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Isolation and some properties of some maltodextrin saccharides

John Anthony Thoma
Iowa State College

ISOLATION AND SOME PROPERTIES OF SOME MALTODEXTRIN SACCHARIDES

by

John Anthony Thoma

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Signature was redacted for privacy.

Head of Major Department

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Dean of Graduate College

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INTRODUCTION

Although the starch-iodine reaction was discovered almost a century and a half ago, it has only been during the last two decades that significant progress has been made toward elucidating its mechanism. The early literature abounds in conflicting evidence and theories concerning the nature of this reaction. Indeed, theories concerning not only the structure of the starch-iodine complex but even of starch itself have run the gamut of speculation. Inadequate methods and theories coupled with the inherent difficulty of preparing and handling starch solutions can account for most of the inconsistencies.

The first unambiguous studies of this problem were begun shortly after the fractionation of whole starch into amylose and amylopectin by Schoch in 1941. Using physical chemical techniques Rundle and coworkers conclusively proved that iodine was bound inside of the amylose helix. However, amylopectin appeared to form an iodine complex both by a helical and an adsorption mechanism. Studies of the interaction of iodine with amylose and starch hydrolysates have led to equivocal results in many cases because the type of complex formed appears to be a function of chain length. It was thought that if the individual members of the maltodextrin series could be isolated, unequivocal results should result from potentiometric and spectrophotometric studies.

This investigation was therefore undertaken to isolate in pure form by chromatographic techniques some of the maltodextrins capable of forming iodine complexes and to study their reactions with iodine. The results of the dextrin-iodine reaction will be compared to the closely related cyclohexaamylose-iodine system. Cyclohexaamylose, a cyclic polymer of glucose, is able to form inclusion complexes with iodine both in the solid state and in solution.

While this study may have some practical application (e.g. determination of chain length of starch hydrolysates), the primary goal was to learn more about the details of complex formation of starch and dextrins with iodine and iodide.

REVIEW OF LITERATURE

Starch-Iodine Complex

Optical studies

Prior to 1935, relatively little progress had been made in understanding the starch-iodine complex in spite of the voluminous amount of literature written about it. The contention that starch-iodine was an adsorption complex gained favor between 1900 and 1920 with the support of Lottermoser (1) and Biltz as cited in Barger (2, pp. 144-145). Lottermoser was able to interpret the results of his potentiometric studies on the basis of typical Freundlich isotherms. In 1925 Murray (3) carried out essentially the same set of experiments but with greater accuracy and interpreted his results as indicative of stoichiometric compound formation. Mellanby (4) on the other hand was inclined to believe that both types of complexes were formed. In the period between 1927 and 1930, three reviews appeared on the starch-iodine complex (2, 5, 6).

Undoubtedly most of the discrepancies and conflicts about the nature of the complex arose because of the employment of whole starch which is currently known to be composed of two discrete components. The fractionation of whole starch by selective precipitation of the linear molecules from the

soluble branched molecules gave new impetus to investigations into the nature of the starch-iodine complex (7).

When Rundle (8) subjected an amylose-iodine solution (linear fraction of starch) to a velocity gradient, he noted that the solution exhibited dichroism parallel to the direction of flow. Theoretically, light with its electric vector parallel to the axes of the iodine molecules should be strongly absorbed. Because the velocity gradient used was insufficient to orient I_2, it seemed reasonable that an amylose-iodine complex was being oriented. This experiment did not differentiate between the possibilities that the complex is helical or that the absorbed I_2 was parallel to extended, essentially, linear amylose chains. Neither amylopectin nor glycogen, stained with I_2, exhibited dichroism of flow which indicated clearly the branched nature of these molecules.

To determine which of the above mechanisms of complex formation was correct, some of the optical properties of butanol precipitated amylose were studied (9). On edge this "crystalline amylose" consisted of small rectangular platelets and was found to retard light with its electric vector parallel to the surface of the platelet to a greater degree than light with its electric vector normal to the platelet. This birefrigence indicated that the starch chains lie parallel to the platelet. When the platelets were stained with

I_2, light with its electric vector normal to the platelet surface was strongly adsorbed, while light with its electric vector in the plane of the platelets was weakly absorbed. This dichroism indicated that the axes of the I_2 molecules were normal to the platelet, and therefore normal to the extended amylose chains. But according to dichroism of flow of amylose-iodine solutions the I_2 molecules must be parallel to the extended chains if this model is adopted. This discrepancy is such that only a helical model will satisfy the observations. In accordance with these observations, Silberstein's (10) theory predicts that an amylose helix would have its greatest polarizability normal to the helix axis.

From X-ray diffraction patterns (11) of amylose which had reacted with I_2 vapors, the unit cell was calculated to be hexagonal; there being six glucose residues per turn of the helix. Moreover, it was noted that only starch producing the "V" modification exhibited this ability to form an iodine complex. The "A" and "B" modifications, generally believed to be linear, stain light brown with I_2 vapors. Because amylose in the "V" modification could adsorb I_2 equal to approximately 26% of its weight it was tempting to postulate that one I_2 molecule was absorbed for each turn of the helix. Inasmuch as extended treatment with I_2 vapors at high temperatures tended to decompose amylose, it was felt that this value

for iodine absorption may be only a practical maximum and fortuitous.

Early spectrophotometric studies of the starch-iodine reaction were handicapped by lack of adequate methods of fractionation and characterization. The butanol fractionation of Schoch (7) and the potentiometric methods developed by Bates (12) alleviated these difficulties. When the spectra of the two starch fractions were examined in the presence of iodine-iodide solutions a marked difference was noted (13). The amylose fraction showed a maximum around 600-630 mμ, while the amylopectin fraction exhibited a maximum in the neighborhood of 525-550 mμ. Spectrophotometric titrations carried out with amylose at various I^- concentrations showed that as the I^- level was increased it was necessary to increase the I_2 concentration to saturate the amylose. Experiments of this type indicated that I^- was in some way competing with I_2 for available binding sites. When the number of glucose residues per molecule of I_2 bound was plotted versus the fourth root of I^- concentration, when the amylose was completely saturated, and this line extrapolated to zero I^- concentration, it intersected the ordinate at a value of six glucose units per I_2 molecule. These results were, however, in consistent disagreement with the results of potentiometric titrations. Because of this fact and the apparently arbitrary choice of the fourth root of the I^- concentration, the

significance of "six glucose residues" remains dubious. Baldwin and coworkers pointed out the dependence of the spectra on both the apparent length of the chain and I^- concentration. Both molar extinction coefficient and absorption maxima in the visible region were found to decrease as the chain length was shortened and/or the I^- concentration increases. Furthermore, it was suggested that the spectra could be used as a qualitative measure of the type of starch present, but since both the variation of the absorption maxima and molecular extinction coefficient were small they could be of little value for quantitative work.

Potentiometric studies

Much of our current information about the starch-iodine complex has come from potentiometric titrations. This method developed by Bates (12) measures the I_2-I^- oxidation potential at a bright platinum electrode against a calomel half cell. In this procedure, starch fractions are titrated with I_2 at constant I^- levels, the EMF measured and the free I_2 concentration determined from standard curves. Bound I_2 is then calculated by subtracting free I_2 from added I_2. Using this method, Bates found the amylose-iodine reaction proceeded in three steps. Initially the iodine activity was raised to a level where amylose begins to absorb it, the activity then

remained nearly constant until a sufficient amount had been added to react with the amylose (helical binding). When the amylose was essentially saturated, the activity again rose rapidly. After the amylose was apparently saturated, it still continued to absorb small amounts of I_2 as its activity in solution was steadily increased. Helical binding was probably responsible for the initial absorption while the secondary absorption followed a Freundlich isotherm. This theory was given added support by Higginbotham (14, 15) who showed that after the helical binding was complete, bound I_2 was a linear function of the log of the free I_2 concentration. When the same experiments were conducted with amylopectin, an entirely different picture was obtained. Except for a very small amount of bound I_2, a Freundlich isotherm could adequately describe the whole of the absorption process. When amylopectin-bound I_2 was plotted versus the log of free I_2, the relationship was linear above 2% adsorption at 25° C. Higginbotham's experiments also revealed a slight shift in the visible absorption maximum to longer wavelengths as the percent of I_2 bound was increased, and he interpreted this information in terms of helical binding.

It has been suggested that the amylose-iodine reaction may be an all-or-none reaction (16). To a dilute amylose solution was added one half of the iodine needed to reach the end point and the complex was precipitated by adding excess

KI. The precipitate when examined contained essentially all of the I_2 but only one half of the amylose. To account for this apparent all-or-none character of the reaction, Rundle has proposed a cooperative effect. He suggested that the entering I_2 molecules were polarized by the permanent dipole moment of the amylose helix. Succeeding I_2 molecules which were introduced acquired a dipole which helped to reinforce the existing field.

Because decreasing either the extent of aggregation or decreasing the chain length lowered both the I_2 affinity and visible absorption maximum, it is desirable to reinvestigate the independent effect of these variables on the amylose-iodine complex. Amylose is not unique in its ability to form highly colored complexes with I_2, but only unique in its ability to form molecular complexes in dilute solutions.

The potential at the midpoint of the iodine titration curves of amylose was found to be a function of chain length (12). Foster (17) considered the bound I_2 molecules as linear harmonic oscillators and showed that the potential at the midpoint of the titration could be related to the chain length by an equation of the form $E = A + B\,(1/\sqrt{n})$ where n is the number of glucose residues and A and B are constants. Since the reaction appeared to be an all-or-none reaction, the possibility existed that if potentiometric titrations of amylose with I_2 could be carried out under equilibrium conditions it

should be possible to determine molecular weight distributions. With this end in mind, Paschall (18) studied some factors which affect the titration behavior of amylose. Using the methods of Bates, they endeavored to determine if amylose-iodine complex was in equilibrium with I_2 in water. A large hystersis effect upon back titration with thiosulfate convinced these workers equilibrium conditions did not prevail. A more extensive examination of the system indicated that even variation in the history of amylose markedly affected its I_2 binding characteristics. However, the shape of the curves seemed to be little influenced by the amylose concentration, as one would expect with an all-or-none reaction possessing high order in I_2. It must be pointed out that this apparent all-or-none reaction may only be a nonequilibrium effect characteristic of large molecules and not at all a reflection of the type of reaction which would occur if equilibrium conditions could be established. Paschall's results also seemed to indicate that aggregation of amylose was in some manner influencing his results. An empirical approach to this problem was made by fractionating amylose into four subfractions with 15% aqueous pyridine and studying their I_2 binding capacities as a function of molecular weight. When the I_2 binding capacity was compared to the weight-average molecular weights found by light scattering following disaggregation, it was learned that binding was proportional to

both molecular size and degree of aggregation. The authors made no claims of molecular dispersion. A decrease in I_2 binding affinity upon disaggregation was accompanied by a downward shift in the wavelength of maximum absorption. A linear relationship between the wavelength of maximum absorption and the log of the I_2 affinity existed suggesting that these properties may be related.

Meyer (19) has gone so far as to reject the helical theory and suggest that micelles may be necessary for the formation of a complex. Undoubtedly neither of these two theories portray the starch-iodine complex in its entirety.

Freudenberg (20) elaborating upon the helical model envisioned by Hanes (21) pointed out that the "lining" of the amylose helix was of a hydrocarbon nature (i.e. devoid of OH groups) and thus could account for the shift in color when amylose had taken up I_2. Experimentally this "solvent" change for I_2 could account for neither the dependence of the absorption maxima on chain length nor for the enhancement of the molecular extinction coefficient of the bound I_2 (17). Two other mechanisms, however, seem adequate to account for these effects. These are the dipole induced dipole forces postulated by Rundle, and resonance of polyiodides inside the helix postulated by Foster (17). In 1944 when these theories

were proposed it was known that I^- played an integral role in the production of the starch-iodine complex.

Stoichiometric relationships

The first quantitative attempt to determine the stoichiometric relationships which existed between I_2 and I^- in the complex was made by Gilbert and Marriot (22) in 1948. They theorized that the following equation could justifiably be written for the reaction:

$$(I_2)^x(I^-)^y = K_{xy}\,(a) \qquad \text{(Eq. 1)}$$

where (a) was the number of moles of complex per gram of amylose and K_{xy} was the equilibrium constant. Then when only one type of complex was present, x and y could be determined by varying first the I_2 concentration at constant I^- concentration, and then the I^- concentration at constant I_2 absorption. Under the first set of conditions:

$$\frac{\partial \log a}{\partial \log I_2} = x \qquad \text{(Eq. 2)}$$

and under the second set of conditions:

$$\frac{\partial \log I_2}{\partial \log I^-} = y/x \qquad \text{(Eq. 3)}$$

or these relations could be expressed in forms for finite changes provided the changes were small:

$$\frac{\Delta \log a}{\Delta \log I_2} = x \qquad \text{(Eq. 4)}$$

and

$$\frac{\Delta \log I_2}{\Delta \log I^-} = y/x \qquad \text{(Eq. 5)}$$

They pointed out that this simplicity disappeared if more than one type of complex was present but the relationships were approximate if one type was predominant. Gilbert and Marriot studied the amylose-iodine system at very small percentage of I_2 absorption to avoid corrections for any decrease in the number of any available binding sites using total I_2 concentrations of the order 10^{-5} to 10^{-7} M. and differential titration methods. When a plot of the log of the absorbed I_2 was made versus the log of the free I_2 concentration for less than 2% absorption, x was found to change from 2 to 3 as the I_2 concentration was increased. Furthermore, this result was independent of the I^- concentration between 10^{-3} and 10^{-4} molar KI. Much higher concentrations of I_2 were required to reach an equivalent absorption at the higher concentrations of KI.

