worked up as described under (A). The resulting product showed the same properties as listed under (A).

Chromatography on Whatman No. 1 paper with 1-butanol–pyridine–water, 6:4:3 resulted in a single spot, Rg value 1.09. The substance is ninhydrin positive.

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[CONTRIBUTION FROM THE DEPARTMENT OF BIOCHEMISTRY, PURDUE UNIVERSITY]

Reaction of Amylose with 1-Acrylamido-1-deoxy-D-glucitol to Introduce Extended Branches[1]

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Amylose reacts with 1-acrylamido-1-deoxy-D-glucitol in aqueous base. Ten molar lithium chloride solution as solvent permits a completely homogeneous reaction and suppresses amide hydrolysis. The product of the reaction is given the trivial name "glucamidoethylamylose." Fractionation of glucamidoethylamylose by ethanol precipitation yields fractions having degrees of molar substitution ranging from 0.20 to 0.82. Solubility in water increases with the amount of substitution. The solutions give a blue color with iodine, but do not show complex formation on titration with iodine. These derivatives of amylose are hydrolyzed by acid and by α-amylase, but are much less susceptible to the action of β-amylase than is amylose. They show no tendency toward retrogradation, nor do they complex with butanol.

A structure consisting of an amylose main chain with grafted poly(1-deoxy-1-β-oxypropionamido-D-glucitol) branches is consistent with these findings and is supported by chromatographic analysis of an acid hydrolyzate.

Introduction of a small amount of neutral substituent into a linear polysaccharide tends to increase its water solubility and its stability in solution. Solubility is further enhanced if the substituent group is hydrophilic. Hydroxyethylation and carbamylethylation are familiar examples of substitution by neutral, hydrophilic groups. The introduction of a sugar or an open-chain polyol as a substituent might provide a still greater enhancement of solubility and solution stability.

Sugar-substituted polysaccharides have recently been prepared by Husemann and Reinhardt,[2] who used the modified Koenigs-Knorr method of Bredereck and co-workers[3] wherein a carbohydrate trityl ether is treated with an acetobromo sugar in the presence of silver perchlorate. The synthesis in this laboratory[4] of 1-acrylamido-1-deoxy-D-glucitol (*N*-acryloyl-D-glucamine) has provided a sugar-substituted acrylamide which has now been shown to undergo reaction, through its activated double bond, with the hydroxyl groups in the linear polysaccharide, corn amylose. The product of the reaction is an *N*-substituted carbamylethylamylose to which the trivial name "glucamidoethylamylose" is given.

In preliminary investigations *N*-acryloyl-D-glucamine reacted with a dispersion of amylose in dilute sodium hydroxide solution. Base concentrations between 0.1 and 1.0*M*, temperatures between 50° and 100° and reaction periods between one half and twelve hours were investigated for mixtures containing one mole of *N*-acryloyl-D-glucamine per D-glucose residue of the amylose. Maximum incorporation of nitrogen to 0.85% *N*, or a molar substitution[5] of 0.12, occurs without the appearance of carboxyl groups in 0.3*M* base at 70° for two hours. An increase in either base strength, temperature, or time brings about partial hydrolysis of amide linkages. Since amylose is not readily soluble in 0.3*M* sodium hydroxide solution, the reaction mixture is not always completely homogeneous. Dissolution of the amylose in 10*M* lithium chloride solution provides a homogeneous reaction mixture, and at the same time reduces the amount of water available for hydrolysis of amide bonds. Consequently, base concentrations up to 0.6*M* in 10*M* lithium chloride solution may be used without the appearance of carboxyl groups in the product. Nitrogen contents up to 2.15%, corresponding to an M.S.[5] of 0.39, are obtained when four moles of *N*-acryloyl-D-glucamine are treated per D-glucose residue of the amylose in this manner.

