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Alginic acids and alginates: analytical methods used for their estimation and characterisation of composition and primary structure

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Contents

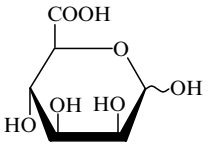
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Abstract. The history and state-of-the-art in the detection, quantitative determination and characterisation of the primary structure of alginic acids and their salts (alginates) are reviewed. A brief survey of the structure and properties of these polysaccharides is given. Numerous analytical methods including chemical, physicochemical and enzymic procedures for the structural analysis of alginates which can also be used for the investigation of other uronic acid-containing polysaccharides are discussed. The bibliography includes 211 references.

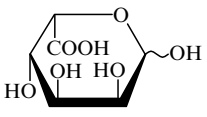
I. Introduction

Alginic acids were first isolated from several brown algae by Stanford more than 100 years ago.[1] Later, these polysaccharides were detected in all brown algal species without exception as cell wall and intercellular matrix components. Their content in the biomass may amount to 40% of the dry weight; this strongly depends on the species and growth conditions.[2–4] Some bacteria (mostly, the representatives of the genera *Azotobacter*[5] and *Pseudomonas*[6]) also produce alginic acids. In 1982, alginates were unexpectedly detected in one of the species of calcareous red algae belonging to the family Corallinaceae.[7] Our recent studies[8,9] have shown that the presence of alginates is virtually a characteristic feature of the representatives of this family. In other plants, these polysaccharides have not been found.

Alginic acids are polyuronides, *i.e.,* polysaccharides the molecules of which are built up of uronic acid residues. At first, D-mannuronic (**1**)[10,11] and then L-guluronic (**2**)[12] acids were identified as the components of alginic acids.



1



2

Partial hydrolysis and isolation of the corresponding disaccharide revealed that the residues of both uronic acids are the components of the same polymeric molecule.[13] According to present-day concepts based on numerous studies including methylation,[14,15] periodate oxidation[16,17] and several physicochemical methods of structural analysis (mostly, NMR spectroscopy[18–21]), molecules of alginic acids are linear and made up of β-D-mannuronic and α-L-guluronic acid residues (in the pyranose form) linked by 1→4 bonds.

Bacterial alginic acids differ from the algal ones in that some of their hydroxy groups are usually acetylated.[22,23] Acetyl groups occupy positions 2 or 3 (sometimes, both simultaneously) in D-mannuronic acid residues.[24] The degree of polymerisation of native polymers is rather high and the number of monosaccharide residues may amount to 1000 – 10 000.

Alginic acids isolated from various sources can differ in the ratios of mannuronic and guluronic acids (M/G)[25] and in the distribution of the monomers along the polymeric chain. Fractionation of oligomeric products formed upon partial hydrolysis has shown that some regions in the molecules of alginic acids are built of only one uronic acid species (M-blocks and G-blocks). These blocks are separated by fragments which contain approximately equal amounts of both monosaccharides arranged into more or less strictly alternating sequences (MG-blocks).[26,27] Such a peculiar structure is a result of stepwise biosynthesis of polysaccharide molecules where the formation of linear mannuronan precursors is followed by epimerisation of some β-D-mannuronic acid residues at the C(5) atom under the action of a specific enzyme, mannuronan-C(5)-epimerase, resulting in the appearance of α-L-guluronic acid residues within the polymer structure.[28]

The size and mutual distribution of individual blocks strongly affect the properties of alginic acids and their salts (alginates). As a matter of fact, β-D-mannuronic and α-L-guluronic acid residues, being structurally very close [they differ only in the configuration at one of the asymmetric centres, *viz*., at the C(5) atom], have opposite conformations of the pyranose rings ($^4C_1$ and $^1C_4$, respectively), which results in completely different shapes of the polymeric molecules in the poly-M and poly-G regions (Fig. 1). This creates spatial conditions for the tight binding of bivalent metal cations with G-blocks. Moreover, the coordination with such cations favours the cooperative binding of different molecules of the polymer with one another,[29] eventually resulting in the formation of ionotropic gels (Fig. 2). Therefore, the total content of α-L-guluronic acids (in the first approximation) or, more precisely, the relative length of G-blocks are important criteria of the most valuable property of alginates, *viz*., their ability to form gels.[30–32] The block structure is responsible for many other characteristics of alginates including their biological activity.[33–35]

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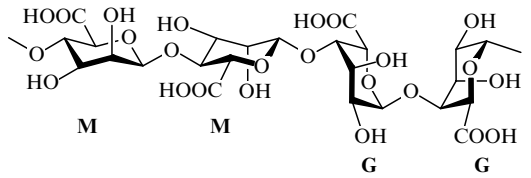


Figure 1. The conformations of monosaccharide units in the molecule of alginic acid.

By virtue of their unique physicochemical properties, alginic acids and their salts have found wide application in the production of foods and in some other branches of industry [36, 37] including biotechnology and medicine.[38] This explains the fact that the majority of reviews devoted to alginic acids provide information about their structure, biosynthetic mechanisms and practical application, but pay insufficient attention to problems concerned with the analytical chemistry of alginates. Thus a special issue of the journal *Carbohydrates in Europe*, which is all devoted to alginates and consists of four reviews describing the chemistry and physical properties,[39] genetics and biosynthesis of alginates,[40] as well as biotechnological and clinical applications of alginate gels [41] and commercial applications of alginates,[42] is laid out on this principle.

Because of considerable practical utility of alginates, their isolation from brown sea weeds has become the main goal of their processing. It is obvious that estimation of alginic acid content in the algal biomass and standardisation of alginic acid preparations and alginate-containing products demand elaboration of convenient analytical procedures, while structural characterisation of these polysaccharides is necessary for determining the most rational approaches to the utilisation of particular preparations containing alginic acids. Proper allowance must be made for the fact that the analytical chemistry of alginates has specific features as compared with that of other polysaccharides.

