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GLUCURONIC ACID, A CONSTITUENT OF THE GUM OF ROOT NODULE BACTERIA[1]

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In a previous paper[2] data were presented which demonstrated that glucose is a product of hydrolysis of the gums produced by the bacteria of red clover, alfalfa and pea, and that uronic acid is also a constituent of these gums and comprises from 4.1 to 25.3% of the weight of the gum. Besides the analytical data, qualitative tests indicated the presence of a uronic acid. When the gum hydrolysate was neutralized with barium hydroxide and poured into ethyl alcohol, a precipitate formed which possessed reducing properties, left a considerable ash on ignition, and gave a strong uronic acid test with naphthoresorcinol. The uronic acid did not yield mucic acid when oxidized with nitric acid and hence was not galacturonic acid.

The nature of the unknown uronic acid has been investigated more thoroughly and, on the basis of the evidence which will be presented in this paper, it is concluded that the unknown acid is glucuronic.

Experimental

Identification of the Uronic Acid.—The gum was produced by pure cultures of pea and red clover bacteria, and was prepared as described in a previous paper.[2] The dried gum was taken into solution with 5% (by weight) sulfuric acid, and was hydrolyzed by heating for two hours in an autoclave at fifteen pounds steam pressure. After neutralizing the hot solution with barium carbonate, the barium sulfate was filtered off and well washed. The filtrate was concentrated to about one-tenth of the original volume and poured into four volumes of 95% alcohol. The precipitate which formed was filtered off. The filtrate was concentrated to a small volume and a second precipitate was obtained by pouring into alcohol. The combined precipitates of crude uronic acid were taken into solution in a small volume of water and an excess of sulfuric acid was added. Upon pouring this solution into four volumes of 95% alcohol, a precipitate of inorganic impurities formed. The alcohol was removed by evaporation and the sulfuric acid precipitated with barium hydroxide. The filtrate from the barium sulfate was neutralized at the boiling point with barium hydroxide in order to insure the complete neutralization of any uronic acid lactone. The solution was then decolorized, concentrated and the barium salt of the uronic acid precipitated with alcohol. The product still retained a slightly yellow color, but it was sufficiently pure for the preparation of the osazone derivative.

The *p*-bromophenylhydrazone of glucuronic acid has been used by Neuberg[3] as a means of identification, but Goldschmiedt and Zerner[4] reported difficulty in the purification of this derivative, and found that the alkaline earth salts of glucuronic acid furnished a better starting material for the preparation of a suitable derivative. They

[1] Herman Frasch Foundation Research in Agricultural Chemistry, Paper No. 15.

[2] E. W. Hopkins, W. H. Peterson and E. B. Fred, THIS JOURNAL, **52**, 3659 (1930).

[3] C. Neuberg, *Ber. deut. Chem. Ges.*, **33**, 3315 (1900).

[4] G. Goldschmiedt and E. Zerner, *Monatsh*, **33**, 1217 (1912).

prepared the *p*-bromophenylosazone and found this compound could be readily purified and that it gave nitrogen and base analyses which checked well with the calculated values. The use of this derivative as a means of identification possessed particular advantages in our work since the separation and purification of free uronic acid from its salt can be accomplished only with considerable loss of material. Therefore the *p*-bromophenylosazone of the barium salt of the uronic acid was prepared and the melting point and the barium and nitrogen contents of this derivative were determined.

Uronic Acid of Preparation 6 (Gum from Red Clover Nodule Bacteria).—The gum was hydrolyzed and the barium salt of the uronic acid purified by the methods already described. This purified barium salt was used in the preparation of the *p*-bromophenylhydrazine derivative, as follows: barium salt of the uronic acid 1 part, *p*-bromophenylhydrazine hydrochloride 3 parts, barium acetate 4 parts and water 70 parts. The mixture was heated for two minutes in a boiling water-bath, shaken well, and rapidly filtered. The turbid yellow filtrate was treated with 3 cc. of glacial acetic acid, and again heated in a boiling water-bath. After ten to twenty minutes' heating yellow crystals began to separate. Further crops of crystals were obtained by continued heating, but the melting point of these later crops varied but little from that of the first. The crystals were filtered off, washed well with water, and with boiling absolute ethyl alcohol. The melting point of the derivative was 216°. Goldschmiedt and Zerner give the melting point of the *p*-bromophenylosazone of barium glucuronate as 215–217°. The same derivative of glucuronic acid was prepared and its melting point found to be 216°.[5] The melting point of a mixture of the known derivative with the unknown was the same as that of the derivatives separately, 216°.

