rate of crystallization of corn amylose at 44 to 45° is shown in Figure 4. The measurement of optical rotation for each of the time intervals was made by withdrawing a small portion of the liquid, centrifuging for twenty minutes in a Sorvall

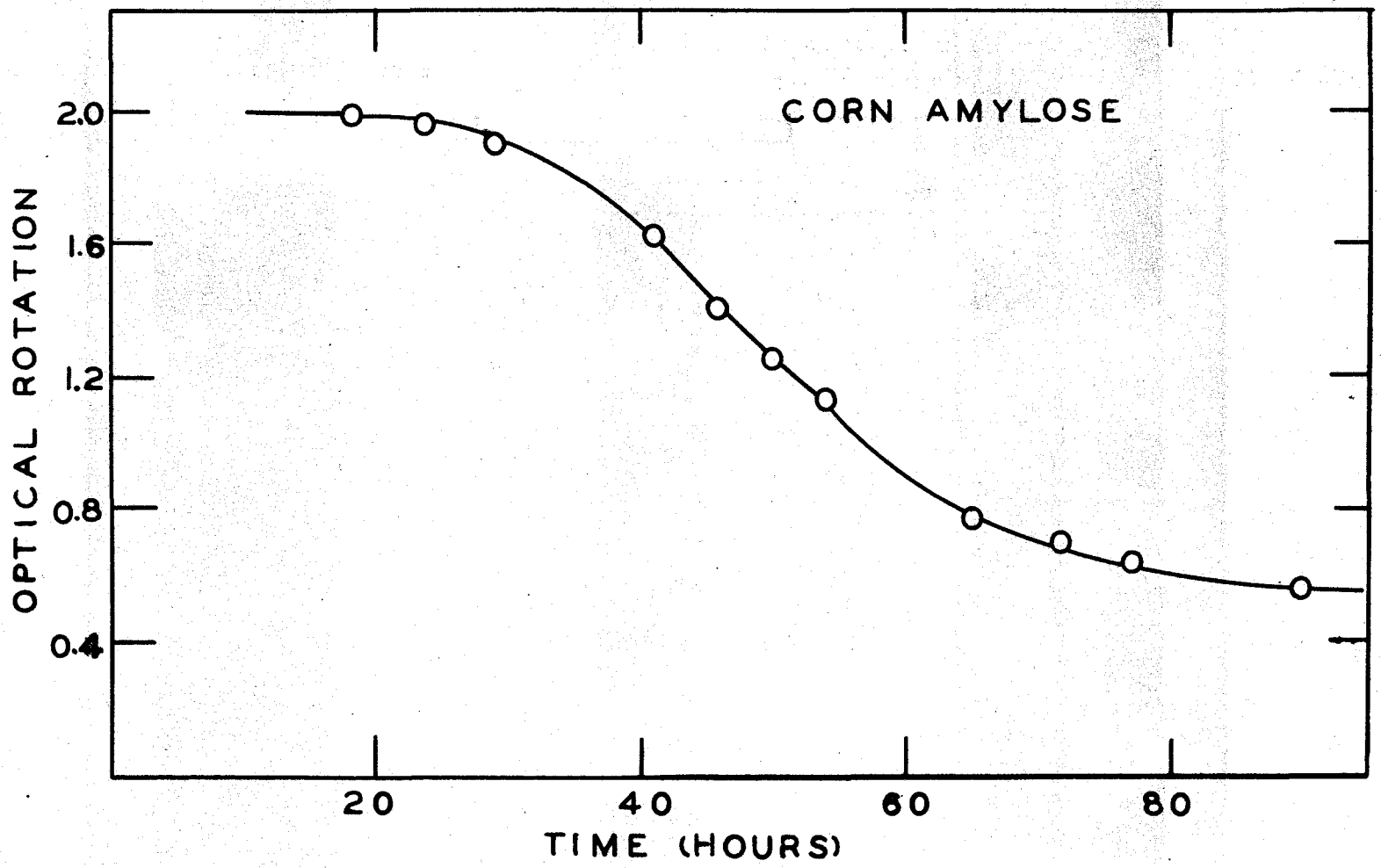

Figure 4. Change in optical rotation with time as corn amylose is allowed to crystallize at 45° in 15 per cent pyridine.

high speed centrifuge and measuring the optical rotation of the supernatant. After each measurement the supernatant and crystallized material were returned to the air tight flask.

B. Removal of the Branched Fraction at 45° With 15 Per Cent Pyridine

A sufficient quantity of pentasol fractionated corn amylose was dispersed in two liters of 15 per cent aqueous pyridine to give a carbohydrate concentration of from 0.6 to 0.7 per cent. Dispersion of the paste was accomplished by refluxing the solution over a steam cone at 80 to 90° until a clear solution was obtained. This required two to three hours. For the dispersing process a three-necked, two-liter flask was used which was fitted with a reflux condenser, air tight stirrer and thermometer. Any insoluble residue remaining after dispersal was removed by centrifugation. The volume was readjusted to two liters.

The completely dispersed solution was again introduced into the three-necked flask and placed in a constant temperature bath maintained at 44 to 45°. The amylose was allowed to crystallize at this temperature until a constant value was observed for the optical rotation of the supernatant liquid after centrifugation. This indicated no further

crystallization. The amylose was removed by crystallization and redispersed in two liters of 15 per cent pyridine in a manner similar to the first dispersion. Only ten or fifteen minutes were now required to disperse the amylose-pyridine complex. The amylose was recrystallized from 15 per cent pyridine at 45° for a second and third time. After each centrifugation the soluble carbohydrate remaining in the supernatant liquid was recovered by reducing the volume to about 200 milliliters by vacuum distillation and precipitating with ethyl alcohol. This residue was thoroughly dried by washing with absolute ethyl alcohol four times and finally heating in a vacuum oven for three hours at 40°.

The total amount of carbohydrate before each recrystallization and the amount of soluble material remaining after each crystallization and centrifugation were accurately followed by optical rotation measurements. Just prior to a centrifugation two samples of 20 milliliters each were withdrawn from the main solution. One was heated in a glass stoppered, volumetric flask to disperse the pyridine complex, and the other was centrifuged in a Sorvall high speed centrifuge to remove the complex. Optical rotations on these clear liquids gave the total amount of carbohydrate as well as the amount of soluble carbohydrate. These data are tabulated in Table IV.

Table IV

Purification of Corn Amylose (Pentasol) With 15 Per Cent Pyridine at 45°

	Crystallization		
	First	Second	Third
Total Carbohydrate (From Optical Rotation) (Grams)	11.2	8.65	8.0
Soluble Fraction (From Optical Rotation) (Grams)	1.25 (2.075)[1]	0.15	0.60
Crystalline Fraction (Grams)	8.95	8.50	7.40
Soluble Fraction Recovered (Grams)	1.63	0.097	0.202
Days Allowed for Crystallization	7	5	3

[1]This value represents the carbohydrate remaining in the supernatant after centrifuging with a low speed centrifuge after the first crystallization.

Not only were the residues from the supernatants available for potentiometric iodine titrations, but amylose samples of the crystallized material were saved after the second and third crystallization. It is unfortunate that a sample after the first crystallization was not saved.

The progress of this procedure in the removal of amylopectin is best seen from the curves shown in Figure 5 which represents the results from potentiometric iodine titrations on the above samples and also on a control of the original material.

The curve in Figure 5 representing the residue from supernatant 1 requires further explanation. Optical rotation measurements after centrifuging an aliquot in a Sorvall high speed centrifuge showed that out of 11.2 grams of total carbohydrate initially present, 1.25 grams or about 11.2 per cent was left in the soluble fraction. However, after centrifuging the main solution in a low speed centrifuge, the supernatant was found to contain a total of 2.075 grams as shown by optical rotation. The supernatant for the latter rotation measurement was heated a few minutes to obtain a clear solution. Apparently the low speed centrifuge was incapable of removing the very small crystallites. This residue then contained about 40 per cent amylose and 60 per cent amylopectin. The potentiometric iodine titration of this mixture also indicated about 40 per cent amylose,

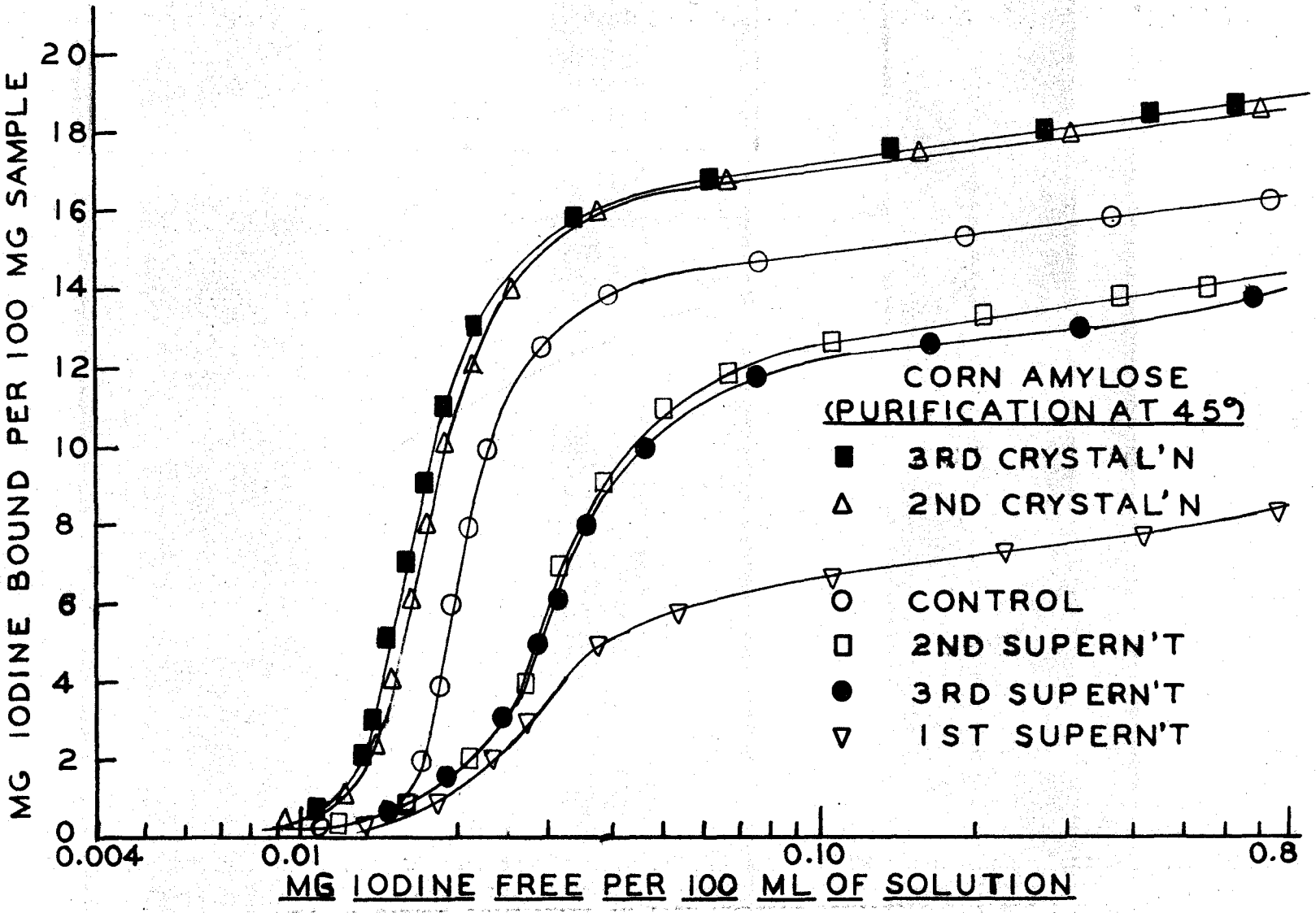

Figure 5. Potentiometric iodine titrations on corn amylose fractions showing the progress of purification with 15 per cent pyridine at 45°.

thus confirming the above value.

It is believed that the soluble carbohydrate in supernatants 2 and 3 contains a negligible amount of amylopectin. A comparison of the titration curves shows very little variation in the binding affinity or even in the per cent of iodine bound. If complete separation of amylopectin had not occurred during the first crystallization, then it is to be expected that a step by step elimination of amylopectin would occur during subsequent crystallizations. This, of course, should be manifest in the curves representing supernatants 2 and 3 in Figure 5. The small amount of soluble material found in supernatant 2 might be attributed to the solubility of amylose in 15 per cent pyridine. The greater amount found in supernatant 3 may result either from the shorter time allowed for crystallization or from an increase in the solubility of amylose upon standing in pyridine. The latter view is given additional support in the results of the subfractionation experiment to be described next.

C. Subfractionation of Purified Corn Amylose With 15 Per cent Pyridine

The subfractionation of the amylose which is free from amylopectin was carried out in an operation similar to the

previous experiment. Originally it was planned to carry out the complete subfractionation at 44 to 45° since at this temperature a period of about five days was required for complete crystallization. However, only the first cut was taken out at this temperature because of a failure in the heating system controlling the temperature of the water bath. The second and third cuts were taken out at room temperature. The fourth cut was obtained by reducing the volume of the supernatant to 200 milliliters and recovering the amylose as was done with the supernatants in the previous section. The first three cuts were taken out by use of the Sharples centrifuge. The centrifuged pyridine complex was dispersed in warm water, precipitated with ethyl alcohol and washed with absolute alcohol followed by drying for four hours in a vacuum oven maintained at 40°.

The effectiveness of the subfractionation is shown in Figure 6. Two controls are given in this figure, one before amylopectin was eliminated and the other after. The large difference in binding affinity and per cent of iodine bound for these is shown in relation to the curves obtained for the subfractions. Comparing this set of curves with those shown in Figure 3, it is seen that the binding affinity as well as the per cent of iodine bound has been increased greatly after elimination of amylopectin. Only with an

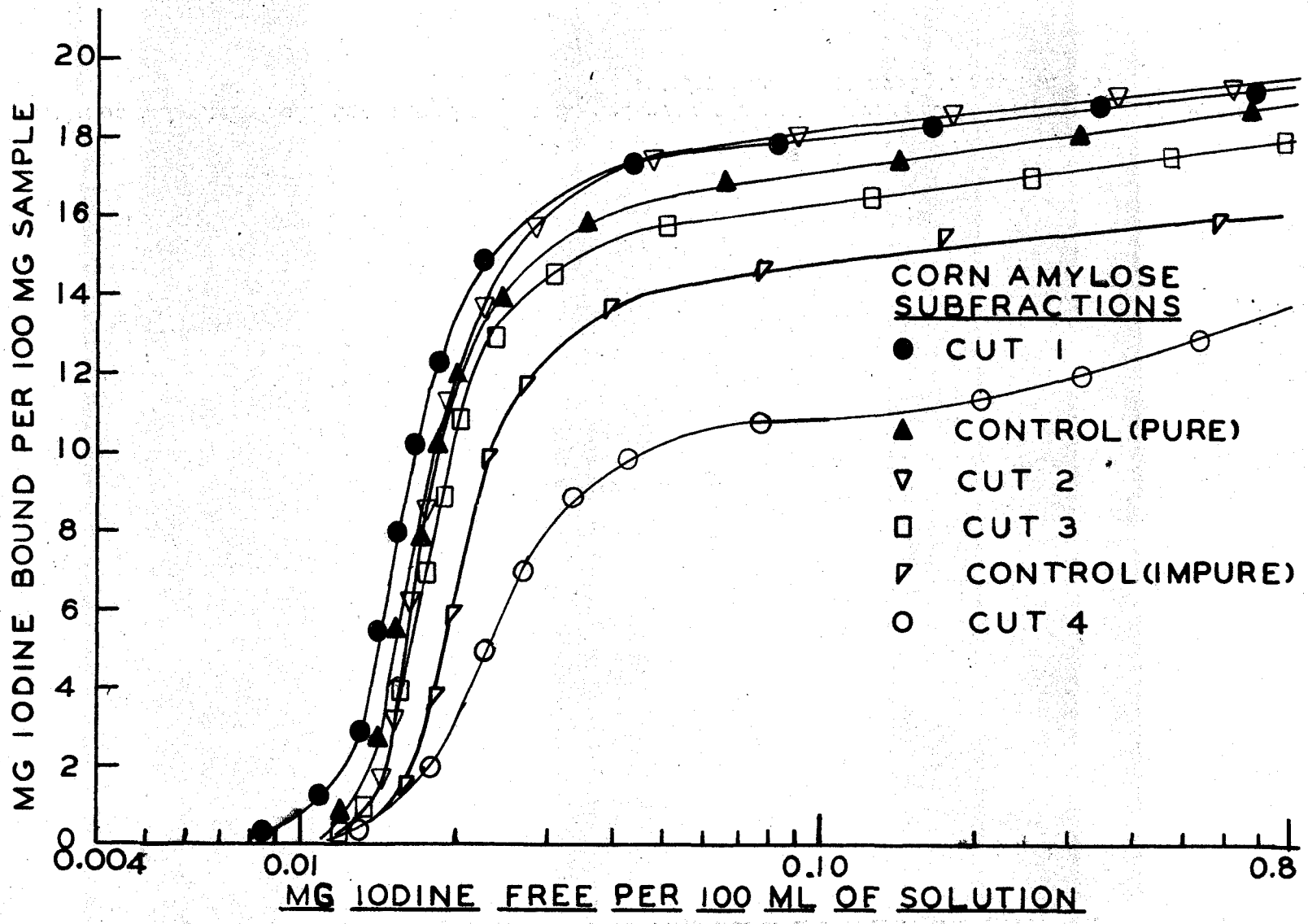

Figure 6. Potentiometric iodine titrations of corn amylose subfractions after purification with 15 per cent pyridine at 45°.

additional subfractionation of the first cut shown in Figure 2 is one able to get a comparable binding affinity and per cent of iodine bound for corn amylose without first eliminating the amylopectin.

The Cut 4 sample which was isolated from the supernatant with alcohol behaves in a manner similar to the residues from supernatants 2 and 3. A close inspection of Figures 5 and 6 reveals that Cut 4 has a slightly greater binding affinity but binds less iodine. It is found that this sample represents about 15 per cent of the starting material which had previously complexed with pyridine during the purification procedure. Apparently considerable modification has occurred during the extended pyridine treatment at room temperature. After the removal of amylopectin, perhaps the carbohydrate which would not complex with pyridine at room temperature is composed of highly aggregated short material which formed during the extended treatment with 15 per cent pyridine. Some allowances, however, must be made for the solubility of amylose in the solvent. It is recalled that this material was able to complex with 15 per cent pyridine during the purification procedure. Perhaps a temperature of 45° prevents the formation of aggregates.

Thus, three somewhat distinct types of carbohydrates are indicated for the pentasol fractionated corn amylose:

(1) An amylopectin impurity which represents about 11 per cent of the starting material, (2) a highly aggregated fraction of short material of rather low binding affinity and greatly reduced capacity for iodine, the amount formed being possibly dependent on the length of time in 15 per cent pyridine, (3) a major fraction (perhaps 80 to 85 per cent) containing an undetermined distribution of chain lengths.

V. FACTORS WHICH INFLUENCE THE IODINE BINDING AFFINITY OF AMYLOSE AS SHOWN BY POTENTIOMETRIC IODINE TITRATIONS

Some of the factors which influence the iodine binding affinity of amylose have been described (4). These factors are temperature, potassium iodide concentration, concentration of other electrolytes, chain length of amylose, concentration of amylose, and time interval between addition of iodine increment and reading of potential. This investigation was carried out to discover what additional factors influence the binding affinity of amylose and to correlate these findings with the results of the spectrophotometric experiments.

The configuration of the amylose molecules in aqueous solutions is of considerable interest. There is little doubt that amylose when complexed exists in the helical configuration in the crystalline state. Little evidence has been presented supporting a particular type of configuration for amylose in aqueous solution. Experiments reported in Section III point toward the presence of aggregates of amylose molecules that remain even after dispersing the amylose in KOH for several hours. However, one is also concerned with the molecules within the suspected aggregate.

It is of particular interest to know whether a helical amylose molecule or some other configuration such as a random coil exists in aqueous solutions.

From a potentiometric iodine titration it is possible to follow the affinity of amylose for iodine at various stages of the titration. This is brought out by plotting per cent of iodine bound versus iodine concentration. The binding affinity has been defined in Section IV as a measure of the free iodine concentration at 50 per cent binding.

The binding affinity, according to the helical theory, should be directly related to the ability of the amylose to form a helical complex and to the orientation of the helices, possibly into bundles. By studying the factors which influence the binding affinity, one may infer after several experiments what kind of configurational changes have taken place from a particular treatment.

A. Experimental Details

The influence of temperature on the potentiometric iodine titrations to be described was eliminated by carrying out the titrations in a temperature bath controlled to $\pm 0.03^{\circ}$ C. A three-necked, 250 milliliter flask flattened on the bottom to accommodate a magnet sealed in glass was used as a container for the solution to be titrated. This flask was

immersed in a low form, cylindrical jar filled with water which was maintained at ±0.03° by pumping water from a thermoregulated water bath through a copper hose coiled to fit the inside of the jar. The cylindrical jar was placed upon a magnetic stirrer. Constant stirring was satisfactorily obtained with this arrangement, which also permitted titrations to be carried out under nitrogen when desired.

A Leeds and Northrup calomel electrode, type 1199-13, was used as a reference electrode in which the concentration of the potassium chloride solution was maintained at 248 grams per liter. A centimeter square of bright platinum welded to a copper wire which in turn was sealed in a glass tube served as the inert electrode. The galvanometer and potentiometer used have been previously described (4).

The amylose solutions were prepared for potentiometric iodine titrations after the amylose was dried in a vacuum oven maintained at 40°. Usually a sample slightly greater than 40 milligrams was weighed and to each 40 milligrams of sample 5 milliliters of 1.000 N. KOH were added and allowed to stand with occasional stirring until the sample was completely dispersed. The basic solution was neutralized with 0.500 N. hydrochloric acid. Fifteen milliliters of the above solution containing 40 milligrams of sample was transferred to a 100 milliliter volumetric flask. To this

were added 10 milliliters of 0.500 N. potassium iodide. Upon diluting to 100 milliliters a total salt concentration of 0.050 N. with respect to both potassium iodide and potassium chloride was obtained. The iodine solution was also prepared to contain this amount of salt so that the potassium iodide and potassium chloride concentration remained constant throughout the titration. The iodine concentration used in all of the titrations was 0.0015 N.

A Leeds and Northrup potentiometer was used to make the final pH adjustment. Since the concentration of the acid and base was known to three significant figures, it was possible to neutralize to within a drop or two of the amount required before using the pH meter.

In all of the titrations three minutes were allowed between adding an increment of iodine solution and reading the potentiometer. Although equilibration was not attained in this time interval, it was necessary to use a set interval so that a comparison could be made with the various titration curves.

B. Effect of pH on the Electrode System

It was necessary to study experimentally the effect of pH on the iodine electrode, particularly in the region of very low iodine concentration. This concentration is of

importance in a potentiometric iodine titration since the formation of the amylose-iodine complex occurs at a free iodine concentration of about 10^{-6} N.