The value of y/x was determined in the following way. The I_2 concentration required at equilibrium for an arbitrary

absorption at one concentration of KI was measured followed by a similar measurement at a different KI concentration. When this experiment was conducted, the ratio of y/x was found to be two:three and the predominant complex then appeared to be amylose$\cdot(I_2)_3\cdot(I^-)_2$. Similar experiments conducted at successively higher KI concentrations showed that the ratio of I_2 to I^- in the complex approached one.

Although there could be no doubt that I^- played an important role in the starch-iodine reaction, its exact role has not yet been elucidated. Baldwin has postulated that I^- could cause a partial replacement of the absorbed I_2 by I^- or I_3^-.

Forster (23) in 1951 examined the possibility that complexes of the starch-iodine type could be formed in solution in the absence of I^-. His study was primarily centered around the interaction of cyclohexaamylose (alternatively referred to as α) and amylose with I_2 in water. Cyclohexaamylose was chosen because it is a cyclic molecule composed of six glucose units linked α- 1→ 4 and served as a model for the starch helix. When I_2 is dissolved in water, it is hydrolyzed with the production of I^- according to the following equation:

$$I_2 + H_2O = H^+ + I^- + HOI \qquad \text{(Eq. 6)}$$

Forster attempted to oxidize the I^- with 0.2 M. potassium iodate at pH 6 as follows:

$$5\ I^- + IO_3^- + 6\ H^+ = 3\ I_2 + 3\ H_2O \qquad \text{(Eq. 7)}$$

The conditions he chose did not significantly oxidize the I^- resulting from the hydrolysis of I_2 because the oxidation potential of iodate at this pH was toc low. Therefore, his experimental results must be judged as inconclusive. He reported that both amylose and cyclohexaamylose formed complexes in the absence of I^- and that their spectra were identical to the spectra of the complexes formed in the presence of I^-. In his unpublished dissertation, there appeared a theoretical quantum mechanical calculation predicting the positions and intensities of the absorption bands of the amylose-iodine complex. The valence electrons alone were considered and treated as an electron gas in a cylinder. The results obtained were in good agreement with the experimental observation.

Because the reaction of cyclohexaamylose with I_2 is more easily interpreted than the reaction of I_2 and starch, it was hoped that the results from the interaction of I_2 with this cyclic material could be applied to the interpretation of the more complex starch-iodine reaction. Formation of crystalline cyclohexaamylose KI and cyclohexaamylose I_2 complexes raised the possibility that they may also be formed in solution (24). This possibility is particularly intriguing because X-ray data from these crystals indicated that they were inclusion complexes. Dube (25) using a modified titration procedure of

Bates investigated the system. After studying the absorption of I_2 at various KI levels, he reached the conclusion that the following set of equations could best explain the interaction of I_2 and KI, with cyclohexaamylose (abbreviated by α).

$$\alpha + I_2 = \alpha I_2 \qquad K_1 = \frac{(\alpha I_2)}{(\alpha)(I_2)} \qquad \text{(Eq. 8)}$$

$$\alpha I_2 + I^- = \alpha I_3^- \qquad K_2 = \frac{(\alpha I_3^-)}{(\alpha I_2)(I^-)} \qquad \text{(Eq. 9)}$$

$$\alpha + I^- = \alpha I^- \qquad K_3 = \frac{(\alpha I^-)}{(\alpha)(I^-)} \qquad \text{(Eq. 10)}$$

$$\alpha I^- + I_2 = \alpha I_3^- \qquad K_4 = \frac{(\alpha I_3^-)}{(\alpha I^-)(I_2)} \qquad \text{(Eq. 11)}$$

The results of similar studies with amylose were difficult to interpret because at the higher KI concentrations there was a phase change of the complex and equilibrium was observed to be only slowly attained.

One of the most recent and refreshing approaches to this knotty problem was invented by Mould and Synge (26) in 1954. Preliminary investigations by Swanson (27) in 1948 had clearly demonstrated the dependence of the absorption maximum of the starch-iodine complex on chain length. In order to understand more completely the effect of chain length on the color of the complex, Mould and Synge separated amylose hydrolysates into

differently staining fractions. For preparative work a continuous electrophoretic fractionation was carried out in an apparatus similar to that devised by Svensson and Brattsten (28). The electrophoretic fractionation separated the hydrolysate into a scarcely staining zone DP < 10, an orange-staining zone DP 10-25, a red staining zone DP 25-40, and a blue-staining zone DP 40-130, the upper limit depended upon the degree of hydrolysis. The zones were separated by gaps in which polysaccharide was absent.

Because paper chromatographic methods had not been successful in separating homologous saccharides larger than DP 10 (29), Mould and Synge developed the method of electrokinetic ultrafiltration in collodian membranes to separate mixtures of polymers. They reasoned that if a block of material of uniform porosity could be substituted for paper, "molecular-sieve" and adsorption effects would work together when a liquid containing molecules of various sizes was forced through the block. For large hydrostatic pressures which would crush the block, they substituted electrokinetically promoted flow of the liquid. Synthetic dextrins of known average DP were placed on collodian membranes and partially separated by electrokinetic ultrafiltration. Curves were constructed showing variations of the R_f with DP for various pore sizes. The DP ranges of the electrophoretic "blue", "red",

and "orange" fractions were then interpolated from the curves after their R_f values were measured.

The number of molecules of I_2 bound by the blue and red-staining dextrins was determined by the potentiometric methods of Gilbert and Marriot. Potentiometric studies of the "orange" fraction were not made because of difficulty in handling the high I_2 concentrations necessary for complex formation. In the case of the blue-staining polysaccharide the initial slope corresponded to x = two and changed to x = three at higher I_2 concentrations in accord with the work of Gilbert and Marriot. For the red-staining polysaccharide the initial slope corresponded to x = one, and changed to x = two at higher concentrations. The red-staining material then contained two molecules of I_2 per molecule of dextrin.

At this point it seems reasonable that the differently colored starch iodine complexes may be a series of polyiodides with the general formula I^-_{2n+1}.

Chromatography

Since the invention of chromatography by Tswett, it has found ubiquitous application in all fields of chemistry. The great value of chromatography lies in its ability to readily separate compounds which can only be separated with difficulty or not at all by standard chemical techniques. Because it has

proven such a versatile tool a number of recent comprehensive reviews (30, 31, 32) and books (33, 34, 35) have discussed chromatography exhaustively.

MATERIALS AND METHODS

Chromatographic Techniques

Paper chromatography

The techniques used for multiple ascending paper chromatography have been described by Jeanes (36). Eaton and Dikeman number 613 filter paper was used for chromatographic analysis. For a rapid ascent rate, a water, ethanol, nitromethane solvent system was selected. From these three components, a set of solvent proportions was selected by the procedure of Thoma (37) to separate homologous oligosaccharides. This solvent contained 28 parts water, 37 parts ethanol and 35 parts nitromethane and was satisfactory for resolving starch oligosaccharides up to about maltodecaose. However, to separate the higher homologs, it was necessary to use solvents with slightly higher water contents. To separate the megalodextrins (DP greater than 10), two solvent systems were selected, one containing 33 parts water, 37 parts ethanol, and 30 parts nitromethane and the other containing 35 parts water, 37 parts ethanol and 28 parts nitromethane. The former was used to separate homologs in the region DP 10-14 and the latter for homologs having DP greater than 14. The symbol G_n

will be used to represent a specific maltodextrin composed of n glucose units linked α-1→4.

All sugar positions were rendered visible by dipping the chromatograms first in $AgNO_3$ dissolved in acetone and then in alkaline methanol (38). Megalodextrins greater than DP 12 were detected alternatively by spraying the chromatograms with 90% methanol containing 0.3 grams of I_2 and 0.15 grams of KI per 100 ml. This spray had the advantage that the developed papergram could be irrigated again with solvent to separate some of the higher homologs since the I_2 moved with the solvent front.

Column chromatography

Cellulose columns were packed by the method of Flodin (39) with some modification and will be described in detail in the next section. The flow rate of the solvent through the column was controlled by the electrolysis of water (hydrogen generator) in an apparatus (not previously described) designed by Dr. Harry Wright. The apparatus consisted of a U tube with a platinum electrode sealed into each arm near the bottom. One arm of the U tube was connected directly to the reservoir by means of tygon tubing with a sodium hydroxide trap inserted to dry the hydrogen and neutralize any acid carried over in the spray. The rate of flow was controlled by adjusting the

rate of electrolysis with a six volt rectifier in conjunction with a Variac adjustable transformer.

Spectrophotometry

For all the spectral studies, a Beckmann model DU spectrophotometer was used with a light path of 1 cm. unless otherwise indicated.

The method of continuous variation for the study of binary systems has been described by Vosburgh (40). The same procedure was essentially used to study ternary systems; only two components were varied while the third was added in a constant amount. The theory for application of the method of continuous variation to ternary systems is developed in the theoretical section.

Potentiometry

The methods for potentiometric titrations used in this study were those developed by Bates (12) but modified to a microscale. The titration vessel was a micro three neck flask. An eye dropper which served as a stirrer was fitted into one neck and a platinum electrode was sealed through the stirrer to make contact with the solution to be titrated. To avoid air oxidation of I^- all of the platinum wire at the air

liquid interface was coated with soft glass. Into the other necks were placed respectively the tip of an "Agla" Micrometer syringe (Burroughs Wellcome and Co., London), capable of delivering 0.5 ml. ± 0.5 microliters, and an agar bridge. The agar bridge, a glass tube drawn out so that the orifice was about 0.1 mm. in diameter, was filled with a hot two per cent solution of Bacto-agar (Difco) containing the same concentration of KI as that used to titrate the dextrin. The space above the agar in the bridge was then filled with saturated potassium chloride solution and the orifice of a calomel half-cell was dipped into it. The potential of the system was measured with a Leeds and Northrop Type K potentiometer in conjunction with a suspension galvanometer capable of detecting differences of 0.02 mv. The temperature was 25.2° C ± 0.1° C.

Amylase

Five times recrystallized sweet potato β-amylase was kindly supplied by Dr. Schwimmer.

Carbohydrates

Amylodextrin

Nägeli amylodextrin was prepared by Dr. Philip Nordin and Mrs. Valerie Piepho Yount in this laboratory.

Amylodextrin hydrolysate

The hydrolysate used for fractionation was prepared by dissolving 50 gms. of Nägeli amylodextrin in 200 ml. of boiling water and adding 15 ml. of 2 N. H_2SO_4 and hydrolyzing for 30-35 minutes at 100° C. Distilled water was added occasionally to maintain an approximately constant volume.

To neutralize the H_2SO_4, amberlite IR-45 was added to the hydrolysate until it reached a pH of 5. The solution was then filtered and evaporated to dryness in vacuo.

Cyclohexaamylose

Cyclohexaamylose was prepared in this laboratory by Dr. Nordin and its purity was determined by measuring its optical rotation in a Rudolph precision polarimeter.

FRACTIONATION OF THE MALTODEXTRIN SACCHARIDES

Preliminary Studies

Fractionation of the maltodextrin saccharides was attempted by ion exchange, adsorption, and partition chromatography.

Zill (41) was successful in separating mono-, di-, and trisaccharides on anion exchange columns in the borate cycle by eluting the sugars with borate buffer. All attempts to separate the maltodextrins larger than DP 3 failed. While glucose and maltose could be resolved, separation progressively decreased as the series was ascended and by the time maltohexaose was eluted separation was hardly discernible. Attempts to separate dextrin-iodine complex on anion exchange resins in a halide cycle were also unsuccessful. When amylodextrin or cyclohexaamylose-iodine complexes were added to the column, the I_2 was strongly absorbed and the dextrins were immediately eluted.

Barker (42) isolated dextrans as large as DP 10 by eluting them from charcoal columns with borate buffers at pH 10 in aqueous ethanol. Although this method demonstrated moderate success with the maltodextrins, it has the disadvantage of eluting the sugars in an alkaline medium and requires that the solution be neutralized and both salts and

boric acid be removed before the dextrins could be obtained pure.

However, with the development of the empirical method for selecting solvents for paper chromatography by Thoma (37), it became a routine matter to separate some of the large saccharides above DP 10 on paper. After this initial success with partition chromatography on paper, the procedure was adapted to cellulose columns for the preparation of laboratory quantities of pure starch oligosaccharides and megalosaccharides, up to DP 18.

Column Chromatography

Preparation of column

A 5 x 50 cm. glass column fitted with a water jacket, stopcock, and coarse sintered glass disc was packed with Whatmann standard grade cellulose powder by the method of Flodin (39) with some modifications. Fifty grams of powdered cellulose, suspended in one liter of water, was sedimented through a 4 x 125 cm. tube into the glass column while the reservoir was agitated constantly. Good "zoning" was achieved only when the rising surface of the cellulose powder was maintained exactly horizontal while the powder was sedimenting. After each 5 cm. of cellulose powder was deposited, the

stopcock was opened for several seconds. This temporarily increased the rate of flow of water and compacted the sedimented cellulose. After the bed reached a height of about 46 cm., the cellulose was placed under one half atmosphere pressure and the stopcock again opened to further compress the packed cellulose. When the water level dropped to within 2 cm. of the bed the stopcock was closed and the pressure was slowly released. When the column is under pressure the liquid level should never be allowed to approach the cellulose surface closer than 2 cm. because the bed expands slightly upon release of the pressure.

