ketoglutaric acid which was isolated from the homogenate was found to carry the isotope entirely in the γ-carboxyl group. These results prove that the asymmetric distribution of isotope in the carboxyl groups of α-ketoglutaric acid which is found in studies of Krebs' citric acid cycle arises from an asymmetric configuration (of the isotope) in the citric acid and an antipodal specificity of the enzymes which catalyze the formation and the dehydration of citric acid.

The enzymatic reactions are discussed in terms of the stereochemical properties of symmetric molecules which are asymmetrically labeled.

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[CONTRIBUTION FROM THE DIVISION OF PLANT NUTRITION, COLLEGE OF AGRICULTURE, UNIVERSITY OF CALIFORNIA, AND THE DEPARTMENT OF PLANT PATHOLOGY, COLLEGE OF AGRICULTURE, UNIVERSITY OF WISCONSIN]

The Structure of Crown-Gall Polysaccharide. I

BY E. W. PUTMAN, A. L. POTTER, ROLAND HODGSON[1] AND W. Z. HASSID

The most abundant polysaccharides in nature, cellulose, starch and glycogen possess the 1,4-glucosidic linkage as their chief linkage; however, polysaccharides with glucose residues united through 1,3- and 1,6-linkages also occur.[2] Granicshstädten and Percival[3] reported a hemicellulose isolated from Iceland moss in which some of the glucose residues are joined through 1,2-glucosidic linkages.

McIntire, Peterson and Riker[4] showed that the crown-gall organism, *Phytomonas tumefaciens*, when grown on sucrose as a source of carbon, produces a low molecular weight water-soluble polysaccharide consisting entirely of D-glucose and having a specific rotation in water, $[\alpha]_D$ -9 to $-10°$. An upward shift in rotation during hydrolysis indicates a predominance of β-linkages, while the rate of hydrolysis suggests that the glucose residues have a pyranose structure. Hodgson, *et al.*,[5] demonstrated that when the crown-gall organism is grown on D-glucose or D-fructose, a polysaccharide is also produced, which is probably identical with the one formed when the bacterium is grown on sucrose.

Reeves[6] found that the shift in optical rotation of this polysaccharide in water and cuprammonium solution closely resembles that of methyl 2-methyl-β-D-glucopyranoside; he therefore suggested that the D-glucose units in the polysaccharide are most likely linked chiefly through the 2-position. It was therefore of interest to examine the structure of this polysaccharide and to ascertain the unique linkage.

The crown-gall polysaccharide was acetylated and the product methylated with methyl sulfate and sodium hydroxide by simultaneous deacetylation and methylation. It was found that methylation of the compound proceeded with difficulty and that it could not be completely methylated. However, its methoxyl content was sufficiently high to attempt the isolation of a trimethyl monosaccharide derivative. On methanolysis of the incompletely methylated polysaccharide a methyl trimethylglucoside was obtained which on hydrolysis of the glucosidic group gave rise to trimethylglucose. The rotation of this trimethylglucose agreed with that of the 3,4,6-trimethylglucopyranose synthesized by Haworth, *et al.*[7] Oxidation of the trimethylglucose with hypoiodite yielded a trimethylglucono lactone. The rate of hydrolysis of this lactone and its final specific rotation showed that it was 3,4,6-trimethylglucono lactone and that it belonged to the δ-lactone series.

On treatment of the trimethylglucose with phenylhydrazine hydrochloride and sodium acetate, an osazone was produced which was identical with that obtained from trimethylfructofuranose derived from hydrolyzed methylated inulin.[8] This constitutes further proof that positions 2 of the trimethyl glucose residues are predominately free and that some of the glucose residues in the crown-gall polysaccharide are united through 1,2-glucosidic linkages. Figure 1 represents the structure of a segment of the polysaccharide chain, consisting of β-glucopyranose units joined through 1,2-glucosidic linkages.

These methylation data confirm Reeves'[6] observation that the direction and magnitude of shift in specific rotation of the D-glucopyranose polysaccharides determined in aqueous and cuprammonium hydroxide solution can furnish information regarding the position of the linkage.

Experimental

Isolation of Polysaccharide.—The polysaccharide was isolated from cultures of *Phytomonas tumefaciens* (Smith and Townsend) Bergey, *et al.*, grown on a sucrose, mineral-salts medium. The techniques used in culturing the or-

(1) Eli Lilly Company, Indianapolis, Indiana.