Alginic acids form water-soluble salts with monovalent cations but are precipitated upon acidification. Alginates of many bivalent cations, particularly, of Ca^{2+}, Sr^{2+} and Ba^{2+}, are insoluble in water. This property is utilised in the isolation of alginic acids from algae.[43] At first, bivalent cations and concomitant water-soluble polysaccharides are extracted from the biomass with a dilute mineral acid; then alginic acids are extracted with an alkaline solution with subsequent precipitation from the extract by treatment with ethanol,[44] acidification or conversion into Ca^{2+} salt.[45] Preparative yields of alginic acids may be used for determination of their content in the algal biomass; it should be remembered, however, that the results obtained with the use of different isolation procedures can give substantially different results for the same algal specimen. This depends on a number of factors, such as reagents used, the number of precipitations and the degree of achieved purity. Thus special experiments aimed at comparing the efficiency of three most popular laboratory procedures [46–48] revealed that the relative scatter in the values of preparative yields of alginates may amount to 20%–40%.[49] Additional problems may arise when this approach is used for the analysis of food compositions where the behaviour of pectin components is similar to that of alginates as regards their extraction and precipitation.

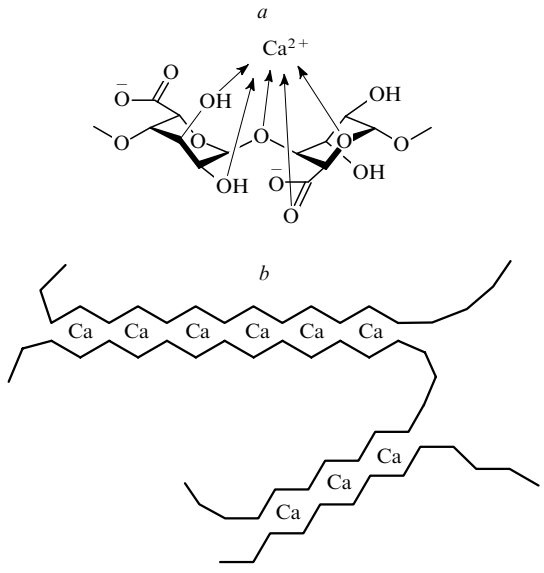


Figure 2. The coordination of calcium ions by α-L-guluronic acid residues in the molecule of an alginate (*a*) and the role of these ions in intermolecular interactions during gel formation (*b*) (the 'egg-box' model).[29]

Some procedures for the estimation of alginic acids are based on specific reactions of uronic acids. These reactions include decarboxylation and degradation under the action of concentrated sulfuric acid with subsequent formation of coloured products in the presence of carbazole, indole, 3-hydroxybiphenyl, *etc*. These methods have found wide application, although it is evident that the presence of other polysaccharides containing uronic acids in the specimen under study will inevitably give erroneous results of alginate assays.

The use of acid hydrolysis of alginates for the preparation of monomeric uronic acids is a rather complex problem. Glycosidic bonds of uronic acid residues are highly resistant to acid hydrolysis. Low solubility of alginic acids in acid media creates an additional problem. Total acid hydrolysis of alginic acids is accompanied by the destruction of some monomers. It is of note that L-guluronic acid residues are destroyed faster than D-mannuronic acid residues and specific losses of each monomer depend on the pattern of their block distribution within the polymeric molecule. Therefore, it is very difficult to select versatile conditions for hydrolysis and to find correction factors for the destruction of monomers that would be equally suitable for the determination of the composition of alginates differing considerably in the ratios and the distribution of uronic acid residues in their molecules.

Nondestructive physicochemical methods, in the first place, ^1H and ^{13}C NMR spectroscopy, have assumed great importance in structural characterisation of alginic acids. These methods can be used both for the determination of the monomer ratio in alginic acid molecules and their characterisation in terms of their arrangement into individual blocks. In the subsequent sections, the above-mentioned analytical approaches will be considered in more detail and illustrated, mostly by the studies of sea weeds, although these methods are equally suitable for the characterisation of bacterial alginates. Additional information can be retrieved from several reviews devoted mostly to alginates of bacterial origin.[50–55]

II. Chemical methods

1. Assays based on cation exchange

If a starting material is used in sufficiently large amounts (*i.e.,* the alginic acid content in a sample under study is no less than 100 mg), alginic acids can be determined by the gravimetric method. Polymeric alginic acids are precipitated upon acidification of weakly alkaline solutions, while calcium alginate is precipitated from neutral sodium alginate solutions upon addition of calcium chloride. Drying of the precipitates is problematic, since these compounds tightly retain water and are readily decomposed on heating. Volumetric analysis is more preferable in experiments with smaller amounts of the material. In this case, the polysaccharide is also precipitated as calcium salt, this is washed with a dilute acid to remove metal cations, and the insoluble alginic acid is washed with water and titrated with

sodium hydroxide in the presence of phenolphthalein or using conductometric [56] or potentiometric [57] control. The formation of viscous sodium alginate solutions in the course of titration can be avoided if an excess of calcium acetate is added to alginic acid, and the liberated acetic acid is titrated.[58] A procedure is described which combines titration with colorimetry and is designed for the determination of the content of alginic acid in algae.[59]

An alternative, rather lengthy procedure for quantitative analysis of alginates is based on the ability of alginic acid-containing algal biomass to bind calcium ions.[60] To this end, samples of a ground plant material are dialysed against dilute hydrochloric acid to remove metal cations; the mineral acid is removed by dialysis against water. Then, alginic acid is converted into calcium salt by dialysis against a calcium acetate solution; the excess of the reagent is removed by repeated dialysis against water. Upon this treatment, all the residues of uronic acids in the alginate are converted into calcium salts where one cation corresponds to two uronic acid residues. Bound calcium ions are then quantitatively extracted by dialysis against dilute hydrochloric acid. The calcium concentration in the diffusate is determined by atomic adsorption spectrophotometry, and the alginate content in the original biomass is calculated. The main drawback of this method is that the presence of other polyanions (*e.g.*, sulfated polysaccharides) in the biomass can overestimate the experimental results.

