ADVANCES IN CHITIN SCIENCE

VOLUME II

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ORGANIZATION

The European Chitin Society -EUCHIS- and the UMR - CNRS 5627 (Lyon - France) organized the 7th International Conference on Chitin and Chitosan. This scientific event was supported by the University of Lyon, the City and the Urban community of Lyon, the Department of Rhône and the Region Rhône-Alpes, the CNRS, MERCK Clevenot, the Société Générale and Systèmes Bio-industries.

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Preface

In volume I of "Advances in Chitin Science", the European origins of the chitin science were recalled. Happily, this science has grown from its initial stage to become one of the most developed in the field of the polymers from the biomass. The particular interest shown in chitin and its derivatives is noteworthy in view of the very limited number of applications actually developed to date. This phenomenon must be related to the fact that these molecules find their applications primarily as additives or in fields where there is a high add-on value but a very limited market. There are two main obstacles to the development of industrial uses for these polysaccharides. The first is related to the cost of the raw material which remains too high, although there has been a continuous decrease of the prices over the last 20 years. The second and more important problem is that of the limitation in the production of these materials from biomass, involving a conversion process of low overall yeld but giving rise to large volumes of highly polluted effluent. Thus, there is much important fundamental research required to be done in the near future to solve these problems and to ensure a long life for chitin science.

The 7th ICCC/EUCHIS '97 joint conference was organized in France, the first time that the ICCC has been held in the country of Henri Braconnot. EUCHIS '97 was the second international meeting organised by the European Chitin Society and the location of the conference, Lyon, was therefore also very appropriate in this respect since the University Claude Bernard is where the European Chitin Society was founded and where it still retains its official address.

The number of delegates attending the Conference was over 230, the largest number ever attending a meeting devoted to chitin science. Furtermore, at least 35 countries were represented. The success of the meeting will be a considerable encouragement and a challenge both to those working in the field of chitin science and to the organisers of future meetings.

The number of scientific publications in the area of chitin science is still increasing, but the number of general texts is very restricted and can now be considered as limited to the monograph "Chitin Chemistry" by George Roberts, the other books now being out of print. As a consequence, books of conference play a very important role, in particular as a record of the most important research taking place in the time between two consecutive conferences. The series "Advances in Chitin Science", which was initiated with the proceedings of EUCHIS '95, will, if it is continued, constitute both a history and an important reference series for chitin science. It is therefore the wish of the editors that Volume II will not be the last of the series.

Alain Domard

CONTENT

CHITIN AND CHITOSAN: MOLECULAR AND BIOLOGICAL FUNCTIONS NEWLY GENERATED BY CHEMICAL MODIFICATION

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Abstract

The present lecture is focused the hydrogels and fibers of chitin and chitosan produced by chemical generating and regenerating reactions, and on some of their applications as novel functional biomaterials.

Keywords: biomaterials, fibers, filaments, generating reactions, hydrogels, regenerating reactions, textiles, wound-healing.

Chitin, a (1→4)-linked 2-acetamido-2-deoxy-β-D-glucan, has two hydroxyl groups, and its N-deacetylated derivative, chitosan, has one reactive amino and two hydroxyl groups per hexosamine residue. These biopolymers are biocompatible, biodegradable and biofunctional. Strong intra- and intermolecular hydrogen bonds exist in natural chitin to form parallel and antiparallel orientations. The dissociation and reorganization of these hydrogen bonds by chemical modification give rise to novel molecular conformations in the forms of solutions, sols, hydrogels, fibers, films, and sponges.

Generating and regenerating reactions

The generating and regenerating reactions are generally used for chemical modifications in the field of carbohydrate chemistry. Table 1 shows some of the well-known generating

Table 1. Some generating and regenerating reactions for chitin and chitosan

Reactions	Examples
$R\text{-}NH_2$ <⟶> $R\text{-}NH_3^+X^-$	chitosan <⟶> chitosan hydroacetate
$R\text{-}NH_2$ <⟶> R-NH(C=O)R'	chitosan <⟶> N-acylchitosan
$R\text{-}NH_2$ <⟶> R-N=CHR'	chitosan <⟶> N-arylidene- & N-alkylidenechitosans
R-NH(C=O)R' <⟶> R-NH(C=O)R'	metal complexes
	N-acylchitosan <⟶> N-acylchitosan in LiCl-DMFA
R-OH <⟶> R-ONa	N-acylchitosan <⟶> Sodium N-acylchitosan salt
R-OH <⟶> R-O(C=O)R'	chitin <⟶> O-acylchitin
R-OH <⟶> R-O-(C=S)SNa[b]	N-acylchitosan <⟶> sodium N-acylchitosan xanthate

[a]⟶>, generating reactions; <⟵, regenerating reactions.

[b]Well known in the viscose rayon industry.

and regenerating reactions, in which the primary structure (glycosidic linkage) is not destroyed (Fig. 1). In these reactions, their original molecular conformation are changed into novel ones having new properties.

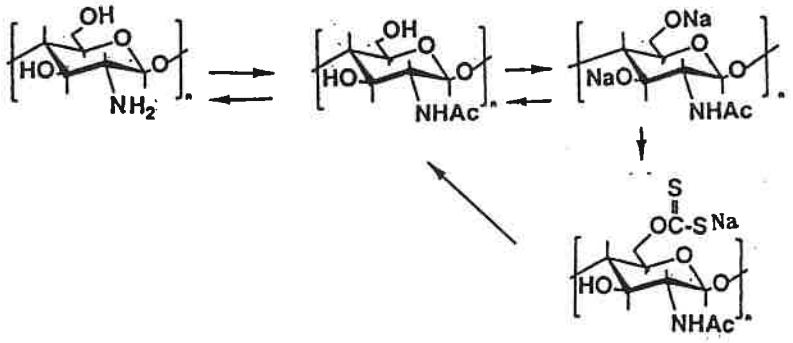

Fig. 1. An example for the chemical modification of chitosan and chitin [1]

Hydrogels formed in a molecular self-assembly way

By generating reactions

1) N-acylchitosan, and N-alkylidene- and N-arylidenechitosan hydrogels: To a solution of chitosan in aqueous acetic acid-methanol, the corresponding carboxylic anhydride (3-5 mol/GlcN) is added to afford a hydrogel [2-9].

2) Chitosan oxalate gel and chitosan gel: Chitosan is dissolved in 3% aqueous oxalic acid by heating, and the solution is allowed to cool at room temperature to afford the chitosan oxalate hydrogel. The gel is soaked in 1 N aqueous NaOH at room temperature to afford a chitosan hydrogel [10, 11].

Table 2. Some properties of hydrogels produced by generating reactions

	Hydrogels					
	N-acyl-chitosan	N-arylidene-N-alkylidene-chitosan	Chitosan oxalate	Chitosan	Chitosan-Ca alginate	N-acetylchitosan-collagen
Color	less	less or brown	less	white	less	less
N-substitute	-NH(C=O)-R	-N=CH-R	$-NH_3^+ -OOC-R$	$-NH_2$	$-NH_2$	-NH(C=O)-R
Transparency	trans.	trans.	trans.	opaque	trans.	trans.t
Stability on						
heat	irrever.	irrever.	rever.	irrever.	irrever.	irrever.
aq. acids	stable	unstable	unstable	unstable	unstable	stable
aq. alkalis	stable	stable	unstable	stable	unstable	stable

3) Chitosan-Ca alginate hydrogel: A mixed solution of chitosan and sodium alginate in a dialysis membrane tube is soaked in 0.1M aqueous $CaCl_2$ solution at room temperature to afford a hydrogel [12].

4) Collagen or silk fibroin-immobilized N-acylchitosan hydrogels: To a mixed solution of chitosan and collagen or silk fibroin in aqueous acetic acid, an carboxylic anhydride (10 moles/GlcN) is added to afford a hydrogel [13].

Some properties of these hydrogels are shown in Table 2 [14]. Only the chitosan oxalate hydrogel is reversible on heating and cooling.

Table 3. Some hydrogels produced by regenerating reactions

Derivatives	Solvent	Hydrogels
Monoconstituent hydrogels		
Sodium N-acetylchitosan salt	A	N-acetylchitosan
Sodium N-acetylchitosan xanthate	A	N-acetylchitosan
N-Acetylchitosan	B	N-acetylchitosan
Chitin	C	Chitin
Sodium N-propionylchitosan salt	A	N-propionylchitosan
Sodium N-propionylchitosan xanthate	A	N-propionylchitosan
N-propionylchitosan	B	N-propionylchitosan
Biconstituent hydrogels		
Sodium N-acetylchitosan salt & sodium cellulose xanthate	A	N-acetylchitosan-cellulose
Sodium N-acetylchitosan xanthate & sodium cellulose xanthate	A	N-acetylchitosan-cellulose
Sodium N-propionylchitosan salt & sodium cellulose xanthate	A	N-propionylchitosan-cellulose
Sodium N-propionylchitosan xanthate & sodium cellulose xanthate	A	N-propionylchitosan-cellulose

=A, 14% aq. NaOH; B, MeOH saturated with $CaCl_2$ $2H_2O$ or LiBr H_2O; C, DMFA or N-methyl-2-pyrolidone in the presence of 5% LiCl.

By regenerating reactions

1) N-acylchitosan hydrogels: Each solution of sodium N-acylchitosan salt and sodium N-acylchitosan xanthates [1,9] in aqueous NaOH is allowed to stand at room temperature for 2-7 days or to warm up at 40-50 °C for up to 1 hr to afford a hydrogel [15].

2) Chitin hydrogel: A chitin solution in DMFA or N-methyl-2-pyrolidone in the presence of 5% LiCl is mixed with pyridine, and the mixture solution is heated at 60 °C for 5 hr to afford a hydrogel [16].

Table 4. Fibers prepared from chitin and chitosan

Derivatives	Solutions[a]		Fibers[b]
	Spinning	Coagulating	
Monoconstituent fibers			
Chitosan hydroacetate	A	C	Chitosan
			* N-acetylchitosan
			* N-propionylchitosan
			* N-butyrylchitosan
			* N-hexanoylchitosan
			* N-(1'-carboxylpropionyl)chitosan
			* N-(4'-hydroxy-3'-methoxybenzylidene) chitosan
			* N-methylenechitosan
			* N-formylmethylenechitosan
			* N-benzylidenechitosan
Sodium N-acetylchitosan salt	B	D	N-acetylchitosan
Sodium N-propionylchitosan salt	B	D	N-propionylchitosan
Sodium N-acetylchitosan xanthate	B	D	N-acetylchitosan
Sodium N-propionylchitosan xanthate	B	D	N-propionylchitosan
Biconstituent fibers			
Sodium N-acetylchitosan xanthate & sodium cellulose xanthate	B	D	N-acetylchitosan-cellulose
Sodium N-acetylchitosan salt & sodium cellulose xanthate	B	D	N-acetylchitosan-cellulose
Sodium N-propionylchitosan salt & sodium cellulose xanthate	B	D	N-propionylchitosan-cellulose

[a]A, 2% aq. AcOH; B, 14% aq. NaOH; C, 10% aq. NaOH-28% Na_2SO_4; D, 10% aq. H_2SO_4-32% Na_2SO_4-1.3% $ZnSO_4$.

