Tritiated Starch Granules

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1. Surface-labelling of starch granules by tritiation seems feasible, and a technique is described that could be useful in structure determination. The impurities that are produced must be taken into account but the fact that a high polymer can be successfully tritiated seems very promising. 2. The surfaces of corn-starch granules must contain both amylose and amylopectin.

A variety of labelled organic compounds can be prepared by exposing them to energetic tritium atoms. The method involves bombarding a thin film of the selected compound with tritium atoms produced by the atomization, at a hot filament, of tritium gas at a pressure of a few microns. Labelled sucrose, glucose and glucose 1-phosphate containing 5.63–8.13 μC of non-exchangeable tritium/mg. have been prepared by this procedure (Moser, Nordin & Senne, 1964).

A characteristic phenomenon associated with this method is that surface-labelling occurs. The kinetic energy of some tritium atoms is sufficient to enable exchange with carbon-bound hydrogen on surface molecules but is not great enough to exchange after penetrating several molecular layers. Many biological materials occur as particles and surface-labelling of such particles could be useful in further work designed to elucidate their structures.

The starch granule was selected as a biological particle for study for two reasons. First, starch has been extensively studied and its basic chemistry is well understood. Methods are available for fractionation into branched and linear components, and enzymic procedures for their degradation are well known. It is thus a good test material for surface-labelling as well as determining whether labelling of a polymer by this method is feasible. Secondly, it was hoped to gain information on the location of amylose and amylopectin in the starch granule. Amylases appear to degrade starch granules by first attacking the outer surfaces (Sandstedt, 1955). Analysis of the amylose and amylopectin at early stages of hydrolysis indicated that amylopectin had been preferentially attacked (Nordin & Kim, 1960; Aspinall, Hirst & McArthur, 1955). Also, simple gelatinization of starch produces sac-like structures that are mainly amylopectin (Ulman, 1957). Thus amylopectin is either in the outer surfaces of the granule or becomes pushed to the surface during gelatinization.

More recently it has been postulated that amylose and amylopectin are either intimately mixed in the granule (Badenhuizen, 1959), or that there is an amylose-to-amylopectin gradient from the inside to the outside of a shell (Badenhuizen, 1960). However, in recent reports it was shown that the shells commonly seen in granules are caused by variations in density (Buttrose, 1963*a,b*) and are best related to variations in starch precursors rather than to an amylose/amylopectin ratio. It was hoped that these present experiments would show whether the surfaces of starch granules contain amylopectin only or contain a mixture of amylose and amylopectin.

Whole starch granules have been irradiated by a variety of means in recent years. The various types of radiation used have been reviewed recently in a paper describing effects of high-energy electrons (Greenwood & MacKenzie, 1963). The radiations have generally been of high energy and degradative effects have been considerable in some cases. It should be emphasized that the energy of the tritium atoms used in the present study is very low compared with the energies of electrons etc. reported by Greenwood & MacKenzie (1963).

MATERIALS AND METHODS

Preparation of tritiated starch. The procedure was as described by Moser *et al.* (1964) for sugars. A 100 mg. sample of defatted corn starch was suspended in a few millilitres of acetone and coated on the inside of the reaction vessel by evaporating the acetone under vacuum. The dried starch granules remained attached to the surface of the reaction vessel firmly enough not to be dislodged when the vessel was handled.

Measurement of activity. Activity was measured with a Packard Tri-Carb liquid-scintillation spectrometer. The starch was solubilized in a few millilitres of 2.5*N*-NaOH. The solution was adjusted to pH 7 with dilute H_3PO_4 and made up to volume with water. Measurements were made with 1 ml. of starch solution and 15 ml. of counting solution of the following composition: 100 g. of naphthalene, 7 g. of

2,5-diphenyloxazole and 50 mg. of 1,4-bis-(5-phenyloxazol-2-yl)benzene/l. of dioxan. The counting efficiency was 10%. When the starch was solubilized in this manner it remained dissolved in the counting solution, and the activity measured was proportional to the amount of starch, at least within the range 0–76 μ g. It was estimated that the total counting error rarely exceeded 5%. In later experiments it was found that measurements could also be made on starch that had been solubilized by autoclaving at 120°.

Specific activity measurements are reported either on a dry-weight basis (vacuum-dried at 60° for 4 hr.) or, for starch hydrolysates, as μ C/mg. of glucose. In the latter, total carbohydrate was measured by a modification of the anthrone method (Stewart & Nordin, 1963).

Purification of starch. After tritiation, exchangeable tritium was removed as follows: the starch was suspended in 5 ml. of water and allowed to stand for several hours or overnight. The water was then evaporated under vacuum and collected in a trap at –78°. The starch was resuspended and the treatment repeated once or twice.

