CHITIN IN NATURE AND TECHNOLOGY

Chitin in Nature and Technology

Edited by

Riccardo Muzzarelli
University of Ancona
Ancona, Italy

Charles Jeuniaux
University of Liège
Liège, Belgium

and

Graham W. Gooday
University of Aberdeen
Aberdeen, Scotland

PLENUM PRESS • NEW YORK AND LONDON

Library of Congress Cataloging in Publication Data

International Conference on Chitin and Chitosan (3rd: 1985: Ancona, Italy)
Chitin in nature and technology.

"Proceedings of the Third International Conference on Chitin and Chitosan, held April 1–4, 1985, in Senigallia, Italy"—Verso of t.p.
Includes bibliographies and index.
1. Chitin—Congresses. 2. Chitosan–Congresses. I. Muzzarelli, Riccardo A. A., 1937– . 2. Jeuniaux, Charles. III. Gooday, G. W., 1942– . IV. Title.
QP702.C5I58 1985 574.19'2482 85-28088
ISBN 0-306-42211-5

Proceedings of the Third International Conference on Chitin and Chitosan, held April 1–4, 1985, in Senigallia, Italy

FOREWORD

Exegi monumentum aere perennius.
The monument I have built will last longer than bronze.

Horace

My previous book, "Chitin", (1977) was listed by the publisher, as a "key research book", among the most requested books by libraries. It received favorable comments from each of the journals which reviewed it, Science, 198, 28 Oct. 1977, Physiological Entomology, 2(4), Dec. 1977, The Canadian Institute of Food Science and Technology Journal, April 1978, The Quarterly Review of Biology, 53:361, 1978, Oceanographic Abstracts, 15:182, 1979, Annales de Zoologie-Ecologie Animale, 11:127, 1979, and Enzyme & Microbial Technology, 2, 1980. The variety of these journals testifies to the interdisciplinary character of chitin studies. "Chitin" has really been a landmark, to use the definition given by Science, because it stimulated interest in the less known polysaccharides and in modified chitins, besides chitin itself, to the point that three International Conferences on Chitin / Chitosan were convened (Boston, U.S.A. 1977, Sapporo, Japan 1982 and Senigallia, Italy 1985).

In convening the 3rd International Conference on Chitin / Chitosan (1-4 April 1985), one of the main objectives was the preparation of the present book. While the proceedings of the previous two Conferences were very valuable, they did not appear in any book catalogs and this severely limited their distribution. Therefore, in view of the need for a full-coverage book accessible world-wide, and in consideration of the large attendance (238 participants from 16 Countries), Plenum Press agreed to publish the present book which wants to be more than a mere proceeding. Its spirit is that of a "Chitin" book for the eighties, of lasting interest for the expert, and of passionate reading for the cultured man.

While offering the essential background, the present book is devoted mostly to advanced aspects of pure and applied research. I think that "Chitin" will remain a major reference for those who seek more basic information.

It is through the competence of the participants who supplied their manuscripts without delay, the help offered by the coeditors, Prof. Charles Jeuniaux and Prof. Graham W. Gooday, who also provided some of the "editor's reports" on interesting material that would have otherwise been inadequate for publication, and the understanding shown by the Government of Regione Marche, Ancona, Italy, which offered a special grant, that the final typescript has been produced, and it is a merit of Plenum Press that the book has been published so shortly after the Conference.

I wish to express my heartful and sincere thanks to all participants in the 3rd International Conference on Chitin / Chitosan, that I had the honor to chair; its success is also a merit of Maria G. Weckx, general manager, Irene Muzzarelli, secretary, Barbara B. Muzzarelli, social activities and Domenico Mencarelli, social relations. I thank Frank Salvi and Catherine Lough who assisted me in editing and producing the final typescript, as well as the staff of Chito-Bios s.r.l., Ancona, including among others Pamela La Marca and Lorella Falcetelli. All of them join me in expressing best wishes to the readers of this book, for fruitful studies and gratifying research activities.

Ancona, June 5th, 1985

Riccardo A. A. Muzzarelli

Professor of Chemistry and Biochemistry
Faculty of Medicine
University of Ancona
I-60100 Ancona, Italy

CONTENTS

Section III

CHITIN SYNTHESIS INHIBITION

Section IV

MOLECULAR RECOGNITION AND BIODEGRADATION

Section V
CHEMISTRY OF CHITIN

Section VI
MEDICAL USES OF CHITIN

PROGRESS TOWARDS SOLVING THE STRUCTURES OF POLYSACCHARIDES

K. D. PARKER

Astbury Department of Biophysics, The University of Leeds
Leeds, LS2 9JT, U.K.

