SELECTIVE CHEMICAL MODIFICATION OF POLYSACCHARIDES
YALPANI, MANSUR
ProQuest Dissertations and Theses; 1980; ProQuest Dissertations & Theses Global

0-315-03937-X

National Library of Canada | Bibliothèque nationale du Canada

Canadian Theses Division | Division des thèses canadiennes

Ottawa, Canada
K1A 0N4

51831

PERMISSION TO MICROFILM — AUTORISATION DE MICROFILMER

• Please print or type — Écrire en lettres moulées ou dactylographier

Full Name of Author — Nom complet de l'auteur

Manssur Yalpani

Date of Birth — Date de naissance	Country of Birth — Lieu de naissance
131/5/1950	Iran

Permanent Address — Résidence fixe

#507-2527 Melfa RD., Vancouver, B.C.

Title of Thesis — Titre de la thèse

SELECTIVE CHEMICAL MODIFICATION OF POLYSACCHARIDES

University — Université

University of British Columbia

Degree for which thesis was presented — Grade pour lequel cette thèse fut présentée

Ph.D.

Year this degree conferred — Année d'obtention de ce grade	Name of Supervisor — Nom du directeur de thèse
1980	Dr.L.D.Hall

Permission is hereby granted to the NATIONAL LIBRARY OF CANADA to microfilm this thesis and to lend or sell copies of the film.

The author reserves other publication rights, and neither the thesis nor extensive extracts from it may be printed or otherwise reproduced without the author's written permission.

L'autorisation est, par la présente, accordée à la BIBLIOTHÈQUE NATIONALE DU CANADA de microfilmer cette thèse et de prêter ou de vendre des exemplaires du film.

L'auteur se réserve les autres droits de publication; ni la thèse ni de longs extraits de celle-ci ne doivent être imprimés ou autrement reproduits sans l'autorisation écrite de l'auteur.

Date	Signature
2/9/1980	M. Yalpani

NL-91 (4/77)

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

National Library of Canada
Collections Development Branch

Canadian Theses on
Microfiche Service

Bibliothèque nationale du Canada
Direction du développement des collections

Service des thèses canadiennes
sur microfiche

NOTICE

The quality of this microfiche is heavily dependent upon the quality of the original thesis submitted for microfilming. Every effort has been made to ensure the highest quality of reproduction possible.

If pages are missing, contact the university which granted the degree.

Some pages may have indistinct print especially if the original pages were typed with a poor typewriter ribbon or if the university sent us a poor photocopy.

Previously copyrighted materials (journal articles, published tests, etc.) are not filmed.

Reproduction in full or in part of this film is governed by the Canadian Copyright Act, R.S.C. 1970, c. C-30. Please read the authorization forms which accompany this thesis.

THIS DISSERTATION
HAS BEEN MICROFILMED
EXACTLY AS RECEIVED

AVIS

La qualité de cette microfiche dépend grandement de la qualité de la thèse soumise au microfilmage. Nous avons tout fait pour assurer une qualité supérieure de reproduction.

S'il manque des pages, veuillez communiquer avec l'université qui a conféré le grade.

La qualité d'impression de certaines pages peut laisser à désirer, surtout si les pages originales ont été dactylographiées à l'aide d'un ruban usé ou si l'université nous a fait parvenir une photocopie de mauvaise qualité.

Les documents qui font déjà l'objet d'un droit d'auteur (articles de revue, examens publiés, etc.) ne sont pas microfilmés.

La reproduction, même partielle, de ce microfilm est soumise à la Loi canadienne sur le droit d'auteur, SRC 1970, c. C-30. Veuillez prendre connaissance des formules d'autorisation qui accompagnent cette thèse.

LA THÈSE A ÉTÉ
MICROFILMÉE TELLE QUE
NOUS L'AVONS REÇUE

Ottawa, Canada
K1A 0N4

NL-339 (Rev. 8/80)

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

SELECTIVE CHEMICAL MODIFICATION OF POLYSACCHARIDES

by

MANSUR YALPANI

B.Sc., Simon Fraser University, 1975

A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF

THE REQUIREMENTS FOR THE DEGREE OF

DOCTOR OF PHILOSOPHY

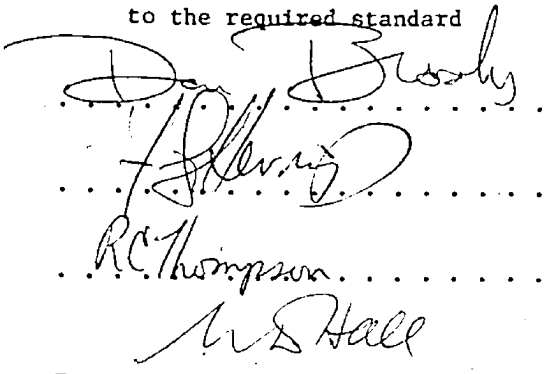
in

THE FACULTY OF GRADUATE STUDIES

(Department of Chemistry)

We accept this thesis as conforming

to the required standard



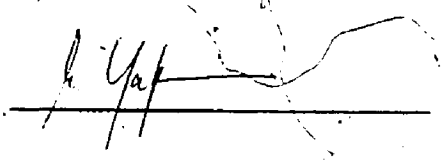
THE UNIVERSITY OF BRITISH COLUMBIA

August 1980

©Mansur Yalpani, 1980

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

In presenting this thesis in partial fulfilment of the requirements for an advanced degree at the University of British Columbia, I agree that the Library shall make it freely available for reference and study. I further agree that permission for extensive copying of this thesis for scholarly purposes may be granted by the Head of my Department or by his representatives. It is understood that copying or publication of this thesis for financial gain shall not be allowed without my written permission.



Department of Chemistry

The University of British Columbia
2075 Wesbrook Place
Vancouver, Canada
V6T 1W5

Date 2/9/1980

DE-6

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

ABSTRACT

Various synthetic procedures were designed for selectively transforming carbohydrate polymers into versatile products with a wide range of applications using as substrates several abundant and industrially important polysaccharides (alginate, cellulose, chitin, chitosan, guaran, locust bean gum, xanthan gum). Thus, alkyl amide, amine, ester, and hydrazine derivatives of alginic acid as well as similar derivatives of cellulose and xanthan gum were prepared. Selective modification of chitosan afforded N-aryl derivatives which were found to be highly efficient metal-chelating agents. Attachment of carbohydrate moieties to the amino groups of chitosan using reductive alkylation yielded a new class of branched, comb-like, 1-deoxyglucit-1-yl derivatives which exhibited a great diversity of useful properties in terms of solubility, gel-formation, compatibility, and interaction with other polysaccharides. The synthetic principles of this procedure were found to be amenable for adaption to other polysaccharide systems and branching types, as exemplified by a guaran derivative with chain-extended trisaccharide branches. Reductive alkylation of chitosan using ferrocenylaldehyde and sodium cyanoborohydride produced a new type of organometallic polysaccharide derivative. Combined enzymic and chemical modifications were found to be high-yielding for guaran and locust bean gum: specific oxidations using galactose oxidase afforded versatile C6' aldehyde intermediates which were reductively aminated to produce a wide variety of useful derivatives including

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

synthetic glycoproteins and glycopeptide analogues.

Various spectroscopic and other instrumental techniques were employed for the characterization of the polysaccharide derivatives and some native polymers in terms of their primary structure, three dimensional shape, and surface structure, as well as the molecular mobility of the hydrated polymer chains. Thus, nitroxide spin-labelling was utilized to monitor the course of many of the modification reactions, and provided evidence for a heterogeneous galactosyl-branch distribution for guaran and locust bean gum which was in agreement with a recently proposed structural model for these polymers. Esr experiments suggested heterogeneities in surface structure for chitosan and cellulose. The structural elucidation of several branched-chain chitosan derivatives and of guaran and locust bean gum was accomplished using ^{13}C-nmr spectroscopy. Electron microscopy (SEM) of xerogels derived from branched-chain chitosan derivatives revealed a wide variety of ultrastructures ranging from smooth, non-porous to microporous.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

TABLE OF CONTENTS

ABSTRACT ii

LIST OF TABLES viii

LIST OF FIGURES ix

ACKNOWLEDGEMENT xi

INTRODUCTION 1

Chapter

I BACKGROUND 7

I-A. Polysaccharide Structure, Origin and Use . . . 7
1. Alginic Acid 7
2. Xanthan Gum 8
3. Cellulose 10
4. Galactomannans (Guar gum and Locust Bean gum) 13
5. Chitin/Chitosan 16

I-B. Solution Properties of Polysaccharides 19
1. Solubility 19
2. Viscosity 22
3. Gels 26
4. Applications 27

I-C. Polysaccharide Modification 29

I-D. Analytical Methods 30
1. Magnetic Resonance 31
(i) Nmr 31
(ii) Esr 31
2. Elemental Microanalysis 47

II CHEMICAL MODIFICATION OF ALGINIC ACID, XANTHAN GUM, AND CELLULOSE 54

II-A. Background 54
1. Introduction 54
2. Covalent Linkages 54
(i) Criteria used in previous studies . . 55
(ii) Criteria used in this study 57
3. Selection Criteria 58

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

II-B. Chemical Modifications 63
1. Alginic Acid 63
(i) Alkyl ether 64
(ii) Alkyl ester 66
(iii) Amides 68
(iv) Amines 71
(v) Hydrazines 75
(vi) s-Triazine derivatives 76
2. Xanthan Gum 77
(i) Amides 78
3. Cellulose 81
(i) Amines 81
(ii) Urethanes 86
(iii) Hydrazine 88
II-C. Summary 89

III BRANCHED-CHAIN CHITOSAN DERIVATIVES 94

III-A. Introduction 94
III-B. Synthesis and Properties of Branched-chain Chitosan Derivatives 95
1. Synthesis 95
2. Solute Interactions of Chitosan 99
3. Gelling Processes 101
(i) Solute-mediated gelation 101
(ii) Gelation of branched-chain derivatives 102
4. Other Properties 102
III-C. ^{13}C nmr 104
III-D. Spin-Labelling of Chitin and Chitosan 112
1. Introduction 112
2. Spin-Labelling of Chitin and Chitosan . . 113
3. Esr Spectra 116
4. Selective Broadening Experiments 122
III-E. Scanning Electron Microscopy 128
1. Introduction 128
2. SEM of Chitosan 130
3. SEM of Branched-Chain Chitosan Derivatives 130

IV COMBINED ENZYMIC AND CHEMICAL MODIFICATIONS 140

IV-A. Introduction 140
IV-B. Galactomannans 141
1. Modification with Galactose Oxidase . . . 141
(i) Guar gum 141
(ii) Locust bean gum 143
(iii) Esr 144
(iv) Applications 150
(v) Nmr 152
(vi) Viscosity 157
(vii) Compatibility 160
2. Periodate Oxidation 161

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

V MODIFICATION OF THE METAL-CHELATING CAPACITY OF CHITIN AND CHITOSAN 167

V-A. Introduction 167
V-B. Modification of Chelating Capacity and Solubility 168
1. Enhancement of Chelating Performance . . . 168
(i) Synthesis and copper complexes . . 168
(ii) Esr of copper complexes 174
2. Reduction of Chelating Capacity 179
3. Solubility Modification 180
V-C. Organometallic Chitosan Derivative 182

VI SUMMARY 186

VII EXPERIMENTAL 194

VII-A. General Methods 194
1. Electron Spin Resonance 194
2. Nuclear Magnetic Resonance 195
3. Synthetic Methods 196
4. Materials 196
5. Product Nomenclature 197
VII-B. Chapter II 198
1. Materials 198
2. Synthesis 198
(i) Carbodiimide coupling 199
(ii) Via propylene glycol esters 199
VII-C. Chapter III 205
1. Scanning Electron Microscopy (SEM) 205
2. Materials 206
3. Preparation of 1-deoxyglycit-1-yl Chitosan Derivatives 206
(i) General procedure 206
(ii) Reactions of chitosan with reducing sugars 206
(iii) Attempted chitosan derivatives . . 210
(iv) Reactions of chitosan with non-reducing sugars 210
(v) [N-cyclohexane] chitosan [16] . . . 211
4. Oxidations with Galactose Oxidase 211
5. Interaction of [5] with Other Polysaccharides 212
6. Spin Labelling of Chitosan and Chitin . . 212
VII-D. Chapter IV 215
1. Viscosity Measurements 215
2. Oxidation Procedures 215
3. Reductive Amination--General Procedure . . 217
4. Borodeuteride Reduction of C6-Aldehyde Derivatives 219

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

VII-E. Chapter V 220
1. Materials 220
2. Synthesis 220
3. Copper Complexation Reactions 224

APPENDIX 226

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

LIST OF TABLES

Table		
I-1.	Viscosities of 1.0% Gum Solutions at 25°C	23
I-2.	Gelling Mechanism of Polysaccharides	27
I-3.	Relationship Between Certain Properties and Applications	28
III-1.	Reactions of Chitosan with Carbohydrates and Other Compounds	97
III-2.	Chemical Shift Assignment of Chitosan and Some Derivatives	105
III-3.	τ_c Values for some Spin-labelled Chitin and Chitosan Derivatives	120
III-4.	Ultrastructure Characteristics	136
IV-1.	Compatibility of some Guaran Derivatives	161
V-1.	Copper Chelation Performance of some Chitin and Chitosan Derivatives	170

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

LIST OF FIGURES

Chapter I

Figure

I-1. Structure of alginic acid 8
I-2. Structure of xanthan gum 9
I-3. Structure of cellulose 11
I-4. Proposed structures for the cellulose microfibril . . 12
I-5. Composition of guaran and locust bean gum 14
I-6. Structural models proposed for guaran and locust bean gum 15
I-7. Structure of chitin 16
I-8. Proposed structures of (a) α-chitin, (b) β-chitin . . 18
I-9. Representation of the space occupied by the gyration of polysaccharides 21
I-10. Rheological phenotypes 24
I-11. Rheogram of pseudoplastic fluid 25
I-12. Rheograms of polysaccharides 25
I-13. Generalized scheme for gelling of polysaccharides . . 26
I-14. Energy level diagram for a nitroxide 33
I-15. Directional dependence of Zeeman and hyperfine interactions 34
I-16. Esr spectra of magnetically dilute di-t-butyl nitroxide showing g- and A-anisotropies 36
I-17. Esr spectra of nitroxide spin-label in aqueous glycerol solutions 37
I-18. Simulated spectra for isotropically tumbling nitroxides 41
I-19. Power spectrum showing the heights d_1 and d and the splitting 2A 42
I-20. Spin state energy level diagram for a copper (II) nucleus 46
I-21. Scheme of copper (II) esr spectrum 46

Chapter II

II-1. Esr spectra of [10] 65
II-2. Esr spectra of [12] 67
II-3. Esr spectra of [16A] 69
II-4. Esr spectra of [16B] 70
II-5. Esr spectra of [18A] 72
II-6. ^{13}C nmr spectrum of p-fluoroaniline alginate derivative 74
II-7. Esr spectrum of [21] 76
II-8. Esr spectrum of [24] 77
II-9. Esr spectrum of [26] 79

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

II-10. Esr spectrum of [27] 80
II-11. Esr spectra of [31] 82
II-12. Esr spectra of [34], [35] 85
II-13. Esr spectra of [37] 87
II-14. Esr spectrum of [40] 89

Chapter III
III-1. ^{13}C-nmr spectra of [1] 106
III-2. ^{13}C-nmr spectrum of [5] 107
III-3. ^{13}C-nmr spectra of [7] and [6] 109
III-4. ^{13}C-nmr spectrum of [16] 110
III-5. Esr spectrum of [25] 117
III-6. Esr spectra of [31] 118
III-7. Esr spectra of [34], [35] 119
III-8. Esr spectra of [33]; [35] in the presence of Ni (II) 123
III-9. Plot of centre-field linewidth of [35] as a function of added nickel sulfate 124
III-10. Esr spectra of [31], [34] in the presence of Ni (II) 126
III-11. Esr spectra of [29], [30] in the presence of Ni (II) 127
III-12. Plot of increase of centre-field linewidth of [31], [35], [33] as a function of added nickel sulfate 127A
III-13. SEM of [1], [2], [3] 131
III-14. SEM of [6], [7], [10] 132

Chapter IV
IV-1. Esr spectra of [3] and [7] 145
IV-2. Proposed structures for guaran and locust bean gum 146
IV-3. Esr spectra of [3] 148
IV-4. Derivatives obtained from aldehyde intermediates [2] and [6] 151
IV-5. ^{13}C-nmr spectra of [1], [5] 153
IV-6. ^{13}C-spectral region of C1, C4, C5, C6 for [1] . . . 155
IV-7. Apparent viscosities of guaran derivatives as a function of shear stress 158
IV-8. Logarithmic plot of apparent viscosities of guaran derivatives versus shear stress 159
IV-9. Rheogram of a 0.3% food-grade guar gum solution . . 160
IV-10. Esr spectra of aqueous solutions of [21], [22] at 298K 162
IV-11. Esr spectra of [21] and [22] at 77K 162

Chapter V
V-1. Esr spectra of Cu(II) complexes of [3], [4], [5] [10], [11], [12], [13] at ambient temperature . . 175
V-2. Esr spectra of Cu(II) complexes of [3], [4], [5], [10], [11], [12] at 77K 176
V-3. Esr spectra of Cu(II) complexes of [1] and [7] . . . 177
V-4. Esr spectra of Cu(II) complex of [5] after various reaction times 178

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

ACKNOWLEDGEMENT

I would like to express my deep appreciation to Dr. L. D. Hall for his interest and constant encouragement throughout the course of this work. It is a pleasure to thank all members and visitors of this lab, particularly Liane Evelyn, Mike Adam, Jeremy Sanders, Subramanian Sukumar, David Verrinder, John Waterton, and Kim Wong, for numerous helpful discussions and inspiration. I am also grateful to Mr. V. Bourne and Drs. D. Brooks, C. Culling, P. Reid, P. Salisbury, L. Srivastava, and I. Whyte for the generous loan of equipment, advice, and assistance, as well as to Dr. S. Ganapathy for obtaining solid state spectra of some derivatives prepared here.

My special thanks are due to my wife, Talieh, without whose support this project could not have been completed, and to my brother, Nasser, whose efforts provided special 'insight' to some aspects of this work.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

INTRODUCTION

Carbohydrates are increasingly being recognized for their immense value in numerous industries ranging from foods,[1] pharmaceuticals,[2] textiles,[3] tertiary oil recovery[4] to the treatment of environmental pollutants.[5] They include the most abundant organic molecules on earth[6] (cellulose and chitin being produced at ca. 10^{11} tonnes/year) and play a vital role in the plant world,[7] where cellulose is the principal structural component and starch the main energy store,[8] in crustacea and insects, where chitin provides skeletal support,[9] in many bacteria, where exocellular polysaccharides constitute a protective mechanism against accidental dehydration,[10] through to many facets of human life where they are components of antibodies, enzymes, nucleic acids, and cell surface glycoconjugates;[11] they are of interest as a renewable source of carbon,[12] for energy storage,[13] and as readily manipulable natural source of chirality.[14]

Considerable attention has been directed[15-17] in recent years towards the development of an understanding of the properties of carbohydrate polymers in aqueous solution where they can naturally occur in a variety of sequences, as free entities as well as in different types of associations, and in a corresponding multitude of conformational states. The overall structural complexity of this situation is frequently added to by structural inhomogeneities of the polymers both in terms of distributions in molecular weight or degree of polymerization (poly-

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

molecularity), or small differences in the sequence or substitution pattern (polydispersity).

Although these factors have contributed to the difficulty of establishing the primary structure and three-dimensional shape of many polysaccharide systems in general, new technology, in the form of chemical and physical methodology, is rapidly being developed which is providing insight into the various hydrated and condensed states of many carbohydrates. Nevertheless, the predictive understanding of the important relationship between their structure and function is still in the state of infancy;[15] its extent can be summarized in the distinction of various conformational families of polysaccharides which are associated with certain sequence families, each of which may produce intrinsic physical properties.[15,16] Clearly, new techniques for determining primary and secondary structure and for studying their function are required.

Another challenge facing the polysaccharide chemist derives from the growing demands for the synthesis of controlled-solubility polymers for special purposes such as blood plasma expanders,[18] drug releasing matrices,[19] or collecting agents for precious or industrially important metals.[5,9] With their rich abundance, low cost, and general versatility, polysaccharides constitute ideal candidates for such applications. At the same time, the limitations of many of the procedures traditionally used[7,8] for the derivatization of polysaccharides are increasingly becoming obvious. Although the specific derivatization of polysaccharide functionalities, such as for example carboxylates,[20] is by no means a novelty nowadays, the prevailing trend in polysaccharide modifications could, until recently, be appropriately termed as "statistical chemistry";

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

the polysaccharides, often activated under strongly alkaline conditions, were subjected to treatment with unselective reagents, affording products whose exact composition could usually be determined only with great difficulty, if at all. Xanthates and carboxyalkyl derivatives[21] typify such products. The intractability of many of the native polymers, such as cellulose and chitin, and the limited range of functional groups available for derivatization (mostly hydroxyl groups, some carboxylate, amine, or sulfate functions) has, of course, greatly contributed to this situtation.

The resolution of the above problems associated with polysaccharide systems is envisaged by the author to require a two-fold strategy incorporating, on the one hand, the design of specific and high-yielding reactions which, simultaneously, are of sufficient versatility to offer the opportunity for systematic modification of polysaccharide structure, and, on the other, the combination of various spectroscopic and analytical techniques with which the induced structural changes can be probed at both the macro- and molecular-levels.

The work to be described in this thesis deals with various aspects of polysaccharide modification using several different naturally-occurring polysaccharides. Before proceeding to the discussion of the results of this investigation, its scope and the organization in this thesis need to be defined.

It was the intention of this study to develop various procedures whereby polysaccharides can be transformed from intractable materials which, for the purposes of derivatization, offer few functionalities with only slightly different reactivity, into versatile polymers with a wide range of applications; it was consequently deliberately decided at

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

the onset to seek a general overview of the subject area in terms of the strategy proposed above, in preference to the detailed and comprehensive pursuit of any one particular of its aspects.

The investigations to be described followed the course outlined below: initially (Chapter II), a series of chemical reactions were evaluated as to their utility for derivatizing several representative types of polysaccharides (alginate, cellulose, xanthan gum). The reductive amination reaction emerged as the most versatile procedure and it was subsequently (Chapter III) applied to chitosan, which, with its prominent amine function, offered an ideal substrate for specific and efficient modifications affording a new class of branched-chain derivatives. Next (Chapter IV), the same reaction was combined with an enzymic modification procedure to prepare, again specifically and in high yield, various galactomannan derivatives. Lastly (Chapter V), an effort was made to direct the experience gained towards a specific application area, exemplified here by the synthesis of metal-chelating derivatives of chitosan. An intercomparison of the various results is given in the short summary (Chapter VI) preceding the Experimental data (Chapter VII). Chapter I provides some background information about the polysaccharide substrates and some of the instrumental techniques used. As will be seen, these techniques have played an important role in helping with the development of the basic chemistry.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

References

1. I. W. Cottrell and K. S. Kang, Development in Industrial Microbiology, 19, 117 (1978); R. K. Robinson and P. Khan, Plant Foods Man, 2, 113 (1978).

2. H. H. Baer and J. L. Strominger, The Amino Sugars, Academic Press, New York, 1A, 1969; L. B. Jaques, Science, 206, 528 (1979).

3. R. L. Whistler, Industrial Gums, Academic Press, New York, (1973); J. D. Reid and R. M. Reinhardt, in Modified Cellulosics, R. M. Rowell and R. A. Young, eds., Academic Press, New York, 11 (1978).

4. J. Mulderink, Erzmetall., 29, 560 (1976); C. T. Githens and J. W. Burnham, Soc. Petr. Eng. J., 17, 5 (1977); B. Sandiford, Energy Comm., 4, 53 (1978).

5. B. Tabushi, Y. Kobuke, and T. Nishiya, Nature, 280, 665 (1979); M. Takahashi, K. Shinoda, T. Mori, and T. Kikyo, Japan Pat., 7803982 (1978), Chem. Abstr., 89, 64708 (1978).

6. R. M. Rowell and R. A. Young (eds.), Modified Cellulosics, Academic Press, New York, (1978); D. A. Rees, Polysaccharide Shapes, Chapman and Hall, London, (1977).

7. R. L. Whistler, ed., Methods in Carbohydrate Chemistry, III, (1963).

8. R. L. Whistler, ed., ibid., IV (1963).

9. R. A. A. Muzzarelli, Chitin, Pergamon Press, New York, (1977).

10. S. A. Barker and P. J. Somers, in The Carbohydrates, IIB, W. Pigman and D. Horton, eds., Academic Press, New York, 573 (1970).

11. M. I. Horowitz and W. Pigman, eds., The Glycoconjugates, Academic Press, New York, (1977); R. C. Hughes, Membrane Glycoproteins, Butterworths, London, (1976); E. G. Brunngraber, Neurochemistry of Aminosugars, C. C. Thomas, Springfield, (1979); N. Sharon, Complex Carbohydrates, Addison-Wesley, Reading, (1975).

12. T. A. Hsu, M. R. Ladish, and G. T. Tsau, Chem. Techn., 315 (1980); K. Selby, in Industrial Aspects of Biochemistry, L. Spencer, ed., 787 (1973).

13. D. J. Manners, Adv. Carbohydr. Chem., 12, 262 (1957).

14. H. Brunner, Angew. Chem. Internat. Edn., 10, 249 (1971).

15. D. A. Rees, Polysaccharide Shapes, Chapman and Hall, London, (1977).

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

16. E. M. Morris, in Techniques and Applications of Fast Reactions in Solution, W. J. Gettins and E. Wyn-Jones, eds., 379 (1979); D. A. Rees, in MTP Intern. Rev. Sci., Org. Chem. Ser. One, VII, G. O. Aspinall, ed., 251 (1973).

17. J. H. Elliott, in Extracellular Microbial Polysaccharides, ACS Symp. Ser., 45, 144 (1977).

18. P. Calvert, Nature, 280, 108 (1979).

19. H. Moldenhauer and H. J. Loh, Pharmazie, 33, 216 (1978).

20. D. Horton and T. Usui, in Carbohydrate Sulfates, R. G. Schweiger, ed., ACS. Symp. Ser., 77, 95 (1978).

21. Y. L. Meltzer, Water-soluble Resins and Polymers, Noyes Data Corp., London, (1976).

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

CHAPTER I

BACKGROUND

I-A. Polysaccharide Structure, Origin, and Use

Polysaccharides can be variously classified in terms of primary structure, overall charge (neutral or polyelectrolyte), origin, etc.[1-5] For the purposes of chemical modification it may be most appropriate to categorize the polysaccharides used in this study according to their most prominent functional group. Thus, alginic acid and xanthan gum are anionic species characterized to a large extent by their C-6 carboxylate functions, chitosan, with its 2-deoxy-2-amino group, belongs to the less commonly encountered class of cationic polysaccharides, while cellulose, guar, and locust bean gum are representative examples of the large group of polysaccharides which offer only hydroxyl groups for derivatization. The following provides a brief summary of some of the most pertinent aspects of the above polysaccharides.

1. Alginic Acid

Alginic acid has been known and utilized for almost a century, while the details of its chemical structure and properties have only recently been established. The principal sources of this watersoluble gum are various species of marine brown algae (Phaeophyceae).[6] The thickening, suspending, stabilizing, gel- and film forming properties of alginic acid have found various applications in the food, cosmetic, coating, paint, pharmaceutical, and other industries.[7]

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

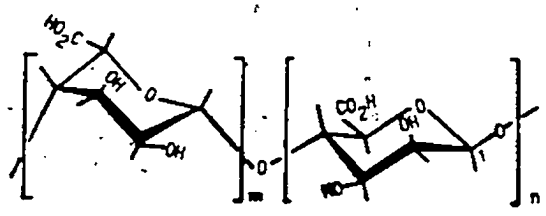


Fig. I-1. Structure of alginic acid.

Alginic acid is a (1–4) linked, linear block polymer of β-D-mannopyranosyluronic (M) and α-L-gulo-pyranosyluronic (G) acid residues, containing sequences of both types (poly M) and (poly G), alternating with regions of mixed structure (poly MG).[8] The M/G ratio varies depending on the source of the material. The molecular weight of alginic acid has been reported to be as high as 10^6, but is commonly around $2.0\text{–}2.5 \times 10^5$ with degrees of polymerization ranging between 180–930.[9] The structure is depicted in Figure I-1.

2. Xanthan Gum

Xanthan gum typifies microbial polysaccharides which, with their unique physical and chemical properties, have found numerous successful applications in food, textile, agricultural, paint, and petroleum industries in recent years.[10] Xanthan gum, produced by some 30 species of the genus Xanthomonas, is a high molecular weight ($> 10^6$) polysaccharide which contains D-glucose, D-mannose, and D-glucuronate as well as acetyl (4.7%) and pyruvate (3%) as depicted below (Fig. I-2).[11]

The secondary and tertiary xanthan structures are still largely unknown. However, evidence obtained from various instrumental techniques has revealed order-disorder transitions of the polymer in solution.[12] The native, probably double-stranded, polymer fibre can be denatured

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

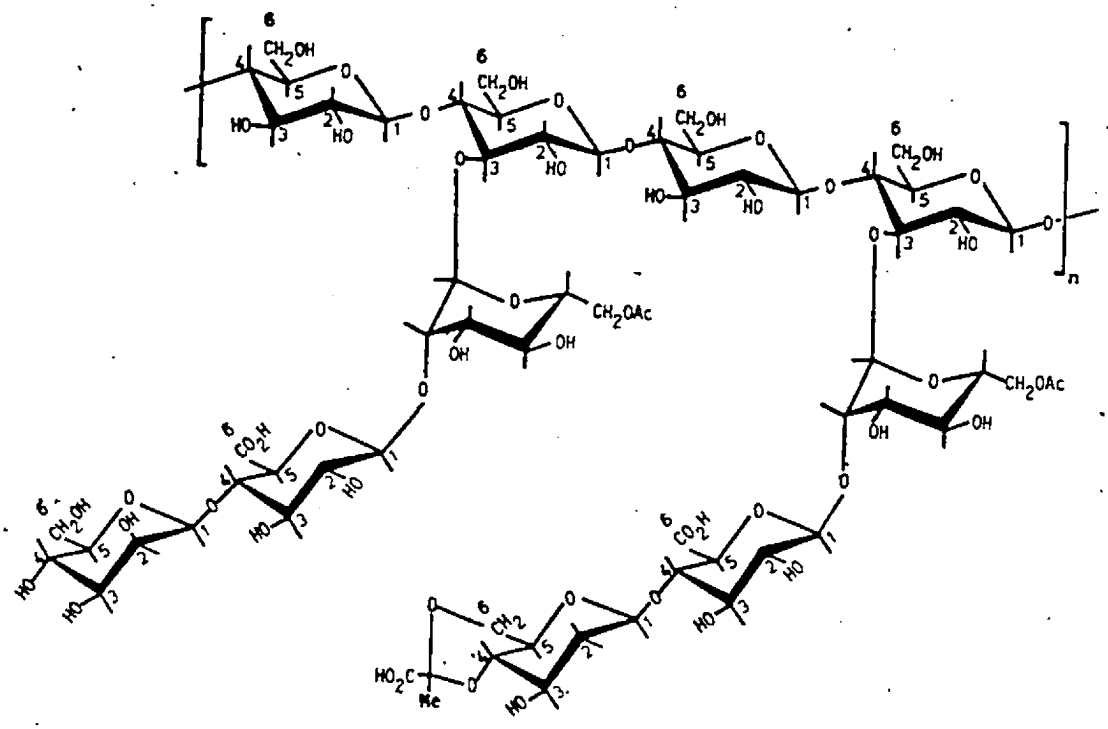
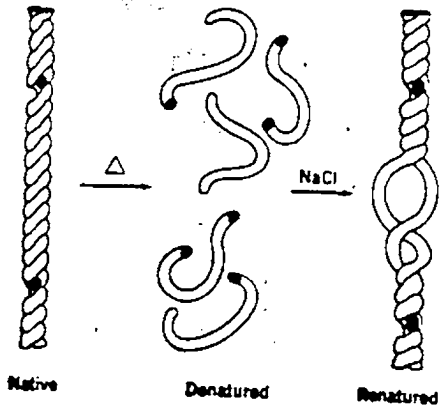


Fig. I-2. Structure of xanthan gum.

into single strands by heat treatment. The denatured xanthan strands can in turn be renatured by addition of sodium chloride as schematically shown below.[13]

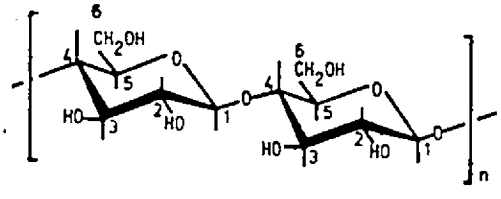


Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

One of the most significant functional properties of xanthan gum is its ability to control aqueous fluid rheology;[14] it forms homogeneous aqueous dispersions and solutions exhibiting high viscosity, as well as having characteristics of both pseudoplastic and plastic polymer systems; thus, a rapid decrease in viscosity is observed with increasing shear rate and, upon the release of shear, total viscosity recovery occurs almost instantaneously.

3. Cellulose

Ubiquitous cellulose is one of the most important natural products on earth.[15] It is produced, consumed and destroyed in nature in tremendous quantities (10^{11} tonnes/year).[1,16] Man has utilized this material for an almost unlimited number of purposes ranging from fibres, textiles, films, plastics, coatings, pharmaceuticals, cosmetics, foods, to tertiary oil recovery.[2,16] More recent interests in cellulose derive from its potential use as a renewable source of carbon.[17]



After more than 150 years of study, the question of the formation and structure of cellulose still remains to be fully resolved. Cellulose is a linear polymer of β(1-4) linked D-glucose with chains assuming a flat ribbon conformation, similar to chitin (Fig. I-3).[1,18] The ribbons, in turn, are packed into sheets. The biosynthesis of cellulose in plant cell walls produces amorphous regions and regions, constituting

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

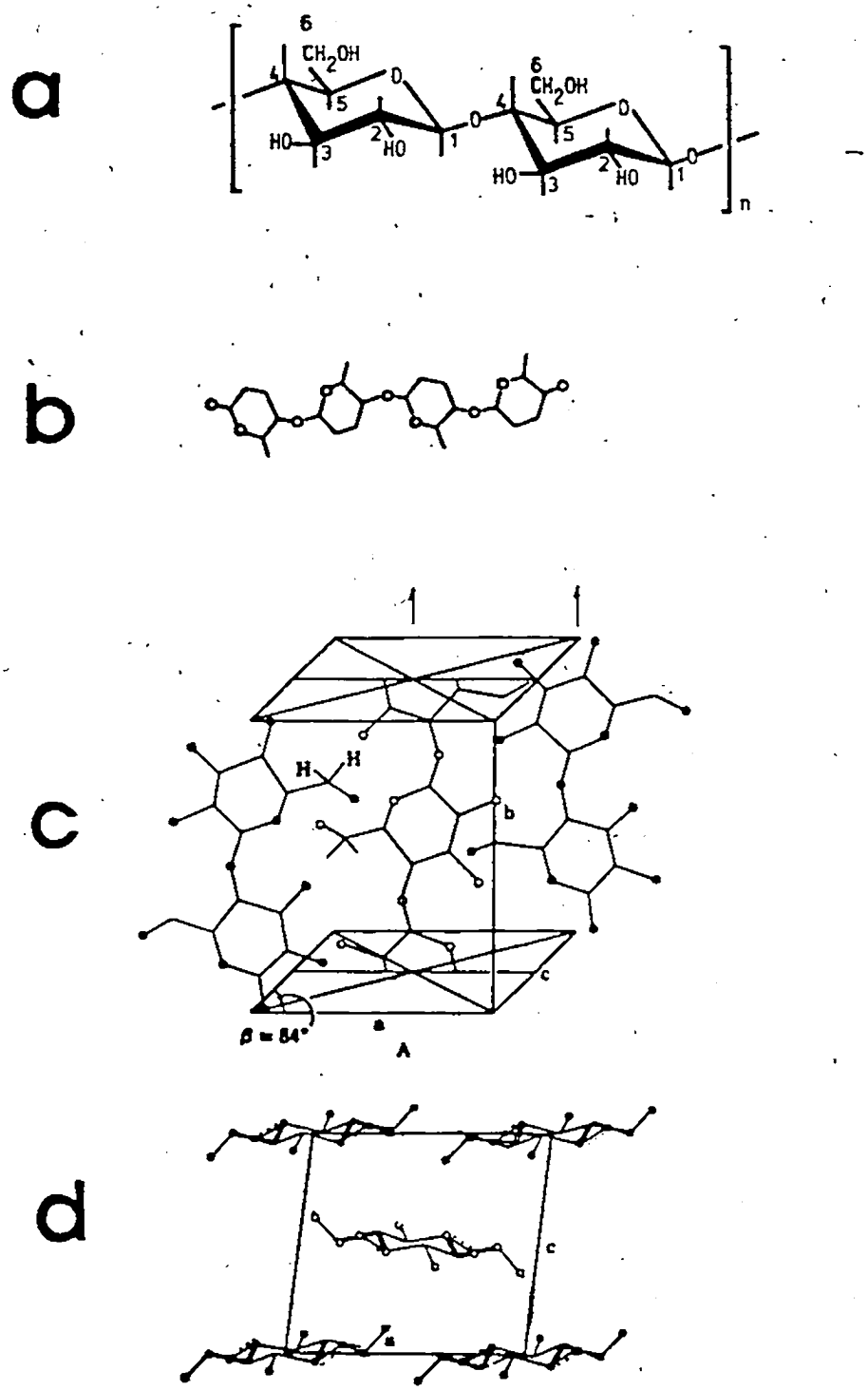


Fig. I-3. Structure of cellulose: (a)sequence; (b)chains resemble a flat ribbon; (c),(d)proposed chain packing

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

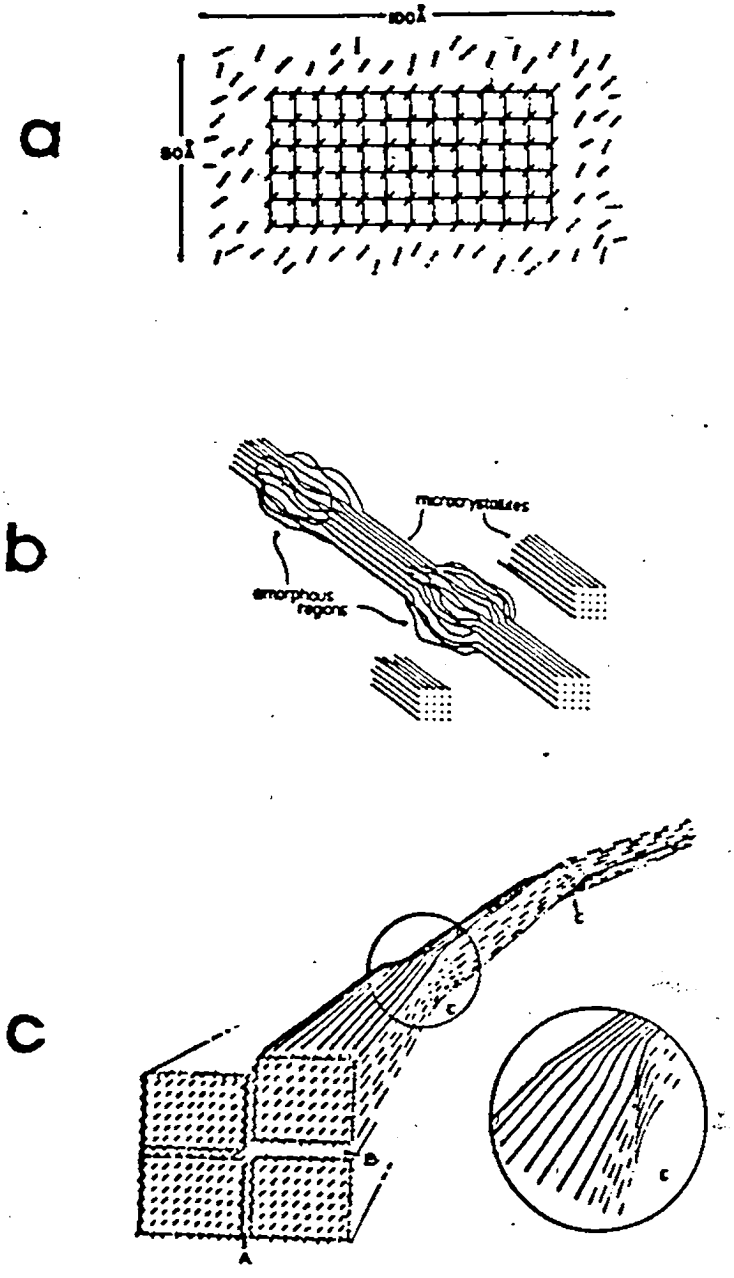


Fig. I-4. Proposed structures for the cellulose microfibril: (a) cross-section of fibril; order increases towards a central core (from ref. 18); (b) amorphous regions and microcrystallites within a single microfibril; (c) model incorporating elements of (a) and (b), three surface types are shown (A,B,C,).

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

50% or more, of high crystallinity (microcrystalline)(Fig. I-4).[19,20] The latter can be characterized by crystallography. The efficient chain packing and the numerous interchain hydrogen bonds account for the insolubility of cellulose in conventional solvents, and its stability and mechanical strength.[21]

The accessibility and reactivity of the hydroxyl groups in the different regions of cellulose have been the subject of considerable attention. The amorphous regions are known to have greater accessibility to solvent or solute penetration than the microcrystalline regions.[16,22] Such, and other findings, have led to the proposal of several models for the three dimensional structure for cellulose.[18,23] The first model envisages an ordered crystalline core surrounded by a region whose order decreases with distance from the core (Fig. I-4a), whereas in the second model, crystalline and amorphous regions alternate along the microfibrillar axis (Fig. I-4b). Figure I-4c shows a model which accommodates features of both a and b, involving three types of surfaces, viz., highly ordered ones (type a), type b which are less ordered but physically still close to crystalline regions, which are likely to contain inter-chain hydrogen bonds. Type c surfaces are much less ordered due to twist and tilt distortions of the fibril.

4. Galactomannans (Guar gum and Locust bean gum)

Galactomannans are assuming an ever increasing role in various branches of industry, notably in foods, pharmaceuticals, paper products, cosmetics, paints, drilling, and explosives.[24] Guar gum and locust bean gum are two of the more important galactomannan polysaccharides which are mainly derived from the seeds of leguminous plants or from microbial sources. Their primary physiological function appears to be the

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

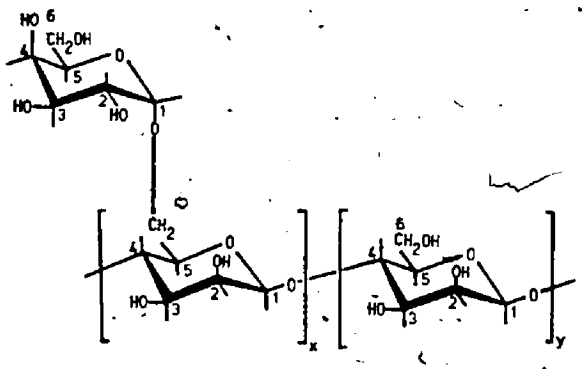


Fig. I-5. Composition of guaran (x/y = 1.8) and locust bean gum (x/y = 4.3) used in this study.

retention of water (by solvation), preventing the drying out of the seeds, and also their capacity as food reserves. In addition, galactomannans assume important roles in the inhibition of viruses and in interferon induction.[25] The synergistic interactions of these non-ionic polymers with other polysaccharides, e.g., with xanthan gum, and with themselves, has attracted considerable attention[1] and efforts directed at the elucidation of their primary structure have so far been only partially successful.

Both guar gum (MW 220,000) and locust bean gum (MW 310,000) contain a β-D (1-4) linked mannan backbone which carries α-D-galactosyl residues at the C-6 positions and assumes a ribbon-like structure. The mannose (M) to galactose (G) ratio varies from 1.8:1 for guaran to 4:1 for locust bean gum.[24] The distribution of galactose bearing mannose units (MG) was until recently, subject to some controversy. Previously it was thought that the structure of these polysaccharides was regular consisting of either a homogeneous distribution of galactose residues[26] (Fig. I-6a) or of alternating sequences of (poly M) and (poly G) blocks[27] (Fig. I-6b); however, recent evidence has suggested otherwise[28-30] as shown by the representative structures in Figure I-6c. Whereas in

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

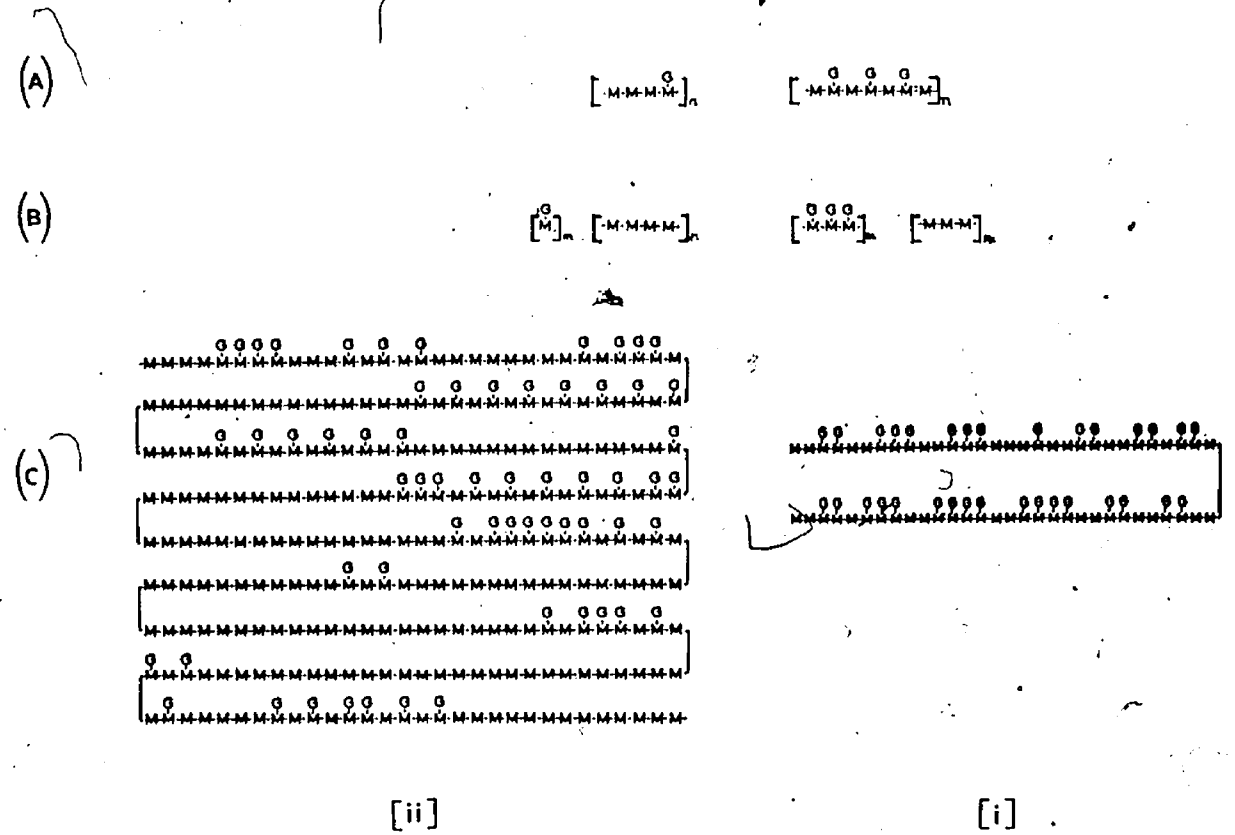


Fig. I-6. Three structural models proposed for guaran and locust bean gum: (a) regular galactosyl distribution,[26] (b) strictly alternating block sequence,[27] (c) most recent model (Painter, Grasdalen, Gonzalez[28-30]) for (i) guaran, (ii) locust bean gum.

guaran MG units occur in groups of mostly two to four, alternating with blocks of two or three contiguous M units, one finds in locust bean gum, long blocks of contiguous M units, together with long sequences of alternating M and MG units, as well as sections with two or three contiguous MG units.

The rheological properties of aqueous solutions of both gums are of particular interest for many reasons: they behave as non-Newtonian solutions which, by themselves, form no gels unless borate or transition metal ions are added.[24] The gums are stable over a wide pH range and are compatible with many salts. The viscosity and shear stability of guar and its derivatives have made these preferred gelling agents for fracturing fluids in the oil industry.[31]

5. Chitin/Chitosan

Chitin was first isolated from mushrooms in 1811[32] and from insects in 1823.[33] Chitosan was discovered in 1859.[34] The development of chitin chemistry has proceeded at an erratic pace. It took almost a century before the chemical constitution of both polysaccharides was firmly established and most of the information presently available on these polymers has been obtained since 1950. The first descriptions of chitosan as a metal-chelating agent appeared only in 1969[35] but comparatively little research has so far been carried out on these important polymers.

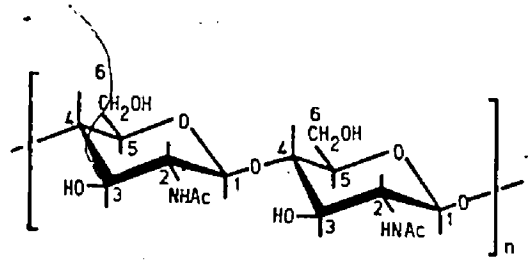


Fig. I-7. Structure of chitin.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

Chitin is very widely distributed in the plant and, to an even greater extent, animal kingdom, where its main function is the provision of structural and skeletal support.[36] Chitin is found most abundantly in fungi, while chitosan is obtained mainly by N-deacetylation of chitin, but also occurs in nature. The importance of chitin is emphasized by its natural abundance, an estimated 10^{10}–10^{11} tons annually,[1] which makes it one of the most abundant organic materials on earth.

Chitin is a linear polymer of β-D (1-4) linked 2-acetamido-2-deoxy-D-glucopyranose units, of which a proportion, typically ∿15%, is N-deacetylated (the fully acetylated polymer is called chitan).[37] The molecular weight of chitin has been estimated at 1.04×10^6, while that of chitosan ranges between $1.45\text{–}1.80 \times 10^5$.[38] Chitin, like cellulose, has a ribbon-like structure (Fig. I-8). Three crystalline forms, α, β, and γ-chitin are distinguished on the basis of different chain arrangements and the presence of bound water (Fig. I-8).[36] In contrast to most other polysaccharides, chitin and chitosan have basic characteristics (pK_a of chitosan is 6.3)[39] which impart them with unique properties in terms of solubility, viscosity, polyelectrolyte behaviour, membrane forming ability and metal chelation.

Both these aminopolysaccharides are insoluble in common organic solvents, water, dilute acids, or cold alkalis of any concentration.[36] There are only a few solvents or solvent systems which do not give rise to hydrolysis, degradation, or N-deacetylation. Chitin dissolves, for example, in 9N HCℓ, or >9N H_2SO_4 with hydrolysis of the glycosidic and amide linkages. The solvent systems for chitin which are more satisfactory include hexafluoroisopropanol, hexafluoroacetone sesquihydrate, certain chloroalcohols, and hot concentrated solutions of

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

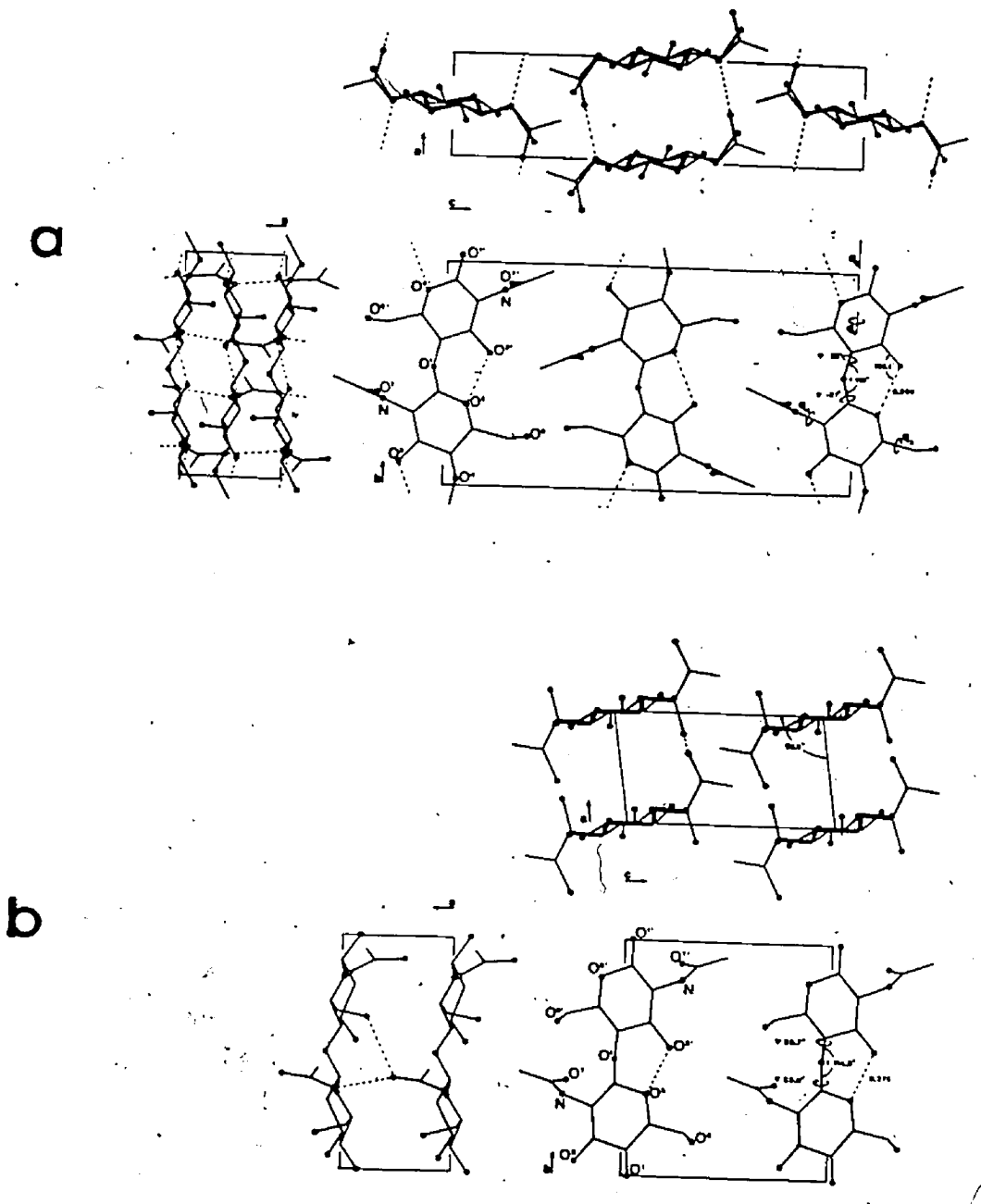


Fig. I-8. Proposed structures of (a) α-chitin, (b) β-chitin.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

neutral salts which are capable of high degrees of hydration, such as saturated lithium thiocyanate solution at 95°C.[40] Chitosan is soluble in a number of organic acids, including formic and acetic acid. Chitosan products have found a wide range of industrial applications and certain derivatives have been implicated in cancer therapy,[41] wound healing,[42] and other medicinal uses.[36]

I-B. Solution Properties of Polysaccharides

1. Solubility

The outstanding industrial importance of polysaccharides arises from their useful interactions with a wide variety of molecules ranging from food ingredients and pharmaceuticals, to inorganic particles, clay slips and oil well muds. These interactions occur mostly in aqueous media and the solubility properties of polysaccharides are therefore of great interest. Their unique solubility properties constitute, at the same time, one of the major problems in the development of polysaccharide chemistry. Although these properties are known to follow certain trends, a systematic understanding of the relation of primary polysaccharide structure to physical behaviour in aqueous solution, and of polysaccharide-solute interactions, etc., remains to be established. In contrast to other synthetic polymers, relatively little effort appears to have been carried out in this area so far, which can presumably be ascribed, to some extent, to the intractability of these materials.

The following is a brief description of some pertinent aspects of 'hydrated' polysaccharides. Their dissolution is envisaged[2,43] to proceed through a process in which, beginning in amorphous regions, i.e., where intermolecular interactions are only partially operative due to

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

the disorganized spatial arrangement of molecules or chain segments, continual hydration of the polysaccharide replaces intermolecular H-bonding. The competition of water molecules for available polymer sites leads eventually to an intermediate "gel state" in which large sections of the polysaccharide are fully solvated while some incompletely solvated areas are still associated with other polysaccharide chains. The completion of this dissolution process facilitates, under appropriate conditions, subsequent arrangement of the polysaccharides into more or less ordered structures such as gels.

Natural polysaccharides, of which some 300 are known to date,[43] can be classed into different solubility groups according to their structure and conformation. Thus, linear polysaccharides with a regular, ribbon-like structure such as cellulose and chitin, form highly ordered, often crystalline arrays which are difficult to dissolve due to strong cohesive forces, whereas branching usually leads to an enhancement in solubility and a reduction in the intermolecular interactions. It can also be envisaged[2] that for equal molecular weight polysaccharides, a branched molecule will require a smaller volume in solution for gyration than an extended linear one, as indicated in Figure I-9. Highly branched polysaccharides are almost always watersoluble. Solubility can also be affected by a series of other factors such as ionic charges, structural irregularities, glycosidic linkages which preclude ribbon structures, low molecular weight, and a wide molecular weight distribution.

The possibility of modifying polysaccharide solubility by chemical derivatization is a relatively novel concept which is gaining increasing importance for a wide variety of medicinal applications,[44] including polymer-mediated drug release.[45] It has long been known that the

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

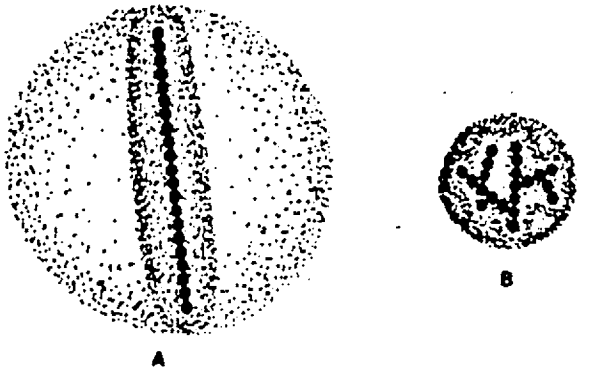


Fig. I-9. Representation of the space occupied by the gyration of (a) an extended linear polysaccharide; (b) a branched polysaccharide of equal molecular weight (from ref. 2).

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

introduction of substituents into the linear cellulose structure can afford soluble derivatives, while, conversely, the removal of branches, as in the hydrolysis of the galactose side-chains of guaran, or the introduction of hydrophobic groups or 3,6-anhydro rings, as for starch sulfates,[46] leads to a reduction of polymer solubility. Pfannemüller et al.[47] have recently converted a series of linear polysaccharides into branched derivatives, which resulted, in the case of amylose, in drastic changes in solution viscosity. However, no precedent was known for applying this concept for the purposes of solubilizing an insoluble polysaccharide. In this study (III-B) facile methods were explored for transforming an insoluble, linear polysaccharide (chitosan) into soluble, branched derivatives, which provides the opportunity for systematic studies of the effects of structure on physical properties. One aspect of such studies, the "tailoring" of the hydrophobicity/hydrophilicity characteristics of polysaccharide derivatives will be demonstrated in V-B.

2. Viscosity

An important property of polysaccharides is their ability to impart a wide range of viscosities to aqueous solutions,[2,48] as demonstrated by the representative selection listed in Table I-1; (the comparable viscosity of water is ~1 cps at room temperature!). The definition of some basic rheological terminology[51] may be useful here.

The ratio of shear stress, τ (shearing force per area sheared) to shear rate, γ (gradient of velocity over clearance or film thickness) is commonly defined as viscosity, η.* The standard units for these are

*or apparent viscosity, μ, for non-Newtonian fluids

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

Table I-1. Viscosities of 1.0% Gum Solutions at 25°C[a]

Gum	cps
Gum arabic (20% by wt.)	50
Locust bean gum	100
Methylcellulose	150
Gum tragacanth	200
Carrageenan	300
Xanthan gum[b]	1000
High-viscosity sodium carboxymethyl cellulose	1200
Gum karaya	1500
Sodium alginate	2000
Chitosan (pH 4.1)[c]	2780
Guar gum	4200

[a]adapted from ref. 2 (except for) b ref. 49; c ref. 50

dynes cm^{-2}, sec^{-1}, and poise or centipoise, respectively. Rheological fluid behaviour is classified in terms of certain phenotypes which are diagrammatically shown in Figure I-10 as idealized curves. Of the various types, only three will be of concern to us, namely (i) Newtonian systems, which exhibit a linear relation between shear stress and shear rate and which are usually confined to low molecular liquids such as water; (ii) pseudoplastic; and (iii) dilatent systems. The latter two can be described by the relation

$$\tau = \eta_N \gamma^n$$

where η_N is the zero shear viscosity; for pseudoplastic or shear-thickening systems $n < 1$, while $n > 1$ for dilatent or shear thinning systems. The most common type of non-Newtonian behaviour is pseudo-plastic flow for which three distinct regions are usually encountered

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

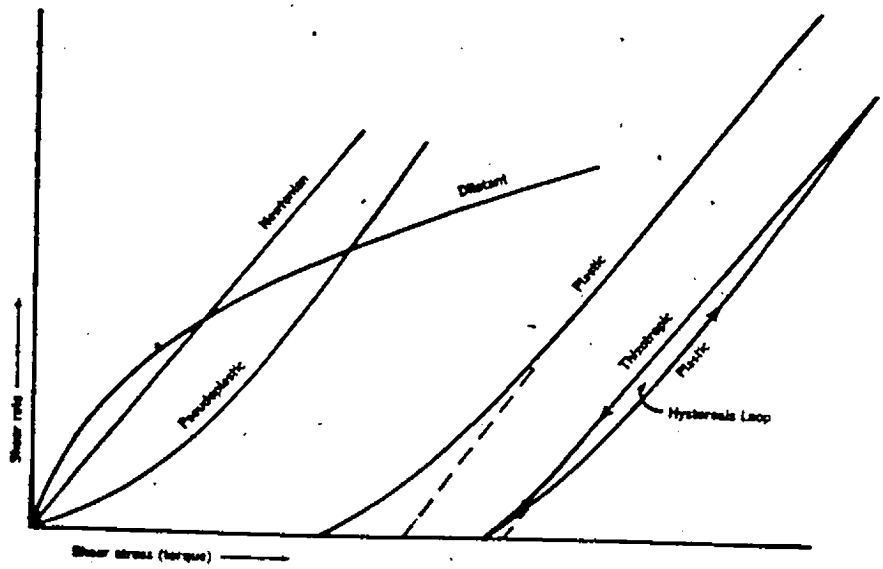


Fig. I-10. Rheological phenotypes (from ref. 49).

as illustrated in the logarithmic plot of apparent viscosity versus shear rate (Fig. I-11).[48]

Most of the industrially important polysaccharides fall into the pseudoplastic category (e.g., xanthan, alginate, guar gum, etc.) while dilatency has been observed for few (e.g., certain starch derivatives);[52] some typical rheograms are shown in Figure I-12.

