COMPARATIVE METHODS FOR THE STUDY OF BACTERIAL POLYSACCHARIDES

WILLIAM J. PAYNE, STEPHEN J. GAGAN, AND ARTHUR L. POLLARD

Department of Bacteriology, University of Tennessee, Knoxville, Tennessee

Received for publication September 24, 1952

In a previous paper by Smith and Pollard (1952) a method of using the sensitive anthrone reagent in connection with paper chromatography was described. The present work is a survey of various methods to identify the presence of uronic acids and glucosamine in bacterial polysaccharides. Specific and accurate methods for the determination of these substances in natural products do not exist. The problem is complicated further by their behavior during hydrolysis. According to Anderson and Sands (1945), furanoside bonds are easily broken whereas pyranoside bonds may survive many hours of heating to 120 C in 4 per cent acid. Moreover, there is evidence of a reaction between the uronic acids and any glucosamine that may be present.

The recent commercial production of synthetic glucuronolactone by the Chemical Division of Corn Products Refining Company probably will stimulate extensive experimentation, and there is immediate need for better analytical procedures. In addition to the well known detoxifying activity of glucuronic acid, there is evidence that it has antibacterial properties (Wooldridge and Mast, 1949). Fishman *et al.* (1951) report anomalous variations in retention, after oral administration, by patients with rheumatic diseases or with cancer of the breast. Of greater importance are the activity of plant uronides in the conditioning of soil and the effect of soil algae and bacteria in this process.

METHODS

Although it has been stated by various workers that neither uronic acids nor glucosamine gives typical color reactions with anthrone, it seemed desirable to confirm this. The reaction was tried on a series of dilutions of glucuronic acid, galacturonic acid, glucuronolactone, and glucosamine. Solutions of glucosamine hydrochloride were brought to neutrality before being placed in reaction. The resulting percentage transmittance was read at 630 mμ on a photoelectric colorimeter.

The crude polysaccharide of *Leuconostoc mesenteroides*, prepared by Smith and Pollard, was used in the present work. No effort was made to remove proteins or amino acids since our interest was in substances which also might be removed by such an attempt. The method of hydrolysis was modified considerably after a number of preliminary experiments. The final procedure was to use 2.5 per cent sulfuric acid but to extend the time to 48 hr at 78 C. Hydrolysis was effected in 500 ml flasks equipped with long air condensers and heated on a hot plate.

The polysaccharide of *Serratia marcescens* was investigated also. It was prepared from a laboratory strain by the method of Shear and Turner (1943). It was hydrolyzed by the method described for *L. mesenteroides*.

Two chromatographic techniques were used. The technique described by Smith and Pollard was modified only to obtain better equilibration of the paper to the solvent vapor by allowing the strips to hang in the chamber overnight. Strips also were run in the same manner, but instead of cutting them into 20 segments and leaching, they were sprayed with the aniline hydrogen phthalate reagent recommended by Partridge (1949). Preparation of this reagent is critical. Aniline should be redistilled and the reagent prepared immediately before use. The products of Distillation Products Industries and of Delta Chemical Works were used to establish Rf values for glucuronic acid, glucuronolactone, galacturonic acid, and glucosamine. These values are shown in table 1.

Total carboxylic acid groups were determined on the polysaccharide from *L. mesenteroides* before hydrolysis by the uranium acetate method of Farrar *et al.* (1951). The method was modified as follows: The polysaccharide was soaked in a saturated solution of uranium acetate in water for 48 hr in the cold. An equal volume of ethanol was added and the solution allowed to stand three days at 8 C. Then it was filtered through a Gooch

crucible, washed thoroughly with 50 per cent ethanol, ignited, and weighed.

Uronic carboxylic acid groups were determined by the Maher (1949) modification of the Lefevre and Tollens (1907) method.