Fractionation of glucamidoethylamylose on the basis of its solubility in water-ethanol mixtures gives rise to fractions having different nitrogen contents (Table I). As the concentration of ethanol is increased, fractions containing an increasing amount of nitrogen are obtained. The fractions are soluble in water, and their solubility increases with increasing amounts of substitution.

(1) Presented before the Division of Cellulose Chemistry at the 138th Meeting of the American Chemical Society, New York, September 1960; Journal Paper No. 1689 of the Purdue Agricultural Experiment Station.

(2) E. Husemann and M. Reinhardt, *Angew. Chem.*, **71**, 429 (1959); Abstracts of Papers, 138th Meeting, American Chemical Society, September 1960, 8D.

(3) H. Bredereck, A. Wagner, G. Faber, H. Ott, and J. Rauther, *Chem. Ber.*, 92, 1135 (1959).

(4) R. L. Whistler, H. P. Panzer, and H. J. Roberts, *J. Org. Chem.*, **26**, 1583 (1961).

(5) Molar substitution (M.S.) is defined as the number of moles of substituent introduced per D-glucose residue.

TABLE I

FRACTIONATION OF GLUCAMIDOETHYLAMYLOSE

Fraction No.[a]	Ethanol Concentration Range Where Precipitation Occurred, % V/V	Yield[b] G./G. Amylose	Nitrogen %	Nitrogen M.S.
1-1	35–40	0.69	1.35	0.20
1-2	40–50	0.21	1.85	0.31
1-3	50–60	0.30	2.73	0.58
2-0	—	—	2.15	0.39
2-1	35–40	0.68	1.75	0.29
2-2	40–50	0.88	2.19	0.40
2-3	50–60	0.10	3.23	0.82

[a]Fractions 1-1, 1-2, and 1-3 are from glucamidoethylamylose prepared with 0.4*M* base in 10*M* lithium chloride solution. The other fractions are from a preparation in which 0.6*M* base was used. Fraction 2-0 is the material from the latter preparation prior to fractionation. None of the fractions contain carboxyl groups. [b] Grams of product per one gram of amylose.

Glucamidoethylamylose fractions 2-1 and 2-2 (Table I) are compared with amylose in tests for retrogradation, butanol complexing ability, iodine binding capacity, and susceptibility to both acid and enzyme hydrolysis.

The tendency of amylose solutions to retrograde is well known. Glucamidoethylamylose fractions unlike amylose[6] are not precipitated by 1-butanol.

Solutions of glucamidoethylamylose give a blue color with iodine. However, when they are titrated with iodine potentiometrically, no inflection is observed in the plot of E.M.F. versus amount of iodine added. In this respect glucamidoethylamylose resembles amylopectin. The wave length of maximum absorption (λ_{max}) and the intensity of the glucamidoethylamylose-iodine color lie between the corresponding values for amylose and amylopectin. Thus, the absorbance of 0.005% solutions at λ_{max} for amylose-, glucamidoethylamylose-, and amylopectin-iodine colors are 0.905 at 630 mμ, 0.180 at 590 mμ, and 0.072 at 565 mμ, respectively. Husemann and Reinhardt[2] report that their sugar-substituted amyloses give no color with iodine above

TABLE II

RETROGRADATION OF AMYLOSE AND GLUCAMIDOETHYLAMYLOSE FRACTIONS

Polysaccharide	Concentration of Solution Before Test, %	Concentration of Solution After Test, %	Remarks
Amylose	0.50	0.015	Heavy white precipitate
Fraction 2-1	0.49	0.50	Trace of precipitate
Fraction 2-2	0.51	0.51	No precipitate

(6) T. J. Schoch, *J. Am. Chem. Soc.*, **64**, 2957 (1942).

a degree of substitution (D.S.) of 0.2, and yellow to red-brown colors when the D.S. is lower.

A marked difference exists between the abilities of amylose and glucamidoethylamylose to serve as substrates for the enzyme β-amylase. Glucamidoethylamylose fractions are hydrolyzed much less extensively than is amylose. On the other hand, α-amylase hydrolyzes amylose and glucamidoethylamylose fractions to the same extent (Table III).