Dilatency[53] can be envisaged to arise from chain entanglement of polymers which, with increasing shear rate, leads to viscosity increases, while shear-thinning can arise from shear degradation of the polymer or from a parallel alignment of the polymer chains in the flow direction resulting in a reduction of the fluid's flow resistance. The molecular origin of these processes, particularly those giving rise to dilatent flow behaviour, is, however, only incompletely understood.

The control of rheological fluid behaviour has obviously important implications for the various applications of polysaccharides in which they provide viscosity, solution stability, suspendability, emulsifying action, gelation, etc. Likewise, the ability to modify polysaccharide

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

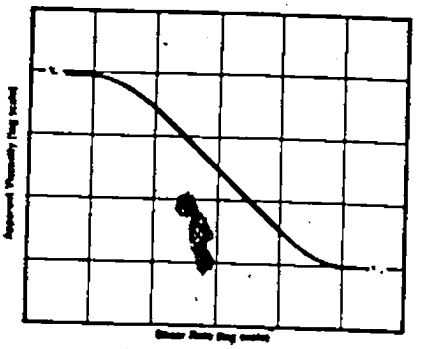


Fig. I-11. Logarithmic plot of apparent viscosity versus shear rate for a pseudoplastic fluid (from ref. 48).

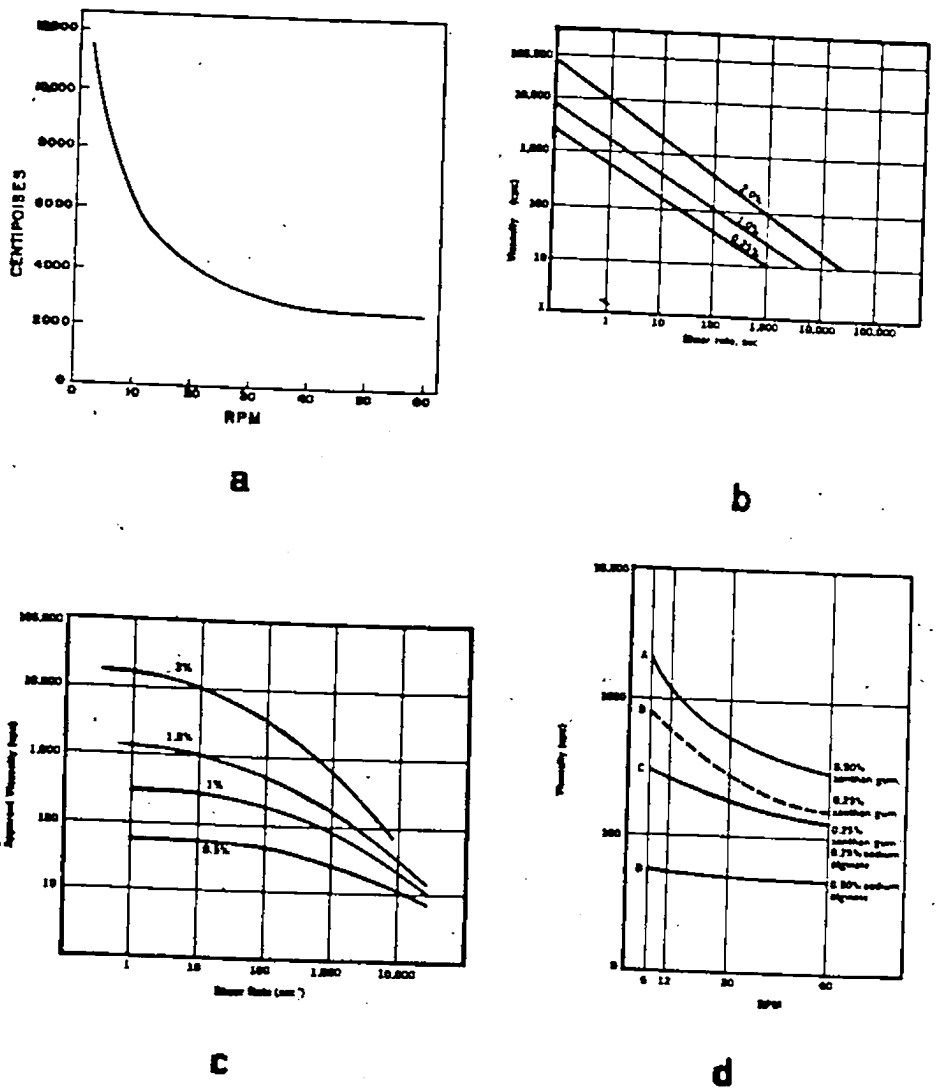


Fig. I-12. Rheograms of (a) 1% aqueous guar gum solution (from ref. 2); (b) xanthan gum (from ref. 49); (c) alginate (from ref. 54); (d) mixture of alginate and xanthan (from ref. 49).

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

rheology by chemical derivatization as for carboxymethylcellulose,[2] or by interaction with other polysaccharides as for the reduction of the pseudoplasticity of xanthan by alginate (Fig. I-11e), will constitute an increasingly important concept in the future.

3. Gels

Many polysaccharides have been shown[1] to adapt ordered gel structures by way of (i) associations into double helices as for e-carrageenan, (ii) bundles of double helices as for agarose, (iii) ribbon-ribbon aggregations as for alginate; or (iv) synergistic helix-ribbon associations as for mixed systems containing agarose and locust bean gum. Rees and coworkers[1] have summarized these different cross-linked networks of chains into a generalized scheme (Fig. I-13). The common feature of all

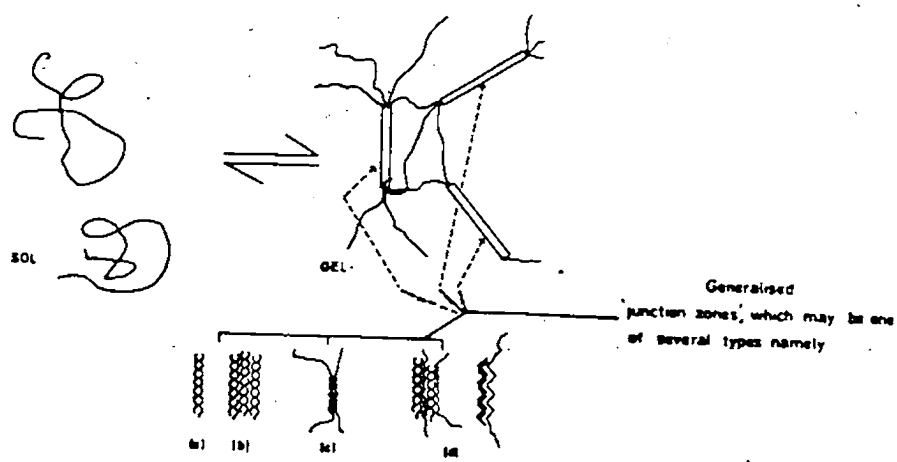


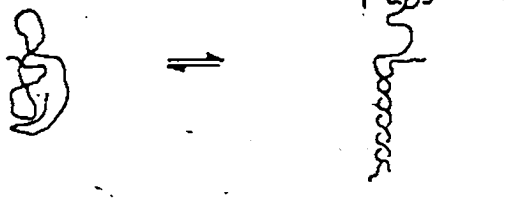
Fig. I-13. Generalized scheme for gelling of polysaccharides (from ref. 1).

these gelling mechanisms is the cooperative association of two or more chain segments which is often terminated by "interrupted sequences," i.e., by changes in the sequence of sugar units, by chain branching, or other altered substitution.

Without going into the details of the various individual gelling mechanisms, it is worthwhile to point out the series of distinguishable

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

Table II-2. Polysaccharide gelling mechanism (from ref.55).

Pictorial view of molecular states				
Name	Random coil	Double helix	Aggregate
Significance for gel structure	Predominant in the sol; can exist as connecting lengths in gel structure and impart elasticity when they do so	Provide cross-linking junctions in the gel	Add cross-linking to consolidate the gel structure, thus acting as super-junctions
Corresponding range of gel properties	Sol ⇌ Incipient gel ⇌ Clear elastic gel ⇌ Stiff gel ⇌ Turbid rigid gel ⇌ Phase separation; syneresed gel			

stages,[55] summarized in Table I-2 in the progressive association of chains which leads to gel networks, by way of, for example, helices. In the sol state, which can be characterized as a state of incomplete hydration of the polysaccharide chains due to aggregation phenomena, the chains assume the random coil conformation. The gel, obtained when sufficient helix has formed to provide crosslinks, becomes increasingly more rigid with further helix associations acting as "super-junctions." At a later stage, this process may be accompanied by a loss of optical transparency, and finally, by synereses, i.e., a contraction of the gel network concomitant with the exclusion of solvent.

4. Applications

It is apparent that the aforementioned polysaccharide properties (and numerous others which could not be discussed here) avail themselves to a variety of applications and Table I-3 (adapated from ref. 56)

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

Table I-3. Relationship Between Certain Properties and Applications

	Viscosity	Pseudoplasticity and yield point	Solubility	Compatibility	Enzyme resistance	Shear resistance	Temperature stability	Suspension stability	Film formation	Crosslinking film	Crosslinking solution	Protein reactivity	Solvent tolerance
Tertiary oil-recovery	+		+	+		+							
Oil-well drilling	+	+		+		+	+	+			+		
Paints		+	+		+			+					
Paper			+	+				+	+	+		+	
Textiles	+							+	+	+			
Explosives	+			+							+		
Photography	+		+	+				+				+	
Cosmetics	+		+					+				+	+
Toothpaste	+				+			+					
Food	+	+	+	+			+	+				+	

indicates some of the major relationships between polysaccharide properties and various end uses. Table I-3 shows that for a particular polysaccharide to be of utility to any one area of application, it has to fullfil in most cases more than a single requirement. It is with this consideration, that throughout this study potential applications of various polysaccharide derivatives are suggested on the basis of their observed characteristics.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

I-C. Polysaccharide Modification

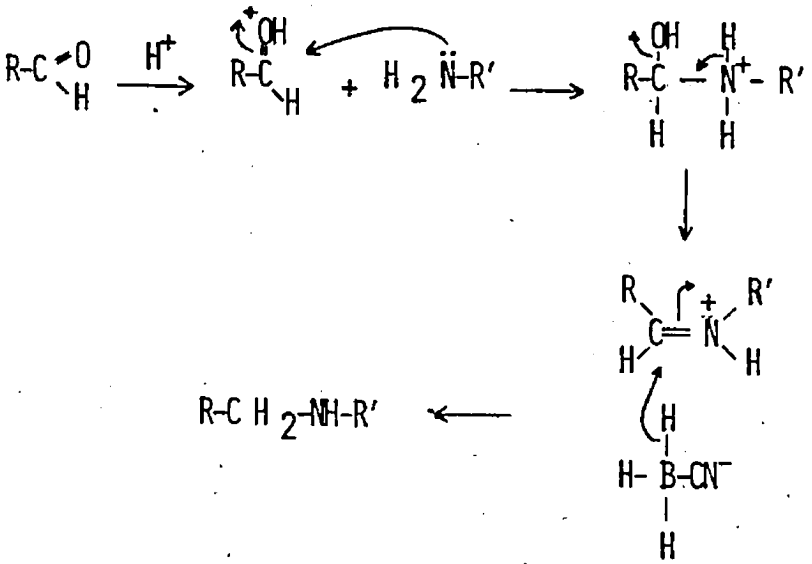
In view of the immense utility of polysaccharides in their native form, a substantial amount of work[57] has been directed at modifying them in order to obtain derivatives with altered chemical and physical properties. Such changes can frequently, particularly for neutral polysaccharides, be provoked by the introduction of only very small amounts of ionic substituents. However, in most of the derivatization procedures employed, the exact composition of the resulting products, in terms of the sequences of substituted and unsubstituted polymer functionality (e.g., hydroxyl groups), remains unknown due to the limitations of conventional analytical techniques, and one is left with the difficulty of characterizing the derivatives. A minimal description of the product can be given by the degree of substitution (d.s.), which is generally defined as the average number of hydroxyl groups derivatized per monosaccharide unit.

In view of the large numbers of, and differently positioned, hydroxyl groups which display very similar reactivities, a major difficulty encountered in polysaccharide modification arises from the fact that the reactions are often heterogeneous and do not proceed to completion. Although a few reactions employed in this study reflect some of these problems, the general conceptual approach chosen herein attempts to minimize such difficulties by exploring novel chemical and other routes, or combinations thereof, for preparing, in high yield, specifically derivatized polysaccharides.

In this context, one reaction in particular, the reductive amination reaction[58] using sodium cyanoborohydride, deserves to be mentioned for its key role in achieving this goal. This versatile reaction, whose

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

mechanism is summarized below; is operative for a range of aqueous or organic solvents and pHs. Requiring only very mild conditions, the reductive amination reaction has been successfully applied to a wide variety of carbohydrates and other biological materials.[58,59]



I-D. Analytical Methods

The difficulties associated with the structural elucidation of polysaccharide derivatives were already alluded to; further complications arise when attempts are made to characterize the polymeric materials in terms of their molecular behaviour in the various solution (sol, gel) or solid states. Fortunately, the development of various modern instrumental techniques in recent years has facilitated an immense progress in this area.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

In this section it is attempted to briefly outline salient features of the magnetic resonance techniques employed without going into the details of several other methods (e.g., Scanning Electron Microscopy and Viscometry). Fuller descriptions of both the former[60,62] and the latter[51,61] techniques are found elsewhere.

1. Magnetic Resonance

(i) Nmr

Nuclear magnetic resonance (nmr) spectroscopy, particularly ^{13}C nmr, is a powerful analytical tool which is routinely used[60] for the elucidation of the primary structure and conformation of polysaccharides in solutions, gels, and more recently, in the solid state; no elaboration of the technique is required here. However, the author would like to direct attention to the utility of nuclei other than carbon and proton[63] which have not yet found extensive application in the analysis of polysaccharide modification products. With the availability of modern instrumentation, nmr studies can be made of a practically unlimited range of magnetic nuclides which can be chemically incorporated into polysaccharides. Thus, the wide chemical shift range of ^{19}F[63] can be exploited to distinguish "products" from "unreacted reagent" as preliminary experiments on alginate derivates have shown. Unfortunately, time did not permit the author to pursue these studies to any extent.

(ii) Esr

The following is a very brief summary of some fundamental aspects of esr spectroscopy pertinent to the concepts and methods employed in this work; such treatment necessarily omits a vigorous quantitative derivatization (which the reader will find elsewhere, e.g., in references 62, 64-68).

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

(a) Nitroxide esr

Esr spectroscopy monitors the net absorption of energy by a paramagnetic molecule whose electron magnetic dipole interacts with an electromagnetic field H_0 of frequency ν. The magnetic moment of the electron, characterized as the magnetic dipole moment μ, can be simplistically considered to derive from the spinning of the electron about an axis through its centre (spin magnetic dipole) with small contributions from the electron's motion about the nucleus of an atom (orbital magnetic dipole).

Resonance occurs when transitions between the Zeeman levels, whose degeneracy may be lifted by the application of a magnetic field H_0, are induced by an electromagnetic field, i.e., when

$$h\nu = g\beta H_0 \qquad (1)$$

where h is Planck's constant, β the Bohr magneton ($e\hbar/2m$), m the electron mass, and g a dimensionless parameter related to the effective magnetic moment of the electron (μ_e)

$$\mu_e = -g\beta\underline{S} \qquad (2)$$

$\underline{S}\hbar$ being the spin angular momentum vector. Differences in the Zeeman energy between different molecules are described as changes in g from its free electron spin-only value of 2.00232, as a result of spin-orbit coupling. The g-value characterizes the position of the resonance in the frequency spectrum. Equation (1) is satisfied over a wide range of frequencies and magnetic fields; most esr experiments including those described here, are conducted at X-band, i.e., at about 9.5 GHz corresponding to an H value of $\sim$3.4 kG. The energy absorption is monitored as the first derivative of the absorption signal.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

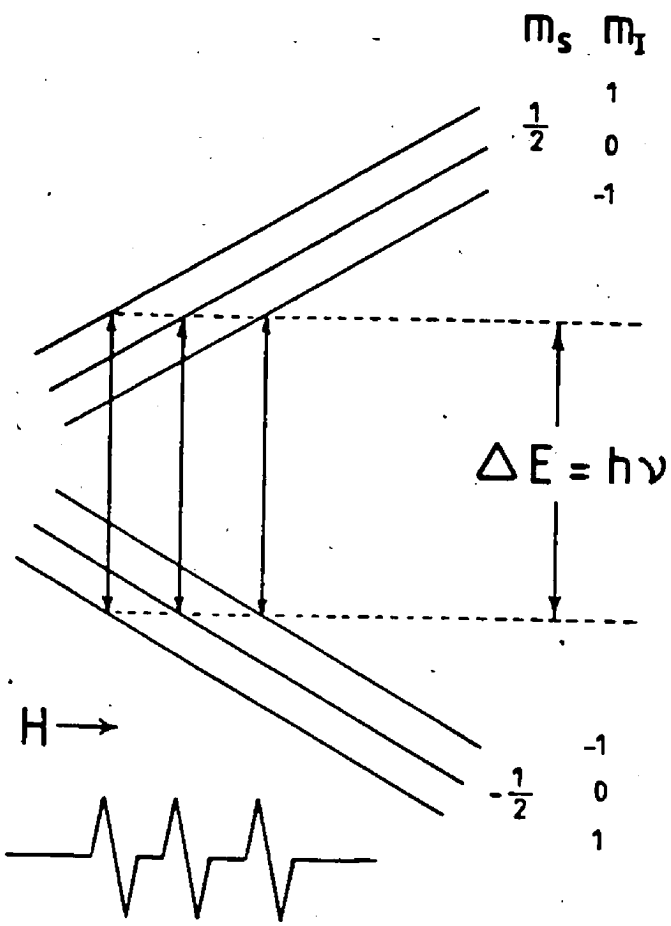


Fig. I-14. Energy level diagram for a nitroxide ($s = \frac{1}{2}$) in a magnetic field with hyperfine coupling to the spin 1 nitrogen nucleus (from ref. 67).

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

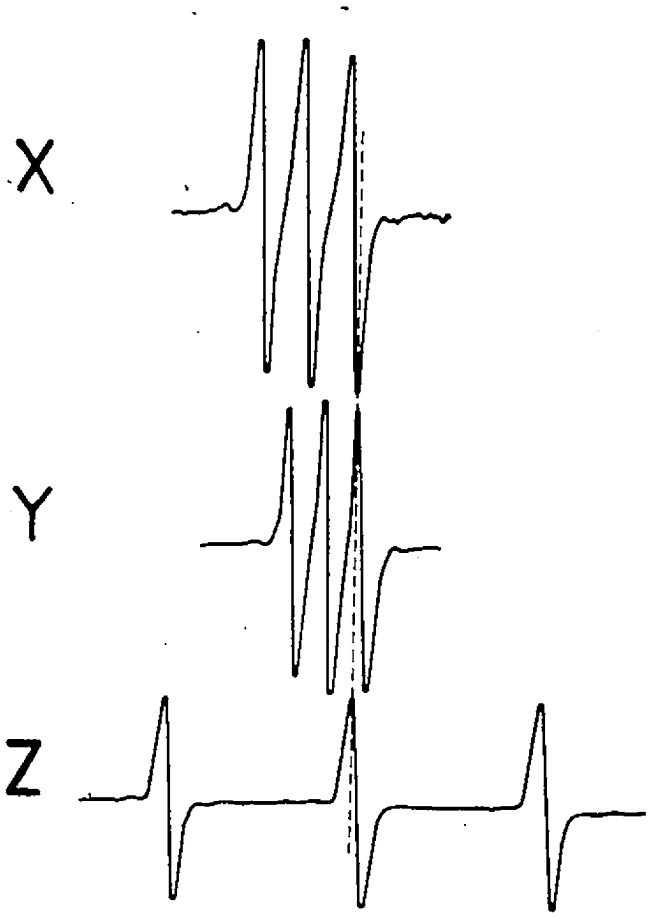
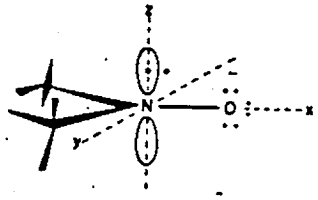


Fig. I-15. Directional dependence of Zeeman and hyperfine interactions in a nitroxide oriented in a diamagnetic host crystal, which was rotated in the molecular xz plane (from ref. 62).

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

For nitroxide free radical molecules such as[67]



the principal hyperfine interactions occurs between the electron, shown in the nitrogen $2p_z$ orbital and the nitrogen nucleus (I = 1), leading to the characteristic three-line spectrum (Fig. I-14) in which transitions obey the selection rules $\Delta m_I = 0$, $\Delta m_S = \pm 1$, (where m_S and m_I are values of the components of the electron and nuclear spin operators along the external field direction).

The Zeeman and hyperfine interactions are direction dependent as demonstrated by the esr spectrum of a diamagnetic host crystal "doped" with nitroxide (Fig. I-15). Both the position (g) and the splitting of the lines (A) are direction dependent leading to the principal values g_{xx}, g_{yy}, g_{zz}; A_{xx}, A_{yy}, A_{zz}. The axial symmetry of both tensors arising from the molecular symmetry, affords

$$\begin{aligned} g_{xx} \simeq g_{yy} \neq g_{zz} \quad &\text{or} \quad g_{xx} \simeq g_{yy} = g_{\perp} \quad \text{and} \quad g_{zz} = g_{\parallel} \\ A_{xx} \simeq A_{yy} \neq A_{zz} \quad &\text{or} \quad A_{xx} = A_{yy} = A_{\perp} \quad \text{and} \quad A_{zz} = A_{\parallel} \end{aligned} \tag{3}$$

A dilute nitroxide solution also produces a 3-line spectrum (Fig. I-16) but due to the averaging of the g and A anisotropies ($g_{\parallel}$, $g_{\perp}$ and $A_{\parallel}$, $A_{\perp}$) as a result of molecular motion only the isotropic splitting constant a_0 remains. Figure I-16 also shows the nitroxide spectrum in a polycrystalline solid (e.g., at 77 K) in which all possible nitroxide orientations contribute to the spectrum which is simply the sum of resonances indicated in Figure I-15. It is evident that while the central maximum contains contributions from all orientations, the outer

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

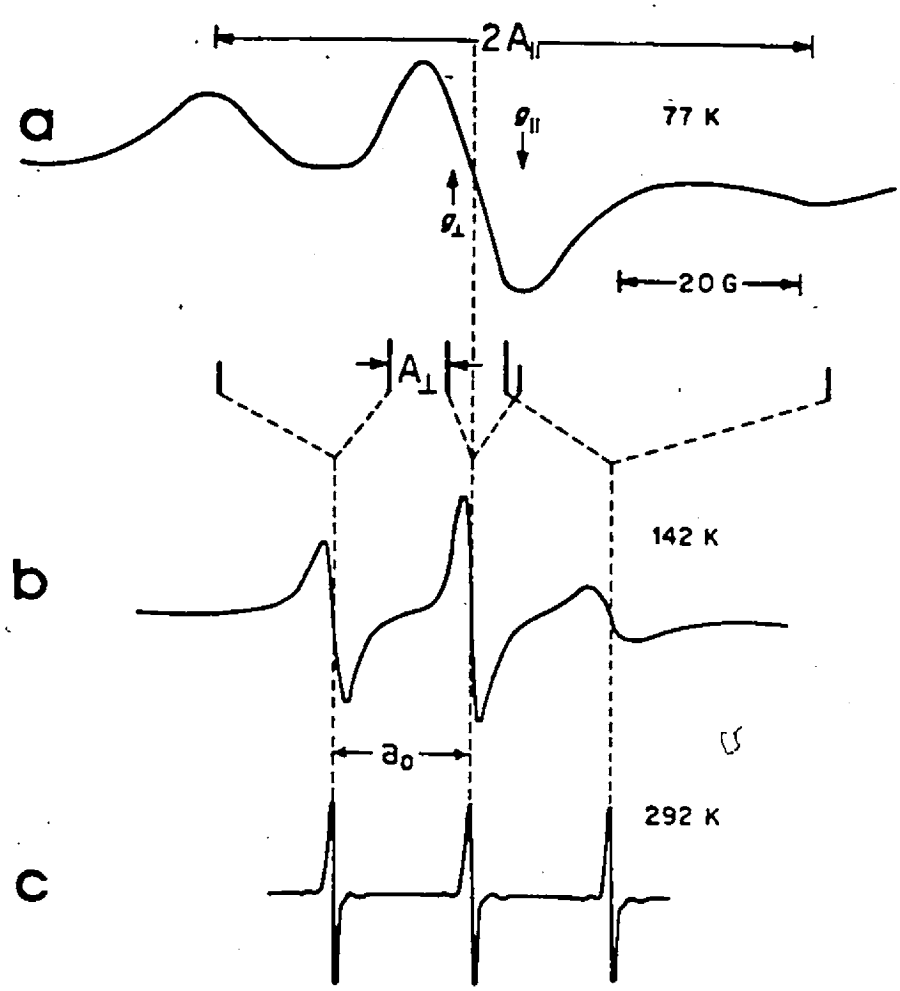


Fig. I-16. Esr spectra of magnetically dilute di-t-butyl nitroxide (a) in a polycrystalline solid, (b) a viscous solution, and (c) a non-viscous solution showing g- and A-anisotropies (from ref. 67).

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

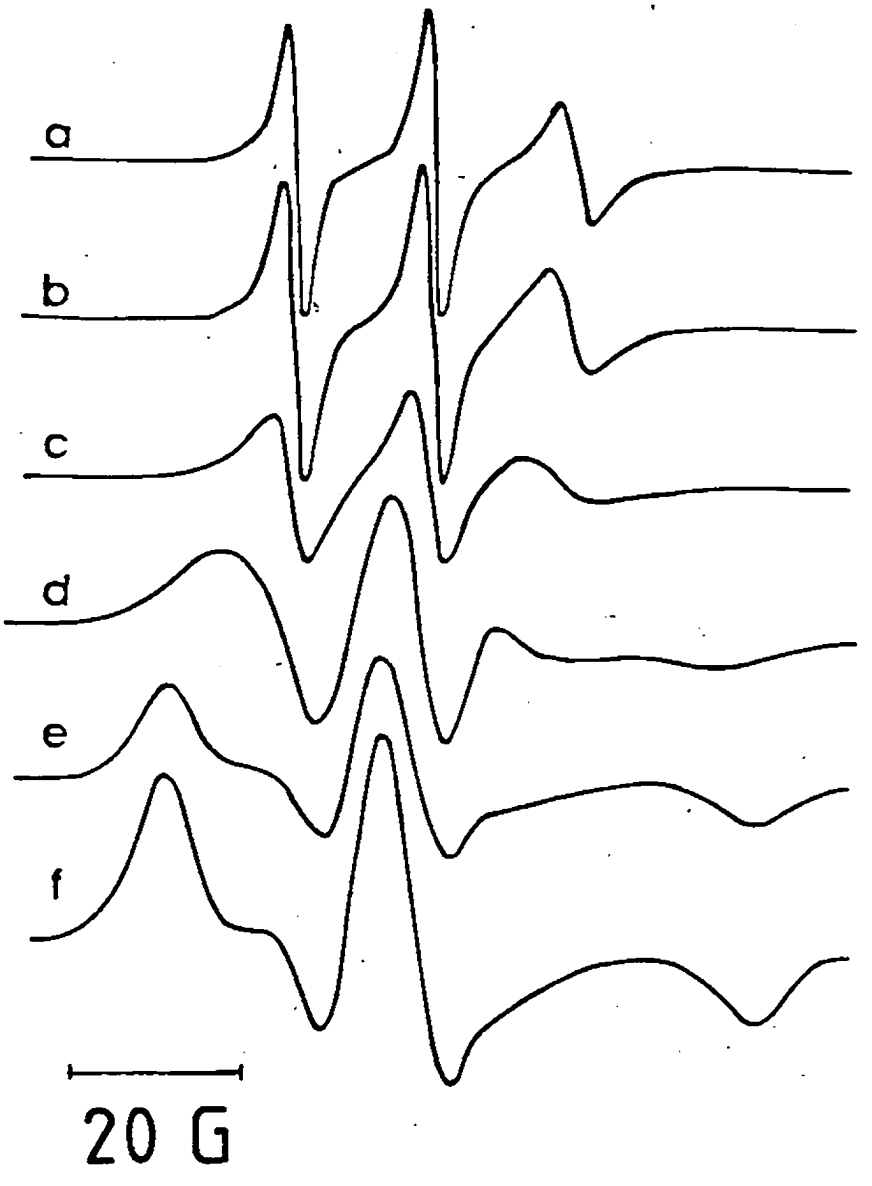


Fig. I-17. Esr spectra of nitroxide spin-label in aqueous glycerol solutions; viscosity increases and temperature decreases from (a)-(f) (from ref. 67).

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

extrema arise from radicals oriented with a molecular z-axis parallel to the external field.

(b) Rotational correlation time, τ_c

It is well known[62] that nitroxide esr lineshapes are a sensitive function of molecular motion on a timescale of 10^{-11} to 10^{-7} sec. In order to affect the lineshape, such motion must partially average A or g tensor anisotropy (Fig. I-16). Reduced molecular motion leads to broadening of the high field line followed by the low field line and lastly the central line. Figure I-17 demonstrates these effects for a system where the isotropic motion decreases and the correlation times (τ_c) increase.

The line shapes of the peaks in the spectra of isotropically tumbling molecules are assumed to be Lorentzian in which case the line width and the peak-to-peak height are a function of the transverse relaxation time T_2 given by

$$[T_2(m_I)]^{-1} = \tau_c\{[3I(I+1) + 5m_I^2]\frac{b^2}{40} + \frac{4}{5}(\Delta\gamma H_0)^2 - \frac{4}{15}b\Delta\gamma H_0 m_I\} + X \qquad (4)$$

where H_0 is the applied field strength and

$$b = \frac{4}{3}\pi(A_{zz} - A_{xx}) \text{ in } H_z \qquad (5)$$

$$\Delta\gamma = \frac{-\beta e}{h}[g_{zz} - \tfrac{1}{2}(g_{xx} + g_{yy})] \qquad (6)$$

X is a function which accounts for other contributions to the line width. This expression is only valid under the following conditions: (1) the hyperfine interaction is axially symmetric, i.e., $A_{xx} = A_{yy}$; (2) the molecular motion is isotropic and sufficiently slow that $\omega^2\tau_c^2 >> 1$ (where $\omega = \beta e H_0/h$ and $b^2\tau^2 << 1$), so that line widths are influenced; and (3) that $\tau^2 a_0^2 << 1$, which ensures that the three lines do not overlap.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

Thus at X band the equations are applicable in the range $5 \times 10^{-1} < \tau_c < 5 \times 10^{-9}$s. Usually for nitroxides in low viscosity solvents these conditions are met so that if we set I = 1 for nitrogen in (4) the following expression is obtained:

$$\frac{T_2(0)}{T_2(m_I)} = 1 - \tau_c T_2(0)\left[\frac{4}{15} b \Delta\gamma H_0 m_I - \frac{b^2}{8} m_I^2\right] \qquad (7)$$

The ratio $T_2(0)/T_2(m_I)$ can be expressed in terms of the ratio of the peak-to-peak heights by

$$\frac{T_2(0)}{T_2(m_I)} = \left(\frac{h_0}{h_{m_I}}\right)^{\frac{1}{2}} \qquad (8)$$

where h_{m_I} is the peak-to-peak height in arbitrary units. $T_2(0)$ is related to the line widths by

$$T_2(0) = \frac{1}{\pi\sqrt{3}\Delta\nu(0)} \qquad (9)$$

where $\Delta\nu(0)$ is the line width for the central peak in H_z. If we then insert (8) and (9) into (7) we obtain

$$\frac{h_0}{h_{m_I}} = 1 - \frac{\tau_c}{\pi\sqrt{3}\Delta\nu(0)}[C_1 m_I + C_2 m_I^2] \qquad (10)$$

where

$$C_1 = \frac{4}{15} b \Delta\gamma H_0 \qquad (11)$$

$$C_2 = b^2/8 \qquad (12)$$

It is now possible to solve for τ_c with $m_I = +1$ and $m_I = -1$ in terms of C_1 or C_2. Values of τ_c derived with C_1 have been found to be very dependent on the microwave power[67] so that it is best to avoid using C_1 unless the microwave dependence is known. Therefore we solve (10) for τ_c in terms of C_2

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

$$\tau_c = [(\frac{h_0}{h_1})^{\frac{1}{2}} + (\frac{h_0}{h_{-1}})^{\frac{1}{2}} - 2] \frac{\pi\sqrt{3}\Delta\nu(0)}{2\ C_2} \qquad (13)$$

$$= [(\frac{h_0}{h_1})^{\frac{1}{2}} + (\frac{h_0}{h_{-1}})^{\frac{1}{2}} - 2] \frac{9\sqrt{3}\Delta\nu(0)}{4\pi(A_{zz}-A_{xx})^2} \qquad (14)$$

Because it is easier to measure the line widths in gauss than in hertz we convert $\Delta\nu(0)$ to $\Delta H(0)$ and $A_{zz} - A_{xx}$ from hertz to gauss by Eq. (1) with g taken as the averaged g value. In gauss, Eq. (14) is:

$$\tau_c = [(\frac{h_0}{h_1})^{\frac{1}{2}} + (\frac{h_0}{h_{-1}})^{\frac{1}{2}} - 2] \frac{9\sqrt{3}h\ \Delta H(0)}{4\pi g\beta_e(A_{zz}-A_{xx})^2} \qquad (16)$$

For anisotropic reorientations of nitroxides, a situation more likely to prevail for a labelled macromolecule, some additional factors have to be noted. Many different and highly complex lineshapes can arise from anisotropic reorientation, and the best to quantitate the motion is by spectral simulation. Programmes are available for axially symmetric reorientation in which two correlation times, $\tau_{\parallel}$ and $\tau_{\perp}$ are derived.[69] In practice, however, at least where motional anistropy is small and motions are rapid ($\tau \sim \tau_{\parallel} \sim \tau_{\perp} < 10^{-9}$S), τ has often been calculated using equation (16) or using simulation methods which assume isotropic reorientation.[62] Some workers have even completely dispensed with τ using other parameters as empirical indices of mobility.[70]

Figure I-18 illustrates lineshapes simulated[71] on the assumption of isotropic tumbling with τ values of 2.0 ns (a), 3.2 ns (b), and 5.0 ns (c). Thus, the appearance of two spectral 'components' at high field (as in (c)) is not, as might be initially thought, <u>necessarily</u> associated with the existence of two distinct correlation times or with the occurrence of motional anisotropy. In the present work, the exact

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

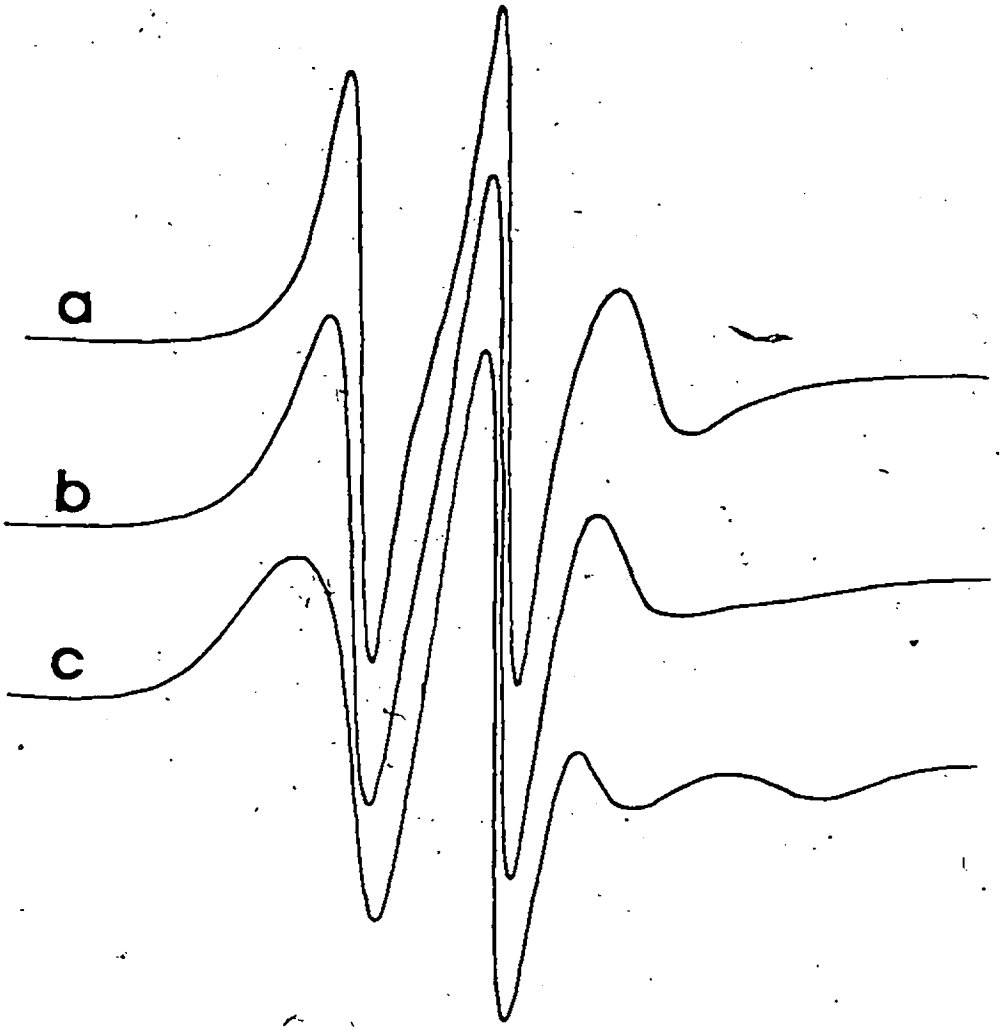


Fig. I-18. Simulated spectra for isotropically tumbling nitroxides with τ_c (a) 2 ns; (b) 3.2 ns; (c) 5 ns (from ref. 71).

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

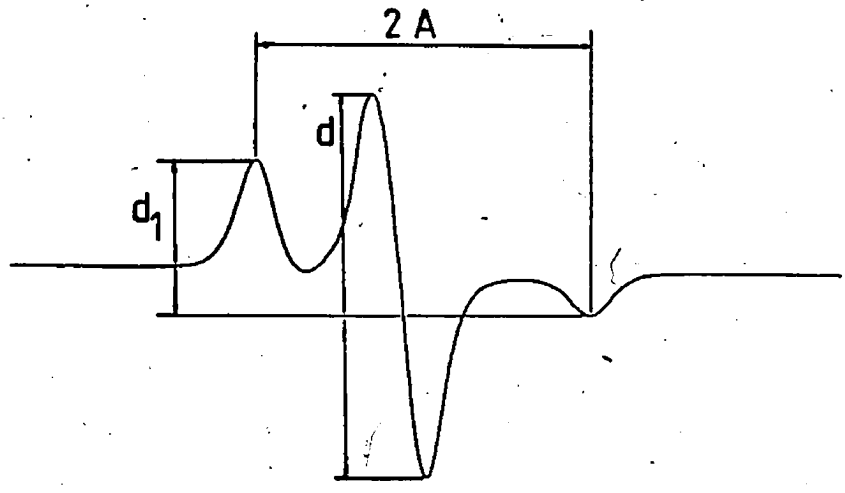


Fig. I-19. Powder spectrum showing the heights d_1 and d and the splitting 2A.

quantitative evaluation of tumbling motions was, in general, considered less important than a qualitative comparison of motional rates of different polysaccharides or of a given system in varying physical states, and, hence, computer simulations were not employed for τ_c determinations.

(c) Distance measurements

The esr spin-labelling technique provides, in principal, a number of independent methods for determining intermolecular distances.[65,71] The method used here is based on dipolar linebroadening. If the relative reorientation of two spin-half radicals is sufficiently slow, the esr signal of each will show a dipolar coupling with the other, whose magnitude is proportional to $(3\cos^2\theta - 1)r^{-3}$ where r is the electron-electron distance and θ is the angle between the distance vector and the magnetic field. In a magnetically dilute sample of radicals neither whose orientations nor whose positions are correlated, the esr signal should suffer a Laurentzian broadening ΔH_{dip} which is proportional to $\bar{r}^{-3}$, where $\bar{r}$ is the mean nearest-neighbour distance. For a random, three dimen-

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

sional spin distribution, $\bar{r}$ can be related to the spin label density, ρ (nm^{-3}), by:

$$\bar{r} = (\frac{4}{3}\pi\rho)^{-1/3}\Gamma(\frac{4}{3}) \qquad (17)$$

with $\Gamma(n) = \int_0^\infty e^{-x}x^{n-1}dx$, $\Gamma(\frac{4}{3}) = 0.89261$, and thus

$$\bar{r} = 0.55373\rho^{-1/3} \qquad (18)$$

The density is related to the molar concentration by:

$$\rho = 0.602\ [C] \qquad (19)$$

where [C] is the molar concentration of spin label. Substitution gives

$$\bar{r} = 0.656\ [C]^{-1/3} \qquad (20)$$

The effective concentration of spin labels cannot be measured directly, since, however, ΔH_{dip} is concentration dependent it can be indirectly obtained. The d_1/d spectral parameter, first introduced by Kokorin et al.,[72] is a measure of ΔH_{dip}, where d_1 and d can be derived from the powder spectrum, as defined in Figure I-19. From calibration studies at 77 K, the concentration dependence of d_1/d has been shown[71] to be given by

$$(\frac{d_1}{d}) = (\frac{d_1}{d})_{\infty dil} + 2.04\ [C] \qquad (21)$$

No dipolar broadening was found to exist for [C] < ∿5 mM below which d_1/d remains constant. This is the value of d_1/d at infinite dilution $(d_1/d)_{\infty dil}$, i.e., where no dipolar interactions occur between spins. This value varies somewhat from system to system and is a function of solvent polarity and the degree of residual motion at 77 K. Typical values are close 0.4. Combination of (21) and (19) yields

$$\frac{d_1}{d} = (\frac{d_1}{d})_{\infty dil} + 0.58\,\bar{r}^{-3} \qquad (22)$$

Eq. (22) can be used to measure distances in the regime of 1.0-2.4 nm.[71]

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

(d) Spin-label-spin-probe experiments

Interactions between spin labels, usually nitroxides, and spin probes, paramagnetic metal ions can provide valuable information about the topography of biological materials.[65,71,73] Two types of interactions may arise from the paramagnetic species: (i) dipole-dipole interactions which arise from induction of local magnetic fields by the magnetic dipole of paramagnetic group at the site of another; and (ii) Heisenberg exchange, which is caused by orbital overlap of unpaired electrons of proximal paramagnetic species. The interactions may be modulated by the spin-lattice relaxation times of the metal ions (T_1), by rotational diffusion of the metal-ion-free-radical assembly, or by translational diffusion of the metal ion with respect to the free radical. It is essential to establish both the nature of the interactions and the significance of the modulation of the dominant interaction. No adequate theory exists to account for the range of possible situations, although for transition metal complexes[68] and nitroxides in low viscosity solutions[73] Heisenberg exchange has been found to be the dominant interaction. Likhtenshtein favours[65] the same mechanism for interactions between metal ions and nitroxide radicals covalently bound to proteins. Previous studies[71] carried out in this laboratory have employed transition metal ions with very short T_1 values, e.g., Ni^{2+} for which T_1 has been estimated[65] at $\sim 10^{-12} - 10^{-13}$ S, in order to minimize dipolar contributions and maximize exchange interactions. The present study uses the same method.

The spin-label-spin-probe method allows, in principal, for the resolution of complex esr spectra arising from a heterogeneous distribution of radicals covalently attached to a macromolecule, since the esr

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

linewidth of the radical ΔH_0 is related to the rate constant for exchange relaxation (K) for encounters between the radical and the paramagnetic metal ion by

$$\Delta H_0 = 6.5 \times 10^{-8} \cdot K \cdot C \qquad (23)$$

where C is the concentration of the spin probe.[65] Hence, any heterogeneities due to different accessibilities of labels to the spin probe will appear as discontinuities in plots of ΔH_0 versus C, because readily accessible spin labels have higher K values than their less accessible counterparts; the esr signals of the former should be broadened at lower probe concentrations. When rapid exchange occurs between the different label sites, the experimental K values should become equal leading to the disappearance of the discontinuities in the ΔH_0 versus C plots.

(e) Copper (II) esr[68]

For the d^9 configuration of copper(II) the effective electron spin $S = \frac{1}{2}$ and the spin angular momentum $m_S = \pm\frac{1}{2}$ give rise to a double degenerate energy state. If the copper ion is located in a site of lower symmetry than a perfect cubic crystal, the g and A values, as before, are orientation dependent (or anisotropic) as expressed in equation (3), where the z-axis is defined as coinciding with the highest-fold notation axis.

When the unpaired Cu(II) electron couples with the nuclear spin (I = 3/2) the absorption signal is split with 2I + 1 components, in this case four lines (Fig. I-20).[74] In a polycrystalline sample no averaging of the g and A anisotropies occurs. With the random distribution of molecular symmetry axis, the observed resonance lines represent the sum of the superimposed individual resonances. The spectrum shows a

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

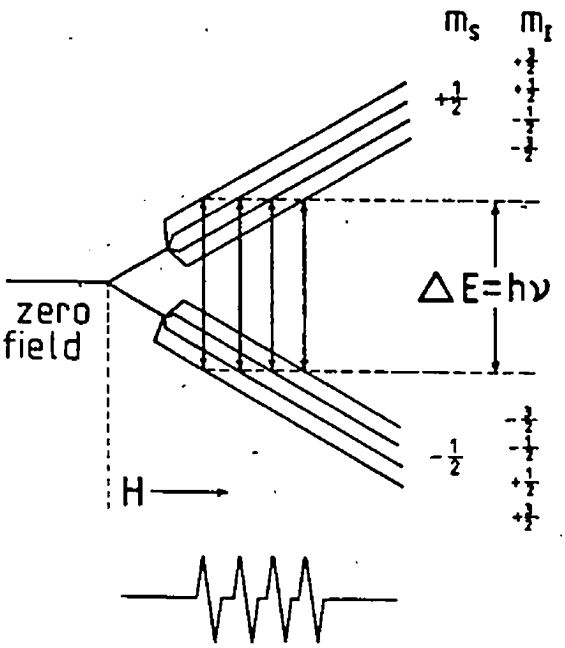


Fig. I-20. Spin state energy level diagram for a copper (II) nucleus in a magnetic field.

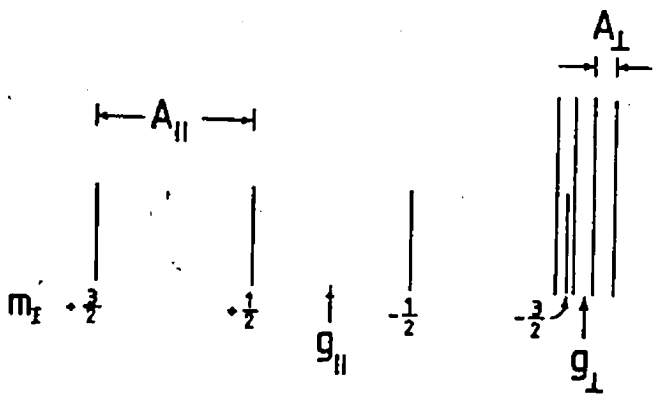


Fig. I-21. Scheme of copper (II) esr spectrum showing both $g_{\parallel}$ and $g_{\perp}$ components.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

weak set of lines at $g_{\parallel}$, corresponding to molecules whose symmetry axis are parallel to the applied field and a set of strong lines at $g_{\perp}$ corresponding to perpendicular alignment as schematically shown in Figure I-21. Superhyperfine structure, resulting from hyperfine interactions of spin 1 nitrogen nucleus and the electron spin, is sometimes resolved. In the copper-complexes discussed in Chapter V no such interactions were observable.

The A and g values can be abstracted directly, if desired, from the field-corrected spectra. The $A_{\parallel}$ values are taken as the separation between the $+\frac{1}{2}$ and $-\frac{1}{2}$ lines, and a_0 values are derived from the $-\frac{1}{2}$, $-3/2$ separation. Both $g_{\parallel}$ and g_0 are obtained from the centre point between the $+\frac{1}{2}$ and $-\frac{1}{2}$ lines. Equation (24) converts the values of these hyperfine constants (units of magnetic field) into frequency units.

$$A(cm^{-1}) = A(gauss) \times \frac{g}{g_e} \times 9.3484 \times 10^{-5} \qquad (24)$$

2. Elemental Microanalysis

Elemental microanalysis is a standard technique for studying polysaccharide modifications since it provides a facile determination of the, otherwise often difficult to establish, degree of substitution (d.s.). It is, however, necessary to explain to the reader the nature of the "fudging" factor which accompanies most of the elemental analysis results in the Experimental section.

Most polysaccharides have a saturation vapour pressure of hydration roughly equivalent to that of phosphorous pentaoxide, and at normal humidities they contain 8-10% water as water of hydration.[43] It will be consequently understood, why, despite even the greatest precautions and the most stringent specimen drying procedures, a certain small percentage

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

of water of hydration is usually associated with elemental analysis data. To illustrate the point, the effect of water on the C, H, N percentages of N-cyclohexylchitosan (d.s. 0.5) is listed below.

Anal. for $[C_8H_{13}NO_5)_{0.02}\ (C_6H_{11}NO_4)_{0.48}\ (C_{13}H_{23}NO_4)_{0.5}]_n$

	$[MW]_n$	H_2O present	C	H	N	C/N
calcd.	209.96	0	54.53	8.18	6.67	8.18
	212.05	0.12 (1%)	53.99	8.21	6.60	8.18
	216.25	0.35 (3%)	52.94	8.27	6.47	8.18
	218.35	0.47 (4%)	52.43	8.30	6.41	8.18
found			52.56	8.36	6.44	8.16

The C/N ratios provide, of course, another measure of the reliability of the analytical results.

Throughout this work two situations were encountered where elemental analysis did not provide useful information. The first was in systems where the degree of substitution was very low and microanalysis proved to be not sensitive enough. The second case was found for a series of alginate derivatives which gave unexplicably low C, H, and, for spin-labelled derivatives, N percentages, despite various efforts to remove any impurities; (it should be noted that alginate and most other polysaccharides contain considerable quantities of various trace metals and were consequently purified prior to use). In such situations, recourse was taken to esr double integration and/or other spectroscopic techniques, such as ^{13}C nmr.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

References

1. D. A. Rees, Polysaccharide Shapes, Chapman and Hall, London, (1977).

2. R. L. Whistler (ed.), Industrial gums, Academic Press, New York, 2nd edition, (1973).

3. G. O. Aspinall, Polysaccharides, Pergamon, New York (1970).

4. G. O. Aspinall (ed.), MTP Intern. Rev. Sci., Org. Chem., Series One, 7 (1973).

5. F. A. Bettelheim, in Biological Polyelectrolytes, (A. Veis, ed.), Marcel Dekker, New York, 131 (1970).

6. E. Percival and R. H. McDowell, Chemistry and Enzymology of Marine Algal Polysaccharides, Academic Press, New York (1970).

7. K. B. Guiseley, 'Seaweed Colloids,' Kirk-Othmer Encycl. Them. Technol., 2nd Edition, 17, 763-784, Wiley, New York, (1968); W. H. McNeely and D. J. Pettit, in ref. 2, p. 49.

8. A. Haug, B. Larsen, and O. Smidsrød, Acta Chem. Scand., 21, 691-704 (1967); A. Haug, B. Larsen, and O. Smidsrød, ibid., 20, 183-190 (1966); A. Penman, and G. R. Sanderson, Carbohydr. Res., 25, 273-282 (1972); J. Boyd, and J. R. Turvey, ibid., 66, 187-194 (1978).

9. F. G. Donnan and R. C. Rose, Can. J. Res., B-28, 105 (1950); A. Haug, Norwegian Inst. Seaweed Res., Trondheim, Norway, Rept. No. 30, (1964).

10. W. H. McNeely and K. S. Kang, in ref. 2, p. 486; R. Moorhouse, M. D. Walkinshaw, and S. Arnott, in Extracellular Microbial Polysaccharides, (P. A. Sandford and A. Laskin, eds.), ACS Symp. Ser. 45, 90 (1977); A. Jeanes, J. Polym. Sci., Symp. No. 45, 209 (1974).

11. G. Holzwarth, Carbohydr. Res., 66, 173 (1978).

12. G. Holzwarth, Biochemistry, 15, 4333 (1976); E. R. Morris, D. A. Rees, G. Young, M. D. Walkinshaw, and A. Drake, J. Mol. Biol., 110, 1 (1977); C. S. H. Chen and E. W. Sheppard, J. Macromol. Sci. Chem., A13, 239 (1979).

13. G. Holzwarth and E. B. Prestridge, Science, 197, 757 (1977).

14. P. J. Whitcomb, B. J. Ek, and C. W. Macosko, ACS Symp. Ser., 45, 160 (1977).

15. F. Hoyle, A. H. Olaveson, and N. C. Wickramasinghe, Nature, 271, 229 (1978).

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

16. R. M. Rowell and R. A. Young (eds.), Modified Cellulosics, Academic Press, New York (1978).

17. T. A. Hsu, M. R. Ladisch, and G. T. Tsau, Chem. Techn., 315 (1980).

18. O. H. Northcote, Essays Biochem., 8, 89 (1972).

19. R. H. Marchessault, and A. Sarko, Adv. Carbohydr. Chem., 22, 421 (1967).

20. A. J. Stipanovic, and A. Sarko, Macromolecules, 9, 851 (1976); A. Sarko, J. Southwick, and J. Hayashi, ibid., 9, 857 (1976); A. D. French, Carbohydr. Res., 61, 67 (1978).

21. A. H. Nissan, Macromolecules, 10, 660 (1977); A. F. Turbak, R. B. Hammer, R. E. Davies, and N. A. Portnoy, ACS Symp. Ser., 58, 12 (1977).

22. H. T. Kokhande, J. Appl. Polym. Sci., 20, 2313 (1976).

23. For a review, see, S. P. Rowland, and E. J. Roberts, J. Polym. Sci., A-1, 10, 2447 (1972).

24. A. M. Goldstein, E. N. Alter, and J. K. Seaman, in ref. 2, p. 303; R. Rol, ibid., p. 323.

25. P. M. Dey, Adv. Carbohydr. Chem. Biochem., 35, 341 (1978).

26. R. L. Whistler, Adv. Chem. Ser., 11, 45 (1954).

27. C. W. Baker and R. L. Whistler, Carbohydr. Res., 45, 237 (1975).

28. J. J. Gonzalez, Macromolecules, 11, 1074 (1978).

29. T. J. Painter, J. J. Gonzalez, and P. C. Hemmer, Carbohydr. Res., 69, 217 (1979).

30. H. Grasdalen and T. Painter, ibid., 81, 59 (1980).

31. C. J. Githens and J. W. Burnheim, Soc. Pet. Eng. J., 17, 5 (1977).

32. H. Braconnot, Ann. Chi. Phys., 79, 265 (1811).

33. A. Odier, Mem. Soc. Hist. Nat. Paris 1, 29 (1823).

34. C. Rouget, Comp. Rend., 48, 792 (1859).

35. R. A. A. Muzzarelli and O. Tubertini, Talanta, 16, 1571 (1969).

36. R. A. A. Muzzarelli, Chitin, Pergamon Press, New York (1977).

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

37. R. L. Whistler, in ref. 2, p. 465; J. N. BeMiller, in Methods in Carbohydr. Chemistry, 5, 103 (1965).

38. R. A. A. Muzzarelli, Natural Chelating Polymers, Pergamon Press, New York (1973).

39. L. A. Nud'ga, E. A. Plisko, and S. N. Danilov, Zhur. Obs. Khim., 43, 2752 (1973).

40. C. L. McCormick and D. K. Lichatowich, J. Polym. Sci., Polym. Lett. Edn., 17, 479 (1979).

41. A. E. Sirica and R. J. Woodman, J. Nat. Cancer Inst., 47, 377 (1971).

42. L. L. Balassa and J. F. Prudden, Proc. First Intern. Conf. Chitin/Chitosan, (R. A. A. Muzzarelli and E. R. Panser, eds.), 296 (1978).

43. R. L. Whistler, in Carbohydrates in Solution, ACS Ser., 117, 242 (1973).

44. P. Calvert, Nature, 280, 108 (1979); C. Schuerch, Adv. Polym. Sci., 10, 173 (1972); L. B. Jaques, Science, 206, 528 (1979).

45. P. Ferruti, M. C. Tanzi, and F. Vaccaroni, Makromol. Chem., 180, 375 (1979); H. Moldenhauer and H. J. Loh, Pharmazie, 33, 216 (1978).

46. R. L. Whistler and S. J. Hirase, J. Org. Chem., 26, 4600 (1961).

47. B. Pfannemuller, G. Richter, and E. Husemann, Carbohydr. Res., 56, 139 (1977); H. Andresz, G. C. Richter, and B. Pfannemuller, Makromol. Chem., 179, 301 (1978).

48. J. H. Elliott, in Extracellular Microbial Polysaccharides, ACS Symp. Ser., 45, 144 (1977).

49. Xanthan Gum, Kelco Co., Los Angeles, 1976.

50. L. J. Filar and M. G. Wirick, in Proc. First Intern. Conf. Chitin/Chitosan, 144 (1978).

51. J. H. Bradbury, in Physical Principles and Techniques of Protein Chemistry, B, (S. J. Leach ed.), Academic Press (1970); R. S. Lenk, Polymer Rheology, Applied Science Publ., London, (1978); J. D. Ferry, Viscoelastic Properties of Polymers, Wiley, (1970).

52. A. R. Eastwood and H. A. Barnes, Rheol. Acta, 14, 795 (1974).

53. R. Darby, Viscoelastic Fluids, Marcel Dekker. New York (1976).

54. Kelco Algin, Kelco Co., Los Angeles, 1976.

55. D. A. Rees, Chem. Ind., 630 (1972).

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

56. R. G. Schweiger, Carbohydr. Res., 70, 185 (1979).

57. J. F. Kennedy, Adv. Carbohydr. Chem. Biochem., 29, 306 (1974), W. G. Overend, Chem. Ind., 61 (1976); J. F. Kennedy, Carbohydrate Chemistry, 11, 445 (1979).

58. R. F. Borch, M. D. Bernstein, and H. D. Durst, J. Am. Chem. Soc., 88, 1024 (1966); G. R. Gray, Methods Enzymology, 50, 155 (1978).

59. L. D. Hall and M. Yalpani, Carbohydr. Res., 78, C4 (1980); L. D. Hall and M. Yalpani, ibid., in press; M. A. Bernstein, L. D. Hall, and W. E. Hull, J. Amer. Chem. Soc., 101, 2744 (1979); M. Brownlee and A. Cerami, Science, 206, 1190 (1979); H. Wiegandt and W. Ziegler, Hoppe-Seyler's Z. Physiol. Chem., 355, 11 (1974).

60. A. S. Perlin, MTP Internat. Rev. Sci., Org. Chem. Ser. Two, 7, 1 (1976).

61. R. G. Kessel and C. Y. Shih, Scanning Electron Microscopy in Biology, Springer, New York (1974).

62. L. J. Berliner, ed., Spin Labelling, I, Academic Press, New York (1976); II, (1979).

63. R. A. Dwek, Nuclear Magnetic Resonance in Biochemistry, Clarendon Press, Oxford, (1975).

64. E. G. Rozantsev, 'Free Nitroxyl Radicals,' Plenum, New York (1970).

65. G. I. Likhtenshtein, 'Spin Labeling Methods in Molecular Biology,' Wiley, New York, (1976).

66. J. R. Bolton, D. Borg and H. Swartz, Biological Applications of Electron Spin Resonance, Wiley, New York, (1972).

67. J. E. Wertz, and J. R. Bolton, 'Electron Spin Resonance, Elementary Theory and Practical Applications,' McGraw-Hill, New York (1972).

68. C. Alleyne, M.Sc. thesis, University of British Columbia, 1973; B. V. McGarvey, Transition Metal Chemistry, 3, 89 (1966); B. J. Hathaway and D. E. Billings, Coordination Chem. Reviews, 4, 143 (1970); S. S. Eaton, and G. R. Eaton, Coord. Chem. Rev., 26, 207 (1978).

69. J. H. Freed, in ref. 62, p. 53.

70. S. A. Goldman, G. V. Bruno, and J. H. Freed, J. Chem. Phys., 59, 3071 (1973); F. J. Sharom, and C. W. M. Grant, Biochem. Biophys. Res. Commn., 74, 1039 (1977).

71. J. D. Aplin, Ph.D. thesis, University of British Columbia (1979).

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

72. A. I. Kokorin, K. I. Zamarayev, G. L. Grigoryan, V. P. Ivanov, and E. G. Rosantsev, Biofizika, 17, 34 (1972) (31 in transl.).

73. J. Hyde, H. M. Swartz, and W. E. Antholine, in Spin Labelling II, (L. J. Berliner, ed.), 71 (1979).

74. M. J. Adam, Ph.D. thesis, University of British Columbia, (1980).

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

CHAPTER II

CHEMICAL MODIFICATION OF ALGINIC ACID, XANTHAN GUM, AND CELLULOSE

II-A. Background

1. Introduction

In light of the consideration discussed in the Introduction, the work described in this chapter was principally aimed at investigating the synthetic aspects of several derivatization procedures, some of them previously documented, using as substrates three important and representative polysaccharides, alginic acid, xanthan gum, and cellulose. For these particular studies esr spectroscopy of the products obtained by reactions with reagents based on stable nitroxide spin labels was used to obtain insight to the general course of the reactions. As will be seen, the particular merit of this approach is that it enabled a facile distinction to be made between material which had been covalently "bound" to the polysaccharide from that which was "unbound" or merely absorbed.

Before discussing the chemistry undertaken it is appropriate to provide some background information concerning covalent chemical linkages, the criteria employed here for establishing their presence, and for selecting the various modification procedures.

2. Covalent Linkages

It is appropriate to begin with a discussion of the general nature of the reactions performed which in all cases afforded covalently linked

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

spin-label conjugates, as monitored by esr. To the organic chemist, unfamiliar with this field, some of the chemical transformations presented here, for instance the amidation or esterification of alginate derivatives, will appear to be rather surprising, at least under the given experimental conditions. It is necessary therefore, to briefly summarize the criteria which were used in this study to determine the existence of covalent linkages as well as evidence obtained from other sources. It is convenient to begin with the latter.

