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MUCO-POLYSACCHARIDES

BY

J. S. BRIMACOMBE

AND

J. M. WEBBER

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MUCOPOLYSACCHARIDES

Chemical Structure, Distribution and Isolation

by

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and

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FOREWORD

The macromolecules of biological tissues and fluids in health and disease continue to attract wide interest in many parts of the world, for it is appreciated that knowledge of their structure, of their mode of biosynthesis and of the way in which they change and break down, will lead to a proper understanding of their function in the complex cycles constituting living systems. Although the nature of the protein constituents remains obscure, study of the carbohydrate components by the classical techniques of carbohydrate chemistry, and the newer enzymatic and physical methods, is giving a clear picture of the structural relationships between the carbohydrate components of the various tissue macromolecules.

Already, two books describing these molecules have appeared from this School of Carbohydrate Chemistry. These previous books give a general picture, (*a*) of the bacterial polysaccharides and (*b*) of the carbohydrates of living tissues. The present book is of a more specialist nature, dealing only with the mucopolysaccharides but giving greater detail especially on methods of preparation and the manner of working out the structures.

Owing to the great activity in the field, new facts are emerging almost every day. My young colleagues have made a particularly vigorous effort in this book to bring up to date all references to these important mucopolysaccharides, particularly the acid mucopolysaccharides. The approach is essentially from the point of view of structural organic chemistry, and is aimed to help the worker who is engaged in dealing practically with these complex macromolecules. The many references will also make the book of value to the newcomer to the field.

Although nomenclature in this field is in a very bad state of confusion and now needs an International Commission to standardise it, readers will have no difficulty in recognising the well-defined mucopolysaccharides. The book will be of value to all those who are interested in the rapidly expanding field of nitrogen-containing carbohydrate substances and I am pleased to commend it.

The University, Birmingham
June 1964

M. STACEY

PREFACE

The structures of the acidic mucopolysaccharides and related glycosaminoglycans have excited interest for many years, but it is only in the last decade, following the development of improved methods of isolation and analysis, that many of the structural details have been elucidated. The present situation is such that, although some details remain obscure, most of the fundamental structures are known with certainty. Future developments are therefore likely to be increasingly concerned with the naturally-occurring, mucopolysaccharide–protein complexes, about which much less in known. Knowledge of the structures of these complexes should contribute to our understanding of their biological roles, both in normal and pathological conditions.

In this book, we present a detailed account of the isolation and structural investigations of the mucopolysaccharides which, in conjunction with Dr. Gottschalk's companion volume on glycoproteins (*B.B.A. Library*, Vol. 5), will, perhaps, help to provide a background for future developments in this field. In discussing the biological properties of these molecules, a selective approach has been adopted since a comprehensive treatment would be beyond the scope of this volume.

It is with pleasure that we acknowledge the advice and encouragement which we have received during the preparation of this book, and, in this respect, we particularly wish to thank Professor M. Stacey and Drs. A. B. Foster, T. D. Inch, C. R. Ricketts and J. J. Willard. We are also grateful to Drs. D. Horton and L. Rodén for supplying results prior to their publication, and to the following authorities and the respective authors for permission to reproduce published material: John Wiley and Sons Ltd. (Table 2); Academic Press Inc. (Table 3); the Commonwealth Scientific and Industrial Research Organisation (Table 4 and passage on p. 24); Nature (London) (Table 6); the British Medical Journal (passage on p. 119); the American Heart Association, Inc. (Table 8); the Journal of Pharmacy and Pharmacology (Table 9); and the Elsevier Publishing Company (Table 10 and Figure 3).

Finally, we are pleased to acknowledge the assistance of Miss Heather Jones, Miss Shirley Martin, and Mrs. Thelma Swash, in typing the manuscript, and Mrs. Eileen Brimacombe and Mr. R. Brueton in proof-reading.

Birmingham, September 1964

J. S. BRIMACOMBE
J. M. WEBBER

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CHAPTER 1

INTRODUCTION AND GENERAL ASPECTS

1. INTRODUCTION

Polysaccharides may be regarded as condensation polymers of monosaccharides resulting from the formation of glycosidic linkages by elimination of water. With many polysaccharides, systematic nomenclature and classification presents considerable problems, and it has therefore been usual to group the polysaccharides in broad divisions according to their source and biological function. In the animal kingdom, a major class obtained in this way comprises the amino sugar-containing polysaccharides, for which the name *mucopolysaccharide* has been adopted.

