TRENT POLYTECHNIC

ACYLATION STUDIES ON CHITOSAN AND SUBSTITUTED CHITOSANS

being a thesis submitted to

The Council for National Academic Awards for

the degree of

DOCTOR OF PHILOSOPHY

by

Graham Kenneth Moore B.Sc., Grad R.I.C.

November, 1978

I wish to declare that, except where stated otherwise, the work described in this thesis is my own and was carried out in the Department of Physical Sciences, Trent Polytechnic, during the years 1975-1978.

G.K Moore.

To

My parents.

Preface

The work described in this thesis was carried out by the author in the laboratories of the Department of Physical Sciences, Trent Polytechnic, Nottingham, between October 1975 and September 1978

The author wishes to thank Dr. G.A.F.Roberts for his excellent supervision and constant encouragement throughout the course of this work. Thanks are also due to Dr. B.W.Marshall for useful guidance and discussions on parts of the work; Mrs. F.A.Wood for the many infrared spectral determinations and for the preparation of many of the figures in this thesis; Mr. M.L.Wood for nmr determinations; Mr.P. Stockham for X-ray photographic prints; Mr. B. Marshall, of the Department of Mechanical Engineering, for advice on the x-ray diffraction work; the Boots Company Limited, for the use of their gelometer in gelation studies; the National Oceanic and Atmospheric Administration and Hercules Incorporated for gifts of chitosan; and to Mrs. B.D.Evans for typing this thesis.

The author is also indebted to the Science Research Council for the provision of a research studentship between the above dates.

Part of this work was presented in a paper given at the 1st. International Conference on Chitin/Chitosan at Boston, Massachusetts in April, 1977.

G. K.Moore
Trent Polytechnic
November, 1978

Abstract

Several detailed aspects of the heterogeneous and homogeneous acylation reactions of chitosan and substituted chitosan have been studied.

Acetylation of chitosan in film or reprecipitated form, using conventional cellulose techniques, were unsuccessful. However, a facile, selective technique was developed for the N-acylation of chitosan film, using methanol/carboxylic acid anhydride mixtures. The solubility parameter values of the solvent system were found to be important in the acylation reactions, no reaction occurring in media having a solubility parameter value $\delta < 13$ Hildebrands.

O-Acetylation studies of various N-acyl chitosan films in pyridine/acetic anhydride mixtures showed that there was a minimum spacing required between the polymer chains to enable the reaction to take place. Increased distance between the chains brought about only slight improvement in the extent of reaction, whilst the presence of bulky N-acyl groups were found to hinder the reaction. O-Acetylation was found to be incomplete, only 50% of the available hydroxyl groups being acetylated after prolonged reaction times in the majority of cases.

O-Acetylation of various linear aliphatic Schiff's base derivatives of chitosan gave a similar pattern to that of the equivalent N-acyl derivatives. The N-substituents were susceptible to hydrolysis and N-acetylation accompanied the hydrolysis. However, the aromatic Schiff's base derivatives showed marked differences to the equivalent N-acyl chitosans. O-Acetylation was found to be virtually complete in the majority of cases whereas only

approximately 50% of the hydroxyl groups had again reacted in the case of the N-acyl chitosans.

A di-O-acetyl-N-acetyl chitosan has been prepared by O-acetylation of a Schiff's base intermediate followed by hydrolysis and N-acetylation. Organosoluble carbamates of some Schiff's base derivatives of chitosan have also been prepared.

Gelation of chitosan solutions in methanol/acetic acid/carboxylic acid anhydride mixtures have been followed by monitoring the reaction viscosity changes during gelation. The viscosity versus time curve was a smooth curve but kinked, indicating that the gelation process was not simple chain aggregation. Variations in temperature, anhydride and polymer concentration and co-solvents have been studied and gelation was found to start after about 60-70% of the primary amines had been acylated. A mechanism has been proposed for the gelation process based on the experimental evidence.

"One day, Professor Wohler partook of lobster for lunch and, bringing back the shell to the laboratory, gave it to his nephew and admonished him to find out what it was.....thus was [chitin] discovered"

Contents

	Page
Introduction	1
Chapter 1 Chitin and Chitosan	4
1.1 Occurrence of Chitin	4
1.1.1. General	4
1.1.2. Occurrence of chitin in lower animals	7
1.1.3. Occurrence of chitin in lower plants	12
1.2. The structure of chitin and chitosan	16
1.2.1. Molecular structure	16
1.2.2. Morphology	21
1.2.2.1. The conformation of α-chitin	23
1.2.2.2. The conformation of β-chitin	24
1.3. Isolation of chitin	25
1.4. Preparation of chitosan	27
1.5. Solubility of chitin and chitosan	37
1.6. Molecular weights of chitin and chitosan	45
1.6.1. Effect of shearing on molecular weight	46
1.7. Viscosity of chitin and chitosan	47
1.8. Preparation of chitin and chitosan membranes	53
Chapter 2 Reactions, properties and utilization of chitin and chitosan	60
2.1 Chemical reactions of chitin	60
2.1.1. Inorganic ester derivatives	61
2.1.1.1. Sulphates	61
2.1.1.2. Nitrates	62
2.1.2. Alkyl derivatives	63
2.1.3. Acyl & related derivatives	66
2.1.4. Miscellaneous derivatives	68
2.2. Chemical reactions of chitosan	70
2.2.1. Inorganic ester derivatives	70

Contents Page

2.2.1.1. Sulphates 70

2.2.1.2. Phosphates and nitrates 71

2.2.2. Alkyl derivatives 72

2.2.3. Acyl and related derivatives 74

2.2.4. Schiff's base derivatives 76

2.2.5. Complex formation with polyelectrolytes 77

2.2.6. Oxidation of chitosan 78

2.2.6.1. Deamination 78

2.2.6.2. Carboxyl group formation 79

2.2.7. Salt formation 79

2.2.7.1. Dye adsorption 80

2.2.8. Chelation properties 80

2.2.9. Thermal properties 84

2.2.10 Optical properties 84

2.3. Utilisation of chitin chitosan and their derivatives 86

2.3.1. Paper additives 86

2.3.2. Textile applications 87

2.3.3. Photographic applications 88

2.3.4. Adhesive applications 89

2.3.5. Coagulating applications 90

2.3.6. Analytical applications 90

2.3.7. Medical applications 91

2.3.8. Miscellaneous applications 93

Chapter 3 Results and discussion 96

Part A Heterogeneous acylation studies 96

3. 1. Acylation of chitin and chitosan 96

3.1.1. General 96

Contents

	Page
3.1.2. N-Acetylation of chitosan in film form	98
3.1.2.1. General	98
3.1.2.2. Methanol/acetic anhydride solutions	102
3.1.2.3. Influence of solvent media on N-acetylation	104
3.1.2.4. N-Acetylation of chitosan in film form	115
3.1.2.5. Studies on the rates of N-acylation of chitosan in film form	116
3.1.3. N-Acylation of chitosan in powder form	124
3.1.3.1. X-Ray diffraction studies on N-acyl chitosans	127
3.2.1. O-Acetylation of N-acyl derivatives of chitosan in film form	131
3.2.1.1. Linear aliphatic anhydrides	134
3.2.1.2. Branched aliphatic anhydrides	141
3.2.1.3. Aromatic anhydrides	144
3.2.2. O-Acylation of N-acyl derivatives of chitosan in powder form	150
3.3. Preparation and acetylation of Schiff's bases and ketoimine derivatives of chitosan	153
3.3.1. Preparation of Schiff's base derivatives	153
3.3.2. Preparation of ketoimine derivatives	161
3.3.3. Acetylation of Schiff's base derivatives in film form	164
3.3.3.1. Linear aliphatic aldehydes	169
3.3.3.2. Branched aliphatic aldehydes	172
3.3.3.3. Aromatic aldehydes	172
3.3.4. Acetylation of Schiff's base derivatives of chitosan in solid form	176
3.3.5. Acetylation of the ketoimine derivatives of chitosan	180
3.4. Comparison of acetylation of N-acyl chitosans and Schiff's base derivatives of chitosan	183

Contents

	Page
3.5. Preparation of N-acetyl - di-O-acetyl chitosan	195
3.6. Miscellaneous derivatives of chitosan	200
Part B Homogenous acylation studies	207
3.7. Studies on the gelation of N-acyl chitosans	207
3.7.1. Introduction	207
3.7.2. Preliminary experiments	208
3.7.3. The use of alternative co-solvents	209
3.7.4. Rate of gelation	211
3.7.4.1. General	211
3.7.4.2. Effect of varying the anhydride	213
3.7.4.3. Effect of temperature	216
3.7.4.4. Effect of variation in anhydride concentration	219
3.7.4.5. Effect of variation in polymer concentration	223
3.7.4.6. Effect of variation in polymer concentration with corresponding increase in anhydride concentration	225
3.7.5. Ultimate gel strength	229
3.7.6. Further studies on gelation	234
3.7.6.1. Relationship between degree of N-acylation and gelation	234
3.7.6.2. Effect of anhydride and temperature	235
3.7.6.3. Effect of polymer concentration	238
3.7.6.4. Determination of energy of activation for N-acylation	239
3.7.7. The mechanism of gelation	243
3.7.8. Optical rotation changes during gelation	247

Contents Page

3.7.9. Syneresis of chitosan gels 247

3.7.10 The use of novel anhydrides for gel formation 252

3.7.10.1 Branched anhydrides 252

3.7.10.2. Toluic anhydrides 252

3.7.11 The use of aldehydes for gel formation 252

Chapter 4 Conclusions 254

Chapter 5 Experimental 258

5.1. Materials used 258

5.1.1. Chitosan 258

5.1.2. Chemicals 258

5.2. Spectroscopic methods 258

5.3. X-Ray diffraction 258

5.4. Dilute solution viscosity 259

5.5. Concentrated solution viscosity 259

5.6. Solubility studies 261

5.7. Characterisation of starting materials 262

5.7.1. Molecular weight 262

5.7.2. Measurement of Limiting Viscosity Number 262

5.7.3. Degree of acetylation 263

5.7.3.1. Acidimetry 264

5.7.3.2. Periodate oxidation 265

5.7.3.2.1. Iodometry 265

5.7.3.3. Infrared spectroscopy 266

5.7.4. Chemical tests 275

5.7.4.1. Rimini test 275

5.8. Preparation of chitosan 277

Contents Page

5.8.1. Film form 277

5.8.2. Solid form 277

5.9. Preparation of aliphatic anhydrides 278

5.9.1. Method of Wallace and Copenhaver 278

5.9.2. Method of Gerrard and Thrush 279

5.10 Preparation of anhydrides of o-,m- and p-toluic acid 280

5.10.1. o-and m- Toluic anhydrides 280

5.10.2. p-Toluic anhydride 280

5.11 Preparation of N- acyl chitosans 281

5.11.1. Film form 281

5.11.2. Solid form 283

5.12. Rates of N-acylation of chitosan film 284

5.13 Attempted O-acetylation of N-acyl chitosan in solid form 285

5.14. Preparation of Schiff's base derivatives of chitosan 286

5.14.1. Film form 286

5.14.2. Solid form 286

5.15. The preparation of ketoimine derivatives of chitosan 287

5.15.1. The benzoylacetone adduct of chitosan 287

5.15.2. The acetylacetone adduct of chitosan 287

5.16. The preparation of N-salicyclidene O-acetylchitosan 288

5.17. Acetylation of ketoimine derivatives of chitosan 288

Contents

	Page
5.18. Preparation of N-acetyl-di-O-acetyl chitosan	289
5.19. Preparation of aromatic carbamates of the Schiff's base derivatives of chitosan.	290
5.19.1. N-Salicyclidene chitosan α-napthlyl carbamate and phenyl carbamate	290
5.19.2. N-Benzylidene chitosan α-napthyl carbamate and phenyl carbamate.	290
5.20 Preparation of the α-naphthyl carbamate and phenyl carbamate derivatives of chitosan.	291
5.2.1. Preparation of N- acyl chitosan gels	292
5.2.2. The isolation and characterisation of partially N-acylated chitosan gels.	292
References	294
Appendices	AI-AVII

Introduction

The existence of chitin in living things has been known for well over 150 years, but it is only in the last thirty years or so that chitin and its modified product chitosan, have been the subject of many widely diversified research programmes. This stems from a need to utilise the large amounts of waste products from shellfish processing plants, from the continuing search for natural and cheap materials, and from the biological analogy and structural similarity of chitin and cellulose.

In the idealised state, chitin is a polysaccharide consisting of β-(1→ 4)-2-acetamido-2-deoxy-D-glucose units and chitosan is its fully de-N-acetylated product, poly[β-(1→ 4)-2-amino-2-deoxy-D-glucose], however due to the methods of isolation it is doubtful whether chitin is ever obtained fully N-acetylated. The preparation of chitosan is based on alkaline treatments which, depending on the conditions, give products with differing degrees of de-N-acetylation. Therefore chitosan is the name applied to any of the intermediate compositions between poly[β-(1→ 4)-2-amino-2-deoxy-D-glucose] and poly[β-(1→ 4)-2-acetamido-2-deoxy-D-glucose]. In the case of analogues of chitin having N-acyl groups other than N-acetyl these are designated as N-acyl chitosans. Figure 1 shows idealised structures of chitin, (1A) and chitosan, (1B).

Chitin occurs naturally in three polymorphic forms which differ in the arrangement of the molecular chains within the crystal cell. The three forms are known as α-, β-, and γ-chitins respectively, and by far the most abundant is α-chitin which is found in arthropod cuticles and certain fungi. The three forms have been found in different parts of the same organism, in the squid Loligo, which indicates the three forms are relevant to different functions and not to animal grouping.

The advancement of knowledge about chitin and chitosan has been erratic due to the wide variability of the starting products and the multi-disiplinary interests of the research workers. However, in the past few years several reviews and books have been published on the wide

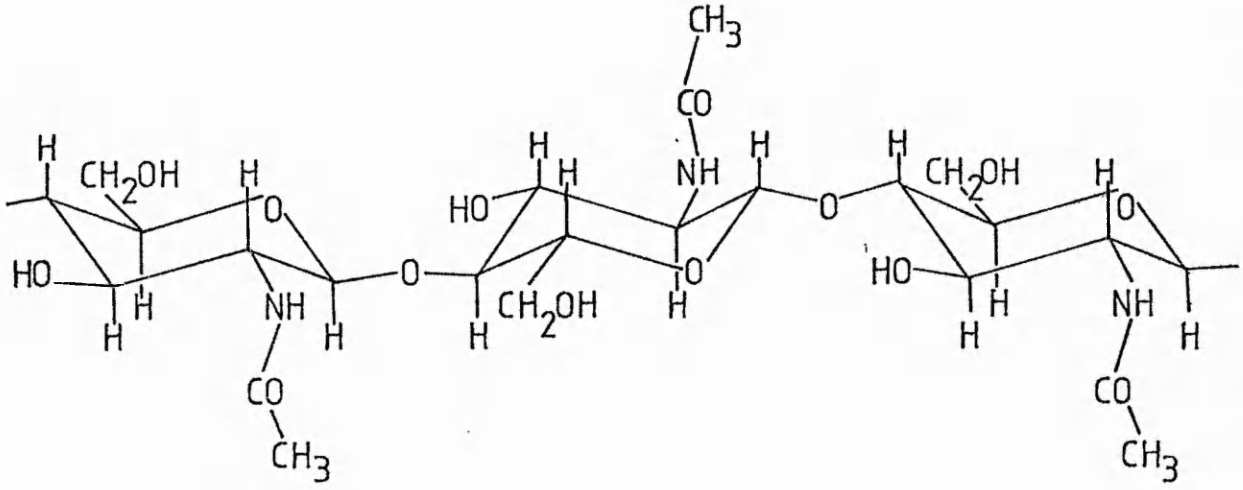


Figure 1A Idealised structure of chitin.

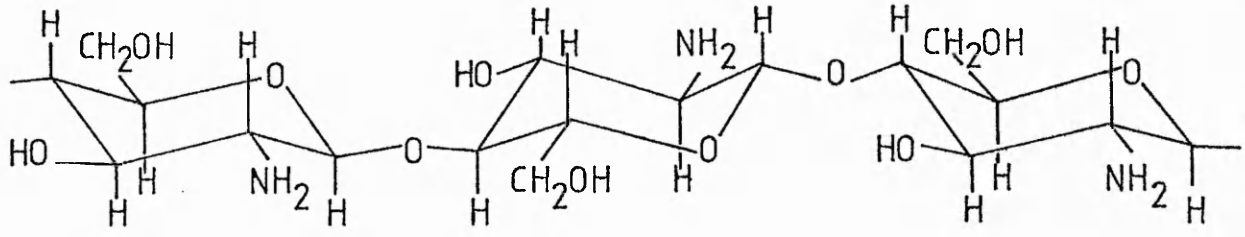


Figure 1B Idealised structure of chitosan

variety of research work that has been undertaken and these include works by Tracey [1], Foster and Webber [2], BeMiller [3], Muzzarelli [4,5], Hepburn [6], and Plisko, Nud'ga and Danilov [7]. Very recently the Proceedings of the First International Conference on Chitin/Chitosan which took place at Boston, Massachussetts, in April 1977, have been published [8]. The Conference was the first inter -disiplinary meeting of scientists, researchers and industrialists of the "chitin-world".

The work reported in this thesis is concerned with chemical aspects of chitosan and is divided into two main parts. The first part contains a detailed study of the N-acylation reactions of chitosan and the acylation reactions of substituted chitosans. In the second part, gel-forming reactions of N-acyl chitosans are examined and the variation in parameters are studied. A mechanism for the gelation reaction of N-acyl chitosan is proposed.

Chapter 1

Chitin and Chitosan

1.1 Occurrence of Chitin.

1.1.1 General

Chitin was first isolated by Braconnot [9] (1811) from Agarious volvaceus and other mushrooms. He named the product "fungine" and stated that it seemed to contain more nitrogen than did wood and concluded that it was a quite distinct substance among those identified in plants.

The name Chitin, from the Greek term χιτών meaning a tunic or envelope, was proposed [10] by Odier (1823) for a product he isolated from the elytra of May beetles. He also established, for the first time, a relationship between the insect cuticle and plant tissue.

The first reviews on the occurence and distribution of chitin were those by Van Wisselingh [11](1898), Wester [12](1910), Von Wettstein [13] (1921), Levene and Lopez-Suarez [14](1925) and Von Franciis [15] (1930).

Chitin is now known to occur widely throughout nature in both the animal and plant kingdoms. A table illustrating the distribution of the diverse chitinous structures in living organisms [16] is given in the Appendix I. It exists also in the less evolved taxonomic groups such as Protozoa. The biosynthesis of chitin appears to be controlled by early established genes and this biosynthetic ability has been retained by numerous diblastic animals and by most of the triblastic Protostomia. However, it was lost at the start of the deuterostomian evolutionary lineage, except for the Tunicata.[17,18].

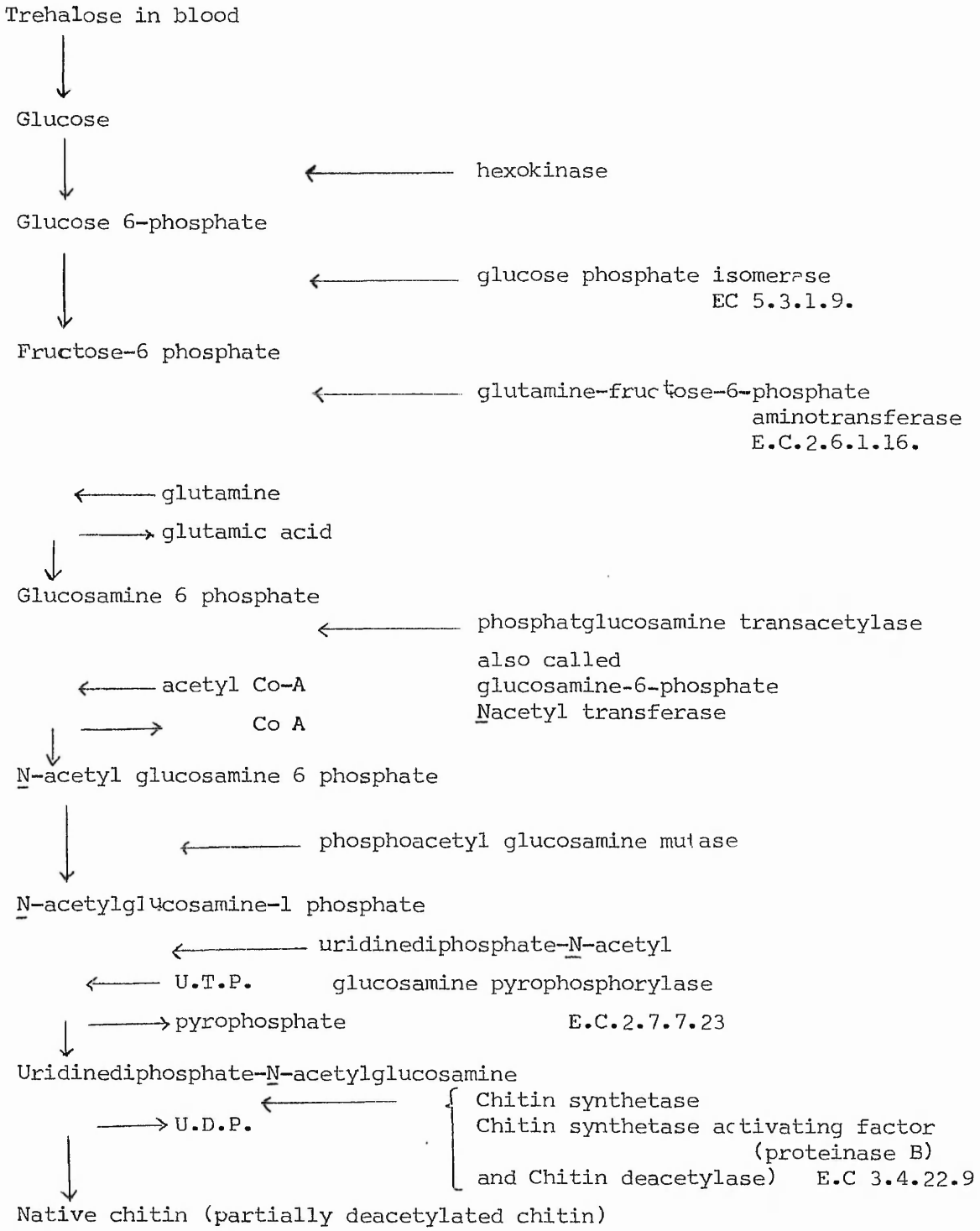
Chitinous cellwalls in plants are only found in those forms, such as fungi and moulds, which like animals have a high percentage of nitrogen in their foods. Photosynthetic plants, on the other hand, use nitrogen-free polysaccharides for supporting structures. Some lower green plants e.g. Chlorophycea however, do utilise chitin for the cell membrane.

In multicellular animals chitinous structures are mainly of ectodermal origin and form the exoskeleton of most of the invertebrates. Exceptionally chitin may constitute over 50% of the total organic matter in chitinous structures. Arthropoda, which are particularly able to synthesise chitin, have concentrations of up to 85%. No correlation between the proportion of chitin and the degree of calcification, hardness, or flexibility of the structures has been found.

The distribution of the three crystallographic forms of chitin does not appear to be related to taxonomy. Striking differences in morphology, chemical composition and physical characteristics are shown by the various chitinous structures. The biosynthesis of chitin is shown schematically in Figure 2 [19] where chitin synthetase (uridine diphosphate-2-acetamido-2-deoxy-D-glucose: chitin-4-β-acetamido deoxy glucosyl transferase, E.C.2.4.1.16) takes part together with other enzymes.

Figure 2

Pathway of Chitin Synthesis



1.1.2. Occurrence of chitin in lower animals

Chitin has been found in a wide range of animal species. The exoskeletons of crabs and lobsters have long attracted attention as a source of raw material for chitin production as the dry arthropod exoskeletons contain from 20-50% chitin [20]. Knowledge of the metabolism of chitin in animals is limited, but it is clear that it acts as a carbohydrate and nitrogen reserve and it is associated with proteins to form glycoproteins. The supporting or protecting function of chitin appears to be of minor importance.[20]

Chitin is found as the major organic skeletal substance of invertebrates in the phyla Annelida, (segmented worms), Arthropoda and Mollusca, and to a lesser extent in Coelenterata and Nematoda (unsegmented worms)[21].

Complilation of the reported occurrence and distribution of chitin amongst animal species has been covered comprehensively by Richards[23](1951), Rockstein[24] (1974), Hepburn [6] (1976) and Jeauniaux[25] (1977).

Richards[23] reports that chitin has been found in the body wall of every species of arthropod tested. Examples of all the major classes (Arachinida, Crustacea, Insecta, etc) and sub-classes have been tested Occurrence of the polysaccharide is not restricted to body coverings and it has been detected in gut linings, trachae, muscle attachments, internal skeletons and wing coverings.[23]

Using various specific methods, Jeauniaux [25] has detected chitin in many different species. In Protozoans mainly Ciliates , chitin is used to build up kyst walls, whilst in lower invertebrates, it makes the bulk of the stalks or stems of most Hydrozoan colonies. However, it is only exceptionally produced by Scyphozoa (Jelly Fish) and Anthozoa.

It is the major structural polysaccharide of most invertebrate classes[25] belonging to the Protostomia. It is not detected in either larvae and adults of free and parasitic Flatworms (Plathyhelminthes) Nermerteans, Sipunculids and Leeches. In other groups, e.g. Nematodes and Rotifers it is only present in the egg envelopes.

Besides Arthropods, other animal sources containing significant amounts of chitin are[25] the setae of Annelids (20-30%) of dry weight, the skeleton of the colonies of Bryozoa (Ectoprocta or "moss animals"), and the shells and other structures (jaws, radula, gastric shield) of many Mollusca species (up to 7% of dry organic matter in Gastropods and Bivalve shells and up to 26% in Cephalopods).[25]

Chitin synthesis has not been observed in Echinoderms and Vertebrates, but the tubes of some Pogonophora contain up to 33% of chitin, while Tunicates secrete a chitin-containing peritrophic membrane.[25]

Marine benthic animals may also be a rich source of chitin[25]. Despite their small size, Bryozoans and Hydrozoans are able to play a role in the auto-epuration of semi-polluted streams together with the production a high biomass of chitin-containing colonies.

A number of investigations on Antarctic Krill (Euphausia superba) as a source of chitin have been carried out[26-28]. The contents of chitin reported by different workers vary considerably depending on the basis of determination. Yanase[28] found 38.7% in the washed exoskeleton, whilst Anderson, de Pablo and Romo[26] found 3.2% based on the whole krill.

The subject of the arthropod cuticle structure has been covered in several reviews.[29-31] The typical arthropod cuticle consists of two major subdivisions,[32-33] a thin chitin-free, outer layer (or epicuticle) and a much thicker inner layer (procuticle) which always contains chitin. The exocuticle is frequently hardened for rigidity by sclerotization, which in non-calcified species (insecta) is effected by a tanning reaction involving the cross-linking of protein chains by certain polyphenolic derivatives.[34-37] The compacting of chitin crystallites may be an additional hardening factor.[38] The hardening in crustaceans occurs largely by deposition of calcium carbonate, but hardening by tanning can occur prior to the onset of calcification.[39] The ratio of chitin to protein in non-calcified species,[23 40] or in areas of non-calcification in otherwise calcified species, is about equal, but with calcification the proportion of protein added to the cuticle decreases considerably. Examples of the proportions of the organic components in arthropod cuticles are shown in Table 1[41].

Recent work has suggested[42] that common marine crabs (Neptunes sanguidantus and uca pugilater) at certain stages of the moult cycle, are a source of very pure chitin that is free from proteinacious impurities. Nitrogen estimations on material obtained from the fresh cuticle and purified with alkali, indicate that the "pillans" stage yields chitin that can be purified to the maximum extent.

The moulted cuticle contains more inorganic than organic constituents, calcium carbonate being the largest fraction, and this is eliminated by acid treatment; a 10% trichloroacetic acid solution is suitable for selective elimination of the inorganic part and for the determination

Table 1

Proportions of Organic Components in Arthropod Cuticles[41]

Source		Proportion (%organic fraction dry weight)	
		Chitin	Protein
Arachnida			
Buthus (scorpion)		31. 9	68. 1
Miygale (spider)		38. 2	61. 8
Insecta			
Locusta, elytra & wings		23. 7	76. 3
Periplaneta (cockroach)	av	35. O	--
Cocleoptera			
Dystiscus (water beetle)	adult elytra	37. 4	62. 6
Lepidoptera			
Bombyx (silkworm)	larvae	44. 2	55. 8
Crustacea (decapoda)			
Cancer (edible crab)		71. 4	13. 3
Eupagurus (hermit crab)	calcified	69. O	31. O
	non-calcified	48. 2	51. 8

of the organic part (protein and chitin) which is known as the "artropodin complex"[43,44].

Crab cuticle are abundantly available, many species being cultivated worldwide. The crabs are collected during the moulting period (April-September) and their cuticles dried and treated chemically[45]. Large amounts of chitin raw material are discarded daily by the crab and shrimp meat industries. The by-products of the South African industry have been considered [46] for the production of chitin and other substances, as have crustacea from the Carribean Sea.[47]
Sediments of chitin in seawater have been estimated to amount to several billion tons per year, due mostly to moulted copepod exoskeletons[48]. Copepods are the most abundant multicellular animals in the world and they produce eleven cast exoskeletons for every adult. Chitinous skeletons are characteristic of fossil forms of trilobites and graptolites.[49] Deposits of graptolites found in Dictyanema shales indicate the essential role of chitin in the formation of the organic mass of shales. Deposits encountered in old rocks (cenozoic) have been found to contain thin organic films with up to 15% chitin content. No large accumulation of undegraded fossil chitin has been detected.

1.1.3. The occurrence of chitin in lower plants.

Chitin is present in the vast majority of fungi [51-53] as the principal fibriller polymer of the cell and, as such, it is responsible for the rigidity and the shape of the cell wall. All fungi, with a few possible exceptions, have chitinous cell walls [54]. The only major classes of fungi [52] which lack chitin are the Schizomycetres Myxomycetes and Trichomycetes. As a group, Euascomycetes are the fungi which contain the highest amounts of chitin, followed by the Zygomycetes, Basidomycetes and the Deuteromycetes. The highest values reported [52] are in the Allomyces macrogynes (58%) and the Sclerotium rolfsii (61%)

Chitin is also found in green algae [55,56] (Chlorophyceae), but not in bacteria and Achnomycetes. Several authors have detected the presence of chitin in yeasts (saccharomycetes).

By use of microchemical methods, chitin has been found in a variety of filamentous and non-filamentous yeasts [57,58], but some findings were not supported by X-ray diffraction studies. Diffraction patterns characteristic of chitin have been obtained from numerous yeasts after purification involving both acid and alkali extractions. Long thin fibres on the inner surface of the yeast phase of Paracoccidiordes Brasiliensis have been shown to be chitin [59]. Crystalline chitin, with a crystal size of about 6 mm, has been reported [60] as being present in the wall complex of the yeast Saccharomyces cerevisiae. A high concentration of poly(hexoasamine), probably chitin, has been detected in isolated and partially degraded bud scars from Sacchromyces cervisiae. This has been further investigated [62] using specific

enzymes and found to contain about 15% chitin.

Chitin is associated with other compounds in the fungal cell wall.[63] Purified chitin is usually recovered in yields of 3-5% from fleshy fungi. The majority of alkali-resistant cell wall material, which may comprise 20-44% of the dry weight of the organism, is non-chitinous but this can be destroyed by permanganate oxidation. Chitin has been isolated in 35% yield from Agarious compestris ,[64] using purification by acidic hydrolysis, and identified by X-ray diffraction. The content of chitin in various fungi has been determined and the results are summarised in Table 2 [65].

Chitin has been isolated [66] from the myclial cell walls of fuscariae in about 18% yields. The accumulation was found to correlate with the rates of fungal growth. Chitin has also been found [67] as a mycobiont hyphal wall component. The mycobionts were isolated from the lichens Xanthoria parientina, Torabenia intricata and Sarcogyne. Chitin has also been suggested [68] as being present in the hyphal cell walls of Oomycetres, Saprolegniaceae and pythiaceae. The occurrence of chitin in cell walls of Neocallimastrix frontalis, Piromeras communis and Sphaeromonas communis has been shown[69] by enzymic analysis.

The identity of fungal chitin with animal chitin has been established using optical measurements[70], viscosity data[71], X-ray diffraction pattern comparisons [72,73], infra-red spectra [74] and enzymatic [75,76] and chemical degradations[77]. However, it has been shown[78] that the capacity for metal ion collection of the two types of chitin are not equivalent. Data published on the collection of uranium from solutions of uranyl sulphate

Table 2 Chitin content of Various Fungi [65]

Organism	Growth Temp. °C	4 days Growth Chitin %	4 days Growth Dry W t mg/100ml	4 days Growth pH	8 day Growth Chitin %	8 day Growth Dry W t mg/100ml	8 day Growth pH
Alternaria species	20	11.5	37	5.6	10.1	338	2.8
Aphanomyces laevis	25	7.0	182	2.9	7.7	620	2.0
Aspergillus flavus	25	13.6	623	2.2	13.2	981	2.2
" "	25	22.4	714	2.4	25.6	990	2.2
" niger	25	8.8	1056	2.0	14.6	830	2.3
" "	25	13.8	332	2.0	11.7	696	2.5
" "	20	8.9	645	2.3	21.2	418	2.3
" "	20	7.2	743	2.4	14.8	725	2.3
" "	20	9.6	700	2.3	22.4	448	2.2
Aspergillus oryzae	25	18.6	577	2.1	10.4	1038	2.0
" parasiticus	25	14.8	930	2.1	17.1	1009	2.1
" "	20	14.4	592	2.8	26.2	845	2.3
" "	25	15.7	795	2.3	24.9	594	2.1
Collybiaspecies	20			5.8	3.1	116	5.2
Dactylium dendroides	20	6.1	31	5.6	9.4	144	3.6
Geotrichium species	20	2.1	116	4.8	10.4	181	2.7
Glomerella cingulata	20	3.8	92	3.5	6.3	424	2.8
Helminthosporium satiuum	20	4.4	58	5.6	6.4	284	3.0
Mucor rhizopodiformis	20	6.7	58	4.9	7.9	598	2.6
Neurospora crassa	20	2.4	200	3.3	2.6	163	3.3
Neurospora tetrasperma	25	4.6	278	2.8	14.7	990	2.2
Penicillium notatum	20	16.1	655	2.4	24.9	716	2.2
Penicillium species	20	5.8	262	3.0	6.5	556	2.3
Rhizopus nigricans	25	8.0	25	5.5	12.2	28	5.6
Rhizopus species	20	5.3	23	5.6	3.1	506	2.5

by fungal and animal chitins showed that fungal chitin removed 65% of the uranium used whilst animal chitin only removed 20%.

1.2. The structure of Chitin and Chitosan

1.2.1. Molecular structure

The presence of nitrogen in chitin was first demonstrated by Children[79] in 1824, but it was not until 1876 that Ledderhose[80] isolated a sugar hydrochloride containing nitrogeon by hydrolysis of chitin with concentrated hydrochloric acid. The product was named glucosamine and he showed[81] that acetic acid was also formed during hydrolysis, but was not formed by treating glucosamine under the same conditions. Subsequently it was shown[82] that these products of hydrolysis are formed in equimolar proportions. Ledderhose concluded that on the basis of the elementary analysis of the chitin used, three acetyl groups were associated with two glucosamine residues in the molecule. The presence of glucosamine was confirmed by Gibson[83] in 1896.

Frankel and Kelly, in 1901, treated[84] chitin for 48 hours with cold concentrated sulphuric acid and isolated five fractions by precipitation with alcohol followed by ether. The last fraction was found to be the most solu ble and was shown to be N-acetylglucosamine, identical with a synthetic specimen. Glucosamine had been previously shown[85] to be configurationally related to either glucose or mannose since on reaction with phenyl hydrazine, D-glucose phenylosazone was formed and the amino group eliminated. The synthesis of glucosamine by Fischer and Leuchs[86] in 1903 confirmed the attachment of the amino group to the C(2) carbon atom but did not settle the configurational problem.

Irvine and co-workers showed [87-9] that, depending on the method of preparation, glucosamine could be derived from either glucose or mannose. Levene commented[90] that"much of the chemical structure of the sugar was formulated correctly rather by instinct than by experimental evidence" and consistently used the name chitosamine for glucosamine in order to avoid prejudging the structural issue. Unequivocal evidence for its structure as 2-amino-2-deoxy-D glucose was provided[91] finally by Haworth, Lake, and Peat in 1939. Peat had previously produced[92] a review of the majority of the previous work, showing that this structure was likely.

Bergmann, Zeras and Silberkweit isolated [93] di-N-acetyl-hexa-O acetylchitobiose (chitobiose octaocetate) from the products of the acetolysis of chitin and the same compound was isolated [94] by Zechmeister and Toth from the products of the partial acid hydrolysis of chitin. From the reactions of chitobiose octaocetate it was concluded that the mode of linkage was most likely to be 1:4, and from X-ray evidence and changes of rotation on hydrolysis a β form was indicated. Enzymic studies by Zechmeister and Toth[95] gave further support to the β linkage and as a result of various acidic and enzymic degradative studies on chitin, there is little doubt that the polysaccharide is a polymer composed of 2-acetamido-2-deoxy-D-glucose residues. Confirmation of the β-D nature of the glycosidic linkages is given by the specificity of such chitnases as 2-acetamido-2-deoxy-β-D-glucosidase . Yields of 60-70% of 2-amino-2-deoxy-D-glucose hydrochloride have been obtained [96] by hydrolyzing crab shells and a patent was granted in 1948 to Matsushima[97] for the production of glucosamine from crab shells.

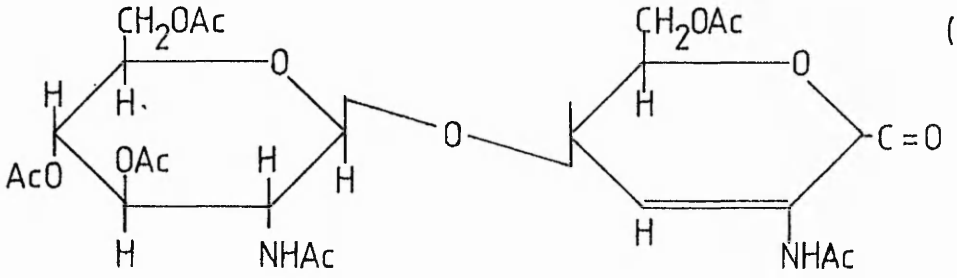
With the advent of chromatography the parent sugars and the higher homologs were isolated in crystalline form. In 1955, di-N-acetyl chitobiose was obtained[98] by carbon-Celite fractionation of a mixture

of five compounds obtained by alkaline de-O-acetylation of chitobiose octaacetate. The presence of numerous deacetylation by-products hindered attempts to prepare a series of chitin oligosaccharides by similar methods and limits the value of acetolysis as a technique for studying amino polysaccharides.

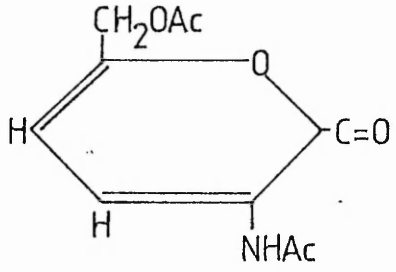
Controlled partial hydrolysis of chitin is difficult due to the need to use concentrated acids to bring about dissolution. Chitosan, however, is amenable to controlled hydrolysis. A chitosan hydrolyzate has been fractionated using ion exchange chromotography giving at least five saccharides, the first two of these having been characterised as 2-amino-2-deoxy-D-glucose hydrochloride and chitobiose hydrochloride.[99,100] Following selective N-acetylation, successful fractionation of chitosan hydrolyzates on carbon-Celite was achieved[101-102] to give the first seven members of a series of chitin oligosaccharides. The properties of these clearly indicate the polymer-homologous nature of the series.

The original proof of the chitin dissacharide structure was provided by studies [93] on its octaacetate which was shown to contain two N-acetyl and six O-acetyl groupings. Iodine oxidation in the presence of sufficient alkali to saponify the O-acetyl groups of the octaacetate gave a di-N-acetyl " aldobionic" acid which on hydrolysis gave 2-amino-2-deoxy-D-glucose and 2-amino-2-deoxy-D-gluconic acid. A free aldehyde group must, therefore, be present in the disaccharide which shows that the C(1) and the C-(2) of the reducing moiety cannot be involved in the glycosidic linkage. Treatment of the disaccharic acid with acetic anhydride-sodium acetate gave an unsaturated compound (1) with a double

bond between the C-(2) and C-(3). Using the same conditions, 2-amino-2-deoxy-D-gluconic acid gave an unsaturated product (2) with double bonds between C-(2) andC-(3) and C-(4) and C-(5). Assuming the presence of a pyranoid ring, it was therefore thought that C-(4) of the disaccharides reducing moiety was the point of the glycosidic linkage. Further studies involving periodate oxidation [103] and the Morgan-Elson[104-107] test have confirmed the presence of β-D-(1- 4) glycosidic linkages in the chitin oligosaccharides.



(1)



(2)

Rate studies of deamination with aqueous nitrous acid have shown that the rate for chitosan is similar to that for methyl 2-amino-2-deoxy-β-D-glucoside, and quite different to that for the α-isomer.[108]

Other physical evidence in support of this structure has been reported. Chitin and chitin oligosaccharides show absorptions in the infrared at 884-890cm^{-1} which are indicative of the β-D-glucopyranosidic linkage.[101,102]

X-ray diffraction studies have been carried out by many workers. The crystalline nature of chitin was shown by Herzog[109] in 1924 and confirmed by Gonell[110]. Meyer and Mark postulated[111] that the 2-acetamido-2-deoxy-D-glucose were linked in the same manner as the D-glucose residues in cellulose, each chain having a diagonal screw axis and β-D-(1-4) glycosidic linkages. These workers also proposed a rhombic unit cell containing eight 2-acetamido-2-deoxy-D-glucose residues. The basis of the commonly accepted model was given by detailed analysis of crustacean chitin by Meyer and Pankov[112] in 1935. They recorded a cell which had dimensions a=0.49; b= 1.046 and c=1.925nm. This was confirmed in subsequent investigations and further refined in more recent work (Chapter 1.2.2.)

1.2.2. Morphology

Chitin is a crystalline polysaccharide and has a highly ordered structure, as shown by X-ray diffraction studies. It occurs in three polymorphic forms, namely α-, β- and γ-chitins, which differ in the arrangement of the molecular chains within the crystal cell. X-ray spectra showing these differences in molluscan samples has been presented by Rudall[31] and in arthropodan samples by Rudall and Kenchington [113]. The tightly compacted α-chitin is the most crystalline polymorphic form with the chains arranged in an antiparallel fashion. The chains are parallel in β-chitin whilst in γ-chitin two chains are "up" to every one "down".[37] The most abundant polymorphic form is α-chitin which is found in arthropod cuticles and in certain fungi. β-chitin exists[114,115] as a crystalline hydrate which accounts for its lower stability since water can penetrate between the chains of the lattice. α-chitin has been found in the cocoons of beetles, Ptinustectus and Phynchaenus fagi. Treatment of γ-chitin[113] with lithium thiocyanate can transform it to α-chitin.

All three forms have been found in the same organism indicating that they are relevant to different functions and not to the animal grouping. In the squid, Loligo, α-chitin has been found in the beak, β-chitin in the pen and γ-chitin in the stomach linings. The existence of the three forms has been thought[21] to occur because of the diversity of its functions. In plants it serves as an alternative to cellulose, in animals as an alternative to collagen. α-chitin is usually found, frequently sclerotized and encrusted with mineral deposits, where extreme hardness is required. β- and γ-chitins seem to be associated

with collagen-type proteins providing toughness, flexibility and mobility, and may have physiological functions other than that of support.

No interconversion between the three forms occurs on boiling in 5% potassium or sodium hydroxide solutions[116] but treatment with 6N hydrochloric acid leads to conversion of the β- and γ- forms to the α-form. Similar conversions have been observed by other workers[21,117].

The structures and possible roles of dislocation and dislocation-like defects that may occur in chitin have been discussed. One such role may be in the interconversion of α-,β- and γ-chitins.[118]

1.2.2.1. The conformation of α-chitin

Many workers have carried out detailed investigations into the conformation of α-chitin. Calstrom proposed[119] the buckled chain structure arranged in an orthorhombic cell with dimensions a=0.476; b= 1.032 and c=1.885 nm, with the space group $P2_12_12_1$. By application of the least square rigid body refinement technique Ramakrishan and Prasad have refined[120,121] the structure of α-chitin.

The unit cell contains[122] disaccharide sections of two chains. Successive residues along the chain form intramolecular O_3-H---- O_5' hydrogen bonds, asin cellulose, and the chains are linked by C=O... H-N bonds parallel to the a axis, so that the structure is an array of hydrogen bonded sheets of chains. The $-CH_2OH$ side chains have different orientations on the two chains; on one chain the $-CH_2OH$ forms a second intramolecular hydrogen bond, $O_6H----O_7'$, to the carbonyl oxygen of the next residue; the $-CH_2OH$ group of the second chain forms an intersheet hydrogen bond $O_6H-----O_6'$. The data indicates that there is a statistical mixture of these two types of bonding, equivalent to half oxygens at the two positions for each residue. The intersheet hydrogen bonding accounts for the inability of α-chitin to swell in water. In addition, the structure contains two types of amide group which have different hydrogen bonding and this accounts for the splitting of the amide I bond in the infrared spectrum.

1.2.2.2. Conformation of β-chitin

Several detailed investigations have been carried out into the structure of β-chitin. The unit cell[122] is monoclinic with dimensions a=0.485 b=1.038 and c=0.926nm β=97.5° and the space group is P2. The unit cell contains two sugar residues related to the twofold screwaxis. The sugar residues are arranged with β (1→4)- glycosidic linkages on 2_1 screw axis. The chain conformation is the same as in α-chitin and the chains form sheets linked by C=O----H-N hydrogen bonds. The $-CH_2OH$ forms intra-sheet hydrogen bonds to the carbonyl of the next chain along the a axis. The absence of hydrogen bonding between the sheets explains the ease with which β-chitin can be swollen in water to produce hydrates. There are numerous points of analogy[122] between the structures of β-chitin and cellulose I. Both structures contain extended parallel chains and can be visualised as an array of hydrogen bonded sheets.

No detail on the conformation of γ-chitin has been found, but some structural considerations have been made[31] based on X-ray data.

1.3. Isolation of Chitin

In the wide variety of animal and plant species in which chitin has been shown to exist, it is usually found in close association with other materials. The isolation of chitin, therefore, requires drastic methods which can cause degradation and it is doubtful whether a pure, undegraded product is normally obtained.

In a critical evaluation of potential sources of chitin as commercial possibilities Allan, Fox and Kong concluded[123] that of all the sources, crustaceans formed the first choice with fungi as the only near alternative. In order to remove calcium carbonate and protein from the raw chitinous material of crustaceans, harsh chemical treatments have been developed over the years. Most methods are based on an acid treatment for decalicification and then an alkali treatment for deproteination.[99,124,125] Milder methods have also been developed using ethylene diamine tetracetic acid for the decalcifying stage[126] and using various proteolytic enzymes at the deproteination step.[127-129] The most important methods are given in detail in the Appendix II.

A variety of other methods have been developed. Rigby treated[130] chitin-containing material with 1% sodium carbonate for 6 hours at the boil. This was followed by steeping in 5% hydrochloric acid at room temperature. On further treatment with 1% sodium carbonate solutions, a "pure" white chitin was obtained. Blumberg, Southall, Van Rensburg, and Volchman carried out a study of chitin production[131] from processing wastes. A high quality chitin product containing 92-96% chitin on a dry basis and less than 2% ash was obtained by treating shells, and in particular the tail shell of rock lobster (fasus lalandii), with hot 5% sodium hydroxide solution, cold sodium hypochlorite solution, and warm 5% hydrochloric acid.

Using crayfish waste, Lovell, Lafleu, and Hoskins prepared chitin[132] by agitating fat-free crayfish meal in 90% formic acid at room temperature for 24 hours. After centrifuging, the acid and solubilized calcium were decanted, the residue washed, recentrifuged with acetone, and refluxed for $1\frac{1}{2}$ hours with 5% sodium hydroxide. The product was then washed and dried. Variations were tried using hot and cold hydrochloric acid instead of formic acid. Kishu obtained[133] chitin from silkworm pupae by powdering and then moistening the dried pupae. Hydrogen peroxide was then added until the chitin and the oily substances floated on the surface and then these were skimmed off. By pressing fresh krill, Euphausia superba, Lagenor, Kryuchova, and Nikolaeva[134] obtained a liquid fraction containing proteins and a solid part containing mostly chitin. Production of chitin on a pilot plant scale was discussed by Peniston, Johnson, Turrill and Hayes[135-137] and its economic viability considered.

Preparation of radioactive chitin has been achieved[138] by injecting ^{14}C glucosamine dissolved in the insect Ringers solution through the muscular footpad of Manduca sexta larvae 10 hours prior to ecdysis from 4th-5th instar. The radioactive cuticles were washed, milled and treated with 1M sodium hydroxide at 80°C to solubilize the protein. Radioactive chitin has also been prepared by treating chitosan with tritiated acetic anhydride[139].

1.4. Preparation of Chitosan

Amide linkages are more difficult to cleave under basic conditions than are ester linkages. Under vigorous basic conditions acetamido groups adjacent to *cis* related hydroxyl groups may undergo *N*-deacetylation, but *trans* related analogues are much more resistant.[140] The amino group in amino polysaccharides is generally *N*-acetylated. Heparin is exceptional in being *N*-sulphated and it may be readily *N*-desulphated by treatment with dilute methanolic hydrochloric acid.[141] However *N*-acetyl groups cannot be removed under acid conditions without hydrolysis of the polysaccharide and so alkaline methods must be employed for *N*-deacetylation.

Anhydrous hydrazine at 100°C can be used to cleave amide linkages in proteins and its use has been suggested as a method for *N*-deacetylation of chondroitin sulphate preparations. It has also been employed for the *N*-deacetylation of blood group A substance from hog gastric mucus. Although this reagent causes little degradation of simple glycans it gives extensively degraded products when applied to amino polysaccharides and *N*-deacetylation is usually incomplete.

Chitin possesses a C(2)-C(3) *trans* arrangement of substituents in its monosaccharide unit and it is remarkably stable to most reagents, including aqueous alkali. Winterstein[142] and Hoppe-Seyler[143] showed that fusion of chitin with potassium hydroxide at 180°C gave chitosan. Von Furth & Rosso in detailed investigations concluded [144] that about three quarters of the acetyl groups of chitin could be readily removed by alkaline treatments.

Lowry considered[145] that material similarly prepared contained one acetyl group per disaccharide unit. Rigby gave an alternative preparation of chitosan[130] involving treatment of chitin with 40% aqueous sodium hydroxide at 110° for 4 hours. Evidence of the chitosan identity was put forward by Clark & Smith who registered X-ray diffraction data[146]. Extended treatment with hot concentrated sodium hydroxide solution gave an almost completely N-deacetylated, but degraded, product. Infra-red spectroscopy has been used to follow the removal of the N-acetyl groups by monitoring the disappearance of the amide carbonyl bond.[147]

Despite early reports that chitin was stable in acidic solutions[71,148] because it could be recovered from solutions in concentrated strong acids, viscosity measurements of chitin in 45% nitric acid and 30% hydrochloric acid showed that extensive degradation occurs even at 0°C. Thus it has been shown by several workers[149,150,151] that any acidic treatment carried out on chitin or chitinous raw material leads to partial or extensive depolymensation, and that the viscosity of the final product produced from chitin will depend on the demineralization scheme followed.

Madhavan and Ramachandran[149] treated prawn waste from the Matapenacus dobsoni species of prawn with boiling 0.5% aqueous sodium hydroxide for 30 minutes in the ratio of 2:3. The alkali was drained off and kept separately for recovery of the protein. The residual protein was removed by heating the residue to boiling with an equal weight of 3% sodium hydroxide solution, the alkali drained off, and the process repeated again. The residue was then immersed in cold sodium hypochlorite solution, containing 0.3-0.5% available chlorine, for 30 minutes in order to bleach most of the pigments contained in

prawn waste. The liquor was drained off and the residue washed and demineralized in 1.25 N hydrochloric acid at room temperature for one hour. Deacetylation was then carried out by dipping in a 1:1(w/w) sodium hydroxide solution for 2 hours at 100°C. The alkali can be used for the deacetylation of a number of subsequent batches. The product was then washed, dried and pulverised to the required size.

The demineralization step of the protein-free mass is particularly important[149] as it greatly affects the characteristics of the chitosan produced particularly the solution viscosity. With progressive increases in the concentration of hydrochloric acid, the degree of demineralization is increased, but use of acid of concentrations above 1.25N adversely affects the solution viscosity of the final product. The relationships between the concentration of acid used, time of treatment, content of acid soluble ash in chitin and solution viscosity of the final product are given in Table 3[149].

Moorjani, Achuta and Khasim have described[150] a process for the preparation of chitosan giving highly viscous solutions using prawn waste from either Panaeus indicus or Metapenaeus dobsani. The process involves demineralization with 5% hydrochloric acid at room temperature followed by deproteinization with 5% sodium hydroxide at 100°C for 30 minutes. Deacetylation was carried out using 60%(w/v) potassium hydroxide solution, 1:65 parts, at 100°C for one hour. A study of the many factors affecting the solution viscosity of the chitosan was made including choice of alkali, concentration, volume used, effects of bleaching, concentration of hydrochloric acid and time of demineralization. The results are summarised in Tables 4-7 [150].

Table 3: Effect of Degree of Demineralization On Viscosity of Chitosan[149]

Concentration of acid used	Time of treatment in minutes	Acid Soluble ash in chitin%	Viscosity of 1% chitosan in 1% acetic acid (Centipoises)
0.75N	30	48.44	14.63
	60	46.30	16.84
	120	41.52	18.86
	180	39.44	18.45
	∞	37.50	18.02
1.0N	30	43.69	32.03
	60	38.28	36.56
	120	33.86	38.19
	180	23.95	39.42
	∞	20.32	40.03
1.25 N	30	24.34	106.85
	60	18.82	97.07
	120	6.33	58.05
	180	2.97	46.44
	∞	1.31	40.89
1.5N	30	15.34	49.28
	60	7.90	43.95
	120	3.14	40.06
	180	1.46	38.84
	∞	1.31	34.58
2.0N	30	2.71	37.66
	60	1.76	31.52
	120	1.03	26.94
	180	0.65	17.79
	∞	0.64	17.20

Table 4

Effect of Time and Concentration of HCl used for Demineralization [150]

Demineralization time (min)	Ash in chitin (%)	Rel. viscosity	Abs.viscosity* (Poises)
HCl concentration 1.57N(5% HA)			
60	1.92	151.5	1.356
120	1.397	148.3	1.326
180	0.3147	140.5	1.257
HCl concn. 1.25N			
60	5.667	139.5	1.248

*Chitin bleached after demineralization, deacetylated to 60% w/w NaOH
1% Chitosan in 2% w/w acetic acid

Table 5

Effect of Sequence of Demineralization and Deproteinization [150]

Sequence of process[(a)]	Rel Viscosity	Abs. viscosity* (poises)
1. Demineralization Bleaching Deproteinization	151.5	1.356
2. Deproteinization Demineralization Bleaching	114.5	1.024

(a) Demineralization - 5% HA for 1hr deacetylated with 60% (w/w) NaOH

* 1% Chitosan in 2% (w/v) acetic acid.

Table 6

Effect on Viscosity of Chitosan when bleaching is carried out at different stages [150]

Bleaching stage	Rel. viscosity	Abs Viscosity* (poises)
Unbleached	226.1	2.023
After demineralization	151.5	1.356
After deproteinization	98.22	0.879
After deacetylation	1.354	0.0121

Process - demineralization (5% HA for 1hr) deproteinization and deacetylation (with 60% (w/w) Na OH)

* 1% Chitosan in 2% (w/v) acetic acid

Table 7

Effect of deacetylating chitin with potassium hydroxide and sodium hydroxide [150]

Chitin:alkali	Alkali concn. (w/w)	Rel. Viscosity	Abs Viscosity* (poises)
1:60	60% KOH	Not fully dissolved in acetic acid	
1:65	"	344.8	3.086
1:70	"	309.4	2.769
1:100	"	381.5	3.414
1:60	60% NaOH	226.1	2.023

No bleaching at any stage

* 1% Chitosan in 2% (w/v) acetic acid

In a study by Lusena and Rose [151] on the preparation and viscosity of chitosan, demineralization of the crude chitin was carried out using hydrochloric acid at a pH not lower than 3. Deacetylation with 55 % (w/v) potassium hydroxide solution for 30 minutes at 100°C (1 part chitin: 100 alkali) gave rise to at least 65% deacetylation. Decreasing the alkali concentration increased the time required to obtain soluble chitosans and the product gave less viscous solutions. Increasing the alkali concentration to saturation had little effect on deacetylation or viscosity. The size of the chitin particle, within the range 20-80 mesh, had no effect on the extent of deacetylation and on the viscosity. However the size of the chitosan molecules was most affected by the demineralization treatment of lobster shells to obtain the crude chitin.[151]. Preparations involving demineralization at 5°C and pH greater than 3 yielded chitosans having a limiting viscosity number of about 20 whilst those demineralized at room temperature and then left overnight in 5% hydrochloric acid gave chitosans with a limiting viscosity number of about 4. Deacetylation in an atmosphere of nitrogen yielded chitosans of higher viscosity than deacetylation in air. Increasing the temperature or time increased the percentage deacetylation and reduced the molecular size, as indicated by viscosity measurements. Deacetylation in two half-hour stages, separated by washing and air drying, was as effective as a single continous 15 hour deacetylation and the product gave more viscous solutions.[151] The percentage deacetylation was found to rarely exceed 80%, although higher figures have been obtained using an alkali fusion procedure.

Nud'ga, Plisko and Danilov prepared chitosan[153] by treating crab chitin with 49% aqueous sodium hydroxide (1:10) at 140°C for various time

periods and in an atmosphere of hitrogen, argon or air. The results obtained are summarised in Table 8. It shows that at a given alkali concentration and temperature, the time of treatment does not affect the degree of deacetylation substantially, but it does affect the viscosity. Increasing the length of alkaline treatment reduces the limiting viscosity number of chitosan by a factor of about 10 both in air and nitrogen. Higher viscosity chitosan was prepared using nitrogen rather than air.

Chitosan has been obtained[154] with a viscosity of 1650 cps (as a 0.5% solution in 0.1% hydrochloric acid at 20°) by deacetylation of crab shell chitin for 15 hours at 50°C with 30% aqueous sodium hydroxide.

In a series of studies on chitin, Sannan, Kurita and Iwakura prepared[155,156] various alkaline treated chitins, with varying degrees of deacetylation, by suspending chitin in aqueous sodium hydroxide (66%w/v) for different time periods, at 25°C, under reduced pressure. The degree of deacetylation increased rapidly to about 75% as the alkaline treatment time increased and began to level off to about 90%. Samples with around 50% of deacetylation were found to be soluble in water.

Many authors have reported methods for preparing chitosan from chitin, and the important ones are given in detail in the Appendix III.[99,129,130,152,157-160] Some involve fusion with potassium hydroxide pellets at high temperatures[99,152] whilst others use solutions of alkali at varying temperatures.[129,130,157,158,159]

Table 8

Preparation of Chitosan by Treatment of Chitin with 49% Sodium Hydroxide solution[153]

Sample	Atmosphere	Time of Heating up to 140°h	Time of Treatment at 140°h	% N Content	Degree of Deacetyln.		Limiting Viscosity Number in 2% acetic acid
				Total	Amine		
1	air	1.0	1.0	6.85	6.83	0.79	8.8.
2	air	1.0	2.0	--	6.79	0.78	6.8
3	air	1.0	6.0	7.66	7.35	0.84	0.9
4	N_2	--	1.0	6.55	6.38	0.73	31.5
5	N_2	0.5	2.0	7.34	7.13	0.82	13.8
6	N_2	1.0	2.0	7.55	7.23	0.83	13.4
7	N_2	1.5	2.0	6.86	6.82	0.78	7.4
8	N_2	2.0	2.0	6.64	7.58	0.87	6.8
9	N_2	1.0	6.0	7.88	6.40	0.74	3.7
10	Ar	--	1.0	8.21	6.37	0.73	12.8
11	Ar	--	1.0	8.14	7.61	0.87	18.9

A small pilot plant has been designed for the production of chitosan[160] which produces it directly from shellfish wastes and permits recovery of proteins, sodium acetate, and lime or calcium carbonate as by-products.

1.5 Solubility of Chitin and Chitosan

Chitin is insoluble in water, dilute acids, cold alkalis of any concentration, and organic solvents. Common solvents are the strong mineral acids and concentrated salt solutions which may degrade or denature chitin. The polymer can be dissolved in concentrated hydrochloric and sulphuric acids, 78-97% phosphoric acid,[161] or anhydrous formic acid[162]. Degradation accompanies the dissolution in mineral acids, although it is not clear whether this occurs with formic acid. It has been reported[163] that chitin will dissolve with difficulty in liquid ammonia but Schweizer's reagent (cuprammonium hydroxide) will not dissolve it[71] because of substitution at C(2) prevents the formation of the required complex.

Colloidal solutions of chitin can be obtained [164] by dispersion in hot concentrated aqueous solutions of certain neutral salts, for example lithium thiocyanate. It has been claimed [146] that regenerated chitin, reprecipitated from aqueous lithium thiocyanate solution after several months, showed no sign of hydrolysis having occurred.

In an attempt to find a better solvent than strong mineral acids, Austin used [165-7] several acid systems modified with organic solvents, such as chloroethanol and sulphuric acid and mixtures containing trichloroacetic acid. The chloroalcohols used in conjunction with aqueous solutions of mineral acids, or with certain organic acids, were found effective for dissolving chitin in any form. They formed relatively low viscosity solutions of chitin, but did dissolve it rapidly at room or mildly

elevated temperatures, and hydrolytic degradation proceeded relatively slowly in them. However, the choice of the system used was a compromise of such aspects as rate of solution, rate of degradation, viscosity of the acid and viscosity of the resulting chitin solution. All the acids have a reduced degree of ionization in the chloroalcohol which leads to a greater stability of the chitin in such solutions.

Further investigations by Rutherford and Austin[168] of over 200 solvent mixtures showed that two tertiary amide systems; N,N-dimethyl-acetamide (DMAc)-lithium chloride and N-methyl-2-pyrrolidone (NMP)-lithium chloride were the best inert solvents for chitin. The solvent power is derived from the addition of lithium chloride, as the two liquids alone are only swelling agents for chitin. The lithium chloride reduces or breaks the inter-chain forces, such as hydrogen bonding, by association with the polymer and solvent.

Capozza reported[169] that hexafluoroisopropanol and hexafluoroacetone sesquihydrate could be used as solvents for chitin and prepared films of chitin from 2% and 1.4% solutions, respectively.

A mixture of dimethylformamide and dinitrogen tetroxide has been examined as a solvent system for chitin and chitosan.[170] The solubilities in a solvent to polymer ratio of 3:1, were found to be 5% for chitin and 100% for chitosan. The absence of chemical modification was shown by infra-red analysis.

The choice of a representative chitin for property determinations in inert solvent systems presents a problem as chitin is not a chemical entity, but a product defined by its source and method of isolation.

Observations on the solubility of different chitins are shown in Table 9 [171].

For example, in 88% formic acid β-chitin swells and then dissolves but α-chitin is only bleached. Polar aprotic solvents such as dimethylsulphoxide are only able to swell β-chitin slightly and are totally ineffective on α-chitin

Table 9.

Solubility of chitin from crab shells and Loligo pen and beak

Origin	Solvent	Observation
Crab shells	hexafluoroisopropanol	dissolves
"	hexafluoroacetone-	dissolves
"	1-2 chloroalcohols	completely dissolves
Loligo pen β	88% formic acid	swells + dissolves
"	98-100% formic acid	completely dissolves does not redissolve when precipitated by water
"	anhydrous trifluoro-acetic acid	No
"	98% formic + 2% acetic anhydride	No
"	dimethylformamide	No
"	dimethyl sulphoxide	V. slight swelling
"	picric acid + dimethyl-formamide	V. slight swelling
"	8 M urea	No
"	8M guanidine HCl	No
"	0.1 LiCNS , boiling	No
"	2-aminoethanol	No
"	cadoxen	No
Loligo beak α	88% formic acid	Bleaches
Crab shells α	88% formic acid	Bleaches, sample rubbery
"	98-100% formic acid	Bleaches, sample separates into layers
Both	polyethylenimine	"coloured" "dispersions"
"	cyclohexanone oxime	coloured "dispersions"

Chitosan is insoluble[172] in water, except when specially prepared as described earlier,[155,156] and in alkaline and organic solvents, but it is soluble in many dilute aqueous organic acids at concentrations in the range of 0.25 - 10% (at pH levels below 6). These acids include formic, propionic, oxalic, malonic, succinic, adipic, lactic, pyruvic, malic, tartaric and citric. Chitosan is also soluble in dilute (1% concentration or less) nitric and hydrochloric acids, marginally soluble in 0.5% phosphoric acid and insoluble in sulphuric acid at any concentration at room temperature. Water saturated with carbon dioxide is not sufficiently acidic to dissolve, or even swell the polymer.[172]

As chitosan is not soluble in any common organic solvent, solvent compatability studies have been carried out[172]. It dissolves readily in a 3:1 glycerol: water system, when the mixture contains 1% acetic acid, to give a clear, colourless, highly viscous solution. A less viscous solution is formed when ethylene glycol is used. A blend of 29% water, 1% acetic acid and 70% sorbitol with 1% chitosan gave a gel when agitated. A summary of the results is given in Table 10.[172]

Organic solvents appear to exert very little effect on chitosan solution viscosity except in the case of the polyols. The composition containing glycerol can be described as a jelly while that containing sorbitol is a semi-rigid gel. Glycerol acidified with acetic acid (99% glycerine 1% glacial acetic acid by volume) functions as a near solvent without any water.[172]

Salt tolerance of 1% acetic acid solutions of 1% chitosan were also carried out[172]. Salt solutions were adjusted to pH4 (pH of 1% polymer in 1% acetic acid) with acetic acid at a final salt concentration of 10%.

Table 10

Chitosan Solution Tolerance for Common Water Mixible Solvents[172]

Solvent	Max % Solvent for complete Compatability	% Acetic Acid (by Vol.)	Solution Properties Viscosity cps	pH
Methanol	50	5	2480	4.1
Ethanol	50	5	2400	4.1
iso-Propanol	40	3	3440	4.15
Acetone	40	3	2020	4.2
Ethylene Glycol	75	5	7600	4.0
Glycerol	80	3	60,000	4.1
Sorbitol	70	1	146,000	4.1
Water (for comparison)	-	1	2780	4.1

A 10 ml. portion of each of the salt solutions was then mixed with 1 ml. of a standard 1% chitosan solution. Since chitosan behaves as a cationic polymer in acid solution, sodium salts were used in compatability studies to explore the effect of various anions. A few nitrates of certain metals recognised as complexing agents were also included to examine the effect of these multivalent cations. Of all the salts tried, only sodium sulphate precipitated chitosan. All of the following were compatible at the 10% concentration level.

Sodium - Acetate; Bromide; Chloride; Citrate; Formate; Nitrate; Nitrite and Phosphate dibasic; Aluminium; Calcium; Chromium (III) ; Copper(II) ; and Iron (III) nitrates.

Organic solvent systems suitable for chitosan have been characterised into four groups based on viscosity versus concentration data[173] The first group contains 2M aqueous solutions of acetic, citric, formic, glycolic, lactic, maleic, malic, malonic, pyruvic and tartaric acids and these all produce non-Newtonian solutions with no clearly defined solubility limit.

The second group, which includes 2M dichloroacetic acid and 10% oxalic acid, give solutions which are very non-Newtonian. Three acids are contained in the third category; 0.041M benzoic, 0.036M salicyclic, and 0.052M sulphanilic acids. Solutions of these show an initial increase in viscosity and therefore some solubility. Solvent-chitosan systems for which the viscosity is independent of concentration of chitosan are included in the last category. These systems are colourless and there is no indication of any solubility. These include aqueous solutions (generally 2M) of DMF, dimethylsulphoxide,

ethylamine, glycine, methylamine, nitrilotriacetic acid, iso-propylamine, pyridine, salicylic acid, trichloroacetic acid, urea and 2M solution of benzoic acid in ethanol.

1.6 Molecular Weights of Chitin and Chitosan

Molecular weight determinations of chitin by light scattering have been reported by Hackman and Goldberg[174] Using 5.55 M lithium thiocyanate as solvent, a sample of chitin isolated from the shell of the crab Scylla serrata was found to have $\overline{M_w}$=1.036 x10^6 Determinations on carboxymethyl chitin and glycol chitin in 0.5M sodium chloride had $\overline{M_w}$ = 1.896 x 10^6 and $\overline{M_w}$=1.819 x 10^6 respectively. These correspond to an average degree of polymerisation of above 5,200.

Lee[171] assuming that the constants of the Mark-Houwink equation obtained for chitosan in 0.2M acetic acid - 0. 1M sodium chloride - 4M urea are not significantly different from the constants for chitin in anhydrous formic acid, obtained a value of 2.5 x 10^6 for the molecular weight of chitin and a degree of polymerisation of 1.38 x 10^4. For various preparations of chitosan obtained from β- chitin by deacetylation in 45% sodium hydroxide under nitrogen for 40(A), 60(B) and 80(C) minutes at 140°C, Lee recorded[171] values for the average molecular weights as chitosan (A) 7.25 x 10^5, chitosan (B) 4.92 x 10^5 and chitosan (C) 2.35 x 10^5 with degrees of polymerisation 4.1 x 10^3, 2.89 x 10^3, and 1.49 x 10^3 respectively. Values of 0.93 x 10^5 for the molecular weight and 0.58 x 10^3 for the degree of polymerisation were recorded for commercial chitosan. It is clear that extended degradation occurs during preparation of samples and derivatives unless precautions are taken.

Muzzarelli, Ferrero and Pizzoli[175] determined the molecular weight of

chitosan by light scattering in 8.5% formic acid and 0.5M sodium formate. The average molecular weight was 1.2×10^5. The same value has been reported by Nagasawa, Tohura, Inoue and Tanoora,[176] who also obtained a value of 3.1×10^4 for the weight average molecular weight of chitosan sulphate.

The use of high-pressure liquid chromatography in the size exclusion mode has been reported [177,178] to be a useful method for estimating the molecular weight distribution of chitosan products. Chitosan dissolved in 2% acetic acid containing 0.2M sodium acetate was fractionated by passage through a sequential combination of columns packed with coated glassbeads having a range of controlled pore sizes from 2500A°--40A°. A survey of commercially manufactured samples showed a molecular weight distribution of $0.1\text{-}4 \times 10^6$ for the weight average, $0.05\text{-}0.8 \times 10^6$ for the number average, and approximately 2-10 for the dispersity or ratio of weight and number average molecular weights.

1.6.1. Effect of shearing on the molecular weight

Two features of the degradation process stand out in shearing chitosan.[171] The molecular weight fall-off is steepest with the initial passes and molecular weight rapidly approaches a limiting size with repeated passes. Shearing the same chitosan in a solution containing 0.2M acetic acid + 0.1N sulphuric acid gave a more rapid fall-off of the initial molecular weight and the limiting degree of polymerisation was lower, suggesting that solvolysis may contribute to the lowering of the activation energy of bond scisson.

The essential features[171] of the shearing of chitosan are that there always appears to be a limiting size attained by shearing, and that this limiting size is lower and attained more rapidly by solvolysis.

1.7 Viscosity of chitin and chitosan

Variations in the procedure for deacetylating chitin cause wide variations in the nature of the final product, chitosan. This is particularly reflected in viscosity data from chitosan. Figure 3 shows viscosity as a function of deacetylation time in 50% sodium hydroxide at 118°C.[179] This illustrates the dramatic change in viscosity and emphasises the problems involved in the production of high molecular weight chitosan.

At present there is a wide variation in the recording of viscosity measurements of chitosan solutions and because of this non-standardisation no direct comparison of data can be made.

Chitosan in solution exhibits the polyelectrolyte effect; in the absence of salt there is an abnormal increase in viscosity of the more dilute solutions because of an enlarged effective volume due to charge repulsion and the osmotic effect. The viscosity behaviour is normal when sufficient salt is added to neutralise these effects.

In a study of the viscosity of chitosans[172] deacetylated for varying lengths of time the effects of various parameters were observed. Figure 4 shows the effect of polymer concentration on solution viscosity. The slopes of the curves for the different viscosity types are similar to those of comparable cellulose ethers. The same chitosan solutions were then used in viscosity-temperature studies.[172] Viscosity was determined at several concentration levels for each chitosan over the temperature range 25-60°C, Figure 5. All solutions appear to undergo

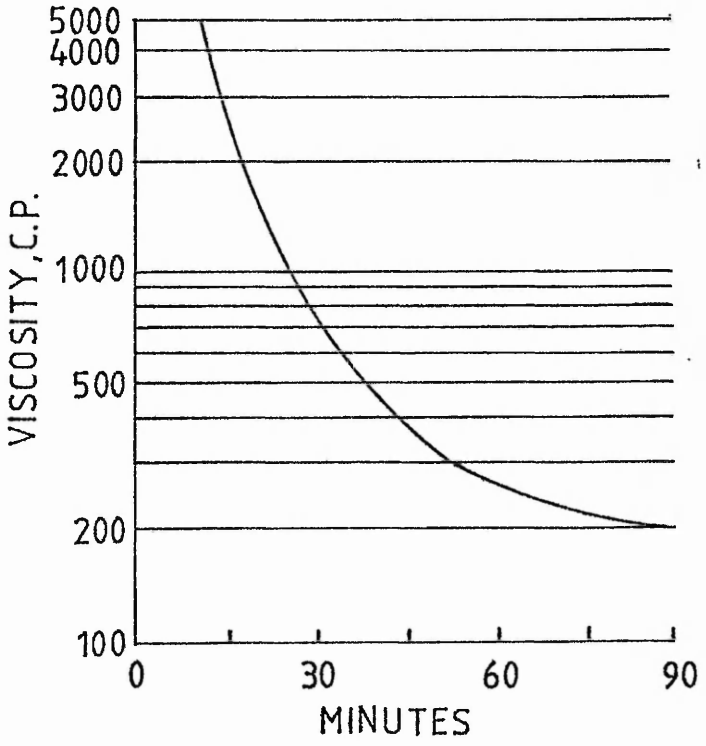


Figure 3. Viscosity as a function of deacetylation time in 50% NaOH solution at 118°C [179]

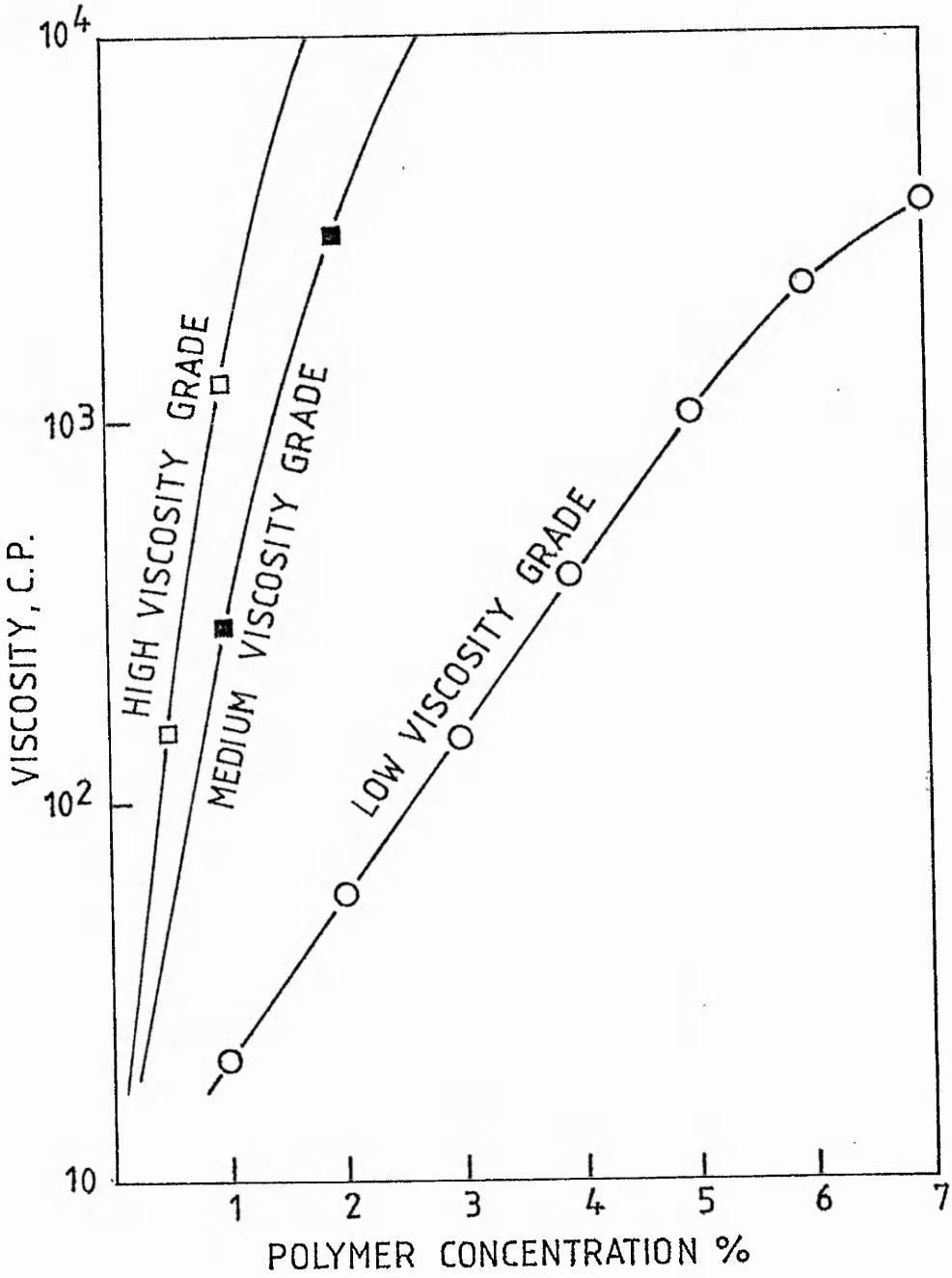


Figure 4. Effect of concentration on viscosity of solutions of chitosan at pH4 and 25°C.[172]

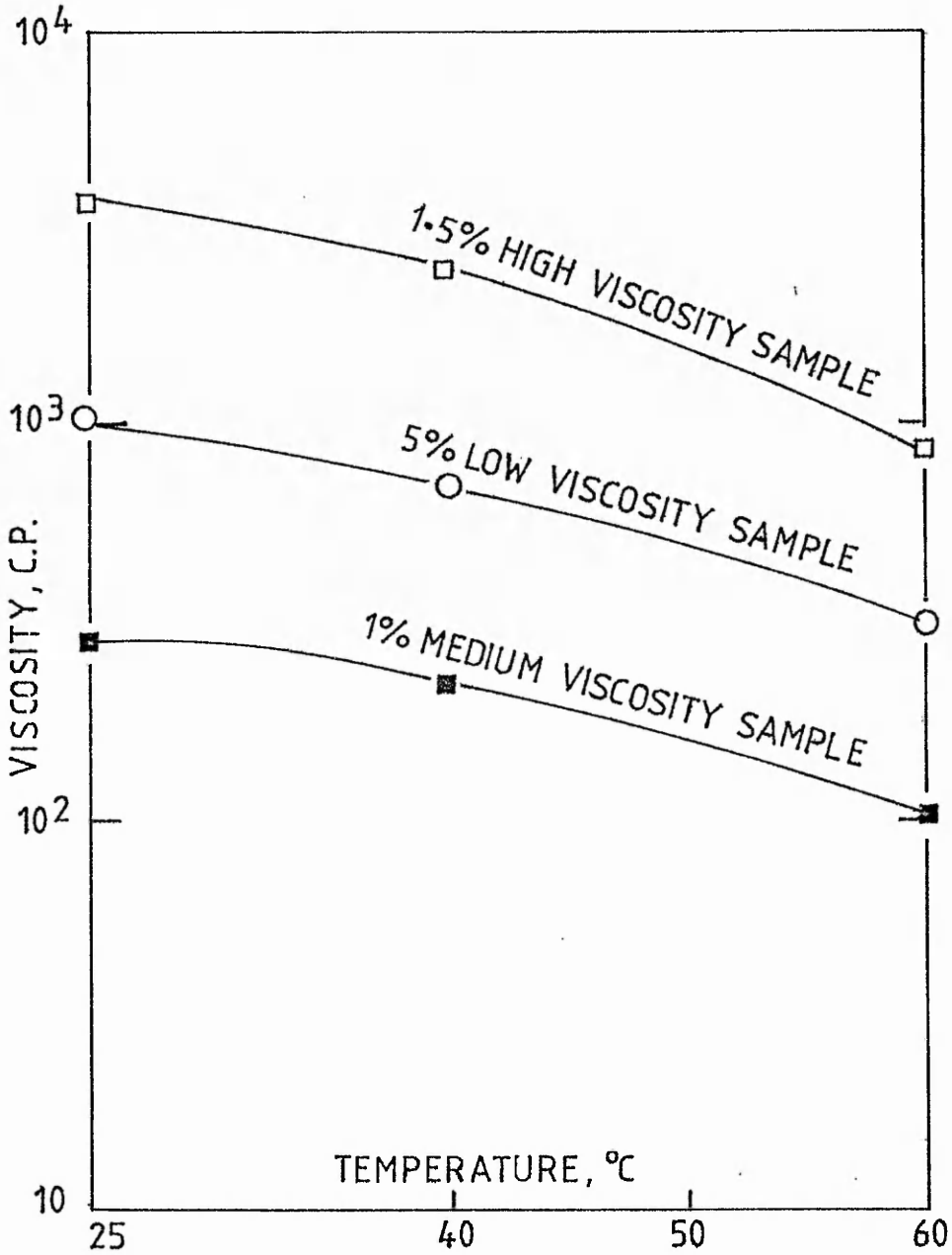


Figure 5. Effect of temperature on viscosity of solution of chitosan at pH4 in aqueous acetic acid.[172]

viscosity loss at about the same relative rate with increasing temperature, regardless of concentration.

The viscosity of chitosan solutions in acetic acid varies with acid concentration[172]. This phenomenon has been attributed to a change in molecular configuration. In a low density ionic environment, as the pH is reduced, chitosan molecules uncoil and assume a more elongated or rod-like shape. The equilibrium $-NH_2 + H^+ \rightleftharpoons -NH_3^+$ is driven to the right and the mutual repulsion of charged groups supplies the uncoiling force. The magnitude of the viscosity change can be reduced by adding a salt to an acetic acid solution.

Figure 6 gives the viscosity behaviour of chitosan in 0.2M acetic acid and various acetate buffers.[171] The limiting viscosity number of chitosan sample in 0.2M acetic acid and 0.1M sodium acetate was found to be 11.46 at 25°C and in 0.2M acetic acid - 0.1M sodium chloride - 4M urea 10.77 at 20°C. By correlation of limiting viscosity number data with ultracentrifugation measurements, Lee obtained [171] values for the constants K and α in the Mark-Houwink equation,

$$\log [\eta] = \log K + \alpha \log Mw$$

The chitosan used in this work was not fractionated but sheared to different extents to give three samples of different molecular weights. A value of 8.93×10^{-4} was obtained for K and 0.71 for α.

Using anhydrous formic acid as solvent, the behaviour and stability of chitin has been studied by viscosity measurements[171]. The polymer solutions show a non-linear dependance on concentration. Above certain concentrations the polymer becomes more entangled and there may be formation of an internal net-work. Lee measured the limiting viscosity numbers and obtained values of 31.7 dlg^{-1} at 2°C and 30dlg^{-1} at 25°C.[171]

Viscosity data for chitin ethyl ether(Mw=66,000) has been recorded by Danilov and Plisko [181]. In different solvents the limiting viscosity number was found to vary between 2.3 and 2.6.

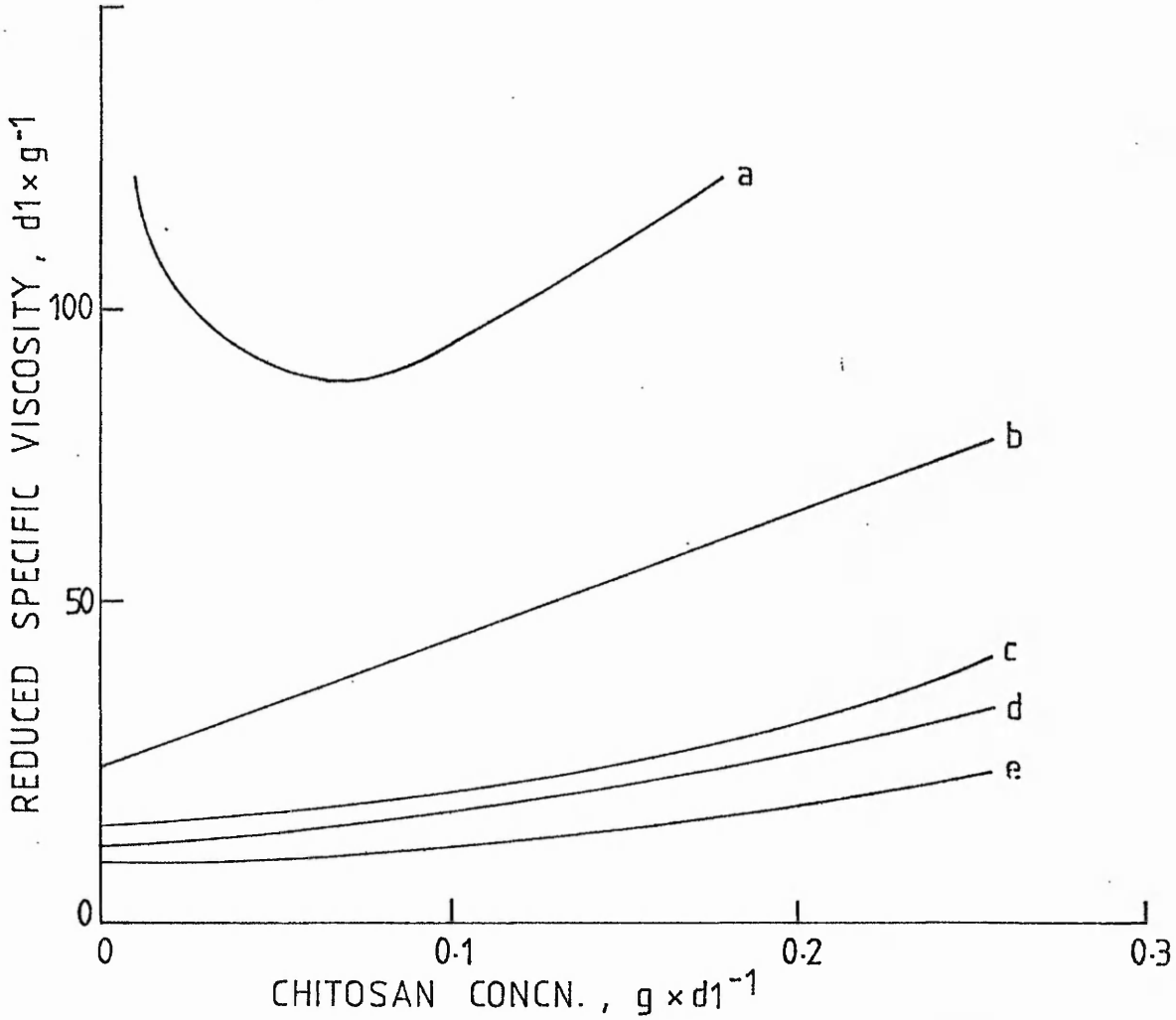


Figure 6. Dependence of the reduced specific viscosity on concentration of chitosan in the absence and presence of salts. a=0.2M acetic acid; b=0.2.M acetic acid +0.01 M sodium acetate; c=0.2 M acetic acid + 0.1 M sodium acetate; d=0.2M acetic acid +0.2 M sodium acetate; e=0.2 M acetic acid + 0.5 M sodium acetate.

1.8 Preparation of Chitin and Chitosan Membranes

Production of chitin membranes is restricted due to its limited solubility. However, a chitinous membrane has been obtained[182] from the cuttlefish, Sepia officinalis by decalcifing the shell and then deproteinising the resulting chitin-protein complex. The form and area of the membrane obtained are those of the shell.

Using a 2% solution of chitin in hexafluoroisopropanol, or a 1.4% solution in hexafluoroacetone sesquihydrate, Capczza prepared[169] transparent flexible chitin films. Brine and Austin produced[183] chitin membranes by using various solvent systems. Use of trichloroacetic acid, methylene chloride and chloral hydrate gave high quality films that were able to be cold drawn to high-tenacity products. The chloral hydrate speeded up solution and dissolved up to 2-3% more chitin. It aided the cold drawing operation but contributed markedly to the chlorine content of the films. The possible reaction of chloral with acetamido or amino groups to form an aldehyde-ammonia or Schiff's base type of structure may account for the difficulty of removing the halogenated impurities. Rutherford and Austin[168], using NN-dimethyl acetamide - 5% lithium chloride or N--methyl-2-pyrrolidone - 5% lithium chloride as solvent systems for chitin , produced films and fibres that could be cold drawn, indicating a good degree of crystalline order.

Acid and water insoluble chitin films having improved mechanical strength, rigidity and clarity have been prepared[184] by casting chitosan films, followed by reacetylation. The cast films were

immersed in an aqueous solution of alkali or an organic base, swollen with water and then acetylated by treatment wqith acetic acid and dicyclocarbodiimide.

Membranes of chitin xanthate have been prepared and found to behave in a similar manner to chitin towards swelling media and solvents.[185-187] Determination of nitrogen content of several regenerated chitin films and of the original chitin flake indicated that no significant deacetylation occurs through the whole process of dispersion, xanthation, and regeneration.

The tensile strength of regenerated chitin films in the dry condition is comparable with that of regenerated cellulose films prepared in the same way from viscose. However, the wet strength is considerably lower. Joffe and Hepburn determined[188] the elastic modulus for regenerated chitin films and found it to be 1.97×10^9 Nm^{-2} and the tensile strength to be $5.10 \times 10^7 Nm^{-2}$. The material tested was a portion of a film originally made by Thor[185] 30 years earlier, who obtained a value of $9.31 \times 10^7 Nm^{-2}$ for the tensile strength.

Table 11

Mechanical Properties of some chitin films

Film	Tensile strength	Elongation	Remarks and Reference
Natural	35	--	Kunike[189] (1926)
Natural (oriented fibre)	58	--	Clark + Smith[146] (1936)
Regenerated (xanthate)	9.49	--	Thor & Henderson[186] (1940)
Renatured	32.5	11	Non crystalline 183 * Brine & Austin (1975)
Renatured	52-58	125	Crystalline. 183 ° Brine & Austin (1975)
Renatured	75-95	4	Pre-oriented by partial cold drawing. 183 Brine & Austin (1975)

*
Cast from 40% trichloro acetic

°
Cast from chloral extracted 12hr with acetone

Chitin xanthate dispersions are miscible in all proportions with regular viscose and such mixtures can be used to cast formed articles. Acidic and basic dyestuffs are retained by regenerated chitin membranes.[188] This is an important quality of the material, in view of the fact that the material has been used in the manufacture of both washable wallpapers and coloured fabrics resistant to laundering.

Danilov and Plisko [181] cast a film of ethyl chitin from an alcohol and benzene mixture and determined its tensile strength. Values ranging from 5.5 and 7.3 kg mm^{-2} were obtained with an extension of between 5 and 23%. Chitosan and chitosan derivatives have been the subject of more extensive research into membrane formation, due to their ease of solubility. Izard [190] and Mima, Yoshikawa, and Miya [191] have produced membranes after the addition of polyvinyl alcohol solutions to chitosan solutions. Membranes of sulphoethylated chitosan have been formed by Nud'ga, Plisko and Danilov [192] for use in medical applications and Kochnev, Moldkin, McHedlishvili and Plisko [193] have used sulphonated chitosan membranes in medical experiments.