(2) V. C. Barry, *Sci. Proc. Roy. Dublin Soc.*, **22**, 59 (1939); W. Z. Hassid, M. A. Joslyn and R. M. McCready, THIS JOURNAL, **63**, 295 (1941); E. C. Fairhead, J. M. Hunter and H. Hibbert, *Canad. J. Research*, Sec. B., **16**, 151 (1938); S. Peat, E. Schlüchterer and M. Stacey, *J. Chem. Soc.*, 581 (1939).

(3) H. Granicshstädten and E. G. V. Percival, *ibid.*, 54 (1943).

(4) W. C. McIntire, W. H. Peterson and A. J. Riker, *J. Biol. Chem.*, **143**, 491 (1942).

(5) R. Hodgson, A. J. Riker and W. H. Peterson, *ibid.*, **158**, 89 (1945).

(6) R. E. Reeves, *ibid.*, **154**, 49 (1944).

(7) W. N. Haworth, E. L. Hirst and L. Panizzon, *J. Chem. Soc.*, 154 (1934).

(8) W. N. Haworth and A. Learner, *ibid.*, 619 (1928).

ganism and in the isolation and purification of the polysaccharide were the same as those already described.[4,5]

Methylation.—The polysaccharide used for methylation was first acetylated by the procedure of McIntire, Peterson and Riker.[4] Thirteen grams of the acetylated polysaccharide was dissolved in 240 ml. of acetone and simultaneously deacetylated and methylated at 55° with 75 ml. of methyl sulfate and 220 ml. of 30% sodium hydroxide. The reagents were added in ten equal portions at ten-minute intervals with vigorous stirring. This procedure was repeated seven times. After the eighth methylation, which was carried out using 40% sodium hydroxide, 6.33 g. of material was obtained (69%) having a methoxyl content, OCH_3 39% (calcd. for $(C_6H_7O_2(OCH_3)_3)_n$, 45.6%). Another methylation did not increase the methoxyl content of the material. The product was then subjected to further methylation, using a modified procedure of Muskat.[9] The partially methylated product was dissolved in anisole and treated with liquid ammonia, sodium and methyl iodide. This treatment did not increase the methoxyl content, but considerably decreased the yield of the material (25% of the theoretical).[10]

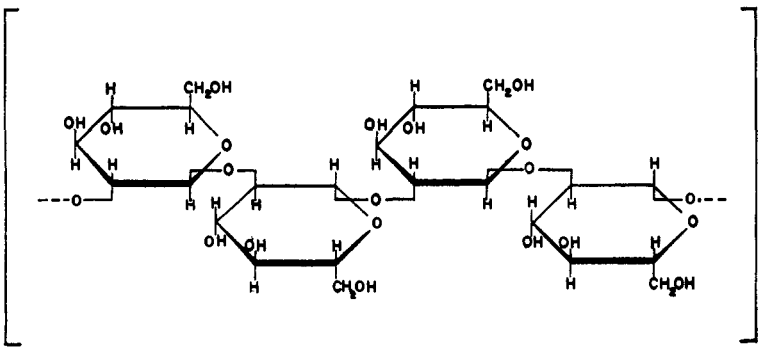

Fig. 1.—Arrangement of the β-glucose residues joined through 1,2-glucosidic linkages

Methanolysis of the Polysaccharide.—Two grams of the methylated product was boiled under a reflux condenser for 18 hours with 60 g. of methanol, containing 15% of dry hydrogen chloride. The hot solution was then neutralized with lead carbonate, filtered when cold and the filtrate evaporated to dryness. The residue was extracted with chloroform, the chloroform removed by evaporation and the sirup distilled at 0.1 mm. pressure and at from 120 to 140°. The yield was 0.77 g. The methoxyl content of this sirup was 51.5% (calcd. for $C_6H_8O_2(OCH_3)_4$, 52.6%). The specific rotation of the methyl trimethylglucoside in water (*c*, 2) was $[\alpha]D$ + 119°. Haworth, Hirst and Panizzon[7] record the specific rotation for crystalline methyl 3,4,6-trimethyl-β-D-glucopyranoside as $[\alpha]D$ −15°. From a calculation, applying Hudson's rule of isorotation,[11] the α-form should have a specific rotation of +143°. Thus, it appears that the methyl trimethyl-D-glucoside was most likely a mixture of both forms of methyl 3,4,6-trimethyl-D-glucopyranoside with the α-form predominating.