The term *mucopolysaccharide* was introduced by Meyer[1] to describe "hexosamine-containing polysaccharides of animal origin occurring either in a pure state or as protein salts". The prefix "muco" was chosen to denote the relationship of this type of substance with mucus, the physiological term for a viscous secretion. Subsequent classifications of materials as mucopolysaccharides involved limitations with regard to protein content (the presence of hexosamine being not necessary)[2], and the absence or presence of uronic acid and/or sulphate residues[3,4]. The term has also been applied to (*a*) protein–polysaccharide complexes of high protein or peptide content, and (*b*) lipid–polysaccharide complexes which are, however, more accurately termed glycoproteins (or glycopeptides) and glycolipids, respectively. Despite this confused situation, there has been no formally accepted definition of a mucopolysaccharide, although it is now common[5] for the term to be applied only to those heteroglycans* which contain residues of both a uronic acid and a hexosamine. Attention is thereby confined to a group of substances including hyaluronic acid, the chondroitin sulphates, and heparin which are conveniently termed *acidic mucopolysaccharides***. The acidic character of many of these substances is enhanced by the presence of sulphate groups.

Most of the acidic mucopolysaccharides are found in the connective tissues of animals, and, although complexed with protein or peptide residues, the pure polysaccharide can be isolated without undergoing appreciable degradation. Little is known about the structure of the protein complexes, and the term mucopolysaccharide is therefore best applied only to the pure polysaccharide. When the latter is complexed with protein, it has been suggested[6] that a non-committal term such as hyaluronic acid–protein complex should be used.

In this volume, attention has not been confined to those substances which are strictly defined as acidic mucopolysaccharides since it is desirable that certain re-

* In the systematic classification of polysaccharides by their chemical structure, those containing only one monosaccharide component are termed *homoglycans*, whilst those containing two or more types of monosaccharide units are termed *heteroglycans*.

** In the interests of systematisation the name *glycosaminoglycuronoglycans* has been proposed[6] for these substances.

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TABLE 1

MUCOPOLYSACCHARIDES AND THEIR MONOSACCHARIDE COMPONENTS

Mucopolysaccharide	*Monosaccharides*
Chitin	2-acetamido-2-deoxy-D-glucose
Hyaluronic acid	D-glucuronic acid, 2-acetamido-2-deoxy-D-glucose
Chondroitin	D-glucuronic acid, 2-acetamido-2-deoxy-D-galactose
Chondroitin sulphate A (chondroitin 4-sulphate)[a]	D-glucuronic acid, 2-acetamido-2-deoxy-4-*O*-sulpho-D-galactose
Chondroitin sulphate B (dermatan sulphate)[a]	L-iduronic acid, 2-acetamido-2-deoxy-4-*O*-sulpho-D-galactose
Chondroitin sulphate C (chondroitin 6-sulphate)[a]	D-glucuronic acid, 2-acetamido-2-deoxy-6-*O*-sulpho-D-galactose
Heparin	D-glucuronic acid, 2-deoxy-2-sulphoamino-D-glucose (both residues containing *O*-sulphate groups)
Keratosulphate (keratan sulphate)[a]	D-galactose, 2-acetamido-2-deoxy-6-*O*-sulpho-D-glucose
Heparitin sulphate (heparan sulphate)[a]	D-glucuronic acid, 2-deoxy-2-sulphoamino-D-glucose (also containing *O*-sulphate groups), 2-acetamido-2-deoxy-D-glucose
Teichuronic acid (teichan)[a]	D-glucuronic acid, 2-acetamido-2-deoxy-D-galactose
Blood group substances	L-fucose, D-galactose, 2-acetamido-2-deoxy-D-glucose, 2-acetamido-2-deoxy-D-galactose

[a] Alternative names suggested by Jeanloz[6].

lated "aminopolysaccharides" should also be considered. These include keratosulphate, which accompanies the acidic mucopolysaccharides in animal connective tissues, and the homoglycan chitin. The inclusion of a chapter on the blood group substances is justified on the grounds that these are predominantly carbohydrate in nature, and that it is the carbohydrate moieties whose structures have been most widely studied. Moreover, it is the carbohydrate residues which determine the serological specificities of the blood group substances. The structures of the blood group substances are more complex than those of the acidic mucopolysaccharides, and their elucidation (as yet only partly achieved) illustrates admirably the complementary use of chemical and immunological techniques. Wider aspects of the blood group substances will be discussed in the companion volume on the glycoproteins, edited by Dr. A. Gottschalk (BBA Library, Vol. 5).