According to the Nernst equation, the observed potential of the iodine electrode is related to the activities of iodine and the iodide ion according to the expression:

$$E = E^{o} - \frac{RT}{nf} \ln a_{I_2}/a^2_{I^-} . \qquad (6)$$

Thus a plot of observed potential versus iodine activity for the iodine electrode in combination with a reference electrode should produce a linear curve if the iodide ion activity is held constant. However, if internal factors are involved which influence the iodine or iodide activities, particularly at the low iodine concentrations, then a linear curve is not obtained.

An influence of pH upon the activity of iodine and iodide would be expected from the hydrolysis of iodine according to the equation:

$$I_2 + H_2O = H^+ + I^- + HIO \qquad (7)$$

where the hydrolysis constant for the forward reaction is 3×10^{-3}. At pH values above 7 hydrolysis is sufficient to cause an appreciable deviation in the calibration curve (plot of E versus a_{I_2}). However, at pH values below 7 a linear curve should be produced.

An examination of the calibration curves shown in Figure 7, however, shows that a large deviation from linearity at the low iodine concentration (10^{-6} N.) occurs at a pH of 4. The direction of deviation represents an increase in iodine concentration. At a pH value of 9 the deviation is in the direction expected from the hydrolysis of iodine represented by equation (7).

The gain in iodine at the pH of 4 at very low iodine concentrations may result from the oxidation of hydriodic acid according to the equation:

$$4HI + O_2 = 2I_2 + 2H_2O. \qquad (8)$$

These results show that care must be exercised in adjusting the pH of the potassium iodide solution for the preparation of a calibration curve. Furthermore, the use of methyl orange indicator in neutralizing basic amylose solutions influences the accuracy of a potentiometric iodine titration to a certain extent. The loss or gain of iodine due to the pH effects would not be nearly so evident in an iodine titration since amylose acts as a buffer to bind iodine at a nearly constant iodine activity. A change in potassium iodide concentration should, however, be as evident here as in the preparation of a calibration curve. Bates (4) has determined experimentally and theoretically the effect of small changes in potassium iodide concentrations upon the observed potential of an iodine electrode.

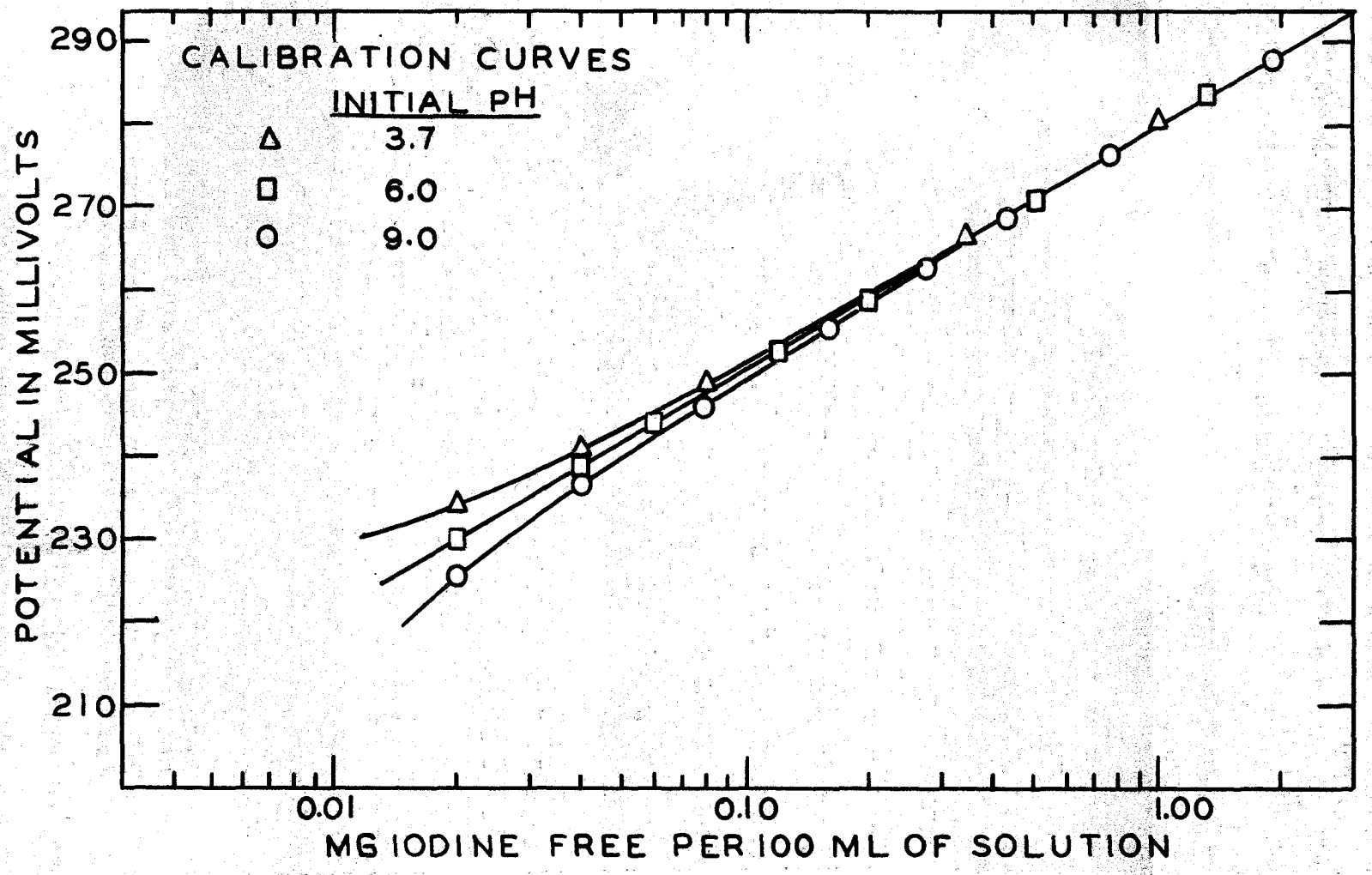

Figure 7. Calibration curves obtained at various pH values.

Curves obtained from a potentiometric iodine titration of potato amylose performed at pH values of 4, 6.5, and 9 are shown in Figure 8. A comparison of the curves at a pH of 6.5 and at a pH of 9 shows that a small variation exists only in the first part of the curves. The sample which was titrated at a pH of 4, however, shows considerable deviation throughout the vertical portion of the curve. Deviations of about the same magnitude were expected at a pH of 4 and at a pH of 9 since variations of about the same degree were found in the calibration curves. This pronounced decrease in binding affinity at the pH of 4 suggested that in addition to the pH effect on the electrode system the physical behavior of amylose was also influenced. This led to additional experiments concerning the pH effects upon amylose.

C. Influence of Time of Standing on the Binding Affinity of Amylose at Various pH Values

Results were presented in the previous section which indicated that a change in iodine binding affinity occurred with amylose which was titrated at a pH initially adjusted to 4. This behavior suggested an investigation of the pH effect on the binding affinity of amylose.

A potato amylose, P5/6A (9c), was chosen since titrations on this material over a period of six months or longer

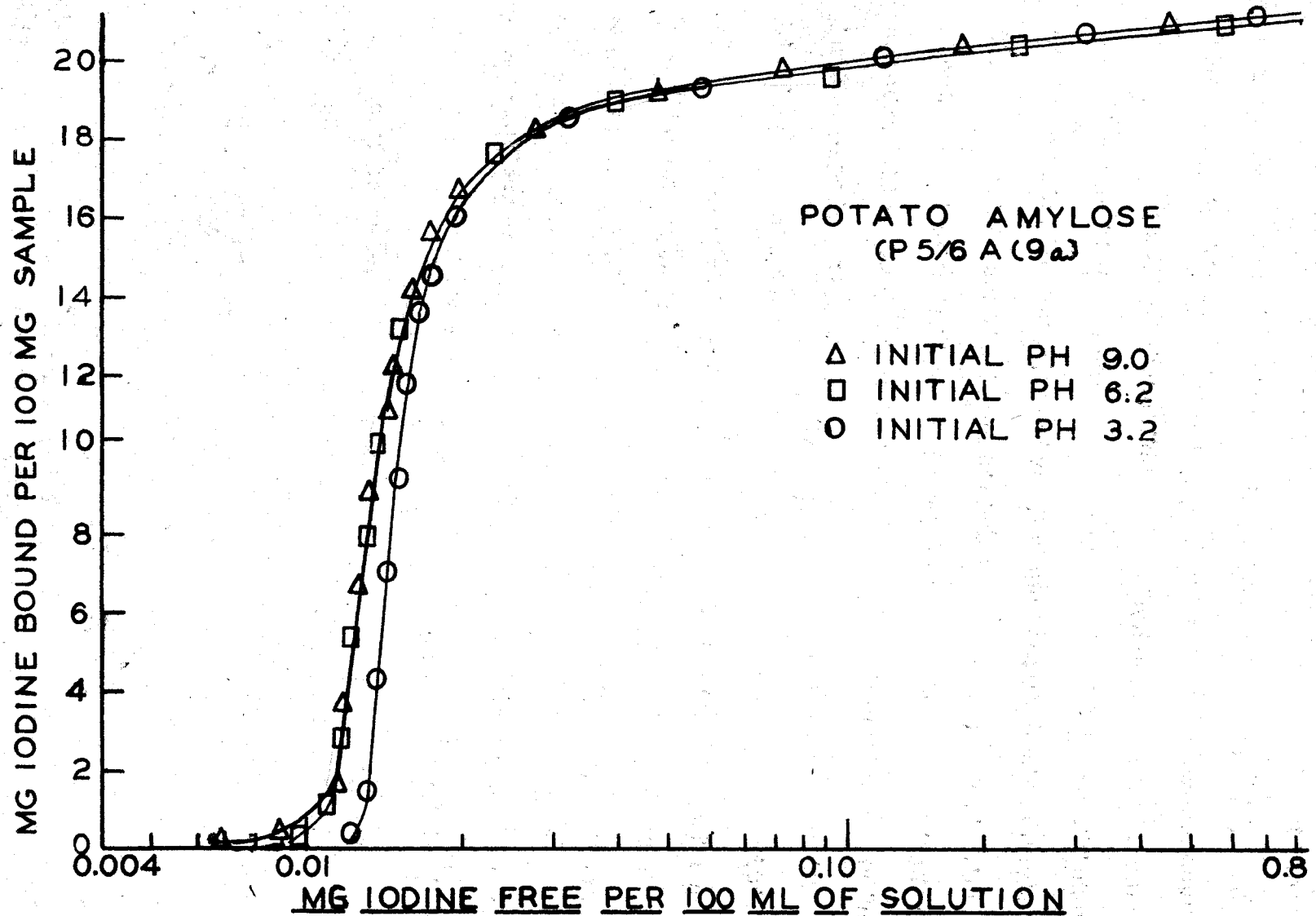

FIG. 8 INFLUENCE OF PH DURING TITRATION ON THE BINDING AFFINITY OF CORN AMYLOSE

had given results that were in good agreement. A maximum deviation of not more than one-half millivolt had been obtained. This is about the maximum accuracy obtainable according to Bates (4).

Amylose solutions were prepared at the usual concentration for a potentiometric iodine titration, adjusted to various pH values between 4 and 9, and allowed to stand. It was observed that after a day or so the material which had been adjusted to a pH of 6.8 had begun to retrograde. After about five days the solution adjusted to a pH of 8 also showed visible evidence of retrogradation. The sample at the low pH of 5.6 showed very little evidence of crystallizing even after ten days. This behavior, especially at the low pH value, was unexpected. Previous to this experiment the view had been held that the retrogradation tendencies increased as the pH of a basic solution of amylose was reduced.

To help clarify this, iodine titrations were run on the amylose discussed above as soon as the solutions became slightly turbid. The results of these titrations are shown in Figure 9. The pH of all the solutions was readjusted to the value of about 6.3 before each titration. The treatment of the samples is recorded in the figure. A most interesting fact is the progressive decrease in binding affinity with decreasing pH of amylose solutions. However,

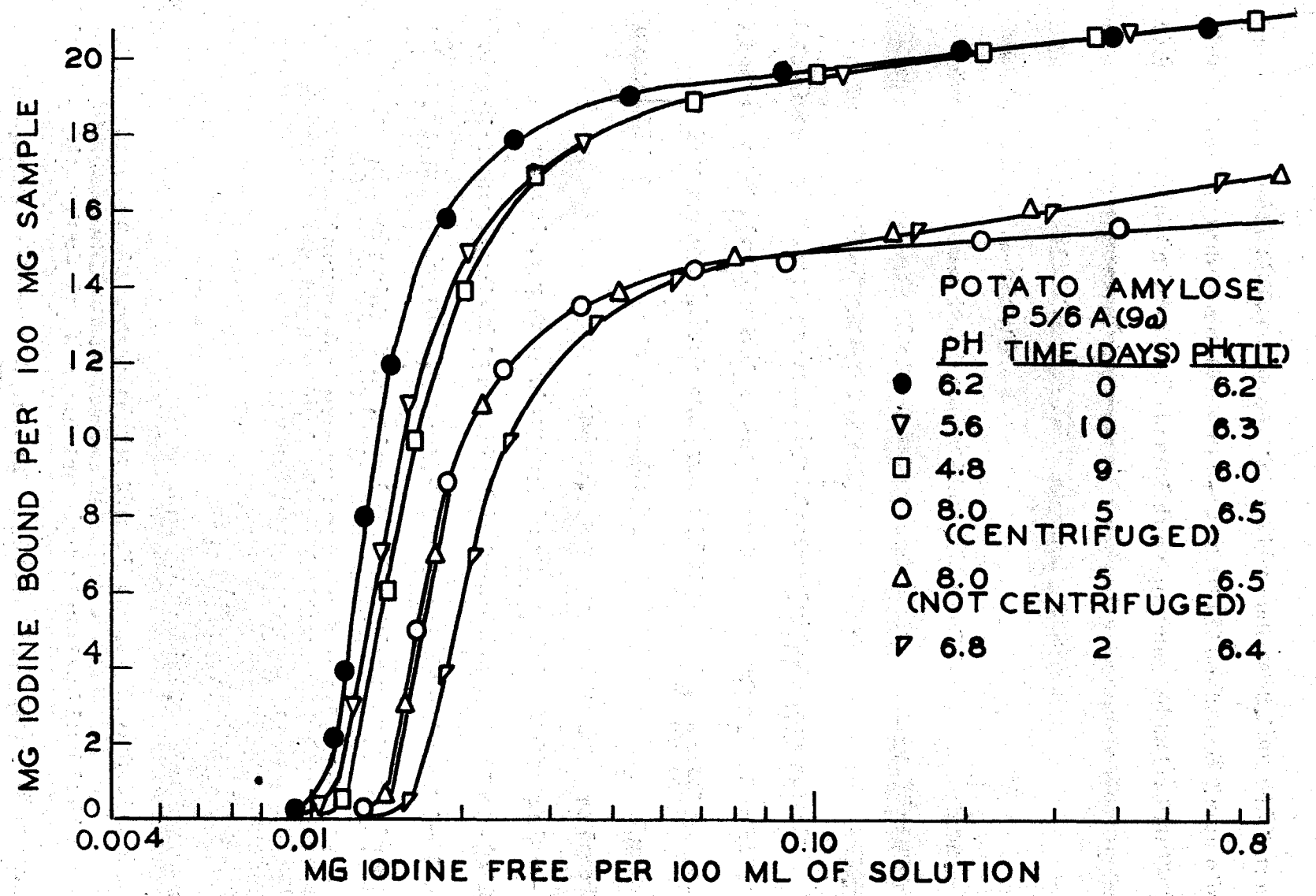

Figure 9. Influence of time of standing at various pH values on the binding affinity of potato amylose. Solutions titrated at pH values between 6.0 and 6.5.

for pH values below 6 the per cent of iodine bound remains essentially the same as in the control which was titrated immediately after adjusting to the neutral pH. It will be recalled that a similar shift was observed in Figure 8.

That the retrogradation tendency is greater near the neutral pH is also apparent from Figure 9. It is noticed that the solution at a pH of 6.8 shows the maximum decrease in binding affinity and requires the shortest time to retrograde (two days), whereas the binding affinity of the solution at a pH of 8 experienced a smaller decrease over a longer period of time (five days). These two samples have the same capacity for iodine, but their binding affinities differ markedly. The explanation originally given for this behavior was that a greater per cent of the amylose at a pH of 6.8 was in some stage of retrogradation and as a result, was incapable of binding iodine at such a low level. Later experiments have shown that this explanation is probably not correct. Instead a disaggregation of amylose micelles is involved.

One additional point of interest is brought out in this experiment. The sample which had been allowed to stand at a pH of 8 for five days was titrated before and after centrifugation. A comparison of the two curves in Figure 9 shows that they can almost be superimposed except in the region of high free iodine concentration. In this

region the uncentrifuged amylose sample binds more iodine than the centrifuged, indicating that particles which are capable of being removed by the high speed Sorvall centrifuge (20,000 g) are also capable of binding iodine.

In summary, these experiments show that retrogradation occurs at a greatly increased rate at the neutral pH. At pH values of 4 the retrogradation tendencies are practically nil, whereas at a pH of 8 the behavior is somewhat intermediate between that at 4 and at 6 to 7. At a pH below 6 the binding capacity (or per cent iodine bound) does not change appreciably, but the binding affinity appears to decrease as the pH decreases. Samples which retrograde show a large decrease in binding affinity and also a decrease in per cent of iodine bound. The large decrease in binding affinity is only partly caused by the shift which is known to occur as the amylose concentration is decreased. This effect will be discussed separately.

D. Influence of Concentration on the Iodine Binding Affinity of Amylose

The decrease in iodine binding affinity caused by a reduction in the concentration of amylose has been described by Bates (4). However, no attempt was made to plot the data in terms of per cent of iodine bound versus free iodine

concentration. This necessitates a correction for the weight of the sample. Potentiometric iodine titrations were performed on samples of various amylose concentration (Figure 10). It is observed that there is a definite though small decline in iodine binding affinity as the amylose concentration is decreased. It is important to note that the decrease in binding affinity caused by halving the initial amylose concentration is small. It is recalled from Figure 8 that the amylose sample which stood for two days at a pH of 6.8 experienced a large decrease in binding affinity though less than a third of the sample was lost by retrogradation. This experiment indicates that only a small fraction of the shifts in binding affinity is caused by a decrease in amylose concentration. The major part of the decrease apparently is due to physical changes in the amylose.

E. Influence of Regeneration on the Iodine Binding Affinity of Amylose

The observation was made that a freshly prepared amylose sample experienced a greater affinity for iodine than the same amylose after it had been allowed to stand in the solid state for several weeks. It appeared that in the progress of regeneration some type of configurational change had occurred with the amylose, and this change was

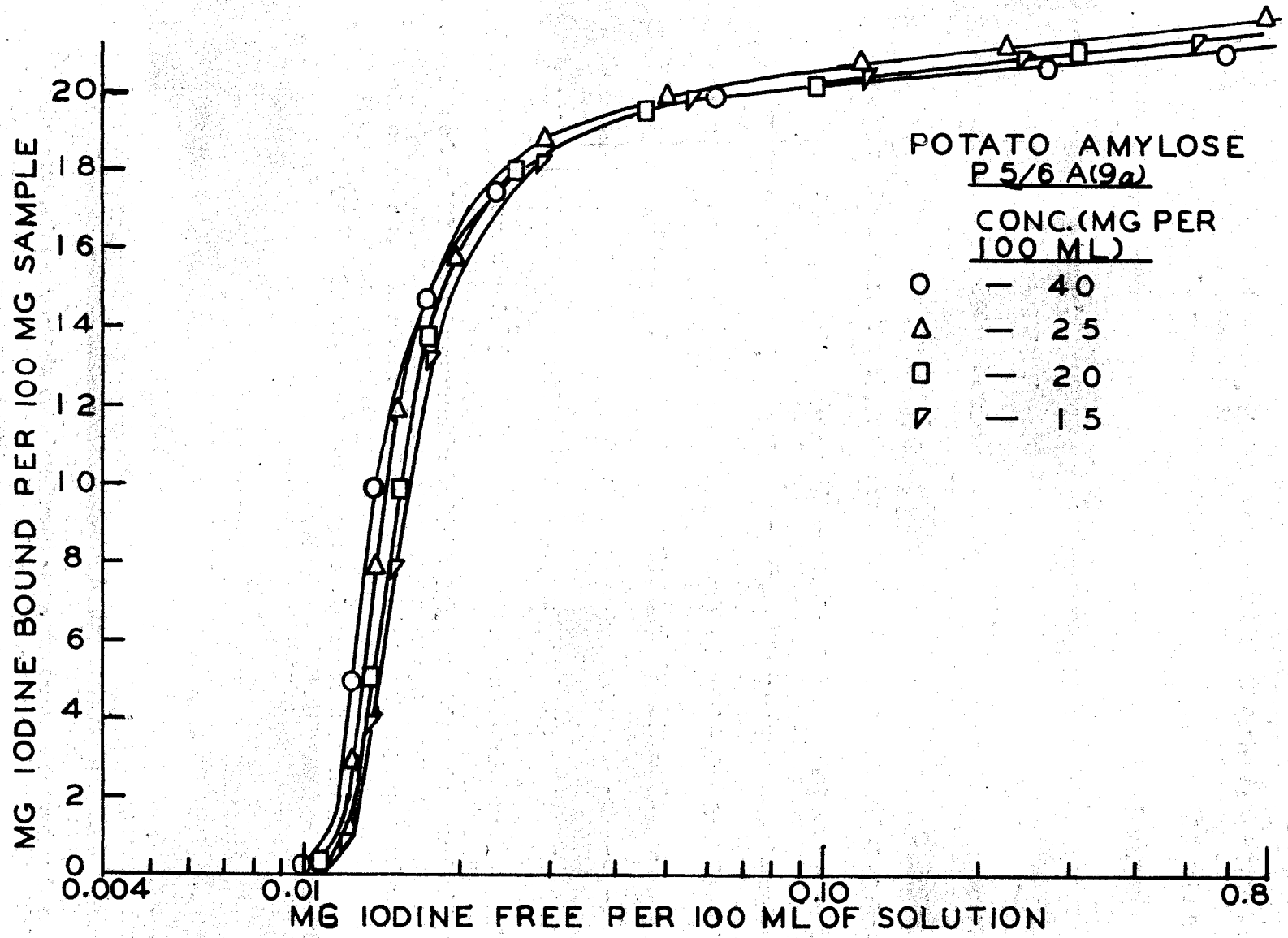

Figure 10. Influence of conc. on the iodine binding affinity of amylose.

subsequently lost during long periods of standing. It was reasonable to assume that the complexing agent was responsible for this change during regeneration.