[b]Prepared from chitosan fiber by treatment with the corresponding carboxylic anhydride or aldehyde.

3) N-acylchitosan-cellulose composite hydrogels: A solution of sodium N-acylchitosan salt or sodium N-acylchitosan xanthate in aq. NaOH mixed with sodium cellulose xanthate in aqueous NaOH is treated as described 1) to afford a hydrogel [15].

The N-acylchitosan hydrogel and N-acylchitosan-cellulose hydrogel are colorless, transparent, stable in aqueous acidic and alkaline solutions, and irreversible on heating and

cooling (Table 3).

A thin slice of these gels is air-dried to afford a transparent film, and the slice is frozen and lyophilized to afford a porous sponge sheet. These products are usable as biomaterials for bandages, sponges, and artificial skin dressings [17-20].

Fibers formed in a spinning molecular assembly way

Each of the solution of chitosan, chitosan and their derivatives is spun through a viscose-type spinneret into the corresponding coagulating solution to afford fibers (Table 4) [21,22]. The chemical N-substitution of the chitosan fibers is carried out by treating with carboxylic anhydrides or aldehydes in MeOH at room temperature to afford a series of N-substituted chitosan fibers (*signed fibers in Table 4).

Table 5 shows some mechanical properties including the titer, tenacity and elongation values of the filaments. The chitosan filaments have a tenacity value similar to that of cellulose, but the elongation value of chitosan filaments is in about 1/7 of cellulose filament, because of a low internal

Table 5. Some mechanical properties of the filaments

Filament (% in constituents)	Preparation method[a]	Titer (denier)	Tenacity (g/d)	Elongation (%)
Chitosan		4.50	1.20	8.2
N-acetylchitosan	A	4.35	0.78	6.9
	B	3.08	1.17	11.2
N-propionylchitosan	A	3.40	0.70	4.9
	B	4.71	0.52	25.9
N-butyrylchitosan	B	3.22	0.70	5.9
N-hexanoylchitosan	B	4.29	0.52	15.2
N-(1'-carboxylpropionyl)chitosan				
	B	5.85	0.63	8.0
N-acetylchitosan-cellulose				
(62:38)	A	5.31	0.18	4.8
(43:57)	A	6.96	0.26	4.5
(28:72)	A	3.62	0.53	19.1
N-propionylchitosan-cellulose				
(61:39)	A	2.03	0.67	21.0
(49:51)	A	2.79	0.91	22.6
(3:97)	A	3.66	0.53	15.9
Cellulose		4.51	0.99	54.7

[a]A, prepared from sodium N-acylchitosan salt; B, prepared from chitosan fiber by N-acylation.

orientation of the filaments [21,22]. The N-acylchitosan filaments, which were prepared by N-acylation of chitosan filaments with carboxylic anhydrides, have relatively good mechanical properties, but the N-arylidene- and N-alkylidenechitosan filaments, which were prepared by N-substitution with aldehydes, have week mechanical properties.

Chitosan fiber has been reported from several groups of Poland, Japan and U.S.A., chitin and chitin-cellulose composite fibers from Japan, and O-butyrylchitin fiber from Poland.

A proposed molecular mechanism for hydrogelation and spinning

In the generating reactions

In a chitosan salt solution in aqueous organic acids, a mutual repulsion of cationized amino groups gives rise to stretched random conformations. During N-substitution reaction, the cationic character is gradually reduced, and the chitosan chains form a new stable framework in a molecular self-assembly way (Fig. 2). The framework architecture is microporous, polyphasic and honeycomb-like as examined by the SEM observation of the xerogel [23], and these pores fill with water droplets to afford the present hydrogel. The dehydration of the water-droplets present in the microporous by air-drying gives rise to a film sheet, in which many fibril layers are detected by the SEM observation of the film cross section [24]. The lyophilization of a hydrogel slice gives rise to a porous sponge sheet.

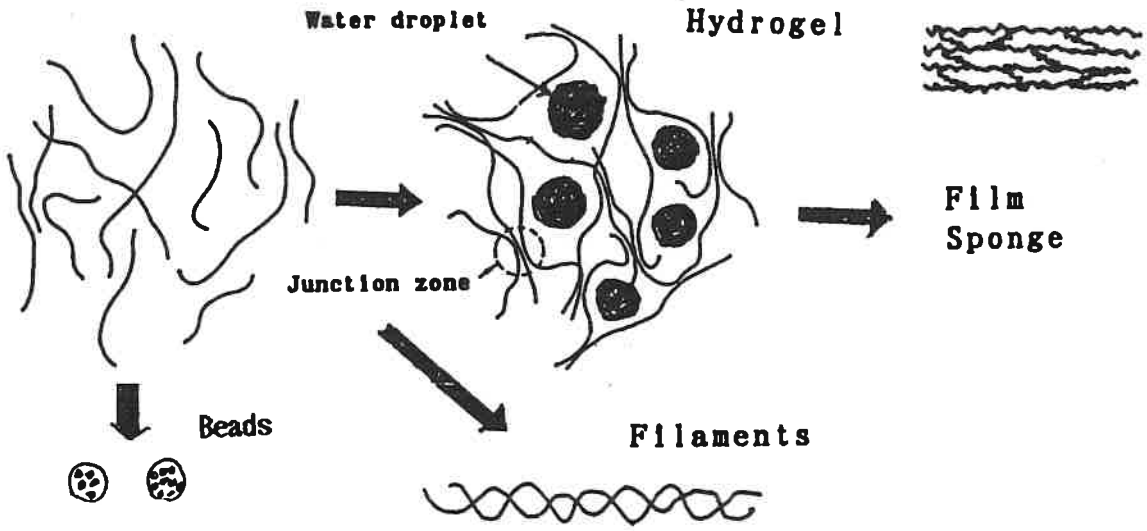

Fig. 2. A proposed molecular mechanism for hydrogelation and spinning (see text)

In the regenerating reactions

1) Each aqueous alkaline solution of sodium N-acylchitosan salts, sodium N-acylchitosan xanthate and their mixtures with sodium cellulose xanthate is allowed to stand at room temperature or to warm up at 40-50 °C. A spontaneous molecular reorganization occurs along with the slow hydrolyses of RO-Na to ROH and NaOH in the sodium N-acylchitosan salts, and RO-C(=S)SNa to ROH and HOC(=S)SNa in both the sodium N-acylchitosan and cellulose xanthates to give rise to a hydrogel. The hydrogelation occurs in a molecular self-assembly way, and the hydrogels consist of 5-10% polysaccharide and about 90-95% water.

2) A solution of the corresponding derivative is spun through a viscose-type spinneret into the corresponding coagulating solution. The filaments have intra- and interhydrogen bonds, and have no space for water droplets in and among the filaments [21,22].

Functions as biomaterials

Antibacterial and wound healing accelerating functions in animal and plant tissues

The wound tissues of animals and plants are dressed with a sheet of the films or sponges of chitin and chitosan, or with their cotton fibers. In plant wound tissues, the cell growth is enhanced, and the biosyntheses of phytoalexins and pathogenesis-related (PR) proteins such as chitinase and phenylalanine ammonia-lyase are stimulated [25]. In animal wound tissues, the cell growth is enhanced, and the macrophages are activated, resulting in the activation of humoral and cell-mediated immunity functions and in the stimulation of the extracellular secretion of lysozyme [19]. The lysozyme and chitinase hydrolyze the cell walls of pathogens, and prevent their growth [19,20]. In addition, free amino groups on chitosan chain form polyelectrolyte complexes with anionic cell walls of pathogens, resulting in an inhibition of pathogen growth [26]. In this way, the wound-healing of plant and animal tissues are accelerated, and the pathogen infection is prevented.

Controlled digestibility by chitinase and lysozyme

Chitin is biodegradable in animal and plant tissues. Its digestibility is controlled by the chemical structure of N-substituents and the degree of substitution for the N-acyl groups [27,28]. As shown in Table 6, the generated and regenerated N-acetylchitosans are more faster digested than natural chitin, because of their weak molecular intra- and interhydrogen bonds. N-acyl (>C6) chitosans are not digested by these enzymes because of their substrate specificity [29].

Thus chitin and chitosan are usable as novel biomedical and biotechnological materials for controlled digestible surgical sutures, and for controlled-release carroers of drugs and agrochemicals [17-20].

Table 6. The *In vitro* digestibility of N-acylchitosan fibers by lysozyme and chitinase

Fiber	Increase of reducing sugar value[a]	
	Lysozyme (μmol/5h)	Chitinase (μmol/2h)
N-acetylchitosan fiber	2.44	2.09
N-propionylchitosan fiber	0.34	0.21
N-hexanoylchitosan fiber	0.00	0.00
Natural crab shell chitin[b]	0.23	0.19

[a]Expressed as μ mole of N-acetyl-D-glucosamine.

[b]Powdered into 100-200 mesh.

Table 7. Some of the other functions and properties as biomaterials

Compounds	Functions and/or properties
Chitin and chitosan	An elicitor for PR-proteins
	Antibacterial and anti-fungal
	Drug- and agrochemical-carrying, and their control releasing
	Hypocholesterolemic
	Media for affinity chromatographies [32]
	Metal chelating
	Moisture retaining, anti-electrostatic, and hair protecting
	Moisture permeability, water-absorbing and desorbing , and odor absorbing
	Polyelectrolyte forming
	Capsule type fertilizer and herbicide
N-Acylchitosan	Media for affinity chromatography [33]
N-Methylenechitosan	Medium for gel chromatography [34]
Sulfated chitin and chitosan	
	Blood anticoagulant [35], lipolytic [36], and tumor metastasis-inhibiting
CM-, HE-, Methylchitin and chitosan	
	Moisture retaining and viscous [37]

Antithrombogenic and hemostatic functions

N-hexanoyl and N-octanoylchitosans are resistant to chitinase and lysozyme reactions, blood compatible, and are usable as antithrombogenic materials for artificial blood vessel and contact lens [30-31]. However, chitosan is not antithrombogenic and hemostatic, and is usable as a hemostatic agent and hemostatic materials in the forms of cottons, fibers, films and sponges [17-20].

Functional textile materials

Chitin and chitosan fibers have non-allergenic, deodorizing, antibacterial, fragrance-slow releasing, and a moisture-controlling functions. A chitin-cellulose composite fiber has been commercialized as a novel functional textile material and is used for the manufacture of textiles including underwear, sportswear and socks, and non-woven fabrics.

The other functions as biomaterials

Some of the other functions and properties as a biomaterial are summarized in Table 7.

Conclusion

The chemical modifications of carbohydrates have been focused so far on the preparation of new compounds by the generating reactions [e.g., 38,39], and little attention has been to the regenerating reactions, which have been used only for the protection of hydroxyl or amino groups in the specific position as an aide of the generating reactions. However, one of the successful applications of the regenerating reactions is the viscose rayon industry. The regenerating reactions are easy to do and will be more usable as a tool for "chemical molding art" in order to develop new functional materials having not only their stable conformations but also their unstable conformations.