3,4,6-Trimethyl-D-glucopyranose.—A sample of 0.60 g. of the methyl 3,4,6-trimethyl-D-glucopyranoside was refluxed for two hours with 20 ml. of 1.5 *M* hydrochloric acid. The solution was cooled, neutralized with silver carbonate, filtered and passed through ion exchange columns. The neutral solution was concentrated to dryness under reduced pressure and the residue extracted with chloroform. After removal of the chloroform and drying of the residue in a vacuum oven at 50°, 0.51 g. of a strongly reducing sirup (trimethylglucose) was obtained. Its reducing value, determined by oxidation with ferricyanide,[12] as compared with glucose on molecular basis, was 106%. Its specific rotation in water (*c*, 3) was $[\alpha]D$ + 73.5°, which is in fair agreement with the specific rotation $[\alpha]D$ +71° for 3,4,6-trimethyl-D-glucopyranose given in the literature.[7]

Preparation of Phenylosazone from 3,4,6-Trimethyl-D-glucopyranose.—The trimethylglucose (0.15 g.) was mixed with an excess (100%) of phenylhydrazine hydrochloride and sodium acetate. Water was added to yield a clear solution and the mixture heated on a steam-bath for half an hour. On cooling, an orange-red oil separated and solidified. The solid product was purified by repeated crystallizations from 50% ethanol. The crystalline osazone weighed 0.05 g. and melted at 80°. *Anal.* Calcd. for $C_{21}H_{28}O_4N_4 \cdot H_2O$: N, 13.4; OCH_3. 22.2. Found: N, 13.5; OCH_3, 21.0.

This osazone proved to be identical with the 3,4,6-trimethylglucosazone (m. p. 80°) prepared in this Laboratory from hydrolyzed methylated inulin. Haworth and Learner[8] record the melting point for trimethyl osazone prepared from the hydrolysis products of the same methylated polysaccharide as 80–82°.

3,4,6-Trimethyl-δ-gluconolactone.—Oxidation of the trimethylglucose (0.35 g.) with hypoiodite[13] yielded trimethylgluconolactone. Goebel's method of oxidation was slightly modified in the following manner: After the inorganic salts had been removed, the solution was passed through a Duolite C-3 column. The resulting solution was concentrated *in vacuo* and the residue extracted with chloroform. The chloroform was removed and the residual sirup distilled at 140° and 0.1 mm. pressure. The yield of 3,4,6-trimethylgluconolactone was 0.30 g. The neutral equivalent of the lactone was 223 (calcd., 220).

A 0.062-g. sample of the lactone was dissolved in 2 ml. of 50% methanol and the rate of hydrolysis followed polarimetrically. The following change in rotation was observed: $[\alpha]D$ +75° (5 min.); +70° (30 min.); +31° (17 hr.); +14° (21 hr.); +13° (24 hr., constant value).

The rate of hydrolysis of this trimethyl gluconolactone was similar to that of other δ-lactones in the glucose series.[14] Its constant value agreed with the value $[\alpha]D$ +15° observed by Haworth, *et al.*,[7] for the synthetic 3,4,6-trimethyl δ-gluconolactone.

Phenylhydrazide of 3,4,6-Trimethylgluconic Acid.—When a 0.2-g. portion of the trimethylgluconolactone was heated on the steam-bath with the calculated amount of phenylhydrazine, the lactone was quantitatively converted to the crystalline phenylhydrazide of 3,4,6-trimethylgluconic acid. After recrystallization from ethyl acetate, its melting point was 131°. The melting point for the compound given by Haworth, *et al.*,[7] is 126°. *Anal.* Calcd. for $(C_{15}H_{24}O_6N_2)$: C, 54.8; H, 7.4; N, 8.5; OCH_3, 28.5. Found: C, 55.0; H, 7.3; N, 8.5; OCH_3, 28.3.

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(10) The authors wish to note that another lot of 9 g. of acetylated polysaccharide, which had been previously methylated eight times with methyl sulfate and 30% sodium hydroxide, had a methoxyl content OCH_3 of 42%, and was obtained in 79% yield. This lot of methylated material was accidently lost.

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continued interest of Dr. A. J. Riker, Department of Plant Pathology, and Dr. W. H. Peterson, Department of Biochemistry, is acknowledged gratefully.

Summary

The polysaccharide produced by the crown-gall organism, *Phytomonas tumefaciens*, was partially methylated. Hydrolysis of this methyl derivative yielded 3,4,6-trimethylglucopyranose.

Oxidation of the trimethylglucose produced 3,4,6-trimethyl-δ-gluconolactone, and on treatment of this lactone with phenylhydrazine, the phenylhydrazide of 3,4,6-trimethylgluconic acid was obtained.