STRUCTURE AT LOW RESOLUTION

The art of determining the structure of a regular fibrous molecule is well illustrated by the example of α-chitin. In 1950 Darmon and Rudall obtained good X-ray fibre diffraction patterns, good polarised infra-red spectra and built analogue molecular models of the stick and ball kind. Their conclusions are now considered to be wrong whilst Carlstrom in 1957 using almost the same data deduced a structure which is widely held to be substantially correct. It appears to us now that both these attempts were based on data which is insufficient to generate a uniquely correct solution. Darmon and Rudall took account of some fine detail in the X-ray and I.R. results and were led astray. Carlstrom, more selective, minimised or put aside these details, used superior model building and gave an acceptable solution. These discarded details still await a satisfactory explanation.

Many polysaccharide structures have been studied since then by similar methods with similar certainty and uncertainty in the results. Progress in protein and nucleic acid structures has tended to lead and inform parallel work on polysaccharides, and whilst the practical techniques have improved over 30 years the general approach has changed very little. Alginic acid in our laboratory provides a good illustration. Astbury in 1945 was hopelessly handicapped by lack of chemical information at that time. Subsequently in 1973 we were able to deduce crystal structures for the two components of alginic acid, p-mannuronic acid and p-guluronic acid, studied separately. The basic conformations of these molecular chains each in a crystalline state is thus known with some confidence. We can also surmise that the manner of packing of chains in the crystal is energetically favourable. We were also able to make tentative suggestions about the dispositions of some of the side groups, but it is at this level that hard evidence weakens and personal judgement or guesswork enters.

In 1950 to study structure was an end in itself; now we do so with the further aim of increasing our knowledge of the physical and chemical potentialities of the substance examined. With nucleic acid the mere outline structure has been sufficient to generate new ideas of molecular biology. With alginic acid very limited structural information has stimulated speculation on the mechanism of gel formation which may be technologically helpful. In both these examples the contribution of structure to the argument is so general that its value is difficult to assess. Trying to be

more specific, from a more detailed knowledge of polymer structures we might hope to understand and predict their detailed physical chemistry, particularly their solubility, and their interaction with enzymes. Some of these topics will be discussed at this meeting. Two less general problems of chitin spring to mind. The helicoidal layer structure of insect cuticle is an ultrastructural phenomenon which may well originate from helicity at the molecular level a subtlety of structure probably beyond the sensitivity of current techniques to demonstrate. The difficulty of explaining the biogenesis of the anti-parallel structure of α-chitin by a mechanism at a cellular level has caused some workers to doubt the uniqueness of this interpretation of the data. It is thus still necessary to establish beyond all doubt that α-chitin is in fact an anti-parallel structure.

THE PURSUIT OF STRUCTURE REFINEMENT

Remarkably these outline structures of polysaccharides have been found without the direct application of computing. Much work has been done to improve results by the use of computing; the gains though useful have not been dramatic.

The X-ray diffraction data has been the subject of rigid-body refinement procedures and difference Fourier maps have been studied to enable the utmost information to be extracted from fibre diagrams. Owing to the limitations of the diffraction data these have proved to be of limited value and have completely resolved very few points. Three independently conducted refinements of α-chitin all came to the conclusion that less symmetrical structures than that of Carlstrom should be considered, probably with statistically distributed disorder, but the data did not allow a more precise specification. Personal preference based on model building must then take over. Blackwell and Gardener found that for cellulose I a parallel chain model agreed more closely with the X-ray data than the best anti-parallel-chain model tested by them. The difference in agreement was relatively small and although considered to be signficant by them one is still left wishing for further confirmation of the conclusion reached.

Model building is being refined by computing structures of least free energy. Such conformational analysis will almost certainly become of great use in future when the techniques are perfected but so far it seems merely to provide circumstantial approval or disapproval to structures which have already been decided upon.

Infra-red spectroscopy (to which Raman spectroscopy has been added) has been taken to its limits with attempts at a full normal coordinate analysis of the vibrational modes of cellulose. However the imperfect data on the force fields made a direct comparison of the observed and calculated frequencies meaningless, whilst the calculation of the intensities was even more rudimentary. This work was valuable in a limited way, in that it gave insight into the general types of the modes to be encountered in a polysaccharide vibrational spectrum and must certainly be taken into account by anyone using these methods.

