HANDBUCH DER HISTOCHEMIE

BAND II

POLYSACCHARIDE

ERSTER TEIL

ERGEBNISSE DER POLYSACCHARIDHISTOCHEMIE
MICROORGANISMEN · INVERTEBRATEN

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A. M. BRESLAU - Los Angeles · M. GABE - Paris

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Polysaccharides in Microorganisms

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With 25 figures

I. Introduction

A. Field of Study

We are interested in identification and localization of carbohydrates in microorganisms and in changes that occur during metabolism, development and in the interrelationships with other organisms (host-parasite). The microorganisms which include protozoa, fungi and bacteria are a heterogeneous assemblage consisting of plant-like, animal-like forms, and forms that do not seem to fit into this type of classification at all. Autotrophic, mesotrophic and heterotrophic patterns of metabolism all are represented. Microorganisms both utilize and synthesize a greater variety of polysaccharides than is found in the entire animal kingdom. There are over 70 different polysaccharides in capsules of Pneumococcus alone.

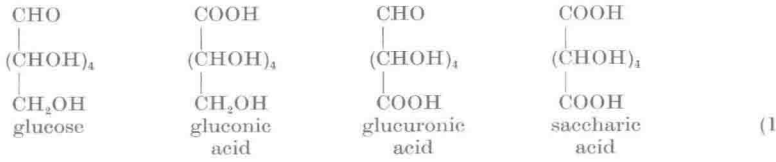
At the present time, we are learning something about their metabolism, but as yet, we know very little of the cytochemistry of microorganisms. With the introduction of new methods for more specific identification and accurate localization of polysaccharides, this group will be a very fruitful field for cytochemical research. We need not disdain the use of any method, be it dyeing, biochemical analysis, application of enzymes, physical or serological methods, if it helps us in this purpose. The application of cytochemical methods to electron microscopy should be especially useful.

B. Chemistry of Polysaccharides found in Microorganisms

1. Classification

Because of their solubility, monosaccharides and oligosaccharides do not lend themselves readily to cytochemical studies. However, many polysaccharides are insoluble or may be fixed so that they may be studied in organisms. They are composed of monosaccharides which are liberated by acid hydrolysis. The structural units are joined glycosidically through loss of a hydrogen atom of the coupling hydroxyl group. The monosaccharides contain a carbon skeleton which generally is unbranched. Each monosaccharide bears a carbonyl oxygen which may reside on a terminal carbon atom producing an aldehyde or upon a nonterminal carbon atom producing a ketone. Hexoses are the most common monosaccharides. Glucose and mannose differ only in configuration about carbon atom 2; glucose and galactose differ only in configuration about carbon atom 4. Microorganisms possess enzymes which permit transformation from one hexose to another. Pentoses also are present and most important are arabinose, ribose, and rhamnose.

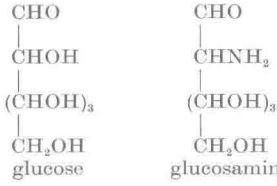
Carboxylic acids, carboxylic alcohols, and amino sugars are derived from monosaccharides. There are three types of carboxylic acid: 1. a type in which the aldehyde group is oxidized to carboxyl, i.e. gluconic acid; 2. one in which a primary hydroxy group far from the aldehyde is oxidized to carboxyl, i.e. glucuronic acid; and 3. one in which both ends of the carbon chain are oxidized to form a dicarboxylic acid as in saccharic acid. Glucuronic acid conjugates insoluble substances, increasing their solubility. It is possible, therefore, that in microorganisms as in animals conjugates may be excreted to prevent possible toxic effects.



(1)

Carboxylic alcohols are isomeric with true monosaccharides sharing with them the empirical formula $(CH_2O)n$. Mannitol is a reserve alcohol stored by yeasts and molds.

Amino sugars are formed from monosaccharides by replacement of an hydroxyl by an amino group.



