chromatogram was dipped in a 0.05 *M* solution of NEM in isopropyl alcohol, allowed to dry for 15 minutes, and then was dipped in a 0.25 *M* solution of potassium hydroxide in isopropyl alcohol. Each reactant gave rise to a single pink spot (R_f 0.95).

The procedure was repeated by incubating for 12 hours 17.5 mg. of L-cysteine hydrochloride monohydrate (100 μmoles), 30 mg. (100 μmoles) of glutathione, 7 mg. (100 μmoles) of imidazole and 19 mg. (100 μmoles) of L-histidine hydrochloride monohydrate separately with 12.5 mg. (100 μmoles) of NEM in 5 ml. of 0.1 *M* phosphate buffer at *p*H 7.4. With the thiols, reaction products (R_f 0.42 and 0.55, respectively) were observed; with the imidazoles, the pink reaction product (R_f 0.95) was observed.

Cysteine hydrochloride monohydrate (17.5 mg., 100 μmoles) was treated at room temperature for 7 days with 12.5 mg. (100 μmoles) of NEM in 5 ml. of 0.1 *M* phosphate buffer at *p*H 7.4 (solution 1). Solutions 2–5 were prepared using cysteine hydrochloride monohydrate (100 μmoles) and increasing amounts (200–500 μmoles) of NEM. After 7 days, solution 1 was homogeneous, and chromatography showed the exclusive presence of the product of R_f 0.42; solutions 2–5 showed increasing separation of pink crystals, and chromatography showed a progressive decrease in concentration of the product of R_f 0.42, and a simultaneous progressive increase in concentration of a second product of R_f 0.95. The first product was reactive to ninhydrin at room temperature, the second was reactive only after drying of the ninhydrin sprayed chromatogram at 100°.

S-(N-Ethylsuccinimido)-L-cysteine (VI).—To a solution of L-cysteine hydrochloride monohydrate (1.75 g., 10 mmoles) in 50 ml. of water was added 1.25 g. of NEM (10 mmoles), followed by 10 ml. of 1.0 *N* sodium hydroxide. The resulting solution (*p*H 5.8) was incubated for 30 minutes, and then was concentrated to 20 ml. under reduced pressure at 35°. On addition of 20 ml. of acetone, 2.1 g. (84%) of crystals separated and were washed with ethanol. Recrystallization from water–acetone yielded 1.2 g. of a product melting at 194–195° dec.

Anal. Calcd. for $C_9H_{14}O_4N_2S$: C, 43.9; H, 5.7; N, 11.4; S, 13.0. Found: C, 43.8; H, 5.7; N, 11.5; S, 13.2.

One mmole (246 mg.) of the product VI was dissolved in 10 ml. of water, and 12.5 ml. of 0.08 *N* potassium hydroxide was added in small portions over a period of 15 hours. The *p*H of the solution at no time exceeded 9.2. After 36 hours, 9.2 ml. of 1.08 *N* hydrochloric acid was added, and the solution was concentrated to dryness *in vacuo* at 35°; 5 ml. of water was added, and the distillation was repeated. The distillation was twice repeated after addition of 20 ml. of absolute ethanol. The residual white crystalline solid was dried, and then extracted 4 times with 20-ml. portions of anhydrous acetone. The combined extracts were concentrated to 10 ml. *in vacuo* at 30°; 10 ml. of petroleum ether (b.p. 30–60°) was added slowly, yielding 104 mg. of a white crystalline product (m.p. 165–166°). Recrystallization of this product from acetone–petroleum ether yielded 85 mg. of pure product VIII (m.p. 169°).

Anal. Calcd. for $C_9H_{14}O_4N_2S$: C, 43.9; H, 5.7; N, 11.4; S, 13.0. Found: C, 43.7; H, 5.8; N, 11.1; S, 12.6.

The acetone mother liquors on addition of 10 ml. of petroleum ether yielded a further 98 mg. of crystals (m.p. 166–167°). The total yield of product (202 mg.) constituted 82% of the theoretical yield.

A solution of 2 mg. of VII in 1 ml. of 0.05 *M* acetate buffer at *p*H 4.8 gave no visible color after heating at 100° with 1 ml. of 0.1% ninhydrin solution. The same initial solution gave no visible color when treated with a solution of potassium cyanide and sodium nitroprusside. An aqueous solution of VII had a *p*H of about 2.

