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Mathematical analysis of amylase action

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MATHEMATICAL ANALYSIS OF AMYLASE ACTION

by

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I. INTRODUCTION

Without attempting a complete definition of biochemistry, it may be stated that it deals, along with other things, with the processes occurring in living tissues. The complexity of the subject is appreciated if one considers the types of compounds and processes that are involved. The former are often large organic molecules of unknown molecular constitution; the latter generally proceeding by intricate reaction mechanisms which are not yet clearly understood.

A common feature of biochemical reactions is that they are enzymically controlled processes. Since enzymes themselves are macromolecules and since they differ widely in their action, it is not surprising that a mathematical formulation of a unified theory of enzyme action has not been accomplished.

The enzymes are classified according to the substrates upon which they can act. The carbohydrases are, therefore, enzymes capable of acting on carbohydrates. Since the discovery of malt amylase in 1833, numerous carbohydrases differing in their substrate specificity, their constitution, their mode of action, etc., have been reported. Inasmuch as amylases are concerned with the hydrolysis of starch, and are widely distributed in plant and animal materials, they have

probably been studied more than any other enzyme type. These studies have shown that amylases consist of several types of enzymes of which alpha, beta and macerans amylase are examples.

It was the purpose of these investigations to study the mechanism of amylase action on low and high molecular weight carbohydrate substrates. Although the action of alpha and beta amylases is irreversible under normal circumstances, that of macerans amylase is readily reversible. Consequently, studies on the equilibria in macerans amylase reactions were also undertaken.

For these studies, substrates of known constitution are most desirable. In this respect, amyloheptaose, the linear heptasaccharide consisting of seven glucose residues joined through alpha 1-4 linkages has been most useful. Amylodextrin, the crystalline degradation product of starch, and amylose, the linear component of starch, though both somewhat heterogeneous, have also been valuable substrates for studying these reactions.

The investigations reported in this dissertation have been conducted with purified amylase enzymes on carbohydrates of known constitution. They have been directed towards (1) elucidation of the mechanisms of amylase action, (2) consideration of equilibria in reactions of macerans amylase, and (3) quantitative interpretation of enzyme action.

II. REVIEW OF PERTINENT LITERATURE

A. Starch Oligosaccharides

A starch oligosaccharide is defined by Levine (51) as a homogeneous glucose polymer of definite molecular constitution containing only those types of linkages normally found in starch. Such compounds may be derived from either amylose or amylopectin; they may be composed of linear, branched or cyclic structures or any combination thereof; they may contain alpha 1-4 or alpha 1-6 linkages or both; in short, they include any single molecular species of disaccharides or higher glucose polymers.

The preparation of a pure oligosaccharide is accompanied by many difficulties (e.g. non-crystallizability of the compounds, non-specificity of precipitating agents, etc.) and has been accomplished only in a few instances.

The Schardinger dextrins representing at least three distinct molecular species - alpha, beta and gamma dextrins, are a notable exception. These compounds are cyclic, non-reducing dextrins formed by the action of macerans amylase on starch or starch-like compounds. They can be separated readily from crude starch digests because they form insoluble crystalline complexes with many organic solvents, such as hydrocarbons or halogenated hydrocarbons. The dextrins,

themselves, readily crystallize from water or water-alcohol solutions; likewise, their derivatives crystallize from appropriate solvents. Consequently, the Schardinger dextrins have been prepared in a high state of purity.

The literature on the preparative procedures have been admirably and thoroughly reviewed by Levine (51), and will not be considered here. The controversy over the molecular size of these compounds by Freudenberg et al. (30, 33, 34) and French et al. (22, 25) is ended. Freudenberg (27) now accepts the hexasaccharide, heptasaccharide and octasaccharide structures for the alpha, beta and gamma compounds.

Although these dextrins are composed of glucose residues joined by typical starch linkages - alpha 1-4 glucosidic bonds (26, 32) they exhibit many chemical and physical properties atypical of starch. They are resistant to the action of most hydrolytic amylases (26) and hence useless for studying the mode of action of these enzymes on low molecular weight compounds. They are, however, useful and convenient starting materials for preparing other starch oligosaccharides that, in turn, may be used for enzymic studies. French, Levine and Pazur (22) have reported on the preparation and properties of amyloheptaose, a linear heptasaccharide obtained on mild acid hydrolysis of Schardinger beta dextrin. Obviously since both molecules are heptasaccharides, the rupture of one alpha-1-4 bond in the beta dextrin molecule

leads directly to the linear amyloheptaose. Similarly, the linear hexasaccharide and octasaccharide may be obtained from the alpha and gamma compounds.

Previous to the report on the preparation of amyloheptaose, several workers claimed that they had succeeded in preparing pure oligosaccharides. Waldschmidt-Leitz and Reichel (98) reported a "crystalline" amylohexaose obtained by pancreatic amylase digestion of erythroamylose. More recently Myrbäck (66, 67) employed a similar procedure, and was not able to obtain the crystalline product. He does claim to have prepared the hexasaccharide in a homogeneous form but only in an amorphous state. Since neither the action of pancreatic amylase on starch is specific nor the alcohol fractionation procedure selective, it is questionable whether Myrbäck's preparation was homogeneous.

Samec and Klemen (84) described a crystalline trisaccharide isolated from a hydrolyzate of pancreatic amylase-erythroamylose digest. Their work could not be verified in this laboratory (78).

Perhaps, the first preparation of quite pure linear oligosaccharide derivatives was that obtained by Freudenberg and Friedrich (28). After the methylation of acetylated starch, they isolated a methylated trisaccharide and tetrasaccharide by distillation of reaction mixture at reduced pressure. These preparations were in all likelihood quite

pure, but since no satisfactory method of demethylation is available their usefulness would be limited.

In the period following the amyloheptaose work, another oligosaccharide, amylotriose (maltotriose), has been prepared in pure form. This compound was reported simultaneously from two laboratories. Wolfrom et al. (35, 89) isolated the trisaccharide from an enzymic digest of starch. The trisaccharide acetate was obtained in crystalline form but the trisaccharide itself only in an amorphous state. French and co-workers (24) have prepared amylotriose by a more direct method. Amyloheptaose, a compound of known constitution is converted to one molecule of amylotriose and two molecules of maltose by the action of beta amylase. The trisaccharide is obtained as a sirup on alcohol fractionation of the resulting mixture.

Other mention of oligosaccharides known to be mixtures is made by Myrbäck (64, 65, 69, 76), in connection with action of alpha type enzymes, by Levine, Foster and Hixon (52) with fractionation of corn sirup dextrins and by Swanson and Cori (91) with activation of phosphorylase by low molecular weight glucose polymers. The resolution of mixtures similar to the previous ones by absorption analysis (99) and by paper chromatography (80) has been accomplished. However, the isolation and characterization of the individual compounds was not attempted.

Crystalline oligosaccharides in the amylodextrin range (ca 20 glucose residues per molecule) have been reported in the early literature by Nägeli (70) and by Brown and Morris (11). These compounds were obtained by an extended action of cold mineral acid on granular starch. Although the authors claimed that their compounds were homogeneous, there is little doubt that in actuality their amylodextrin preparations were also mixtures. Compounds similar to amylodextrin are reported by Köhler-Hollander (48) and Koehler (47). These were obtained on fractionation of a salivary amylase digest of erythrodextrin. When one considers that the molecular weight of the crystalline dextrin was determined by reducing methods and found to be 17.5 glucose residues, one must view Koehler's contention that these preparations are homogeneous with considerable skepticism.

B. Macerans Amylase

The earliest report of an organism which produced non-reducing crystalline dextrins from starch was that by Villiers (97). Later Schardinger (85, 86) isolated the organism, Bacillus macerans, which was responsible for this action and characterized the alpha and beta dextrins resulting from its action on starch. A review of this early work has been prepared by Norberg (72).

In the period following this early work from about 1910 to 1930, no reliable work on the organism or on the crystalline dextrins was reported. En passant, reference should be made to the voluminous arguments now known to be erroneous presented by Pringsheim (82, 83) for tetrasaccharide and hexasaccharide structures of alpha and beta dextrin and for the miraculous interconversion of these compounds through their respective monomeric units, diamylose and triamylose, by reversible polymerizations.

After Schardinger, the next careful study on culturing the organism and on the crystalline dextrins was made by Freudenberg and Jacobi (30). Employing a complicated separation scheme to crude starch digests they isolated five crystalline substances which they named alpha, beta, gamma, delta and epsilon dextrins. Since this report only the first three compounds have been obtained by other workers (23) and the existence of the delta and epsilon compounds is now questioned even by Freudenberg (33, 34).

While Freudenberg et al. (30, 33, 34) directed their studies primarily towards the characterization of the cyclic dextrins, others were interested in morphological aspects of Bacillus macerans and in the enzymes elaborated by the organism. Porter, McCleskey and Levine (81) report the most complete study of the former type. Hudson and associates (53, 93, 94, 95) investigated the isolation of the enzyme which

was responsible for cyclic dextrin formation. The techniques of culturing the organism for obtaining high enzyme activities are described by Tilden and Hudson (94). Details on the partial purification of the enzyme are presented in a later paper by the same workers (95). Studies of this type were also conducted by Norberg (72) who obtained a relatively pure but non-crystalline enzymic preparation of very high activity. That the preparation contained only a single enzyme was indicated by electrophoretic analysis. More recently, Hale and Rawlins (36) reported that an active macerans amylase preparation which they have purified, thus far shows no signs of being other than a single enzyme.

As several products are formed by macerans amylase action, and evidence is accumulating for the existence of only one enzyme, here, then, is an example of an enzyme capable of catalyzing several types of reactions. These reactions include in addition to the cyclizing reactions, the coupling reactions - the reverse of cyclizing reactions, described by Levine (51) and the redistribution reactions, redistribution of glucose residues among linear substrate molecules, described by Norberg (72). Qualitative equilibrium studies by Norberg (72) on enzyme digests of amyloheptaose, alpha dextrin plus glucose and beta dextrin plus maltose, showed that a common equilibrium composition of cyclic and linear molecules was approached. Quantitative verification

of this observation awaits further experimentation.

C. Alpha Amylase

The amylase enzymes capable of catalyzing the hydrolysis of starch are widely distributed in nature occurring in the digestive secretins and within the cells of many animals, plants and microorganisms. The alpha or dextrinogenic amylases effect a rapid loss of viscosity of starch pastes and a gradual increase in the reducing power by hydrolyzing the starch initially into dextrin fragments of high molecular weights. The stage in which rapid loss of viscosity occurs is termed "dextrinization" whence the name dextrinogenic. The use of the Greek letter alpha for naming these enzymes originates from the fact that hydrolytic products exhibiting laevo mutarotation are produced in the reaction.

The most common enzymes cited as typical examples of this class are malt alpha amylase, pancreatic amylase and salivary amylase. Originally, most of the investigations on the amylase problem have dealt with these enzymes. At present microbiol enzymes are used in several industrial processes and the number of publications on bacterial and fungal amylases has increased continually during the past few years.

The mode of action of the alpha amylases on amylo-type

compounds is interpreted by most investigators as a random hydrolysis of the alpha 1-4 glycosidic linkages. The evidence for this mechanism has been of the deductive type and two examples will be cited. Ohlson (74, 75) investigated the osmotic pressure behaviour of the products during the initial phase of starch breakdown by malt alpha amylase. The reaction was stopped when the iodine color was violet and the reduction value corresponded to 23% theoretical maltose. The solution was placed in a collodion sac osmometer and after two days the pressure stood at 407 mm. of water as compared to 58 mm. for the undegraded substrate. Ohlsson's observations established that a great increase (seven fold) in non-dialyzable particles had occurred during the initial part of alpha amylase action.

Freeman and Hopkins (20) subjected similar products to an alcohol fractionation scheme. The major portion (80%) of these products was alcohol insoluble and consisted of dextrins. However a relatively large amount (20%) of low molecular weight sugars (including maltose) was present in the initial phases of amylase action. Other evidence also of the deductive type for random hydrolysis is excellently reviewed by Hanes (37).

According to Hanes postulation of the action of the enzyme, an amylose-alpha-amylase digest would contain a gradation of intermediate products beginning with high molecular

weight reducing dextrins through low molecular weight reducing dextrins, hexasaccharide fragments, and eventually maltose. These products would account for the progressive decrease in the average chain-length of the reactions products and would be formed by random cleavage of the amylose chains.

Recently the random concept has been propagated by Meyer et al. (9, 54, 56) and by Caldwell et al. (1, 2, 61) from studies with purified enzymes and substrates. From Meyer's laboratories, Bernfeld and Studer-Pecha (9) report on the degradation of amylose by malt alpha amylase. They state that the enzyme hydrolyzes the glucosidic bonds in the amylose equally with the exception of the terminal linkage. Alfin and Caldwell (2) concluded that purified pancreatic amylase causes the random hydrolysis of both the straight and the branched chain components.

At this point it should be emphasized, again, that the conclusions are of the deductive sort since specific intermediates were not isolated in any of the investigations. The separation and identification of these intermediates in the digests would conclusively establish the theory of random hydrolysis.

The identification of glucose in the enzymic digests of starch or starch-like compounds has been reported by many investigators (9, 51, 66, 88). That alpha amylase does pro-

duce glucose from starch is an unquestioned fact. However, the mode of glucose formation, the time of formation, the type of molecules from which it is formed are all points of disagreement in the most recent literature.

Bernfeld and Studer-Pecha (9) claimed glucose is produced only in the later stages of reaction and only from amylotriose. They presented data in which a 13% yield of glucose from an amylose digest agrees with the calculated value of 13%. Levine (51) reported that glucose is formed in the early stages of salivary amylase action on amyloheptaose. It was formed from amylotriose and also from compounds larger than amylotriose. Myrbäck (66), on the other hand, showed that amylotriose is not attacked by the enzyme and therefore the source of glucose must be molecules larger than amylotriose. This brief summary shows the present state of knowledge on the problem of glucose formation in starch-enzyme digests and illustrates the discrepancies in the literature.

Much of the early amylase work was performed with crude enzyme preparation of unknown purity. The persistence of Meyer and associates in attempts to crystallize the amylase enzymes and the final accomplishment should be commended. Pancreatic amylase (18, 55, 56), salivary amylase (8, 57, 58) and bacterial alpha amylase of Bacillus subtilis (59) have all been crystallized.

Thus uncertainties in the mode of action of this enzyme which could be introduced by impurities in the crude preparation may be eliminated. Work along this line is being continually reported by Meyer and coworkers (4, 19, 54). Another useful result of the crystallization experiments is that the number of enzymes in the starting materials is now known. For example, Meyer et al. (54, 58) have shown that saliva, the source of salivary amylase contains only a single enzyme. Independent work of Koehler (47) has confirmed this observation. Consequently a supply of a pure homogeneous alpha amylase is available in saliva, and tedious purification procedures to remove contaminating enzymes are not necessary.

The stability of salivary amylase under widely different conditions is reported by Bernfeld et al. (8). The enzyme may be kept at 20° C. and at pH between 4 and 11 for as long as 20 hours with no loss in activity. The optimum conditions of pH, temperature and salts required by the various alpha amylases are discussed in a review article by Caldwell and Adams (12).

D. Beta Amylase

Beta amylase, also known as saccharogenic or maltogenic amylase is more commonly found in plant than in animal tiss-

ues. It produces from starch or starch-like compounds beta maltose which is responsible for the dextro mutarotation observed in beta amylase-starch digests. It is thermolabile and acid stable in contrast to the plant alpha amylases which are thermostable and acid labile. This difference facilitates the separation of the two enzymes in malt extracts.

The action of beta-amylase on starch is a type of step-wise degradation beginning at the non-reducing end of the molecular chains splitting off successive maltose units. Linear molecules are completely degraded by the enzyme while non linear molecules only up to the points of structural irregularities. Evidences for this type of action are of several kinds, and will now be considered.

That maltose is the only low molecular weight product is most strongly supported by the work of Freeman and Hopkins (20). They showed that the low molecular weight compounds isolated by alcohol extraction of starch-beta amylase digests at any stage of hydrolysis is exclusively maltose by derivatization of the product, rotation measurements and reducing values. That the action is step-wise is indicated by several indirect evidences. Since maltose is the sole low molecular weight product, then an ordered type of reaction must be proceeding for otherwise, the production of other reducing short chain fragments would seem inevitable. Furthermore according to Ohlsson (74) and Hanes (37) the gross

structure of the residual dextrin after beta-amylase action on starch is essentially the same as the starting material. Ohlsson (74) investigated the osmotic pressure behaviour of the products when soluble starch is degraded to the extent of 31% conversion to maltose. After equilibrium has been established by diffusion of the maltose through the collodion osmometer, the osmotic pressure of the degraded products was practically the same as that of the undegraded material. It was accordingly clear that no increase in the number of nondialyzable particles had occurred.