Chitosan films can be prepared simply by the evaporation of an aqueous carboxylic acid from a chitosan solution spread on a glass plate. They can also be produced from the Sepia officinalis shell[182] by including, in the isolation procedure, treatment for 3-4 days with 10% hydrochloric acid, an ether extraction, and deacetylation with potassium hydroxide in ethanol/monoethylene glycol mixtures.

The films are of high tensile strength, a value of 7kg.mm^{-2} has been reported[194] and no appreciable elongation observed. The burst strength

was found to be 1.8kg.cm^{-2} Permeabilities to gases and vapours have been noted and compared with those of cellophane membranes. They have comparable permeabilities to water moisture but chitosan films have an outstanding ability to prevent the passage of oxygen, nitrogen and carbon dioxide.

The film forming ability of chitosan has been noted by Averbach[195,196]. Tough flexible films, with a tensile strength of 20,000 psi and an elongation of 3% have been cast from dilute acetic acid solutions. The films were found to be impervious to air, but easily platicized by water. The film-forming qualities appeared to correlate well with the structure as defined by X-ray diffraction. More recent results have shown elongation of the films to be much greater[197].

The strengths of various chitosan films prepared from chitin deacetylated at 100°C for various time periods with 50.9% sodium hydroxide have been reported[198], the tensile strength of the film decreasing with increasing deacetylation time.
Water-impermeable membranes with excellent mechanical properties have been obtained[199] by mixing 3 parts chitosan and 7 parts polyhexamethyleneadipamide in 99% formic acid solution and evaporating the resulting homogeneous solution spread on crystal plates. Membrane potentials were measured on 10-20μm thick membranes using a potentiometer connected to a membrane through saturated calomel electrodes, saline bridges, and sodium chloride solutions of variable concentrations. Determination of the ionic potentials in cells:

0,1M NaCl | membrane | 0.1M NaX

where X= thiocyanate, iodide, nitrate, bromide, chloride, formate, iodate or acetate

were also carried out. The osmotic flow through these films 12-18μm thick was found to be between 100-250 mm^{3}x 100 cm^{-2}x hr^{-1}.

Chitosan membranes have been noted as the first natural polymer to chelate.[194] Crystallisation occurs upon collection of transition metal ions and this is accompanied by the appearance of colour. They have a lower capacity than chitosan powder, due to the reduced contact surface; however the collection is good, particularly for molybdenum, chromium, and mercury.

Semipermeable membranes prepared from glycol chitosan and/or heparin and/or chondroitin sulphate have been found to be stable[200] at high pH values and not to compress under high pressures.

Chitosan has been used as a reverse osmosis membrane[201], having selective permeability for water and hence useful for water purification or as an artificial kidney. When a 0.2% sodium chloride solution was passed through a 6μm thick membrane under a pressure of 40kg/cm^2, water permeation was 0.55 ton/m^2/day and 72% of sodium chloride was removed.

An acetylated chitosan membrane has also been produced[202]. The 0.02mm thick chitosan membrane was acetylated by treatment with a solution of pyridine, 100; dicyclohexylcarbodiimide,5; and acetic anhydride 10g; at room temperature for seventy hours. When 2.5% sodium chloride solution was poured through the membrane and subjected to a pressure of 100kg/cm^2, water permeated at 1.4 x 10^{-3} cm^3/sec and contained 0.15% sodium chloride.

The chitosan membrane possesses a high permeability towards water even when the thickness (severalμm) makes it self supporting.[203] A cellulose acetate membrane with an effective layer thickness of 0.2μm had

a water permeability of 0.8 tons/ m^2/ day under a pressure of 40 kg/cm^2. Under similar conditions a chitosan membrane possesses a water permeability of 2 tons/m^2/ day. The high water permeability of this membrane can be attributed to the chitosan molecules being swollen with water and possessing a stereospecific structure.

Chapter 2

Reactions, Properties and Utilization of Chitin and Chitosan

2.1. Chemical reactions of Chitin

Chitin is more difficult to modify chemically than is cellulose. It is insoluble in the usual cellulose solvents, only swells slightly in basic solvents, and does not swell at all in the usual media for esterification reactions. It has been claimed, from measurements of the amount of non-freezing water[204] and of the heats of swelling[204] of chitin and cellulose, that the former has a smaller active surface area and stronger interchain hydrogen bonds.

There are only two hydroxyl groups in chitin which can be involved in chemical reactions and the derivatives that are formed usually require drastic chemical operations which often lead to degradation.

2.1.1. Inorganic Ester Derivatives

2.1.1.1. Sulphates

Because of their possible use as synthetic blood anticoagulants, sulphate diesters of chitin have been widely prepared by treatment of chitin with chlorosulphonic acid in differing media.

In pyridine[205] the reaction gave a product containing 14.4% sulphur after 7 hours, whilst after 2 hours in 1,2-dichloroethane,[206] a product containing 13-15% sulphur was obtained. The molecular weights of the esters were determined and found to be 14,000-17,000 depending on the conditions of synthesis. Formamide has also been suggested[207] as a suitable reaction medium to avoid possible degradation of chitin.

By the use of sulphur trioxide complexes with pyridine, dioxane, NN-dimethylaniline or bis (2-chloroethyl) ether, Jones prepared sulphated chitins [208] for use as thickeners in pastes, adhesives and drilling muds.

Following a study of the reaction of carbohydrates with sulphuric acid, Nagasawa, Tohura, Inove and Tanoura[176] found that drastic depolymerisation and sulphation occurred when solid chitin was treated with sulphuric acid, followed by gradual depolymerisation and sulphation; the temperature during the reaction markedly influencing the degree of depolymerisation

2.1.1.2. Nitrates

Chitin nitrates were obtained for the first time by nitration of chitin with fuming nitric acid.[209] Detailed investigations by Schorigin and Hait showed that by using concentrated nitric acid alone, to avoid the extensive degradation caused by sulphuric acid, chitin nitrates with approximately 1.5 nitrate groups per sugar unit were formed in 1 - 2 hours. The products were partially soluble in formic acid, were quite stable, and burnt vigorously at 163°C. Denitration was brought about by treatment in sodium hydrogen sulphide solutions for 3 hours at 16°C. Clark and Smith[146] and Meyer and Wehrli[71] have also prepared chitin nitrate. The product is by no means homogeneous, as a result of the severe hydrolytic conditions used in nitration, and the mixture of relatively short chain substances can be readily fractionated by the use of formic and acetic acids. X-ray analysis has shown that dispersion in lithium thiocyanate and reprecipitation causes no change to chitin nitrate.

2.1.2. Alkyl Derivatives

Treatment of chitin[165] with sodium in liquid ammonia gives a sodium derivative containing one sodium atom per sugar unit. An alkali chitin has also been prepared by steeping the polysaccharide in 50% aqueous sodium hydroxide for 2 hours at 25°C.[186,211] By operating at a lower temperature (0-15°C) less degradation and deacetylation occurs.[212,213] When alkali chitin is extracted with water it regains the appearance of the original chitin.

Sodium chitin can be dispersed in water by mixing it with the desired quantity of crushed ice.[185] A dispersion prepared in this manner is stable for several days at 0°C.

Alkali chitin films allowed to stand at room temperature for 2 days were observed[155] to become soluble in water and to swell in m-cresol. This was due to the chain degradation and destruction of the secondary structure. Prolonged treatment with alkali gave a decrease in viscosity. Acetylation of the partially deacetylated water-soluble chitin under homogeneous or almost homogeneous conditions gave a pure chitin.[213] Dispersions of alkali chitin are suitable for the preparation of a number of alkyl derivatives.

Methyl ethers containing 9.34% of methoxyl groups have been obtained[214] by treating alkali chitin with dimethylsulphate. Preliminary activation of chitin with hydrochloric acid, and methylating in the presence of alkali, made it possible to increase the content of methoxyl groups to 16%. Monomethylchitin dissolves in formic acid, swells strongly and

partially dissolves in glacial acetic acid.

Ethyl ethers of chitin have been obtained [181] by the action of ethylchloride on alkali chitin. The products were almost completely soluble in organic solvents. Ethyl chitin decomposed at 180°-190°C in air, and at 200-210° in vacuo. Hydroxyethyl ethers have been obtained by treatment of alkali chitin with ethylene oxide under heterogeneous or homogeneous conditions [181,215,216]. In both cases the formation of a water-soluble ether required a considerable excess of ethylene oxide. A nitrated derivative containing 5.71% of nitro-nitrogen has been obtained[181] from the hydroxyethyl ether. Shimahara, Nagahata and Takiguchi have reported [217] a 6-O-hydroxypropylchitin.

Alkali chitin treated[218] with glycerol monochlorohydrin forms glyceryl ethers. Treatment of chitin with glycerol monochlorohydrin did not give rise to a water soluble ether, despite a high content (49%) of glycerol residues. The ether obtained did not dissolve in hydrochloric or phosphoric acids indicating crosslinking due to the presence of traces of glycerol dichlorohydrin in the monochlorohydrin.

Carboxymethyl chitin and carboxyethyl chitin have been obtained by treatment of alkali chitin with the corresponding chloroacid. These chitin derivatives have been studied by Okimasu,[219] Danilov and Plisko[220,221] Miyazaki and Matsushima[222], Hayashi, Imoto and Funatsu[223] and by Trujillo[224] among others.

A study of the kinetics of carboxymethylation of carboxymethyl chitin dissolved in solutions of alkali[225] has shown that with increase in concentration of sodium hydroxide to 15-20%, and increase in temperature of 20-30°C, there is a sharp increase in the rate of reaction. However

an increase in reaction temperature entails the formation of a precipitate from the alkaline solution owing to the hydrolysis of N-acetyl groups and the formation of chitosan. During carboxymethylation under heterogeneous conditions the acetyl groups are not hydrolysed. At room temperature the reaction proceeds almost to completion and a degree of substitution of 0.8-1.0 is obtained. An increase of reaction temperature to 40-60°C accelerates the hydrolysis of monochloroacetic acid, so that the main reaction requires a smaller amount of alkylating agent.

A claim to have determined the structure of sodium carboxymethyl chitin by periodate oxidation and acid hydrolysis has been made.[222] Chromatographic analysis of the hydrolysis product showed that substitution takes place mainly at C(6) as indicated by the susceptibility of sodium carboxymethyl chitin to periodate oxidation. However this indicates that de-N-acetylation must have occurred during the carboxymethylation step.

A new method has been described for the preparation of carboxymethyl chitin involving the preliminary activation of chitin with dimethylsulphoxide.[224] Carboxymethyl chitin with a degree of substitution of 0.99 and which is readily soluble in water has been synthesised in this way. Acidification of sodium carboxymethyl chitin [220] with dilute hydrochloric acid at room temperature yielded a chitino-glycolic acid which swelled strongly and partially dissolved in water. The viscosity of carboxymethyl chitin varies with conditions in the same manner as that of ordinary polyelectrolytes, except that an isoelectric point is observed at pH6.3. Capozza has used carboxymethyl chitin[169] as a membrane for the controlled release of drugs.

2.1.3. Acyl and Related Derivatives

A number of acetylation techniques have been examined by Schorigin and Hait[226] but in only one case, where dry hydrogen chloride gas was passed through acetic anhydride at room temperature for 120 hours, was a completely acetylated product (2.99 acetyl groups per sugar unit) obtained. The product was soluble in formic acid and 50% resorcinol but insoluble in other organic solvents. It dissolved slowly in concentrated hydrochloric and sulphuric acids but decomposition prevented recovery of the product by dilution. Dissolution occurred rapidly in concentrated nitric acid and dilution of the solution gave a crumbly precipitate of nitro-acetyl chitin containing 2.14% nitro-nitrogen.

Acetylation for 3 months[71] with a mixture of acetic acid and acetic anhydride in the presence of zinc chloride gave a chitin acetate containing 2.5 acetyl groups per residue. The product was strongly degraded insolution in 90% formic acid.

By dissolving chitin in phosphoric acid acetylation has been carried out[227] both in the presence and absence of perchloric acid as catalyst. In the presence of 1% perchloric acid, 27.5% of the acetyl groups were formed in 4 hours at 75°C corresponding to a degree of substitution of 1.8; in the absence of a catalyst, the maximum degree of substitution was 1.7 at 80°C. The products obtained dissolved in 50% resorcinol, phenol and partially in m-cresol.

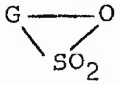
In order to study the properties of films and fibres of acetyl chitin, Nogushi, Tokura and Nishi acetylated[228] chitin to nearly 30% by treatment of alkaline chitin with acetic anhydride at 0°C for 16 hours. Acetyl

chitins, acetylated to varying extents, were obtained by treatment of chitin with a mixture of acetic anhydride and methane sulphonic acid at $0^{o}C$. Fibres and films obtained from 99% formic acid solution were found to be more flexible with increasing degree of acetylation. Diacetyl chitin was synthesised by using the following methods - (i) acetylation in excess of acetic anhydride with hydrogen chloride gas at $0^{o}C$ for 10 days; (ii) excess acetic anhydride in methane sulphonic acid at $0^{o}C$ for 16 hours and (iii) excess acetic anhydride in the presence of perchloric acid at $0^{o}C$ for 3 hours.

Methane sulphonate and toluene - p - sulphonate esters of chitin have been prepared.[229] The mono methane sulphonate was prepared by treatment of chitin with methane sulphonyl chloride and pyridine in the ratio of 1:10:40, at room temperature for 7 days. The mono toluene -p-sulphonate ester was prepared using chitin and tosylchloride in pyridine in the ratio of 1:10:50 at room temperature for 10 days.

2.1.4. Miscellaneous Derivatives

Chitin xanthate was prepared by Thor and Henderson by treatment of alkali chitin with crushed ice and carbon disulphide.[186] The chitin could be regenerated by acidification. Chitin xanthate can be further reacted with sultones,[230] having the general formula



(where G is a divalent hydrocarbon radical of three to six carbon atoms containing lower alkyl substituents) to produce polymeric sulphonic acids containing xanthate ester groups.

Using an enzymic process which cleaves sucrose and grafts the resulting glucose on to chitin or cellulose, Neely has obtained[231] glycosylated chitin.

An alcoholized chitin has been claimed by György , Kuhn and Zilliken.[232] A suspension of chitin in anhydrous methanol was kept at just above 0^{o} and saturated with hydrogen chloride gas for 2 hours until nearly all the chitin had dissolved. Evaporation gave chitin methanolate a white powder which is readily soluble in water.

Austin has prepared[233] various chitin complexes with alcohols and carbonyl compounds. Preparation is straightforward, although increased rate of reaction is observed with water or solvent swelled chitin. The complexes formed have a voluminous physical form which makes them easy to handle and filter for the preparation of highly purified chitin for pharmaceutical and other purposes. They are easily dissolved in chitin solvents and hence are useful for preparing chitin solutions for the fabrication of films fibres and other shaped articles.

Harmon, De and Gupta have prepared [234] trimethylsilyl chitin by swelling chitin in formamide for 5 hours at 80°C and then, on cooling, adding hexamethyldisilizane. The silicon content was 6.19% and the degree of substitution 0.6. Cleavage of the trimethysilyl groups was readily brought about by treatment in water at room temperature.

2.2. Chemical Reactions of Chitosan

The presence of two hydroxyl groups and a primary amino group in chitosan extends the scope of possible chemical modification in comparison with the possible modifications of either chitin or cellulose.

2.2.1. Inorganic Ester Derivatives

2.2.1.1. Sulphates

Considerable interest has been shown in the possible use of N-sulphated polysaccharides as synthetic blood anticoagulants. This interest has arisen from the belief [235,236] that the N-sulphate in heparin contributes toward the activity of the polysaccharide in its function as an anticoagulant. Sulphation of chitosan has been achieved by treatment[237] with a mixture of sulphur trioxide and sulphur dioxide at the boiling point of the mixture. The resulting ester contained 14.8% of sulphur and exhibited anticoagulant activity *in vivo* and *in vitro*. Subsequently chlorosulphonic acid in pyridine,[157] sulphur trioxide- pyridine complex,[238] and sulphuric acid have[176,239] been used to sulphate chitosan. In the last case, a fully N- sulphated chitosan has been prepared.

Selective sulphation of chitosan has been developed [240] by altering the reagent and media. N-Sulphatochitosan containing one sulphate group per glucosamine unit has been obtained using a pyridine-sulphur trioxide complex in an aqueous alkaline medium at pH 9-10. Subsequent

treatment of the N-sulphatochitosan with a mixture of sulphur-dioxide and sulphur trioxide, in the cold, yielded the O-sulphated derivative (75% of hydroxyl groups sulphated). No anticoagulation activity was observed with the N-derivative but a high activity was found for the O,N- derivative of chitosan.

It has been found[241] that selective O-sulphation of chitosan, to give a reproducible product of given molecular weight range, may be easily carried out by converting chitosan into one of its salts prior to sulphation. The salts of inorganic or organic monovalent acids have been employed, the preferred salts being formate, acetate, perchlorate, nitrate, chloride or bromide. The chitosan salt is sulphated by dissolving it in formamide and treating the solution with chlorosulphonic acid.

2.2.1.2. Phosphates and Nitrates

Chitosan has been phosphorylated[205] by treatment with phosphoryl chloride at 40°C for 5 hours. Nitration of chitosan[158] with a mixture of acetic and nitric acids or with absolute nitric acid gave the free ester and its nitrate salt. The perchlorate salt of this ester was also prepared[158] but it was unstable.

2.2.2. Alkyl Derivatives

The synthesis of chitin ethers in alkaline medium, in the absence of special precautions, entails a degree of deacetylation the extent of which depends on the reaction conditions. For this reason the final products are sometimes the corresponding chitosan ethers. Thus, for example, in the synthesis of ethyl[214] and carboxymethyl ethers [242] of chitin, the degree of deacetylation is 60% and more. In view of the simultaneous occurrence of deacetylation and alkylation processes, the resulting compound cannot be regarded solely as an O-derivative. The formation of chitosan ethers with substituents located in specific positions requires the introduction of N-protecting groups.

The sulphoethylation of chitosan has been carried out by treatment[243] with sodium β-chloroethane sulphate in the presence of alkali using isopropyl alcohol, and mixtures of o-xylene with propyl and isopropyl alcohols, as reaction media. The highest degree of substitution obtained was 0.35. Both O - and N- sulphoethylation were shown to take place by analysis of the amino-nitrogen content and by conductometric titrations.

For specific O-alkylation of chitosan the amino group was protected by reaction with an aromatic aldehyde,[244,245] resulting in the formation of a Schiff's base. Salicylaldehyde was found to react fully with the amino groups of chitosan and the resulting salicyclidene chitosan is stable in alkaline medium but decomposes in an acidic medium. The salicyclidene chitosan was sulphothylated using the same conditions as for the sulphoethylation of chitosan[243]. The product was treated with 2% sodium hydroxide in order to convert it to the basic form. The final product was an O-sulphoethyl chitosan with a degree of

substitution of 0.31, as determined by analysis of the amino-nitrogen content and by conductometric titration. It was soluble in water and 2% acetic acid solution. With a lower degree of substitution it was only soluble in dilute acids.

O- Carboxylation of chitosan has also been achieved[244] using salicyclidene chitosan. Treatment with sodium monochloroacetate (9 moles per mole of chitosan) followed by acidification and neutralisation gave a mono-substituted carboxymethyl chitosan with free amino groups.

Cyanoethyl chitosan was obtained[246] by the reaction of alkali treated chitosan with acrylonitrile in order to obtain complete O-substitution without hydrolysis of the cyanoethyl groups. The reaction was carried out at 20°C for 24 hours. Increase of temperature caused hydrolysis of the cyanoethyl groups. No reaction occurred in neutral medium, but in acidic medium a degree of substitution of 0.31 was obtained at 20°C over a period of 24 hours.

Methylation of chitosan with dimethyl sulphate in alkaline medium gave a product with a methoxyl content of 29% of the theoretical methoxyl content maximum[247].

A limited degree of N-methylation of the exposed free amino groups occurred. The product was then partially acetylated in formamide-acetic anhydride and then further methylated with methyl iodide in DMF in the presence of barium oxide-barium hydroxide. This gave a product with a methoxyl content of 66%. Repetition of the acetylation

and methylation stages finally gave a product with a methoxyl content of 92%.

Anion exchange polymers have been obtained by[212,248] treatment of chitosan and chitosan derivatives with epichlorohydrin. The epichlorohydrin reacts with hydroxyl groups and some amine groups. The resulting epichlorohydrin chitosan is insoluble in both acidic and basic media. Epichlorohydrin benzyl chitosan and epichlorohydrin oxystyrene chitosan have also been prepared. Both products are insoluble in acidic and basic media.

Fully substituted N-alkyl derivatives of chitosan have been prepared[249] by heating chitosan under reflux in a mixture of the corresponding alkyliodide and absolute ethanol in the presence of organic bases. NNN— Trimethyl- and NNN-triethyl chitosans are readily soluble in water. Treatment of hydroxyethyl chitosan with methyl iodide has given[250] N-methoxyethyl chitosan. Alkylation of both the amine group and the hydroxyl groups with allyl bromide gives[251] allyl chitin which has been used industrially to give chitinous coatings. Numerous other derivatives of this type have been produced using unsaturated aliphatic halides containing more than 2 carbon atoms[251].

Chitosan has been N-acetylated and then treated with various alkyl halides in the presence of alkali to give alkyl derivatives of chitin.[252]

2.2.3. Acyl and Related Derivatives

Treatment of chitosan with acetic anhydride at 100-135°C in a sealed tube yielded a product with properties resembling chitin.[143,144] Acylation of chitosan by treatment with formic acid and propionic and butyric anhydrides at 110°- 125°C led to the formation of the corresponding N-acyl chitosan[253]. Alkali-treated chitosan, reacted with

benzoic anhydride under the same conditions, gave the N-benzoyl derivative.

New acyl derivatives of chitosan have been obtained by using saturated and unsaturated dicarboxylic acid anhydrides followed by addition reactions involving the double bond[254]. The heterogeneous reactions are carried out at room temperature in inert solvents, such as dioxane or tetrahydrofuran, to give chitosan derivatives having up to 70% of the amine groups acylated. Most of the reported addition products were soluble and required ethanol for precipitation.

N- acetyl chitosan gels and partially O-acetylated N-acetylchitosan gels have been produced by a facile acetylation of chitosan with acetic anhydride in 10% acetic acid and in aqueous acetic acid/methanol solutions at room temperature.[255-260] A series of N-acylchitosan gels were produced, under the same conditions, by using the other carboxylic anhydrides. The gels produced were colourless, transparent, rigid and stable on heating. The structure of the N-acyl groups influenced the gelation;[261] the minimum requirement for gelation was defined as about 0.4N-lauroyl(C12); about 0.6 N-fatty acyl(C_3- C_{10}) or about 0.7 N-benzoyl groups per hexosaminide residue. Gelation was not observed with higher fatty acyl groups.

A study has been made of methods for the rapid determination of the degree of acetylation[262] in chitosan prepared by different procedures. Titration of chitosan hydrochloride and the comparison of the mass spectrometer fragmentation patterns have been found to be very useful techniques for characterizing chitosan.

The sulphonic acid esters of chitosan have been produced by the reaction of benzene and naphthalene sulphonyl chlorides with alkali treated chitosan.[253] The derivatives contained approximately one substituent per glucosamine unit. Benzene sulphonyl chitosan is partly soluble in dilute hydrochloric acid and readily soluble in alkali solutions in contrast to naphthalene sulphonyl chitosan which is only sparingly soluble in alkali.

2.2.4. Schiff's Base Derivatives

The preparation of Schiff's bases from chitosan and aromatic aldehydes has been reported.[244] Chitosan was treated in a neutral medium at room temperature for 3 hours with either salicylaldehyde, benzaldehyde, anisaldehyde or o-nitro benzaldehyde. The Schiff's bases obtained did not melt, decomposed above 220°C, and were insoluble in organic solvents.

Several Schiff's base derivatives of chitosan have been characterised by differential scanning calorimetry and studied in connection with their chromatographic performances.[263] The reaction products of chitosan with "Amidox", a dialdehyde starch, have been reported.[171,264] The products were yellowish brown and their characteristics depended on the mole ratios used. Aldehydes and dialdehydes have been used to cross link chitosan with other substances. Moshy and Gemino used limited amounts of glyoxal to crosslink a chitosan tobacco sheet.[265] Masri and Randall insolubilized enzymes by mixing acid-solubilized chitosan with an enzyme solution of either lactase, invertase or glucose oxidase, and a difunctional or polyfunctional crosslinking agent such as glyoxal or glutaraldehyde.[266] Glutaraldehyde has also been used to covalently

link enzymes to chitin.[267,268]

The preparation and some physicochemical properties of the reaction products obtained by the reaction of chitosan and glutaraldehyde have been studied.[264] Generally mixing glutaraldehyde solutions with chitosan acetate solution gives rigid gels which do not undergo syneresis easily and occupy the total volume of the solution from which they are prepared.

Novel chitosan-aldehyde gels have been prepared by Hirano, Miura and Kondo[269] by reacting chitosan in aqueous acetic acid-methanol solution with aldehydes. Gelation occurred with formaldehyde, glutaraldehyde, acetaldehyde, acrolein, benzaldehyde, cinnamaldehyde, propionaldehyde and p-tolualdehyde.

2.2.5. Complex formation with polyelectrolytes

Chitosan reacts with negatively charged polyelectrolytes like heparin, alginic acid and sodium dextran sulphate. Hydroxyethyl chitosan and alginic acid have been used to produce anisotropic gels. The polyelectrolyte reaction products of chitosan with heparin, obtained by mixing a dilute aqueous solution of heparin with glycol chitosan or with dilute chitosan acetate solution, have been reported.[270-273] From the sulphur content of the product, the mole ratios of chitosan to heparin repeating units were in the range 6:1 to 3:1 for several different samples. From the reaction of chitosan and sodium dextran sulphate[274-277] the product was found to contain 1.6:1 - 1.3:1 of chitosan to sodium dextran sulphate. No solvent was found for the product which was obtained as a fine colloidal precipitate. Sodium cellulose acetate sulphate and ammonium cellulose sulphate were treated with chitosan to give polyelectrolyte complexes in the

form of transparent membranes.[278] The interactions of hydroxyethyl chitosan and chondroitin sulphates or hyaluronic acid have been studied. A form of pyranose-pairing aggregate structure was indicated for the complexes.[272]

2.2.6. Oxidation of Chitosan

2.2.6.1. Deamination

Chitosan has been deaminated in hydrochloric acid solution by addition of silver nitrite; evolution of nitrogen and a decrease in viscosity were immediately observable.[279] The residual nitrous acid was destroyed by addition of a small amount of urea and the silver chloride removed by filtration.

Changes in the optical rotations of solutions have shown the half time of the deamination of chitosan by nitrous acid to be of the order of 60 seconds. The reaction of chitosan with sodium nitrite is a cleavage reaction and entails deamination at the point of cleavage.[280] The products are chitosan fragments consisting of 2-deoxy-2-amino-anhydroglucose for all units in the chain, except for the unit at the reducing end of the chain which is an amine-free sugar. The extent of deamination is greater in small fragments, from 50% in the dimer to 3% in a 33-unit fragment.

The deamination of chitin has been mentioned briefly by Katz, Fishman and Levy[281] who used deaminated chitin as a chromotographic support to isolate lysozyme.

Periodate oxidation of chitosan gives rise to 0.6-0.7 mol of ammonia per glucosamine unit, according to Jeanloz and Forchielli.[103]

Hypochlorite, chlorine and lead dioxide have been proposed as oxidising agents for glucosamine[282] but all bring about extended degradation of the polymer chain. Deamination of a suspension of chitosan in water using gaseous dinitrogen trioxide has been attempted.[279]

2.2.6.2. Carboxyl Group Formation

Partial C(6) carboxylation has been achieved using dinitrogen tetroxide or oxygen-platinum.[283] However, only a small proportion of the primary hydroxyl group were oxidised and there was some concomitant degradation of the amine functionality and partial oxidative depolymerisation.

Complete and specific oxidation of the C(6)- hydroxyl group to a carboxylic acid group has been achieved by oxidising chitosan, in the form of the perchlorate salt, with chromium trioxide in acetic acid.[284] The resultant (1→4)-2-amino-2-deoxy-β-D-glucopyranuronan, obtained as its perchlorate, was N-sulphated with chlorosulphonic acid in pyridine to afford a (1→4)-2-deoxy-2-sulphoamino - β-D-glucopyranuroran, isolated as its amorphous sodium salt. The latter displayed moderate blood-anticoagulant activity and both showed marked *in vitro* growth inhibition of leukemia L-1210 cells.

2.2.7. Salt Formation

Chitosan forms numerous salts, many of which are water soluble. Chitosan can be reprecipitated from solution by several acidic precipitating agents including sulphuric, phosphotungstic, iodomecuric, iodobismuthic, molybdic, tungstic, picric and tannic acids. A table of many of the salts found can be found in the Appendix IV. Rigby

prepared[130] 68 different salts from substantially undegraded chitosan, containing 0.8 free amine groups per glucosamine residue. In addition salts were also prepared from chitosans having different amounts of free amine content.

A process for the purification of chitosan by means of its salicylic acid salt has been described.[285] The process allows chitosan to be purified from extraneous material including inorganic salts, proteinaceous materials, and gums. The procedure consists of adding an excess of a soluble salicylate, preferably an alkali metal salicylate, to an aqueous solution of a chitosan salt. After cooling in an ice bath, the salicylic acid salt of chitosan precipitate out and is collected by centrifugation, adjusted to pH 9 with a water soluble base. The resulting precipitate is filtered off, washed by a water-miscible organic solvent, and dried to give chitosan in a purified form.

2.2.7. 1. Dye Adsorption

A series of studies of the adsorption of sulphonated azo dyes, other dyes, and organic solutes by chitin was made.[286-8] It was concluded that chitin adsorbs by hydrogen bond formation with the solute in the absence of water, by van der Waals attraction alone in the presence of water, and by ion exchange. The extent of adsorption of dyestuffs was correlated with the dye structures.

2.2.8. Chelation Properties

Although a number of workers had previously reported[289-292] the interaction of chitin and chitosan with metal ions, Muzzarelli[4] was the first to point out that in the case of chitosan such interactions

are due to chelation. Since then considerable work has been carried out on the chelating ability of chitosan[4,293-295] and Table 12 lists some of the metal ions studied.

The nature of the anions present in the solutions carrying the transition metal ions play an important role. Chloride and thiocyanate ions reduce the amount of metal ion taken up whilst sulphate ions increase it. Acetate ions alter the surface of the chitosan grains because chitosan acetate is soluble, but the effect on metal ion uptake was not reported.

Enhanced transition metal ion capacity occurs with chitosan previously conditioned in sulphate media. Chitosan bonded with silver and zinc ions has been used [296] to collect traces of cyanide from 0.44M ammonium sulphate solutions. The collection of various metal ions on chitosan [297] which has had no conditioning has been compared with the collection on chitosans which have been conditioned with 0.1M sulphuric acid, conditioned with 0.1M ammonium sulphate, and conditioned with both. At pH 3.0 in the absence of ammonium sulphate, sulphuric acid conditioned chitosan is less efficient than chitosan as a collecting agent. The collection of manganese is enhanced by the presence of 0.1M ammonium sulphate on chitosan, but sulphate conditioned chitosan does not collect manganese. Collection is slightly more efficient at pH5.0, whereas sulphuric acid at pH 1.0 prevents collection of transition metal ions on chitosan and conditioned chitosan. As far as metal oxyanions such as metavanadate, molybdate, or chromate are concerned, the presence of sulphate depresses the degree of collection especially at pH 5.0.

Various comparitive studies have been made of the chelating ability of chitosan and synthetic chelating polymers.[298-300]

Table 12

Examples of Metals Chelated with Chitin-Chitosan

		Metal	Comments
Chitin and Chitosan	e.g.	Alkali-alkaline earths Li,Na,K,Cs Th,NH_4,Mg,Ca Ba,Rb, St	Not collected to any extent. Do not prevent collection of transition metal ions when simultaneously present
		1st row transition metals	Colour appearance
Chitin and Chitosan		Ti metavanadate	Red) Colour formation quite detectable even at low concentrations.
		Cr	Orange)
		Cr	Green)
		Fe	Orange)
		Co	Yellowish brown)
		Ni	Pink)
		Cu	Green)
			Blue)
Chitosan		Zn	Powder collects more than flake
Chitin		Mn,Fe,Zn,Ni, Co,Cu,Pb,and other transition metals	Mn,Fe and Mg less stable complexes than remainder.
Chitosan		Ni and Cd	Rates compared in single and mixed solutions. Cd suppressed Ni uptake.

Comparison of the uptake of metal ions between chitosan[301] and poly(p-aminostyrene) shows that chitosan has a higher binding capacity with all metals tested. The greater effectiveness of chitosan for metal binding may be due, in part, to the greater basicity of the aliphatic primary amino group compared to the aromatic amino group.

The chelating ability of chitin has important implications in ecology.[302] The concentrations of cadmium, copper, cobalt, iron, magnesium, manganese, nickel, lead, strontium and zinc in surface zooplankton samples have been compared with values determined on samples collected at depths of 100 metres or more in the ocean. The average values of copper, iron, manganese, nickel, lead, strontium and zinc were higher in samples collected at depth because food-dependent moulting rates were lower, therefore more time was available for metal collection to take place.

The role of chitin in the transport of metals in the oceans has been noted.[303] Considering the volume of biodegradable chitins in the skeletons of anthropods, the quantity of metals associated with them will be high. When the chitin-metal complexes break down they release large amounts of these metals to the seawater, and decomposition of such complexes can be considered as one of the sources of localised metalliferous sediments in marine environments. Detailed reviews of the chelating properties of chitin and chitosan have been given by Muzzarelli.[4,5]

2.2.9. Thermal Properties

At high temperatures in air, chitin and chitosan undergo degradation. According to Bihari-Verga et al ,[304] the thermal decomposition of N-acetyl-D-glucosamine takes place with maximum rate at 200°C, followed by a second process at 400-450°C. The polymers showed increased thermal stability, water loss occurring at 60° and the main thermal process occurring at 275° and 280° for chitin and chitosan respectively.

Chitosan has been utilised[265] as an adhesive for tobacco particles in reconstituted tobacco sheet formulation. The low tar yield in the smoke of chitin has led to its trial as a tobacco extender and prompted a study[305] of chitin pyrolysis.

The major basic components of chitin pyrolysis were picolines and pyrazine. Acetic acid and C2,C3 and C5 acids were also produced. In comparison, chitosan produced relatively large amounts of butyric acid and less than half of the acetic acid obtained from chitin.[306]

2.2.10. Optical Properties

The infra-red spectra of chitin and chitosan have been published by many authors.[31,119,146,147,307] The most significant parts of the spectrum are those illustrating the amide bands at 1665,1555 and 1313cm^{-1}, all of which show perpendicular dichroism and which are, respectively, assigned to the C=O stretching, to the N-H deformation in the CONH plane, and to the CN bond and CH_2 wagging.

The chitosan spectrum differs from that of chitin in that the new band at 1590 cm^{-1} predominates over the one at 1665cm^{-1} and the band at 1555cm^{-1} is absent.

On N-acetylation of chitosan films a gradual shift of the amide band from 1595 cm^{-1} to 1550cm^{-1}, as the reaction proceeds, has been observed.[308]

Chitin and chitosan have no conjugation in their structures and show no ultra violet absorption above 260nm.[171]

2.3. Utilization of Chitin, Chitosan and their Derivatives

The number and variety of attempts to utilize chitin and chitosan, and their derivatives, has steadily grown in recent years. A wide range of the properties of these polymers are used; their ability to form fibres and films, their capacity for ion exchange and complex formation, and their physiological activity with absence of toxicity.

2.3.1. Paper Additives

Chitosan has attracted attention in the paper industry for a long time, both as an adhesive[309,310] and as a sizing agent.[311] Addition of the grart copolymer of chitosan with 2-acrylamido-2-methyl propane, sulphuric acid, acrylamide, or acrylic acid to paper pulp gives improved printing and mechanical properties to the paper.[312,313] A coating[314] for lithographic paper plates which accepts and hold both water and ink is based on chitosan, colloidal silica or clay fillers, a cross-linking agent, and a catalyst. It gives increased sensitivity to the paper for the formation of an image. Cardboard has been prepared[315] by sizing pulp, cotton linters and chrome leather shavings with chitosan acetate.

When chitosan is[310,316,317] added to paper pulp or when paper is subjected to a surface treatment with chitosan solutions, improvements in many of the properties are observed, e.g. tensile strength, breaking strength, the resistance to breaking and puncturing, and wet strength. A similar effect is observed on the addition of cyanoethyl chitosan to paper; improved dielectric properties have also been noted.[318] The applications of chitosan in the paper industry have been mentioned by Brine and Austin[319] and by Foster.[320]

2.3.2. Textile Applications

The possibility of forming films and fibres from chitin dispersions was investigated as early as 1926.[189,321] In later studies[186] chitin films were produced by depositing dispersions of chitin xanthate on to a glass support, with subsequent regeneration of chitin in a coagulation bath containing 40% ammonium sulphate and 5% sulphuric acid in water. Using this basic method, mixed chitin-cellulose fibres have been formed[228,322] from chitin and cellulose xanthates. The fibres exhibited increased dyeability and had ramie-like properties.

Aqueous solutions of blends of poly(vinyl alcohol) and chitosan acetate were spun to introduce amino groups into "Vinylon" fibres.[323] The chitosan- poly(vinyl alcohol) fibre produced can be dyed with direct or acid dyes, burns without melting and has a handle similar to that of linen. Fabrics sized with chitosan salts have certain highly desirable properties like greater stiffness and fullness. The treatment also improved the appearance of fabrics by adding lustre and brightening their colours.

It has been found that chitosan, when applied to glass fibre or fabrics,[324,325] forms a permanent coating with many sites available for the adsorption of a wide variety of dyes. In addition, when the coating is applied to fabrics it imparts a high degree of fibre-to-fibre bonding and improves the distortion stability, the abrasion resistance, and the general appearance of the fabric.

Dabrowski has applied[326] aqueous solutions of chitosan and a reactive chromium complex e.g. p-aminobenzoate chromic chloride to fibres, films, and fabrics which are difficult to dye using commercially available dyes.

The process gives a film of the reaction product bonded to the base material, which has good affinity for commercial dyestuffs. Chitosan has also been used to give improved[327] and simplified dyeings on polyester and cotton fibres.

Polymeric dyes based on polymers like chitosan have been prepared[328] by reacting the polymers with organic dyes or intermediates having chromogenic properties.

Chitosan salts have been used as print resisting materials[329] for various knitted or woven fabrics, such as wool, cotton, silk etc. Sulphonated polymeric alcohol derivatives[230] of chitosan have been found effective for use in providing antistatic, soil repellant and/or soil release coatings to a wide range of materials, including textiles, metals, ceramics, etc.

Interfacial deposition of cross-linked chitosan on woven wool fabric has been shown to impart laundering-shrinkage control to the fabric.[330] Results showed that the fabric had a marked resistance to machine washing. Flame retardants and moth proofing agents could also be introduced in the cross-linking procedure. Salts of chitosan have also been used to shrink-proof wool.[251]

Chitosan has been used as a thickener for printing and for finishing various fabrics[331] whilst chitosan acetate has been used as a fixing agent and thickener in the preparation of printing pastes.[332]

2.3.3. Photographic Applications

Chitosan has found important applications in the field of photography[333-338]

with the advent of rapid development photography e.g. Polaroid. Chitosan has been chosen because of its resistance to abrasion, optical characteristics, film forming ability, and its behaviour with silver complexes which are not appreciably retained by chitosan. Because of its regularly distributed amine groups it is also suitable for forming mixtures with gelatin and for preventing lateral diffusion of acidic dyes. While products containing chitosan have not yet been marketed, it is clear that the photographic field is a potentially important area for chitosan applications.

2.3.4. Adhesive Applications

Chitin occurs naturally in several systems quite well known for their adhesive properties. It has, for instance, been found in barnacle cement [339,340] and in an extremely hydrolysis-resistant substance[341] that attaches the eggs of hog-lice to hog bristles.

The use of chitosan in cements for electrically non-conducting surfaces has been studied.[130] The cements were found to develop good strength and water resistance. Satisfactory joints have been formed with paper and between wood and a wide variety of materials.[342] Chitin xanthate has been found to be a good adhesive[343] for decorative and commercial plywood, hardboard and furniture joints.

Chitosan is an effective sealer and primer for wood, asbestos, asbestos-cement board, paper, plaster, brick and tile.[344,345] Derivatives of chitosan also possess cementing ability and the use of chitosan sulphate as a thickener and adhesive in drilling muds has been proposed.[208,346]

Fertilizer and feeds have been pelletized by using chitosan.[347]

2.3.5. Coagulating Applications

Chitosan effectively coagulates suspended solids in the wastewaters from the processing of poultry,[348] eggs,[349] cheese,[350] meat, seafood,[351,352] fruit cakes and vegetables.[353] The nutritional value of the coagulated solid as an animal feed stuff has been examined.[354] In addition, it functions as a conditioning agent for dewatering activated sludge suspensions from biological treatment of vegetable and brewery wastes.[355] It has also been found effective[356] for conditioning municipal and industrial sludges and is finding wide use in Japan for sludge dewatering.

Treatment of suspensions of montmorillonite with partially deacetylated chitin in conjunction with alum and lime showed deacetylated chitin to be as effective as commercial flocculating agents.[357] Similar results were observed with kaolinite clay suspensions.

2.3.6. Analytical Applications

The use of chitin and chitosan in various forms of chromatography has been explored. Examples of general chromatographic applications of chitin have been reported by Iwata and Nakabayaski[358], who removed coloured substances from tea, coffee, apple juice, dry mushroom infusions, caramel, sugar and other solutions by column chromatography. Cellulose[359] and paper[360] impregnated with chitosan have been used in the ion exchange chromatography of nucleic acids.[95] Chitin powder has been used as a chromatographic support in thin layer chromatography for the separation of amino acids[361] and nucleic acids.[362] Using chitosan thin

layers, the chromatographic behaviour of nucleic acid constituents,[363] phenols[363] and inorganic ions[364] have been studied.

Chitin can be used as a specific adsorbent in affinity chromatography for the isolation,[365,367] purification, and concentration of lysozyme.[368] Improvement of the holding capacity and reproducibility of the adsorbent has been achieved by using carboxymethyl chitin and deaminated chitin.[365] Chitin columns equilibriated with hydrochloric acid at pH.6.8 have been used in chromatography of tobacco mosaic virus[369].

Chitosan[250] and hydroxyethyl chitosan[370,371] have been used in the colloidal titrations of pectin and pectic acid[250,370] and of lignosulphonic acid.[371] Trimethylammonium hydroxyethyl chitosan iodide has been used to titrate maleic acid-methyl vinyl ether co-polymers.[372] Since chitosan forms complexes with heavy metals it has been used for the determination of such metals in sewage, sea water and aqueous brines. It has also been used as a chromatographic solid carrier for the selective extraction and accumulation of mercury, cobalt, gold, antimony, silver, chromium, iron zinc, iridium, palladium, copper, cadmium, nickel and lead ions. The use of chitosan in the concentration of waste from nuclear fuels has been suggested because of its ability to retain its chelating properties after irradiation.[4,5]

2.3.7. Medical Applications

Chitosan and its derivatives have found wide ranging uses in the treatment and diagnosis of various diseases. This has been promoted, in part, by the low toxicity of chitosan even in high doses.[373]

The use of sulphochitin and sulphochitosan as blood anticoagulants has been the subject of several investigations. In terms of their activity they are comparable to the natural anticoagulant, heparin. A study of the influence of the structure of O- and N- sulpho derivatives of chitosan on its anticoagulation activity revealed that only when all the amino groups are sulphated is its full activity realised, and this increases rapidly with degree of O-sulphation.[283] In the intravenous administration of chitosan its molecular weight is important; a decrease in the latter increases the toxicity of the preparation.[157] By sulphating chitosan films anti-thrombogenic surfaces have been obtained.[193] Complexes of chitosan with heparin and dextran sulphate also exhibit anticoagulation activity.[274,374]

Chitosan's ant-acid activity[375] and its ability to suppress the activity of pepsin[376] has suggested the use of chitosan in the treatment of gastric ulcers. A formulation containing chitosan has been recommended[377] for use under conditions of increasing acidity. Chitin, chitosan and partially depolymerized chitin have been found to be higher in wound healing acceleratory activity than the standard acid-pepsin digested cartilage preparations.

Increases in the rate of wound healing were found to be in the range of + 30% - +75% depending on the source of the chitin.[378-383] Chitosan has been found to have anti-sceloracic activity due to the activation of lipoprotein lipase.[384,385] The influence of chitosan on the activities of enzymes has been observed in a number of cases; inhibition of deoxyribonuclease[386], and activation of hyaluronidase[387] and β-glucuronidase.[388] The complex salt of chitosan with the 1,4 lactone of D-glucaric acid also

exhibits physiological activity.[389]

Chitin, 6-O-carboxymethyl chitin, 6-O- hydroxyethyl chitin and 6-O-ethyl chitin have been prepared and used as enzyme-degradable pharmaceutical carriers.[169] The membranes prepared from the polymers carried pilocarpine nitrate and were slowly degraded by lysozyme, thereby releasing the drug at a controlled rate. A recent patent has suggested[390] the use of the same derivatives of chitin, and chitin itself, in the form of finely divided biodegradable powders, as lubricants for surgical gloves, tubing, and catheters.

Reports have also appeared recently of studies on the behaviour of chitosan in the inhibition of cell growth[386] and in the decomposition of cells [391] of certain types of malignant tumours. The complex of chitosan with iododeoxy-cytidylic acid penetrates cancer cells selectively, facilitating the diagonosis of cancer.[392] Chitosan causes the selective aggregation of the cells L 1210 leukaemia *in vitro*.[393]

2.3.8. Miscellaneous Applications

Microcrystalline chitin produced by controlled acid hydrolysis is suitable for use as a food thickener and stabilizer. The viscosity and emulsion stability of the products containing commercial microcrystalline chitin were found to be 10-20 times higher than those containing microcrystalline cellulose. This fact suggests applications in mayonnaise, peanut butter and other emulsion type foods. [394]

Powdered chitin suspended in a non-toxic, volatile, liquid carrier e.g. ethanol, has been used as a dry shampoo for cleaning hair.[395] Chitosan salt solutions, such as the acetate, have been used in conjunction with acetic acid, sorbic acid, and water as hair setting lotions.[396]

O-Carboxyalkyl chitin, N-carboxyalkyl chitosan, and N-carboxyacyl chitosan have been suggested as suitable agents for removing fog from transparent surfaces, such as condensation on car windows.[397] The chill haze from beers has been removed by papain immobilized on chitin.[398]

Finely ground chitin is used in the cosmetic industry as an additive to creams and gels and other cosmetic agents.[399] Chitosan has been used in the food industry to clarify plant juices and extracts[400] and cane sugar solutions.[401] Carboxymethyl chitin, carboxylethyl chitin and chitin sulphate have been used in the mining industry for the preparation of drilling compositions.[346]

A method has also been described for filtering fluids through a filtration bed of chitosan.[402] Phenols, organic acids, aldehydes, and negatively charged particles can be removed from liquid mixtures and from smokes by this technique. When used as a cigarette filter material it reduces the level of tar in the smoke considerably.

A study has been made of the use of chitosan in tanning, paste drying, and finishing of leather.[403] The results are encouraging; chitosan having good potential as a filling material for poor quality leather. In the field of reconstituted tobacco sheet[265] chitosan has been found to give good smoking characteristics such as burn, aroma, taste, and ash to tobacco compositions.

Chitin has been used as a solid support for immobilized enzymes such as acid phosphatase, α-glucoamylase, chymotrypsin, glucose isomerase, β-galactosidase, D-glucose oxidase, trypsin, subtilisin. pronase, yeast invertase and papain.[264,267,268,398,404-411]

The amine groups present on these polymers can be of help for anchoring bridge molecules, like glutaraldehyde, for the purpose of establishing covalent bonds with the enzymes.

A recent review of new applications of natural polysaccharides including chitin and its derivatives has been published.[412]

Chapter 3 Results and Discussion

Part A Heterogeneous Acylation Studies

3.1 Acylation of Chitin and Chitosan

3.1.1. General

Drastic conditions have normally been employed in the acetylation of chitin and chitosan. Chitin has been acetylated[210] by treatment with acetic anhydride and dry hydrogen chloride gas for 120 hours at room temperature to give a product with a degree of substitution (D.S.) of 2.99. The use of acetic acid and acetic anhydride in the presence of zinc chloride gives a highly degraded chitin acetate product with a D.S. of 2.5.[71] Chitin dissolved in phosphoric acid was acetylated[227] with perchloric acid as catalyst for 4 hours at 75°C to give a product with a D.S. of 1.8. Early reports of the acetylation of chitosan with acetic anhydride at 100-135°C in a sealed tube, claimed the product to resemble chitin but to be highly degraded.[143,144] A very recent paper has reported[228] the acetylation of chitin to varying degrees by reaction with a mixture of acetic anhydride and methane sulphonic acid for 16 hours at 0°C.

The chitosan used in this work was supplied in flake form and was found to be of a fairly intractable nature. Early attempts were made to acetylate it in this form using conventional cellulose acetylation techniques,[413] with

acetic anhydride/glacial acetic acid mixtures and perchloric acid (85%) as catalyst. Variations in reaction time and temperature were tried but even with reaction times in excess of 120 hours, no success was achieved.

The failure of the reaction was attributed to the nature of the starting material and so,in an attempt to obtain a more open structure, the chitosan flake was dissolved in aqueous acetic acid and reprecipitated in ammonical methanol. The fluffy white polymer was acetylated under the same conditions as before, but again little success was achieved.

Chitosan film was then tried as in this form the polymer should be accessible,and had the advantage that the reactions could be easily followed by infrared spectroscopy. Samples of desalted film were placed in solutions of glacial acetic acid/acetic anhydride/ perchloric acid. They were removed at various time intervals and the extent of reaction checked. Little reaction was observed even when reaction times were extended beyond 120 hours.

Two possible explanations for this lack of reactivity were considered to be i) the limited accessibility of the functional groups,due to the close packing of the chains, restricts the extent of reaction and ii) protonation of the amine groups, due to the acidic nature of the reaction medium, causes a reduction in the extent of their acetylation and in the extent of O-acetylation through destabilisation of the transition state structures required for the acetylation of the adjacent C(3) and C(6') hydroxyl groups.

Various attempts were made to overcome these problems. Acetylation was attempted on the cast polymer film before it had dried by steeping the glass plate, with the gelled film on it, in the acetylating mixture. No

satisfactory results were obtained as the method proved to be unworkable.

Steeping the chitosan film in various methanol/aqueous acetic acid mixtures prior to acetylation was also unsuccessful for similar reasons. Immersion of the film in distilled water and then in methanol and glacial acetic acid before reacting in the acetylating medium showed promise, with some O-acetylation evident, Figure 7 . Presteeping the film in methanol for 300 hours and then placing in acetic anhydride gave N- acetylation with some O-acetylation, Figure 8 . Treatment with acetic anhydride at room temperature for 120 hours followed by refluxing the film in acetic anhydride gave good N-acetylation and some O-acetylation, Figure 9. However extension of reaction times gave no noticable increase in the degree of acetylation, nor did the use of triethylamine as an acid scavenger.

3.1.2. N-Acetylation of Chitosan in Film Form

3.1.2.1. General

In order to obtain chitin, N-acetyl chitosan, in a workable form such as a film, it is necessary to de-N-acetylate it to afford chitosan, which readily forms films when cast from aqueous carboxylic acid solutions, and re-N-acetylate. Yaku and Tamashita have reported [184] the acetylation of chitosan films with acetic acid and dicyclohexylcarbodiimide (DCC) in aqueous DMF but the acetylation was incomplete, giving only a partially N-acetylated chitosan. Pure chitin has been prepared recently[213] by acetylating 50% deacetylated chitosan with acetic anhydride/pyridine or with acetic acid/DCC mixtures. However, in both cases, the 50% deacetylated

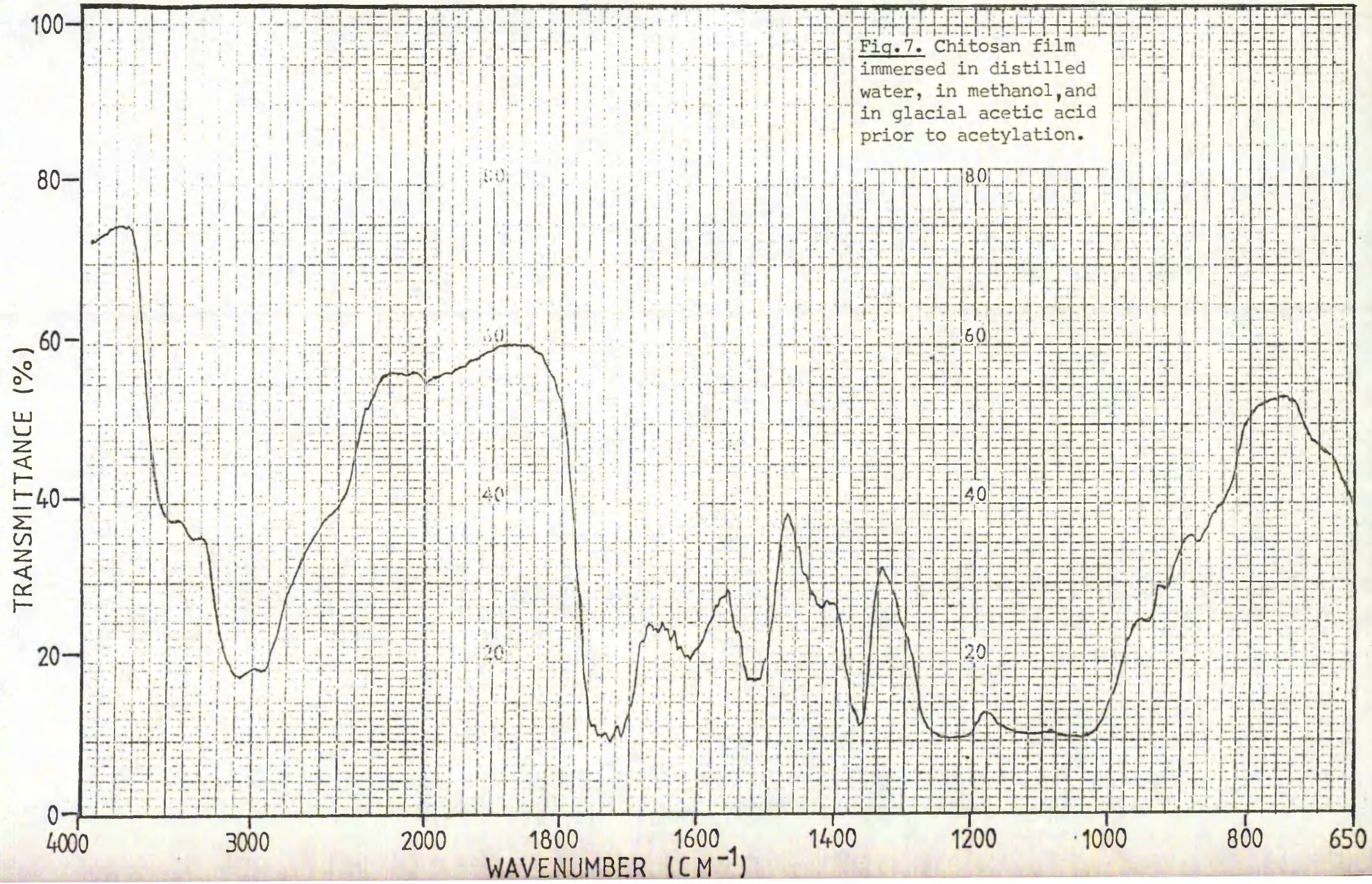


Fig.7. Chitosan film immersed in distilled water, in methanol, and in glacial acetic acid prior to acetylation.

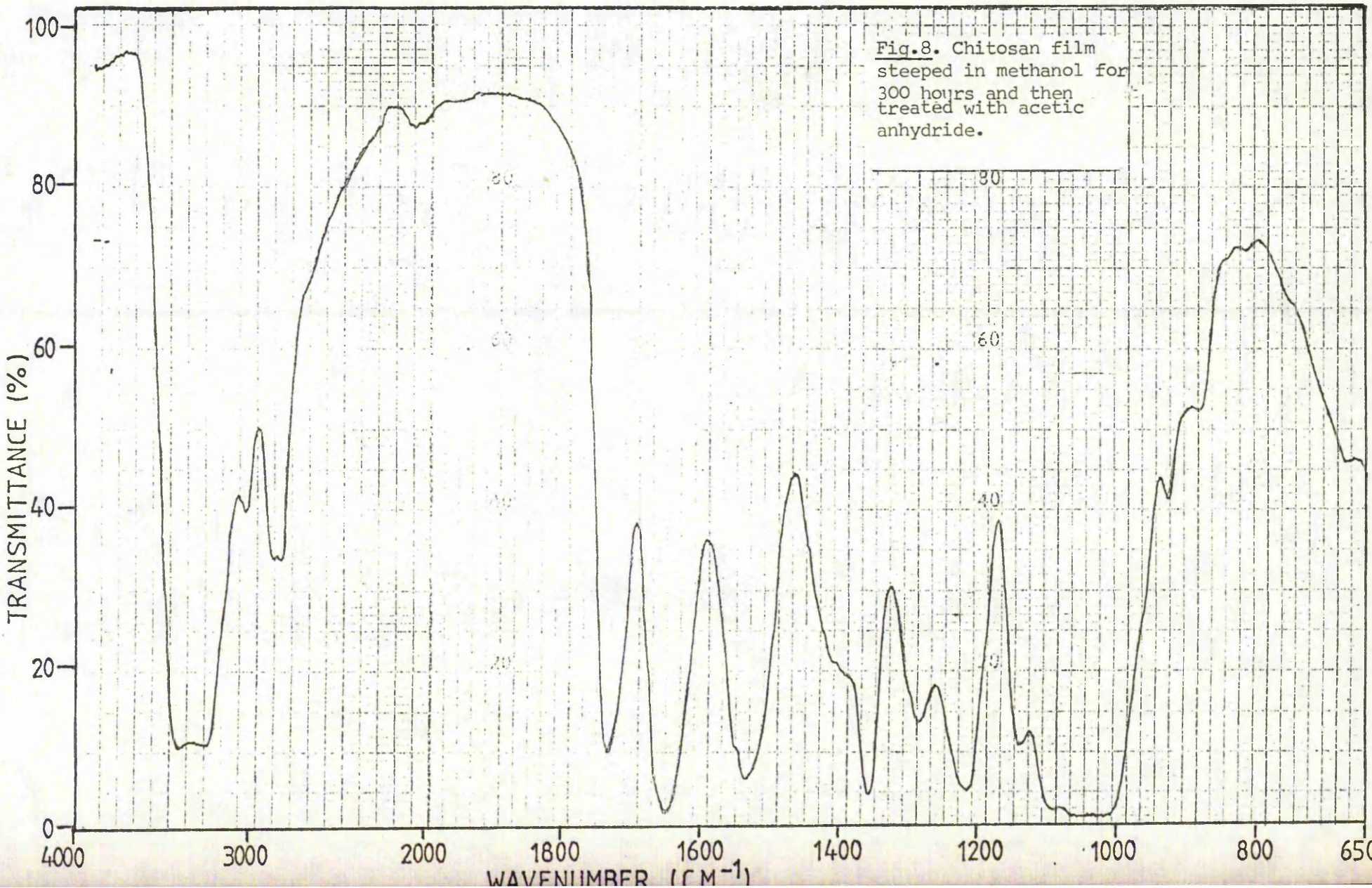


Fig.8. Chitosan film steeped in methanol for 300 hours and then treated with acetic anhydride.

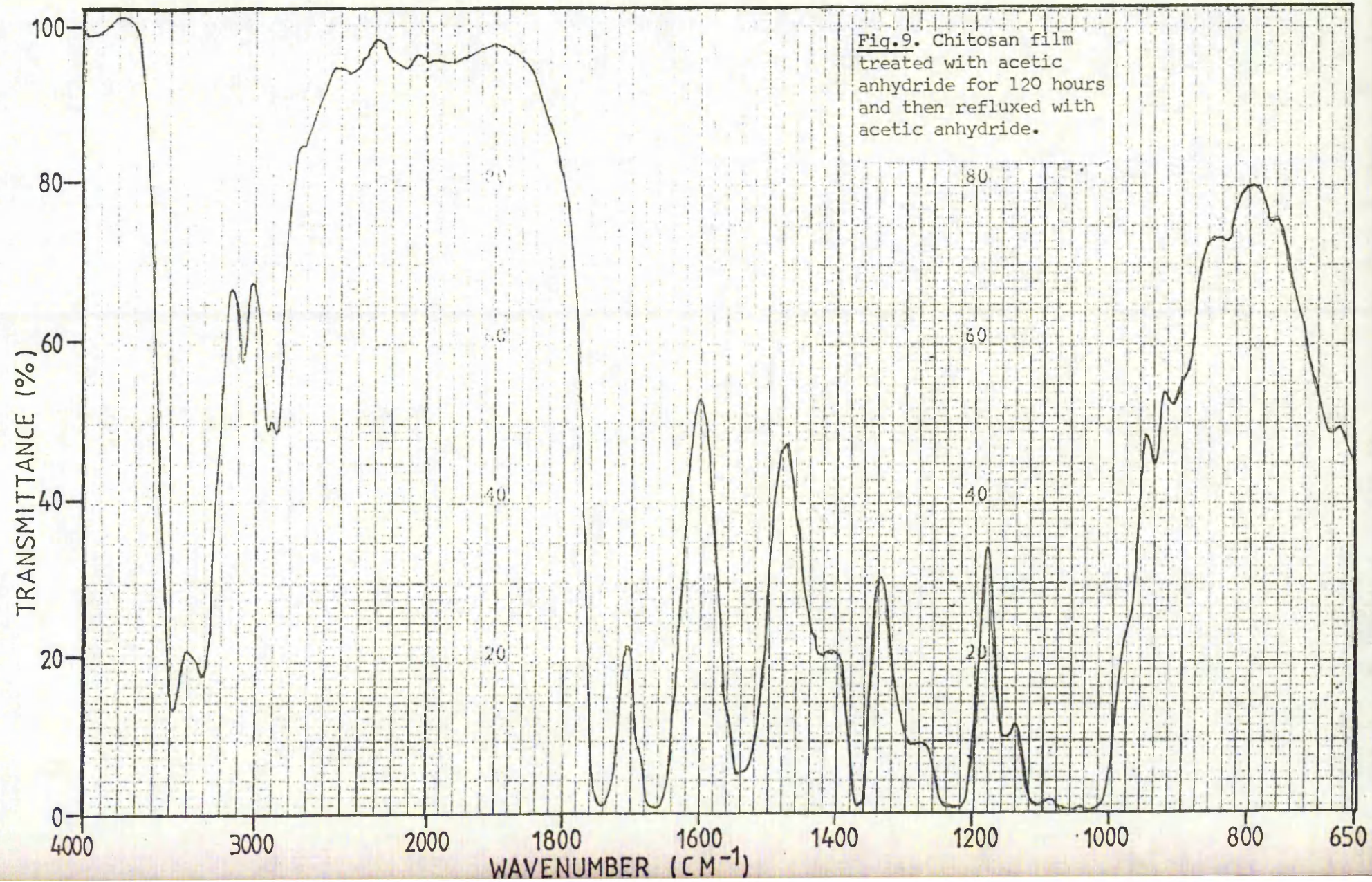


Fig.9. Chitosan film treated with acetic anhydride for 120 hours and then refluxed with acetic anhydride.

water-soluble chitosan has to be prepared first by treatment in alkali for about 65 hours.

Methanol is well known as a reaction medium for selective N- acetylation in the presence of hydroxyl groups and from the earlier work on acetylation it was shown to be the most promising of the solvent systems tried. The presence of methanol as a co-solvent in the facile acetylation of chitosan with acetic anhydride in aqueous acetic acid/methanol solutions has been reported.[255-260] N-Acetyl chitosan gels and partially O-acetylated N-acetyl chitosan gels were obtained depending on the reaction conditions used.

3.1.2.2. Methanol/acetic anhydride solutions.

To further investigate the use of methanol in the acetylation of chitosan films, two pieces of film were prepared. One was used with no further treatment and the other refluxed in methanol prior to use. Each piece was divided into three and reacted in the following acetylating media:-
i) acetic anhydride alone; ii) acetic anhydride and triethylamine; and iii) acetic anhydride and methanol. The reactions were carried out at room temperature for 24 and 48 hours, and followed by infrared spectroscopy. The results are summarised in Table 13.

Table 13 Effect of differing acetylation media on extent of N-acetylation of chitosan film

Film	Acetylating Media	24 hours	48 hours
Untreated	Acetic anhydride	No evidence of acetylation	No change
	Acetic anhydride/ triethylamine	No evidence of acetylation	No change
	Acetic anhydride/ methanol	Good N-acetylation; some O-acetylation	Further O-acetylation
Film refluxed in methanol prior to use	Acetic anhydride	No evidence of acetylation	No change
	Acetic anhydride/ triethylamine	No evidence of acetylation	No change
	Acetic anhydride/ methanol	Good N-acetylation; Some O-acetylation	Some increase in O-acetylation

Figures 10,11 and 12 show the infrared spectra of the film, refluxed in methanol prior to use, after acetylation in the different media for 48 hours. It is very evident that in the absence of methanol no reaction occurs, whilst in the presence of methanol N-acetylation occurred together with limited O-acetylation.

Initial experiments with methanol/acetic anhydride mixtures showed that oomplete N-acetylation was obtained in less than 12 hours at room temperature. O-acetylation occurred to a small extent with increased reaction times. The O-acetyl groups formed could be readily and preferentially hydrolysed by treatment of the film in ethanolic potassium hydroxide solution (0.5M) for 16 hours at room temperature.

3.1.2.3. Influence of Solvent Media on N-Acetylation

It was evident from the initial work that the reaction media is important in the N-acetylation of chitosan. A range of organic solvents was therefore examined to determine their influence on N-acetylation. The solvents studied included alcohols up to hexanol, glacial acetic acid, DMF, formamide, pyridine, DMSO, various ethers and chlorinated hydrocarbons. A full list is given in Appendix V.

Samples of desalted chitosan film were left steeping in acetic anhydride: solvent mixtures (1:2) for 24 hours at room temperature. The films were then removed, washed well in methanol and dried. Examination of the films by infrared spectroscopy showed that only in methanol and formamide did any appreciable N-acetylation occur. Thus it would appear that N-acetylation

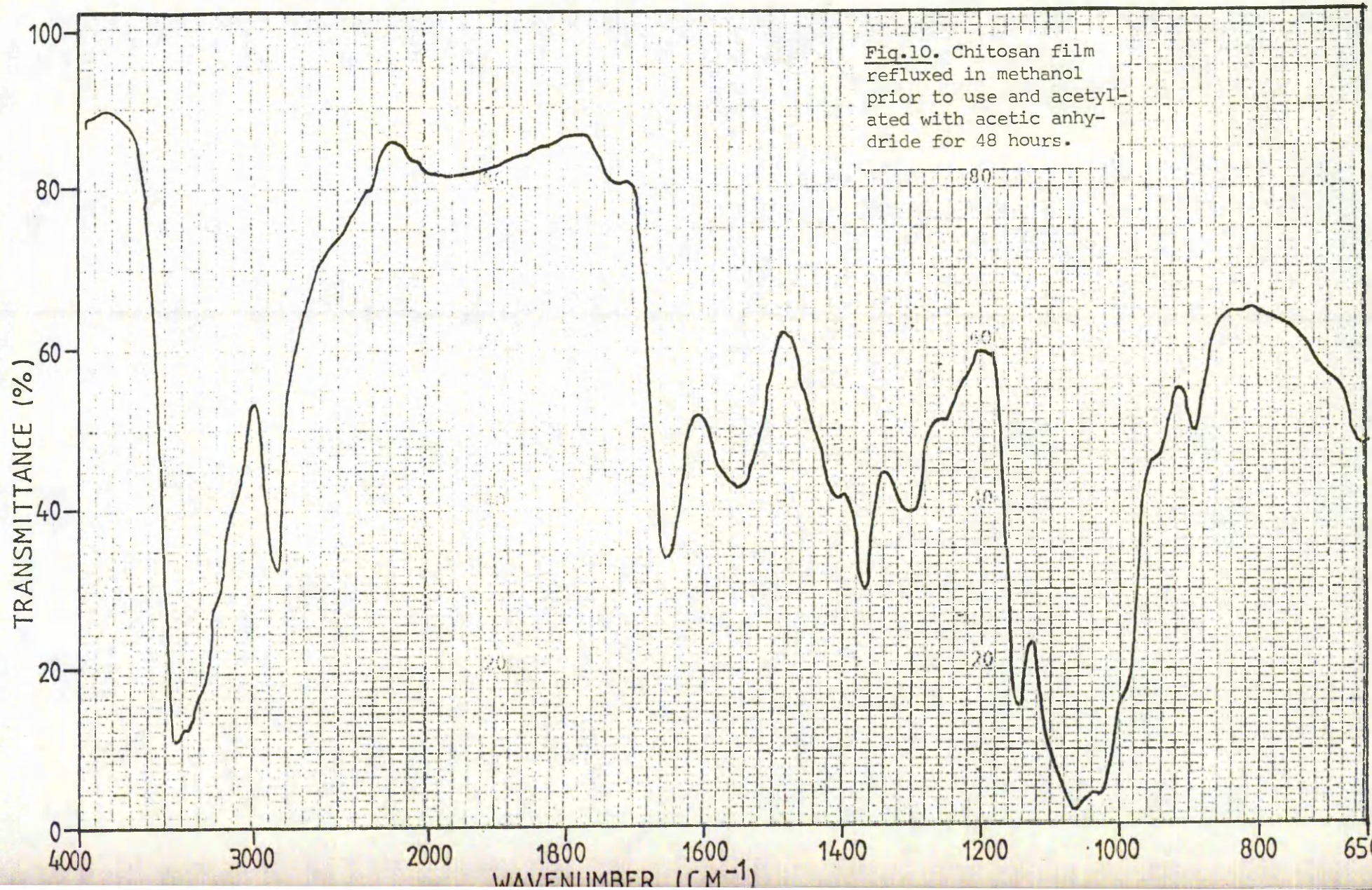


Fig.10. Chitosan film refluxed in methanol prior to use and acetylated with acetic anhydride for 48 hours.

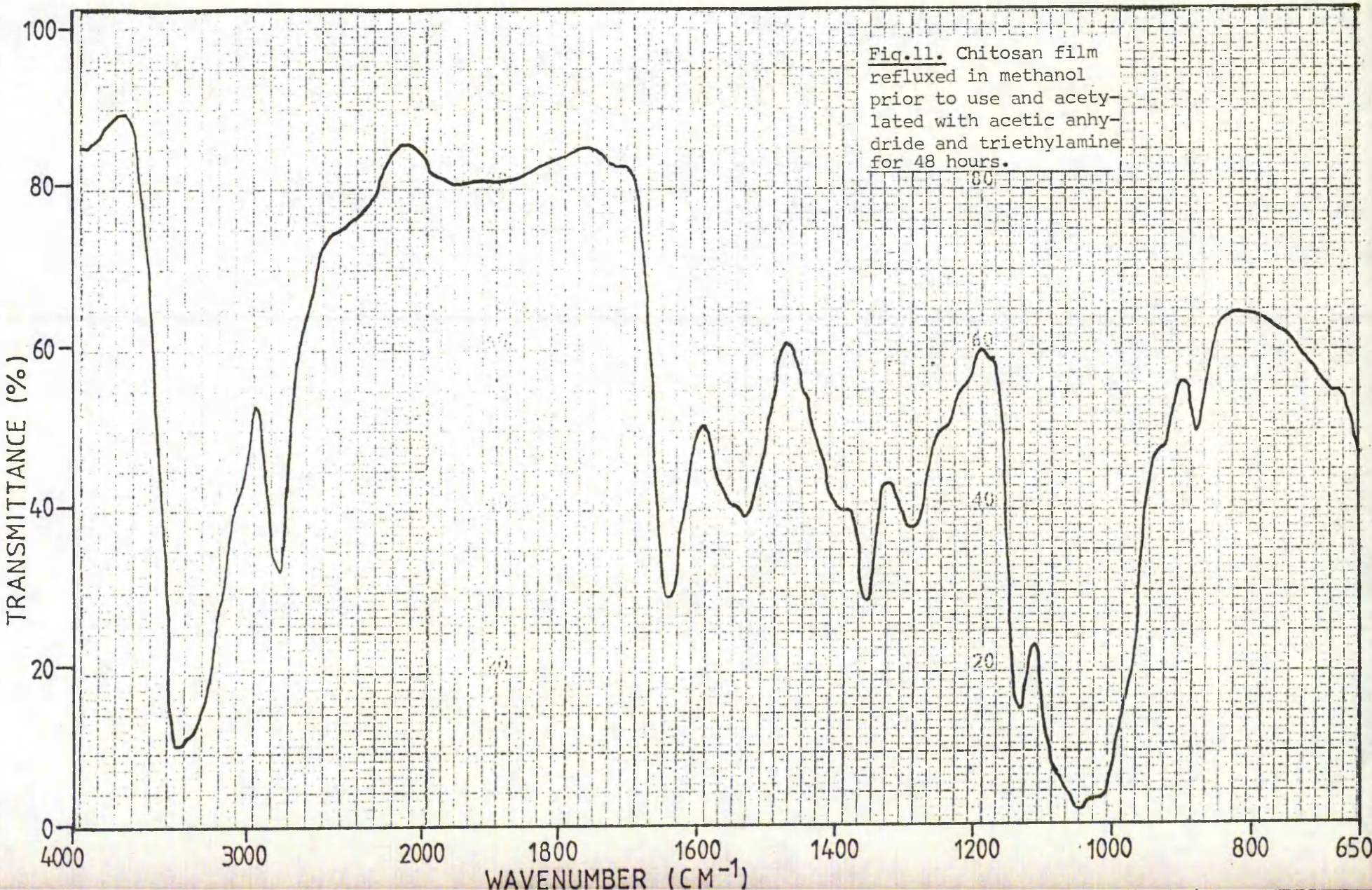


Fig.11. Chitosan film refluxed in methanol prior to use and acetylated with acetic anhydride and triethylamine for 48 hours.

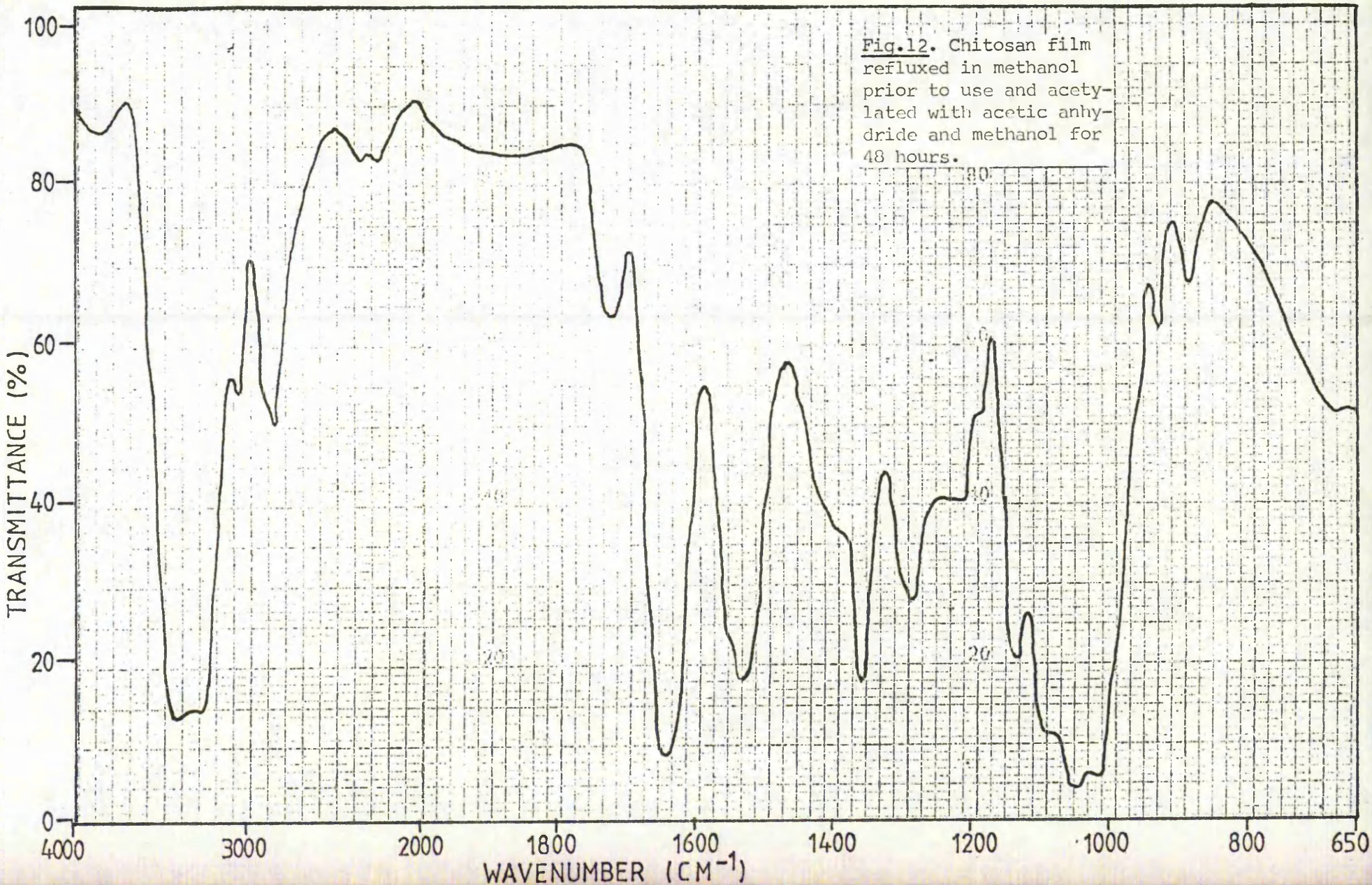


Fig.12. Chitosan film refluxed in methanol prior to use and acetylated with acetic anhydride and methanol for 48 hours.

would only take place in a reaction media having a solubility parameter value greater than 13 Hildebrands. To investigate this further, a series of binary mixtures of ethanol/methanol and of methanol/formamide were prepared to cover the solubility parameter range 12.05 to 15.3 Hildebrands. All the solubility parameter values have been adjusted to take into account the contribution of the acetic anhydride present. Samples of chitosan film were left to steep in acetic anhydride: binary solvent mixtures (1:2) for 30and 60 minutes, at 25°C. The films were removed, washed well in methanol, dried, and the extent of reaction determined by infrared spectroscopy. The extent of acetylation was calculated by measuring the increase in the absorbance of the amide I band at 1655 cm^{-1}, using the absorbance of the hydroxyl band at 3450 cm^{-1} as an internal standard to take into account variations in film thickness.

Figure 13 is a plot of the extent of N — acetylation after 30 and 60 minutes reaction time, expressed as a percentage of amine groups acetylated, against the solubility parameter values of the reaction media.
It can be seen that there is a very considerable increase in the rate of N-acetylation in reaction media having solubility parameter values in the range $12.7 < \delta < 14.1$ and it reaches a maximum at $12.9 < \delta < 13.5$.

A series of binary mixtures to cover the same solubility range were then made with ethanol and formamide only. The experiment was repeated as before. From the results shown in Figure 14 it can be seen that very little reaction occurs in any of the solvent mixtures at 30 minutes. There is some increase in the percentage of amine groups acetylated as the solubility parameter value reaches $14.3 < \delta < 14.8$. The results at

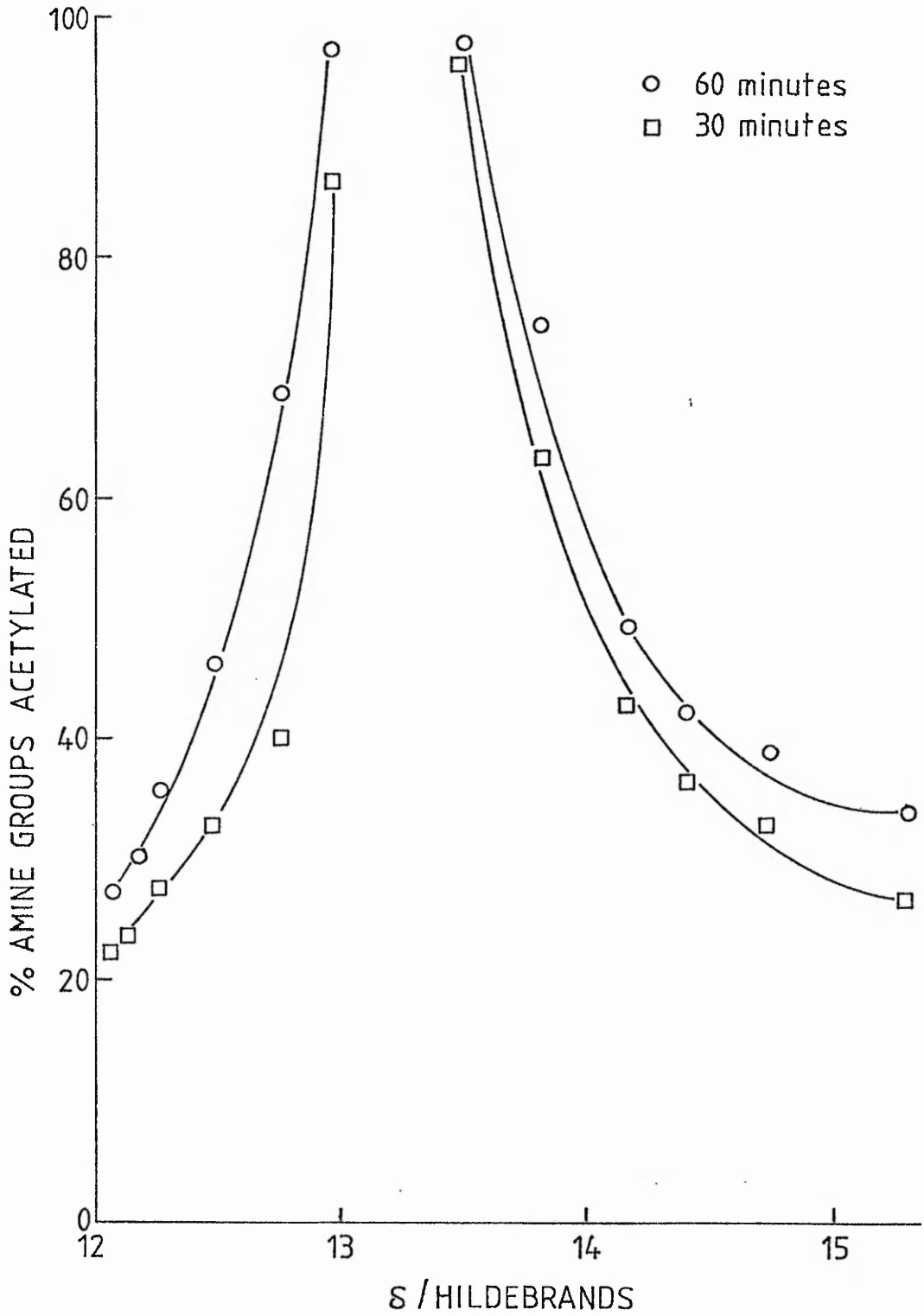


Figure 13. The extent of N-acetylation of chitosan film in binary mixtures of ethanol/ methanol and methanol/formamide at 30 and 60 minutes reaction time.

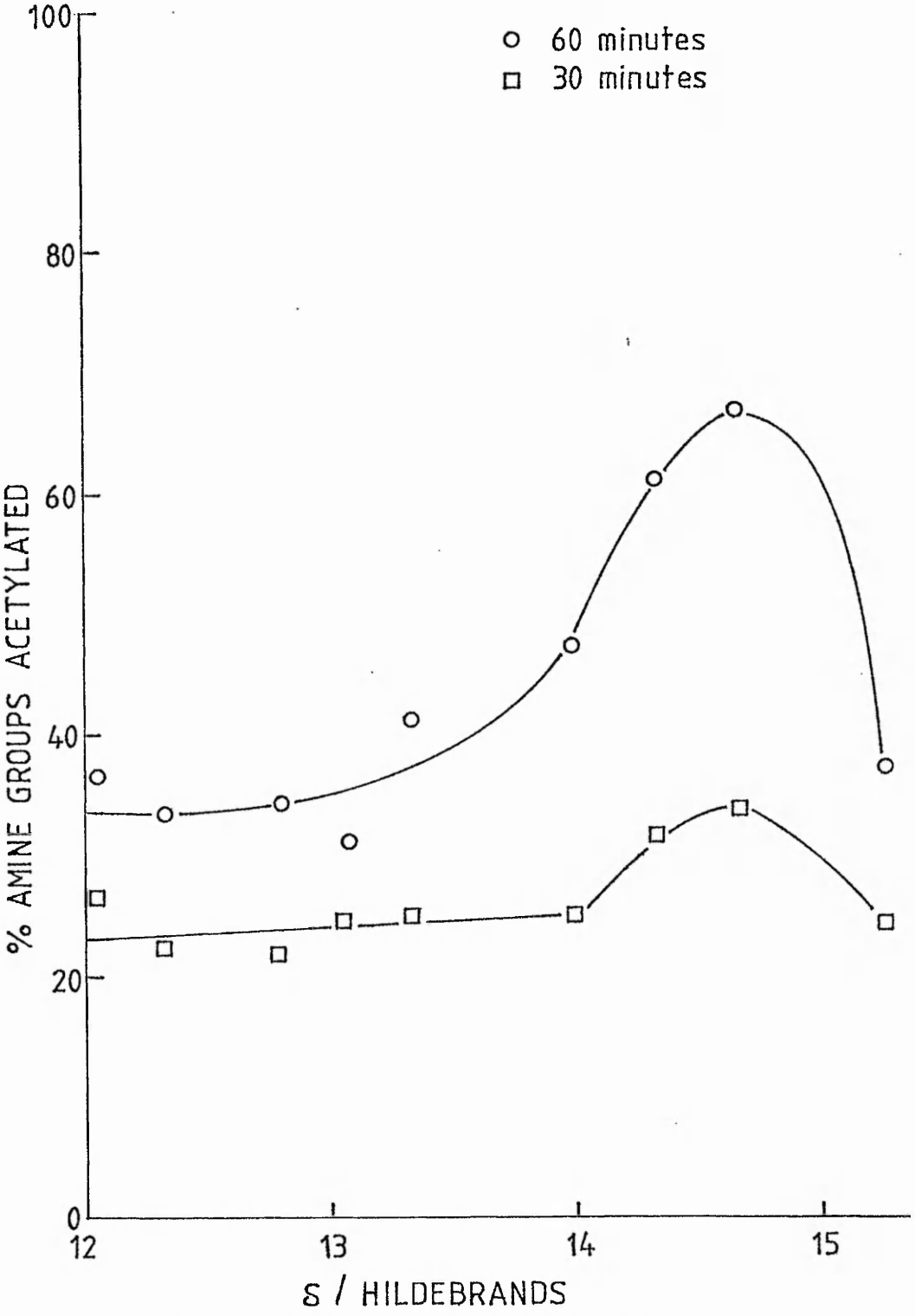


Figure 14. The extent of N-acetylation of chitosan film in binary mixtures of ethanol/formamide at 30 and 60 minutes reaction time.

60 minutes show a similar trend, again the largest number of amines acetylated occurs in the solubility parameter value range of $14.3 < \delta < 14.8$.

Comparison of these results with those obtained with ethanol-methanol and methanol-formamide, Figure 13 ; clearly show the marked influence of methanol in the N-acetylation reaction medium. For example, at $\delta = 13.54$ for the methanol- formamide and acetic anhydride solution, the degree of N-acetylation is equal to approximately 95% at 30 minutes, whereas at $\delta = 13.35$ with the ethanol-formamide and acetic anhydride solution, only 25% of the amine groups are acetylated after the same time period.

Binary mixtures of n-propanol and methanol were also prepared to study the effect of varying the alcohol in the binary mixtures. The same reaction conditions were used as before and the reaction carried out for 30 minutes. Figure 15 shows the percentage of amine groups acetylated against the solubility parameter values of the reaction media. The curve obtained shows a large increase in the number of amines acetylated as the solubility parameter value of the reaction media approaches 12.7 Hildebrands and parallels that obtained for ethanol-methanol mixtures, Figure 13.

It would appear that acetylation is dependent on the ability of the solvent media to swell the chitosan sufficiently for the reaction to take place. Hence to eliminate differential times of swelling the film by different solvents, the following experiments were carried out with the chitosan film presteeped for 24 hours in the given solvent system prior to use.

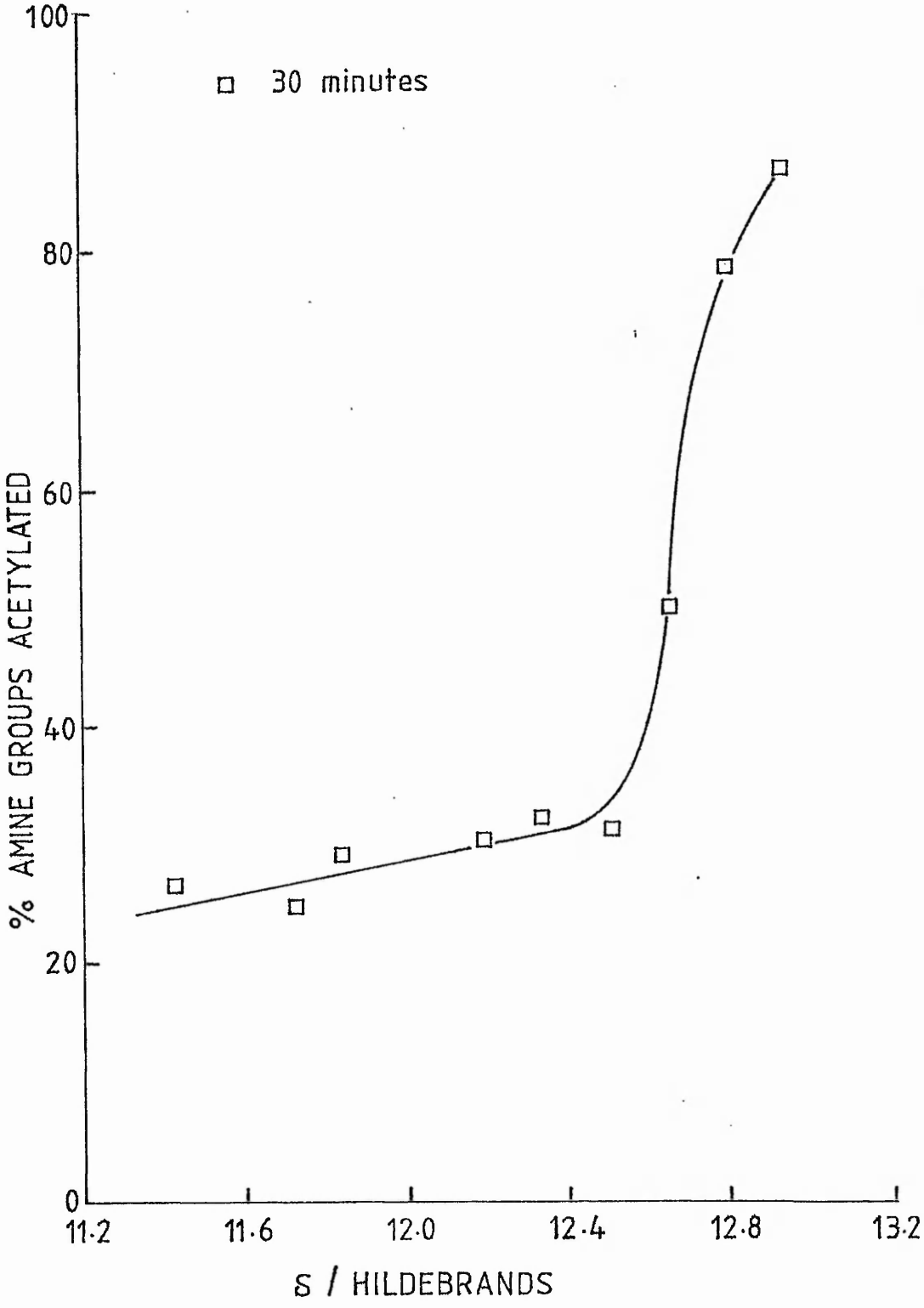


Figure 15. The extent of N-acetylation of chitosan film in binary mixtures of n-propanol/ methanol at 30 minutes reaction time.