The regeneration was accomplished by performing several successive crystallizations of the amylose in a 50 per cent aqueous ethyl alcohol solution. After each crystallization the complex was centrifuged and dispersed in water. Enough ethyl alcohol was again added to give a 50 per cent solution. This treatment effectively removed the salts from the amylose after the third crystallization. The water was conveniently eliminated from the salt-free complex by washing four or five times with absolute ethyl alcohol. Finally the complex was dried in a vacuum oven for three or four hours.

It was of interest to regenerate an amylose sample after first complexing with an excess of iodine, which was removed by reduction with sodium thiosulfate. The belief that iodine forms a more stable complex with amylose than does ethyl alcohol is based upon the observation that a temperature of 80° is required to destroy the iodine complex while the alcohol complex is destroyed at only 50°. If the increased binding affinity after regeneration is caused by the relatively weak ethyl alcohol complexing agent, then a pretreatment with iodine should induce a greater change.

That this idea is correct may be seen from Figure 11. The control curve represents a potentiometric iodine titration on an amylose aged for several months. The curve for the iodine pretreated sample falls on the left and shows a very high binding affinity. The regenerated sample not pretreated with iodine is seen to have an intermediate binding affinity. Thus it seems to be reasonable to assume that the complexing agent is responsible for this change, and a more pronounced effect is caused by stronger complexing agents.

It is interesting at this point to speculate as to the type of change occurring in view of the results of some of the preceding experiments. The spectrophotometric experiment indicated that aggregates of amylose were responsible for high wave length of maximum absorption for the amylose iodine complex. It is presumed that the same factors which cause the high value for wave length of maximum absorption are also those which cause an increased binding affinity for iodine. Work by Baldwin (1) showed that amylose samples which showed high binding affinities for iodine showed high values for wave length of maximum absorption. Whether this correlation is due to a difference in chain length or to a difference in aggregate size or to a combination of both is as yet not clear.

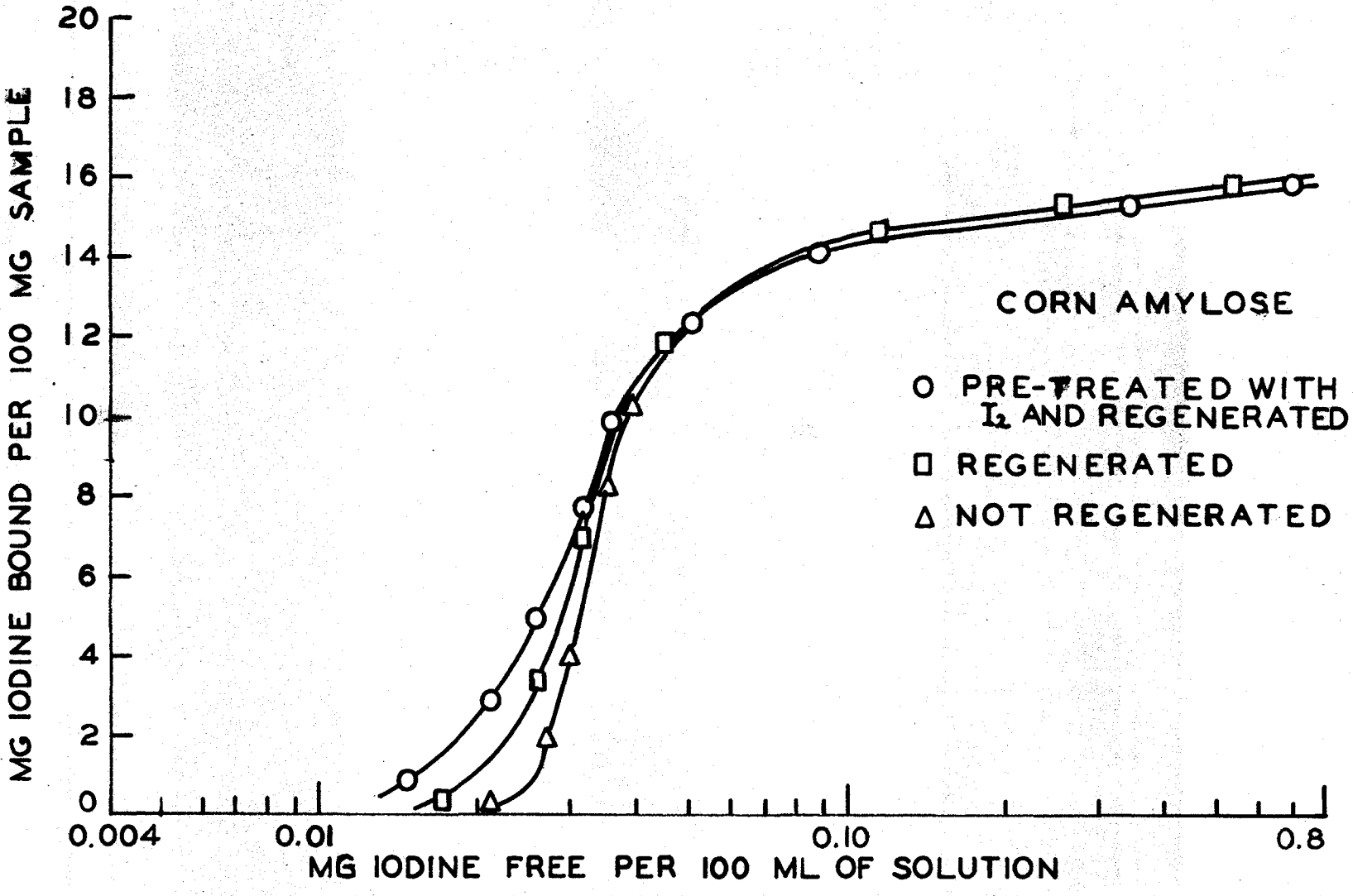

Figure 11. Influence of regeneration on the iodine binding affinity of corn amylose.

The argument is presented that complexing agents may not only orient the amylose helices linearly within the aggregate, but also stabilize the helical structure by forming a more compact helix. This applies particularly to stronger complexing agents. The increased stabilization may result from the formation of a greater number of intramolecular hydrogen bonds. It should be emphasized that this latter effect might occur whether or not one assumes that aggregates of amylose are initially present. A more definite proof for or against the existence of aggregates is required.

The changes that are produced in amylose when aged are not clear. It perhaps should be noted that this aging effect has also been found to influence the turbidity results. The humidity of the sample as well as the time factor influences the physical behavior of amylose.

F. Back-Titration of the Amylose-Iodine Complex

In the preceding sections it was shown that the iodine binding affinity of an amylose was not constant but was easily changed without using conditions which lead to oxidative degradation or cleavage of the glucosidic linkages. This behavior strongly suggested that the forces involved in increasing the binding affinity were physical rather than

chemical. The increased binding affinity caused by regeneration was explained as being due to the formation of more compact helices and also to the linear orientation of the helices within the aggregate.

In performing potentiometric iodine titrations it was found that after adding an increment of iodine a steady potential was not reached even after several hours of standing. The rate of potential drop was greatest directly after the addition of the iodine increment. In trying to determine a minimum potential by letting the solution of the complex stand for several hours, the difficulty of maintaining a constant iodine concentration was encountered. It was of interest to investigate the behavior of the binding affinity during a back-titration with sodium thiosulfate. The complex was first formed by the addition of an excess of iodine. The iodine complex was then gradually destroyed by reducing the iodine with standard sodium thiosulfate. The results of a forward and back-titration are shown in Figure 12. An exceptionally large increase in binding affinity is observed for the back-titration. The potential after each addition of thiosulfate was much more steady than in the forward-titration. However, after about half of the complex was destroyed, the potential experienced a small rise after the addition of an increment of thiosulfate. The increase was more pronounced during the last

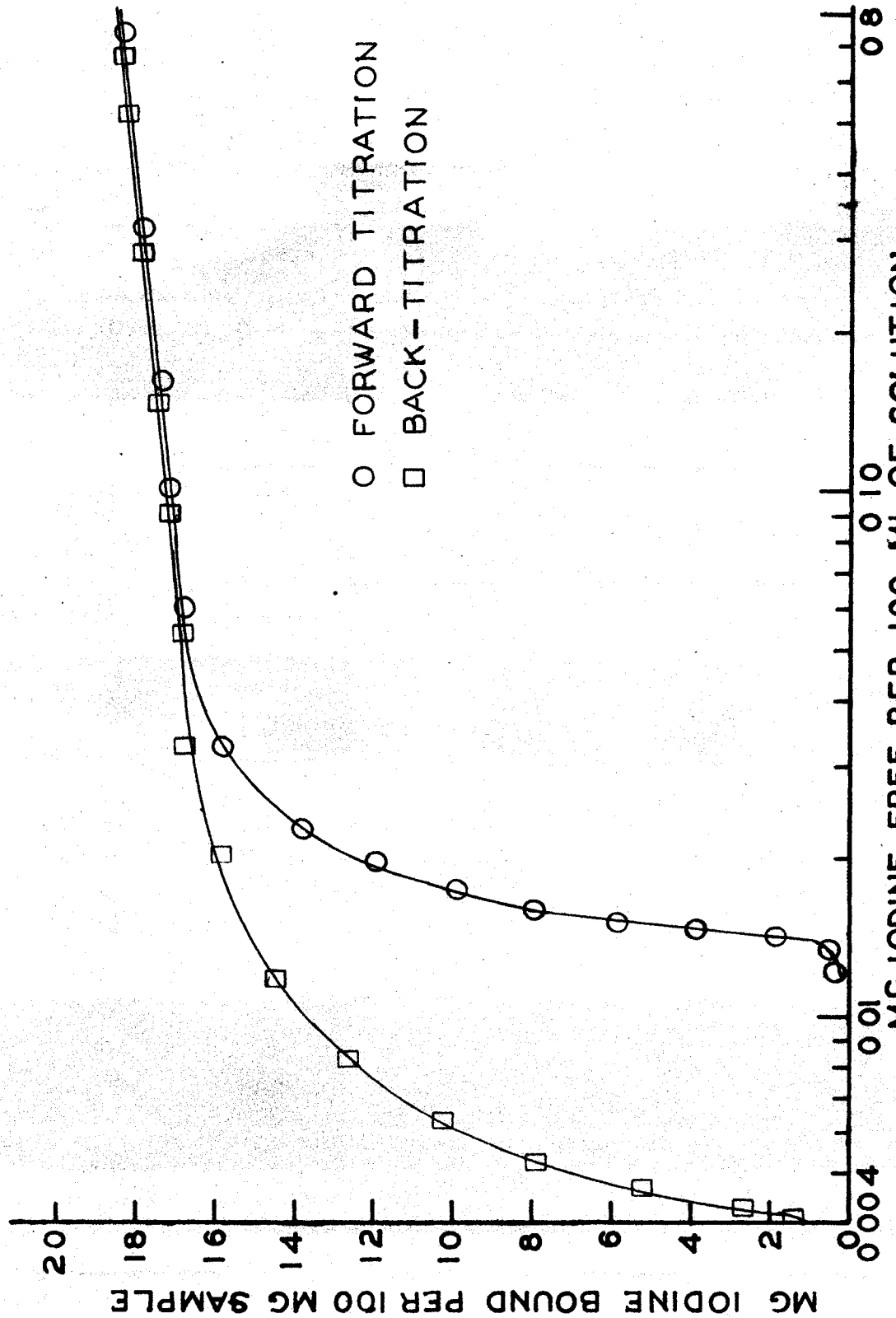

Figure 12. Back-titration of the sorghum iodine complex.

part of the back-titration.

This behavior was not due to the salts formed by the reduction of iodine with thiosulfate. This was shown by adding an equivalent amount of iodine reduced with thiosulfate at the beginning of a normal titration. Essentially the same curve was obtained as in the normal titration.

Thus it appears that once the complex is formed it has a greater tendency to hold the iodine in the complex. The same factors which were considered as causing an increase in binding affinity of the regenerated amylose may be involved here. The increase in binding affinity during a back-titration is greater than that found with a regenerated sample of amylose pretreated with iodine. This is expected since in the latter case the sample is dispersed in KOH and neutralized with hydrochloric acid before a titration is begun. This might allow some entanglement and also a certain amount of disorganization of an oriented micelle.

G. Influence of Time of Standing in KOH on the Binding Affinity of Amylose

It was found from the spectrophotometric investigation of the amylose-iodine complex that amylose which was allowed to stand for two or three weeks in 1 N. KOH experienced a reduction in wave length of maximum absorption of about 30

millimicrons. Previous experiments also showed that the iodine binding affinity of amylose steadily decreased as amylose stood in N. KOH. The remarkable feature of this latter experiment was that upon regeneration of the amylose the binding affinity was nearly restored to the original value which was obtained after thirty minutes in KOH. It may be significant that the amylose preparation which stood in KOH three weeks and was regenerated gave a wave length of maximum absorption of 620 which was about the same value obtained before regeneration.

The curve showing the behavior of corn amylose after standing in N. KOH for various time intervals is shown in Figure 13. To prevent evaporation of the solvent during standing, the solution was kept in a stoppered burette. The sample which stood in KOH thirteen days was regenerated, and a large increase in binding affinity was found. The curve representing this increase is also to be compared with that representing a regenerated sample after an hour's standing in KOH. This type of comparison is necessary because of the increased binding affinity caused by regeneration.

It was of interest to determine if the decrease in binding affinity was due to oxidative degradation and whether or not it could be eliminated by keeping the solution under nitrogen. The experiment was therefore

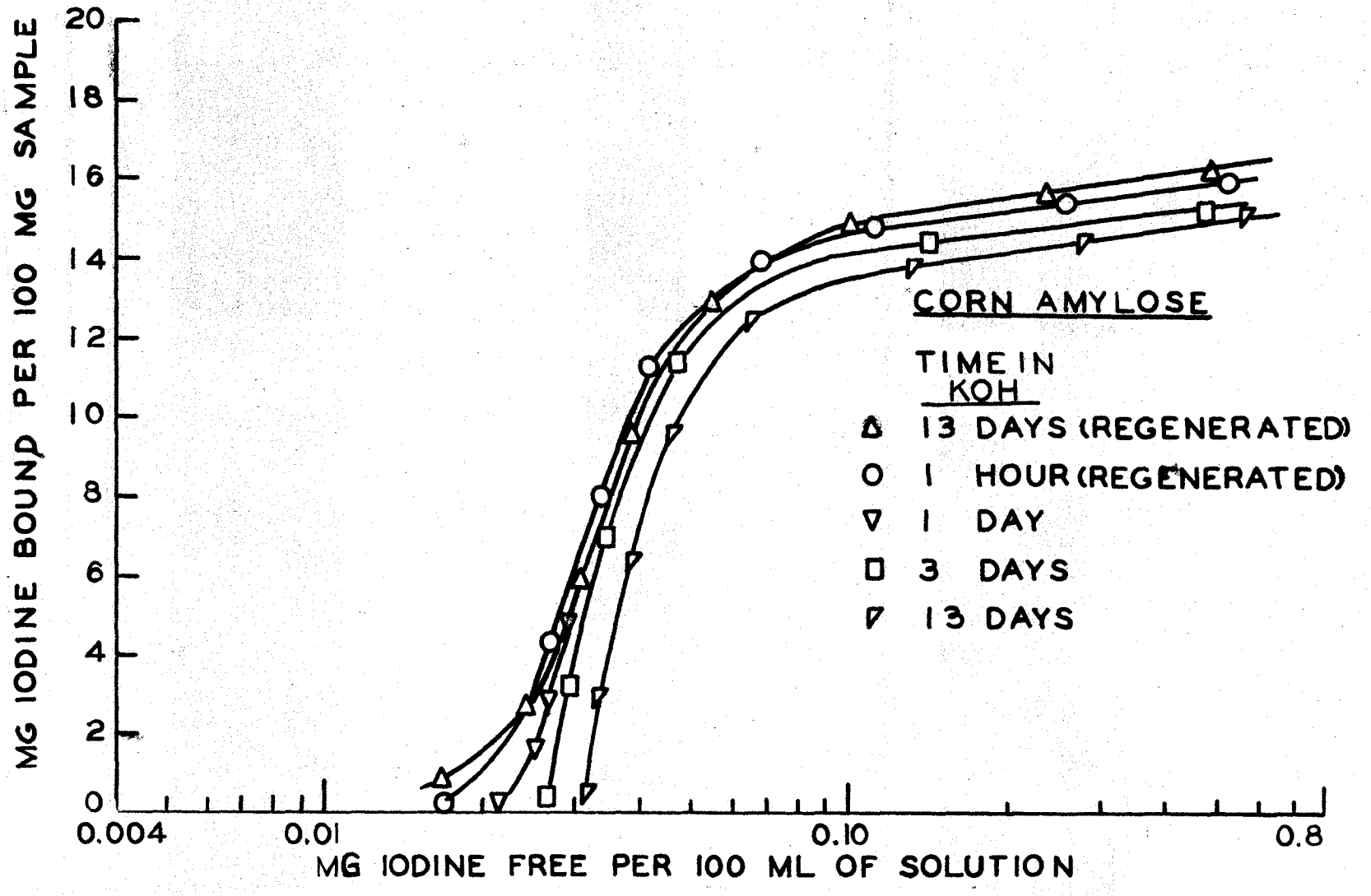

Figure 13. Influence of time on the binding affinity of amylose in KOH.

repeated using conditions outlined in Section VI for the exclusion of oxygen. Figure 14 shows that with this procedure a decrease in binding affinity is also encountered. The total decrease appears to have diminished somewhat; however, it is not certain whether the difference is significant.

A decrease in binding affinity is also observed with a subfractionated corn amylose preparation shown in Figure 15. In addition to potentiometric iodine titrations the change in apparent molecular weight was also followed from turbidity measurements on this amylose. This phase of work will be discussed in Section VI where a correlation will be drawn between the change in binding affinity and the apparent molecular weight as the amylose stands in N. KOH under nitrogen.

This behavior of amylose suggests that KOH may be functioning in more than one manner. If aggregates of amylose are present, as has been indicated in previous experiments, and the aggregates are partly responsible for high iodine binding affinity, then the KOH may be functioning as a dispersing agent for the aggregates. If amylose helices are present in aqueous solution, the KOH could conceivably operate in a manner to partially destroy or at least extend the helix. This might arise from the repulsion of negative charges spaced along the helix under

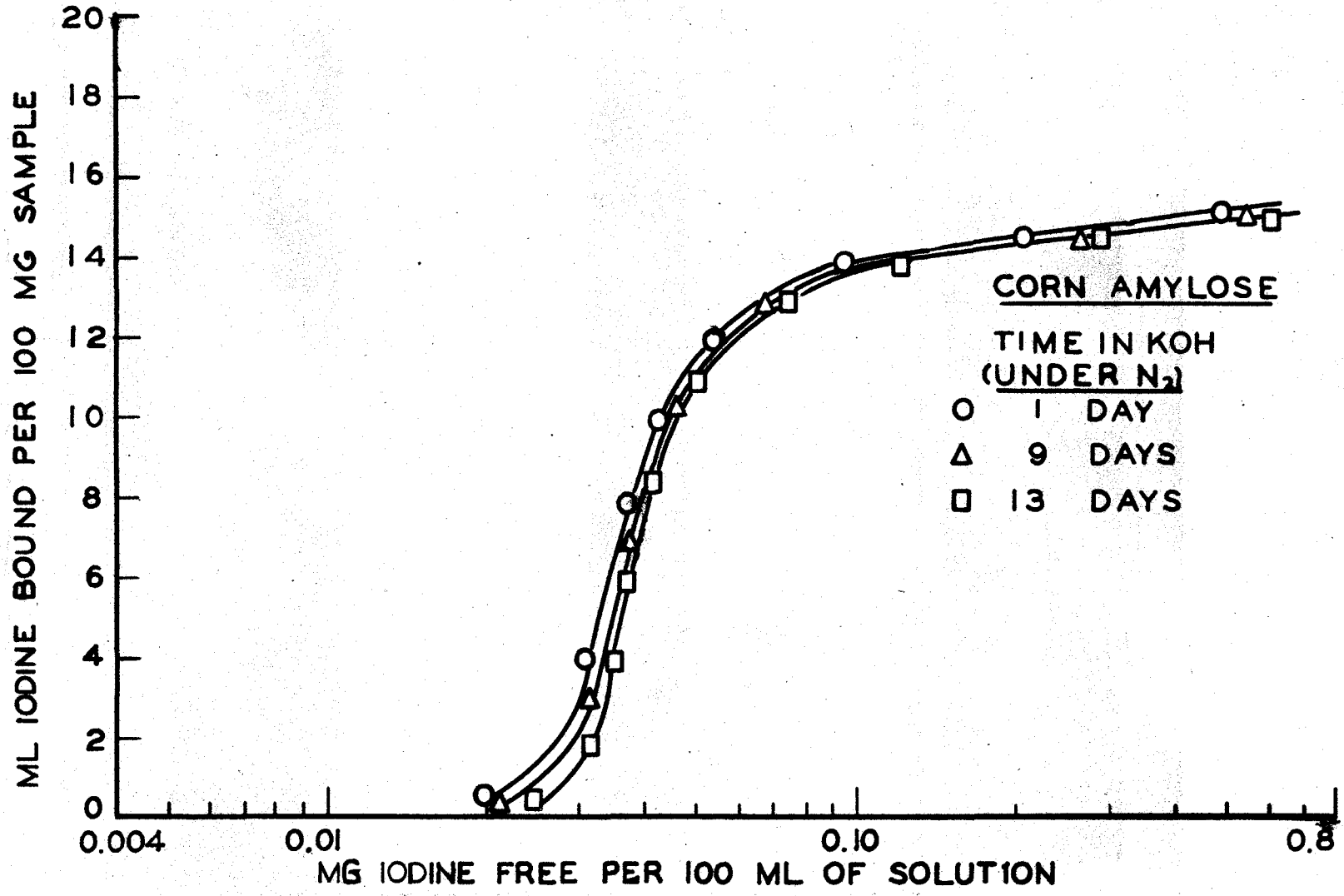

Figure 1h. Influence of time on the binding affinity of amylose in KOH under nitrogen.