TABLE III

ACTION OF α- AND β-AMYLASE ON AMYLOSE AND GLUCAMIDOETHYLAMYLOSE FRACTIONS

Milligrams of Reducing Sugars as Maltose

Polysaccharide	Per 100 Mg. of Substrate α-Amylase	Per 100 Mg. of Substrate β-Amylase	Per 162 Mg. of Amylose α-Amylase	Per 162 Mg. of Amylose β-Amylase
Amylose	31.8	77.1	51.5	125.
Fraction 2-1	25.4	2.6	49.3	5.9
Fraction 2-2	20.3	2.8	51.9	7.1

The rates of hydrolysis of amylose and glucamidoethylamylose in dilute sulfuric acid solution, as measured by the change in reducing power of the solutions, are similar. However, the extent of hydrolysis appears to be somewhat less in the case of glucamidoethylamylose. Thus, after two and a half hours in 1.4*N* sulfuric acid at reflux temperature the hydrolyses are nearly complete. The dextrose equivalents of the hydrolyzates indicate 86% conversion for amylose, but only 60% and 78% for the two glucamidoethylamylose fractions. A reaction between reducing groups of the sugars and free amino groups from the hydrolysis of amide linkages might explain the apparent difference.

Three courses for the reaction between amylose and *N*-acryloyl-D-glucamine may be envisaged. Homopolymerization of *N*-acryloyl-D-glucamine through a reaction between one of the hydroxyl groups of the D-glucamine moiety and the double bond, with no participation of the amylose, would result in a mixture of amylose and homopolymer. Homopolymerization with accompanying reaction of hydroxyl groups on the amylose molecule would result in the grafting of extended branches onto the amylose chain. Reaction between hydroxyl groups on the amylose molecule and *N*-acryloyl-D-glucamine without homopolymerization would result in single-unit substitution. That the amylose does react is demonstrated by performance of the reaction in the absence of amylose and, after neutralization, addition of amylose in a solution of 10*M* lithium chloride. Precipitation of this known mixture by methanol results in a product which contains 0.94% nitrogen, an apparent M.S. of 0.13. However, this mixture is not soluble in water, whereas glucamidoethylamylose preparations having a M.S. greater than 0.1 are water soluble.

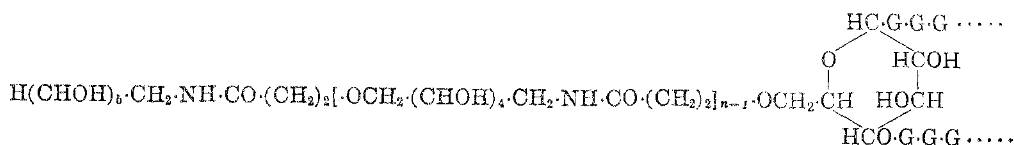

Fig. 1. Suggested repeating unit of glucamidoethylamylose. G = anhydro-D-glucose unit; n = average branch length

These results support a course of reaction involving concurrent homopolymerization and substitution.

Several additional facts indicate that substitution of amylose occurs. No amylose is detected in glucamidoethylamylose by potentiometric iodine titration. Very little reducing sugar is obtained when glucamidoethylamylose is subjected to the action of β-amylase, while α-amylase hydrolyzes glucamidoethylamylose and amylose to the same extent. No amylose is obtained from solutions of glucamidoethylamylose by retrogradation or upon butanol precipitation.

The variation in composition of glucamidoethylamylose fractions (Table I) is consistent with a course of reaction involving concurrent homopolymerization and substitution. In this homogeneous reaction the number of hydroxyl groups substituted should be the same for each amylose molecule, but the nitrogen content of the substituted molecules will depend upon whether the substituent is a polymer chain or a single N-acryloyl-D-glucamine molecule. The isolation of fractions of glucamidoethylamylose which range in M.S. from 0.20 to 0.82 (Table I) is thus explainable on the basis of a distribution of branch lengths. A glucamidoethylamylose molecule in agreement with these observations would consist of an amylose main chain with grafted poly(1-deoxy-1-β-oxypropionamido-D-glucitol) branches (Fig. 1).