Hanes (37) measured the extinction coefficients of the complexes formed by addition of iodine to the starch-beta amylase reaction mixtures. During the degradation, the extinction values decrease from the beginning over the whole spectral range, but the positions of maximal absorptions and the general form of the extinction curve remain unchanged. Other experiments along similar lines and with similar results have been conducted by Swanson (90) and by Cleveland and Kerr (14). On the basis of this evidence it is highly probable then that beta amylase acts on starch by liberating successive terminal maltose units and leaving a residual dextrin whose gross structure approximates that of the original substrate.

There remains to be considered the evidence for the initial attack at the non-reducing ends of the substrate mole-

cules. Brown and Millar (10) and Örtenbland and Myrbäck (77) reasoned that since dextrinic acids are hydrolyzed by the enzyme at the same rate as unoxidized dextrins, the free aldehyde group is not essential for the action of the enzyme, and most likely the enzyme attacks the non-reducing end of the molecule. Although this reasoning is by elimination processes it has nevertheless proven correct. French and coworkers (24) have shown that amyloheptaonic acid is converted into two molecules of maltose and one of amylotrionic acid by beta amylase. Accepting the concept of a regular mode of action of beta-amylase, it must be concluded that amyloheptaonic acid is degraded at the non-reducing end. The gross aspects of beta-amylase action are thereby established by all these evidences.

At present, a heated controversy on whether the action of beta-amylase proceeds through the "single-chain" process or a "multi-chain" process has developed between the Kerr and the Hopkins schools. In 1947, Cleveland and Kerr (14) reported a series of experiments on the action of beta amylase on corn amylose. Their data showed that when a hydrolysis of amylose by beta amylase is interrupted, only maltose and unconverted residues which have nearly the same average size as the original samples are present. This result is interpreted as evidence for the "single-chain" theory which asserts that once enzyme makes contact with the amylose mole-

cule, this chain is hydrolyzed completely to maltose before the enzyme attacks another chain.

Hopkins, Jelinek and Harrison (43) have studied the hydrolysis of potato amylose. From an analysis of the products at various stages of reaction and the fact that the hydrolysis proceeds at a continually diminishing rate, they concluded that longer amylose chains are hydrolyzed at a faster rate than short chains. By this mechanism all the chains are progressively shortened, and the process termed "multi chain". Since the short chains are attacked at a slower rate, a diminishing rate for the hydrolysis as the reaction proceeds is observed. Both Kerr (44) and Hopkins (42) have published recent notes in which they defend their respective theories.

III. PREPARATION OF MATERIALS

A. Carbohydrates

Schardinger dextrins

The original supply of Schardinger alpha, beta and gamma dextrins was acquired by applying the separation procedures of Freudenberg and Jacobi (30) to crude enzymic digests. The individual dextrins were purified by repeated crystallizations from n-propyl alcohol. In later work the scheme described by French and coworkers (23) in which selective organic precipitants are used for separating and purifying the dextrins has been utilized. Since this scheme does not require the acetylation and saponification steps, it has proven to be much superior to the older procedures.

Amyloheptaose

Amyloheptaose, the heptasaccharide of the amylose series, was prepared from cycloheptaamylose by a mild acid hydrolysis. The experimental details for the preparation of this compound were described in a recent publication from this laboratory (22). In the present investigation, in order to obtain larger quantities of the compound in a shorter time, larger batches (500 gms.) of cycloheptaamylose were hydrolyzed by refluxing the dextrin in .001 N hydrochloric acid for seven

hours. An additional modification was introduced as after the removal of the major part of the unchanged cycloheptaamylose, the hydrochloric acid was removed by passing the cooled hydrolyzate through an acid absorbing column of Amberlite resin. The final purification of the amyloheptaose was accomplished as previously by several precipitations from 95% ethyl alcohol. The optical rotation of the product was 176.5; theoretical, 179.6° (51) and the molecular weight by hypoiodite titration (45) was 1206; theoretical molecular weight, 1152.

Amylohexaose

Since cyclohexaamylose is more resistant to acid hydrolysis, a longer hydrolysis period was used for preparing the linear hexasaccharide. One hundred grams of cyclohexaamylose were dissolved in 400 mls. of .001 N hydrochloric acid and the mixture refluxed for ten hours. At the end of this time the hydrochloric acid was neutralized with an equivalent amount of lithium carbonate. Tetrachloroethane was added to the solution and the mixture agitated for 12 hours. The insoluble dextrin tetrachloroethane complex was collected on a filter. The clear filtrate was concentrated in vacuo to a volume of 40 mls. Seven such concentrates were combined and again treated with tetrachloroethane. The filtrate from this precipitation was concentrated under vacuum to 40 mls. The alpha dextrin was again removed in the

tetrachloroethane complex and the filtrate taken to dryness under vacuum. The residue was dissolved in water, decolorized with carbon, and filtered. The filtrate was concentrated to 10 mls. and 190 mls. of hot absolute ethyl alcohol were added. The amylohexaose was purified by two more precipitations from 95% ethyl alcohol. The yield of amylohexaose was 4.6 gms. The specific rotation was 174° and molecular weight by the hypoiodite titration (45) was 1040; theoretical values, 178° (29) and 990, respectively.

Amylodextrin

Amylodextrin was prepared according to directions of Nägeli (70) by the extended action of cold 15% sulfuric acid on granular starch. Two thousand grams of starch were subjected to acid hydrolysis for a period of several months. After removal of the acid by decantation, the residue was washed with water, neutralized with ammonium hydroxide and washed four more times with water. The yield of air dried residue was 800 gms. This was dissolved in four parts of water, decolorized with carbon and clarified by centrifugation in supercentrifuge. The latter step was introduced since filtration under pressure was slow even with the aid of earth. To the clear filtrate an equal volume of hot methanol was added. After standing for several days at room temperature, the crystalline amylodextrin was collected in a

Büchner funnel and allowed to air dry. The weight of air dry material was 350 gms. In order to reduce the molecular size, the amylodextrin was refluxed in .001 N hydrochloric acid for several hours. On completion of the hydrolysis period, the solution was neutralized with lithium carbonate, treated with carbon and filtered. On adding an equal volume of hot methanol, the amylodextrin crystallized in granular white crystals. On two recrystallizations from 50% methanol the yield of dextrin was 240 gms. The specific rotation of the amylodextrin was 190.5 and the average molecular weight calculated from its reducing power (17) was 3750.

Amylose

A sample of potato amylose prepared by the alcohol precipitation procedure (87) and recrystallized three times from n-butyl alcohol was available in the laboratory. This material was used without any further purification.

Glycogen beta amylase limit dextrin

To 10 gms. of glycogen dissolved in 200 mls. of water, 1600 units (71) of beta amylase in 2 mls. of acetate buffer were added. The reaction was allowed to continue for two days in an incubator at 40° C. At the end of this time, the mixture was boiled for several minutes, decolorized with carbon and filtered. To the clear filtrate, two volumes of 95%

ethyl alcohol were added whereupon the dextrin precipitated. After several days, the supernatant liquid was decanted; the limit dextrin redissolved in water and again precipitated with alcohol. On standing for several days, the insoluble material settled to the bottom and was collected on a filter. The yield of air dried limit dextrin was 4.0 gms.

Starch beta amylase limit dextrin

A sample of starch beta amylase limit dextrin previously prepared by a procedure similar to the preceding was used in this investigation.

B. Enzymes

Macerans amylase

Macerans amylase solutions were obtained essentially according to procedure of Norberg (72). Bacillus macerans cultures were grown alternately on increasing amounts of potato media, or oatmeal media until a sufficient volume for purification was obtained. In early experiments enzyme solutions were purified by filtration, acetone precipitation, adsorption on starch, etc. by Norberg (72). In later work, crude bacterial filtrates dialyzed and pervaporated have been used. These filtrates are shown to be free of hydrolytic amylase activity by both the reducing sugar test (23)

and the homologizing action on maltose. Thus the tedious purification steps needed to remove hydrolytic amylases are eliminated. The activity of some of the enzyme solutions was as high as 40 units per ml. Generally, the solutions have been preserved until required under toluene in a refrigerator.

Beta amylase

The beta amylase used was prepared from soybeans by Newton (71). This preparation after storage for 10 years at refrigerator temperatures had retained 80% of its original activity when it was assayed by Newton's method. No further purification of this material was attempted or deemed necessary.

Salivary amylase

Saliva was collected as needed for the individual experiments. Any suspended particles in the saliva were removed by filtration. For rate studies aliquots of this clear filtrate were diluted to appropriate volumes with water or buffer.

Further purifications of the material was unnecessary in view of the results of Meyer *et al.* (58) and confirmed by Koehler (47). These investigations have shown that only a single amylase is present in saliva.

Pancreatic amylase

Commercial pancreatin was the source of this enzyme. Weighed quantities of the commercial preparation were extracted with water. The clear filtrate from these extractions was used in the experiments.

Malt amylase

A commercial malt amylase preparation was used. Water solutions of the enzyme were prepared following the directions of the preceding section.

IV. EXPERIMENTAL

A. Analytical Methods

Solubility of Schardinger dextrins in the presence of organic precipitants

Aqueous solutions of pure alpha, beta and gamma dextrins were treated with sufficient amounts of precipitant to form two distinct layers. Generally about 5 mls. of precipitant were required per 25 mls. of ca 1% dextrin solution. In the cases where no precipitation occurred at this dextrin concentration increasing amounts of dextrin were used. After equilibration by shaking overnight and standing for a few days at room temperature, the precipitates were removed by filtration. The concentration of the dextrin in the filtrate was calculated from the rotation of the solution. The results are presented in Table 1.

The additivity of solubilities of alpha, beta and gamma dextrins

Since one object of the precipitation studies was to find solvents which could be used for quantitatively separating dextrin mixtures, information on the additivity of solubilities is also necessary. These experiments were conducted concurrently with the precipitation studies and consequently a large number of solvents were used. However, only the data

for those solvents used in the separation scheme which finally evolved is pertinent and is reported.

The mixtures of dextrins were shaken with an excess of precipitants for 24 hours. After equilibration at room temperature, the dextrin-solvent complex was removed by filtration. The rotation of the clear filtrate was measured in a polarimeter. Using the data of Table 1, the sum of rotations of the individual compounds in solution after precipitation with a particular solvent was obtained. These values along with the observed rotations for the mixture are recorded in Table 3.

Quantitative separation of cyclic saccharides by selective precipitants

The organic precipitants have been doubly useful in Schardinger dextrin studies. Firstly, by using selective precipitants for the individual dextrins, a new scheme for the purification of the dextrins was devised and has been recently reported from this laboratory (23). Secondly, the same selective precipitants have been used for quantitative separation and recovery of the dextrins from known mixtures.

Ten grams of alpha, ten gms. of beta and four grams of gamma dextrin-alcohol complex containing 70, 85, 80 percent respectively of the cyclic dextrins were dissolved in excess water. The solution was boiled until the alcohol was re-

moved. The volume of the cooled solution was adjusted to 100 mls., and an excess trichloroethylene was added. After adequate equilibration the precipitate was quantitatively removed and fractionated by the scheme in Table 2. The volume of solution of each step was recorded. From these and the solubility data, the amounts of dextrins remaining in solution are calculated. The values were added to the dry weights of dextrin, and total recovery values are shown in Table 4.

Qualitative separation of linear and cyclic saccharides by paper chromatography

Droplets of ca 0.01 ml. of the substance being tested were placed at intervals of 2.5 cms. along a line ruled 2.5 cms. from one edge of a rectangle generally 20 cms. by 22 cms. of filter paper. After drying, the paper was rolled into a cylinder and held in this form by a wire staple at each end, thus giving a cylinder capable of supporting itself even when wet with solvent. The cylinder, with the sample spots near the bottom, was then placed in a shallow layer of solvent (3 parts water, 4 parts pyridine, 6 parts n-butanol by volume) (13) such that the sample spots were above the solvent level. The solvent vessel was kept away from marked thermal disturbances, out of direct light, and tightly sealed. After the solvent had climbed by capillary attrac-

tion to the top of the cylinder, it was removed from the vessel, air dried and oven dried, then returned to the solvent for additional climbs. The number of climbs for resolution of the linear saccharide varied from four to ten depending on the molecular weights of the compounds being resolved. The resolution of cyclic dextrin generally required eighteen to twenty climbs.

The reducing sugars showed up as blue spots on the paper chromatogram after the following treatment. The dried cylinder was sprayed lightly with alkaline copper reagent (without KIO_3 and KI) (79) heated in an oven at 105° C. for about five minutes, then sprayed with phosphomolybdic acid reagent (92) to locate the areas in which reduction of the copper has taken place. The sucrose containing compounds were located on the chromatogram by spraying the chromatogram with phloroglucinol reagent (1% phloroglucinol in .1 N HCl). The brown spots indicating the sucrose compounds appeared after heating a sprayed chromotogram for about 5 to 10 minutes. For locating the cyclic dextrins, the chromotograms were sprayed with iodine solution. Characteristic iodine complexes, blue for alpha, yellow for beta and orange for gamma were observed immediately after spraying.

A partially hydrolyzed amylodextrin solution containing all the low molecular weight members of the amylose series was routinely used for identification purposes. The presence

and identity of the saccharides (G_1, G_2, ---) in this mixture was established by using pure glucose and maltose as standards (see Figure 1).

Quantitative separation of linear and cyclic saccharides by paper chromatography

A measured amount, generally 0.2 to 1 mls., of saccharide solution was introduced in a continuous streak (1.5 cms. x 16 cms.) about 2.5 cms. from the bottom of a rectangle (20 cms. x 22 cms.) of filter paper. After adequate development two vertical strips of 1.6 cm. width corresponding to 20% of the total area over which the carbohydrate moved were cut at distances of 6 cms. and 13 cms. from the right hand edge of the chromatogram. The carbohydrate regions were located on the strip by spraying with appropriate reagents. If the development had been adequate each saccharide was concentrated in a localized streak across the paper and completely separated from the adjacent members. These strips were used as markers for sectioning the remainder of the chromatogram. The sections containing a single saccharide were cut into small pieces and extracted four times with boiling water. The final volume was adjusted to 10 mls. and carbohydrate concentrations on the aliquots of this solution were determined by either the copper or the diphenylamine method. The grams of saccharide were calculated using appropriate cali-

bration curves (51, 79) with corrections being made for the amounts of saccharides present on the test strips. A correction factor was not necessary in the determination of cyclic dextrins as the dextrin region on the paper chromatogram was found by spraying the whole chromatogram with iodine. Recovery data for mixtures of known composition is recorded in Table 5.

B. Action of Macerans Amylase

Coupling reactions

Expt. 1. - Pure cyclohexaamylase, 2.0 gms. and C. P. maltose, 0.7 gms., were dissolved in about 50 mls. of water, heated to complete mutarotation of the maltose, cooled and treated with 10 units of macerans amylase. The solution was made up to 100 mls. and the change in rotation was followed in a polarimeter: initial rotation, + 7.87°; after two hours, + 8.10°. At this point the enzyme was inactivated by boiling and the reaction products were separated by alcohol fractionation procedures.

Unreacted cyclohexaamylose was removed from the digest as the insoluble tetrachloroethane complex. The clear filtrate from this precipitation was concentrated to a sirup by distillation of the solvent under vacuum. The residue was dissolved in 5 mls. of water, decolorized with carbon and fil-

tered through a glass sintered filter with pressure. To reduce transferring losses to a minimum, the filtrate was collected directly in the beaker in which precipitation by alcohol was to be effected. Ninety-five mls. of hot absolute ethyl alcohol were added whereupon the carbohydrate precipitated as a sirup. After standing for several days, the alcohol layer was removed by decantation and the residue dissolved in 2.5 mls. of water. The carbohydrate material was again precipitated from 95% ethyl alcohol. In a few days, the insoluble fraction was collected on a filter, washed with a little of dry n-butanol, and dried in a vacuum at 70° C. The yield of oven-dried material was 0.14 gms., the specific rotation = + 163° (c 1.4, water). The reducing value was calculated from ferricyanide titers (17) of 2 ml. aliquots of the 1.4% solution.

The specific rotation was low if the material was pure octasaccharide (theoretical rotation, + 185°). Attempted purification of 6 mls. of the 1.4% solution by ion exchange resins yielded 50 milligrams of purified sample. The specific rotation of this was + 172° (c 0.5, water).