Hot solvent was then layered gently over the liquid inside the column, pressure was applied and the stopcock opened. This procedure was repeated until no further compression was evident. Whenever the surface was slightly agitated upon addition of solvent, tapping the column gently but firmly with a large rubber stopper and reapplication of pressure effected removal of these minor blemishes.

Before fractionation was begun, the column was equilibrated with 1 l. of developing solvent and the "zoning" characteristics of the column were judged. After 300 to 400 ml. of solvent had percolated into the bed a mixture of dyes (pH indicators were satisfactory) was added to the surface and eluted with the remainder of the solvent.

When columns were packed by dry or other slurry techniques, the dyes were eluted in the shape of cones. This was readily apparent from the patterns of the dyes on the sintered glass disc as they were eluted from the bottom of the column. However, visual observation along the length of the column indicated even horizontal bands.

To avoid channeling or rupture of the bed during operation the following precautions were scrupulously adhered to:

1. All irrigating solvents and water used to pack the column were brought to a brisk boil immediately prior to use to remove dissolved gases.
2. When the column temperature was changing, liquid was kept flowing through it.
3. The liquid level was never allowed to pass below the bed surface.

Before addition of the amylodextrin hydrolysate to the column, it was heated to $53^{\circ} \pm 1^{\circ}$ C. During operation of the column, the solvent was preheated in a water-jacketed spiral condenser to 53° C immediately prior to entering the column. A second preheater, maintained at 75° C, was placed between the solvent reservoir and the condenser directly above the column.

The purpose of this heater was to remove any gas which dissolved in the solvent while in the reservoir. When this

precaution was not followed air pockets and channels formed in the cellulose bed.

A sample of 2.5 gms. of hydrolyzed amylodextrin was dissolved in 2 ml. of boiling water and enough irrigating solvent was added to form two phases. The sirup was distributed evenly and gently on the bed; no solvent was flowing. After application of the carbohydrates, the flow rate was adjusted to one drop per three seconds and when the liquid level had just reached the packed surface, 5 ml. of irrigating solvent was added. When the solvent just reached the packed surface, the space above the cellulose was filled with irrigating solvent and the flow rate was adjusted to 1 ml. per minute by means of the hydrogen generator. Liquid between the stopcock and sintered glass disc was removed to prevent mixing of the eluates and samples of 25 ml. were collected with a Technicon fraction collector.

Because of the large volumes of pure organic solvents required for elution, practical considerations led to the choice of ethanol and 1-butanol as the organic components of the solvent. The solvent proportions for cellulose column chromatography were chosen along a line of the ternary phase diagram containing ten per cent ethanol in excess of miscibility (phase discontinuity determined at 27° C). Elution of the maltodextrins was then begun with a solvent which had previously been found to be just effective in separating glucose and maltose.

The water content was increased approximately two per cent as each of the first five maltodextrins were eluted and one per cent thereafter (see Figure 1).

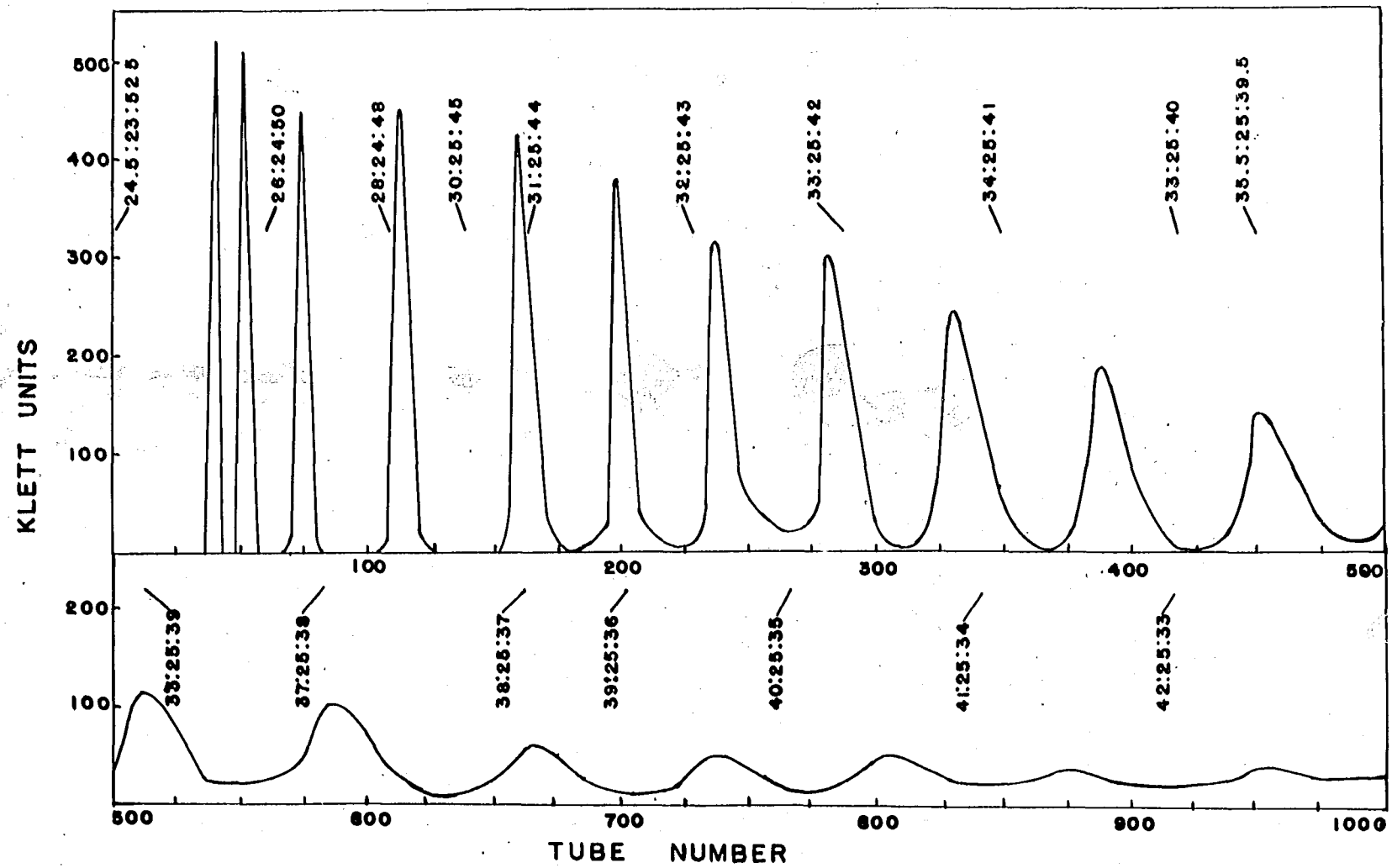
Analysis of eluant

In a set of ten Klett tubes, including a blank, 2 ml. of anthrone reagent (43) was layered under 1 ml. of eluant from each fraction to be tested. The tubes were shaken vigorously to ensure thorough mixing and after the contents had reacted for three minutes the tubes were cooled in running tap water. To each tube, 2 ml. of concentrated sulfuric acid was then added, the contents were stirred, and the absorbancy was read immediately in a Klett-Summerson photoelectric colorimeter employing a red filter.

Purification

After the tubes from each fraction were pooled and evaporated in vacuo to about 50 ml. two phases appeared. The aqueous phase was separated and extracted twice with both 1-butanol and ethyl ether and then evaporated to dryness in in vacuo. Contaminants of the sugars were extracted from the tygon tubing by the solvent as it flowed from the reservoir to the column.

Figure 1. Separation of the maltodextrin saccharides


KLETT UNITS
500
400
300
200
100
24.5:23:52.5
26:24:50
28:24:48
30:25:45
31:25:44
32:25:43
33:25:42
34:25:41
35:25:40
35.5:25:39.5
100
200
300
400
500
200
100
36:25:39
37:25:38
38:25:37
39:25:36
40:25:35
41:25:34
42:25:33
500
600
700
800
900
1000
TUBE NUMBER

Identification

The separated dextrins were identified by paper chromatography employing amylose and amylodextrin hydrolysates as reference materials. When 1/2 to 1 mg. of sugar was spotted on a papergram, irrigated, and then developed with silver nitrate reagent the sugars were found to be chromatographically pure up to DP 12. All the megalodextrins above DP 12 were accompanied by traces of impurities from the next lower homolog. When the dextrins were hydrolyzed by β-amylase to 50% conversion to maltose (44) both the even and odd member saccharides appeared in the digest for all fractions above DP 12 indicating contamination.

THEORY

Stoichiometry

Applications of the method of continuous variation and the implications and validity of the assumptions made in the theory of this method have been critically reviewed by Woldbye (45). Vosburgh (40) and Katzin (46) have extended the method to binary systems in which successive dissociation constants for the complexes differ by several orders of magnitude. In this thesis, the method has now been extended to some special ternary systems which may occur when starch and iodine and iodide react. The most general case which is treated involves the completion of one binary system with a ternary system and is represented below.

$$A + B = AB \qquad \text{(Eq. 12)}$$

$$aA + bB + cC = A_aB_bC_c \qquad \text{(Eq. 13)}$$

To determine c/b experimentally the absorbancy (or some other property of the ternary complex) is measured in a set of solutions containing an amount x of B and an amount $C_o - x$ of C. Each solution also contains a fixed amount of A in large excess over that required for complex formation. When the

absorbancy due to the ternary complex is plotted against x, the value of c/b is given by $(x_{max})/(C_o - x_{max})$, where x_{max} is the value of x which gives the maximum amount of complex.

For the ternary system at equilibrium the following equations may be written:

$$\underline{A} = A_o - \text{complex} \approx \underline{A_o} \qquad \text{(Eq. 14)}$$

$$\underline{B} = x - p - bq \qquad \text{(Eq. 15)}$$

$$\underline{C} = C_o - x - cq \qquad \text{(Eq. 16)}$$

$$K' = (\underline{A})(\underline{B})/p \qquad \text{(Eq. 17)}$$

$$K'' = (\underline{A})^a(\underline{B})^b(C)^c/q \qquad \text{(Eq. 18)}$$

where $\underline{A}$, $\underline{B}$, $\underline{C}$, p, and q represent respectively the equilibrium concentrations of A, B, C, binary complex and ternary complex; and K' and K'' are the dissociation constants for the binary and termary complexes.

Substituting (Eq. 14), (Eq. 15), (Eq. 16), and (Eq. 17) into (Eq. 18) differentiating with respect to x, and setting $dq/dx = 0$, the condition for a maximum, one obtains after simplification $c/b = (x_{max})/(C_o - x_{max})$. Similarly, when A and C are continuously varied in the presence of a large

excess of B the combining ratio of A to C in the complex may be evaluated.

However, when A and B are continuously varied in a large excess of C their apparent ratio will vary between a/b and one depending upon the magnitudes of the dissociation constants of the binary and ternary complex and the amount of C in the system. Only if a/b equals one or the amount of binary complex formed is insignificant may the ratios of any two reactants in the ternary complex be found by continuously varying them in the presence of a large excess of the third component. Similarly, if $K'' \gg K'$ and the ternary reaction is the preponderating equilibrium, the combining ratio of any two components in the complex may be found by continuously varying these components in the presence of added constant amount of the third (not necessarily a large excess).

Even though equilibrium constants may be unfavorable it is sometimes possible to choose conditions under which the method of continuous variation is applicable to ternary systems. The α-I_2-I^- system will serve as an example, and may be represented by the following series of equations:

$$I_2 + I^- = I_3^- \qquad K_I = \frac{I_3^-}{(I_2)(I^-)} \qquad \text{(Eq. 19)}$$

$$I_2 + \alpha = \alpha I_2 \qquad K_1 = \frac{\alpha I_2}{(\alpha)(I_2)} \qquad \text{(Eq. 8)}$$

$$\alpha I_2 + I^- = \alpha I_3^- \qquad K_2 = \frac{\alpha I^-}{(\alpha I_2)(I^-)} \quad \text{(Eq. 9)}$$

$$\alpha + I^- = \alpha I^- \qquad K_3 = \frac{\alpha I_3^-}{(\alpha)(I^-)} \quad \text{(Eq. 10)}$$

$$\alpha I^- + I_2 = \alpha I_3^- \qquad K_4 = \frac{\alpha I_3^-}{(\alpha I^-)(I_2)} \quad \text{(Eq. 11)}$$

Then setting complex,

$$C = \alpha I_3^- + \alpha I_2 \quad \text{(Eq. 20)}$$

$$\text{free } \alpha, \; F = \alpha + \alpha I^- \quad \text{(Eq. 21)}$$

$$\text{and analytical } I_2, \; A = I_2 + I_3^- \quad \text{(Eq. 22)}$$

a pseudo-equilibrium constant exists:

$$K_\psi = \frac{(C)}{(F)(A)} \quad \text{(Eq. 23)}$$

Then substituting (Eq. 8) through (Eq. 11), and (Eq. 19) through (Eq. 22) into (Eq. 23)

$$K_\psi = \frac{(\alpha I_2)}{(\alpha)(I_2)} \cdot \frac{(1+K_2 I^-)}{(1+K_3 I^-)(1+K_I I^-)} = \frac{K_1(1+K_2 I^-)}{(1+K_3 I^-)(1+K_1 I^-)} \quad \text{(Eq. 24)}$$

Now if conditions are used such that $K_3 I^-$ and $K_I I^-$ are insignificant compared to 1, then

$$K\psi = K_1 (1 + K_2 I^-) \qquad \text{(Eq. 25)}$$

Then letting $$y = \alpha I_3^- + \alpha I_2^- \qquad \text{(Eq. 26)}$$

$$K\psi = \frac{y}{(\alpha_{free})(I_{2_{free}})} \qquad \text{(Eq. 27)}$$

let amount $$\alpha \text{ added} = x \qquad \text{(Eq. 28)}$$

and amount $$I_2 \text{ added} = t - x \qquad \text{(Eq. 29)}$$

and amount of $$I^- \text{ added} = \text{constant} \qquad \text{(Eq. 30)}$$

then $$\alpha_{free} = x - y \qquad \text{(Eq. 31)}$$

$$I_{2_{free}} = t - x - y \qquad \text{(Eq. 32)}$$

and $$K\psi = \frac{y}{(t - y)(t - x - y)} \qquad \text{(Eq. 33)}$$

but $$I^- = I^-_{added} - \alpha I_3^- \qquad \text{(Eq. 34)}$$

So $$y = \alpha I_3^- \left[1 + \frac{1}{K_2(I^-_{added} - \alpha I_3^-)} \right] \qquad \text{(Eq. 35)}$$

and $$K_1 \left[1 + K_2(I^-_{added} - \alpha I_3^-) \right] = \frac{y}{(x - y)(t - x - y)} \qquad \text{(Eq. 36)}$$

then differentiating with respect to x and setting $\frac{d(\alpha I_3^-)}{dx} = 0$ after simplification one obtains $x = t/2$.

