

## MINI-REVIEW

### INSECT CHITIN

#### PHYSICAL STATE, SYNTHESIS, DEGRADATION AND METABOLIC REGULATION\*

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#### INTRODUCTION

"The shock arose not from the creatures abilities (intelligence, calculation, cunning and aggressiveness) but from the fact that they possessed internal skeletons, leathery skins and flexible bodies. They moved like primitive animals of the jungles but their intelligence was undeniable. The discovery had caused consternation in the... scientific community, which had postulated that *no creature lacking a protective exoskeleton would survive long enough to evolve true intelligence.*"

From *Nor Crystal Tears* by Alan Dean Foster (1982).

The above quote from Foster's science fiction novel expresses the idea that creatures with an exoskeleton are less primitive than those with an endoskeleton. So far of course, in evolutionary time, that is not the case except in certain specialized ecological niches. The evolution of a light yet mechanically strong exoskeleton (cuticle) has contributed greatly to the success of insects in nature. The cuticle and attached epidermis are one of the largest and most important organs of the insects' body. It is defined as a supra-molecular assembly of chitin, protein, minerals, lipid, catechols, pigments, water and other components that determines body shape and allows the insect to grow, move, communicate, reproduce and cope with environmental hazards such as predators, pathogens and toxic substances. The cuticle serves as an anchorage to skeletal muscles and protects against physical damage, penetration of pathogens and desiccation.

As a consequence of the exoskeleton's rigidity, insects must form new cuticles and shed old confining

ones periodically in order to grow and develop into larger larvae, pupae or adults. The stabilization of a new cuticle and destabilization of the old one are critical events in the molting process. Extracellular assembly of cuticle involves many complicated biochemical reactions that cause changes in mechanical properties including chitinization, sclerotization, mineralization, dehydration, water-proofing and other processes (Hepburn, 1985; Andersen, 1985). Although the relative importance of the individual cuticular biopolymers in imparting physical and chemical properties is not quantitatively known, it is generally believed that the polysaccharide chitin and sclerotized proteins play major roles in conferring various properties to the exoskeleton. Molting fluid which destabilizes the cuticle contains proteases and chitinases as the major degradative enzymes.

The subject of this review is the former biopolymer which is the simplest glycoaminoglycan, a  $\beta$ -1,4-homopolymer of *N*-acetyl-D-glucosamine. This natural polymer is distributed widely, especially in fungi, yeast and the exoskeleton of invertebrates. The unusual combination of toughness, flexibility and resistance to biodegradability makes chitin very attractive for utilization as a biological coating material. It has been the subject of many basic biochemical and physiological studies in developmental biology as well as a focus of applied research in the biochemical, pharmaceutical and diet supplement fields (Neville, 1975; Muzzarelli, 1977; Austin *et al.*, 1981).

Insects make excellent use of chitin in structural tissues. The assembly of a chitin-protein-crosslinking agent-mineral complex cuticle is one of the unsolved mysteries of insect development. The main factors contributing to the mechanical properties of cuticle are the organization and length of the chitin microfibril as well as chitin-protein and protein-protein interactions. Covalent crosslinks are thought to occur between chitin and protein although the exact nature of the interaction has not yet been defined. In some insects, i.e. certain flies, mineral salt interactions play a prominent role in cuticular mechanical properties (Darlington *et al.*, 1983; Roseland

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*et al.*, 1985). Hepburn and Roberts (1975) suggest that the stabilized matrix protein rather than chitin dominates the functional properties of cuticle. No doubt there are exceptions depending on the degree and type of physical strength (flexible, soft, brittle, hard, elastic) required in the exoskeleton.

The discovery of a class of insecticides in the early 1970s that specifically interferes with macromolecular (probably chitin) deposition in insect cuticle has generated a renewed interest in chitin metabolism (Verloop and Ferrell, 1977). We now have seen a practical experience that demonstrates how a disruption of chitin biosynthesis and subsequent fibrillogenesis, as well as its untimely degradation can be detrimental to insects.

This article is a review of papers published on insect chitin metabolism and is primarily an update of our review published in 1985 which covered most of the literature into the first quarter of 1982 (Kramer *et al.*, 1985). It also includes some references that we overlooked in the earlier review. Although the subject of this article is insect chitin biochemistry, the biochemistry of chitin present in other organisms is occasionally referred to for comparative purposes and also in certain topics where little information is available from insect research. In a relatively short period of a few years, significant new information has been developed concerning this fascinating field of research involving insects.

#### PHYSICAL STATE OF CHITIN

Chitin is found in the procuticle of body wall, the foregut and hindgut lining, tracheal tubes and muscle attachment points in assorted combinations with protein and other components depending on the species and desired physical properties. When observed at high magnification in thin section, arthropod cuticle often shows a fibrillar pattern, indicating that chitin is deposited in fiber-containing layers (Noble-Nesbitt, 1963a,b; Rudall, 1965; Neville, 1967a,b, 1970; Retnakaran and Hackman, 1985). It has often been assumed that this pattern corresponds to the spatial association of chitin and protein with the opaque region representing the protein fraction and the clear rods the chitin crystallites. However, there is no definitive evidence that the clear rods are indeed composed of pure chitin. The usual interpretation of the microfibril molecular structure of cuticle has recently been questioned (Filshie, 1982; Giraud-Guille, 1984). The diameter of the transparent rods and their spacing show large variations among groups of arthropods. Thus, a general molecular model of the cuticle microfibril arrangement is unavailable at present. More direct chemical evidence of the microfibril composition is desired. Chitin (or chitosan) can be localized ultrastructurally in microorganisms using either a conjugated lectin-gold or chitinase-gold complex technique (Tronchin *et al.*, 1981; Arroyo-Begovich and Carabez-Trejo, 1982; Chamberland *et al.*, 1985). These techniques have not yet been applied to insect tissues.

Chitin-containing microfibrils originate at the epidermal cell border and are probably surrounded primarily by cuticular proteins, the matrix consisting

of varying relative amounts of each (Ogawa *et al.*, 1982). In general higher levels of chitin are found in lesser elastic cuticles which suggests a brittle nature to the polysaccharide. The rate of chitin deposition varies and may be correlated with thickness, being more rapid in regions of the body where cuticle is thicker (Koulis and Gould, 1983). Although it is difficult to attribute mechanical properties to individual components in a composite material, it has been demonstrated that removal of chitin from crab cuticle results in a small decrease in elongation capacity and no change in tensile strength (Dendinger and Alterman, 1983). Extraction of soluble protein and minerals leads to a decrease and increase, respectively, in both physical parameters.

Efforts to investigate the three-dimensional structure of chitin or its protein complex have a history of over 60 years and include the use of electron microscopy (Hall *et al.*, 1981; Bal *et al.*, 1981; Blackwell *et al.*, 1981), nuclear magnetic resonance (Saito *et al.*, 1981a,b; Tsukada and Inoue, 1981; Gagnaire *et al.*, 1982; Peter *et al.*, 1984), electron diffraction (Gemerle *et al.*, 1982) and X-ray diffraction (Gardner and Blackwell, 1975; Minke and Blackwell, 1978; Blackwell *et al.*, 1980). Some of the recent work in diffraction analysis has been concerned with a refinement of chitin structures utilizing least squares techniques and new information on polysaccharide stereochemistry. Chitin can be regarded as a reinforcing fiber element of cuticle. It is structurally anisotropic as a long polymer but the anisotropy does not necessarily carry through to complex cuticle. The diffraction methods have been the most successful for structural analysis, providing structures of the three naturally occurring polymorphic forms known as  $\alpha$ -,  $\beta$ -, and  $\gamma$ -chitins. Basic to the proposed structures for all three forms is the presence of sheets of chains linked by  $-N-H \cdots O=C-$  hydrogen bonds through the amide groups. The forms make up microfibrils that differ in the sense of the chains in successive sheets. In  $\beta$ -chitin the sheets are all arranged in a parallel manner, whereas in the  $\alpha$ -form successive sheets are anti-parallel. The  $\gamma$ -structure is grouped in sets of three chains, two parallel and one anti-parallel.  $\alpha$ -Chitin occurs in arthropod cuticles;  $\beta$ - and  $\gamma$ -chitins are found in cocoons (Kenchington, 1971). After various treatments the latter forms convert to  $\alpha$ -chitin which apparently is the most stable form (Rudall and Kenchington, 1973). These models represent only those parts of the chitin molecule which are highly ordered and should not be interpreted as a picture of chitin structure in general. The less ordered chitin chains may have altogether different arrangements and the contribution of associated components such as protein is not at all clear. Mechanically chitin has been classified as a viscoelastic polymer with stiffness, strength and extensibility varying among polymorphs (Hepburn and Chandler, 1978; Hepburn, 1985).

Morphologically distinct forms of chitin may exist in cuticle. For example, in the dermatophyte *Trichophyton mantagrophytes*, two-thirds of the wall chitin is a microfibrillar and chitinase-sensitive form (Pollack *et al.*, 1983). The remaining chitin is a non-fibrillar form which is insensitive to chitinase. The presence of a highly crystalline matrix of chitin or of protective

layers of protein, pigment or other components may impart chitinolytic enzyme resistance to the cuticle. Certain entomopathogenic agents such as fungi may attack only those insects whose cuticular chitin is fibrillar and therefore susceptible to hydrolysis by chitinases (Ishikawa *et al.*, 1983).

Blackwell and coworkers (Blackwell and Weih, 1980, 1984; Blackwell *et al.*, 1981) have used X-ray diffraction and electron microscopy to derive a model for the arrangement of the chitin-protein complex in the cuticle of the ovipositor of the ichneumon fly *Megarhyssa*. This tissue is one of the most ordered structures ever studied and the cuticle shows a high degree of periodicity with both the chitin and protein ordered. Earlier Rudall and Kenchington (1973) had reported a hexagonal lattice of chitin chains in the same tissue. The Blackwell model also consists of a hexagonal array of chitin microfibrils, each surrounded by a sheath of protein. The sheath consists of a helix of protein subunits. Fourier filtration and reconstruction of electron micrographs of uranyl acetate stained cuticle showed a hexagonal array of chitin microfibrils ( $\alpha$ -chitin) in a matrix of stained protein. A globular protein of molecular weight  $2.7 \times 10^4$  daltons would have the approximate dimensions of the subunits. Neville *et al.* (1976) also reported microfibrils arranged on a hexagonal lattice in a survey of a variety of insect cuticles. The chitin-protein fibrils of *Megarhyssa* also exhibit a long fiber repeat which may be due to supercoiling of the chitin-protein complex fiber.

In recent years one of the most dramatic advancements in biopolymer research has been the use of solid state nuclear magnetic resonance spectroscopy for non-destructive structural analysis of complex insoluble material such as insect cuticle. The techniques of single and double cross-polarization (CP) and magic angle sample spinning (MASS) yield high resolution spectra of dilute nuclei (e.g.  $^{13}\text{C}$  and  $^{15}\text{N}$ ) in the solid state (Yannoni, 1982). CPMAS-NMR involves cross-polarization to enhance the  $^{15}\text{N}$  or  $^{13}\text{C}$  signal, higher power dipolar decoupling to eliminate dipolar line broadening due to protons, and rapid spinning of the sample about an axis at a particular angle to the static field to eliminate chemical shift anisotropy. The chemical shift values of solid state spectra are isotropic values which are similar to those obtained in solution and may be used to provide information about chemical identity, microstructure and conformation of compounds. This structure has already been used to demonstrate that certain naturally occurring and regenerated crab and insect chitins have nearly identical conformations, presumably  $\alpha$ -chitin (Saito *et al.*, 1981a,b, 1982; Peter *et al.*, 1984; Kramer K., Speirs R., Schaefer J., Garbow J. and Jacob G. unpublished data) and that native and regenerated celluloses exist in several distinct forms (Dudley *et al.*, 1983; Atalla and Vanderhart, 1984; Herbert *et al.*, 1985). Line-broadening effects were attributed to imperfect crystalline packing in some preparations. Chitin, protein, catechol and lipid carbon signals dominate the natural abundance  $^{13}\text{C}$ -solid state NMR spectrum of *Manduca sexta* pupal exuviae which are composed of approx. 20% chitin (Fig. 1). The chitin sample was prepared by using the traditional method of hot alkali digestion (Odier,

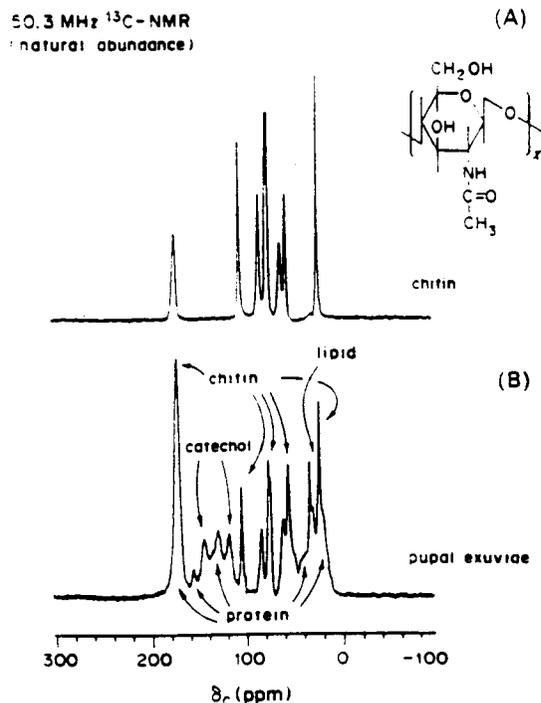


Fig. 1. Cross polarization magic angle sample spinning 50 MHz- $^{13}\text{C}$ -NMR spectra of *Manduca sexta* chitin (A) and pupal exuviae (B). The assignments for the carbohydrate carbons are carbonyl, 172 ppm; C-1, 102 ppm; C-4, 82 ppm; C-5, 74 ppm; C-3, 72 ppm; C-6, 60 ppm; C-2, 54 ppm; methyl, 21 ppm (Schaefer J., Kramer K., Jacob G., Grabow J., Fukamizo T. and Speirs R., unpublished data).