The polysaccharides discussed in this volume are listed in Table 1, together with the constituent monosaccharide components. Recent reviews on various aspects of the chemistry of the mucopolysaccharides are available[5, 7–10].

2. GENERAL ASPECTS

(a) Nomenclature

Attempts at systematic nomenclature of structurally complex polysaccharides are of little value because of the cumbersome nature of the names involved. Indeed, even glycosaminoglycuronoglycan, the systematic name proposed[6] for the acidic mucopolysaccharides, is very unwieldy. In general, therefore, the mucopolysaccharides have retained the trivial names which were allotted at the time of their discovery. Factors involved in the nomenclature of mucopolysaccharides have been discussed by Jeanloz[6] who has proposed several modifications (see Table 1). There has, as yet, been no formal acceptance of these proposals, and, in this volume, the original names have been retained because of their greater familiarity. Mono-, di-, and oligo-saccharide components have been named systematically[11], and, where necessary, the appropriate conversion to this system has been made.

(b) Biological function

The mucopolysaccharides fulfil diverse functions in the animal body, and, before embarking on a detailed study of their chemistry, it is desirable to have an overall picture of their function. Chitin, for example, serves in a similar structural capacity in many invertebrates as does cellulose in the plant world. Another group of acid mucopolysaccharides (Table 1, hyaluronic acid through to heparitin sulphate) form the carbohydrate constituents of connective tissues, which support and bind together the organs of the body. The three principal components generally recognised in all connective tissues are (*a*) the cells, (*b*) the extracellular fibres, and (*c*) the extracellular amorphous ground substance. The fibrillar (collagen, reticular and elastic fibres) and ground-substance materials, which are present in great abundance, give connective tissues their main characteristics. The toughness and flexibility of cartilage, for example, have been attributed[12] to macromolecular aggregates of chondroitin sulphate–protein complexes linked to the fibrillar substance collagen. Similarly, tendon owes its tensile strength to the dense bundles of collagen fibres of which it is composed. Acidic mucopolysaccharides occur as one of several components of the amorphous ground substance which is interspersed between the fibres and the cells.

All substances going to and from the cells must pass through the ground substance so that changes in its state and composition exert a profound influence on the life of individual cells and tissues. It has been suggested[13] that heparin may play an important part in the breakdown and the synthesis of ground substance. Considerable interest has been shown with regard to the physiological role of heparin since it is not established that the polysaccharide's ability to act as a blood anticoagulant and as an antilipaemic agent are principal natural functions. In general, it has become increasingly apparent that connective tissues have physiological functions other than that of a supporting medium. Dorfman[14], in a recent review, has suggested that the acidic mucopolysaccharides of connective tissues have a role in a number of physiological and pathological processes including calcification, control of electrolytes and water in extracellular fluids, wound healing, lubrication, and the maintenance of the stable transport medium of the eye. A possible role of sulphated mucopolysaccharides in hair growth has also been suggested[15]. The participation

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of acidic mucopolysaccharides in a number of these roles is undoubtedly associated with their polyanionic nature, resulting from the presence of carboxyl and sulphate residues.

(c) Isolation and purification

The principal problem encountered in the isolation of pure mucopolysaccharides concerns removal of bound protein under conditions which do not significantly degrade the polysaccharide. In reviews of available methods[16,17], it has been concluded that the only procedures which are of *general* value involve digestion of the tissue with proteolytic enzymes and/or alkali. In the past, widely varying methods of extraction and purification have been used with individual mucopolysaccharides, and these are dealt with severally in the appropriate chapters. However, more recently, valuable techniques have been developed which are of general application in the isolation and purification of crude polysaccharides obtained in tissue digests and extracts. The general principles of these methods, which are based on the polyanionic character of the acidic mucopolysaccharides, are outlined below.