By similar considerations it can be shown that if I_2 and I^- are continuously varied in the presence of a constant amount of α the method of continuous variation would show a maximum where the ratio of I_2 to I^- equals one.

Dissociation Constant

The dissociation constant for a 1:1 complex can be determined graphically by plotting the optical densities of a set of solutions, containing a constant amount of one reagent, against the log of the added concentration of the other reagent which is added in variable amounts. Consider the following equilibrium:

$$AB = A + B \qquad K = \frac{(A)(B)}{AB} \qquad \text{(Eq. 37)}$$

then if $$A = A_{added} - AB \qquad \text{(Eq. 38)}$$

and $$B = B_o - AB \qquad \text{(Eq. 39)}$$

substituting (Eq. 38), (Eq. 39) into (Eq. 37) and taking the log of both sides

$$\log K = \log (A_{added} - AB) + \log \frac{(B_o - AB)}{AB} \qquad \text{(Eq. 40)}$$

then at the midpoint of the titration curve

$$K = (A_{added} - AB) = A_{added} - B_o/2 \qquad \text{(Eq. 41)}$$

The assumptions made in this derivation are that the complex and the reagent added in constant amount are the only absorbing species and obey Beer's Law, the law of mass action is operative, and there are no competing equilibria.

EXPERIMENTAL

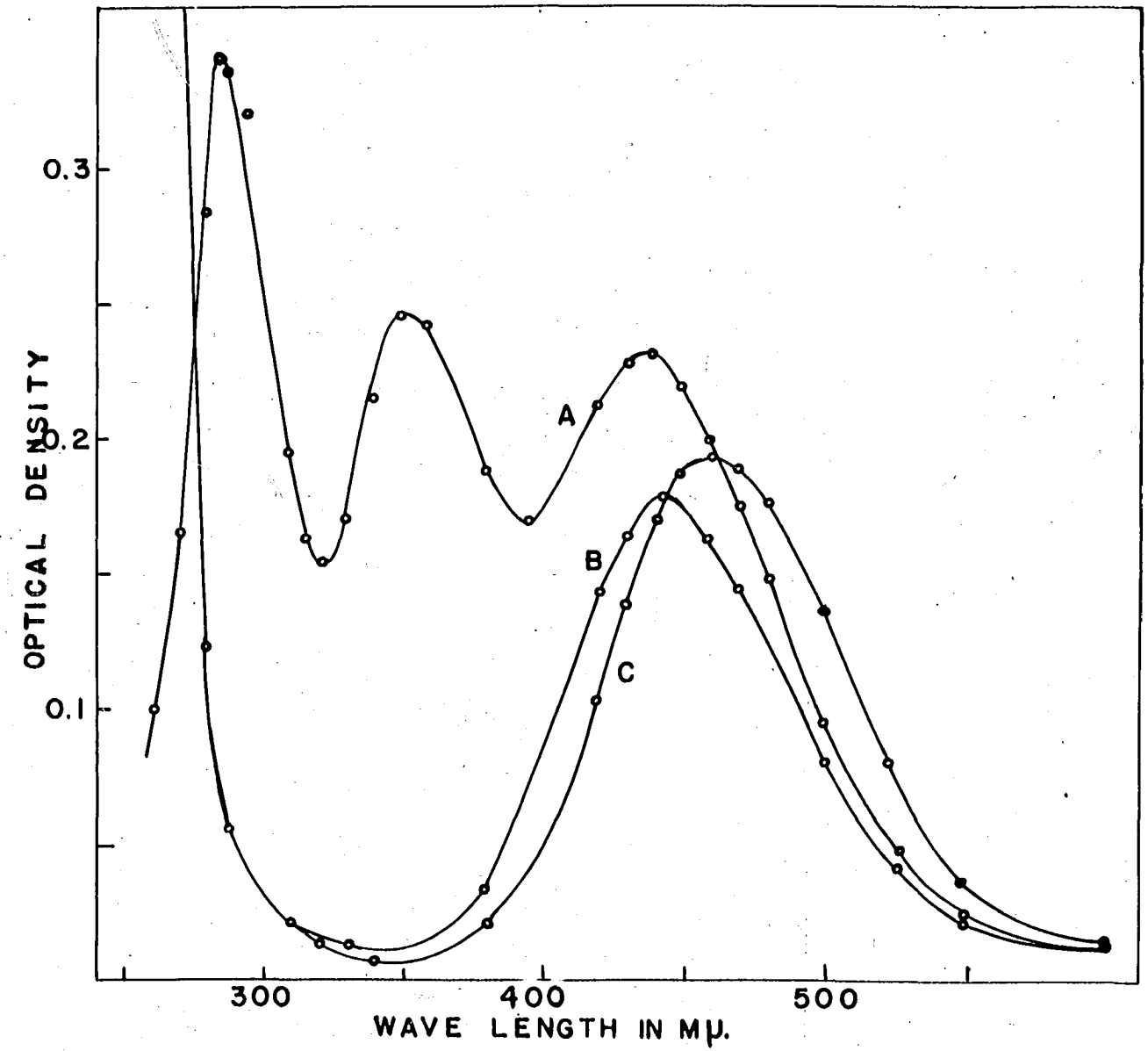
Cyclohexaamylose-Iodine-Iodide Interactions

The ultraviolet and visible absorption spectra of the α-I_2 and I_2 systems in the presence and absence of HIO_3 are depicted in Figure 2. Curve A is the spectrum of a solution containing 2.6×10^{-4} M. I_2 and 4.8×10^{-3} M. α ; curve B is the spectrum of a solution containing 2.6×10^{-4} M. I_2, 4.8×10^{-3} M. α and 0.08 M. HIO_3; curve C is the spectrum of a solution containing 2.6×10^{-4} M. I_2 and 0.08 M. HIO_3. Distilled water was used as a blank.

The shift of the I_2 spectrum upon addition of α suggested the formation of an α-I_2 complex. The stoichiometry of complex formation was studied at 420 mμ by the method of continuous variation for binary systems (Figure 3). The total I_2 and α concentrations were varied between 0 and 4.57×10^{-4} M. and the optical densities of the solutions were plotted against the ratio $I_2/I_2 + \alpha$ to give curve A. The differences between the optical densities of the α-I_2 solutions and that of identical solutions minus α, magnified 5 times, are represented by curve B. To repress the formation of I^- by the hydrolysis of I_2, these experiments were conducted in 0.2 M. HIO_3, which also served as a blank in the absorption measurements.

Figure 2. The ultraviolet and visible adsorption spectra of I_2 and α-I_2 systems in the presence and absence of iodic acid

A, α-I_2; B, α-I_2-HIO_3; C, I_2-HIO_3


0.3
0.2
0.1
OPTICAL DENSITY
A
B
C
300
400
500
WAVE LENGTH IN Mμ.

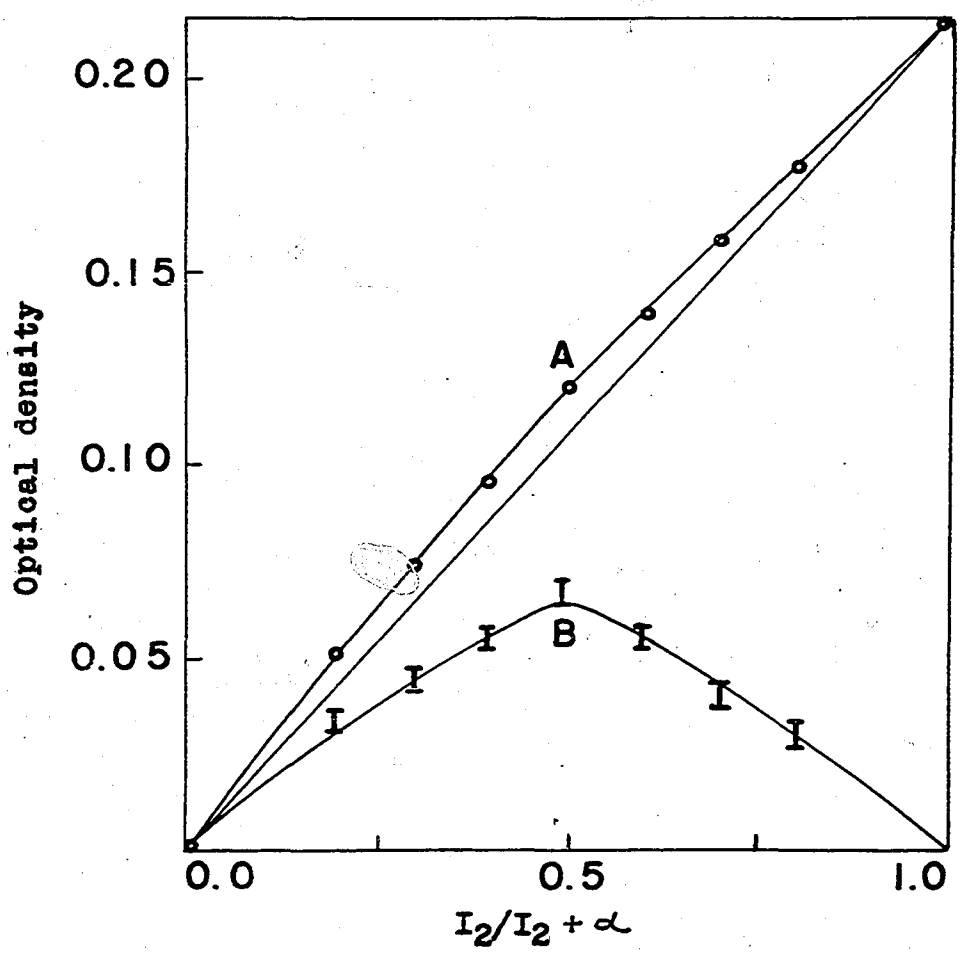


Figure 3. Continuous variation plot of the α -I_2 system

A, α -I_2; B, 5 x [A-blank]

The ultraviolet and visible spectrum of a solution containing 1.15×10^{-5} M. I_2, 2.5×10^{-5} M. I^- and 1.2×10^{-3} M. α (Curve B) is compared to the spectrum of a solution containing the same amount of I_2 but 800 times the amount of I^- (curve A) in Figure 4. Distilled water was used as a blank.

To determine the ratio of I_2 to α in the ternary complex the α-I_2-I^- system was studied by a modified method of continuous variation. The optical densities of a set of solutions containing varying amounts of I_2 and α, between 0 and 4.0×10^{-4} M., were measured at 288 mμ. (curve A), 350 mμ. (curve B), and 440 mμ. (curve C) and plotted against the ratio $I_2/I_2 + \alpha$ in Figure 5. The total concentration of I^- in each solution was held constant at 2.5×10^{-5} M. The blank was 0.8 M. $HClO_4$ which was the concentration in all of the solutions in this experiment.

The results of a similar study holding α constant (1.3×10^{-4} M.) and varying I_2 and I^- between 0 and 1.0×10^{-4} M. are represented graphically in Figure 6. Curve A, B, and C represent respectively the absorbancies at 288 mμ., 350 mμ., and 440 mμ. These experiments were also conducted in 0.8 M. $HClO_4$ which served as a blank.