A 3,4,6-trimethylphenyl osazone was also prepared from the trimethylglucose, which proved to be identical with that obtained from the hydrolysis products of trimethylinulin.

These data show that a substantial proportion of the glucopyranose residues in the crown-gall polysaccharide are united through 1,2-glucosidic linkages.

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[CONTRIBUTION FROM THE NOYES CHEMICAL LABORATORY, UNIVERSITY OF ILLINOIS]

Polyalkylene Sulfides. IV. The Effect of *p*H on Polymer Size[1,2]

BY C. S. MARVEL AND GENE NOWLIN

The primary purpose of the present work was to investigate the effect of *p*H on emulsion polymerization of dithiols and dimercaptans in the hope of finding optimum conditions for producing high molecular weight polyalkylene sulfides. Earlier work[3,4] in this Laboratory has shown that emulsion polymerization gives more satisfactory results than solution polymerization. However, the polymer size failed to reach the magnitude desired and the results were not consistent.

The emulsion polymerization of hexamethylenedithiol with biallyl (1,5-hexadiene) was studied in emulsions in which MP-189-EF[5] was used as an emulsifier and a persulfate was employed as the initiator with copper and bisulfite activators as previously recommended by Bacon[6] and Morgan[6] for the reduction activated polymerization of acrylonitrile.

In the first series of experiments the *p*H of the emulsifier solution, containing the initiator and activators, was varied within the range 3.0 to 8.6 by addition of proper amounts of sodium hydroxide solution. Duplicate runs were made in all polymerization studies reported here. The yields and inherent viscosities are summarized in Table I.

It can be seen that polymer size, as determined from inherent viscosities, is essentially constant when the polymers are prepared in emulsions initially in the *p*H 3.0–6.9, and per cent. conversions are approximately equal (90–93%); furthermore, the *p*H dropped to the same value, 2.4–2.6, at the end of the polymerization period. However, when the initial *p*H was 8.6, there was a smaller *p*H change (to 6.4), the conversion (75%) and polymer size (inherent viscosity, 0.22) were considerably decreased. This might be expected, since the oxidation of mercaptans to disulfides is a competing reaction in alkaline medium.[7,8] In a preliminary experiment it was found that the inherent viscosities of polymers prepared at a *p*H of 2.0 to 1.0 dropped appreciably (0.28 to 0.16).

In a variation of these experiments the *p*H of the emulsifier solution was adjusted to the desired value in the range 3.0–5.4 prior to introduction of the initiation and activators. On addition of the latter agents to the adjusted emulsifier solution the *p*H in each instance dropped to 2.6–2.7. At the conclusion of the

TABLE I

THE EFFECT OF *p*H ON POLYMER SIZE OF POLYHEXAMETHYLENE SULFIDE IN UNBUFFERED EMULSION

Number	0.1 *N* NaOH, ml.[a]	*p*H,[b] initial	*p*H, final	Conversion, %[c]	Inherent viscosity[d]
1	2.93	3.0	2.4	92	0.77
2	3.89	3.5	2.4	93	.74
3	4.27	4.3	2.5	91	.74
4	4.40	5.0	2.5	92	.66
5	4.70	6.0	2.5	93	.74
6	5.10	6.9	2.6	90	.70
7	7.30	8.6	6.4	75	.22

[a] This is the volume of hydroxide solution necessary to give the desired initial *p*H. [b] All *p*H determinations reported throughout this work were measured with a Beckman *p*H meter, model H2. [c] Based on recovery of polymer after one reprecipitation as described in the experimental. [d] All inherent viscosities reported throughout this paper were determined from solutions containing 0.4 g. of polymer/100 ml. of chloroform.

(1) This investigation was carried out under the sponsorship of the Office of Rubber Reserve, Reconstruction Finance Corporation, in connection with the Government Synthetic Rubber Program.

(2) This is the fourth paper on Polyalkylene Sulfides; for the third, see Marvel and Baumgarten, *J. Polymer Sci.*, in press.

(3) Marvel and Chambers, THIS JOURNAL, **70**, 993 (1948).

(4) Marvel and Aldrich, *ibid.*, **72**, 1978 (1950).

(5) MP-189-EF is an electrolyte-free emulsifier which consists essentially of mixed alkanesulfonic acids. We are indebted to Dr. Stanley Detrick of Jackson Laboratory, E. I. du Pont de Nemours and Company, for this material.

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(8) Gilman, "Organic Chemistry," 2nd edition, John Wiley and Sons, Inc., New York, N. Y., 1943, p. 835.