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The nature of the starch-iodine complex

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NOTE TO USERS

THE NATURE OF THE STARCH-IODINE COMPLEX

by

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I. INTRODUCTION

The starch-iodine reaction has attained considerable significance in the past as a valuable means of identifying starch and of following the course of enzymic and hydrolytic degradation of starch. As the starch is broken down, the iodine color changes from a deep blue through violet to red and finally becomes brown. The use of starch pastes in volumetric work with iodine is well known.

In spite of long familiarity of chemists with starch-iodine colors and the common use to which these are put in describing various native and modified starches, there exists today little real basis for an understanding of the meaning of these colorations in terms of starch molecular or colloidal organization.

In the past attempts to explain the varied colors of the complex have been based upon formation of addition or adsorption compounds, phosphorus content of the starch, colloidal dispersion of the iodine in starch, or a combination of these factors. There is a definite lack of experimental evidence for the proof or disproof of any of the theories proposed, and the literature on the subject has been confusing and contradictory. To a large extent this has been due to the heterogeneous character of naturally occurring

starches.

Only within the last few years has our concept of the chemical structure of starch reached a stage where a scientific explanation for the existence of a straight-chain and a branched-chain fraction could be made. The introduction of the idea of branching in the starch molecule, as opposed to the tendency to consider starch as a linear molecule similar to cellulose, as well as the idea of a helical and an extended-chain configuration of starch, makes it desirable to reinvestigate starch materials in the light of these concepts.

As will be shown, the starch iodine complex is extremely useful in the qualitative and quantitative determinations of starch components. The present investigation offers information gathered in a survey of the iodine-complexes of various polysaccharide materials, chosen to test the more recent theories of the starch structure.

II. DEVELOPMENT OF THE CONCEPT OF TWO COMPONENTS AND A HELICAL CONFIGURATION FOR STARCH

The best evidence that starch exists in an essentially branched or an essentially straight-chain configuration is derived from methylation studies on the starch fractions separated by physico-chemical methods. Meyer has suggested the term amylose to designate the unbranched chains, and amylopectin, the branched molecules (57). The literature is abundant with methods of separating the two fractions. Among the more satisfactory techniques are those of Pacsu and Mullen (70) using cotton to adsorb the amylose, and Schoch (83) using butanol to precipitate the amylose.

Upon methylation and hydrolysis of the amylose fraction, the sugar formed consists of mostly 2,3,6-trimethyl glucose from which it follows that the glucose residues are combined by 1:4-glucosidic linkages (42). The molecular weight of the methylated amylose as determined by osmotic pressure corresponds to 250 glucose units (61). Since only 0.4% of the glucose units (or one out of the 250 units consists of 2,3,4,6-tetramethyl glucose) may be considered as an end group, and since the molecule contains 250 units, it cannot be branched.

Furthermore, amylose is hydrolyzed completely by beta-amylase, is adsorbed readily on other straight-chain

materials such as cellulose, and its ethers and esters form strong extensible films.

In the case of amylopectin, it has been shown that the end group content (determined from amounts of 2,3,4,6-tetramethyl glucose) of 4 to 5% corresponds to one terminal unit to 25 glucose residues (42). Haworth who made the discovery but believed that starch consisted of associated chains of 25 glucose units, accepted the concept of branching only after such investigators as Staudinger, Freudenberg, Hirst and Young had pointed out that osmotic pressure determinations on soluble amylopectin derivatives gave molecular weights almost 100 times as large as a 25 glucose unit. Further evidence for branching may be summarized as follows: The slight reducing power of amylopectin indicates only one aldehyde group to 1500 to 3000 glucose units. Beta-amylase breaks it down only to residual dextrins of high molecular weight, and the ethers and esters form brittle inextensible films.

In his viscosity studies Staudinger attempted to explain certain dissimilarities between starch and cellulose by suggesting a helical configuration for starch (89).

At about the same time Hanes (37) suggested a helical configuration for starch in order to explain some of the peculiarities of the digestion of starch by alpha-amylase. He also stated that such a configuration might well explain

the iodine reaction with starch and dextrins, the iodine being enclosed within the spiral and more than one complete turn of the helix required for the production of color. Although his mechanism for enzyme action is somewhat doubtful, the concept of a helical configuration is strongly indicated by a study of the starch-iodine complex.

Freudenberg (32) made use of the theory to explain the formation of the cyclic Schardinger dextrins produced by B. macerans amylase. Upon the examination of space-filling models of amylose which show a natural tendency to form a helix, Freudenberg suggested that starch was coiled into spirals of periodicity approximately equal to six glucose residues; within these spirals the atom groups are chiefly hydrocarbon and there is room for iodine molecules. Freudenberg also suggested that since iodine produces a color in starch similar to that produced in hydrocarbon solvents, the iodine in starch-iodine was simply "dissolved" in the hydrocarbon phase of starch, and so dissolved produced a blue color. He gave a similar explanation for the formation of iodine addition products with Schardinger dextrins-- an iodine molecule was held in the center of the cyclic dextrin.

Caesar and Cushing (12) built space models of amylose chains which indicated that helical chains were sterically necessary. Their models were similar to a "stretched" coil

or an extended helix in contrast to the tightly packed structure of Freudenberg. However, it should be stated that evidence from space models cannot be rigidly accepted, particularly in the case of sugar polymers where the true configuration of the pyranose ring and the glucosidic bond angles are still in doubt.

Meyer has objected to the helical configuration of starch on the basis that the evidence for extended chains in starch is incontrovertible (59). Bear has pointed out that it needn't be a question of an extended chain or a helical chain, but may be both depending upon the treatment of the starch (7). Interpretations of X-ray diffraction patterns of starch in the "V" configuration are not in disagreement with a helical configuration.

III. CONCEPTS OF THE STARCH-IODINE COMPLEX

Since the first report of a colored product produced by the action of iodine on starch by Colin and deClaubry (15) in 1814, the literature on the subject has been controversial in regard to the exact nature of the complex. These first workers in the laboratory of Gay Lussac studied the effect of iodine on various starches as well as the effect of various reagents, mostly electrolytes on the starch-iodine complex.

In his review of the starch-iodine reaction, Samec (82) has summarized the early concepts of the complex. With regard to the formation of the complex within the granule, the reaction goes faster when iodine is dissolved in water than when dissolved in organic solvents or when iodine vapor is used. In the same reagent, the higher the temperature, the less intense is the coloration of the complex. An iodine-colored and water-permeated granule of starch will change its color from blue to red to yellow as the water is removed by evaporation or addition of alcohol. Hence the color is dependent upon the water content of the granule. In solution other factors such as degree of dispersion and presence of electrolytes have an effect on the iodine color.

Effect of Water

In 1834, Leroy found that water was necessary for the formation of the complex (54). Although his finding was confirmed by a number of investigations, there are now known conditions under which the complex can be formed in the absence of water. French (31) obtained a blue product by treating dry amylose (from a butanol precipitation) with iodine vapor.

Effect of Iodide Ions

Whether or not iodide ions are necessary for the formation of the complex has been a subject of controversy ever since they were first found present in the analysis of the complex. Roberts (76), Hale (36), Stocks (90) and Mylius (64) all claim that hydriodic acid or at least iodide ions are essential to the complex. Their results are based on chemical analyses of the complex in which iodide ions are invariably found present, or upon the fact that some native starches are not colored by iodine absolutely free of iodide ions. Mylius compared the properties of starch-iodine with iodocholic acid and came to the conclusion that it was a definite compound with fixed ratios of starch, iodine, and hydriodic acid. Roberts also favored the formula proposed by Mylius. From conductivity measurements on starch, iodide ions, and iodine,

Padoa and Savare concluded that the complex was an additive product of all three, but at the same time could not establish a constant relation between iodine and hydriodic acid (71). Lottermoser (55) carried out an extensive potentiometric study of the starch-iodine complex. He first measured the potential of the iodine-iodide system for different concentrations of iodine in 0.1 N potassium iodide, and determined the concentration of iodine. Assuming the iodide ion was not adsorbed, he made similar measurements in the presence of starch and calculated the amount of iodine adsorbed. From this datum he plotted a typical adsorption curve. To test the validity of the assumption, he determined the distribution ratio of iodine between 0.1 N potassium iodide and carbon tetrachloride, and then did the same in the three phase system, starch, 0.1 N potassium iodide and carbon tetrachloride. The two methods checked nicely for concentrations of potassium iodide equal to or greater than 0.1 N. However, with smaller concentrations, more iodine was adsorbed in the distribution experiment than in the potentiometer experiment. In further potentiometric experiments in which the iodine concentration was held constant while the iodide was varied, the presence of starch caused a change in the e.m.f.-concentration curve although it was small compared to the change in the iodine curve. From these data it was concluded that iodide ions must play an integral part in the

formation of the complex. Murray (62) also made an extensive study of the complex using the iodine electrode on aliquot portions from distribution experiments in the system, starch, carbon tetrachloride, iodine, and potassium iodide. His conclusions were much the same as those of Lottermoser, but a few points are worthy of mention. There was an absence of a direct relationship between the iodine taken up by the starch and the concentration either of free iodine or the tri-iodide ion. In dilute iodine solutions the quantity of iodine taken up was approximately double that of the combined iodide ion, suggesting the formation of the compound, starch.2 $I_2.I^-$. However, at higher iodide concentrations the iodide ion was taken up in excess of the requirements of this compound. Nearly all workers who have experimented with the distribution of iodine between an organic solvent and a starch solution have come to the conclusion that iodide ions are necessary for the formation of a blue color (28), but it should be emphasized that they have all used pastes of native starches or solubilized starches which normally exist in a straight chain configuration.

Barger (3), who studied a great number of blue adsorption compounds of iodine, assumed the necessity of iodide ions in the formation of colored iodine complexes, and suggested this necessity might result from these compounds being periodides.

Investigators who insist that iodide ions are not necessary for the formation of the complex are just as numerous. From chemical studies Toth found that potassium iodide was not absorbed in forming the blue compound (91).

Studies of the adsorption of iodine by corn starch using potassium iodide in various concentrations indicated that the adsorption took place according to the exponential formula $C_a = KC^p$ in which C_a is the iodine adsorbed by 1 gram of starch, C the concentration of iodine remaining in the solution after adsorption, and K and p are constants. Since K and p are independent of the concentration of potassium iodide, the investigators assumed iodide was not adsorbed by starch (2).

Berczeller (8) was able to produce a blue color with starch and iodine solutions under conditions in which no potassium iodide could possibly be present. His data indicate an adsorption compound consisting of starch and iodine alone. He concedes that addition of iodide ions causes an intensification of color of starch-iodine, but ascribes this to a change in the colloidal state of the starch produced by the presence of the ions (9).

More recently Meyer has carried out light absorption experiments on the starch-iodine complex, measuring the concentration of the components and the blue compound colorimetrically (59). He comes to the conclusion that iodide ions

are neither necessary for the production of the blue color nor for the precipitation of the iodine compound.

Some investigators have come to the conclusion that iodide ions play an intermediate role in forming the blue complex. When alcoholic iodine, which contained no iodide ions, was added to starch solution, the conductivity of the substance formed was much greater than the sum of the conductivities of the individual substances. The complex behaved something like an unstable iodide. It seemed probable that micellar ions were given out in solution and that the presence of outside iodide ions was not necessary for the complex formation. Electrolytes had the general effect of intensifying the blue color by the coalescence of smaller particles into bigger ions caused by their coagulating effect. These are the conclusions from the conductivity experiments of Dhar (23).

Gorbatscheff and Winogralowa (35) explain the function of iodide ions as follows: The influence of electrolytes, especially iodides, on the adsorption process is due to the fact that their anions neutralize the positive charge of the hydrogen ions on the surface of the starch and thereby increase its negative field of force, leading to a considerable increase in its adsorbing power. The positive temperature coefficient of the adsorbing of iodine by starch the authors attribute to the great influence of temperature on the union of hydrogen

ions with the adsorbing starch surface. If the influence of the hydrogen ions is suppressed, the temperature coefficient is negative as would be expected of a simple adsorption process.

It has also been proposed that iodide ions are necessary for the production of tri-iodide ions which are absorbed more readily than iodine itself by starch (1).

Effect of Heat

One of the most difficult properties of starch-iodine to explain is the loss of color with increase in temperature and the return, or at least partial return, of color upon cooling. The phenomenon was reported by Lassaigne in 1833 (53). One of the first explanations regarding the loss of color upon heating was that hot water had a greater affinity than starch for iodine whereas with cold water the reverse was true (74). Personne suggested the decoloration of starch-iodine was due to volatilization of a portion of the iodine, to formation of a colorless compound of starch with iodine and to conversion of a portion of the starch to sugar (73). Payen noted that starch grains swell to 25-30 times their original volume when placed in a saturated solution of potassium iodide or potassium bromide. To him this indicated that the disappearance of the blue starch-iodine reaction

upon heating was due to the expansion of groups of particles, and the reappearance of the coloration upon cooling was due to a contraction of these particles (72). However, Baudrimont (5) showed that a blue color could be obtained while hot if enough iodine were present. His explanation of the fact was that when heated, a certain amount of iodine formed a vapor and left the starch. Upon cooling the iodine vapor redissolved. Staiger further showed that the addition of hydrochloric acid to starch-iodine permitted the blue color to be retained even at the boiling point (88). Nagai attempted a more systematic study of the effect of heat on the complex (65). The color reaction of starch-iodine disappeared on heating to 78°; on cooling the blue color was restored, but not to its original intensity, a phenomenon believed analogous to hysteresis. Since the complex was found to be electro-negative (oriented toward the positive pole), it was heated to 80°, subjected to cataphoresis at 60° and allowed to cool. The starch did not carry iodine with it. Yet, Sonstadt (87) found that with strongly dried starch-iodine, four-fifths of the iodine was driven-off by charring at red heat, but one-fifth of the iodine remained with the charcoal formed.

Effect of Other Materials

A great number of electrolytes have the effect of changing the sensitivity and the color of the starch-iodine complex; with increased concentration of salts the color changes from blue through red to yellow (35). This observation has been made by numerous investigators. It has also been observed that the complex is prevented or destroyed by such material as phenolic compounds, gum arabic and several proteins (56). The starch-iodine color is removed by alkali, arsenous acid, sulfurous acid, sodium thiosulfate, alcohol, chloroform and chloral hydrate. A great many of these reagents form the basis of starch-iodine analyses.

Sensitivity of the Reaction

Kolthoff has shown that the sensitivity of this reaction varies considerably with pH, temperature and presence of foreign materials, both electrolytes and non-electrolytes (49). Nevertheless rough limits of sensitivity may be described. Chretien and Vandenberghe found that at 19°C 0.15 milliliter of 0.01 N iodine gave a distinct color to a solution of one part of starch to 50,000 parts of distilled water containing 50 parts of potassium iodide (14). Murray, varying the starch and potassium iodide concentrations over wide ranges, found the iodine threshold concentration was

0.15×10^{-15} moles per liter (62). These sensitivity studies were made with solutions of native starches or solubilized starches. As will be shown, pure amylose is much more sensitive to iodine coloration than amylopectin. Since all starches have a high amylopectin content and varied amylose content the term sensitivity in a rigid sense is meaningless.

Analysis of the Complex

It would require several pages to tabulate the results on the analyses of the starch-iodine complex as reported in the literature. Samec has made a table of the results obtained by the more outstanding workers in the field of starch chemistry (82). A few of the analytical results are tabulated on the following page as an indication of the wide range of results recorded. With the exception of the last two analyses in Table I, all results are from purely chemical analyses.

Table I

Analyses of the Starch-Iodine Complex

Author	Method of Analysis	% Iodine in Complex	Formula Proposed
Mylius (63)	Starch-iodine precipitated; iodine determined volumetrically	17-19.7	$(C_6H_{10}O_5)_{16}\ I_4 \cdot HI$
Rouvier (78)	Precipitation and combustion of starch-iodine	8.6-9.1	$(C_6H_{10}O_5)_{16}\ I_2$
Rouvier (79)	Analyses of supernatant liquid after starch-iodine precipitation	12.8	$(C_6H_{10}O_5)_{16}\ I_3$
Rouvier (80)	Titration of precipitated complex	19.6	$(C_6H_{10}O_5)_{16}\ I_5$
Toth (91)	Titration of precipitated complex	22.6-22.8	-
V. Euler & Myrback (25)	Distribution experiments in system; Benzene, H_2O, Starch, I_2	7.4 13.5	$(C_6H_{10}O_5)_{18}\ I_2$ $(C_6H_{10}O_5)_{18}\ I_4$
Murray (62)	Iodine Electrode		$(C_6H_{10}O_5)_{15}\ I_5$
Field (26)	Radiomicrometric titration	4.5-25.3	None

Proposed Theories

Names of some of the investigators favoring the theory of compound formation between iodine and starch may be obtained from Table I. Other theories and their supporters are listed below.

A great many workers assumed the complex to be a simple adsorption phenomenon because of the close analogy to other blue adsorption compounds of iodine (1,3). Meyer pointed out that not only starch but polyvinyl alcohol, cellulose swollen with zinc chloride and certain colloidal suspensions or finely divided precipitates show color reactions with iodine. Meyer does not commit himself as to whether the complex formed is an adsorption compound or a chemical compound, but states that such terms become meaningless in the light of our present knowledge of forces of attraction and the formation of compounds. He believes that secondary valences are responsible for the binding of iodine to micellar aggregates of starch (57).

Jackson and Hudson prepared, by treating starch with periodic acid, an oxidized derivative of starch which was devoid of iodine coloration in spite of being highly colloidal in nature (44). In this product the individual pyranose rings were opened as a result of the formation of aldehydic groups in positions 2 and 3, but little fragmentation of the chains

themselves appeared to have occurred. These observations suggested that the coloring reaction depended upon residual affinities associated with the pyranose rings of the chain structure. This view was supported by the observation of Bergman (10) that certain cyclo-acetals formed blue-black iodine compounds, suggesting the oxygen bridge as an integral part of the coloring group. The fact that fully methylated starch derivatives retained the property of iodine coloration (indicating that the hydroxyl groups were not involved) also supported this view. Barger (3) demonstrated, however, that a bridge oxygen atom was not enough to insure adsorption of iodine, there being a great divergence of behavior among closely allied compounds.

Hanes summarized the available data on the relationship of chain length to iodine color (40). Fragments of chain length up to 6 units do not give color with iodine. From 7 to 12 units are required to give a brown to red color. At the time the paper was written there seemed to be little correlation between molecular size and iodine color inasmuch as waxy maize gave a red violet color with iodine, but had a molecular weight comparable with starches which gave a blue color with iodine. He then proposed two mechanisms for the complex formation: (a) The coloring groups might be considered to be uniform in character, the absorption properties of the iodine compounds being altered in some way by the

organization of the chains into molecular associations; and (b) two or more coloring groups might be present, of which one type at least is peculiar to molecular associations. Either theory could explain a change in iodine color as the starch was degraded.

Freudenberg (32) believes the blue color of the complex due to iodine molecules dissolved in the hydrocarbon interior of starch in a helical configuration, just as iodine in other non-polar solvents produces a somewhat blue color.

Other theories present in the literature are easily proven untenable. The hypothesis that iodine forms a colloidal solution protected by starch is erroneous, because in the presence of cholic acid, the color appears at the time the acid separates from solution as a colloid, and the protective power should have reached a maximum when the acid was in true solution. The coloration of starch cannot be regarded as a protection phenomenon (1).

IV. PHYSICAL STUDIES OF THE STARCH-IODINE COMPLEX

The wide range of results obtained from chemical analyses of the complex has led a number of investigators to attempt physical or physico-chemical methods to obtain additional information. Osmotic pressure measurements were attempted with little success (77). By this method the molecular weight of the complex varied from approximately 37,000 to 40,000 while that of the starch alone was about 33,000. Friedenthal found that the addition of iodine to starch solutions caused no marked variation in the freezing point, whereas addition of soluble starch to a strong iodine-potassium iodide solution considerably lowered the freezing point (33). Katayama was among the first of the investigators to make a colorimetric study of the starch-iodine complex (45). He varied the concentrations of starch, iodine and iodide but came to no definite conclusions. A more recent colorimetric study has been carried out by Muller and McKenna who examined the starch-iodine system by a photoelectric method and found that beyond a preliminary dissociation stage, Beer's law was obeyed. They suggested the use of the Duboscq type colorimeter in the analyses. Field employed a silver-bismuth thermocouple to measure the intensity of light passing through a starch-iodine solution while changing the concentration of iodine (27). These "radiomicrometric titrations"

indicated a wide variation in the amount of iodine which would combine with native starch solutions. However, since cold water-soluble fractions of ground corn and wheat starches showed approximately the same combining capacity with iodine, Field concluded that the starch-iodine reaction was not specific for different types of starches.

Dhar, Murray and others have made electrical studies of the starch-iodine complex, measuring the conductivity and the iodine, iodide, tri-iodide concentrations by means of the iodine electrode. Interpretation of their results has been doubtful because of the heterogeneity of the starch samples used and variation of conditions.

Recently Bates, French and Rundle have developed a method, using the iodine electrode, for determining the amount of amylose in a starch sample (4). The method indicates without a doubt that amylose is a distinct component, sharply different from the amylopectin fraction of the starch. In their method the starch was thoroughly dispersed in dilute standard alkali and then neutralized with hydriodic acid. The starch was then titrated with dilute standard iodine in potassium iodide of the same concentration as the iodide in the starch solution. The authors found that the iodine-iodide potential remained constant as long as there was any free amylose in the solution. With amylopectin there was a continual rise in the iodine-iodide potential. A great many

properties of both the starch and the starch-iodine complex can be determined by this method. A few of these are listed:

(a) The purity of amylose and amylopectin samples can be determined.