Expt. 2. - Maltose, .042 grams, cyclohexaamylose, .120 grams and macerans amylase, 2 units, in a total volume of 6 mls. were incubated at 40° C. At 0, 5, 15, 30, 60, 120, 180 and 300 minute periods, 1/2 ml. aliquots were transferred to pyrex test tubes and the enzyme inactivated by boiling. The

resulting solutions were analyzed for reaction products by qualitative paper chromatography methods.

Expt. 3. - The following reaction mixture ; .084 grams of sucrose, .24 grams of cyclohexaamylose, and 4 units of m-amylase was incubated at 40° C. Aliquots of 1 ml. were removed at 0, 1, 1 1/2, 3, 5, 22, 42, 66, 90 hour periods and were concentrated by boiling. Reaction products were identified on paper chromatograms of the aliquots.

One-half mls. of the 42 hr. sample was treated with excess beta-amylase and 1/2 ml. of the 66 hr. sample with excess salivary amylase. After 16 hours the digests were analyzed for products of action of these enzymes by the standard chromatography methods.

Expt. 4. - Cyclohexaamylose, .12 grams, and isomaltose (63) (kindly supplied by Dr. Montgomery), .075 grams were dissolved in 5 mls. of water. To the solution, 1 ml. of m-amylase (2 units per ml.) was added. The reaction mixture was incubated at 40° C. One-half ml. samples were removed at 0, 1/4, 3/4, 1 1/2, 3, 6, 18, 25, 72 hour periods, concentrated by boiling and examined for products by paper chromatography. Two additional aliquots had been removed at the 3 hr. period for experiments with hydrolytic amylases. One aliquot was treated with excess of beta amylase and the other with excess salivary amylase. At the end of 24 hours reaction period, these enzymes were inactivated by heat and

the reaction products separated and identified on paper chromatograms.

Homologizing reactions

Expt. 1. - Several samples (ca .2 mls.) of 2% amylohexaose solution were placed on a spot plate. To each sample .2 mls. of macerans amylase were added. The spot plate was kept in an incubator (40° C.) for 1 hour. At the end of this time, the digests were examined for characteristic alpha dextrin-iodine complexes and alpha dextrin-n-propanol crystals.

Expt. 2. - Amylotriose experiments were carried out in the analogous manner to Experiment 1.

Expt. 3. - To 5 mls. of a .292 M maltose solution in a glass stoppered Erlenmeyer flask 5 mls. of macerans amylase (20 units per ml.) were added. To prevent microbiol growth a few crystals of thymol were included and the flask and contents kept at 40° C. The concentrations of saccharides were determined on 0.4 mls. aliquots at the end of 2000 and 6000 conversion periods (72) by the paper chromatography procedure.

Equilibria in macerans amylase reactions

Expt. 1. - Twenty-five grams of amylodextrin (12.2% moisture) were dissolved in 500 mls. of water. Ten mls. of .1 M sodium cyanide were added and the pH adjusted to 6.5

with acetic acid. The mixture was treated with two hundred units of macerans amylase and diluted to a volume of 1 liter. The reaction was allowed to proceed at 40° C. in a tightly stoppered flask.

Since at the time of this experiment there was no convenient way of following the course of the reactions, and in view of results of McClenahan, Tilden and Hudson (53) it was assumed that equilibrium had been reached after an incubation period of several months. At this point the digest was boiled to destroy the enzyme activity and the volume adjusted to 1 liter. Twenty-five mls. of this solution were removed for "i" value determination. The Schardinger dextrins were determined in the remainder of the digest as follows. First, the major portion of the dextrins was removed from the solution by precipitation with trichloroethylene. The filtrate from this precipitation was concentrated by vacuum distillation to 100 mls. The dextrins were once again precipitated with trichloroethylene. The two precipitates were combined and the whole fractionated by the scheme of Table 2. As previously, the amounts of dextrin remaining in solution after each precipitation were calculated from the solubility data. The yields of dextrin in one liter of original digest, and the percent of amylodextrin converted into cyclic dextrins are recorded in Table 7.

The reducing power of the digest before and after beta-

amylase hydrolysis was determined on 5 ml. aliquots of original digest by the ferricyanide procedure. The "i" value for the system can be determined by approximation methods as shown in the discussion section.

Expt. 2. - To 3.5 mls. of 9.35% solution of amyloheptaose in a ground glass stoppered flask, 8.2 mls. of macerans amylase (40 units of per ml.) were added. Microbiol growth in the digest was prevented by the addition of a few crystals of thymol. At the end of 3000 conversion periods the following aliquots were removed; one ml. samples for paper chromatographic determination of the linear saccharides and four mls. for cyclic dextrin determinations. The latter sample was mixed with 1/2 ml. of 2.64 N sodium hydroxide and heated in a boiling water bath for 1/2 hour. The cooled solution was neutralized with 1/2 ml. of 264 N sulphuric acid. Cyclic dextrin concentrations were determined on one ml. aliquots by the chromatography procedure. These values have been included in Table 7.

C. Action of Salivary Amylase

On amylotriose and amylotetraose

Five mls. of salivary amylase solutions prepared by diluting 1 ml. of filtered saliva to 100 mls. were added to ten mls. of 0.87% amylotriose solution. The resulting solu-

tion was thoroughly mixed and an aliquot transferred to a polarimeter tube. The rotation changes were followed in a polarimeter. Experiments were conducted in which 5 mls. of enzyme solutions of malt alpha amylase and pancreatic amylose were used instead of the salivary amylase solution.

After the rotation had become constant the enzyme action was stopped by boiling the enzymolyzates. The coagulated material in the salivary digest was removed by filtration and the clear filtrate taken to dryness. The solid residue was dissolved in 0.25 mls. of H_2O and 4.75 mls. of absolute ethyl alcohol were added. The solution was examined for products by column chromatography procedures (22). A plot of the concentrations of carbohydrate versus ml. portion of eluate showed peaks at 36 and 42 mls. corresponding to the glucose and the maltose.

Fifty milligrams of amylotetraose were dissolved in 2.5 mls. of water. One-half ml. of diluted salivary amylase was added and the mixture incubated at 40° C. Samples were removed at 0, 2.5, 5, 10, 15 and 30 minutes and examined for reaction products by paper chromatography methods.

On amylodextrin

The salivary amylase solutions were prepared as needed by diluting one ml. of filtered saliva to 25 mls. with appropriate buffer, either of pH 10.3, (.08 M Na_2CO, and .02 M

$NaHCO_3$) or of pH 7.0 (.06 M K_2HPO_4 and .04 M KH_2PO_4). One ml. of the enzyme solution was added to 4 mls. of substrate solution, .0053 M in amylodextrin and .02 M in sodium chloride. The digest was kept at 40° C. in a constant temperature water bath. One ml. samples were removed at 0, 5, 10, 15, 30 minute periods, concentrated to 1/2 the original volume and examined by paper chromatography for intermediate products. Typical paper chromatograms at a stage just prior to the achromic point are shown in Figure 2 for the two pH values.

To test for viscosity effects on the rate of enzyme reactions, salivary amylase-amylodextrin experiments were conducted at varying viscosities. The viscosities of the digests were increased by adding weighed amounts of a 95% glycerol solution. The enzyme solution was prepared by mixing 1 part of filtered saliva with 1 part of phosphate buffer of pH 7.0. The weights of digests in the experiment were 25.0 gms. comprised of 0.5 gms. amylodextrin, 0.5 gms. of enzyme solution, and water-glycerol mixtures. The viscosities of the digests which were set up are shown in Table 11. The reducing power at various time intervals of 5 ml. aliquots was determined by the ferricyanide procedure. The increases in reducing power in milligrams of maltose for the experiment are shown in Table 10.

On amylose

One hundred and thirty-five mls. of a 0.5% amylose solution, 3 mls. of 1 M sodium chloride, and 10 mls. of phosphate buffer (pH 7.0 and .1 M total phosphate) were mixed and allowed to equilibrate in a temperature bath of 40° C. To this solution 2 mls. of salivary amylase solution, prepared by diluting 1 ml. of filtered saliva to 10 mls. with water, were added. At time intervals indicated in Table 9, the following aliquots were removed. A twenty ml. sample was pipetted into 25 mls. of ferricyanide reagent and the reducing power of the aliquot determined (17). One ml. of digest was added to 49 mls. of iodine solution (final iodine concentration of .0002 M). The optical density in the visible range of these samples was measured in a spectrophotometer. These values along with the reducing values expressed as milligrams of maltose are recorded in Table 9. One ml. samples of the digests were concentrated by boiling in order to inactivate the enzyme. These samples were examined for low molecular weight saccharides by the paper chromatography method.

An experiment at equivalent amylose and salt concentrations but at unfavorable pH was also performed. Ten mls. of .08 M Na_2CO_3 and .02 M $NaHCO_3$ buffer of pH 10.3 and 2 mls. of filtered saliva were the buffer and enzyme solutions, respectively, employed in this experiment. The results are

given in Table 9.

On limit dextrins

Four mls. of a 4% beta amylase starch limit dextrin were treated with 1 ml. of salivary amylase solution (1 part of filtered saliva and 25 parts of phosphate buffer). Samples were removed periodically, concentrated and analyzed by paper chromatography.

A beta amylase glycogen limit dextrin was substituted for the starch limit dextrin and the previous experiment repeated.

Affinity constants

The velocities of action of salivary amylase with amyloheptaose and amylodextrin were measured by recording the changes in optical rotation of the reaction mixtures. Two mls. of salivary amylase preparation which assayed 90 units per ml. (72) and 2 mls. of phosphate buffer of pH 7.0 were used in each digest. The total volume of the reaction mixture was 15 mls. Amyloheptaose concentrations, amylodextrin concentrations and the rotatory changes are recorded in Tables 12 and 13.

D. Action of Beta Amylase

On amylohexaose

Ten mls. of 1.60% amylohexaose solution previously boiled and cooled to room temperature was stirred with 10 mls. of beta amylase solution (8000 units). An aliquot of the mixture was transferred to a 2 dcm. polarimeter tube. The rotations of this solution and of a blank consisting of 10 ml. of the amylohexaose solution and 10 mls. of water were checked periodically. The rotation of the blank remained constant at 2.87° while that of the digest dropped to 2.21°. When the reaction had stopped, as judged by the constancy of the rotation, the reducing value of 2 ml. aliquots of the digest was determined by the ferricyanide procedure. The titer was 4.40 mls. of .0585 N ceric sulphate solution.

On amyloheptaose

Expt. 1. - Amyloheptaose sample (.100 gms.) was dissolved in 4 mls. of water, boiled, cooled, and adjusted to a volume of 4 mls. One-tenth of a milligram of beta amylase preparation suspended in 1 ml. of acetate buffer of pH 4.5 was added and the whole incubated at 40° C. One half ml. samples were removed at 0, 2.5, 5, 10, 15, 30, 60 minutes. Qualitative paper chromatograms on these samples were ob-

tained. From the intensity of the carbohydrate spots on the chromatogram the percentage of each saccharide was approximated. Molar concentrations of the saccharides in the digest for the various times were calculated and are shown in Table 15.

Expt. 2. - This experiment was similar to the Experiment 1 except that carbonate buffer of pH 10.3 maintained the pH at unfavorable conditions.

Expt. 3. - The concentrations of materials in the experiment were the same as in Experiment 1, but the incubation temperature was 65° C. The molar concentrations of reaction products in Experiments 2 or 3 were not obtained.

Expt. 4. - Two mls. of amyloheptaose solution (10.3%), 2 mls. of acetate buffer (pH 4.5), and 0.1 mgs. of beta amylase preparation suspended in 1 ml. of water were thoroughly mixed and incubated at 40° C. At 0, 2.5, 10, 40, 80 minutes 0.2 ml. sample were introduced on paper chromatograms and immediately heated in oven at 105° C. The carbohydrate concentration at the various times was determined by the quantitative paper chromatography method. These are presented in Table 16.

Expt. 5. - One and half ml. of amyloheptaose (13.8%), 0.5 mls. of carbonate buffer (pH 10.3), 3 mgs. of beta amylase in 3 mls. of water were introduced into a pyrex tube, mixed and incubated at 40° C. The procedure followed in this

experiment was the same as in Experiment 4. The results are also recorded in Table 16.

On amylodextrin

Nine mls. of a 2% amylodextrin solution 0.1 mgs. of beta amylase in acetate buffer (pH 4.5) were thoroughly mixed and incubated at 40° C. One ml. samples were removed at 0, 2.5, 5, 10, 15, 20, 30, 60, 180, 4320 minute intervals, concentrated, and examined for reaction products by qualitative paper chromatography methods.

On starch

The velocities of enzyme action on starch under conditions of high pH (10.3) low temperature (5° C.) and high temperatures (65° C.) and of optimum pH and temperature (4.5 and 40° C.) were determined by the method of Newton and Naylor (71). The rate of increase of maltose in the digests is recorded in Table 17. The velocities under the different conditions are proportional to the enzyme activities and are shown in units per milligram of preparation in Table 17.

Affinity constants

The affinity constants at pH values of 4.5, 7.0 and 10.3 were determined for amyloheptaose-beta amylase mixtures. The buffer solutions for the three pH values were, acetate,

phosphate and carbonate respectively. The same amount of enzyme in units of activity was used in the three experiments. Molar concentrations of amyloheptaose are shown in Table 18.

The velocity of reaction was calculated from the increase in reducing power in 5 ml. aliquots of the mixtures. The experimental data is presented in Tables 18 and 19.

One hundred mls. of amylodextrin solutions were introduced into four 250 ml. Erlenmeyer flasks. The flasks were immersed in a constant temperature water bath at 40° C. On temperature equilibration 1 ml. of acetate buffer of pH 4.5 and 1 ml. of beta amylase solution containing 0.1 mgs. of enzyme preparation were added to each flask. The amylodextrin concentrations on these solutions were 1.33×10^{-4} M, 2.13×10^{-4} M, 3.20×10^{-4} M and 5.34×10^{-4} M.

The contents of the flasks were thoroughly mixed and 25 mls. aliquots were transferred within 1 minute after addition of the enzyme into 300 ml. Erlenmeyer flask containing 25 mls. of alkaline ferricyanide reagent. The reducing power of the aliquots was determined. Subsequent aliquots were removed 5, 10 and 15 minutes. The results are recorded in Table 20.

The affinity constant of maltose-enzyme complex is found by a slightly different procedure. Since maltose is a competitive inhibitor, the affinity constants can be calculated

from the velocities of reaction in the presence and in the absence of maltose. The latter information was available from the preceding experiments. To obtain the other necessary data, the velocity of reaction of a solution 1.33×10^{-4} M in amylodextrin and 1.11×10^{-3} M in maltose was determined.

V. RESULTS AND DISCUSSION

A. Analytical Methods

Organic precipitants for the Schardinger dextrins

The formulae for calculating equilibrium constants for macerans amylase reactions are derived in a later section. However, before a numerical evaluation of the constants was possible, analytical methods for determining the Schardinger dextrin concentrations and "i" value in an equilibrated digest were needed. The first methods devised for this purpose involved the use of selective precipitants for the former and the determination of reducing values of the system before and after beta-amylase digestion for the latter.

Levine (51) found that the efficiency of organic solvents for precipitating Schardinger beta dextrin varied markedly. Accordingly solubility data on the precipitation efficiency of several additional organic liquids was collected and is presented in Table 1. These data and the differences of solubilities of the dextrins in water and in 60% n-propanol have been incorporated into a scheme (Table 2) for separating the Schardinger dextrins.

For testing this analytical scheme, it was necessary to obtain quantitative data on the additivity of solubilities and recovery of known mixtures of Schardinger dextrins.