1823). CPMAS-NMR should be very useful for studying the entire supramolecular structure of many intractable biological structures.

The interaction of chitin and protein in cuticle appears to be of both a covalent and non-covalent nature. Upon extraction by certain mild solvents, X-ray patterns associated with ordered components disappear, indicating that at least some of the proteins interact via non-covalent bonding (Rudall and Kenchington, 1973). Chitin affinity chromatography has been used to determine whether proteins bind chitin non-covalently. The adsorbents available include dispersed chitin (Cherkasov and Kravchenko, 1967; Roberts and Cabib, 1982), chitin-coated cellulose (Imoto and Yagishita, 1973), *N*-acetyl- $\beta$ -D-glucosaminide agarose (Junowicz and Charm, 1975), *N,N',N''*-triacetyl- $\beta$ -D-glucosaminide agarose (Cornelius *et al.*, 1974), *N,N',N''*-triacetyl- $\beta$ -D-glucosaminide cellulose (Yamasaki and Eto, 1981) and chitin-coated celite (Yamada *et al.*, 1986), the latter being the only adsorbent with physical properties suitable for HPLC. Eighty-five per cent of the *Drosophila* larval proteins and 70% of the pupal cuticle proteins extracted in 7 M urea bind to dispersed chitin when renatured (Fristrom *et al.*, 1978; Silvert *et al.*, 1984).

The possible existence of covalent protein (amino acid)-chitin linkages has been investigated by a number of laboratories (Attwood and Zola, 1967; Hackman and Goldberg, 1971; Lipke, 1971; Lipke and Geoghegan, 1971; Lipke and Strout, 1972; Hillerton,

1980; Brine and Austin, 1981a,b; Lipke *et al.*, 1981; Sugumaran *et al.*, 1982; Kramer and Fukamizo, unpublished data). Protein from newly ecdysed cuticle is more extractable than that from stabilized cuticle. The presence of residual amino acids in chitin preparations following extraction from sclerotized cuticle suggests a small degree of covalent bonding at irregular intervals in these chitin-protein complexes. Jacobs (1978, 1985) has proposed that  $\beta$ -alanine binds not only to protein during sclerotization, but also to chitin which results in compaction of cuticular chitinous lamellae in *Drosophila*. The non-covalent bonding is thought to be non-specific (Hackman and Goldberg, 1978). Although its idealized chemical structure is a homopolymer of  $\beta(1,4)$ -linked *N*-acetylglucosamine residues, chitin (or chitosan depending on the relative abundance of *N*-acetylglucosamine versus glucosamine) may actually be a heteropolymer containing deacetylated residues. Fungal cell wall may contain polymers that are as much as 70% deacetylated (Davis and Bartnicki-Garcia, 1984a,b). It is very probable that there are glucosamine residues in insect chitin chains which may impart chitosanic-like properties to the polysaccharide. Chitin deacetylation (presumably catalyzed by a deacetylase) has been associated with enlargement of the abdomen of the queen termite *Macrotermes esterae* (Rajula *et al.*, 1982). The degree of deacetylation changes from 0% in the alate queen abdomen to approx. 90% in the fully expanded physogastric queen abdomen. Whether deacetylation facilitates elongation of the cuticle remains open to question. The degree of deacetylation of chitin has been estimated to be a small but significant percentage of residues (1–20%) in other types of cuticle by product analysis after enzymatic digestion (Hackman and Goldberg, 1965), titration, i.r. spectroscopy (Hackman and Goldberg, 1974), thermal analysis (Alonso *et al.*, 1983) and pyrolysis-mass spectrometry (Mattai and Hayes, 1982; Van der Kaadem *et al.*, 1984). The peak intensities of amino, amide,  $\text{CH}_3$ , carbonyl and C-1 atoms in CPMAS-NMR can be used to quantitate the extent of chitin deacetylation (Saito *et al.*, 1982).

A gravimetric determination of chitin is possible although harsh chemical treatments are usually required to remove protein, minerals and pigments from raw material (Muzzarelli, 1977; Hackman and Goldberg, 1981; Hackman, 1982). The preferred methods include prolonged digestion in alkali, acid and oxidizing solutions to eliminate protein, minerals and pigments, respectively. In nearly all cases, a partially deacetylated product is obtained. Reacetylation with acetic anhydride may be necessary to generate a more native-like chitin product.

Chitin chemical analysis is not straightforward because assays generally lack specificity due to potential interference from carbohydrates present in other biopolymers and also from the presence of amino compounds such as amino acids and hexosamines. Chitin has been traditionally measured colorimetrically in terms of *N*-acetylglucosamine equivalents by the Morgan-Elson reaction (Ghuysen *et al.*, 1966) after digestion of the polymer with chitinase and  $\beta$ -*N*-acetylglucosaminidase (Molano *et al.*, 1980). However, enzymatic digestions are not quan-

titative if chitin chains are inaccessible to those enzymes. Alternatively, chitin can be acid hydrolyzed and the resultant glucosamine quantified by the Elson-Morgan procedure (Davidson, 1966) or by the Morgan-Elson procedure after reacetylation of glucosamine (Ghuysen *et al.*, 1966). After separation from amino compounds, glucosamine can be measured by ninhydrin, fluorescamine or *o*-phthaldialdehyde reaction (Lui, 1972; Chang and Hash, 1979; Carroll and Nelson, 1979; Chen and Mayer, 1981). Gas chromatographic and high performance liquid chromatographic procedures are available for precise analyses of chitin hydrolytic products and, when these techniques are coupled to mass spectrometry, the identification is unequivocal (Coduti and Bush, 1977; Van Eikeren and McLaughlin, 1977).

Although a good deal of progress has been made in studies of chitin architecture and the quarternary structure of chitin-protein complexes, we still need a more detailed picture in order to understand the contribution of these components to cuticle ultrastructure. In particular information about the degree of polymerization of insect chitin, its "chitosanic" nature if any, the structure of cuticular protein and the precise covalent and non-covalent interactions of proteins, catechols and minerals with insect chitin is lacking. Naturally occurring chitin-protein complexes change between one type of cuticle and another depending on the physical properties desired of the composite material. In some instances the whole architecture of the chitin changes yet still allows a precise molecular fit to occur with surrounding protein (Atkins, 1982).

#### CHITIN SYNTHESIS

Despite the importance of chitin in nature, its biosynthesis is imperfectly understood. Biomass reutilization is important for energy conservation in invertebrates. Chitin from cuticle of the previous instar is degraded by molting fluid chitinolytic enzymes to GlcNAc, some of which is reutilized for chitin synthesis of new cuticle. Radioactivity from GlcNAc of locust larval cuticle was subsequently found in the new imaginal cuticle of the adult (Surholt, 1975a). Gwinn and Stevenson (1973a) demonstrated the reutilization of GlcNAc derived from old cuticle of the crayfish for new cuticle formation.

The biosynthetic pathway for chitin in invertebrates has been delineated primarily by studies using radiolabeled compounds (Fig. 2). Over 20 years ago, the pathway from glucose to uridine diphosphate *N*-acetylglucosamine (UDPGlcNAc) was established using a wing extract of the desert locust (Candy and Kilby, 1962). It was suggested that seven enzymes catalyze reactions in the chitin biosynthetic pathway including hexokinase, phosphohexose isomerase, glutamine transaminase, phosphoglucosamine transacetylase, acetyl coenzyme-A synthetase, phosphoacetylglucosamine mutase and uridine diphosphate *N*-acetylglucosamine pyrophosphorylase. *N*-acetylglucosamine kinase catalyzed the formation of *N*-acetylglucosamine-6-phosphate in crayfish epidermis (Gwinn and Stevenson, 1973b). The pathway from UDPGlcNAc to chitin was determined by

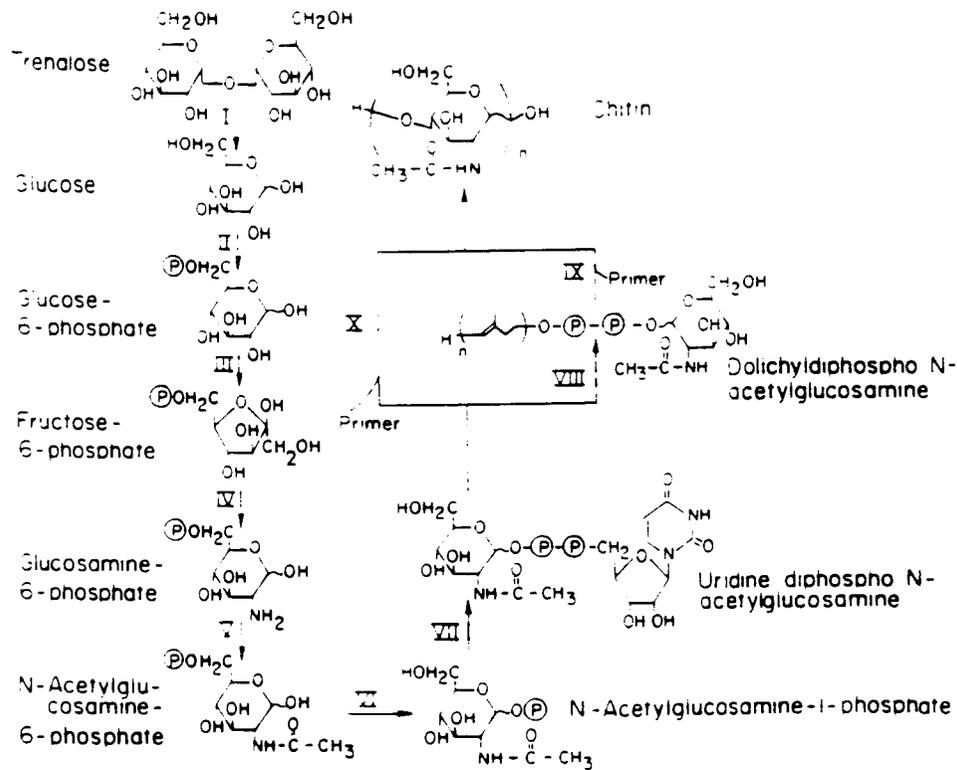


Fig 2. Pathway for chitin biosynthesis. Reactions catalyzed by enzymes are I, trehalase; II, hexokinase; III, glucose phosphate isomerase; IV, glutamine-fructose-6-phosphate aminotransferase; V, glucosamine-6-phosphate *N*-acetyltransferase; VI, phosphoacetylglucosamine mutase; VII, uridinediphospho-*N*-acetylglucosamine pyrophosphorylase; VIII, uridinediphospho-*N*-acetylglucosamine dolichyl phosphate transferase; IX and X, chitin synthase.

Jaworski *et al.* (1963) using the southern armyworm preparation and by Carey (1965) using the blue crab and the brine shrimp preparations. UDPGlcNAc and primer chitin oligosaccharides were excellent promoters or precursors utilized by the synthase enzyme for chitin. The polymerizing enzyme is probably the most unique and also the most critical in the entire sequence of reactions. Thus, the general pathway for chitin synthesis is fairly well established and is supported by many studies (Table 1). Several carbohydrates besides glucose (Hornung and Stevenson, 1971; Post *et al.*, 1974; Surholt, 1975a,b; Vardanis, 1976; Hajjar and Casida, 1978, 1979; Mayer *et al.*, 1980a) can serve as precursors for chitin including trehalose (Quesada-Allue *et al.*, 1976), fructose (Mayer *et al.*, 1979), glucosamine (Speck *et al.*, 1972; Oberlander and Leach, 1975; Mayer *et al.*, 1979; Vardanis, 1976, 1979; Hajjar and Casida, 1979; Turnbull and Howells, 1982; Quesada-Allue, 1982) and *N*-acetylglucosamine (Gwinn and Stevenson, 1973a,b; Sowa and Marks, 1975; Surholt, 1975a,b; Mayer *et al.*, 1979, 1980b; Ferkovich *et al.*, 1981; Turnbull and Howells, 1982).