As polyanions, alginic acid molecules form salts with coloured inorganic (bivalent copper cations [61] or ruthenium red [62]) and organic cations (cationic dyes of the Methylene Blue type [63–65]). In this case, quantitative assays are based on the measurement of the decrease in the optical density of the original coloured solution upon formation of poorly soluble salts or on the changes in the spectral characteristics of the dye (metachromasia) due to its binding to the polymer. This method, which is characterised by high sensitivity and precision, has been recently proposed for quantitative determination of alginates in microcapsules used in cell transplantation.[66] The experimental procedure is based on the analysis of metachromatic changes in the spectrum of 1,9-dimethyl-Methylene Blue as a result of its interaction with the alginate. This type of reactions also includes precipitation of polyanions (as salts) with cationic detergents (Cetavlon precipitation).[67] Polyhexamethylenebiguanidinium chloride used for the precipitation of alginic acids is the most studied reagent of this class.[68] Precipitation of alginic acids with this reagent is virtually independent of the presence of salts (< 2.5%), pH (5.5–7.6), temperature (10–60 °C) and the M/G ratio. The analytical procedure is based on the measurement of the decrease in the reagent absorption at 235 nm. Although this method was recommended for the analysis of industrial extracts of brown algae,[69, 70] other acid polysaccharides can be precipitated by cationic detergents together with alginic acids. It was proposed [71] to use coloured hydrazides formed by the reaction of 2-nitrophenylhydrazine in the presence of water-soluble carbodiimide in colorimetric assays of alginates and their fragments instead of coloured salts;[71] however, this method has not found wide application.

2. Assays based on degradation of uronic acids

Heating of alginates results in decarboxylation and evolution of CO_2.[72] The samples under study are usually heated with 19% HCl, and the CO_2 evolved is absorbed and weighed as in the microanalytical determination of carbon in organic compounds by combustion.[73, 74] Although this method was described in a popular practical guide to biochemical assays of algae,[75] it requires special skill and equipment (*cf.* Refs 76–78). This is also true for a modified decarboxylation procedure in which polysaccharide samples are heated with 57% HI and the CO_2 evolved is assayed by gas-liquid chromatography (GLC)[79] or direct non-aqueous titration.[80] It is obvious that other uronic acid-containing polysaccharides can interfere with the alginate assays.

Colorimetric assays of polyuronides based on acid degradation of their constituent monosaccharides have found much wider application. The main reaction product formed upon heating of uronic acid derivatives with concentrated sulfuric acid is 5-formylfuran-2-carboxylic acid, which is rather stable under these conditions.[81] The formation of degradation products of neutral sugars (5-hydroxymethylfurfural and furfural) from hexoses and pentoses, respectively, prevents its direct spectrophotometric analysis. It is therefore more expedient to use the ability of 5-formylfuran-2-carboxylic acid to form coloured products with various organic reagents, particularly with some aromatic nitrogen heterocycles and phenols, *e.g.*, carbazole,[82, 83] indole,[84] 3-hydroxybiphenyl [85] and 3,5-dimethylphenol.[86]

Along with indisputable advantages (in the first place, the possibility to experiment with microquantities of material), colorimetric assays have obvious drawbacks, such as interference due to neutral sugars and different reactivities of individual uronic acids. This is the reason why the analytical procedure was repeatedly modified, although the reaction with carbazole had long been used for the determination of polyuronides including alginic acids.[87] Thus it was proposed to supplement the reaction mixture with boric acid in order to intensify the colour formation and to equalise the reactivities of different uronic acids.[88, 89] The addition of sulfamate [90, 91] or different temperature regimes [92] were used in order to minimise the effects of neutral sugars. It was demonstrated that the composition of a specimen containing three uronic acids (glucuronic, galacturonic and mannuronic) can be calculated by conducting the reaction with carbazole in four different regimes.[93]

The effect of ballast neutral sugars was estimated during the elaboration of a procedure for pectin assays.[94, 95] It was found that 3,5-dimethylphenol [86] and 3-hydroxybiphenyl [96] have salient advantages over carbazole in pectin and hemicellulose assays.

In a more recent study,[97] Filisetti-Cozzi and Carpita proposed a method for the colorimetric assay of polyuronides including alginic acids, which took account of recommendations from previous investigations. This method utilised 3-hydroxybiphenyl in combination with the borate anion and sulfamate. Our recent studies have shown that 3,5-dimethylphenol is a more preferable specific reagent for alginic acids.[8] We modified the procedure proposed by Scott [86] for the analysis of pectins and hemicelluloses to fit with the specific composition of alginates using greater amounts of borate and more prolonged heating of reaction mixtures at 70 °C. The results of these experiments weakly depended on changes in the ratios of D-mannuronic and L-guluronic acids in different alginate specimens.

The problem of selectivity of the colour reaction and the necessity to eliminate interference due to the presence of neutral sugars in the specimen under study is of prime importance in the analysis of heteropolysaccharides, such as plant hemicelluloses and complex pectins, animal mucopolysaccharides and many bacterial and fungal polysaccharides. In the case of alginic acids, the effect of neutral sugars can be eliminated by preliminary removal of ballast substances,[98] although this procedure complicates the experimental protocol. For example, ion-exchange chromatography on Dowex 1 × 2 was used for purification of an alginate isolated from the extracellular material of mucoid strains of *Pseudomonas aeruginosa*,[99] although DEAE-cellulose or DEAE-Sephadex are evidently more suitable.[100, 101] In alginate assays in food products, it is recommended to conduct enzymic deproteination with subsequent precipitation of calcium alginate. The latter is carefully washed off from soluble carbohydrates and dissolved in cold 80% sulfuric acid or in hot sodium hexametaphosphate solution; alginates are assayed in the resulting solutions using naphthoresorcinol,[102] phenol [103, 104] or trivalent iron sulfate [105] in concentrated sulfuric acid. The selectivity of these reagents is lower than that of 3-hydroxybiphenyl, 3,5-dimethylphenol and carbazole. The reaction with $Fe_2(SO_4)_3$ was successfully used for the study of seasonal changes and anatomic distribution of alginates in some species of brown algae.[106] In a more complicated procedure described by Toyoda *et al.*,[107] naphthoresorcinol-HCl was added to an alginate after its

precipitation as Cu^{2+} salt; the coloured reaction product formed thereupon was extracted with butyl acetate and quantitatively assayed using HPLC.