Table 1

Solubilities of the Schardinger Dextrins
in the Presence of Precipitants

Precipitant	alpha g./100 mls.	beta g./100 mls.	gamma g./100 mls.
Cyclohexane	.15	.06	-
Toluene	.8	.06	.04
p-Xylene	.9	.02	.06
p-Cymene	3.3	.04	.17
Trichloroethylene	.26	.03	.03
Tetrachloroethane	.08	.12	.03
Tetrachloroethylene	.7	.004	.01
Tetrabromoethane	.10	.02	-
Bromobenzene	2.4	.03	.01
Iodobenzene	-	.03	.03
p-Chlorotoluene	-	.02	.05
o-Bromotoluene	-	.02	.02
Nitrobenzene	-	.03	.04
Aniline	-	.3	.4
Cyclohexanol	.68	-	-
Cyclohexane	.15	.06	-
Nitrobenzene	-	.03	.04
Aniline	-	.3	.4

Table 2

Fractionation Procedure for the Quantitative Separation of Schardinger Dextrins

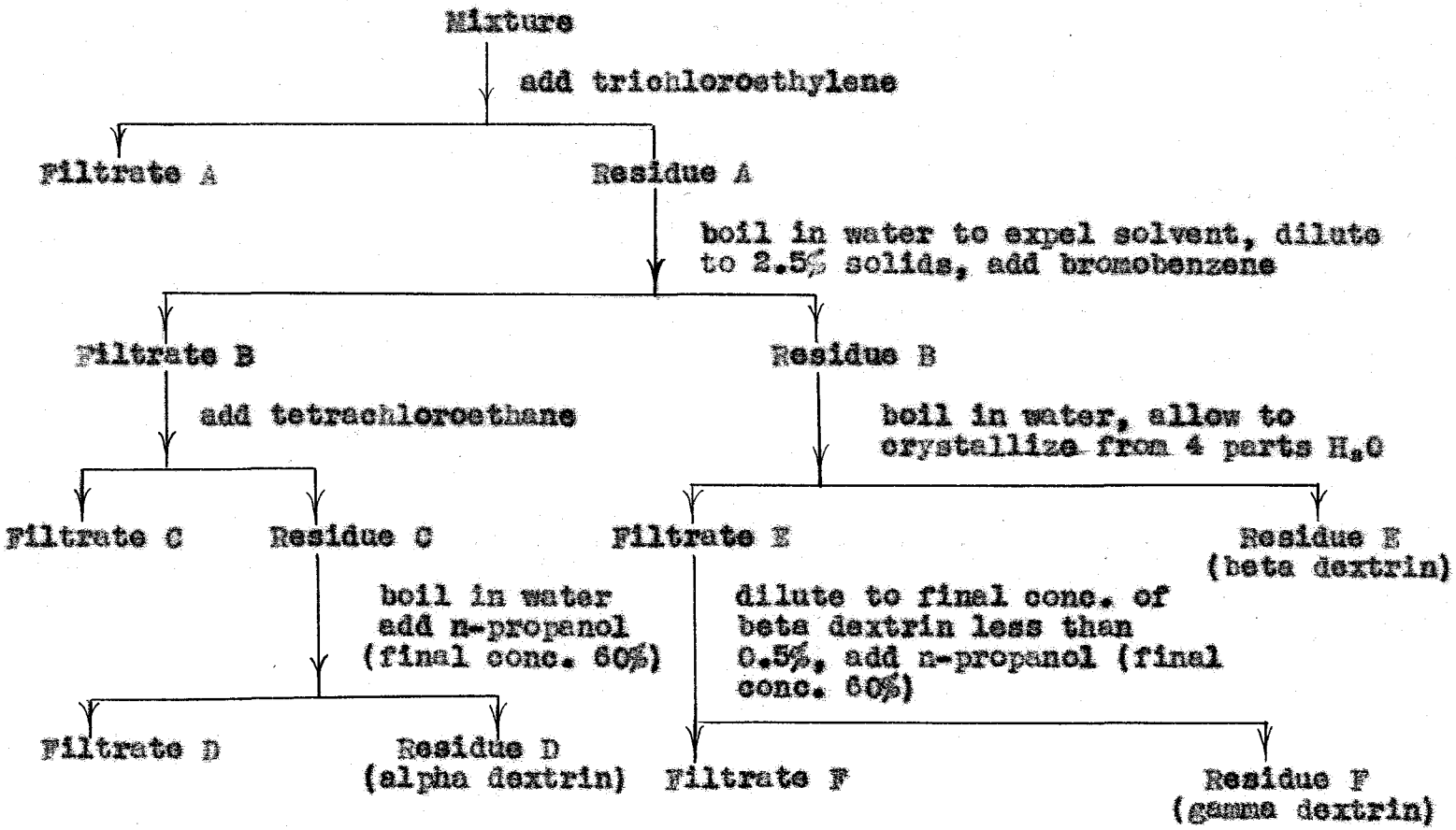

Table 3

Rotation of Filtrates from the Precipitation of Alpha, Beta and Gamma Dextrin Mixtures by Organic Solvents

Precipitant	Rotation Observed	Rotation Calculated*
Trichloroethylene	1.02	0.98
Bromobenzene	6.84	7.15
Tetrachloroethane	0.80	0.80
n-Propanol (60%)	5.21	5.22

*From solubility data of Table 1.

Table 4

Recovery of the Schardinger Dextrins by the Selective Precipitant Scheme

Dextrin	Dextrin added gms.	Dextrin recovered gms.	Recovery %
Alpha	7.00	6.80	97.2
Beta	8.50	8.38	98.7
Gamma	3.20	3.17	99.2

This information is contained in Tables 3 and 4. It is evident that the solubilities are additive and that recoveries of dextrins are quantitative. The recovery values have been corrected for the amounts of dextrins remaining in solution after each precipitation and crystallization step. The purity of the recovered fractions was established by specific rotations and by their crystal forms from 60% n-propyl alcohol.

Paper chromatography of the linear and cyclic saccharides

The paper chromatographic procedures were the second used for determining the concentration of linear and cyclic dextrins at equilibrium. These are superior to the previous since not only are they less tedious but also they are more accurate particularly for determining "i" values. Their development was considerably later and consequently the first equilibrium constants were calculated from values obtained by the selective precipitant scheme. In later experiments analyses of the mixtures have been by means of paper chromatography.

Of necessity, experiments on the separation and recovery of known amounts of linear and cyclic compounds, individually and in mixtures were the first to be performed. Typical recovery values are shown in Table 5. The reducing oligosaccharides were determined by an alkaline copper (79) procedure and the non-reducing cyclic saccharides by the di-

Table 5

Recovery of Linear and Cyclic Oligosaccharides by the Paper Chromatographic Procedure

Sample no.	Compounds	Treatment	CHO Added mgs.	CHO Recovered mgs.	Recovery %
1	glucose	none	10.0	10.0	100
2	maltose	"	9.50	9.80	103
3 *	glucose	"	5.00	4.90	98
	+ maltose		4.75	4.70	99
4 *	alpha dextrin	"	1.65	1.58	96
	beta dextrin		2.99	2.88	97
	gamma dextrin		1.82	1.82	100
5 *	alpha dextrin	alkali	1.65	1.70	103
	beta dextrin		2.99	2.94	98
	gamma dextrin		1.82	1.81	99
	linear oligos.				

*Mixtures of the compounds listed.

phenylamine method (51). Values for mixtures of pure cyclic compounds and for mixtures of cyclic dextrins and linear oligosaccharides are reported. The latter are important since in the equilibria experiments mixtures of this type were analyzed. In these cases the linear compounds must first be removed before the cyclic dextrins can be determined.

An alkali treatment has been used to degrade the linear compounds in the samples which were used for cyclic dextrin determinations. The first members of the linear series, on the other hand, were determined directly in the mixture, since cyclic compounds are non-reducing.

The paper chromatographic methods for the linear saccharides have also been very useful for studying reactions of hydrolytic amylases. The products and intermediates in both alpha and beta amylase digests have been identified by these procedures.

B. Action of Macerans Amylase

Coupling reactions

That macerans amylase has a synthetic as well as a degradative action has been indicated by the studies of Levine (51). The evidence for synthetic action was based on changes in the physical properties of a system composed of crystalline alpha dextrin, C.P. maltose and macerans amylase. Extensive data on the rotation and iodine coloration of such systems was recorded by Levine. All the findings indicated that the action of macerans amylase was reversible. However, Levine did not isolate the products of the reverse action in any of his experiments. In this investigation the reverse action has been tested and verified by identifying the re-

action products.

The initial coupling reaction, a term applied to the reverse action of macerans amylase, for alpha dextrin and maltose substrates is shown in equation (1).

alpha dextrin + maltose (G_2) $\rightleftharpoons$ amylooctaose (G_8) (1)

From the reaction mixture of the experiment outlined in the experimental section, 0.14 grams of an alcohol insoluble fraction which in view of homologizing reactions (see page 55) was undoubtedly a mixture of oligosaccharides were obtained. The specific rotation of the material was + 163° (c 1.4, water) and is low for an octasaccharide. Partial purification was effected by passing a solution of the material through ion exchange resins. The rotation of the purified preparation was + 172° (c 0.5, water). The theoretical rotation of amylooctaose calculated from Freudenberg's rule (29, 51) is + 185°. The average molecular size of the isolated fraction calculated from reducing power was 8.9 glucose residues. Analysis by paper chromatography showed that the sample contained, in addition to amylooctaose, small amounts of other oligosaccharides. The isolation and identification of these compounds in the reaction mixture is the most convincing argument for reversible action of macerans amylase.

Similar results were obtained with sucrose and isomal-

tose as co-substrates. A series of sucrose containing amylo-oligosaccharides formed by first coupling and then homologizing action of macerans amylase was observed in the reaction mixture by paper chromatography means. A corresponding series containing isomaltose and similarly identified was obtained with isomaltose. The incorporation of these co-substrates in the amylo-oligosaccharide chains is additional evidence for reversibility. These coupled products have been most useful in enzymic studies where irregularities in the structure of the amylo-compounds are desired. In particular they have been used for elucidating the mode of action of the hydrolytic amylases - alpha and beta, in the vicinity of branch points in the molecule. These studies are discussed in a later section.

The coupling experiments 2, 3 and 4 were conducted at equal concentrations of substrates and enzyme. The rate of coupling reactions with these co-substrates can be compared. The times at which coupled products appeared in the different reaction mixtures were 1/4, 3 1/2 and 1 1/2 conversion periods for the maltose, sucrose and isomaltose experiments respectively. Using maltose as the arbitrary standard the rate of coupling with isomaltose was 1/6 and with sucrose 1/14 that with maltose. Such studies could be extended to a large variety of co-substrates for ascertaining the importance of spatial configuration of the co-substrates in coupling

reactions.

The reactions of macerans amylase which were established by these experiments are expressed by equations (2), (3) and (4).

$$G_n \rightleftharpoons G_{n-m} + \text{alpha} \qquad \begin{matrix} n = 7, 8, 9, 10, .. \\ m = 6 \end{matrix} \qquad (2)$$

$$G_n \rightleftharpoons G_{n-m} + \text{beta} \qquad \begin{matrix} n = 8, 9, 10, M, .. \\ m = 7 \end{matrix} \qquad (3)$$

$$G_n \rightleftharpoons G_{n-m} + \text{gamma} \qquad \begin{matrix} n = 9, 10, 11, 12, .. \\ m = 8 \end{matrix} \qquad (4)$$

They involve a type of "glycosidic exchange" between linear and cyclic molecules. It was believed that amyloheptaose (G_7) was the smallest linear molecule from which cyclic dextrins could be synthesized by the enzyme.

Homologizing reactions

When amylohexaose became available, a digest of macerans amylase and amylohexaose was set up. It was indeed surprising to find that large amounts of cyclic dextrins were formed from amylohexaose. The cyclic dextrins were identified by their characteristic iodine complexes and n-propanol complexes (23). Not only were Schardinger dextrins formed from amylohexaose, hitherto believed to be impossible, but also they were formed from amylotriose.

In order that cyclic dextrins of 6, 7 and 8 glucose

residues may be formed from these compounds, the short substrate molecules must first be built up into longer ones. Therefore, macerans amylase is capable of effecting a redistribution of the glucose residues among linear molecules. These reactions will hereafter be termed homologizing reactions denoting that homologs of the amylose series are formed. Norberg and French (73) described a series of experiments in which the course of homologizing reactions in amyloheptaose and in maltose digest was followed by an electrophoretic technique. These studies established the presence of low molecular weight saccharides (G_1, G_2, G_3 and G_4) in a maltose-macerans digest, and the absence of cyclic dextrins. The results of paper chromatograms of a similar maltose digest have verified this observation and have shown that high molecular weight saccharides are also present (see Figure 1).

The perplexing observations of Levine (51) that G_1, G_2, G_3, molecules and long chain molecules were present early in coupling reaction mixtures, are now readily interpreted. Their formation was the result of homologizing reactions on the initial coupled product and not the result of the type of reactions proposed by Levine.

A study of the disproportionation of amyloheptaose by macerans amylase using the paper chromatography technique have been informative on the mode of action of macerans

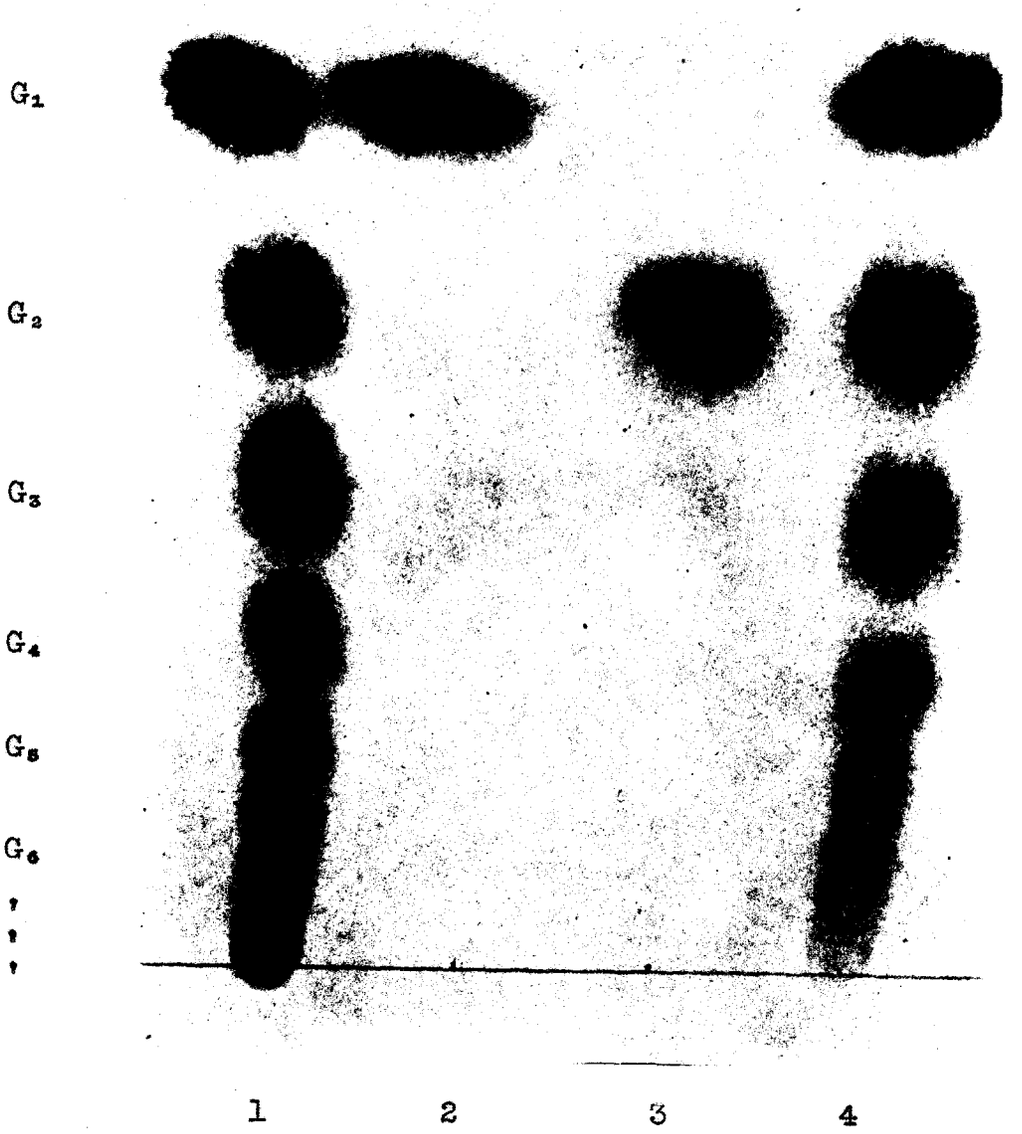

Fig. 1. Paper chromatograms of reducing sugars.
1. Reference oligosaccharide mixture.
2. Glucose. 3. Maltose. 4. Macerans amylase-maltase digest.

amylase. Although there is no reason for expecting a preferential disproportionation of the substrate molecules, Norberg and French (73) have made this claim. From electrophoretic analyses of the reactions products in an amyloheptaose digest they concluded that a preferential reaction was proceeding according to equation (5).

$$2G_7 \rightleftharpoons G_4 + G_{10} \qquad (5)$$

An examination of the products of reaction conducted under the same conditions (concentrations, time, temperature, etc.) did not verify this claim. Whereas in the experiment by Norberg and French maltose was not observed in the reaction mixture until 9 conversion periods (C.P.) and glucose not until 500 C.P., in the present experiment these compounds as well as G_3, G_4, G_5, G_6 and higher unrevolved saccharides could be detected on the paper chromatogram at 1/4 C.P. and unquestionably identified at 1/2 C.P. As judged from the intensity of the saccharide spots on the chromatogram, redistributed reactions occurred with equal probability at any glycosidic bond of the heptasaccharide molecule until equilibrium was reached. This contention was substantiated by the results of the alpha dextrin-sucrose coupling experiment. The coupled product in this experiment was disproportionated in a manner such that probabilities of breaking any glycosidic bond in the molecule are equal. The tri-, tetra-,

penta-saccharides etc., formed by disproportionation of the coupled compound were observed in the reaction mixture within the same reaction period.