Various modifications of the naphtho-resorcinol test for uronic acids were tried on the unhydrolyzed polysaccharide of *L. mesenteroides* and on the eluates from segmented chromatographic strips. The Kapp (1940) method was the only one which gave reproducible results. This method when carefully standardized distinguishes between glucuronic and galacturonic acid. The distinction is delicate and requires precise observance of the published procedure.

TABLE 1

Chromatographic behavior of uronic acids and glucosamine, solvent butanol-pyridine-water 3:2:1.5 spray, aniline hydrogen phthalate

SUBSTANCE	CONDITION	Rf
Galacturonic acid	acid	0.09
	neutral	0.05
Glucuronic acid	acid	0.09, 0.55
	neutral	0.05
Glucuronolactone	acid	0.09, 0.55
	neutral	0.05
Glucosamine	neutral	0.26
Glucosamine + glucuronic acid	neutral	0.05

Glucosamine was determined by the method of Elson and Morgan as given by Browne and Zerban (1948). This method is not particularly sensitive but does allow the detection of significant quantities.

Although the present investigation was not concerned with sugars, these were identified easily both on the sprayed strips and by the anthrone reagent in the eluates from segmented strips.

RESULTS

Data in figure 1 show the sensitivity of glucose, galacturonic acid, glucuronic acid, glucuronolactone, and glucosamine to anthrone. Within the range 0 to 60 μg per ml, glucose alone gives a significant reaction; but above 200 μg per ml there is a color produced by the other compounds which is indistinguishable from that given by glucose. It is difficult to explain this result because the curves are not what might be expected from impurities. The reaction is obviously of no utility for the detection of uronic acids in bacterial products, but does indicate the necessity for care in using it on products containing large quantities of uronic acid or glucosamine.

Experiments to determine Rf values, using commercially available chemicals, demonstrated a somewhat curious behavior of these substances in solution. This is particularly true in the case where glucosamine is run in the presence of glucuronic acid; the glucosamine spot being completely suppressed.

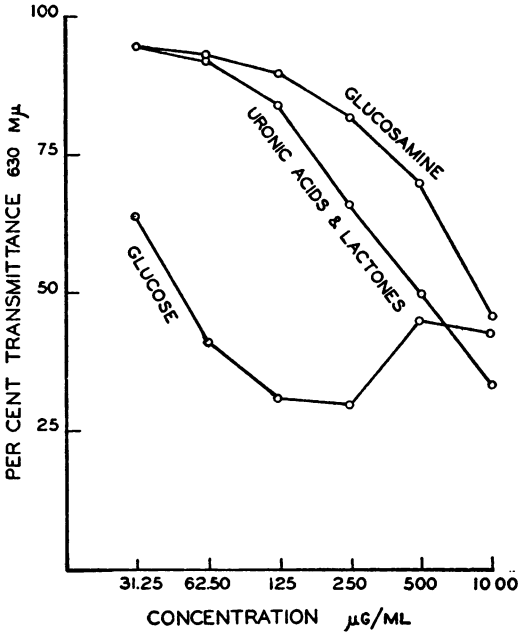

Figure 1. Comparative sensitivity of the anthrone reaction to glucose, uronic acids, glucuronolactone, and glucosamine.

The crude polysaccharide of *L. mesenteroides* gave a positive Kapp test for glucuronic acid both before and after hydrolysis. This test also was given by section no. 1 of the segmented strips, representing an Rf value of 0 to 0.05. The unhydrolyzed polysaccharide, containing a considerable amount of protein, showed 55 mg per g of CO_2 by the uranium acetate method. A determination of the uronic acid content of the same material by the Maher method gave an

average value of 3.48 per cent. This value checked well with a roughly quantitative estimation by the Kapp method. Neither the uronic acids nor glucosamine could be detected positively on the sprayed chromatograms.

The polysaccharide of *S. marcescens* was not available in sufficient quantity to attempt the Maher determination of uronic acid, but its presence was detected easily in the hydrolyzate by the Kapp method. Glucosamine was found to be present in the hydrolyzate by the Elson and Morgan method. It could not be demonstrated in the chromatographic segments, probably due to its low concentration.