Other Properties of Compound VI.—A solution of 10 mg. of VI in 2 ml. of water was treated with a few drops of an aqueous solution of sodium nitroprusside. Addition of 5% ammonia solution produced no purple color, even on addition of a solution of potassium cyanide. After VI (0.2 *M*) had been incubated for 2 hours at room temperature in *N* sodium hydroxide, or after boiling this solution for 30 seconds, addition of sodium nitroprusside solution produced a strong purple coloration.

A solution of 10 mg. of VI in 2 ml. of water was added to 1 ml. of 0.5 *N* hydroxylamine hydrochloride solution, then 0.1 *N* sodium hydroxide was added to *p*H 9. After incubation at room temperature for 5 minutes, acidification with dilute hydrochloric acid and addition of a few drops of 5% ferric chloride solution produced a deep red color. When the incubation was carried out at *p*H 7, no red color resulted on repeating the procedure.

NOTE ADDED IN PROOF.—After this article had been submitted for publication, we noted the abstract[24] of a paper to have been read at the April, 1960, meeting of the Federation of American Societies for Experimental Biology. In this abstract, the authors state that "NEM can react both with glycine ethyl ester and with imidazole at *p*H 7.3."

(24) R. Benesch and R. E. Benesch, *Federation Proc.*, **19**, 78 (1960).

NEW HAVEN, CONN.

[CONTRIBUTION FROM CHEMISTRY DEPARTMENT, KANSAS STATE UNIVERSITY, MANHATTAN, KANSAS]

The Reaction of Amylases with Starch Granules[1,2]

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The reaction of α-amylase, β-amylase and a mixture of these with starch granules was studied. Measurements made on the residual starch and separated fractions after enzymic degradation included iodine potentiometric titration, β-amylase assay, periodate oxidation, alkali number, iodine absorption and electrophoretic studies. A method of isolating the products of enzymic digestion was developed. Evidence was presented that the enzymes in the initial stages degrade only the outer chains of amylopectin. This is best explained in terms of availability rather than enzyme mechanism. It is postulated that the surfaces of starch granules which are accessible to enzymes are the termination points of the outer chains of the amylopectin.

Introduction

Amylases react very slowly with starch granules in contrast with gelatinized starch. Nevertheless, in plants, starch granules are continually being synthesized and hydrolyzed. Reports on the nature of amylase reaction with starch granules have for the most part been confined to microscopic studies. Sandstedt[3] examined starches which had been partially digested by various enzymes. Concentric layers were clearly shown in some granules. More recently the electron microscope has been used[4,5] to reveal greater detail of partially digested starches. Observations by the

(1) Contribution No. 592, Department of Chemistry, Kansas Agricultural Experiment Station, Kansas State University, Manhattan.

(2) Part of this paper was taken from a Masters' thesis by Yee Sik Kim, Kansas State University.

(3) R. M. Sandstedt, *Cereal Chem., Suppl.*, **32**, 17 (1955).

(4) E. S. Turner, Ph.D. Thesis, Purdue University Library, Lafayette, Indiana, 1958.

(5) Z. Nikuni, *Kagaku (Tokyo)*, **27**, 283 (1953); [*C. A.*, **51**, No. 21, 17210i (1957)]; Z. Nikuni and S. Hizukuri, *Mem. Inst. Sci. and Ind. Research, Osaka Univ.*, **14**, 173 (1957); [*C. A.*, **52**, No. 1, 766d (1958)].

latter authors[3] indicate that the granule is composed of a large number of microgranules, or crystallites, and that these are held together rather loosely by material which is digested more readily by the enzyme.

There is evidence, too, that the granule is not of uniform chemical composition. In an examination of the starch from malted barley, Aspinall, *et al.*,[6] concluded that only the amylopectin component was affected. Furthermore, the evidence indicated that only the outer chains had been shortened. This was ascribed to the high concentration of β-amylase present in barley.

We have studied the reaction of some amylases with sorghum starch. Some preliminary experiments in this area are reported below. In contrast to the work of Aspinall, *et al.*,[6] our reactions were carried out with the isolated enzymes and starch. We have employed only one starch, namely that from Pink Kafir sorghum. The enzyme systems studied were salivary amylase, β-amylase and the mixture of α- and β-amylase from germinated Pink Kafir sorghum. The latter system is high in α-amylase but contains traces of β-amylase.[7]

Experimental

Preparation of Starch.—Starch was prepared from Pink Kafir sorghum using a modified pilot plant procedure devised by Johnston.[8] The procedure consisted of soaking the grain overnight in distilled water, then grinding it under water with a mortar and pestle. Purification steps consisted of screening and several washings in distilled water. The final air-dried preparation always contained less than 0.05% nitrogen.