One measure of the average branch length, n, is the ratio M.S./D.S. It might be expected that the D.S. of a glucamidoethylamylose fraction could be obtained by alkaline hydrolysis of all amide linkages and determination of the carboxyl groups in the resultant carboxyethylamylose salt. However, when conditions sufficiently vigorous to give a nitrogen-free product are used, the product is also devoid of carboxyl groups. It is suggested that, as is the case with the closely related Michael reaction,[7] the reaction of N-acryloyl-D-glucamine with a hydroxyl group is reversible, and that an excess of base favors the reverse reaction. As a result, treatment of glucamidoethylamylose with strong base merely regenerates the amylose.

The D.S. of a glucamidoethylamylose fraction may also be obtained by measurement of the ratio of carboxyethyl-D-glucose to D-glucose in an acid hydrolyzate. Quantitative paper chromatography indicates a ratio of 1:50 in glucamidoethylamylose fraction 2-1. The average branch length, n, of this fraction is thus 0.29/0.02 or 14–15 units.

(7) E. S. Gould, *Mechanism and Structure in Organic Chemistry*, Henry Holt and Co., New York, N. Y., 1959, p. 393.

EXPERIMENTAL

Amylose. Defatted corn starch was fractionated by the butanol method of Schoch.[6] The butanol-amylose complex was recrystallized from butanol-water three times. The amylose was obtained by treatment of the purified complex first with acetone and then with absolute ethanol. Ethanol was finally removed under reduced pressure in the presence of calcium chloride. The product was a fine, white powder.

1-Acrylamido-1-deoxy-D-glucitol. 1-Acrylamido-1-deoxy-D-glucitol (N-acryloyl-D-glucamine) was synthesized from acryloyl chloride and D-glucamine as described earlier.[4]

Glucamidoethylamylose. A solution of amylose in $10M$ lithium chloride was prepared by addition of 10 ml. of the lithium chloride solution per g. of polysaccharide, followed by stirring for 15–20 min. at 70°. The solution was blanketed with nitrogen. In this solution 4 mmoles of N-acryloyl-D-glucamine per 162 mg. of amylose was then dissolved, and sufficient $10M$ sodium hydroxide solution was added to give the desired base concentration (0.4 or $0.6M$).[8] Stirring was continued at 70° for 2 hr., with the nitrogen atmosphere maintained. The reaction mixture was diluted with water and neutralized to phenolphthalein with acetic acid. Hydrochloric acid was added to pH 2, and the solution was poured into 5–6 volumes of methanol. After 2 hr. the turbid supernatant liquid was poured off and discarded, and the white precipitate either redissolved in water for fractionation (see below) or washed free from lithium chloride with methanol, washed once with anhydrous ether, and dried under reduced pressure in the presence of phosphorus pentoxide.

Fractionation of glucamidoethylamylose. An aqueous solution of glucamidoethylamylose was prepared using 100 ml. of water for each g. of amylose present. Ethanol, 99.5%, was added slowly with stirring until a precipitate was visible. There was no precipitate up to an ethanol concentration of 35% by volume. At 40% ethanol the suspension was centrifuged to yield the first fraction. Second and third fractions were obtained similarly at ethanol concentrations of 50% and 60%, respectively. No additional precipitate formed up to an ethanol concentration of 80%.