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CHITIN CRYSTALS

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Abstract

A series of well characterized specimens of α and β crystalline chitin were investigated by electron microscopy imaging, electron and X-ray diffraction analysis together with solid state ^{13}C NMR spectroscopy. These samples which were all of the fibrillar form allowed to confirm the $P2_12_12_1$ space group and therefore the antiparallelism of the chitin chains in α chitin. For β chitin, the one chain $P2_1$ parallel structure was persistently observed in the anhydrous as well as in the hydrated state. The solid state conversion chitin β → chitin α under the influence of strong acid was also investigated. During the conversion, the β chitin crystals were seen to dissolve slowly under the influence of the acid and to reprecipitate epitaxially as α chitin on the underlying remaining β chitin when the samples were washed subsequently.

The occurrence of native chitin in the form of parallel or antiparallel structure raises the fundamental question of the mode of biogenesis of these two types of allomorphs for fibrillar crystals.

Keywords: α chitin, β chitin, crystals, biogenesis, ^{13}C NMR spectroscopy, ultrastructure, swelling, X-rays, electron microscopy.

Introduction

Chitin, the most abundant biopolymer after cellulose, occurs chiefly as structural material in arthropod cuticles, fungal and yeast cell walls as well as in a number of other aquatic and terrestrial living organisms [1-4]. Besides some rare systems where granular chitin has been found [5], most chitin occurs as crystalline fibrillar entities. In terms of X-ray, infrared and ^{13}C NMR spectroscopy, one distinguishes two main types of crystalline chitin: α chitin the most common and β chitin which is less frequent [3, 4]. A third allomorph, γ chitin has also been reported in the past [6] but its existence appears to be controverted today [7].

A survey of the crystallography of chitin indicates that the crystals of β chitin can undergo a reversible intra-crystalline hydration. In their anhydrous form, these crystals contain only one chain per unit cell [8, 9] and thus they consist of a parallel chain arrangement. This unidirectional organization of the β chitin microfibrils is consistent with a biogenesis model where these microfibrils would be biosynthesized, spun and crystallized in a continuous process. Such mechanism is supported by ultrastructural observations which have revealed striking images showing β chitin microfibrils being extruded out from synthesizing organelles still anchored in the plasma membranes of the corresponding specimens [10, 11]. The status of the crystallography of β chitin hydrates is less clear as a two chain unit cell has been tentatively proposed [12]. Since the β chitin hydrates can be converted reversibly into the β anhydrous, the molecules of chitin must be also parallel in these hydrates.

In α chitin, there is no crystalline hydrates. It is currently believed that this allomorph is crystallized along a two chain unit cell and a space group $P2_12_12_1$ that implies an antiparallel packing of the chains within the crystals [13, 14]. The occurrence of an antiparallel crystalline arrangement in α chitin microfibrils raises the important question of the mode of their biogenesis. A two step model with a delay between the biosynthesis of the chains and their crystallization has to be envisaged. It is during this lapse of time that the chains must rearrange to yield the antiparallel situation that is more favourable for chitin in terms of packing considerations. It is interesting to notice that the space group - and therefore the antiparallelism - of α chitin is still challenged today as the usual α chitin X-ray fiber diagrams present some diffraction spots that should be absent if the $P2_12_12_1$ symmetry was enforced [14, 15]. Also, the solid state conversion of β chitin crystals into α chitin under the influence of strong acid [6, 16] appears at first incompatible with a parallel to antiparallel crystalline transformation.

This paper summarizes years of work with colleagues, post-docs and students on the ultrastructure of various chitin systems. By selecting α and β chitin samples of high crystallinity we were able to clarify some of the mysteries of chitin and give some details on their behaviours. Some comments on the biogenesis of α and β chitin are also presented.

Materials and Methods

Throughout this study we have tried to characterize the various chitin samples by a series of solid state techniques, namely electron microscopy -using low dose imaging and electron diffraction analysis- X-ray and synchrotron analysis together with solid state ^{13}C NMR spectroscopy.

Results and Discussion

β chitin crystals

Despite the limited number of specimens where β chitin is present, highly crystalline samples of this chitin allomorph have nevertheless been identified and described. In order to gain more information on the crystallography of β chitin and its hydrates, we have undertaken a series of investigations on some of these samples, namely the spines of the diatom *Thalassiosira fluviatilis* [17, 18] and the chitinous components of the tubes of *Tevnia jerichonana*, a deep sea vestimentiferan worm [19, 20]. It was found that this last sample was more susceptible toward crystalline hydration than the diatom spines. A study of the diffraction diagrams of anhydrous and hydrated *T. jerichonana* chitin revealed that a one chain unit cell was persistently observed. Under the influence of hydration, essentially the b parameter of the unit cell increased, in going from b = 0.918 nm for the anhydrous sample to b = 1.092 nm for the hydrated material. The other parameters : a, c and γ remained almost the same. In both cases, the $P2_1$ space group was maintained and there was no data indicative a two chain unit cell. These observations were confirmed by solid state ^{13}C NMR spectroscopy where samples of dry and hydrated *T. jerichonana* were analyzed. In both cases the spectra revealed only one signal per carbon atom, as expected from a one chain unit cell with chitin molecules positioned on the 2_1 screw axes.

Figure 1 shows a typical crystal of β chitin from *T. jerichonana* together with its electron diffractogram. Figure 2 presents two electron diffraction patterns corresponding to hydrated and anhydrous *T. jerichonana* crystals. Figure 3 presents the corresponding ^{13}C NMR solid state spectra.

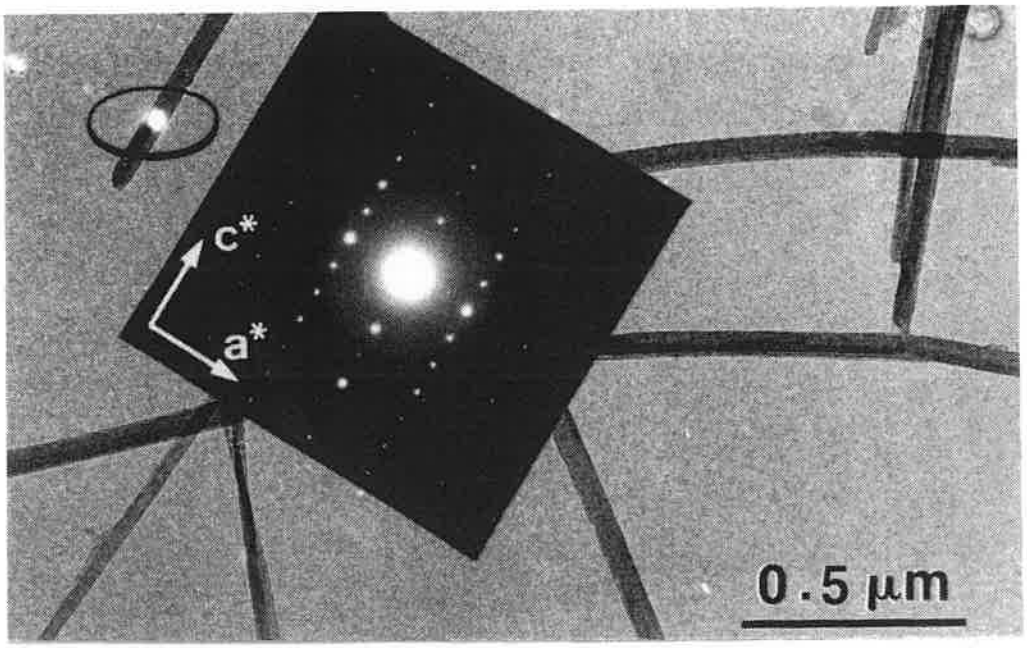


Figure 1. Low dose transmission electron micrograph of microfibrils of β chitin from deproteinized tubes of *T. jerichonana.* Insert: Typical electron diffraction pattern recorded on the illuminated area. This diagram which can be indexed along the a^*c^* reciprocal net of β chitin denotes the single crystal characteristic of each microfibril [19]

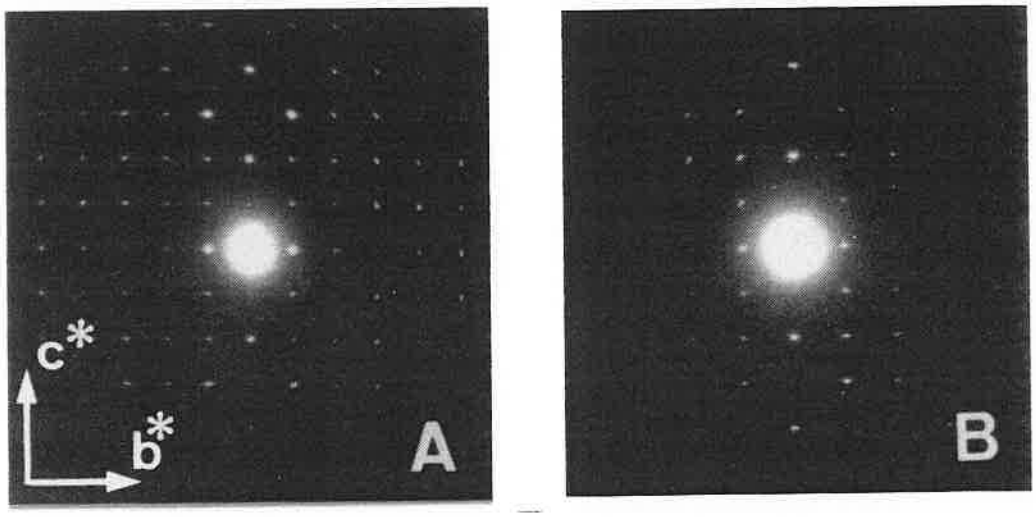


Figure 2. Typical electron diffraction diagrams of β chitin from *T. jerichonana* , recorded as in the insert in Figure 1, but showing the b^*c^* orientation . A corresponds to the hydrated sample whereas B is from the anhydrous material [19]

α chitin crystals from *Sagitta*

Following the description given by Rudall [21], the oral spines of *Sagitta* have a remarkable crystallinity. They are therefore ideal specimens to verify the crystallographic features of α chitin. Our approach has followed two different routes. In one of them [23], thin layers of *Sagitta* chitin have been investigated by electron diffraction analysis. As observed