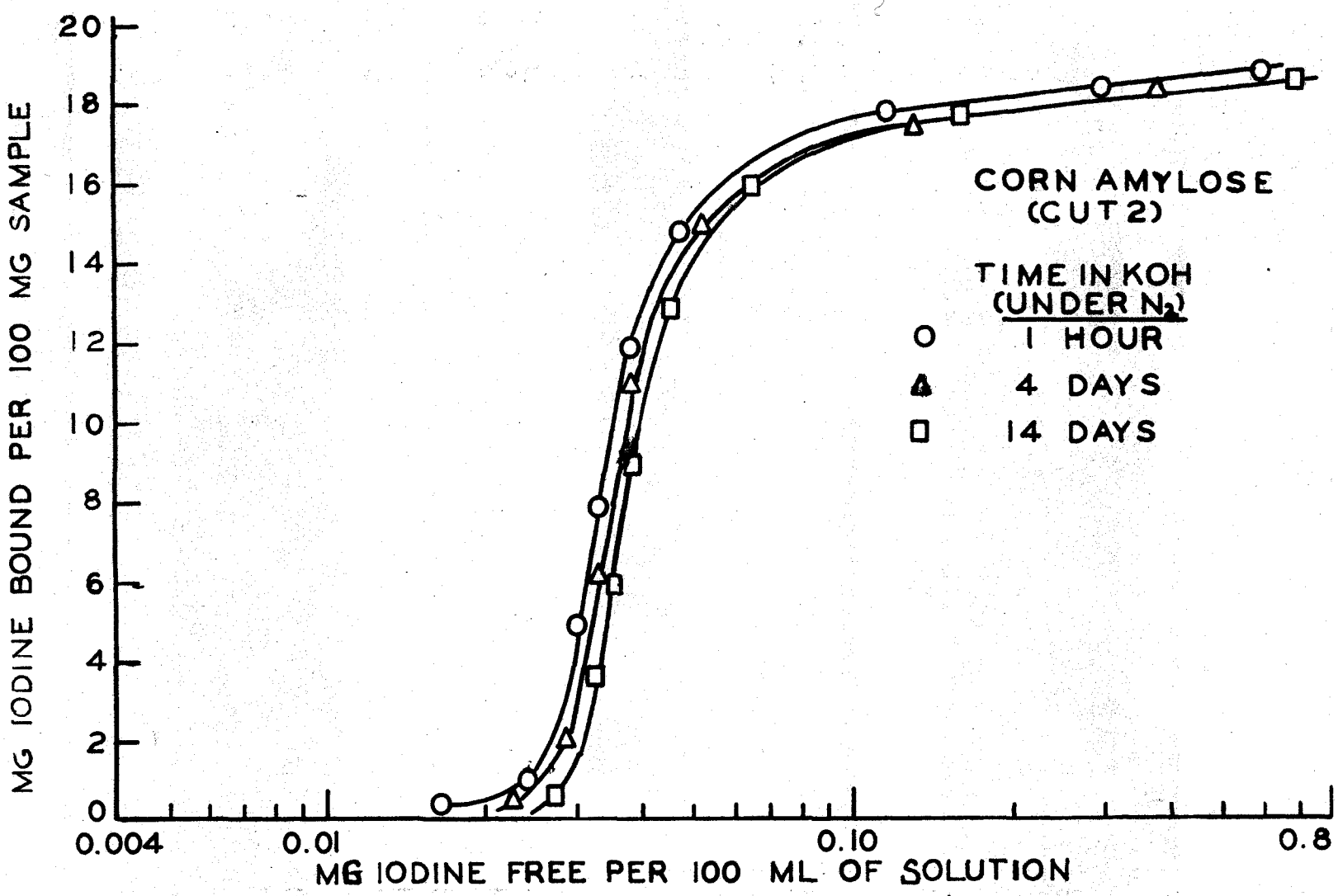

Figure 15. Influence of time on the binding affinity of corn amylose, Cut 2, in KOH under nitrogen.

the influence of KOH. The behavior of the regenerated amylose after several days in N. KOH is in harmony with this idea, since in the process of regeneration the complex is dispersed and reformed several times in neutral media.

If both a dispersion of amylose aggregates and the extension or destruction of the helix is caused by KOH, then one would expect the titration curve of a regenerated sample after several days in KOH to reflect both of these effects: that is, a decrease in binding affinity due to the KOH treatment and an increase due to the regeneration. The observed binding affinity would depend on the relative magnitude of the two effects. In Figure 13 the small decrease in binding affinity after three weeks in KOH and regeneration is then perhaps due to the KOH treatment, since the curve with which it is compared represents a regenerated amylose sample.

To understand better the function of KOH on amylose it was necessary to perform additional experiments to establish more conclusively the existence of amylose aggregates and their influence upon the binding affinity of amylose for iodine.

VI. BEHAVIOR OF AMYLOSE IN AQUEOUS SOLUTIONS AS REVEALED BY LIGHT SCATTERING[1]

A. Turbidity as a Function of Time

1. Influence of time of standing on the turbidity of amyloses at various pH values

It was shown previously by the potentiometric iodine titration method that both the rate and extent of retrogradation were influenced by the pH of the amylose solution. The greatest retrogradation tendencies were found to occur at pH values of between 6 and 7. In contrast amylose solutions which stood at a pH of 4 showed a very small loss of soluble amylose. This was evidenced by the small change in the per cent of iodine bound. There was also, however, a definite decrease in the iodine binding affinity. The behavior at a pH of 9 was somewhat intermediate between that at a pH of 4 and at a pH of 6 to 7. These varied pH effects at first suggested the possibility of an isoelectric point similar to that found for proteins. To substantiate further this somewhat strange behavior, light scattering experiments

[1]The light scattering instrument was built by Dr. Raymond Rhees, Iowa State College, 1951.

were undertaken to follow the change in particle size as a function of time and pH.

Rather elaborate precautions must be taken in the preparation of amylose solutions for light scattering measurements. In particular this applies to the clarification of the solutions immediately prior to turbidity measurements. Dust or any other foreign material must be eliminated in order that reliable turbidity results may be obtained.

The drying and the dispersion of the amylose follows the same procedure as was outlined for the preparation of solutions for potentiometric iodine titrations in Section V-A. The initial concentration of the amylose was generally 0.267 per cent. This value was reduced in certain cases where extremely high turbidities were encountered. After the amylose was completely dispersed in KOH and neutralized with hydrochloric acid if desired, the solutions were centrifuged in the Sorvall high speed centrifuge for one hour (20,000 g). The top half or two-thirds of the solution was carefully withdrawn by pipette and transferred to the cell in which turbidity readings were to be made. All of the glassware and centrifuge tubes to be used were thoroughly washed and finally rinsed with doubly-distilled water. The turbidity measurements as a function of time of standing were made on solutions contained in capped, weighing vials.

Dilution series were performed on solutions kept in six-sided, scattering cells which permitted dissymmetry measurements to be made.

Turbidity measurements on the various amylose solutions led to the early demonstration that a turbidity drop was generally observed with solutions that were allowed to stand at a pH of 6 to 7. The magnitude of the drop was dependent on the value of the initial turbidity. Solutions of high initial turbidities displayed a much greater drop than those of low initial turbidities. The variation of the minimum turbidity values for the various amyloses was much less than the variation of the initial values. An examination of Figures 17, 18, 19 and 20 will readily substantiate this.

At a pH of 4 the behavior of the turbidities on standing was unexpectedly different. A drop in value below that initially observed was never achieved. With amylose solutions of rather high initial turbidities the value would either remain constant or increase slightly on standing. However, a preparation of Kerr's crystalline amylose, which was extracted from starch with hot water and recrystallized with butyl alcohol, retrograded completely at a pH of 4. This took place at a much slower rate than was observed at a pH of 6 to 7. This material will be discussed in greater detail later. It need only be pointed out here that the amylose showed a very low initial turbidity, and the speed

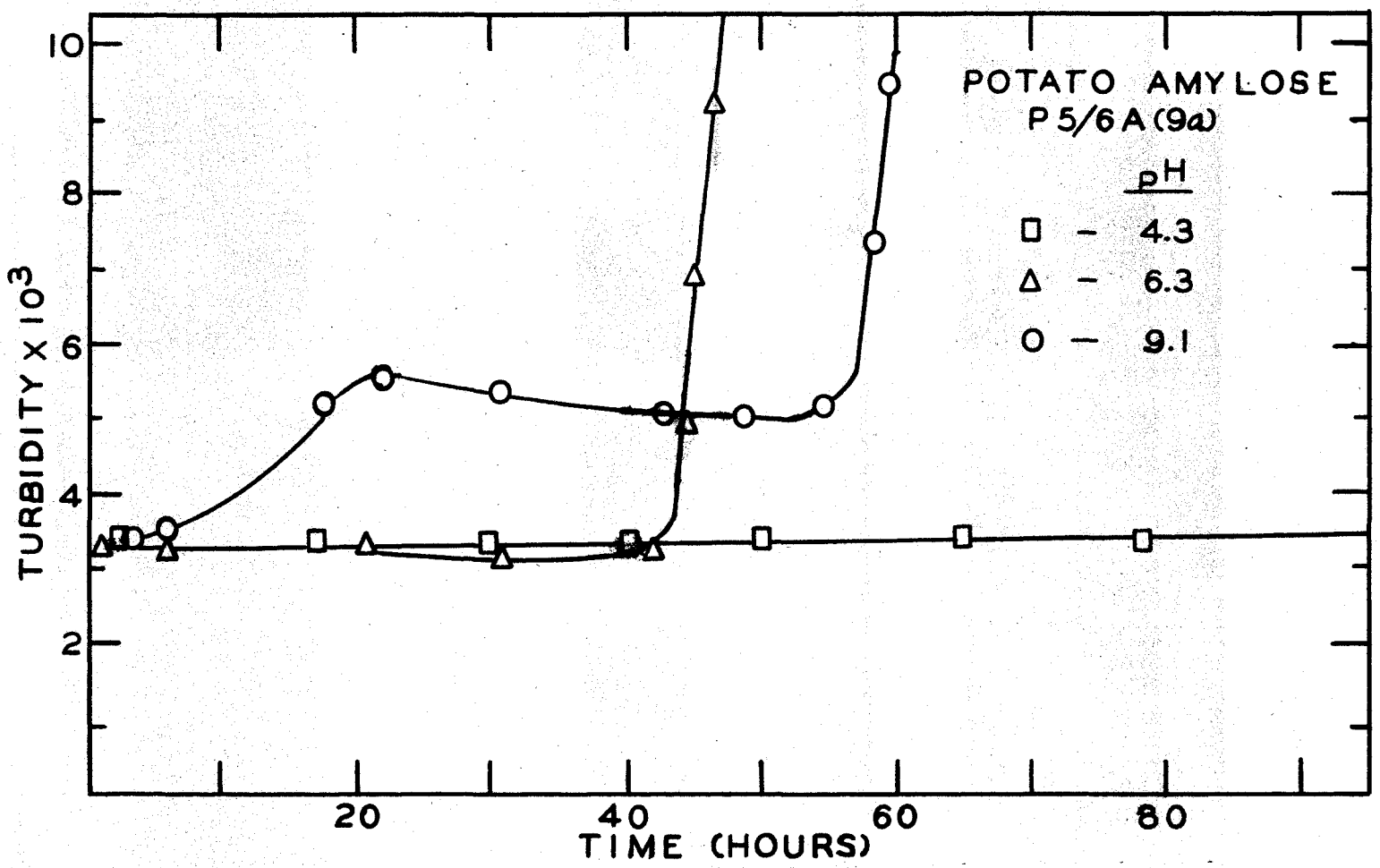

Figure 16. Turbidity vs. time of standing for amylose solutions at various pH values. Intrinsic viscosity — 2.05.

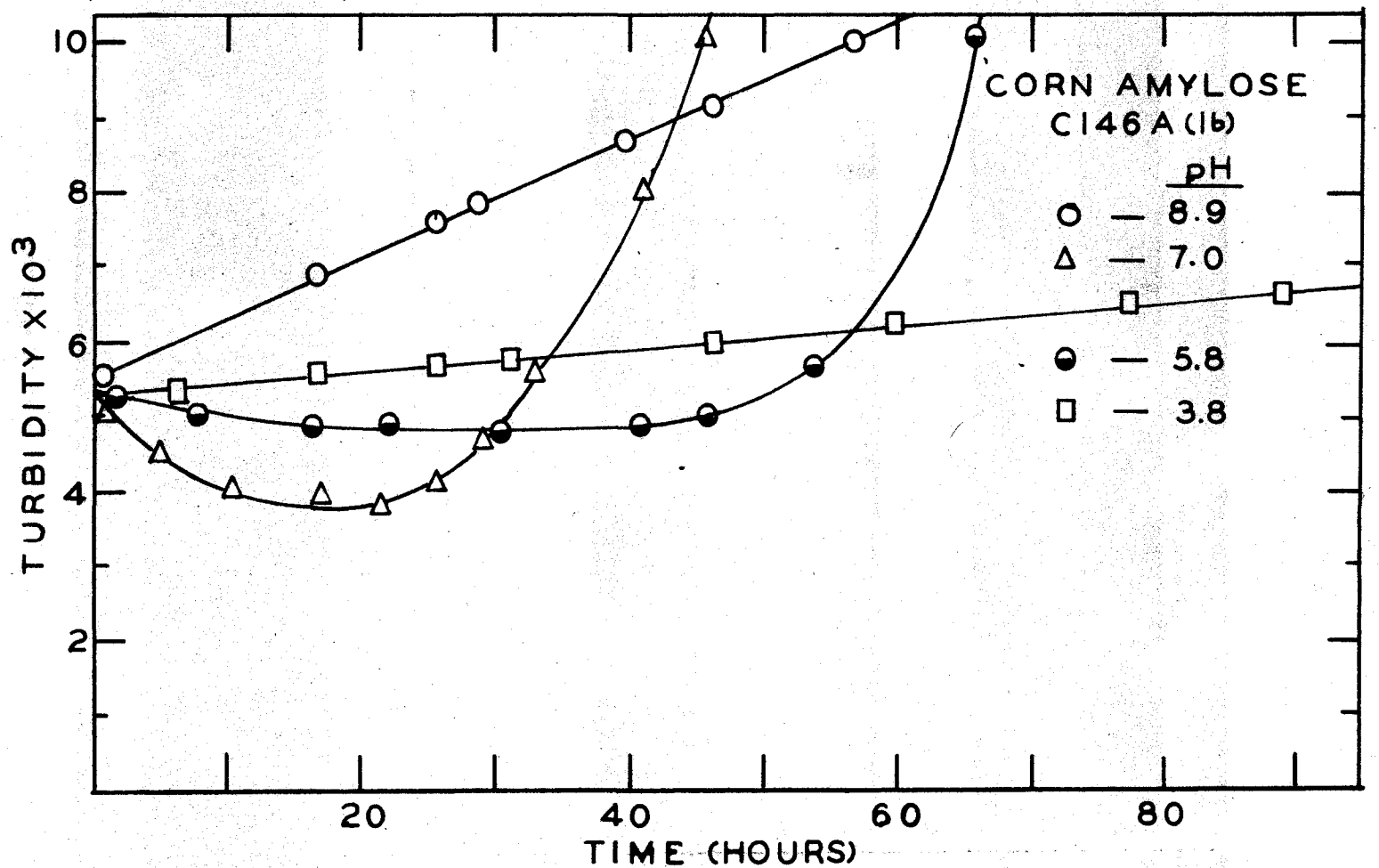

Figure 17. Turbidity vs. time of standing for amylose solutions at various pH values. Intrinsic viscosity — 0.9k.

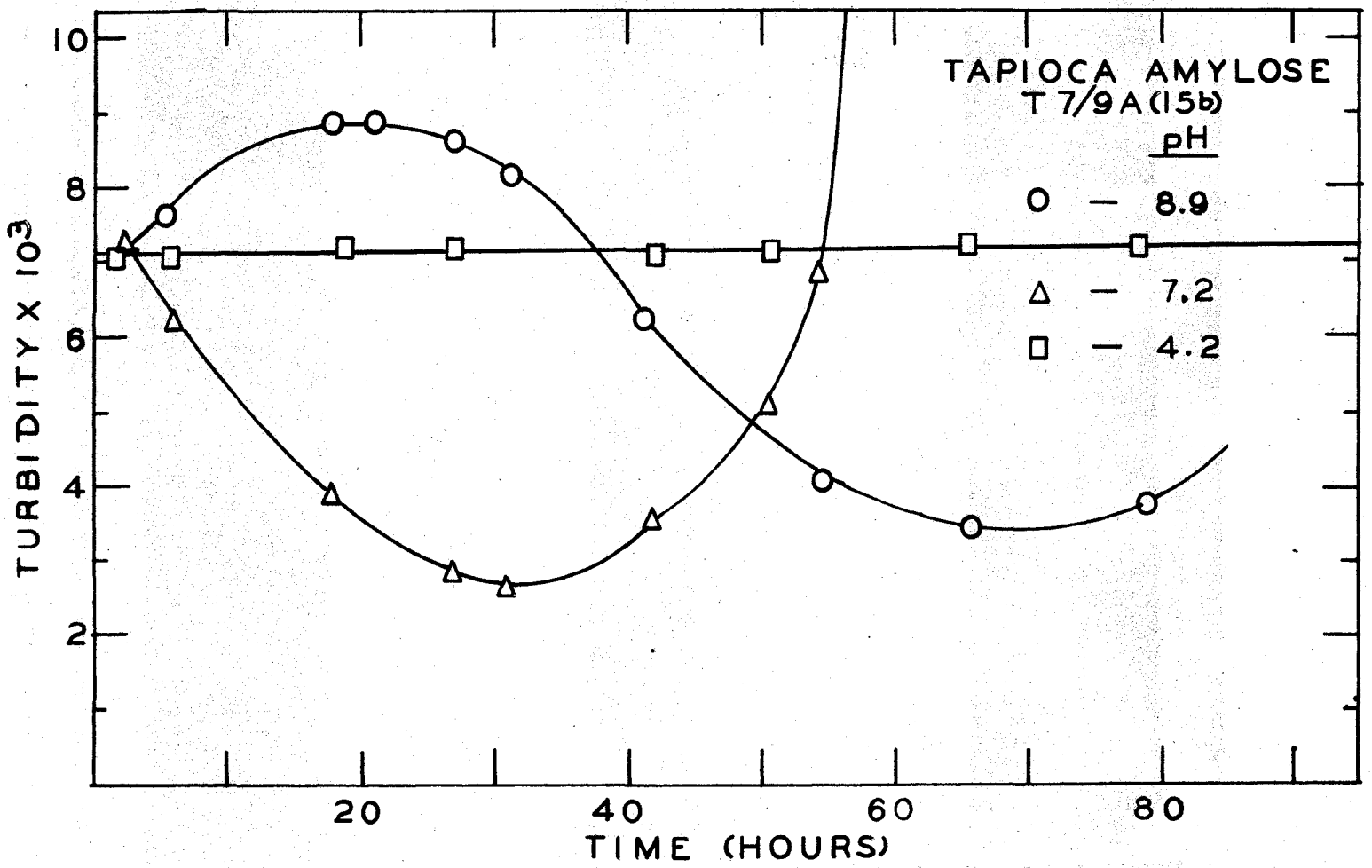

Figure 18. Turbidity vs. time of standing for amylose solutions at various pH values. Intrinsic viscosity — 2.98.

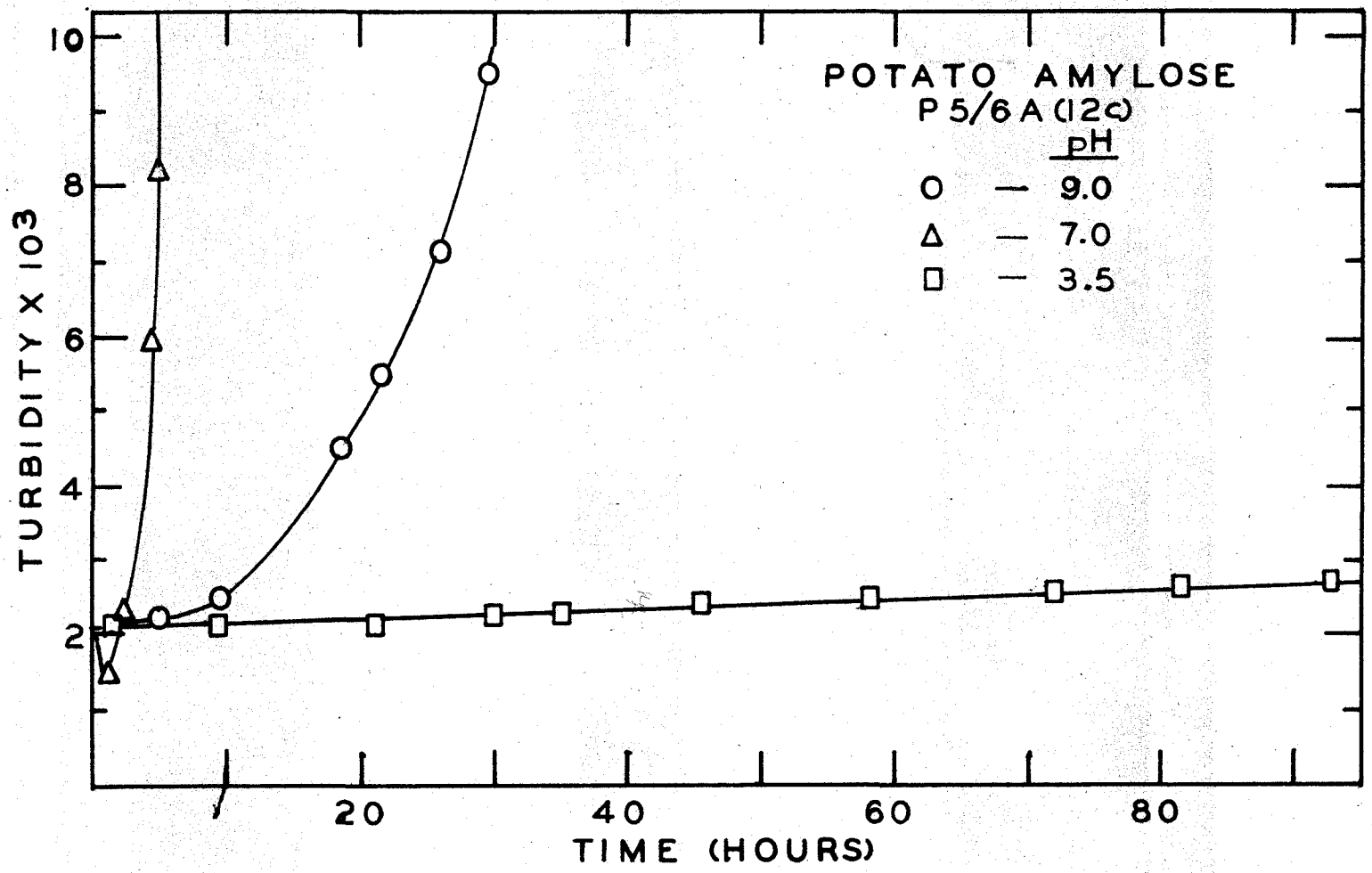

Figure 19. Turbidity vs. time of standing for amylose solutions at various pH values. Intrinsic viscosity — 2.05.

