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Insecticides Based on Differences in Metabolic Pathways

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I. INTRODUCTION

Insects have been major pests of humankind at least since the beginning of recorded history. To this day insects continue to cause problems in domestic, agricultural, and health situations. It is no wonder that people have continually sought new solutions to controlling insect pests. Even when new control methods are discovered and established, insects evolve into resistant species so that the method is only of real value for a few brief years. Modern science and technology are now enabling scientists to tear away the fabric that has so long masked physiological and biochemical events critical to insects. Armed with this new knowledge, researchers should be able to develop novel control strategies that focus on key physiological, biological, and biochemical events such that they can be altered, influenced, disrupted, and/or inhibited. Three promising areas that may lead or are currently leading to new insect control methods are the cuticle, prostaglandins, and steroids. We discuss each of these areas in regard to their biological significance, current research, metabolic inhibitors and their modes of action.

II. CHITIN SYNTHESIS INHIBITORS

One of the major observable differences between arthropods and vertebrates is that arthropods possess an exoskeleton (cuticle). The insect cuticle serves as a first-line defense against predators, pathogens, dehydration, etc. Cuticle is also important in locomotion as it serves as a site for muscle attachment. Cuticle is a composite

structure consisting of chitin, protein, lipids, waxes, and pigments that are secreted by the underlying epithelial cells [1]. Because of this apparent difference between insects and mammals, the cuticle present itself as a prime target for controlling insect pests. Ebeling [2] has referred to the cuticle and its underlying epidermis (together termed the integument) as "a vulnerable organ system."

Physical agents such as dusts and clays were the first insecticides that were used to specifically attack the cuticle [3]. Initially, abrasion of the cuticular lipid layer was thought to be necessary to cause water loss from and the subsequent death of the insect [4,5]. This thinking persisted for about 30 years, until it was conclusively demonstrated that sorptive dusts had greater insecticidal activity than abrasive dusts [6,7]. Although insecticidal dusts are quite effective, their use is restricted to stored products or out-of-the-way places, such as storage rooms, attics, etc., because they have a tendency to float in the air and create films on floors and furnishings.

Chemicals are also quite effective in altering the insect's existing cuticle or affecting the deposition of cuticle. Besides the obvious use of oil, solvent, and surfactant sprays to dissolve or disturb the wax layer orientation [5,8], chemicals can affect epidermal cells such that the systems producing the cuticle are adversely influenced or inhibited. Most of the latter chemicals fall under the term "insect growth regulators" (IGRs) because the cuticle is directly associated with the growth and development of the insect. Of particular interest are the IGRs called chitin synthesis inhibitors (CSIs). CSIs represent several classes of compounds that variously affect the deposition of chitin in the cuticle. Although all of the CSIs being commercially explored inhibit deposition of chitin in the cuticle, the exact mode of action for any one of these compounds has not been established. Because several different theories have been suggested for the mode of action, it is necessary to describe the biochemical and physiological events associated with chitin metabolism in insects.

A. Chitin Biosynthesis

Chitin is a long-chain carbohydrate polymer that may reach molecular weights of 400,000 or more. The chitin biopolymer is comprised of about 90% repeating units of N-acetyl-D-glucosamine (GlcNAc) and 10% D-glucosamine (GlcN) interspersed in the chain [9]. The carbohydrate units are in β-1,4-linkages in the polymer. In nature, chitin occurs in yeast, fungi, molluscs, protozoans, and most protostomian vertebrates [3,9–12]. Crystallographic analysis of chitins isolated from various sources reveal that there are three different types, that is, α, β, and γ. All three types are found in insects, with α-chitin being the most predominant [9,10].

Candy and Kilby [13] proposed a metabolic pathway for the synthesis of chitin (Fig. 1) based on their work with homogenates from

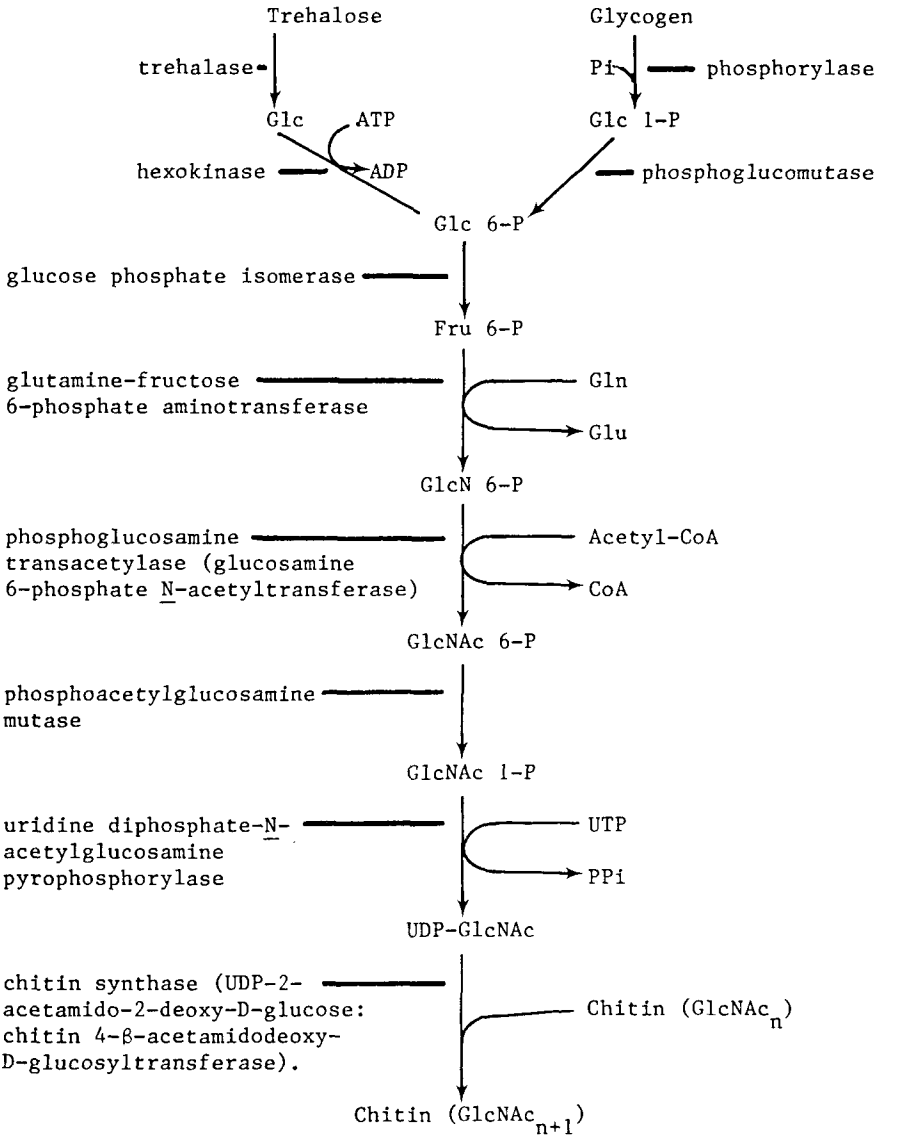

Fig. 1 Chitin biosynthetic pathway.

in vivo studies with the desert locust, Schistocerca gregaria Forskal. All of the enzymes leading to the synthesis of UDP-GlcNAc, an obligate substrate of chitin synthase* (UDP-2-acetamido-2-deoxy-D-glucose: chitin 4-β-acetamidodeoxy-D-glucosyl-transferase; EC 2.4.1.16) [14], were demonstrated in in vitro experiments. Attempts at incorporation of [^{14}C] UDP-GlcNAc into chitin with in vitro conditions failed, which may have been indicative of the sensitive nature of the chitin synthase from this insect. Incorporation of labeled precursors into chitin of the desert locust could be achieved only in vivo.

Jaworski et al. [15] and Porter and Jaworski [16] were able to achieve chitin synthesis in vitro utilizing UDP-GlcNAc and mitochondrial and microsomal fractions obtained from homogenates of Spodoptera (Prodenia) eradania in various developmental stages. Chitin synthase activity was clearly shown to be associated with particulate fractions [16]. Although activity was found in mitochondrial and microsomal fractions, the highest yield was isolated in the cellular debris, which indicated inefficient homogenization techniques. Chitinase and acid digestions of the product followed by carbohydrate analysis of the digests by paper chromatography indicated that the product was chitin.

Further reports on insect cell-free chitin synthase systems subsided for more than a decade, perhaps because of reports that indicated low chitin synthase activity in tissues [17] and enzyme stability problems [18,19]. In vitro organ culture and tissue culture systems were developed and provided information on chitin synthesis in insects [3,11,17,18,20-26]. All of these chitin synthesis systems required activation by prior exposure of the insect or tissues to ecdysone or 20-hydroxyecdysone before incorporation of radio-labeled carbohydrates into chitin could be observed.

Most of what is known about the biochemistry of chitin synthase during this period came from in vitro cell-free studies with yeast and fungi. This information has had an effect on shaping some of the theories on the modes of action of CSIs.

The location of chitin synthase activity in vitro varies depending on the organisms and techniques used to isolate the enzyme. Some chitin synthase activity is found in all subcellular fractions; however, there are a number of reports that suggest the enzyme is attached to plasma membranes or plasma membrane-derived fractions [9,20,27-30]. In other instances, chitin synthase may be contained in discrete cytoplasmic containers called "chitosomes" [31].

The yeast and fungal chitin synthases have many common properties besides being membrane-bound and requiring UDP-GlcNAc. The enzymes exist in a zymogenic form and must be treated with proteases

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*Synthase is used as recommended by the International Union of Biochemistry, Enzyme Nomenclature, Academic Press, New York, 1979.

before becoming active [32,33]. A primer molecule in the form of a small oligosaccharide or chitodextrin may be necessary to initiate the chain elongation reaction [22,32,34]. The presence of GlcNAc in the reaction mixture (approximately 20–25 mM) stimulates activity in many cases [35], and divalent cations, for example, Mg^{++}, are necessary for activity [9]. Additions of phospholipids and glycerin may also enhance incorporation of GlcNAc into the chitin polymer [34,35]. UDP and UMP, byproducts of the reaction, and nucleoside antibiotics (e.g., polyoxins and nikkomycin) competitively inhibit the chitin synthase reaction [30,32,35]. The Km for UDP-GlcNAc is usually in the range of 1–5 mM [9,20,29,30,35].

Beginning in 1980, reports on in vitro cell-free chitin synthase systems from insects began to appear. Thus far, four cell-free chitin synthase systems have been reported that include preparations from Stomoxys calcitrans [36], Trichoplusia ni [37], Hyalophora cecropia [37], and Tribolium castaneum [38]. The first three preparations are considered to be integumental in origin, whereas the latter is from gut and is involved with the synthesis of peritrophic membranes. Chitin synthases from insect tissues appear to be more diverse in their characteristics than those of yeast and fungi. The insect enzymes are membrane-bound because enzyme activity is associated with the mitochondrial and microsomal pellets [36–38]. Whether or not the insect chitin synthases exist as proenzymes or zymogens has not been conclusively demonstrated. Trypsin pretreatment of chitin synthase from S. calcitrans and T. castaneum increased activity 20–40% [36,38]. Divalent cations were required for activity in T. ni, T. castaneum and H. cecropia, but not for S. calcitrans. Monosaccharides (i.e., GlcNAc) increase chitin synthase activity when present in reaction mixtures from T. castaneum [38] and T. ni [37], but not from S. calcitrans [36] and H. cecropia [37].