Analyses. Nitrogen analyses were performed according to a micro-Kjeldahl technique.[9]

A semimicro potentiometric titration was devised for the determination of carboxyl groups. A 20–50-mg. sample was dissolved, by heating on a steam bath if necessary, in $10M$ lithium chloride solution previously adjusted to pH 5.5–6.0. This solution was diluted to 5 ml. with water and titrated with $0.01M$ sodium hydroxide in $5M$ lithium chloride

(8) In the preliminary experiments the amylose and N-acryloyl-D-glucamine were triturated with a solution of sodium hydroxide of the desired concentration (0.3 to $1.0M$) until a smooth dispersion was obtained. For each g. of amylose 12.5 ml. of base solution was used. The dispersions were then heated with stirring under nitrogen.

(9) *Official Methods of Analysis of the Association of Official Agricultural Chemists*, 7th Edition, Association of Official Agricultural Chemists, Washington, D. C., 1950, p. 745.

solution using a Radiometer automatic titration apparatus.[10] The titer was read from the inflection point on the titration curve and corrected by subtraction of the volume of base required to reach the same *p*H in a blank titration. The base was standardized by titration of 3–5-ml. aliquots of a standard solution of 0.01*M* potassium hydrogen phthalate mixed with 5 ml. of 10*M* lithium chloride solution. By this method it was possible to determine carboxyl groups in the concentration range 0.1–1% CO_2. The method is also applicable, by adjustment of sample size, to other acidic polysaccharides. Carboxymethylcellulose and two hemicelluloses have been titrated in this manner. Carboxyl contents of the hemicelluloses were identical with the values obtained by the decarboxylation method of Whistler, Martin, and Harris.[11]

Retrogradation. A solution of 250 mg. of glucamidoethylamylose in 50 ml. of 0.5*M* potassium chloride was refrigerated for 5 days at about 4°. A solution of 250 mg. of amylose in 25 ml. of 1*M* potassium hydroxide was neutralized with an equal volume of 1*M* hydrochloric acid and similarly refrigerated. The change in concentration was determined by measurement of the optical rotation of solutions after centrifugation. The specific rotations, $[\alpha]^{25}_{D}$, of glucamidoethylamylose fractions 2-1 and 2-2 were found to be +140° (*c*, 2.64 in water) and +121° (*c*, 2.41 in water), respectively. The specific rotation of amylose is about +200°.[12]

Iodine sorption. The potentiometric titration method of Bates, French, and Rundle[13] as modified by Wilson, Schoch, and Hudson[14] was used.

The absorption spectra of the polysaccharide-iodine colors were obtained by treatment of amylose, glucamidoethylamylose, and amylopectin according to the method of McCready and Hassid[15] and measurement, at a concentration of 0.005% polysaccharide, with a Cary recording spectrophotometer[16] in 1-cm. cells.

Enzymic hydrolysis. The amylase assay of Kneen and Sandstedt[17] was adapted as follows. Glucamidoethylamylose substrates were prepared by the dissolution of 500 mg. of the polysaccharide in 20-ml. portions of 0.5*M* potassium chloride solution, the addition of 1 ml. of acetate buffer,[17] and dilution to 25 ml. with distilled water. Amylose substrate was prepared by allowing 500 mg. to dissolve in 10 ml. of 1*M* potassium hydroxide solution under nitrogen at 4°, and adding 1 ml. of acetate buffer and 4 ml. of water. Immediately before use the amylose solution was neutralized with 10 ml. of 1*M* hydrochloric acid. Ten milliliters of each substrate was incubated for 15 min. at 30° with 10 ml. of either of the α- or β-amylase solutions described below. The reaction was stopped by the addition of 10 ml. of 1% sulfuric acid. Five-milliliter aliquots were analyzed for total reducing sugars by a macro version[18] of the alkaline ferricyanide method.[19]

The α-amylase used was prepared from commercial malt amylase. The preparation was heat-treated to destroy all β-amylase activity. By the amylase assay of Kneen and Sandstedt[17] 5 ml. of the enzyme solution converted 137 mg. of corn starch.

The β-amylase solution was prepared from crystalline sweet potato amylase.[20] Five milliliters converted[17] 208 mg. of corn starch.