Comparison was made of different alcohol/ formamide systems by preparing binary mixtures of methanol/formamide, ethanol/formamide, n-propanol/formamide and n-butanol/formamide. Samples of chitosan film were placed in each for 24 hours at 15°C. The lower temperature was adopted so that the reaction would be sufficiently retarded for monitoring purposes. Aliquots of acetic anhydride were added, in the ratio of 1:2 (acetic anhydride:solvent), and the reaction maintained with stirring for 5 minutes at 15°C in a shaking water bath. The films were then removed, rinsed in ice-cold methanol, dried, and the extent of reaction determined by infrared spectroscopy.

Figure 16 shows the percentage of amine groups acetylated versus the solubility parameter values of the reaction media for the different solvent systems. The solubility parameter values have again been adjusted to take into account the contribution of acetic anhydride present to maintain continuity. However, it may be more accurate in this case to consider only the solubility parameter values of the solvent-systems. The two possible solubility parameter-values for each result are given in the Appendix VI.

The effect of presteeping in the various solvent systems is clearly shown. Comparison of the curve obtained with ethanol-formamide mixtures as solvent with no presteeping, Figure 14, with that obtained with presteeping shows that once the solubility parameter of the solvent system increases towards that of methanol (δ= 1428), N-acetylation occurs at a much greater rate in the presteeped solvent media than in the non-presteeped solvent media.

It is evident in all cases that as the solubility parameter values approach that of methanol, the degree of N-acetylation in these solvent systems is

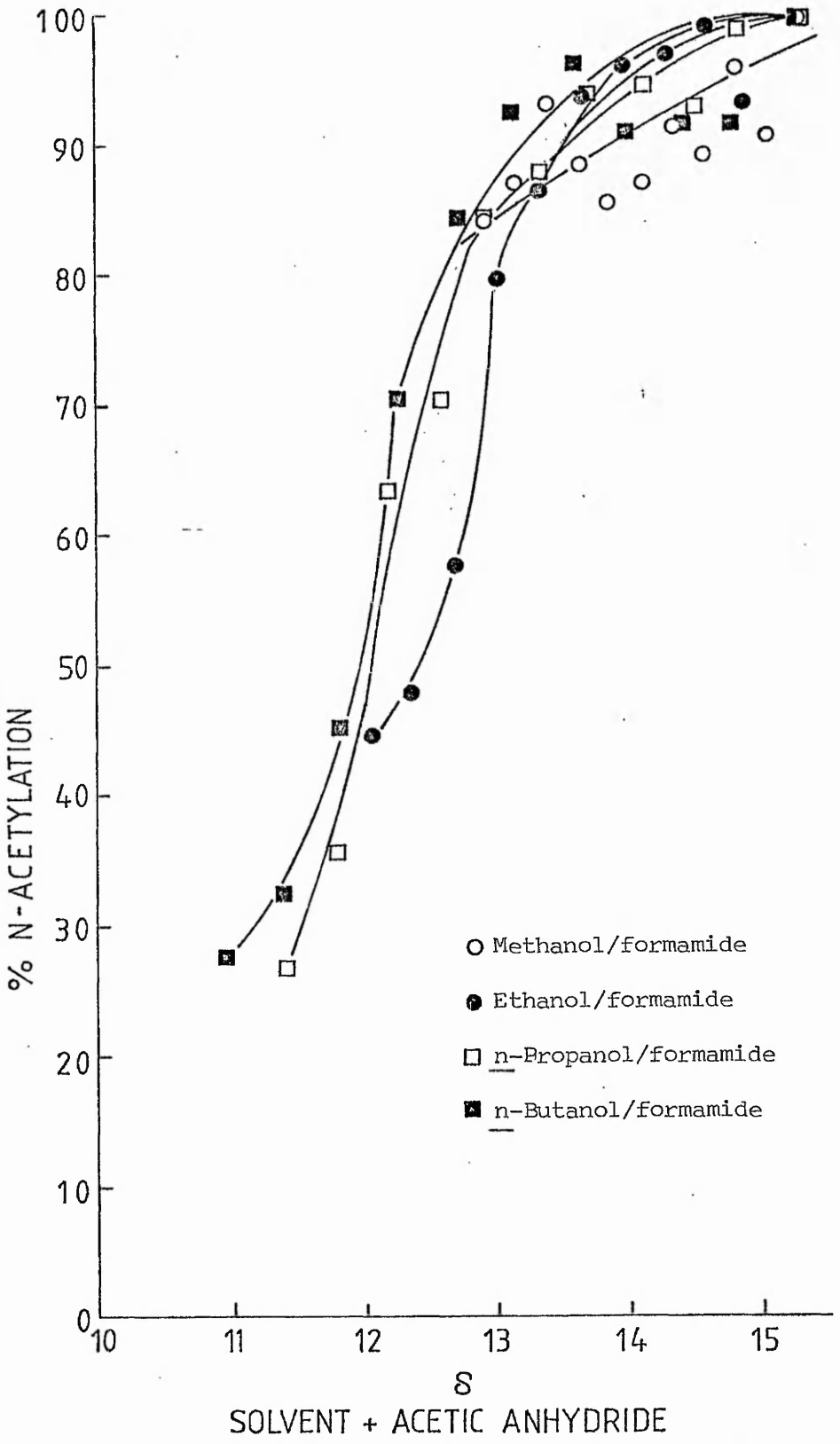


Figure 16. The extent of N-acetylation of chitosan film in different alcohol/ formamide systems at 5 minutes reaction time, 15°C.

similar to that in methanol. In ethanol alone, the degree of N-acetylation was 44.4%, whilst in n-propanol and n-butanol the figures obtained were 26.7% and 27.5% respectively,which is just above that of the starting material, 25%. In formamide virtually all the available amines were acetylated in the course of 5 minutes, whereas in methanol, only 85% were acetylated.

It is clear, therefore, that N-acetylation of chitosan film is very dependent on the nature and solubility parameter value of the reaction media. Diffusion of the solvent into the polymer is a controlling factor in the N—acetylation reaction, and presteeping the film in the chosen solvent-system before reaction is advantageous, and leads to much shorter reaction times. Thus given sufficient time, solvent systems of similar solubility parameter values to that of methanol, (δ=14.28) will allow N-acetylation of chitosan to occur to the same degree. Methanol seems to be unique in that the diffusion time required to penetrate the polymer is very short and readily allows N-acetylation whether presteeped or not. Chitosan presteeped in formamide appears to give a higher degree of acetylation over short time periods than methanol, but the overall effect may not be as great as it appears due to the higher percentage errors involved with the larger absorbances at 1655 cm^{-1} in the infrared spectrum.

3.1.2.4. N-Acylation of Chitosan in Film Form

Using the method for the N-acetylation of chitosan film, a large range of N-acyl derivatives were prepared from chitosan film. Initially linear

aliphatic carboxylic acid anhydrides, up to n—decanoic anhydride, were taken and reacted with the film in methanol. The films were checked periodically by infrared spectroscopy for completion of reaction. Reaction times varied but increased with increasing molecular weight of the anhydride, and it was found that with the higher fatty acid anhydrides, C6 and above, the reaction mixtures had to be refluxed to afford complete reaction. With the large excess of anhydride used in each case some O-acylation was observed in all cases. De-O-acylation was carried out by steeping the film in ethanolic potassium hydroxide (0.5M). The infrared spectra were all similar, except for the absorptions at 2990 cm^{-1}, which increased considerably with increasing molecular weight of the anhydride.

Several branched aliphatic carboxylic acid anhydride derivatives were prepared by similar methods. These included N-iso-butyryl—, N-iso-valeryl—, N-pivalyl-, and N-3,3— dimethylbutyryl chitosans. The N—3,3- dimethylbutyryl derivative had to be prepared in formamide due to the rapid hydrolysis of the anhydride in methanolic solutions.

The following aromatic N-acyl derivatives were also made, N-benzoyl, N-o-, N-m-, and N-p-toluyl chitosans. These were all prepared by refluxing the film in methanol for 120 hours, the methanolic anhydride solutions being replaced periodically as the anhydrides decomposed.

3.1.2.5. Studies on the Rates of N-Acylation of Chitosan in Film Form

A comparison of the rates of N-acylation of chitosan film by several carboxylic acid anhydrides, with methanol as the reaction medium, was then made. The acid anhydrides used were acetic, propionic, butyric, hexanoic and benzoic. The reactions were followed by monitoring the change with

time of absorbance of the amide I band at 1655 cm^{-1}, until acylation was complete. The absorbance of the 3450 cm^{-1} band was used as an internal standard.

It was evident that apart from changes in their intensity the majority of the absorption bands in the infrared spectrum of chitosan are unaffected by increasing the extent of N-acylation. A major exception to this is the amide II band at 1595 cm^{-1} which shifts to 1550 cm^{-1} as N-acylation proceeds, Figure 17. The presence of hydrogen bonding in simple secondary amides[429] has been found to increase the absorption frequency of the amide II band and this shift to lower frequencies as N-acylation proceeds indicates gradual disruption of hydrogen bonding, involving the amide group, present in the original chitosan film.

The results for the reaction of butyric anhydride are shown in Figure 18 and similar rate curves were obtained for the other linear aliphatic anhydrides. The extent of N-butyrylation is expressed as At-Ao where At and Ao are the absorbances of the amide I band at time t and time t=0 respectively, both values being corrected for film thickness. There is evidence of an induction period for the reaction which is particularly prominent in the rate curve obtained at 25°C. In general the induction period increases with increasing size of the acid anhydride molecule and decreases with increases in temperature. All the aliphatic acid anhydrides required approximately similar reaction times for complete N-acylation, of the order of 10-12 hours, but benzoic anhydride was considerably slower and reaction times in excess of 72 hours were required for full N-benzoylation at 25°C.

The rates of N-acylation determined in this way gave reasonable results

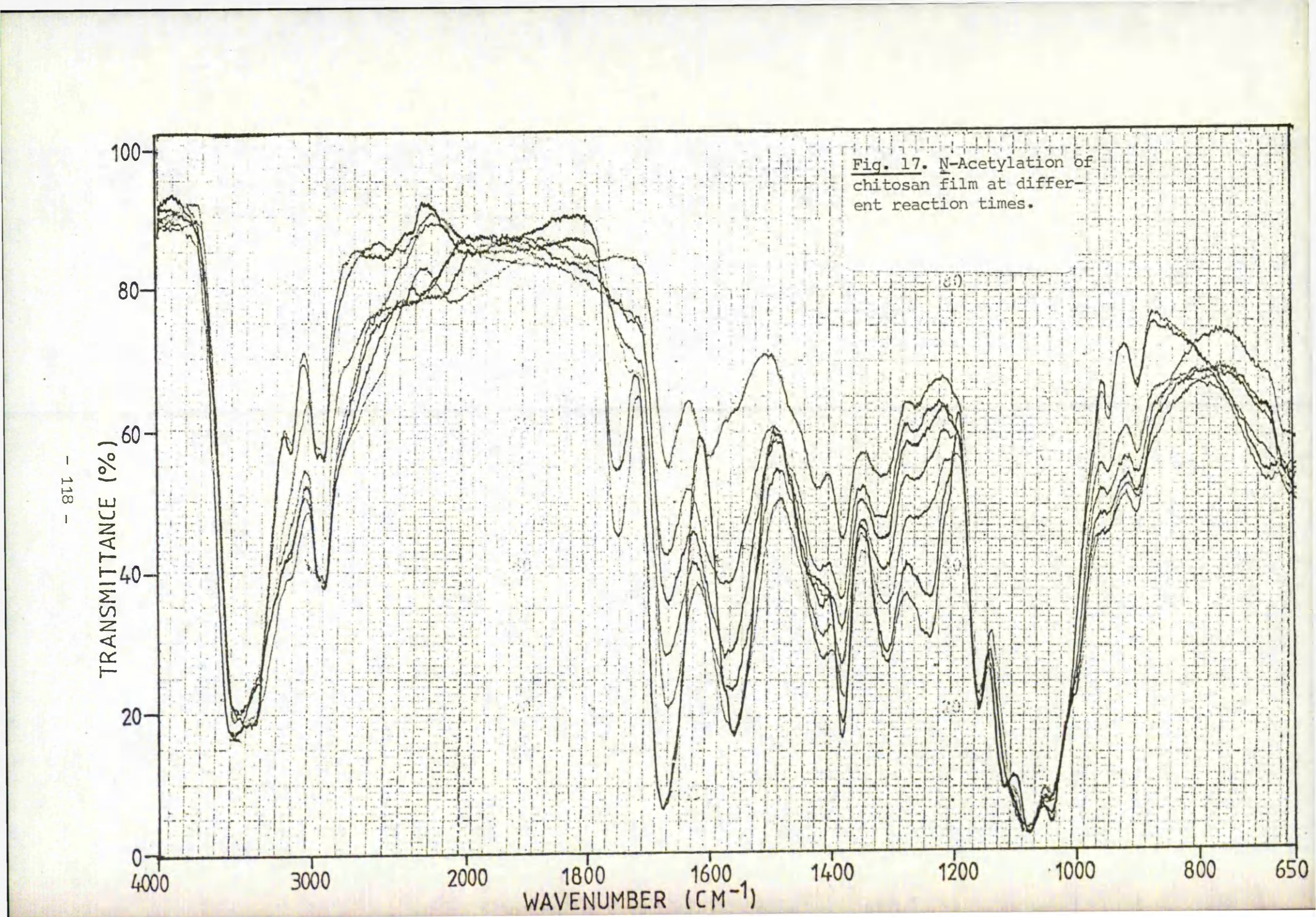


Fig. 17. N-Acetylation of chitosan film at different reaction times.

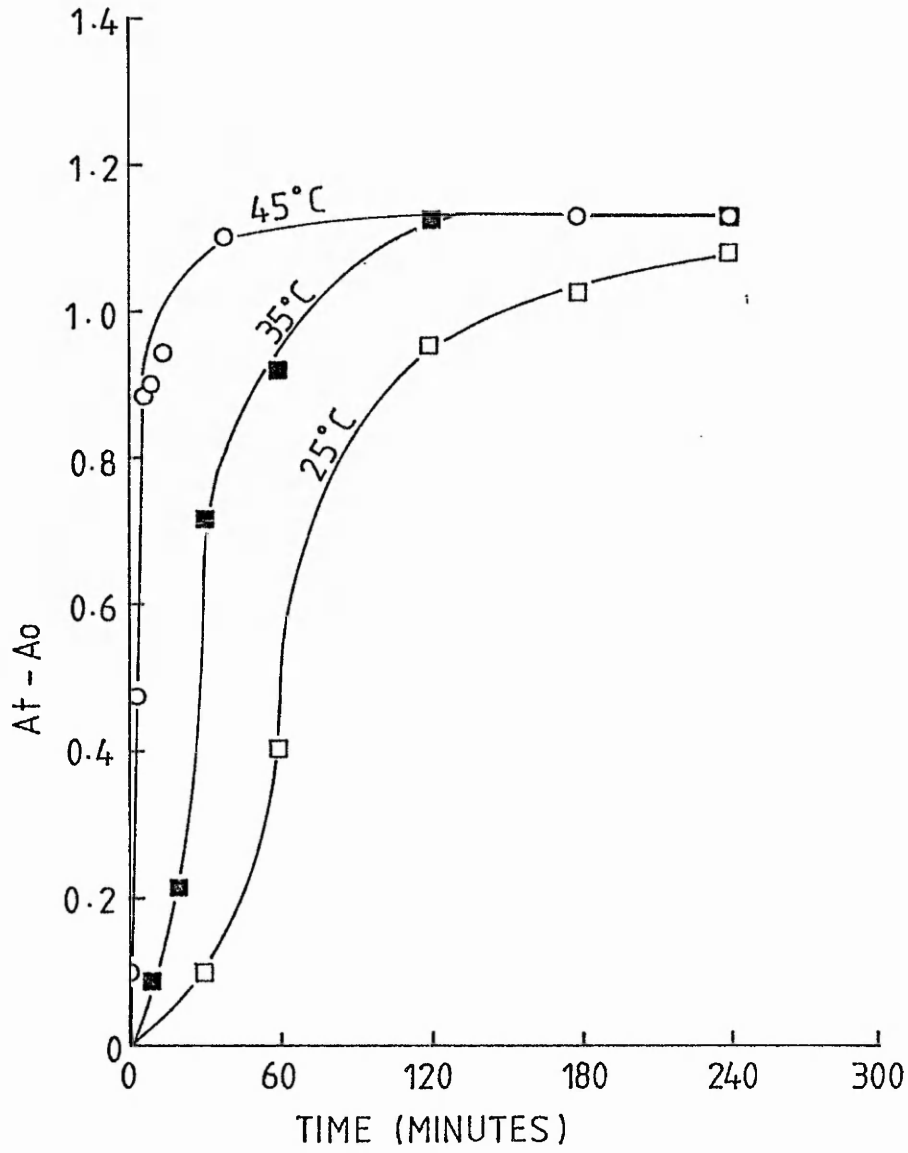


Figure 18. Rates of reaction for the N-butyrylation of chitosan film at 25, 35 and 45 ^{o}C.

for comparitive purposes, but it was felt improvements were necessary if energies of activation, Ea, were to be calculated with any degree of accuracy from the measured rates. However very approximate values were calculated from the measured rates at 25°C, 35°C and 45°C. These were of the order of 95-90kJ mol^{-1} for acetic, propionic and butyric anhydrides, and about 75 kJ mol^{-1} for hexanoic anhydride. Although the values for acetic, propionic, and butyric are close together, they do form, together with that for hexanoic anhydride, a series in which the energy of activation decreases with increase in size of the anhydride molecule.

It was found that pretreatment of the film samples in methanol for 24 hours at the temperature at which acylation was to be carried out, completely eliminated the induction period. Thus the induction period was due to the time required for the methanol to swell the film before diffusion of the anhydride into chitosan could take place.

In an attempt to improve the accuracy of measuring the rates of reaction and hence to obtain a more accurate energy of activation for the process, the following modifications were made. Individual samples of film presteeped in methanol for 16 hours were used for each measurement, and each measurement timed from the point of addition of the anhydride to that of the first washing. All film samples were washed well with methanol at less than 5°C to ensure that the reaction was stopped as quickly as possible.

The results obtained were disappointing; Figure 19 shows the rate curve obtained for the N—acetylation of chitosan film at 20°C and it is very evident that there is a large scattering of the results. This is mirrored in the results obtained at 15°C, 25°C and 30°C. The scattering of the

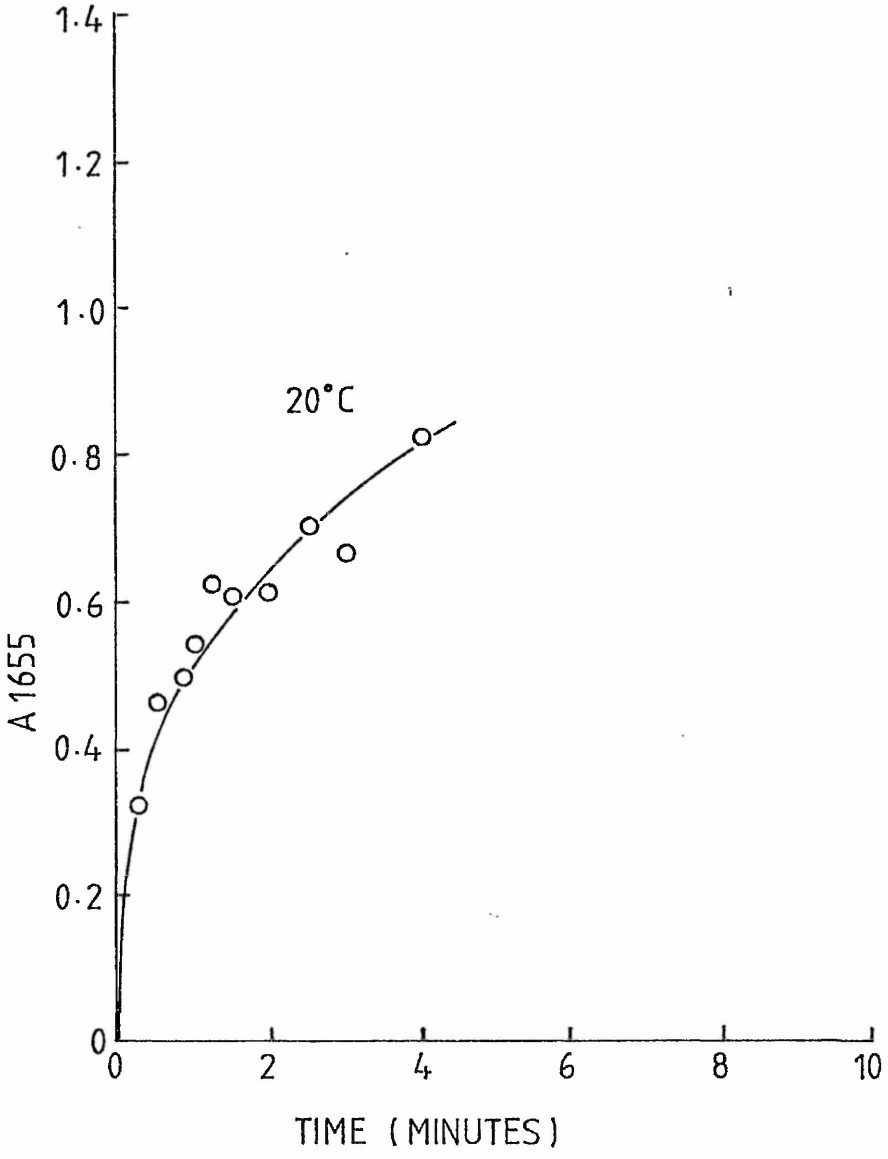


Figure 19. Rate of reaction for the N-acetylation of chitosan film at 20°C.

results would appear to be inherent, due to the nature of the starting material, i.e. the chitosan film. The method of casting the film is such that variability in the thickness of the polymer film samples exists, which would lead to uneveness of reaction across the sample. Further, the effect of evaporation of the polymer solution will lead to differential drying across the film. In addition, the technique of stopping the reaction may also be suspect, as acylation may continue beyond the time recorded. Thus the results at best should be treated only as an approximate picture and the energy of activation as a guide.

To determine the Ea of the N-acetylation of chitosan film from these results, it was decided to plot log $(^{1}/t)$ versus $^{1}/T$, where t=time taken to reach 60% N-acetylation, and T= temperature, degrees absolute. This was used since i) the rate curves that could be drawn through the points obtained were suspect and hence the values of the slopes would be suspect, and ii) the percentage N-acetylation taken would then enable some comparison to be made with the work carried on gelation studies of N-acyl chitosans (3.7.6.4.)

Figure 20 is the plot of $\log(^{1}/t)$ against $^{1}/T$ and the slope = -3.024×10^{3}, and hence Ea = 57.9 kJ mol^{-1} for the N-acetylation of chitosan film. However, from the standard deviation of the slope there is an error of $\pm$ 38%, which again emphasises the inherent inaccuracies involved in the technique.

Attempts to repeat the experiment using hexanoic anhydride were unsuccessful. Little or no reaction was observed and it is possible that insufficient anhydride was present and hence the anhydride was reacting preferentially with the methanol. Insufficient time was available to repeat this experiment again using larger volumes of hexanoic anhydride.

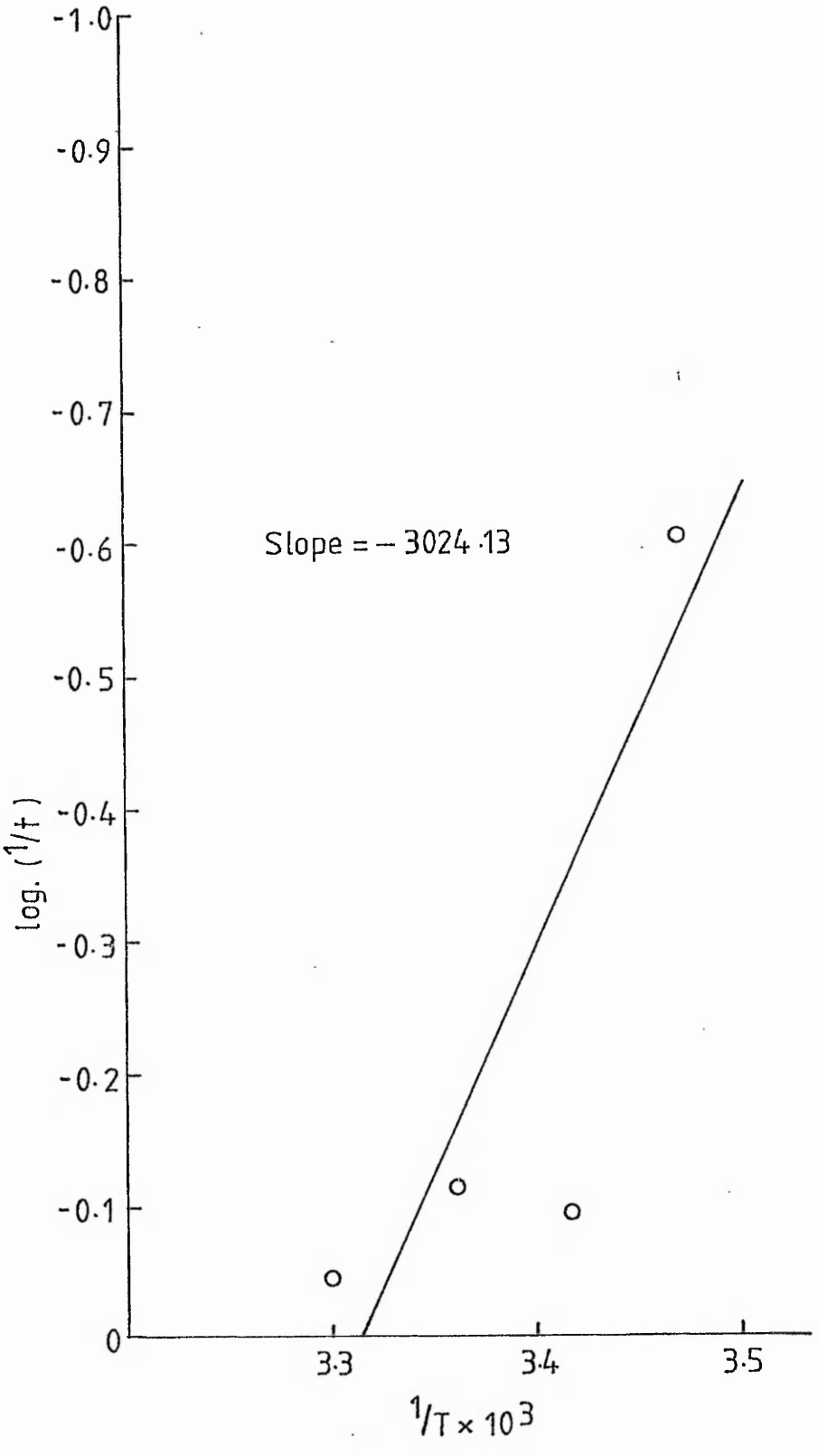


Figure 20. Graph of log ($1/t$) against $1/T$ for the N- acetylation of chitosan film.
(t=time taken to reach 60% N-acetylation and T=temperature degrees absolute).

3.1.3. N-Acylation of Chitosan in Powder Form

The method of N-acylation of chitosan films was then adapted for use on reprecipitated chitosan. Thus chitosan was reprecipitated in the normal way and dispersed in a large excess of methanol. Acetic anhydride (2.3mol per anhydro-D-glucosamine residue) was then added to the stirred dispersion and the mixture left for 16 hours at room temperature. The resulting product was off-white and fluffy in appearance.

In order to check the completion of the reaction a number of techniques were adopted. (i) Solubility in aqueous acetic acid (5%) - the starting product was soluble in this media, but the final product was not. There was no evidence of any swelling, indicating a complete reaction. (ii) Application of the Rimini test for primary amines - chitosan gave the characteristic purple colouration but no such colour was observed with the N-acetyl chitosan, further indicating that the primary amines had reacted. iii) The infrared spectrum of the product showed characteristic absorptions — 2900cm^{-1}; CH_3 of the acid, and 1655 and 1540 cm^{-1} due to the amide I and amide II bands, respectively. The spectrum differed markedly from that of the original spectrum of chitosan.

The method was then applied successfully to other acid anhydrides to give a range of N-acyl chitosans. It was found that for the N-acyl chitosans of the higher fatty acids and benzoic acid it was necessary to reflux the methanolic dispersions for 6-8 hours with the appropriate anhydride to afford reactions. This is similar to the reaction conditions needed for the N-acylation of film. All the products were off-white and fluffy in appearance. They were all insoluble in cold and boiling water, aqueous acetic acid, methanol, DMSO, DMF, acetone and other organic solvents tried.

An attempt was made to prepare the N-acyl chitosans directly from the starting material, i.e. in flake form,using the same method as before. Results from chemical tests, however, showed that as the molecular weight of the carboxylic acid anhydride increased, the degree of reaction was less. Thus it would appear that only a surface reaction was occurring on the flake, further reaction being precluded because of the nature of the starting material.

Table 14 gives details of the various N-acyl chitosans prepared from both reprecipitated and flake chitosan.

In order to improve the N-acylation of chitosan in flake form, attempts were made to increase the reactivity of the starting material. In one case, the flake was ground as finely as possible in methanol with a Waring Blendor and allowed to steep in methanol overnight, prior to reaction.

In another, the flake was again ground finely in methanol but this time in the presence of sufficient aqueous acid (5%) to cause the flake to swell. Various methanol: acetic acid ratios were tried and a ratio of 4:1 was found to give the best results.
The pretreated flake was then reacted in methanol and acetic anhydride for 16 hours at room temperature. Characterisation of the products was again difficult due to the intractable nature of the material; however it was found that both products were insoluble in aqueous acetic acid and gave negative results with the Rimini test. This would seem to suggest a complete reaction but it is difficult to differentiate between this and just a surface reaction.

Table 14 The various N-acyl chitosans prepared

Acyl Group	Reprecipitated	Flake
Acetyl	Rimini test-ve.Insol. in aq. acetic acid. Sol. in formic acid (85%) after 24 hours. Off-white in colour	Rimini test —ve Insol. in aq. acetic acid (5%)
Propionyl	Rimini test —ve.Insol. in aq. acetic acid and formic acid. Off-white in colour.	Rimini test :- some purple spots formed Insol in aq. acetic acid
Butyryl	Rimini test-ve.Insol. in aq. acetic acid Sol. in formic acid after 24 hours. Off-white in colour.	Rimini test:- some purple spots. Insol in aq. acetic acid.
Valeryl	Rimini test -ve. Insol. in aq acetic acid and formic acid Beige in colour.	Rimini test:- purple colouration. Some swelling in aq. acetic acid.
Hexanoyl	Rimini test-ve.Insol. in aq. acetic acid and formic acid Beige in colour.	Rimini test:- purple colouration. Swelling in aq. acetic acid
Heptanoyl	Rimini test-ve.Insol. in aq. acetic acid. Sol. in formic acid after 48 hours.	Rimini test:- purple coloration. Swelling in aq. acetic acid.
Octanoyl	Rimini test -ve.Insol. in aq. acetic acid and formic acid White in colour.	Rimini test:- purple coloration Swelling in aq. acetic acid.
Nonoyl	Rimini test-ve.Insol. in aq. acetic acid and formic acid White in colour	Rimini test:- purple coloration. Swelling in aq. acetic acid.
Decanoyl	Rimini test-ve.Insol. in aq. acetic acid and formic acid Off-white in colour.	------
Benzoyl	Rimini test-ve.Insol. in aq. acetic acid and formic acid Off-white in colour	------

3.1.3.1. X-Ray Diffraction Studies on N-Acyl Chitosans

In a study of renaturated chitin fibrils, films, and filaments , Brine and Austin [183] compared chitin, renatured from trichloroacetic acid solutions by precipitation with acetone, with natural chitin, using X-ray diffraction and other techniques. The results from the X-ray patterns showed that although the pattern obtained for the renatured chitin was not quite as sharp as for the natural chitin, the product was still a crystalline ordered structure.

It was therefore decided to carry out X-ray diffration studies on the N-acyl chitosans, N - acetyl-, N-propionyl-, N-butyryl-, N - hexanoyl-, and N - benzoyl, and see whether or not a crystalline ordered structure remained on changing the N - acyl group. Figures 21-25 show the X-ray diffraction patterns obtained.

N-Acetyl chitosan gave a pattern with well defined, concentric rings, Figure 21, indicating that the product obtained by N--acetylating chitosan is as crystalline as the natural chitin. On changing the N-acyl group, the degree of crystallinity decreases as shown by the increasingly diffuse patterns obtained for N-propionyl, N-butyryl-, and N-hexanoyl chitosans, Figures 22,23 and 24.
Thus it would appear the structures become more amorphous as the length of the N-acyl group increases. The N-benzoyl chitosan also gives a diffuse X-ray pattern but does not appear to be as diffuse as the N-butyryl- or N-hexanoyl chitosans, Figure 25.

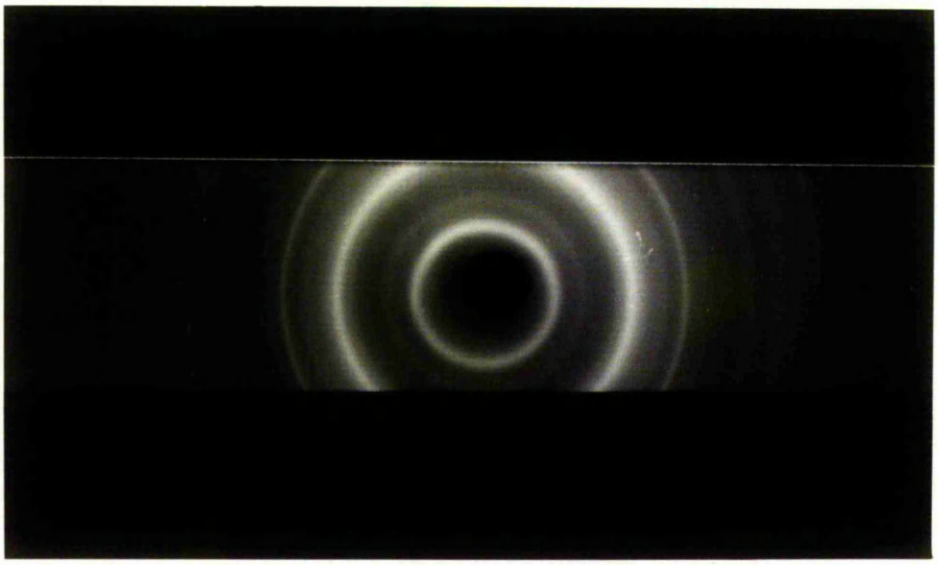


Figure 21. X-Ray diffraction pattern of N-acetyl chitosan.

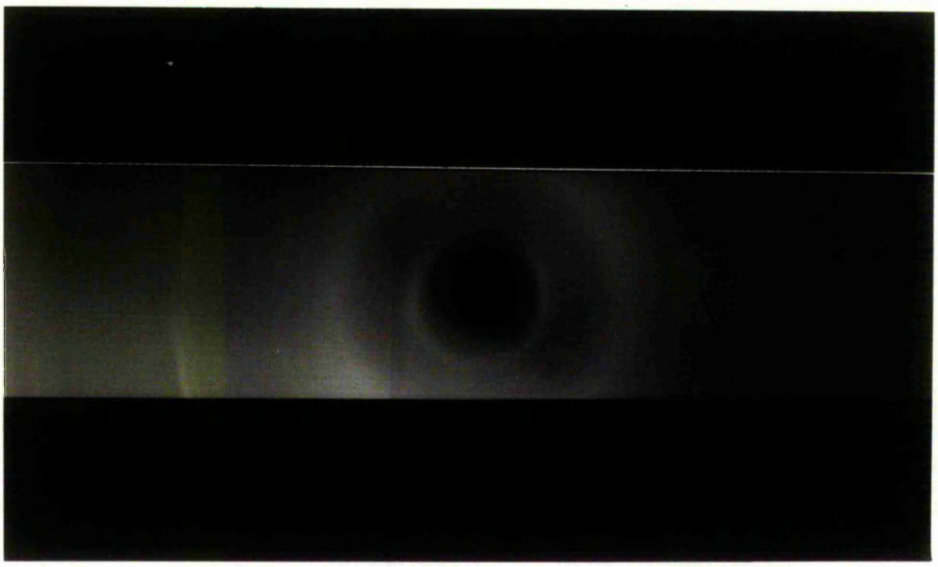


Figure 22. X-Ray diffraction pattern of N-propionyl chitosan.

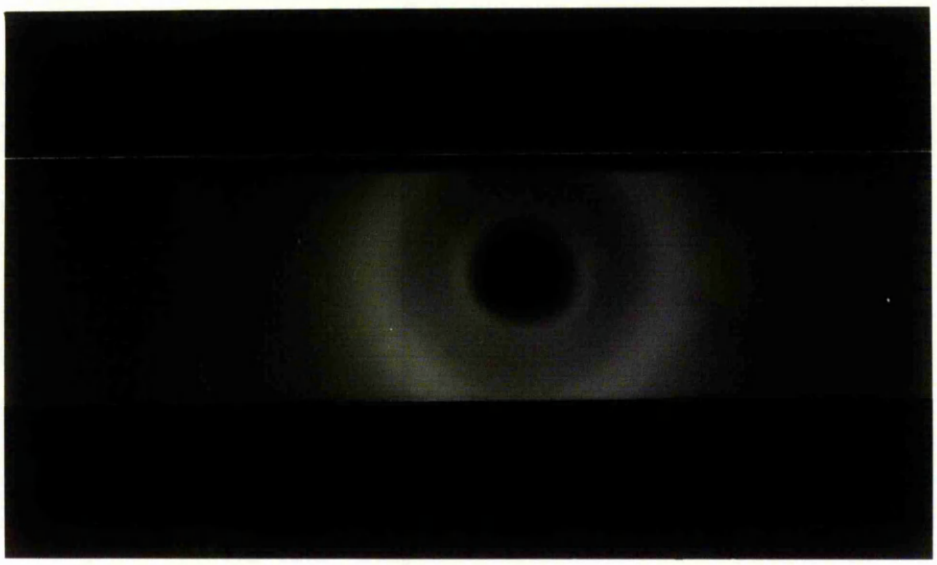


Figure 23. X-Ray diffraction pattern of N-butyryl chitosan.

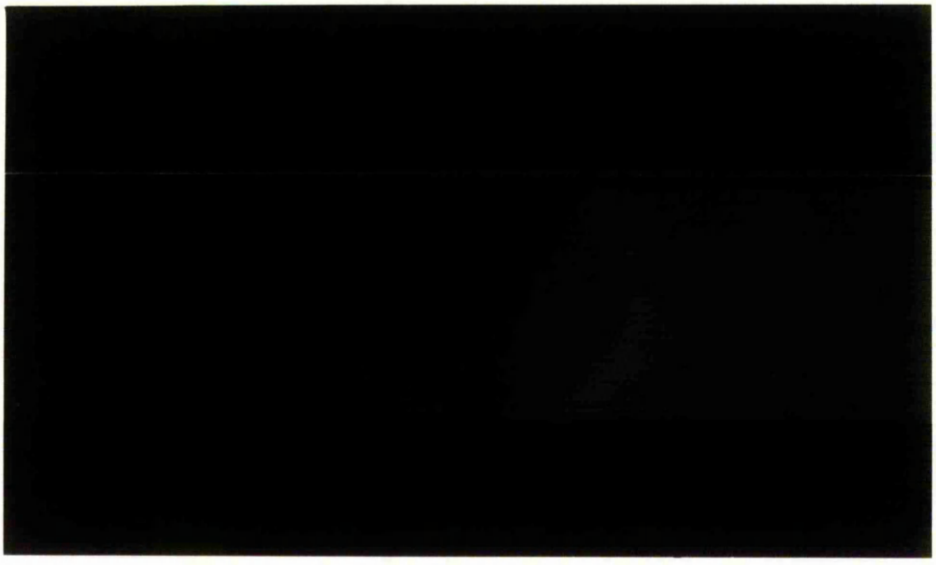


Figure 24. X-Ray diffraction pattern of N-hexanoyl chitosan.

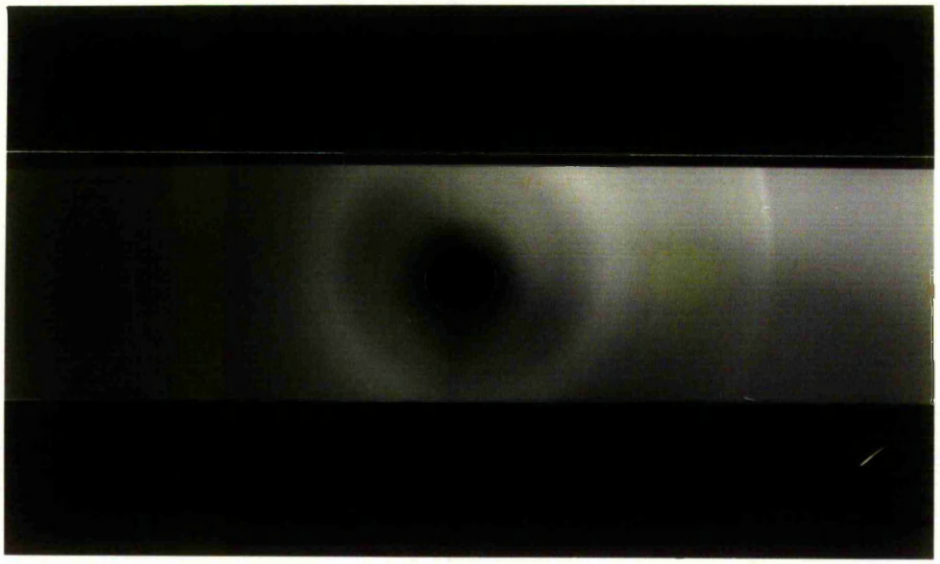


Figure 25. X-Ray diffraction pattern of N-benzoyl chitosan

3.2.1. O-Acetylation of N-Acyl Derivatives of Chitosan in Film Form

A preliminary study was carried out on the O-acetylation reactions of N-acetyl-, N-propionyl-, N-butyryl-, N-hexanoyl- and N-benzoyl chitosans in film form. All the N-acyl chitosans were de-O-acylated and the infrared spectrum of each sample recorded prior to use. The samples were then acetylated for 1 hour at room temperature in either acetic anhydride/glacial acetic acid/perchloric acid or acetic anhydride/pyridine. The films were removed, washed thoroughly in methanol, dried, and the infrared spectrum of each re-recorded. The extent of O-acetylation was measured using the absorbance of the carbonyl band at 1740 cm^{-1} and the 3450 cm^{-1} band at t=0 as an internal standard.

Figure 26 shows the values of A 1740 (corrected for film thickness) plotted against the calculated lengths of the respective N- acyl groups. It is observed that in both acetylation media the extent of reaction of N-acetyl chitosan is very small and that there is only a slight increase in the extent of O-acetylation when the substrate is changed to N-propionyl chitosan. However, on changing the substrate to N- butyryl chitosan there is a 12-15 fold increase followed by a further small increase with N-hexanoyl chitosan. These results indicate that the spacing between the polymer chains is the controlling factor in the O-acetylation of chitosan and that there is a critical separation between chains, below which O-acetylation does not occur to any appreciable extent. Further separation of the chains above this critical distance has only a small effect upon the ease of acetylation. Although the N-benzoyl group is intermediate in length between the N-butyryl and the N-hexanoyl groups, its influence on the ease of O-acetylation is less than that of either of these, but greater

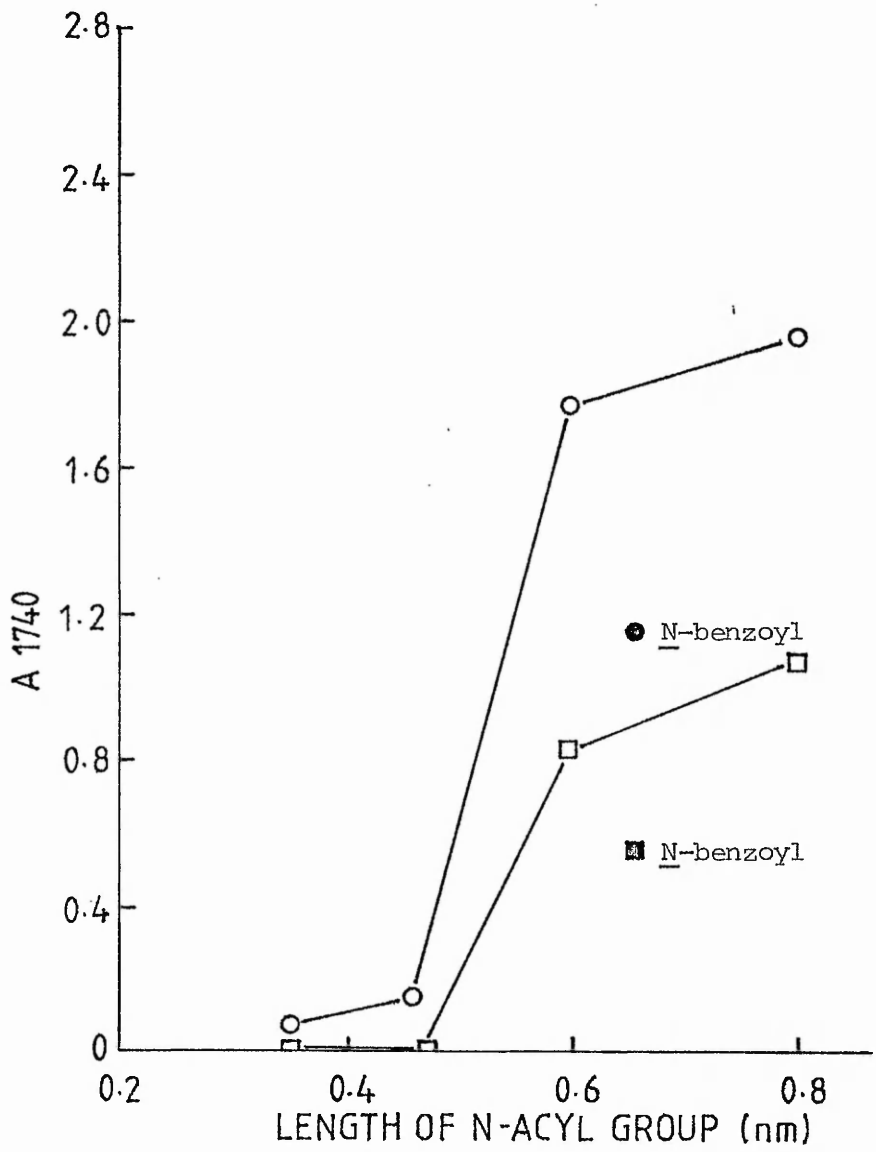


Figure 26. O- Acetylation of N-acyl chitosans

O - in acetic anhydride/glacial acetic acid/ perchloric acid, □ - in acetic anhydride/pyridine for 1 hour at room temperature

than that of the N-propionyl group. This is possibly due to the planar nature of the benzene ring which means that the N-benzoyl group will only separate the polymer chains in one direction whilst the aliphatic acyl group will cause disruption in two directions.

It was decided to enlarge the study of O-acetylation by using larger linear aliphatic anhydrides, branched aliphatic anhydrides, and other aromatic anhydrides. Acetylation, however, would only be carried out in acetic anhydride/ pyridine solutions since it was felt that although both media gave similar trends, the acetylation in pyridine would give more reproducible results. The acidic media is catalysed by perchloric acid and small variations in the amount of catalyst used might tend to give large variations in the results. In addition, prolonged treatment of chitosan films in acidic media makes them brittle, causing fragmentation on handling. The extent of O-acetylation was monitored at regular time intervals up to 6 hours, and then after 24 hours. All samples were presteeped for 24 hours in pyridine prior to use.

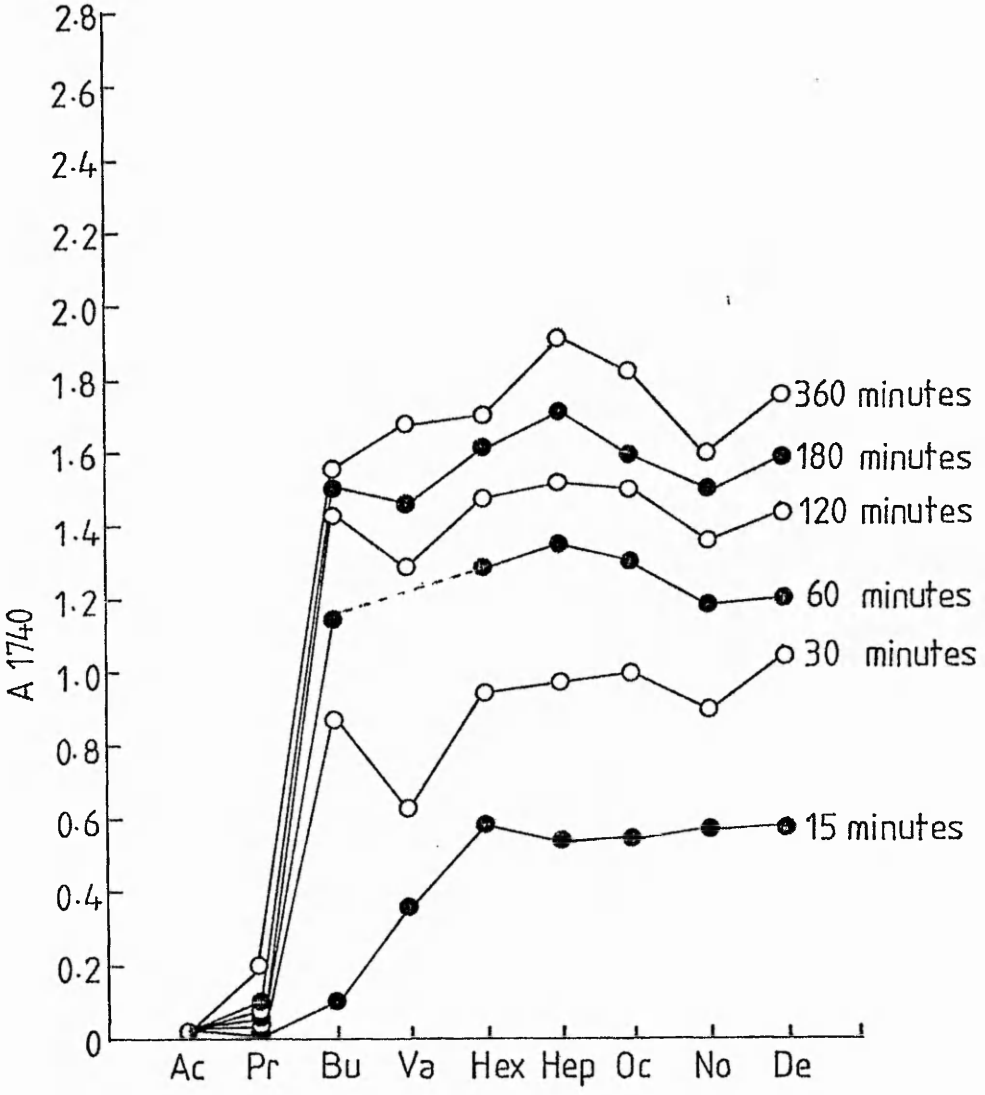
3.2.1.1. Linear Aliphatic Anhydrides

Samples of N-acyl chitosans in film form, prepared from the linear aliphatic anhydrides acetic to decanoic, were used. Aliquots of acetic anhydride were added to each sample steeping in pyridine and at the required time interval the films were removed, washed, dried, and the infrared spectra recorded. The samples were then replaced in the acetylating media and the process repeated.

Figure 27 is a plot of A1740, corrected for film thickness, against each of the linear anhydrides used at the different time periods. It is evident that with N-acetyl chitosan little or no O-acetylation occurs even after 6 hours, but with N-propionyl chitosan there is a very small, but steady, increase in O-acetylation as the reaction times increases. On passing to N-butyryl chitosan there is, again, a very large increase in the extent of O-acetylation. Further increase in the size of the anhydride brings about some increase in the acetylation but this generally levels out. As the time of the reaction increases, the increase in the extent of O-acetylation decreases.

The values of A1740 were then plotted against time for the different linear anhydrides used. The effect of time on the reaction is more clearly emphasised; the rate of O-acetylation of N-propionyl chitosan is slowly increasing, whilst the rate of O-acetylation of the higher N-acyl chitosans decreases and slowly levels off with time. A small induction period is present in the N-butyryl chitosan, but this appears to last only for the first 15 minutes of the reaction, Figure 28.

Results from an extended run, 30 hours, showed only very small increases in



Fiqure. 27. The extent of O-acetylation of linear aliphatic N-acyl chitosans at different time periods. (ac=acetyl; pr=propionyl; bu= butyryl; va=valeryl; hex=hexanoyl; hep=heptanoyl; oc=octanoyl; no=nonyl; de= decanoyl.)

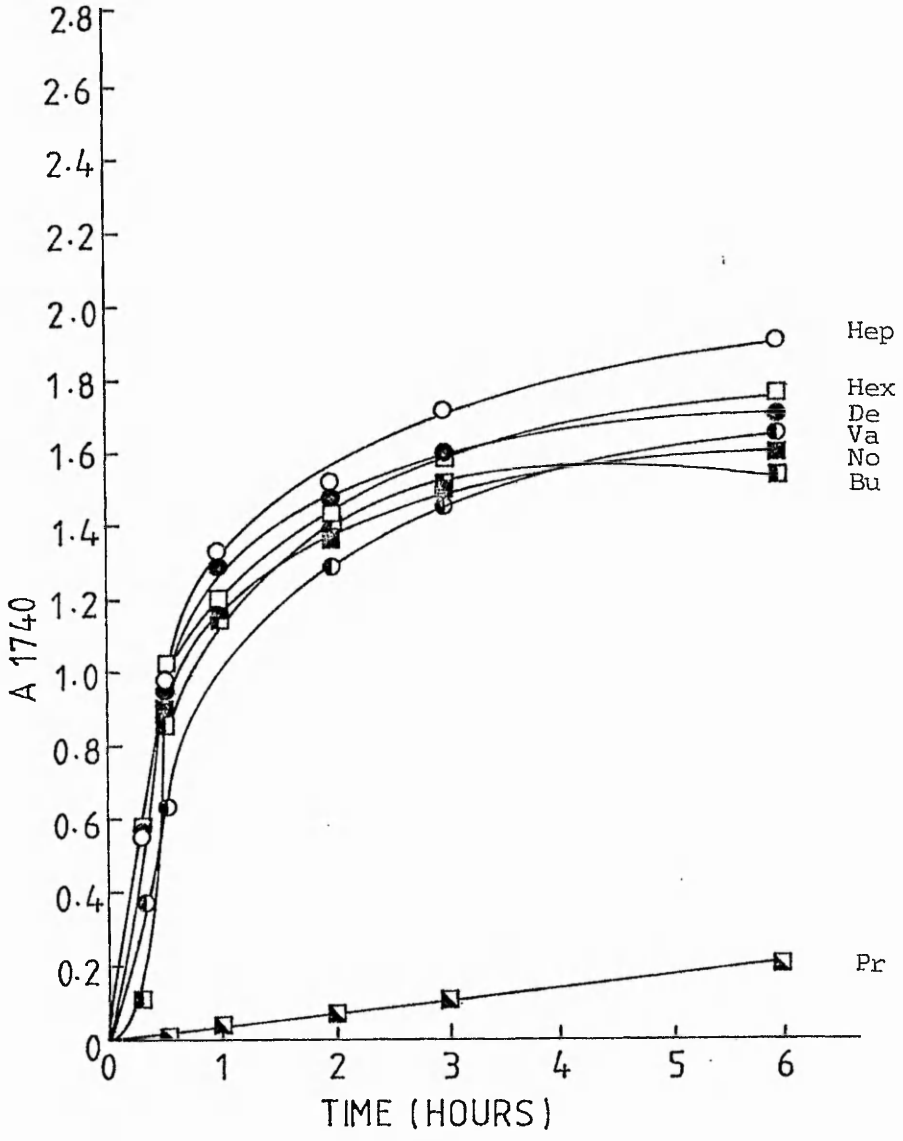


Figure 28. Rate of O-acetylation of linear N-acyl chitosans.

(ac=acetyl; pr=propionyl; bu=butyryl; va=valeryl; hex=hexanoyl; hep=heptanoyl; oc=octanoyl; no=nonyl; de=decanoyl.)

the extent of O-acetylation of all the N-acyl chitosans. Figure 28 shows the infrared spectrum of O-acetylated N-decanoyl chitosan, after 6 hours and 30 hours reaction, and illustrates the very small increase in O-acetylation after 30 hours. The extent of absorption of the hydroxyl band, 3450 cm^{-1}, for each N-acyl chitosan was measured and compared with that of each sample prior to O-acetylation. In virtually all cases the ratio of the hydroxyl absorbance at 30 hours to that at zero time was approximately 0.6. It would appear, therefore, that O-acetylation in pyridine/acetic anhydride media occurs at a reasonable rate over the first 2 hours or so, but then slows down markedly after about 40% of the hydroxyl groups are acetylated.

The acetylating medium was then changed to see if any further improvement could be made in the extent of O-acetylation. A sample of N-decanoyl chitosan which had previously been acetylated for 30 hours in pyridine/acetic anhydride was placed in glacial acetic acid/ acetic anhydride/ perchloric acid for a further 72 hours. The infrared spectra of the film was then recorded, Figure 29 , and the absorption of the hydroxyl band at 3450 cm^{-1}, compared with that at 30 hours reaction time and at zero time, Table 15.

Table 15 Absorptions of the hydroxyl band of N-decanoyl chitosan O-acetylated for different lengths of time

	A 3450 at 0	A3450 at 30hr	A3450 at 102hr
	0.83	0.54	0.20
$\frac{A3450^t}{A3450^o}$	1	0.64	0.24

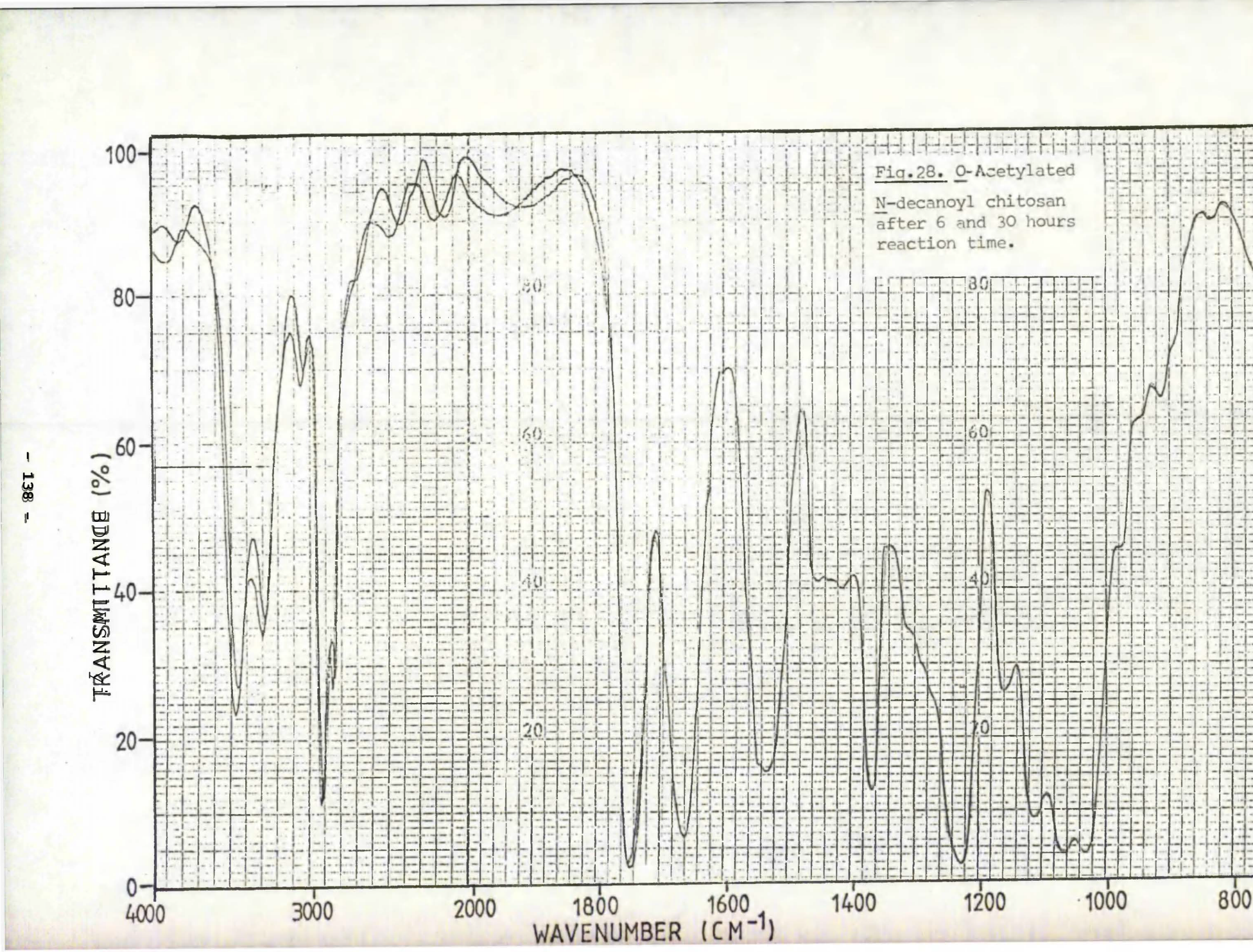


Fig.28. O-Acetylated N-decanoyl chitosan after 6 and 30 hours reaction time.

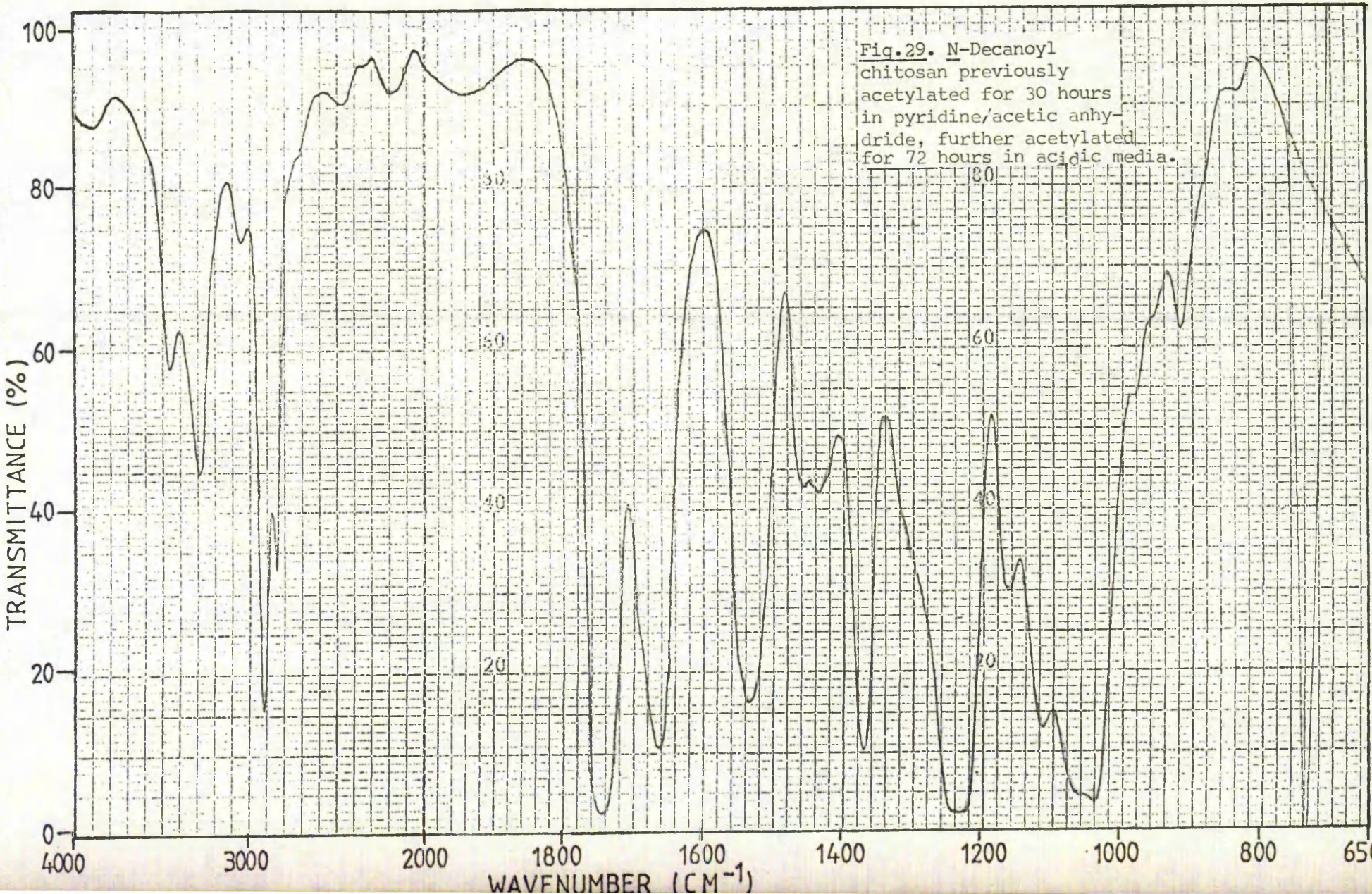


Fig.29. N-Decanoyl chitosan previously acetylated for 30 hours in pyridine/acetic anhydride, further acetylated for 72 hours in acidic media.

Thus under more stringent acetylating conditions further O-acetylation was achieved, but it was still far from complete. It would appear, therefore, that O-acetylation is controlled by steric considerations in addition to the spacing of the polymer chains. It is presumed that O-acetylation is occurring preferentially on the C(6) in pyridine/acetic anhydride media.

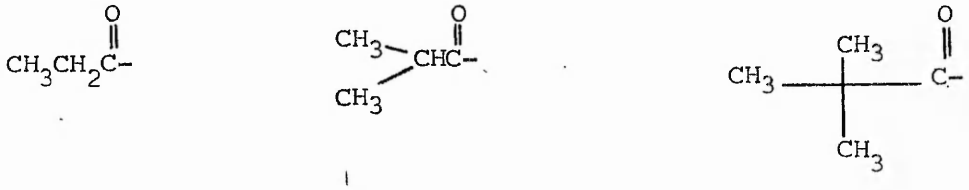
In early experiments on O—acetylation of the linear N-acyl chitosans the results for N-heptanoyl and N-nonyl chitosans were much lower than those for N-hexanoyl, N-octanoyl, or N-decanoyl chitosans, such that when A1740 was plotted against the various N-acyl chitosans a zig-zag plot was obtained, the peaks corresponding to C6,C8 and C10 N-acyl chitosans and the troughs to C_7 and C_9 N-acyl chitosans. No reasonable explanation for this effect could be made and it was decided to check the starting products by ir and nmr. It was found that the n—heptanoic and n-nonoic anhydrides, which had been prepared using the method of Wallace and Copenhaver,[414] where the appropriate carboxylic acid is refluxed with an excess of acetic anhydride and the products then separated by vacuum distillation, were contaminated with acetic anhydride. Thus, when the appropriate N-acyl chitosans were prepared the percentage contamination of acetic anhydride present in the higher anhydrides used, although small, was sufficient for N-acetyl chitosan to form preferentially. Thus the derivative formed was a heterogeneous N-acyl chitosan, with large areas of the polymer chain containing N-acetyl groups and so little or no O-acetylation occurred in these areas.

By using the method of Gerrard and Thrush[415] for the preparation of the higher anhydrides, in which the anhydride is prepared from the fatty acid

by the reaction of thionyl chloride in pyridine, the use of acetic anhydride was avoided and hence the above erroneous results were eliminated. Attempts to prepare pivalic anhydride by this method were unsuccessful, and so the pivalic anhydride produced by the method of Wallace and Copennaver[414] was redistilled under vacuum, several times, to obtain a pure sample.

3.2.1.2. Branched Aliphatic Anhydrides

Two series of N--acyl chitosans were made, each with increasing substitution of methylene groups in the penultimate methyl group. Thus the first series was made up of N-propionyl-, N-iso-butyryl-, and N-pivalyl chitosans.



and the second consisted of N-butyryl-, N-iso-valeryl-, and N-3,3-dimethylbutyryl chitosans.

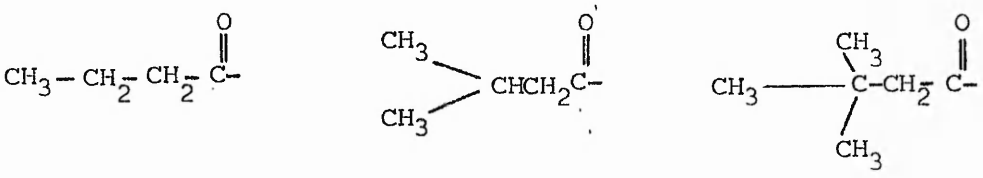


Figure 30 gives A1740, corrected for film thickness, plotted against the first series of branched N-acyl chitosan used and Figure 31 gives A1740 plotted against time for each of the N-acyl chitosans. N-propionyl chitosan, as before, was O-acetylated to a small extent, which increased

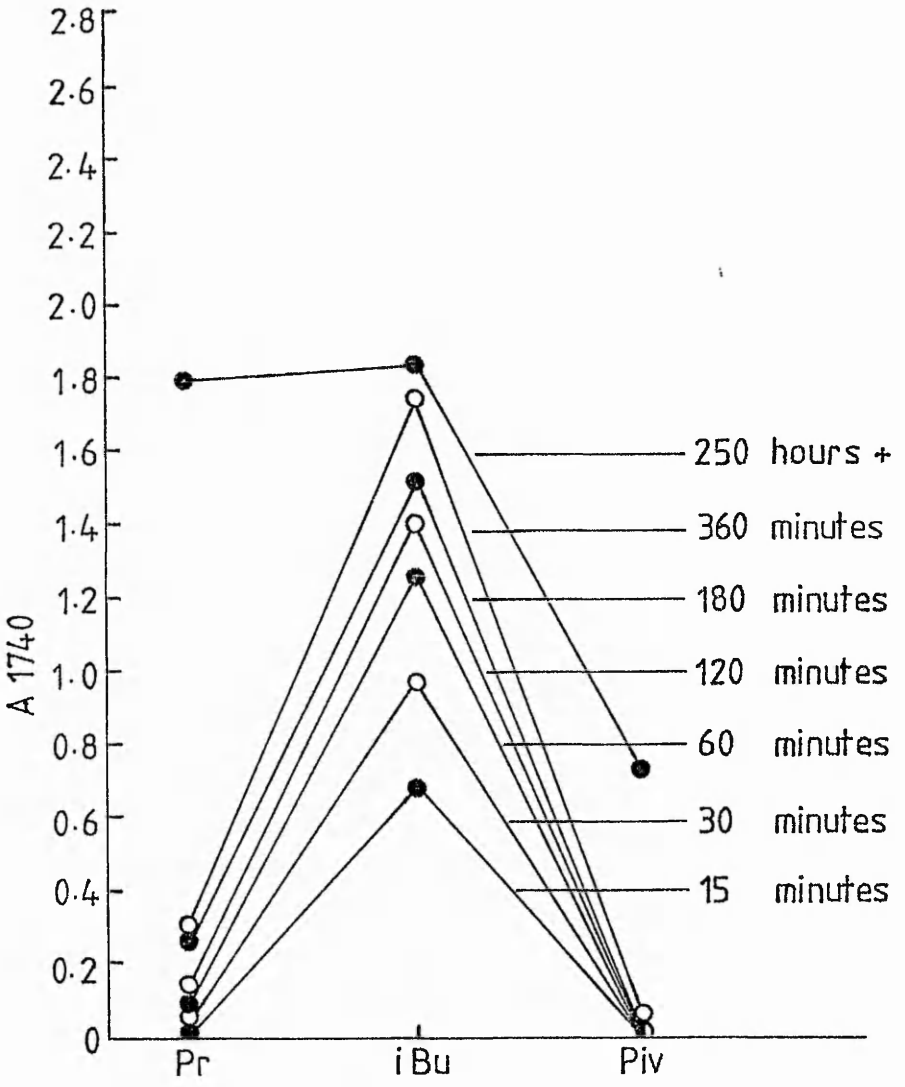


Figure 30. The extent of O-acetylation of branched aliphatic N-acyl chitosans at different time periods.

(pr=propionyl; ibu=iso- butyryl; piv=pivalyl.)

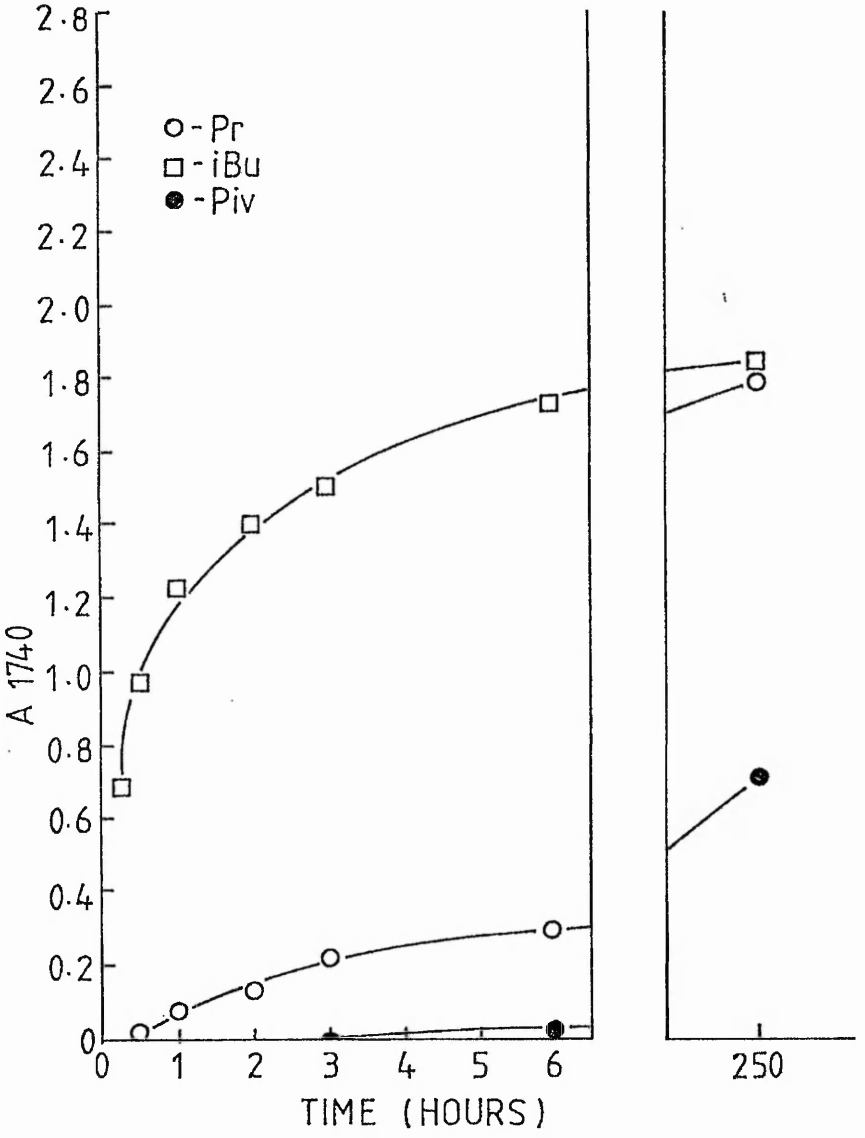


Figure 31. Rate of O-acetylation of branched aliphatic N-acyl chitosans.

(pr=propionyl; ibu= iso- butyryl; piv=pivalyl.)

slowly with time. With the introduction of a second methyl group, i.e. N-iso-butyryl chitosan, a large increase in the extent of O-acetylation is observed, corresponding to a 6 fold increase over N-propionyl chitosan. Thus the branched nature of the N-acyl group has allowed sufficient spacing of the polymer chains for O-acetylation to occur. The N-pivalyl derivative, however, shows only a very small amount of O-acetylation indicating that the very bulky nature of the N-acyl group is preventing O-acetylation from taking place.

Similar effects are observed with the second series of branched N-acyl chitosans, Figures 32 and 33. This time however, the first member of the series, N- butyryl chitosan, is of sufficient length to allow O-acetylation to occur, and hence on changing to N-iso-valeryl chitosan, there is only a small increase in the extent of O-acetylation. The bulky nature of the third member of the series, N- 3,3-dimethylbutyryl chitosan, again hinders reaction and little or no O-acetylation occurs.

Prolonged reaction times, of the order of 250 hours, in pyridine/acetic anhydride gave only small increases in the extent of O-acetylation of N-propionyl and N-iso butyryl chitosan. It was estimated that only about 40% of the available hydroxyl groups were acetylated.

3.2.1.3. Aromatic Anhydrides

The extent of O-acetylation of benzoic and o, m, and p- toluic anhydride derivatives of chitosan was compared. From the results obtained, Figures 34 and 35, there is over all very little difference in the extent of O-acetylation of any of the N- acyl chitosans after 6 hours reaction time, indicating that the presence and position of the substituent on the aromatic ring, in

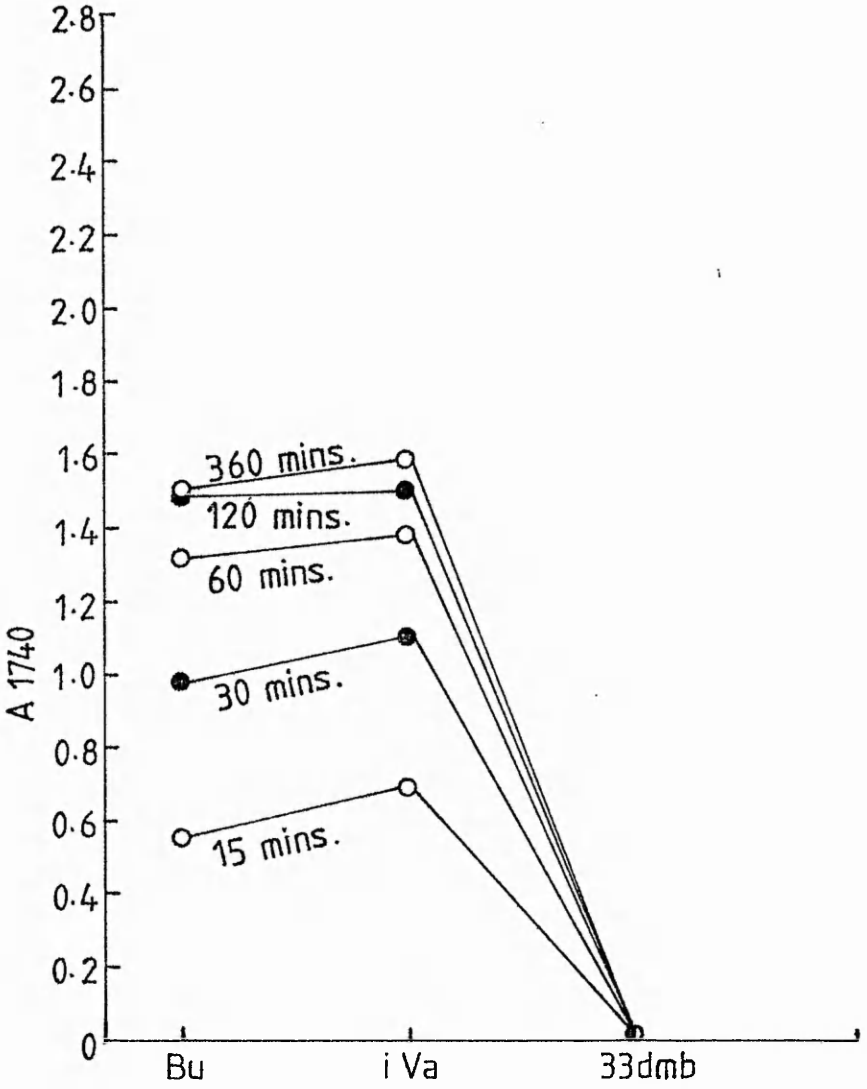


Figure 32. The extent of O-acetylation of branched aliphatic N-acyl chitosans at different time periods.

(bu=butyryl; iva=iso-valeryl; 33dmb= 3,3-dimethylbutyryl.)

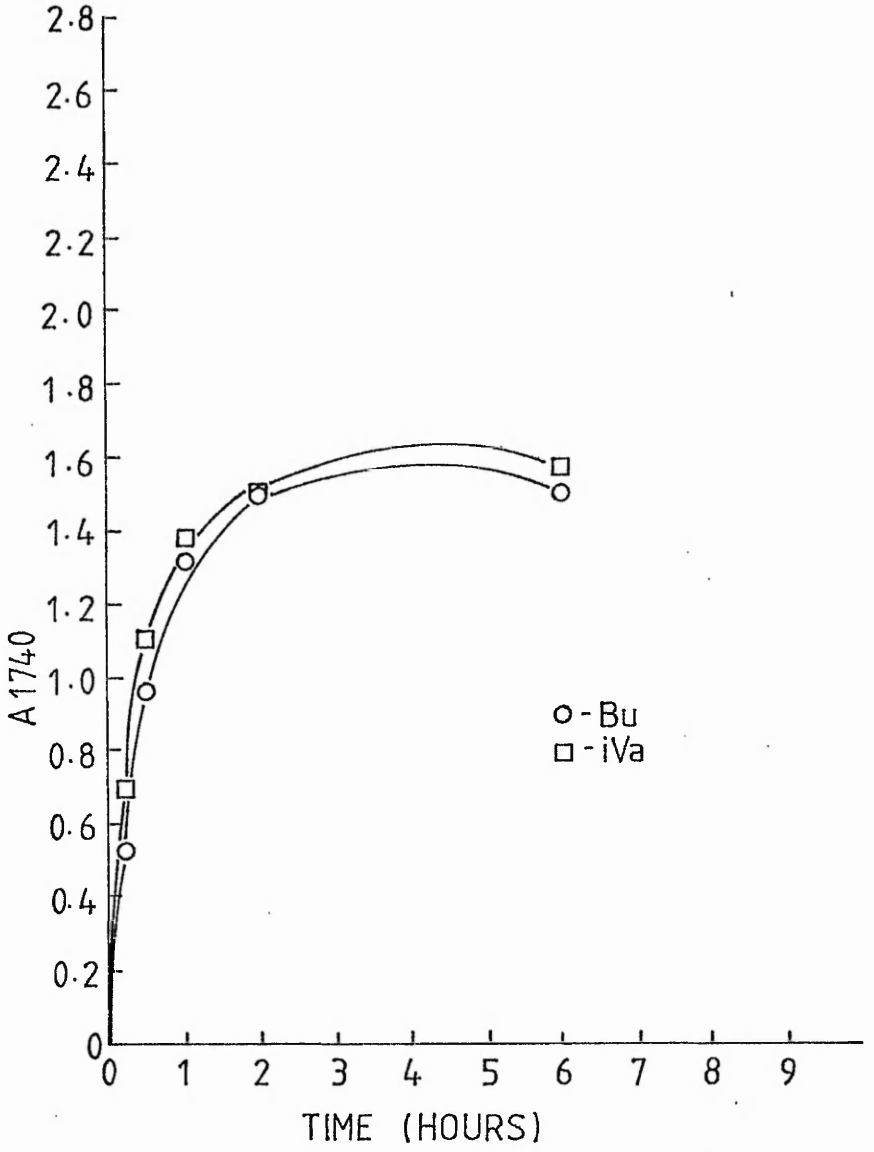


Figure 33. Rate of O-acetylation of branched aliphatic N-acyl chitosans.

(bu=butyryl; iva=iso- valeryl.)

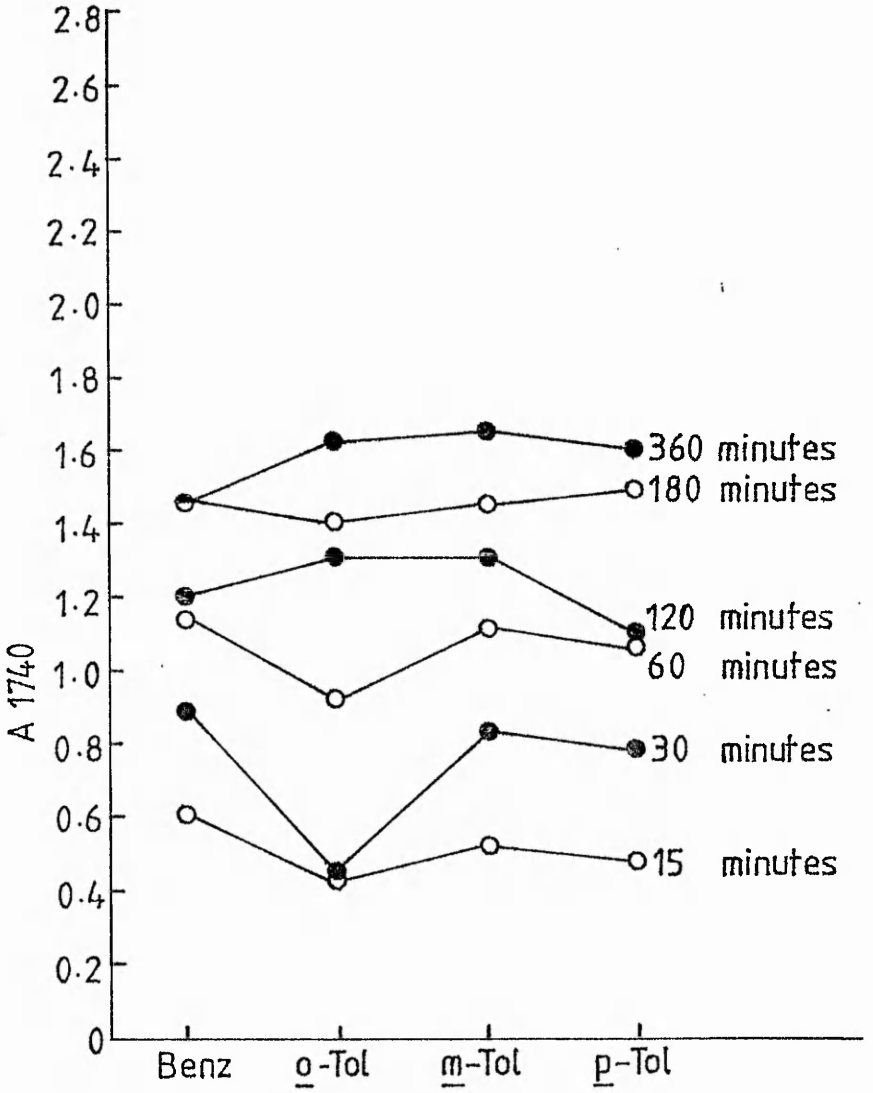


Figure 34. The extent of O-acetylation of aromatic N-acyl chitosans at different time periods. (benz=benzoyl; o-tol= o-toluyl; m-tol=m-toluyl; p-tol=p-toluyl.)

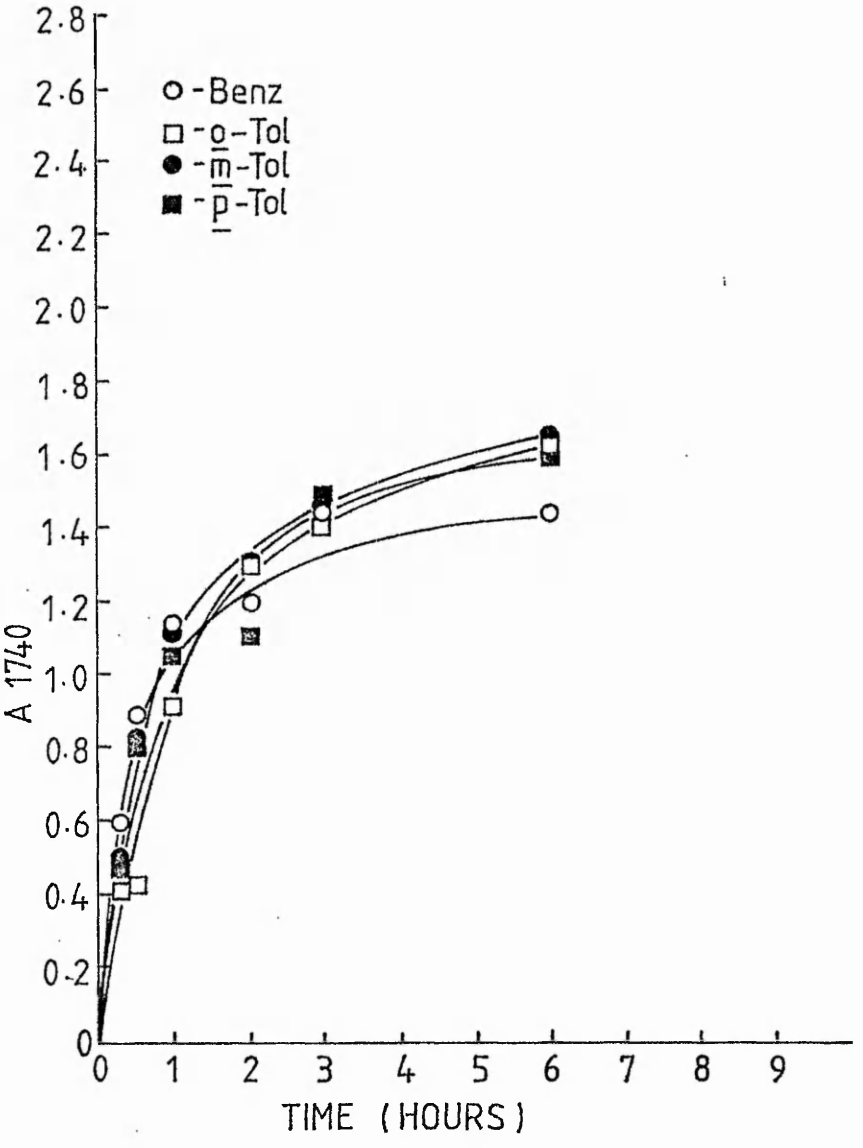


Figure 35. Rate of O-acetylation of aromatic N-acyl chitosans.

(benz=benzoyl; o-tol=o-toluyl; m-tol=m-toluyl; p-tol=p-toluyl.)

this case, has little effect on the overall reaction. However it is of interest to note that initially the o-substituted N-toluyl chitosan is less readily O-acetylated than the other three.

Comparison of the hydroxyl absorbance at 3450 cm^{-1}, after 78 hours treatment, again shows that approximately 40% of the hydroxyl groups have been O-acetylated in each case.

3.2.2. O-Acylation of N-Acyl Derivatives of Chitosan in Powder Form

It was evident from the work on N-acyl chitosan film that O-acetylation could be achieved to some extent on the higher fatty acid anhydride derivatives. Attempts were made, therefore, to produce O-acetylated derivatives of some of the N-acyl chitosans using various acetylation techniques.

Samples of N-butyryl-, N-hexanoyl-, and N-decanoyl chitosans were treated with glacial acetic acid/acetic anhydride/ perchloric acid solutions for up to 72 hours at room temperature. The products formed were found to be soluble in formic acid and DMSO, and to swell in DMF. They were insoluble in other organic solvents tried. Figure 36 shows the infrared spectrum of the O-acetylated N-decanoyl derivative of chitosan and the characteristic bands due to the O-acetyl group are present at 1735 and 1230 cm^{-1}. However O-acetylation is not complete as there are still some hydroxyl groups present as shown by the absorbance at 3450 cm^{-1}. Similar spectra were obtained for the other derivatives. Extension of the reaction times brought little improvement in the extent of O-acetylation.

Using the same method an almost totally substituted O-propionyl — N-hexanoyl chitosan was produced. The white powdery product was soluble in formic acid and DMSO, and swelled in DMF, dimethylacetamide and hexamethylphosphoric acid. A film was cast from the DMSO solution and the infrared spectrum, Figure 37 , of the film showed the characteristic absorptions at 1735 and 1230 cm^{-1} corresponding to the O-propionyl group. A small number of hydroxyl groups remained unreacted, as shown by the absorbance at 3450 cm^{-1}.