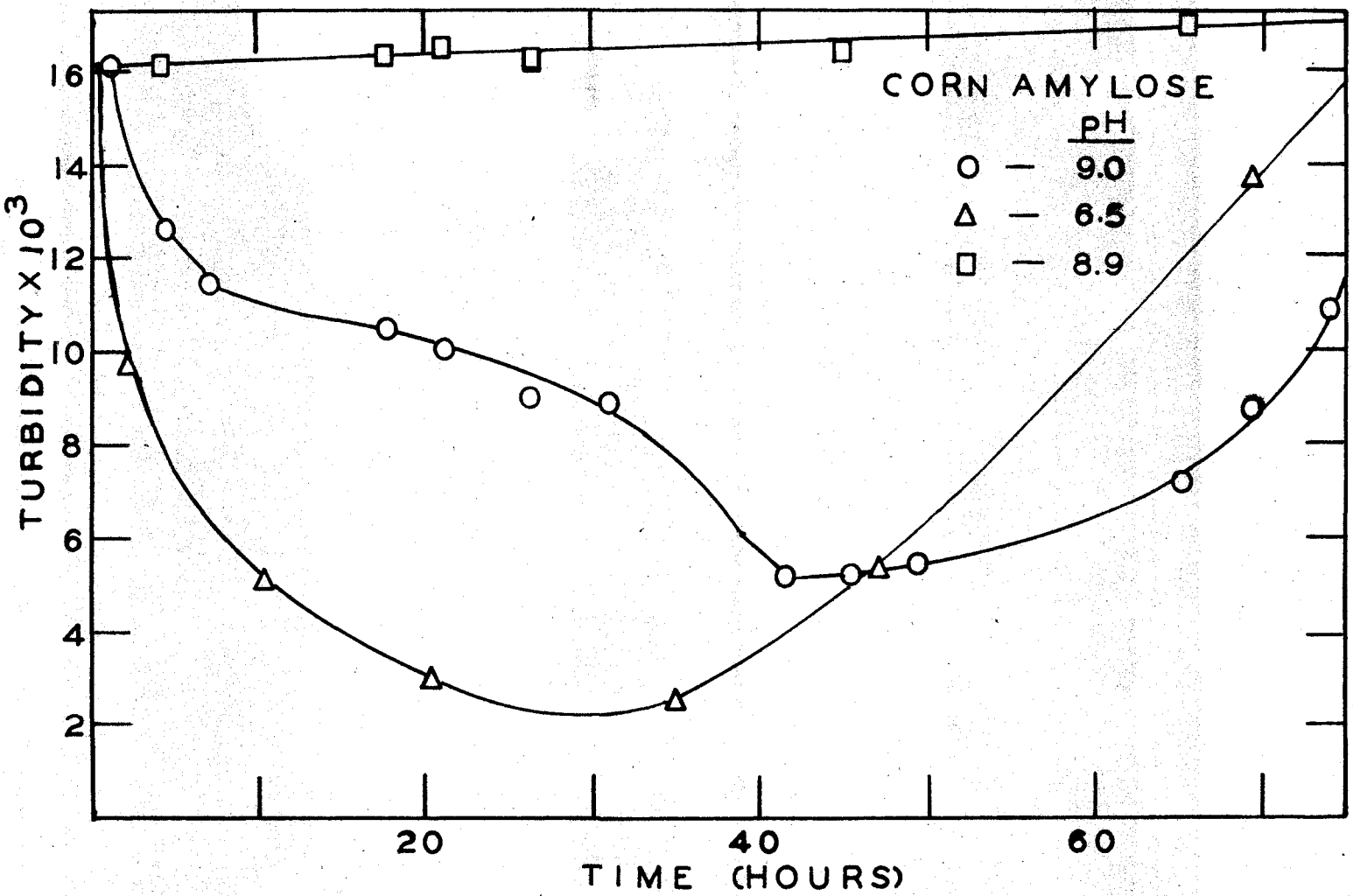

Figure 20. Turbidity vs. time of standing for pentanol fractionated amylose solutions at various pH values.

of retrogradation was such that it was impossible to follow accurately the turbidity as a function of time.

The behavior of amyloses at a pH of 9 was found to be somewhat complicated with both a rise and drop in turbidity before final retrogradation. In the event the amylose solution had a strong tendency to retrograde, the hump in the curve due to a turbidity rise was not evident. This is seen to be the case in Figure 19.

The discussion so far has concerned the general behavior of amyloses at various pH values. It is desirable to discuss the individual behavior of amylose from various sources from the standpoint of reducing value and intrinsic viscosity. The data for several amyloses are recorded in Table V. These data infer the following order of decreasing chain lengths: tapioca T 7/9 A(15b), potato P 5/6 A(9a), potato P 5/6 A(12c) and corn C 146(1b).

It was found that the initial turbidity does not always correlate with the length of the amylose as inferred from its intrinsic viscosity. For example, the sample of potato amylose, P5/6A(9a), with an intrinsic viscosity of 2.05 showed a low initial turbidity of (2.7 to 3.2) x 10^{-3} (Figure 16). This is in sharp contrast to an initial value of 5.4 x 10^{-3} for the corn amylose, C146(1b), with a low intrinsic viscosity of 0.94 (Figure 17) and is only slightly greater than the value of 2.5 x 10^{-3} for the potato amylose,

Table V

The Relationship of Turbidity to Intrinsic Viscosity and Reducing Value of Various Amyloses[1]

	Initial $\tau \times 10^3$	Minimum $\tau \times 10^3$	$[\eta]$	Reducing Value
P5/6A(9a)	2.7 - 3.2	1.9 - 3.1	2.05	2.64
P5/6A(12c)	2.5	1.35	1.37	3.28
T7/9A(15b)	7.9	2.34	2.98	1.76
C1A6(1b)	5.4	4.0	.94	6.11

[1]These samples as well as the reducing values and viscosity data were supplied by Dr. Thomas Schoch, Corn Products Refining Company.

P5/6A(12c), with an intrinsic viscosity of 1.37 (Figure 19). However, it was found that the long potato amylose retrograded with only a small drop in turbidity, whereas the apparently shorter potato amylose with about the same initial turbidity dropped from 2.7×10^{-3} to 1.4×10^{-3} before retrogradation. Furthermore, the tapioca amylose which showed an initial turbidity of 8×10^{-3} and an intrinsic viscosity of 2.98 (Figure 18) underwent a drop in value to 2.34×10^{-3} before retrogradation began, and a sample of pentasol fractionated corn amylose (Figure 20) with a very high initial turbidity of 18×10^{-3} also experienced a drop to 2.34×10^{-3} before retrogradation.

Several conclusions may be drawn from these results. With most of the amyloses under consideration a drop in turbidity was necessary before retrogradation. A pH somewhere in the range of 6 to 7 was required for this drop. Two exceptions have been encountered. As mentioned previously, Kerr's crystalline amylose showed a very low initial value of 5 and retrograded immediately without a decrease in turbidity. This occurred also at a pH of 4 although at a slower rate than at the neutral pH. A similar behavior is noted above with the longer potato amylose sample with little or no turbidity drop required for retrogradation. The minimum turbidity value perhaps depends on the length of the amylose molecules, with the shorter

species experiencing a lower minimum before retrogradation. It is obvious that large deviations from the neutral pH will greatly influence the turbidity behavior. The exact pH required for a maximum drop in turbidity is not easily ascertained, but small deviations from the optimum pH apparently raise the minimum and thus the time required for retrogradation. The rate of disaggregation may be greater for a particular species of aggregates in a heterogeneous amylose solution. Part of the amylose may have reached the retrograded stage while the remainder still is aggregated to a certain extent. The effect on the observed turbidity as a function of time would be a high minimum as observed with the corn amylose sample, G146A(1b) in Figure 17.

Another observation which may be of considerable importance in clarifying the cause of the turbidity drop was the gradual increase in pH upon standing. Apparently protons were adsorbed by the amylose. This behavior generally required a readjustment of the pH after a few hours. In some cases the readjustment caused an increase in turbidity which was decreased to the original value by recentrifugation. The effect of changing the pH will be discussed further in the next section.

These findings lend additional support to the belief that aggregates of amylose are present in aqueous solutions of amylose. Furthermore, these aggregates are of

considerable size where high initial turbidities are observed. A dispersion of these aggregates is necessary before retrogradation can occur. This explains why the apparently disaggregated crystalline amylose retrogrades without experiencing a drop in turbidity.

That the initial high turbidity observed with some of the samples was not due to the manner of KOH dispersion and centrifugation was shown by the fact that almost identical initial values were obtained with the various amylose samples in a large number of experiments carried out over a period of six or eight months.

2. Influence of time of standing on the turbidity of amylose in N. KOH

Previously it was observed that a decrease in iodine binding affinity occurred as amylose stood in KOH. Upon regeneration of the amylose the binding affinity was partially restored to the initial value. It was of interest to investigate the turbidity as a function of time of standing in N. KOH in connection with this experiment and also to observe what relationship existed between the turbidity behavior in neutral solution and in N. KOH solution.

For this experiment corn amylose (pentasol fractionated) and a potato amylose, P5/6A(9a), with a relatively high intrinsic viscosity, were chosen. Both samples were used

in the previously discussed potentiometric iodine titration and turbidity experiments. The corn amylose was found to have a high initial turbidity and experienced a large turbidity drop on standing at the neutral pH. The potato amylose showed a relatively low initial turbidity and retrograded with very little if any turbidity drop.

The results of the turbidity change as the amyloses stood in N. KOH are shown in Figure 21. The corn amylose showed a gradual decrease in turbidity with time, whereas the potato amylose showed very little change. This behavior implies that the turbidity drop at the neutral pH and that in N. KOH are both due to the dispersion of amylose aggregates. The potato amylose experienced very little change in either case, whereas corn amylose showed a drop with both treatments in the time interval used.

It should be pointed out that a regeneration and redispersion in KOH of the corn amylose after the turbidity drop does not restore the turbidity to the initial value. In fact the same turbidity at the point of regeneration is encountered upon redispersion in KOH. This again points toward an irreversible dispersion of amylose aggregates.

One is able to postulate from these results that a drop in turbidity at the neutral pH is due to the same type of disaggregation as a drop in N. KOH. Amyloses which are in a low state of aggregation, such as the above potato amylose,

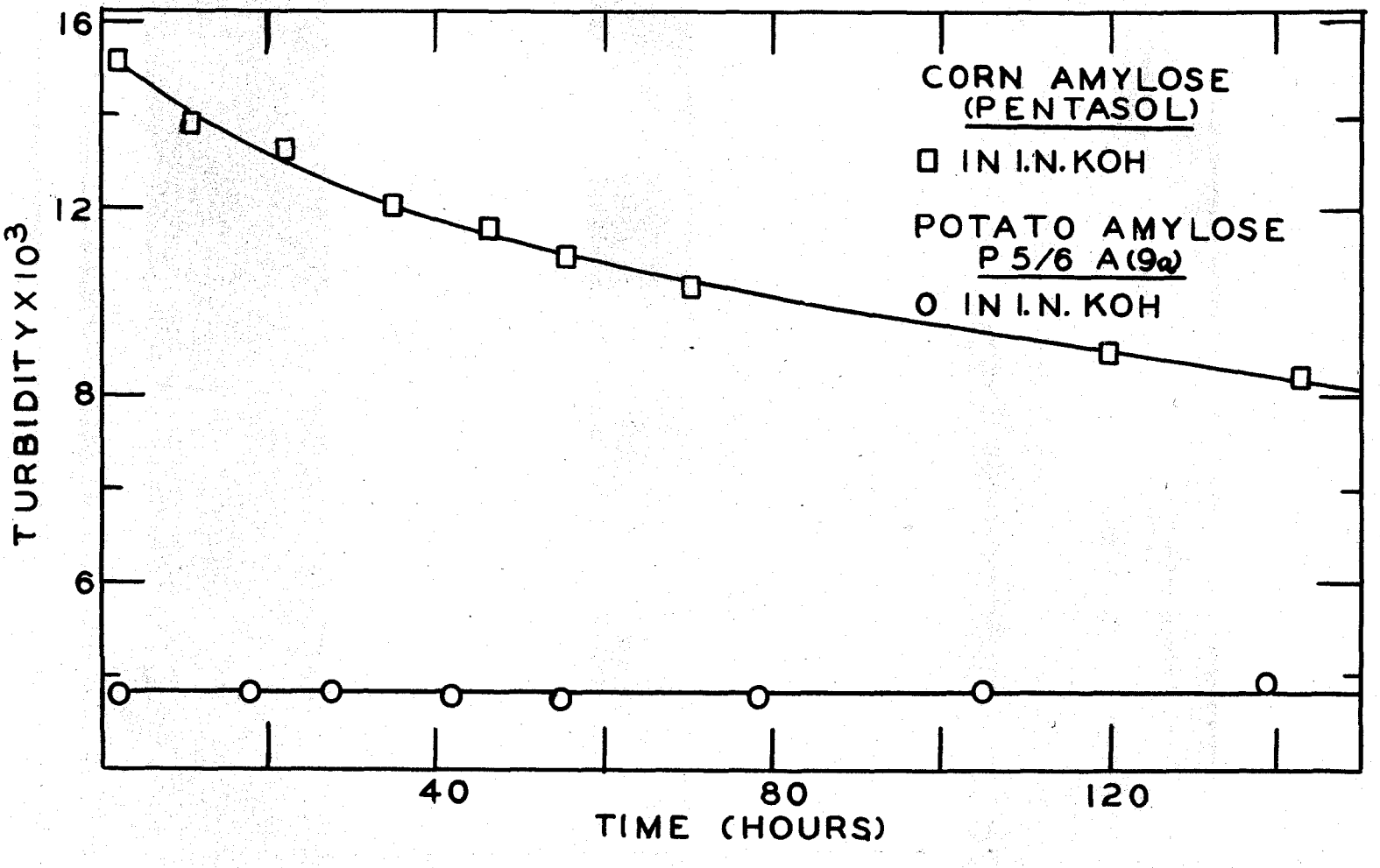

Figure 21. Turbidity vs. time of standing of amyloses in N. KOH.

do not reflect the turbidity change that is encountered with the highly aggregated preparations. Also, the greater change in iodine binding affinity observed for the above corn amylose is in part due to a disaggregation of amylose.

3. Effect of iodine pretreatment on the turbidity of amylose

It was observed previously that if a sample of amylose were pretreated with iodine and regenerated after reducing the iodine with sodium thiosulfate, an increase in iodine binding affinity was produced. This was explained as due principally to an orientation under the complexing influence of iodine of the amylose molecules composing an aggregated network. The final state of the aggregated network was conceived as being a linear array of helical amylose molecules clumped together to form a helical micelle complex. These complexes in turn were envisioned as associating further under the influence of salt. Upon regeneration the orientation of the amylose molecules in the original aggregate was preserved so that the amylose experienced a greater binding affinity for iodine.

With this in mind experiments were carried out with two potato amylose preparations to determine the behavior of the turbidity after complexing the amylose with iodine. The amylose preparations used were two which were discussed in

the preceding section -- Schoch's P5/6A(9a) and P5/6A(12c), the first of which retrogrades slowly and the latter rather rapidly. To one aliquot of each potato amylose an excess of iodine solution was added and neutralized with sodium thiosulfate. To the second aliquot of each sample the same amount of iodine already neutralized with thiosulfate was added. These solutions were centrifuged and turbidity measurements run as a function of time. The pH at all times was kept at a value of about 6.5.

It was observed that both the rate and magnitude of retrogradation were affected by the iodine treatment. The samples which were treated with an equivalent amount of iodine neutralized with sodium thiosulfate retrograded completely. In the case of the iodine-pretreated, short amylose, P5/6A(12c), the time required for retrogradation was increased by about ten hours as shown by Figure 22. More important, however, was the observation that prolonged standing of the iodine-pretreated sample produced very little retrograded material, as shown by the thin layer of material on the bottom of the cell. This obviously meant that only a fraction of the total amylose retrograded. The formation of the iodine complex, then, greatly retarded the ability of this short material to retrograde. With the longer P5/6A(9a) sample the time required for retrogradation was practically doubled. The same factors which operate to increase the

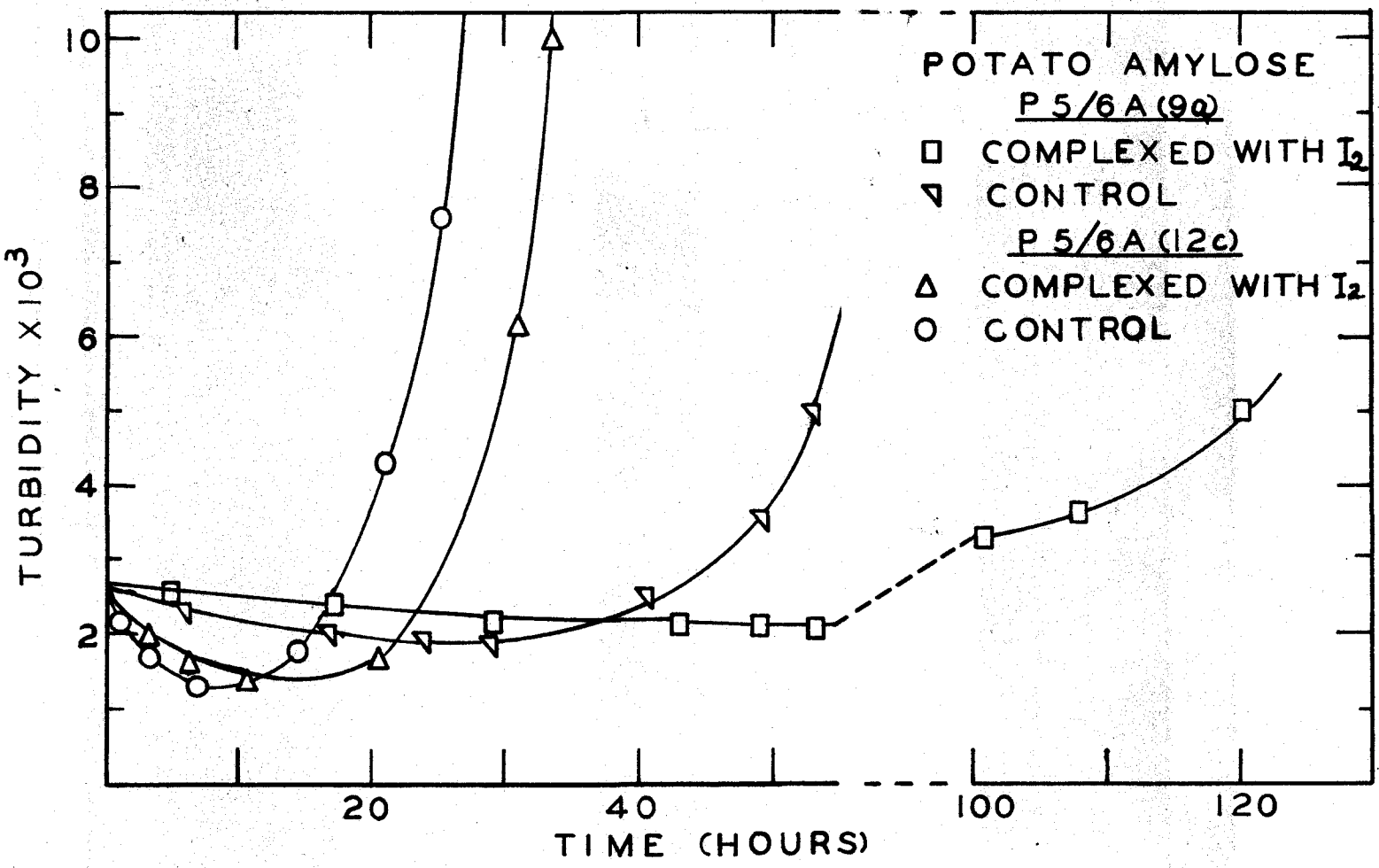

Figure 22. Turbidity vs. time of standing for amyloses pre-treated with iodine. Controls pre-treated with iodine reduced with thiosulfate.

iodine binding affinity after an iodine treatment and regeneration also operate to retard the retrogradation of amylose that has been complexed with iodine.

It is evident from Figure 22 that the initial turbidities for the iodine treated and non-treated samples are nearly the same, indicating very little change in the size of the aggregates after pretreatment with iodine. This is in agreement with the previously expressed concept that iodine orients the molecules within the aggregate and does not necessarily increase or decrease the size of the aggregate.

A difference in the rate of turbidity drop would ordinarily be expected for experiments of this nature because of the reduced tendency toward retrogradation. It was unfortunate that these samples were chosen for the experiment since their initial turbidities are low and a large drop is not necessary for retrogradation. Nevertheless, a greater turbidity drop with both amyloses which were not pretreated with iodine was observed. It is expected that a large difference in minimum would be observed if a more highly aggregated sample were chosen.

The conclusion is reached that the formation of the amylose-iodine complex not only orients the helical amylose molecules linearly within the aggregate but also causes an additional stabilization of the aggregate. It is probable

that this orientation aligns the molecules in a manner so as to cause a greater interaction of the hydroxyl groups with those of adjacent molecules to form additional hydrogen bonds with no appreciable change in size of the aggregate. It also seems reasonable to expect increased intramolecular attraction between the polar hydroxyl groups, causing an increased stabilization of the helical structure. This perhaps would account for the slow rate of retrogradation and diminished turbidity drop.

4. Influence of a change in pH on the turbidity of amylose

It was observed in a few cases that a turbidity drop did not occur at the pH of 6.5. This was perplexing since apparently the same precautions had been used in neutralizing and adjusting the pH to the required value. Nevertheless, experiments were done to show that solutions which stood at a pH of 4 for several hours and then were adjusted to the neutral pH experienced the usual drop in turbidity. Also, there was found to be little difference in either the rate of the turbidity drop or the amount of material retrograded after the change. A similar experiment was performed in which the pH was changed from 6.5 to 4 after a drop in turbidity. The behavior in both experiments is shown in Figure 23 in curves 1 and 2.