Generalising the position with respect to techniques we note that the X-ray method is not directed at any particular detail in a structure so that in the case of fibres, lacking the extreme accuracy possible with single crystal studies, it cannot give detailed information at the atomic level. Moreover X-ray diffraction is not particularly well suited to studying either static or dynamic variability of structure. Vibrational spectroscopy gives information which is in part localised and in part non-localised but the theory of interpreting spectra in all but the simplest cases is very difficult. High resolution nuclear magnetic resonance which is by far the

most informative spectroscopic tool could not previously be applied to solid specimens.

THE WAY AHEAD?

Progress towards a more complete understanding of polysaccharides through their structure has thus been a difficult and piecemeal process. Whilst a number of hopeful technical developments have recently appeared it is worth remembering that fibre structure determinations have often in the past been advanced by preparing or by discovering in nature improved specimens for examination. Thus Loligo pen gives a β-chitin of modest crystallinity but the same structure has been discovered in a highly crystalline form in Pogonophore tubes and in diatom spines. Similarly crab tendon gives an α-chitin of good crystallinity but more recently Rudall has discovered that the grasping spines of Sagitta are α-chitin of a very high degree of crystallinity. The latter has not yet been fully exploited because of technical difficulties but its very existence is reassuring. This kind of research contains too large a sporting element for most researchers, but with patience, skill or luck better specimens can be obtained in vitro as shown by the improvements obtained by annealing films and fibres cast from solutions of mannuronic acid.

The determination of its crystal structure, however accurate, gives just one configuration of a molecule in one particular environment. From a physical point of view this represents just one point on a multi-dimensional energy graph. To understand properties under different conditions (in solution perhaps) we should like to have information on more such points. The α, β (and γ) forms of chitin and the modifications of cellulose do give us limited variation for these substances but the opening of research into the structures of their oligomers could give us much more information about the force fields which are operating. This approach is promoted on the one hand by improved methods of preparing and purifying the oligo-saccharides and on the other hand by the availability of fast direct methods for single crystal structure determination. Structures related to but different in detail from the polymer may thus be studied with reasonable speed and with full single crystal accuracy.

Although N.M.R. techniques have been improving steadily since their inception the application of N.M.R. at high resolution to polysaccharides has increased rapidly in significance only during the last few years. (Notice the paucity of N.M.R. references in the Sapporo meeting in 1982.) In solution (where applicable) improved resolution and sensitivity, double resonance methods and the use of other nuclei have all been exploited. ^{13}C spectroscopy using cross polarisation and magic angle spinning allows high resolution spectra of solids to be observed for the first time thus linking up with the infra-red observations of 35 years ago. Moreover magnetic resonance methods are capable of giving information about the motion of molecules. In the fields of molecular enzymology and nucleic acids the possibility of variations of structure within limits is now seen to be vital to their functions. Perfectly static material is dead. Whilst it would be stretching analogy too far to argue for equally important effects in polysaccharides it may be that some of their properties and some of the effects by which we observe them will turn out to be influenced by fluctuations of their structure.

In conclusion the role of the structure of biological molecules in determining their properties remains paramount as originally postulated by Astbury but the concept of structure has now to be extended beyond the static and highly symmetrical arrangements with which he was chiefly occupied.

Section I

CHITIN IN SKELETAL STRUCTURES OF ANIMALS

CHITINOPROTEIC COMPLEXES AND MINERALIZATION IN MOLLUSK SKELETAL STRUCTURES

M. POULICEK, M. F. VOSS-FOUCART and Ch. JEUNIAUX

Laboratory of Animal Morphology, Systematics and Ecology
Zoological Institute, 22, Quai Van Beneden
B-4020 Liège, Belgium

STRUCTURE AND CHEMICAL COMPOSITION OF CHITINOPROTEIC MATRICES OF MOLLUSK SHELLS

The deposition of shell material by Mollusks is one of the more fully studied processes of extracellular calcification, and a large amount of data has been collected on shell structure, formation and regeneration, mineralogy, trace elements and organic compounds biochemistry. Like in many other invertebrates, the nucleation and growth of mineral crystallites occur on a well defined structure of organic components, these components being more or less "frozen" within developing crystals : this constitutes the "organic matrix template theory".

Both vertebrate and invertebrate organic matrices seem to be built on the same general scheme: a core of structural macromolecule (polysaccharide or protein) sheathed by one or several envelopes of acidic proteins, glycoproteins and mucopolysaccharides. All these components may be stabilized by structural bonding, mostly quinone-tanning and sulfur bonds.