(b) Amyloses from various starches take up iodine at different characteristic potentials. This fact has been attributed to variations in chain length.

(c) The percent amylose in native starches can be determined by the method. No amylose was found in the waxy starches; 17 to 24 percent in the commonly occurring starches; and the high value of 34 percent in lily bulb starch.

(d) The amount of iodine taken up by amylose in the complex formation can be determined. Amylose was found to take up 18.7 percent iodine at the end-point in 0.05 N potassium iodide solution.

The literature has very few papers containing information regarding the spectral distribution of light absorption involved in the starch-iodine complex; particularly are lacking sufficient data for precise definitions of the terms blue, violet, red and brown, so often used in describing iodine colors.

In 1939, Simerl and Browning confirmed by a spectrophotometric study of the starch-iodine complex in the visible region that different starches and starch fractions gave

absorption spectra which differed considerably from one to another (86). They recognized two components of starch (called alpha- and beta-amylose) and reported a difference in their absorption spectra although suitable methods of fractionation at that time were not available. They also found that oxidation and hydrolysis led to changes in the absorption spectrum; the breakdown of starch to dextrins resulted in almost complete disappearance to the characteristic absorption band in the longer visible wave lengths. Modification of starch by acid oxidizing agents caused the intensity of the starch-iodine color to decrease. Other observations made by Simerl and Browning are significant. With an iodine concentration of 0.1 gm. per liter and a potassium iodide ratio of 1.5 to 1 by weight, the starch concentration does not affect the position of the maximum absorption; neither does small changes in the potassium iodide concentration. They also confirmed the fact that a deviation from Beer's law exists at certain concentrations of iodine, but at higher concentrations and by the use of suitable filters, the logarithm of the transmission becomes proportional to the starch concentration. Using different types of starches, they found by varying the starch concentration that each starch has its own transmission curve having a slope which is characteristic of the starch type.

Das Gupta followed the action of methyl-alcoholic sulfuric acid on starches by determining the colors given by the products of the reaction with iodine by means of the Lovibond tintometer (22). He found that the blue component of the color decreased, the yellow remained unchanged and the red first increased to a maximum and then decreased. Except for this, little quantitative data are available with regard to the effects of modification by heat or by acids on the color or absorption spectrum of the starch-iodine complex.

Lampitt, Fuller and Goldenberg made an extensive spectrophotometric study of the starch-iodine complex with special emphasis upon the effect of grinding the starch before forming the iodine complex (52). In their interpretation of results they assumed a laminar structure of starch as advanced by Hirst and co-workers (6,41,42). In this structure starch consisted of units of 24 to 30 anhydroglucose units joined together by "lateral linkages", these "lateral linkages" being rather easily broken. Their investigation showed that a decrease in molecular weight of the starch fractions was accompanied by a decrease in absorption at the longer wave lengths only.

By comparison of the absorption spectrum of the iodine complex of ground wheat starch with that produced by amylose action, they concluded that the depolymerization of wheat

starch during grinding in the ball mill occurred only by breakage of the "lateral linkages" joining the repeating units of 24 to 30 anhydroglucose units to one another. Lampitt also made a comparative study of the absorption spectra of iodine solutions in potassium iodide and in starch over the region from 2000 A to 8000 A. It is significant that, with an excess of starch present, iodine in the starch-iodine complex is a much better light absorber than iodine of the same concentration in potassium iodide alone. The extinction coefficient of the starch-iodine complex at its maximum absorption is from 4 to 5 times as great as the extinction coefficient of the iodine in potassium iodide solution at its maximum.

V. DEGRADATION AND SYNTHESIS OF STARCH BY ENZYMES

Degradation of Starch

In recent years many workers have used the iodine color to follow changes in the starch brought about by the action of enzymes on starch. Unfortunately the colors obtained were either not measured quantitatively or were measured by empirical and rather unsatisfactory methods. The results, however, showed that differences exist especially in the actions of amylases on starch. Blom, Bak and Braae (11) suggested the use of iodine-color along with viscosity and reducing power to determine whether a given amylase preparation contained one, two or three differently acting enzymes.

By spectrophotometric studies Hanes and Cattle found that beta-amylase, which splits off maltose units from the non-aldehydic end of the starch molecule, affected the absorption spectrum of the starch-iodine complex differently from alpha-amylase, which first "disaggregates" the starch molecule into low molecular weight dextrins. By a combination of reducing power determinations and spectrophotometric measurements, they were able to differentiate not only between alpha and beta-amylase but also between alpha-amylase from different sources. Hanes and Cattle concluded that alpha-amylase resulted in a degradation product whose iodine complex showed

a shift in the absorption maximum toward the lower wave lengths, accompanied by a decrease in the extinction coefficients over the entire wave lengths as the degradation proceeded. The iodine complex of the degradation products from the action of beta-amylase on starch showed a uniform decrease in extinction coefficients, but the absorption spectrum retained a general similarity to that of the original starch (40).

Synthesis of Starch

Almost simultaneously, several investigators found that the reaction

$$\text{Starch} + H_3PO_4 \xrightleftharpoons{\text{phosphorylase}} \text{glucose-1-phosphate}$$

was reversible. Schaffner and Specht (85) first reported the reversibility of the reaction with phosphorylase from yeast. Kiessling also found phosphorylase in yeast and, in a more detailed study, showed the reaction reversible (47,48). Cori and his co-workers obtained an active phosphorylase first from muscle of well-fed rabbits and then obtained a slightly different type of phosphorylase-- apparently similar to that obtained from yeast-- from liver (20,21). Ostern also isolated phosphorylase from liver, and with it prepared a synthetic polysaccharide similar to glycogen (68,69).

Hanes found phosphorylase to be quite abundant in several types of plants, notably, pea seeds and potatoes (38,39).

One of the essential differences in the nature of the synthetic polysaccharides prepared from glucose-1-phosphate and phosphorylase from these various sources is the way they react with iodine. The synthetic polysaccharide prepared from muscle, potato and peas stains a deep blue with iodine while that prepared from liver and yeast stain brown. Since the iodine complex bears a definite relation to the structure and configuration of starch it would seem worth while to study more quantitatively the complex at various stages of the synthesis and degradation of starch by phosphorylase.

From the changes produced in the starch-iodine absorption spectrum during degradation of starch by enzymes and the similarity of these changes to those produced by acid hydrolysis of starch, it appears quite evident that there is a definite relationship between chain length of starch and iodine color. It is also evident that polysaccharides with a high degree of branching produce a brown to red-violet complex with iodine. Methylation studies on glycogen and waxy starches indicate them to be highly branched; they all form brown to red-violet complexes with iodine.

Hanes (39) observed that potato phosphorylase, during a short induction period, produced some red-staining polysaccharide if considerable maltose was present in the digest with glucose-1-phosphate. Thus it becomes questionable

whether or not synthetic starch contains branched chains or straight chains or both.

In the preparation of phosphorylase various other enzymes are encountered and must be removed. The commonly occurring ones are here listed along with their activities:

(a) Alpha-amylase: A dextrinogenic enzyme which degrades starch to dextrins of 6-7 glucose units. It rapidly destroys the iodine coloration properties of starch.

(b) Beta-amylase: A saccharogenic enzyme which degrades natural starches to maltose and a residual dextrin. During the process the iodine color changes from a blue to a red.

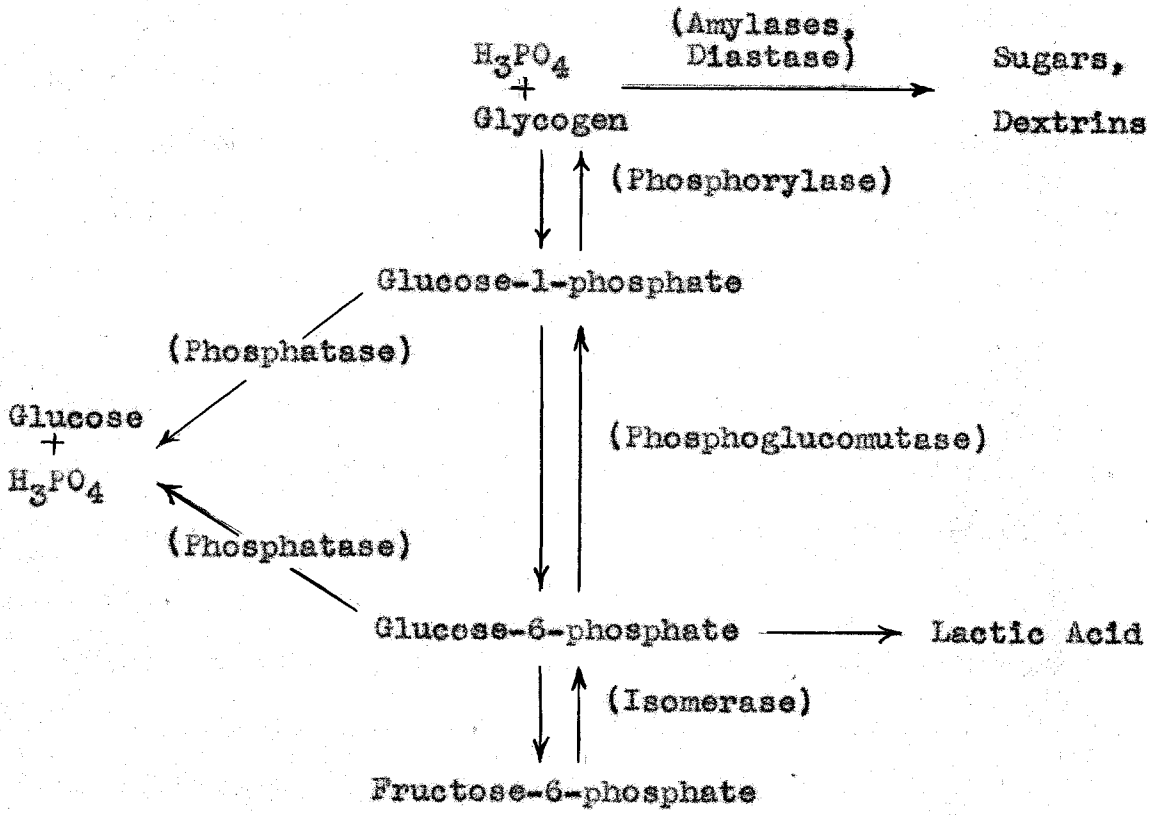
(c) Phosphatase: An enzyme which splits phosphate from organic phosphate esters. It interferes by removing phosphate from glucose-1-phosphate.

(d) Phosphoglucomutase: An enzyme which converts glucose-1-phosphate to glucose-6-phosphate. The 6-phosphate will not synthesize starch.

(e) Diastase: An enzyme which converts glycogen and starch to dextrins, non-fermentable reducing substances and maltose.

(f) Isomerase: An enzyme which converts glucose-6-phosphate to fructose-6-phosphate.

The enzyme actions may be represented as follows:



Although the equilibria involved in these enzyme reactions are somewhat affected by pH, their values in neutral or near-neutral solutions may be represented by the following equations:

Starch + Phosphate 77% $\rightleftharpoons$ Glucose-1-phosphate 23%

Glucose-1-phosphate 6% $\rightleftharpoons$ Glucose-6-phosphate 94%

Glucose-6-phosphate 85% $\rightleftharpoons$ Fructose-6-phosphate 15%

In the study of synthetic starches, Cori and his co-workers found that a purified phosphorylase solution required a small amount of adenylic acid as a coenzyme (17,18, 19); on the other hand, Kiessling declared that adenylic acid was not necessary inasmuch as enzyme preparations freed of adenylic acid by dialysis were active. Cori believed that dialysis was incomplete. This controversy between Cori and Kiessling has led certain investigators to believe that the enzyme system might be more complex than originally pictured, and that enzymes from different sources might vary in composition as well as in catalytic action. On this assumption Meyer tested the activity of phosphorylases from various sources on different starch types. Although he carried out very few experiments, his results seem to indicate the existence of at least two phosphorylases, one capable of breaking a 1:4-glucosidic linkage and another capable of breaking a 1:6-glucosidic linkage (58,60).

Meyer first pointed out that the enzyme beta-amylase degrades starch by splitting off maltose units until a branch point is reached. The enzyme action then ceases, leaving a limit dextrin with a high percentage of 1:6-glucosidic linkages, the linkages involved in branching, and no branches over 2 or 3 glucose units in length. Meyer prepared the limit dextrin from corn starch and found that phosphorylase from yeast would attack such a limit dextrin producing

glucose-1-phosphate. Phosphorylase from potatoes had no effect on the limit dextrin. He also showed that after yeast phosphorylase had loosened the 1:6-glucosidic linkages in the limit dextrin, beta-amylase would further degrade the dextrin, producing more maltose.

It is of particular interest that yeast phosphorylase when allowed to act upon glucose-1-phosphate produces a high molecular weight polysaccharide which stains brown with iodine. Thus iodine color can be used as an index of chain length (short-chain dextrins produce a brown color) or degree of branching, which ultimately involves the length of the branches.

VI. EXPERIMENTAL AND DISCUSSION

Preparation of Materials

Phosphorylases.

For these experiments, phosphorylase was first prepared either from rabbit muscle or from potatoes in order to synthesize a blue-staining polysaccharide, and second from yeast to synthesize a brown staining polysaccharide. With some modifications, the phosphorylase from rabbit muscle was prepared according to the methods of Cori (16). The perfusion of the muscles, as suggested by Cori for the purpose of getting rid of traces of diastase, could be omitted. Diastase, if present, would interfere in the process by converting any synthetic starch formed by phosphorylase to dextrins, reducing sugars and maltose. Rabbit muscle, however, contained so little diastase that it did not affect the process of synthesis to any extent. Muscles from the hind limbs of a freshly-killed rabbit were first chilled, then passed through a meat grinder and triturated with an equal volume of ice water. The extraction of phosphorylase required two hours at zero degrees after which time the residue was centrifuged and again extracted with cold water. Dialysis of the combined extracts at this stage caused a loss of activity due to loss of adenylic acid, the coenzyme

apparently necessary for activity of rabbit phosphorylase. To the combined centrifugates from the cold water extraction, 0.5 volume of C_γ aluminum hydroxide was added with stirring and then centrifuged. The adsorbate was washed with water in the centrifuge tube and the residue eluted with 0.5 volume of 0.25 molar sodium glycerophosphate. Addition of solid ammonium sulfate to the elutions to 0.3 saturation gave a flocculent precipitate upon standing overnight in the ice box. This precipitate, when separated by centrifugation and dissolved in 0.5 percent potassium chloride, made an enzyme solution sufficiently pure-- free of phosphatase and phosphoglucomutase-- to prepare synthetic starch.

Phosphorylase from potatoes was prepared according to the method of Hanes (39). Although Hanes suggested the use of small King Edward potatoes, almost any variety of potatoes, new or old, could be used for the preparation of an active phosphorylase. Old potatoes seem to have slightly less phosphorylase activity, and preparations made from them lose their activity more rapidly. One kilogram of potatoes (best results obtained using peeled potatoes) were passed through a meat grinder with an equivalent amount of ice. From this stage on the materials must be kept at zero degrees and saturated with toluene to prevent discoloration, an indication of loss of activity. The ground mass was pounded to a pulp in a large mortar and the juice pressed through canvas in a

screw press. Any starch present settled out rapidly and could be separated by decantation. Addition of solid ammonium sulfate (in place of the saturated solution used by Hanes) to 0.5 saturation brought down a voluminous precipitate which was filtered off by gravity in an ice box and dissolved in an amount of ice water equivalent to one-fourth of the volume of the original press juice. To this solution, solid ammonium sulfate was added until 0.25 saturated. The precipitate which formed at this concentration contained most of the interfering enzymes and very little phosphorylase; the liquid, separated by filtration, contained most of the phosphorylase activity. Further purification was brought about by repeated fractionations with ammonium sulfate, discarding the protein material which precipitated at 0.25 saturation and keeping the fraction which then precipitated by increasing the concentration to 0.5 saturation. The enzyme solution best retained its activity in a 1 percent ammonium sulfate solution.

Kiessling determined suitable conditions for the preparation of phosphorylase from yeast (48). A Lebedeff maceration juice, prepared by adding 33 grams of dried brewer's yeast to 100 milliliters of water and allowing to stand for three hours at 37 degrees, was adjusted to a pH of 7.8 by the addition of about 7 milliliters of 2 N sodium

hydroxide. The precipitated proteins were removed by gravity filtration in an ice box (the process required several hours). The pH of the filtrate must be adjusted to 6.5 before precipitation of the phosphorylase fraction by the addition of solid ammonium sulfate (or a saturated solution of ammonium sulfate) to 0.5 saturation. Further purification was brought about by fractional precipitations, retaining only the material which precipitated in the concentration range of from 0.2 to 0.4 saturation with ammonium sulfate. The enzyme from yeast was best preserved by suspending it in a 0.3 saturated solution of ammonium sulfate. Because of the multitude of enzymes present in yeast juice, great precautions must be taken in the control of pH and salt concentration in order to prepare an active phosphorylase free of other enzymes.

Glucose-1-Phosphate.

The method devised by Cori for the preparation of synthetic glucose-1-phosphate was used to obtain this material in a pure state (16). The reactions involved are as follows: Glucose $\longrightarrow$ Glucose pentaacetate $\xrightarrow{HBr}$ Acetobromoglucose $\xrightarrow{Ag_3PO_4}$ Tri-(tetraacetyl glucose-1)-phosphoric acid $\xrightarrow{HCl}$ Glucose-1-phosphoric acid $\xrightarrow{Ba(OH)_2}$ Barium salt of glucose-1-phosphate.

The 1-phosphate has been prepared from the action of phosphorylase on starch according to the method of Hanes (38), but it was rather difficult to isolate a pure product by this procedure.

Adenylic Acid.

The coenzyme adenylic acid was required in the synthesis of starch only when highly purified and dialyzed enzyme solutions from muscle were used.

Adenylic acid suitable for this work was prepared from rabbit muscle by a procedure somewhat similar to Ostern's (68); however, the acid was precipitated only once as the lead salt, decomposed with hydrogen sulfide, filtered and the filtrate concentrated in a vacuum to remove excess hydrogen sulfide.

Synthetic Starch.

The digests used in the preparation of synthetic starches varied somewhat in accordance with the purity of the enzyme solution. The following was typical of quantities used with muscle phosphorylase:

5 ml. purified enzyme
100 mg. glucose-1-phosphate
2.5 mg. adenylic acid
5 mg. glycogen or soluble starch

The synthetic Ba-salt of glucose-1-phosphate was always converted to the Na-salt by addition of sodium sulfate. In using potato phosphorylase, the adenylic acid could be omitted. Digests of yeast phosphorylase worked better upon the addition of 5 milliliters of a buffer solution prepared

as follows:

2 ml. 0.12% $MnSO_4$

4 ml. 5% $NaHCO_3$

20 ml. water saturated with CO_2

When the reaction was followed in the reverse direction, $starch + H_3PO_4 \xrightarrow{phosphorylase}$ glucose-1-phosphate, the digest approximated the following:

5 ml. 1% starch paste

5 ml. enzyme solution

1 ml. 0.3 M Na_2HPO_4, adjusted to a pH of 7 with HCl

This reaction was usually followed by analyzing for inorganic phosphate colorimetrically by the method of Fiske and Subbarow (29), using either a Duboscq colorimeter or a Coleman spectrophotometer.

The synthetic starch could be isolated by a variety of methods. That prepared from muscle phosphorylase was highly retrograded and could be obtained in fair purity by merely centrifuging. It was further purified by repeated solution in sodium hydroxide and precipitation with alcohol.

The synthetic product from potato phosphorylase was best isolated by precipitation with an excess of iodine solution. The iodine was removed from the precipitated complex by boiling and washing with the organic solvents, benzene and alcohol.

Schardinger Dextrin.

The Schardinger dextrin, cyclohexaamylose, used in this investigation was prepared by Dexter French.

Glycogen.

The glycogen used was the C.P. grade from the Pfanstiehl Chemical Company.

Waxy Maize Starch.

Waxy maize starch was milled from Iowa Waxy 939 hybrid corn by B. E. Starr.

Amylose, Amylopectin Fractions.

Amylose and amylopectin fractions from corn, potato and tapioca starch were obtained from T. J. Schoch who prepared them by means of the butanol precipitation method (84). F. L. Bates prepared the butanol precipitated fractions from lily bulb starch following the procedure of Schoch. In all cases the amylopectin fractions were freed from traces of amylose by adsorption on cotton.

The crystalline amylose was prepared by R. W. Kerr and has been described by him (46).

Amylodextrin Fractions.

Dextrins resulting from the action of strong acids on granular starch in the cold contain practically no branched chains. Apparently the branches are preferentially hydrolyzed away. Such dextrins have been prepared by French and subjected to butanol fractionation by Bates.

Limit Dextrins.

Limit dextrins from corn and waxy maize starch have been prepared and described by C. G. Caldwell (13). The products were precipitated with 60 percent alcohol after beta-amylase digestion.