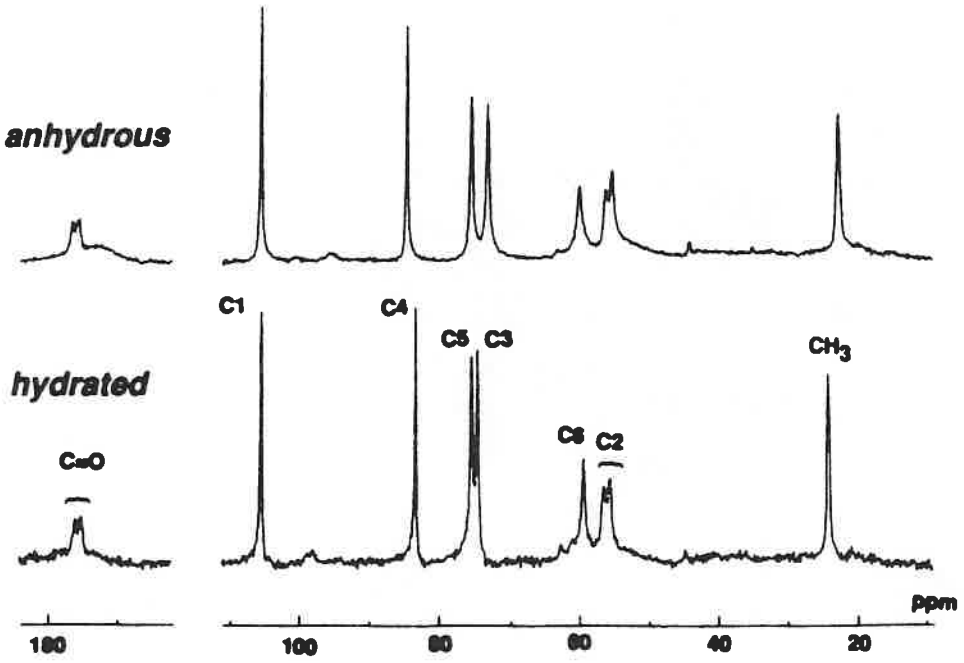


Figure 3. ^{13}C NMR solid state spectra of deproteinized tubes of *T. jerichonana* [22]

by Atkins *et al.* [15], the electron diffraction patterns contain indeed spots that are forbidden by the $P2_12_12_1$ symmetry. However, upon tilting around the major axis of the diagram, these forbidden reflection disappear, to reappear if one comes back to the initial un-tilted situation. Thus, these extra reflections are a clear example of a double diffraction effect. From electron diffraction data, the crystalline chitin system appears therefore consistent with the $P2_12_12_1$ space group.

In an other approach, we have also scanned fragments of spines from *Sagitta gazellae* with a microfocus synchrotron beam having 10 μm in diameter [24]. In this experiment, we could collect about 300 independent reflections that allowed to confirm the orthorhombic unit cell of α chitin. Systematic absences along 0k0 and 00l were clearly observed, but such absences could not be identified along h00 due to overlaps. Thus the antiparallelism of the chains in the α chitin crystals could be confirmed. In addition the data set is being analyzed to give a refinement of the structure of α chitin. A fragment of *S. gazellae* spine and two of its oscillation patterns are presented in Figure 4.

The solid state transformation β chitin → α chitin under the influence of strong acid.

If one accepts the parallelism of the chitin chains in β chitin and their antiparallelism in α chitin, the solid state transformation where the fibrillar β chitin gets converted into α chitin appears at first impossible. It has nevertheless been described in several instances [6, 16]. We have re-investigated this transformation by preparing dispersed β chitin microfibrils from *T. jerichonana* and subjecting them to aqueous solutions of HCl of various strengths [25]. With acid strength below 6N, the chitin microfibrils remained un-affected. With an acid strength between 6N and

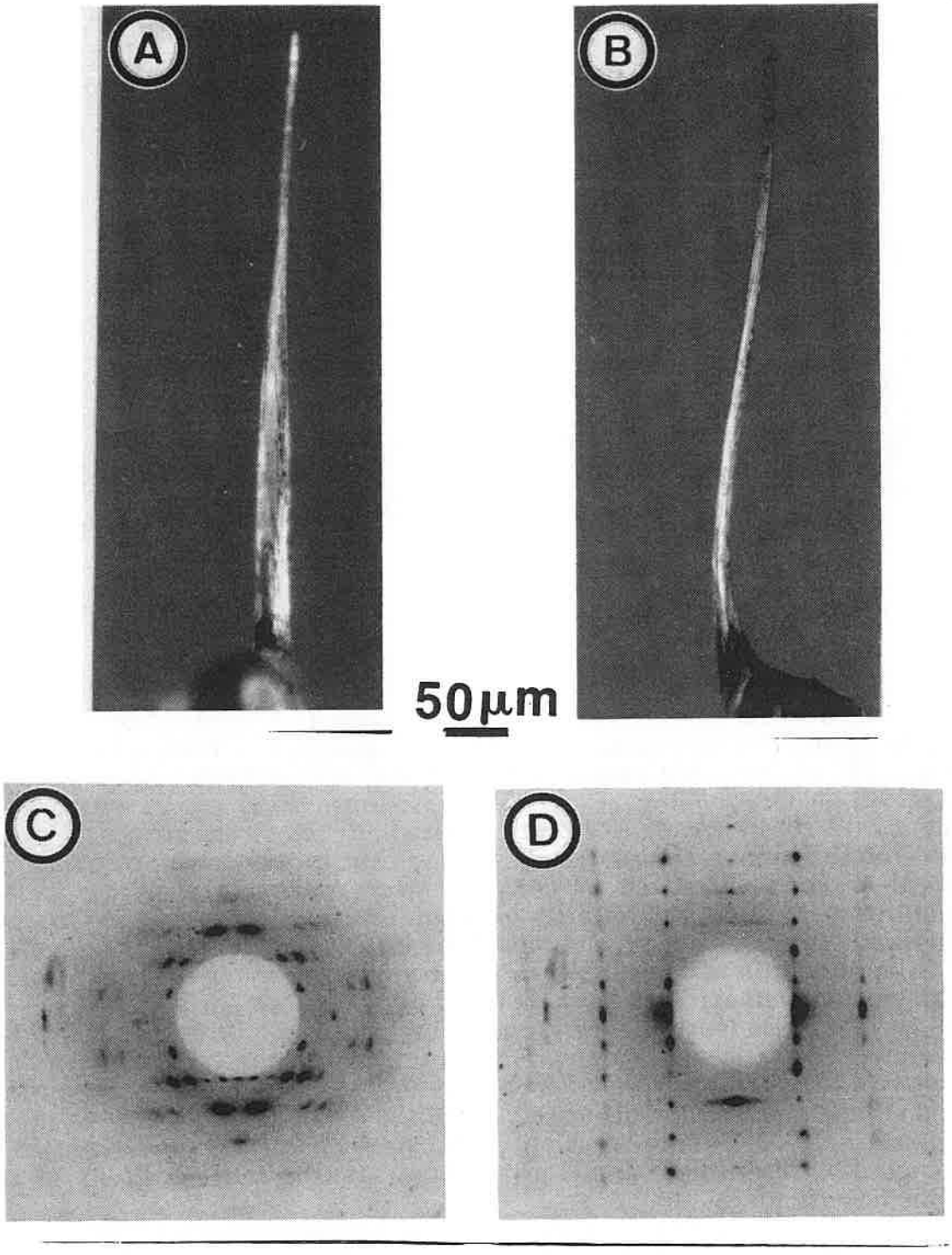


Figure 4. Well oriented fragments of grasping spine of an Antarctic arrow worm : *S. gazellae*. 4A and 4B are rotated with respect to one another by 90° around the spine axis. 4C and 4D : oscillation patterns recorded at ESRF with a synchrotron X-ray beam having 10 µm in diameter. 4C corresponds to an orientation as in 4A and 4D as in 4B [24].

7N, a total decrystallization was observed when the samples were immersed into the acid medium (Figure 5). During the subsequent

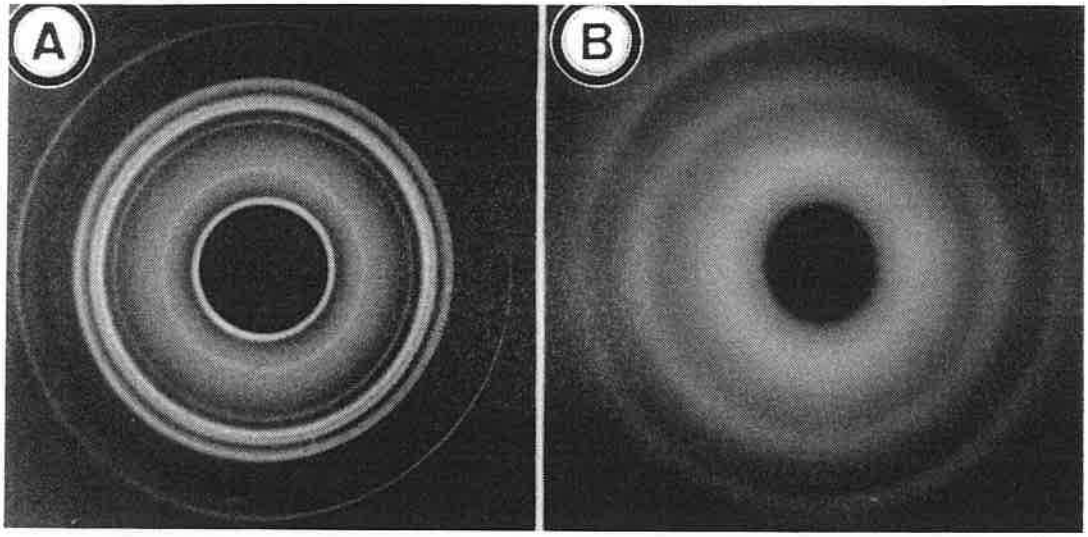


Figure 5. X-ray powder diagrams of β chitin from *T. jerichonana*. 5A of initial anhydrous sample. 5B as in 5A, but its immersion into 6N HCl leads to a total decrystallization [25].

washing, β chitin crystals were restored, but the initial microfibrils (Figure 6A) became split into smaller sub-fibrils (Figure 6 B). With acid strength from 7N to 8N, decrystallization of the microfibrils together with significant depolymerization were also observed. Upon washing, a substantial amount of α chitin resulted. By electron microscopy and electron diffraction analysis, it was found that this phase corresponded to small crystals organized as epitaxial overgrowths (Figure 6C) on the remaining β chitin microfibrils or whatever was left of them. This is a classical "shish-kebab" type of morphological organization with "kebab" or α chitin crystals recrystallized in epitaxy on underlying "shish" β chitin. This mechanism explains how dispersed microfibrils made of parallel chain chitin can be converted slowly into microfibrils containing a substantial amount of anti parallel chains. It is only when all the crystals have been converted that the fibrillar morphology is lost. This occurs here with an acid strength of 8 N and above (Figure 6D).

The conversion β chitin → α chitin is schematically depicted in Figures 7 and 8 that illustrate the various steps of the transformation.

Comments on the biogenesis of α and β chitin crystals

The morphogenesis of fibrous polysaccharide has been extensively studied in the case of cellulose I [26, 27]. In this case, it is believed that the native microfibrils originate from terminal complexes TCs consisting of organized clusters of cellulose synthases. There is a simultaneous polymerization, extrusion and crystallization of the cellulose molecules, with the result of organized microfibrils made of parallel cellulose chains. Some biological systems produce the antiparallel cellulose II, but the products are never organized as regular microfibrils [28].

