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An investigation of the configuration of starch and its crystalline degradation products

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NOTE TO USERS

AN INVESTIGATION OF THE CONFIGURATION OF STARCH AND ITS CRYSTALLINE DEGRADATION PRODUCTS

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I. INTRODUCTION

It is generally recognized that knowledge of the physical configuration of starch would be of considerable value in interpretation of the physical and chemical phenomena associated with this high molecular weight substance. Whereas starch has been fairly extensively investigated from the organo-chemical point of view, it has been the purpose of this investigation to apply physical methods to the problem of starch structure, supplemented by such chemical methods as were considered necessary. The use of x-ray diffraction, which is the most direct method for the determination of physical configuration, forms the foundation of this research. Optical properties have been of great assistance, especially in determining general molecular orientations in crystals or other oriented preparations.

Some problems of starch chemistry which may be at least partially explained by a more complete knowledge of starch configuration are the phenomena of solubility behavior of starch, the nature of the starch granule, the blue iodine addition compound, and the variation of behavior of starches from different plant and animal sources. It was with these problems in mind that the investigation here reported was undertaken.

Since starch is a high molecular weight substance of great complexity, it has been considered expedient to examine some of the simpler and more homogeneous preparations such as the crystalline degradation products of starch. Glucose and simpler products of degradation have not been examined here, the scope of the investigation covering only the gross features of starch configuration at the expense of attention to the finer details.

Maltose may be considered to be the building unit of starch, as cellobiose is of cellulose. Maltose contains the characteristic α-1,4-glucosidic linkage which differentiates starch from cellulose. Thus a detailed examination of the structure of maltose might be expected to yield much worthwhile information regarding the ultimate configuration of starch. Since this compound is of fairly high molecular weight and presents little symmetry, the experimental difficulties of a complete investigation by means of x-ray diffraction place such a study outside the range of practicability. However, a few useful facts have been found by a brief examination of maltose hydrate.

The non-reducing cyclic dextrins discovered by Schardinger have been given a place of importance in this study. Although these compounds are of sufficient interest in themselves to have excited considerable chemical study, the fact that they form stable, intensely blue crystalline iodine addition products which appear to be quite analogous to the starch-iodine

addition product more than justifies a detailed physical investigation.

Other low molecular weight crystalline degradation products filling the gap between starch and maltose would be of great value. Such few as have been reported in the literature have either completely failed to produce single crystals, or have presented crystals of such exceedingly small size that methods of examining single crystals were useless. It should be emphasized that much is to be hoped for from single crystals of, say, hexaamylose.

Finally, it should be pointed out that raw starch is a mixture of at least two components which are considerably different in their chemical and physical properties. In order to interpret the properties of whole starch, it has been considered necessary to examine the properties of its unlike components. A failure to recognize the need for working on a homogeneous fraction of starch has caused considerable confusion in the older literature. However, with improved methods of fractionation which are now available, the investigator may hope to tackle problems one at a time rather than be confused by the heterogeneity of the material used.

II. THE DEVELOPEMENT OF IDEAS REGARDING THE CONFIGURATION OF STARCH AND ITS CRYSTALLINE DEGRADATION PRODUCTS

Although our knowledge of the chemical structure of starch has been rather indefinite and incomplete, especially until rather recently, any definite information of the configuration of the starch molecule is even more fragmentary. In contrast to the situation with cellulose, the use of x-ray diffraction methods has not resulted in a satisfactory picture of starch configuration (1). Undoubtedly the difference is due to the fact that cellulose forms fibers, from which excellent oriented x-ray diffraction patterns may be obtained. On the other hand, native starch occurs as tiny spherocrystals which are not capable of giving oriented diffraction patterns. Neither is it possible to form oriented fibers from starch pastes, or at least all attempts have been unsuccessful (2). Partially compensating for the lack of oriented preparations has been the variety of crystalline forms presented by starch (3), but even here any evidence for the orientation of the molecules within the unit cell has been completely lacking.

Since most attempts to arrive at the configuration of starch by physical methods have been unsatisfactory, more reliance has come to be placed on chemical methods of attack on this problem. The method of methylation has been of primary importance in this connection. The glucose polymer

being investigated is first completely methylated, and the resulting product is hydrolyzed to the corresponding partially methylated glucoses. By separation and identification of the hydrolytic products, it is possible to determine the position of linkage in the original substance. In all starch substances thus far investigated by this method, the bulk of the hydrolysis product is 2,3,6-trimethyl glucose. Other products, such as 2,3,4,6-tetramethyl glucose and dimethyl glucose, are present in smaller amounts. The large proportion (usually more than 90%) of 2,3,6-trimethyl glucose is most simply interpreted on the basis of a chain structure for the starch molecule (4). Each 2,3,4,6-tetramethyl glucose is produced by the glucose residue at the non-reducing end of the starch chain, and therefore the proportion of tetramethyl glucose is an indication of the average chain length of the starch molecule (5). The fact that small quantities of dimethyl glucose are produced indicates that starch has a branched structure. On hydrolysis, each branch point in completely methylated starch results in 2,3-dimethyl glucose (6).

Methylation studies on raw starch have indicated an average chain length of about 24--30 glucose residues, independent of the kind of starch examined (7). Recently, however, Meyer (8) has prepared starch fractions which produce a very small amount of tetramethyl glucose, indicating an average chain length of about 250 glucose units. Meyer showed that this straight-chain fraction of starch is

completely degraded to maltose by the action of beta-amylase, whereas ordinary whole starch digests only to the extent of about 60%.

The portion of the starch remaining after removal of the straight chain component represents the bulk of the starch (85 - 95%) and has chemical properties similar to those of raw starch. Methylation experiments by Meyer indicate an average chain length of about 25 glucose residues for this component, and the degradation by beta amylase proceeds to the extent of about 55%. Evidence for the high molecular weight of this fraction is its very low reducing power to the ordinary reagents (alkaline copper, silver, and iron). Although it is not possible to measure very low reducing values with any great degree of precision, the values for molecular weights so obtained are probably about as reliable as those obtained from osmotic pressure determinations, etc. (9). The very low reducing power indicates an average molecular magnitude in excess of 1000 glucose units. In order to account for the apparent discrepancy between chain length determined by methylation and molecular size as determined by reducing power and other methods, it is necessary to assume a branched chain structure (10). It should be pointed out that for the straight chain fraction estimates of molecular size by methylation and by reducing power agree fairly closely (8).

Methylation studies on glycogen indicate an even more

highly branched structure. Although the molecular weight is known to be very high (100,000 to 1,000,000), the average chain length as determined by methylation is about 12 glucose units (11). On degradation by beta amylase, about 50% of the material is converted to maltose (12).

It is evident that there is a considerable degree of correlation between the results of methylation and digestion with beta amylase. Therefore, the degree of degradation by beta amylase may be taken as a criterion of the relative amount of branching in starch chains (13).

The Crystallization of Starch

It has long been known that starch granules are capable of giving crystalline x-ray patterns. The birefringence of these tiny particles also indicates a crystalline structure. Katz (14) was able to show that the type of x-ray pattern obtained varied according to the type of starch used, and that there were three main types which he designated "A", "B", and "C". The A patterns were obtained from the cereal starches and represent one extreme, while the B patterns were obtained from the tuber starches and lie at the other extreme. Patterns whose characteristics were in between the A and B types were called C.

A different type of x-ray pattern was obtained when starch pastes were rapidly precipitated with alcohol. This pattern was considerably more simple than the A--B type of

patterns and was designated "V" (Verkleisterungspektrum) by Katz (15). Starch precipitated in this way was found to be more readily soluble than the granular starch.

An advance was made when Katz (16) prepared specimens giving A, B, or C type patterns by drying starch pastes slowly at different temperatures. Pastes crystallized below 30° gave the typical B pattern; between 40° and 60° C type patterns were obtained, and above 60° starch crystallized in the A modification. Thus it appears that the type of pattern obtained with starch granules is dependent on the conditions under which the starch granules are formed, rather than on any genetic characteristics of the starch. This is further confirmed by the fact that starch removed from solution by freezing, or retrogradation, always exhibits the B type pattern, regardless of the type or source of the starch.

Frey-Wyssling (17) has shown that the birefringence of native starch granules is in accord with a radial arrangement of extended starch chains, rather than helices as proposed by Hanes (41) and Freudenberg (20). It is therefore evident that the A--B modification of starch represents an extended chain configuration (3).

Katz showed that amylodextrin was capable of giving A, B, and V type patterns. Since the amylodextrin molecule is much smaller than starch, and probably contains no branching, it is evident that the starch x-ray diagrams are produced by crystallites which are quite small in size and include

only the straight chain portion of starch. The fact that all fractions of starch give the same types of x-ray pattern indicates the presence of the same structural unit. Glycogen, however, could not be induced to give crystalline x-ray diagrams under any condition (18). As Meyer (9) has pointed out, this is probably due to the fact that the chains in glycogen are too short to form crystallites.

In a preliminary investigation, Bear (19) arrived at the conclusion that the V pattern represents a helical configuration of the starch chain. The similarity of the V pattern to the powder pattern of the Schardinger alpha dextrin was pointed out by Katz (15), and starch-iodine precipitated by ammonium sulphate gives a semi-crystalline pattern resembling the V pattern. Since the alpha dextrin was considered to be a ring shaped molecule and a helical configuration had been postulated for the starch-iodine addition compound (20), the similarity of their patterns with the V pattern was considered to be strong evidence of a similarity of structure.

A new type of crystallization of starch was discovered by Schoch (21). When starch pastes are treated with butanol, a portion of the starch crystallizes out in the form of six sided rosettes or dumbbell shaped platelets. These crystals give an entirely new type of crystalline x-ray pattern, which somewhat resembles the V pattern but is much sharper and more complex (19). On removal of the volatile material from these crystals, the x-ray diagram becomes almost identical to the V pattern.

Interpretation of the x-ray patterns in terms of crystal unit cells.

Although several early writers had attempted to interpret the x-ray patterns of native starch granules in terms of crystal unit cells, no great amount of success was met. The main difficulty was that the crystalline interferences were relatively weak and diffuse, and it was difficult to assign exact spacing values to the diffraction rings. Moreover, the number of clearly resolved lines on the powder pattern was insufficient for the use of accurate analytical methods.

Bear and French (3) were able to obtain improved patterns of native starch by using wet starch granules and finer diffraction methods. The resolution of broad lines into close doublets and the sharpening of weaker lines made it possible to observe about 24 lines in the powder pattern. Using patterns obtained from potato starch, it was possible to determine interplanar spacings with a degree of accuracy hitherto impossible, and thus apply analytical methods to the determination of the unit cell constants. By using a series of starches of the A, B, and C types it was found that the indices assigned for the potato starch unit cell could be transferred directly to all other patterns, and the agreement between observed and calculated interplanar spacings indicated the correctness of the unit cell assignments. There are four glucose residues or two maltose residues per unit cell, but

due to a lack of oriented preparations it was impossible to determine the arrangement of molecules in the cell. The presence of four glucose residues is analogous to the case with cellulose, and probably the A--B type of crystallization is similar to cellulose. On the other hand, a helical configuration of the starch molecule would require some multiple of six or seven glucose residues per cell.

The only attempt to assign a unit cell or other crystal symmetry to the V pattern has been made by Bear (19). The simple V pattern contains only about four rings, which are, however, fairly sharp and strong. By comparison with powder patterns of the Schardinger alpha dextrin it was decided that the pattern was most like that of the hexagonal modification of the alpha dextrin. Moreover it was possible to observe a $\sqrt{3}$ relationship between the spacings of two of the lines. These considerations indicated the possibility that the V pattern represents some sort of a hexagonal structure which is related in a simple way to the alpha dextrin.

Degradation Products of Starch

Glucose.

After many years of exploratory work by Cox and others (22), any exact knowledge of the configuration of the glucopyranose ring was still lacking. Although the general size and shape of the pyranose ring had been deduced by Astbury and Marwick (23), the difficulties involved in a complete

analysis of structure prohibited a detailed examination of any simple sugar. Using chitoseamine hydrochloride and hydrobromide, Cox and Jeffrey (24) were able to carry out complete Fourier crystal structure analyses. They found that in these compounds the glucose ring has a chair configuration, rather than the planar configuration earlier proposed by Cox (22).

Maltose.