Studies concerning insect chitin synthesis generally have not been definitive primarily because they were conducted using crude extracts or preparations of enzymes and poorly characterized substrates, intermediates and products. Chitin synthases from different sources are not all alike and it is doubtful whether they are necessarily closely related, despite

the evident similarity in their substrates and products. Until more information is available about heterogeneity and structure of the enzymes, substrates, intermediates and products, any conclusion should be regarded with caution. Such is the case with chitin synthase which has not yet been purified to homogeneity. It is a rather large unstable protein or complex of proteins which apparently exist as a particulate entity whose integrity requires the interaction with other particulate components found in membranes or cell walls.

Although many studies have been conducted on chitin synthases (UDP-2-acetyl-amino-2-deoxyglucosyltransferase; EC 2.4.1.16) from yeast and fungi (Glaser and Brown, 1957; Cabib, 1972; Cabib and Ulane, 1973; Ruiz-Herrera and Bartnicki-Garcia, 1974; Ruiz-Herrera *et al.*, 1977; Bracker *et al.*, 1976; Duran and Cabib, 1978; Duran *et al.*, 1975, 1979), relatively few have been done using an insect enzyme preparation. Common features of most of the chitin synthases are their localization on vesicular membranes and slight activation after treatment with proteases. Chitin synthase preparations from both yeast and mycelial *Candida albicans* were chiefly in a zymogenic form, activated by trypsin treatment (Hardy and Gooday, 1983; Kang *et al.*, 1984). Several *in vitro* insect organ cultures or *in vivo* studies have been carried out (Candy and Kilby, 1962; Marks and Leopold, 1971; Oberlander and Leach, 1975; Surholt, 1975a; Vardanis, 1976). Recently it was reported that

Table 1. Biosynthetic studies of chitin using carbohydrate precursors

Precursor	Organism	Tissue	Reference	
Glucose	<i>Lucilia cuprina</i> (crayfish)	Third instar larval integument	Retnakaran and Hackman (1985)	
	<i>Locusta migratoria</i> (locust)	Larvae, <i>in vivo</i>	Surholt (1975a, b)	
	<i>Melanoplus sanguinipes</i> (grasshopper)	Adult abdominal integument tissue	Vardanis (1976)	
	<i>Oncopeltus fasciatus</i> (milkweed bug)	Newly emerged adult abdominal tissue	Hajar and Casida (1978, 1979)	
	<i>Orconectes obscurus</i> (crayfish)	All stages, <i>in vivo</i>	Horning and Stevenson (1971)	
	<i>Stomoxys calcitrans</i> (stable fly)	Imaginal epidermal tissue from 4-day-old pupae	Mayer <i>et al.</i> (1980a)	
	Glucose → UDPGlcNAc	<i>Schistocerca gregaria</i> (desert locust)	Fifth larval instar and adult, <i>in vivo</i> and wing extract	Candy and Kilby (1962)
		Glucose → GlcNAc → UDPGlcNAc	<i>Pieris brassicae</i> (cabbage butterfly)	Fifth instar larvae, <i>in vivo</i>
Trehalose	<i>Triatoma infestans</i> (blood sucking bug)		Newly molted imago, <i>in vivo</i>	Quesada-Allue <i>et al.</i> (1976)
Fructose	<i>Stomoxys calcitrans</i> (stable fly)	Imaginal epidermal tissue from 4-day-old pupae	Mayer <i>et al.</i> (1980a)	
Glucosamine	<i>Drosophila melanogaster</i> (fruit fly)	Imaginal disc	Fristrom <i>et al.</i> (1982)	
	<i>Leucophaea maderae</i> (cockroach)	Leg regenerate	Sowa and Marks (1975)	
	<i>Lucilia cuprina</i> (sheep blowfly)	Third instar larval integument	Turnbull and Howells (1982) Retnakaran and Hackman (1985)	
	<i>Melanoplus sanguinipes</i> (grasshopper)	Abdominal integument	Vardanis (1976, 1979)	
	<i>Plodia interpunctella</i> (Indianmeal moth)	Wing imaginal disc from last larval instar	Oberlander and Teach (1975)	
	<i>Oncopeltus fasciatus</i> (milkweed bug)	Abdomen	Hajar and Casida (1979)	
	<i>Stomoxys calcitrans</i> (stable fly)	Imaginal epidermis from 4-day-old pupae	Mayer <i>et al.</i> (1980a)	
	<i>Triatoma infestans</i> (blood sucking bug)	Abdominal epidermis from newly molted imago	Quesada-Allue (1982)	
	GlcNAc → UDPGlcNAc	<i>Lucilia cuprina</i> (sheep blowfly)	Third instar larval integument	Turnbull and Howells (1982)
		GlcNAc → GlcNAc-6-phosphate → GlcNAc-1-phosphate → UDPGlcNAc	<i>Orconectes obscurus</i> (crayfish)	Epidermal homogenate and <i>in vivo</i>
	<i>Orconectes sanborni</i> (crayfish)		<i>In vivo</i>	Stevenson and Hettick (1980)

GlcNAc	<i>Galleria mellonella</i> (greater wax moth)	Last instar larvae and pupae	Ferkovich <i>et al.</i> (1981)
	<i>Leucophaea maderae</i> (cockroach)	Leg regenerate, <i>in vitro</i>	Sowa and Marks (1975)
	<i>Locusta migratoria</i> (locust)	Larval-adult molting cycle from 4 days before to 8 days after ecdysis	Surbolt (1975)
	<i>Lucilia cuprina</i> (sheep blowfly)	Third instar larval integument	Retnakaran and Hackman (1985)
	<i>Manduca sexta</i> (tobacco hornworm)	Embryonic cell line	Marks <i>et al.</i> (1984)
	<i>Oncopeltus fasciatus</i> (milkweed bug)	Abdomen	Hajar and Casida (1979)
	<i>Orconectes limosus</i> (crayfish)	<i>In vivo</i>	Speck <i>et al.</i> (1972)
	<i>Plodia interpunctella</i> (Indianmeal moth)	Last instar larvae and pupae	Ferkovich <i>et al.</i> (1981)
	<i>Plodia interpunctella</i> (Indianmeal moth)	Wing imaginal disc	Oberlander <i>et al.</i> (1983)
	<i>Stomoxys calcitrans</i> (stable fly)	Pupae	Mayer <i>et al.</i> (1979-1980)
	<i>Triatoma infestans</i> (blood sucking bug)	Microsomal fraction from larval integument	Quesada-Allue <i>et al.</i> (1976)
UDPGlcNAc	<i>Artemia salina</i> (brine shrimp)	Microsomal fraction from larvae	Horst (1981)
	<i>Callinectes sapidus</i> (blue crab)	Subcellular particles from the epidermis of molting blue crab	Carey (1965)
	<i>Hemigrapsus nudus</i> (purple shore crab)	Extract of dorsal integument	Hohnke (1971)
	<i>Hyalophora cecropia</i> (giant silkworm)	Microsomal fraction from pupal wing tissue	Cohen and Casida (1982)
	<i>Locusta migratoria</i> (locust)	Larvae, <i>in vivo</i>	Surbolt (1975a)
	<i>Lucilia cuprina</i> (Australian sheep blowfly)	Crude homogenate of larval integument	Turnbull and Howells (1983) Retnakaran and Hackman (1985)
	<i>Melanoplus sanguinipes</i> (grasshopper)	Abdominal integument after final molt	Vardanis (1976)
	<i>Prodenia eridania</i> (southern army moth)	Late prepupae	Jaworski <i>et al.</i> (1963)
	<i>Tribolium castaneum</i> (flour beetle)	Microsomal fraction from last instar larval gut	Cohen and Casida (1980a) Cohen (1982)
	<i>Trichoplusia ni</i> (cabbage looper)	Microsomal fraction from fifth instar larval integument	Cohen and Casida (1982)
UDPGlcNAc	<i>Melanoplus sanguinipes</i> (grasshopper)	Abdominal integument	Vardanis (1976, 1979)
UDPGlcNAc → (dolichyl diphosphate GlcNAc)	<i>Triatoma infestans</i> (blood sucking bug)	Microsomal fraction from larval integument	Quesada-Allue <i>et al.</i> (1976)
UDPGlcNAc → lipid-linked intermediate (dolichyl diphosphate GlcNAc)	<i>Artemia salina</i> (brine shrimp)	Microsomal fraction from larvae	Horst (1983)

an embryo-derived insect cell line synthesizes chitin (Marks *et al.*, 1984). The use of an insect cell free preparation has proven difficult because enzyme activity is not stable and can be lost by treatments such as freezing and thawing (Jaworski *et al.*, 1963; Porter and Jaworski, 1965; Surhoit, 1975a; Vardanis, 1976; Retnakaran and Hackman, 1985). Chitin synthase from the stable fly, *Stomoxys calcitrans* (L.) is a particulate enzyme with a pH optimum of 6.5 that utilizes UDPGlcNAc as substrate (Mayer *et al.*, 1980a). The enzyme appears to be synthesized as a zymogen since incubation with trypsin was found to increase the original activity level slightly. However, definitive evidence for precursor forms of chitin synthase in insects is lacking, even though information about the zymogenic nature of the enzyme from microbial sources is available (Hardy and Gooday, 1983).

For studies of the conversion of UDPGlcNAc to chitin, most experiments were performed *in vitro* using a microsomal enzyme preparation (Table 2). Chitin synthase activity has been detected in the microsomal fraction of epidermal cells, integument and gut. There are differences in effector requirements and in inhibition patterns. Cohen and Casida (1982) observed differences in the requirement of a primer such as GlcNAc and in the effect of the UDPGlcNAc analogues, polyoxin and nikkomycin, on chitin synthesis in the giant silkworm and the cabbage looper. Horst (1981) reported that polyoxin *D* did not inhibit chitin synthesis in a crustacean but that diflubenzuron did. Turnbull and Howells (1983) obtained a Dipteran chitin synthase preparation which is sensitive to inhibition of both polyoxin *D* and diflubenzuron. Since polyoxin and nikkomycin are antibiotics as well as structural analogues of UDPGlcNAc, they probably inhibit chitin synthase directly as competitive inhibitors (Gooday, 1972). In isolated larval integument of *Lucilia*, polyoxin *D* and diflubenzuron appear to interfere with the final polymerization step in the chitin biosynthetic pathway since the final intermediate (UDP-*N*-acetylglucosamine) was found in the presence of inhibitors (Turnbull and Howells, 1982). Leighton *et al.* (1981) proposed that benzoylphenylurea derivatives act indirectly on chitin synthesis by inhibiting a proteolytic enzyme required for the activation of chitin synthase. The variable effects of inhibitors of chitin synthase appear to be due in part to the conditions of enzyme preparation and the presence of potential effectors. There may exist multiple types of chitin synthase, and modulating factors may also be present in heterogeneous preparations.

Quesada-Allue *et al.* (1976) have reported the involvement of a lipid-linked intermediate in the reaction from UDPGlcNAc to chitin (Parodi and Leloir, 1979). Tunicamycin inhibited chitin synthesis in larval abdominal epidermis of a blood sucking bug. This antibiotic inhibits the condensation of dolichyl phosphate and UDPGlcNAc in protein glycosylation reactions (Takatsuki *et al.*, 1975; Schwartz *et al.*, 1979). Horst (1983) showed that a microsomal preparation from larval brine shrimp catalyzes the transfer of GlcNAc to a lipid acceptor. The carbohydrate components of the intermediate contained oligosaccharides ranging from two to eight GlcNAc

units which are susceptible to chitinase digestion. These results suggested that the conversion of UDPGlcNAc to chitin proceeds via the formation of dolichyldiphospho-GlcNAc as an intermediate. However, using a subcellular particulate preparation as the source of crude enzyme from 4-day-old pupae of the stable fly, Mayer *et al.* (1981) reported that tunicamycin did not inhibit chitin synthesis. In a more recent study, Mayer and Chen (1985) suggest that tunicamycin may competitively inhibit chitin synthase or *N*-acetylglucosaminyl transferase which catalyzes the formation of dolichyldiphospho-*N*-acetylglucosamine. Bade (1983) obtained negative results with tunicamycin *in vitro* using cuticle epithelial tissue from the tobacco hornworm. There may be two pathways for chitin synthesis, one being a direct conversion of UDPGlcNAc to chitin and the other also involving dolichyldiphospho-GlcNAc. Whether those pathways for chitin synthesis reflect the differences in the effects of inhibitors is unknown. The possible existence of multiple forms of chitin synthase which differ in inhibitor specificity needs further investigation.