Kinetic data are available only for S. Calcitrans preparations; the apparent Km and V_{max} for UDP-GlcNAc were, respectively, 3.7 ± 10.3 pmol GlcNAc incorporated $hr^{-1} \cdot mg^{-1}$ protein [36]. The specific activity for T. castaneum gut chitin synthase was reported as 11 pmol GlcNAc incorporated $min^{-1} \cdot mg^{-1}$ protein [38]. Polyoxin D inhibited gut chitin synthase preparations from T. castaneum [39] by almost three orders of magnitude greater (I_{50} = μM) than enzyme preparations from S. calcitrans (I_{50} = 1 mM) [36]. Uridine nucleotides have been reported as inhibitors for chitin synthase preparations from both T. castaneum [39] and S. calcitrans [36].

B. Chitin Degradation

Periodically, developing insects must molt and construct a larger exoskeleton to accommodate the insect during its next growth stage. Degradation of the insect endocuticle is a necessary, orchestrated, biological event. The degradative process is necessary, first because

the old cuticle must be weakened enough so that it can be ruptured along ecdysial lines and the insect can exit and, second, because many of the degradative products are recycled and utilized in the synthesis and deposition of the new cuticle. During the pupal instar of S. calcitrans [40], degradation of the larval endocuticel is accomplished by a molting fluid (contains proteases and chitinolytic enzymes) that is secreted into the space between the epithelial cells and the endocuticle after apolysis. A prepupal cuticle and an ecdysial membrane are formed during apolysis, which may act as a barrier to the molting fluid. As the molting fluid gradually digests the old endocuticle, the products are reabsorbed and incorporated into the imaginal cuticle that will be deposited by the newly formed imaginal epidermal cells during the fourth day of the pupal instar.

There is more than one protease present in molting fluids; Katzenellenbogen and Kafatos [41] isolated two similar proteases from Antherea polyphemus. Proteolytic activity was trypsinlike and the enzymes were inhibited by soybean trypsin inhibitor. Differences exist in molting fluid proteases isolated from different insects. For example, Bade and Shoukimas [42] isolated a trypsinlike protease and neutral protease from Manduca sexta that required metal ions for activity.

The molting fluid proteases may be necessary for the chitinolytic system to operate. Bade and Stinson [43] have reported that in M. sexta chitinases will not degrade intact cuticle, that is, cuticle that has not had the protein removed from it. Removal of the protein by proteolytic treatment (either molting fluid or trypsin) allows chitinase to hydrolyze the chitin. Degradation of chitin to monosaccharides is performed by a chitinolytic system that contains two enzymes (Fig. 2). Chitinase [poly-β-1,4-(2-acetamido-2-deoxy)-D-glucoside glycano-hydrolase, EC 3.2.1.14] hydrolyzes the chitin polymer to the dissacharide, N, N'-diacetylchitobiose. In turn, the disaccharide is hydrolyzed to monosaccharide units by β-N-acetylglucosaminidase (EC 3.2.1.30) or chitobiase. In insects, both enzymes appear to be soluble enzymes and therefore somewhat easier to work

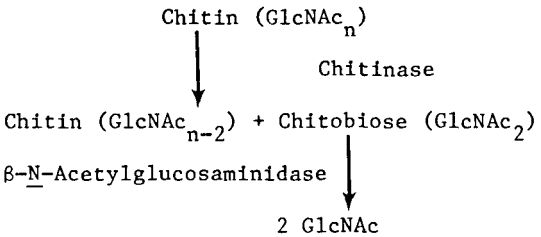


Fig. 2 Enzymatic degradation of chitin.

with. Both enzymes have been purified and characterized from several insect sources [3,11,12,44-52]. With the exception of the intestinal enzymes, many of the enzymes have peak activities at or about the time of the molt that coincides with the appearance of chitin synthase.

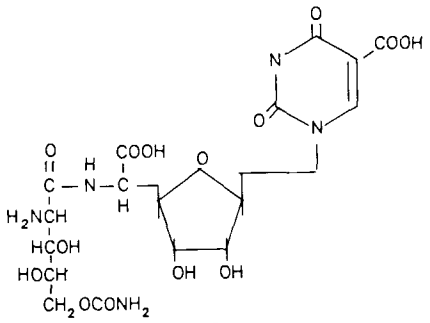
Insects appear to have several chitinases (endo- and exochitinases) involved with chitin hydrolysis. The chitinases operate at the same time and work in concert with β-N-acetylglucosaminidases, forming a binary system to degrade the chitin polymer as much as six times faster than the sum of the individual enzymes [51]. The chitinases may exist as proenzymes [41] that are activated by proteolytic action in the molting fluid or elsewhere. Not all of the chitinases exist initially as proenzymes [45,49]; however, it is difficult to prove that proteolytic degradation did not occur before or during homogenization and preparation of the enzyme. The pH optimum for maximal chitinolytic activity is usually in the acid range. Molecular weights vary from approximately 20,000-150,000 daltons [11,12,53]. Kinetic data are difficult to compare because of the different calculation methods. For the chitinase isolated from S. calcitrans [45] Michealis-Menten constants (Km) and the V_{max} were, respectively, 33 mM and 1.21 μmol · min^{-1} · mg^{-1} protein using acetylated chitosan as the substrate. Insect chitinases generally do not have cation requirements and are inhibited by 1-10 mM Hg^{2+} [44,45].

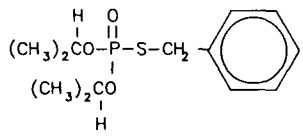
β-N-Acetylglucosaminidases have been isolated from several insect species including Locusta migratoria [50], S. calcitrans [54], B. mori [55,56], M. sexta [46], and Drosophila [57]. There is a great deal of variability between the β-N-Acetylglucosaminidases. The enzymes are usually soluble and can be found distributed in different tissues such as hemolymph, integument, gut, etc. The molecular weights range from about 50,000-150,000 daltons [11,12]. The pH optima for enzymatic activity are on the acid side, which is to be expected since the β-N-acetylglucosaminidases work in concert with chitinases. It is not unusual for the enzyme to exhibit substrate inhibition [50, 57,58]. Kinetic data are available for a number of different substrates [11,12].

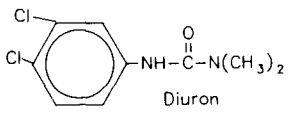
Mazzone [59] has suggested an interesting approach to controlling insects by exploiting the chitinase gene. Using genetic engineering techniques, viral and bacterial control agents would be produced that would permanently contain the chitinase gene within their genomes. Besides their usual infectivity then, these biological agents would have chitinolytic properties that would make them more effective.

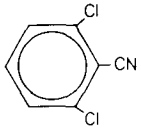
C. Chitin Synthesis Inhibition

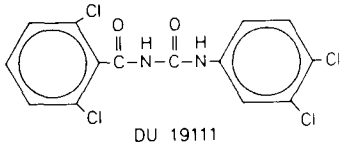
In reviewing the literature, one finds that there are many different chemicals that inhibit the synthesis of chitin in vivo and in vitro, and in cell-free preparations of the chitin synthase. Figures 3a and b show the structures and names of representative chemicals that

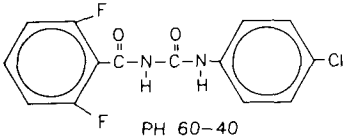


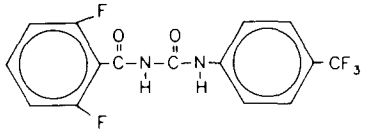


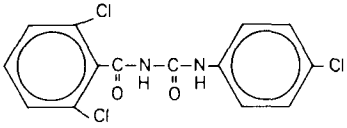












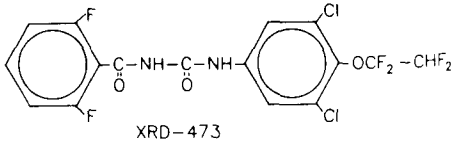
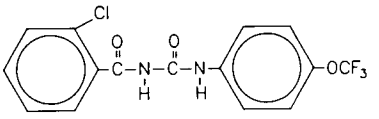


Fig. 3(a) Natural and synthetic chitin synthesis inhibitors.



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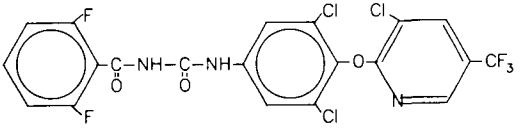
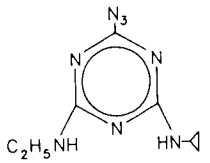
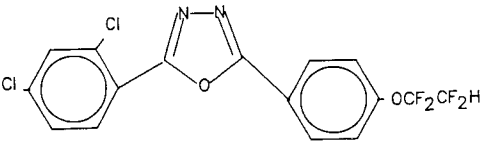


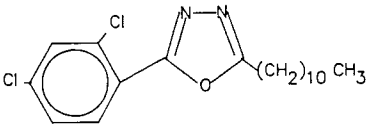
Chlorfluazuron
IKI-7899/CGA112913



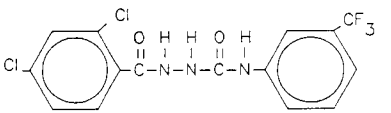
CGA-19255



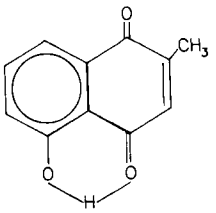
2-(2,4-Dichlorophenyl)-5-
(4-tetrafluoroethoxyphenyl)-1,3,4-oxadiazole



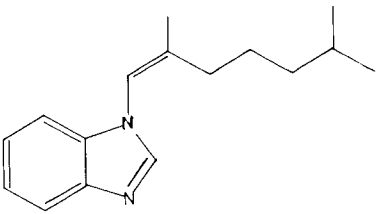
2-(2,4-Ddichlorophenyl)-5-
(n-undecyl) 1,3,4-oxadiazole



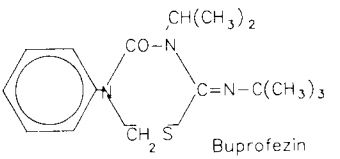
4-(2,4-Dichlorobenzoyl)-1-
(3-trifuloromethylphenyl)-semicarbazide



Plumbagin



1-Nerylbenzimidazole



Buprofezin
Applaud

Fig. 3(b) Natural and synthetic chitin synthesis inhibitors.

interfere with chitin synthesis. More extensive lists are available [3,11,12,20,60–62]. Polyoxin D and Kitazin P are used extensively as fungicides in Japan and other parts fo the Orient and were the first compounds to be categorized as chitin synthesis inhibitors [63–67]. The polyoxins have been shown to strongly inhibit chitin synthesis in cell-free systems of Neurospora crassa [62] and Piricularia oryzae [63] by competing with UDP-GlcNAc for active sites on the chitin synthase. The polyoxins are also inhibitors of chitin synthesis in insect tissues [3,11,12,18,20,22–25,36,61,68–71]; however, it has been demonstrated that polyoxins are competitive inhibitors of the insect enzyme system. The inhibitory effects of polyoxin D vary widely in cell-free insect preparations, ranging from little inhibition (300 μM = 14% inhibition) with N. cecropin (37) to great inhibition (4–14 μM = 50% inhibition) with T. castaneum.

The variation in the effectiveness of polyoxin D inhibition for insect chitin synthase systems may be directly related to the Km for UDP-GlcNAc. The Km for UDP-GlcNAc for fungal systems is in the mM range, and the Ki's for polyoxins range from approximately 3–30 μM [64]; polyoxins can then compete quite effectively with UDP-GlcNAc. The chitin synthase cell-free system from S. calcitrans exhibits a Km of about 30 μM for UDP-GlcNAc [36], meaning that the polyoxins would not be as good inhibitors here as they are for the fungal system; 1 mM polyoxin D resulted in about 40% inhibition with S. calcitrans.