DISCUSSION

Although in this survey project a great many replicate determinations were made which checked well, it is doubtful that any of the methods for uronic acids are very precise when applied to bacterial polysaccharides. The extreme stability of the pyranose form of a uronic acid when linked to a sugar through an aldehyde group tends to make the Maher determination too low. On the other hand the Maher determination on unhydrolyzed material may be too high due to amino acids which are degraded readily by acid. The Maher method is reproducible, however, for any particular polysaccharide and should be valuable in comparing the results of various methods of purification and hydrolysis.

The Kapp naphtho-resorcinol method also is valuable in connection with eluted chromatographic segments. It seems to offer a method of distinguishing between galacturonic and glucuronic acids.

The determination of glucosamine in the presence of uronic acids by any of the methods used in this study is highly uncertain. The Elson and Morgan test may give an indication of its presence in hydrolyzates, but it is doubtful that the result gives any indication of the content of unhydrolyzed material.

It is apparent that a method of removing proteins and amino acids without at the same time removing quantities of uronic acids and glucosamine would be an ideal solution of the problem. Such a method does not seem to be available. A possible approach might be to conduct hydrolysis under conditions which would form a stable and separable metal complex of the uronic acids. An unsuccessful attempt to do this with manganese was made in this laboratory.

SUMMARY

A survey was made of various chromatographic, gravimetric, and colorimetric methods for the determination of uronic acids and glucosamine in bacterial polysaccharides, using polysaccharides from *Leuconostoc mesenteroides* and from *Serratia marcescens*.

It was found that several methods for uronic acids were useful but of doubtful accuracy.

No useful method of determining small amounts of glucosamine in the presence of uronic acids is available.

REFERENCES

ANDERSON, E., AND SANDS, L. 1945 A discussion of methods of value in research on plant polyuronides. Advances in Carbohydrate Chem., **1**, 329–341.

BROWNE, C. A., AND ZERBAN, F. W. 1948 *Sugar analysis*. John Wiley and Sons, Inc., New York, p. 962.

FARRAR, J., NEALE, S. M., AND WILLIAMSON, G. R. 1951 A gravimetric estimation of the carboxylic acid group in cellulose. Nature, **168**, 566.

FISHMAN, W. H., SMITH, M., THOMPSON, D. B., BONNER, C. D., HASDON, S. C., AND HOMBURGER, F. 1951 Investigation of glucuronic acid metabolism in human subjects. J. Clin. Invest., **30**, 685–696.

KAPP, E. M. 1940 Microestimation of uronic acids. J. Biol. Chem., **134**, 143–150.

LEFEVRE, K. U., AND TOLLENS, B. 1907 Untersuchungen über die glucuronsaure, ihre quantitative bestimmung und ihre farbenreaktionen. Ber. deut. chem. Ges., **40**, 4513.

MAHER, G. G. 1949 Technique in semimicroestimation of uronic acids. Anal. Chem., **21**, 1142–1144.

PARTRIDGE, S. M. 1949 Aniline hydrogen phthalate as a spraying agent for chromatography of sugars. Nature, **164**, 443.

SHEAR, M. J., AND TURNER, F. C. 1943 Clinical treatment of tumors. V. Isolation of the hemorrhage-producing fraction from *Serratia marcescens* (*Bacillus prodigiosus*) culture filtrate. J. Natl. Cancer Inst., **4**, 81–97.

SMITH, P. B., AND POLLARD, A. L. 1952 Chromatographic methods in the study of a polysaccharide. J. Bact., **63**, 129–132.

WOOLDRIDGE, W. E., AND MAST, G. W. 1949 Effects of uronic acids, pectins and pectinates on the enteric flora, alone and in combination with antibiotics. I. *In vitro* studies. Am. J. Surg., **78**, 881–886.