Anal. Calcd. for $C_{31}H_{38}O_6N_3Cl \cdot HCl$: N, 6.77; Cl, 11.4. Found: N, 6.6; Cl, 11.3.

***p*-Hydroxyacetanilide hydroxyethylapocupreinium chloride** separated as a gum during the reaction and was obtained as an amorphous solid in a 74% yield after the precipitate from the alcoholic solution treated with four volumes of anhydrous ether was dried.

***p*-Hydroxyacetanilide hydroxyethylapocupreinium chloride hydrochloride** was obtained as an amorphous powder by pouring an alcoholic solution of the dry hydrochloride into anhydrous ether, and crystallized from either absolute ethyl or methyl alcohol; $[\alpha]_D$ −87.6° (l = 1, c = 0.525 in distilled water).

Anal. Calcd. for $C_{29}H_{34}O_5N_3Cl \cdot HCl$: N, 7.29; Cl, 12.3. Found: N, 7.21; Cl, 12.1.

Acetanilide hydroxyethylapocupreinium chloride did not separate in the course of the reaction. It was obtained as an amorphous solid in a 70% yield after the alcohol–ether treatment of the acetone residue.

Acetanilide hydroxyethylapocupreinium chloride hydrochloride separated as an amorphous powder from two volumes of absolute ethyl alcohol in the course of continued heating; $[\alpha]_D$ −89.2° (l = 1, c = 1.051 in distilled water).

Anal. Calcd. for $C_{29}H_{34}O_4N_3Cl \cdot HCl$: N, 7.50; Cl, 12.66. Found: N, 7.50; Cl, 12.3.

***p*-Hydroxyacetanilide cinchonidinium chloride hydrochloride.**—U. S. P. cinchonidine $[\alpha]^{22}_D$ −49.9° (l = 1, c = 1 in pyridine) was suspended in 50 volumes of dry acetone, containing the calculated equivalent of *p*-chloroacetylaminophenol, and refluxed at 61° for fifty-two hours. A quaternary salt gradually crystallized on the walls of the flask; after twenty-four hours of refluxing the yield was 39% of theoretical, and after fifty-two hours of refluxing the yield was 57%. The quaternary salt was recrystallized from 35 volumes of absolute ethyl alcohol; $[\alpha]^{21}_D$ −30.3° ± 0.5 (l = 1, c = 1.012 in pyridine).

The hydrochloride of the quaternary salt was obtained as an amorphous solid, having been prepared by titrating an alcoholic solution to an end-point acid to methyl orange; $[\alpha]^{21}_D$ −47.0° (l = 1, c = 1.085 in distilled water).

Anal. Calcd. for $C_{27}H_{30}O_3N_3Cl \cdot HCl$: N, 8.14. Found: N, 7.93, 7.96.

Conclusion

Quaternary salts of hydroxyethylapocupreine have been prepared and tested for biological action in comparison with dihydroquinine-*p*-chloroacetylaminophenol hydrochloride.[1] The antipneumococcic action of dihydroquinine was enhanced but that of hydroxyethylapocupreine was greatly decreased, in the quaternary derivatives tested.

PITTSBURGH, PENNA. RECEIVED JUNE 7, 1941

[CONTRIBUTION FROM THE DIVISION OF PLANT NUTRITION, COLLEGE OF AGRICULTURE, UNIVERSITY OF CALIFORNIA]