Crystalline maltose hydrate (25) has been known since 1819, but nothing has been published concerning its crystal structure or configuration (26).

Reducing dextrins.

Crystalline hexaose. The hexaose described by Waldschmidt-Leitz and Reichel (27) was reported to crystallize in tabular aggregates. No crystallographic properties were given by these writers, however, and subsequent efforts to produce the compound in the crystalline state have not succeeded.

Amylodextrin. The amylodextrins described by A. Meyer (28), Kohler-Hollander (29), and other writers have very similar properties and are undoubtedly very similar chemically. Their molecular weights (as determined by reducing power) correspond to a chain length of 14--25 glucose residues. By the action of beta amylase they are converted to maltose to the extent of at least 75%, according to Klason and Sjoberg

(30), leaving a residue containing about 4 or 5 glucose residues. Amylodextrin crystallizes in the form of spherocrystals which are similar in appearance to starch granules (28), giving the characteristic dark cross between crossed Nicols and having the same sign of birefringence (31). By careful crystallization, amylodextrin may be made to crystallize in the form of microscopic needles.

Since amylodextrin gives rise to x-ray patterns which are identical with those obtained from starch, it may be assigned the same configuration as that prevailing in starch crystallites.

Non-reducing dextrins (Schardinger dextrins).

The crystalline non-reducing dextrins discovered by Schardinger (32) have been investigated from the chemical point of view by Pringsheim (33), Irvine (34), Karrer (35), and Freudenberg (36). Hudson and Tilden (37) showed that the dextrins can be produced in fairly high yield by the action of an enzyme from B. macerans on starch. Although early theories of the structure of these compounds were badly confused, partly because of unreliable molecular weight determinations and partly because of the pronounced polymorphism of one of the dextrins, Freudenberg (36-a) has greatly clarified the problem by introducing a systematic method of fractionation of the enzymolysis mixture. The main product, alpha dextrin, was characterized by a fairly

high solubility in water, a specific rotation of about 148°, the formation of crystalline greenish needles on the addition of iodine, and the low solubility of the crystalline acetate in toluene and ethyl acetate. On the basis of approximate molecular weight determinations and methylation evidence, the compound was assigned a ring structure having about five glucose residues arranged in the form of a ring and connected by α-1,4-glucosidic linkages (36-b). A second product, the beta dextrin, was thought to be a similar ring-shaped molecule containing six glucose residues. The beta dextrin is much less soluble in water, has a higher specific rotation ($[\alpha]_D$ = 158°), and forms reddish brown prisms on the addition of iodine. Other dextrins, called gamma, delta, and epsilon, were not obtained in sufficient quantity to characterize very definitely, but they were tentatively assumed to be similar rings of higher molecular weight.

The crystalline iodine and bromine addition products of the alpha and beta dextrins were prepared and analyzed by Pringsheim and Steingrover (38). According to their analyses, there is about one halogen molecule for every six glucose residues. A small amount of halide was also found. Since the reducing dextrins of similar size do not form iodine addition products, Freudenberg (20) has inferred that each ring shaped molecule encloses an iodine molecule, thus making a stable complex.

Hess, Trogus and Ulmann (39) examined by means of x-ray powder diagrams the different forms in which the alpha dextrin crystallizes. By varying the solvent or the conditions of crystallization, it was found possible to obtain at least 10 different types of crystal patterns. A few of the crystal modifications were obtained in the form of single crystals, but these were not investigated by single crystal methods.

In view of the importance of this class of compounds, and the unreliability of molecular weights determined from colligative properties, Kratky and Schneidmesser (40) attempted to determine the molecular weight of the alpha dextrin by the x-ray method. Although these writers claim to check Freudenberg's molecular weight, it is apparent that a serious error was made in the crystal space group interpretation. The unit cell given is said to contain two five-membered molecules, while the space group requires that each molecule have a two-fold axis of symmetry. Such symmetry is clearly impossible for a five-membered ring molecule. Since the validity of all molecular weight determinations on the Schardinger dextrins is in serious doubt, the problem of the molecular weights of the Schardinger dextrins and their configurations remains incompletely solved.

The Helical Configuration of Starch and the Starch-Iodine Reaction.

The concept of the helical ("spiral") configuration for starch was first introduced by Hanes (41) to explain some of the peculiarities of the digestion of starch by alpha amylase. He also observed that such a configuration might explain the iodine reaction with starch and dextrins, with more than one complete turn of the helix being required for the production of the characteristic color. Although there is considerable evidence that his mechanism for the attack of starch by alpha amylase is incorrect, the concept of the helical configuration appears to be useful in explaining certain other properties of starch.

Freudenberg (20) has extended the theory to include the degradation of starch by B. macerans amylase with the formation of cyclic dextrins (Schardinger dextrins). On the inspection of space filling models it was observed that the starch chain had a natural tendency to form a helix with a periodicity of about six glucose residues, and that such a helix had a lining essentially hydrocarbon in nature, i. e., protected from hydroxyl or other strongly polar groups. Since iodine produces a color in starch similar to that produced in hydrocarbon solvents, Freudenberg concluded that the iodine in starch-iodine was associated with or "dissolved" in the hydrocarbon phase. Freudenberg also postulated a

similar mechanism for the formation of iodine addition products with the Schardinger dextrins.

Interesting as the helical hypothesis is, it has not met with complete approval. Caesar and Cushing (42) propose an extended helix configuration rather than the tightly packed structure of Freudenberg. On the other hand, Meyer (43) believes it is possible to account for the formation of starch-iodine on a micellar basis. By comparison with colloidal precipitates which take up iodine, it is assumed that the iodine is adborbed in the interstices of the crystallites. Accordingly, the degree of iodine coloration should vary with the crystallizing power of the preparation.

The role of water and certain negative ions in the formation of starch-iodine is rather obscure. At various times it has been claimed that each is necessary to the formation of the blue color. Meyer claims that iodide ions are not essential, and may be replaced effectively by acetate, bromide, and other negative ions. He asserts that water is necessary, since starch loses its power to become colored by iodine vapor when thoroughly dried.

Configuration and the Fractionation of Starch

In studying a complex mixture such as starch, it has long been recognized to be advantageous to deal with the simpler constituents. Methods of fractionating starch into

unlike components have been invented which differentiate starch on many bases: solubility, phosphorous content, ease of crystallization, digestion by enzymes, iodine coloration, and various others. It is now apparent that all these methods are in fact dependent on the difference in configuration of the starch fractions, the so-called basis of separation being a logical consequence of the difference in behavior between straight and branched chain starch.

Electrophoresis (Electrodialysis).

On attempting to remove inorganic ions from starch pastes by electrodialysis, it was noted (44) that a considerable amount of the starch deposited at the anode, while the remainder of the starch had no tendency to migrate in the electrical field. Undoubtedly the most important negative ion attached to starch is the phosphate ion, which occurs only to the extent of one phosphorous for every 250 - 500 glucose residues (45). It is probable that the phosphorous is concentrated in the complex, high molecular weight branched chain fraction. Thus the electro separation results in drawing a complicated dragnet of interlaced and interlinked starch chains towards the anode, while the simple molecules of low molecular weight and simple structure are left in solution (46).

Freezing of starch pastes.

Starch pastes which have been frozen and thawed form

voluminous sediments, which when filtered off and dried are difficult to redissolve (47). There remains in the filtrate a small portion of the starch which is more readily soluble. Here it is not obvious that a separation of straight chains from branched molecules is effected, since both fractions contain straight and branched chains. In the freezing process the straight chain material of high molecular weight becomes closely associated with the large branched molecules, while the smaller branched molecules as well as straight chains are free to dissolve on thawing of the mass.

Water extraction of granules.

On the warm water swelling of the starch granule, a loose network of interlaced starch chains is formed, through which the molecules of simple structure are free to pass and diffuse into solution (48). By continued extraction of the swollen granules with hot water it is possible to remove approximately 10% of the starch substance. Although the hot water extract is apparently more soluble than the rest of the granule, after it has been separated from solution and dried it becomes quite insoluble. Apparently the straight chains are able to form super-molecular aggregates of higher organization than that existing in the starch granule, and being more stable are extremely difficult to redisperse in water (9).

Cellulose adsorption of starch.

On treating starch pastes with cellulose (cotton or

filter paper) one fraction of the starch is strongly adsorbed, as evidenced by the change in iodine coloration of the resulting solution (49) (50). The blue-staining component is fixed to the cellulose, while a red-staining material remains in solution. Since cellulose is a straight chain carbohydrate similar to starch, it is possible that the straight chains tend to crystallize out on the cellulose or otherwise be held by secondary valence forces.

Alcohol crystallization of starch.

Among the more recent techniques for fractionating starch, the crystallization with higher alcohols, e. g. butanol and pentanol, appears most promising (21) (13). In this method, starch is autoclaved with a saturated butanol solution and allowed to cool slowly. One fraction of the starch crystallizes out with the butanol, apparently producing a fairly clean-cut separation. The straight chains are able to form fairly perfect helices which then pack together to form a crystal (19). A highly branched chain structure is unable to participate in this type of crystallization because the side chains are too short to allow the formation of helices of sufficient length to have the same packing characteristics as helices formed from long straight chains.

III. THE RELATIONSHIP BETWEEN STRUCTURE AND OPTICAL PROPERTIES

Birefringence

Present views on the relationship between structure and the index of refraction are developed from the principles laid down by Silberstein (51). Bragg (52) was able to calculate the indices of refraction for calcite and a few other simple compounds of well known structure. Wooster (53) has correlated optical and x-ray data for a number of crystalline organic compounds. The known facts concerning the relationship between crystal structure and index of refraction may be summarized as follows: The higher index of refraction corresponds to the direction of higher polarizability in the molecule, which for most molecules is along the greater average length of the molecules.

Pleochroism

The relationship between crystal structure and double absorption of light has received little attention. Krishnan and co-workers (54) have investigated the pleochroism of several crystals in the ultraviolet region, and Raman (55) has investigated the relationship between pleochroism and structure of some colored organic compounds.

A knowledge of the double absorption of light by halogens, especially iodine, is of considerable importance in the interpretation of the dichroism of iodine addition products of starch and related compounds. Spedding (56) found that the maximum absorption of light by halogen crystals (bromine and iodine) takes place when the electric vector is parallel to the direction of greatest average length of the halogen molecules. Almost no absorption occurs when the electric vector is normal to all the halogen molecules. Observations in this laboratory show that light is strongly absorbed by ammonium triiodide crystals when the electric vector is coplanar with the linear triiodide ions and only weakly absorbed when the electric vector is normal to the triiodide plane.

Optical Rotation

The principle of additivity of molecular rotation, set forth by Hudson (57), has been developed and employed by Freudenberg (58) to relate the specific rotations of compounds in the cellulose and amylose series. The so-called Freudenberg equation

$$[M]_n = [M]_2 - (n-2)\cdot[M]_\infty / \infty$$

formulates the relationship between the molecular rotation of an n-membered dextrin and the molecular rotations of the appropriate disaccharide and infinite glucose chain. Foster (66) has successfully applied the equation to relate reducing values and specific rotations of various corn-syrup dextrins.

IV. NOMENCLATURE

In view of the confused system of nomenclature used at present by carbohydrate chemist, the terminology used throughout the remainder of this thesis is here defined. The writer has followed Meyer's suggestion that the terminology be based on chemical constitution.

Amylose.

The term amylose is used to designate a chain of glucose residues linked together exclusively by α-1,4-glucosidic bonds. The term is used without any implication of physical properties such as solubility, viscosity, or tendency to retrograde; no reference is intended concerning the method of preparation, nor is the term supposed to imply any chemical or biochemical properties, such as the ability to convert in 100% yield to maltose.

In order to define the number of glucose residues in the amylose chain, the appropriate Greek prefix or Arabic numeral may be used, e. g., hexaamylose, dekaamylose, 50-amylose, etc. The term amylose with no delimiting prefix may be used to represent a straight amylose chain of sufficient length to form a blue color on the addition of dilute iodine solution, while an amylose chain too short to give the characteristic blue color may be designated amylodextrin.

Isoamylose (Amylopectin).