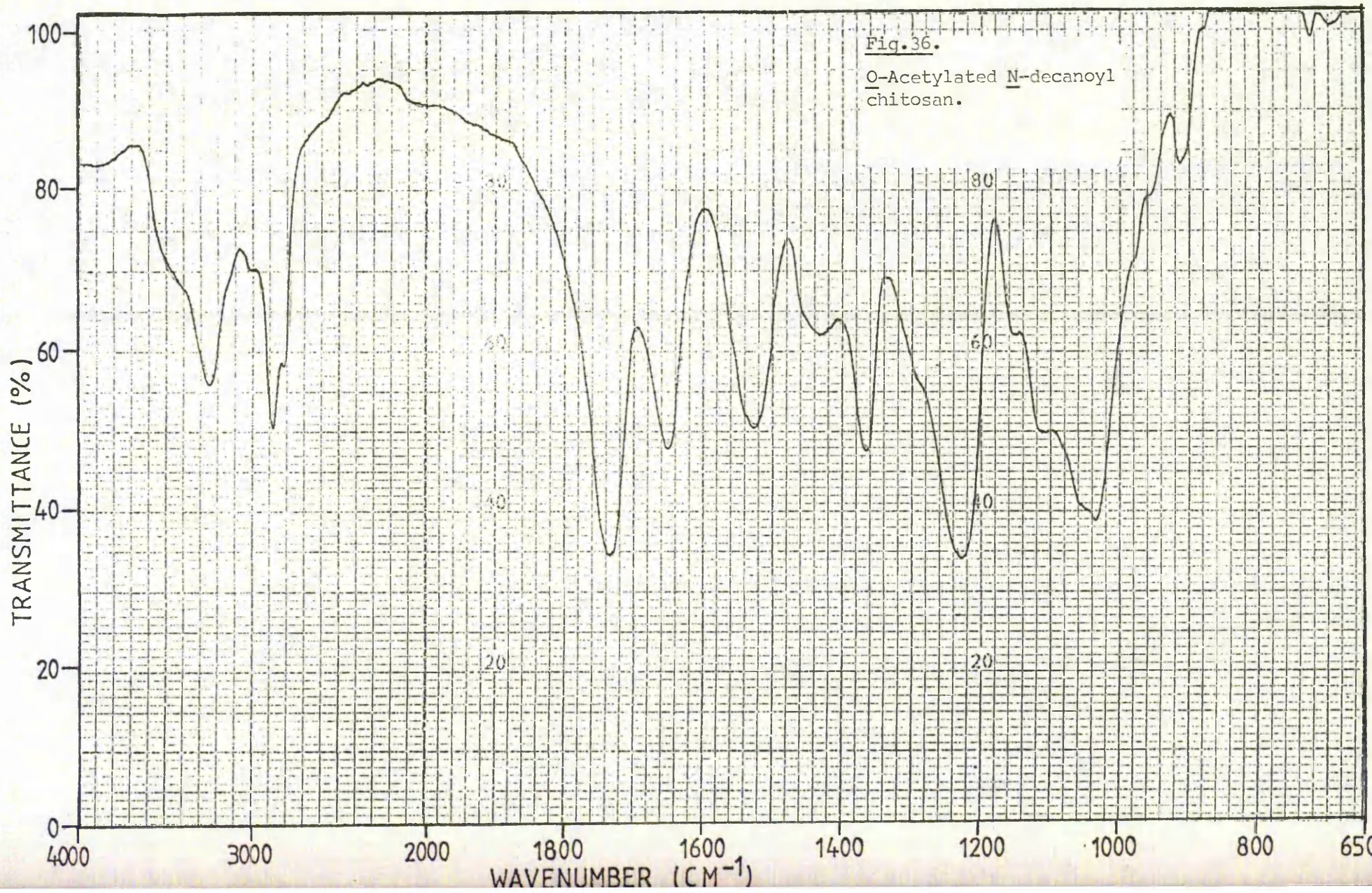


Fig.36.

O-Acetylated N-decanoyl chitosan.

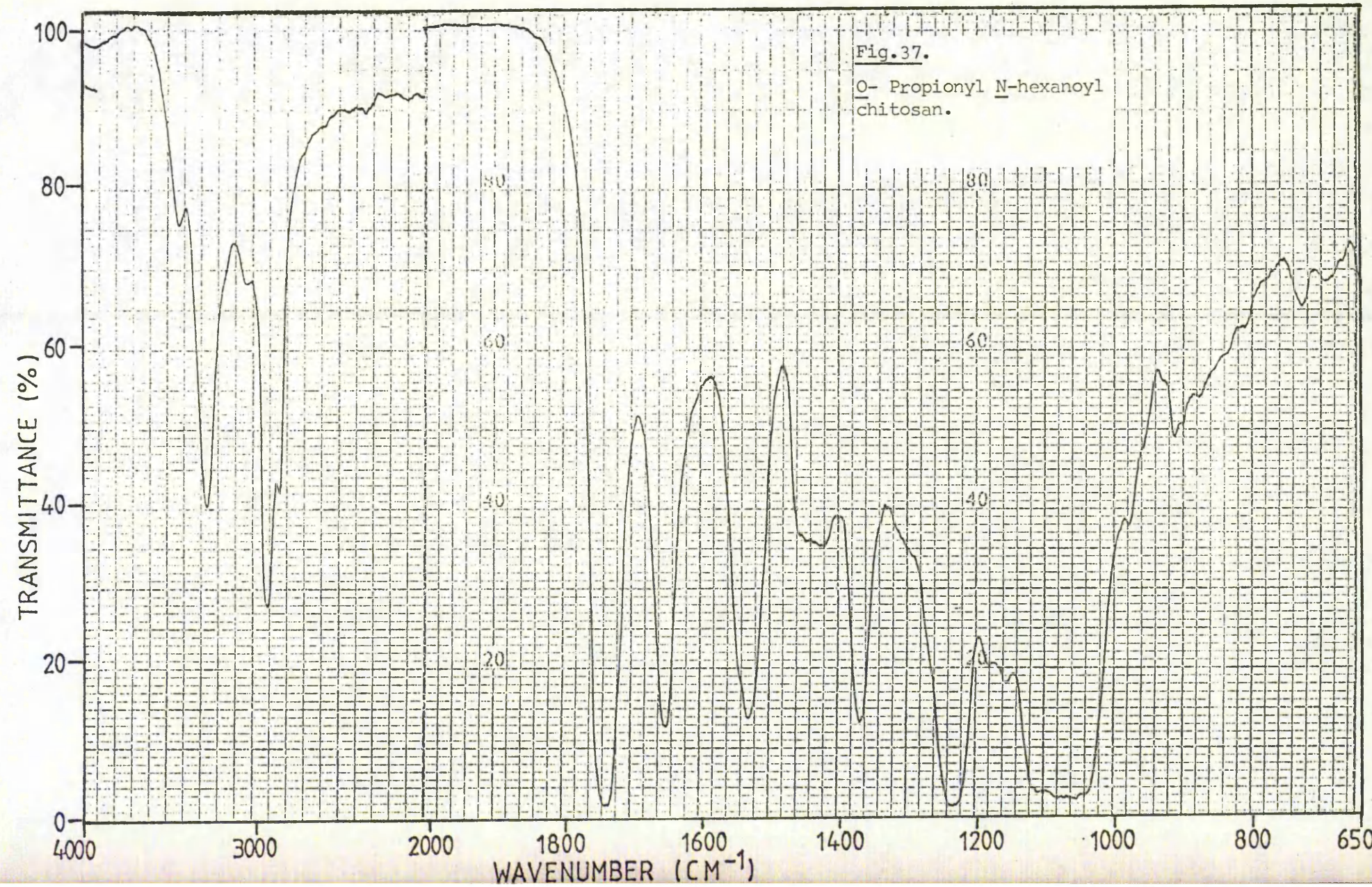


Fig.37.

O- Propionyl N-hexanoyl chitosan.

3.3. Preparation and Acetylation of Schiff's Bases and Keto-imine Derivatives of Chitosan

3.3.1. Preparation of Schiff's base Derivatives

Several Schiff's base derivatives of chitosan have been reported in the literature. The reaction of salicylaldehyde with chitosan in neutral medium was used as a protecting group for the primary amine in the specific O-alkylation of chitosan.[244,245] The use of benzaldehyde, anisaldehyde and O-nitrobenzaldehyde was also reported, but it was claimed that these did not react completely. The reaction products of chitosan with 'Amidox', a dialdehyde starch, have been reported[171,264] and glutaraldehyde has been reacted with chitosan in several studies [267,268] on the immoblisation of enzymes. Gel formation has been observed recently[269] on treatment of chitosan in aqueous acetic acid/methanol solutions with acetaldehyde, acrolein, benzaldehyde, cinnamaldehyde, propionaldehyde, p-tolualdehyde, fomaldehyde and glutaraldehyde. Studies have been carried out on the Schiff's base derivatives of chitosan to determine their capacity for metal ions[263] and their ability to bind on enzymes in the preparation of polymer-supported enzymes.[263,267,268].

A wide range of aliphatic and aromatic aldehydes were chosen and reacted with chitosan in film form. Once complete reaction was established, as shown by their infrared spectra, samples of each film were checked for reaction in the Rimini test. The Schiff's base derivatives prepared included those from the aliphatic aldehydes, acetaldehyde up to decanaldehyde, from the branched aliphatic aldehydes, iso-butyric, iso-valeric and

trimethyl acetic aldehyde and from the aromatic aldehydes salicylaldehyde, benzaldehyde, o-m-p-tolualdehydes, p-dimethylaminobenzaldehyde, o-and p - nitrobenzaldehyde, and pyridine — 4 — aldehyde. These derivatives were prepared for subsequent O-acetylation studies and comparison with the corresponding N-acyl derivatives. Figures 38-42 show infrared spectra of the acetaldehyde, decanaldehyde, iso-valericaldehyde, benzaldehyde, and salicylaldehyde derivatives of chitosan. Other derivatives gave similar spectra.

It has been reported[416] that when a Schiff's base is mixed with an aromatic aldehyde in absolute alcohol the aldehyde component of the Schiff's base is exchanged. The ease of exchange of aromatic aldehyde to Schiff's base is in the order

$$o\text{-}HO\text{-}C_6H_4CHO \quad > \quad p\text{-}HO\text{-}C_6H_4CHO \quad > \quad C_6H_5CHO$$

A sample of N-benzylidene chitosan film was placed in methanol and to this was added salicylaldehyde and the film slowly went from colourless to yellow. Complete replacement of the Schiff's base by the salicylaldehyde was confirmed by the infrared spectrum of the film.

Several Schiff's base derivatives were prepared using reprecipitated chitosan dispersed in methanol and these included the products formed from salicylaldehyde, benzaldehyde, p-hydroxybenzaldehyde, p-nitrobenzaldehyde, p-methoxybenzaldehyde and pyridine-4-aldehyde. All were powdery, some coloured, and all were insoluble in water, alcohols, ether and other organic solvents.

The X-ray diffraction patterns were recorded for N-salicyclidene and N-benzylidene chitosan. The patterns, Figures 43 and 44, show that both derivatives

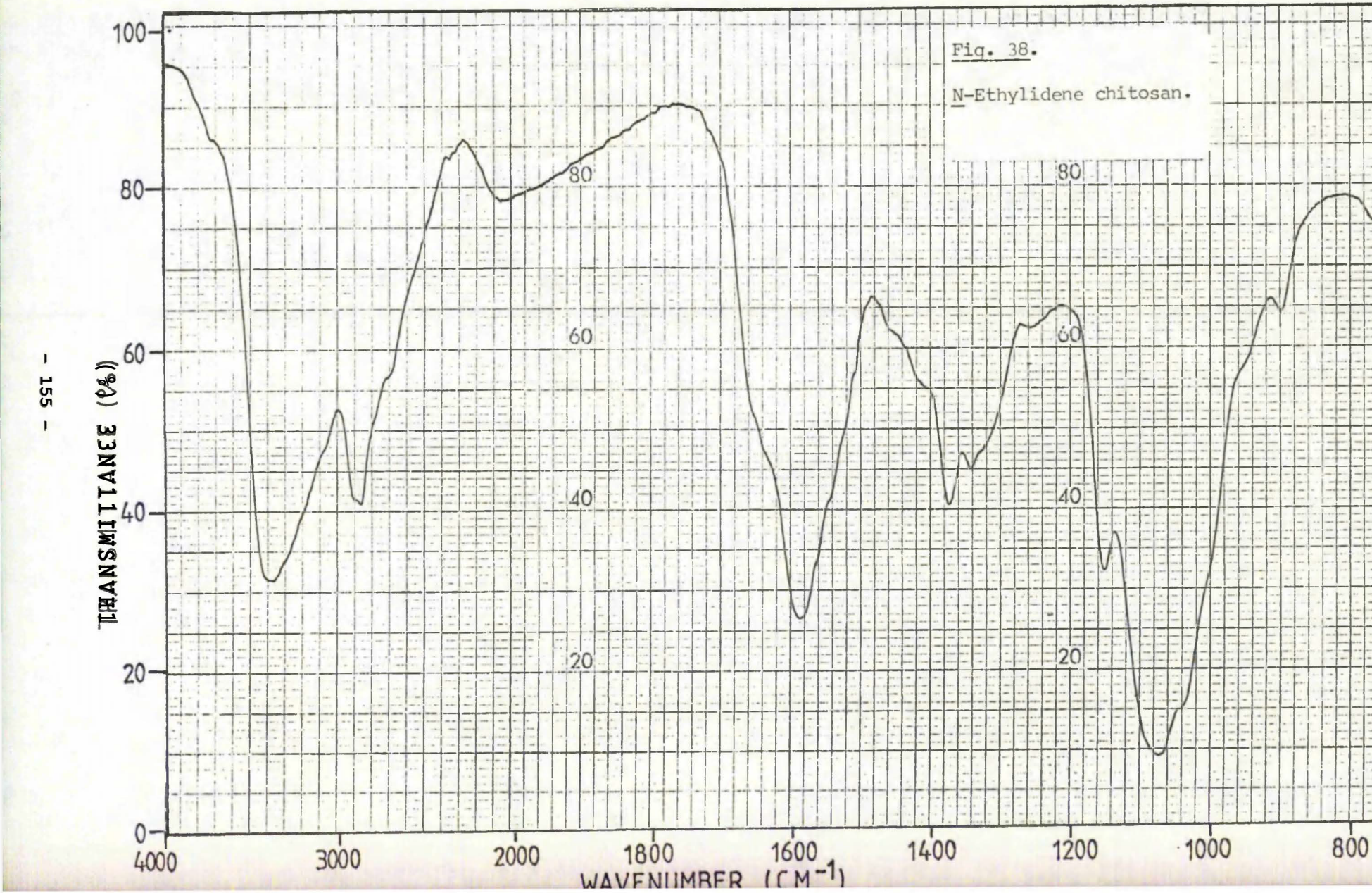


Fig. 38.

N-Ethylidene chitosan.

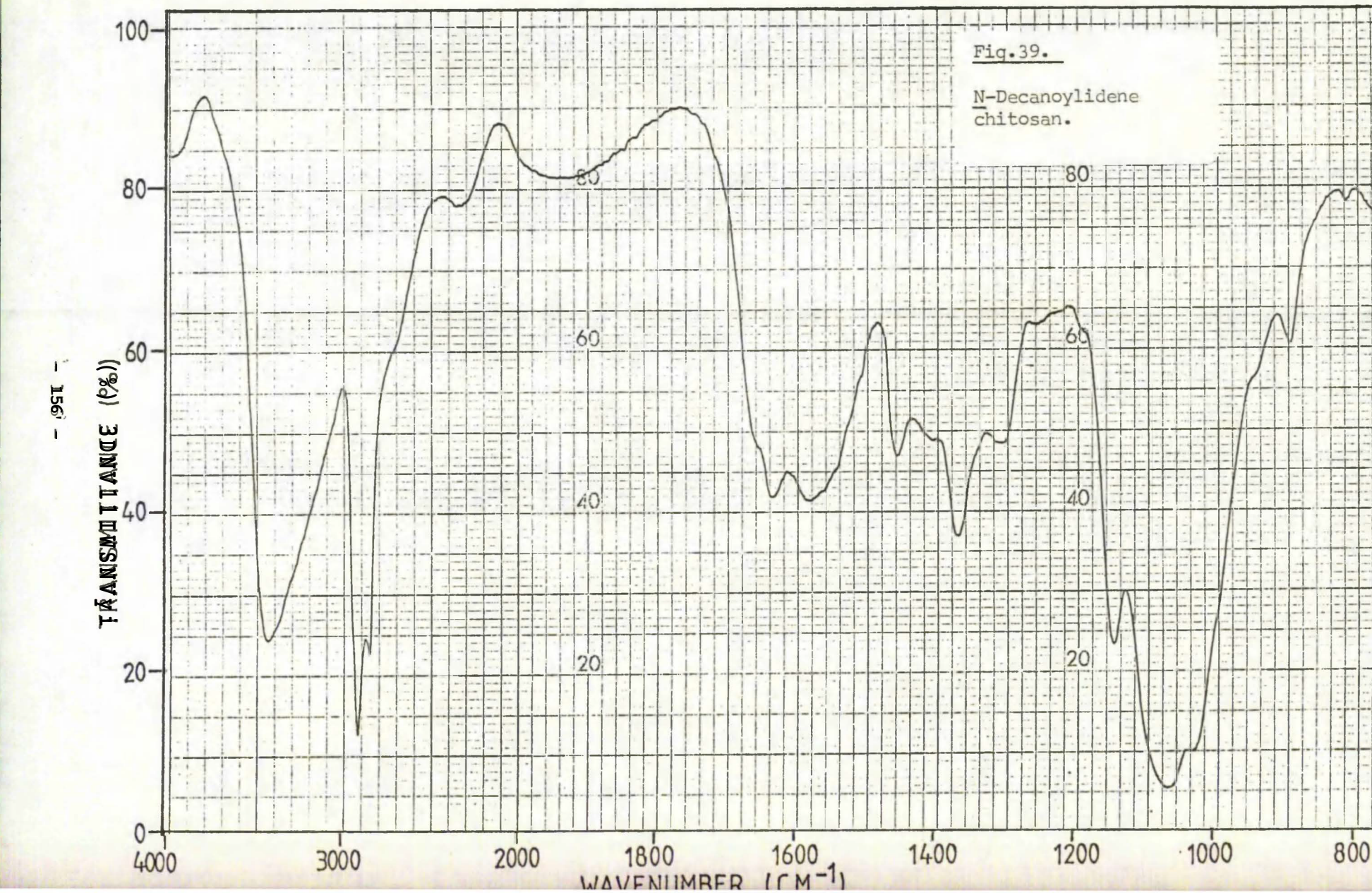


Fig.39.

N-Decanoylidene chitosan.

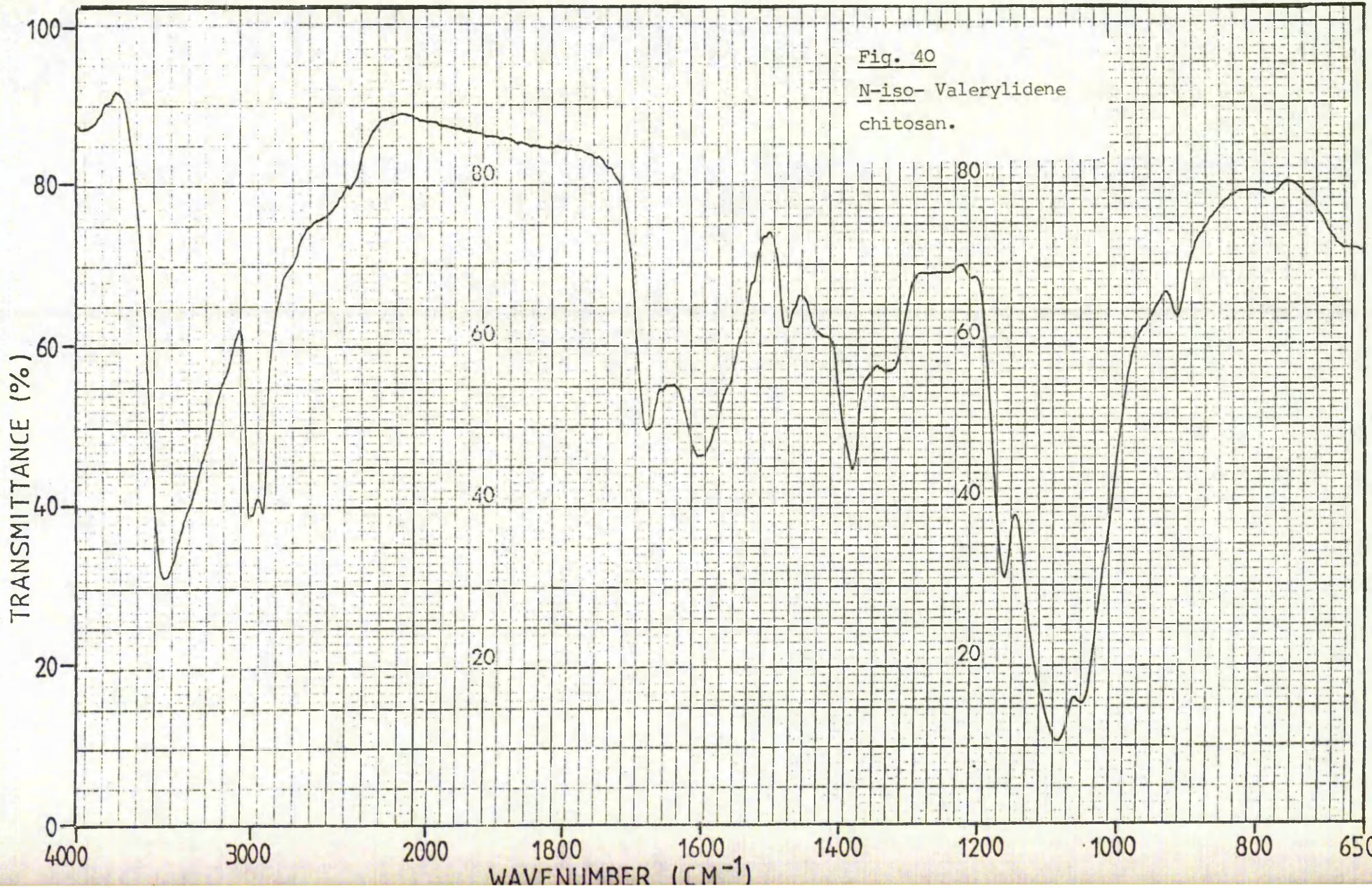


Fig. 40

N-iso- Valerylidene chitosan.

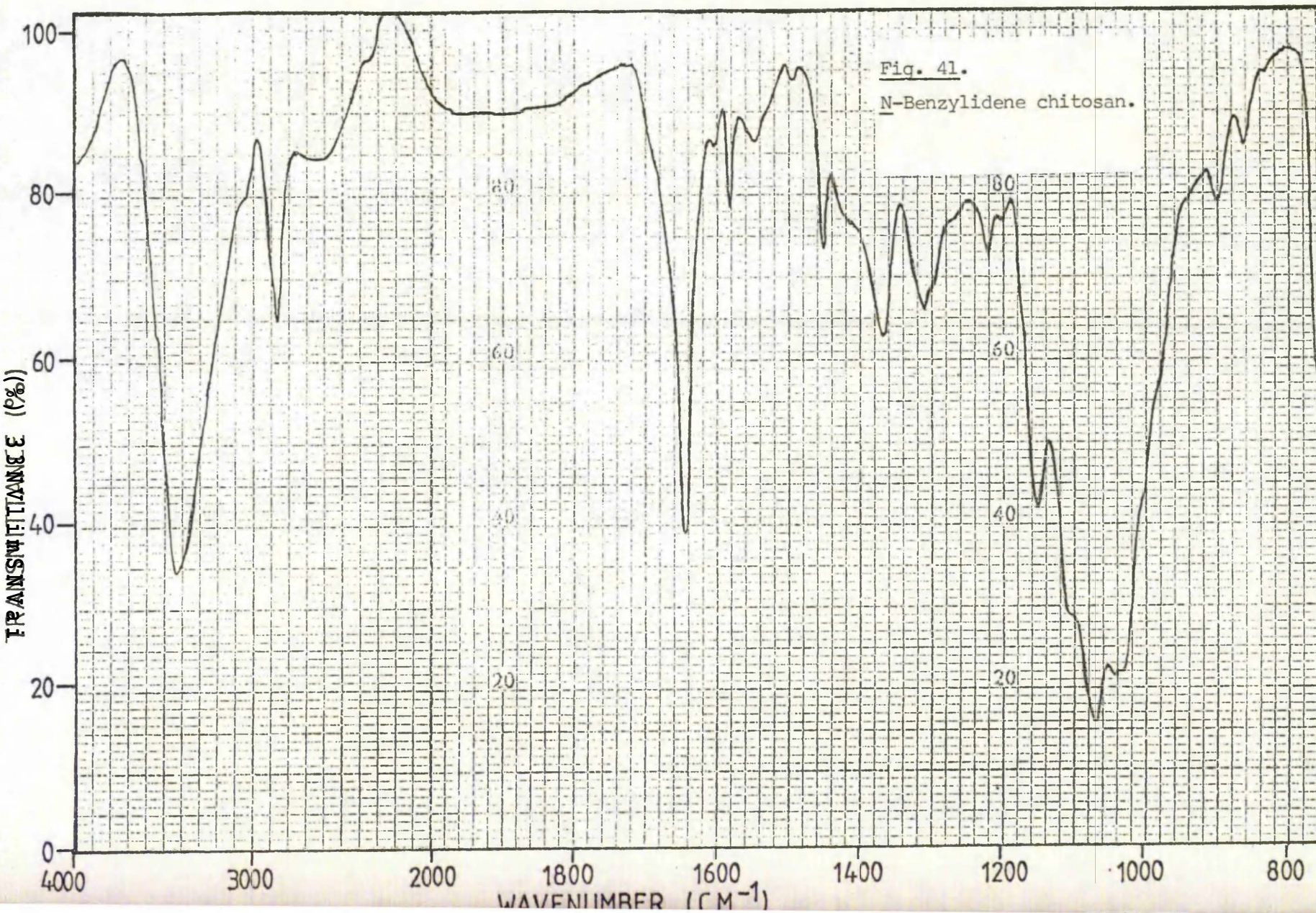


Fig. 41.

N-Benzylidene chitosan.

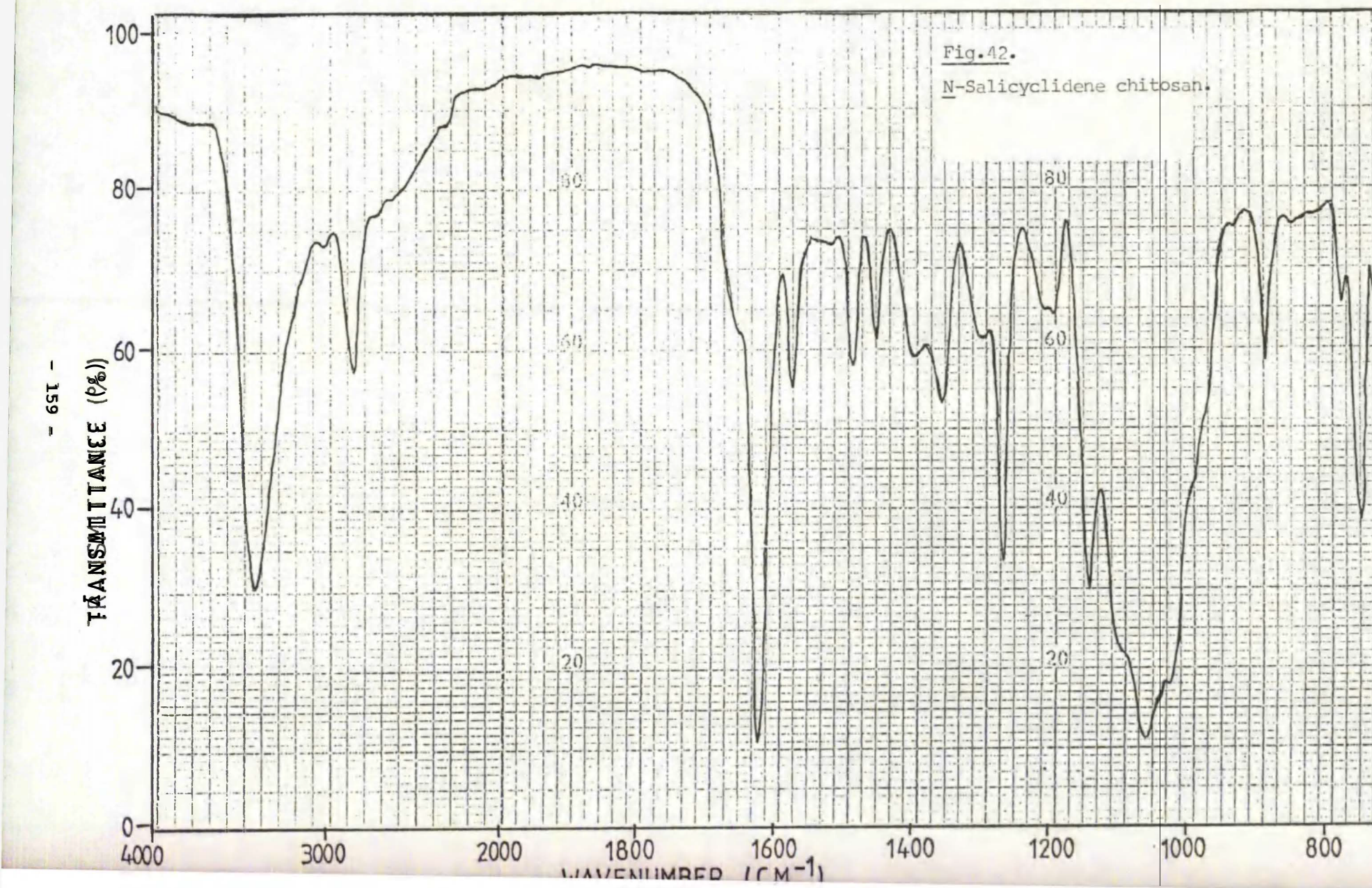


Fig.42.

N-Salicyclidene chitosan.

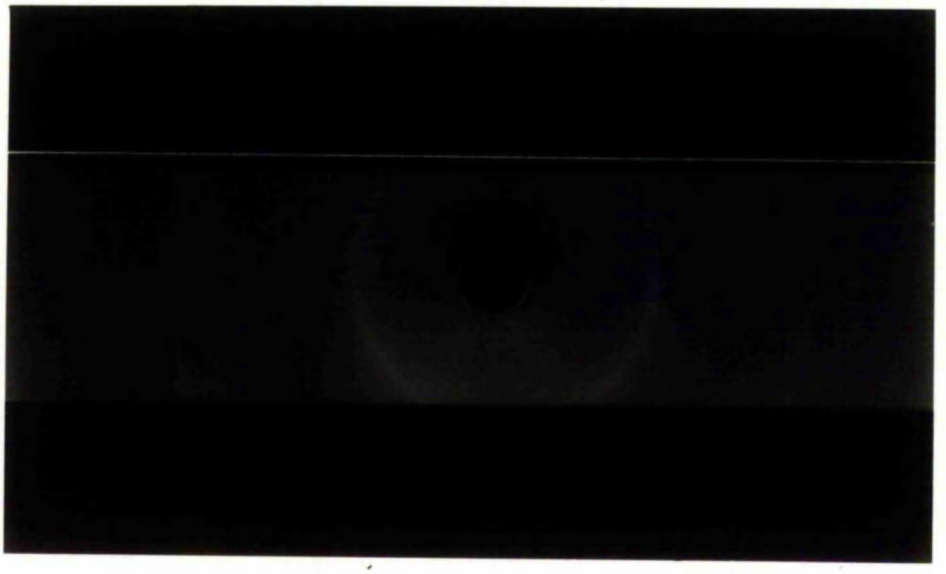


Figure 43. X-Ray diffraction pattern of N-salicyclidene chitosan.

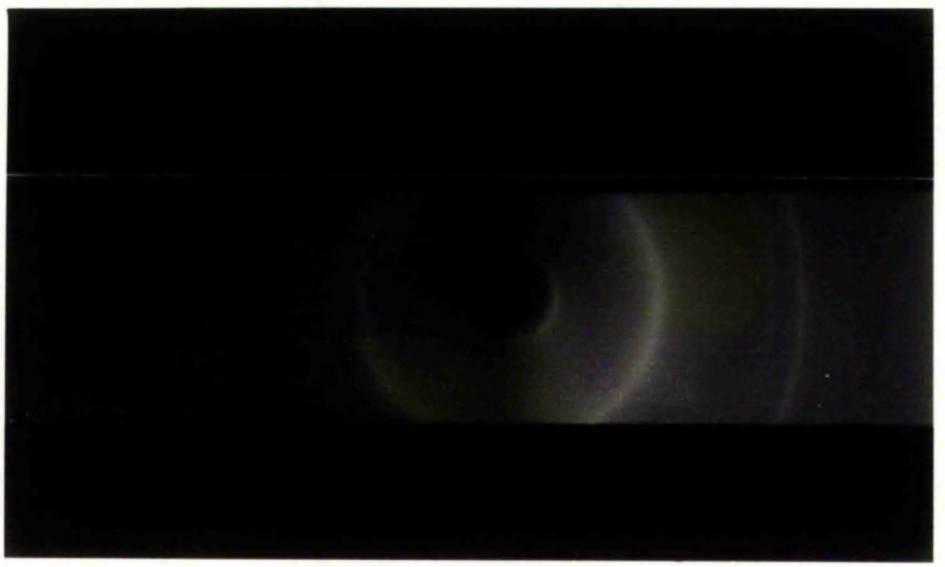


Figure 44. X-Ray diffraction pattern of N-benzylidene chitosan.

are fairly amorphous giving diffuse X-ray patterns. Comparison with N-benzoyl chitosan shows that the Schiff's base derivatives give slightly sharper X-ray patterns, indicating a slightly more ordered structure than the N-acyl derivative.

3.3.2. Preparation of Keto-imine Derivatives

Attempts were made to prepare simple keto-imine derivatives by reaction of acetophenone, ethylmethylketone, benzylacetone with chitosan in film or reprecipitated form, but were unsuccessful. Reaction with chitosan dissolved in aqueous acetic acid-methanol solutions was also unsuccessful, no evidence of reaction or gelation being observed in any case.

It has been reported[417] that 2-amino-2-deoxy-D-glucose reacts readily with benzoylacetaldehyde and benzoylacetone. However no reaction was observed with chitosan in either film or reprecipitated form. when treated with benzoyl acetone in methanol for up to 96 hours. On treatment of chitosan dissolved in aqueous acetic acid-methanol, the solution gelled and went opaque and subsequent characterisation of the product by infrared spectroscopy, Figure 45, showed that reaction had occurred, the strongest absorptions being at 1605 cm^{-1}, the carbonyl stretching frequency. After similar treatment of chitosan with acetylacetone, no gelation took place, but on casting a film from the reaction mixture and subsequent examination of the film, Figure 46, by infrared spectroscopy, it was found that reaction had taken place. Precipitation of the polymer in ether gave an off-white product. Both derivatives were insoluble in water, alcohols, ether and other organic solvents tried. The benzoylacetone adduct was scluble in formic acid and greatly swollen in DMSO

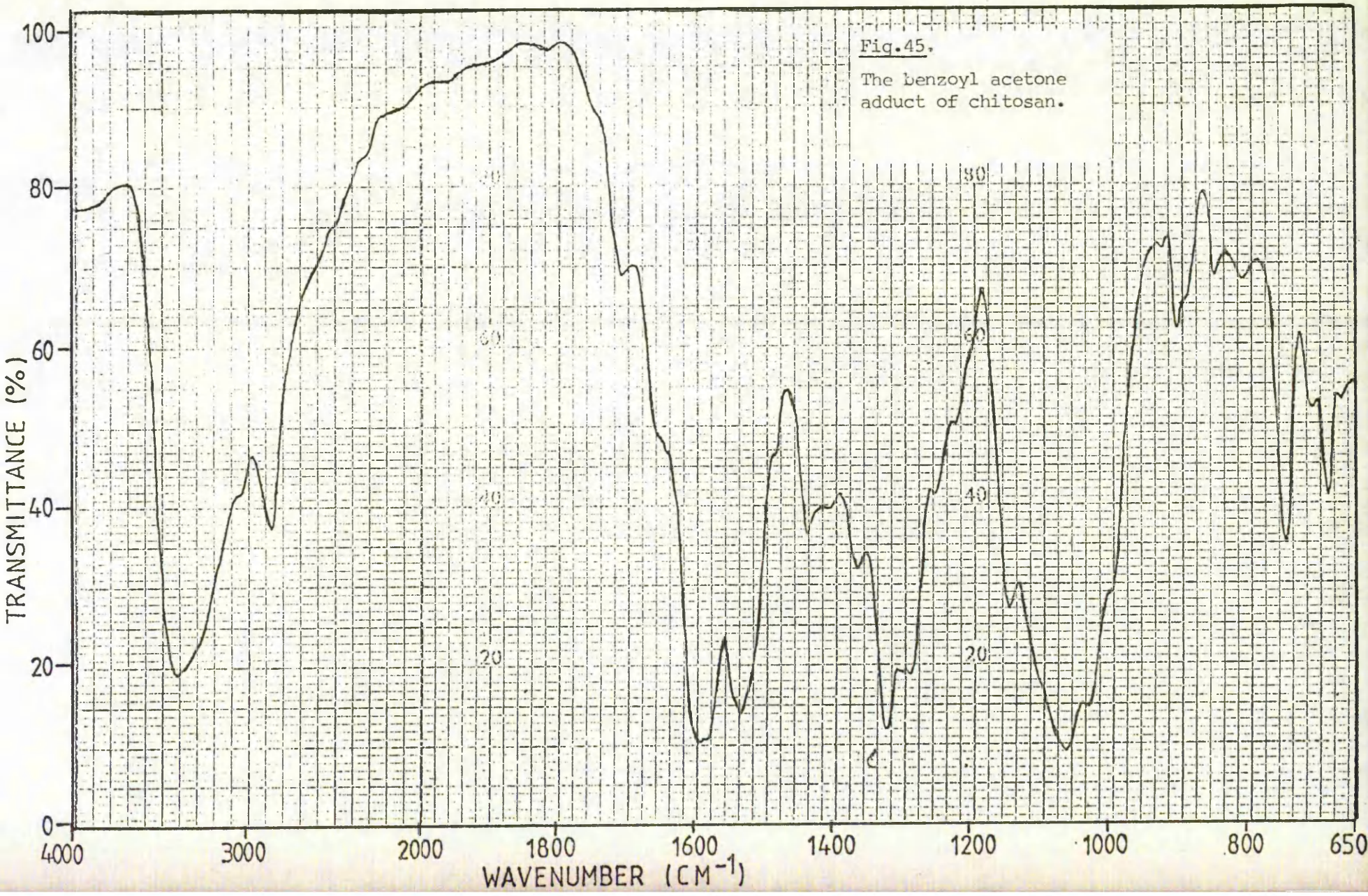


Fig.45.

The benzoyl acetone adduct of chitosan.

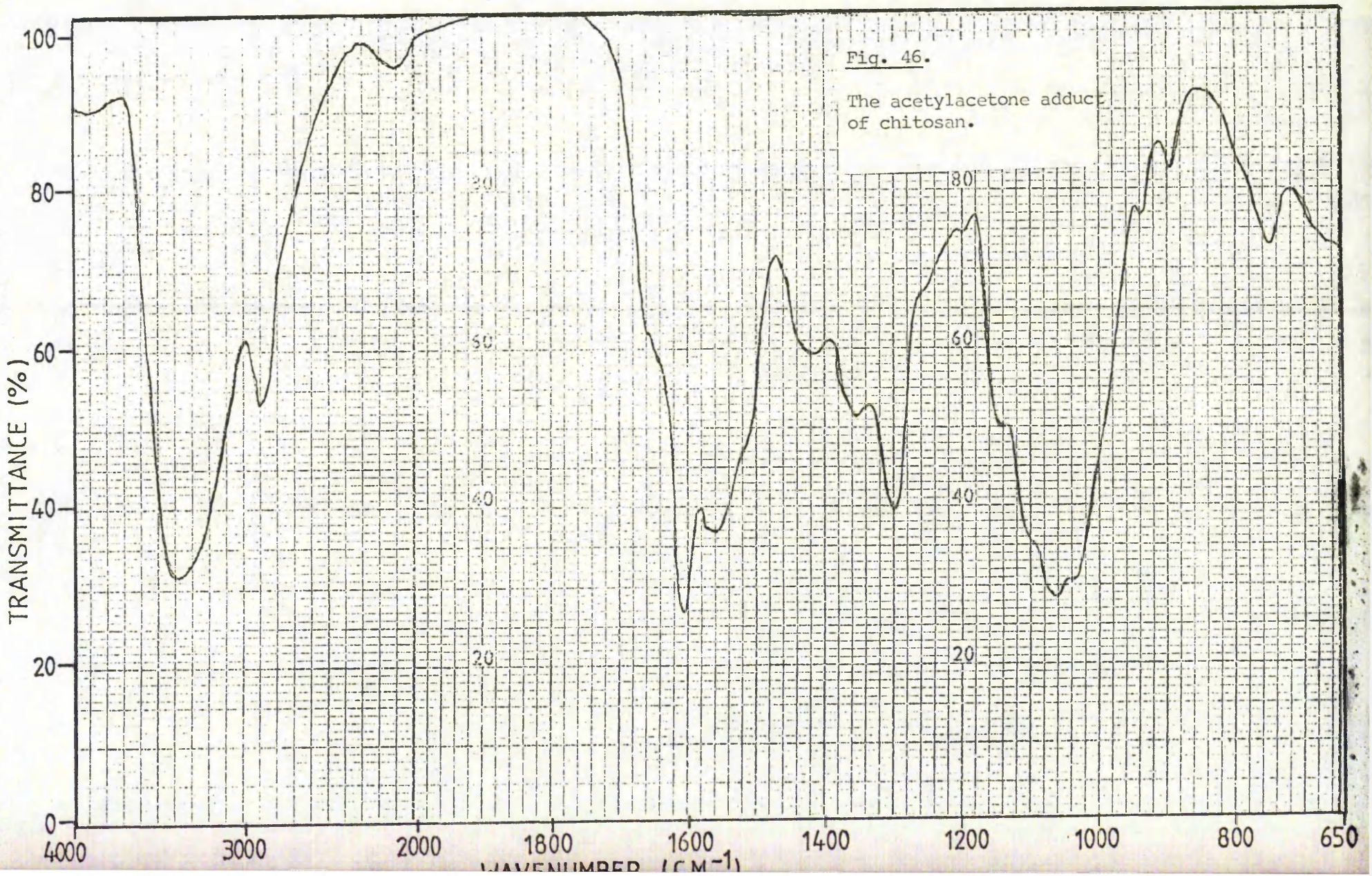


Fig. 46.

The acetylacetone adduct of chitosan.

3.3.3. Acetylation of Schiff's base Derivatives in Film Form

Initially, samples of various Schiff's base derivatives of chitosan were taken and the film samples steeped in acetic anhydride for 24 hours, then examined by infrared spectroscopy. The results obtained are summarised in Table 16.

Table 16 Schiff's base derivatives of chitosan acetylated for 24 hours

Derivative	Observation
Acetaldehyde	No reaction
Benzaldehyde	Cleavage of Schiff's base
Cinnamic aldehyde	Some cleavage of Schiff's base
Crotonaldehyde	Some O-acetylation
p-Dimethylaminobenzaldehyde	Some cleavage of Schiff's base
o-Nitrobenzaldehyde	Cleavage of Schiff's base & N-acetylation
p-Nitrobenzaldehyde	Cleavage of Schiff's base& N-acetylation
Salicylaldehyde	Some O-acetylation cleavage of Schiff's base

It was evident that although some O-acetylation was occurring it was accompanied by cleavage of the Schiff's base in the majority of cases. Thus any advantage of having the amine protecting group was lost and eventually N-acetylation was complete. No reaction at all was observed with the acetaldehyde derivative.

The study was extended, therefore, to three different acetylating media, and the reaction of the acetaldehyde, benzaldehyde, and salicylaldehyde Schiff's base derivatives of chitosan were examined in the three media. The solutions

used were i) acetic anhydride in the presence of triethylamine as an acid scavenger; ii) glacial acetic acid/acetic anhydride/perchloric acid, and iii) acetic anhydride/pyridine. Samples of each derivative were placed in the acetylating solutions for 24 hours at room temperature. The infrared spectrum of each sample was recorded and the results are summarised in Table 17.

Table 17 Acetylation of various Schiff's base derivatives of Chitosan in different media

Schiff's Base Derivative	Acetylating Solution		
	Acetic anhydride Triethylamine	Glacial Acetic Acid Acetic anhydride	Pyridine Acetic anhydride
Acetaldehyde	No reaction	Some O-acetylation Cleavage of aldehyde; amine salt formed	No reaction
Benzaldehyde	Good O-acetylation	Good O-acetylation Cleavage of aldehyde	Good O-acetylation
Salicylaldehyde	Good O-acetylation	Some O-acetylation Cleavage of aldehyde	V-good O-acetylation

The acetaldehyde derivative was the most unreactive and O-acetylation occurred only to a limited extent in acidic media. Benzaldehyde and salicylaldehyde derivatives underwent O-acetylation to a large extent in base media, and to some extent in acidic media, but this was accompanied by hydrolysis of the aldehyde. N-Salicyclidene chitosan gave the best results overall, and Figures 47,48 and 49, show the infrared spectra of N- salicyclidene chitosan

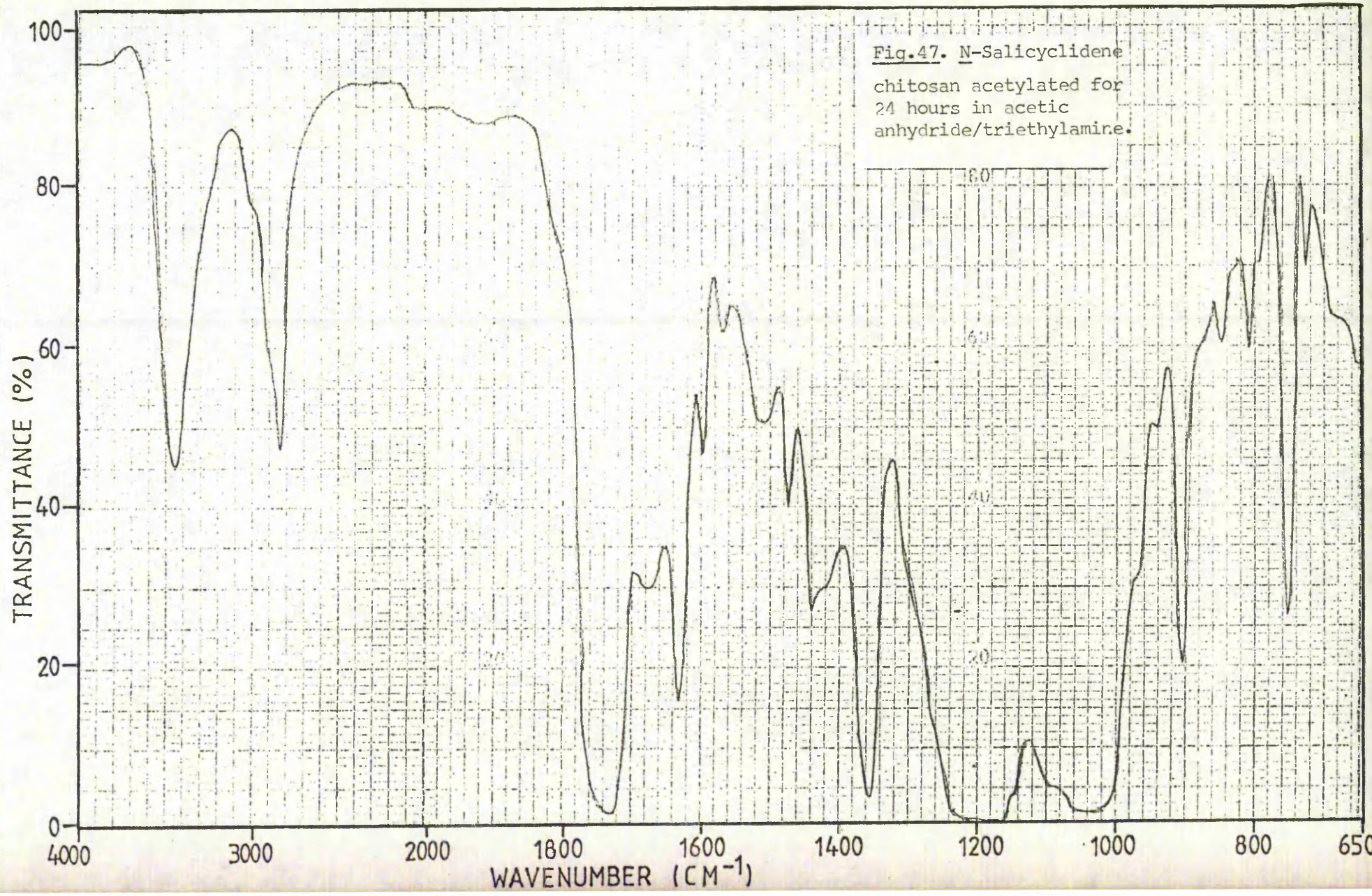


Fig.47. N-Salicyclidene chitosan acetylated for 24 hours in acetic anhydride/triethylamine.

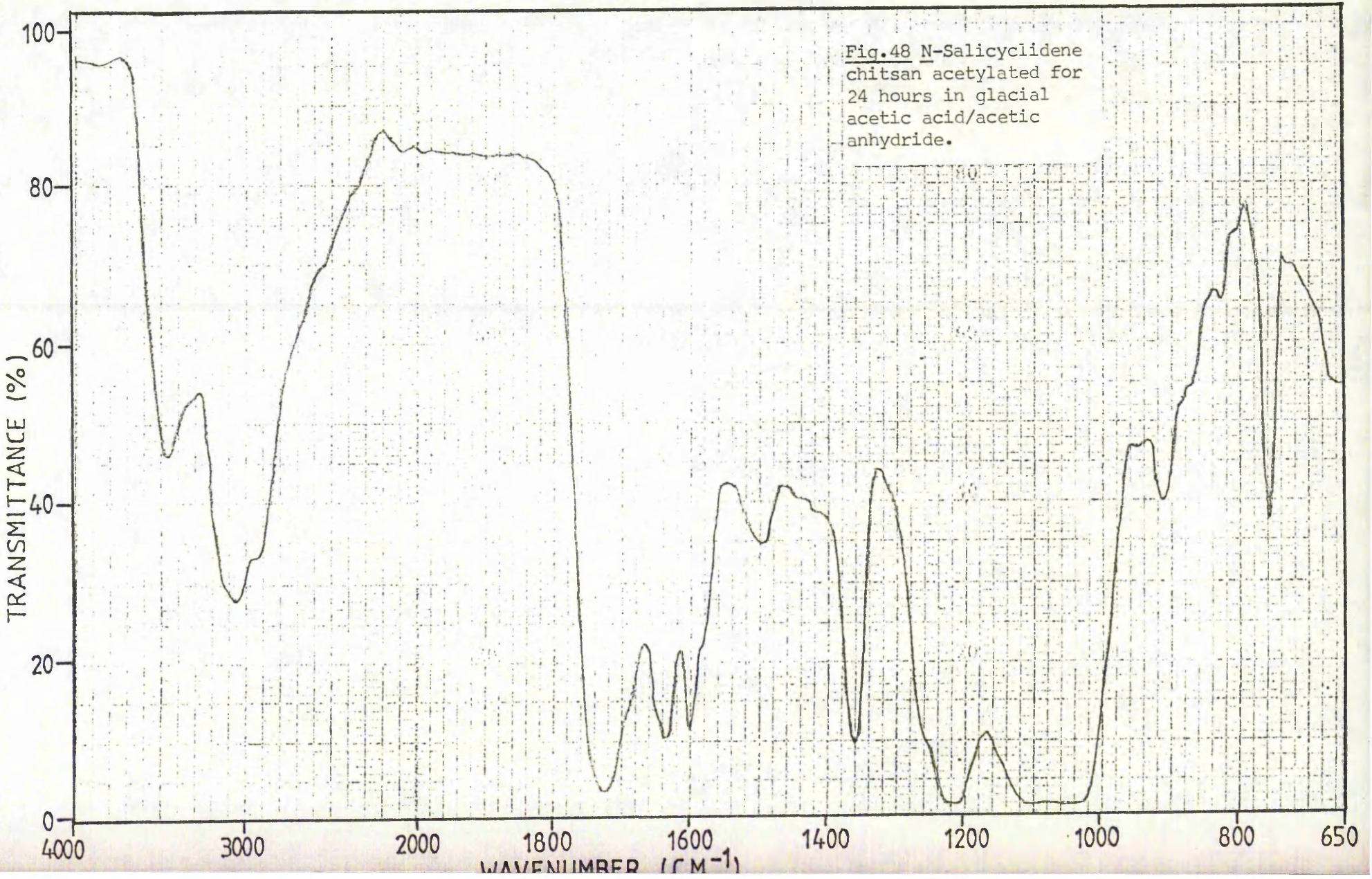


Fig.48 N-Salicyclidene chitsan acetylated for 24 hours in glacial acetic acid/acetic anhydride.

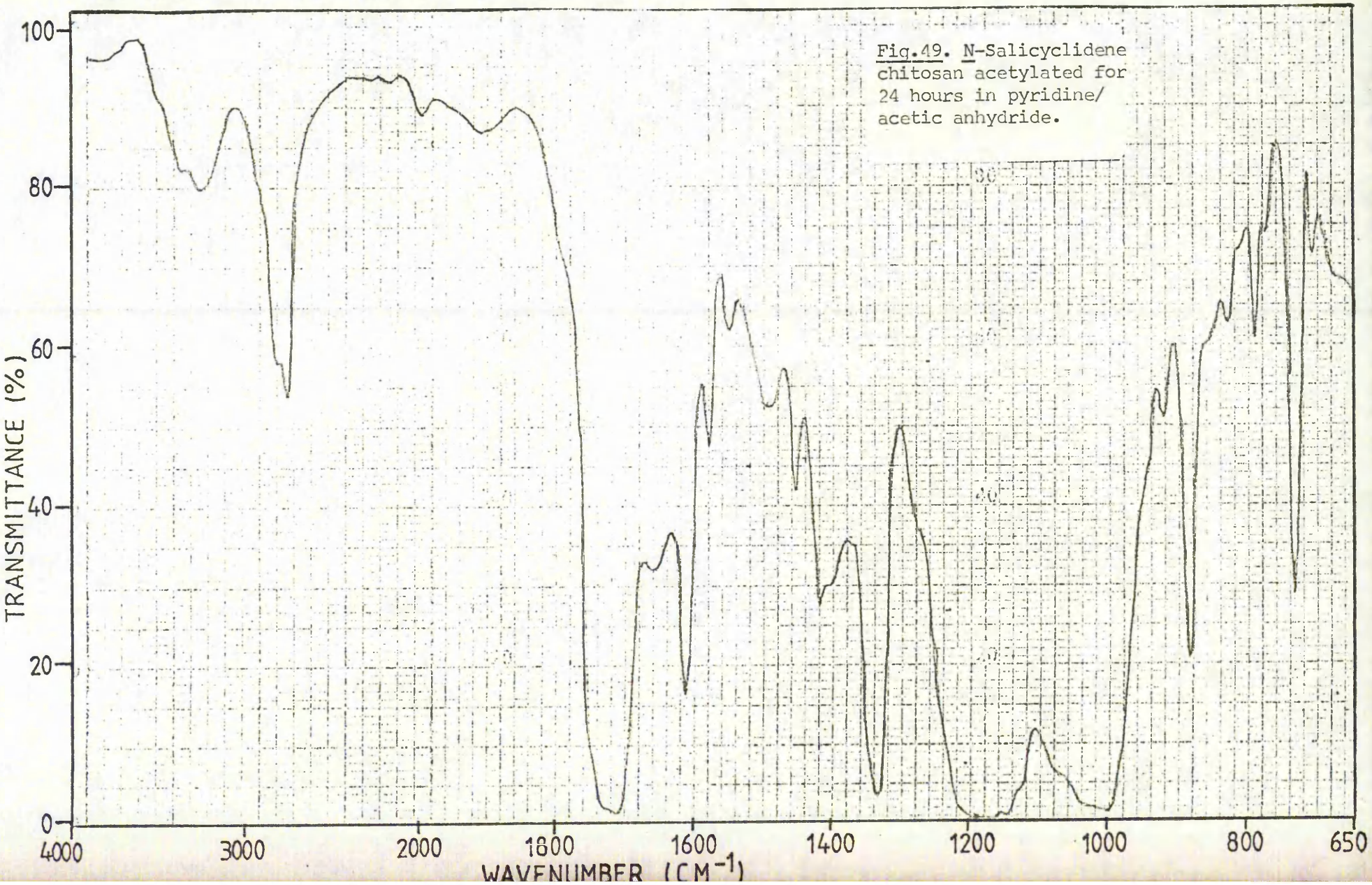


Fig.49. N-Salicyclidene chitosan acetylated for 24 hours in pyridine/ acetic anhydride.

after treatment for 24 hours in the different media.

Comparable ranges of aldehyde derivatives of chitosan with those of the N-acyl derivatives were then prepared and the O-acetylation of these studied in greater detail in acetic anhydride-pyridine mixtures. All samples were pre-steeped in pyridine prior to use.

3.3.3.1. Linear Aliphatic Aldehydes

Samples of the Schiff's base derivatives of chitosan prepared from the linear aliphatic aldehydes, acetaldehyde to decanaldehyde, were taken and to each sample in pyridine was added acetic anhydride. The reactions were then followed as before.

Figure 50 is the plot of A1740, corrected for film thickness, against the different Schiff's base chitosans used, and Figure 51 is A1740 plotted against time for each of the derivatives. Hydrolysis of the Schiff's bases occurred during the course of the reactions, together with N-acetylation in all cases. It is evident that no O-acetylation occurs with the N-ethylidene chitosan but on changing to the N-propylidene derivative there is a large increase in O-acetylation. Introduction of higher aliphatic aldehyde derivatives increases the extent of reaction but in non-uniform manner. Increased time periods show that the extent of O-acetylation increases, the rate decreasing with increasing time. After 30 hours, comparison of the hydroxyl absorptions at 3450 cm^{-1} with those of the starting films indicates that between 55-60% of the hydroxyl groups remain unacetylated. Fairly extensive hydrolysis of the Schiff's bases has also occurred.

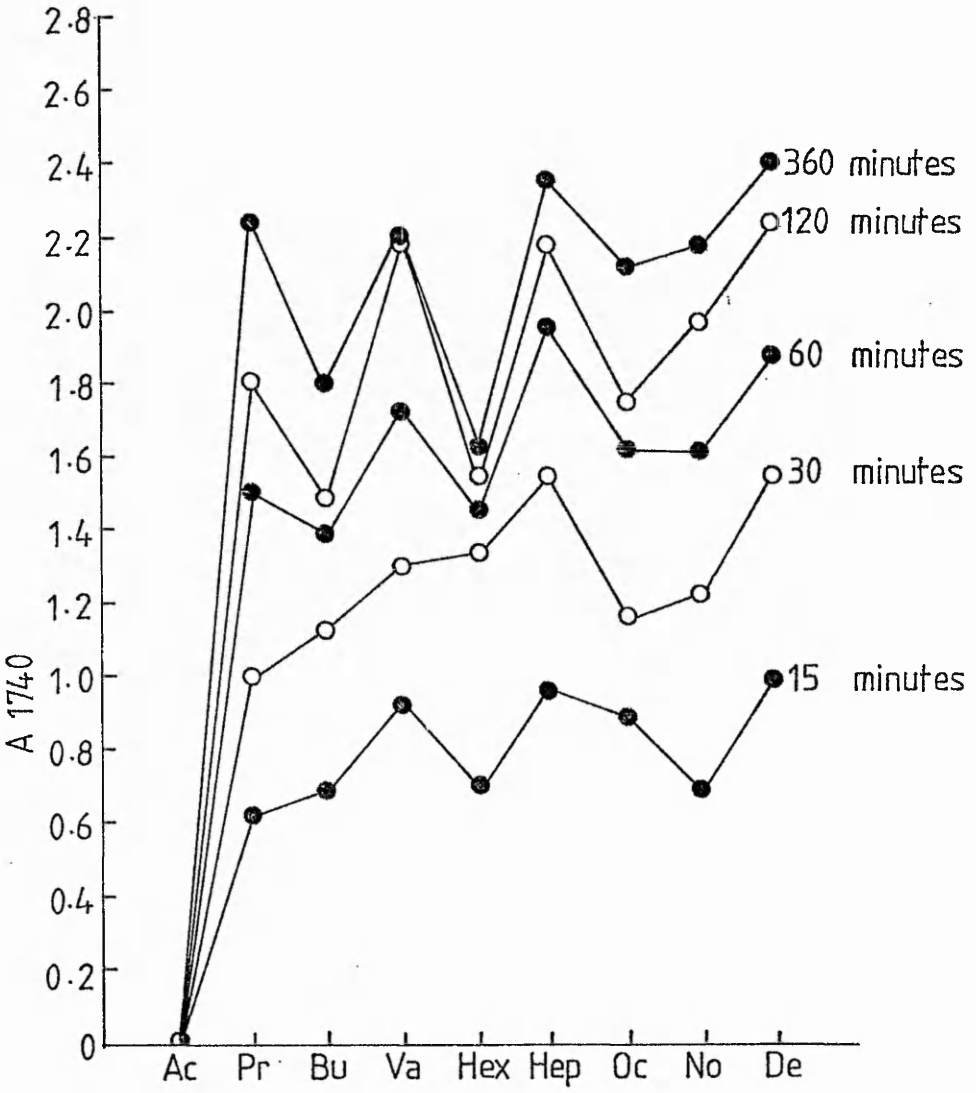


Figure 50. The extent of O-acetylation of linear aliphatic Schiff's base derivatives of chitosan at different time periods.

(ac=acetaldehyde; pr=propionaldehyde; bu=butyraldehyde; va=valeraldehyde; hex=hexanaldehyde; hep= heptanaldehyde; oc=octanaldehyde; no=nonanaldehyde; de= decanaldehyde.)

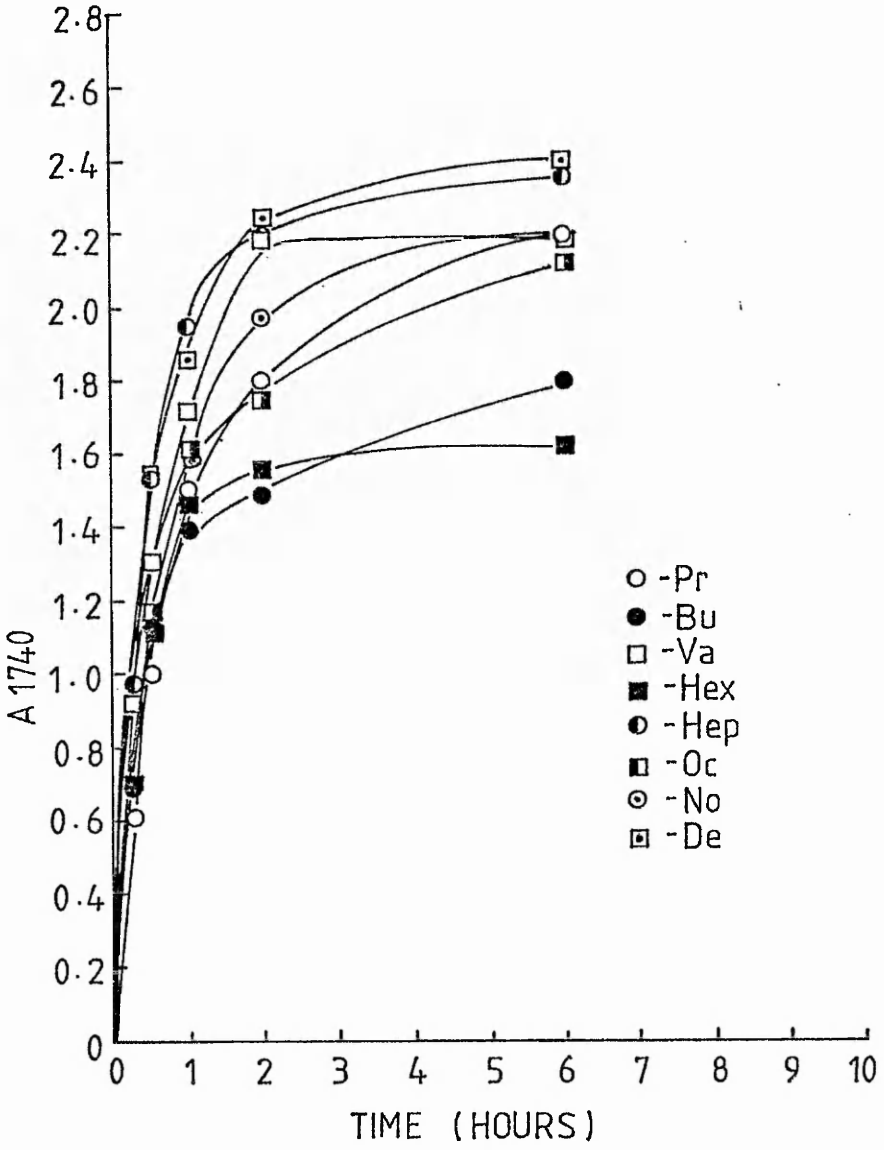


Figure 51. Rate of O-acetylation of linear aliphatic Schiff's base derivatives of chitosan.

(ac=acetaldehyde; pr=propionaldehyde; bu=butyraldehyde; va=valeraldehyde; hex=hexanaldehyde; hep=heptanaldehyde; oc=octanaldehyde; no-nonanaldehyde; de=decanaldehyde.)

3.3.3.2. Branched Aliphatic Aldehydes

A series of Schiff's base derivatives of chitosan prepared by reaction with propionaldehyde, iso-butyraldehyde and trimethylacetaldehyde were formed and O-acetylated in the normal way. The results obtained are shown in Figure 52. The unbranched member of the series, N-propylidene chitosan, is the most readily O-acetylated and N-trimethylethylidene chitosan the least O-acetylated. Extensive hydrolysis of the Schiff's bases was again observed and N-acetylation occurred in all cases.

A comparison was also made of the butyraldehyde and iso-valerylaldehyde derivatives of chitosan. Very similar results were obtained, Figure 52, the branched derivative having a slightly greater extent of O-acetylation.

It is thought that any steric advantages these derivatives might have were lost due to the susceptibility to hydrolysis in the acetylating medium.

3.3.3.3. Aromatic Aldehydes

Earlier results had shown that the N-salicyclideneand N-benzylidene chitosans were amenable to O-acetylation in acetic anhydride/pyridine. These products, together with the o-,m-,p- tolualdehyde derivatives, were therefore taken and their O-acetylation studied in more detail.

Figures 53 &54 show the results from the O-acetylation experiments expressed as A 1740 against the different Schiff's base derivatives and as A 1740 against time. The N-benzylidene chitosan and N-p-toluylidene chitosan have very similar degrees of O-acetylation, showing that, as expected, the p-substituent creates no more useful space than the unsubstituted derivative. The o-substituted tolualdehyde derivative has a similar

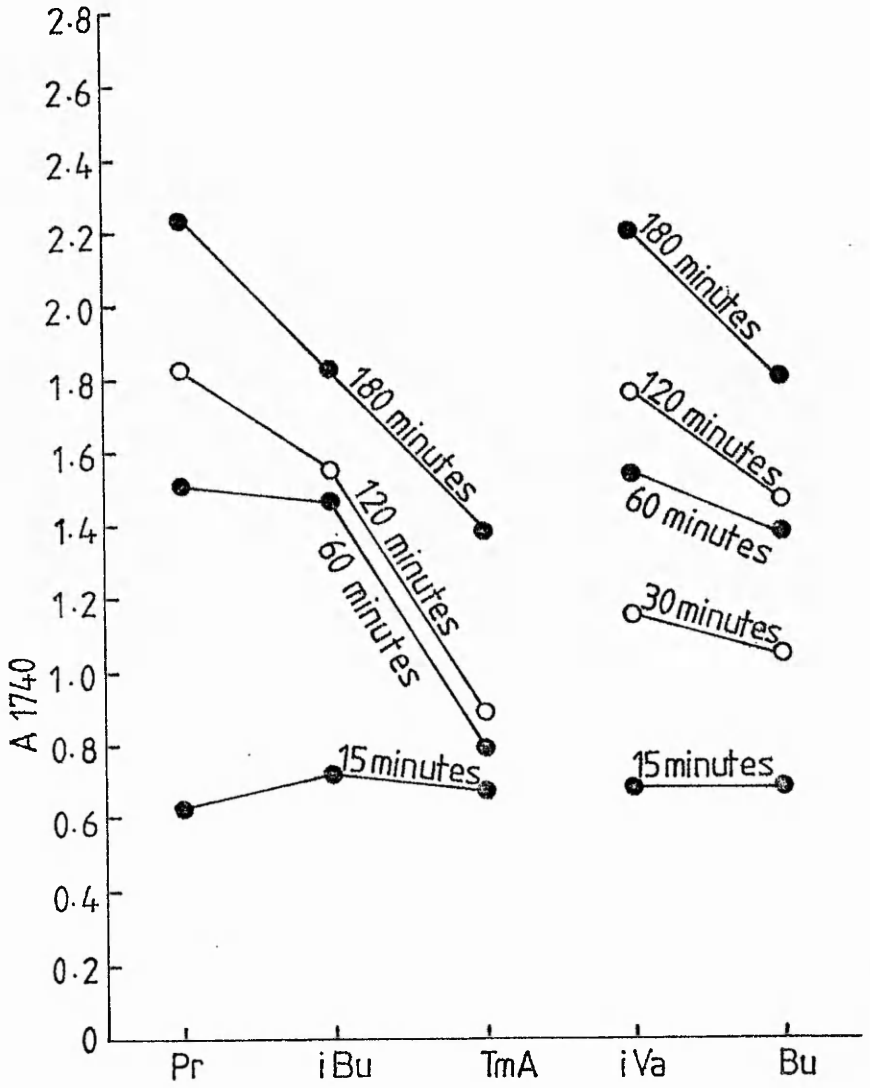


Figure 52. The extent of O-acetylation of branched aliphatic Schiff's base derivatives of chitosan at different time periods.

(pr=propionaldehyde; ibu=iso-butyraldehyde; tma=trimethylacetaldehyde; iva=iso-valeryläldehyde; bu=butyraldehyde.)

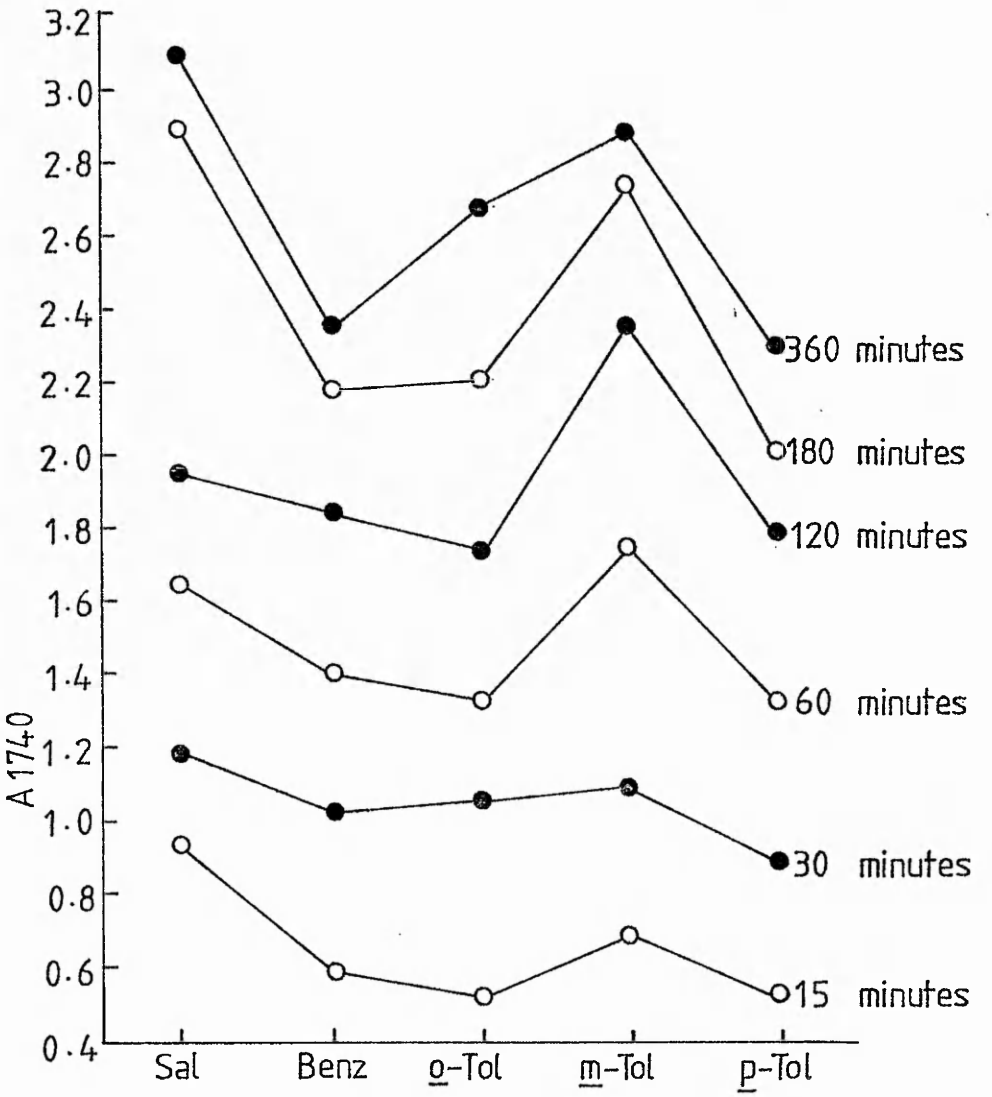


Figure 53. The extent of O-acetylation of aromatic Schiff's base derivatives of chitosan at different time periods.
(Sal= salicylaldehyde ; benz= benzaldehyde; o-tol=o-tolualdehyde; m-tol=m-tolualdehyde; p-tol=p-tolualdehyde.)

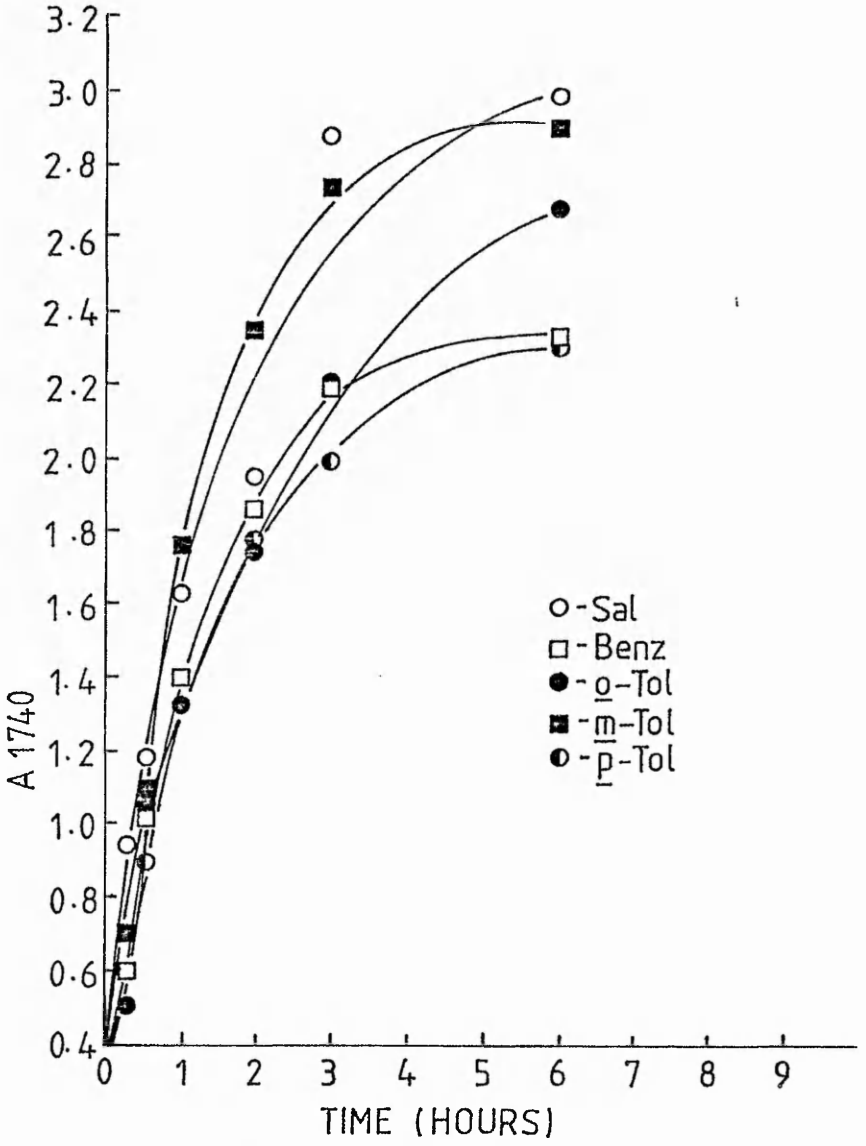


Figure 54. Rate of O-acetylation of aromatic Schiff's base derivatives of chitosan.

(Sal= salicylaldehyde ; benz= benzaldehyde; o-tol=o-tolaldehyde; m-tol=m-tolualdehyde; p-tol=p-tolualdehyde.)

degree of reaction also, suggesting that any size advantage is offset by increased hinderance to the C(3) and C (6') hydroxyl groups.

The m- substituted tolualdehyde derivative of chitosan has a similar degree of O-acetylation as the salicylaldehyde derivative and is greater than the other substituted toluylidene chitosans. Presumably the m-substituted methyl group creates more space around the reaction centre and is sufficiently far away to avoid steric hinderance to the reaction. The N-salicyclidenechitosan has the best degree of O-acetylation overall and this may be due to several factors. The hydroxyl group present in salicylaldehyde forms a chelate ring with the nitrogen and hence may create more space around the reaction centre. The N-salicyclidene chitosan was the least susceptible to hydrolysis of the Schiff's base and hence the advantage of the amine protecting groups was maintained throughout the course of the reaction. Prolonged treatment with acetic anhydride and pyridine for 80 hours gave a virtually fully substituted O-acetyl-N-salicyclidene chitosan and an approximately 85% substituted O-acetyl-N- m-toluylidene chitosan. The remaining derivatives were found to be about 60% O-acetylated.

3.3.4. Acetylation of Schiff's base Derivatives of Chitosan in Solid Form

From the evidence of the film work, it would seem apparent that fully O-acetylated Schiff's base products of aromatic aldehyde derivatives of chitosan could be prepared. N-salicyclidene chitosan had given the best results on film and this was used, therefore, as a starting point.

N-salicyclidene chitosan, prepared using low molecular weight chitosan, was treated with a solution of acetic anhydride/triethylamine/pyridine and a clear, brown, viscous solution formed. This solution was poured into

ether. and the reprecipitated product collected by filtration as a pale yellow powder. The product was soluble in methylene chloride, chloroform, DMF, DMSO, glacial acetic acid and formic acid, and had an inherent viscosity, ηinh, of 2.36 dlg^{-1}. Clear, coherent films were cast from methylene chloride and chloroform solutions and the infrared spectra of these films, Figure 55, showed total O-acetylation, with strong absorptions at 1745 cm^{-1} due to C=O stretching and at 1225 cm^{-1} due to C-O stretching.

Preparation of the same derivative, using high molecular weight chitosan, was not as successful when using the same method as for the low molecular weight product. However increased reaction times brought about further O-acetylation and eventually a virtually completely substituted product was achieved. The solubility of the product was not as good as the low molecular weight derivative, but films could be cast from methylene chloride solutions. The product was highly swollen in DMF and soluble in DMSO.

Attempts to prepare the O-propionyl- and O-hexanoyl-analogues were only partially successful. The products formed only swelled in methylene chloride, chloroform, DMF, and DMSO, and no suitable solvent was found to prepare films. Infrared spectra of the products showed that only partial O-acylation had occurred and that increased reaction times brought about little improvement.

The O-acetylation of N- benzylidene chitosan, prepared from high molecular weight chitosan, was attempted using the same method as for N-salicyclidene chitosan. The product formed was soluble in formic acid, highly swollen in chloroform, DMSO and DMF, and partially swollen in methylene chloride and epichlorohydrin. A clear coherent film could be cast from the formic acid solution and the infrared spectrum, Figure 56, showed that O-acetylation was virtually complete, but that hydrolysis of the Schiff's base had

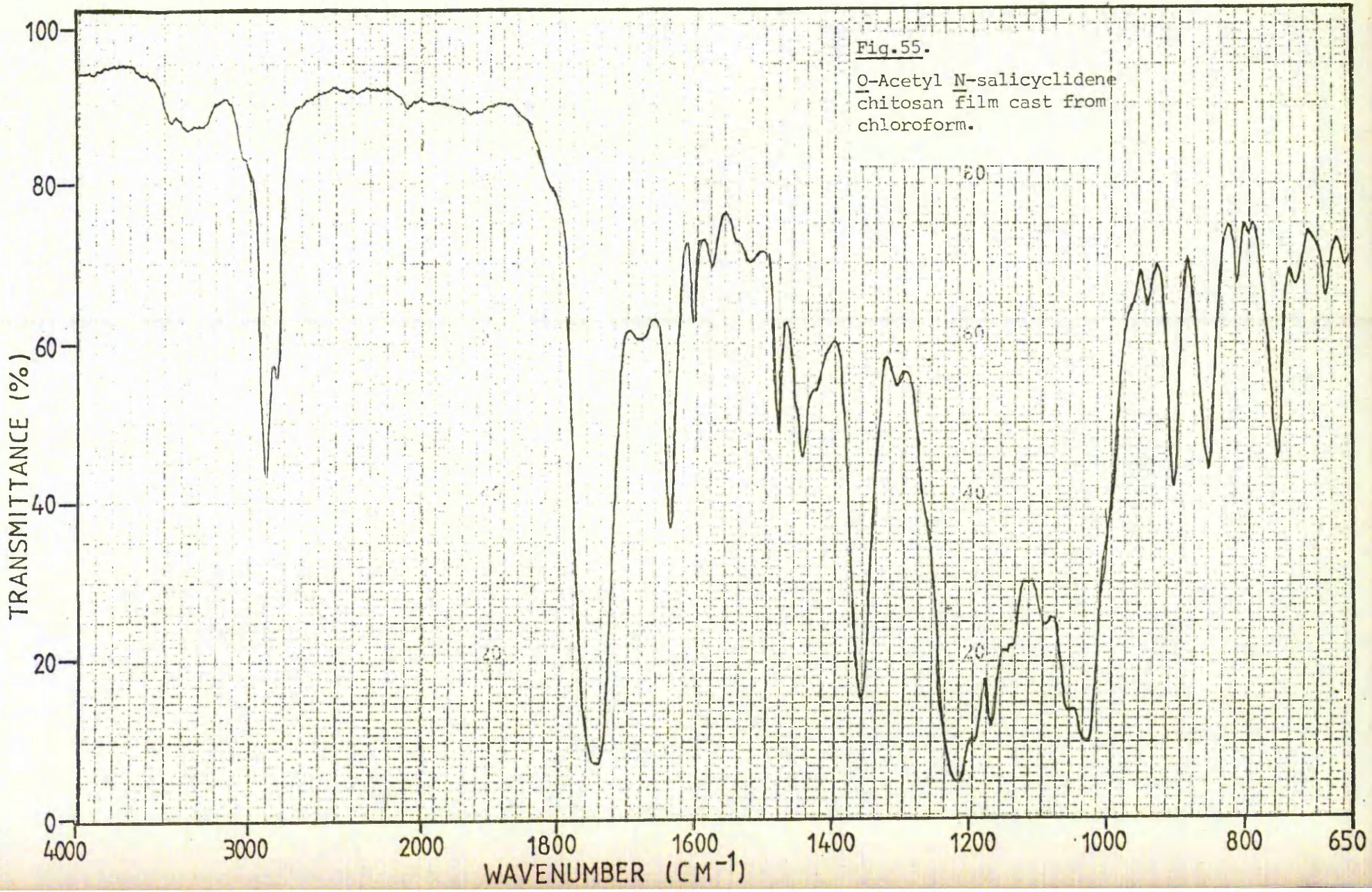


Fig.55.

O-Acetyl N-salicyclidene chitosan film cast from chloroform.

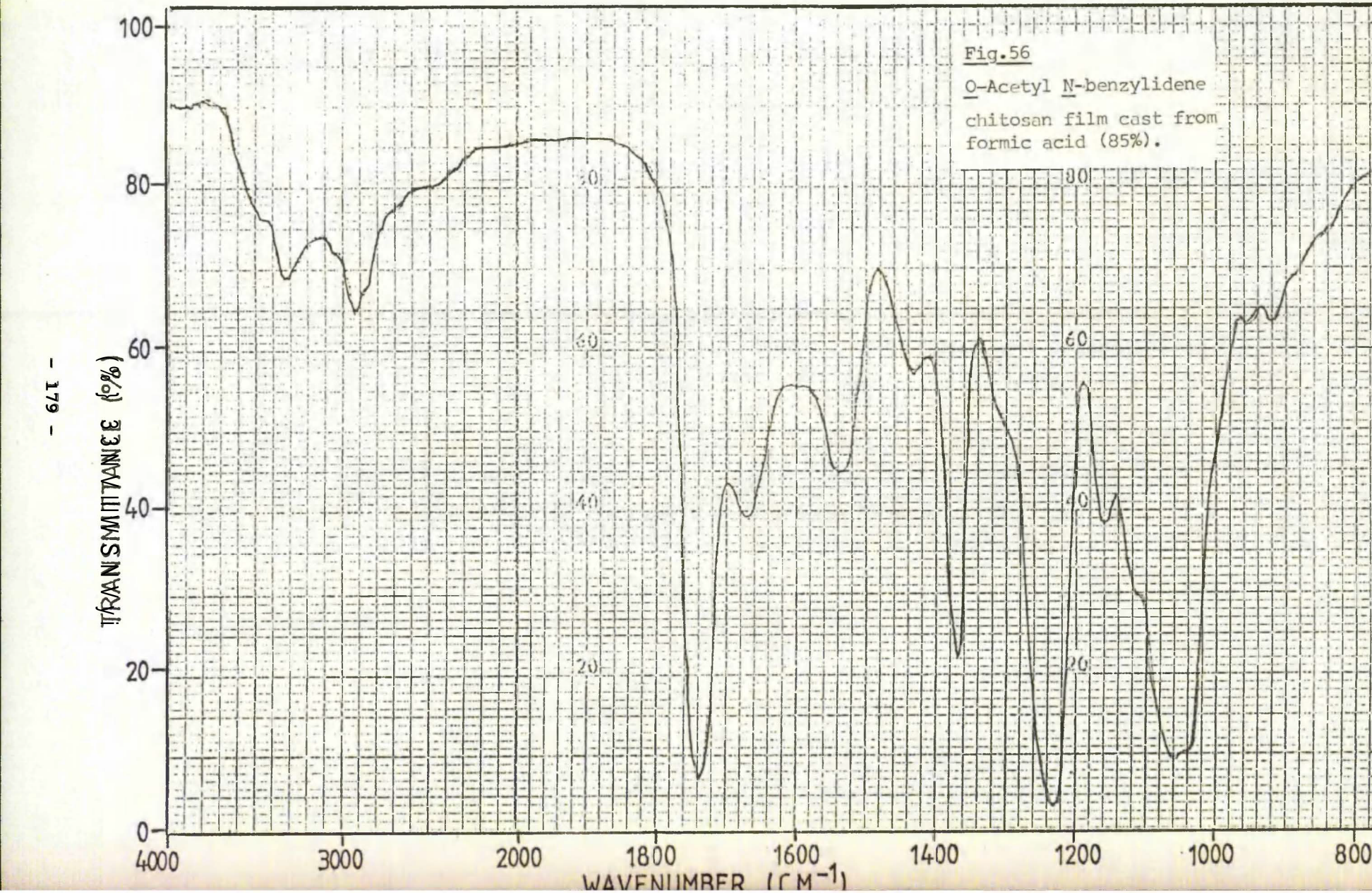


Fig.56

O-Acetyl N-benzylidene chitosan film cast from formic acid (85%).

occurred. The infrared spectrum of a very fibrous film cast from chloroform, Figure 57, showed good O-acetylation with only minimal hydrolysis of the Schiff's base suggesting that the hydrolysis had occurred in the solution in formic acid.

3.3.5. Acetylation of the Keto-imine Derivatives of Chitosan

Treatment of samples of the benzoylacetone adduct and the acetylacetone adduct of chitosan with pyridine-acetic anhydride solutions afforded viscous solutions which on pouring into ether yielded the O-acetylated derivatives. Both products were soluble in formic acid and swelled greatly in epichlorohydrin, DMF and DMSO. Clear, coherent films were cast from formic acid solutions. The infrared spectrum of the O-acetylated benzoylacetone adduct, Figure 58 , showed good O-acetylation, as evidenced by absorption bands at 1735 and 1230 $cm.^{-1}$ Some hydrolysis of the ketoimine group had occurred, but this may have been due to the formic acid solution. A similar spectrum was obtained with the acetylacetone adduct.

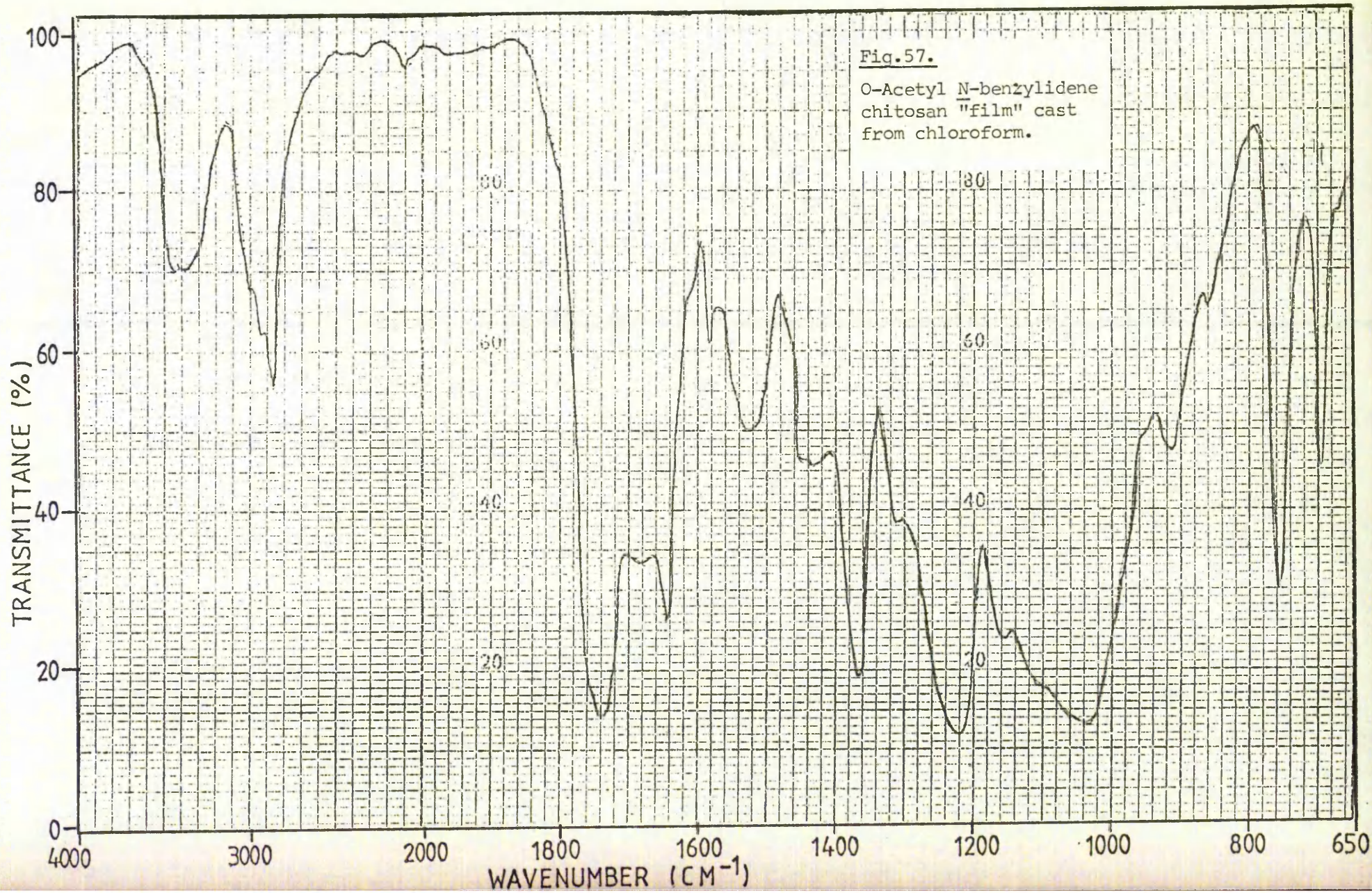


Fig.57.

