[CONTRIBUTION FROM THE DEPARTMENT OF BIOCHEMISTRY AND NUTRITION, UNIVERSITY OF NEBRASKA]

The Preparation and Characterization of 1-C^{14}-Amylotriose[1]

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1-C^{14}-Amylotriose has been prepared from cyclohexaamylose and 1-C^{14}-glucose by the coupling action of *macerans* amylase and the hydrolytic action of β-amylase. The radioactive oligosaccharide was isolated, in pure form, from the enzymatic digest by a paper chromatographic procedure. The specific rotation, the molecular weight, the mobility on paper chromatograms and X-ray diffraction data on the crystalline flavazole derivative identified the oligosaccharide as amylotriose. Degradation of the pure compound and its crystalline flavazole derivative followed by activity measurements on the products showed that the compound is 1-C^{14}-amylotriose or *O*-α-D-glucopyranosyl-(1->4)-*O*-α-D-glucopyranosyl-(1->4)-D-1-C^{14}-glucose.

Pure oligosaccharides of the amylose series have proven to be valuable substrates for the elucidation of the mode of action of the amylase enzymes.[2-5] In recent studies from our laboratory it has been shown that α-amylase preparations from saliva[4] and malt[6] liberate glucose from amylotriose (maltotriose) and from amylotetraose (maltotetraose). Since the enzyme preparations were without action on maltose, the liberation of glucose cannot be attributed to maltase activity but rather to α-amylolysis of terminal α-1,4-glucosidic bonds of the oligosaccharides. It was not possible to determine whether the enzyme can differentiate between the terminal bonds at the reducing and the non-reducing ends of the oligosaccharide molecule. In order to determine which terminal bond is susceptible to hydrolysis, the action of the enzymes should be tested on oligosaccharides labeled in the terminal glucose residue. 1-C^{14}-Amylotriose has now been prepared; the methods of preparation and characterization of the compound are presented in this paper.

Cyclohexaamylose (Schardinger α-dextrin) and 1-C^{14}-glucose were subjected to the action of an enzyme solution from *Bacillus macerans*. The amylase of this bacterium effects a coupling reaction in which the glucose residues of the cyclic dextrin are linked to a cosubstrate (1-C^{14}-glucose) yielding a new oligosaccharide.[7] Subsequently a redistribution of the glucose residues of the oligosaccharide occurs resulting in the production of a series of α-1,4-glucosyloligosaccharides.[8,9] A separation of the first six members of the series (glucose, maltose, amylotriose, amylotetraose, amylopentaose and amylohexaose) in an aliquot of the digestion mixture followed by radioactivity measurements showed that all of the compounds were radioactive (Table I).

In order to increase the yield of radioactive amylotriose the mixture of oligosaccharides was subjected to the action of wheat β-amylase. This enzyme hydrolyzed the oligosaccharides with an odd number of glucose residues to maltose and amylotriose and those with an even number of glucose residues to maltose.[6] Paper chromatographic examination of the digestion mixture showed that glucose, maltose and amylotriose were present at this stage of the preparation. All of these compounds were radioactive (Table I). A separation of the glucose, maltose and amylotriose was effected by paper chromatography as previously described.[4] Radioautographs of the paper chromatograms were obtained and were used for locating the position of the oligosaccharides on the paper chromatograms. The individual compounds were cut from the paper, extracted with water and worked up with carrier amounts of pure non-radioactive compounds. The procedure included removal of the water by vacuum distillation and precipitation of the oligosaccharides with acetone as described in detail in the experimental section.

TABLE I

RADIOACTIVITIES OF 1-C^{14}-OLIGOSACCHARIDES

Compound	Apparent R_f value	After *m*-amylase treatment, c.p.m.	After β-amylase treatment, c.p.m.
Glucose	0.80	2100	2244
Maltose	.58	1553	3082
Amylotriose	.36	1073	2385
Amylotetraose	.24	1192	23
Amylopentaose	.13	1010	9
Amylohexaose	.08	1081	16

The amylotriose obtained by this procedure was an amorphous yellow solid. Sufficient material was not available to permit a purification to constant specific activity. The R_f value of the reducing compound in the preparation was identical with that for pure amylotriose in the several solvents used. Under all the chromatographic conditions, the radioactivity of the preparation was always found in the amylotriose spot on the paper strips. The molecular weight of the trisaccharide was calculated from its reducing power and was 490; the specific rotation of the oven-dried sample was +157°. A 1-phenylflavazole derivative[10] of the compound was prepared in crystalline form and was found to be radioactive. An X-ray diffraction pattern of the flavazole was identical with that of a flavazole prepared from pure amylotriose. Hydrolysis of the radioactive flavazole in mineral acid yielded radioactive glucose 1-phenylflavazole and non-active glucose. The radioactivity of the amylotriose is, therefore, located in the reducing glucose moiety of the trisaccharide.