Other types of antibiotics have been used to inhibit chitin synthesis including inhibitors of RNA synthesis such as  $\alpha$ -amanitin and actinomycin *D*, inhibitors of protein synthesis such as cycloheximide and puromycin, and cytochalasin *B*, colcemid and vinblastine which are cytoskeletal microtubule or microfilament inhibitors (Oberlander, 1976; Ferkovich *et al.*, 1981; Oberlander *et al.*, 1980, 1983). In *Drosophila* imaginal discs (Fristrom *et al.*, 1982), chitin synthesis is sensitive to inhibitors of protein (cycloheximide), RNA ( $\alpha$ -amanitin), and chitin (polyoxin *D*, diflubenzuron) syntheses, but had little sensitivity to inhibitors of DNA synthesis (cytosine arabinoside) and dolichol-dependent glycosylation (tunicamycin).

Using the microsomal fraction from last instar larvae of the flour beetle and UDPGlcNAc, MgCl<sub>2</sub>, DTT and GlcNAc, chitin was synthesized *in vitro* and observed in a form of long coiled microfibrils (10–80 nm thick) by electron microscopy (Cohen, 1982). Other particles became associated with the microfibrils to form spheroidal complexes that range from about 50 to 250 nm in diameter.

In microorganisms (primarily yeasts and fungi) chitin synthase has been found in the cell wall, plasma membrane, chitosome (a cytoplasmic organelle), and cytoplasm (Cabib, 1981; Ruiz-Henera *et al.*, 1984). High resolution autoradiography revealed that most of the chitin synthesized by *Mucor rouxii* is located within the cytoplasm and only a small amount of enzyme product appears at the cell surface (Sentandreu *et al.*, 1984). Apparently the majority of *M. rouxii* chitin synthase is located in the cytoplasm. In *Neurospora crassa*, chitosomal chitin synthase is found in a wall-less mutant (Bartnicki-Garcia *et al.*, 1984). The enzyme requires a divalent cation, usually Mg<sup>2+</sup>, and is inhibited by nikkomycin and polyoxin (K<sub>i</sub> = 0.5–1.5  $\mu$ M; Keller and Cabib, 1971; Duran and Cabib, 1978; Ruiz-Herrera *et al.*, 1977; Hanseler *et al.*, 1983a,b). Chitin synthase from the stable fly has a higher affinity for UDPGlcNAc (K<sub>m</sub> = 31.7  $\mu$ M) than does the enzyme from microbial sources (K<sub>m</sub> = 0.5–1.5 mM). The requirement of

Table 2. Characteristics of chitin synthase in cell free systems

Organism	Tissue	Requirement	Inhibitor	Comment	Reference
<i>Artemia salina</i> (brine shrimp)	Larvae	MgCl <sub>2</sub> , endogenous acceptor or exogenous chitin	UDP, diflubenzuron, chitodextrin	pH opt. ~7.0	Horst (1981, 1983)
	Larvae (3 day old)	MgCl <sub>2</sub> , NaCl, chitodextrin		pH opt. ~7.7	Carey (1965)
<i>Callinectes sapidus</i> (blue crab)	Epidermis	MgCl <sub>2</sub> , NaCl, chitodextrin		pH opt. ~7.7	Carey (1965)
<i>Hemigrapsus nudus</i> (purple shore crab)	Dorsal integument	MgCl <sub>2</sub>	Chitodextrin	pH opt. ~8.0, K <sub>m</sub> = 0.17 mM for UDPGlcNAc	Hobbs (1971)
<i>Hyalophora cecropia</i> (giant silk worm)	Pupal wing	MgCl <sub>2</sub>	Captan	pH opt. ~7.2	Cohen and Casada (1982)
<i>Lucilia cuprina</i> (Australian sheep blowfly)	Larval integument (early third instar)	MgCl <sub>2</sub>	Polyoxin D (K <sub>i</sub> = 0.04 μM), diflubenzuron (K <sub>i</sub> = 5.8 μM)	K <sub>m</sub> = 0.8 mM for UDPGlcNAc	Turnbull and Howells (1983)
<i>Stomoxys calcitrans</i> (stable fly)	Pupae (4 day old)		GlcNAc, UDP, βGlcNAc <sub>2</sub> , UTP, glycerine, polyoxin D at high conc.	pH opt. ~6.8, K <sub>m</sub> = 31.7 μM, V <sub>max</sub> = 135 pmol/hr per mg for UDPGlcNAc	Mayer <i>et al.</i> (1980a), Mayer <i>et al.</i> (1981)
<i>Triatoma infestans</i> (blood sucking bug)	Larval integument	MgCl <sub>2</sub> , GlcNAc	Tunicamycin	pH opt. ~7.2	Quesada-Allue <i>et al.</i> (1976)
<i>Tribolium castaneum</i> (flour beetle)	Larval gut	MgCl <sub>2</sub> , GlcNAc	Polyoxin D, UDP, UTP, CDP, CTP, captan, nikkomycin	pH opt. ~7.2	Cohen and Casada (1980a,b), Cohen (1982)
<i>Trichoplusia ni</i> (cabbage looper)	Larval integument	MgCl <sub>2</sub> , GlcNAc	UDP, UTP, polyoxin B and D, nikkomycin, captan	pH opt. ~7.2	Cohen and Casada (1982)

GlcNAc as a primer appears to be dependent on the enzyme source in both invertebrates and microorganisms. Phospholipid is required for chitin synthase from *Schizophyllum commune* (Vermeulen and Wesseis, 1983). In *Candida albicans* membrane Chiew *et al.* (1982) found that chitin synthase has two binding sites for GlcNAc with  $K_m = 0.3$  and 20 mM. The synthase from *Agaricus bisporus* has at least two substrate binding sites and exhibits a homotropic-heterotropic mixed-type regulation (Hanseler *et al.*, 1983a). The chitosome appears to be a cytoplasmic conveyor of chitin synthase to the cell surface (Bracker *et al.*, 1976; Bartnicki-Garcia *et al.*, 1978; Ruiz-Herrera *et al.*, 1977; Hanseler *et al.*, 1983a,b). *Tetranychus urticae* (Acari) chitosomal and membrane-bound chitin synthases are located in oocytes and the hypodermis, respectively (Mothes and Seitz, 1981). Whether a chitosomal synthase is present in insect tissues is unknown. To date the only purified chitin synthase preparations are those from the yeast *Saccharomyces cerevisiae* (Kang *et al.*, 1984) and the toadstool *Coprinus cinereus* (Montgomery *et al.*, 1984). The former is a plasmalemma-bound enzyme that is present in the cell as a zymogen which can be converted into an active form by partial proteolysis. After solubilization with digitonin, the enzyme is trapped in the chitin formed is the reaction and extracted in 50% yield, the entrapment being a crucial step in the isolation procedure. Yeast chitin synthase exhibits an apparent molecular weight of  $5.7 \times 10^5$  under non-denaturing conditions and may be composed of subunits with apparent molecular weights of  $6.3 \times 10^4$  and  $7.4 \times 10^4$ . *N*-Acetylglucosamine and chitin oligosaccharides do not participate as primers in the yeast reaction when UDPGlcNAc is used as substrate. Apparently yeast chitin synthase is capable of initiating and terminating the polymerization process. The toadstool enzyme appears to be an  $8 \times 10^5$  molecular weight aggregate of  $6.7 \times 10^4$  molecular weight subunits. The subunit structure of insect chitin synthase is unknown.

A zymogen of chitin synthase may exist in insects. The activity of the enzyme increased when the microsomal fraction from pupae of the stable fly was incubated with trypsin (Mayer *et al.*, 1980a), suggesting that an endogeneous activating factor may regulate tissue enzyme levels. In the red flour beetle, most of the chitin synthase activity is associated with microsomes which may form a chitosome-like particle composed of chitin synthase aggregates (Cohen, 1982). However, the role and source of chitosome structures in insects is controversial. In yeast a zymogen for chitin synthase has been partially purified from the cytoplasmic membrane, as well as an activating factor from particulate matter and an inhibitor of the activating factor from the cell wall (Cabib and Farkas, 1971; Cabib and Keller, 1971; Braun and Calderone, 1979; Fernandez *et al.*, 1982). Zymogens have also been found in a plasma membrane fraction from *Saccharomyces cerevisiae* (Duran *et al.*, 1975; Duran and Cabib, 1978), a wall membrane fraction from *Aspergillus nidulans* (Ryder and Peberdy, 1979), chitosomes from *Mucor rouxii* (Ruiz-Herrera *et al.*, 1977) and *Agaricus bisporus* (Hanseler *et al.*, 1983a), and digitonin-treated preparations from cell and my-

celium of *Candida albicans* (Hardy and Gooday, 1983). The yeast form of *Blastomyces dermatitidis* contains a latent form of chitin synthase that requires trypsin treatment for maximal *in vitro* activity (Shearer and Larsh, 1985). The mycelial enzyme is extracted in an active form which is rapidly inactivated by trypsin. These zymogens were activated commonly by proteolytic enzymes (trypsin, neutral or acid proteinase, pronase). A clostripain-like protease may be involved in the endogenous activation of yeast chitin synthase (Georgopapandakou and Smith, 1985).

Chitin deacetylase has been detected in microorganisms (Davis and Bartnicki-Garcia, 1984a,b). It provides a mechanism for synthesizing chitosanic regions of carbohydrate into the chitin polymer which occurs by the tandem action of chitin synthase and chitin deacetylase. Whether a similar deacetylating enzyme is present in insect tissues is unknown.

#### CHITIN DEGRADATION

Since the discovery of chitinase in association with larval exuviae of the silkworm, *Bombyx mori*, by Hamamura and Kanehara (1940), the existence of enzymes which are involved in cuticular chitin degradation has been demonstrated in several arthropods, including the silkworm (Jeuniaux and Amanieu, 1955; Kimura, 1974, 1976a), cockroach (Waterhouse *et al.*, 1961; Waterhouse and McKellar, 1961; Powning and Irzykiewicz, 1963), fruit fly (Winicur and Mitchel, 1974; Spindler, 1976), tobacco hornworm (Bade, 1975; Dziadik-Turner *et al.*, 1981a; Koga *et al.*, 1982, 1983), spider (Mommson, 1978, 1980), locust (Zielkowski and Spindler, 1978), stable fly (Chen *et al.*, 1981), house fly (Singh and Vardanis, 1984) and flour beetle (Aoki and Kramer, unpublished data). A substantial percentage of the old cuticle is digested and resorbed to be utilized for subsequent development. For example, only about one-third of the pupal cuticle of *M. sexta* is left over as the exuvium after dissolution by molting fluid (Kramer, unpublished data). The enzymatic hydrolysis of chitin is a complex mechanism modulated by many parameters including biophysical properties of chitin, interactions between chitin and other cuticular components, enzyme adsorption, enzyme cooperativity and competitive or uncompetitive inhibition, all of which are capable of influencing any analytical procedure for assessing chitinolytic enzyme activity. In microorganisms, chitinolytic enzymes have been isolated from several bacteria (Berger and Reynolds, 1958; Jeuniaux, 1966; Monreal and Reese, 1969; Skujins *et al.*, 1970; Tarentino and Maley, 1974; Roberts and Cabib, 1982; Charpentier and Percheron, 1983). Those enzymes are secreted out of the cell and apparently degrade media chitin to GlcNAc which is subsequently taken up as a nutrient. Insect cell lines also secrete chitinolytic enzymes into media (Dziadik-Turner *et al.*, 1981b; Boden *et al.*, 1985). The fungi, whose cell wall is composed of chitin, synthesize chitinolytic enzymes as well as chitin synthetase for cell division and nutrient uptake (Ohtakara, 1961, 1981; Jones and Kosman, 1980; Ishikawa *et al.*, 1981; Correa *et al.*, 1982; Elango *et al.*, 1982). Some entomopathogenic fungi such as

*Conidiobolus lamprauges* utilize both chitin binding proteins and degrading enzymes to infect insects (Ishikawa *et al.*, 1981, 1983). The former proteins apparently participate in the initial adhesion of fungi to cuticle while the latter assist in the penetration of cuticle by the pathogen. Other pathogenic fungal and hydrozoan species do not appear to utilize chitinases to penetrate the cuticle of prey (Coudron *et al.*, 1984; Klug *et al.*, 1984). In plants, chitinase has been found in beans (Powning and Irzykiewicz, 1965; Abeles *et al.*, 1970; Boller *et al.*, 1983), trees (Wargo, 1975), tomato leaves (Pegg and Vessy, 1973; Pegg and Young, 1981), wheat germ (Molano *et al.*, 1979) and yam (Tsukamoto *et al.*, 1984), and appears to play a defensive role for protection of the plant against chitin containing predators and pathogens such as fungi and insects (Abeles *et al.*, 1970; Pegg, 1977). Chitin, chitosan and their oligosaccharide fragments may act as signals to activate plant defense enzymatic responses such as proteinase inhibitors, chitinase and chitosanases (Walker-Simmons and Ryan, 1984). Chitinase has also been detected in mammalian serum, particularly from ruminants such as goat and bovine (Lundblad *et al.*, 1974, 1979). However, the physiological role of mammalian chitinases is unknown. Thus, chitinolytic enzymes are widely distributed and are not unique enzymes for arthropods. However, the enzymes seem to be especially important for organisms with external skeletons because they are necessary for the cuticular molting process.