Reaction of Amylases with Starch.—Salivary amylase was freshly collected and filtered. The β-amylase was a commercial preparation.[9] The sorghum amylase preparation was obtained by extracting germinated Pink Kafir sorghum.[10]

Reaction with the starch granules was carried out as follows: 10 g. of starch was suspended in 20 ml. of enzymic solution. To the starch–enzyme mixture was added 0.2 ml. of 1 *M* acetate buffer and the flask was covered with toluene to inhibit bacterial growth. The flask was placed in a 30° water-bath with occasional shaking. After a three day incubation period, the starch was centrifuged out at 2000 r.p.m. for 20 min. The starch was then extracted with water and finally with 80% methanol v./v.[11] to remove lipid materials. This was followed by drying for 24 hr. in a vacuum oven at 30°. Analyses were performed on the dry material. As small amounts of fatty acids influence the iodine potentiometric titration, it was necessary to remove this contaminant as completely as possible. Consequently, after each enzymic treatment the extraction and drying process was repeated. While this treatment does influence the chemical reactivity of the granules,[12] enzymic attack appeared under the microscope to be quite uniform.

Methods of Analyses and Characterization.—The iodine potentiometric titration was performed according to the method of Bates, French and Rundle[13] using a Leeds and Northrop potentiometer. A saturated calomel cell was used as reference electrode.

For the β-amylase assay the starch was brought into solution with a small amount of 0.1 *N* potassium hydroxide, then adjusted to *p*H 4.5 with acetic acid and the enzyme added. Samples were withdrawn and analyzed for maltose by the method of Somogyi.[14] For the starch fractions described later as A and B, the analyses were performed directly on the clear solutions. The required acetate buffer (*p*H 4.5) was added directly in this case. Maximum hydrolysis was usually reached in 30 minutes, but samples were taken over a 2 hr. period or until a constant value had been reached.

The hot water extraction method of Montgomery and Senti[15] was used with some modifications to fractionate the starch. This method involved a soaking period in 80% glycerol–water followed by centrifugation at 20,000 r.p.m. for 1 hr. in the Spinco ultracentrifuge. The precipitated starch which was mainly amylopectin was washed twice in the centrifuge with distilled water, dehydrated with *n*-butanol and dried at room temperature. This fraction is referred to throughout as amylopectin. The combined supernatant and washings were precipitated with methanol and air dried. This preparation is referred to in Table I as crude amylose. Normally, the amylose is obtained from this fraction by complexing with *n*-butanol or amyl alcohol. In this case, however, we wished to study the properties of the whole supernatant.

The fractionation method of Montgomery and Senti[15] was preferred because of its ability to separate the enzyme modified amylopectin (fraction B) from the unreacted amylopectin. As is shown in the Results section, the supernatant from the centrifugations appeared to contain the whole of this fraction. Other advantages also are the mild conditions used and very good reproducibility. In the electrophoretic studies the supernatant from the first centrifugation was merely dialyzed against the appropriate borate buffer[16] and resolved at 15 milliamps for 45 minutes in the Aminco electrophoresis apparatus.

Periodate oxidation was carried out with excess sodium periodate in the dark for 25 hr. at 0–2°.[17] The excess periodate was decomposed with ethylene glycol and the formic acid titrated to a bromothymol blue end-point. Average chain length is expressed as: sample wt. of starch × 1/162 × 1/eq. formic acid produced, for amylopectin and sample wt. of starch × 3/162 × 1/eq. formic acid produced, for amylose.

The carbohydrate content of starch fractions was estimated by refluxing a sample in 0.2 *N* hydrochloric acid for 4 hr., then carefully neutralizing with sodium hydroxide. The glucose content was then determined by the method of Somogyi.[14]

Alkali number measurements were made according to the procedure of Schoch and Jensen.[18] It is used here to denote gross changes in molecular weight.