Absorption Spectrum of the Iodine Complex of Cyclohexaamylose

The iodine addition product of cyclohexaamylose is of great interest because of its close analogy to the starch-iodine complex. This dextrin is a cylic polymer consisting of six glucose residues joined by 1:4-glucosidic linkages. The solution of the cyclic dextrin is only colored a darker brown by the addition of iodine. If, however, the water is allowed to evaporate from a concentrated dextrin-iodine solution, blue hexagonal plates are formed. Although these plates are microscopic in size, they may be grown large enough to completely fill the field of a microscope with a 20X objective and 10X ocular. D. French (31) made a thorough study of these crystals. From their physical properties and X-ray patterns, he concluded that the iodine molecules were enclosed by the cyclohexaamylose, as in the Freudenberg model, and each iodine atom lay along the axis of the cyclic compound. A Patterson projection was in agreement with the proposed arrangement. Thus if the absorption spectrum of

such a known compound could be shown similar to the absorption spectrum of starch-iodine, it should afford further evidence for a corresponding configuration of starch in its complex with iodine.

A microspectrograph had proven satisfactory for obtaining absorption spectra of microscopic amounts of colored plant products (66); a similar set-up was used for obtaining the absorption spectrum of the crystalline dextrin-iodine complex. A Leitz microscope was used with a direct-vision Leitz microspectroscope attached to the ocular. The lens and shutter system attached to the spectroscope were from a 35 millimeter Leica camera for taking photomicrographs. Since it was found that the microspectroscope was overcorrected, a tubular plate holder was made and attached in place of the ordinary roll film container of the Leica camera. The plate holder consisted of three concentric and tightly-fitting tubes, the inner two of which were open along the lower side to allow light to pass through. The innermost cylinder which held the plate could be rotated and set with a set screw to the middle cylinder when all portions of the spectrum were in focus. Thus clamped together, the two inner cylinders could be moved forwards or backwards to allow a total of 14 spectra to be photographed on the same plate. Spectrographic plates, 4½ by 6 centimeters, from the Eastman Kodak Company were used. A mercury arc served as a reference; the principal lines

being photographed at two or more places on every plate. The spectra obtained were 3 millimeters in width, and a distance of about 17 millimeters separated the yellow (5790.7 A) and violet (4046.7 A) mercury lines.

Intensities were measured by a specially constructed photometer. The plates were held by a stage micrometer and could be accurately moved in two directions. The light source consisted of a straight-wire filament Mazda 3 volt bulb operated by a storage battery. An image of the straight-wire filament was focused on the plate with a microscope objective of 12 millimeter focal length. A selenium photocell attached to a sensitive Leeds and Northrup galvanometer was used to record intensities (in galvanometer deflection) as the plate was moved across the beam of light. To avoid stray light, the entire process was carried out in a dark room.

The poor resolution of the instrument in the vicinity of maximum absorption made it impossible to carry out the experiments on anything but a roughly qualitative basis. It has also been impossible to measure the thickness of the Schardinger dextrin-iodine crystals with any degree of accuracy. Nevertheless the complex was shown to have a broad band of absorption quite similar to that of starch-iodine with a maximum in the vicinity of 600 millimicrons.

The Dichroism of Flow of Starch-Iodine Solutions

The basis of most objections to the helical configuration for starch has been the lack of experimental evidence in favor of such a configuration. Assuming a helical model of starch-iodine as shown in Figure 1, dichroism of flow of starch-iodine solutions is to be expected. The iodine molecules in the complex are located in the center of the helix with their long axes coincident with the helix axis. A velocity gradient, such as that produced by a rotating cylinder in a larger concentric cylinder, would be expected to orient the long helical iodine-containing starch molecules preferentially along the flow lines. This would result in the preferred orientation of the iodine molecules with their long axes parallel to the flow lines. The type of dichroism to be expected from such orientation can be understood from the pleochroism of iodine crystals. According to the unpublished work of Dr. F. H. Spedding, the pleochroism of the crystals indicates that light with its electric vector parallel to the long axis of the iodine molecule is strongly absorbed, while light with its electric vector normal to the long axis of the molecule is but slightly absorbed (Figure 2). This is in agreement with existing theories, and with observations of dichroism and pleochroism in other crystals (50,51,75). Hence, light with its electric vector parallel to the long axis of the

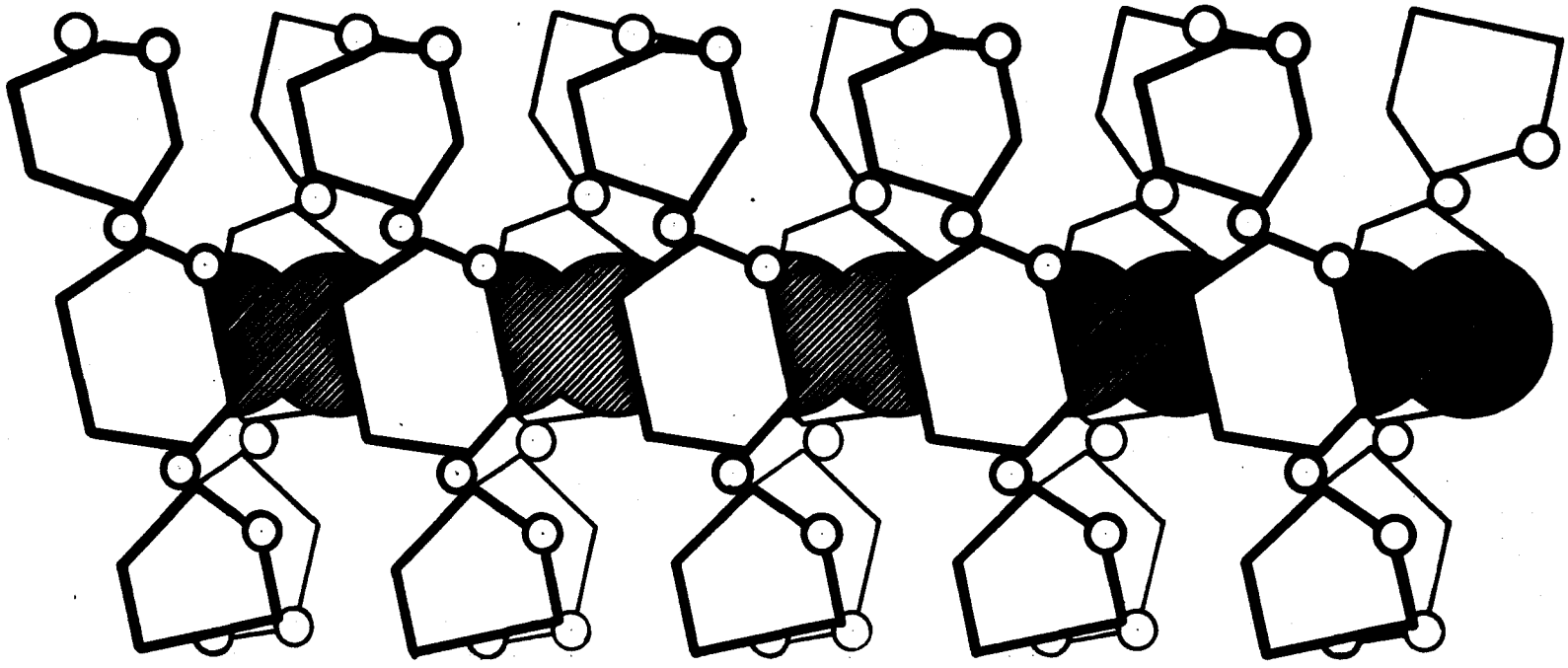
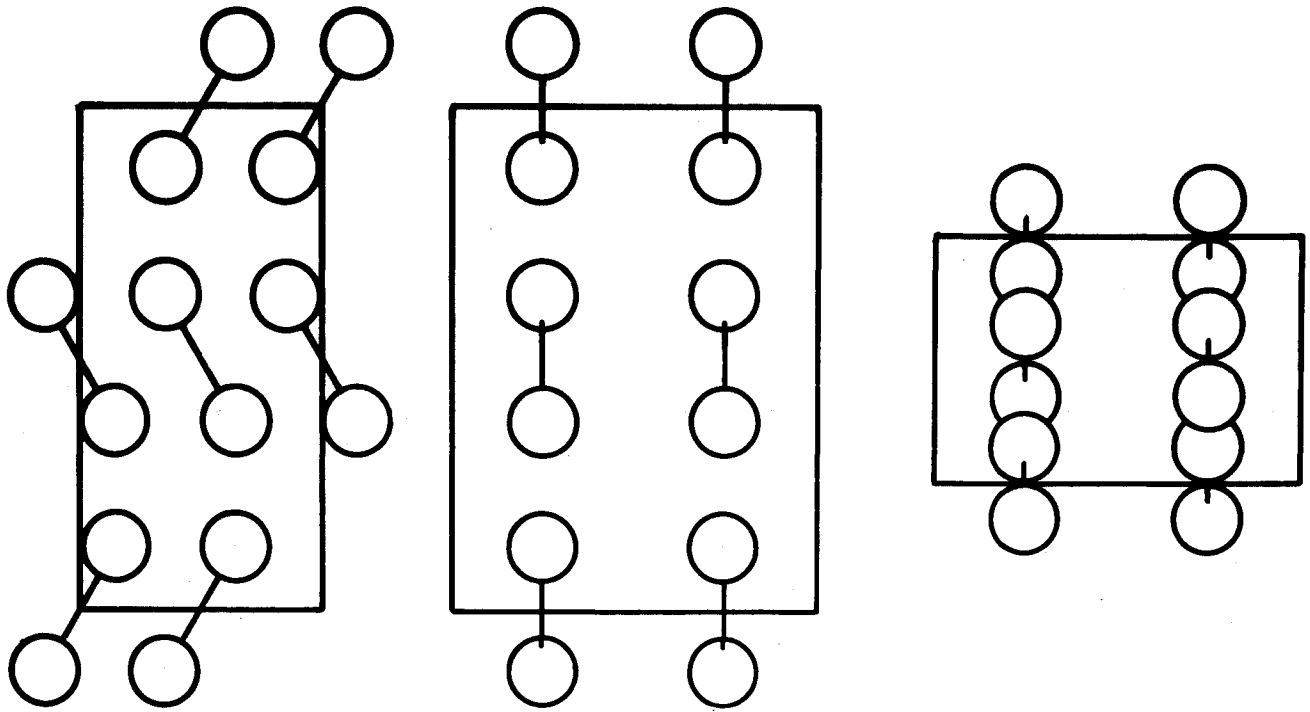


Fig. 1

Model of helical starch chain with iodine molecules in the center of the helix.



E ↑ more strongly absorbed
E → less strongly absorbed

E ↑ very strong absorption
E → very weak absorption

E ↑ rather strong absorption
E → very weak absorption

Fig. 2

Projections on (010) (100) and (001) faces of the orthorhombic iodine unit cell. Light is considered to be travelling normal to the plane of the projection. Direction of electric vector is given by arrow following E.

helical starch-iodine complex should be absorbed strongly while light with its electric vector normal to the helix axis should be but slightly absorbed. In accordance with this, starch-iodine solutions, under the influence of a velocity gradient, should absorb light with its electric vector parallel to the flow lines more strongly than light with its electric vector normal to the flow lines. Investigations of starch-iodine solutions during flow reveal that this is the case.

A qualitative observation of the dichroism of flow was made with the apparatus shown in Figure 3 in which i is the inner cylinder and o is the outer one. This concentric cylinder apparatus was similar to those described for observation of birefringence of flow (24). Although great care was taken in aligning the cylinders, the apparatus was crude in comparison with those used in more quantitative studies. The optical system was simpler, and, inasmuch as starch-iodine solutions were found to be strongly absorbing, the length of the cylinders was less than usually employed in birefringence of flow experiments.

The most satisfactory apparatus was constructed with an inner cylinder of 2.27 centimeters diameter, a distance between cylinders of 0.29 centimeters and a cylinder length of 2.20 centimeters. The excess overall length was found convenient in eliminating bubbles from the line of vision.

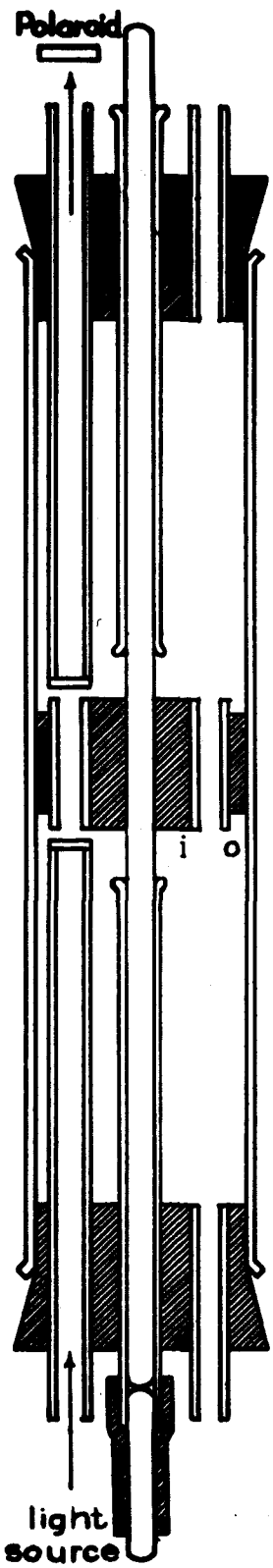


Fig. 3. Apparatus for Observation of Dichroism of Flow.

A variable speed motor with a flexible drive was used to drive the inner cylinder up to speeds of 5000 revolutions per minute. With viscous pastes stained with iodine, dichroism of flow was readily observed at 1500 revolutions per minute. Observations were made parallel to the axis of rotation through windows provided for the purpose. An intense unpolarized beam of light was directed through the solution, and the components of the emerging beam with electric vectors parallel and normal to the flow lines were examined with polaroid or Nicol prism. The difference in absorption of the two components was easily observed, but was confirmed with a selenium photocell connected to a galvanometer. For most solutions reliance was placed on visual observation which was quite sensitive if the Nicol or polaroid was rotated rapidly through 90 degrees.

Dichroism of flow was most readily observed using heavy (3 to 5 percent) potato starch pastes stained with iodine. Swollen starch granules increased the viscosity of the solutions. To be sure the dichroism was due to the dispersed starch and not the swollen granules, well dispersed starch solutions were prepared from starch fractions in which the granules no longer existed. Dilute solutions of such fractions showed dichroism of flow which was made much more easily visible by adding corn sirup or some other medium to increase the viscosity of the solution. Swollen granules

provided an extraordinarily high viscosity, and their effect on the dichroism could be attributed to that property.

In the study of starch fractions, care was taken to disperse the unbranched component thoroughly. In all cases, the unbranched fractions showed dichroism of flow while the branched fractions showed little or no dichroism. The complete results may be summarized as follows:

(a) The butanol precipitated fractions from corn and potato starches showed a noticeable dichroism of flow even in dilute solutions.

(b) For ordinary native blue-staining starches, dichroism of flow was easily observed.

(c) The amylopectin from a butanol precipitation of corn and potato starches showed a doubtful dichroism of flow. If all traces of straight-chain material were removed by passing the solutions through cotton columns, the dichroism disappeared completely.

(d) Glycogen stained with iodine showed no dichroism of flow.

(e) Iodine-stained pastes from waxy maize, glutinous rice and red staining starches in general showed little if any dichroism of flow.

A great many investigators have tried to compare the blue adsorption compounds of iodine with the starch-iodine complex. To make sure the dichroism shown by the starch pastes stained

with iodine was a unique function of the starch-iodine complex, a blue solution of the adsorption compound, alpha-naphthoflavane-iodine, made viscous by the addition of corn sirup was placed in the concentric cylinder apparatus and tested for dichroism. None was apparent. Likewise iodine solutions were tested for dichroism. Again none was apparent.

From the above evidence it is possible to explain the observed dichroism by any structure in which the long axes of the iodine molecules and the long axis of the starch-iodine complex are parallel. In place of the helical structure of Figure 1, it is possible that the starch-iodine complex consists of a linear starch chain with the long axes of the associated iodine molecules parallel to the chain. Although the dichroism of flow of starch-iodine solution is not in itself sufficient to decide between the two alternative structures, any acceptable structure must be in accord with the observed dichroism.

To decide between the two possible structures, Rundle and French (81) have investigated the optical properties of crystals of the starch-iodine complex formed from the crystalline amylose of Kerr and Severson (46). As is shown, the optical properties of helical-chain starch should be quite different from starch in the "A" or "B" modification where the chains are known to be extended. The crystalline

amylose can be stained with iodine with no apparent loss of crystalline properties and presumably, therefore, with no appreciable change in the configuration of the starch chains. Before staining the amylose platelets are uniaxial and, on edge, are quite birefringent. The retardation of light with its electric vector parallel to the surface of the platelet is greater than the retardation of light with its electric vector normal to the platelet. That is, the platelets are optically negative.

On edge, the platelets stained with iodine are extremely dichroic; light with its electric vector normal to the plate is very strongly absorbed, while light with its electric vector in the plane of the platelet is very weakly absorbed.

Any attempt to interpret the optical properties in terms of an extended chain fails. If an extended chain were adopted as a model, it would be expected that light with its electric vector parallel to the chain would be retarded more than light with its electric vector normal to the chain[1].

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1. For a discussion of polarizability see Silberstein, Phil. Mag., 33, 92, 521 (1917).

The birefringence of crystalline amylose would then indicate that the starch chains lie in the plane of the platelet while the dichroism would require that the iodine molecules be normal to the platelet, and therefore normal to the extended chain. But according to the dichroism of flow of starch-iodine solutions, the iodine molecules must be parallel to the extended chain if the extended-chain model is adopted.

On the basis of existing theories of polarizability, it would seem probable that a starch helix would show its greatest retardation of light in a direction normal to the helix axis. If so, the dichroism and birefringence of crystalline amylose would be quite understandable. The birefringence would indicate that the axes of the helices were normal to the plane of the platelet, and the dichroism would indicate that the iodine molecules have their long axes normal to the plane of the platelet. The iodine molecules then have their long axes parallel to the long axis of the starch molecule, the helix axis, in agreement with the requirements of the dichroism of flow. The helical model would also account for the uniaxial, optical negative character of the platelets. The optically negative character is hard to understand on the basis of an extended-chain model inasmuch as fibers of starch showing the "B" X-ray

diffraction pattern are optically positive just as are cellulose fibers.

Recently French and Rundle[1] have shown that the X-ray diffraction pattern of the amylose-iodine complex, formed by the treatment of amylose with iodine vapor, could be indexed using a hexagonal cell, a_o 12.97, c_o 7.91, d_{100} 11.23A. The amylose-iodine pattern showed a high symmetry of structure. A hexagonal lattice for so complicated and unsymmetrical a substance as starch would be hard to understand unless the starch chains approximated cylinders which were then packed together in closest packing.

The space-filling model of a helical starch chain built by Freudenberg (32) contained six glucose residues per helix turn. The exterior diameter of his helix was within a few tenths of an Angstrom of the 12.97 Angstroms required by the observed lattice translation in the X-ray study. The length of the turn in the helix should be equal to the width of a glucose residue which, from the model, appeared to be about 7.5 to 8.0 Angstroms. The periodicity along the amylose-iodine was found to be 7.91 Angstroms, in good agreement. The observed cell dimensions were in full accord with a helix of six glucose residues per turn.

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1. This work has just been completed and will be published in the Jour. Am. Chem. Soc.

Hydrocarbon Lining of Starch in the Helical Configuration

As has already been stated, Freudenberg has attributed the blue color of the starch-iodine complex to the iodine dissolved in the hydrocarbon lining of the starch in a helical configuration. This hypothesis was based on a qualitative comparison of the color of iodine in starch and in organic solvents. In the light of further evidence for the helical configuration, a more quantitative investigation of the iodine color in organic solvents and in starch is indicated. Absorption spectra have been taken for iodine in starch and in organic solvents and transmission curves plotted. The instrument used for obtaining the transmission data was a Coleman Model 10S double monochromator spectrophotometer with an accompanying vacuum tube electrometer (Coleman pH meter) serving as an intensity measuring device. A slit selecting a 15 millimicron band of light was used for all transmission data. The solution to be tested was placed in a square cuvette 13 millimeters in thickness and compared with a control solution placed in a matched square cuvette. For highly absorbing solutions a glass prism with optically parallel sides was placed in the cuvette to reduce the thickness from 13 millimeters to 1.63 millimeters. With the exception of certain dextrins and glycogen, all absorption and transmission curves in this investigation were

made from solutions in which the final polysaccharide concentration was 0.01 percent. The iodine concentration was 0.0001 N in most cases; the temperature was kept at 23 degrees. All absorption curves were made using the 13 millimeter cuvettes, one containing the starch-iodine solution, the control containing a concentration of iodine equal to that used in preparing the starch-iodine solution. This procedure, adopted in other spectrophotometric studies, overcorrects for the iodine not involved in the complex; but the error is slight because of the negligible absorption of a 0.0001 N iodine solution over the wave length range used. Experimental points for all transmission curves were taken at 25 millimicron intervals and curves drawn through the points plotted directly from electrometer readings. The glass prism for reducing cuvette thickness, because of its change in light transmission with wave length, proved satisfactory only in spectrophotometric titrations in which the wave length was held constant.