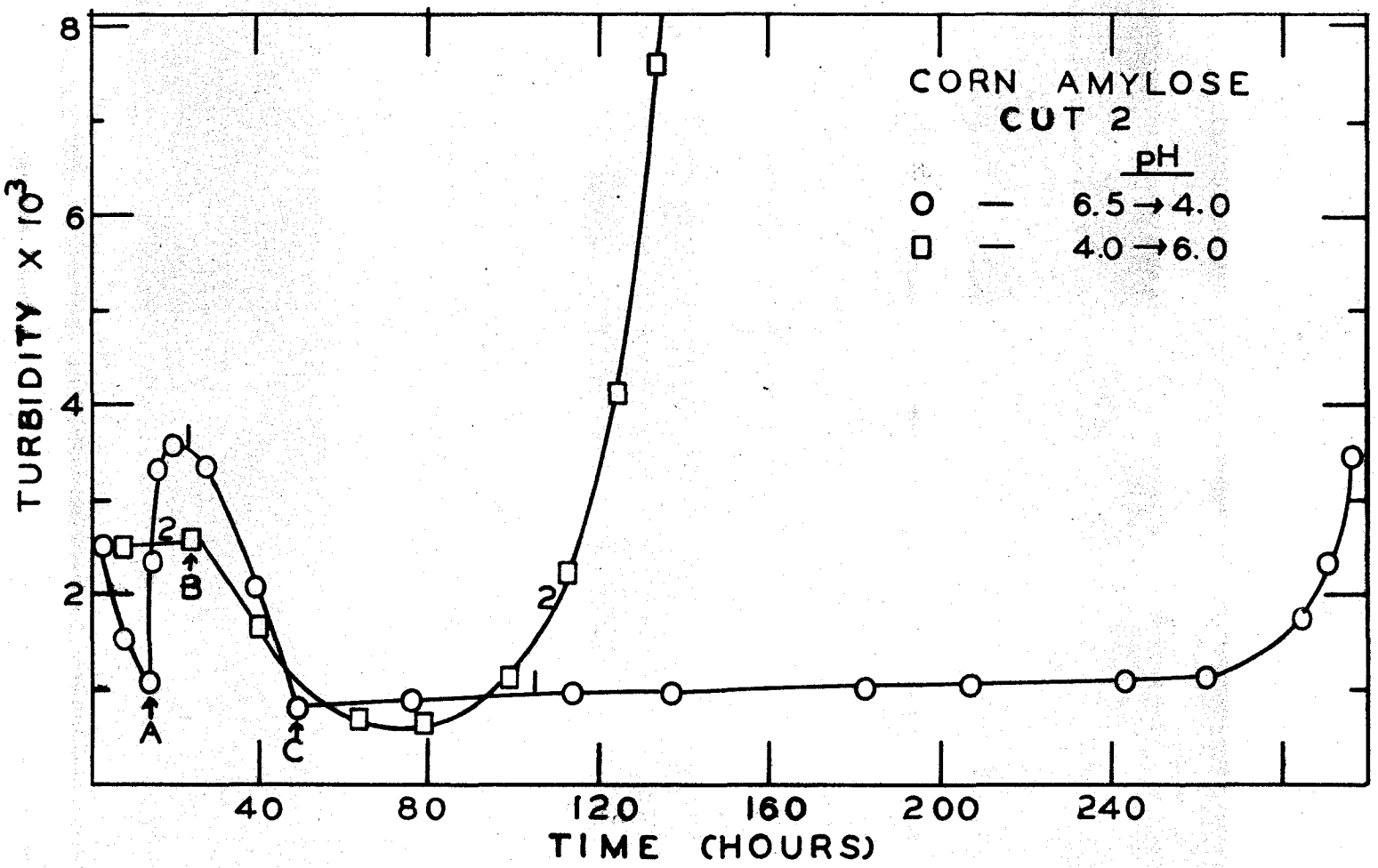

Figure 23. Influence of pH change on the turbidity of amylose as a function of time. (A) pH changed from 6.5 to 4. (B) pH changed from 4.0 to 6.5. (C) Solution centrifuged.

A sample of corn amylose Cut 2 from the pyridine subfractionation experiment was dispersed in KOH and divided into two portions. The concentration of amylose in this experiment was half that used in the previous turbidity experiments. To compare the turbidities the values of this experiment must be doubled. One sample was adjusted to an initial pH of 4 and the other to 6.5. The turbidities were followed for a few hours at each pH and then these were reversed by adding the required amount of KOH and hydrochloric acid. The solution at an initial pH of 6.5 showed the usual turbidity drop, but when this was adjusted to 4, a sharp rise was observed as shown in curve 1. This rise exceeded the initial turbidity. That the increase in turbidity was due to the formation of large particles was evidenced by the slow decrease in turbidity as the material settled. Stirring caused the turbidity to again rise to the peak value first observed after the pH change. Upon centrifugation the turbidity was lowered to 0.8×10^{-3} as shown by curve 2. It should be noted that this value is not as low as the minimum observed in curve 1 but is somewhat lower than the value at which the pH was changed from 6.5 to 4. This latter difference probably represents the amylose lost as a result of the pH change. From curve 2 it is seen that the turbidity at the pH of 4 continues to rise slowly until at 260 hours retrogradation occurs rapidly. A similar

behavior at a pH of 4 was ovserved with Kerr's crystalline amylose without the turbidity drop and pH change required for the Cut 2 preparation.

The turbidity of the amylose which was at a pH of 4 remained constant as expected. Upon readjusting the pH to 6.5 the turbidity dropped in the usual manner and was followed by retrogradation.

Several important results can be gathered from this experiment. Amylose which does not ordinarily retrograde at a pH of 4 because of its high initial turbidity may be caused to do so by first allowing the turbidity of a neutral solution to drop and then readjusting to a pH of 4. A pH change from 6.5 to 4 causes the aggregation of at least some of the amylose. Solutions which are initially at a pH of 4 may be adjusted to the neutral pH without any apparent change in turbidity drop or time of retrogradation. It may be inferred that retrogradation will occur more rapidly at a pH of 4 than at the neutral pH provided the amylose is completely disaggregated.

B. Apparent Molecular Weights As a Function of Time and pH

1. Influence of N, KOH as a function of time

Results were presented in the preceding section showing that amylose solutions of high initial turbidity experienced a drop in turbidity upon standing in KOH. No attempt was made to exclude oxygen, and the possibility existed that oxidative degradation may have been responsible for the turbidity drop. It was desired to perform a similar experiment in which the amylose was kept under nitrogen and the turbidity drop followed in terms of apparent molecular weights. By plotting Hc/T versus c, the B term in the expression $Hc/T = 1/M + 2Bc$ is eliminated and Hc/T is then equal to the reciprocal of the molecular weight.

The amylose samples not only were kept under nitrogen but were dispersed by bubbling nitrogen through the KOH solution as the amylose was added. A separatory funnel fitted with a two-holed rubber stopper served as a container for the sample. One hole was used as an inlet for nitrogen and the other as an outlet. Nitrogen was bubbled through the KOH solution for about thirty minutes before adding the amylose and for about one hour during and after the addition of amylose. The solution was sealed and allowed to stand

under a positive pressure of nitrogen. Nitrogen was bubbled through the solution as aliquots were withdrawn.

Two experiments were carried out on corn amylose preparations, one with an initial turbidity of 18×10^{-3} and another with an initial turbidity of 5.4×10^{-3}. The choice of the former was made in part so that a direct comparison of the potentiometric iodine titration could be made with a previous titration not using nitrogen. The iodine titrations on the above amyloses after standing under nitrogen were included in Section V-G.

Dilution series with both of the amylose preparations showed a decrease in the apparent molecular weight with time of standing. This showed that the turbidity drop was real and represented a decrease in particle weight. These results are shown in Figures 24 and 25. Two additional points of interest are brought out in the dilution series curves. From Figure 25 it is seen that the apparent molecular weight decreases until a certain value is reached. Further standing does not appear to decrease the particle weight although the slope of the curve may show a more positive value upon standing. Oster (59) maintains that the slope in a dilution series, which is a measure of B, is related to the size and shape of the solute molecules, and the more extended the particles become, the greater is the

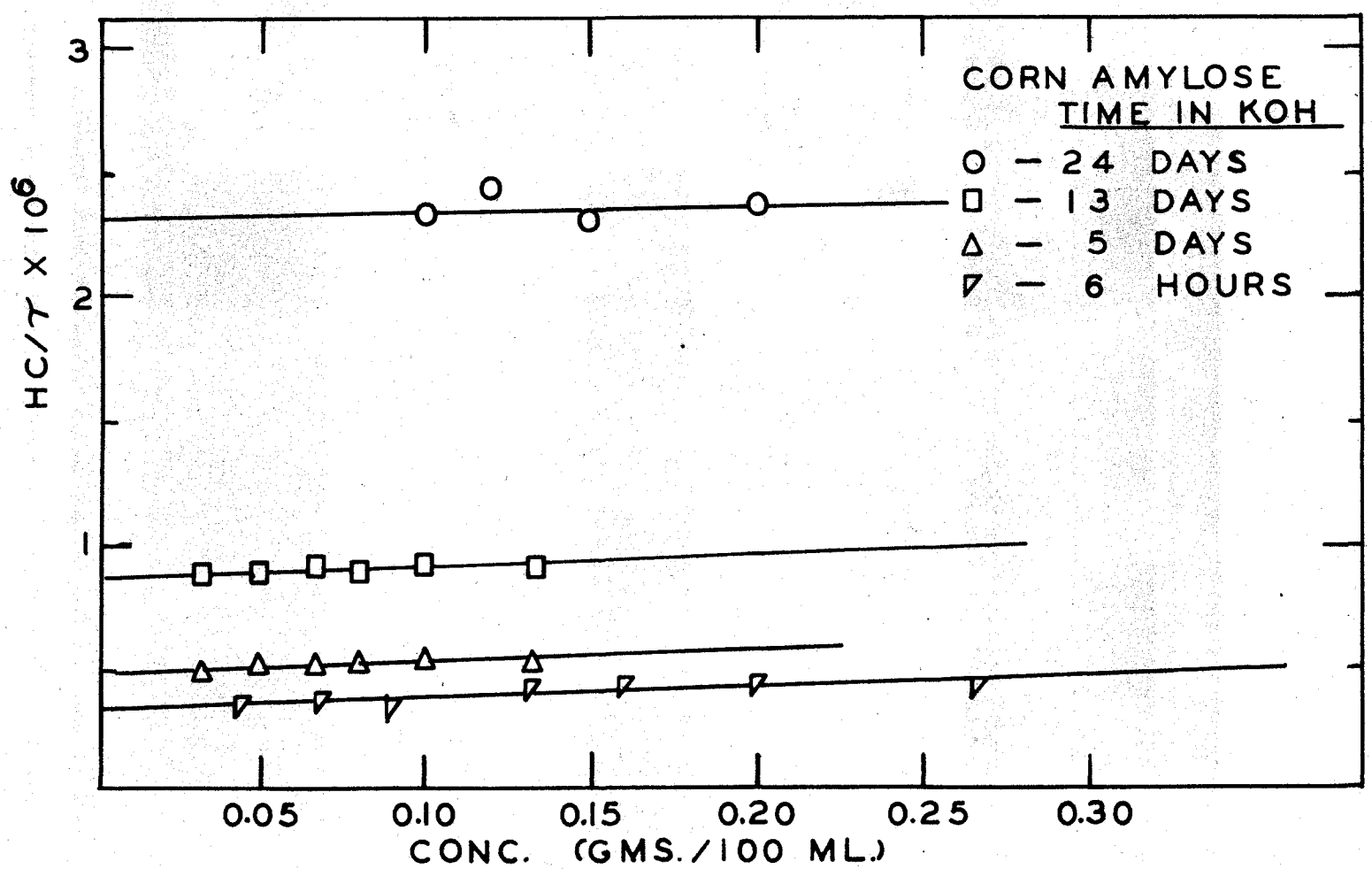

Figure 24. $\frac{Hc}{\tau}$ vs. c for amylose in N. KOH under nitrogen for various time intervals.

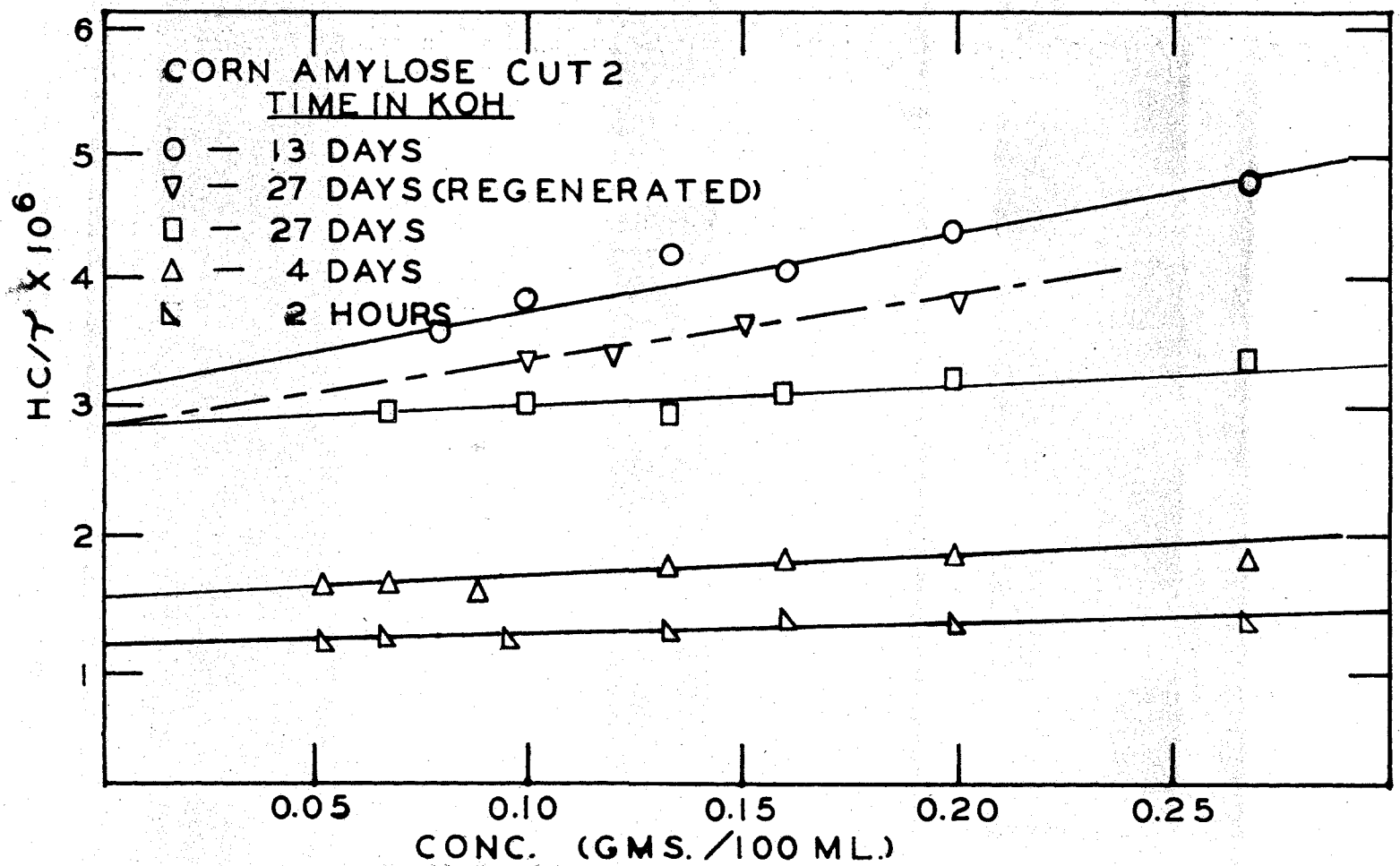

Figure 25. $\frac{Hc}{\tau}$ vs. c for amylose in N, KOH under nitrogen for various time intervals.

slope. This arises from the increased solubility of the solute particles as they are extended.

The more positive value for the slopes in Figure 25 may arise from two causes. First, the outer regions of the amylose aggregates may be dispersed until only the more compact interior remains after eight or nine days treatment with N. KOH. Further standing may swell and extend this dense region without much decrease in particle weight but with an increase in the solubility. The other view is that KOH serves a two-fold purpose when in contact with aggregates of amylose. The slow dispersion of amylose aggregates may also be accompanied by an extension or stretching of the helical amylose molecules contained within the aggregate. The latter effect may not be manifest until the aggregate is nearly dispersed. It is seen from both Figures 24 and 25 that the slope of the curves is practically zero, at least during the early stages of dispersion and, in the case of the amylose in Figure 25, apparently does not assume a positive value until after eight or nine days. Partial dispersion could presumably cause a better exposure of the individual helices to the KOH environment to produce the extension and consequent increased solubility of the amylose molecules. The extension might result from the repulsion of negative charges spaced along the helices due to the KOH.

An additional point of interest is the increased rate of dispersion manifest by the amylose in Figure 24. The rate is very slow initially but increases upon standing. As a result of this somewhat unorthodox behavior, amylose may be kept several hours in KOH with only a slight decrease in turbidity. However, longer periods cause rather rapid disaggregation.

It is observed that a minimum particle weight is not reached with this amylose preparation. When it is recalled that the initial turbidity was about 18×10^{-3}, it is obvious that larger aggregates are encountered and a longer time will be required to reach a limiting value.

An interesting point is that the apparent minimum particle weight reached by dispersion in KOH is about the same as the particle weight obtained from a dilution series after the turbidity is allowed to reach a minimum value at the neutral pH.

It is concluded from these experiments that amylose disaggregates in KOH under nitrogen perhaps to a certain limited value. The rate of disaggregation is at first slow but increases with time of standing. The total decrease in turbidity due to disaggregation is a function of the initial turbidity or initial size of the aggregates. With high initial turbidities, large drops are observed on standing.

2. Influence of turbidity drop at the neutral pH on dilution series

When it was found that amylose showed the unique behavior of a turbidity drop followed by retrogradation at the neutral pH, it was necessary to determine whether this drop represented a configurational change or whether it was due to a disaggregation of amylose aggregates. As stated earlier in this section it is possible to follow the turbidity drop in terms of particle weight by running dilution series and extrapolating to zero concentration.

Dilution series were carried out on solutions which were neutralized to a pH of 4 and a pH of 6.5. For the latter value dilution series were run before and after the turbidity drop. Since a measurable turbidity drop generally required an hour or longer and the time required for a dilution series was normally about forty-five minutes, it was apparently possible to carry out dilution series at the neutral pH and thus obtain the particle weight before disaggregation.

The results of the dilution series are shown in Figure 26. It is observed that only at a pH of 4 is a linear extrapolation to zero concentration possible using all of the points from a dilution series. At the neutral pH large variations in particle size are seen to occur especially

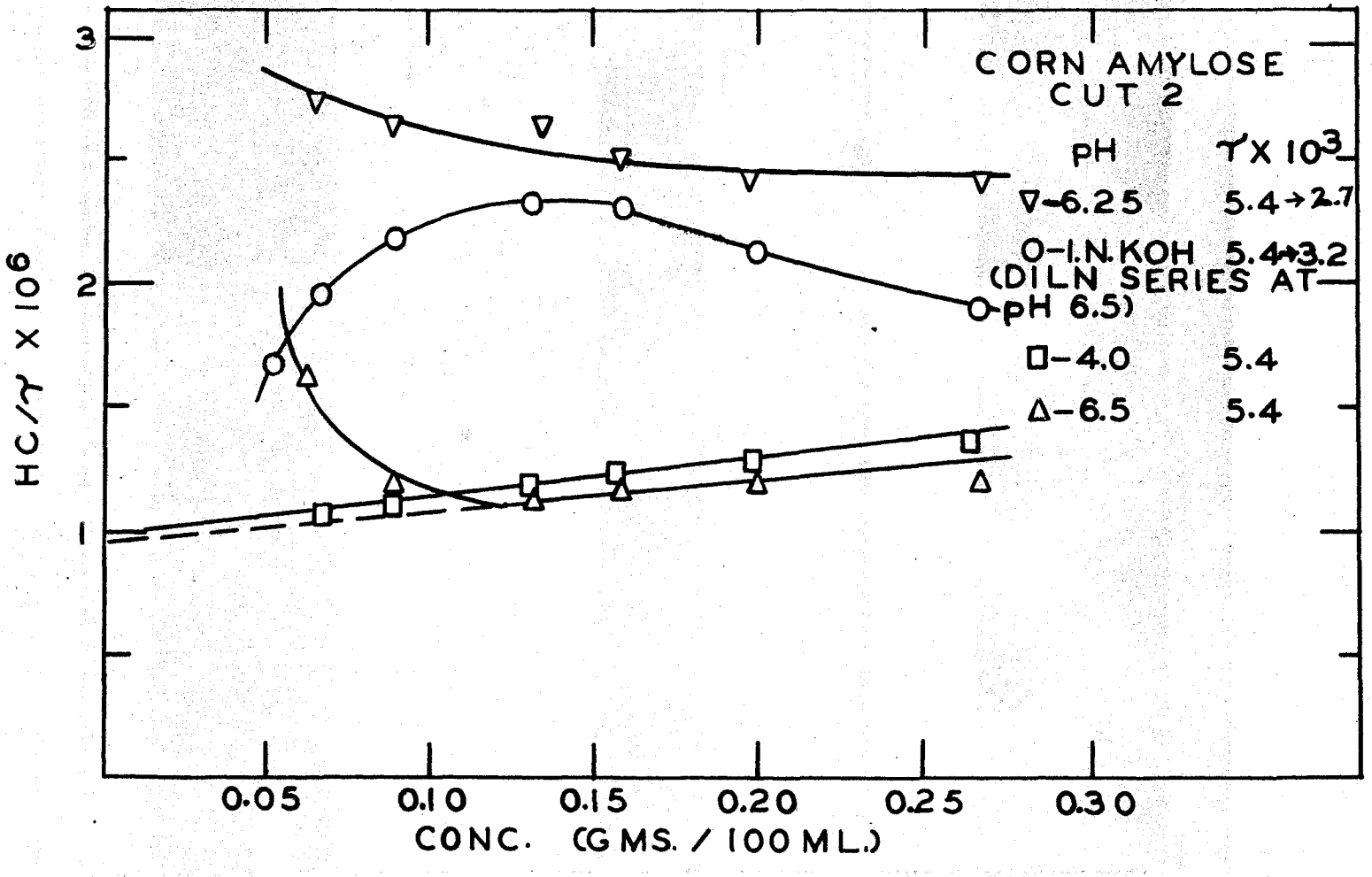

Figure 26. $\frac{Hc}{\tau}$ vs. c for amyloses at various pH values and intervals of standing.

during the latter stages of the dilution series. At the pH of 4 an apparent molecular weight of 1,000,000 was obtained. The particle weights are conveniently calculated from the ratio 1,000,000 to ordinate intercept.

The same particle weights were obtained for a solution at a pH of 4 as for a solution at a pH of 6.5 before a turbidity drop if the last two dilutions of the neutral solution were not included in the extrapolation.

Evidence indicates that disaggregation at the neutral pH is greatly accelerated as the dilutions are made. In later experiments it was found that agitation produced by stirring will promote a turbidity drop and retrogradation. The dilution effect is also evident from the curve obtained after the turbidity dropped from 5.4×10^{-3} to 2.7×10^{-3}. The Hc/T value increases during the dilutions, indicating a continued turbidity drop and disaggregation.

Also shown in Figure 26 is the result of a dilution series run after first allowing the turbidity to drop from 5.4×10^{-3} to 3.2×10^{-3} in N. KOH and then neutralizing with hydrochloric acid in the usual manner. It is seen that as the dilution proceeds both disaggregation and retrogradation are observed within the time required for a dilution series. Apparently long periods in KOH help to increase the rate of disaggregation and decrease the time required for retrogradation. As previously mentioned long treatments with

N. KOH not only slowly disperse the aggregate but also may assist in the extension of the helical amylose molecules, thus helping to produce the linear molecules necessary for retrogradation.

It was not shown by these experiments that the extrapolations of the various curves to zero concentration would lead to apparent molecular weights proportional to the turbidity before the dilutions were started. However, the general appearance of the curves with respect to the initial turbidity suggests this to be true. To illustrate this it was necessary to find conditions which would stabilize the solutions at the desired turbidity. A pH of 4 apparently stabilizes the turbidity of solutions of relatively high initial turbidities. However previous experiments indicate that this pH will not prevent retrogradation of solutions which are wholly or partially disaggregated. A method for performing dilution series at any turbidity value will be discussed next.

In summary these results suggest that the aggregates of amylose are initially of the same size in solutions of both a pH of 4 and a pH of 6.5. Dilutions of amylose solutions with accompanying agitation promote disaggregation and retrogradation. If amylose is allowed to stand in N. KOH for long periods of time before neutralization, the rate of disaggregation and retrogradation is further increased.