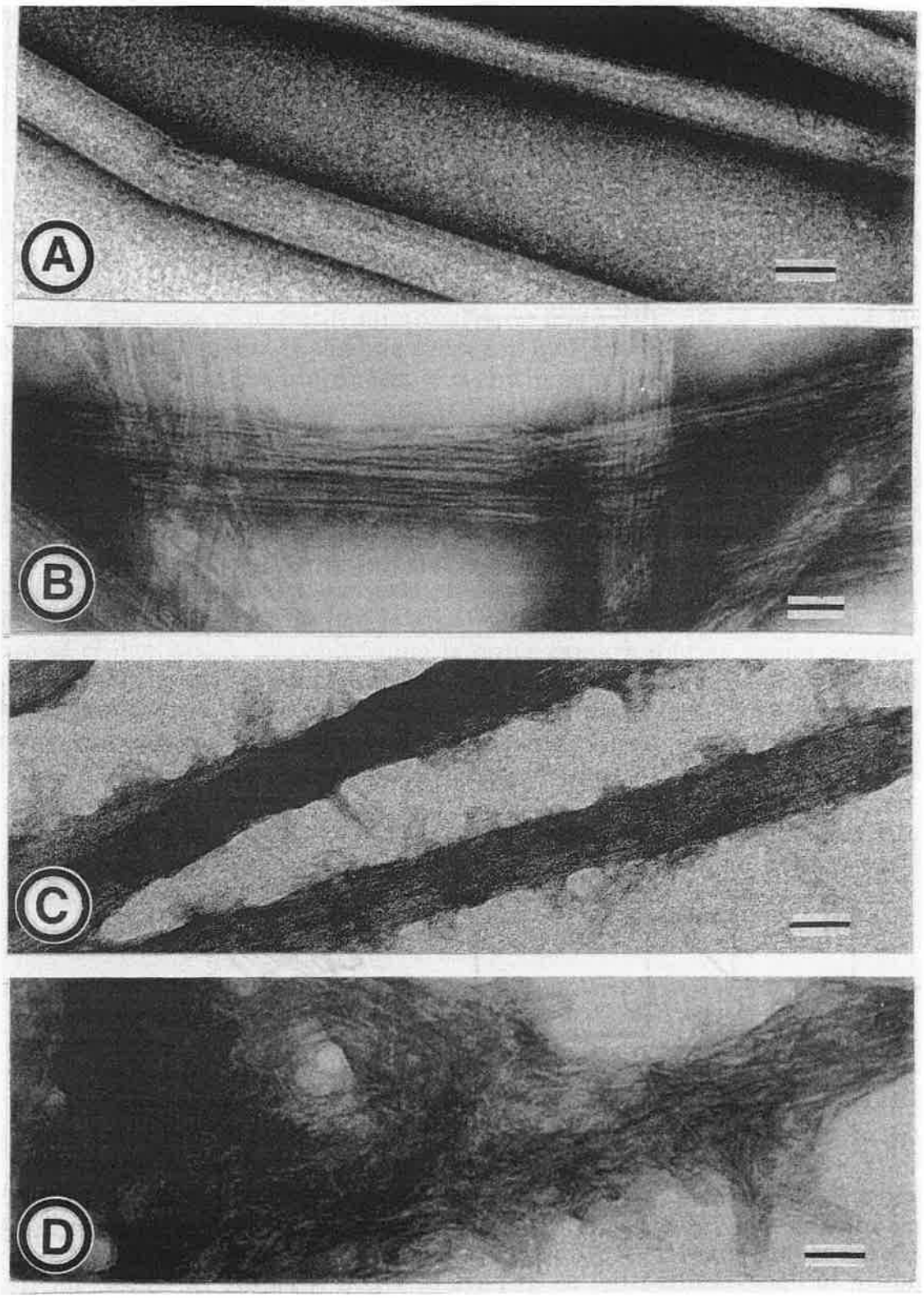


Figure 6. A series of negatively stained electron micrographs describing the swelling of β chitin microfibrils from T. *jerichonana* into HCl. The bars correspond to 50 nm. 6A: initial sample. 6B : sample after 30 min. in 7 N HCl, followed by washing in water. 6C: sample after 2 h in 7N HCl followed by washing in water. 6D : sample after 30 min in 8N HCl followed by washing in water [25].

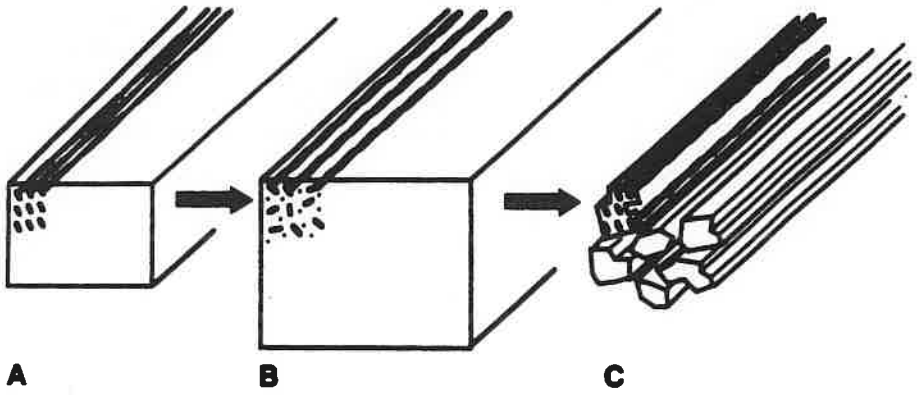


Figure 7. Schematic diagram describing the swelling of β chitin from *T. jerirchonana* in 6-7 N HCl. Upon HCl uptake, the initial sample in part A undergoes an intracrystalline swelling without chain scission leading to decrystallization as in part B. Upon washing in water, partial recrystallization occurs as in part C, leading to subfibrillation of the chitin microfibrils. In this scheme, step C reverts entirely to β chitin [25].

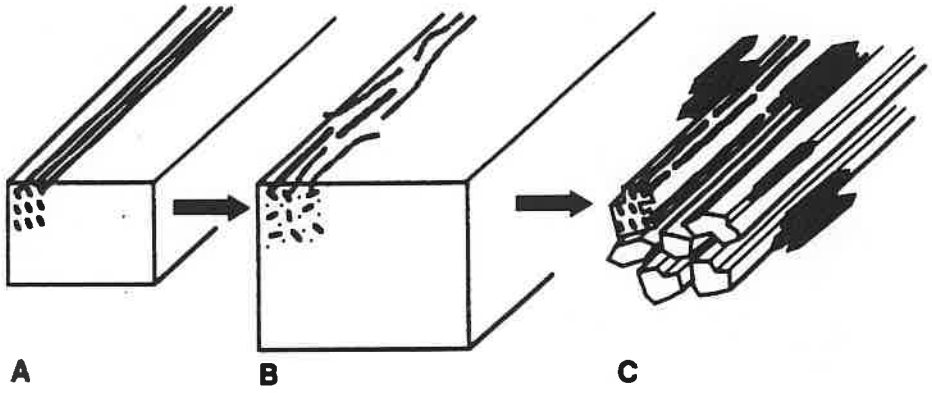


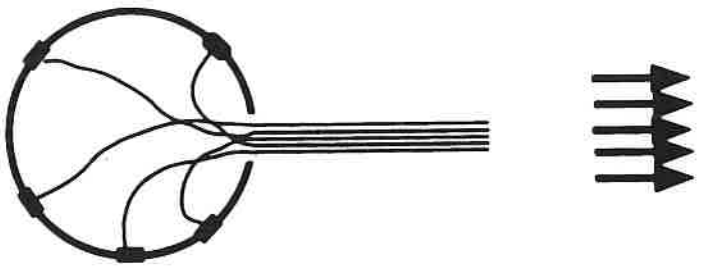
Figure 8. Schematic diagram describing the swelling of β chitin from *T. jerichonana* in 7.5-8 N HCl. Upon HCl uptake, the initial sample A undergoes an intracrystalline swelling together with chain scissions leading to decrystallization as in part B. Upon washing in water, partial recrystallization occurs as in part C, leading to a subfibrillation of the chitin microfibrils. In addition, the low molecular weight fragments that were partially solubilized in B will recrystallize in epitaxy on the surface of the chitin microfibrils, leading to a "shish kebab" arrangement [25].

The biogenesis mechanism of the parallel β chitin can be compared to

that of cellulose I. Some ultrastructural observations of growing β chitin microfibrils have been made in the case of diatom spines [10] and vestimentiferan chitin microfibrils [11]. In both cases, the chitin elements are seen as originating from hollow organelles located in the plasma membrane of the corresponding organism. These organelles, are opened toward the cell exterior, whereas toward the inside, a number of vesicles have been observed. The vesicles are believed to contain the chitin precursors which are being assembled in the organelles. In such case, the production of β chitin microfibrils appear as a continuous process where the chitin chains are being polymerized, spun and crystallized in a continuous fashion. Such mechanism is therefore consistent with the production of microfibrils with a parallel chain organization.

The case of α chitin microfibrils is more difficult to envisage. At present, the best model is the one proposed by Ruiz- Herrera *et al.* [29, 30] who have described the biogenesis of α chitin as a two step mechanism. In the first step, chitin is biosynthesized in a close organelle or chitosome where it occurs in a somewhat fluid manner. In a second step, the chitosomes burst open and the chitin microfibrils are unravelled. During the first step, which is somewhat disconnected from the second, the chains have time to reorient and to adopt the most thermodynamically stable arrangement, namely the antiparallelism. The chitosome system works well *in vitro* and has been operated also in our laboratory. It remains to be seen whether such mechanism is also operative *in vivo*. At any rate, the state of chitin inside the chitosome is quite remarkable as under normal condition chitin should precipitate readily as soon as it is produced, even at a very low degree of polymerization. This precipitation does not occur during the biosynthesis inside the chitosomes where for some unknown reason, chitin keeps a liquid-like mobility within the confinement of the chitosome.

Schematic drawings of the one step biogenesis process of β chitin and the possible two step mechanism for α chitin are shown in Figures 9 and 10.



β - chitin biogenesis

Figure 9. Schematic representation of the "one step" process leading to the biogenesis of β chitin microfibrils consisting of parallel chains. In this model, there is a continuous biosynthesis coupled with chitin extrusion out of the synthesizing organelle and simultaneous crystallization.