A substance which departs from ideal amylose in that occasional linkages of other types are present, is designated isoamylose. The foreign linkages may be assumed to be those that give rise to branching in starch. This term is proposed in place of amylopectin for the following reasons. Pectin infers that the substance will set to a gel, but the gel-forming tendency is now believed to be a characteristic of straight-chain amylose, whereas the branched-chain fraction lacks rigidity even in solutions of fairly high concentration. Moreover, the term amylopectin as originally defined was understood to name a compound that was not degraded by beta amylase. It is now known that no fraction of starch is entirely resistant to the action of this enzyme.

On the other hand, isoamylose implies no such chemical or physical properties, but only that the substance is like amylose in many respects. By definition, amylose is much simpler than isoamylose, and when the chemical structure of the latter is better understood, it will be appropriate to change the name entirely or add qualifying and descriptive terms to the base isoamylose.

Cycloamyloses (Schardinger dextrins).

The cyclic non-reducing dextrins are named cyclopentaamylose, cyclohexaamylose, cycloheptaamylose, etc., according to the number of glucose residues in the molecule. In

case the number of glucose residues per molecule is in doubt, the proposed nomenclature may be supplemented by the nomenclature of Freudenberg and Jacobi (36-a).

V. EXPERIMENTAL PART

Methods Used

X-ray diffraction methods.

Laue patterns. Laue patterns were taken using a tungsten target and a peak voltage of about 45 K.V. Higher voltages were not considered necessary because of the large size of the unit cells involved in this investigation. The wave length range was limited to the shorter wave-lengths by placing a sheet of aluminum .5 mm. thick between the film and crystal. By this device, all wave lengths above $\lambda = 1$ Å. were reduced to less than 15% of their original intensity. A flat film was used at a distance of 5 cm. from the sample. Gnomonic projections were made using a gnomonic ruler and a projection distance of 1 cm. Murdock's (59) suggestions for the examination of Laue patterns were followed.

Oscillation patterns. Oscillation patterns were made using a cylindrical camera of 5 cm. radius using copper radiation filtered through nickel foil .015 mm. thick. The crystal examined was mounted on a goniometer head equipped with centering slides. Oscillations of 20° were effected by means of a constant angular velocity cam. Indexing of patterns was carried out by Bernal's method (60), and primitive

translations were calculated by means of the Polanyi equation

$$n\lambda = x \sin X$$

where x is the primitive translation and X is the layer line angle.

Reciprocal lattice goniometer patterns. A reciprocal lattice x-ray goniometer of the type developed by Clark and Gross (61) was employed. This instrument is similar to the one described by deJong and Bouman (62) but is much simpler in construction and also more versatile. A brief description of the instrument used is given in Appendix 1. Using copper radiation filtered through nickel foil, the relationship between lattice spacings (d in A.) and distances measured on the film (s in cm.) is

normal incidence: $d = \frac{4.617}{s}$

60° incidence: $d = \frac{5.331}{s}$

Reciprocal lattice angles, which are the supplements of the lattice angles in the monoclinic system and systems of higher symmetry, may be accurately measured directly on the x-ray film by measuring the angles between rows of points.

Powder patterns. For exploratory work, powder patterns were taken using a 3 cm. sample to film distance and a flat film. For more accurate spacing measurements and better resolving power, a 10 cm. radius cylindrical camera was used, together with a slit system including two .25 x 2 mm. slits 10 cm. apart. Copper radiation filtered through nickel foil

.015 mm. thick was used exclusively. In order to avoid changes in moisture content or to prevent loss of a volatile constituent, samples were sealed in thin-walled pyrex glass capillaries. This procedure was absolutely necessary in many cases to avoid deterioration of the sample.

Oriented patterns. Patterns from oriented specimens (other than single crystals) were taken using powder pattern equipment by mounting the sample directly on the pinhole. The highest degree of microscopic orientation of amylose obtained in this investigation is that of uniaxial films. Although little work has been done on oriented films, the patterns are fairly readily interpreted if the organization of the specimen approaches the ideal limit. A preparation with only a small amount of orientation is little better than a randomly oriented powder.

A perfectly oriented film bears the same relationship to a fiber that a fiber bears to a single crystal. In a single crystal, corresponding coordinate axes of all crystallites are parallel. In the crystallites of a fiber, one set of axes lies parallel to the fiber axis, while the remaining axes are arranged at random about the fiber axis. Thus a fiber is equivalent to a crystal rotating about a special crystallographic axis. In the crystallites of a film, the only constraint upon the orientation of the crystal axes is that one particular axis be parallel to the film surface. / Such orientation is equivalent to a fiber rotating about a normal

to the fiber axis. A specimen containing any less orientation, such as a spherocrystal or powder, is incapable of producing oriented x-ray diffraction patterns.

Projection methods. Intensity data, visually estimated and corrected for polarization, were handled by standard projection methods for F and F^2 series (63). The summations of terms in the Fourier series were carried out by the method of Beevers and Lipson (64) using printed strips.

Optical methods.

In the examination of all oriented and crystalline specimens, use was made of an ordinary polarizing microscope equipped with a selenite plate (551 mμ retardation) and a revolving cap type analyzer. In determining the sign of double refraction, an attempt was made to use only first order interference colors by limiting observations to the smallest crystals. Usually it was possible to select a needle-shaped crystal tapering off at one end, and thus exclude the possibility of mistaking higher order interference colors for the more reliable first order colors.

For observation of pleochroism (double absorption) effects, the lower Nicol (polarizer) was removed and the effects observed by rotating the analyzer. This procedure proved much more effective than using the polarizer alone and rotating the stage.

Maltose

Preparation of crystals.

Maltose was obtained in the form of single crystals by adding 95% ethanol to a concentrated aqueous solution of C. P. maltose until the precipitated maltose no longer redissolved on shaking. The mixture was then heated to dissolve all the precipitate and set aside to cool. Amorphous maltose separated out and slowly crystallized. By slow evaporation of the solvent, clusters of maltose crystals were built up, with comparatively large flakes of crystalline maltose growing out from the clusters. The crystals used in this investigation were some of the larger and more perfect flakes.

Crystal data.

The crystal unit cell was found to be:

$$a_o = 4.9\ \text{Å.},\quad b_o = 15.2\ \text{Å.},\quad c_o = 10.7\ \text{Å.},\quad \beta = 82.5^\circ$$

$$(\sin\beta = .991)$$

Monoclinic symmetry was indicated by the symmetry of oscillation patterns about [100] and [010] as well as by Laue patterns taken along the b axis. Since the compound is optically active it must belong to the point group C_2--2. The regular absence of odd orders of (010) indicated that the space group was C_2^2--$P2_1$. The density was determined by flotation in a mixture of carbon tetrachloride and chloroform and was found

to be 1.540 ± .004. The molecular weight of maltose hydrate is 360.2, and the number of maltose molecules per unit cell is

$$N = \frac{(1.540)x(4.9)x(15.2)x(10.7)x(.991)x(.606)}{360.2} = 2.05 \sim 2$$

The crystals were tabular on (001), and were elongated along (100). Some of the crystals were examined microscopically and found to be biaxial, negative, with the acute bisectrix parallel to a and the obtuse bisectrix normal to (001).

Arrangement of the molecules within the unit cell.

Astbury and Marwick (23) have pointed out that the spatial requirements of a glucopyranose ring are approximately 4.5 Å. by 5.5 Å. by 7.5 Å., corresponding to the thickness, length and breadth respectively. The length is taken along a line joining the first and fourth carbon atoms, this being the long direction in a polysaccharide chain. If two such glucose residues are linked together as in maltose, the combination would require a space 4.5 Å. by 11.0 Å. by 7.5 Å. This is so nearly one half of the maltose unit cell that it becomes apparent that the 10.7 Å spacing represents the length of the maltose molecule (somewhat shortened from 11 Å. due to the glucosidic linkage between glucose residues), while one half of the 15.2 Å. spacing represents the breadth of the molecule. Beyond doubt the 4.9 Å. dimension represents the thickness of the molecule, since it is too small for length or breadth.

The optical properties of the maltose crystal are in accord with the above interpretation. The greatest index of refraction, which should correspond to the length of the molecule, is along the 10.7 Å. axis, or more strictly along the normal to the plane (001). The least index is along the 4.9 Å. axis, which corresponds to the thickness of the molecule.

It is significant that the thin dimension of the unit cell leaves very little room for departure from a parallel orientation of the planes of the glucose residues in the maltose molecule. If the maltose molecule possessed a folded structure, it would be impossible to pack molecules into a lattice having such a small primitive translation.

Molecular Weights of the Cycloamyloses

Molecular weights of high molecular weight compounds have been calculated by the use of cryoscopic methods, osmotic pressure methods, and other methods which depend directly or indirectly upon the number of molecules, large or small, in a weighed sample of the substance examined. Values obtained by such methods are known to be unreliable, especially in the case of compounds which are difficult to obtain in a state of complete purity or which tend to decompose. X-ray diffraction provides a method which is particularly suitable for the exact determination of the molecular weights of high molecular weight crystalline compounds. The size and symmetry of the unit cell are first determined, and in favorable cases the number of molecules per unit cell is determined by the symmetry of the space group. In this way, the volume occupied by each molecule may be calculated, and if the crystal density is determined, the molecular weight is simply calculated. This method is one of the most direct methods of arriving at the molecular weight of a compound, and by sufficient refinement of the unit cell and density determinations it is capable of a high degree of precision.

Preparation of crystals of cyclohexaamylose (alpha dextrin).

The cycloamyloses were prepared and separated by the method of Freudenberg and Jacobi (36-a), and also by Schoch's

(65) modification of the method described by Hudson (37). Cyclohexaamylose (designated alpha by Schardinger) is characterized by its fairly high solubility in water, its ability to form greenish needles and blue-black hexagonal plates on the addition of iodine and potassium iodide, and its low specific rotation. The specific rotation, using sodium light and about 1% solutions, was found to be $[\alpha]_D^{24}$ = + 151.1°, + 151.3°, + 151.8°, or an average value of $[\alpha]_D^{24}$ = +151.4°. The value given by Freudenberg is $[\alpha]_D$ = + 148°; perhaps his slightly lower value is due to incomplete drying of the sample.

Single crystals of cyclohexaamylose were prepared by adding 95% ethanol to a hot, aqueous solution of cyclohexaamylose until the alcohol concentration reached 60--80%. On cooling, large glass-clear orthorhombic prisms were formed. Determinations of volatile matter, ash and density on large crystals gave values of 12.2%, 1.6% and 1.436 respectively. The actual carbohydrate density was then calculated by multiplying the observed crystal density by the fraction of carbohydrate in the crystal (1.436 x .862 = 1.238).

X-ray data on cyclohexaamylose.

The lattice constants were determined by the use of 5 cm. oscillation patterns. By measurement of the layer line separations, the following primitive translations were obtained: a_o= 15.49 Å., b_o= 24.06 Å., c_o= 13.93 Å., $a_o b_o c_o$= 5195 cu. Å.

The crystals are orthorhombic, as evidenced by symmetry of oscillation, goniometer, and Laue patterns. The compound is optically active in solution so the crystal structure must be isomorphous with the point group V---222. Oscillation patterns showed the presence of all types of reflections except odd orders of (100), (010), and (001). Accordingly reciprocal lattice goniometer patterns were taken for the reciprocal lattice nets (hk0) and (0kl). All odd orders of (100) through (15,0,0) were missing; all odd orders of (010) through (0,25,0) were missing; all odd orders of (001) through (0,0,13) were missing. The only space group in the orthorhombic system having these characteristic absences is the space group V^4---P $2_12_12_1$. The unit cell for this space group requires four molecules, regardless of molecular symmetry.

Molecular weight of cyclohexaamylose.

The number of glucose residues per unit cell is

$$N = \frac{(1.238)\times(5195)\times(.606)}{162.14} = 24.03 \sim 24$$

where 1.238 is the carbohydrate density, 5195 is the unit cell volume in cu. Å., .606 is the product of Avogadro's number times 10^{-24} (to convert cu. Å. to cc.), and 162.14 is the weight of a glucose residue in molecular weight units. Since the unit cell contains 24 glucose residues and requires four molecules, there are six glucose residues per molecule. On this basis the compound called alpha dextrin by Schardinger and Freudenberg is now named cyclohexaamylose. The molecular

weight of the compound may be found to the highest degree of accuracy by multiplying the residue weight of glucose by six. The molecular weight so obtained is 972.84.

Preparation of crystals of cycloheptaamylose (beta dextrin).