To express the reactions catalyzed by macerans amylase in general equations, equation (6) for homologizing reactions must be added to equations (2), (3) and (4).

$$G_n + G_m \rightleftharpoons G_{n-x} + G_{m+x} \qquad \begin{matrix} n = 2, 3, 4, 5, .. \\ m = 1, 2, 3, 4, .. \\ x = 1, 2, 3, 4, .. \end{matrix} \qquad (6)$$

Thus in macerans amylase-substrate digests, homologizing, cyclizing, and coupling reactions are proceeding concurrently. In view of the scope of the macerans amylase action, it is indeed an inviting system for equilibria studies.

Mathematical derivation of equilibrium expressions

In the derivation of equilibrium expressions for cyclizing reactions of macerans amylase, the more convenient equations (7, 8, 9) will be used.

$$G_{n+6} \rightleftharpoons G_n + \text{alpha} \qquad (7)$$

$$G_{n+7} \rightleftharpoons G_n + \text{beta} \qquad (8)$$

$$G_{n+8} \rightleftharpoons G_n + \text{gamma} \qquad (9)$$

G_n represents a linear molecule or a linear portion of

a branched molecule and, "n" the number of glucose residues per molecule . Assuming activity coefficients of unity, the equilibrium constant for equation (7) is expressed in equation (10)

$$K_{alpha} = \frac{[alpha][G_n]}{[G_{n+6}]} \qquad (10)$$

Similar expressions and subsequent arguments hold for equations (8) and (9) and mathematical detail for only equation (7) will be presented.

Of the quantities in equation (10) only the alpha dextrin concentration is readily measureable. The ratio G_n/G_{n+6} must be evaluated by indirect methods. That this ratio is equal to a readily measurable quantity $(1-i)^{-6}$ can be shown mathematically.

Since the enzyme is capable of breaking any 1-4 glycosidic bonds and synthesizing new ones, at equilibrium a random distribution of linear oligosaccharides will result. The distribution function from probability considerations is similar to that for random hydrolysis of polysaccharides and treated by Kuhn and coworkers (31, 49, 50).

Let the probability that a glucosidic bond be broken be "i". Then the probability (P_1) that a certain glucose residue is found in G_1 molecule is

$$P_1 = i^2$$

The probabilities that it is found in G_2, G_3, G_4, ... mole-

cules are

$$P_2 = 2\, i^2\, (1-i)$$
$$P_3 = 3\, i^2\, (1-i)^2$$
$$P_4 = 4\, i^2\, (1-i)^3$$
$$\vdots$$

This analysis for the general case leads directly to Kuhn's distribution function

$$G_n = n\, i^2\, (1-i)^{n-1}$$

The probabilities of obtaining molecules G_2, G_3, G_4, ... are proportional to

$$P_2,\ 1/2\ P_2,\ 1/3\ P_3,\ 1/4\ P_4,\ \ldots$$

On normalizing they become

$$P_1 = i$$
$$P_2 = i\,(1-i)$$
$$P_3 = i\,(1-i)^2$$
$$P_4 = i\,(1-i)^3$$
$$\vdots$$
$$P_n = i\,(1-i)^{n-1}$$
$$\vdots$$
$$P_{n+6} = i\,(1-i)^{n+5}$$
$$\vdots$$

If the total molar concentration of saccharides in the system is M_0, the molar concentration (G_n) of the nth component is given by equation (11)

$$G_n = i\,(1-i)^{n-1}\,M_o \qquad (11)$$

The molar ratio

$$\frac{G_n}{G_{n+6}} = (1-i)^{-6}$$

By this analysis a parameter "i" has been introduced into the equilibrium expression. It is defined as follows. If "a" is the average number of reactive glucose units per reducing group at completion of reaction, then 1/a is equal to "i". Thus "i" is the probability that a glucosidic linkage is broken and is the reciprocal of the average chain length of linear saccharides in the system. The quantity "a" is readily measurable by any one of the procedures described in the experimental section. The equilibrium expressions for the formation of cyclic dextrins reduce to

$$K_{alpha} = [alpha]\,(1-i)^{-6} \qquad (12)$$

$$K_{beta} = [beta]\,(1-i)^{-7} \qquad (13)$$

$$K_{gamma} = [gamma](1-i)^{-8} \qquad (14)$$

Equilibria in maltose systems

When maltose is used as a substrate for the enzyme, the only measurable reactions proceeding are those of Equation (6). Cyclizing reactions are not proceeding since under normal experimental conditions, negligible amounts of Schardinger

dextrins are formed. Therefore, an analysis for the saccharides present in an equilibrated maltose-macerans system should lead to values for equilibrium constants of homologizing reactions.

Studies by Norberg and French (73) on homologizing reaction in a maltose digest indicated that the system was in equilibrium in about 1000 C.P. Saccharide concentrations were determined at 2000 C.P. and 6000 C.P. in a maltose digest. Undoubtedly equilibrium had been established since the saccharide concentrations were the same at the two times of sampling. These concentrations are recorded in Table 6.

Table 6

Concentration of Linear Saccharides in a Macerans-Maltose Digest Originally 0.146 M in Maltose

Saccharide	Concentration mgs. per ml.	Molar	"i" Value
G_1	12.94	.0719	.49
G_2	12.32	.0360	.50
G_3	9.56	.0189	.49
G_4	6.02	.00905	.50
G_5	3.87	.00467	.50

The "i" value for a system is the reciprocal of the average chain length of linear saccharides in solution. The

"i" values in Table 6 were calculated by substituting the appropriate saccharide concentrations in equation (15) and solving for "i".

$$G_n = i\,(1-i)^{n-1}\,M_o \qquad (15)$$

If the equilibrium constants for homologizing reactions (equation 6) in which m, n, x, are any possible combination were not equal then the "i" values would not be equal. The latter would result since different concentrations of each saccharide would be present depending on specific equilibrium constants. However constant "i" values were obtained for the system; therefore the equilibrium constants for homologizing reactions with linear substrates are equal.

Equilibria in amylodextrin systems

The concentrations of cyclic dextrins at equilibrium in an amylodextrin digest were determined by the selective precipitant scheme and are recorded in Table 7. This table also contains values for the dextrins concentrations obtained in the amyloheptaose experiment.

The "i" value for the amylodextrin digest was calculated from the $Ce(SO_4)_2$ titers before and after beta amylase digestion, which were 8.80 mls. and 12.55 mls. of .0595 N $Ce(SO_4)_2$. As a first approximation for the quantity of linear saccharides in solution, calculate the maltose equiv-

Table 7

The Equilibrium Concentrations of Cyclic Dextrins in Amylodextrin and in Amyloheptaose Digests

Compound	Amylodextrin Digest gms.	moles	% yield	Amyloheptaose Digest gms.	moles	% yield
alpha	2.24	.00231	10	1.68	.00173	6
beta	5.82	.00514	26	3.52	.00307	13
gamma	3.25	.00251	15	1.65	.00127	6

alent of the digest from its reducing power using equation (16).

$$\text{wt. of maltose} = \frac{[\text{mls. of } Ce(SO_4)_2] \times [\text{N of } Ce(SO_4)_2]}{30.2} \quad (16)$$

The factor 30.2 is the reducing equivalent of maltose (79). When numerical values (8.80 and .0595) are inserted in equation (16), the maltose value obtained is .0173 grams. This figure is only approximately equal to the total weight of saccharides since it was assumed in the calculation that each aldehyde group corresponds to a maltose molecule. In the digest several molecular species of linear saccharides including glucose and molecules larger than maltose are present. To obtain the weight of these additional glucose residues substitute 3.75, the increase in .0595 N $Ce(SO_4)_2$ in equation (16). A value of .0074 grams is obtained on solving the equation. Therefore the total weight of linear

saccharides in solution is .0247 grams, the sum of the two values. The reducing equivalent of the linear saccharides in the system is

$$\frac{(8.80)\ (.0565)}{.0242} = 21.2$$

This reducing equivalent of 21.2 corresponds to an average chain length of 3.6 glucose residues (79), and an "i" value of .28 for the system.

The equilibrium constants for the cyclizing reactions were obtained by substituting appropriate values in equations (12), (13) and (14) and solving for the K values.

$$K_{alpha} = 1.70 \times 10^{-2}$$

$$K_{beta} = 5.14 \times 10^{-2}$$

$$K_{gamma} = 3.48 \times 10^{-2}$$

Equilibria in amyloheptaose systems

The equilibrated digest was analyzed by the paper chromatographic procedures for both linear and cyclic compounds. The data for the cyclic saccharides have already been presented in Table 7. The concentrations of the linear saccharides were determined by the standardized copper procedure (79) and are shown in Table 8. As in the maltose digest, the "i" value was calculated by substitution in equation (15).

Table 8

Concentrations of Linear Saccharides in Amyloheptaose *Macerans* Amylase Digest

Saccharide	Concentration mgs. per ml.	molar	"i" value
G_2	1.49	.00826	.32
G_3	2.07	.00606	.36
G_5	2.03	.00403	.34
G_4	2.02	.00303	.28

For this experiment M_o = .0262 M.

Since the analytical values for the higher molecular weight compounds varied because of poor resolution on the paper chromatograms, the "i" value employed in the calculations was .34, the average of the first three values of Table 8. This value was also obtained when two simultaneous equations using the G_3 and G_5 concentrations were solved. The equilibrium constants were computed from the results and are:

$$K_{alpha} = 2.10 \times 10^{-2}$$

$$K_{beta} = 5.64 \times 10^{-2}$$

$$K_{gamma} = 3.53 \times 10^{-2}$$

The agreement between the two sets of equilibrium constants is good especially so since two different analytical

methods were used. The preferred values for the three equilibrium constants are 2.10×10^{-2}, 5.64×10^{-2} and 3.53×10^{-2}, the values for the heptasaccharide digest. This digest was undoubtedly at equilibrium, as shown by glucose measurements at various times of reaction. Glucose values were used as a measure of extent of reaction because disproportionation studies (73) indicated that the velocity of formation of glucose was the smallest. Thus at the point where glucose values became constant, equilibrium among the other reactions had been attained.

Utility of "i" value concept

The determination of equilibrium constants is not novel by any means as the literature abounds with examples of determinations of equilibrium constants for both organic and inorganic reactions. However for several reasons very few equilibrium constants for enzyme reactions have been reported. In the first place many enzyme reactions are essentially irreversible and useless for equilibrium studies. Secondly, a complete analysis for all reactions products have been an unsurmountable obstacle in the past since the reaction products are generally complex organic molecules. In the amyloheptaose and the amylodextrin experiments all the products were not determined; but the equilibrium constants could be calculated by using a parameter in "i" in

the equilibria equations. The concept of "i" values is new and has evolved in the course of this investigation. Not only is it applicable to the macerans enzyme but also to several other enzymes of the trans-glucosidase group.

The trans-glucosidases are enzymes which bring about a redistribution of the glucose residues of the substrate molecules to form new molecules. In addition to macerans amylase, this group includes phosphorylases (15, 16, 38, 39), isophosphorylase (5, 6, 7), viscosucrases (3), amylomaltase (62, 96) and amylosucrase (40, 41). To illustrate the utility of the "i" value concept let us consider briefly the phosphorylase and amylomaltase enzymes.

The phosphorylase enzymes of animal origin have been most thoroughly studied by the Coris (15, 16) and that of plant origin by Hanes (38, 39). The enzymes catalyze the reactions of type in equation (17).

$$G_n + H_3PO_4 \rightleftharpoons G\text{-}1\text{-}p + G_{n-1} \qquad n = 4, 5, 6, 7, \ldots \qquad (17)$$

The mathematical derivation of an expression for the equilibrium constant for this reaction parallels that for the macerans reactions. The final result is

$$K = \frac{[G\text{-}1\text{-}p][1\text{-}i]^{-1}}{(H_3PO_4)} \qquad (18)$$

The Coris (15, 16) and Hanes (38, 39) showed that the

equilibrium state, defined by the values of the ratio of organic to inorganic phosphate, was not significantly affected by alterations of the starch or glycogen concentrations. As a result they disregarded the contribution of the ratio G_{n-1}/G_n to the equilibrium expression. Though this contribution may be small, it must be included in the equilibrium expression from theoretical considerations. The experimental values for the ratio of organic and inorganic phosphate and the "i" value in a phosphorylosis mixture are needed for evaluating the equilibrium constant for the reaction.

Amylomaltase catalyzes the following reaction:

$$(n+1)\ G_2 \rightleftharpoons n\ G_1 + G_{2+n} \qquad n = 1, 2, 3, 4, \ldots \qquad (19)$$

The enzyme was first reported by Monod and coworkers (62, 96). They observed that high molecular weight polysaccharides staining blue with iodine were produced in the reaction only in the presence of notatine, a glucose oxidase, In the absence of notatine the polysaccharides produced would at the most stain a faint red with iodine.

"We cannot yet offer an interpretation of this observation" is the comment of these investigators.

The "i" value concept can be used to interpret this observation. The removal of glucose by notatine oxidation from the system decreases the "i" value of the system and equilibrium is not reached until high molecular weight poly-

saccharides are produced. In the absence of notatine, the glucose which is formed remains in the system and a rapid attainment of equilibration results. Under these conditions low molecular weight compounds staining red with iodine are produced in the reaction mixture.

C. Action of Salivary Amylase

Question of enzyme specificity

The term "enzyme specificity" commonly used by enzyme chemists supposedly divides the compounds which are potential substrates for an enzyme into two distinct classes, those upon which the enzyme can act and those upon which the enzyme cannot act. To be sure enzymes are specific in that they require substrates possessing structural similarities, as for example, ester linkages, glycosidic bonds, peptide bonds, etc. However, it is this group of similar type compounds which has been further subdivided according to "enzyme specificity".

Proponents of this view would lead us to believe that on account of specificity the action of the enzyme is restricted to a few specific compounds. These workers have failed to appreciate the significance of reaction kinetics in enzyme action. The so-called "enzyme specificity" may be regarded equally well as a kinetically controlled phenomena.

The problem thereby resolves to one of a study of reaction kinetics. Many compounds are classed as poor substrates because the enzyme acts on them at a very slow rate which has not been observed under the experimental conditions and not because the enzyme cannot act upon them. That velocity of enzyme reactions is dependent on the type of substrate is illustrated by the salivary amylase system. Here, at least six, and probably more, compounds of the amylose series are hydrolyzed at different rates. It is proposed that enzyme reactions in which several substrates are possible are interpreted more fundamentally in terms of reaction kinetics than in terms of enzyme specificity.

The formation of glucose

That glucose is one product of the action of salivary amylase on starch or starch-like compounds has been agreed upon by most investigators. However, its manner of formation, the time of its formation, the amounts formed are all points of disagreement. For example, Bernfeld and Studer-Pecha (9) claimed that it was formed in the late stages of starch-amylase hydrolyzates whereas Levine (51) interpreted his data as evidence that it was formed in the early phases of salivary amylase action. Further, Bernfeld and Studer-Pecha stated that it was formed only from amylotriose; Levine that it was formed from molecules larger than maltose includ-

ing amylotriose; and Myrbäck (66) that it was formed from molecules larger than amylotriose per se. Whether glucose is formed from amylotriose or from larger oligosaccharide molecules, or both can be unambiguously determined by using pure amylotriose and amylotetraose substrates. As it has been repeatedly demonstrated that salivary amylase acts on maltose at an infinitesimal rate, the only possible reaction routes for these two substrates are indicated by equations (20), (21) and (22).

$$G_3 \longrightarrow G_1 + G_2 \qquad (20)$$

$$G_4 \longrightarrow G_1 + G_3 \qquad (21)$$

$$G_4 \longrightarrow 2\ G_2 \qquad (22)$$

Salivary amylase, malt amylase, and pancreatic amylase do act on amylotriose since the rotations of trisaccharide digests dropped from initial values of 1.85° to a final value of 1.23°. The actual rotation drop in the experiment of 0.62° agreed well with the theoretically expected drop calculated for equation of 0.60°. Glucose and maltose were identified in the reaction mixture by column chromatography (51). The hydrolysis of amylotriose to one molecule of glucose and one molecule of maltose is therefore demonstrated and is in harmony with contentions of Bernfeld *et al.* and Levine. Myrbäck's conclusion that it was not hydrolyzable was based

on fermentation experiments which have proven to be unreliable at times (66, 67, 68) and is obviously erroneous.