Other antibiotic materials besides polyoxins have been reported to be competitive inhibitors of yeast and fungal chitin synthases with respect to UDP-GlcNAc, including nikkomycin [72] and tunicamycin [73]. In insect tissue, the extent of chitin synthases inhibition by nikkomycin was dependent on the enzyme source. For example, with integumental enzyme from H. cecropia, 50% inhibition was achieved with 1 mM nikkomycin [37], whereas with the T. ni integumental enzyme [37] or T. castaneum gut enzyme [39], 50% inhibition was observed at 6 and 23 nM nikkomycin, respectively.

Contradictory reports exist for tunicamycin [39,74,75]. It is possible that the reported inhibition of insect chitin synthesis in Triatoma infestans [75] by tunicamycin may be the result of the antibiotic blocking the production of lipid-linked oligosaccharides [76] that can act as primer molecules for chitin synthases.

Kitazin P (Fig. 3a) inhibits chitin synthesis in fungi [20,65] and in cockroach leg regenerate organ cultures [23]. The chitin synthesis inhibition in fungi resulted from UDP-GlcNAc not being available to chitin synthase; that is, Kitazin P affected membrane permeability [65–67]. Chitin synthesis inhibition was not the primary mode of action of Kitazin P, but merely the most readily observable result. Kitazin P is not an inhibitor in cell-free preparations of insect chitin synthase [39], presumably because the substrate is freely available to the enzyme and no longer transports across a cell membrane.

In the early 1970s a new benzoylphenyl urea insecticide, DU 19111 [1-(2,6-dichlorobenzoyl)-3-(3,4-dichlorophenyl)urea], was reported [77]. The structure of Du 19111 was derived from the structures of the herbicides diuron and dichlobenil (Fig. 3a). It is interesting to note that the mode of action of dichlobenil is the inhibition of glucose incorporation into cellulose components of plant cell walls [78]. Diuron is known to inhibit electron transfer occurring during photosynthesis [77]. Numerous other benzoylphenyl urea homologs and analogs, as well as unrelated structures, were subsequently synthesized and tested [3,11,60–62,78–86]. There are almost as many proposed modes of action for the benzoylphenyl ureas as there are reported homologs and analogs. (A few representative structures are shown in Figs. 3a and b.)

Mulder and Gijswijt [82] and Post el at. [83], working with Du 19111 and diflubenzuron (PH 60-40), demonstrated that the benzoylphenyl ureas interfered with cuticle deposition. Post et al. [83] performed experiments in which D-[^{3}H]-glucose was injected into either normal or Du 19111-treated larvae of Pieris brassicae. Their results showed that glucose was not incorporated into endocuticular chitin in the Du 19111-treated insects. Further experiments were conducted to determine if UDP-GlcNAc levels were influenced by Du 19111 treatments. Low levels of UDP-GlcNAc would indicate that Du 19111 treatments disrupted the metabolic pathways leading to UDP-GlcNAc, whereas high levels would indicate that the polycondensation step in chitin synthesis was blocked, causing accumulation of UDP-GlcNAc. No differences in UDP-GlcNAc levels were observed between normal and treated P. brassicae larvae; however, Du 19111-treated larvae had elevated levels of GlcNAc. To explain this observation, Post et al. [83] suggested that Du 19111 inhibits the polycondensing enzyme (chitin synthase) in such a way that the enzyme can accept UDP-GlcNAc, but in the process of adding GlcNAc to the chitin polymer, GlcNAc is dropped or lost.

Deul et al. [84] found that diflubenzuron increased GlcNAc levels in P. brassicae larvae with virtually complete inhibition of chitin synthesis occurring within 15 min of diflubenzuron injection into the larvae. In another series of experiments, Gijswijt et al. [85] ran parallel experiments with polyoxin D and diflubenzuron to compare the effects of these chemicals on chitin deposition in P. brassicae larvae. Histological examinations, gravimetric measurements, and determinations of the amounts of [^{14}C] glucose incorporated into cuticle were made; no differences were observed between polyoxin D and diflubenzuron treatments.

Thus, it would appear that the benzoylphenyl ureas are CSIs and that they interact with chitin synthase in such a way to prevent and/or inhibit chitin synthesis. Many reports have been published, documenting the fact that the benzoylphenyl urea CSIs do inhibit insect cuticle deposition in vivo and in vitro for a wide variety of

insects [3,11,12,17,20,22-26,60-62,68-70,86-90]. Whether or not the benzoylphenyl ureas actually interact with chitin synthase and whether or not chitin synthesis is the primary mode of action are matters of discussion [91-93].

Immediately following the report of Post et al. [83] that DU 19111 interferes with chitin synthase, Ishaaya and Casida [94] presented data that indicated diflubenzuron elevated housefly larval cuticle chitinase and phenol oxidase levels to approximately 180 and 155%, respectively, when the larvae had 1 ppm diflubenzuron in the diet. Chitinase levels of 240% of the control levels were attained when dietary levels of diflubenzuron were increased to 2.5 ppm. Such elevated enzyme levels could explain the reduced amounts of chitin deposited in the cuticle and the weakened cuticle structure of insects exposed to diflubenzuron. Ishaaya and Casida [94] also recognized that diflubenzuron may be affecting other physiological systems, for example, hormone systems, that activate the chitinase and phenol oxidase.

Deul et al. [84] questioned the possibility that defects in the cuticle caused by exposure to diflubenzuron were due to activation of chitinase. No effect on chitinase activity could be demonstrated in fifth instar P. brassicae larvae-fed cabbage leaves treated with either diflubenzuron or Du 19111. Moreover, almost a complete inhibition of chitin synthesis (measured by the amount of [^{14}C] glucose incorporated into chitin) was observed in the absence of appreciable chitinase activity. Chen et al. [45] found that diflubenzuron had no effect on the activity of chitinase purified from S. calcitrans. Therefore, it would seem that activation of chitin degradation mechanisms can be ruled out as possible modes of action for benzoylphenyl ureas. Different modes of action may exist for different benzoylphenyl ureas. Duel et al. [84] concluded that diflubenzuron inhibits the polymerization of chitin but with a different action than that of Du 19111, because GlcNAc did not accumulate in treated P. brassicae larvae.

Several other investigators have reported on the levels of UDP-GlcNAc and GlcNAc in relation to diflubenzuron treatments. Marks and Sowa [23] reported that the presence of either polyoxin D or diflubenzuron in cultured cockroach leg regenerates results in a buildup of UDP-GlcNAc. Hajjar and Casida [61] showed a buildup of UDP-GlcNAc in isolated abdomens of Oncopeltus fasiatus when treated with diflubenzuron, whereas van Eck [24] showed the same result with diflubenzuron and Du 19111 in cultured integumentary tissues from third instar larvae of Musca domestica. All of these investigators recognized the difficulty in interpreting the results of CSIs on the chitin synthesis system when tissue culture or in vivo techniques are used. This point was aptly made by van Eck [24], who stated that "Final proof that chitin synthetase in insects is the target for benzoylphenyl ureas can only be given when chitin synthetase can be isolated from insect tissue and studied in vitro."

The first cell-free insect chitin synthesis assays were reported in 1980. Mayer et al. [36] used whole pupae from S. calcitrans as the tissue source for their cell-free system. The pupae were obtained at the time imaginal cuticle was being deposited during the pupal instar and, therefore, the chitin synthase was considered to be integumentary in origin. Cohen and Casida [38] described a cell-free chitin synthase from gut tissue of T. castaneum that was considered to be associated with the synthesis of the peritrophic membrane. Both systems failed to be inhibited by a variety of benzoylphenyl urea and triazine (including CGA-19255) chitin synthesis inhibitors (Fig. 3b) [36,39,73,95]. Integumental, cell-free chitin synthase preparations from H. cecropia and T. ni also were not inhibited by benzoylphenyl ureas [37]. Conversely, polyoxin D, a known competitive inhibitor of chitin synthase, inhibited the chitin synthases in all of the insect cell-free systems [36,37,39,74,95]. Consequently, the mode(s) of action of the benzoylphenyl urea and triazine CSIs lies elsewhere than in a direct interaction with chitin synthase.

CSI effects on other physiological systems such as hormonal systems have already been mentioned as a possibility. Ecdysteroids and juvenile hormones and the metabolism of these hormones would seem to be likely targets for diflubenzuron effects, since they are known to influence chitin metabolism [23,96–98]. Indeed, Yu and Terriere [99] found that inclusion of benzoylphenyl urea CSIs in the diets of housefly larvae reduced the activity of 20-hydroxyecdysone (β-ecdysone)-metabolizing enzymes. In addition, dietary supplements of the cecropia juvenile hormone of benzoylphenyl urea-treated insects partially restored pupal-adult ecdysis, but 20-hydroxyecdysone supplements did not. Yu and Terriere proposed that inhibition of 20-hydroxyecdysone-metabolizing enzymes resulted in elevated levels of 20-hydroxyecdysone, which subsequently stimulated chitinases to degrade the cuticle (the possibility of this latter aspect has been discussed above). O'Neill et al. [100] observed no differences in 20-hydroxyecdysone levels in similar experiments conducted on S. calcitrans. Further, the metabolism of injected doses of ecdysone and 20-hydroxyecdysone was not altered in diflubenzuron-treated milkweed but nymphs [61]. Diflubenzuron effects were not alleviated but instead were synergized when milkweed bug nymphs were injected with juvenile hormone analogs. Increased ecdysial failures also resulted when boll weevils (Anthonomus grandis) were treated with juvenile hormone and diflubenzuron as opposed to diflubenzuron alone [101]. Ecdysteroid levels were decreased in Teneberio molitor pupae after dipping in acetonic solution of diflubenzuron [102]; in some cases, injection of 20-hydroxyecdysone alleviated the diflubenzuron effects but the new cuticle was of an abnormal architecture. Soltani et al. [103] using epidermal explants from T. molitor pupae reported inhibition of cuticle deposition and ecdysteroid titers in the culture media for tissues that had been exposed to diflubenzuron.

In addition, they were able to suppress inhibition of cuticle synthesis by diflubenzuron with 20-hydroxyecdysone treatments. They concluded that diflubenzuron and 20-hydroxyecdysone were mutally antagonistic with partially reversible effects on epidermal cells synthesizing cuticle.

It seems likely that the primary mode of action of diflubenzuron would involve hormones, not only because of the evidence presented above on hormone levels, hormone metabolism, and hormonal activation of chitinases, but simply because diflubenzuron and other benzoylphenyl ureas inhibit chitin synthesis so rapidly in vivo and in tissue culture situations that any long-term hormone involvement would be unlikely. The hormone experiments discussed here in regard to hormonal effects were with extended diflubenzuron exposure periods.

Another possible explanation for the inhibition of chitin synthesis in vivo and in tissue/organ cultures, but not in cell-free chitin synthase systems, is that benzoylphenyl ureas are metabolized to potent chitin synthase inhibitors [37,39]. Thus, in a cell-free chitin synthase preparation the required metabolic system may not be present to transform the benzoylphenyl urea into a potent inhibitor. Although this proposal cannot be ruled out as a possibility, there are many facts that argue against it. First, Cohen and Casida [39] have tested some diflubenzuron metabolites in T. castaneum cell-free chitin synthase system without observing any inhibition. Second, many vastly different benzoylphenyl ureas (see Figs. 3a and b) have been found to be excellent chitin synthesis inhibitors: Is it probable that they would all be metabolized to potent CSIs? Third, the triazine CSIs appear to share many of the same chitin synthesis-inhibiting characteristics of benzoylphenyl ureas, that is, they inhibit chitin synthesis in intact cells in vivo and in vitro but not in cell-free systems; and for this group, also, to be metabolically activated is improbable.