The compound designated beta dextrin by Schardinger and Freudenberg is characterized by its low solubility in water, the formation of dark red-brown crystals on the addition of iodine and potassium iodide, and its higher specific rotation. Four determinations of the specific rotation of anhydrous samples gave $[\alpha]_D^{24}$ = +161.9°, + 161.8°, + 161.9°, + 162.2°; average $[\alpha]_D^{24}$ = +161.9°. The value given by Freudenberg, $[\alpha]_D$ = 158°, is somewhat lower, as was also observed with cyclohexaamylose.

Cycloheptaamylose may be obtained in the form of large, clear crystals by slow evaporation of an aqueous solution at room temperature. The crystals present monoclinic symmetry and are definitely polar in habit. When freshly prepared, the crystals have a density of 1.444 ± .004 and a moisture content of 14.18%. The carbohydrate density is therefore 1.239 g./cc. On standing in dry air for a length of time, water is lost and the crystals become shattered in appearance. Smaller crystals appear perfectly stable in air, indicating that the water lost from large crystals may be merely interstitial water rather than water of crystallization.

X-ray data on cycloheptaamylose.

The lattice constants for cycloheptaamylose are: a_o= 15.27 Å., b_o= 10.24 Å., c_o= 20.93 Å., β = 68.0° ($\sin\beta$ = .9272), $a_o b_o c_o \sin\beta$ = 3034 cu. Å. The crystals are monoclinic, as shown by the symmetry of oscillation, goniometer, and Laue patterns. The monoclinic crystals must belong to the point group C_2--2 since the compound is optically active in solution. Oscillation patterns showed all possible types of reflections to be present except odd orders of (010). Accordingly an intense goniometer pattern of the reciprocal lattice net (0kl) was taken. The range of the film included the first nine orders of (010), and all (0kl) planes except odd orders of (010) were clearly registered. The only space group isomorphous with C_2--2 which has these characteristic missing absences is C_2^2--$P2_1$. This space group requires the presence of two molecules per unit cell, regardless of molecular symmetry.

Molecular weight of cycloheptaamylose.

The number of glucose residues per unit cell is

$$N = \frac{(1.239)x(3034)\ x\ (.606)}{162.14} = 14.05 \sim 14.$$

Since the unit cell requires the presence of two molecules, each molecule contains seven glucose residues. The exact molecular weight of cycloheptaamylose (beta dextrin) is then seven times the weight of a glucose residue or 1134.98.

The significance of the molecular weight determination.

Since it is well known that the cycloamyloses are ring molecules containing glucose residues linked in α-1,4-glucosidic bonds, it is now possible to assign definite structural formulas to these compounds. Such a step is very important in establishing the spatial configuration of the molecules.

Moreover, the individual compounds become valuable starting products for synthesis or degradation. The possibility of preparing heptaamylose, a straight chain dextrin containing seven glucose residues, will be discussed in a following section.

Polymorphism of Cyclohexaamylose

By various methods of preparation, it has been found possible to prepare single crystals of at least five different modifications of cyclohexaamylose. In addition it is well known that other crystalline modifications exist, as well as crystalline addition products with many organic and inorganic compounds. The modifications investigated have been confined to those forms crystallized from water or dilute ethanol solutions.

Modification 1.

This modification is discussed in the preceding section.

Modification 2.

Dilute aqueous alcoholic solutions of cyclohexaamylose deposit blade-shaped orthorhombic crystals on standing at room temperature or lower. These crystals contain alcohol as well as water of crystallization, but are nevertheless fairly stable. Some small and well-formed crystals kept for over a year in an unstoppered bottle have remained perfectly clear. The specific rotation on the basis of the undried crystals is $[\alpha]_D^{24} = 130^\circ$, and the crystal density is about 1.44. The carbohydrate density is 1.23, found by multiplying the crystal density by the ratio of the rotations of the compound in the crystalline and anhydrous form.

The unit cell data as determined from layer line separations on oscillation patterns are: a_o = 9.5 Å., b_o = 38 Å., c_o = 14.4 Å., $a_o b_o c_o$ = 5200 cu. Å.

The crystals show orthorhombic symmetry and the diffraction patterns have the characteristic missing reflections of the space group V^4--$P2_12_12_1$. The number of molecules per unit cell is

$$N = \frac{1.24 \times 5200 \times .606}{972.84} = 4.02 \sim 4$$

The space group V^4--$P2_12_12_1$ requires 4 molecules per cell.

Modification 3.

Small hexagonal plates are formed on precipitating cold aqueous solutions of cyclohexaamylose with 95% ethanol, or by the slow evaporation of dilute alcoholic solutions. The crystals were rather difficult to work with, being very small, and since any evidence of the Laue symmetry was lacking, a determination of the space group was impossible. The unit cell dimensions obtained from oscillation patterns are:

a_o = 23.4 Å., C_o = 15.9 Å., $(a_o)^2 c_o \times \sqrt{3}/2$ = 7540 cu. Å.

Modification 4.

By crystallization from water at about 80°, cyclohexaamylose forms rhombus-shaped orthorhombic crystals. The unit cell dimensions are:

a_o = 15.9 Å., b_o = 16.1 Å., c_o = 24.0 Å., $a_o b_o c_o$ = 5370 cu. Å.

The crystal probably belongs to the space group V^4--$P_{2_1 2_1 2_1}$ with four molecules per cell. The crystals were of poor appearance and quality. Because of the great imperfection of the crystals, each diffration spot being multiple in nature, no great reliance is placed on the space group determination. There is little doubt, however, concerning the crystal class and the true size of the unit cell.

Modification 5.

On evaporation of aqueous solutions of cyclohexaamylose at room temperature, large rhombus-shaped crystals are formed. These crystals may also be formed from dilute ethanol solutions, i. e. at concentrations of 40% alcohol. The lattice constants are:

a_o = 22.0 Å., b_o = 16.8 Å., c_o = 8.3 Å., $a_o b_o c_o$ = 3070 cu. Å.

The unit cell is orthorhombic and contains only two molecules of cyclohexaamylose. Since odd orders of (100) and (010) are absent while odd orders of (001) are definitely present, the space group is V^3--$P_{2_1 2_1 2}$. The symmetry of this space group requires the presence of four molecules per unit cell, or if the molecule has a two-fold axis only two molecules are required. The crystals are biaxial negative, with the acute bisectrix parallel to c and the obtuse bisectrix parallel to b. The optic axial angle is very small.

Configuration of the Cycloamyloses

Cyclohexaamylose.

Methylation studies as well as the kinetics of hydrolysis indicate that cyclohexaamylose is a ring shaped molecule containing glucose residues linked together as in starch. Accurate molecular weight determinations now show that the ring contains six glucose residues. Such a molecule is capable of higher symmetry than most carbohydrate molecules, and in modification 5 of the preceding section, it is evident the molecule has a two-fold axis of symmetry. Higher symmetry, e. g. three-fold and six-fold, is theoretically possible for a six-membered molecule, but has not as yet been observed in any crystal modification or derivative of cyclohexaamylose.

The significance of the two-fold axis can hardly be overestimated. In addition to the novelty of being the most symmetrical of all saccharides thus far investigated (pentaerythritol not taken as belonging to this class), the presence of a two-fold axis makes possible the exact orientation of the molecule in those unit cells which require for the molecule a two-fold axis of symmetry. Furthermore, it is important supporting evidence that the glucose residues in cyclohexaamylose are linked together in exactly the same way. If any linkage other than the α-1,4- glucosidic bond is present, it must be present to the extent of at least two of the six

linkages. The best methylation evidence at present indicates that at least five of the six linkages are α-1,4-glucosidic bonds, and there is no evidence for any other kind of linkage. It is plain, therefore, that all the linkages in cyclohexaamylose are the same type as those in starch.

It is possible to deduce the approximate packing size of cyclohexaamylose by an inspection of the unit cell size and symmetry of modification 5. The two-fold axis of the ring must coincide with a two-fold axis of the unit cell. The optical properties are in accord with this arrangement.

The primitive translation along the Z axis is 8.3 Å., which is then the packing thickness of the cyclohexaamylose ring. This thickness is about 10% more than the breadth of the maltose molecule. The approximate diameter of the ring may be found by taking half the diagonal of the projection of the unit cell on the xy plane. The diagonal is $\sqrt{a_o^2 - b_o^2} = 27.68$; the molecular packing diameter is then about 13.84 Å.

One interesting feature of this crystal is its tube-like structure. Since the unit cell is only one molecule thick, the molecular rings are superposed producing a tube extending the length of the crystallite. This tube structure is of importance in the interpretation of the reaction with iodine vapor discussed in a following section.

Cycloheptaamylose.

All chemical evidence points to the close similarity between cyclohexaamylose and cycloheptaamylose. However, the cycloheptaamylose molecule is incapable of any crystallographic symmetry (a seven-fold axis not being a crystal symmetry element), and a further investigation of symmetry by x-ray methods was not considered practical. Here it is not possible to show so conclusively that all the linkages between glucose residues are α-1,4- glucosidic linkages, but the molecule may be tentatively assumed to be a ring of seven glucose residues connected by α-1,4- glucosidic bonds.

Other cycloamyloses.

According to Freudenberg and Jacobi, the gamma and delta dextrins are compounds similar to cyclohexaamylose and cycloheptaamylose, but of larger size. Such a conclusion is supported in part by the higher specific rotations. If it is found possible to prepare these higher molecular weight cycloamyloses, they would be very valuable for crystal structure work. Cyclooctaamylose would be capable of showing two-fold and four-fold axes, while cyclononamylose might show a three-fold axis.

The Preparation of Low Molecular Weight Amylodextrins from the Cycloamyloses.

The initial product formed in the acid hydrolysis of a cycloamylose is the corresponding amylodextrin. By using pure cycloheptaamylose for example, it should be possible to prepare heptaamylose in good yield and high purity. Assuming the velocity constant of hydrolysis for the glucosidic bond is the same in cycloheptaamylose as in heptaamylose, it is possible to calculate the relative amounts of cycloheptaamylose, heptaamylose, and lower molecular weight degradation products present in the hydrolysis mixture. The amount of cycloheptaamylose (C) after a time t will be $C = C_o e^{-kt}$, and if one knows the velocity constant, the proportion of the starting material may be calculated for any valve of t.

The rate of formation of heptaamylose (H) is the rate of destruction of cycloheptaamylose, or $KC_o e^{-kt}$. The rate of destruction of heptaamylose depends on the amount of heptaamylose present as well as the velocity constant of hydrolysis. Since heptaamylose contains only six glucosidic bonds, the velocity constant of hydrolysis is assumed to be 6/7 k. The rate of increase of heptaamylose is then the rate of formation minus the rate of destruction, or

$$\frac{dH}{dt} = kC_o e^{-kt} - \frac{6}{7} kH.$$

Solving, $H = 7\ C_o \times (e^{-\frac{6}{7}kt} - e^{-kt})$.

Table 1 shows the relative amounts of cycloheptaamylose, heptaamylose, and degradation products corresponding to various stages in the hydrolysis process. As the heptaamylose is the desired product, the amount of lower molecular weight

Table 1. Conversion of Cycloheptaamylose

Amount of cyclohepta-amylose	Amount of hepta-amylose	Amount of lower M. Wt. products	Per cent impurity in hepta-amylose	Time required (kt)
1.00	0	0	----	0
.99	.010	.00004	.4	.010
.95	.049	.001	2.0	.051
.90	.096	.004	4.1	.105
.80	.181	.019	9.8	.224
.70	.256	.044	14.5	.357
.60	.316	.084	20.9	.511
.50	.364	.146	29.2	.694
.30	.394	.306	43.8	1.204
.10	.272	.628	69.7	2.303
.00	.000	1.000	100.0	∞

product is calculated as the percentage "impurity" on the basis of the amount of heptaamylose which has been further broken down. The appropriate time to interrupt the acid hydrolysis is determined by the degree of purity of raw product desired.