(i) Criteria used in previous studies

In two pre-1960 patents[1,2] pertaining to the preparation and use of alginamides from esters of alginic acid under mild conditions the following evidence is given in support of the reactions: (i) the solubility properties of the amide products depend on the hydrophilicity/hydrophobicity of the starting amine, i.e., the former yield water-soluble compounds while the latter afford water-insoluble compounds which are soluble in organic solvents; when two different amines are used the resulting mixed amides have solubility features intermediate to the above ones; (ii) the products can be characterized by nitrogen analysis and, more importantly, the theoretical amounts of alcohol can be isolated from the reaction of alginic esters and ammonia.[1] In another study,[3] nitrogen analysis was also used to confirm the quantitative conversion of esters into amides. In both of the above patents,[1,2] the inventors consider it probable that some of the alginic esters could be saponified to form the corresponding alginate salts; no evidence is, however, cited why the trans-esterifications or amidations should not go to completion. In a more recent survey, McDowell[4] has demonstrated that the physicochemical properties of alginamides derived from alginic esters are consistent with the

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

formation of covalent products rather than salts. McDowell proposed the co-existence of several competing reactions, including cross-link formations, where the conditions for the breakdown of links (i.e., of alginic esters) are not markedly different from those for their formation (i.e., of products); thus, a narrow range of conditions has to be found for the formation rate to be greater than the destruction rate.

Having dwelt on the formation of alginamides and esters, evidence for other reactions can next be considered. Incorporation of radioactive labels has been used to demonstrate the carbodiimide-mediated, quantitative esterification of polyuronides.[5] A wealth of information about covalent linkages has been established for various industrially important processes such as the dyeing of textiles and cellulose derivatives.[6] As will be subsequently discussed, alkylations of cellulose using chloroacetic acid or other halogenated compounds, requires prior alkali-activation of the polymer for the reaction to proceed. Thus, it has been demonstrated for chlorotriazine-dye-treated cellulose that the dye is removed by washing with water if the reaction had been conducted in a neutral bath, whereas the dye cannot be washed off if alkali treatment precedes the reaction. Organic solvents do not remove the dye in the latter case, whereas they do if cellulose is treated with an unreactive vat- or azoic-dye under similar conditions. Dyed cellulose is insoluble in cuprammonium solutions whereas the undyed material is soluble. Furthermore, the dyed portion of treated materials can be chemically cleaved to produce a reactive moiety for further derivatizations; e.g., diazotation, amine or phenol coupling with new dyes. In a recent Ph.D. thesis[7] chlorotriazine-based nitroxide labels have been used in similar fashion to demonstrate covalent couplings to several poly-

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

saccharides. In addition, for many polysaccharide reactions the equivalent monosaccharide chemistry has been established.

Numerous studies have used various combinations of chemical and spectroscopic techniques to characterize the products obtained from (often multi-step) derivatizations of polysaccharides. The above examples clearly reveal the possibility of demonstrating the covalent nature of various polysaccharide derivatives although for this proof to be unambiguous several direct and indirect methods rather than a single and facile spectroscopic one are required in most cases. The advantages of the esr technique over other spectroscopic techniques for these purposes have already been alluded to. Before detailing the criteria used in this study for establishing covalent linkages, it should be mentioned that similar criteria have been employed in another recent Ph.D. thesis, which exclusively deals with the spin-labelling of polysaccharides.[8]

(ii) Criteria used in this study

The evidence derived in this study from esr spectroscopy was based on the following factors:

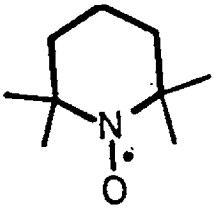
1. The spectral lineshapes and widths obtained from, in most cases, aqueous solution are a good measure of the degree of motional immobilization of nitroxide moieties attached to the polymers.

2. The spectral lineshapes obtained from solid or frozen (77°K) samples allow for a facile distinction of "bound" and "free" labels (in the former case) and they provide additional information about interactions between proximate spin labels (in the latter case).

3. Control experiments in which the unreactive spin label [1] was employed under identical conditions as the reactive counterparts, demonstrated that very little, if any, non-covalent associations or other,

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

phenomena such as trapping, occur for the various polysaccharides used. Alternatively, the leakage of any "free" spin labels from the polymeric matrices was monitored with time, whenever such species were suspected to coexist with bound labels.



[1]

Complimentary evidence was gathered from other spectroscopic techniques and methods, wherever possible. In many cases it would have been desirable to characterize functional groups by infra red (ir) spectroscopy. This technique, however, in my hand proved to be somewhat unsatisfactory largely due to the many limitations imposed by the polysaccharide derivatives themselves. In many cases, specific spectral bands could not be detected due to overlapping bands, or the degree of substitution was so small that positive identification was impossible with the available instrumentation. Similar difficulties have presumably also contributed to the lack of ir-evidence in any of the above cited references.

From the aforementioned it is clear that none of the criteria by itself can unambiguously establish covalent linkages, but, in toto, their combination together with other available evidence from the literature should provide sufficient support for the presence of such bonds.

3. Selection Criteria

Turning now to the strategy employed for the syntheses, previous

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

experience of this laboratory had demonstrated[9] that the reductive amination reaction promised the greatest general versatility for the purposes of this work. As will be seen, most of the modifications described herein were consequently designed to introduce appropriately either carbonyl or amine functionalities into the polysaccharides for subsequent derivatizations. The choice of synthetic procedures was further guided by the following main considerations: (i) to maintain the polysaccharide chain largely, if not completely intact and to avoid acid hydrolysis of the nitroxide spin labels; and (ii) to form covalent linkages between the nitroxide labelled reagents and the polymers in the simplest and most direct way. As a consequence, strongly acidic or basic conditions were not employed and the reactions were conducted at ambient temperature and pressure.

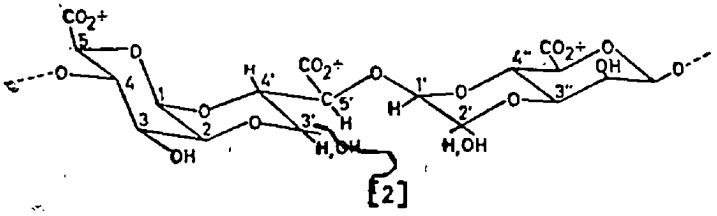
Of the numerous available modification procedures two, periodate oxidation[10] and carbodiimide coupling,[11] were found to be particularly useful and deserve further mention here.

Dialdehyde derivatives of polysaccharides obtained by periodate oxidation are widely used; for example, dialdehyde dextran derivatives have been combined with various amine-bearing pharmaceuticals,[12] and dialdehyde starch and cellulose have been employed for graft polymerization,[13] medical uses,[14] and enzyme immobilization.[15] For alginate, Painter and coworkers[16,17] have established carefully controlled conditions for preparing periodate-oxidized alginate with degrees of oxidation ranging from 0 to 60%. These dialdehyde derivatives constitute versatile intermediates since they can be further derivatized,* say with

*Another interesting variant has been demonstrated by the oxidation ($NaClO_2$) of the aldehyde groups to the corresponding carboxylic acid derivatives.[12a]

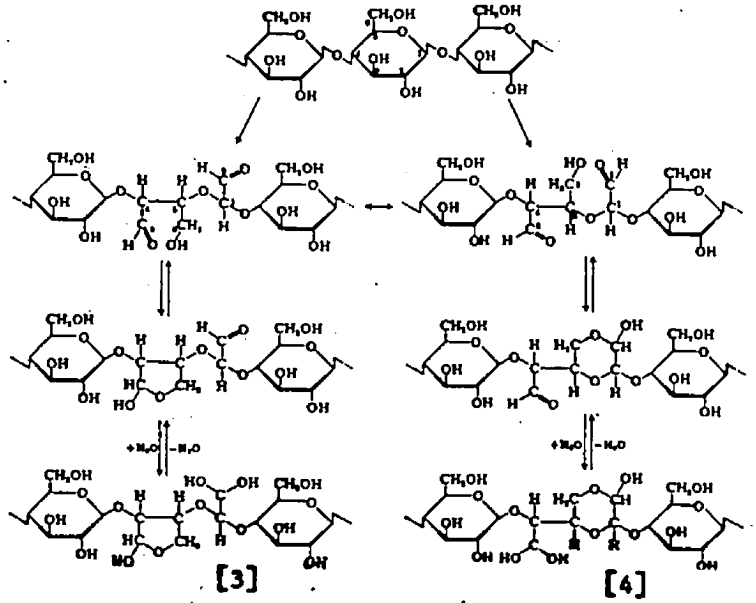
Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

amines, to afford products which are associated with varying degrees of structural perturbation and substitution. It should be noted, however, that the periodate oxidation method can be complicated by a number of factors such as radical initiated-chain scission, and formation of inter-residue hemiacetals and hydrated species.[10] Thus, Painter et al.[16] have shown that six-membered hemiacetal rings can be formed between aldehyde groups of hexuronic acid residues of alginate and the closest hydroxyl groups on adjacent unoxidized residues [2], while Nadzhimutdinov et al.[18]



[2]

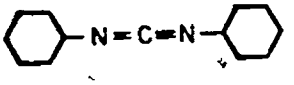
found dialdehyde cellulose to exist in two forms in aqueous solutions ([3], [4]).



[3] [4]

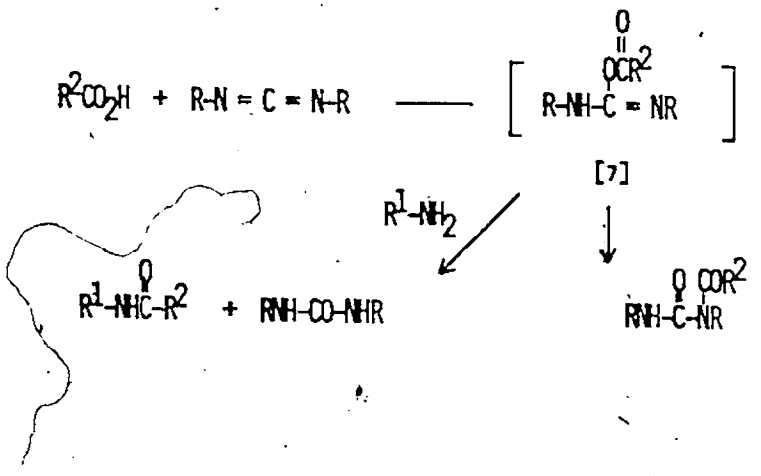
Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

Carbodiimides, particularly N,N'-dicyclohexyl-carbodiimide (DCC) [5], have found widespread applications as condensing agents in amide and ester syntheses.[19,20] More recently, watersoluble derivatives such as N-ethyl-N'-dimethyl aminopropyl carbodiimide hydrochloride (EDG) [6] have been developed which are well suited for polysaccharide[5] derivatizations since they offer the additional advantage of watersolubility of

[5] 

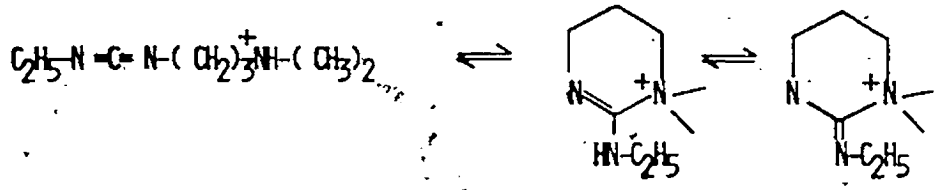
[6] $CH_3CH_2N=C=N(CH_2)_3\overset{H}{\underset{+}{N}}(CH_3)_2\ Cl^-$

the N-acyl urea byproducts which may otherwise complicate the isolation of the desired products. The condensation of amines ($R'NH_2$) with carboxylic acids ($R''CO_2H$) in the presence of DCC is envisaged to proceed via the activated ester intermediate [7]:[21]



Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

The details of the reaction mechanism for EDC coupling have not yet been fully elaborated, but it is known[22] that in neutral aqueous solution a ring-chain tautomerism exists which predominantly (93%) favors the ring tautomers as shown below:



Lastly, a comment is required concerning the reaction yields obtained in this chapter. While some reactions proceeded in essentially quantitative fashion, for many others the conversion yields were relatively low. However, no attempts were made to fully optimize the reaction conditions in such cases since some of these studies were designed to initially prove the formation of covalent derivatives. It would have been relatively easy in many instances to substantially improve the results simply by varying the reaction conditions such as temperature, use of surfactants, and catalysts, etc. Reference can be made here to a recent patent which exclusively describes procedures for the preparation of high d.s. polysaccharides.[23]

In view of the low d.s. obtained in many reactions, esr double integration was used to determine the levels of spin-label incorporation; microanalysis was performed, wherever possible on products not containing nitroxide moieties.

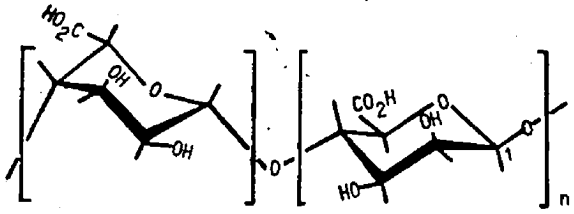
The reactions to be described are organized into separate sections based on the polysaccharide substrates used.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

II-B. Chemical Modifications

1. Alginic Acid

Of the three polysaccharides discussed here, alginic acid [8],



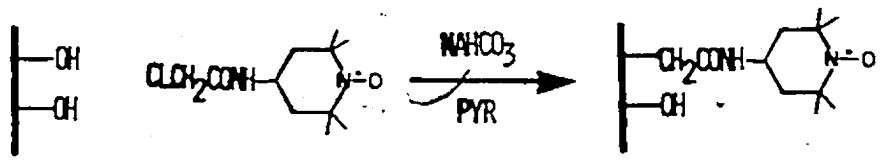
[8]

provided the greatest challenge for modifications as a result of its "fragility" and low reactivity. Some previous attempts[8] at spin-labelling of [8] had met with little success and it was found necessary to activate [8] prior to several modification reactions. Various workers[24] had previously noted that the acetylation of alginic acid never proceeded to completion, introducing no more than one acetyl group per uronic acid residue. However, Schweiger[25] discovered that a peracetylated product could be obtained with minimal polymer degradation if alginic acid was initially partially (80-90%) dehydrated (complete dehydration leading to inactivation of [8]). Thus, following Schweiger's method partially dehydrated alginic acid was obtained by treatment of [8] with cold, glacial acetic acid; this procedure reduces the extensive hydrogen-bonding in [8] thereby rendering the hydroxyl groups available for reaction.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

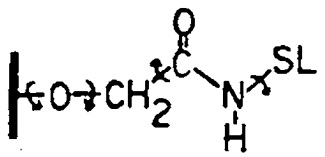
(i) Alkyl ether

Following the above procedure, stable acetamido ethers [10] of alginic acid [8] were prepared using 4-chloroacetamido-2,2,6,6-tetramethylpiperidine-1-oxyl [9], in either pyridine or aqueous acetone (65%) using solid sodium bicarbonate as base. The mild conditions which were employed to avoid degradation of [8] led to very low d.s. (0.03-0.04).



[8] [9] [10]

The esr spectrum of [10] in aqueous solution (Fig. II-1a) reveals a very mobile nitroxide moiety. The relatively high mobility can be ascribed to some extent to the rotational freedom associated with the four-bond linkage between the polymer and probe.



The spectrum of solid [10] (Fig. II-1b) resembles that of a polycrystalline sample and shows no presence of free label.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

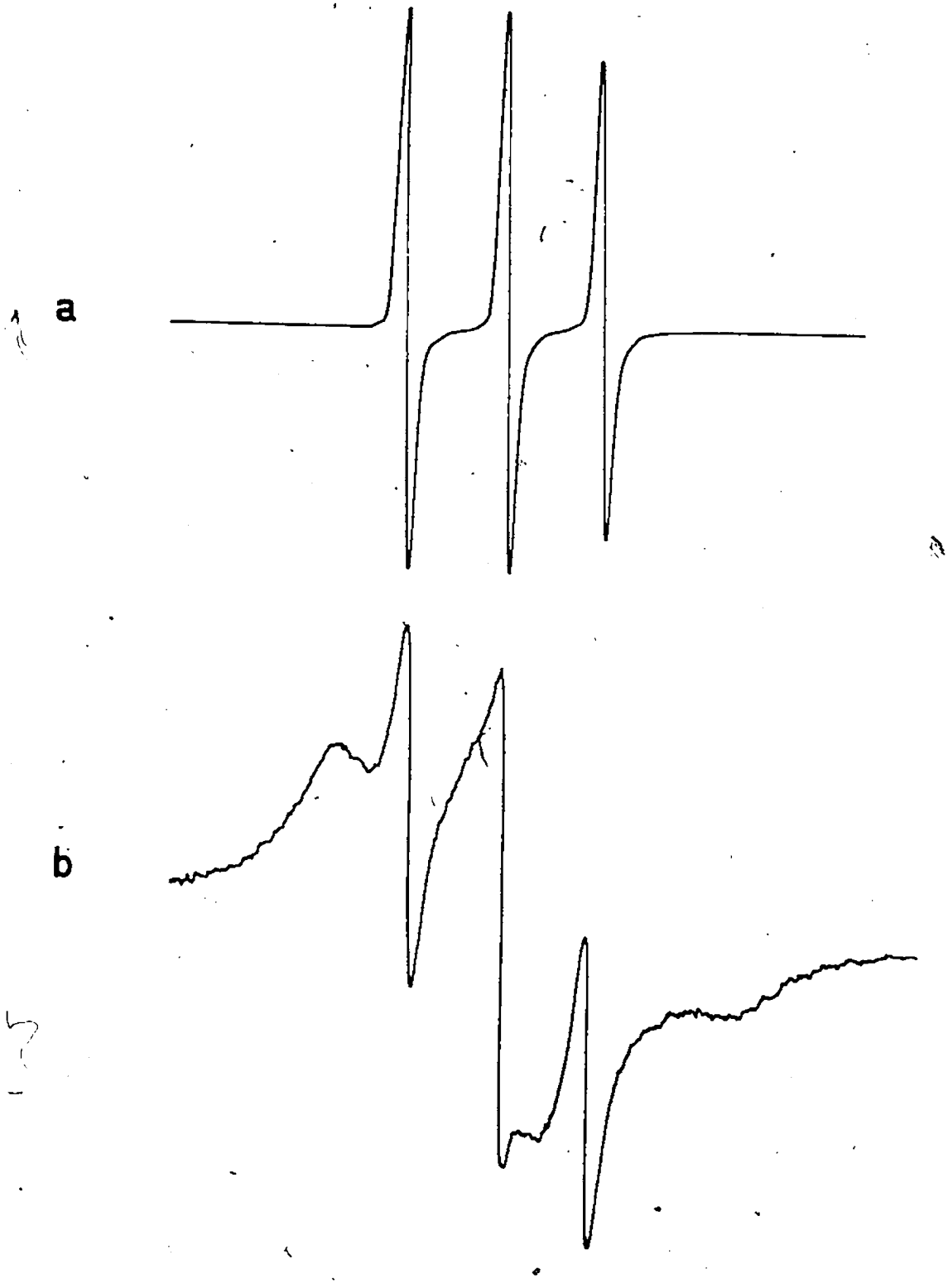
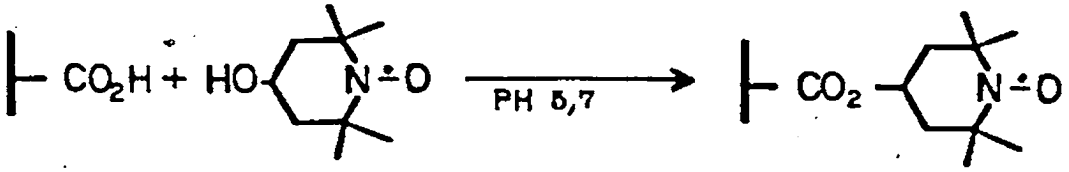


Fig. II-1. Esr spectra of [10] (a) in aqueous solution; (b) solid; (at 298 K).

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

(ii) Alkyl ester

The esterification of partially dehydrated alginic acid [8] was accomplished using 4-hydroxy-2,2,6,6-tetramethylpiperidine-1-oxyl [11] in aqueous acetone affording the ester [12] (d.s. 0.05).



[8] [11] [12]

Evidence for the formation of [12] was derived from the esr spectrum of the solid under methanol (Fig. II-2b) which revealed a broad "bound" component together with a superimposed signal arising from "free" label (despite extensive dialysis of the product!). This "free" label may be present as the salt which previous workers have alluded to (see II-B 2(i)). The τ_c value of [12] in aqueous solution was 20 ns (Fig. II-2a). Additional evidence for the occurrence of esterification was derived from a control experiment in which the spin label [11] was replaced by the unreactive analogue [1]. Only a very small proportion of [1] remained in the sample after dialysis, as indicated in Figure II-2c.

The above reaction appears to provide a viable alternative to the known esterification procedures for alginic acid which involve the use of alkalyne oxides[26] or the acid chloride intermediate [14] proposed by Wypych.[27]

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

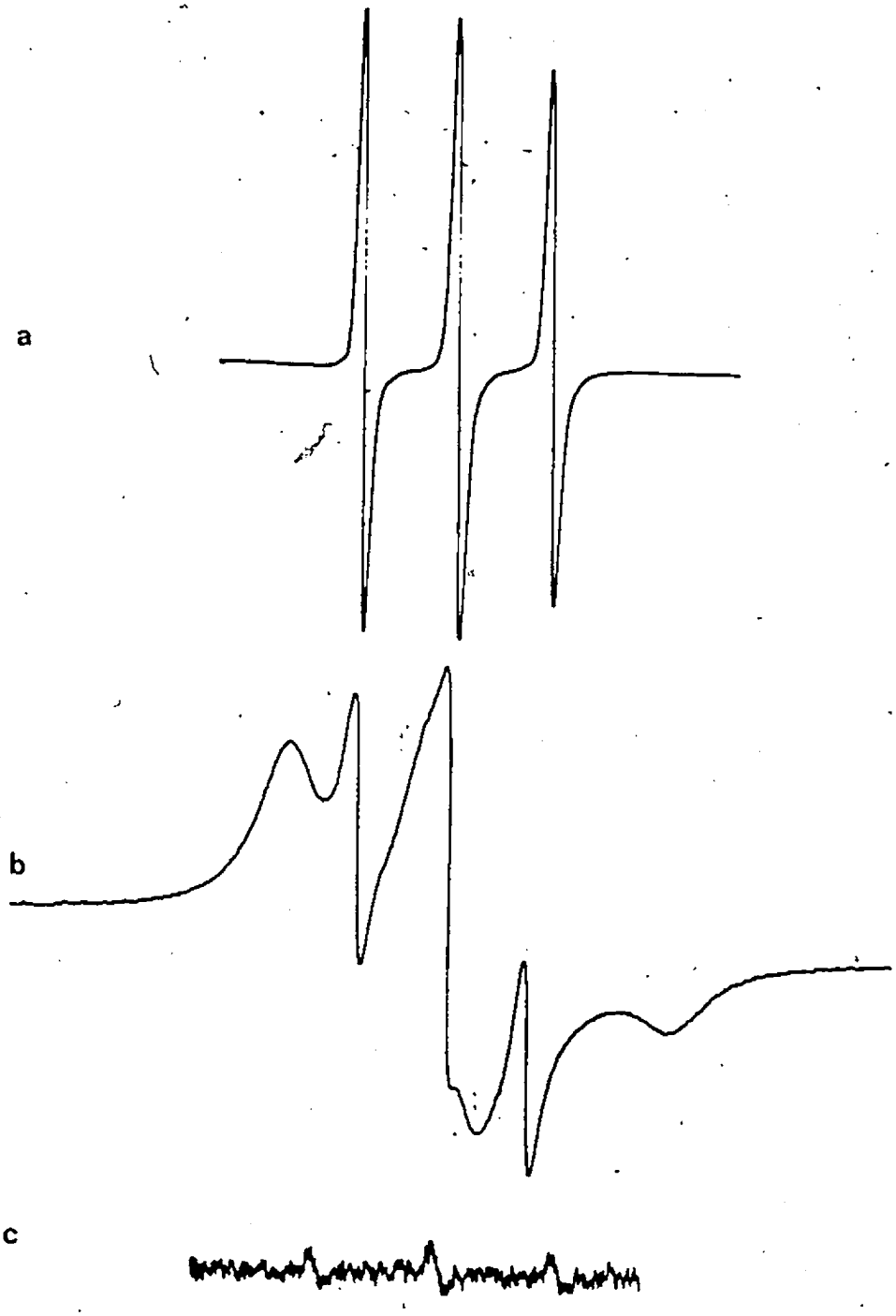


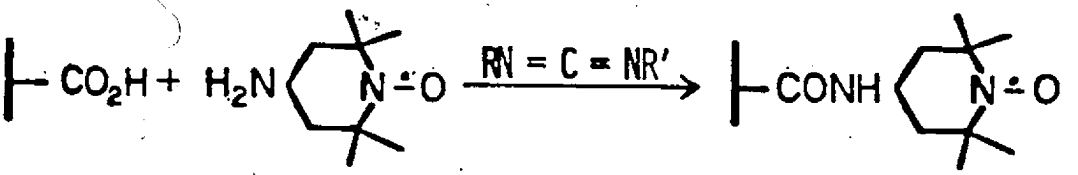
Fig. II-2. Esr spectra of [12] (a) in aqueous solution; (b) solid; (at 298K); (c) control experiment: [8] treated with [1] under identical conditions.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

(iii) Amides

(a) via carbodiimide coupling

Amide derivatives of alginic acid could be readily prepared by carbodiimide-mediated coupling in organic or mixed aqueous-organic media. As in the previous reactions, however, alginic acid is only reactive in its partially dehydrated form. Amidation can be achieved using DCC in dimethylformamide (DMF), or with EDC in 65% aqueous acetone to afford the amide [16A] with d.s. 0.10 and 1.00, respectively. Clearly, EDC is the



[8] [15] [16A]

preferred reagent for high d.s. products. The esr spectra of [16A] provide evidence for the covalent nature of the linkage (Fig. II-3); in the control experiment with label [1] no signal was detectable after identical treatment (Fig. II-3c). The nitroxide moiety of [16A] is moderately immobile in aqueous solution, (τ_c 15 ns) (Fig. II-3a).

(b) via nucleophilic substitution of alginic esters

Propylene glycol esters of alginate [17] have been reported to react with amines to form amides in high yields at ambient temperature.[1-4] Commercial propylene glycol alginate (PGA) samples with different degrees of esterification (d.s. 0.80-0.85 and d.s. 0.50-0.60, respectively) were condensed with amine spin label [15] in DMF in the presence of small amounts of water to yield the amide [16B].

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

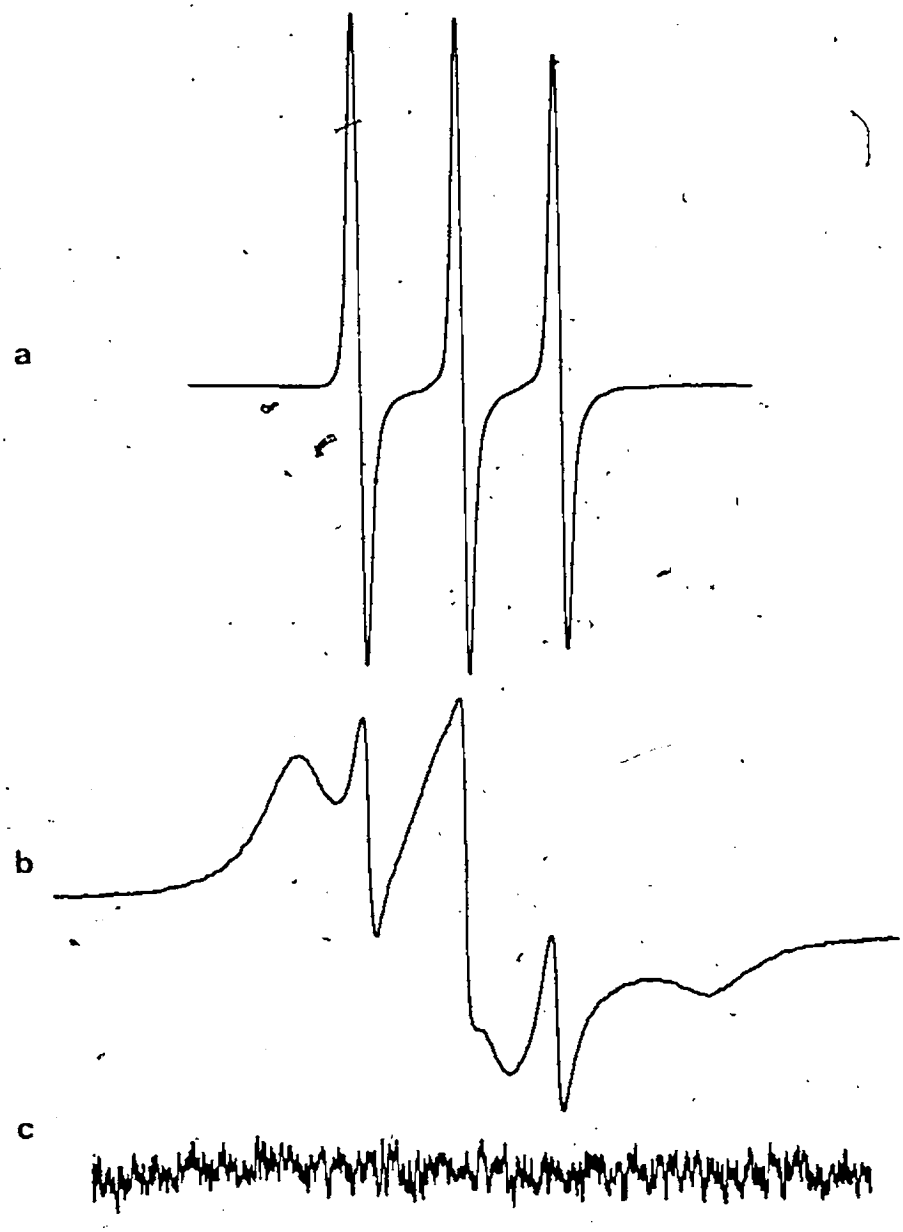


Fig. II-3. Esr spectra of [16A] (a) in aqueous solution; (b) solid; (at 298K); (c) control experiment: [8] treated with [1] under identical conditions.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

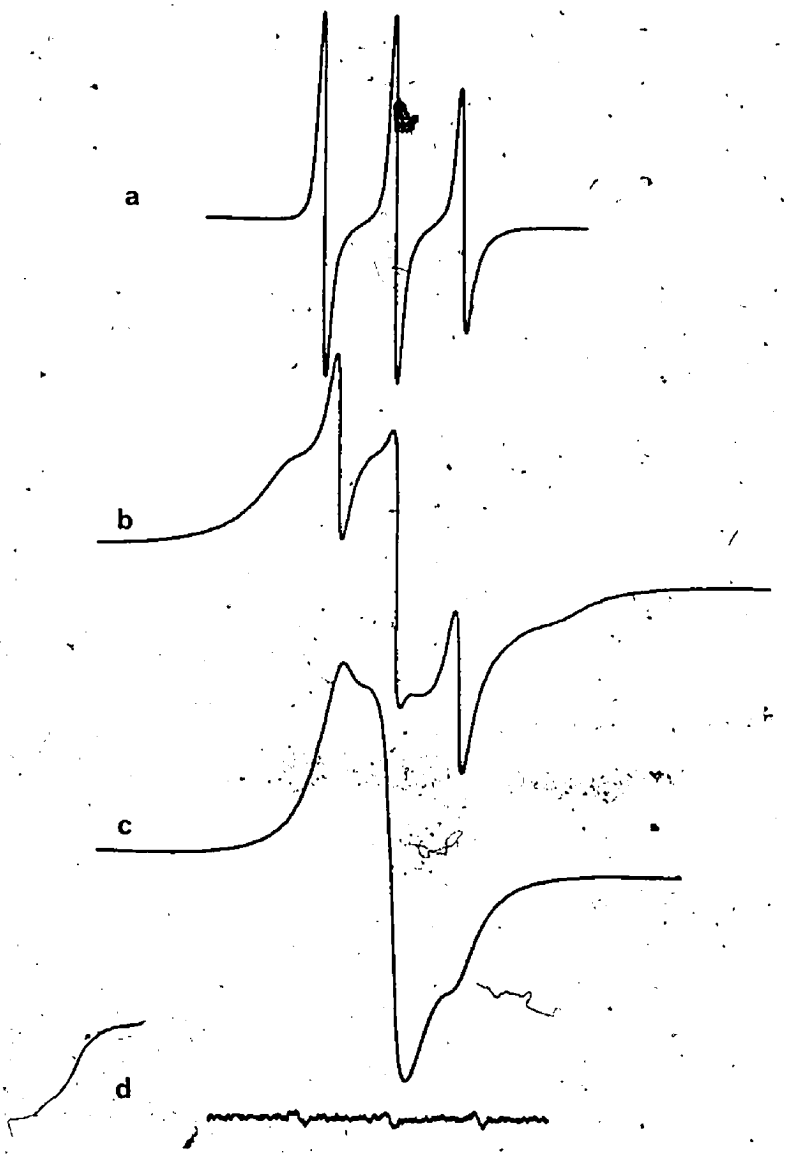
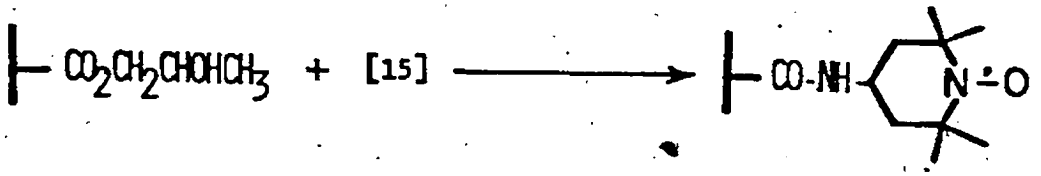


Fig. II-4. Esr spectra of [16B] (a) in aqueous solution; (b) solid; (at 298K); (c) frozen (77K); (d) control experiment: [17] treated with [1].

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.



[17] [16B]

This reaction is of considerable utility since it can be performed with a wide range of amines such as primary or secondary aliphatic, cycloaliphatic, aromatic, and diamines.[1] The resulting products are of interest for their gel-forming properties.

The esr spectra of amides of the type [16B] in aqueous solution (Fig. II-4a) indicate moderately immobile nitroxide moieties (τ_c 33 ns) and the corresponding spectra of the solid samples reveal not only the covalent nature of the linkages to the polymer but also strong dipolar interactions (Fig. II-4b). Similar information can be derived from rapidly frozen (77K) samples (Fig. II-4c). The control experiment with label [1] revealed no detectable esr signal (Fig. II-4d).

(iv) Amines

Following the methods of Painter and coworkers,[17] dialdehyde alginates with varying degrees of oxidation (d.o.) were prepared and subsequently converted to the corresponding amine derivatives [18] using reductive amination and label [15]; typically, amines with d.s. 0.05 [18A] and 0.13 [18B] were obtained from dialdehyde precursors with d.o. 0.10 and 0.44, respectively. The esr spectra of [18A] and [18B] in aqueous solution (Fig. II-5a) revealed substantial line broadening and τ_c values of 51 ns and 57 ns, respectively. When these solutions were

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

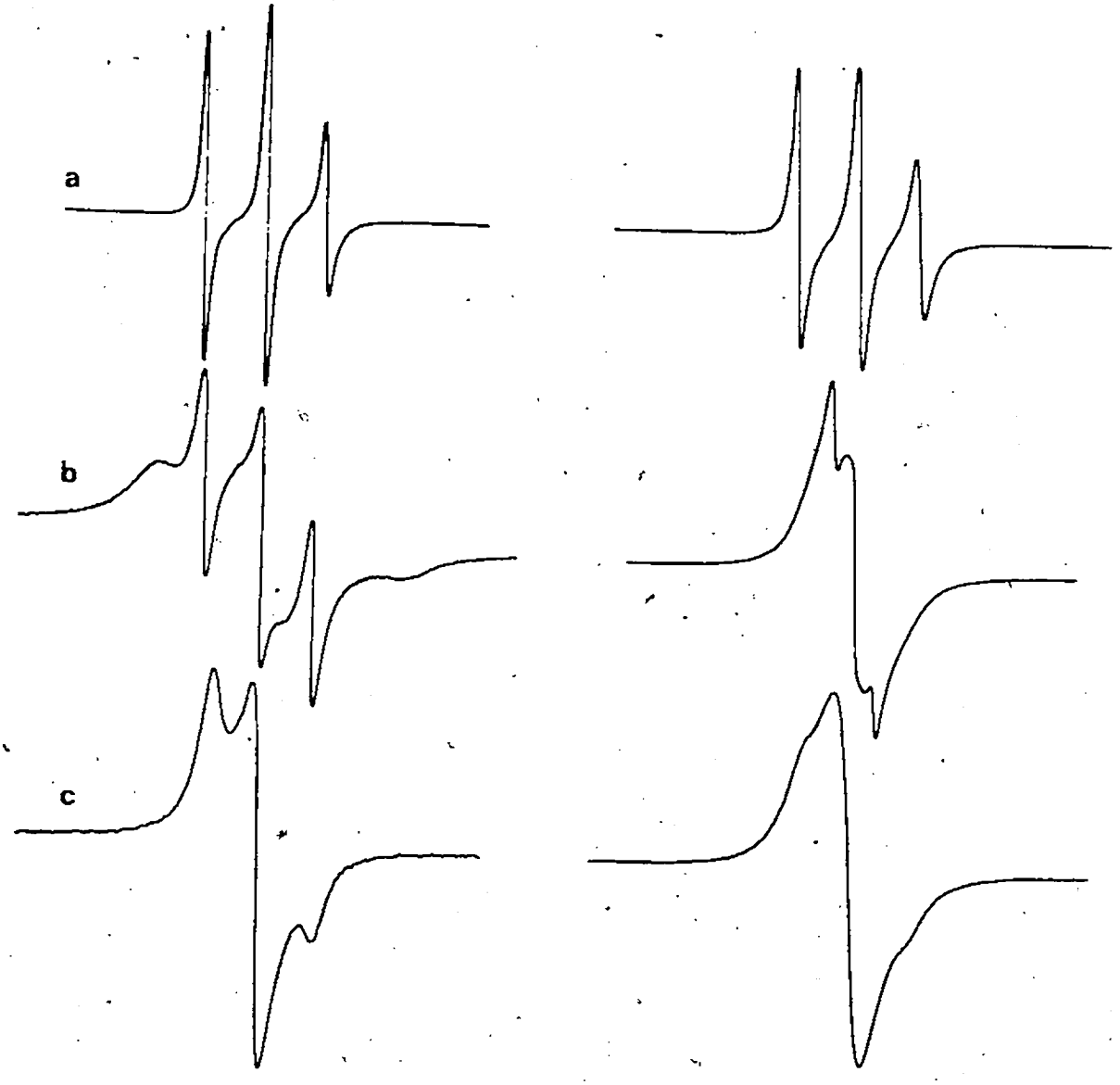
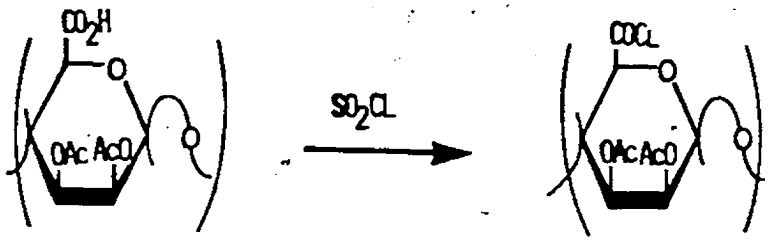


Fig. II-5. Esr spectra of [18A] (left) and [18B] (right) (a) in aqueous solution; (b) solid; (at 298K); (c) frozen (77K).

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.



[13] [14]

frozen, dipolar broadening was again apparent (Fig. II-5c). The spectra derived from the solid materials under methanol show the presence of small proportions of free label (Fig. II-5b). The above findings seem to implicate the presence of two spin labels units per periodate-cleaved hexuronic acid residue of [18] since even at low d.s., dipolar interactions are evident. Possible contributions to the observed lineshapes from interactions between nitroxides attached to different chains or to neighbouring uronide residues, should be negligible, at least for derivatives of [18] with low overall d.s. If the above inference is correct, it would be in contrast to several observations of other dialdehyde reaction products for which incorporation of only one amine-bearing unit per dialdehyde has been found.[28] The reaction of equivalent dialdehyde cellulose derivatives will be discussed in II-B 3.

A sample of periodate oxidized alginate (d.o. 0.10) was treated with p-fluoroaniline under identical conditions as above to produce a derivative which could be characterized by ^{13}C nmr. Figure II-6 demonstrates the utility of ^{13}C nmr in detecting even low (between 5-10%) levels of substituent incorporation. Similarly, preliminary studies of this derivate have shown that ^{19}F nmr is well suited for distinguishing

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

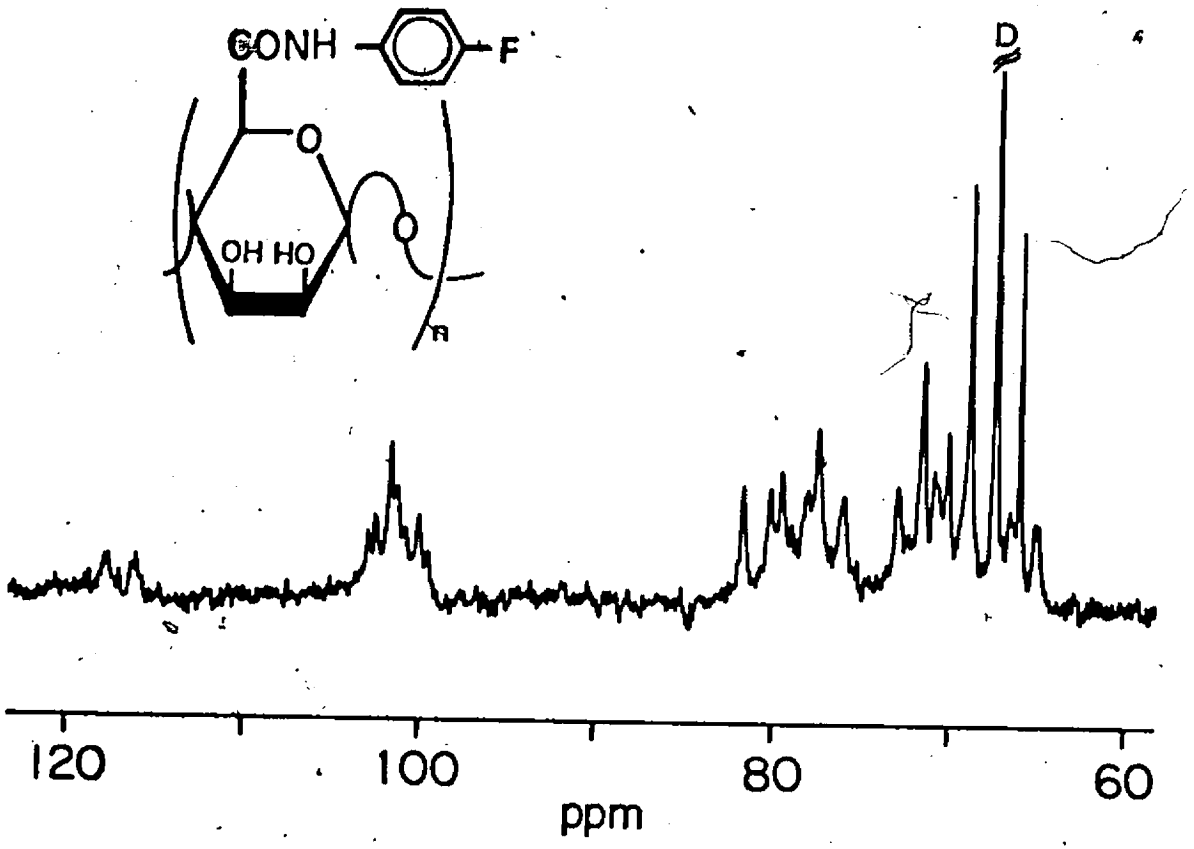
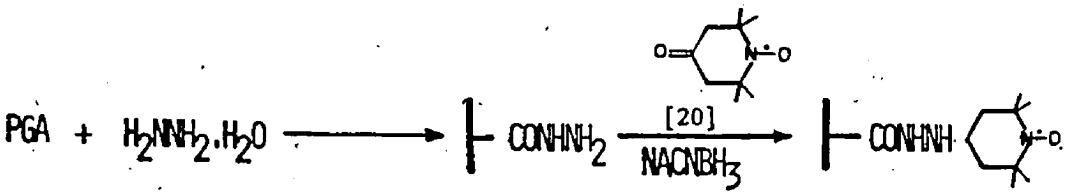


Fig. II-6. 100.6 MHz ^{13}C nmr spectrum of p-fluoroaniline alginate derivative, (4%) in D_2O at 308K, showing aromatic resonances at 117 ppm.

covalently bound material from reactant; when p-fluoroaniline was added to an aqueous solution of the fluoroaniline alginate derivative, the two species were found to be separated by ca 1 ppm in chemical shift.

(v) Hydrazin[illegible]

Pfannemüller et al.[29] have recently reported a series of polysaccharide hydrazine derivatives which were employed for the synthesis of branched-chain derivatives. Thus, alginic acid hydrazine [19] was prepared, in adaption of their method, from propylene glycol alginate [17]



[17] [19] [21]

and hydrazine hydrate. Reductive amination of [19], using 4-oxy-2,2,6,6-tetramethylpiperidine-1-oxyl [20] afforded the spin labelled derivative [21] (d.s. 0.21), whose aqueous solution spectrum is shown in Figure II-7. This method is potentially useful for the preparation of polysaccharide intermediates since it introduces a very short spacer group between the polymer and other molecules to be attached to it.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

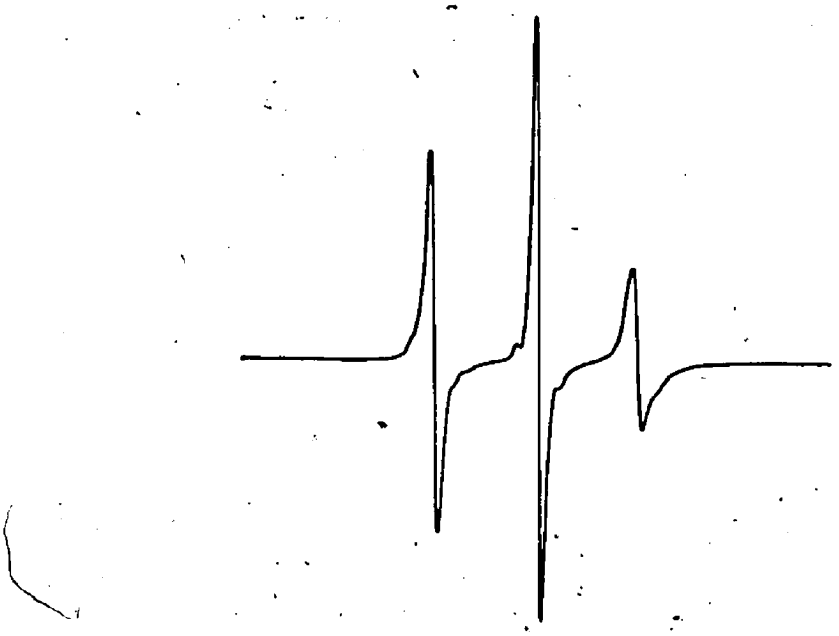
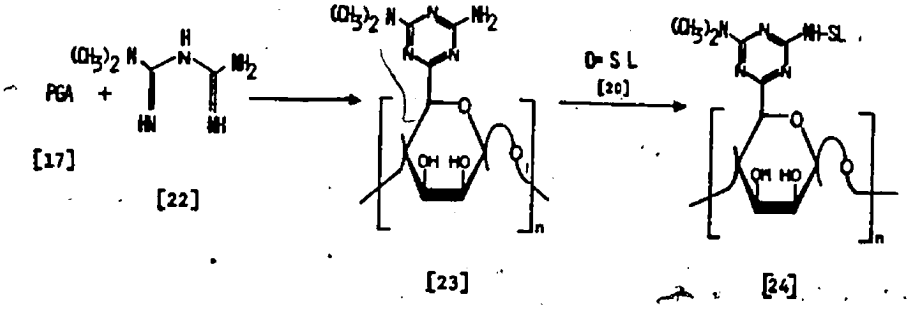


Fig. II-7. Esr spectrum of [21] in aqueous solution (at 298K).

(vi) s-Triazine derivatives

Lee and Maekawa[30] have reported a method for incorporating the carboxyl groups of polyuronides into s-triazine rings using dimethylbiguanidine hydrochloride [22]. Following their method [22] (d.s. 0.12) was prepared from propylene glycol alginate [17] and subsequently labelled using [20] to afford a product [24] (d.s. 0.02), whose aqueous solution



Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

spectrum (Fig. II-8) revealed a relatively slow tumbling nitroxide moiety (τ_c 31 ns).

The s-triazine type derivative may be of interest for applications in which a partial masking of carboxylate groups of polyuronides is desired.

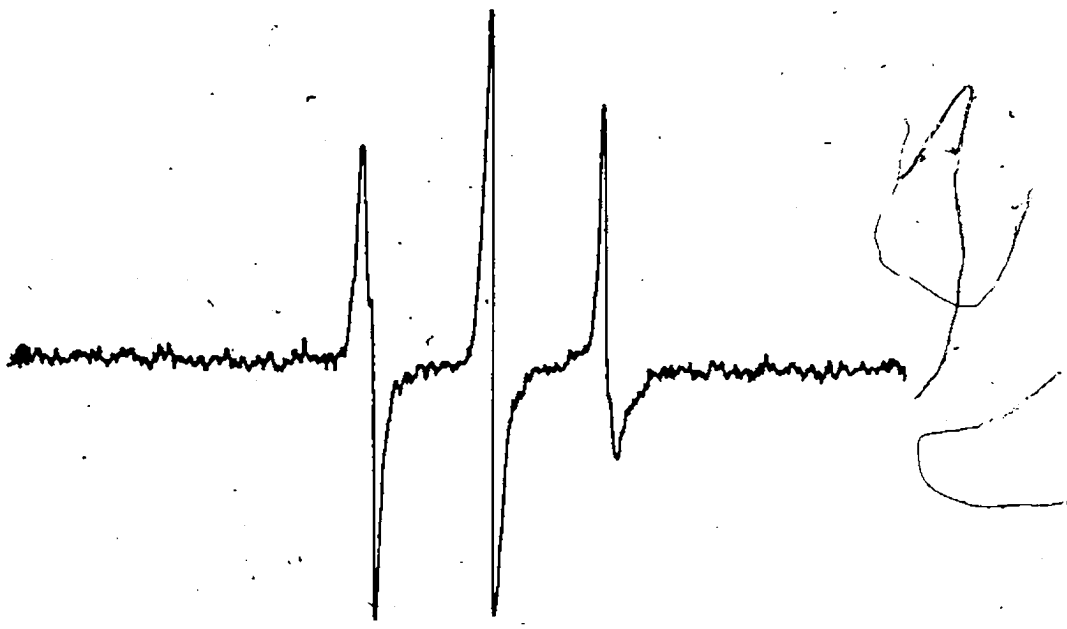


Fig. II-8. Esr spectrum of [24] in aqueous solution (at 298K).

2. Xanthan Gum

In view of its recent discovery relatively very few derivatives of xanthan gum are presently known.[31] However, significant developments in this area can be expected considering the wide range of industrial applications which the native polymer has already found. The carboxyl functions of the glucuronic acid residues are the most prominent candidates for specific derivatizations of xanthan gum and should undergo the same reactions as their counterparts in alginate, as demonstrated here by

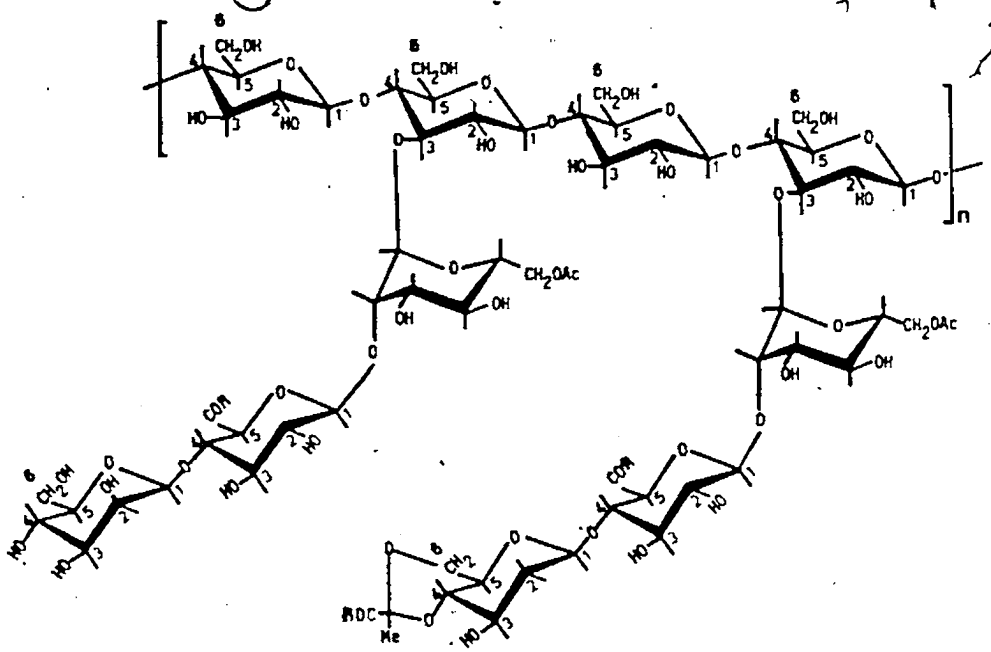
Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

the carbodiimide-mediated preparation of the carboxamide derivative.

Amides

(a) via EDC coupling

Purified xanthan gum [25] was activated via the carbodiimide procedure using EDC and subsequently condensed with amine label [15] to afford the amide [26] in which 43% of the carboxyl groups had been



[25] R = OH

[26], [27] R =

[28] R = $NH(CH_2)_3CH_3$

transformed. In light of the mild reaction conditions employed, one could also expect derivatization of the pyruvic acid acetal groups to occur.

The esr spectrum of [26] in aqueous solution reveals a fast tumbling (τ_c 6.1×10^{-11} s) nitroxide moiety which is presumably attributable to the motional freedom enjoyed by the side-chains (Fig. II-9).

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

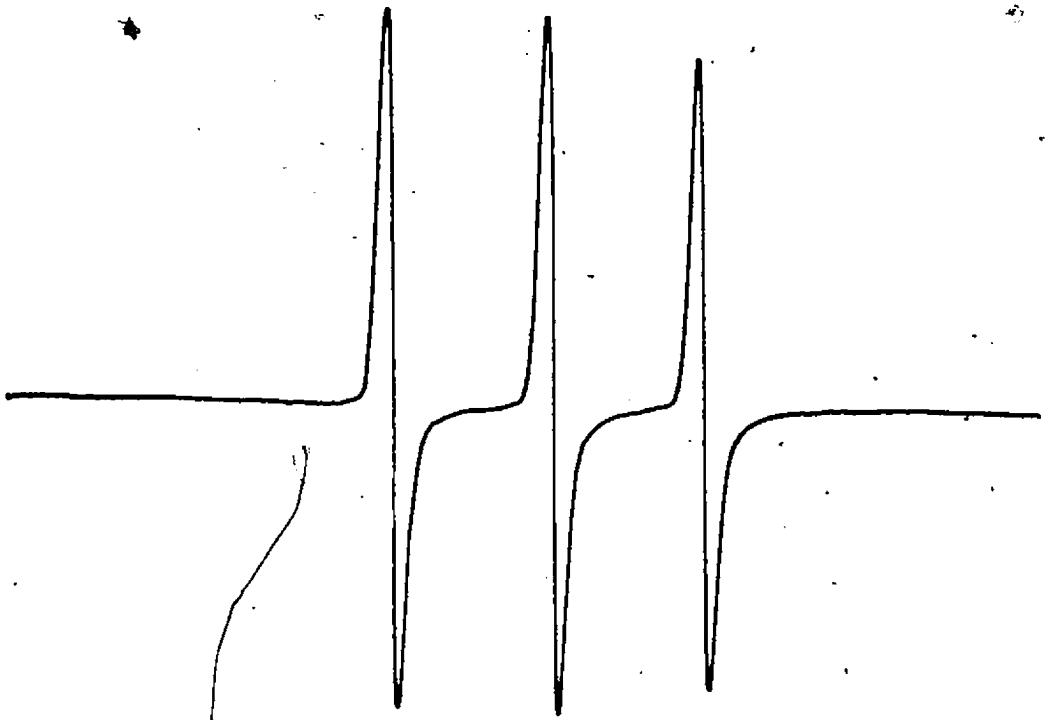


Fig. II-9. Esr spectrum of [26] in aqueous solution (at 298K).

(b) via direct condensation

A procedure has been described[32] for the preparation of "amine" alginates which involves the condensation of amines with solid alginate in the presence of small amounts of water. It is unclear, however, from this report what the term "amine alginates" actually implies in terms of the structure of such derivatives, since no further information is provided; assuming the formation of covalent linkages between the amines and hexuronide carboxylates, such products should be more appropriately classified as amides.

Nevertheless, the procedure appeared to be interesting enough to warrant some preliminary investigations using xanthan gum as substrate. Thus, xanthan gum [25] was condensed with

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

amine label [15] following this procedure to yield a product [27] whose esr spectrum (Fig. II-10) revealed the presence of both "bound" and some "free" label, after purification by dialysis.

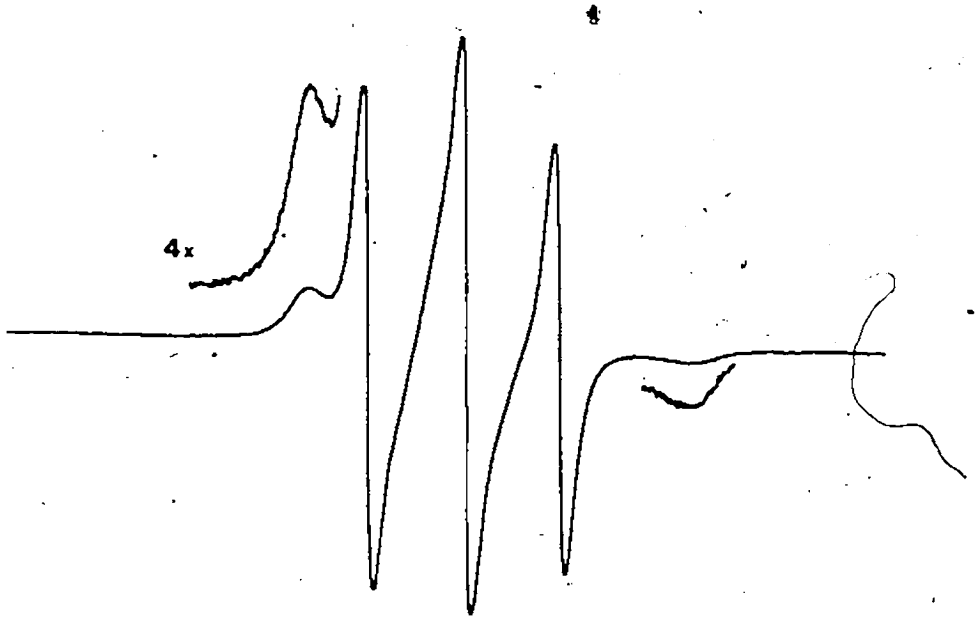


Fig. II-10. Esr spectrum of solid [27] under methanol (at 298K).

Encouraged by this evidence for the formation of a covalent derivative, the reaction was performed using a diamagnetic amine, n-octadecylamine $[CH_3(CH_2)_{17}NH_2]$. The d.s. of the resulting product [28] could not, however, be exactly established by microanalysis in view of the presence of lower molecular weight fractions contained in the amine (which was 90% technical grade). The C/N ratio found corresponded roughly to a fully substituted product and preliminary ^{13}C nmr experiments of aqueous solutions of [28] (at 37°C) confirmed the presence of the amine substituent with a broad, unresolved peak, centered at 32 ppm which can be assigned to the methylene carbons (see Appendix).

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

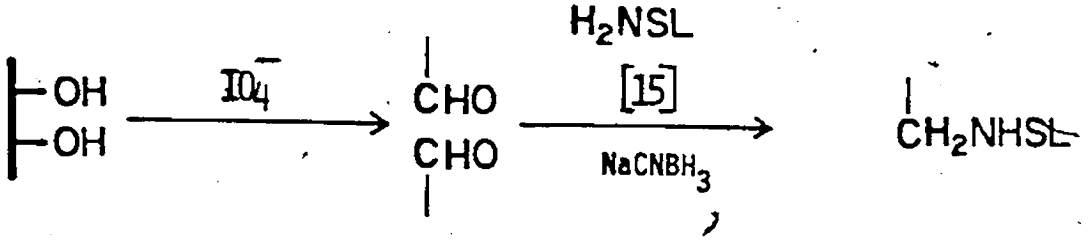
Pending further investigations, this low-cost modification procedure may be of substantial industrial benefit.

3. Cellulose

(i) Amines

(a) via reductive amination of dialdehyde cellulose

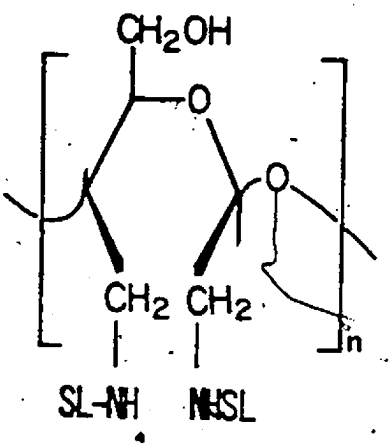
Following Nevell's procedure,[33] cellulose [29] was oxidized with periodate to the dialdehyde derivative [30] to obtain products with three different degrees of oxidation (d.o.), 0.05, 0.19, and 0.34, respectively. Subsequent reductive amination of these materials produced the spin-labelled derivatives [31]. As in the case of alginate, the conversion into spin-labelled amines [31] did not seem to linearly correspond to the increasing d.o. of the precursors. D.s. values of 0.01, 0.03, and 0.03 were obtained for [31] from the dialdehyde samples with d.o. 0.05, 0.19,



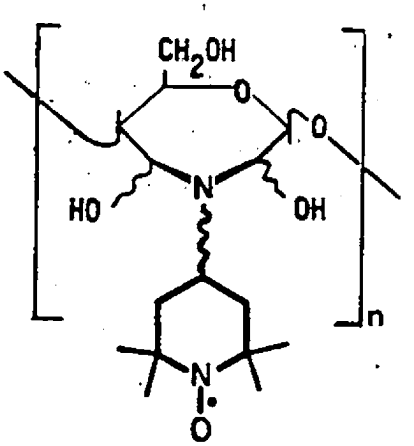
[29] [30] [31]

and 0.34, respectively. The low overall spin label incorporations can probably be attributed to the fact that periodates, in contrast to most other oxidizing agents, can penetrate both microcrystalline and amorphous regions of cellulose fibres.[33] The low yields may consequently be merely a reflection of the inability of the bulky nitroxide molecules to fully penetrate the microcrystalline regions. Such reasoning seems justifiable in light of the otherwise great efficiency of the reductive

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.