Cohen and Casida suggested another explanation for benzoylphenyl urea inhibition of chitin synthesis in intact cells and tissues, but not of cell-free chitin synthase systems [39]. They suggest that the spatial and organizational properties of the chitin synthases require that the cell integrity must be intact for inhibition to occur. Again, this proposal cannot be ruled out, but there is evidence that argues against it. First, if spatial and organizational properties of the chitin synthase were that critical, one would expect the cell-free enzyme activity to be nonexistent. Second, other inhibitors, such as the polyoxins, function as inhibitors of chitin synthesis in vivo and in vitro and in cell-free chitin synthase systems, which suggests that spatial and organizational properties are not critical factors.

Another proposal suggests that CSIs and certain fungicides and insecticides prevent chitin synthesis by interfering with the proteo-

lytic activation of the chitin synthase zymogen [20,104]. These investigators showed that these compounds are serine protease inhibitors, with the benzoylphenyl ureas exhibiting a preference for chymotrypsin-like proteases. Several problems prevent the acceptance of this hypothesis as the primary mode of action of benzoylphenyl ureas. Thus far, only slight evidence exists that suggests insect chitin synthases exist in a zymogenic form, because some activation occurs when cell-free preparations are pretreated with proteases [36,38]. Inclusion of phenylmethylsulfonylfluoride (10 μM) in the homogenization and reaction buffers to cell-free preparations from S. calcitrans did not affect chitin synthase activity (unpublished data). Moreover, this hypothesis does not account for the fact that benzoylphenyl ureas and other CSIs inhibit chitin synthesis when added to insect tissues already actively synthesizing chitin [24,25, 60,84], that is, when chitin synthase has already been activated.

A seemingly unrelated action of diflubenzuron and other benzoylphenyl ureas to the subject of chitin synthesis inhibition is the observed sterilizing effect on eggs oviposited by treated insects [105–108]. Mitlin et al. [109] suggested that diminishment of sexual function of adult boll weevils dipped in diflubenzuron solution may, in part, result from the inhibition of DNA synthesis. Later, Meola and Mayer [110] reported that both larval and imaginal epidermal cells of pupae of S. calcitrans were affected by diflubenzuron treatments. Larval epidermal cells did not undergo normal programmed cell death events, and imaginal epidermal histoblasts did not undergo mitosis, indicating an effect on nucleic acid synthesis [102]. In addition, although the larval epidermal cells of treated insects were viable, no chitin synthesis occurred. Of course, no imaginal cuticle would be deposited because there were no imaginal epidermal cells. DeLoach et al. [111] reconfirmed the effects of diflubenzuron on larval epidermis and imaginal epidermal histoblasts and demonstrated that diflubenzuron treatments specifically inhibited DNA synthesis in imaginal epidermal histoblasts; other cell types divided normally and were unaffected by diflubenzuron (see Fig. 4). Similar effects on DNA synthesis have now been confirmed in T. molitor [112]. DeLoach et al. [111] suggested that the inhibition of DNA synthesis in S. calcitrans pupae, that is, DNA polymerase, was probably not the primary action of diflubenzuron, because DNA synthesis in other cell types appeared to occur at the normal time intervals. Direct inhibition of DNA polymerase was finally ruled out when diflubenzuron was tested in a cell-free DNA polymerase system isolated from M. sexta cells and no inhibition was observed [113].

Could these effects, that is, effects on DNA synthesis and chitin synthesis somehow be related? It was suggested [111] that the primary action of diflubenzuron could be similar to that of the fungicide Kitazin P, which was previously thought to be a chitin synthesis

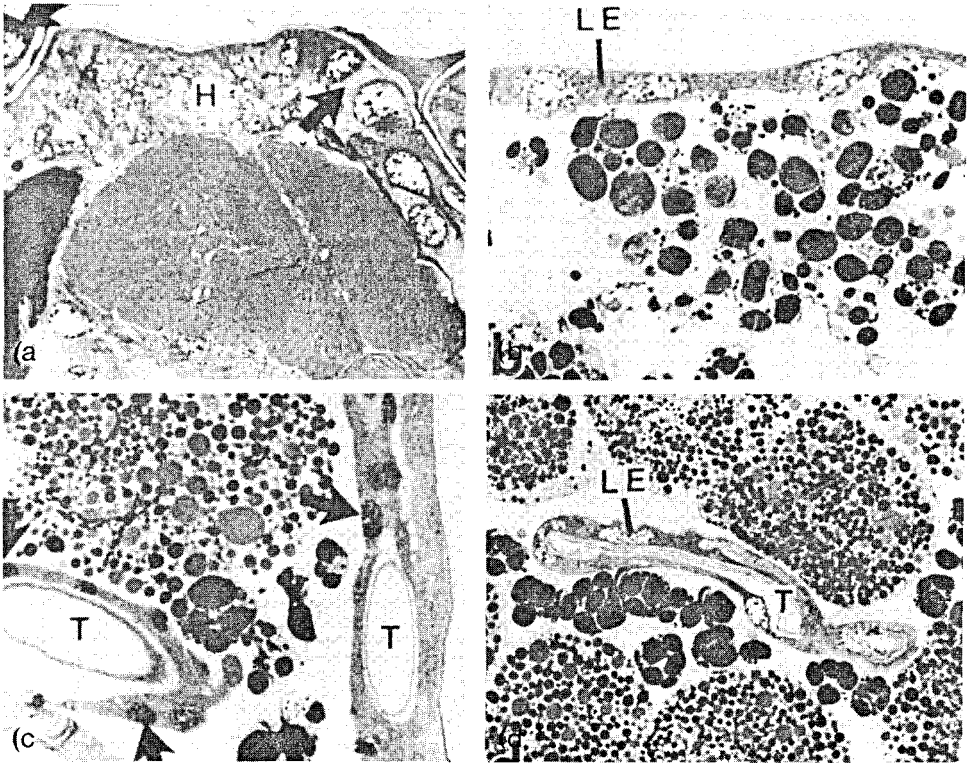


Fig. 4 Effects of topically applied diflubenzuron on pupal tissues of Stomoxys calcitrans. Pupae were topically treated at the prepupal stages (O hr) with either 0.5 µl acetone or 0.5 µl acetone with 0.1 µg diflubenzuron. (a) histoblast area (H) in the abdominal region of a 32–36 hr diflubenzuron-treated pupa. No labeled thymidine is present in the nuclei of these cells and no histogenesis of adult epidermal cells has begun even at this late stage, as shown by the larval epidermal cells (arrows) bordering the histoblast region. (b) Section through thoractic region of 32–36 hr diflubenzuron-treated pupa with larval epidermis (LE) intact and no adult epidermis being formed. (c) Histogenesis of adult tracheal system (T) occurring in the abdominal region of a 32–36 hr acetone-treated pupa. Discrete clusters of silver grains (arrows) from [^{3}H]thymidine incorporated into DNA are clearly discernible over the nuclei of epithelial cells that have recently undergone mitosis. (d) Trachea (T) in the abdominal region of a 32–36 hr diflubenzuron-treated pupa still retains the larval epithelium (LE)-no [^{3}H]thymidine incorporation was ever observed in these cases. (From Ref. 103.)

inhibitor but is now known to affect membrane permeability [66,67]. As discussed above, because cell membrane permeability is the primary action of Kitazin P, one would expect this compound to inhibit chitin synthesis in intact cells but not in cell-free systems, which is actually the case in insects not only for Kitazin P but also for benzoylphenyl ureas [39,104].

Other evidence to support the hypothesis that CSIs alter membranes comes from the work of Kessel and McElhinney [114], who demonstrated that dithiocarbonilates (see Fig. 5) inhibited facilitated diffusion of nucleosides and active transport of amino acids across leukemia L1210 cell membranes. Diflubenzuron was tested in vitro on Harding-Passey melanoma cells to determine if affected membrane properties [115]. The presence of diflubenzuron in the leukemia L1210 cells cultures significantly inhibited the uptake of nucleosides, which is an indication of membrane alterations, but did not alter cell-growth patterns [115]. Tests conducted in vivo showed that injections of diflubenzuron into C57 BL/6 mice with B-16 melanoma tumors significantly reduced tumor mass as compared to controls, whereas in vitro incubations of diflubenzuron with B-16 melanoma cells had no effect on cell growth [116]. Tests with several benzoylphenyl ureas and the triazine compound, CGA 19255, were conducted on a chitin-producing cell line isolated from M. sexta to determine the effects on incorporation of nucleosides into DNA and RNA [113]. All the compounds inhibited nucleoside incorporation to some extent, with CGA 19255 being the best overall inhibitor (60% inhibition for cytidine and 49% inhibition for adenosine).

Klitschka et al. [117] examined the cellular and subcellular binding characteristics of DFB to cultured M. sexta cells. Scatchard

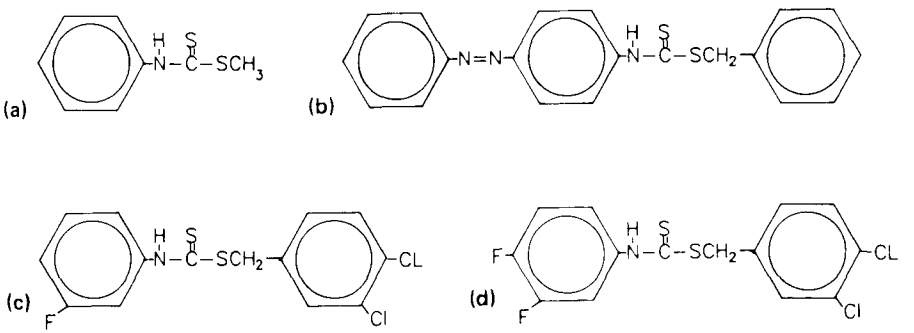


Fig. 5 Dithiocarbonilates (or dithiocarbamates) that alter membrane properties of leukemia L1210 cells. (a) N-phenyl-S-methyl-dithiocarbamate; (b) N-(4-phenyldiazophenyl)-S-(benzyl)-dithiocarbamate; (c) N-(3-fluorophenyl)-S-(3,4-dichlorobenzyl)-dithiocarbamate; (d) N-(3,4-difluorophenyl)-S-(3,4-dichlorobenzyl)-dithiocarbamate.

analysis of diflubenzuron binding to viable and nonviable cells after short-term incubations did not reveal any specific, saturable uptake mechanisms. When diflubenzuron binding was measured in subcellular fractions, it was found that the plasma membrane fraction bound about 3-fold more diflubenzuron than did equivalent amounts of cell homogenates. This observation becomes more significant when one realizes that chitin synthase is probably attached to the plasma membrane of insect epidermal cells. Leopold et al. [101] stated that the accumulation of large secretory bodies in boll weevil epidermal cells during pupal-adult transformation indicated that the secretory commitment of the cells was unaffected, but that either the transport, utilization, and/or assembly of the cuticle precursors had been inhibited. In addition, Mitsui et al. [118–122] suggest that the transport system for UDP-GlcNAc across midgut epithelial cell membranes in larvae of Mamestra brassicae is inhibited by diflubenzuron.

Thus, it appears that benzoylphenyl ureas and triazine CSIs alter epidermal cell membranes such that transport of nucleic acid precursors, chitin synthesis precursors, etc. is prevented. This hypothesis is appealing because it would explain most of the observed effects of these compounds on insects and other organisms, particularly the observation that chitin synthesis is inhibited in intact cells but not in cell-free systems. What remains to be discovered is exactly how these compounds alter the cell membrane. One mechanism that has been suggested is the possible inhibition of glycosyltransferases by CSIs [74]. These are membrane-bound enzymes that have been shown to be involved with the synthesis of glycoproteins, glycolipids, plasma and cell membranes, etc. [123,124]. Also, N-acetylglucosaminyl transferases have UDP-GlcNAc as an obligate substrate and are probably involved with chitin synthesis by providing primer oligosaccharides to chitin synthase [125]. Inhibition of N-acetylglucosaminyl transferases then may result in an accumulation of UDP-GlcNAc (which has been observed in insect tissues treated with various CSIs), inhibit chitin synthesis by altering the composition of cell membranes and subsequently preventing chitin precursor transport, and/or reducing the number of primer oligosaccharides. Both mannosyl and N-acetylglucosaminyl transferases have been investigated; diflubenzuron had no effect on either enzyme [76,126, 127].