O-Acetyl N-benzylidene chitosan "film" cast from chloroform.

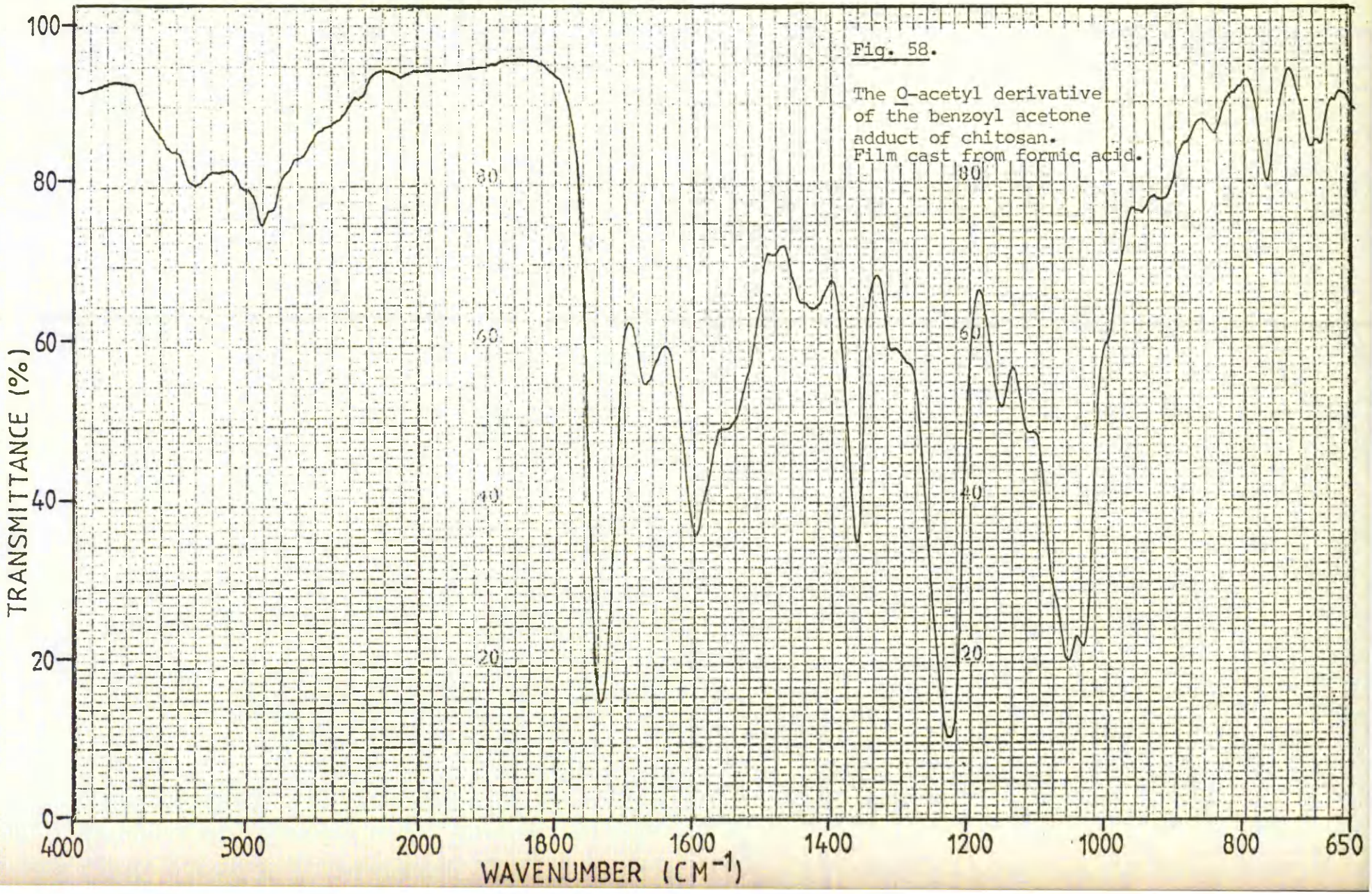


Fig. 58.

The O-acetyl derivative of the benzoyl acetone adduct of chitosan. Film cast from formic acid.

3.4. Comparison of Acetylation of N-Acyl Chitosans and Schiff's Base Derivatives of Chitosan

The acetylation of the linear aliphatic N-acyl chitosans and the linear aliphatic Schiff's base derivatives of chitosan at first sight appears very similar. However a more detailed look at the results shows several major differences. Figure 59 gives the extent of O-acetylation of the different substituted chitosans expressed as A1740 against the different chitosans after 6 hours reaction time.

The extent of O-acetylation of N-propylidene chitosan is about 10 times greater than that of the N-propionyl chitosan, and the majority of the Schiff's base derivatives have a higher extent of acetylation than the corresponding N-acyl derivatives. The first member of each series, N-ethylidene and N-acetyl chitosan, show little or no reaction.

After extended treatments the N-acyl chitosans are, on average, 40-45% O-acetylated whereas the Schiff's base derivatives are 45-50% O-acetylated. However, the Schiff's base chitosans have in all cases undergone hydrolysis of the Schiff's base with accompanying N-acetylation. Any size advantage of the N-substituent is therefore lost as hydrolysis proceeds, and it is suggested that if hydrolysis did not occur the extent of O-acetylation might be greater. No hydrolysis occurs with the N-acyl chitosans.

Very similar results are observed on comparison of the branched aliphatic derivatives; the overall O-acetylation of the Schiff's base derivatives being slightly higher in all cases, but again hydrolysis of the N-substituent occurred.

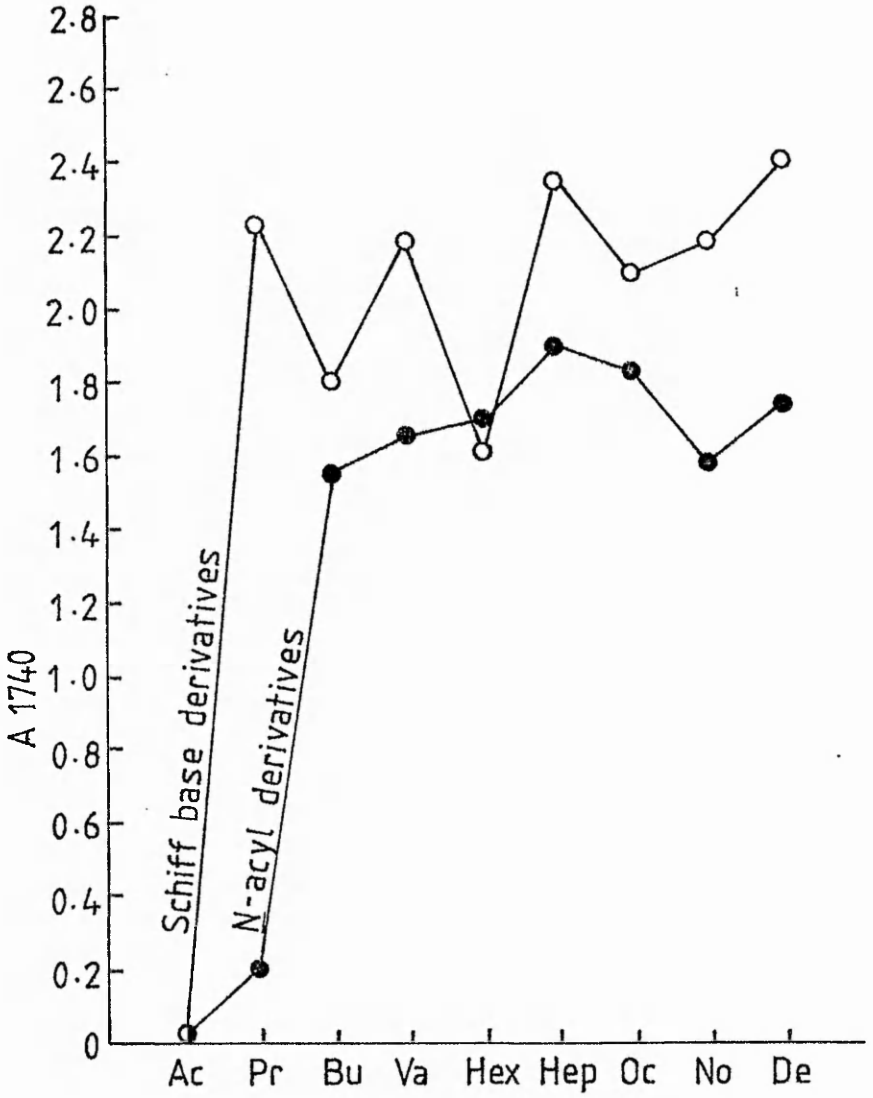


Figure 59. Comparison of the extents of O-acetylation of the linear aliphatic N-acyl chitosans and the linear aliphatic Schiff's base derivatives of chitosan after 6 hours reaction time.

It is with the aromatic derivatives that the advantageous nature of the Schiff's base derivatives becomes clearer. Figures 60 & 61 show the extent of O-acetylation of the m-toluic anhydride and aldehyde derivatives and of the benzoic anhydride and benzaldehyde derivatives of chitosan plotted against time. In each case the initial rate of O-acetylation of both the anhydride and aldehyde derivatives is equivalent, but after 30 minutes the rate of O-acetylation of the anhydride derivative falls away and levels off. The aldehyde derivative, on the other hand, continues at a comparable rate and only after about 2 hours does the rate start to decrease. The overall extent of O-acetylation of the aldehyde derivatives is approximately twice that of the equivalent anhydride derivatives. Some hydrolysis of the N-substituent was observed in the case of the Schiff's base derivatives, but almost complete O-acetylation was achieved in both cases. The N-salicyclidene chitosan gave complete O-acetylation.

From the point of view of overall size, the equivalent anhydride or aldehyde substituent should be of approximately the same size and so no advantage would be gained from the Schiff's base derivative. However, as shown by the early results on the O-acetylation of N-acyl chitosans, the spacing between the polymer chains is the controlling factor in the O-acetylation of chitosan. There is a critical separation between chains below which O-acetylation does not occur to any appreciable extent and this is shown both by the N-acetyl- and N-ethylidene chitosans which undergo little or no O-acetylation. Further, evidence from the X-ray diffraction studies shows the N-acetyl chitosan to be a well ordered crystalline product but, on increasing the length of the N-acyl substituent, the polymer becomes less ordered and more amorphous, so allowing easier access to chemical reagents.

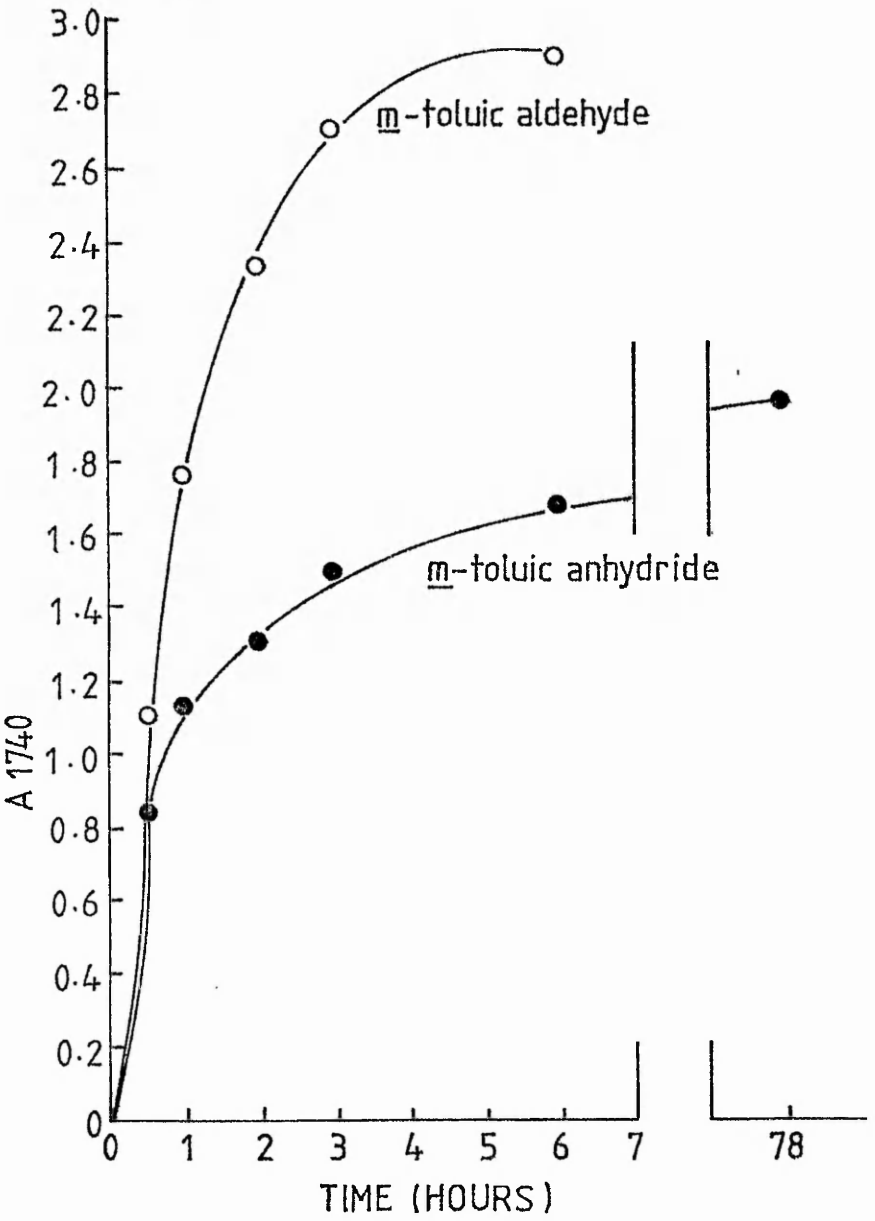


Figure 60. Comparison of the rate of O-acetylation of the m-toluic aldehyde and m-toluic anhydride derivatives of chitosan.

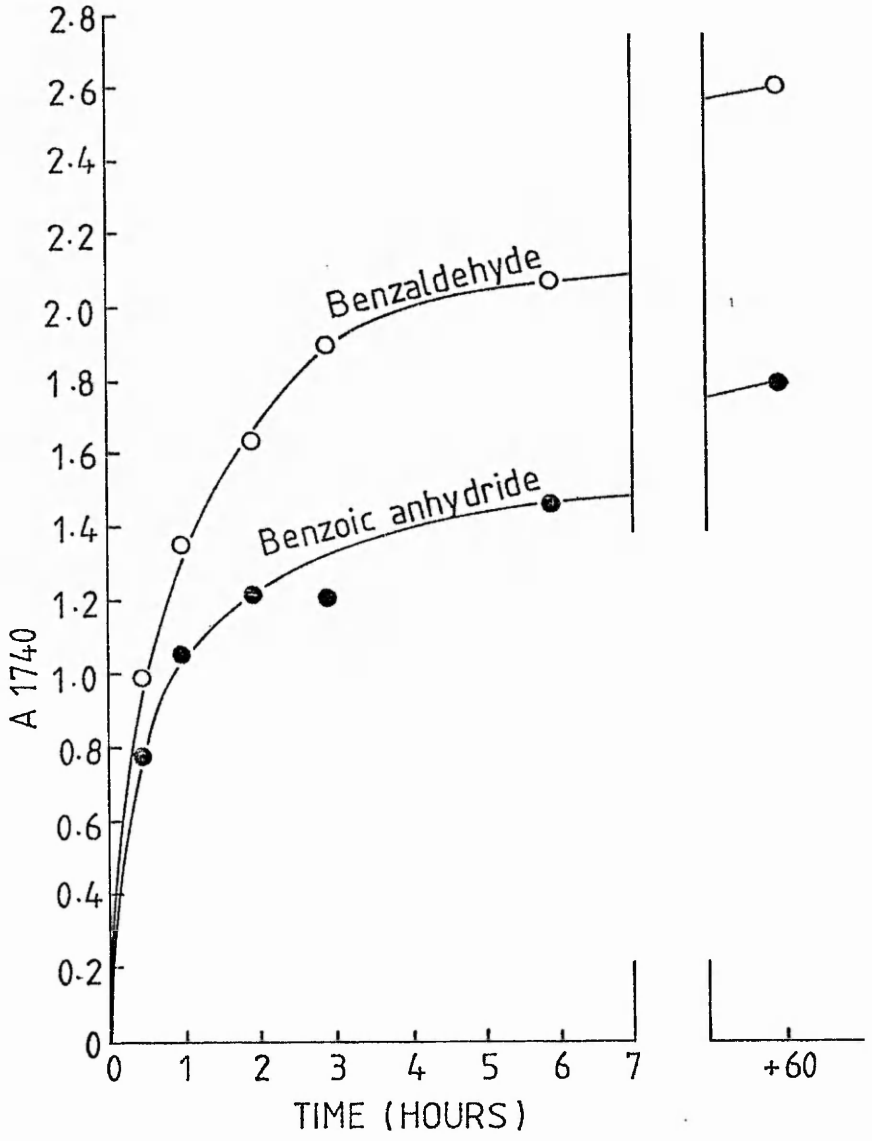
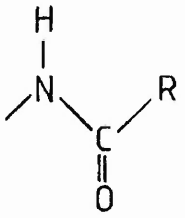
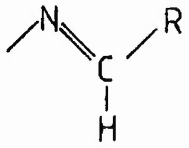


Figure 61. Comparison of the rate of O-acetylation of the benzaldehyde and benzoic anhydride derivatives of chitosan.

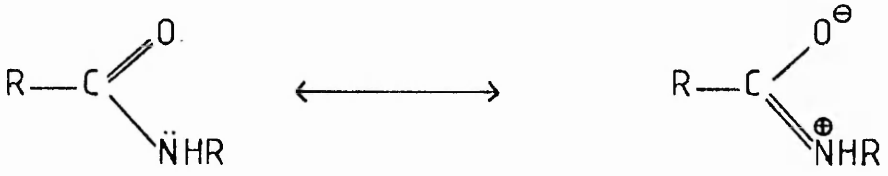
Results from the extended acetylation reactions indicate that below 50% of the hydroxyl groups are acetylated in the aromatic N-acyl chitosans but in the equivalent aromatic Schiff's base derivatives of chitosan up to 100% of the hydroxyl groups are acetylated. The overall size of the N-substituent has already been discounted for this marked difference in the extent of O-acetylation and hence a more detailed examination is required of each substituent.

Comparison of the two different N-substituents shows that the major





differences are in the double bond of the imine and the hydrogen present in the amide function. Any restriction of rotation around the -C=N- bond in the imine can be discounted as a similar restricted rotation may exist in the amide due to resonance hybridisation,



The hydrogen present in the amide would seem unlikely to cause steric hinderance due to its small size. However, under the conditions within the polymer chains and the very tight packing present, it is possible that this may cause disruption and restrict reaction.

The carbonyl group would be larger than the -C-H group of the imine and this may also cause hindrance to the reaction, but this would seem unlikely as there are no such problems associated with the acetylation of cellulose under similar conditions. However, under the conditions present in chitosan, the presence of the bulkier carbonyl group and the hydrogen in the -N-H hinder the reaction sufficiently to cause the steric hindrance which is inhibiting full acetylation from occurring in the N-acyl chitosans. This was emphasised by molecular models of the N-acyl chitosans and Schiff's base derivatives of chitosan which illustrated the steric hindrance present in the N-acyl chitosans.

Some attempts were made to determine whether the C(6) hydroxyl group was preferentially acetylated in the N-acyl chitosana. Horton and Just[284] have reported that the oxidation of chitosan perchlorate with chromium trioxide in acetic acid gives specific reaction at C(6), without oxidation of the C(3) hydroxyl group. The primary alcohol groups are oxidised rapidly to aldehydes in the absence of water and the subsequent oxidation of the aldehydes to carboxylic acid occurs on addition of water to the reaction mixture.

A sample of N-benzoyl chitosan was taken and acetylated in the normal way with acetic anhydride in pyridine. A further sample of N-benzoyl chitosan was taken and used without further treatment. Oxidation of both these samples was performed under relatively anhydrous conditions in acetic acid with an excess of chromium trioxide, water being introduced after 1 hour of the reaction. The N-benzoyl chitosan which had not been previously acetylated was then treated with acetic anhydride in pyridine. Infrared spectra of all the samples, before and after oxidation, were recorded, Figures 62-65.

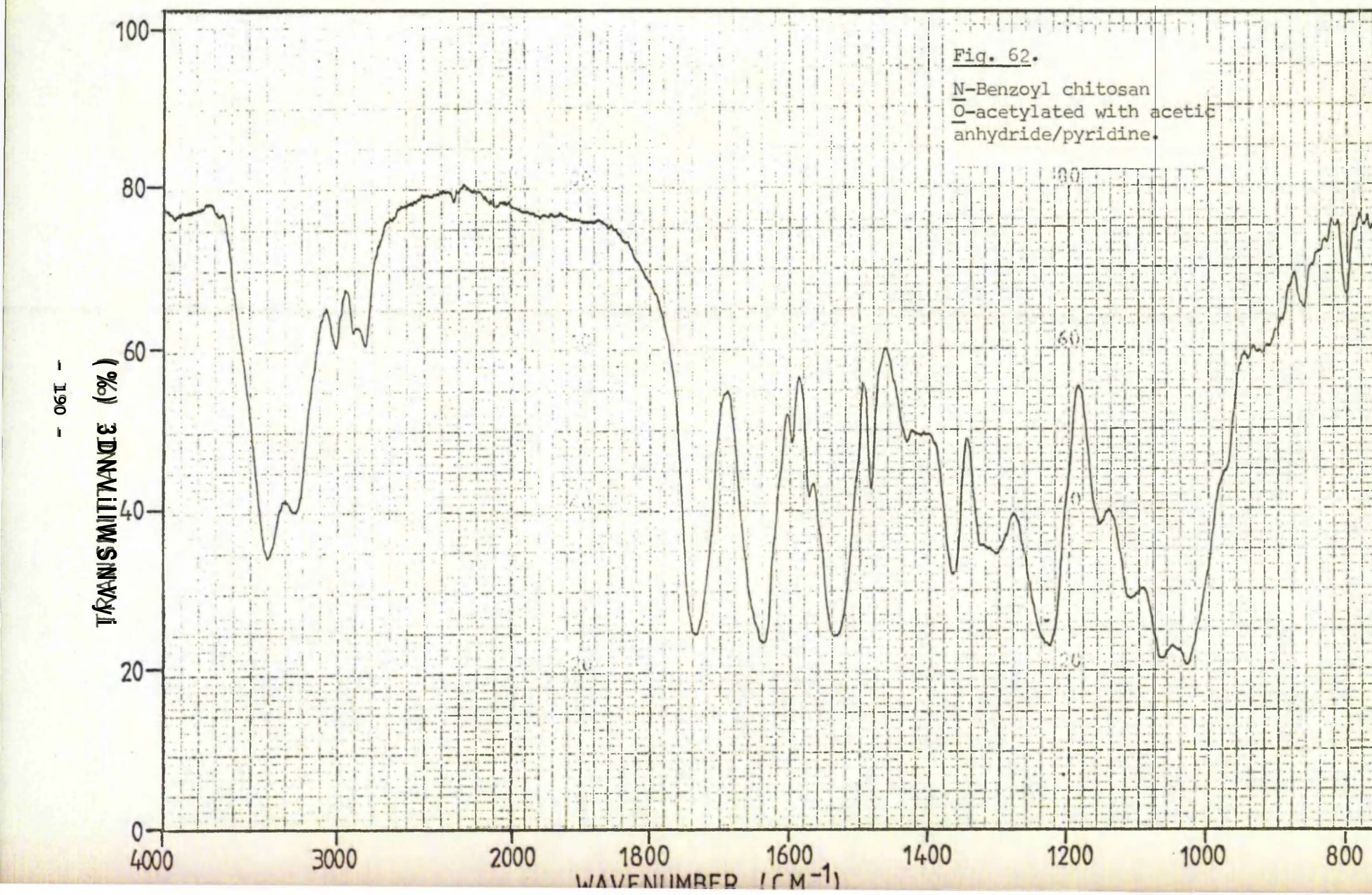


Fig. 62.

N-Benzoyl chitosan
O-acetylated with acetic anhydride/pyridine.

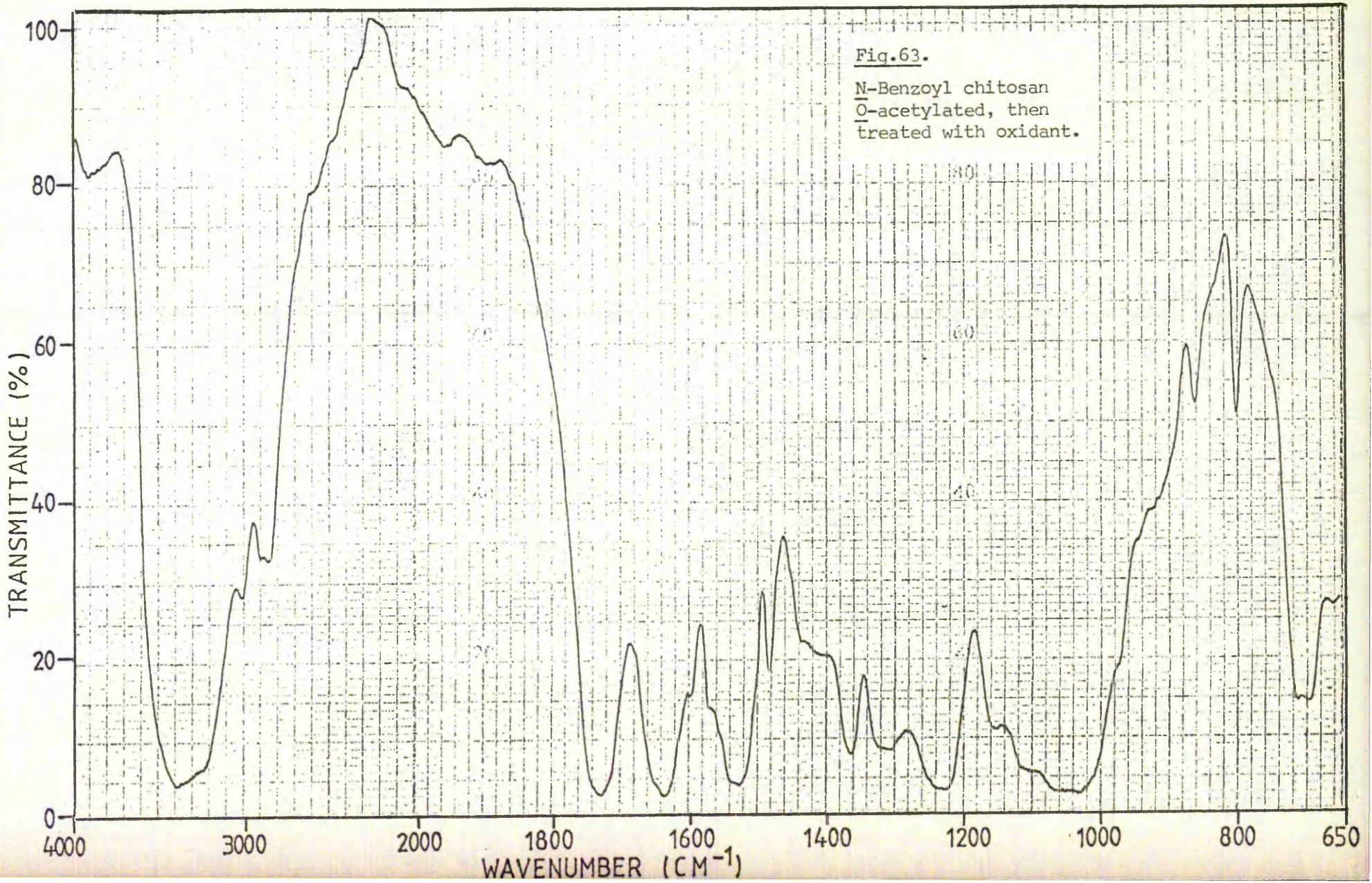


Fig.63.

N-Benzoyl chitosan
O-acetylated, then
treated with oxidant.

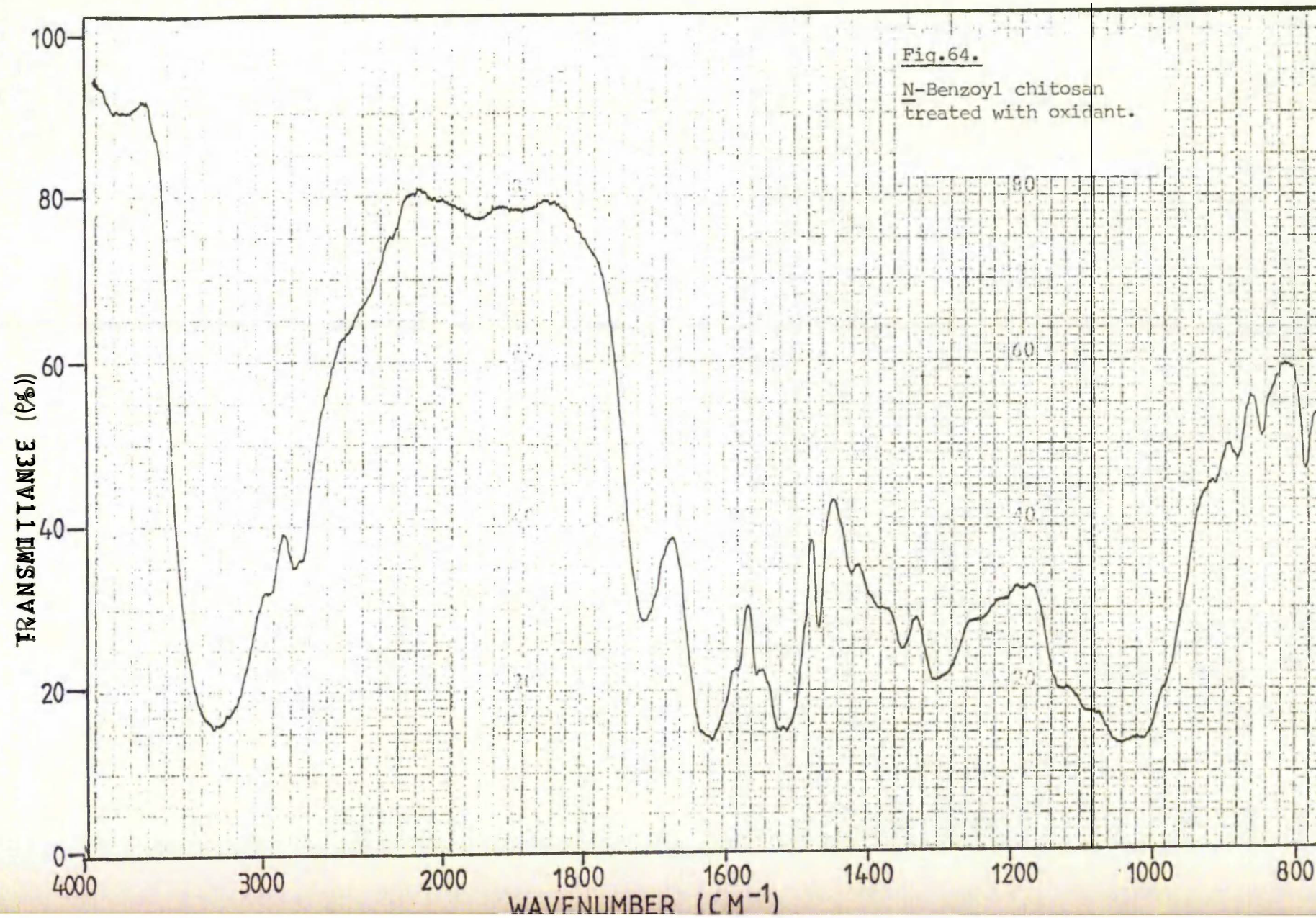


Fig.64.

N-Benzoyl chitosan treated with oxidant.

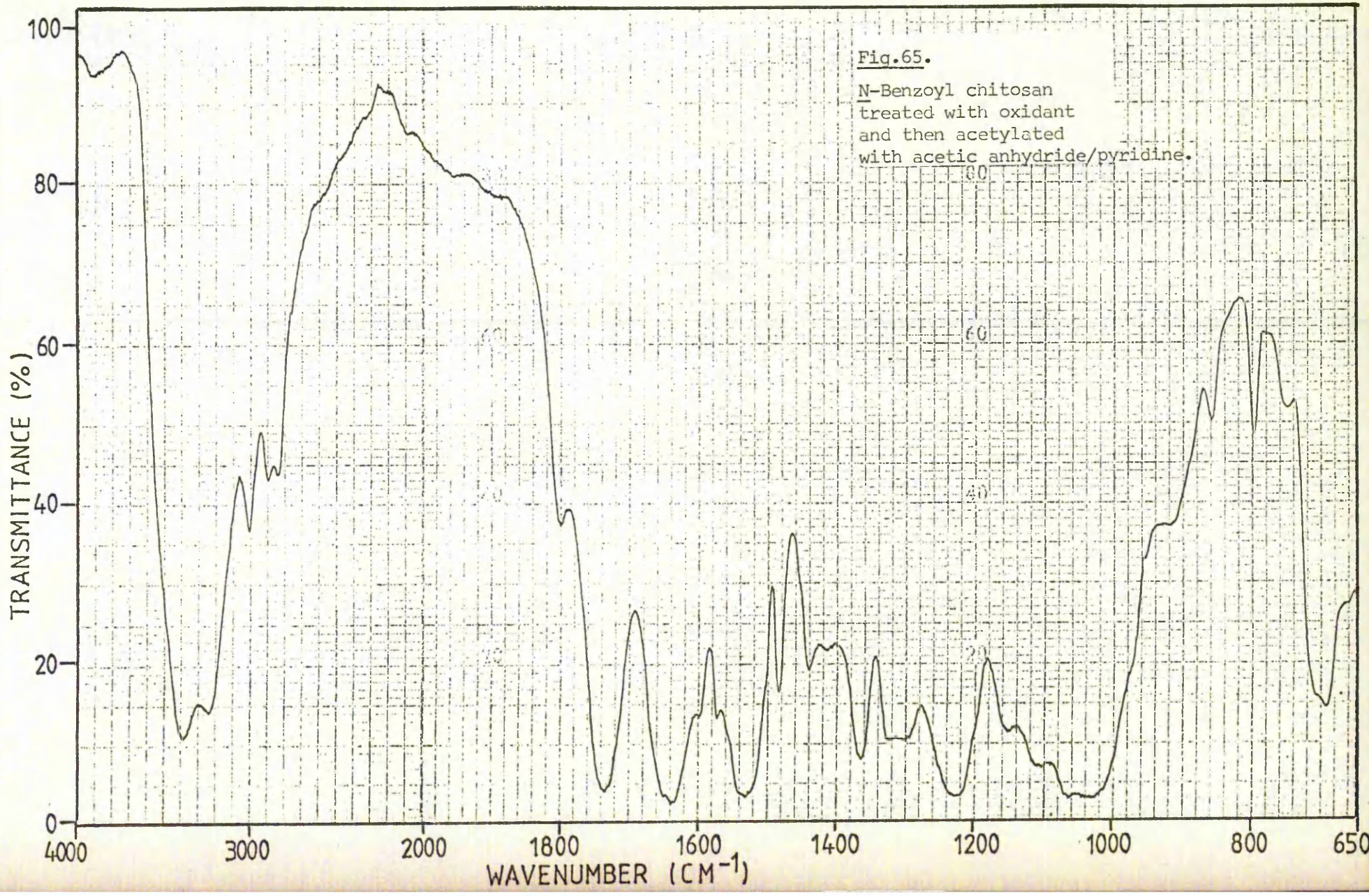


Fig.65.

N-Benzoyl chitosan treated with oxidant and then acetylated with acetic anhydride/pyridine.

The results obtained were inconclusive. Examination of the spectra of the sample O-acetylated and then oxidised, Figures 62 & 63 , reveals no apparent change at all. However the spectra of the sample oxidised and then acetylated shows the appearance of a band at 1730 cm^{-1} C=O, after oxidation which on acetylation increased in intensity, Figures 64 & 65. The intensity of the band at 1730 cm^{-1} prior to acetylation was such that it appeared that oxidation was incomplete and if, as supposed, acetylation occurs preferentially at the C(6) hydroxyl, the increase in intensity, after acetylation is due to the acetylation of the remaining C(6) hydroxyls. However, this presupposes no acetylation has occurred on the C(3) and hence is not conclusive. Unfortunately, due to the shortage of time, no further headway was made in this direction.

3.5. The Preparation of N -Acetyl - Di - O - Acetyl Chitosans

One of the original aims of this work was to prepare the fully acetylated derivative of chitosan although it soon became apparent that conventional techniques would be unsuccessful. It has been claimed recently [228] that a diacetyl chitin has been produced; (i) by reaction with acetic anhydride/ hydrogen chloride gas at $0^{o}C$ for 10 days; (ii) by reaction with acetic anhydride/ methanesulphonic acid at $0^{o}C$ for 16 hours; and (iii) by reaction with acetic anhydride in the presence of perchloric acid at $0^{o}C$ for 3 hours. However examination of some of the infrared spectra of the reported products showed the presence of a fairly large hydroxyl band at 3450 cm^{-1}.

Results from the O-acetylation studies of the aromatic aldehyde derivatives of chitosan suggested a possible synthetic route to a fully acetylated product. It was observed that in the acetylation of N-benzylidene chitosan, in different media, hydrolysis of the Schiff's base occurred to a large extent in acidic media, but only to a small extent in basic media. Thus if O-acetylation was first carried out in pyridine/acetic anhydride, the resulting product treated to hydrolyse the Schiff's base and then N-acetylated, a fully acetylated derivative should be produced.

Figure 66 gives the scheme adopted; a sample of N-benzylidene chitosan, prepared from high molecular weight chitosan, was treated with pyridine/ acetic anhydride to bring about O-acetylation and then hydrolysis of the Schiff's base was carried out with acetylation of the free amine in

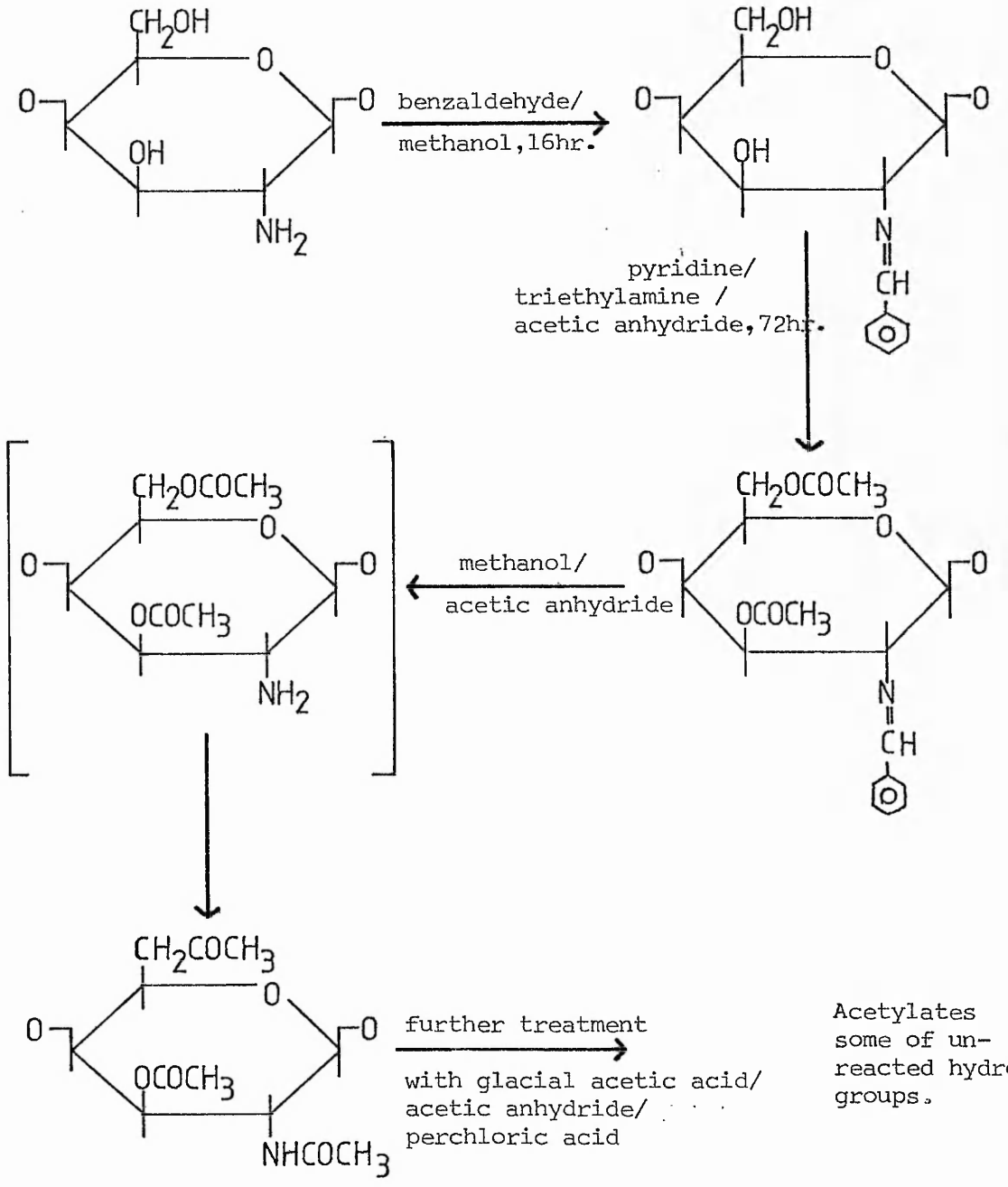


Figure. 66 Scheme for the preparation of N-acetyl-di-O-acetyl chitosan.

methanol/acetic anhydride solutions. Further treatment of the product with glacial acetic acid/acetic anhydride/perchloric acid was made to ensure any unreacted hydroxyl groups were acetylated. The off-white powdery product formed was readily soluble in formic acid and DMSO, and was swollen in DMF and ethylene chlorohydrin. It was not soluble in any other organic solvents tried. Clear, coherent films could be cast from formic acid and DMSO solutions and the infrared spectra of these showed it to be a virtually totally acetylated product with strong absorptions at 1740 (C=O band), 1665, 1545 (the Amide I and II bands) and 1230 cm^{-1} (C-O band). Figure 67 shows the infrared spectrum of the product cast from DMSO solution. Attempts at measuring the inherent viscosity were unsuccessful due to the high molecular weight of the product. However it was estimated that $\eta inh \triangleq 25 dlg^{-1}$.

To see if any improvement in the solubility of N- acetyl-di-O-acetyl chitosan could be made, the preparation was repeated using low molecular weight chitosan. Unfortunately the same limited solubility was found, the product being readily soluble in formic acid and DMSO only. However the inherent viscosity of the product could be determined and was found to be 2.24 dlg^{-1}. The limited solubility of the final products restricted attempts to carry out compatability studies of N-acetyl-di-O- acetyl chitosan with cellulose triacetate. However a film was cast from a 50:50 mixture of N-acetyl-di-O-acetyl chitosan and cellulose triacetate in formic acid. The film was fairly clear, indicating that the two were compatible, but extremely brittle. It had been intended to fractionate a sample of the fully acetylated chitosan and determine its molecular weight distribution, but this was also abandoned because of the restricted solubility of the product.

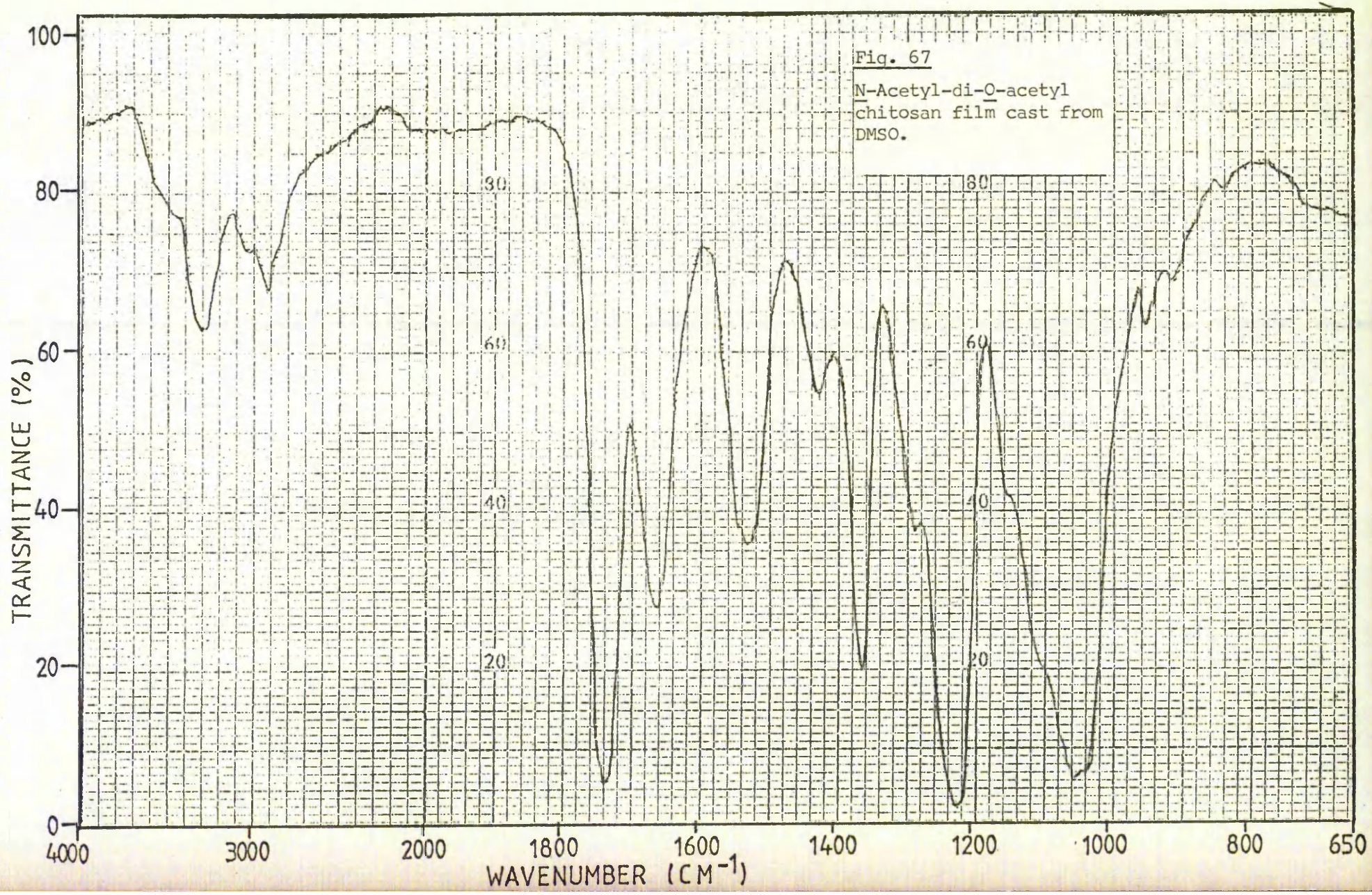


Fig. 67

N-Acetyl-di-O-acetyl chitosan film cast from DMSO.

The restricted solubility of the N-acetyl-di-O-acetyl chitosan prepared from either high or low molecular weight chitosan is not as restricted as it appears, when compared with cellulose triacetate itself, which is only soluble in[418] 1,4-dioxan , methylene chloride, chloroform, tetrachloroethane and nitromethane. Further the degree of polymerisation, DP, of high grade cellulose triacetate is between 220-250,[418] whereas an approximate figure for the DP of N-acetyl-di-O-acetyl chitosan, prepared from low molecular weight chitosan, is about 600, which would also limit the solubility of the product.

3.6. Miscellaneous Derivatives of Chitosan

The preparation of various carbamates of cellulose and cellulose acetate has been reported [419] using aliphatic and aromatic isocyanates. An attempt was made, therefore, to produce carbamates of N-salicyclidene and N-benzylidene chitosans, prepared from low molecular weight chitosan. Any organic isocyanate reacts readily with water to give the primary amine, which in turn reacts with more isocyanate to yield a symmetrical urea as the final product, according to the equations.

$$RNCO \xrightarrow{H_2O} RNH_2 + CO_2 \qquad \xrightarrow{RNCO} \qquad RN(H)-C(=O)-N(H)R$$

Since two moles of isocyanate are used for every mole of water, all components of the reaction mixture had to be kept as anhydrous as possible.

It was reported [419] that in contrast with aliphatic reagents, aromatic isocyanates reacted readily and completely with cellulose materials, and so phenyl and α-napthyl isocyanates were chosen as suitable reagents. Treatment of the dry N-substituted chitosans in anhydrous pyridine with the appropriate aromatic isocyanate gave rise to clear viscous solutions. Reprecipitation of the polymer solutions in suitable solvents gave the different products.

The N-salicyclidene chitosan gave, as the α-napthyl carbamate, an off-white fibrous product and as the phenyl carbamate, a yellow product. The α-napthyl carbamate was soluble in methylene chloride, chloroform, DMF and DMSO and gave clear, coherent films when cast from methylene chloride and chloroform solutions. The inherent viscosity was found to be 1.05 dlg^{-1}. The phenyl

carbamate was less soluble in chloroform, but was soluble in methylene chloride, DMF and DMSO. A clear, coherent film was cast from a methylene chloride solution and its inherent viscosity was found to be 1.49 dlg^{-1}. Infrared spectra of the product, Figures 68 & 69 show the characteristic bands at 1730 (-C(=O)-N) and 1215 cm^{-1} (N-C-O) and the presence of only small absorptions at 3450 cm^{-1} (-O-H) indicates a virtually complete reaction in both cases.

The N-benzylidene chitosan formed a fine powdery, magnolia coloured, α-napthylcarbamate and a buff coloured phenyl carbamate. The former was soluble in methylene chloride, chloroform, DMF and DMSO, and the latter in DMF and DMSO. A clear coherent film was cast from a methylene chloride solution of the α-napthyl carbamate. The phenyl carbamate proved to be more difficult to produce films from; a brittle film was prepared from DMF and a slightly better film from DMSO. The inherent viscosities were recorded; that for the α-napthyl carbamate was equal to 1.34 dlg^{-1} and that for the phenyl carbamate equal to 2.56 dlg^{-1}.

Only small hydroxyl band absorptions were observed in the infrared spectra of the products, indicating a virtually complete reaction, Figures 70 &71.

Application of the method to unreacted low molecular weight chitosan was reasonably successful, but much longer reaction times were necessary to afford reaction. The solubilities of the resulting products were not as good, both being soluble in DMF and DMSO only. The inherent viscosities were 1.79 dlg^{-1} for the α-napthyl carbamate and 2.56 dlg^{-1} for the phenyl carbamate. The infrared spectra, Figures 72 & 73 , of the products indicated a trisubstituted carbamate derivative in both cases.

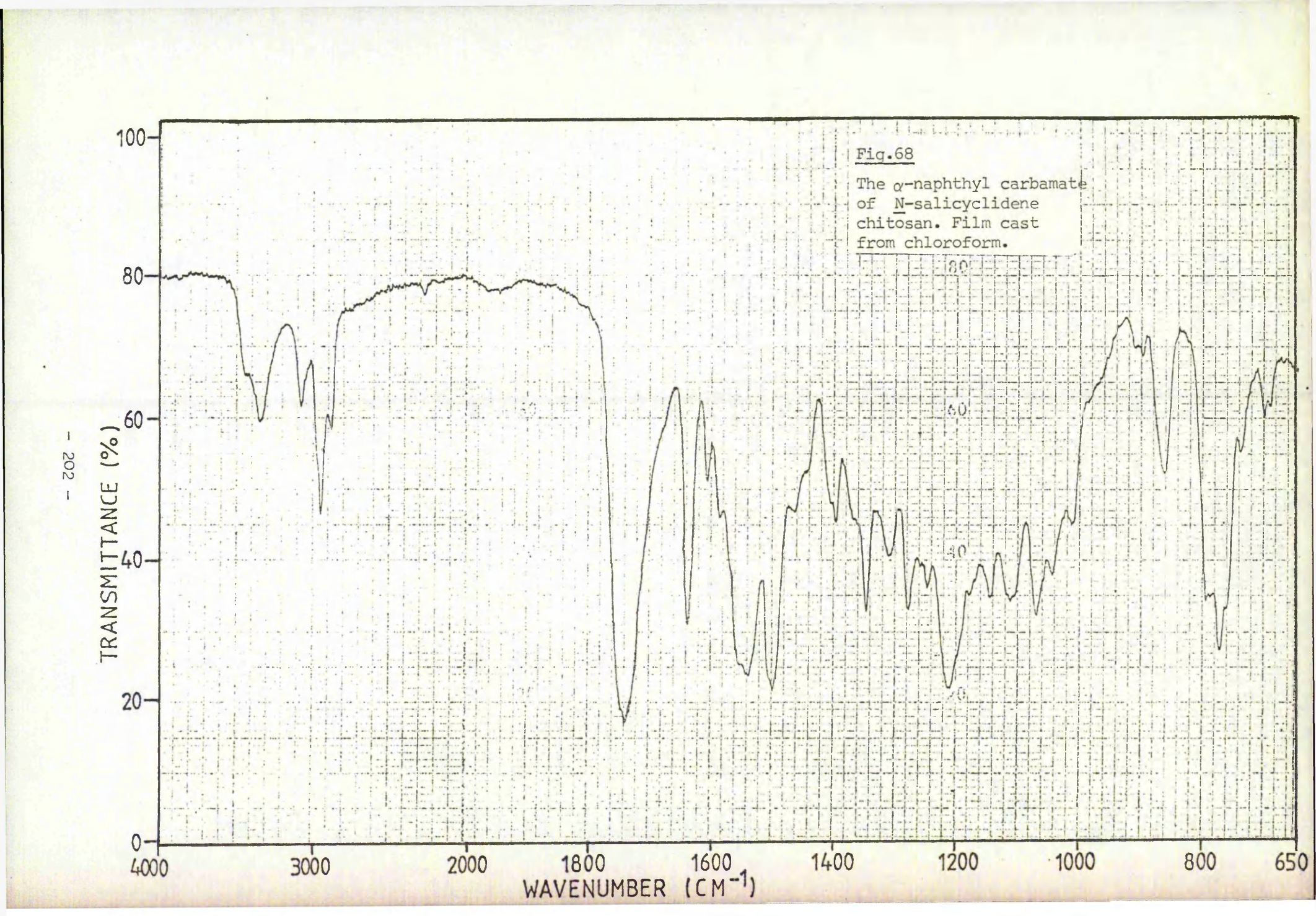


Fig.68

The α-naphthyl carbamate of N-salicyclidene chitosan. Film cast from chloroform.

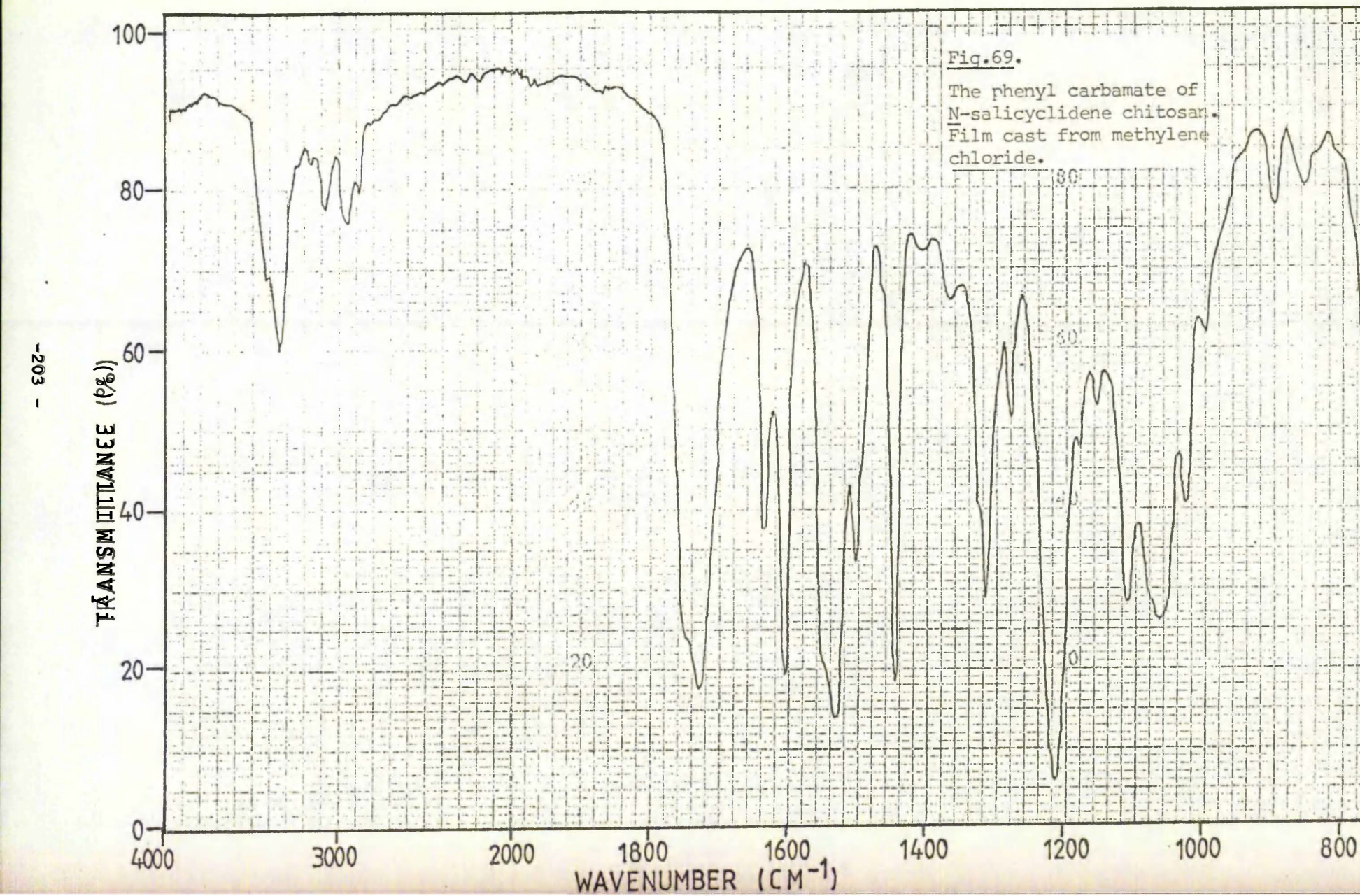


Fig.69.

The phenyl carbamate of N-salicyclidene chitosan. Film cast from methylene chloride.

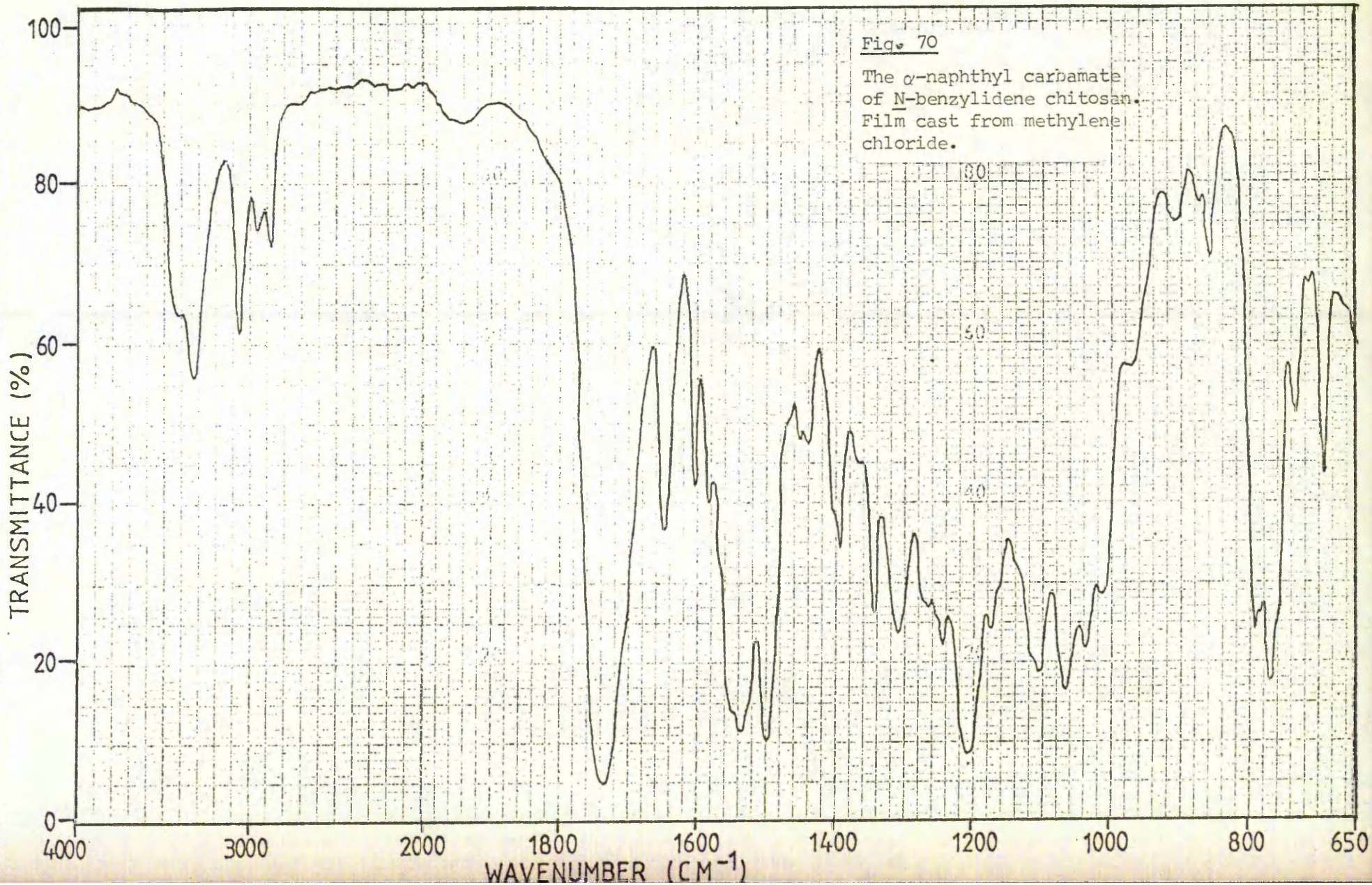


Fig. 70

The α-naphthyl carbamate of N-benzylidene chitosan. Film cast from methylene chloride.

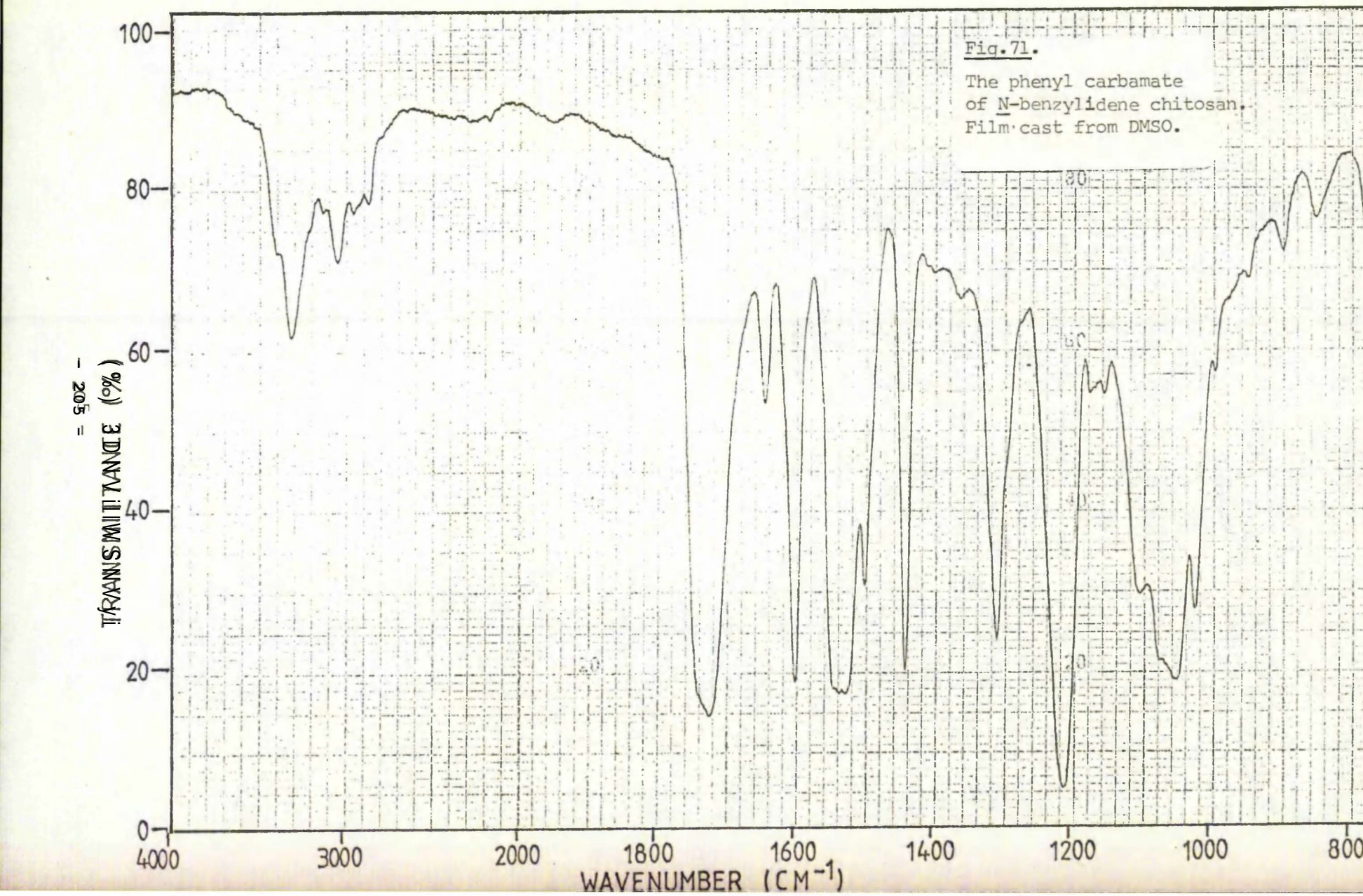


Fig.71.

The phenyl carbamate of N-benzylidene chitosan. Film cast from DMSO.

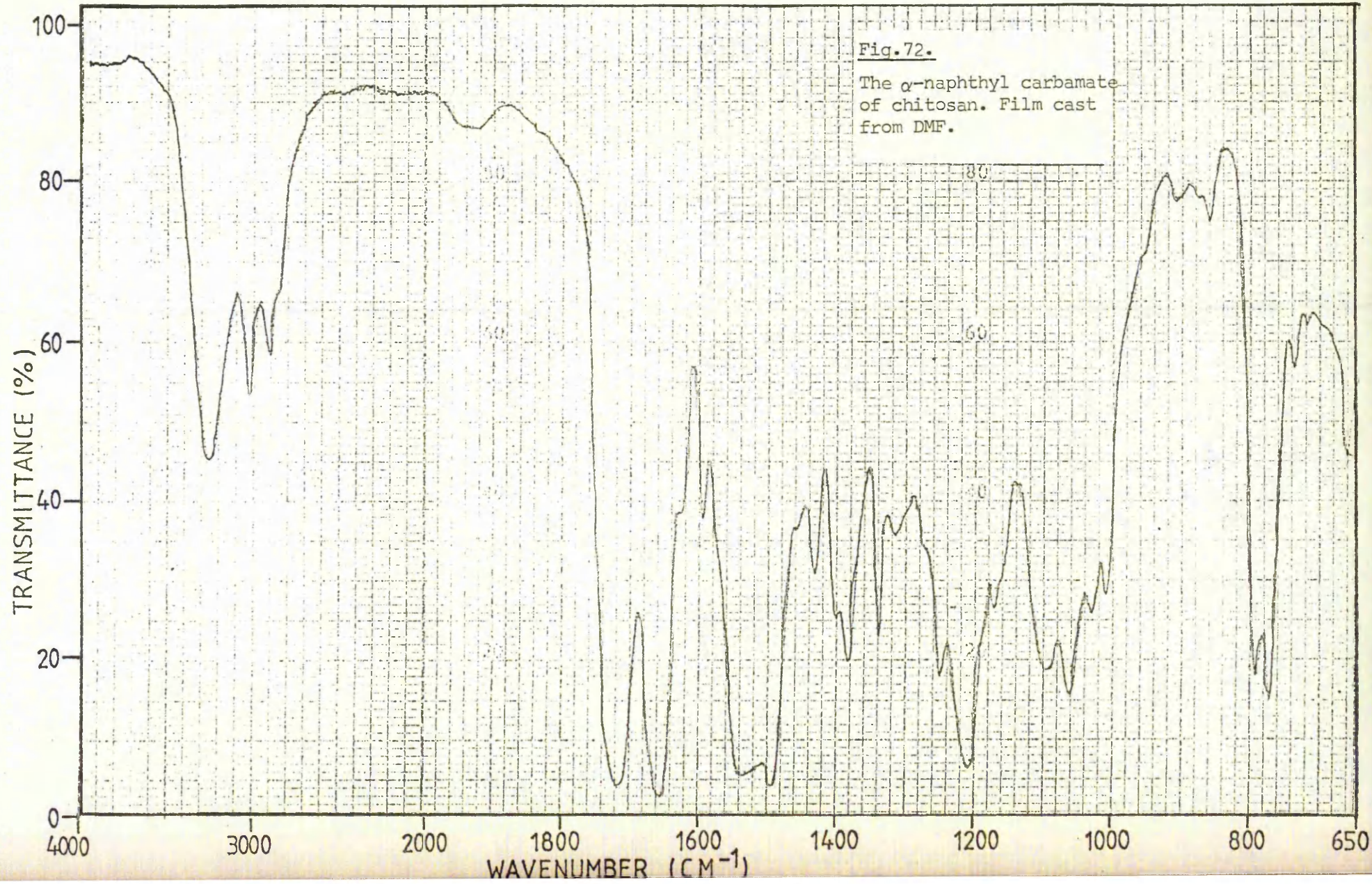


Fig.72.

The α-naphthyl carbamate of chitosan. Film cast from DMF.

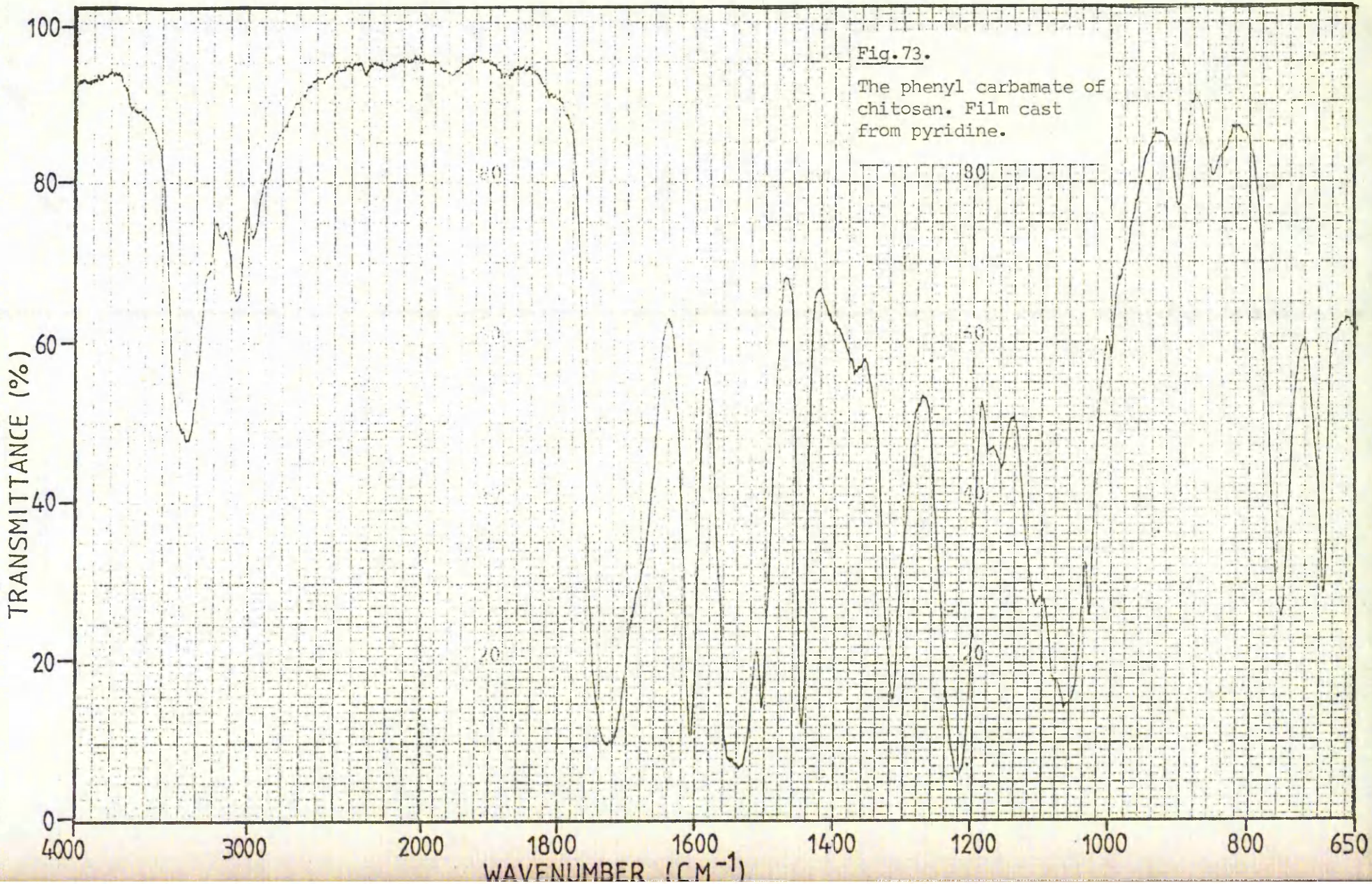


Fig.73.

The phenyl carbamate of chitosan. Film cast from pyridine.

Part B Homogeneous Acylation Studies

3.7 Studies on the Gelation of N-Acyl Chitosans

3.7.1. Introduction

Non-reversible N-acetyl chitosan gels and partially O-acetylated N-acetyl chitosan gels have been produced [255-260] by facile acetylation of chitosan with acetic anhydride in acetic acid (10%) and in aqueous acetic acid-methanol solutions at room temperature. Using the same conditions a series of N-acyl chitosan gels have been produced [255-60] by reaction with other carboxylic anhydrides. The structure of the N-acyl groups has been found to influence gelation,[261] and the minimum requirements for gelation to occur has been defined as approximately 0.4N-lauroyl (C_{12}), approximately 0.6N-fatty acyl (C_3- C_{10}) or about 0.7N-benzoyl groups per hexosaminide residue. Gelation does not occur with high fatty acyl groups (C_{14}- C_{18}),[261] Gels have also been produced [269] by Schiff's base formation on treatment of solutions of chitosan, in aqueous acetic acid-methanol media, with aldehydes. The architecture of the solid phase of the N-acetyl chitosan gels has been examined by scanning electron microscopy,[420] which shows it to be polyphasic gel with small droplets of water held in pores which are 30-50 μm in diameter and 80 -300 μm in length.

Aqueous oxalic acid solutions of chitosan have been found to form thermo-reversible gels.[421] The effects of a range of other acids was studied but similar gel formation was only observed with dichloroacetic acid, the gel in this case being much weaker. It has been proposed that in solution the

chitosan molecules normally exist in a random coil configuration but that in the presence of oxalic acid double helices are formed, thus creating cross-links which eventually lead to gel formation.

3.7.2. Preliminary Experiments

Hirano reported[258] that the addition of a 2-3 mol. excess of carboxylic anhydride, based on the free amine concentration, to a solution of chitosan in aqueous methanolic acid would bring about gelation. A later, more detailed report [259] reported that a rigid gel was formed with 1.7-3.3 mol. equivalents of acetic anhydride. Therefore, it was decided that for the present work a 3 mol. excess of carboxylic anhydride would be taken as standard. The standard ratio of methanol: aqueous acetic acid (5%) was taken as 5:2.

Initially, varying concentrations of chitosan in aqueous acetic acid (5%) were mixed with methanol and acetic anhydride (3mol.) added, with stirring, to each solution. The solutions were then left at room temperature and the time taken for gelation to occur noted, Table 17.

Table:17 Effect of Chitosan Concentration on time for Gelation

Concentration of Chitosan	Time for Gelation
0.5%	No gelation up to 24 hrs.
1.0%	Within 10 mins.
1.5%	" 5 "
2.0%	" 3 "
2.5%	" 2 "

Evidently polymer concentrations in excess of 0.5% are necessary for gelation to occur and the speed of gelation increases with increase in polymer concentration.

3.7.3. The Use of Alternative Co-Solvents.

In Hirano's work[255-261] methanol was the only co-solvent used, except for the addition of small quantities of pyridine to aid solubilisation of the higher fatty acid anhydrides. The influence of other co-solvents was therefore examined.

Standard 2.5% solutions of chitosan in aqueous acetic acid (5%) were made up and these were diluted with a range of co-solvents, Table 18; similar volumes of the liquid co-solvents being used in each case. In the case of the polyethylene glycol (P.E.G.) 1000 and 6000 a 1:1 solution was prepared in the equivalent volume of distilled water. A 3 mol. excess of acetic anhydride was stirred into each mixture and the gelation behaviour noted.

Table: 18 The Effect of Varying Co-Solvent

Solvent	Observation
Methanol	Gelation occurred in 2 mins.
Ethanol	Solution cloudy, with formation of lumps
n-Propanol	Precipitation of polymer
n-Butanol	Formation of gelationous lumps
Formamide	Gelation occurs very rapidly
Acetone	Precipitation of polymer
Ethylene Glycol	Gelation occurred
Digol	Gelation occurred
Trigol	Very rapid gelation
P.E.G. 200	No gelation
P.E.G. 400	No gelation
P.E.G. 1000	No gelation
P.E.G. 6000	Gelation occurred within 60 mins.

The gels formed with methanol as a co-solvent were found to undergo syneresis and eventually dried out to give a hard, inflexible product. However, gels formed with formamide, ethylene glycol, digol and trigol, although subject to some syneresis, eventually reached a constant volume to give a rubbery, flexible gel. No marked change has been observed in these gels over a period of 12 months.

3.7.4. Rate of Gelation

3.7.4.1. General

Since little is known of the mechanism or rate of gelation of chitosan with carboxylic acid-anhydrides, some attempts were made to follow the rate of gelation of chitosan with acetic anhydride, by monitoring viscosity changes using the Brookfield Synchro-Lectric Viscometer LVF. Based on the earlier work on concentration effects, a 1.0% solution of chitosan in aqueous acetic acid was taken as the standard solution. Methanol was added, a 3 mol. excess of acetic anhydride stirred in and viscosity readings were taken every minute.

Results from four runs were in reasonable agreement and showed that the viscosity increases rapidly after an induction period of 8-9 minutes and rises steadily, eventually going off the scale of the viscometer. Discontinuities, however, were observed when the speed of the viscometer, or the spindle used were changed. The gelation was also subject to fluctuations in room temperature. The experiment was modified, therefore, by continuous monitoring of the solution viscosity using the lowest speed (6 r.p.m.) and the smallest spindle (No.4) available. In addition, variation due to temperature fluctuations were avoided by use of a thermostatically controlled water bath. Results from several runs carried out under these conditions were found to be in reasonable agreement.

From Figure 74, it can be seen that there is a rapid rise in viscosity over a period of 7-8.5 minutes, followed by a slight levelling off period, after

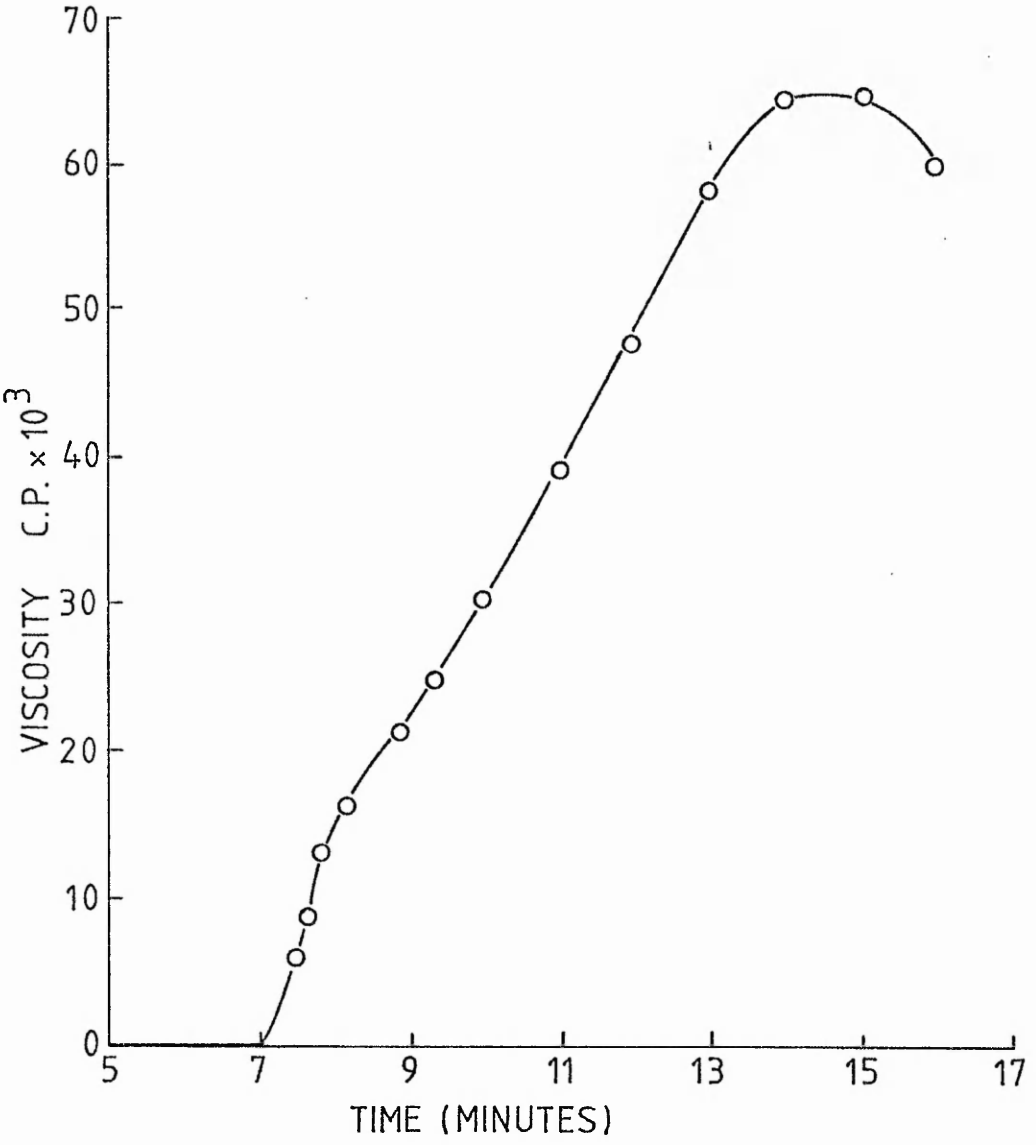


Figure.74. The viscosity versus time graph for the gelation of N-acetyl chitosan at 25°C.

which the viscosity increases steadily to about 60,000 centipoises (cps) and then decreases. It was thought that this decrease was due to the gel breaking away from the spindle towards the end. Although the viscosity appears to drop, it was presumed that the actual strength of the gel continued to increase. The kink due to the levelling off period was observed in all cases and appeared just after the initial very rapid rise in viscosity.

In order to determine whether the observed levelling-off period was due to the technique used to follow gelation,or whether it is inherent in the gelation process itself, the gelation behaviour of Oxidol-Agar was monitored in the same manner. Oxidol-Agar was dissolved in boiling water and allowed to cool. The viscosity of the solution was continuously monitored as the temperature dropped and gelation occurred. The curve obtained, Figure 75, shows a steady increase in viscosity up to a maximum, followed by a decrease as the gel breaks away from the spindle. There is no evidence of any kink in the curve showing that its appearance is not due to the technique used but is due to some stage in the N-acyl gelation process.

3.7.4.2. Effect of Varying the Anhydride

The effect of a number of carboxylic acid anhydrides was then examined using a 3 mol. excess of the appropriate anhydride in every case. The anhydrides studies were propionic, butyric, valeric, hexanoic, heptanoic, octanoic, nonoic, decanoic and benzoic. Marked variations in the time of the start of gelation were observed for the different anhydrides.

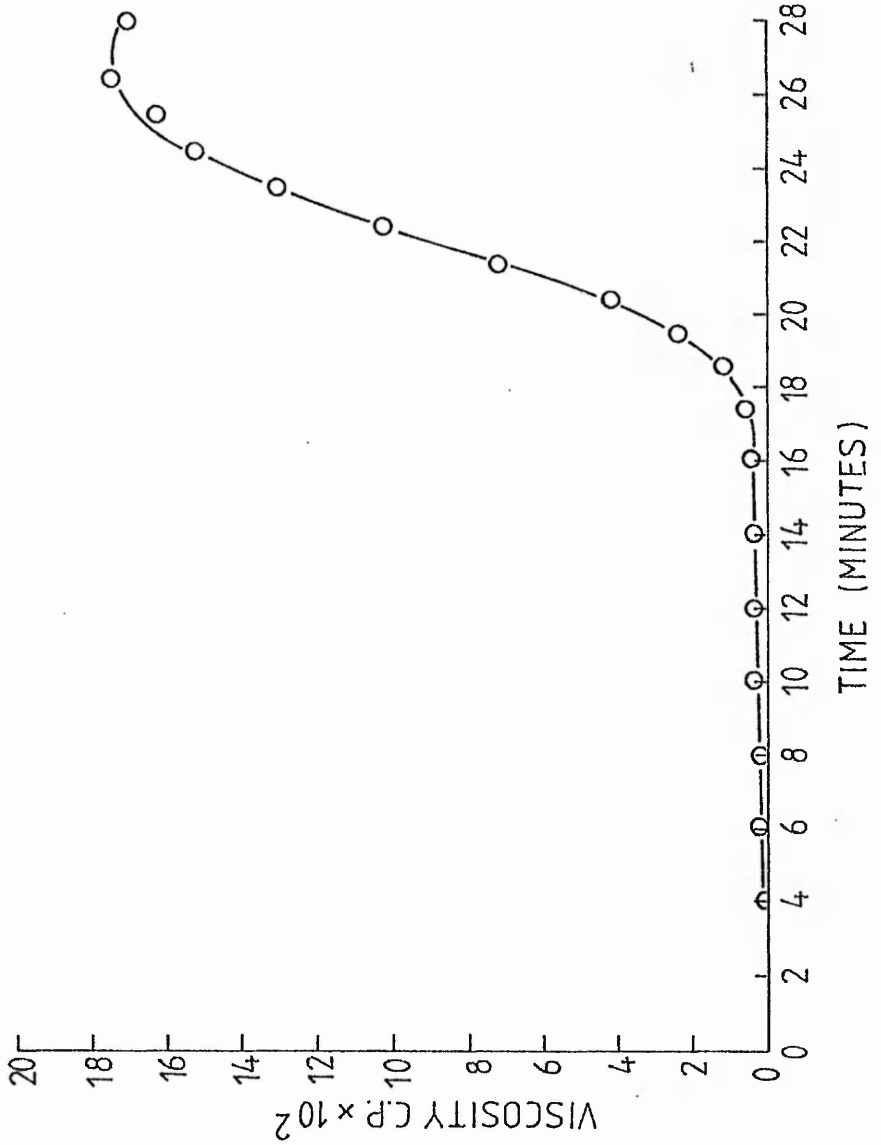


Figure 75. The viscosity versus time graph for the gelation of Oxidol-Agar.

Table: 19 Average Time to Start of Gelation for the different Anhydrides

Anhydride	Average Time to start of gelation (mins)
Acetic	7
Propionic	19
Butyric	64
Valeric	30
Hexanoic	33
Heptanoic	35
Octanoic	--
Nonoic	80
Decanoic	260
Benzoic	280

Agreement between runs of the same anhydride decreased with increase in the molecular weight of the anhydride. However, as can be seen from Table 19 there is a general trend towards increasing time to gelation with increase in molecular weight of the anhydride, butyric anhydride being an exception to this. The pattern of viscosity versus time was the same in all cases, i.e., an initial rapid increase in viscosity, followed by a kink and then a steady increase in viscosity to the breaking off point.

Complete gelation did not occur with octanoic anhydride. This may have been due to impurities in the anhydride, since nonoic and decanoic anhydrides gave satisfactory gels.

The effects of varying different parameters in the gelation procedure was then studied, using acetic and hexanoic anhydrides. Hexanoic anhydride was chosen since the time taken to the start of gelation under standard conditions, approximately 33 minutes, allowed wider variations in some parameters to be examined.

3.7.4.3. Effect of Temperature

Standard Polymer Solution:	1% chitosan in aqueous acetic acid and methanol in the ratio 2:5 3 mol. excess of appropriate anhydride.
Brookfield Viscometer:	Speed: 6 rpm. Spindle: No.4.
Temperature Ranges:	Acetic anhydride: 15,20,25,30,45,45°C. Hexanoic anhydride: 20,25,30,35,40,45°C.

From Figure 76 it is evident that with acetic anhydride, increasing temperature causes a decrease in the time to gelation. Gelation occurs in under 2 minutes at 45°C the reaction taking place so rapidly that accurate measurements were impossible to make. At 35°C gelation occurred after 4.5 minutes and at 25°C, 7.5 minutes. Further decrease in temperature brings about a much greater increase in the time of gelation, such that at 15°C, gelation occurs at 32.5 minutes. The maximum viscosity recorded before the spindle breaks away from the gel seems to occur at lower viscosities with higher temperatures.

Figure 77 shows a plot of the time to gelation against temperature. The results obtained fit on a reasonable curve and emphasise the temperature

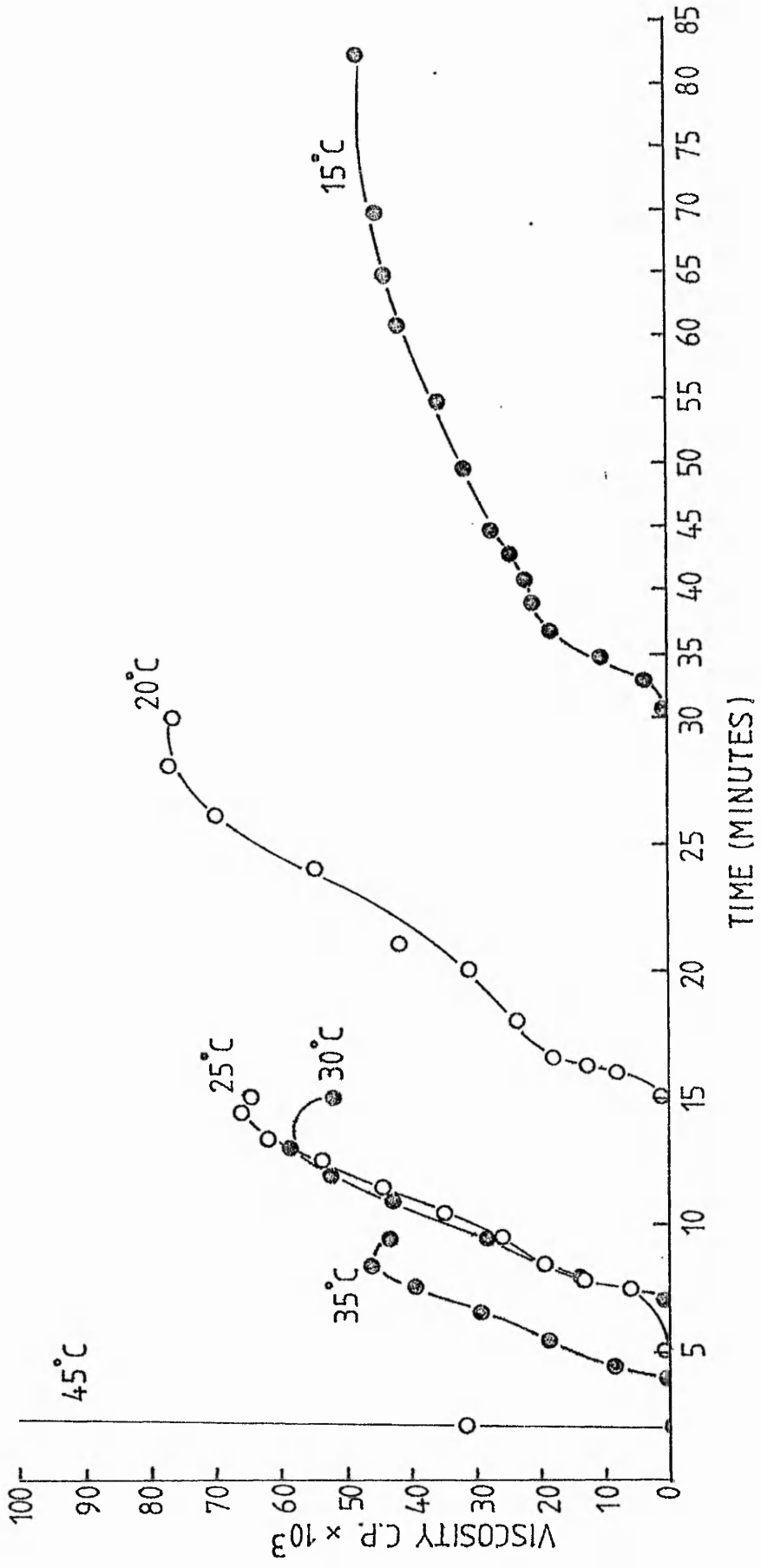


Figure 76. The effect of temperature on the gelation of chitosan solution with acetic anhydride.