The amylotetraose was isolated in small quantities from phosphorylase-heptasaccharide digest in our laboratories. The tetrasaccharide-amylase experiments were conducted on a micro scale and using only one alpha type enzyme, salivary amylase. Maltose was the only product of reaction identified on paper chromatograms of the digest. Consequently, the action of salivary amylase on amylotetraose is expressed by equation (22). It is highly improbable that under normal conditions glucose is formed from the end of molecular chains larger than G_4. These observations are in disagreement with Myrbäck's and with Levine's contentions that glucose was produced from molecules larger than amylotriose. Once again, Myrbäck's fermentation experiments are subject to the criticism already mentioned. Levine, on the other hand, used amyloheptaose as a substrate and failed to realize that this molecule would be rapidly degraded by salivary amylase to give relatively large amounts of amylotriose, from which, in turn, glucose could be formed in relatively high concentrations. It is, therefore, unnecessary to postulate that some of this glucose results from the action of salivary amylase on molecules larger than amylotriose.

Another line of evidence for the proposed mechanism for glucose formation was obtained from the amylodextrin experi-

ments. The initial products of action of salivary amylase on this substrate are maltose, amylotriose and amylotetraose. Periodic analyses for the products of reaction with time revealed that after the bulk of the substrate was hydrolyzed to G_2, G_3 and G_4 molecules, the amylotetraose concentration began to decrease and the maltose concentration to increase correspondingly. The amylotriose was hydrolyzed next for glucose began to appear in the mixture and the amylotriose to disappear. Ultimately the original substrate is completely converted to glucose and maltose. Glucose was formed only in the later stages of the reaction and apparently from amylotriose.

Evidence for non-random hydrolysis

The action of alpha amylases on amylo-type compounds is interpreted by most investigators as a random hydrolysis of the alpha-1-4 glycosidic bonds. Consequently during the course of reaction one would expect to find the whole array of linear amylooligosaccharides in enzymic digests. When amylodextrin, amyloheptaose, amylose or starch were used as substrates for the enzyme under optimum conditions of temperature, pH, salts, etc., the only significant reaction intermediates present at any time were maltose (G_2), amylotriose (G_3) and amylotetraose (G_4). A typical chromatogram of an amylodextrin digest at ca 50% hydrolysis is reproduced in

Figure 2. Analyses by qualitative paper chromatographic methods of the digests at varying reaction times, showed that during reaction the concentrations of these low molecular weight saccharides increased and of the substrate decreased with time. In such digests intermediates of the penta, hexa, hepta-saccharide type which would be expected if random hydrolysis occurred were not observed at any stage of the reaction. If these compounds had been present, they would have been easily identified by the analytical methods. Obviously the action of the enzyme on these substrates under optimum conditions is not random. Before proposing a new mechanism for the mode of action of salivary amylase, the results of several other pertinent experiments will be considered.

If salivary amylase experiments were conducted under conditions unfavorable for enzyme action, e.g. high pH, low pH, high temperature, absence of essential ions, then not only the rate of the reaction but also the amounts and nature of intermediate products was altered. A paper chromatogram of salivary-amylase-amylodextrin digest at pH 10.3 at which the enzyme is stable but acts at a slow rate (4) is shown in Figure 2. Compounds G_1, G_2, G_3, G_4, G_5, G_6, G_7 and unresolved higher molecular weight saccharides are present in this digest. In digests of pH 4.5, intermediates up to G_9 were resolved and identified on paper chromatograms.

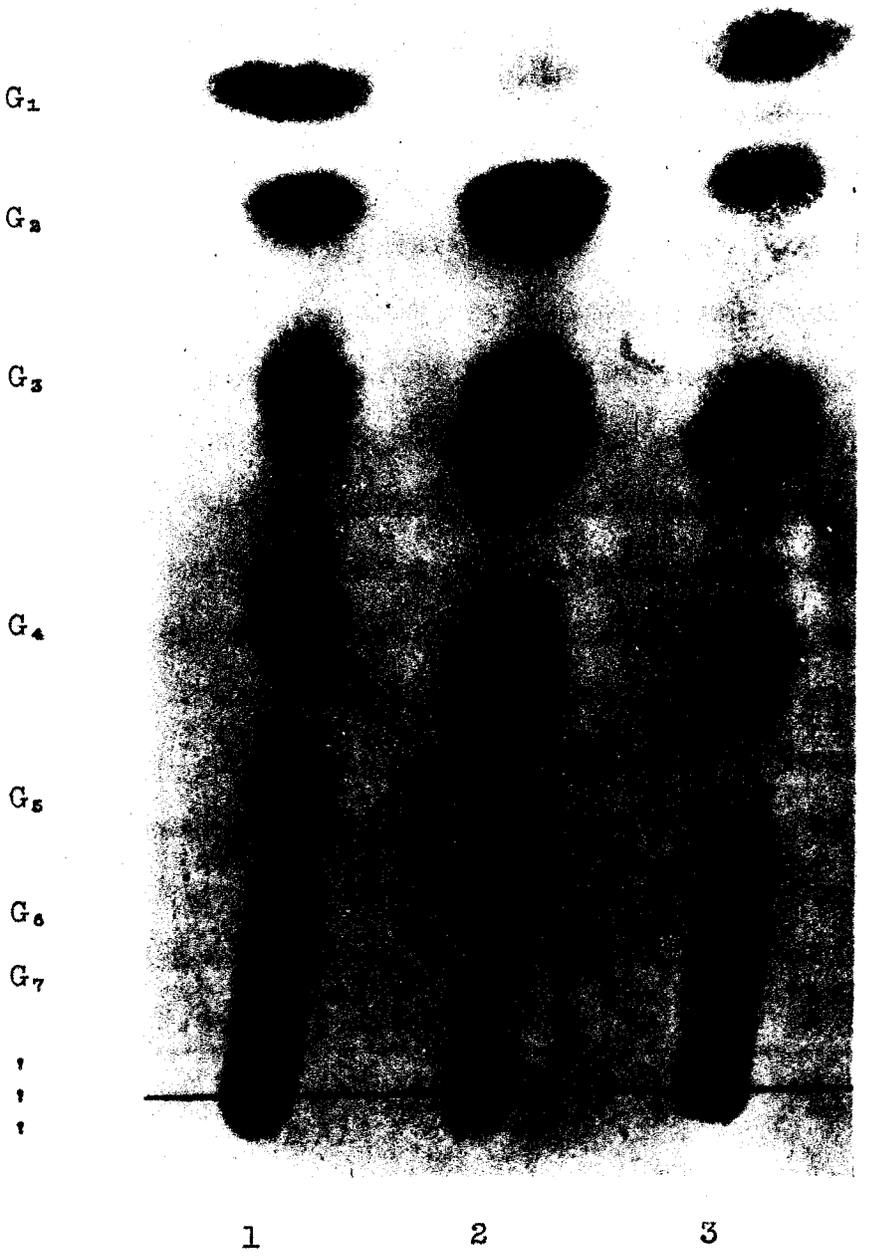

Fig. 2. Paper chromatogram of salivary amylase-amylodextrin digests. 1. Reference oligosaccharides. 2. Digest at pH 7.0. 3. Digest at pH 10.3.

Similar results were obtained in experiments conducted at pH 7.0 and 60° C.

The intermediate compounds in the action of salivary amylase on amylose were studied by their characteristic migration on paper chromatograms, by the absorption spectra of their iodine complexes and by their reducing power. Only under favorable conditions were the intermediates G_2, G_3 and G_4 present in sufficient concentrations to be observed on paper chromatograms. The total reducing power of the digests expressed in milligrams of maltose and the absorption measurements on the iodine complexes are recorded in Table 9.

Table 9

The Reducing Power and Iodine Coloration of Amylose-Salivary-Amylase Digests as a Function of Time

Time Minutes	mgs. of maltose per 20 mls. pH 7.0	pH 10.3	Optical Density* pH 7.0	pH 10.3
2	3.89	0.72	0.92	1.30
5	8.30	1.05	0.38	1.14
9	-	-	0.02	-
15	16.3	3.08	-	0.56
30	25.9	4.88	-	0.32
60	33.0	7.04	-	0.04

*Optical density values are for wave lengths of maximum absorption and for 1 ml. aliquots of the digests diluted to 50 mls. in a final iodine concentration of .0002 M.

The rate of change of these properties can be compared from the plot of data on semi log paper (Figures 3 and 4).

It is noted that the optical density of the alkaline digest decreased at 1/5 the rate of the neutral digest while the reducing power increased by only 1/11. If the course of action of the enzyme under the two conditions was the same, the rate of change in the two properties would be the same. The change in iodine coloration of the digests results from the production of shorter but still large molecular fragments in the digest while the increase in reducing power from these fragments as well as from the low molecular weight products (G_2, G_3 and G_4). That the latter were present in the neutral digest and not in the alkaline digests was shown by paper chromatographic methods. Evidently the action of the enzyme on the same substrate but under different conditions proceeds by different reaction routes. Here, as in the amylodextrin experiments, not only the rate but also the nature and amount of intermediate products are determined by reaction conditions.

Role of diffusion

The prevailing theory of enzyme kinetics due to Michaelis and Menten (60) asserts that laws of first order kinetics are followed. The velocity of reaction consequently depends on the concentration and the rate of disintegra-

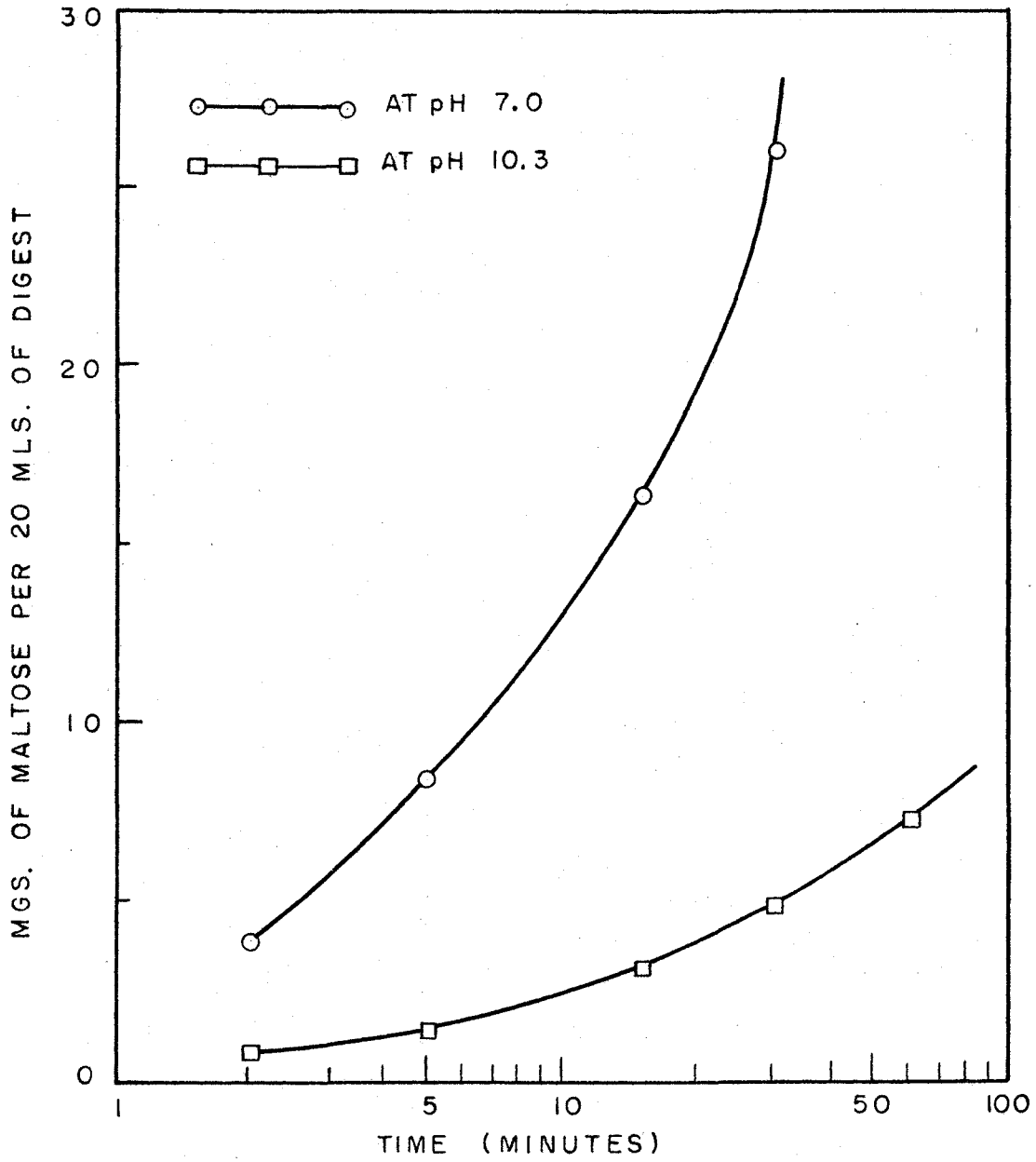

Fig. 3. The reducing power of salivary amylase-amylose digests.

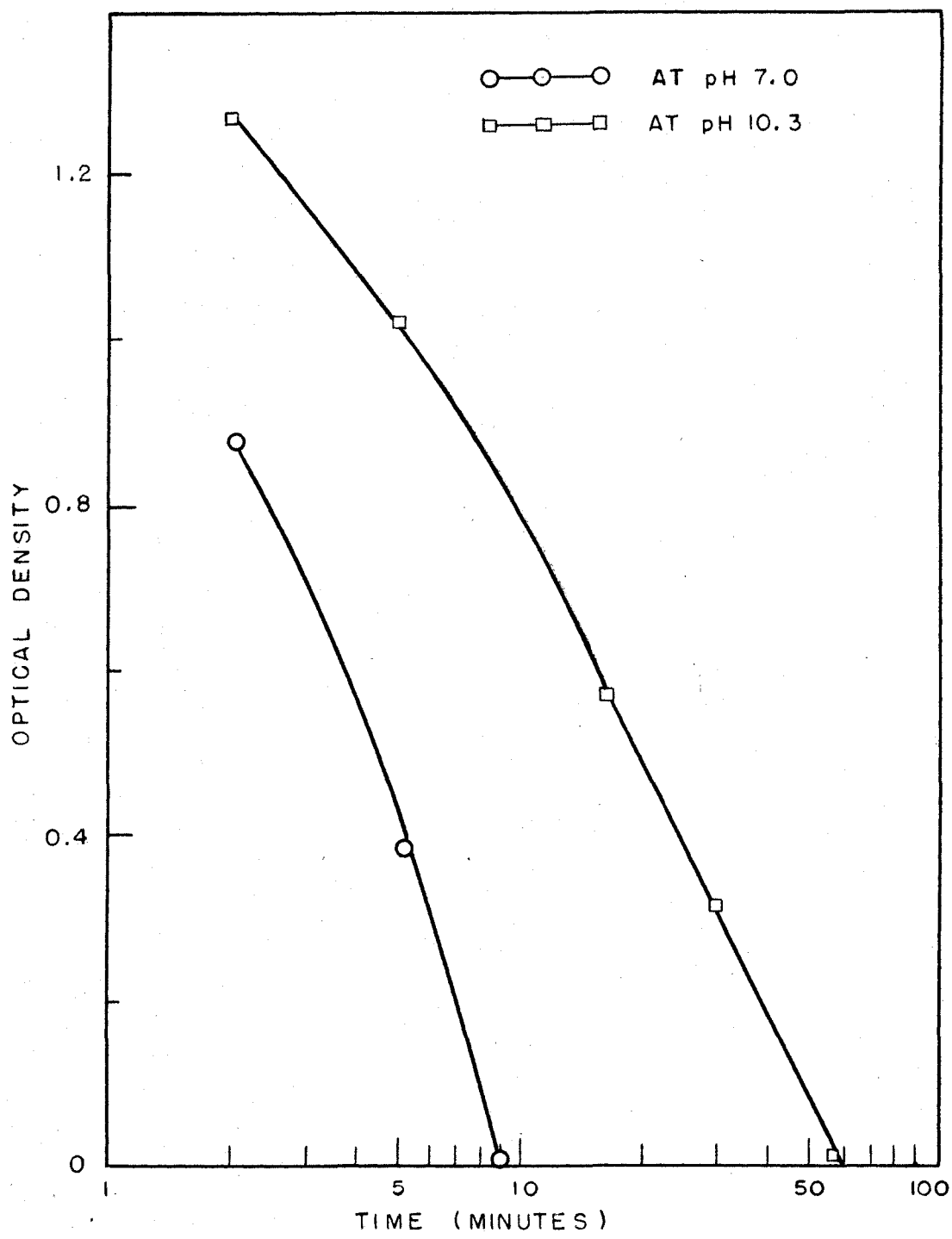

Fig. 4. The absorption spectra of salivary amylase-amylose digests in the presence of iodine-iodide.

tion of a reactive enzyme-substrate complex. Using symbols of Michaelis and Menten the salient points of this treatment are condensed in equations (23), (24) and (25).

$$E + S \rightleftarrows ES \longrightarrow E + P \qquad (23)$$

$$v = k [ES] \qquad (24)$$

$$v = \frac{V_m [S]}{K_m + [S]} \qquad (25)$$

K_m is the dissociation constant for the enzyme-substrate complex (ES).