Marks et al. [20] and Marks and Ward [97] suggest a similar action for diflubenzuron, that is, an intact cell structure is required for inhibition of chitin synthesis. They propose that diflubenzuron partitions into the cell membrane, consequently disrupting the lipoprotein lattice of the plasma membrane. The resulting effects could either prevent activation of the chitin synthase zymogen or prevent the UDP-GlcNAc precursor from reaching the site of polycondensation, or both.

There are recent reports on a few new CSIs that are structurally unrelated to the benzoylphenyl ureas and triazines that appear to directly inhibit insect chitin synthases in cell-free systems. One is a natural product, plumbagin (Fig. 3b), isolated from an African medicinal plant [128]. Another group of compounds is the benzimidazoles (Fig. 3b), although they are less inhibitory than polyoxin D [129]. The benzimidazoles primarily seem to be inhibitors of the respiratory chain, with chitin synthesis inhibition being a secondary effect [130].

Buprofezin (Applaud; 2-tert-butylimino-3-isopropyl-5-phenyl-3,4,5,6-tetrahydro-2H-1,3,5-thiazin-4-one) and its analogs have been shown to be effective in controlling several insect pests [131–133]. Although these compounds are structurally unrelated to the benzoylphenyl ureas (Fig. 3b), they exhibit many of the same effects. Izawa et al. [81] have shown that buprofezin and its analogs inhibit chitin synthesis and to a lesser extent nucleic acid synthesis in the brown rice planthopper, Nilaparvata lugens Stal. It is not known at this time whether or not buprofezin will inhibit chitin synthesis in cell-free chitin synthase preparations. In addition, Uchida et al. [134] have reported that 20-hydroxyecdysone has an antagonistic effect on N. lugens nymphs treated with buprofezin; nymphocidal and oviposition-inhibitory effects of buprofezin on nymphs were countered by 20-hydroxyecdysone. Recently, a new mode of action, that is, inhibition of prostaglandin biosynthesis, has been reported for buprofezin [135].

Finally, the fermentation product avermectin inhibits chitinase activity in Streptomyces antibioticus in vitro and chitin synthesis in the brine shrimp Artemia salina and the fungus Mucor miehi [136]. Avermectin also appears to interfere with DNA synthesis in Mucor miehi. Avermectins have been shown to be potent acaricides, insecticides, and antihelminthics [137], whose primary action has been proposed as being interference of γ-aminobutyric acid binding to synaptic receptors [138].

III. PROSTAGLANDINS

A. Background

Since prostaglandins exhibit a wide range of biological activities in mammals, a great deal of research exists in the development of pharmacologically active compounds related to prostaglandins. However, because of the limited information on the effects of prostaglandins on insects, research on the potential use of prostaglandin synthesis inhibitors and prostaglandin analogs for insect population control is limited.

Several investigators have noted that aspirin, acetaminophen, indomethacin, and other mammalian inhibitors of prostaglandin syn-

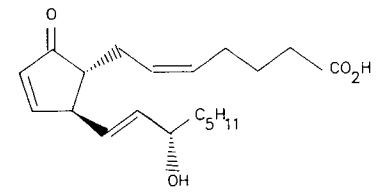
thesis also inhibit prostaglandin synthesis in insects, and in some insects, the decrease in synthesis results in a marked lowering of oviposition activity. The success of the development of compounds that have a biological effect on insects depends on continued research on the roles and mechanisms of action of prostaglandins in insects.

The clinical use of prostaglandins preceded their identification and nomenclature by over 50 years. The term prostaglandin was applied by Von Euler in 1935 because of his belief that these compounds were biosynthetic products of the prostate gland. Actual isolation and structural identification came about through the excellent work of Bergstrom et al. in 1962 [139]. At the present time, nine classes of naturally occurring prostaglandins have been isolated and identified (see Fig. 6). All these compounds are 20 carbon acids with a cyclopentane ring in the middle. Synthesis of prostaglandins occurs from 20 carbon polyunsaturated fatty acids.

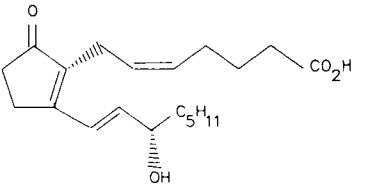
It is not the purpose of the current review to extensively cover the literature on prostaglandins, but rather to concentrate on recent published reports on this group of compounds in insects. Extensive coverage of the experimental data on prostaglandins can be found in several reviews [140–143]. Also, an excellent review of prostaglandins in insects was written by Brady in 1983 [144]. Prostaglandins are widely distributed in mammalian tissues, with the highest concentrations found in seminal fluids of sheep and man (approximately 300 μg/g). They have been found in numerous other tissues at much lower levels. These tissues include the kidney, pancreas, eye, brain, and uterus. Nonmammalian sources of the prostaglandins include the A_2-15 acetate from Plexaura homomella, a coral, Prostaglandin A, in onions, and Prostaglandins E_2 and F_2 in Gracilania lichenoids, a red algae [140]. At the present time, prostaglandins of the E and F series have been reported in ten insect species representing six orders [145,146].

B. Biosynthesis of Prostaglandins

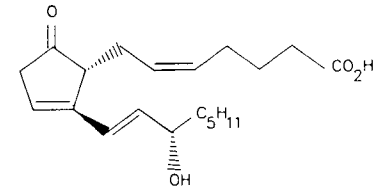
The synthesis of prostaglandins in mammalian systems has been studied extensively [147,148]. Metabolic pathways leading to the E and F series prostaglandins from either arachidonic acid, homo-7-linolenic acid, and 5,8,11,14,17 eicosapentaenoic acid have been elucidated (Figs. 7 and 8). Although synthesis has now been documented in other vertebrate and invertebrate systems, much less is known about the reactions leading to the final product. Only recently has information on the enzymes involved in the synthesis of prostaglandins in insects been presented [144–146,149,150]. Polyunsaturated fatty acids (PUFA) are essential components of the diets of most higher animals. Over 50 years ago, their essential role for normal growth and development was demonstrated in rats. It was not until 1964 that the role of the essential fatty acids (EFA) in the



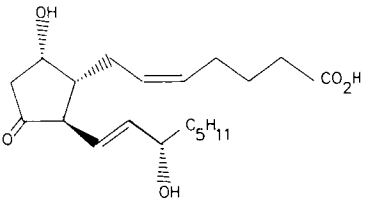
Prostaglandin A_2



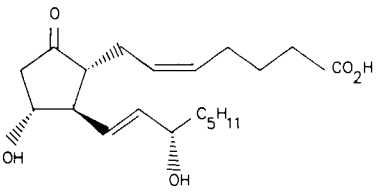
Prostaglandin B_2



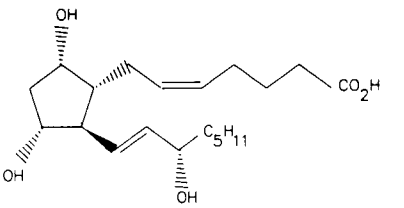
Prostaglandin C_2



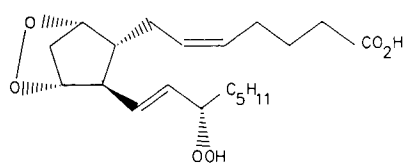
Prostaglandin D_2



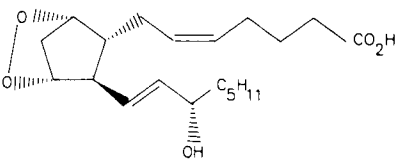
Prostaglandin E_2



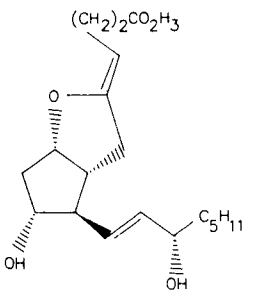
Prostaglandin $F_{2\alpha}$



Prostaglandin G_2



Prostaglandin H_2



Prostaglandin I_2

Fig. 6 Structure of naturally occurring prostaglandins.

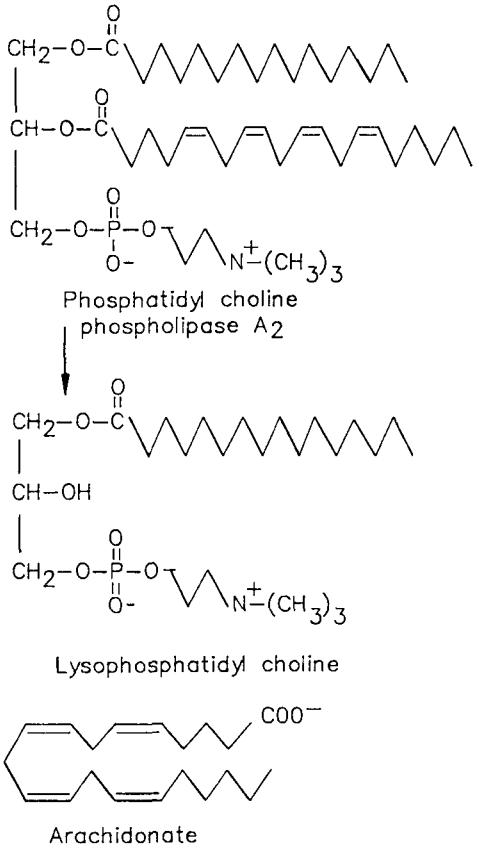


Fig. 7 Arachodonic acid release from phosphatidylcholine.

synthesis of prostaglandins was established. Many mammals have the capability of converting 18C PUFA (linoleic and linolenic acids) to 20C PUFA with four or five double bonds. The most intensively investigated of these has been arachidonic acid [143].

As noted in the review by Brady, and in a published report by Dadd, most insects appear to be unable to synthesize the EFA and therefore require them in their diet [144,151]. In some insects, these 18C EFA are suitable substrates for the synthesis of the 20C tetraenoic acids, such as arachidonic acid, but are unsuitable in others. For example, Dadd [152] demonstrated that dietary arachidonic acid was necessary for the development and normal flight of Culiseta incidens and could not be substituted for by the EFA. In addition, Dadd [153] indicated that arachidonic acid and/or docosahexaenoic acids could not substitute for EFA in the diets of certain Lepidoptera and Orthoptera.

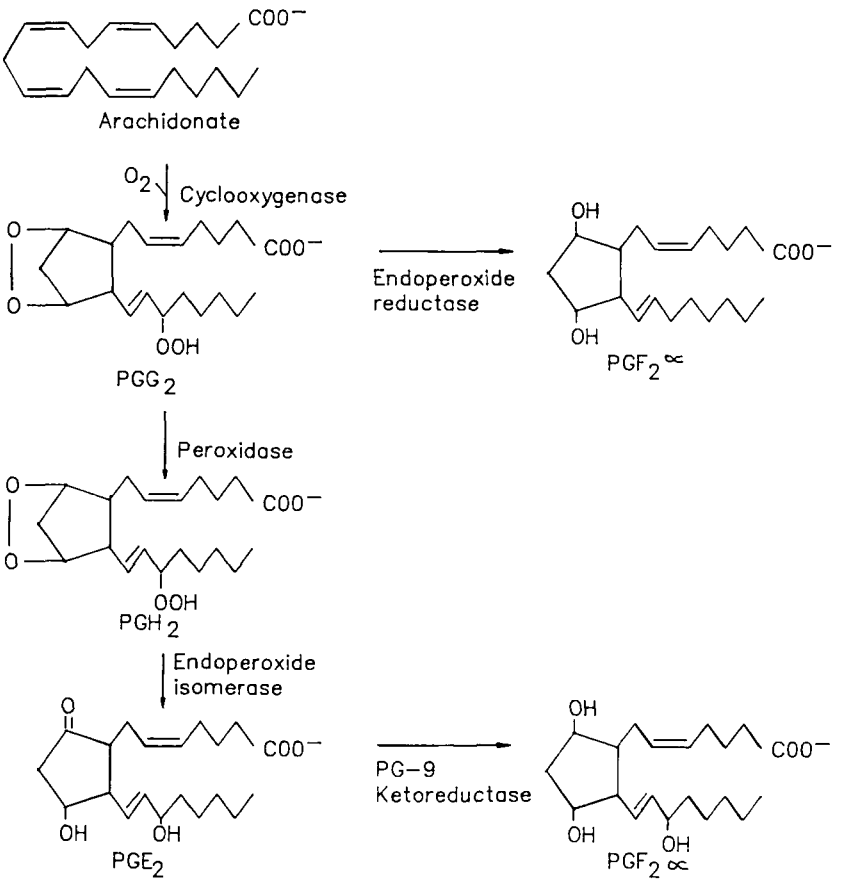


Fig. 8 Biosynthetic pathway for prostaglandins PGE, PGF2Q', and related prostaglandins.