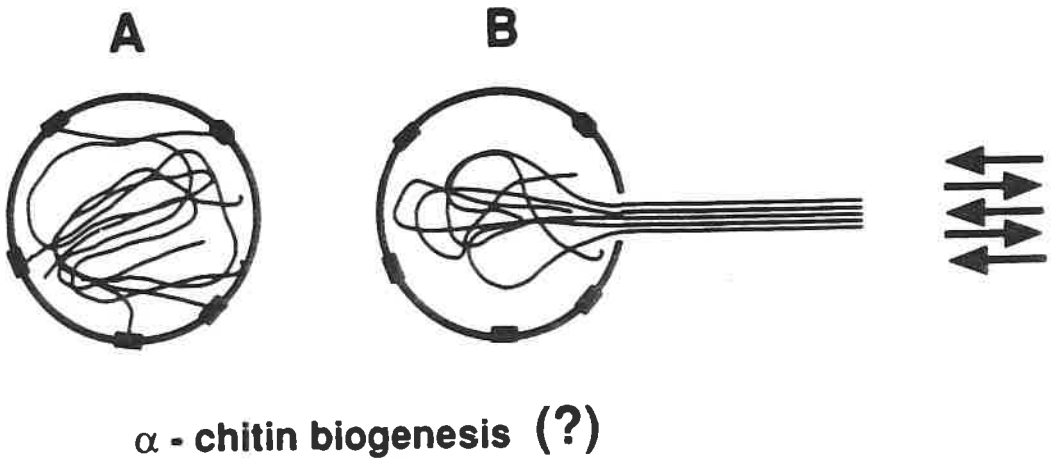


Figure 10. Schematic representation of the "two step" process leading to the biogenesis of α chitin microfibrils consisting of antiparallel chitin chains. In this model, the chitin chains are first synthesized in a close environment as in A. In B, they are then extruded in a second step, leading to crystalline microfibrils where the chains are crystallized according to the antiparallel α allomorph.

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CHITOSAN PRODUCTION ROUTES AND THEIR ROLE IN DETERMINING THE STRUCTURE AND PROPERTIES OF THE PRODUCT

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Abstract
The main processes for isolation of chitin from crustacean shell and its conversion to chitosan, together with a number of process variations, are reviewed, and their possible influence on the properties of the chitosan produced considered. The concept of a Polydispersity Index for F_A measurements is introduced and proposed as the explaination for the observed differences in bulk properties of chitosans prepared by different routes. A nomenclature system to deal with the range of possible structures is proposed.

Keywords: Chitin, isolation, deacetylation, chitosan, structural variations, polydispersity, nomenclature.

Introduction

Currently most, if not all, of the chitin used as the starting material for production of chitosan is obtained from crustacean exoskeletons produced as waste material in the seafood processing industry[1]. Isolation of chitin from such waste material involves three main processes:

- removal of residual protein
- removal of inorganic materials, principally $CaCO_3$ and $Ca_3(PO_4)_2$
- removal of colouring matters

In conventional processing protein removal by treatment with a relatively dilute alkaline solution is the first step, followed by removal of inorganic materials by treatment with acid. Decolourisation is normally carried out by a conventional textile bleaching treatment with H_2O_2 or NaOCl. Unless carefully controlled, any one of these processes may cause degradation of the chitin and hence of the chitosan produced in the subsequent deacetylation process. The choice of processing conditions may be governed to some extent by the intended end use of the chitin, since partial deacetylation during deproteinisation is not a disadvantage if it is intended for subsequent conversion to chitosan, while some hydrolysis of the chain during demineralisation is acceptable if it is intended for production of low molecular weight chitosan, chitosan oligomers, or **D**-glucosamine salts.

Results and discussion

Isolation of chitin

Deproteinisation: Conditions that have been used range from 0.1 M Na_2CO_3 + soap to 5.0 M NaOH, both for 4 hours at 100°C, with the most commonly reported conditions involving use of approximately 1 M NaOH at 100°C and times ranging from 0.5-72 hours.

Obviously the greater the severity of the treatment the more likely it is that deacetylation and/or chain cleavage will occur, but it would appear that efficient removal of protein at this stage is an important factor in determining the quality of the final chitosan. In a study shrimp waste was deproteinised by a series of treatments of varying severity and after demineralisation and deacetylation, under standard conditions, the chitosans were assessed for quality on the basis of clarity, colour and viscosity of their solutions. In general the quality was found to improve with increase in severity of the deproteinisation step[2].

Demineralisation: This is normally carried out by treatment with HCl at ambient temperature to minimise the risk of hydrolysis of the chitin chains. Again there is a wide range of HCl concentrations, temperatures and treatment times reported in the literature, ranging from 0.275 M HCl at ambient for 16 hours to 2.0 M HCl at ambient for 48 hours or 11.0 M HCl at -20°C for 4 hours. Generally it may be assumed that the milder the treatment the higher the molecular weight of the purified chitin and of the chitosan prepared from it[3-5]. However too mild a treatment will result in incomplete demineralisation and high concentrations of inorganic material may be left in the chitin through a combination of too low a concentration of HCl and too short a treatment time.

Decolouration: In principle the simplest method of decolouration is a straightforward textile bleaching process using H_2O_2 or NaOCl. However such bleaching agents will attack free amine groups, causing chain cleavage; indeed early patents[6,7] claimed the use of oxidative bleaching agents for production of low molecular weight chitosan under both heterogeneous and homogeneous conditions. This means that bleaching is best carried out as early on in the process as possible to ensure the lowest possible content of amine groups (highest possible mole fraction of *N*-acetylated units, F_A). The position of the bleaching step in the processing sequence has been studied for H_2O_2. In this study[4] demineralisation was carried out before deproteinisation which is not the usual sequence. The relative viscosities of the chitosans produced from the various chitins are given in Table 1.

Table 1.

Position of bleaching	Unbleached	Bleached after demineralisation	Bleached after deproteinisation	Bleached after deacetylation
F_A	⟵ Decreasing F_A ⟶			
η_{rel}	226	151.5	98	1.35

Process variations

Several variations on this basic chemistry have been examined by a number of researchers with the aim of either reducing processing costs or increasing the molecular weight of the resultant chitosan, or both.

The use of enzymes as a non-degradative method for removal of residual protein has been examined quite extensively[8-12]. This had been foreseen in the early patents[6,7] where the use of "certain putrefactive bacteria" was claimed but no details given. Although a range of enzymes have been examined, including bacteria specifically cultured to provide good proteolytic activity, complete removal of protein by enzymes has not been achieved. In all

cases between 1 - 7% protein was not removed, even with several treatments using freshly inoculated baths[12], and this has been attributed to a portion of the protein being inaccessible to the proteolytic enzymes.

The use of sulphurous acid as an alternative to HCl has been proposed[13]. If present in excess of the calcium salts present $Ca(HSO_3)_2$ is formed and this can be washed out. The sulphurous acid used can be recovered in two stages:

$$Ca(HSO_3)_2 \xrightarrow{\Delta} CaSO_3 \Downarrow + H_2SO_3$$

$$CaSO_3 + H_2SO_4 \longrightarrow CaSO_4 \Downarrow + H_2SO_3$$

so that the overall process is equivalent to demineralisation with H_2SO_4, which is a cheaper acid than HCl.

Extraction with EDTA at alkaline pH has been used as a non-degradative demineralisation process[10,14,15]. It has been found possible to reduce the inorganic content to approximately 1.5% by this means, and although EDTA is a relatively expensive chemical it would be possible to recover the EDTA from the complex and recycle it. A further mild acid treatment would be required to remove the remaining inorganic material.

The most interesting and attractive new approach to the production of chitin from crustacean shell is the lactic acid bacterial fermentation process developed by Hall and co-workers[16-18]. In this, well ground shellfish waste is inoculated with a lactic acid producing culture and a carbohydrate source and mixed thoroughly together. Acidification occurs to lower the pH and dissolve the $CaCO_3$ while at the same time the residual protein undergoes proteolysis by the enzymes from the shellfish viscera. The fermented waste may be separated into a protein-rich liquor and solid chitin from which about 70% of the protein and 35% of the calcium salts have been removed. This chitin can then be further purified by the standard treatments with NaOH and HCl but with a much lower consumption of chemicals.

Deacetylation of chitin

There is no "standard" deacetylation process as such but most work has been carried out using concentrated NaOH solutions (35 - 50 wt.-%) at temperatures of 80 - 140°C for reaction times of 0.5 - 10 hours at liquor ratios of approximately 10:1. Regardless of the particular conditions used there is a rapid initial decrease in both F_A and molecular weight, followed by a continuing but slower decrease in both parameters. The decrease in molecular weight can be reduced by carrying out the deacetylation under an inert atmosphere (N_2 or Ar) or by the addition of an oxygen scavenger such as thiophenol or a reducing agent such as sodium borohydride to reduce "end-peeling"[19]. There are a number of variations to this basic process:

- use of a multi-step process
- use of lower temperatures
- use of reduced amounts of alkali

Multi-step processes: Deacetylation in excess of 80-85% is rarely achieved by the basic process and higher values, up to and including 100% deacetylation, require a multi-step process which may involve:

- washing with water between successive deacetylation treatments[3,20]
- dissolution/reprecipitation of the chitosan between successive deacetylation treatments[20-22].

The effectiveness of intermediate washing and drying has been known for more than 40 years since Lusena and Rose[3] reported that two 0.5 hour deacetylation treatments in 55 wt.-% KOH solution, separated by washing and air-drying, were as effective in terms of deacetylation as one continuous 15 hour treatment, and additionally gave a higher molecular weight product. This was later shown also to be the case for deacetylation using NaOH solutions[20], where three separate 1 hour treatments with 47 wt.-% NaOH at 110°C were found to give a product having $F_A = 0.04$ (96% deacetylation) compared to a single 4 hour treatment, under the same conditions, which gave a product having $F_A = 0.18$ (82% deacetylation).

Two explanations have been offered for the effect of intermediate washing and drying[19,23]. The first, which is based on the effects of NaOH concentration on the swelling of cellulose[24], suggests that during washing the concentration of NaOH within the chitin/chitosan particles gradually decreases through the maximum swelling concentration, which by analogy with cellulose should be at about 18-20 wt.-%. This increased swelling increases the accessibility of the chains in the subsequent deacetylation treatment[19]. The second explanation proposes that chitin forms a complex with the alkaline medium and that the rate constant for the deacetylation step of this chitin/medium complex is very much smaller than that for the deacetylation step of the uncomplexed chitin. Washing and drying are considered to destroy the complex thereby converting the remaining *N*-acetylated units back to the much more reactive form for the subsequent deacetylation treatment[23].

It is generally accepted that the reason that the deacetylation process does not normally go beyond 80-85% is that the morphology of the chitin makes the remaining *N*-acetyl groups inaccessible to the NaOH unless drastic conditions are used. Dissolving and reprecipitating the chitosan alters the morphology and so should make these *N*-acetyl groups more accessible, and several groups have used this approach, albeit with different deacetylation protocols, to produce fully deacetylated chitosan[20-22].

Lower temperatures: The use of lower temperatures has been reported by a number of researchers[25-28] and the most detailed report is that of Alimuniar and Zainiaddu[28] who studied the deacetylation process at 30°C using NaOH concentrations covering the range 23 wt.-% - 39 wt.-% NaOH and a liquor ratio of 14:1 as standard. Acid-soluble products were only obtained using NaOH solutions of 33 wt.-% or higher. Using this minimum concentration a reaction time of 6 days was required to obtain completely acid-soluble material, the required reaction time dropping to 5 days with 35 wt.-% NaOH and 4 days with 37.5 or 39.5 wt.-% NaOH. Increasing the liquor ratio reduced the required reaction time and at a liquor ratio of 56:1 a reaction time of only 1 day was needed to give an acid-soluble product using 33 wt.-% NaOH.