To graphically determine the dissociation constant for the αI_2 complex the absorbancies at 480 mμ. of a series of α-I_2 solutions, containing a constant total amount of I_2 (2.45×10^{-4} M.), and varying amounts of α, are plotted

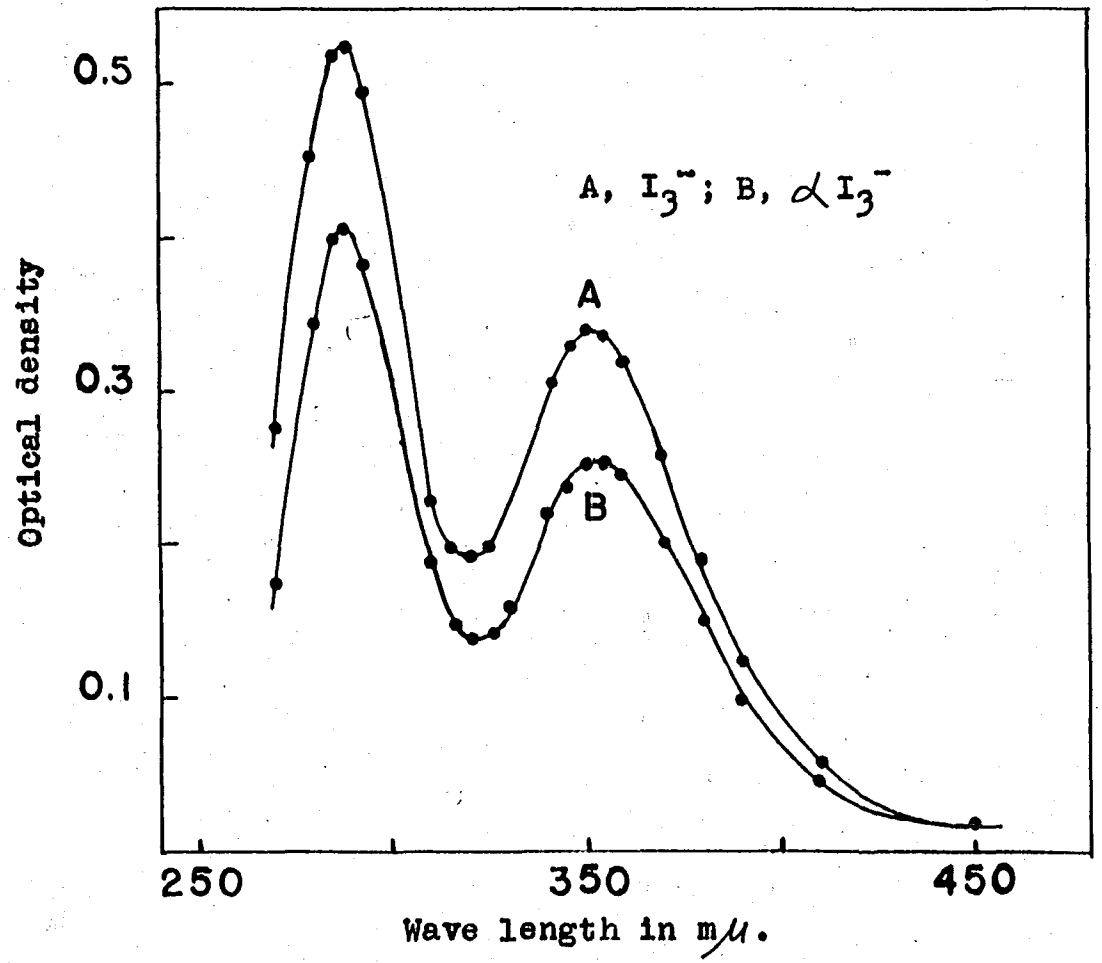


Figure 4. The ultraviolet and visible absorption spectra of the I_2-I^- and α-I_2-I^- systems

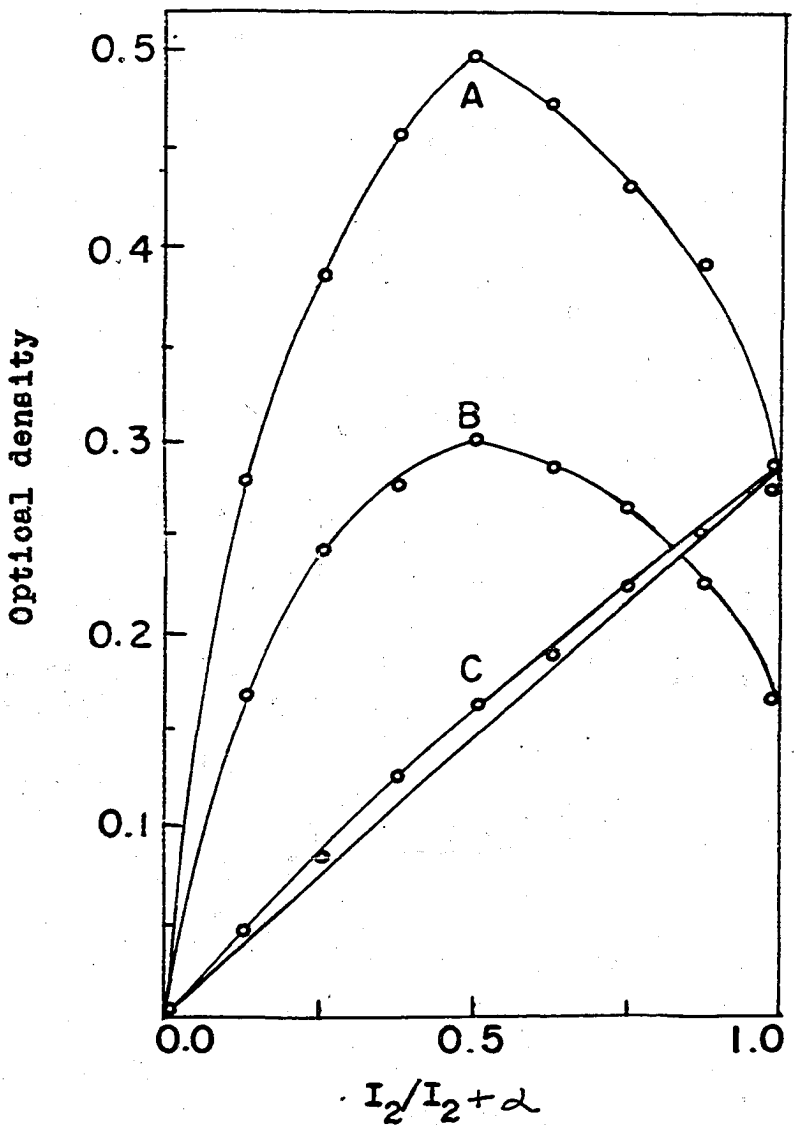


Figure 5. Continuous variation plot for the α-I_2-I^- system in constant KI

A, 288 mμ.; B, 350 mμ.; C, 440 mμ.

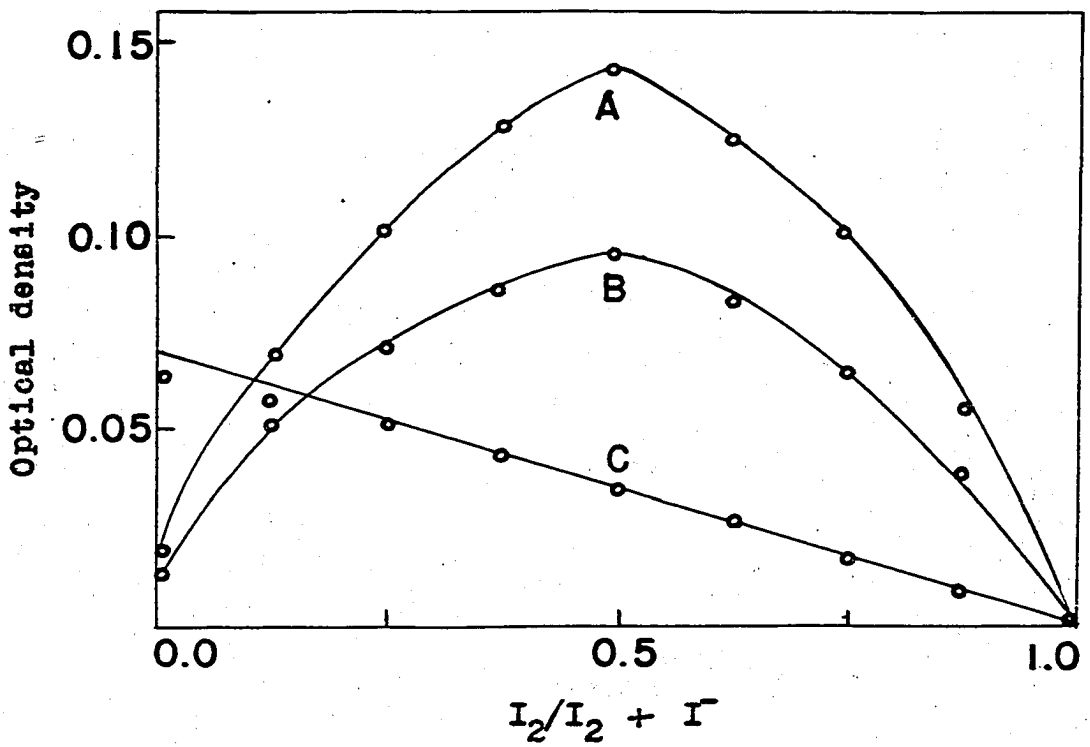


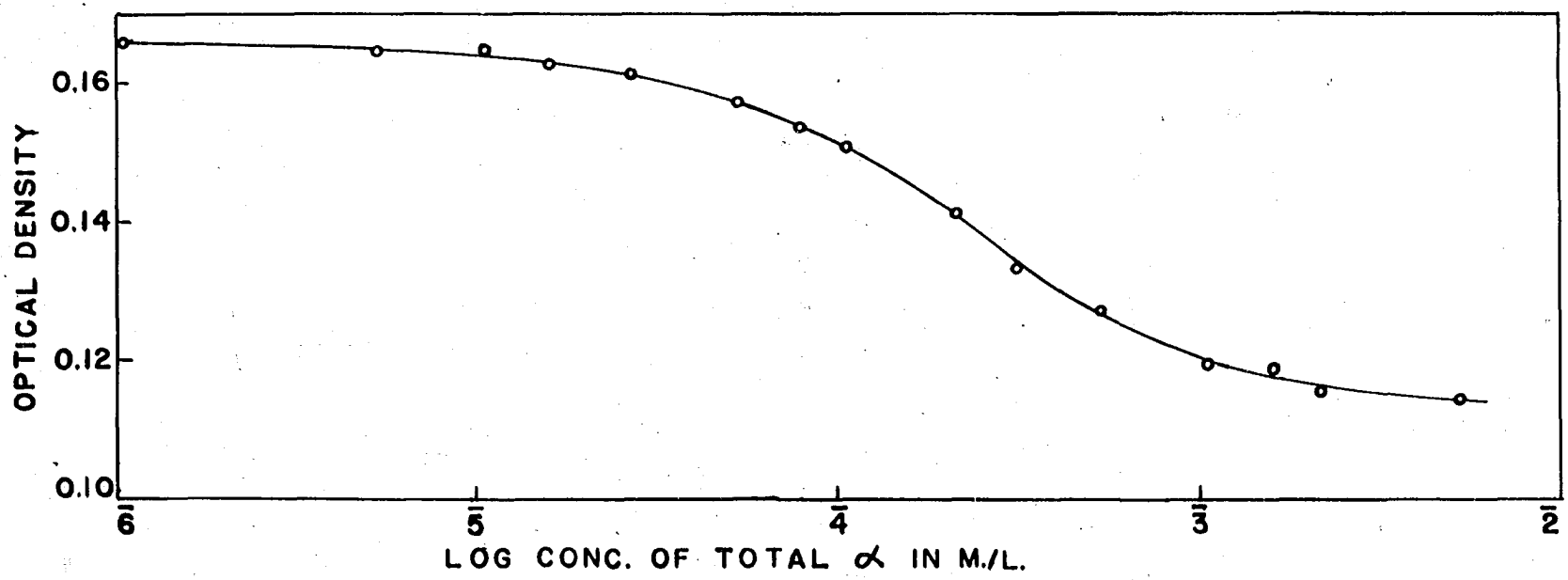
Figure 6. Continuous variation plot for the α-I_2-I^- system in constant

A, 288 mμ.; B, 350 mμ.; C, 440 mμ.

against the log of the total α concentration (see Figure 7). This experiment was conducted in 0.2 M. HIO_3 which served as a blank.

All of these spectral studies were conducted at room temperature which was $24^{\circ} \pm 2^{\circ}$ C.

Figure 7. Variation of the absorbancy of the α-I_2 system at 420 mμ. at differing α levels


OPTICAL DENSITY
0.16
0.14
0.12
0.10
6̄
5̄
4̄
3̄
2̄
LOG CONC. OF TOTAL α IN M./L.

Maltodextrin-Iodine-Iodide Interactions

Spectrophotometry

The ultraviolet and visible absorption spectra of solutions containing 1.2×10^{-4} M. I_2, and 2.4×10^{-4} M. KI and in addition 0.40% G_{10} (curve A), 0.43% G_8 (curve B), and 0.54% G_6 (curve C), and no dextrin (curve D) are shown in Figure 8. The blanks contained an equivalent amount of dextrin, weighed on an air dry basis and dissolved in distilled water. The total volume of the solutions was 1 ml., the optical path was 2 mm., and the measurements were made at room temperature.

The cuvettes were rinsed with methanol and air dried after each set of measurements. When acetone was used to rinse the cuvettes a residue was deposited on the cell wall which reacted with significant quantities of the added I_2.

Figure 9 depicts the spectra of solutions containing 2.6×10^{-4} M. I_2, and 1.2×10^{-4} KI, and in addition 0.96% G_{11} (curve A) 0.8% G_{12} (curve B), 0.87% G_9 (curve C), 0.32% mixture containing G_{18}, G_{19}, and G_{20} (curve D), and no dextrin (curve E). The other experimental conditions were identical to those listed above.