In all spectrophotometric work it is essential to have perfectly clear solutions. This is often difficult in the case of starch, particularly in solutions containing an appreciable amount of starch, but in very dilute solutions, 0.01 to 0.1 percent, a molecular dispersion can be closely approached if not actually attained by the following procedures. Native starches were dispersed by pouring a paste

containing the required weight of starch into boiling water. The solution was boiled for 5 minutes and then autoclaved for 1 hour at 18 pounds pressure. There are indications that long autoclaving causes a partial degradation of starch, but there is no apparent effect after one hour of autoclaving in a strictly neutral solution. Perfectly transparent solutions could be prepared in this manner from nearly all native starches if the concentration was kept at a few hundredths of a percent. It was particularly difficult to disperse the amyloses prepared by butanol precipitation. The solubility of the butanol precipitate from corn starch in boiling water or under 18 pounds steam pressure in the autoclave was found to be less than 0.01 percent. The butanol precipitate from potato starch, a substance of higher molecular weight, was considerably more soluble in boiling water. The process of solution is one of breaking up of starch crystallytes. The shorter straight chains in corn amylose are much better building blocks for crystallytes than the longer potato amylose chains; hence it is easier to disperse the potato amylose. In order to disperse the amylose samples, the amylose was first thoroughly dried in a vacuum oven at 60 degrees for 48 hours. Dried samples were found to be more readily soluble than those wet with butanol. A weighed amount of the dried amylose was treated with dilute potassium hydroxide and allowed to stand at room temperature until

the solution was completely transparent. Five milliliters of 0.5 N potassium hydroxide disperse up to 100 milligrams of amylose material within an hour. Such solutions can then be neutralized with hydriodic acid and diluted to any convenient volume. Amylose was allowed to stand several days in potassium hydroxide of this concentration to determine whether or not the material was degraded. Practically no degradation was apparent as long as the mixture was kept at room temperature. Warming or boiling for any length of time produced discoloration of the solution due to air oxidation of the starch.

When potassium hydroxide solutions of certain amylose materials are neutralized, they show a strong tendency to retrograde, and in general the shorter the straight chain, the more rapid is the retrogradation. Therefore, the solutions for spectrophotometric studies were not neutralized until ready for use.

For absorption curves the starch was usually kept in excess; so that absorption depended upon concentration of iodine. As will be shown, most starch materials stop adsorbing iodine when the ratio is 1 iodine molecule to 6 glucose residues. The iodine used was 0.001 N with respect to iodine and 0.00125 N with respect to iodide concentration. This iodine solution was prepared by dilution of 0.1 N iodine solution (which was repeatedly standardized) in calibrated

volumetric apparatus. For most of the following work, the required amount of the dilute standard iodine was added to 25 milliliters of 0.02 percent starch solutions in a 50 milliliter volumetric flask. The mixture was then diluted to volume and allowed to come to equilibrium.

The higher concentration of iodine in organic solvents required to produce a solution of absorbing power comparable to iodine in starch makes it unreasonable to compare transmission curves of the solutions. True enough the shapes of the curves are quite similar. For the purpose of comparing the relative absorbing ability of iodine in organic solvents and in starch, the curves in Figure 4 and in Figure 5 have been plotted as molecular extinction coefficients against wave length. The molecular extinction coefficient K has been defined as follows:

$$K = \frac{-\log T}{cd},$$

where T is equal to the percent transmission divided by 100, c is equal to the concentration of iodine in moles per liter and d is equal to the thickness in centimeters of the absorbing liquid.

Figure 4 shows the absorption curves of iodine in typical organic solvents. These solutions were prepared from iodine which had been sublimed twice and from organic solvents which had been carefully dried and redistilled. Particular care was taken with the alcoholic solution inasmuch as the

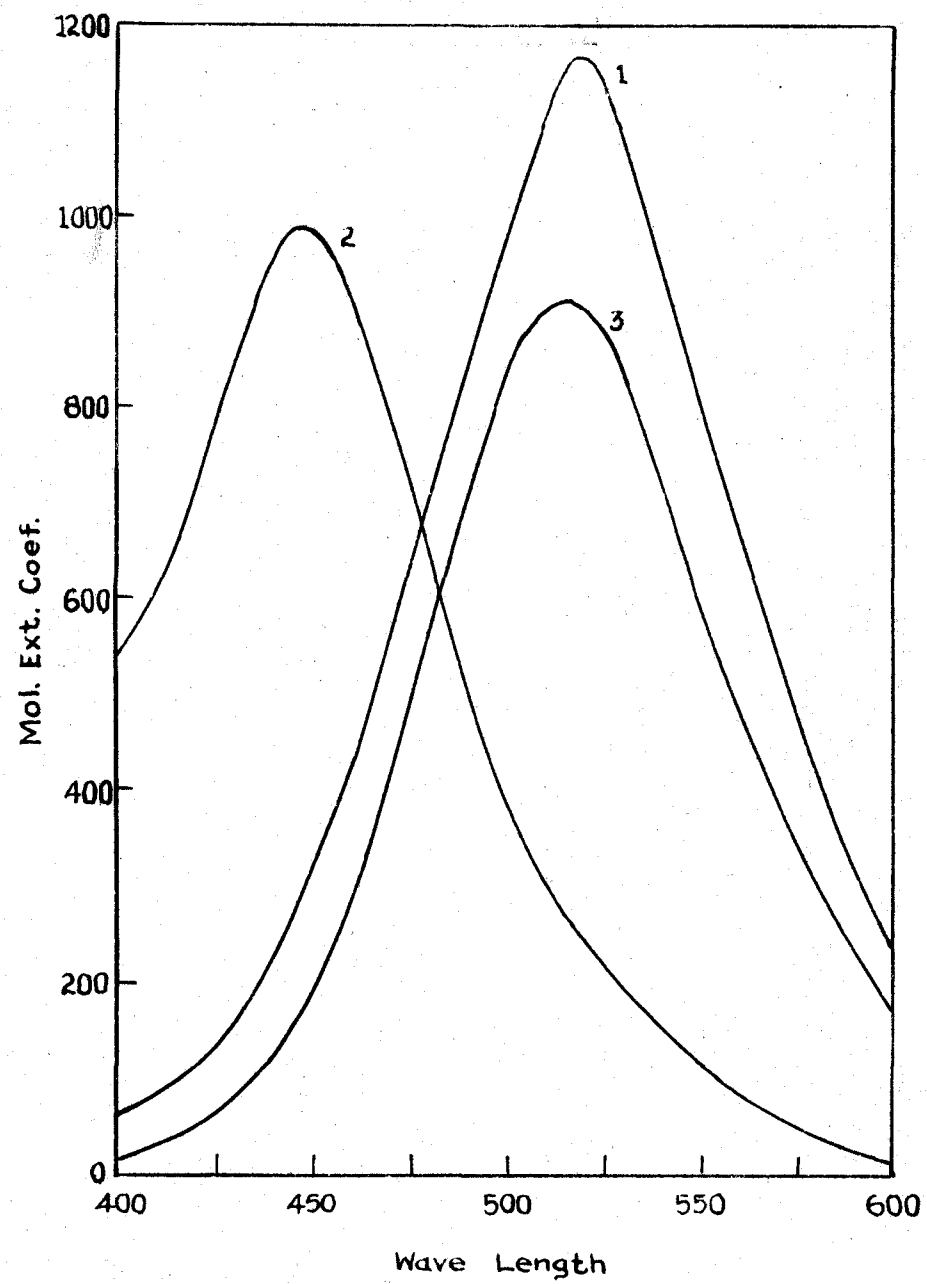

Fig. 4. Absorption Curves of 0.001 N Iodine in Organic Solvents

1, Iodine in carbon disulfide; 2, iodine in carbon tetrachloride; 3, iodine in ethyl alcohol.

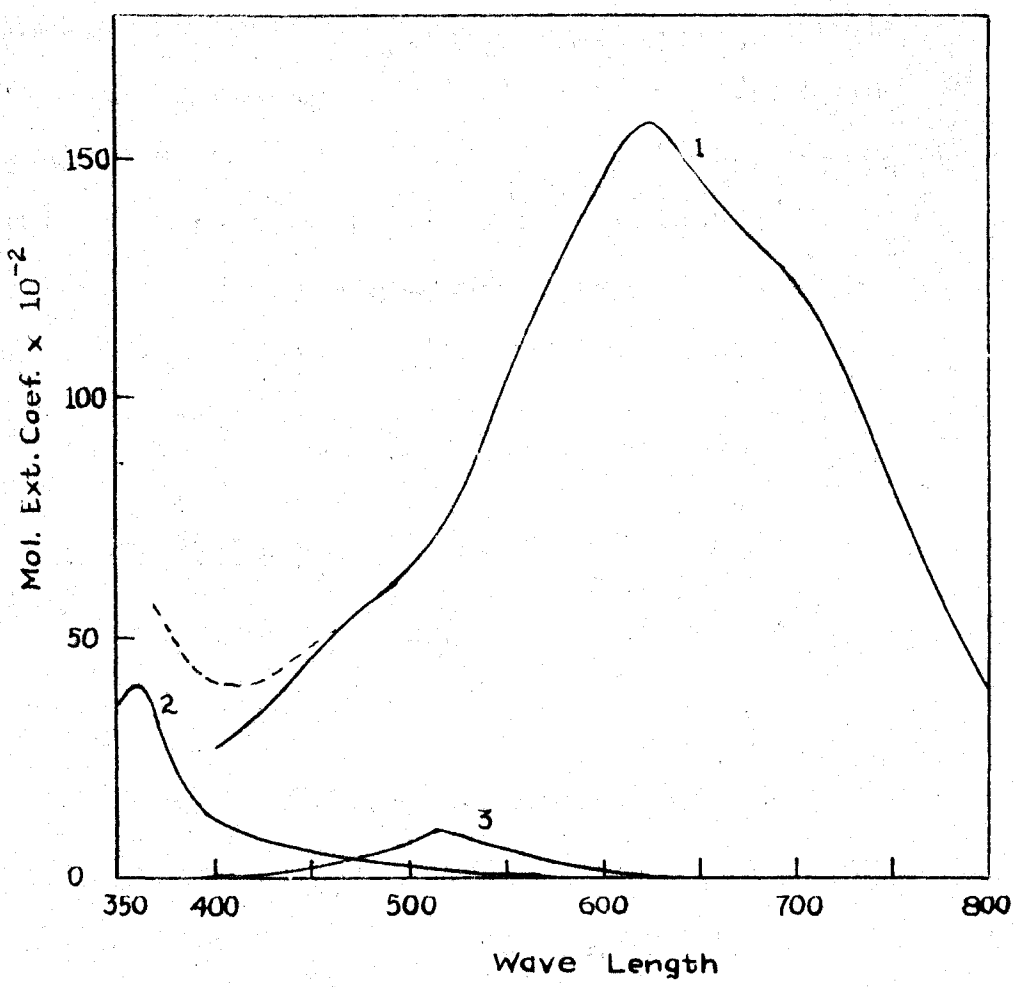

Fig. 5. Absorption Curves

1, 0.0001 N iodine in 0.01 percent potato starch; 2, 0.0002 N iodine solution; 3, 0.001 N iodine in carbon tetrachloride solution.

presence of one percent water shifted the absorption maximum from approximately 450 millimicrons to 380 millimicrons.

To avoid confusion, only three curves were plotted in Figure 4. All curves taken were in agreement with often repeated observations (92) that iodine in nonpolar solvents exhibits purple color caused by a broad absorption band with maximum at about 520 millimicrons, and that with increasing polarity of solvent this maximum is shifted progressively to shorter wave lengths. The results are tabulated in Table II along with molecular extinction coefficients.

Table II

Characteristics of Iodine Solutions

Solvent	Dipole Moment X 10^{18}	Absorption Maximum	Molecular Extinction Coefficient
CCl_4	0	520	910
CS_2	0	520	1170
C_6H_6	0	500	1100
$(C_2H_5)_2O$	1.12	456	1030
CH_3COOH	1.4	470	800
C_2H_5OH	1.70	450	995
H_2O	1.84	360	4000

The similarity in the shape of the iodine curves to the starch-iodine curves can be seen by comparing them with curve 1 of Figure 5 or the curves for synthetic starches in Figure 12 which are plotted on the same basis. Figure 5 is a comparison of iodine curves in starch, water and carbon tetrachloride. It is obvious that iodine in starch absorbs much more light than does iodine in water or organic solvents. The curves also vary in two other respects: (a) The principal absorption of starch-iodine is broader and has a maximum effect at somewhat larger wave lengths; and (b) the starch-iodines exhibit much more complex curves than the iodine solutions. There are present at least three more or less distinctly visible absorption bands: the principal one in the neighborhood of 600 millimicrons, and two lesser ones at about 450 millimicrons and slightly above 700 millimicrons. The lesser bands are particularly apparent in the blue staining starches, while they become progressively weaker as one goes through the red-staining starches to glycogen.

Getman (34), in a study of iodine solutions, showed that with increasing displacement to short wave lengths the intensity of the principal absorption band is weaker. If this were true it might be argued that in blue solutions, corresponding to the position of this absorption band shifted toward even longer wave lengths than shown in ordinary nonpolar solutions of iodine, a marked increase in

absorption might be exhibited by a given amount of iodine taken up by starch. However, Getman used a selected series of solvents to illustrate his point; there are apparently exceptions to the rule as shown in Figure 4. Iodine in alcohol shows a stronger absorption band than iodine in carbon tetrachloride. Moreover, the change in strength of absorption bands of iodine in organic solvents is insignificant in comparison to the change produced by dissolving iodine in starch. Hence it is to be expected that any mechanism to explain the formation of a blue color is more complex than one of simple solution of iodine in starch.

It is best to investigate other factors which affect the starch-iodine complex before attempting a quantitative explanation.

Effect of Iodine Concentration

Starch iodine solutions were prepared in which the starch concentration was held constant while the concentration of iodine was varied. Absorption curves were drawn for each solution from transmission data. In all cases the final starch concentration was 0.01 percent while the iodine varied from 0 to 48 x 10^{-5} equivalents per liter. With amylose fractions from any source, the absorption maximum did not change within these limits of iodine concentration. The

same was true of amylopectin fractions; there was no change in the absorption maximum. The effect of iodine concentration on absorption of light was best observed by plotting the logarithm of transmission against iodine concentration. The transmission of such solutions was taken at intervals of 4×10^{-5} equivalents of iodine between the concentration limits. All spectrophotometric titrations were made on solutions of 1.63 millimeters thickness. The light absorption measurements were made in every case at the wave length of maximum absorption. Again, the temperature was kept at 23 degrees.

In their spectrophotometric study of starch-iodine, Lampitt, Fuller and Goldenberg (52) found that an increased iodine concentration caused an increased value of extinction coefficients. If, however, the absorption is calculated as molecular extinction coefficients, the greater values are obtained with lower iodine concentrations. That is, the greater the ratio of starch to iodine, the greater is the molecular extinction coefficient.

The spectrophotometric titrations of a typical amylopectin and a typical amylose are shown in Figure 6. Iodine was taken up slowly in the case of amylopectin, with no sharp break in the titration curve. On the other hand, the amylose took up iodine rapidly during the first part

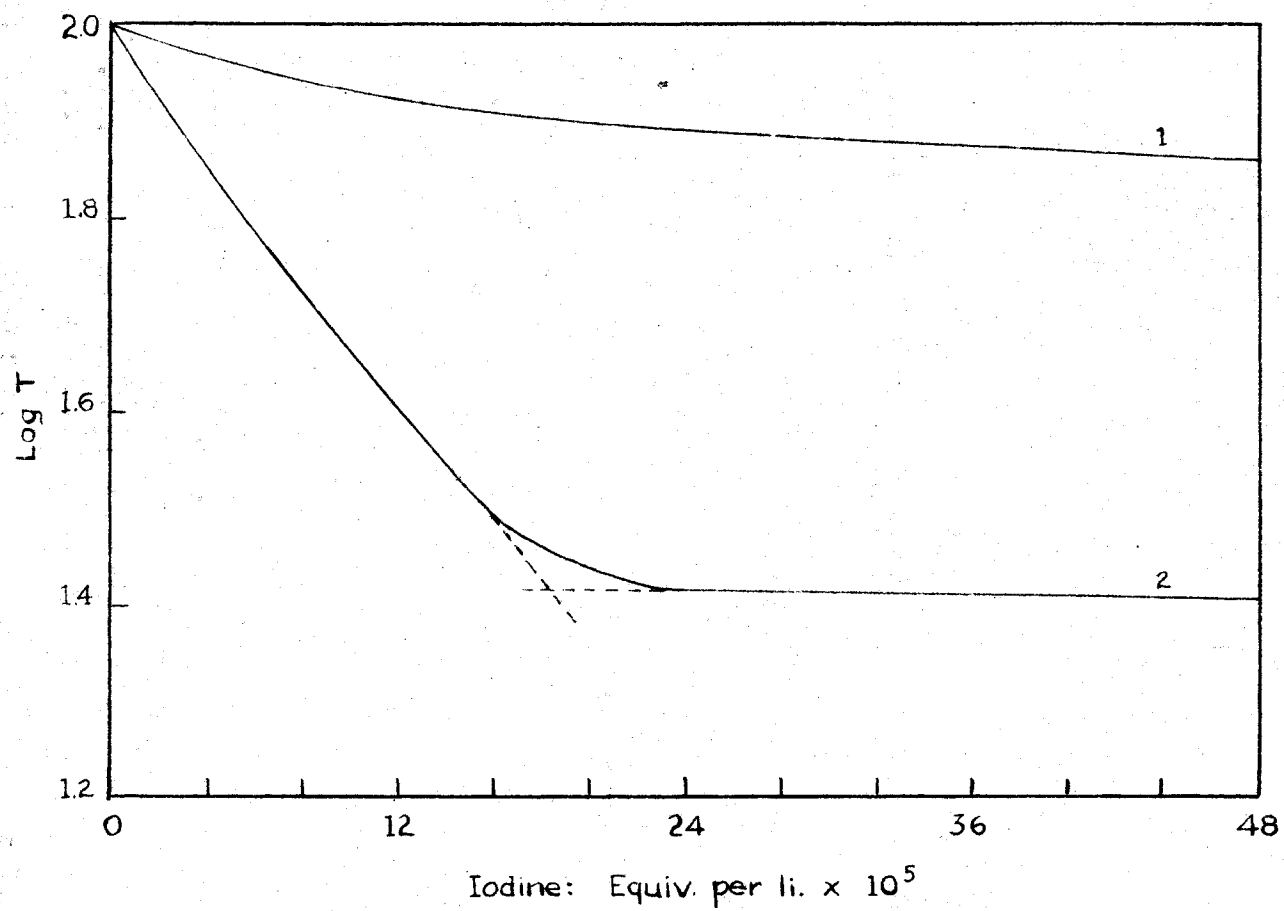

Fig. 6. Spectrophotometric Titrations of Typical Amylopectin and Amylose Materials

1, Waxy maize starch; 2, Kerr's "crystalline amylose". Points were taken at wave length of maximum absorption; 540 millimicrons for waxy maize and 605 millimicrons for the amylose. Iodide concentration was 0.005 N.

of the titration. The curve then broke rapidly indicating a rather definite amount of iodine associated with the amylose-iodine complex.

The straight line portions of the amylose-iodine curve were extrapolated (dotted lines of curve 2 in Figure 6) to find the exact end-point. This end-point corresponded to one iodine molecule for every 6.7 glucose units. Since all the titrations were run with the final starch concentration equal to 0.01 percent, the following table is included for the purpose of reading ratios of iodine to glucose residues at the break in the titration curves.