In the majority of the above-described reactions, concentrated sulfuric acid was used to destroy uronic acids; however, similar results can be obtained in polyuronide assays utilising the Bial reagent (orcinol in concentrated hydrochloric acid, usually supplemented with $FeCl_3$ [108]). In the case of alginates, the reaction with orcinol has certain advantages over the reaction with carbazole,[109] although analytical results also depend on the composition of the original polymer.[110] Obviously, this dependence is the main limitation of the majority of alginic acid assays, since chemical properties of uronic acid residues strongly depend on whether it forms a part of a homo- or a heteropolymeric block. This circumstance is the main obstacle on the way to the design of versatile calibration curves for the determination of alginic acids with any ratio and any distribution of β-D-mannuronic and α-L-guluronic acids along the polymeric chain.

3. Assays based on acid hydrolysis of glycosidic bonds

As mentioned above, alginic acids, like other polyuronides, are hydrolysed with difficulty and under drastic conditions. This leads to partial degradation of the monosaccharides liberated, in the first place, of guluronic acid. In the hydrolysis products, the monomeric uronic acids are in an equilibrium with lactones. This all makes the analysis of alginic acid hydrolysates a rather complicated procedure. The method for determining the M/G ratio by polarimetry of the total hydrolysate of alginates in the form of brucine salts [111] has not found wide application, since it requires large quantities of the starting material and its high purity, for ballast carbohydrates significantly distort the results of polarimetric analysis. Therefore, in the overwhelming majority of cases the hydrolysates are analysed following preliminary separation of the hydrolysis products.

Some time ago, it was proposed to use paper chromatography for separation of the hydrolysis products; this procedure allowed for the first time the proof of the presence of guluronic acid residues in the polymers.[12] Later, conditions were elaborated for the separation of guluronic, mannuronic, galacturonic and glucuronic acids by paper electrophoresis.[112] However, these methods are suitable for qualitative rather than for quantitative analysis of hydrolysates. In more recent studies, thin-layer chromatography (TLC) on silica gel was used for the identification of depolymerisation products of alginic acids. This method is especially convenient for detecting small amounts of alginates in beer [113–115] and other foodstuff; acid methanolysis is more preferable than hydrolysis. Thus TLC of the methanolysis products allows unambiguous identification of alginates even in the presence of other uronic acid-containing food polysaccharides.[116, 117] High-performance TLC was used to study alginate biosynthesis in *Pseudomonas aeruginosa*.[118]

A procedure based on ion-exchange chromatography has been developed for quantitative determination of uronic acids in hydrolysates and, correspondingly, for the calculation of the M/G ratio.[110, 119] The conditions of hydrolysis proposed by Haug and Larsen [110, 119] were used (almost without modification) in a great number of more recent studies. After treatment of alginate with 80% sulfuric acid at 0 °C, the mixture was incubated for 18 h at 20 °C, then water was added up to the acid concentration of 1 mol litre^{-1} and the resulting mixture was heated for 5 h at 100 °C. After cooling, the hydrolysate was neutralised with calcium carbonate, alkalified to pH 8.0, incubated for 30 min for lactone opening and chromatographed on a Dowex-1 column (acetate form) in a linear gradient of acetic acid (from 0.5 to 2 mol litre^{-1}). Under these conditions, guluronic acid was eluted from the column before mannuronic acid. The uronic acid content in the fractions was determined using the reaction with orcinol; the experimental value of M/G was multiplied by 0.66 in order to correct for guluronic acid losses due to its predominant degradation during hydrolysis. This method was used to characterise the alginates isolated from a great number of algal species.[25]

Some more recent modifications were aimed at the improvement of hydrolysis conditions [120] and refinement of the correction coefficients.[121] In particular, it was proposed to use 88% formic acid instead of sulfuric acid for diminishing the degradation of monomeric uronic acids.[22] However, the main progress in this field is connected with elaboration of new technologies for separation and identification of the hydrolysis products. Thus high-performance liquid chromatography was widely used for ion-exchange separation of uronic acids;[122–128] quantification of these compounds in the eluate can efficiently be performed by measuring intrinsic absorption at 210 nm,[127] by using refractometric [124] or pulse amperometric detectors [125] or in a reaction with cyanoacetamide [126] with subsequent spectrophotometry or spectrofluorimetry.[129]

The new separation technique for uronic acids markedly accelerated and simplified the experimental procedure, but did not eliminate the distortions introduced by hydrolysis of alginates. A method for the analysis of real specimens of alginic acids isolated from different brown algal species [127] which utilises ion-exchange chromatography in an isocratic regime and UV-detection for monitoring elution, has gained especially wide popularity.

Yet another popular procedure for the quantification of hydrolysis of products of alginic acids is GLC, which requires additional chemical conversion of uronic acids into their volatile derivatives. Hydrolysis products normally represent a mixture of uronic acids with lactones. Substitution of methanolysis for hydrolysis yields a multicomponent mixture of methyl esters of methyl glycosides. Analysis of such mixtures (*e.g.*, in the form of trimethylsilyl derivatives) is a rather complex problem,[130–132] and the results obtained are of limited value for quantitative assays.