Chitinolytic enzymes are usually products from cells in epidermis, gut, salivary glands or fat body of insects. However, bacterial, fungal and other microbial organisms that are embedded in animal tissues may also be repositories for such enzymes. A *Pseudomonas* bacterium was isolated from screw-worm cuticle that elicited chitinase activity (Gassner *et al.*, 1983). Scanning electron microscopy showed that the bacteria degraded screw-worm cuticles *in vivo*. Entomopathic fungi are also sources of chitinolytic enzymes detected in insect tissues (Ishikawa *et al.*, 1979, 1983). Chitinolytic enzymes have been isolated from molting fluid which appears in the space between the old and the new cuticles during ecdysis (Locke and Krishnan, 1973), from the integument at the prepharate stage (Bade, 1975; Kimura, 1976; Spindler, 1976; Zielkowski and Spindler, 1978; Dziadik-Turner *et al.*, 1981a; Koga *et al.*, 1983), and from the alimentary canal of the silkworm which contains the peritrophic membrane (Kimura, 1981a; Koga, unpublished data). Furthermore, the enzymes were present in the digestive juice of a spider (Mommsen, 1978, 1980), saliva of a cockroach (Waterhouse *et al.*, 1961) and labial glands of an ant (Febvay *et al.*, 1984). These latter enzymes are probably used for digestion of chitin containing foods. The feeding stages of a mycophagous grain beetle, *Ahasverus advena*, have more elevated levels of chitinolytic enzymes than comparable stages from a non-mycophagous beetle, *Oryzaephilus surinamensis* (Fukamizo *et al.*, 1985c). Apparently the mycophagous beetle utilizes chitin from the fungi upon which it feeds nutritionally. The feces of attine ants contains chitinase (Martin *et al.*, 1973). The fecal enzyme is thought to serve a beneficial role in the fungus-culturing activities of the ant by contributing to the

degradation of chitinous substrates and by lysing potentially competitive chitinous fungi. Chitinolytic enzymes have been detected in insect haemolymph (Bernier *et al.*, 1974; Dziadik-Turner *et al.*, 1981; Chen *et al.*, 1982; Koga *et al.*, 1983) and the spent medium of insect cell cultures (Dziadik-Turner *et al.*, 1981; Boden *et al.*, 1985), but the physiological roles of those enzymes are unknown.

The early studies of the enzymes participating in the molting process in arthropods, in particular of degradation mechanisms of chitin in cuticle, were performed using crude enzymes without significant purification. It is unclear whether the results obtained in those studies are due to a simple enzyme reaction or to a mixed order of reactions catalyzed by multiple enzymes. The digestion of chitin involves the action of a composite chitinase system where the two individual enzymes operate at various rates, depending not only on their ability to bind to the surface of insoluble and soluble saccharides, but also on the crystallinity, surface area and conformation of substrates. The microbial chitinase system from *Serratia marcescens* also consists of two hydrolase fractions: an endoglycanase that randomly cleaves chitin to produce oligosaccharides and an *N*-acetylglucosaminidase that yields 2-acetamido-2-deoxy-D-glucopyranoside (Young *et al.*, 1985a,b). The cellulase system from microorganisms which consists of at least three separate enzymes is similar in many respects to the chitinase system from insects (Klesov, 1982; Klesov and Grigorash, 1982; Matsuno *et al.*, 1984; Fukamizo and Kramer, 1985a,b; Chanzy and Henrissat, 1985; Beldman *et al.*, 1985).

More recent studies have used purified molting enzyme preparations, and the properties and mechanisms of action of chitinolytic enzymes are becoming better understood. Some of the characteristics of these enzymes are shown in Table 3. In 1976, three chitinases and two  $\beta$ -*N*-acetylglucosaminidases were isolated from the molting fluid of the silkworm on an anion-exchange column (Kimura, 1976). The isolated chitinase degraded colloidal chitin to mainly *N,N'*-diacetylchitobiose which was subsequently cleaved to GlcNAc by a so-called chitobiase (probably a  $\beta$ -*N*-acetylglucosaminidase; Kimura, 1976a). Such a degradation mechanism had been previously proposed in a study of cockroach chitinase (Waterhouse *et al.*, 1961). Similar enzymes were also partially purified from the integument of fruit fly and locust (Spindler, 1976; Zielkowski and Spindler, 1978). A rather detailed analysis was subsequently conducted on insect chitinolytic enzymes purified to homogeneity from molting fluid, integument and hemolymph of the tobacco hornworm, *Manduca sexta* (Dziadik-Turner *et al.*, 1981; Koga *et al.*, 1982, 1983; Fukamizo and Kramer, 1985a,b). Kinetic analyses were performed using intact chitin, colloidal chitin, glycol chitin, chitin oligosaccharides and nitrophenylated 2-acetamido-2-deoxy-D-glucopyranoside for the determination of cleavage patterns and reactivity, and 2,4-dinitrophenyl chitin tetrasaccharide was utilized for the determination of the cleavage direction for the exoenzyme.

Chitinolytic enzymes can be classified into three distinct types, depending on their properties. The first type is the endoenzyme chitinase, which hydrolyzes

Table 3. Characteristics of chitinolytic enzymes from arthropods

Organism	Enzyme	Occurrence	Mol. wt*	pI	pH opt.	Comments	Reference	
<i>Bombyx mori</i> (silkworm)	Chitinase	Integument	65,000			Inhibited by HgCl <sub>2</sub>	Koga (unpublished)	
		Alimentary canal	53,000 49,000		10 (glycolchitin) 7 ( $\beta$ GlcNAc <sub>4</sub> )	Inhibited by HgCl <sub>2</sub>	Koga (unpublished)	
		Molting fluid					Kimura (1973a, 1973b, 1976, 1981)	
	$\beta$ -N-acetyl- glucosaminidase	Integument	67,500	5.0	6 (pNp $\beta$ GlcNAc)	$K_m = 0.38$ mM, $k_{cat} = 564$ sec <sup>-1</sup> (pNp $\beta$ GlcNAc) $K_m = 0.8$ mM, $k_{cat} = 449$ sec <sup>-1</sup> (GlcNAc <sub>2</sub> )	Inhibited by HgCl <sub>2</sub>	Koga (unpublished)
		Alimentary canal	119,000 (67,500 + 57,500)	4.8	5.5-6 (pNp $\beta$ GlcNAc)	$K_m = 0.49$ mM, $k_{cat} = 1367$ sec <sup>-1</sup> (pNp $\beta$ GlcNAc) $K_m = 0.397$ mM, $k_{cat} = 803$ sec <sup>-1</sup> (GlcNAc <sub>2</sub> )	Inhibited by HgCl <sub>2</sub>	Koga (unpublished)
		Molting fluid	150,000	4.9	5-6 (pNp $\beta$ GlcNAc, chitobiose)	$K_m = 6.3$ mM, $V_{max} = 43.5$ $\mu$ mol/min per mg (pNp $\beta$ GlcNAc) $K_m = 6.7$ mM, $V_{max} = 0.5$ $\mu$ mol/min per mg (GlcNAc <sub>2</sub> )	Inhibited by HgCl <sub>2</sub>	Kimura (1974, 1977)
$\beta$ -N-acetyl- glucosaminidase	Hemolymph	125,000 (61,000)	5.1	5 (pNp $\beta$ GlcNAc)	$K_m = 3.6$ mM, $V_{max} = 276.6$ $\mu$ mol/min per mg (pNp $\beta$ GlcNAc) $K_m = 20.0$ mM, $V_{max} = 0.6$ $\mu$ mol/min per mg (GlcNAc <sub>2</sub> )	Inhibited by HgCl <sub>2</sub> and glycoprotein	Kimura (1974, 1976, 1977)	
<i>Cupiennius salei</i> (spider)	Chitinase	Digestive juice	48,000		7.2 (colloidal chitin)		Mommsen (1980)	
	$\beta$ -N-acetyl- glucosaminidase	Digestive juice	108,000		6.5 (chitobiose) 5.4 (pNp $\beta$ GlcNAc)	$K_m = 0.93$ mM, $V_{max} = 63.6$ $\mu$ mol/min per mg $K_m = 0.35$ mM, $V_{max} = 67.6$ $\mu$ mol/min per mg	Mommsen (1980)	
<i>Drosophila hydei</i> (fruit fly)	Chitinase	Integument	40,000		5.5-6.2 ([ <sup>14</sup> C]chitin)	$K_m = 5$ mg/ml	Spindler (1976)	
	$\beta$ -N-acetyl- glucosaminidase	Integument	>100,000		5.5-6.2 (pNp $\beta$ GlcNAc)	$K_m = 4$ mM	Spindler (1976)	

<i>Locusta migratoria</i> (locust)	Chitinase	Integument			5 ( $^{14}\text{C}$ )chitin		Zielkowski and Spindler (1978)
	$\beta$ -N acetylglucosaminidase	Integument			4.5 (pNp $\beta$ GlcNAc)	$K_m = 5 \text{ mM}$	Zielkowski and Spindler (1978)
<i>Manduca sexta</i> (tobacco hornworm)	Chitinase	Integument, molting fluid	50,000 62,000 75,000		6 (oligosaccharide) 9-10 (glycolchitin)	$K_m = 0.2 \text{ mg/ml}$ , $k_{cat} = 2 \text{ sec}^{-1}$ (glycol chitin)	Koga <i>et al.</i> (1983), Fukamizo and Kramer (1985a,b)
	$\beta$ -N-acetylglucosaminidase	Integument, molting fluid, pupal hemolymph	61,000 (53,000 10,000)	5.9	$\sim 6$ , $pK_1 = 3.7$ , $pK_2 = 8.07$ (oligosaccharide)	$K_m = 0.247 \text{ mM}$ , $k_{cat} = 392 \text{ sec}^{-1}$ (pNp $\beta$ GlcNAc) $K_m = 0.128-0.063 \text{ mM}$ , $k_{cat} = 265-77 \text{ sec}^{-1}$ (GlcNAc $_2$ ) $K_m = 0.14 \text{ mM}$ , $k_{cat} = 285 \text{ sec}^{-1}$	Dzadik-Turner <i>et al.</i> (1981), Koga <i>et al.</i> (1982, 1983)
<i>Musca domestica</i> (house fly)	$\beta$ -N-acetylglucosaminidase	Pupal and larval hemolymph	(61,000, 10,000)	5.1	$\sim 6$ (pNp $\beta$ GlcNAc)		Dzadik-Turner <i>et al.</i> (1981), Koga <i>et al.</i> (1982)
<i>Periplaneta americana</i> (cockroach)	Chitinase	Wandering larvae, pupae	42,000 120,000		6.5 (chitin)		Singh and Vardanis (1984)
	Chitinase	Hemolymph, saliva, digestive juice, midgut cuticle brei, extract of cast skins			5.6 (chitin)		Waterhouse <i>et al.</i> (1961), Waterhouse and McKeller (1961), Powning and Irzykiewicz (1963, 1964)
	$\beta$ -N-acetylglucosaminidase	Hemolymph, gut cuticle			$\sim 4.5$ ( $\beta$ GlcNAc $_2$ )	$K_m = 0.31 \text{ mM}$ , $V_{max} = 8 \mu\text{mol/min per mg}$	
<i>Sitophilus oryzae</i> (rice weevil)	Chitinase	Ovary, whole body	150,000		4.7 (pNp $\beta$ GlcNAc)	$K_m = 0.2-0.8 \text{ mM}$ (pNp $\beta$ GlcNAc)	Nardon (1978)
<i>Stomoxys calcitrans</i> (stable fly)	Chitinase	Pupae	48,000	4.85	$\sim 5$ ( $^{14}\text{C}$ )chitin)	$K_m = 33 \text{ mM}$ , $V_{max} = 1.2 \mu\text{mol/min per mg}$ (chitin)	Chen <i>et al.</i> (1981)
	$\beta$ -N-acetylglucosaminidase				4.5-5.5 (pNp $\beta$ GlcNAc)		Deloach and Mayer (1979)
<i>Tegenaria atrica</i> (spider)	Chitinase	Digestive juice			6.8 (chitin)	$K_m = 0.9 \text{ mg/ml}$ , $V_{max} = 0.9 \mu\text{mol/min per mg}$	Mommsen (1978)
	$\beta$ -N-acetylglucosaminidase		111,000 (55,000)	5.5 (pNp $\beta$ GlcNAc)		$K_m = 0.6 \text{ mM}$ , $V_{max} = 11 \mu\text{mol/min per mg}$	Mommsen (1978)
<i>Tribolium confusum</i> (flour beetle)	Chitinase	Pupae	77,000	4.7	$\sim 6$ ( $\beta$ GlcNAc $_4$ )	$K_m = 0.3 \text{ mg/ml}$ , $V_{max} = 1.8 \text{ sec}^{-1}$ (glycol chitin)	Aoki and Kramer (unpublished)
	$\beta$ -N-acetylglucosaminidase	Pupae	150,000 (73,000 + 64,000)	4.9	4.5-5 (pNp $\beta$ GlcNAc, chitobiose)	$K_m = 0.071 \text{ mM}$ , $k_{cat} = 320 \text{ sec}^{-1}$ (pNp $\beta$ GlcNAc)	Aoki and Kramer (unpublished)