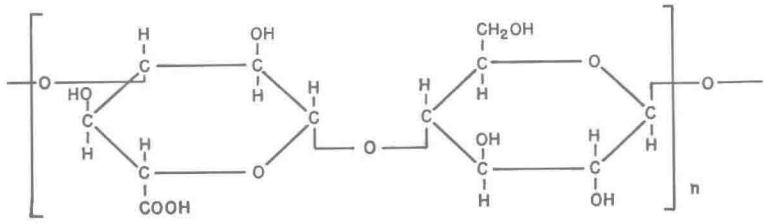
(2)

Monosaccharides also may be acylated, esterified with phosphoric or sulfuric acids, or methylated. They exist in cyclical form. If the ring is composed of one oxygen atom and five carbon atoms, the sugar is called a pyranose; if composed of one oxygen and four carbon atoms it is a furanose.

Polysaccharides are composed of polymerized di-, tri-, or tetrasaccharides. Sugar residues may be all of one kind in which case the carbohydrate is called a homopolysaccharide or the residues may be of more than one type of sugar in which case the carbohydrate is a heteropolysaccharide. The compound may be linear or branched. Sometimes two or more polysaccharides may be closely associated, as amylose and amylopectin are in starch. Frequently, the carbohydrate is part of a complex containing protein or lipid or both. If polysaccharide is predominant, the complex is called mucoprotein; if protein is predominant, the complex is called glycoprotein.

In the first class, true polysaccharides, the compound is composed only of sugars and contains neither carboxylic acids nor amino-sugar residues. In this group are found many of the most important storage and structural carbohydrates: amylose, glycogen, cellulose, etc.

In the second class are carbohydrates that contain carboxylic acid but no amino-sugar. Carboxylic acid, which is rarely found among the microorganisms, may be sulfated. But, such a sulfated polysaccharide (STOLOFF 1959) extracted from algae has strong anticoagulant, antithrombin, and lipemia clearing activity. Cellobiuronic acid, the capsular polysaccharide of Type III Pneumococcus, is a polymerized dimer of glucuronic acid and glucose bound to one another by 1–4 linkage and bound to the following dimer through the first carbon in the glucose to the third carbon in the following glucuronic acid.



CELLOBIURONIC ACID (3)

In the third class are the aminopolysaccharides, containing amino sugars with or without other sugars. They range from neutral homopolysaccharides such as chitin containing N-acetyl-D-glucosamine residues, through acid polysaccharides such as hyaluronic acid, a polymerized dimer of N-acetyl-D-galactosamine and D-glucuronic acid, to the heterotetrasaccharides.

2. Storage Polysaccharides

Starch is a mixture of polymerized amylose, amylopectin, and amylocellulose. Amylose is a straight chain of glucose units attached to one another by 1–4 glucosidic linkages. Amylopectin, in addition to the 1–4 glucosidic linkages, also contains 1–6 linkages for the side chains. Paramylon, the reserve carbohydrate of Euglenoids, probably is neither amylose nor amylopectin; it is similar to glucan of fungi. Amylocellulose is composed of units similar to cellobiose.

Glycogen is similar to amylopectin in being a straight chain of glucose units bound glucosidically by 1-4 linkages and side chains bound to the straight chain by 1–6 linkages. In yeast glycogen, one eleventh of the glucose units have both 1–4 and 6–1 bonds. Glycogen is soluble in water and in alkali but is precipitated by ethanol. Paraglycogen (Brand 1935) which is found in many protozoa, especially in Sporozoa, is fairly resistant to salivary digestion; it is not soluble in cold water; it is soluble, however, in hot water. By polarized light it appears birefringent. On acid hydrolysis it yields glucose. The glycogens vary in solubility because of number and length of the side chains.

Trehalose is found as a free sugar in bakers' yeast and as part of a lipid complex in acid-fast bacteria (Stacey and Kent 1948). It is composed of polymerized 1-(alpha-D glucopyranose) alpha-D-glucopyranoside, is very soluble in water and trichloracetic acid, slightly soluble in aqueous alcohol when free, but is precipitated by 80 per cent alcohol. Trehalose is found in 142 of 212 fungi studied (Myrbäck 1949) including *Mucor*, *Aspergillus* and *Rhizopus*. It is also found in *Mycobacterium phlei* and *Mycobacterium tuberculosis*.

Many *aminopolysaccharides* are found in protozoa, bacteria, and fungi. They are of many kinds. A few have been studied biochemically; however, very little cytochemical work has been done on the storage aminopolysaccharides of microorganisms. Much confusion is caused by nucleic acids and polymerized inorganic phosphates which, like the acid aminopolysaccharides, give a metachromatic reaction with basic stains, such as toluidine blue. But Ebel et al. (1958a) have shown how to differentiate the inorganic polyphosphates from the acid mucopolysaccharides.