In order to simplify the composition of the mixtures to be separated, it was proposed to convert uronic acids into aldonic acid lactones (by reduction of the aldehyde groups with subsequent lactonisation [133, 134]) or alditols (by more profound reduction) with subsequent chromatographic separation as acetates.[134–136] In the latter case, several approaches are used to distinguish uronic acid derivatives from the reduction products of neutral monosaccharides. For example, uronic acids are separated by treatment of hydrolysates with ion-exchange resins prior to reduction,[136] otherwise sodium borodeuteride is used for reduction followed by chromato-mass spectrometry for the identification of reaction products.[137] It was also recommended to separate hydrolysates into two portions, in one of which neutral sugars are converted into aldononitrile acetates (the procedure employed results in degradation of uronic acids), and the other portion is used for complete reduction. Comparison of analytical data allows one to calculate the amounts of both neutral monosaccharides and uronic acids.[138] Neutral sugars and uronic acids can also be assayed simultaneously by converting the former into alditol acetates and the latter into *N*-propylaldonamide acetates *via* aldonolactones.[139]

Attempts have been made to circumvent difficulties in alginic acid hydrolysis by reducing the carboxy groups in the polymeric molecule. This can be done either by preliminary esterification of the carboxy groups [140, 141] or by their activation with water-soluble carbodiimide with subsequent treatment with sodium borohydride at controlled pH.[142] The reduction procedure for the determination of the composition of alginic acids was proposed in 1981;[143] however, further studies of the preparative reduction of alginic acids with water-soluble carbodiimide revealed that this reaction cannot be brought to completion.[144, 145] Thus treatment with an excess of carbodiimide caused a significant destruction of the original polysaccharide [144] concomitantly with the preferential reduction of the L-guluronic acid carboxy groups;[145] in the deficiency of this reagent, preferential reduction of the D-mannuronic acid carboxy groups took place.[144]

A more recent study was specially devoted to comparison of different methods of reduction of the carboxy groups in polymers containing uronic acids.[146] The method based on activation of the carboxy groups with 1-cyclohexyl-3-(2-morpholinoethyl)carbodiimide *metho-p*-toluenesulfonate in 8 M urea [147] was found to be the most efficient procedure; however, in this case subsequent treatment with sodium borohydride caused only partial reduction of the carboxy groups and the results obtained for polysaccharides yielding viscous solutions were poorly reproducible. We have simplified the reduction procedure [8] by using prolonged treatment of alginates with the so-called magic methanol [140] (a methanol – chloroform – concentrated HCl mixture, 10 : 1 : 1) for esterification and by reducing the formed ester with a large excess of sodium borohydride in a concentrated imidazole buffer (pH 7.0, 0 °C), by analogy with a method proposed for the identification of esterified galacturonic acid residues in pectins.[148] The hydrolysis products of reduced polysaccharides were converted into aldononitrile acetates and analysed by GLC. The chromatograms revealed two peaks corresponding to gulose derivatives (1,6-anhydro-β-L-gulose and L-gulononitrile acetates) and a peak of acetylated D-mannononitrile. Despite incomplete esterification and, correspondingly, incomplete reduction, the M/G values calculated from the peak areas of the corresponding mannose and gulose derivatives coincided with the results of analysis of the ^{13}C NMR spectra.

A procedure for the determination of the M/G ratio which consists in the reduction of the carboxy groups with subsequent methylation, reductive splitting of the polymer with triethylsilane in the presence of trimethylsilyl methanesulfonate and boron trifluoride etherate and identification of the anhydroalditols (in the form of the corresponding derivatives) using HPLC or GLC, proposed by Zeller and Gray,[149] seems to be too complicated for routine assays of alginic acids.

4. Partial hydrolysis for determining the block composition

As stated in the introduction, physicochemical properties of alginates are largely determined by the mode of distribution of the two uronic acid residues along the polymeric chain. This distribution pattern can be characterised chemically using partial acid hydrolysis.[26,27] After short-term heating of alginic acid suspensions in dilute mineral acids (20 min, 100 °C, 0.3 M HCl), an oligomeric fraction with an average degree of polymerisation of ~ 20 and containing approximately equal amounts of both uronic acid residues (MG-blocks) passes into the solution. The insoluble residue additionally heated for 20 h with acid is separated into two fractions, *viz*., a fraction soluble at pH 2.85 and insoluble one. These fractions also represent oligomers with average degrees of polymerisation of ~ 20, but the former contains exclusively M-blocks (the mannuronic acid content exceeds 90%), while the latter consists of G-blocks (the mannuronic acid content is less than 10%). From the yields of all the three fractions, one can establish the distribution pattern of uronic acid residues in the linear chain of alginates. Using partial hydrolysis, it was demonstrated for the first time that alginates of different origin differ in fact in their block composition.[150] In subsequent studies, a similar approach was repeatedly used for characterisation of alginates (see, *e.g*., Ref. 151).

A simplified procedure for the determination of the block composition of alginates was described by Penman and Sanderson.[18] These authors proposed to use a one-step treatment of alginates with an acid (5 h, 100 °C, 0.3 M HCl) instead of two-step hydrolysis and to determine the yields of soluble and insoluble fractions colorimetrically, by reaction with phenol and concentrated sulfuric acid. The insoluble fraction obtained was analysed by ^1H NMR spectroscopy as a solution in D_2O at 80 °C rather than separated into blocks of different composition. The low-field region of the spectrum has a singlet for H(1) of the residue M at $\tau = 5.34$ (peak *B*), a doublet for H(1) of the residue G at $\tau = 4.96$ (peak *A*) and a singlet for H(5) of the residue G at $\tau = 5.56$ (peak *C*). The ratios of integral intensities B/A or B/C (these values can be averaged) and the yield of the soluble fraction permit one to calculate the block composition of the original polysaccharide.