(i) Complex formation with quaternary ammonium salts

With detergents such as cetyltrimethylammonium bromide and cetylpyridinium chloride, the acidic mucopolysaccharides form quaternary ammonium complexes which are insoluble in aqueous solutions of low ionic strength. These complexes are soluble in strong salt solutions, but the charge density on the polysaccharide determines the salt concentration (critical salt concentration) at which a significant change in solubility occurs. As a result of their different critical salt concentrations, mucopolysaccharides may be fractionated into groups based on charge density, and therefore on the degree of sulphation[17] (Table 2).

Selective precipitation of sulphated mucopolysaccharides is enhanced at pH values which are sufficiently low to suppress ionisation of carboxyl groups[18]. Precipitation of neutral polysaccharides (for example keratosulphate) may be achieved[19] in the presence of borate at an alkaline pH because of the formation of negatively-charged sugar–borate complexes. It should be noted that the precipitation efficiency of an ammonium salt is governed by the number of —CH_2— groups in the longest

TABLE 2

GROUP FRACTIONATION OF ACIDIC MUCOPOLYSACCHARIDES AS QUATERNARY AMMONIUM SALTS[17]

	Ionised anionic groups per sugar residue	*Ratio of sulphate to carboxyl groups*
Group I. Hyaluronic acid, chondroitin	0.5	0
Group II. Chondroitin sulphates, heparitin sulphate	1.0	1.0
Group III. Heparin	1.5–2.0	2.0–3.0

alkyl chain. Commercial detergents are rarely homogeneous, and improved separations have been reported using pure materials.

The use of aliphatic ammonium salts in extraction and purification of acidic polysaccharides has been reviewed at length by Scott[17].

(ii) Ion exchange

Although use of Dowex-1 [Cl^-] permitted[2] separation of chondroitin from chondroitin sulphate, a similar fractionation of heparin on conventional anionic resins was apparently accompanied by structural changes in the mucopolysaccharide[21]. However, satisfactory separations of hyaluronic acid, chondroitin sulphate, and heparin have been achieved using Ecteola cellulose* as anion exchanger[22]. A clear-cut separation of hyaluronic acid, heparitin sulphate, chondroitin sulphate, and heparin has recently been reported[23] using DEAE–Sephadex**. This material is claimed to have a high capacity as an ion exchanger but to show only low non-specific adsorption. The gel-filtration action of Sephadex presumably assists the ion-exchange effect in this type of fractionation.

(iii) Preparative electrophoresis

Separation of hyaluronic acid and chondroitin sulphate B has been reported using slab electrophoresis[24]. Column electrophoresis may also be employed.

It is essential that structural investigations of mucopolysaccharides should be performed on preparations which have undergone rigorous proof of homogeneity. Failure to do this in the past has led to description of non-existent entities such as hyaluronic acid sulphate and mucoitin sulphate. Further, with a mucopolysaccharide such as heparin, the degree of sulphation should be carefully ascertained since this is vitally concerned in subsequent structural interpretations. Methods for proof of homogeneity include analytical ultracentrifugation, electrophoretic analysis (moving-boundary, paper, cellulose acetate), paper chromatography, and immunological techniques. Evidence of homogeneity from two such tests would strongly indicate the presence of a single molecular species.

Although essentially complete removal of protein is required before structural investigation of a mucopolysaccharide is commenced, isolation of polysaccharide–protein complexes is likely to be of increasing importance from the biological and medical viewpoint. This requires use of less drastic conditions and, amongst various methods reported, chromatography on DEAE–Sephadex promises to greatly facilitate the isolation of such complexes from unrelated protein[25].

(d) Structural methods

(i) Determination of constituents

The mucopolysaccharides considered in the ensuing pages are, in general, built of

* Ecteola cellulose is a modified cellulose of uncertain composition obtained by condensation of cellulose, epichlorohydrin, and triethanolamine.

** DEAE–Sephadex (Pharmacia) is obtained by the introduction of diethylaminoethyl groups into Sephadex which itself is a cross-linked dextran.

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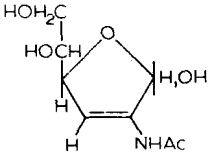
alternate units of hexosamine (usually *N*-acetylated) and hexuronic acid; in certain of the mucopolysaccharides these residues carry sulphate ester groups. Methods that give a quantitative measure of the amounts of these components are therefore of particular value in the analysis of mucopolysaccharides.

