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INSECT PROTEASES AND PEPTIDASES

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I. Introduction

It has been estimated that four of every five living animals are insects and that five-sixths of the named animal species are insect

species (1,2). Among the members of this immensely diverse group one finds bizarre specializations that allow insect species to survive in nearly any imaginable ecological niche. Comparative biochemistry of insects is in its infancy, but the field is gaining impetus not only because of its inherent interest, but because of the potential contribution of such studies to the development of novel and specific controls on the growth of the insect populations.

While the insect proteases so far described hold little promise as targets for insect control agents, they do add to our understanding of insect biochemistry, physiology, and ecology, and of the structure and function of proteolytic enzymes. It seems likely that as we learn more about minor proteases of specific insect tissues, a rich new field in selective control of function will open, analogous to the developments in mammalian proteases (3).

This review is an attempt to summarize our present knowledge of insect proteases and peptidases and to indicate future directions of research. We discuss the role of proteases in food digestion as well as enzymes used for highly specialized functions, such as cocoon opening. The fragmentary material indicating the role of proteases and peptidases in insect development and tissue restructuring is summarized and ecological interactions involving insect proteases are noted. Our hope is that this introduction will stimulate future investigation of these areas.

II. Digestive Proteases

A. THE INSECT DIGESTIVE SYSTEM

Detailed descriptions of the anatomy and physiology of the insect digestive tract can be found in Wigglesworth (4) and House (5). The following brief summary is based on their accounts.

The digestive system of the insect is composed of the alimentary canal and associated glands. The anatomy is extremely varied to fit the individual needs of each group of insects, for feeding techniques and preferences are remarkably diverse. Figure 1 shows a generalized diagram that may be helpful for the following discussion. For any particular insect many features may differ completely from the drawing, however.

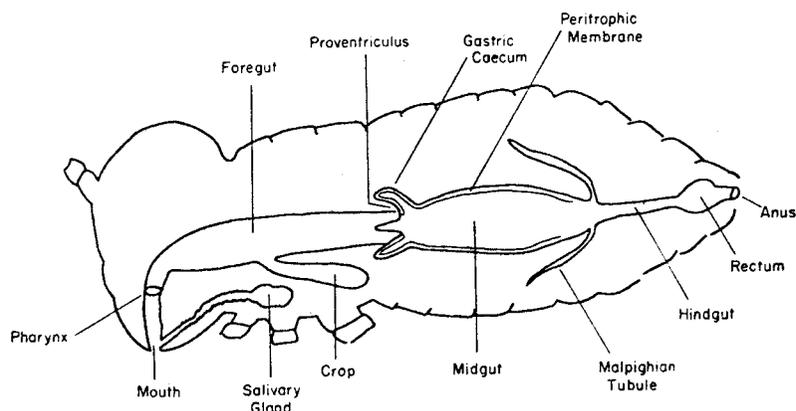


Fig. 1. Generalized diagram of the insect alimentary canal. Modified from E. Melville DuPorte. *Manual of Insect Morphology*, Reinhold, New York, 1959. Copyright © 1959 Litton Educational Publishing, Inc. Reproduced by permission Van Nostrand Reinhold Company.

Food enters the mouth and is mixed with secretions of the salivary glands. These may or may not contain digestive enzymes. In some cases saliva is used for some specialized purpose, for example, lubrication of piercing mouthparts, injection of anticoagulants, and external digestion of food. Food is sometimes chopped by mouthparts into smaller particles as it enters the mouth and before it passes into the foregut.

The foregut (stomodeum) and the hindgut (protodeum) are ectodermal in origin, and the epidermal cell layers of these organs are lined with cuticle—the tough protein–chitin layer that forms the insect exoskeleton (see below, p. 415). The foregut serves chiefly as a storage depot for raw foodstuffs and its capacity is often increased by the crop—a diverticulum that in some exceptional cases can be of enormous size. The crop of honeybees, for example, is the site of honey storage and processing, while in honey-gathering ants, certain individuals serve as honey pots, storing the liquid in a crop of gigantic proportions.

From the foregut discrete packages of food pass into the midgut (mesenteron or ventriculus) through the proventriculus—a valve that may contain a grinding apparatus for reducing the particle size. The midgut is the site of digestion and absorption of foodstuffs. It arises from the endoderm, in contrast to the fore-

and hindgut. It is lined with a layer of thick cubical or columnar cells interspersed with goblet cells. The columnar cells have both the function of secretion of digestive enzymes and that of absorption of soluble metabolites. There is no phagocytosis of food particles and so all foodstuffs must be digested to small soluble components capable of traversing the cell membrane. Individual epithelial cells may cycle between secretion of enzymes and absorption of nutrients, but it is not known if they are capable of both processes simultaneously (6). Since the midgut is not covered by a chitinous layer, the epithelial cells are usually protected by a thin membrane, the peritrophic membrane, that is continuously formed at the anterior end of the midgut and passes with the food residue into the hindgut. The peritrophic membrane consists of a matrix composed of chitin and other polysaccharides laid upon a fibrous layer or layers and varies in thickness from less than 1 to 12 μm (6).

The process of secretion of digestive enzymes from midgut epithelial cells is poorly understood. Like mammalian pancreas cells, the insect cells have a high content of rough endoplasmic reticulum, but the Golgi apparatus is poorly developed and no evidence has been obtained for secretory vacuoles emptying into the lumen (6). This is in contrast to the situation in the specialized cells that secrete the zymogen of cocoonase, as is discussed later (see p. 404).

The surface area of the midgut is often increased at the anterior end by evaginations or diverticula called gastric caecae. Discrete regions of the midgut can often be marked by characteristic luminal pH values, which may vary markedly as one moves along the organ. These may correspond to functional differences in terms of digestion and absorption, as well as other processes. Undigested residue leaves the midgut and enters the hindgut where, in the anterior end, the soluble nitrogenous waste products from the hemolymph (blood) enter via the Malpighian tubules. The main function of the hindgut is water absorption and preparation for excretion.

B. PROTEASES OF INSECT MIDGUT

A large number of qualitative studies aimed at characterization of digestive proteases have been carried out with crude extracts or

homogenates of midgut tissue or contents. In some cases even whole animals have been homogenized. Proteases were usually detected by their action on some protein substrate, such as gelatin, casein, and hemoglobin, or by the hydrolysis of small "specific" substrates, such as chromophoric esters of amino acid derivatives. Specific inhibitors of serine, metallo, and sulfhydryl proteases have been tested, as well as the alkylating pseudosubstrates TLCK and TPCK.

Even the most complete of these studies have been hampered by difficulties inherent in working with crude extracts, especially the inability to distinguish separate specific enzymes from single enzymes of broad specificity. In a few cases enzymes have been separated, and in a very few instances, homogeneous enzymes have been characterized. The sometimes surprising results have justified the considerable effort.

Much thought and experimentation have been given to inhibitors of digestive enzymes that enter the gut of insects along with food. When protease inhibitors are present in plants at relatively high concentrations (>5% of the soluble protein), they are toxic to insects (7). For example, Gooding has reported inhibition of trypsin from the tsetse fly (*Glossina morsitans*) (8) and other bloodsucking insects (9) by a factor in mammalian sera, and Gooding et al. (10) have shown that honey and nectar of certain flowers can inhibit mosquito trypsin.

Since many seeds (e.g., soybean) contain specific trypsin inhibitors, the proteolytic enzymes of insects that feed on such materials were a natural choice for investigation. In 1964 Applebaum et al. (11) undertook a study of the digestive enzymes of the yellow mealworm, *Tenebrio molitor*, as it was known that the insect failed to grow on raw soybean meal. Proteolytic enzymes were shown to be most abundant in the lumen of the larval midgut, which was isolated by dissecting out the food material enclosed in the peritrophic membrane. Specific substrates and inhibitors gave evidence for a carboxypeptidase B and "aminotripeptidase" activity, as well as a typical trypsin-like enzyme. The latter, partially purified by chromatography on an ion-exchange column, attacked basic amino acid esters and polylysine. It was inhibited by soybean trypsin inhibitor, although the complex formed had a higher dissociation constant than that of the bovine trypsin-inhibitor complex.

Tenebrio digestive proteases were later investigated by Pfleiderer and Zwilling (12,13), who isolated two proteases from adult beetles. The first of these ("a-protease") had a molecular weight of 24,000 and was inhibited by PMSF, but attacked neither derivatives of arginine nor those of tyrosine (BAEE or ATEE). It was also unaffected by TLCK and TPCK. The second (β -protease) had a molecular weight of 60,000, but otherwise bore all the earmarks of a typical trypsin. It was inhibited by several protein trypsin inhibitors and by TLCK and PMSF, but not by TPCK. β -Protease had good activity toward BAEE, but none toward ATEE, and it hydrolyzed polylysine. The authors therefore concluded that it was probably identical to the larval "*Tenebrio* trypsin" of Applebaum et al. (11).

When the activity of the β -protease was tested against a protein substrate, however, no typical tryptic activity was observed (14). The B chain of insulin was cleaved at the Glu₄-His₅, Tyr₁₆-Leu₁₇, and Phe₂₅-Tyr₂₆ linkages. A second test on a dodecapeptide devoid of lysine and arginine yielded a single cleavage between Leu and Asp. Thus the activity of the β -protease on protein substrates is essentially chymotryptic, but in all other ways the enzyme displays tryptic activity. The experiments clearly show that conclusions drawn from studies with crude enzyme mixtures and with nonnatural substrates must be regarded with caution and further serve to blur the distinction between these two types of serine proteases.