Removal of unchanged cycloheptaamylose. Cycloheptaamylose may be removed practically completely from aqueous solutions by the action of organic precipitants, such as trichloroethylene, ethylene dibromide, toluene, and xylene. The degree of completeness of removal is illustrated by the following experiment: 5 g. of cycloheptaamylose were dissolved in 100 ml. of hot water and allowed to cool to room temperature. After standing two days at room temperature (24°) the crystals were filtered off (about 3 g.). The filtrate was put in a 2 dm. tube and the rotation taken ($\rho = 5.98^{\circ}$). The same solution was now stored under xylene with frequent shaking for several days at room temperature. A considerable bulk of precipitate (nicely crystalline and easily filterable) was formed. After filtering off the precipitate, the rotation of the solution was taken as before. ($\rho = .28^{\circ}$). Since the specific rotation of cycloheptaamylose is about 162°, the concentration before addition of xylene was about 1.85 g./100 ml.; after precipitation with xylene, about .086 g./100 ml. Thus the xylene removed more than 95% of the amount of cycloheptaamylose left in solution. By using small volumes of solutions it is possible to remove all but an insignificant amount of cycloheptaamylose.

An example of the proposed experiment. Dissolve 20 g. of cycloheptaamylose in about 100 ml. of aqueous sulfuric

acid and allow it to hydrolyze to the extent of 10%, or until the specific rotation has increased about 2°. The solution is now neutralized by pouring into calcium carbonate, the precipitated calcium sulfate together with unchanged calcium carbonate filtered off (hot), and the precipitate washed with boiling water. The filtrate and washings are now concentrated to a volume of about 50 ml. and allowed to crystallize. The crystals are removed, and the solution is covered with xylene or toluene. After standing several days with frequent shaking, the precipitate is filtered, and the xylene is separated from the filtrate. After concentrating the solution to a thin syrup, alcohol is added to precipitate the raw heptaamylose. Let us suppose a quantitative precipitation on the addition of alcohol; the raw product then contains 1.92 g. of heptaamylose, .08 g. of lower degradation products, and about .04 g. of cycloheptaamylose, or the product is about 94% pure. It is not unreasonable to suppose that a product of this purity should crystallize.

The value of heptaamylose. If heptaamylose can be obtained in the crystalline state, it would be a very valuable compound from the viewpoint of starch structure. Crystal structure work would be expected to reveal a great deal more about the intimate nature of the starch chain than has yet been found from starch or its derivatives. It should be possible to establish the presence or absence of the straight

chain configuration, and the amount of "kinking" in the starch chain.

The compound would be a primary standard for the calibration of molecular weight methods depending on reducing value or other end-group assay. Studies with enzymes would be expected to reveal the minimum chain length upon which any particular amylolytic enzyme will work. The nature of the iodine color (if any) and the value of the specific rotation would be informative.

Desirable starting product. It is necessary to start with a pure material, since the impurities in the starting product are all preserved through the treatment. Cycloheptaamylose is more easily obtained in a state of high purity than cyclohexaamylose. It is much less soluble than cyclohexaamylose, and can be removed from the hydrolysis mixture very completely by means of precipitants. Furthermore, it can be readily obtained in high yield from starch by adding toluene or other stable precipitants to the enzymolysis mixture.

Iodine Addition Products of Cyclohexaamylose

The iodine addition products of the cycloamyloses, noticed by Schardinger, are of unusual interest because of their close analogy to starch-iodine. Therefore three modifications were prepared and analyzed, and other modifications were observed under the microscope. The conditions under which these addition products are formed are most important in determining the modification which results. Frequently more than one modification is present in the same crystallization mixture, as for example when a solution is examined on a microscope slide. Blue-black hexagons are to be found around the border of the evaporating solution, while long needles extend into the bulk of the solution.

Modification 1.

This modification is formed when a concentrated aqueous solution of cyclohexaamylose is treated with a concentrated iodine-potassium iodide solution. It is essential to have a high iodide ion concentration. The crystals are blue-black hexagonal plates or prisms, and may be grown to fairly large size by slow evaporation of an appropriate solution. For chemical analysis, crystals were prepared by mixing together 5 g. of cyclohexaamylose in 15 ml. of water with 1.5 g. of iodine and about 1.1g. of potassium iodide dissolved in 5 ml. of water. The mixture was heated until no particles

were left undissolved, and allowed to cool. The precipitated sludge was filtered off, and microscopic examination showed it to consist almost entirely of short stubby hexagonal prisms. Since these crystals decomposed at 110° C., they were allowed to dry at room temperature.

For analysis, a weighed sample of about 0.3 g. was mixed with about 10 ml. of water and heated to dissolve. The resulting solution was rapidly titrated with 0.1 N thiosulfate without the use of any indicator other than the color of the iodine itself. After determination of the free iodine in this way, the solutions were transferred quantitatively to a 25 ml. volumetric flask and made up to volume at 24°. The amount of cyclohexaamylose in the solution was calculated from the rotation. The results of four determinations are given below:

Sample	Per cent of I_2	Per cent of cyclohexa-amylose	Ratio of I_2 to cyclo-amylose
1	16.4	62.5	.262
2	16.7	62.5	.267
3	16.7	62.5	.267
4	16.4	63.1	.260
Average	16.5	62.7	.263

The potassium iodide was determined by ashing weighed samples at about 500° - 600° (somewhat below the m. p. of

potassium iodide). The per cent ash in two samples was 11.2 and 10.5, or an average of 10.8%. The weight ratio of potassium iodide to iodine in the crystal is then .654.

Since the addition product occurs as a crystalline compound the cyclohexaamylose, iodine and potassium iodide must be present in definite proportions corresponding to a chemical formula. The ratio of the molecular weight of iodine to that of cyclohexaamylose is .2609, while the observed weight ratio is .263. Considering the lack of precision in the iodine determination caused by the use of small volumes and the necessity of titrating without an indicator, the results are considered to agree reasonably well with the calculated ratio of an equimolar mixture of iodine and cyclohexaamylose. Likewise, the ratio of potassium iodide to iodine (.654) is in good agreement with the calculated ratio for an equimolar mixture ($\frac{166.03}{253.86} = .6545$). The molecular formula neglecting water of crystallization is then $(C_6H_{10}O_5)_6 \cdot I_2 \cdot KI$.

For crystal structure work crystals were prepared by slow evaporation of a solution containing a high concentration of iodine and potassium iodide. The crystals were found to have a density of 1.776 $\pm$ .004, as measured by flotation in a mixture of carbon tetrachloride and ethylene dibromide. The unit cell constants are as follows:

$a_o = 16.00$ Å., $c_o = 39.7$ Å., $(a_o)^2 c_o \sqrt{3}/2 = 8810$ cu. Å.

The crystal density indicates that the unit cell contains 6

molecules each of cyclohexaamylose, iodine, and potassium iodide.

Careful examination of oscillation patterns and Laue patterns indicates a Laue symmetry of D_{6h}. Since the crystal is composed in part of optically active molecules, it must belong to the point-group D_6-62. Differentiation between space groups isomorphous with this point group must be made on the basis of present and missing orders of (0001). Therefore, a goniometer pattern of the reciprocal lattice net (HO$\bar{H}$L) was taken, and orders of (0001) examined. Although 21 orders were in position to reflect, only those orders whose indices were divisible by three appeared on the pattern. The space group is therefore D_6^4--$C6_22$ or its enantiomorph D_6^5--$C6_42$. Unit cells in these space groups require the presence of 12 asymmetric molecules, or 6 molecules containing two fold symmetry axes.

Although macroscopic crystals of this modification of the iodine addition product of cyclohexaamylose are completely opaque, smaller crystals have very interesting optical properties. If one observes the tiny hexagonal prisms under polarized light, a very strong dichroism is noticed. The ordinary ray is almost completely absorbed while the extraordinary ray is passed with practically no absorption. These striking optical properties are due to the presence of iodine, since crystals of cyclohexaamylose are perfectly colorless. In view of the fact that the iodine molecule is known to

absorb light most strongly when the electric vector is parallel to the axis of the iodine molecule (cf. p.22), it is possible to assume that in this crystal the axis of the iodine molecule is normal to the hexagonal axis. Such an arrangement would account for the almost complete absorption of the ordinary beam.

The two-fold axis of the cyclohexaamylose molecule must coincide with one of the two-fold axes of the crystal in order to satisfy the symmetry requirements of the space group. The most probable packing of these large ring shaped molecules is with the molecular axis coinciding with the a axis, or a crystallographically equivalent arrangement. Since the primitive translation along the a axis is 16 A., the packing thickness of the molecule is about 8 A. and the packing diameter is then about $8\sqrt{3}$ or about 13.8 A. In this arrangement of the cyclohexaamylose molecules, long tube-like structures are formed, similar to those formed by modification 5 of cyclohexaamylose.

If the iodine molecules are enclosed by the cyclohexaamylose, as in Freudenberg's model, each iodine atom lies along the a axis, and the symmetry requirements of the space group are fulfilled. Such an arrangement is in accord with the observed dichroism of the crystal.

In order to place the iodine molecules more accurately, and perhaps determine the position of the iodide ions, a

Patterson projection on the plane (0001) was constructed (appendix 2). Due to the rapid falling off of intensity with increasing diffraction angle, the resolution was poor. Nevertheless, significant maxima are present about equally spaced along each axis of the map, which is in agreement with the proposed arrangement of iodine molecules. The iodide ions as well as the potassium ions are unresolved.

Modification 2.

Crystals of an iodine potassium iodide addition product of cyclohexaamylose are formed when a rather dilute solution of iodine and potassium iodide are added to a dilute cyclohexaamylose solution. Solutions of cyclohexaamylose containing as little as 1% dry substance will give a voluminous precipitate on the addition of the iodine solution. The needle shaped crystals appear greenish bronze by reflected light, while under the microscope they appear colorless, or at most somewhat brown, depending on the size of the crystal. In polarized light the crystals show an extreme dichroism.

Fairly large crystals may be obtained by dissolving 5 g. of cyclohexaamylose in 100 ml. of hot water, adding 1.5 g. of iodine and 1.1 g. of potassium iodide, and by allowing the mixture to cool slowly to room temperature or lower. On examining the crystals under the microscope, no black hexagonal prisms (modification 1) are to be found, even though the proportions of iodine, potassium iodide and cyclohexa-

amylose used are exactly the same as used to prepare modification 1. Furthermore, crystals of modification 1 may be dissolved in hot water, and on cooling modification 2 is formed. Conversely, if a concentrated solution of potassium iodide is added to crystals of modification 2, the crystals dissolve and crystals of modification 1 are formed.

The crystals were analyzed in exactly the same manner as that used for modification 1. The results are as follows:

Sample	Per cent of I_2	Per cent of cyclohexa-amylose	Ratio of I_2 to cyclo-hexaamylose
1	17.0	66.0	.257
2	16.9	66.2	.255
3	16.9	67.1	.252
4	17.1	67.1	.256
Average	17.0	66.6	.255

The potassium iodide contents of two samples were 5.7% and 6.1%; average 5.9%. The ratio of potassium iodide to iodine is then .348 (calculated for KI/I_2, .327). The molecular formula for the second modification is therefore $[(C_6H_{10}O_5)_6 \cdot I_2]_2 \cdot KI$.

Crystals prepared as above have not been satisfactory for crystal structure work. The crystals are multiple in nature, some crystals being so imperfect that complete

rotation patterns were obtained from stationary specimens. Occasionally crystals were found from which goniometer patterns could be obtained, but even here the diffraction spots were highly streaked.

Inasmuch as the crystals were of very small size and of poor quality, no Laue patterns could be obtained, and the symmetry is undetermined. Although the symmetry of the goniometer patterns is approximately hexagonal, a microscopic examination of many smaller, more perfectly formed crystals indicates the symmetry to be orthorhombic or lower. the crystals are most frequently lath shaped needles, which are approximately rectangular in cross section.

The equatorial layer line of "fiber" patterns or rotation patterns about the needle axis may be indexed on the basis of a hexagonal unit cell, a_o= 13.8 Å., while the primitive translation along the pseudo-hexagonal axis (needle axis) is 15.4 Å. Such a pseudo-cell would contain two molecules of cyclohexaamylose and iodine and one molecule of potassium iodide. The true cell is undoubtedly several times larger.

As mentioned before, the crystals are extremely dichroic. Plane polarized light with its electric vector parallel to the needle axis is completely absorbed except in the very thinnest crystals. Such a dichroism indicates that the iodine molecules are aligned parallel to the needle axis.