\*Apparent molecular weight of subunits given in parentheses.

chitin, colloidal chitin, glycol chitin and oligosaccharides of GlcNAc to smaller oligosaccharide products. In *M. sexta* there are three chitinases found in prepupate pupal integument and molting fluid which are able to cleave polysaccharides and oligosaccharides larger than disaccharide (Koga *et al.*, 1983). When comparing large substrates to small ones, *M. sexta* chitinases are more catalytic towards a substrate with a high degree of polymerization such as chitin itself. Moreover, those enzymes exhibit significant inhibition by soluble substrates and products. The second type of chitinolytic enzyme is the exoenzyme  $\beta$ -*N*-acetylglucosaminidase, which liberates GlcNAc from the non-reducing end of oligosaccharides (Koga *et al.*, 1982). It is found in molting fluid, integument and pupal hemolymph.  $k_{cat}$  values are inversely proportional to chain length up to the tetrasaccharide, but the affinity of the enzyme for the substrate ( $1/K_m$ ) increases with size of substrate so that the reaction turnover for all oligosaccharide substrates is relatively constant. The smaller saccharides are hydrolyzed slightly faster than larger ones.  $\beta$ -*N*-Acetylglucosaminidase is susceptible to both substrate inhibition and product inhibition. A third type of chitinolytic enzyme is also an exo- $\beta$ -*N*-acetylglucosaminidase and is found in larval and pupal hemolymph but not integument and molting fluid (Koga *et al.*, 1982). It hydrolyzes pNp $\beta$ GlcNAc as well as oligosaccharides of GlcNAc, but is distinguished from the other  $\beta$ -*N*-acetylglucosaminidase by a greater reactivity toward nitrophenylated GlcNAc than toward chitin oligosaccharides. The latter enzyme does not exhibit substrate inhibition behavior.

Chitinase and the first type of  $\beta$ -*N*-acetylglucosaminidase are localized in molting fluid and integument where they may directly participate in cuticular chitin degradation. The GlcNAc supplied by chitinolysis of the old cuticle is reabsorbed with the molting fluid and recycled for synthesis of new cuticle (Gwinn and Stevenson, 1973a; Surholt, 1975b). Resorption may occur through the integument or the midgut via the foregut and hindgut (Cornell and Pan, 1983). Chitin degradation in *M. sexta* cuticle is carried out by a binary mixture of chitinolytic enzymes, chitinase and  $\beta$ -*N*-acetylglucosaminidase (Fukamizo and Kramer, 1985a,b). The tandem combination of two enzymes results in a synergistic effect on the catalysis that is as much as six times faster than the sum of individual enzyme rates depending on the concentration ratio of chitinase to  $\beta$ -*N*-acetylglucosaminidase. The greatest synergism occurs at a ratio of enzymes (6:1) typically found in molting fluid. A similar synergistic phenomenon occurs in the solubilization of cellulose by cellulolytic enzymes from certain fungi (Wood and McCrae, 1978, 1979). In spite of a complex stoichiometry of soluble and insoluble substrates and intermediates as well as multiple enzymes, the mechanism of chitin degradation in *M. sexta* can be analyzed quantitatively within the framework of a simple kinetic theory. The catalysis is dominated by the endo-splitting chitinase which initiates hydrolysis by attacking the insoluble polymeric substrate in random fashion. The larger oligosaccharide intermediates are either insoluble or occur at low levels in

solution except for *N,N',N''*-triacylchitotriose and *N,N'*-diacylchitobiose. The latter intermediates are converted to *N*-acetylglucosamine by the exo-splitting  $\beta$ -*N*-acetylglucosaminidase. The chitin-degrading enzyme system of *Streptomyces* and *Serratia* also consists of both chitinases and  $\beta$ -*N*-acetylglucosaminidases (Charpentier and Percheron, 1983; Young *et al.*, 1985a,b).

During the ecdysial cycle chitinase and not  $\beta$ -*N*-acetylglucosaminidase appears to be the rate limiting enzyme for the digestion of chitin in cuticle. Chitinase titer is elevated in *M. sexta* integument more than 10-fold by injection of ecdysteroid into isolated larval abdomens while the level of  $\beta$ -*N*-acetylglucosaminidase is elevated less than 2-fold (Fukamizo and Kramer, unpublished data).

Rather than chitin degradation the physiological role of the  $\beta$ -*N*-acetylglucosaminidase that is present in hemolymph is probably to hydrolyze GlcNAc  $\beta$ -linked compounds such as glycoprotein and glycolipid. The exo- $\beta$ -acetylglucosaminidase purified from *B. mori* hemolymph by Kimura (1977, 1981b, 1983) appears to be similar to the glucosaminidase in *M. sexta* hemolymph, while *B. mori* chitobiase may be identical in properties to the integumental enzyme from *M. sexta*. Chitobiase (EC 3.2.1.29) has been deleted from the enzyme classification nomenclature and now is included with the  $\beta$ -*N*-acetyl-D-glucosaminidases (EC 3.2.1.30). The insect exo- $\beta$ -*N*-acetylglucosaminidases have some properties similar to those of other  $\beta$ -*N*-acetyl-D-glucosaminidases. However, the insect enzymes have distinct substrate specificities as mentioned earlier and they probably should be discriminated.  $\beta$ -*N*-Acetylglucosaminidase is called "chitobiase" or "exochitinase" in some publications. However, chitobiase may be an unsuitable name because that type of enzyme may exhibit significant activity toward large chitin oligosaccharides in addition to disaccharide.

With regard to endochitinase, that enzyme is best known as "chitinase" (EC 3.2.1.14). Plant, microbial and insect chitinases exhibit endo-cleavage patterns during chitin hydrolysis (Powning and Irzykiewicz, 1965; Molano *et al.*, 1979; Correa *et al.*, 1982; Koga *et al.*, 1983; Charpentier and Percheron, 1983). On the other hand, Chen *et al.* (1982) purified a chitinase from stable fly pupae that produced no oligosaccharides of GlcNAc during chitin hydrolysis other than *N,N'*-diacylchitobiose. Because of this apparent non-random cleavage pattern, stable fly chitinase was called "exochitinase". A microbial chitinase from *Streptomyces* exhibited a random cleavage pattern toward soluble chitodextrin substrates, but a so called "exochitinase" pattern toward insoluble chitin (Berger and Reynolds, 1958). Apparently there are several kinds of chitinases that exhibit different cleavage patterns depending on the substrate.

Chitinases that exhibit either or both random and non-random cleavage patterns have been detected in different microorganisms. Those enzymes that release only *N,N'*-diacylchitobiose from chitin are thought to follow a processive degradation mechanism where a single polymeric substrate is hydrolyzed to completion before releasing a small, resistant oligosaccharide (usually disaccharide) and then initiating hydrolysis of another chain. In *Escherichia coli* poly-

ribonucleotide chains are apparently cleaved by ribonuclease following such a mechanism (Nossal and Singer, 1968). Yeast chitinase does not follow a processive-type mechanism but instead exhibits an equilibrium Langmuir isotherm binding behaviour in its catalysis (Barrett-Bee and Hamilton, 1984). A randomly acting chitinase was purified from *Saccharomyces cerevisiae* (Correa *et al.*, 1982) while an exochitinase which apparently releases only disaccharide was isolated from *Serratia marcescens* (Roberts and Cabib, 1982). However, the latter enzyme was probably contaminated with  $\beta$ -*N*-acetylglucosaminidase. Even an endochitinase may degrade chitin finally to the disaccharide and a small amount of GlcNAc via oligosaccharide intermediates. In some reports it is unclear whether the focus of a study is an exochitinase or endochitinase (Kimura, 1976; Mommsen, 1980). Detailed kinetic analysis is needed to distinguish the mode of hydrolytic attack by those enzymes.

It appears that there may be two kinds of exochitinase involved in chitin degradation. One liberates GlcNAc from the non-reducing end of mainly oligosaccharides. The other excises the disaccharide of GlcNAc from terminal positions. In order to determine substrate specificity and cleavage patterns, the use of homogeneous enzymes and both soluble and insoluble polymeric-oligomeric substrates is advised for kinetic studies. Two enzymes related to chitinolytic enzymes are  $\beta$ -*N*-acetyl-D-hexosaminidase (EC 3.2.1.52) and endo- $\beta$ -*N*-acetylglucosaminidase (EC 3.2.1.96). Both hydrolyze  $\beta$ -linked GlcNAc compounds. However, the former has a rather wide substrate specificity, while the latter is involved in the endohydrolysis of  $\beta$ -linked GlcNAc in mannosylated glycoproteins (Tarentino and Maley, 1974). Another enzyme, lysozyme (EC 3.2.1.17) cleaves not only chitin oligosaccharides but also the 1,4- $\beta$ -linkage between *N*-acetylmuramic acid and GlcNAc which is found in gram positive bacterial cell walls. Although it is difficult to determine whether a certain  $\beta$ -*N*-acetylglucosaminidase is an exoenzyme that either participates in chitin degradation of some other kind of carbohydrate metabolism, a variety of substrates can be used to discriminate chitinases from  $\beta$ -*N*-acetylglucosaminidases from lysozymes.

In terms of physical size the molecular weights of chitinases from invertebrates range between approx.  $4 \times 10^4$  and  $1.2 \times 10^5$ . Mammalian serum chitinases have molecular weights of  $4.7 \times 10^4$  (bovine) and  $6 \times 10^4$  (goat; Lundblad *et al.*, 1974, 1979). In plants, the enzyme size is somewhat smaller, about  $3 \times 10^4$  for wheat (Molano *et al.*, 1979), red kidney (Boller *et al.*, 1983) and yam (Tsukamoto *et al.*, 1984). Microbial chitinases vary from  $2 \times 10^4$  to  $1 \times 10^5$  (Roberts and Cabib, 1983; Corea *et al.*, 1982; Charpentier and Percheron, 1983).

The isoelectric points of invertebrate chitinases are between pH 4 and 5 in all cases except for the enzymes present in the digestive juice of a snail and spider where they range from 5.7 to 7.3 (Lundblad *et al.*, 1976; Mommsen, 1980). Acidic chitinases also occur in yam (pI 3.8, 4.0 and 4.05; Tsukamoto *et al.*, 1984), *Streptomyces* bacteria (pI 4.25 and 4.2; Trimble *et al.*, 1979; Charpentier and Percheron,

1983), goat and bovine (pI 4.85 and 5.3; Lundblad *et al.*, 1974, 1979). Basic chitinases are found in wheat (pI 7.5-9.2; Molano *et al.*, 1979) and *Streptomyces* species (pI 7.5 and 8.2; Charpentier and Percheron, 1983).

The optimum pH for catalysis by invertebrate chitinases ranges from pH 4.5 to 6.0 and depends on the substrate utilized. However, the optimum pH value for tobacco hornworm chitinases using glycol chitin as a substrate is basic, pH 9-10, while the optimum pH for the same enzymes toward the oligosaccharides of GlcNAc is pH 6 (Koga *et al.*, 1983). Similar results were obtained with silkworm chitinase (Koga, unpublished data) and yam chitinases (Tsukamoto *et al.*, 1984). Acidic pH optima are also exhibited by chitinases from plants, microorganisms and mammals (Pegg and Vessey, 1973; Molano *et al.*, 1979; Roberts and Cabib, 1982; Lundblad *et al.*, 1974, 1979). The most acidic pH optimum is that of *Saccharomyces cerevisiae* chitinase, pH 2.5 (Correa *et al.*, 1982).