[31A]



[31B]

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

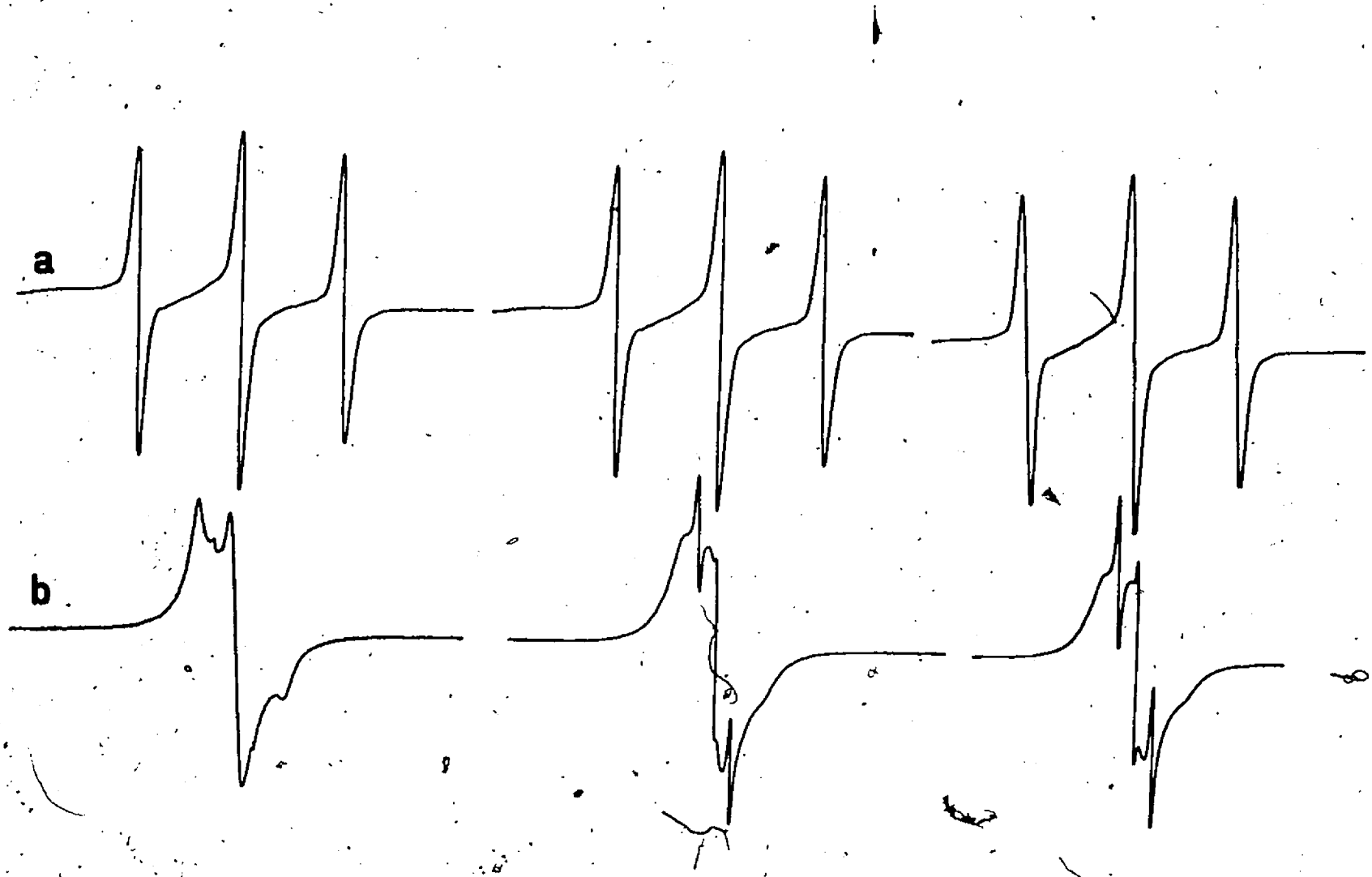


Fig. II-11. Esr spectra of [31] derived from dialdehyde cellulose samples ([30]) with d.o. 0.05 (left), 0.19 (centre), 0.34 (right), respectively; (a) aqueous suspension; (b) solid; (298K)

amination procedure, and of a recent esr study of the accessibilty of cellulose which came to similar conclusions.[8,34] Interestingly, the esr spectra of [31] in aqueous suspension (Fig. II-11a), indicate two distinct nitroxide populations of which one enjoys a substantially greater freedom of motional reorientation than the other; this finding gives further credence to the above "accessibility" arguments.

As for the corresponding alginate derivatives ([18]), the esr spectra (Fig. II-11b) of the cellulose derivatives [31] reveal dipole-dipole interactions between neighbouring spins from which it may again be inferred that structures containing two nitroxides per periodate-cleaved glucose residue [31A] predominate over those bearing only one label, as exemplified (from a series of other possibilities) by [31B]. The strongest evidence for this inference derives from the observation of dipolar interactions for cellulose derivatives with low d.o. Assuming random oxidation and subsequent labelling of the polymer and with due consideration of the aforementioned "accessibility" factor, it is reasonable to expect the separation between single nitroxides attached to different hexose units to be too great for significant contributions to the dipolar interactions to arise from this source. Thus, these findings are best accommodated by structural units of the type [31A].

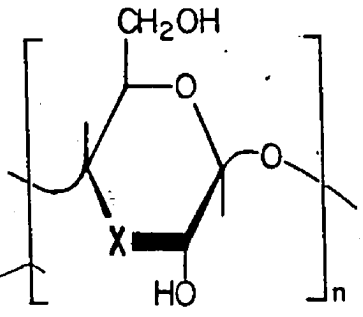
(b) via reductive amination of C2- and C3-oxycellulose

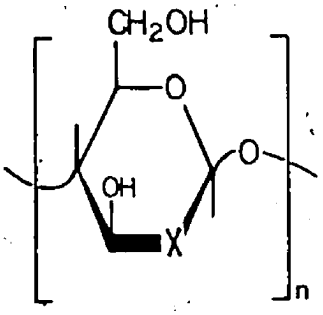
In the next round of experiments cellulose was oxidized* without any ring cleavage, using acetic anhydride-DMSO,[35] to afford C2- and C3-oxycellulose, [32] and [33], respectively. The degrees of oxidation of these derivatives, as determined by microanalysis, were 0.85 and 0.80,

*These samples were kindly provided by Dr. J. Defaye, C.N.R.S., Grenoble, France.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

respectively. Reductive amination in methanolic medium afforded the corresponding amine derivatives [34] and [35] (d.s. 0.28 and 0.09,





[33] X= C=O

[35] X= CH-NH-SL

[32] X= C=O

[34] X= CH-NH-SL

respectively). The lower d.s. value obtained for [34] may be due to steric hindrance by the bulky C6-trityl group.

The two products exhibited interesting differences in their solubility properties; the C3-labelled [35] is watersoluble but insoluble in organic solvents, whereas [34] is water-insoluble but slowly dissolves in chloroform (see I-B). The esr spectra of these materials clearly demonstrate these solubility differences (Fig. II-12): the aqueous solution spectrum of [35] reveals a slow-tumbling nitroxide moiety while [34] produces in the same medium a "polycrystalline" spectrum (Fig. II-12a) (see I-C (i)); on the other hand, the nitroxide moiety of [34] in chloroform solution is highly mobile in comparison to that of [35] (Fig. II-12b).

The hydrophobicity of the C6-trityl of [34] is mainly responsible

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

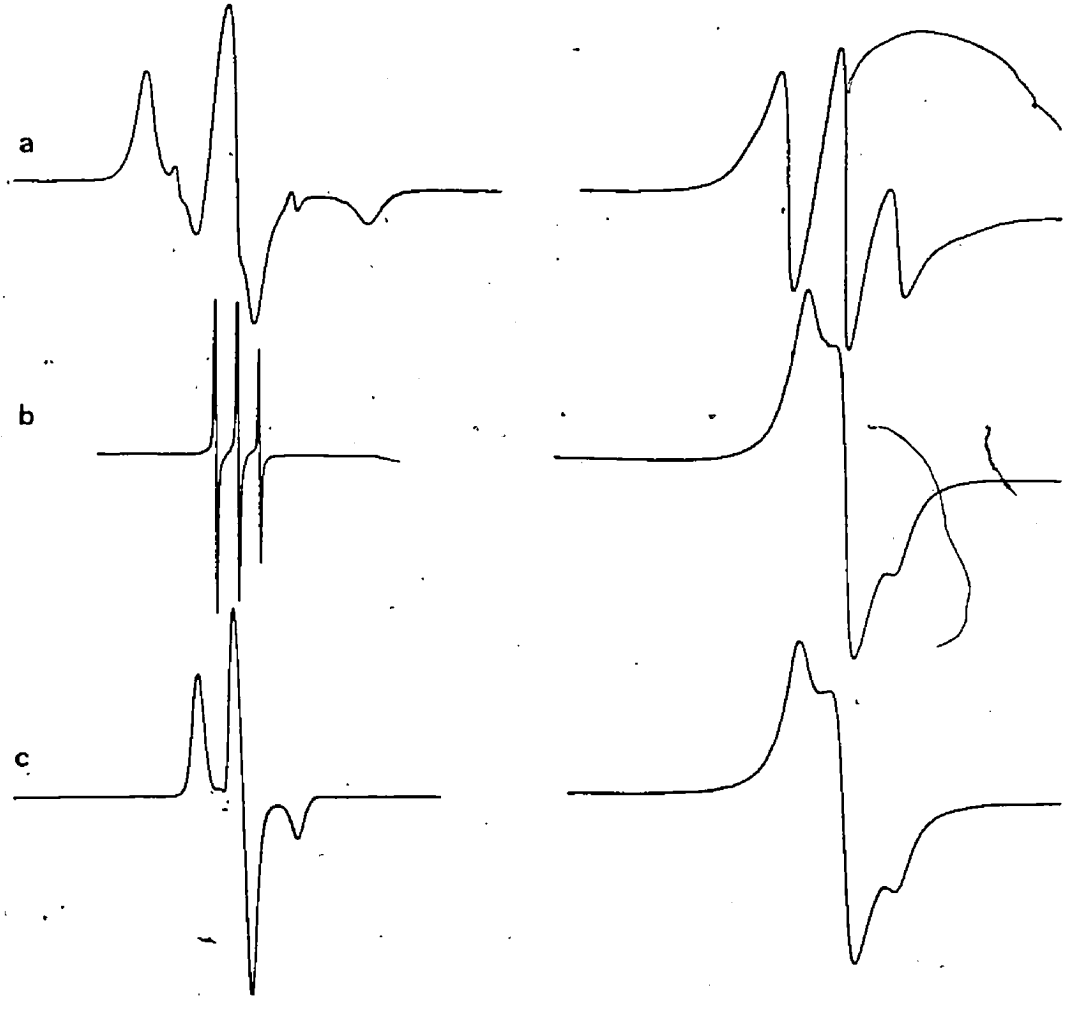


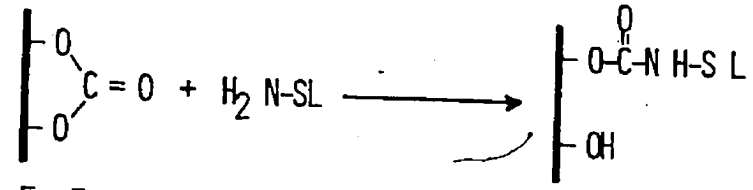
Fig. II-12. Esr spectra of [34] (left) and [35] (right), (a) in water; (c) in $CHCl_3$; (at 298K); (c) frozen (77K).

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

for the observed solubility differences. It is quite obvious that these oxycellulose derivatives, in conjunction with the reductive amination procedure, would be ideally suited for various studies some of which will be discussed in Chapter III.

(ii) Urethanes

Five-membered cyclic carbonate derivatives, obtained from the reaction of ethylchloroformate with vicinal diols, have been reported for a number of carbohydrates including polysaccharides.[36] These derivatives form stable urethane products upon reaction with amine nucleophiles. Commercial cellulose carbonate (d.s. 0.21) [36] was derivatized in aqueous medium to afford the spin labelled derivative [37] (d.s. 0.08).



[36] [15] [37]

It is interesting to note that (as for [31]) an aqueous suspension of [37] produced a complex spectrum consisting of a partially resolved broad component and a more mobile (τ_c 9.1×10^{-11}s) one. Differences in the morphology of [37] are therefore again indicated; indeed the properties of [37] varied with its history: when prepared from methanol, the product had a lower d.s. and its esr spectrum (Fig. II-13b) resembled that of a polycrystalline sample, whereas the product obtained from reaction in aqueous medium revealed strong dipolar interactions. It can therefore be concluded that these differences are attributable to the greater accessibility of [36] to aqueous solvent.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

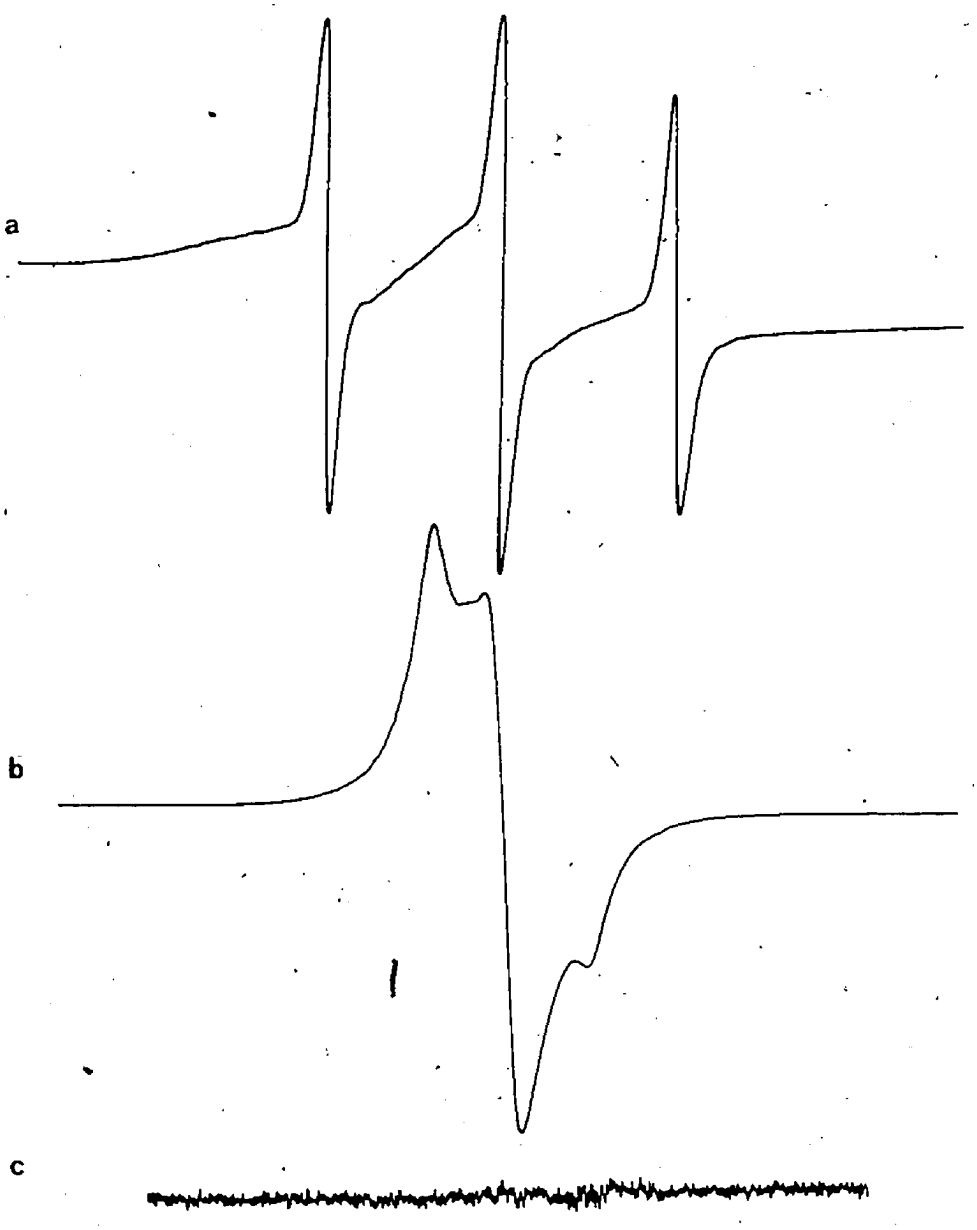
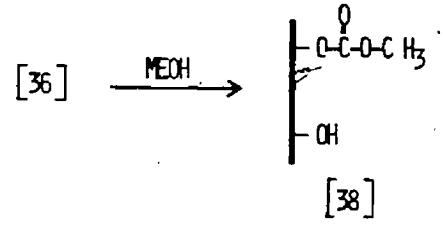


Fig. II-13. Esr spectra of [37] (a) aqueous suspension, (298K); (b) frozen (77K); (c) control experiment: [36] treated with [1] under identical conditions.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

A control experiment in which [36] was treated with the unreactive label [1], proved that no adsorption phenomena were present in this set of experiments (Fig. II-13c).

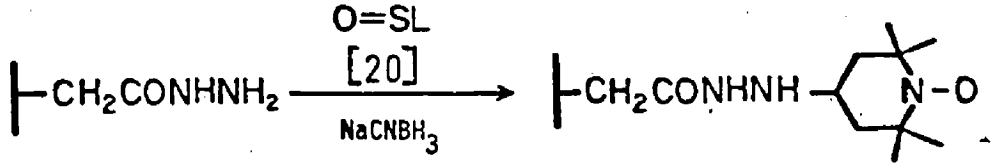
Finally, it should be noted that [36] in methanol may possibly undergo side-reactions[37] (formation of O-methoxycarbonyl derivatives [38]) which could be partially responsible for the lower d.s. of [37] obtained from this medium.



[38]

(iii) Hydrazine

Cellulose hydrazine derivatives have found various applications as reactive intermediates,[29] for enzyme immobilization,[38] or for metal-collection.[39] Their synthesis can be achieved via the C6-chlorodeoxy derivative[39] or, more conveniently, via commercially available carboxy-methyl cellulose.[29,40] Using the latter method, cellulose hydrazine [39]



[39] [40]

(d.s. 0.5) was prepared and subsequently reductively aminated, using [20], to yield the hydrazine [40] (d.s. 0.003) whose aqueous solution spectrum is shown in Figure II-14. The nitroxide moiety of [40] displayed a relatively slow motional reorientation time (τ_c 17 ns).

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

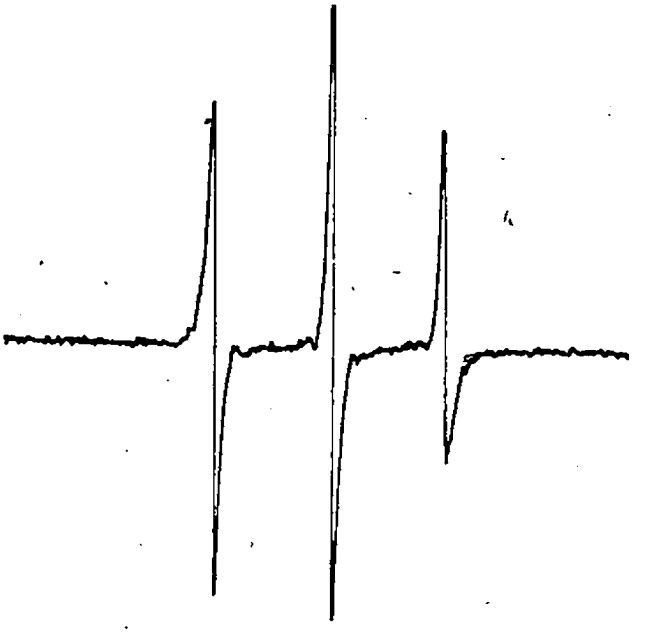


Fig. II-14. Esr spectrum of [40] in aqueous solution (298K).

II-C. Summary

It is evident from the preceding section that a number of reactions satisfied the requirements for specific and medium to high efficient conversion of polysaccharides. The carbodiimide-mediated amidation falls into this category, as does the formation of reactive intermediates via hydrazidation or specific oxidation of suitably-blocked cellulose. The modification of vicinal diols via periodate-oxidation or cyclic carbonate formation is less specific; but the former procedure may, if performed under controlled conditions, in some cases be useful (see, e.g., V-B 2). Among the other methods examined, the esterification of alginic acid with alcohols seems to be promising and should be investigated for some other polysaccharide systems. The same holds true for the amidation reactions of alginic esters.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

It is obvious that the use of sodium cyanoborohydride is mainly responsible for the utility and versatility of almost all of the above modification procedures.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

References

1. R. Köhler and W. Dierichs, U.S. Pat., 2,881,161 (1959); Chem. Abstr., 54, 7926d (1960).

2. Brit. Pat., 768,309 (1957), (cited in ref. 4).

3. K. W. Kirby, Ph.D. thesis, Purdue University, (1958).

4. R. H. McDowell, J. Soc. Cosmet. Chem., 21, 441 (1970).

5. R. L. Taylor and H. E. Conrad, Biochemistry, 11, 1383 (1972); see also I. Danishefsky and E. Siskovic, Carbohydr. Res., 16, 199 (1971).

6. R. L. M. Allen, Color Chemistry, Appleton-Century-Crofts, New York, 1971.

7. M. J. Adam, Ph.D. thesis, University of British Columbia (1980).

8. J. D. Aplin, Ph.D. thesis, University of British Columbia, (1979).

9. J. D. Aplin, M. A. Bernstein, C. F. A. Culling, L. D. Hall, and P. E. Reid, Carbohydr. Res., 70, C9 (1979); L. D. Hall and J. C. Waterton, J. Am. Chem. Soc., 101, 3697 (1979); L. D. Hall and M. Yalpani, Carbohydr. Res., 78, C4 (1980).

10. G. O. Aspinall, in MTP Intern. Rev. Sci., Org. Chem. Ser. One, 7, 295 (1973).

11. H. G. Khorana, Chem. Rev., 53, 145 (1953).

12. (a) K. P. Khomyakov, M. A. Penenzhik, A. D. Virnik, and Z. A. Rogovin, Vysokomol. Soyed., 7, 1030, 1035 (1965), (1140, 1145 in transl.); (b) A. Gabert, F. Kretzschmann, W. D. Wiezorek, and P. Wolf, Brit. Pat., 1,069,820 (1967).

13. A. Takahashi, T. Omata, and S. Takahashi, Sen-I Gakkaishi, 35, 70 (1973); Chem. Abstr., 81, 39195 (1974).

14. Neth. Pat. Appl., 77,11034 (1977); Chem. Abstr., 91, 63007 (1980).

15. L. Goldstein, M. Pecht, S. Blumenberg, D. Atlas, and Y. Levin, Biochemistry, 9, 2322 (1970); T. Jaraslava, V. Jiri, and V. Drahomira, Collect. Czech. Chem. Commun., 44, 3411 (1979).

16. T. Painter and B. Larsen, Acta Chem. Scand., 24, 813 (1970).

17. I. L. Andresen, T. Painter, and O. Smidsrød, Carbohydr. Res., 59, 563 (1977); and references therein.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

18. S. Nadzhimutdinov, A. A. Sarymsakov, and K. U. Usmanov, Dokl. Akad. Nauk SSSR, 219, 1371 (1974); (892 in transl.).

19. G. E. Means and R. E. Feeney, Chemical Modification of Proteins, Holden-Day Inc., San Francisco, (1971).

20. K. Holmberg and B. Hansen, Acta Chem. Scand., B33, 410 (1979).

21. A. F. Hegarty, M. T. McCormack, G. Ferguson, P. J. Roberts, J. Amer. Chem. Soc., 99, 2015 (1977).

22. T. Tenforde, R. A. Fawwaz, and N. K. Freeman, J. Org. Chem., 37, 3372 (1972).

23. C. P. Iovine and D. K. Ray-Chaudhuri, U.S. Pat., 4,129,722 (1978).

24. A. Wassermann, Nature, 158, 271 (1946); N. H. Chamberlain, G. E. Cunningham, and J. B. Speakman, ibid., 158, 553 (1946).

25. R. G. Schweiger, J. Org. Chem., 27, 1786 (1962).

26. A. B. Steiner and W. H. McNeely, Ind. Eng. Chem., 43, 2073 (1951).

27. Z. Wypych, Roczniki Chemii, 43, 1616 (1969).

28. See for example, J. C. Lee, H. L. Weith, and P. T. Gilham, Biochemistry, 9, 113 (1970); Z. I. Kuznetsova, E. G. Ivanova, and A. I. Usov, Izvest. Akad. Mauk SSSR, Ser. Khim., 1858 (1973).

29. H. Andresz, G. C. Richter, and B. Pfannemüller, Makromol. Chem., 179, 301 (1978).

30. C. S. Lee and K. Maekawa, Agr. Biol. Chem., 40, 785 (1976).

31. W. H. McNeely and K. S. Kang, in Industrial Gums, R. L. Whistler ed., 473 (1973); G. Holzwarth, Carbohydr. Res., 66, 173 (1978).

32. Kelco Algin, (second edn.), Kelco Co., San Diego, California, 41 (1976).

33. P. T. Nevell, Methods Carbohydr. Chem., 3, 164 (1963).

34. L. D. Hall and J. D. Aplin, J. Amer. Chem. Soc., in press.

35. N. Pravdic and H. G. Fletcher, Carbohydr. Res., 19, 353 (1971).

36. W. M. Doane, B. S. Shasha, E. I. Stout, C. R. Russell, and C. E. Rist, ibid., 11, 321 (1969); J. F. Kennedy and H. C. Tun, Antimicrob. Ag. Chemother., 3, 575 (1975).

37. E. I. Stout, W. M. Doane, B. S. Shasha, C. R. Russell, and C. E. Rist, Tetrahedr. Lett., 45, 4481 (1967).

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

38. E. Junowicz and S. E. Charm, Biochim. Biophys. Acta, 428, 157 (1976).

39. S. Machida and Y. Sueyoshi, Angew. Makromol. Chem., 49, 171 (1976).

40. H. L. Weith, J. L. Wiebers, and P. T. Gilham, Biochemistry, 9, 4396 (1970).

41. H. Grasdalen, B. Larsen, and O. Smidsrød, Carbohydr. Res., 56, C11 (1977).

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

CHAPTER III

BRANCHED-CHAIN CHITOSAN DERIVATIVES

III-A. Introduction

The unique properties and importance of chitin and chitosan have already been briefly indicated in Chapter I. Despite the serious limitations imposed by their insolubility, various derivatives of both polymers have been successfully prepared in analogy to equivalent cellulose derivatives.[1] More recently, the primary amine functions of chitosan have been derivatized with a number of anhydrides,[2] and common aliphatic and aromatic aldehydes,[3,4] invariably affording insoluble products. Although certain watersoluble ether and salt derivatives of chitosan are known,[1] no attempts had previously been made to affect the solubilization of chitosan by introducing suitable hydrophilic moieties into the polymer. The main impetus for the work described in this chapter derived from the goal to solubilize these rather intractable materials using the latter method. We were also interested in studying the molecular properties of the branched-chain derivatives obtained by covalent attachment of various carbohydrate moieties to these polymers.

Considerable efforts have been directed at the conversion of linear polysaccharides into branched-chain analogues which are of interest for a variety of reasons,[5] including the investigation of lectin-carbohydrate reactions,[6] the preparation of model compounds in the fields of allergy,[7] enzymology,[8] and immunology,[9] and the study of the physical properties of

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

branched-chain derivatives.[10] Previous workers have applied various synthetic routes[11] such as copolymerization,[12] orthoesters,[10] acetobromo-sugars,[13] hydrazones,[14] or enzymic glycolysations[15] to cellulose, amylose, alginic acid, and other polysaccharides. These procedures, however suffer from various limitations since they require (i) specific protection of the linear polysaccharide, such as in the reaction of 1,2-orthoacetate sugars with 2,3-di-O-phenylcarbomoyl derivatives of amylose and cellulose; (ii) activation of the sugar which is to form the side chain; or (iii) reaction conditions which lead to partial or extensive polysaccharide degradation, e.g., using hydrazinehydrate. Most of the reactions are also laborious and low-yielding, all reasons which mitigate against routine or large-scale adaption.

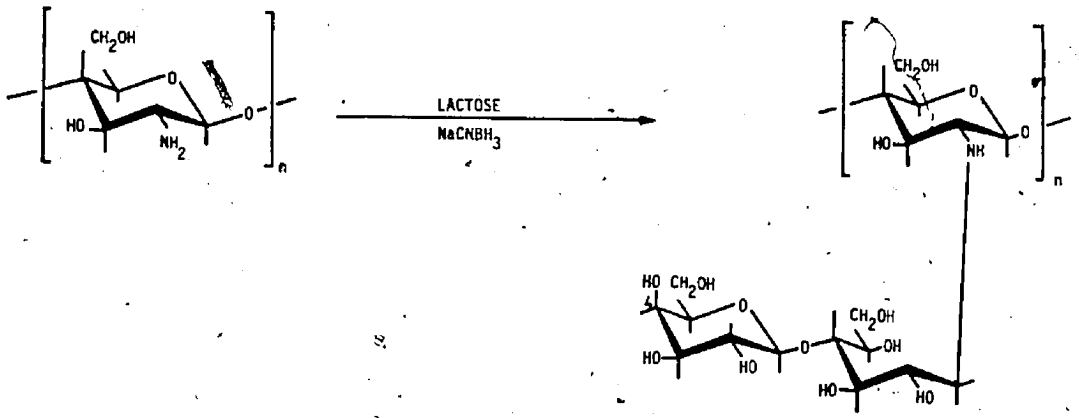
The work described in this chapter demonstrates the utility of the reductive amination reaction for the synthesis of comb-like chitosan derivatives, whose solution and molecular properties were examined. Although the study performed herein constitutes only a beginning, it, nevertheless, clearly indicates the immense variety of unique and useful properties which these derivatives exhibit and their significance for the potential application in various areas.

III-B. Synthesis and Properties of Branched-chain Chitosan Derivatives

1. Synthesis

Using chitosan as an exemplar, a method was devised which is suitable for the transformation of linear, amine-containing polysaccharides into stable, branched-chain derivatives. The reaction itself, summarized in scheme 1, represents a further example of the reductive amination procedure which is compatible with essentially any aldehydo

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.



scheme 1

sugar. Under typical conditions, chitosan [1], dissolved in a mixture (1:1) of dilute (1%) aqueous acetic acid and methanol, was reductively alkylated using a solution of the carbonyl-containing sugar [1.1-3.2 molar equivalents per hexosamine residue (mol/GlcN)] at room temperature. The reactions of chitosan with various aldehyde-, keto-, lactone, and non-reducing sugars and cyclohexanone are summarized in Table III-1.

Table III-1 reveals the following important features. The reactions of chitosan with aldehydo sugars proceed, in general, smoothly yielding products with mostly high degrees of substitution (d.s.) in almost all cases, these reactions were accompanied by the formation of soft to very rigid, transparent or milky-white gels with, in the latter case, attendant synereses. Monosaccharides produced gels at almost twice the rate of disaccharides and the d.s. of the chitosan products increased with increasing amounts of aldehydo sugar used (with one exception for lactose). It may, in passing, be mentioned that the

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

Table III-1. Reactions of Chitosan with Carbohydrates and Other Compounds

Compound added	Conditions (mol/GlcN)	Time (hr)	Product Gel[a]	[code]	d.s.[b]
glucose	1.33	8	6	[3]	n.d.[c]
	3.00	8	1,5		0.9
galactose	2.03	4	1,3	[2]	0.7
	2.22	4	1,3		1.0
glucosamine[e]	1.3-1.6	8	4,5	[18]	0
	2.7	72	4,5		0
galactosamine[e]	1.17-2.73	72	6	[19]	0
N-acetylglucosamine	1.6	12	1,3	[4]	n.d.
	1.6	24	1,5		1.0
cellobiose	1.17	12	7	[5]	n.d.
	2.2	36	7		0.3
lactose	1.17	10	6	[6]	0.25
	1.5	30	6		0.14
	1.54	144	1,5		0.8
	2.92-2.94	24	1,5	[7]	"2.0"[d]
maltose	1.7	12	7	[9]	n.d.
	1.7	30	1,5		0.6
melibiose	1.11	4	1,4	[10]	n.d.
	1.11	18	1,5		0.6
maltotriose	1.3	12	6	[20]	0
fructose	2.2-3.2	30	7	[13]	0
α-glucoheptonic lactone	3.2	72	6	[23]	0
melizitose	1.05	24	7	[12]	n.d.
	1.05	240	2		0
trehalose	1.3	72	6	[11]	0
cyclohexanone	4.5	24	7	[16]	0.5

[a](1-5): gels formed; (1) rigid, (2) ropy, (3) transparent, (4) very soft, (5) white; (6) gel not formed, (7) very viscous solution

[b]obtained from microanalysis [c]not determined

[d]see text [e]see Experimental section

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

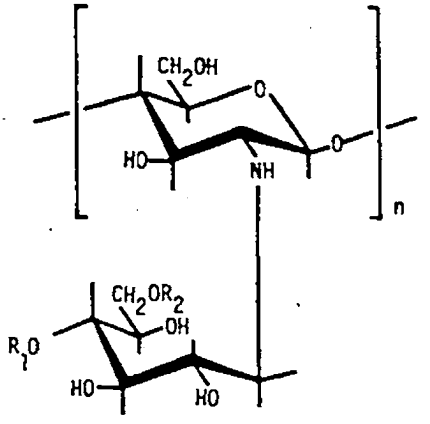
amounts of aldehydo sugars (as well as aromatic aldehydes, in Chapter V) required for the formation of fully (or highly) substituted chitosan derivatives were found to be far less (by a factor of 2-40) than those reported by Hirano et al.[3] for their Schiff's base derivatives. A rather unusual situation was encountered for the reaction of relatively large amounts of lactose (2.9 mol/GlcN) which gave products with d.s. corresponding to 2.0 as determined by microanalysis. The question as to whether this value arose from two lactose residues covalently linked to each glucosamine unit or from a mixture of bound and "free" lactose unit could be readily solved by both ^{13}C-nmr and electron microscopy in in favour of the latter case as will be subsequently discussed (III-C, E).

In this series of experiments, no products were obtained for the reactions with glucosamine and galactosamine despite the fact that very soft white gels were produced with glucosamine. Coulombic repulsion between the protonated amine groups of chitosan (pH ~4.5) and those at C2 of the respective hexosamines seems to be primarily responsible for the lack of product formation in the latter two cases since a fully substituted chitosan derivative [4] was obtained using N-acetglucosamine. The amine function at C2 of the side-chain of [4] provides, upon N-deacetylation, a convenient locus for further reductive alkylation reactions which could afford branched, tree-like derivatives.

The facility with which chitosan can be selectively modified is also demonstrated by its reaction with cyclohexanone to yield [16] (d.s. 0.5). The versatility of this procedure was further exemplified by the specifically oxidized C6'-aldehydo derivatives [14] and [15] which were derived from the corresponding lactityl- and melobiityl derivatives [6] and [10] using galactose oxidase (see IV-B 1). Derivatives [14]

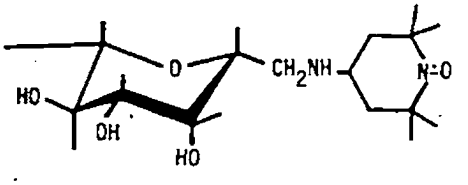
Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

and [15] are useful intermediates as demonstrated by their conversion into the spin-labelled derivatives [34] and [35], respectively (see III-D). [14] and [15] could similarly be used, if desired, to prepare chitosan derivatives with even longer side-chains via reductive amination.



[34] R_1=X, R_2=H
[35] R_1=H, R_2=X

X=



The apparent failure of the reactions with maltotriose, fructose, and α-glucoheptonic lactone is considered to be spurious in view of the ease with which the previous reactions proceeded. These reactions were, however, not further investigated. Noteworthy is, nevertheless, the fact that the reaction of fructose afforded very viscous solutions.

A more detailed account of the observed gelling processes will be given in III-B 3.

2. Solute Interactions of Chitosan

Previous studies had shown that acidified blends of chitosan and polyols such as sorbitol and glycerol, produce high viscosity solutions,[16] while aqueous oxalic acid solutions of chitosan afford gels.[17] This, in

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

conjunction with our observation that both fructose and glucosamine significantly altered the solution viscosity of chitosan, appeared to indicate that in the reactions studied here, processes other than the chemical reaction between chitosan and sugar substrate alone, were, at least in these specific cases, responsible for the solution properties of the reaction mixtures. In order to test this hypothesis, two non-reducing saccharides, melizitose and trehalose, were added to chitosan solutions and it was found that, in the former case, the viscosity gradually increased leading eventually (10 days) to the formation of a ropy gel (see Table III-1).

Next, an aqueous solution of sodium cyanoborohydride (700 mg, 11.1 mM, 15 ml) was added to a 1% solution of chitosan (30 ml) and a sharp increase in solution viscosity was observed within 5 minutes; no gel was obtained, however, within 30 days.* When, on the other hand, the reaction of chitosan with lactose (3.9 mol/GlcN) was performed under omission of sodium cyanoborohydride, no gels were formed and a product [8] with very low d.s. (0.1) was obtained. These facts would seem to indicate a specific interaction of chitosan and the reducing agent which significantly contributes to the formation of gels.

In control experiments with solutions of chitosan itself or of mixtures (1:2 w/w) of chitosan and a series of other solutes, including alkali earth, lanthanide, and transition metal salts, no viscosity increases or gel formations were observed over periods of up to 30 days.

---

*Gel formation could also not be induced by cooling (5°C).

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

3. Gelling Processes

From the results discussed above it is obvious that a complex series of phenomena contribute to the observed gelling processes, and we will now attempt to identify some of these.

(i) Solute-mediated gelation

Although the interactions of solutes with some acidic polysaccharides have been investigated[18] no equivalent work exists for cationic polysaccharides, with the exception of several reports on the formation of polyelectrolyte complexes of chitosan and anionic polysaccharides.[19] Assuming that the processes involved for both types of ionic polysaccharides are fairly similar in nature, two types of solute effects can be considered:

(a) Electrolytes have been implicated in the gelation of various polysaccharides, most notably in those of alginate[20] and carrageenan,[21] where they mediate helix aggregations by way of specific bonding interactions with carboxyl and hydroxyl groups. In general however, the presence of metal ions has unpredictable effects on polysaccharides in solution, which cannot be simply rationalized in terms of coulombic attractions.[22] Thus, some electrolytes decrease the viscosity of alginate and guaran but have the opposite effect for carrageenan and hypnean.[22,23]

The action of sodium cyanoborohydride (and oxalic acid) on chitosan solutions may therefore be described as specific interactions of these relatively large anions with chitosan.

(b) Non-electrolytes, such as sugars and alcohols are known[23,24] to induce gelation of hypnean dispersions, with only small quantities of sugar being required for the formation of very strong

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

gels. In such systems sugars are considered[21,25] to act, on the one hand, as competing agents for solvent thereby leading to a decreased solvation of the polysaccharide chains and, on the other, as promoters of inter-chain hydrogen bonding; both modes of action resulting in gel formation.

Similar phenomena must be also operative for the case of the viscosity increase or gelation of chitosan solutions in the presence of various carbohydrates, such as fructose, glucosamine, and melizitose, which did not form chitosan derivatives.

(ii) Gelation of branched-chain derivatives

The gelation of the branched-chain chitosan derivatives probably involves very similar factors as discussed above. Noteworthy is the generally greater gel strength of these derivatives in comparison with that of the solute-mediated products. It can be invoked that the covalently-linked saccharides allow for a more proximate interchain-association than in the latter case. In addition to these factors, the extent and regularity of the substitutions on chitosan appear to be of importance for gelation, since all derivatives with high d.s. formed rigid gels, whereas for derivatives with d.s. ≤ 0.3 no gels (or only viscosity increases) were observed, e.g., for cellobiose and lactose. In a related study, Schweiger[26] has attributed the unusual compatibility of cellulose sulfate derivatives to the uniformity of substitution.

4. Other Properties

The utility of the branched-chain chitosan derivatives is further illustrated by the properties which they exhibit after isolation from the reaction mixtures. In contrast to other known derivatives, e.g., the arylidene chitosan derivatives discussed in Chapter V, all of these

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

samples retained their gelling properties when the dry powders were reconstituted in either neutral aqueous or slightly acidic media. Thus, the derivatives [3], [6], [7], and [9], at concentrations above 3-5% (some even at much lower concentrations), formed rigid gels in aqueous solutions, while [2] and [4] gelled in slightly acidic (pH 5-6) solutions; [10] was soluble in the latter medium but produced gels on addition of base. Gels obtained from [2] thinned out with time (10-12 h).

Derivatives [5] and [7] exhibited stability to alkaline media, and [5] and [10] were also compatible with 50% aqueous ethanol, the latter forming very viscous solutions. Aqueous solutions of [6] or [7] did not gel or precipitate when mixed with calcium chloride, chromium chloride, tin chloride, potassium chromate, boric acid, or several combinations of these.

Interestingly, the derivative [5], which by itself did not gel, was found to form rigid white gels (which contracted after a few hours), when mixed with alginate, and very viscous solutions, when mixed with either guaran or locust bean gum.

Our initial evaluation has demonstrated the great facility with which an intractable material, such as chitosan, can be converted, using this modification procedure, into products which, even at relatively very low d.s. (e.g., for [6] d.s. 0.14), are soluble in aqueous media. The wide range of solubility, gelling, and compatibility properties of these branched-chain derivatives appears to be rather unique for polysaccharide products and promises to be of potential utility for a variety of applications, considering the low cost of combining two surplus carbohydrates such as, for example, lactose and chitosan.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

III-C. ^{13}C nmr

^{13}C nmr was employed for the spectral assignment and structural elucidation of chitosan and a selected number of the branched-chain derivatives; the spectra are shown in Figures III-1-4, and the proposed chemical shift assignments are recorded in Table III-2.

The spectral assignment of chitosan [1] itself (Fig. III-1a) was readily accomplished by comparison with previous data reported for the monomeric aminoglycosides.[27,28] The anomeric signals of both the aminoglucose and acetamidoglucose residues of [1] were clearly resolved; the signal at lower field was attributed to the acetamido derivative while that of the amino-derivative appeared 3 ppm upfield. The signals of the other ring carbons of the two types of hexosamine residues were indistinguishable at this pH (pD 4.0) and were assigned, based on the relative proportions of acetamidoglucose and glucosamine residues in [1], to the latter (Table III-2). On lowering the pH (to pD 1.5) the resonances of [1] experienced an upfield shift of 2-3 ppm (Fig. III-1b), which was accompanied by the appearance of several additional signals. No previous ^{13}C-data for equivalent monomeric aminoglycosides (at pD 1.5) were available to confirm the proposed assignments (Table III-2) of these additional resonances.*

---

*The degradation of [1] under strongly acidic conditions (pD 1.5) could be observed only after prolonged periods of time (3-4 weeks) at 298K with the appearance of additional resonances in the anomeric region and between 60-77 ppm (C3,C4,C6).

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

TABLE III-2. Chemical Shift Assignment of Chitosan and some Derivatives

	Compound (d.s.)[h]	Chemical shift[a], ppm C1	C2	C3	C4	C5	C6	C1'	C2'	C3'	C4'	C5'	C6'
[1][b] pD4.0	β1,4-(2-deoxy-2-amino-glucopyranosyl)[k]	101.8	56.6	75.3	71.1	77.8	60.9						
	β1,4-(2-deoxy-2-acetamido-glucopyranosyl)[k]	98.6											
pD1.5	β1,4-(2-deoxy-2-amino-glucopyranosyl)	98.3	53.1	72.0	68.4	74.3	57.5						
	β1,4-(2-deoxy-2-acetamido-glucopyranosyl)	94.9	52.9	71.6	67.8								
[5] (0.3)	β1,4-(2-deoxy-2-amino-glucopyranosyl)	102.0	56.9	75.2	71.5	77.6	61.3						
	β-glucopyranosyl	103.0	74.1	76.3	n.r.	76.5	61.3						
	1-deoxyglucit-1-yl							51.3	73.9	75.5	80.6	71.5	62.6
[6] (0.9)	β1,4-(2-deoxy-2-amino-glucopyranosyl)	102.8	56.8	75.7[c]	72.0[d]	77.7	61.8						
	β-galactopyranosyl	104.0	72.0	73.5	69.0	75.7[c]	n.r.						
	1-deoxyglucit-1-yl							51.3	72.0[d]	72.0[d]	79.0[e]	n.r.	62.9
	lactose	n.r.	n.r.	73.5	69.6	76.1	n.r.	92.6[i]	n.r.	n.r.	79.0[e]	75.7[c]	n.r.
								96.5					
[6] (0.25)	β1-4-(2-deoxy-2-amino-glucopyranosyl)	102.7	57.0	74.9	n.r.	77.5	61.9						
	β-galactopyranosyl	104.0	72.0	72.8	69.1[f]	76.0	n.r.						
	1-deoxyglucit-1-yl							51.1	72.0	72.0	79.0	69.1[f]	63.0
[16] (0.5)[b]	β-1,4-(2-deoxy-2-amino-glucopyranosyl)	102.1	56.6	75.3	71.0	79.0	61.9						
		98.5					60.6						
pD4.0	cyclohexyl							59.8	30.5	25.0[g]	30.0	24.8[g]	30.5

[a] in D_2O relative to internal p-dioxane (67.40 ppm) at 315 K ; [b] solvent DOAc-d_4/D_2O; [c-f] coinciding resonances; [g] assignment may be reversed; [h] degree of substitution; [i] α,β respectively; [k] resonances coincide except as indicated; n.r. = not resolved.

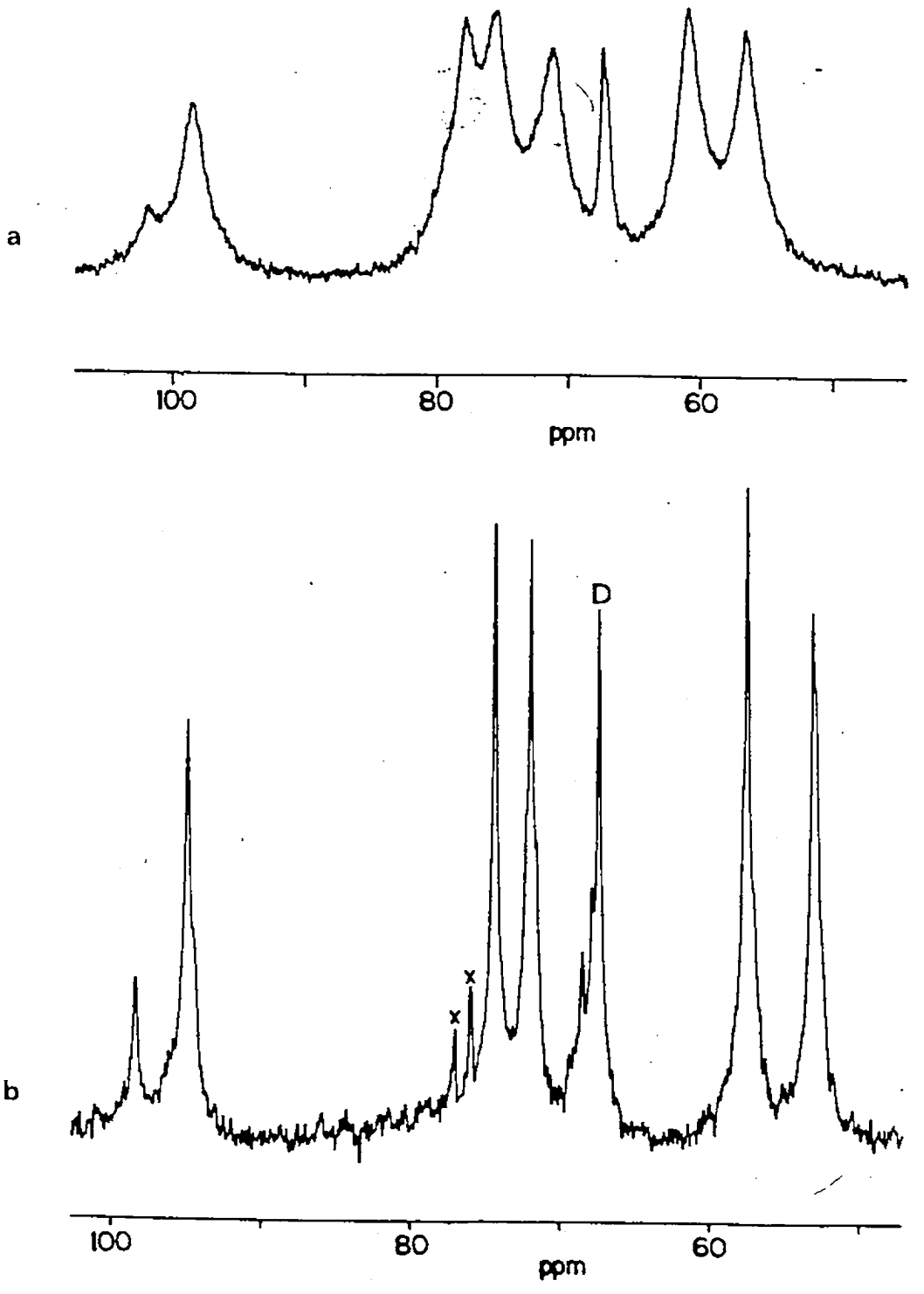


Fig. III-1. 100.6 MHz ^{13}C-nmr spectra of [1] (4% in DOAc-d_4/D_2O) at 315K (a) at pD 4; (b) at pD 1.5; (sweep width 26,000, ^{2} pulse width 13 μsec, delay 0.1 sec, 50 000 scans).

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

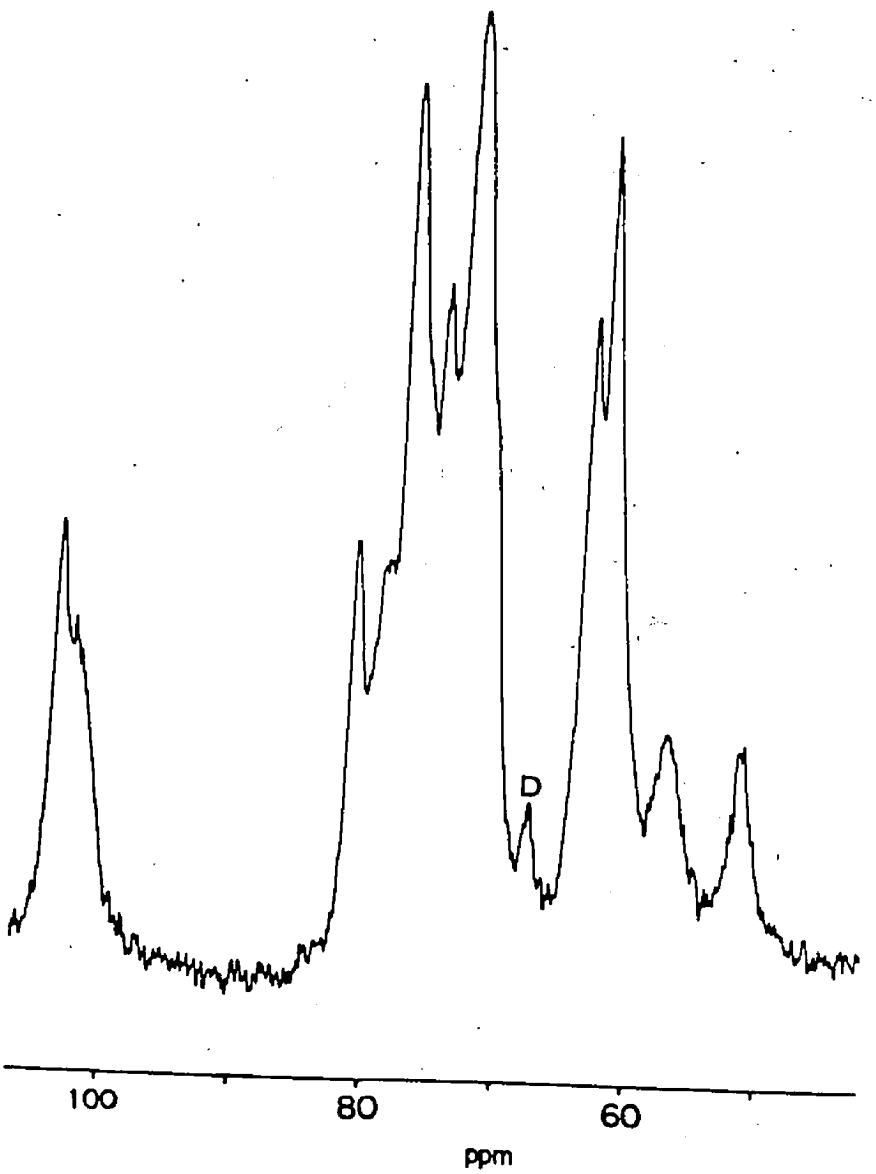


Fig. III-2. 100.6 MHz ^{13}C-nmr spectrum of [5] in D_2O (5%) at 315K; (same conditions as for [1], 81 000 scans).

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

The resonances of the polysaccharide backbone were, upon substitution, found to be displaced by small increments only. For example, the hexosamine signals of C1, C2, C4, and C6 of derivative [5] were shifted downfield by approximately 0.3 ppm, while those of C3 and C5 were shifted upfield by 0.2 ppm (Fig. III-2).* The resonances of the pending cellobiityl residues could be identified by comparison with the chemical shifts reported for cellobiose[29] and 4-[(1-Deoxycellobiit-1-yl)-amino] benzophenone (prepared analogously by reductive amination of cellobiose, see reference 30). The chemical shift of the deoxyglucityl C1' was shifted upfield by more than 40 ppm from that of the parent cellobiose. The assignments of the terminal glucopyranosyl residue of [5] were found to be in excellent agreement with the previous results of several workers.[29]

The reductive alkylation of chitosan with excess lactose (3 mol/GlcN residue) produced, as mentioned earlier, a derivative [7] whose elemental analysis indicated a d.s. of "2.0". The ^{13}C nmr spectrum of [7] (Fig. III-3a), however, revealed quite clearly that this d.s. value derived from one equivalent of "free" lactose per hexosamine equivalent, as evidenced by the presence of the two anomeric glucopyranose signals (both α and β) in the region between 90-100 ppm. The spectrum of [7] was dominated by the sharper signals of the trapped lactose and was not further assigned. The lactose complex [7] was subsequently dialyzed for five days in order to remove the unbound lactose but the resulting product [6] was found to still contain residual quantities of the trapped lactose (ca. 50%) as shown by the anomeric C1' signals at 92.6 ppm and

*Note, however, pH differences between the two samples [1] and [5]; the spectrum of the latter was recorded at pD 7.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

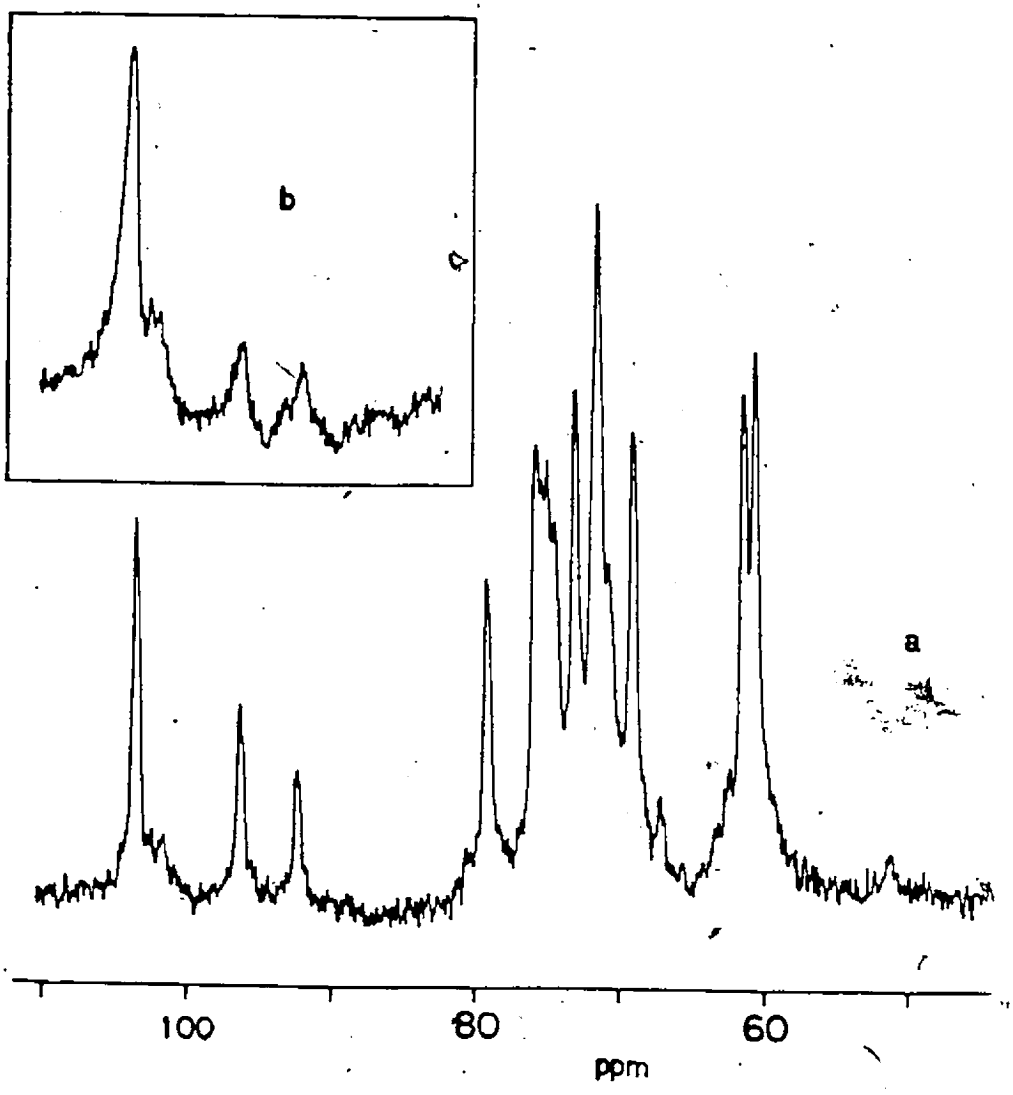


Fig. III-3. 100.6 MHz ^{13}C-nmr spectra of (a) [7] and (b) [6] (d.s. 0.9) obtained from [7] (a) after 5d dialysis; (contd.)

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

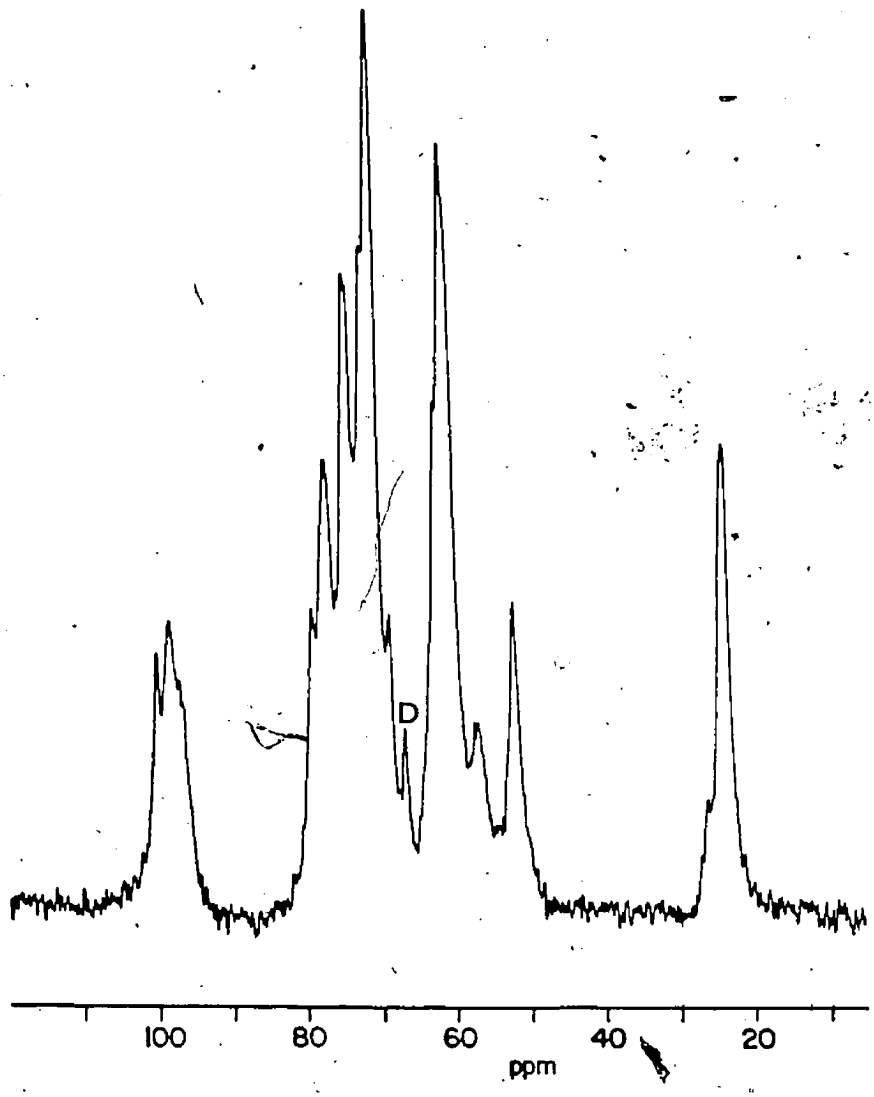


Fig. III-3. (continued) (c) ^{13}C-nmr of [6] (d.s. 0.25); at 315K in D_2O.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

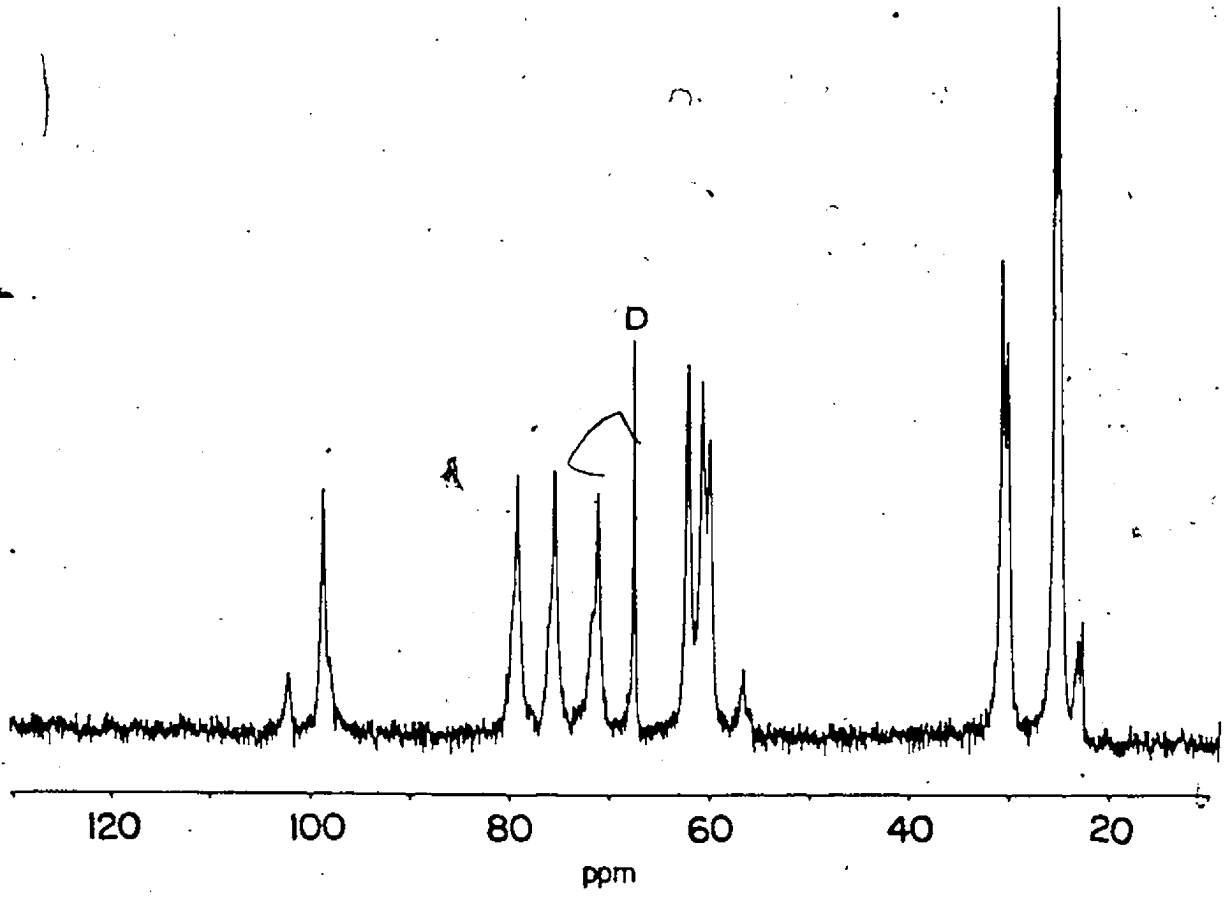


Fig. III-4. 100.6 MHz ^{13}C-nmr spectrum of [16] in DOAc-d_4/D_2O at pD 4.0 and 315K, 60,000 scans.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

96.5 ppm, respectively (Fig. III-3b). These findings, consequently, provide evidence for the formation of a strong complex between 1-deoxylactit-1-yl chitosan and lactose from which the "free" lactose is only incompletely released upon extensive dialysis. From this it may be postulated that [7] constitutes an inclusion complex in which lactose may reside in the interstices of multistranded helices of [6], somewhat similar to the well-known iodine-amylose inclusion complex. It appears that this type of association is specific to [6], since no similar phenomena have so far been observed for any of the other branched-chain chitosan derivatives.