A sample of the C^{14}-labeled amylotriose was hy-

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drolyzed completely to glucose. A Ruff degradation of the glucose was effected. Carbon 1 of the glucose was recovered as barium carbonate while carbons 2, 3, 4, 5 and 6 were recovered as arabinose. Radioactivity was found in the carbonate but not in the arabinose fraction.

The evidence presented shows that the radioactive oligosaccharide is 1-C^{14}-amylotriose or *O*-α-D-glucopyranosyl-(1- > 4)-*O*-α-D-glucopyranosyl-(1- > 4)-D-1-C^{14}-glucose.

Experimental

Enzymes.—*Macerans* amylase was used for the synthesis of radioactive oligosaccharides. A dried viable culture of *Bacillus macerans* was provided by Dr. Dexter French, Iowa State College, Ames, Iowa. Five grams of potato slices, 1.5 g. of calcium carbonate and 0.2 g. of ammonium sulfate in 50 ml. of water were sterilized, cooled to room temperature and inoculated with the culture. The sample was incubated at 37° for five days. At the end of this time the mixture was centrifuged in a Sorvall high speed centrifuge. The clear supernatant was assayed for enzyme activity by the Tilden–Hudson method.[11] This solution contained 1.5 units[11] of *macerans* amylase per ml. and was used as the enzyme source in our experiments.

Wheat β-amylase was prepared from wheat malt by the method of Kneen and Sandstedt.[12] The enzyme material was obtained in dry form and assayed 0.15 unit[12] of β-amylase per 1 mg. of preparation. The solution of β-amylase used in the preparative procedure contained 2 mg. or 0.3 unit of β-amylase per ml.

Preparation of 1-C^{14}-Labeled Amyloöligosaccharides.—Samples of 2 mg. of 1-C^{14}-glucose (5 microcuries) and 12 mg. of cyclohexaamylose[13] were dissolved in 0.1 ml. of water and mixed with 0.1 ml. of *macerans* amylase solution (1.5 units per ml.). The digest was mixed thoroughly and placed in an incubator at 37°. After an 18-hour reaction period, the enzyme was inactivated by heat. The oligosaccharides in a 0.005-ml. aliquot of the digest were separated on paper in *n*-butyl alcohol–pyridine–water solvent (6:4:3 by volume) by the multiple ascent technique.[14] Copper sulfate and molybdic acid reagents were used for locating the position of the compounds on the paper. The radioactivities of the oligosaccharides were measured directly on the paper strip with a thin-walled mica tube and Tracerlab scaler. The values are recorded in Table I.

Preparation of 1-C^{14}-Amylotriose.—The solution of C^{14}-oligosaccharides (0.2 ml.) was mixed with 0.2 ml. of β-amylase solution. Enzyme action was allowed to proceed for four hours at room temperature. At the end of the reaction period, the enzyme was inactivated by heat and 0.01 ml. of the digest was analyzed for reducing sugars by paper chromatography. Examination of the finished chromatogram showed that glucose, maltose and amylotriose were present in the mixture. The radioactivities of these compounds are recorded in Table I.

Two chromatograms each containing 0.2 ml. of the digest were prepared and developed in the solvent system. The finished chromatograms were placed in contact with Kodak no-screen X-ray film for 72 hours. The radioautographs obtained were used for locating the position of the compounds on the paper chromatograms.

The amylotriose from one chromatogram was extracted with 20 ml. of water. Fifty mg. of pure amylotriose was added as a carrier and the solution was concentrated to a sirup under vacuum. Ten ml. of acetone was added to precipitate the trisaccharide. The precipitated material was collected on a filter and dried in a vacuum oven at 60°.