Table III

Ratio of Glucose Residues to Iodine in Titrations in which the Final Starch Concentration is 0.01 percent

Iodine: Equivalents per liter X 10^5	Glucose Residues per iodine molecule
10	12.36
12	10.29
14	8.83
15	8.25
16	7.72
17	7.27
18	6.87
19	6.51
20	6.17
21	5.88
22	5.61
24	5.14
26	4.75
28	4.41
30	4.12

The titration curves in Figure 7 show the relative absorbing ability of iodine in various amyloses as compared with iodine in carbon tetrachloride, a typical organic solvent. Curve 1 of this figure is iodine in carbon tetrachloride whose absorbing power does not approach that of the short chain amylose (curve 2), a butanol precipitate from amylodextrin whose chain length was found, from viscosity measurements and reducing values, to be about 44 glucose residues. Curves 3, 4, 5, 6 and 7 are for the amyloses from corn (Kerr's crystalline amylose), corn, lily bulb, tapioca and potato respectively. The curves were drawn as straight lines between 0 and 10^{-5} equivalents of iodine. Intermediate points were taken and always fell in the proper order; so that certainly the curves do not cross within this region, although they should bulge downward to a slight extent as shown in following curves (see Figure 20). The effect of changing iodine concentrations on the shape of the absorption curves in the region from 400 to 800 millimicrons is shown in Figure 8. Curve 1 is for 0.0001 N iodine in 0.01 percent corn starch while curve 2 is for 0.0002 N iodine in 0.01 percent starch. With the higher concentration of iodine the absorption maximum is shifted to a slight extent to the lower wave length. This is in disagreement with the observation that the absorption maximum of amylose-iodine does not change over wide variations in

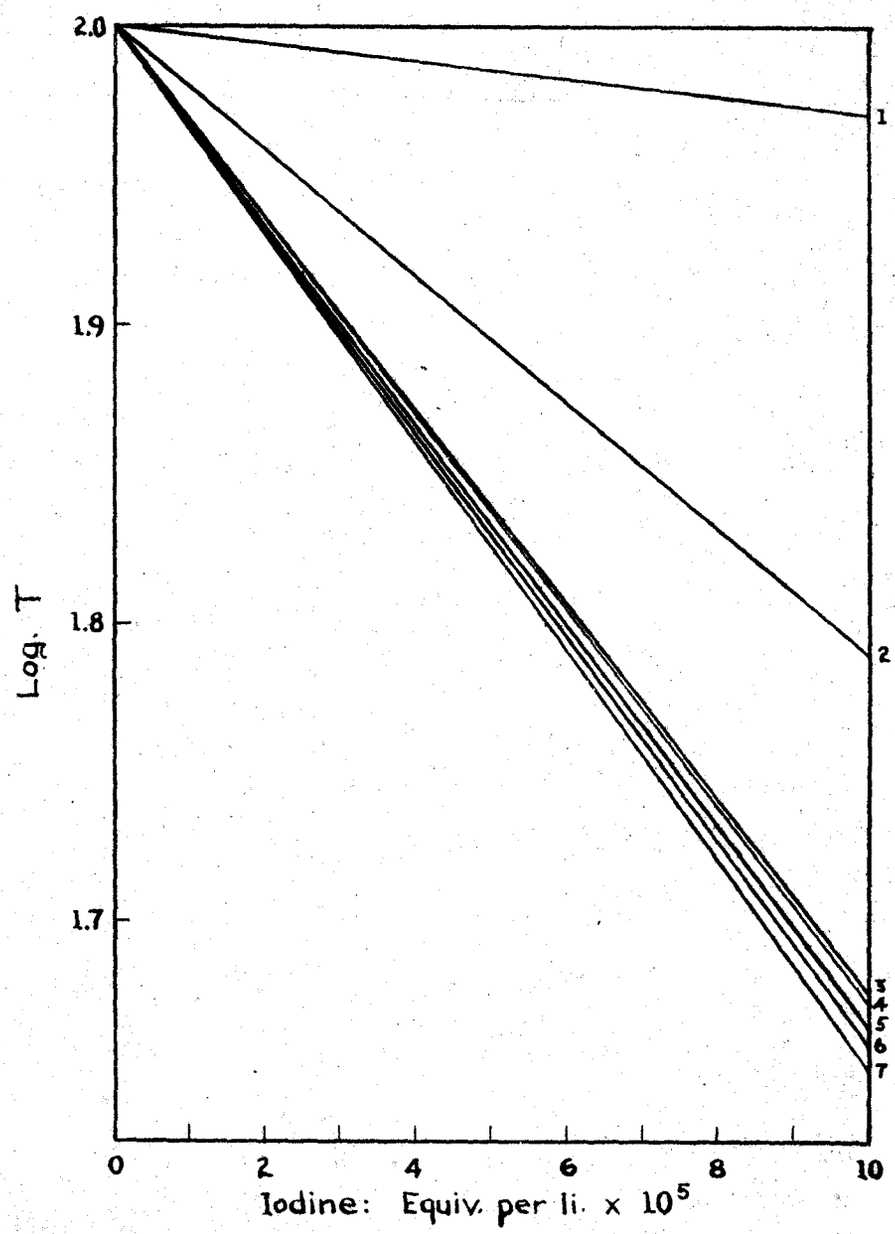

Fig. 7. Spectrophotometric Titrations of Carbon Tetrachloride and Amylose Materials

1, Carbon tetrachloride; 2, amylodextrin amylose; 3, "crystalline amylose"; 4, corn amylose; 5, lily bulb amylose; 6, tapioca amylose; 7, potato amylose.

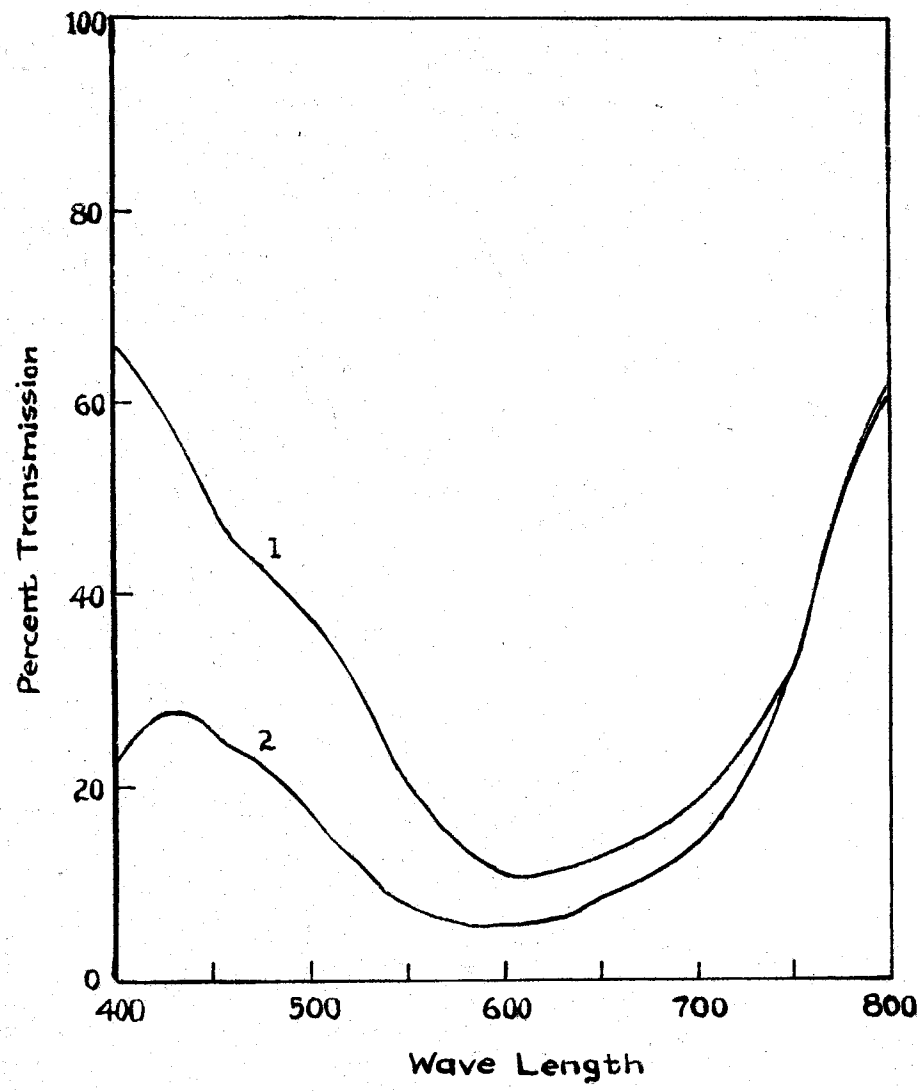

Fig. 8. Transmission Curves of 0.01 Percent Corn Starch at Different Iodine Concentrations

1, 0.001 N iodine; 2, 0.002 N iodine.

iodine concentration. However, Figure 8 merely indicated that iodine is first taken up by the amylose present in the corn starch. Afterwards the amylopectin takes up iodine. As will be shown, the amylopectin fraction shows an absorption maximum at a lower wave length than the amylose fraction. The fact that curve 2 breaks downward at 400 millimicrons indicates that part of the iodine was not taken up by the starch. Iodine itself has an absorption maximum at 360 millimicrons. An interesting effect of changing iodine concentration was observed in the titration of amylopectin materials which had not been freed of traces of amylose. The first color to appear was blue, followed by the characteristic violet color of amylopectin-iodine with increased iodine concentration. The phenomenon was not observed in amylopectin solutions passed through cotton which would adsorb the traces of amylose. This fact would indicate that amylose preferentially takes up iodine and should afford a basis for separation of the two components. Separations were attempted by precipitation of the complex with potassium fluoride at various iodine concentrations. By this method amylose could be sharply separated from glycogen, but other separations were only partially successful.

It was also observed that amylose could be fractionally precipitated by the addition of less than the calculated

amount of iodine (approximately 1 iodine molecule to 6 glucose residues). The addition of one-half this calculated amount of iodine to an amylose solution (containing potassium fluoride for complete precipitation) brought down the complex leaving the supernatant liquid clear and devoid of iodine color. Further addition of iodine caused more complex to precipitate until the amylose was completely removed, as indicated by the yellow iodine color of the supernatant liquid. This result is incompatible with the concept that the stability is due to interaction between iodine and amylose in which case the amylose helices would tend to fill up uniformly, all becoming saturated at the same time. The above result would, however, be predicted on the basis that the stability of the complex is due to interaction of iodine molecules. Obviously there is a tendency for an amylose helix to fill up completely once an iodine molecule enters that helix. Subsequent iodine molecules go into the complex more readily than the first.

Effect of Iodide Concentration

The effect of iodide ions on the starch-iodine complex has been discussed in another section of this paper. Little was concluded as to the exact nature of the role the iodide

ions play in the formation of the complex. Figure 9 shows the effect of different iodide concentrations on the spectrophotometric titrations of amylose. It is obvious that an increased iodide ion concentration causes a decrease in general light absorption. Of greater significance is the fact that the break in the titration curve occurs at a higher ratio of amylose to iodine with higher iodide concentrations. In 0.5 N iodide, the ratio (see Table III) of glucose residues to 1 iodine molecule is 8.0; in 0.05 N iodide 7.2; in 0.005 N iodide 6.7; and in 0.0025 N iodide 6.4. The crystalline amylose used in these determinations could not be dispersed without the use of potassium hydroxide; hence it was impossible to obtain results at lower iodide concentrations. Nevertheless potato amylose could be dispersed to the extent of 0.005 percent in water by autoclaving. The break in the titration curves of such solution corresponded to exactly 6 glucose residues to 1 iodine molecule. Similar results were obtained with dilute solutions of amyloses from other sources and synthetic polysaccharides. The ratio of glucose to iodine does not bear a simple mathematical relationship to the iodide concentration. In an attempt to show this, the ratios of starch to iodine were plotted against iodide concentration in order to find the iodine ratio at zero iodide concentration by extrapolation. The shape of the curve did not justify extrapolation. A log concentration of iodide

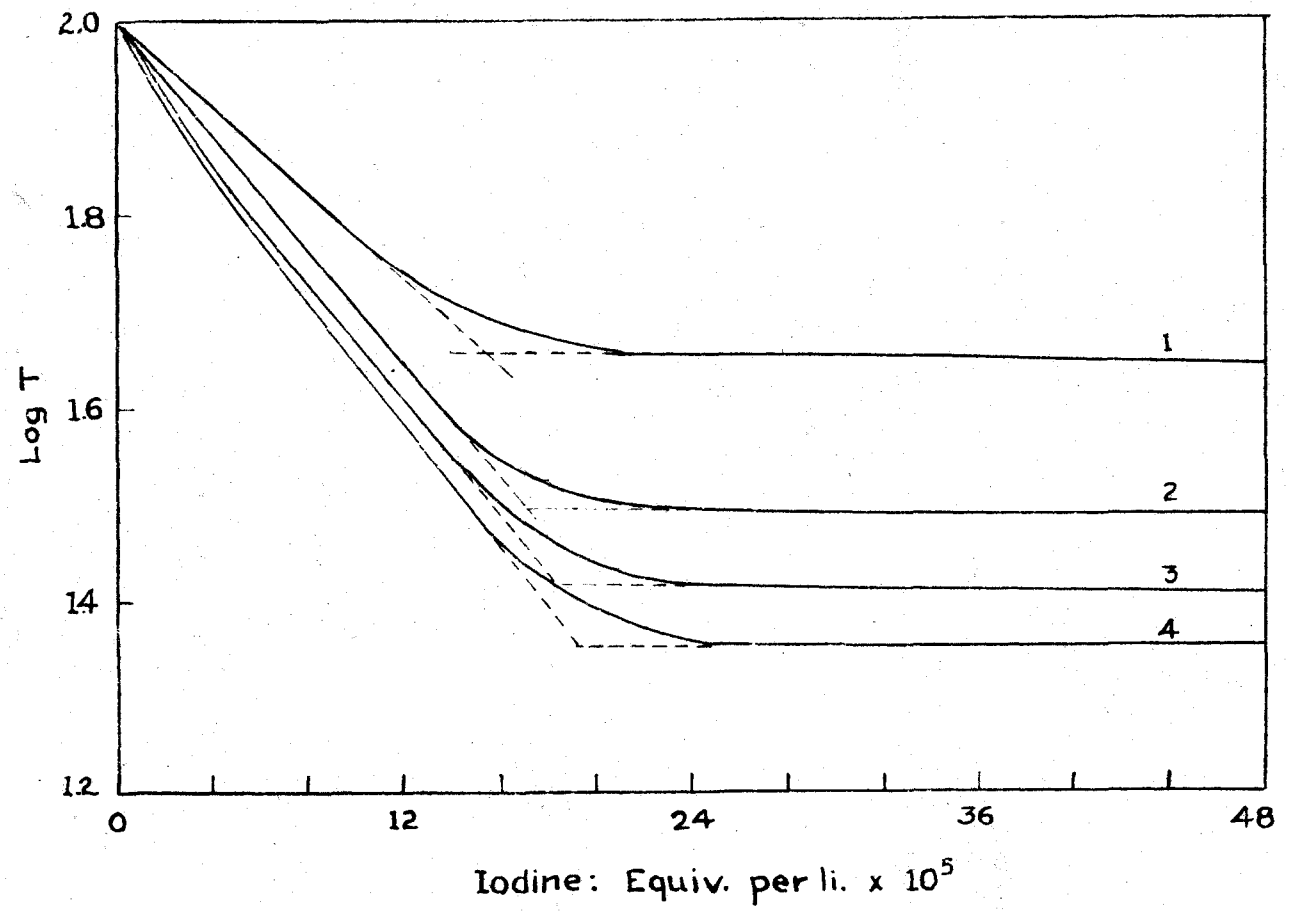

Fig. 9. Spectrophotometric Titrations of Amylose at Various Iodide Concentrations

1, 0.5 N iodide; 2, 0.05 N iodide; 3, 0.005 N iodide; 4, 0.0025 N iodide.

versus starch-to-iodine ratio curve approached a straight line with an extrapolation value of approximately 6 glucose units to 1 iodine molecule. This fact in addition to the fact that in no experimental case was the ratio of glucose to iodine greater than 6 to 1 seems to indicate there is a limiting value to the amount of iodine starch can take up. The exact role of iodide may be that of affecting the configuration of starch or of taking part in the complex itself.

Although many investigators have analyzed the starch-iodine complex by chemical means for iodine as well as iodide, they have always used native or solubilized starch or starch fractions separated by methods which are now known to be poor. For that reason a series of iodine-iodide analyses were run on the iodine complex of potato amylose separated by butanol precipitation. The following procedure proved satisfactory for the analysis of the precipitated complex: a 20 milligram sample of perfectly dry potato amylose contained in a 100 milliliter volumetric flask was dispersed in a volume of standard potassium hydroxide which when neutralized with hydriodic acid would give the required concentration of potassium iodide. Addition of an excess of iodine caused the complex to precipitate. With low iodide concentration, the complex failed to precipitate, but could be made to do so by addition of a small amount of potassium fluoride; for

that reason, 50 milligrams of potassium fluoride were added to all amylose solutions before the addition of the iodine.

Since drying of the precipitated amylose-iodine complex resulted in considerable loss of iodine, the analyses were run on the freshly-precipitated complex with more emphasis on the ratio of iodine atoms to iodide ions than the actual amounts of these materials present in a given amount of amylose. Unless a given set of conditions are rigidly adhered to, the results are not reproducible. The amount of iodine and iodide in the precipitated complex formed from 20 milligrams of amylose were determined by the following steps:

(a) 10 milliliters of 0.005 N arsenite solution were added to the complex buffered with sodium bicarbonate and the mixture allowed to stand until the complex completely decomposed.

(b) The excess arsenite was then back titrated with a standard iodine solution in which the total iodine plus iodide concentration had previously been determined. This step gave the amount of iodine in the complex.

(c) To the mixture containing only amylose and iodide were added 2 milliliters in excess of neutral of 2 N hydrochloric acid. At this acid concentration the mixture could be refluxed for 5 minutes-- sufficient time to hydrolyze all amylose present, but insufficient to oxidize

any iodide to iodine.

(d) Upon cooling, the excess acid was partially neutralized before the addition of 3 drops of bromine. This amount of bromine oxidized all iodide to iodate.

(e) The excess bromine could be destroyed by the addition of formic acid, but boiling the mixture served the purpose equally well.

(f) To the clear solution was added one-half gram of solid potassium iodide which formed six equivalents of iodine for each iodate present. The iodine liberated was titrated with standard thiosulfate. From this step the total iodide plus iodine in the original complex could be calculated.

Figure 10 shows the effect of washing the complex, precipitated in 0.05 N iodide, before the analysis. The iodide curve shows that there is a large amount of iodide loosely associated with the complex, most of which disappears upon washing. The iodine curve indicates that iodine is rather slowly removed from the complex, probably due to diffusion out the ends of the helix. It seems plausible that iodide ions could take part in the complex formation by simply going into the starch helix. This would explain the flat portion of the iodide curve which indicates that all iodide can not be washed out of the complex. The maximum ratio of greater than 4 to 1, indicates that the tri-iodide ion is not an essential part of the complex. The following table shows

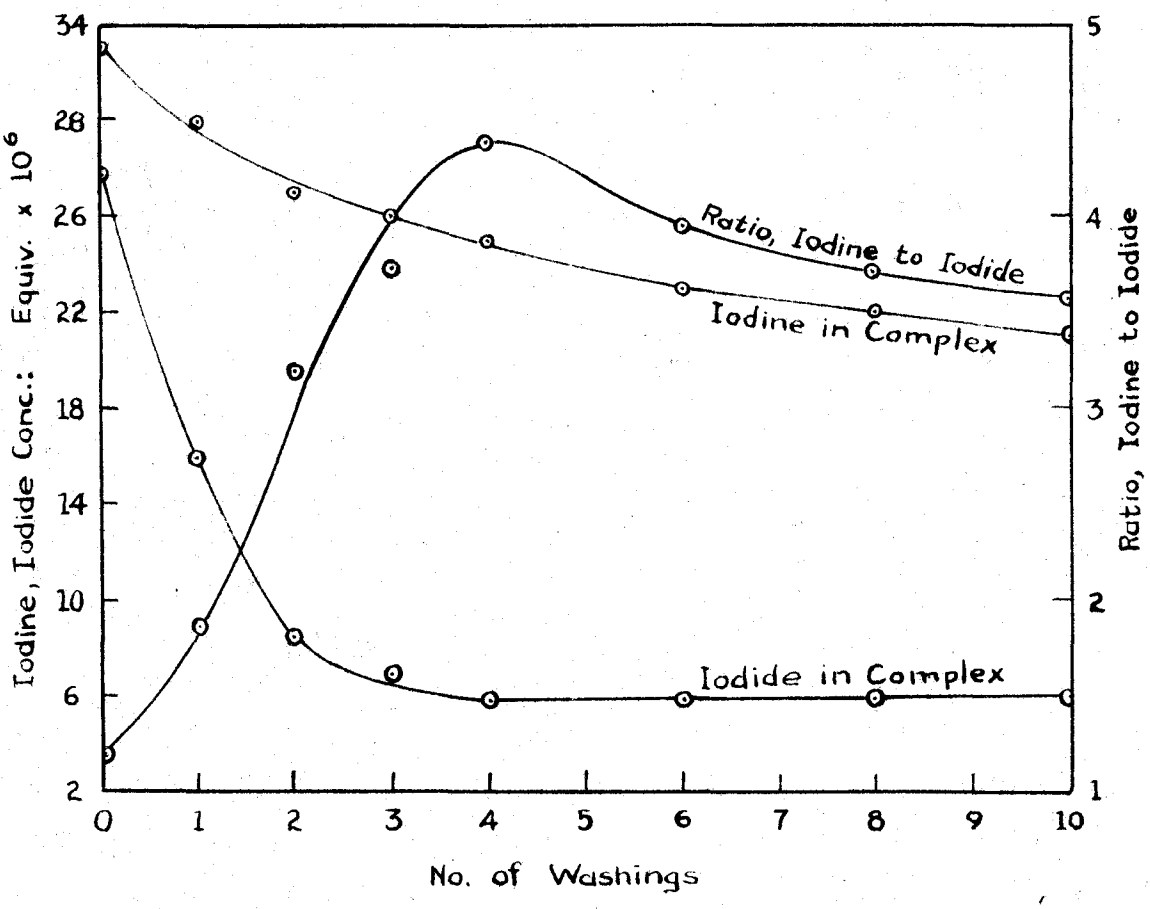

Fig. 10. Effect of Washing the Precipitated Amylose-Iodine Complex before Analysis for Iodine and Iodide. Analyses were made on 20 milligram samples of amylose.

the results of analyses made on the complex precipitated at various iodide concentrations, and washed 3 times.

Table IV

Relative Amounts of Iodine and Iodide in the Starch-Iodine Complex Precipitated at Different Iodide Concentrations

Iodide Concentration	Ratio Iodine Atoms to Iodide Ions
0.5	2.1
0.05	3.7
0.005	5.2
0.0025	5.5

The approach of the ratio to the value 2 at high iodide concentrations might indicate the maximum iodide concentration possible was limited to the formation of triiodide ions.