The pseudo-cell g axis is equal to the packing diameter

of the cyclohexaamylose molecule, indicating that the two-fold axis of the molecule may coincide with the needle axis. Such an orientation would account for the strong tendency to crystallize in needles (a characteristic tendency of planar molecules). In support of such an arrangement, needles are sometimes observed to grow out from the hexagonal prisms of modification 1 with the needle axis extending along the a and b axes of the hexagon.

Modification 3.

When iodine is added to cyclohexaamylose solutions containing no iodide ions or substances which are capable of reacting with iodine to form any appreciable amount of such ions, a yellow-orange precipitate containing iodine and cyclohexaamylose is formed. Under the microscope the crystals are seen to be tiny needles tapered at both ends. Individual crystals are practically colorless, but have a slight yellow tinge. Under polarized light they are seen to be somewhat dichroic, changing from colorless to light orange as the plane of polarization is turned through 90°. The crystals were too small to allow any single crystal x-ray diffraction work or other determination of symmetry.

The crystals of modification 3 were analyzed for iodine and cyclohexaamylose by the procedure already given. The results are:

iodine: 17.6%, cyclohexaamylose: 63.8%.

The ratio of iodine to cyclohexaamylose is .276 (calculated

for an equimolecular mixture, .261). The molecular formula is then $(C_6H_{10}O_5)_6 \cdot I_2$.

When the crystals are suspended in pure water, they are stable and do not tend to change into other modifications. However, if a trace of iodide ion is added, the suspension immediately turns greenish-black with the precipitation of needle-shaped crystals. The iodide ion added may be in the form of potassium iodide, hydriodic acid, barium iodide, or apparently any other source of iodide ions. This simple experiment demonstrates in a striking way the importance of the iodide ion in the other crystal modifications.

Further modifications.

Other interesting modifications are formed by the substitution of other positive ions for potassium in the iodine-iodide mixture. One such modification that should be of interest is the barium iodide iodine addition product of cyclohexaamylose. At high iodide ion concentrations, small trigonal crystals were observed to form. The crystals were observed to be strongly dichroic, similar to crystals of the hexagonal modification (modification 1) but of the opposite sign of dichroism. The ordinary ray is freely transmitted, while the extraordinary ray is almost completely absorbed. Trigonal symmetry is indicated by the pronounced tendency to crystallize in triangular transparent prisms. Under polarized light, the crystals were uniaxial, both with respect to double refraction and dichroism.

The Crystalline Forms of Amylose

The amylose used in this investigation was prepared by Schoch and Kerr by butanol precipitation of starch or starch fractions (13) (21). This material has a very strong tendency to crystallize out of solution, forming the characteristic "retrograded" starch. Evidence that this material approaches ideal amylose is its high conversion with beta amylase, conversions of about 90 - 95% being obtained (66).

Oriented amylose.

The tendency of amylose solutions to form strong insoluble skins was noticed by Schoch (21). When these skins are carefully removed from the surface of the solution, rinsed well with distilled water and allowed to dry on a flat surface (aluminum sheet or ferrotype), they appear strongly birefringent under the microscope. The films are uniaxial negative, giving the characteristic negative uniaxial interference figure. Irregularities in the film structure, such as wrinkles, edges, etc., appear as especially birefringent regions, with the sign of the birefringence being positive with respect to the irregularity. The sign of birefrigence of the film itself indicates that the amylose molecules are extended parallel to the film surface.

Oriented x-ray diffraction patterns. Since the films of amylose are anisotropic, x-ray patterns taken with the

x-ray beam parallel to the surface show a considerable degree of orientation. Due to the diffuseness of the diffraction arcs and the lack of perfect orientation it was not possible to index the patterns without the help of the indices used by Bear and French (3) for B-type native granule patterns. The appearance of the oriented x-ray patterns agreed closely with that expected on the assumption that the 9 Å. axis of the B-type unit cell corresponds to the length of the amylose molecule. On this basis, the 16 Å. spacing is the double width of the amylose chain while the 6 Å. dimension is the packing thickness of amylose.

The relationship to maltose. The maltose unit cell, given before, is approximately 5 Å. by 15 Å. by 11 Å., these dimensions corresponding to the maltose molecule thickness, double breadth, and length respectively. These dimensions resemble the amylose unit cell dimensions quite closely, except that dimension which corresponds to the length of the respective molecules. This discrepancy is undoubtedly due to the closer packing allowed by chemical linkage between the glucose residues of adjacent maltose molecules.

The significance of oriented amylose. By the use of better oriented preparations and finer diffraction methods, it will undoubtedly be possible to investigate the configuration of amylose within the unit cell to a higher extent. However, the mere fact that oriented samples and oriented diffraction patterns may be obtained is of considerable importance.

In the first place, oriented patterns confirm that the amylose A--B diffraction pattern is due to a three-dimensional lattice rather than to one or two dimensional lattices, which are not capable of giving diffraction patterns showing two-dimensional orientation.

In the second place, the fact that oriented films are uniaxial negative indicates that the amylose molecules lie parallel to the film surface. As the film grows, it increases in thickness rather than in area. Since it is a well known tendency of highly asymmetric molecules to pack with the large dimension of the molecule normal to the direction of fastest growth of the crystal, an extended chain configuration rather than a compact helix is indicated. The 9 Å. periodicity of amylose along the film surface corresponds to the 10 Å. periodicity of cellulose along the fiber. Each of these periodicities must be that of the disaccharide building unit of the corresponding polysaccharides, i. e., maltose and cellobiose. The difference between the molecular periodicities of amylose and cellulose is undoubtedly due to the difference in linkages, being approximately that expected on the basis of models.

The swelling of amylose films. If oriented amylose films are treated with a swelling agent such as calcium nitrate solution, the films increase about 50% in surface area, while the thickness increases to about three to five times its original value. The only explanation of these

facts which is consistent with the physical structure of the amylose film is that the amylose molecule swells in a direction normal to the axis of the molecule without any decrease in length of the molecule. Since the amylose film is constrained by the molecular valence forces of the extremely long amylose chains, the bulk of the swelling must occur in a direction normal to the film surface. This direction is normal to the molecular chains, and there is no constraint to swelling.

The Absorption of Iodine by Amylose and Cyclohexaamylose

The theory of the formation of iodine addition products of starch and the cycloamyloses is rather poorly developed at present. Hanes (41) and Freudenberg (20) feel that the presence of starch residues which are capable of forming helices is the most important essential, while Meyer (43) states that iodine molecules are held in the crystal interstices and that the presence of water is of utmost importance, since ordinary dry starch does not absorb iodine vapor. The following simple experiment shows the correctness of Freudenberg's views and also that probably the function of water is to enable the amylose to assume the helical configuration.

The absorption of iodine vapor by dry starch types.

Various starch types including starch fractions, amylose crystallized in the A--B and V modifications, and various modifications of cyclohexaamylose were thoroughly dried in the oven at 110°. After cooling to about 80° in dry air, a small crystal of iodine was added to each sample and the mixture warmed for a few minutes to remove excess iodine. Although most of the samples remained white, or assumed a very light tan color, a few were turned deep purple or black. The results are indicated in table 2. It is notable that ordinary starch in the A--B or extended configuration is

incapable of absorbing iodine vapor, while amylose in the V configuration absorbs a large amount of iodine. Of the five modifications of cyclohexaamylose examined, only modification 5 absorbed iodine to any appreciable extent.

Table 2. Absorption of Iodine by Dry Starch

Starch Type	Iodine Color
Native starch granules (corn, potato)	None
Retrograded starch	Light tan
Amylose films	None
Amorphous amylose	None
Isoamylose (fraction of starch not precipitated by butanol)	None
Soluble starch granules	Light tan
Amylodextrin spherocrystals (A--B configuration)	None
Ethanol precipitated soluble starch (V configuration)	Dark brown
Butanol precipitated amylose (V configuration)	Dark blue-black
Cyclohexaamylose, modifications 1, 2, 3, and 4	No color or light tan
Cyclohexaamylose, modification 5	Dark purple--black
Amylodextrin, ethanol precipitated (V configuration)	Dark rust-brown

It should be noted that the preparations which absorb iodine have tube-like structures (the helix in the V configuration and the tube in cyclohexaamylose modification 5). It is apparent that the tube structure, or the ability to form such a structure, is the essential feature of the starch-iodine addition. Any necessity of water, iodide ions, etc., is incidental to the formation of the tube structure, since in this experiment insignificant quantities of water or iodide were present.

Dichroism of cyclohexaamylose 5--iodine.

Although the addition products of iodine with dry amylose showed no organization under the microscope, the dry iodine addition product with cyclohexaamylose 5 had very interesting optical properties. If the crystals are warmed with an excess of iodine, they become completely opaque, but if only a trace of iodine is used the crystals become strongly dichroic, similar to crystals of the addition product formed in solution. The dichroism of the dry iodine addition compound indicated that the iodine molecules were oriented parallel to the crystal two-fold axis. This observation is considered good supporting evidence that in all strongly colored iodine addition products of iodine with amylose or cyclohexaamylose the axes of the iodine molecules coincide with the axis of the tube-shaped carbohydrate structure.

The absorption of iodine by aqueous suspensions of starch types.

If suspensions of crystalline preparations of amylose types are placed under the microscope and treated with a small amount of iodine solution, there is a considerable difference in behavior. While amylodextrin spherocrystals (A---B configuration) remain uncolored, native starch granules are colored a deep blue. Although lightly stained granules are highly birefringent, there is no noticeable dichroism, indicating a random orientation of the iodine molecules. The fact that amylodextrin spherocrystals remain uncolored is in direct discord with Meyer's views on the mechanism of starch-iodine formation. Amylodextrin is a crystalline material with crystallites about the same size as those of starch, and presumably has interstices like those supposed to exist in starch. If the iodine coloration were dependent upon the presence of interstices, amylodextrin should be colored as well as native starch granules.

Crystals of amylose precipitated by butanol. Although there is little external similarity between crystalline amylose and cyclohexaamylose modification 5, their optical properties and behavior with iodine are closely related. Amylose crystals, which occur as rectangular platelets, are approximately uniaxial negative, with the optic axis normal to the platelet. On the addition of a small amount of iodine to a suspension of amylose crystals, they become extremely

dichroic, similar to the iodine addition product of cyclohexaamylose modification 5. Both crystals are uniaxial in their absorption characteristics, the optic axis of cyclohexaamylose coinciding with the acute bisectrix before adding the iodine. Such a correspondence in the optical properties is a strong indication of a similarity of structure. The only structure for the amylose crystals which will explain these optical properties is a packing of helical molecules with the helix axis normal to the platelet surface. On absorbing iodine, the iodine molecules are constrained to lie normal to the platelet surface, producing the observed dichroism. As before mentioned, amylose crystals when dry give typical V patterns, and may therefore be considered to possess the helical configuration. Further evidence that this is the case is presented in the following section.

The Amylose--Iodine Addition Product

When dry amylose in the V configuration is allowed to absorb iodine vapor, it may take up as much as 30% of its weight of iodine. This amylose--iodine addition product now gives a new x-ray diffraction pattern. The pattern appears similar to the V pattern, and although there are many more lines present the pattern maintains a simple appearance. The pattern is in no way related to the iodine crystal pattern.

Indexing the amylose--iodine x-ray pattern.

The $\sin^2\theta$ values were calculated for the first 10 lines of the powder pattern and are given in the second column of table 3.

Table 3. Powder Data for Amylose--Iodine.

Relative intensity	$\sin\theta$	$\sin^2\theta$ /.00469	Index
st.	.06845	1.00	100
m.	.11872	3.01	110
m.	.13682	4.00	200
v. st.	.18100	6.99	210
m. w.	.20552	9.02	300
v. w.	.23698	11.99	220
m. w.	.24694	13.01	310
v. w.	.27347	15.98	400
w.	.29838	19.01	320
m. w.	.31387	21.02	410

On calculating the ratio of the $\sin^2\theta$ values, it is seen that the values are all very close to whole numbers. This particular set of integers is characteristic of the hexagonal lattice, being the ratio of the $\sin^2\theta$ values for the prism zone reflections (63). Since all the reflections are from the prism zone ($l = 0$), it is evident that the crystal has a two-dimensional structure, or rather that the reciprocal lattice is a two-dimensional net. The primitive translation of this lattice is 13.0 Å.