Non-exchangeable impurities in the starch were removed by washing in water and methanol or by solubilizing the starch in NaOH and precipitating it three times with methanol. The latter treatment removed more impurities, and three precipitations provided starch of nearly constant specific activity. The washing procedure was used for the granule-digestion experiments where it was necessary to keep the starch granule intact. Washing was performed as follows: starch was placed in a small tube containing a sintered-glass fitting, an arrangement was made for continuous flow and fractions of various size were collected. Combinations of methanol and water were used to wash the starch because it was found that, after elution for some time with one solvent, a fresh burst of activity could be eluted by switching to the other. The active material eluted was largely non-volatile impurities. After 3 weeks of almost continuous elution, the eluate had not yet reached background, but was negligible in total activity eluted.

Fractionation of starch. A 3% solution of methanol-precipitated starch was autoclaved for 1 hr. at 120°. It was saturated with butan-1-ol and allowed to cool slowly for 24 hr. The amylose–butanol complex was separated by centrifugation (10000 rev./min., 12000g for 20 min.) and recrystallized from water-saturated butan-1-ol. The amylopectin was recovered from the supernatant by precipitating with methanol.

Enzyme digestion experiments. Whole starch granules were digested with a mixture of α - and β -amylase (diastase; Nutritional Biochemicals Corp., Cleveland, Ohio, U.S.A.). The reaction was carried out in a vessel as described for washing starch. The granules were placed in the vessel and suspended in 3 ml. of the diastase solution (5 mg./ml. in 0.1M-sodium acetate buffer, pH 4.6) and placed on the shaker for 30 min. at room temperature. (The activity of the diastase, which was stored in a refrigerator, was such that 1 ml. of a solution hydrolysed 125 mg. of solubilized starch to the achroic point in 39 min. at pH 5 and 30° in 0.01M-acetate buffer.) The solution was then filtered through sintered glass under vacuum, rinsed with 3 ml. of buffer and replaced by a fresh charge of enzyme. The eluates from successive 30 min. washings were collected and analysed for carbohydrate content and for radioactivity. The results are shown in Table 1. Amylose and amylopectin solutions were hydrolysed with salivary amylase (filtered

Table 1. *Reaction of amylases with starch granules*

A 15.4 mg. sample of whole starch granules (30 μ C/mg.) was digested with malt diastase. The reaction was carried out in a small tube containing a sintered-glass fitting. The granules were suspended in 3 ml. of the diastase solution, capped and placed on a shaker at room temperature for 30 min. The sample was then filtered under vacuum and washed with water. Sample numbers represent successive 30 min. washings.

Sample no.	Total activity eluted (μ C)	Total carbohydrate eluted (mg. of glucose)	Specific activity (μ C/mg.)
1. Buffer only	1.17	0	—
2. Buffer only	0.89	0	—
3. Enzyme + buffer	70.0	0.78	91.0
4. Enzyme + buffer	13.6	0.90	15.4
5. Enzyme + buffer	6.4	1.3	4.9
6. Enzyme + buffer	2.70	0.77	3.5
7. Enzyme + buffer	2.18	0.68	3.2

saliva) and with crystalline β -amylase (Nutritional Biochemicals Corp.). Solutions were buffered at pH 7. The reaction was followed by the disappearance of the iodine stain.

The products of hydrolysis were examined by paper chromatography [Whatman no. 1 paper; solvent mixture, water–pyridine–butan-1-ol (3:4:6, by vol.)]. Spots were revealed with the silver nitrate dip (Trevelyan, Procter & Harrison, 1950). α -Amylase hydrolysates were fractionated on Sephadex G-75 (medium grade). When the desired state of conversion had been reached, digestion was interrupted by heating, and the hydrolysates were filtered through cotton and made up to volume. The specific activities were determined and samples of the above solutions applied to the column (1.5 cm. $\times$ 60 cm.).

After enzymic action a low percentage of new exchangeable tritium was found in the hydrolysates. Presumably it arose through the formation of reducing groups and consequent exchange of hydrogen bonded to C-1 and C-2 with the solvent. In some experiments this exchangeable tritium was removed before being applied to the column. In other cases it was removed from the fractionated samples before analysis.

Purification. The total activity incorporated into the starch varied somewhat from one experiment to another owing to variations in the conditions of exposure to tritium atoms, such as pressure of T₂ gas, length of exposure etc. The relative proportion of exchangeable and other impurities to fixed tritium was fairly reproducible. In the experiment involving a 3-week washing period, approx. 15% of the activity remained in the starch and its specific activity was 30 μ C/mg. It could be further decreased to 16 μ C/mg. by dissolving in NaOH and precipitating with methanol. Generally, 5–8% of the activity remained after purification by precipitation with methanol. The proportion of impurities removed as exchangeable tritium was 20–25% of the total incorporated.

Reaction of amylases with amylose and amylopectin. Tritiated starch was fractionated into amylose and amylopectin. In every case activity was found in both fractions.

Fig. 1. Fractionation of approx. 30 mg. of tritiated amylopectin on a Sephadex G-75 column (60 cm. $\times$ 1.5 cm.). The column was eluted with water and 5 ml. fractions were collected. Fractions were analysed for carbohydrate by the anthrone method and for radioactivity in a liquid-scintillation counter. O, Concn. of carbohydrate; Δ , radioactivity.