This, of course, should not be taken to mean that all insect digestive enzymes that appear to be trypsin-like in their specificity toward ester substrates and inhibitors will have anomalous action on proteins and peptides. Table I summarizes the properties of a number of partially or fully purified digestive trypsins from a variety of insects (see also discussion of cocoonase, p. 409). In all cases these enzymes appear to have normal tryptic specificity on protein substrates, and although minor cleavage products have sometimes been observed, this is likely to result from contamination by small amounts of other proteases. Perhaps anomalous trypsins are characteristic of beetles, but unfortunately we have no information about proteases from beetles other than *Tenebrio*. Although *Pterostichus melananis* has been examined by Gooding and Huang (23), only ester substrates were used to establish the presence of a trypsin and a chymotrypsin.

TABLE I
Properties of Trypsin-Like Enzymes Isolated from Insect Midgut

Species	Molecular weight	Ester substrates		Inhibitors		Action on peptides	Ref.
		Cleaved	Not cleaved	Effective	Not effective		
<i>Pieris brassica</i> (cabbage butterfly)	32,000	<i>N</i> -Benzoyl argininamide	Z-Phe Phe	DFP	PCMB	Glucagon insulin, tryptic + other activities	15
				SBTI			16
<i>Apis mellifera</i> (honeybee)	20,000	BAEE	ATEE APNE	TLCK	TPCK	Insulin B chain, tryptic	17
				PMSF			18
308 <i>Vespa orientalis</i> (hornet)	17,000	BAEE BANA APNE	ATEE Glu Phe naphthylamide	Ovomucoid		Insulin B chain, tryptic	19
				TLCK			
<i>Locusta migratoria</i> (locust)	17,000	BAEE	BTEE	PMSF	TPCK	Insulin B chain, tryptic	20
				TLCK			
<i>Manduca sexta</i> (tobacco hornworm)	24,000	BAEE NPGB		Ovomucoid	TPCK PCMB	Glucagon, mainly tryptic	21
				TLCK			
<i>Tineola bisselliella</i> (clothes moth)		BAPA BANA		DFP		Insulin B chain, mainly tryptic	22

While some isolated digestive enzymes appear to be quite stable to autodigestion (see, for example, ref. 12), others are quite labile and must be protected during isolation by reversible inhibitors (21). Ward (22,24) has reported the presence of several trypsin-like enzymes in the webbing clothes moth, *Tineola bisselliella*. It seems likely that some of these are derived from a parental polypeptide chain by proteolytic cleavage, much the same as the families of trypsins and chymotrypsins that can be found in mammalian pancreatic juice. Ultimately, this will probably be unraveled only by isolation of zymogens. Ward (24) has reported failure in detecting proenzymes in homogenates of whole *T. bisselliella*, but they may be present only in small amounts in the midgut epithelial cells.

Insect digestive trypsins have been most extensively studied, although the activity of chymotrypsins, pepsins, carboxypeptidases, and aminopeptidases have been detected in several insect species. Of those enzymes that have been isolated and rather carefully studied, an example is the chymotrypsin of the hornet *Vespa orientalis*.

Digestive enzymes and gluconeogenic enzymes in *Vespa* have been postulated to play a role in social organization (25). Adults appear to be incapable of degrading protein and converting it to sugar. Larvae contain high levels of proteases—the chymotrypsin and carboxypeptidase A and B activities are at least 10 times as high in larvae as in adults. Adults forage for protein foods that they feed to the larvae. The protein is degraded in the larval midgut and converted to sugars (26) that appear in the saliva. The adults collect larval saliva, which is rich in sugars and amino acids, as a food source. Thus a biochemical division of labor exists in the social organization and the interdependence created by this division probably contributes to social cohesion (27).

A chymotrypsin was isolated from midguts of *V. orientalis* and *V. crabo* larvae (28). Only the former has been extensively studied. A combination of chromatography on cation-exchange columns and affinity chromatography yielded a pure enzyme of molecular weight 13,000–14,000—a much smaller molecule than most known chymotrypsins (29). The molecular weight was determined by several methods—ultracentrifugation, gel filtration, polyacrylamide gel electrophoresis with and without SDS (29), and stoichi-

ometric reactions with isotopically labeled PMSF and ZPCK (30)—all with very good agreement. Amino acid analysis showed that the enzyme contained only four cysteine residues and no methionine (30). The properties of this chymotrypsin and other partially characterized enzymes of similar specificity isolated from insects are summarized in Table II.

Of the remaining studies of midgut proteases the most complete are those of Ward (24) on the enzymes from *Tineola bisselliella*. Interest in this organism stems from its ability to digest keratin—an insoluble and refractory protein. Much early work on wool-digesting insects has been reviewed by Waterhouse (31) and by Gilmour (32). The key to the digestion of keratin was discovered by Linderstrøm-Lang and Duspiva 40 years ago (33,34). Wool-eating insects maintain strongly reducing conditions in the larval midgut ($E_h = -190$ to -280 mV) and thus are capable of reducing the disulfide bonds that contribute to the insoluble and indestructible nature of keratins. Once the structures are unfolded by the reduction of disulfides, proteolytic enzymes can digest the chains. Gilmour (32) has suggested that the low redox potential is the result, rather than the cause, of disulfide reduction, but the fact that the low redox potential can be measured even in larvae fed a nonwool diet (34) strengthens the idea that reducing conditions are somehow maintained.

In *T. bisselliella* Ward (24) has identified a chymotrypsin-like enzyme, a family of aminopeptidases and carboxypeptidases, and two novel metalloendopeptidases in addition to a family of trypsin-like enzymes already mentioned. The aminopeptidases have not been extensively purified and they are a complex group differing in molecular weight and charge (35,36). Again, since these enzymes have been studied in homogenates of whole animals, they may constitute a family of proteolytic degradation products. More interesting are the two metalloproteinases of molecular weight 24,000 that have novel endopeptidase activity (37,38). Both are inhibited by metal chelators. Metalloproteinase-2, which appeared to be homogeneous by polyacrylamide gel electrophoresis, attacked *S*-carboxymethylated insulin chains as shown in Table III. The cleavage pattern indicates a specificity clearly different from those of metalloendopeptidases of bacteria or snake venom.

TABLE II
Chymotrypsin-Like Digestive Proteases Isolated from Insects

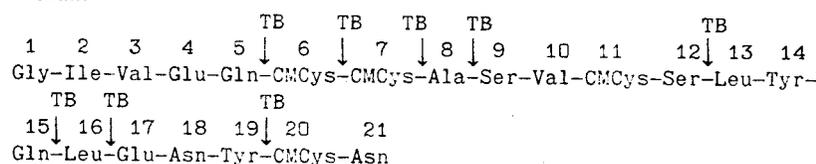
Species	Molecular weight	Substrate		Inhibitors		Peptide substrates	Ref.		
		Cleaved	Not cleaved	Effective	Not Effective				
<i>Pieris brassica</i> (cabbage butterfly)	32,000	Z-Phe-Phe	Bz Arg NH ₂	DFP		Glucagon-, chymotryptic Insulin-, chymotryptic	15		
		Z-Phe-Leu		SBTI			16		
		Z-Trp-Tyr		(weak)			17		
		Z-Leu-Phe							
<i>Apis mellifera</i> (honeybee)	19,500	A'EE	BAEE	PMSF	TLCK	Insulin A or B chain, mainly chymotryptic	18		
		APNE	BANA	TPCK					
<i>Vespa orientalis</i> (hornet)	13,600	A'EE	BAEE	PMSF	TLCK	Insulin B chain, chymotryptic	28		
		APNE		TPCK				polyvalent trypsin inhibitor	29
				SBTI					
				LBTI					
				Ovomucoid					
				α-Anti-chymo- trypsin of serum					

TABLE III

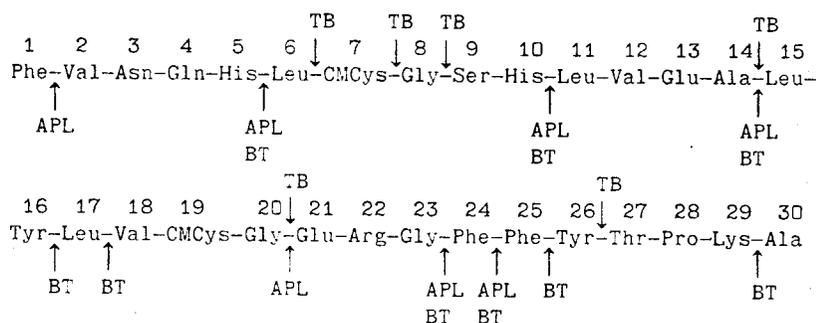
Cleavage of Reduced, Carboxymethylated Insulin Chains by Metalloproteinase-2 of *Tincola bisselliella*.

The cleavage points for the *Tincola* enzyme are shown in the A chain. In the B chain, cleavage points for the *Tincola* enzyme (TB) are compared with those for metalloproteases from the bacterium *Bacillus thermoproteolyticus* (BT) and from the viper, *Aghistrodon piscivorus leucostoma* (APL). From references 37, 44, and 45.

A chain



B chain



Two carboxypeptidases were separated from the extracts of whole *T. bisselliella* and the major form was purified to homogeneity (39). It was shown to have a molecular weight of 72,000 by gel filtration (compared to 34,000 for pancreatic enzymes). With the pancreatic enzyme it shared susceptibility to inhibition by thiol reagents and 1,10-phenanthroline, but it was also inhibited by DFP, as is the yeast enzyme. The substrate specificity, investigated with di- and tripeptides, was similar to the pancreatic enzymes, that is, peptides with *N*-terminal lysine, arginine, and proline are not cleaved, while *Z*-Glu-Leu or Leu-Gly-Leu are very rapidly hydrolyzed.

It is hardly surprising that insect digestive enzymes display some properties different from those of mammals. Insects gener-

ally lack an acid digestive process analogous to the peptic action in the stomach preceding the alkaline processes of the small intestine. The diptera sometimes have regions of quite acid pH in the midgut, and there are several reports of pepsin-like enzymes in flies (40-43). In no case, however, has any one of these enzymes been purified and characterized to the point where we could be confident that it shares properties of mammalian pepsin other than optimum activity at acidic pH.