The iodine absorption spectra of the fractions were measured as for the blue value,[15,19] *i.e.*, 1 mg. of starch, 2 mg. of iodine and 20 mg. of potassium iodide per 100 ml. The absorbance was measured in a spectronic 20 Bausch and Lomb colorimeter using the standard 1 cm. cuvette.

Preparation of A and B Fractions.—The supernatant and washings from the ultracentrifuge were combined and an excess of *n*-butanol was added with thorough agitation. After standing at room temperature for 2 to 3 hr., the solution was centrifuged at ordinary speeds (2000 r.p.m.). The precipitated amylose-*n*-butanol, or A fraction, was usually dissolved in boiling water and sparged with nitrogen until all the *n*-butanol had been eliminated. The supernatant, or B fraction, was similarly treated. Measurements were made directly on the clear solutions to avoid retrogradation effects. The terms A and B fractions are used by the authors to

(6) G. O. Aspinall, E. L. Hirst and W. McArthur, *J. Chem. Soc.*, 3075 (1955).

(7) E. Kneen, *Cereal Chem.*, **22**, 113 (1945).

(8) R. W. Johnston, M.Sc. Thesis, Kansas State University Library, Manhattan, 1942.

(9) Obtained from the Nutritional Biochemicals Corporation, Cleveland 28, Ohio.

(10) Article in preparation—sorghum germinated using the Ragdoll principle [E. Kneen, *Cereal Chem.*, **21**, 304 (1944)] but using aseptic conditions. Visible traces of mold growth were not evident.

(11) T. J. Schoch and C. B. Williams, THIS JOURNAL, **66**, 1232 (1944).

(12) R. L. Whistler, J. L. Goatley and W. W. Spencer, *Cereal Chem.*, **36**, 84 (1959).

(13) F. L. Bates, D. French and R. E. Rundle, THIS JOURNAL, **65**, 142 (1943).

(14) M. Somogyi, *J. Biol. Chem.*, **160**, 61 (1954).

(15) E. M. Montgomery and F. R. Senti, *J. Polymer Sci.*, *XXVIII*, 1 (1958).

(16) D. H. Northcote, *Biochem. J.*, **58**, 353 (1954).

(17) A. L. Potter and W. Z. Hassid, THIS JOURNAL, **70**, 3488 (1948).

(18) T. J. Schoch and C. C. Jensen, *Ind. Eng. Chem., Anal Ed.*, **12**, 531 (1940).

(19) E. J. Bourne, G. H. Donnison, W. N. Haworth and S. Peat, *J. Chem. Soc.*, 1687 (1948); R. M. McCready and W. Z. Hassid, THIS JOURNAL, **65**, 1154 (1943).

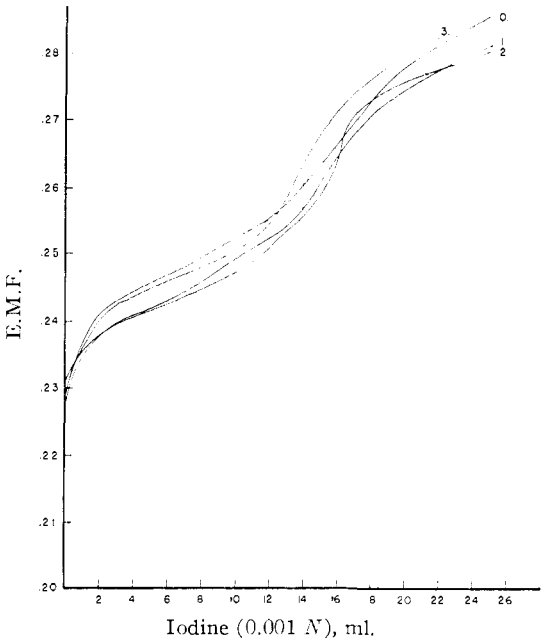

Fig. 1.—Changes in iodine absorption of sorghum starch after granule digestion by sorghum enzymes. Numbers indicate successive enzymic treatments. Iodine affinities obtained by plotting bound iodine *vs.* free iodine. 0, 4.6%; 1, 4.6%; 2, 4.7%; 3, 4.2%. (E.m.f. measured against saturated calomel half-cell.)