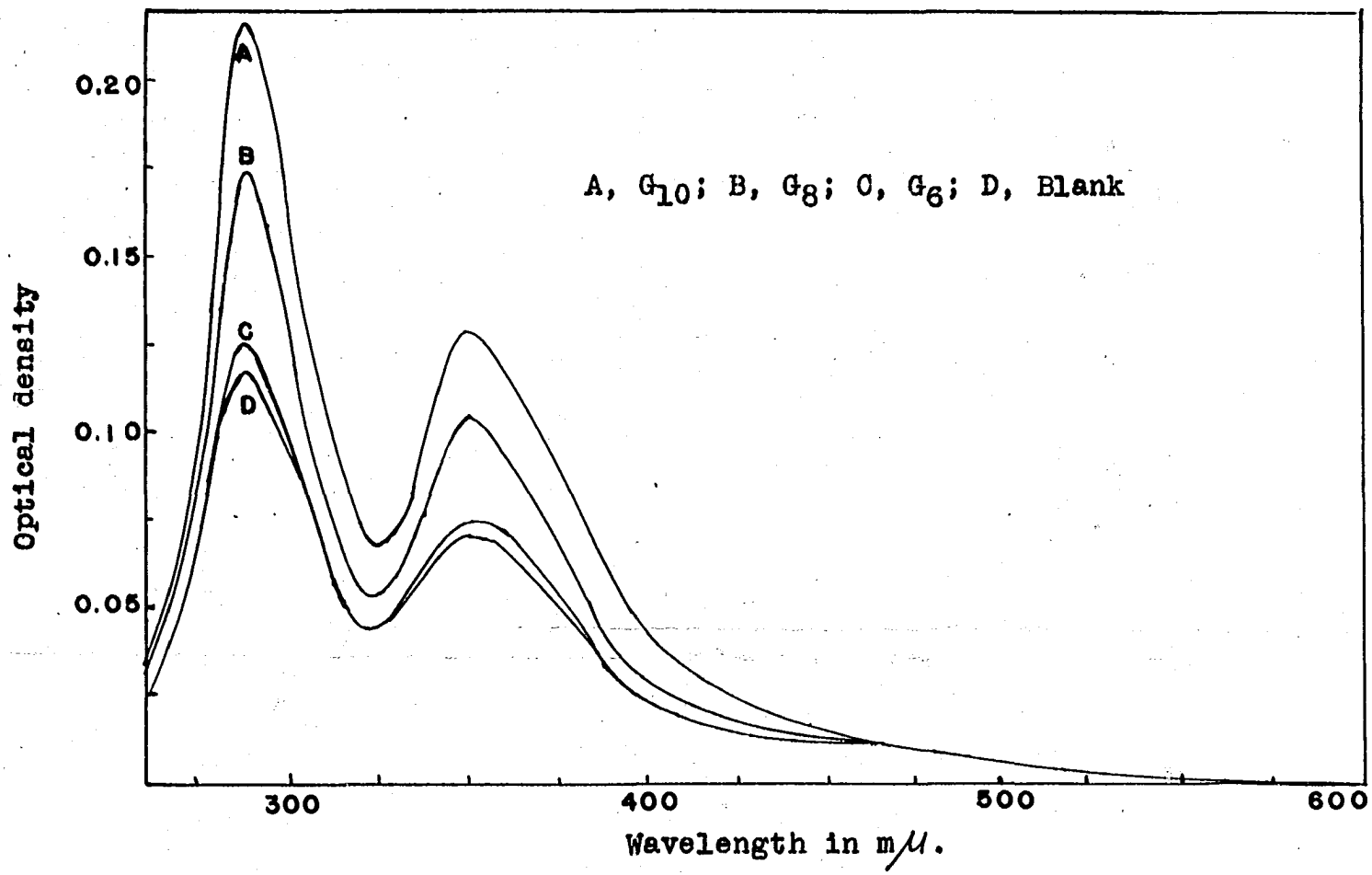
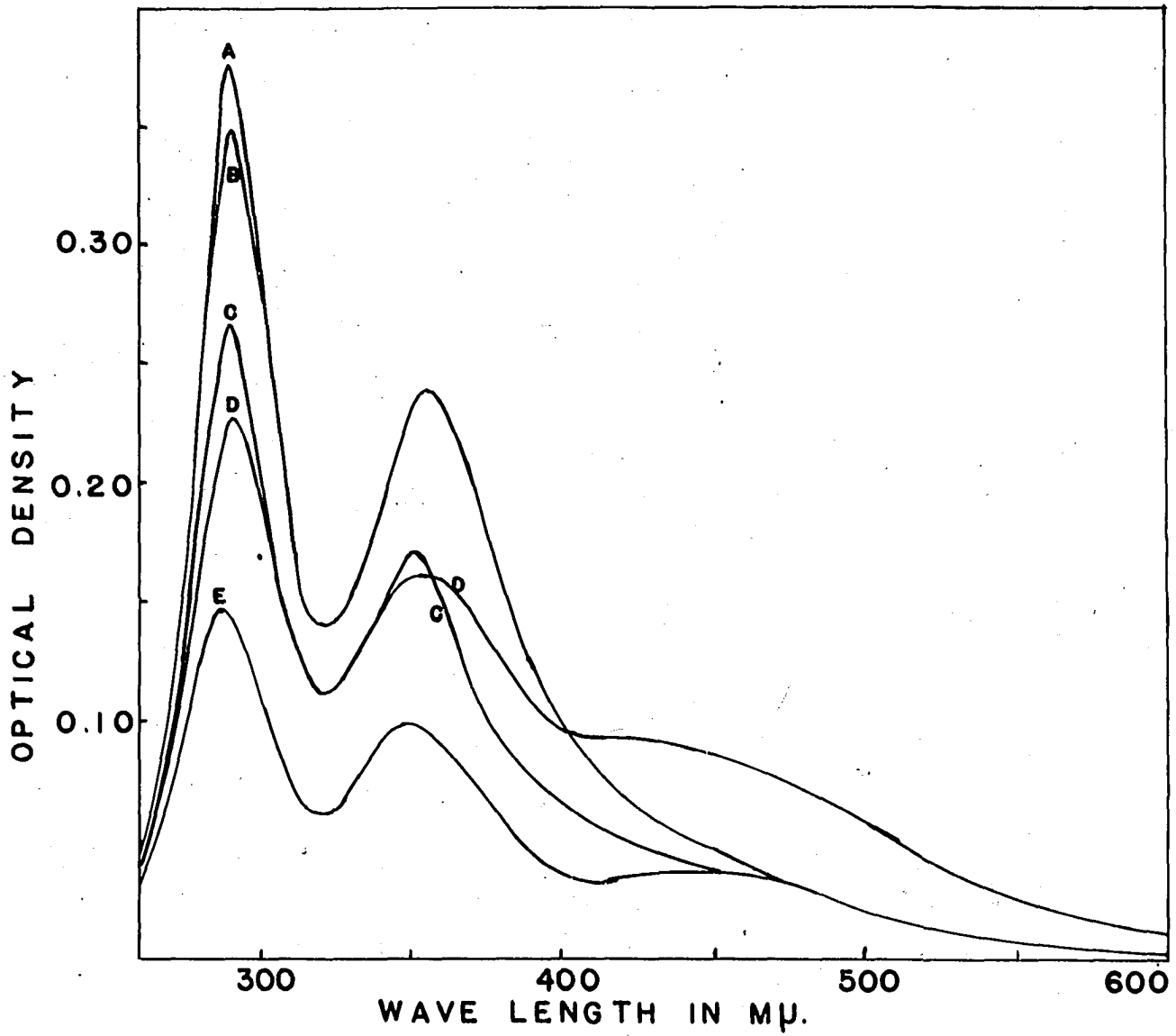


Figure 8. Ultraviolet and visible absorption spectra of dextrin-I_2-I^- solutions

Figure 9. Ultraviolet and visible absorption spectra of dextrin-I_2-I^- solutions

A, G_{11}; B, G_{12}; C, G_9; D, Mixture G_{18}, G_{19}, G_{20}; E, Blank


A
B
C
D
E
C
D
0.30
0.20
0.10
OPTICAL DENSITY
300
400
500
600
WAVE LENGTH IN Mμ.

Potentiometry

Agar bridges were prepared daily and stored in 0.25 M. KI. The average deviation for standard I_2 curves measured with bridges prepared simultaneously was generally within 0.1 millivolt. However, the average deviation for the standard I_2 curves measured with bridges made on different days was as much as 0.4 millivolt. All EMF measurements were made at $25.1^{\circ} \pm 0.1^{\circ}$ C. while the solution was gently agitated with the eye dropper.

The dextrins weighed on an air-dry basis all assayed between 87 and 92% and were dissolved in exactly 1.2 ml. of 0.25 M. KI to give approximately 1% solutions. Exactly 0.5 mm. of the dextrin solution was pipetted into the micro three-neck flask and titrated with 2.00×10^{-2} M. I_2 dissolved in 0.25 M. KI. The average deviation between duplicate samples was always within 0.3 millivolt.

Sample transformations of the EMF data into a form suitable for plotting and graphical determination of the apparent dissociation constants are shown in Table 1 and Table 2.

A plot of the log of the I_2 ($I_2 + I_3^-$) bound against the log of the I_2 free ($I_2 + I_3^-$) of the dextrins G_4 through G_9 is depicted in Figure 10. A theoretical curve is fitted to the experimental points. The curves labeled G_4, G_5, G_6, G_7, G_8, and G_9 correspond respectively to the titration behavior

Table 1. Titration of 5.46 milligrams of maltohexaose in 0.50 milliliters of 0.250 molar potassium iodide with 2.00×10^{-2} molar iodine in 0.250 molar potassium iodide[a]

(Temperature $25.1^{\circ} \pm 0.1^{\circ}$ C.)

1	2	3	4	5	6	7
.2463	.2461	2.10	0.51	1.09	2	0.91
.2555	.2552	4.27	0.52	2.22	4	1.78
.2647	.2645	8.65	0.54	4.67	8	3.33
.2689	.2686	13.0	0.56	7.28	12	4.72
.2764	.2762	20.5	0.60	12.8	20	8.2
.2813	.2811	31.4	0.65	20.4	30	9.6
.2846	.2845	40.6	0.70	28.4	40	11.4
.2871	.2870	49.0	0.75	36.8	50	13.2
.2891	.2890	57.3	0.80	45.8	60	14.2
.2907	.2906	63.8	0.85	54.3	70	15.7
.2920	.2920	70.0	0.90	63.0	80	17.0
.2941	.2940	82.0	1.00	82.0	100	18.0

[a] 1 = Electromotive force in millivolts.
2 = Electromotive force of duplicate sample in millivolts.
3 = Iodine free concentration $\times 10^4$ (average of sample 1 and 2).
4 = Total volume.
5 = Free iodine in millimoles $\times 10^4$ (average of sample 1 and 2).
6 = Added iodine in millimoles $\times 10^4$
7 = Bound iodine in millimoles $\times 10^4$ (average of sample 1 and 2).

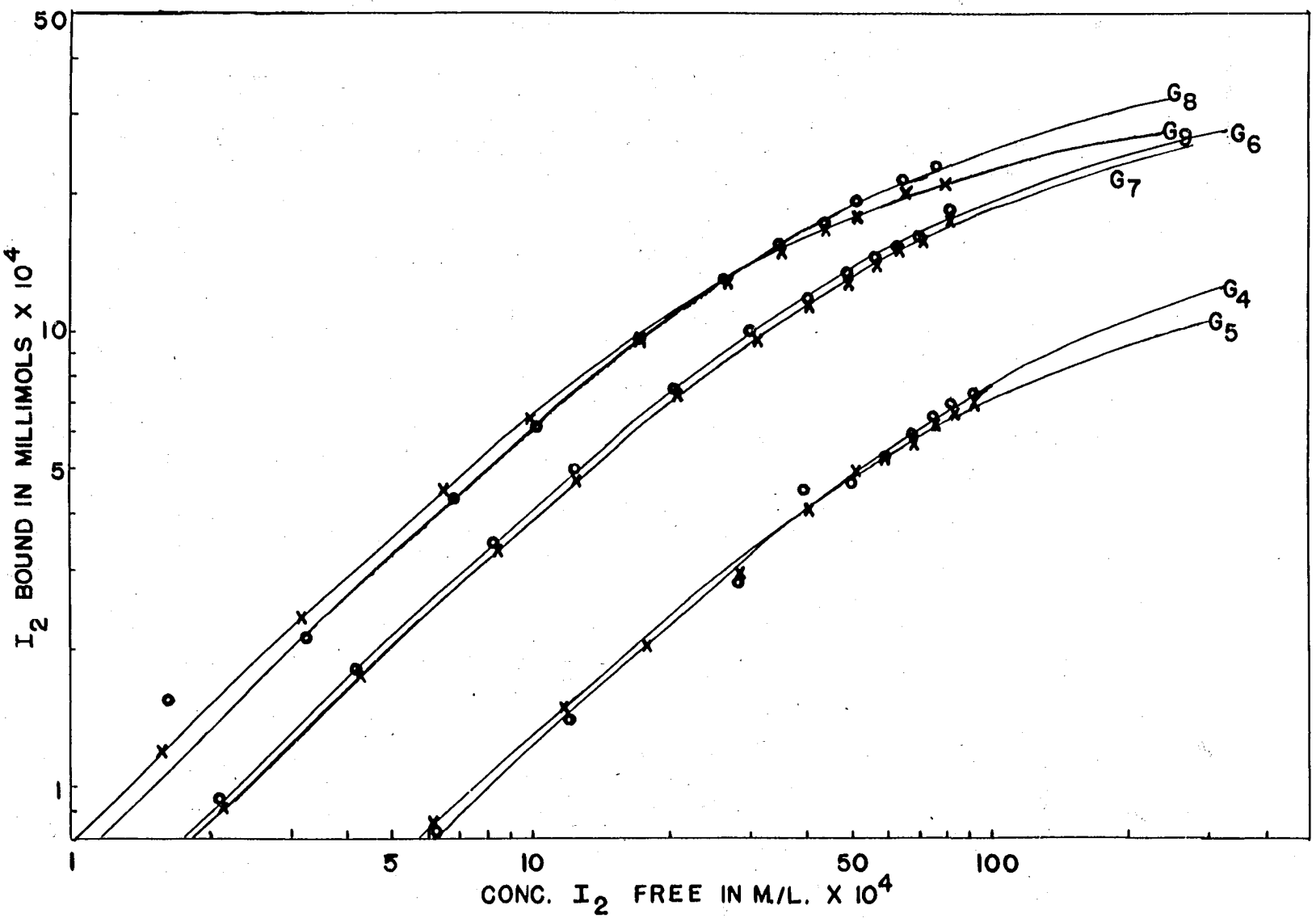
Table 2. Titration of 4.05 milligrams of maltopentadecaose in 0.50 milliliters of 0.250 molar potassium iodine with 2.00×10^{-2} molar iodine in 0.250 molar potassium iodide[a]

(Temperature 25.1° ± 0.1° C.)