C. CONTROL OF THE SECRETION OF DIGESTIVE PROTEASES

Proteases are secreted only in response to dietary protein entering the midgut. A number of studies have shown a correlation between the total amount of protein entering the midgut and the amount of protease secreted. It has been reported that engorgement of the crop in the tsetse fly, *G. morsitans*, was necessary for secretion of high levels of protease and that the response was mediated through visceral nerves and a neurosecretory substance (46). A similar neurosecretory response has been implicated in the blowfly, *Calliphora erythrocephala* (47,48). Engelmann (49) has reviewed this work and pointed out possible pitfalls in the interpretation of the experiments.

Engelmann's study on the roach, *Leucophaea maderae*, (49) clearly showed that the controlling factor in protease secretion is the amount of protein in the gut. Removal of the brain, corpora cardiaca, and corpora allata (the latter two organs are part of the insect neuroendocrine system)—simply or all together—did not affect the rise in protease following feeding of a protein-rich meal; thus neurosecretion from these organs does not play a role. A similar conclusion was reached by Persaud and Davey (50) working with a bloodsucking bug, *Rhodnius prolixus*. In this case destruction or removal of the brain neurosecretory cells following feeding did not stop the secretion of proteases, although decapitation depressed the level of protease secretion.

Engelmann (49) showed that only certain proteins, and not free amino acids, were effective in stimulating protease secretion in the roach. This line of investigation was extended by Briegel and Lea (51) using mosquitos and an ingenious experimental technique in which test substances were administered by enema to the midgut. Fluids were injected by means of a small tube attached to a

microsyringe through the anus into the posterior midgut. After appropriate times the midgut was removed and homogenized, and protease levels were determined. It was shown that the protein content, not the volume of solution, determined protease levels. Protease secretion was only stimulated by intact globular proteins and not, for example, by gelatin, histone, or protein hydrolysates (52,53).

Gooding (53) has studied the secretion of a trypsin-like protease in the midgut of the mosquito *Aedes aegypti* in response to a blood meal. The level of protease secretion was depressed by administration of the antibiotics puromycin and actinomycin D (Dactinomycin), which interfere with protein synthesis. After mosquitoes were fed on blood that contained ^{14}C -amino acids, isotopic label was found in partially purified protease from the midgut. This indicates that the protein meal stimulates *de novo* synthesis of protease. A similar conclusion was reached by Fuchs and Fong (54), also using actinomycin D and α -amanitin administered to *A. aegypti*.

D. NONINTESTINAL DIGESTION OF FOOD

Food digestion may occur outside the midgut, for example, in the crop or foregut. Digestive enzymes in this case either come from the salivary gland or are passed forward from the midgut, since there is neither secretion of enzymes nor absorption in the crop and foregut. There is very little compelling evidence for the production in the salivary gland of proteases used for digestion of food.

Price (55) investigated an acid protease in blowfly (*Calliphora erythrocephala*) salivary glands and found maximal activity after larvae normally cease to feed. In this case the protease seems to leave the glands internally rather than being ejected in the saliva. Carnivorous insects often inject into their prey proteolytic enzymes that carry out digestion before ingestion (see ref. 4, p. 505), but it is unclear whether the salivary gland or the midgut is the source of the proteolytic enzymes.

Proteases are sometimes detected in feces (see, for example, refs. 49, 51, and 56) and probably represent stable enzymes that survive digestion and are excreted. In some cases, however, excretion plays a role in the ecology of the insect. Fly larvae that

feed on meat excrete proteases (57) that may enable them to carry out digestion and dissolve passageways in the flesh.

A most remarkable instance of fecal proteases is found in the leaf-cutting attine ants. Attine ants are chiefly tropical species with subterranean nests. Workers cut the leaves of jungle vegetation, sometimes causing extensive defoliation. The leaf material is not used directly for food, but as a substrate for the cultivation of fungal gardens (ref. 27, Chap. 4). The biochemistry of the symbiotic relationship between ants and fungi has been investigated by Martin and his colleagues (58-61). Fecal proteases were separated and were shown to consist of a serine protease (mol. wt. ~ 70,000) and two metalloproteases (mol. wt. ~ 41,000 and 15,000). Fungus isolated from the ant gardens and grown in pure culture also produced three proteases, and these appeared to be identical to the fecal proteases by several criteria. Thus the ants seem to ingest fungal proteases and later excrete them. Ants separated from the colony and fed a nonfungal diet showed a fivefold decrease in fecal digestive enzymes. These observations led Martin et al. to postulate that the fungus produces intracellular proteases that are released when fungus is eaten by ants. The ants can be seen to defecate on freshly masticated vegetation in a newly established area of the fungal garden and thus appear to transfer fungal proteases to the new nutrient supply. These fecal enzymes digest the leaf material and aid in the establishment of new fungal cultures. The ants apparently have lost the ability to produce their own digestive enzymes and depend on the fungus to supply them with amino acids. Reminiscent of the case of *Vespa orientalis*, we again find a relationship cemented by the production of digestive enzymes in only one partner.

III. Cocoonases

A. INTRODUCTION

Cocoonases, as the name implies, are enzymes used for softening insect cocoons to permit escape of the adult moths. They have evolved in certain of the giant saturniid silkmoths, including the silkworm of commerce, *Bombyx mori*. Thus the observation that a digestive fluid was used for cocoon softening is probably as old as

sericulture. Certainly, it was known to that indefatigable observer and chronicler of insect behavior, J. H. Fabre, who recorded his observations nearly a century ago. His account, as translated by deMattos (62) is as follows:

At the moment of her deliverance, the Mulberry Bombyx has in her stomach a particular solvent which the new-born moth disgorges against the wall of the cocoon to soften it, to dissolve the gum that sticks the threads together and in this way to force an exit by the mere pressure of her head. With the aid of this reagent, the recluse is able triumphantly to attack her silken prison at the fore-end, the rear-end or the side, as I discover by turning the chrysalis in its cocoon, which I slit with a pair of scissors and then sew up again. Whatever the spot to be perforated for the emergence, a spot which my intervention varies at will, the liquid disgorged promptly soaks into and softens the wall, whereupon the captive, struggling with her fore-limbs and pushing her forehead against the tangle of unstuck threads, makes herself a passage with the same ease as in her natural liberation.

The active principle of the fluid used for softening the cocoon was variously supposed to be an acid (63), potassium hydroxide (64), or an enzyme (65,66, see also ref. 82 and references therein). Duspiva (66), who carried out preliminary characterization of the enzyme from *B. mori*, believed that it was regurgitated from the crop and that it dissolved the amorphous protein sericin, which cements the fibrous silken threads that form the body of the cocoon. Thus he termed the enzyme sericinase. As we were later able to show, the enzyme is a trypsin and does not have a strict specificity for sericin, and thus we have used the functional term cocoonase. So far, these enzymes are well documented from four species, *B. mori*, *Antheraea polyphemus*, *A. pernyi*, and *A. mylitta* (67-69).

B. SYNTHESIS AND SECRETION OF PROCOCOONASE

Cocoonases are not products of the digestive tract, but are secreted by a specialized tissue derived from a modified mouthpart, the galea. The galeae are part of the laterally paired maxillae in most insects, and in many lepidoptera join to form the feeding tube or proboscis. The adult saturniid moths do not feed,

however, and in the cocoonase producers the galeae undergo hypertrophy and striking development during formation of the adult form (pharate adult). Kafatos (70,71 and references therein) has made a detailed study of the production of prococoonase in the galea. The galea is a cone-shaped organ consisting of a layer of epidermal cells enclosing a cavity exposed to the hemolymph. During adult development the epidermal cells on the lateral face of the galea undergo a series of mitotic divisions that give rise to three cell types—epidermal, zymogen, and duct cells. The epidermal cells are at the surface and secrete cuticle in the normal fashion (see below, p. 417).

One duct cell associated with two zymogen cells forms a complex "organule" that consists of the duct and valve apparatus supplied by the duct cell penetrating a large extracellular vacuole contributed by a cooperative effort of the two zymogen cells. During the development of the galea secretory apparatus, the polyploid zymogen cells become gigantic and the synthesis of prococoonase is so prodigious that it becomes the predominant soluble protein of the whole galeae (72).

As discussed earlier, the secretion of digestive enzymes by midgut cells does not seem to follow the pattern of zymogen synthesis in the mammalian pancreas (73,74); however, prococoonase production follows the mammalian model quite faithfully. Cells acquire rich endoplasmic reticulum and well-developed Golgi zones. Spirals of 9–12 ribosomes are found and these could represent polysomes of appropriate size for prococoonase (mol. wt. $\sim 28,000$) synthesis (71). Indirect evidence has been reported (75) for the formation of a prococoonase messenger RNA that is considerably more stable than average cellular messenger RNA. This is often characteristic of cells that produce a single major product.

Secretion of zymogen increases the size of the storage vacuole until it reaches a length of 110–200 μm and a volume of $11\text{--}20 \times 10^3 \mu\text{m}^3$ (71). On a precise schedule the zymogen solution is secreted through the duct provided by the duct cell onto the newly formed galea cuticular surface. It is not known how extrusion is accomplished nor when zymogen activation takes place, but the coalesced droplets on the galea surface contain active enzyme.

Prococoonase is efficiently activated by the cleavage of a single peptide bond catalyzed by bovine trypsin or by enzymes from the insect molting fluid (76) (see below, p. 417). Autoactivation or activation by porcine enterokinase is very much slower (77). It is believed that either a residual enzyme remaining on the cuticular surface after the resorption of the molting fluid or some proteolytic enzyme provided by the duct-cell apparatus is responsible for the initiation of zymogen activation, after which autoactivation may complete the process. Cocoonase is extremely stable to autodigestion at neutral pH (78) and thus, once formed, it remains active during subsequent dehydration and storage.