The chemical shifts of the hexosamine residues of [6] followed the same trend as were established for those of [5] and the signals of the covalently-linked lactityl residues could be readily identified by comparison with previous assignments of lactose,[29] as well as of a series of related alkyl-deoxylactitylamine derivatives reported by Pfeffer et al.[31] and of two fluorescent deoxylactitylamine derivatives.[30] The C1' signal of the 1-deoxyglucit-1-yl residues of [6] appeared at 51.3 ppm and was in line with the corresponding signals of the alkyl derivatives of Pfeffer et al.[31] Good agreement was also obtained between the other chemical shift assignments of [6] and the results reported elsewhere.[29,30]

Figure III-3c shows the spectrum of a sample of [6] with lower d.s. (0.25) which contained no trapped lactose. The observed chemical shifts of this product agreed with the results of the previous sample, revealing only minor displacements of some resonances.

The cyclohexyl derivative [16] produced a well-dispersed spectrum (at pD 4.0) in which the methylenic ring carbons were observed in the

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

region between 24 to 31 ppm in close agreement with the corresponding signals of N-methylcyclohexylamine[32] (Fig. III-4); the cyclohexyl C1' resonance appeared at 59.8 ppm, 1.2 ppm downfield from that of the corresponding N-methyl derivative.

The chemical shifts of the carbons of the polymer backbone were found to be again in good agreement with those of the previous derivatives with the exception of the C5 signal which was displaced downfield by 1.2 ppm with respect to that of [1]. The hexosamine resonances in the spectral region between 70 to 80 ppm were further characterized by the presence of incompletely resolved signals at lower field which can presumably be assigned to the unbranched hexosamine residues since the branched residues produced, in general, sharper signals (compare the ^{13}C nmr spectra of galactomannans, discussed in Chapter IV). The C6 resonances of both the glucosamine and acetamidoglucose residues were clearly resolved and could be assigned on the basis of the corresponding monomeric aminoglycosides.[27]

The carbonyl and methyl resonances of the N-acetate groups were observed at 175 ppm (±0.6) and at 22 ppm (±0.9), respectively for all derivatives discussed here.

III-D. Spin-Labelling of Chitin and Chitosan

1. Introduction

Concomitant with the advent of novel chitin based products, the need has arisen for the development of spectroscopic labelling methods that will facilitate detailed analysis of the molecular parameters responsible for the solution and gel behaviour of both the native polymers and their derivatives. The intractability of the aminopoly-

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

saccharides is mainly responsible for the paucity of such information in the literature; only recently have spectroscopic studies begun to shed some light on the shape and molecular properties of these materials. Buffington and Stevens[33] have concluded from circular dichroism data that inter-, rather than intramolecular interactions between amide groups play an important factor in the gel formation of chitan and chitin. Hirano et al.[3] have observed pronounced changes in specific rotation of chitosan during Schiff's base formation and the attendant gelation, but did not draw any structural conclusion from these. As an extension of our investigations of the gelling and metal chelating properties and ultrastructure of chitin and chitosan derivatives it was essential to evaluate procedures for preparing nitroxide spin-labelled derivatives.

The work described in this section covers three aspects: (i) the synthesis of a number of spin-labelled chitin and chitosan derivatives; (ii) the assessment of several structural parameters of these materials, such as the mobility of the label moieties and, by inference, of the polymers; and (iii) the exploration of interactions between paramagnetic ions and spin-labels bound to the polysaccharide backbone.

2. Spin-Labelling of Chitin and Chitosan

The spin-labelling proved to be a somewhat greater challenge than other reactions of these materials due to the acid lability of the nitroxide probes. Several strategies were explored to resolve this problem.

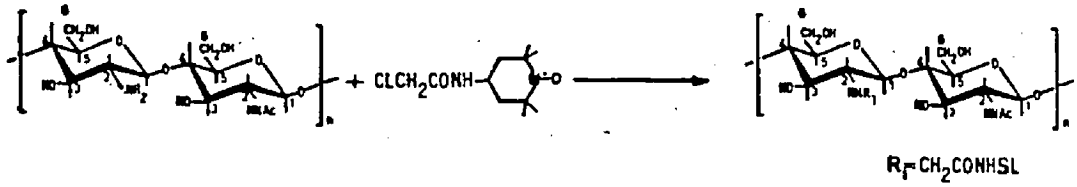
Firstly, chitin [21],* pretreated with methyl sulfoxide and aqueous sodium hydroxide, reacted with 4-chloroacetamido-2,2,6,6-tetramethyl-

---

*Derived from crab shell and containing 10% free NH_2.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

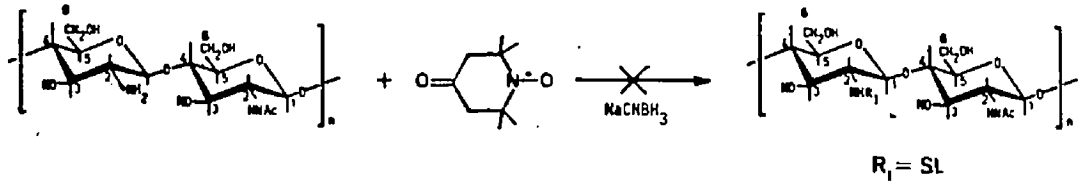
piperidine-1-oxyl [24] to produce the spin-labelled chitin [25] (d.s.



[21] [24] [25]

0.04). The derivative [25] swelled to some extent when suspended in methanol or aqueous solution.

Alternative attempts to specifically label chitin in solution (using the LiCl/DMAc solvent system)[34] by reductive alkylation of the free amine groups with 4-oxy-2,2,6,6-tetramethylpiperidine-1-oxyl, [26] were unsuccessful. The expected product [27] was obtained in such low yields* (from several attempts) that this route was not further pursued.



[21] [24] [25]

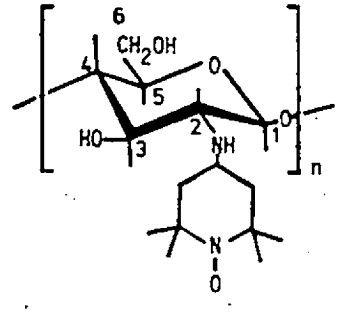
Spin-labelling of chitosan [1] was conducted similarly using the labels [24] and [26]. Since, however, acidic media are required for the dissolution of [1], a number of routes were investigated to avoid or minimize acid-degradation of the nitroxide labels. The first approach consisted of converting chitosan into the N-sulfate derivative

*As judged by its esr spectrum.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

[28] which is moderately watersoluble at elevated temperatures, and subsequently labelling [28] under mild conditions using [26] or [24] to afford the products [30], (d.s. 0.45) and [29] (d.s. 0.5), respectively. Thus, the derivative [28] is a versatile intermediate and has recently also been employed for enzyme immobilizations.[35] Both derivatives [29] and [30], which were partially N-sulfated, formed gels in aqueous solutions.

In a different approach, chitosan was next dissolved in a mixture of methanol and very dilute (0.4%) aqueous acetic acid and reductively alkylated using [26] to afford a product [31] which gelled considerably in methanol or aqueous solution. This feature combined with the fortuitously low substitution (d.s. 0.1) (which ensured a minimal perturbation of the polymer structure) made [31] of greater utility than [29] or [30].*



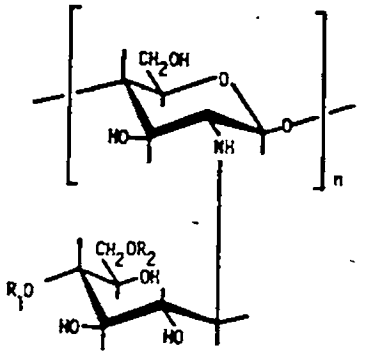
[31]

The spin-labelled derivatives [34] and [35] were obtained by reductive amination of the corresponding C6'-aldehydo, branched chitosans [14] and [15] (which were described in III-B) using 4-amino-2,2,6,6-tretramethylpiperidine-1-oxyl [32]. Both]34] and [35] were soluble in water, forming viscous solutions.

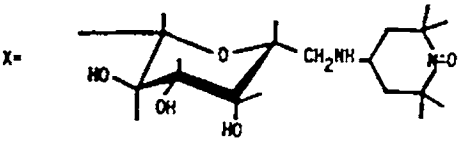
---

*Several other chitosan derivatives, obtained from reactions performed in solvent systems such as aqueous pyridine or aqueous acetic acid, were produced in very low yields and were consequently considered to be unworthy of further attention.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.



[34] R_1=X, R_2=H
[35] R_1=H, R_2=X



3. Esr Spectra

The esr spectra of the derivatives described above are shown in Figures III-5-7 and the corresponding motional correlation times are listed in Table III-3; the τ_c values represent approximate values* since they were derived with the assumption of isotropic motion. Nevertheless, they do provide a measure of the relative differences in the degrees of motional freedom between the derivatives. From Table III-3 it is evident that the chitin derivative enjoys a smaller freedom of rotational reorientation than the chitosan derivatives. Intuitively, this is not unreasonable since the former only swelled in solution, while the latter, particularly [31], formed voluminous gels which consisted of

*Particularly for the complex spectra of [30] and [31]; an accurate quantitation of τ_c's would require use of spectral simulation.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

Fig. III-5. Esr spectrum of [25] in aqueous solution (298K).

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

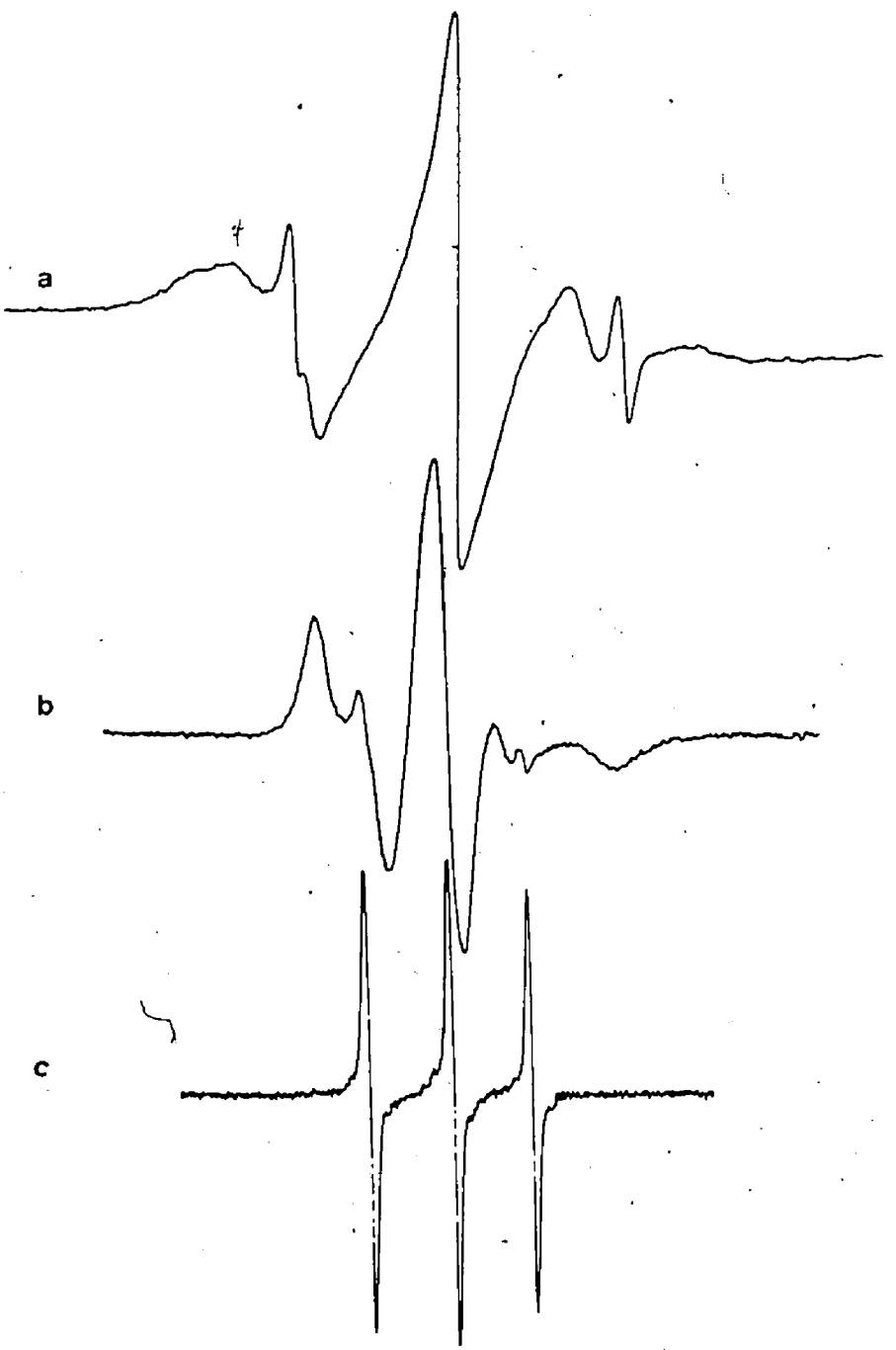


Fig. III-6. Esr spectra of [31] (a) gel in water, (b) gel in methanol, (c) gel (b) transferred into water (after 2 weeks equilibrization); (at 298K).

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

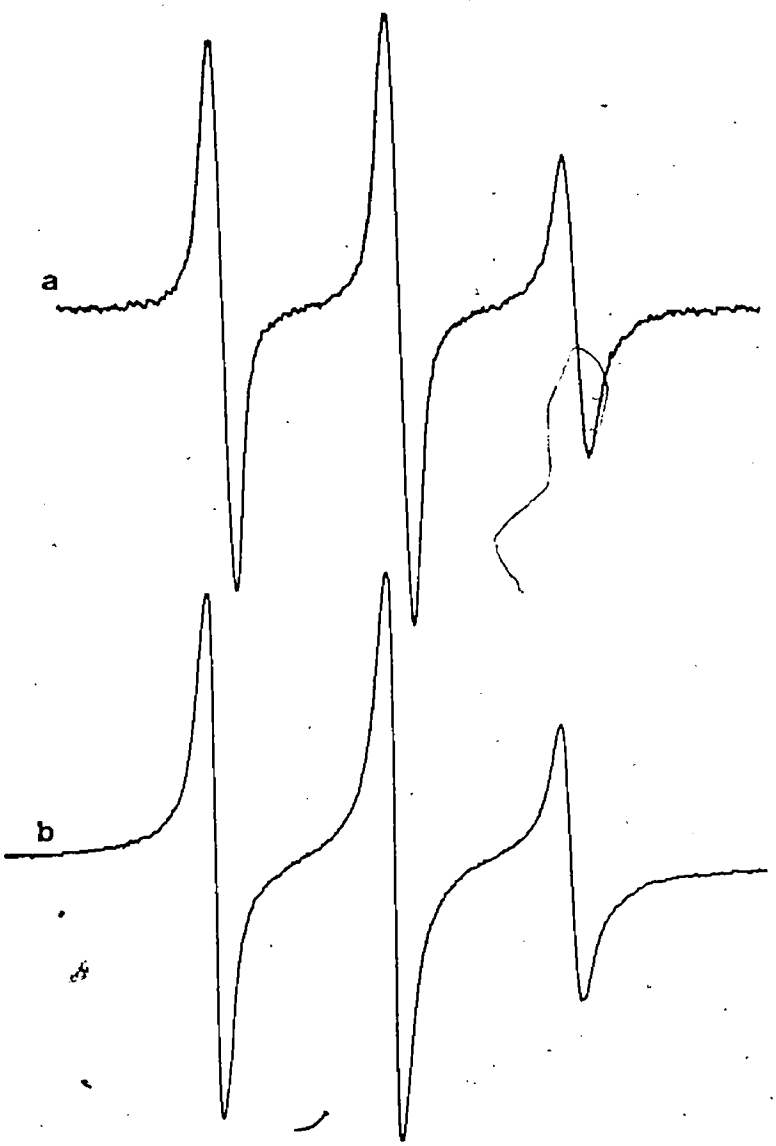


Fig. III-7. Esr spectra of (a) [34], (b) [35], in aqueous solution (at 298K).

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

Table III-3. τ_c Values for some Spin-labelled Chitin and Chitosan Derivatives

Compound	τ_c (ns) in H_2O
[25]	2
[30]	35
[31]	61-400[a]
[34]	68
[35]	43

[a]Values arise from different components, see text

large proportions of solvent (>95%). The solvent pockets provided a matrix in which the nitroxide had a greater mobility than in [25]. It is also interesting to note the substantial differences, both in line-shape and τ_c, between [30] and [31], seemingly similar derivatives, differing mainly in their degree of substitution.

Within the series of chitosan derivatives, [34] and [35] revealed a somewhat greater mobility than [30] which is presumably attributable to the fact that (i) [34] and [35] were watersoluble, while [30] formed gels; and (ii) the nitroxide moieties of [34] and [35] are separated from the polymer backbone by a spacer group of two sugars, while in [30] the nitroxides are separated from the polymer by a four bond linkage only. It might be expected that the latter factor should lead to a much greater nitroxide mobility in [34] and [35]. That this effect is apparently mitigated can be possibly ascribed to the entanglement of polysaccharide chains and/or intra-chain interactions. This argument is also supported by the substantial (50%) differences in τ_c of [34] and [35], which in all likelihood, can only arise from the different types

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

of linkage (1→4 and 1→6, respectively) between the pendant sugar moieties. The more extended chain of [35] allows for a more ordered, less mobile, solution structure, whereas the 1→4 linkage of [34] leads to less ordered conformation in which the nitroxide moiety enjoys a greater mobility.

The esr spectra of gelled [31] (Figs. III-6 a,b), in either methanol or aqueous solution, reveal a very interesting lineshape which is strongly indicative of several partially resolved spectral components with vastly (∿ order of magnitude) different τ_c values and hyperfine linewidths. It should be noted here that the "sharp" signal could not be removed despite extensive washing of the material and may originate from a bound but very mobile nitroxide population, although the possibility for it to derive from a small quantity of adsorbed label cannot be totally excluded. The esr spectra of [31] seem to indicate the presence of nitroxide populations residing in more or less accessible sites. The likelihood that the observed lineshapes simply arise from an isotropically tumbling, homogeneous population of spin labels can be ruled out on the following grounds: (i) similar lineshapes (with approximately equal proportions of spin populations in the respective sites) are obtained for [31] in two different solvents; and (ii) data obtained from experiments involving paramagnetic ions show a heterogeneous distribution of spins over the polysaccharide matrix (discussed below). Precedence for the proposed existence on chitosan of several types of sites with varying degrees of accessibility exists in the literature similar phenomena having previously been observed for cellulose[36] and agarose[37,38] using nmr and esr methods. The heterogeneity of spin-label distribution in [31] was also demonstrated by an experiment in which one solvent

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

(methanol) in the interior of the gelled [31] was replaced by another one (water) resulting, after sufficient time for equilibrization (2 weeks), in changes in the relative proportions of the spectral components (Fig. III-6c). Figure III-6c reveals a predominance of a very mobile nitroxide population over a less mobile, and barely detectable, one. Although this observation seems to conflict at first sight with the similarity in lineshape displayed by freshly prepared gels in each of the two solvents (Figs. III-6 a,b), it can be considered to arise from a slow structural reorganization of the gel which results in an overall less ordered, more mobile assembly. Support for such "solvent-mediated" gel expansion derives from the observation that the macroscopic gel volume increased significantly ($\sim$ 2x) with time (weeks) when methanol was replaced by water. This volume increase was not, however, accompanied by a loss in overall gel rigidity over extended periods of time (several months) eliminating the possibility for the increased mobility to arise from a slow dissolution of the gel.

4. Selective Broadening Experiments

The "multi-site" model proposed above was next tested using the spin-probe-spin-label method. Previous workers[38-41] have shown that paramagnetic "probe" ions, such as transition metal ions, in solution lead to exchange broadening of the esr lines of spin labels both in solution and attached to macromolecules. The line width of the nitroxide esr signal of [33] increased rapidly with increasing probe ion concentration, as shown in Figure III-8a, no signal being detectable at Ni^{2+} concentration above 3 mM. In contrast, for the derivative [35] one finds (Fig. III-8b) that again the signal decreases with increasing concentration of probe ion. However, a residual signal was

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

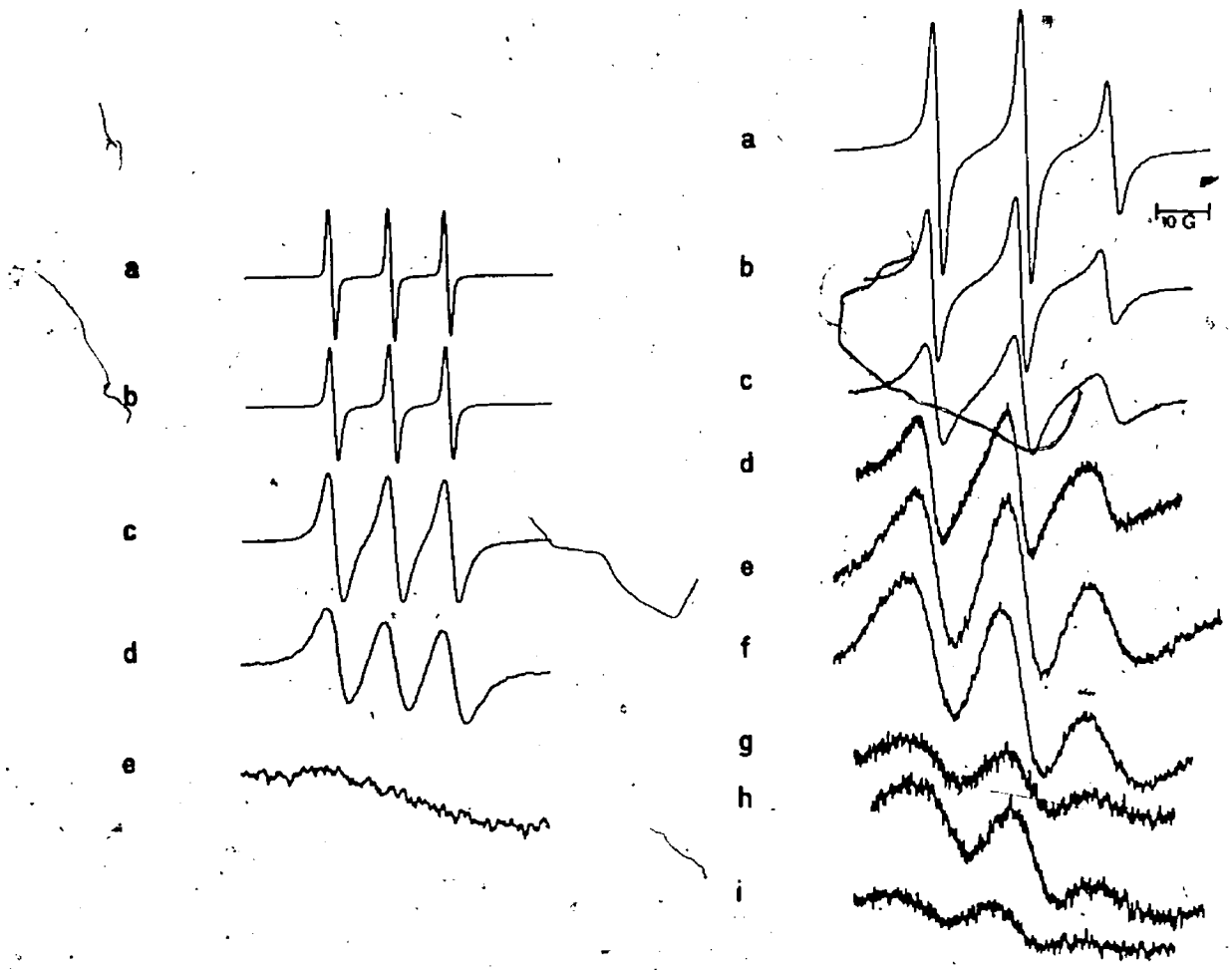


Fig. III-8. Esr spectra of [33] (left) and [35] (right) in the presence of Ni(II); Ni(II) concentrations were (mM): [33] (a) 0,(b) 0.12,(c) 0.23,(d) 0.45, (e) 1.12; [35] (a) 0,(b) o.22,(c) 0.45,(d) 2.90,(e) 5.14,(f) 9.61,(g) 11.84,(h) 20.2,(i) 30.0.

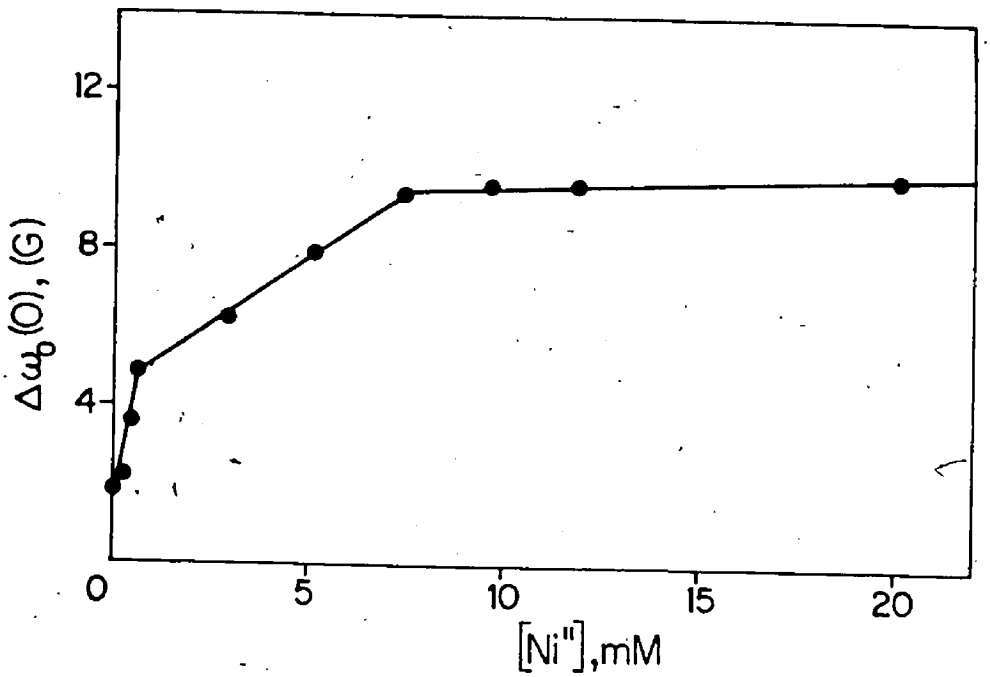
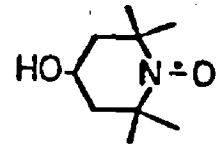


Fig. III-9. Plot of centre-field linewidth of [35] as a function of added nickel sulfate.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.



[33]

observable even after addition of 30 mM Ni^{2+}, although reduced in intensity by approximately two orders of magnitude in comparison to the signal obtained in the absence of metal ion. The plot of the line width, ($\Delta\omega_0(c)$) (peak-to-peak) against concentration of added nickel reveals discontinuities which indicate[40] the existence of several different sites occupied by labels (Fig. III-9). At concentrations above 12 mM, nickel seems to exert little or no influence on $\Delta\omega_0(c)$. (A similar overall $\Delta\omega_0(c)$ dependency on (Ni^{2+}) was observed for the directly labelled chitosan [31].)

The line broadening of the spin-labelled derivatives in the presence of paramagnetic ions (Figs. III-10, 11) can also be expressed in terms of the dependency of the increase in peak-to-peak width of the centre line on metal concentration as illustrated in Figure III-12 for [31], [33], and [35]. For both [31] and [35] rapid broadening was observed at low (Ni^{2+}), whereas at higher concentrations this effect was reduced (for [31])* or virtually absent (for [35]); 7 mM concentrations of metal, on the other hand, broadened the signal of [34] essentially beyond detection.

*Similar behaviour was exhibited by [29] which showed a residual signal even at concentration of 26 mM Ni^{II}(Fig. III-11); peak overlap, however, precluded line width measurements.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

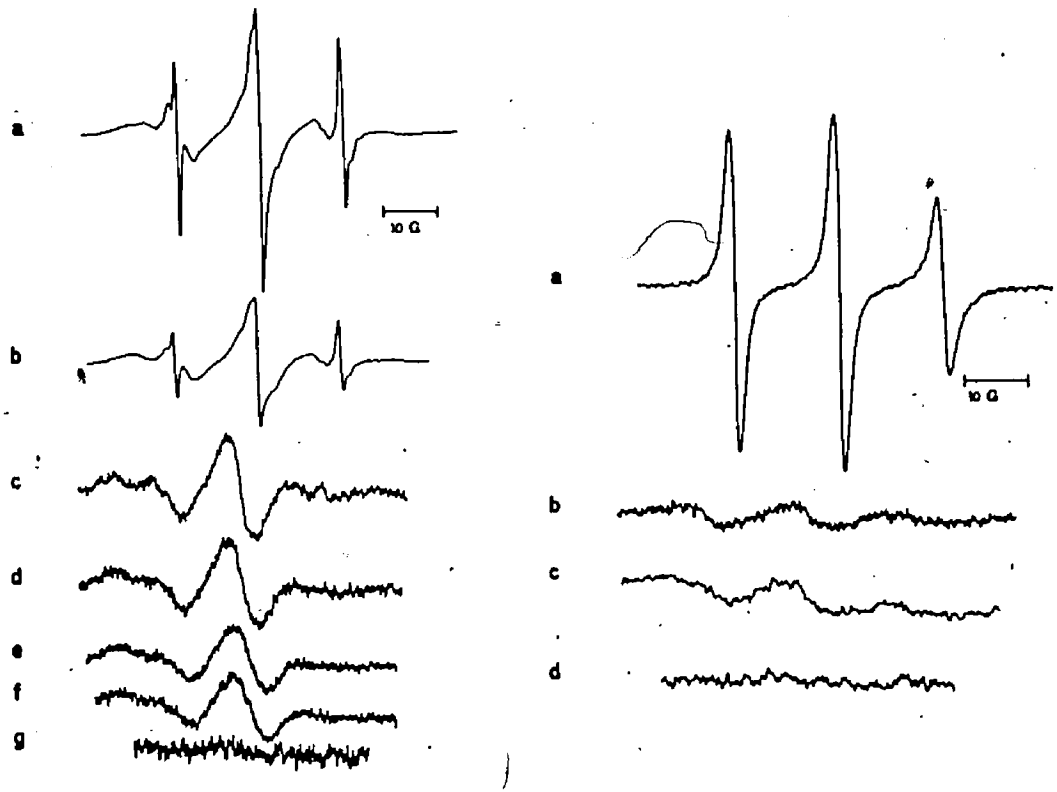


Fig. III-10. Esr spectra of [31] (left) and [34] (right) in the presence of Ni(II); Ni(II) concentrations were (mM): [31] (a) 0,(b) 2.23, (c) 4.47,(d) 6.70,(e) 8.94,(f) 13.40,(g) 31.28; [34] (a) 0,(b) 2.23,(c) 4.47,(d) 6.70.

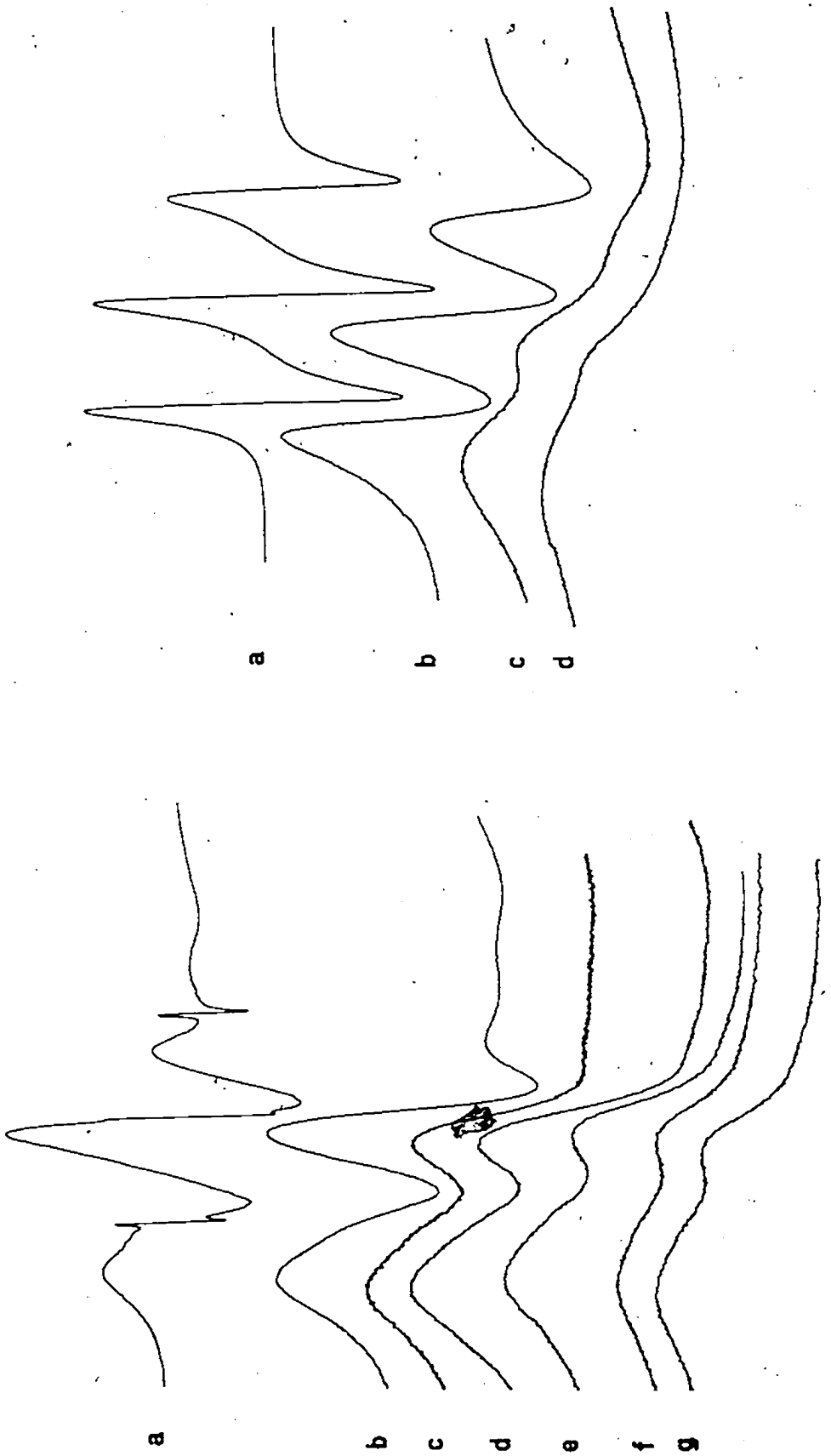


Fig. III-11. Esr spectra of [30] (left) and [29] (right) in the presence of Ni(II); Ni(II) concentrations were (mM): [30] (a) 0,(b) 1.12, (c) 2.23,(d) 3.35,(e) 5.59,(f) 16.76,(g) 25.69; [29] (a)0,(b) 2.23,(c) 3.35,(d) 4.47.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

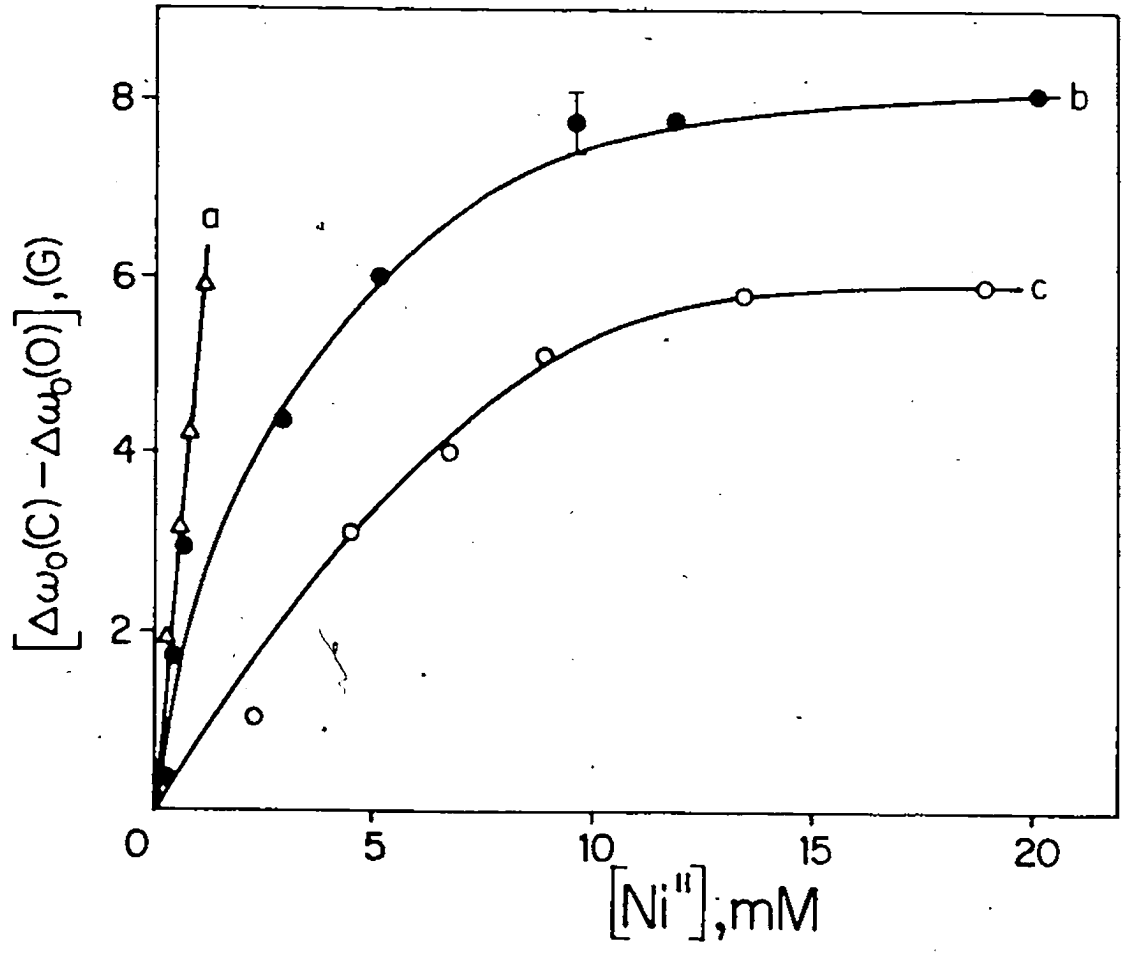


Fig. III-12. Plot of increase of centre-field linewidth of (a) [31], (b) [35], (c) [33] as a function of added nickel sulfate.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

Clearly, each of the former two systems seemed to contain nitroxides in several different sites which were accessible to different extents to metal ions. For the case of [31], other evidence for this phenomenon was already cited.

In conclusion then, it can be said that there is sufficient evidence to indicate the presence of several different sites on chitosan and some of its derivatives in which the randomly distributed labels reside. A physical description which would accommodate such evidence can be sought in the existence of either pores or pockets within the individual polysaccharide chains or junction zones (in gels) between the chains containing nitroxide labels which are more or less accessible to solvents or solutes. Electron microphotographs of xerogels derived from a series of chitosan derivatives bearing sugar-side chains exhibited (see III-D) polyphasic microporous ultrastructures, in light of which the proposed "multi-site" model for the spin-labelled derivatives would seem to be even more probable. Unfortunately, there was no opportunity to obtain SEM microphotographs of these materials.

III-E. Scanning Electron Microscopy

1. Introduction

Scanning electron microscopy (SEM) is a new and powerful technique which has already found widespread applications in such areas as material science, biology, and medicine. More recently, it has facilitated correlations between structural organization and functional significance of synthetic materials such as polymers. SEM studies have established crucial links between macromolecular polymer properties, including mechanical strength, solvent adsorption, solute separation,

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

etc., and ultrastructure of various materials, e.g., polyacrylamides,[42] silica gel,[43] gelatin,[44] and polystyrene.[45] Polysaccharides such as curdlan,[46] alginate,[47] and chitin[48] have also been examined in their native forms, while only relatively few polysaccharide derivatives, with the notable exception of cellulose products, have so far been morphologically characterized. Thus, Masri and Jones[49] reported micrographs of commercial chitosans derived from crab shells which revealed lamellar phases with interconnective transverse fibrils. Hirano and coworkers[48] have noted the similarity between cellulosic and N-acetylated chitosan gels both of which featured microporous polyphases;* a similar morphology was found for xerogels** of N-methylene chitosan. These studies have, however, provided little information about the gel formation of the chitosan products.

The work presented in this section*** portrays some initial attempts to characterize the architecture of several chitosan derivatives described in the previous sections and to establish correlations with their gelling properties. It can be anticipated that the SEM results will also facilitate a better understanding of other aspects of the molecular properties of these materials, such as metal-chelating efficacies. The following discussion will focus on some novel features of chitosan itself as well as on four branched-chain, gel-forming derivatives thereof (see III-B).

---

*I.e., consistent of several structural types, in this case, a mixture of membraneous and microporous regions.

**I.e., dried gels which retain most of their three dimensional morphology.

***SEM studies were performed by Mr. Nasser Yalpani.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

2. SEM of Chitosan

Masri and Jones[49] have, as already mentioned, characterized commercial, crab shell-derived chitosan. We examined chitosan derived from shrimp shell and found many similarities between these two materials as well as some novel features, typical aspects of which are shown in the photomicrographs (Fig. III-13 a, b). Chitosan flakes (Fig. III-13a) exhibited flat lamellar phases with some lamellae extending at an angle of between ca. 45-90° with respect to the outer surface of the flakes. A large number of protruding microfibrils, ca. 20-40 nm in diameter and up to 0.2 μm long, are evident on these lamellae; these microfibrils are oriented either parallel or perpendicular to the lamellar plane. Masri and Jones[49] concluded from similar findings that the former assemble to form individual lamellae while the latter act as support and link between the lamellar layers. Another interesting view of chitosan is illustrated in Figure III-13b where relatively large (ca. 10 μm height, 10-50 μm width, 5-10 μm depth) dome-shaped orifices are seen to be incorporated into the membraneous surface. Microfibrils, 20 nm in diameter and up to 10 μm in length, are again evident as are some crystallites presumably from mineral sources.

3. SEM of Branched-Chain Chitosan Derivatives

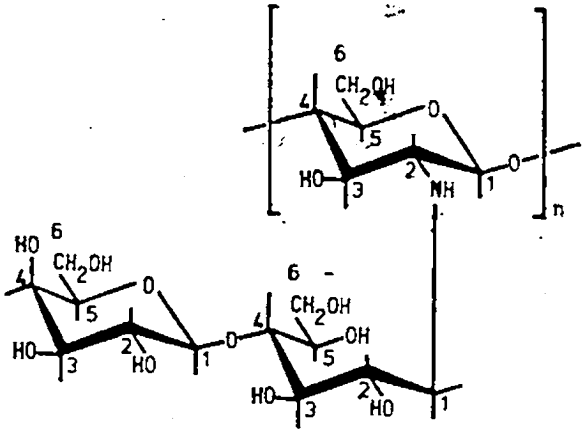
The xerogels of the branched-chain chitosan derivatives synthesized in this work displayed a vast range of ultrastructures. Galactityl chitosan [2] xerogels have non-porous, smooth membraneous phases (Fig. III-13 e,f) of 2-4 μm thickness. The enlargement in Figure III-13f discloses a highly-ordered microfibrillar substructure. The non-porous surface structure of [2] is rather unique among the chitosan derivatives studied and correlates well with the observation that no metal-chelation could be accomplished with this derivative (see V-B 2).

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

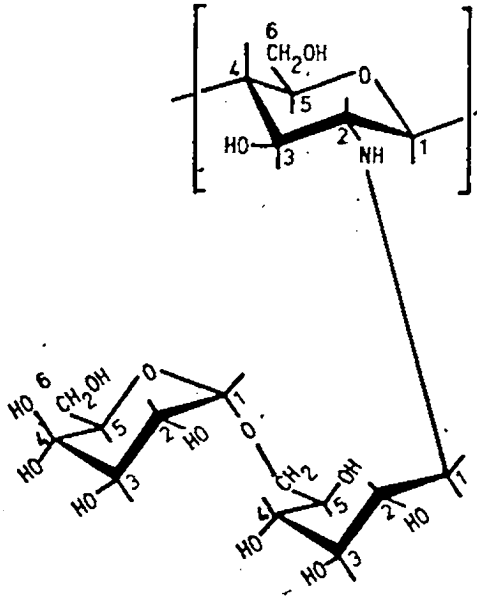
Fig. III-13. SEM of [1] (a), (b); [3] (c), (d); [2] (e), (f); bars under the micrographs indicate 10 μm (a-d,f) and 100 μm (e).

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.



[6]

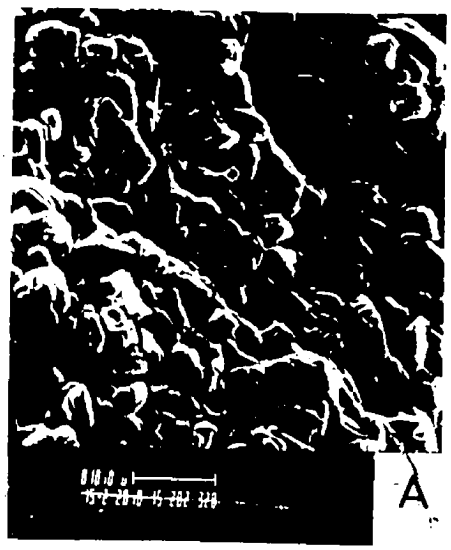


[10]

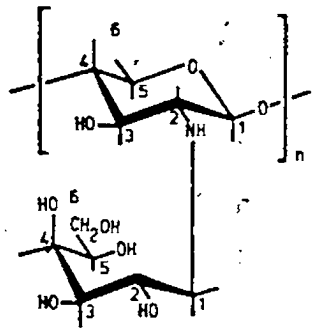
Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

Fig. III-14. SEM of [7] (a)-(c); [10] (d); [6] (e), (f); bars under the micrographs indicate 10 μm.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.



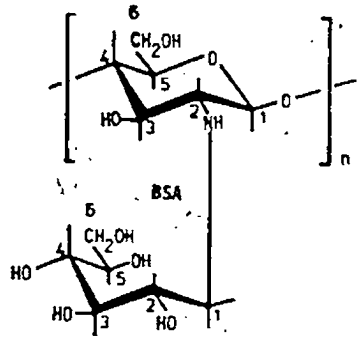
Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.



[2]

In contrast to the above, the glucityl chitosan [3] exhibits a polyphasic, microporous ultrastructure (Fig. III-13 c,d). The pore dimensions are non-uniform ranging from 20-35 × 40-70 μm (Fig. III-13d), with very thin membrane walls. The ultrastructure of [3] has certain similarities to that of the N-methylene chitosan reported by Hirano et al.,[4] the pore dimensions of the latter being somewhat larger (see Table III-4).

Photomicrographs of xerogels derived from the reaction products [7] of chitosan with three molar equivalents of lactose per glucosamine residue reveal a rather unusual polyphasic topography. Figure III-14a shows



[3]

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

contiguous membraneous surfaces, structured in irregular honeycomb-fashion, which are covered with crystallites. The honeycomb regions consist mainly of hexagonal, sometimes triangular, formations with typical sizes of ca. 2-5 × 2-7 μm. The crystallites (Figs. III-14 a,c) vary substantially in size (ca. 0.4-7 × 13 μm) and shape and appear to consist of a microfibrillar substructure. When the above lactityl chitosan gels were purified by extensive dialysis instead of by washings only, the resulting product [6] displayed a drastically altered micro-architecture as shown in Figures III-14 e and f. A polyphasic, micro-porous structure is obtained containing non-uniformly sized pores (9-17 × 10-28 μm) with thin walls; some individual microfibrils, ca. 20-140 nm in diameter, are also evident (Fig. III-14f).

These findings confirm earlier conclusions (see III-B) based on ^{13}C nmr spectroscopy that lactityl chitosan, in the presence of excess lactose, produces gels which "trap" this reagent (in a 1:1 ratio of the latter per lactityl glucosamine residue) to afford materials with "closed" (i.e., non-porous) ultrastructure; upon removal of the "trapped" reagent by dialysis "open" (microporous) materials are obtained. These particular observations have so far only been made for the reaction of chitosan with lactose.

Xerogels derived from melibiityl chitosan [10] displayed a highly ordered microfibrillar ultrastructure. The edge view shown in Figure III-14d reveals a parallel array of microfibrils which have diameters of ca. 150 nm. It will be recalled from the previous section that, in explanation of the τ_c value observed for the C6'-spin labelled derivative [35], an extended conformation of the 1-6 linked side chains had been invoked. Assuming that the solution structures of [10] and [35]

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

are not very dissimilar, one is tempted to correlate the esr results with the gel structure described here.

The dimensions of the various xerogels are assembled in Table III-4 together with the data obtained for some other materials. The microporous, branched-chain chitosan derivatives reported here have pore sizes intermediate between N-acetyl chitosan and N-methylene chitosan and the other synthetic polymers listed in Table III-4.

The SEM results obtained so far do not indicate consistent trends in the relation of the micro-architecture and the chemical structure of the side-chains of the chitosan derivatives. The α-1,6 linkages of the side-chains of [10] are presumably partially responsible for the observed highly regular microfibrillar ultrastructure, which, in comparison with the β-1,4 linkages of [6], would allow for a more extended and ordered assembly of the side-chains. It is, however, unclear why the small conformational difference between the side-chains of [2] and [3] should result in such drastic changes in ultrastructure as evidenced here. A more likely, less simplistic explanation for the variety in the micro-architecture of the chitosan derivatives must consequently be sought in factors other than the differences in chemical structure of the side-chains alone. A combination of factors, such as those leading to gel formation, i.e., H-bonding and a variety of associative phenomena of polysaccharide chains in solution, and the extent and uniformity of substitution, etc., (see III-B) presumably contributes to the observed topographies of the xerogels.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

Table III-4. Ultrastructure Characteristics

Material	Pore Structure: Main Feature	Dimensions (μm)	Wall Thickness (μm)	Reference
[3]	polyphasic, microporous	20-35 × 40-70	very thin	-
[2]	polyphasic, non-porous	-	-	-
[6]	polyphasic microporous	9-17 × 10-28	0.2-1.9	-
[7]	polyphasic, non-porous	-	-	-
[10]	polyphasic, microfibrillar	-	-	-
N-methylene-chitosan	polyphasic, microporous	40-70 × 50-100	very thin	4
N-acetyl-chitosan	polyphasic microporous	30-50 × 80-300	very thin	48
polyacrylamide	microporous	2 × 10-15	0.3-1.0 up to 200 in sections	42
polyvinylalcohol	polyphasic, microporous	0.1-0.3	very thick	42
polyethylene oxide	polyphasic, microporous	3-20	1.5-2.0	42

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

References

1. See R. A. A. Muzzarelli, Chitin, Pergamon Press, New York, (1977).

2. S. Hirano, Y. Ohe, and H. Ono, Carbohydr. Res., 47, 315 (1976).

3. S. Hirano, N. Matsuda, O. Miura, and H. Iwaki, ibid., 71, 339 (1979).

4. S. Hirano, N. Matsuda, O. Miura, and T. Tanaka, ibid., 71, 344 (1979).

5. See, e.g., W. W. Graessley, Acc. Chem. Res., 10, 332 (1977); H. Grisebach and R. Schmid, Angew. Chem. Intern. Ed., 11, 159 (1972).

6. I. J. Goldstein, R. D. Poretz, L. L. So, and Y. Yang, Arch. Biochem. Biophys., 127, 787 (1968).

7. S. F. Grappel, Experientia, 27, 329 (1971).

8. E. T. Reese and F. W. Parrish, Biopolymers, 4, 1043 (1966).

9. J. Cisar, E. A. Kabat, M. M. Dorner, and J. Liao, J. Exp. Med., 142, 435 (1975).

10. B. Pfannemüller, G. C. Richter, and E. Husemann, Carbohydr. Res., 56, 139 (1977).

11. For reviews, see A. F. Bochkov and G. E. Zaikov, Chemistry of the O-Glycosidic Bond, Pergamon Press, New York, (1979); W. A. Szarek, in MTP Intern. Rev. Sci., Org. Chem. Ser. One, 7, 80 (1973).

12. H. Ito and C. Schuerch, J. Amer. Chem. Soc., 101, 5797 (1979).

13. B. Pfannemüller, G. Richter, and E. Husemann, Carbohydr. Res., 47, 63 (1976).

14. H. Andresz, G. C. Richter, and B. Pfannemüller, Makromol. Chem., 179, 301 (1978).

15. W. B. Neely, U.S. Pat., 3,133,856 (1964); Chem. Abstr., 61, 5899h (1964).

16. L. J. Filar and M. G. Wirick, Proc. First Intern. Confer. Chitin/Chitosan, R. A. A. Muzzarelli and E. R. Pariser, eds., 169 (1978).

17. E. R. Hayes and D. H. Davies, ibid., 193 (1978).

18. F. A. Bettelheim, in Biological Polyelectrolytes, A. Veis, ed., Marcel Dekker, New York, 131 (1970).

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

19. Y. Kikuchi and H. Fukudu, Makromol. Chem., 175, 3593 (1974).

20. G. T. Grant, E. R. Morris, D. A. Rees, P. J. C. Smith, and D. Thom, FEBS Lett., 32, 195 (1973).

21. G. Robinson, E. R. Morris, and D. A. Rees, J.C.S. Chem. Comm., 152 (1980).

22. R. J. Dobbins, in Industrial Gums, R. L. Whistler, ed., Academic Press, New York, 19 (1973).

23. R. E. Sand and M. Glicksman, ibid., 147 (1973).

24. H. J. Humm and L. G. Williams, Amer. J. Bot., 35, 287 (1948).

25. E. Pippen, T. H. Schultz, and H. Owens, J. Colloid Sci., 8, 97 (1953); H. Harvey, Chem. Ind., 7, 29 (1960).

26. R. G. Schweiger, Carbohydr. Res., 70, 185 (1979).

27. N. Yamaoka, T. Usui, H. Sugiyama, and S. Seto, Chem. Pharm. Bull., 22, 2196 (1974).

28. S. J. Perkins, L. N. Johnson, D. C. Phillips, and R. A. Dwek, Carbohdr. Res., 59, 19 (1977).

29. L. D. Hall, G. A. Morris, and S. Sukumar, J. Amer. Chem. Soc., 102, 1745 (1980); P. E. Pfeffer, K. M. Valentine, and F. W. Parrish, ibid., 101, 1265 (1979).

30. L. D. Hall and M. Yalpani, Carbohydr. Res., 78, C4 (1980); L. D. Hall and M. Yalpani, in preparation.

31. P. D. Hoagland, P. E. Pfeffer, and K. M. Valentine, Carbohydr. Res., 74, 135 (1979).

32. L. F. Johnson and W. C. Jankowski, Carbon-13 NMR Spectra, Wiley, New York (1972).

33. L. A. Buffington and E. S. Stevens, J. Amer. Chem. Soc., 101, 5159 (1979).

34. C. L. McCormick and D. K. Lichatowich, J. Polym. Sci., Polym. Lett. Ed., 17, 479 (1979).

35. M. S. Masri, V. G. Randall, and W. L. Stanley, in Proc. First Intern. Conf. Chitin/Chitosan, R. A. A. Muzzurelli and E. R. Pariser, eds., 364 (1978).

36. M. F. Froix and R. Nelson, Macromolecules, 8, 726 (1975).

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

37. L. D. Hall and J. D. Aplin, J. Amer. Chem. Soc., 100, 1934 (1978); L. D. Hall and J. D. Aplin, ibid., in press.

38. J. D. Aplin, Ph.D. thesis, University of British Columbia, (1979).

39. E. Odgaard, T. B. Melo, and T. Henriksen, J. Magn. Res., 18, 436 (1975); G. I. Likhtenstein, Adv. Mol. Relax. Int. Proc., 10, 47 (1977).

40. G. I. Likhtenstein, Spin Labels in Molecular Biology, Nauka, Moscow, (1974).

41. J. S. Hyde, H. M. Swartz, and W. E. Antholine, in Spin Labeling II, L. J. Berliner, ed., 71 (1979).

42. Z. Blank and A. C. Reimschussel, J. Mater. Sci., 9, 1815 (1974).

43. J. C. Dennis, J. Colloid and Interf. Sci., 28, 32 (1968).

44. E. S. Halberstadt, H. K. Henisch, J. Nickl, and E. W. White, ibid., 29, 469 (1969).

45. P. Bajaj and S. K. Varshney, Polymer, 21, 201 (1980).

46. A. Koreeda, T. Harada, K. Ogawa, S. Sato, and N. Kasai, Carbohydr. Res., 33, 396 (1974).

47. E. S. Obolonkova, E. M. Belavtseva, E. E. Braudo, and V. B. Tostoguzo, Colloid. Polymer Sci., 252, 526 (1974).

48. S. Hirano, R. Yamaguchi, and N. Matsuda, Biopolymers, 16, 1987 (1977).

49. M. S. Masri and F. T. Jones, in Proc. First Intern. Conf. Chitin/Chitosan, R. A. A. Muzzarelli and P. E. R. Pariser, eds., 483 (1978).

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

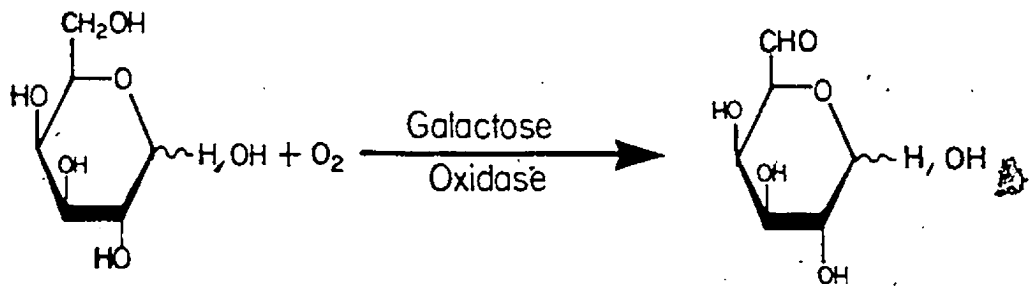
CHAPTER IV

COMBINED ENZYMIC AND CHEMICAL MODIFICATIONS

IV-A. Introduction

The work described in the previous chapters involved polysaccharide modifications by chemical means and the reader will be now be familiar with some of the problems encountered in accomplishing the task of specific functional transformations. Enzymes provide in many cases a facile alternative for polysaccharide modifications.

From the numerous enzyme systems available,[1] galactose oxidase[2] (D-galactose : oxygen 6-oxidoreductase, EC 1:1.3.9) seemed particularly attractive for its specificity and overall simplicity of reaction (eqn. 1). This enzyme exists as a single polypeptide chain (MW 68,000) with a



single metal ion (copper) as its sole cofactor and is produced by a fungus.[3] The enzyme has been employed for a range of mono,[4] oligo-[5] and polymeric[6] galactose substrates; inactivity is apparently only observed for C4 substitution of galactose or galactosides and for C3

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

substitution in the case of 2-deoxy-2-aminogalactose derivatives.[2,5,7] The enzyme displays a much greater affinity for polymeric than for monomeric substrates but its metabolic functions remain obscure.[2] A further useful aspect of this enzyme consists in its stereospecific oxidation of the C6 primary alcohol group in the course of which the (pro-S)-hydrogen is abstracted.[8] These features of galactose oxidase have been successfully exploited for applications to a number of polysaccharides,[6] including guaran[2,5,14] and agarose,[9] and to cell surface glycoproteins;[10,11] various spectroscopic and other probes, such as deuterium,[11] tritium,[10,12] and nitroxide spin-labels,[13] have been incorporated into biological materials of different complexity using this procedure. The substantial utility of this enzymic modification can be fully appreciated if one considers equivalent chemical methods for the introduction of C6 aldehyde functions into polysaccharides.[15]

The work presented in this hapter was aimed at demonstrating the potential of enzymic treatment of D-galactose containing polysaccharides, exemplified here by guaran and locust bean gum, two representative and important galactomannans for affecting specific and high-yielding transformations.