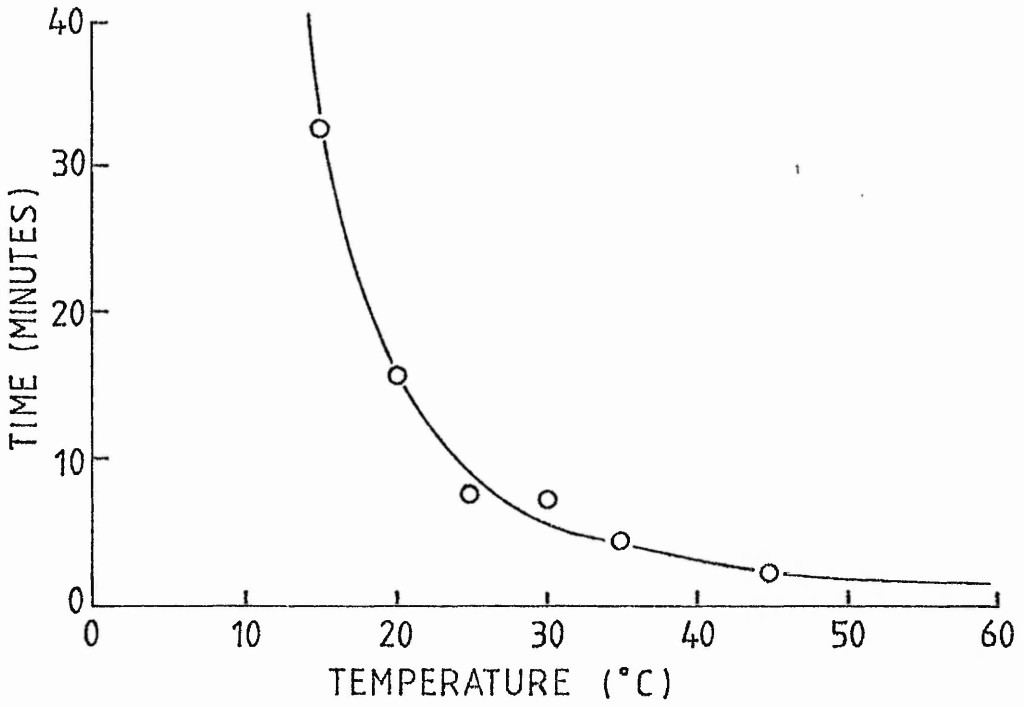


Figure 77. The time to gelation versus temperature graph for the gelation of chitosan solutions with acetic anhydride.

dependance of the gelation process.

The results for hexanoic anhydride show a similar trend (Figures 78&79) At 45°C gelation occurred after approximately 9 minutes and after approximately 12 minutes at 40°C. A steady increase in time to gelation occurred with decreasing temperature. The kink in the viscosity curve was not as apparent at the higher temperatures with either anhydride, but was very evident at 15°C with acetic anhydride and at 25°C with hexanoic anhydride.

3.7.4.4. Effect of Variation in Anhydride Concentration

Standard Polymer Solution:	1% chitosan in aqueous acid and methanol in the ratio 2:5
Temperature:	25 °C
Brookfield Viscometer:	Speed: 6rpm Spindle: No.4
Anhydride concentrations used:	Acetic: 7 mol. excess (xs) 5 mol xs, 3mol xs.
	Hexanoic: 8 mol xs, 7 mol xs, 5 mol xs,
	4 mol xs, 3 mol xs, 1 mol equiv.

The results with hexanoic anhydride , Table 20 , Figure 80 , show that an increase in anhydride concentration causes a reduction in the time to gelation

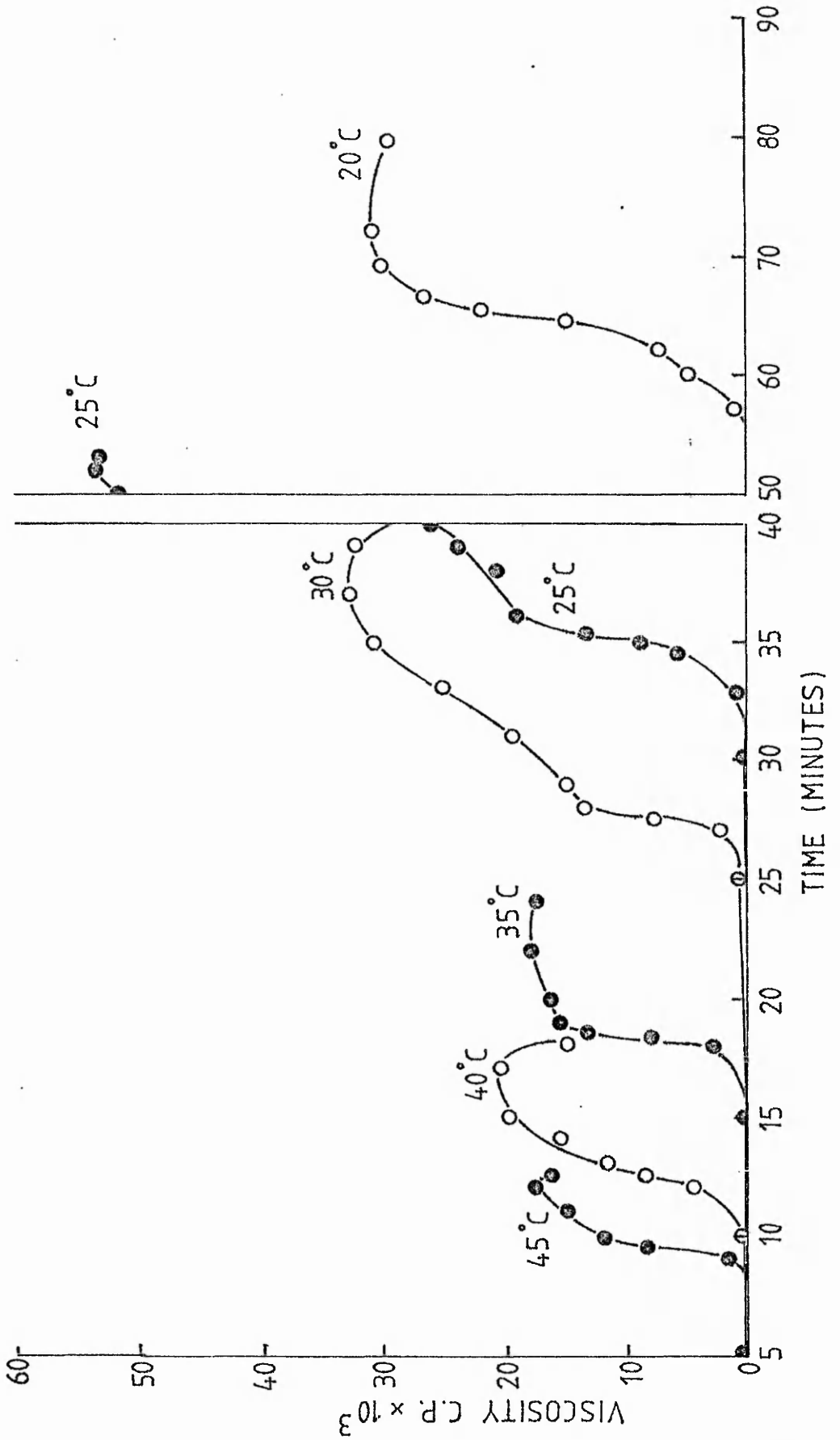


Figure 78. The effect of temperature on the gelation of chitosan solutions with hexanoic anhydride.

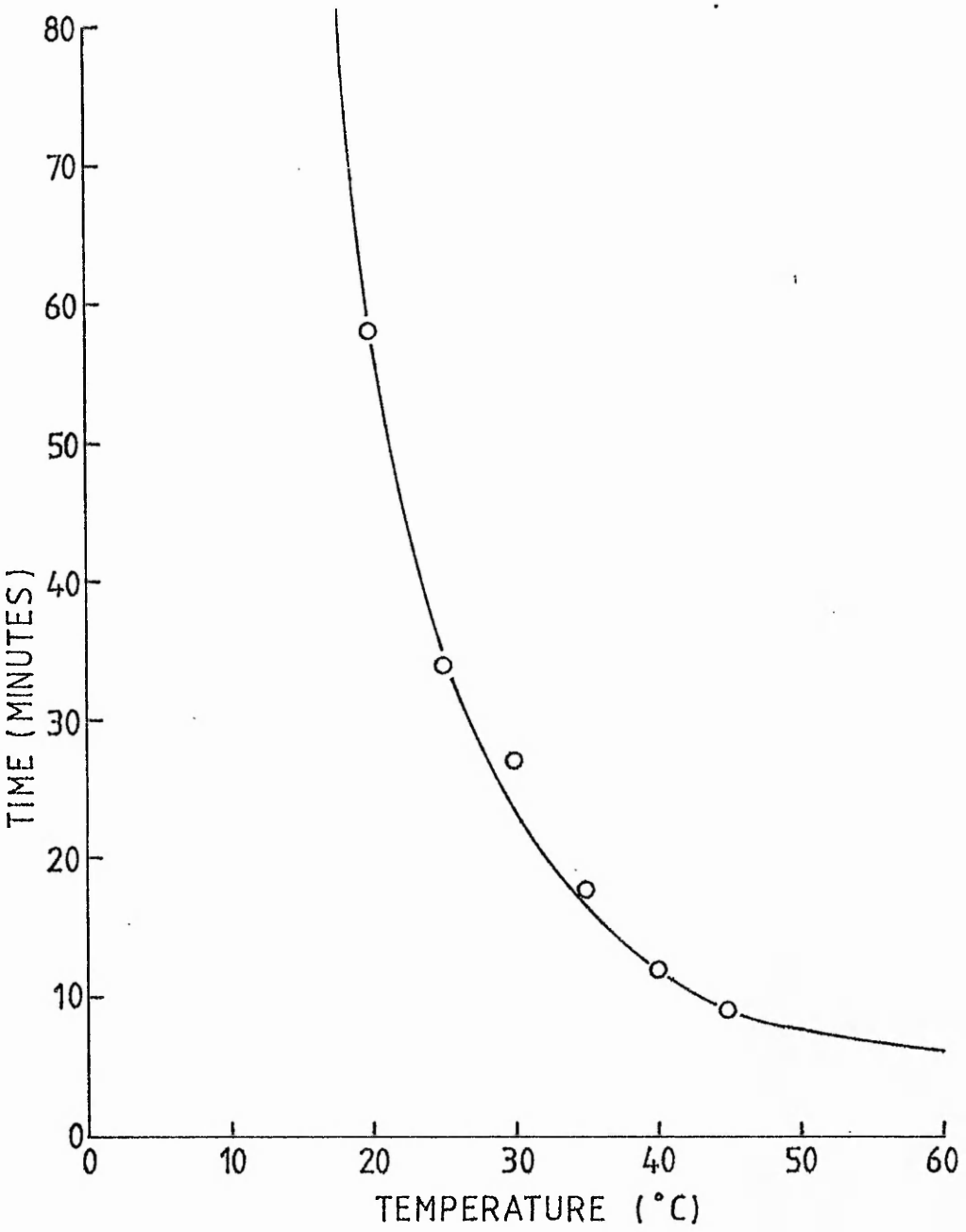


Figure 79. The time to gelation versus temperature graph for the gelation of chitosan solutions with hexanoic anhydride.

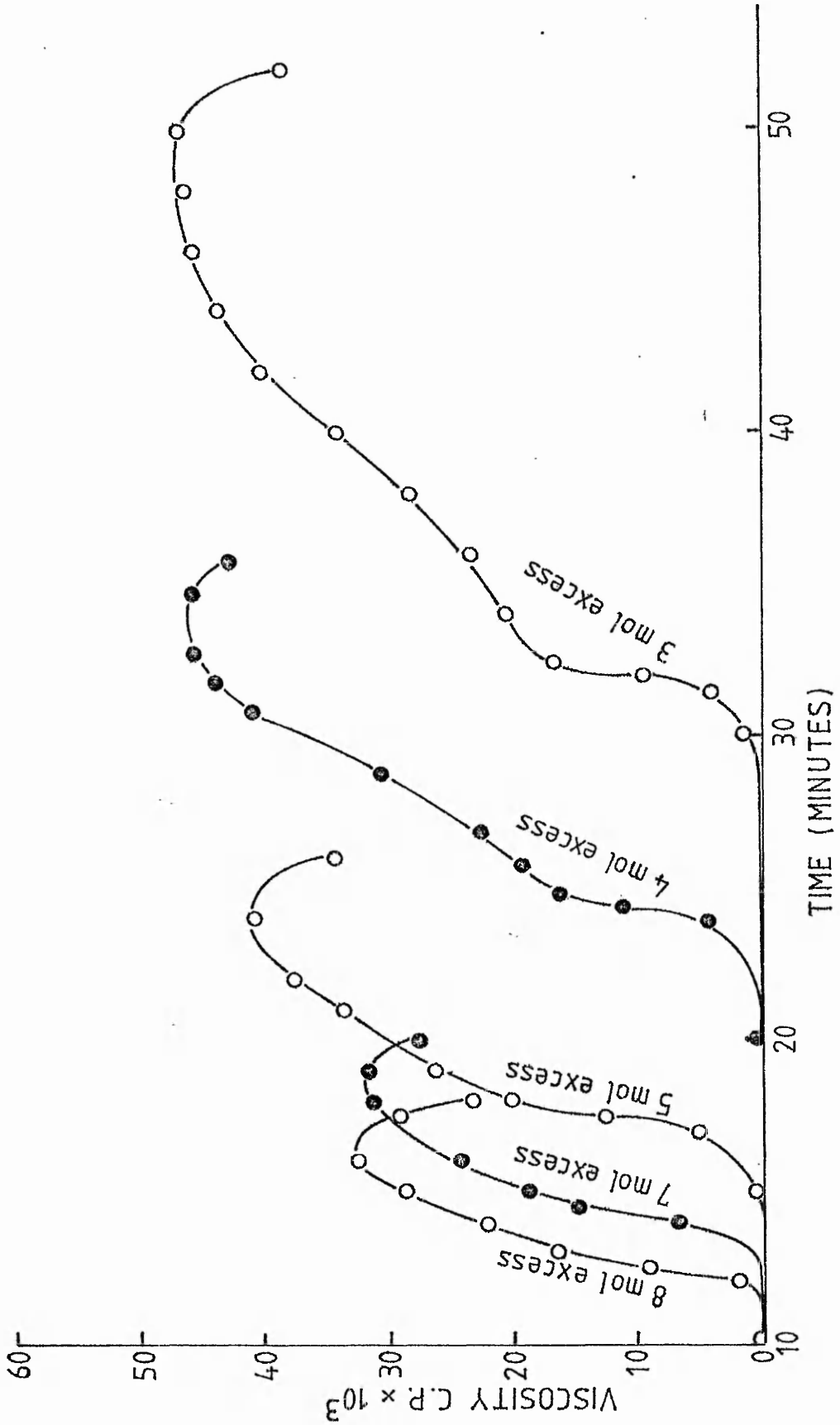


Figure 80. The effect of anhydride concentration on the gelation of chitosan solutions with hexanoic anhydride.

Table: 20 Effect of Varying Anhydride Concentration on time to Gelation

Anhydride Concentration (mols)	Time to Gelation (mins)
8	12
7	14
5	17
4	24
3	32
1	—

No gelation was observed with a 1 mol. equivalent of hexanoic anhydride.

Similar results were obtained with acetic anhydride, Figure 81 . The viscosity rise with the 7 mol. excess and 5 mol. excess was very rapid, gelation starting in under 2 minutes and 4 minutes respectively. Very high maximum viscosity readings were obtained in both cases.

3.7.4.5. Effect of Variation in Polymer Concentration

Constant volume of anhydride taken, equivalent to a 3 mol. excess for a 1% polymer solution.

Temperature: 25°C

Brookfield Viscometer: Speed 6 r.p.m. Spindle: No.4.

Polymer concentrations used: 0.5%; 1.0%, 2.0%, 3.0% in aqueous acetic acid + methanol in the ratio (2:5)

Only hexanoic anhydride was used in this case, as acetic anhydride caused

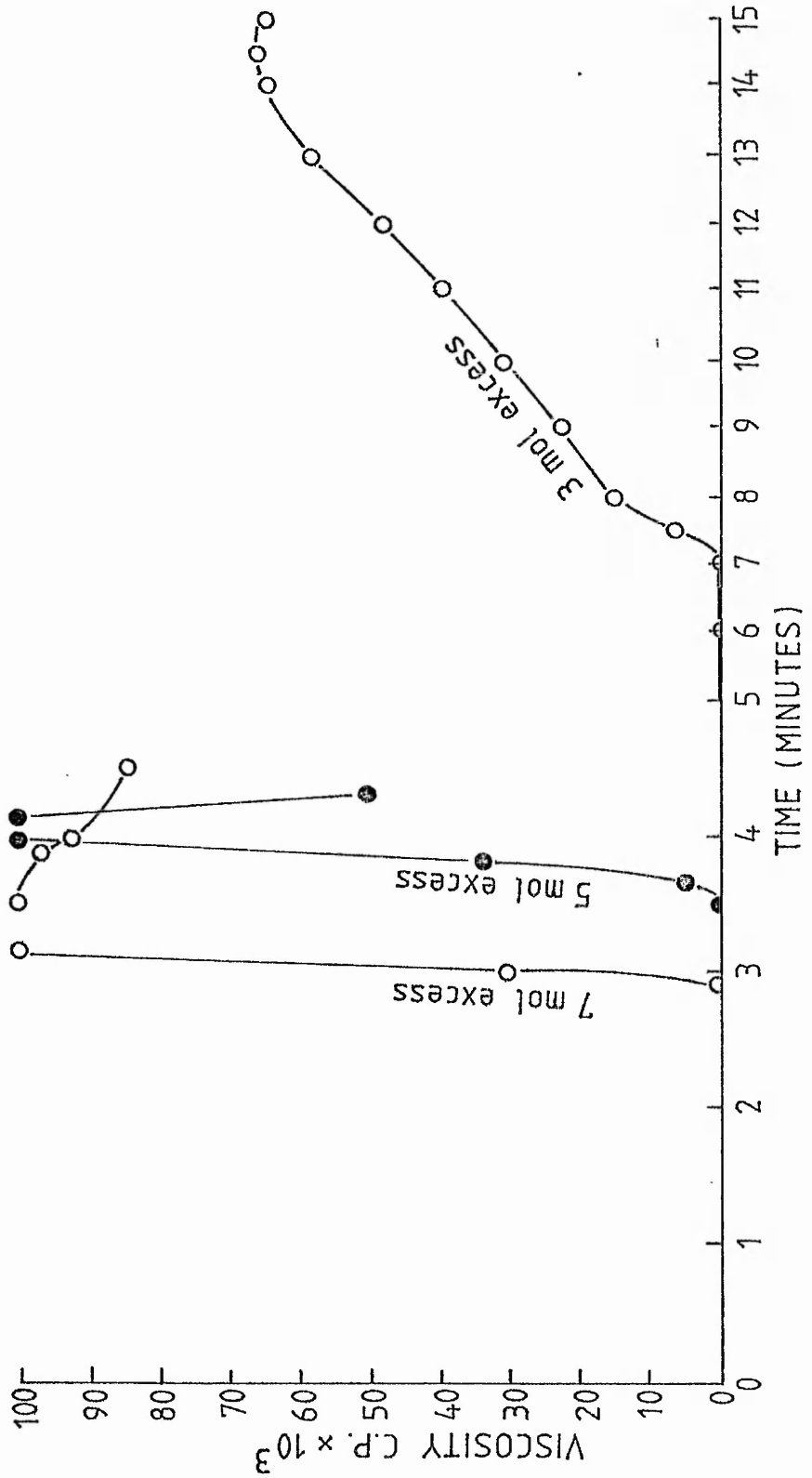


Figure 81. The effect of anhydride concentration on the gelation of chitosan solutions with acetic anhydride.

too rapid gelation at the higher polymer concentrations.

Increase in polymer concentrations gave a decrease in gelation times, Figure 82. The 3% polymer solution gelled in 11 minutes and the 2% in 14 minutes. The 0.5% solution, however, took over 58 minutes to show any increase in viscosity and even then, the increase was very small. Thus, although in this case the comparitive anhydride concentration was equivalent to a 6 mol. excess, the polymer concentration was not sufficiently high for gelation to occur. A kink in the viscosity curve was evident in all cases, except for the 0.5% polymer concentration.

3.7.4.6. Effect of Variation in Polymer Concentration with Corresponding Increase in Anhydride Concentration

Anhydride concentration: 3 mol. excess

Temperature: 25°C

Brookfield Viscometer: Speed 6.r.p.m. Spindle: No. 4

Polymer concentrations used: 0.75%, 1.0%, 1.25%, 1.5% and 1.75% with acetic anhydride - 1.0%, 1.25%, 1.5%, 2.0% and 2.5% with hexanoic anhydride
all in aqueous acetic acid and methanol in the ratio (2:5)

The results, as expected, showed that with increased polymer concentration decreased times to gelation are obtained, together with higher maximum viscosities, Figures 83 & 84. The results for acetic anhydride did not give as smooth a variation as did the hexanoic anhydride, but this may be due, in part, to the much faster reaction times of the former which would give greater inaccuracies in the measurements.

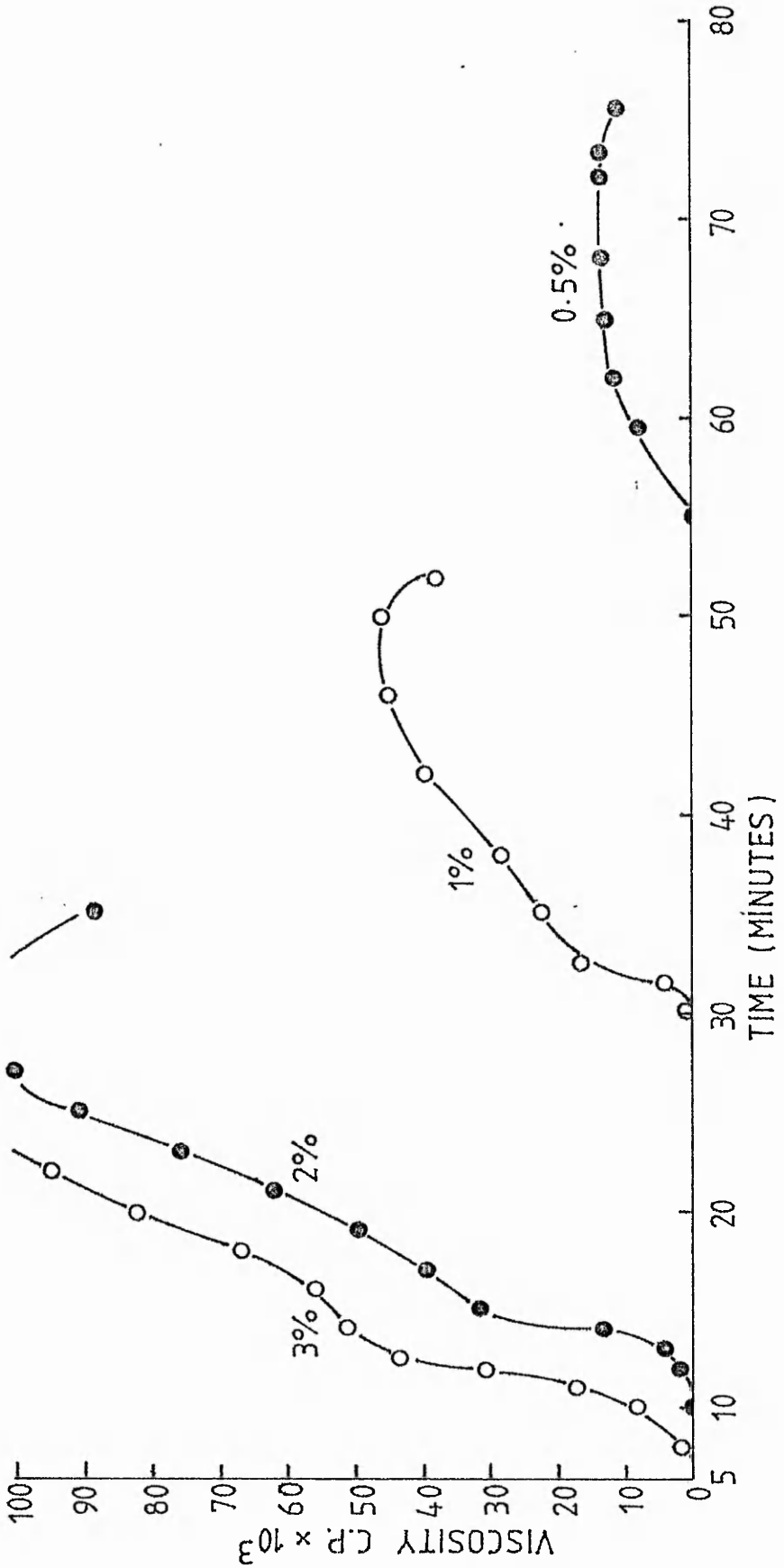


Figure 82. The effect of polymer concentration on the gelation of chitosan solutions with hexanoic anhydride.

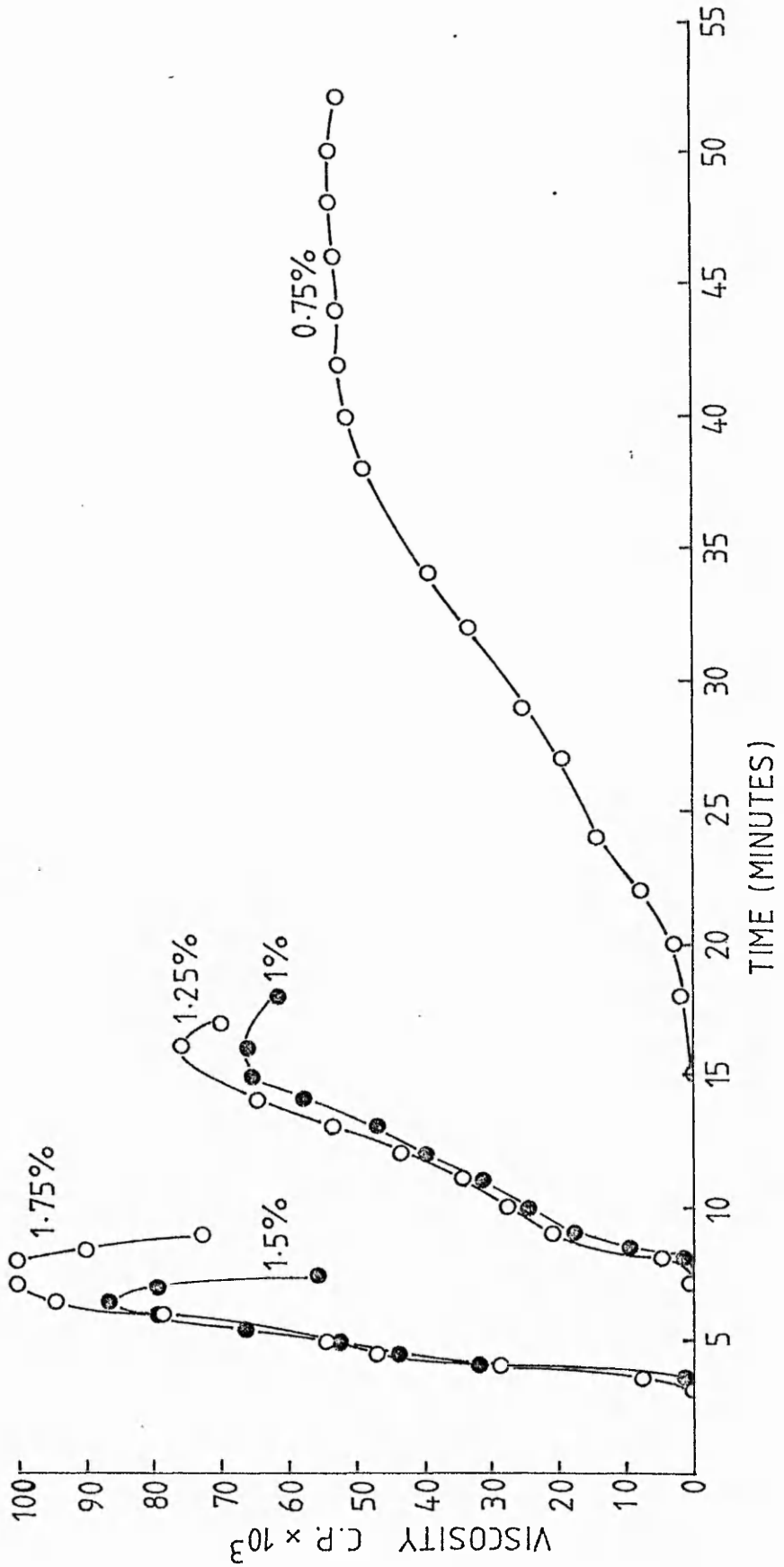


Figure 83. The effect of variation in polymer concentration with the corresponding increase in anhydride concentration on the gelation of chitosan solutions with acetic anhydride.

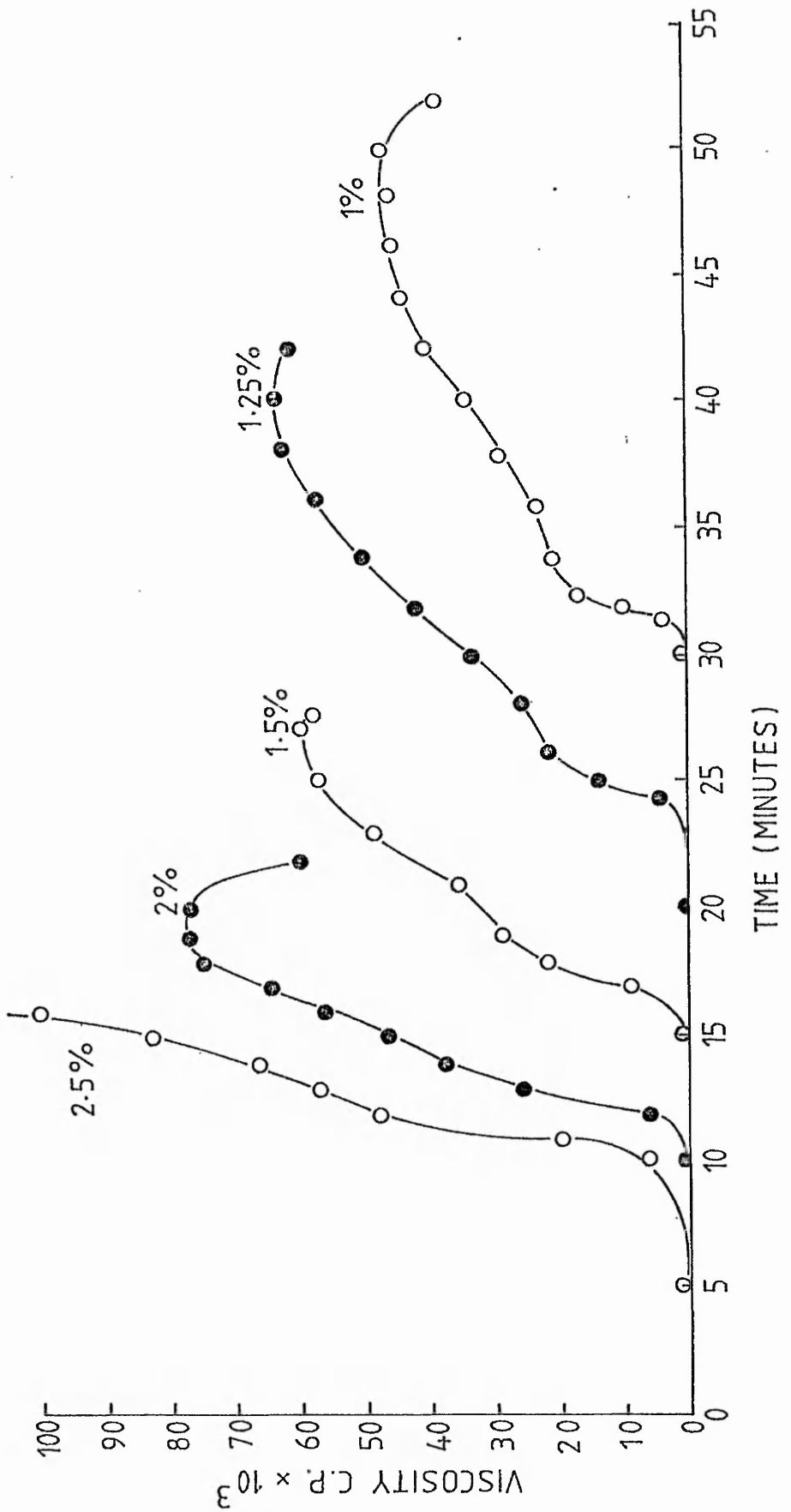


Figure 84. The effect of variation in polymer concentration with the corresponding increase in anhydride concentration on the gelation of chitosan solutions with hexanoic anhydride.

3.7.5. Ultimate Gel Strength

From the results obtained it was evident that the final gel strength could not be measured using the Brookfield Viscometer as the gel eventually breaks away from the spindle. Available equipment for measuring gel strengths is limited:, however, one such instrument is the Boucher Electronic Jelly Tester which is used in the food and pharmaceutical industries. The accuracy of this instrument is limited but its use showed some useful trends in the gelation of N-acyl chitosans.

Using the standard 1% solution of chitosan in aqueous acetic acid and methanol in the ratio (2:5) with a 3 mol. excess of acetic or hexanoic anhydrides, the effect of increasing time on the strength of the gel was noted. With both anhydrides, Figures 83 & 84 , a reasonably linear relationship was observed between the strength of the gel and the time, indicating increasing gel strength with increasing time, with no evidence of a decrease in gel strength.

The effect on the gel strength of using a different co-solvent was also studied, using acetic anhydride as the anhydride. The standard 1% chitosan solution in aqueous acetic acid was prepared and equivalent volumes of methanol, ethylene glycol, digol and trigol added. The strength of the resulting gel was noted after one hour, Table 21.

Table: 21 The Effect of Varying Co-solvent on Gel Strength

Solvent	Gel Strength (Boucher Units)
Methanol	110
Ethylene Glycol	No gelation within one hour
Digol	304
Trigol	436

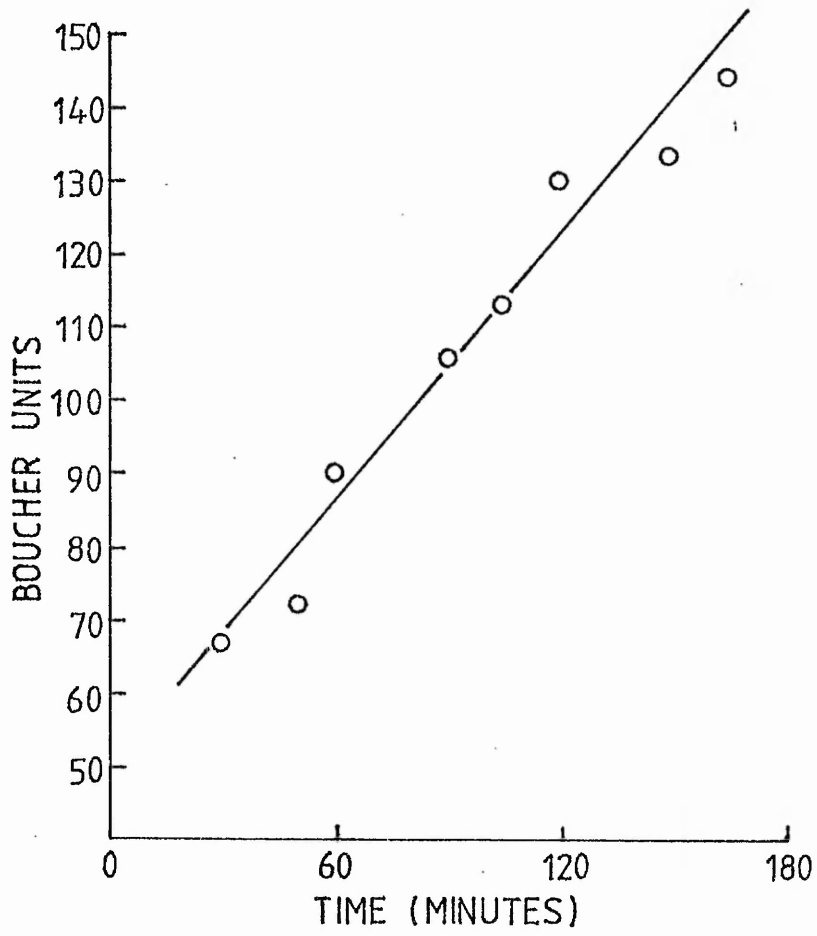


Figure 83. The effect of increasing time on the strength of the gel formed by the N-acetylation of chitosan.

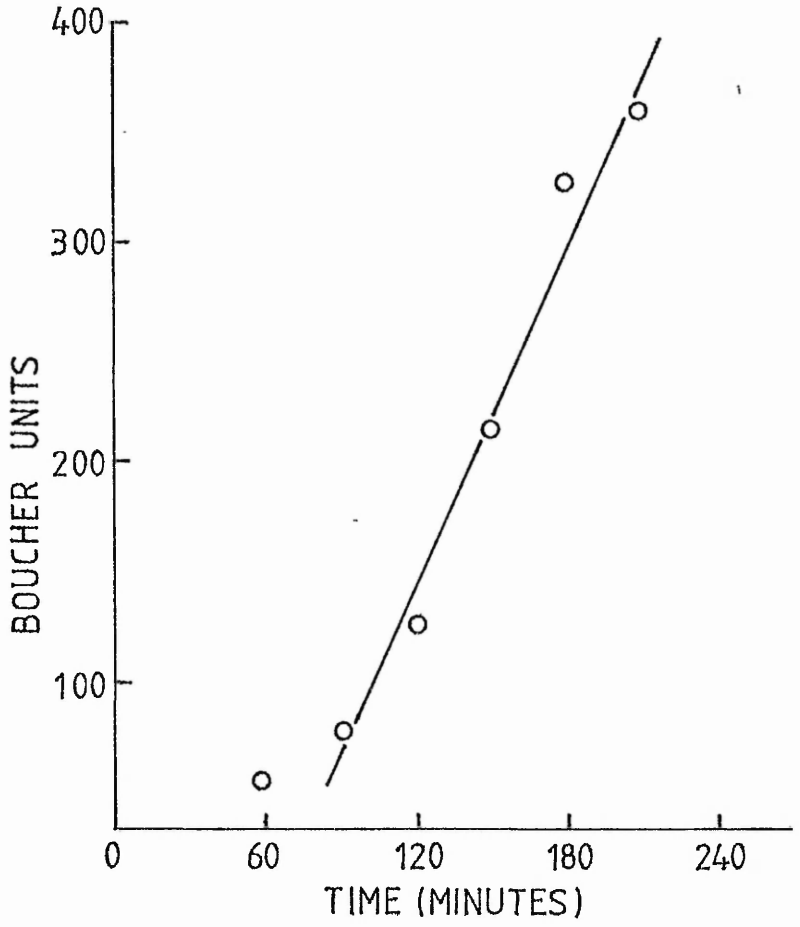


Figure 84. The effect of increasing time on the strength of the gel formed by the N-hexanoylation of chitosan.

There would appear to be an increase in gel strength with increase in molecular weight of the added co-solvent. The limiting factor with this technique is that after a period of time the N-acyl chitosan gels start to undergo syneresis, resulting in contraction of the gels and hence the formation of more rigid and stronger products.

Some attempts were made to follow gelation using the Helipath stand attachment for the Brookfield Viscometer. The standard 1% solution of chitosan in aqueous acetic acid and methanol in the ratio (2:5) was used with a 3 mol. excess of acetic anhydride.

Results again showed the rapid rise in viscosity, followed by the kink and then a steady increase in viscosity, Figure 85. No break off point was observed but, unfortunately, as the lowest spindle speed obtainable with the Brookfield model being used was 6 r.p.m. the readings eventually went off the scale.

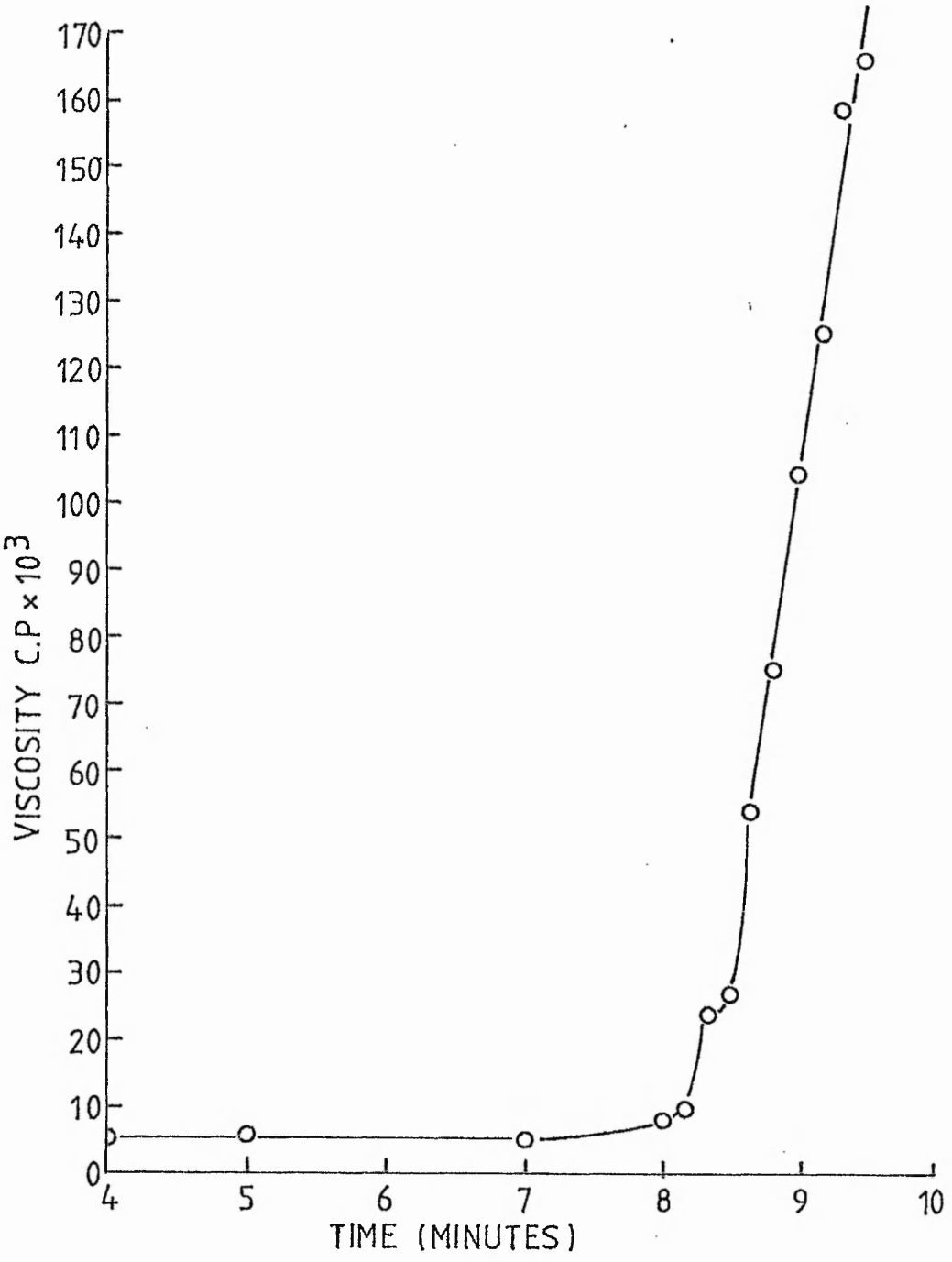


Figure 85. The viscosity versus time graph for the gelation of chitosan solution with acetic anhydride followed by the Helipath stand attachment for the Brookfield Viscometer at 25°C.

3.7.6. Further studies on gelation

3.7.6.1. Relationship between Degree of N-Acylation and Gelation

From the results of the experiments carried out so far it was evident that gelation was occurring at specific times depending on the anhydride used, conditions of reaction, etc., and it was of interest to see if some correlation could be made between the degree of acylation and gelation.

A series of partially and mixed N-acylated chitosans have been prepared [261] for the purpose of studying the gelation mechanism. Analysis of the dried gel by g.l.c., n.m.r., and elemental analysis indicated that the minimum requirement for gelation is approximately 0.4N lauroyl (C_{12}) approximately 0.6N fatty acyl (C_3-C_{10})or about 0.7N benzoyl groups per hexosaminide residue. The distribution of N-acyl groups in these partially N-acylated chitosan is unknown.[261]

For the purposes of this study hexanoic anhydride was chosen as it has the advantage of having a relatively long time to gelation. Standard 1% polymer solutions in aqueous acetic acid (5%) were mixed with methanol and a 3 mol. excess of hexanoic anhydride. The reactions were then allowed to proceed at 25^{o}C for certain lengths of time, and were monitored by the Brookfield Viscometer. At the required time the reactions were stopped and the degree of N-acylation of the product at that stage determined. The procedure of isolation and characterisation of the products is described in the experimental Chapter 5.22.

The results are summarised in Table: 22

Table: 22 Results showing the Relationship between Degree of N-acylation and Gelation

Time of Reaction (mins)	Viscosity of Solution -when stopped(cps)	% N-acylation	Solubility of Product
0	0.2	19.5	5% acetic
10	0.4	46.8	5% "
20	0.3	61.0	5% "
30	0.5	70.0	85% formic
34	5.0	73.0	85% "
37	15.4	72.0	85% "
38	18.3	77.0	85% "
39	22.3	83.0	85% "

By comparison of the N-acylation versus time curve and the viscosity versus time curve, Figure 86 , it is evident that gelation occurs when about 70% of the amine groups are acylated. The kink in the viscosity curve occurs when the degree of acylation is about 80%.

The variation of different parameters was then studied and their effect on the correlation between N-acylation and gelation observed.

3.7.6.2. Effect of Anhydride and Temperature

Standard Polymer Solution:	1% chitosan in aqueous acetic acid and methanol in ratio (2:5)
Carboxylic Anhydride:	3 mol. excess of acetic or hexanoic anhydride
Temperature Range:	25,35,45^{o}C
Brookfield Viscometer:	Speed 6 r.p.m. Spindle: No.4.

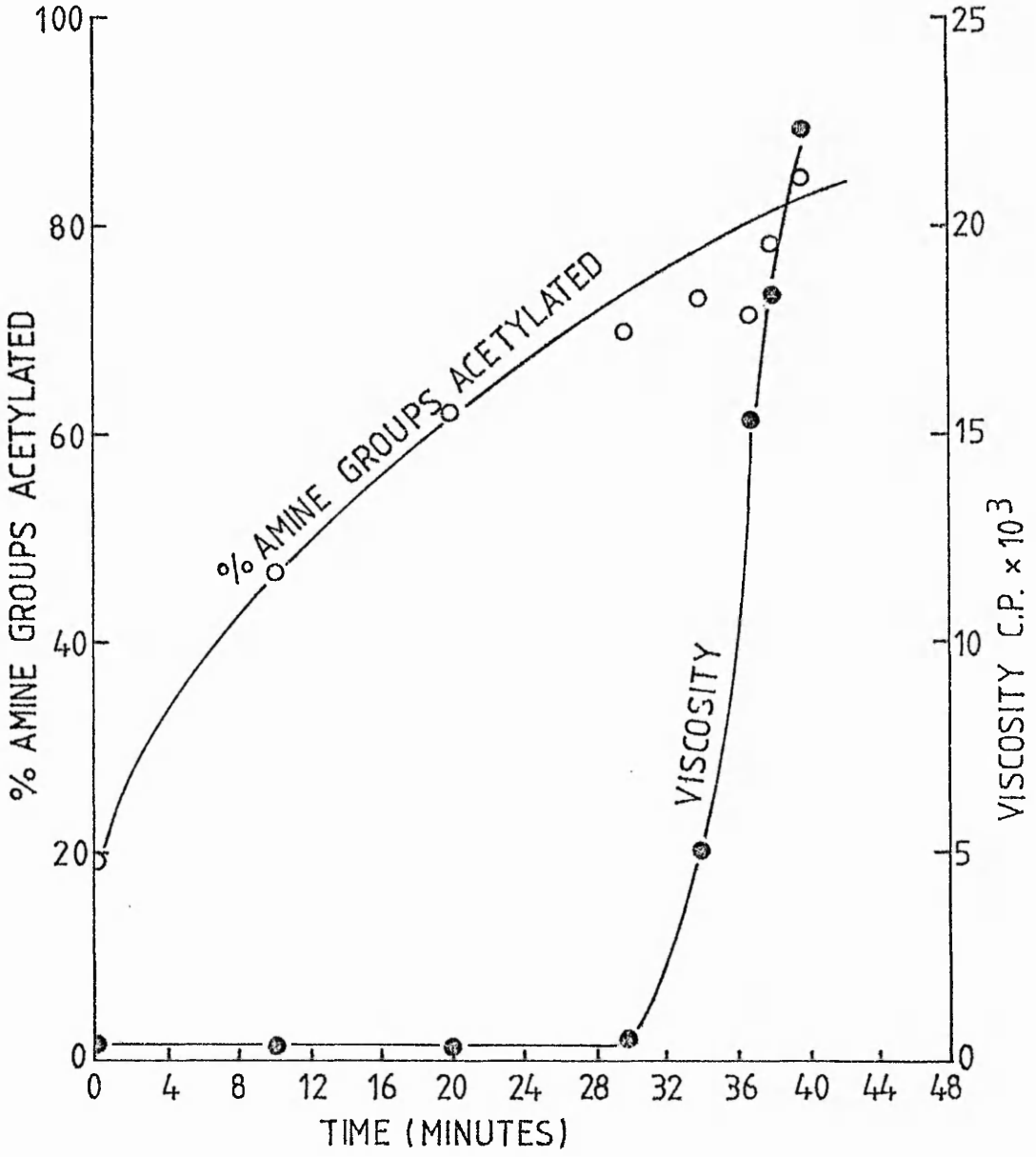


Figure 86. The N-acylation versus time curve and the viscosity versus time curve for the reaction of chitosan solution with hexanoic anhydride at 25°C.

The reaction was followed on the Brookfield Viscometer to the point where the viscosity started to increase and the products were then isolated and characterised as described in the experimental chapter, 5.22.

Table: 23 The Effect of Varying Anhydride and Temperature on the Degree of the N- acylation/Gelation Relationship

Anhydride	Temperature °C	Time Stopped (mins)	Viscosity (cps)	% N- acylation	Average %
	25	11.5	3.0	80	
	25	8.0	2.5	76.8	82.3
	25	11.5	3.0	90	
Acetic	35	6.5	3.5	90	85.0
	35	6.5	3.0	80	
	45	3.8	3.9	85	82.5
	45	2.5	3.0	80	
	25	34.3	2.0	76.5	
	25	29.0	3.5	77.6	77.0
	25	36.0	1.0	76.8	
Hexanoic	35	20.0	2.0	80.2	79.1
	35	21.0	1.0	78	
	45	9.5	4.0	74.2	74.2
	45	8.0	1.0	74.2	

The results would suggest that as the molecular weight of the acyl group increases, the degree of acylation required for gelation decreases, but the effect of temperature is not significant.

3.7.6.3. Effect of Polymer Concentration

Constant volume of anhydride taken equivalent to a 3 mol. excess for a 1% polymer solution

Temperature: 25°C

Polymer solutions: 0.5%; 1.0%; 2.0%; 2.5%; 3.0% in aqueous acetic acid and methanol (2:5)

Brookfield Viscometer: Speed 6.r.p.m. Spindle: No.4.

Only hexanoic anhydride was used as acetic anhydride causes too rapid gelation

Table: 24 Effect of Polymer Concentration on Degree of N-acylation/ gelation Relationship

Polymer Concentration	Time stopped (mins)	Viscosity (cps)	% Acylation	Average
0.5	--	--	--	--
1.0	29	3.5	77.6	73.5%
1.0	30	2.5	70.0	
2.0	17	1.7	58.2	62%
2.0	18.5	1.7	66.0	
2.5	6	2.8	61.8	65%
2.5	5.5	4.1	67.6	
3.0	7.0	2.5	61.1	60%
3.0	6.5	2.7	58.9	

The results indicate that at higher polymer concentrations the degree of acylation is less at the point of gelation, such that a 3.0% solution of chitosan in aqueous acetic acid (5%) is only 60% N-acylated at this stage.

This agrees, in part, with a figure of approximately 60% that Hirano et al determined[261] as the minimum requirement for gelation with acyl anhydrides (C_3- C_{10}) for a 5.0% solution of chitosan in aqueous acetic acid (10%).

3.7.6.4. Determination of the Energy of Activation for N-Acylation

From the results of the effect of anhydride and temperature on the relationship between the degree of N-acylation and gelation, it would seem evident that the degree of acylation required for gelation varies as the molecular weight of the acyl group varies, but that the effect of temperature is not significant. Thus by recording the time taken to the onset of gelation for N-acyl chitosan at different temperatures and then plotting the logarithm of the reciprocal time, t, against the reciprocal of the absolute temperature, T, the energy of activation for the N-acylation of chitosan in methanolic aqueous acetic acid solution may be determined.

Figures 87 &88 give $\log (1/t)$ against $1/T$ for N-acetyl chitosan and N-hexanoyl chitosan, respectively. The slope of the N-acetylation of chitosan is - 3530 and hence the energy of activation is 67.6 kJ mol^{-1} The results from the N-hexanoylation of chitosan give a slope of -2999 and hence the energy of activation is 57.4 kJ mol^{-1}

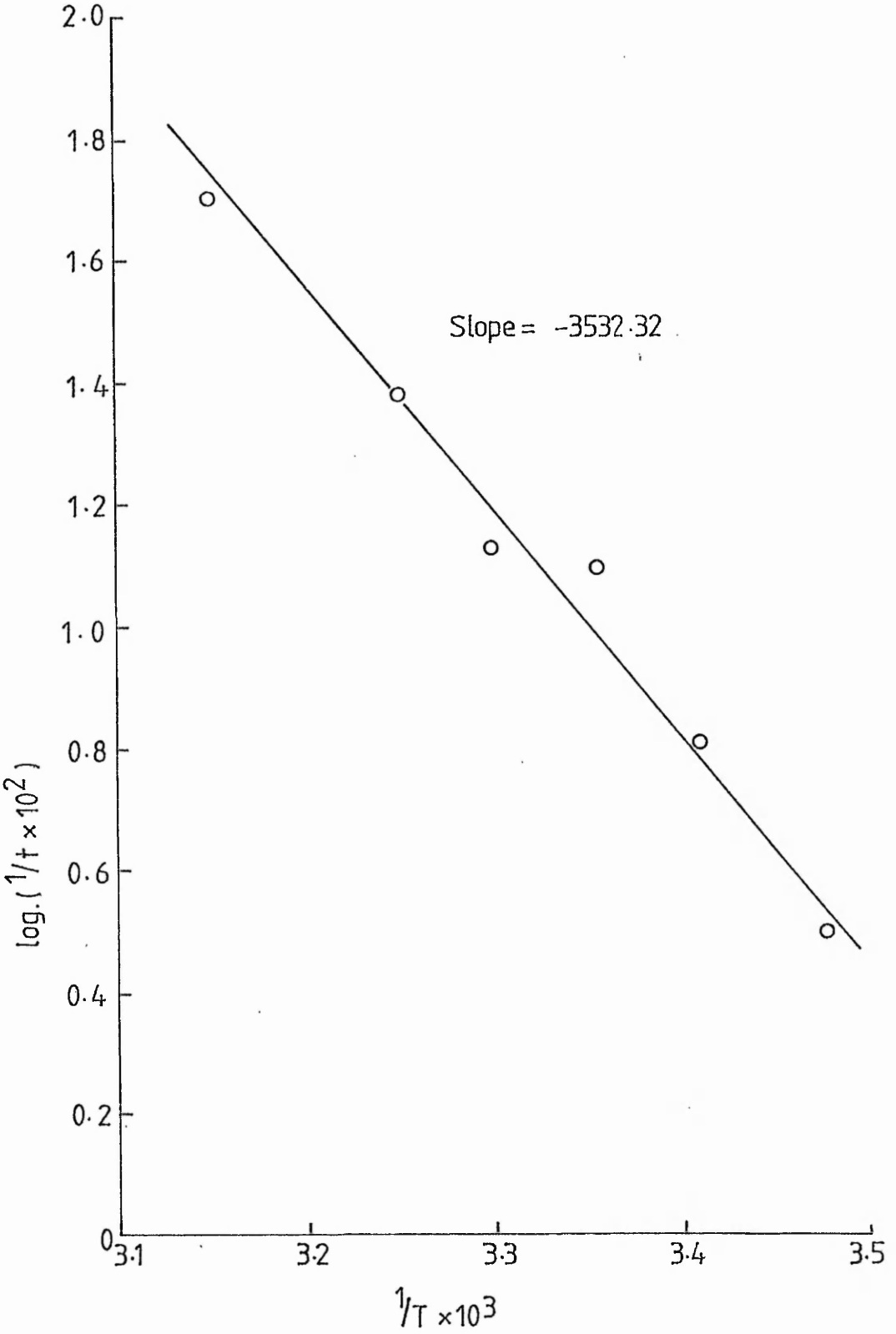


Figure 87. Graph of log (1/t) against 1/T for the N-acetylation of chitosan in solution.
(t=time to the onset of gelation and T= temperature, degrees absolute).

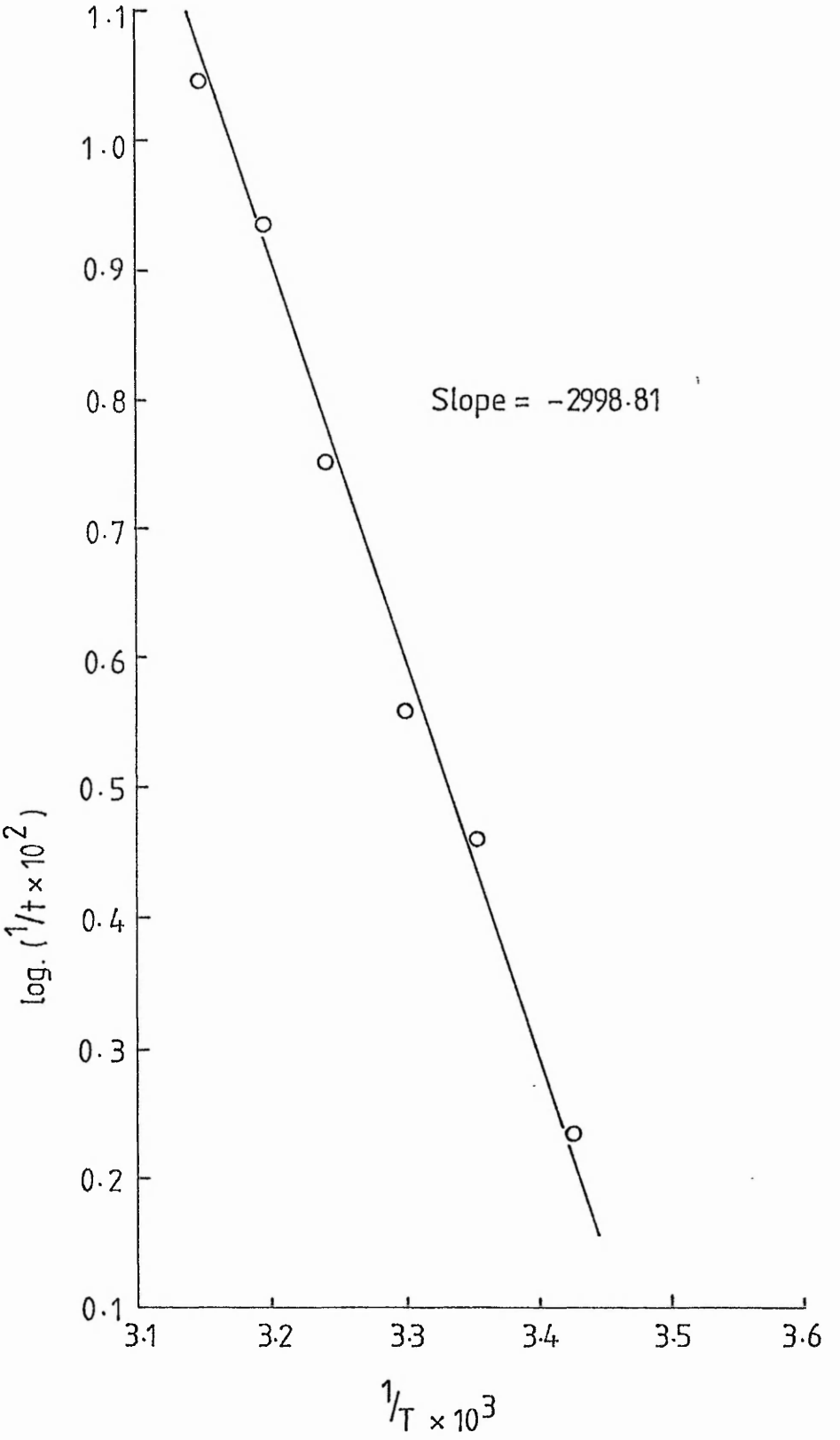


Figure 88. Graph of log (1/t) against 1/T for the N-hexanoylation of chitosan in solution.

(t=time to the onset of gelation and T=temperature, degrees absolute).

Application of regressional analysis to the results shows the standard deviations of the slope to be less than $\pm$ 10%.

It appears that the energy of activation decreases with increase in size of the N-acyl group and this agrees with the earlier approximate results obtained for the N-acylation of chitosan film, where Ea was again found to decrease with increase in the size of the N-acyl group. This apparent decrease in Ea of the acylation reaction is presumably due to increased steric hindrance created by increased size of the acyl reactant.

The method of determining Ea in this case appears to give reasonable results. Very accurate figures should be obtainable by increasing the number of results and by improving the accuracy of recording the results. This is in contrast to the determination of Ea of N-acetylation of chitosan film which, as already stated, is unable to be refined because of the intractable problems associated with the polymer film.

Although direct comparison of the Ea values obtained for the N-acetylation of chitosan is not possible because of the different solvent media in which the N-acetylation was carried out and the inaccuracy of the film method, it can be seen that the figures obtained, 67.6 kJ mol^{-1} for N-acetylation in solution, and 57.9 kJ mol^{-1} on film are of the same order.

3.7.7. Mechanism of Gelation

From the results obtained it appears that the gelation process of N-acyl chitosan solutions occurs in different stages. From the viscosity versus time curves obtained it is evident that with the onset of gelation there is a rapid rise in viscosity, which then levels off momentarily and then continues to rise steadily. The gelation of Oxidol-Agar was compared and this gave a smooth curve for the viscosity versus time relationship, indicating the kink was not an artefact of the technique but corresponded to a particular stage in the gelation process.

It has been shown that the rapid rise in viscosity at the onset of gelation corresponds to about 70% of the amine groups being acylated. Variations in the time of the initial increase in viscosity were found for the different N-acyl chitosans and these increased, for the most part, with increase in molecular weight of the acyl reactant. Increases in reaction temperature brought about decreases in the time to gelation.

Thus the gelation process of N-acyl chitosan solutions can be considered as two interdependent reactions, (i) the acylation reaction of the primary amines present and (ii) the formation of the gel. The chitosan molecules in the methanolic aqueous acetic acid solutions are changed from uncharged polymer chains to chains carrying ionized groups through protonation of the primary amine groups. The mutual repulsion of the fixed positive charges will lead to chain expansions giving a conformation where the chains are as far a part as possible, Figure 89. In addition, the overall size of each charged polymer chain may be increased by acetate counter ions lying close to the chain and the protonated amine groups and creating a "sausage-shaped" region around each chain.

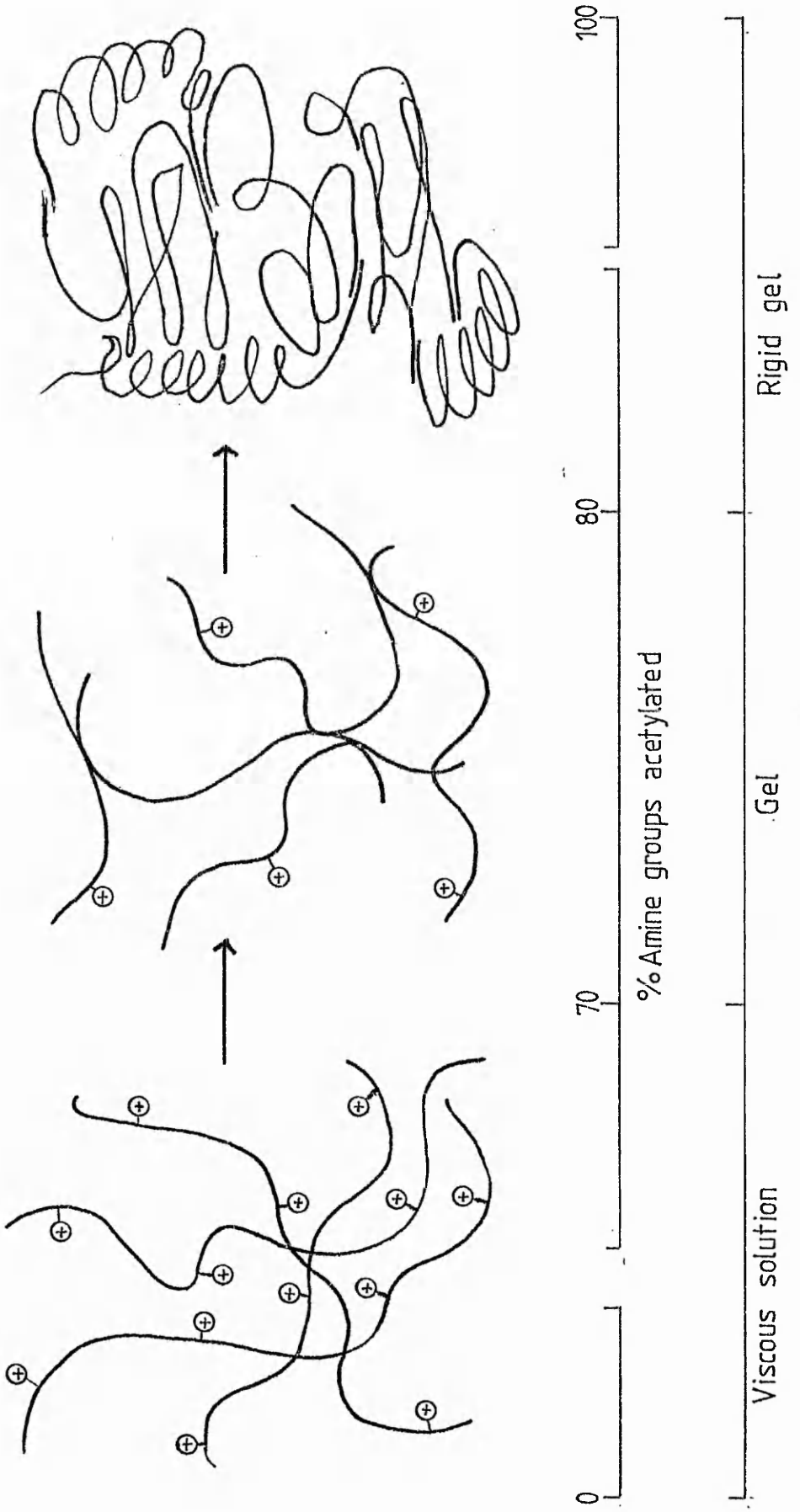


Figure 89. The changes in the conformations of the polymer chains of chitosan in solution with increasing N-acylation.

This region may then act like a membrane, as the solvent tries to equalise the acetate ion concentration on either side of the membrane, through osmotic effects.

On addition of the carboxylic acid anhydride N-acylation occurs which reduces the number of protonated amine groups present and hence reduces the mutual repulsion effects along the polymer chains and causes the "sausage-shaped" region of counter-ions to diminish. The chain expansions will then presumably contract and as the extent of N-acylation approaches 70% gel formation is initiated through molecular aggregation of the chains at various junction points, the precise nature and position of which is unknown. Hirano[259] has postulated that molecular aggregation occurs in three dimensions.

After approximately 80% of the amine groups are acylated, the rate of increasing viscosity levels off and remains at constant value for a while and then starts to rise sharply again. If molecular aggregation occurs through a network of junction points, this apparent stationary stage in the rate of viscosity increase could be considered as a momentary collapse of the polymer chains between these junction points followed by a rearrangement of these chains giving rise to randomly coiled polymer chains linked through a series of junction points. As these chains begin to compact then the gel changes from a state of flexibility to one of rigidity.

Examination of the time at which gelation occurs in the N-acetylation of chitosan shows that it corresponds to a similar point at which

Hirano,[259] under similar experimental conditions, has observed significant changes in the specific rotation of the N-acetyl chitosan solutions. It was considered[259] that this stage corresponded to the overlap of the N-acetylation reaction and aggregation process.

3.7.8. Optical Rotation Changes during Gelation

Hirano has reported [259] that changes occur in the specific rotation of chitosan on N-acylation with both acetic and benzoic anhydrides. Significant changes were recorded as the solutions began to solidify.

An attempt was made to follow the gelation of N-hexanoyl chitosan by optical rotation and correlate any results obtained with those of viscosity. However, using a polarimeter, no worthwhile results were obtained, the changes in optical rotation being extremely small.

A major problem is the difficulty in making up sufficiently concentrated solutions of the polymer to give a reasonable measured change in the rotation. Also, the time taken to obtain a reading made continuous monitoring difficult; the use of an automatic polarimeter might give more useful information.

3.7.9. Syneresis of Chitosan Gels

It was observed that the N-acyl chitosan gels undergo syneresis on standing at room temperature. Hirano and Yamaguchi reported[259] that syneresis occurred after 24 hours with N-acetyl chitosan gels prepared from mixtures containing 13 or more mol. equivalents of acetic anhydride. As there was no report of any observations at shorter time periods an attempt was made to record the rate of syneresis by measuring the volume of liquid produced at varying time intervals.

A 2.5% chitosan solution in aqueous acetic acid was mixed with methanol and a 3 mol. excess of acetic anhydride added. The solution was poured into a jar, sealed to avoid evaporation losses, and placed in a thermostatically controlled water bath at 25°C. Once gelation had occurred any liquid formed around the gel was removed, measured, and the time noted. The experiment was repeated, but this time the gel was cut away from the side of the jar immediately on gelation.

Figure 90 gives the two rate curves obtained. It is evident that cutting the gel away from the side of the vessel causes an initial increase in the rate of syneresis. This is presumably due to the increased surface area through which syneresis can occur. This rate then slowly levels off and approaches that of the rate of uncut gel so that after 21 hours an equivalent volume of liquid has been lost from each.

A more accurate rate curve was obtained with an increased number of readings and with the time starting from the point of mixing. The results give a typical rate curve with a steady rise in the volume of liquid expelled over the first hour followed by slow levelling off; Figure 91 .

Comparison with other N-acyl gels showed that similar rates of syneresis were obtained for all the gels tried. However, it was felt that the method used was not of sufficient accuracy for any detailed work to be carried out. Variability of the surface area of the gel, time of starting the measurements, and measurements of the initial volume of liquid produced would all need standardisation, and improvements were necessary in recording.

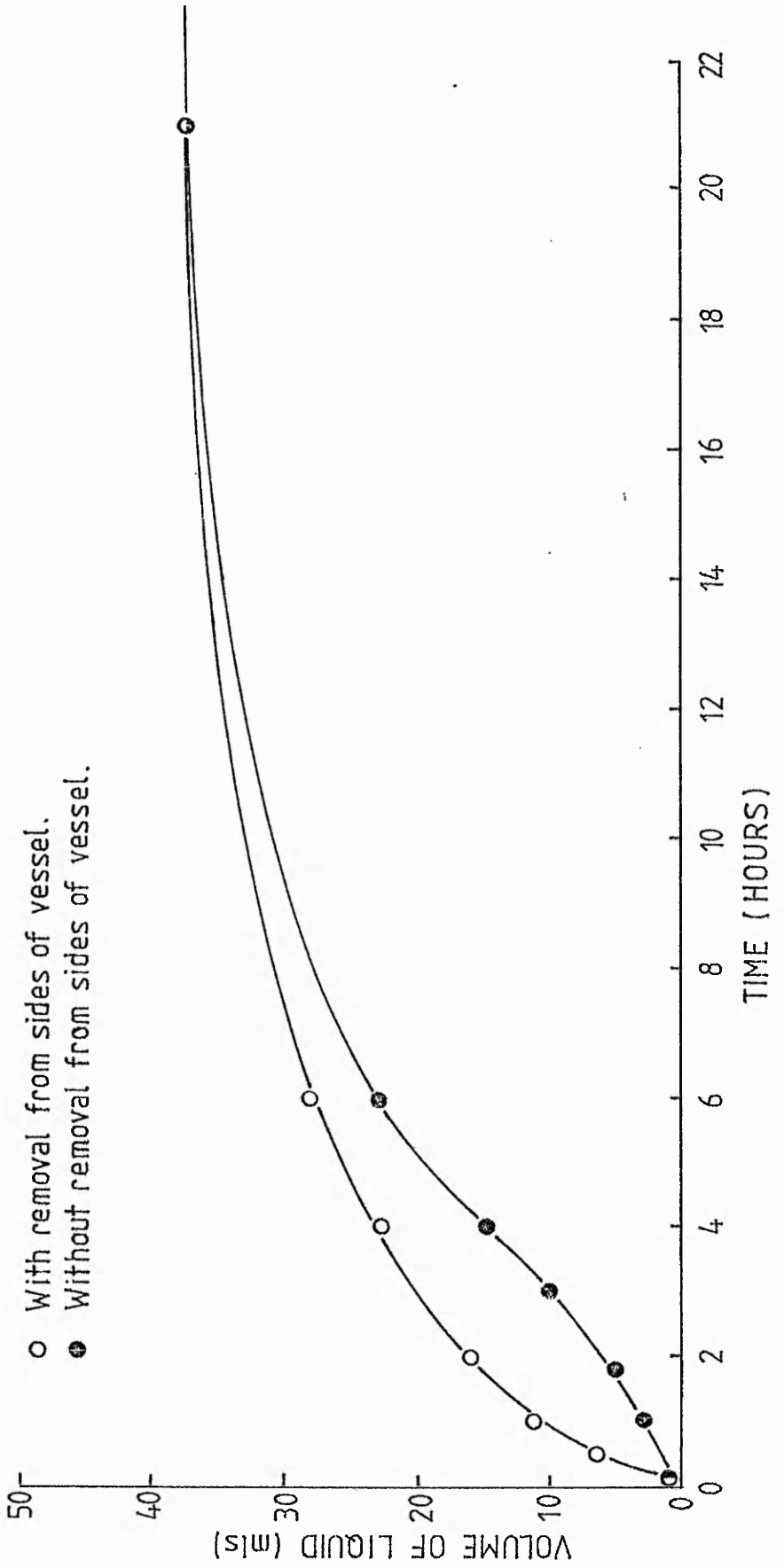


Figure 90. The rate of syneresis of N-acetyl chitosan gel at 25°C.

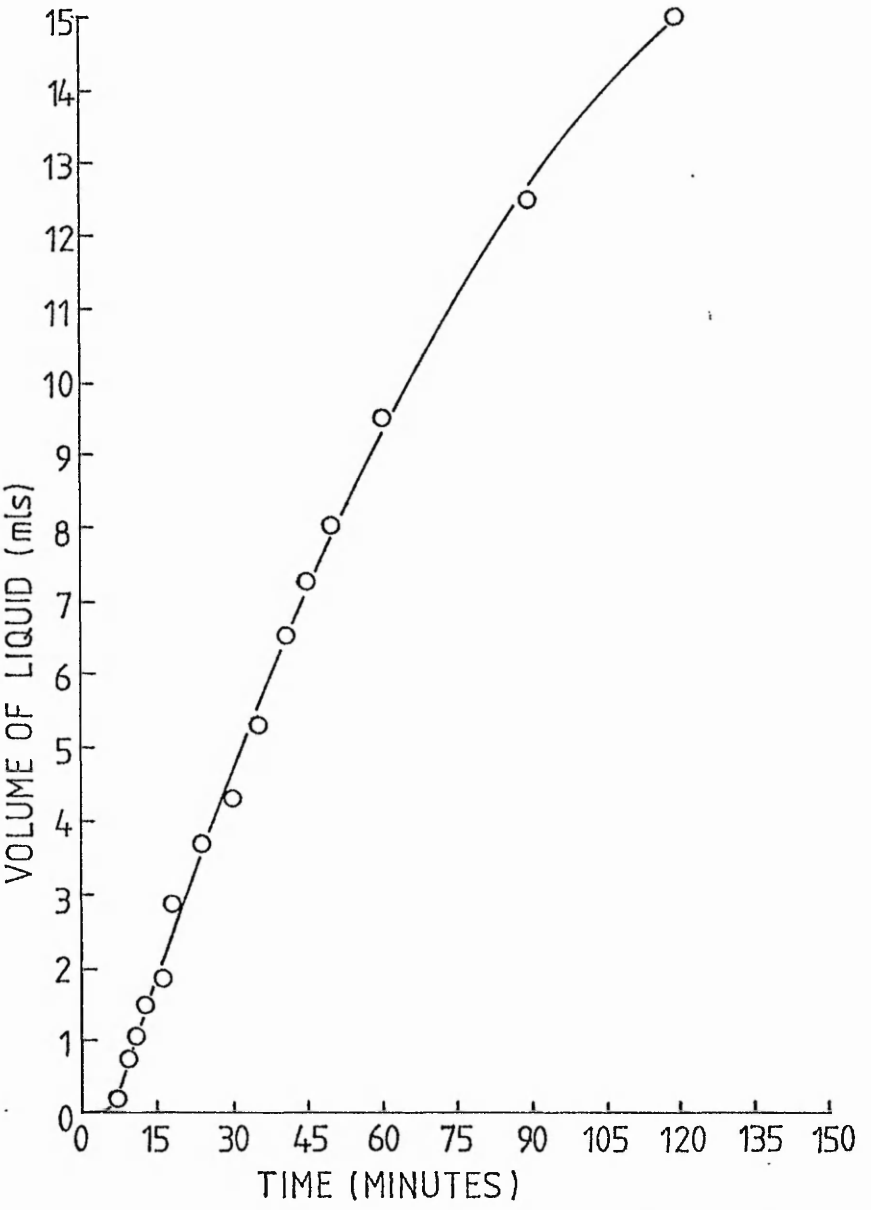


Figure 91. The rate of syneresis of N-acetyl chitosan gel at 25°C, with the time starting from the point of mixing.

Despite this, it was evident that syneresis begins soon after gelation has occurred and the extent of syneresis rises steadily so that with N-acetyl chitosan gels about 50% of the initial volume of liquid within the gel has been lost after 21 hours. Although no further measurements were recorded after 21 hours, it was observed that the remaining 50% was lost over a period of 5 days, leaving a hard dry inflexible product.

As previously mentioned (Chapter 3.2.3.) a number of co-solvents give gels in which syneresis stops, leaving a rubbery, flexible product.

3.7.10. The Use of Novel Anhydrides for Gel Formation

3.7.10.1 Branched Anhydrides

Gelation was attempted with standard polymer solutions containing a 3 mol. excess of iso-butyric, iso-valeric or pivalic anhydride. The results show that gelation occurs with both iso-butyric and iso-valeric anhydrides after four hours but that no gelation was observed with pivalic anhydride for up to 72 hours at room temperature. This may be due to the decomposition of the anhydride in the aqueous, alcoholic medium.

3.7.10.2. Toluic Anhydrides

On addition of o- and p-toluic anhydrides to the polymer solutions, dense white precipitates were formed and no gelation was observed. The precipitates formed were presumably the decomposition products of the respective anhydrides for the infrared spectrum of the recovered chitosan showed no evidence of any N-acylation having occurred.

3.7.11. The Use of Aldehydes for Gel Formation

The formation of chitosan-aldehyde gels in aqueous acetic acid-methanol solutions, with acetaldehyde, acrolein, benzaldehyde, cinnamaldehyde, propionaldehyde, p-tolualdehyde, formaldehyde, glutaraldehyde and salicylaldehyde has been reported.[269]

An attempt was made to follow the rate of gelation, with the Brookfield Viscometer, of chitosan salicylaldehyde using a standard 1% polymer solution in aqueous acetic acid -methanol and a 2 mol. excess of salicylaldehyde , at 25°C.

The reaction took place very rapidly and accurate readings could not be taken, but it was observed that gelation occurred at 6 minutes from the time of mixing. Reductions in the concentrations of aldehyde gave minor increases in the time to gelation, but still did not allow the taking of accurate readings. No gelation occurred with a 1 mol. equivalent of salicylaldehyde, although the formation of the Schiff's base product was observed through the appearance of a yellow colouration.

Attempts to prepare chitosan-aldehydes gels with straight-chain aliphatic aldehydes (C_4-C_{10}) were unsuccessful, even though Hirano has prepared [269] gels with acetaldehyde and propionaldehyde.

Chapter 4 Conclusions

1. The major obstacle to the ready acetylation of chitosan is the restricted accessibility of the polymer chains. Protonation of the amine groups of chitosan through an acidic acetylation media would be expected to reduce the extent of N-acetylation. However this appears to have little effect on the extent of O-acetylation, as evidenced by the similar difficulty in O-acetylating chitosan film both before and after N-acetylation.

2. Facile, selective N-acylation of chitosan in film or powder form can be carried out using carboxylic acid anhydride/methanol mixtures. Binary mixtures of n-alcohols/formamide solutions having the same solubility parameter values, δ, as methanol give similar degrees of N-acetylation of chitosan film, but on decreasing the value of δ there is a corresponding decrease in the extent of N-acetylation. Presteeping the film in the appropriate solvent mixture prior to N-acylation removes the induction period and brings about much faster reaction times.

3. Approximate experimental activation energies for the N-acylation of chitosan film have been determined and found to decrease with increase in the size of the anhydride molecule. Attempts to obtain more accurate figures for the energy of activation were hindered by the inherent problems associated with the nature of the chitosan film.

4. X-ray diffraction studies of the N-acyl chitosans produced by treatment of chitosan with acyl anhydride/methanol show N-acetyl chitosan

to be a well ordered crystalline polymer resembling natural chitin. Increasing the length of the N-acyl substitent gives increasingly diffuse X-ray patterns indicating that the higher N-acyl chitosans are more amorphous.

5. The ease of O-acetylation of films of the various N-acyl chitosans with acetic anhydride/pyridine mixtures increases with increase in size of the N- acyl group, although very bulky substituents hinder O-acetylation through steric effects. However only partially O-acetylated products, with approximately 50% of the hydroxyl groups remaining unreacted, are obtained even with prolonged reaction times.

6. Almost fully O-acetylated derivatives of N-butyryl,N-hexanoyl and N-decanoyl chitosans in powder form can be prepared by prolonged treatment with glacial acetic acid/acetic anhydride/perchloric acid mixtures. Films of these N- acyl chitosans disintegrate on similar treatment.

7. Aromatic Schiff's base derivatives allow complete O- acetylation but aliphatic Schiff's base derivatives are susceptible to hydrolysis and only partial O- acetylation is achieved together with N- acetylation in these cases. Fully O- acetylated derivatives of the N- salicyclidene and N-benzlidene chitosans can be prepared in powder form.

8. Comparison of the O-acetylation behaviour of the N-acyl chitosans and the equivalent Schiff's base derivatives indicates that in general all the aliphatic derivatives show a similar extent of reaction which is limited to approximately 50% of the hydroxyl groups. The Schiff's base

derivatives undergo hydrolysis, with accompanying N-acetylation, during the O-acetylation. The aromatic derivatives show marked differences, the Schiff's base products having very high extents of reaction whereas the N-acyl chitosans allow only about 50% O-acetylation. The results suggest that steric hinderance of the C(3)-hydroxyl group by the C(2)-amide group is the factor which limits the extent of O- acetylation that can be obtained within reasonable time periods, regardless of the nature of the N-acyl group.

9. Di- O-acetyl-N-acetyl chitosan, prepared via an aromatic Schiff's base derivative intermediate, has limited solubility, being soluble only in formic acid and DMSO. Clear, coherent films can be cast from formic acid and DMSO solutions. The product prepared from low molecular weight chitosan has an estimated D.P. of 600, compared with high grade cellulose acetate which is reported to have a D.P of about 250, and this would reduce the solubility.

10. Reaction of N-salicyclidene and N-benzylidene chitosans with aromatic isocyanates gives organosoluble derivatives of chitosan.

11. The process of gelation of N- acyl chitosans in methanol/acetic acid/acyl anhydride solutions has been examined by monitoring viscosity changes. In all cases there is an initial rapid rise in viscosity as gelation starts, followed by a slight levelling-off period and then a further steady increase in viscosity. Increased temperatures and higher anhydride and polymer concentrations bring about decreased times to gelation.

12. Variations in the nature of the co-solvent in the gelation process give variations in the nature of the resulting product. With methanol

as co-solvent the gels are found to undergo syneresis and eventually dry out, giving a hard, inflexible product; but with formamide, ethylene glycol, digol, and trigol syneresis does not go to completion and a stable, rubbery, flexible product is formed.

13. Gelation begins when about 70% of the amine groups are acylated. The degree of acylation required for gelation varies as the molecular weight of the acyl group varies, but is independent of temperature.

14. The experimental energies of activation of N-acetylation and N- hexanoylation of chitosan are approximately 67 kJ mol^{-1} and 57 kJ mol^{-1} respectively. The energy of activation again decreases with increase in size of the acyl anhydride molecule, in agreement with the trend in N-acylation of chitosan film.

15. A mechanism has been proposed for the gelation process based on the experimental evidence obtained from the viscosity changes.

Chapter 5 Experimental

5.1. Materials used

5.1.1. Chitosan

The chitosan used in earlier work was supplied by the U.S. National Oceanic and Atmospheric Administration (Batch No.1-74) and that used in the later work by Hercules Incorporated (Batch No. SPX-5350)

5.1.2. Chemicals

The chemicals and solvents were used as supplied and were of general purpose reagent grade, except for those used in the study of the effect of solubility parameters values on N-acetylation, which were of Analar grade.

5.2. Spectroscopic methods

Infrared spectra were recorded using either a Perkin Elmer 137 sodium chloride or a Perkin Elmer 137G grating spectrophotometer.

Proton magnetic resonance spectra were recorded on a Jeol JNMC-60 HL 60MH_z spectrophotometer with tetramethylsilane as the internal standard.

5.3. X-Ray diffraction

X-ray diffraction patterns were recorded using a Raymax RX3D-X-ray generator and a flat film camera supplied by the Picker X-ray Corporation. The samples were prepared by mixing the powdered samples with moistened gum tragacanth powder and rolling into a thin cylindrical shape. The X-ray tube was operated on 40kV and 15mA for $1\frac{1}{2}$-2 hours.

5.4. Dilute Solution Viscosity

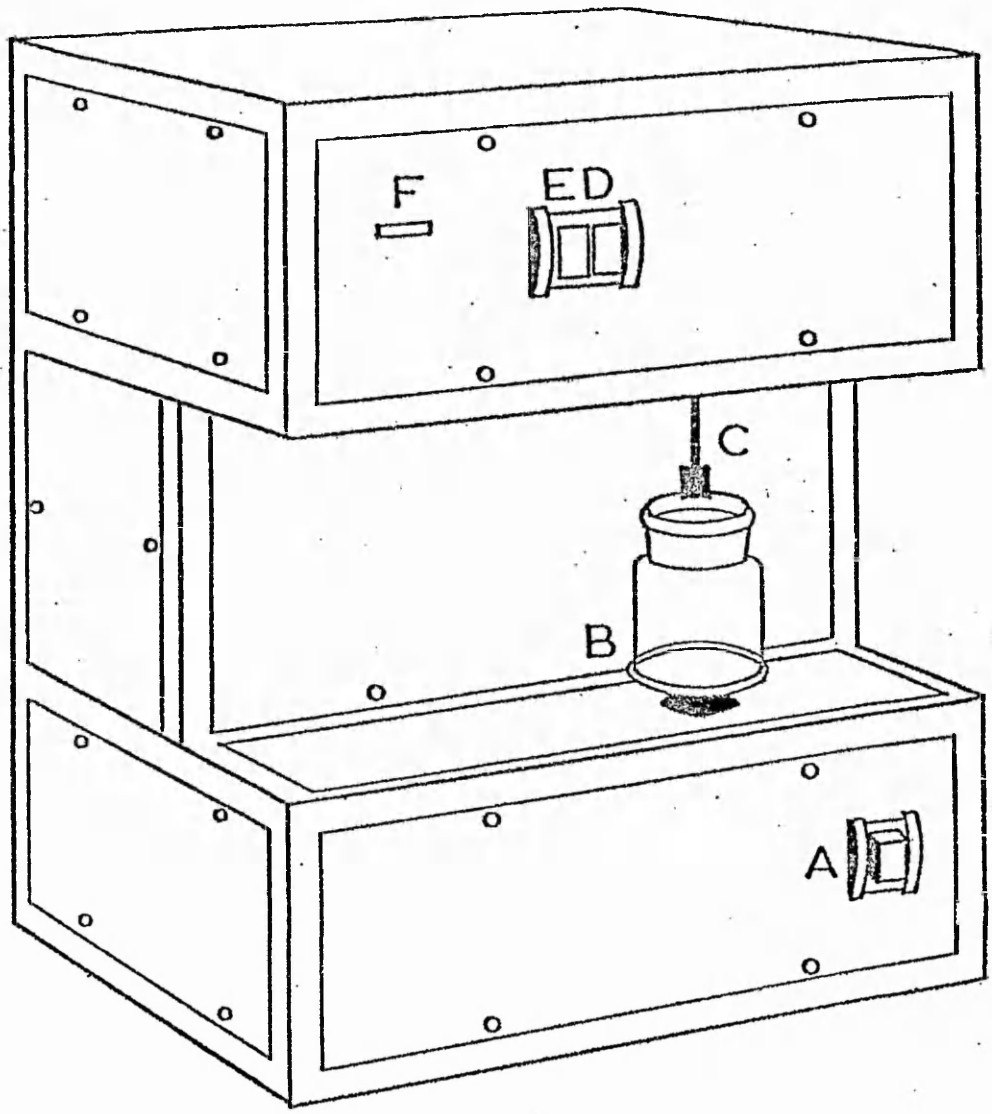
Viscosity measurements were made using a suspended level dilution viscometer with a sintered glass filter, as supplied by Polymer Consultants Limited. All measurements were carried out in a thermostatically controlled water bath at 25°C ± 0.05°C unless otherwise stated, and timed by a stop watch accurate to 0.2 second. Inherent viscosities, η inh = $\ln(\eta soln/\ \eta solv)/C$, were determined in dimethylsulphoxide at 25° at a concentration (C) of approximately 0.5g of polymer per 100ml; results are expressed as $dlg.^{-1}$

5.5. Concentrated Solution Viscosity

A Brookfield Synchro-Lectric viscometer, model LVF, with speeds of 6,12,30 and 60 r.p.m. and spindles numbered 1,2,3 & 4, was used for gelation studies.