*Hexosamine**. The analytical procedures most frequently used for determination of hexosamines released by acidic hydrolysis of mucopolysaccharides are those based on the Elson–Morgan[27] and Morgan–Elson[28] methods for free hexosamines and *N*-acylhexosamines, respectively. The Elson–Morgan reaction for the determination of 2-amino-2-deoxy-D-glucose and 2-amino-2-deoxy-D-galactose involves treatment with alkaline 2,4-pentanedione (acetylacetone) followed by reaction of the resulting chromogens with *N*,*N*-dimethyl-*p*-aminobenzaldehyde (Ehrlich's reagent) in acid solution to produce a red colour which is measured spectrophotometrically. Although several chromogens are formed in this procedure, 2-methylpyrrole is responsible for about two-thirds of the colour developed[29]. The Elson–Morgan reaction has been widely investigated[30], and has been modified to increase its sensitivity, specificity, and reproducibility; the absorption spectra produced vary according to the conditions of reaction. In the procedure developed by Svennerholm[31], a red colour with an absorption maximum at 530 mμ is produced, and the colour is stable for at least one hour at room temperature. Extinction coefficients for 2-amino-2-deoxy-D-glucose and 2-amino-2-deoxy-D-galactose do not differ significantly in this procedure. Non-nitrogenous sugars and amino acids do not interfere in the Elson–Morgan estimation *singly*, but high values are often obtained when compounds of both kinds are present[32,33]; some of the interference can be eliminated by dichromatic readings at 450 mμ and 530 mμ. In the presence of borate buffer[34], the colours given by the two hexosamines are reduced to 75% and 50%, respectively, and this permits a differential determination. Another procedure which differentiates between 2-amino-2-deoxy-D-glucose and 2-amino-2-deoxy-D-galactose is recommended by Brogan[35] and is based on a modification of the Elson–Morgan reaction by Schloss[36]. Since one of the four chromogens formed in this procedure with 2-amino-2-deoxy-D-glucose (λ_{max} 512 mμ) is not formed in the reaction with 2-amino-2-deoxy-D-galactose (λ_{max} 535 mμ), the two hexosamines show significant differences in absorption maxima.

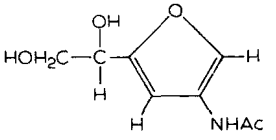
The Morgan–Elson reaction[28] has been used extensively for estimation and detection of 2-acetamido-2-deoxyhexoses. In this procedure, a solution of the sugar, which has been heated for a short time under alkaline conditions, is treated with Ehrlich's reagent in acid solution and the resultant purple colour measured. In contrast to its behaviour in the Elson–Morgan reaction, 2-acetamido-2-deoxy-D-galactose produces only 23% of the colour given by 2-acetamido-2-deoxy-D-glucose[37]. In the original Morgan–Elson procedure, 2-acetamido-2-deoxy-D-glucose was treated with hot, dilute sodium carbonate solution. During this treatment, three chromogens are formed, one of which has been identified[38] as 3-acetamido-5-(1,2-dihydroxyethyl)-furan (II). The other chromogens were thought to be anhydro-derivatives of 2-acetamido-2-deoxy-D-glucose (for example, I) which gave II on further dehydration. The chromogens are also formed on heating the

* An extensive review of the amino sugars has recently been published[26].

sugar in high-boiling solvents, and alkali seems merely to accelerate the reaction. Anhydro-sugar (I) and 3-acetamido-5-(1,2-dihydroxyethyl)-furan (II) have recently been shown[39] to react directly with an acid *N*,*N*-dimethyl-*p*-aminobenzaldehyde reagent to give, at similar rates, similar amounts of colour with absorption maxima



(I)



(II)

at 545 mμ and 584 mμ. It appears that under the acidic conditions of the reagent, anhydro-sugar (I) is converted almost quantitatively to furan derivative (II). Treatment with borate instead of carbonate has been advocated[40] to increase the sensitivity and the reproducibility of the method. Quantitative studies[39] indicate that the amounts of 2-acetamido-2-deoxy-D-glucose converted to anhydro-sugar (I) by the carbonate and borate treatments were 20% and 50%, respectively.