3. Dilution series during and after retrogradation

Since a satisfactory dilution series could not be made with amylose solutions at the neutral pH, it was necessary to use a method which would permit the turbidity to drop and still allow a dilution series to be run. From previous experiments it was found that the rate of the turbidity drop in N. KOH was much slower than at the neutral pH. This suggested the procedure of allowing the turbidity to drop to the desired value at the neutral pH and then adding sufficient N. KOH to make the solution 0.5 N. with respect to KOH.

Dilution series carried out by this method gave linear curves which extrapolated smoothly to zero concentration and did not show evidence of any unusual change in the particle size during the dilutions.

The apparent molecular weights obtained by this method in 0.5 N. KOH were compared to those obtained by performing dilution series at a pH of 4 and at 1 N. KOH. In each case nearly the same value was obtained, indicating little if any change in the size of the amylose aggregates. This is seen to be true by observing the lower two curves in Figures 26, 27, and 28. It was also shown that an addition of salt was not necessary in carrying out dilution series in N. KOH since the curves proved to be superimposable.

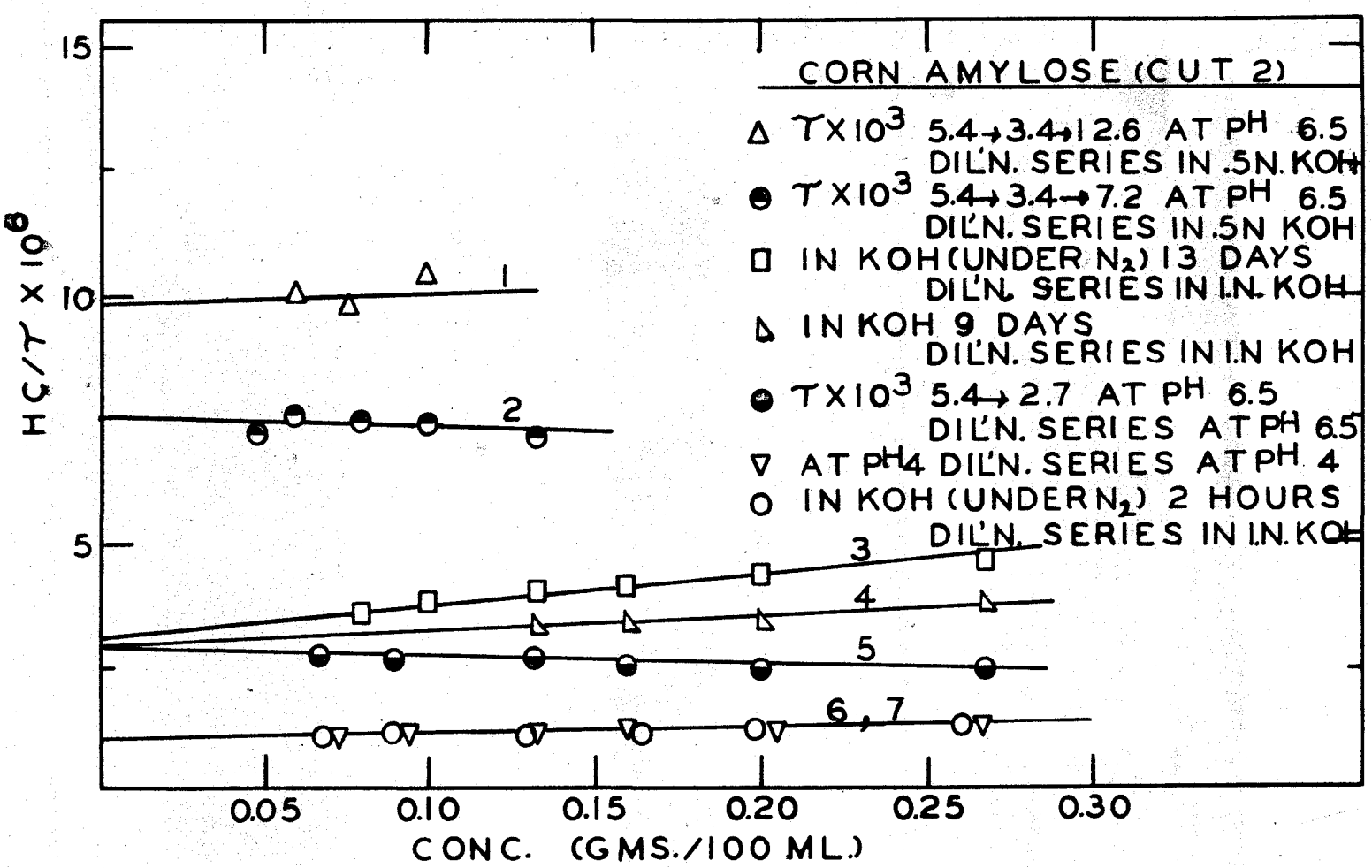

Figure 27. A composite of $\frac{Hc}{\tau}$ vs. c curves.

The turbidity of a corn amylose preparation initially at 5.4×10^{-3} was allowed to drop to a minimum value of 2.7×10^{-3}, and a dilution series was run after adjusting to 0.5 N. KOH as shown in curve 5, Figure 27. The apparent molecular weight was lowered from a value of 1,000,000 to 300,000. This value of 300,000 also represented the minimum apparent molecular weight obtained by allowing amylose to stand in KOH for eight days or longer. This shows the relative effect of the two treatments.

The most important fact gained from this experiment is that the value of 300,000 obtained at the minimum in turbidity does <u>not</u> represent the minimum apparent molecular weight that could be obtained for this amylose. It was observed that, by allowing the amylose to retrograde and then by adjusting to 0.5 N. KOH, a much lower particle weight could be obtained. In fact, the particle weight observed is dependent upon the time allowed for retrogradation before adding KOH. The lowest apparent molecular weights were obtained for those amyloses which were allowed to retrograde the longest.

Figure 27 represents a composite of several dilution series performed initially and during various stages of the turbidity drop and retrogradation. The value of 300,000 mentioned above was lowered to a particle weight of 110,000 by allowing the turbidity to undergo the change 5.4×10^{-3}

→ minimum → 7.2×10^{-3} and was decreased still further to 100,000 for the turbidity change 5.4×10^{-3} minimum 12.6×10^{-3}. The results of the dilution series are shown in curves 1 and 2 in Figure 27.

The view is taken that the process of disaggregation at the neutral pH does not stop when retrogradation begins. Rather the material which has disaggregated to a particular particle size is retrograding. The particles which have not yet disaggregated to this stage will continue to do so and in time will also retrograde.

Since the material used above was aggregated to a considerable extent as evidenced by the comparatively high initial turbidity of 5.4×10^{-3}, it was deemed desirable to perform similar experiments on the much shorter crystalline amylose of Kerr. This material, as previously mentioned, was found to retrograde rapidly at the neutral pH and rather quickly even at a pH of 4. It was found to have an initial turbidity of about 0.9×10^{-3}.

The results of the experiment are shown in Figure 28. Curves 4 and 5 represent dilution series before retrogradation. Both are included to show that no more difference in apparent molecular weight is encountered by performing a dilution series in KOH directly after dispersing in KOH than is obtained by first neutralizing and then adjusting to 0.5 N. KOH.

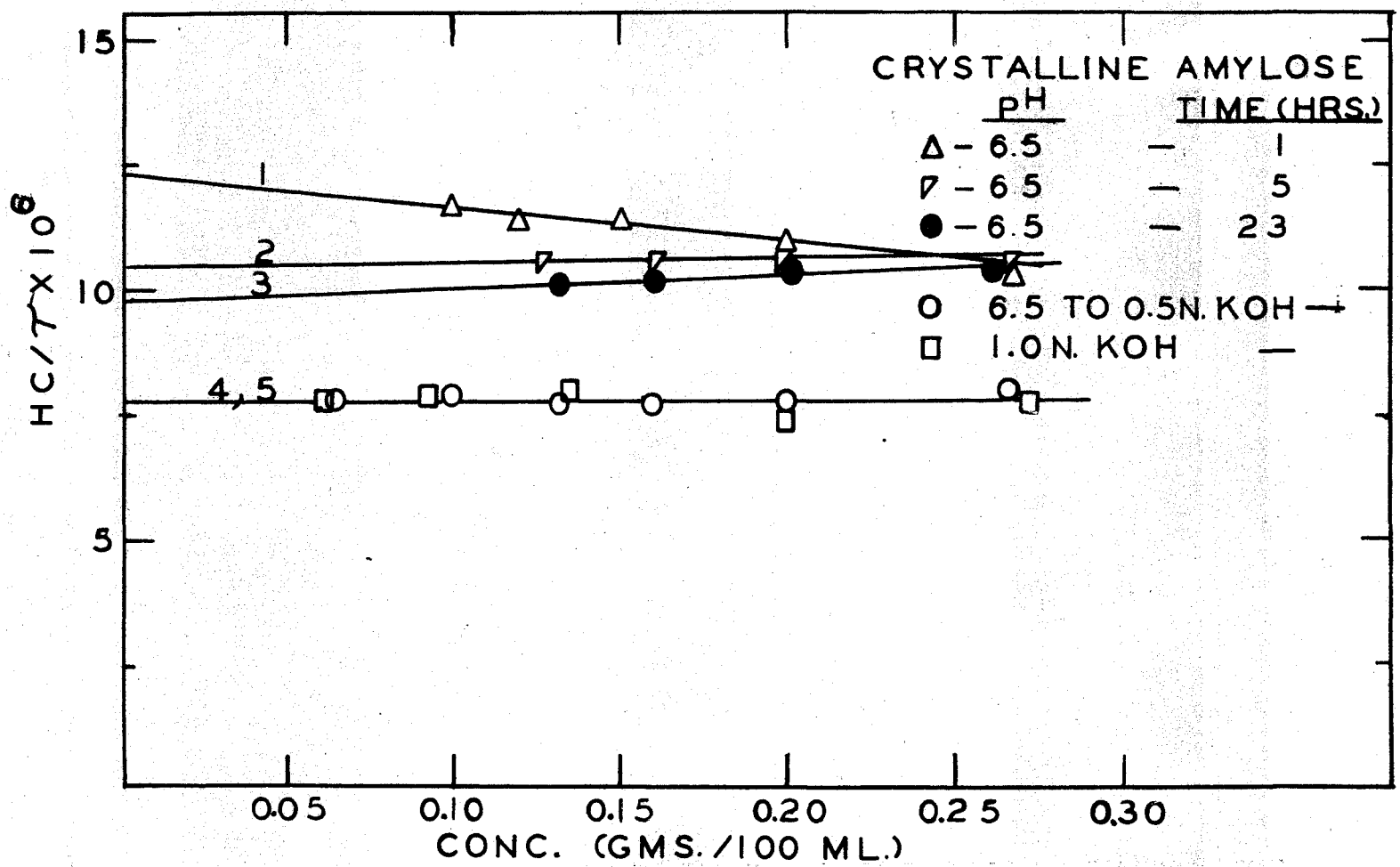

Figure 28. $\frac{\pi c}{\tau}$ vs. c for Karr's crystalline amylose at various stages of retrogradation.

Curves 1, 2, and 3 in Figure 28 represent dilution series performed after allowing the material to retrograde for various time intervals. It was impossible to observe the usual turbidity drop because of the speed of retrogradation. Nevertheless, the results show that disaggregation occurred as evidenced by the drop in apparent molecular weight from 127,000 (curves 4, 3) to 80,000 (curve 2). Kerr[1] obtained a value of 37,000 for the molecular weight from osmotic pressure measurements on the amylose acetate. The value of 80,000 is still considerably higher, but the fact that the value from osmotic pressure is a number average and that from light scattering is a weight average would account for at least part of this discrepancy. Also there are reasons to believe that a monomolecular state is not always reached before retrogradation. These reasons arise from the inability to get a single value for the minimum molecular weight of a particular amylose preparation. Also, retrogradation can be forced at temperatures near zero degrees C. with very little change in apparent molecular weight. This is true even though the amylose is completely retrograded.

An experiment which may bear upon this observation was performed by allowing a corn amylose preparation to alternately stand at 3° and at room temperature for various

[1]Private communication from Dr. Ralph Kerr, Corn Products Refining Company.

time intervals. A control of the same material was allowed to stand at room temperatures only. Both solutions were kept at a pH of 6.5. The turbidity of each was followed as a function of time. When the amylose preparation was allowed to stand at 3°, the turbidity generally experienced a slight increase after about an hour. However, when the solution was warmed to room temperature, a rapid drop was observed which closely approached the low value experienced by the control sample. This behavior is shown in Figure 29. The broken line connects turbidity readings observed at room temperature a few hours after the amylose was warmed to room temperature. The discontinuous curve represents turbidity readings taken alternately at 3° and at room temperature.

The expectation that the average rate of the turbidity drop would be less than that observed with the control is not realized. Possibly low temperatures force at least some of the particles to retrograde in a metastable aggregate. Enough heat energy is supplied by warming to room temperature to cause a dispersion of these aggregates and in addition permit the usual turbidity drop.

Several important conclusions may be drawn from these experiments. (1) The rapid turbidity drop and subsequent retrogradation which is observed in a dilution series at the neutral pH may be arrested by making the solution 0.5 N. in

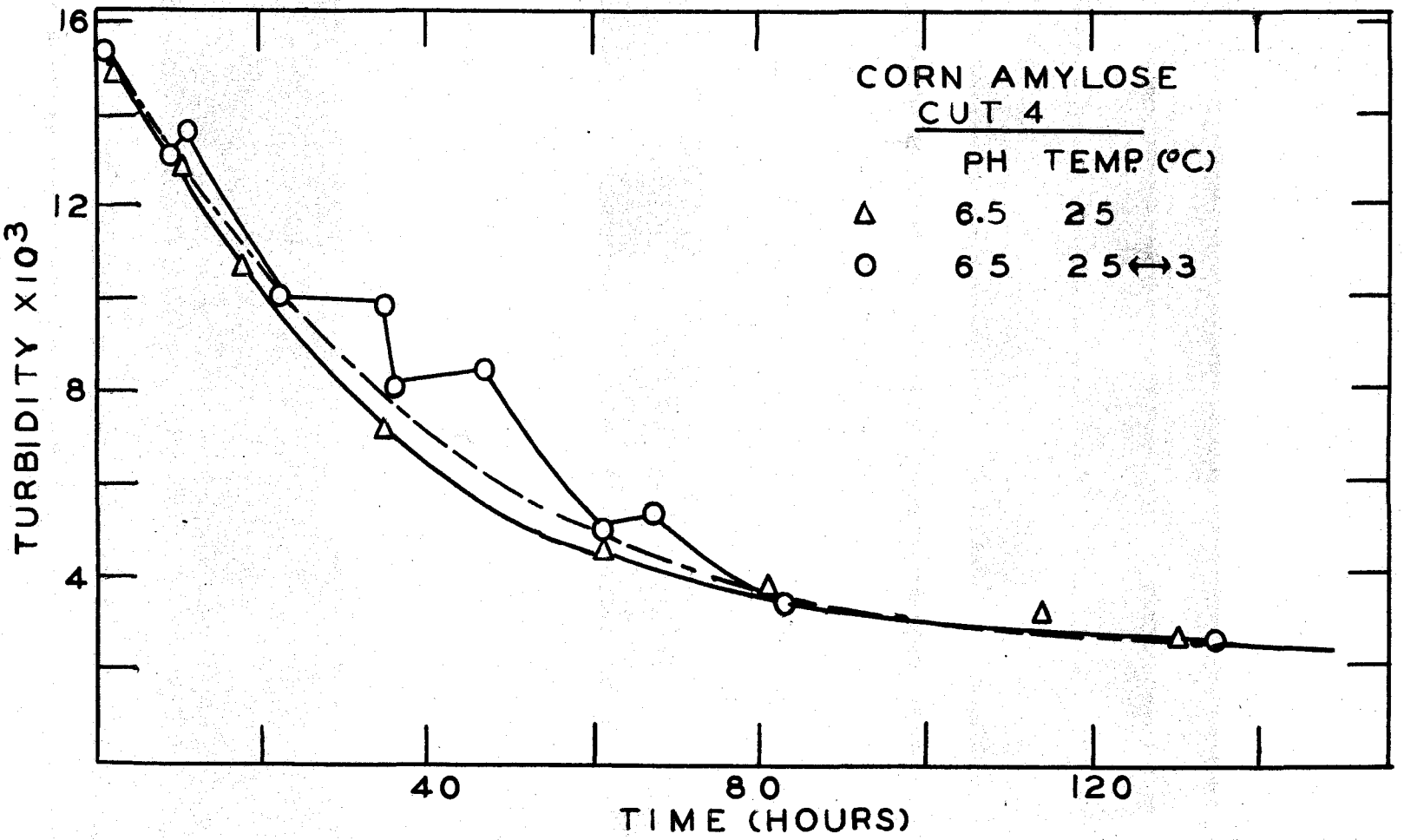

Figure 29. Turbidity vs. time of standing for amylose alternately at 3° C. and room temperature. Comparison with turbidity curve of amylose at room temperature only.

KOH. Dilution series may be conveniently performed on this solution to give particle weights corresponding to the turbidity at the time the KOH was added. (2) Disaggregation continues after retrogradation sets in. (3) Solutions which are not highly aggregated will retrograde without showing a turbidity drop but disaggregate during retrogradation as evidenced from the reduction in particle weight obtained from a dilution series.

VII. CORRELATION OF POTENTIOMETRIC IODINE TITRATIONS AND SPECTROPHOTOMETRIC DATA OBTAINED FROM CORN AMYLOSE SUBFRACTIONS BEFORE AND AFTER DISAGGREGATION

The experiments to be described were undertaken with a two-fold purpose. The first was to correlate the binding affinity and wave length of maximum absorption with the particle weight of amylose before and after disaggregation. For this purpose the subfractions from the pyridine subfractionation were convenient. The second was to further investigate the physical nature of the amyloses from the various subfractions obtained with 15 per cent pyridine and to correlate the titration results before and after disaggregation with the corresponding apparent molecular weights.

The existence of amylose aggregates was repeatedly postulated during the many phases of this investigation. In the preceding section it was shown quite conclusively from light scattering data that aggregates were present. With some amyloses the degree of aggregation was found to be small. In none of the amyloses investigated did it appear that complete molecular dispersion occurred with the short KOH treatment generally used for dispersion. In fact, a

complete molecular dispersion was not evident after three or four weeks in KOH if the minimum values obtained for the apparent molecular weight for retrograded amylose are taken as a criterion.

A. Correlation of Binding Affinity and Wave Length of Maximum Absorption Before and After Disaggregation

Even though the results of the spectrophotometric and potentiometric iodine titration experiments were interpreted in terms of aggregates of amylose, there was no definite proof available to show that the presence of amylose aggregates would actually cause an increase in binding affinity and wave length of maximum absorption. This assumption had been made after many experiments were performed in which chemical changes were kept at a minimum. In order to definitely establish this supposition the binding affinity and wave length of maximum absorption were determined on amylose before and after disaggregation.

Iodine titrations and spectrophotometric determinations were performed on the partially or wholly disaggregated amylose after allowing the amylose to retrograde thoroughly at the neutral pH. The turbidity behavior was followed prior to retrogradation to make certain disaggregation occurred. This retrograded material was made 0.5 N. with

respect to KOH and a dilution series run to determine the particle size. After this operation, which usually required not more than an hour, the basic solution was neutralized with hydrochloric acid and the amylose regenerated. It is believed that this relatively short period in KOH has very little influence on the binding affinity or the wave length of maximum absorption.

The binding affinities, maximum wave lengths, and apparent molecular weights before and after disaggregation are shown in Figures 30, 31, 32 and 33. It is seen that a large decrease occurs in each of these values after the amylose has aged at the neutral pH. The increased capacity for iodine observed with Cuts 3 and 4 after disaggregation will be discussed later in connection with the pyridine subfractionation experiment. The main interest in these results arises from the proof that aggregates are instrumental in causing high binding affinities and high wave length of maximum absorption. Both of these are evidently dependent upon the size of the aggregate. It is of interest that a plot of the logarithm of binding affinity versus the wave length of maximum absorption demonstrates a linear relationship, indicating a progressive decrease in the maximum wave length with an increase in binding affinity.

In Figure 31 the titration curve representing amylose which was allowed to retrograde in the presence of

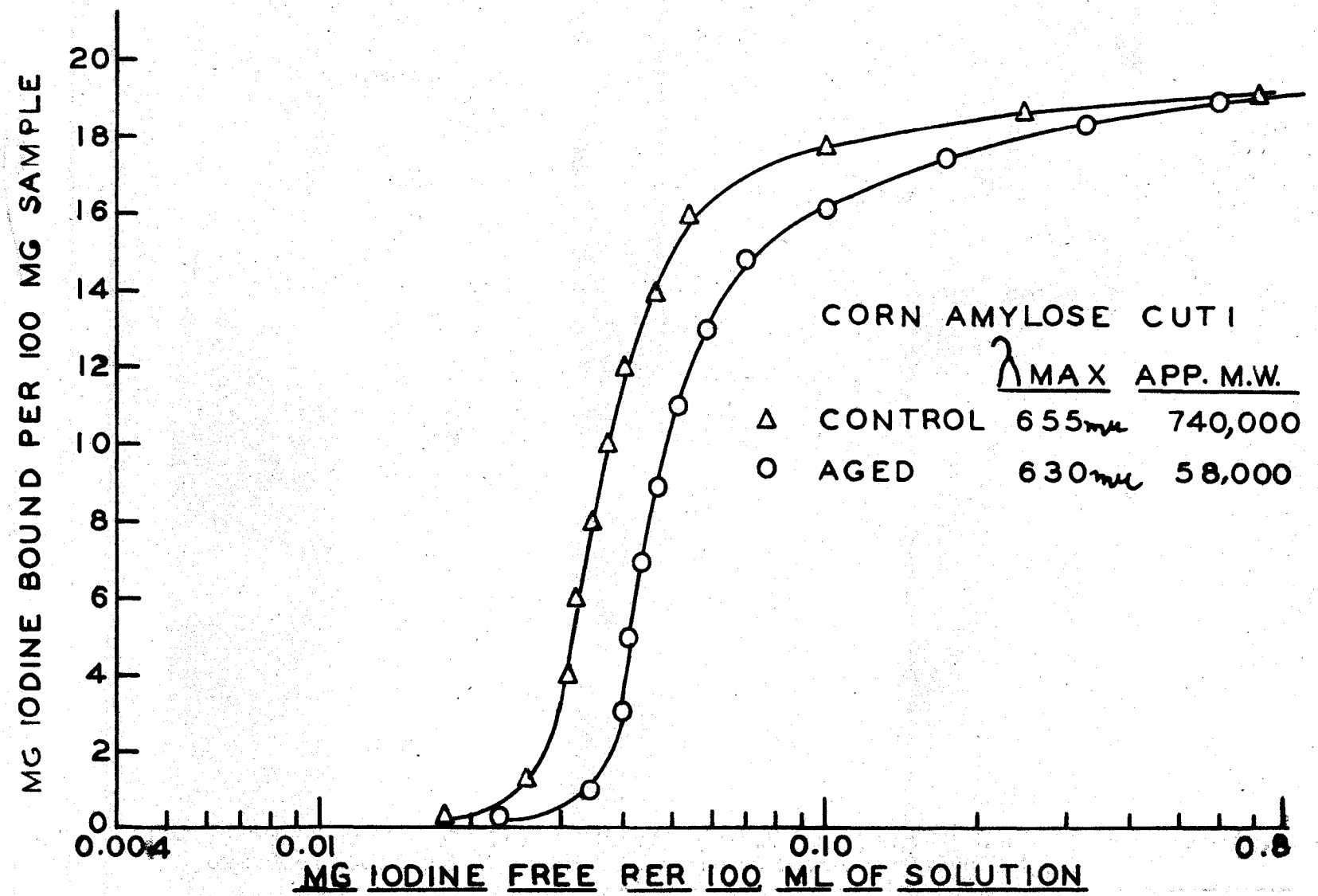

Figure 30. Potentiometric iodine titrations before and after aging at the neutral pH. Correlation with λ_{max} and apparent molecular weight.