Fig. 2. Fractionation of products of α -amylolysis of 42 mg. of tritiated amylopectin ($0.19 \mu\text{Ci}/\text{mg.}$) on a Sephadex G-75 column (60 cm. $\times$ 1.5 cm.). The column was eluted with water and 5 ml. fractions were collected. Fractions were analysed for carbohydrate by the anthrone method and for radioactivity in a liquid-scintillation counter. O, Concn. of carbohydrate; Δ , radioactivity.

The specific activity of the fractions varied from exactly equal to almost twice as high a value for amylopectin as for amylose. The variability in the results may have been due to limitations of the separation procedures in removing all labelled impurities or to variations in surface impurities of the starch. The enzymes are highly specific, and the activity of the products of amylase action should be a reliable criterion of labelling in the starch. The fractionated amylose

Fig. 3. Fractionation of products of α -amylolysis of approx. 28 mg. of tritiated amylose on a Sephadex G-75 column (60 cm. $\times$ 1.5 cm.). The column was eluted with water and 5 ml. fractions were collected. Fractions were analysed for carbohydrate by the anthrone method and for radioactivity in a liquid-scintillation counter. O, Concn. of carbohydrate; Δ , radioactivity.

and amylopectin were accordingly treated with excess of β -amylase and fractionated by paper chromatography. The spot corresponding to maltose was eluted and its specific activity was determined. The following values were found: maltose from amylopectin, 19% of original amylopectin specific activity; maltose from amylose, 16% of original amylose specific activity; i.e., the specific activity of the maltose was less than one-fifth of the starting material, reflecting the presence of labelled impurities in both fractions.

Samples of labelled amylose and amylopectin were digested with salivary amylase to the iodine achroic point and the products fractionated on Sephadex (Figs. 1-3). The oligosaccharides obtained from the Sephadex column (peak 3 of Fig. 2) were pooled and maltose, maltotriose and maltotetraose were separated by paper chromatography. The specific activity of each, expressed as a percentage of that of the amylopectin substrate, was as follows: maltose, 21%; maltotriose, 31%; maltotetraose, 52%.

DISCUSSION OF RESULTS

Tritiation of starch at 77°K is probably a surface phenomenon. A thin surface layer of starch becomes labelled as some of the hydrogen atoms are replaced by tritium. Only on the surface layer would the kinetic energy of the tritium atoms be great enough to permit exchange. This requirement has been demonstrated by the results of tritium atom bombardment of films of saturated hydrocarbons at 77°K (Moser & Shores, 1962). Exchange of T for H occurred when the atoms were energetic. When the atoms were thermalized to the temperature of the film (77°K), exchange did not take place.

The enzymic degradation experiments with whole starch verify that the granule is surface-labelled. Thus the first treatment with enzyme yields soluble carbohydrate material, mainly glucose and maltose, of high specific activity. In subsequent

treatments the specific activity declined to lower and lower values. Amylase action on starch granules is apparently a surface phenomenon (Sandstedt, 1955). The first treatment therefore hydrolysed most of the labelled starch that had not been altered very much chemically during the tritiation process. However, after several treatments the residue represented impurities inaccessible to the enzyme, and the enzyme now selected to hydrolyse that portion of the granule which was not labelled.

If the tritium is bonded to oxygen, it is exchangeable. Experiments with D₂O (Mann & Marrinan, 1956) verify that hydrogen bonded to oxygen is exchangeable. Some non-exchangeable activity is incorporated. It evidently consists of various products ranging from starch not appreciably altered chemically to degradation products that can be extracted with water or methanol.

Fractionation of hydrolysis products on Sephadex. Sephadex G-75 can be used to fractionate starch dextrins according to molecular weight (Nordin, 1962). In Figs. 1-3 the products of amylolysis of tritiated amylose and amylopectin have been fractionated. Amylopectin contains a high-molecular-weight impurity that is not hydrolysed by the enzyme. Its very high specific activity shows that only a small fraction of the starch has become labelled. The starch was not greatly damaged because oligosaccharides of fairly high specific activity were produced.

Of secondary interest in the curves is the mobility of tritiated water, which was found to be the same as the high-molecular-weight material and to come through at approximately tube 10. The activities reported in Figs. 1-3, however, are on a non-exchangeable basis and contain no tritiated water.

The amylose, despite crystallization with butan-1-ol, a process that is known to be selective compared with precipitation with methanol, still contained a high-molecular-weight impurity. Attempts to remedy it by repeated crystallization were unsuccessful. In one experiment amylose was recrystallized six times from butan-1-ol-water and the amylopectin likewise precipitated six times

with methanol. The specific activities, however, were not diminished significantly beyond the second crystallization and the third precipitation. It was concluded that the impurity in the amylose was only slightly modified amylose and was partly susceptible to α -amylase because dextrins of high specific activity were produced. At present we have no information on the type of modification that occurred during tritiation. However, a free-radical process probably occurs and inversion of configuration, as well as rearrangements and degradative reactions, might be expected. Salivary amylase is very selective and might be expected to be unable to hydrolyse a portion of the starch molecule containing an abnormality. They would accumulate as dextrins.

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