C. COCOONASE OCCURRENCE, PURIFICATION, AND DEPLOYMENT

Deposits of active cocoonase can be found on the galeae surface just prior to the emergence of the adult moth from the pupal cuticle (67,78,79). Up to 200 μg of material can be harvested from a single animal simply by lifting off the dry deposit with a pair of fine forceps. Active-site titration of the material with a trypsin titrant, NPGB, indicated that 68% of the dry weight can be accounted for as active enzyme (80). In most cases polyacrylamide gel electrophoresis of the crude material reveals only one protein band (78). The remaining material consists of a complex mixture of peptides, probably representing degraded zymogen peptide plus autodigestion products.

For use in cocoon softening, the adult silkworm produces an alkaline fluid in which dry cocoonase deposits are dissolved to yield the digestive fluid to be applied against the inner surface of the cocoon. In *A. polyphemus* the fluid is isotonic KHCO_3 , pH 8.3—close to the optimum for cocoonase activity. The buffer solution is the product of the paired labial glands. These glands have a remarkable history. In the larva they form part of the silk gland and produce the cuticular duct through which the liquid silk flows to the spinnerets. The cells of the gland are greatly reduced in size in the pupa, but become active and form during adult development the cytoplasmic and membranous machinery necessary for intensive ion transport (70,81).

In *B. mori* the origin of the solvent is less clear. No labial glands are present and the solvent seems to be secreted in the crop (78). Eguchi et al. (82,83) have shown that pharate adults of *B. mori*

have a high content of proteases in the midgut and that these enzymes are capable of digesting silk proteins. The fact remains, however, that *B. mori* galeae are well developed in the pharate adult and dry deposits of cocoonase can be harvested from them (69,79). It appears that the cocoon-softening fluid in this species consists of a mixture of enzymes from the galea and the midgut (83).

Cocoons are composed primarily of two proteins—the insoluble silk protein, fibroin, and an amorphous protein, sericin, that cements the fibroin threads into a tough layer (84). From a microscopic analysis it seems that cocoonase digests only sericin, leaving the fibroin fibers intact (70). It is difficult to test chemically whether one or the other protein is indeed the preferred substrate for the enzyme because both proteins are insoluble under mild extraction conditions and are degraded by the usual extraction techniques (85). However, a pure source of sericin can be obtained from a mutant silkworm Nd *B. mori* or “naked pupa” (86). This animal spins a very flimsy cocoon that is highly enriched in sericin (>99%) because the posterior silk gland that produces fibroin has degenerated. The same peptide products are obtained when the normal or Nd cocoon is digested by cocoonase (87), supporting the hypothesis that only sericin is hydrolyzed.

As noted by Fabre (62) and confirmed by Eguchi and Iwamoto (83), *B. mori* is capable of escaping from any surface of the cocoon. In *A. polyphemus* normal escape can only be accomplished from the end that the larva faces when it completes its spinning (69). Larvae of *A. pernyi* terminate cocoon spinning pointed head upwards, an orientation cued by gravitational and tactile stimuli (88). This direction is decided upon during a critical period of construction that occurs several turning cycles before conclusion of the spinning. In this manner the animal somehow properly prepares the anterior end in the weaving process for the eventual enzyme-assisted escape.

On the other hand, escape can sometimes be accomplished by *A. polyphemus* from the opposite end of the cocoon when the animal has been experimentally removed and inverted or when cocoonase has been removed from the animal (69). In these cases cocoons are still wetted with solution, which may be pure buffer

or buffer mixed with regurgitated crop or midgut fluid. The cocoons are also shredded by clawing of the animal, a phenomenon never observed in normal escape (69). Similar experiments have been carried out on *B. mori* by Eguchi and Iwamoto (83), who derived similar conclusions.

In summary, prococoonases are produced by giant polyploid zymogen cells in the galeae of the pharate adult moth. During or shortly after secretion, the zymogen is activated and the solution of active enzyme dries to a solid. The dry deposit is dissolved in an alkaline solvent at the appropriate moment, and the active digestive solution is applied to the interior of the cocoon, where it acts upon sericin to release the cemented fibroin threads, allowing the adult moth to force its way from the cocoon.

D. CHEMISTRY OF PROCOCOONASE

Prococoonase is easily purified from homogenates of *A. polyphemus* galea removed just prior to the emptying of the zymogen cell storage vacuoles (76). The original molecular weight estimate, 33,000, was corrected to 28,000 when it was recognized that the protein contained sugar residues (77) and that these led to anomalous molecular weight estimates. Both *A. polyphemus* and *A. pernyi* enzymes and zymogens contain glucosamine and mannose; the *A. mylitta* enzyme is not glycosylated (89). As mentioned earlier, cocoonase is capable of activating the proenzyme, though less efficiently than bovine trypsin. The product in either case is identical to native cocoonase.

The *N*-terminal sequence of the *A. polyphemus* and *A. mylitta* proenzymes have identical 13 amino acid residue zymogen peptides, as shown in Table IV. Also shown are the activation peptides of some other trypsin-like enzymes for comparison. The prococoonase peptide is somewhat larger than that of vertebrate trypsinogens (6–8 residues). There are only three homologous amino acids with bovine trypsinogen, and two of the acidic amino acids that precede the carboxyl terminal lysines of all previously examined trypsinogen activation peptides are absent. Since mammalian enterokinase appears to recognize the tetraanionic sequence (90), it is not surprising that it is relatively ineffective in prococoonase activation.

TABLE IV
Comparison of NH_2 -Terminal Sequences of Prococonases with Other Trypsinogens^a

	← Activation peptide →										Trypsin																				
	1	2	3	4	5	6	7	8	9	10	10	11	12	13	14	15	16	17	18	19	20	...									
<i>Antheraea polyphemus</i> prococonase	Lys	Lys	Thr	Pro	Gln	Arg	Thr	Gln	Asp	Asp	Gly	Gly	Lys	Ile	Val	Gly	Gly	Phe	Thr	Ile	Gly	Ile	Asp	Thr	Val	Pro	Tyr	...			
<i>Antheraea mylitta</i> prococonase	Lys	Lys	Thr	Pro	Gln	Arg	Thr	Gln	Asp	Asp	Gly	Gly	Lys	Ile	Val	Gly	Gly	Tyr	Ser	Trp	Gly	Leu	Asn	Thr	Ala	Pro	Tyr	...			
Bovine trypsinogen									Val	Asp	Asp	Asp	Asp	Lys	Ile	Val	Gly	Gly	Tyr	Thr	Cys	Gly	Ala	Asn	Thr	Val	Pro	Tyr	...		
Porcine trypsinogen									Phe	Pro	Thr	Asp	Asp	Asp	Asp	Lys	Ile	Val	Gly	Gly	Tyr	Thr	Cys	Ala	Ala	Asn	Ser	Val	Pro	Tyr	...
Dogfish trypsinogen									Ala	Pro	Asp	Asp	Asp	Asp	Lys	Ile	Val	Gly	Gly	Tyr	Glu	Cys	Pro	Lys	His	Ala	Ala	Pro	Trp	...	
Lungfish trypsinogen									Phe	Pro	Ile	Glu	Glu	Asp	Lys	Ile	Val	Gly	Gly	Tyr	Glu	Cys	Pro	Lys	His	Ser	Val	Pro	Trp	...	

^a Numbering corresponds to bovine trypsinogen. Sequences were obtained from references 89 (prococonases), 98 and 99 (dogfish and lungfish trypsinogens), and 100 (others).

E. CHEMISTRY OF COCOONASES

1. *Substrate Specificity and Inhibitors*

Cocoonases act readily only upon ester and amide derivatives of lysine and arginine (91). For small ester and amide substrates K_M and k_{cat} values, substrate activation effects, and presteady-state kinetics are virtually identical to those of bovine trypsin (69,77,80,91). The enzymes react readily with DFP, TLCK, and NPGB with kinetics similar to those of bovine trypsin, and the resulting derivatives are devoid of enzymatic activity (69,80,91). No inhibition was observed with TPCK, metal chelators, or sulfhydryl inhibitors (91). The enzymes therefore appear to have an active-site histidine, susceptible to alkylation by TLCK, and a serine hydroxyl, capable of forming stable esters on reaction with NPGB and DFP. Reaction with isotopically labeled DFP produced an inactive enzyme with a covalently linked labeled group. This permitted isolation and sequence determination of the active-site peptide (69,89).

Cocoonases are inhibited by soybean trypsin inhibitor (91), but the dissociation constant is higher for the cocoonase-inhibitor complex than for the trypsin-inhibitor complex, and the cleavage of inhibitor by trypsin is tenfold faster than by cocoonase (92). This quantitative difference in the action of the two enzymes on proteins is also seen with citraconylated lysozyme as a substrate (69). In the case of chymotrypsinogen activation, a specialized function of trypsin, the cocoonase-catalyzed reaction is 1000-fold slower than that of trypsin (69). For prococoonase activation the second-order rate constant for trypsin is approximately 500-fold larger than that for cocoonase (77). Peptide maps produced from proteolytic digest of glucagon, insulin B chain, and *S*-carboxymethylated lysozyme by *A. polyphemus* and *A. mylitta* cocoonases and bovine trypsin are essentially identical (68,69). These experiments leave no doubt that cocoonases are extremely similar to mammalian trypsin in terms of their specificity and catalytic mechanisms. Nonetheless, when solutions of cocoonase and trypsin of the same concentration were tested qualitatively as cocoon-softening agents, cocoonase solutions were superior (69). This may result from the presence of small amounts of surfactants in the cocoonase preparations.