Hexuronic acid. The amount of hexuronic acid in mucopolysaccharides may be determined quantitatively by spectrophotometric measurement of the coloured complex formed upon the addition of alcoholic carbazole after heating the mucopolysaccharide with strong sulphuric acid[41]. The orcinol method [42] has also been modified[43] to give satisfactory results. A rapid method for the analysis of small quantities of materials containing uronic acid is based on determination of the carbon dioxide liberated during treatment with hot 12%-hydrochloric acid[44]. Comparison of the values obtained by these methods may also serve to indicate the nature of the uronic acid. L-Iduronic acid, for example, gives a significantly low value in the carbazole reaction and this led to recognition of its presence in chondroitin sulphate B. The carbazole procedure is known[45] to give abnormally high values for D-glucuronic acid in heparin, but the reason for this is obscure; heparin also gives abnormal values in the orcinol and anthrone reactions (see p. 106).

Sulphate. A number of sensitive analytical procedures have been developed for determination of the sulphur content of sulphated polysaccharides. Release of sulphate ions under oxidative conditions of hydrolysis with nitric acid, followed by determination of barium sulphate with ethylenediaminetetraacetic acid, gives results of ±1% accuracy with *ca.* 50-mg samples[46]. In a recent procedure[47], which appears to be of general application, the sample (*ca.* 5 mg) is digested with nitric acid and hydrogen peroxide, followed by determination of sulphate with 4′-chlorobiphenyl-4-yl-amine using a spectrophotometric method[48]. Inorganic sulphate liberated by hydrolysis with hot, dilute hydrochloric acid may be determined spectrophotometrically after addition of dilute barium chloride solution and potassium dichromate reagent[49]. The benzidine[50] and chloranilate[51] procedures have been applied[52] to determine the release of sulphate following enzymatic treatment of certain sulphated mucopolysaccharides. Both methods have been scaled-down and modified to give reproducible results[52].

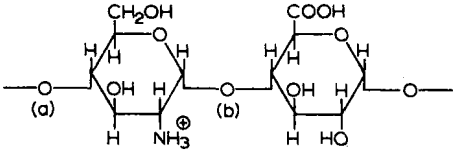
Determination of the degree of sulphation of heparin is particularly important, since apparently conflicting results derived from oxidation of the mucopolysaccharide with periodate may be attributed, in part, to different degrees of sulphation of the materials used.

(ii) Acidic hydrolysis

Total acidic hydrolysis of a mucopolysaccharide yields a mixture of the constituent monosaccharides, although extensive decarboxylation of uronic acid residues usually results. This has caused difficulty in the characterisation of uronic acid residues, but, recently, the use of new reagents (potassium borohydride, diborane) has permitted reduction of carboxyl groups (after esterification) in the mucopolysaccharide, and identification of the uronic acid as the parent hexose following acidic hydrolysis. Application of paper chromatography and paper electrophoresis is useful as a rapid analytical tool to precede isolation of larger quantities of the sugars which can then be identified unequivocally as crystalline derivatives.

Chromatographic techniques have also facilitated the isolation of larger fragments released by controlled, acidic (or enzymatic) hydrolysis of mucopolysaccharides. The numerous methods that are available for determining the structures of oligosaccharides have been reviewed recently[53], and attention here is confined to brief mention of those methods which have been applied to oligosaccharides released from mucopolysaccharides. With oligosaccharides, information is required on such structural features as (*a*) the identity and sequence of constituent monosaccharides; (*b*) position and sequence of glycosidic linkages; (*c*) configuration of the glycosidic linkages; and (*d*) size of the monosaccharide rings. Direct comparison on paper chromatograms and paper electrophoretograms of an unknown oligosaccharide with an authentic specimen will often suffice for its preliminary characterisation. Identification, however, should be considered as tentative until a comparison of the properties (melting point, infrared spectrum, optical rotation) of crystalline derivatives has been made.

The pattern of controlled, acidic hydrolysis of some mucopolysaccharides (*e.g.* hyaluronic acid) is governed by the presence of alternate residues of uronic acid, the carboxyl function of which shields the glycuronidic linkage from attack by



(III)

hydrions. The major product of hydrolysis is, therefore, usually the constituent aldobiouronic acid (see pp. 50, 66). With mucopolysaccharides such as heparin, which contain sulphoamino residues, acidic hydrolysis involves rapid cleavage of *N*-sulphate groups followed by slower release of *O*-sulphate groups. Subsequent hydrolysis of the de-*N*-sulphated heparin is essentially controlled by the positive charges acquired by the free amino groups in acid solution. These charges shield