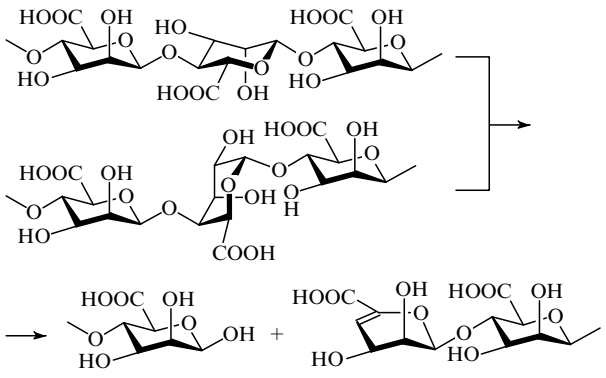
Yet another modification of the partial hydrolysis procedure is the use of ion-exchange and gel-permeation chromatography for the separation of oligomeric products.[152,153] Using several commercial alginate preparations as examples, it was shown that the size of M-blocks varies in the range of 3 – 10, that of G-blocks varies in the range of 4 – 15 monosaccharide residues, while the distribution of monomers within MG-blocks is random.[152]

Electrophoresis in polyacrylamide gel [155] was recommended for separation and quantitative determination of oligomannuronic and oligoguluronic acids with a degree of polymerisation varying from 2 to 9 obtained by partial hydrolysis of alginates.[154] In order to increase the electrophoretic mobility of the oligosaccharides and sensitivity of their assays, these were fluorescently labelled by reductive amination with 8-aminonaphthalene-1,3,6-trisulfonic acid.

III. Enzymic and immunological methods

All currently known enzymes able to depolymerise alginate molecules are lyases, *i.e*., they catalyse β-elimination of the substituent from position 4 of the uronic acid residue rather than hydrolysis of glycosidic bonds.[156–158] This cleavage results in the formation of a 4,5-unsaturated uronic acid residue (Scheme 1) at the newly formed end of one of the fragments. Since elimination results in disappearance of asymmetric centres at C(4) and C(5), the same unsaturated uronic acid is formed from both β-D-mannuronic and α-L-guluronic acids. This unsaturated residue has an intense absorption band at 230 – 240 nm, and the increase in optical density at 230 nm can be used as a convenient method for determining the enzymic activity of alginate lyases.[159] A mixture of oligomers with low degrees of polymerisation is a final product of enzymic treatment of polymeric alginic acids with alginate lyases.[160]

Scheme 1



Alginate lyases have been detected in many organisms, *e.g*., sea molluscs, echinoderms, bacteria and fungi.[161] Molluscs (Gastropoda) [162] and bacteria [163–165] are the most convenient sources for the isolation of these enzymes. Alginate lyases of different origin differ essentially in their stabilities,[166] specificities [167–169] and abilities to hydrolyse acetylated bacterial alginates.[170,171] Thus M-lyases (polymannuronate lyases) normally isolated from molluscs are endoenzymes, which easily hydrolyse M- and MG-blocks without any effect on G-blocks.[172,173] In contrast, G-lyases (polyguluronate lyases) hydrolyse G- and MG-blocks but are inert with respect to M-blocks.[162] The substrate specificities of alginate lyases can be examined using a recently proposed procedure for depolymerisation of alginates on agar plates in the presence of calcium chloride [174] or using homopolymeric substrates containing a terminal fluorescent label.[175]

A convenient and versatile procedure which takes into account the specificity of alginate lyases and is suitable for detection and analysis of alginates in solutions at concentrations

of 0.01 to 1 mg ml^{-1} (see Ref. 176) was developed for quantitative determination of various alginic acids. The material under study was treated with a mixture of two enzymes of different origin, *viz*., a mixture of poly-M-lyase isolated from the mollusc *Haliotis tuberculata* and poly-G-lyase isolated from the bacterium *Klebsiella pneumoniae*; the absorption of reaction products was measured at 230 nm. With the use of an excess of enzymes it was shown that the final values of optical density were proportional to the initial concentration of the substrate. A combination of two enzymes made it possible to eliminate the effect of the variable composition of different alginates. It is noteworthy that the separate application of these enzymes can provide information about the block structure of various alginates. This recently developed procedure holds much promise, especially for future studies provided alginate lyases become commercially available.

Alginate lyases of known specificity are also used for obtaining information about the distribution of uronic acid residues and the sizes of individual blocks in alginates. It is bacterial enzymes that made it possible to establish, for the first time, the deviation of the uronic acid sequence in poly-MG from a strictly alternating pattern.[160, 177] Some alginates were found to contain poly-M-blocks that are homogenous in size and have an average degree of polymerisation of 24;[160] however, more recent studies with other subjects established more variable distribution patterns of poly-G- and poly-M-blocks according to their size.[178, 179]

The specificity of action of the enzyme isolated from the sea mollusc *Haliotis tuberculata* was studied in most detail. High-performance liquid chromatography was specially adapted for the separation of saturated and unsaturated oligoguluronates and oligomannuronates formed upon enzymic hydrolysis of alginates[180] and different procedures of NMR spectroscopy[181] were specially developed for the elucidation of the structure of these oligosaccharides. It was found that alginate lyase effectively cleaves M−M and G−M bonds (but not G−G or M−G bonds) and that the pentamer of mannuronic acid is best bound to the enzyme active centre.

In an analogous study of recombinant bacterial alginate lyase,[182] it was established that the enzyme cleaved exclusively M−M bonds. The enzymic action is blocked by the presence of the acetyl groups in mannuronic acid residues, which is characteristic of bacterial alginates. This enzyme was recommended for the preparative isolation of poly-M-blocks from acetylated polymannuronate of *Pseudomonas aeruginosa* and of poly-MG-blocks from another bacterial alginate containing residues of both uronic acids.[183]

Alginates possess antigenic activity. Several attempts were undertaken to raise antibodies against definite structural elements of alginate molecules and to use these antibodies for the analysis of alginates by standard immunological procedures.[184] Specific antibodies (both polyclonal[185, 186] and monoclonal[187]) against alginates were used to localise the polysaccharide in various algal tissues; however, this assay was complicated by gel formation. Considering that gel formation decreases the ability of the corresponding sites in the alginate molecule to bind to antibodies and that the reaction conditions (pH, temperature, accessibility of Ca^{2+} ions and the nature of other buffer components) can strongly influence the results of immunological assays,[188] it may be concluded that these studies are still far from being completed and that the immunological behaviour of alginates and the specificity of the corresponding antibodies need additional investigation.