2. Structural Studies

Amino acid analyses of cocoonases are generally similar to those of mammalian trypsin and chymotrypsins. An exception is the cysteine content, which is either 4 or 6 residues per molecule for cocoonases, but 12 for bovine trypsin. Contrary to early indications (78), all cysteines of cocoonases are involved in disulfide bridges (89). The presence of fewer disulfide bridges probably contributes to the instability of cocoonase at extremes of temperature and pH and in the presence of urea (91). Peptide maps of tryptic digests of S-methylated cocoonases and bovine trypsin indicated that some trypsin peptides had map positions identical to those derived from cocoonases. The cocoonases of *A. polyphemus* and *A. pernyi* shared 20 of 25 peptides in common. As mentioned earlier, these cocoonases contain a small carbohydrate substituent composed of 6 residues of mannose and 2 residues of glucosamine (89).

Similarity between cocoonases and other serine proteases was also suggested by immunological evidence, at least in those regions of the polypeptide chain that determine antigenicity (93). Anticocoonase crossreacted with trypsin, chymotrypsin, elastase, and subtilisin while other types of sulfhydryl or carboxylic acid proteases and nonproteolytic serine enzymes (acetyl cholinesterase) show no crossreactivity.

In contrast, the zymogens of the vertebrate proteases are not recognized by antibodies directed toward prococoonase, which indicates that the structures of the antigenic region of the zymogen proteins are rather different. Sequence of the zymogens discussed earlier may help to explain the immunological observations. There are marked differences between the activation peptides of prococoonase and trypsinogen as shown in Table IV, and if antigenic determinants are located in this region, these differences may explain the lack of crossreactivity.

As Table IV also shows, two cocoonases share with other trypsin the first four residues of the N-terminus. One might expect extensive sequence similarities in regions critical for tryptic enzyme function, and if one assumes mechanistic identity for cocoonase and trypsin, the cleavage of prococoonase probably generates enzymatic activity by allowing the liberated α -amino

group of isoleucine to participate in an ionic interaction with the side-chain carboxylate group of an acidic residue near the active-site serine (94-96). Beyond the first four residues homology breaks down, even between two cocoonases.

As shown in Table V, the active-site peptide of cocoonase is highly homologous with those of other trypsin enzymes. Eleven of the 16 residues are identical to bovine trypsin, including the NH_2 -terminal binding aspartic acid adjacent to the nucleophilic serine residue. The specificity site of trypsin contains another negatively charged aspartic acid (residue 177) that electrostatically binds the positively charged groups of substrates and inhibitors (97). Aspartic acid is also found in the corresponding position in cocoonase (the first residue in the active-site serine peptide). Another homology is the basic amino acid that immediately precedes the active-site peptide and is the site of a tryptic cleavage (residue 176). The active-site homology underscores the preservation of the basic trypsin mechanism during the span of evolution in such phylogenetically diverse groups as bacteria, insects, and mammals (98).

IV. Proteases and Peptidases in Insect Development

A. GROWTH AND DEVELOPMENT IN INSECTS

The growth and development of insects proceeds discontinuously through a series of programmed stages. Progress is regulated by the titers of the molting hormone (ecdysone) and the juvenile hormone. The processes of growth and development commence with embryogenesis following the fertilization of the ovum. When embryonic development is completed, the insect emerges from the egg (hatching) as a larva or nymph and proceeds to feed. The immature insect feeds and grows until its integument is completely expanded. At this time the cuticle must be shed (molted) and a new, larger one synthesized to allow further growth. In the more primitive orders (ametabola, hemimetabola), the nymph undergoes gradual metamorphosis at each molt so that the newly formed exoskeleton resembles more closely the adult form of the species. Larval and adult stages are more cleanly separated in the more highly evolved orders (holometab-

TABLE V
Comparison of Sequence of Active-Site Serine Peptides from *Antheraea polyphemus* Cocoonase and Several Other Trypsin-like Enzymes^a

	180					Ser	185					190				
<i>A. polyphemus</i> cocoonase	Asp	Ala	Cys	Gln	Gly	Asp	Ser	Gly	Gly	Pro	Val	Gln	Asn	Ala	Gly	Arg
Bovine trypsin	Asp	Ser	Cys	Gln	Gly	Asp	Ser	Gly	Gly	Pro	Val	Val	Cys	Ser	Gly	Lys
Sheep trypsin	Asn	Ser	Cys	Gln	Gly	Asp	Ser	Gly	Gly	Pro	Val	Val	Cys	Ser	Gly	Lys
Pig trypsin	Asn	Ser	Cys	Gln	Gly	Asp	Ser	Gly	Gly	Pro	Val	Val	Cys	Asn	Gly	Gln
Lungfish trypsin	Asn	Ser	Cys	Gln	Gly	Asp	Ser	Gly	Gly	Pro	Val	Val	Cys	Asn	Gly	Gln
<i>Streptomyces griseus</i> protease	Asp	Thr	Cys	Gln	Gly	Asp	Ser	Gly	Gly	Pro	Met	Phe	Arg	Lys	Asp	Asn
Bovine thrombin	Asp	Ala	Cys	Glu	Gly	Asp	Ser	Gly	Gly	Pro	Phe	Val	Met	Lys	Ser	Pro
Starfish trypsin		Ser	Cys	Gln	Gly	Asp	Ser	Gly	Gly	Pro	Val	Val	Cys			
Shrimp trypsin	Asp	Ser	Cys	Glu	Gly	Asp	Ser	Gly	Gly	Pro	Leu	Ala	Cys	Cys	Ser	Asn

^a Numbering corresponds to bovine trypsinogen. Sequences were obtained from references 84 (cocoonase), 101 (starfish trypsin), 102 (shrimp trypsin), and 99 (others).

bola) and each succeeding larval instar resembles its predecessor. After a series of larval molts, the animal undergoes metamorphosis, first to a pupa and, finally, to the adult form. Development in holometabolous insects, then, may be described as progressing through the developing embryo, the growing larva, the transitional pupa, and the reproductive adult. The molts that punctuate this process are controlled by the titer of ecdysone, and the form of the insect that emerges after the molt is determined by the titer of the juvenile hormone. As its name suggests, a high titer of juvenile hormone maintains the insect in larval form and a decrease in hormone titer releases the processes of metamorphosis that lead eventually to the reproductive adult.

The existence of such a controlled, discontinuous program of growth and development requires that very specialized metabolic processes occur during defined periods. Many—possibly all—of these processes involve the action of specialized proteases and peptidases. In the following sections, we discuss lysosomal enzymes and also proteases and peptidases involved in the molting cycle.

B. LYSOSOMAL PROTEASES

1. Introduction

The occurrence of lysosomes in insects has been reviewed by Lockshin (103) and Lockshin and Beaulaton (104). Lysosomes have been observed in cells that are involved in the transport and degradation of materials in nonmetamorphosing insects and in cells that are involved in the process of programmed tissue destruction in metamorphosing insects. For the purposes of this discussion, we consider proteases with acid pH optima that appear in cells concurrently with lysosomes and that sediment with particulate fractions after homogenization of biological samples to be lysosomal. None of the enzymes that are described has been purified to homogeneity and studied in detail.

Reports of "cathepsin-like" acid proteases in homogenates of whole insect bodies at different times during development have appeared. Kuk-Meiri et al. (105) have studied the variation in the activity of an acid protease (pH optimum 3.5–4.1) in homogenates of developing eggs of the African migratory locust, *Locusta*

migratoria migratorioides. The enzyme is inhibited by iodoacetate, activated by mercaptoethanol, and may function in the utilization of yolk protein by the embryo. Similarly, acid proteases have been reported to occur during egg development in the housefly, *Musca domestica* (40). However, lysosomes have not yet been observed in such developing eggs.

An essential feature of metamorphosis is the histolysis of larval tissues and their replacement by tissues characteristic of the adult organism. This process of genetically determined histolysis has been termed "programmed cell death" (106). Among the many larval tissues that undergo histolysis during metamorphosis are the larval salivary glands of the diptera (flies) and the larval intersegmental muscles of lepidoptera (butterflies and moths).

2. Salivary Gland Regression

During the end of the last larval instar of the midge, *Chironomus tentans*, before the larval-pupal molt and the onset of regression, large acid phosphatase staining, lysosome-like bodies begin to appear in the salivary glands and increase in number as the pupal molt approaches (107). At the onset of pupation, a protease appears in gland sonicates with a pH optimum of 3.4 and this activity persists until gland degeneration has been completed (108). More careful study of this protease (109) revealed that it was insensitive to inhibitors of serine and thiol proteases and to chelating agents. At the onset of gland regression, the protease sediments with particulate fractions, but near the end of cell disruption appears free in homogenates. The appearance of free enzyme in extracts coincides with the apparent bursting of lysosomes and the appearance of nonsedimentable acid phosphatase activity in gland extracts. When extracts of early pupal glands, which contain low levels of acid protease, were centrifuged, and the particulate fraction was treated with trypsin, protease activity with a pH optimum of 3.5 was produced. From these data it was concluded that lysosomes that contain an inactive acid protease are produced in the salivary glands of *Chironomus* prior to the larval-pupal molt. At the onset of gland histolysis during pupation, the acid protease is activated and participates in gland-cell degeneration. The activation process can be prevented by cyclo-

heximide treatment and is therefore assumed to require the synthesis of protein.

3. Degeneration of Intersegmental Muscles

The larval abdominal intersegmental muscles of the silkworm, *Antheraea pernyi*, persist through the pupal period and the 3 weeks required for the development of the adult moth, but undergo complete histolysis during the first 48 hr after the emergence of the adult. Both hormonal and neural triggers are involved in the control of the onset of this tissue degradation. Histolysis of the intersegmental muscle is prepotentiated by the ecdysone pulse that occurs in the absence of the juvenile hormone and initiates adult development (110). The actual onset of histolysis is triggered 3 weeks later by a sudden cessation of motor nerve impulses to the muscles (111). During the pharate adult period, the muscle becomes populated by lysosomes (112), and homogenates contain acid phosphatase and a protease with a pH optimum of 3.9–4.0 that sediment with a particulate fraction, but may be released by osmotic shock or detergents (113). As in the case of the degenerating dipteran salivary gland, the onset of intersegmental muscle-cell lysis can be prevented by cycloheximide (114), and cell disruption is preceded by the apparent bursting of lysosomes with concurrent release of free acid phosphatase and acid protease (113).