The strength of N-acyl chitosan gels was determined using a Brookfield Model D Helipath Stand with six spindles, T-A to T-F, and a Boucher Electronic Jelly Tester.

The Boucher Electronic Jelly Tester is used in the pharmaceutical and food industries to determine the strength of gel preparations. Its mode of operation is straightforward and based on the following principles. The test gel is centrally positioned on the platform (Figure 92) and the test started. The platform is raised so that the gel surface is brought into contact with the plunger, and contrives to rise through a few mm, lifting the whole plunger assembly and activating a reed switch which simultaneously stops the platform. This activates another motor which rotates the torsion wire connected to the beam carrying the plunger. The rotational force depresses the assembly and plunger through 4 mm,



A On-Off switch: red
B Platform
C Plunger for Bloom Gelometer
D Commence test switch: green
E Zero recorder switch: amber
F Digital indicator

Figure 92. The Boucher Electronic Jelly Tester.

after which a further reed switch, which stops the motor driving the torsion wire, is activated. A counter records the number of turns of the electric motor and is adjusted so as to be related to a force in grammes Bloom. Jelly strength in grammes Bloom = Boucher reading -40.

5.6. Solubility Studies

In the characterisation of novel derivatives of chitosan, the following list of solvents were used to determine the solubility of the product. Acetone, chloroform, carbontetrachloride, dimethylformamide, dimethylsulphoxide, dioxan, diethylether, dimethylacetamide, ethylacetate, ethanol, ethylene chlorohydrin, epichlorohydrin, formamide, methanol, methylene chloride, toluene, tetrahydrofuran and water. This covers a solubility parameter range of 6.9 - 23. 4 Hildebrands ($cal^{\frac{1}{2}}\ cm^{-3/2}$). In addition glacial acetic acid, formic acid (85%), aqueous acetic acid (5%), dilute hydrochloric acid (1%), aqueous sodium hydroxide (5%) and hexamethylphosphoric acid were also examined as solvents.

5.7. Characterisation of Starting Materials

Chitosan is prepared from chitin by deacetylation in alkali media. However, under the conditions employed in the deacetylation stage the extent of acetyl removal can vary, thus giving chitosan products with different characteristics of acetyl content, viscosity, and molecular weight distribution. The variable nature of chitin sources and the variability of the deacetylation stage make it virtually impossible to have a "standard chitosan". Hence, there is a need for characterisation of any chitosan materials used in research work.

5.7.1. Molecular Weight

Molecular weight determinations of chitosan have been carried out by a variety of methods. In a survey of the molecular weight distribution of commercially prepared chitosan samples Wu and Bough[177,178] using high-pressure liquid chromatography, found the weight average molecular weights to range in value from 110,000 to 3,999,000 and the number average molecular weights to vary from 51,000 to 839,000. Lee[171] using ultracentrifugation as a technique for measuring molecular weights of chitosan samples in conjunction with Limiting Viscosity Number data, determined the constants K and α in the Mark-Houwink equation. The values obtained were $K=8.93 \times 10^{-4}$ and $\alpha = 0.71$. Thus the molecular weights of the different samples could be determined from Limiting Viscosity Number data.

5.7.2. Measurement of Limiting Viscosity Number

A standard solution of chitosan (0.2g) in 0.2M acetic acid - 0-1M sodium

chloride - 4M urea(100ml) was prepared. The viscosity measurements were made in a suspended level dilution viscometer with a sintered glass filter at 25°C ± 0.05°C in a thermostatically controlled water bath. Repeat measurements of the time of flow of the solution were made until readings agreed to within 0.2 seconds. Dilutions were made using 0.2M acetic acid - 0.1M sodium chloride - 4M urea.

6.7.3. Degree of Acetylation

A variety of methods have been used to determine the extent of N-acetylation of chitosan samples. It has been suggested that measurement of the primary amine present has several advantages over the measurement of the residual acetyl.[172] Firstly it is simple and accurately measured by titration and is a direct measure of the functionality of the polymer, and secondly it is a much more sensitive measure of the degree of deacetylation than is analysis for nitrogen. Broussignac has described[129] a straightforward method for the determination of the free amine content. Chitosan samples are dissolved in a known excess of acid and the solutions titrated potentiometrically. A curve is obtained with two points of inflexion, the value of the difference of these points corresponds to the quantity of acid needed for neutralisation of the primary amines. However the method has the disadvantage that the samples need to be soluble in the acid solutions, and this would be a problem with sampled with high degrees of N- acetylation. Similar methods have been used by other workers.[156,172]

From the evaluation of several methods [262]for the rapid determination of the degree of acetylation in chitosan, it has been claimed that determination of the free amine group by potentiometric and argentimetric titrations is promising. In addition, the evaluation of the mass spectra of chitosan was suggested as a technique for the determination

of the degree of acetylation.

In the present work, three techniques were used to determine the degree of N-acetylation.

5.7.3.1. Acidimetry

To about 0.5g (accurately weighed) of chitosan was added 50ml of 0.5M hydrochloric acid. The flask was then stoppered and shaken and left for 72 hours, with occasional shaking. After this time, a 10ml aliquot of the acid was taken and titrated against 0.5M sodium hydroxide with phenolphthalein as indicator. This was repeated with the other samples of chitosan. A 10ml aliquot of the original acid solution was also titrated against the standard sodium hydroxide solution. From the differences in volume of the sodium hydroxide used to neutralise the varying acid solutions, the volume of acid used to neutralise the primary amine present in each sample of chitosan could be determined.

By assuming an idealised chitosan structure made up of 10 sugar units, the equivalent weight of hydrochloric acid necessary to neutralise the 10 available amine groups present can be calculated. By subsequent replacement of each primary amine group by an acetamido group, the corresponding equivalent of hydrochloric acid can be determined. A calibration curve of percentage of amines against equivalent weight of hydrochloric acid necessary to neutralise the free amine groups in 1g of chitosan can be constructed. Thus, from the titration results, the equivalent weight of hydrochloric acid used in each can be determined and the percentage of amine groups acetylated read off from the curve.

5.7.3.2. Periodate Oxidation

The oxidation of cellulose[422] by periodate leads, under suitable conditions, to the conversion of anhydro-D-glucose units to dialdehyde units without the simultaneous occurrence of side reactions to any great extent. Determination of the oxygen consumption of the cellulose sample gives the percentage of chain units that have been oxidised. It was thought that adaption of this method to chitosan should give an estimation of the extent of acetylation. The presence of the primary amine group on C(2) would allow cleavage of the α-amino alcohol bond by the periodate whereas the presence of an acetamido group on C(2) would prevent cleavage. Thus, determination of the periodate used should give a measure of the primary amine groups present.

5.7.3.2.1. Iodometry

To about 0.5g (accurately weighed) of chitosan was added 50ml of approximately 0.1M sodium periodate. The flask was then stoppered, shaken, and left in the dark for 5 days. A sample of the periodate solution was reserved for determination of the initial concentration of periodate.

Determination of the concentration of periodate left after reaction was carried out by titration against standard sodium arsenite solution. Thus a 10ml aliquot of the periodate was pipetted and 10ml of a saturated solution of sodium hydrogen carbonate, followed by 10ml of a 10% solution of potassium iodide, was added. The liberated iodine was then titrated with sodium arsenite solution, using 'Thyodene'(starch substitute) as indicator.

By assuming an idealised chitosan structure of 10 units the equivalent weight of periodate required to cleave the α-amino alcohol units could be calculated. By subsequent replacement of the primary amine group by an acetamido group, the corresponding equivalent weight of periodate that would be required to attack the remaining C(2)-C(3) bonds can be calculated. A calibration curve of the percentage of amine groups present against the equivalent weight of periodate required at each step can be drawn and from the titration results the equivalent weight of periodate can be determined and hence the percentage of amine groups acetylated read off.

5.7.3.3. Infrared Spectroscopy

From the work carried out on the N-acylation of chitosan it seemed evident that infrared spectroscopy could be used as a simple and rapid method for determining the extent of acetylation in a given sample of chitosan. The average value of A 1655/ A 3450 (where A 1655 = the absorbance of the amide I band and A 3450 = the absorbance of the hydroxyl band) for a number of completely N- acetylated chitosan samples was found to be 1.33. Thus by calculating A1655/A3450 of a given sample of chitosan and dividing through by 0.0133 the percentage acetylation could be determined.

A very recent paper[423] has suggested a similar method for determining the degree of acetylation, but using the ratio of the absorbances at 1550 cm^{-1}, the amide II band, and 2878 cm^{-1}, the C-H band. However,

from the studies on the rate of N-acetylation (Chap.3.1.2.5.) it was found that the amide II band only appears at 1550 cm^{-1} as N -acetylation proceeds and is originally present at 1595 cm^{-1}. Thus, this gradual shift of the amide II band must have some effect on the accuracy of this method. Further, the current work has shown that the value of A1655/A3450 for fully N-aceylated chitosans is approximately constant, at 1.33, regardless of the particular N-acyl group used. Thus this ratio enables the extent of N-acylation to be determined readily whilst the A1550/A2878 ratio would not; the value of A2878 varying considerably with the chain length of the N-acyl group.

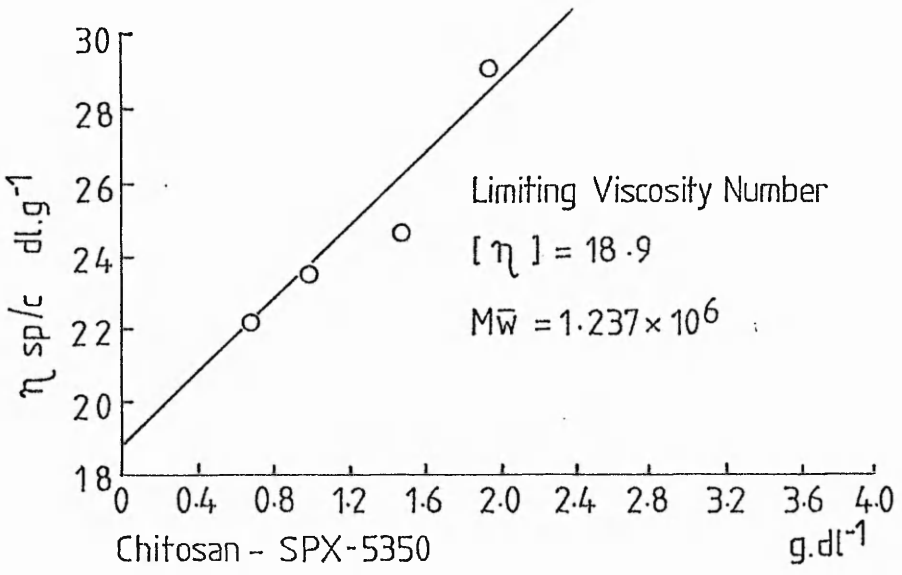
The technique was carried out as follows:-
The infrared spectrum of the sample, prepared either as a film cast from aqueous acetic acid (5%) or formic acid (85%) with subsequent treatment in methanol-ammonia to de-salt it, or as a KBr disc, was run. The absorbances of the bonds at 1655 and 3450cm^{-1}, corresponding to the amide I band and hydroxyl adsorptions respectively, were calculated by the base line method, and the ratio of the absorbance at 1655cm^{-1} (A1655) to that at 3450 cm^{-1} (A3450) determined. From a large number of completely N-acetylated chitosan samples the average value of A1655/A3450 was found to be 1.33. Hence the percentage of amine groups acetylated in a given sample

$$= \quad (A1655/A3450) \text{ of the sample} \times 100/1.33$$

The chitosan used in this work was characterised by the methods outlined. From the Limiting Viscosity Number results, Table 25, Figure 93, the molecular weight of the chitosan sample NOAA-1-74 was 1.24×10^5 and that of the

Table: 25 Limiting Viscosity Number data of the chitosan samples used

Sample	Conc, g dl^{-1}	Time, sec	Specific Viscosity, ηsp	Viscosity Number $\eta sp/c$, dl g^{-1}
Hercules Inc SPX-5350	0.198	1563.9	5.73	29.01
	0.148	1124.3	3.66	24.71
	0.099	800.7	2.32	23.48
	0.074	638.7	1.65	22.24
N.O.A.A.				
1 - 74	0.201	456.7	0.89	4.44
	0.151	392.1	0.63	4.15
	0.101	344.9	0.43	4.28
	0.075	307.4	0.28	3.65
Blank	-	241.2	-	-



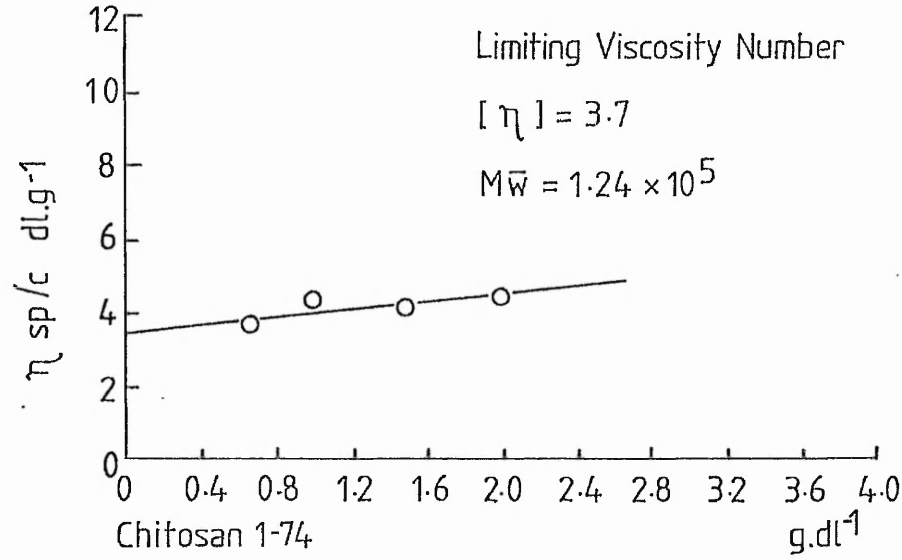


Figure 93. The Limiting Viscosity Number data for the chitosans used in this work.

(Chitosan - SPX- 5350 from Hercules Incorporated;

Chitosan - 1-74 from U.S. National Oaceanic and Atmospheric Administration).

Table 26 Extent of N-acetylation of the chitosan samples used

Acidimetry

Sample	Wt.	Wt. corrected for moisture content	Titre mls	Blank-titre	% N-acetylation
SPX-5350	0.5178	0.4595	8.3	1.6	33.5
1-74	0.5233	0.4644	8.5	1.4	38

Iodometry

Sample	Wt.	Wt. corrected for moisture content	Titre mls	Blank-titre	% N-acetylation
SPX-5350	0.5415	0.4806	0.2	10.07	13
1 -74	0.6100	0.5414	0.2	10.07	21

Infrared

Sample	Absorbance 3450 cm^{-1}	Absorbance 1655 cm^{-1}	1655/3450	% N-acetylation
SPX-5350	0.589	0.191	0.325	24.4
1 - 74	0.957	0.297	0.31	23.3

chitosan sample H-SPX-5350 was 1.237×10^6. The results of measuring the extent of N-acetylation are shown in Table 26. There is a fairly wide discrepancy shown between the methods, particularly the results from the acidimetric titrations. However, on averaging the values obtained, it would appear that chitosan, NOAA 1-74, was 27% N-acetylated and chitosan, H SPX-5350, was 23% N-acetylated.

To investigate the apparent discrepancy between the methods of determining the extent of acetylation, samples of chitosan prepared by reprecipitation from solutions of sample H-SPX-5350 were taken and N-acetylated under differing conditions, Table 27, to obtain samples of N - acetylated chitosan, which were then analysed using the three methods. The results are summarised in Table 28 and show that the

Table 27 : Conditions for N-acetylation of chitosan

Sample	Amount of acetic anhydide used	Time of reaction
1	3 mol excess	24 hours
2	1 mol-equivalent	$2\frac{1}{2}$ mins.
3	1 mol equivalent	1 min.
4	$\frac{1}{2}$ mol equivalent	1 min.
5	0	0

iodometric technique gives, on average, lower results than the other methods. This may be due to side reactions occurring which give an artificially high figure for the amount of periodate used, which in turn gives a lower figure for the degree of acetylation. It has been shown[424] that the primary reaction of cellulose with periodate at pH 10 is the same as that occurring under slightly acid conditions, namely the conversion

Table 28 Results from Samples of Chitosan, Kytex H, N-acetylated to differing degrees

Acidimetry

Sample No.	Wt., g.	Wt. corrected. for moisture content, g.	Titre, mls.	Blank titre	% N-Acetylation
1	0.5984	0.53108	9.9	--	100
2	0.5686	0.5046	9.65	0.25	87.5
3	0.5628	0.4995	9.60	0.30	84.5
4	0.5394	0.4787	9.45	0.45	77.0
5	0.5548	0.4924	8.4	1.5	34.0

Iodometry

Sample No.	Wt., g.	Wt. corrected for moisture content, g.	Titre, mls.	Blank titre	% N-Acetylation
1	0.5420	0.4810	10.05	0.22	98.5
2	0.5368	0.4764	6.6	3.67	63.5
3	0.5391	0.4785	6.4	3.87	62.0
4	0.5262	0.4670	5.45	4.82	53.0
5	0.5599	0.4969	0.2	10.07	17.0

Table 28 (Cont(d)

Sample No.	Absorbance 3450 cm^{-1}	Absorbance 1655 cm^{-1}	A1655/ A3450	% N-acetylation
1	0.855	0.942	1.1	83.0
2	0.5996	0.602	1.00	75.5
3	0.4384	0.416	0.939	70.6
4	0.5901	0.531	0.899	67.6
5	0.4973	0.164	0.329	24.7

Averages

Sample No.	Acid Titration(A)	Iodine Titration(B)	Average (A+B)	Infrared
1	100	98.5	99.3	83.0
2	87.5	63.5	75.5	75.5.
3	84.5	62.0	73.3	70.6
4	77.0	53.0	65.0	67.6
5	34.0	17.0	25.5	24.7

of anhydro-D—glucose units to dialdehyde units. Cellulose chain molecules containing such units are, however, extremely alkali-sensitive and undergo alkaline hydrolysis with the production of new end groups and soluble fragments. Whatever the precise nature of the fragments produced, they will almost certainly contain actual or potential α-glycol groups capable of further oxidation, leading to a progressively increasing consumption of periodate. Since one of the products of the periodate oxidation of chitosan is ammonia,[103] such alkaline hydrolysis would be expected to take place during periodate oxidation in unbuffered solutions. This would lead to increased consumption of periodate and give erroneously low values for the degree of N-acetylation.

The anomalous results of sample I which gives a higher figure for the degree of N-acetylation, may be due to the inability of the periodate to diffuse into the more highly N-acetylated samples.

The acid titration results are correspondingly higher than those obtained by the other methods and this may be explained on the basis of the Donnan membrane theory.[425] In the external phase $[Cl^{\ominus}]_{ext} = [H^{\oplus}]_{ext}$, whilst in the internal phase $[Cl^{\ominus}]_{int} = [H^{\oplus}]_{int} + [-NH_3^{\oplus}]_{int}$. Since $[Cl^{\ominus}]_{int} \times [H^{\oplus}]_{int} = [Cl^{\ominus}]_{ext} \times [H^{\oplus}]_{ext}$ it follows that $[H^{\oplus}]_{int} < [H^{\oplus}]_{ext}$

Thus the internal aqueous phase will have a lower concentration of hydrogen ions than will the external aqueous phase and hence titration of aliquots of the latter will indicate a lower free amine group concentration in the polymer than actually exists.

Another contributory factor to the high figures may be lack of accessibility of the polymer as indicated by its low reactivity.

However, in all cases the value given by the infrared technique was close to the average figure for the other two techniques and it seems reasonable to take this value as the correct one.

5.7.4. Chemical Tests

The intractable nature of many of the N-substituted derivatives of chitosan made estimation of the extent of reaction difficult. Thus although the solubility of chitosan in various acidic media is altered through N-substitution, this is not a definite sign of complete substitution of the primary amine groups. Application to chitosan of the Rimini test for primary amines was found to be very successful and a visual estimation of the extent of reaction could be made. Chitosan in film, reprecipitated, or flake form, was amenable to the test. It was observed that a sample of chitosan film treated under the conditions of the Rimini test then placed in the beam of an infrared spectrophotometer turned brown in colour and remained this colour for up to 100 hours, gradually returning to the original colour. However, if a sample was heated with an iron, the film went a blue-mauve in colour and remained this colour. Variation of the ketone used caused some differences in the colouration; with methyl ethyl ketone a pink coloured film was produced, whereas with cyclopentanone a brown coloured film was formed.

5.7.4.1. Rimini Test

To a sample of chitosan or substituted chitosan dispersed in distilled

water (5ml) is added pure acetone (1ml). Then 2-3 drops of aqueous sodium nitroprusside solution (1%), freshly prepared, is added and the mixture shaken. If any primary amine groups are present a violet-red colour develops in 2-3 minutes.

5.8. Preparation of Chitosan

5.8.1. Film Form

Chitosan flake (1g) was dissolved in aqueous acetic acid (5%, 100ml). The viscous solution was filtered through fine polyester cloth to remove any undissolved particles and portions were then spread on a glass plate to the required thickness by means of a glass rod. The plate was covered and the solution allowed to evaporate to dryness. After drying, the film was removed by means of a razor blade and steeped in ammonical-methanol (30%) for 16 hours to obtain salt-free chitosan film. The film was then washed in methanol, dried between filter paper, and kept until required.

5.8.2. Solid Form

Chitosan flake (10g) was dissolved in aqueous acetic acid (5%, 1l). The viscous solution was filtered through fine polyester cloth to remove any undissolved particles and the filtrate then poured into ammonical-methanol (30%, 2.5l) The white precipitate was stirred thoroughly to ensure complete precipitation, then filtered off and washed thoroughly with methanol until neutral to litmus. The product was dispersed in methanol (500ml) and chopped up finely in a Waring Blendor. The finely chopped white polymer was filtered off, washed with ether, and allowed to air dry to give a finely divided, fluffy product.

5.9. Preparation of Aliphatic Anhydrides

5.9.1. Method of Wallace and Copenhaver [414]

This method is described fully for the preparation of n-octanoic anhydride. n-Heptanoic, n-nonoic, n-decanoic and pivalic anhydrides were prepared by the same method using the appropriate acid.

One equivalent of n-octanoic acid (100ml) was mixed with 3 equivalents of acetic anhydride (212ml) in a round bottom flask fitted with a reflux condenser and the mixture heated under reflux for 8 hours. The apparatus was then set up for vacuum distillation and the excess acetic anhydride and acetic acid distilled off (48-50°C under reduced pressure). A fraction was collected at 90°C and checked for the presence of the anhydride by infrared spectroscopy. The remainder was distilled and collected as a clear, slightly yellow liquid. The product was identified as the anhydride by infrared spectroscopy and nmr.

Yield: 45g (53.3%)

ν max (thin film) 1830(C=O), 1755 (C=O)cm^{-1}

δ ($CDCl_3$) 2.4 (CH_2CO), 1.3 (CH_2) 0.9 (CH_3) ppm

% Yields of the other anhydrides prepared were -

n- heptanoic: 63.6%

n-decanoic: 43.3%

n- nonoic: 68%

pivalic : 14.5%

Similar infrared and nmr spectra were obtained.

5.9.2. Method of Gerrard and Thrush[415]

This method is described in full for the preparation of iso-butyric anhydride. n-Heptanoic, n-octanoic, n-nonoic, and 3,3-dimethyl butyric anhydrides were prepared by the same method using the appropriate acid.

Thionyl chloride (35.9g,0.5mol) in ether (50ml) was added dropwise to an ethereal solution (300ml) of iso-butyric acid (92.9ml, 1 mol) and anhydrous pyridine (80.5ml, 1mol) at -10°C. Immediately a dense white precipitate formed which was filtered off. Ether was removed from the filtrate by distillation and the ether-free residue vacuum distilled to yield iso-butyric anhydride as a clear colourless liquid. The nature of the product was confirmed by infrared and nmr.

Yield: 75ml (90.5%)

ν max (thin film) 1830 (C=O), 1755 (C=O)cm^{-1}

δ ($CDCl_3$) 2.55 (CHCO) 1.2 (CH_3) ppm

% Yields of the other anhydrides prepared were

n-heptanoic: 58%

n-octanoic: 98%

n-nonoic: 70%

3,3-dimethybutyric: 24%

Similar infrared and nmr spectra were obtained.

5.10. Preparation of Anhydrides of o-,m- and p- Toluic Acid

5.10.1. o- and m- Toluic Anhydrides

The appropriate toluic acid (20g) was refluxed with a 3 mol. excess of acetic anhydride (45ml) for 4 hours. After vacuum distillation, the crude product crystallised out on cooling, and was recrystallised from ether.

o - Toluic anhydride: yield 8.5g; (45.7%)

m.p: 37.5°, Lit,[426] 38.9°C ν max (KBr Disc) 1830 (C=O), 1755 (C=O) cm^{-1}

m- Toluic anhydride: yield 11.6g; (62.3%)

m.p. 69°, Lit,[427] 71° C ν max (K Br Disc) 1830 (C=O), 1755 (C=O) cm^{-1}

5.10.2. p- Toluic Anydride

p- Toluic acid (20g) was treated, as in the method of Gerrard and Thrush[415] with anhydrous pyridine (48.2ml) and thionyl chloride (5.3ml) in ether. The resulting product was recrystallised twice from acetone.

Yield: 5.1g, (27.4%)

m.p: 88°, Lit[428] 95° C

ν max (KBr Disc) 1830 (C=O), 1755 (C=O) cm^{-1}

5.11. Preparation of N-Acyl Chitosans

5.11.1. Film Form

A portion of salt free chitosan film, prepared as previously described, was taken and steeped in methanol (100ml) at room temperature for 16 hours. A large excess of the appropriate carboxylic acid anhydride (2.5 ml) was then added to the methanol, stirred, and the reaction mixture left until N-acylation was complete as indicated by infrared measurements. Reaction times varied with the nature of the anhydride and in some cases it was necessary to heat to reflux to obtain complete reaction. Table 29 gives the typical reaction times and the reaction temperatures.

De-O-acylation of the film was carried out by steeping in 0.5M potassium hydroxide in aqueous ethanol, (95%, 100ml) for 16 hours. The film was then removed, washed twice in methanol, and infrared spectra recorded.

ν max (thin film) 1650 (AmideI), 1545 (Amide II) cm^{-1}

Table: 29 Typical reaction times and temperatures of anhydrides with chitosan

Anhydride	Reaction Time,hrs.*	Reaction Temperature °C
Acetic	16	25°
Propionic	16	"
Butyric	"	"
Valeric	"	"
Hexanoic	24	65°
Heptanoic	"	"
Octanoic	"	"
Nonoic	"	"
Decanoic	"	"
iso-Butyric	"	"
iso-Valeric	"	"
Pivalic	"	"
3-3 Dimethylbutyric	150	60
Benzoic	120	"
o-Toluic	"	"
m-Toluic	"	"
p-Toluic	"	"

* These are only approximate times, much shorter times being required in some cases.

5.11.2 Solid Form

Chitosan flake (1g) was dissolved in aqueous acetic acid (5%,100ml) the viscous solution filtered to remove any undissolved particles and then poured into ammonical methanol (30%,250ml). The white precipitate was stirred to ensure complete precipitation, then filtered off and washed thoroughly with methanol , until pH 7.0. The product was dispersed into methanol (150ml) and chopped up finely with a Waring Blendor. The appropriate carboxylic acid anhydride(2-3mol excess per anhydro-D-glucosamine residue) was added to the dispersion in methanol and the mixture stirred for 16 hours at room temperature. In the case of the higher fatty acid anhydrides , C6 onwards,the reaction was carried out under reflux for 16 hours. The product was filtered off, washed twice with methanol then with ether and air dried.

Removal of any O-acyl groups that may have been introduced was carried out by steeping the product in 0.5M potassium hydroxide in aqueous ethanol (95%) for 16 hours at room temperature. The product was then filtered off, washed twice with methanol and then ether and air-dried.

ν max (KBr Disc) 1650 (Amide I), 1545 (Amide II) cm^{-1}.

5.12. Rates of N-Acylation Of Chitosan Film

Samples of desalted chitosan film, prepared as previously described, were placed in methanol A.R (10ml) and left in stoppered flasks, in a thermostatically controlled water bath set at the required temperature, for 16 hours. The appropriate anhydride (2.5ml) was added to each sample and the reaction continued for the required length of time. The film was then removed, placed in ice-cold methanol and the time noted. The film was then rinsed in fresh methanol, dried in an oven at 60°C and then placed in a desiccator, until the infrared spectra was recorded. The extent of N-acylation of each sample was then determined, as described before, and corrected by subtraction of the average value of N-acetylation in a number of blank samples.

5.13. Attempted O-Acetylation of N-Acyl Chitosan in Solid Form

The procedure is described for the preparation of N-hexanoyl — O-acetyl chitosan. A similar procedure was used for the preparation of N-butyryl-O-acetyl, N-decanoyl— O-acetyl and N-hexanoyl-O-propionyl chitosans.

N-hexanoyl chitosan (0.5g) was dispersed into a solution of glacial acetic acid (20ml), acetic anhydride (10ml) and perchloric acid (85%,0.2ml) and left for 72 hours at room temperature, with occasional stirring. The product was filtered off and washed exhaustively with water, until neutral to litmus, then with methanol and ether, and air dried to afford a white granular product (0.45g).

ν max (KBr disc) 1740 (C=O), 1230 (C-O) cm^{-1}.

Similar infrared spectra were obtained for the other derivatives

5.14. Preparation of Schiff's Base Derivatives of Chitosan

5.14.1. Film Form

A portion of salt-free chitosan film, prepared as described, was taken and steeped in methanol (100ml) at room temperature for 16 hours. A large excess of the appropriate aldehyde in methanol was then added and the reaction mixture left for 24 hours at room temperature. The film was then removed, washed twice in methanol and the infrared spectra recorded.

ν max (thin film) 1625 (C=N-) cm^{-1}

5.14.2. Solid Form

The standard procedure is described in detail for the preparation of N-salicyclidene chitosan. A similar process was used to prepare Schiff's base derivatives of chitosan with benzaldehyde, p-hydroxybenzaldehyde, p-nitrobenzaldehyde, p-methoxybenzaldehyde and pyridine-4-aldehyde. Reprecipitated chitosan (1g) was dispersed in methanol and a 3 mol excess of salicylaldehyde (2.5ml) added. The mixture was then stirred for 16 hours at room temperature, the product filtered off, washed with methanol and extracted with methanol in a Soxhlet for 4 hours. The yellow powder was then washed with ether and air dried.

5.15. The Preparation of Keto-imine Derivatives of Chitosan

5.15.1. The Benzoylacetone Adduct of Chitosan

Chitosan flake (1g) was dissolved in aqueous acetic acid (5%,100ml) and methanol (150ml) added with stirring, followed by a 3 mol. excess of benzoylacetone (3g) dissolved in methanol (10ml). After standing for 24 hours at room temperature the resultant gel was chopped up finely in a Waring Blendor, washed twice with methanol and ether, then air dried to give a fine yellow product (1.1g).

ν max (thin film) 1605 (C= O and phenyl ring) cm^{-1}.

5.15.2. The Acetylacetone Adduct of Chitosan

The method was repeated as for the benzoylacetone derivative but no gelation occurred. The solution was therefore poured into ether (500ml) and the reprecipitated polymer filtered off. The product was washed twice with methanol and ether and air dried.

ν max (thin film) 1605 (C = O) cm^{-1}.

5.16. The Preparation of N-Salicyclidene O-Acetyl Chitosan

N-salicyclidene chitosan (2.5g) dispersed into a solution containing pyridine (100ml), acetic anhydride (20ml) and triethylamine (5ml) was placed on a water bath for 6 hours. A clear, viscous, yellow-brown solution formed after 16 hours and this was poured into ether (500ml) to reprecipitate the polymer. The precipitate was then thoroughly washed with methanol and ether, and air dried to give a pale yellow, powdery product (2.7g).

ηinh = 2.36 dlg-1

ν max (thin film) 1745 (C=O), 1225 (C-O) cm^{-1}

5.17. Acetylation of Keto-imine Derivatives of Chitosan

Samples (1g) of the appropriate chitosan derivative were dispersed in pyridine (50ml) and acetic anhydride (10ml) and left to stand at room temperature for 120 hours. The gelled products were poured into ether and then filtered off, washed twice with methanol and ether, and air dried.

The O-acetyl derivative of the benzoylacetone adduct (0.95g) was pale yellow in colour.

ν max (thin film) 1735 (C=O), 1230 (C-O) cm^{-1}

The O- acetyl derivative of the acetyl acetone adduct (0.9g) was beige in colour.

ν max (thin film) 1740 (C=O) 1230 (C-O) cm^{-1}

5.18. Preparation of N-Acetyl Di-O-Acetyl Chitosan

The benzaldehyde adduct of chitosan (5g) was placed in pyridine (100ml), acetic anhydride (15ml) and triethylamine (1ml) and left to stand at room temperature for 96 hours. The gelatinous clear product was then poured into ether (500ml) filtered, washed well with ether, and dried. The off-white product was then placed in methanol (150ml) and acetic anhydride (15ml) at room temperature for 24 hours. After filtration, the product was washed well with methanol and ether and dried. It was then finally treated with a solution of glacial acetic acid (100ml) acetic anhydride (15ml) and perchloric acid (85%, 0.2ml) for 24 hours. The N-acetyl-di-O-acetyl chitosan was filtered off, washed well with water, methanol, and ether, and dried in an oven at 60°C. The product formed was off-white in colour.

Yield 5.7g ηinh $\triangleq$ 25dlg^{-1}

ν max (thin film) 3300, (N-H) 1740 (C=O), 1665 (Amide I), 1545 (AmideII), 1230 (C-O) cm^{-1}.

N-acetyl-di-O-acetyl chitosan prepared from low molecular weight chitosan

η inh = 2.24 dlg^{-1}

5.19.

Preparation of aromatic carbamates of the Schiff's base derivatives of chitosan

The salicylaldehyde and benzaldehyde derivatives of chitosan were dried by slurrying in toluene followed by azeotropic distillation until all the water was removed. The solvent and appropriate reagent were then added to the N-substituted chitosan and residual toluene without changing the flask.

5.19.1.

N-salicyclidene chitosan α-napthyl carbamate and phenyl carbamate

Dried N-salicyclidene chitosan (1g) prepared from low molecular weight chitosan was dispersed in anhydrous pyridine (15ml) and a 2-3 mol. excess of the appropriate aromatic isocyanate in anhydrous pyridine (15ml) added. The flask was fitted with a drying tube (calcium chloride) and placed on a water bath at 70°C for 6 hours. The clear, coloured solution that formed was poured into denatured alcohol (500ml) and a pale yellow precipitate formed. This was recovered by filtration then re-dissolved in pyridine (50ml) and reprecipitated.

N-salicyclidene α-napthyl carbamate: yield: 2g ; ηinh = 1.05 dlg^{-1}.

ν max (thin film) 1735 (-C(=O)-N), 1210 (NC-O) cm^{-1}.

N-salicyclidene phenyl carbamate: Yield 1.6g ; η inh = 1.49 dlg^{-1}

ν_m(thin film) 1730 (-C(=O)-N) 1215 (N-C-O) cm^{-1}.

5.19.2.

N-benzylidene chitosan α-napthyl carbamate and phenyl carbamate

The α-napthyl and phenyl carbamates were prepared using the same method

as for the analogous N-salicyclidene chitosan derivatives

N-benzylidene chitosan α-napthyl carbamate : Yield: 1.4g;

η inh= 1.34 dlg^{-1}; ν_{max}(thin film) 1735 (-C(=O)-N), 1210 (N-C-O) cm^{-1}.

N-benzylidene phenyl carbamate: Yield: 1.5g ηinh = 2.56 dlg^{-1}

ν_{max}(thin film) 1725 (-C (=O)-N) , 1215 (N-C-O) cm^{-1}.

5.20.

Preparation of the α-napthyl carbamate and phenyl carbamate derivatives of chitosan

Dried, low molecular weight chitosan (1g) was dispersed in anhydrous pyridine (25ml) and a 2-3mol. excess of the appropriate aromatic isocyanate in anhydrous pyridine (25ml) added. The flask, fitted with a drying tube (calcium chloride) was placed on a water bath at 70°C and kept at this temperature for 120 hours. Clear, light tan coloured, viscous solutions were formed in both cases. The products were precipitated out by pouring the solutions into methanol (500ml) and filtered off. The recovered polymers were then dissolved in pyridine (50ml), filtered to remove insoluble by-products, and reprecipitated.

Chitosan α-napthyl carbamate: Yield: 1.9g, ηinh = 1.79 dlg^{-1}

ν max (thin film) 3250 (n-H) 1720 (-C(=O)-N), 1210 (N-C-O) cm^{-1}.

Chitosan phenyl carbamate: Yield: 1.8g , ηinh = 2.56dlg^{-1}

νmax (thin film) 3350 (N-H), 1725 (-C(=O)-N), 1220 (N-C-O) cm^{-1}.

5.21. Preparation of N-Acyl Chitosan Gels

Chitosan flake of high molecular weight, (0.6g) was dissolved overnight in aqueous acetic acid (5%, 60ml). The solution was filtered to remove any undissolved particles and then methanol (150ml) added with stirring. The beaker containing the polymer solution was placed in a thermostatically controlled water-bath to bring the solution to the required temperature. After the desired temperature had been reached, a 3mol. excess of the appropriate carboxylic acid anhydride (based on the amine content) was added, with stirring,to the solution. Stirring was maintained for 2 minutes and then the solution left to allow the gel to form.

5.22. The isolation and characterisation of partially N-acylated chitosan gels

To enable a correlation to be made between the degree of acylation and gelation it was necessary to isolate a partially N- acylated chitosan gel, at a given time, and characterise the product and compare this with the viscosity of the gel at this point.

Thus, after the required time, the viscous/gelatinousproduct was poured into a large excess of ether (1l) and chopped up. The liquid was filtered off and the residue placed in fresh ether (1l). After filtration the product was placed in a denatured alcohol (500ml) and then finely chopped in a Waring Blendor. The finely divided product was filtered, washed well with denatured alcohol, and dried.

Samples of the dried product were dissolved in either aqueous acetic acid

5% or formic acid (85%) and films cast from the solutions, dried, removed from the glass plate and placed in methanol/ammonia solutions for 16 hours to deprotonate the amine groups. The films were then rinsed well in methanol, dried, and the infrared spectrum of each film recorded.

The absorbance of the amide I band at 1655 cm^{-1}, (A1655), was determined and divided by the absorbance of the hydroxyl band at 3450cm^{-1}, (A3450). The corrected figure was then divided through by 1.33 (the value of A1655/A3450 for a fully N-acylated product) and expressed as a percentage of the fully N - acylated product. (See Chap. 5.7.3.3.)

References

1 Tracey, M.V., Reviews Pure and Appl. Chem. Royal Aust. Chem. Inst., 1957, 7,(1),3.

2 Foster,A.B. and Webber, J.M., Adv. in Carbohydr. Chem., 1960, 15,371.

3 Be Miller, J.N., Methods Carbohdr. Chem., 1965, 5,103.

4 Muzzarelli, R.A.A., "Natural Chelating Polymers", Pergamon Press, Oxford, 1973.

5 Muzzarelli, R.A.A., "Chitin", Pergamon Press, Oxford, 1977.

6 "The Insect Integument", Hepburn, H.R., (Ed.), Elsevier,Amsterdam, 1976.

7 Plisko, E.A., Nud'ga, L.A. and Danilov, S.N., Russian Chemical Reviews, 1977, 46,(8),764.

8 "Proceedings of the First International Conference on Chitin/Chitosan", Muzzarelli, R.A.A. and Panser, E.R., (Eds), MIT Sea Grant Report,MITSG 78-7, 1978.

9 Bracannot, H., Ann chim (Paris), 1811, 79, 265.

10 Odier, A., Mem soc histoire nat., Paris, 1823, 1, 29.

11 Van Wisselingh, G., Jahrb Wiss Bot., 1898, 31,619.

12 Wester, D.H., Geogr. Biol Terre, 1910, 28, 53.

13 Von Wettstein, F., sitzber, Akad Wiss. Wien., 1921, 130, 3.

14 Levene, P.A. and Lopez-Suarez, J., "Hexosamines and Mucoproteins", Longmans, London, 1925.

15 Von Franciis, ., "Chitins, Lichenins and Cellulose", Zurich, 1930.

16 Muzzarelli, R.A.A., ref. 5, p.6.

17 Jeauniaux, C., "Chitine et chitinolyse", Masson, Paris, 1963.

18 Jeauniaux, C., Bull.Soc. Chim. Biol., 1965, 47, 2267.

19 Muzzarelli, R.A.A., ref. 5, p.8.

20 Muzzarelli R.A.A., ref.4, p.84.

21 Rudall, K.M., Symposia Soc. Exptl. Biol., 1955,9,49.

23 Richards, A.G., "The Integument of Arthropods" Univ. of Minnesota Press, Minneapolis, Minn, 1951.

24 Rockstein, M., "The Physiology of Insecta", Academic Press, New York, 1974.

25 Jeauniaux, C., ref. 8, p.5.

26 Anderson,C.G., de Pablo, N. and Romo. C.R, ref. 8, p.54.

27 Mori, M. and Yasuda, S., Nippon Suisan Jabushiki Kaisha Chuo Kenkyusho Hokoku, 1976, 11, 1.(C.A., 1977, 86, 136622n).

28 Yanase, M., Tokaiku Suisan Kenkyusho Kenkyu Hokoko, 1975, 83, 1. (C.A., 1976, 85, 17428y).

29 Pryor, M.G.M., "Comparative Biochemistry", (Florkin, M. and Mason, H.S., Eds.) , Academic Press, London, 1962, 4, 371.

30 Dennel, R., "The Physiology of Crustacea", (Waterman, T.H., Ed.), Academic Press, London, 1960, 1, 447.

31 Rudall, K.M., Adv. Insect. Physiol., 1963, 1, 257.

32 Wigglesworth, V.B., Quart J. Microscop. Sci., 1933, 76, 269.

33 Wigglesworth, V.B., Biol Revs Cambridge Phil Soc., 1948, 23, 408.

34 Pryor, M.G.M., Proc. Roy. Soc., (London), 1940, B128, 378.

35 Pryor, M.G.M., Russell, P.B. and Todd, A.R., Nature, 1947, 159, 399.

36 Dennell, R., Proc. Roy. Soc. (London), 1947, B134, 79.

37 Fraenkelm G., and Rudall, K.M., Proc. Roy. Soc. (London), 1947, B134,111.

38 Fraenkel, G., and Rudall, K.M., Proc. Roy. Soc (London), 1940, B129, 1.

39 Dennell, R., Proc. Roy. Soc. (London), 1947,B134, 485.

40 Lafon, M., Ann. Sci. Nat. Zool. et biol. animale,1943, 11, 5, 113.

41 Brimacombe, J.S., et al, "Mucopolysacc harides", Elsevier, Amsterdam, 1964.

42 Karuppaswamy, S.A., ref. 8, p.437.

43 Lafon, M., Bull. Inst. Oceanogr., 1948, 45, 1.

44 Neville, A.C., Adv. Insect Physoil.,1967, 4, 213.

45 Varagnolo, S., Arch. Oceanogr. Limnol.,1968, 15S, 83.

46 Molteno, C,C., S. African Ind. Chem.,1951, 5,2.

47 Nicolo Acosta, O.M., and Henriques Rodriguez, R.D., Prikl. Biokhim. Mikrobiol.,1977, 13,(4), 610. (C.A. 1977, 87, 197638 q)

48 Brisou. J., Tysset, C., De Rautlin de la Roy, Y., Curcier R and Moreau, R., Ann. Inst. Pasteur, 1964, 106, 469.

49 Vinogradov, A.P., "Elementary Chemical Composition of Marine Organisms", ANUSSR, Moscow, 1944.(referred to in ref. 4, p.90).

50 Muzzarelli, R.A.A., ref. 4, p.92. (references citied therein).

51 Sharon, N., "The amino sugars", (Bataza. E.A. and Jeanloz, R.W.,Eds.), Academic Press, London, 1965.

52 Ruiz-Herrera , J., ref. 8, p.11.

53 Kharkerich, E.S. and Koval, E.Z., Ukr. Bot. Zh.,1977, 34,(3), 309. (C.A. 1977, 87, 98496k).

54 Foster, J.W., "The Chemical activities of fungi", Academic Press, New York, 1949.

55 Tracey, M.V., "Modern methods of plant analysis", (Paech, K. and Tracey, M.V., Eds.), Springer Verlag, Berlin, 1955,2,264.

56 Foster, A.B., and Stacey M., "Encyclopaedia of plant physiology", (Ruhland, W., Ed.), Springer Verlag, Berlin, 1958.

57 Roelofsen, P.A. and Hoette, I., Antonie van leewenhoek, J. Microbiol. Serol., 1951, 17, 297.

58 Frey, R., Ber. Schweiz. Botan. Ges., 1950, 60, 199.

59 Carbonell, L.M., Kanetsura, F., and Gil, F., J. Bacteriology, 1970, 101, 636.

60 Beran, K., Holan, Z., and Baldrian, J., Folia Microbiol., 1972, 17, 322.

61 Bacon, J.S.D., Davidson, E.D., Jones, D., and Tay or, I.F., Bioch. J., 1966, 101, 36C.

62 Cabib, E., Ulaine, R. and Bowers, B., Curr. Topics. Cell. Regul., USDHEW-NIH, 1975, 8,1.

63 Aronson, J.M., and Machlis, L., Am. J. Botany,1959, 46, 292.

64 Kreger, D.R., Biochem. Biophys. Acta, 1954, 13, 1.

65 Bluementhal, H.J. and Roseman, S., J. Bacteriol, 1957, 74, 222.

66 Koval, E.Z., Tr' S'ezda Mikrobiol., Ukr 4th ,(Zalula, D.G., Ed.), "Naukova Dumka", 1975,46.

67 Galum, M., Braum, A., Frensdorff, A. and Galun, E., Arch. Microbiol., 1976, 108, (1), 9.

68 Dietrich, S.M.C., An. Acad. Bras. Genc., 1975, 47,(1), 155.

69 Orpin, C., J. Gen. Microbiol., 1977, 99,(1), 215.

70 Diehl, J.M. and Van Iterson, G., Kolloid Z., 1935, 73,142.

71 Meyer, K.H., and Wehrli, H., Helv. Chim. Acta., 1937,20, 353.

72 Van Iterson, G., Meyer, K.H. and Lotmar, W., Rev. Trav. Chim., 1936, 55,61.

73 Heyn, A.N.J., Protoplasma, 1936, 25, 372.

74 Heller, K., Claus, L., and Huber, J., Z. Naturforsch, 1959, 14-B, 476.

75 Karrer, P. and Hoffmann, A., Helv. Chim. Acta., 1929, 12,616.

76 Karrer, P. and Von Francois, G., Helv. Chim. Acta., 1929, 12, 986.

77 Zechmeister, L. and Toth, G., Physiol. Chem., 1934, 223, 53.

78 Manskaya, S.M. and Drozdova, T.V., "Geochemistry of organic substances," Pergamon Press, Oxford, 1968.

79 Children, J.G., Zool. J., 1824, 1, 101.

80 Ledderhose, G., Ber. 1876, 9, 1200.

81 Ledderhose, G., Hoppe-Seiler. Z., Physid.Chem., 1878, 2,213.

82 Brach, H., Biochem.Z., 1912,38. 468.

83 Gilson, E., Bull.Soc.Chim., Paris, 1894,3,1099.

84 Frankel,S. and Kelly,A., Sitzber Akad.Wiss.Wien. Math-Naturw. Kl. Abt., 1901, 11B, 110.

85 Tiemann, F., Ber., 1886, 19, 49.

86 Fischer,E. and Leuchs, H., Ber., 1903, 36, 24.

87 Irvine, J.C. and Hynd, A., J. Chem. Soc., 1912, 101, 1128.

88 Irvine,J.C. and Hynd,A., J. Chem. Soc., 1914, 105, 698.

89 Irvine, J.C., McNicoll,D. and Hynd, A., J. Chem. Soc., 1911, 99, 250.

90 Levene, P.A., "Hexosamines and mucoproteins", Longmans Green and Co., London, 1925.

91 Haworth, W.N., Lake, W.H.G., and Peat, S., J. Chem. Soc., 1939,271.

92 Peat,S., Ann. Repts. on Progr. Chem., Chem. Soc., London, 1937,34,289.

93 Bergmann, M., Zervas, L. and Silberweit, E., Ber. 1931, 64, 2436.

94 Zeichmeister, L. and Toth,G., Ber., 1931, 64, 2C28.

95 Zeichmeister, L. and Toth, G., Forlscher Chem. org. Naturstoffe., 1937,2,212.

96 Purchase, E.R. and Braun, C.E., Org. Synth., 1946, 26, 36.

97 Matsushima, S., Japan 175, 981, 1948. (C.A. 1950, 45, 7141f)

98 Zilliken, F., Braun, G.A., Rose C.S. and Gyorgy, P.G., J.Am. Chem.Soc., 1955, 77, 1296.

99 Horowitz, S.T., Roseman S. and Bluementhal, H.J., J.Am. Chem. Soc., 1957, 79, 5046.

100 Horowitz, S.T., Bluementhal, H.J., Seppla, R. and Roseman, S. Federation Proc., 1955, 14, 299.

101 Barker, S.A., Foster, A.B., Stacey, M. and Webber, J.M., Chem and Ind., (London), 1957, 208.

102 Barker, S.A., Foster, A.B., Stacey, M and Webber, J.M., J. Chem. Soc., 1958, 2218.

103 Jeanloz, R. and Forchielli, E., Helv. Chim. Acta., 1950, 33, 1690.

104 Aminoff, D., Morgan, W.I.J. and Watkins, W.M. Biochem. J.,1952,51,379.

105 Kuhn, R., Gauhe, A. and Baer, H.H., Chem. Ber., 1954,87,1138.

106 Jeanloz, R. and Tremege, M., Federation Proc., 1956, 15,282.

107 Juhn,R. and Jruger, G., Chem. Ber., 1956, 89, 1473.

108 Foster, A.B., Martlew, E.F. and Stacey, M., Chem and Ind., (London), 1953, 825.

109 Hertzog, R.O., Naturwissenschaften, 1924, 12, 995.

110 Gonell, H.W., Z. Physoil. Chem., 1926, 152, 18.

111 Meyer, K.H. and Mark, H., Ber., 1928, 61, 1936,

112 Meyer, K.H. and Pankow, G.W., Helv. Chim. Acta, 1935, 18, 589.

113 Rudall, K.M. and Kenchington, W., Biol. Rew., 1973, 48, 597.

114 Dweltz , N.E., Bioch. Biophys. Acta, 1961, 51, 283.

115 Blackwell, J., Biopolymers, 1969, 7, 28.

116 Rudall, K.M., J. Polym. Sci. C., 1969, 28, 63.

117 Lotmar, W. and Picken, L.E.R., Experientia, 1950, 6, 58.

118 Chandler, H.D., and Hepburn, H.R., ref. 8, p.144.

119 Calstrom, D., J. Biophys. Bioch. Cytol., 1957, 3, 669.

120 Ramakrishnan, C. and Prasad, N., Bioch. Biophys. Acta , 1972, 261,123.

121 Prasad, N. and Ramakrishnan, C., Indian J. Pure Appl. Phys., 1972,10,501.

122 Blackwell, J., Minke, R. and Gardner, K.H., ref. 8 p.108.

123 Allan, G.G., Fox, J.R. and Kong, N., ref. 8, p.64.

124 Hackman, R.H., Austrl. J. Biol. Sci., 1954, 7, 168.

125 Whistler, R.S. and Be Miller, J.N., J. Org. Chem., 1962, 27, 1161.

126 Foster, A.B. and Hackman, R.H., Nature, 1957, 180, 40.

127 Takeda, M. and Abe, E., Norisho Suisan Koshusho Kenkyu Hokoku, 1962, 11, 339. (C.A. 1963, 59, 11795g).

128 Takeda, M. and Katsuura, H., Suisan Daigaku Kenkyu Hokoku, 1964, 13,109 (C.A., 1965, 62, 12067).

129 Broussignac, P., Chim. Ind. Genie Chim., 1968, 99, 1241.

130 Rigby, G.W., U.S. 2,072,771, 1936.

131 Blumberg, R., Southall, C.L., Van Rensburg, N.J. and Volkman, O.B., J. Sci. Food Agric., 1951, 2,571.

132 Lovell, R.T., Lafleur, J.R and Hoskins, F.H., J. Agr. Food Chem., 1968, 16, 204.

133 Kishi, Y., Japan 157, 624, 1943. (C.A. 1949, 43, 8417h)

134 Lagunov, L.L., Kryuchkova, M.I. and Nikolaeva, N.E., Fr. 2,024,710, 1970 (C.A., 1971, 74, 110570w).

135 Peniston, Q.P., Johnson, E.L., Turrill, C.N. and Hayes, M.L., Eng. Bull. Purdue Univ. Eng. Ext. Serv., 1969, 135,(1), 402.

136 Johnson, E.L. and Peniston, Q. , ref. 8, p.80.

137 Peniston, Q.P., and Johnson, E.L., U.S. 4,066, 735, 1977.

138 Bade, L.M., Bioch. Biophys. Acta, 1974, 372 , 474.

139 Molano, J., Duran, A. and Cabib, E., Anal. Biochem., 1977, 83,(2),648.

140 Thompson, A., Wolfrom, M.L. and Pacsu, E., Methods Carbohydr. Chem, 1965, 2, 215.

141 Danishefsky, I., Methods Carbohydr. Chem., 1965, 2, 407.

142 Winterstein, E., Ber., 1894, 27, 3113.

143 Hoppe-Seyler, F., Ber., 1894, 27, 3329; 1895, 28, 82.

144 Von Forth, O. and Russo, M., Beitr. Chem. Physoil. Pathol., 1906,8, 163.

145 Lowy, E., Biochem Zeitschr., 1909, 23, 47.

146 Clark, G.L. and Smith, A.F., J. Phys. Chem., 1936, 40, 863.

147 Darmon, S.E. and Rudall, K.M., Disc. Fara. Soc., 1950, 9, 251.

148 Wester, D.H., Arch. Pharm., 1909, 247, 282.

149 Madhavan, P. and Ramachandran, N.K.G., Fish Technol., 1974, 11, 50.

150 Moorjani, M.N., Achutha, V. and Khasim, D.I., J. Food Sci. Technol., 1975, 12, 187.

151 Lusena, C.V. and Rose, R.C., J. Fish Res. Board Can., 1953, 10, 521.

152 Horton, D. and Lineback, D.R., Methods Carbohydr. Chem., 1965, 5, 403.

153 Nud'ga, L.A., Plisko, E.A. and Danilov, S.N., Zhur. Obshch. Khim., 1970, 41, 2555. (C.A. 1972, 76, 127305c).

154 Numazaki, S., Oono, K., Kito, H. and Nakano, S., Japan Kokai, 75, 126,784, 1975. (C.A., 1976, 84, 76100n).

155 Sannan, T., Kurita, K. and Iwakura, Y., Die Makromol. Chemie, 1975, 176, 1191.

156 Sannan, T., Kurita, K. and Iwakura, Y., Makromol. Chem., 1976, 177, 3589.

157 Wolfrom, M.L. and Shen Han, T.M., J.Am. Chem. Soc., 1958, 81, 1764.

158 Wolfrom, M.L., Maker, G.G. and Chaney, A., J. Org. Chem., 1957, 23, 1990.

159 Fujita, T., Japan, 7,013,599, 1970. (C.A., 1970, 73, 56369b).

160 Peniston, Q.P. and Johnson, E.L., U.S. 3,862, 122, 1975.

161 Danilov, S.N. and Plisko, E.A., Zhur. Obshch. Khim., 1954, 24, 1761. (C.A. 1955, 49, 3280)

162 Schulze, P. and Kunike, G., Biol. Zentr., 1923, 43, 556.

163 Schmid, L., Waschkav, A. and Ludwig, E., Montash, 1928, 49, 107.

164 Von Weimarn, P.P., Ind. Eng. Chem., 1927, 19, 109.

165 Austin, P.R., U.S., 3, 892, 731, 1975.

166 Austin, P.R., U.S., 3, 879, 377, 1975.

167 Austin, P.R., Ger., 2,707,164,1977. (C.A. 1978, 88, 63496d)

168 Rutherford, F.A and Austin, P.R., ref. 8, p.182.

169 Capozza, R.C., Ger. 2,505,305,1975.

170 Allan, G.G., Johnson P.G., Lay, Y.Z. and Sarkanen, K.V, Chem. Ind., 1971, 127.

171 Lee, V.F., Univ. Microfilms, Ann Arbor, 1974, 74/29446.

172 Filar, L.J. and Wirick, M.G. ref. 8, p.169.

173 Hayes, E.R. Davies, D.H., and Munroe, V.G., ref. 8, p.103.

174 Hackman, R.H. and Goldberg, M., Carbohydr. Res., 1974, 38, 3545.

175 Muzzarelli, R.A.A., Ferrero, A. and Pizzoli, M., Talanta, 1972, 19,1222.

176 Nagasawa, K., Tohura, Y., Inoue, Y. and Tanoura, N., Carbohydr. Res., 1971, 18, 95.

177 Wu, A.C.M., and Bough, W.A., ref. 8 p.88.

178 Wu, A.C.M., Bough, W.A., Conrad, E.C. and Alden, K.E. Jr., J. Chromatogr., 1976, 128, (1), 87.

179 Muzzarelli, R.A.A. ref. 5, p.62.

180 Morawetz, H., "Macromolecules in Solution", Chap.7., Interscience Publishers, 1965.

181 Danilov, S.N. and Plisko, E.A., Zhur. Obshch. Khim., 1958, 28, 2217, (C.A. 1959, 53, 3747i).

182 Okafor, N., Biochim. Biophys. Acta, 1965, 101, 193.

183 Brine, C.J. and Austin, P.R., A.C.S. Sympy. S., 1975, 18, 505.

184 Yaku T. and Yamashita, I. Japan, 73, 19, 213, 1973. (C.A.1974,80,722 91v)

185 Thor, C.J.B., U.S., 2, 168, 375, 1939.

186 Thor, C.J.B., and Henderson, W.F., Am. Dyestuff Rep., 1940, 29, 488.

187 Zimmermann, A., Ger. 856,146, 1952.

188 Joffe, I. and Hepburn, H.R., J. Mats. Science, 1973, 8, 1751

189 Kunike, G., J. Soc. Dyers Colourists, 1926, 42, 318.

190 Izard, E.F., Ger. 712, 328, 1941.

191 Mima, S., Yoshikawa, S. and Miya, M., Japan Kokai, 75, 130, 870, 1975. (C.A. 1976, 84, 752 39c).

192 Nud'ga, L.A., Plisko, E.A. and Danilov, S.N., Zhur. Prikl. Khim., 1974, 47, 872. (C.A. 1974, 81, 4164b).

193 Kochnev, I.G., Molodkin, V.M., McHedlishuili, B., V. and Plisko, E.A., Zhur. Prikl. Khim., 1973, 46(5), 1141, (C.A. 1974, 80 6884g).

194 Muzzarelli, R.A.A., Isolati, A. and Ferrero, A., Ion Exchange Membranes, 1974, 1, 193.

195 Averbach, B.L., U.S. N.T.I.S., P.B., Rep. 1975, PB-246876.

196 Averbach, B.L., ref. 8, p.199.

197 Averbach, B.L., personnel communication.

198 Du Pont de Memours., Co., Brit. 458,839,1936

199 De Chirico, A. and Gallo, E., Ric. Sci., 1958,28,1856.

200 Matsuda, H. and Shinoda, K., Japan, 154, 182, 1975. (C.A.1976,84,155721u)

201 Matsutani, H., Kankichi, K., Shinoda, K., Nakagawa,T., and Koike, M., Japan Kokai, 74, 91,079,1974. (C.A. 1975,82,173048t)

202 Arai, S. and Akiya, F., Japan Kokai, 74, 115, 084, 1974. (C.A., 1975 82, 173051p)

203 Muzzarelli, R.A.A., Ref. 5, p.76.

204 Klenova, N.I. and Plisko, E.A., Zhur. Obshch, Khim, 1957, 27,399. (C.A. 1958, 52, 14876h)

205 Karrer, P., Koenig, H. and Usteri, E., Helv. Chim. Acta., 1943, 26,1296.

206 Cushing, I.B. Davis, R.V., Kratovil, E.J. and MacCorquodale, D.W. J.Am. Chem. Soc., 1954, 76, 4590.

207 Pulver, R., U.S. 2,612,499, 1953.

208 Jones, R.V., U.S. 2,689,244,1954.

209 Fuhrt,O. and Sollas, B.J., Chem. Zentr., 1907,2,910.

210 Schorigin, P., and Hait, E., Ber, 1934, 67B, 1712.

211 Senju, R. and Okimasu, S., Nippon Nogei, Kagaku, Kaishi, 1950,23,432. (C.A. 1952, 46, 9018i).

212 Noguchi, J., Arato, K. and Komai, T., Koggo Kagaku Zasshi, 1969, 72, (3), 796 (C.A. 1969, 71, 952 82W)

213 Kurita, K., Sannan, T. andIwakura, Y., Makromol Chim., 1977,178,2595.

214 Schorigin, P. and Makarova - Semljanskaja, N., Ber., 1935, 68, 969.

215 Okujama, T., Protein Nucleic Acid Enzyme, 1970,15,47.

216 Okimasu,S.,J.Agric. Chem. Soc., Japan, 1956,30,36. (C.A. 1958,52,19344g).

217 Shimahara, K., Nagahata, N. and Takiguchi, Y., Seikei Daigaku Koyakubu Kogaku Okoku, 1974,18,1371.(C.A. 1975,82,166329g).

218 Danilov, S.N. and Plisko, E.A., Zhur. Obshch. Khim., 1954, 24,2071 (C.A. 1955, 49, 14650f).

219 Okimasu, S., Nippon Nogei Kagaku Kaishi, 1958, 32, 383, 387,471. (C.A. 1959, 53, 21049a).

220 Danilov, S.N. and Plisko, E.A., Zhur. Obshch. Khim., 1961, 31,469 (C.A. 1961, 55, 23356f).

221 Plisko, E.A. and Danilov,S.N., Mat. Uses. Konf. po.Probl."Khim Obmen Ugledov", 3rd Moscow, 1963, 141. (referred to in ref: 7)

222 Miyazaki, T. and Matsushima, Y., Bull. Chem. Soc., Japan, 1968, 41, (11),2723. (C.A. 1969, 70, 58188d).

223 Hayashi, K., Imoto, T. and Funatsu, M., J. Bioch., 1968,63,550.

224 Trujillo, R., Carbohydr. Res., 1968, 7,483.

225 Okimasu, S., J. Agric. Chem. Soc., Japan, 1958, 32,303.

226 Schorigin,P. and Hait,E.,Ber.,1935, 68, 971.

227 Plisko,E.A. and Danilov,S.N., "The chemistry and metabolism of carbohydrates" Izd. Nauka, Moscow, 1965, (referred to in ref.7)

228 Noguchi,J.,Tokura,S. and Nishi,N., ref.8,p.315.

229 Danilov,S.N.,Plisko,E.A. and Pyaivien,E.A., Izd. Akad. Nauk., USSR, Ord. Khim. Nauk., 1961,1500. (referred to in ref.7).

230 Bridgeford,D.J.,Turbak,A.F. and Burke,N.I., US 3,649,346 , 1971.

231 Neely,W.B., US 3,133,856,1964.

232 Gyorgy,P., Kuhn,R. and Zilliken,F., US 2,783,148, 1957.

233 Austin,P.R., US 4,063,016, 1977.

234 Harmon,R.E., De,K.K. and Gupta,S.K., Carbohydr. Res.,1973,31,407.

235 Wolfrom,M.L.,Shen Han,T.M. and Summers,C.G., J.Am.Chem.Soc.,1953,75,1519.

236 Foster,A.B. and Huggard,A.J., Adv. Carbohydr. Chem., 1955, 10, 363.

237 Upjohn Co. Ltd., Brit. 746,870, 1956.

238 Hoffman La Roche Co., Swiss. 326,792, 1958.(C.A. 1959,52,18245).

239 Nagasawa,K. and Tanoura,N., Chem. and Pharm. Bull.,(Japan),1972,20, 157.(C.A.1972,76,113456).

240 Warner,D.T. and Coleman,L.L., J.Org.Chem., 1958, 23, 1133.

241 Hoffman La Roche Co., US 2,831,851, 1958.

242 Zimmermann,A., Swed. 136,717, 1952. (C.A. 1953, 47, 12420).

243 Nud'ga,L.A., Plisko, E.A. and Danilov, S.N., Zhur. Obshch. Khim.,1974, 47,(4),872 (C.A. 1974, 81,4164b).

244 Nud'ga, L.A., Plisko, E.A. and Danilov,S.N., Zhur. Obshch. Khim.,1973, 43,(12),2752. (C.A. 1974, 80, 96252).

245 Plisko,E,A., Nud'ga, L.A. and Danilov,S.N., USSR, 352, 234, 1970. (C.A. 1972, 76, 154705).

246 Nud'ga, L.A., Plisko, E.A. and Danilov, S.N., Zhur. Obshch.Khim., 1975, 45, (5), 1145. (C.A. 1975, 83, 97749).

247 Wolfrom, M.L., Vercellotti, J.R. and Horton,D., J. Org. Chem.,1964,29, 547.

248 Haga,H., Japan, 71, 39,322,1971 (C.A. 1972,76, 100682).

249 Nud'ga.L.A., Plisko, E.A. and Danilov, S.N., Zhur. Obshch. Khim., 1973, 43, 2756 (C.A. 1974,80,96251).

250 Okimasu,S., J.Agric. Chem. Soc.,Japan, 1956, 30,29.

251 Rust,J.B., U.S. 2,669,529,1949.

252 Matsuda,H., Kauki, K., Nakagawa,T., Koike, M. and Yamashita K., Japan, Kokai, 76,68689,1976. (C.A. 1976,85,108939).

253 Karrer,P. and White, S.M.,Helv. Chim.Acta., 1930, 13, 1105.

254 Vanlerberghe,G. and Sebag,H., Ger. 2,222,733,1972 (C.A.,1973,78,47665).

255 Hirano,S. and Ohe,Y., Agric. Biol. Chem., 1975,39,(6),1337.

256 Hirano, S and Ohe, Y., Carbohydr. Res., 1975, 41,C1-C2.

257 Hirano, S., Ohe, Y. and Kondo, S., Polymer, 1975, 16,(8),622.

258 Hirano, S.,Ohe, Y. and Ono, H., Carbohydr. Res.,1976, 47,315.

259 Hirano, S. and Yamaguchi, R., Biopolymers, 1976, 15, 1685.

260 Hirano, S., Tampakushitu Kakusan Koso, 1977,22, (13), 1431, (C.A. 1978, 88,59458).

261 Hirano,S., Miura, O. and Yamaguchi, R., Agric. Biol. Chem., 1977,41,(9),1755

262 Hayes, E.R., and Davies, D.H., ref. 8, p.406.

263 Muzzarelli, R.A.A., ref. 8, p.335.

264 Muzzarelli, R.A.A., Barontini, G., and Rochetti, R., Biotech. Bioeng., 1976,18,(10),1445.

265 Moshy, R.J. and Gemino, R.J. U.S. 3,421,519,1969.

266 Masri, M.S. and Randall, V.G., ref. 8, p.277.

267 Stanley W.L. and Watters, G.G., U.S. 3,909,358,1975.

268 Stanley, W.L. Watters,G.G., Chan.B. and Mercer, J.M.,Biotechnol. Bioeng., 1975,17,351.

269 Hirano, S., Yamaguchi, R., Matsuda, N., Muira, O. and Kondo,Y., Agric. Biol. Chem., 1977, 41, (8),1547.

270 Thiele, H. and Langmaack, L., Z.Physik. Chem., 1957, 207,118.

271 Kikuchi, Y., Nippon Kagaku Kaishi, 1973,12,243. (C.A. 1974,81,34798).

272 Shinoda,K. and Nakashima, A., Bull.Inst. Chem.Res. Kyoto Univ., 1975,53,392,400.(C.A. 1976,84,150869-70).

273 Kikuchi,Y.and Noda, A., J. App. Polym. Sci., 1976,20,(9) 2561.

274 Kikuchi,Y. and Fukuda, H., Makromol. Chem., 1974,175,3593.

275 Kikuchi, Y and Toyota T., Hiroshima Daigaku Kogakubu Kenkiju Hokoku, 1976,24,(2),7.(C.A. 1976,85,10389).

276 Kikuchi, Y. and Fukuda, H., Nippon Kagaku Kaishi, 1976,9,1505.(C.A., 1976 85,166546).

277 Fukuda, H. and Kikuchi, Y., Makromol.Chem., 1977,178,(10), 2895.

278 Hata,K.Y., Yokota, K. and Sakai,T., Japan, 75,123,179,1975.(C.A. 1976, 84,75024).

279 Matsuhima,Y. and Hujii, N.,Bull.Chem.Soc., Japan, 1957, 30, 48.(C.A.1958, 52,8973).

280 Peniston, Q.P.and Johnson, E.L., U.S., 3,922,260, 1975.

281 Katzi,F., Fishman L. and Levy,H.,Brit. 1,418,738,1975.

282 Matsushima, Y., Sci. Papers, Osaka Univ., 1951, 31, 1.(C.A.1952, 46,7052).

283 Whistler, R.J. and Kossik,M., Arch. Bioch. Biophys.,1971, 142, 106.

284 Horton, D. and Just, E.K., Carbohydr. Res.,1973, 29,173.

285 Doczi, J., U.S. 2,795,579,1957.

286 Giles, C.H., Hassan, A.S.A., Laidlaw, M. and Subramanian, R.V. R., J.Soc. Dyers Colourists, 1958,74,647.

287 Giles C.H., Hassan, A.S.A. and Subrananian, R.V.R., J.Soc. Dyers Colourists, 1958,74,682.

288 Giles, C.H. and Hassan A.S.A., J. Soc. Dyers Colourists, 1958,74,846.

289 Sadov,F.I.,Text. Prom., 1941, 2,52,(C.A.1944,38,2215).

290 Manskaya, S.M., Drozdova, T.V. and Emel'yanova, M.P., Geokhimiya, 1956,4,10.(C.A. 1957,51,7967).

291 Andreev,P.F., Plisko, E.A. and Rogozin, E.M.,Geokhimiya, 1962,536. (C.A., 1962,57,10942).

292 Glowacka, D. and Popowicz,J., Roczniki Akad. Med. Bialymstoku, 1965,11,47. (C.A. 1967, 67,28952).

293 Yoshinari, T. and Subramanian V., "Environmental Biogeochemistry" Vol.2., Metal Transfer and Ecological Mass Balance, Nirogu,J.O., (Ed.) Ann. Arbor. Sci. Publ., Ann Arbor, 1976.

294 Yaku,F. and Koshijima, T., ref. 8, p.386.

295 Madhavan, P. and Ramachandran Nair, K.G., ref. 8, p.444.

296 Muzzarelli, R.A.A., and Spalla, B., J. Radioanal. Chem., 1972,10,27.

297 Muzzarelli, R.A.A. and Rochetti, R., Talanta, 1974,2,1137.

298 Haver, H. ref. 8, p.263.

299 Masri, M.S. and Friedman M., Environ. Sci. Technol.,1972, 6,745.

300 Koshijima, T., Tanaka, R., Muraki, E., Yamada, A. and Yaku,F., Cellul. Chem. Technol.,1973, 7,197.

301 Masri, M.S., Friedman,M. and Reuter, F.W., J. Appl. Polym. Sci., 1974, 18, (3), 675.

302 Martin, J.H., Limnol. Oceanogr., 1970, 15, 756.

303 Subrananian, V., ref. 8, p.288.

304 Bihari Varga, M., Sepulchre, C., and Moczar,E., J. Thermal Anal., 1975, 7,675.

305 Austin, P.R., U.S. 3,987, 802, 1976.

306 Schlutzhaver, W.S., Chortyk, O.T. and Austin P.R., J.Agric. Food Chem., 1976, 24, 177.

307 Pearson, F.G., Marchessault, R.H., and Liang,C.Y., J.Polym. Sci., 1960,43,101.

308 Moore G.K., and Roberts, G.A.F., ref. 8. p 427.

309 Rigby, G.W.,U.S., 2,047,226, 1936.

310 Allan, G.G., Grosly, G.D., Lee,J.H., Miller, M.L., and Weif, M.M., Proc Symp. Man-Made Polym., Papermaking, 1972, 85.

311 Sadov, F.I. and Markova, G.B., Nauk. Issled. Trudy Moscow, Tekstil. Inst., 1954, 13,70. (C.A. 1956, 50, 11676).

312 Slagel, R.C. and Di Marco Sinkowitz, G., U.S. 3,709, 780, 1973.

313 Slagel, R.C. and Di Marco Sinkowitz, G, U.S., 3,770,673,1974.

314 Oxford Paper Co., Brit. 930, 765, 1963.

315 Fel'dman, R.I., Makarov, Y.Y., Reutov, O.S. and Goldovskii,E.A., U.S.S.R. 125, 127, 1959. (C.A. 1960, 54, 11476).

316 Plisko, E.A.,Bavanova, V.N. and Nud'ga, L.A., U.S.S.R., 428,053, 1972. (C.A. 1975, 82, 18903).

317 Allan, G.G.,Friedhoff, J.F., Koppela, M., Laine, J.E. and Powell, J.C., A.C.S., Sym. Ser., 1975, 10, 172.

318 Plisko, E.A., Bavanova, V.N., and Nud'ga, L.A., U.S.S.R. 424,933,1972. (C.A. 1974,81, 181815).

319 Brine, C.J. and Austin P.R., Rep. COM-75-10195/6GA, 1974.

320 Foster, W.A., Am. Chem. Soc. Div. Org. Coating Plat, Chem. Pap., 1972, 32, 210.

321 Knecht, E. and Hibbert, E., J. Soc. Dyers and Colourists, 1926, 42,343.

322 Noguchi, J., Wada, O., Hirochi, S., Tokura, S. and Norio, N., Kobunshi Kagaku, 1973, 30, 320 (C.A. 1973, 79, 93253).

323 Muzzarelli,R.A.A., ref.5, p.229.

324 Hurd, I.S. and Hayes, G.M., U.S. 2,961,344, 1960.

325 Hamiter,W.L. and Collins, J.M., U.S. 3,108,897, 1963.

326 Dabrowski, J., U.S. 3,023,072, 1962.

327 Mody, A.V., Ger. 2,438,454, 1975.

328 Ciba, S.A., Belg., 609, 054,1962.

329 Adachi,S. and Fujita,T., Japan 70,02,799,1970 (C.A. 1970 73, 16203).

330 Masri, M.S., Randall, V.G. and Pittman, A.G., Ref.8, p.306.

331 Viktorov, P.P. and Maiofis, I.M., Khlopchatobumazhnaya Prom., 1940, No.11-12, 52. (C.A. 1944, 38, 874).

332 Sadov, F.I. and Vil'dt , E.O., Tekst.Prom., 1958,18,38. (C.A. 1958,52,211176).

333 Ryan,W.H. and Yankowski, E.L., Ger. 1,116,969,1961.

334 Ryan,W.H., U.S. 3,003,875,1958.

335 Bongard, S.A. and Pruglo, N.V., Zhur. Nauchi. Prikl. Fotogri. Kimematogr., 1973,18,(2), 132, (C.A. 1973, 79, 131327)

336 Haberlin, R.J., U.S. 3,674, 482, 1972.

337 Delangre, J.P., U.S. 2, 842, 049, 1958.

338 Scott, K.G., U.S., 4,056, 392, 1977.

339 Otness, J.S. and Medcalf, D.G., Comp. Bioch. Physiol., 1972, B-43,443.

340 Holland, D.L.and Walker, G., J. Cons. Con. Int. Explor. Mar., 1975, 36, 162.

341 Barat, S.K. and Scania, K.Y., Bull. Centr. Leather Res. Inst., 1962, (9),73.

342 Du Pont de Nemours, Co., Brit. 458,818, 1936.

343 Sundara Rajulu, G., and Gowri, N., ref. 8, p.430.

345 Du Pont de Memours, Co., Brit., 458, 813,1936.

346 Jones,R.V., U.S. 2,670, 329, 1954.

347 Yoshida, T. and Yamashita,H., Japan 75,136,287,1975. (C.A. 1976, 84, 104434).

348 Bough,W.A., et al, Poultry Sci., 1974, 54, 992.

349 Bough,W.A., Poultry Sci., 1975, 54, 1904.

350 Bough, W.A. and Landes, D.R., J. Dairy Sci., 1976,59, 1874.

351 Bough, W.A., Process Biochem., 1976, 11, (1), 13.

352 Fujita, T., Yamaguichi, T., Yanagisawa, I and Hirai,O., Nippon Suisan Kabushiki Kaisha Chuo Kenkyusho Hokoku, 1976,11, 49 (C.A. 1977, 87, 172265).

353 Bough. W.A., Food Sci., 1975,40, 297.

354 Bough,W.A. and Landes, D.R., ref. 8, p.218.

355 Bough,W.A., et al, Proc. 6th Nat. Sym. on Food Proc. U.S. Enviro. Prot.Agency, 1976, EDA 600/2-76-224,22.

356 Asano, T., Hayakawa, N. and Suzuki, T., ref. 8, p.231.

357 Peniston, Q.P. and Johnson, E.L., U.S. 3,533,940, 1970.

358 Nakabayasji, T. and Ono, M., Japan Kokai,74, 110, 588,1974. (C.A. 1975, 83, 7635)

359 Nagasawa,K., Watanabe, H. and Ogamo,A.L.,Chromatogr., 1970, 47,408.

360 Nagasawa, K., Kaneko, T. and Ogamo,A., Seikagaku, 1970, 42,(5), 255 (C.A., 1971, 74, 38997).

361 Takeda, M., and Tomida, T., J. Shim Univ. Fish, 1969,18,36.

362 Takeda, M. and Tomida,T., J.Shim Univ. Fish, 1972,20,107.

363 Lepri,L., Desideri, P.G. and Muzzarelli, R.A.A., J. Chromatogr., 1977,139,(2),337.

364 Lepri,L., Desideri, P.G. and Tanturti, J. Chromatogr., 1978, 147,375.

365 Cherkasor, I.A. and Kravchenko, N.A., Biokhimiya, 1969, 34,(5),1089. (C.A. 1970, 72,86535).

366 Cherkasov, I.A. and Kravchenko, N.A., Biokhimiya, 1970,35(1),182. (C.A. 1970, 73,396).

367 Muzzarelli, R.A.A., Barontini,G and Rochetti, R., Biotechnol. Bioeng., 1978,20,(1),87.

368 Weaver G.C., Kroger, M. and Katz, F., J.Food Sci.,1977,42,(4),1084.

369 Townsley, P.M., Nature, 1961, 191, 626.

370 Hiranto, J., J.Chem. Soc., Japan., Ind. Chem. Sect., 1962,65,1367.

371 Senju,R., Bull. Chem. Soc., Japan, 1953, 26, 143. (C.A.1954,48,6689).

372 Kokujuta,E. and Iwai, S., Bull Chem. Soc., Japan, 1977,50,(11),3043. (C.A. 1978,88,38233h).

373 Arai, K., Fuita, F. and Kinumaki,T., Tokaiku Suisan Kenkyasho Kenkyu Hokoku, No.56, 1968, 89. (C.A. 1970, 73,86001).

374 Kikuchi,Y., Makromol. Chem., 1974, 175, 2209.

375 Hilbjard, J.W., Doczi, J. and Kierman, P.B., Proc. Soc. Exp. Bio. Med., 1964, 115,1108.

376 Doczi, J., Ninger, F.C. and Silverman, H.I., U.S. 3,155,575,1964.

377 Weisberg, M. and Gubner, R.S., U.S. 3,257,275,1966.

378 Balassa, L.L., Brit. 1,252,373,1971.

379 Balassa, L.L., U.S. 3,632,754,1972.

380 Balassa, L.L., U.S. 3,903, 268, 1975.

381 Balassa, L.L., U.S. 3,911,116, 1975.

382 Balassa, L.L., U.S. 3,914,413, 1975.

383 Balassa, L.L. and Prudden J.F., ref. 8,p.296.

384 Fujita, H., Sogo Igaku, 1956, 13,740.(C.A. 1960,54,22997).

385 Bernfield, P. and Kelley, T.F., J.Biol.Chem., 1963, 238, 1236.

386 Regelson, W., Tunis, M. and Kuhar S., Acta Unio. Intern. contra Cancrum, 1960, 16, 729.(C.A.,1960,54,23051).

387 Acino, M., Japan, Zool., 1960, 12,527.

388 Bernfield, P., Bernfield, H.C. and Nessellboum. J.S., J.Am. Chem. Soc., 1954, 76,4872.

389 Anikeeva,A.N. Plisko,E.A., Nud'ga L.A., Vlasov, N.N. and Pliss, G.B., U.S.S.R. 419, 529, 1971.(C.A.1975,82, 45565)

390 Casey, D.J., U.S. 4,059,097,1978.

391 Turlan,M. and Moroson, H., Radiat. Prot. Sensitisation Proc. Int. Symp. 2nd., 1969, 233.

392 Woodman, R.J., Cancer Res., 1968, 28,2007.

393 Sinka, A.E. and Woodman, R.J., J.Nat. Cancer Inst., 1971,47,377.

394 Dunn H.J. and Farr, M.P., U.S. 4,034,121,1977.

395 Glecker, G.C. and Gochel, J.C., U.S. 4,035, 267,1977.

396 Gross, P., Konrad,E. and Mayer, H., Ger. 2,627,419,1977.

397 Saad, H.Y., U.S. 4,027,068,1977.

398 Finley, J.W., Stanley,W.L. and Watters, G.G., Biotechnol. Bioeng., 1977,19,(12, 1895.

399 Bernadet, M., Fre. 1,552,076, 1963.

400 Boehringer, C.H., Ger. 1,015,764,1957

401 Shinohara,K.,Japan Kokai, 77,57,342,1977. (C.A.1977,87,54842r)

402 Peniston Q.P. and Johnson E.L., U.S. 4018, 678,1977.

403 Babu, K., Satish Rao, K. and Panduranga, J.K.T.,Leather Sci.,1975, 22, 244.

404 Schade, J.E., Biotech. Bioeng.,1976, 18, 439.

405 Kennedy, J.F. and Doyle, C.E., Carbohydr. Res., 1973,28,89.

406 Masri, M.S., Randall, V.G. and Stanley,W.L., Polymer Prepr., 1975,16,70.

407 Stanley, W.L., Watters, G.G., Kelley, S.H. and Olson,A.C., Biotechnol. Bioeng., 1978, 20, (1), 135.

408 Maekawa, Y., Inoue.E., Otaki M. and Matsunoto, H.,Japan Kokai, 77,130,979-80,1977. (C.A. 1978,88,77092-3).

409 Masri, M.S., Randall,V.C. and Stanley,W.L., U.S. 706, 980, 1977.

410 Iwaski, T., Hashiba,H. and Kikuchi,T., Japan Kokai, 77,03 892, 1977. (C.A. 1977, 86,152093w).

411 Leuba, J.L. Ger. 2,522,484,1976.

412 Tokura, S. and Nishi,N., Kapaku To Seibutso, 1977, 15, (12),766. (C.A. 1978,88,170396t).

413 Tanghe, L.J., Genung,L.B. and Mench, J.W., Methods in carbohydrate chemistry, 1963, Whistler R.A.(Ed.), Academic Press Inc., (London), Ltd., 3, 196.

414 Wallace, J.M. and Copenhaver,J.E., J.Am. Chem.Soc., 1941, 63, 699.

415 Gerrard, and Thrush, ,J.Chem. Soc., 19 ,52, 741.

416 Harada,K., "Chemistry of the carbon-nitrogen double bond", 1970 Patai,S.(Ed.), Interscience Pub., 265.

417 Goméz-Sanchez,A. and Goméz-Guillen,M, Carbohydr. Res., 1967,3,486.

418 Gloor,W.E. and Klug,E.D., Cellulose Data, Interscience Pub., Inc., 1955, 34.

419 Hearon,W.M., Hiatt,G.D. and Fordyce,C.R., J.Am. Chem. Soc., 1963,65,829.

420 Hirano, S., Yamaguchi, R., and Matsuda, N., Biopolymers, 1977,16,1987.

421 Hayes,E.R. and Davies, D.H., ref. 8, p.193.

422 Nevell, T.P., Methods in carbohydrate chemistry, 1963,Whistler R. A., (Ed), Academic Press Inc., (London),Ltd., 3, 164.

423 Sannan,T., Kurita, K., Ogura, K. and Iwakura,Y., Polymer, 1978, 19, 458.

424 Nevell, T.P., J. Text. Inst., 1957,48,(12),T484.

425 Donnan, F.G., 1924, Chem. Rev., 1, 73.

426 Beilstein, 9^2,319.

427 Beilstein, 9^2,324.

428 Beilstein, 9^2,329.

429 Bellamy,L.J., "The infrared spectra of complex molecules", John Wiley & Sons,Inc.,1958.

Appendix I

The occurrence of chitin: the distribution of the diverse chitinous structures in living organisms

Organism	Structures
Fungi:	
Ascomyceta	Cell walls and structural membranes of mycelia, stalks and spores.
Basidiomyceta	
Phycomyceta	
Imperfecti	
Algae:	
Chlorophyceae	
Protozoa:	
Rhizopoda	cystwall or shell
Cnidaria:	
Hydrozoa	
Hydroidea	
Milleporina	coenosteum
Siphonophora	pneumatophore
Anthozoa	"skeleton"
Scyphozoa	
Aschelmintes:	
Rotifera	egg inner membrane
Nematoda	egg middle membrane
Acanthocephala	egg capsule
Priapulida	cuticle
Endoprocta:	cuticle
Bryozoa:	ectocyst
Phoronida:	tubes

Brachidopoda:	
Articulata	stalk cuticle
Inarticulata	shell
Echiurida:	hooked chaetae
Annelida:	
Polychaeta, Eunicidae, Oligochaeta	peritrophic membrane
Mollusca:	
Polyplacophora	shell plates, radula
Gastropoda	mother of pearl, radula, jaws
Cephalopoda	calcified shell
Lamellibranchia	shells, gastric shield
Arthropoda:	
Crustacea	calcified cuticle
Insecta	hardened cuticle
Arachnida	peritrophic membrane
Pogonophora:	tubes

Appendix II

Methods for the isolation of chitin

Method of Hackman [124] (1954)

Lobster shell, cleaned by washing and scraping under running water is dried at 100°C. The prepared shell (200g) is digested with 2N hydrochloric acid (2l) for 5 hours at room temperature, washed well, dried at 100°C and ground to a fine powder (91g). Extraction of the powder for 48 hours with cold 2N hydrochloric acid (500ml) with frequent agitation, is followed by centrifugation, washing with water and extraction at 100°C with 1N sodium hydroxide (500ml) for 12 hours with frequent agitation.

The insoluble material is collected by centrifugation and extracted with alkali a further four times. The chitin is washed well with water to neutralise the reaction, and washed with ethanol and ether and dried in vacuo over phosphorous pentoxide.

Yield: 37g (17%) of a cream coloured powder

(Found N: 68% Ash nil Calculated for $(C_8H_{13}O_5N)$ x N: 6.9%)

Method of Horowitz, Roseman and Bluementhal [99] (1957)

Concentrated formic acid (90%) solution (100mls) and decalcified powdered lobster shell (10g) are mechanically shaken together for 18 hours and the mixture filtered and washed with water. Treatment of the residue with 10% sodium hydroxide solution (500ml) on a steam bath for $2\frac{1}{2}$ hours is followed by filtration and washing with water. The precipitate is dried under vacuo in a desiccator.

Yield: 60-70% of a white product

(Found N:6.95% $COCH_3$: 19.2%; calculated for N:6.90% $COCH_3$ 21.2%)

Method of Foster and Hackman[126] (1957)

This method was developed as a milder method for the isolation of chitin, as the authors felt that earlier, more drastic, methods might well effect structural changes in the chitin, such as the removal of the pendant groups (proteins and peptides), de-N-acetylation and fragmentation.

Fresh, clean, air dried fragments ($1cm^2$) of the cuticle of the edible crab Cancer Pagurus are attacked slowly (50% loss in weight in two weeks) by ethylene diamine tetraaceticacid (EDTA) at pH9. Similar treatment using powdered shell (particle size 1-10μ) gives a decalcified product in less than 15 minutes. No carbohydrate material is extracted in this process. After a further treatment with EDTA at pH 3, the residue is extracted with ethanol and ether. Spectrographic analysis gives SiO_2 1.07% Mg O 0.08% error ± 10-20% (before treatment SiO_2 5.6% MgO 4.5% CaO 77.0% P_2O_5 8.2% error ± 10-20%) Elemental analysis corrected for 2.6% ash as oxide, C: 43.5 H: 6.6 N: 7.6 The high value of N indicates that the chitin contains protein and this is confirmed by hydrolysis with 6N hydrochloric acid for 24 hours, followed by paper electrophoresis. Protein is estimated to be 5% of the product and it can not be removed by extraction at room temperature with dimethyl formamide (DMF) or phenol-water mixture. Extraction with 98-100% formic acid gives a soluble and an insoluble fraction, both with the same proportion of protein. The soluble fraction can be reprecipitated by dilution with water and resembles chitosan. The insoluble fraction can be dispersed in saturated aqueous lithium thiocyanate at 100°C. Fractionation gives fractions with similar protein contents that can be substantially reduced by repeated hot alkali extractions.

Method of Whistler and BeMiller[125] (1962)

Cleaned lobster shells are dried at 50°C in a vacuum oven. After grinding the shells (500g) they are soaked for three days at room temperature in de-aerated 10% sodium hydroxide solution; the sodium hydroxide solution being renewed daily. The deproteinized chitin is washed until free of alkali, then washed with 95% ethanol (6 litres) to remove the pigments from the product. Next, the chitin is washed with acetone (1 litre) absolute ethanol (2.5 litres) and ether (500ml). The product is dried under vacuo and introduced into a 37% hydrochloric acid solution at -20°C for 4 hours. The particles are then washed with water ethanol and ether. The cold hydrochloric acid treatment may be repeated.

Yield: 100g (20%) of a white powder

Found sulphated ash 0.15% N: 7.1%

Method of Takeda and Abe[127] (1962); Takeda and Katsuura[128] (1964)

Decalcification of king crab shells with EDTA at room temperature and pH10 is followed by digestion with a proteolytic enzyme, such as tuna proteinase (pH8.6 37.5°C), papain (pH 5.5-6; 37.5°C) or a bacterial proteinase (pH 7, 60°C) for over 60 hours. The chitin obtained by this method contained less than 5% protein. Residual protein is removed by various treatments, sodium dodecylbenzene sulphonate and DMF have been found to be very effective.

Method of Broussignac[129] (1968)

Decalcification of crab shells that have been ground and sieved, is

carried out using hydrochloric acid (50g $HCl\,l^{-1}$). The operation takes about 24 hours and the end of the reaction is indicated by the cessation of carbon dioxide evolution. Ash content is determined at the end and found to be in the range 0.4-0.5%. Papain, pepsin or trypsin are then used to remove proteins from the residue and this gives a product with little deacetylation. When chitosan is required as the end product, the enzyme treatment is replaced by an alkali treatment at high temperatures.

Appendix III

Methods for the Preparation of Chitosan

Method of Horowitz, Roseman and Bluementhal[99] (1957) and of Horton and Lineback[152] (1965)

Chitin (30g) is fused with potassium hydroxide pellets (150g) at 180° for 30 minutes under nitrogen in a nickel crucible. The melt is poured cautiously into ethanol and the gelatinous precipitate washed with ethanol and then water until neutral to litmus. Purification by dissolving the crude chitosan in 5% acetic acid and reprecipitating the gel with dilute alkali, is carried out three times. The acetyl value is quoted as a trace or insignificant and the chain length after dialysis is about 20 units.

Method of Rigby[130] (1936) of Wolfrom, Maher and Chaney[158] (1957) and of Wolfrom and Shen Han[157] (1958)

Chitin (50g) is heated in 40% aqueous sodium hydroxide solution (2.4 litres) at 115°C for 6 hours with the exclusion of air. The cooked mixture is filtered and washed with water until neutral to phenolphthalein. A deacetylation value of 82% is obtained for chitosan by this method.