Three chromatograms of the radioactive oligosaccharide and reference amylotriose were developed in three different solvents: (a) *n*-butyl alcohol–pyridine–water (6:4:3 by volume), (b) ethyl acetate–pyridine–water (2:1:2 by volume) and (c) *n*-butyl alcohol–ethyl alcohol–water (4:1:2 by volume). The apparent R_f values (three ascents of the solvent) for the radioactive and the non-active trisaccharide were: solvent a, 0.36 and 0.35; solvent b, 0.61 and 0.63; solvent c, 0.24 and 0.23. The radioactivities of the trisaccharide spots on the chromatograms were measured directly on the paper strips and were 1050, 980 and 1120 c.p.m. for the three chromatograms.

The specific rotation of the amylotriose preparation was +157° (*c* 0.2, water); literature value +158°[2] and +160°.[15] The reducing equivalent[16,17] of the trisaccharide was 10.3 while that of glucose and maltose was 32.0 and 15.5, respectively. The molecular weight of the trisaccharide calculated from its reducing equivalent and the reducing equivalent of glucose is 490; theoretical molecular weight 504.

Preparation of 1-C^{14}-Amylotriose 1-Phenylflavazole.—The radioactive amylotriose from the second chromatogram was extracted with 5 ml. of water. One hundred mg. of pure amylotriose was added for carrier purposes. Thirty mg. of *o*-phenylenediamine, 180 mg. of phenylhydrazine hydrochloride and 0.1 ml. of glacial acetic acid were added to this mixture.[18] The reaction mixture was heated at 98° for 5 hours, then cooled to room temperature. The precipitate in the mixture was collected on a filter and air-dried. It was crystallized from 3 ml. of hot *n*-propyl alcohol.

A sample of the crystalline flavazole was redissolved in *n*-propyl alcohol and chromatographed in a solvent of *n*-butyl alcohol saturated with water. The R_f value of the derivative was identical with that of the flavazole prepared from pure amylotriose.[4] The radioactivity of the flavazole was measured directly on the paper and was 422 c.p.m.

An X-ray diffraction pattern[19] of the radioactive flavazole was obtained. Comparison of this pattern with that obtained for the flavazole of pure amylotriose showed that the two patterns were identical.

Hydrolysis of 1-C^{14}-Amylotriose 1-Phenylflavazole.—Ten mg. of the trisaccharide flavazole was dissolved in 2.0 ml. of *n*-propyl alcohol. The solution was acidified with 0.1 ml. of 12 *N* hydrochloric acid and refluxed for 3 hours. Ten chromatograms each containing 0.2 ml. of the hydrolysate were prepared and developed in a solvent of *n*-butyl alcohol saturated with water. The flavazole on the chromatograms was extracted with 50 ml. of hot *n*-propyl alcohol. The solution of the flavazole was concentrated to 3 ml. and allowed to crystallize at room temperature. A sample of the crystalline compound was rechromatographed on paper. The R_f value of the material was identical with that of the flavazole prepared from pure glucose. The activity of the sample was measured on paper and was 253 c.p.m. X-Ray diffraction patterns for the radioactive flavazole and for non-active glucose 1-phenylflavazole were identical.

Since the R_f values of the glucose and of glucose 1-phenylflavazole in the *n*-butyl alcohol–water solvent are 0.09 and 0.84, respectively, complete separation of the two was achieved. Consequently, the glucose liberated on acid hydrolysis of the phenylflavazole was readily recovered from the chromatograms by extraction with water. The water extract was concentrated to 5 ml. under vacuum. A paper chromatogram of this solution showed that a reducing compound with the same R_f value as glucose was present. Radioactivity was not detected in this compound. The concentration of the reducing carbohydrate in this solution was determined by the diphenylamine method[4] and found to be 0.28 mg. per ml. The specific rotation of the substance was +54°; the specific rotation of glucose is +52°. The crystalline osazone of the reducing compound formed at the same rate as the osazone of glucose and appeared identical with glucosazone.