The organic matrix isolated through decalcification of calcified layers of Mollusk shells (the so-called "conchyolins") is very complex in composition and structure. Proteins are the main component and account for 50 to 80% of matrix dry weight (1). Chitin was demonstrated in the shells of several species (2,3) and recent investigations made obvious its presence in every calcified layer of Mollusk shell so far examined (ca. 150 species). Its amount, estimated by the enzymatic method of Jeuniaux (2,4), is quite variable : from 0.01 to 40 % of the matrix dry weight (1 - 3).

The conformation and orientation of the matrix macromolecules were relatively poorly understood until the studies of Weiner and co-workers (5,6, 7,8). As stressed by Weiner (8), the main difficulty was the fact that X-ray and electron diffraction studies need to remove the minerals and, during this operation, some hydrophilic components are lost. The use of non-calcified material (for example, *Loligo* pen) may lead to misinterpretation (9). X-ray and electron diffraction studies (5) of the insoluble components of the matrix reveal that there is an association of chitin in its β-form (parallel chains crystalline form) with proteins that, similarly, adopt an antiparallel β-sheet conformation (8). The chitin polymer would be oriented approximately perpendicular to the protein-polypeptide chains (5,6,7), so that this crossed construction presumably contributes to the mechanical strength of the matrix (6).

This theory is consistent with transmission electron microscopy results

(shadowcast preparations, ultrathin sections) (10 - 12). The representative ultrastructure of the matrix is a micro-meshwork made of dense grains (2.3 - 4.0 nm average diameter) united by short, straight and thin organic connections (10). The sheets of the matrix are composed of several (up to five) different layers. The two surface layers are composed mainly of more soluble, acidic constituents. The core comprises a thin layer of chitin sandwiched between two thicker layers of proteins (12,13).

Bearing in mind those structural results, the calcophillic matrix of Mollusk shells appears, from a chemical point of view, as composed of two structural units :

1. An acidic polypeptide fraction with strong affinity to Ca^{++} ions, mostly soluble in decalcifying reagents (1, 14, 15, 16, 17). Its most probable arrangement involves a spiraled peptide chain (18). It is generally called "Mineralization Matrix"(MM).

2. A high molecular weight chitinoproteic complex with no affinity to calcium, arranged in the form of sheets and layers (1, 18), called "Carrier Protein" (CP).

The attachment of the soluble "mineralization matrix" to the "carrier protein" complex will activate the mineralizing substrate, leading to epitaxial $CaCO_3$ deposition (18). A "sandwich" structure thus appears with the chitinoproteic CP embedded between two sheets of the mineralization matrix, as presented on Figure 1. Subsequently, quinone-tanning can stabilize this whole molecular framework (1).

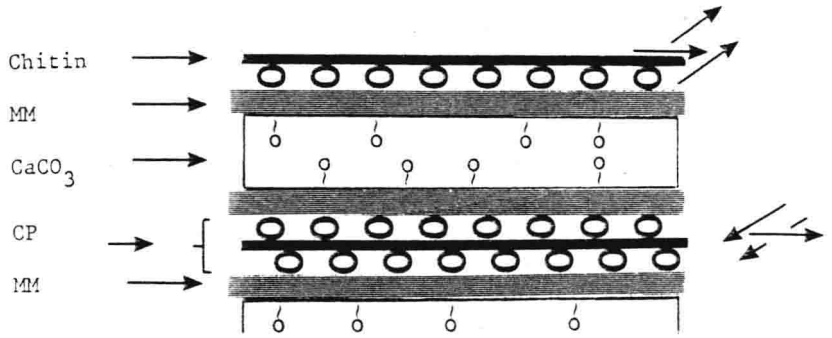

Fig. 1 : Schematic interpretation of the structure of the organic matrix in Mollusk shells (not drawn to scale).

CHITIN-PROTEIN BOND IN MOLLUSK SHELLS

The nature of the chitin-protein association in Mollusk calcified shells is still poorly understood, particularly as far as linkage is concerned. As chitin always appears accompanied by proteins, it is obvious that there is/are site/s in the polysaccharide polymer through which covalent binding to protein occurs. This proteoglycan may be accompanied by some other proteins or glycoproteins, specifically or not specifically associated with it by weak forces (9, 19).

According to the generalized high aspartic acid content of the insoluble protein complex (1), Hackman datas on chitin-protein relations may tentatively be extended to calcified Mollusk shells.

N-acetylglucosamine and chitin can react with α-aminoacids and peptides to give stable complexes and it seems that chitin can form more or less stable glycoproteins, probably through aspartyl and histidyl residues(20,21). Similarly, Brine and Austin (22) showed that predominant aminoacids in the residual chitin after vigorous alkaline hydrolysis were aspartic acid,