3. Wall, Slime and Capsular Polysaccharides

Glucan is a homopolysaccharide built of glucose units in pyranose form, primarily with 1–3 linkages (STONE 1958). Yeast glucan exists in two forms: one is readily soluble in cold dilute alkali, but is acid resistant and gives a sharp distinctive x-ray diffraction pattern; the other, alkali resistant, is a highly branched compound which gives only a diffuse x-ray diffraction pattern similar to the above. Glucan (TREVELYAN 1958) is soluble in boiling 3 per cent sulfuric acid and in warm acetic acid.

Mannan (TREVELYAN 1958) is a homopolysaccharide in which mannose units are combined to form long primary and short secondary chains. Mannan (MEIER 1958) also exists in two forms. Mannan A is soluble in dilute potassium hydroxide and is composed of 10–15 polymerized sugar units. Mannan B is composed of 40–80 sugar units. It is not soluble in dilute potassium hydroxide, but after hydrolysis in one percent hydrochloric acid it is soluble in 24 per cent alkali solution. It may exist in the form of microfibrils. Like cellulose, it is soluble in cuprammonia and reacts with the zinc chlor-iodide test. It combines with Fehling's solution, but instead of reducing the copper, the adjacent hydroxyl groups of the mannose form a complex with alkaline copper. Mannans also form insoluble complexes with borax. The zymosan of yeast wall (DI CARLO and FIORE 1958) is composed of glucan, mannan, and protein plus a small quantity of chitin. Yeast mannan is a highly branched compound having 1–2, 1–3, and 1–6 linkages.

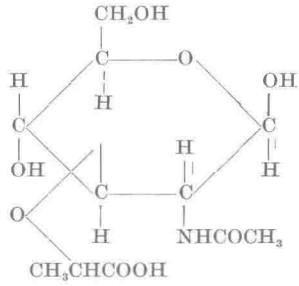
Cellulose is a simple polysaccharide built of the disaccharide units, cellobiose, which is itself composed of two residues of glucose. It is soluble in concentrated (above 72 per cent) sulfuric acid, but is not soluble in water, alcohol, hot dilute mineral acids, or alkali. It is soluble in cuprammonia or cupra-di-(ethylene diamine) hydroxide. Cellulose does not fluoresce, but when examined under crossed NICHOL prisms, it is anisotropic. Cellulose is frequently associated with various other polysaccharides. Some may be removed by water or by acids or by alkalis. In many cases it is very difficult to purify cellulose even by chlorite treatment (PRESTON 1959).

Chitin is a neutral polyaminosugar formed of polymerized N-acetylglucosamine units bound to one another by beta glycosidic 1–4 linkages. It is soluble in concentrated potassium hydroxide only when very high temperatures are applied for a long time, under which circumstances chitin is degraded to chitosan (SCHULZE 1924). This can be performed better in an autoclave at 15 pounds pressure for three hours, after which the organisms should be washed in several changes of alcohol, followed by running water and finally, one per cent acetic acid to neutralize the residual alkali. This treatment, of course, destroys the structure of the organism and therefore is not suitable for cytochemistry. Chitin is not soluble in water, alcohol, ether, dilute acid, or dilute alkali. It is soluble in formic acid, in lithium thiocyanate (STACEY 1958), in concentrated mineral acids, and in strong sodium hypochlorite. Like cellulose, it is anisotropic. Unfortunately, one of the surest methods for differentiating cellulose from chitin, x-ray diffraction, is not applicable in cytochemistry because the organism is destroyed first. Other polysaccharides than chitin contain acetylglucosamine, for example the S XIV Pneumococcal heteropolysaccharide (STACEY 1958).

Hyaluronic acid is found in capsules of some hemolytic streptococci and in capsules of some pneumococci. It is a linear polymer of alternating units of N-acetyl-D-galactosamine and D-glucuronic acid joined by alternating beta 1–3 and beta 1–4 linkages.