Work by Blomquist et al. [154] demonstrated the de novo synthesis of linoleic acid from acetate in vivo and in isolated tissues of Periplaneta americana, Zooptermopis angusticollis, and Acheta domesticus.

In an extensive survey of long-chain PUFAs in insects, Stanley-Samuelson and Dadd [155] found that these acids were regular components of the tissues of insects. Twelve different species of insects representing five orders were used in the investigation and arachidonic acid or other long-chain fatty acids were found in all species at least in trace amounts. The phospholipid fraction of tissues contained the highest level of PUFA. Research with mammals similarly

indicates a higher proportion of PUFA in the phospholipids, specifically in the 8 position. Stanley-Samuelson and Loher [156] reported that arachadonic acids comprised 24% of the fatty acids in phosphatidyl choline and 4% of the fatty acids in phosphatidyl ethanolamine, but was undetectable in neutral lipids.

As Brady [144] noted, the EFA concentration appears to be closely associated with synthesis of prostaglandins and related compounds. However, there is also the possibility that the EFA have a distinctly separate role as well. A comparative study of EFA roles in insects and vertebrates was conducted by Dadd in 1983 [157]. A recent review has been written by Prabhu and Jacob on the role of dietary essential fatty acids in tissue prostaglandins synthesis [158].

Prostaglandins appear to be synthesized in the tissue in which they act. Synthesis begins with the mobilization of the free acid from the phospholipids (Fig. 8). In mammals, this release occurs through the action of phospholipase A_2 on the phospholipids. Many different types of stimulations of tissues of animals can lead to the release of free arachidonic acid [159]. Antiinflammatory steroids such as the corticosteroids appear to exert their inhibitory effect on prostaglandin synthesis by inhibiting the release of PUFA from phospholipids. As mentioned above, data from insects indicate that the most abundant source of arachidonic acid and other PUFA is the phospholipid fraction [156,157,160]. This would indicate that the source of the EFA and arachidonic acid in insects may be through the action of phospholipase similar to that in mammals.

The released fatty acid can be converted to prostaglandin by an enzyme system often referred to as prostaglandin synthase. Prostaglandin synthase is a group of enzymes bound in an organized unit and located in the microsomal cell fraction. Each step in the synthesis involves a different part of this enzyme complex. The first two steps involve the enzyme prostaglandin endoperoxide synthase. The cyclooxygenase and peroxidase activities are all part of the same protein in some mammalian systems [143]. The cyclooxygenase catalyzes the addition of two molecules of oxygen to arachidonic acid to produce the precursors of all or many of the prostaglandins, thromboxanes, and prostacyclins, but not the leukotrienes. The peroxidase converts the peroxide to a hydroxyl functional group. This product is then converted to a variety of other prostaglandins through the action of other isomerases, peroxidases, and dehydrogenases [143,159]. The capability to carry out the synthesis described above is found in most mammalian systems and in many lower vertebrates and invertebrates [161,162].

Nonsteroid antiinflammatory agents, aspirin, acetaminophen, etc., are reported to act by inhibiting cyclooxygenase but not the hydroperoxidase activity of the prostaglandin endoperoxide synthase [163]. Aspirin is reported to acylate the cyclooxygenase, thus resulting in irreversible inhibition of the cyclooxygenase [163].

The first report of prostaglandin synthase activity in insects was by Destephano et al. [164] in A. domesticus. Prostaglandin synthase activity was found in the testes, seminal vesicles, and spermatophores of this insect species [150,165]. Since that time, synthesis has been demonstrated in several insect species.

Wakayama and co-workers have extensively studied the enzyme complex in the housefly, Musca domestica [145,146,149]. Prostaglandin synthesis capability was demonstrated in homogenates of the whole insects, as well as in homogenates of the head and thorax, abdomen, ovaries, and male reproductive tissues. The highest level of activity was found in the microsomal fraction of these tissues. Conversion of radio-labeled arachidonic acid and 8,11,14 eicostrienoic acid was demonstrated. Recently, Stanley-Samuelson et al. injected labeled arachidonic and eicosapentanenoic into wax moth larvae, Galleria mellonella, and found that these compounds were converted into prostaglandins [166].

Nonsteroid antiinflammatory agents (e.g., aspirin and acetaminophen), when incubated with the microsomal tissue preparation, inhibited prostaglandin synthesis; however, no inhibition was noted when these same drugs were fed to houseflies [145,146]. Prostaglandin synthase inhibitors, aspirin and acetaminophen, were first reported to inhibit PGE and $PGF_{2\alpha}$ synthesis in the house cricket, A. domesticus. A significant inhibition was demonstrated when the drugs were fed to house crickets in time periods ranging from 5-20 days [167].

C. Catabolism of Prostaglandins

Prostaglandins are metabolized rapidly in mammalian tissues. For example, radio-labeled PGE_2 was 97% deactivated in 15 min [146] after being injected into the bloodstream of mammals. However, there appears to be some difference in the capability of tissues to metabolize prostaglandins, with the lungs being the most active in this catabolic process. Catabolism begins by an oxidation of the 15 hydroxyl position to the corresponding keto functional group [159]. This is often followed by reduction of the 13-14 double bond and then the carbon chain is shortened from the carboxyl group by beta oxidation. Finally, the ω position is hydroxylated and then oxidized to a carboxyl group. Beta oxidation can then proceed from the ω carbon.

Very little data are available on the breakdown of prostaglandins in insects. Insects do not have the forced circulatory systems that are associated with rapid deactivation. Stanley-Samelson and Loher [156] found that radio-labeled PGE_2 injected into the abdomen of adult virgin female Australian field crickets, Teleogryllus commodus, remained in significant quantities for up to 2 hr. The decrease in the level of radioactivity in the hemolymph was associated with increases in radioactivity of the Malpighian tubules hind gut complex,

ovaries, fat bodies, and a much smaller amount in the ventral nerve cord and flight muscles. These investigators have suggested that this differential uptake may be related to an important but presently unknown physiological function.

Data collected from research on the housefly also indicated a much slower rate of metabolism than in mammals [146]. PGE_2 was metabolized much more rapidly than $PGF_{2\alpha}$ in both male and female houseflies. In the housefly, PGE_2 is metabolized rapidly in the first 20 min, whereas there is a slow gradual decline in the levels of $PGF_{2\alpha}$ with 10% of radioactivity remaining at the end of 60 min. In females, approximately 30% of radioactivity injected as PGE_2 remained after 60 min and 50% of the $PGF_{2\alpha}$. One of the breakdown products in the housefly was identified as PGB_2.

D. Physiological Actions of Prostaglandin in Insects

Extensive information on the physiological effects of prostaglandins in mammals can be found in several recent reviews [168,169]. These reports document the effects of prostaglandins on reproduction, gastric secretions, thermoregulation, pain and inflammation, and control of blood pressure, to name a few.

Although prostaglandins have been found in numerous insects, only two documented effects of prostaglandins have been presented. An increase in oviposition activity of adult virgin crickets, Acheta domesticus, occurred after injection with PGE_2 [166]. Similar effects on egg-laying activity have been shown with Bombyx mori [170,171] and with the field cricket, T. Commodus [156]. Second, evidence for an involvement of prostaglandin in flight capability of mosquitoes was presented by Dadd and Kleinjan [160].

Work by Destephano et al. [164] indicates that prostaglandin-synthesizing capability was transferred from the male to the female by way of the spermatophores during mating. After mating, a significant increase in PGE and $PGF_{2\alpha}$ was noted. This was followed by an immediate increase in the oviposition activity of the female. Similar results were reported for the Australian field cricket, T. commodus, by Loher et al. [172]. In this insect, the prostaglandin synthase enzyme complex is again transferred from the male to the female during mating by way of the spermatophore, and prostaglandins are then synthesized from existing substrates within the spermatheca of the female. As with A. domesticus, oviposition activity was stimulated after the mating process. It is not clear how the synthase complex is regulated. Buprofezin, an insect growth regulator, was found by Uchida et al. to suppress egg laying by N. lugens [135]. The suppression was presumably due to an 84% reduction in biosynthesis of prostaglandin S. Utilizing T. commodus, Tobe and Loher [173] presented strong evidence to indicate that the major prosta-

glandins synthesized were PGE_2 and $PGF_{2\alpha}$. The synthesis of PGE_2 and $PGF_{2\alpha}$ was inhibited by the feeding of aspirin to the crickets.

Investigations by Murtaugh and Denlinger [167] also demonstrate the effectiveness of known mammalian prostaglandin inhibitors on the enzyme complex in A. domesticus. When aspirin, acetaminophen, and indomethacin were fed to male and female crickets for periods ranging from 5 to 20 days, a significant reduction in endogenous levels of PGE_2 and $PGF_{2\alpha}$ was observed. The greatest reduction was observed in the female where PGE_2 and $PGF_{2\alpha}$ levels were reduced to less than 10% of controls. Similar reductions were noted in untreated females when they were mated to males fed a diet containing aspirin and acetaminophen. In a study of the long-term regulation of oviposition in the house cricket, Murtaugh and Denlinger [167] found that a single mating early in life was sufficient to induce egg laying for the entire life of the female. With removal of the spermatophore, the egg-laying response was greatly decreased [167]. Their results indicate that the factor produced by the male may not be the same as $PGF_{2\alpha}$, which has previously been shown to give an immediate oviposition response.

A transfer of prostaglandin synthase activity from the male to the female has been noted in Locusta migratoria [174]. The enzyme complex responsible for the synthesis of prostaglandin was found in the opalescent gland and in the seminal vesicle of the male accessory reproductive gland. The complex was transferred via the spermatophore as in the cricket. The major prostaglandins synthesized were PGE_2 and $PGF_{2\alpha}$. Injections of PGE_2 and $PGF_{2\alpha}$ into the hemocoel of virgin females did not cause an increase in the oviposition in locusts, as it did in crickets. It is possible that the PGE_2 and $PGF_{2\alpha}$ may be metabolized too rapidly in the hemocoel so that levels do not rise high enough to exert any effect. Even though prostaglandin-like compounds were found in the reproduction tissue of the cabbage looper, Trichoplusia ni, no effect on oviposition activity was shown [175]. Still, after mating, a 3-fold increase in PGE_2 and in a $PGF_{2\alpha}$-like compound were noted. Injections of PGE_2 and $PGF_{2\alpha}$ exerted no oviposition effects on the female moths. Control experiments were performed using virgin females of A. domesticus, in which a positive effect had already been demonstrated [176]. These female crickets were injected with the same solutions of prostaglandins used with T. ni, and ovipostional activity in these virgin females was equal to that seen in mated female crickets. This is in contrast to the lack of response of the female moth of T. ni. Casas et al. [177] reported that the presence of the female cricket, Gryllus bimaculatus, stimulated the production of PGE_2 in the male cricket. No similar effects were noted in the cockroach, Blattela germanica. In addition, Hagan and Brady [175] measured the effect of the injected prostaglandins on the calling behavior of the female moths. Calling behavior has

been noted to decrease after mating. However, the behavior of the females injected with prostaglandins was not different from that of saline-injected females. The lack of effectiveness of injected prostaglandins in this experiment could be due to an impermeability of the prostaglandins used, or to a rapid inactivation of the injected compound. In addition, T. ni, could use a prostaglandin different from those used in this study for regulating reproductive behavior. These investigations found no response to the feeding of the prostaglandin inhibitor, N-acetyl-p-aminophenol, on oviposition activity or calling behavior of T. ni. This inhibitor did retard the growth of one of the larval stages of development [175].