Effect of Other Ions

The larger the amount of iodide ions that enter the starch helix at a given iodide concentration, the smaller is the amount of room left in the helix for iodine molecules. The spectrophotometric curves indicate this to be possible. Therefore, the presence of other ions of different physical sizes, if these ions enter the helix, should cause the amylose to take up varying amounts of iodine, in accordance

with the size of the ions. With this in mind, potassium hydroxide solutions of amylose were neutralized by the series of acids: hydrofluoric, hydrochloric, hydrobromic, hydriodic and acetic. The negative ions of these are of increasing physical size. Amylose solutions, 0.05 N with respect to these ions, were titrated with iodine and the absorption taken at regular intervals. At this ion concentration the end points were as expected-- the smaller the actual size of the ion, the more iodine required to reach the end point. However, the results had to be somewhat discounted inasmuch as the solutions containing fluoride and bromide showed a strong tendency to precipitate. Figure 11 shows the spectrophotometric titrations at lower ion concentrations (0.005 N). Solutions containing fluoride, chloride or bromide do in general require more iodine for the complete formation of the complex. Curve 1 for the solution containing the acetate ion shows no break whatsoever. Its solution also shows the poorest absorbing qualities.

A more plausible explanation of the effect of other ions is their effect on configuration of the starch molecule. A few simple experiments indicated this to be possible.

To a 1 percent potato starch paste was added an equivalent amount of various 2 N salt solutions. This made the starch concentration one-half percent and the salt concentration 1 N. The starch in such solutions was always

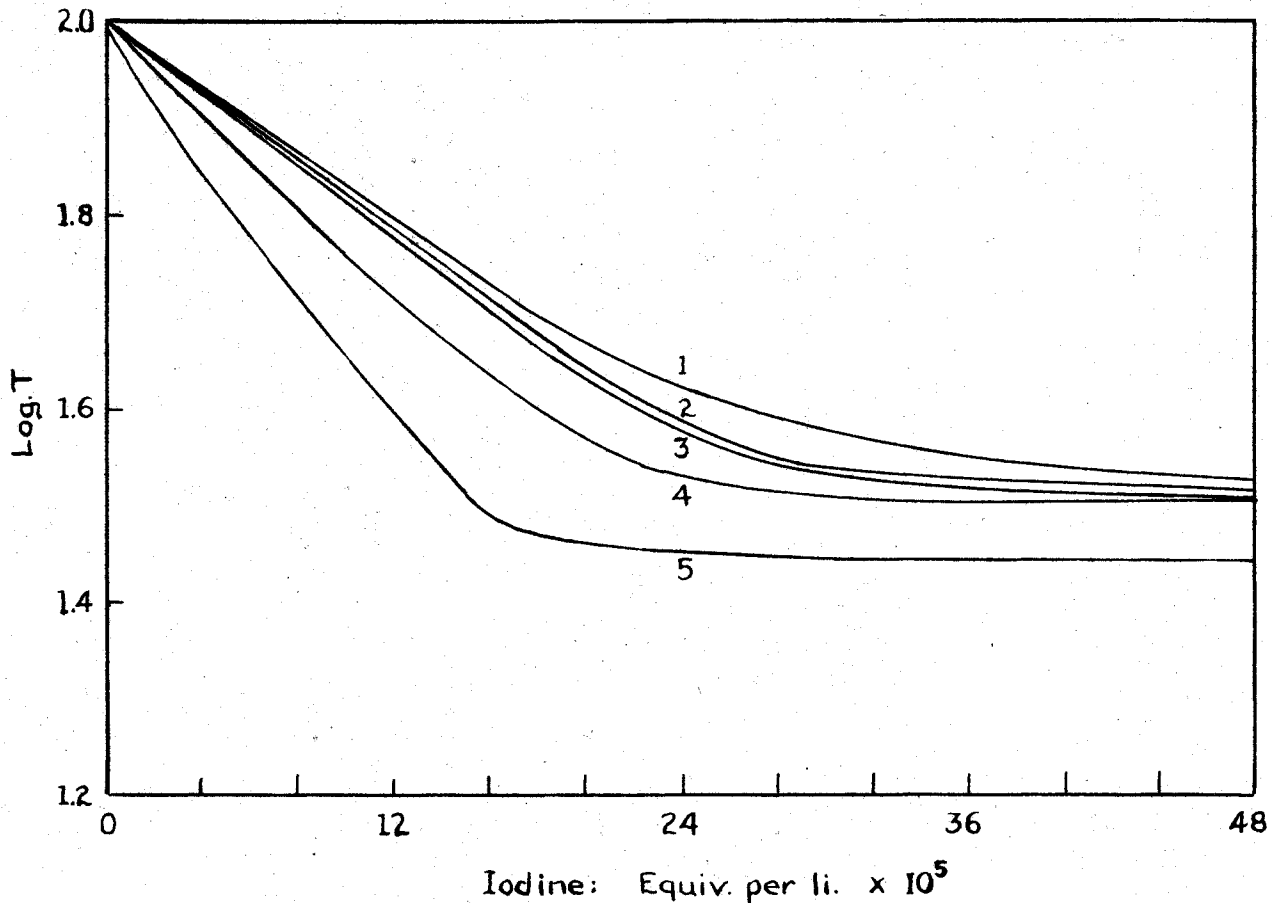

Fig. 11. Spectrophotometric Titrations of Amylose in the Presence of Different Ions

1, 0.005 N acetate; 2, 0.005 N fluoride; 3, 0.005 N bromide; 4, 0.005 N chloride; 5, 0.005 N iodide.

precipitated with much less alcohol then required to precipitate a control solution with no salt. For example, a solution containing potassium iodide required 11 milliliters of alcohol for complete precipitation while a corresponding control required 30 milliliters. The nature of the starch must have changed inasmuch as non-electrolytes such as glucose did not have a similar effect upon the precipitability of the starch with alcohol.

The change in starch configuration by ions was best shown by the addition of fluoride ions to a 0.1 percent potato starch paste. Upon standing for a few hours, a precipitate settled out. After centrifugation, the supernatant liquid stained violet with iodine while the precipitate stained blue. Furthermore the precipitate when thoroughly dried could be stained a brownish black with iodine vapor. The only other materials known to stain with iodine vapor are amyloses which exhibit the "V" configuration.

Effect of Chain Length of Starch on Absorption Maximum

Examination of a great number of absorption and transmission curves of the iodine complex of starches and starch fractions and comparison with existing data on chain length of starches indicated a close relationship between the length of the starch chain and the absorption maximum exhibited by the complex. Curves were carefully plotted from transmission data for the amyloses available, and the absorption maxima obtained.

Table V shows the correlation between the wave length in millimicrons of the absorption maximum and the chain length of the amylose material used. The molecular weights of the amyloses in glucose units per amylose chain were obtained from the viscosity and osmotic pressure measurements of Foster (30) which, as he has shown, were in fair agreement with molecular weights determined by other means. Also included in the table are molecular extinction coefficients of the amylose-iodine complexes, calculated for solutions 0.0001 N with respect to iodine and 0.01 percent with respect to starch, and the characteristic potentials at which these amyloses take up iodine.

Table V

Comparison of Molecular Weights and Characteristics of the Iodine Complex of Various Amyloses

Amylose	Molecular Size	Absorption Maximum	Molecular Extinction Coefficient	Characteristic Potential
Potato	500	628	43,000	0.197
Tapioca	450	625	41,600	0.200
Lily	310	622	41,400	0.202
Corn	250	618	40,400	0.203
Crystalline	175	605	40,100	0.205
Synthetic	85	590	32,900	0.204
Amylodextrin	44	580	25,400	0.218

The fact that the principal absorption band is wide and the shift of the absorption maximum is small makes a quantitative determination of chain length on this basis impractical. The calculation of molecular extinction coefficients from transmission data is a more sensitive criterion of chain length than the wave length shift as seen in Table V. This holds true as long as the amylose is kept in excess, in which case the absorption is a function of iodine concentration, and the molecular extinction coefficient increases with an increase in amylose chain length.

More conclusive evidence for the shift in the absorption maximum with increasing chain length may be seen in Figure 12. The synthesis of starch from glucose-1-phosphate by the enzyme phosphorylase can be stopped at various stages, the product isolated and transmission curves taken for the

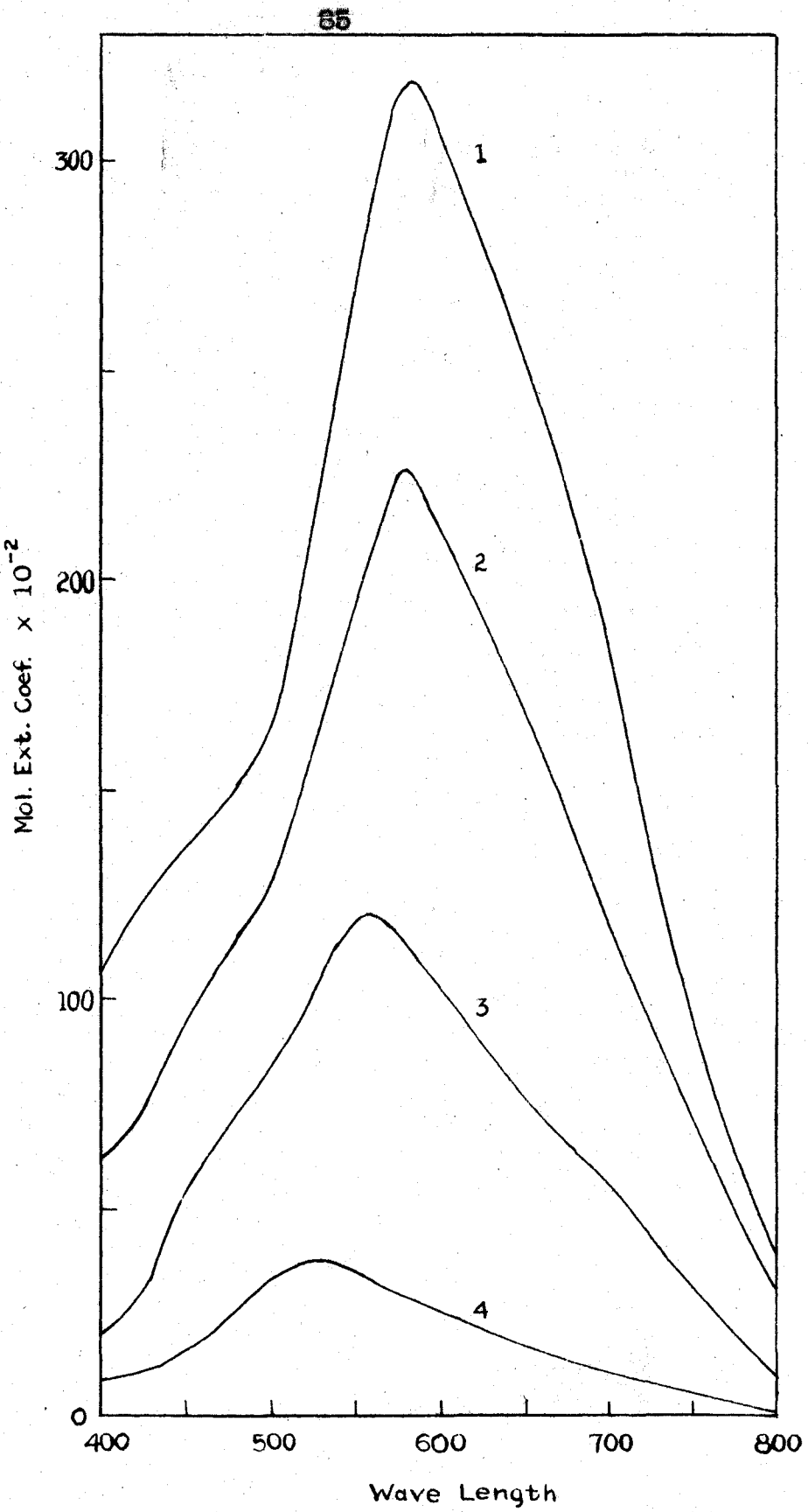

Fig. 12. Absorption Curves of Synthetic Polysaccharides

iodine complex. Ordinarily the synthesis of starch takes place so rapidly that it would be impossible to isolate separate fractions. However, a crude sample of glucose-1-phosphate, prepared by the action of phosphorylase on starch, contained traces of dextrins and possibly some maltose. The dextrins present were only sufficient to cause a slight darkening of iodine solution. Synthesis of starch with this sample of glucose-1-phosphate was sufficiently slow, due to the inhibitory action of the dextrins and maltose, that three fractions could be isolated. A similar but lesser inhibitory effect had been noted by Hanes (39) when maltose was added to the glucose-1-phosphate. Curve 1 of Figure 12 represents the starch-iodine complex of synthetic starch prepared with a pure sample of glucose-1-phosphate. All digests used for the preparation of these synthetic starches were exactly as previously described. For the preparation of the red-staining synthetic starch represented by curve 4 of Figure 12, the synthesis was stopped after five minutes. The synthesis was stopped at one-half hour and two hours for the preparation of the red-violet and violet-blue staining starches represented by curves 3 and 2 respectively. All digests of this series were run at a pH of 6.6, slightly acidic, for the highest yield of synthetic starch. The reaction in each case was stopped by the addition of two

parts of ethyl alcohol which precipitated some protein in addition to the synthetic material. Purification was brought about by repeated solution in 0.5 N sodium hydroxide and precipitation with alcohol. The absorption data were taken from solutions of the dried samples dispersed with potassium hydroxide and neutralized with hydriodic acid, such that the final iodide concentration was 0.005 N. The iodine concentration was somewhat different for the different samples, a factor necessary because of the sensitivity range of the spectrophotometer used. The iodine concentrations were 0.00004 N, 0.00007 N, 0.00014 N and 0.00021 N for curves 1, 2, 3 and 4 respectively. The absorption maximum shifted progressively from 525 millimicrons to 590 millimicrons, as the iodine color changed from red to blue.

Meyer has shown that purified potato phosphorylase synthesizes only straight chain amylose molecules (60). Therefore, the longer the synthesis was allowed to proceed, the longer the straight chain; the longer the straight chain, the higher was the wave length of the absorption maximum. To make sure all fractions of synthetic starch isolated were straight-chain material, a portion of each was subjected to a beta-amylase digestion. Each was completely digested, leaving no limit dextrin whatsoever. The red-staining fractions could not, therefore, be branched, inasmuch as branched starch leaves a residue when treated with

beta-amylase.

Figure 13 shows the effect of phosphorylase on potato starch. Curve 1 shows the transmission curve of the potato starch-iodine control, while curve 2 shows the transmission curve of the iodine complex of the material left after the action of phosphorylase on potato starch. There is the expected shift of the maximum absorption band to the lower wave length, accompanied by a decrease in absorbing power. Phosphorylase is known to break off glucose units in the form of glucose-1-phosphate. The process continues only until equilibrium is reached between glucose-1-phosphate and inorganic phosphate.

The results of this investigation of wave-length shift with chain length lend weight to the suggestion that the color of the amylose-iodine complex is a function of the interaction of iodine molecules rather than the interaction of amylose and iodine. Foster (30) attempted a quantitative explanation on such a basis, assuming a reaction of the type

$$\text{amylose} + n\ I_2 = \text{complex}\ .$$

If iodine behaves as a simple harmonic oscillator, its characteristic vibration frequency can be expressed by the equation (valid for any harmonic oscillation)

$$\nu_{I_2} = 1/2\pi\sqrt{k/m}$$

where m is the mass of the iodine molecule and k the restoring constant. Increasing the number of iodine

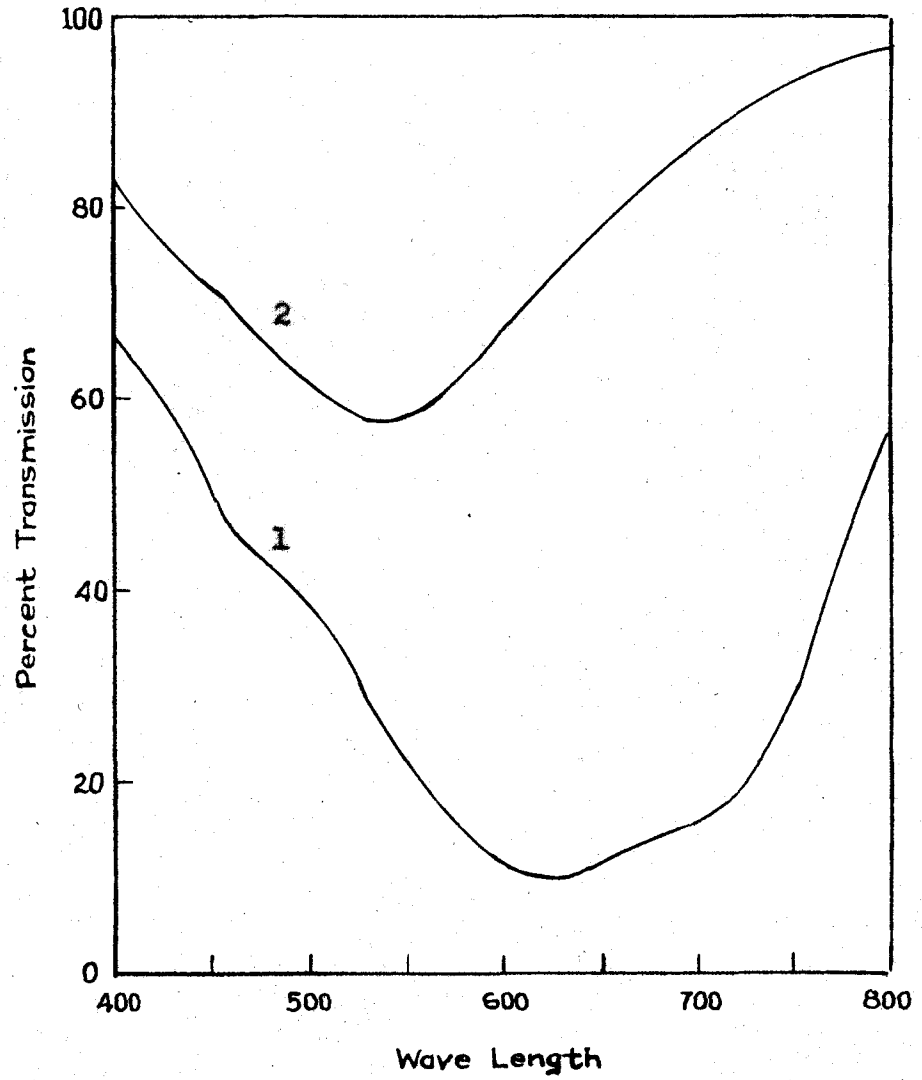

Fig. 13. Transmission Curves of Potato Starch-Iodine

1, Before phosphorylase treatment; 2, after phosphorylase treatment.

molecules in an amylose helix modifies the equation to

$$\nu_{I_2 \text{ in complex}} = 1/2\pi\sqrt{k/nm}$$

where n is the number of iodine molecules. Changing to wave length, the equation becomes

$$\lambda_{I_2 \text{ in complex}} = \frac{2\pi c}{\sqrt{k/nm}} .$$

This equation predicts such a shift in wave length as was actually found. The experimental values, however, were not nearly as great as the calculated values. This might be explained by the fact that a chain of 40 iodine molecules, as would be the case in corn amylose, might be interrupted by iodide ions or by bending of the amylose molecule such that only portions of the iodine chain acted as harmonic oscillators. This type of oscillator might also produce the characteristic absorption band in the infrared. The examination of infrared spectrum of the complex is impossible with equipment at hand.

Effect of Branching of Starch on Absorption Maximum

A comparison of transmission curves of the iodine complex of amylopectin (branched-chain material) and amylose clearly indicates the effect of branching on the absorption maximum; there is a definite shift to the lower wave lengths with increased branching. Figure 14 shows the

curves for 1, potato amylopectin; 2, potato starch; 3, potato amylose. For this set of curves, the fractionation was made by adding cotton to 0.1 percent potato starch paste. The amylose fraction, adsorbed on the cotton, was removed by elution with boiling water. Amylose and amylopectin were obtained from solution by precipitation with alcohol.

Figure 15 shows the transmission curves of the same three fractions separated, however, by the method of butanol precipitation. The greater shift of the absorption band of the amylopectin-iodine complex would indicate a more efficient separation. The amylose from this separation was so highly absorbing when iodine was added in the same concentration that the solution had to be diluted ten times to produce a colored solution suitable for spectrophotometric analysis (curve 1, Figure 15).

The similarity of the shape of the transmission curve of amylodextrin-iodine to that of the transmission curve of the iodine complex of an amylose prepared from the amylodextrin indicated that the degree of branching in the original material was slight (Figure 16). In all probability the butanol precipitation used to obtain the amylose merely separated longer chains from shorter chains, resulting in an increased light absorption of the amylose-iodine, but practically no shift in absorption maximum.