The Molecular Constitution of Enzymatically Synthesized Starch

BY W. Z. HASSID AND R. M. MCCREADY

Cori and Cori[1] demonstrated that animal tissues contain an enzyme, phosphorylase, which acts upon glycogen to produce glucose-1-phosphate (Cori ester). From a consideration of the structural configurations for this hexosephosphate, Cori, Colowick and Cori[2] suggested that it was α-glucopyranose-1-phosphoric acid. Later, Wolfrom and Pletcher[3] proved that the structure of the ester was the same as originally proposed by Cori and co-workers. Kiessling[4] and others[5] showed that when phosphorylases, isolated from yeast, muscle or liver, act on the Cori ester, it is transformed reversibly into a polysaccharide. Hanes[6] subsequently demonstrated that many plants contain phosphorylase which catalyzes the reversible reaction: starch + free phosphate ⇄ glucose-1-phosphate. According to these investigators, similar reactions occur in the breakdown and formation of glycogen or starch *in vivo*. Hanes[6b] showed that enzymatically synthesized starch is indistinguishable in certain of its characteristics from natural starch. Astbury, Bell and Hanes[7] also showed that the X-ray pattern of synthetic starch is essentially the same as that of natural starch. However, certain differences exist between the natural and synthetic starch. The latter is less soluble in water, and rapidly retrogrades in solution; it gives a more intense color with iodine and is quantitatively hydrolyzed with β-amylase to maltose. With natural starches the enzymic hydrolysis ceases when approximately 60% has been converted into maltose. This behavior toward β-amylase is similar to the amyloamylose fraction of natural

(1) C. F. Cori and G. T. Cori, *Proc. Soc. Exptl. Biol. Med.*, **34**, 702 (1936).

(2) C. F. Cori, S. P. Colowick and G. T. Cori, *J. Biol. Chem.*, **121**, 465 (1937).

(3) M. L. Wolfrom and D. E. Pletcher, THIS JOURNAL, **63**, 1050 (1941).

(4) W. Kiessling, *Biochem. Z.*, **302**, 50 (1939).

(5) C. F. Cori, *Endocrinology*, **26**, 285 (1940); *J. Biol. Chem.*, **135**, 733 (1940).

(6) C. S. Hanes, (a) *Proc. Roy. Soc.* (London), **B128**, 421 (1940); (b) **B129**, 174 (1940).

(7) W. T. Astbury, F. O. Bell and C. S. Hanes, *Nature*, **146**, 558 (1940).

starch, prepared from the latter by autoclaving and electrodialysis.[8]

Haworth, Hirst and collaborators[9] have shown that the molecule of natural starch is made up of a large number of unbranched chains, each consisting of 24 to 30 anhydroglucose units joined by α-glucosidic linkages through the first and fourth carbon atoms. In view of the differences in properties between the natural and synthetic starches, especially in their behavior toward β-amylase, it was of interest to study the structure of the enzymatically synthesized starch and to determine whether these differences bear any relation to the molecular constitution.

In the present investigation the acetylated and methylated derivatives of synthetic starch were prepared and studied. They were found to be similar in properties to those of natural starches. The synthetic starch acetate was soluble in acetone and chloroform and had a specific rotation in chloroform $[\alpha]_D$ +170°. The molecular weight of the acetylated derivative, determined by viscosity measurements, corresponded to 85,000. The starch, regenerated from the acetate by treatment with alcoholic potassium hydroxide, was similar in its properties to the original starch. The methylated starch was soluble in acetone and chloroform and had a specific rotation in chloroform $[\alpha]_D$ +216°. Its molecular weight, estimated by the viscosity method, corresponded to 54,000. The starch was methylated by the process of simultaneous deacetylation and methylation. Hirst and Young[9b] showed that regardless of the method of preparation of the methyl derivative—whether the starch is methylated directly in air or nitrogen, or whether the methyl derivative is prepared from the starch acetate—the percentage of end-group (tetramethylglucose) obtained on hydrolysis remains unchanged. The method of simultaneous deacetylation and methylation in a medium of acetone, the starch acetate being readily soluble in this solvent, has the advantage over direct methylation that the reaction proceeds more rapidly, and also a better yield of methylated starch is obtained.

On hydrolysis of the methylated synthetic starch 2,3,6-trimethylglucose was obtained as the sole product. No tetramethylglucose (end-group) and dimethylglucose could be detected. The apparent absence of an end-group in the synthetic starch molecule indicates that it does not consist of relatively short repeating chains, and therefore differs in molecular constitution from natural starch. It may be assumed that either the chains in synthetic starch form continuous loops, or the glucose units in the chains are too numerous to allow the isolation of tetramethylglucose under the conditions employed.