The fact that the amylose--iodine addition product forms a two-dimensional hexagonal lattice is very strong evidence that the molecules are cylindrical in form. The primitive translation is approximately equal to the diameter of a starch helix with about six glucose residues to the turn. It is impossible to differentiate between trigonal and hexagonal two-dimensional lattices on the basis of x-ray data alone. In fact, it is quite possible that the lattice is really a two-dimensional orthorhombic lattice or a lattice of even lower symmetry. Since all the observed reflections correspond to a hexagonal lattice, and all the possible reflections of a two-dimensional hexagonal lattice are present (within the range examined), the crystal may be assumed to have at least an effective hexagonal lattice.

Fourier synthesis of amylose--iodine.

If the iodine atoms are at the two-dimensional lattice

points, the phase constants are all + 1 and a Fourier synthesis of electron density becomes possible. A preliminary synthesis along a line joining two-dimensional lattice points indicated a large maximum at the origin and a lesser maximum 4.2 Å. from the origin. A complete synthesis covering the entire two-dimensional unit cell has not as yet been completed. Such a synthesis is expected to demonstrate clearly the presence of the helical amylose molecule in amylose-iodine.

The Action of Macerans Amylase

Schoch has indicated that acid modified starches give much poorer yields of the cycloamyloses than unmodified starch. In order to determine the nature of the macerans amylase reaction, and to find a practical substrate for the production of cycloamyloses, several different starch types were submitted to enzymolysis and the reaction product tested for cycloamyloses by the addition of iodine solution. The formation of the characteristic needles and black hexagonal prisms was considered a positive test for the production of cyclohexaamylose.

Production tests by various starch types.

For the testing of starch types, 3% aqueous solutions were prepared by autoclaving 30 min. at 20 lb. pressure. To 10 ml. of the starch solution were added 2 ml. of the concentrated enzyme solution (activity = 0.3, Tilden scale) and the mixture was incubated 12 hr. at 40° C. Three drop portions of each digest were withdrawn and each mixed with 1 drop .1 N iodine solution on a spot plate. After standing a few minutes, the borders of the evaporating solutions were examined for the characteristic blue-black hexagons of cyclohexaamylose-iodine addition product (modification 1). The results are summarized in table 4. A "+" sign indicates the formation of the characteristic crystals, while a "?" sign

indicates that possibly the crystals were produced in small quantity, and a "-" sign indicates no crystals whatsoever. A blank containing the same amount of enzyme and iodine solution showed absolutely no crystals, even on complete evaporation of the solution.

Table 4. Action of Macerans Amylase

Starch type	Production of cyclo-amyloses	Appearance of solution on addition of iodine
Whole potato starch	+	Clear
Whole corn starch	+	Dark
Whole waxy maize starch	+	Clear
Butanol precipitated amylose	+	Black ppt., supernate clear
Non-precipitated amylose	+	Clear
Acid solublized potato starch	+	Slightly dark
Glycerol solublized potato starch	+	Clear
Glycogen	+	Slightly dark
Amylodextrin from potato starch	+	Clear
Beta amylase limit dextrin from corn starch	-	Dark
Oxidized corn starch	?	Very dark
Corn syrup dextrins	+	Clear

All starch types tested with the exception of the beta amylase limit dextrin and possibly also strongly oxidized starch produced cycloamyloses. Since the most strongly acid-hydrolyzed preparations (amylodextrin and the corn syrup dextrins) yielded considerable amounts of cyclohexaamylose, it must be assumed that solubilization of starch by acid treatment

does not break those units in starch which are responsible for the formation of cycloamyloses. A more plausible explanation for the failure of thin-boiling starch to give sizable yields of the cycloamyloses is its high tendency to precipitate or retrograde.

On the other hand, the limit dextrin is the only starch type which lacks the presence of a non-reducing unbranched chain. It is therefore apparent that the macerans amylase and beta amylase work on the same part of the starhh molecule. In this connection, a correlation of the yields of maltose and cycloamyloses from various starch types would be of interest.

The use of toluene during enzymolysis.

Toluene is known to be an effective anti-mold agent as well as a precipitant of the cycloamyloses. When enzymolysis of starch by macerans amylase is carried out in the presence of an excess of toluene, an abnormal amount of cycloheptaamylose is produced. Whereas the normal ratio of cycloheptaamylose to cyclohexaamylose produced is about 1:4, by the use of toluene as a preservative during enzymolysis practically no cyclohexaamylose is formed. In one trial run using potato starch, three 50 g. samples of starch yielded respectively 13 g., 18 g., and 19 g. cycloheptaamylose by the use of toluene. These yields amount to about 35% on the basis of the weight of starch taken, which is approximately the normal yield of

cyclohexaamylose and cycloheptaamylose combined.

Since it is seen possible to alter the normal course of the enzymolysis by the addition of suitable precipitants, it is herewith suggested that precipitants might be found which would induce a higher yield of the higher members of the cycloamylose group (cyclooctaamylose, cyclononamylose, etc.).

Optical Rotation in the Amylose Series

By the use of Freudenberg's equation (p. 22), it is possible to calculate the specific and molecular rotation of any amylodextrin if the specific rotations of both amylose and maltose are known. The molecular rotation of an amylodextrin is considered to be the sum of the molecular rotations of each glucose unit in the chain. The sum of the molecular rotations of the first and last glucose units of the chain is assumed to be equal to the molecular rotation of maltose, while the molecular rotation of any intermediate glucose residue is equal to the molecular rotation per glucose residue of an infinite amylose chain.

The specific rotation of amylose.

Values given in the literature for the specific rotation of whole starch range from about $[\alpha]_D = +190^\circ$ to $+230^\circ$. A determination of the specific rotation of amylose was made by Meyer, who dissolved amylose in alkali, neutralized with acid and quickly measured the rotation. He reports a value of $[\alpha]_D = +220^\circ \pm 5^\circ$, which is somewhat higher than most values reported for whole starch. Accordingly an amylose solution was prepared by dissolving potato starch butanol precipitated amylose in boiling water, and the solution was allowed to cool to room temperature. The rotation was quickly taken, and the solid content of a 25 ml. sample was determined. A value

of $[\alpha]_D = 200^\circ$ was obtained for the specific rotation of this particular sample.

The turbidity of 1% amylose solutions in water makes an accurate determination of the specific rotation difficult and rather inaccurate, as the wide range in values reported must indicate. It may well be asked if another method for the determination of this important value is available .

Since it is possible to calculate the specific rotation of an amylodextrin given the specific rotations of maltose and amylose, it is equally possible to calculate the specific rotation of amylose given the specific rotation and chain length of an amylodextrin. The molecular rotation of an amylodextrin containing n glucose residues is:

$[M]_n = [M]_{maltose} + (n-2) \times [M]_{amylose}$ per glucose residue from which the value of the specific rotation of amylose may be calculated. Using a value of $[\alpha]_D = +136^\circ$ for maltose and $[\alpha]_D = +193^\circ$ for an amylodextrin containing 22 glucose residues, one obtains a value of $[\alpha]_D = +201^\circ$ for an infinite amylose chain, or about $+32,600^\circ$ for the molecular rotation per glucose residue.

The molecular rotations of the cycloamyloses.

The specific rotation of cyclohexaamylose is $+151.4^\circ$, and the molecular rotation is $+147,300^\circ$. The corresponding rotations of cycloheptaamylose are $+161.9^\circ$ and $+183,900^\circ$.

On the basis of Hudson's additivity rule it might be assumed that the specific rotation of the cycloamyloses would be the same as that of amylose, since each glucose residue in the cyclic molecule is equivalent to a glucose residue in an infinite amylose chain. However, since the difference between the molecular rotations of cycloheptaamylose and cyclohexaamylose is approximately the molecular rotation of a glucose unit in an amylose chain, it appears that the specific rotation of any cycloamylose may be calculated by a modification of Freudenberg's equation:

$$[M]_{\text{cyclo-n-amylose}} = [M]_{\text{cyclohexaamylose}} + (n-6) \times [M]_{\text{amylose per glucose residue.}}$$

or

$$[M]_{\text{cyclo-n-amylose}} = 147{,}300^{\circ} + (n-6) \cdot 32{,}600^{\circ}.$$

The specific rotations of higher cycloamyloses so calculated are given in table 5.

Table 5.

Optical Rotations of Cycloamyloses.

n	M. Wt.	$[M]$	$[\alpha]_D$
4	648	+ 82,100	+ 127°
5	811	114,700	141°
6	973	147,300	151°
7	1135	179,900	159°
8	1297	212,500	164°
9	1459	245,100	168°
10	1621	277,700	171°
20	3243	603,700	186°
50	8107	1,581,700	195°
100	16214	3,211,700	198°

By way of comparison with these calculated values, the specific rotations given by Freudenberg and Jacobi (36) for the alpha, beta, gamma, delta, and epsilon dextrins are respectively: +148°, +158°, +160°, +166° and +171°. These values resemble closely calculated values for cyclo-n-amyloses, where n is 6, 7, 8, 9, and 10. Unfortunately the higher cyclo-amyloses reported by Freudenberg and Jacobi were not available for accurate molecular weight determination or characterization by means of iodine addition products.

Acid Hydrolysis of Starch and its Relationship to Granule Structure

Although it is well established that the action of dilute acids on starch is to break the primary glucosidic linkages and break down the large molecules to smaller and perhaps more simple molecules, the exact nature of the process deserves considerably more attention. Whereas the ultimate product of hydrolysis of starch pastes is glucose, starch granules are never completely hydrolyzed by dilute acids in the cold. The end product, called amylodextrin, contains about 20 - 25 glucose residues, and it may be obtained in amounts up to about 50% of the original starch. Another indication of the essential difference in the course of the hydrolysis reaction in starch pastes and granules is the ability of the partially hydrolyzed starch to digest with beta amylase. Amylodextrin has a very high conversion limit (30), 75% or more being converted to maltose, while a corresponding dextrin obtained by acid hydrolysis of starch pastes converts only to the extent of about 30% (67). Furthermore, there is a pronounced difference in the crystallinity of the two products. Amylodextrin can readily be obtained in spherocrystals, but dextrins produced by hydrolysis of starch pastes stubbornly refuse to crystallize.

These facts point to a constitutional difference between amylodextrin and the corresponding product from starch paste degradation. The most logical interpretation is that the

amylodextrin is essentially a straight-chain compound whereas the paste dextrin has a branched structure.

Acid hydrolysis of the starch granule.

Quantitative measurements of the birefringence of starch granules indicate that during the course of mild acid hydrolysis the birefringence remains unaffected, or may even increase somewhat. This indicates that it is not the crystalline portion of starch which is being attacked by the acid, but rather the amorphous material which holds the crystallites together. Since branch points in the starch chain and other structural irregularities are incapable of participating in crystallite formation, these amorphous regions are hydrolyzed by the acid with the production of easily soluble low molecular weight compounds, which diffuse into the bulk of the solution. The organization of the crystallites protects them against rapid hydrolysis, since dilute acid is unable to penetrate the crystalline regions. Only the straight chains and straight portions of branched chains are found in the original crystallites, and so by recovering the crystalline portions of the original starch granule it is possible to obtain a purely straight chain amylodextrin. It is important to note that this point of view does not require a selective rupture of the characteristic bonds of branching, but rather the eventual rupture of all bonds in the amorphous regions of the starch granule.

The action of acid on granules during the solubilization process may be considered the first step in the above process. Since the acid affects only those regions containing branches, the product resulting from brief acid treatment behaves more nearly like amylose than the original starch. In this regard, the well known tendency of acid modified starches to undergo precipitation or retrogradation may be pointed out. Such behavior is characteristic of amylose.

Acid hydrolysis of starch pastes.

Measurements of the kinetics of hydrolysis of starch indicate that there is no linkage present which is hydrolyzed more rapidly than the main type of linkage present, i. e., α-1,4- glucosidic bonds. Therefore, the other linkages which are known to occur in starch must hydrolyze at least as slowly as the main linkages. For the purpose of this discussion, it will be assumed that the velocity constants for hydrolysis of all types of glucosidic linkages present are equal.

Methylation studies indicate that the average "chain length" for raw starch is about 25--30 glucose residues. Since the reducing power of starch is very low, it may be assumed that approximately one linkage out of every 25 is a branch-type linkage, or the probability that any one bond in starch will be a characteristic α-1,4-glucosidic bond is 24/25. The probability that a dextrin containing 25

glucose residues will contain only α-1,4-linkages is then $(24/25)^{25}$ or .36. If amylodextrin is present to the extent of only two or three per cent of the total solid content of a solution, a large amount of it may be crystallized by freezing. However, the dextrin from paste hydrolysis appears to be impossible to crystallize by this method. This indicates that practically all of the dextrin molecules are branched, which in turn points to the stability of the branch linkages to acid hydrolysis.