IV-B. Galactomannans

1. Modification with Galactose Oxidase

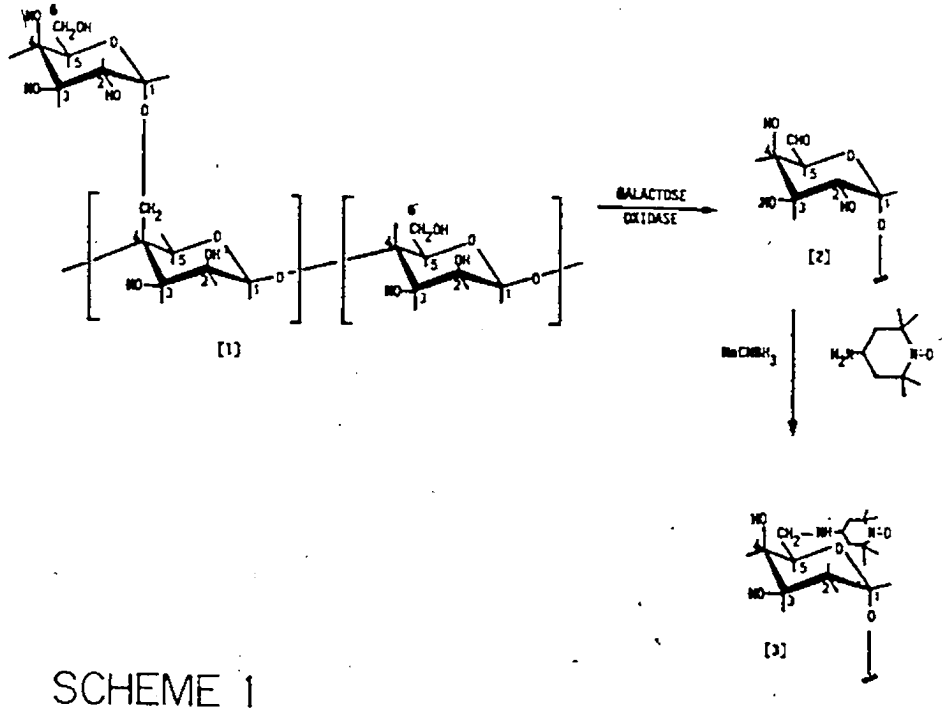
(i) Guar gum

Following the prescient suggestion of Avigad et al.[2] and of Schlegel et al.,[5] galactose oxidase was used to introduce an aldehyde group at C6 of the pendant galactose units of guar gum [1], reductive amination of which, using any primary or secondary amine and sodium

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

cyanoborohydride affords a polymer bearing a substituent of choice at C6.

The general sequence of reactions is summarized in scheme 1, and the spin-labelling of [1] typifies the conditions used; pure[16] guar gum (60 mg, 0.12 mM equivalents galactose) in phosphate buffer (pH 7, 25 mM, 15 ml) reacted with galactose oxidase (90 units) in the presence of catalase (E.C. 1.11.1.6, 10500 units) for 24 hours, affording a very viscous, ropy material. Omission of catalase led to an approximately four-fold reduction in the yields of [3]*.[17] Reductive amination could be performed in situ by the addition of aqueous solutions of 4-amino-2,2,6,6-tetramethylpiperidine-1-oxyl ([4], 92 mg, 0.54 mM) and sodium cyanoborohydride (300 mg, 4.4 mM) over 36 hours; alternatively the aldehyde derivative [2] could be isolated by ethanol precipitation, followed



SCHEME 1

*by consuming inhibiting H_2O_2 in the reaction mixture.[17]

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

by centrifugation. Purification of the product [3] could be achieved either by dialysis (4 days) or by ethanol precipitation* followed by careful washing. Although no attempts were made to ascertain complete removal of the small amounts of enzyme, no interference from this source could be detected by nmr, or even prior to purification, by esr; were this to be a problem it could be ideally resolved by an immobilized enzyme system.[18]

Although only some preliminary attempts** have so far been made to fully optimize the reaction conditions, the yields (based on galactose content) obtained are rather encouraging, typically varying between 60-70% as determined by elemental microanalysis, ^{13}C nmr, or by esr double integration in the case of [3].

(ii) Locust bean gum

Following the above procedures locust bean gum [5] was oxidized to the corresponding C6 aldehyde [6]. The oxidation in this case proceeded with a greater efficiency as judged by elemental analysis and esr double integration of the spin-labelled derivative [7], typical yields ranging from 70-90%. It is uncertain at this point, whether the higher yields obtained for locust bean gum have any significance in terms of the structural differences of the two gums affecting enzyme activity. However, the high degrees of oxidation obtained in both cases are in line with previous, less accurate findings of Schlegel et al.[5] who, using colorimetric and other analytical procedures, reported degrees of oxidation for guaran ranging from 3% to 76% and 95%. Avigad et al.[2]

---

*Resulted usually in insolubization of the product.

**See Experimental section.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

found for guaran an enzyme substrate affinity constant (Km) value of 3.1×10^{-4}M which is three orders of magnitude smaller than that for galactose (Km = 2.4×10^{-1}M). Similarly, the relative rate of guaran oxidation was 50% higher than for galactose itself,[2] both factors clearly indicating the greater enzyme affinity for the high molecular weight substrate. No corresponding data were available for locust bean gum.

The application of galactose oxidase to some synthetic chitosan derivatives was already discussed in the previous chapter (II-D).

(iii) Esr

The esr spectra of the spin-labelled guaran [3], and locust bean gum [7] in aqueous solution were very similar, revealing relatively mobile nitroxide moieties with τ_c values of 7.70×10^{-10} sec and 7.61×10^{-10} sec, respectively (Fig. IV-1a). It is noteworthy that the corresponding spin-labelled gums prepared via periodate oxidation of the native polymers displayed similar spectral lineshapes as [3] and [7] (see Fig. IV-1b).

Esr spectroscopy of [3] and [7] at 77K provided novel information concerning the distribution of the nitroxide groups and, by inference, that of the pendant galactose units of the gums themselves whose structures are illustrated in Figure IV-2. Thus, from calculation of the mean nearest-neighbour distance between spins, $\bar{r}$, derived from the spectral parameter d_1/d at 77K(see I-C), a value of 1.36 nm (±5%) was obtained for [3]. This value is most consistent with a structure in which the pendant galactose units are arranged in "blocks" as recently proposed by Painter, Gonzalez and coworkers[19-21] (Fig. IV-2); this conclusion is based on the following arguments.

From molecular model building studies, a rough distance estimate

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

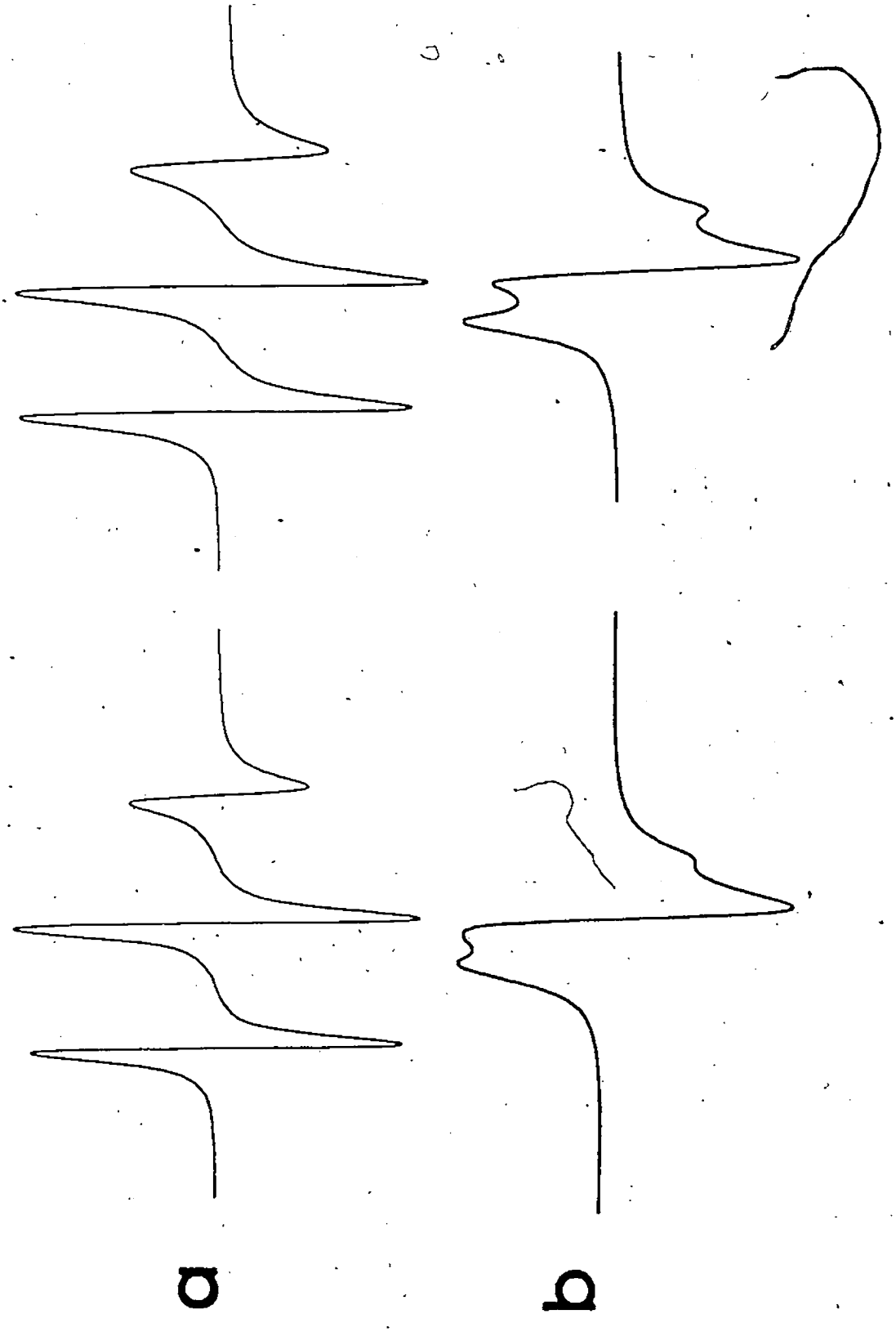


Fig. IV-1. Esr spectra of [3] (left) and [7] (right) (a) in aqueous solution (298K), (b) frozen at 77K.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

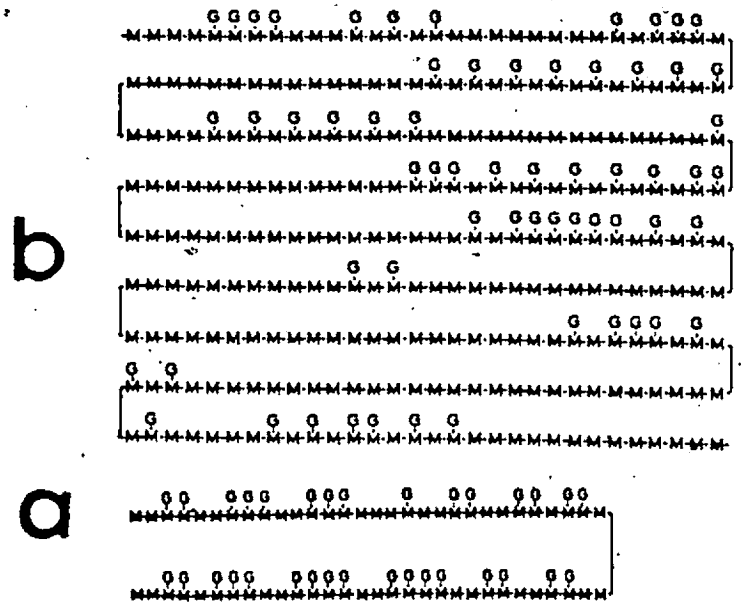
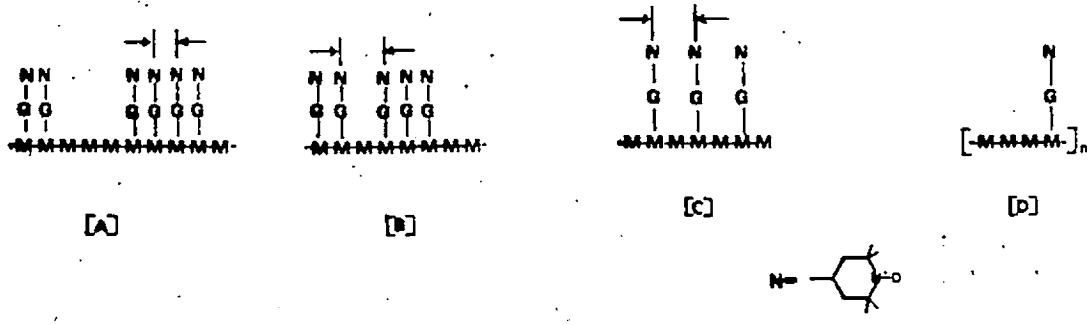


Fig. IV-2. Proposed structures for guaran (a) and locust bean gum (b) (from ref. 20).

of 1.1-1.7 nm was obtained for the separation of the nitroxide moieties of [3] within blocks of between two to four contiguous, branched mannose



(MG) residues [A]. The nearest-neighbour distance of nitroxides of adjacent MG blocks which are separated by one unbranched mannose (M) unit [B], was found to be ~1.8-2.3 nm by the same method, whereas a value of ~1.5-2.0 nm was obtained for a regular M-MG sequence, i.e., for a uniform galactose distribution [C].

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

It should be noted here that, fortuitously, the maximal distances of the two latter estimates approach the upper detection limit (~2.2 nm) for which the d_1/d parameter provides reliable distance information (see I-C). Consequently, contributions of dipolar interactions from distances greater than the above limit can be effectively ignored in light of the r^{-6} dependency of dipolar couplings; dipolar contributions arising from interactions close to the detection limit should be small in comparison with short-range interactions. Thus, in view of the above, a regular guaran structure appears unlikely. However, lacking knowledge of the exact conformations of either of the polymer derivatives [3] and [7] in solution or in the solid state, the above distance estimates were derived by assuming a range of, what was deemed, preferred conformations of a single polymer chain with a twofold conformation of the mannose backbone; nitroxide interactions due to chain-folding or other inter- and intra-molecular sources were also not taken into account. It is therefore obvious that no firm conclusions could be reached, based solely on these estimates. Further supporting evidence was, however, obtained from additional experiments.

In the first set of experiments the extent of spin-labelling of guar gum was continuously reduced for a comparison of the esr spectra of the resulting products. Figure IV-3 illustrates the fact that the pronounced line broadening, observed for derivatives with high degrees of substitution (d.s. 0.71, Fig. IV-3a), is largely retained for samples bearing one half (d.s. 0.34, Fig. IV-3b) or only one quarter (d.s. 0.14, Fig. IV-3c) the number of nitroxide residues. From these results, assuming random labelling of the polysaccharide, a non-regular nitroxide distribution can be inferred, since the nitroxides of low d.s. derivatives

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

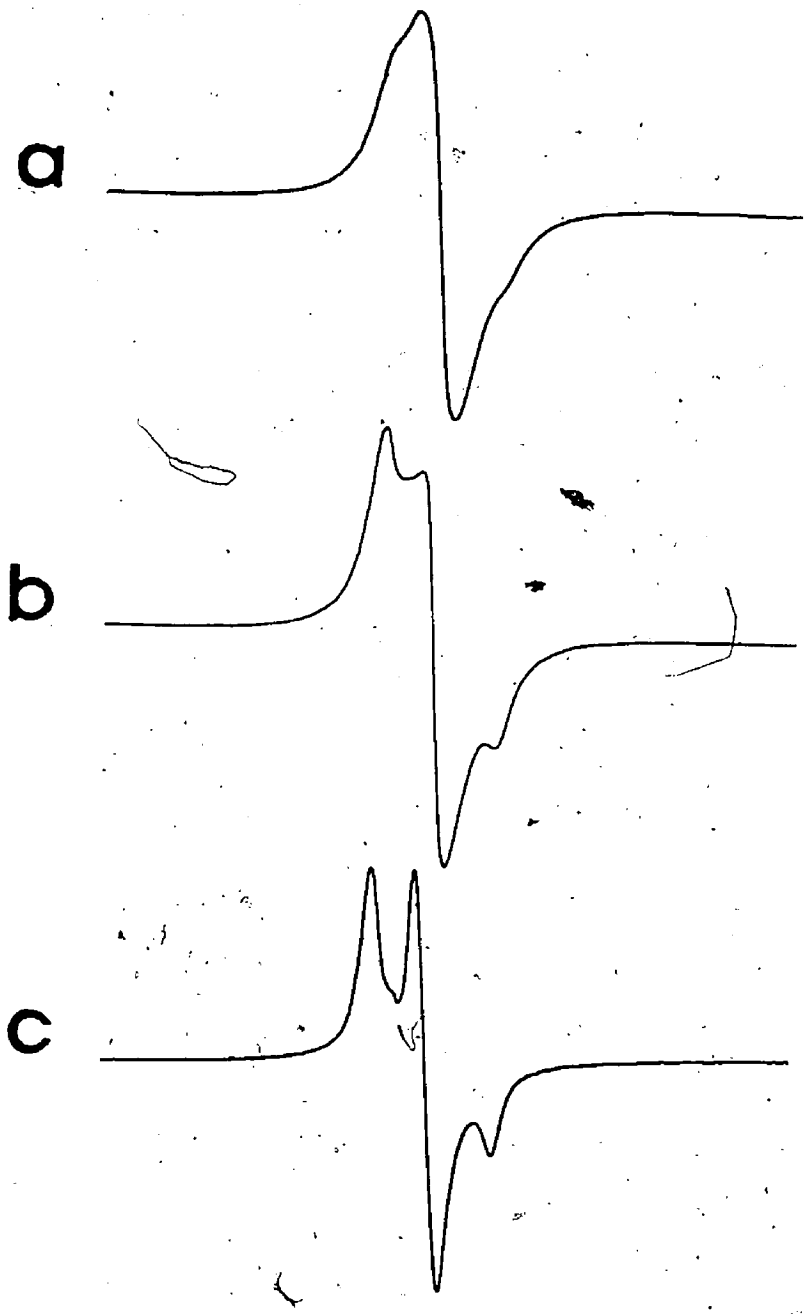


Fig. IV-3. Esr spectra of [3] (a) d.s. 0.71, (b) d.s. 0.34, (c) d.s. 0.14; at 77K.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

of [3] would be too far apart in a strictly regular structure for the above interactions to occur, even if intermolecular interactions between chains were operative. These results alone do not, however, rule out another proposed[22] structure which is based on alternating long blocks of contiguous MG and M units; but further results obtained from locust bean gum and those most recently reported by Grasdalen and Painter,[21] exclude this possibility as well.

Thus, significantly, for [7] a value of $\bar{r}$ = 1.75 nm (±5%) was found in similar fashion (Fig. IV-1b); this value is ~30% greater than that of [3], clearly reflecting the proposed structural differences between guaran and locust bean gum as expressed in Figure IV-2. According to Painter et al.[20] locust bean gum has, in comparison to guaran, only one fifth and one quarter the number of doublet and triplet frequencies for two and three consecutive MG units, respectively, while its triplet frequency for alternating M and MG units, i.e., M-MG-M or MG-M-MG, is greater than that of guaran by a factor of 4.5. In other words, the observed greater $\bar{r}$ value of [7] arises from a relatively smaller contribution from consecutive MG blocks and a larger contribution from alternating sequences. The $\bar{r}$ value of [7], which is also in agreement with the molecular model calculations discussed earlier, rules out the

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

possibility of structures with regular galactosyl distribution [D] since the distances involved would fall outside the detection range of the esr method, as discussed earlier.

Thus, in conclusion, it can be said that the esr data are in good qualitative agreement with the galactomannan structures of Painter and coworkers,[19-20] although, strictly speaking, separations between nitroxides rather than galactose units were measured here with the assumption that the conformations of [3] and [7] are not very dissimilar to those of their respective native polymers.

(iv) Applications

The extreme versatility of the aldehyde intermediates [2] and [6] could be demonstrated by performing the reactions, summarized in Figure IV-4, which range from reductive amination to oxidation and reduction. The reductive amination reaction, exemplified here by a variety of products, including [3], [7]-[11], has obviously many interesting variants, such as the possibility to introduce amine functions into the polysaccharide by use of ammonium acetate affording [12], a cationic key intermediate. Conversely, anionic derivatives can be prepared by oxidation of the aldehyde function using bromine water at pH 6, to yield the carboxylic acid [14].

Reduction of the aldehyde intermediate with sodium borohydride or its deuterated analogue affords the original polymer which in the latter case has incorporated a probe with minimal structural perturbations, [15], [16].

Of the products obtained by reductive amination the BSA-derivative [11] constitutes a novel type of polysaccharide-protein conjugate which typifies a wide range of potentially useful derivatives, including

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

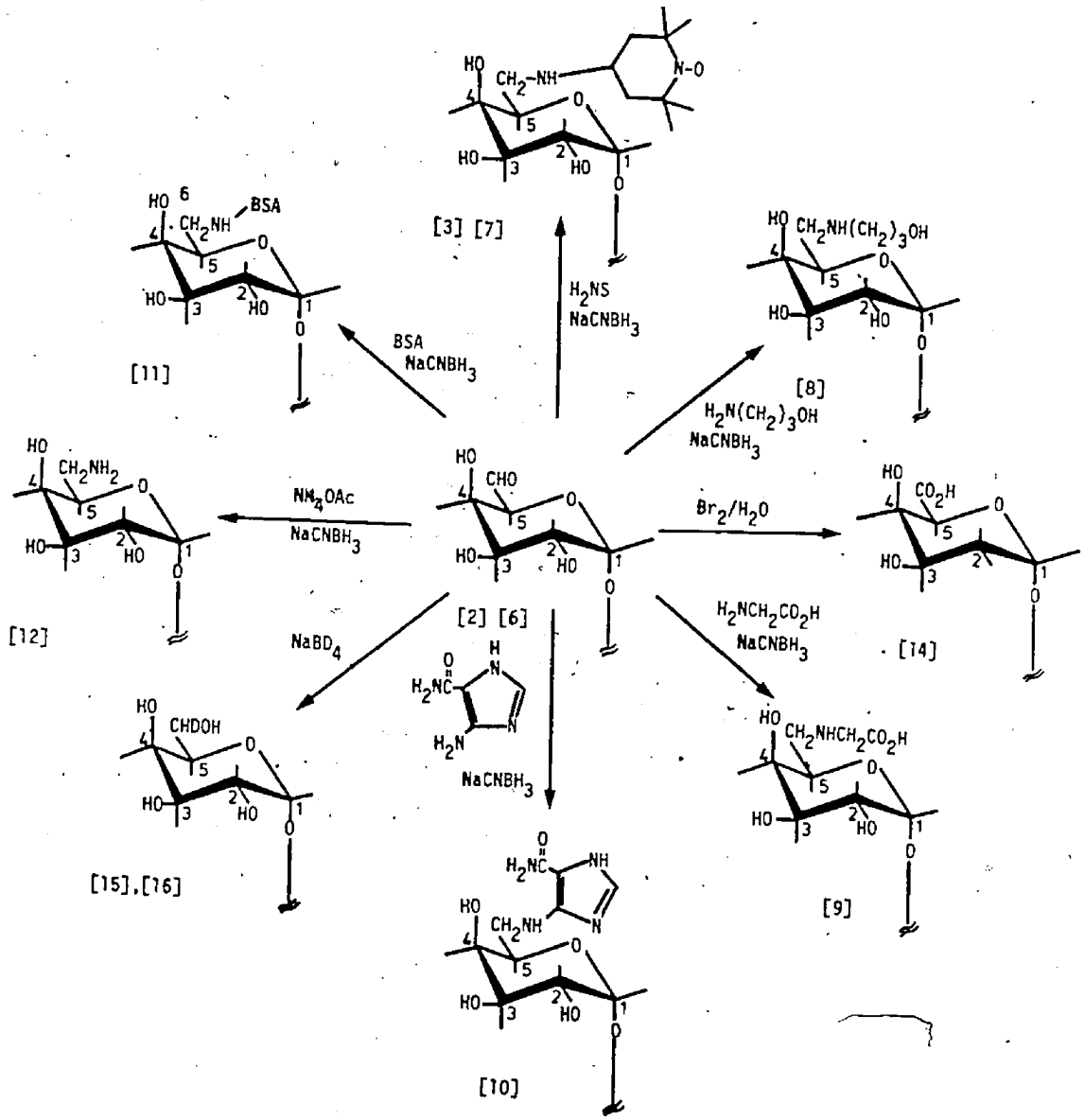
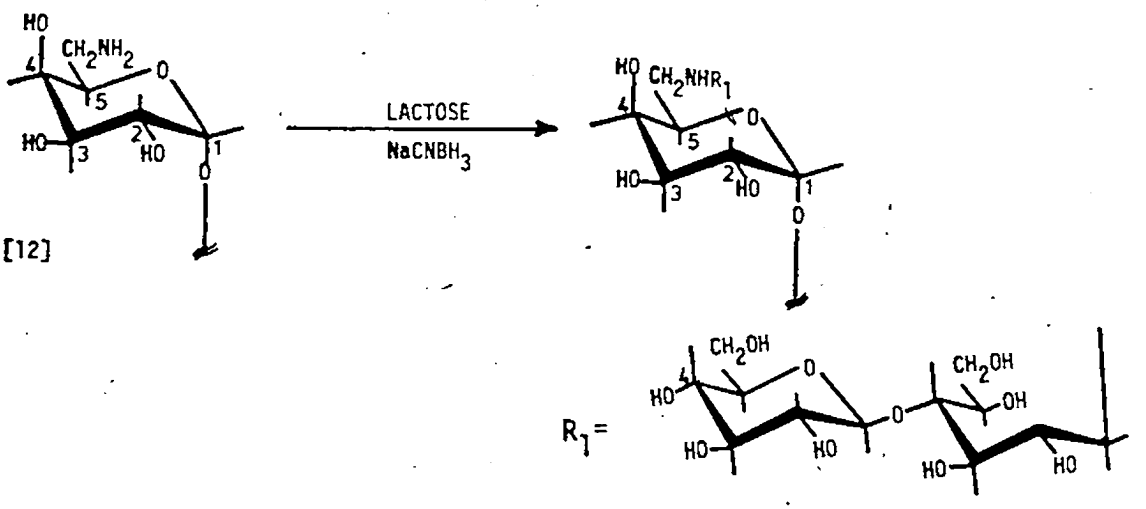


Fig. IV-4. Derivatives obtained from aldehyde intermediates [2] and [6].

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

immobilized enzymes. Similarly, reaction of [2] with glycine to yield [9], another anionic species, illustrates the preparation of a new class of glycopeptides. The hydroxypropylamine guar derivative [8] (d.s. ~0.8) is of interest for its similarity to hydroxyethyl and hydroxypropyl derivatives of guar, prepared from the respective alkylene oxides, which have applications as efficient fracturing fluids[23] for oil well stimulation.[24] In contrast to the latter, [8] is specifically substituted at C_6 of the galactose units which seems to result in substantial differences in the rheological properties (see vi). The imidazole guaran [10] could serve as a model for medicinal derivatives.[25]

Lastly, the utility of the intermediates derived from [2] and [6] is exemplified here by the reductive alkylation of the amino guaran derivative [12] using lactose to afford the branch-extended guaran derivative [13] in acceptable yield (d.s. 0.4).



(v) Nmr

High resolution ^{13}C nmr was employed in an effort to elucidate the structures of the native galactomannans and their derivatives. Figure IV-5 shows the proton-decoupled, natural abundance 100.6 MHz ^{13}C nmr

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

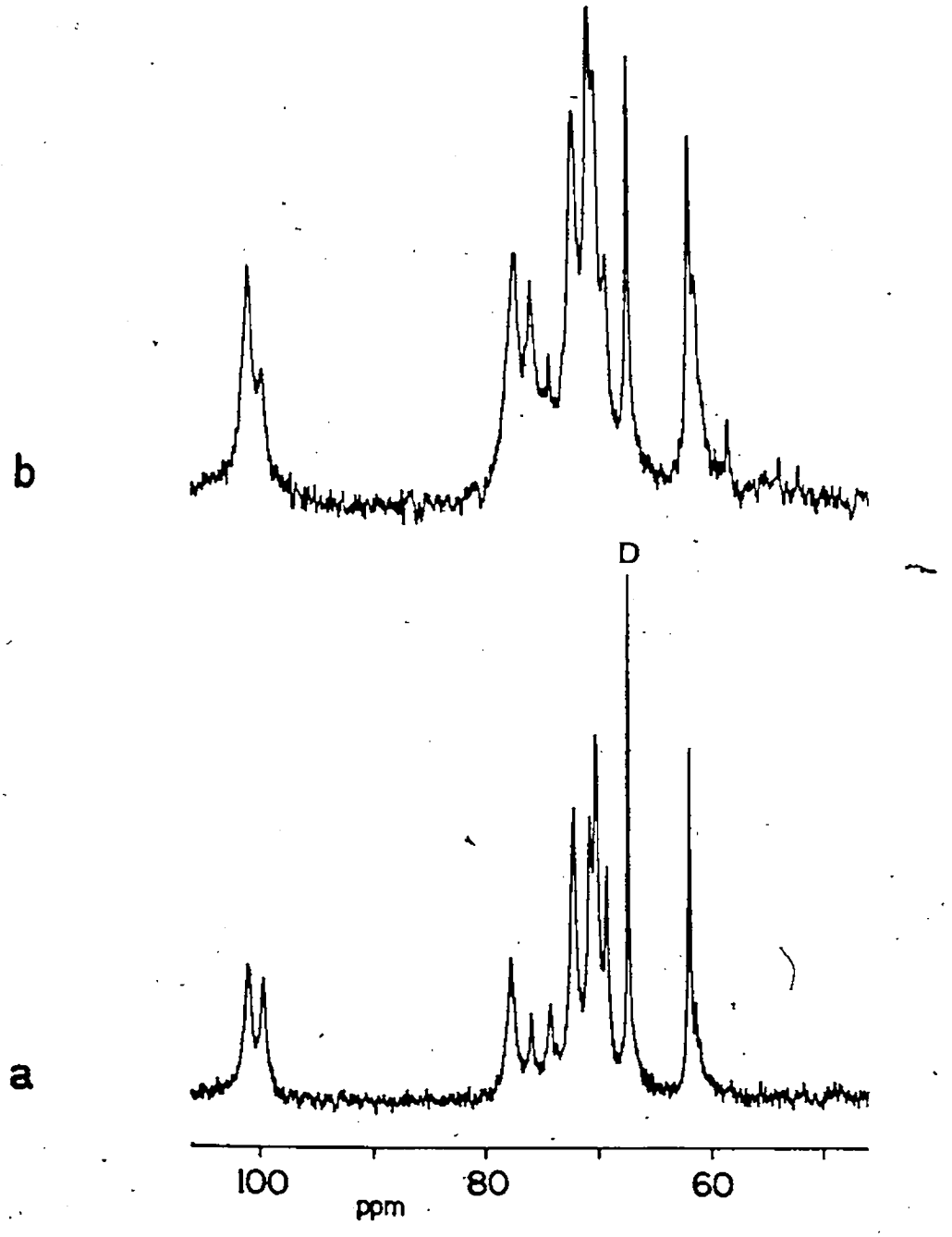


Fig. IV-5. 100.6 MHz ^{13}C-nmr spectra of (a) [1] and (b) [5] in D_2O; (sweep width 23,000 Hz, pulse width 14 μs, delay 0.1 s, 50,000 scans).

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

spectra of guaran and locust bean gum at a probe temperature of 30°C.

From the known mannose to galactose ratios of the two polymers, an initial comparison of these spectra allows for facile identification of several resonances: for guaran (values of [5] are given in parenthesis) the anomeric signals at 100.1 ppm (100.6 ppm) and 99.4 ppm (99.6 ppm) can be assigned to C1 of the β-D-mannose and α-D-galactose residues, respectively, and the methylene resonances at 61.1 ppm (61.1 ppm) and 60.6 ppm (60.7 ppm) are attributable to C6 of the α-D-galactose and β-D-mannose residues. These values agree with those reported for the α-D-galactopyranosyl unit of raffinose[26] and the 1,4-linked β-D-mannopyranose residues of an unbranched mannan[27] (which will henceforth be referred to as α-D-galactosyl unit and 1,4-mannan, respectively).

The resonances at 63.3 ppm (69.5 ppm), 69.9 ppm (69.9 ppm), and 71.3 ppm (71.4 ppm) can be assigned to C2, C3, and C5, respectively of the galactosyl units based on the close agreement with the values of the monomeric equivalent (69.30 ppm, 70.25 ppm, 71.83 ppm). The C4 resonance of the galactose residues expected at 70.03 ppm is unresolved in the guaran spectrum, but partially resolved in the locust bean gum spectrum at 70.6 ppm.

The resonances at 72.9 ppm (unresolved in the spectrum of [5]) and 73.4 ppm (73.5 ppm) can be readily assigned to C2 and C3 of the mannose units, respectively, from comparison with the values for the 1,4-mannan (72.0 ppm, 73.2 ppm).

The deduction of the remaining mannose signals in Figure IV-5 is less trivial in view of the expected complexity[27] arising from branching at C6 of approximately half the mannose units. Thus, the multiplet centred at 76.7 ppm (76.6 ppm) is shifted upfield by 1.6 ppm (1.7 ppm)

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

relative to the position of the corresponding C4 resonance of 1,4-mannan. The resonance at 74.2 ppm (74.4 ppm), shifted upfield by 2.5 ppm (2.3 ppm), is more readily identified as originating from C5 of the unbranched mannose residues in light of its greater relative intensity in the spectrum of locust bean gum compared to that of [1]. From this it can be implied that the resonance at 73.4 ppm (73.5 ppm) arises from C5 of the branched mannose units; in both spectra (Fig. IV-5) the ratio of the relative intensities of the latter two signals is almost identical to that of the respective anomeric signals confirming thereby the above inference.

The ^{13}C nmr spectra, at least in the case of guaran, appear to contain information concerning the sequences of branched and unbranched mannose units. The position of the carbon resonances of the unbranched mannose units is seemingly sensitive to branching at the neighbouring mannose residue as indicated by the splitting of the resonances of C1, C4, C5, and C6 (Fig. IV-6).

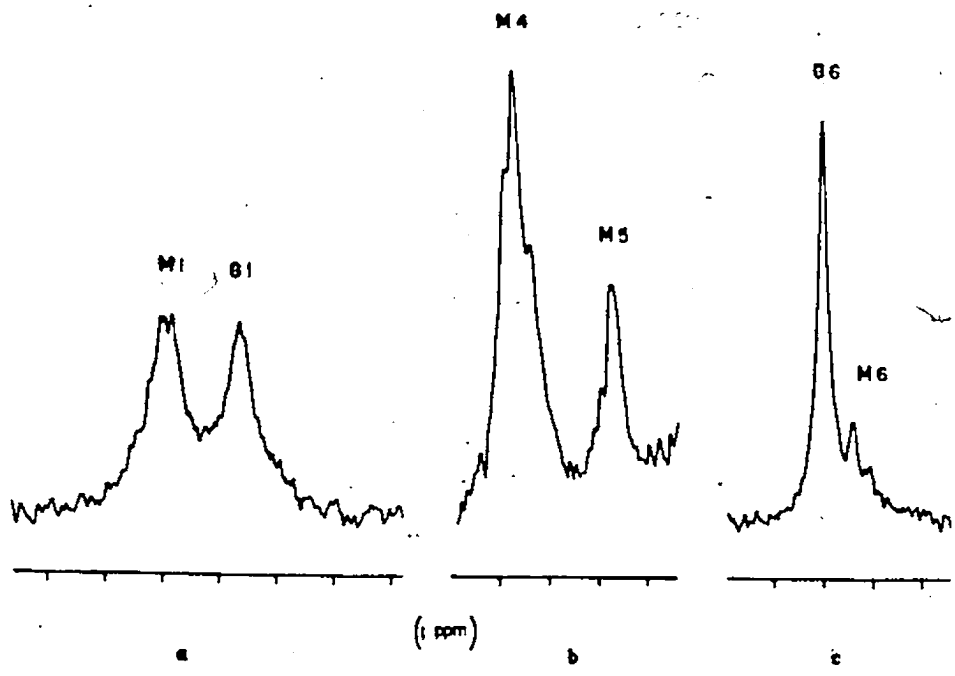


Fig. IV-6. ^{13}C-spectral region of (a) C1, (b) C4, C5, (c) C6 for [1].

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

After completion of these assignments, Grasdalen and Painter[21] reported recently on ^{13}C nmr studies of partially degraded guaran and locust bean gum. The authors obtained a complete spectral assignment, recording their spectra at lower magnetic field (25 MHz) and higher temperature (90°C). Their data are in good qualitative agreement with those described here, from which they differed only in two aspects: (i) the C6 resonance of the branched mannose units was resolved in their spectra (69.7 ppm); and (ii) differences in chemical shift (downfield shifts of up to 2 ppm) of some of the corresponding resonances were observed, particularly for those of the galactose residues. The latter observation derives presumably from the combined effect of various factors, such as differences in probe temperature and sample integrity.*

Some of the postulates advanced here were confirmed by Grasdalen and Painter who, from the splitting pattern of the C4 resonance of the mannose residues, derived nearest-neighbour probabilities for both polymers which were in good agreement with their previous findings.[19,20] In contrast to the guaran spectra discussed here, these authors did not, however, observe peak splitting for any other carbon of the unbranched mannose units.

Thus, in conclusion, the experiments performed here have demonstrated that high resolution ^{13}C spectra of high-molecular weight polysaccharides can be obtained, using admittedly high-field spectrometers, at relatively low temperatures and without extensive degradation of the materials. Although such investigation can be associated with the disadvantage of slightly lower resolution, obvious advantages accrue from the study of the native, undegraded polysaccharides.

---

*Chemical shift displacements, albeit to higher field, were observed in this study when the experiments were conducted at higher temperatures (55°C).

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

(vi) Viscosity

The variety of chemical modifications of the gums can induce a correspondingly large range of interesting rheological properties of the resulting derivatives, as demonstrated by a selection of the guar derivatives discussed above. Some of the observed rheological features are similar in nature to those of the native polymer, while others appear to be novel and unique. Thus, the plot of apparent viscosity versus shear rate (Fig. IV-7) of 1% aqueous solutions of the aldehyde derivative [2] shows the same characteristics of pseudoplasticity as guar gum itself, albeit at far lower viscosities (see I-B). In marked contrast, the carboxylic acid derivative [14] and both the hydroxy propylamine [8] and the spin-labelled ([3]) derivatives seem to exhibit dilatent behaviour which is less pronounced at higher shear rates (>10 sec^{-1}). A better understanding of the latter behaviour is gained when the flow curves are plotted on a logarithmic scale (Fig. IV-8) since (as illustrated in I-B 2) pseudoplasticity and dilatency can be expressed (within a limited shear stress range) in terms of a power law. Comparison of Figures IV-7 and 8 reveals a new feature. Derivatives [3], [8], and [14] exhibit two distinct rheological regions: shear rates below 10-15 sec^{-1} produce dilatent flow, while at higher shear rates no viscosity changes are apparent (Newtonian region). Derivative [2], on the other hand, reveals the expected features of pseudoplastic flow (see I-B). The nature of the viscosity plots of either of the above derivatives was not affected by changes in concentration between 0.1-1.0%.

The apparent dilatent flow behaviour observed here is uncommon for guar or its derivatives; the shear stability of the latter has, however, found many industrial applications. Goldstein et al.[29] found a reduced

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

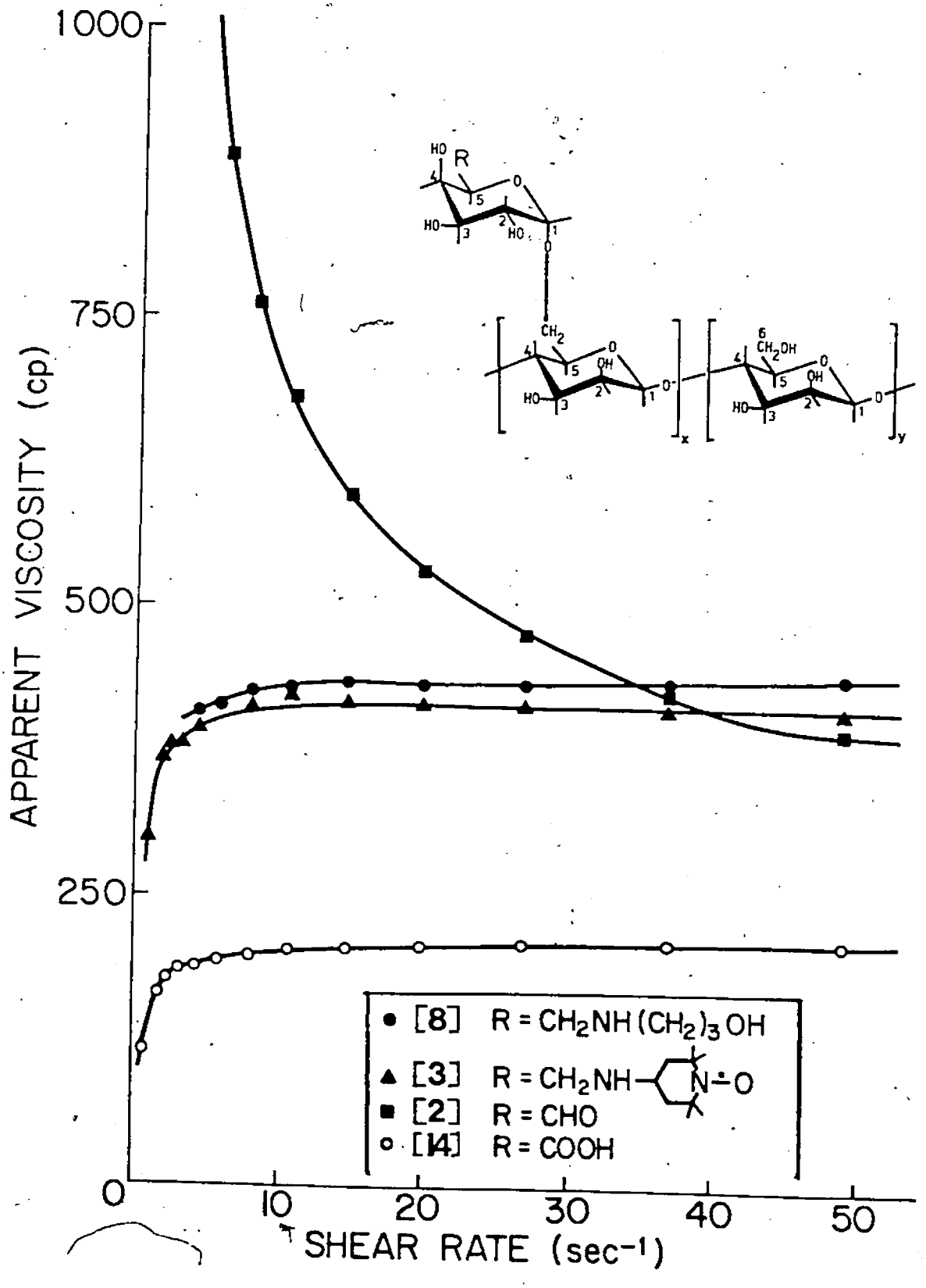
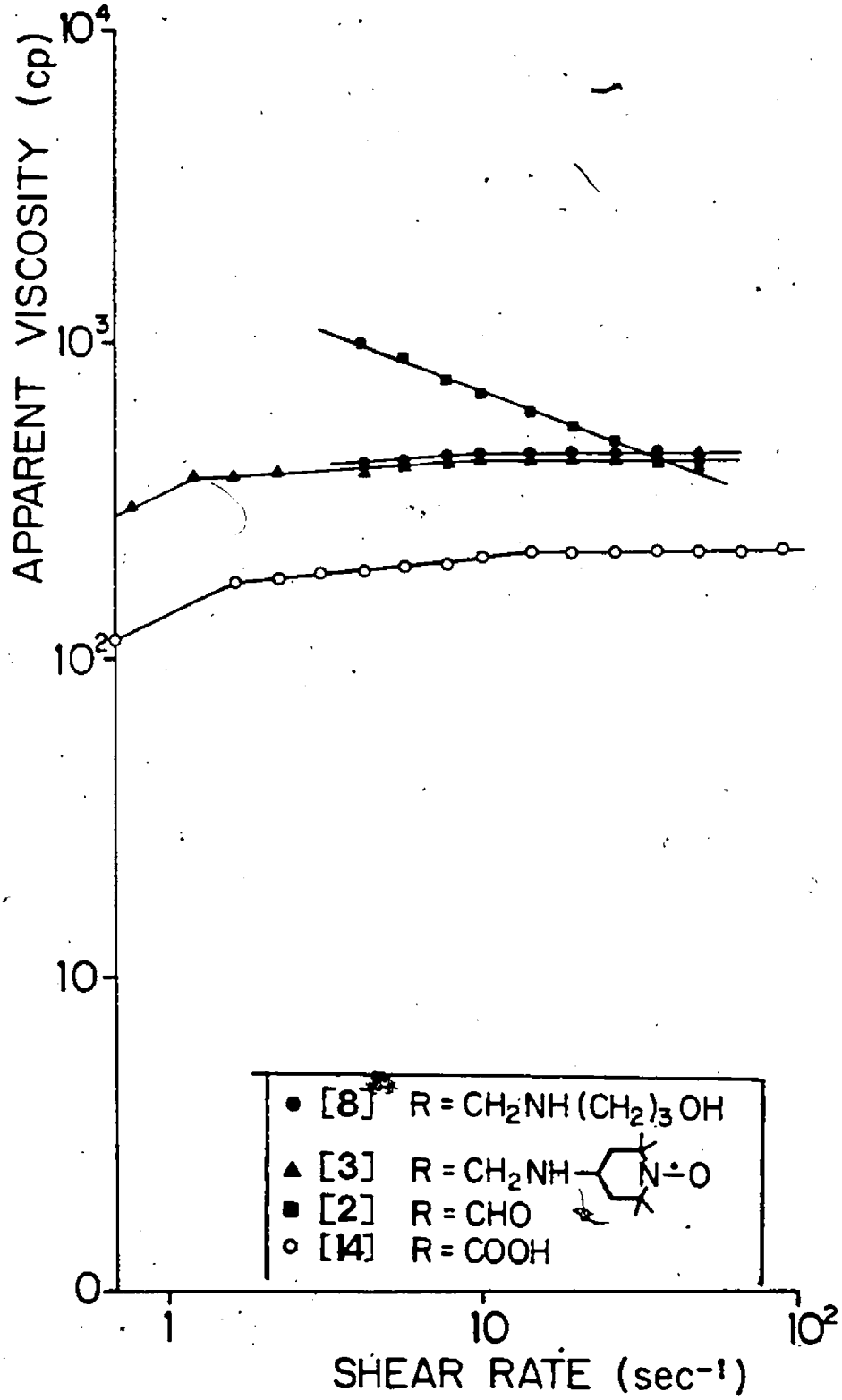


Fig. IV-7. Apparent viscosities of 1% aqueous solutions of guaran derivatives as a function of shear stress.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.



Fig, IV-8. Logarithmic plot of apparent viscosities of guaran derivatives versus shear stress.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

pseudoplastic flow response for dilute (0.3%) aqueous solutions of food-grade guar gum at shear rates between 40-60 rpm (Fig. IV-9), while Holocomb and Smith[23] reported a reduced sensitivity to changes in shear rate for certain cross-linked hydroxy alkyl guar derivatives.

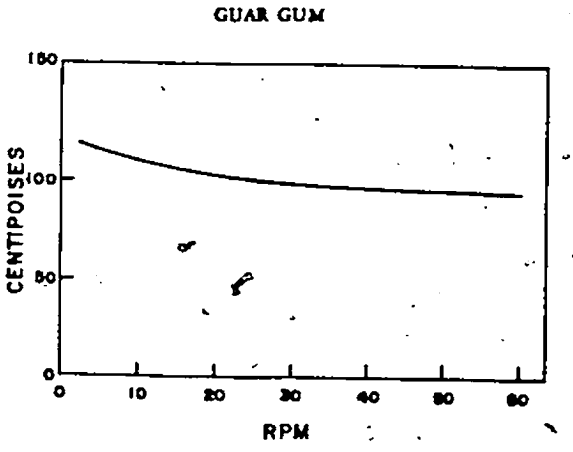


Fig. IV-9. Rheogram of a 0.3% food-grade guar gum solution at 25°C (from ref. 29).

The interesting changes in the rheological properties which were induced here coupled with the ease with which further chemical variations can be made, suggests a number of potentially important applications for these and similar guaran derivatives. This will be further illustrated by the experiments discussed in the subsequent section. It should, however, be pointed out that, for the derivatives described here to be of use in specific areas, a fuller rheological evaluation of the respective derivative would, of course, be required.

(vii) Compatibility

Some of the above guar derivatives were tested for their compatability with aqueous salt solutions, boric acid as well as with absolute ethanol

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

Table IV-1. Compatibility[a] of some guaran derivatives

Compound	Salt $CaCl_2$	$SnCl_2$	K_2CrO_4	$CrCl_3$	H_3BO_3	Ethanol
[1]	+	+	+	-	-	-[c]
[2]	+	-[b]	+	+	+	+
[3]	+	-[b]	+	+	+	-[c]
[8]	+	-	+	+	+	+
[13]	+	-[b]	+	+	+	-[c]

[a] 1% aqueous solutions of polysaccharide with saturated, aqueous solutions of metal salts; + indicates compatibility, - incompatibility; [b] gel formed; [c] precipitate.

and the results are compared with the native guaran [1] in Table IV-1. The guaran derivatives show good compatibility with metal ions, none being gelled in contrast to [1] by either chromium chloride or boric acid. Unlike the native guaran, however, they are gelled by tin chloride. Noteworthy, also is the stability of the aldehyde and hydroxypropylamine derivatives [2] and [8] towards ethanol.

These complementary compatibility properties found here could serve to greatly extend the range of useful applications, particularly in the oil industries, of guar gum.

2. Periodate Oxidation

The foregoing oxidation procedures using galactose oxidase were shown to be high-yielding and specific, but may, nevertheless be not directly compatible with industrial applications in view of the costs involved. Enzyme immobilization and controlled periodate oxidation can be suggested as two alternative, less expensive routes, of which the feasibility of the latter was investigated to some extent here.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

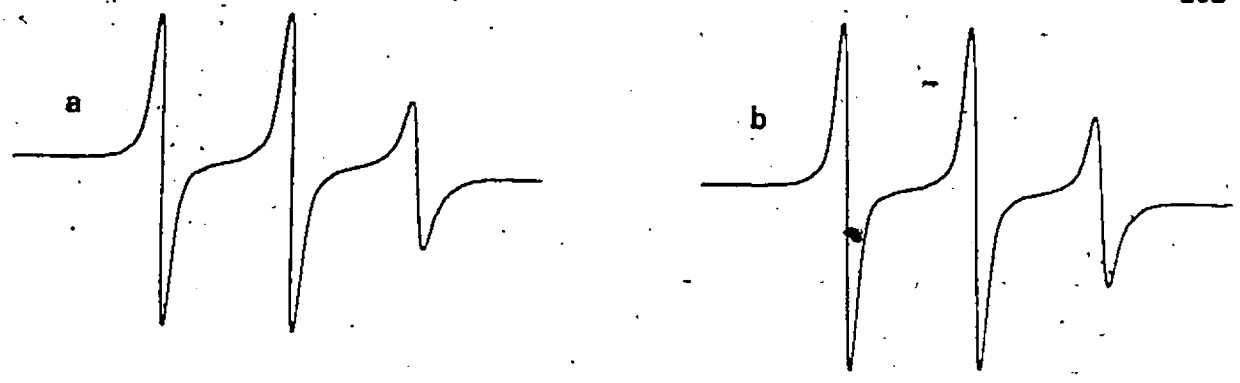


Fig. IV-10. Esr spectra of aqueous solutions of (a) [21], (b) [22] at 298K.

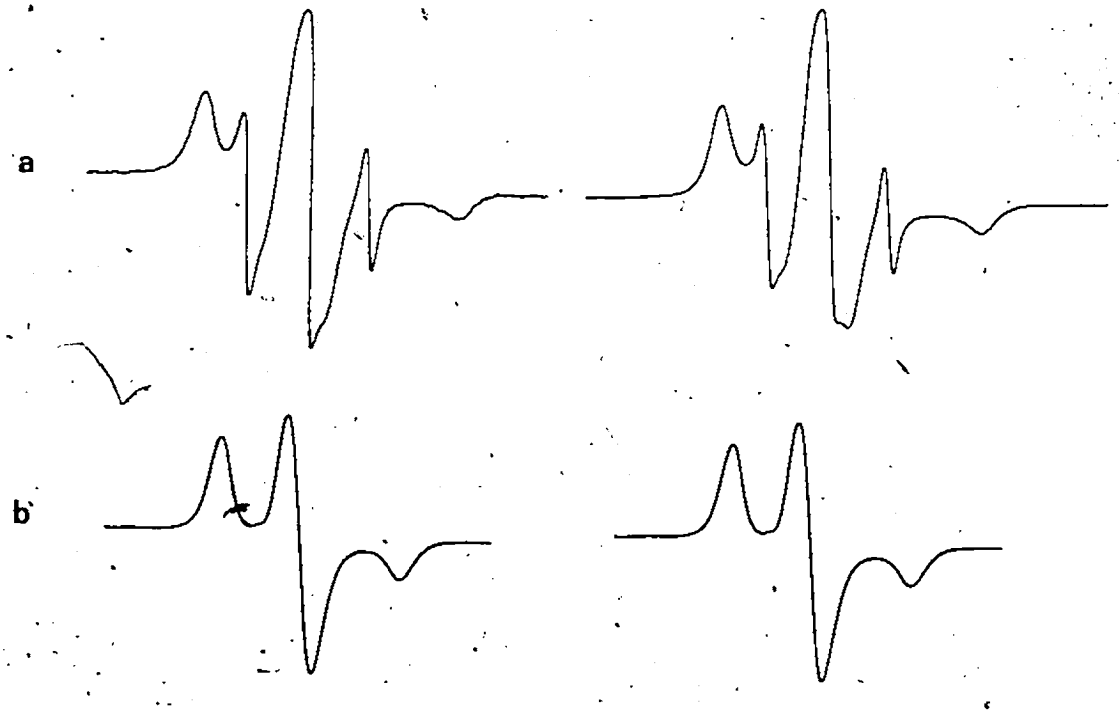


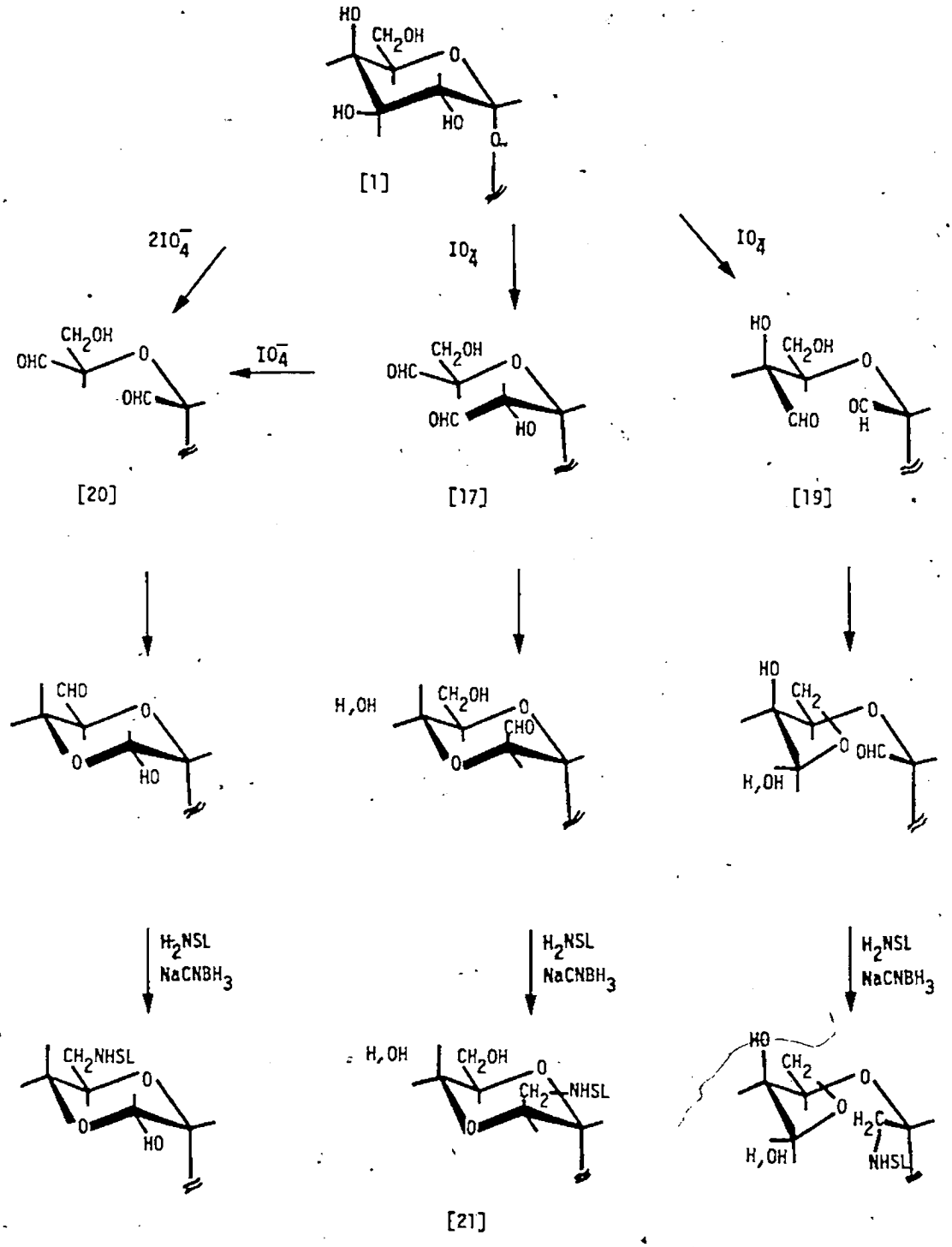
Fig. IV-11. Esr spectra of [21] (left) and [22] (right) (a) solid (at 298K), (b) frozen at 77K.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

The preferential oxidation of the galactopyranosyl groups of guar gum and locust bean gum by limited quantities of sodium metaperiodate had been previously reported to leave the mannose backbone of these polymers largely intact.[28] Thus, Opie and Keen,[28] using 0.25 mole $NaIO_4$ per mole of guaran hexose unit, found 77% of the oxidized hexose units of the product to derive from the galactose residues. Following their method, guar gum and locust bean gum were oxidized using even smaller amounts of periodate (0.19 mole and 0.14 mole $NaIO_4$/mole of hexose, respectively) which correspond to 14% and 12%, respectively, of the theoretical requirement. If, as assumed, periodate consumption is confined to the galactose units in each case, the expected degrees of oxidation (d.o.) of these units would be 0.53 and 0.70, respectively, i.e., the polymers would be incompletely oxidized at these periodate levels.

Another limitation of this method becomes apparent when one proceeds to examine the structure of the dialdehyde products. Although it is known that the reactivity of the galactosyl cis-diol (C3 and C4) is much greater than that of the trans-diol (C2 and C3), and that, hence, the product should predominantly be [17] rather than [19], complications arise from the variety of possible intra-residue cyclic hemiacetal structures (inter-residue hemiacetal formation is not favoured)[19] and the small (in view of the very low levels of periodate used) but finite probability for a monooxidized galactose residue to consume a second mole of periodate[20] affording [20]. Thus, upon reductive amination of the dialdehyde products derived from guaran ([17]) and locust bean gum ([18]), using spin-label [4], a series of spin-labelled product structures can be envisaged to exist for each of the polymers some of which are indicated in scheme 2.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.



SCHEME 2

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

In view of the above, extensive esr studies of the spin labelled guar gum [21] and locust bean gum [22] derivatives were considered to be not worthwhile. Suffice it to say that the esr spectra of [21] and [22] in aqueous solution (Fig. IV-10) resembled in lineshape those of the corresponding galactose oxidase-derived samples (Fig. IV-1) and had similar apparent τ_c values (for [21], $\tau_c = 6.36 \times 10^{-10}$ sec and for [22], $\tau_c = 5.31 \times 10^{-10}$ sec). The esr spectra of the solid derivatives reveal two components of which one appears to arise from a more mobile label population (Fig. IV-11a). This interpretation is not unreasonable in view of the possibility of different oxidation products and the fact that the products were purified by extensive (5 days) dialysis. No dipolar interactions are evident in either the above spectra or in those obtained at 77K (Fig. IV-11b), indicating a larger mean separation between spins than in the corresponding enzyme-treated materials. This can presumably be ascribed to a more extended, sterically less crowded conformation of the side-chains in the ring-cleaved derivatives in comparison to the enzyme-derived samples.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

References

1. See, for example, J. F. Kennedy, in Carbohydrate Chemistry, Specialist Periodical Reports, 11, 371 (1979).

2. G. Avigad, D. Amaral, C. Asensio, and B. L. Horecker, J. Biol. Chem., 237, 2736 (1962).

3. D. Amaral, L. Bernstein, D. Morse, and B. L. Horecker, ibid., 238 (1963); D. J. Kosman, M. J. Ettinger, R. E. Weiner, and E. J. Massaro, Arch. Biochem. Biophys., 165, 456 (1974).

4. J. M. Sempere, C. Gancedo, and C. Asensio, Anal. Biochem., 12, 509 (1965); J. N. C. Whyte and J. R. Englar, Carbohydr. Res., 57, 273 (1977).

5. R. A. Schlegel, C. M. Gerbeck, and R. Montgomery, Carbohydr. Res., 7, 193 (1968).

6. S. M. Rosen, M. J. Osborn, and B. L. Horecker, J. Biol. Chem., 239, 3196 (1964); W. Jack and R. J. Sturgeon, Carbohydr. Res., 49, 335 (1976).

7. A. Maradufu and A. S. Perlin, ibid., 32, 43 (1974).

8. A. Maradufu, G. M. Cree, and A. S. Perlin, Can. J. Chem., 50, 768 (1971); 49, 3429 (1971).

9. M. W. C. Hatton and E. Regoeczi, Biochim. Biophys. Acta, 438, 339 (1976).

10. K. Y. Chen, R. H. Kramer, and E. S. Canellakis, ibid., 507, 107 (1978).

11. M. A. Bernstein, L. D. Hall, and W. E. Hull, J. Amer. Chem. Soc., 101, 2744 (1979).

12. G. Avigad, Carbohydr. Res., 3, 430 (1967).

13. J. D. Aplin, M. A. Bernstein, C. F. Culling, L. D. Hall, and P. E. Reid, ibid., 70, C9 (1979).

14. L. D. Hall and M. Yalpani, ibid., 81, C10 (1980).

15. See, for example, D. M. Clode and D. Horton, ibid., 19, 329 (1971).

16. R. L. Whistler and J. W. Marx, Methods Carbohydr. Chem., 5, 143 (1965).

17. G. A. Hamilton, P. K. Adolf, J. de Jersey, G. C. Du Bois, G. R. Drykacz, and R. D. Libby, J. Amer. Chem. Soc., 100, 1899 (1978).