(8) C. S. Hanes, *New Phytologist*, **36**, 199 (1937).

(9) E. L. Hirst and G. T. Young, (a) *J. Chem. Soc.*, 951 (1939); (b) 1471 (1939).

Although, in general, it may be considered that the mechanism of starch formation in the living plant involves an enzymic system similar to that functioning in plant extracts, the fact that synthetic starch possesses a molecular constitution different from that of natural starch indicates that the two systems are not identical. Also, since the synthetic starch granules do not possess the characteristic shape of natural starches, the formation of these granules may be considered a specific property of the plastid, which is the site of starch formation. It is likely that the starch in living plant tissues is transformed by phosphorolysis into the Cori ester by enzymes similar to those acting *in vitro*, but the formation and deposition of starch must involve a more highly organized enzyme system of which these phosphorylases are only a part.

Experimental

Properties of Synthetic Starch.—Synthetic starch was prepared from the Cori ester and partially purified potato phosphorylase according to the method of Hanes.[6b] The starch gave a blue-black color with iodine, did not reduce Fehling solution but, when oxidized with ferricyanide, gave a reducing value of 1% calculated as glucose.[10] When treated with barley β-malt amylase or salivary amylase, maltose was produced as the result of hydrolysis. It was almost insoluble in water (less than 0.1%) and soluble in dilute sodium hydroxide. Its ash content was 3.5%; N, 0.24%; and P, 0.09%. Its specific rotation (c, 0.25) in 1 N sodium hydroxide was $[\alpha]_D$ +170° (analyses calculated on ash free basis).

Acetylation.—Two grams of synthetic starch was boiled with 100 cc. of water for about ten minutes and reprecipitated by the addition of 95% ethanol. The precipitate was washed with ethanol and ether, and the slightly moist starch suspended in 25 cc. of pyridine and stirred for twenty-four hours. The starch was then acetylated by gradual addition of 20 cc. of acetic anhydride and the mixture stirred at 60° for twelve hours. The starch acetate was precipitated by pouring the solution into an excess of cold water. The acetylated starch did not produce a blue coloration with iodine and was soluble in chloroform and acetone. Its specific rotation (c, 1) in chloroform was $[\alpha]_D$ +170°. The acetyl content, $COCH_3$, 44.6%

(10) W. Z. Hassid, *Ind. Eng. Chem., Anal. Ed.*, **9**, 228 (1938).

(calculated $COCH_3$ content for the triacetate, $(C_6H_7O_5(CH_3CO)_3)_n$, 44.8%).

The specific viscosity $\eta_{sp.}$ at 22° of a 0.4% solution of acetylated starch in *m*-cresol was 0.17. This corresponds to a molecular weight of 84,000, determined by using Staudinger's formula with $K_m = 10^{-4}$.[9a]

Regeneration of Synthetic Starch from its Triacetate.—One gram of the acetylated starch was treated with 25 cc. of 0.5 *N* alcoholic potassium hydroxide and allowed to remain at room temperature for twelve hours. The alkali was neutralized with dilute acetic acid and the regenerated starch filtered off, washed with ethanol, then with ether and dried in vacuum at 80°. The properties of the regenerated product were similar to those of the original starch. It stained blue with iodine and was practically insoluble in water. Its specific rotation (*c*, 0.25) in 1 *N* sodium hydroxide was $[\alpha]_D$ +168°.

Methylation.—The starch acetate used for methylation was prepared from crude starch containing proteins and salts. Since the proteins were not acetylated when treated with acetic anhydride, the insoluble starch acetate was thus freed from nitrogenous matter. The inorganic salts were removed by washing with water. The specific rotation of the acetylated product (*c*, 1) in chloroform was $[\alpha]_D$ +170°. It had a N content of 0.1% and was free of phosphorus.