Acid hydrolysis. One hundred milligrams of polysaccharide, glucamidoethylamylose, or amylose, was dissolved in 2 ml. of 72% sulfuric acid at ice bath temperature. The clear solution was diluted with 30 ml. of water. After removal of 1 ml. for analysis, the remainder was refluxed. Aliquots of 1 ml. were removed periodically for analysis. All aliquots were neutralized with the calculated amount of 1*M* sodium hydroxide solution and diluted to 25 ml. Two-milliliter portions of these dilutions were analyzed for total reducing sugars by the alkaline ferricyanide method.[19,21]

Chromatography. The acid hydrolyzate of glucamidoethylamylose fraction 2-1 was examined qualitatively and quantitatively by chromatography on Whatman No. 1 and No. 3MM papers, respectively. The solvent systems used were neutral, 1-butanol : ethanol:water (40:11:19 v/v); acidic, ethyl acetate:acetic acid:formic acid : water (18:3:1:4 v/v); and basic, ethyl acetate:pyridine:water (8:2:1 v/v). To locate the sugars on the chromatograms the silver nitrate reagent of Trevelyn, Procter, and Harrison[22] was used, but modified by the substitution of a dilute solution of sodium thiosulfate for the ammonium hydroxide. A 2.5% solution of aniline hydrogen phthalate in water-saturated 1-butanol[23] and a 1% solution of ninhydrin in 1-butanol were used to detect reducing sugars and amino sugars, respectively.

In the glucamidoethylamylose hydrolyzate three sugars were found which were not present in an amylose hydrolyzate. One of these sugars traveled more rapidly than D-glucose in the acid solvent but failed to move in the basic solvent. It gave a positive test with aniline hydrogen phthalate and a negative test with ninhydrin. On the basis of the proposed structure (Fig. 1) it is suggested that this sugar is carboxyethyl-D-glucose. Upon elution it was found[21] to be present in $^1/_{50}$th of the concentration of the D-glucose present.

The other two sugars gave negative tests with aniline hydrogen phthalate and positive tests with ninhydrin. One was present in much lower concentration than the other, and this minor component corresponded in $R_{glucose}$ value to D-glucamine in all three solvent systems. The major amino sugar was tentatively assigned the structure of carboxyethyl-D-glucamine.

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LAFAYETTE, IND.

(10) Product of Radiometer, Copenhagen, Denmark.

(11) R. L. Whistler, A. R. Martin, and M. Harris, *J. Research Natl. Bur. Standards*, **24**, 13 (1940).

(12) J. A. Radley, *Starch and Its Derivatives*, John Wiley & Sons, Inc., New York, N. Y., 3rd Ed., 1954, Vol. II, p. 364.

(13) F. L. Bates, D. French, and R. E. Rundle, *J. Am. Chem. Soc.*, **65**, 142 (1943).

(14) E. J. Wilson, T. J. Schoch, and C. S. Hudson, *J. Am. Chem. Soc.*, **65**, 1380 (1943).

(15) R. M. McCready and W. Z. Hassid, *J. Am. Chem. Soc.*, **65**, 1154 (1943).

(16) Product of Applied Physics Corp., Pasadena, Calif.

(17) E. Kneen and R. M. Sandstedt, *Cereal Chem.*, **18**, 237 (1941).

(18) R. M. Sandstedt, *Cereal Chem.*, **14**, 603 (1937).

(19) H. C. Hagedorn and B. N. Jensen, *Biochem. Z.*, **135**, 45 (1923).

(20) Kindly supplied by Professor A. K. Balls.

(21) F. J. Bates, *Polarimetry, Saccharimetry and the Sugars*, United States Government Printing Office, Washington, D. C., 1942, p. 198.

(22) W. E. Trevelyn, D. P. Procter, and J. S. Harrison, *Nature*, **166**, 444 (1950).

(23) L. Hough, *Methods of Biochem. Anal.*, **1**, 205 (1954).