IV. Nondestructive physicochemical methods

1. IR spectroscopy

The total amount of polymers consisting of uronic acids can be determined from the intensity of the absorption band of the ionised carboxyl at 1607 cm^{-1} in the IR spectrum of a sample solution in a D_2O-phosphate buffer.[189] It was recommended to determine semi-quantitatively the M/G value in alginates by measuring the ratio of absorption band intensities at 808 (M) and 787 cm^{-1} (G) in the IR spectra of specially prepared films.[190] Comparison of band intensities at 1320 (M) and 1290 cm^{-1} (G) can also be used for this purpose.[191] However, because of low precision and interference due to different factors these two methods have not found wide application. On the other hand, IR spectra provide valuable information about the presence of acetyl groups in bacterial alginates[22, 99, 101] judging from intense absorption bands at 1250 and 1730 cm^{-1}.

Recently, it was proposed to use spectroscopy in the near IR region (400–2500 nm) for determination of the alginate content in brown algae biomass.[192] The reflectance spectra recorded for powdered biomass samples are computer-processed at 9 parameters. The experimentally determined calibration curves allow the determination of alginate in the starting material with high precision. This method was successfully used for characterisation of biodegradation of the alga *Laminaria hyperborea* in which the alginate content varies from 2.2% to 40.8%. Horn *et al*.,[192] who have developed this procedure, gave it a high estimate, although this analysis requires the use of a specially designed spectrophotometer. It was noted also that variable content of moisture and polyphenols in the specimens under study interfere with the analysis of alginates.

2. Circular dichroism

The three types of blocks present in alginate molecules differ essentially in their circular dichroism (CD) spectra; the poly-G spectrum lies completely in the region of negative molar ellipticity values, $[\theta]$, the poly-M spectrum has an intense positive band, while the poly-MG spectrum is characterised by intermediate values of $[\theta]$.[193, 194] The CD spectra of natural alginates are characterised by a peak at 200 nm and a trough at 215 nm. It was found that the M/G ratio may be calculated from the observed ratio of peak height to trough depth using simple equations.[195] Therefore, this important parameter can be determined from the CD spectra even for milligram quantities of the alginate without any destruction of the polymer.

Since the CD spectra of mixed blocks (poly-MG) are not identical with the spectra of equimolar poly-M and poly-G mixtures, the determination of the block composition of alginates from their CD spectra is possible. A relatively simple computer-assisted calculation was proposed which mimics the real spectrum owing to a linear combination of three types of spectra characteristic of isolated blocks.[195] Although the shape of poly-MG spectra depends on the distribution of uronic acid residues within the blocks, which is not taken into consideration in the calculation procedure, the results obtained with known alginate specimens showed good coincidence with those earlier established using partial hydrolysis or NMR spectra. The CD spectra were subsequently used to characterise the block composition of alginates isolated from many species of brown algae or from different types of algal tissues.[47, 196, 197]

3. ^{13}C NMR Spectroscopy

A preliminary report concerning the interpretation of ^{13}C NMR spectra of alginates was published in 1977 by Grasdalen *et al*.[20] who showed that the alginate spectra recorded at 25 MHz contained four signals in the anomeric resonance region rather than two signals, as might be expected for a polysaccharide made up of two uronic acid residues. This implies that the position of C(1) signals of both monosaccharides (M and G) depends on the nature of the neighbouring residue. The intensities of these signals could be used for estimating both M/G and the relative content (F) of pairs of monomers (MM, MG, GM and GG). Moreover, the signal corresponding to the C(5) atom of the residue M was represented in the spectrum by three lines, which suggests the dependence of the signal position on the nature of both neighbouring residues. This phenomenon could be used for quantification of the number of triplets with a central M residue within the polymer. It was found that the central line of the signal corre-

sponds to the central residues M of the MMM and GMG triplets, whereas the low-field and high-field lines represent the corresponding signals for GMM and MMG, respectively. Thus, the relative content of the latter two triplets in the alginate molecule can be measured directly, from the intensity of the corresponding signals, and the probability of the presence of triplets MMM and GMG in the polymeric chain can be calculated from the ratios: $F_{MMM} + F_{GMM} = F_{MM}$ and $F_{GMG} + F_{MMG} = F_{MG}$. The data calculated for several alginates showed good correlation with the results of chemical analysis. However, no possibility to determine the number of triplets with a central (G) residue was found in this pioneering study.

Later, in detailed studies Grasdalen *et al.*[21] used a higher-resolution NMR spectrometer (50 MHz) and carefully selected the conditions for recording NMR spectra. The signals for the anomeric carbon atoms gave eight lines corresponding to the central units of eight theoretically possible triplets of the primary structure. The intensities of these lines were used to calculate the content of each type of triplets in several specimens of natural alginates. The validity of these calculations was partially confirmed by an analysis of signals corresponding to the carboxyl carbon atoms and to C(4) and C(5) atoms of the residue M, which also have multiplet structures, and by comparison of these data with the results of chemical assays. The average lengths of homopolymeric blocks can be calculated from the triplet content: $N_{G>1} = (F_G - F_{MGM})/F_{GGM}$ and $N_{M>1} = (F_M - F_{GMG})/F_{MMG}$.