The relationship to starch granule structure.

The size of fragments of the original starch molecules which are isolated by granule hydrolysis give a clue to the original crystallite size. Estimates of the molecular size of various preparations of amylodextrin range from about 14 to 25 glucose residues, depending on the length of acid treatment. Most estimates of chain length carried out in connection with this research range between 20 and 25 glucose residues for amylodextrin from potato starch. The original crystallite may then be assumed to contain about 25 glucose residue portions of starch molecules, or assuming a length of 4.5 Å. per glucose residue, the crystallite would be about 110 Å. long. This value agrees well with the value of 100 Å. calculated from the width of the x-ray diffraction pattern lines (9).

It may well be that the crystallite sizes of different

starches may be quite different. For this reason, a determination of the chain length of amylodextrin prepared from, say, waxy maize starch or glutinous rice starch would be of interest. These starches apparently contain almost no purely straight-chain starch, as shown by their failure to stain blue on the addition of iodine. However, the molecules must contain straight chain regions, since the granules are strongly birefringent and give good x-ray diffraction patterns.

VI. CONCLUSIONS

1. Single crystals of maltose hydrate may be prepared by slow evaporation of a dilute alcoholic solution. The crystals are biaxial, negative. The unit cell constants are $a_o = 4.9$ Å., $b_o = 15.2$ Å., $c_o = 10.7$ Å., $\beta = 82.5^\circ$. The monoclinic unit cell contains two molecules and it belongs to the space group C_2^2. The packing dimensions of the maltose molecule are approximately 5 Å. by 7.6 Å. by 11 Å.

2. The molecular weights of the Schardinger alpha and beta dextrins are 973 and 1135, corresponding to six and seven glucose residues per molecule. The compounds have been named cyclohexaamylose and cycloheptaamylose, and their specific rotations are $[\alpha]_D^{24} = +151.4^\circ$ and $[\alpha]_D^{24} = +161.9^\circ$.

3. The cyclohexaamylose molecule shows a two-fold axis of symmetry in one of five crystal modifications examined (modification 5). The molecule is ring-shaped with a packing diameter of about 13.8 Å. and a packing thickness of about 8 Å.

4. Cyclohexaamylose combines with iodine and varying amounts of iodide to form crystalline compounds. Modification 1, $(C_6H_{10}O_5)_6 \cdot I_2 \cdot KI$, forms blue black hexagonal crystals; modification 2, $[(C_6H_{10}O_5)_6 \cdot I_2]_2 \cdot KI$, forms greenish-bronze needles; modification 3, $(C_6H_{10}O_5)_6 \cdot I_2$, forms orange-yellow needles. Other compounds are formed in

the presence of barium ions, etc.

5. In the strongly colored iodine addition products 1 and 2, the cyclohexaamylose molecules form long tube-shaped structures, while the iodine atoms form a linear chain through the center of the tube. This structural feature is responsible for the extreme dichroism of both modification 1 and modification 2.

6. Oriented films of amylose are strongly anisotropic. The birefringence is uniaxial, negative. Oriented x-ray diffraction patterns from amylose films confirm previous unit cell determinations for granular starches. The 9 Å. dimension of the unit cell corresponds to the length of the fundamental repeating unit in the amylose chain, i. e., maltose. The swelling of amylose films is due to a lateral expansion rather than to a molecular contraction.

7. Dry amylose in the V configuration and modification 5 of cyclohexaamylose take up large amounts of iodine vapor. Amylose crystals and cyclohexaamylose modification 5 have similar optical properties before and after taking up iodine, indicating a structural similarity. In the iodine addition products of these pre-formed crystals, the iodine molecules enter the tube-shaped structures and are held on the tube axis (helix axis of amylose and molecular axis of cyclohexaamylose).

8. Amylose-iodine gives an x-ray diffraction pattern similar to the V pattern. The pattern may be indexed on

the basis of a two dimensional hexagonal lattice, a_o = 13.63 Å. A Fourier synthesis of electron density distribution is in agreement with a helical amylose molecule with iodine molecules lying on the helix axis.

9. B. macerans amylase acts on all starch types except strongly oxidized starch and beta amylase limit dextrin. B. macerans amylase and beta amylase act in the same manner on the starch molecule, i. e., the enzyme works from the non-reducing end of an amylose chain and breaks the chain with the formation of a cycloamylose molecule. Degradation of the amylose chain continues in a similar manner until the action is stopped because:

(a) the amylose chain is degraded to a low molecular weight stub or to a branch point, or

(b) the amylose chains are made unavailable to the action of the enzyme by retrogradation or crystallization.

10. The specific rotation of starch is approximately $[\alpha]_D^{24} = +201^\circ$. The specific rotations of the cyclo-n-amyloses may be calculated by a modification of Freudenberg's equation.

11. Acid hydrolysis of the starch granule results ultimately in the degradation of all intercrystalline or amorphous starch, leaving practically intact the original crystallites of the starch granule. Acid hydrolysis of starch pastes tends to increase the ratio of branch linkages

to amylose linkages. Acid modification of starch granules tends to eliminate branching, and therefore to make starch more like amylose.

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IX. VITA

Dexter French was born in Des Moines, Iowa, February 23, 1918, the second child of Minnie E. Ormerod and Raymond Albert French. After graduating from Dubuque High School in 1935, he entered the University of Dubuque, Dubuque, Iowa. In 1938 he received the degree B. A. with majors in Chemistry and Mathematics. From 1938 to 1942 he was a graduate student at Iowa State College, Ames, Iowa. His study of the investigation of structure in the carbohydrate field was under the direction of Dr. R. S. Bear until 1941, when Dr. Bear's place was taken by Dr. R. E. Rundle. In 1939 he married Mary Catherine Martin.

APPENDIX 1.

DESCRIPTION OF THE RECIPROCAL LATTICE X-RAY GONIOMETER

Since the Clark-Gross modification of the x-ray goniometer described by de Jong and Bouman (62) is considerably more versatile, simple, and useful than the original model, it is described here in sufficient detail to allow duplication. In the goniometer constructed by de Jong and Bouman it was possible to photograph only rather high order layer lines, but with the instrument developed by Clark and Gross it is possible to obtain patterns from all layer lines from the equatorial to the highest allowed by the wave length of the radiation used.

The instrument is supported by a $\frac{1}{2}$ in. o. d. brass collimator, which in turn is rigidly fastened directly to the x-ray tube. The body of the goniometer is merely a small brass block with four $\frac{1}{2}$ in. holes drilled for the collimator, crystal support bearing and film support bearing. The bearings for crystal support and film support are accurately parallel and may conveniently be placed 3 cm. apart. Two holes are provided for the collimator, one at an angle of 90° to the axes of rotation of the crystal and film and another at an angle of 60°. It is important that the axes of all four holes be coplanar and that the axes of the two holes for the collimator coincide at a fixed

point on the axis of the crystal support. The crystal support is designed to allow the crystal to be mounted at this fixed point in order to enable one to use the same mounting of the crystal with different orientations of the collimator. A support is made for a small flat photographic film normal to the crystal support axis and about 5/16 in. above the crystal. The main film support is a circular disk 1/16 in. thick to which a circular film may be fastened by means of a ring-shaped clamp. The film support is mounted on the film support bearing rod by means of a collar and set screw. The support and clamp should be so constructed that the film will be as flat as possible and accurately normal to the crystal and film rotation axes.

The crystal and film are rotated by a small synchronous motor to which both crystal support bearing rod and film support bearing rod are geared. It is essential that both crystal and film rotate smoothly and synchronously.

A removable crystal support is almost a necessity. The crystal may be oriented on the crystal support by the ordinary methods and the support may then be mounted in a fixed position on the crystal support bearing. To test for accurate orientation of the crystal and to enable one to cut masks which will exclude the undesired layer lines, a rotation pattern is taken using the small auxiliary film. If the crystal is accurately oriented, the pattern consists of concentric rings of diffraction spots, each ring corresponding

to a layer line on a cylindrical film. A mask to remove the undesired layer lines is cut from thin sheet metal (lead foil) using the small auxiliary film as a guide. Since the position of the equatorial layer line does not vary, a permanent mask for this layer line was turned out from a flat ¼ in. plate of brass. With this mask it is possible to resolve layer lines having a separation of only 2°. The undiffracted x-ray beam tends to fog the film and may be removed by a narrow strip of lead.

The correct position of the film holder for any layer line is calculated from the primitive translation along the axis of rotation, which is determined by the usual methods, or can be found with sufficient accuracy from the auxiliary film by a graphical construction method.

Calculation of lattice constants. The origin of the reciprocal lattice is the intersection of the x-ray beam with the axis of rotation of the film. The scale of the reciprocal lattice thus depends on the distance between this point and the crystal. For 90° incidence this is 3 cm., while for 60° incidence it is 2 3 cm. Therefore, for normal incidence, $\lambda/2$ (.77 Å. for Cu Kα) is equivalent to 6 cm. and to find the distance on the reciprocal scale, divide $6\lambda/2$ (4.62) by the distance in cm. Similarly, for 60° incidence, $\lambda/2$ is equivalent to $4\sqrt{3}$ cm., and to find the distance on the reciprocal scale, divide $4\sqrt{3}\lambda/2$ (5.33) by the distance in cm. Since the reciprocal lattice

pattern is a "photograph" of the reciprocal lattice net, the angle between reciprocal axes may be measured directly on the film.

APPENDIX 2.

PATTERSON MAP OF CYCLOHEXAAMYLOSE-IODINE ADDITION PRODUCT (MODIFICATION 1)

Intensities of the prism zone reflections of cyclohexaamylose-iodine (modification 1) were estimated visually on a reciprocal lattice goniometer pattern. The intensities were corrected for polarization and values proportional to F^2 obtained.

Table 6.

F^2 Values for the Prism Zone Reflections

(hkl)	cF^2
(100)	Unobserved
(110)	5.1
(200)	1.8
(210)	0.0
(220)	0.0
(300)	4.2
(310)	0.4
(320)	0.0
(330)	2.7
(400)	7.4
(410)	0.1
(420)	3.3
(430)	0.8
(500)	2.6
(510)	1.2
(520)	0.0
(600)	4.4
(610)	0.7
(700)	0.5

The F^2 values given in Table 6 were handled by the method of Lipson and Beevers (64). A contour map of the result is shown in Fig. 1.

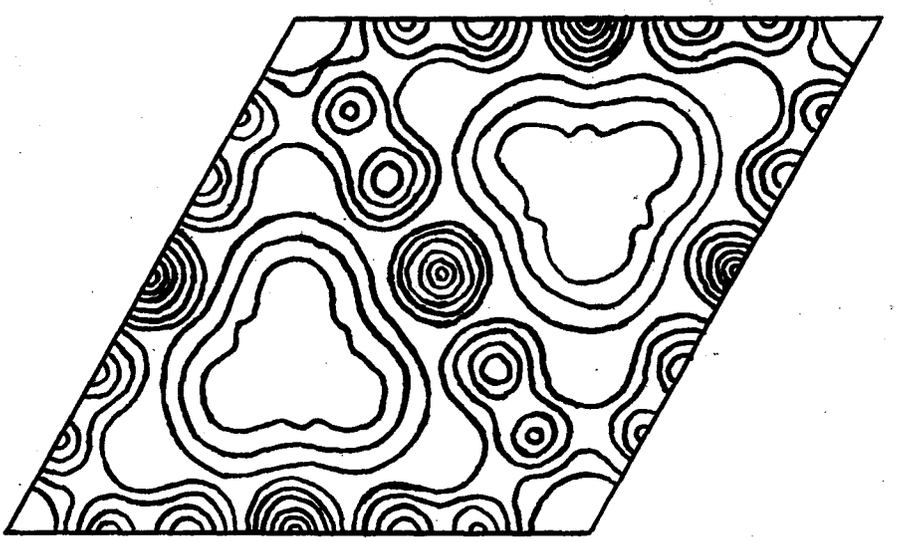

Fig. 1. Patterson Map of
Cyclohexaamylose-iodine Modification 1.