Degradation of 1-C^{14}-Amylotriose.—A sample of 5 mg. of amylotriose was dissolved in 0.2 ml. of 0.5 *N* hydrochloric acid. The mixture was heated at 100° for 2 hours in which time the amylotriose was hydrolyzed to glucose. Four

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tenths of a ml. of 2 *M* potassium hydroxide and 2 mg. of iodine were added to the hydrolysate. The glucose in the solution was completely oxidized to gluconic acid in a 6-hr. reaction period. Next, the reaction mixture was acidified with hydrochloric acid and a Ruff degradation of the gluconate was effected according to the procedure of Hockett and Hudson.[20] Five mg. of barium acetate, 3 mg. of ferric sulfate and 0.5 ml. of hydrogen peroxide were employed in the degradation. The carbon dioxide produced in the reaction was recovered as barium carbonate. Total counts per minute in the oven dried sample of barium carbonate were 686. The reaction mixture from the degradation was chromatographed with arabinose and glucose standards. The finished strip showed that a reducing compound with the same R_f value as arabinose had been produced. Radioactivity was not detected in this compound.

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Maltopentaose and Crystalline Octadeca-*O*-acetylmaltopentaitol[1]

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A pentasaccharide isolated from corn sirup by chromatography on charcoal and cellulose columns is characterized as maltopentaose by periodate and hypoiodite oxidations and by hydrolysis with β-amylase. Hydrogenation to maltopentaitol followed by acetylation yields a crystalline octadeca-*O*-acetyl derivative. $R_{glucose}$ values at 25° are given for members of the malto-homologous series up to seven D-glucose units using as eluting agent butanol–ethanol–water (3:2:1 v./v.).

Aside from their chemical significance the maltooligosaccharides are of particular interest to enzymologists. Members of this series are produced by hydrolytic enzymes acting on starches and glycogens and are used as primers and substrates for enzymatic syntheses. This importance of the series in general metabolic investigations provides impetus to the isolation and characterization of the individual members. The richest and most available source of maltoöligosaccharides is commercial corn sirup. From this source individual members can be isolated by chromatographic separations on successive carbon[2] and cellulose[3] columns. It is unfortunate that the maltoöligosaccharides are poor crystallizers. Presumably the presence of the repeating α-D-1 → 4 linkages connecting the D-glucopyranose units coupled with the activity of the potentially reducing group cause the molecules to assume shapes which do not readily fit into crystalline lattices. As a consequence the oligosaccharides are obtained in amorphous form. The acetylated glycitols crystallize from ethanol and hence constitute excellent reference points.

Several members of the maltoöligosaccharides have been isolated, from corn sirups[4,5] and from an acid hydrolyzate of potato amylose.[6] The structures of maltose,[7] maltotriose[8,9] and maltotetraose[5] have been determined and evidence for the structures of still higher homologous has been given.[6] Here is described the isolation and characterization of maltopentaose.

Maltopentaose is present to an extent[4] of about 8% in a standard commercial corn sirup of 42 dextrose equivalent. A concentrate of maltopentaose mixed with small amounts of its homologous neighbors can be obtained by chromatography from a carbon column. Final purification is effected by subsequent separation on a cellulose column. The specific optical rotation of the pure amorphous powder, $[\alpha]^{25}D+179.4°$ agrees with the calculated value[10,11] $[\alpha]D +178°$. A fivefold increase in reducing power upon acid hydrolysis demonstrates that five monosaccharide units are present in the oligosaccharide. On periodate oxidation a mole of pentasaccharide consumes 7.8 moles of periodate and produces 3.0 moles of formic acid and 1.4 moles of formaldehyde.

Enzymic hydrolysis illustrates the nature of the glycosidic linkages present. β-Amylase is known[12] to hydrolyze specifically α-D-(1 → 4)-glucosidic linkages if three or more such linkages occur successively. Hydrolysis of the pentasaccharide with crystalline sweet potato β-amylase[13] gives maltose and maltotriose in isolated yields of 34.3 and 52.9%, respectively. Similar results are obtained by Whelan, Bailey and Roberts.[6] The known structure of the isolated maltose and maltotriose aids in establishing that the pentasaccharide is composed of five D-glucopyranose units linked by four α-D-1 → 4 bonds in a linear chain.

Low pressure hydrogenation converts maltopentaose to maltopentaitol which can be isolated as a crystalline octadecaacetate. The optical rotation of the maltopentaitol (+158.5°) agrees with the value (+160°) calculated by means of the Freudenberg, Friedrich and Baumann equation.[10] Likewise the rotation of the maltopentaitol acetate agrees with the calculated value. Thus the optical rotation data also support the view that maltopentaose is a linear molecule of D-glucopyranose units connected by α-D-1 → 4 links.

The $R_{glucose}$ values of chromatographically pure

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