In this theory, the size of the enzyme and substrate molecules which in reality is generally enormous in comparison to that of ions or molecules involved in ordinary chemical reactions has not been considered. The importance of molecular size lies in the fact that both enzyme and substrate molecules must first diffuse through the solution by thermal motion until they collide and form an enzyme-substrate complex. If the rate of diffusion is less than the rate of disproportionation of the complex, then diffusion will be a rate controlling process.

Diffusion may also effect kinetics of reaction in another manner. After the enzyme substrate complex is formed it is surrounded by a cage of solvent molecules. The initial

reaction products can be held in the proximity of the enzyme for a short time by the solvent cage and can be further attacked before they diffuse out of the sphere of influence of the enzyme.

If diffusion is a rate controlling process, then an inverse relationship between viscosity and reaction rate exists. Data on the relationship between viscosity and reaction rate was determined for a salivary amylase-amylodextrin system. Glycerol was added to the digests in appropriate amounts for increasing the viscosities. The glycerol did not inactivate the enzyme since the velocity of reaction did not decrease with time and since all the substrate was completely degraded in the presence of glycerol. To allow for comparisons, the concentrations of enzyme and substrate were the same in the digests of different viscosities. Neither the concentrations of amylodextrin nor of water were rate limiting factors. The former was such that the enzyme could operate at maximum velocity until 50% of reaction had occurred and the latter was 2000 times greater than the dextrin concentration.

The velocities of reaction are expressed in milligrams of maltose per minute in Table 11 and have been obtained from the rate of increase of reducing power of the digests (Table 10).

A plot of the reciprocal of velocity versus viscosity

Table 10

Reducing Power of Salivary Amylase-Amylodextrin Digests of Varying Viscosities

Sample	Time Minutes	Titer .0498 N $Ce(SO_4)_2$ per 5 mls.	mgs. Maltose per 5 gms.
1	1	5.38	8.90
	3	13.60	22.4
	6	22.33	36.4
	9	30.22	49.8
2	1	1.62	2.29
	15	4.43	6.28
	30	7.48	10.7
	60	11.71	16.6
3	1	0.10	0.13
	15	0.78	1.08
	30	1.18	1.64
	60	2.44	3.38
4	1	-	-
	25	0.13	0.17
	50	0.88	1.20
	100	1.50	2.06

Table 11

Velocity of Salivary Amylase Action on Amylodextrin at Different Viscosity Conditions

sample	viscosity centipoises	velocity mgs. of maltose per min.	$\frac{1}{v}$
1	1.00	7.64	.131
2	10.8	0.30	3.37
3	25.5	0.057	17.5
4	37.2	0.021	48.8

is shown in Figure 5. If the reaction rate was dependent only on the rate of diffusion of the reactants towards each other then a straight line should have been obtained. However, the "cage effect" of viscous solvent on the rate of dissociation of the products and enzyme must also be considered since the reaction products are susceptible to further attack. A reaction rate greater than that obtained under normal circumstances might be anticipated. The deviation from linearity of the curve in Figure 5 was in this direction.

The formation of branched saccharides

The branched oligosaccharides containing 1-6 and 1-4 glucosidic linkages are formed along with the linear oligosaccharides by the action of salivary amylase on branched

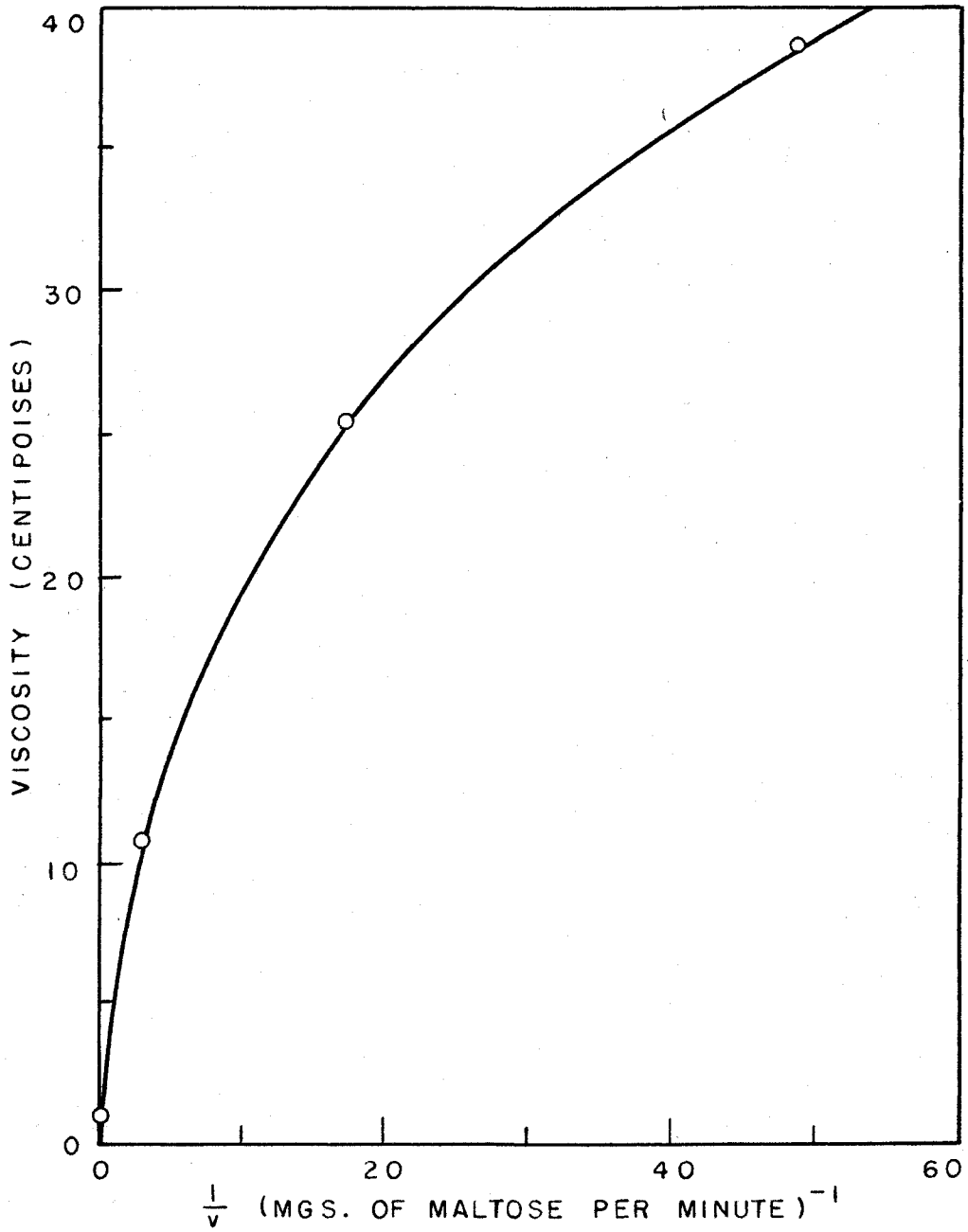

Fig. 5. The effect of viscosity on the velocity of salivary amylase action.

substrates such as beta amylase limit dextrin. The first member of the branched series is the tetrasaccharide. Its rate of formation was comparable to the rate of formation of glucose. The branched pentasaccharides, hexasaccharides and heptasaccharides were formed earlier than the tetrasaccharide and have been observed in both starch and glycogen limit dextrin enzymolyzates by the paper chromatography methods.

The branched saccharides were identified only by their characteristic migration velocities on paper and not by isolation procedures. The presence of a 1-6 linkage in these molecules retards their migration in comparison to the linear compounds. Thus isomaltose migrated with a velocity midway between that for maltose and amylotriose; the branched tetrasaccharide between that of the linear tetrasaccharide and linear pentasaccharide, etc.

Two methods are suggested for isolating the pure branched saccharides. In the first method the compounds in crude enzyme digests would be resolved on paper chromatograms and recovered by election techniques. In the second method, alcohol fractionations of enzymic digests would be used. The digests would be prepared by reacting branched limit dextrins successively with salivary amylase and clostridium maltase (46). The latter enzyme would degrade the linear compounds to glucose and large branched saccharides

to tetrasaccharide. The linear compounds would be removed by fermentation and the tetrasaccharide separated by alcohol precipitation.

An interesting problem in carbohydrate research still unsolved is the degree of branching in starch and glycogen molecules. Only approximate values for the degree of branching in these molecules have been reported in the literature. The extent of branching may be found by quantitatively determining the branched saccharides present in salivary amylase digests of starch or glycogen. From the percentage of branched saccharide, it should be possible to calculate the degree of branching in the original compounds.

Affinity constants

The affinity constants of salivary amylase for different substrates varied with the molecular size of the substrate. For amyloheptaose (G_7) the value obtained was 1.0×10^2 and for amylodextrin (ca G_{33}) it was 2.3×10^3.

In the determination of these constants the velocities of reaction were followed by rotation measurements. The data are given in Tables 12 and 13. The velocity of reaction in degrees per minute was calculated from the initial slope of the curve obtained on plotting the rotation data. Using these values (Table 14) and the Michalles-Menten equation (25) in the reciprocal form

$$\frac{1}{v} = \frac{K_m}{V_m} \cdot \frac{1}{(S)} + \frac{1}{V_m} \qquad (26)$$

the affinity constants can be evaluated. A plot of 1/(v) and 1/(S) gave a straight line. Maximum velocity (V_m) corresponds to the intercept on the 1/v axis and the K_m/V_m to the slope of the line. The affinity constant, the reciprocal of the dissociation constants, (K_m) were calculated from these values.

Table 12

Rotations (degrees) of Digests of Salivary Amylase and Amyloheptaose

Time Minutes	Amyloheptaose Concentration .00192 M	.00383 M	.0115 M
2.0	0.80	1.58	4.86
3.0	0.78	1.55	4.83
5.0	0.75	1.51	4.77
10.0	0.72	1.45	4.61
15.0	0.70	1.43	4.52
30.0	0.70	1.37	4.33

Table 13

Rotations (degrees) of Digests of Salivary Amylase and Amylodextrin

Time Minutes	Amylodextrin Concentration .00116 M	.00233 M	.00349 M
2.0	1.75	3.47	5.29
3.0	1.70	3.45	5.27
5.0	1.68	3.40	5.21
10.0	1.60	3.26	5.08
15.0	1.55	3.17	4.93
30.0	1.47	2.97	4.63

Table 14

Velocities of Salivary Amylase Action on Amyloheptaose and Amylodextrin

Substrate	Concentrations (S) molar	1/S	Velocity (v) degrees per min.	1/v
amyloheptaose	.00192	521	.00925	108
	.00383	261	.0155	64.5
	.0115	87	.0312	32.0
amylodextrin	.00116	862	.0218	45.8
	.00233	430	.0254	39.4
	.00349	287	.0274	36.5

Action mechanism

Let us consider a possible mechanism by which the pertinent data is interpretable for the action of salivary amylase. Under favorable conditions (i.e., the enzyme can act rapidly on the substrate) several effective collisions between enzyme and substrate molecules can occur in a single encounter since the complex is surrounded by a solvent cage. As a result the substrate molecule is cleaved several times in a localized area, and products such as the di-, tri- and tetra-saccharides are produced. These intermediates accumulate in the digest and can be identified on the paper chromatograms. Under unfavorable conditions, either the number of collisions is reduced or a smaller percentage of the collisions are effective and consequently the initial hydrolytic products can diffuse out of the sphere of influence of the enzyme. It seems probable that at unfavorable conditions only one linkage is broken in each encounter and therefore the enzyme acts in a random fashion under these circumstances.

To illustrate the reactions under these two conditions, the action of salivary amylase on a single molecule (G_{10}) is shown by equation (27) for favorable and equation (28) for unfavorable conditions.

$$G_{10} + E \rightleftharpoons G_{10}{\cdot}E \longrightarrow G_7{\cdot}E + G_3 \longrightarrow G_5{\cdot}E + G_5 \longrightarrow G_5 + G_5 + E$$
$$\downarrow\uparrow \qquad\qquad \downarrow\uparrow$$
$$G_7 + E \qquad\qquad G_5 + E \qquad\qquad (27)$$

$$G_{10} + E \rightleftharpoons G_{10}{\cdot}E \longrightarrow G_7{\cdot}E + G_3$$
$$\downarrow\uparrow$$
$$G_7 + E \qquad\qquad (28)$$

A reversible equilibrium can be established between enzyme-substrate complex, free enzyme and hydrolytic products at all stages of the reaction. That under unfavorable conditions this equilibrium is shifted towards free molecules has been demonstrated for the beta amylase (page 103) but it is only speculative for salivary amylase. If this speculation is correct, then the reaction would be shifted towards free enzyme and initial products. Experimental observations are in harmony with this speculation. It would appear that this theory in which diffusion and the cage effect are important processes more adequately interprets the experimental findings than the random hydrolysis theory.

D. Action of Beta Amylase

On linear and branched substrates

Beta amylase acts on amylaceous substrates by removing maltose units from the substrate chains beginning at the non-

reducing ends. Linear molecules containing an even or an odd number of glucose residues are degraded respectively to maltose or to maltose and amylotriose. The glucose residue containing the original free aldehyde group constitutes one residue of the amylotriose molecule. That linear molecules are hydrolyzed in this manner has been proven by experiments with amyloheptaose and amylohexaose. The former have been described in a recent publication by French and co-workers (24) and the latter in the experimental section.

In the hexasaccharide experiment the observed rotation drop during beta-amylase action of 0.66° agreed reasonably well with the theoretical expected drop of 0.63° for complete conversion of the hexasaccharide to maltose. The reducing power of the digest after reaction had subsided was 30.4 m.e.; theoretical value for maltose, 30.2 m.e. (79). The action of the enzyme on linear molecules is illustrated in equations (29) and (30).

$$G_{2n+1} \longrightarrow (n-1)\ G_2 + G_3 \qquad (29)$$

$$G_{2n} \longrightarrow n\ G_2 \qquad (30)$$

If the amylaceous substrate is branched, that is contains 1-6 linkages, then the enzyme action ceases when one or two glucose residues, depending on whether the number of glucose residues per chain is even or odd, remain intact beyond the glucose residue containing the branch point. Neither the isomaltose nor the sucrose containing tetrasaccharide

was attacked by beta amylase. Branched compounds larger than the tetrasaccharide were hydrolyzed to maltose and to either the trisaccharide or tetrasaccharide. Here is another method of enzymically synthesizing new carbohydrate compounds. The 1-6 glucosidic and sucrose containing tri- and tetra-saccharides should be present in beta amylase digests of the coupled product obtained by the action of macerans amylase on alpha dextrin and appropriate co-substrate. These should be separable from the mixture by paper chromatography procedures.

The single-chain and multi-chain theories

The controversy between the Kerr and the Hopkins schools on whether beta-amylase action proceeds by a single chain or the multi-chain process is still in progress. Amyloheptaose experiments (24) supported the single chain theory since amylopentaose, an intermediate that should be present if the enzyme acts by the multi-chain theory was not observed in the enzymic mixture. However, an analysis of an amyloheptaose digest at several stages of reaction by a more sensitive analytical procedure - paper chromatography, indicated that the pentasaccharide was present in the mixture. On further application of both qualitative and quantitative chromatography methods its presence was definitely established. The amount of amylopentaose in these digests reached

a maximum corresponding to 15 mole percent of the total carbohydrate and at about 75% hydrolysis stage (see Table 15). Admittedly the amount of pentasaccharide was small but it was nevertheless significant. It is not surprising that its presence was not detected by the less sensitive electrophoretic procedure (24) especially as the analysis of the digest by the latter procedure was conducted at 50% hydrolysis stage.