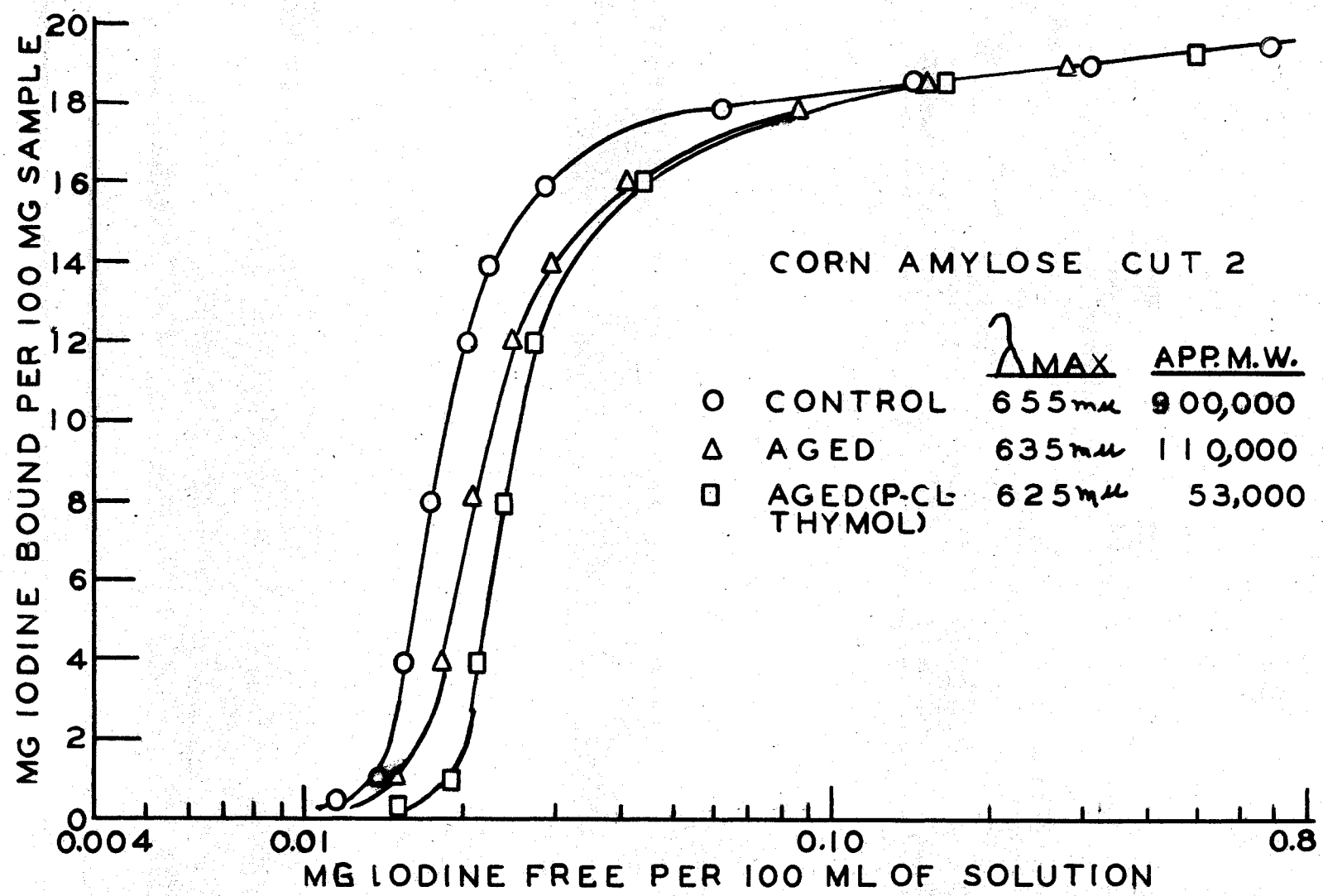

Figure 31. Potentiometric iodine titrations before and after aging at a neutral pH. Correlation with λ_{max} and apparent molecular weight.

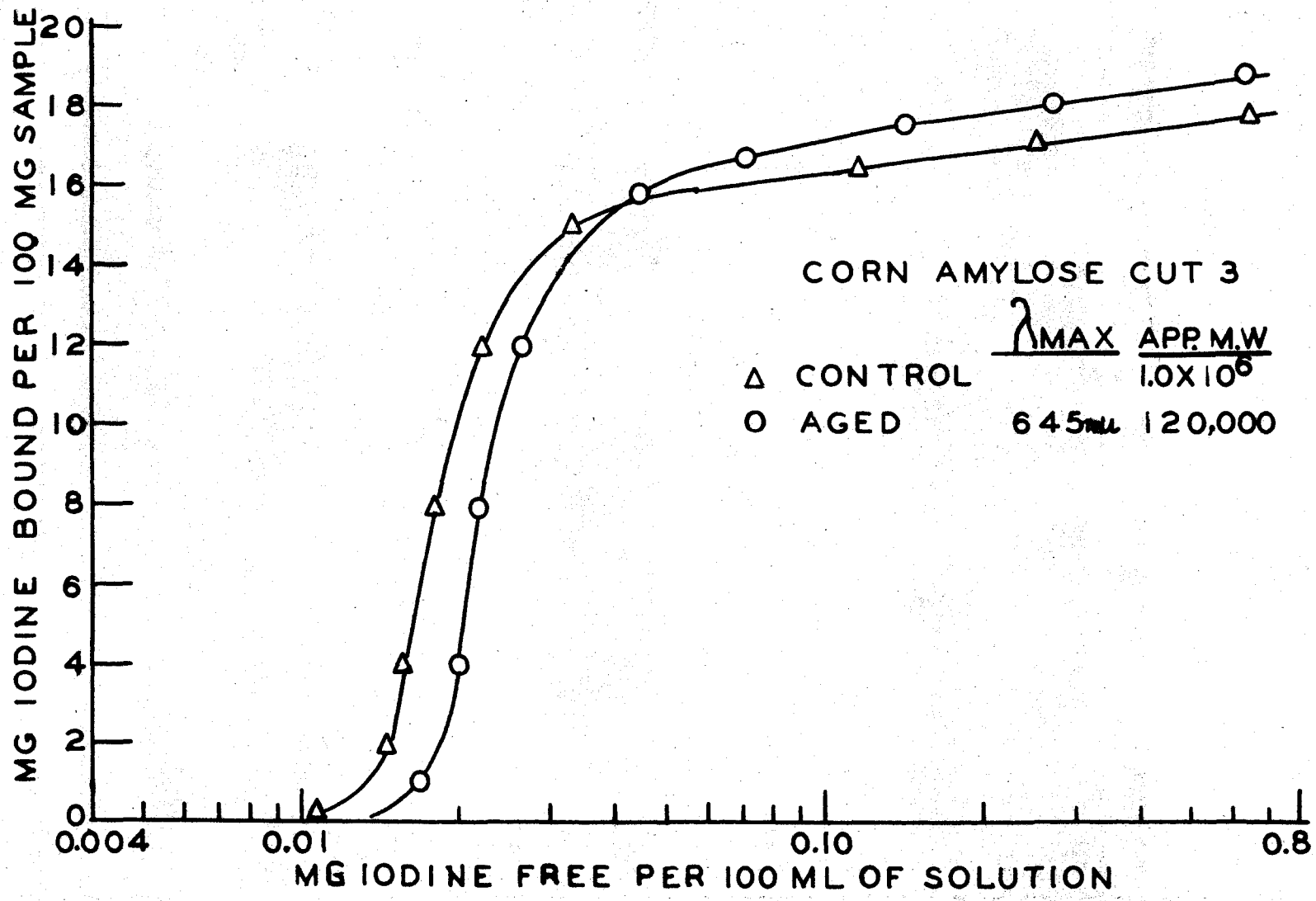

Figure 32. Potentiometric iodine titration before and after aging at the neutral pH. Correlation with λ_{max} and apparent molecular weight.

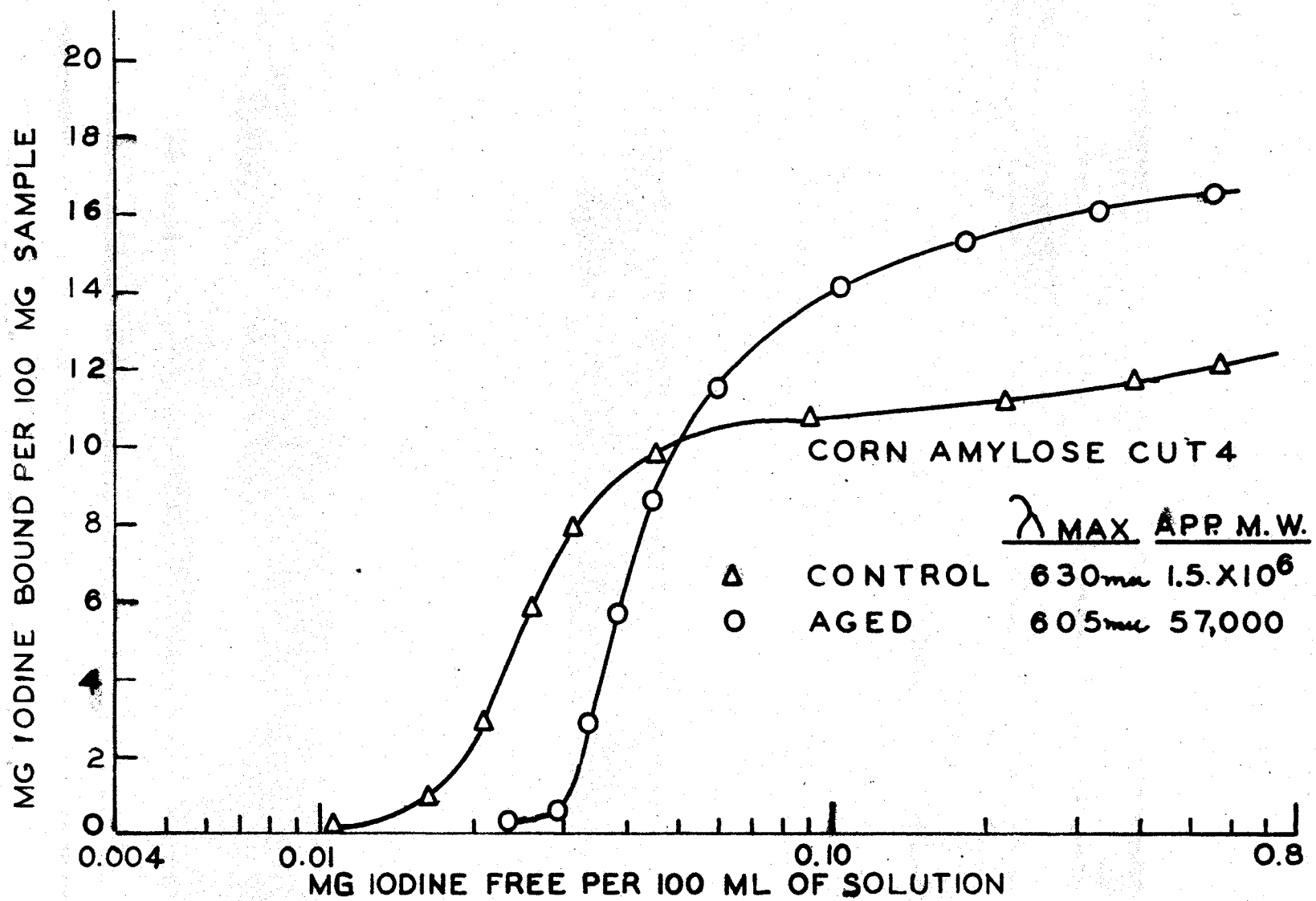

Figure 33. Potentiometric iodine titration before and after aging at the neutral pH. Correlation with λ_{max} and apparent molecular weight.

p-chloro-thymol, an antibacterial agent, is included as evidence that bacterial contamination is not a factor in causing the observed turbidity drop. In following the turbidity as a function of time it was observed that the sample treated with this antibacterial agent actually experienced a faster drop than an untreated sample. Other evidence may be cited which precludes bacterial contamination as a factor in the initial stage of the turbidity drop. A bacterial growth in most cases would be expected to cause an increase in turbidity because of large particle weights even though a breakdown of amylose occurs simultaneously. More sensitive yet to a few large particles is the dissymmetry, which is a ratio of the light intensities obtained at 45° and 135°. A very small amount of foreign material, such as dust, causes a large increase in the dissymmetry. Actually it is observed in the experiments described that the dissymmetries drop as the turbidity falls. Other factors which would serve to eliminate bacteria are high speed centrifugation and the treatment of amylose with KOH. It is recognized that contamination from the air is a possibility even though the solutions are kept in vials with ground glass covers. However, an appreciable drop is usually observed in an hour. Thus it is reasonable to suspect that disaggregation is taking place directly after neutralization of the basic solution.

To correlate these results with the data from the preceding experiments it is necessary to assign a special configuration to the aggregates of amylose. These aggregates are believed to remain at least partially intact during the dispersion of the native starch granule. The molecules within the aggregate are considered to be oriented so that the helices formed upon complexing are spaced in such a manner that a bundle of closely packed helices results.

The regions of crystallinity within the starch granule perhaps provide the necessary orientation. The molecules within the crystallite are not helical since they exhibit an A or B type X-ray diffraction pattern, which is considered to be an arrangement of linear molecules. The helices are not formed until a complexing agent is added to the dispersed starch granule.

In view of this picture the question arises concerning what is accomplished during a subfractionation experiment. There are two factors which one must consider. One involves the extent of aggregation of the molecules and the other, the length of the molecules within the aggregates. The increase in apparent molecular weight as the subfractionation with 15 per cent pyridine progressed would indicate that the size of the aggregates must be considered.

B. Significance of Potentiometric Iodine Titrations Before and After Disaggregation

The demonstration that aggregates of amylose definitely exist in aqueous solution causes concern as to the function of aggregates during complex formation in a subfractionation. If aggregates are responsible for increased binding affinity, then their role in a subfractionation experiment is of primary importance. In fact, the amylose aggregates may be playing the part reserved in the past for long amylose molecules. This is not meant to imply that long molecules are not a factor in complex formation. Rather it is the contention that aggregates composed of long molecules are responsible for greater binding affinity than aggregates composed of short molecules. Potentiometric iodine titrations on the subfractions obtained by the use of 15 per cent pyridine before and after disaggregation should offer a means of determining experimentally the binding affinity and per cent of iodine bound in terms of particle weight at the various stages of the subfractionation.

In addition to the decrease in binding affinity after disaggregation with each of the subfractions, there is an increase in the per cent of iodine bound in Cuts 3 and 4 shown in Figures 32 and 33 respectively. This increase is

greatest with Cut 4. It is recalled that this cut would not form a complex with 15 per cent pyridine during the subfractionation although it had complexed earlier during the purification procedure. This was attributed to the aggregating influence of aqueous pyridine at room temperature over long periods of time. More evidence substantiating this belief is furnished by the gradual increase in the apparent molecular weights of the cuts as the subfractionation proceeds (Figures 30, 31, 32 and 33). Special emphasis has been placed on Cut 4 since it undoubtedly represents the extreme case of aggregation. The same effect to a lesser extent is observed with Cut 3.

It might appear from the gradual decrease in binding affinity and increase in apparent molecular weight of the various cuts as subfractionation proceeds that larger aggregates of amylose have a lower iodine binding affinity and capacity to bind iodine. Since the aggregates formed under the influence of pyridine are probably different in crystal structure from those of comparable size formed in the native starch granule, there is little reason to believe that they should behave in the same manner toward complexing agents. It is probable that the aggregate formed under the influence of pyridine is dense or entangled to such an extent that the formation of the helices is greatly restricted.

It is the belief that once the aggregates which are formed in the starch granule are dispersed, they may not reform in the same orderly arrangement to give a high binding affinity and wave length of maximum absorption.

VIII. SUMMARY AND CONCLUSION

1. The influence of temperature on the amylose-iodine complex was studied by determining the wave length of maximum absorption at various temperatures. A decrease in the wave length of maximum absorption of from 30 to 40 millimicrons is found to occur between room temperature and 40°. Heating the complex at 80° for several hours produces little additional change. The change is temperature irreversible since upon cooling, an overall decrease of about 20 millimicrons is found for the complex.

2. The treatment of amylose with KOH for several days decreases the wave length of maximum absorption. Regeneration of the amylose after extended KOH treatment does not increase the wave length maximum for the complex.

3. The amylopectin impurities in a pentasol fractionated corn amylose were removed by complexing the amylose with 15 per cent pyridine at 45°. The progress of the purification was determined by performing potentiometric iodine titrations on both the carbohydrate remaining in the supernatant and on the crystallized amylose.

A procedure was developed for the subfractionation of corn amylose with 15 per cent pyridine. Using this procedure

fractions of increasing particle size and decreasing iodine binding affinities and capacity for iodine are obtained as the subfractionation progresses.

4. Amyloses which are allowed to stand in N. KOH show a progressive decrease in iodine binding affinity with time. Upon regeneration the binding affinity is partially restored to the original value. A decrease in binding affinity is also observed with samples which are kept in N. KOH under nitrogen. Experiments of this nature indicate that oxidative degradation is not responsible for all of the change produced in amylose by the KOH treatment.

5. Fresh amylose samples prepared by regeneration are found to possess a greater binding affinity than those which have been aged. The complexing agent used in the regeneration procedure is found to influence the binding affinity. Samples pretreated with iodine show very high binding affinities after regeneration.

6. The iodine binding affinity and retrogradation tendencies of amylose are found to be greatly influenced by the pH at which solutions are allowed to stand. Amylose solutions are stabilized at a pH below 5 and may be kept for several days in the unretrograded state. Solutions at a neutral pH show a strong tendency to retrograde. The binding affinity is also greatly reduced. The behavior of amylose at a pH of 9 is intermediate between that at 4 and

at 7. The findings presented in paragraphs (1) through (6) strongly suggest that aggregates of amylose are present in aqueous solutions of amylose.

7. Turbidity measurements as a function of time were made on amylose solutions adjusted to various pH values. Solutions at a neutral pH and of high initial turbidities experience a large turbidity drop. This drop apparently is necessary for retrogradation to occur. The initial turbidity is generally stabilized at the pH of 4. The behavior at a pH of 9 is unpredictable.

8. The turbidity behavior at the neutral pH was studied after an amylose iodine complex had been formed and destroyed. The retrogradation tendencies are found to be greatly reduced.

9. Turbidity measurements as a function of time were made on N. KOH solutions of amylose. With solutions of high initial turbidities, a large decrease with time is observed. With an amylose preparation which shows a relatively low initial turbidity and retrogrades at the neutral pH without experiencing a turbidity drop, a negligible drop is observed in KOH. The turbidity behavior is not changed appreciably by storing the solution in KOH under nitrogen.

10. Amylose solutions of low initial turbidities may retrograde without experiencing a turbidity drop. Also, if solutions of high initial turbidity are allowed to undergo a

turbidity drop at the neutral pH and then the pH is readjusted to 4, retrogradation will occur. These turbidity experiments also tend to confirm the previous hypotheses that amylose exists as aggregates in aqueous solution.

11. The apparent molecular weights were determined on various amylose solutions by the light scattering method. Weights were determined initially and during the various stages of the turbidity drop. The apparent molecular weight is found to decrease as the turbidity decreases. This behavior definitely establishes the presence of aggregates.

12. The apparent molecular weight found at the turbidity minimum does not represent a minimum molecular weight. By allowing the amylose solutions to retrograde thoroughly, particle weights are secured which are much lower. These weights are obtained after dispersing the retrograded material in one-half N. KOH. The apparent molecular weight of a corn amylose preparation decreases from an initial value of 900,000 to 80,000 by this procedure.

13. The apparent molecular weights were determined on solutions of amylose in N. KOH under nitrogen for various time intervals. The rate of disaggregation is found to be initially slow but increases upon standing until after ten to fifteen days a constant minimum particle weight is obtained. Longer periods in KOH cause the Hc/T versus c curve to become more positive. The positive slope is

explained as due to an extension of amylose helices.

14. The apparent molecular weights of amylose before and after disaggregation are correlated with the binding affinity and wave length of maximum absorption before and after disaggregation. A decrease in particle weight upon aging at the neutral pH causes a corresponding decrease in both the binding affinity and wave length of maximum absorption.

15. It is concluded, by considering the results of all of the experiments, that the aggregates responsible for high molecular weights, high binding affinities and high wave length of maximum absorption are present in the native starch granule and remain at least partially intact during the dispersion of starch.

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Also I would like to thank my wife, Elizabeth, for her able assistance in the preparation of this thesis.

XI. APPENDIX

A. Carbohydrate Samples Used

Several of the amylose subfractions used in this investigation were obtained through the courtesy of Thomas J. Schoch. The primary separation of the amylose was effected by selective precipitation with pentasol[1] (76). Subfractionation was accomplished by partial precipitation with small amounts of n-octyl alcohol. The subfractions referred to in this thesis are designated by the code numbers C146A(1b), P5/6A(9a), P5/6A(12c) and T7/9A(15b).

The crystalline amylose was supplied by Ralph Kerr and represents amylose which was extracted from starch with hot water (70°) and recrystallized with n-butyl alcohol (44).

The pyridine subfractionated amyloses designated as Cuts 1, 2, 3 and 4 were obtained according to the method described in Section IV.

The potato amylopectin samples were prepared by Richard L. Smith, and the method of preparation has been

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[1]Commercial mixture of amyl alcohol marketed by Sharples Solvents.

described (79).

The pentasol fractionated corn amylose was prepared in these laboratories. The method of preparation was essentially the same as that described by Schoch (76). A 2 to 3 per cent starch suspension was autoclaved for two to three hours at 15 to 18 pounds pressure. The dispersed starch was crystallized twice with pentasol.