Pectin-like substances are present in microorganisms and may play a part as protective enclosure of some protozoans. They are polygalacturonic acid compounds. Methylated forms are quite soluble; their salts may be insoluble. More frequently, galacturonic acid is a constituent of heteropolysaccharides.

Muramic acid is the structural polysaccharide of bacterial cell walls. Spore walls of *Bacillus cereus* (STRANGE and DARK 1957) contain an enzyme which acts on bacterial cell walls liberating a glucosamine, 3–0 alpha carboxy-ethylhexosamine. This substance is present in walls of almost all bacteria studied (WORK 1957).



MURAMIC ACID (4)

II. Cytochemical Identification of Polysaccharides in Microorganisms

A. Fixatives

Microorganisms should be so fixed that the polysaccharides are not degraded or removed during processing and are ready for interaction with the test substances. Most monosaccharides or oligosaccharides are too soluble to be studied cytochemically. Polysaccharides are insoluble or can be precipitated. Some difficulty in studying polysaccharides is caused by the inertness of some of these substances.

It has been suggested that freeze-drying be used to preserve polysaccharides. By itself, this method will only preserve those substances that are already insoluble. Aqueous media should not be used before fixation with alcohol as is done after sectioning and deceration. Covering the sections with celloidin will also help preserve some of the soluble polysaccharides.

When organisms are fixed in liquid solution at room temperature, the polysaccharides migrate toward the side or surface through which the fixative enters. This polarization is inhibited (LISON and VOKAER 1949) by fixing at very low temperature. Different fixatives may be used, most of which fix the proteins and thereby imprison the polysaccharides that are associated with them. Many carbohydrates are precipitated by alcoholic fixatives, but since some of them remain soluble, water must be avoided in later treatment.

Those fixatives that are especially recommended are:

Rossman's Fluid, which is composed of 90 volumes of absolute ethanol saturated with picric acid and 10 volumes of neutral formalin. This fixative is especially useful when acids are contraindicated.

Gendre's Fluid is composed of 80 volumes of 95 per cent ethanol saturated with picric acid, 15 volumes of formalin, and five volumes of glacial acetic acid.

Carnoy's Fluid, which is composed of six parts absolute ethanol, three parts chloroform, and one part glacial acetic acid, is a very powerful fat solvent which may be advantageous in unmasking polysaccharides. It penetrates and fixes very rapidly and should not be used for more than a few hours.

Formol-Alcohol-Acetic Acid is composed of 85 volumes of 80 per cent ethyl alcohol, 10 volumes of formalin, and five volumes of glacial acetic acid. Organisms may remain in this fluid for a long time without adverse morphological effect.

Neutral formalin and calcium chloride-formalin have been recommended. If enzymes are to be used, formalin-containing fixatives should be avoided; and if the polysaccharide is insoluble, the enzyme should be employed before the fixative.

Mannan forms a complex with Fehling's solution. Many polysaccharides form insoluble complexes with borax. WILLIAMS and JACKSON (1956) recommend pretreatment with ribonuclease and then fixing for 48 hours in the following mixture:

1. Ethanol, 50 ml
2. 5-aminoacridine hydrochloride, 0.4 g
3. Water, 50 ml

Perhaps, ethanol and acidulated acridine orange alone should fix acid mucopolysaccharides and stain them at the same time for fluorescent microscopy. Cetavlon and cetylperidinium chloride also precipitate acid mucopolysaccharides to form water- and alcohol-insoluble complexes. Cetavlon and cetyltrimethyl ammonium bromide (STACEY 1958) at pH 7.0 precipitate acid polysaccharides containing carboxyl and sulfate groups but not those containing simple uronic acid radicals. At pH 8.5 in borate buffer they precipitate mannan complexes and at pH 10.0 glycogen complexes, but they do not precipitate dextran complexes. It is claimed that these fixatives are superior to formol, Carnoy's fluid and basic lead acetate for fixing acid mucopolysaccharides. Fixing smears by heat or air drying should be avoided as they produce marked artifacts and do not preserve the more labile polysaccharides.