The osazone was analyzed for nitrogen and barium by the micro methods of Pregl.[6]

Anal. Subs., 6.090 mg.: N_2, 0.504 cc. (24°, 746 mm.). Subs., 7.335 mg.: $BaSO_4$, 1.430 mg. Calcd. for $(C_{18}H_{17}O_5N_4Br_2)_2Ba$: N, 9.37; Ba, 11.49. Found: N, 9.35; Ba, 11.47.

Uronic Acid Preparation 8 (Gum from Pea Nodule Bacteria).—The gum was hydrolyzed, the barium salt of the uronic acid was purified, and the *p*-bromophenylosazone prepared in the manner already described. The melting point of the preparation was 216° and no depression in the melting point of the same derivative of glucuronic acid occurred when the two were mixed together. Analyses for nitrogen and barium gave the following results.

Anal. Subs., 7.300 mg.: N_2, 0.615 cc. (24°, 746 mm.). Subs., 4.960 mg.: $BaSO_4$, 0.980 mg. Calcd. for $(C_{18}H_{17}O_5N_4Br_2)_2Ba$: N, 9.37; Ba, 11.49. Found: N, 9.52; Ba, 11.62.

The melting point and the barium and the nitrogen content of the *p*-bromophenylosazone agree with those of the same derivative of glucuronic acid.

Further Evidence of the Identity of the Sugar Produced by Hydrolysis of the Gum.—In a previous paper[2] it was reported that the sugar constituent of the gums was glucose, as was indicated by fermentation tests and the melting point of the phenylosazones. In the preparation of the glucuronic acid considered in this paper, the sugar obtained on hydrolysis was a matter of minor concern, but since material was available, it seemed worth while again to determine the identity of the sugar.

Sugar of Preparation 8 (Gum from Pea Nodule Bacteria).—After concentra-

[5] The authors desire to express their gratitude to Dr. Armand J. Quick of the Cornell University Medical College for placing at their disposal the glucuronic acid used in this test.

[6] The authors are indebted to Professor Karl Paul Link of the Agricultural Chemistry Department for the micro analyses given in this paper.

tion of the sugar solution to a small volume, glacial acetic acid was added. Crystallization of the sugar took place rapidly and the sugar was purified by recrystallization from acetic acid. The pure product was dried thoroughly in a vacuum oven and a polarization of the sugar made: 0.5005 g. was dissolved in water and after adding a drop of strong ammonia the volume was made up to 25 cc. The rotation in a 200-mm. tube at 24.5° was found to be +6.1°. Ventzke; specific rotation: found, +52.7°, required for *d*-glucose, +52.7°.[7]

The phenylosazone was prepared and was found to have a melting point of 204°. The nitrogen content of the phenylosazone was as follows.

Anal. Subs., 4.515 mg.: N_2, 0.6566 cc. (24°, 741.7 mm.). Calcd. for $C_{18}H_{22}N_4O_4$: N, 15.65. Found: N, 16.25.

Although the nitrogen content of the phenylosazone does not agree as well with the calculated value as is desirable in a good derivative, the other evidence presented, *i. e.*, the specific rotation of the crystallized sugar, and the results of the fermentation tests reported in the previous paper[2] give clear evidence that the sugar is glucose.

Sugar of Preparation 6 (Gum from Red Clover Nodule Bacteria).—An attempt was made to crystallize the sugar obtained by hydrolysis of this preparation, but without success. One of the solutions was clarified with basic lead acetate and another was dehydrated in vacuo and the friable residue taken up in absolute methyl alcohol, but both treatments failed to remove some substance which interfered with crystallization. The phenylosazone, however, was prepared without difficulty. The melting point of the osazone was 204°. Analysis gave the following figures for nitrogen.