Reduced amounts of alkali: The large volumes of concentrated alkali required in the normal deacetylation process present problems in terms of effluent disposal, corrosion effects and chemical costs and so attention has been directed towards reducing the volumes required. In one approach chitin is either mixed by kneading with about 2-5 parts NaOH solution[25] or steeped in excess NaOH solution then filtered or centrifuged to remove the surplus liquid and leave a mixture containing 0.5 - 2.5 parts NaOH to 1 part chitin[26]. The chitin/NaOH mass is then heated at 40-80°C and 45 - 110°C respectively. In the second approach use is made of a diluent system and these have included both water-miscible liquids such as IPA, TBA or acetone[22], and water-immiscible liquids such as paraffin oil[29] or polyethylene glycol dimethylether[30]. The diluents act both as the reaction medium to enable the mass of particles to be stirred and to distribute the alkali uniformly throughout the chitin.

Dependence of structure on processing route

In most of the work discussed above the chitosan products have been characterised in terms of their bulk properties such as solubility, F_A, and viscosity or molecular weight. However there is a growing awareness that at the molecular level there are more subtle structural variations that may have significant influence over a number of properties of chitosan. This was intimated over 15 years ago by Muzzarelli and co-workers in a study of 14 chitosans of varying provenance[31]. Noting that there was no apparent correlation between F_A and pK values they commented that "the reason for so wide a range of pK values is not clear, but possibly depends on the structure". A greater understanding of the varying structural effects arising from different processing routes would enable chitosan products to be tailored more closely to their end-use requirements.

There are four basic ways of preparing a chitosan sample having a given F_A:

- heterogeneous deacetylation of chitin suspended in NaOH solution[19]
- homogeneous deacetylation of chitin dissolved in NaOH solution[32]
- heterogeneous re-*N*-acetylation of chitosan of lower F_A[33, 34]
- homogeneous re-*N*-acetylation of chitosan of lower F_A[35]

and there are a number of differences in properties such as crystallinity, solubility and swelling for chitosans having the same F_A value but prepared by different routes[32, 36, 37]. Kurita *et al.* proposed[32], on the basis of x-ray diffraction data and solubility, that chitosan prepared by homogeneous deacetylation has a random copolymer structure while chitosan prepared by heterogeneous deacetylation has a block copolymer structure. This interpretation received support from Aiba's studies[36,37] of the stability of solutions in alkaline media, the swelling behaviour in water, and the x-ray diffraction patterns of chitosans prepared by heterogeneous deacetylation and homogeneous re-*N*-acetylation, and from the studies of Ogawa and Yui[38].

Other workers have reached different conclusions. Varum *et al.* found, using high field ^1H and ^{13}C NMR spectroscopy, that the distribution of *N*-acetylated residues in chitosans prepared by both heterogeneous and homogeneous deacetylation of chitin were the same, showing a nearly random (Bernoullian) distribution[27,39]. Similar conclusions were reached by Sashiwa *et al.* following GPC analysis of the nitrous acid degradation products of chitosans prepared by heterogeneous and homogeneous deacetylation[40,41]. Thus the bulk properties suggest that chitosans prepared under homogeneous conditions have a different

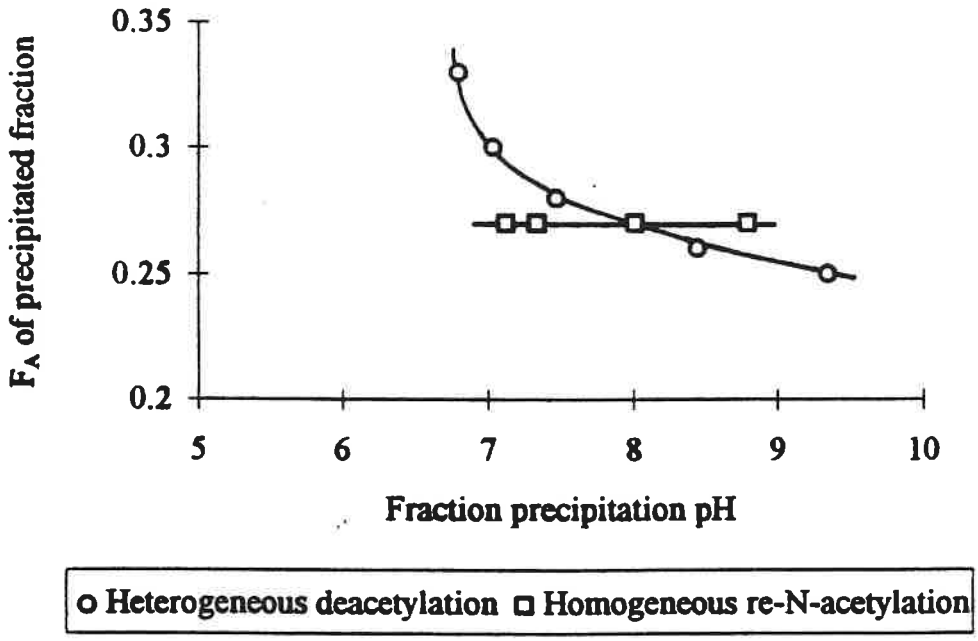
structure than have chitosans prepared under heterogeneous conditions, whereas chain structure studies indicate that both types have the same structure.

This problem has recently been investigated using a pH-solubility fractionation technique and chitosans prepared by both heterogeneous deacetylation and homogeneous re-*N*-acetylation[42]. Typical results are given in Table 2 and Figure 1.

Table 2. Fractionation results for chitosans prepared under heterogeneous and homogeneous routes.

Chitosan prepared by heterogeneous deacetylation (F_A = 0.25)					
pH	6.80	7.04	7.47	8.45	9.35
Fraction wt./%	20.5	46	74	91	100
F_A	0.33	0.30	0.28	0.26	0.25
Chitosan prepared by homogeneous re-*N*-acetylation (F_A = 0.27)					
pH	6.81	7.13	7.34	8.02	8.80
Fraction wt./%	16	50	60	87	98
F_A	-	0.27	0.27	0.27	0.27

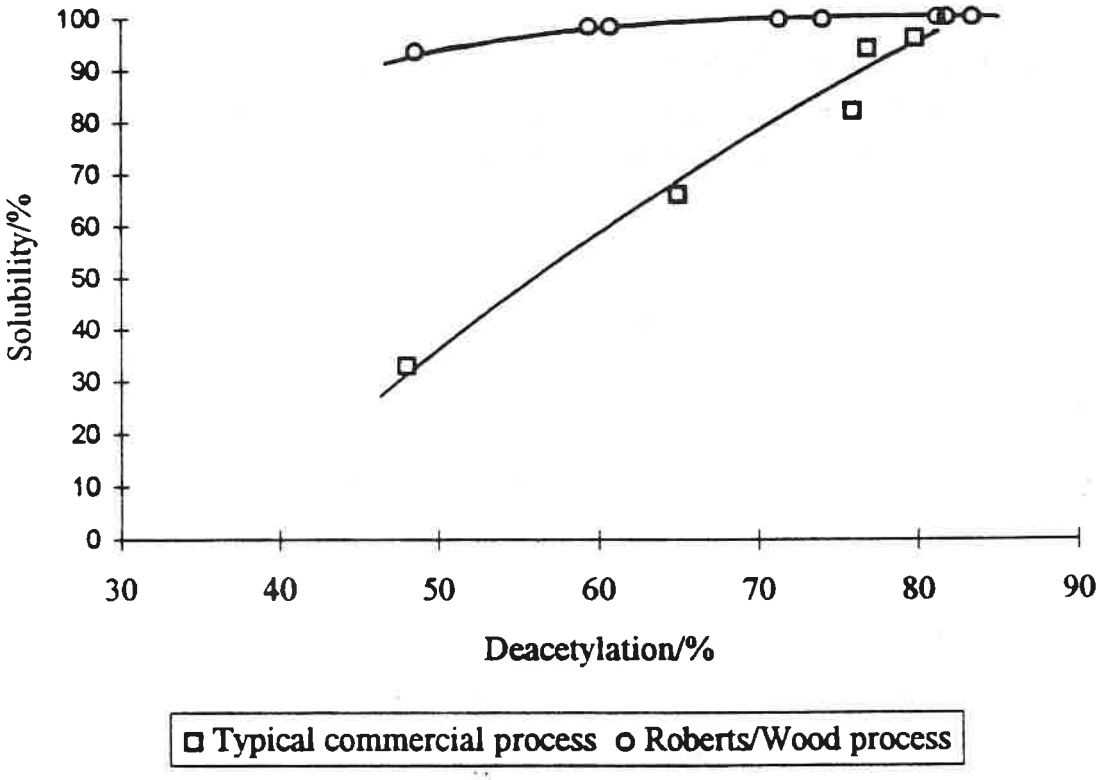
Figure 1. F_A values of fractions obtained from chitosan samples prepared under heterogeneous conditions (F_A = 0.25) and homogeneous conditions (F_A = 0.27).



These results show clearly that the polymer chains of a sample prepared under typical heterogeneous conditions will have considerable variation in F_A, and that the value determined for the bulk sample is an average value, whereas the chains of a chitosan sample prepared under homogeneous conditions will not have this interchain variation in F_A, each chain having the same value as that of the bulk sample. By analogy with polymer molecular weight terminology the former chitosan is *polydisperse* in respect of F_A while the latter is *monodisperse*, and a "Polydispersity Index" may be calculated from $F_{A[max]}/F_{A[bulk]}$, where $F_{A[max]}$ is the highest value of F_A for any of the isolated fractions and $F_{A[bulk]}$ is the F_A value of the bulk sample.

The monodisperse character of F_A in chitosans prepared under homogeneous conditions, compared to its polydisperse character in chitosans prepared heterogeneously, offers an explanation in molecular structure terms for observed differences in bulk properties[32, 36-38] between the two types of material, despite the overall similarity in distribution patterns of the *N*-acetylated residues[22, 39-41].

Figure 2. Solubilities as a function of the % deacetylation for chitosans produced by different heterogeneous processes.



The proposed Polydispersity Index is a new and important structural parameter for characterising chitosan materials, since the extent of polydispersity in F_A would be expected to influence both physical and biological properties of chitosans. It is therefore important to develop an understanding of how conditions used in the various deacetylation processes affect it. Obviously homogeneous deacetylation will give an Index value approaching 1.0 but it is possible that this can also be achieved in heterogeneous deacetylation under certain conditions. A novel deacetylation process has been developed recently[43] that yields products having a high level of solubility, even at relatively high F_A values. The solubility at different extents of deacetylation for chitosans prepared by this process are shown in Figure 2, which also shows similar data for chitosans prepared by a conventional heterogeneous deacetylation process. It is clear that the new process gives much more soluble products and this may be attributed to the greater structural uniformity, i.e. lower Polydispersity Index, of these products. In other words it is a heterogeneous process that gives a product having the characteristics of one produced by a homogeneous process and is an example of how the molecular structure and properties of chitosans may be controlled through control of the deacetylation process.