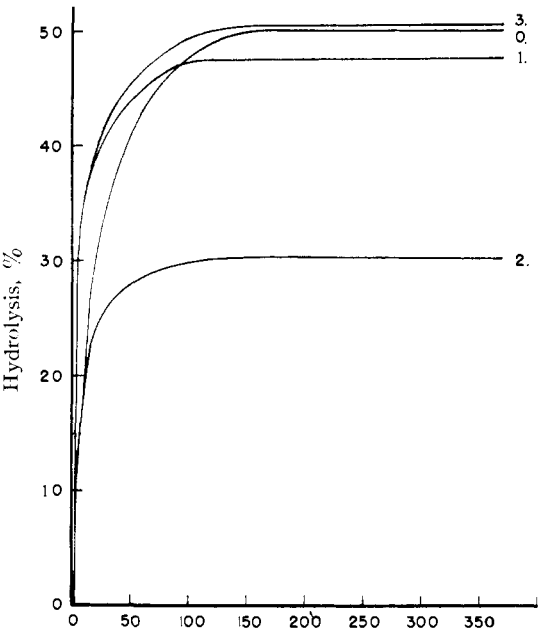

Fig. 2.—Degree of hydrolysis by β-amylase after granule digestion by sorghum enzymes. Numbers indicate successive enzymic treatments.

distinguish the components of the supernatant and are arbitary terms.

Results and Discussion

All granules, when viewed under the microscope, appeared to be attacked. The appearance was somewhat similar to that reported for other cereal starches by Sandstedt[3] and Badenhuizen.[20] In the early stages hydrolysis appeared to be confined mainly to the surfaces although an occasional granule was found showing the concentric layering reported by Sandstedt.[3]

In Fig. 1 are shown the changes in the amount of iodine absorbed by the starch with successive sorghum amylase treatments. The results of the β-amylase assay for these samples are shown in Fig. 2.

It should be noted that there is an apparent increase in amylose in the early stages of hydrolysis as measured by the potentiometric titration. In the later stages this is reversed. Furthermore, the shapes of the curves clearly indicate that the hydrolysis has not been accompanied by extensive dextrinization. Also, with respect to digestibility by β-amylase, the changes noted here can be correlated with the potentiometric titration curves. Similar type curves have been recorded for salivary amylase and for β-amylase. The shifts shown in Figs. 1 and 2 occurred regardless of the species of enzyme used although the following individual characteristics should be noted. The changes for β-amylase were even smaller than for sorghum amylase and with salivary amylase there was a much greater tendency to fragmentation of the granules. The degradation products found in solution when the sorghum enzyme was used, consisted entirely of low molecular weight products. Paper chromatography revealed spots corresponding in R_f to glucose and maltose with traces of isomaltose and one other compound estimated to have three glucose units. These compounds, however, were not identified further.

That the changes while small, are significant, and are caused by enzymic action could be shown by carrying sorghum starch through the extraction and drying process. Changes in the potentiometric titration values on this starch blank were not significant.

It seemed to us that the changes noted could be explained best in terms of granule structure rather than in terms of the known mechanisms of α- and β-amylase. According to the theory advanced by Ulman,[21] the outer layers of the granule are richer in amylopectin than is the center. Hence, chiefly amylopectin will be degraded in the early stages. The increase in amylose is thus only an apparent one. The β-amylase assay results (Fig. 2) are compatible with this view if one assumes that only the outer chains of amylopectin are attacked, as shown by Aspinall, *et al.*,[6] for β-amylase. If this is also true for α-amylase, then one must assume that this requirement is imposed by the manner in which amylopectin is laid down in the granule, *i.e.*, only the outer chains are exposed.

To test these ideas further, the crude amylose and amylopectin fractions were analyzed (see Table I). For this a sample was chosen showing the maximum change in the potentiometric iodine titration. It is at once apparent that the amylopectin fraction is very similar in the two starches.

(20) N. P. Badenhuizen, *Protoplasmatologia*, II, B, 2, b, δ, Springer-Verlag (Vienna) 1959.

(21) M. Ulman, *Kolloid Z.*, **150**, 128 (1957), [*C. A.*, **51**, No. 18, 14301a (1957)].

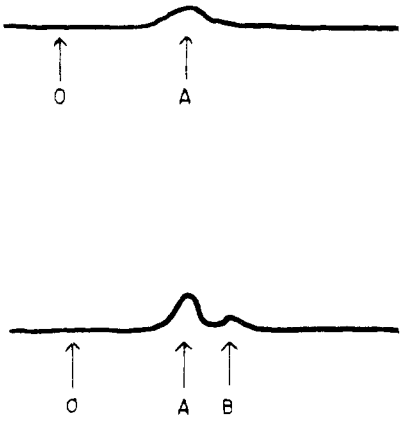

Fig. 3.—Electrophoretic mobility of the amylose fraction from: upper, normal sorghum starch; lower, enzyme digested sorghum starch. O, Origin; A, amylose fraction; B, modified amylopectin component.