C. MOLTING CYCLE PROTEASES

1. Insect Molting

As was mentioned above, the discontinuous development of insects is punctuated by shedding of the integument. During a molt the insect digests most of its exoskeleton, synthesizes a new one that may be morphologically quite different, and sheds the remains of the old.

The events of the molting cycle are shown schematically in Figure 2. Insect integument is comprised of cellular and extracellular layers. Starting from the inside of the insect (hemocoel), there is a basement membrane and then a layer of epidermal cells. The remainder of the integument is extracellular, is produced

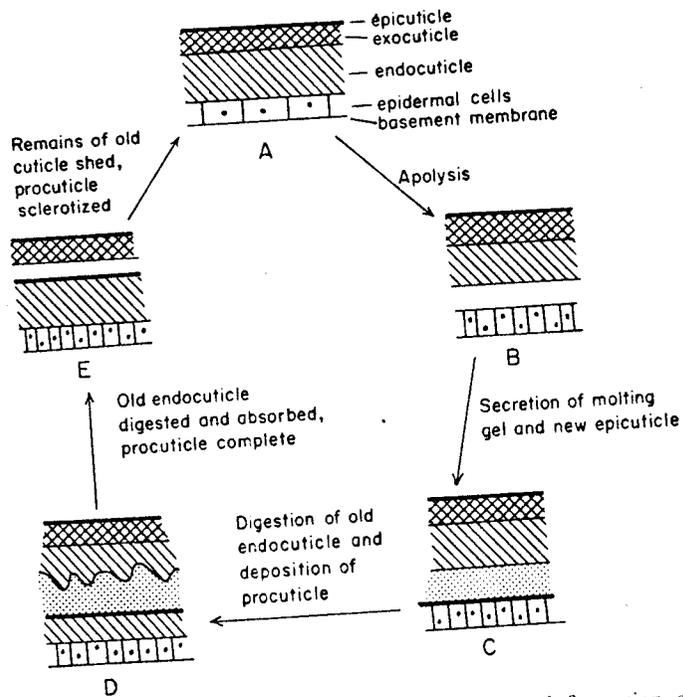


Fig. 2. Sequence of steps in the process of molting and formation of insect cuticle.

and secreted by the epidermal cells, and is replaced at each molt. The extracellular portion (cuticle) is composed of several layers, the innermost of which is the endocuticle, composed of chitin and protein. The middle layer is the exocuticle, which, in a mature integument, consists of a crosslinked protein matrix (sclerotin) that gives the cuticle its rigidity. Freshly molted insects possess a soft, white, pliable cuticle that is rapidly hardened in the process of sclerotization. In a series of poorly understood reactions, crosslinks are formed between the proteins of the exocuticle, using catechols derived from the amino acid tyrosine. The outermost layer of the cuticle is the epicuticle, which consists of a lipoprotein layer (cuticulin), a layer of wax esters and hydrocarbons, and the outermost cement layer. This outer lipid coat serves

to waterproof the insect—it is the major barrier against transcuticular water loss.

The molting cycle commences with a period of mitotic cell division in the epidermis after which the cells become closely packed and columnar. Following cell division the cuticle becomes detached from the epidermal cells (apolysis), producing a space (exuvial space) that is soon filled with a mixture of inactive proteolytic enzymes called the molting gel. A new cuticulin layer is produced outside the epidermal cells, and the molting enzymes are activated to produce the molting fluid, which proceeds to digest the old endocuticle. As the endocuticle is digested, the released metabolites are resorbed by the epidermal cells and used to synthesize and deposit a new undifferentiated procuticle inside the cuticulin layer. When the digestion is completed, only the old exocuticle and epicuticle remain and a new procuticle has been produced. Just prior to ecdysis, wax is added to the cuticulin layer. The remains of the old cuticle are then shed, and the new cuticle is expanded, returning the epidermal cells to their normal flat form. Finally, the expanded cuticle is sclerotized to produce the rigid exocuticle. Proteases and peptidases have roles in the digestion of the endocuticle, the activation of sclerotizing enzymes, and the mobilization of tyrosine for sclerotization.

2. Molting-Fluid Proteases

The molting gel that first fills the exuvial space after apolysis contains no proteinase or chitinase activity. However, after deposition of a new cuticulin layer, which protects the underlying epidermal cells, both proteolytic and chitinolytic activities appear as the molting gel is transformed into molting fluid (115–117). Katzenellenbogen and Kafatos (118) have examined the molting fluid of pharate adults of the silkworm, *Antheraea polyphemus*, and have identified two trypsin-like serine proteases. The enzymes were partially purified by sucrose-density-gradient centrifugation and DEAE-cellulose chromatography and have molecular weights of approximately 30,000 and 34,000, respectively. In addition to their proteinase activity, both enzymes readily hydrolyze the ester substrates BAEE and TAME, but hydrolyze BLME more slowly. However, the *N*-benzoyl methyl esters of alanine, glycine, histi-

dine, and valine and the *N*-acetyl esters of phenylalanine and tyrosine are not hydrolyzed by either enzyme. The optimum pH for ester hydrolysis is 7.8 for both proteases. DFP, benzamidine, SBTI, and high Hg^{2+} concentrations inhibit the enzymes but TLCK, EDTA, and iodoacetamide have no effect on the amino acid esterase activities. In addition to a DFP-sensitive proteinase with alkaline pH optimum (pH 7.7), Bade and Shoukimas (119) have reported an additional neutral (pH optimum = 7.0) metal-chelator-sensitive protease in the molting fluid of pharate adults of the sphingid moth, *Manduca sexta*. This enzyme is not sensitive to DFP or organic mercurials, but is inhibited by several metal chelators (8-hydroxyquinoline, 1,10-phenanthroline, EDTA) and requires calcium ions for activity. The authors also report that the chelator-sensitive activity is inhibited by ovomucoid and soybean trypsin inhibitors. However, it is difficult to assess the significance of these data, since the experiments were performed with unfractionated molting fluid. As yet no information is available concerning the mechanism of the activation process in which molting gel becomes molting fluid.

It appears that in addition to digesting the protein of the endocuticle, molting fluid proteases also activate a prochitinase already present in the endocuticle (120).

3. Prephenoloxidase Activator

Once the old endocuticle has been digested and the remains of the old cuticle shed, the newly formed procuticle is expanded and sclerotized. While the chemistry of the formation of crosslinks between proteins in the exocuticle remains uncertain, it is clear that the process of sclerotization consumes free tyrosine that originates from some pool in the hemolymph (121-125) or fat body (126) and involves the oxidation of at least some of that tyrosine by the enzyme phenoloxidase (o-diphenol: O_2 -oxidoreductase, EC 1.10.3.1). Phenoloxidase is present in the hemolymph (127) of the silkworm, *Bombyx mori*, and in other insects in the form of a proenzyme (prephenoloxidase) that can be activated by an endogeneous activator, by a protein present in the integument of the insect, or by α -chymotrypsin (128). Prephenoloxidase has been purified from the hemolymph of *B. mori* (129). The purified protein readily aggregates, but seems to have a monomer molecu-

lar weight of 36,000–40,000 and contains 0.15–0.16% copper in the cupric oxidation state. Prephenoxidase-activating enzyme (PPAE) has also been purified from the larval cuticle of *B. mori* (130) and has a molecular weight of 33,000–35,000. Since it had been shown that prephenoxidase could be activated by α -chymotrypsin, the purified activating enzyme was assayed for esterolytic activity. Purified PPAE readily hydrolyzes the tryptic substrates BAEE and TAME, but not the chymotryptic substrate BTEE. The esterase activity of PPAE is optimal from pH 7.8 to 10 and is inhibited by DFP, PMSF, and NPGB. The enzyme is not inhibited by sulfhydryl reagents (PCMB or *N*-ethylmaleimide) or by EDTA. A single peptide was released from the prephenoxidase during activation by the purified cuticular activating enzyme (131). It was suggested that the released peptide may inhibit further activation by PPAE. The authors believe that this is the first case discovered in which an inactive precursor of an oxidase or oxygenase is activated by a specific hydrolytic enzyme.

4. Tyrosine Mobilization

The sclerotization of the procuticle after a molt consumes large quantities of L-tyrosine (132,133), perhaps more than can be accumulated in solution in the hemolymph of the insect because of the low solubility of tyrosine. As a result, at least some insect genera have evolved alternative methods for accumulating tyrosine during larval feeding for later use. Several small molecules that sequester tyrosine or its equivalent have been described, including β -alanyl-L-tyrosine [(*Sarcophaga bullata*, (121)], tyrosine-*O*-phosphate [(*Drosophila melanogaster* (122)], γ -glutamyl-L-phenylalanine [(*Musca domestica* (123)], L-tyrosyl-*O*-acetyl-dopamine [(*Cel-erio euphorbiae* (124)], and protocatechuic acid-4-*O*- β -glucoside [(*Periplaneta americana* (134)]. The biosynthesis, accumulation, and utilization of β -alanyl-L-tyrosine during the development of the fleshfly, *Sarcophaga bullata*, have been extensively studied. The peptide is synthesized in the fat body from its component amino acids by a soluble ATP-dependent enzyme (135) and accumulates in the hemolymph during larval feeding (121). In the fully grown larva it is the predominant nonprotein, ninhydrin-positive material in the hemolymph, sequestering up to 90% of the nonprotein tyrosine in the insect (121,136). From the data of Levenbook et al.