Table 15

Molar Concentrations of Saccharides in the Reaction Mixture of Amyloheptaose and Beta Amylase

Time Minutes	G_7	G_6	G_5	G_4
0	.0174	0	0	0
2.5	.0156	.0012	.0006	.0024
5.0	.0122	.0015	.0037	.0089
10	.0087	.0022	.0065	.0152
15	.0070	.0029	.0075	.0179
30	.00174	.0017	.0140	.0297
60	0	0	.0174	.0348

That the enzyme is acting on amyloheptaose solely by a single chain process (equation 31) is eliminated since amylopentaose was present in the reaction mixture. That only the multi-chain process (equations 32 and 33) is involved is also

eliminated since on calculating psuedo velocity constants for equations (32) and (33) using the data of Table 15, a wide discrepancy between the calculated and observed maximum concentrations of pentasaccharide was obtained.

$$G_7 \longrightarrow G_3 + 2\,G_2 \qquad (31)$$

$$G_7 \longrightarrow G_5 + G_2 \qquad (32)$$

$$G_5 \longrightarrow G_3 + G_2 \qquad (33)$$

That the two processes expressed in equations (31), (32) and (33) were involved has been verified by solving three simultaneous differential equations. In setting up the differential equations, k_1, k_2 and k_3, were defined as the psuedo first order velocity constants for the three reactions, respectively. The rate of change of G_7, G_5 and G_3 may be expressed by the differential equations

$$\frac{dG_7}{dt} = -(k_1 + k_2)\,G_7 \qquad (34)$$

$$\frac{dG_5}{dt} = k_2 G_7 - k_3 G_5 \qquad (35)$$

$$\frac{dG_3}{dt} = k_1 G_7 + k_3 G_5 \qquad (36)$$

with corresponding solutions

$$G_7 = G_7^o\, e^{-(k_1+k_2)t} \qquad (37)$$

$$G_3 = \frac{k_2\, G_7^0}{[k_3-(k_1+k_2)]}\left[e^{-(k_1+k_2)t} - e^{-k_3 t}\right] \qquad (38)$$

$$G_3 = G_7^0\left[1 + \frac{k_2-k_3}{[k_3-(k_1+k_2)]}\, e^{-(k_1+k_2)t} + \frac{k_3}{[k_3-(k_1+k_2)]} e^{-k_3 t}\right] \qquad (39)$$

From the data in Table 15 and the fact that maximum concentration of pentasaccharide was attained in 15 minutes, constants k_1, k_2, and k_3 were found to be .039, .028, .065 min^{-1}, respectively. The calculated maximum for G_3 concentration for this experiment was .0027 M and the experimental value was .0029 M.

Beta amylase action on a molecule as small as amyloheptaose appears to proceed to a greater extent by a single chain process and to a lesser by the multi-chain process. For molecules as large as amylodextrin the reaction appears to proceed mainly by the single chain theory. At no time have intermediate products G_4 up to G_{10} which would be expected by multi-chain action been observed in amylodextrin-beta amylase digests.

At favorable and unfavorable conditions

In Section V-C, the action of salivary amylase under unfavorable conditions was considered. Since beta-amylase like salivary amylase is a hydrolytic enzyme it seemed con-

ceivable that similar effects would be observed for this enzyme. At first qualitative experiments on the action of beta amylase on amyloheptaose at pH 10.3 and 40° C. and at pH 4.5 and 65° C. were conducted. The prediction proved correct. The course of beta amylase-heptasaccharide reaction was dependent on reaction conditions. Under favorable conditions, pH 4.5 and temperature 40° C., the initial products of reaction were maltose and amylotriose; however, under unfavorable conditions the initial products were maltose and amylopentaose (Table 16). In the latter digest the amount of amylopentaose accumulated in the initial phase of reaction and only when its concentration decreased did significant amounts of amylotriose appear. That the enzyme is not inactivated by unfavorable conditions was proven since the amyloheptaose was converted quantitatively into two moles of maltose and one mole of amylotriose (Table 16).

The effect of reaction conditions on the velocity of reaction may be found from the values in Table 17, which contains the data of the starch-beta amylase experiment. For comparative purpose the velocity is expressed in units of enzyme activity per milligram of dried enzyme preparations. It is noted for example that the velocity at pH 4.5 is 50 fold that at pH 10.3

Table 16

Molar Concentrations of Saccharides
in a Reaction Mixture of Amyloheptaose and Beta-Amylase
at Favorable (I) and Unfavorable (II)
Reaction Conditions

	Time Minutes	G_7	G_6	G_5	G_4
I	0	0.0363	0	0	0
	2.5	0.0321	0.0016	0.0021	0.0070
	10	0.0263	0.0037	0.0055	0.0164
	40	0.0069	0.0057	0.0210	0.0514
	80	0	0	0.0331	0.0703
II	0	0.0375	0	0	0
	1.0	0.0264	0.0078	0.0031	0.0137
	2.5	0.0180	0.0123	0.0059	0.0277
	5.0	0.0128	0.0142	0.0100	0.0355
	10	0.0056	0.0135	0.0164	0.0490
	20	0.0019	0.0069	0.0250	0.0651
	80	0	0	0.0360	0.0723

Table 17

Reducing Power of Beta Amylase-Starch Mixtures at Different Reaction Conditions

Reaction conditions pH	temp. C.	Velocity units per mg.	Time minutes	.0546 N $Ce(SO_4)_2$ titer mls.	Maltose mgs.
4.5	40	1000	1.0	1.65	3.0
			10.0	9.40	17.0
			20.0	17.62	31.8
			40.0	28.65	51.8
10.3	40	20	1.0	0.91	1.7
			10.0	2.42	4.4
			20.0	3.80	6.9
			40.0	5.55	10.1
4.5	65	194	1.0	18.50	33.5
			10.0	34.65	62.6
			20.0	-	-
			40.0	-	-
4.5	5	106	1.0	2.50	4.4
			10.0	10.35	18.5
			20.0	19.15	34.7
			40.0	29.50	53.4

The effect of pH on affinity constants

The affinity constants at pH values of 4.5, 7.0 and 10.3 were determined as described in the experimental section. The analytical data are shown in Table 18. The maximum velocities (V_m) and dissociation constants (K_m) were evaluated

Table 18

Maltose Concentrations in Amyloheptaose-Beta Amylase Digests of Varying pH

Amyloheptaose Concentrations M x 10^4	pH	Mgs. of Maltose per 25 mls. 2 minutes	5 minutes	8 minutes
1.73	4.5	1.0	1.8	2.5
3.46	4.5	1.2	2.5	-
5.19	4.5	1.6	3.2	4.3
6.92	4.5	2.1	3.6	5.0
1.73	7.0	2.0	2.5	-
3.46	7.0	2.3	4.1	-
5.19	7.0	2.6	4.4	6.0
6.92	7.0	2.8	6.3	9.2
1.73	10.3	1.1	1.4	2.5
3.46	10.3	1.5	3.3	-
5.19	10.3	2.0	3.6	-
6.92	10.3	2.9	5.6	7.1

as previously from the slope and the intercept on the 1/v axis of the straight line resulting on plotting 1/v versus 1/S values of Table 19. The affinity constant, the recip-

Table 19

Velocities of Beta Amylase Action on Amyloheptaose Under Varying pH Conditions

pH	Substrate Concentrations (S) M x 10^4	1/S	Velocity (v) mgs. Maltose per M units	1/v
4.5	1.73	5770	.485	2.06
	3.46	2890	.685	1.46
	5.19	1930	.795	1.26
	6.92	1450	.900	1.11
7.0	1.73	5770	.785	1.27
	3.46	2890	1.10	0.91
	5.19	1950	1.32	0.76
	6.92	1450	1.77	0.57
10.3	1.73	5770	.545	1.83
	3.46	2890	.810	1.24
	5.19	1930	1.00	1.00
	6.92	1450	1.53	0.65

rocal of the dissociation constants, for pH values of 4.5, 7.0 and 10.3, are 4.0×10^3, 2.4×10^3 and 1.2×10^3. The decrease in affinity constants at pH 4.5 and pH 10.3 was only 3 1/2 fold and accounted for only a small fraction of the difference in reaction velocities under the two conditions. Quite clearly, other factors must also be considered. Reasoning by analogy from the results of the salivary amylase system, it seemed probable that the activity of the enzyme substrate complex under unfavorable conditions was decreased. Since the velocity was first order in the complex, the velocity under unfavorable conditions would therefore be decreased. Preliminary work by French and Knapp (21) on the velocity rates at very high substrate concentrations indicated that the activity of the complex was markedly decreased at pH 10.3.

Affinity constants at optimum conditions

The affinity constants of beta amylase and amylodextrin, amyloheptaose and maltose substrates were 8.7×10^3, 4.0×10^3 and 3.5×10^2 at 40° C. and pH 4.5. The amylodextrin data contained in Tables 20 and 21 was used to prepare Figure 6. The dissociation constant and, in turn, the affinity constant was evaluated from equation 26 and Figure 6. The amyloheptaose and maltose data has been recorded in a previous section.

Table 20

Reducing Power of Beta Amylase-Amylodextrin Mixtures

Substrate Concentration M x 10^4	mgs. of Maltose per 25 mls. 5.0 min.	10 min.	15 min.
1.33	2.51	5.10	6.72
2.12	2.95	6.00	8.70
3.20	3.58	6.81	9.95
5.34	3.94	7.44	10.84

Table 21

Velocities of Action of Beta Amylase on Amylodextrin

Concentrations (S) M x 10^4	1/S	Velocity mgs. Maltose per minute	1/v
1.33	7520	.495	2.02
2.13	4690	.601	1.67
3.20	3120	.692	1.45
5.34	1870	.754	1.33

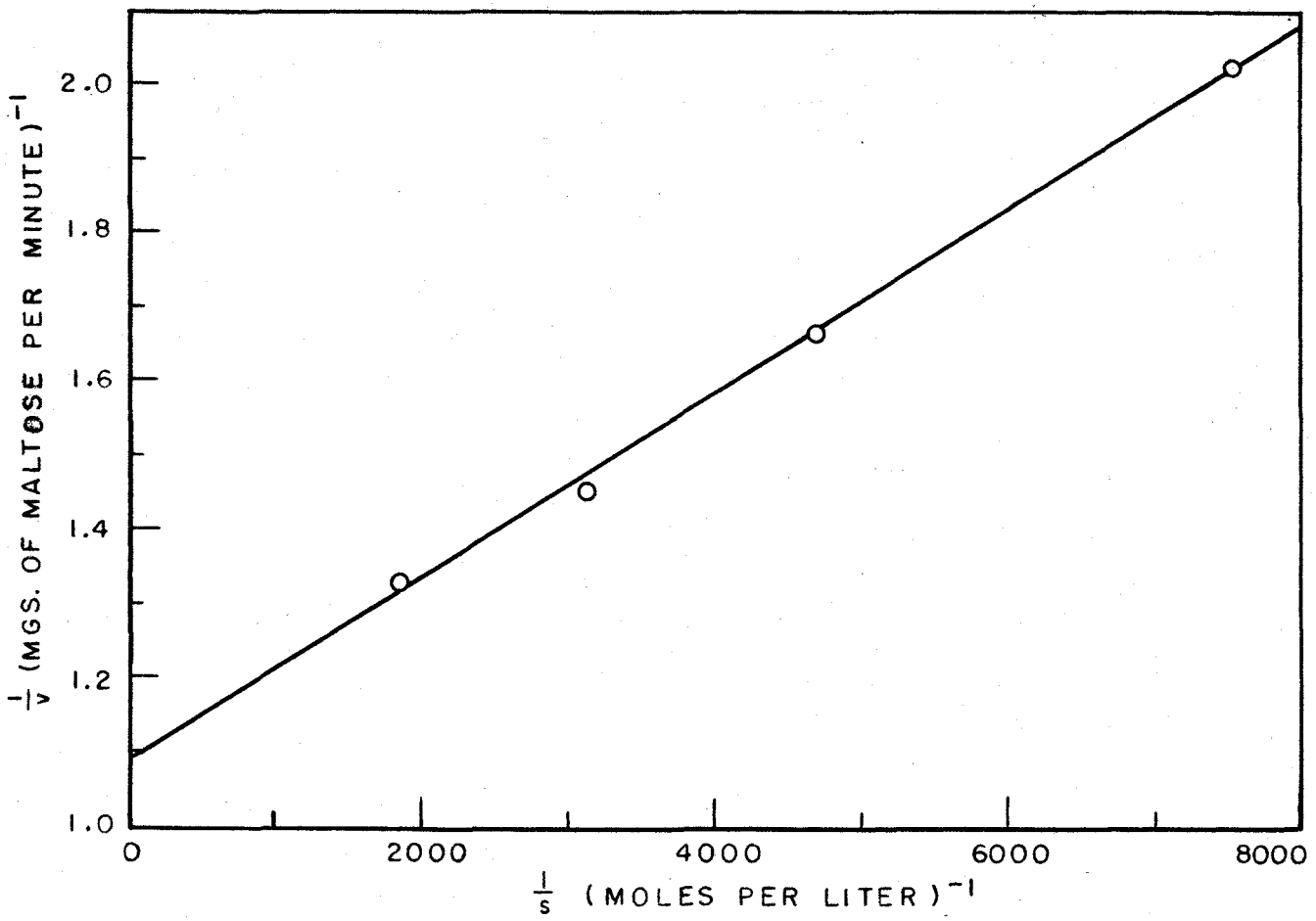

Fig. 6. The velocity of action of beta amylase at varying concentrations of amylodextrin.

It is indeed remarkable that under optimum conditions an enzyme molecule should degrade one molecular chain completely before it attacks another chain. According to Kerr (44) the absence of intermediate molecules in beta amylase digests is the result of a preferential hydrolysis of the short chain molecules. Such differences in hydrolysis rates should be reflected in the affinity constants of the enzyme for various substrate molecules. However, Hopkins and Jelinek (42) failed to obtain a significant difference for the affinity constants of the enzyme for amylodextrin and amylose. For this reason they rejected all of Kerr's arguments for a single-chain theory and claimed that beta amylase action was explained by the multi-chain theory.

The kinetic studies by Kerr and by Hopkins and Jelinek were conducted with amylose substrates and uncertainties would be introduced from limited solubility of amylose and from the approximate molecular weight values of the amyloses. When affinity constants were determined for compounds of proven constitution, maltose, amyloheptaose and amylodextrin, the values substantiated neither Kerr's nor Hopkins' contentions. In the range G_2 to G_{∞}, the affinity constants decreased markedly with decreasing molecular weights. The interpolations from the results of amylose experiments to low molecular weight compounds are obviously erroneous. Therefore, that short chain molecules are preferential hydrolyzed

is questionable and that they are intermediates in enzymic digests is impossible since their presence was not detected by chromatography procedures.

These observations would seem to invalidate the multi-chain process and some of the interpretations of the single chain process. The latter, however, appears to more adequately explain the mode of action of beta amylase. It is tentatively suggested that the enzyme acts by single chain process because the energy of the glucosidic bonds is utilized to maintain the enzyme and substrate in oriented positions. The net result is a complete hydrolysis of one molecular chain by a type of chain reaction before the enzyme and substrate dissociate. This suggestion is attractive when it is remembered that in muscular tissues enzymes appear to be responsible for the conversion of chemical energy into mechanical work.

VI. CONCLUSIONS

1. The quantitative separation of cyclic dextrins using differential precipitants and differential solubilities in water and 60% n-propanol is described.

2. Paper chromatography procedures have been developed for the quantitative analysis of mixtures of linear and cyclic saccharides.

3. Macerans amylase has been shown to have a trans-glucosidation action in which the exchangeable glucose residues are redistributed among linear and cyclic saccharides. Enzymic mixtures of linear substrates approached a state of dynamic equilibrium at which the cyclic dextrin and linear saccharides (G_1, G_2, G_3, ...) were shown to be present. Equilibrium constants for the reactions in the formation of cyclic alpha, beta, and gamma dextrin are 2.1×10^{-2}, 5.6×10^{-2} and 3.5×10^{-2}, respectively.

4. Salivary amylase acts by hydrolyzing several linkages of the substrate in a localized area before the enzyme and substrate molecules dissociate by diffusion.

5. The affinity constants of beta amylase for substrates are dependent on pH and on the molecular size of the substrate.

6. Not only the rate but also the nature and amounts of intermediate products in amylase (alpha and beta) action are

influenced by reaction conditions. The initial products of the action of beta amylase on amyloheptaose at pH 4.5 and 40° C. are maltose and amylotriose, and at pH 10.3 and 40° C. they are maltose and amylopentaose. In the intermediate stages of salivary amylase acting at pH 7.0 and 40° C. the only significant intermediate products of low molecular weight are maltose, amylotriose and amylotetraose while at pH 10.3 and 40° C. the whole array of linear amylo-oligosaccharides are present.

7. It is suggested that beta amylase acts by the single chain process because the energy of the glucosidic bonds can be utilized to maintain the enzyme and substrate in oriented positions.

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