The amylose fraction, however, is greatly different both with regard to composition and to yield. An increased yield of over 50% was observed in some of the fractionations. The nature of this difference was demonstrated by the electrophoretic studies. Here the supernatant or amylose fraction obtained from the centrifuge was dialyzed directly against the borate buffer[16] and resolved in the

TABLE I

COMPARISON OF THE CRUDE AMYLOSE AND AMYLOPECTIN FRACTIONS FROM PARTIALLY DIGESTED AND UNTREATED SORGHUM STARCH

Characteristic properties	Untreated starch		Partially digested starch	
	Crude amylose	Amylo-pectin	Crude amylose	Amylo-pectin
Amylose content (pot. iodine tit.)	75%	6.2%	56%	6.2%
Degree of hydrolysis by β-amylase	88%	40.2%	63%	43.2%
Chain length (periodate)	352	29.8	168	32.5
Alkali number	22.0	4.8	17.9	5.6

Aminco electrophoresis apparatus. The marked difference between the two fractions is shown in Fig. 3. When *n*-butanol was added to both fractions followed by centrifugation and re-examination in the electrophoresis apparatus, then in both cases the A fraction had entirely disappeared, showing that these components were amylose although they were not identical in shape. The amylose component of the enzyme digested sample was always found to have a more symmetrical electrophoretic pattern. The B fraction which could not be complexed with *n*-butanol is thus an amylopectin-like material which is found only in the enzyme digested sample.

Why fraction B is so readily separated by centrifugation from the remaining amylopectin could be a matter of some conjecture. Certainly it would not be expected on the basis of changes in molecular weight, as measured by alkali number (Table II).

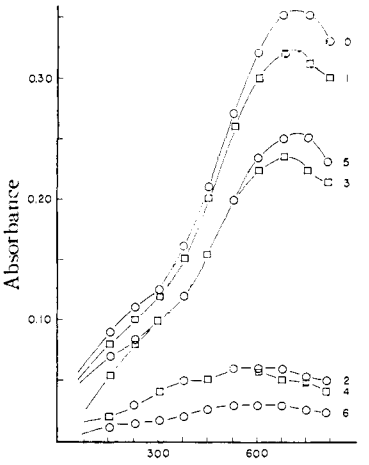

Fig. 4.—Absorption spectra of various starch fractions with iodine. All samples contain 1 mg. of carbohydrate, 2 mg. of iodine and 20 mg. of potassium iodide per 100 ml. 0, Commercial amylose;[9] 1, amylose from untreated sorghum starch; 2, amylopectin from untreated sorghum starch; 3, amylose from enzyme digested starch; 4, amylopectin from enzyme digested starch; 5, A fraction; 6, B fraction.

On the other hand, amylopectin prepared by this method may be highly aggregated. The loss of the outer chains of fraction B might be expected to reduce the tendency to aggregate.

TABLE II

COMPARISON OF A AND B FRACTIONS OBTAINED FROM PARTIALLY DIGESTED STARCH

Characteristic properties	A fraction	B fraction
Degree of hydrolysis by β-amylase	89.5%	26.4%
Chain length (periodate)	289	20.6
Alkali number	21.6	7.6

Other differences in the properties of the various starch fractions, reported in Table II, are shown in Fig. 4. Taken together these data support the statement that the B fraction is an amylopectin with short outer chains. The hypothesis advanced in the introduction was thus supported. The amylases appear to attack a portion of the amylopectin reacting mainly with the outer chains. This must mean that for some reason the outer branches are more readily available, otherwise α-amylase would be expected to hydrolyze linkages which are between branches as well as outer chains. A possible explanation is that this is inherent in the manner in which the starch granule was synthesized in the plant. For a starch granule to be synthesized by phosphorylase it is necessary that the outer chain be available and at the surface. Even if it is assumed that the enzymes penetrate into the granule in fissures, phosphorylase action would necessitate the outer chains being available in the surface of these fissures. These points are being further investigated