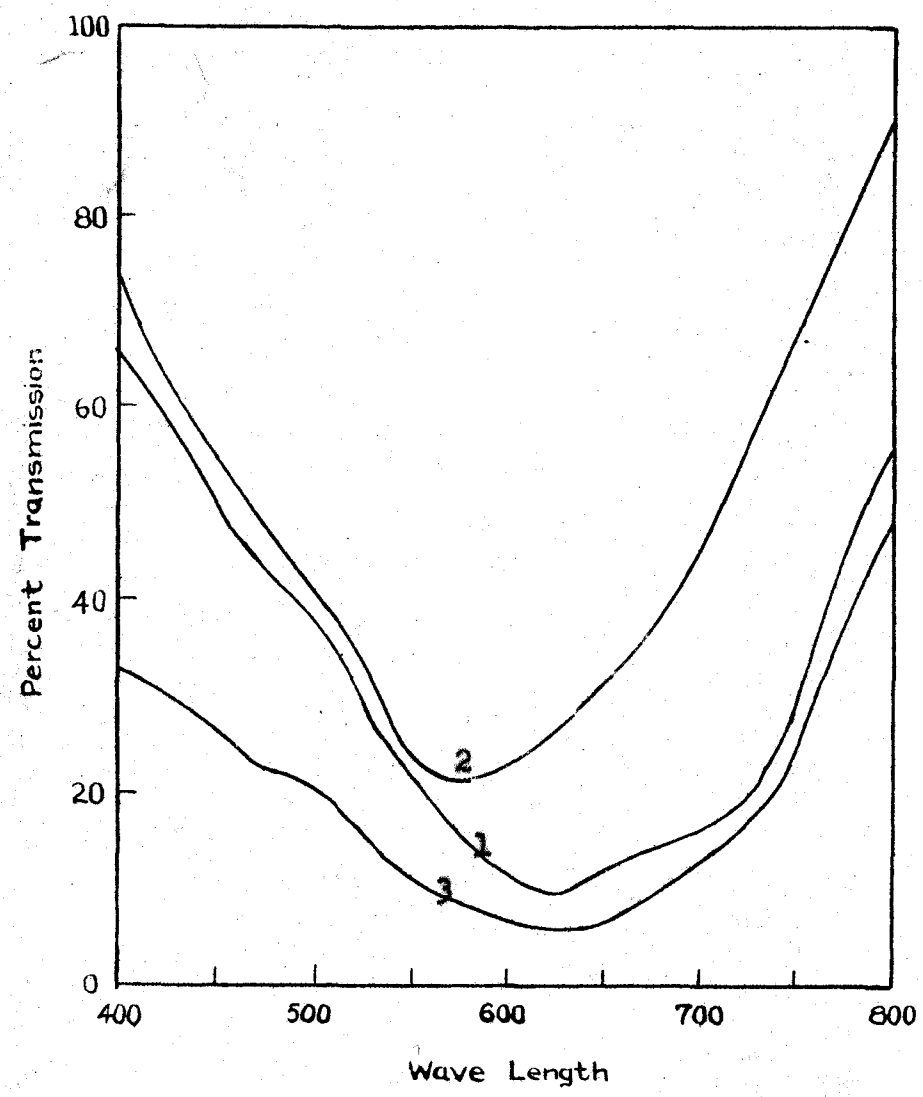

Fig. 14. Transmission Curves of Iodine Complex of Potato Starch and Components Separated by Adsorption on Cotton

1, Original potato starch; 2, fraction passed through cotton; 3, fraction adsorbed on cotton.

Iodine concentration 0.0001 N in all cases.

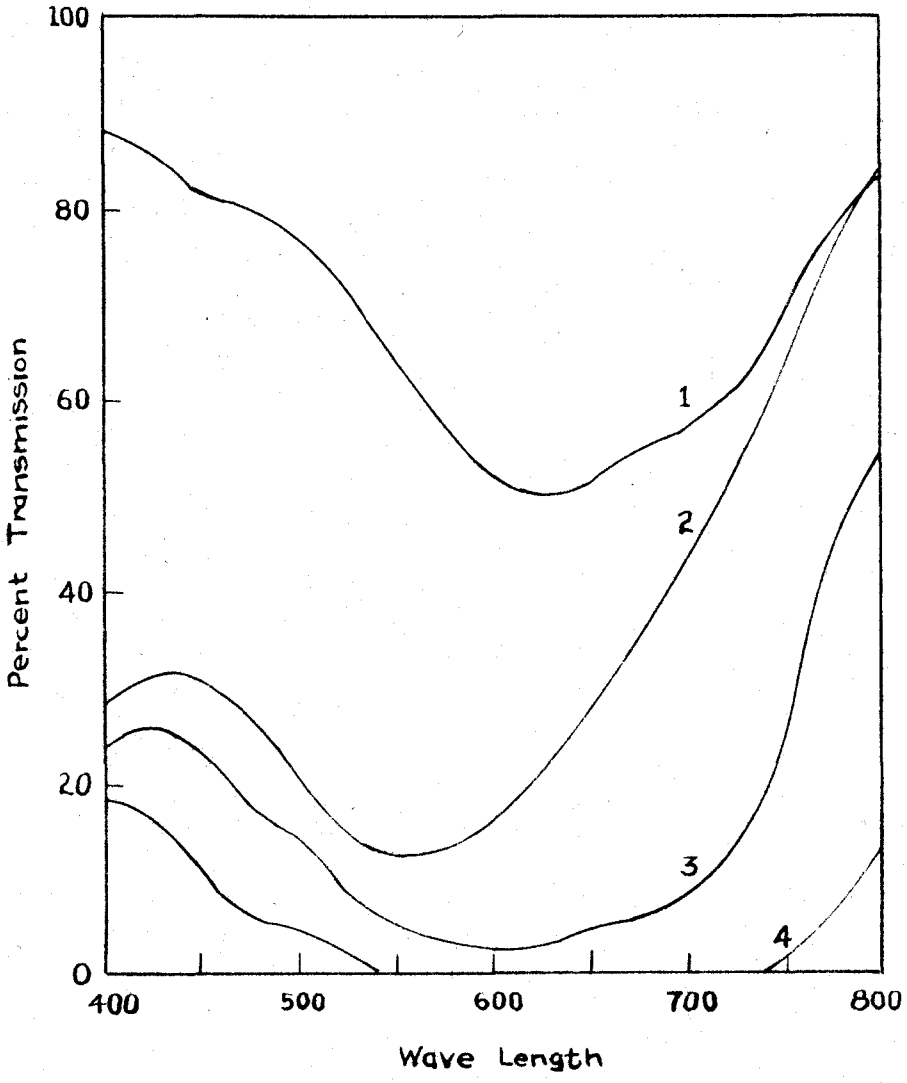

Fig. 15. Transmission Curves of Iodine Complex of Potato Starch and Components Separated by Butanol Precipitation

1, Butanol precipitate, 0.00002 N iodine; 2, soluble fraction, 0.0002 N iodine; 3, potato starch, 0.0002 N iodine; 4, butanol precipitate, 0.0002 N iodine.

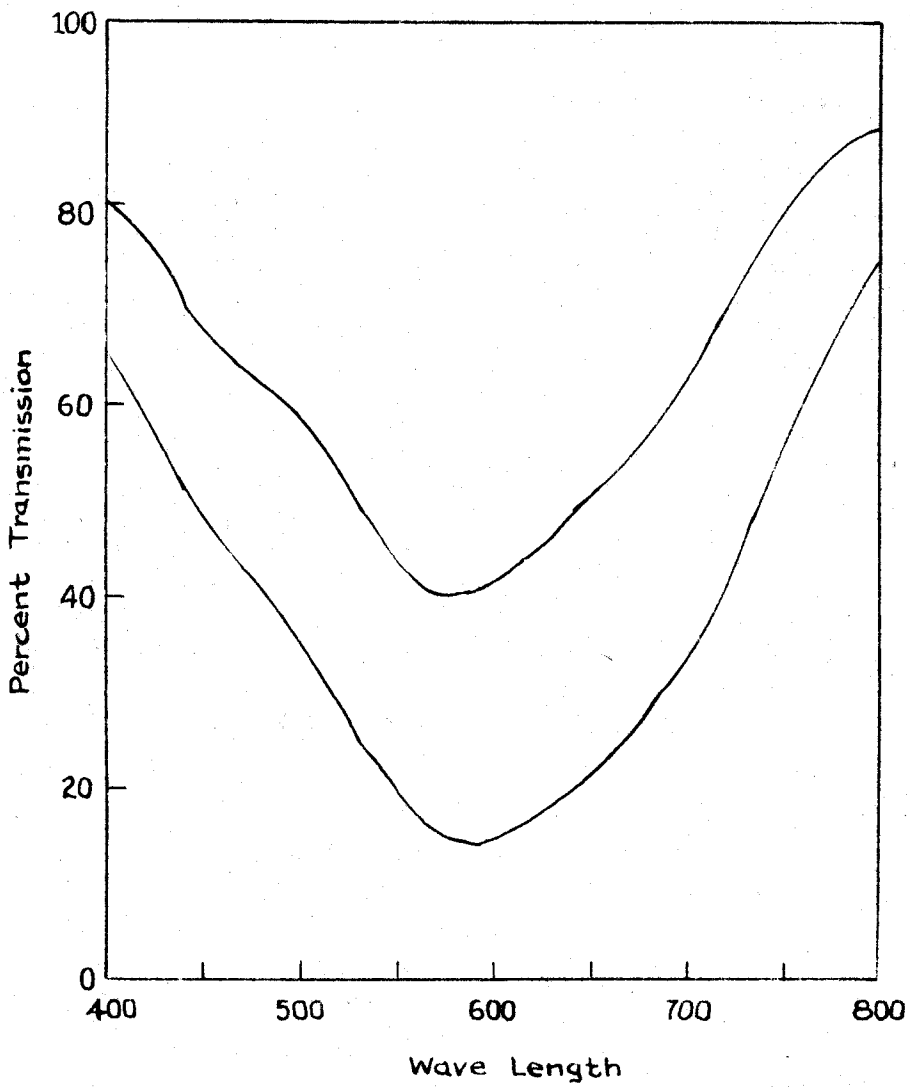

Fig. 16. Transmission Curves of Iodine Complex of Amylodextrin and its Butanol Precipitate

1, Amylodextrin, 0.0001 N iodine; 2, butanol precipitate from anylodextrin, 0.0001 N iodine.

A still greater effect on the starch-iodine complex produced by branched-chain materials is seen in Figure 17. Curve 1 is the transmission curve for 0.0005 N iodine solution. Curve 2, with but a slight indication of an absorption band, is for glycogen-iodine, in which the transmission data was obtained by balancing the glycogen-iodine solution against an iodine solution of equivalent concentration. Curve 3 represents the transmission curve of a branched dextrin of 20-40 glucose residues. Its absorption band is more pronounced than that of the high molecular weight glycogen. Curve 4, of Figure 17, is the transmission curve of the iodine complex of waxy maize starch, an amylopectin with longer branches. These curves show a correlation between absorption maximum and length of branches.

Effect of Branching of Starch on Light Absorption

In taking transmission curves of iodine complexes of starch fractions it was observed that the straight chain fractions were always more highly absorbing. The amylose separated from amylodextrin showed a greater absorption over the entire concentration range of iodine as shown in Figure 18. It is interesting to note that the straight-chain amylose fraction of this material shows a break in the titration curve corresponding to 6 glucose residues to 1

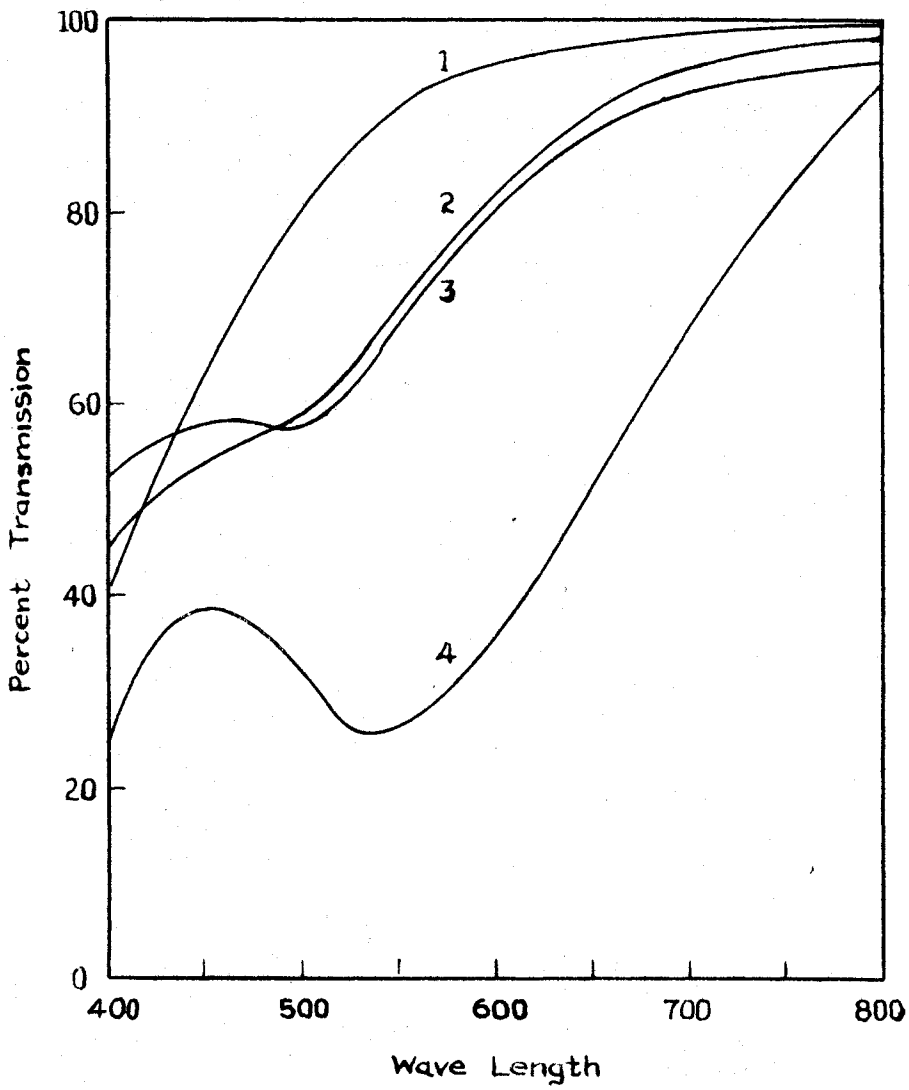

Fig. 17. Transmission Curves of Iodine Complex of Branched-Chain Polysaccharides

1, 0.0005 N iodine; 2, glycogen, 0.0005 N iodine; 3, dextrin of 20-24 glucose units, 0.0005 N iodine; 4, waxy maize, 0.0005 N iodine.

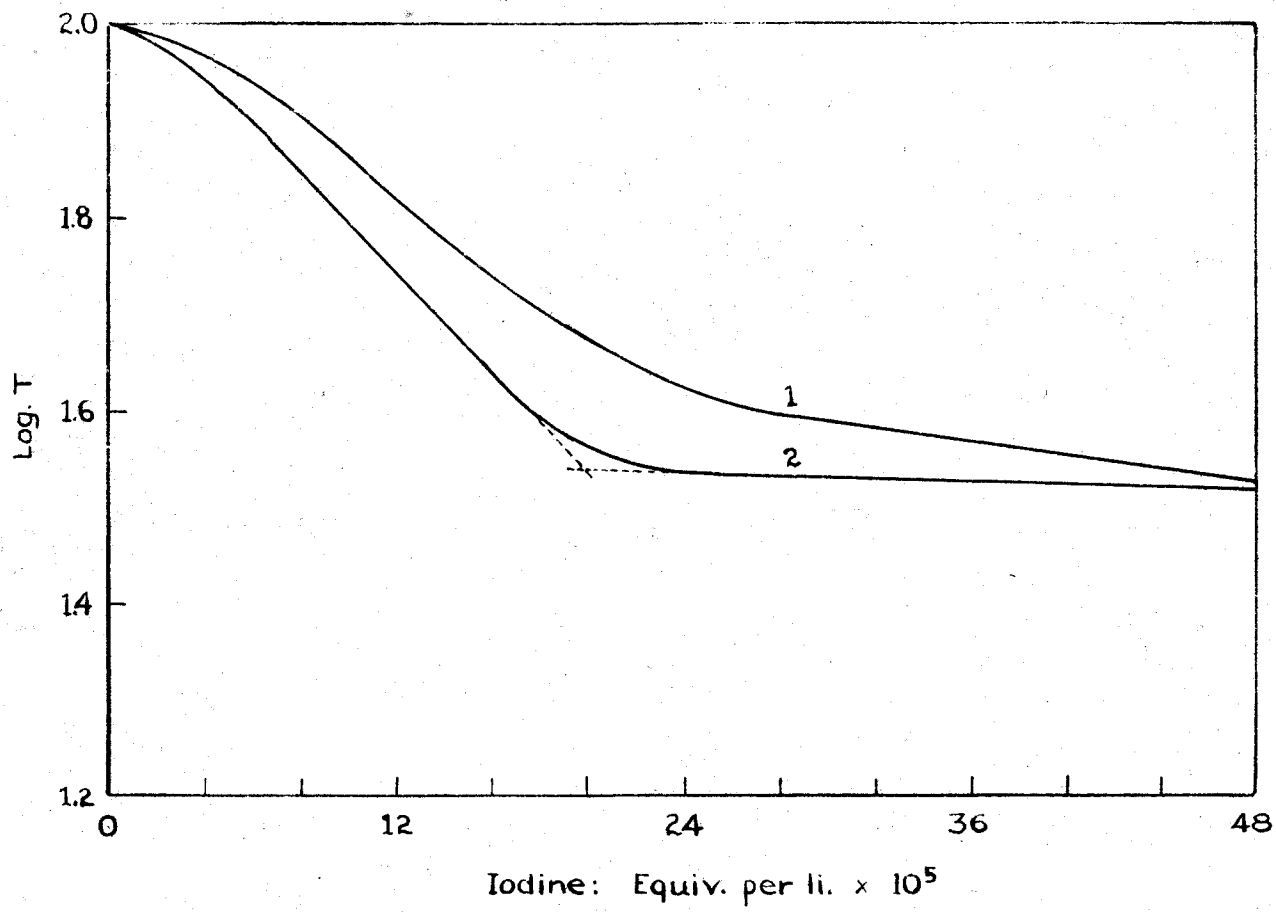

Fig. 16. Spectrophotometric Titration of Amylodextrin and its Butanol Precipitate

1, Amylodextrin; 2, butanol precipitate from amylodextrin.

iodine molecule, while the amylodextrin curve (curve 1), containing a variety of chain lengths and possibly some branched-chain material, shows no break in the titration curve.

A more pronounced effect of branching on light absorption of the iodine complex was observed by titrating the amylose and amylopectin fractions from native starches. A typical example is shown in Figure 19 in which curve 1 represents the titration of potato amylose, curve 2 the titration of potato starch and curve 3 the titration of potato amylose. Again it is obvious that the two components of starch are quite different; the complexes formed with iodine vary considerably in light transmission; therefore, it would seem possible to determine amylose, amylopectin content from transmission data. The logarithm of the transmission of light at the wave length of maximum absorption is an additive effect in mixtures of this type where the absorption bands are broad and overlap one another. Hence, the absorption level of potato starch-iodine (flat portion of curve 2, Figure 19) should represent the additive effect of the flat portions of curves 1 and 3. The following equation could be used to calculate amylose percentage:

$$x \log T_{\text{amylose-iodine}} + (1-x) \log T_{\text{amylopectin-iodine}} = \log T_{\text{starch-iodine}},$$

where x is the fraction amylose and (1-x) the fraction

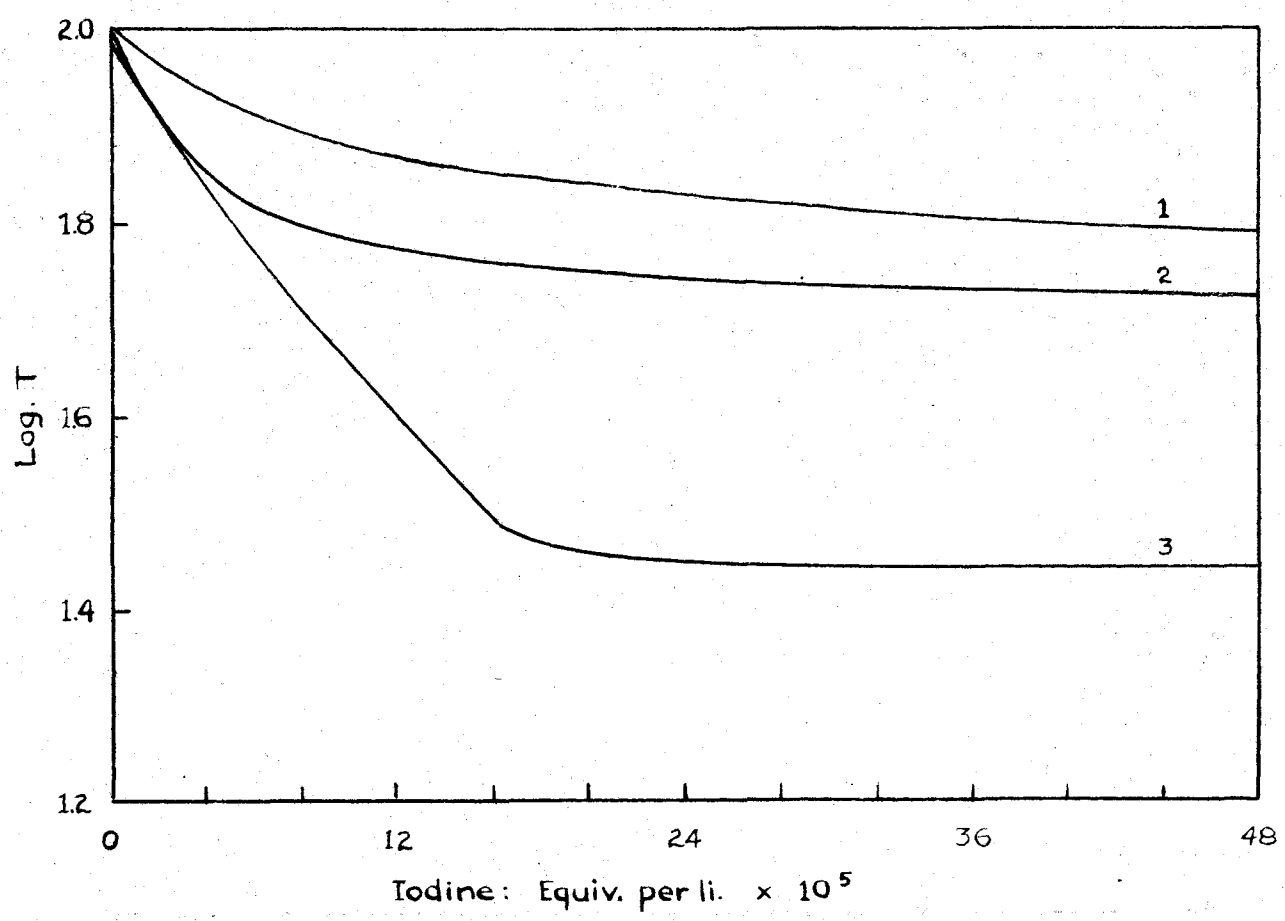

Fig. 19. Spectrophotometric Titrations of Potato Starch and its Components

1, Potato amylopectin; 2, potato starch; 3, potato amylose.

amylopectin. For potato starch and its components the equation becomes

$$x\ 1.44 + (1-x)\ 1.79 = 1.72 \quad .$$

Solving for x indicates there to be 20 percent amylose in potato starch, in fair agreement with the value of 22 percent obtained by Bates (4). In a like manner, other starches and starch components were titrated and amylose content determined. Several of these titration curves are shown in Figure 20. The amylose content calculated from such curves is as follows: lily bulb 29 percent, potato 20 percent, corn 18 percent, and tapioca 16 percent. The results are all slightly lower than values ordinarily obtained. The reason for this is probably due to traces of impurities in the amylose fraction. It is, for example, easy to remove traces of amylose from the amylopectin fractions. The process can be accomplished only by repeated precipitations with butanol. The corn and potato amylose used in this investigation were shown, by the iodine titration, to be only about 90 percent straight chain material. Beta-amylase digestions with corn and potato amylose left traces of violet-staining limit dextrins--further evidence of the presence of small amounts of branched material.