Works by Howard and Mueller on the flour beetle, Tribolium brevicornis, has resulted in the isolation of 12 organic compounds in the defensive secretions [178]. Two of these compounds, 2-hydroxy-4-methyoxyacetophenone and 2'-hydroxy-4'-methoxypropiophenone, were potent inhibitors of prostaglandin synthetase. The role of these compounds in the defensive secretions is not known. Jurenka et al. have reported that five compounds found in the defensive secretion of insects are inhibitors of prostaglandin synthesis in a bovine seminal vesicle microsome system and in a cockroach (Periplaneta americana) fat-body microsome system [179]. These compounds were methyl anthranilate from male ants; O-aminoacetophenone from male seed bugs and ants; and methyl salicylate, 2,5-dibydroxyphenylacetic acid gamma lactone, and salicylaldehydes from beetles. The amount of these compounds produced appeared in the defensive secretions to be sufficient to interfere with the physiological actions that involve prostaglandins. Again, no known function of these prostaglandin synthesis inhibitors was found [179].

The extensive distribution of prostaglandins in different types of insect tissue may indicate an important role of prostaglandin in physiological functions other than reproduction. However, except for the work of Dadd and Kleinjan [144], which indicated a possible role of prostaglandin in flight behavior of mosquitoes, the documented effects of the prostaglandins in insects have been in the area of reproduction. A review by Stanley-Samuelson and Loher in 1986 summarizes the importance of prostaglandins in insect reproduction [180]. This review also covers some of the investigations of the relationships of prostaglandins and the long-chain essential fatty acids.

IV. STEROID ANALOGS AS INSECT MOLTING HORMONE INHIBITORS

Another interesting approach to insect control involves the chemistry of sterols that insects require for growth, development, and repro-

duction [181]. Unlike mammals and most other animals, insects must go through a molting process that has been shown to be closely regulated by various steroids and steroid-related substances [182]. Due to the uniqueness of this process, a potentially safe and selective method for the control of insect populations may one day be available. Insects are not normally able to synthesize steroids from simple precursors and must obtain them by way of their diet. They are, however, able to metabolize the dietary sterols by various means to the appropriate steroidal structure for the regulation of their specific molting process. As a result, a significant amount of work has been carried out to understand the biochemistry of insect molting hormones for a variety of species while also looking for substances that will interfere with this process in a specific and predetermined manner.

A considerable amount of work has been done by Svoboda et al. [183] on the tobacco hornworm, Manduca sexta, which indicates that sitosterol, stigmasterol, and campesterol (Fig. 9) are the important dietary sterols for this species. These phytosterols are subsequently converted to cholesterol, which is the precursor of most molting hormones.

An important consequence of this work was the identification of desmosterol (Fig. 9) as the final intermediate in the conversion of the phytosterols to cholesterol. Desmosterol is important since the dealkylation of the C-24 substituent in the phytosterol metabolism of insects is not analogous to sterol metabolism in higher animals. Although all the details of this particular metabolic scheme have not been worked out, it appears that there are a variety of mechanisms by which insects utilize phytosterols. For example, the milkweed bug, Oncopeltus fasciatus [183], does not dealkylate the C_{28} and C_{29} plant sterols and convert them to cholesterol.

The next important biochemical process is the conversion of cholesterol or related substances to the appropriate molting hormones, which subsequently are involved in tissue stimulation and ultimately the growth, development, and metamorphosis of the insect. Some of the insect molting hormones that have been isolated [183] and characterized are ecdysone, 20-hydroxyecdysone, 20,26-dihydroxyecdysone, 26-hydroxyecdysone, 3-epi-20-hydroxyecdysone, and 2-deoxyecdysone (Fig. 10).

All of these substances are thought to be metabolically derived from ecdysone, with 20-hydroxyecdysone possibly being the most active in terms of hormonal function. For example, recent work by Kiss and Molnar [184] has shown that 20-hydroxyecdysone elicited metamorphic changes in wild type and mutant Drosophila tissues when cultured in vitro, although the responses were weaker and slower than those in vivo.

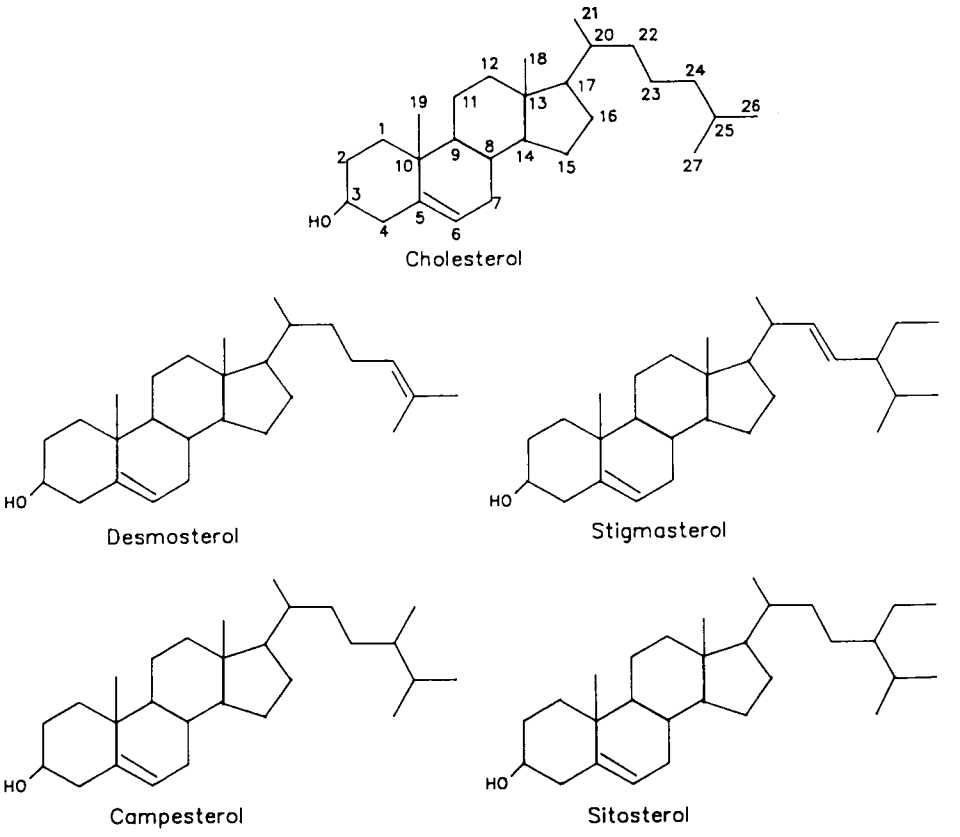
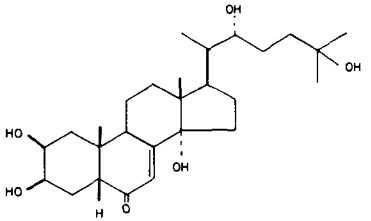


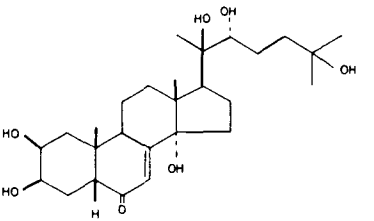
Fig. 9 Important dietary sterols for Manduca sexta.

Thompson et al. [185] have shown that 26-hydroxyecdysone may be playing a physiological role in the molting process of the tobacco hornworm; high concentrations as the conjugates have been found in the ovaries and newly laid eggs of this species. This is interesting in light of the absence of molting activity for this compound in the housefly bioassay. Recently, Thompson et al. [186] have examined tobacco hornworm eggs that were 48–64 hr old, and these scientists were able to identify 26-hydroxyecdysone-26-phosphate as the major ecdysteroid conjugate. Thompson has suggested that such steroid conjugates allow for the storage of large amounts of ecdysteroids that could be released later in a developmental stage.

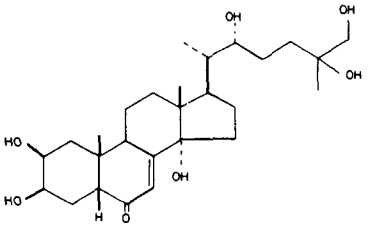
Feldlaufer et al. [187] have also examined the ecdysteroids of the gypsy moth via high-performance liquid chromatography and mass spectroscopy techniques and have found that 20-hydroxyecdysone and ecdysone are the major components found for 4-day-old pupae.



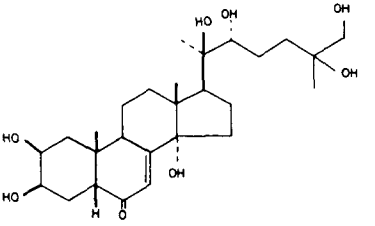
Ecdysone



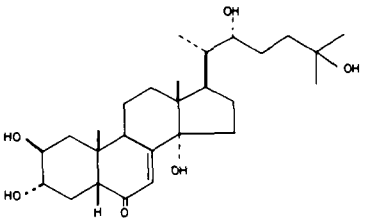
20-Hydroxyecdysone



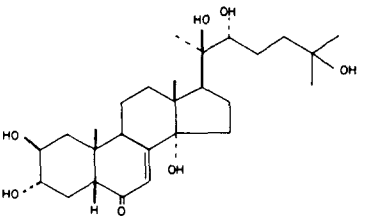
26-Hydroxyecdysone



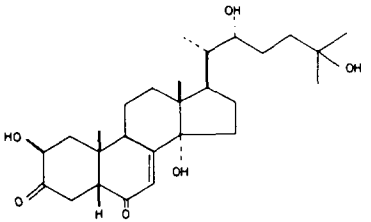
20,26-Dihydroxyecdysone



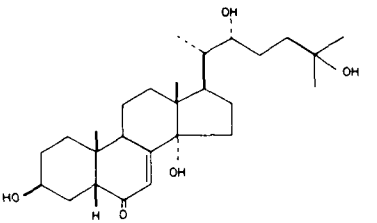
3-Epiecdysone



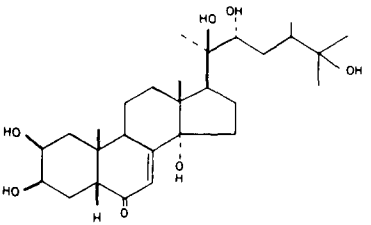
3-Epi-20-Hydroxyecdysone



3-Dehydroecdysone



2-Deoxyecdysone



Makisterone A

Fig. 10 Some known insect ecdysteroids and metabolites.