It is of note that the most probable distribution of residues along the polymeric chain in synthetic binary copolymers formed by polymerisation of two different monomers can be calculated, taking into account the initial concentrations and relative reactivities of the monomers. However, the block composition of alginates usually differs from the calculated sequence, which is at variance with the mechanism of formation of these polysaccharides by copolymerisation of two monosaccharide precursors and is a convincing argument in favour of an alternative route of their biosynthesis involving mannuronan-C(5)-epimerase.[198, 199]

^{13}C NMR spectroscopy is an extremely convenient procedure for structural characterisation of alginates isolated from different natural sources (see, *e.g.,* Refs 8, 9, 200) or modified by enzymic treatment.[201] This was also used to monitor alginate biosynthesis in bacteria.[202] However, recent experiments have shown [49, 203] that the reliability of determination of the block composition depends on M/G of the specimen under study. More adequate results were obtained for alginates with a high content of one of the monomers; if M/G is close to unity, the calculated values of individual block lengths can differ substantially from the results obtained upon chemical or enzymic hydrolysis.

^{13}C NMR spectra were obtained not only for solutions but also for solid alginate samples.[204] In the latter case, the resolution of peaks was much lower, especially for the anomeric carbon signals. Nevertheless, a complex spectral pattern could be represented as a sum of individual symmetric signals corresponding to the C(2)–C(5) atoms of both uronic acid residues, which made it possible to estimate the M/G ratio with a sufficiently high accuracy.[204]

4. ^{1}H NMR spectroscopy

The ^{1}H NMR spectra of alginates were studied in parallel with ^{13}C NMR spectra in the hope to elaborate a more sensitive and accurate method for the determination of the content of monomers and their block distribution. It was shown [19] that informative spectra of alginates can be obtained using a 100 MHz spectrometer after short-term mild acid treatment of samples, which reduces the viscosity of solutions. Although not all signals could be resolved in these spectra, the low-field region contained three well-defined peaks, *viz.*, *A* [H(1) of the residue G], *B* [a sum of H(1) of the residue M and H(5) of the residue **M**G] and *C* [H(5) of the residue **G**G], which made it possible to calculate the following values: $F_G = I_A/(I_B + I_C)$ and $F_{GG} = I_C(I_B + I_C)$. Taking into account that $F_G + F_M = 1$, $F_{GG} + F_{GM} = F_G$ and $F_{MM} + F_{MG} = F_M$ (for lengthy chains, $F_{MG} = F_{GM}$), the M/G ratio and the content of the four possible monomeric pairs in alginate molecules can be calculated. The parameters established for well-known specimens coincided with the results obtained by chemical methods and ^{13}C NMR spectroscopy.

In a continuation of these studies, ^{1}H NMR spectra of alginates and their fractions were recorded at 400 MHz.[205] As in the above-cited paper,[19] the M/G values and contributions of the dimeric sequences (MM, MG = GM and GG) were calculated from the analysis of ^{1}H NMR spectra. In addition, higher resolution made it possible to isolate four signals corresponding to the H(5) atom of the central residue of α-L-guluronic acid in the four possible triads (GGG, MGG, GGM and MGM). The content of these trimers showed a good correlation with the values obtained by other analytical methods.

The ^{1}H NMR spectra of poly-M and poly-G, the components of a bacterial alginate produced by *Azotobacter vinelandii*, were further analysed using both one-dimensional and modern two-dimensional NMR techniques.[206, 207] The unambiguous assignment of signals was performed on the basis of chemical shift theory, analysis of spin–spin coupling constants and measurements of the nuclear Overhauser effects. These data were used to study the interaction of poly-M- and poly-G-blocks with Ca^{2+} ions in aqueous media. A model for binding of calcium ions was proposed, which is based on the measurement of the relative intensity of cross-peaks in the two-dimensional spectra (NOESY) of the free polymer and its calcium salt. The experimental results are important for interpreting the gel-forming ability of alginates in the presence of calcium ions and elucidation of the role of calcium as a cofactor essential for the functioning of mannuronan-C(5)-epimerase.

High-resolution ^{1}H NMR spectroscopy was efficiently used for characterisation of many natural alginates, of both algal [196, 208] and bacterial [209, 210] origin, particularly for establishing the degree of acetylation and localisation of acetyl groups in bacterial alginates.[24] It was noted, however,[204] that partial hydrolysis traditionally used for decreasing the viscosity of solutions of high-molecular-weight alginates prior to recording their proton NMR spectra can induce the precipitation of a portion of alginate and thus significantly distort the experimental results (M/G ratio).

V. Conclusion

As may be inferred from the survey, the analytical chemistry of alginic acids is a well-grounded and well-studied field. Numerous approaches have been proposed for the solution of analytical problems of different degrees of complexity, from quantitative determination of alginates in mixtures with other natural compounds to the analysis of uronic acid sequences in the linear molecules of these polysaccharides.

Alginic acids differ from other natural polyuronides in that they are made up of unique monosaccharides and are characterised by a peculiar distribution of monomers along the polymeric chain. Special approaches were elaborated for investigation of these structural features among which partial acid hydrolysis, enzymic degradation and some nondestructive physicochemical methods (in the first place, NMR spectroscopy) have proved to be especially efficient. The experimental findings have made it possible to formulate a concept of the primary block structure of alginic acids and to gain a deeper insight into the pathways of their biosynthesis leading to this structure. These results not only have a great theoretical significance, but are of great practical value, since they permit direct alteration of physicochemical properties of alginates by enzymic treatment.[201, 211] However, it should be remembered that specific analytical approaches were elaborated for the solution of concrete practical problems and have certain limitations which have been mentioned in the review. Further in-depth studies into the structure and properties of alginates will

probably demand further improvement of existing methods of structural analysis of these polysaccharides.

The significance of analytical chemistry of alginates goes far beyond the confines of this class of biopolymers. As uronic acid-containing polysaccharides are widely spread in nature, many chemical methods designed for the detection and quantitative determination of alginates, particularly, the approaches based on specific degradation of uronic acids or chromatography of acid hydrolysates, are equally efficient in the analysis of plant mucilages, pectins, hemicelluloses, bacterial and fungal polysaccharides and animal glycuronoglycans.

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