Method of Broussignac[129] (1968)

Following a systematic study the following anhydrous deacetylation mixture is used, potassium hydroxide (50%) ethanol (25%) and monoethylene glycol (25%) by weight. The reaction is carried out in a stainless steel reactor consisting of a steam heating system

and a reflux condenser. Dried chitin (27kg) is heated at 120°C in the deacetylation mixture (360kg), for the required length of time and after filtration, the chitosan is washed with water until neutral. A reaction time of 16 hours gives chitosan with 83% deacetylation.

Method of Fujita [159] (1970)

Chitin (10 parts) are mixed with 50% sodium hydroxide (10 parts), kneaded, mixed with liquid paraffin (100 parts) and stirred for 2 hours at 120°C. The mixture is poured into cold water (80 parts) filtered and washed thoroughly with water. Yield of 8 parts chitosan is obtained with 92% deacetylation.

Method of Peniston and Johnson [160] (1975)

This method eliminates the need to use hydrochloric acid to demineralize the crab shell, recycles most of the processed chemicals, and dispenses with the intermediate isolation of chitin. Recovery of protein, sodium acetate, and calcium carbonate as by-products is also possible.

The scheme is shown in Fig 94 . Shellfish waste ground to particles 3-6mm in size is applied at (1) to a protein extraction apparatus (2) where the shell is moved counter-currently to a flow of dilute sodium hydroxide (0.5-2%) at (3) The sodium proteinate formed is extracted at (4) The temperature is kept at 50-70°C, but extraction times vary from 1-4 hours depending on the porosity of the shell.

The sodium proteinate solution is clarified by centrifugation or filtration (5) and treated with refining agents to remove lipids or

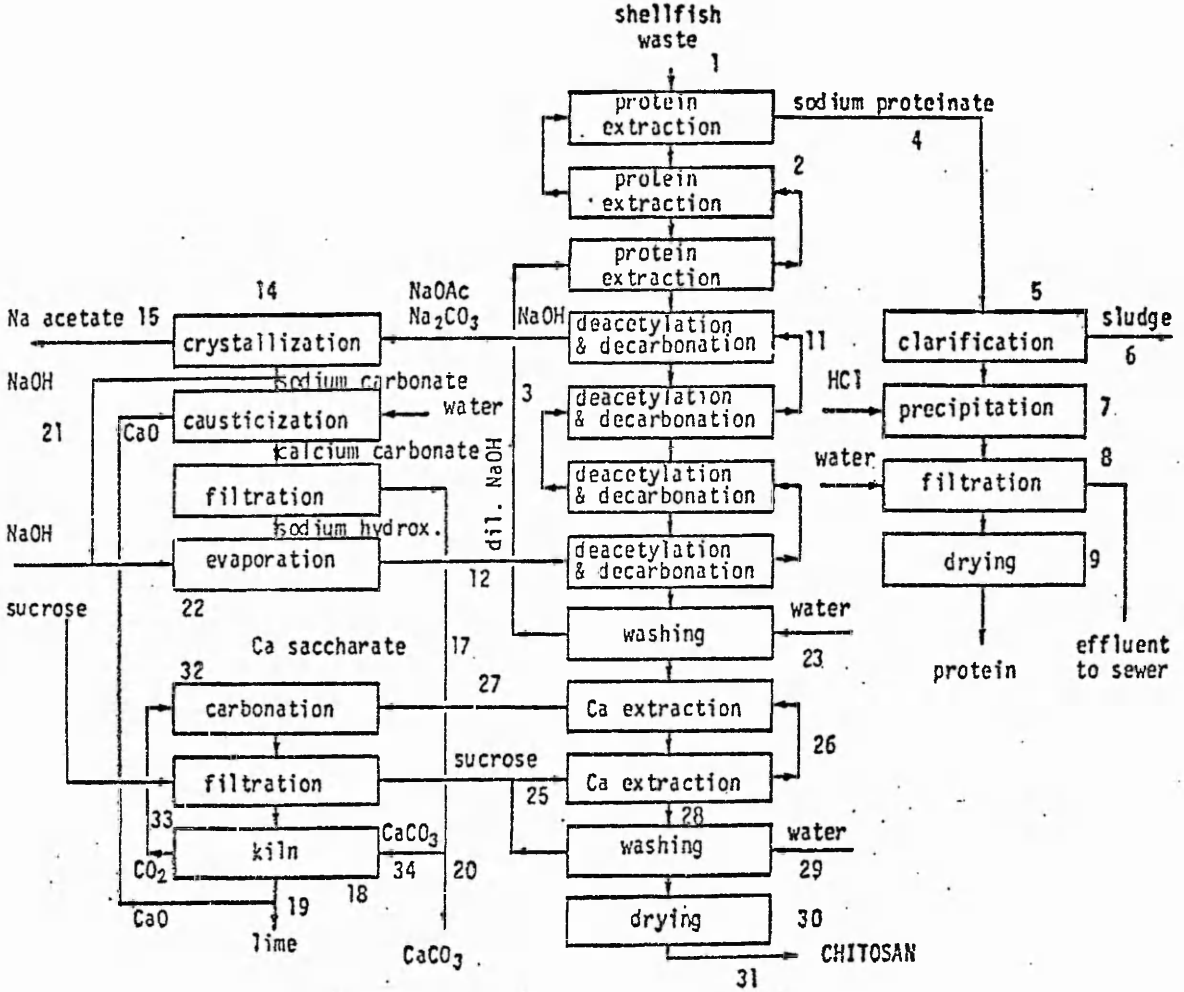


Figure 94 Chitosan production process scheme

pigments. These are removed at (6) Neutralization of the clarified product with hydrochloric acid to pH 4.5-3.4 is then carried out. The resulting precipitated protein at (7) is collected at (8) and washed and dried at (9) by reslurrying and spraydrying to provide the protein by-product at (10).

Following protein removal, the shell is extracted again, counter-currently in extraction cells (11) with strong sodium hydroxide (12). The effluent from this stage (13) contains excess sodium hydroxide, sodium acetate and sodium carbonate. It flows to a crystalliser (14) to precipitate out the sodium acetate (15) together with the majority of the sodium carbonate.

The molten liquor is diluted with water and treated with lime (16) to convert the remaining sodium carbonate back to sodium hydroxide. The precipitated calcium carbonate is collected at (17). The regenerated sodium hydroxide solution at (21) is combined with added strong alkali to replace that removed at (15) as sodium acetate and is concentrated by evaporation at (22) to the desired strength for application in the extraction process, (at 12).

Following deacetylation and decarbonisation, the residual shell, consisting of calcium hydroxide and chitosan, is washed at (23) with carbonate-free water to remove residual sodium hydroxide. This washing gives the dilute sodium hydroxide used at (3) for the protein extraction.

Aqueous sucrose solution is applied at (25) to extract the chitosan and calcium hydroxide mixture. Calcium saccharate is formed and removed (27) leaving chitosan (28) which is washed to neutrality at (29) and

dried (30). The saccharate is decomposed by carbonisation and calcium carbonate is precipitated at (34) and is washed and passed to the lime kiln (18). The sucrose solution is evaporated to the desired concentration and reused (25). Other substances capable of chelating calcium e.g. glycols, EDTA, sorbitol or gluconates, may be used.

Salt	Solubility	Salt	Solubility
Acetate	s	cyanate	ss
monochloro	s	Dilactate	s
dichloro	s	Dithiocarbonate	ss
monoiodo	s	Formate	s
phenyl-	s	Fumarate	ss
trimethyl-	s	Glycinate	ss
Acetacetate	s	phenyl-	ss
Acrylate	s	Glycolate	s
ethacrylate	s	di-	s
furacrylate	s	thio-	s
hydracrylate	s	Hydrobromide	s
Adipate	s	Hydrochloride	s
Anthranilate	s	Hydroiodide	s
Azelaate	s	Hypochlorite	ss
Benzenesulphonate	s	Lactate	s
Benzoate	s	Laurate	ss
o-benzoyl	ss	Levulinate	s
Borate	s	Linoleate	ss
Iso-butyrate	s	Malate	s
αchloro-	s	Maleate	s
hydroxy-	s	Malonate	s
n-butyrate	s	diethyl-	s
α-bromo-	s	Mandelate	s
Caproate	s	Molybdate	ins
Chromate	ins	phospho	ins
Cinnamate	ss	Naphthenate Mw186	s
Citrate	s	Mw450	s
Crotonate	s	Nitrate	ss

Salt	Solubility	Salt	Solubility
Oxalate	ss	Sulphanilate	s
Palmitate	ss	sulphate	ins
Phosphate	ss	Sulphite	s
Phthalate	ss	sodium bisulphite	ss
Picrate	ins	Sulphos algcylate	s
Propionate	s	Tartrate	s
α-bromo-	s	Terephtalate	ss
α-chloro-	s	Tetrachloroaurate	s
α-iodo	s	Tetraiodomecurate	
Pyruvate	s	Thiocyanate	ss
Salicylate	ss	Tungstate	ins
Sebacate	s	-phospho-	ins
Stearate	s	meta-Vanadate	ss
Succinate	ss		

s- soluble ss-slightly soluble ins- insoluble

Appendix V

Solvents used in studies of influence of solvent media on N-acetylation

Solvent	δ Hildebrands ($cal^{\frac{1}{2}} cm^{-3/2}$)
Acetic anhydride	10. 3
Acetic acid (glacial)	10. 1
Acetone	9. 9
Carbon tetrachloride	8. 6
Chloroform	9. 3
Dimethylformamide	12. 1
Dimethylsulphoxide	12. 0
Dioxan	10. 0
Ether	7. 4
Ethyl acetate	9. 1
Formamide	19. 2
Pyridine	10. 7
Toluene	11. 6
Tetrahydrofuran	9. 1
Methanol	14. 5
Ethanol	12. 7
iso-Propylalcohol	11. 5
n-Butanol	11. 4
iso-Butanol	10. 5
t-Butanol	10. 6
Cyclohexanol	11. 4

Appendix VI

N-Acetylation in ethanol-methanol and methanol-formamide

(not presteeped)

Ratio of ethanol:methanol		Ratio of Methanol:formamide		δ solvent	δ solvent + acetic anhydride	% N-acetylation at 30 mins	% N-acetylation at 60mins
10	0			12.92	12.05	23.5	28.3
8.75	1.25			13.09	12.16	26.6	31.3
7.5	2.5			13.26	12.27	28.0	35.2
5	5			13.6	12.5	33.4	46.1
2.5	7.5			13.94	12.73	41.6	69.5
0	10			14.28	12.95	86.5	97.5
		7.5	2.5	15.16	13.54	95.6	97.5
		6.25	3.75	15.6	13.83	63.1	74.4
		5	5	16.04	14.13	43.0	48.0
		3.75	6.25	16.48	14.42	37.5	42.7
		2.5	7.5	16.92	14.71	33.0	39.7
		0	10	17.8	15.3	26.5	34.3

N-Acetylation in ethanol-formamide (not presteeped)

Ratio of ethanol:formamide		δ solvent	δ solvent + acetic anhydride	% N-acetylation at 30 mins	% N-acetylation at 60 mins
10	0	12.92	12.05	26.3	36.1
9	1	13.41	12.37	21.8	33.8
8	2	13.90	12.7	21.1	34.6
7	3	14.38	13.02	24.1	30.8
6	4	14.87	13.35	25.6	41.4
4	6	15.85	14.0	25.6	47.4
3	7	16.34	14.32	31.6	61.2
2	8	16.82	14.64	34.6	66.2
0	10	17.8	15.3	24.8	37.6

N-Acetylation in n-propanol-methanol (not presteeped)

Ratio of n-propanol:methanol		δ solvent	δ solvent + acetic anhydride	% N-acetylation
10	0	11.97	11.41	26.4
8	2	12.43	11.72	25.8
7	3	12.60	11.83	28.3
5	5	13.13	12.19	28.9
4	6	13.36	12.34	31.8
3	7	13.59	12.49	30.5
2	8	13.82	12.65	51.2
1	9	14.05	12.8	79.7
0	10	14.28	12.95	86.5

N-Acetylation in different alcohol-formamide systems (Presteeped)

Methanol-Formamide

Ratio of Methanol:Formamide		δ solvent	δ solvent+ aceticanhydride	% N-acetylation
10	0	14.28	12.95	84.2
9	1	14.63	13.19	87.2
8	2	14.98	13.43	93.2
7	3	15.34	13.66	88.7
6	4	15.69	13.89	85.7
5	5	16.04	14.13	87.2
4	6	16.39	14.36	91.7
3	7	16.74	14.59	89.4
2	8	17.10	14.83	96.2
1	9	17.45	15.07	91.0
0	10	17.8	15.3	100

Ethanol-Formamide

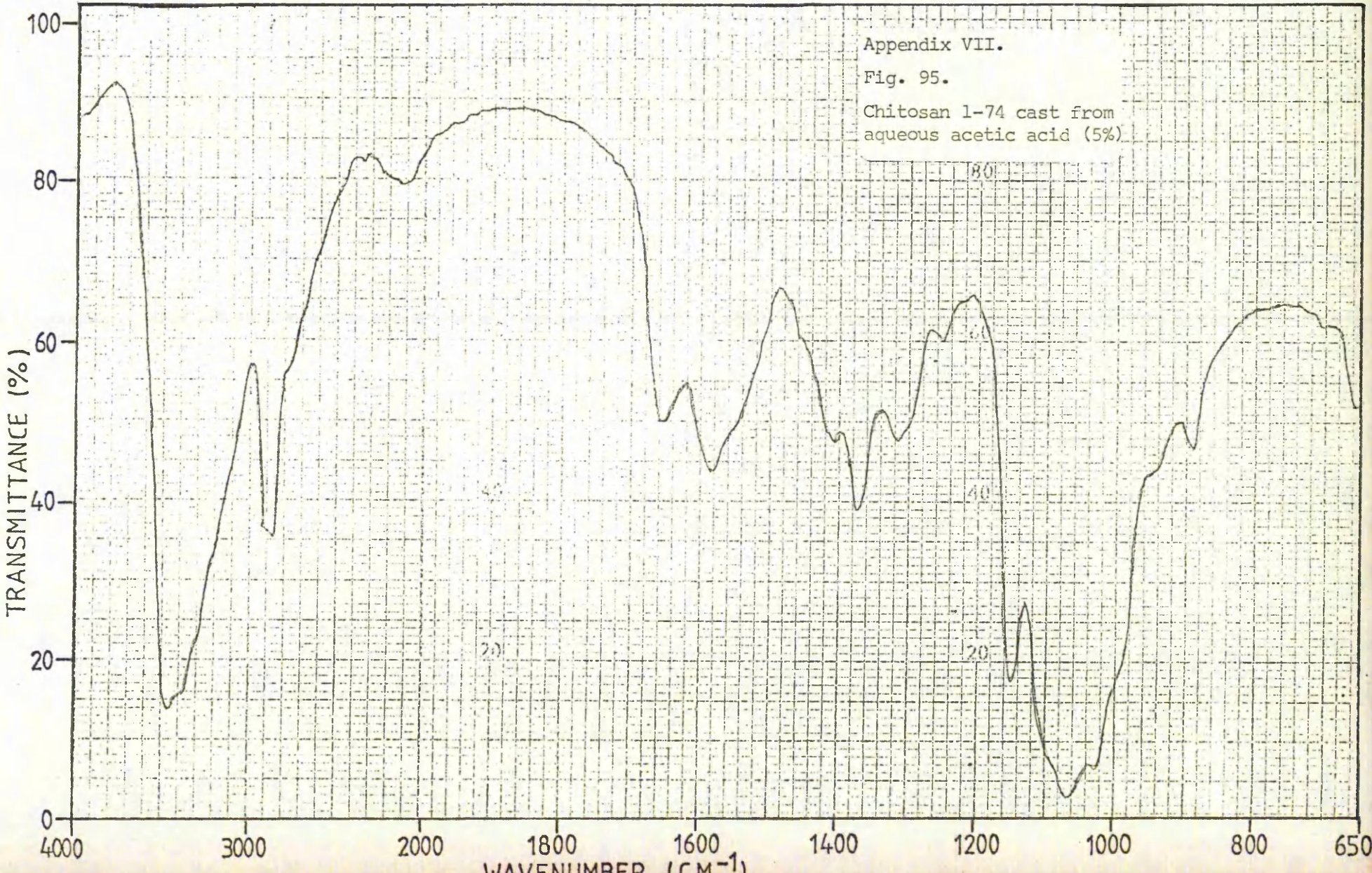
Ratio of Ethanol:Formamide		δ solvent	δ solvent+ acetic anhydride	% N-acetylation
10	0	12.92	12.05	44.4
9	1	13.41	12.37	47.9
8	2	13.90	12.7	57.5
7	3	14.38	13.02	79.7
6	4	14.87	13.35	86.4
5	5	15.36	13.67	94.0
4	6	15.85	14.0	96.2
3	7	16.34	14.32	97.0
2	8	16.82	14.64	99.2
1	9	17.31	14.97	93.2
0	10	17.8	15.3	100

n-Propanol-Formamide

Ratio of n-propanol-formamide		δ solvent	δ solvent + acetic anhydride	% N-acetylation
10	0	11.97	11.41	26.7
9	1	12.55	11.8	35.5
8	2	13.14	12.19	63.4
7	3	13.72	12.58	70.2
6	4	14.30	12.97	84.2
5	5	14.89	13.36	87.9
4	6	15.47	13.74	93.9
3	7	16.05	14.13	94.7
2	8	16.63	14.52	93.2
1	9	17.22	14.91	99.2
0	10	17.8	15.3	100

n-Butanol-Formamide

Ratio of n-butanol-formamide		δ solvent	δ solvent+ acetic anhydride	% N-acetylation
10	0	11.30	10.96	27.5
9	1	11.95	11.40	32.5
8	2	12.60	11.83	45.1
7	3	13.25	12.27	70.4
6	4	13.96	12.74	84.2
5	5	14.55	13.13	92.5
4	6	15.2	13.58	96.2
3	7	15.85	14.00	91.0
2	8	16.5	14.43	91.7
1	9	17.15	14.87	91.7
0	10	17.8	15.3	100



Appendix VII.

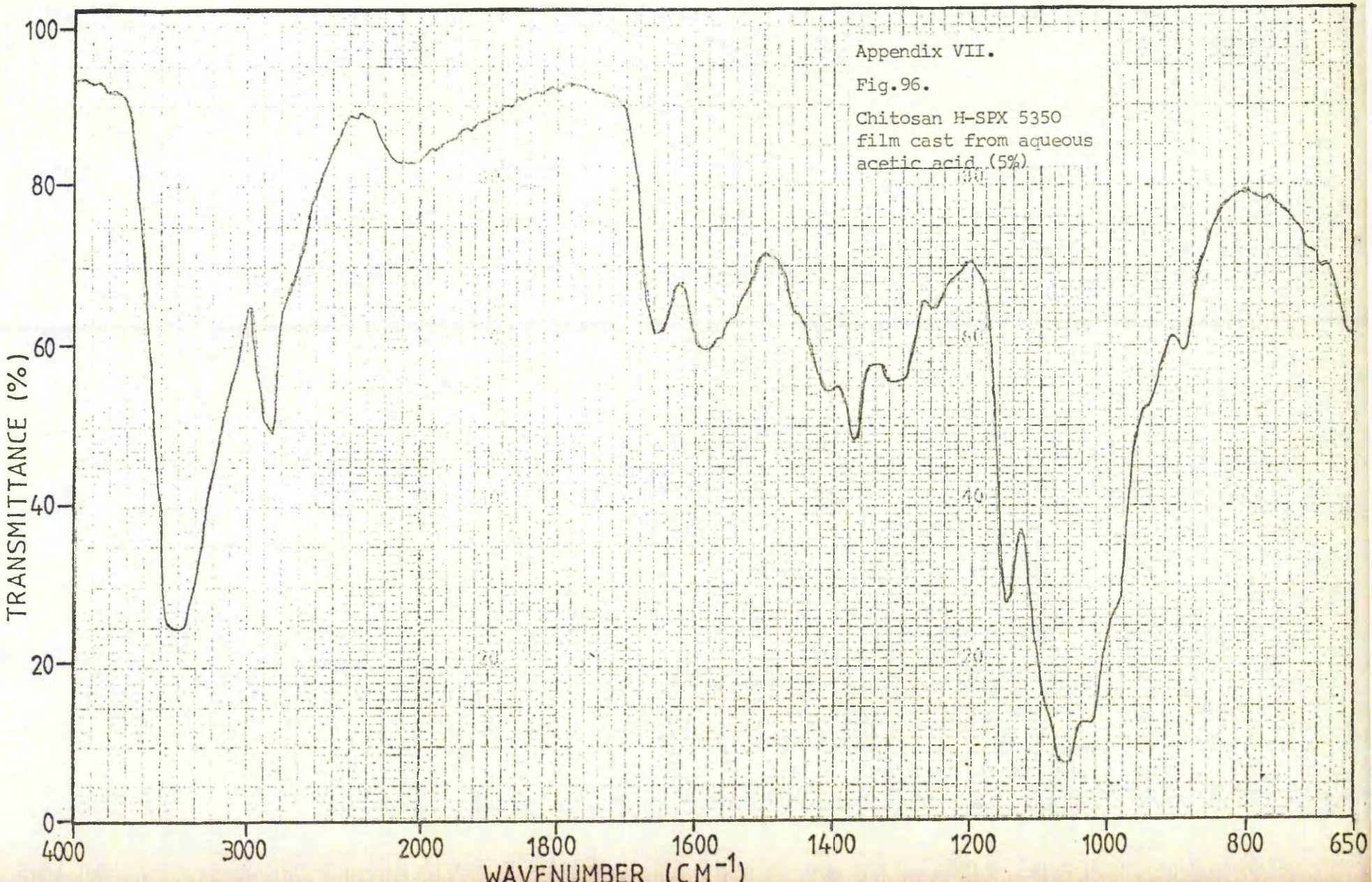
Fig. 95.

Chitosan 1-74 cast from aqueous acetic acid (5%)

Appendix VII.

Fig.96.

Chitosan H-SPX 5350 film cast from aqueous acetic acid (5%)



4

Derivatives of Chitin and Chitosan

4.1 INORGANIC ESTERS AND RELATED DERIVATIVES

4.1.1 Nitrates

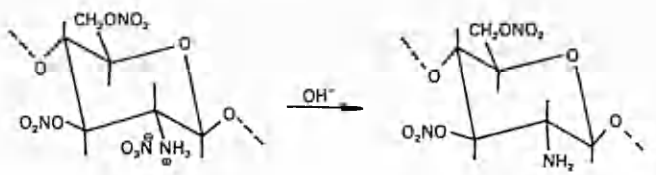
Compared with the extensive research that has been carried out on both the production and the properties of cellulose nitrate,[1] because of its commercial importance as an explosive and as a plastic, very little research has been carried out on the nitration of chitin and chitosan.

Von Fürth and Scholl[2] found that chitin can be nitrated by fuming nitric acid, the product being partially soluble in acetic acid. Their work was repeated some 30 years later by Schorigin and Hait[3] who concluded that the HNO_3–H_2SO_4 nitrating mixtures used for preparing cellulose nitrate are unsuitable for use with chitin, owing to the extensive chain degradation brought about by H_2SO_4. The use of concentrated HNO_3 (*d*, 1.5) on its own was recommended instead, nitration occurring in 1–2 h after which the chitin nitrate was isolated by pouring into a large volume of water. Analysis showed the maximum *DS* to be ~1.5 and complete denitration could be achieved by treating with NaSH at room temperature. The product was in general quite stable, although it ignited at ~163°C and burned very vigorously. A considerable portion was soluble in formic acid, the % N contents of both the soluble and the insoluble fractions being similar. This fact led Schorigin and Hait to suggest that the difference in solubility is due to the effect of different molecular weights but, since the elemental analysis figures show the material to be less than completely nitrated an alternative explanation is that the two fractions have different patterns of substitution within the same overall *DS* value.

At about the same time Clark and Smith[4] also prepared chitin nitrate by dissolving chitin in fuming nitric acid and precipitating in water after reaction times of 1–2 h. These latter workers found, contrary to their expectations, that it was not possible to estimate the *DS* by determining the increase in weight of the sample, the values obtained by this method being less than those obtained from determination of the % N content. Based on the observation that the solubility of the chitin nitrate in concentrated HCl was similar to that of the original chitin, it was concluded that very little deacetylation takes place during the nitration step, a view supported by its lack of solubility in dilute aqueous acids. They did not give any analytical details for their chitin nitrate but Hackman,[5] using a similar process, obtained a product having a *DS* of 1.25.

Clark and Smith also carried out heterogeneous nitration of chitin,[4] using a 5:1 mixture of fuming HNO_3:concentrated HNO_3, to obtain an oriented sample for X-ray diffraction studies, the results of which indicated an orthorhombic cell with a = 9.0 Å, b = 10.3 Å and c = 23.0 Å. The increase in the c axis was stated to be close to that expected for substitution of nitrate groups for hydroxyl groups along the chain.

Wolfrom *et al.*[6] examined the preparation of chitosan nitrate and its nitrate and perchlorate salts. Two routes were investigated; in the first, chitosan[0.15] was dissolved in absolute HNO_3, while in the second it was suspended in a 1:1:1.3 mixture of glacial acetic acid:acetic anhydride:absolute nitric acid for 5.5 h at < 5°C. The products from both processes were very similar, being the nitric acid salt of chitosan nitrate (**4.1**) having a $DS_{(ONO_2)}$ value of 1.65. These were converted to the chitosan nitrate (**4.2**),



4.1 **4.2**

with the *O*-nitrate content unchanged, by addition of dilute alkali to a solution of the salt in 50 vol.-% aqueous acetone. Care must be taken to avoid the solution becoming alkaline since the analogous cellulose nitrate is very readily degraded by alkali. The chitosan nitrate (**4.2**) was then converted to the perchlorate salt by suspending it in glacial acetic acid containing perchloric acid. The perchlorate salt, unlike the nitrate, was found to be unstable at room temperature, decomposing slowly in most cases but with detonation in one instance.

The most comprehensive study to date is that of Hirano and Yano[7] who

TABLE 4.1 *Substitution values of* N-*acylchitosan* O-*nitrate derivatives*[7]

N-*acyl group*	$DS_{(ONO_2)}$	
	N-*acylated chitosan* O-*nitrate*	O-*nitrated* N-*acyl-chitosans*
Acetyl	1.4	1.9
Propionyl	1.5	2.0
Butyryl	1.5	1.7
Hexanoyl	1.5	2.0
Octanoyl	1.5	2.0
Decanoyl	1.5	2.0
Dodecanoyl	1.6	1.9
Tetradecanoyl	1.7	1.9
Hexadecanoyl	1.7	1.9
Benzoyl	1.8	3.0

have reported the preparation and properties of *O*-nitrated chitosan and of the *O*-nitrate derivatives of a number of *N*-acylchitosans. These latter products were prepared by two routes:

(a) nitration of chitin and its *N*-acylchitosan analogues using fuming nitric acid–acetic anhydride mixtures;
(b) nitration of chitosan[0.0], followed by conversion to the free base[6] and *N*-acylation of the *O*-nitrated chitosan[0.0].

The $DS_{(ONO_2)}$ was 1.7–2.0 for the products obtained by nitration of *N*-acylchitosans and 1.5–1.8 for those obtained by *N*-acylation of *O*-nitrated chitosan[0.0] (Table 4.1). The values of 1.7–2.0 for the *DS* of samples prepared by route (a) are in agreement with the results obtained by Marchenko *et al.*[8] who reported *DS* values close to the theoretical maximum of 2.0 for chitin nitrate samples. The high *DS* values obtained by these latter workers are somewhat surprising since they used aqueous HNO_3 as the nitrating reagent and it is well known, from studies carried out on the nitration of cellulose, that the presence of water reduces the extent of nitration.[1]

Nitration[7] of *N*-benzoylchitosan gave a product having a *DS* value of 3.0, and although it was assumed that the additional NO_2 group was a $C\text{–}NO_2$ group located on the aromatic ring, this could not be confirmed since the IR absorption bands at 750 and 720 cm^{-1} are similar to those in the spectrum of the *N*-benzoyl derivative of *O*-nitrated chitosan.

O-Nitrated chitosan ($DS_{(ONO_2)}$ = 1.5–1.8) was flammable at 151–156°C but no ignition point was observed for the *N*-acylated derivatives, all of which decomposed at temperatures in excess of 240°C. This stability contrasts with the instability of cellulose nitrate and is presumably due to the reduction in the proportion of high-energy bonds. The solubilities of the nitrated derivatives of the various *N*-acylchitosans differ considerably from that of cellulose nitrate. Nitrated *N*-benzoyl- and the *N*-acylchitosans up to *N*-butyrylchitosan were found to be soluble in DMSO, while up to the *N*-hexanoylchitosan were soluble in DMF. All products up to *O*-nitrated *N*-hexadecanoylchitosan were soluble in methanesulphonic acid but none was soluble in either acetone or ether–ethanol mixtures, both of which are common solvents for cellulose nitrate.[1]

4.1.2 *Phosphates*

Karrer *et al.*[9] were the first to attempt the phosphorylation of either chitin or chitosan. They treated chitosan with 15 parts pyridine and 5 parts phosphorous oxychloride at 40°C for 5 h, but although they claimed that products having 24% P were obtained, Hackman[5] was unable to obtain phosphorylated derivatives of chitin using their method. More recently interest in phosphate esters of chitin and chitosan has increased, mainly because of their metal ion binding capabilities (section 5.2.6) and two preparative techniques have been developed. The first is based on a method for preparing cellulose phosphates, originally through heating with mixtures of phosphoric acid and urea by an impregnation-baking sequence[10] but subsequently modified by the use of an inert liquid as the reaction medium.[11] Both DMF[12, 13] and toluene[14] have been used as the inert reaction medium for preparation of chitin and chitosan phosphates.

The second method, which has been developed by Nishi *et al.*,[15–18] involves low temperature (0–5°C) reaction of phosphorus pentoxide with chitin or chitosan dissolved in methanesulphonic acid. A phosphorylated chitin having a *DS* of 1.6 was obtained using 2 equivalents of P_2O_5, but the efficiency of the reaction decreases sharply at higher levels and the *DS* was only increased to about 1.75 on doubling the amount of P_2O_5 used.[16, 17] Analysis by ^{13}C NMR spectroscopy indicated that the C(3)OH group was at least as reactive as the C(6)OH group. Although the *DS* obtained increases steadily with time of reaction, the molecular weight of the product decreases concomitantly and the preferred reaction conditions were stated to be[17] 1–2 h at 5–8°C.

Since the preparation process described in the literature[16, 17] does not involve a neutralisation step the products obtained from chitosan, or from a chitin having an appreciable concentration of amine groups, may have quite complex structures. The phosphate ester group will be present in the free acid form while many of the amine groups will be in the form of the methanesulphonate salt. Zwitter-ion type structures may also be formed between adjacent phosphate ester groups and protonated amine groups, with elimination of a molecule of methanesulphonic acid (Figure 4.1), if the affinity of the phosphate ester anion for the protonated amine group is greater than that of the methanesulphonate ion. This will introduce

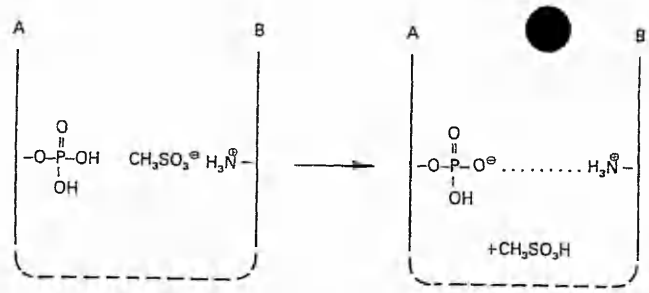


FIGURE 4.1 Formation of salt linkage in chitosan phosphate. Broken line indicates that chain sections A and B may be sections of the same chain or of two different chains

inter- or intrachain ionic bonds – salt linkages -- the likelihood of formation increasing with increase in either the *DS* of the product or the extent of deacetylation of the starting chitin or chitosan.

Such salt linkages have been invoked to explain the dependence of the solubility on the *DS* in the case of chitosan phosphates.[17] Although chitin phosphates are water-soluble at any *DS* level above a minimum value, chitosan phosphates are insoluble in water at high *DS* values and this has been attributed to the formation of such interchain salt linkages, thereby producing a polyelectrolyte complex (section 6.1.4). It would be of interest to evaluate the solubility of the fully neutralised product sodium chitosan phosphate.

Aqueous solutions of both chitin phosphate and chitosan phosphate show the viscosity behaviour typical of polyelectrolytes, the viscosity number increasing rapidly with decreasing polymer concentration in the absence of added low-molecular-weight electrolyte, but giving a rectilinear decrease in viscosity number with decrease in polymer concentration in the presence of low concentrations of electrolyte. However the LVN value for a chitin phosphate sample was considerably lower than that for a chitosan phosphate sample prepared under similar conditions, the values being approximately 15 $cm^3\ g^{-1}$ and 100 $cm^3\ g^{-1}$ respectively for a chitin phosphate having $DS = 1.0$ and a chitosan [0.55] phosphate having $DS = 0.4$, when measured in 0.05M NaCl solution. Furthermore, while the viscosities of chitin phosphate solutions were found to increase with increase in pH because of increasing dissociation of the phosphate group, those of chitosan[0.55] phosphate solutions decrease. This latter decrease has been attributed to a decrease in the extent of protonation of the amine groups as the methanesulphonate salt is neutralised. Nishi *et al.* concluded[17] that the difference in LVN values between the chitin phosphate and chitosan phosphate samples was due to the inherent low pH of the system rather than to differences in molecular weight. They calculated the $\bar{M}_v$ value for chitin phosphate using the Mark–Houwink equation constants for heparin[19] and obtained a value of approximately 1×10^4. However, application of the constants for chitosan[20] to the LVN for chitosan[0.55] phosphate gives $\bar{M}_v = 1.26 \times 10^5$ or 1.49×10^5 depending on whether the constants for 0.2M NaCl or for 0.02M NaCl are used, the NaCl concentration used in determining the LVN for chitosan[0.55] phosphate being 0.05M.

The much larger LVN value for chitosan[0.55] phosphate may be an artefact arising from interchain ionic interactions between $-O\text{-}P(O)(OH)O^{\ominus}$ and $-\overset{\oplus}{N}H_3$ groups, as suggested by Nishi *et al.*,[17] or it may be a true difference arising from the much greater stability of chitosan towards acid hydrolysis compared with that of chitin, so that the former would be expected to undergo much less chain scission during reaction in methanesulphonic acid. Again it would be of interest to compare the LVN values for neutral products, sodium chitin phosphate and sodium chitosan phosphate.

The same workers have also measured the titration curves for these two products and for D-glucose 6-phosphate. All three show two pK_a values for the phosphate group, those for the polymeric phosphates being lower than those for the monomeric phosphate. A comparison of the values for the chitin and chitosan phosphates shows that the lower pK_a values for each polymer are similar (3.65 and 3.7 respectively, 4.2 for D-glucose 6-phosphate) but there is a much larger difference between the two higher pK_a values (8.2 and 8.5 respectively, 8.65 for D-glucose 6-phosphate). Furthermore, the titration curve for chitosan[0.55] phosphate shows an additional, broad inflection point in the pH 6–7 region due to the presence of the amine groups.

Insoluble derivatives of chitin phosphate and chitosan phosphate were prepared by gradual addition of adipoyl chloride to the reaction mixture. The crosslinked products precipitated out of the reaction mixture and could be easily collected and purified.[18]

4.1.3 Sulphates

This has been studied more extensively than any of the other inorganic esterification reactions mainly, but not exclusively, in a search for heparin substitutes.

Non-specific sulphation reactions

The earliest attempt at sulphation was that of Karrer *et al.*[9] who sulphated chitin using a mixture of 3 parts chitin:30 parts pyridine:7 parts chlorosulphonic acid at 0°C. The product was isolated as its sodium salt and had 14.4% S, which is equivalent to a *DS* of 1.7. However its physiological

activity was very low, presumably because the sulphate groups were predominantly, if not completely, *O*-sulphate ester groups. Once the suggestion[21] that the anticoagulant behaviour of heparin depends on the presence of the *N*-sulphate groups* had been confirmed,[22, 23] attention switched to sulphation of chitosan, two papers being published simultaneously.[23, 24] Dozzi *et al.*[23] demonstrated that the contribution of *N*-sulphate groups to anticoagulant activity is far greater than that of *O*-sulphate groups but gave no description of their preparation route. At the same time Wolfrom *et al.*[24] described the preparation of chitosan sulphate by the heterogeneous reaction between pyridine-swollen chitosan and chlorosulphonic acid in pyridine, the reaction being carried out at 100°C for 1 h. The water-soluble product was isolated in the sodium salt form and found to contain two *N*-sulphate groups and one *O*-sulphate group per anhydrochitobiose unit. The anticoagulant activity was 56 International Units per mg, heparin has an anticoagulant activity ≥ 100 IU mg^{-1}, and the toxicity was approximately twice that of heparin. This increased toxicity was attributed to the high molecular weight of the chitosan sulphate.

Hackman[5], using similar reaction conditions to those used by Wolfrom *et al.*,[24] obtained chitin sulphate having a *DS* of 1.1. Enzymatic hydrolysis of the sodium chitin *O*-sulphate yielded D-glucosamine in slight excess over that obtained on hydrolysis of the starting chitin, indicating that a limited amount of de-*N*-acetylation occurs during sulphation under these conditions. The use of chlorosulphonic acid–pyridine mixtures to sulphate chitin was also examined by Cushing *et al.*[25, 26] and rejected on the grounds of lack of reproducibility and the poor colour of the product. Instead they used $ClSO_3H$ in an inert solvent, 1,2-dichloroethane, at 25°C. Products having 13.2–15% S (*DS* = 1.45–1.8) and $\bar{M}_w$ values of 1.2×10^4–1.7×10^4 were obtained. The sodium chitin *O*-sulphate could be bleached with H_2O_2 without any apparent degradation. The anticoagulant activity of freshly prepared material was 22–34 IU mg^{-1} but there was a drop on storage in the solid state, while in unbuffered solution there was a gradual fall in pH, owing to hydrolysis of the sulphate groups, leading to a decrease both in the anticoagulant activity and in the solution viscosity. Omission of the dialysis step in the initial purification process gave products having improved stability. The sulphation process caused considerable chain cleavage which was considered beneficial since it reduces the toxicity problem associated with higher-molecular-weight materials.[24]

A patent issued the same year as the paper of Cushing *et al.*[25] claims the use of SO_3 complexed with pyridine, dioxane, *N,N*-dimethylaniline or 2,2′-dichlorodiethylether for sulphating chitin.[27] The SO_3–pyridine complex was the preferred one since pyridine could also be used as the reaction medium. The reaction mechanism proposed is shown in Figure 4.2.

*The product formed on sulphation of the amine groups of chitosan is more correctly designated as an *N*-substituted sulphamic acid but the normal convention, which is used here for convenience, is to distinguish between the two by use of the terms *O*-suphate and *N*-sulphate.

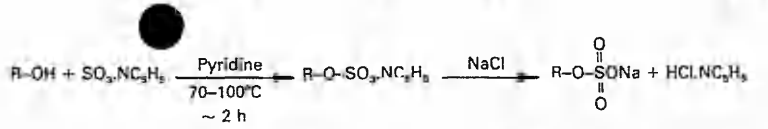


FIGURE 4.2 Proposed mechanism of formation of chitin *O*-sulphate by reaction of chitin with an SO_3–pyridine complex[27]

Although Cushing's patent[26] includes chitosan in a list of polysaccharides suitable for modification by sulphation, no examples were given. However within a couple of years patents were issued dealing with the sulphation of chitosan[28] and of *N*–formylchitosan.[29, 30] The first of these patents claimed the use of SO_3–SO_2 mixtures at their reflux temperature (~ −10°C). Products having 9–20.6% S (*DS* = 0.75–3.0) were obtained. The other two patents[29, 30] describe the preparation of *N*-formylchitosan sulphates either by sulphation of *N*-formylchitosan, prepared by treating chitosan with concentrated HCOOH at ~ 100°C in the presence of pyridine, or by *N*-formylation of chitosan sulphate. Sulphation was carried out at 20°C using either $ClSO_3H$–$CHCl_3$ or $ClSO_3H$–$HCONH_2$ mixtures. The use of H_2O_2 to bring about a reduction in the molecular weight was also claimed.

Wolfrom and Shen Han[31] reprecipitated chitosan[0.1] and subjected it to solvent exchange through the series water→ethanol→absolute ethanol→diethyl ether→pyridine. The final product was suspended in pyridine and treated with a $ClSO_3H$–pyridine mixture for 1 h at 100°C to give, after neutralisation and purification, a product containing two sulphate groups per anhydro-D-glucosamine unit. The presence of acid-sensitive *N*-sulphate groups and the absence of free amine groups was demonstrated, indicating that the product was a chitosan *N*-sulphate-*O*-sulphate derivative – chitosan *N,O*-disulphate. Wolfrom and Shen Han also reported a homogeneous sulphation process by treatment of chitosan, reprecipitated and solvent-exchanged through the same series except that the pyridine was replaced by DMF, with an SO_3–DMF complex in an excess of DMF, the reaction taking place at room temperature. The proposed reaction mechanism involves formulation of the SO_3–DMF complex as a dipolar ion which is susceptible to nucleophilic attack by either the amine or hydroxyl groups of chitosan (Figure 4.3). Again the product contained one *N*-sulphate and one *O*-sulphate groups per anhydro-D-glucosamine residue.

The chitosan sulphate obtained using $ClSO_3H$–pyridine had an anticoagulant activity of 56 IU mg^{-1} but its acute LD_{50} was 380 mg kg^{-1}, more than twice the toxicity of heparin (LD_{50} = 750 mg kg^{-1}), whereas that

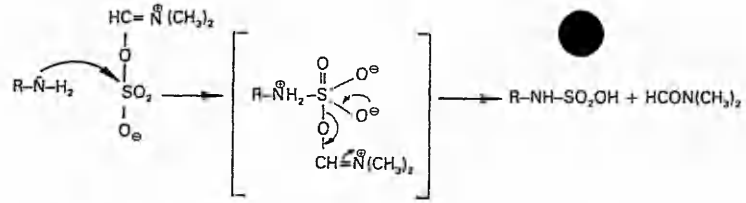


FIGURE 4.3 Proposed mechanism of formation of chitosan *N*-sulphate and *O*-sulphate by reaction of chitosan with SO_3–DMF[31]

prepared using the SO_3–DMF complex had an anticoagulant activity of 50 IU mg^{-1} and an acute LD_{50} of 775 mg kg^{-1}. The major difference between the two products was their molecular weights, being 4.56×10^5 and 1.86×10^5 respectively (DP = 1280 and 530). The lower toxicity of the second sample was attributed to its lower molecular weight. A patent issued to Wolfrom the previous year also deals with sulphation of chitosan with $ClSO_3H$–pyridine and with SO_3–DMF in DMF but gives no additional information.[32]

Nagasawa and co-workers have published a series of papers[33–35] dealing with the reaction between H_2SO_4 and a number of polysaccharides including chitin[33, 34] and chitosan.[33, 35] Treatment of chitin with 96 wt-% H_2SO_4 for 2 h at −5°C gave a product which could be separated into a dialysable and a non-dialysable fraction, both fractions having a $DS \sim 1.2$. Similar treatment of a chitosan[0.08] sample yielded only a non-dialysable product having a DS of ~2. The hydrolysis constant for the acid-labile sulphate of chitosan sulphate in 0.1M HCl at 99.5°C was found to be 1.17×10^{-3} s^{-1}, very similar to the value of 1.03×10^{-3} s^{-1} reported previously for heparin.[36] This was taken as evidence of the presence of N-sulphate groups in the product. It was shown that neither extending the reaction time from 2 to 10 h, nor varying the reaction temperature between 0 and 30°C, had any noticeable effect on the level of substitution of the product, but that reducing the H_2SO_4 concentration from 96 to 80 wt-% reduced the DS from 2 to 0.7.

Selective 6-O-sulphation reactions

More recently two methods for the selective 6-*O*-sulphation of chitosan have been developed. The first[37] involves the use of a 2:1 mixture of 95 wt-% H_2SO_4 and 98 wt-% $ClSO_3H$ at 0–4°C. Reaction times of about 1 h are used and the product is isolated by precipitation. Chitosan[0.20] was used and characterisation of the product by conductiometric titration, IR and ^{13}C NMR spectroscopy showed the DS to be 0.95–1.0 and that sulphation had taken place at the C(6)OH group. The spectrum in D_2O

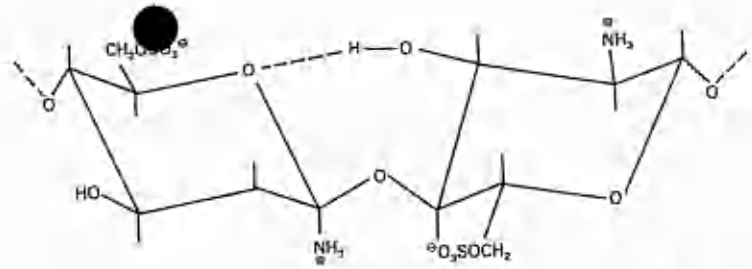


FIGURE 4.4 Proposed Zwitter-ionic interaction between C(6′)OSO_3^- and C(2)$\overset{\oplus}{N}H_3$ leading to increased rigidity of chitosan 6-*O*-sulphate chains at low pH values[37]

solution was compared with that for a low-molecular-weight chitosan (DP ~30) and whereas the signals for C(1)–C(5) of chitosan 6-*O*-sulphate were shifted by relatively small amounts compared with their position in the spectrum for the chitosan oligomer, the C(6) signal is shifted significantly downfield (−6.2 ppm). The more or less complete absence of any C(1) signal attributable to reducing end-groups indicates that the sulphation reaction did not involve concomitant hydrolysis of the polymer chain.

The authors argued that in alkaline solution chitosan 6-*O*-sulphate should behave like a typical polyanion and that the local conformation of its chains should be similar to that of cellulose or chitin, with ribbon-like segments stabilised by C(3′)OH . . . O(5) intramolecular hydrogen bonds. However at low pH values there is the possibility of forming Zwitter-ionic structures having intramolecular electrostatic bonds between C(6′)OSO_3^- . . . $\overset{\oplus}{N}H_3$ (2)C and these should lead to an increase in chain rigidity (Figure 4.4).

The second method[38, 39] involves protecting the amine group by complexing it with Cu(II) ions, followed by treatment of a DMF suspension of the Cu(II)–chitosan complex with SO_3–pyridine at 25–75°C for 8–48 h. Elimination of Cu(II) ions from the Cu(II)–chitosan 6-*O*-sulphate complex was achieved by percolating an aqueous solution of the complex through a selective cation exchange resin. At 25°C the reaction shows an induction period of about 16 h followed by a very rapid increase in reaction rate and a levelling off after about 24 h (Figure 4.5). At a mole ratio of SO_3–pyridine:Cu(II)–chitosan of 6:1, a reaction time of 16 h at 25°C gives a product having a DS of approximately 1. The selectivity of the reaction was confirmed by IR and ^{13}C NMR spectroscopy. Higher DS values than 1 could be achieved by increasing the temperature, a product having DS = 1.8 being obtained after 16 h reaction at 75°C using a 6:1 mole ratio of reactants to substrate. The additional sulphation occurred partially at C(3)OH but mainly at $C(2)NH_2$, as shown by ^{13}C NMR spectroscopy,

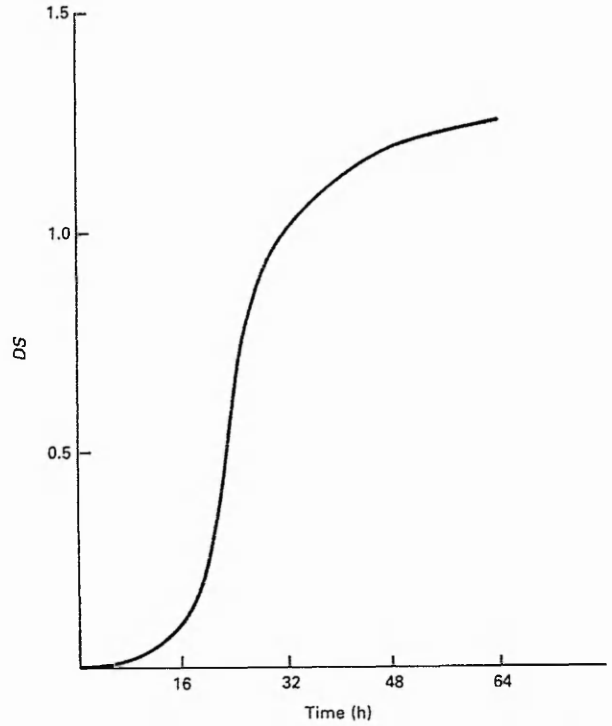


FIGURE 4.5 Rate of formation of chitosan 6-*O*-sulphate by treatment[39] of chitosan–Cu(II) complex with SO_3–pyridine complex at 25°C. Mole ratio of SO_3:Py = 6:1

while the molecular weight (light scattering) of a product obtained after 24 h reaction time at 25°C was 1.3×10^6 with an LVN of 270 $cm^3\ g^{-1}$ measured in 0.2M NaCl solution.

A very elegant study of chitosan 6-*O*-sulphate by Stivala *et al.*[40] using small-angle X-ray scattering led to the conclusion that the sample, as prepared by Naggi *et al.*,[37] is quite highly branched. The study determined the solution parameters of chitosan 6-*O*-sulphate and compared them with those of heparin;[41] the most important parameters are shown in Table 4.2.

The total length L, both that calculated on the basis of the molecular weight, monomer molecular weight and length per monomer unit, and that determined experimentally, are very similar for heparin but differ considerably for chitosan 6-*O*-sulphate. In the latter case the calculated value of L is more than twice the experimentally measured value. Similarly the experimental and calculated mass per unit length (M_u) of heparin are very similar while those for chitosan 6-*O*-sulphate differ, the experimentally determined value being more than twice the calculated value, the latter being the value calculated on the basis of a linear, unbranched structure.

TABLE 4.2 *Solution parameters of chitosan 6-O-sulphate and heparin*[40]

	Chitosan 6-O-sulphate	*Heparin*
$\bar{M}_w$	3.16×10^4	1.29×10^4
L(exp.)	271 Å	237 Å
L(calc.)	620 Å	260 Å
Persistence length	20.3 Å	21.1 Å
M_u(exp.)	117 Å	54.5 Å
M_u(calc.)	51 Å	52.7 Å
$[\eta]$	80 $cm^3\ g^{-1}$	417 $cm^3\ g^{-1}$

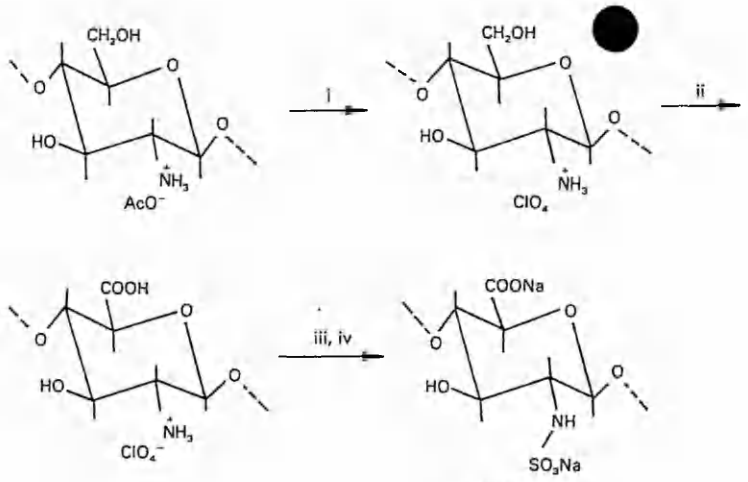
Finally, since $\bar{M}_w$ for the chitosan 6-*O*-sulphate sample is more than twice that for the heparin sample, the LVN of the former would be expected to be greater than the LVN of the heparin whereas in fact the latter is considerably greater. These three factors all support a branched structure for the chitosan 6-*O*-sulphate investigated.

Branching was explained as arising from chain degradation through acid hydrolysis, and linking of the oligomer chains to the backbone through sulphate diester groups formed between a backbone C(6)OH and an oligomer C(6)OH. Evidence of sulphate diester groups was obtained by IR spectroscopy, a weak band at 1390 cm^{-1} being observed in addition to the much stronger band at about 1230 cm^{-1} characteristic of the sulphate monoester group. Consideration of the M_u (calc.) value, together with the reported potentiometric results,[37] enabled a structure to be proposed in which one oligomer chain having a DP ~15 is attached for every ten monomer units in the backbone chain (a DS of ~0.1).

The preparation of chitin sulphate under homogeneous conditions has recently been reported.[42] Chitin dissolved in DMAc–LiCl (50 g dm^{-3}) was treated with SO_3–pyridine complex, in one instance after subjecting the chitin–DMAc–LiCl solution to ultrasonic treatment. In both cases ^{13}C NMR spectroscopy showed selective substitution of the C(6)OH groups, the DS values being 0.3 and 0.9.

Sulphation of chitin and chitosan derivatives

Other workers have studied the preparation and properties of sulphate derivatives of modified chitins and chitosans. The first work of this type was that of Horton and Just[43] who prepared the *N*-sulphate derivative of poly[β(1→4)-2-amino-2-deoxy-D-glucopyranuronic acid] (**4.3**). The synthesis route, which is outlined in Figure 4.6, involved formation of the perchloric acid salt to protect the amine group, followed by oxidation of the C(6)OH with CrO_3–glacial acetic acid, sulphation by the procedure of Wolfrom and Shen Han[31], and neutralisation. The product had an anticoagulant activity of 25.8 IU mg^{-1} and an LD_{50} of 237 mg kg^{-1}. The low



4.3

Reagents: i = $HClO_4$; ii = CrO_3–CH_3COOH; iii = $ClSO_3H$–pyridine
iv = NaOH

FIGURE 4.6 Route for the preparation of the *N*-sulphate of poly[β(1→4)-2-amino-2-deoxy-D–glucopyranuronic acid][43]

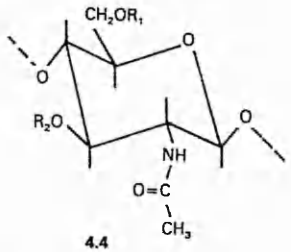
anticoagulant activity and high toxicity relative to heparin was attributed to its molecular weight, 4.3×10^5, being very high compared with that of therapeutic grade heparin which is $\sim 1.3 \times 10^4$.

Okiei *et al.*[44] prepared chitin *O*-sulphate and carboxymethyl chitin *O*-sulphate by reaction of chitin or carboxymethyl chitin respectively, with $ClSO_3H$–pyridine. The products (Table 4.3) were examined for their inhibitory effect on thrombin activity and it was found that the carboxymethyl chitin *O*-sulphate (**4.4c**) had similar thrombin inhibition activity to that of heparin while chitin *O*-sulphate (**4.4d**) was much less effective.

Hirano *et al.*[45] prepared sulphate derivatives of chitosan and *N*-acylated chitosans (Table 4.4). The *N*-acetylchitosan di-*O*-sulphate (chitin di-*O*-sulphate, **4.5c**) was approximately twice as active as heparin as an anticoagulant but had much less lipoprotein lipase activity, while the chitosan *N*-sulphate-*O*-sulphates (**4.5a, b**) were 10–60% more active as anticoagulants than heparin and had 2–3 times the lipoprotein lipase activity.

Finally Muzzarelli *et al.*[46, 47] have prepared *N*-carboxymethyl chitosan sulphates and examined their behaviour as blood coagulants. The starting *N*-carboxymethyl chitosan was prepared by the reductive alkylation technique[48] (section 4.3.3) and this was then sulphated with either a 1:1 H_2SO_4:$ClSO_3H$ mixture[37], SO_3–DMF in DMF[31], or $ClSO_3H$–pyridine, to give the mono-, di- and trisulphate derivatives respectively (Table 4.5). It was found that sulphation of the C(3)OH group, which is required in the preparation of the *N*-carboxymethyl chitosan 3,6-*O*-*N*-trisulphate, necessi-

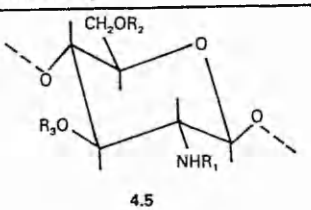
TABLE 4.3 *Structures of sulphated chitin derivatives evaluated for thrombin inhibition activity*[44]



4.4

	R_1	R_2	*Molecular weight*
a	0.8 CH_2COONa 0.2 H	H	6.3×10^4
b	0.8 CH_2COONa 0.14 SO_3Na 0.06 H	H	1.8×10^4
c	0.56 CH_2COONa 0.44 SO_3Na	0.37 SO_3Na 0.63 H	2.4×10^4
d	0.69 SO_3Na 0.31 H	H	2.0×10^4

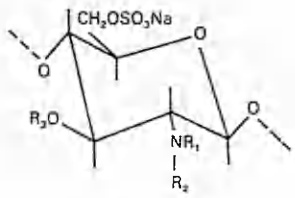
TABLE 4.4 *Structures of sulphated derivatives of chitosan and* N*-acylchitosans evaluated for anticoagulant activity*[45]



4.5

	R_1	R_2	R_3
a	SO_3Na	SO_3Na	0.63 SO_3Na 0.37 H
b	0.92 SO_3Na 0.08 CH_3CO	0.73 SO_3Na 0.27 H	H
c	CH_3CO	SO_3Na	SO_3Na
d	$CH_3(CH_2)_4CO$	SO_3Na	0.8 SO_3Na 0.2 H
e	H	0.74 SO_3Na 0.26 H	H
f	SO_3Na	0.54 CH_2COONa 0.36 H	H

TABLE 4.5 *Structures of sulphated N-carboxymethyl chitosans evaluated for anticoagulant activity*[47]



4.6

	R_1	R_2	R_3	Reagent
a	0.5 CH_2COONa 0.5 CH_3CO	H	H	H_2SO_4–$ClSO_3H$
b	0.58 CH_2COONa 0.42 CH_3CO	H	0.8 SO_3Na 0.2 H	SO_3–DMF in pyridine
c	0.50 CH_2COONa 0.50 CH_3CO	0.5 SO_3Na 0.5 H	SO_3Na	$ClSO_3H$–pyridine

tated prior hydrolysis of the *N*-carboxymethyl chitosan by boiling in 6M HCl for at least 10 minutes (since the starting chitosan in this case was a chitosan[0.50], the maximum *DS* for sulphate groups is actually 2.5 and not 3.0). This increase in the extent of substitution with decrease in the molecular weight of the chitosan was also observed on fractionating the *N*-carboxymethyl chitin disulphate on Bio-gel P-100, when fractions having molecular weights from 4.5. $\times 10^4$–10 $\times 10^4$ had sulphur contents of 12.4–9.5%.

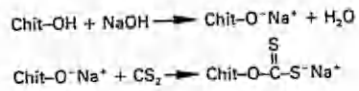
All showed very good anticoagulant activity with the low-molecular-weight fraction of the disulphate the best, being equal in effectiveness to heparin. The better activity of the *N*-carboxymethyl chitosan disulphate was considered to be due to the presence of a high proportion of *N*-acetyl groups, in agreement with the results of Hirano *et al.*[45] None of the samples exhibited adverse effects on red blood cells.

A naturally occurring chitin sulphate–chitin 6-*O*-sulphate, $DS = 1$, molecular weight (GPC) = 4×10^4, has been isolated from the test of tunicates *H. roretzi* and *S. plicata* and its interaction with lectins studied.[49]

4.1.4 *Xanthates*

Formation of alkali chitin

Preparation of chitin xanthate requires the initial formation of alkali chitin followed by reaction between the alkali chitin and carbon disulphide:



Thus the formation of alkali chitin is of considerable importance in the preparation of chitin xanthate and of a number of chitin ethers (section 4.3.1).

Three patents granted to Thor[50–52] cover both the preparation of alkali chitin and the xanthation step. Thor found that the amount of NaOH bound/*N*-acetylglucosamine residue increases with increase in the concentration of the steeping solution and with decrease in the steeping temperature. A plot of his tabulated results shows (Figure 4.7) that the Absorption *versus* Concentration curve is S-shaped and similar to those reported for various cellulose substrates,[53] although the concentration at which the

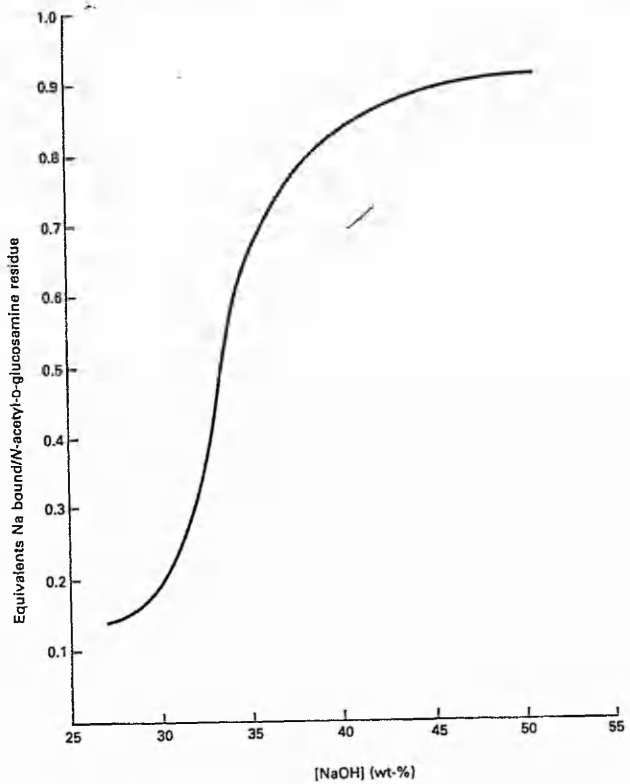


FIGURE 4.7 Relationship between NaOH combined with chitin and the concentration of the NaOH steeping solution at 25°C[50–52]

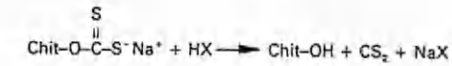
steepest rise occurs, 30–35 wt-% NaOH, is higher than for cellulose which shows this rapid increase in NaOH absorption between 8–16 wt-% NaOH depending on the particular cellulose substrate. Since Thor worked with chitin from shrimp, that is α-chitin, this decreased readiness to swell may be attributed to the greater extent of interchain hydrogen bonding in α-chitin compared with either cellulose I or cellulose II (section 1.4.2).

Thor[50–52] emphasised the advantage of working at low temperature stating that it is 'generally desirable to work at somewhat below the maximum permissible temperature,' but it was Danilov and Plisko[54] who appear to have been the first actually to freeze the alkali chitin mixture, their process involving subjecting the alkali chitin to up to three cycles of freezing and thawing and the removal of any residual aqueous liquor after each freezing treatment. This technique of freezing alkali chitin has been used by a number of workers[55–60] including in some instances freezing under vacuum.[56, 58] Tokura *et al.*[60] claim that high alkali concentrations, addition of a surfactant such as sodium dodecylsulphonate, and freezing for at least 10 h gives the best alkali chitin, while Nishi *et al.*[56] commented that 'generally chitin seems to differ from cellulose in the respect that treatment at low temperatures increases its reactivity.' Noguchi *et al.*[55] reported that if temperatures of 20°C are used in preparation of alkali chitin, or even temperatures of 11–13°C for prolonged periods, the regenerated fibres tend to be water-soluble or else gel and adhere to each other during spinning. They suggested that this was due to some degree of deacetylation of the chitin and it is possible that they were the first to obtain the randomly deacetylated chitin subsequently extensively studied by Sannan and Kurita (section 2.5.7).

Xanthation of alkali chitin

Less work has been carried out on the xanthation step itself. Thor[50–52] claimed two methods of preparation; in the first the shredded, relatively dry alkali chitin is treated in a closed vessel for 1–6 h with 0.25–0.5 parts CS_2 per 1 part original chitin, followed by addition of an ice–water mixture to bring about solution of the chitin xanthate at a temperature <15°C. The second method involves mixing the shredded alkali chitin with about 10 parts ice per 1 part original chitin in order to dissolve or disperse the alkali chitin, the mixture then being reacted with CS_2 at <15°C to form the xanthate.

Either method produces a solution of the xanthate from which chitin may be regenerated by extrusion of the solution into a bath containing acid and a low-molecular-weight electrolyte such as $(NH_4)_2SO_4$.



Chitin xanthate is not normally used in its own right but mainly as an intermediate in the preparation of regenerated chitin, although its use as an adhesive has been reported.[61] It has been used in preparation of chitin fibres,[50–52, 62, 63] chitin films[50–52, 62] and chitin sponges.[62] Tokura *et al.*[57, 63] have stated that the properties of chitin fibres produced from chitin xanthate are inferior to those spun from solutions of chitin in 99% HCOOH but this may be due to the use of non-optimum conditions, particularly in the coagulation/regeneration steps.

4.2 ORGANIC AMIDES, ESTERS AND RELATED DERIVATIVES

4.2.1 N-Acyl derivatives of chitosan

N-Acylation using carboxylic acids

N-Acylation may be brought about in a number of ways, not all of which are necessarily applicable to the preparation of all *N*-acyl derivatives.

The simplest procedure is reaction between a carboxylic acid and chitosan, and the preparation of *N*-formylchitosan by heating a solution of chitosan in 100% HCOOH at 90°C, with the gradual addition of pyridine, has been claimed,[29] while Aiba[64] has followed the course of *N*-acetylation of chitosan in 20 vol.-% acetic acid. This latter method was much slower, the extent of *N*-acetylation being approximately 50% after 300 h at 80°C.

Alternatively, heating the as-cast films prepared from solutions of chitosan in the appropriate dilute aqueous acid has been shown to bring about some degree of *N*-acylation. Thus a film cast from a mixture of equal proportions of chitosan and phthalic acid in 0.33M acetic acid became insoluble in water, 0.33M acetic acid or dilute NH_4OH on heating at 50°C for 48 h, and similar behaviour was found with the as-cast film prepared from a solution of chitosan in maleic acid.[65] Insolubilisation of chitosan acetate films on heating at temperatures >60°C has also been observed.[66]

N-Acylations involving carbodiimide-mediated reactions have also been carried out. Yaku and Yamashita[67] acetylated chitosan film with acetic acid and dicyclohexylcarbodiimide (DCC) using aqueous DMF as the reaction medium, but did not achieve complete acetylation when the reaction medium contained more than 40 vol.-% H_2O. Kurita *et al.*[68] carried out homogeneous reactions using water-soluble chitosan[0.5] and a 20-fold excess of both acetic acid and DCC, in aqueous DMF. Again the extent of *N*-acetylation achieved was dependent on the water content of the reaction medium; with either 55 or 60 vol.-% H_2O the products remained in solution in the reaction mixture and on isolation were found to be soluble in dilute HCl, indicating incomplete *N*-acetylation, but with 40 vol.-% H_2O the product precipitated out as a highly swollen gel that on working up was

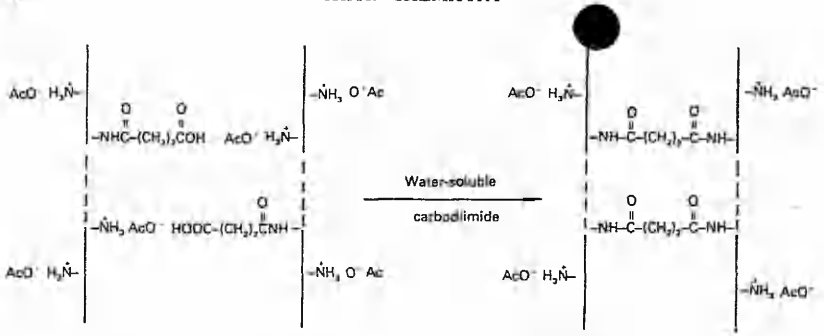


FIGURE 4.8 Crosslinking of partially *N*-succinylated chitosan through carbodiimide-mediated amide formation[69]

found to be chitin[1.0]. The IR spectrum showed no evidence of any *O*-acetylation having occurred. The use of a water-soluble carbodiimide, 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide, and no DMF was also examined but negligible *N*-acetylation was achieved, a result that was taken as support for the contention that the reaction is hampered by too high a water content.

Yamaguchi *et al.*[69] used a water-soluble carbodiimide to induce gelation in solutions of partially *N*-succinylated chitosan and hydroxyethyl chitosan through crosslink formation by reaction between pendant carboxylic acid groups and amine groups on the chitosan chain (Figure 4.8). Roberts and Taylor[70] used carbodiimide-mediated reactions to couple chitosan to preformed crosslinked polymer beads of poly(methacrylic acid) resin and carboxymethyl Sephadex. Three techniques were investigated: direct coupling by treatment of the beads with a water-soluble carbodiimide, 1-cyclohexyl-3-[2-(*N*-methylmorpholino)ethyl]carbodiimide, followed by the addition of a solution of chitosan; coupling through active ester formation[71] by treatment of the beads with 1-hydroxybenzotriazole and the carbodiimide; and coupling by the Sundaram technique[72] using 2-ethoxy-1-ethoxycarbonyl-1,2-dihydroquinoline. Both chitosan[0.14] in 0.1M acetic acid and water-soluble chitosan[0.58] in distilled water were used with each technique and, in the case of direct coupling, the effect of incorporating a spacer was also examined. The results showed that chitosan was coupled more efficiently under neutral conditions and that the amount bound to the beads increased with increase in the concentration of added NaCl. It was concluded that the effect of acid is to increase both the chain dimensions and interchain repulsion through protonation of the amine groups, thereby increasing the extent of screening of adjacent potential sites by chains already attached to the surface.

N-Acylation using acyl chlorides

Acid chlorides, because of their high reactivity and hence their lack of discrimination, have found little application in the selective *N*-acylation of chitosan. Kurita *et al.*[68] attempted to *N*-acetylate water-soluble chitosan[0.50] by an interfacial reaction technique but only a very limited amount of *N*-acetylation was obtained. This was attributed to the steric arrangement of the chitosan molecules at the liquid interface limiting the accessibility of the amine groups to the acetyl chloride molecules. In a later paper[73] acetyl chloride was used to *N*-acetylate a highly swollen chitosan precipitate prepared by addition of a chitosan solution in aqueous acetic acid–methanol to a large excess of pyridine, the acetyl chloride being added to the stirred mixture. The efficiency of the reaction was very low, at a mole ratio of 307:1 for CH_3COCl:$-NH_2$ the starting chitosan[0.12] was converted to chitosan[0.34]. The efficiency was improved by solvent exchange of the precipitate five times with pyridine, followed by addition of the acetyl chloride in THF. Under these conditions a mole ratio of 25:1 gave chitin[0.84]. In both series the acetylation reaction, although carried out at 0°C, was not specific for the amine group, the presence of a weak band at 1730 cm^{-1} in the IR spectra indicating some esterification of the hydroxyl groups. The ester groups were removed by treatment with 1M methanolic KOH at room temperature for 4 h.

N-Acylation using acyl anhydrides

The most common reagents for *N*-acylation of chitosan are, without doubt, the acyl anhydrides and these have been used under both heterogeneous and homogeneous conditions, principally the latter. One detailed study has been made of heterogeneous *N*-acylation.[74,75] Three systems were examined: (a) acetic anhydride–glacial acetic acid–$HClO_4$; (b) acetic anhydride at room temperature for 120 h followed by refluxing in acetic anhydride for 2 h; (c) acetic anhydride–methanol at room temperature. Of these, the last method was found to be the most efficacious.

The effect of solvent on the ease of *N*-acetylation of films of chitosan[0.22] was examined with a number of solvents covering the solubility parameter range of 7.4–19.2 Hildebrands and only methanol and formamide gave any appreciable *N*-acetylation. *N*-Acetylation was also carried out in a series of binary solvent mixtures; methanol–ethanol, methanol–formamide, methanol–propanol and ethanol–formamide, and the extent of reaction determined after 30 minutes. The results suggest that although there is a considerable increase in the rate of *N*-acetylation in the solubility parameter value range $12.75<\delta<14.75$ Hildebrands, with a maximum at $13.1<\delta<13.5$ Hildebrands, the crucial factor is not the

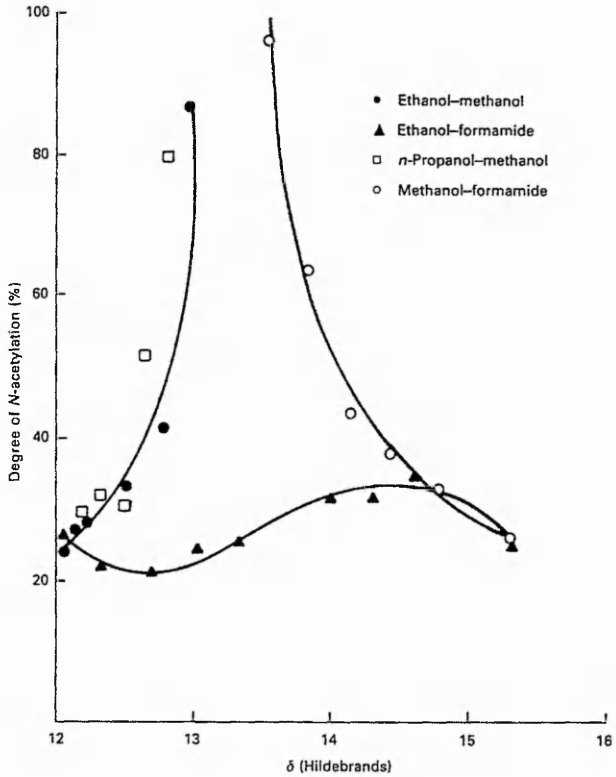


FIGURE 4.9 Degree of *N*-acetylation, after 30 minutes' reaction, as a function of the solubility parameter value (δ) of the reaction mixture. Films not presteeped[74, 75]

δ value of the reaction medium but rather the presence of methanol (Figure 4.9).

However on presteeping films in the reaction media for 24 h prior to the addition of acetic anhydride, and measuring the extent of *N*-acetylation after 5 minutes, it was found that reaction media having the same δ value give the same degree of *N*-acetylation, and that those having $13<\delta<15.5$ Hildebrands allow complete *N*-acetylation within 5 minutes. This shows that the degree of swelling of chitosan, and hence the accessibility of the amine groups, depends primarily on the δ value of the reaction medium provided sufficient time is allowed for the solvent molecules to penetrate and swell the chitosan. Methanol appears to be unique among the solvents examined in that only a very short time is required for its diffusion into

chitosan so that rapid *N*-acetylation can take place without the need for a presteeping treatment.

The rate of *N*-acylation of chitosan[0.22] with different acyl anhydrides in methanol – acetic, propionic, butyric, hexanoic and benzoic anhydrides – was also examined.[74] The results for butyric anhydride, typical for the aliphatic acyl anhydrides, are shown in Figure 4.10. The induction period decreases with increase in temperature and with decrease in the molecular size of the anhydride, typical of a diffusion-controlled reaction, and could be completely eliminated by a pretreatment in methanol. The Arrhenius energies of activation decreased with increase in molecular size of the anhydride, from 95–90 kJ mol^{-1} for the series acetic, propionic and butyric, to ~75 kJ mol^{-1} for hexanoic anhydride.

Heterogeneous *N*-acylation using highly swollen precipitated chitosan has been extensively studied by Kurita's group. In their first paper on this technique,[68] water-soluble chitosan[0.5] was precipitated out by addition of its aqueous solution to a large volume of pyridine. The precipitate, in the form of a highly swollen gel, was solvent-exchanged several times with

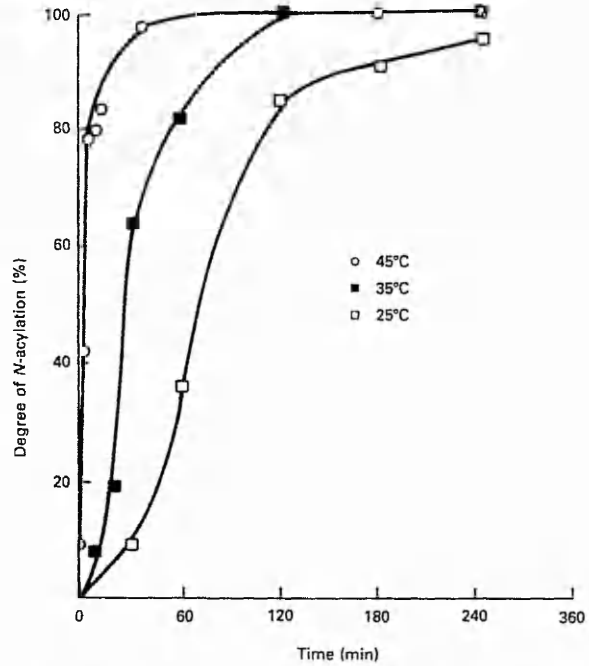


FIGURE 4.10 Rates of reaction for the *N*-butyrylation of chitosan film at different temperatures[74]

fresh portions of pyridine, then suspended in pyridine and acetic anhydride added (60 cm^3 of acetic anhydride for 0.3 g chitosan, a [redacted]-fold excess based on the amine group concentration) and the mixture stirred for 3 minutes at room temperature. The solid, without intermediate drying, was steeped in a saturated $NaHCO_3$ solution to hydrolyse any *O*-acetyl groups and give chitin[1.0] as the final product.

In a subsequent paper[73] a swollen precipitate of chitosan[0.12] was prepared by addition of a solution in aqueous acetic acid–methanol to pyridine. The mixture was not solvent-exchanged and much smaller quantities of acetic anhydride were used so it is not surprising that complete *N*-acetylation was not achieved. The highest mole ratio used was 31:1 which gave chitosan[0.62] after a reaction time of 5 h, but despite incomplete *N*-acetylation some *O*-acetylation also occurred.

In a third paper[76] water-soluble chitosan was prepared by *N*-acetylation of a swollen precipitate of chitosan[0.1]. The required chitosan[0.50] was obtained by treatment of the precipitate with a 20 to 25-fold excess of acetic anhydride and a reaction time of 5 h. Better efficiency was attained if the precipitate was produced by pouring the chitosan solution into pyridine containing the required amount of acetic anhydride, rather than adding the acetic anhydride to the chitosan–pyridine mixture. Use of this latter technique enabled chitosan[0.5] to be produced with only a 6 to 7-fold excess of anhydride.

Prior to these last two papers[73, 76] the group reported the use of this technique to prepare amic acid and imide derivatives of chitosan by reaction with aromatic cyclic carboxylic acid anhydrides: phthalic, trimellitic and pyromellitic anhydrides.[77] The starting material was water-soluble chitosan[0.5] and after precipitation into excess pyridine and several solvent exchanges with pyridine, reaction was carried out at room temperature for 24 h. Pyridine was used as the reaction medium for phthalic anhydride and a 1:1 mixture of pyridine: DMAc for mellitic and pyromellitic anhydrides. In each case an 18-fold excess of anhydride was used.

The products were amic acid derivatives of chitosan (**4.7**, Figure 4.11) with some of the hydroxyl groups esterified. The amic acid groups could be further reacted to form the corresponding imide derivatives by heating under vacuum (**4.8**, Figure 4.11). At temperatures below 150°C cyclisation was incomplete, while above 190°C discolouration due to decomposition occurred. Heating for 3 h at 170°C and 0.1 mbar pressure allowed cyclisation to the imide to proceed smoothly and, in addition, eliminated the ester groups by a cyclisation step that regenerated the acid anhydrides and hydroxyl groups. The cyclisation reaction could be followed by IR spectroscopy.

Both the amic acid (**4.7a**) and imide (**4.8a**) derivatives from phthalic anhydride and the amic acid derivative (**4.7b**) from trimellitic anhydride were soluble in DMSO and these three, together with the imide from

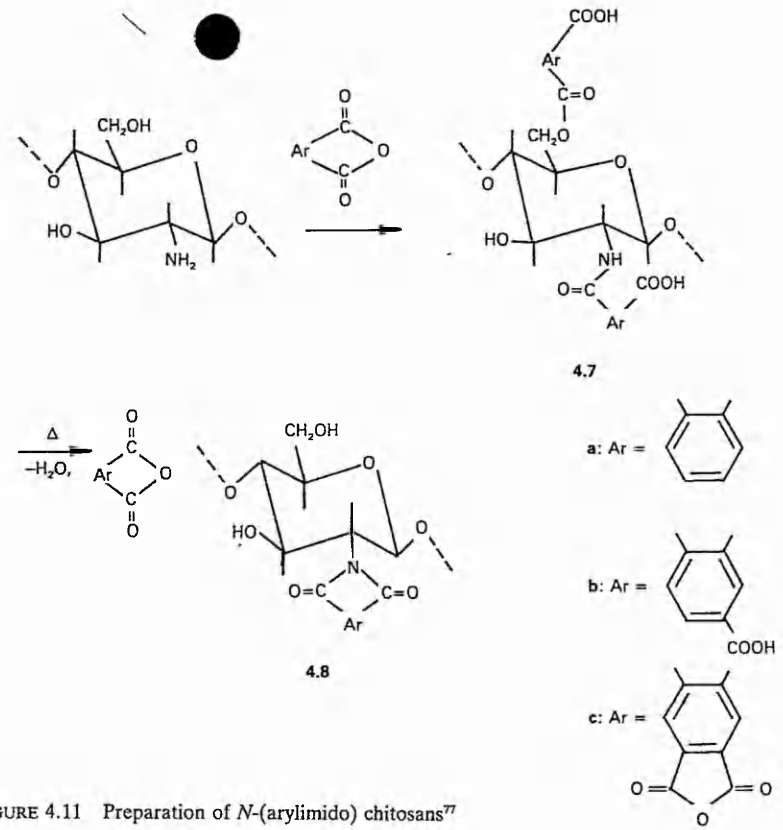


FIGURE 4.11 Preparation of *N*-(arylimido) chitosans[77]

trimellitic anhydride (**4.8b**) were soluble in 0.5M NaOH and saturated $NaHCO_3$ solutions. Both the amic acid and imide derivative from pyromellitic anhydride (**4.7c** and **4.8c**) were insoluble in all the solvents tested, indicating the probable formation of interchain crosslinks by the dianhydride. Derivatives **4.8b** and **4.8c** could be further reacted with amines such as *p*-chloroaniline.

The technique of *N*-acylation under homogeneous conditions was pioneered by Hirano and much of the subsequent work has been carried out by his group. However a considerable number of their papers deal with the production of *N*-acylchitosan gels which are considered in Chapter 6.

Combining the selective *N*-acetylation of chitosan oligomers in aqueous methanol,[78] which is a non-solvent for chitosan, and the facile *N*- and *O*-acetylation of chitosan in aqueous acetic acid[79] led Hirano to investigate the acylation of chitosan in aqueous acetic acid-methanol mixtures.[80] Two solvent systems were examined, the first being a 1:9:40 mixture of acetic acid:water:methanol. Addition of a carboxylic acid anhydride to a solution

TABLE 4.6 *Values of DS and yield for N-acylchitosans prepared under homogeneous conditions in two solvent systems*[80]

N-*acyl group*	*Solvent system*			
	10 vol.-% aqueous acetic acid–methanol		*10 vol.-% aqueous carboxylic acid*	
	DS	*Yield (%)*	DS	*Yield (%)*
Acetyl	1.0	92	1.39[a]	93[a]
Propionyl	1.0	92	1.30[b]	104[a]
Butyryl	1.0	92	1.10[c]	103[c]
Hexanoyl	1.0	96		
Octanoyl	0.83	92		
Decanoyl	1.0	93		
Dodecanoyl	0.92	88		
Tetradecanoyl[d]	0.87	77		
Hexadecanoyl[d]	0.82	87		
Octadecanoyl[d]	0.83	80		
Benzoyl	0.82	90		

[a] 10 vol.-% acetic acid

[b] 10 vol.-% propionic acid

[c] 10 vol.-% butyric acid

[d] precipitate formed

of chitosan[~0] in this solvent, using a mole ratio of 2–3:1 based on the amine group concentration, gave selectively *N*-acylated products with from 82% to 100% of the amine groups acetylated (Table 4.6). The products showed strong amide I and II bands at ~1650 cm^{-1} and ~1540 cm^{-1} but no absorption band at ~1750 cm^{-1}, demonstrating the absence of *O*-acyl ester groups. The second solvent system was a 10 vol.-% solution of the appropriate carboxylic acid and in this case much higher mole ratios of acyl anhydride were required, 20–40:1 based on the amine group concentration. The products showed absorption bands at ~1750 cm^{-1} (C=O) and ~1240 cm^{-1} (C–O), indicating the presence of ester groups, in addition to the amide I and II bands (Table 4.6). The ester groups could be removed by treatment overnight at room temperature in 0.5M ethanolic KOH, the products after this treatment being identical with the appropriate derivatives produced in the first solvent. All of the dried *N*-acylchitosans were gelatinous and hygroscopic, insoluble in cold or boiling water, 50 vol.-% formic acid, 10 vol.-% acetic acid, acetic acid, formamide, DMSO, 50 vol.-% aqueous resorcinol and inorganic acids and alkalis. However the *N*-acetyl-, *N*-propionyl- and *N*-butyrylchitosans were soluble in formic acid.

In the second paper Hirano *et al.*[81] prepared mixed *N*-acyl derivatives of chitosan in a two-step process in aqueous acetic acid–methanol. In the first step 0.25–0.75 molar equivalents of anhydride were added for each mole of amine and the reaction left at room temperature overnight, after which the

partially *N*-acylated chitosan was either isolated and purified or else 1.5–1.75 molar equivalents of a second acyl anhydride were added and the reaction continued for another period of time before isolating the products, many of which gelled during the second acylation step. A wide range of mixed *N*-acyl derivatives was prepared, for all of which complete *N*-acylation and no *O*-acylation was claimed. None of these second stage products were soluble in 10 vol.-% acetic acid which was the only solvent examined, although the first stage products, with *DS* <0.35, were all soluble.

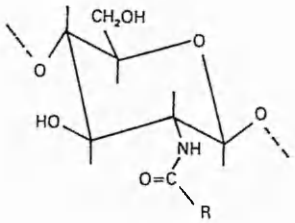
Hirano's group have used this technique, or modifications of it, to produce a range of *N*-acylchitosans – *N*-formyl-, *N*-chloroacetyl-, *N*-glycyl-, *N*-(2-methylpropionyl)- and *N*-pentanoylchitosan – to study the effect of variation in the *N*-acyl group on the susceptibility to biodegradation by chitinase,[82] to prepare a slow-release aspirin carrier by *N*-acylating chitosan with 2-acetoxybenzoic anhydride,[83] and to prepare *N*-acyl derivatives of chitosan *O*-nitrate.[7] They have also studied the distribution of *N*-acetyl groups in partially *N*-acetylated chitosans, their results indicating that the groups are distributed uniformly along the chain.[84]

Another area that this group has looked at is the use of cyclic anhydrides for homogeneous *N*-acylation of chitosan[0.0].[85] A range of derivatives were prepared (**4.9**, Table 4.7) with the extent of *N*-acylation ranging from 43% obtained with diphenic anhydride (**4.9i**) to 80% with *cis*-tetrahydrophthalic andydride (**4.9g**). In all cases the remaining amine groups could be subsequently *N*-acetylated. The products were isolated as the methyl or ethyl ester, free acid or sodium salt. No crosslink or imide formation was observed (see Kurita *et al.*).[77] In another paper[69] the reaction between succinic anhydride and chitosan or glycol chitosan (hydroxyethyl chitosan) was studied in some detail. In all cases *N*-succinylation was incomplete, even with a molar ratio of 14.6:1 only 79% of the amine groups were acylated and there was no evidence of any *O*-acylation. The products from the reaction of chitosan, together with their solubilities, are given in Table 4.8. The *N*-succinyl derivatives of the glycol chitosans were soluble in all three solvents.

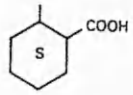
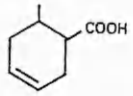
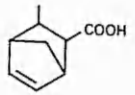
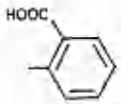
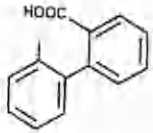
In the work of Hirano's group the *N*-acylation reactions have been allowed to go to completion and no attempt has been made by them to follow the course of partial *N*-acylations. A brief study of this aspect has been made by Aiba[64] who followed the degree of *N*-acetylation at 23°C in a 1:50:20 mixture of acetic acid:water:methanol containing 0.56 molar equivalents of acetic anhydride. This demonstrated that the reaction is quite fast, reaching completion in under 3 h. Other workers have used the technique of partial *N*-acetylation of chitosan in aqueous acetic acid–methanol to prepare water-soluble chitosan (section 2.5.7).

A water-soluble *N*-acylchitosan has been prepared by reaction of D-glucoheptonic acid-γ-lactone with chitosan in aqueous acetic acid–methanol.[86] A reaction time of 144 h was required and the product had a

TABLE 4.7 *N-acylchitosans produced by reaction with cyclic anhydrides under homogeneous conditions*[85]



4.9

	R		*R*
a	$-CH=CH-COOH$	f	
b	$-CH_2-CH(SAc)-COOH$	g	
c	$-C(=CH_2)-CH_2COOH$	h	
d	$-CH_2CH_2CH_2COOH$		
e		i	

DS of ~0.75. Other workers[87] have reacted aldonic acid lactones of D-glucose, D-maltose and D-cellobiose with chitosan hydrochloride at 70°C using ethylene glycol containing triethylamine as the reaction medium. Surprisingly, in view of Yalpani and Hall's later work,[86] these products were stated to be insoluble in water.

A novel form of *N*-acylation, the formation of chitosan–polypeptide graft copolymers through reaction of chitosan with the *N*-carboxyanhydride (NCA) derivative of an amino acid, has been reported.[88, 89] Aqueous solutions of water-soluble chitosan[0.05] were treated at 0°C for 2 h with varying amounts of γ-methyl-L-glutamate *N*-carboxyanhydride in ethyl acetate and the resultant graft copolymers coagulated and washed with

TABLE 4.8 DS *values and solubilities of* N-*succinylchitosans and hydroxyethyl* N-*succinylchitosans*[69]

Substrate	*Mole ratio anhydride: amine group*	DS[a]	*Solubility*[b]		
			Water	*0.5M CH_3COOH*	*0.5M NaOH*
Chitosan	0.24	0.12	–	+	–
	0.49	0.17	S	+	–
	0.74	0.29	±	+	S
	0.98	0.35	+	+	+
	3.67	0.63	+	±	+
	14.6	0.8	+	–	+
Hydroxyethyl chitosan	1.2	0.1	All soluble in all three solvents		
	2.9	0.22			
	5.9	0.53			
	8.8	0.65			
	14.7	0.77			

[a] Average result from saponification, free amine group determination and elemental analysis.
[b] –, insoluble; +, soluble; S, swollen.

aqueous DMF and acetone, and then dried (**4.10a**, Figure 4.12). Treatment of the copolymers with 1M Na_2CO_3 solution at 70°C for 1 h caused hydrolysis of either the *N*-acetyl groups on the chitosan backbone nor the peptide bonds. The resultant copolymers (**4.10b**, Figure 4.12) were purified and isolated by dialysis and freeze drying.

The average *DP* values of the polypeptide side chains were calculated from weight gain and IR spectroscopic results on the assumption that all the amine groups had undergone reaction. Very high grafting conversions were obtained (Table 4.9) and since no homopolypeptide was detected in any of the copolymerisation runs the grafting efficiency was thought to be almost 100%. The IR spectra of the unhydrolysed graft copolymers agree with the proposed structures and at *DP* values ≥ 10 a band at 610 cm^{-1}, due to the presence of the α-helix structure, becomes apparent. Evidence of such a structure is also obtained from X-ray diffraction studies where a peak at $2\theta = 8.3°$ is observed in the diffractogram of the $DP = 10.4$ sample and is more intense in that for a sample having $DP = 20$.

The initial products (**4.10a**) were found to be readily soluble in hexafluoro-2-propanol giving clear, viscous solutions from which tough, flexible, transparent films could be cast. They were also soluble in dichloroacetic acid but insoluble in DMF, DMAc, DMAc–LiCl, HMPA and DMSO. Some of these graft copolymers swelled in DMSO, the extent of swelling depending on the *DP* of the side chains and reaching a maximum at an average *DP* of 4. The decrease in swelling with further increase in side chain *DP* was ascribed to the formation of ordered structures by the polypeptide chains, including the adoption of an α-helix conformation