Another important development in the biochemistry of insect molting has been the isolation of a 28-carbon ecdysteroid called makisterone A (Fig. 10) from 96-hr-old eggs of the milkweed bug [188]. Until now, all the insect molting hormones that had been identified contained 27 carbon atoms and were presumably derived from cholesterol metabolism. As mentioned earlier, many phytophagous insects can convert 28- and 29-carbon phytosterols by a dealkylation procedure to the 27-carbon cholesterol. Apparently, the milkweed bug lacks this mechanism, but has the ability to use the C-24 methylated derivative of 20-hydroxyecdysone. It was subsequently shown by Aldrich et al. [189] that makisterone A is 10 times more active than 20-hydroxyecdysone with regard to stimulating cuticle synthesis and inhibition of vitellogenesis when injected into 3-day-old virgin female milkweed bugs. It has been suggested that campesterol is probably the 28-carbon phytosterol precursor to makisterone A.

Svoboda et al. [190] have recently shown that three species of Pentatomomorpha (Hemiptera) have a high content of C-28 and C-29 phytosterols and produce makisterone A as their major molting hormone. In addition, these same coworkers, along with Feldlaufer et al. [191], have recently isolated and identified makisterone A as the major pupal ecdysteroid in the honeybee. Barbier and Schindler [192] has earlier reported that the honeybee contained relatively large amounts of 24 methylene-cholesterol, and since subsequently it was also shown to be unable to convert C-28 and C-29 phytosterols to cholesterol, this was not a surprising result.

A recent review article by Feldlaufer and Svoboda [193] summarizes the work on makisterone A to date. This report indicates that makisterone A has been found in seven species of insects representing three orders. These authors also conclude that this ecdysteroid probably has a larger distribution among insects than originally anticipated.

Another interesting development has been the report by Thompson et al. [194] that a C-21 steroid conjugate has been recently isolated from the tobacco hornworm. These workers reported the isolation and characterization of 5-[^{14}C]pregnen-3, 20-diol-glucoside from eggs following injection of [^{14}C] cholesterol into 16-day-old pupae of female Manduca sexta. Thompson et al. [173] suggest that this is the first piece of strong evidence indicating that C-21 steroids may be playing an important physiological role in this insect.

As a consequence of the growing information relating to the biochemistry of insect molting hormones, several substances have been found that interfere with this process. For example, Svoboda and Robbins [195] reported in 1967 that certain azasteroids inhibit the Δ^{24}-Sterol reductase system in insects. This actually resulted from the knowledge that desmosterol was an intermediate in insect sterol metabolism for the production of cholesterol. The Δ^{24} reductase

system was shown to be responsible for this transformation. The in vivo studies on these azasteroids showed the disruption of larval molts, formation of precocious fourth instar prepupae, and inhibition of pupation. For example, when 22, 25-diazacholesterol (Fig. 11) was fed to the tobacco hornworm in combination with the dietary sitosterol, inhibition of larval growth was observed [183].

Interestingly, although a number of azasteroids and related compounds have been found that inhibit the Δ^{24}-sterol reductase system, not all of these substances disrupt the molting process in insects. This indicated that a number of steroid metabolic pathways were being affected. Subsequent work by Svoboda et al. [183,195,196] demonstrated that the most active azasteroids were 25-azacholesteryl methyl ether, 25-azacholestane, and 25-azacoprostane (Fig. 11). The active azasteroids exhibit physiological effects at the ppm level and a number of different morphological changes can be observed. For example, in the yellow fever mosquito first and second instar larval molts are blocked. In the case of the housefly, unusual morphological changes take place between puparium formation and adult emergence.

Svoboda et al. [183] were subsequently able to find even simpler analogs (Fig. 12) that also exhibited inhibitory effects. For example, the following acyclic amines were found to be inhibitors of the insect molting process: N,N-dimethyl-3,7,11-trimethyldodecanamine and

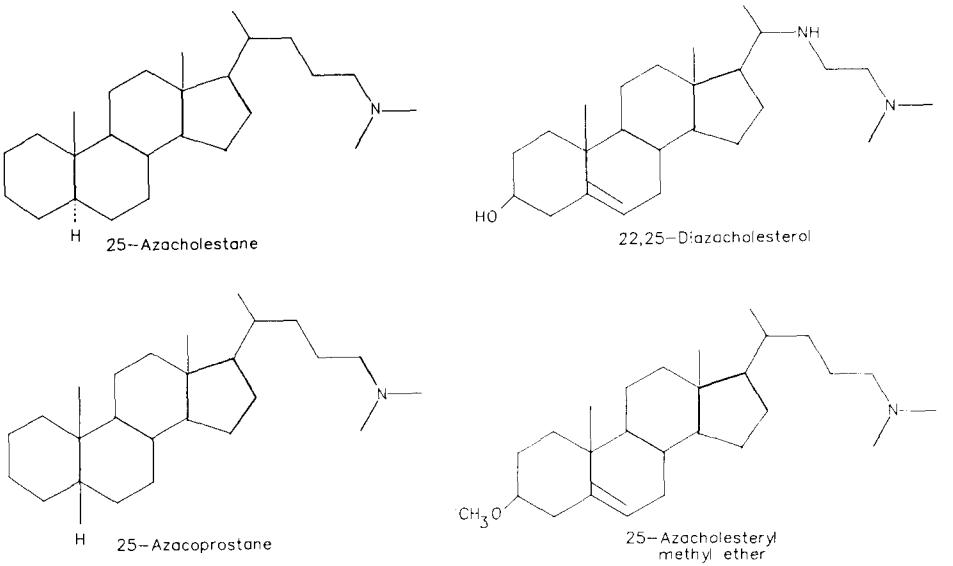
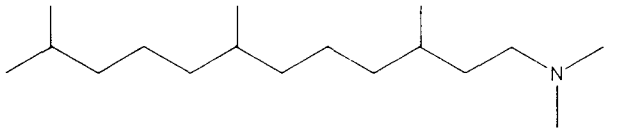
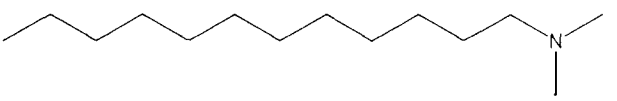


Fig. 11 Biologically active azasteroids that interfere with insect molting.



N,N–Dimethyl–3,7,11–trimethyldodecanamine



N,N–Dimethyldodecanamine

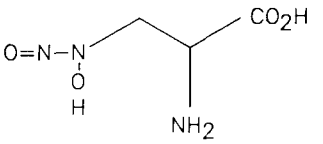
Fig. 12 Simple analogs that interfere with insect molting.

N,N-dimethyldodecanamine. Svoboda et al. [183] also showed that many of these inhibitors affect the system that hydroxylates the C-20 position of ecdysone in certain in vitro systems. Recently, Chitwood et al. [197] and Bottjer et al. [198] have extended this work to nematodes that also have a dietary requirement for sterols. Their findings suggest that azasteroids were at least, in part, responsible for producing a cholesterol deficiency by inhibiting the conversion of phytosterols to cholesterol. Other possible modes of action suggested by these investigations involve (1) inhibition of cholesterol uptake, (2) interference with the utilization of cholesterol as a membrane component, and (3) inhibition of ecdysteroid synthesis.

Apparently, these azasteroids have a general inhibiting effect on systems that require sterols for growth-related processes and have also been found to be fungistatic with regard to sexual reproduction in Phytophthora cactorum [199]. Lozano et al. [200] have shown that N,N-dimethyldodecanamine is also an inhibitor of phytosterol metabolism in nematodes such as Caenorbditis elegans. This compound is thought to inhibit (1) sitosterol dealkylation and (2) Δ^{24}-sterol reductase in certain species.

Another interesting development has been the isolation and structure determination of L-alanosine (Fig. 13), which is a naturally occurring compound that has been found to inhibit the larval ecdysis of the armyworm [201].

In conclusion, due to the extensive studies carried out on the role of sterols in the molting process of insects, several compounds are emerging as potential materials to serve as model compounds in the development of safe and selective control of insect populations.



L–Alanosine

Fig. 13 Naturally occurring insect molting inhibitor.

V. CONCLUSIONS

What is immediately evident from reading this review is that there are many possibilities for the development of insecticides that either interfere with or affect metabolic pathways that are unique to arthropods. What is also obvious is that an enormous amount of research remains to be performed. All three areas discussed, that is, chitin synthesis inhibitors, prostaglandins, and steroid analogs, are just in their infancy and have yet to be fully exploited.

In regard to the putative chitin synthesis inhibitors, we are just now beginning to understand how these compounds function at the molecular level. Probably we know more about what the benzoylphenyl urea CSIs do not do than what they do since most of the research has been directed toward projects that have assumed that there is direct involvement with the chitin synthesis process. Current research findings indicate that the benzoylphenyl ureas and triazines are much more interesting compounds than originally thought. Here are a group of chemicals that appear to specifically affect insect epidermal cells, the results being that larval epidermal cells do not follow normal programmed cell-death events during metamorphosis [102], that chitin synthesis is disrupted [78,81,82,102], that imaginal epidermal histoblasts do not divide, and that DNA synthesis is inhibited in these cells [102–104]. Investigators are now delving into the biochemistry of insect epidermal cell membranes to determine the mode of action of the benzoylphenyl ureas and triazines. These efforts may yield new information on insect immunological systems (e.g., cell recognition) and metamorphosis in general and may provide new approaches for insect control strategies.

Even though the compounds originally termed chitin synthesis inhibitors apparently only indirectly affect the chitin metabolic pathway, this remains as an ideal process as a target for insect control. The recent reports on the natural products of plumbagin [118] and benzimidazoles [119] indicate that it is possible to develop and synthesize chemicals that directly inhibit insect chitin synthases. This is especially so since there are cell-free assay systems for the insect chitin synthases [36,38] that can be used to test new inhibitors.

Efforts to develop chemicals that interfere with chitin metabolism should not be entirely focused on chitin synthesis. The catabolic side of this pathway is just as important as are those processes involved with sclerotization and tanning of the cuticle and regulation of chitin metabolism, all of which we know very little about.

Chemicals that could possibly be used as insecticides and whose mode of action is involved with prostaglandin metabolism and actions have not been reported. To our knowledge, no one has attempted to synthesize analogs of aspirin, acetaminophen, idomethacin, known inhibitors of prostaglandin synthesis, and specifically test them as potential insecticides. Possibly the reason for this is that prostaglandin metabolism and action appear to be similar in insects and mammals. However, this remains to be determined, as much of the research to date has been mainly confirmatory. Certainly differences exist on the physiological effects of prostaglandins between mammals and insects. It may be here that new chemicals with more specificity to insect prostaglandins will prove most effective.

There is an enormous amount of knowledge on insect molting hormones including identification, synthesis, catabolism, physiological and biochemical effects. General statements made in regard to the metabolism and effects of molting hormone should not be made as various insects possess different metabolic pathways leading to the synthesis of cholesterol [162] and different molting hormone requirements [172]. Even with all of this knowledge, little effort has been expended on the development of compounds that interfere with the metabolism and physiological action of edysteroids. Nevertheless, good progress has been made on compounds that effectively inhibit the Δ^{24}-sterol reductase system [183,195]. Evidence that other compounds may be effective in disrupting other places in the metabolic pathway or physiological action comes with the discovery that L-alanosine interferes with larval ecdysis in the fall armyworm. Many enzymes have been identified that are involved with various aspects of steroid metabolism in insects, including mixed function oxidases, oxidases, epimerases, kinases, and glucosyltransferases. Some of these enzymes are quite specific in their activities, others are not. In vitro, cell-free assays for most of the enzymes have been developed and therefore are available for the testing of candidate inhibitors, insecticides, and/or insect growth regulators. Few have stepped forward to develop such compounds, probably because most believe that steroids that are homologous or analogous in structure to the naturally occurring materials would have to be synthesized. No doubt this would be a difficult task, but may not be absolutely necessary if one remembers the success with the simple amine inhibitors of Δ^{24}-sterol reductase [183]. Other areas associated with

ecdysteroid metabolism and action that will probably yield new control chemicals are receptor sites and bioregulators such as the prothoracicotropic hormone (PTTH).

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