In terms of kinetic parameters the  $K_m$  values obtained using pure chitinase together with chitin or a chitin derivative as substrate include 0.2 mg/ml for the hornworm integument enzyme (Koga *et al.*, 1983), 0.9 mg/ml for the spider digestive juice enzyme (Mommsen, 1978, 1980), and 5 mg/ml for the fruit fly integument enzyme (Spindler, 1976). Tomato leaf chitinase exhibits a relatively low  $K_m$  value of 0.4 mg/ml (Pegg and Vessey, 1973).

Many of the insect  $\beta$ -*N*-acetylglucosaminidases appear to be dimeric aggregates with apparent molecular weights larger than  $1 \times 10^5$  with subunits about equal in size. Plants, microorganisms and vertebrates also possess large molecular weight  $\beta$ -*N*-acetylglucosaminidases (Li and Li, 1970; Sellinger *et al.*, 1973; Verpoorte, 1974; Ohtakara and Tran Thi, 1978; Jones and Kosman, 1980; Ohtakara *et al.*, 1981; Dolhofer *et al.*, 1982). The isoelectric point of insect  $\beta$ -*N*-acetylglucosaminidase is acidic, pI 4.8-5.9 (Dziadik-Turner *et al.*, 1981a).

#### REGULATION OF CHITIN METABOLISM

This section will consider recent work on the regulation of chitin metabolism by endogenous and exogenous modulators. The former materials include primarily hormones and enzymes while the latter include insecticides and broad spectrum antibiotics. The molt cycle including chitin metabolism may also be regulated by nutrition, photoperiod, temperature and other environmental factors (Herrera-Estrella and Ruiz-Herrera, 1983; Quackenbush and Herrnkind, 1983) but those topics will not be considered here.

One of the more obvious ways of regulating an anabolic enzyme is with a catabolic enzyme and *vice versa*. In microorganisms some inhibitors of chitin synthase are chitinases (Lopez-Romero *et al.*, 1982; Zarain-Herzberg and Arroyo-Begovich, 1983). Another microbial chitin synthase inhibitor that is also a protein apparently lacks chitinase activity (Craig *et al.*, 1981). Conversely, the presence of chitin synthases would have a negative effect on chitinase activity. However, no evidence for the latter type of regulation has been noted in the literature. Whether

chitin metabolism in insects is directly modulated by catabolic-anabolic enzyme interactions remains an unanswered question.

Because molting is indispensable for growth and morphological transformation in arthropods, it must be regulated precisely by certain hormones. Cuticle production as well as mechanical properties are directly influenced by ecdysteroid, juvenile hormone and peptide hormone levels (Riddiford and Truman, 1978; Sedlak and Gilbert, 1979; Reynolds, 1985), and there are alternate phases of production of enzymes involved in cuticle synthesis and enzymes involved in cuticle degradation in epithelial cells (Mitchell *et al.*, 1970). Both chitin synthesis and degradation in cuticle, trachea and peritrophic membrane are affected by fluctuations in hemolymph ecdysteroid titers.

The timing of the period of chitin synthesis and deposition has been investigated by many researchers and there seems to be a rather complex enzyme level regulation of chitin synthesis. A rapid increase in the formation of chitin was observed during and immediately after the molting of the fifth instar of the desert locust (Candy and Kilby, 1962). A high chitin synthase activity in the cell free extract from prepupae of the southern armyworm was reported by Jaworski *et al.* (1963). There was a correlation between changes in chitin levels and chitin synthase activity, and maximum levels of both occurred in late last instar larvae during early stages of the larval-pupal transformation (Porter and Jaworski, 1965). Chitin synthase activity increased in early premolt and early postmolt periods of the shore crab (Hohnke, 1971). Uptake of radiolabeled chitin precursors into cuticle of the blood sucking bug was attained immediately after ecdysis (Quesada-Allue *et al.*, 1976). Incorporation of radiolabeled glucose and GlcNAc into the cuticle of the crayfish occurred from the premolt through the intermolt stages and maximum uptake was at postmolt (Hornung and Stevenson, 1971; Gwinn and Stevenson, 1973a). Apparently the earlier acting enzymes in the chitin synthetic pathway increase in activity at the premolt stage, causing accumulation of GlcNAc-6-phosphate and UDPGlcNAc, while later acting enzymes increase at the postmolt stage, reducing levels of accumulated precursors with a concomitant rise in chitin levels (Stevenson and Hettick, 1980). Studies of *in vivo* and *in vitro* chitin synthesis during the nymphal-adult transformation of the migratory locust revealed that the timing of the uptake of GlcNAc after ecdysis occurs before that of glucose, and incorporation of GlcNAc is at least 5-fold higher than that of glucose (Surholt, 1975a). Apparently chitin is *de novo* synthesized immediately after ecdysis using GlcNAc from old cuticle that was generated by chitinolytic enzyme action before ecdysis and from fat body glycogen. This is consistent with observations in the silkworm that glycogen derived from injected [<sup>14</sup>C]glucose was decomposed and used for chitin synthesis immediately after larval-larval ecdysis (Kimura, 1974), and with the observations in the crayfish by Speck *et al.* (1972) that GlcNAc and glucosamine but not glucose are incorporated after the molt. A key regulatory enzyme for chitin synthesis in crayfish may be glutamine-fructose-6-phosphate amino transferase. A similar

profile of the uptake of precursors into pupal cuticle of the fruit fly *Drosophila melanogaster* was reported by Sparrow and Chadfield (1982).

Studies on cuticle deposition and chitin synthesis in the pupal instar of the stable fly showed that there are two distinct periods of GlcNAc incorporation into cuticle at 1 and 4 days postpupation (Mayer *et al.*, 1979). Electron micrographs showed a correlation between the first peak on day 1 postpupation and the production of ecdysial membrane and underlying imaginal disc cuticle, as well as between the second peak on day 4 postpupation and the production of imaginal disc cuticle. Judging from its shape, location and timing of appearance, the ecdysial membrane of the stable fly appears to be similar to the subcuticle observed in the larval integument of the silkworm (Kimura, 1974).

There are many reports about the molting hormone stimulation of insect chitin synthesis. Ecdysteroid accelerated the molting stage in the crayfish (Stevenson and Tschantz, 1973) and cuticle deposition in leg regenerates of cockroach (Marks and Leopold, 1970, 1971; Marks, 1972, 1973) and in larval trachea explants of the skipper butterfly (Ryerse and Locke, 1978). Molting hormone stimulated *in vitro* synthesis of the peritrophic membrane in the adult blow fly but juvenile hormone was inhibitory (Becker, 1978). Juvenile hormone also inhibited *in vitro* cuticle formation in pupal wing of cecropia silkworm and *in vivo* cuticle formation in last instar larvae of the milkweed bug (Willis, 1981). Ecdysteroid also induced chitin synthase in diapausing *Hyalophora cecropia* (Cohen and Casida, 1982). In imaginal discs and epidermis of the Indianmeal moth, *Plodia interpunctella*, and the greater wax moth, *Galleria mellonella*, ecdysteroid stimulated incorporation of glucose and GlcNAc into chitin (Oberlander and Leach, 1975; Oberlander, 1976; Oberlander *et al.*, 1980, 1983; Ferkovich *et al.*, 1981). Stimulation was blocked by actinomycin D and cycloheximide which are inhibitors of RNA and protein syntheses, respectively, and also by cytochalasin B, vinblastine and colcemid which are inhibitors of microtubule formation. Apparently ecdysteroids act to stimulate protein synthesis and also the uptake of precursors for chitin synthesis in the imaginal disc and epidermis. Ecdysteroid may also affect the permeability of the cell membrane to allow uptake of chitin precursors, the biosynthesis of enzymes involved in chitin synthesis, and also the translocation of chitinolytic enzymes or their precursors.

The regulatory effectors of insect chitin synthase which may contain many subunits and be a multi-enzyme complex are virtually unknown. A microbial chitin synthase exhibits homotropic-heterotropic type enzyme regulation and has at least two binding sites for substrates (Hanseler *et al.*, 1983b).

Sequential activity of hydrolytic enzymes may lead to chitin degradation. A trypsin-like protease has been proposed to unmask chitin from structural protein so that chitinolytic enzymes have access to their substrate (Bade and Stinson, 1978; Lapierre *et al.*, 1983). Since a binary enzyme mixture, chitinase and  $\beta$ -N-acetylglucosaminidase, acts to degrade chitin completely to GlcNAc, a tri-enzyme system

that includes a protease may be ultimately required for chitin degradation *in vivo* (Fukamizo and Kramer, 1985a,b). Sequential proteolytic and chitinolytic enzyme activities also appear to be required for microbial penetration of corn earworm integument (Smith *et al.*, 1981).

A few studies of the interaction of ecdysteroid with chitinolytic enzymes during the larval-pupal transformation have been conducted. Chitinolytic enzyme activities sharply increase just before apolysis and generally reach a maximum at apolysis, and decrease just before exuviation. The profile has been found in the integument of the silkworm, *Bombyx mori* (Kimura, 1973a), the locust, *Locusta migratoria* (Zielkowski and Spindler, 1978) and the tobacco hornworm, *Manduca sexta* (Bade, 1975). There is a correlation between the titer of ecdysteroid and the chitinolytic enzymes in *M. sexta* and other species (Bollenbacher *et al.*, 1975). Furthermore, experiments where ecdysone is injected into the isolated larval abdomen of the silkworm and hornworm clearly demonstrate that there is a positive relationship between molting hormone and chitinolytic enzymes (Kimura, 1973a; Fukamizo and Kramer, unpublished data). However, there is a difference in the timing of increase in activity between the two different types of chitinolytic enzymes.  $\beta$ -N-Acetylglucosaminidase titer increases before chitinase titer (Kimura, 1973a; Zielkowski and Spindler, 1978; Fukamizo and Kramer, unpublished data). Chitinase activity is also stimulated by ecdysone to a greater degree than  $\beta$ -N-acetylglucosaminidase (Kimura, 1973a; Fukamizo and Kramer, unpublished data). These findings suggest that there are differences in the stimulation mechanisms of chitinolytic enzymes by molting hormone. Chitinase may be activated by a later large pulse of ecdysteroid that occurs in the prepupal stage of *M. sexta* while  $\beta$ -N-acetylglucosaminidase may be induced by an earlier small pulse of hormone that occurs during the larval feeding period (Bollenbacher *et al.*, 1975; Fukamizo and Kramer, unpublished data).

Morphological changes in the larval integument of the silkworm were investigated with light and electron microscopy by Kimura *et al.* (1974). It was observed that a subcuticle forms between the endocuticle and the epidermis before apolysis is induced by ecdysone. About 3 hr later, an epicuticle forms beneath the subcuticle and the inner layer of the endocuticle proceeds to be digested. The subcuticle is thought to prevent chitinase from attacking the epidermis. Dense granules in the subcuticle are gradually divided into smaller spherical bodies in the endocuticle during apolysis. Chitinase is apparently carried by the dense granules to the endocuticle in an inactive form, perhaps a proenzyme. Ecdysteroid may affect the permeability of the cell membrane to allow the transfer of inactive chitinase from the epidermal cell to the endocuticle where the enzyme is activated, perhaps by proteolysis. The increase in chitinase activity induced by ecdysteroid is not completely blocked by actinomycin D or puromycin which inhibit RNA and protein syntheses, respectively (Kimura, 1973a). The synthesis of chitinase as a zymogen would allow the proenzyme to pass through newly forming cuticle without digesting it.

The inactive precursor form may also afford protection of the enzyme from autodigestion, in a manner much like zymogen forms of proteases. *M. sexta* chitinases are glycoproteins that may degrade carbohydrate portions of the macromolecule (Koga *et al.*, 1983). However, a proenzyme for insect chitinases has not yet been detected even though evidence for zymogenic forms of fungal chitinases is available (Humphreys and Gooday, 1984a,b; Kang *et al.*, 1984). Indirect evidence for an insect chitinase zymogen comes from studies where incubation of proteolytic enzymes with crude preparations of fly or beetle chitinases leads to substantial elevation in activity (Chen *et al.*, 1982; Aoki and Kramer, unpublished data).

Chitinolytic enzymes have also been found in the alimentary canal of the silkworm, *Bombyx mori* (Kimura, 1981; Koga, unpublished). The peritrophic membrane of the alimentary canal is mainly composed of chitin, protein and hyaluronic acid, and is also shed just prior to cocoon spinning. High chitinase activity is observed in the white pupae stage while chitobiase increases earlier during spinning behavior and formation of the white pupae. The developmental profile of enzyme activity in the alimentary canal is similar to that observed in integument. Apparently ecdysteroid regulates peritrophic membranous and epidermal chitinolytic enzymes in a similar manner. Since gut epithelium is replaced with regenerated cells instead of larval type cells (columnar and goblet) in the process of the larval-pupal transformation, activities in the alimentary canal may function in the regeneration as follows (Kimura, 1981). Proenzymes may be present in the gut epithelium or goblet cells and be activated to digest the larval gut. The increasing GlcNAc concentration found in hemolymph in the early pupal stage could be supplied from the degenerated larval gut and reutilized for chitin synthesis in developing tissues.