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

18. See, for example, W. Hartmeier and G. Tegge, Starch, 31, 348 (1979).

19. J. J. Gonzalez, Macromolecules, 11, 1074 (1978).

20. T. J. Painter, J. J. Gonzalez, and P. C. Hemmer, Carbohydr. Res., 69, 217 (1979).

21. H. Grasdalen and T. Painter, ibid., 81, 59 (1980).

22. C. W. Baker and R. L. Whistler, ibid., 45, 237 (1975).

23. D. L. Holcomb and M. O. Smith, Southw. Petrol. Short Course, 22, 129 (1975).

24. C. J. Githens and J. W. Burnham, Soc. Pet. Eng. J., 17, 5 (1977).

25. H. Guglielmi and A. Jung, Hoppe-Seyler's Z. Physiol. Chem., 358, 1462 (1977); J. A. Hickman and D. H. Melzack, Biochem. Pharmacol., 25, 2489 (1976).

26. G. A. Morris and L. D. Hall, submitted for publication.

27. P. A. J. Gorin, Carbohydr. Res., 39, 3 (1975).

28. J. W. Opie and J. L. Keen, Germ. Pat., 1,262,756 (1968); Chem. Abstr., 68, 88358K (1968).

29. A. M. Goldstein, E. N. Alter, and J. K. Seaman, in Industrial Gums, R. L. Whistler (ed.), Academic Press, New York, 303 (1973).

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

CHAPTER V

MODIFICATION OF THE METAL-CHELATING CAPACITY OF CHITIN AND CHITOSAN

V-A. Introduction

The application of polymers as support matrices for chelation,[1] clinical use,[2] catalysis,[3] as well as for synthesis has grown rapidly since their use in peptide synthesis was demonstrated by Merrifield. One facet of this effort has been directed at incorporating metal ions or metal complexes into polymers using a variety of chelating groups. Some problems encountered in many of these studies derive from the often complex and costly synthesis, from inefficient metal chelation, and from the leaching into solution of the metal complex from the polymer. The search for new and efficient chelating polymers constitutes therefore a major area of research. Numerous other reasons exist for interest in metal-polymer conjugates including the study of metal complexes in biological systems,[4] metal-based affinity chromatography,[5] and the treatment of environmental pollutants.[6]

The work described in this chapter represents an effort to direct some of the previously gained experience towards some specific application. Chitin and chitosan appeared to be well-suited for advancing the development of metal chelation since both materials are inexpensive, abundant 'natural' chelating agents[7] and since an extensive knowledge of the chelating properties of their monomeric constituent has been established for many years.[8] We found, surprisingly, that, in spite of a number of studies of metal chelation by the native polymers,[7,9] only a few very recent attempts to

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

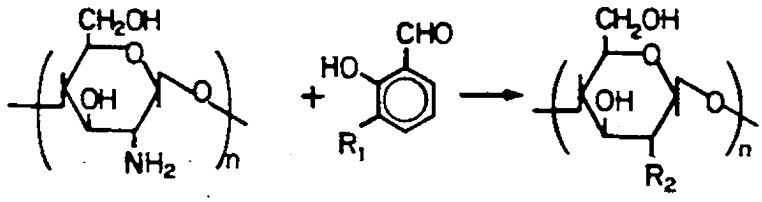
improve their chelating capacity by means of chemical derivatization have been described in the literature.[10] Thus, as an extension of a long-standing interest in this laboratory in "metal-conjugation" by monosaccharides,[11] including amino-sugars,[12] methods were first evaluated whereby the metal-chelating performance of both chitin and chitosan can be enhanced or modified. The incorporation of a relatively novel concept, namely the "tailoring" of the solubility properties of the polymeric derivatives, was demonstrated for these procedures. Lastly, a method was investigated for the preparation of a new type of organometallic polysaccharide derivative.

V-B. Modification of Chelating Capacity and Solubility

1. Enhancement of Chelating Performance

(i) Synthesis and copper complexes

Chitosan [1] was condensed with salicylaldehyde [2] following the methods of Nudga et al.[12] and Hirano et al.[13] to afford the Schiff's base derivative [3] (scheme 1):



[1] R_1= R_2=

[2] H [3] N=CH- HO

[6] $-CO_2H$ [4] NH-CH_2- HO

N=CH- HO CO_2H

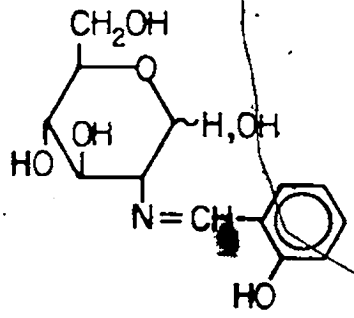
scheme 1

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

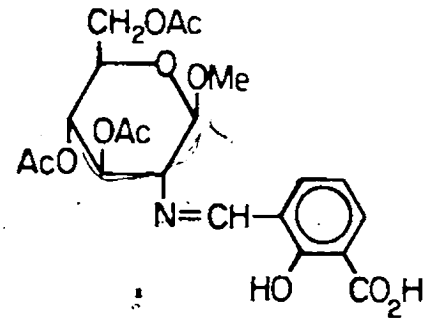
Interestingly, reduction of the acid-labile* azomethine function of salicylidene-chitosan [3], with sodium cyanoborohydride simultaneous with its formation produced a very soft, ivory coloured gel, which after dialysis and lyophilization, gave the amine [4] (d.s. 0.6) as a fluffy, off-white material. (Attempts to carry out the reduction consecutive to the formation of [3] were only partially successful as indicated by the retention of most of the yellow colour and rigidity of the gel initially produced.)

The Schiff's base derivative [5] (d.s. 1.0) was produced in similar fashion from 3-formyl-2-hydroxy benzoic acid [6].**

The salicylidene chitosans [3], [4], and [5], like chitin [7] and chitosan [1], readily reacted with copper(II) acetate in either aqueous or methanolic*** solution to produce, by analogy with the monosaccharide equivalents [8] and [9],[14] coloured complexes (see Table V-1), which could be characterized by esr spectroscopy.



[8]



[9]

*The derivative is, however, stable to base.[13]

**Kindly provided by Dr. M. J. Adam.

***No substantial differences in Cu-chelation capacity between these media were observed.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

Table V-1. Copper Chelation Performance of some Chitin and Chitosan Derivatives[a]

Cmpd.	Time[b] (hr)	Copper content mmol g^{-1}[c]	Copper content % of theory	Colour parent polymer	Colour Cu(II) complex
[3]	1	0.54	23	deep yellow	light green
	12	0.62	26		dark green
[4]	1	2.19	72	white	green
	12	3.03	100		dark green
[5]	1	0.02	1	deep yellow	deep yellow
	12	0.06	2		deep yellow
	20	-	-		green yellow
[10]	1	0.42	13	white	light blue
	12	2.64	80		turquoise
[11]	1	0.26	7	light yellow	light green
	12	0.40	11		turquoise
[1]	12	0.06	1	white	blue
[7]	12	0.18	4	yellow	light blue
[12]	12	0.07	1	yellow	light green
[13]	12	0.01	0.3	light yellow	light yellow

[a] in methanol at 25°C

[b] contact time

[c] millimole per gram polymer

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

Atomic absorption spectroscopy was used to quantitatively determine the amounts of Cu(II) incorporated into the derivatives. Table V-1 shows that the copper chelation capacity of the amine [4], sampled after 12 hr reaction time, was enhanced by a factor of four over that of [3], and a 50 factor over that of [5] or chitosan.

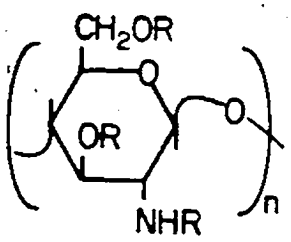
This increased chelating capacity of [4] over [3] is in line with the observed stability constants of the copper(II) complexes of related ligand systems (for a comparison, see ref. 16). Furthermore, the greater porosity[7a] of [4], a fluffy, water-insoluble material which in contrast to its solid analogue [3] and the other derivatives, swelled considerably in aqueous or alcoholic solution, is presumably also partly responsible for this observation. More information was gained from experiments in which attempts were made to elute the copper ions from the complexes of [3] and [4], using 0.1M EDTA solution at pH 8, this proceeded successfully for the former case, whereas ~30-40% of the copper was retained by the latter complex, which was, however, completely "demetallated" by treatment with aqueous acid (pH 2). These findings testify again to the greater chelating ability of [4].

It is interesting to note (see Table V-1), that the amine [4], its analogue [3], and chitosan* chelated a relatively large proportion (72%, 87%, and 86%, respectively) of their total uptake within a short period (1 hr), whereas [5] complexed a relatively smaller amount (30%) of copper. The high chelating rate of [4] was also reflected in the almost instantaneous colouration (green) when this material was added to a solution of cupric or nickelous ions.

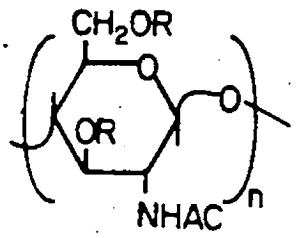
---

*From reference 7a (obtained under equivalent conditions).

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.



[11] R=CH_2CO_2H,H



[10] R=CH_2CO_2H,H

A number of studies[17] have suggested the use of chitosan for metal-based chromatography and from the foregoing discussion it is apparent that the salicyldimine type chitosan derivatives may be of interest for similar applications, two aspects of which are suggested here:

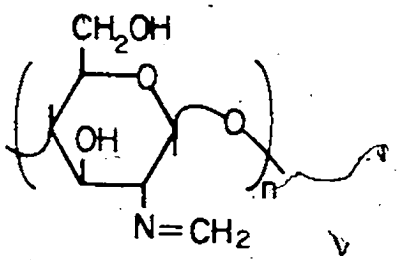
1. Metal ions can be collected on columns packed with these materials and subsequently eluted therefrom using complexing agents, such as EDTA. The metal ions can be alternatively removed with acidic solutions (pH2) as the salicylaldehyde complex and recovered after degradation of the complex. Overall, it is therefore possible to regenerate both the chitosan column and the salicylaldehyde derivative. This latter procedure is of importance because it could be utilized for the large-scale purification of salicyladehyde derivatives in analogy to established methods.[18]
2. Reduction of the Schiff's base derivatives affords versatile derivatives which are hydrolytically stable to acidic and basic conditions.

We have also evaluated a number of other derivatives of chitin and chitosan which may be preferable complexing agents for large scale applications since they are less expensive to prepare. The carboxymethyl derivatives of chitin [10] (d.s. 1:0) and chitosan [11] (d.s. 1.2)

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

both prepared* by a procedure similar to that of Trujillo,[19] formed turquoise copper(II) complexes. Both derivatives had a greater chelation efficacy than their underivatized precursors [1] and [7] (20 times and 11 times, respectively) (see Table V-1). The carboxymethyl chitosan derivative [11] is therefore an excellent candidate for commercial metal complexation.

Another chitosan derivative tested was the N-methylene compound [12] which was recently described[20] as material for gel chromatography. Reaction of [12] with copper(II) acetate produced a blue gel. The chelating capacity of [12] was, however, found to be not significantly greater than that of chitosan (Table V-1).



[12]

It should be noted that due to the intrinsic variations in porosity and grain size (e.g., chitosan flakes were employed) of the materials studied, absolute levels of metal chelation are not as informative as the relative (or enhanced) values. These materials should also not be evaluated solely on the basis of their absolute chelation capacity since, under suitable conditions, various synthetic polymers may also exhibit high metal complexing efficacies. Other factors, in addition to the ones

*The degrees of substitution of both products were found to be quite sensitive to experimental conditions, i.e., reaction time with alkali and chloroacid--see Experimental.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

previously alluded to, such as the stability and rate of formation of the polymer bound metal complex, its selectivity to and general sensitivity towards various classes of metal ions (e.g., alkali earth, and transition metals), and the versatility of the materials, should be taken into consideration. Although time did not permit the author to pursue some of the above aspects, the findings described here clearly indicate the utility of these aminopolysaccharides as hydrophilic support matrices for metal chelating agents; we suggest that systematic studies might be rewarding.

(ii) Esr of copper complexes

The esr spectra of the copper complexes of the native polymers and of the derivatives unambiguously established the presence of polymer-bound copper complexes (Figs. V-1-4). The spectra of all the samples reveal closely related features: the nitrogen hyperfine couplings are unresolved but an axial symmetry about the copper atom is displayed. The spectrum of [5] is distinguished by an additional $g_{\perp}$ band (Fig. V-4).

Attempts to identify the coordination geometry of these copper complexes were undertaken, but were inconclusive due to the complexity and variety of possible chelation sites (i.e., -OH, $-NH_2$, -NHAc, $-CO_2H$, etc.) of these materials. Thus, an impression that the predominance of one or more coordination geometries could be dependent on metal ion concentration (very vaguely apparent in the case of the chitosan-Cu(II) complex, Fig. V-3) could not be confirmed when the derivative [5] was exposed to increasing amounts of metal ions; the induced esr spectral changes were unrevealing (Fig. V-4). The esr spectra of the frozen (77K) Cu(II)-complexes did likewise not provide

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

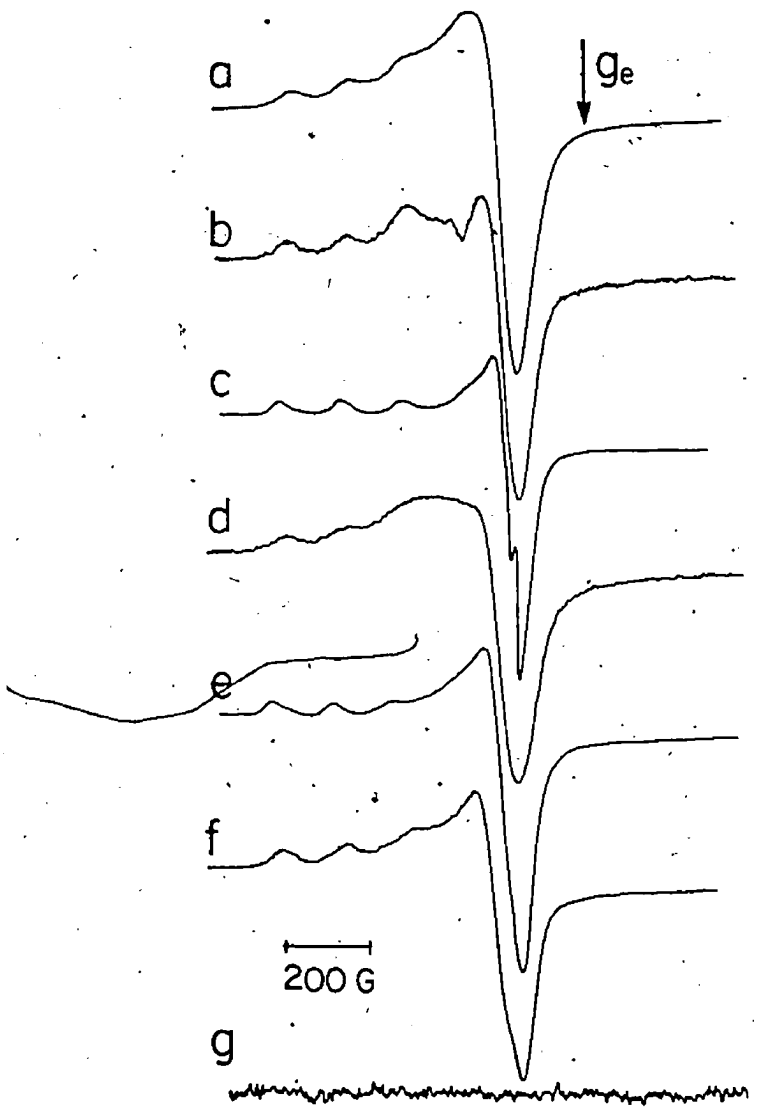


Fig. V-1. Esr spectra of Cu(II) complexes of (a) [3], (b) [4], (c) [5], (d) [10], (e) [11], (f) [12], (g) [13] at ambient temperature.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

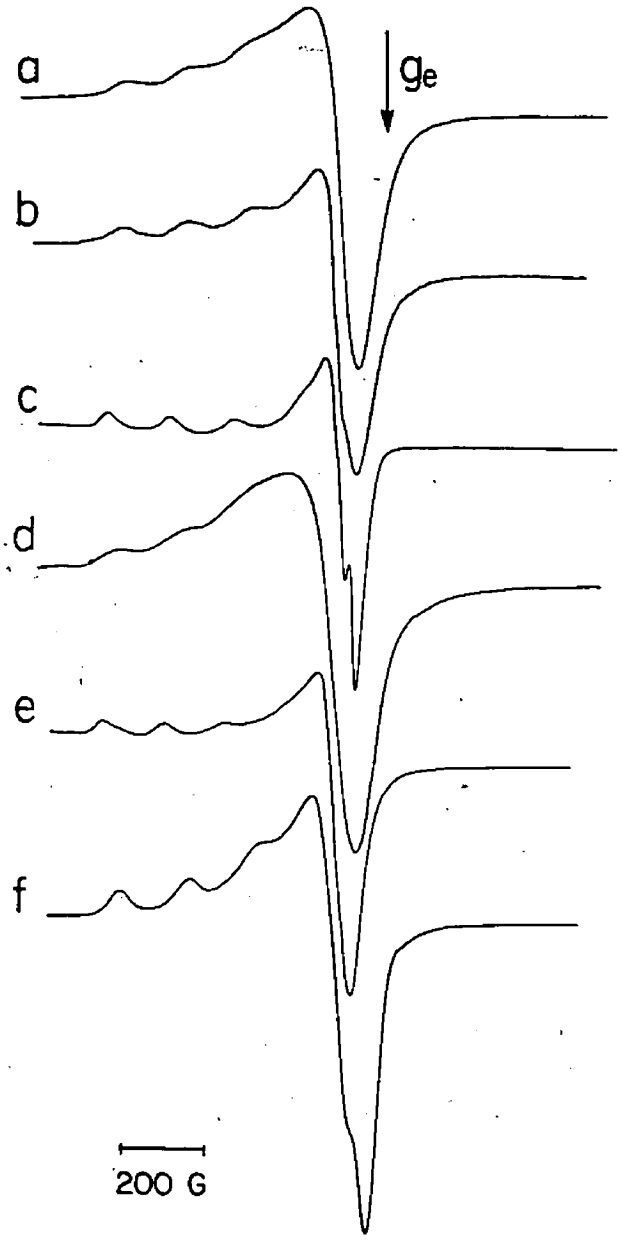


Fig. V-2. Esr spectra of Cu(II) complexes of (a) [3], (b) [4], (c) [5], (d) [10], (e) [11], (f) [12] at 77K.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

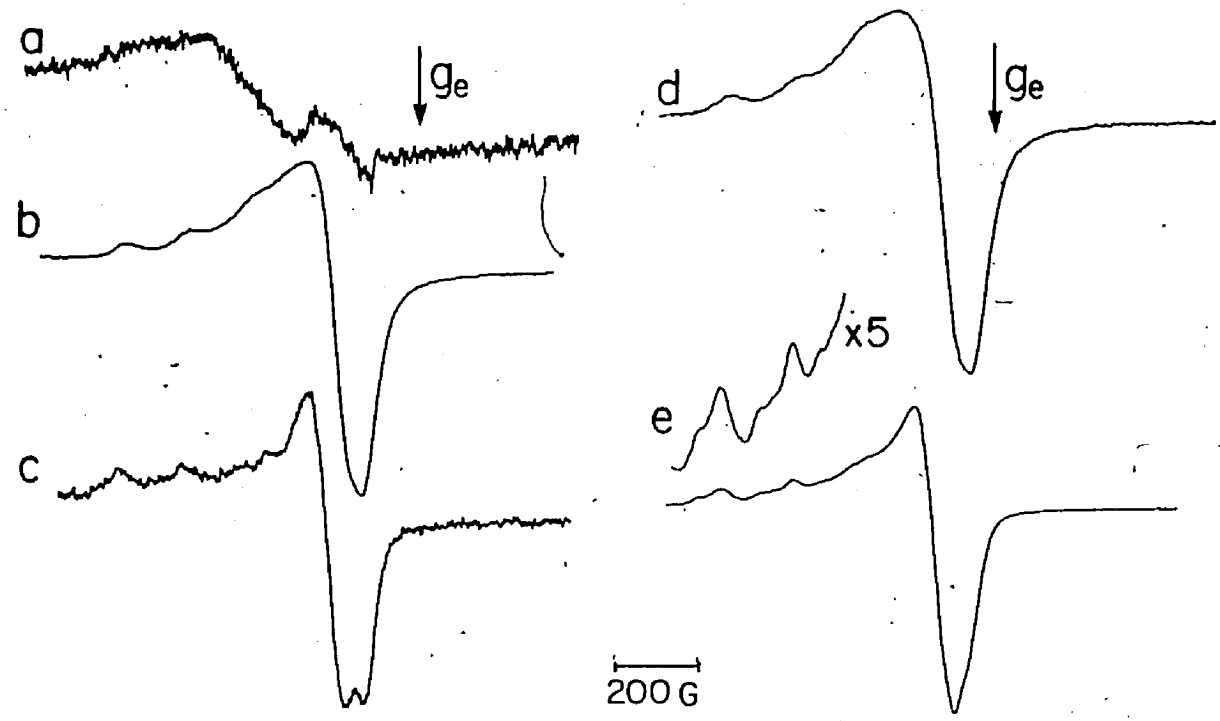


Fig. V-3. Esr spectra of Cu(II) complexes of (a) [1] after 8 hr reaction time (298K), (b) [1] after 20 hr reaction time (298K), (c) [7] after 8 hr reaction time, (d) sample (b) at 77K, (e) sample (c) at 77K (inset: 5× amplification).

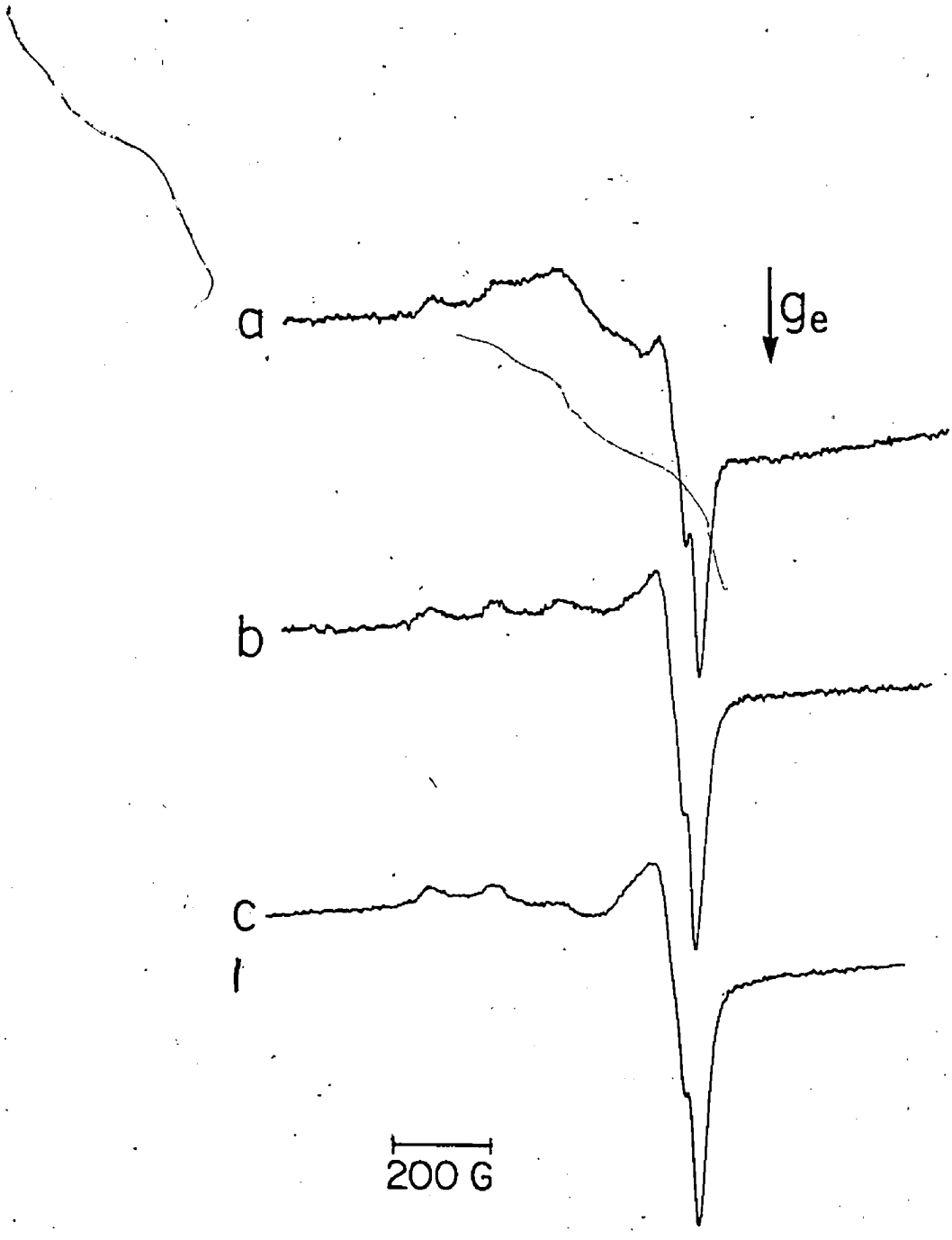
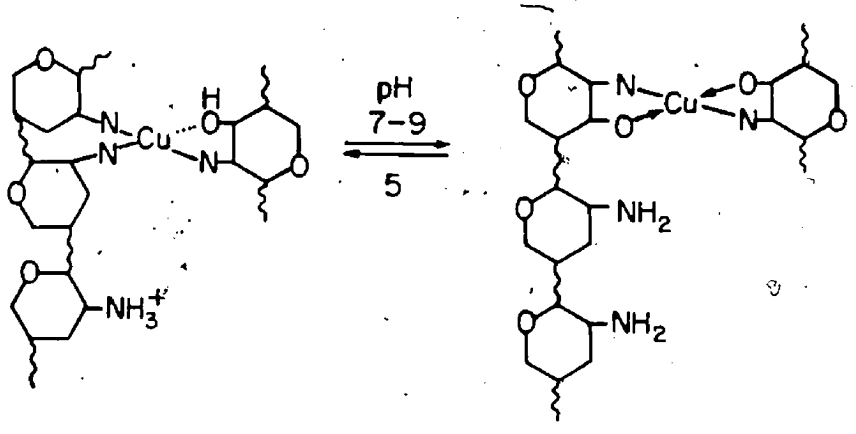


Fig. V-4. Ambient temperature esr spectra of Cu(II) complex of [5] after (a) 8 hr, (b) 16 hr, (c) 28 hr reaction time.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

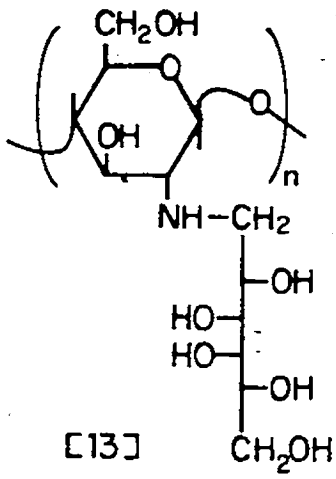
any additional information (Fig. V-2). It is most likely that a mixture of geometries rather than one pure coordination geometry prevails. It can nevertheless be noted that the corresponding mono- and oligosaccharide copper conjugates (of glucosamine) reportedly form binuclear complexes[15] and species consisting of one Cu(II) ion and four moles of glucosamine units,[22] respectively. For the native chitosan in aqueous solution, Inaki et al.[23] have recently proposed an equilibrium between Cu(II) complexes which, depending on the pH of the medium, involves two or three glucosamine units per metal atom and is accompanied by conformation changes of the polymer as shown below.



2. Reduction of Chelating Capacity

Insofar as all the above derivatives possessed enhanced chelating capacities, it is interesting to also observe the converse phenomenon, i.e., reduction or complete inhibition of metal complexation. For example, chitosan reductively aminated with galactose affords a branched-chain derivative [13] (see III-B) which, on exposure to Cu(II) ions does not alter its colour nor does it produce a detectable esr signal (Fig. V-19). This chelating "inhibition," also determined by atomic absorption spectroscopy (Table V-1), is presumably due, to a large extent, to

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.



[13]

the molecular architecture of this material as evidenced by scanning electron microscopy (see III-E). The smooth, non-porous surface structure of [13] appears to be impervious to metal ions. In contrast, the amine [4], along with most other aminopolysaccharide derivatives, is a porous material.

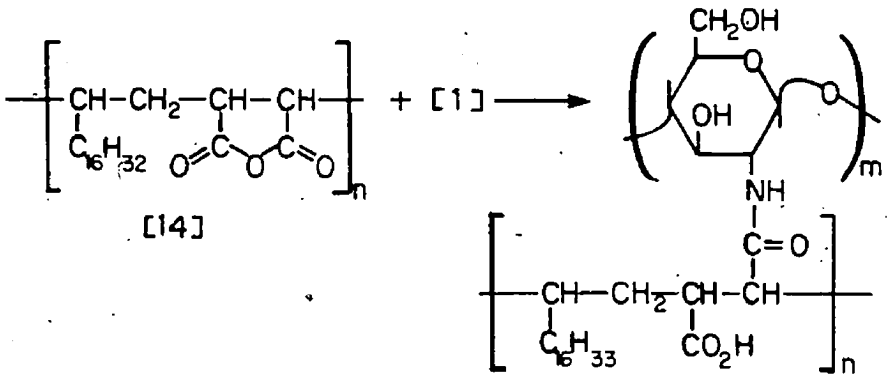
3. Solubility Modification

The versatile combination of Schiff's base-formation and reductive amination provides a convenient route for attaching and/or controllably releasing a wide range of medicinally or otherwise important molecules to chitosan, which itself is a biodegradable material. Medicinal application,[7b,24] in particular, would clearly benefit from the additional options of selectively either solubilizing the polymer backbone, (an example of which will be described subsequently) or conversely, reducing its solubility by reaction of chitosan [1] with another polymeric species.

As an example of the latter effect, the polyanhydride [14] (Gulf PA-18)* was reacted to afford the white insoluble derivative [15] (d.s. 1.0):

*A sample of which was provided by Gulf Specialty Chemicals.

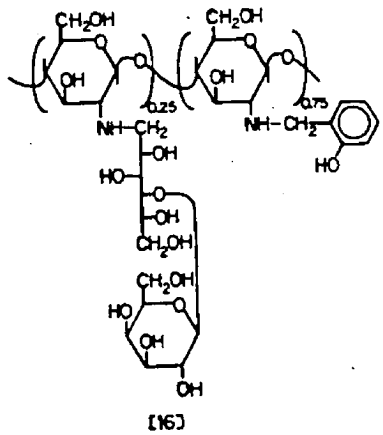
Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.



[14] [15]

Reactions of this type have been used to insolubilize enzymes using copolymers of ethylene and maleic anhydride.[25]

On the other hand, it is feasible to prepare polymeric compounds derived from [1] which have enhanced solubility. The amine [4], as already mentioned, was not watersoluble, whereas reductive amination of chitosan with 1.2 molar equivalents of salicylaldehyde [2] and 0.3 molar equivalents of lactose produced a slightly watersoluble product [16] (d.s. 1.0) which contained a salicyldimine to sugar ratio of 3:1. This product, in view of its solubility properties and structural similarity to certain poly (vinylsalicylic acid) derivatives of the type [17],[24] was selected for some preliminary tests of antibacterial activities.

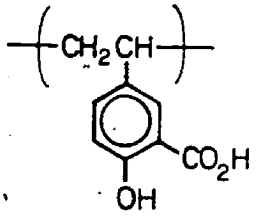


[16]

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

Derivatives of the type [17] have been reported[24] to have antibacterial activities against gram-positive and gram-negative bacteria. The derivative [16] was tested* for antibacterial activity against Bacilius mycoides, Staphylococcus aureus and Pseudomonas putida by the agar-plate test. The test consists of applying the sample on small filter discs to the respective cultured bacteria; the development and size of a zone of growth inhibition can then be used as a qualitative measure of the antibacterial potency of the sample.

Although the test results indicated no detectable activity of derivative [16] against either of the gram-positive B. Mycoides and S. aureus or the gram-negative P. putida, the preliminary nature of these tests, e.g., only one derivative was investigated, should be noted. In view of the potency and specificity of the poly- (vinyl-salicylic acid) derivatives, further detailed studies, involving, for instance, a variety of structurally related derivatives, could well prove more successful.



[17]

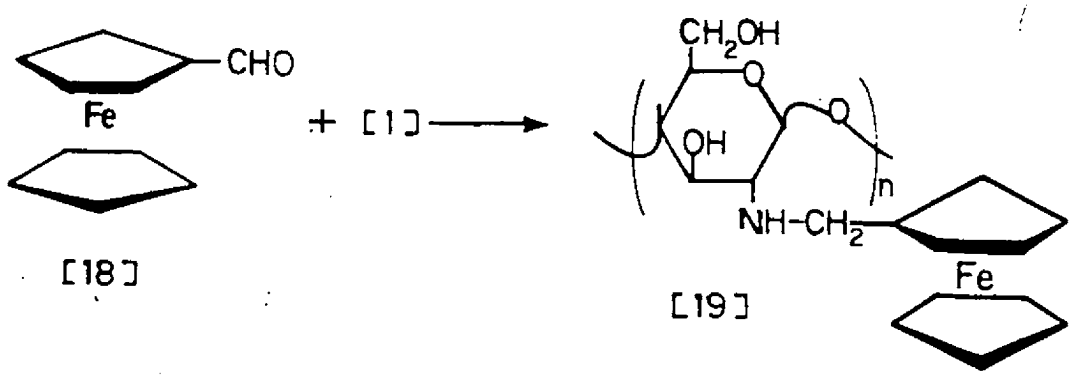
*With the kind assistance of Dr. P. J. Salisbury.

**A sample of which was kindly provided by Dr. M. J. Adam.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

V-C. Organometallic Chitosan Derivative

Complimentary to the foregoing procedures are methods for covalently attaching metals to polysaccharides. This can be exemplified by the reductive alkylation of chitosan using ferrocene-aldehyde** [18] which produces the brown organometallic derivative [19] (d.s. 0.455). Compound [19] constitutes, to our knowledge, the first such organometallic polysaccharide derivative. Polymer attached metallocenes are potentially of interest for a variety of reasons including catalysis,[26] and medical applications.[27]



[18] [19]

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

References

1. See for example, H. Rapoport, Acc. Chem. Res., 9, 135 (1976); R. S. Card and D. C. Neckers, J. Am. Chem. Soc., 99, 7734 (1977); L. D. Rollman, ibid., 97, 2132 (1975); R. S. Drago, J. Gaul, A. Zombeck, and D. K. Straub, ibid., 102, 1033 (1980).

2. R. S. Ramirez and J. D. Andrade, in Polymer Grafts in Biochemistry, (H. F. Hixson and E. P. Goldberg, eds.), Marcel Dekker, New York, 309 (1976).

3. F. R. Hartley and P. N. Vezey, Adv. Organometallic Chem., 15, 189 (1977); E. Kalalova and J. Kalal, F. Svec, Angew. Makromol. Chem., 54, 141 (1976).

4. J. F. Kennedy, Chem. Soc. Rev., 8, 221 (1979); R. B. Martin, in Metal Ions in Biological Systems, (H. Sigel, ed.), Marcel Dekker, New York, Vol. 8, (1979).

5. B. Lonnerdal, J. Carlsson, and J. Porath, FEBS Letters, 75, 89 (1977).

6. I. Tabushi, Y. Kobuke, and T. Nishiya, Nature, 280, 665 (1979).

7a. R. A. A. Muzzarelli, Natural Chelating Polymers, Pergamon Press, New York, (1973).

7b. R. A. A. Muzzarelli, Chitin, Pergamon Press, New York, (1977).

8. J. C. Irvine and J. C. Earl, J. Chem. Soc., 121, 2376 (1922); D. Horton, in The Amino Sugars (R. W. Jeanloz, ed.), Academic Press, New York, 1A, 70 (1969).

9. F. Yaku, and T. Koshijima, in Proceedings of the First International Conference on Chitin/Chitosan, (R. A. A. Muzzarelli and E. R. Pariser, eds.), 386 (1978).

10. M. Takahashi, K. Shinoda, T. Mori, and T. Kikyo, Japan Pat., 78 03982 (1978), [Chem. Abstr., 89 (1978) 64708g]; T. Sakaguchi, A. Nakajima, and T. Horikoshi, Nippon Nogei Kagaku Kaishi, 53, 149 (1979), [Chem. Abstr., 91 (1970) 126598y].

11. V. G. Gibb and L. D. Hall, Carbohydr. Res., 63, C1 (1978); L. D. Hall, P. R. Steiner, and D. C. Miller, Can. J. Chem., 57, 38 (1979); P. R. Steiner, Ph.D. thesis, University of British Columbia (1971); A. M. Slee, M.Sc. thesis, University of British Columbia (1973); D. C. Miller, M.Sc. thesis, University of British Columbia (1977).

12. M. J. Adam and L. D. Hall, Chem. Comm., 234 (1979).

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

13. L. A. Nud'ga, E. A. Plisko, and S. N. Danilov, Zh. Obshch. Khim., 43, 2752 (2729 in transl.) (1973).

14. S. Hirano, N. Matsuda, O. Miura, and H. Iwaki, Carbohydr. Res., 71, 339 (1979).

15. M. J. Adam, Ph.D. thesis, University of British Columbia (1980).

16. Stability Constants, Supplement No. 1, The Chemical Society London, Specialist Publication 24, 521; 551 (1971).

17. See R. A. A. Muzzarelli, in Proceedings of the First International Conference on Chitin/Chitosan (R. A. A. Muzzarelli and E. R. Pariser, eds.), 335 (1978); and references cited therein.

18. A. I. Vogel, Practical Organic Chemistry, Longmans, Greens and Co., London, 704 (1956).

19. R. Trujillo, Carbohydr. Res., 7, 483 (1968).

20. S. Hirano, N. Matsuda, O. Miura, and T. Tanaka, ibid., 71, 344 (1979).

21. B. J. Hathaway and D. E. Billing, Coordin. Chem. Rev., 5, 143 (1970); H. Yokoi, Bull. Chem. Soc. Japan, 47, 3037 (1974); R. S. Drago, J. Gaul, A. Zombeck, and D. K. Straub, J. Amer. Chem. Soc., 102, 1033 (1980).

22. F. Yaku, E. Muraki, K. Tsuchiya, Y. Shibata, and T. Koshijima, Cell. Chem. Technol., 11, 421 (1977).

23. Y. Inaki, M. Otsuru, and K. Takemoto, J. Macromol. Sci. Chem., A12, 953 (1978).

24. O. Vogl and D. Tirrell, ibid., A13, 415 (1979).

25. M. S. Masri, V. G. Randall, and A. G. Pittman, in Proceedings of the First International Conference on Chitin/Chitosan (R. A. A. Muzzarelli and E. R. Pariser, eds.), 306 (1978).

26. H. Brunner, Angew. Chem. Internat. Edn., 10, 249 (1971).

27. E. I. Edwards, R. Epton, and G. Marr, J. Organometallic Chem., 107, 351 (1976); J. C. Johnson, Metallocene Technology, Noyes Data Corp., New York, 230 (1973) and references therein.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

CHAPTER VI

SUMMARY

It will be recalled that the objectives for this thesis were to develop versatile synthetic methods for selective and efficient modifications of carbohydrate polymers for a wide variety of different applications using modern analytical tools for the characterization of the products. Since this study was intended only to provide an overview in preparation for subsequent more detailed investigations it is important in this chapter to draw together the experiences gained for comparison.

It was demonstrated in the different chapters that the task of transforming abundant and industrially important carbohydrate polymers (alginate, cellulose, chitin, chitosan, guaran, locust bean gum, xanthan gum) into a great variety of readily manipulable products can be successfully accomplished using, in many cases, inexpensive synthetic methods.

From the synthetic aspect, it was demonstrated in Chapter II that a range of representative polysaccharides can be readily modified in a selective and efficient manner from which it may be inferred that the methods used are applicable to other polysaccharides as well. The derivatization of carboxylate groups was accomplished in several ways affording amide, ester, hydrazine, and triazinyl derivatives. Of these, the amidation methods were found to be high-yielding where amines were either directly coupled, as for the xanthan amides, or through carbodiimide mediation, as for both alginate and xanthan gum, or via alkyl

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

esters, such as the propylene glycol alginates. The simplicity of the esterification of the carboxylic acid moieties of alginic acid with alcohols appeared to be rather promising despite the low yields presently obtained. Hydrazide derivatives of polysaccharides, exemplified here for alginate and cellulose, constitute useful intermediates for the attachment of carbonyl-containing molecules via reductive alkylation. Carbonyl functions can, conversely, be introduced into polysaccharides by several equally facile routes for subsequent derivatizations as exemplified by the oxycellulose derivatives and the various periodate-cleaved materials.

The syntheses performed in Chapter III, transformed an intractable polysaccharide, chitosan, into soluble products which comprise a novel class of branched-chain derivatives and exhibited a wide range of useful properties in terms of gel-formation, solubility, compatibility, and interactions with other polysaccharides. The spin-labelling of chitin and chitosan illustrated several alternative methods of derivatization using comparatively milder conditions.

It may be pointed out that the synthetic principles employed for the preparation of the branched-chain chitosan derivatives are amenable to adaption for the systematic syntheses of branched polysaccharides with both comb-like and tree-like structures. This method is also more facile than any of the known procedures for the preparation of branched derivatives;[1,2] although its utility was demonstrated here only by the attachment of mono- and oligosaccharides to chitosan, one can envisage the combination of different polysaccharides or polysaccharide fragments to produce another novel class of polysaccharides using similar procedures. The key for such reactions is the introduction, if not already

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

present, of suitable prominent functional groups into the polymeric materials, a task which can be usually achieved in a variety of ways.

This was nicely born out by the galactomannan modifications where galactose oxidase treatment in conjunction with the reductive amination reaction afforded a variety of novel polysaccharide conjugates ranging from chain-extended guaran, to synthetic glycoproteins and glycopeptides. Several of the polysaccharide derivatives displayed an interesting range of rheological and compatibility properties. Although this enzymic procedure is, in its present form, incompatible with large-scale applications, its optimization, using existing enzyme reactor technology,[3] should not pose too difficult a task. The controlled periodate oxidation may, for some applications, also provide a viable alternative.

In Chapter V the advantages of selective modification were illustrated in terms of one specific polysaccharide application and it was found that salicylidene chitosan derivatives constituted highly efficient metal chelating agents, the hydrolytic stability of which could be controlled using sodium cyanoborohydride. Procedures were designed to tailor the solubility of these derivatives in analogy with the branched-chain chitosan derivatives. The reductive amination reaction was also employed for the preparation of new organometallic polysaccharide derivatives.

Various spectroscopic and other instrumental techniques were employed throughout this study for the characterization of the polysaccharide derivatives and some native polymers in terms of their primary structure, three dimensional shape, and surface structure, as well as their molecular mobility in solution.

The nitroxide spin-labelling method was successfully utilized for

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

determining various reaction parameters, such as yield and purity of product, as well as extracting information about motional correlation times and, to some extent, structures of the products obtained. The distance measurements performed on guaran and locust bean gum were found to be in good qualitative agreement with the structural models proposed for these polysaccharides by Painter and coworkers.[4,5] For the spin-labelled amine derivatives of alginate and cellulose, derived from the periodate-oxidized dialdehyde precursors, the esr results suggested the presence of two nitroxide residues per cleaved hexose unit. Indications of the heterogeneity in surface structure and accessibility of cellulose and chitosan could be obtained with the aid of esr methods, which also provided evidence for the formation of stable copper(II) complexes of several chitosan derivatives. It should be noted that esr spectroscopy and most of the esr techniques used in this study have only recently found application to polysaccharides[6] and can be expected to provide a wealth of information for such systems in future.

High resolution ^{13}C nmr was employed in efforts to elucidate the structure of several branched-chain chitosan derivatives and of guaran and locust bean gum. Using a 100.6 MHz (for ^{13}C) spectrometer, it could be demonstrated that the ^{13}C nmr spectra of high molecular weight polysaccharides are adequately resolved at relatively low probe temperatures without the need for extensive prior depolymerization as is commonly practiced at lower fields.[5] For guaran, the ^{13}C nmr spectra appeared to contain information concerning the sequences of branched and unbranched mannose units, which was independently confirmed by the work of Painter and coworkers for partially depolymerized guaran samples.[5]

The nmr studies performed in this study are representative of the

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

enormous potential of this method as applied to polysaccharides. The multitude of observable nuclei in conjunction with new nmr techniques,[7] of which some have only recently been developed in this lab,[8] ensure an increasingly important role to nmr spectroscopy for studies in this area. This is exemplified by the preliminary results obtained by ^{13}C magic angle spinning-cross polarization and ^{13}C spin-echo nmr experiments of polymers which cannot be analyzed by conventional techniques (see Appendix). It is regrettable that I could not pursue these studies to any great extent, particularly since many of the polysaccharide derivatives which were prepared were well-suited for model studies.

SEM provided evidence for a wide variety of ultra-structures for several of the branched-chain chitosan derivatives and revealed interesting aspects of shrimp shell-derived chitosan itself. The microarchitecture of the chitosan derivatives ranged from smooth non-porous to microporous with evidence, in most cases, for microfibrillar substructures. SEM studies proved valuable in establishing, together with ^{13}C nmr, the formation of the lactose inclusion complex of 1-deoxy-lactit-1-yl chitosan. Although the results obtained so far do not allow for strict correlations between ultrastructure and the conformation of the polysaccharide side-chains, certain relationships seemed to be indicated for 1-deoxymelibiit-1-yl chitosan whose α1,6-linked side-chain gave rise to a highly ordered parallel array of microfibrils. The non-porous surface structure of 1-deoxy-galactit-1-yl chitosan appeared to correlate with the observed lack of metal-chelating capacity.

During the course of this study several underlying concepts were apparent, the significance of which extends beyond the merely synthetic aspects discussed so far. Perhaps the most important, and at the same

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

time generally least documented so far,[9] idea is that of inducing systematic changes in polysaccharide structure and physical properties. The difficulties encountered in this area originate mainly from the lack of facile chemical methodology for the preparation of suitable model compounds the structures of which can be systematically varied in terms of, for example, branching. The synthesis of the branched-chain chitosan derivatives, as already alluded to, seems to provide access to such model polysaccharides, particularly in view of the possibility of reducing the size of this high molecular weight polymer to the level of oligosaccharides. The ease with which branching can be affected for polysaccharides other than chitosan was demonstrated for guaran and should be equally facile for xanthan gum, cellulose, and alginate in analogy with the various non-saccharidic derivatives prepared (e.g., octadecyl xanthan amide, C2 and C3 spin-labelled cellulose derivatives, and spin-labelled algin amides or esters).

The concept of solubilizing intractable, but abundant polysaccharides by way of reactions with other surplus carbohydrates, such as lactose, is certain to find applications to cellulose, chitin, and other important polymers. It is equally certain that the ability to modify the solubility, chelating efficacy, viscosity, and surface structure of polysaccharide derivatives will, if properly understood, be of great utility to industry and other areas.

The relevance of the above ideas is, of course, not confined to the presently known polysaccharides. Other types of polysaccharides, particularly bacterial varieties,[10] could be screened and developed with the aid of such concepts to drastically expand the scope of carbohydrate polymer applications. Such developments, together with the

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

obvious extension of many of the chemical reactions which are presented here in preliminary form only, should in due course, serve to place the present studies into context.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

References

1. For reviews, see A. F. Bochkov and G. E. Zaikov, Chemistry of the O-Glycosidic Bond, Pergamon Press, New York, (1979); W. A. Szarek, in MTP Intern. Rev. Sci., Org. Chem. Ser. One, 7, 80 (1973).

2. H. Ito and G. Schuerch, J. Amer. Chem. Soc., 101, 5797 (1979); B. Pfannemüller and A. Berg, Makromol. Chem., 180, 1201 (1979); W. B. Neely, U.S. Pat., 3,133,856 (1964), Chem. Abstr., 61, 5899h (1964).

3. W. Hartmeier and G. Tegge, Starch, 31, 348 (1979).

4. J. J. Gonzalez, Macromolecules, 11, 1074 (1978); T. J. Painter, J. J. Gonzalez, and P. C. Hemmer, Carbohydr. Res., 69, 217 (1979).

5. H. Grasdalen and T. J. Painter, ibid., 81, 59 (1980).

6. J. D. Aplin, Ph.D. thesis, University of British Columbia, (1979); J. D. Aplin and L. D. Hall, J. Amer. Chem. Soc., in press; L. D. Hall and J. D. Aplin, ibid., 100, 1934 (1978); D. Gagnaire and L. Odier, Bull. Soc. Chim. Fr., 2325 (1974).

7. W. L. Earl and D. L. VanderHart, J. Amer. Chem. Soc., 102, 3251 (1980).

8. L. D. Hall and S. Sukumar, ibid., 102, 1745 (1980).

9. D. A. Rees, Polysaccharide Shapes, Chapman and Hall, (1977).

10. P. A. Sandford and A. Laskin, Extracellular Microbial Polysaccharides, ACS Symp. Ser., 45, (1977).

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

CHAPTER VII

EXPERIMENTAL

VII-A. General Methods

1. Electron Spin Resonance

Esr spectra were recorded at X-band using a Varian E-3 instrument in the derivative absorption mode and integrated using a Pacific Precision Co. MO-1012A integrator. The second integration was performed by peak cutting and weighing and comparison with freshly-prepared standard solutions of 4-hydroxy-2,2,6,6-tetramethylpiperidine-1-oxyl. Spectrometer settings--modulation amplitude, filter time constant and scan rate--were chosen in each case to avoid distortion of the spectral lines, and power levels were non-saturating. The ambient temperature was always 25°C $\pm$ 1, and the field always increased from left to right. Line widths were measured using the smallest possible scan range (generally not shown in the diagrams) and at least two measurements were made using different scans in each case. The field was calibrated using a proton nmr magnetometer and the X-band microwave frequency was monitored on a Hewlett-Packard 5245-L electronic counter equipped with a 8-18 GHz frequency converter.

Spectra at 77K were obtained using a Dewar insert containing liquid nitrogen, at 0.16 mW microwave power, the lowest available. Oxygen was prevented from condensing in the sample tube by sealing the top with a rubber septum cap. All low temperature spectra were recorded in 3 mm

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

i.d. quartz tubes when non polar organic solvents were used and in 1 mm i.d. Pyrex tubes for aqueous solutions. At room temperature, both these types of tubes were used along with a flat high-quality quartz cell, capacity 73 μL, with ground glass joints at both ends (J. Scanlon Co.). For aqueous suspensions and gels of polysaccharides a teflon insert designed by Dr. F. G. Herring* was used. This consisted of a cylinder of diameter 10 mm with a half-cylinder section 30 mm long cut away in the center, forming a flat surface 10 × 30 mm upon which the wet polysaccharide was placed beneath a glass cover slip, the latter retained by surface tension. Solution of nitroxides, whose spectra were to be used for correlation time (τ_c) measurements, were deoxygenated by bubbling nitrogen through for several minutes; in aqueous solutions this was found to be unnecessary.*

2. Nuclear Magnetic Resonance

^{1}H nmr. Proton nmr spectra were measured at 270 MHz with a prototype of a home-built spectrometer based on a Bruker WP-60 console, a Nicolet 1180 computer (32K), a Nicolet 293A pulse controller unit, a Diablo Disk, and an Oxford Instruments Superconducting solenoid, or at 400 MHz on a Bruker WH-400 spectrometer.

^{13}C nmr. Proton-decoupled carbon nmr spectra were recorded at 20 MHz with a Varian CFT-20 spectrometer or with a Bruker WP-80

---

* Spectra of solid polysaccharide derivatives were recorded under under deoxgygenated chloroform unless otherwise indicated.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

instrument, or at 100.6 MHz with a Bruker WH-400 spectrometer; the latter two instruments being equipped with a variable temperature control unit. Polysaccharide samples were dissolved in D_2O directly in the 10 mm nmr tubes to avoid handling of the viscous or gelling materials. Final sample concentrations ranged between 3-8% (w/v). Unless otherwise indicated, spectra were obtained at 305-310°K and were referenced to internal dioxane.

^{19}F. Fluorine nmr spectra were recorded at 94.08 MHz on a Varian XL-100 spectrometer and were referenced to external trifluoroacetic acid.

3. Synthetic Methods

All reactions involving polysaccharides were conducted at ambient temperature. All concentrations were performed on a Büchi rotary evaporator. Soluble polysaccharide reaction products were purified by dialysis using Spectropore #1 or #2 tubing from Spectrum Medical Inc. (Los Angeles, California), against 0.01M EDTA / 0.01M NaCl solutions or distilled water for 4-6 days, and subsequently lyophilized. All samples were stored refrigerated with desiccation.

C, H, N microanalyses were carried out by Mr. P. Borda of this department; Cu and Fe microanalysis was performed by Canadian Microanalytical Service Ltd. (Vancouver) using nitric acid digestion of the polysaccharides and atomic absorption.

4. Materials

The following alginate samples were a gift from Kelco Co., San Diego, California: sodium alginate (Keltone), alginic acid (Kelacid), and propylene glycol alginate esterified 50-60% (Kelcoloid-HVF) and 80-85% (Kelcoloid-O). Sodium alginate was purified following Schweiger's[1] method. Cellulose carbonate was purchased from Sigma Chemical Co.;

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

Cellulose powder (Whatman CF11) from W. & R. Balston Ltd., U.K. Samples of 2- and 3-oxycellulose were gifts from Dr. J. Defaye, Centre de Recherches sur les Macromolécules Vegétales, Grenoble. Carboxymethylcellulose (12M31P) was a gift from Hercules Inc., Willington, Delaware. Xanthan gum samples were gifts from Kelco Co. (Keltrol), and Tate & Lyle Ltd., London ($77A_3$). The former samples were purified according to the method of Holzwarth[2] while the latter were supplied in purified form. Guar gum and locust bean gum were gifts from Kelco Co. and were purified according to Whistler's[3] method with slight modifications. Chitosan (from shrimp shell) and chitin (from crab shell) were purchased from Sigma and used without further purification. Sodium cyanoborohydride was from Aldrich Chemical Co.

4-amino-2,2,6,6-tetramethylpiperidine-1-oxyl, 4-oxo-2,2,6,6-tetramethylpiperidine-1-oxyl, and the spin label analogue 4-amino-2,2,6,6-tetramethylpiperidine-1-oxyl were purchased from Alrich Chemical Co. The other spin labels, 4-hydroxy-2,2,6,6-tetramethylpiperidine-1-oxyl, and 4-chloroacetamido-2,2,6,6-tetramethylpiperidine-1-oxyl were synthesized by L. Evelyn of this lab using the method of McConnell.[4]

5. Product Nomenclature

The application of systematic nomenclature to most of the polysaccharide derivatives reported here is not feasible due to the heterogeneity in the structure of the original polymers or in the substitution pattern of the reaction products; in a number of cases, such as for the products derived from periodate-oxidized materials, the exact structure of the product is unknown (see text). The nomenclature system adapted here attempts to indicate the substituent(s) introduced followed by the name of the native polysaccharide.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

VII-B. Chapter II

1. Materials

1-Ethyl-3-(3-dimethyl-aminopropyl)-carbodiimide HCl (EDC) was purchased from Sigma Chemical Co., and dicyclohexylcarbodiimide (DCC) from Eastman Chemical Co. Dimethylbiguanidine HCl was from Aldrich Chemical Co., sodium periodate was from Fisher Scientific Co. Hydrazine hydrate was purchased from Mallinckrodt Co.

2. Synthesis

[4-(Acetamido-2,2,6,6-tetramethylpiperidine-1-oxyl)] alginic acid [10]

Alginic acid was partially dehydrated following Schweiger's[5] method with some modifications. Alginic acid (1 g) was swelled in glacial acetic acid (5 ml) and then centrifuged in a disk-top centrifuge. The supernatent was decanted, and the procedure repeated several times. The wet material thus obtained was used for subsequent reaction.

A solution of chloroacetamide label [9] in dry pyridine (130 mg, 5.3 mM, 4 ml) was added to partially dehydrated alginic acid (40 mg, 0.23 mM) and the pH of the dispersion was adjusted with solid $NaHCO_3$ to ~7.5. The reaction mixture was gently shaken for 18 hr. The resulting product [10] had d.s. 0.04. When 65% aqueous acetone was used instead of pyridine the d.s. of [10] was lowered by one fourth.

[4-(2,2,6,6-tetramethylpiperidine-1-oxyl)] alginate [12]

Alginic acid (150 mg, 0.85 mM) was partially dehydrated and suspended in 65% aqueous acetone (5 ml). After addition of hydroxy label [11] (300 mg, 1.7 mM) the pH was adjusted to 6 using $NaHCO_3$ and the mixture was shaken for 18 hr. The product [12] had d.s. 0.05.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

[4-(2,2,6,6-tetramethylpiperidine-1-oxyl)] alginamide [16]

(i) Carbodiimide coupling

(a) EDC coupling. To partially dehydrated alginic acid (150 mg, 0.85 mM) was added a 65% aqueous acetone solution of EDC (240 mg, 1.25 mM, 5 ml), and after adjustment of the pH to 6.5 using Na_2CO_3, a solution of amine label [15] (270 mg, 1.6 mM, 2 ml). The mixture was shaken for 18 hr and the resulting product [16A] had d.s. 1.00.

(b) DCC coupling. The reaction conditions were the same as in the EDC coupling procedure with the exception of using DCC (100 mg, 0.48 mM), and DMF (3 ml) as solvent. This reaction, however, proceeded in lower (~10x) yields than the EDC coupling.

(ii) Via propylene glycol esters

A solution of amine label [15] (276 mg, 1.6 mM) in DMF (2 ml) was added to a suspension of propylene glycol alginate (PGA), with a degree of esterification 0.80-0.85 (193 mg, 0.86 mM) in DMF (2 ml). The mixture was shaken for 14 hr to yield a product [16B] with d.s. ~0.15 (18% conversion of available esters groups). For PGA with a lower degree of esterification (0.5-0.6) a product with d.s. ~0.10 (18% conversion) was obtained.

[4-N-(2,2,6,6-tetramethylpiperidine-1-oxyl)] algin amine [18]

Sodium alginate was periodate-oxidized according to the procedure of Painter et al.[6] In a typical experiment, a 0.78% aqueous solution of sodium alginate was oxidized with 0.25M $NaIO_4$ in the presence of 1-propanol at 4°C in the dark for 24 hr. The oxidation was terminated by addition of ethylene glycol. Reductive amination was performed by addition of an aqueous solution of amine spin-label [15] (3-5 fold molar

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

excess) and sodium cyanoborohydride (7-10 fold molar excess). The reaction mixture was shaken for 24 hr. For alginate samples oxidized 10%, products with d.s. 0.05 (52% conversion of available aldehyde groups) were obtained, while for samples oxidized 44% the products [18] had d.s. 0.13 (30% conversion).

[4-N-(2,2,6,6-tetramethylpiperidine-1-oxyl)] algin hydrazine [21]

(a) The method of Andresz et al.[7] was modified to prepare hydrazine derivatives. Samples of propylene glycol alginate (Kelcoloid-O, 0.5 g, 2.2 mM), were mixed with hydrazine hydrate, (10 ml, 0.2 M), in a 50 ml Erlenmeyer flask. The reaction mixture was gently shaken overnight at 22°C, affording a viscous solution which was diluted with water (25 ml). The product was precipitated in methanol (300 ml), the precipitate was collected after centrifugation (9000 rpm, 50 min.), and dialyzed against 0.01 M EDTA for 2d, before lyophilization. Elemental analysis showed a product [19] with d.s. 0.76, i.e., 92% conversion of the ester.

Anal. for $[(C_6H_8O_6)_{0.17}(C_9H_{14}O_7)_{0.07}(C_6H_{10}N_2O_5)_{0.76}]\cdot 0.88\ H_2O$; calcd. C 36.25, H 5.74, N 10.35, (C/N3.53); found C 36.01, H 5.37, N 10.36, (C/N 3.48).

Similarly, for PGA with lower degrees of esterification, products [19] with d.s. ~0.45 (i.e., ~80% conversion) were obtained (C/N 5.71). These d.s. obtained here correspond well with those of Andresz et al.[7] (d.s. 0.67).

(b) Reductive alkylation of hydrazine alginate [19] (d.s. 0.76) was carried out by dissolving it (27 mg, 0.13 mM) in water (10 ml) and adding a solution (7 ml) of keto spin label [20] (102 mg, 0.58 mM) and $NaCNBH_3$ (140 mg, 2.2 mM). The reaction mixture was left on a shaker overnight. The hydrazine product [21] had d.s. 0.21.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

[(2-amino-4-N-dimethylamino)-s-triazin-6-yl] alginate [23] and [4-(N-dimethylamino)-2-(4-N-amino-2,2,6,6-tetramethylpiperine-1-oxyl)-s-triazin-6-yl] alginate [25]

(a) The s-triazinyl derivate was prepared according to Lee and Maekawa[8] using propylene glycol alginate, (Kelcoloid-O) instead of the methyl ester of alginate. Alginate (1 g, 5.44 mM) was suspended in MeOH (100 ml) at 0°C and dimethylbiguanidine hydrochloride [22] (1 g, 6.1 mM) in THF/MeOH (1:1) (60 ml) was added with stirring to the suspension over a 20 min period. After one hour, the reaction mixture was allowed to warm to room temperature and left stirring for four days. The mixture was reduced to dryness, and distilled water (20 ml) was added affording a gel, which was purified. The product had d.s. ~0.12 (i.e., 15% conversion of esters) as determined by microanalysis.

Anal. for $[(C_6H_8O_6)_{0.2}(C_9H_{14}O_7)_{0.68}(C_{10}H_{15}N_5O_4)_{0.12}]\cdot 2.01\ H_2O$; calcd. C 38.88, N 3.20, (C/N 12.2); found C 38.59, N 3.05 (C/N 12.7)

(b) The s-triazinyl alginate [23] was labelled by dissolving 40 mg (~0.18 mM) in water (10 ml) and adding a solution of keto label [20] (80 mg, 0.47 mM) and $NaCNBH_3$ (100 mg, 1.6 mM) to the mixture, which was shaken for 13 hr. The product [24] had d.s. 0.02.

[4-N-(2,2,6,6-tetramethylpiperidine-1-oxyl)] xanthan amide [26]

To xanthan gum (150 mg, 0.16 mM) dissolved in water (10 ml) was added EDC (100 mg, 0.78 mM) and the reaction mixture (pH 5) was cooled to 5°C for ten minutes before the amine label [15] (80 mg, 0.47 mM) was added with stirring. The reaction was continued for twelve hours, yielding a product [26] with d.s. 0.43 (based on carboxyl groups) after dialysis (4 d).

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

[4-N-(2,2,6,6-tetramethylpiperidine-1-oxyl)] xanthan amide [27]

Following a method reported for the synthesis of "amine alginates,"[9] xanthan gum (50 mg, 0.05 mM) was mixed in a 25 ml Erlenmeyer flask with solid amine spin label [15] (57 mg, 0.33 mM) using a spatula. Three drops of water were added and mixed in to produce a heavy paste, which was shaken for 11 hr, and then dialysed (4 d). The product [27] had d.s. 0.05 (based on carboxyl groups).