1	2	3	4	5	6	7	8
.2296	.564	0.505	0.280	1	0.720	0.005	4.25
.2371	1.04	0.51	0.531	2	1.467	0.097	3.98
.2446	1.81	0.52	0.942	4	3.06	0.206	3.74
.2491	2.57	0.53	1.36	6	4.64	0.309	3.59
.2527	3.35	0.54	1.81	8	5.19	0.349	3.47
.2578	4.98	0.56	2.79	12	9.21	0.614	3.30
.2620	6.64	0.58	3.85	16	12.2	0.810	3.18
.2657	9.13	0.60	5.47	20	14.5	0.968	3.04
.2732	15.4	0.64	9.85	28	18.2	1.19	2.91
.2774	22.4	0.68	15.2	36	20.8	1.39	2.65
.2813	30.0	0.72	21.6	44	22.4	1.50	2.52
.2842	37.7	0.76	28.7	52	23.3	1.56	2.42
.2865	45.3	0.80	36.3	60	23.7	1.57	2.34
.2884	51.8	0.84	43.5	68	24.5	1.62	2.29
.2899	57.9	0.88	50.9	76	25.1	1.67	2.24
.2913	64.3	0.92	59.2	84	25.8	1.72	2.19

[a] 1 = Electromotive force in millivolts (average of duplicate results).
2 = Free iodine concentration x 10^4.
3 = Total volume.
4 = Free iodine in millimoles x 10^4.
5 = Iodine added in millimoles x 10^4.
6 = Iodine bound in millimoles x 10^4.
7 = $\bar{n}$, ratio of iodine bound to dextrin.
8 = Negative log of free iodine.

Figure 10. Titration behavior of the oligodextrins


50
10
5
1
I_2 BOUND IN MILLIMOLS X 10^4
1
5
10
50
100
CONC. I_2 FREE IN M/L. X 10^4
G_8
G_9
G_6
G_7
G_4
G_5

of 6.25 mg. of maltotetraose, 4.80 mg. of maltopentaose, 5.00 mg. of maltohexaose, 4.76 mg. of maltoheptaose, 5.48 mg. of maltooctaose, 5.80 mg. of maltononose.

Polyiodine complex formation becomes measurable when the maltodextrin series is ascended to ten glucose units. At this point the method outlined by Bjerrum (47) was used to graphically analyze the data for apparent successive dissociation constants. The negative log of the free I_2 ($I_2 + I_3^-$) is plotted against $\bar{n}$, the ratio of bound I_2 to total dextrin. The curves in Figure 11 labeled G_{11}, G_{12}, G_{13}, G_{14}, and G_{15} represent respectively the titration behavior of 4.25 mg. of maltodecaose, 5.42 mg. of maltohendacaose, 4.88 mg. of maltododecaose, 5.42 mg. of maltotridecaose, 4.17 mg. of maltotetradecaose, and 4.14 mg. of maltopentadecaose.

Figure 12 is a plot of the log of the apparent dissociation constants versus DP for the dextrin-iodine complexes. Curve A is drawn through the apparent equilibrium constants for the polyiodine (dextrin $I_6^=$ or dextrin I_5^-) reaction and curves B (G_3-G_6) and C (G_7-G_{15}) are drawn through the apparent equilibrium constants for the dextrin I_3^- complex.

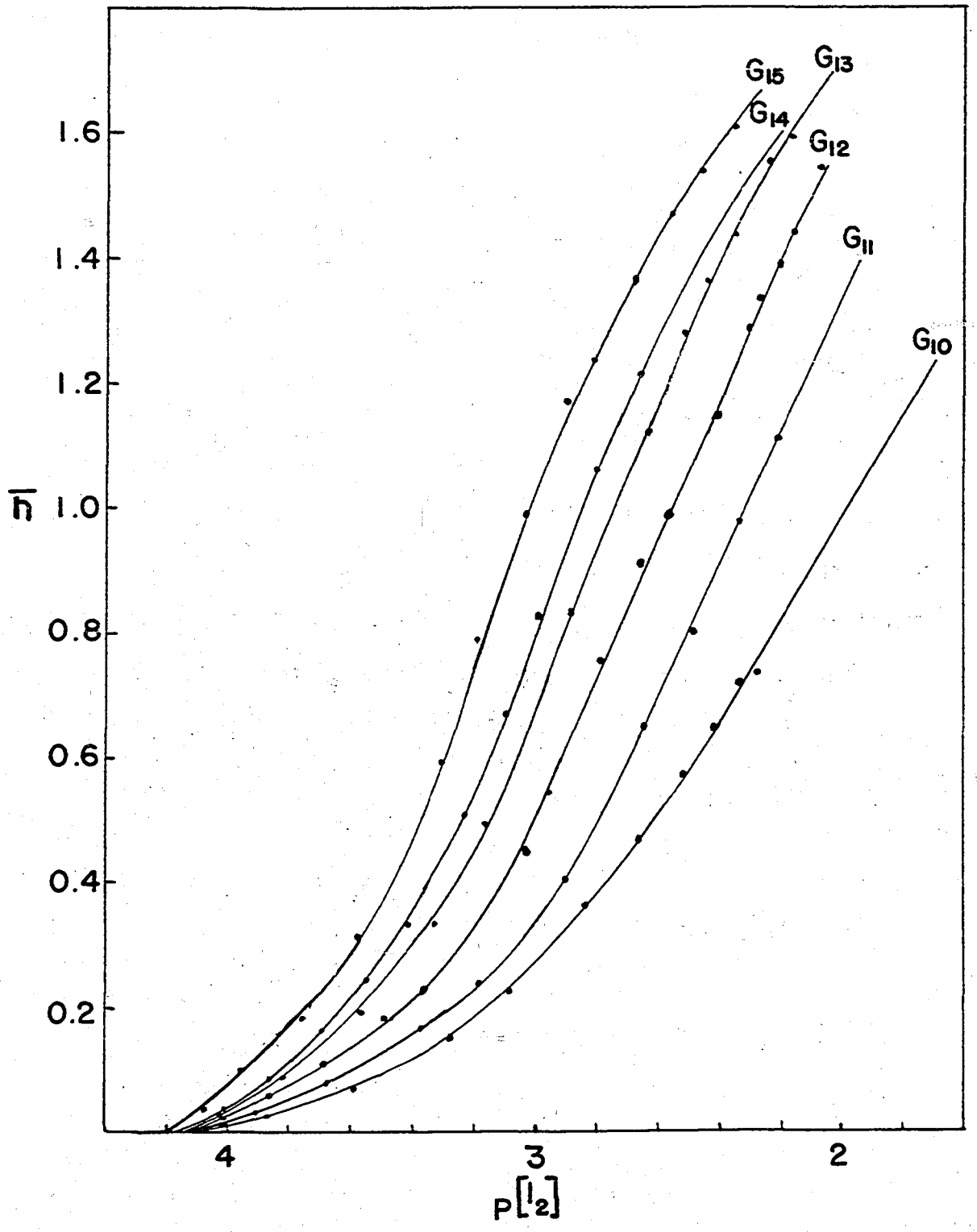


Figure 11. Titration behavior of the megalodextrins

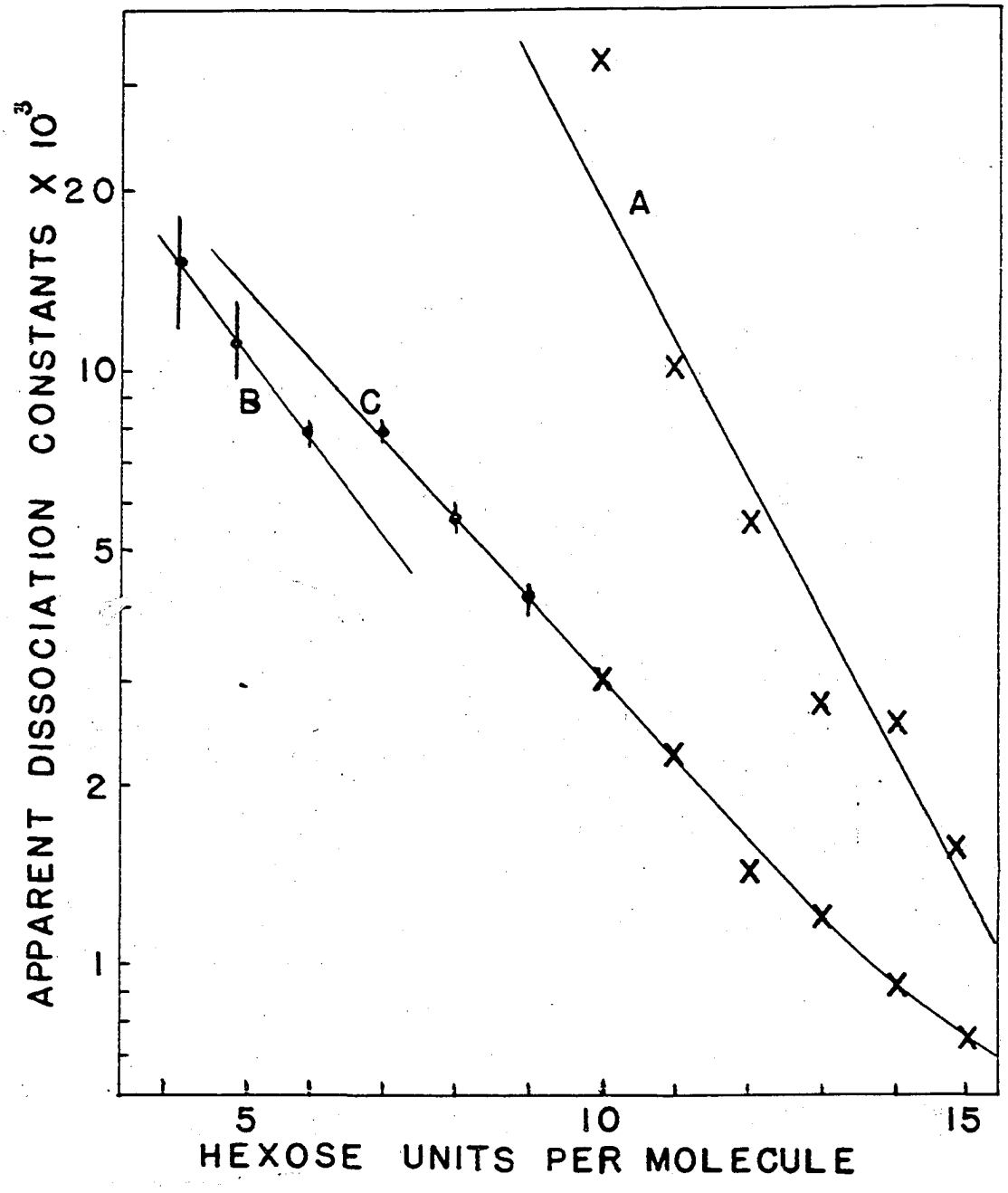


Figure 12. Variation of the apparent dissociation constants with DP

A, Second apparent dissociant constants for G_{10}-G_{15}; B, First apparent dissociation constants for G_3-G_6; C, First apparent dissociation constant for G_7-G_{15}

RESULTS AND DISCUSSION

Cyclohexaamylose

Binary system

When α was added to a solution containing I_2 in iodic acid, a spectral shift was observed indicating that an α-I_2 complex was formed. When the method of continuous variation was applied to the system, maximum complex formation occurred when the ratio of I_2: α was 1:1 indicating that this is the formula of the binary complex.

Dube attempted to measure the dissociation constant for this complex by partitioning I_2 between benzene and an aqueous α solution. Because the Schardinger dextrins are notorious complexing reagents, it seems likely that Dube measured an "average" dissociation constant (i.e. for the dissociation of αI_2 and αbenzene). Moreover he did not take into consideration the interaction of I^-, formed by the hydrolysis of I_2, with the other chemical species in solution. A spectrophotometric study was therefore undertaken to measure the dissociation constant for the αI_2 complex at room temperature. Iodic acid was added in this experiment to prevent competing equilibria involving I^-.