B. Staining Reactions

1. Iodine Tests for Starch and Glycogen

Many protozoa and bacteria can be suspended in a drop of water, and without further treatment can be studied under the light microscope. It was discovered early that adding a small drop of iodine kills the organisms and improves the differentiation of structures, and, of greater importance for our purpose, stains amylose blue, erythrodextrin red, and glycogen mahogany brown. Glucan (paramylon) is not colored blue by iodine (DEFLANDRE 1934). The staining reaction is the result of the imprisonment of iodine by the host compound; the color is due to the length of the host molecule. TAKEUCHI and GLENNER (1960) stain in dilute solution of iodine : potassium iodide : water = 1 : 2 : 3,000 until the color reaction occurs. They then replace the above with Gram's iodine in glycerol = 1 : 10, cover, and seal with paraffin or varnish. PEARSE (1960) recommends bringing paraffin sections to alcohol and staining with a saturated alcoholic iodine solution, clearing, and mounting in balsam.

In *Donaldson's Method* (1917), a small particle of feces is emulsified in equal parts of Lugol's iodine and saturated aqueous eosin. The parasites are localized because of the presence of glycogen. This method is easily modified to study various microorganisms, but it produces a temporary mount which cannot be studied again at one's leisure. This difficulty can be overcome to some extent by mounting the organisms in a more permanent medium.

In *Pacaud's Method* (1949), the organisms are mounted in gum arabic. Chunks of gum arabic are suspended in a dark bottle containing Lugol's iodine at 37° until a syrupy consistency is produced. The iodinated gum is then hardened, blocks cut from it, and resuspended in fresh Lugol's iodine. Procede as follows: Wet smears or sections fixed in Regaud's bichromate-formol may be used. Stain in acidified aniline blue, differentiate rapidly in 70 per cent ethanol. Without washing, place the slide in a Petri dish and cover the organisms with a piece of iodinated gum, cover the dish, and let stand for 10 minutes. Remove the excess gum and cover with a glass slip. Place the slide in a drying oven for a few days to differentiate the stain. After removal from the oven, permit the gum to harden, seal, and keep in a closed box.

In *Guégen's Method* (1905), 0.1 per cent Sudan III and 0.1 per cent cotton blue are dissolved in lactic acid. Ten to thirty drops of tincture of iodine are added to 100 cc. lactic acid. The coverslip is sealed with lacquer to make a relatively permanent slide. Amylose is stained blue; erythrodextrin, red; amylopectin, violet; glycogen, brown; and, lipids, red.

2. Iodine Tests for Cellulose and Chitin

Iodine tests have long been used for the identification of cellulose. Interpreting the results of these tests must be done with great caution because other polyasccharides, such as paraglycogen and mannan, may give the same or similar reaction.

Cellulose frequently is encrusted by substances which interfere with test results. Eau de Javelle, hypochlorite (Frey-Wyssling and Mühlethaler 1951) and phloroglucin are used to remove these substances. The cellulose must be partially degraded to hydrocellulose which is accomplished with a strong zinc chloride solution. This, however, is not effective on the walls of some lower organisms for which cuprammonia is substituted. The treated cellulose is then soaked in Lugol's iodine. When a few drops of dilute (5 per cent) sulfuric acid are added, the presence of cellulose is indicated by the development of a blue color. Mannan gives a reaction similar to cellulose with the iodine tests. Before a substance is identified, it is recommended (Preston 1959) that it meet a number of criteria. In addition to the iodine staining test, it should also pass the test for anisotropy and x-ray diffraction analysis. In the last test the structure of the organism is destroyed.

Solvents and enzymes should be especially valuable supplements to the color tests for cellulose, if we knew more about their action on complexes and knew how to control their activity to insure specificity.

The more specific tests for chitin are notoriously destructive of organisms. Chitin is frequently associated with carbohydrates that interfere with staining reactions. These substances are best removed by diaphanol (Schulze 1924), chlorine dioxide dissolved in 50 per cent acetic acid. The methods for dissolving chitin in chlorine solution and its degradation to chitosan in hot concentrated potassium hydroxide have been described in the section on chemistry. The best of the iodine methods, and yet far from satisfactory, is to treat the organisms with strong zinc chloride solution followed by soaking in Lugol's iodine. When one per cent sulfuric acid replaces the iodine, chitin gives a violet color. We need better cytochemical tests that will give specific identification, good localization, and yet, not destroy the structure of the organism being studied. Thus a technique such as powder x-ray diffraction studies (Frey 1950; Blank 1953) cannot be used alone in cytochemistry.