Anal. Subs., 4.100 mg.: N_2, 0.5831 cc. (24°, 741.7 mm.). Calcd. for $C_{18}H_{22}N_4O_4$: N, 15.65. Found: N, 15.89.

The nitrogen content of this osazone is within the limits of error usual in such preparations. This evidence together with the results of the fermentation tests previously reported[2] indicates that here, as in the pea gum, glucose is a constituent of the gum.

Discussion

Although glucose and glucuronic acid were the products of hydrolysis of both pea and red clover gums, it does not appear that these two gums are identical chemical entities. Certain differences between them were observed. Less reducing sugar as glucose was obtained from pea gum than from the red clover gum. After fermentation of the sugars by yeast and bacteria, the hydrolysate of pea gum contained a larger amount of unfermentable reducing substance. A further point of difference was evidenced by the unsuccessful attempts to crystallize the sugar of red clover gum in contrast to the comparative ease with which crystallization of the pea gum sugar took place. Thus, when considered from the point of view of their hydrolytic products, there are differences in the gums in spite of their apparent similarity. These products of hydrolysis are the same as those obtained by Heidelberger and associates[8] from the soluble specific substance of Type III Pneumococcus. However, these workers found in the

[7] C. A. Browne, "A Handbook of Sugar Analysis," New York, 1912, p. 173.

[8] (a) M. Heidelberger, W. F. Goebel and O. F. Avery, *J. Exptl. Med.*, **42**, 727 (1925); (b) M. Heidelberger and W. F. Goebel, *J. Biol. Chem.*, **74**, 613 (1927); (c) M. Heidelberger, *Physiol. Rev.*, **7**, 107 (1927).

hydrolytic products an aldobionic acid precipitable by basic lead acetate. Since in our work the sugar solution was purified by repeated precipitation with 95% ethyl alcohol, such an acid might have been overlooked. Ehrlich and von Sommerfeld[9] report a di-uronic acid obtained by the hydrolysis of pectinic acid. This acid appeared as an insoluble mass in the hydrolysate. In our work a very small amount of insoluble matter was observed in the acid solution after hydrolysis and this material apparently did possess acidic properties. However, the method of hydrolysis used was more drastic than that employed by either Heidelberger and Goebel or Ehrlich and von Sommerfeld, as it was desired to obtain only the products of complete hydrolysis. If compounds of either of these types were present, they must have constituted only a small part of the products of hydrolysis.

Summary

Glucuronic acid has been identified as a constituent of the gum produced by the root nodule bacteria of pea and red clover. The gum was subjected to acid hydrolysis and the glucuronic acid separated from the hydrolysis products as the barium salt. The *p*-bromophenylosazone of this salt had the melting point and the barium and the nitrogen content required for this derivative of glucuronic acid.

The sugar produced by hydrolysis of the gum of the pea bacteria was crystallized, and its specific rotation agreed with that of glucose. In the case of the sugar from the gum of the red clover bacteria, crystallization could not be effected. However, the phenylosazone of this sugar possessed the melting point and the nitrogen content required for phenylglucosazone.

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THE MIGRATION OF ACYL FROM SULFUR TO NITROGEN

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The work of Raiford,[1] from a study of sixteen different substituted *o*-aminophenols, has shown that when the radicals acetyl and benzoyl are introduced, identical diacyl derivatives are obtained regardless of the order of introduction. This clearly involves a migration of acyl between oxygen and nitrogen during acylation. The present investigation was undertaken to determine whether a similar migration of the radicals acetyl and benzoyl would take place between sulfur and nitrogen in an *o*-aminothiophenol.

The base employed, 2-amino-4-chloro-thiophenol is readily prepared and isolated in the form of the hydrochloride, by reducing 4,4'-dichloro-2,2'-

[9] F. Ehrlich and R. von Sommerfeld, *Biochem. Z.*, **168**, 263 (1926).

[1] See papers of Raiford, THIS JOURNAL (1919–1926).