Little work has been done on hormonal effectors of chitinase in other types of organisms. In many plants chitinase is induced by ethylene where the enzyme may play a role in the plant's defense against pathogens (Boller and Vogeli, 1984). Fungal pathogens also cause chitinase to increase in plants (Mauch *et al.*, 1984).

Little is known about the genetic regulation of insect chitin metabolism. A mutant phenotype *lethal-cryptocephal (crc)* of *Drosophila melanogaster* was initially proposed to result from excess chitin synthesis which produced a stiffer cuticle than normal and prevented eversion of the cephalic complex from within the thorax during early pupal development (Fristrom, 1965). That proposal was challenged when no difference in total chitin content of wild-type and mutant pupal cuticles was measured (Sparrow and Chadfield, 1982). However, the possibility that the *crc* mutation specifically increases chitin content in the small localized regions of head and anterior thorax which must flex for head eversion still remains. No other viable dominant mutations are known that might specifically affect genes that code for proteins involved in chitin metabolism. The chitin system appears to be so vital that any genetic defect leading to defective chitin or proteins required for chitin

synthesis, deposition or degradation is likely to be lethal at an early stage of development.  $\beta$ -*N*-Acetylglucosaminidase levels in hemolymph of *Bombyx mori* appear to be under the control of a gene regulating association of enzyme subunits to the active enzyme molecule (Kimura, 1981). Silkworm strains of high and low enzyme titer share the same genetic background except for a gene controlling differential expression of  $\beta$ -*N*-acetylglucosaminidase.

Posttranslational modification of proteins is a way of modulating enzyme activity. In terms of proteolytic processing no zymogenic forms of insect chitin metabolizing enzymes have been detected to date. In microorganisms the zymogens of chitin synthase and chitinase are rather hard to work with and appear to be membrane-bound particulate proteins that are made soluble by detergent extraction or proteolysis (Hardy and Gooday, 1983; Humphreys and Gooday, 1984a,b; Koga *et al.*, 1984). The gene sequence for a bacterial  $\beta$ -*N*-acetylglucosaminidase suggested that a proenzyme or, more probably, a preproenzyme is the primary product of translation (Robbins *et al.*, 1984). The existence of multiple chitinolytic enzyme forms that differ in molecular size also suggests that proteolytic processing may play a role in the regulation of chitin metabolism (Spindler, 1976; Zielkowski and Spindler, 1978; Koga *et al.*, 1983).

In terms of glycosylation and phosphorylation, there are suggestions in the literature that chitinase-type enzymes may be so modified. Mammalian  $\beta$ -hexosaminidases are lysosomal glycoproteins that are synthesized as higher molecular weight precursors and are also phosphorylated on mannose residues (Hasilik and Neufeld, 1980a,b). *M. sexta* chitinases are glycosylated (Koga *et al.*, 1983). Glucosylation of bovine kidney  $\beta$ -*N*-acetylglucosaminidase causes inactivation (Dolhofer *et al.*, 1982). Protein phosphorylation may play a role in the activation of insect molting fluid chitinase. Incubation of *M. sexta* molting fluid *in vitro* with ATP, calcium ion and a factor from boiled molting fluid leads to chitinase activation presumably via a calcium ion-calmodulin couple and phosphorylation (Bade *et al.*, 1983a,b). However no enzyme proteins were covalently phosphorylated during *in vitro* activation. Whether insect chitinolytic enzymes are phosphorylated or glycosylated for regulatory purposes remains unknown.

A number of insecticidal chemicals have been found to have antimolting activity in insects (Kramer *et al.*, 1985). In fact most of the insect growth regulators have target sites located in the integument (Chen and Mayer, 1985). The most notable are diflubenzuron and related substituted urea derivatives. Several hypotheses have been proposed concerning their mode of action. As one of the final precursors of chitin synthesis was found to accumulate in insects treated with diflubenzuron, it was suggested that the compound blocks the terminal polymerization step in the chitin synthetic pathway (Deul *et al.*, 1978; Hajjar and Casida, 1979). However, diflubenzuron did not directly inhibit chitin synthase from integument of *Trichoplusia ni* and *Hyalophora cecropia* whereas the fungicide polyoxin D did (Cohen and Casida, 1982). Similar observations were obtained with insect gut or whole body

enzyme preparations in *Tribolium castaneum*, *Stomoxys calcitrans* and *Mamestra brassicae* (Cohen and Casida, 1980a,b; Mayer *et al.*, 1981; Mitsui *et al.*, 1981). It has also been proposed that diflubenzuron prevents chitin synthesis by interfering with the proteolytic activation of the chitin synthase zymogen (Leighton *et al.*, 1981). In the latter study, chlorinated hydrocarbons, triazines, nitrophenols, organophosphates, sulfenimides and thiolones inhibited chitin deposition in fungal and cockroach bioassay systems. Morphologically diflubenzuron inhibits cuticular tanning by eliminating or reducing the lamellar structure of cuticle such that chitin content and resistance to fracture are decreased (Grosscut, 1978; Ker, 1977; Leopold *et al.*, 1985). Whether inhibition of chitin synthesis is a primary or secondary effect of the substituted urea insecticide treatments is unknown.

Another possible mechanism involves disruption of the necessary spacial arrangement of the enzyme complex which is important in chitin polymerization, deposition and fibrillogenesis (Cohen and Casida, 1980a,b). The most recent hypothesis suggests that diflubenzuron disrupts transport of the final precursor UDPGlcNAc through biomembranes after UDPGlcNAc is enzymatically synthesized from glucosamine or GlcNAc in epithelial cells (Mitsui *et al.*, 1984, 1985). Diflubenzuron may interfere with other biochemical processes besides chitin metabolism. In *Tenebrio molitor*, *S. calcitrans* and *Anthonomus grandis*, it inhibits DNA synthesis (Mitlin *et al.*, 1977; Deloach *et al.*, 1981; Soltani *et al.*, 1984). Diflubenzuron has also been reported to enhance chitinase, polyphenol oxidase and mixed function oxidase activities, and to depress glycosylation of cuticular proteins (Sacher, 1971; Ishaay and Casida, 1974; Yu and Terriere, 1975). The mode of action of the benzoylphenylurea compounds is controversial at this time because the primary insecticidal site of action remains to be determined.

Cultured integument and a cell free chitin synthase preparation have been used to screen chemicals for anti-chitin activity. The most potent insecticidal compounds in the rice stem borer integument bioassay were halogenated benzoylphenylureas with ID<sub>50</sub> values < 1  $\mu$ M (Nishioka *et al.*, 1979; Kitahara *et al.*, 1983). The integument system is very sensitive in identifying cuticle formation inhibitors such as chitin synthase inhibitors but it is also susceptible to compounds that inhibit basic cellular processes such as protein synthesis and respiration. A cell free chitin synthase system circumvents the complications of cellular bioassays, and both insect and mushroom solubilized enzymes are available for testing (Adams and Gooday, 1980; Cohen and Casida, 1983). Polyoxin D was an order of magnitude more potent as an inhibitor of *Tribolium* chitin synthase than were benzimidazoles with a terpene moiety, the best being 1-geranylbenzimidazole (Cohen *et al.*, 1984; Cohen, 1985). Imidazole derivations exhibit anti-molting activity in the house fly and anti-juvenile hormone activity in the silkworm (Kuвано *et al.*, 1982, 1983). Buprofezin, a thiadiazinone derivative, is another insect growth regulator that causes poisoning symptoms similar to those observed with diflubenzuron-type compounds and has also been

labeled as a chitin biosynthesis inhibitor (Uchida *et al.*, 1985).

Plumbagin, a natural product phenol quinone derivative from the African medicinal plant, *Plumbago capensis*, is a naturally occurring inhibitor of chitin synthase preparations from *Trichoplusia ni* integuments (Kubo *et al.*, 1982, 1983). However, plumbagin may not be very specific as a chitin synthase inhibitor since quinones are highly reactive electrophilic molecules capable of chemically modifying most proteins. Another naturally occurring insect ecdysis inhibitor is azadirachtin which is also a potent anti-feedant limonoid (Kubo and Klocke, 1982). Insect larvae are incapable of completing a molt after feeding on a diet containing azadirachtin, remaining in a pharate condition still encased by the exuvium and head capsule. No inhibitory activity of azadirachtin was found in an *in vitro* assay utilizing chitin synthase extracted from integument. No experiments testing the effects of azadirachtin on chitinolytic enzymes have been attempted. Azadirachtin may not directly interact with chitin metabolizing enzymes. When injected it suppresses ecdysteroid titer in *Locusta migratoria* and perhaps inhibits the central nervous system (Sieber and Rembold, 1983). 2-Acetylpyridine thiosemicarbazones are ecdysis inhibitors in *Oncopeltus fasciatus* (DeMilo *et al.*, 1983). The latter compounds have not been tested for activity against chitin metabolizing systems. Calcofluor white, a 4,4'-diamino-2,2'-stilbene-disulfonic acid derivative used commercially as a fabric bleaching agent, appears to inhibit chitin synthase and chitin microfibril assembly in microbial cell free systems (Mulisch *et al.*, 1983; Selitrennikoff, 1984). In fungi and protoplasts the rate of chitin synthesis *in vivo* is enhanced by calcofluor white and Congo red (Romcero and Duran, 1985). Neither dye has been tested for activity towards insect chitin synthase.

Practically none of these compounds have been screened for activity against chitin degrading enzymes. Recently avermectin, a macrocyclic lactone neurotoxin with acaricidal, insecticidal and antihelminth activities has been shown to inhibit fungal and shrimp chitin synthesis *in vivo* as well as bacterial chitinase activity *in vitro* (Calcott and Fatig, 1984). It was proposed that avermectin may kill susceptible organisms not only by a neurotoxic mechanism but also by inhibiting chitin turnover and synthesis at relatively low concentration. However, using purified *M. sexta* chitinase and  $\beta$ -*N*-acetylglucosaminidase, we were unable to show any inhibition of chitinolytic enzyme activity by avermectin at levels 10-fold greater than those effective against bacterial chitinase preparations (Fukamizo and Kramer, unpublished data).

#### CONCLUDING REMARKS

This review points out our limited knowledge of the metabolism of chitin in insects. It shows the uneven coverage that the field has received. Aspects of chitin biosynthesis and degradation have been rather extensively researched but the regulation and genetics of chitin metabolism, orientation and interactions with other cuticular components have re-

ceived much less attention. Insect biochemists and physiologists sometimes adopt a simplistic view of chitin and have not always appreciated its biophysical complexities. This has led to some unsound work being published and a rather simplistic view of chitin metabolism. There are many kinds of insect cuticles and perhaps diverse chemical interactions of chitin depending on the type of cuticle. The scientific literature on chitin contains its share of speculations that are listed as explanations of how chitin is assembled into and disassembled out of the insect cuticle. At the present time it is impossible to propose a scientifically worthy model for the anabolism and catabolism of chitin in cuticle because we still do not understand the precise molecular interactions (covalent and non-covalent) between chitin and other cuticular components.

Many difficulties have been encountered in the study of chitin biosynthesis and assembly, chief among them being the apparent lability of the chitin synthase systems. Little is known about the physical, chemical and kinetic properties of that enzyme. It may exist as a multienzyme complex with both catalytic and regulatory subunits embedded in a membrane somewhere. The mechanisms of polymerization and fibrillogenesis are also little understood. We know a bit more about chitin catabolic enzymes. Chitinolytic enzymes have proven to be more stable than chitin synthetase and to exhibit rather simple kinetic behavior *in vitro*. The roles of other molting fluid enzymes such as proteases in the activation process of zymogen forms of metabolizing enzymes and in the unmasking of chitin remains controversial. Unanswered questions include: Are chitin synthases synthesized as proenzymes? Is sclerotin (crosslinked structural protein in cuticle) a "defensive" mechanism that prevents the interaction of chitinolytic enzymes with chitin? Is there a chitin-sclerotin crosslink in cuticle? What is the extent of chitin deacetylation *in vivo*?

We know virtually nothing about the genetics of chitin metabolism or about the regulatory mechanisms that operate at the level of DNA replication, RNA transcription and protein translation. There are some data available on regulation at the hormonal, enzyme and substrate levels, but many questions concerning metabolic regulation remain unanswered. If the enzymatic and regulatory mechanisms involved in the synthesis and degradation of chitin *in vivo* would be better understood, then our chances of manipulating chitin levels for insect pest control by insect growth regulators would be enhanced. It is hoped that reference to the many unanswered questions about insect chitin biochemistry should stimulate further research. Such study requires a combination of careful scientific analyses and imagination. Research on chitin biochemistry can profit from the addition of more scientists possessing those qualities.

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