[18-N-(n-octadecyl)] xanthan amide [28]

Following the above procedure, octadecylamine (60 mg, 0.22 mM) was admixed with xanthan gum (75 mg, 0.08 mM) to afford, after dialysis (3 d), [28]. The microanalytical results (C 48.04, H 7.99, N 2.27) could not be matched with a corresponding molecular formula, presumably because of the presence of lower molecular weight fractions in the starting amine (90%, technical grade--Aldrich Chemical Co.), but the C/N ratio corresponds roughly to a d.s. 1.0.

[4-N-(2,2,6,6-tetramethylpiperidine-1-oxyl)] cellulose amine [31]

(a) The periodate oxidation method of Nevell[10] was used. Three samples (500 mg, 3.1 mM) of cellulose powder were each reacted with $NaIO_4$ (0.01M, 0.05M, and 0.1M) in distilled water (25 ml) in the dark for 60 hours at 20°C in a constant temperature shaker to afford products which had a degree of oxidation (d.o.) of 5%, 19%, and 34%, respectively. The reaction was quenched by dumping the dispersions into distilled water (250 ml) and decanting the supernatent. This process was repeated 6-7 times. The samples were left in distilled water overnight, then washed with water (100 ml) and subsequently dried in vacuo at 56°C.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

(b) Periodate-oxidized cellulose samples (1 mM) were labelled in MeOH (7 ml) by adding 2.6 equivalents of amine spin label [15] and 6.4 equivalents $NaCNBH_3$ and a few drops of water. After gentle shaking (28 hr) the samples were successively washed on a fritted glass filter with 100 ml of water, ethanol, acetone, and ether, and subsequently dried under vacuum for 24 hr at 56°C. The following d.s. were obtained (d.o.): (A) d.s. 0.03 (5%); (B) d.s. 0.063 (19%); (C) d.s. 0.065 (34%).

Anal. for (A) $[(C_6H_{10}O_5)_{0.95}(C_6H_8O_5)_{0.04}(C_{13}H_{27}N_2O_5)_{0.03}]\cdot 0.13\ H_2O$; calcd. C 44.30, H 6.43, N 0.50; found C 44.01, H 6.38, N 0.51.

Anal. for (B) $[(C_6H_{10}O_5)_{0.81}(C_6H_8O_5)_{0.127}(C_{13}H_{27}N_2O_5)_{0.063}]\cdot 0.14\ H_2O$; calcd. C 44.81, H 6.49, N 1.02; found C 44.70, H 6.49, N 1.03.

Anal. for (C) $[(C_6H_{10}O_5)_{0.66}(C_6H_8O_5)_{0.275}(C_{13}H_{27}N_2O_5)_{0.065}]\cdot 0.21\ H_2O$: calcd. C 44.61, H 6.37, N 1.05; found C 44.33, H 6.25, N 1.04.

[2-deoxy-2-(4-N-amino-2,2,6,6-tetramethylpiperidine-1-oxyl)] cellulose [34] and [3-deoxy-3-(4-N-amino-2,2,6,6-tetramethylpiperidine-1-oxyl)-6-trityl] cellulose [35]

(a) 2-oxycellulose [32] and 3-oxy-6-trityl cellulose [33] were prepared (Dr. J. Defaye)[11] by oxidation of cellulose using acetic anhydride in DMSO/Ac_2O and subsequent precipitation from water. Detritylation was accomplished by stirring the product in acetone (100 ml) containing conc. HCl (4 ml) for 16 hr to obtain a product [32] with d.p. 150, d.s. 0.8.

Anal. for [32] $[(C_6H_8O_5)_{0.8}(C_6H_{10}O_5)_{0.2}]\cdot 0.18\ H_2O$; calcd. C 44.00, H 5.39; found C 43.78, H 5.20.

Anal. for [33] (D.P. 150, d.s. 0.85) $[(C_6H_{10}O_5)_{0.15}(C_{25}H_{23}O_5)_{0.85}]\cdot$

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

0.2 H_2O; calcd. C 71.71, H 5.83; found C 71.94, H 5.63.

(b) Spin-labelling of 2-oxycellulose [32] was achieved by reacting it (378 mg, 2.36 mM) in methanol (7 ml) with the amine spin label [15] (412 mg, 2.41 mM) and $NaCNBH_3$ (400 mg, 6.4 mM) and a few drops of water for 33 hr on a shaker at 20°C. The products were filtered, and successively washed with water (100 ml), ethanol (150 ml), acetone (50 ml), diethylether (50 ml), and subsequently dried in vacuo at 50°C. The product was found to have d.s. ~0.28.

Anal. for $[(C_6H_{10}O_5)_{0.2}(C_6H_8O_5)_{0.52}(C_{15}H_{27}N_2O_5)_{0.28}]\cdot 1.59\ H_2O$; calcd. C 43.93, N 3.37, (C/N 13.04); found C 43.73, N 3.37, (C/N 12.98). (Esr double integration gave d.s. 0.12.)

3-oxy-6-trityl cellulose [33] was labelled under the same conditions yielding a product with d.s. ~0.09.

Anal. for $[(C_6H_{10}O_5)_{0.15}(C_{25}H_{23}O_5)_{0.764}(C_{34}H_{42}N_2O_5)_{0.086}]\cdot 0.3\ H_2O$: calcd. C 71.33, H 6.08, N 0.62; found C 71.22, H 5.54, N 0.61. (Esr double integration gave d.s. 0.04.)

[4-N-(2,2,6,6-tetramethylpiperidine-1-oxyl] cellulose urethane [37]

A solution of the amine label [15] (366 mg, 2.1 mM) in phosphate buffer (0.5M, pH 7, 2 ml) was added to a suspension of cellulose carbonate (d.s. 0.21, 80 mg, 0.48 mM) in the buffer (10 ml). The reaction mixture was stirred overnight at 5°C, and then dialyzed (5 d) at the same temperature to afford a product [37] with d.s. 0.08.

Anal. for $[(C_6H_{10}O_5)_{0.74}(C_7H_8O_6)_{0.18}(C_{16}H_{27}N_2O_6)_{0.08}]\cdot 0.75\ H_2O$; calcd. C 43.22, H 6.50, N 1.16; found C 43.08, H 6.54, N 1.10.

When the reaction was carried out in methanol a product with lower d.s. (0.05) was obtained.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

Anal. for $[(C_6H_{10}O_5)_{0.8}(C_7H_8O_6)_{0.15}(C_{16}H_{27}N_2O_6)_{0.05}]\cdot 0.34\ H_2O$; calcd. C 44.06, H 6.25, N 0.77; found C 43.86, H 6.16, N 0.75.

[4-(2,2,6,6-tetramethylpiperidine-1-oxyl)] cellulose hydrazine [40]

(a) The cellulose hydrazine derivative [39] was prepared following the method of Andresz et al.,[7] using sodium carboxymethylcellulose (d.s. 1.2-1.4) as starting material. The product had a d.s. value of ~0.5 (0.68 lit.)[7] as calculated from elemental analysis.

Anal. for $[(C_8H_{12}O_7)_{0.5}(C_8H_{14}N_2O_6)_{0.5}]\cdot 2.9\ H_2O$: calcd. C 34.37, N 5.01, (C/N 6.86); found C 34.20, N 4.90, (C/N 6.98).

(b) Cellulose hydrazine (40 mg, 0.16 mM) was labelled in phosphate buffer (0.5M, pH 7, 10 ml) using keto spin label [20] (97 mg, 0.6 mM) and $NaCNBH_3$ (100 mg, 1.6 mM). The mixture was shaken for 15 hr and then dialyzed (4 d), to afford [40], d.s. 0.003 (from esr double integration).

VII-C. Chapter III

1. Scanning Electron Microscopy (SEM)*

The methods of Hirano et al.[12] were followed for the preparation of the gel samples. Portions of the chitosan derived gels were cut, washed, and rapidly frozen (ca -60°C), before lyophilization. The xerogels were dried at 80°C under reduced pressure (0.01 mm) for 12 hr prior to examination. Specimens were mounted on metal holders with double-coated sticky cellophane tape and silver cement and coated with gold at a vacuum of $<10^{-4}$ mm. An Etec Autoscan electron microscope was used, operated at 20 kV. Unless otherwise indicated, only those

*These experiments were performed by Mr. Nasser Yalpani.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

portions of the specimens were viewed which were not damaged by handling.

2. Materials

The carbohydrates used in this section were purchased from the following suppliers: maltose, cellobiose, maltotriose, galactosamine hydrochloride, glucose (Aldrich Chemical Co.); melizitose, trehalose, α-glucoheptonic lactone (Pfanstiehl Lab.); lactose, melibiose (Eastman Chemicals); fructose (BDH Chemicals); galactose (Merck); glucosamine hydrochloride (Sigma Chemical Co.).

3. Preparation of 1-deoxyglycit-1-yl Chitosan Derivatives

(i) General procedure

Chitosan (500 mg, 3 mM) was dissolved with stirring in a mixture (1:1) of methanol and 1% aqueous acetic acid (solvent A) or in the latter medium[13] (solvent B) (30 ml). To the resulting viscous solution was added with vigorous stirring a solution (10-20 ml) of the carbonyl-containing compound (3.3-10 mM) and sodium cyanoborohydride (20 mM). The reaction mixture was left stirring at room temperature for 3-18 hr until a gel had formed. The solvent excluded by the gel was decanted, the gel was broken up, repeatedly washed with methanol (150 ml) and finally with diethyl ether (150 ml). The solid products thus obtained were first air-dried for several hours, then dried in vacuo at 56°C for 12-18 hr , and finally crushed into a fine powder. In the cases where no gel was formed, the reaction mixture was dialyzed in dialysis bags against distilled water for periods of 4-6 d with frequent (~15-20) changes of water to obtain, after lyophilization, mostly white materials.

(ii) Reactions of chitosan with reducing sugars

[1-deoxy-1-galactit-1-yl] chitosan [2]

Addition of galactose (1.20 g, 6.7 mM) led to the formation of a

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

stiff, glassy gel within 1-2 hr. The product (d.s. 0.9) was ivory coloured.

Anal. for $[(C_8H_{13}NO_5)_{0.1}(C_{12}H_{23}NO_9)_{0.9}]\cdot 1.9\ H_2O$: calcd. C 40.07, H 7.49, N 4.03; found C 40.09, H 7.58, N 3.97.

When a smaller amount of galactose (1.10 g, 6.1 mM) was used, the resulting product had a lower d.s. (0.7).

Anal. for $[(C_6H_{11}NO_4)_{0.3}(C_{12}H_{23}NO_9)_{0.7}]\cdot 0.61\ H_2O$; calcd. C 42.65, H 7.24, N 4.88; found C 42.59, H 7.20, N 4.97.

[1-deoxy-1-glucit-1-yl] chitosan [3]

Addition of glucose (0.72 g, 4 mM) in aqueous methanol produced no gel. Further addition of 0.9 g (5 mM) afforded a firm white gel which was washed and dried. The solid obtained (0.86 g) appeared to be inhomogeneous and was dialyzed for 4 days. After work-up the product had d.s. 0.9 (0.64 g, 71%).

Anal. for $[(C_8H_{13}NO_5)_{0.02}(C_6H_{11}NO_4)_{0.08}(C_{12}H_{23}NO_9)_{0.9}]\cdot 0.51\ H_2O$; calcd. C 43.05, H 7.22, N 4.39; found C 42.80, H 7.10, N 4.60.

[1-deoxy-1-(2-deoxy-2-N-acetylamino) glucit-1-yl] chitosan [4]

Addition of N-acetylglucosamine (1.07 g, 4.84 mM) produced an elastic clear gel after standing overnight, which hardened and turned white after 24 hr. The product had d.s. 0.97.

Anal. for $[(C_8H_{13}NO_5)_{0.03}(C_{14}H_{25}N_2O_9)_{0.97}]\cdot 2.9\ H_2O$: calcd. C 40.20, H 7.44, N 6.69; found C 39.99, H 6.90, N 6.55.

[1-deoxy-1-cellobiit-1-yl] chitosan [5]

Addition of cellobiose (3.5-6.6 mM) produced no gel after 2 d. The product had d.s. 0.3.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

Anal. for $[(C_8H_{13}NO_5)_{0.05}(C_6H_{11}NO_4)_{0.65}(C_{18}H_{33}NO_{14})]\cdot 0.72\ H_2O$; calcd. C 42.47, H 7.04, N 5.11; found C 42.38, H 7.06, N 5.15.

[1-deoxy-1-lactit-1-yl] chitosan [6]

Addition of lactose (1.2 g, 3.5 mM) produced a milky solution but no gel when the reaction mixture was left stirring for 10 hr. This product (A) had d.s. 0.23. Similarly, no gel was formed when the lactose to glucosamine (L/G) ratio was increased to 1.5 (1.5 g, 4.5 mM lactose); the product isolated after 30 hr had d.s. 0.12 (B), while the same L/G ratio produced a white gel when the reaction mixture was left undisturbed for 144 hr (with occasional addition of reducing agent). This product (C) had d.s. 0.78 after dialysis. When the L/G ratio was increased to 2.94 (3.0 g lactose) a white soft gel was formed within 24 hr, which, after nine washes with methanol (150 ml) and ether (150 ml), produced a material (D) whose elemental analysis indicated a fully substituted (d.s. 0.94) product [7] containing one equivalent of unreacted lactose per repeating unit. Subsequent extensive dialysis (5 d) of (D) produced a clear sol (E) with d.s. 0.94.

When the reaction was carried out in the absense of sodium cyanoborohydride, using an L/G ratio of 3.90, no gel formed after 28 hr and the resulting product (F), [8] had d.s. 0.1.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

Anal. for $[(C_8H_{13}NO_5)_m(C_6H_{13}NO_4)_n(C_{18}H_{23}NO_{14})_p] \times H_2O$

Product (d.s.)	Formula m	n	p	x	C calcd.	C found	H calcd.	H found	N calcd.	N found
A (0.23)	0.07	0.7	0.23	-	44.01	44.29	6.99	7.27	5.85	5.96
B (0.12)	0.07	0.81	0.12	0.79	41.85	41.69	7.12	7.03	6.44	6.60
C (0.78)	0.07	0.15	0.78	2.9	39.52	39.29	7.30	6.88	2.98	3.00
F (0.1)	0.03	0.87	0.1	0.92	41.22	41.01	7.13	7.01	6.63	6.71

Anal. for (E) $[(C_8H_{13}NO_5)_{0.01}(C_{18}H_{33}NO_{14})_{0.94} + 0.05\ (C_{12}H_{22}O_{11}) \cdot 1.62\ H_2O$; calcd. C 41.72, H 7.06, N 2.63; found C 41.44, H 7.06, N 2.81.

Anal. for (D) $[(C_8H_{13}NO_5)_{0.01}(C_{18}H_{33}NO_{14})_{0.94} + C_{12}H_{22}O_{11}] \cdot 1.9\ H_2O$; calcd. C 41.80, H 6.86, N 1.59; found C 41.49, H 6.96, N 1.55.

[1-deoxy-1-maltit-1-yl] chitosan [9]

Addition of maltose (1.74 g, 5.09 mM) produced a relatively viscous solution after standing of the reaction mixture for 12 hr, and a stiff, white gel was formed within 24 hr. The product had d.s. 0.6.

Anal. for $[(C_8H_{13}NO_5)_{0.01}(C_6H_{11}NO_4)_{0.39}(C_{18}H_{33}NO_{14})_{0.6}] \cdot 1.98\ H_2O$: calcd. C 40.38, H 7.23, N 3.56; found C 40.14, H 6.72, N 3.67.

[1-deoxy-1-melibiit-1-yl] chitosan [10]

Addition of melibiose (1.20 g, 3.5 mM) led to the formation of an initially (4 hr) soft gel which hardened on standing (12 hr). The product had d.s. 0.6.

Anal. for $[(C_8H_{13}NO_5)_{0.01}(C_6H_{11}NO_4)_{0.39}(C_{18}H_{33}NO_{14})_{0.6}] \cdot 0.59\ H_2O$; calcd. C 43.12, H 6.96, N 3.81; found C 43.05, H 6.87, N 3.80.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

(iii) Attempted chitosan derivatizations

Reaction with glucosamine [18]

Addition of glucosamine hydrochloride (0.86 g, 4 mM) in a 1:1 mixture of methanol and aqueous $NaHCO_3$ (10 ml) produced a viscous solution but no gel after 8 hr. Upon further addition of glucosamine (0.86 g) the reaction was stirred for 0.5 hr, and then left standing for 1 d. A very soft gel formed, to which methanol (150 ml) was added, leading to a hardening of the white gel. This gel, which constituted only ~1/3 of the reaction mixture, was filtered and worked up as before to yield 0.20 g of a white product and the filtrate was dialyzed (3 d) to afford a fluffy material. Microanalysis revealed that no product was formed in either of the above cases; similarly, no reaction occurred when smaller quantities (1.06 g, 4.93 mM) of glucosamine were used. No firm gel was obtained initially after overnight stirring, but when the reaction mixture was left undisturbed for 2 weeks, a white, soft gel was formed.

Reaction with galactosamine [19]

No reaction occurred when galactosamine hydrochloride (3.5-8.2 mM) was used under the above conditions.

Reaction with maltotriose [20]

To chitosan (170 mg, 1 mM) dissolved in solvent A was added a solution of maltotriose (670 mg, 1.3 mM) and sodium cyanoborohydride (5 mM). No gel was formed and no reaction occurred after 8 hr as indicated by elemental analysis.

(iv) Reactions of chitosan with non-reducing sugars

Reaction with trehalose [11]

Trehalose (1.5 g, 4 mM) dissolved in solvent A (10 ml), was added to chitosan (3.0 mM) and mixed for 12 hr. No gel was formed.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

Reaction with melizitose [12]

A yellow, viscous solution was obtained when melizitose (1.70 g, 3.2 mM) was mixed with chitosan in solvent A. After standing for 11 d a ropy gel was produced from the reaction mixture.

Reaction with fructose [13]

No gel was obtained on addition of a solution of fructose (9.6 mM) in solvent A (10 ml) after 24 hr.

Reaction with α-glucoheptonic lactone [23]

Addition of α-glucoheptonic lactone (9.6 mM) produced no derivative after 3 d.

(v) [N-cyclohexane] chitosan [16]

The milky-white solution obtained on addition of cyclohexanone (4.8-13.5 mM) produced no gel. The product [16] was isolated after dialysis (10 d) (d.s. 0.5).

Anal. for $[(C_8H_{13}NO_5)_{0.02}(C_6H_{11}NO_4)_{0.48}(C_{13}H_{23}NO_4)_{0.5}]\cdot 0.41\ H_2O$; calcd. C 52.68, H 8.28, N6.44; found C 52.56, H 8.36, N 6.44.

4. Oxidations with Galactose Oxidase.

[1-deoxy-6'-aldehydo -lactit-1-yl] chitosan [14]

Compound [6] (103 mg, 0.13 mequiv. galactose) was dispersed in phosphate buffer (25 mM, pH 7, 10 ml) and formed a soft glassy gel which was purged with O_2 for 1 minute. Catalase (14400 units) and galactose oxidase (90 units) solutions were added and a viscous, ropy material formed after a few hours. The polysaccharide was diluted with water (10 ml) after 2 d and then poured into absolute ethanol (150 ml). The precipitate was collected by centrifugation (9000 rpm, 40 min) and dried, yielding 93 mg of the oxidized product [14].

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

[1-deoxy-6'-aldehydo -melibiit-1-yl] chitosan [15]

Compound [10] (100 mg, 0.13 mequiv. galactose) was dissolved in dilute acetic acid and the pH was raised to 4.5 by addition of aqueous $NaHCO_3$ solution yielding a gel which was treated as above. 95 mg of the oxidized product [15] were isolated.

5. Interaction of [5] with Other Polysaccharides

To each of three portions of the [cellobiit-1-yl] chitosan derivative [5] dissolved in distilled water (10 ml) was added a solution (0.2 g, 25 ml) of (i) sodium alginate, (ii) guar gum, and (iii) locust bean gum. The mixtures were vigorously stirred and diluted to 40 ml. A white gel formed immediately for alginate; the gel volume decreased considerably (~15×) over a 12 hr period, the surrounding solution being very viscous. No gels were produced in the other cases, but the guar gum mixture developed a considerable viscosity.

6. Spin Labelling of Chitosan and Chitin

[4-N-(2,2,6,6-tetramethylpiperidine-1-oxyl)] chitosans [30], [31]

Method A. Chitosan (500 mg, 3 mM) was dissolved in a mixture (1:1) of methanol and 0.4% aqueous acetic acid (50 ml) to which was added, with stirring, a solution of 4-oxy-2,2,6,6-tetramethylpiperidine-1-oxyl [26] (1.0 g, 5.8 mM) and $NaCNBH_3$ (0.9 g, 15 mM) in methanol (10 ml). The solution was stirred for 12 hr at which point a soft white gel had formed. The product [31] had a d.s. 0.1.

Anal. for $[(C_8H_{13}NO_5)_{0.02}(C_6H_{11}NO_4)_{0.88}(C_{15}H_{27}N_2O_7)]\cdot 0.49\ H_2O$; calcd. C 44.72, H 7.38, N 8.27; found C44.47, H 7.10, N 8.15.

Method B. Via chitosan N-sulfate [28].[14] Chitosan (250 mg, 1.5 mM) was dissolved in 10% aqueous acetic acid (50 ml) and precipitated by addition of an aqueous ammonium sulfate solution. The precipitate was

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

collected by centrifugation, and then suspended in phosphate buffer (20 ml, pH 7). After adjusting the pH to 6.5, the solution was heated to 50° to dissolve the sulfate salt upon which keto spin label [26] (340 mg, 2.0 mM), and $NaNCBH_3$ (0.44 g, 7.2 mM) were added with stirring. The reaction mixture was kept at 50°C for 5 hr, then aqueous sodium hydroxide solution was added to precipitate the product at slightly alkaline pH. The product, [30] had d.s. 0.45 (d.s. 0.1 from esr double integration).

Anal. for $[(C_8H_{13}NO_5)_{0.1}(C_6H_{12}NO_7S)_{0.45}(C_{15}H_{27}N_2O_5)_{0.45}]\cdot 0.6\ H_2O$; calcd. C 43.63, H 7.17, N 7.20; found C 43.55, H 6.99, N 7.44.

The spin labelling was also attempted by reacting chitosan with spin label in 2M aqueous pyridine and 1% aqueous acetic acid resulting, however, in a much lower yield.

[4-acetamido-2,2,6,6-tetramethylpiperidine-1-oxyl] chitosan [29]

The labelling was carried out via the chitosan sulfate intermediate (200 mg, 0.8 mM) described above, which was dissolved in hot water (20 ml, pH 6.5). A solution of 4-chloroacetamido-2,2,6,6-tetramethylpiperidine-1-oxyl [24], (176 mg, 1 mM) in 65% aqueous acetone (2 ml) was added with stirring and the reaction mixture was kept at 40-50°C for 6 hr at which time gelation occurred. The gel was separated, washed consecutively with water, ethanol, acetone, and ether and then dried; (d.s. 0.25).

Anal. for $[(C_8H_{13}NO_5)_{0.1}(C_6H_{12}NO_7S)_{0.65}(C_{17}H_{30}N_3O_6)_{0.25}]\cdot 1.2\ H_2O$; calcd. C 36.73, H 6.55, N 7.18; found C 36.60, H 6.49, N 7.14.

The same reaction carried out in the presence of 4-dimethylamino-pyridine (DMAP) catalyst (100 mg, 0.8 mM) gave a product with greater d.s. (0.5).

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

Anal. for $[(C_8H_{13}NO_5)_{0.1}(C_6H_{12}NO_7S)_{0,4}(C_{17}H_{30}N_3O_6)_{0.5}]\cdot 1.35\ H_2O$; calcd. C 42.87, H 7.32, N 8.55; found C 42.64, H 7.28, N 8.88.

Reductive Amination of C-6 Aldehyde Chitosan Derivatives

(a) The C6' aldehydo lactityl chitosan [14], (43 mg, 0.06 mequiv. galactose) was suspended in aqueous methanol (15 ml) to which was added a solution of amine spin label [32] (150 mg, 0.9 mM) and $NaCNBH_3$ (0.1 g, 2 mM) in the same solvent (5 ml). The amination was carried out for 12 hr and the product was purified by dialysis (3 d); esr double integration gave a d.s. ~0.7 (the microanalytical results could not be exactly matched with a molecular formula; found C 40.82, H 6.53, N 3.87).

(b) The C6' aldehydo melibiityl chitosan [15] (58 mg, 0.08 mequiv. galactose) was treated as above yielding a product [35] with d.s. ~0.15 (from esr); (found C 41.47, H 6.77, N 3.92).

[4-acetamido-2,2,6,6-tetramethylpiperidine-1-oxyl] chitin [25]

Chitin (180 mg, 0.85 mM) was soaked in DMSO (15 ml) for 48 hr, then filtered and suspended in aqueous (65%) NaOH solution (40 ml) for 1 hr, after which the alkalichitin was filtered, pressed, and resuspended in iso-propanol (25 ml). A solution of the label [24] (250 mg, 0.92 mM) was added with stirring. After 2 hr the product was filtered, washed (water 100 ml, methanol 150 ml), and dried. The product had d.s. 0.04.

Anal. for $[(C_8H_{13}NO_5)_{0.96}(C_{19}H_{32}N_3O_7)_{0.04}]\cdot 0.47\ H_2O$; calcd. C 45.77, H 6.70, N 6.83; found C 45.58, H 6.45, N 6.78.

Attempted Reductive Amination

Chitin (1 g, 4.72 mM) was dissolved in a mixture containing 3.6% (w/v) LiCl and dimethylacetamide (DMAC) (40 ml) and the undissolved

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

material was separated by filtration through glass wool. To the filtrate was added a solution of keto spin label [26] (3.6 g, .21 mM) in DMAC and $NaCNBH_3$ (20 g, 33 mM). The reaction mixture was warmed (50°C) for 24 hr, after which aqueous methanol (70 ml) was added to separate the product out as a gel. The gel was filtered, washed (methanol 200 ml), and dried, and was found to contain no bound label.

VII-D. Chapter IV

1. Viscosity Measurements

Viscosity determinations were performed on a Contravese Low Shear 2/Rheomat-30 viscometer using a Fisher 5000 chart recorder and a Haake constant temperature bath. The instrument was calibrated using a Brookfield viscosity standard (10.3 cps). Measurements were conducted for three polysaccharide concentrations (0.01, 0.1, and 1.0%) at 25.00°C (± 0.05), a minimum of three readings were recorded for each data point.

2. Oxidation Procedures

(a) Galactose oxidase. The galactomannan (70-200 mg) was dissolved in phosphate buffer (25 mM, pH 7, 20 ml) by shaking for several hours. The resulting solution was purged with oxygen for several minutes before adding catalase (90000 units/0.1 mM galactose equivalents), and galactose oxidase (100 units/0.1 mM galactose equivalents). The samples were kept at 24°C on a constant temperature shaker for 24-36 hr. The viscosity of the reaction mixture increase sharply during the course of the oxidation affording a ropy gel for both polysaccharides after a few hours. The gel formation could be avoided by performing the reaction at greater dilutions (~50 ml solution volume). The aldehyde products were isolated by (i) diluting the sample with an equal volume of

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

phosphate buffer prior to ethanol precipitation (250 ml). The precipitate was then collected by centrifugation (7000 rpm, 40 min); or (ii) extensive (5 d) dialysis and lyophilization. Efforts to avoid possible coprecipitation of the small amounts (few mg) of enzyme by fractional precipitation were not very successful, although no interference in either the esr or nmr spectra could be detected.

Several attempts to optimize the oxidation conditions, such as variations in temperature (23-31°C), time (22-36 hr), or enzyme to substrate ratio (100-150 units/0.1 mM galactose equivalents) produced no significant changes in the degree of oxidation of [2] as judged by analysis of the d.s. of its spin-labelled derivative [3].

(b) Periodate oxidations. These were carried out, with slight modification, according to the method of Opie and Keen.[15] The polysaccharide (0.3 mM galactose equivalents) was dissolved in phosphate buffer (20 ml) as before and 1-propanol (1 ml) was added followed by aqueous solutions (2 ml) of sodium metaperiodate (0.23 mM /mol. hexose unit for guaran and 0.17 mM/mol. hexose unit for locust bean gum). The oxidation was conducted at 5°C in the dark for 15 hr after which it was stopped by addition of ethylene glycol (1 ml). When the oxidation was carried out in smaller solution volumes (~8 ml) the same ropy gels were obtained as in the case of galactose oxidase treatments. The dialdehyde products were isolated after dialysis (3 d).

(c) Bromine oxidation of [2]. The bromine oxidation of the C6 aldehyde guaran derivative [2] was similar to that of Avigad et al.[16] An aqueous solution of [2] (75 mg, 0.15 mM, 7 ml) was added to bromine water (0.38 mM, 2 ml), the pH was adjusted with barium benzoate to ~6 and the reaction mixture was kept at 26°C in a constant temperature

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

shaker for 14 hr. The galacturopyranosyl product [14] was precipitated with ethanol (50 ml), and collected after centrifugation and dialysis (3 d).

3. Reductive Amination--General Procedure

The reductive aminations of the C6 aldehyde derivatives [2] and [6] were carried out in situ after oxidation or by dissolving the isolated aldehyde products in aqueous solution followed by treatment with the amine (4-8 mol./galactose equivalent) and sodium cyanoborohydride (20-40 mol./galactose equivalent) at ambient temperature for 24-36 hr. The products were isolated by dialysis (4-6 d) and lyophilization.

6-[N-4(-amino-2,2,6,6-tetramethylpiperidine-1-oxyl)] guaran derivative [3]

The yields of the spin-labelled derivative [3] ranged between 60-70% as determined by esr double integration and microanalysis (the latter being generally more reliable). A typical sample (d.s. 0.61) gave the following analytical results.

Anal. for $[(C_6H_{10}O_5)_{0.64}(C_6H_{10}O_5)_{0.14}(C_{15}H_{28}N_2O_6)_{0.22}]\cdot 0.78\ H_2O$; calcd. C 44.86, H 7.32, N 2.89; found C 44.83, H 7.15, N 2.71.

6-[N-3 -amino-1-propanol] guaran derivative [8]

Reaction of [2] with 3-amino-1-propanol (7.5 mM/aldohexose equivalent) afforded [8]. The d.s. was estimated from ^{13}C-nmr to be ca. 0.8. Microanalysis gave a C/N ratio of 17.33; (calcd. for d.s. 1.0; C/N 16.86).

6-(N-glycine) guaran derivative [9]

Reaction of [2] with glycine (4.0 mM/aldohexose equivalent) afforded [9], d.s. 0.2.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

Anal. for $[(C_6H_{10}O_5)_{0.64}(C_6H_9O_6)_{0.16}(C_8H_{13}NO_7)_{0.2}]\cdot 0.89\ H_2O$; calcd. C 39.35, H 6.31, N 1.43; found C 39.14, H 6.16, N 1.37.

6-[N-(4-amino)-5-imidazolecarboxamide] guaran derivative [10]

Reaction of [2] with 4-amino-5-imidazolecarboxamide HCl (Chemical Dynamics Corp.) (4.3 mM/aldohexose equivalent) afforded [10], d.s. 0.05.

Anal. for $[(C_6H_{10}O_5)_{0.64}(C_6H_9O_6)_{0.31}(C_{10}H_{15}N_4O_5)_{0.05}]\cdot 1.05\ H_2O$; calcd. C 38.94, H 6.35, N 1.48; found C 38.83, H 6.28, N 1.46.

6-[N-4-(amino-2,2,6,6-tetramethylpiperidine-1-oxyl)] locust bean gum derivative [7]

Spin-labelled derivatives of locust bean gum with d.s. ranging between 0.7-0.8 were obtained, as before; a sample with d.s. 0.7 had the following data.

Anal. for $[(C_6H_{10}O_5)_{0.81}(C_6H_{10}O_6)_{0.06}(C_{15}H_{28}N_2O_6)_{0.13}]\cdot 0.56\ H_2O$; calcd. C 44.33, H 6.99, N 1.91; found C 44.07, H 6.76, N 1.79.

Spin-labelled guaran [21] obtained from periodate oxidized guaran, [17].

The product [21] had d.s. 0.85 (based on d.o. = 0.53 for [17]) according to elemental analysis (calculated with one spin-label per dialdehyde unit).

Anal. for $[(C_6H_{10}O_5)_{0.64}(C_6H_9O_6)_{0.20}(C_{15}H_{28}N_2O_6)_{0.16}]\cdot 1.34\ H_2O$; calcd. C 41.32, H 7.16, N 2.10; found C 41.08, H 6.88, N 2.03.

Spin-labelled locust bean gum [22], obtained from periodate oxidized locust bean gum, [18].

The product [22] had d.s. 1.0 (based on d.o. 0.70 for [18]) as for [21].

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

Anal. for $[(C_6H_{10}O_5)_{0.81}(C_6H_9O_6)_{0.06}(C_{15}H_{28}N_2O_6)_{0.13}]\cdot 0.93\ H_2O$; calcd. C 42.70, H 7.02, N 1.84; found C 42.50, H 6.94, N 1.97.

6-[N-amino] guaran derivative [12]

Reductive amination of [2] with ammonium acetate (6 mol/aldohexose unit) for 3 d yielded [12], d.s. 0.55.

Anal. for $[(C_6H_{10}O_5)_{0.64}(C_6H_{10}O_5)_{0.16}(C_6H_{12}NO_5)_{0.20}]\cdot 0.56\ H_2O$; calcd. C 40.48, H 6.52, N 1.56; found C 40.27, H 6.46, N 1.54.

6-[N-1-deoxy-1-lactit-1-yl amine] guaran derivative [13]

C6-N-amino guaran [12] (55 mg, 0.33 mM) was reductively alkylated with lactose (400 mg, 1.17 mM) to afford, after 2 d, [13], d.s. 0.4. (based on [12]).

Anal. for $[(C_6H_{10}O_5)_{0.64}(C_6H_{10}O_6)_{0.16}(C_6H_{12}NO_5)_{0.12}(C_{18}H_{34}NO_{15})_{0.08}\cdot 1.82\ H_2O$; calcd. C 38.94, H 6.74, N 1.41; found C 38.65, H 6.49, N 1.29.

Bovum Serum Albumin--conjugate of guaran [11]

To a solution of the aldehyde [2] in distilled water (119 mg, 0.71 mM, 20 ml) was added an aqueous solution of BSA (96.1 mg, 1.4 μM) and sodium cyanoborohydride (250 mg, 4 mM, 5 ml). After stirring the reaction mixture for 2 d, the product [11] was isolated after dialysis.

4. Borodeuteride Reduction of C6-aldehyde Derivatives

Sodium borodeuteride (0.1 g, 2.4 mM) was added to the dissolved C6 aldehyde derivatives [2] and [6], respectively (0.3 mM aldohexose equivalent, 15 ml) and the aqueous reaction mixture was stirred for 1 d, after which excess reducing agent was destroyed by addition of 2N HCl (0.5 ml). The monodeuterated products [15] and [16] (respectively), were subsequently dialyzed (3 d) and lyophilized.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

VII-E. Chapter V

1. Materials

The chemicals were purchased from the following suppliers: salicylaldehyde was vacuum distilled before use (Fisher); salicylic acid (Malinckrodt); cupric acetate (Fisher); PA-18 polyanhydride was a gift of Gulf Specialty Chemicals; ferrocene aldehyde [18] was kindly provided by Dr. M. J. Adam.

2. Synthesis

[N-Salicylidene] chitosan [3]

The methods of Hirano et al.[13] and Nud'ga et al.[17] were employed with some modifications: to chitosan (500 mg, 3.0 mM), dissolved in a mixture (1:1, 25 ml) of methanol and 1% aqueous acetic acid, was added dropwise and with vigorous stirring, salicylaldehyde [2] (0.35 ml, 3.5 mM). The resulting yellow, initially very viscous solution turned into a thick gel within minutes. A saturated aqueous solution of $NaHCO_3$ was added (2 ml) to prevent acid hydrolysis of the Schiff's base. A further portion of [2] (0.35 ml, 3.5 mM) was added dropwise resulting in a further stiffening of the gel, to which was then added methanol (80 ml) and $NaHCO_3$ solution (1 ml). After a few minutes the solvents were decanted and the wash was repeated twice, the pH of the final wash being neutral. The gel was left standing in methanol (100 ml) for 4 hr, then filtered on a sintered glass funnel, washed with methanol and diethyl ether (100 ml each), air-dried for several hours and finally dried in vacuo at 56°C. Yield 0.75 g, d.s. 0.97.

Anal. for $[(C_8H_{13}NO_5)_{0.02}(C_{13}H_{15}NO_5)_{0.98}]\cdot 0.65\ H_2O$; calcd. C 56.20, H 5.94, N 5.08; found C 56.36, H 5.84, N 5.09.

Addition of a total 4.2 mM [2] resulted in a product with lower

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

d.s. (0.7).

Anal. for $[(C_8H_{13}NO_5)_{0.03}(C_6H_{11}NO_4)_{0.27}(C_{13}H_{15}NO_5)_{0.7}]\cdot 0.72\ H_2O$; calcd. C 53.02, H 6.21, N 5.64; found C 52.84, H 5.97, N 5.67.

[N-(2-cresol)]-chitosan [4]

The same procedure as for [3] was employed using $NaCNBH_3$ (0.2 g, mM) concommitant with the addition of [2]. Addition of the reducing agent caused the yellow colour of the product to fade and no gel was formed initially. The solution was left stirring overnight resulting in a soft gel. Further addition of the same quantities of [2] and $NaCNBH_3$ afforded a soft, ivory coloured gel which was dialyzed (3 d) against distilled water, and lyophilized to give a fluffy white material (0.44 g), d.s. 0.7.

Anal. for $[(C_8H_{13}NO_5)_{0.03}(C_6H_{11}NO_4)_{0.27}(C_{13}H_{17}NO_5)_{0.7}]\cdot 0.76\ H_2O$; calcd. C 52.55, H 6.76, N 5.59; found C 52.30, H 6.50, N 5.55.

When the reduction was carried out after the Schiff's base gel had formed, the latter retained its rigidity and, to a large extent, its yellow colour. After the usual workup, a yellow product with d.s. 0.85 was obtained (0.70 g).

Anal. for $[(C_8H_{13}NO_5)_{0.02}(C_6H_{11}NO_4)_{0.13}(C_{13}H_{17}NO_5)_{0.85}]\cdot 0.63\ H_2O$; calcd. C 54.63, H 6.66, N 5.32; found C 54.42, H 6.34, N 5.49.

[N-(3-carboxy-)salicylidene] chitosan [5]

As before, chitosan (0.33 g, 2.0 mM) was condensed with 3-formyl-2-hydroxy-benzoic acid [6] (0.39 g, 2.35 mM) dissolved in methanol (10 ml) to produce a bright yellow and very rigid gel within 3 min. The product was yellow and odourless and had a d.s. 1.0 (0.53 g).

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

Anal. for $[(C_8H_{13}NO_5)_{0.02}(C_{14}H_{15}NO_7)_{0.98}]\cdot 1.01\ H_2O$; calcd. C 51.24, H 5.26, N 4.31; found C 51.26, H 5.20, N 4.17.

Carboxymethyl derivatives of chitosan [10] and chitin [11]

The method of Trujillo[18] was adopted with slight modifications. Chitin was suspended in DMSO (15 ml) for 1 d prior to the treatment which was used for the preparation of both [10] and [11]. The polysaccharides (0.5 g) were suspended in an aqueous (65%) NaOH solution (50 ml) for 0.5 hr to produce the alkali derivatives which were filtered, pressed, and then added to a solution of monochloroacetic acid (2.6 g) in i-propanol (50 ml). The suspensions were stirred for 1 hr, filtered, resuspended in water (100 ml) and the solution pH was raised (from 3.5-4.0) with conc. NaOH solution to neutral. The chitosan derivative [10] formed a gel at this stage, which was lyophilized. The solid carboxymethyl chitin [11] was filtered, pressed and dried.

Anal. for [10] d.s. 1.2 $[(C_{12}H_{17}NO_{10})_{0.1}(C_8H_{13}NO_6)_{0.9}]\cdot 1.02\ H_2O$; calcd. C 40.46, H 6.25, N 5.62; found C 40.29, H 6.67, N 5.56.

Anal. for [11] d.s. 1.0 $[(C_8H_{13}NO_6)_{0.1}(C_{10}H_{15}NO_7)_{0.9}]$; calcd. C 45.78, H 5.81, N 5.45; found C 45.89, H 6.86, N 5.46.

N-Methylene Chitosan [12]

This derivative was prepared following the procedure of Hirano et al.[19] Chitosan (500 mg, 3.0 mM) was dissolved in 2% aqueous acetic acid (25 ml). Dropwise addition of 37% formaldehyde solution (2 ml, 24 mM) produced a colourless gel in less than 3 hr, which was broken up and suspended in methanol (100 ml) for 1 d, and then filtered. This washing procedure was repeated twice, before the gel was finally suspended in diethyl ether (100 ml) for 1 d. Filtration and drying yielded

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

the product [12].

Cross-linked polyanhydride chitosan [15]

To chitosan (500 mg, 3 mM), dissolved in a mixture (1:1, 45 ml) of methanol and 2% aqueous acetic acid, was added polyanhydride [14] (Gulf PA-18) (1.15 g, 3.3 mM) dissolved in benzene (8 ml). The white viscous solution formed a very soft gel after standing overnight which was suspended in methanol (100 ml) for 1 d. After two such washes with methanol, and with diethyl ether, the product was filtered and dried yielding 0.08 g of a white powder (A). Alternatively, chitosan dissolved in 10% aqueous acetic acid (15 ml) was mixed with a solution of the polyanhydride (5.05 g, 14.4 mM) in ethyl acetate (30 ml) to produce a milky gel within seconds, which was worked up as before. 0.25 g white powder was obtained (B) (d.s. ~1.0).

Anal. for $[C_{50}H_{87}NO_{12}]\cdot 1.49\ H_2O$; calcd. C 65.18, H 9.85, N 1.52; found C 65.20, H 9.89, N 1.53.

Solubilized salicylidene chitosan [16]

To chitosan (0.50 g, 3.0 mM), dissolved in a mixture (1:1) of methanol and 1% aqueous HOAc, was added lactose (0.30 g, 0.9 mM) in MeOH (4 ml) and subsequently, salicylaldehyde [2] (0.35 ml, 3.5 mM) and $NaCNBH_3$ (0.3 g, 4.8 mM) dissolved in water (4 ml). The vigorously stirred mixture lost its yellow colour after a short time and produced a soft, faintly yellow gel. The product had an overall d.s. 1.0 with 25% sugar substitution.

Anal. for $[(C_8H_{13}NO_5)_{0.05}(C_{13}H_{17}NO_5)_{0.71}(C_{18}H_{33}NO_{14})_{0.24}]$; calcd. C 52.85, H 6.57, N 4.42; found C 52.51, H 5.95, N 4.20.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

N-Ferrocenyl chitosan [19]

To chitosan (0.20 g, 1.2 mM), dissolved in a mixture of methanol and 1% aqueous acetic acid (1:1, 50 ml) was carefully added, with stirring, a solution of ferrocenealdehyde [18] (0.30 g, 1.4 mM) and $NaCNBH_3$ (0.9 g, 14.4 mM) in methanol (10 ml). The initially red reaction mixture was left stirring overnight yielding a fine brown precipitate which was filtered, washed (methanol), and dried. 0.38 g of the brown product (d.s. 0.445) was isolated.

Anal. for $[(C_8H_{13}NO_5)_{0.02}(C_6H_{11}NO_4)_{0.54}(C_{17}H_{21}FeNO_4)_{0.44}]$; calcd. C 48.83, H 5.83, N 5.26, Fe 9.37; found C 48.60, H 5.90, N 5.32, Fe 10.06.

3. Copper Complexation Reactions

The polysaccharides were complexed by dispersing portions (0.1 g) in methanol (20 ml) with vigorous magnetic stirring. Aqueous solutions were not used due to the water-solubility of some derivatives and in order to keep reaction conditions constant. (However, no substantial differences were observed when the complexation was carried out in water.) After the desired times, the polysaccharides were filtered and washed (150 ml) before being dried in vacuo at 80°C for 24 hr. The samples were kept desiccated before elemental analysis. The copper determinations were obtained by nitric acid digestion of the polysaccharide samples followed by atomic absorption measurements.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

References

1. R. G. Schweiger, J. Org. Chem., 41, 90 (1976).

2. G. Holzwarth, Biochemistry, 19, 4333 (1976).

3. R. L. Whistler and J. W. Marx, Methods Carbohydr. Chem., 5, 143 (1965).

4. W. G. Struve and H. M. McConnell, Biochem. Biophys. Res. Commun., 49, 1631 (1972), H. W. Wien, J. D. Morrisett, and H. M. McConnell, Biochemistry, 11, 3707 (1972).

5. R. G. Schweiger, J. Org. Chem., 27, 1786 (1962).

6. I. L. Andresen, T. J. Painter, and O. Smidsrød, Carbohydr. Res., 59, 563 (1977); and references cited therein.

7. H. Andresz, G. C. Richter, and B. Pfannemüller, Makromol. Chem., 179, 301 (1978).

8. C. S. Lee and K. Maekawa, Agr. Biol. Chem., 40, 785 (1976).

9. Kelco Algin (Second Edn.), Kelco Co., San Diego, California, 41 (1976).

10. P. T.Nevell, Methods Carbohydr. Chem., 3, 164 (1973).

11. Dr. J. Defaye, private communication to Dr. L. D. Hall.

12. S. Hirano, R. Yamaguchi, and N. Matsuda, Biopolymers, 16, 1987 (1977); Int. J. Biochem., 9, 501 (1978).

13. S. Hirano, N. Matsuda, O. Miura, and H. Iwaki, Carbohydr. Res., 71, 339 (1979).

14. M. S. Masri, V. G. Randall, and W. L. Stanley, in Proc. First Intern. Conf. Chitin/Chitosan, (R. A. A. Muzzuralli and E. R. Pariser, eds.), 364 (1978).

15. J. W. Opie and J. L. Keen, Germ. [illegible], 1, 262, 756 (1968); Chem. Abstr., 68, 88, 358k (1968).

16. G. Avigad, D. Amaral, C. Asensio, and B. L. Horecker, J. Biol. Chem., 237, 2736 (1962).

17. L. A. Nud'ga, E. A. Plisko, and S. N. Danilov, Zh. Obshch. Khim., 43, 2752 (1973), (2729 in transl.).

18. R. Trujillo, Carbohydr. Res., 7, 483 (1968).

19. S. Hirano, N. Matsuda, O. Miura, and T. Tanaka, ibid., 71, 344 (1979).

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

APPENDIX

^{13}C nmr spectroscopy of natural polysaccharides has estblished itself as a very powerful tool in recent years. However, with the exception of permethylated derivatives, modified polysaccharides have not yet been widely studied. Both native and modified derivatives are, unfortunately, not always amenable to analysis by conventional nmr techniques, e.g. for xanthan gum (MW 10^6) [1] no ^{13}C nmr spectra have so far been observed due to its extremely high solution viscosities.[2] This Appendix contains the preliminary results from ^{13}C nmr experiments which were designed to remedy such situations.

In the first set of experiments we foud that a ^{13}C nmr spectrum of xanthan gum in aqueous solution could be obtained using conditions (pulse width 32 μs, ca. 144°- delay 2 s) which were essentially those for the spin-echo technique.[3] Figure A-1 illustrates the complex (yet unassigned) spectrum of xanthan gum which presumably derives only from the pendant, more mobile trisaccharide side-chains.

Another, very powerful recent development is ^{13}C Magic Angle Spinning-Cross Polarization (MAS-CP) nmr[4], which allows for the analysis of solid materials. Figure A-2 demonstrates the enormous utility of this high resolution technique for both native xanthan gum (unpurified Keltrol) and the octadecyl xanthan amide derivative whose sythesis was discussed in Chapter II-B. The spectrum of xanthan gum (Fig. A-2A) shows the carbonyl signals at 173 ppm, the anomeric signals (partially resolved) at 102 ppm, the overlapping signals from C2 to C5 of the various monosaccharide residues at 73 ppm, and the pyruvate methyl resonance at 21 ppm.[2] The spectrum of the amide (Fig. A-2B) reveals an

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

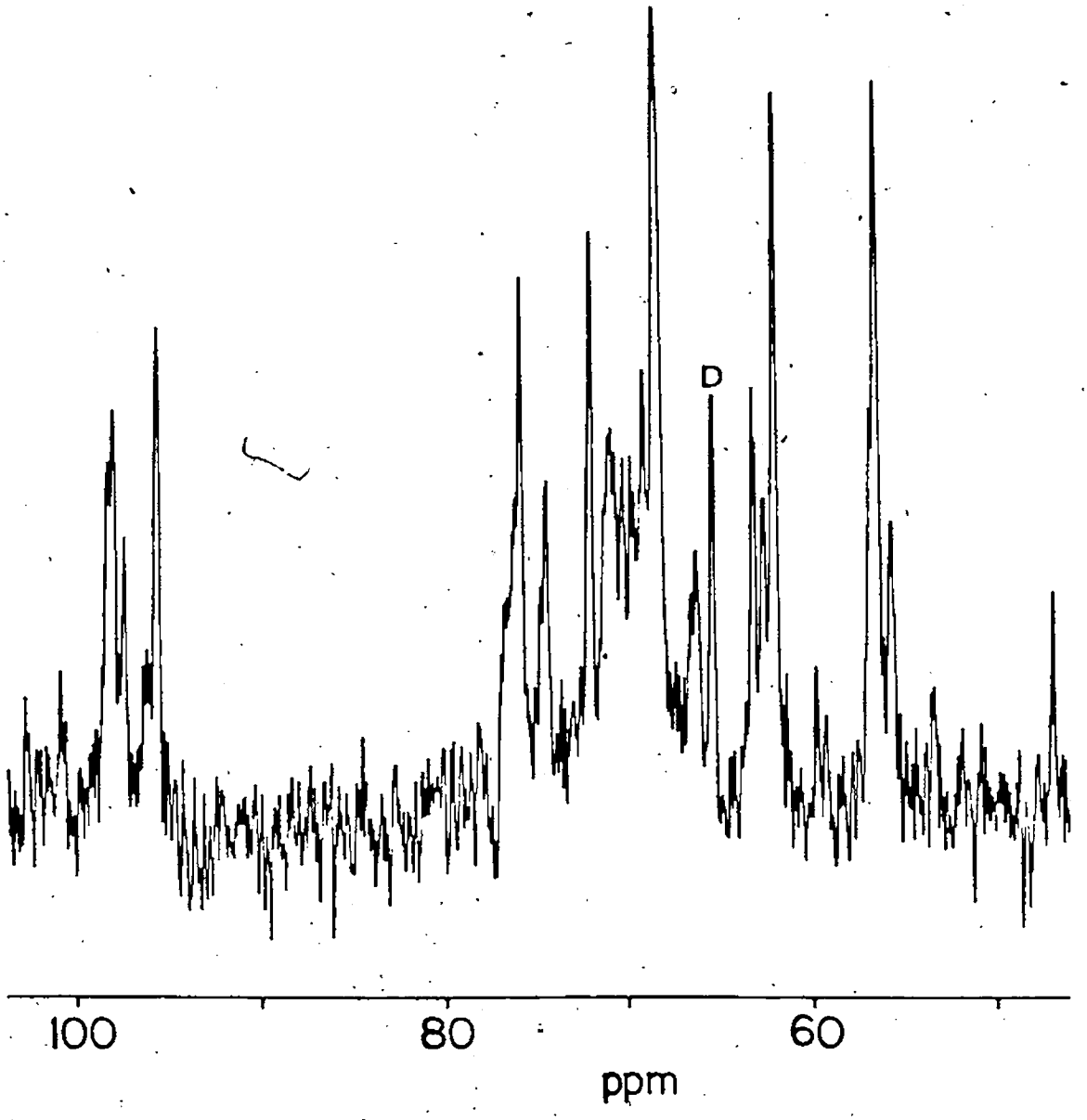


Fig. A-1. 100.6 MHz ^{13}C-nmr spectrum of xanthan gum (5%) in D_2O at 338K; (sweep width 26,000 Hz, 25,000 scans).

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

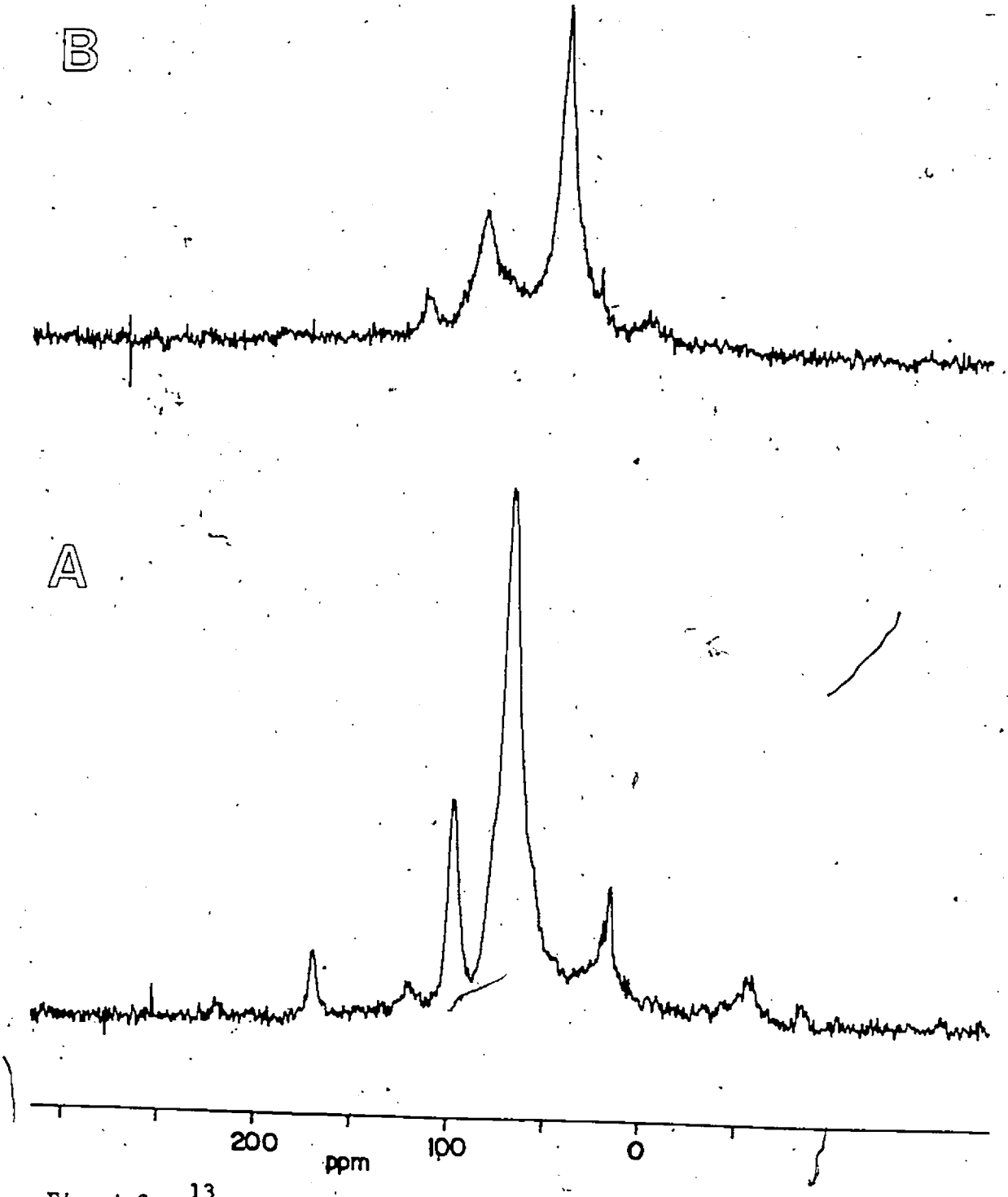


Fig. A-2. ^{13}C-MAS-CP nmr spectra of (A) xanthan gum (unpurified Keltrol), 10,000 scans, 2 s repetition, 0.5 ms contact time, 2 KHz spinning 10 G ^{1}H decoupling; (B) octadecyl xanthan amide, 100 mg sample, same conditions. Spectra were obtained on a Bruker CXP-200 spectrometer (by Dr. S.Ganapathy).

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

additional resonance at 33 ppm which can be assigned to the methylene carbons of the hydrophobic side-chains. The spectrum required a total of three hours experimental time providing considerably more detail than the corresponding ^{13}C solution experiment in an equivalent period (using conventional nmr techniques).

The MAS-CP method is, of course, of particular importance for substances which are not soluble in common solvent systems, as exemplified here by the ^{13}C nmr spectra of cellulose powder (Whatman CF11) and [N-(3-fluoro)-benzyl] chitosan (prepared analogously to the salicyldimine chitosan derivatives in Chapter V) (Fig. A-3). The spectrum of the former (Fig. A-3a) appears to be intermediate to those of purely microcrystalline and amorphous cellulose samples[5] providing support for the esr results (see II-B 3) which were indicative of heterogeneities in accessibility for this material. The aromatic signals of the chitosan derivative (Fig. A-3b) are observed between 120 to 145 ppm and the N-methylene signal appears at 23 ppm.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

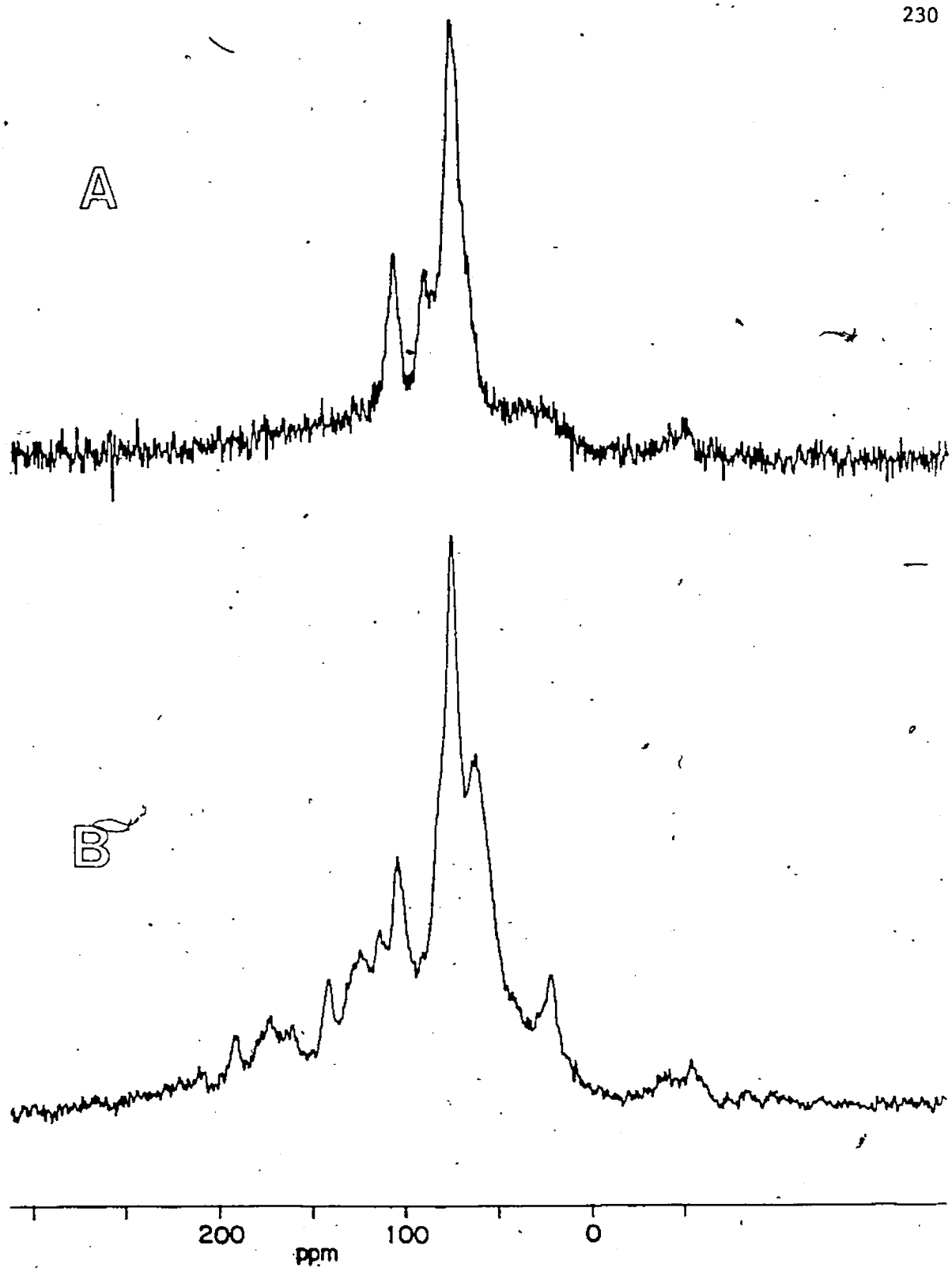


Fig. A-3. ^{13}C-MAS-CP nmr spectra of (A) cellulose (Whatman CF11), 10,000 scans, 2 s repetition, 0.5 ms conact time, 2 KHz spinning, 10 G ^{1}H decoupling; (B) [N-(3-fluoro)-benzyl] chitosan, 20,000 scans, same conditions.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

References

1. G. Holzwarth, Carbohydr. Res., 66, 173 (1978).

2. E. R. Morris, D. A. Rees, G. Young, M. D. Walkinshaw, and A. Darke, J. Mol. Biol., 110, 1 (1977); P. J. Garegg, P. E. Jannson, B. Lindberg, F. Lindh, J. Lönngren, I. Kvarnström, and W. Nimmich, Carbohydr. Res., 78, 127 (1980).

3. L. D. Hall and S. Sukumar, J. Magn. Res., 38, 559 (1980); and references therein.

4. J. Schaefer and E. O. Stejskal, in Topics in Carbon-13 NMR Spectroscopy, G. Levy, ed., 283 (1979); W. E. Earl and D. L. Hart, Macromolecules, 12, 762 (1979).

5. R. H. Atalla, J. C. Gast, D. W. Sindorf, V. J. Bartuska, and G. E. Maciel, J. Amer. Chem. Soc., 102, 3249 (1980).

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

Biographical Information

NAME: Manssur Yalpani

PLACE AND DATE OF BIRTH: Tehran 31/5/1950

EDUCATION (Colleges and Universities attended, dates, degrees)

Simon Fraser University, B.Sc.(Hon.), 1975

POSITIONS HELD:

PUBLICATIONS:

L.D.Hall and M.Yalpani, Carbohydrate Res., 81, C10, 1980.

L.D.Hall and M.Yalpani, Carbohydrate Res., 78, C4, 1980.

L.D.Hall and M.Yalpani, Carbohydrate Res., in press.

AWARDS:

Mcmillan Bloedel Limited Graduate Scholarship

University of B.C. Graduate Fellowship

This form is to be completed by candidates for the Master's or higher degree and submitted to the University Library Special Collections Division with the thesis.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.