From the amount of added α at the midpoint of the titration curve and a knowledge of total III_2, the dissociation constant, $1/K_1$, was calculated to be $1.15 \times 10^{-4} \pm 0.15 \times 10^{-4}$ (Figure 7). Using the value of 4.3×10^{-9}, reported by Dube, for $1/K_1K_2$ (Eq. 8 and 9), $1/K_2$ was calculated to be $3.8 \pm 0.6 \times 10^{-5}$.

No spectral evidence was obtained supporting the formation of an αI^- complex even though solutions containing as much as 2% α were studied. Failure to note any spectral shifts may be attributed to a large dissociation constant and/or a close similarity of the I^- and αI^- spectra.

Ternary systems

When α and I_2 were allowed to react in the absence of iodic acid (Figure 2, curve A), two new maxima were observed at 290 and 353 mμ. The similarity of curve A to the I_3^- spectrum (16) suggested that a complex of α with I_3^- might be responsible for these new peaks. The peak at 420 mμ is attributed to the μ I_2 complex. To test the hypothesis that an α-I_3^- complex was formed, the spectrum of a solution containing I_2, KI and α was compared to the spectrum of a solution containing the same amount of I_2, but 800 times the amount of KI. The spectra of these solutions are essentially identical (Figure 4). The possibility that the spectrum of

the α-I_2-KI solution resulted from free I_3^- was dismissed because at this dilution, without added α, the calculated (16) optical density at 288 mμ. would be only 0.006, compared to the observed optical density of 0.41.

When I_2 and α were continuously varied in the presence of I^- and the absorbancies plotted against the ratio $I_2/I_2+\alpha$ (Figure 5), it was apparent that the I_2 and α reacted in the ternary complex in a 1:1 ratio. Similarly when the optical densities of solutions continuously varied in I_2 and I^- in constant α were plotted against the ratio $I_2/I_2 + I^-$ (Figure 6), it was apparent that I_2 and I^- reacted in a 1:1 ratio in the complex. This data supported the inference arrived at from the shape of the absorption spectrum that the complex had the formula $\alpha\ I_3^-$. Perchloric acid (0.8 M.) was added to all solutions in this group to suppress the hydrolysis of I_2 according to Eq. 6.

One of the requirements for the application of the method of continuous variation is that the system studied be in a state of equilibrium. The lack of an hysteresis effect upon back titration of the $\alpha\ I_3^-$ complex with sodium thiosulfate and the fact that the potential of the system was stable immediately upon thorough mixing indicated that the α-I_2-I^- system was in a state of rapidly reversible equilibrium.

When α was varied between 10^{-3} and 10^{-5} M. and I_2 and I^- were independently varied over as large a range as was

suitable for spectrophotometric measurement, all the maxima (other than that of I_2) in the region of 350 to 650 mμ. could be attributed to I_3^-, αI_3^- or αI_2.

Because it has been shown that α can form an I_2 complex in the absence of I^- and because α serves as a simple model for the amylose helix it will be of interest to examine the spectra of amylose-iodine solution in the absence of I^- to see if amylose and I_2 complex. If amylose does form an I_2 complex it may be inferred that amylose exists predominantly in the helical configuration in aqueous solution.

From the results of this study, it may be inferred that the absorption spectra of starch-iodine complexes will be markedly changed in the presence of I^- and that varying ratios of I_2 to I^- in the complex may produce continual spectral shifts; moreover, it may be possible to determine this ratio quite unambiguously by the method of continuous variation, if a system can be found in which equilibrium exists.

Maltodextrin-Iodine-Iodide Interactions

Spectrophotometry

Because color has long been associated with the formation of starch-iodine complexes, many carbohydrate chemists had tacitly assumed that "achroic" dextrins did not form complexes with iodine. Recently, however, Dube using electrometric methods demonstrated that the Schardinger dextrins formed molecular complexes with iodine without any accompanying color change. By analogy, low molecular weight maltodextrins would also be expected to form complexes with iodine which might be detectable spectrophotometrically.

When the spectra of I_2-I^- solutions were examined in the presence of maltodextrins of DP six and above, enhancement of the I_3^- peaks was observed suggesting that they formed complexes (Figures 8 and 9).

Because the spectra of the G_6 and G_8 complexes changed with time (not the position of the maxima but only the intensity), it was questionable whether the enhancement of the spectra resulted from complex formation or merely reflected a change from the I_2 to I^- ratio by reducing contaminants. To eliminate this possibility, the added I_2 to I^- ratio was made two to one. Under these conditions, reduction of I_2 can only effect a decrease in the absorbancy and any enhancement of the

optical density can be directly ascribed to a dextrin-iodine interaction.

It has been reported that dextrins in the range of 10-12 glucose units enhance the visible color of the iodine solutions. To test this hypothesis spectrophotometrically, the I_2 to I^- ratio was changed to two to one, a condition which should favor polyiodine formation. Because of the limiting availability of samples, only the dextrins G_9, G_{11}, and G_{12} and a mixture of G_{18}, G_{19}, and G_{20} were examined. Under experimental conditions no visible enhancement for the dextrins through G_{12} could be detected although the G_{18}-G_{20} mixture exhibited visible enhancement (Figure 9). The ratio of the optical density at 288 mμ to dextrin concentration increased steadily as the molecular weight of the sample increased indicating a gradual increase in binding affinity.

When two percent solutions of G_8 through G_{12} were spotted on filter paper and sprayed with the methanol I_2-KI solution, G_9 through G_{12} stained brown while the staining capacity of G_8 was questionable. Papergrams developed with the iodine spray stained yellow brown in the region of 10 to 12 glucose units depending upon the concentration of saccharide, and the color changed to plum when the series was ascended to 15 glucose units. These experiments suggested that dextrins as small as G_9 and possibly G_8 could form polyiodine complexes under favorable conditions. It also appeared that ability to

form polyiodine complexes changed gradually rather than sharply, as had been previously supposed (26, 48), as monomer units were added to the dextrin polymer. This inference has received support from potentiometric titrations.

Potentiometry

In order to formulate a more quantitative picture of the dextrin-I_2-I^- interactions, the system was studied by the electrometric methods of Bates. The maximal practical limit of I_2 concentration was 0.01 M. At higher concentrations, the standard I_2 curves demonstrated marked deviations from linearity while the measuring system became increasingly more insensitive to changes of the I_2 molarity possibly because of reaction of I_2 with the agar bridge. Because of this experimental restriction and the relatively small binding affinity of the oligodextrins, it was necessary to employ graphical methods to evaluate the equilibrium constants.

If the reactants, dextrin and I_2, form a one to one complex then the shape of a plot of the log of $I_2(I_2 + I_3^-)$ bound versus log $I_2(I_2 + I_3^-)$ free will be independent of both the equilibrium constant (for the dextrin-iodine complex) and the maximum amount of I_2 which can be bound. In addition to allowing evaluation of the dissociation constants (when 1/2 of the maximum I_2 is bound), the molecularity of I_2 in the complexes

may be noted from the initial slopes of the curves. One additional piece of information can be gleaned from this graphical analysis: the maximum amount of I_2 which can be bound. This value will correspond to the amount of saccharide which has been titrated and furnishes an additional check on the experimental results. The apparent dissociation constants determined potentiometrically are related to the dissociation constant for the reaction:

$$G_n + I_2 + I^- = G_n I_3^-$$

by a factor of $1/K_I$ (see Eq. 19 and 43).

The apparent dissociation constants, determined for G_4 through G_9 by this method, are presented graphically in Figure 12. The apparent dissociation constants for G_{10} and G_{11}, determined by the template method, were both greater than the dissociation constant for G_9. Because the spectrophotometric experiments indicated that the binding affinity increased rather than decreased, it was suspected that polyiodine formation was becoming significant at the higher I_2 levels. To establish values for the apparent successive dissociation constants for the saccharides having a DP greater than nine the method outlined by Bjerrum (47) and extended by Irving (49) was used. The two constants for each of the

dextrins G_{10} through G_{15} are presented graphically in Figure 12. Assuming that the iodine bound at low concentrations is all in the form of I_3^-, a value for the first apparent dissociation constant can be determined by matching the template (theoretical curve for 1:1 complex) to the first few experimental points and the point of maximum iodine binding. Apparent equilibrium constants determined for G_{10} and G_{11} agree closely with those determined by the method of Bjerrum.

When the maximum amount of I_2 which could be bound as determined graphically was compared to the calculated value the results agreed within experimental error for G_8 and G_9 but rose above the experimental error for all saccharides containing more than nine glucose units. Furthermore, when $p[I_2]$ was plotted versus $\bar{n}$ the titration curves were reasonably symmetrical around the point $\bar{n} = 1$ which means that the reaction is bimolecular in I_2 (Figure 11).

Saccharides composed of seven or less glucose units also showed marked deviations from the calculated maximum amount of I_2 which could be bound. The experimental values were low which is reasonable if the reactions are polymolecular with respect to the dextrin. However, steric considerations militate against such a conclusion. Further work need be done in order to resolve this discrepancy.

Although complex formation for G_3 could be detected, the scatter in the experimental points was too great to allow a

determination of the equilibrium constant. The EMF measurements from the titrations of glucose and maltose were identical with blanks within experimental error and therefore it cannot be conclusively established that they exhibit a binding affinity for iodine.

At constant I^- levels no information can be gained about the molecularity of I^- in the complex. For example, it is impossible to differentiate between the following sets of equilibria:

$$G_n + I_2 = G_nI_2^- \qquad \text{(Eq. 42)}$$

or

$$G_n + I_3^- = G_nI_3^- \qquad \text{(Eq. 43)}$$

and

$$G_nI_3^- + I_3^- = G_nI_5^- + I^- \qquad \text{(Eq. 44)}$$

or

$$G_nI_3^- + I_3^- = G_nI_6^= . \qquad \text{(Eq. 45)}$$

Nevertheless, consideration of the α-I_2-I^- interactions, electrostatic repulsions and thermodynamics lend strong support to the accuracy of Eqs. 43 and 44.

Dube demonstrated that α reacts not only with I_3^- but also with I_2 and I^- to form inclusion complexes. If α is a reliable model of the amylose helix, then it is reasonable that the enthalpy for the formation of a G_6 complex would be comparable to the enthalpy for the corresponding reaction

with α. If this hypothesis is valid then one can calculate the entropy of coiling to be 15.2 entropy units from a knowledge of the dissociation constants for the αI_3^- and $G_6I_3^-$ complexes. Considering this large entropy term it appears extremely unlikely that a G_6I_2 or a G_6I^- complex could be detected under experimental conditions employed. If it is reasonable, that the free energy of the reaction to form a complex can be represented by the following equation:

$$\Delta F = n\Delta F' + \Delta F'' \qquad \text{(Eq. 46)}$$

where n is the number of glucose units in the polymer and $\Delta F'$ is the free energy of reaction per glucose and $\Delta F''$ is a constant, then analogous reasoning indicated that the concentrations of G_nI_2 and G_nI^- would remain below the limits of experimental detectability. This treatment has neglected stabilization of the helix by hydrogen bonding which is most likely insignificant for one or two turns of the helix but may be an important factor for a large molecule like amylose.

According to Eq. 46, a plot of the log of the apparent dissociation constants against DP should be linear if each monomer has equal "accessibility" to the bound I_2. The constant $\Delta F''$ or the intercept will represent the end effects. Figure 12 is in harmony with this hypothesis.

The discontinuity in the curve of the first apparent dissociation constants between G_6 and G_7 can readily be interpreted as a configurational transformation from a "loop" to a helical arrangement. This argument is also supported by the relative values of the equilibrium constants when the ratio of G_6 to G_7 is compared to those of the corresponding Schardinger dextrins, α and β. Dube reports the binding affinity of α is 100 times larger than binding affinity of β. Now if G_7 is actually in a "loop" configuration rather than in a helical configuration, the ratios of the apparent dissociation constants would be expected to differ by a factor of approximately 100 while the measured values are the same within experimental error.

The values for the first apparent dissociation constants appear to be leveling off in the neighborhood of two to two and a half turns of the helix. This result would be expected when the length of the helix approaches the length of the I_3^- species. However, the values for the second apparent dissociation constant become steadily smaller. It appears that as chain length increases, the stability of the polyiodines complex increases more rapidly than the stability of the monoiodine complex. Indeed, a DP will be reached when the second apparent dissociation constant will become smaller than the first apparent dissociation constant. This pattern then may be reasonably expected to repeat itself for higher

complexes as the maltodextrin series is ascended, and can account for the spectral shifts reported in the literature when starch is hydrolyzed.

SUMMARY

1. The method of continuous variation has been extended to some special ternary systems and used to study the α-I_2-I^- system. A spectral analysis of this system revealed that αI_2 and αI_3^- complexes were formed.
2. The dissociation constant for the αI_2 complex was found to be $1.15 \pm 0.15 \times 10^{-4}$.
3. Maltodextrin saccharides up to a DP of 18 have been isolated by partition chromatography on cellulose columns.
4. A spectrophotometric examination of the dextrin-I_2-I^- systems indicated that all maltodextrins of DP six and above could form dextrin I_3^- complexes.
5. The first apparent dissociation constants for the dextrin complexes in the range of DP 4 to DP 15 and the second apparent dissociation constants for the dextrin I_5^- complexes in the range of DP 10 to DP 15 were found electrometrically.
6. The results of these potentiometric titrations are interpreted in terms of helical binding.

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