Sixteen grams of the starch triacetate was simultaneously deacetylated and methylated at 55° with methyl sulfate and 30% sodium hydroxide as previously described.[11] After eight methylations the methyl starch was dissolved in chloroform, the solution filtered, evaporated to a small volume and reprecipitated by the addition of petroleum-ether. The specific rotation of the methylated product (*c*, 1) in chloroform was $[\alpha]_D$ +216°. Its methoxyl content, OCH_3 was 44.7% (calculated for $(C_6H_7O_2(OCH_3)_3)_n$, 45.6%).

The specific viscosity, $\eta_{sp.}$ at 22° of a 0.4% solution of the methylated derivative in *m*-cresol was 0.17. Applying Staudinger's formula with $K_m = 1.6 \times 10^{-4}$,[9a] a molecular weight of 54,000 was obtained for the methylated starch.

Hydrolysis.—Five grams of the methylated starch was hydrolyzed with 215 cc. of 1.5% methyl-alcoholic hydrogen chloride for eight hours under a reflux condenser. The methylglucosides (yield 94%) were fractionally distilled at 10^{-4} mm. pressure into fractions shown in Table I.

TABLE I

HYDROLYSIS PRODUCTS OF METHYLATED SYNTHETIC STARCH

Fraction	Temp., °C.	Weight, g.	$[\alpha]_D$ in water	η^{16}_D	OCH_3
I	90	0.741	60.8	1.4584	52.3
II	90–100	1.862	68.3	1.4588	52.0
III	100–120	1.110	71.8	1.4588	52.2
IV	120–150	0.673	81.5	1.4588	52.3
V	150–180	0.474	81.0	1.4588	52.2
		4.860			

The indices of refraction and the methoxyl contents show that all five fractions represent one constituent, trimethylmethylglucoside (calculated OCH_3 content for $C_6H_8O_2(OCH_3)_4$, 52.6%). Using the criteria of purity established by Hirst and Young[12] for mixtures of tetramethyl- and trimethylmethylglucosides, it can be concluded that no tetramethylmethylglucoside was present in the hydrolysis products of the methylated synthetic starch. Dimethylmethylglucoside was also not detected. Employing the same fractionating column, mixtures of about 5 g. containing different proportions of pure tetramethylmethylglucoside and trimethylmethylglucoside could be separated quantitatively by the Hirst and Young method.

Identification of 2,3,6-Trimethylglucose.—About 0.5 g. of the sirup (combined fractions I and II) was hydrolyzed with 6 *N* sulfuric acid on a steam-bath for six hours, and the product isolated in the usual manner.[11] The methoxyl content of the crystalline product was 41.5% (calculated for $C_6H_9O_3(OCH_3)_3$, 41.9%). The specific rotation (*c*, 1) in water was $[\alpha]_D$ +70° (equilibrium value). When treated with methanol containing 1% hydrogen chloride the trimethylglucose gradually underwent inversion of sign, a behavior regarded characteristic for 2,3,6-trimethylglucose.

The authors are indebted to Mr. W. H. Dore for his interest and suggestions during the course of this investigation.

Summary

The molecular structure of enzymatically synthesized starch was studied. On hydrolysis of the methylated starch, followed by a quantitative separation of the cleavage products, 2,3,6-trimethylglucose was obtained as the sole product of hydrolysis. Unlike the natural starches, no end-group (tetramethylglucose) could be detected among the hydrolysis products. This suggests that the synthetic starch molecule is either made up of long chains in which the number of anhydroglucose units is too great to allow the isolation of tetramethylglucose, or the chains exist in the form of continuous loops.

BERKELEY, CALIFORNIA RECEIVED MAY 13, 1941

(11) W. Z. Hassid and R. M. McCready, THIS JOURNAL, **63**, 1632 (1941).

(12) E. L. Hirst and G. T. Young, *J. Chem. Soc.*, 1247 (1938).