(121), it can be estimated that the hemolymph of *S. bullata* prepupae contains at least 50 mM β -alanyl-L-tyrosine (135). At pupariation the concentration of the peptide drops precipitously as a result of the ecdysone-induced appearance of a specific dipeptidase (136-138). The liberated β -alanine and tyrosine are incorporated into the forming puparium (136,139,140). The peptidase is a soluble, fat-body enzyme with an alkaline pH optimum and a molecular weight of approximately 90,000 (138). A purification of 140-fold has been reported using a combination of ammonium sulfate fractionation, gel filtration, ion-exchange chromatography, and affinity chromatography on PCMB-Se-pharose. The partially purified enzyme was inhibited by several divalent cations, including Hg^{2+} , and was activated by EDTA and β -mercaptoethanol. From these data it was proposed that the peptidase contains an essential sulfhydryl in its active site. Among dipeptide substrates tested, only those with *N*-terminal β -alanine were cleaved. No ester or protein substrates were tested. Further studies of the mechanism of ecdysone stimulation of β -alanyl-L-tyrosine hydrolase production may contribute to our understanding of the mode of action of this developmentally important hormone.

V. Concluding Remarks

Even a casual perusal of this review will show that studies on insect proteases are, for the most part, fragmentary and incomplete. We hope that it will also show that the time is ripe for biochemical studies on a host of interesting biological problems that involve insect proteases and peptidases, such as hemolymph coagulation, phagocytosis, egg fertilization, tissue restructuring during development, and cellular differentiation. Perhaps what we do know will serve as an appetizer and entice the reader to partake of the main course.

Abbreviations

APNE
ATEE

N-Acetyl-L-phenylalanine naphthyl ester
N-Acetyl-L-tyrosine ethyl ester

BAEE	<i>N</i> -Benzoyl-L-arginine ethyl ester
BANA	<i>N</i> -Benzoyl-L-arginine naphthylamide
BAPA	<i>N</i> -Benzoyl-L-arginine <i>p</i> -nitroanilide
BLME	<i>N</i> - α -Benzoyl-L-lysine methyl ester
BTEE	<i>N</i> -Benzoyl-L-tyrosine ethyl ester
DEAE cellulose	Diethylaminoethyl cellulose
DFP	Diisopropylphosphorofluoridate
EDTA	Ethylenediaminetetraacetic acid
LBTI	Lima bean trypsin inhibitor
NPGB	<i>p</i> -Nitrophenyl- <i>p</i> '-guanidinobenzoate
PCMB	<i>p</i> -Chloromercuribenzoate
PMSF	Phenylmethane sulfonylfluoride
SBTI	Soybean trypsin inhibitor
SDS	Sodium dodecyl sulfate
TAME	<i>N</i> - α -Tosyl-L-arginine methyl ester
TLCK	1-Chloro-3-tosylamido-7-amino-2-heptanone
TPCK	1-Chloro-3-tosylamido-4-phenyl-2-butanone
ZPCK	1-Chloro-3-benzoyloxycarbonylamido-4-phenyl-2-butanone

References

1. Chauvin, R., *The World of an Insect*, World University Library, McGraw-Hill, New York, 1967.
2. Wigglesworth, V. B., *The Life of Insects*, New American Library, New York, 1968.
3. Reich, E., Rifkin, D. B., and Shaw, E., Eds., *Proteases and Biological Control*, Cold Springs Harbor Conference on Cell Proliferation, Vol. 2, Cold Spring Harbor Laboratory, Cold Spring Harbor, N. Y., 1975.
4. Wigglesworth, V. B., *Principles of Insect Physiology*, 7th ed., Chapman and Hall, London, 1972.
5. House, H. L., in Rockstein, M., Ed., *The Physiology of Insecta*, Vol. 5, 2nd ed., Academic Press, New York, 1974, p. 63.
6. Richards, A. G., *Acta Trop.*, 32, 83 (1975).
7. Ryan, C. A., *Ann. Rev. Plant Pathol.*, 24, 173 (1973).
8. Gooding, R. H., *Can. Entomol.*, 106, 39 (1974).
9. Gooding, R. H., *Comp. Biochem. Physiol.*, 43B, 815 (1972).
10. Gooding, R. H., Cheung, A. C., and Rolseth, B. M., *Can. Entomol.*, 105, 433 (1973).
11. Applebaum, S. W., Birk, Y., Harpez, I., and Bondi, A., *Comp. Biochem. Physiol.*, 11, 85 (1964).

12. Pfeleiderer, G., and Zwilling, R., *Biochem. Z.*, **344**, 127 (1966).
13. Zwilling, R., *Z. Physiol. Chem.*, **349**, 326 (1968).
14. Zwilling, R., Medugorah, I., and Mella, K., *Comp. Biochem. Physiol.*, **43B**, 419 (1972).
15. Lecadet, M.-M., and Dedonder, R., *C.R. Acad. Sci.*, **258**, 3117, 3380 (1964).
16. Lecadet, M.-M., and Dedonder, R., *Bull. Soc. Chim. Biol.*, **48**, 631 (1966).
17. Lecadet, M.-M., and Dedonder, R., *Bull. Soc. Chim. Biol.*, **48**, 660 (1966).
18. Giebel, W., Zwilling, R., and Pfeleiderer, G., *Comp. Biochem. Physiol.*, **38B**, 197 (1971).
19. Hagenmaier, H. C., *J. Insect Physiol.*, **17**, 1995 (1971).
20. Knecht, M., Hagenmaier, K. E., and Zebe, B., *J. Insect Physiol.*, **20**, 46 (1974).
21. Miller, J. W., Kramer, K. J., and Law, J. H., *Comp. Biochem. Physiol.*, **48B**, 117 (1974).
22. Ward, C. W., *Biochem. Biophys. Acta*, **391**, 201 (1975).
23. Gooding, R. H., and Huang, C.-T., *J. Insect Physiol.*, **15**, 3251 (1969).
24. Ward, C. W., *Aust. J. Biol. Sci.*, **28**, 1 (1975).
25. Ikan, R., Bergmann, E. O., Ishay, J., and Gitter, S., *Life Sciences*, **7**(2), 929 (1968).
26. Ishay, J., and Ikan, R., *Ecology*, **49**, 169 (1968).
27. Wilson, E. O., *The Insect Societies*, Harvard University Press, Cambridge, Mass. 1971.
28. Sonneborn, H.-H., Pfeleiderer, G., and Ishay, J., *Z. Physiol. Chem.*, **350**, 389 (1969).
29. Jany, K.-D., Pfeleiderer, G., and Molitoris, H. P., *Eur. J. Biochem.*, **42**, 419 (1974).
30. Jany, K.-D., Tabatabai, M. S., and Pfeleiderer, G., *FEBS Lett.*, **48**, 53 (1974).
31. Waterhouse, D. F., in *Advances in Pest Control Research*, Vol. 2, Metcalf, R. L., Ed., Interscience, New York, 1958.
32. Gilmour, D., *Biochemistry of Insects*, Academic Press, New York, 1961.
33. Linderstrøm-Lang, K., and Duspiva, F., *Nature*, **135**, 1039 (1935).
34. Linderstrøm-Lang, K., and Duspiva, F., *Compt. Rend. Trav. Lab. Carlsberg, Ser. Chim.*, **21**, 53 (1936).
35. Ward, C. W., *Biochim. Biophys. Acta*, **410**, 361 (1975).
36. Ward, C. W., *Aust. J. Biol. Sci.*, **28**, 447 (1975).
37. Ward, C. W., *Biochim. Biophys. Acta*, **384**, 215 (1975).
38. Ward, C. W., *Aust. J. Biol. Sci.*, **28**, 439 (1975).
39. Ward, C. W., *Biochim. Biophys. Acta*, **429**, 564 (1976).
40. Greenberg, B., and Paretsky, D., *Ann. Entomol. Soc. Am.*, **48**, 46 (1955).
41. Lambremont, E. N., Fisk, F. W., and Ashrafi, S., *Science*, **29**, 1484 (1959).
42. Fraser, A., Ring, R. A., and Steward, R. K., *Nature*, **192**, 999 (1961).
43. Sinha, M., *Appl. Entomol. Zool.*, **10**, 313 (1975).
44. Morihara, K., and Tsuzuki, H., *Biochim. Biophys. Acta*, **118**, 215 (1966).
45. Spiekman, A. M., Fredericks, K. K., Wagner, F. W., and Prescott, J. M., *Biochem. Biophys. Acta*, **293**, 464 (1973).
46. Langley, P. A., *J. Insect Physiol.*, **13**, 1921 (1967).
47. Thomsen, E., and Møller, I., *Nature*, **183**, 1401 (1959).