Chitosan nomenclature

It is now universally recognised that the names 'chitin' and 'chitosan' describe a continuum of copolymers covering a wide range of compositions in terms of the ratio of the anhydro-2-acetamido-2-deoxy-**D**-glucopyanose units to the anhydro-2-amino-2-deoxy-**D**-glucopyranose units. It is therefore essential to have a nomenclature system that can simply and unambiguously identify any sample of chitin or chitosan. This problem has been discussed in several articles[44-46] and the following proposals are largely based on the author's earlier suggestions[44, 45].

1. All β-(1→4)-linked copolymers of anhydro-2-acetamido-2-deoxy-**D**-glucopyranose and anhydro-2-amino-2-deoxy-**D**-glucopyranose units should be designated as chitosan or chitin on the basis of their solubility or insolubility, respectively, in 0.1 M acetic acid. There is no need to introduce a new, artificial name to identify materials intermediate between the two homopolymers.
2. All of the confusing multitude of terms currently used in the literature to describe what is essentially the mole fraction of anhydro-2-acetamido-2-deoxy-**D**-glucopyranose units - extent of deacetylation, degree of deacetylation, level of deacetylation, % deacetylation, % *N*-acetylation, degree of residual *N*-acetylation, level of residual *N*-acetylation - should be replaced by the symbol F_A representing the mole fraction of anhydro-2-acetamido-2-deoxy-**D**-glucopyranose units. In cases where it is necessary to express the mole fraction of anhydro-2-amino-2-deoxy-**D**-glucopyranose units, this can be done either by use of the symbol F_D or, preferably, $(1-F_A)$.
3. The F_A, if known, should be given in brackets after the name e.g. chitin[0.94], chitosan[0.25].
4. If deacetylation has been carried out under homogeneous conditions this can be indicated by an italicised '*h*' after the figure giving the F_A e.g. chitosan[0.5*h*] which would be expected to differ markedly in properties from chitosan[0.5]. If the distributions along the chain of the two co-monomers are known this can also be indicated by italicised letters: *r* (random), *b* (block), *a* (alternating).

5. A distinction should be made between material prepared directly from chitin by deacetylation (chitosan) and material prepared by re-*N*-acetylation of chitosan (*N*-acetylchitosan). In the case of the latter material the value of F_A is given in two parts; the first represents the value of the starting chitosan and the second represents the value of the increase due to the *N*-acetylation process. Thus *N*-acetylchitosan[0.1/0.4*h*] would unambiguously identify a material having a total F_A of 0.5 that had been prepared by homogeneous *N*-acetylation of a chitosan sample previously prepared by heterogeneous deacetylation and having an F_A value of 0.1.

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An attempt to estimate crustacean chitin production in the hydrosphere

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Abstract

Chitin is widely distributed in nature and its annual production is thought to be huge. Crustaceans, which are currently the major industrial source of chitin [1], are probably one of the main chitin producers in the hydrosphere. However, their chitin production in aquatic ecosystems has been rarely estimated [2-7]. Nevertheless, a correct evaluation of the chitin production by crustaceans in the different marine and freshwater ecosystems is needed to understand their importance in the biogeochemical cycles of carbon and nitrogen and to achieve a rational exploitation of these organisms as a source of chitin for the industry.
For several years, our laboratory has been involved in the study of the chitin production in marine and, more recently, freshwater environments. On the basis of the results of our laboratory and a large data set from literature, our aim here is to initiate a comprehensive database about chitin production by crustaceans in the hydrosphere. A global estimation of the chitin production in the major parts of the hydrosphere will be discussed and some aspects to be further studied will be highlighted.

Keywords: crustacea, chitin, production, aquatic resources, plankton, benthos, meiofauna.

Methodology

Studies concerning simultaneously chitin and secondary production are scarce [3-7]. However, for a given organism or community, annual chitin production can be estimated as a fraction of the total production of tissue based on its mean chitin content. In the present paper, we have classified the production data considering six different parts in the hydrosphere (rivers and streams; lakes and ponds; saline lakes and inland seas; shores and continental shelf; continental slope and deep sea) and two different communities in each part (benthos and plankton).

Chitin levels in crustaceans

The proportion of chitin, with respect to the whole body mass, has only been estimated in a limited number of different orders (Table 1). On the whole, the chitin levels in crustaceans range from 2 to 12 % of the whole body dry mass but no general pattern can be set up in relation to phylogeny or ecology [8]. It must be pointed out that data are lacking for some major group such as freshwater amphipoda, freshwater isopoda and ostracoda.
In the studies carried out by our laboratory [3,7-9], chitin has been quantified using the method described by Jeuniaux [10]. Dry material is demineralized in 0.5 N HCl at 25 °C during 4 hours, washed and treated with 0.5 N NaOH at 100 °C during 6 hours. The

residue left after the successive acid and alkaline treatments is then hydrolysed by specific enzymes, chitinase and chitobiase. The resulting *N*-acetylglucosamine is assayed colorimetrically.

Table 1: Chitin levels in crustaceans

Order	Chitin content (Mean [range]) (% of whole body mass)	Reference(s)
Amphipoda (sea water)	7.3 [5.8 - 9.4]	[7,11]
Cladocera (fresh water)	4.9 [3.5 - 6.4]	[9]
Cladocera (sea water)	12.2	[3]
Anostraca (fresh water)	2.2 [1.5 - 3.3]	[8]
Anostraca (brackish water)	1.5 [0.9 - 2.1]	[8]
Copepoda (fresh water)	12.4 [10.8 - 13.9]	[12]
Copepoda (sea water)	5.8 [3.1 - 8.6]	[3]
Decapoda (sea water)	8.8	[13]
Euphausiacea (sea water)	2.6 [2.1 - 3.6]	[14-17]

In the other studies, chitin has been isolated in similar ways but assayed differently. Anderson *et al.* [14] and Clarke [17] have estimated chitin gravimetrically in the residues. Nicol *et al.* [16] have converted the concentration of acetic acid being formed after orthophosphoric acid digestion of the residues into chitin concentration. Rakusa-Suszczewski and Dominas [11] and Calvo-Carrillo *et al.* [13] have calculated the chitin content on the basis of the nitrogen concentration in the residues. Brzeski [15], Berthon [12] and Calvo-Carrillo *et al.* [13] have considered the chitin content of the organisms to be equal to the ash-free dry mass of the residues ("crude fiber"). Since significant quantities of proteins, lipids or pigments can be still contained in the residues, the use of methods which lack specificity like the gravimetric method, the measure of nitrogen content or the crude fiber method, leads thus to overestimation of the chitin content.

Annual tissue production

Over 140 production data were collected in the international literature and transformed into a same unit, *i.e.* grams of dry mass produced by square meter and by year. These data are presented in table 2. The production value were transformed on the basis of information (water column depth, dry mass:wet mass ratio,...) given in the original paper.
From a geographical point of view, production data are heterogeneously distributed (figure 1). In fresh waters, the majority of production estimates has been done in North America and Western Europe. The intertropical zone has received little attention. In marine environment, the annual production by crustaceans has been mainly estimated along the east coast of North America and the west coast of Europe. The only major exception is the estimations of the euphausiid production in North and Southwest Atlantic Ocean [134,138]. To our knowledge, the continental slope and the deep sea floor has not been investigated for production.
In both freshwater and marine environments, the majority of the crustacean production values are between 1 to 40 $g.m^{-2}.yr^{-1}$. This wide variation in production values seems to be mainly due to great differences in food and predator abundance among the studied

ecosystems. However, we don't have sufficient data to establish a general pattern of production variation in function of biotic or abiotic factors. So, in our first attempt to estimate crustacean chitin production in the hydrosphere, we shall only consider a mean production value for each type of community, regardless of trophic or geographical differences.

Table 2: Annual production by freshwater crustaceans

CLASS ORDER Community	Annual production ($g.m^{-2}.yr^{-1}$) mean	(min - max)	Reference(s)
FRESH WATER			
WHOLE CRUSTACEAN COMMUNITY			
Lake plankton	181.2	(9.0 – 471.5)	[18-21]
BRANCHIOPODA			
ANOSTRACA			
Temporary pool plankton	6.2	(1.0 – 11.3)	[22]
CLADOCERA			
River plankton	0.6	(0.02 – 0.1)	[23 – 24]
Lake plankton	40.1	(0.4 – 251.9)	[26-41]
Waste stabilisation ponds	261.3	(47.0 – 446.1)	[25, (1)]
COPEPODA			
Lake plankton	33.2	(0.2 – 414.6)	[29,34-36,42-50]
MALACOSTRACA			
AMPHIPODA			
River benthos	6.6	(3.0 – 12.8)	[52,53,55-57]
Lake benthos	2.0	(0.8 – 6.0)	[51,52,54,58-61]
DECAPODA			
River benthos	4.2	(3.3 – 4.9)	[63,66]
Lake benthos	9.7	(4.8 – 17.4)	[62-65]
ISOPODA			
River benthos	33.9	(12.9 – 55.0)	[68]
Lake benthos	0.02		[67]
MYSIDACEA			
Lake plankton	0.8	(0.01 – 3.2)	[61,69-72]
OSTRACODA			
Lake benthos	0.05		[67]
SEA WATER			
COPEPODA			
Coastal plankton	26.7	(0.3 – 139.3)	[73,74,76,79,82,83,85,87,90-95]
Sea plankton	46.0	(11.4 – 100.0)	[96-99]
Estuarine plankton	9.5	(1.3 – 22.5)	[75,88,89]
Coastal meiofauna	2.7	(0.06 – 7.2)	[77,78,80,81,84,86]

Table 2 : (continued)

MALACOSTRACA			
AMPHIPODA			
Coastal benthos	5.3	(0.006 – 672.0)	[100-103,106,108-118,121-128,131]
Sea benthos	9.3	(0.1 – 39.8)	[104,105,107,108]
Estuarine benthos	15.2	(6.3 – 22.0)	[119,120]
Beach wreck fauna	660.4		[129]
Salt marsh benthos	1.1	(0.8 – 1.4)	[130]
DECAPODA			
Coastal benthos	1.5	(0.3 – 3.0)	[132,133]
EUPHAUSIACEA (= "KRILL")			
Coastal plankton	2.1	(0.3 – 3.8)	[135-137]
Sea plankton	57.9	(0.2 – 205.2)	[134,138]
ISOPODA			
Estuarine benthos	14.0	(5.6 – 22.3)	[139]
Coastal benthos	0.8		[140]
LEPTOSTRACA			
Coastal benthos	3.7	(0.8 – 7.0)	[118,131,141]
MYSIDACEA			
Coastal plankton	1.6	(0.04 – 3.8)	[142-144]
OSTRACODA			
Salt marsh benthos	9.2		[145]

(1) Cauchie *et al.*, unpublished data

Figure 1: Geographical distribution of the production data