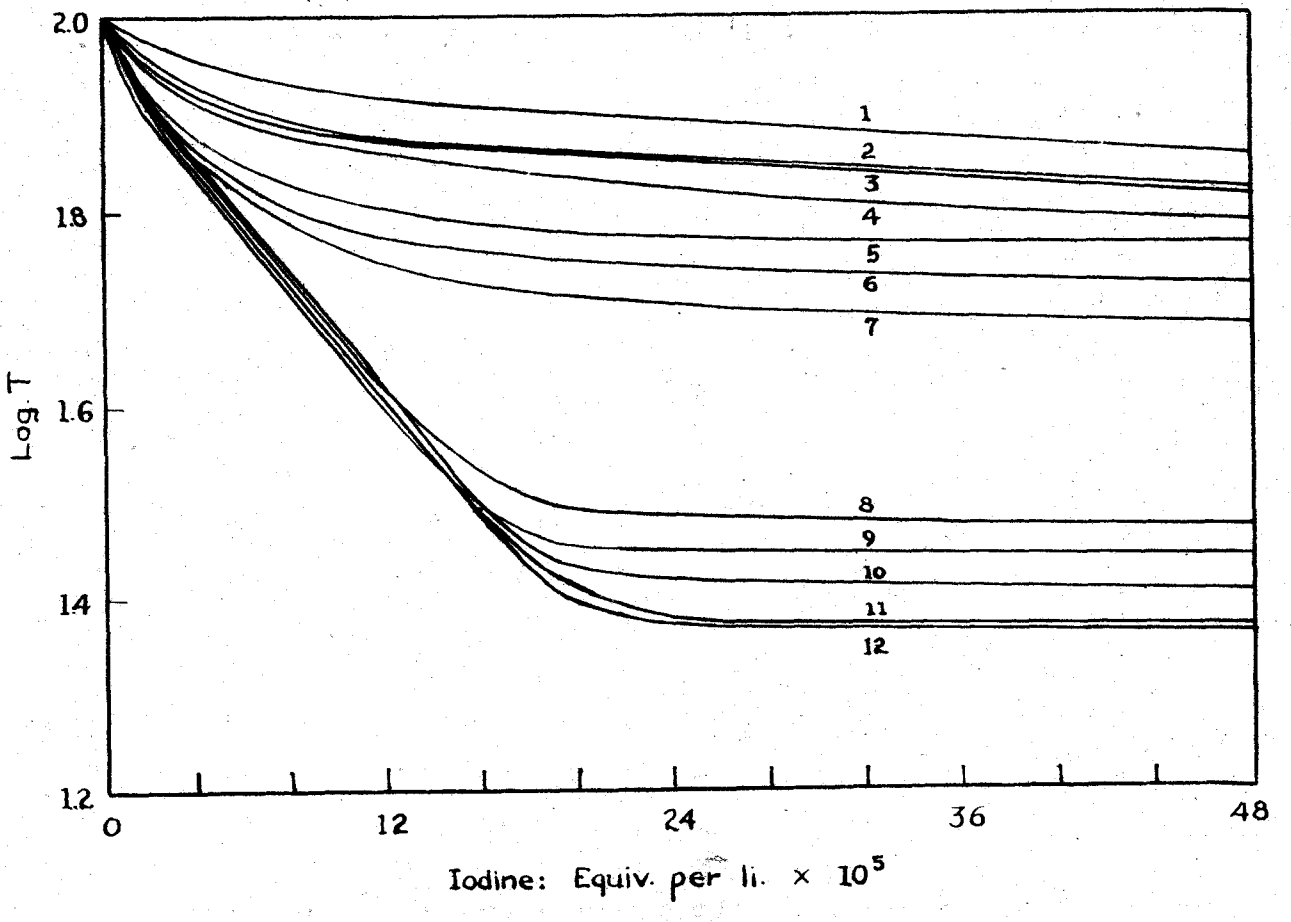

Fig. 20. Spectrophotometric Titrations of Starches and Starch Components

1, Waxy maize; 2, corn amylopectin; 3, lily amylopectin; 4, potato amylopectin; 5, corn starch; 6, potato starch; 7, lily starch; 8, corn amylose; 9, potato amylose; 10, Kerr's "crystalline amylose"; 11, tapioca amylose; 12, lily amylose.

The Linkages in Starch

Enzyme reactions with phosphorylase have been shown by such investigators as Cori, Kiessling and Hanes, to be entirely reversible. The enzyme produces a synthetic starch and inorganic phosphate from glucose-1-phosphate and vice versa. The process can be followed in either direction by analyzing for inorganic phosphate, a procedure proved justifiable by the experiments of Cori (17). In effect, he showed that the disappearance of inorganic phosphate could be quantitatively accounted for by the glucose-1-phosphate formed. A study of the reaction during the formation of synthetic starch indicates that two types of starch may be formed-- one which stains brown with iodine, the other which stains blue with iodine. The previously described experiments of Meyer indicate two types of phosphorylase. Meyer, however, was able to isolate only one of these from potatoes and that one could synthesize only a straight-chain amylose. Experiments in this investigation with purified potato phosphorylase confirmed the finding of Meyer inasmuch as all synthetic starches obtained from pure potato phosphorylase consisted of 100 percent straight-chain material, whether they stained red or blue with iodine. The question arises as to where the 80 percent amylopectin in potato starch comes from.

There was no action of purified phosphorylase on amylase-digested, highly-branched dextrins (as the limit dextrin from waxy maize), indicating no branched-chain material could be synthesized from this type phosphorylase. Using a crude potato phosphorylase, prepared by grinding and extracting the juice from potatoes, the results were somewhat different. This enzyme solution was capable of cleaving the 1:6-glucosidic linkages in starch, and, therefore, should be capable of synthesizing branched-chain materials. The results are indicated diagramatically in Figure 21 which shows the initial and the equilibrium concentrations of inorganic phosphate when preparations of crude or purified potato phosphorylase were allowed to act on various starch types. For these experiments all digests were made as follows: 50 milligrams starch in 4.9 milliliters water; 1.1 millliliters 0.3 M phosphate buffer; 5 millliliters of enzyme solution; and 5 milligrams of potassium fluoride, added to poison phosphatase action in the crude extracts. The pH was always adjusted to 7.4 at which point the equilibrium constant is

$$K = 2.8 = \frac{\text{concentration of inorganic phosphate}}{\text{concentration of glucose-1-phosphate}} .$$

Since the equilibrium involves only the concentrations of inorganic phosphate and glucose one phosphate (19), the disappearance of inorganic phosphate represents the percent cleavage of glucose units possible in the digests used.

Experimental conditions were chosen such that the disappearance of 0.1 milligram of inorganic phosphorus represented 1 percent cleavage. That is, in Figure 7, curve 3 indicates that crude phosphorylase was able to cleave the limit dextrin from waxy maize to the extent of 6 percent. Further purification resulted in less and less cleavage of the limit dextrin. Starch materials containing long chains or branches of 1:4-glucosidic linkages were much more readily cleaved as shown in curves 4, 5, 6 and 7 in Figure 21. It is interesting to note that glycogen, a highly branched material is quite readily cleaved by pure potato phosphorylase. Evidently the branches are sufficiently long to allow the action of phosphorylase and, of more importance, are joined together by 1:4-linkages. The results of the action of yeast phosphorylase of starch materials are shown in Figure 22. The fact that this phosphorylase will attack the limit dextrin from waxy maize indicates it cleaves 1:6-glucosidic linkages; the fact that it cleaves amylose indicates its action is not limited to 1:6-glucosidic linkages, but it also cleaves 1:4-glucosidic linkages. Obviously this enzyme is different from potato phosphorylase and the difference can best be explained by the existence of 2 enzymes, both present in yeast. This would explain the production of a high molecular weight but brown-staining polysaccharide with yeast phosphorylase. The product is simply branched as is glycogen.

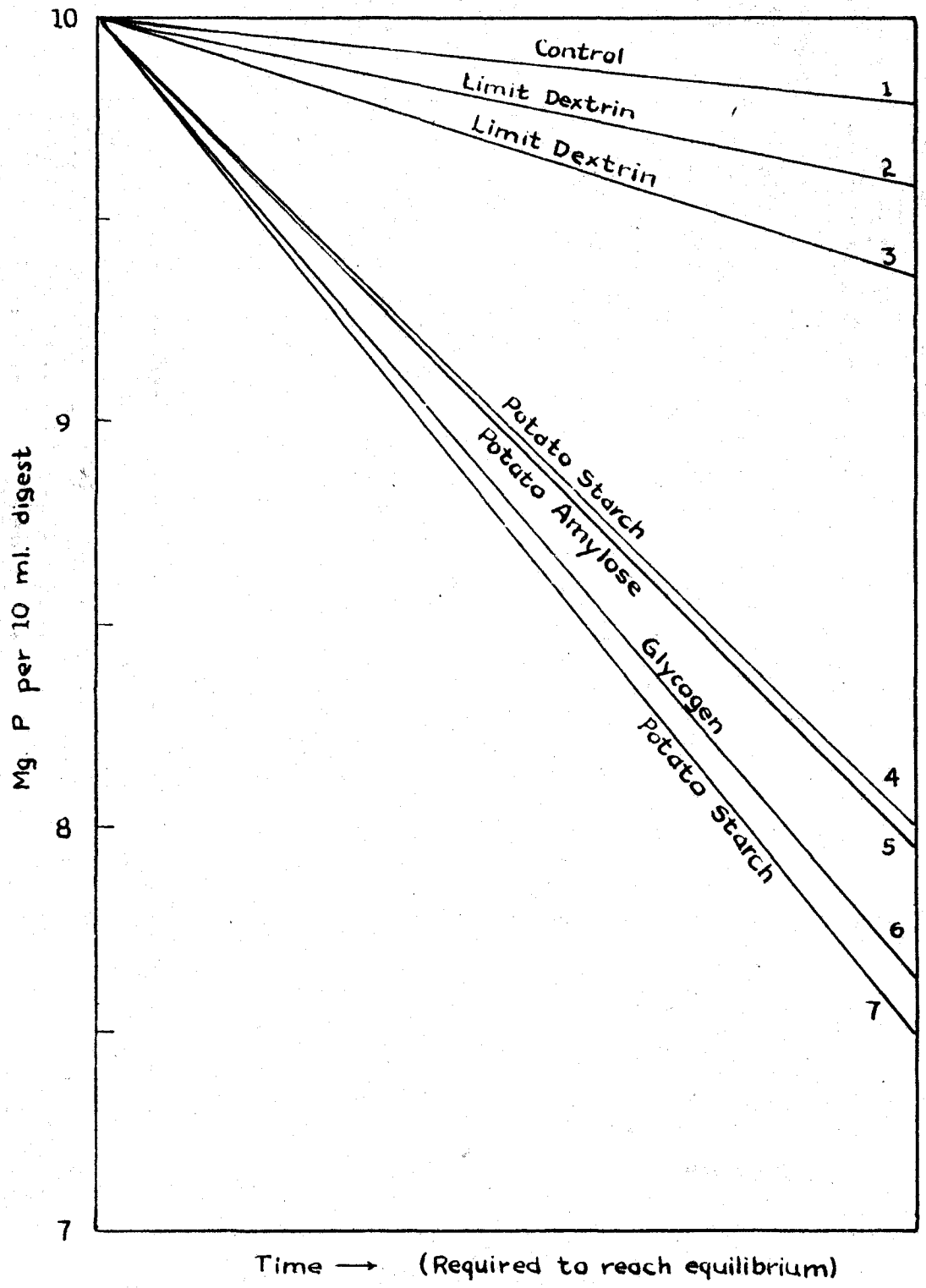

Fig. 21. Action of Potato Phosphorylase on Various Polysaccharides

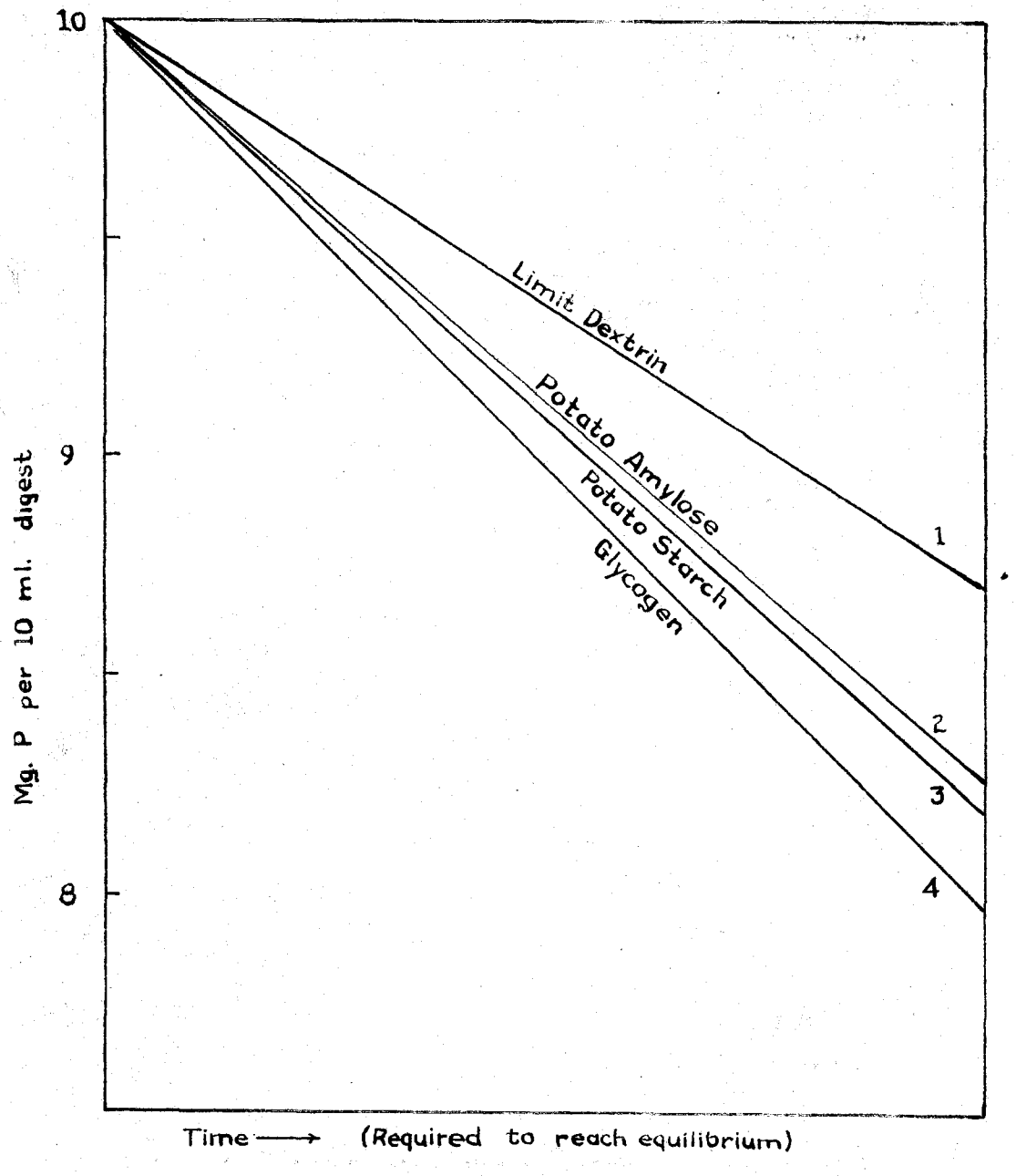

Fig. 22. Action of Yeast Phosphorylase on Various Polysaccharides

VII. SUMMARY AND CONCLUSIONS

1. An extensive investigation of the starch-iodine complex and its relation to the structure and configuration of starch is made.

2. The absorption spectrum of starch-iodine is shown to be similar to the absorption spectrum of the iodine complex of cyclohexaamylose whose structure has been established as a six-membered cyclic glucose-polymer which encloses an iodine molecule.

3. The dichroism of flow of starch-iodine solutions is such that the iodine molecules involved in the complex must have their long axes parallel to the length of the starch molecule. Optical studies of crystalline amylose-iodine complex make any but a helical configuration of amylose unfeasible. X-ray patterns of the amylose-iodine complex are in accord with a helical configuration with 6 glucose residues per helix turn.

4. The absorption curves of iodine in non-polar solvents and in starch are compared on the basis of molecular extinction coefficients. The comparison discredits the hypothesis that the blue color of the starch-iodine complex is due to iodine merely dissolved in starch.

5. A change in iodine concentration does not change

the absorption maximum of the amylose-iodine complex. Iodine is taken up more readily by amylose than by amylopectin, and the absorption is much greater in the case of amylose-iodine. The spectrophotometric titration of amylose with iodine shows an end point when the ratio of glucose residues to iodine molecules is approximately 6 to 1.

6. The amylose-iodine complex can be formed in the absence of iodide ions. In their presence, amylose takes up iodide ions in addition to iodine, with a limiting value of 1 iodide ion to 2 iodine atoms.

7. The presence of other ions affects the iodine-complex formation. This effect is attributed to changes in the configuration of the starch itself.

8. There is a direct relationship between the chain length of starch and the absorption maximum of the corresponding iodine complex. With a decrease in chain length, the absorption maximum shifts to shorter wave lengths. The width of the absorption band and the small degree of the shift make a quantitative analysis on this basis impractical. There is also a direct relationship between the molecular extinction coefficient of the amylose-iodine complex and the molecular size of the amylose, if the coefficient is calculated for solutions in which the amylose is in excess.

9. A relationship exists between the degree of branching in starch and the absorption maximum of the corresponding iodine complex. There is a shift to shorter wave lengths with an increased amount of branching. The color of a starch-iodine complex is not a function of molecular weight, but a function of the length of straight chains or the length of branches.

10. From the difference in light absorption qualities of amylose-iodine and amylopectin-iodine, it is possible to estimate quantities of these materials present in starches.

11. Yeast phosphorylase is capable of cleaving (and presumably synthesizing) a 1:6-glucosidic linkage-- the linkage involved in branching-- as well as a 1:4-glucosidic linkage. This explains the synthesis of a high molecular weight but brown-staining polysaccharide with yeast phosphorylase. Potato phosphorylase, as ordinarily prepared, is capable of cleaving and synthesizing only a 1:4-glucosidic linkage. There is evidence that an enzyme capable of forming a 1:6-glucosidic linkage is present in potatoes but is lost in the process of purification.

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X. VITA

Robert Russel Baldwin was born in Chicago, Illinois, on November 15, 1916, the first child of Susan Mabel (King) and William Russel Baldwin. His early education was in the public schools of Kokomo, Indiana, and in the Kokomo High School. In 1934 he entered De Pauw University, Greencastle, Indiana, and in 1938 received the degree of Bachelor of Arts with major in Chemistry and minors in Mathematics and Physics. In 1938 he entered the Chemistry Department of Iowa State College, Ames, Iowa, investigating the structure of starch under the direction of Dr. R. S. Bear. Dr. Bear left Iowa State College in 1941, but the work was continued under the direction of Dr. R. E. Rundle. During his graduate work he held the position of Instructor in the Chemistry Department.