48. Thomsen, E., and Møller, I., *J. Exp. Biol.*, **40**, 301 (1963).
49. Engelmann, F., *J. Insect Physiol.*, **15**, 217 (1969).
50. Persaud, C. E., and Davey, K. G., *J. Insect Physiol.*, **17**, 1429 (1971).
51. Briegel, H., and Lea, A. O., *J. Insect Physiol.*, **21**, 1597 (1975).
52. Briegel, H., *J. Insect Physiol.*, **21**, 1681 (1975).
53. Gooding, R. H., *Can. Entomol.*, **105**, 599 (1973).
54. Fuchs, M. S., and Fong, W.-F., *J. Insect Physiol.*, **22**, 465 (1976).
55. Price, G. M., *J. Insect Physiol.*, **20**, 329 (1974).
56. Yang, Y. J., and Davies, D. M., *J. Insect Physiol.*, **17**, 2119 (1971).
57. Moser, J. G., *Biochem. Z.*, **344**, 337 (1966).
58. Martin, M. M., Gieselmann, M. J., and Martin, J. S., *J. Insect Physiol.*, **19**, 1409 (1973).
59. Boyd, N. D., and Martin, M. M., *Insect Biochem.*, **5**, 619 (1975).
60. Boyd, N. D., and Martin, M. M., *J. Insect Physiol.*, **21**, 1815 (1975).
61. Martin, M. M., Boyd, N. D., Gieselmann, M. J., and Martin, J. S., *J. Insect Physiol.*, **21**, 1887 (1975).
62. Fabre, J. H., *The Insect World*, E. W. Teale, Ed., trans. by A. T. de Mattos, Dodd, Mead and Co., New York, 1966.
63. Trouvelot, L., *Am. Naturalist*, **1**, 30 (1863).
64. Lutter, O. H., *Trans. Entomol. Soc. London*, 1895, 399 (1895).
65. Honda, M., *Zent. Bakteriolog. Abt.*, **2**, 67, 365 (1926).
66. Duspiva, F., *Z. Naturforsch.*, **5**, 273 (1950).
67. Kafatos, F. C., and Williams, C. M., *Science* **146**, 538 (1964).
68. Felsted, R. L., Law, J. H., Sinha, A. K., and Jolly, M. S., *Comp. Biochem. Physiol.*, **44B**, 595 (1973).
69. Hruska, J. F., Felsted, R. L., and Law, J. H., *Insect Biochem.*, **3**, 31 (1973).
70. Kafatos, F. C., in *Problems in Biology: RNA in Development*, E. W. Hanly, Ed., Univ. of Utah Press, Salt Lake City, 1970, p. 111.
71. Kafatos, F. C., *Cur. Top. Dev. Biol.*, **7**, 125 (1972).
72. Berger, E., and Kafatos, F. C., *Dev. Biol.*, **25**, 377 (1971).
73. Jamieson, J. D., and Palade, G. E., *J. Cell Biol.*, **34**, 577 (1967).
74. Jamieson, J. D., and Palade, G. E., *J. Cell Biol.*, **51**, 135 (1971).
75. Kafatos, F. C., and Reich, J., *Proc. Nat. Acad. Sci. U.S.A.*, **60**, 1458 (1968).
76. Berger, E., Kafatos, F. C., Felsted, R. L., and Law, J. H., *J. Biol. Chem.*, **246**, 4131 (1971).
77. Felsted, R. L., Kramer, K. J., Law, J. H., Berger, E., and Kafatos, F. C., *J. Biol. Chem.*, **248**, 3012 (1973).
78. Kafatos, F. C., Tartakoff, A. M., and Law, J. H., *J. Biol. Chem.*, **242**, 1477 (1967).
79. Hruska, J. F., and Law, J. H., *Methods Enzymol.*, **19**, 221 (1970).
80. Hruska, J. F., Law, J. H., and Kézdy, F. J., *Biochem. Biophys. Res. Commun.*, **36**, 272 (1969).
81. Kafatos, F. C., *J. Exp. Biol.*, **48**, 435 (1968).
82. Eguchi, M., Furukawa, S., and Iwamoto, A., *J. Insect Physiol.*, **18**, 2457 (1972).
83. Eguchi, M., and Iwamoto, A., *J. Insect Physiol.*, **21**, 577 (1975).
84. Lucas, R., Shaw, J. T. B., and Smith, S. G., *Adv. Protein Chem.*, **13**, 108 (1958).

85. Tokutake, S., and Okuyama, T., *J. Biochem. (Tokyo)*, **71**, 737 (1972).
86. Iijima, T., *J. Insect Physiol.*, **18**, 2055 (1972).
87. Kramer, K. J., unpublished observation.
88. Lounibos, L. P., *Ann. Entomol. Soc. Am.*, **69**, 567 (1976).
89. Kramer, K. J., Felsted, R. L., and Law, J. H., *J. Biol. Chem.*, **248**, 3021 (1973).
90. Maroux, S., Baratti, J., and Desnuelle, P., *J. Biol. Chem.*, **246**, 5031 (1971).
91. Kafatos, F. C., Law, J. H., and Tartakoff, A. M., *J. Biol. Chem.*, **242**, 1488 (1967).
92. Hixson, H. F., Jr., and Laskowski, M., Jr., *Biochem.*, **9**, 106 (1969).
93. Berger, E., and Kafatos, F. C., *Immunochemistry*, **8**, 391 (1971).
94. Keil, B., in *The Enzymes*, P. Boyer, Ed., Vol. 3, Academic Press, New York, 1971, pp. 249.
95. Lazdunski, M., Delange, M., Abita, J. P., and Vincent, J. P., in *Structure-Function Relationships of Proteolytic Enzymes*, P. Desnuelle, H. Neurath, and M. Ottesen, Eds. Academic Press, New York, 1970, p. 42.
96. Stroud, R. D., Kay, L. M., and Dickerson, R. C., *Cold Spring Harbor Symp. Quant. Biol.*, **36**, 125 (1971).
97. Eyl, A. W., Jr., and Inagami, T., *J. Biol. Chem.*, **246**, 738 (1971).
98. de Haen, C., Neurath, H., and Teller, D. C., *J. Mol. Biol.*, **92**, 225 (1975).
99. Hermodson, M. A., Tye, R. W., Reeck, G. R., Neurath, H., and Walsh, K. A., *FEBS Lett.*, **14**, 222 (1971).
100. Davhoff, M. O., *Atlas of Protein Sequence and Structure*, Vol. 5, National Biomedical Research Foundation, Silver Spring, Md., 1972.
101. Camocho, Z., Brown, J. R., and Kitto, G. B., *Comp. Biochem. Physiol.*, **54B**, 27 (1976).
102. Gates, B. J., and Travis, J., *Biochim. Biophys. Acta*, **310**, 137 (1973).
103. Lockshin, R. A., "Lysosomes in Insects" in *Lysosomes in Biology and Pathology*, Dingle, J. T. and Fell, H. R., Eds. Vol. 1, North Holland, Amsterdam, 1969, p. 363.
104. Lockshin, R. A., and Beaulaton, J., *Life Sci.*, **15**, 1549 (1974).
105. Kuk-Meiri, S., Lichtenstein, N., Shulov, A., and Pener, M. P., *Comp. Biochem. Physiol.*, **18**, 783 (1966).
106. Lockshin, R. A., and Williams, C. M., *J. Insect Physiol.*, **11**, 123 (1965).
107. Schin, K. S., and Cleaver, U., *Z. Zellforsch.*, **86**, 262 (1968).
108. Rodems, A. E., Henrikson, P. A., Clever, U., *Experientia*, **25**, 686 (1969).
109. Henrikson, P. A., and Clever, U., *J. Insect Physiol.*, **18**, 1981 (1972).
110. Lockshin, R. A., and Williams, C. M., *J. Insect Physiol.*, **10**, 643 (1964).
111. Lockshin, R. A., and Williams, C. M., *J. Insect Physiol.*, **11**, 601 (1965).
112. Lockshin, R. A., and Williams, C. M., *J. Insect Physiol.*, **11**, 123 (1965).
113. Lockshin, R. A., and Williams, C. M., *J. Insect Physiol.*, **11**, 831 (1965).
114. Lockshin, R. A., *J. Insect Physiol.*, **15**, 1505 (1969).
115. Passonneau, J. V., and Williams, C. M., *J. Exp. Biol.*, **30**, 545 (1953).
116. Katzenellenbogen, B. S., and Kafatos, F. C., *J. Insect Physiol.*, **16**, 2241 (1970).
117. Bade, M. L., *Federation Proc.*, **34**, 705 (1975).
118. Katzenellenbogen, B. S., and Kafatos, F. C., *J. Insect Physiol.*, **17**, 775 (1971).
119. Bade, M. L., and Shoukimas, J. J., *J. Insect Physiol.*, **20**, 281 (1974).

120. Bade, M. L., Abstracts, 10th International Congress of Biochemistry, Hamburg, W. Germany, 1976, p. 644.
121. Levenbook, L., Bodnaryk, R. P., Spande, T. F., *Biochem. J.*, **113**, 837 (1969).
122. Mitchell, H. K., and Lunan, K. D., *Arch. Biochem. Biophys.*, **106**, 219 (1964).
123. Bodnaryk, R. P., *J. Insect Physiol.*, **16**, 909 (1970).
124. Sienkiewicz, F., and Piechowska, M. J., *Bull. Acad. Polon. Sci. Ser. Sci. Biol.*, **21**, 797 (1973).
125. Levenbook, L., and Dinamarca, M. L., *J. Insect Physiol.*, **12**, 1343 (1966).
126. Price, G. M., *Insect Biochem.*, **2**, 175 (1972).
127. Ashida, M., and Ohnishi, E., *Arch. Biochem. Biophys.*, **122**, 411 (1967).
128. Ohnishi, E., Dohke, K., Ashida, M., *Arch. Biochem. Biophys.*, **139**, 143 (1970).
129. Ashida, M., *Arch. Biochem. Biophys.*, **144**, 749 (1971).
130. Dohke, K., *Arch. Biochem. Biophys.*, **157**, 210 (1973).
131. Ashida, M., Dohke, K., and Ohnishi, E., *Biochem. Biophys. Res. Commun.*, **57**, 1089 (1974).
132. Karlson, P., and Sekeris, C. E., *Comp. Biochem.*, **6**, 221 (1964).
133. Hackman, R. H., in M. Rockstein, Ed., *The Physiology of Insecta*, 2nd ed., Academic Press, New York, 1964.
134. Brunet, P. C. J., and Kent, P. W., *Proc. Roy. Soc. (London) Ser. B*, **144**, 259 (1955).
135. Dunn, P. E., Ph.D. Thesis, Purdue University (1973).
136. Bodnaryk, R. P., and Levenbook, L., *Comp. Biochem. Physiol.*, **30**, 909 (1969).
137. Bodnaryk, R. P., *Gen. Comp. Endocrinol.*, **16**, 363 (1971).
138. Fader, R. G., Ph.D. Thesis, Purdue University (1973).
139. Bodnaryk, R. P., *Comp. Biochem. Physiol.*, **35**, 221 (1970).
140. Bodnaryk, R. P., *J. Insect Physiol.*, **17**, 1201 (1971).

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